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## FUNCTIONAL REGULATION OF YAP BY AURORA A KINASE IN TRIPLE-NEGATIVE BREAST CANCER

Shih-Shin Chang

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**FUNCTIONAL REGULATION OF YAP BY AURORA A KINASE IN TRIPLE-  
NEGATIVE BREAST CANCER**

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**FUNCTIONAL REGULATION OF YAP BY AURORA A KINASE IN  
TRIPLE-NEGATIVE BREAST CANCER**

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

M.D. Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

**DOCTOR OF PHILOSOPHY**

by

**Shih-Shin Chang, *M.S.***

Houston, Texas

May, 2016

## **ACKNOWLEDGEMENTS**

I started to pick up a pipetman and immerse myself in research since 2002. Since then, labs and benches consist most of my physical world. Staying on benches, however, my imagination is broadened immensely by a deeper understanding of research. Science has made me humble. Of course God makes me humble always. The more I learned, the tinier I felt about myself. Though I have not yet achieved something truly significant thus far, I am confident that I have learned ways to identify meaningful issues and to strategically tackle problems. I will keep learning and moving forward in hopes that one day my works will have benefited people in some way.

Having the opportunity to receive a higher education is a privilege. I do not take it for granted. There are so many other talent people who never have the chances to do so. I am very grateful for everything given to me, from my parents, advisors, friends and even a stranger on a train. They have made a meaningful me.

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Some say that the road to becoming a PhD is very long. After my college education, it took me 14 years to get the chance to write this thesis. However, I feel like it all happened in the blink of an eye. Some say that we don't feel time when we work on things that we love. I agree with that wholeheartedly.

# **FUNCTIONAL REGULATION OF YAP BY AURORA A KINASE IN TRIPLE-NEGATIVE BREAST CANCER**

**Shih-Shin Chang, M.S.**

**Advisory Professor: Mien-Chie Hung, Ph.D.**

The Yes-associated protein (YAP) is an effector that transduces the output of the Hippo pathway to transcriptional modulation. Considering the role of YAP in cancers, this protein has emerged as a key node in malignancy development. In this study, we determined that Aurora A kinase acts as a positive regulator for YAP-mediated transcriptional machinery. Specifically, YAP associates with Aurora A predominantly in the nucleus. Activation of Aurora A can impinge on YAP activity through direct phosphorylation. Moreover, aberrant expression of YAP and Aurora A signaling is highly correlated with triple-negative breast cancer (TNBC). We herein provide evidence to establish the functional relevance of this newly discovered regulatory axis in TNBC. This study not only has advanced the understanding of the intricate network of YAP in cancers but also has revealed a potential route to intervene oncogenic YAP function.

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**CHAPTER 1**  
**INTRODUCTION**

## **1.1: Overview of Breast Cancers**

Breast cancer is the most frequent cancers in U.S. women population with estimated 230,000 new cases and 40,000 deaths in 2015 (1). Types of breast cancers can be classified depending on histopathological origins of the disease, including ductal carcinoma, lobular carcinoma and inflammatory breast cancer. Among them, ductal carcinoma is the most commonly found. There are several risk factors associated with the onset of breast cancers; however, at this moment, hereditary BRCA1/2 gene mutation represents the most predictive markers for breast cancer.

The stages and types of cancers determine treatments of breast cancer. There are four major routes of treatments, including surgical removal of part or whole breast (mastectomy), radiation therapy, chemotherapy, hormone therapy and target therapy. Usually the treatment procedure would combine at least two of abovementioned options (Information was obtained from National Cancer Institute; <http://www.cancer.gov/types/breast/patient/breast-treatment-pdq>).

Hormone receptors play key roles to stimulate and maintain breast cancer growth. Once a breast cancer is diagnosed, the cancer cells are closely examined for their molecular signatures based on the protein expression or gene amplification of estrogen receptor (ER), progesterone receptor (PR) or ERBB2 (HER2/neu). After the examination of these three markers, breast cancers are classified as ER/PR-positive but HER2-negative, HER2-positive (Lumina A subtype with ER/PR-positive;

Luminal B with ER/PR-negative) and triple-negative breast cancer (TNBC; ER/PR/HER2-negative) (2).

In addition to surgery, radiation and systemic chemotherapies, breast cancer with specific expression of receptor receives corresponding treatment to block hormone effect. This procedure is called hormone ablation or endocrine therapy. Tamoxifen and aromatase inhibitors are used to block action of ER or to lower down the production of estrogen. HER2-positive patients would receive anti-HER2 therapy such as monoclonal antibody (Herceptin; chemical name: trastuzumab) and small molecular compounds (i.e. Lapatanib) that inhibit HER2.

The discovery of hormone ablation therapies has greatly advances the survival rate of breast cancer patients. In general, around 60-70% of ER-positive and HER2-positive patients will respond to anti-estrogen or anti-HER2 therapy. According to the statistics conducted from 2005 to 2011 by Surveillance, Epidemiology, and End Results Program (SEER) at National Cancer Institute, the current overall 5-year survival rate of breast cancer reaches 89.4%. Although overall breast cancer is more manageable than many other cancer types, to eliminate breast cancer is still the final goal as each year there are still nearly 40,000 lives in US deceased from it. Particularly, triple-negative breast cancer (TNBC) is a subtype of breast cancers that currently lacks effective treatments and the overall survival of TNBC is worst among all breast cancers. Identification of the cause of aggressiveness and the targetable targets represents an urgent challenge for breast cancer research field.

## **1.2: Background of Triple-Negative Breast Cancer (TNBC)**

In general, TNBC is more aggressive and has a poor prognosis compared to ER-positive breast cancers, regardless of the stages when cancers were diagnosed (3, 4). Among total new breast cancer patients diagnosed, around 15-20% are TNBC. Due to the lack of expression of ER, PR and HER2, TNBC does not respond to endocrine or anti-HER2 therapy. TNBC has higher recurrence rate within the first 5-year after treatment and the recurrent TNBC is hardly manageable.

TNBC shares a common morphological and genetic status with basal-like breast cancer (BLBC). BLBC is a subtype of breast cancers defined by a unique gene profile. Usually BLBC expresses basal cytokeratin 5, 14 and 17. Around 70% TNBC can be classified as BLBC (5). The rest 30% of TNBC is consisted of unclassified subtypes with different gene expression profiles, indicating TNBC should be considered as a complex type of disease.

Though recurrent TNBC is hardly manageable, chemotherapy is effective as first line treatment for TNBC. In general, TNBC responds well to anthracycline-based and taxane-based therapies (6, 7). Alternative treatments for TNBC may include EGFR inhibitors, Src inhibitors and poly-ADP-ribose polymerase (PARP) inhibitors that are currently under clinical trials. EGFR is expressed in up to 70% of TNBC (8-10). Clinical trial data so far shows low efficacy when anti-EGFR monoclonal antibody, Cetuximab, was used alone. However, in a small study enrolled 18 patients

with metastatic TNBC, 5 patients showed a complete or partial response to the combination of taxane with cetuximab (11).

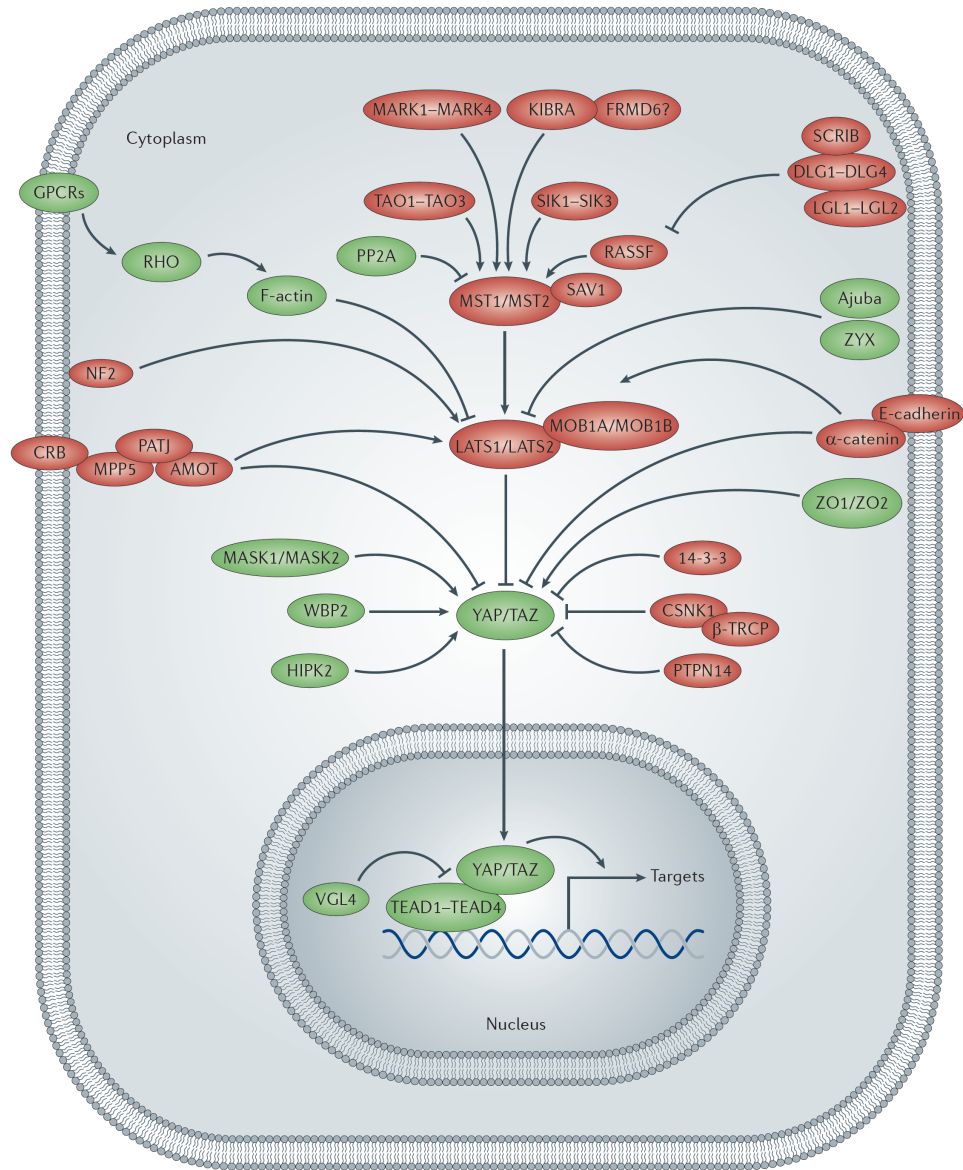
As mentioned earlier, BRCA1/2 gene mutation represents the most predictive genetic marker for breast cancers. Most breast cancers associated with BRCA mutation fall into TNBC and BLBC. However, the relationship between BRCA mutation and the onset of TNBC or BLBC is still unclear. BRCA and PARP are two major pathways that maintain a functional DNA repair system. In BRCA-mutant cancers, PARP becomes essential to maintain sufficient DNA repair machinery for the cell survival. Therefore, the combination of PARP inhibitors with DNA-damaging drugs (for example, platinum-based compounds) has shown promising results in BRCA-mutant ovarian cancer. The encouraging results have led to an approval from FDA (Food and Drug Administration) to use Lynparza, a PARP inhibitor, in the treatment of advanced ovarian cancer with defective BRCA genes. Similar trials are ongoing in TNBC patients and the results are encouraging thus far.

### **1.3: Hippo Pathway- Discovery and Overview**

The Hippo pathway was discovered to regulate the organ size by the screening of mutant *Drosophila melanogaster*. Mutation of a kinase induced the outgrowth of target organs and the phenotype is like hippopotamus, therefore this kinase was named “hpo” (12-14). Later discoveries have supported a solid role of Hippo pathway in the regulation of tissue homeostasis, apoptosis, proliferation and

environmental mechanosensing (15, 16). Very similar to other canonical signal pathways in the cells, the Hippo pathway consists of two major serine/threonine kinases (Hpo and Wts in fly; MST1/2 and LATS1/2 in mammals) in the cascade (15, 16). The major role of these two kinases is to sense environmental or cellular cues and transduce them to the most downstream effectors that control expression of a variety of genes. Hippo pathway is quite conserved from fly to mammals. The major difference is that, in fly, there is only one downstream effector called Yorkie (Yki) while in mammals, there are two paralogue effectors, named Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ or WWTR1) (15, 16). Hereafter we will mainly focus on the mammalian Hippo pathway.

**Figure 1.3: Illustration of the Mammalian Hippo Pathway**



Reproduced with permission from **Johnson R, Halder G.** 2014. The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment. *Nat Rev Drug Discov* **13**:63-79.



#### **1.4: Regulators of the Hippo Pathway**

Hippo pathway translates the signal output from membrane to the nucleus by inhibiting YAP and TAZ activity. Unlike other signaling pathways, there are no ligand or peptide that can directly activate Hippo cascade. Instead, Hippo pathway can sense the activity from cell-cell junction, cell-matrix junction and cytoskeleton dynamics (15). Upon the activation of Hippo pathway, MST1/2 kinase (mammalian STE20-like protein kinase) phosphorylates and activates LATS1/2 kinase (large tumor suppressor homolog). Two major proteins SAV1 (Salvador homolog 1) and MOB1A/B (Mps One Binder Kinase Activator-Like) serve as scaffolding proteins to support and bridge function of MST-LATS axis. LATS1/2 kinase subsequently phosphorylates YAP and TAZ protein. After YAP and TAZ protein are phosphorylated by Hippo pathway, their localization is switched to the cytoplasm and therefore the transcriptional activity is shut off. The more detailed mechanisms of how MST, LATS kinases and YAP/TAZ are regulated by upstream modules are described below.

#### **1.5: From Environmental Cues to the Hippo Pathway**

Activation of MST and LATS pathway can attribute to four major types of signals: Cell polarity complex, direct regulators of MST or LATS kinase from cell junctions, stiffness of cell-extracellular matrix (ECM) attachment and cell-cell contact. Several of these signalings more or less affect Hippo pathway through affecting organization of cytoskeleton.

Cell polarity complex regulates Hippo pathway. Crumb protein complex (CRB) is critical to define apical phase of a cell (17). CRB is a transmembrane protein with a short intracellular domain that serves as protein docking platform. CRB has been shown to regulate Hippo pathway (18-22). For example, CRB complex recruits angiomin, a cell junction protein, to the apical membrane (22) and angiomin can affect Hippo pathway by two ways. One is to directly bind with YAP and thus it restricts YAP to the membrane; the other is to interact with and activate LATS kinase, therefore it induces YAP nuclear export (23-27). Although angiomin was shown to cause inhibition of YAP activity, however, in an angiomin-knockout mouse cancer model, angiomin appears to be essential for transactivation activity of YAP in the nucleus (28). It appears angiomin may possess positive and negative effects on YAP activity in a cell-context-dependent manner.

KIBRA (kidney and brain protein) and TAO kinase (thousand and one amino acid protein) are apical junction proteins that influence Hippo pathway. Association with KIBRA promotes Hippo pathway to restrict cell growth (29-32). TAO is a cell-junction kinase downstream of KIBRA. It can directly phosphorylate, activate LATS kinase and thus inhibit YAP-mediated cell growth (33-35).

Mechanical force was recently found to regulate Hippo pathway, linking the mechanical sensing of cells to signal transduction. Stiffness of cell-extracellular matrix (ECM) attachment was indicated to influence Hippo pathway. Briefly, a softer matrix can activate Hippo pathway and make YAP shuttle to the cytoplasm; while a

stiff matrix can promote nuclear import of YAP that stimulates cell proliferation (36-38). F-actin dynamics controls the mechanosignaling to YAP and TAZ (38).

Although there is no clearly defined receptor that directly influences Hippo pathway, some G protein-coupled receptors (GPCRs) were discovered to activate YAP and TAZ through the actin reorganization. GPCR stimulators like lysophatic acid (LPA) and sphingosine-1-phosphate (S1P) were shown to inhibit Hippo pathway (39-41).

High confluence of cell-cell contact also activates Hippo pathway and moves YAP, TAZ to the cytoplasm (42). Although the detail mechanism is still unclear, it is possible that high cell density engages complex formation of junctional proteins such as CRB, PATJ, PALS, E-cadherin, alpha-catenin and angiomotin, which were shown to directly or indirectly promote nuclear export of YAP and TAZ (43-46).

### **1.6: Effectors of the Hippo Pathway - Discovery, Structural Features, Working Mechanisms**

The most downstream effectors of the Hippo pathway are transcriptional co-activators, YAP and TAZ, which transduce upstream signals to gene expression. In flies, mutation of the Hippo kinase induces abnormal enlargement of target organs, and this phenotype is rescued by deletion of Yki (Yorkie, YAP orthologue in fly) (12-14). In humans, YAP and the PDZ-binding protein, TAZ, are the two paralogues that seem to have a redundant role in terms of Hippo signaling (16). The full length of

YAP protein contains 504 amino acids. Structurally, YAP protein contains a transcriptional factor-binding domain in its N-terminal half and one to two WW domains depending on which isoforms. The C-terminus of WW domains is followed by the transactivation domain (TD). The last five amino acids of YAP protein comprise a PDZ-binding motif (PDZ-bm). WW domain is composed by around 40 amino acids that direct the binding of YAP with PPxY motif on other proteins. For example, YAP interacts with LATS kinase and angiotensin through the WW domain (26, 47). Transactivation domain of YAP protein is used to turn on the expression of the downstream genes. PDZ-binding motif can interact with protein with PDZ domains. PDZ-domain containing proteins are usually found to help anchor receptors to the membrane and supporting cytoskeleton. Of note, though PDZ-binding motif of YAP protein only consists of five amino acids, it was reported PDZ-bm is very critical for the nuclear localization and transactivation activity of YAP (48).

YAP protein itself does not contain a DNA-binding domain. In order to achieve the activity on gene regulation, YAP interacts with numerous transcriptional factors that possess DNA-binding ability. Plenty of works have demonstrated that YAP can exert its transactivation effect by interacting with TEADs (TEA domain-containing sequence-specific transcription factor), p73, beta-catenin, Smad, TBX5, c-fos (49-55).

Although the determinants that decide the association of YAP with other specific protein partners are yet to be investigated, the interaction between YAP and these DNA-binding factors at least reflects a complex role of YAP in various cellular scenarios. Among YAP-interacting DNA-binding proteins, TEADs are the major accomplice for YAP's function as a transcriptional co-factor (52). It was revealed that mutation of the TEAD-binding motif on YAP at serine 94 severely compromised its biological output (52). Also, through systematic chromatin-IP sequencing analyses, several studies revealed that the occupancy of TEADs highly coincides with YAP-binding genes, corroborating the role of TEADs in YAP's function (52, 56).

### **1.7: YAP and TAZ – Phenotypes in Developmental Stages**

YAP and TAZ play key roles in embryonic development of mice. Whole body knockout (KO) of YAP causes embryo death between E9.5 to E10.5. Differentially, whole body knockout of TAZ reduces litter size, body size and it only causes partial postnatal death or prematuration. This suggests that YAP and TAZ play different roles in the development, though structurally they are similar. Besides the phenotypes observed from the whole body knockout experiment, there were plenty of works indicate the key roles of YAP and TAZ in the development of different specific organs, including liver, pancreas, salivary glands, kidney, lung, heart, intestine, skin and nervous system (57). Of note, it is not just the expression level of YAP and TAZ controls the development, the subcellular localization of YAP and TAZ appears more important.

## 1.8: Regulation of YAP and TAZ

So far, the major control that regulates YAP activity is through modulating its subcellular localization. YAP protein is shuttled between the nucleus and cytoplasm in a well-controlled manner. YAP activity is largely regulated by upstream Hippo kinases. In humans, MST1/2 and LATS1/2 consist of a central kinase cascade that, upon activation, inhibits YAP activity by direct phosphorylation on multiple sites of the YAP protein (14, 42, 58). Upon phosphorylation from the LATS kinase on serine 127 of YAP, YAP is sequestered by 14-3-3 to the cytoplasm, and the transcriptional program in the nucleus is subsequently shut off (14, 42). Furthermore, LATS kinase phosphorylation of YAP on serine 397 has been shown to decrease YAP stability (59). LATS phosphorylation of YAP primes YAP for a sequential phosphorylation on amino acids 401 and 403 by CK1 kinase and this eventually leads to beta-TrCP-directed proteasomal destruction in the cytoplasm (59). In addition to the negative regulatory circuit from the Hippo pathway, YAP can be inactivated by a direct sequestration to the cytoplasm from angiomotin (an F-actin-binding protein located in cell junctions) and alpha-catenin (23, 25, 26, 44, 45, 60). In general, most of studies have identified signaling that negatively regulates YAP function; however, signaling and mechanisms that directly elevate YAP transcriptional activity remain less characterized.

In the nucleus, YAP acts as a transcriptional co-factor and it has been reported to promote expression of several growth-related genes, for example, *CTGF*, *Birc5*, *Amphiregulin*, *Cyr61*, and *EGFR* (16, 61). Although the very determinant genes that

directly influence cell proliferation are yet to be investigated, several other studies have shown that DNA replication proteins, MCM family and cell cycle-regulating genes may be downstream genes of YAP (62-65).

### **1.9: Relevance of the Hippo Pathway in Cancers**

The relevance of YAP and TAZ in cancer development has been widely reported (16). Dysregulation of YAP expression has been shown in several cancer types, for example, in breast, ovarian, lung, pancreatic, liver, colorectal cancers and in malignant mesothelioma and medulloblastomas (66-68). In experimental models, YAP was reported to mediate tumorigenesis, anti-apoptosis, drug resistance, and metastatic programming (16, 69-71). For example, overexpression of YAP in the liver causes liver enlargement and hepatocellular carcinoma in mice, proving a strong oncogenic property of YAP *in vivo* (72, 73). In these studies, short-term overexpression of YAP in the liver can obviously increase the liver size in a reversible manner; however, a long-term expression of YAP leads to a dramatic formation of liver cancer. Expression of YAP reduces anoikis and promotes chemoresistance in cancer cells (74). In melanoma, YAP promotes resistance to BRAF and MEK1/2 inhibitors (75). YAP and TAZ can facilitate epithelial-mesenchymal transition (EMT), increase cancer metastasis, and endow cells with cancer-initiating properties (52, 55, 76-79). Furthermore, YAP has been shown crucial for Kras-induced PDAC development (80). Expression of YAP is sufficient to alleviate the dependence of Kras in a PDAC mouse model (63). These expanding reports support an intriguing role of YAP in cancer biology.

### **1.10: Aurora A Kinase - Discovery and Structure**

Aurora family kinases were discovered first in yeast where mutation of Ip1 (increase-in-ploidy) causes the failure in normal chromosome segregation (81). Later findings in *Xenopus* and fly all revealed crucial roles of Aurora kinase in the mitotic control (82-84). The phenotypes of Aurora kinase deficiency in model systems were similar. For example, in fly, the mutation of Aurora A kinase is coupled with pupal lethality, mitotic arrest, defect in centrosome segregation and the loss of spindle polarity (85-88).

### **1.11: Regulation of Aurora A Kinase**

Structurally, Aurora A is consisted of 403 amino acids. The canonical kinase domain is nearby within amino acids 133 to 383. Regarding the activity control of Aurora A, the amino acids 274 to 299 are so-called “mobile loop”, whose conformational change will determine the kinase active or not. In the mobile loop, the threonine 288 residue represents the most well-studied and critical site for the kinase activity. Threonine 288 is an auto-phosphorylation site and its phosphorylation can be promoted by the interaction of Aurora A kinase with other co-activators (discussed below). In addition to be auto-phosphorylated, threonine 288 or residue 287 can be a direct substrate for PAK, protein kinase A and atypical protein kinase C.

Although several kinases have been shown to direct phosphorylate on threonine 288 of Aurora A kinase and therefore activate Aurora A activity, the



binding of specific protein co-activators with Aurora A controls the major enzymatic output of Aurora A. Aurora A kinase belongs to the AGC family of kinases (PKA, PKG, PKC families) and, unlike receptor tyrosine kinases (RTKs), its activation is achieved by interacting with specific protein co-factors. Activation of Aurora A kinase has been reported to attribute to the interaction with TPX2 (Targeting Protein for XKLP), Nedd9, Ajuba, Bora, Calmodulin and PAK1 (89). TPX2 represents the most well-characterized allosteric activator for Aurora A. Upon binding with Aurora A, TPX2 induces a conformational change in Aurora A toward its active state, and this substantially elevates its kinase activity (90, 91). Intermolecular autophosphorylation occurs on Thr288 of Aurora A and then further pushes its maximum kinase activity (92, 93). It was nicely demonstrated in a comprehensive structural study that binding of TPX2 and phosphorylation of Aurora A-T288 can independently elevate Aurora A kinase activity (94).

### **1.12: Roles of Aurora A in The Cell Cycle Control**

In human, there are three family members of Aurora kinase, Aurora A, B and C. Aurora A and B regulate general mitosis and Aurora C kinase regulates meiosis (95). Since Aurora A is more relevant to cancers and is our focus in this research, we will mainly focus on the role of Aurora A hereafter.

Aurora A is pivotal in the regulation of cell cycle progression, particularly for the G2-M phase transition. Mutation of Aurora A kinase renders a multi-aspect defect in chromosome segregation resulted from inaccurate number of centrosomes

and mis-positioning of mitotic spindles (89). In non-transform cells, the mRNA level of Aurora A starts to accumulate in the S-phase and then peaks at G2/M phase (96). Accumulated Aurora A protein begins to concentrate on centrosomes and they help increase the diameter of centrosomes by nucleating relevant tubulins and other protein complexes, like PCM (pericentriolar mass) (97). Once the mitosis is completed, Aurora A protein is subjected to the proteasomal degradation mediated by APC/C (anaphase-promoting complex) (98). The phosphorylation of serine 51 residue of human Aurora A seems to control this degradation process. Expression of PP2A (phosphatase 2A) that removes phosphorylation of serine 51 can decrease the degradation of Aurora A in late mitosis (91, 99).

Activation of Aurora A kinase during G2/M cell cycle is largely induced by TPX2. TPX2 is a nuclear protein and it is released upon the breakdown of nuclear envelope. The released TPX2 therefore not only begins to assemble spindles but also binds with Aurora A for kinase activation (100-104). Once Aurora A is activated, it phosphorylates its own threonine 288 to further potentiate kinase activity and it also phosphorylates surrounding centrosome complex, including TPX2, PLK1 kinase, LATS kinase. Overall the phosphorylation event coming from Aurora A helps the maturation of centrosome, spindle assembly/positioning and ignites the mitotic entry. Aurora A activates CDK1/cyclin B around the centrosome and this facilitates of the nuclear import of CDK1/ cyclin B that can promote cell cycle progression (105, 106). Also, the Aurora A downstream PLK1 kinase can further phosphorylate on WEE1, a CDK1/cyclin B inhibitor, to decrease the inhibitory effect of WEE1 on

CDK1, therefore the CDK1/cyclin B activity can sustain during the cell cycle (107, 108). In the end of G2 phase, Aurora A directly phosphorylates CDC25B, an activator of CDK at mitosis, to promote M phase entry (109). Interestingly, Aurora A can also phosphorylate BRCA1 at the serine 308 and this event somehow is important for transitioning G2 to M phase (110, 111). The downstream event of BRCA1 in this case is still not clear.

### **1.13: Relevance of Aurora A in Cancers**

Genome instability is a hallmark of cancers. Abnormal expression or activation of Aurora A causes aneuploidy (112). Aurora A is highly expressed or amplified in breast, colon, skin, ovary, head and neck cancers (112-116). Besides its roles of in the cell cycle, Aurora A has been shown to affect various other cellular behaviors, including cell signaling (117, 118), migration/metastasis (119-122), drug resistance (123-126), and stem cell properties (125, 127, 128). Aurora A kinase is highly relevant in human cancers because its expression and gene amplification correlate with malignant phenotypes in cancers (95, 129). Aberrant activation of Aurora A increases chromosomal instability (CIN) in cells (130). However, one reports indicated that Aurora A may exert oncogenic functions independent of CIN (131).

### **1.14: Aurora A Substrates and Its Non-Mitotic Roles**

In addition to those kinase substrates in mitotic regulation, expanding discoveries have shown that Aurora A also phosphorylates numerous substrates (more than 22) participating in various cellular functions (89). For example, Aurora

A phosphorylates p53 and induces degradation of p53 protein (132). Also, it can phosphorylate p73 and this leads to the cytoplasmic retention of p73 (133). Aurora A phosphorylates AP-2alpha, a tumor suppressor, and induce AP-2alpha degradation (134). Phosphorylation of Ikb-alpha, inhibitor of NF-kB pathway, by Aurora A leads to the destruction of IKB-alpha and elevates NF-kB signaling (117, 135). Aurora A also involves in cell signaling. For example, it phosphorylates AKT and RALA and this consequently increases the activation of AKT and Ras activity (124, 136, 137).

#### **1.15: Aurora A Kinase As A Cancer Target**

Owing to the oncogenic role of Aurora A kinase supported by a wealth of studies, considering Aurora A kinase as a therapeutic target is of intensive interest in cancer treatment. According the information from the ClinicalTrials.gov, to date, there are 26 ongoing cancer clinical trials using the Aurora A inhibitor, Alisertib (MLN8237), alone or in combination with other drugs. Many more trials have been completed in the past 5 years. Thus far, only limited partial responses have been observed from the clinical trials, therefore the identification of biomarkers or resistant mechanisms may greatly help guide the use of Aurora A inhibitors, assuming Aurora A is still a promising target in cancers.

### **1.16: Rationale and Hypothesis**

Functional studies of the pathogenesis of TNBC are emerging topics in breast cancer research. The discoveries of key genes critical for TNBC growth or survival may help reveal the TNBC vulnerability and overall this direction is in hope to bring out targeting therapeutics in the future. We have identified that the Hippo pathway effector, YAP, is critical to support TNBC cell growth in the three-dimensional (3D) culture condition. However, it is still not clear how other targetable pathways might involve in YAP function in TNBC. We hypothesized that there are oncogenic kinases that can directly enhance YAP activity. In this study, we utilized proteomic approach, bioinformatics analysis and *in vitro* functional characterization to provide a better understanding on YAP, seeking to provide an alternative way for managing the oncogenic role of YAP in TNBC.

## **CHAPTER 2**

### **RESULTS**

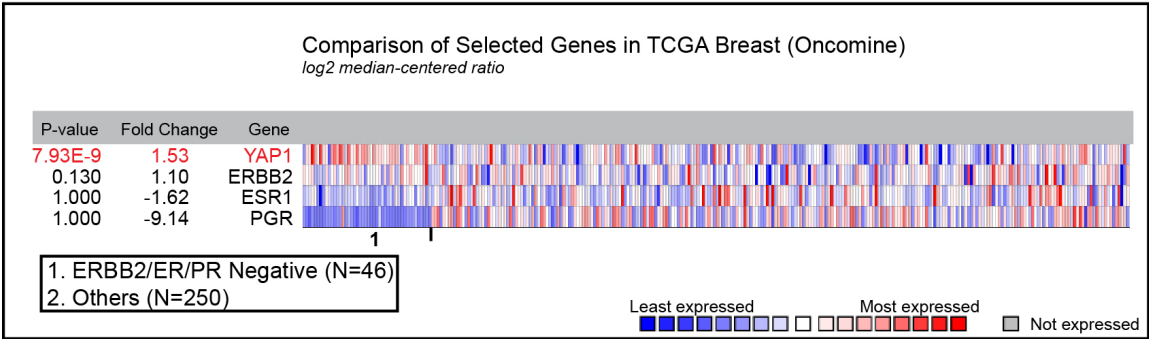
## **2.1: Expression of YAP Correlates with TNBC**

### ***2.1.1: mRNA Expression of YAP from the Public Databases***

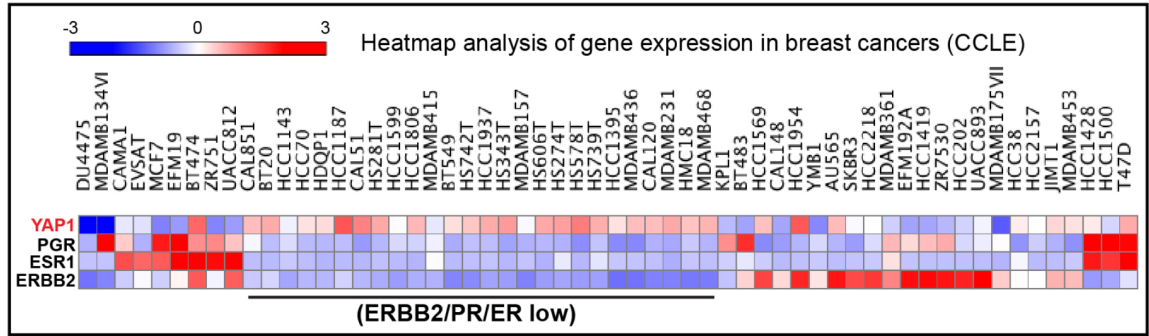
Although higher YAP expression (protein and/or mRNA) has been correlated with malignant phenotypes in many other cancer types, the correlation of YAP in breast cancer subtypes is not clear. To address whether there is a differential expression of YAP in breast cancer types, especially in TNBC, we analyzed the mRNA expression level of YAP from public databases of both patients (TCGA, The Cancer Genome Atlas) and cell lines (CCLE, Cancer Cell Line Encyclopedia) by a simple bioinformatics analysis. From the results, YAP expression significantly associates with the TNBC subtype (Figures 2.1.1A and B) both in patients ( $n = 296$ ;  $P = 7.93 \times 10^{-9}$ ) and in cell lines ( $n = 58$ ).

Figure 2.1.1 Expression of YAP mRNA Correlates with TNBC

A



B



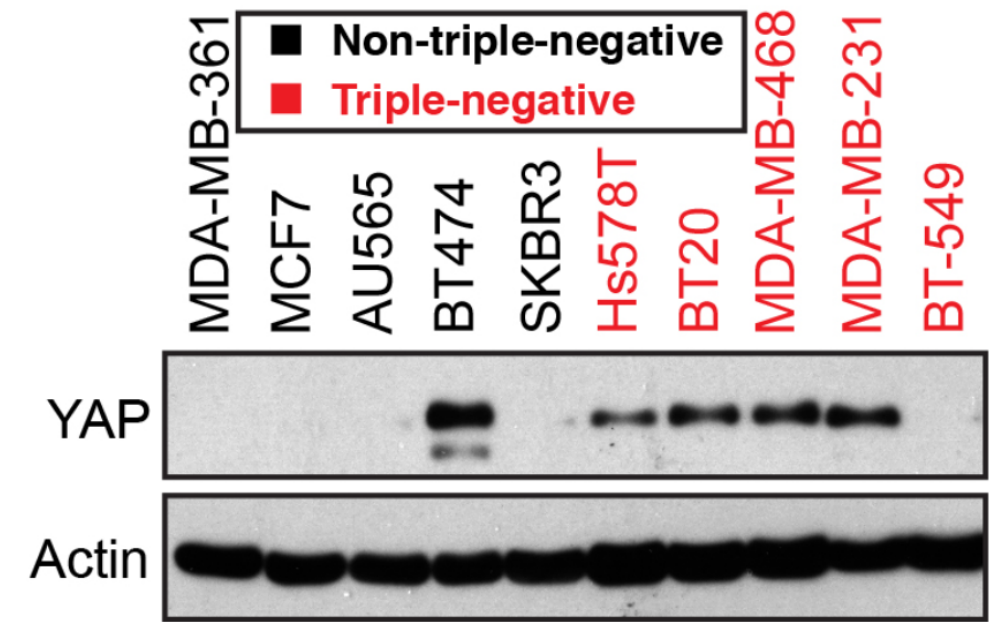
- (A) Heatmap analysis of mRNA expression of YAP and breast cancer markers in TCGA database (analyzed by Oncomine® platform).
- (B) Heatmap analysis of mRNA expression of YAP and breast cancer markers in CCLE database (analyzed by GENE-E platform).



### ***2.1.2: Protein Expression of YAP in Cell Lines***

Since the mRNA expression of YAP is significantly correlated with TNBC subtypes from the public databases. We wonder if the protein level of YAP has a similar trend of expression in TNBC. To test this, we selected a panel of commonly used cell lines in the lab, containing five TNBC and five non-TNBC cells to explore the protein expression level of YAP. Interestingly, TNBC cell lines in general express higher amount of YAP protein in immunoblotting (Figure 2.1.2).

Figure 2.1.2: Expression of YAP Protein Correlates with TNBC

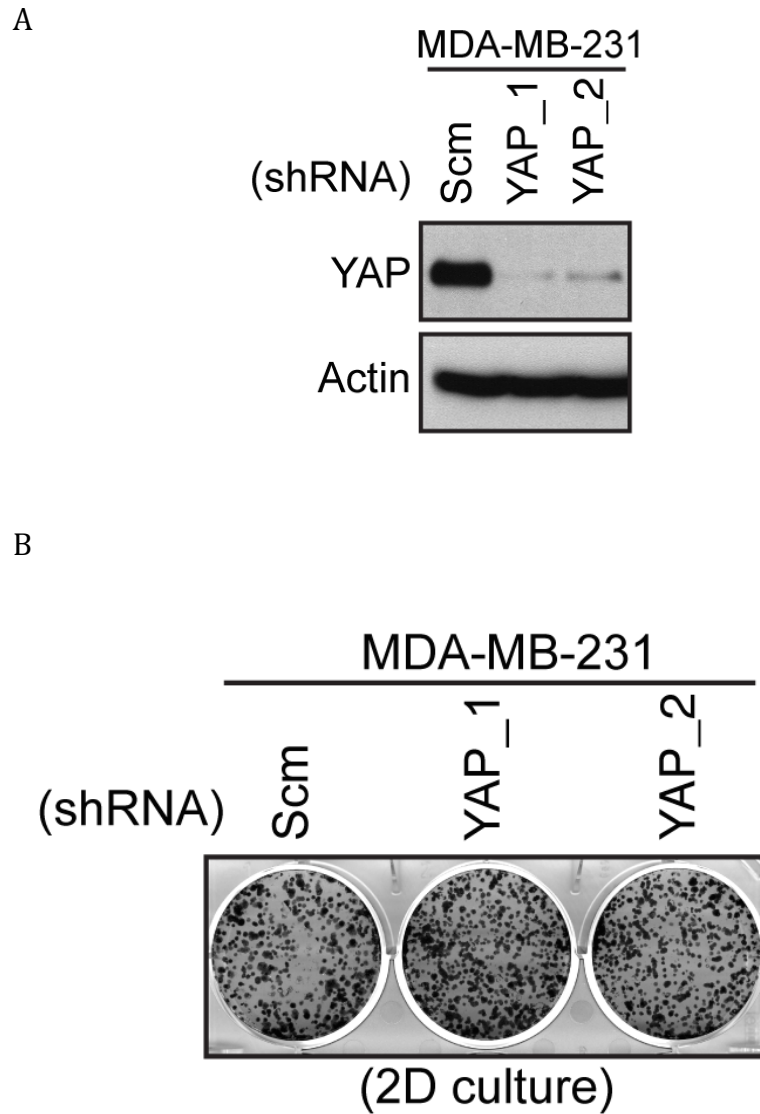


Immunoblot assay to detect YAP expression in a panel of breast cancer cell lines.

### **2.1.3: Knockdown of YAP Reduces 3D Cell Growth**

Knowing that both the mRNA and protein expression level of YAP are indeed more profound in TNBC subtypes, the biological role of YAP in terms of TNBC cell growth is not clear. It is of our interest to know whether YAP plays an oncogenic role in TNBC or not. To answer this question, we used three TNBC cells (MDA-MB-231, MDA-MB-468 and Hs578T) for the manipulation of YAP expression level by short interference RNAs (siRNAs) or CRISPR-cas9 knockout system and then monitored the cell growth. Several stable YAP-knockdown (KD) or knockout (KO) cell lines were established. However, from the results, we did not observe a noticeable growth difference of these stable cell lines in the two-dimensional (2D) colony-formation assay. Since it is generally believed in the cancer field that three-dimensional (3D) cell culture can better mimic tumor growth properties *in vitro*, we then tested the growth of these stable clones on either matrigel or soft agar. Interestingly, we found that in all three cell lines, knockdown or knockout of YAP expression has significantly reduced the number of colonies formed in 3D culture condition (Figures 2.1.3.1 to 2.1.3.4). Reconstituting the expression of YAP in YAP-KO cells rescues the cell growth on matrigel (Figure 2.1.3.4 C). The quantification results of soft agar assay were shown in Figure 2.1.3.5. Overall, these lines of evidence suggest that YAP plays an oncogenic role in TNBC cell growth.

**Figure 2.1.3.1: Knockdown of YAP Does Not Affect 2D Cell Growth in MDA-MB-231 cells**



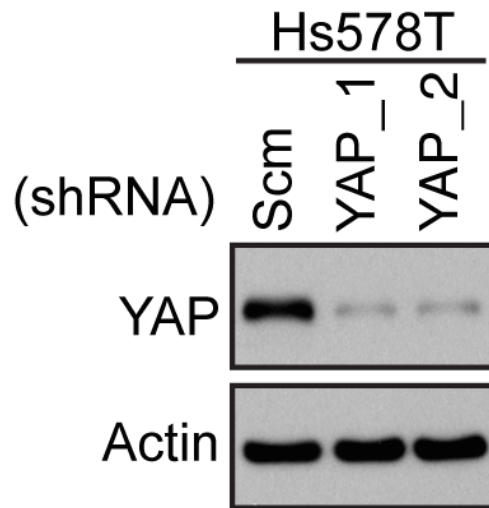
(A) Immunoblot of YAP to show shRNA efficiency in MDA-MB-231 cells.

(B) Colony growth of MDA-MB-231 cells knocked down with YAP shRNAs in 2D culture. Scm, scrambled shRNA control.

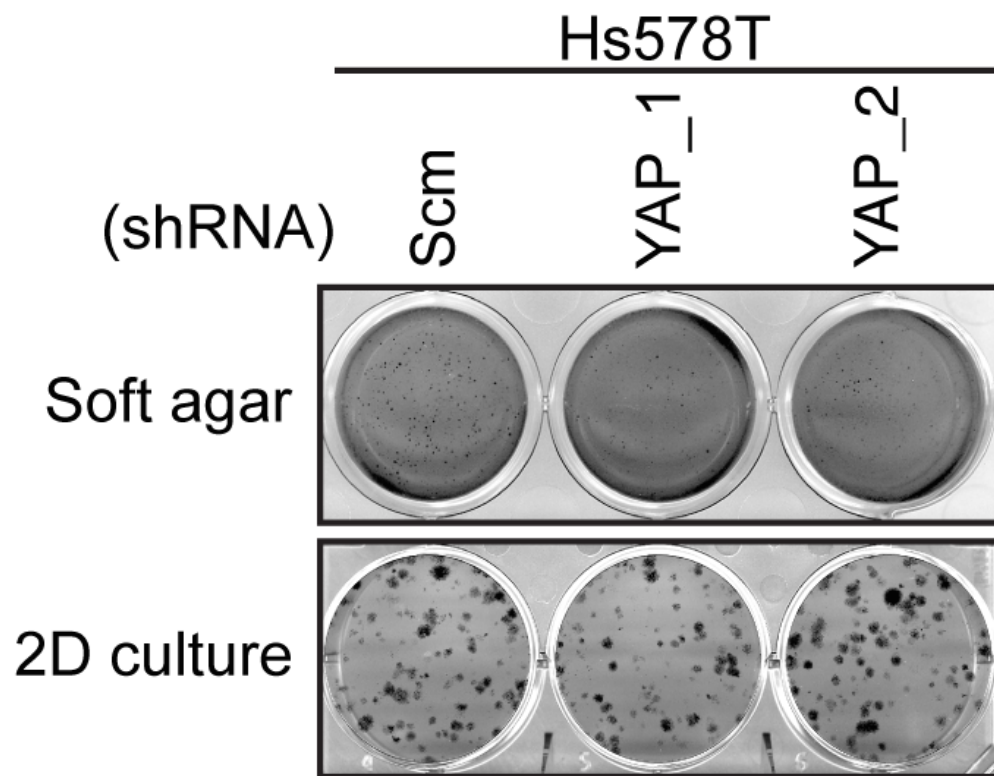
**Figure 2.1.3.2: Knockdown of YAP Does Not Affect 2D Cell Growth in Hs578T**

**Cells**

**A**



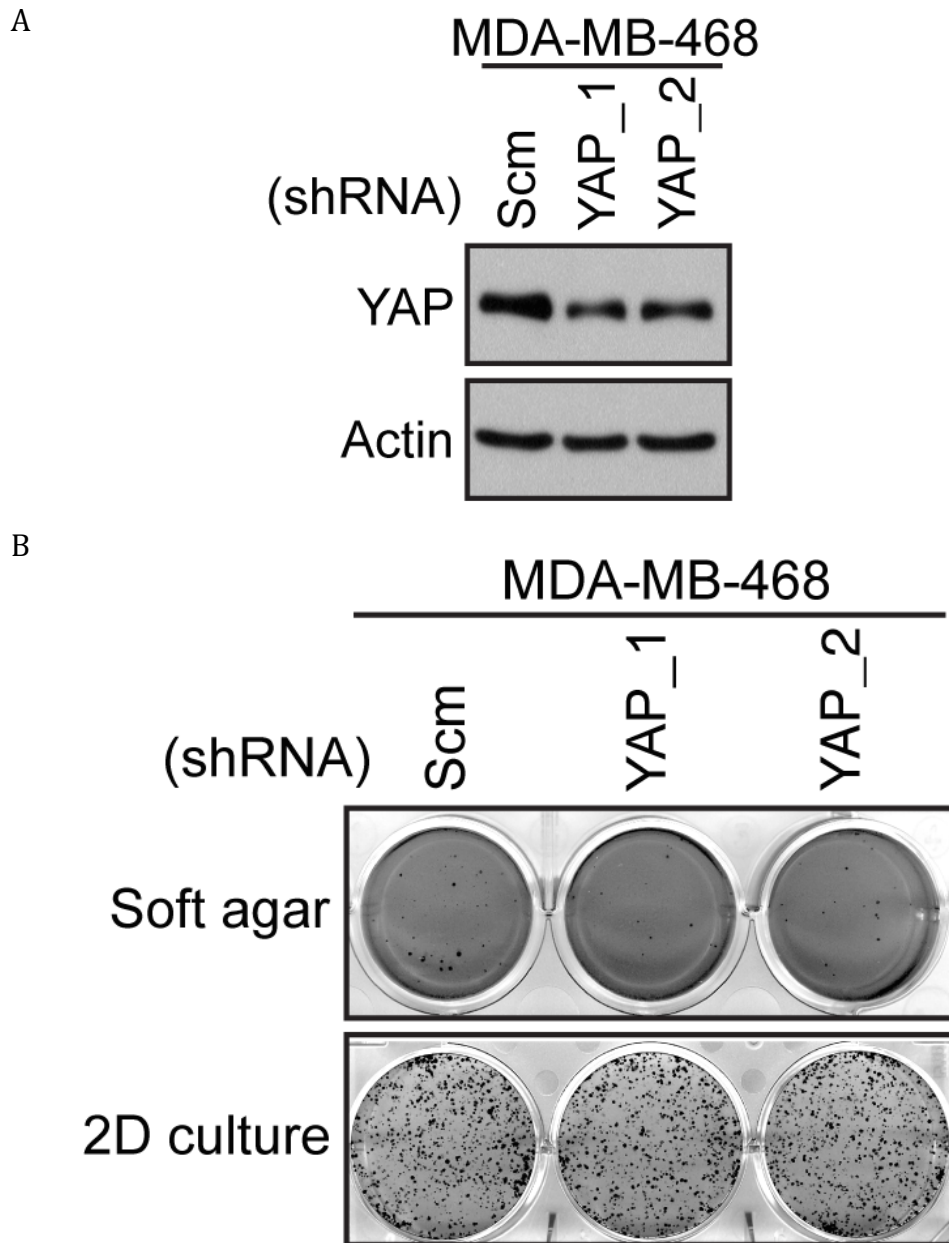
**B**



(A) Immunoblot of YAP to show shRNA efficiency in Hs578T cells.

(B) Colony growth of Hs578 cells knocked down with YAP shRNAs in 2D and soft agar culture.

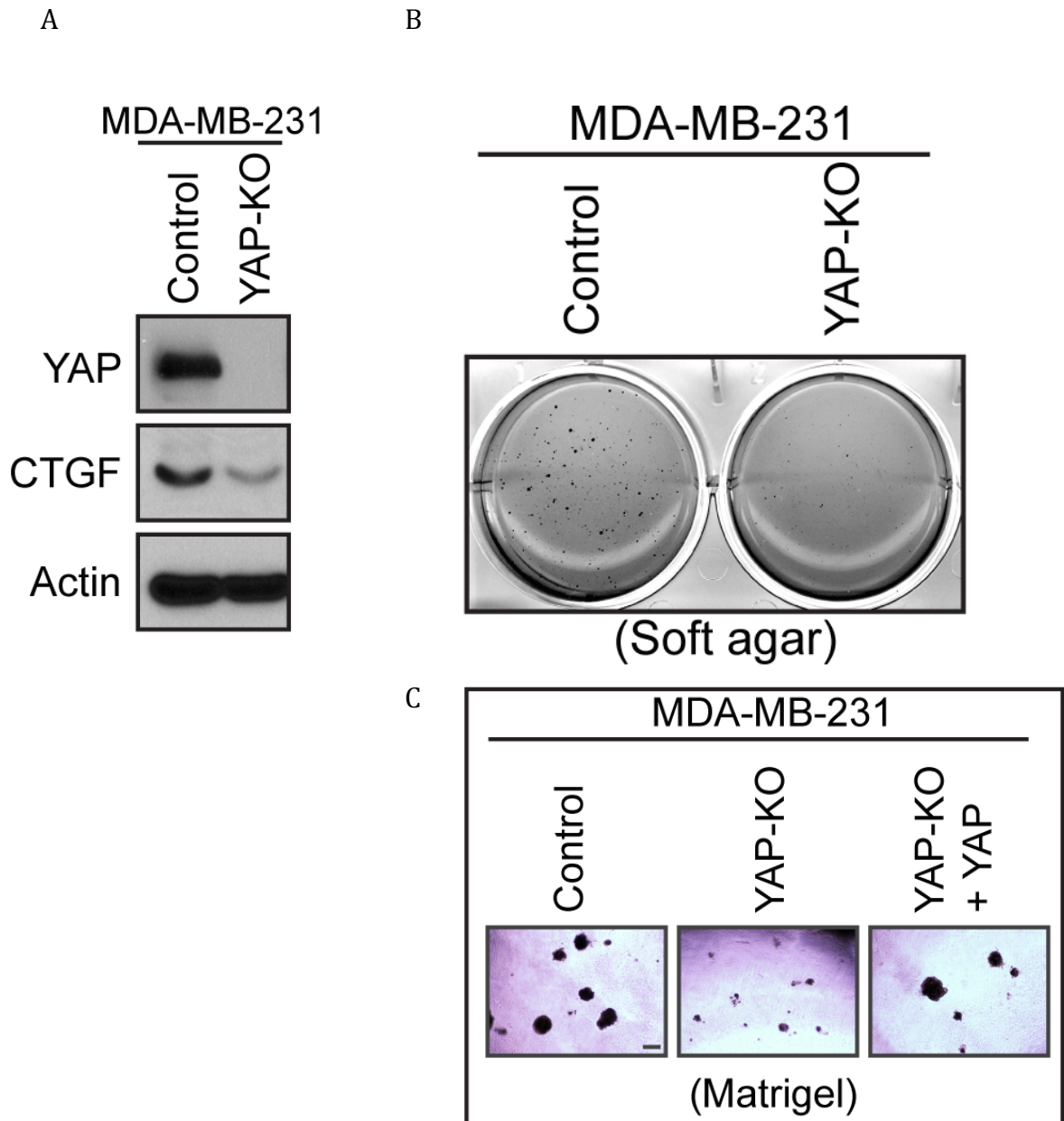
**Figure 2.1.3.3: Knockdown of YAP Does Not Affect 2D Cell Growth in MDA-MB-468 Cells**



(A) Immunoblot of YAP to show shRNA efficiency in MDA-MB-468 cells.

(B) Colony growth of MDA-MB-468 cells knocked down with YAP shRNAs in 2D and soft agar culture.

**Figure 2.1.3.4: Knockout of YAP Reduces 3D Cell Growth**



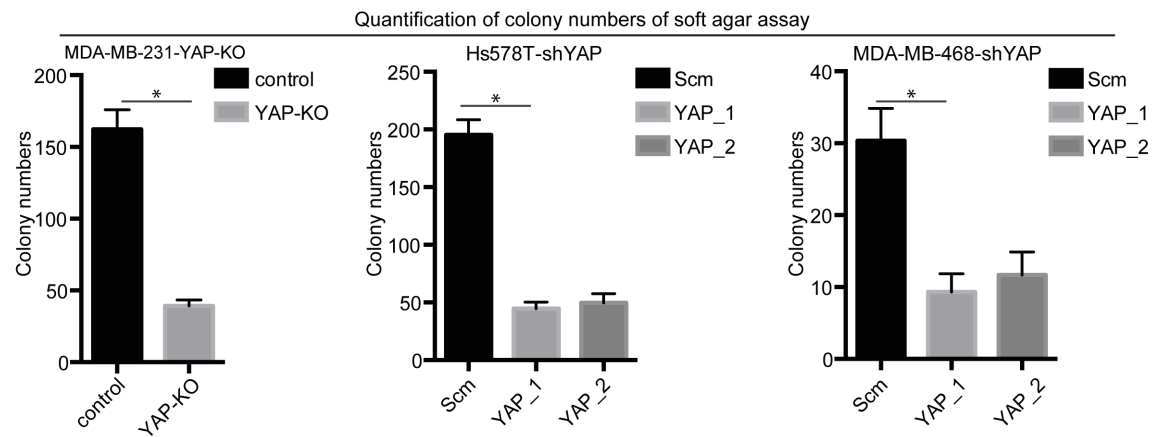
(A) Immunoblot of YAP and CTGF in MDA-MB-231 YAP-KO cells.

(B) Colony growth of MDA-MB-231 YAP-knockout (YAP-KO) cells in soft agar culture.

(C) Images of MDA-MB-231 cell growth on matrigel. KO indicates knockout.

Control, CRISPR-sgRNA targeting EGFP. (Scale bar is 200  $\mu$ m)

**Figure 2.1.3.5: Quantification of 3D Cell Growth from Three TNBC Lines**



Quantification of visible colony numbers in soft agar assays. Error bars indicate the SD (n=3). \* $P < 0.05$ , Student's t-test.



## **2.2: Aurora A Kinase Interacts with YAP**

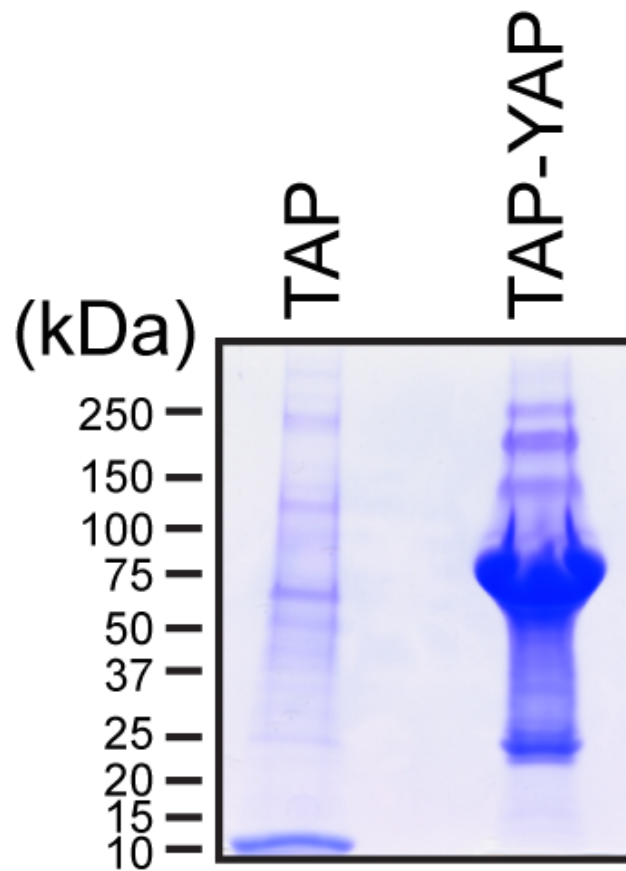
### ***2.2.1: Identification of YAP-Interacting Proteins by a Proteomic Approach***

Since the oncogenic role of YAP in terms of supporting the cell growth in TNBC has been determined, next we would like to know how YAP activity may be regulated by other interacting proteins. To tackle this task, we first need to know what are other proteins that show physical interaction with YAP in TNBC cells. We took advantage of the tandem-affinity-purification system (TAP) and coupled it with mass spectrometry analysis (M/S) for the identification of YAP-interacting proteins. TAP system is good for identification of protein-protein interaction as this system uses double affinity-elution process to reduce most of the nonspecific binding , compared to the regular immunoprecipitation method. First, we constructed a TAP-YAP-expressing lentiviral vector (with protein A and calmodulin-binding domain tags). Then we established a stable clone of TAP-YAP in MDA-MB-231 cells. Using a stable clone for protein purification is beneficial, as a long-term expression of bait protein would allow the bait protein to restore the stoichiometric ratio with other endogenous proteins. We then purified the YAP-interacting protein complexes from a huge amount of protein lysates under a non-denaturing condition. The eluted proteins were separated by SDS-PAGE and stained by coomassie blue on the gel (Figure 2.2.1.1). In the figure, a strong band, which corresponds to the size of YAP was shown, indicating a successful purification.

The eluted protein complexes were subjected to M/S analysis through a collaboration laboratory located in Taiwan. A total of 184 unique protein IDs have

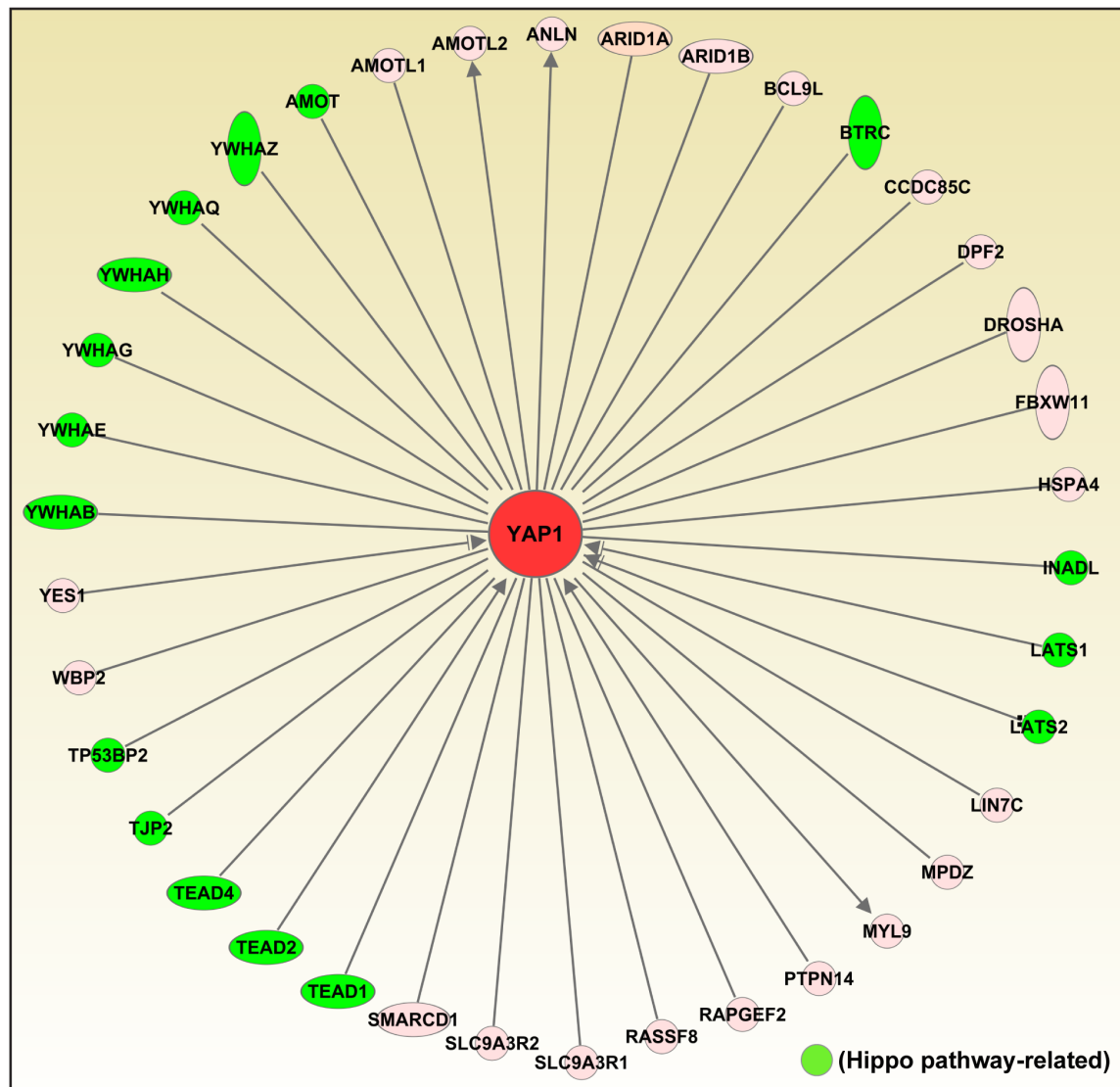
been identified through M/S. We then input the protein list to Ingenuity Pathway Analysis (IPA; from Qiagen) platform to analyze the association of these proteins with cellular signaling pathways. As expected, 21 of 184 proteins have been reported to relate to Hippo pathway. 38 of 184 proteins have been reported to interact with YAP (Figure 2.2.1.2). These results have indicated the faithfulness of our purification system and condition that can well preserve YAP-associated complexes.

**Figure 2.2.1.1: Pull-Down of YAP Complex**



Coomassie blue staining of the pulled-down elution of MDA-MB-231 stable cells. TAP indicates a no-bait control (Tandem-affinity-purification tag only). TAP-YAP, YAP1 protein N-terminus-tagged of TAP sequence.

**Figure 2.2.1.2: Network of YAP-Interacting Proteins from M/S**

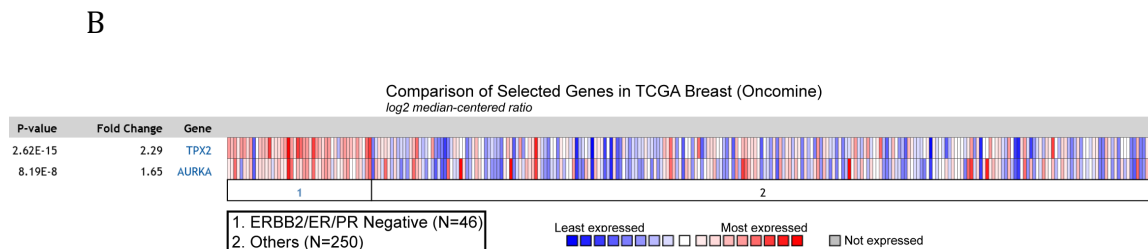
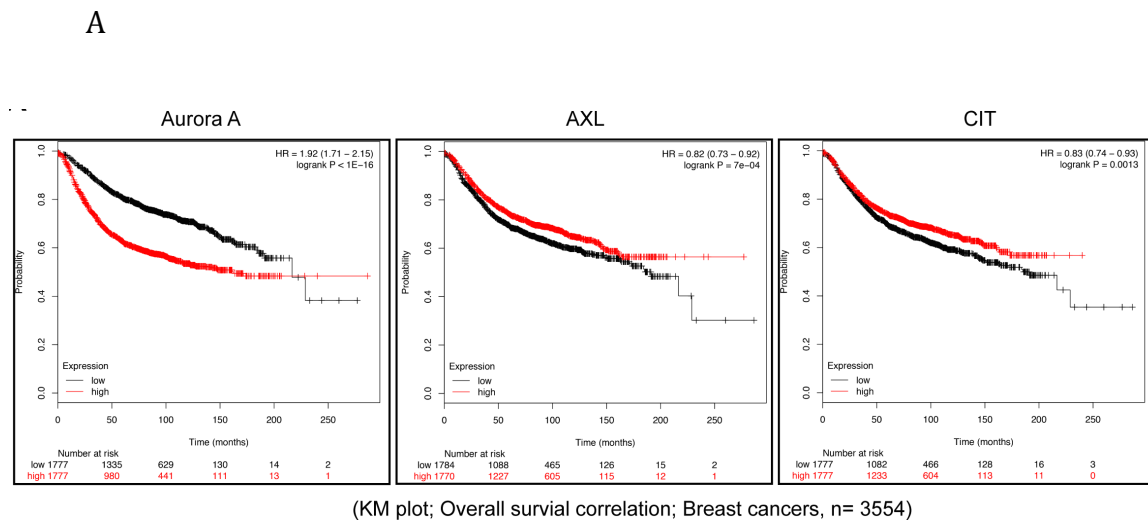


Interaction map of YAP-associated protein complexes from our study. Only proteins reported with direct interaction with YAP were shown. Green color indicates those reported in the Hippo pathway. Arrows indicate the kinase/substrate relationship. The results were analyzed and drawn by the IPA® platform.

### ***2.2.2: Clinical Relevance of AXL, CIT and Aurora A kinases in Breast Cancers***

From the newly identified YAP-associated proteins in MDA-MB-231 cells, we found three kinases, which were not reported in the literatures. These three kinases are Aurora A kinase, CIT kinase (Citron Rho-Interacting Serine/Threonine Kinase) and AXL tyrosine kinase. Kinases are particularly of our research interest as there are many small compound inhibitors readily available and many of them are now in clinical trials. Identification of kinase that regulates YAP activity may decipher a new use from existing drugs to control YAP activity. In order to select kinases more relevant to the malignancy of breast cancer, we analyzed the patient survival data based on the mRNA expression level of these three kinases. We used an online tool, Kaplan Meier plotter (138), to find the correlation in 3,554 breast cancer patients. From the results, only the expression of Aurora A correlates with a poor prognosis in overall breast cancers (Figure 2.2.2 A). This result prompted us to focus on investigating further of potential regulation of Aurora A on YAP protein. Interestingly, after analyzing the expression correlation of Aurora A and its activator, TPX2, from a TCGA breast cancer database, we also observed a nice correlation of Aurora A with TNBC subtype (Figure 2.2.2 B). These results suggests Aurora A and TPX2 are relevant to TNBC.

**Figure 2.2.2: Clinical Relevance of Identified Kinases in Breast Cancers**

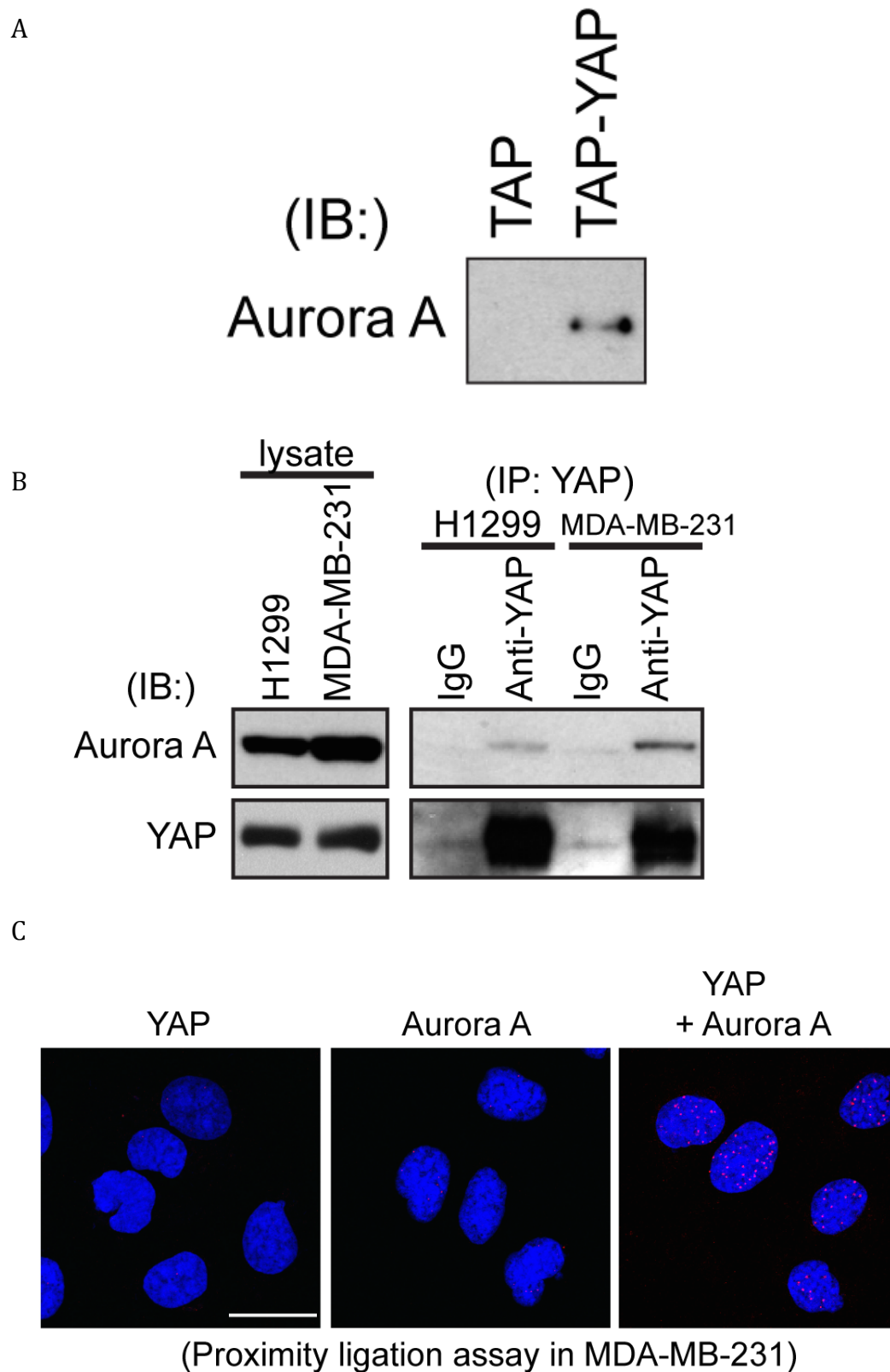


- (A) Analysis of the correlation of kinases and breast cancer patient survival in Kaplan Meier plotter (<http://kmplot.com/analysis/>).
- (B) Heatmap analysis of mRNA expression of TPX2, Aurora A, and breast cancer markers in TCGA database (analyzed by Oncomine® platform).

### ***2.2.3: Aurora A kinase Physically Interacts with YAP***

The presence of Aurora A kinase in the YAP-containing complex encouraged us to further validate whether Aurora A indeed can interact with YAP protein. First, we used immunoblot to check if Aurora A was indeed in the sample subjected to M/S analysis. From the result, we can clearly see that Aurora A was found in the sample (Figure 2.2.3 A). Then we tested whether YAP can interact with endogenous Aurora A in cells. MDA-MB-231 and H1299 cells were tested by immunoprecipitation assay to pull down YAP and immunoblotted Aurora A. From the result, YAP can interact with Aurora A (Figure 2.2.3 B). Furthermore, in order to detect where the interaction between Aurora A and YAP occurs inside the cells, we performed a proximity-ligation assay in MDA-MB-231 cells. Interestingly, the majority of interaction happened in the nucleus (Figure 2.2.3 C).

**Figure 2.2.3: Aurora A kinase Interacts with YAP**





**Figure 2.2.3: Aurora A kinase Interacts with YAP**

- (A) Immunoblot of Aurora A in elution from (Figure 2.2.2.2).
- (B) Co-immunoprecipitation of YAP and Aurora A protein from two different cells.
- (C) Proximity ligation assay to analyze co-localization of YAP and Aurora A in MDA-MB-231 cells. The scale bar represents 10  $\mu\text{m}$ .

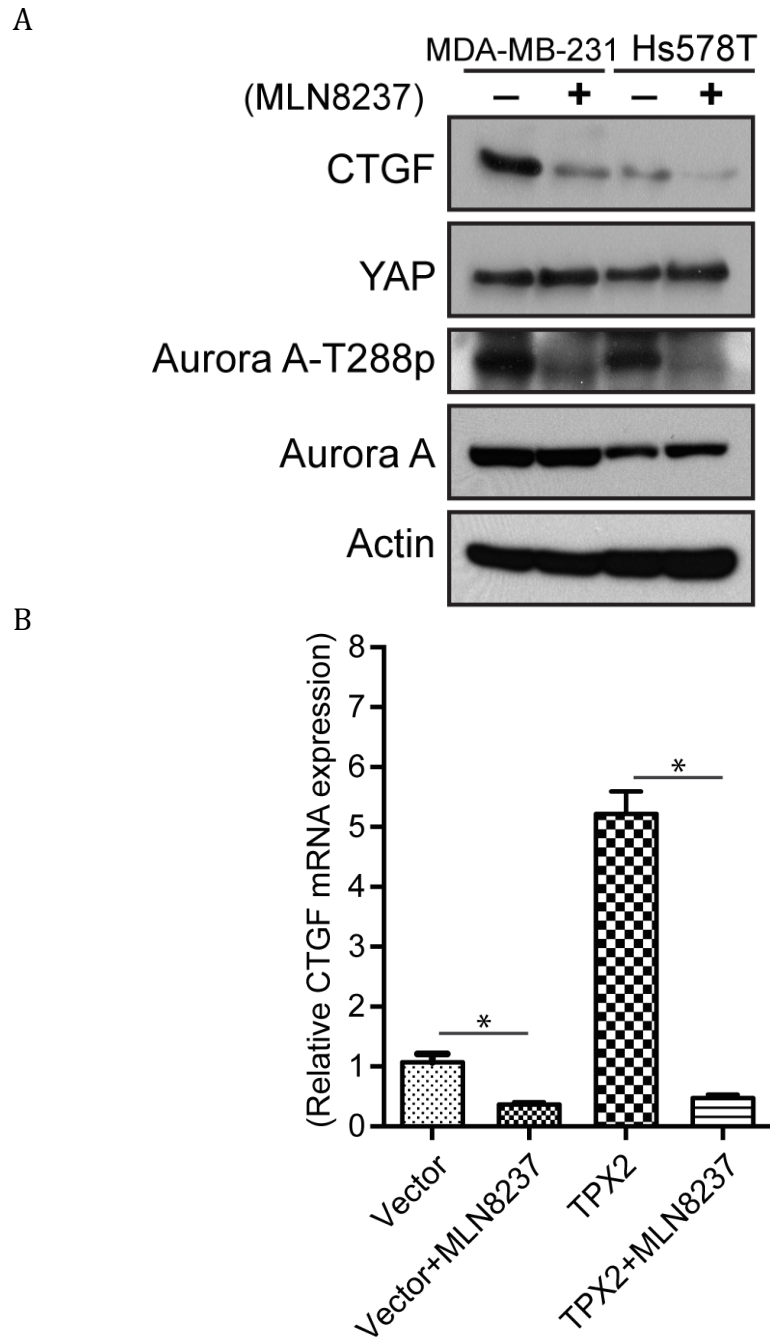
## **2.3: Aurora A Kinase Regulates YAP-Mediated Transcriptional Activity**

### ***2.3.1: Inhibition of Aurora A Decreases CTGF Expression***

As YAP functions as a transcriptional co-activator in the nucleus and Aurora A happens to interact with YAP in the nucleus (Figure 2.2.3 C), we hypothesized that Aurora A may affect the transcriptional activity of YAP. To quickly tested this possibility, we treated an Aurora A kinase inhibitor, MLN8237, in two TNBC cells, MDA-MB-231 and Hs578T, to see if it has any effect on YAP target gene, CTGF. Interestingly, upon the inhibition of Aurora A kinase activity, the CTGF expression level went down (Figure 2.3.1.1 A). Examination of mRNA expression of CTGF also indicated a downregulation by Aurora A inhibitor (Figure 2.3.1.1 B).

Chemical inhibitors may possess off-target effects. To consolidate our findings that Aurora A is required for YAP-mediated CTGF expression, we used siRNAs to test the hypothesis again. In Hs578T cells, knockdown of Aurora A decreases the expression of CTGF (Figure 2.3.1.2 A). Similarly, knockout of Aurora A by CRISPR-cas9 system in MDA-MB-231 cells also decrease the expression of CTGF (Figure 2.3.1.2 B). These results suggested that Aurora A kinase is required for optimal YAP transcriptional activity.

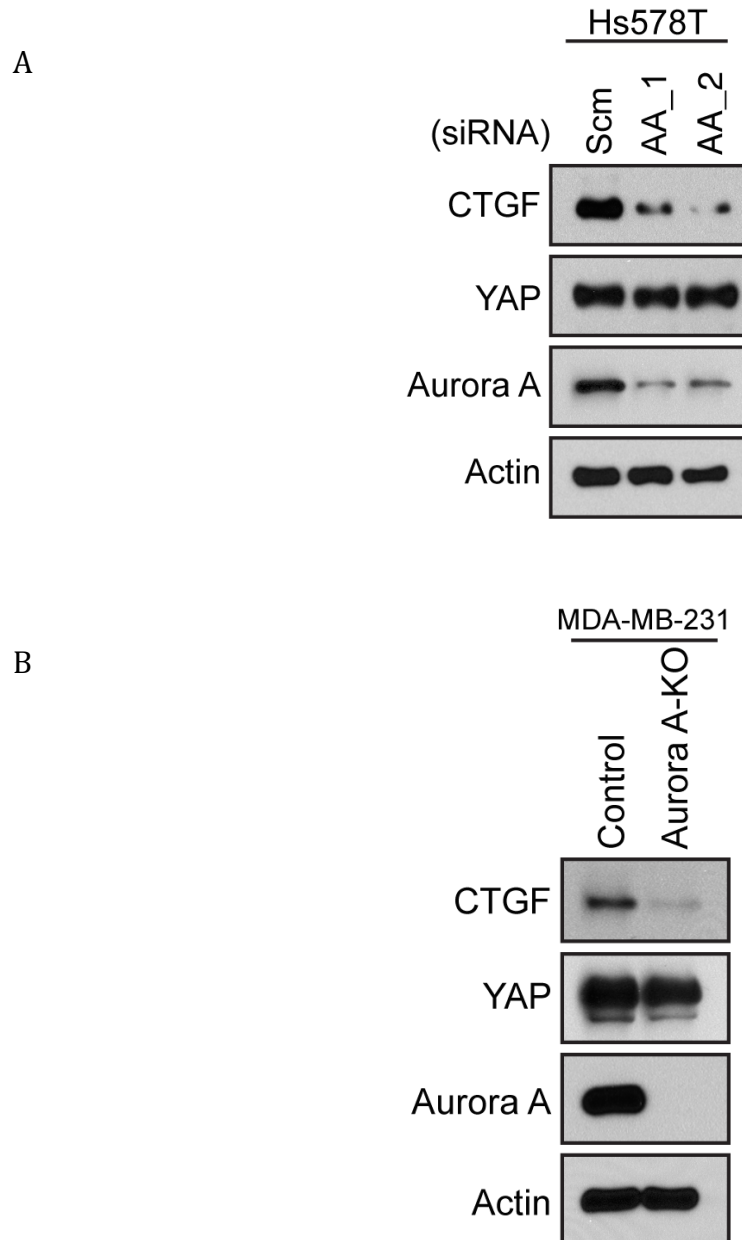
**Figure 2.3.1.1: Aurora A Inhibitor Reduces Expression of CTGF**



(A) Immunoblot of CTGF expression from lysates of MDA-MB-231 and Hs578T cells treated with or without Aurora A kinase inhibitor, MLN8237.

(B) RT-qPCR analysis of CTGF mRNA level from MDA-MB-231 and MDA-MB-231-TPX2 cells treated with MLN8237.

**Figure 2.3.1.2: Knockdown of Aurora A Reduces Expression of CTGF**



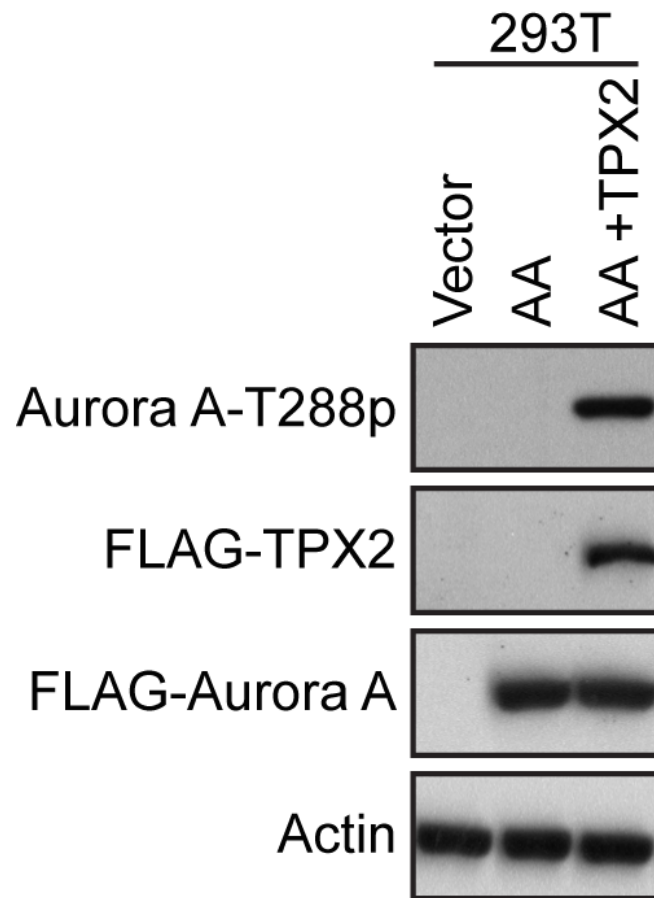
- (A) Immunoblot of CTGF expression in Hs578 cells transfected with siRNAs against Aurora A. (AA, Aurora A)
- (B) Immunoblot of CTGF expression in MDA-MB-231-Aurora A-KO cells. (KO, knockout)

### ***2.3.2: Activation of Aurora A Increases Expression of YAP-Target Gene***

Activation of Aurora A is demonstrated by monitoring the phosphorylation status of its own threonine 288 (T288) residue. However, when we overexpressed Aurora A alone, it did not cause an activation of Aurora A (Figure 2.3.2.1). To efficiently activate Aurora A, we chose to stimulate Aurora A activity by expressing TPX2, a strong co-activator of Aurora A. Indeed, expression of TPX2 induces a robust activation of Aurora kinase, supported by the auto-phosphorylation at T288 (Figure 2.3.2.1). Because of this result, we later only used exogenously expressed TPX2 to serve as a stimulus for Aurora A activity.

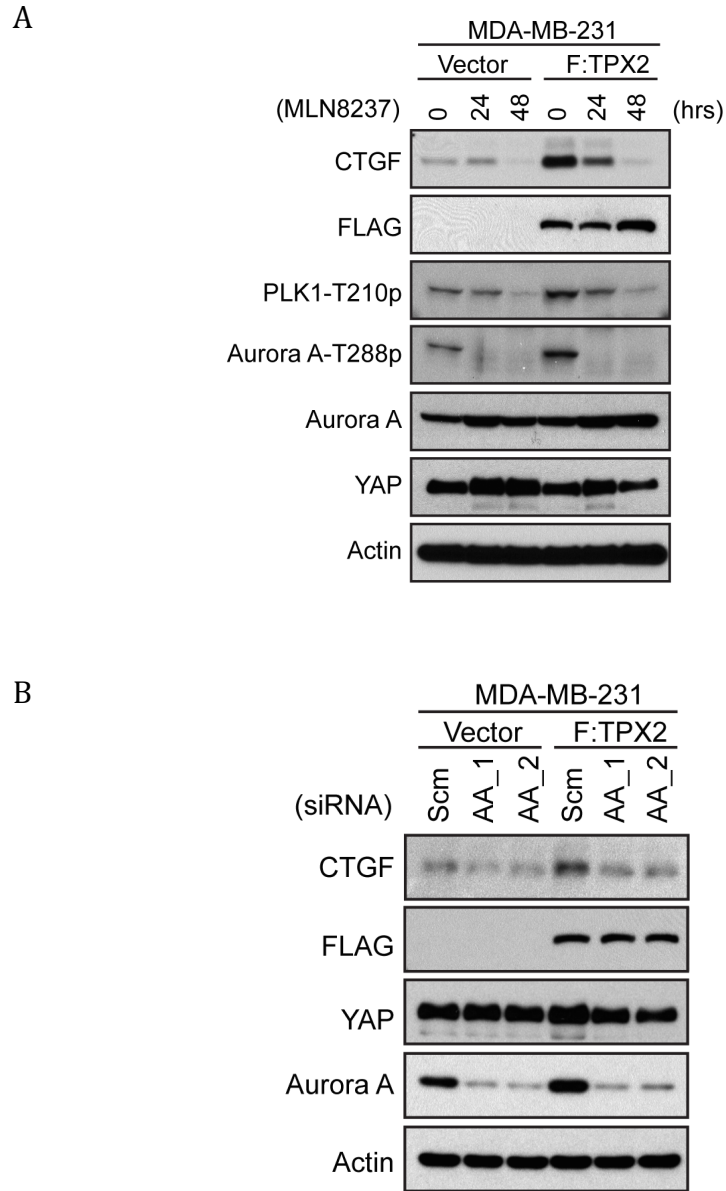
We wonder whether activation of Aurora A signaling can increase YAP activity. To test this hypothesis, we established a stable TPX2-expressing MDA-MB-231 line and used immunoblotting to detect the expression level of YAP-target gene, CTGF. From the results, it shows that expression of TPX2 is sufficient to induce a higher expression of CTGF, compared to the vector control. Similar to the result in section 2.3.1, inhibition of Aurora A by kinase inhibitor or siRNAs has also diminished TPX2-induced CTGF in both mRNA and protein level, suggesting that TPX2 induces CTGF level through Aurora A (Figure 2.3.2.2 A and B; Figure 2.3.1.1 B). These results demonstrated that Aurora A can positively regulate YAP activity.

**Figure 2.3.2.1: TPX2 Activates Aurora A Kinase Activity**



Immunoblot assay to detect the activation of Aurora A from lysates of transfected 293T cells.

**Figure 2.3.2.2: Activation of Aurora A Induces Expression of CTGF**



- (A) Immunoblot of CTGF expression from MDA-MB-231 cells with stable expression of FLAG-TPX2. Cells were treated with/without MLN8237 and collected at indicated time point. (F:TPX2, FLAG-TPX2)
- (B) Immunoblot of CTGF level in MDA-MB-231-TPX2 cells knocked down with siRNAs against Aurora A (AA).

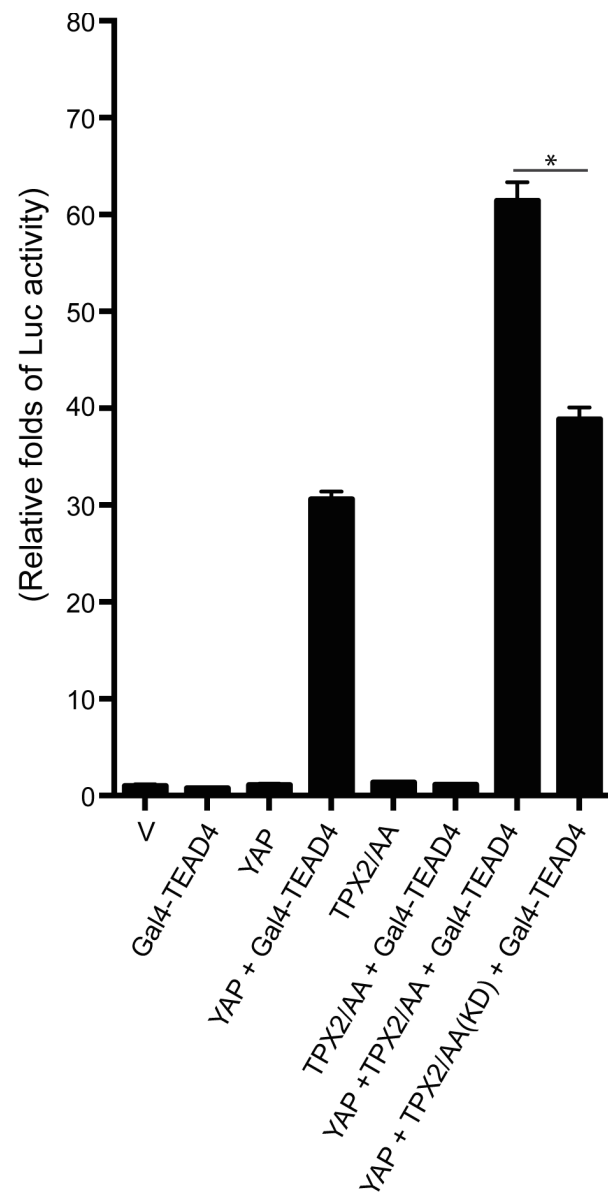
### ***2.3.3: Aurora A Potentiates YAP-Regulated Promoter Activity***

To further delineate whether Aurora A can directly affect the transcriptional activity of YAP, we used a luciferase reporter system to test the activity of YAP. The reporter system contains three functional elements, including an activator, a GAL4-containing DNA-binding protein and a UAS-driven luciferase reporter. How does it work? For example, when YAP is expressed with GAL4-TEAD in the presence of UAS-luciferase reporter, YAP will complex with TEAD and the YAP/TEAD complex will specifically bind on UAS sequence due to GAL4. If the binding protein on the UAS possesses activity toward transcriptional regulation, the luciferase gene will be expressed and the result can be quantified biochemically.

From our result, expression of YAP can induce TEAD-dependent promoter activation in about 30 folds compared to the control (Figure 2.3.3). Expression of YAP or TEAD alone was not sufficient to activate the reporter, indicating this system has very low background in cells tested. Interestingly, co-expression of Aurora A/TPX2 can further potentiate YAP activity to around 60 folds more than controls. Expression of a kinase-dead mutant of Aurora A losses this potentiation of YAP activity, suggesting the kinase activity of Aurora A is an essential factor to activate YAP (Figure 2.3.3).



**Figure 2.3.3: Aurora A Activates YAP-Mediated Transcription**



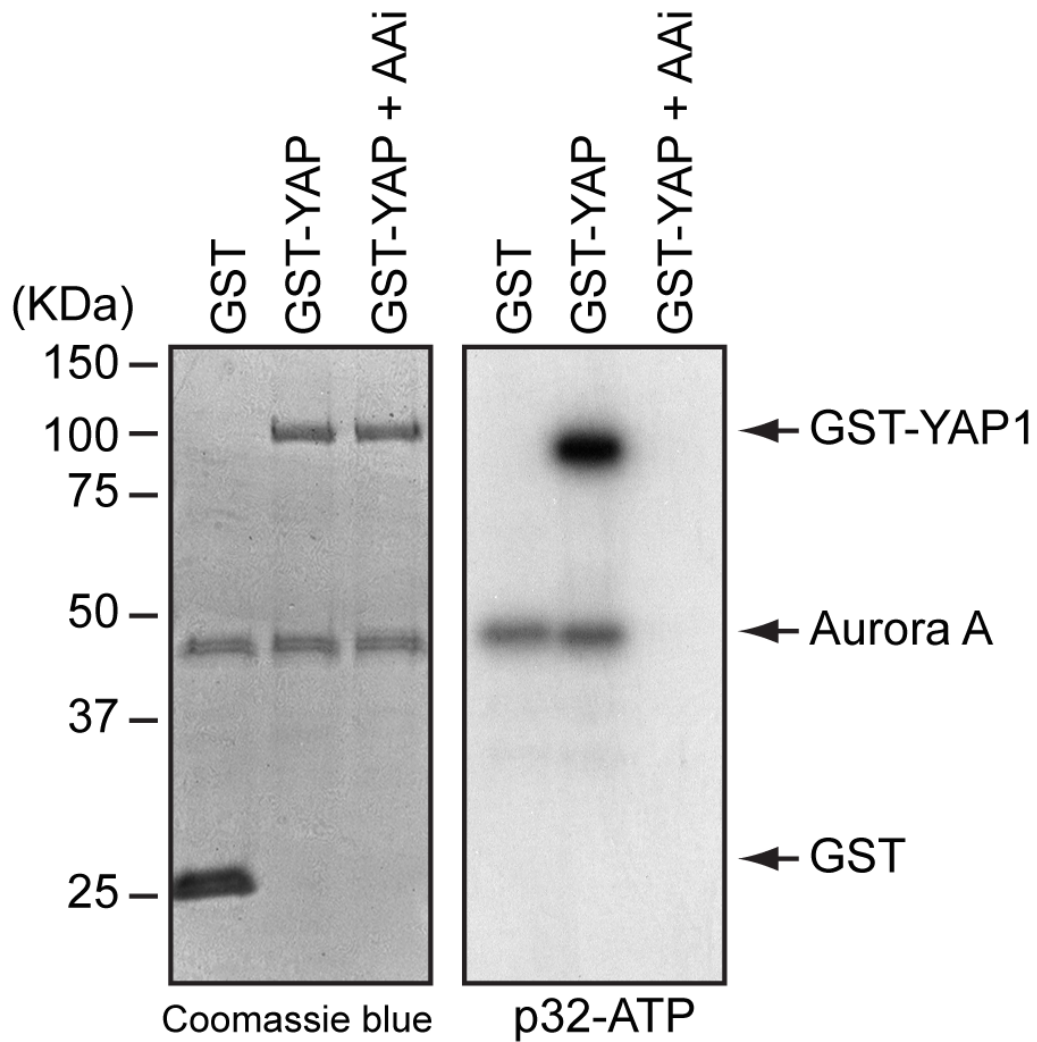
Luciferase reporter assay to evaluate activity of YAP under the influence of TPX2 plus Aurora A (AA). AA-KD represents a kinase-dead mutant (K162R) of Aurora A. Error bars indicates the SD (n = 3). \* $P < 0.05$ , Student's t-test.

## **2.4: YAP Is A Novel Substrate of Aurora A Kinase**

### ***2.4.1: Aurora A Phosphorylates YAP in vitro***

Since Aurora A not only interacts with YAP in the nucleus but also activates YAP's transcriptional activity, we wondered if Aurora A can serve as a kinase that directly phosphorylates YAP protein. To test this possibility, we performed an *in vitro* kinase assay by using recombinant Aurora A kinase and YAP protein. In the presence of gamma-p32-ATP, we can observe a strong phosphorylation event on YAP. Adding the Aurora A kinase inhibitor in the reaction completely abolished the phosphorylation on YAP (Figure 2.4.1). This result demonstrated, *in vitro*, YAP protein can be a substrate of Aurora A kinase. This was a novel discovery related to YAP regulation.

**Figure 2.4.1: Aurora A Kinase Phosphorylates YAP *in vitro***

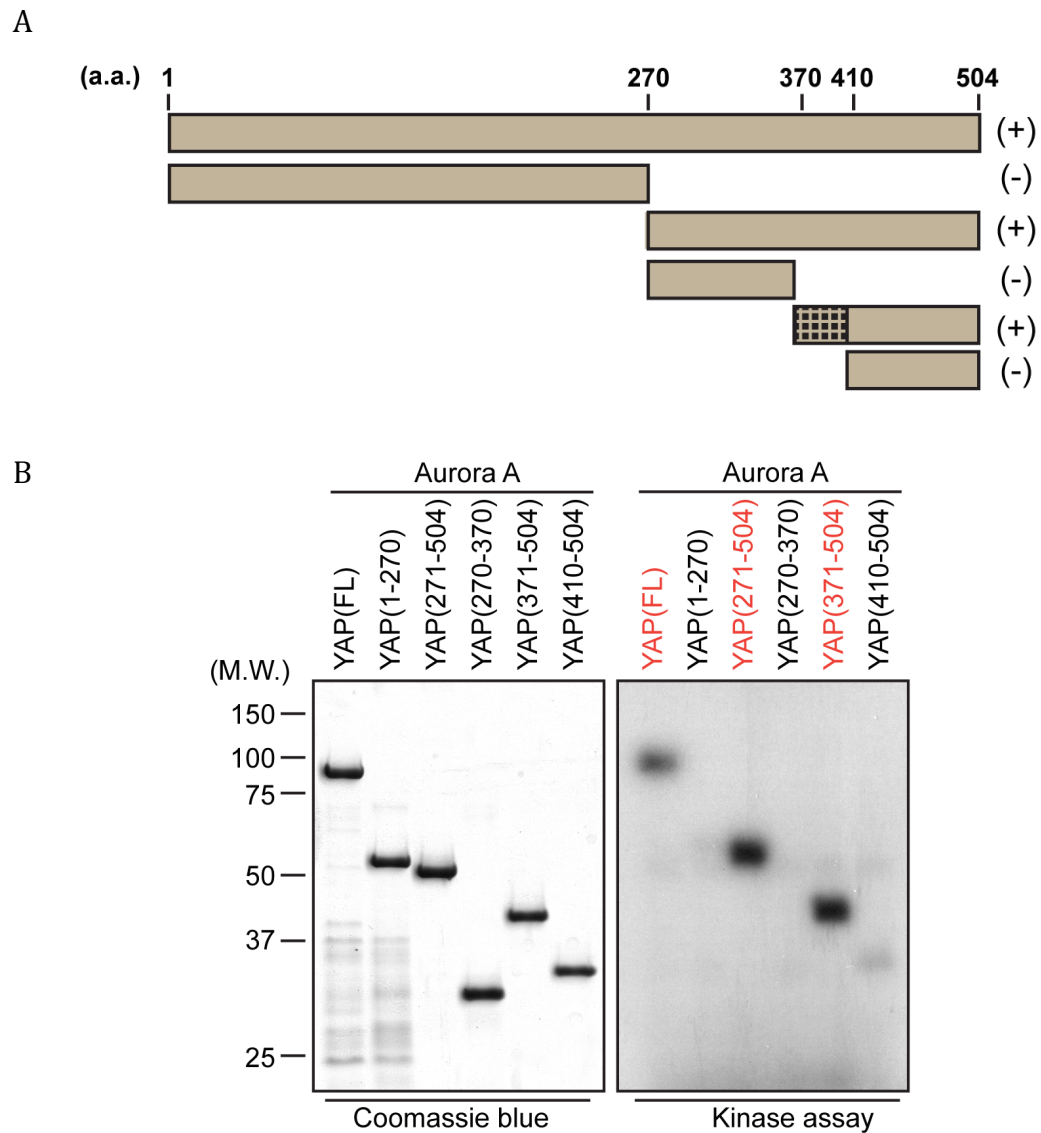


*In vitro* kinase assay using p<sup>32</sup>-ATP. Recombinant Aurora A kinase and GST-tagged YAP proteins are used. (AAi: Aurora A inhibitor)

#### **2.4.2: Protein Domains of YAP Phosphorylated by Aurora A**

Since Aurora A can phosphorylate the full-length YAP protein, next we would like to identify which specific domains of YAP are responsible for phosphorylation coming from Aurora A. Again, we performed an *in vitro* kinase assay using several truncated protein fragments of YAP protein (schematic presentation of protein domains are listed in Figure 2.4.2 A). The results showed that only the C-terminal half (amino acids 270-504) can be phosphorylated by Aurora A. Further dissection showed that amino acids 371-410 is indispensable for phosphorylation. Deletion of amino acids 371-410 has lost majority of phosphorylation signal from Aurora A, suggesting this small area may contain the important residues for the phosphorylation event.

**Figure 2.4.2: Protein Domains of YAP Phosphorylated by Aurora A Kinase**

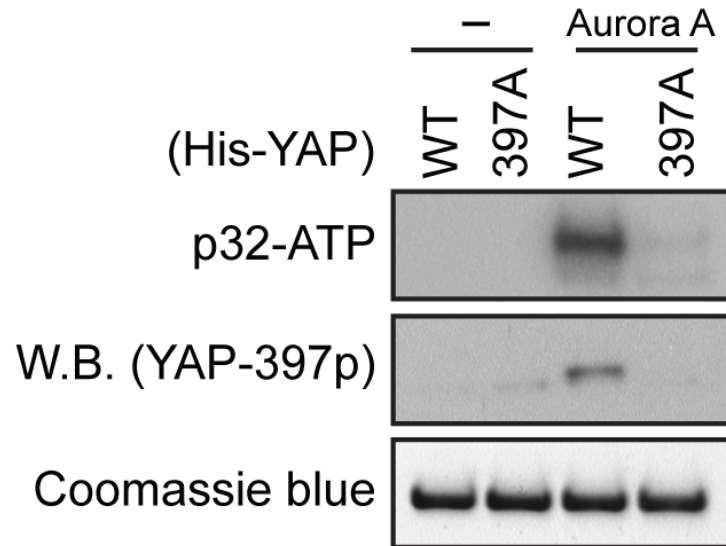


- (A) Schematic illustration of different YAP fragments used in (E). Labeled part represents a potential region that contributes to the phosphorylation signal. (+) and (-) represent whether the protein fragments was phosphorylated or not, respectively from (C).
- (B) *In vitro* kinase assay to evaluate the phosphorylation status of truncated YAP proteins.

### **2.4.3: Identification of The Phosphorylation Site of YAP**

Knowing that protein domain encompassing amino acids 371 to 410 of YAP is the critical area to receive phosphorylation from Aurora A kinase, next we sought to identify whether there is a critical serine or threonine residue for this event. We combined several criteria, including our experimental results, known Aurora A consensus substrate sequence, our own M/S analysis and M/S results from a public database (Phosphosites; Cell Signaling™) to narrow down the candidates. By overlapping these criteria together, only the serine 397 residue is a candidate that fits. We performed an *in vitro* kinase assay using YAP protein mutated its S397 to alanine as the substrate. Interestingly, mutation of S397 has lost most of the phosphorylation signal from Aurora A (Figure 2.4.3). Furthermore, using a commercially available monoclonal antibody which recognizes YAP-S397 phosphorylation, we can also detect a specific signal from the reaction of the *in vitro* kinase assay. The antibody cannot detect the signal when S397 site was mutated to alanine (Figure 2.4.3). These results suggested that serine 397 of YAP is the major site to be phosphorylated by Aurora A kinase.

**Figure 2.4.3: YAP-S397 Is A Aurora A Phosphorylation Site**



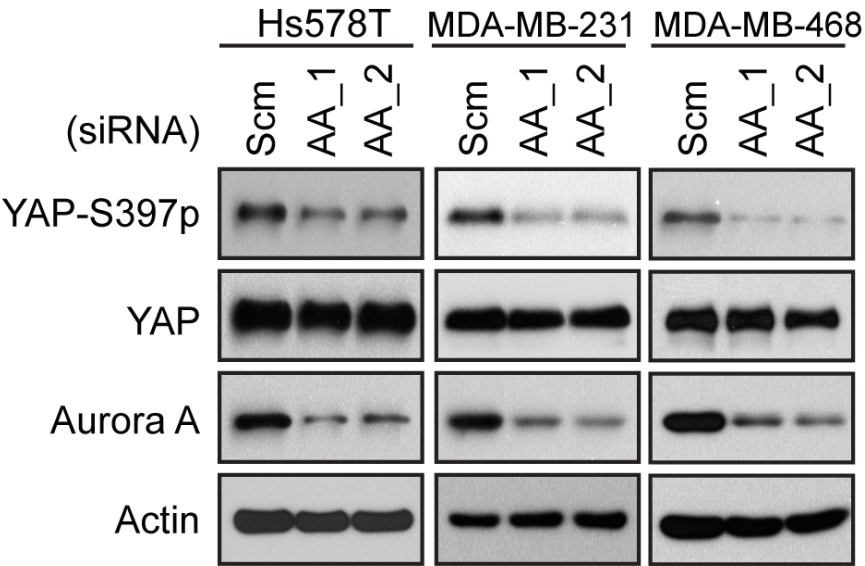
Immunoblot and *in vitro* kinase assay to evaluate the phosphorylation status of Serine 397 of YAP protein (YAP-S397) in the presence of Aurora A kinase. His-YAP represents a recombinant fragment of His-tagged YAP protein (a.a. 271-504). WT: wild-type; S397A: alanine mutant.

#### ***2.4.4: Endogenous YAP Is Phosphorylated by Aurora A***

Although YAP is a substrate of Aurora A kinase in test tubes, we wonder if the cellular endogenous phosphorylation of S397 of YAP can be influenced by Aurora A. To test this notion, endogenous Aurora A was knocked down by siRNAs in three TNBC cell lines, MDA-MB-231, Hs578T and MDA-MB-468. As we expected, knockdown of Aurora A significantly reduces the phosphorylation of YAP-S397 site (Figure 2.4.4.1). In contrast, expression of TPX2 to activate endogenous Aurora A increases the phosphorylation of YAP-S397 in MDA-MB-231 and BT-549 cells (Figure 2.4.4.2 A and B). The phosphorylation signal detected by the antibody was specific as mutation of YAP-S397 to alanine lost the signal (Figure 2.4.4.2 B).

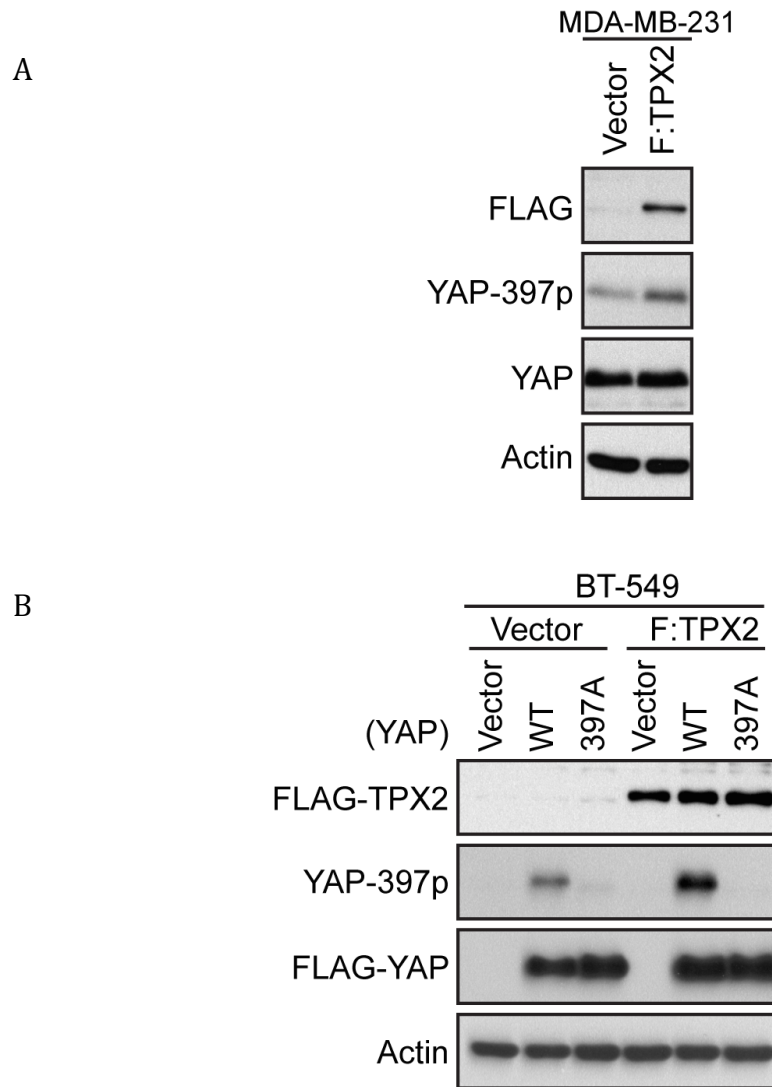


**Figure 2.4.4.1: Phosphorylation of YAP-S397 Is Regulated by Aurora A *in vivo***



Evaluation of phosphorylation of YAP-S397 by immunoblot in cells knocked down of Aurora A (AA).

**Figure 2.4.4.2: Activation of Aurora A Increase Phosphorylation of YAP-S397**



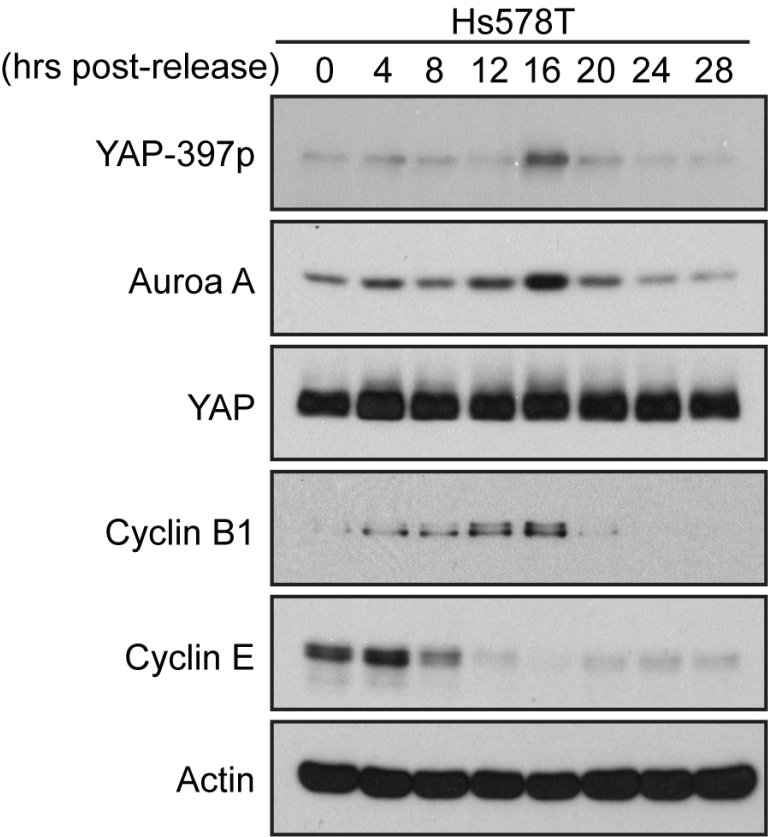
(A) Immunoblot of YAP-S397 phosphorylation from lysate of MDA-MB-231-TPX2 cells.

(B) Immunoblot to evaluate the level of YAP-S397 phosphorylation in the presence of TPX2 or not.

#### ***2.4.5: Cell Cycle-Dependent Phosphorylation of YAP***

One of the major physiological regulations of Aurora A activity/expression is the cell cycle. We wondered if physiology-relevant activation of Aurora A can induce the phosphorylation of YAP-S397. To test this question, we used double-thymidine block to synchronize the cell cycle of Hs578T cells. After released, the cell lysates were collected following a time course. Expressions of cyclin B1 and cyclin E were used as markers to show the status of the cell cycle. From the result (Figure 2.4.5), cell cycle was indeed synchronized at G1/S boundary and then it gradually progressed into G2 and M phase, as indicated by the expression pattern of corresponding cyclin(s). Expression of Aurora A peaked at G2/M phase as reported in the literatures. Interestingly, phosphorylation of YAP-S397 peaked mostly at the same time point with Aurora A. This result strongly supported that Aurora A can phosphorylate YAP on S397 site and this might postulate that the activity of YAP can be regulated through a cell-cycle-dependent manner.

**Figure 2.4.5: Cell-Cycle Dependent Phosphorylation of YAP-S397**



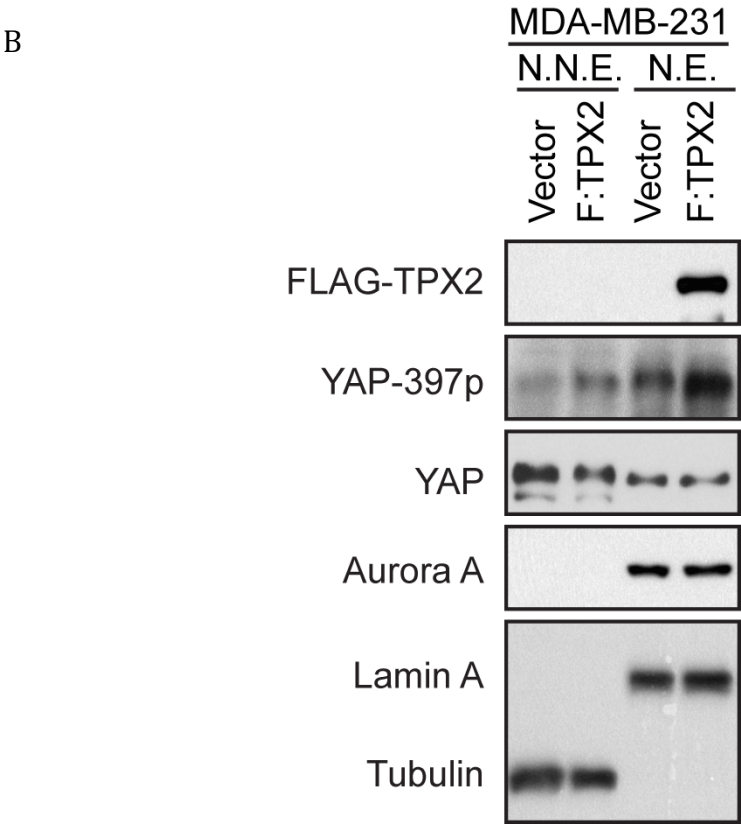
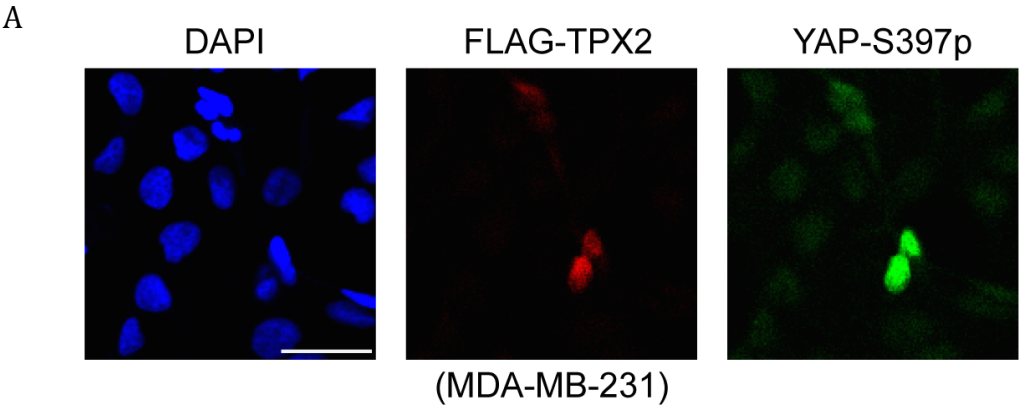
Immunoblot of phospho-YAP-S397 in Hs578T cells that are cell cycle synchronized by double-thymidine block.

## **2.5: Phosphorylation of YAP by Aurora A Occurs Predominantly in the Nucleus**

### ***2.5.1: TPX2 Induces YAP Phosphorylation Mainly in the Nucleus***

Since YAP and Aurora A predominantly interacts with each other in the nuclear compartment, we wonder if the phosphorylation of YAP-S397 occurs mostly in the nucleus. To test this, we transiently transfected TPX2-expressing construct into MDA-MB-231 cells and performed an immunofluorescent staining on YAP-S397 phosphorylation. From the result, the phosphorylation signal of YAP-S397 indeed mainly localized in the nucleus of TPX2-transfected cells (Figure 2.5.1 A). Through cell fractionation assay to separate nuclear and cytoplasmic proteins, we can also observed that in TPX-2 stable cells, YAP-S397 phosphorylation occurs mainly in the nucleus (Figure 2.5.1 B). Hippo-LATS kinase was shown to phosphorylate the same site in the cytoplasm. Our finding suggests that the regulation of YAP-S397 site by Aurora A is spatially distinct from LATS regulation (detail discussion is in the Discussion section).

**Figure 2.5.1: Aurora A Induces Phosphorylation of YAP-S397 in the Nucleus**



### **Figure 2.5.1: Aurora A Induces Phosphorylation of YAP-S397 in the Nucleus**

(A) Immunoblot of YAP-S397 phosphorylation in subcellular extracts of MDA-MB-231 cells, in the presence of TPX2 or not. N.N.E., non-nuclear extract; N.E. nuclear extracts.

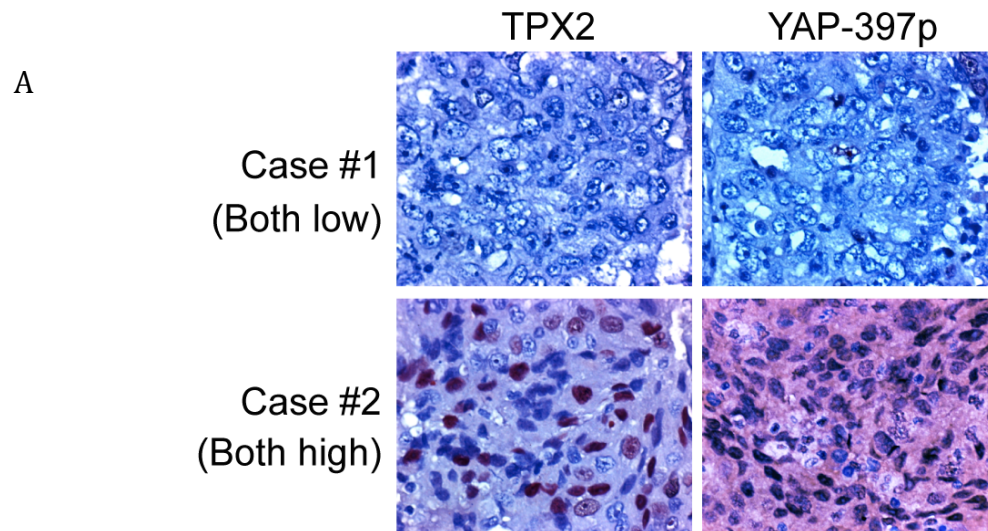
(B) Confocal microscopy analysis of YAP-S397 phosphorylation in MDA-MB-231 cells transfected with FLAG-TPX2 plasmids. For FLAG antibody, Alexa ® 594 secondary antibody (red) was used; for YAP-S397p, Alexa ® 488 secondary antibody (green) was used.

### ***2.5.2: Clinical Correlation of YAP Phosphorylation with TPX2***

To understand whether Aurora A-mediated phosphorylation of YAP-S397 occurs in patient samples, TNBC tissue microarrays were used for immunohistochemistry staining to detect the expression level of TPX2 and phospho-YAP. TPX2 was used as a marker because TPX2 represents the activity control of Aurora A. Aurora A expression itself does not guarantee the activity. From the IHC result performed by a pathologist, Dr. Weiya Xia, we do see a nice correlation between expression of TPX2 and phospho-YAP from a total of 114 patient samples ( $p=0.0001$ ) (Figure 2.5.2 A and B). These results suggested that activation of Aurora A has a significant contribution to the phosphorylation of YAP-S397 site.



**Figure 2.5.2: Expression of TPX2 Correlates with Phospho-YAP in Patient Samples**



B

		Expression of TPX2			Total	P value
		-/+	++	+++		
Phospho-Yap	-/+	19 (67.9%)	9 (40.9%)	15 (23.4%)	43 (37.7%)	$P = 0.0001^*$
	++	2 (7.1%)	9 (40.9%)	12 (18.8%)	23 (20.2%)	
	+++	7 (25%)	4 (18.2%)	37 (57.8%)	48 (42.1%)	
	<b>Total</b>	28 (100%)	22 (100%)	64 (100%)	114 (100%)	

**Figure 2.5.2: Expression of TPX2 Correlates with Phospho-YAP in Patient Samples**

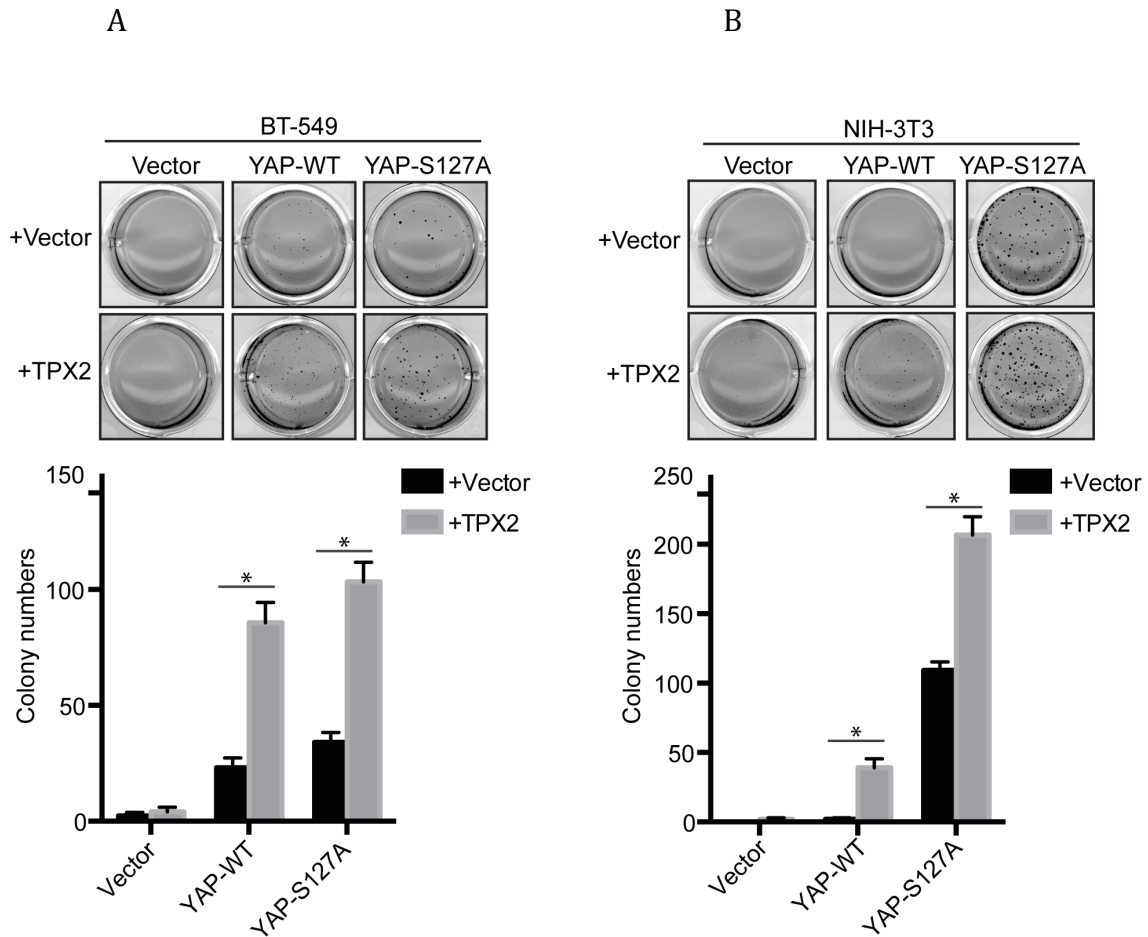
- (A) Representative images of immunohistochemistry staining from specimens of breast cancer patients. Antibodies against human TPX2 or YAP-S397p were used.
- (B) Relationships between TPX2 and phospho-YAP-S397 expression in surgical specimens of triple-negative breast cancer. \*Correlation between TPX2 and phospho-YAP-S397 was analyzed by using the Pearson Chi-Square test ( $P < 0.0001$ ). A  $P$  value of  $< 0.05$  was set as the criterion for statistical significance. Thank Dr. Weiya Xia for performing and grading the results.

## **2.6: Dependence of Aurora A Kinase Activity for YAP-Mediated Transforming Ability**

### ***2.6.1: Aurora A Increases 3D Colony Formation***

We have demonstrated in earlier results that Aurora A kinase can regulate the transcriptional activity of YAP. However, until now, whether Aurora A can influence YAP-mediated biological function is still unclear. Regarding cell growth, YAP was only critical in the 3D culture condition. So we then tested the effect of Aurora A on YAP-mediated cell growth in the soft agar assay. From the results, expression of YAP-WT (wild-type) or YAP-S127A (a constitutively active form of YAP which has lost the inhibitory regulation from Hippo pathway) in BT-549 cells increases the colony growth. Interestingly, Co-expression of TPX2 in these cells can further increase the colony numbers to around 3 folds more (Figure 2.6.1 A). Similarly, in a non-transformed mouse fibroblast cell line, NIH-3T3, co-expression of TPX2 can potentiate the colony-forming numbers mediated by both YAP-WT and YAP-S127A (Figure 2.6.1 B). These results indicated that functionally Aurora A kinase serves as an enhancer to boost YAP-induced biological phenotypes.

**Figure 2.6.1: Activation of Aurora A Enhances Transforming Ability of YAP**



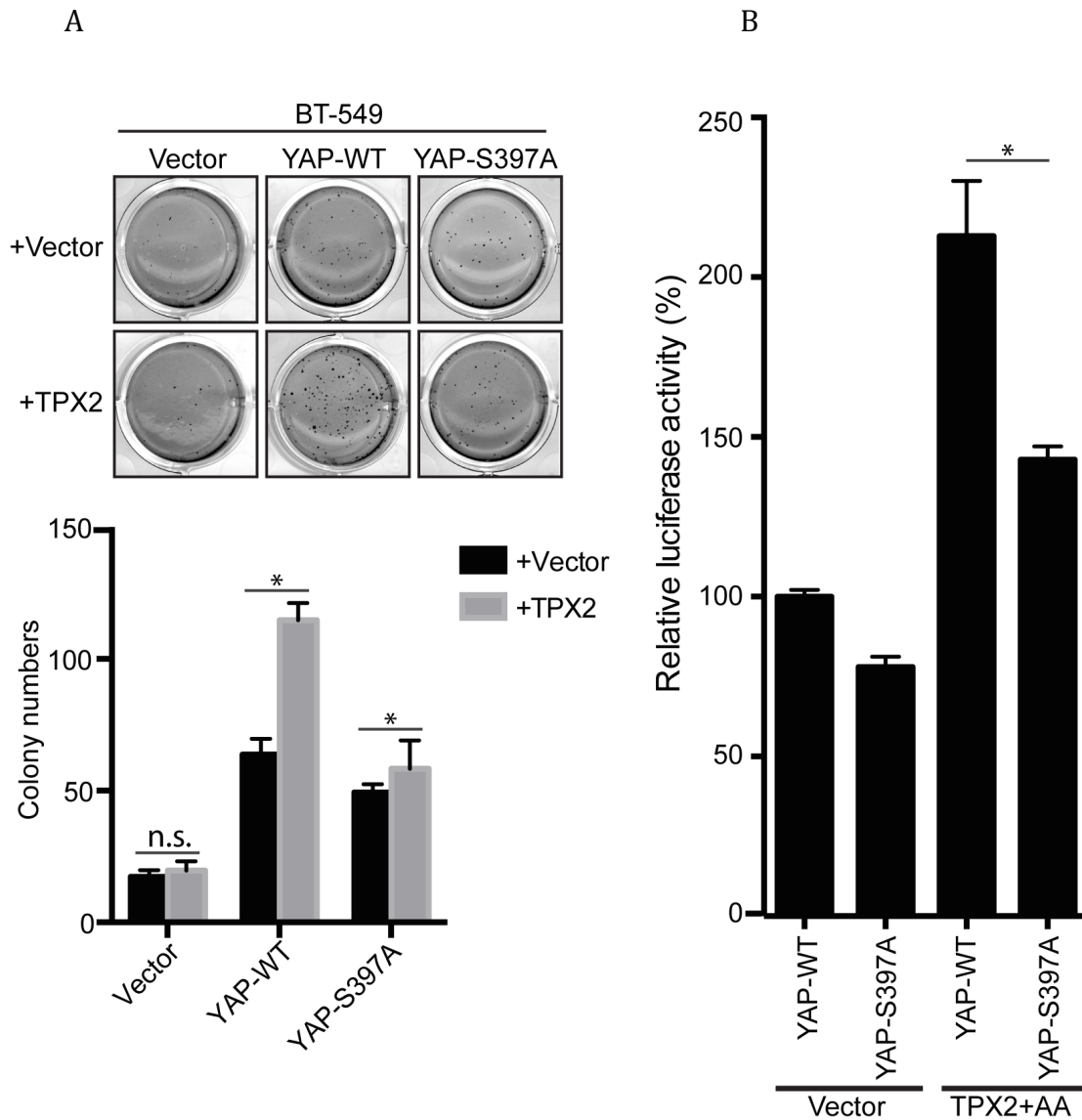
(A) Colony growth of BT-549 cells in soft agar assay (18 days after plating). BT-549 cells were manipulated to stably co-express wild-type YAP (YAP-WT) or S to A mutant of YAP-S127 (YAP-S127A) and FLAG-TPX2. Quantified result (n=3) of visible colonies is shown.

(B) Same as (A) but NIH-3T3 cells were used in the assay.

### ***2.6.2: YAP-S397 Is Required for TPX2-Mediated Activity and Transformation***

Does Aurora A-mediated YAP function go through the serine 397 residue of YAP? As we have identified that S397 is the major site to receive phosphorylation from Aurora A, we wondered if this phosphorylation event accounts for the Aurora A and YAP-mediated biological output. To test this, we used a luciferase reporter assay and a soft agar assay to examine the possible importance of serine 397 of YAP. From the soft agar assay, the result indicated that activation of Aurora A can no longer enhance the colony-forming ability mediated by YAP, when its S397 site is mutated to alanine (Figure 2.6.2 A). Similarly in the reporter assay, mutation of YAP-S397 to alanine also greatly reduces the responsiveness to Aurora A-directed potentiation (Figure 2.6.2 B). These results suggests that serine 397 of YAP is functionally important and Aurora A kinase regulates this site.

**Figure 2.6.2: YAP-S397 Is Critical for Aurora A-Mediated Activity**



(A) Similar to (Figure 2.6.1 A), but cells expressing YAP-S397A was assayed and quantified.

(B) Luciferase reporter assay (system is same as Figure 3G) to evaluate the transcriptional activity of YAP-WT or YAP-S397A. Error bars indicate the SD.

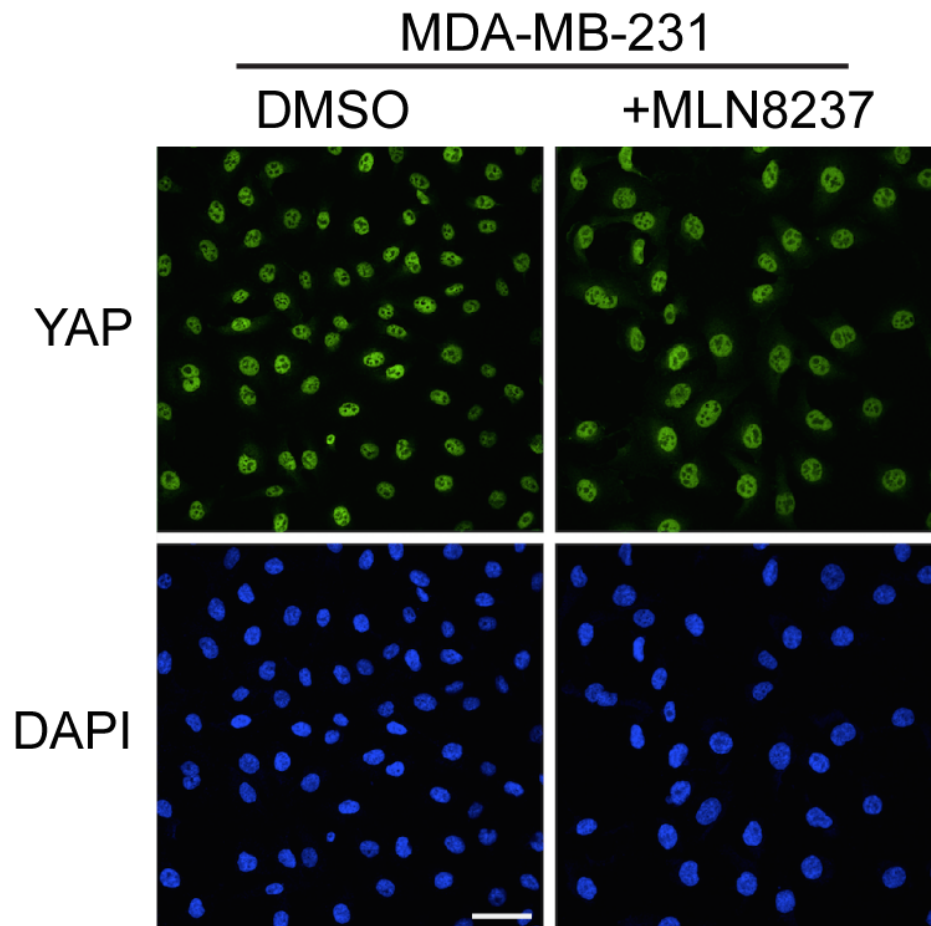
\*  $P < 0.05$ , Student's t-test. n.s., not significant.

## **2.7: Mechanisms of Aurora A Regulation of YAP Function**

### ***2.7.1: Inhibition of Aurora A Does Not Affect Localization of YAP***

The regulation of YAP activity by canonical Hippo pathway is through promoting nuclear export of YAP protein. We wonder if Aurora A can regulate YAP activity through a similar fashion. To test this, we treated MDA-MB-231 cells with an Aurora A kinase inhibitor, MLN8237, and examined the localization of YAP protein by the immunofluorescent staining. As shown in the result, inhibition of Aurora A kinase activity did not affect the localization of YAP (Figure 2.7.1). Most of YAP proteins can still stay inside the nucleus while the transcriptional output of YAP was diminished. This result suggested that Aurora A and Hippo kinases may regulate YAP activity through a distinct control.

**Figure 2.7.1: Aurora A Does Not Affect Subcellular Localization of YAP**



Confocal microscopy of YAP in MDA-MB-231 cells treated with/without MLN8237.

Scale bar represents 20  $\mu\text{m}$ .



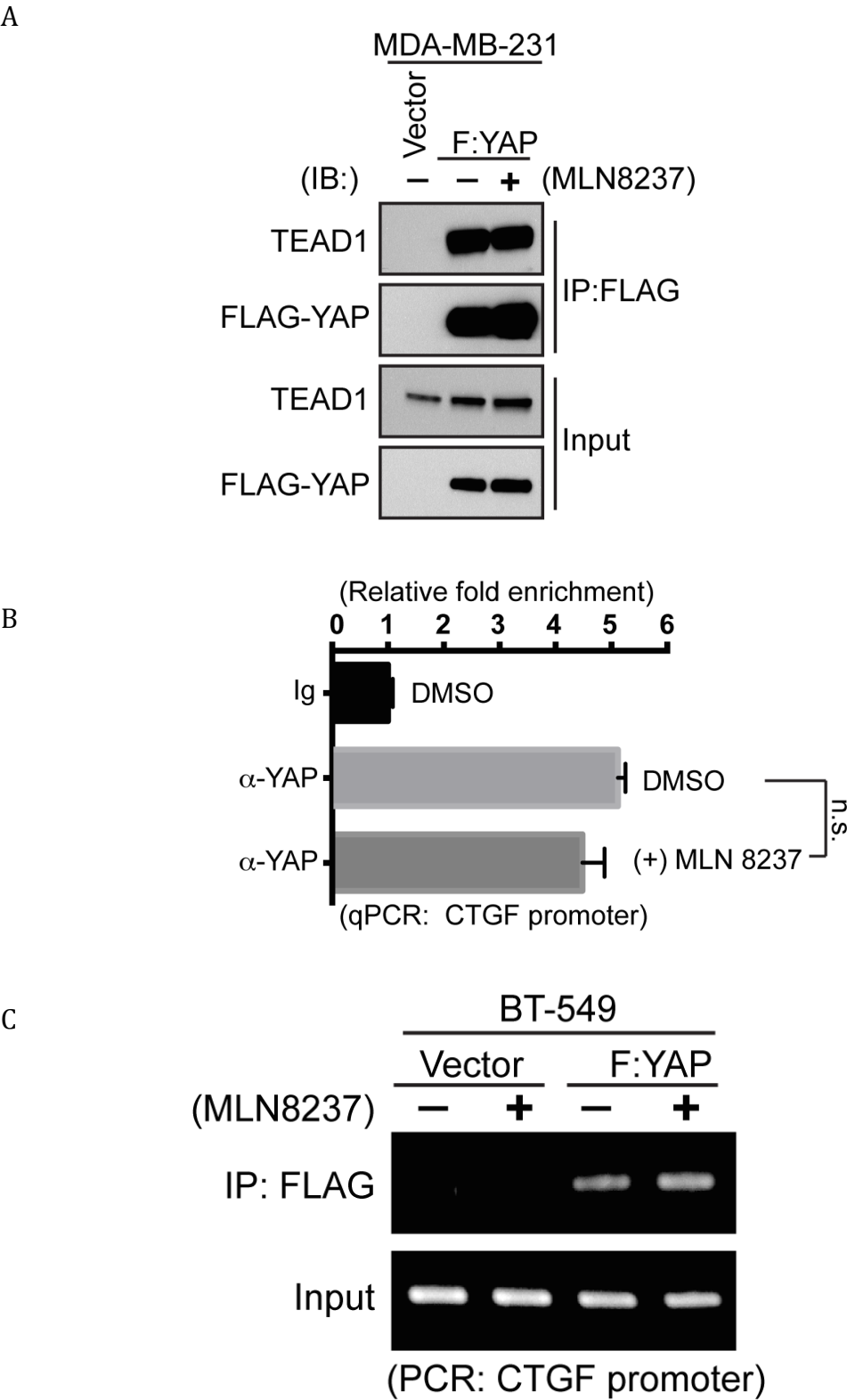
### ***2.7.2: Inhibition of AA Doesn't Affect TEAD-YAP Interaction and Its Loading on the CTGF Promoter***

YAP protein has no direct DNA-binding domain and thus it relies on the interaction with other DNA-binding transcriptional factors to control gene expression. TEADs family proteins are currently considered as the most important partners for YAP's function, although many other factors have been reported critical in a cell-context-dependent manner. Since Aurora A kinase can regulate TEAD-mediated YAP activity, we wondered whether can Aurora A affect the interaction of YAP and TEAD? To test this possibility, MDA-MB-231 cells were treated with an Aurora A kinase inhibitor. The cell lysates were collected for immunoprecipitation assay to examine the interaction of YAP and TEAD protein. As shown in the result, YAP strongly interacts with TEAD1 while inhibition of Aurora A did not affect this interaction, suggesting Aurora A may not regulate YAP-TEAD interaction (Figure 2.7.2 A).

We then wondered if Aurora A can affect the loading of YAP-TEAD complex to the promoter region of target genes. To test this, MDA-MB-231 and BT-549-YAP-expressing cells were treated with MLN8237 to inhibit Aurora A kinase activity. We performed a chromatin-immunoprecipitation assay (ChIP) in treated cells to monitor the occupancy of YAP on TEAD site of the CTGF promoter. As shown in the results (Figure 2.7.2 B and C), compared to the non-treated control group, the occupancy of YAP protein on CTGF promoter is not altered, suggesting that Aurora A

kinase does not regulate the loading of YAP complex to the DNA, at least not in the two cell lines tested.

**Figure 2.7.2: Aurora A Does Not Affect YAP-TEAD Association and Its Loading on CTGF Promoter**



**Figure 2.7.2: Aurora A Does Not Affect YAP-TEAD Association and Its Loading on CTGF Promoter**

(A) YAP and TEAD interaction was accessed by immunoblot. MDA-MB-231-FLAG-YAP cells treated with/without MLN8237 were used. Anti-FLAG antibody was used in pull-down, and the complexes were eluted with FLAG peptide.

(B) Chromatin immunoprecipitation (ChIP) of YAP followed by qPCR analysis of *CTGF* promoter. MDA-MB-231 cells treated with/without MLN8237 were used. Error bars indicate the SD. n.s., not significant, Student's t-test.

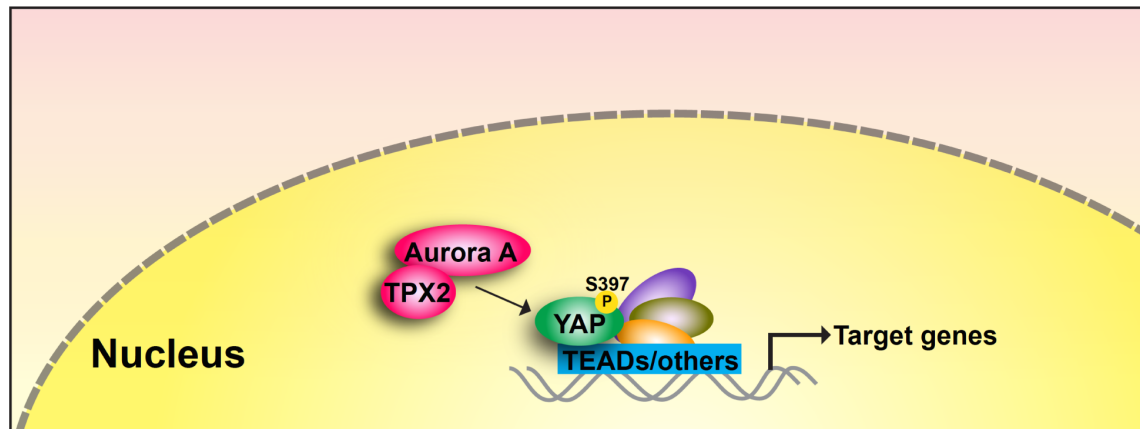
(C) Same as (B), ChIP analysis was performed to check for the presence of YAP on the *CTGF* promoter in BT-549-FLAG-YAP cells. Amplified DNA was separated in agarose gel after a PCR. Chromatin immunoprecipitation (ChIP) of YAP followed by qPCR analysis of *CTGF* promoter. MDA-MB-231 cells treated with/without MLN8237 were used. Error bars indicate the SD. n.s., not significant, Student's t-test.

**CHAPTER 3**  
**DISCUSSION AND FUTURE WORKS**

### **3.1: Summary of Current Findings**

In the current study, through a proteomic approach, we have newly determined that Aurora A kinase acts as a positive regulator for the transcriptional machinery of the Hippo pathway effector, YAP. Specifically, Aurora A interacts with YAP primarily in the nucleus. This interaction may cause specific phosphorylation on the YAP protein and it further elevates YAP's transactivation ability through unknown mechanisms (Figure 3.1). Activation of Aurora A signaling also promotes the transforming ability of YAP in cell models. This discovery provides a deeper understanding of the intricate regulatory network of the YAP oncoprotein. Moreover, through analysis of YAP mRNA expression from both patients and cell line databases, we revealed that the expression of YAP well correlates with an aggressive subtype of breast cancers, TNBC. Our results also indicate that YAP is indispensable for TNBC cell growth in 3D culture, suggesting that YAP may exemplify a key node that regulates malignant development of TNBC. Although further studies on the biological role of YAP in TNBC are necessary, targeting the Aurora A-YAP axis may lead to therapeutic benefits in this disease subset.

**Figure 3.1: Schematic Model of Our Findings**



Nuclear Aurora A regulates the phosphorylation of YAP-S397 and promotes the transcriptional activation of YAP.

### **3.2: Potential Translational Values from the Findings**

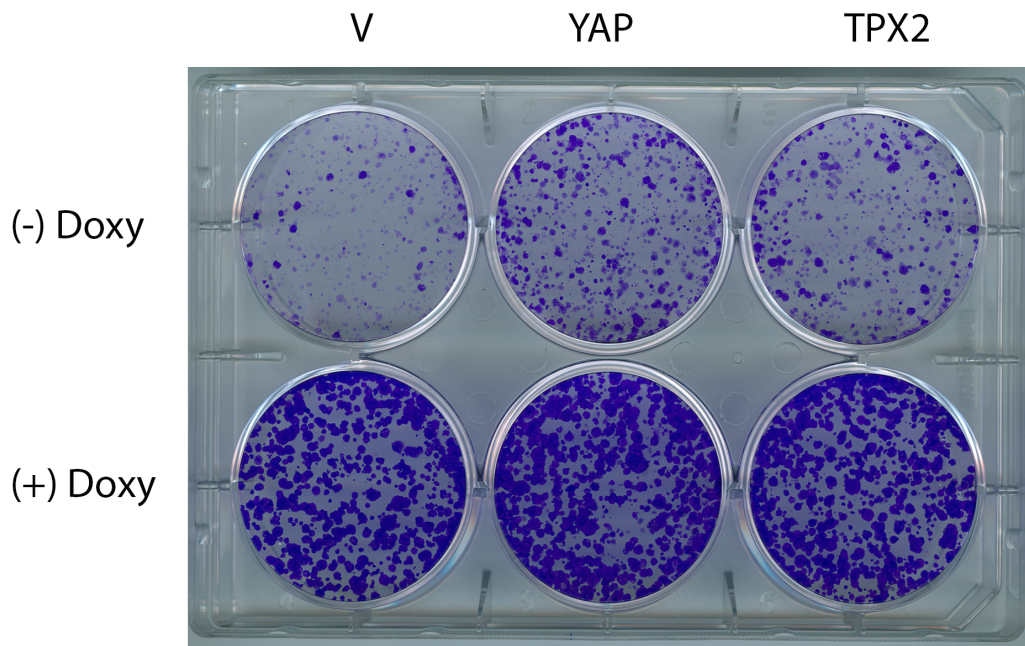
Dysregulation of developmentally conserved Hippo pathway has been shown in multiple cancer types and overall it has profoundly contributed to worsen the disease progression. For example, in liver, overexpression of YAP or mutation of Hippo cascade is sufficient to induce hepatocellular carcinoma (HCC). The identification of Aurora A as a novel kinase that positively regulates YAP activity may provide a way to manage the malignant phenotypes induced by YAP. There are several well-defined small molecule inhibitors for Aurora A kinase available. Some of them have gone through clinical toxicity tests and currently been tested in advanced clinical trials for cancer treatment. If YAP represents an important downstream effector of Aurora A kinase, stratifying cancer patients based on high YAP expression in their tumors may help guide the treatment of Aurora A inhibitor to certain population and increase the drug response rate.

Regarding the pancreatic cancer model, it was reported that, in experimental mice, expression of YAP can alleviate the restrain from Kras-dependency (63). And also, YAP has been found at the downstream of Kras signaling and YAP is indispensable for PDAC development in one Kras-driven mouse model (80). Active Kras mutation was found in more than 85% of patients with pancreatic adenocarcinoma (PDAC). Therefore, identifying direct or indirect strategies to intervene Kras signaling is of great interest in PDAC treatment. If Aurora A kinase can also increase the activity of YAP in PDAC cells, it can be speculated that activation of Aurora A may also help tumor cells escape from Kras inhibition.



Interestingly, in our preliminary result, overexpression of TPX2 to activate Aurora A indeed promotes the PDAC cell survival when Kras was shut down (Figure 4.2). This experiment used an inducible Kras-driven mouse cell line, AK196, developed by Dr. Haoqiang Ying and Dr. Ronald DePinho groups (63). Whether this TPX2-induced phenotype in PDAC cells relies on Aurora A or YAP is still awaited for more investigation, this result at least provided a potential clue to be considered for future Kras-targeting therapy in PDAC. Intriguingly, TPX2 expression has been reported critical for maintaining PDAC growth and TPX2 protein is also frequently overexpressed in PDAC lines (139). The TPX2-Aurora A-YAP axis has a good potential waiting for further examination.

**Figure 3.2: Activation of Aurora A Releases Kras-Dependency of PDAC Cells**



Colony formation of mouse PDAC cells. This mouse cell line relies on Kras to grow. Adding doxycycline (Doxy) induces the expression of Kras. Expression of YAP has been shown to let PDAC escape Kras-dependency. Here, TPX2-expressing PDAC is tested for its dependency on Kras.

### **3.3: Similarity of Aurora A- and YAP-Mediated Cellular Phenotypes**

Identification of YAP as a downstream substrate of Aurora A kinase expands our understanding of Aurora A-mediated malignancies. Aurora A has been reported to enhance not only the cell growth, but also cell migration/invasion, cancer metastasis, properties of cancer-initiating cells, and drug resistance in cancers (119-122, 124-126). Of interest, YAP has also been shown to be involved in many similar functions (52, 55, 77-79), suggesting that the newly identified Aurora A-YAP axis may possess a key role yet to be explored in cancer development.

### **3.4: Enzymatic Regulators of YAP (Negative and Positive Regulators)**

Canonically, YAP activity is controlled strictly by LATS kinase from the Hippo pathway; however, there are several lines of new evidence that YAP activity can also be regulated by other kinases or protein methyltransferase. Here we review the literatures about this aspect to provide a better understanding of post-translational regulation of YAP activity. Each modification can engage a distinct transcriptional program of YAP, depending on how YAP-surrounding complexes are altered.

#### **Negative Regulators:**

LATS inhibits YAP activity by direct phosphorylation on five sites of YAP. Overall, it renders a sequestration of YAP in the cytoplasm or causes the protein degradation of YAP (59).

5' AMP-activated protein kinase (AMPK) has recently been shown to inhibit YAP activity when cells are under energy stress. In one study, AMPK directly phosphorylates on serine 94 residue of YAP and this abolishes the interaction between YAP and TEADs (140). In another study, AMPK directly phosphorylates S61 while this inhibits YAP activity by an unknown mechanism (141). Of note, the second paper did also identify S94 of YAP is an AMPK site but the study did not focus on it. Mutation of S61 to alanine significantly diminishes YAP activity but it did affect neither the localization of YAP nor the YAP-TEAD interaction. The authors suspected that other unknown dynamics on nuclear YAP-complex might be altered by AMPK. This mechanism is similar to our findings in this thesis. Addressing the detail alteration of nuclear YAP-complex under different stimuli may possess the key to understand the modulation of YAP activity.

Protein methylation of YAP can also inhibit YAP activity. A research has discovered that SET7 methyltransferase can monomethylate YAP protein on its lysine 494. The methylation promotes the cytoplasmic retention of YAP protein and therefore the transcriptional program of YAP is turned off (142).

### **Positive Regulators:**

ABL tyrosine kinase was shown to directly phosphorylate YAP on Y357 residue. This phosphorylation occurs upon the DNA damage response. Mechanically, the phosphorylation of YAP-Y357 promotes the complex formation of YAP and p73

and this complex later recruits p300 to help activate apoptosis-related genes during DNA damage (143).

YES kinase was the first kinase found to interact with YAP protein while the functional regulation of YES on YAP activity was not intensively pursued in earlier times. In 2004, one study showed that YES kinase can phosphorylate YAP protein and this brings the association of Runx2 and YAP. YAP functions as a co-suppressor of Runx2 protein. Upon the interaction, YAP suppresses Runx2-mediated gene transcription. For example, osteocalcin gene is a target. Inhibition of YES kinase dissociates the YAP-Runx2 complex and therefore those suppressed genes can be reactivated (144). In 2011, one study showed that leukemia inhibitory factor (LIF) pathway regulates YAP-TEAD2 activity and regulates self-renewal of mouse embryonic stem cells (145). This activation requires YES kinase. In 2012, another phenomenal paper showed that, in colorectal cancer model, YES kinase enhances the loading of YAP/beta-catenin complex to promoters of BCL2L1 and BIRC5. In this study, TPX5 is a critical transcriptional activator to conduct signal output of YAP/beta-catenin complex. Interestingly, YES kinase does not affect the binding of YAP and beta-catenin. The exact mechanism of how YES kinase regulates the loading of YAP-complex on target genes is still unclear (54).

Recently, Homeodomain-interacting protein kinases (HIPKs) have been shown to positively regulate YAP activity in flies. The two reports all revealed a critical role of HIPKs in the potentiation of YAP activity with genetic study. One study showed

that HIPK can regulate the nuclear accumulation of YAP, while the other study showed that YAP remains in the nucleus regardless of HIPK. Inhibition of HIPK kinase activity reduces the YAP-mediated organ growth (146, 147). Although the detail studies in human cells were not conducted yet, HIPK may be also a promising candidate to be targeted in cancers.

### **3.5: Chromatin Remodeling Complex and the Activity of YAP, TAZ**

Gene transcription is a complicate process that involves numerous protein machineries to determine on or off of one particular promoter. Chromatin remodeling factors are essential to control the epigenetic status of promoter areas. Human YAP homolog, TAZ, has been reported to interact with SWI/SNF complex. In that study, TAZ recruits SWI/SNF complex to promote the lineage differentiation of breast epithelial cells from luminal to basal characteristics (148). YAP protein is structurally very similar to TAZ. It is possible that YAP also uses similar machinery to control gene expression. Interestingly, from our own M/S analysis of YAP-interacting proteins, we also observed many of SWI/SNF members (Table 3.5). In another study, drosophila Yorkie promotes gene expression through recruiting the histone methylation complex. YAP interacts with NcoA6 to induce H3K4 methylation on target genes (149). Preliminarily, we have done a sucrose gradient separation of nuclear YAP complex from MDA-MB-231 cells treated with an Aurora A inhibitor. The result suggested that Aurora A may alter the size of nuclear YAP-complex (Figure 3.5). Taken together, whether Aurora A may regulate the

transcriptional activity of YAP through affecting the recruitment of SWI/SNF or histone-modifying proteins is certainly an interesting topic for further study.

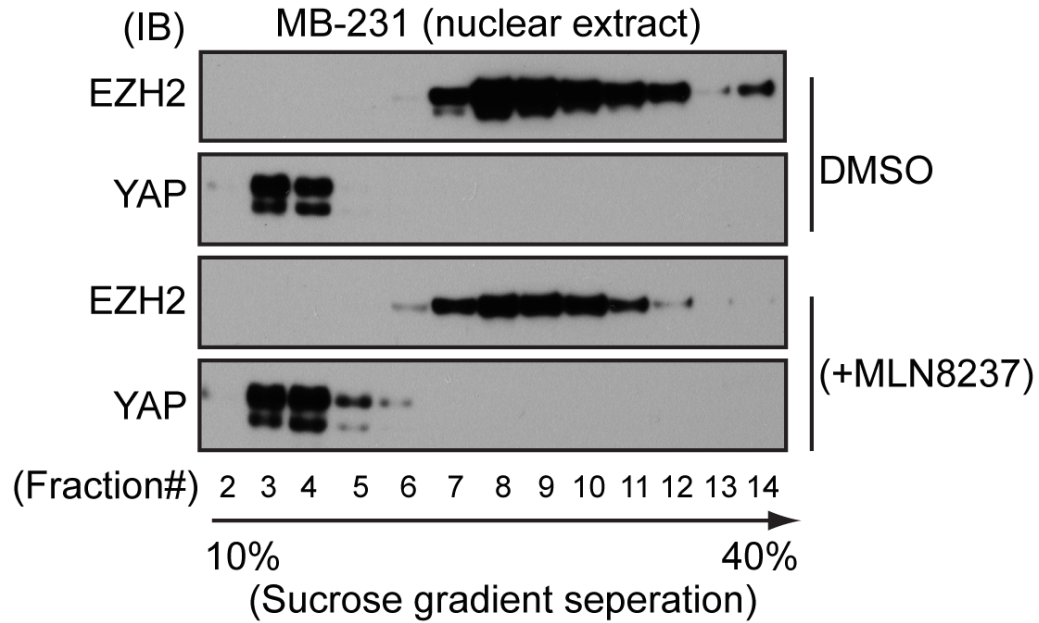
**Table 3.5: Members of SWI/SNF Complex from YAP-Interacting Proteins**

SWI/SNF complex
ARID1A
ARID1B
SMARCA2
SMARCB1
SMARCC1
SMARCC2
SMARCD1
SMARCD2
SMARCE1

This protein list was generated from our M/S analysis of YAP-interacting proteins.



**Figure 3.5: Aurora A Affects the Size of Nuclear YAP Protein Complex**



Immunoblot of YAP proteins in fractions collected from the sucrose gradient separation. Nuclear extracts of MDA-MB-231 cells treated with/without MLN8237 were used. EZH2 is a nuclear protein and it serves as an irrelevant control.

### **3.6: The Interplay of Aurora A and the Upstream Hippo Pathway**

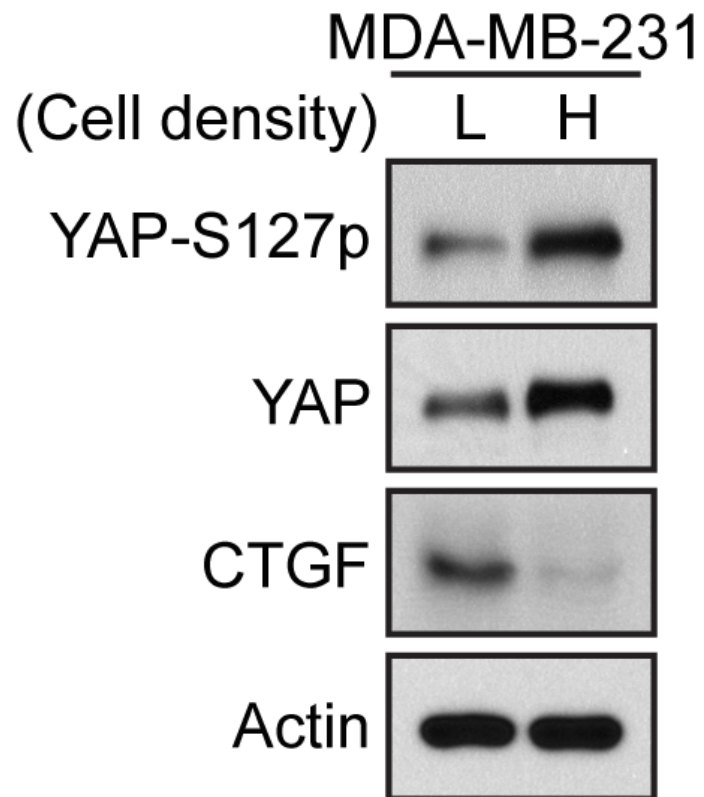
Aurora A kinase, in addition to interacting with YAP from our discovery, has been shown to interact with several upstream Hippo regulators. MST1/2 phosphorylates Aurora A to regulate ciliogenesis in epithelial cells (150). LATS2 kinase was shown to be a substrate of Aurora A, and this regulation is critical for cytokinesis (151, 152). Moreover, KIBRA, which associates with LATS and regulates the Hippo pathway, was also reported as a substrate and regulator of Aurora A (29-32, 153). These lines of evidence indicate a complex interplay between Aurora A and the Hippo upstream cascade. However, how Aurora A may directly or indirectly affect the output of the Hippo pathway remains unanswered. It is plausible that Aurora A may also regulate YAP activity through its influence on Hippo kinases, and if so, one would expect to see a change in localization of the YAP protein when Aurora A activity is manipulated. However, our results showed that YAP protein remains in the nucleus even when Aurora A activity is abrogated, suggesting that, at least in our system, Aurora A could not significantly impinge on the Hippo kinases cascade.

### **3.7: Distinct Functions of Serine 397 Phosphorylation on the YAP Protein**

Our study has determined that the serine 397 residue is a major Aurora A phosphorylation site on YAP. This phosphorylation seems to elevate YAP activity. Of note, however, a previous report clearly showed that the same site could be phosphorylated by LATS kinase and that this phosphorylation primes YAP protein for sequential phosphorylation by CK1, eventually resulting in  $\beta$ -TrCP-mediated

protein degradation of YAP (59). Different output from the same phosphorylation site may reflect the intricate regulation of YAP protein in a cell context-dependent or environment cues-dependent manner. The previous report was conducted in noncancerous NIH-3T3 fibroblast cells, and YAP protein stability was assessed in a scenario of high cell density. In our results (Figure 4.7), when MDA-MB-231 cells were grown at a high density, total YAP protein was even elevated but not decreased. However, in accordance with the previous report (59), we also noticed that high cell density indeed increased the phosphorylation of YAP-S127 and consequently reduced the expression of a YAP target gene, *CTGF*, suggesting activation of Hippo kinases (Figure 4.7). Whether the conserved machinery that regulates YAP protein stability can be seen in both normal and cancerous cells awaits further investigation. From another aspect, we observed that phosphorylation of YAP by Aurora A predominantly occurs in the nuclear compartment, whereas the MST/LATS axis conducts YAP phosphorylation in the cytoplasm (15, 154, 155). It is possible that YAP protein, even with the same site phosphorylated, may be differentially regulated in the nucleus and cytoplasm.

**Figure 3.7: Cell Density Does Not Decrease YAP Protein Level**



Immunoblot of YAP and YAP-S127p from lysates of MDA-MB-231 cells cultured at different densities.

### **3.8: Roles of the Nuclear Aurora A kinase**

In normal cells, Aurora A expression is mainly in the cytoplasm. However, in cancerous cells and a model system, some reports have clearly shown that a large portion of Aurora A protein localizes in the nucleus. In head and neck cancers, nuclear Aurora A was shown to mediate oncogenic transformation (156). In a skin tumor model, protein expression of Aurora A was shown in the nucleus (115). Moreover, in Tetrahymena model system, nuclear Aurora A was shown to regulate the phosphorylation of serine 10 on histone H3. This histone phosphorylation is critical for the onset of mitosis and the control of accurate chromosomal segregation (157). In our results, we also found that Aurora A protein is almost exclusively present in the nucleus of MDA-MB-231 cells. The interaction of YAP and Aurora A is also in the nucleus, suggesting a new function of nuclear Aurora A in oncogenic progression.

### **3.9: When Hippo and Aurora A Collide on YAP Protein**

In our study, we found that Aurora A kinase regulates YAP only in the nucleus. However, canonical Hippo pathway was reported to influence YAP in the cytoplasm. Though we do not have a clear answer for how these two regulations can coordinate with each other, it is reasonable to speculate that Hippo pathway may be still more dominant over Aurora A-mediated YAP activity. The main reason is that Hippo regulates YAP localization. Once YAP is trapped in the cytoplasm due to the activation of Hippo, Aurora A can no longer enhance YAP activity. Cytoplasmic YAP

has been long thought non-functional; however, one interesting paper reported that cytoplasmic accumulated YAP/TAZ protein can function as a scaffold to bridge beta-catenin to the destruction complex (158). Whether cytoplasmic Aurora A may have any role to affect this new regulation remains to be determined.

### **3.10: Future Directions**

Based on our current findings, Aurora A kinase was identified as a new kinase that positively regulates YAP activity through a direct phosphorylation event. Regarding this direction, many extended questions remain to be tested for a more comprehensive understanding of this new axis. The potential future works can span from basic researches to the translational avenue, including the following:

- (1) Which subset of YAP-targeted genes is under the control of Aurora A kinase?
- (2) What are the major biological outputs, in addition to cell growth, from Aurora A-YAP axis?
- (3) There are a variety of transcriptional factors that interact with YAP. How does Aurora A affect YAP activity through the interplay of different transcriptional factors? At which scenarios, is Aurora A most critical for YAP function?
- (4) What is the exact mechanism for Aurora A to augment transcriptional activity of YAP? How is the dynamics of nuclear YAP complex engaged in the process?
- (5) In addition to TNBC, does this pathway occur in other cancer types?

(6) Is Aurora A important to regulate YAP-mediated malignancy in animal models? In this case, does the treatment of therapeutic Aurora A inhibitor improve the disease control?

**CHAPTER 4**  
**MATERIALS AND METHODS**



#### **4.1: Cell Lines, Plasmids, and Inhibitors**

All cell lines, except NIH-3T3, were maintained in DMEM medium with 10% FBS plus streptomycin and penicillin. NIH-3T3 was purchased from ATCC and maintained in 10% bovine serum in DMEM. Plasmids that express FLAG-YAP, FLAG-Aurora A, FLAG-TPX2, and NTAP-YAP were constructed on pCDH-CMV-MCS-EF1-Puromycin vector (#CD510B-1; System Biosciences) or pCDH-EF1-MCS-IRES-Neo vector (#CD533A-2; System Biosciences) when a double-selection was required. Plasmids used for recombinant protein production were constructed on a pET-32a vector. Site-directed mutagenesis was induced according to a QuikChange protocol (Agilent Technologies). Primer sequences and inserting sites are listed in Table 4.1. Other plasmids that were purchased from Addgene are listed in the Plasmid section. For YAP and Aurora A knockout cells used in the experiment, each line is a pool of at least 4 individual knockout clones to minimize clonal variation. The knockout efficiency was confirmed by immunoblotting. A CRISPR plasmid targeting GFP was used as a control. The chemical inhibitor for Aurora A kinase, MLN8237, was obtained from Selleck Chemicals. MLN8237 (100nM) was used throughout the study.

**Table 4.1: Primers for Cloning and Mutagenesis**

Name	Purpose	Site	Sequence (5' to 3')
<b>FLAG-Aurora A/F</b>	Mammalian expression	XbaI	ATA TAT TCT AGA CCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGA TGG ACC GAT CTA AAG AAA ACT GCA TTT CAG G
<b>Aurora A/R</b>	Mammalian expression	BamHI	ATA TAT GGA TCC CTA AGA CTG TTT GCT AGC TGA TTC TTT GTT TTG GC
<b>FLAG-YAP1/F</b>	Mammalian expression	EcoRI	ATA TAT GAA TTC CCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGA TGG ATC CCG GGC AGC AGC CGC
<b>YAP1/R</b>	Mammalian expression	NotI	ATA TAT GCG GCC GCC TAT AAC CAT GTA AGA AAG CTT TCT TTA TCT AGC TTG G
<b>NTAP/F</b>	Mammalian expression	XbaI	ATA TAT TCT AGA ATG GCA GGC CTT GCG CAA CAC G
<b>NTAP/R</b>	Mammalian expression	XbaI	ATA TAT TCT AGA TCC AAC GTT AAC TGC GGT ACC TAG C
<b>YAP1(for NTAP)/F</b>	Mammalian expression	EcoRI	ATA TAT GAA TTC CCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGA TGG ATC CCG GGC AGC AGC CGC
<b>YAP(1-270)/F</b>	Bacterial expression	EcoRI	ATA TAT GAA TTC ATG GAT CCC GGG CAG CAG CCG C
<b>YAP(1-270)/R</b>	Bacterial expression	NotI	ATA TAT GCG GCC GCG TTC ATG GCA AAA CGA GGG TCA AGC
<b>YAP(271-504)/F</b>	Bacterial expression	EcoRI	ATA TAT GAA TTC AAC CAG AGA ATC AGT CAG AGT GCT CC
<b>YAP(271-504)/R</b>	Bacterial expression	NotI	ATA TAT GCG GCC GCT AAC CAT GTA AGA AAG CTT TCT TTA TCT AGC TTG G
<b>YAP(270-370)/F</b>	Bacterial expression	EcoRI	ATA TAT GAA TTC AAC CAG AGA ATC AGT CAG AGT GCT CC
<b>YAP(270-370)/R</b>	Bacterial expression	NotI	ATA TAT GCG GCC GCC ATC CCG GGA GAA GAC ACT GGA TTT TGA G
<b>YAP(371-504)/F</b>	Bacterial expression	EcoRI	ATA TAT GAA TTC TCT CAG GAA TTG AGA ACA ATG ACG ACC
<b>YAP(371-504)/R</b>	Bacterial expression	NotI	ATA TAT GCG GCC GCT AAC CAT GTA AGA AAG CTT TCT TTA TCT AGC TTG G
<b>YAP(410-504)/F</b>	Bacterial expression	EcoRI	ATA TAT GAA TTC CCT CGA ACC CCA GAT GAC TTC C
<b>YAP(410-504)/R</b>	Bacterial expression	NotI	ATA TAT GCG GCC GCT AAC CAT GTA AGA AAG CTT TCT TTA TCT AGC TTG G
<b>FLAG-TPX2/F</b>	Mammalian expression	NheI	ATA TAT GCT AGC CCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGA TGT CAC AAG TTA AAA GCT CTT ATT CC
<b>FLAG-TPX2/R</b>	Mammalian expression	BamHI	ATA TAT GGA TCC TTA GCA GTG GAA TCG AGT GGA G
<b>YAP-397A/F</b>	Mutagenesis		GAT GAG GCT ACA GAC AGT GGA CTA AGC ATG
<b>YAP-397A/R</b>	Mutagenesis		GTC TGT AGC CTC ATC TCG AGA GTG ATA GGT G
<b>YAP-S127A/F</b>	Mutagenesis		GCT CAT GCC TCT CCA GCT TCT CTG C
<b>YAP-S127A/R</b>	Mutagenesis		TGG AGA GGC ATG AGC TCG AAC ATG CTG

<b>Aurora A-K162R/F</b>	Mutagenesis	GCT CTT CGA GTG TTA TTT AAA GCT CAG CTG G
<b>Aurora A-K162R/R</b>	Mutagenesis	TAA CAC TCG AAG AGC CAG AAT AAA CTT GCT TTG C

#### **4.2: Transfection, RNAi, CRISPR, and Lentiviral Infection**

Plasmid DNA transfection was conducted with use of FuGENE6 (Promega) according to the manufacturer's instructions. siRNA transfection was conducted with use of Amexa Nucleofactor II (Lonza Group Ltd., Basel, Switzerland) according to the manufacturer's instructions. Lentiviral shRNA targeting *YAP* was purchased from the GIPZ lentiviral shRNA library (shRNA and OFRome Core, MD Anderson Cancer Center, Houston, TX). The GIPZ lentiviral vector with a non-targeting shRNA was used as a scramble control. All siRNAs were obtained from Sigma-Aldrich. The plasmid used for cloning a custom-designed CRISPR system was obtained from Addgene (#52961. For YAP knockout cells (sequence: catcagatcgtgcacgtccg) used in the experiment, 4 individual knockout clones were pooled to minimize clonal variation. The knockout efficiency was confirmed by immunoblotting. A CRISPR plasmid targeting GFP was used as a control.

Sequences of RNA interference are listed in the Table 4.2. Lentiviral packaging was conducted in 293FT cells (Thermofisher Scientific) with co-transfection of pCMV-VSV-G and pCMV-dR8.2 dvpr. 72 hours after transfection, and medium that contained viruses was used to infect target cells (with 8 µg/ml Polybrene) for 2 days. After infection, cells were selected with 2 µg/ml puromycin or 1 mg/ml G418 until the non-infected control cells were eliminated.

**Table 4.2: Information about qPCR, ChIP, RNAi, and CRISPR Systems**

<b>Name</b>	<b>Purpose</b>	<b>Sequences or other information</b>
<b>CTGF/F</b>	qPCR	CGTGTGCACCGCCAAAGATG
<b>CTGF/R</b>	qPCR	TGCTCTGGAAGGACTCTCCG
<b>GAPDH/F</b>	qPCR	GGT GAA GGT CGG AGT CAA CGG
<b>GAPDH/R</b>	qPCR	ACC AGA GTT AAA AGC AGC CCT GG
<b>CTGF/F (for q-PCR)</b>	ChIP-qPCR	TCT GTG AGC TGG AGT GTG CC
<b>CTGF/R (for q-PCR)</b>	ChIP-qPCR	CTC GCC AAT GAG CTG AAT GG
<b>CTGF/F (for PCR)</b>	ChIP-PCR	TCT GTG AGC TGG AGT GTG CC
<b>CTGF/R (for PCR)</b>	ChIP-PCR	ACG CGC CGG GCT GTC G
<b>YAP_1</b>	siRNA	SASI_Hs01_00182403; Clone ID (From Sigma-Aldrich)
<b>YAP_2</b>	siRNA	SASI_Hs01_00182405; Clone ID (From Sigma-Aldrich)
<b>Aurora A_1</b>	siRNA	SASI_Hs01_00241477; Clone ID (From Sigma-Aldrich)
<b>Aurora A_2</b>	siRNA	SASI_Hs01_00241478; Clone ID (From Sigma-Aldrich)
<b>YAP_1</b>	shRNA	from pGIPZ library #247011
<b>YAP_2</b>	shRNA	from pGIPZ library #65508
<b>YAP1</b>	CRISPR-sgRNA	C ATC AGA TCG TGC ACG TCC G
<b>Aurora A</b>	CRISPR-sgRNA	T GAG TCA CGA GAA CAC GTT T
<b>EGFP</b>	CRISPR-sgRNA	GGG CGA GGA GCT GTT CAC CG

### **4.3: Immunoblotting and Antibodies**

An immunoblot assay was conducted according to standard procedures. The blocking agent used throughout the experiment was 4% bovine serum albumin (BSA). Detailed information about all of the antibodies used in the current study is listed in the Table 4.3.

**Table 4.3: List of Antibodies Used in this Study**

<b>Protein</b>	<b>Company</b>	<b>Purpose</b>	<b>Catalog number</b>
<b>YAP</b>	Cell Signaling	WB, IF, IP, ChIP	14074S
<b>YAP</b>	Santa Cruz	WB, IF	Sc-271134
<b>YAP-S397p</b>	Cell Signaling	WB, IHC	13619S
<b>YAP-S127p</b>	Epitomics	WB	2209-1
<b>Actin</b>	Sigma Aldrich	WB	A2066
<b>CTGF</b>	Santa Cruz	WB	sc-14939
<b>Aurora A</b>	Cell Signaling	WB, IF	4718S
<b>Aurora A-T288p</b>	Cell Signaling	WB	3079S
<b>MCM5</b>	GeneTex	WB	114090
<b>FLAG</b>	Sigma Aldrich	WB, IF, IP, ChIP	F3165
<b>PLK1-T210p</b>	Epitomics	WB	3646-1
<b>TPX2</b>	Cell Signaling	IHC	12245S
<b>Cyclin B1</b>	Santa Cruz	WB	sc245
<b>Cyclin E</b>	Santa Cruz	WB	sc247
<b>Lamin A</b>	Santa Cruz	WB	sc20680
<b>Tubulin</b>	Sigma Aldrich	WB	T5168
<b>TEAD1</b>	Cell Signaling	WB	12292S
<b>EZH2</b>	Cell Signaling	WB	5246S

#### **4.4: Purification of Recombinant Proteins and Kinase Assay**

Recombinant proteins (all are his-tagged proteins) were produced in-house. Basically, one colony of transformed BL21-CodonPlus (DE3)-RILP cells (Agilent Technologies) was inoculated in 2xYT medium and shake-incubated at 30°C until O.D. 600 reached 0.6. Recombinant proteins were then purified according to a Qiaexpressionist™ protocol (Qiagen). Eluted proteins were semi-quantified on SDS-PAGE and compared with a BSA control. Enzyme active Aurora A kinase was purchased from EMD Millipore (Cat. #14-511). GST-tagged YAP1 protein was purchased from Novus (Cat. # H00010413-P01). The *in vitro* kinase assay was performed at 30°C for 20 minutes by mixing 25 ng of Aurora A kinase with 750 ng of YAP proteins in kinase buffer (50 mM HEPES-7.3; 15 mM MgCl<sub>2</sub>; 20 mM KCl; 2 mM EGTA; 1 mM DTT; 100 μM ATP) containing ATP gamma-<sup>32</sup>P. Reactions were quenched by heating at 95°C for 5 minutes in the presence of SDS-loading buffer and then analyzed by SDS-PAGE followed by exposure on x-ray films.

#### **4.5: Tandem-Affinity Purification**

To purify YAP-binding proteins, we used the tandem affinity purification (TAP) tag system, which consists of protein A and calmodulin-binding peptide sequences separated by TEV protease cleavage site (159). The N-terminal TAP-tagged YAP or an empty vector was stably expressed in MDA-MB-231 cells. Then, the cells were grown in 50 plates of 150 mm culture dishes, and lysed in TAP lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EGTA,



protease inhibitor mixture, and phosphatase inhibitor mixture). After sonication and centrifugation, the cleared cell lysates were subjected to purification as described previously (159). In brief, the lysates were applied to the first affinity purification with rabbit IgG agarose beads. After the beads were washed, the YAP protein complex was eluted with TEV protease and applied to second affinity purification with calmodulin beads in calmodulin binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM  $\text{Mg}(\text{CH}_3\text{COO})_2$ , 2 mM  $\text{CaCl}_2$ , and 0.1% Triton X-100). After the beads were washed, the YAP protein complex was eluted with EGTA elution buffer (10 mM  $\beta$ -mercaptoethanol, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM  $\text{Mg}(\text{CH}_3\text{COO})_2$ , 1 mM imidazole, 3 mM EGTA). The eluted proteins were then concentrated with Ultrafree® Centrifugal Filter (Millipore), separated by SDS-PAGE, and stained by Coomassie blue. The bands were cut out from the gel and subjected to trypsin digestion and subsequent mass spectrometry analysis.

#### **4.6: Plasmids**

The plasmids for the reporter assay, pGL2-GAL4-UAS-Luc (Addgene plasmid #33020)(160) and pCMX-Gal4-TEAD4 (Addgene plasmid # 33105) (52), were obtained from Addgene. A  $\beta$ -actin promoter-driven renilla luciferase construct was used as an internal control in the reporter assay. The plasmid used for cloning a custom-designed CRISPR system was obtained from Addgene (#52961) (161). The CRISPR targeting sequences of YAP and Aruroa A were listed in the Table 4.2.

#### **4.7: Immunoprecipitation, ChIP assay, and Mass Spectrometry**

For immunoprecipitation, cell lysates were obtained with modified RIPA buffer (25 mM Tris-HCl-pH 7.6, 150 mM NaCl, 1% NP40, 1 mM DTT). We used 1 mg of cell lysate in each IP reaction. Protein complexes were pulled down from antibody/lysate (overnight incubation at 4°C) by protein A/G beads. Protein complexes were then washed 3 times and subsequently released by SDS-loading buffer or eluted by FLAG peptide (100 µg/ml), if FLAG-tag is used. A ChIP assay was conducted as described earlier (162). A total of  $2 \times 10^7$  cells were used for one reaction. Cellular DNA was sheared in Bioruptor® (Diagenode) until average DNA sizes were about 500 bp. After eluting the DNA from washed beads, the DNA was further purified by Qiaquick spin columns (Qiagen), and 5% of precipitated DNA was used for q-PCR analysis. Primers for ChIP are listed in Table 4.2. Mass spectrometric analysis was performed as previously described (163).

#### **4.8: RT-PCR, q-PCR, and PCR**

Total cellular RNAs were extracted by Trizol reagent (Thermofisher Scientific). We converted 1 µg of RNA to 20 µl cDNA with a SuperScript® VILO™ cDNA Synthesis Kit, according to the manufacturer's instructions (Thermofisher Scientific). cDNA that has been converted from 1 µg of RNA can be used for a total of 60 reactions in q-PCR analysis. q-PCR was conducted with iQ™ SYBR® Green Supermix (Bio-Rad) and analyzed by a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Regular PCR amplification used Phusion® polymerase (NEB). Primers that were used are listed in Table 4.2.

#### **4.9: Reporter Assay**

The reporter assay was conducted as previously described(164) by co-transfection of plasmids in 293FT cells (Thermofisher Scientific).

#### **4.10: Immunohistochemistry**

IHC staining was performed by Dr. Weiya Xia as previously described (165). Human triple-negative breast cancer tissue-microarray slides (n=114) were stained with antibodies against TPX2 and YAP-397p (Cell Signaling). The tissue microarrays were obtained and used according to the guidelines approved by the Institutional Review Board at MD Anderson Cancer Center (IRB Protocol # LAB05-0127). The Pearson Chi-Square test was used for statistical analysis with SPSS software. According to histological scoring, the intensity of staining was ranked into four groups: high (score 3), medium (score 2), low (score 1), and negative (score 0).

#### **4.11: Soft Agar Assay**

Cells ( $5 \times 10^3$  to  $1 \times 10^4$ ) were first mixed with 0.3% agar/DMEM at 42°C and were then laid on top of 0.5% solidified agar/DMEM in 12-well plates. After the top layer solidified, we added 1 ml of fresh medium on top of it. Cells were cultured in the incubator with continuous changes of fresh medium every 5 days until the end point was reached. MTT (thiazolyl blue; 1 mg/ml) diluted in fresh medium was added to stain colonies embedded in the agar.

#### **4.12: Confocal Microscopy**

Cells were seeded on cover slides, treated, and then fixed in 4% paraformaldehyde/PBS at room temperature for 12 minutes. Fixed cells were permeabilized with 0.5% Trion-X100/PBS for another 5 minutes. Antibodies against proteins of interest were added to the cells, followed by an overnight incubation at 4°C. Secondary antibodies were applied for 1 hour at room temperature. PBS was used during the washing process to remove traces of paraformaldehyde, Triton, or unbound antibodies. Slides were mounted with mounting solution that contained DAPI. Images were captured by a multiphoton confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY, USA).

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