MAMMALIAN ENABLED (MENA): A POSSIBLE REGULATOR OF YAP ONCOPROTEIN

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To the people whom I inspired by...

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ABSTRACT

MAMMALIAN ENABLED (MENA): A POSSIBLE REGULATOR OF YAP ONCOPROTEIN

Mammalian Enabled (Mena) induces unbranched actin polymerization and it acts as an antagonist for actin capping proteins in the cell. As an actin related protein, Mena has an important role on both tumor initiation and metastasis. It has been showed that Mena is upregulated in several types of cancers such as breast, colorectal and cervical cancers. In this study, we aimed to find out the signal transduction pathway of Mena which promotes cancer development. Our primary focus was the dysregulation of the Hippo pathway and one of its effector molecules known as an oncoprotein called YAP. The Hippo signaling pathway is one of the main regulators of tissue growth and it is evolutionary conserved from *D. melanogaster* to mammals. Understanding the regulation of the Hippo signaling pathway is one of the recent focuses in cancer research field. By using HEK293FT cells, we showed that Mena negatively regulates TEAD promoter binding activity of YAP, which indicates the inhibition of YAP. Both upregulation and downregulation of Mena also led to change in total YAP protein levels but this effect seemed to be independent from the Hippo signaling. By using real-time PCR, we found that the level of regulation is on protein levels since RNA levels of YAP homologs did not change by Mena downregulation. For *in vitro* experiments overexpression and knockdown vectors for Mena were used and around 50% reduction in Mena expression was achieved by the knockdown vector. In order to verify this regulation in flies, UAS-GAL4 system is used to cause overexpression of Mena homolog Ena in both wing tissue and follicle cells in the ovary. It is found that Ena overexpression does not have any effect neither on the activitiy of the Hippo signaling nor on the activity of YAP homolog Yki. Further studies may be conducted to find the mechanism behind this regulation and the affected cellular processes by this regulation.

ÖZET

MAMMALIAN ENABLED (MENA): MUHTEMEL BİR YAP ONKOPROTEİN DÜZENLEYİCİSİ

Mena proteini hücrede dallanmamış aktin proteini polimerleşmesini tetikleme görevine sahiptir ve bu yüzden aktin kaplamada görev alan proteinlerin antagonisti olarak işlev görmektedir. Aktin ile ilişkili bir protein olması nedeniyle Mena hem tümör başlangıcında hem de metastaz oluşumunda bir rolü olduğu varsayılmaktadır. Daha önce Mena proteininin anlatımının göğüs, kolorektal ve boyun gibi farklı kanser türlerinde arttığı gösterilmiştir. Bu çalışmada, Mena proteinine ait sinyal iletim mekanizmasının bulunması amaçlanmıştır. Odak noktasında Hippo sinyal yolağının ve bu yolağın kanser tetikleyici olarak bilinen efektörlerinden biri olan YAP proteininin regülasyonunun bozulması bulunmaktadır. Hippo sinyal yolağı doku büyümesinin temel düzenleyicilerinden biridir ve evrimsel olarak meyve sineğinden memelilere kadar korunmuştur. Hippo sinyal iletim yolağının düzenlenmesinin anlaşılması kanser araştırmaları alanında son dönemdeki odak noktalardan biri olmuştur. Bu nedenle, HEK293FT hücrelerini kullanarak, Mena proteininin TEAD transkripsiyon başlatıcı bölge bağlanma aktivitesini azalttığı gösterilmiştir. Mena proteininin farklı anlatımı toplam YAP protein seviyesinin değişimine neden olmuş ancak bu etkinin Hippo sinyal yolağından bağımsız olduğu tespit edilmiştir. RT-PCR metodu kullanılarak, YAP homolog genlerinin RNA düzeylerinde Mena'nın farklı ifadesi ile bir değişiklik olmadığı ve bu yüzden düzenleyici etkinin protein düzeyinde olup RNA düzeyinde olmadığı keşfedilmiştir. Bahsedilen düzenleyici etkinin sineklerde de varlığının kanıtlanması için UAS-GAL4 sistemi kullanılarak Mena'nın homoloğu olan Ena geninin aşırı anlatımı hem kanat dokusunda hem de yumurtalıkta bulunan follikel hücrelerde sağlanmıştır. Ena'nın aşırı ifadesinin ne Hippo sinyal yolağının aktivitesinde ne de YAP proteininin homologu olan Yki proteininin aktivitesinde bir etkiye sahip olmadığı bulunmuştur. İleride yapılacak çalışmalar, hem bu düzenleyici etkinin hücrede hangi süreçleri etkilediği hem de etkinin mekanizmasının bulunmasını üzerine olacaktır.

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LIST OF SYMBOLS

Bp	Base Pairs
Cm	Centimeter
G	Gravity
Gr	Gram
kDa	Kilodalton
Mg	Milligram
Min	Minute
Ml	Milliliter
Mm	Millimeter
mM	Millimolar
Ng	Nanogram
Nm	Nanometer
Rpm	Revolution per minute
U	Unit
V	Volt
°C	Centigrade degree
µg	Microgram
µl	Microliter
B	Beta

LIST OF ACRONYMS / ABBREVIATIONS

APS	Ammonium persulfate
BCA Assay	Bicinchoninic Acid Assay
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
cDNA	Complementary DNA
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanosine Monophosphate
CO_2	Carbon dioxide
DAPI	4’6-diaminido-2-phenylindole
DMEM	Dubecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleosidetriphosphate
EDTA	Ethylenediaminetetraacetate
EMT	Epithelial to Mesencymal Transition
EtOH	Ethanol
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
GPCR	G-protein Coupled Receptor
GTP	Guanosine-5’-Triphosphate
HEK	Human Embriyonic Kidney
HRP	Horseradish Peroxidase
JNK	c-Jun N-Terminal Kinase
LB	Luria-Bertani
$MgCl_2$	Magnesium chloride
miRNA	microRNA
mTOR	Mammalian Target of Rapamycin
PAGE	Polyacrylamide Gel Electrophoresis

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
PKB	Protein Kinase B
PKG	Protein Kinase G
PVDF	Polyvinylidene Fluoride
RNA	Ribonucleic Acid
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
Runx2	Runt-related Transcription Factor 2
SDS	Sodium dodecyl sulfate
ShRNA	Short Hairpin RNA
SH3	SRC Homology 3 Domain
TEAD	TEA Domain
TEMED	Tetramethylethylenediamine
UAS	Upstream Activating Sequence
Yap	Yes-associated Protein
Yki	Yorkie

1. INTRODUCTION

1.1. The Hippo Signaling Pathway

The Hippo signaling pathway was recently discovered as one of the key regulators of cell proliferation and apoptosis during development in *D. melanogaster*. Initially, it was identified by genetic studies in *Drosophila* in which mutations in Hippo pathway genes caused tissue overgrowth (Badouel and McNeill, 2009).

In *Drosophila*, the pathway contains two kinases that lie at the center of the Hippo pathway, the Ste-20 like kinase called Hippo (Hpo) and a member of NDR family kinases called Warts (Wts), and these kinases form a kinase cascade (Harvey *et al.*, 2003). The core of the Hippo pathway is formed by these two kinases together with their co-factors Salvador (Sav) (Tapon *et al.*, 2002) and Mats (Lai *et al.*, 2005) and the transcriptional co-activator Yorkie (Yki) (Huang *et al.*, 2005). The signal transduction of this pathway is well understood. If the pathway becomes activated, Hpo in complex with the co-factor Sav phosphorylates Wts and its co-factor Mats (Wei *et al.*, 2007). This phosphorylation events cause activation of Wts kinase activity (Wu *et al.*, 2003). Activated Wts/Mats complex causes phosphorylation of Yki at three different sites S111, S168 and S250 (Oh and Irvine, 2009). Phosphorylation of these sites causes an inhibitory effect on the function of Yki because phosphorylation of S168 causes 14-3-3 protein to bind to Yki and this interaction causes retention of Yki in the cytoplasm, so that it suppresses the transcriptional activity of Yki (Ren *et al.*, 2010). When Yki is not phosphorylated, it localizes to the nucleus and forms complexes with several transcription factors such as Scalloped (Sd) (Goulev *et al.*, 2008). The complex between Yki and Sd causes induction of the expression of several genes such as *cyclin E* known as a cell cycle regulator, *diap1*, which is known as the inhibitor of apoptosis (Pantalacci *et al.*, 2003), *Myc*, known as one of the growth promoters (Neto-Silva *et al.*, 2010) and *bantam miRNA*, which is responsible for cell survival (Hamaratoglu *et al.*, 2006). It can be deduced that Yki is a growth promoter and inhibitor

of apoptosis whereas upstream members of the pathway act as tumor suppressors since they have the function of inhibiting Yki.

Genetically modified mouse models demonstrate that the Hippo pathway is conserved in mammals as well and it functions in a very similar manner (Zhao *et al.*, 2010). In mammals, the core kinase cascade is formed by MST1/2 (Creasy and Chernoff, 1995) and LATS1/2 (Yabuta *et al.*, 2000), which are the mammalian orthologs of Hpo and Wts, respectively. MST1/2 in complex with its co-factor Sav1, which is the Sav homolog, phosphorylate LATS1/2 and Mob1 (the homolog of Mats), the co-factor of LATS1/2. This phosphorylation leads to the activation of LATS1/2 kinases. Active LATS1/2 kinases phosphorylate Yap, the main effector of the mammalian Hippo pathway, and Taz (the homologs of Yki). This phosphorylation results in the inhibition of the transcriptional activities of both Yap and Taz (Halder and Johnson, 2011). Therefore, it can be easily said that the main components of the mammalian Hippo pathway act very similar to that of *Drosophila*.

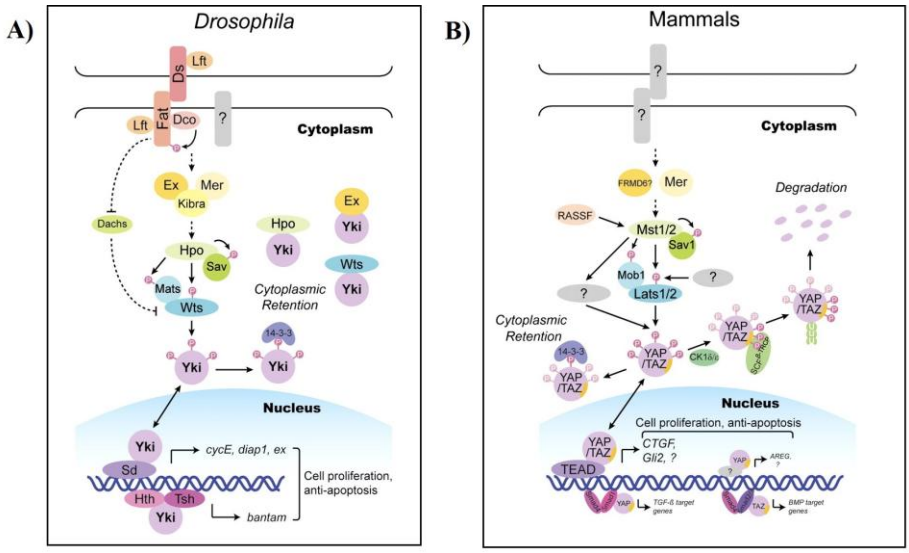

Figure 1.1. The Hippo signaling pathway in (A) *Drosophila* and (B) Mammals. When the pathway is inactive, Yki/Yap enters the nucleus and induces expression of target genes. When the pathway is active, Yki/Yap is phosphorylated and sequestered by 14-3-3 protein and leads to degradation of Yki/Yap (Adapted from Zhao *et al*., 2010).

1.2. Importance of the Regulation of the Hippo Signaling Pathway

Many of the known members of the signaling cascade were identified by genetic screens in *Drosophila*. Loss of function of the two kinases in the center of the Hippo pathway Hpo and Wts cause severe overgrowth of corresponding adult structures in *Drosophila*. In 2003, Georg Halder's group showed that flies with *hpo* mutation in eye discs produce severely overgrown eyes and heads as it can be seen in Figure 1.2 (Udan *et al.*, 2003).

In mammals, initial studies which show the connection between the Hippo pathway and the regulation of organ size had the strategy of Yap overexpression which mimics the inactivation of the mammalian Hippo pathway. Yap overexpression led to very dramatic enlargement of the liver in a way the liver mass is increased three-to-four folds as it can be seen in Figure 1.2 (Camargo *et al.*, 2007). Moreover, consistent with this result, depletion of the components of the Hippo pathway in mice results in the formation of tumors (Lee *et al.*, 2010). Recently, it was shown that abnormal activation of Yap and Taz is observed in many types of cancers (Steinhardt *et al.*, 2008).

The implication of the Hippo pathway with variety of cancers and its unique role in the regulation of tissue growth indicates that a tight regulation of Hippo signaling and its effector molecules Yap and Taz should be present. Recent studies show that the Hippo signaling pathway is dynamically regulated by a variety of signals which lie either upstream of the core kinase cascade or directly target the effectors Yap and Taz.

In *Drosophila*, the core kinase cascade of the Hippo pathway is regulated by four different proteins, which are called Fat, Expanded, Kibra and Merlin. Fat is a member of the Cadherin superfamily and it is found to regulate growth and planar cell polarity (Bryant *et al.*, 1988). Independently from its upstream role, Fat can also act directly on the second kinase of the pathway, Warts, since Dachs, a protein known to accumulate when Fat is inactive (Matakatsu and Blair, 2008), can bind to Warts and causes its degradation (Cho *et al.*, 2006). Another upstream regulator for the core kinase cascade of the pathway is the Expanded/Merlin/Kibra (Ex/Mer/Kibra) complex. All of these proteins are adaptor pro-

teins and flies carrying mutations in one of these genes have weak phenotypes in their imaginal discs compared to *hpo* and *wts*, but double mutant combinations of these three genes show severe phenotypes similar to those of *hpo* and *wts*. The effect of the complex is shown to be via multiple interactions such as Ex and Hpo, Mer and Sav, and Kibra and Sav (Yu *et al.*, 2010).

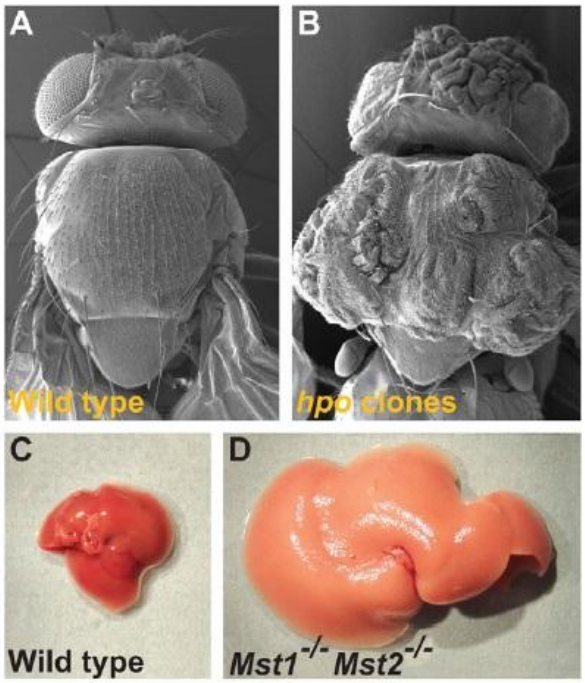

Figure 1.2. Phenotypes of Hippo mutation in flies and mice.(A, B) Electron micrograph scans for (A) wild type fly and (B) *hpo* mutant fly. (C) The liver of 8-weeks old wild type mouse. (D) The liver of 8-weeks old mouse carrying mutation in both MST1 and MST2 (Halder and Johnson, 2011).

The Hippo pathway is also regulated by the input from cell polarity in *Drosophila*. Crumbs, a single pass transmembrane protein which is known to establish and maintain apical-basal cell polarity (Bazellieres *et al.*, 2009), was recently identified as a possible regulator of the pathway (Robinson *et al.*, 2010). It is found that Crb is required for the proper localization of Crb in neighboring cells and it recruits Expanded to the membrane both autonomously and non-autonomously (Chen *et al.*, 2010). Moreover, the Lethal giant larvae (Lgl), a protein involves in the organization of apical-basal cell polarity, has been

linked to the pathway in which Lgl mutant clones in eye discs mimic Yki overexpression but the phenotype is not as severe as the loss of core Hippo pathway components (Grzeschik *et al.*, 2010).

Recently, it is shown that Jub, an Ajuba family protein that localizes in adherens junctions and by linking the adhesive properties of the cell with its nuclear responses (Marie *et al.*, 2003), operates as a negative regulator of the Hippo pathway (Langer *et al.*, 2008). The mechanism of action for Jub lies genetically upstream of Wts but downstream of Hpo. Jub physically interacts with Wts and Sav and causes inhibition of the phosphorylation of Yki by Wts (Das Thakur *et al.*, 2010).

In mammals, there are several other regulatory mechanisms for the Hippo pathway. It was shown that oncogenic stress signals such as activated Ras, induces MST1/2 activation that leads to apoptosis. This process includes the interaction of MST1/2 with RASSF family proteins (Khokhlatchev *et al.*, 2002) and this prevents MST1/2 inhibition by Raf1 (O'Neill *et al.*, 2004). Moreover, recent studies show that Hippo signaling is modulated by DNA damage (Hamilton *et al.*, 2009), contact inhibition (Zhao *et al.*, 2007), F-actin depolymerization (Densham *et al.*, 2009), and a cell surface hyaluronan receptor called CD44 (Xu *et al.*, 2010). As well as these regulators, GPCR signaling was found to be a regulator of the Hippo signaling. The regulatory function of GPCR can be either inhibitory or activating depending on the type of GPCR. Independent from their types, GPCR acts directly on the activity of LATS1/2 kinases.

1.3. The Effector Molecule of the Hippo Signaling Pathway, Yap (Yes-associated Protein) and Its Regulation

Yap is a protein which was discovered as an interaction partner for c-Yes, a non receptor tyrosine kinase (Sudol, 1994). Yap can be localized both in cytoplasm and nucleus which indicates that its shuttling is quite important for the regulation of this unique protein. Yap is known to be a transcriptional co-activator and can interact and modulate several transcription factors such as NFE2 (Gavva *et al.*, 1997), TEAD family transcription factors

(Vassilev *et al.*, 2001), Smad7 (Ferrigno *et al.*, 2002), and p73 (Strano *et al.*, 2001). The interactions of Yap with TEAD family transcription factors and p73 are very important, since the outcome of the interactions have opposite roles. If Yap interacts with TEAD family transcription factors, this interaction leads to increase in the expression of genes related to proliferation and survival (Pan, 2007), whereas if Yap interacts with p73, this interaction leads to the induction of genes related to apoptosis (Strano *et al.*, 2005).

Because of these very different and distinct roles of Yap, it must be tightly regulated. The main regulation for Yap is about physical sequestration inside the cytoplasm since it shuttles between cytoplasm and nucleus to show its activity. This sequestration event is mainly achieved by binding of 14-3-3 protein to phosphorylated Yap. Yap can be phosphorylated by several other kinases. It has been shown that Yap is phosphorylated by Akt/PKB kinase in response to DNA damage and this phosphorylation causes suppression of Yap-p73 interaction and causes suppression of apoptosis (Basu *et al.*, 2003). Moreover, as implied by the name of the protein, Yap can be phosphorylated by c-Yes and c-Src tyrosine kinases and these phosphorylation events are important for recruitment of Runx2 transcription factor to subnuclear sites, so that Runx2 activity is suppressed (Zaidi *et al.*, 2004). Furthermore, it was shown that c-abl kinase can phosphorylate Yap on Y357 and this phosphorylation event increases both stability of Yap and its affinity to p73 transcription factor and it results in induction of apoptosis upon DNA damage (Levy *et al.*, 2008). Last but not least, Yap is known as an effector of the Hippo signaling pathway and its phosphorylation by LATS kinases causes its cytoplasmic retention and binding of 14-3-3 protein. As a result, Yap cannot induce the transcription of genes related to proliferation together with TEAD family transcription factors (Hao *et al.*, 2008).

1.4. MENA (Mammalian Homologue of Enabled) and Its Role in Actin Remodeling and Carcinogenesis

Mena is a mammalian homolog of Enabled protein of *Drosophila* and a member of Ena/Vasp protein family. It is associated with actin based processes and causes unbranched

actin polymerization by associating with free barbed ends of actin filaments and antagonizing filament capping (Krause *et al.*, 2003).

It contains 3 domains with the unique repeat sequence in its structure. EVH1 domain is the domain present at the N-terminus of Mena and it was shown to be important for its interaction with Zyxin, vinculin, and ActA protein of intracellular pathogen *Listeria monocytogenes* (Laurent *et al.*, 1999). The proline-rich domain present in the center is the part for interaction with the proteins which contain SH3 and WW domains as well as small actin-monomer binding proteins called profilin (Ermekova *et al.*, 1997) (Krugmann *et al.*, 2001). Near this central proline-rich region, Mena and the other members of the Ena/Vasp family members contain sites for phosphorylation by PKA and PKG (Urbanelli *et al.*, 2006). The carboxy terminus of Mena protein contains EVH2 domain including 3 regions which are conserved in all Ena/Vasp family members: starting from the amino terminus of the domain, a G-actin-binding site, an F-actin-binding site, and a coiled-coil motif required for oligomerization (Bachmann *et al.*, 1999). As well as these domains, Mena has unique LERER repeat sequence after EVH1 domain which is not present in any other member of Ena/Vasp proteins. This repeat sequence was thought to be functioning as protein-protein binding interface and recently it has been shown that LERER repeat binds to α5 integrin and this interaction is important for α5β1 integrin-dependent processes in fibroblasts (Gupton *et al.*, 2012).

Mena as well as the other members of Ena/Vasp protein family localize to tips of protruding lamellipodia and filopodia and adhesion foci. These proteins are involved in controlling cell motility and cell-to-cell adhesion (Kwiatkowski *et al.*, 2003). Mena has been shown to interact with various proteins such as Abi-1, which promotes c-Abl-mediated phosphorylation of Mena at Tyr-296 (Tani *et al.*, 2003); Zyxin, which is important for proper localization of Mena (Hoffman *et al.*, 2006); TES, a putative tumor-suppressor gene localized in focal adhesions (Coutts *et al.*, 2003); Profillin, an important regulator for microfilament polymerization (Kang *et al.*, 1997); IRSp53, a Rac-binding protein that forms complexes with Cdc42 and Mena in kidney podocytes (Yanagida-Asanuma *et al.*, 2007). Moreover, Mena was also shown to be important in nervous system development where Mena-null mice show subtle deficiencies in forebrain commissure

formation (Menzies *et al.*, 2004). Lastly, Mena has also been linked with schizophrenia (Kahler *et al.*, 2008)

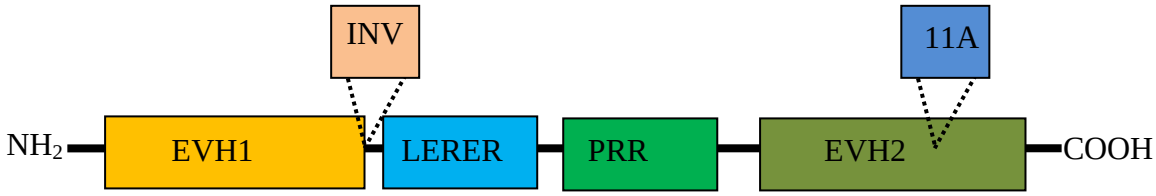

Figure 1.3. Schematic representation of domains present in Mena and differences within isoforms produced from the same mRNA by alternative splicing (Adapted from Goswami *et al.*, 2009).

Human Mena (hMena) was shown to be overexpressed in ~75% of primary breast cancers (Di Modugno *et al.*, 2004). Detailed analysis showed that hMena has at least 4 different specific splice variants, which are referred to as +, ++ and 11a isoforms. $Mena^{++}$ is also known as invasive isoform and causes metastasis. $Mena^{11a}$ isoform is found to be downregulated in invasive tumor cells (Goswami *et al.*, 2009). From these findings, it can be deduced that in invasive migratory tumor cells, Mena isoforms that have increased protrusive and migratory abilities are upregulated, while other isoforms are selectively downregulated (Roussos *et al.*, 2011a). Therefore, Mena can be used as useful biomarker for evaluating the metastatic potential in human tumors. It is also noteworthy that Mena is upregulated in an invasive carcinoma cell that lies in the center of a tumor microenvironment of metastasis together with a macrophage and an endothelial cell. This microenvironment is an important anatomical structure for prediction of risk for metastatic breast cancer (Roussos *et al.*, 2011b). In 2010, it was found that Mena deficiency caused decreased morbidity and metastasis. Moreover, loss of Mena caused delay in tumor progression in polyoma middle-T transgenic mouse mammary tumors (Roussos *et al.*, 2010).

1.5. *D. melanogaster* as a Model Organism

D. melanogaster has been widely used a model organism to study cancer, aging and neurological disorders for decades. Conservation of main signaling pathways between flies and mammals makes *D. melanogaster* a great model organism. Moreover, its sequenced genome and well-characterized developmental stages attracts scientist to use this animal for *in vivo* analysis of what they have been studying such as cancer (Vidal and Cagan, 2006). Recent advances in fly genetics generated very valuable tools to study the differential expression of a gene of interest very easily, such as the UAS (Upstream Activating Sequence)-GAL4 system.

The UAS-GAL4 system is a bipartite system, which is widely used to achieve differential expression of a gene of interest in a variety of tissues and cells. Depending on the promoter of the GAL4 line that is used, activation of the gene cloned downstream of the UAS sequence is obtained in a specific tissue or a group of cells in a specific pattern, that reflects the promoter that has been used.

The UAS-GAL4 system is based on the binding of yeast GAL4 transcriptional activator to the UAS sites present in the genome. The gene encoding GAL4 is inserted randomly into the *Drosophila* genome to drive GAL4 expression from one of the specific enhancers. When these flies are mated with flies containing a transgene, which includes the gene of interest and UAS in the promoter region, it becomes possible for GAL4 to bind UAS sites and cause activation of transcription selectively in the cells containing the GAL4. This makes it possible to see the effects of differential expression as well as misexpression of the gene of interest in a specific tissue (Duffy, 2002).

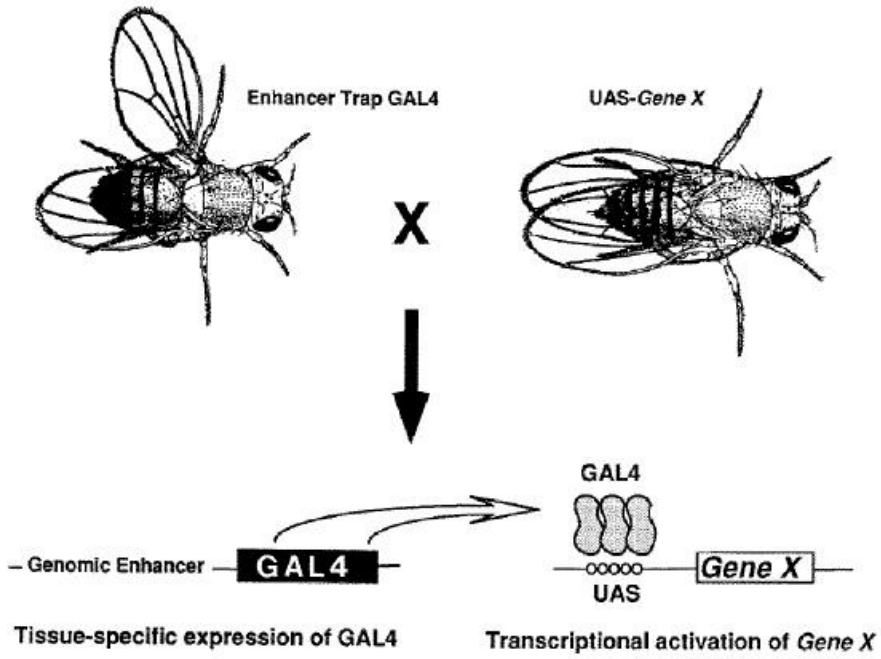

Figure 1.4. Schematic representation of UAS-GAL4 system in *D. melanogaster* (Brand and Perrimon, 1993).

There are several advantages to employ this method to study targeted gene expression in *D. melanogaster*. Firstly, ectopic expression of the gene of interest can be achieved in different tissues and different cell types so that the effect of specific genes in different tissues can be studied. Secondly, since it is a bipartite system, both of the parental lines are viable. In one parental line the gene of interest cannot be differentially expressed since there is no transcriptional activator, while in the other parental line transcriptional activator cannot induce the differential expression because there is no upstream activating sequence. Last but not least, differential expression can be achieved in a specific tissue with very diverse patterns so that the number of cells containing GAL4 in the tissue can be changed to see the effect of the gene of interest (Brand and Perrimon, 1993).

2. PURPOSE

The main aim of this study is to elucidate the regulatory functions of Mammalian Enabled (Mena) in cellular signaling especially on the Hippo signaling pathway and one of its main effectors molecules, Yap.

The Hippo signaling pathway is one of the main regulators of the tissue growth and it is dysregulated in many cancer types. Although, the upstream regulators of the Hippo signaling pathway is poorly understood, several upstream events or proteins that regulate the Hippo signaling pathway were identified recently such as F-actin accumulation, the junction protein Zyxin and a small GTPase called Rac1. When these proteins and the events are analyzed, it can be seen that each has a distinct relationship with Mammalian Enabled (Mena). First of all, Mena promotes the formation of unbranched actin. Therefore, it is an antagonist for actin-capping proteins and it may have a regulatory role on the Hippo signaling pathway in an F-actin dependent manner. Secondly, Zyxin was found to be an important regulator of the Hippo pathway by regulating the effects of the Cadherin Fat on Warts protein levels; is an important interaction partner of Mena and promotes the proper localization of Mena. This data suggest a model in which Mena acts as regulator of the Hippo pathway in a Zyxin-dependent fashion. Thirdly, Mena has been found to have inhibitory function on the activity of Rac1 GTPase, a protein known to regulate one of the upstream regulators of the Hippo pathway called NF2. These evidences suggest Mena as a possible regulator of the Hippo signaling pathway.

Dysregulation of the Hippo signaling pathway results in the activation of its effectors Yap and Taz which are known to have oncogenic effects. In many types of cancer, dysregulation of the Hippo signaling pathway and consequent activation of Yap and Taz were shown so far. Therefore, finding the upstream regulators will help up gaining a better understanding of this unique pathway and give us information related to how its dysregulation leads to cancer.

3. MATERIALS

3.1. General Kits, Enyzmes and Reagents

Table 3.1. List of kits, enzymes and reagents.

Name	**Supplier**
BCA Protein Assay Kit	Thermo, USA
DMEM	PAN, Germany
DNA Molecular Weight Marker	GeneRuler 1 kb DNA Ladder, Fermentas, USA
Dual-Glo Luciferase Assay System	Promega, USA
FBS	PAN, Germany
Genopure Plasmid Midi Kit	Roche, Switzerland
High Pure Plasmid Isolation Kit	Roche, Switzerland
High Pure RNA Isolation Kit	Roche, Switzerland
ImProm-II Reverse Transcription System	Promega, USA
Maxima SYBR Green/ROX qPCR Master Mix (2x)	Thermo, USA
MinElute Gel Extraction Kit	QIAGEN, Germany
MinElute PCR Purification Kit	QIAGEN, Germany
Penicillin/Streptomycin	PAN, Germany
Protein Molecular Weight Marker	PageRuler Prestained Protein Ladder, Fermentas, USA
Restriction Enzymes	Fermentas, USA
T4 DNA Ligase	NEB, USA
Taq DNA polymerase	Fermentas, USA
Trypsin-EDTA (0.5 mM EDTA, 0.025% Trypsin)	PAN, Germany
Turbofect	Thermo, USA
Western Blotting Luminol Reagent	Super Signal West Femto Maximum Sensitivity Kit, Thermo, USA

3.2. Biological Materials

3.2.1. Bacterial Strains

Escherichia coli TOP10 strain was used for the transformation experiments. The genotype of *Escherichia Coli* TOP10 strain is F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsl (StrR) endA1 nupG.

3.2.2. *Drosophila melanogaster* Lines

All the fly lines used in this study were kindly provided by Prof. Georg Halder, KU Leuven and the genotype and the description for each line can be seen in Table 3.2.

Table 3.2. Genotypes of the used fly lines and their description.

Genotype of the Line	**Description**
yw; ex^{697}; ptc-Gal4, UAS-GFP/Cyo, Roi	Expresses Gal4, GFP and ex-lacZ transgene in cells in A/P border of wing imaginal discs
yw; ex^{697}; nubbin-Gal4, UAS-GFP/Cyo, Roi	Expresses Gal4, GFP and ex-lacZ transgene in the cells present in wing pouch
;ex^{697}/Cyo; GR1-Gal4/TM6B	Expresses Gal4 and ex-lacZ transgene in the follicle cells in the ovary.
;nubbin-Gal4, UAS-Hpo^{RNAi}/Cyo;	Expresses Gal4 and dsRNA for *Hpo* in the cells present in wing pouch
;nubbin-Gal4, UAS-Fat^{RNAi}/Cyo;	Expresses Gal4 and dsRNA for *Fat* in the cells present in wing pouch
;nubbin-Gal4/Gla; UAS-Ex^{RNAi}/TM3	Expresses Gal4 and dsRNA for *Expanded* in the cells present in wing pouch
w; nubbin-Gal4, UAS-*Ex*/Cyo Roi;	Expresses Gal4 and *UAS-Expanded* in the cells present in wing pouch
w; nubbin-Gal4, UAS-*Fat*/Cyo-GFP;	Expresses Gal4 and *UAS-Fat* in the cells present in wing pouch
w^{1118}; P{UAS-ena.His6}3;	His tagged *Ena* overexpression
yw; UAS-*Yki*/TM6B;	*Yki* overexpression

3.2.3. Embedding Media

Vectashield Embedding Medium (Vector Laboratories, USA) was used for mounting of adult ovary and larval wing imaginal discs. Canadian Balsam was used for the dissection of adult wings (Sigma-Aldrich, USA).

3.2.4. Cell Lines

Human embryonic kidney cell line HEK293FT was kindly provided by Dr. Nesrin Özören, Boğaziçi University.

3.2.5. Plasmids

pEGFP-N2 (Clontech, CA, USA), pSuper.Neo.GFP (Invitrogen, CA, USA), 8xTGIIC-Luciferase (Addgene, USA) and pRL-SV40 Renilla (Promega, USA) plasmids were commercially obtained and used in this study. Both 6xHis Mena Wild Type and 6xHis Mena S376A plasmids were kindly provided by Prof. Thomas Renne, Karolinska Institute.

3.2.6. Oligonucleotides

Table 3.3. Oligonucleotides used in this study for ShRNA cloning.

Oligonucleotide	**Sequence**
Mena ShC_F	5'-GATCCCCGCCCTTCAAGAGGTTAGGATTAACATTCAAGA-GATGTTAATCCTAACCTCTTGAAGGGCTTTTTA-3'
Mena ShC_R	5'-AGCTTAAAAAGCCCTTCAAGAGGTTAGGATTAA-CATCTCTTGAATGTTAATCCTAACCTCTTGAAGGGCGGG-3'
Mena Sh1_F	5'-GATCCCCGCCATTCCTAAAGGGTTGAAGTACATTCAAGAGATG-TACTTCAACCCTTTAGGAATGGCTTTTTA-3'
Mena Sh1_R	5'-AGCTTAAAAAGCCATTCCTAAAGGGTTGAAGTACATCTCTTGAATG-TACTTCAACCCTTTAGGAATGGCGGG-3'

3.2.7. Primers

Table 3.4. Primers used in this study.

Primer ID	Sequence	Application
MENA_F	5’-GCTGGCACCCACTTCTTATT-3’	Q-RT-PCR
MENA_R	5’-CTGGTGGGGAAGCCTCTG-3’	Q-RT-PCR
18S_F	5’-CTGAAACTTAAAGGAATTGACGGA-3’	Q-RT-PCR
18S_R	5’-GTTATCGGAATTAACCAGACAAATC-3’	Q-RT-PCR
YAP1_F	5′-GTGAGCCCACAGGAGTTAGC-3′	Q-RT-PCR
YAP1_R	5′-CTCGAGAGTGATAGGTGCCA-3′	Q-RT-PCR
YAP2_F	5′-TCTTCCTGATGGATGGGAAC-3′	Q-RT-PCR
YAP2_R	5′-GGCTGTTTCACTGGAGCACT-3′	Q-RT-PCR
TAZ_F	5′-GTATCCCAGCCAAATCTCG-3′	Q-RT-PCR
TAZ_R	5′-TTCTGAGTGGGGTGGTTC-3′	Q-RT-PCR
BAX_F	5'-CTGGACAGTAACATGGAGCTG-3'	Q-RT-PCR
BAX_R	5'-GGCGTCCCAAAGTAGGAGA-3'	Q-RT-PCR
CTGF_F	5’-GTTACCAATGACAACGCCTC-3’	Q-RT-PCR
CTGF_R	5’-TCCACAGAATTTAGCTCGGT-3’	Q-RT-PCR
BIRC2_F	5’-TGTTGTGATGGTGGCTTGAG-3’	Q-RT-PCR
BIRC2_R	5’-AACTCTTGGCCTTTCATTCG-3’	Q-RT-PCR
BIRC5_F	5’-TTGGTGAATTTTTGAAACTGGA-3’	Q-RT-PCR
BIRC5_R	5’-CTTTCTCCGCAGTTTCCTCA-3’	Q-RT-PCR
XmaI_F	5’-AACCCGGGCTGTATGAGACCACAGATCT-3’	Cloning
XbaI_R	5’-AATCTAGAGAGGTCGACGGTATCGAT-3’	Cloning

3.3. Chemicals

Table 3.5. Chemicals used in this study.

Name	**Supplier**
Acrylamide	AppliChem, Germany
Agar	Conda, Spain
Agarose E	Conda, Spain
Ammonium Persulfate (APS)	Sigma-Aldrich, USA
Ampicillin	AppliChem, Germany
β-Mercaptoethanol	Merck, USA
Boric Acid	Sigma-Aldrich, USA
Bovine Serum Albumin (BSA)	AppliChem, Germany
Bromophenol Blue	Fluka, USA
Calcium chloride dehydrate	AppliChem, Germany
Canadian Balsam	Sigma-Aldrich, USA
DMSO	Sigma-Aldrich, USA
EDTA	AppliChem, Germany
Ethanol	Emsure, Germany
EZMix Tryptone	Sigma-Aldrich, USA
Formaldehyde	Sigma-Aldrich, USA
Glycerol	Sigma-Aldrich, USA
Glycine	Fisher Scientific, USA
Isopropanol	Emsure, Germany
Kanamycin	Fluka, USA
Methanol	Emsure, Germany
N, N, N', N'-tetramethylethylenediamine (TEMED)	AppliChem, Germany
N,N'-Methylenebisacrylamide	Sigma-Aldrich, USA
NP-40	Roche, Switzerland
Phosphate Saline Buffer (PBS) - Mol. Biology Grade	Gibco, UK
Sodium Chloride (NaCl)	Fisher Scientific, USA
Sodium Fluoride	Merck, USA
Sodium Hydroxide	Sigma-Aldrich, USA
Sodium Dodecyl Sulfate (SDS)	AppliChem, Germany
Tris-Base	AppliChem, Germany
Tris-Cl	AppliChem, Germany
Triton X-100	AppliChem, Germany
Tween 20	Sigma-Aldrich, USA
Xylene Cyanol	Sigma-Aldrich, USA
Yeast Extract	Conda, Spain

3.4. Buffers and Solutions

Table 3.6. Buffers and solutions used in this study.

Name	**Ingredients**
10X SDS Running Buffer	1% SDS 1.92M Glycine 250 mM Tris-Base
10X Transfer Buffer	1.92M Glycine 250 mM Tris-Base
1X Transfer Buffer	10% 10X Transfer Buffer 20% Methanol
20X SB Buffer	730 mM Boric Acid 200 mM NaOH
4X Protein Loading Dye	200 mM Tris-Cl (pH: 6.8) 8% SDS 40% Glycerol 4% β-mercaptoethanol 50 mM EDTA 0.8% Bromophenol Blue
Cell Lysis Buffer	137 mM NaCl 20 mM Tris-Cl (pH: 7.4) 2 mM EDTA 0.2 % NP-40 5 mM NaF
DNA Loading Buffer	10 mM Tris-Base (pH: 7.4) 0.03% Bromophenol Blue 0.03% Xylene Cyanol 60% Glycerol 60 mM EDTA
LB	5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast Extract
LB Agar	5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast Extract 15 g/l Agar
PBT	1X PBS 0.3% TritonX-100
TBS-T	50 mM Tris-Base (pH: 7.4) 150 mM NaCl 0.1% Tween 20

3.5. Antibodies and Fluorescent Dyes

Table 3.7. Antibodies and fluorescent dyes used in this study.

Name	Species	Dilution	Source
Primary Antibodies			
Anti-phospho-Akt(Ser473) (#4060)	Rabbit	1/1000	Cell Signaling
Anti-Akt (pan) (#4961)	Rabbit	1/1000	Cell Signaling
Anti-Mst1 (#3682)	Rabbit	1/1000	Cell Signaling
Anti-MOB1 (#3863)	Rabbit	1/1000	Cell Signaling
Anti-YAP (#H00010413-M01)	Mouse	1/1000	Abnova
Anti-phospho-MOB1 (Thr35) (#8699)	Rabbit	1/750	Cell Signaling
Anti-LATS1 (#3477)	Rabbit	1/1000	Cell Signaling
Anti-phospho-LATS1 (Thr1079) (#8654)	Rabbit	1/1000	Cell Signaling
Anti-GAPDH (#sc-25778)	Rabbit	1/1000	Santa Cruz
Anti-beta actin (#sc-47778)	Mouse	1/1000	Santa Cruz
Anti-Mena (#2075)	Rabbit	1/1000	Cell Signaling
Anti-phospho-Mst1 (Thr183)/Mst2 (Thr180) (#3681)	Rabbit	1/750	Cell Signaling
Anti-phospho-SAPK/JNK (Thr183)/(Tyr185) (#4668)	Rabbit	1/750	Cell Signaling
Anti-Vimentin (#ab8069)	Mouse	1/1000	Abcam
Anti-E-cadherin (#ab1416)	Mouse	1/1000	Abcam
Anti-beta galactosidase (#Z3783)	Mouse	1/1000	Promega
Anti-His (#R932-25)	Mouse	1/1000	Life Technologies
Secondary Antibodies			
Anti-mouse Cy3 (115-166-003)	Goat	1/500	Jackson Laboratories
Anti-mouse IgG, HRP linked (#7076)	Horse	1/2500	Cell Signaling
Anti-rabbit IgG, HRP linked (#sc-2004)	Goat	1/2500	Santa Cruz
Fluorescent Dyes			
Anti-Phalloidin Alexa Fluor 555 (#A34055)		1/50	Life Technologies
DAPI (#D9542)		1/500	Sigma-Aldrich

3.6. Disposable Labware

Table 3.8. List of disposable labwares used in this study.

Name	**Supplier**
Cell culture plates, 10 cm	Thermo, USA
Cell culture plates, 145 mm	Thermo, USA
Cell culture plates, 60 mm	Thermo, USA
Cell culture plates, 6 well	Thermo, USA
Cell culture plates, 96 well	Thermo, USA
Cell scraper	TPP, Switzerland
Cryo tubes	Greiner Bio One, UK
Filtered tips	CAPP, Denmark
Insulin syringes	Set Medikal, Turkey
LightCycler Capillaries	Roche, Switzerland
PCR tubes, 0.2 ml	Axygen ,USA
Pipette tips	CAPP, Denmark
Test tubes, 1.5 ml	CAPP, Denmark
Test tubes, 15 ml	CAPP, Denmark
Test tubes, 50 ml	CAPP, Denmark

3.7. Equipment

Table 3.9. List of equipment used in this study.

Name	**Supplier**
Agarose Gel Electrophore-sis System	Mini-sub Cell GT, BioRad, USA
Autoclave	Midas 55,Prior Clave, UK
Carbon dioxide Tank	2091, Habaş, Turkey
Cell Culture Incubator	Hepa Class 100, Thermo, USA
Centrifuges	J2-21, Beckman Coulter, USA Allegra X-22, Beckman Coulter, USA 5415R, Eppendorf, USA
Documentation System	GelDoc XR System, Bio-Doc, Italy
Freezers	−20°C, Arçelik, Turkey −80°C ULT Freezer, ThermoForma, USA
Freezing Container	Nalgene Cyro 1°C, Thermo, USA

Table 3.9. List of equipment used in this study (cont.).

Heat Block	DRI-Block DB-2A, Techne, UK
Laminal Flow Cabinet	Labcaire BH18, UK
Luminometer	Fluoroskan Ascent FL, Thermo Electron, USA
Micropipettes	Finnpipette, Thermo, USA
Microplate Reader	680, Biorad, USA
Microscopes	Confocal Microscope, FV1000, Olympus, Japan Inverted Microscope, CKX41, Olympus, Japan Fluoroscence Microscope, Observer.Z1, Zeiss, Germany Light Microscope, Axioplan, Zeiss, Germany Stereo microscope, MZ6, Leica, Germany
Microwave	MD554, Arçelik, Turkey
pH Meter	WTW, Germany
Pipettor	Pipetus-Akku, Hirschmann Labogerate, Germany
Power Supply	Biorad, USA
Real Time PCR	LightCycler 1.5, Roche, Switzerland
Rotors	Beckman JS-7.5, USA Beckman JA-14, USA
SDS Gel Electrophoresis	Biorad, USA
Shaker	VIB Orbital Shaker, InterMed, Denmark
Spectrophotometer	NanoDrop 1000, USA
Stella	Raytest, Germany
Thermocycler	Gene Amp. PCR System 2700, Applied Biosystems, USA
Vortex	Vortexmixer VM20, Chiltern Scientific, UK
Water purification	WA-TECH Ultra-pure water purification system, Germany

4. METHODS

4.1. SDS/PAGE and Western Blotting

4.1.1. Cell Lysis and Protein Extraction from HEK293FT Cell Line

To obtain proteins from HEK293FT cells transfected with knockdown or overexpression constructs, cells in 6-well plate or 60 mm plates were washed once with 1X PBS. Lysis Buffer was added onto plates as 150 µl for 6-well plate and 200 µl for 60 mm plate. Plates were placed onto ice for 15 minutes. Cells were scraped using cell scrapers, collected into 1.5 ml micro centrifuge tubes and incubated on ice for half an hour. Insulin syringes were used to homogenize cells and get rid of genomic DNA. Cell lysates were centrifuged at 14000g for 10 minutes at 4°C. Supernatants obtained after centrifugation were transferred into fresh 1.5 ml micro centrifuge tubes. Protein lysates were stored at -20°C for short term storage or directly used.

4.1.2. Quantification of Protein Lysates

Protein quantification was performed by using a BCA Protein Assay Kit (Thermo). BSA standards were prepared via serial dilution with 1X PBS to obtain different concentrations (125, 250, 500, 750, 1000, 1500, and 2000 µg/ml). Protein samples were prepared as 1/5 dilution in cell lysis buffer for the assay. All samples were prepared in triplicate. To prepare the BCA Working Reagent, 50 parts Reagent A were mixed with 1 part Reagent B. For each sample, 150 µl of BCA Working Reagent was prepared and added onto a 96 well plate. The plate was placed on ice. BSA standards and protein samples were added onto each well as 5 µl. The plate was then incubated at 37°C for 30 minutes and afterwards cooled at RT for 5 minutes. The absorbance of the samples was measured at 562 nm on the plate reader. Blank values were subtracted from the average value measured at 562 nm absorbance. To calculate the concentration of the samples, a standard curve was prepared by plotting blank corrected BSA measurements at 562 nm versus their corresponding concen-

trations in µg/ml. By using a standard curve, the concentration of each sample was calculated.

4.1.3. Preparation of Protein Lysates

Concentrations of protein samples were adjusted according to the lowest amount of protein sample by means of addition of cell lysis buffer. 4X Protein Loading Dye was added into each sample and denaturation of the proteins was achieved via boiling the samples at 95°C for 5 minutes.

4.1.4. SDS-PAGE

SDS-PAGE gels were cast and run by using a Mini-Protean Tetra cell (BioRad). 10% resolving gels (with 37.5:1, acrylamide: bis-acrylamide ratio) were cast first. In order to avoid any bubbles, isopropanol was added on top of the resolving gel until the end of polymerization reaction. Then, isopropanol was removed and a 5% stacking gel (with 37.5:1, acrylamide: bis-acrylamide ratio) was added on top of resolving gel. The comb was inserted at the top and the polymerization of stacking gel was awaited.

Table 4.1. Preparation of SDS-PAGE gels.

	Resolving Gel	**Stacking Gel**
ddH_2O	3.65 ml	1.825 ml
1M Tris-Cl (pH:6.8)	-	313 µl
1.5M Tris-Cl (pH:8.8)	2.25 ml	-
Acrylamide:Bisacrylamide (30% / 0.8% w/v)	3 ml	335 µl
SDS (20% w/v)	45 µl	13 µl
APS (10% w/v)	56.5 µl	21 µl
TEMED	13.5µl	6 µl

4.1.5. Western Blot

Prepared protein samples were loaded into wells generated in the stacking gel, after the removal of the comb. Depending on the efficiency of the antibody that was used, 30-70 µg protein was loaded to each well. The protein ladder was loaded as 5 µl for a single well. Gel running was performed at 100 V for around 90-120 minutes with 1X running buffer. After completion of the run, PVDF membrane was activated in methanol for 45 seconds and transferred to ddH_2O. Gels were placed into transfer cassettes for the transfer of proteins onto the membrane. Transfer was performed at 100 V for 90 minutes with 1X transfer buffer with 20% methanol in the cold room using the Trans-blot cell (BioRad). The system was supplied with an ice-block to prevent heating during the run. After the completion of the transfer, the membrane was removed from the cassette and it was blocked with 5% non-fat dry milk in TBS-T for 1 hour at room temperature. After blocking, the membrane was washed three times with TBS-T, for 5 minutes for each wash. Primary antibodies were applied according to the manufacturer's protocol and prepared in 5% BSA in TBS-T. Primary antibodies were incubated overnight at 4°C. After the incubation with primary antibodies, the membrane was washed three times with TBS-T for 5 minutes each. Then, secondary antibodies, HRP-conjugated anti-rabbit or anti-mouse IgG depending on the source of the primary antibody was prepared in 5% non-fat dry milk at a 1:2500 dilution. Secondary antibodies were incubated at room temperature for 2 hours. After the incubation with a secondary antibody, the membrane was washed three times with TBS-T for 5 minutes each. All processes after the transfer were performed on a shake at 100 rpm. To visualize the proteins, Super Signal West Femto Maximum Sensitivity Kit (Thermo) was used and the detection solution was prepared mixing Solution A and Solution B (1:1 ratio). The protein bands were visualized in bio/chemi-luminescence system (Raytest - Stella).

4.2. Molecular Techniques

4.2.1. Preparation of Plasmids

Plasmid DNA was isolated according to the manufacturer's instructions. Transformed bacteria were inoculated in LB medium, using 5 ml for a mini prep and 50 ml for

midi prep, and the final volume for the elution was 80 µl for a mini prep and 200 µl for midi prep. In all cases, sterile double distilled water was used for elution. Mini prep was done for sequencing and cloning experiments while for transfection experiments, plasmids were prepared at a larger scale using a Midi kit.

4.2.2. Restriction Enzyme Digestion of Plasmids

In cloning experiments, plasmid DNA should be digested using restriction enzymes. 3 µg of pSuper.Neo+GFP plasmid was double digested with 10 U of BglII and 8 U of HindIII restriction enzymes for 4 hours and the R Buffer was used for this reaction at final concentration of 1X in total reaction volume of 30 µl. After completion of digestion reaction, the sample was prepared for agarose gel electrophoresis.

4.2.3. Agarose Gel Electrophoresis

Double digested pSuper.Neo+GFP and its undigested version were run together on 1% agarose gel in a Mini-sub Cell GT (BioRad) using 1X SB Buffer (Table 3.5).

4.2.4. Extraction of DNA Samples from Agarose Gels

DNA samples were run in agarose gel. Bands were visualized, cut with a sterile razor blade, and placed within a 1.5 ml micro centrifuge tube. The weight of the gel was determined and its volume was calculated. QIAGEN MinElute Gel Extraction Kit was used for DNA extraction, which was performed according to the manufacturer's instructions.

4.2.5. Oligo-Annealing

Oligonucleotides were designed according to the instructions of the manufacturer of pSuper.Neo+GFP RNAi system. The designed oligonucleotides were dissolved in sterile water with a final concentration of 3 mg/ml. 1 µl of each complementary oligonucleotide

was added to 48 µl of oligo annealing buffer. This mixture was incubated at 95°C for 2 minutes and then gradually cooled to 25°C over 45 minutes. Annealing of the oligonucleotides was confirmed by agarose gel electrophoresis since annealed oligonucleotides migrate faster and give brighter bands compared to that of non-annealed ones. Not annealed oligonucleotide was used as a negative control for agarose gel electrophoresis.

4.2.6. Ligation

Ligation of plasmid and annealed oligonucleotides were performed with the aid of T4 DNA Ligase (NEB) according to the manufacturer's protocol. The ligation reaction was performed with 1 unit of enzymes, 100 ng of double digested vector and 1:25 vector to insert ratio in a final volume of 30 µl.

4.2.7. Transformation of Chemically Competent TOP10 Cells

A vial of competent cells was thawed on ice for 20 minutes and 50 ng of plasmid or 6 µl of ligation mix was added into the vial. Cells were incubated on ice for 20 minutes. The vial was placed onto a 42°C heat-block for a minute and then put onto ice for 2 minutes. 250 µl of LB medium was added into the vial and cells were grown at 37°C for an hour by shaking. 100 µl of cell suspension was spread out onto antibiotic containing LB agar plates and incubated at 37°C for overnight in inverted orientation.

4.2.8. Colony PCR

To test the presence of plasmid carrying the desired insert, colony PCR was used for validation. First, several colonies were chosen from the agar plate and they were picked with a pipette tip and placed into PCR tubes which contains 25 µl of PCR solution mixture with the content of 1X Taq Buffer, 2 mM $MgCl_2$, 0.5 mM dNTP mix, 0.4 µM of each primer (XmaI_F and XbaI_R), 8% DMSO, 1 u of Taq polymerase (Fermentas). After mixing with PCR solution, 1 µl of the solution mix was dropped onto antibiotic resistant plate to create a backup plate. A PCR was performed with the help of Thermocycler and the fol-

lowing procedure: initial denaturation at 94°C for 5 minute; 35 cycles of denaturation, annealing and elongation at 94°C for 10 seconds, 55°C for 30 seconds and 72°C for 30 seconds respectively; final elongation at 72°C for 7 minutes. PCR products were run on a 1% agarose gel.

4.2.9. Sequencing of the Created Clones

Plasmids were prepared with a final concentration of 100 ng/µl and sequencing was performed by Macrogen Inc. (South Korea).

4.2.10. Extraction of Total RNA from HEK293FT Cells

For RNA extraction, cells in 6-well or 60 mm cell culture plates were washed once with 1X PBS and then treated with trypsin. Trypsinized cells were collected in a micro centrifuge tube and RNA extraction was performed by using High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. The integrity of extracted RNA was tested by Agarose Gel Electrophoresis and the RNA concentration was measured by Nanodrop.

4.2.11. Reverse Transcription and cDNA Synthesis

Reverse Transcription and cDNA synthesis were performed with Promega, Im-Prom-II Reverse Transcription System according to the manufacturer's protocol. 1 µg of total RNA was incubated with 0.5 µl of oligo-dT primers in a total volume of 5 µl at 70°C. Then, the protocol of the manufacturer was followed. Extracted RNA was stored at -80°C for later use.

4.2.12. RT-PCR

RT-PCR was performed with the help of Taq DNA polymerase (Fermentas). Briefly, 1X Taq buffer, 8% DMSO, 2 mM of $MgCl_2$, 0.4 µM of each primers, 0.5 mM of dNTP mix, 1 u of Taq DNA polymerase, 50 ng of cDNA was prepared. Total volume of the reaction was adjusted to 25 µl and PCR was performed with the aid of a Thermocycler.

4.2.13. Real Time PCR

Real Time PCR reaction was performed using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo) according to manufacturer's protocol. 12.5 µl of SYBR Green mix (2x), 1 µl of primer mix, 30 ng of cDNA was prepared as a reaction mixture and dH_2O was added to complete the volume to 25 µl. This mixture was added into the LightCycler capillary. A standard curve was constructed for each primer and cDNA sample for calculating the efficiency. Obtained results were analyzed by using Light Cycler 4.0 Analysis Software (Roche) and Microsoft Excel (Microsoft). Reaction conditions were as follows: Initial denaturation at 95°C for 10 minutes; amplification cycles consist of 3 steps which were denaturation at 95°C for 10 seconds, annealing at 57°C for 5 seconds and elongation at 72°C for 10 seconds. The number of cycles for amplification was 45 and the reaction was terminated by a melting curve.

4.2.14. Luciferase Assay

Luciferase assay was performed to see whether there is any change in the binding activity of TEAD family transcription factors to their binding promoter. This assay was performed using Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol. In order to get signals for the assay, HEK293FT cells were co-transfected with the desired plasmid for differential expression together with 650 ng 8XTGIIC Luciferase plasmid and 100 ng of pRL-SV40 Renilla (for internal control). 48 hours after transfection, cells were washed once with 1X PBS and then treated with trypsin. Trypsinized cells were collected by using 800 µl of 1X PBS and added into a 1.5 ml micro centrifuge

tube. Cells were centrifuged at 0.5 g for 4 minutes and the pellet was washed once with 1 ml 1X PBS and centrifuged again at 0.5 g for 4 minutes. Then, the pellet was resuspended in 100 µl of 1X PBS and put into a well of 96-well plate. 100 µl of Firefly luciferase substrate was added onto the well and measurements were taken after 10 minutes of incubation in the dark at room temperature by Fluoroskan Ascent FL (Thermo Electron). Then, Renilla luciferase substrate diluted 1:100 in Stop&Glo^{TM} buffer was added to the well. The buffer caused the quenching of the Firefly luciferase signal and allowed the detection of Renilla luciferase signal only. After the addition of Renilla substrate, the plate was incubated in the dark at room temperature for 10 minutes and then measurement was performed. Obtained Firefly luciferase signals were normalized to Renilla luciferase signals and graphs were plotted using Microsoft Excel (Microsoft). A two-sided t-test is used for the statistical analysis in order to calculate the significance of the obtained differences in the experiment.

4.3. Cell Culture Techniques

4.3.1. Growth Conditions of HEK293FT Cells

Cells used in cell culture were grown in DMEM – High Glucose containing 10% FBS, 1% of penicillin/streptomycin (complete DMEM). Cells were incubated in 5% CO_2 incubator at 37°C and growth medium was kept at 4°C and warmed at 37°C before use. In order to avoid any contamination, all materials used related to cell culture were wiped with 70% ethanol.

4.3.2. Passaging

The cells were passaged before reaching full confluency on a 100 mm cell culture plate. First, growth medium present in the plate was aspirated and cells were washed with 1X PBS. Then, 1X PBS was aspirated and cells were removed with the addition of 1 ml trypsin-EDTA solution (0.5mM EDTA, 0.025% trypsin). Cells were incubated in the incubator for 2-3 minutes and then collected by 5 ml growth medium, which caused inactiva-

tion of trypsin. Cells were centrifuged at 1600 rpm for 4 minutes and the supernatant was aspirated. 6 ml of growth medium was added onto the pellet and the pellet was resuspended via pipetting. 1/4 of the cells were transferred into new 100mm cell culture plates.

4.3.3. Cryopreservation

In order to prepare stocks for HEK293FT cells, growth medium onto the cells were aspirated and cells washed once with 1X PBS. 1X PBS was removed by aspiration and cells were treated with trypsin-EDTA solution. Then, cells were incubated in the incubator and later collected in 5 ml growth medium and centrifuged at 1600 rpm for 5 minutes. After centrifugation, cells were resuspended in 90% growth medium and 10% DMSO. Fully confluent 10 cm plates were divided into five and 1 ml of solution containing cells were put into 2 ml screw capped-cyrotubes. Tubes were immediately placed into NALGENE Cyro 1°C Freezing Container filled with isopropanol which is immediately placed in -80°C freezer. The container has a unique property which provides cells to freeze -1°C/min.

4.3.4. Thawing

Frozen cells were taken from the -80°C freezer and placed into incubator at 37°C. Just after thawing of the cells, they were added into a 15 ml falcon tube containing 5 ml of growth medium and centrifuged at 1600 rpm for 5 minutes. The pellet was resuspended in 5 ml of growth medium and added into a 10mm cell culture plate. Since DMSO is toxic for the cells, the centrifuge step was performed immediately after the thawing of the cells in the cryovial.

4.3.5. Transient Transfection of Cells

Transfection experiments were performed either in 6 well plates or 60 mm plates *in vitro* by using Turbofect (Thermo) reagent. Cells were plated the day prior to transfection as the confluency of the cells should be around 50-60% at the time of transfection. For 6-well plates, 3 µg of total plasmid DNA was added into 1.5 ml micro centrifuge tube con-

taining 350 µl of serum free medium and mixed by vortexing. 5 µl of transfection reagent was added onto the plasmid-medium mixture. For a 60 mm plate, 6 µg of plasmid DNA and 600 µl of serum free medium were mixed in a sterile tube. 12 µl of transfection reagent was added onto plasmid-medium mixture. Final mixture was vortexed, spinned down and incubated at RT for 15 minutes. The mixture was added onto the cells and the plate was swirled. The growth medium of the transfected cells was replaced with fresh growth medium 5 hours after the transfection.

4.3.6. Cisplatin Treatment

Cells transfected with ShRNA constructs were treated with cisplatin 24 hours after the transfection. Cells were treated with cisplatin for 16 hours and RNA extraction and subsequent steps were performed after that. 2 mM Cisplatin stock was kindly provided by İbrahim Yaman, Boğaziçi University and the treatment performed as the final concentration of the drug is 10 µM.

4.4. *In vivo* Techniques

4.4.1. Maintenance of Drosophila Stocks

Stocks were reared in cylindrical vials (28.5 mm x 95 mm) containing the fresh medium. All flies were maintained in the incubator at 25°C. Adult flies were transferred into new vial containing fresh medium in every two weeks.

4.4.2. Crosses

In order to obtain flies with *Ena* or *Yki* overexpression, 12 virgin females carrying the GAL4 driver were collected and crossed with the males of UAS-*Ena*.His6 or UAS-Yki (3:2 female to male ratio). All the crosses were performed in 25°C. Parental flies were transferred to a new vial three-times a week to obtain proper progeny. Flies that did not

have the phenotype of the balancer, which was mainly the curly wing (CyO) in this study, were selected and analyzed under the stereomicroscope by anaesthesia with CO_2.

4.4.3. Immunohistochemistry for Larval Wing Imaginal Discs and Adult ovary

For the wing imaginal discs, *Drosophila* larvae were collected in 1X PBS on a dissection pad. Larvae were cut from one third of their size in a way that one was the side including the mouth hook. These larval sections were turned inside out.

Larval sections or adult ovaries were collected in a 1.5 ml tube containing ice-cold 1X PBS. 1X PBS was removed and replaced by 4% paraformaldehyde in PBT buffer and tissues were fixed for 10 minutes in this solution. Then, tissues were washed three times with 500 µl PBT for 10 minutes per wash. After washing, blocking solution was prepared as 10 µl of Normal Donkey Serum (Santa Cruz, USA) in 490 µl of PBT. Blocking was performed for an hour. At the end of blocking, 0.5 µl of primary antibody was added into the blocking solution and tissues were incubated with primary antibodies for overnight at 4^oC. Then, tissues were washed three times with 500 µl PBT for 10 minutes per wash. After washing PBT was replaced with secondary antibody mixture, containing 490 µl PBT, 10 µl Normal Donkey Serum, 1 µl DAPI and 1 µl anti-mouse Cy3 antibody and tissues were incubated at room temperature for 2 hours. Then, tissues were washed with 500 µl PBT three times for 10 minutes per wash. Both wing imaginal discs present in the larval sections and adult ovaries were mounted in Vectashield (Vector Laboratories).

4.4.4. Dissection of Adult Wing Tissues

Adult flies with the correct genotypes were collected and stored in 70% ethanol at room temperature for the collection and imaging of the wing tissues. Wings were mounted in Canadian Balsam and imaging was performed by using Zeiss Axioplan Microscope.

4.4.5. Image Analysis

Analysis of the images was performed by using ImageJ software and UCSD Confocal Microscopy Plugins that were utilized for color channel merging and color adjustments.

5. RESULTS

5.1. Effect of Mena on the Hippo Signaling Pathway and Its Effector Molecule Yap in HEK293FT cells

In order to study a possible regulatory function of Mammalian Enabled (Mena) *in vitro*, we used HEK293FT cells. As we noted earlier, Mena has different isoforms present in different types of tissues. In order to eliminate the effect of other isoforms, we used this cell line which primarily expresses the classic isoform of Mena (Benz *et al.*, 2009). In order to show possible regulatory function, we took advantage of several experimental methods such as luciferase assay, western blotting and real-time PCR.

5.1.1. Mena Overexpression Leads to Decrease in TEAD-Promoter Binding Activity

When the Hippo signaling pathway is active, Yap will be phosphorylated by LATS1/2 kinases and this phosphorylation leads to the degradation of Yap. For TEAD family transcription factors to bind their target promoter, they need to form a complex with Yap, the main effector molecule of the Hippo signaling pathway. In order to check whether Mena has any role in the regulation of the Hippo signaling pathway and its components, we performed a luciferase reporter assay to measure the activity of the promoter to which TEAD transcription factors bind. For this purpose, HEK293FT cells were transfected with either pEGFPN2 or pcDNA3 6xHis Mena plasmids. The cells were cotransfected with 8xTGIIC luciferase plasmid and pRL-SV40 Renilla plasmid. 8xTGIIC luciferase plasmid contains the binding site for TEAD family transcription factors and enables us to measure the activity of TEAD binding activity and pRL-SV40 Renilla plasmid was used as an internal control for the experiment. 48 hours after the transfection cell lysates were obtained and luminometric measurements were performed using Fluoroskan Ascent FL (Thermo Electron). The data were analyzed by using Microsoft Excel and two-sided t-test was used to analyze the statistical significance. The results of three independent experiments are shown in Figure 5.1. The result showed that Mena overexpression leads to a significant

decrease (% 70) in the activity of the promoter (p=0.0117). Thus, Mena seems to regulate the Hippo signaling pathway positively, which leads to a negative regulation of Yap onco-protein.

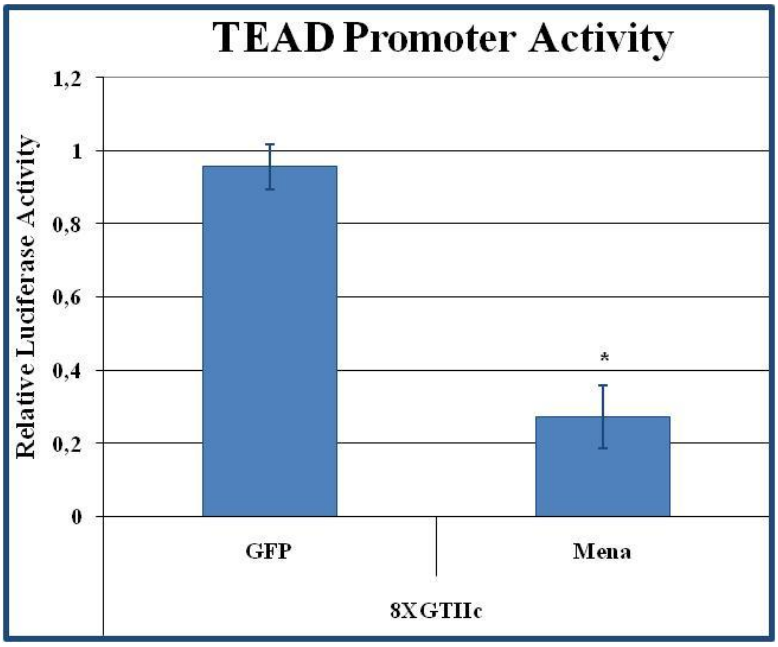

Figure 5.1. Mena severely reduced the binding of YAP to TEAD promoter.

In order to further verify the effect of Mena on Yap, we wanted to test whether there is any change in the expression of several genes whose transcriptions are known to be mainly modulated by the nuclear activity of Yap. We selected *BIRC2*, *BIRC5* and *CTGF* as Yap targets and analyzed their expression levels by performing real-time PCR. For this purpose, HEK293FT cells were transfected either with control shRNA vector (ShC) or shRNA targeting Mena (Sh1) and total RNA of transfected cells was obtained 48 hours after transfection. For the verification of knockdown, Mena primers were also used and the result indicated that expression of Mena mRNA was reduced by 60% in the cells transfected with shRNA targeting Mena. Two independent experiments were performed with experimental duplicates. 18S was used as an internal control and the fold differences were normalized by 18S expression. A two-tailed t-test was used to check the significance of the observed differences and obtained p-values are as follows: For *CTGF* p=0.0047, for *BIRC2* p=0.0049, and for *BIRC5* p=0.020.

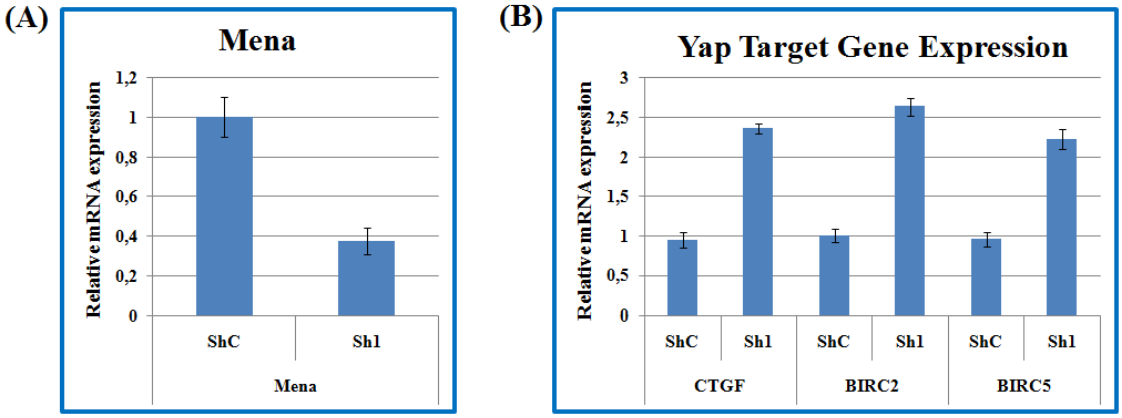

Figure 5.2. Mena knockdown causes induction of the expression of Yap target genes *BIRC2*, *BIRC5* and *CTGF*.(A) Verification of Mena knockdown. (B) Increase in the expression of Yap target genes.

It was observed that Mena knockdown led to around 2.5 half fold increase in the expression of Yap target genes and this indicated that nuclear activity of Yap increased when Mena levels decreased. This result is also consistent with the previous result. Taken together, both results indicate that Mena decreases the activity of Yap, which is possibly due to the positive regulation of the Hippo signaling pathway.

The significant reduction in the binding activity of TEAD family transcription factors to their target promoter by Mena overexpression and the increase in mRNA levels of Yap target genes by Mena knockdown suggested that Mena is affecting the nuclear level and the activity of Yap protein. Since this effect is possibly due to the degradation of Yap after its phosphorylation by LATS1/2 kinases, we wanted to see whether the total protein levels for Yap are changing when expression of Mena changes. For this purpose, Mena and Yap protein levels were analyzed by western blot after overexpressing or downregulating Mena and β-actin was used as a loading control. The result of the western blots for both Mena overexpression and Mena knockdown can be seen in Figure 5.3. For overexpression HEK293FT cells were transfected either with pEGFPN2 or pcDNA3 6xHis Mena plasmids and for downregulation cells were transfected with either control shRNA (ShC) or shRNA targeting Mena (Sh1).

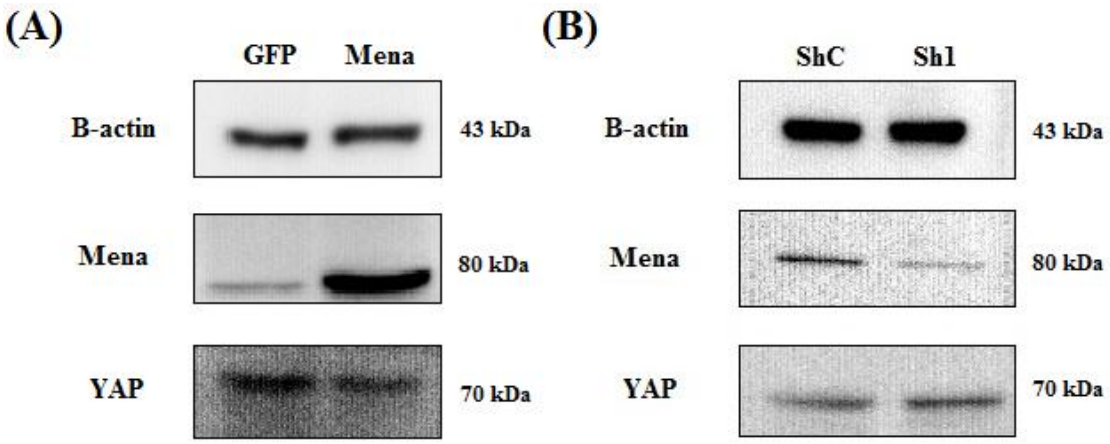

Figure 5.3. Changes in Mena expression levels result in changes in total Yap protein levels. (A) Mena overexpression decreased total Yap protein levels. (B) Mena knockdown increased total Yap protein levels.

As it can be seen in Figure 5.3, Mena overexpression led to a decrease in the total Yap protein levels whereas Mena knockdown led to increase in total Yap protein levels. These results are consistent with the reduction in TEAD-promoter binding activity by Mena overexpression and induction of Yap target gene expression upon Mena knockdown.

All of these data suggested that Mena leads to a decrease in the levels of Yap protein. In order to elucidate whether this is due to the suppression of Yap mRNA production or degradation of Yap protein, we checked whether the expression levels of Yap homologs are changing or not upon Mena knockdown by using real-time PCR. For this purpose, HEK293FT cells were transfected either with control shRNA vector (ShC) or shRNA targeting Mena (Sh1) and total RNA of transfected cells obtained 48 hours after transfection. For the verification of knockdown, Mena primers were also used and the result indicated that expression of Mena mRNA was reduced by around 60% in the cells transfected with shRNA targeting Mena. The results of two independent experiments were performed with experimental duplicates. 18S was used as an internal control and the fold differences were normalized by 18S expression. A two-tailed t-test was used to check the significance of the observed differences and obtained p-values are as follows: for *YAP1* p=0.063, for *BIRC2* p=0.93, and for *BIRC5* p=0.082.The result can be seen in Figure 5.4.

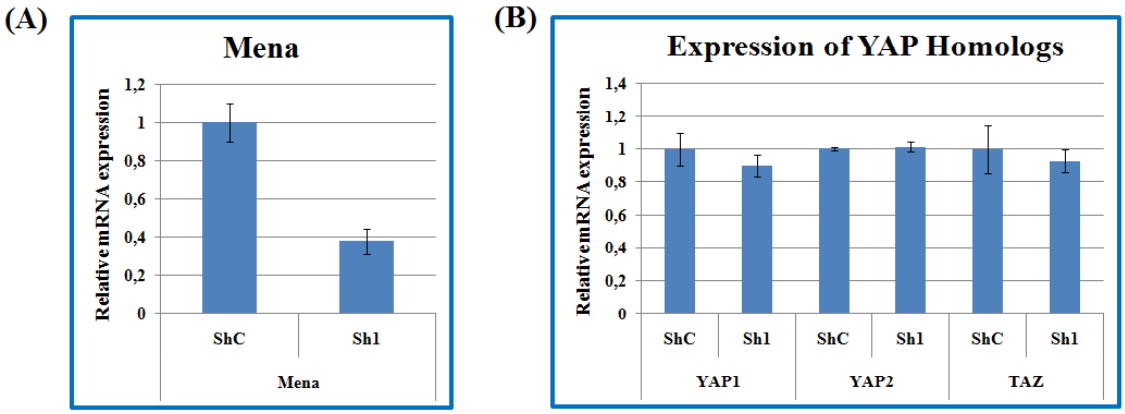

Figure 5.4. Mena knockdown causes no change in the expression of Yap homologs *YAP1*, *YAP2* and *TAZ*. (A) Verification of Mena knockdown. (B) Expression levels of Yap homologs *YAP1*, *YAP2* and *TAZ*.

5.1.2. Mena Regulates Yap Independently from the Hippo signaling and F-actin production

Our data suggest that Mena causes degradation of Yap protein. Phosphorylation of Yap by LATS1/2 kinases due to the Hippo signaling pathway activity is suggested to be the reason. Therefore, we wanted to check whether Mena causes any activation in Hippo signaling. In particular, we wanted to check if it has an effect on the activity of core kinases of the Hippo signaling pathway. For this purpose, protein levels for MST1 and LATS1 and their activated versions were analyzed by western blot. Lysates were obtained from HEK293FT cells transfected with either control shRNA (ShC) or shRNA targeting Mena (Sh1) and GAPDH was used as a loading control.

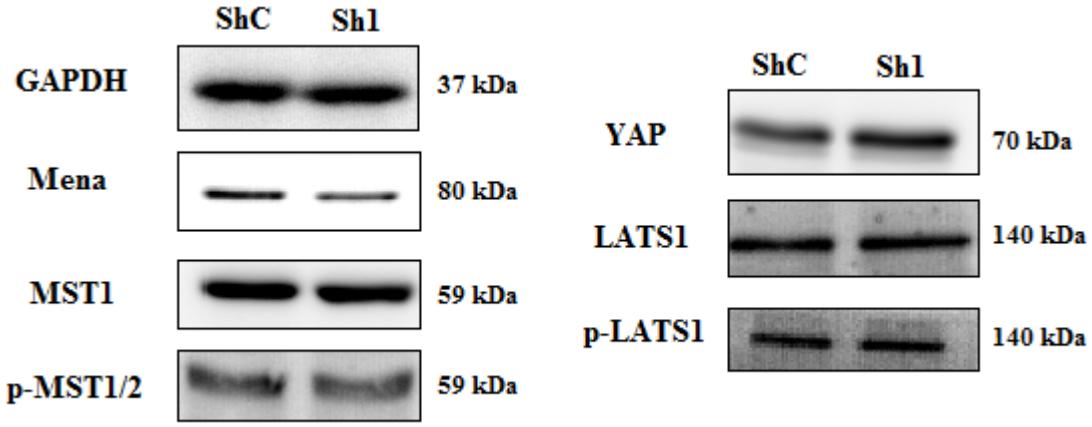

Figure 5.5. Mena knockdown has no effect on the activity of Hippo signaling whereas it induces an increase in total Yap protein levels.

As can be seen in Figure 5.5, Mena knockdown led to an increase in Yap protein levels. However, the levels of core kinases of the Hippo signaling pathway MST1 and LATS1 did not change. Moreover, phosphorylation levels of both MST1/2 and LATS1 also did not change which are both the mark for the activation of the kinases and of the Hippo signaling pathway. This result suggests that regulatory activity of Mena on Yap protein is independent of the Hippo signaling pathway.

Recent studies showed that F-actin can affect the Hippo signaling pathway mainly by affecting the activity of LATS kinases (Sansores-Garcia *et al.*, 2011). Since Mena did not cause any change in the activity of the Hippo signaling pathway, we wanted to verify that the regulatory role of Mena on the Yap protein is independent of the actin related function of Mena. We used a construct containing a phosphorylation mutant of Mena named S376A. S376 is the site where c-AMP- and c-GMP-dependent kinases phosphorylate Mena and this phosphorylation impairs the actin production ability of Mena (Benz *et al.*, 2009). Therefore, this S376A mutant cannot be phosphorylated by c-AMP and c-GMP kinases and can cause the constitutive formation of unbranched F-actin. For testing the effect of F-actin related function of Mena on Yap, protein levels for Mena and Yap were analyzed by western blot. Lysates were obtained from HEK293FT cells transfected with pEGFPN2 (GFP), pcDNA3 6xHis Mena (WT) or pcDNA3 6xHis Mena S376A, 48 hours after transfection and GAPDH was used as a loading control. The western result can be seen in the Figure 5.6.

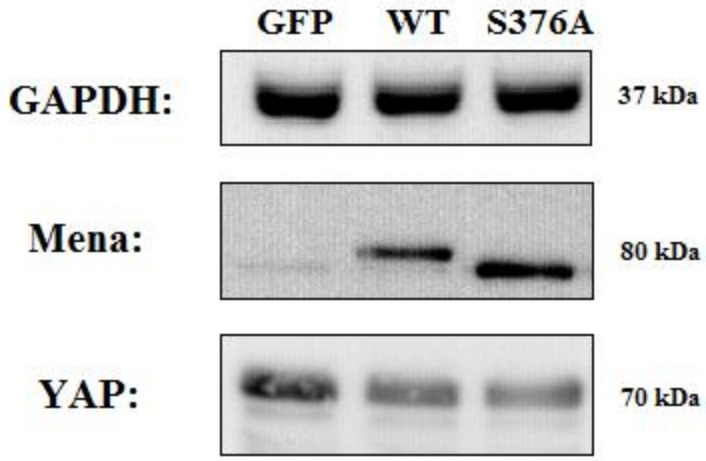

Figure 5.6. Regulatory action of Mena on Yap is independent from its F-actin related function.

It was observed that the mutant version of Mena also led to a decrease in total Yap protein levels compared to the control and the observed decrease was very similar to the decrease caused by the wild-type version of Mena. This result indicates that the effect of Mena on Yap seems to be independent of its F-actin related function.

5.1.3. Mena Knockdown Mimics the Oncogenic Roles of Yap

Yap is known to have a lot of different functions in the cell depending on the activities of its upstream regulators. On one hand, Yap is a transcription co-factor that can interact with p73 and this interaction causes induction of apoptosis whereas on the other hand, Yap is known to act as an oncogene and causes induction of EMT, proliferation and suppression of apoptosis.

First, we analyzed the effect of Mena on the activation of the mTOR pathway which is known to be active when the cell is in a proliferative state. Moreover, it was shown that Yap activates the mTOR pathway (Overholtzer *et al.*, 2006). For this purpose, protein levels for AKT and its mTORC2 phosphorylated version; p-AKT Ser473 were analyzed by western blot. HEK293FT cells were transfected either with control shRNA (ShC) or shRNA targeting Mena (Sh1) and lysates were obtained 48 hours after transfection. β-actin was used as a loading control. The result for this western blot can be seen in Figure 5.7.

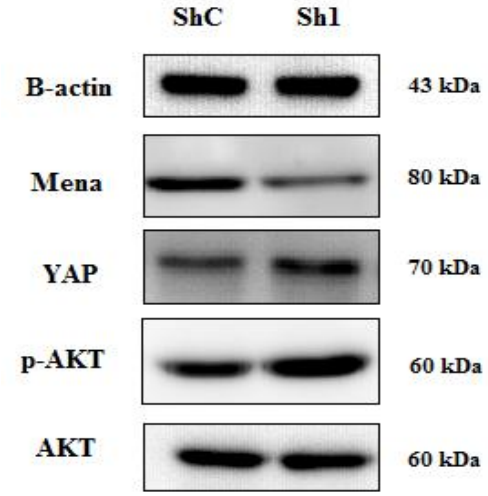

Figure 5.7. Mena knockdown induces mTOR pathway activity and AKT phosphorylation at Ser473.

This experiment showed that, Mena knockdown led to an increase in activity of the mTOR pathway, which also happens upon Yap upregulation in the cell. Total AKT levels did not change, which indicates that increase in S473 phosphorylated AKT levels are directly related to the upstream activity and independent of translation of AKT proteins. Therefore, it can be deduced that Mena knockdown mimicked Yap upregulation in terms of activation of the mTOR pathway.

Second, Yap is known to induce EMT when it is upregulated although the mechanism of this action is poorly understood. Therefore, by using the same approach we employed in testing mTOR pathway activity, we tested whether Mena knockdown causes any induction in EMT by checking for the levels of the epithelial marker E-cadherin and mesenchymal marker Vimentin. HEK293FT cells were transfected either with control shRNA (ShC) or shRNA targeting Mena (Sh1) and lysates were obtained 48 hours after transfection. β-actin was used as a loading control and the result is shown in Figure 5.8.

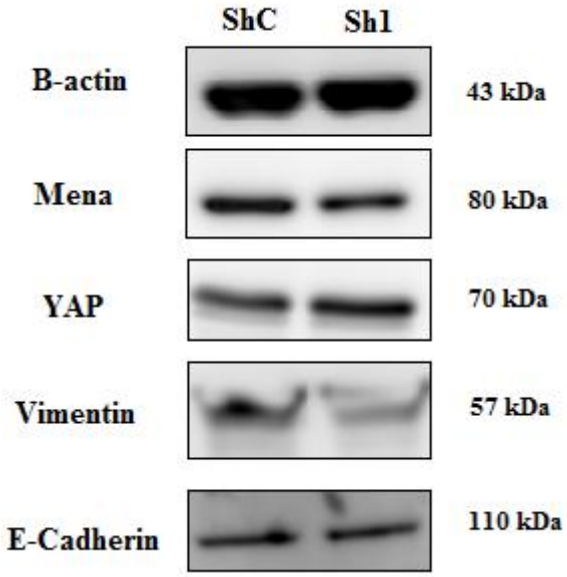

Figure 5.8. Mena knockdown did not induce EMT.

The data showed that protein levels of epithelial marker E-cadherin did not change by Mena knockdown whereas the protein levels of Vimentin decreased. This result suggested that Mena knockdown did not mimic the oncogenic role of Yap as an inducer of EMT. However, there is a possible explanation for this result. Since it is known that the primary function of Mena in the cell is the formation of unbranched F-actin, Mena itself is one of the important proteins for cancer cells to migrate and metastasize, which requires the induction of EMT. Therefore, it can be speculated that Mena is more important for the induction of EMT than Yap protein.

Third, when Yap acts like an oncogene and it is upregulated, it suppresses apoptosis. Therefore, we wanted to see whether Mena knockdown, which causes upregulation of Yap protein leads to suppression of apoptosis. For this purpose, HEK293FT cells were transfected either with control shRNA (ShC) or shRNA targeting Mena (Sh1) and lysates were obtained 48 hours after transfection. We checked the protein level of phosphorylated JNK protein by western blot analysis and β-actin was used as a loading control. JNK signaling is known to be activated and leads to induction of apoptosis (Dhanasekaran and Reddy, 2008). The result is shown in Figure 5.9.

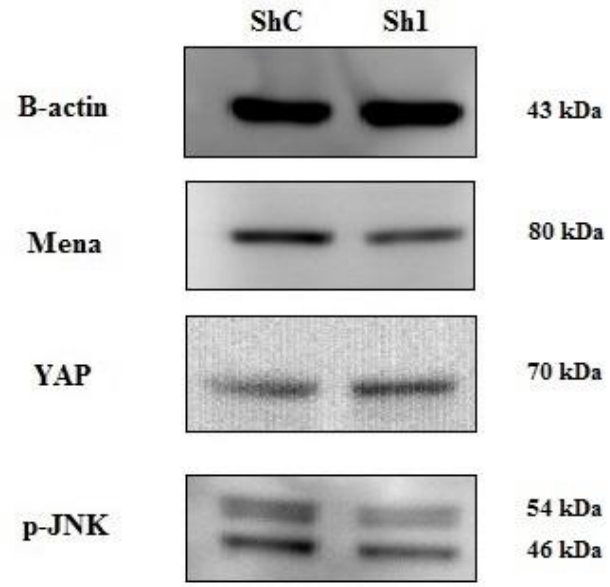

Figure 5.9. Mena knockdown causes a decrease in p-JNK level, a protein that is involved in induction of apoptosis.

Although this result indicated that Mena knockdown causes suppression of JNK-mediated apoptosis, there are other proteins that can induce apoptosis independent of JNK. Therefore, we also checked the levels of Bax mRNA using real-time PCR to see whether there is any suppression on Bax-mediated apoptosis as well. For this purpose, HEK293FT cells were transfected either with control shRNA vector (ShC) or shRNA targeting Mena (Sh1) and total RNA of transfected cells was obtained 48 hours after transfection. For the verification of knockdown, Mena primers were also used and the result indicated that expression of Mena mRNA was reduced by around 50% in the cells transfected with shRNA targeting Mena. Two independent experiments were performed with experimental duplicates. 18S was used as an internal control and the fold differences were normalized to 18S RNA expression. A two-tailed t-test was used to check the significance of the observed differences and the calculated p-values are as follows: For *MENA*, $p=1.13 \times 10^{-5}$ and for *BAX*, p=0.00013. The results are shown in Figure 5.10.

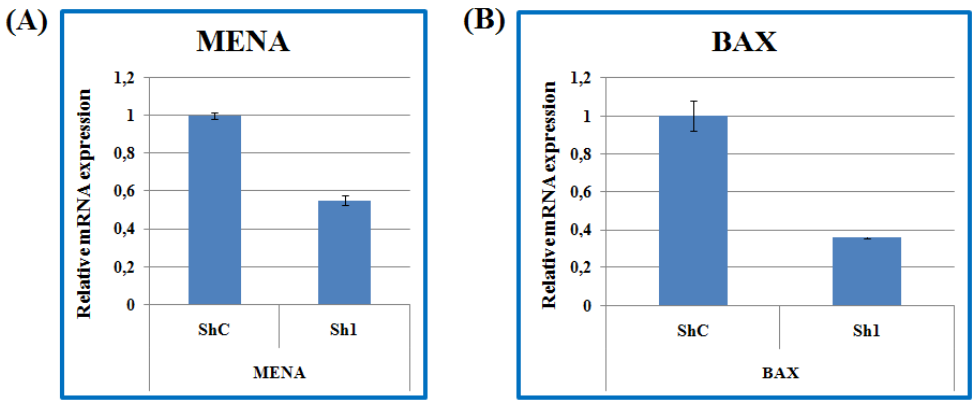

Figure 5.10. Mena knockdown causes reduction in the expression of Bax mRNA.

A 70% decrease in the mRNA levels of Bax was observed by Mena knockdown and indicated that Mena knockdown mimicked Yap upregulation, which results in suppression of apoptosis. The reason why Bax was selected as a marker for apoptosis is that it can provide a basis for future studies. Yap can also induce apoptosis by interacting with p73. Previous studies showed that p73-Yap complex is mainly recruited to the Bax promoter and induces Bax-mediated apoptosis.

5.1.4. Mena Knockdown Does Not Mimic Tumor-Suppressor Function of Yap

As it was stated earlier, Yap can operate as a tumor-suppressor in case of DNA damage. In this scenario, upstream kinases like c-abl phosphorylate Yap and this phosphorylation increases the stability of the Yap protein. Then, Yap can interact with p73 and this complex induces apoptosis by inducing the expression of Bax. In order to get an idea about signaling downstream of Yap when it is regulated by Mena, we wanted to test whether Mena knockdown can mimic the tumor-suppressor function of Yap as it did for the oncogenic roles of Yap. In order to create DNA damage, cells transfected with either control shRNA (Sh1) or shRNA targeting Mena (Sh1), were treated with 10μM cisplatin for 16 hours and total RNA was isolated 48 hours after transfection. mRNA levels of Bax were analyzed by using real-time PCR as well as Mena for the verification of effectiveness of knockdown. It appears that the expression of Mena was reduced by half in cells that were transfected with Mena shRNA. The fold differences were normalized to 18S levels, which

was used as internal control. A two-tailed t-test was used to check the significance of the observed differences and obtained p-values are as follows: for *MENA*, p=0.0075 and for *BAX*, p=0.00013. The result is shown in Figure 5.11.

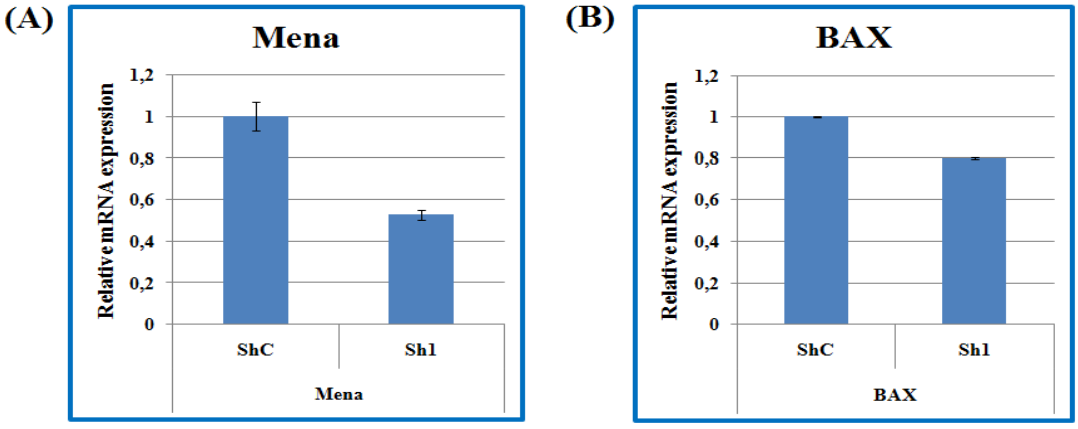

Figure 5.11. Mena knockdown does not mimic the tumor-suppressor function of Yap. mRNA levels for Bax decreases by Mena knockdown, which is known to be upregulated with upregulation of Yap.

The observed 20% decrease in Bax levels by Mena knockdown in case of DNA damage indicates that regulation of Yap by Mena has no relation to the tumor-suppressor function of Yap. Since Mena knockdown causes upregulation of Yap, upregulation of Bax would be expected if the regulatory function of Mena on Yap is related to the tumor-suppressive function of Yap.

Taken all experimental results together, we can say that Mena has no regulatory activity on the Hippo signaling pathway, but it has a regulatory function on the Hippo signaling pathway effector Yap. It seems that Mena suppresses the activity of Yap and the mode of action is independent of both the core kinases of the Hippo signaling pathway and the F-actin related function of Mena. Moreover, although it needs to be further verified, it seems that the regulation is on the oncogenic roles but not on the tumor-suppressive function of Yap.

5.2. Effect of Ena on the Hippo Signaling Pathway and Its Effector Molecule Yki in *D. melanogaster*

5.2.1. Ena Has No Effect on the Development of Wing Tissue

After the promising data we got from mammalian cells *in vitro*, we wanted to test our hypothesis *in vivo* by using *D. melanogaster*. By using the UAS-GAL4 system, overexpression of Ena, the homolog of Mena in flies, was achieved in wing tissue. We mainly used ptc-GAL4 and nub-GAL4 lines in which ptc-GAL4 drives the expression of gene of interest only in the cells located in anterior-posterior border in wing imaginal discs, while nub-GAL4 drives the expression of gene of interest in the cells located in wing pouch in wing imaginal discs. In order to verify the Ena overexpression, wing imaginal discs from 3rd instar larvae were stained with anti-his antibody since Ena contruct has His tag. The verification of Ena overexpression under patched-GAL4 in wing tissue can be seen in Figure 5.12.

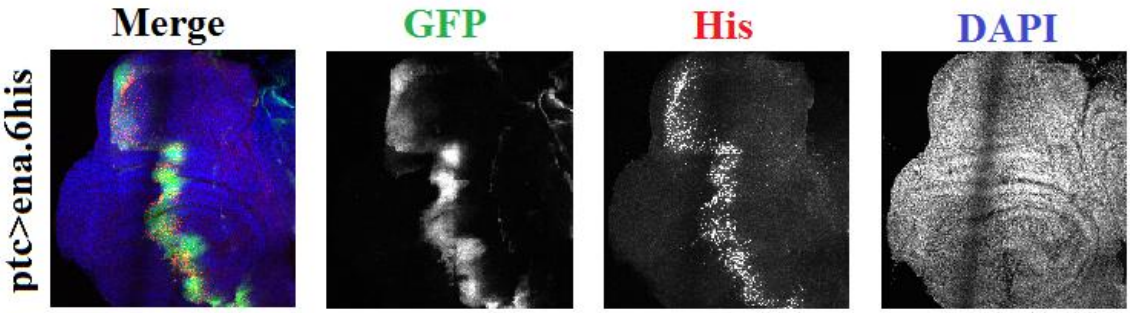

Figure 5.12. Verification of Ena overexpression in wing imaginal discs of third instar larvae by His staining.UAS line contains 6xHis tagged Ena. GFP (green) marks the cells containing GAL4 and His staining (Red) was present only in the cells with GAL4. DAPI (blue) was used for nuclear staining.

After the verification of Ena overexpression in the system, we wanted to see whether there is any effect of Ena overexpression on the development of wing tissue in flies. A line carrying UAS-Ena was crossed with nubbing-GAL4 and the wings of at least 20 adult females in the progeny were analyzed.

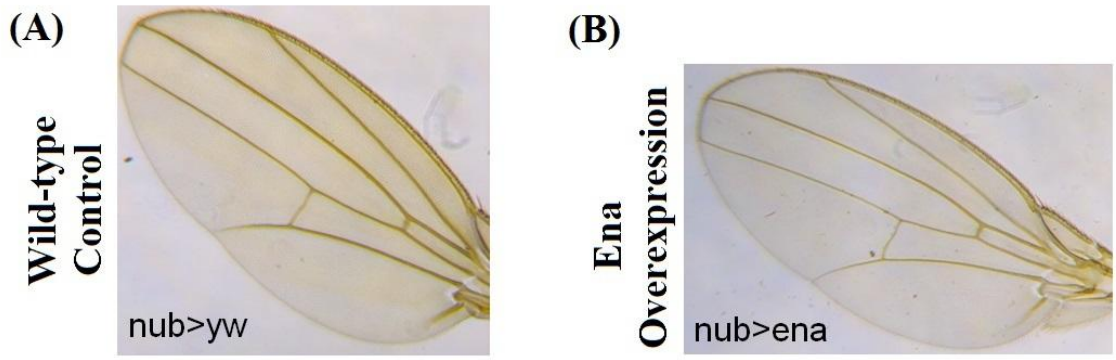

Figure 5.13. Ena overexpression did not change the size of the wings. (A) Wing from control Wild type fly (B) Wing of a fly with Ena overexpression. Both lines contain the nubbin-GAL4.

Although, individual variations were present between the sizes of the wings, it was observed that wing size of the flies carrying Ena overexpression was very similar to size of the wings from wild type flies. This result indicated that either Ena has no effect on the Hippo signaling pathway and on the activity of Yki or the effect of Ena is very small in the wing tissue. In order to differentiate these two possibilities, we crossed UAS-Ena flies with flies containing both nubbin-GAL4 and either knockdown or overexpression of one of the upstream Hippo signaling pathway components such as Hpo, Ex and Fat. Wings of at least 20 adult females were analyzed in each progeny and the result is shown in Figure 5.14.

5.2.2. Ena Has No Effect on the Activity of Yki in the Wing Tissue

After the observation that Ena has no effect on the development of the wing tissue in flies, we wanted to see whether there is any effect of Ena on the activity of Yki, the fly homolog of Yap. For this purpose, we analyzed the expression of *Expanded*, a known transcriptional target of Yki in flies by performing β-gal staining since ptc-GAL4 line also has ex-lacZ transgene in its genome.

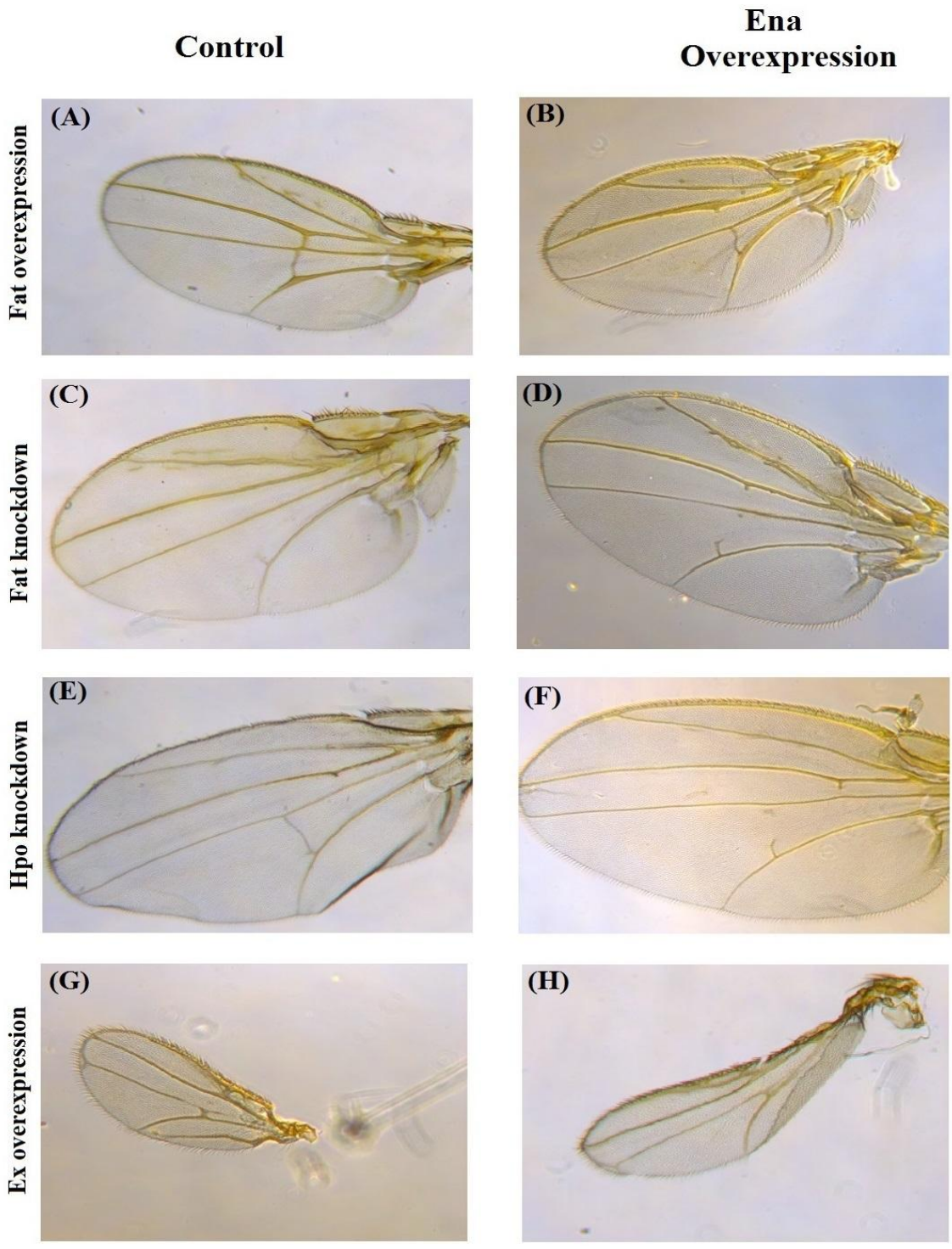

Figure 5.14. Wings from flies carrying differential expression of one of the upstream Hippo pathway components with or without Ena overexpression. (A) Fat overexpression (B) Fat and Ena overexpression (C) Fat knockdown only (D) Fat knockdown with Ena overexpression (E) Hpo knockdown only (F) Hpo knockdown with Ena overexpression (G) Expanded overexpression only and (H) Expanded and Ena overexpression.

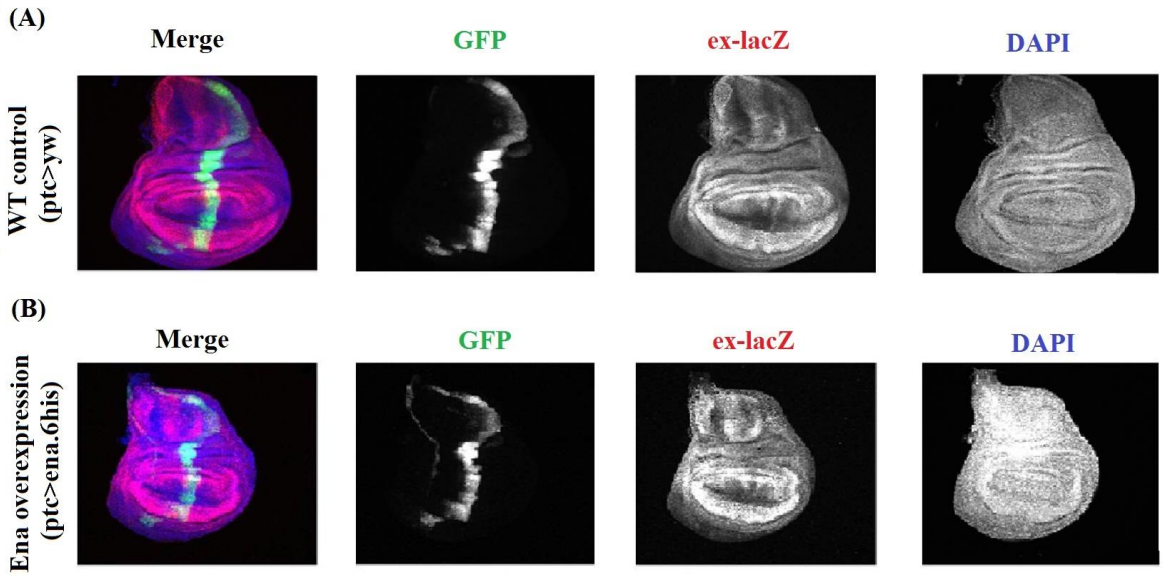

Figure 5.15. Ena had no effect on the expression levels of Expanded (red), a known Yki target gene. (A) Wing imaginal disc of wild-type third instar larvae. (B) Wing imaginal disc of third instar larvae overexpressing Ena. GFP (green) marked cells contain GAL4. DAPI (blue) is used for nuclear staining.

As can be seen from Figure 5.15, Ena overexpression did not cause any change in the expression of *Expanded* in the cells containing both GFP and GAL4. The Expression pattern of *Expanded* in the wing discs in the Ena overexpression background is very similar to that of wild type. If there were any change in the activity of Yki, cells marked with the GFP would not have the same *Expanded* levels with the neighboring cells and would be differentiated in β-gal staining since they have GAL4.

5.2.3. Ena Has No Effect on the Organization of Follicle Cells

After obtaining results from the wing tissue, we thought that the wing tissue could be unsuitable to work on the function of Ena even though it is widely used to study the activity of Yki and the Hippo signaling pathway. Therefore, we used another GAL4 line called as GR1-GAL4, which induces differential expression of the gene of interest only in follicle cells in the ovary. The results related to the effect of Ena and its possible effect on Yki in follicle cells are shown in Figure 5.16.

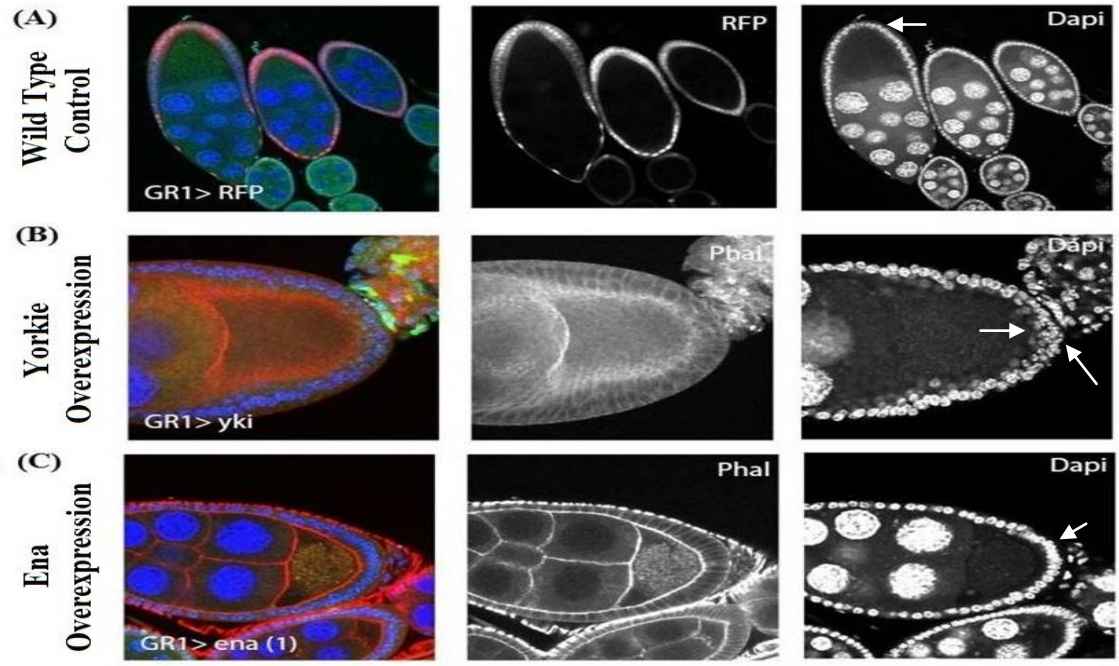

Figure 5.16. Ena had no effect on the organization of the follicle cells. (A) GR1-GAL4 RFP was used as a wild type control (B) GR1-GAL4 Yki as a positive control and (C) GR1-GAL4 Ena. DAPI (blue) was used for nuclear staining and Phalloidin (red) was used to stain F-actin.

In the ovary, the organization of the follicle cells is quite like a columnar epithelium as it can be seen in panel A of Figure 5.16. Increase in the activity of Yki by Yki overexpression led to the disruption of this organization and follicle cells were aligned as several lines. However, the phenotype of Ena overexpression was very similar to that of wild type and the organization of follicle cells was a single columnar layer.

In *D. melanogaster*, it is observed that Ena has no effect on both wing development and on the organization of follicle cells. Moreover, it is shown that Mena does not have any effect on the activity of Yki in wing tissue. The only observed positive result is related to enhancement of the effect of Expanded in wing tissue development. However, this result could be unrelated to the Hippo signaling regulation since Hpo knockdown together with Ena overexpression did not cause any phenotype and Hpo acts downstream of Expanded.

6. DISCUSSION

The main aim of this study was to elucidate the signaling properties of Mammalian Enabled (Mena), a protein known to induce F-actin polymerization. Previously, our group showed that Mena is a transcriptional target of the Wnt/B-catenin signaling pathway (Najafov *et al.*, 2012). Mena is known to be upregulated in several types of cancer such as breast, cervical and colorectal (Roussos *et al.*, 2010). How Mena promotes tumor formation is poorly understood. Mainly, it is characterized as an important protein for EGF-mediated metastasis (Philippar *et al.*, 2008); other signaling properties should be dissected to get better understanding for its ability to promote tumor formation.

Hippo signaling pathway is a recently identified signaling cascade that is crucial for tissue growth (Halder and Johnson, 2011). Several recent studies suggest that dysregulation of the Hippo pathway is present in many types of cancers. Therefore, we thought that there could be a link between Mena upregulation and dysregulation of the Hippo signaling pathway in tumor formation. Further analysis of the relationship between these two showed that there are several possible links between the Hippo signaling pathway and Mena in the literature as well.

In order to find out whether Mena has a regulatory function on the Hippo signaling, a luciferase reporter assay was performed to measure the activity of the promoter containing TEAD binding sites (Dupont *et al.*, 2011). Since TEAD transcription factors need to form a complex with Yap in the nucleus to bind their binding sites, this assay was designed to monitor the nuclear activity of Yap. When Mena was overexpressed in HEK293FT cells, the activity of the promoter was severely reduced. This result indicated that Mena causes reduction in the nuclear activity of Yap, which mimics the activation of the Hippo signaling pathway. This result is unexpected since it was thought that there could be a possible link between Mena upregulation and dysregulation of the Hippo signaling pathway.

In order to further verify the effect of Mena on the nuclear activity of Yap, expression levels of genes whose transcription is mainly dependent on Yap were analyzed by real-time PCR. *CTGF*, *BIRC2* and *BIRC5* were selected as Yap targets and it was found that their expressions increased by Mena knockdown which indicated that Mena downregulation leads to increase in nuclear activity of Yap. This result was consistent with the result obtained from the luciferase reporter assay.

When Yap is out of the nucleus, it is sequestered by 14-3-3 protein and this leads to the degradation of Yap. Therefore, if Mena has an effect on the nuclear activity of Yap, it is expected from Mena to affect the total Yap protein levels in the cell. In order to verify this, western blot analysis was performed by using both knockdown and overexpression vectors for Mena and it was observed that Mena is reversely affecting total Yap protein levels, which means that Mena knockdown led to an increase in total Yap protein level whereas Mena overexpression led to reduction in total Yap protein level. This result is also consistent with the previous results and indicates that Mena acts as an inhibitor for Yap protein.

Although, all of these results indicated that Mena has an effect on Yap protein, it is possible that Mena could affect the transcription of *YAP1* and *YAP2* rather than affecting the stability of the protein. By performing real-time PCR, it was observed that expression levels for Yap homologs *YAP1*, *YAP2* and *TAZ* were not affected by Mena knockdown. This result indicates that Mena affects Yap protein and causes decrease in the stability of Yap protein.

The obtained results indicated that Mena mimics the effect of the Hippo signaling pathway on Yap. However, whether the effect of Mena is dependent on the Hippo signaling pathway, in other words, whether Mena regulates the Hippo signaling pathway and the effect on Yap is a consequence of this regulation needs to be answered. In order to elucidate the effect of Mena on the Hippo signaling pathway, western blot analysis was performed and the levels of the core kinases of the Hippo signaling pathway and their activated versions were analyzed. It was observed that Mena knockdown had no effect on the

activity of the core kinases, which indicated that the effect of Mena on Yap is directly related to Yap but not related to the Hippo signaling pathway.

The main function of Mena is to induce unbranched F-actin formation. Recent studies suggested that F-actin accumulation in the cell affects the activity of Yki in flies and Yap in mammals (Yi *et al.*, 2013). Depending on the modulator of F-actin, this effect could be either dependent or independent of the Hippo signaling pathway. In order to test whether the effect of Mena depends on its function related to F-actin, western blot analysis using both wild type and mutant versions of Mena was performed. The mutant version was named as S376A which indicates the aminoacid substitution from Serine to Alanine at the 376th position. The serine residue at this position is the site of phosphorylation for several kinases and the phosphorylation impairs the F-actin production ability of Mena (Benz *et al.*, 2009). Therefore, this mutant acts as a constitutively active version of Mena in terms of F-actin production. It was observed that S376A also led to a decrease in the levels of total Yap protein just similar to that of wild type. It can be deduced that the effect of Mena on Yap is also independent of its F-actin related function.

In addition to its role in tissue growth, Yap can act as an inducer of apoptosis. Therefore, it can be said that Yap has a dual role in the cell. This dual role is regulated by several upstream kinases and according to the signals from those kinases; Yap decides which way it will take. Although, Mena seems to affect the stability of Yap, the downstream of this effect needs to be elucidated. While, the obtained results need to be further verified by using Yap siRNA and shown that the effect is purely dependent on Yap upregulation, we differentiated that Mena affects the oncogenic function of Yap, but it has no significant effect on the tumor suppressive function. It is known that when Yap acts as an oncogene, it induces proliferation and EMT; and it suppresses apoptosis.

It is known that Yap activates mTOR pathway to induce proliferation (Overholtzer *et al.*, 2006). Since Mena knockdown causes Yap upregulation, the presence of any change in the activity of mTOR pathway upon Mena knockdown was analyzed. For doing this, western blot analysis was performed and the levels of AKT protein which is phosphory-

lated by mTORC2 at Serine residue located at the 473th position were measured. It was observed that Mena knockdown led to an increase in the levels of phosphorylated AKT protein and therefore just mimicked the oncogenic role of upregulated Yap. Moreover, total AKT protein level was analyzed as well and it was found to be similar to that of the control and this result indicates that Mena knockdown specifically induces the phosphorylation event.

Yap is known as an inducer of EMT in the cell. The levels of E-cadherin, an epithelial marker, and Vimentin, a mesenchymal marker, were analyzed by western blot analysis to see whether Mena knockdown can mimic the EMT related function of Yap. It was observed that Mena knockdown had no effect on the levels of the epithelial marker E-cadherin and it reduced the levels of the mesenchymal marker Vimentin. These results indicated that Mena knockdown did not induce EMT and therefore it could not mimic the EMT related function of Yap. However, there is a possible explanation for this. Since Mena is an actin modifier in the cell, it is very important for the actin-based processes such as migration. Therefore, it is possible for Mena to be more important than Yap for the induction of EMT and in this scenario, Mena knockdown causes suppression of EMT.

Proteins which can act as an oncogene mainly suppress the apoptosis in the cell. Therefore, one of the oncogenic roles of Yap is to suppress apoptosis. In order to analyze whether Mena knockdown mimics this function of Yap, first western blot analysis was performed to check the levels of phosphorylated JNK protein. JNK phosphorylation indicates the activation of JNK and when it is active, it induces mainly apoptosis. It is shown that Mena knockdown decreased the p-JNK levels in the cell and this is an indicator of suppression of apoptosis in the cell. Therefore, this result suggests that Mena knockdown mimicked the apoptosis related function of Yap when it acts as an oncogene. As well as p-JNK, Bax mRNA levels were analyzed as well since Bax is one of the main responsible proteins to induce apoptosis in the cell. It is observed that Mena knockdown reduced the Bax mRNA levels and therefore again mimicked the apoptosis related function of oncogenic Yap.

In order to have a better understanding for the downstream of the effect of Mena on Yap, tumor suppressive function of Yap needs to be analyzed as well. It is known that Yap interacts with p73 and forms a complex upon DNA damage. When this complex is formed, it induces the expression of Bax and leads to apoptosis. For this purpose, cisplatin was used as an inducer of DNA damage and Bax mRNA levels were analyzed by Mena knockdown. Since Mena knockdown causes upregulation of Yap, induction of Bax mRNA levels would be expected if the effect of Mena is on the tumor suppressive function of Yap. When cells transfected with knockdown vectors and then treated with cisplatin, it was observed that Mena knockdown caused reduction in the Bax mRNA levels compared control. This result indicated that effect of Mena on Yap is not related to the tumor suppressive function of Yap and knockdown of Mena could not mimic the upregulation of Yap in this manner.

All in all, it can be concluded that Mena suppresses the activity of the Hippo signaling pathway effector, Yap and this effect is on the protein level. Moreover, it seems that observed effect is independent from the core kinases of the Hippo signaling pathway and from the F-actin related function of Mena. Furthermore, Mena knockdown mimics the oncogenic functions of Yap such as induction of proliferation and suppression of apoptosis but it could not mimic the tumor suppressive function of Yap which is normally induced upon DNA damage. Schematic representation of the conclusions is shown in Figure 6.1.

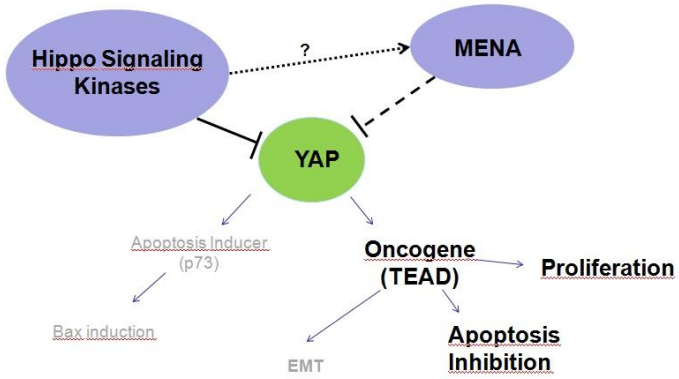

Figure 6.1. Schematic representation for a possible regulatory action of Mena on Yap.

After the results related to the effect of Mena on Yap in HEK293FT cells, *D. melanogaster* was used as *in vivo* model to test the same hypothesis. Since most of the elements of the Hippo signaling pathway were found by using flies particularly by using the wing tissue of the flies. For this purpose, UAS-GAL4 system was used to induce overexpression of Ena, the fly version of Mena and the effect of Ena was analyzed firstly in wing tissue. It is observed that Ena overexpression did not affect the development of the wing and the sizes of the wings in flies with Ena overexpression are almost the same as those of wild type flies.

Weak phenotypes could be masked in the development of wings in flies. Therefore, obtained result could be an indicator of a weak effect. Therefore, the effect of Ena on wing tissue development needs to be further analyzed to be sure whether it has any effect at all. In order to do that, flies which carry either overexpression or knockdown transgenes specific for a particular upstream component of the Hippo signaling pathway were used. Since these flies are sensitized for the Hippo signaling pathway, any effect even the very weak ones can be observed. When these sensitized flies were crossed with flies carrying Ena overexpression construct, progeny with Ena overexpression and sensitized background was obtained. When wing tissues were analyzed, it was observed that Ena had no effect in the development of wing tissue apart from the enhancement of the effect of Expanded. However, since there was no enhancement on the wing tissue development in flies carrying Hippo kinase knockdown, this synergistic effect on Expanded is quite inconclusive.

Obtained results suggest that Ena has no effect on the development of the wing tissue in flies. Since *in vitro* results suggested that Mena has effect on only Yap but not on the Hippo signaling pathway, the activity of Yki, fly homolog of Yap, was monitored with the aid of ex-lacZ transgene which enables us to measure the expression levels of *Expanded*, a transcriptional target of Yki. It is observed that the expression levels of *Expanded* is quite similar to wild type and the cells carrying GAL4, so those have the overexpression of Ena, have the same Yki activity with the cells do not carry GAL4 in the same tissue.

Since Ena had no effect in the wing tissue, it was thought that wing tissue could not be a proper tissue to study the signaling properties of Ena although it is mainly used for studying the Hippo signaling pathway. Therefore, we used follicle cells in the ovary to find out whether Ena has any regulatory action on Yki in flies by using GR1-GAL4. This GAL4 line induces the overexpression of gene of interest only in the follicle cells. When adult female flies were dissected and the ovary structures were analyzed, it was observed that the organization of the follicle cells is a single layer of columnar epithelium in flies with Ena overexpression and this organization was very similar to the phenotype of the wild type flies. Yki overexpression was also used as positive control and the organization of follicle cells was disrupted in these flies.

It can be said that the overexpression of Ena may not lead to the induction of Yap activity and therefore it is not proper to use to see the effect on Yki. Several fly lines with Ena RNAi were also used for the same experiment and the results were exactly the same. Those results were not added into this thesis since Ena knockdown was not verified in those lines due to the absence of Ena antibody.

All the results obtained from flies indicate two possible explanations. First the effect found in this study is probably mammalian specific and the action of Ena on Yki got lost during the evolutionary process. Second possibility is about the tissues which were benefited during the study on flies. It is possible that both wing tissue and the follicle cells in the ovary are not appropriate tissues to study the effect of Ena although those are widely used in studying the Hippo signaling pathway.

In conclusion, this study showed that Mena, a protein known to be upregulated in cancer, has a unique role in the cell to suppress the oncogenic activity of Yap protein. This is quite interesting in a sense that a protein which is known to promote cancer can also inhibit an oncoprotein. Therefore, this study could be a base for therapies that can selectively target the isoforms of Mena but not Mena itself.

Although the effect of Mena on the oncogenic role of Yap is elucidated in this study, questions related to the mechanism of this action and the upstream factors that are involved in this regulation are still unclear. Therefore, future studies will aim to find out the mechanism of the effect of Mena on the stability of Yap and the upstream factors that are involved in this regulation.

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