HEAT SHOCK PROTEIN 27 INHIBITS THE HIPPO TUMOR SUPPRESSOR PATHWAY BY FACILITATING MST1 PROTEASOMAL DEGRADATION

by

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Abstract

Heat shock protein 27 (Hsp27) is a molecular chaperone highly and ubiquitously expressed in aggressive cancers where it controls a variety of pro-tumorigenic signaling pathways. Using gene expression profiling in prostate cancer cells with loss of Hsp27 function, we identified for the first time that Hsp27 regulates target genes in signaling pathways dependent on YAP and TAZ. Suppression of these transcriptional co-activators occurs via their phosphorylation and cytoplasmic retention by the Hippo tumor suppressor pathway. Mechanistic studies revealed that Hsp27 expression is associated with reduced YAP phosphorylation and enhanced transcription of YAP/TAZ target genes. Examination of the core components of the Hippo kinase cascade revealed that Hsp27 facilitates the proteasomal degradation of the core Hippo kinase, MST1, leading to reduced phosphorylation/activity of other main kinases responsible for YAP phosphorylation/inactivation, LATS1 and MOB1. Importantly, our data from cell lines was supported by data from human tumors; clinically, high expression of Hsp27 correlates with increased expression of YAP target genes in prostate cancer as well as reduced phosphorylation of YAP in lung and invasive breast cancer clinical samples. Together, our data reveal a novel mechanism by which Hsp27 regulates the Hippo tumor suppressor pathway, providing further rationale to target Hsp27 in multiple cancers.

Preface

The data presented in this thesis are based on the work that I carried out during the completion of my MSc program. A version of this thesis has been submitted to be published and is currently under review as noted below:

Hsp27-dependent degradation of MST1 leads to inactivation of the Hippo tumor suppressor pathway in cancer. Sepideh Vahid, Daksh Thaper, Kate Gibson, Martin E. Gleave, Jennifer L. Bishop, Amina Zoubeidi

Dr. Zoubeidi was the principal investigator in this study. The study concept and design as well as data interpretation was conducted by myself, Daksh Thaper, Dr. Bishop and Dr. Zoubeidi. I performed all of the experiments presented in this thesis, while Daksh Thaper and Kate Gibson provided technical support. I drafted the original manuscript, which was reviewed by Daksh Thaper, Dr. Bishop and Dr. Zoubeidi.

Dr. Gleave provided Hsp27 antisense oligonucleotide (OGX-427) for one of the experiments in this thesis. He is listed as one of the inventors on the patent for OGX-427 submitted by the University of British Columbia and licensed to OncoGenex Technologies, a Vancouver-based company that Dr.Gleave has founding shares in.

I and others involved in the following thesis project declare no conflict of interest.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AKT	Protein kinase B
ANKRD1	Ankyrin repeat domain-containing protein 1
AR	Androgen receptor
ARHGAP29	Rho GTPase Activating Protein 29
ATP	Adenosine triphosphate
AXL	Gene code for Tyrosine-protein kinase receptor UFO
BMP	Bone morphogenetic proteins
CHIP	C terminus of Hsc70-interacting protein
CRPC	Castration-resistant prostate cancer
CTGF	Connective tissue growth factor
CYR61	Cysteine-rich angiogenic inducer 61
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
FGF2	Fibroblast growth factor 2
GLI2	Zinc finger protein GLI2
GPCR	G-protein coupled receptor
HSF	Heat shock factor
HSP	Heat shock protein
Hsp27	Heat shock protein 27 kDa
Hsp60	Heat shock protein 60 kDa
Hsp70	Heat shock protein 70 kDa
Hsp90	Heat shock protein 90 kDa
HSPB1	Heat shock protein beta-1, gene code for Hsp27
ILK	Integrin-linked kinase
IP	Immunoprecipitation
IPA	Ingenuity pathway analysis
LATS1/2	Large tumour suppressor kinases 1 and 2

MOB1A/B	Mps-one binder kinase activator 1 A and B
MST1/2	Mammalian STE20-like protein kinase 1 and 2
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PI3 kinase	Phosphatidylinositol-4,5-bisphosphate 3-kinase
p27Kip1	Cyclin-dependent kinase inhibitor
RAF or c-RAF	proto-oncogene serine/threonine-protein kinase
RPPA	Reverse phase protein lysate microarray
SAV1	Salvador homologue 1, WW45
Scr	Scrambled
sHSP	Small heat shock protein
siRNA	Small interfering RNA
SMADs	Contraction of Sma and Mad (Mothers against decapentaplegic) family of transcription factors
STAT3	Signal transducer and activator of transcription 3
STK4	Serine threonine kinase 4, gene code for MST1
STR	Short tandem repeat
TAZ	Transcriptional co-activator with PDZ-binding motif
TCF	T cell factor family of transcription factors
TCGA	The cancer genomic atlas
TEADs	TEA domain containing family of transcription factors , also known as TEF Transcription enhancer factor
TGF-β	Transforming growth factor beta
WT	Wild type
WW45	Another name for SAV1
WWTR1	Gene code for TAZ
YAP	Yes Associated protein
µM	Micro molar
nM	Nano molar

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First and foremost, I would like to express my deepest appreciation to my supervisor, Dr. Amina Zoubeidi, for giving me the opportunity to be a part of such a great team, regardless of my different educational background. I would like to deeply thank her for encouraging me, granting me the scientific independence in this journey and believing in me, even when I didn't believe in myself. This work was not possible without her constant guidance and supervision.

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To my dearest parents,

For their endless love and support…

&

To Hooman,

My soulmate…

تقدیم به پدر و مادر عزیزم

به پاس محبت ها و پشتیبانی بی دریغشان...

و

تقدیم به بهترین دوست و همسرم

هومن

1. Introduction

1.1. Heat Shock Proteins

1.1.1. Biology, function and transcription

Heat shock proteins (HSPs) are highly conserved molecular chaperones with indispensable roles in protein homeostasis, transport processes and signal transduction. Although expressed under normal conditions, the expression of HSPs is upregulated in response to different cellular stresses, including but not limited to, hyperthermia, hypoxia, nutrient deprivation, oxidative stress, UV light and hormone- or chemo-therapy. Stress conditions result in abnormal cellular mechanisms such as protein misfolding and aggregation which if not corrected will eventually lead to cell apoptosis; therefore, HSPs are upregulated and can assist cell survival via multiple mechanisms. Firstly, HSPs catalyze the proper folding of misfolded proteins and avoid their aggregation. Secondly, HSPs associate with key modulators of the apoptotic machinery and interfere with the programmed cell death at different stages. There is tremendous evidence on how heat shock proteins like Hsp27, Hsp70 and Hsp90 conduct their anti-apoptotic effects. Thirdly, HSPs are involved in and regulate protein degradation machinery (ATP- and ubiquitin-dependent proteasome systems as well as autophagy machinery), providing either the stability or the proteasomal degradation of selected proteins under stress conditions [1-3].

Mammalian HSPs are classified in to 4 major families based their electrophoretic characteristics (molecular weight). Heat shock protein families are Hsp90, Hsp70, Hsp60 and the small HSPs like Hsp27 and αB-crystallin. While the activities of high molecular weight HSPs are ATP-dependent, the small HSPs act in an ATP-independent manner. Transcription of HSPs is primarily regulated by heat shock transcription factors (HSFs), mainly HSF1, which senses protein damage and increases the transcription of chaperones [4, 5]. However aside form stress induction, the expression of HSPs is regulated by cell cycle and in response to growth factors, where the need for HSPs increases significantly [5]. As a result, HSPs are ubiquitously induced in

malignancies where uncontrolled proliferation, hypoxia and cytotoxic reagents put cancer cells in constant exposure to cellular stress [6].

1.1.2. Small Heat Shock Proteins

Small Heat Shock Proteins (sHSPs) are ATP-independent low molecular weight chaperones (between 12-43 kDa) that are defined by a conserved α-crystallin domain, a sequence of approximately 90 amino acid residues which is constrained by less conserved amino- and highly variable carboxy-terminal extensions [7]. **Figure 1.1** shows a general structure of sHSPs. In addition to α-crystalline, some sHSPs have one or two more domains which are located in N-terminus preceding α-crystalline domain [8].

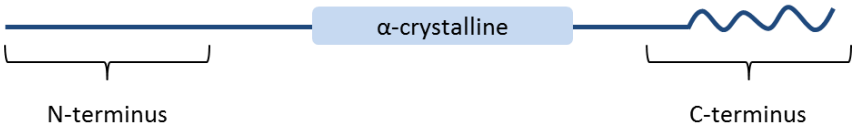


Figure 1.1. General structure of small heat shock proteins.

All sHSPs are structurally characterized by an α-crystalline domain.

The primary role of sHSPs is to bind denaturing proteins and prevent their aggregation. In order to function properly, almost all sHSPs need to dimerize and then oligomerize into multimeric structures. The α-crystallin domain is responsible for dimerization of sHSPs [8, 9]. The amino (N)-terminal extension is thought to influence higher-order oligomerization, subunit dynamics and chaperoning, and finally the carboxy (C)-terminal domain is flexible and amorphous and is important in chaperon activity and solubility [10, 11].

Extensive studies in recent years showed that some of sHSPs are upregulated in diseases, especially in cancers, where they play important part in inhibition of cell death pathways, and modulation of pro-survival signaling networks [12].

1.1.3. Heat Shock Protein 27

Among the stress chaperons, Hsp27 (HSPB1), an ATP independent sHSP has been extensively studied due to its crucial involvement in diverse physiological and pathological cellular

processes. Human Hsp27 consists of 205 amino acid residues forming a α-crystallin domain, a flexible C-terminal tail and a N-terminal domain with WDPF motif which is crucial for oligomerization [13]. Like other HSPs, Hsp27 is regulated by HSF1 at the transcription level; however its function is also thought to be regulated by posttranslational modification including phosphorylation on multiple sites. Ser15, Ser78 and Ser82 have been characterized as the main phosphorylation sites of Hsp27 **(Figure 1.2)** [14]. Hsp27 is phosphorylated by various kinases in response to different stimuli including some growth hormones and cytokines [1]. Hsp27 phosphorylation controls its oligomerization and ultimately its functions. Larger oligomers are mostly involved in protein folding and the prevention of protein aggregation while smaller oligomers are responsible for ubiquitination and proteasomal degradation of client proteins [27].

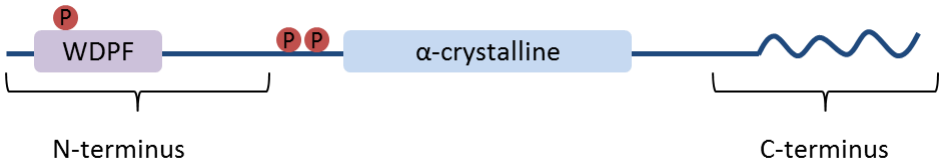


Figure 1.2. Structure of Hsp27.

In addition to the α-crystalin domain, Hsp27 has a WDPF domain in the N terminus. Hsp27 is phosphorylated on multiple sites mainly Serine 15, Serine 78 and Serine 82.

Hsp27 is expressed in almost all human tissues in normal conditions however its expression dramatically increases under certain pathological conditions such as cancer [1]. Under stress conditions, Hsp27 levels increase to prevent protein aggregation and facilitate elimination of misfolded proteins [15]. More importantly, Hsp27 has been reported to modulate apoptosis and interact with its regulators such as procaspase-3 and cytochrome C to inhibit cell death [16, 17].

1.1.4. Hsp27 in human cancer

Due to the wide range of intra- and extracellular insults which malignant cells are constantly subjected to, Hsp27 is overexpressed across different cancers like prostate, breast, ovarian and gastric [18-22] and this overexpression has been associated with poor prognosis

and chemo-resistance [23-27]. Hsp27 contributes to cancer progression via different mechanisms; it is a major anti-apoptotic and pro-survival chaperone which plays crucial parts in tumorigenesis [28-30], increasing proliferation by facilitating cell cycle progression [31] and enhancing migration and invasion via multiple mechanisms [32, 33]. In addition, Hsp27 also participates in the maintenance of cancer stem cells [34, 35] which are also associated with aggressive tumors and poor prognosis [36].

1.1.5. Hsp27 and oncogenic signaling pathways

The pleiotropic roles of Hsp27 underscore its position at hubs of cell signal cascades across multiple cancers. Hsp27 has been shown to cooperate closely with oncogenic signaling pathways like transforming growth factor beta (TGF-β) [37], WNT/β-catenin [32, 38], STAT3 [33] and NF-κB [39]. Hsp27 stabilizes and activates AKT, the infamous oncogene in the PI3 kinase and insulin signaling pathway [28, 30]. Additionally, Hsp27 is required for trafficking and transcriptional activity of sex steroid receptors including the androgen receptor (AR), the central oncogenic driver in prostate cancer progression [40, 41]. Importantly, modulation of these pathways by Hsp27 has been linked to therapy resistance in different cancers [23-25].

Since Hsp27 is central to many upregulated signaling pathways in cancer, it is an attractive therapeutic target; Hsp27 inhibition may concurrently suppress several pathways implicated in cancer advancement and more importantly overcome resistance to hormone- and chemo-therapies.

1.1.6. Targeting Hsp27

The ultimate goal of translational cancer research is to identify molecular mechanisms that contribute to tumor progression and to develop novel and effective therapies that can target these mechanisms to improve patient outcome. However, although we have a relatively thorough understanding of how molecules such as Hsp27 contribute to cancer, targeting such molecules remains difficult. For example, designing small molecule inhibitors for Hsp27 is more difficult compared to some other HSPs like Hsp70 and Hsp90, due to the lack of an ATP binding

domain. As an alternative, Hsp27 suppression is achieved by using antisense oligonucleotides, which prevent its expression. Antisense oligonucleotides are synthetic single stranded nucleic acid structures that bind to RNA and interfere with gene expression by altering RNA function [42]. OGX-427 (Apatorsen), a second-generation antisense oligonucleotide for Hsp27, is currently in phase II clinical trials for prostate, bladder and lung cancers (ClinicalTrials.gov, NCT01454089, NCT01829113, and NCT01120470). Studies suggest that knockdown of Hsp27 using OGX-427 suppresses prostate tumour growth and sensitizes prostate cancer cells to chemo-therapy [40, 43, 44]. OGX-427 also sensitize non-small cell lung cancer cells to Erlotinib and chemotherapy [45].

1.2. Hippo Tumor Suppressor Pathway

1.2.1. Core components

The Hippo tumor suppressor pathway is an evolutionarily conserved pathway which was first characterized in *Drosophila melanogaster*. Using genetic screens in fruit flies, a series of genes were isolated that when inactivated, allowed excessive tissue growth in developing wings or eyes. These proteins make up the core components of the Hippo tumor suppressor pathway and their function is highly conserved in mammals and *D. melanogaster*. The mammalian Hippo pathway's core consists of: Mammalian STE20-like protein kinase 1 and 2 (MST1/2), Large tumour suppressor kinases 1 and 2 (LATS1/2) and adaptor proteins WW45 (SAV1) and Mps-one binder kinase activator 1 A and B (MOB1A/B). The role of these kinases and scaffold proteins is to phosphorylate and inactivate Yes associated protein (YAP) and Transcriptional co-activator with PDZ-binding motif (TAZ), two co-activators which are able to partner with oncogenic transcription factors such as TEA domain-containing sequence-specific transcription factors (TEAD1-4) [46, 47]. Other transcription factors that use YAP and TAZ as their co-regulators are: SMADs (TGF-β signaling), β-catenin/TCF (WNT pathway), RUNXs (blood and bone formation), PAX3 (neural crest formation), KLF4 (terminal differentiation of goblet cells in colon), p63/p73 (apoptosis) [48-53].

Table 1.1 compares core components of the Hippo pathway in *D. melanogaster* and in humans.

Table 1.1. Core components of the Hippo pathway in human and fruit fly.

Homo sapiens	Gene name Human	Gene name Drosophila melanogaster
MST1/2*	STK3/ STK4	HPO (HIPPO)
WW45 (SAV1)	WW45	SAV (SALVADOR)
LATS1/2*	LATS1/2	WTS (WARTS)
MOB1 (A AND B)	MOBKL1A, MOBKL1B	MATS
YAP/TAZ	YAP1, WWTR1	YKI (YORKI)
TEAD1-4	TEAD1-4	SD (SCAPOLLED)

* Functions of MST1 and MST2 as well as LATS1 and LATS2 to inactivate YAP are redundant [54].

1.2.2. Biology and function in mammals

Growing interest to this pathway is driven by tremendous evidence showing the fundamental role of this pathway in organ growth, stem cell renewal and tumor suppression. The activation of the Hippo tumor suppressor pathway begins with the activation of MST1/2 which in turn activates LATS1/2 by phosphorylating it on T1079. Scaffold protein SAV1 tethers MST1/2 to LATS1/2 for optimal interaction. MOB1A and MOB1B potentiate the kinase activity of LATS1/2. LATSs are responsible for phosphorylation of the transcriptional co-activators YAP and its paralogue TAZ. Phosphorylation of YAP and TAZ results in their cytoplasmic sequestration with the 14.3.3 proteins and prevents their nucleus translocation. When the Hippo cascade is deregulated, absence of YAP/TAZ phosphorylation allows for their translocation to the nucleus leading to activation of pro-survival and metastatic pathways such as TGF-β/SMAD, WNT/β-Catenin and ILK/TEADs pathways [46, 55]. **Figure 1.3** shows a schematic view of the Hippo kinase cascade.

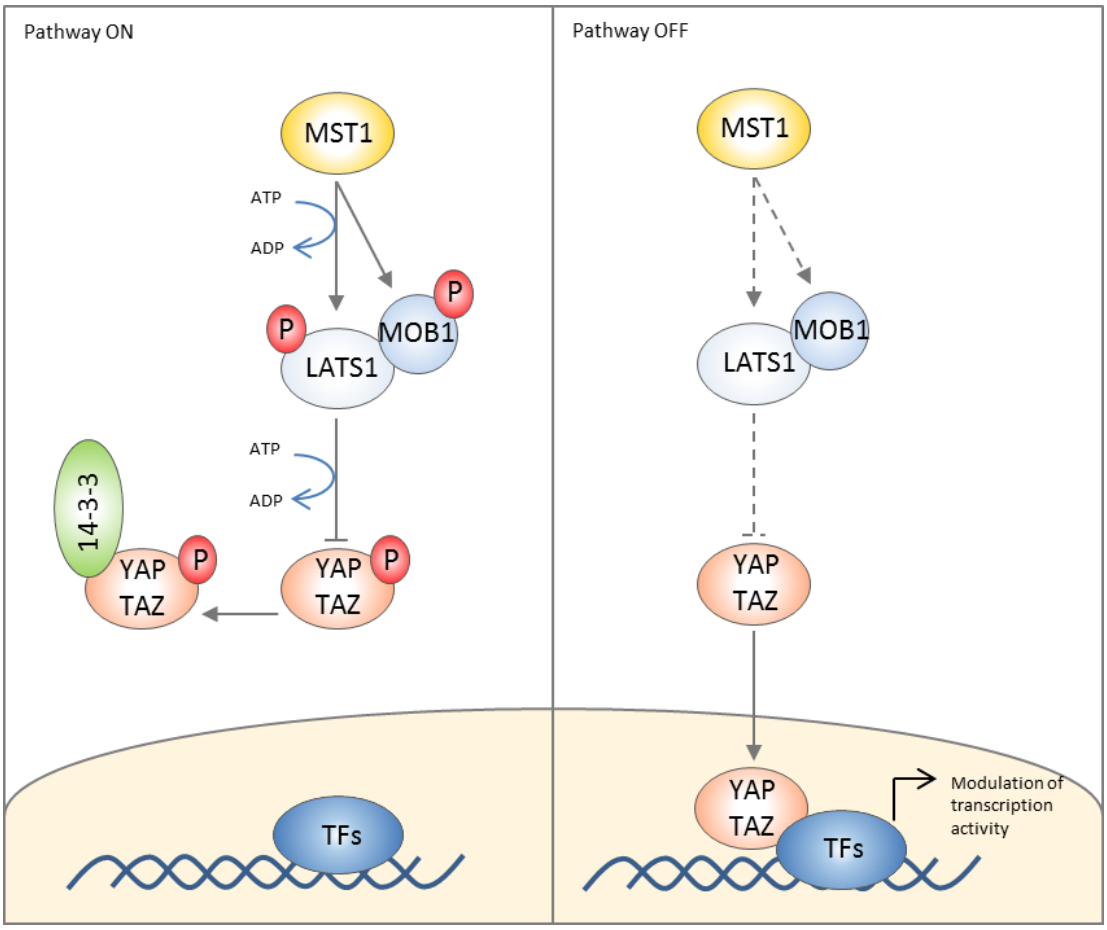


Figure 1.3. Schematic of Hippo tumor suppressor pathway.

When active, a series of phosphorylation events lead to phosphorylation of YAP and TAZ and their cytoplasmic sequestration with 14.3.3 proteins. When the Hippo pathway is inactive, the kinase cascade is not functional; therefore, YAP/TAZ are free to translocate into nucleus and regulate the activity of their partner transcription factors.

1.2.3. Upstream regulators of the Hippo pathway

Although the core components of this pathway appear to form a simple linear cascade, recent work has led to the realization that in fact the Hippo pathway is part of an interconnected signaling web which allows the cell to elicit the appropriate response to an external stimulus. Several cellular processes provide input into this catalytic torrent including mechanical compression [56], cell-cell contact [57], cellular polarity [58, 59], as well as signaling pathways such as G-protein coupled receptor (GPCR) [60], TGF-β [61], WNT [62], Notch [63], EGFR [64] and ILK [55].

1.2.4. Hippo tumor suppressor pathway in cancer

The Hippo tumor suppressor pathway regulates and is regulated by cellular mechanisms that if defective, can lead to tumorigenesis. Inactivation of the Hippo pathway correlates with poor patient outcome, increase of migration, invasion and metastasis [65].

1.2.4.1. Cell proliferation

The Hippo pathway restricts cell proliferation, the most fundamental deregulated feature of cancer cells. Cell proliferation can be controlled via several parameters such as cell-cell contact, loss of which one of the hallmarks of cancer [66]. Defective function of the Hippo components leads to hyper-activation of YAP and TAZ causing ectopic cell proliferation [46, 67]. For example, YAP overexpression causes ovarian and prostate cancer cells to overcome contact-inhibition [57, 68].

1.2.4.2. Cell survival

Aside from increasing proliferation, downregulation of the Hippo tumor suppressor pathway causes insensitivity to apoptosis. Studies in cancer cell lines showed YAP overexpression promotes resistance to apoptosis induced by chemotherapeutic agents [68, 69]. This could be one of the mechanisms by which, deregulation of the Hippo pathway leads to therapy resistance.

1.2.4.3. Treatment-resistance

Inactivation of the Hippo tumor suppressor pathway results in activation of YAP and TAZ which are known to affect tumor cells' resistance to chemotherapy. A recent study evaluated YAP1 to be a major regulator of resistance to RAF (a proto-oncogene serine/threonine-protein kinase) inhibitor. Silencing of YAP1 increased cell sensitivity to RAF inhibitors across different tumor models—lung, melanoma, colon, thyroid and pancreatic cancer cell lines [70].

1.2.4.4. Stem cell features

It is well established that some cancer cells have stem cell capabilities including extensive replicative potential and loss of mature differentiation markers. These cancer stem cells are

hypothesized to persist in tumors as a distinct population; they could cause relapse and metastasize, giving rise to new tumors. Several studies have linked components of the Hippo pathway to stem cell features. YAP and TAZ can promote pluripotency characteristics of embryonic stem cells [71]. Gene expression profiling studies have shown that YAP1 and the TEAD transcription factors are enriched in multiple types of stem cells [72]. Also TAZ regulates mesenchymal stem cell differentiation [49, 73]. Mesenchymal cells are more invasive and therefore, YAP and TAZ hyperactivity might promote tumorigenic potential by enhancing stem-cell-like properties.

1.3. Hypothesis Formation

The Hippo pathway has been shown to cross talk with a number of other molecular pathways commonly altered in human cancers. Similarly, Hsp27 acts as a central hub connecting networks that promote tumor growth and progression. While there is extensive overlap between the cell survival, anti-apoptotic and metastatic pathways that Hsp27 and Hippo components may regulate, a relationship between the two has not yet been described.

1.3.1. Gene expression profiling as a method to paint a global picture of cellular function

Gene expression profiling quantifies expression patterns of genes in the cell. It is a particularly useful tool in studying cancer cells, where DNA microarrays in combination with statistical analysis have enabled researchers to determine the important genes in tumorigenesis or build classifiers based on expression profiling for many types of cancer such as breast, lung and prostate [74]. The oncogenic capacities of Hsp27 present scientists with a vast puzzle of cellular pathways interconnectedness. Our lab has been working extensively on tumorigenicity of Hsp27 in prostate cancer; we have shown that Hsp27 assists prostate cancer progression by increasing cell proliferation and survival, enhances migratory and invasive properties of prostate cancer cells and promotes AR signaling, the key player of this disease [28, 32, 33, 40]. Although these studies helped us put pieces of the puzzle of Hsp27 oncogenicity together, however, to better picture and understand its roles, we performed a gene expression

microarray in small interfering RNA (siRNA) Hsp27 treated PC3 prostate cancer cells and analyzed the results using Ingenuity Pathway Analysis (IPA).

1.3.2. Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) is a web-based software application that allows analysis of large datasets like microarray-based gene expression. IPA places genes into pathways based on previous published information and therefore by analyzing altered genes, it can be used to identify the most affected pathways.

Unbiased comparison of the gene expression signature in siScrambled (siScr) versus siHsp27 treated samples using IPA verified downregulation of major signaling pathways where Hsp27 has been shown to play a role **(Figure 1.4)**. Importantly, as illustrated in **Figure 1.5**, the most highly downregulated signaling pathways, including TGF-β, BMP, WNT/β-Catenin and ILK all utilize YAP/TAZ as transcriptional co-activators [48, 53, 55]. However, a link between YAP/TAZ and Hsp27 had not been established.

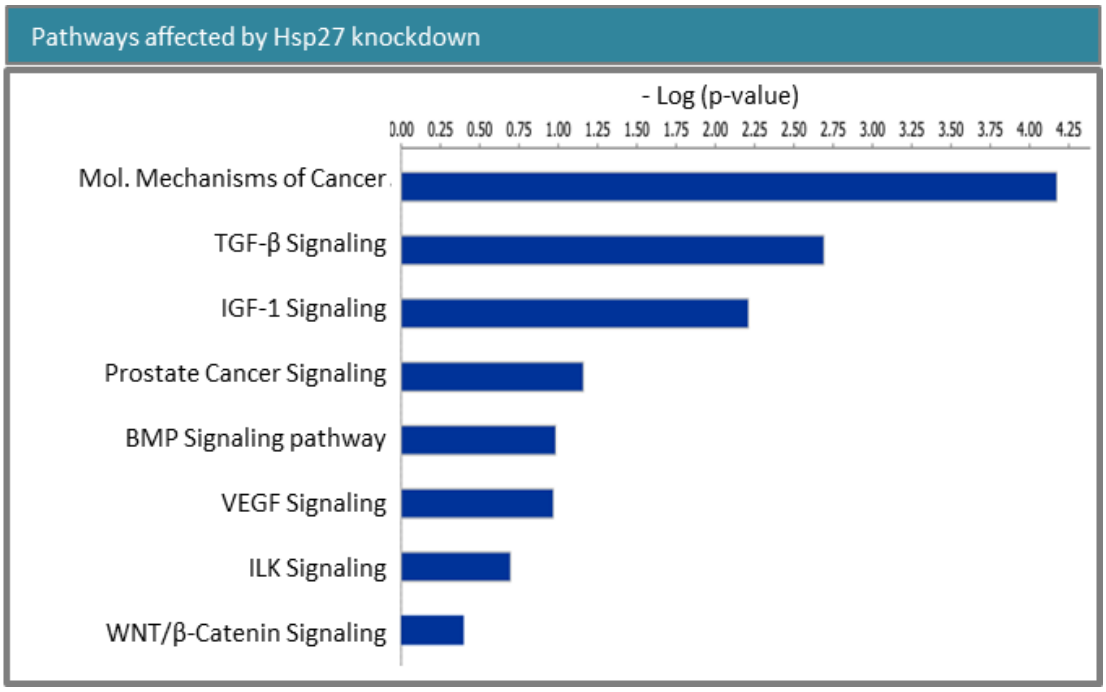


Figure 1.4. IPA analysis of top pathways affected in si Hsp27 PC3 cells compared to si Scr.

Pathway analysis shows knocking down Hsp27 in PC3 cells affects these pathways the most.

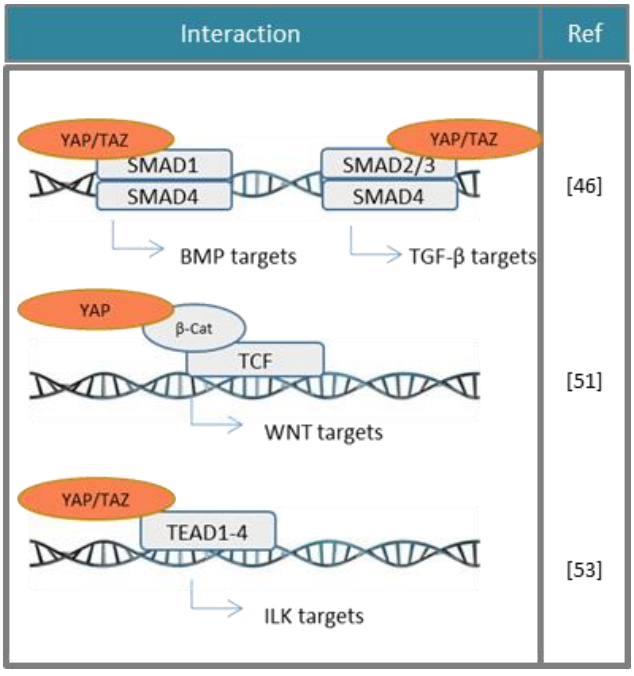


Figure 1.5. Previously reported regulation of TGF-β, BMP, WNT and ILK pathways by YAP/TAZ

1.3.3. Hypothesis

Gene expression profiling of PC3 prostate cancer cells showed that transient knock down of Hsp27 downregulates pathways dependent on YAP and TAZ. Since YAP and TAZ are predominantly inhibited by the Hippo pathway, we hypothesized that Hsp27 negatively regulates the Hippo tumor suppressor pathway.

2. Materials and Methods

2.1. Cell culture

Prostate cancer PC3 and lung cancer cell line A549 were purchased from the American Type Culture Collection (ATCC) and authenticated by isoenzymes analysis in 2008 and short tandem repeat (STR) profile in 2013 respectively. Both cell lines were maintained in RPMI media supplemented with 10% fetal bovine serum (FBS, Invitrogen-Life Technologies). Breast cancer cell line MDA-MB-453 (ATCC) and HSF1 knock-out murine embryonic fibroblasts (MEF-HSF1$^{-/-}$) (a kind gift from I. J. Benjamin, University of Utah) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies) containing 10% FBS.

2.2. Transfections

2.2.1. Small interfering RNA transfection

PC3, A549 and MDA-MB-453 were transfected with 20 nM Hsp27 siRNA (siHsp27) or control siRNA (siScr) using OligofectAMINE (Invitrogen-Life Technologies, Inc.) in Opti-MEM (Gibco) following the manufacturer procedures.

2.2.2. Plasmid transfection for overexpression

pHR-CMV Empty and wild type Hsp27 vectors (WT) were used as previously described [28]. Cells were seeded at 5 X 10^5 cells and 10^6 cells in 10 cm^2 dishes for siRNA and plasmid transfections respectively. Overexpression of MST1 (STK4) was achieved using pJ3H-MST1 plasmid purchased from Addgene (ID: 12203).

2.3. Gene Expression profiling

For differential expression profiling of siScr and siHs27 treated PC3 cells, total RNA was extracted using TRIzol® and the quality of RNAs were measured using Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA). Samples were prepared following Agilent's One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling v6.0. An input of 100ng of total RNA was used to generate Cyanine-3 labeled cRNA. Samples were then hybridized on Agilent

SurePrint G3 Human GE 8x60K Microarray (AMDID 028004) and arrays were scanned with the Agilent DNA Microarray Scanner at a 3um scan resolution. Data was processed with Agilent Feature Extraction 11.0.1.1. and processed signal was quantile normalized with Agilent GeneSpring 12.0. Normalized log2 values of YAP/TAZ target genes were between siHsp27 and siScr treated cells. All microarray profiling analyses were carried out in triplicate.

RNA quality control as well as gene profiling was done by Dr. Collins laboratory at Vancouver Prostate Centre, Vancouver, British Columbia, Canada.

2.4. Western blotting

RIPA lysis buffer was mixed with protease and phosphatase inhibitors and was used to lyse total proteins as previously described [40]. Nuclear/ cytoplasmic fractions were extracted using the CelLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich, St Louis, MO) according to manufacturer's protocol.

2.5. Immunoprecipitation

Immunoprecipitation was performed using ImmunoCruz™ IP/WB Optima B System (Santa Cruz) based on the manufacturer's guideline. 2 µg of primary antibody, or immunoglobulin G (IgG) was used for immunoprecipitation and control respectively. Blots were incubated overnight at 4 °C with designated primary antibodies at 1:1000 dilution, unless noted otherwise. Proteins were visualized using the Odyssey system (Li-Cor Biosciences) and densitometric analysis was performed using ImageJ software (National Institutes of Health, USA).

2.6. Reagents and antibodies

Antibodies against YAP (#4912), YAP/TAZ (# 8418), phospho-YAP S127 (# 4911), LATS1 (# 3477, 1:200), phospho-LATS1 Thr1079 (# 9159, 1:200), MOB1 (#3863), phosphor-MOB1T35 (#8699), MST1 (#3682) were purchased from Cell Signaling, USA, anti-14:3:3 ε (sc-393177), TAZ (sc-48805), Ubiquitin (sc-8017) and Lamin B1 (sc-377001) were from SantaCruz Biotechnology. Anti-LATS1 ab70562 (abcam) and anti-MOB1A/B sc-161867 (Santa Cruz) were used in the

mouse cell line with 1:500 dilution. Anti-Hsp27 (1:5000) was from ENZO lifesciences. MG132 was from Millipore (#474791).

2.7. Immunofluorescence

PC3 and MEF-HSF1$^{-/-}$ **c**ells were grown and transfected with siScr/siHsp27 and mock/Hsp27 respectively, in 10cm^2 plates. Then the cells were trypsinized and plated in 12 well plates at 5000 cells/cm^2 on coverslips submerged in RPMI+FBS 10% for 24 hours. Cells were then fixed and the Immunofluorescence was performed as we previously reported [40] using antibodies against YAP/TAZ (1:500) and 14.3.3 epsilon (1:200). DAPI was used to visualize nuclei and then the pictures were taken by 63x objective lens using Zeiss Axioplan II fluorescence microscope. Results are representative of random pictures taken from three independent experiments.

2.8. Quantitative real time PCR (qRT-PCR)

TRIzol® reagent (Invitrogen) was used to extract total RNA from cultured cells. 2 µg of total RNA was reversed transcribed using random hexamers (Applied Biosystems) as previously reported [33]. q-RT PCR amplification of cDNA was performed using the following primer pairs (sequences listed in table 2): Hsp27, YAP1, WWRT1, STK4, CTGF, CYR61, GLI2, FGF2, ARHGAP29, ANKRD1, GAPDH with FastStart Universal SYBR Green Master (ROX; Roche Applied Science) on the ABI PRISM 7900 HT Sequence Detection System. Target gene expression was normalized to GAPDH levels. The results represent three independent experiments with each sample run in triplicate.

2.9. Luciferase assay

For TEAD, TCF and SMAD transcriptional activity**,** 2 x 10^4 cells were plated in triplicate in six-well plates and transfections were carried out using TransIT®-2020 (Mirus Bio.) and 9 µg of indicated luciferase reporter for each plate: pGL3-OT for TCF [32], 8xGTIIC-luc for TEAD and SBE4-Luc for SMAD1-4. TEAD and SBE4 luciferase reporter were purchased from Add gene (IDs: 34615 and 16495 respectively). Luciferase activities were measured 48 hours after using the Dual-Luciferase Reporter Assay System (Promega) and a microplate luminometer, Tecan Infinite® 200 PRO (Tecan, Männedorf, Switzerland). The signal of firefly luciferase was

normalized to the total protein concentration of each well with the control condition set as one. All experiments were carried out in triplicate wells and repeated three times each triplicate well.

2.10. Public data mining

Two Publically available databases were used in this study:

2.10.1. CBioportal for Cancer Genomics

This website allows the researcher to visualize, download and analyze large-scale cancer studies such as The Cancer Genome Atlas (TCGA) studies for different cancers [75]. TCGA provisional studies (2015) for lung adenocarcinoma and invasive breast cancer were analyzed in order to illustrate the correlation between mRNA expression of HSPB1 (based on z score) and the score of phospho-YAP S127 in human tumor samples. Z-score for mRNA expression data is the number of standard deviations away from the mean of expression in the reference population. This measure is useful to determine whether a gene is up- or down-regulated relative to the normal samples or all other tumor samples.

2.10.2. The UCSC Xena Genome Browser (http://genome.ucsc.edu/)

This website is developed and maintained at the University of California Santa Cruz (UCSC) not only makes large-scale studies available for further analysis but also allows the researcher to visualize and analyze his/her data in the context of larger datasets [76]. Xena software was used to visualize the clinical correlation between mRNA expression of HSPB1 and following YAP/TAZ target genes in the prostate cancer TCGA dataset. Gene names: TAGLN, CCND2, AXL, CYR61, MET, FGF2, CAV1, RND3, IFI16, SNRPG, IFRD1, ID2, RHOU, MYOF, NT5E, CTGF, THBS1.

2.11. Statistical analyses

Data are representatives of three independent experiments and are expressed as mean ± standard error of the mean (SEM). P-values were calculated using Student *t*-test to compare control and treated groups and p-values less than 0.05 were considered statistically significant (*P< 0.05; **P< 0.001; ***P< 0.001).

3. Results

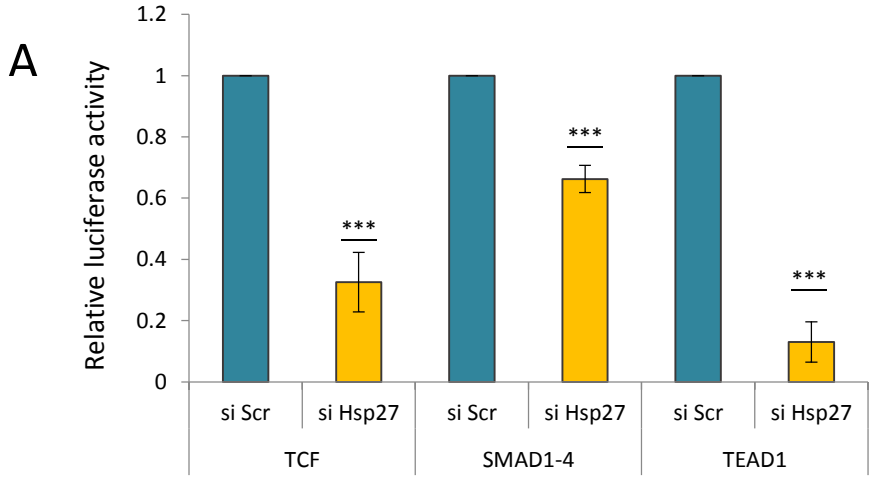
3.1. Identification of Hsp27 as a possible regulator of YAP/TAZ activity

3.1.1. The effect of Hsp27 knockdown on YAP/TAZ activity in prostate cancer cells

Hsp27 is known to regulate multiple molecular pathways that contribute to prostate cancer progression; however an investigation of a global gene expression pattern regulated by Hsp27 in prostate cancer cells has not been reported. In order to identify novel pathways affected by Hsp27 expression, we performed a gene expression microarray in small interfering RNA (siRNA) Hsp27 treated PC3 prostate cancer cells. As illustrated in **Figure 1.5** and **Figure 1.6**, the gene expression signature in siScrambled (siScr) versus siHsp27 treated samples showed downregulation of major signaling pathways that utilize YAP/TAZ as transcriptional co-activators including TGF-β, BMP, WNT/β-Catenin and ILK However a link between YAP/TAZ and Hsp27 had not been established.

3.1.2. Verification of WNT, TFG-β and TEAD pathway downregulation

Confirming IPA pathway analysis results, we showed that the transcriptional activities of TCF, SMAD1-4 and TEAD1, well-established readouts of YAP/TAZ activity [48, 53, 55] were significantly decreased in PC3 cells after Hsp27 knockdown compared to control **(Figure 3.1A)**.



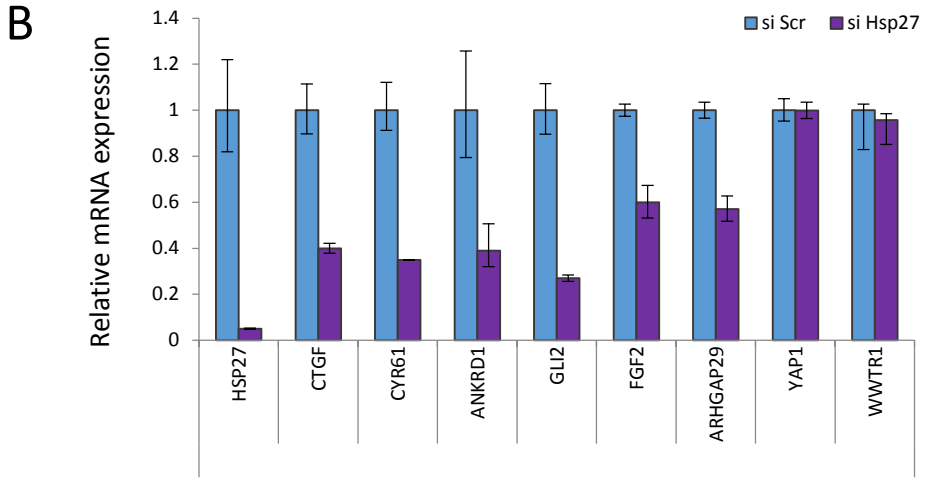


Figure 3.1. Hsp27 regulates YAP/TAZ transcriptional activity and downstream targets in Prostate Cancer.

(A) Relative activity of TCF, SMAD1-4 and TEAD1 assessed by luciferase assay in si Hsp27 PC3 cells compared to si Scr (=1), Graph represents pooled data from three independent experiments. **(B)** Relative mRNA expression of Hsp27 and YAP target genes in si Hsp27 PC3 cells compared to si Scr (=1). Graph is a representative of three independent experiments.

To further validate the regulation of YAP and TAZ by this chaperone, we tested the effect of siHsp27 on the expression of well-known YAP/TAZ target genes including CTGF, CYR61, ANKRD1, GLI2, FGF1 and ARHGAP29 expression in PC3 cells using qRT-PCR. Inhibition of Hsp27 resulted in significant downregulation of these genes, but not YAP and TAZ (WWTR1) themselves **(Figure 3.1B)**, suggesting that Hsp27 modulates YAP and TAZ activity but not their expression.

3.1.3. Generation of a heat map for YAP/TAZ gene signature in prostate cancer cells with Hsp27 loss of function

To further investigate the impact of Hsp27 on YAP/TAZ targets, a comprehensive list of YAP/TAZ target genes was compiled [73, 77, 78] **(Table 3.1).**

Table 3.1. List of YAP/TAZ target genes used to generate the heat map.

ACSL4	CDH2	EPB41L2	IDI1	MSMO1	RAB3B	SMAD6
ADAMTS1	CENPF	ERRFI1	IFI16	MYBL1	RGS4	SNAI2
ADAMTS12	CHST9	ESM1	IFIT2	MYC	RHOU	SNAPC1
ADAMTS5	CLDN1	EXPH5	IFRD1	MYOF	RIMKLB	SNORA75
ADRB2	COX6C	F2RL1	IL8	NID2	RND3	SNRPG
AHNAK	CPA4	F3	INSIG1	NT5DC3	S1PR1	SOX9
AMPH	CRIM1	FBXW11	IRS1	NT5E	SCD	SP1
ANXA1	CSNK2A1	FGF2	ITGBL1	OPN3	SCD5	SQLE
ANXA3	CTGF	FRZB	JPH1	PCLO	SCHIP1	STXBP1
AOX1	CTNNAL1	FSCN1	KIT	PDCD1LG2	SCML1	SYT14
AREG	CTNNB1	FST	KRT5	PDP2	SDPR	TFPI2
ARHGAP29	CYP1B1	FSTL1	LCP1	PHLDA1	SEMA3C	TGFA
ARHGEF28	DAAM1	GADD45A	LGALSL	PITX2	SERPINB7	TGFB2
ASAP1	DAB2	GCNT4	LHFP	PMAIP1	SERPINE1	THBS1
AXL	DDAH1	GGH	LMBRD2	PMP22	SGK1	TMEM154
BCL2	DIXDC1	GLI2	LPIN1	PRICKLE1	SHROOM3	TMEM27
BDNF	DUT	GLS	LRP6	PRRG1	SLC16A6	TOP2A
BICC1	ECT2	GPCPD1	LUM	PRSS23	SLC2A3	
CAV1	EMP1	HEXB	MACC1	PSAT1	SLIT2	
CCL28	EMP2	HMGCS1	MDFIC	PSG5	SMAD1	
CCND2	ENC1	HMMR	MET	PTPN14	SMAD3	
CD55	EP300	ID2	MID1	PYGO1	SMAD5	

Microarray expression of each target gene in siScr versus siHsp27 treated PC3 cells was compared. In accordance with our initial in vitro results, more than 70% of YAP/TAZ targets were downregulated in siHsp27 samples compared to control **(Figure 3.2)**.

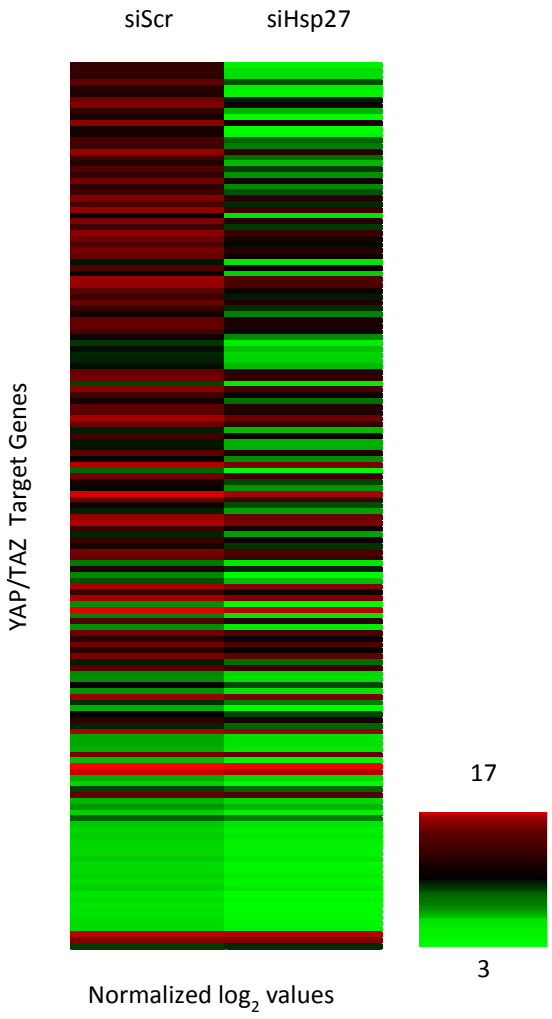


Figure 3.2. Gene expression profiling reveals that Hsp27 knockdown decreases YAP/TAZ transcriptional activity.

Microarray heat map showing expression of YAP/TAZ target genes in si Hsp27 PC3 cells compared to si Scr. Normalized log2 values were used to generate the heat map. (to see values please refer to Appendix 1)

3.1.4. Correlation of Hsp27 expression and YAP/TAZ target genes in human prostate cancer tissues

These in vitro findings were supported by clinical data showing that expression of Hsp27 (HSPB1) and a subset of these YAP/TAZ target genes were positively correlated in the publicly available TCGA database for prostate cancer tissues [76]. Figure 3.3 shows gene expression of YAP/TAZ targets stratified by the expression of Hsp27 in the same patient (**Figure 3.3**, The Cancer Genome Atlas, https://genome-cancer.ucsc.edu/).

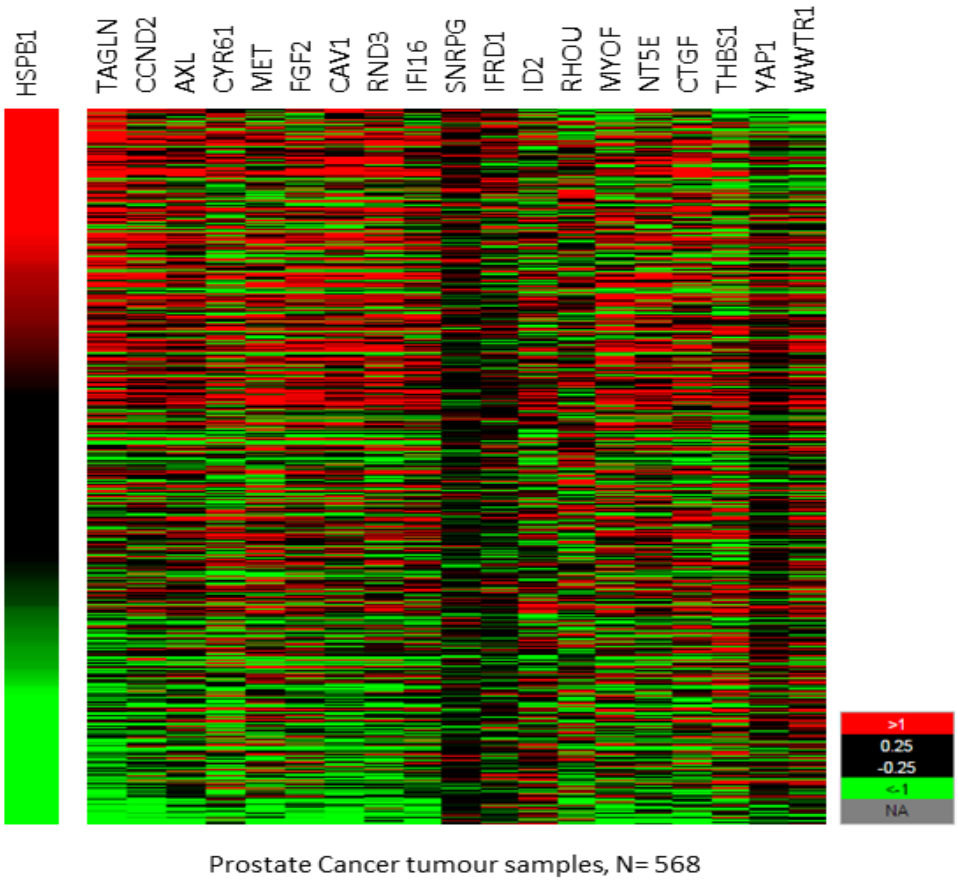


Figure 3.3. : Expression of Hsp27 correlates with YAP/TAZ target genes in prostate cancer clinical samples.

mRNA expression of Hsp27 (HSPB1) and YAP/TAZ target genes obtained from the Prostate Cancer TCGA data set show a positive correlation.

Taken together, our data suggest that Hsp27 knock-down induces a decrease in the activity of YAP/TAZ cooperating transcription factors leading to a downregulation of their targets, a hypothesis supported by both in vitro model and human tumor samples.

3.1.5. The effect of Hsp27 knockdown in lung and breast cancer cells

Similar to what we had illustrated in PC3 prostate cancer cells, we investigated if knocking down Hsp27 negatively affects YAP activity in other cancer types. For that we used a luciferase assay to measure transcription activity of TCF, SMAD1-4 and TEAD1 in A549 lung cancer and MDA-MB-453 triple negative breast cancer cells **(Figure 3.4)**.

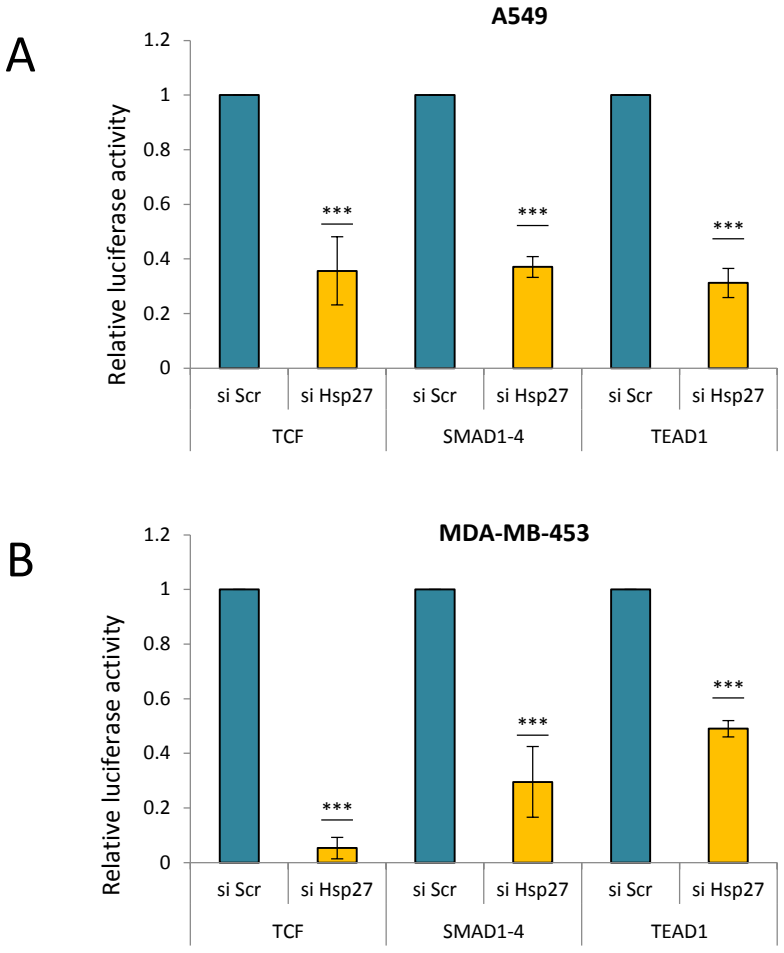


Figure 3.4. Hsp27 regulates YAP/TAZ transcriptional co-activity in Lung and Breast Cancer cell lines.

Relative transcriptional activity of TCF, SMAD1-4 and TEAD1 assessed by luciferase assay in **(A)** si Hsp27 A549 and **(B)** MDA-MB-453 cells compared to si Scr (=1), Graph represents pooled data from three independent experiments.

Similar to what we observed in PC3 cells, the transcriptional activity of TCF, SMADs and TEAD1 decreases upon Hsp27 siRNA treatment in A549 and MDA-MB-453 cells.

Next we confirmed that YAP/TAZ target genes decreased upon Hsp27 knockdown in lung and breast cell lines while the transcription levels of YAP and TAZ are not affected **(Figure 3.5)**.

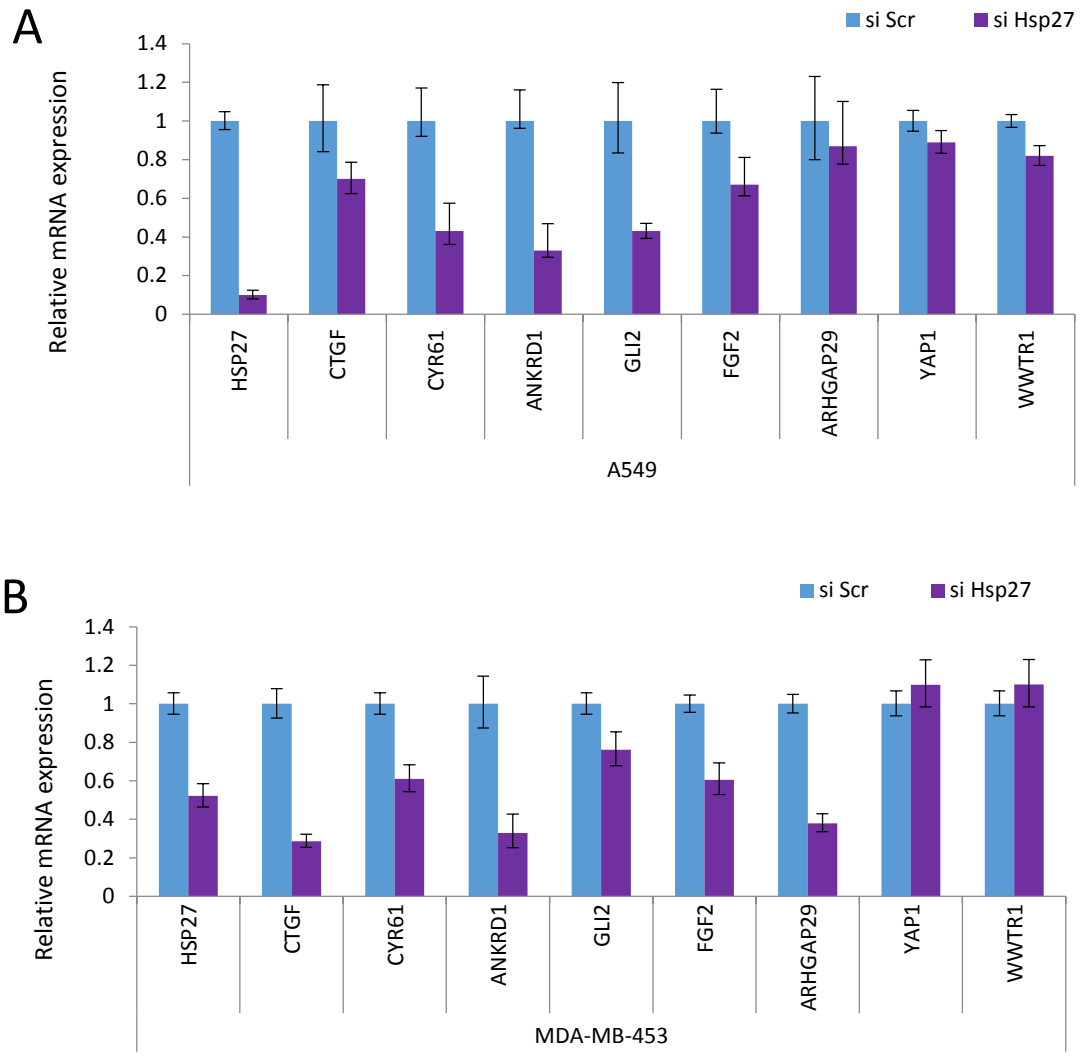


Figure 3.5. Hsp27 regulates YAP/TAZ target genes in Lung and Breast Cancer cell lines.

Relative mRNA expression of Hsp27, YAP, TAZ (WWTR1) and YAP/TAZ target genes in **(A)** si Hsp27 A549 and **(B)** MDA-MB-453 cells compared to si Scr (=1). Graphs are representatives of three independent experiments for each cell line.

3.2. Hsp27 is required for YAP activation and nuclear translocation in cancer cells

3.2.1. Investigating the effect of Hsp27 on YAP phosphorylation in vitro

Our results suggested that Hsp27 affects YAP activity without affecting its transcription **(Figure 3.1B)**. Post-translationally, YAP activity is inhibited by phosphorylation on S127 which prevents its nuclear translocation [46]. As shown in **Figure 3.6A**, siRNA inhibition of Hsp27 lead to increased S127 phosphorylation of YAP in PC3 prostate, A549 lung and MDA-MB-453 triple negative breast cancer cells. Reciprocally, Hsp27 overexpression decreased the inhibitory phosphorylation of YAP on S127 in PC3 and A549 cells compared to control **(Figure 3.6B)**. We further tested this phenomenon using a mouse embryonic fibroblast cell line that lack heat shock factor protein 1 (HSF 1) (MEF-HSF1$^{-/-}$), a transcription factor required to initiate transcription of all HSPs; these cells therefore do not express Hsp27 and provide a useful model to study the effects of Hsp27 overexpression on the Hippo pathway. In accordance with our observations in PC3 and A549 cells, phosphorylation of YAP on S127 decreased upon Hsp27 plasmid transfection **(Figure 3.6B)** in MEF-HSF1$^{-/-}$ cells.

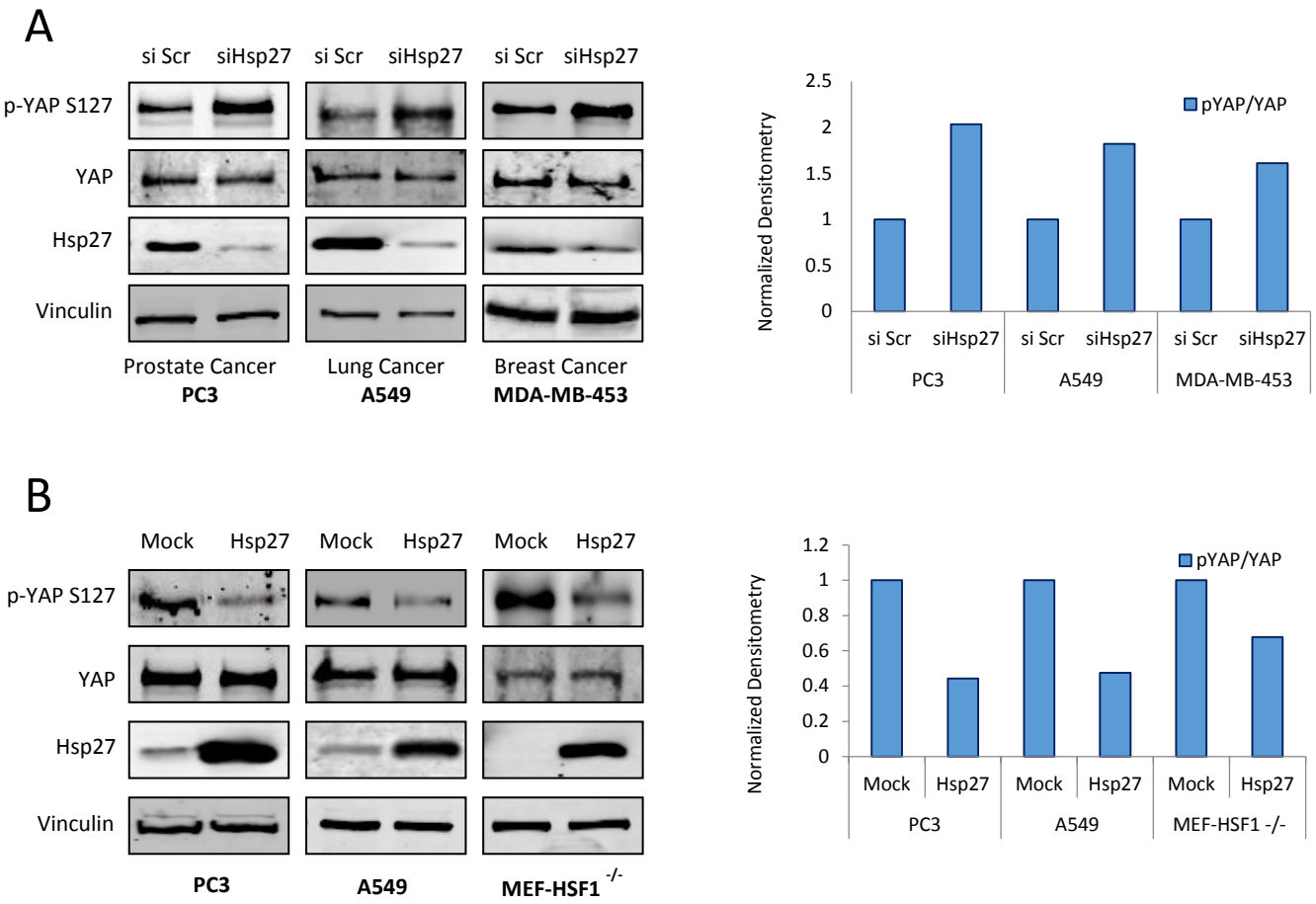
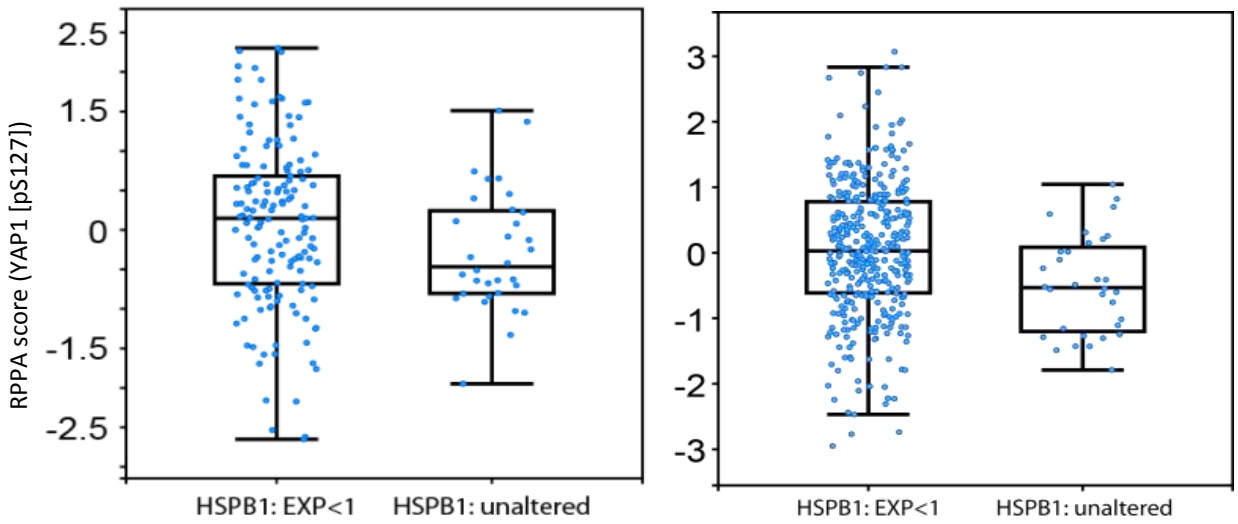


Figure 3.6. Hsp27 is required for YAP activation in vitro.

(A,B) Protein expression of p-YAP S127, YAP, Hsp27 and Vinculin in PC3, A549, MDA-MB-453 and/or MEF-HSF1$^{-/-}$ cells transfected with **(A)** si Hsp27 or si Scr or **(B)** Hsp27 or vector control (mock). Densitometry shows fold expression of p-YAP S127 compared to total YAP (=1).

3.2.2. Investigating the effect of Hsp27 on YAP phosphorylation in human tumor samples

To validate the clinical relevance of our findings, we interrogated the RPPA (reverse phase protein lysate microarray) score of p-YAP S127 in Hsp27 overexpressing tumors found in the cBioportal repository (www.cbioportal.org) [75]. **Figure 3.7** illustrates statistically significant up-regulation of p-YAP S127 in lung **(Figure 3.7 left)** and breast **(Figure 3.7 right)** tumor samples that have lower Hsp27 (EXP<1) compared to samples with unaltered Hsp27.



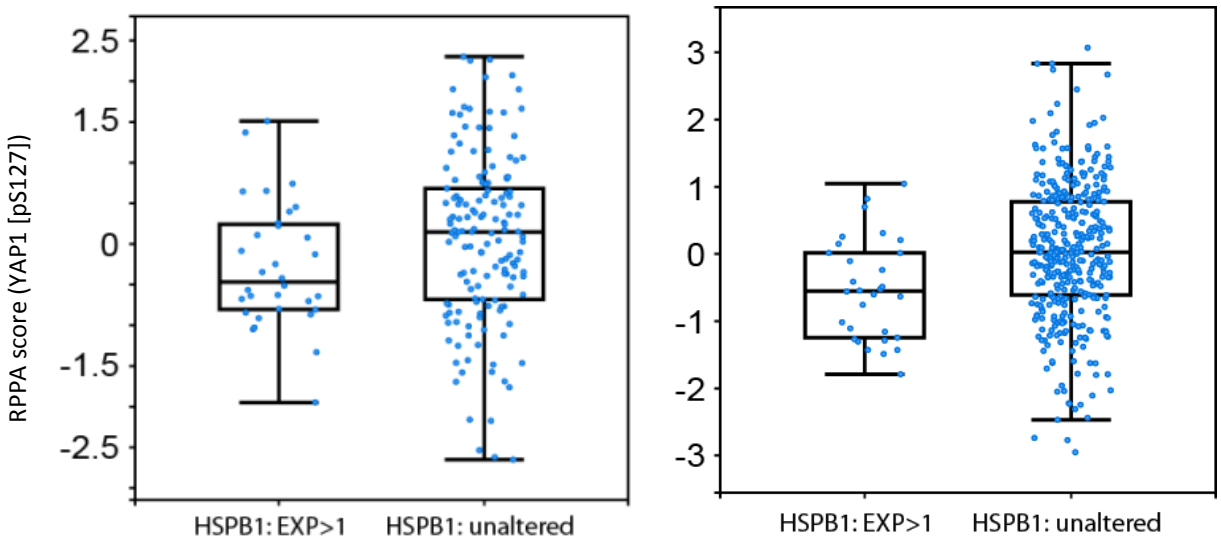
Lung Adenocarcinoma
(TCGA, Provisional)
N= 578 samples
p-value: 0.0421869467

Breast Invasive Carcinoma
(TCGA, Provisional)
N= 1104 samples
P-value: 0.0003983248

Figure 3.7. Low expression of Hsp27 is correlated with higher YAP phosphorylation in human tumors.

RPPA score of p-YAP S127 in Lung Adenocarcinoma and Invasive Breast Carcinoma in tumors with Hsp27 (HSPB1) expression less than 1 z-score obtained from TCGA data sets. (For the definition of z score please refer to 2.10.1)

The opposite trend was observed in tumors that have higher Hsp27 expression (EXP>1); lung **(Figure 3.8 left)** and breast **(Figure 3.8 right)** cancer tissue samples with over-expression of Hsp27 showed significantly lower phosphorylation on YAP S127 **(Figure 3.8)**.



Lung Adenocarcinoma (TCGA, Provisional)
N= 578 samples
p-value: 0.0421869467

Breast Invasive Carcinoma (TCGA, Provisional)
N= 1104 samples
P-value: 0.0006086191

Figure 3.8. High expression of Hsp27 is correlated with lower YAP phosphorylation in human tumors.

RPPA score of p-YAP S127 in Lung Adenocarcinoma and Invasive Breast Carcinoma in tumors with Hsp27 (HSPB1) expression more than 1 z-score obtained from TCGA data sets. (For the definition of z score please refer to 2.10.1)

These data combined with our in vitro findings further support a role for Hsp27 in the negative regulation of the Hippo pathway in human cancer.

3.2.3. The effect of Hsp27 expression on YAP nuclear localization

Phosphorylation of YAP on S127 prevents its translocation to the nucleus. Based on our results showing Hsp27 affects p-YAP levels **(Figure 3.6)**, we investigated the localization of YAP upon changes in Hsp27 expression. In accordance with our results showing Hsp27 knockdown increases YAP S127 phosphorylation, immunofluorescence showed extensive cytoplasmic localization of YAP in PC3 upon Hsp27 knockdown compared to siScr treated cells, in which YAP remained mostly nuclear **(Figure 3.9 top)**. This phenomenon was also captured by western blot

of samples which recieved Hsp27 siRNA (+), compared to control (-) **(Figure 3.9 bottom)**. Laminb1 was used as a loading control for nuclear fraction.

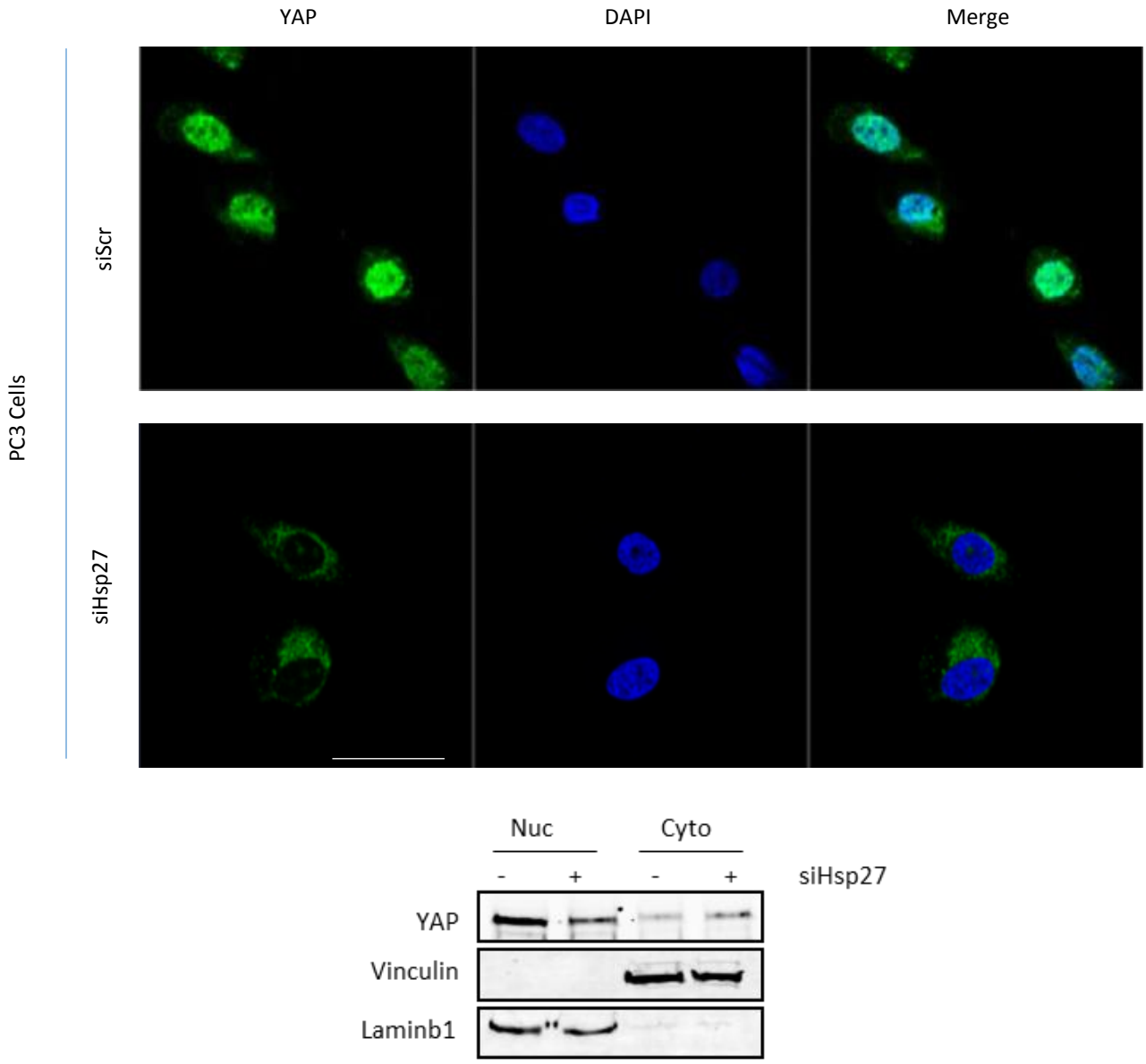


Figure 3.9. Hsp27 knockdown decreases YAP nuclear translocation.

(Top) Immunofluorescence of total YAP (green) and the nucleus (DAPI, blue) in PC3 cells transfected with si Hsp27 or si Scr. Scale bar: 50 µm. **(Bottom)** Western blots show nuclear and cytoplasmic levels of total YAP, Vinculin and Laminb1 in siRNA knockdown of Hsp27.

Reciprocally, in MEF-HSF1$^{-/-}$ cells, immunofluorescence showed nuclear localization of YAP increased after Hsp27 over-expression compared to mock transfected cells **(Figure 3.10**

top), and cyto/nuclear fractionation of MEF-HSF1$^{-/-}$ cells clearly demonstrated the translocation of YAP upon Hsp27 overexpression (+), compared to control (-) **(Figure 3.10 bottom)**.

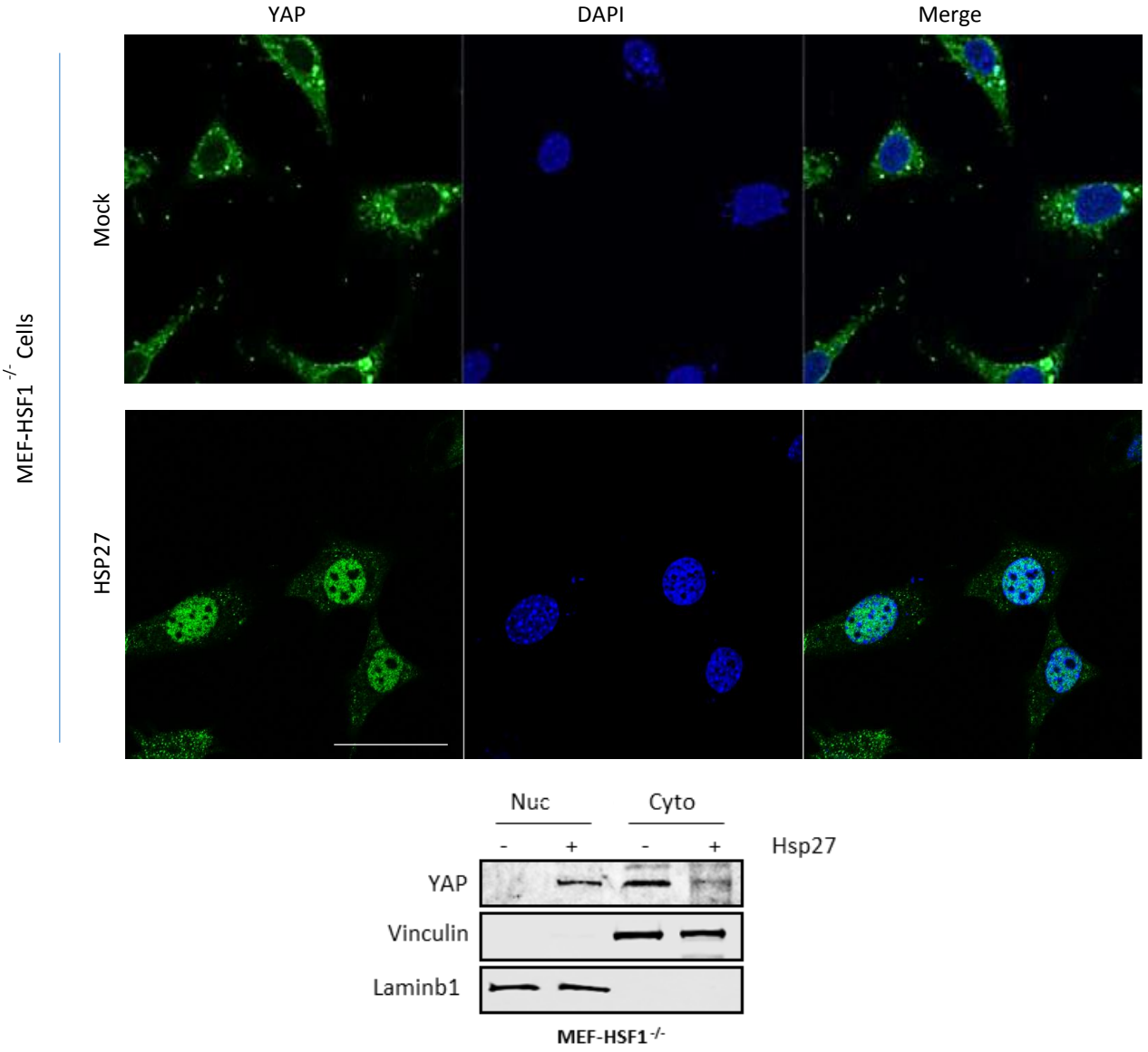


Figure 3.10. Hsp27 expression increases YAP nuclear translocation.

(Top) Immunofluorescence of total YAP (green) and the nucleus (DAPI, blue) in PC3 cells transfected with Mock or Hsp27 plasmid. Scale bar: 50 µm. **(Bottom)** western blots show nuclear and cytoplasmic levels of total YAP, Vinculin and Laminb1 in overexpression of Hsp27.

Taken together, these findings indicate that expression of Hsp27 controls YAP activity by affecting its phosphorylation and subsequent nuclear translocation.

3.3. Hsp27 expression regulates YAP/TAZ cytoplasmic sequestration

Phosphorylation of YAP and its paralogue TAZ lead to their sequestration in the cytoplasm by the 14.3.3 proteins [46]. Concordantly, we observed that Hsp27 silencing resulted in not only increased cytoplasmic sequestration, but also in significant co-localization of YAP/TAZ with 14.3.3 in PC3 cells **(Figure 3.11A)**. An increase in interaction between 14.3.3 and TAZ was also observed upon Hsp27 siRNA treatment **(Figure 3.11B)**.

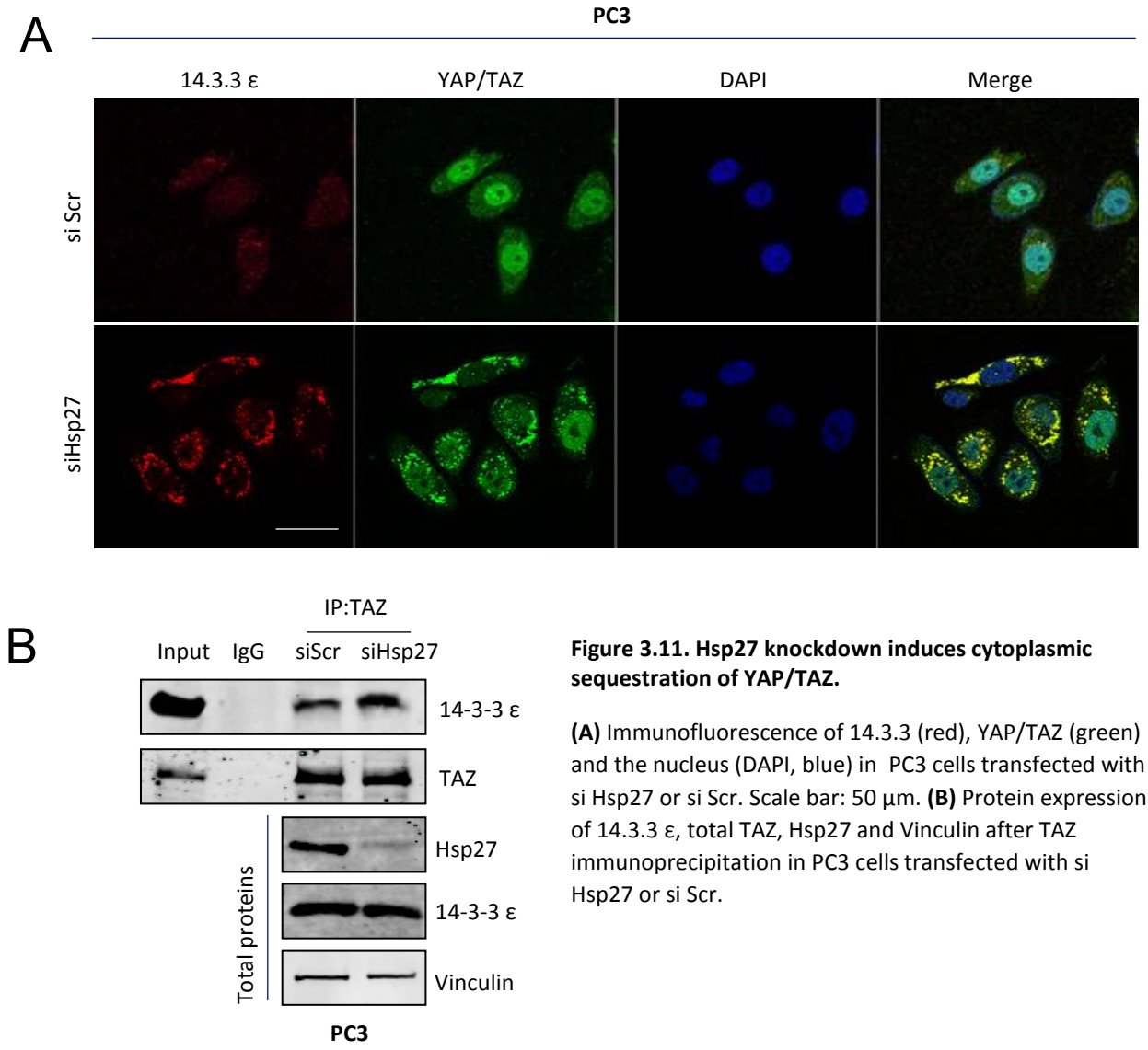


Figure 3.11. Hsp27 knockdown induces cytoplasmic sequestration of YAP/TAZ.

(A) Immunofluorescence of 14.3.3 (red), YAP/TAZ (green) and the nucleus (DAPI, blue) in PC3 cells transfected with si Hsp27 or si Scr. Scale bar: 50 µm. **(B)** Protein expression of 14.3.3 ε, total TAZ, Hsp27 and Vinculin after TAZ immunoprecipitation in PC3 cells transfected with si Hsp27 or si Scr.

Reciprocally, YAP/TAZ were more nuclear and less sequestered with the 14.3.3 proteins upon Hsp27 overexpression in MEF-HSF1$^{-/-}$ cells **(Figure 3.12A)**. This decreased interaction between TAZ and 14.3.3 proteins was again observed by immunoprecipitation in MEF-HSF1$^{-/-}$ cells after Hsp27 overexpression **(Figure 3.12B)**.

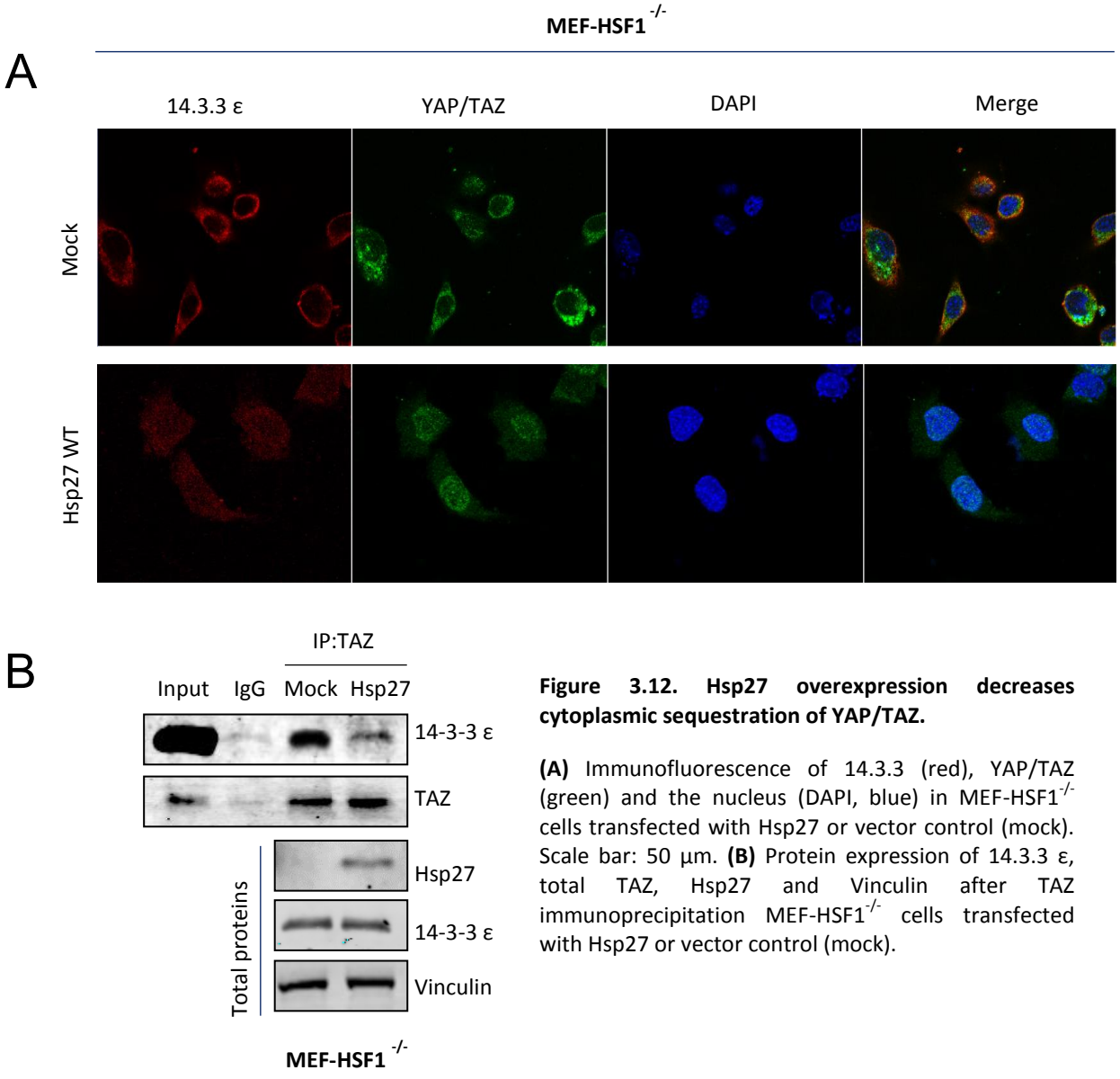


Figure 3.12. Hsp27 overexpression decreases cytoplasmic sequestration of YAP/TAZ.

(A) Immunofluorescence of 14.3.3 (red), YAP/TAZ (green) and the nucleus (DAPI, blue) in MEF-HSF1$^{-/-}$ cells transfected with Hsp27 or vector control (mock). Scale bar: 50 µm. **(B)** Protein expression of 14.3.3 ε, total TAZ, Hsp27 and Vinculin after TAZ immunoprecipitation MEF-HSF1$^{-/-}$ cells transfected with Hsp27 or vector control (mock).

Together, these findings suggest that Hsp27 regulates YAP and TAZ activity through inhibition of phosphorylation, decreasing their sequestration by 14.3.3 proteins and promoting their translocation to the nucleus.

3.4. Hsp27 regulates the Hippo pathway by affecting core kinase components

3.4.1. The effect of Hsp27 knockdown on LATS1 and MOB1

Our results indicate that Hsp27 modulates YAP phosphorylation status, which is known to be controlled by the Hippo pathway through a core kinase cascade involving LATS1, MOB1 and MST1; however, a relationship between these kinases and Hsp27 has never been described. To examine the effect of Hsp27 in regulating the phosphorylation of YAP in more detail, PC3, A549 and MDA-MB-453 cells were treated with Hsp27 siRNA and functional changes in the Hippo pathway core components were examined. YAP is directly phosphorylated and restrained by LATS1, which is active when it is phosphorylated on T1079 [79]. As shown in **Figure 3.13**, knocking down Hsp27 resulted in increased p-LATS1 (T1079) across three cell lines, while the total protein remained unchanged. Looking further upstream in the Hippo pathway, we observed that phosphorylation of MOB1 on T35, which is required for activation of LATS1 [80] was also increased in siHsp27 treated samples compared to siScr **(Figure 3.13)**.

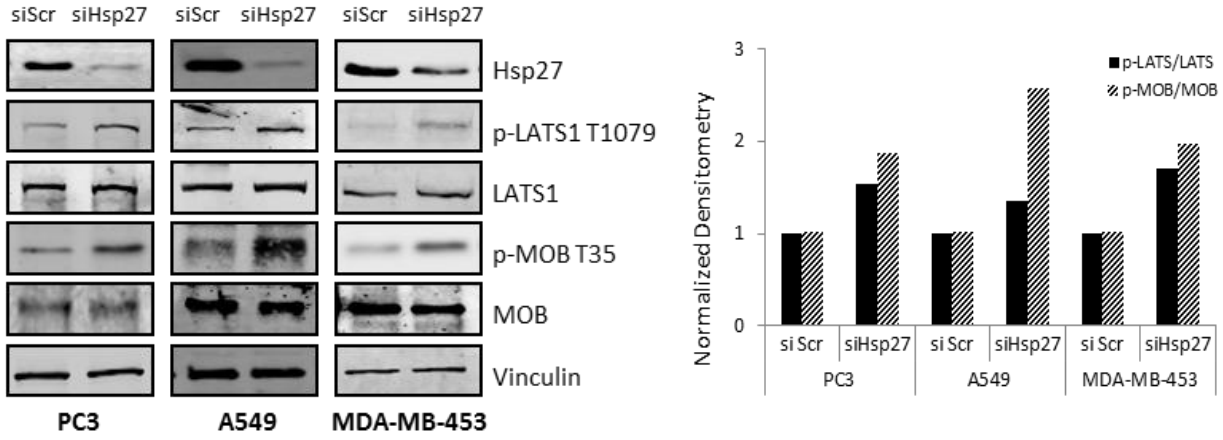


Figure 3.13. Hsp27 knockdown activates the Hippo pathway kinases.

Protein expression of Hsp27, p-LATS1 T1079, total LATS1, p-MOB T35, total MOB1 and Vinculin in PC3, A549 and MDA-MB-453 cells transfected with control or Hsp27 siRNA. Densitometry shows fold expression of p-LATS1 T1079 compared to total LATS1 (=1) and p-MOB1 T35 compared to total MOB1 (=1).

3.4.2. The effect of Hsp27 overexpression on LATS1 and MOB1

Reciprocally, we observed that Hsp27 overexpression In PC3 and MEF-HSF1$^{-/-}$ cells drastically decreased phosphorylation of LATS1 (T1079) and MOB1 (T35) in both cell lines **(Figure 3.14)**.

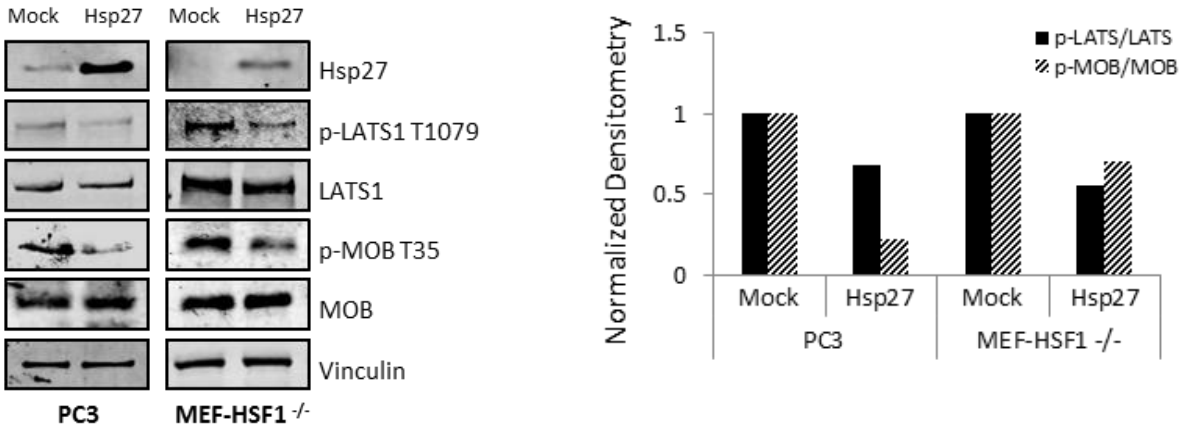


Figure 3.14. Hsp27 overexpression inactivates the Hippo pathway kinases.

Protein expression of Hsp27, p-LATS1 T1079, total LATS1, p-MOB T35, total MOB1 and Vinculin in PC3 and MEF-HSF1$^{-/-}$ cells transfected with control or Hsp27 plasmid. Densitometry shows fold expression of p-LATS1 T1079 compared to total LATS1 (=1) and p-MOB1 T35 compared to total MOB1 (=1).

3.4.3. The effect of Hsp27 expression on MST1, the core Hippo kinase

Finally, we examined the effects of Hsp27 modulation on MST1, the mammalian homologue of Drosophila's Hippo kinase that is directly responsible for phosphorylation of LATS1 and MOB1 [80]. In accordance with changes observed in pLATS1 and pMOB1, we found that Hsp27 loss of function increased MST1 total protein **(Figure 3.15 left)**, while overexpression of Hsp27 decreased total MST1 across multiple cell lines **(Figure 3.15 right)**. Importantly however, RT-PCR analysis of MST1 mRNA in these conditions showed no change in transcription **(Figure 3.16)**.

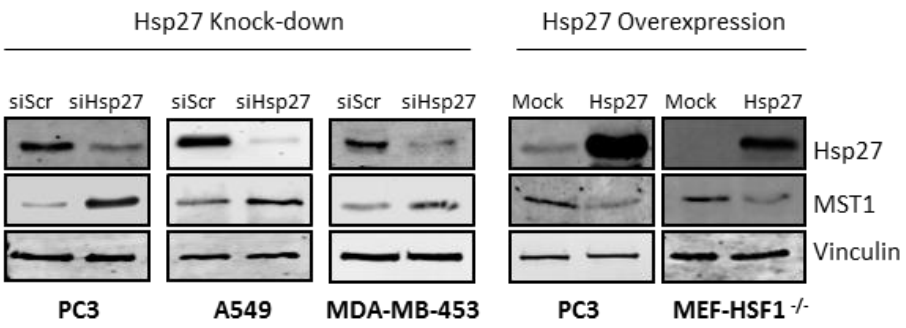


Figure 3.15. Hsp27 expression regulates MST1 at protein level.

Protein expression of Hsp27, MST1 and Vinculin in PC3, A549, MDA-MB-453 and/or MEF-HSF1-/- cells transfected with si Hsp27 **(left panel)** or Hsp27 plasmid **(right panel)** compared to control (si Scr or Mock transfected cells).

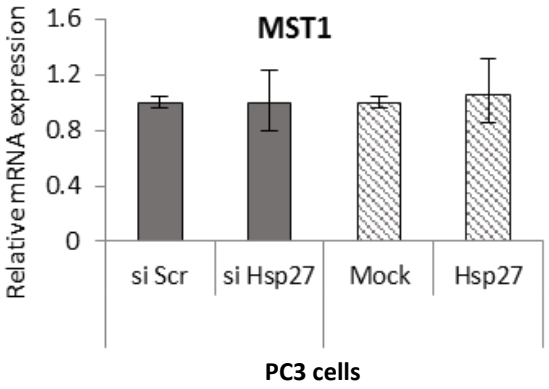


Figure 3.16. Hsp27 expression does not affect MST1 transcription.

Relative mRNA expression of MST1 in PC3 cells transfected with si Hsp27 or Hsp27 plasmid compared to control (si Scr or Mock transfected cells=1). Graph is a representative of three independent experiments.

Levels of MST1 mRNA were also tested in lung and breast cancer cell lines upon Hsp27 knockdown **(Figure 3.17).** These data clearly demonstrate that Hsp27 inhibits the Hippo tumor suppressor pathway by regulating MST1 at the protein level.

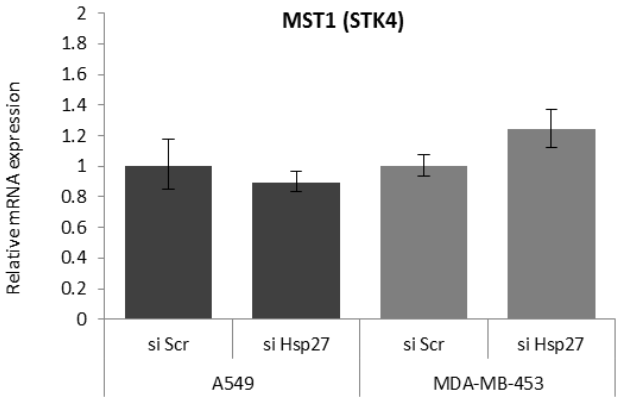


Figure 3.17: Hsp27 regulation of MST1 mRNA in Lung and Breast Cancer cells.

Relative mRNA expression of MST1 (STK4) in si Hsp27 A549 and MDA-MB-453 cells compared to si Scr (=1), Graph represents pooled data from three independent experiments.

3.4.4. The effect of antisense oligonucleotide OGX-427 on the Hippo pathway

OGX-427 is an antisense oligonucleotide designed for Hsp27 and is already in phase 2 clinical trials for different cancers. In order to verify our findings on YAP phosphorylation we tested the effects of OGX-427 on PC3 and A549 cell lines. Similar to our results in **Figure 3.6** and **Figure 3.15** left, knockdown of Hsp27 by OGX-427 increases p-YAP S127 and MST1 respectively (**Figure 3.18**).

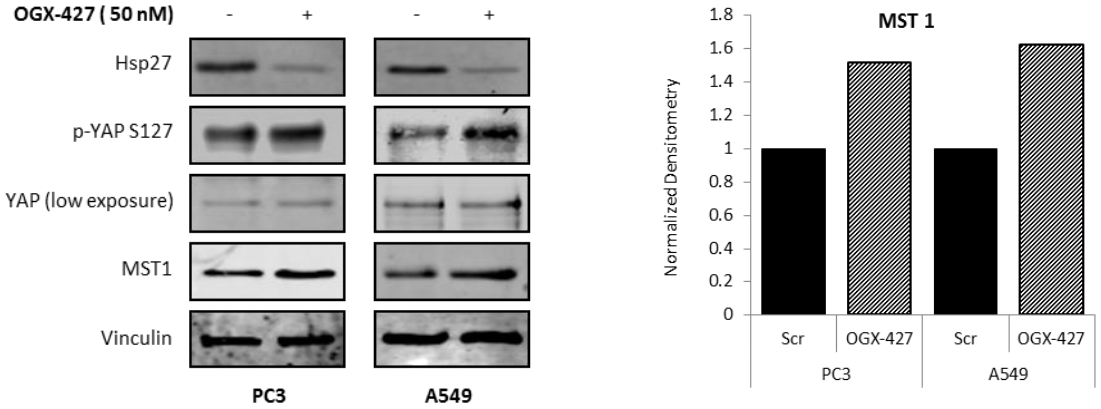


Figure 3.18. OGX-427 activates the Hippo tumor suppressor pathway.

Protein expression of Hsp27, p-YAP S127, YAP, MST1 and Vinculin in PC3 and A549 cells transfected with scrambled oligonucleotide or OGX-427. Densitometry shows fold expression of MST1 in OGX-427 treated cells compared to control (=1)

3.5. Hsp27 forms a complex with MST1 and facilitates proteasomal degradation of ubiquitinated-MST1

3.5.1. Hsp27 forms a complex with MST1 in cancer cells

In an effort to understand the exact mechanism by which Hsp27 regulates protein levels of MST1, we first performed immunoprecipitation (Co-IP) to unveil protein-protein interactions between MST1 and Hsp27. We found that endogenous Hsp27 formed a complex with MST1 in PC3 cells **(Figure 3.19A, top)**. This interaction was confirmed with the reciprocal immunoprecipitation **(Figure 3.19A, bottom)** and was also observed in A549 cells **(Figure 3.19B)**.

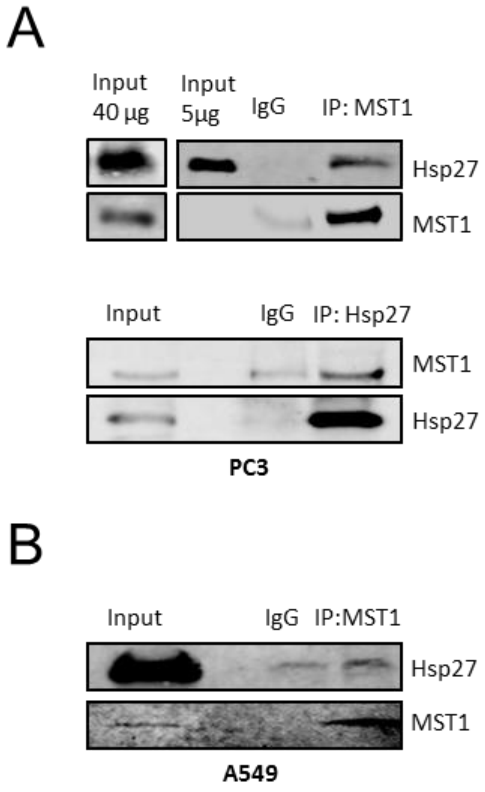


Figure 3.19. Endogenous Hsp27 forms a complex with MST1 in cancer cells.

(A) Protein expression of Hsp27, MST1 and IgG after MST1 **(top)** and Hsp27 **(bottom)** immunoprecipitation in PC3 cells. **(B)** Protein expression of Hsp27, MST1 and IgG after MST1 immunoprecipitation in A549 cells.

3.5.2. Hsp27 regulates the proteasomal degradation of MST1

Since Hsp27 can enhance the proteasomal degradation of ubiquitinated proteins [34, 39, 81] and MST1 degradation is ubiquitin-mediated [82, 83] we tested if Hsp27 overexpression or silencing affects levels of ubiquitinated MST1. By pulling down MST1 and blotting for ubiquitin, we observed that overexpression of Hsp27 in PC3 cells lead to decreased amount of ubiquitinated MST1 **(Figure 3.20A, left**) while knocking down Hsp27 resulted in increased levels of ubiquitinated MST1 **(Figure 3.20A, right)**. Moreover, a decrease in ubiquitinated MST1 was observed in MEF-HSF1$^{-/-}$ cells upon Hsp27 introduction **(Figure 3.20B)**. These findings demonstrate that Hsp27 binds to MST1 and facilitates the proteasomal degradation of ubiquitinated MST1.

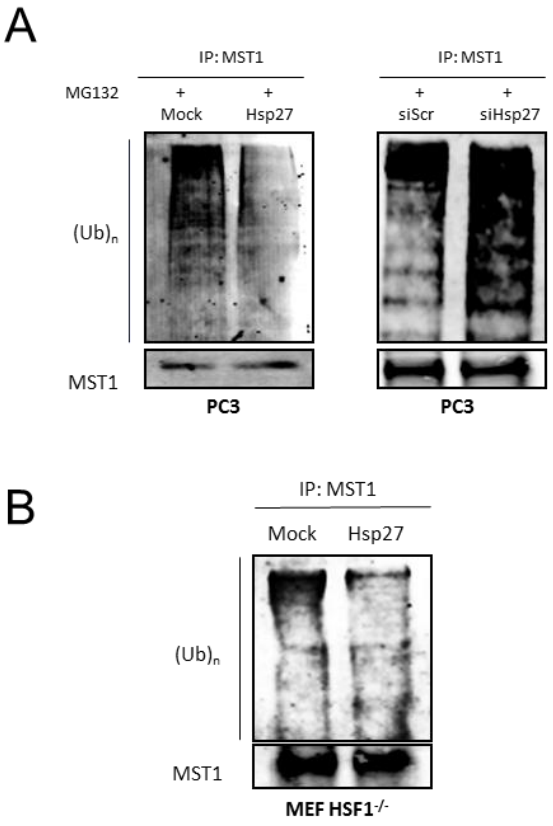


Figure 3.20. Hsp27 facilitates the proteasomal degradation of MST1 in cancer cells.

(A) Protein expression of ubiquitin and MST1 after MST1 immunoprecipitation in Hsp27 plasmid (left) or si Hsp27 (right) transfected PC3 cells treated with MG132. **(B)** Protein expression of MST1 and ubiquitin after MST1 immunoprecipitation in MEF HSF1-/- cells transfected with Hsp27 or vector control (mock).

3.5.3. The effect of Hsp27 on MST1 is not reciprocal

To better understand the interaction between Hsp27 and MST1, we overexpressed MST1 in PC3 cells and looked at the protein changes by western blotting. In harmony with what was reported previously [84] MST1 overexpression resulted in YAP inactivation in PC3 cells displayed by increased p-YAP S127, however no change was observed in the expression of Hsp27 **(Figure 3.21A)**, suggesting that the effect of Hsp27 on MST1 is not reciprocal. We further confirmed inactivation of YAP by demonstrating decreased transcriptional activity of YAP's transcriptional partners: TCF, SMAD1-4 and TEAD1 **(Figure 3.21B)**.

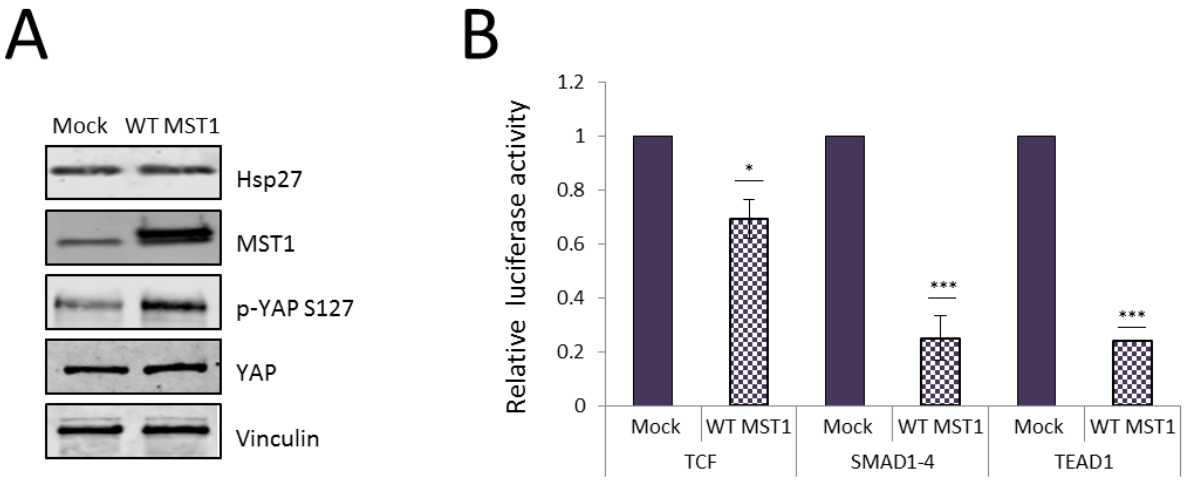


Figure 3.21. Hsp27 facilitates the proteasomal degradation of MST1 in cancer cells.

(A) Protein expression of Hsp27, MST1, p-YAP S127, total YAP and Vinculin in PC3 cells transfected with MST1 (WT MST1) and vector control (mock). **(B)** Relative activity of TCF, SMAD1-4 and TEAD1 assessed by luciferase assay in PC3 cells transfected with MST1 compared to vector control (mock=1). Graph represents pooled data from three independent experiments.

3.5.4 Hsp27 knockdown does not affect Hsp70 levels

MST1 is reported to be regulated by Hsp70 [83], therefore we investigated the effect of Hsp27 siRNA on levels of Hsp70. **Figure 3.22** shows that knocking down Hsp27 does not affect protein expression of Hsp70.

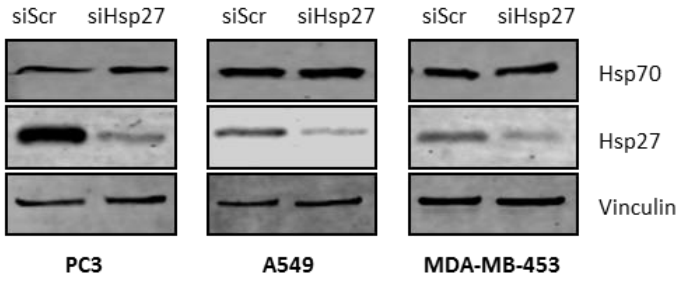


Figure 3.22. Hsp27 knockdown does not affect Hsp70 expression in Prostate, Lung or Breast Cancer.

Protein expression of Hsp70, Hsp27 and vinculin in PC3, A549 and MDA-MB-453 cells transfected with si Hsp27 or si Scr.

3.6. Hsp27 negatively regulates the Hippo tumor suppressor pathway: A schematic summary

Based on our accumulated in vitro data, we propose the following mechanism on how Hsp27 negatively regulates the Hippo tumor suppressor pathway. Overexpression of Heat shock protein 27 in cancer cells inactivates the Hippo tumor suppressor pathway via facilitating the degradation of ubiquitinated MST1 resulting in disruption of the Hippo kinase cascade. Degradation of MST1 protein results in decreased LATS1 and MOB1 phosphorylation/activation leading to decreased YAP/TAZ phosphorylation/inactivation, and ultimately increased YAP/TAZ nuclear localization. Translocation of YAP and TAZ from cytoplasm to nucleus drives transcription of genes associated with malignant cell phenotypes **(Figure 3.23)**.

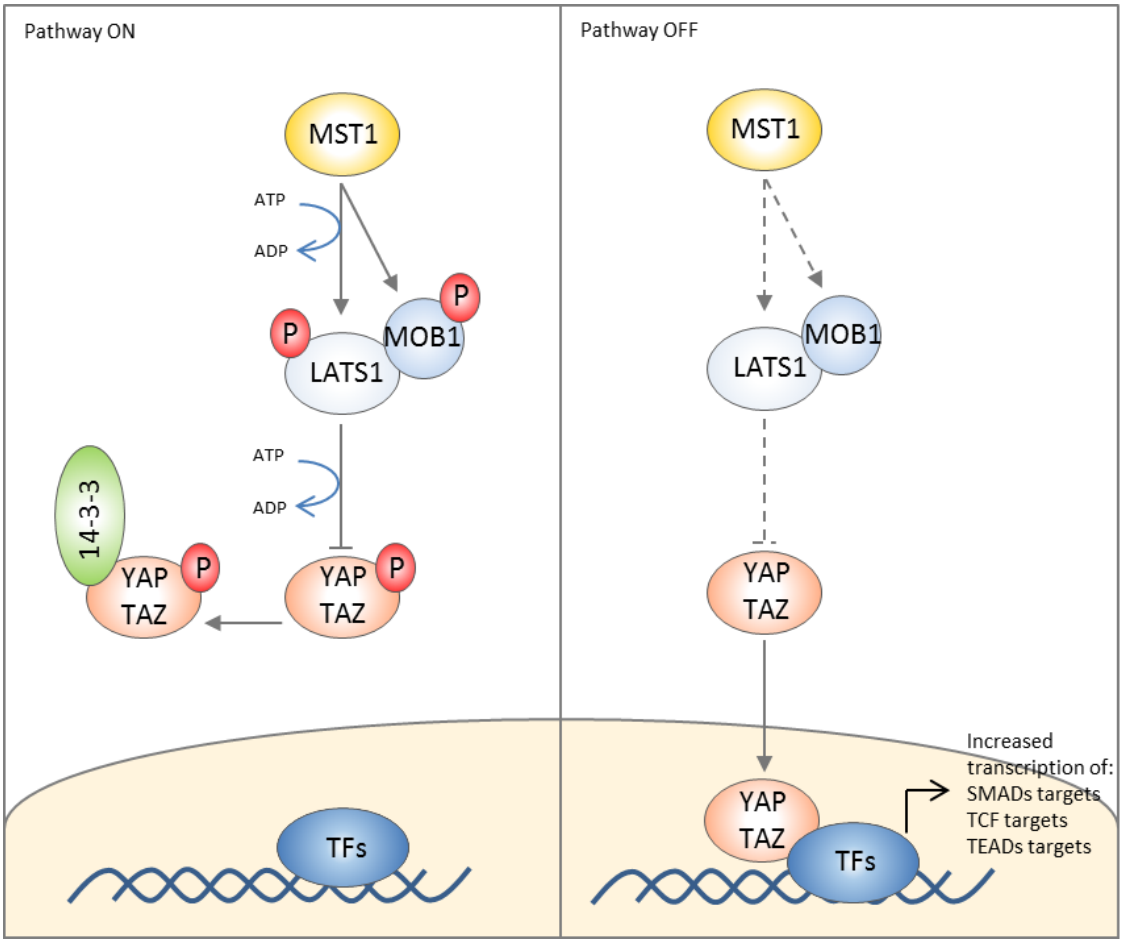


Figure 3.23: Proposed mechanism of negative regulation of the Hippo tumor suppressor pathway by Hsp27.

(Pathway On) The Hippo tumor suppressor pathway is activated by the core kinase MST1, which phosphorylates LATS1 and MOB1, leading to YAP/TAZ phosphorylation and their sequestration in the cytoplasm by 14.3.3 proteins. This prevents YAP/TAZ nuclear translocation and transcription of genes associated with malignant phenotypes such as those downstream of SMADs, TCF and TEADs. **(Pathway Off)** Hsp27 binds MST1 and promotes proteasomal degradation of ubiquitinated MST1. This prevents phosphorylation of LATS1 and MOB1, and phosphorylation of YAP/TAZ, which allows their nuclear translocation and their transcriptional activation of genes associated with malignant phenotypes such as those downstream of SMADs, TCF and TEADs.

4. Discussion

The ability of cancer cells to survive in an environment filled with cellular stress inducers like hypoxia, toxic radicals, DNA damaging reagents, glucose deprivation, etc. depends on how they respond to these stimuli; In the context of such toxic stress, cancer cells activate survival pathways including overexpression of heat shock proteins, especially Hsp27.

Extensive investigation of Hsp27 expression in human tumors has shown that Hsp27 is elevated across different cancers and is associated with aggressive and treatment-resistant malignancies. Research driven by our laboratory and others showed that Hsp27 promotes cell proliferation and tumor growth in different cancers including prostate and bladder [85, 86] and that targeting Hsp27 not only reduces tumor progression, but also opposes chemotherapy resistance in prostate and lung cancer [44, 45, 85]. Moreover, we showed that Hsp27 drives β-catenin-mediated Epithelial Mesenchymal Transition (EMT) that is required for invasion and metastasis in prostate cancer [32, 33]. EMT is a process by which epithelial cells gain migratory and invasive properties and can initiate metastasis. Finally, our efforts to decipher the roles of Hsp27 in cancer led us to perform a gene expression profiling in prostate cancer cells with Hsp27 loss of function.

Using an un-biased approach, we found that in prostate cancer PC3 cells, transient knockdown of Hsp27 reduces the gene signature of several pathways including WNT/β-catenin, TGF-β/SMADs and ILK signaling. Detailed examination of gene transcription activities in these pathways revealed that they all share YAP and TAZ as transcriptional co-activators [48, 53, 55]. YAP and TAZ intertwine closely with other oncogenic pathways and are key modulators in organ size, cell proliferation and apoptosis by regulating the activity of different transcription factors such as β-catenin/TCF, SMAD1-4 and TEAD1-4. Numerous studies have shown that YAP and TAZ dysregulation contributes to cancer development and progression [87]; interestingly increased expression of YAP/TAZ, their nuclear localization, as well as elevation of their target genes, are reported in many types of cancers and are known to be involved in malignant phenotypes such as enhanced cell proliferation, EMT and drug resistance. For example, activation of YAP1 is highly associated with poor prognosis and treatment resistance in colorectal cancer [88] and

promotes migration and invasion in prostate cancers cells [89]. In addition, 90% of metaplastic (a subtype of triple negative) breast cancers with EMT morphology stained positive for nuclear TAZ [90]. YAP and TAZ have also been associated with progression and metastasis in lung cancer [91] and their direct transcription targets, CTGF and AXL, were linked to EMT and drug resistance in this disease [92]. Comparison of pro-tumorigenic characteristics of YAP/TAZ transcriptional co-activators and Hsp27 suggested the possibility of intersection between their oncogenic pathways; however a functional relationship between the two has not been established yet.

Therefore, we compiled an elaborate list of YAP/TAZ-dependent genes regardless of the conventional pathways they were attributed to and investigated their changes when Hsp27 is lost in PC3 cells. We found that Hsp27 knockdown significantly attenuated the gene signature of YAP/TAZ without affecting YAP and TAZ transcript levels. To validate the clinical relevance of our findings we interrogated TCGA data of prostate cancer tumors for mRNA expression of YAP/TAZ target genes in tumors with Hsp27 upregulation or downregulation. Similar to our *in vitro* results, the positive correlation between the expression of Hsp27 and YAP/TAZ target genes was also observed in 568 prostate tumor samples, suggesting the clinical relevance of this association. This prompted us to look at YAP/TAZ regulation more closely.

YAP/TAZ activity and protein levels are predominantly controlled by the Hippo tumor suppressor pathway. First discovered in *Drosophila melanogaster*, the Hippo pathway has a fundamental role in organ growth control, stem cell function, regeneration and tumour suppression [65]. The ability of this evolutionary conserved pathway to inhibit proliferation and promote apoptosis has fascinated cancer researchers in the past decade. Recent work has led to the realization that in fact the Hippo pathway is part of an interconnected web which allows the cell to elicit the appropriate response to an external stimulus [93]. In fact, decreased activity of the Hippo pathway is associated with poor outcome across multiple cancer types. Similarly, numerous studies on cancer cell survival emphasized on the essential role of small heat shock proteins and have shown that Hsp27 acts as a central hub connecting networks that promote tumor growth and progression [1]. While there is extensive overlap between the cell survival, anti-apoptotic and metastatic pathways that Hsp27 and the Hippo components may

regulate, a relationship between the two has not been described yet. Here, for the first time, we report that Hsp27 negatively regulates the Hippo tumor suppressor pathway across different cancers.

Mechanistic investigations revealed that Hsp27 regulates YAP by decreasing the inhibitory phosphorylation of YAP on Serine 127, an established read out for Hippo pathway's tumor suppressive activity. Interestingly, this phenomenon was observed across three different cancers, suggesting a pan-cancer mechanism for regulation of the Hippo pathway by Hsp27. Strikingly, protein-based patient data analysis of 578 lung and 1104 breast adenocarcinoma tumor samples from TCGA demonstrated the same pattern where tumors with higher expression of Hsp27 showed lower p-YAP (S127) and samples with reduced expression of Hsp27 had a higher score for p-YAP (S127). These findings suggest that the interaction between Hsp27 and the Hippo pathway we see in vitro also occurs in human samples.

We then demonstrated that the functional consequence of Hsp27 overexpression and the subsequent decrease in p-YAP, is the reduced cytoplasmic retention and increased nuclear translocation of this transcriptional co-activator, allowing it to interact with pro-tumorigenic transcription factors.

Most importantly, we follow the effects on YAP phosphorylation through the Hippo kinase cascade and report for the first time that downregulation of this tumor suppressor pathway is dependent on Hsp27 negative regulation of the core Hippo kinase, MST1.

MST1 (STK4) is a multifunctional kinase with tumor suppressive roles and is considered an independent prognostic factor in different cancers, where its reduction or loss of expression is associated with poor prognosis [94-96]. For example, a study on more than 1000 colorectal cancer samples showed that loss of cytoplasmic MST1 expression (and not transcriptional changes) was an independent adverse prognostic factor in this disease [94]. In prostate adenocarcinoma, MST1 protein, but not mRNA, expression is significantly downregulated compared to paired normal tissue, pointing to the importance of post-translational modifications of this kinase in prostate cancer [97]. Another independent study revealed that levels of MST1 also decreased with progression of the disease to CRPC [96]. These findings, combined with our previous work showing that transition from hormone naïve prostate cancer

to CRPC is accompanied by an increase in the expression of Hsp27 [33], suggest that Hsp27 may control MST1 in CRPC progression.

It is important to note that in prostate cancer MST1 also interacts with androgen receptor (AR), the main driver of this disease. Different studies showed that MST1 directly binds to AR and antagonizes AR transcriptional activity [98-100]. In addition, we previously showed that cooperative interactions between AR and Hsp27 facilitate AR transcriptional activity [40]. Taken together, these findings are encompassing evidence on how Hsp27 regulation of MST1 can be a player in prostate cancer progression; meaning Hsp27-dependent decrease of cellular MST1, a negative regulator of AR activity, may be another mechanism by which Hsp27 promotes AR activity. Even though these connections are important in an AR-driven model, we strongly believe that what we observe in our PC3 model (AR negative prostate cancer cell line) as well as non-androgenic lung and breast cancer cells is independent of AR signaling and is more reflective of a pan-cancer phenomenon.

Furthermore, our study provides mechanistic insight into how MST1 levels are decreased in aggressive cancers. Although MST1 and its tumor suppressive roles have been studied in multiple cancers, there is little known about the underlying mechanism by which MST1 is reduced, except that its degradation is ubiquitin-mediated through C terminus of Hsc70-interacting protein (CHIP) [82]. CHIP is a HSP co-chaperone with E3 ubiquitin ligase activity that promotes ubiquitination. The ubiquitination system brands proteins to be degraded by the 26S proteasome and HSPs, especially Hsp27, can promote the degradation of proteasomal client proteins. Containing an ubiquitin-like domain, Hsp27 directly binds to one of the subunits of 26S proteasome and enhances the catalytic activity of the 26S proteasome machinery in response to stressful stimuli [39]. Here for the first time, we show that Hsp27 forms a complex with MST1 and enhances its proteasomal degradation. This finding is in concordance with previous studies describing the role of Hsp27 in enhancing the degradation of other tumor suppressor proteins, I-κB (inhibitor of kappa B) and p27Kip1 (cyclin-dependent kinase inhibitor) [39, 81]. Moreover, our data compliment other results showing that another heat shock protein, Hsp70, regulates MST1 degradation [83]. This study by Ren et al. showed that overexpression of Hsp70 in stress conditions, such as chemotherapy, leads to degradation

of MST1 in PCa cells. Importantly however, we did not find that Hsp27 knockdown affects the expression of Hsp70, suggesting that there is an independent role for Hsp27 in MST1 regulation **(Figure 3.22)**.

Although Hsp27 and Hsp70 share several properties such as apoptosis inhibition, resistant induction and tumorigenesis enhancement, they are different in terms of how they regulate these processes. For example, Hsp27 is and ATP independent molecular chaperone where Hsp70 is ATP dependent. They also differ based on their interaction with the proteasomal machinery [101]. Parcellier et al. showed that although overexpression of Hsp70 can enhance proteasomal activity in response to stress, this effect does not depend on a direct interaction with ubiquitin chains, as it is the case with Hsp27 [39]. Comparison of our results to findings of Ren et al. further confirms that Hsp27 has a distinct mode of connection with the proteolytic machinery and that enhancement of client proteins' degradation via Hsp27 is independent of Hsp70.

The novel link our data establish between Hsp27 and the Hippo pathway adds to the mechanisms by which Hsp27 may control tumor size, development and phenotype. For example, Hsp27 has been shown to affect tumor growth in different cancer models like breast and prostate [29, 40]. A study in breast cancer showed that high Hsp27 expression is linked to larger tumor volume, more proliferation and aggressive tumor phenotype. Xenografts originated from stably Hsp27 knock-downed cells were smaller and less angiogenic compared to the ones with normal Hsp27 expression. Also analysis of tissues from human patients suggested that patients with less Hsp27 expression have better survival rate [29]. Although justified through the role of Hsp27 in angiogenesis and increase of VEGF, the findings of this study raises the question whether Hsp27 contributes to breast cancer tumor size via other mechanisms like inactivation of the Hippo pathway, the most important pathway in organ size regulation, particularly because a recent study showed that YAP knockdown reduces VEGF transcripts in a breast cancer cell line [102].

The anti-apoptotic and cyto-protective roles of Hsp27 as well as the fact that it is ubiquitously overexpressed in different cancers, makes this chaperone an attractive target in cancer therapy. Indeed, multiple inhibitors of Hsp27 have been screened or developed. For

example, RP101 (Brivudine), an Hsp27 binding compound, is in phase II for pancreatic cancer [103]. Also depletion of Hsp27 using antisense technology in several preclinical animal models resulted in tumor regression [40, 43, 44]. OGX-427 (Apatorsen), a second-generation antisense oligonucleotide for Hsp27, is currently in phase II clinical trials for prostate, bladder and lung cancers (ClinicalTrials.gov, NCT01454089, NCT01829113, and NCT01120470). Our work suggests that especially in tumor types that depend heavily on inactivation of the Hippo pathway, inhibition of Hsp27 may be efficacious in preventing tumor progression.

In summary, our study reveals an original role for Hsp27 in the negative regulation of the Hippo tumor suppressor pathway in cancer cells. For the first time we show that Hsp27 facilitates the proteasomal degradation of ubiquitinated MST1 and therefore interrupts the Hippo pathway kinase cascade. Consequently, YAP and TAZ oncoproteins are less phosphorylated, free to translocate into the nucleus and promote a malignant phenotype **(Fiure 3.23)**. These findings, as well as corresponding clinical correlations we found in multiple tumor types underscore the central importance of Hsp27 in regulating multiple signaling pathways that promote tumor aggressiveness and provide further rationale for targeting this molecular chaperone in human cancers.

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Appendix

Appendix 1

List of YAP/TAZ gene signature and respective normalized log2 values in si Scr and si Hsp27 treated PC3 cells.

Gene	si Scr	si Hsp27
PSAT1	17.01364523	16.1642907
SMAD1	15.77624513	14.04485355
PDCD1LG2	15.41523747	13.03842473
MSMO1	14.44154268	13.5796855
SMAD6	14.09740834	13.86459485
SCHIP1	13.83917042	11.42588929
LGALSL	13.48697463	12.36662794
MACC1	13.38562382	11.33284504
UGCG	13.35147678	11.49528249
EPB41L2	13.13598257	10.42346027
PITX2	13.08989616	11.32308609
DAB2	12.73120113	9.442547146
CPA4	12.6546593	11.14469644
SGK1	12.45230107	10.3728517
GPCPD1	12.42749121	9.09193641
PYGO1	12.42169003	8.412915021
CENPF	12.25494483	7.459307484
NT5E	12.09808253	8.255554475
ANXA1	12.00732237	6.890668068
MID1	11.77436835	8.955273931
CHST9	11.55329618	11.39165048
CAV1	11.51225568	7.556603253
PCLO	11.43639944	7.630748029
ARHGAP29	11.43577666	6.087261559
ENC1	11.2918425	9.665932932
TGFA	11.22669414	10.22174955
PRSS23	11.17193986	7.576703769
SERPINB7	11.17093091	9.214762959
MYBL1	10.96697517	8.530321332
BCL2	10.94477655	6.761392428
SERPINE1	10.93055913	8.142799633
THBS1	10.92734813	9.371137888
RND3	10.84163479	5.350830328

Gene	si Scr	si Hsp27
CTNNB1	10.80275149	9.240144947
CTNNAL1	10.79686734	6.947355114
EP300	10.71243002	7.898946112
CRIM1	10.63554954	8.386907855
SHROOM3	10.41196544	8.431558379
FRZB	10.40232472	7.362189017
SLIT2	10.35295578	7.58824035
PTPN14	10.27103809	6.666341606
EMP2	10.2659359	9.57971603
SNAPC1	10.26458358	7.145387872
SCD5	10.22401325	7.076027669
MYOF	10.14549825	7.461591073
BICC1	10.10906223	7.341722295
TOP2A	10.08226216	4.789844276
CDH2	9.911701168	7.153638113
LHFP	9.883599282	6.750840879
OPN3	9.814634293	8.469460352
SNAI2	9.583307336	6.189412359
CCND2	9.554704646	7.913117608
ESM1	9.498819395	4.834771394
TGFB2	9.360629972	6.459271661
LRP6	9.21220502	5.925356234
RIMKLB	9.183667138	5.320722922
ID2	9.178417119	4.52703121
TSC22D2	9.177835418	5.627012519
MET	8.949237843	3.821837258
AHNAK	8.898692163	6.254458186
ECT2	8.806316323	3.835926993
GGH	8.804056141	4.43331799
LMBRD2	8.700756036	5.765337189
TMEM154	8.62234022	5.528784582
ARHGEF28	8.599561719	6.656254082
LUM	8.468867234	5.038729883
CCL28	8.467728622	6.398733417
ADAMTS12	8.441855426	6.549500705
ITGBL1	8.376938801	2.12801484
PRRG1	8.352987282	2.224120624
MDFIC	8.345931973	2.094483875
IFIT2	8.197555527	2.264627753
TMEM27	8.177187891	3.354978572
F3	8.115716005	6.928779886
GLS	8.045898437	3.696395629

Gene	si Scr	si Hsp27
PDP2	8.011860896	6.620456942
ACSL4	7.933785474	2.662645565
GADD45A	7.701168511	6.037925031
IFI16	7.622054602	2.050958104
FGF2	7.511950981	1.995879439
SDPR	7.462854366	4.965308957
CLDN1	7.239845759	5.320962217
FSTL1	7.083340184	5.046671197
SCML1	7.074799546	1.903302376
EXPH5	7.039980488	4.218589926
AXL	6.945869383	5.133898067
HMMR	6.936588828	2.002870378
PMAIP1	6.880130696	4.199617898
SEMA3C	6.734954858	1.896036602
PHLDA1	6.70416787	3.880824834
IRS1	6.253338848	3.421702896
DAAM1	6.192013225	2.476968698
FST	6.04905035	4.96256993
ASAP1	6.010676018	2.918076275
INSIG1	5.996509531	3.41756635
RHOU	5.991762634	3.120563863
BDNF	5.957348829	2.186108342
HMGCS1	5.844367976	2.159839427
IDI1	5.784216829	3.639358338
CYP1B1	5.763458318	3.870782342
AOX1	5.760320681	5.30405597
SNORA75	5.739108559	3.52797127
PSG5	5.650150278	2.265399203
JPH1	5.603233315	3.438913103
F2RL1	5.597450724	4.187126117
DIXDC1	5.574666968	3.871582299
SP1	5.564356308	3.687062782
SMAD5	5.327253844	4.402483257
LPIN1	5.295987629	2.184630145
PRICKLE1	5.184416781	4.49580554
SNRPG	5.179853968	2.054330556
DDAH1	5.169179416	2.620085155
FSCN1	5.131463136	5.427053199
DUT	4.8357267	2.088257542
EMP1	4.807664862	2.510870108
LCP1	4.683229889	2.353511348
RAB3B	4.467957492	3.081287668

Gene	si Scr	si Hsp27
CTGF	4.462551023	4.503400582
SMAD3	4.38810679	4.10214715
S1PR1	4.174602962	2.572433119
FBXW11	3.958380475	2.234871706
IFRD1	3.725963596	1.924834297
CD55	3.711125816	1.916858113
ANXA3	3.627528632	1.996193033
TFPI2	3.607848585	3.986906103
AMPH	3.573904048	2.29338142
TSC22D1	3.569048688	2.286186518
SYT14	3.565704521	1.962384886
ERRFI1	3.511753982	2.239459703
RGS4	3.509068722	2.251786479
SQLE	3.440047241	2.142507146
CSNK2A1	3.397488108	2.125744639
PMP22	3.382857986	2.074582587
GLI2	3.376551556	2.108634709
ADAMTS1	3.371090433	2.62306717
ADAMTS5	3.368382694	2.130463824
SLC2A3	3.366874704	2.221180596
ADRB2	3.351806111	2.072713945
HEXB	3.351496604	2.054180811
GCNT4	3.338375777	2.085630121
SCD	3.283995386	2.003441001
IL8	3.28391955	2.046099953
COX6C	3.261657177	2.024147335
NT5DC3	3.240491564	1.977508755
STXBP1	3.234171572	2.016912687
SLC16A6	3.226513616	2.035211092
KIT	3.222433331	2.037982755
AREG	3.202016281	2.010265667
KRT5	3.177729672	1.961008123
SOX9	3.137527623	1.921058386
MYC	3.105650424	1.882593394
UPK1B	3.088504261	1.863256292
NID2	3.086198317	1.863334638