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REGULATION OF HIPPO SIGNALING FOR GROWTH CONTROL

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by

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ABSTRACT

In multicellular organisms, the coordination of cell proliferation, cell death and cellular growth are crucial for the organ size control and the maintenance of organ function. The mechanisms that regulate these crucial processes provide insight into diseases, such as cancer. The Hippo (Hpo) signaling regulates cell number mainly by inhibiting cell proliferation and promoting cell apoptosis, and this signaling is highly conserved from *Drosophila* to mammals. Hpo is the key kinase of Hpo signaling; however, the way in which Hpo kinase activity is regulated remains less understood. In this project, I investigated how Hpo kinase is activated and regulated by upstream molecules both *in vivo* and *in vitro*. I found that Hpo dimerization could facilitate its activation by auto-phosphorylation. Moreover, membrane association appears to increase Hpo dimerization efficiency, and upstream molecules Expanded/Merlin/Kibra promote Hpo membrane association. Therefore, both dimerization and membrane association are critical for Hpo kinase to be activated. This mechanism provides essential insight to reveal the mystery that how upstream molecules transduce signal to Hpo signaling.

In another project, I investigated Yap1 (a major downstream effector of mammalian Hpo signaling) activity regulation in mammalian pancreatic beta β-cells under free fatty acids (FFAs) treatment. Mammalian pancreatic β-cells are responsible for the production of insulin and therefore play a pivotal role in development and glucose homeostasis. Among many factors, high concentrations of saturated free fatty acids (FFAs) such as palmitate are known to have a negative effect on β-cell viability, which might induce type 2 diabetes. In this study, I demonstrated that Hpo signaling effector Yap1 plays a crucial role in regulating β-cell survival under FFA treatment. I found that Yap1 is activated through F-actin accumulation in a time-delayed manner to enhance β-cells viability during palmitate-induced apoptosis. Moreover, Connective Tissue Growth Factor (CTGF), one of the downstream targets of Yap1, was identified to repress palmitate-induced β-cell apoptosis. These discoveries support a model in which Yap1 could

positively regulate β-cell survival under FFA treatment, and this model might lead to the

development of new strategies for potential treatment of diabetes.

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Chapter 1

Introduction

1.1 Organ development and organ size control in multicellular organisms

In multicellular organisms, organ development is regulated by both cell patterning and organ size control. Cell patterning is achieved by cell differentiation, by which a less specialized stem cell differentiates to a specialized cell type. Cell differentiation is regulated by a number of signaling pathways, including Notch, Hedgehog (Hh), Wnt, BMP/TGF-beta, JAK/STAT, and EGF (Neto-Silva et al., 2009; Barolo and Posakony, 2002), while organ size is affected by cell growth and cell number regulation.

Cell growth affects the size and mass of individual cells, and is influenced by mRNA translation, autophagy, ribosome synthesis and endocytosis, which are regulated by signaling pathways, including Insulin/Tor, Akt/PKB, Myc, and E2F pathways (Edgar, 2006; Jorgensen and Tyers, 2004).

To regulate organ size, cell number control is also crucial. Cell number is regulated through the coordination of cell proliferation and cell death (Guo and Hay, 1999). Proliferation results in a cell number increase while cell death results in a cell number decrease. These two processes must be tightly balanced to produce a proper organ size and to maintain tissue homeostasis, while the deregulation of either process can lead to diseases such as cancer (Vogelstein and Kinzler, 2004). There are multiple signaling pathways have been identified to regulate organ size, among which the Hippo (Hpo) signaling pathway coordinates proliferation and cell death by inhibiting proliferation and promoting cell death (Pan, 2007). Our lab is interested in understanding the mechanisms of Hpo signaling and their regulation in order to provide new insights into organ development and related diseases.

1.2 Tumorigenesis

Tumorigenesis is the process during which normal cells are transformed into tumor cells and exhibit unregulated cell division and cell growth. The primary cause of cancer formation is DNA mutation (reviewed in Bernstein, et al., 2013). While a single gene mutation might not be sufficient to change cell behavior, the accumulation of a series of gene mutations might eventually cause cancer, and each individual cancer might have its own genetic features (Bissell and Radisky, 2001). The genes involved in cancer formation are usually those that regulate cell number and cell growth, and they include oncogenes and tumor suppressor genes (Lodish et al., 2000).

Normal versions of oncogenes usually promote cell growth and proliferation. In tumor cells, gain-of-function oncogenic mutations upregulate cell proliferation, and some activated oncogenes may prevent cells from undergoing programmed apoptosis, leading to uncontrolled cell proliferation and cell growth (Wilbur et al., 2009).

In contrast to oncogenes, tumor suppressor genes normally negatively regulate cell growth and cell number. These genes facilitate apoptosis and DNA damage repair and inhibit cell proliferation and cell growth in normal tissues (Weinberg, 2014). Loss-of-function of tumor suppressor genes causes cell overgrowth and uncontrolled division and eventually leads to tumor formation (reviewed in Sherr, 2004).

Mutations of tumor suppressor genes can be classified as neoplastic and hyperplastic mutations (Bryant et al., 1993). Neoplastic mutations cause tissue structure disruption by influencing cell differentiation in addition to causing tissue overgrowth (Humbert et al., 2008). On the other hand, hyperplastic mutations only cause tissue overgrowth without disrupting tissue architecture. Mutations of the components of Hpo signaling are hyperplastic mutations (Reddy and Irvine, 2008). Studies of the Hpo pathway both in *Drosophila* and mammals have provided

new mechanisms for cancer research. For instance, loss-of-function mutations in *Large Tumor Suppressor (LATS)* genes were found in human cancer patient samples and were studied to elucidate their role in cancer formation (Yu, et al., 2013).

1.3 The *Drosophila* Hippo signaling pathway

The Hippo (Hpo) signaling pathway was first identified in *Drosophila* to control organ size mainly by restricting cell proliferation and promoting apoptosis (Tapon et al., 2002). This pathway is highly conserved from *Drosophila* to mammals (Dong et al., 2007; Harvey et al., 2003; Zhao et al., 2007). Deregulation of Hpo signaling has been implicated in the formation of several types of human cancers (Camargo et al., 2007; Harvey and Tapon, 2007), suggesting that understanding the Hpo pathway is crucial for addressing questions of tissue growth control and cancer formation.

Multiple proteins have been identified in this pathway. They are classified as upstream regulators, core components, and downstream effectors of the Hpo pathway (Figure 1-1, Grusche et al., 2010). The core components of the Hpo pathway include Ser/Thr kinase Warts (Wts) (Justice et al. 1995), Hpo (Harvey et al. 2003, Jia et al. 2003, Udan et al. 2003, Wu et al. 2003), Salvador (Sav) (Tapon et al. 2002) and Mob as tumor suppressor (Mats) (Lai et al. 2005). On the one hand, when the Hpo pathway is turned "on," Hpo kinase is phosphorylated and thus activated by its upstream regulators, as shown in Figure 1-1 (reviewed in Hariharan and Bilder, 2006; Harvey and Tapon, 2007). Activated Hpo then phosphorylates its target proteins Mats and Sav. The scaffold proteins Sav and Mats facilitate formation of the core component complex, in which Wts kinase is activated by phosphorylation. The core components of the Hpo pathway repress tissue growth mainly by phosphorylating and thereby inhibiting their downstream effector Yorkie (Yki), a transcription co-activator. On the other hand, when Hpo signaling is turned "off," Hpo

kinase is inactivated and the core components complex is disassociated by Mats and Sav

dephosphorylation. Yki is thus activated and released from the cytosol by dephosphorylation and

translocated into the nucleus to facilitate transcription. Yki, together with its DNA binding

partner Scalloped (Sd), promotes tissue growth by modulating the transcription of the genes

related to cell proliferation, apoptosis, and other signal molecules (Zhao et al., 2008).
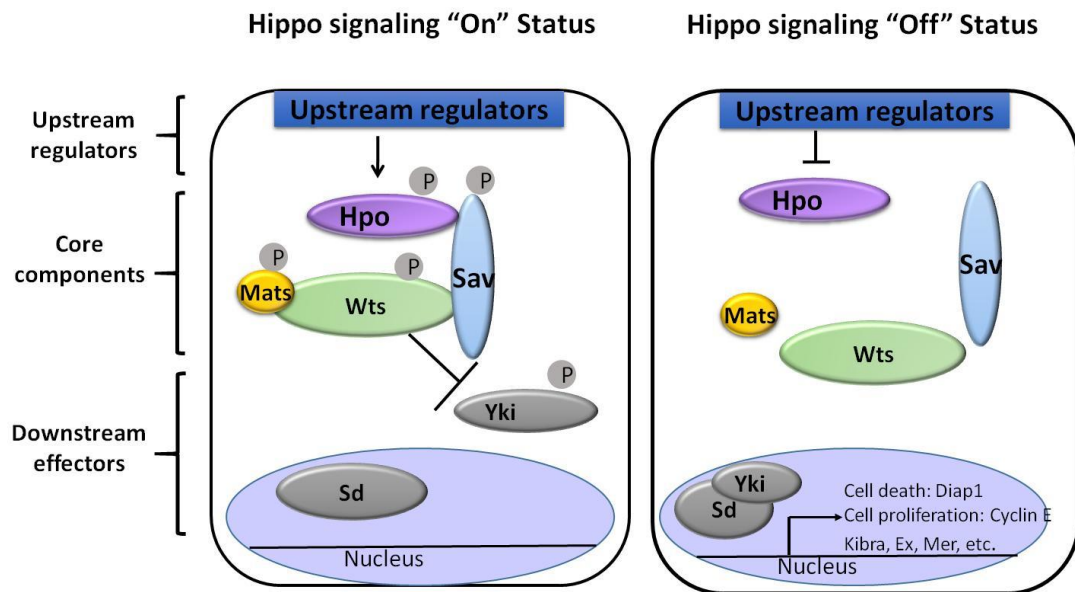
Figure 1-1 Overview of the *Drosophila* Hpo signaling pathway mechanism. When Hpo signaling is turned "on," Hpo kinase is activated by its upstream regulators. Activated Hpo then phosphorylates its target proteins Mats and Sav. The scaffold proteins Sav and Mats facilitate formation of the core component complex, in which Wts kinase is activated by phosphorylation. The core components of the Hpo pathway repress tissue growth mainly by phosphorylating and thereby inhibiting their downstream effector Yki, a transcription co-activator. While when Hpo signaling is turned "off", the core components complex is disassociated. Yki is thus activated and released from the cytosol by dephosphorylation and translocated into the nucleus to facilitate transcription. Yki, together with its DNA binding partner Sd, promotes tissue growth by modulating the transcription of the genes related to cell proliferation, apoptosis, and other signal molecules.

1.4 Regulation of the Hpo signaling pathway in *Drosophila*

Growing numbers of proteins have been identified as upstream components of the Hpo

pathway, which can be mainly grouped into four clusters (Grusche et al., 2010). As shown in

Figure 1-2 (marked by pink frames), the four upstream clusters include 1) Fat (Ft) and Dachsous

(Ds), which function through atypical cadherin transmembrane receptors (Silva et al., 2006); 2),

the Kibra/Expanded/Merlin complex (Baumgartner et al., 2010; Genevet et al., 2010), which

provides a direct link from the apical membrane to the core components of the Hpo pathway; 3)

several apical-basal polarity proteins, including Lgl, aPKC, Crumbs etc. (Grzeschik et al., 2010);

and 4) kinase Tao-1 which activate Hpo directly by phosphorylation (Boggiano et al., 2011; Poon

et al., 2011).

Although Hpo pathway core components are constant, the upstream regulators vary

depending on the environment. In fact, Hpo signaling has been found to play different roles under

different environments and in different tissues due to its regulation complexity (Grusche et al.,

2010; Mo, et al., 2014; Park and Guan, 2013). The core components of Hpo signaling are

regulated by the upstream clusters through different mechanisms. Thus, the regulation mechanism

of the core components of Hpo signaling by its upstream regulators is the key for understanding

how the Hpo pathway is involved in tissue growth and organ size control during development.

Among these upstream regulator clusters, Kibra/Ex/Mer complex is identified as a

primary platform for signal integration among upstream molecules genetically (reviewed in

Grusche et al., 2010). Ex and Mer proteins are located at the sub-apical region in *Drosophila*

epithelia cells and are believed to transmit extracellular signals to the Hpo pathway (reviewed in

Boggiano and Fehon, 2012). Kibra, which interacts with both Mer and Ex, serves as a link

between Mer and Ex (Grusche et al., 2010; Genevet et al., 2010; Yu et al., 2010). Interestingly, an

individual *ex, mer* or *kibra* mutation exhibits weak loss of Hpo signaling phenotypes, while a

double mutant among *mer, ex* and *kibra* induces strong mutant phenotypes, suggesting that these

three molecules regulate the Hpo pathway in a partially redundant manner (Baumgartner et al.,

2010; Genevet et al., 2010; Pellock et al., 2007; Grusche et al., 2010). However, the mechanism

by which these scaffold proteins transduce signals to Hpo signaling core components and activate

Hpo kinase was not clearly understood. Therefore, this study examines the role of the

Kibra/Ex/Mer complex in regulating Hpo kinase activity.
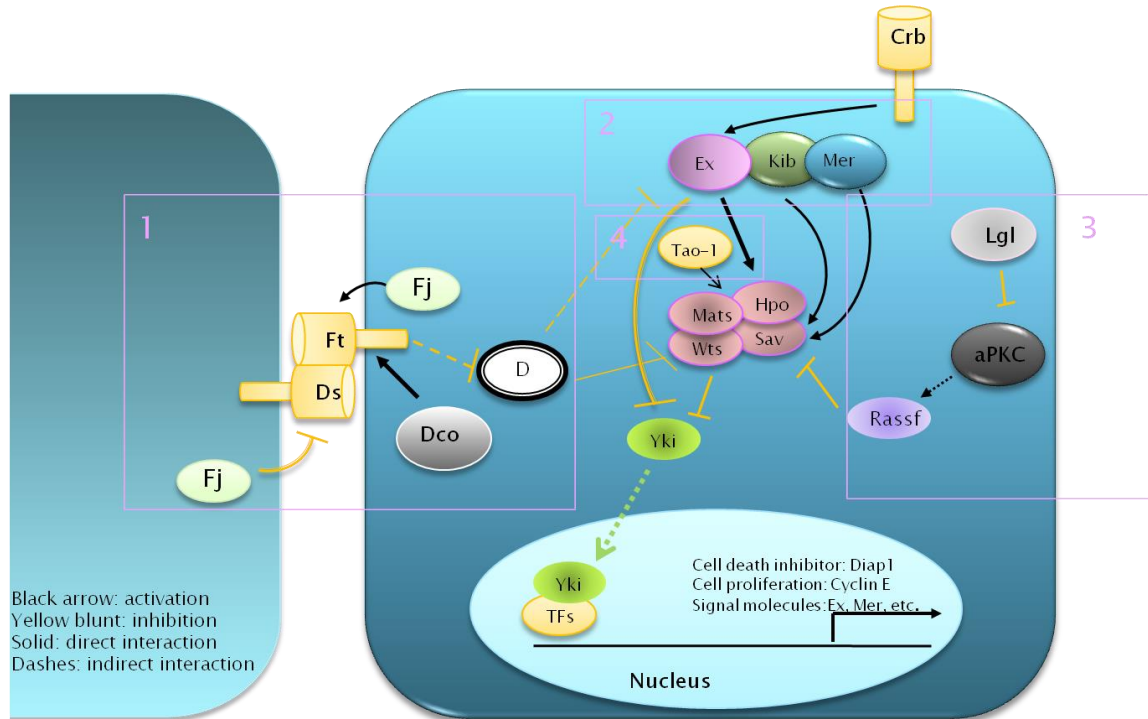
Figure 1-2 Overview of *Drosophila* Hpo signaling upstream regulation molecules. Pink frames mark four upstream regulator clusters of Hpo signaling. Black arrows indicate activation, while yellow blunt lines indicate inhibition. Solid lines show direct interaction, while dashes indicate indirect interaction. Green arrow represents translocation.

1.5 *Drosophila* Hpo kinase structure

Hpo is the key kinase among the core components of the Hpo pathway. Thus,

understanding the regulation mechanism of Hpo kinase is crucial for studying Hpo pathway

regulation. Hpo kinase belongs to the Ste-20 Ser/Tyr protein kinase family and is composed of an

N-terminal catalytic domain (kinase domain) and a C-terminal SARAH domain that mediates

interaction with itself or other SARAH-domain-containing proteins (Sav/Rassf/Hpo) (Figure 1-3,

Tapon et al., 2002). The N-terminal kinase domain of Hpo is highly conserved from *Drosophila*

to mammals, and K71 is recognized as the catalytic site (Wu et al., 2003). Hpo kinase is activated

by phosphorylation (Wu et al., 2003). There are two identified phosphorylation sites for Hpo kinase: T189 and T195; however, only T195 phosphorylation is proven to be critical for Hpo kinase activity (Jin et al., 2012).

Hpo kinase can be phosphorylated by other kinases, such as Tao-1 (Boggiano et al., 2011; Poon et al., 2011), or by auto-phosphorylation like its mammalian homolog Mst1/2 (Jin et al., 2012). Hpo autophosphorylation is achieved by forming a homo-dimer. Two sites have been shown to be critical for self-dimerization: M242 in the kinase domain and I634 in the SARAH domain. However, the SARAH domain association appears not to be critical for auto-phosphorylation, suggesting that it might be crucial for the SARAH domain to associate with other proteins, such as Sav and Rassf (Jin et al., 2012).

Hpo kinase activity is also regulated by its subcellular localization. Hpo is inactivated when maintained in the nucleus (Jin et al., 2012). Two nuclear export sequences (NES) have been discovered that appear to regulate Hpo nucleocytoplasmic transportation via a CRM1 dependent nuclear export mechanism (Jin et al., 2012). In our study, we investigated the Hpo auto-phosphorylation mechanism and Hpo homo-dimer regulation by upstream molecules.

Hpo kinase structure

Catalytic site

Dimerization site

Phosphorylation site

K71 T189 T195 M242 I634

N- Kinase Domain NES1 NES2 SARAH -C
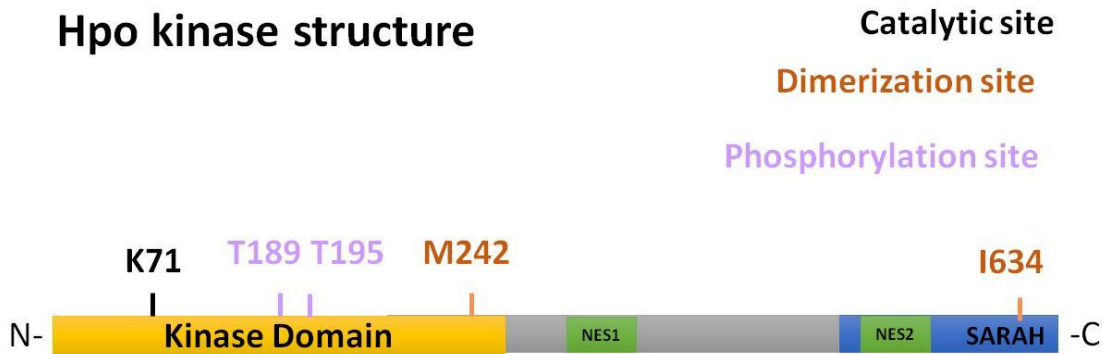
Figure 1-3 *Drosophila* **Hpo kinase structure. Hpo kinase is a protein with 669 Amino Acids. Yellow and blue bars illustrate kinase domain and SARAH domain. Green boxes indicate nucleus export sequences (NES).**

1.6 The Hpo signaling pathway in mammalian systems

Hpo signaling is evolutionarily conserved from fly to mammals (Pan et al., 2007). The mammalian Hpo signaling pathway plays a crucial role in restricting tissue growth during embryo development (Zhao et al., 2007). Loss-of-function mutations in mammalian Hpo signaling components lead to overgrowth and contribute to tumorigenesis (Camargo et al., 2007; Dong et al., 2007; Zhou et al., 2009). Similar to the *Drosophila* Hpo pathway, Mst1/2 (homologs of Hpo) is activated by auto-phosphorylation by forming a homo-dimer (Figure 1-4, Lee et al., 2002). As shown in Figure 1-4, activated Mst1/2 phosphorylate scaffold proteins Sav1 (homolog of Sav) and Mob1 (homolog of Mats), which facilitates the interaction between Mob1 and Lats1/2. Lats is thus phosphorylated and activated. When activated, Lats1/2 can phosphorylate and inactivate Yap/Taz (homologs of Yki), keeping Yap/Taz in the cytosol. On the other hand, when Mst is inactivated, dephosphorylated Yap/Taz are translocated from the cytosol to the nucleus and associate with the transcription activator TEAD (homolog of Sd) or other transcription factors to facilitate transcription (Zhao et al., 2010; Pan et al., 2007).

The regulation of the mammalian Hpo pathway is less understood than in the *Drosophila* system. Although similar upstream regulatory molecules have been discovered, such as NF2 (homolog of Merlin) and Ex (homolog of *Drosophila* Ex) (McClatchey and Giovannini, 2005; Pan, 2007), the mammalian regulatory mechanism is more complicated than in the *Drosophila* system. Mammalian Ex has many isoforms, each of which has a specified function (Hamaratoglu et al., 2006). NF2 has been shown to regulate the mammalian Hpo pathway by inhibiting Yap activity; however the mechanism has yet to be identified (McClatchey and Giovannini, 2005). In addition, Yap may associate with P73, leading to apoptosis under disease conditions (Zhang et al., 2011; Downward and Basu, 2008; Lapi et al., 2008). Recently, the mammalian Hpo pathway has been shown to be regulated by the G-protein coupled receptor (GPCR) pathway, opening new insights into mammalian Hpo pathway regulation, which attracted our great attention (Yu et al., 2013).
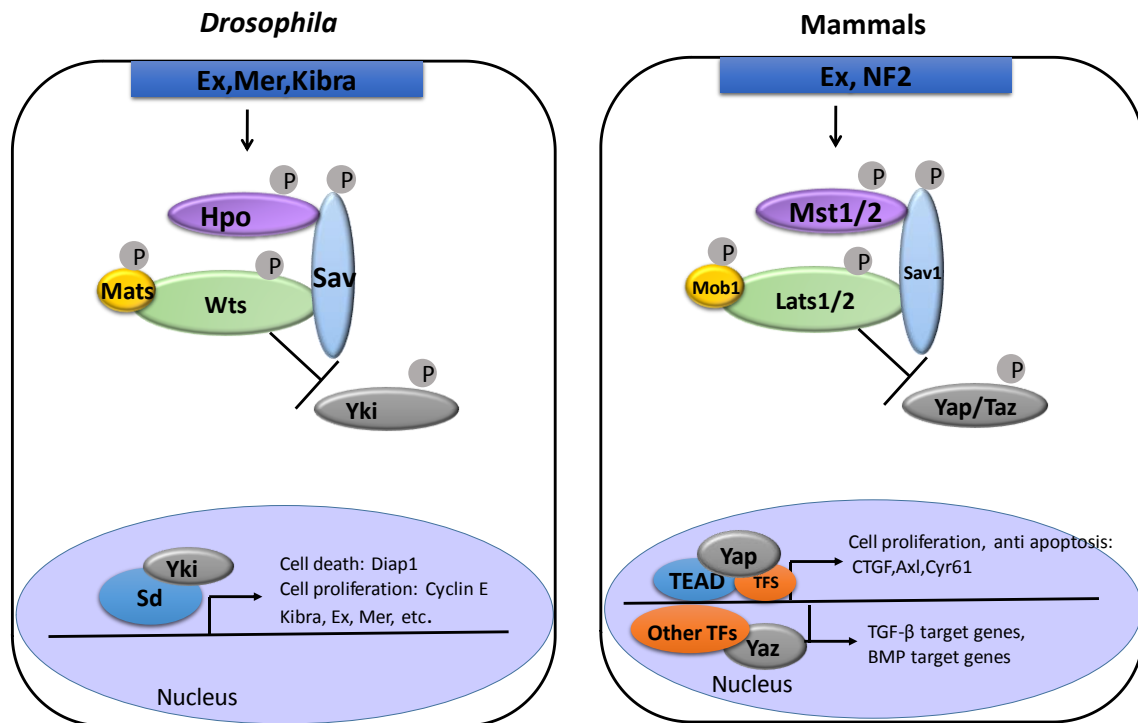
Figure 1-4 Comparison of the Hpo pathway in *Drosophila* and mammals. Components in the same color represent homologs.

1.7 The regulation of mammalian Hpo signaling by the GPCR pathway

Dr. Guan's group recently discovered that Hpo signaling might be regulated by the GPCR pathway in the mammalian system (Yu et al., 2012). In their study, lipid ligands, Serum-borne lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) act through $G\alpha_{12/13}$ coupled receptors to inhibit Lats1/2 activity and thus activate Yap/Taz, bypassing Mst1/2 to facilitate cell migration and proliferation. They also proposed that Lats1/2 kinase might be inhibited by $G\alpha_{12/13}$, $G\alpha_{q/11}$, and $G\alpha_{i/o}$-coupled receptors, while they might be activated by Gs-coupled receptors. In later studies, Rho GTPases and actin cytoskeleton were shown to regulate the mammalian Hpo pathway and were put between $G\alpha$ and Lats1/2 (Zhao et al., 2012; Yu et al., 2013). However, it remains unknown by which mechanism Rho and cytoskeleton actin regulate Lats1/2 activity.

GPCRs are coupled with different G proteins and represent a large family of cell surface receptors (Dorsam et al., 2007). The connection of Hpo signaling to the GPCR pathway offers a new perspective for understanding Hpo signaling regulation by extracellular signals. Many ligands may act through GPCR and regulate Yap/Taz activity, thus influencing tissue growth or related diseases. Due to the complexity of the GPCRs family, they may have different roles in Hpo signaling regulation. It is possible that one ligand may activate Yap in one condition but inactivate Yap in another condition, depending on which receptor and which G protein is involved in specific cases (Figure 1-5, Yu et al., 2013). Thus, multiple possibilities for Hpo pathway regulation by GPCRs need to be considered, depending on the environmental conditions (Yu et al., 2013; Codella and Irvine, 2012). Interestingly, GPCR mutations and G protein mutations have been found in many human cancer samples (Kan et al., 2010; Prickett et al., 2011). Thus, elucidation of this mechanism might demonstrate that Yap/Taz misregulation contributes to cancer formation in these cancer cases (Yu et al., 2013).

1.8 Regulation of Hpo signaling by F-actin cytoskeleton in mammalian systems

Mammalian Hpo signaling can be regulated by cell contact, extracellular attachment, cell morphology and mechanical tension (Codella and Irvine, 2012). Upstream regulators identified are generally cell junction association proteins, linking Hpo signaling regulation to F-actin cytoskeleton (Zhao et al., 2007; Yu et al., 2013; Wada et al., 2011; Aragona et al., 2013). Moreover, the connection of F-actin cytoskeleton to Hpo signaling brings new insight for understanding how G protein activity is linked to Yap/Taz regulation (Yu et al., 2012).

Studies in *Drosophila* cell polarity determination first reveal that the disruption of genes limiting F-actin polymerization leads to activation of Yki (Richardson, 2011; Codella and Irvine, 2012). Later in mammalian cell culture systems, F-actin is also discovered to regulate Lats1 phosphorylation by cell attachment or cell morphology alteration (Ferna´ndez et al., 2011; Sansores-Garcia et al., 2011). Moreover, different cytoskeleton tension is found to modulate Yap/Taz activity (Zhao et al., 2012; Wada et al., 2011). The regulation mechanism by which F-actin dynamics modulate Hpo signaling has yet to be uncovered. The process appears to be Mst independent, but via Lats activity. Overall Yap/Taz activity appears to be regulated by a wide range of F-actin regulators, including GPCRs (Figure 1-5, Yu et al., 2012; Yu et al., 2013; Codella and Irvine, 2012). In addition, the regulation of actin structures on Yap/Taz activity is mostly transient due to the transient and dynamic features of actin structure modulation. These new discoveries excited our lab's interests and triggered us to investigate the GPCRs/F-actin involved metabolism mechanisms.
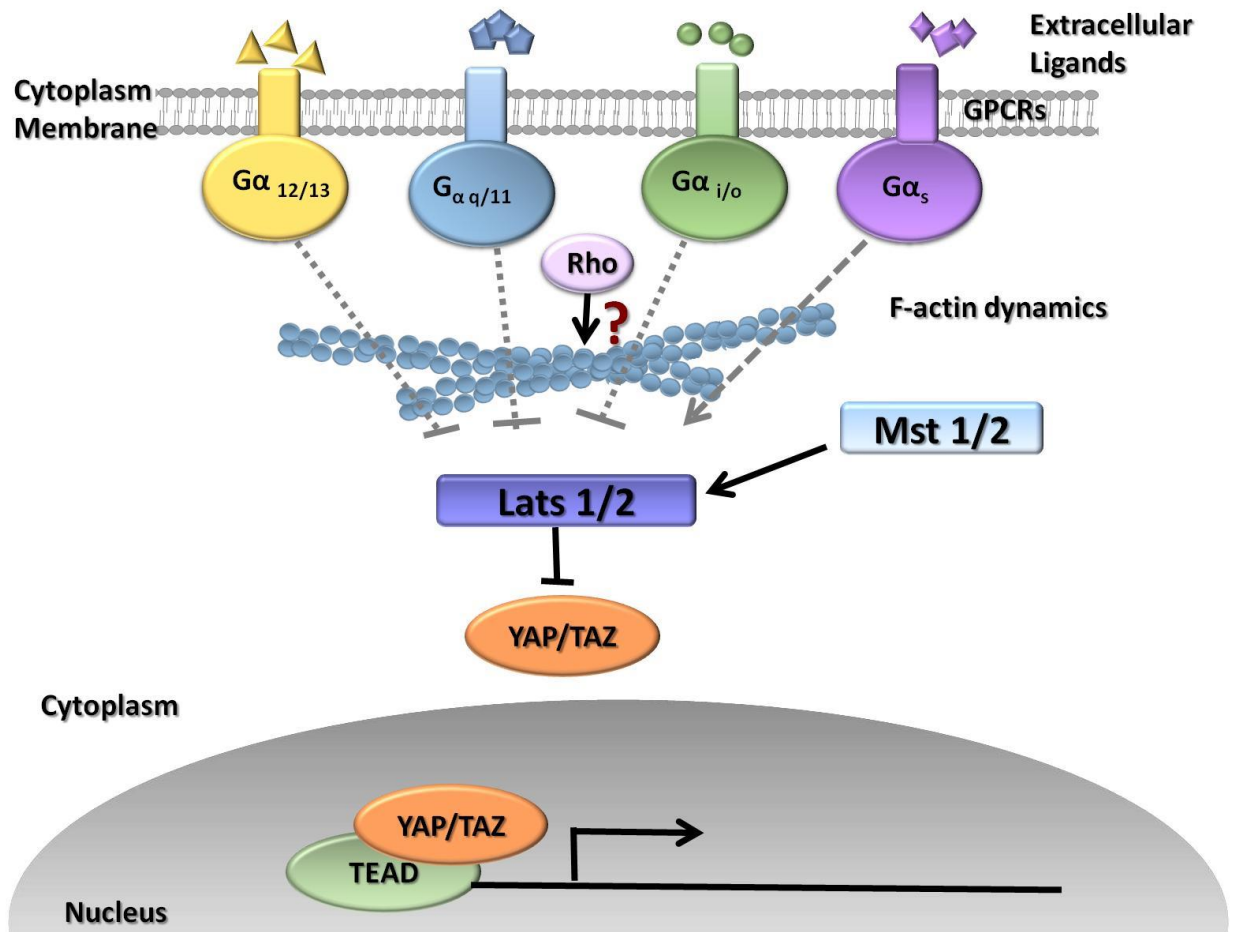
Figure 1-5 GPCRs and cell skeleton regulate mammalian Hpo signaling. Lats1/2 can be activated or inactivated by different G_α and GPCRs bypassing Mst1/2. Actin dynamics and Rho family appear to be involved, and are put between G_α and Lats1/2.

1.9 FFA-induced apoptosis in mammalian pancreatic β-cells

GPCRs are learned to be involved in the regulation of insulin and glucagon secretion pathways in mammalian pancreatic cells. Among GPCRs expressed in pancreatic cells, Free Fatty Acid (FFA) receptors drew our attention, which are involved in insulin/glucagon signaling and FFA-induced β-cell apoptosis.

The insulin signal pathway is important for glucose metabolism, particularly the maintenance of glucose homeostasis (reviewed in Jiang et al., 2007). The hormone insulin is

secreted by pancreatic β-cells to the blood stream and helps peripheral tissues to absorb glucose from the blood to maintain blood glucose concentration (reviewed in Paulmann et al., 2009). However, failure in the insulin signaling pathway might cause blood glucose concentration to increase and lead to diabetes diseases. Type 2 diabetes is a chronic metabolic disease, which might take with up to ten years for development. It is caused by insulin resistance in peripheral tissues and β-cell failure (reviewed in Kumar et al., 2005). Insulin resistance is the insensitivity of peripheral tissues to normal levels of insulin. Under insulin resistance, β-cells need to secret more insulin to compensate for the increasing demand of peripheral tissues. However, chronic exposure to unhealthy environments, such as high plasma free fatty acid (FFA) or high glucose concentration, causes β-cell dysfunction and even cell death, leading to type 2 diabetes (Boden and Shulman, 2002; Karpe, et al., 2011).

Both insulin resistance and β-cell failure (dysfunction and death) contribute to the development of type 2 diabetes, and both processes are related to increased concentration of FFA (Boden, 2008; Boden and Shulman, 2002). Type 2 diabetes is an obesity-related disease. Plasma FFA concentration in the blood is usually elevated in obese people since more FFA is released from enlarged adipose tissue mass, and FFA clearance is decreased (Boden, 1997; Tataranni et al., 2005; Moller and Kaufman, 2005). FFA-induced insulin resistance will be compensated for by insulin over secretion in β-cells under normal conditions. However, chronically elevated plasma FFA disrupts β-cell function by inducing β-cell secretory failure (lipotoxicity, Haber et al., 2002; Lupi et al., 2004; Zhou and Grill, 1995) and causing β-cell death (lipoapoptosis, Shimabukuro et al., 1998; Lupi et al., 2004).

Plasma FFA induces β-cell death by both apoptosis and necrotic mechanisms in type-2 diabetes (Prentki and Madiraju, 2011). FFA induced β-cell apoptosis involves nitrite oxide (NO) production, which leads to mitochondrial DNA (mtDNA) damage (Rachek et al., 2006) and

Endoplasmic Reticulum (ER) stress responding to Ca^{2+} channels (Cunha et al., 2008). However, the molecular mechanism by which FFA induces β-cell apoptosis is not yet well known.

Studies of FFA receptors provide significant perspectives on lipotoxicity and lipoapoptosis. FFA receptors are mainly from the GPCR family, which is widely expressed in different cell types of the islets, including α, β, δ and pancreatic polypeptide cells (reviewed in Lavden et al., 2010). GPR40 (coupled with $G\alpha_{q/11}$) has been identified as long-chain (c>12) FFA receptors while GPR41 (coupled with $G\alpha_i$) and GPR43 (coupled with $G\alpha_i$) are activated by short-chain (c<6) FFAs. Recently, GPR119 (coupled with $G\alpha_s$) was also shown to be involved in FFA-induced insulin secretion (Lavden, et al., 2010; Winzell and Ahrén, 2007).

Among these receptors, GPR40 is mostly studied for its role in regulating insulin secretion, lipotoxicity and lipoapoptosis (Itoh et al., 2003; Salehi et al., 2005; Tomita et al., 2006). GPR40 stimulates Ca^{2+} release from ER via $G\alpha_{q/11}$ signaling and thus influences insulin's secretory response to FFA (Steneberg et al., 2005). GPR40 is also suggested to regulate the lipotoxic effect caused by the chronic exposure of islets to high concentrations of FFA (Haber et al., 2002; Zraika et al., 2002). GPR40 -/- mice were protected from the lipotoxic effects caused by a high fat diet (Steneberg et al., 2005). Moreover, disruption of GPR40 can also repress saturated FFA-induced apoptosis in human and mouse cultured β-cells (Steneberg et al., 2005; Zhang et al., 2007; Wu et al., 2012). However, the mechanism by which GPR40 regulates β-cell viability is still not well understood. In this study, we explored the downstream signaling of GPR40-$G\alpha_{q/11}$ and observed a potential protection signaling via Yap1 activity modulation.

1.10 Abbreviations used in this study

Table 1-1 Abbreviations

Abbreviations	
BiFC	Bimolecular fluorescence complementation
Crb	Crumbs
CytoD	Cytochalasin D
CTGF	Connective Tissue Growth Factor
D	Dachs
Dco	Discs overgrown
Diap1	*Drosophila* inhibitor of apoptosis-1
Dlg	Discs large
Ds	Dachsous
ER	Endoplasmic Reticulum
Ex	Expanded
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid
Fj	Four-jointed
FRT	FLPase recombination target
Ft	Fat

Hpo	Hippo
KEM	Kibra/Ex/Mer
Krb	Kibra
Lats	Large Tumor Suppressor
Lgl	Lethal (2) giant larvae
MARCM	Mosaic analysis with repressible cell marker
Mats	Mob as tumor suppressor
Mer	Merlin
mtDNA	mitochondrial DNA
NF2	Neurofibromin 2
NO	nitrite oxide
RASSF	Ras association domain family protein
Sav	Salvador
Sd	Scallpoped
TEAD	Transcriptional enhancer factor TEF
Wts	Warts
YAP	Yes-associated protein
Yki	Yorkie

Chapter 2

Materials and methods

2.1 Molecular cloning

In molecular biology, molecular cloning is broadly used to construct recombinant DNA molecules. In traditional molecular cloning experiments, a target DNA fragment is cloned from a genome or from another plasmid to a new vector.

The general steps in molecular cloning are summarized in Figure 2-1:

1. Choose the DNA fragment of interest and the target vectors.

2. Design the primers for target genes and use polymerase chain reaction (PCR) to amplify the DNA fragment flanking with chosen restriction enzyme sties (followed by DNA clean up).

3. Apply enzyme digestion to both the DNA fragments and the target vectors (followed by DNA clean up).

4. Ligate the DNA fragments and the vectors with DNA ligase.

5. Transform the ligase product to E.coli for plasmid amplification.

6. Verify the plasmid product by Colony PCR or enzyme digestion.

7. Confirm the plasmid by DNA sequencing.
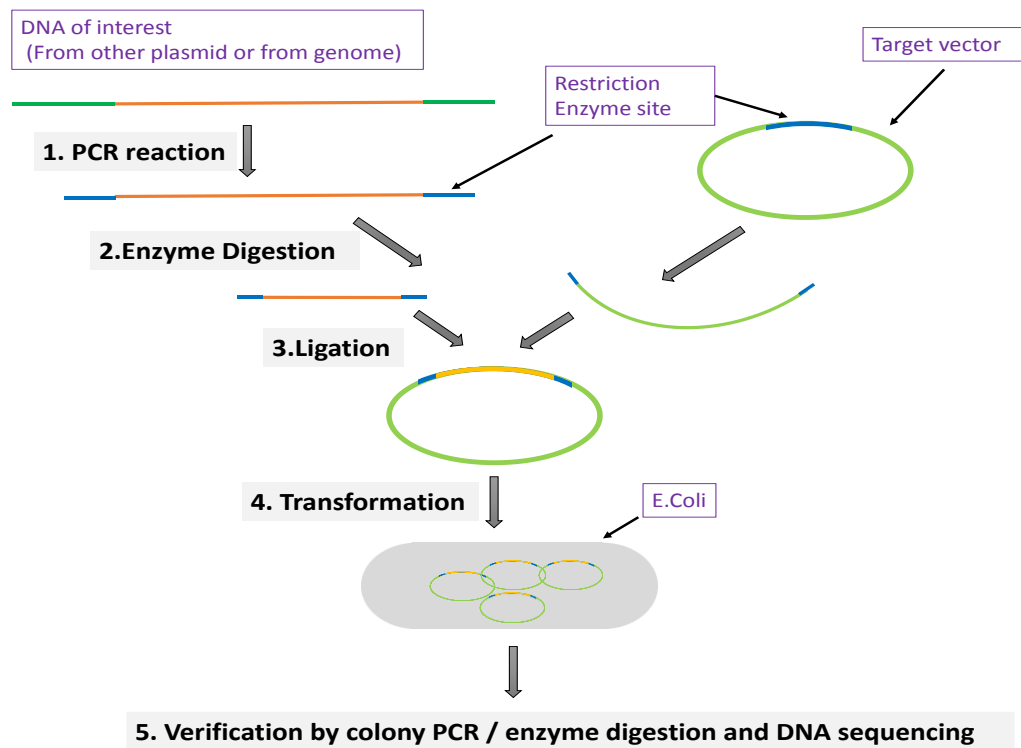
Figure 2-1 Overview of molecular cloning

2.1.1 Primer design

Primer is a strand of nucleic acid that serves as starting point for PCR reactions. It is required for the DNA duplication process. Generally, in molecular cloning, we design a pair of primers that can amplify our DNA of interest starting and finishing at the positions needed. We usually add two restriction enzyme sites at the end of the primers, following at least four nucleic acids for enzyme docking.
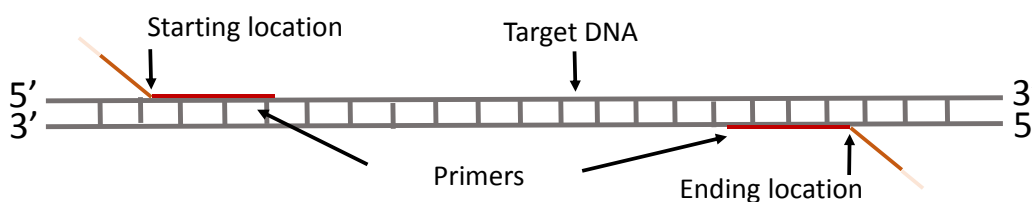
Figure 2-2 Primers for molecular cloning

When designing primers for cloning, we follow the rules referred from

http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html. Online software tools can

be used, such as http://molbiol-tools.ca/.

2.1.2 PCR reaction from plasmid DNA

PCR is used to amplify a specific DNA fragment. A basic PCR set up requires several

components and reagents: DNA template, primers, DNA polymerase, polymerase buffer, and

dNTP. The PCR reaction component differs by different polymerase. DMSO and $MgCl_2$ will be

added according to need. The PCR reaction components and running programs are shown below.

Table 2-1 PCR reaction component for 20ul reaction

Polymerase Type	Taq	pfu	phusion
Enzyme	0.5ul	0.5ul	0.2ul
Buffer	(10x) 2ul	(10x) 2ul	(5x) 4ul
Primers	1ul (100mM) x 2	1ul (100mM) x 2	1ul (100mM) x 2
DNA Template	1ul (plasmid)	1ul (plasmid)	1ul (plasmid)
dNTP	0.5ul (10mM)	0.5ul (10mM)	0.4ul

ddH$_2$O	Make up to 20ul	Make up to 20ul	Make up to 20ul

Table 2-2 PCR reaction component for 50ul reaction

Polymerase Type	Taq	pfu	phusion
Enzyme	1ul	1-2ul	0.5ul
Buffer	(10x) 5ul	(10x) 5ul	(5x) 10ul
Primers	2ul (100mM) x 2	2ul (100mM) x 2	1ul (100mM) x 2
DNA Template	1ul (plasmid)	1ul (plasmid)	1ul (plasmid)
dNTP	2ul (10mM)	2ul (10mM)	1ul
ddH$_2$O	Make up to 50ul	Make up to 50ul	Make up to 50ul

Table 2-3 Basic PCR running program

Polymerase Type	Taq	pfu	Phusion
1.Denature	94°C for 5min	94°C for 5min	98°C for 30s
2. Denature Repeating	94°C for 45s	94°C for 45s	98°C for 10s
3.Annealing (Temp According to primers Tm)	30-45s	45s	30s
4. Elongation	72°C (1Kb/min)	72°C (0.5-1Kb/min)	72°C (1-2Kb/min)
5. Repeat from step 2 -4for 25-30 cycles			
6. 4°C for ever			

2.1.3 PCR reaction from genomic DNA

Genomic PCR is used to clone the whole length of the gene (including introns or regulation elements) from a living organism (*Drosophila* in our study). cDNA of the cells or

organism can be used as template resources only if the gene coding region is needed. The

genomic DNA preparation procedure could be followed as below:

http://francois.schweisguth.free.fr/protocols/Quick_Fly_Genomic_DNA_prep.pdf

Genomic PCR Reaction Components (pfu does not work well for genomic DNA PCR)

Table 2-4 PCR reaction component for genomic DNA

Polymerase Type	phusion	Taq
Enzyme	0.25ul	0.5ul
Buffer	(5x) 5ul	(10x) 2.5ul
Primers	1ul (100mM) x 2	1ul (100mM) x 2
DNA Template	1ul (genomic DNA)	1ul (genomic DNA)
DMSO	0.75ul	0.75ul
$MgCl_2$(50mM)	0.75ul	0.75ul
dNTP	0.5ul (10mM)	0.5ul (10mM)
ddH_2O	Make up to 25ul	Make up to 25ul

2.1.4 Restriction enzyme digestion and DNA fragment ligation

We need to choose at least one (two is better) restriction enzyme site for both DNA

fragment and target vector digestion. Online software can be used to determine which buffer to

use when doing two-enzyme digestion (https://www.neb.com/tools-and-resources/interactive-

tools/double-digest-finder).

DNA ligation is performed using T4 ligase. The mole ratio of the DNA fragment and the

vector should be at least 7:1. The reaction can be done overnight under 16°C or in 0.5-1 hour in

room temperature conditions.

2.1.5 Transformation

Transformation is the process that transforms the intact plasmid into the appropriate *E.coli* strain and is used for ligation selection or DNA amplification. Bacteria will only take the plasmid when it is intact, so transformation is used to select out the successfully ligated plasmid. DH5α is a commonly used strain for basic transformation. Top10 is used to carry large plasmids (>10Kbp), but it grows more slowly.

Transformation procedure:

1. Mix 10ul ligation product (DNA clean-up is not necessary) with 50ul competent cells, or mix 1ul DNA plasmid with 20-30ul competent cells when doing pure plasmid transformation. Put the mixture on ice for 30min.

2. Heat-shock the mixture for 90s in 42°C. This step is critical for transformation efficiency.

3. Add 300ul LB medium (without any antibiotics) to the mixture and incubate them on 37°C shaker for 10-45min. For ligation product transformation, 10-45mins is needed, and for pure plasmid transformation, 10mins incubation is sufficient.

4. Spin down the bacteria by 5000 rpm for 2-3 min and get rid of the extra medium, leaving only 100ul.

5. Resuspend the bacteria in the left 100ul medium and spread them on the LB plates with appropriate antibiotics.

6. Incubate the LB plates in 37°C incubator for 12-18 hours.

2.1.6 Colony PCR verification

We might get lots of bacteria colonies on the plates after transformation; however, not all the colonies carry the right plasmid. Colony PCR is used to verify whether the colony carries the plasmid with the right insertion. We can use both the primers from the inserted DNA fragment (double enzyme digestion cloning) or use one primer from the vector and another one from the DNA insert (double or single enzyme digestion cloning). Each colony is picked by tips or toothpick and is then mixed in 5ul LB medium. 1-2ul mixed culture can be used as DNA template for colony PCR. Add 500ul LB medium to the rest of the bacteria culture and incubate them on 37°C shaker to make stocks.

Table 2-5 PCR reaction component for colony PCR

10 ul Reaction	Taq or pfu
Supper mix	8ul
DNA	1-2ul
Primer	0.5ul(100mM) x 2

Table 2-6 Go Taq supper mix component for 1ml PCR reaction

GoTaq polymerase (Promega)	12.5ul
Go Taq buffer (5x)	200ul
dNTP	25ul
DMSO	30ul
H$_2$O	582.5ul

2.1.7 Site directed mutagenesis

Mutagenesis is the method to introduce a point mutation into the target gene. In our project, site directed mutagenesis is most commonly used.
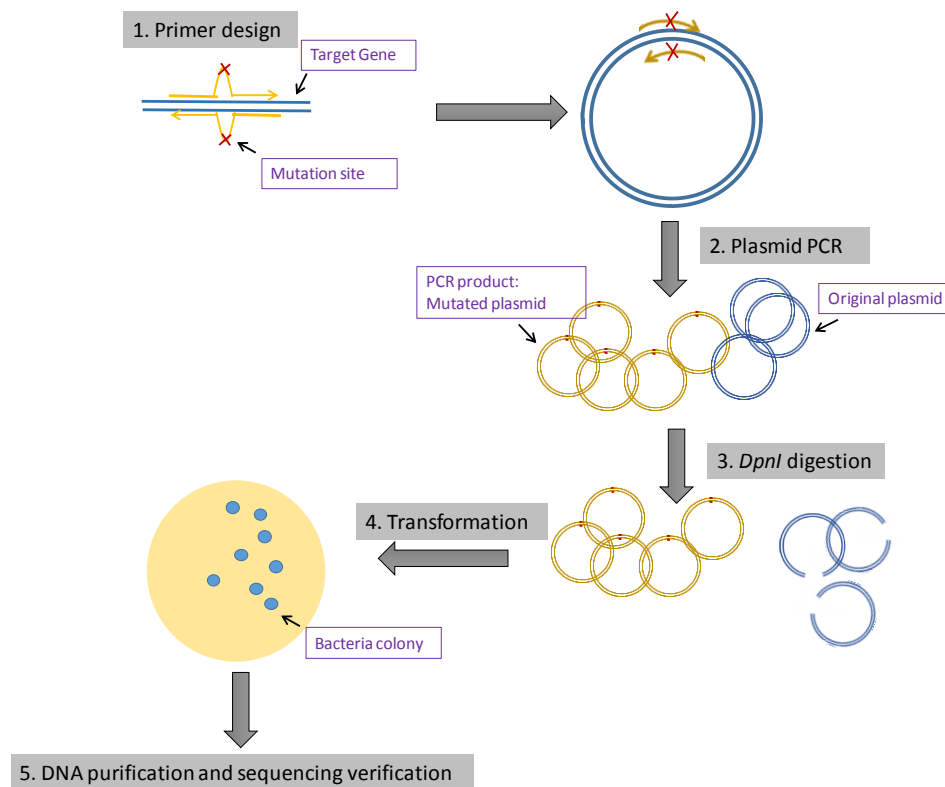
Figure 2-3 Overview of site directed mutagenesis

1. Primer design: We need two primers around 15-20 bp to cover the region you want to mutate. Try to put the mutation site in the middle of the primers. Both primers need to carry the mutation site. Other primer design rules need to be followed.

2. Perform PCR using a long-length DNA polymerase (such as phusion). Original plasmid needs to be purified from bacteria (bacteria-produced plasmid is methylated).

3. Use *DpnI* enzyme (from *New England Biolbas*) to digest original plasmid (methylated plasmid will be digested).

4. Perform transformation and grow bacteria colony.

5. Purify the plasmid product and verify the mutation by DNA sequencing.

2.2 Cell biology experiments

2.2.1 Cell culture work

Cell culture is commonly used in cell biology, which is a complex process by which cells derived from multi-cellular eukaryotes are grown under controlled conditions. In general, cell lines grow exponentially. We use tissue culture-grade Petri dish or T flasks for routine maintenance of the cell lines. T flasks can be sealed, and are therefore convenient for carrying.

The volume of the culture in a given vessel is determined by the surface area, and the medium should be about 2-3 mm deep.

Table 2-7 Culture vessel characteristics

Culture vessel	Medium volume (ml)	Surface area (cm^2)	Approx cell yield
24 well plate	0.5	2	5×10^5
12 well plate	1	4.5	1×10^6
6 well plate	2	9.6	2.5×10^6
30mm dish	1.5	6.9	1.7×10^6
$25cm^2$ T flask	5	25	5×10^6
60mm dish	4	21	5.2×10^6
100mm dish	10	55	13×10^6

Cell culture condition:

Schneider's 2 R+ (S2R+) cell culture

S2R+ cells were purchased from the *Drosophila* Genomics Resource Center (DGRC). Cells are cultured in *Drosophila* Schneider's Medium (Sigma) with 10% fetal bovine serum and 0.5% penicillin streptomycin (optional) in a 24^oC incubator. Split the cells when they grow confluent. S2R+ cells do not have intact inhibition; therefore it is important to dilute them before they grow to over confluent. To remove the S2R+ cells from the culture dish, trypsin can be used or medium blown at the cells using a 5ml pipet.

Human Embryonic Kidney (HEK 293T) cell culture

HEK 293T cells were a kind gift of Dr. Aimin Liu. Cells are cultured in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 0.5% penicillin streptomycin (optional) in a 37^oC incubator with 5% CO_2. The cells are split with 3-10 times dilution when they grow to 80-90% confluent. To maintain the cells over the long term, DMEM without FBS can be used to slow down the cells' growing speed. However, FBS-containing medium must be used after splitting the cells to stop trypsin reaction. HEK 293T cells can grow in lower temperatures; however they are only considered as attached cells under 37^oC or higher. If they are observed suspended in the medium after prolonged exposure at room temperature, they should be put back to 37^oC at once to get attached.

Rat beta (INS-1 832/13) cells culture

Rat β cell INS-1 832/13 was kindly gifted from Dr. Douglas Cavener. INS-1 832/13 cells are cultured in RPMI 1640 medium (Lonza) with 10% FBS (Gemini), 10mM HEPES (Lonza), 1x NaPyruvate (Thermo Scientific), and 1xβ-ME (gibco in Invitrogen). The cells are cultured in 37^oC with 5% CO2, and the medium is changed every two days. 1:3 dilution is used to pass the cells when they grow to 80% -90% confluence.

Mouse beta (MIN6) cells culture

Mouse β cell MIN6 was kindly gifted from Dr. Douglas Cavener. MIN6are cultured in DMEM medium (Invitrogen) with 15% FBS, 10mM HEPES, and 1xβ-ME. The cells are cultured in 37^oC with 5% CO2, and the medium is changed at least every four days. 1:3 dilution is used to pass the cells when they grow to 60%-70% confluence. MIN6 cells tend to form clusters;

therefore it is hard to estimate their confluence. It is not good for them to grow to complete confluence.

Thaw the cells from frozen stocks

If the cells are preserved in -80°C or liquid nitrogen, we need to thaw and recover them before using them in an experiment.

1. Thaw frozen cells rapidly (within 30s) in 37°C water, shaking the tube when thawing them.

2. Transfer all the cells in 25 cm2 T flask or 60mm culture dish with pre-warmed growth medium.

3. Change the medium as long as the cells are attaching to the bottom. Use 20% FBS-containing medium if the cells are recovered too slowly.

Freeze the cells to make stocks

1. Start with a healthy culture (confluent cells from a75cm2 T flask can be collected into 3 x1ml cryovials). Collect the cells by trypsin.

2. Resuspend the cells in freezing medium (Culture medium with no antibiotics and with 20% FBS and 10% DMSO).

3. Dispense the cell suspension into cryovials, 1 ml per vial. Tightly seal vials of cell suspension under room temperature.

4. Place the cryovials into a -80°C freezer in a freezing container. Transfer the cells to liquid nitrogen after 2-3 days if possible.

2.2.2 Transient transfection of culture cells

DNA for transfection should be prepared with a high quality prep kit, such as the Promega Wizard Prep kit. Dissolve DNA in ddH$_2$O or nuclease free water provided by kit. Transfection was carried out following the transfection reagent manufacture's instructions.

For HEK 293 cells general transfection, PolyFect Transfection Reagent (Qiagen) was used. Lipofectamine TM2000 (Invitrogen) can be used for high efficiency plasmid transfection. Effectin Transfection Reagent (Qiagen) can be used for cells under sensitive conditions (such as under RNAi conditions).

For S2R+ cells, Cellfectin II reagent (Invitrogen) was used for plasmid transfection. Effectin Transfection Reagent (Qiagen) was used for RNAi treatment transfection.

2.2.3 Transient expression of Yap1 shRNA in INS-1 832/13 cells by Lentivirus induction

INS-1 832/13 is a cell type that is difficult to be transfected. Therefore, protein expression silencing was done by lentiviral shRNA. Mission shRNA (kindly gift from Dr. Guan Kun-Liang) plasmids were used together with pMD2.G and psPAX2 to produce lentivirus in HEK 293T cells. The lentivirus was used to transfect the target cells with 8ug/ml polybrene.

Lentivirus production and purification:

Day 0: Seed HEK 293T cells at 80% confluent.

Day1: 1) Change cell culture medium and add 5ml of complete medium with serum 30-60 minutes before transfection.

2) For each 10cm dish, dilute total 5ug DNA (viral vector: pMD2g:PsPAX2 = 2:1:2) into 500ul serum free DMEM with high Glucose.

3) For each 10cm dish, dilute 20ul Polyjet *In Vitro* DNA Transfection Reagent

(SignaGene Laboratories) into 500ul serum free DMEM with high Glucose.

4) Add the diluted Polyjet Reagent immediately to the diluted DNA solution all at once.

(Do not mix the solutions in the reverse order!) Immediately pipette up

and down 3-4 times to mix followed by incubation for ~10 min at RT.

5) Add all 1000ul mixture to 10cm culture dish.

6) Change medium 5 hours after transfection.

Day 2: Change medium 24 hours later, discard/bleach the old medium (since it contains low

concentrations of virus, we usually do not use it). See targeting cells for infection at low density.

Day3: Collect virus after 24 hour, and filtrate the virus with 0.45 uM filter (virus can be collected

again after another 24 hours). Purify the virus by centrifuge at 24K g at 4^oC for 1.5-2 hours.

Resuspend the virus in dPBS (virus from 30ml culture medium can be resuspended in 60ul

dPBS). The virus efficiency needs to be titrated each time it is used.

Lentiviral infection:

Lentiviral infection procedure is referred from:

http://www.cincinnatichildrens.org/WorkArea/DownloadAsset.aspx?id=85779

2.2.4 Chemical treatment to culture cells

Palmitate and oleic acid (Sigma) were dissolved in ethanol and stored as a 50mM

solution in -20°C. To assess the effects of FFAs, the correct amount of palmitate or oleic acid was

first added to 50uL culture medium and heated to 70°C for complete dissolution, then the whole

50ul medium was added to the experiment cells. Cytochalasin D (sigma) was dissolved in DMSO

and stored as 2mM solution in -20°C. 0.5uM CytoD was used in the experiments.

2.2.5 Annexin V apoptosis assay

For apoptosis analysis, cells were trypsinized from 24 well plates and staining was performed via Alex Fluor ®488 Annexin V kit (Invitrogen) following the manufacturer's protocols (Annexin V and PI reagent was used in half dosage as the manufacturer suggested for 24 well plates). Data were performed and collected by FC 500 Beckman Coulter, Inc. Apoptotic cells are considered to be Annexin V positive and PI negative cells.

2.2.6 Yki/Sd activity detection using Luciferase Assay

When carrying on luciferase assay, seed the S2R+ cells in 24 well plates. All the samples need be co-transfected with 10ng of the *copia-Renilla* luciferase (an internal control) and 250 ng of 3x*Sd_luc* (Gift from Dr. Jin Jiang). In the project described in Chapter 4, total amount of 50ng of *pAc-ykiV5*, *pUAST-HA-sd* and *pAc-Gal4* were used in each well to induce luciferase expression. Cells were lysised and cell extracts were made after 48 hours incubation and the Dual-Luciferase assay was performed using the Dual-Glo^{TM} luciferase assay kit (Promega) according to the manufacturer's protocols.

2.2.7 RNAi treatment in Drosophila S2R+ cells

1. PCR amplification to generate DNA Templates

a. Primer design: Select the target DNA sequence from public DRSC screening database. Design the primer sequence and add the T7 promoter sequence (TAATACGACTCACTATAGGG) to the 5' end of both primers.

b. Perform a PCR reaction using cDNA extract from S2R+ cells as the DNA template.

c. Perform the second round of PCR using T7 primers and using the PCR product from the last step as the DNA template. Verify the PCR product by running DNA gels. The PCR product should be ~500bp nucleotide.

2. Synthesize dsRNA by reverse transcription using Ambion 5x T& MEGASCRIPT Kit according to the manufacturer's protocols.

3. Purify the dsRNA using RNeasy Purification Kits (Qiagen) and measure the dsRNA concentration.

4. Using Effectene^{TM} (Qiagen) reagent to transfect the dsRNA (together with DNA plasmid if needed) into S2R+ cells according to the manufacturer's protocols. The nucleic acid concentration added in each sample should be kept equal and for a 12 well plates, no more than 500ng of nucleic acid (DNA + RNA) should be use. The ratio of RNA:DNA should be around 1:10.

2.2.8 Immunostaining for Flow Cytometry

1. Trypsinize the cells and collect them in 1.5ml tubes. Centrifuge in 5000 rmp for 5min and wash them by PBS.

2. Fix them by 4%PFA for 10-20min. Vortex when fixing. Wash the cells by 3x PBS after fixation. Use the highest speed (>13000 rpm) to spin the cells down for at least 10min.

3. Dilute the primary antibody in 0.1%PBST (Triton-100) and incubate the cells in the primary antibody for 1-2hr, followed by washing with 0.1% PBST three times. (Use the highest speed to spin them down after each wash)

4. Dilute the secondary antibody in 0.1%PBST and incubate them in Abs for 1hr, followed by washing with 0.3%PBST three times. The incubation must be down in the dark. If the target proteins are in the nucleus, use 0.3% PBST to dilute both the first and second Ab. If the target proteins are membrane associated, and are in low expression level, use 0.05%PBST to dilute both the first and second Abs and use 0.1% PBST for washing. If the cells get lost during the procedure, elongate the centrifuge time to 20-30min using the highest speed.

2.3 Western blot

2.3.1 Protein sample preparation

Protein Sample preparation from cultured cell

1. Wash cells twice with cold 1xPBS.

2. Add the appropriate amount of Lysis Buffer to the culture dish/plates directly, and collect the lysate in 1.5ml tubes with a scraper on ice. In general, add 80ul Complete Lysis Buffer per well for 12 well plates (scale up or scale down for other culture dishes). Concentrate the cell lysate by adding less Lysis Buffer as needed. Complete Lysis Buffer should be made right before use, and can be stored at -20°C for no more than month. Lysis Base Buffer can be premade and stored at 4°C.

3. Keep the tubes on ice for 30min. Vortex every 10min.

4. Spin down cell extracts at max speed for 10min at 4°C and collect supernatant.

5. Add 6x protein loading buffer to the lysate and boil the protein under at least 80°C for 10min.

The protein sample is then good to use and can be stored at -20°C.

Table 2-8 1ml Complete Lysis Buffer component

Lysis Base Buffer	1ml
2mM DTT	2ul
Protease Inhibitor (Sigma)	2ul
1M Glycen-2 phosphate	60ul
50uM PMSF 2	4ul
1M NaF	10ul
200mM Na$_3$VO$_4$	10ul
Lysis Base Buffer: 150mM Nacl. 50mM Tric-HCl (PH=7.4), 2mM EDTA (PH=8.0), 1% Triton-X100, 10% Glycerol	

Table 2-9 10ml 6x SDS Loading Buffer component

6X SDS Loading Buffer	10ml
0.5M Tris (pH 6.8)	7ml
Glycerol	3ml
SDS	1g
DTT	0.93g
Bromophenol blue	1.2mg

Protein Sample preparation from *Drosophila* wing disc

1. Dissect the wing disc from third instar larva (20-30 discs from each line for one WB experiment) and collect the wing disc in PBS in 1.5ml tube.

2. Spin down the tissues and clean the tissue with 20ul PBST (0.1% Triton-X100 in PBS).

3. Add 5ul 6x SDS loading buffer, then boil the sample for 5-10min.

4. Centrifuge the sample at the maximum speed for 10min at RT and collect supernatant.

2.3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-page is performed before the western blot in order to separate the protein from the tissue/cell extract according to their mass. The SDS gel is composed of stacking gel, which is at least 2cm from the bottom of the well, and the separating gel. We need to choose the appropriate concentration for separating the gel according to our needs. 10% separating gel is most commonly used in our project; however the lower the mass of your target protein, the higher the concentration you might want to choose.

The SDS Gel is run at 160V for the stacking gel. When the protein line passes the stacking gel and goes into the separating gel, it is advised to adjust the voltage to 60-80V to produce a high quality protein band. To estimate the amount of target protein, stain the gel with Coomassie Blue and the protein amount can be estimated by the intensity of the Coomassie Blue staining.

2.3.3 Western blot

Transfer: To perform a western blot, proteins on the SDS gel is transferred to the nitrocellulose membrane at 100V for 1 hour (or 20V overnight in a 4°C room). The voltage and the time can be adjusted according to the target protein mass. It is better to use 20V overnight in a 4°C room if the protein is larger than 120KD.

Blotting: The nitrocellulose membrane with proteins is then blocked with 5%-10% nonfat dry milk in 0.1%TBST. The concentration of the milk can be adjusted by the quality of your antibody. If the antibody produces unspecific bands, higher concentration milk should be used, while if the antibody's affinity to the target protein is weak, a lower concentration of milk should be used.

Primary antibody incubation: After blocking, the membrane is incubated in primary antibody overnight in a 4°C room (it can also be incubated in RT for 2 hours on a shaker). Usually the 2 hour RT incubation produces more background. The primary antibody should be diluted in the blocking buffer with the same milk concentration. After the primary antibody incubation, the membrane should be washed 3 times with 0.1% TBST on a shaker.

Secondary antibody incubation: After primary antibody incubation, the membrane will be incubated in HRP-conjugated secondary antibody in RT for 2 hours. The secondary antibody should be diluted in the same blocking buffer as the primary antibody. The membrane should be washed with 0.1% TBST three times after incubation. The secondary antibody is usually diluted 2000 times in a blocking buffer.

ECL reaction: Perform ECL reaction and detection according to the manufacturer's instructions (ECL^{TM} Glycoprotein Detection kit from sigma: gerpn2190).

Stripping: If necessary, the membrane can be stripped and re-stained with other antibodies. The membrane should be washed with 0.1TBST three times before stripping. The stripping is performed by incubating the membrane in a stripping buffer (2% SDS, 62.5mM Tris-HCl pH 6.8, and 100mm β-mercaptoethanol) for 30-45 min at 50°C with agitation. After stripping, the membrane should be washed three times by 0.1%TBST before re-blocking with the milk buffer.

Table 2-10 Antibodies used in western Blot in this study

Antibody	Species	Dilutions	Source
p-Hpo (T195)	rabbit	1:500	Genemed Synthesis, Inc
Hpo	rabbit	1:1000	Dr. Helen McNeil
Ex	rabbit	1:1000	Dr. Gerge Halder
α-Tubulin	mouse	1:2000	Sigma
HA	mouse	1:1000	Sigma
HA	rabbit	1:1000	Sigma
Flag	rabbit	1:1000	Sigma

Myc	mouse	1:1000	Sigma
V5	moudr	1:1000	Invitrogen
Akt	rabbit	1:1000	Cell Signaling
Cleaved -Caspase3	rabbit	1:1000	Cell Signaling
YAP	rabbit	1:1000	Santa Cruze
p-YAP(S127)	rabbit	1:500	Cell Signaling
Lats1	rabbit	1:500	Cell Signaling
p-Lats1(S909)	rabbit	1:50	Cell Signaling
p-MST1(T183)/MST2(T180)	rabbit	1:500	Cell Signaling
Anti-mouse lgG 2nd Ab	donkey	1:2000	Amersham
Anti-rabbit lgG 2nd Ab	donkey	1:2000	Amersham

2.4 Reverse Transcription Real Time PCR

Reverse Transcription -Real time PCR refers to the PCR reaction that can quantify the template cDNA amount, which was reversely transcripted from the total mRNA extract from the sample. This method is used to quantify the relative mRNA amount of a certain gene from the cells or tissue sample.

Total mRNA isolation: Extract and purify the total mRNA using RNAeasy Purification Kit (Qiagene) following the manufacturer's instructions.

Reverse Transcription: Synthesize cDNA using Quanta qScript cDNA superMix (Quanta) following the manufacturer's instructions (1ug mRNA is used as template).

RT-PCR Reaction: Prepare PCR reaction mixture using PerfeCta SYBR Green FastMix (Quanta) in 96 PCR-plates (PCR-96-LP-AB-C fromAxygen). The final reaction mixture should contain: FastMix 10ul; Primers stock (2.4uM) 5ul; cDNA (1:5-1:10 dilution) 5ul. Seal the plates with Optical Adhesive Covers (Applied Biosytems). Run the RT-PCR in ABIprism 7000 SD8.

2.5 *Drosophila* animal experiments

   Drosophila is a simple multicellular organism, and it works as a powerful and efficient

model system in genetics, cell biology, biochemistry and developmental biology. They are easy to

take care of, having four pairs of chromosomes, breed quickly and produce lots of eggs. With

abundant genetic information and powerful tools available, researchers are able to set up large

scale genetic screenings and manipulate the chromosomes and genes easily. During our study,

we made full use of fly genetics and made transgenic flies to study gene function and regulation.

2.5.1 Microinjection for transgenic flies

   To generate site-directed transgenic flies (Bischof et al., 2007; Fish et al., 2007),

phiC31flies were used to make transgenic lines with insertions on the second chromosome (51C)

or third chromosome (86F).

Cloning: The target gene was first cloned into pUAST vector with attB sequence inserted

upstream of the UAS-binding sites.

DNA preparation: High quality DNA is needed for microinjection. The Qiagen Plasmid Midi

Kit is recommended for cleaning up DNA. It's preferable but not required to resolve the DNA in

DEPC water.

Embryos collection: Choose the phiC3 fly line according to insertion sites as needed. (Reference

the efficiency of phiC3 lines at http://www.rainbowgene.com/RTFLineCollection.html). Make

syrup plates using 10cm Petri dishes. Cap at least 100 flies in the chamber and put the syrup

plates at the bottom.

Microinjection: The microinjection procedure must be carried out in a constant 20°C room.

Fresh eggs are collected every 30-45min and washed with distilled water. The eggs are lined up

on the slides with their posteriors directed to the outside. The eggs will stick to the slides once

they are dried out. Cover the eggs with halocarbon oil mix (700 halocarbon oil: 27oil = 9:1), and

perform microinjection under a microscope. The injected embryo will be put on fly food.

Get transgenic flies by genetic cross: G_0 adult flies are crossed with *w-* wild type flies or with

double balanced lines *Adv/SM1; Sb/TM6* or *Sp/Cyo, Tyrd/TM6.* Pick up the pink/orange eyed

progenies with transgenic chromosome balanced with a balancer. These G1 adult flies can be kept

as fly stocks.

2.5.2 Balancer chromosome and Drosophila genetic cross

Balancer chromosomes are modified chromosomes with genetic markers that can prevent

homologous chromosomes recombination during meiosis. Therefore, balancers could be used in

Drosophila genetics to keep transgenic fly stocks, or they can serve as chromosome marker when

making genetic crosses. Balancer chromosomes suppress recombination with their homologous

chromosomes, carrying dominant markers and most of them are homozygous lethal. The

compound chromosome is one special kind of balancer chromosome, which is formed by the

fusion of two separate chromosomes. Thus during meiosis, due to the fact that compound

chromosome cannot separate, the corresponding homologous chromosome will be forever kept in

the same progeny. Different from balancer chromosomes, some other chromosomes with visible

markers, cannot suppress chromosome recombination.

Table 2-11 Commonly used balancer chromosome and mutation chromosomes in our research

Chromosome type	Balancer chromosome	Mutation chromosome
X	FM1	w-
II	CyO, SM1, SM3	Adv, Sp
III	TM1,TM3, TM6, TM6B	Sb, Tyrd,
Compound chromosome	ST (SM1.TM6B)	

Genetic cross to combine two genes (mutations) from different chromosomes

In order to combine the genes (mutations) from different chromosomes in different animals to the same progenies, we need to balance both chromosomes carrying the target genes. The genetic cross is shown in the (Figure2-4). For G1, there is no difference to choose which line is female and which one is male; however, it is critical for G2 to pick up only males (GeneA/Adv; GeneB/Sb), since male flies do not have recombination during meiosis, and in this case both genes have mutation chromosomes on their homologous chromosomes. In each step, we used a genetic maker on each balancer chromosome or mutation chromosome to pick up the right genotype progeny. It is important to use white eyes balancer tool lines (Adv/SM1; Sb/TM6 and w-/ST), and your mutations or genes should carry colored eyes; thus you can differentiate the genes/mutations from wild type chromosome.

G0 GeneA (II) **X** Adv/SM1; Sb/TM6   GeneB (III) **X** Adv/SM1; Sb/TM6

G1 ♂ GeneA/ SM1; Sb/+ **X** ♀ Adv/ +; GeneB/TM6

G2 ♂ GeneA/Adv; GeneB/Sb **X** ♀ W-/ ST (SM1.TM6B)

G3: Stocks GeneA; GeneB/ST
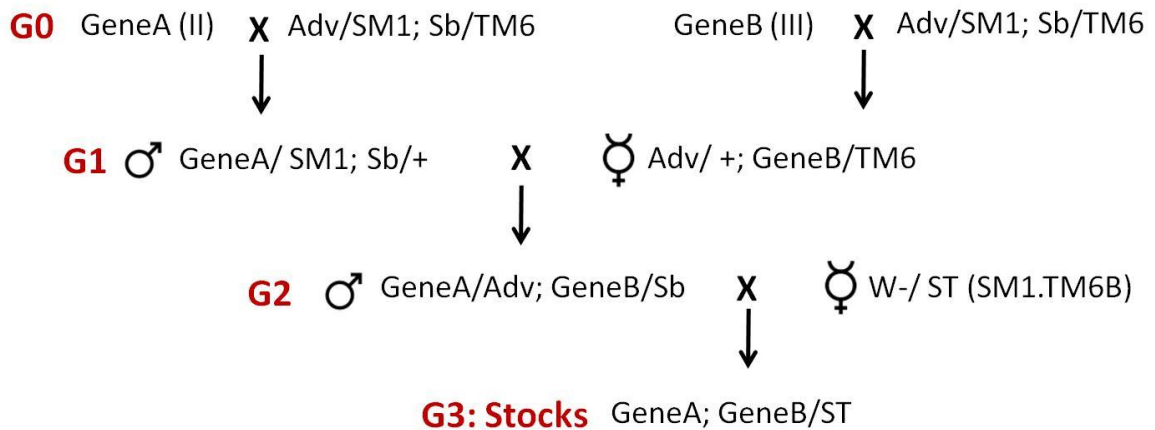
Figure 2-4 Example of genetic recombination between two different chromosomes

Genetic cross to combine two genes (mutations) on the same chromosome

In order to combine two genes (mutations) on the same chromosome, first we need to estimate the recombination rate between these two sites. There would be no recombination if these two genes (mutations) are on the same chromosome locus. Online tools can be used (http://petrov.stanford.edu/cgi-bin/recombination-rates_updateR5.pl). We then set up a cross (Figure 2-5) to put these two genes on homologous chromosomes. It is critical to pick up the female progeny from G2, since only female flies will have recombination during meiosis. From G2 to G3, we needed some genetic marker to identify GeneAGeneB chromosome, such as eye color intensity. Otherwise we need to set up a genetic screening to select the GeneAGeneB chromosome by their phenotype.

G0 GeneA (II) **X** Adv/SM1   GeneB (II) **X** Adv/SM1

G1 ♂ GeneA/ Adv   **X** ☿ Gene B/SM1

G2 ☿ GeneA/GeneB **X** ♂ Adv/SM1

G3: Stocks GeneA,GeneB / SM1
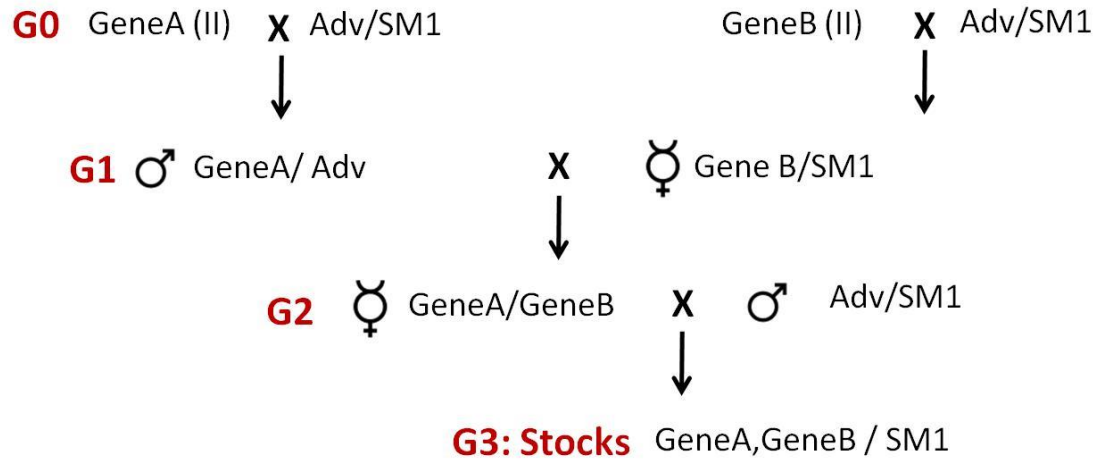
Figure 2-5 Example of genetic recombination on the same chromosome

2.5.3 Conditional Over-expression or knocking down using UAS-Gal4 system

The Gal4 is a modular protein derived from yeast, and UAS is the enhancer element that can be activated by the Gal4 protein (Figure 2-6). The UAS-Gal4 system is a powerful technique to study the expression genes. The system allows one to separate the problems of defining which cells express a gene and what people want to do with the gene. People created varieties Gal4 lines in the *Drosophila* system, each of which expresses Gal4 in some subset of the animal's tissues, allowing conditional expression of the target genes.
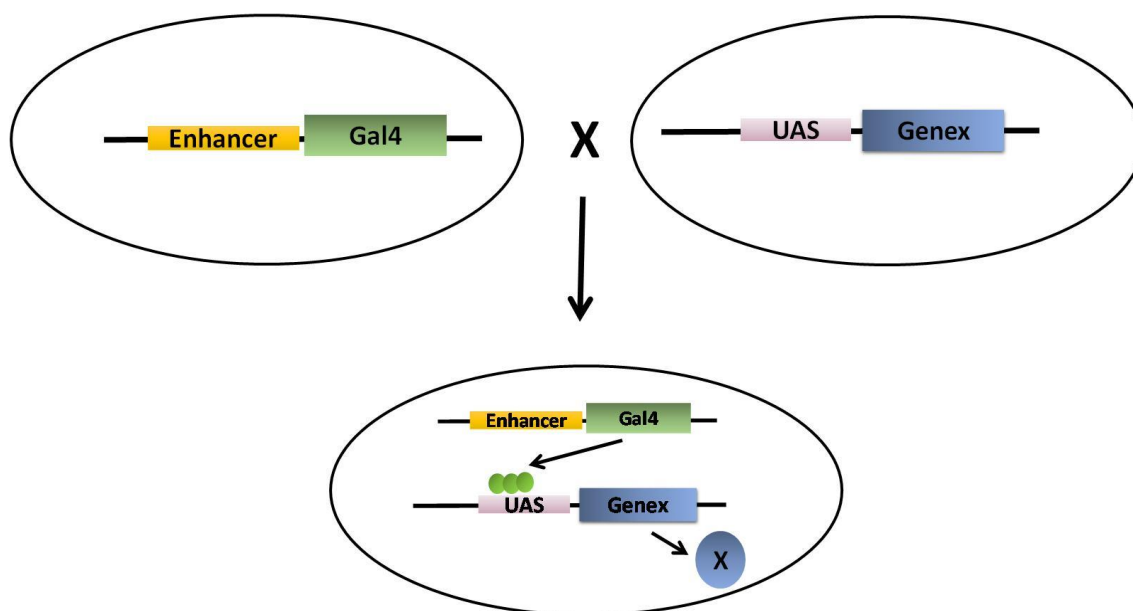
Figure 2-6 Overview of UAS-Gal4 system

Table 2-12 Commonly used Gal4 lines in our study

Gal4 lines	Expression condition
dpp-Gal4	Drive expression between third and fourth veins of wings
en-Gal4	Drive expression in posterior region from embryonic stage; high expression level
C5-Gal4	Drive expression in the whole wing and the wing porch region in wing disc, low expression level
ms1096-Gal4	Drive expression in the whole wing , high expression level
actin-Gal4	Drive expression ubiquitous in the whole body from embryonic stage; low expression level

2.5.4 Immunostaining of mid-pupal wing discs

Dissection: Dissect larval wing disc in RT in PBS. Use pipette to transfer the tissues. The tips need to be coated with a protein-rich buffer (milk buffer, FBS, BSA buffer, etc.) to prevent the tissues from sticking on the tips.

Fixation: Fix the tissue on ice for 60min or under RT for 30-45min in PLP or 4% PFA. Cover the

tissue with foil if the tissues have GFP expressed. Add 0.1% Tx-100 to fixtive if the target gene is

in the nucleus. Wash the tissues with PBS three times.

Primary staining: Incubate the tissues in primary antibody overnight under 4^oC or for 2 hours

under RT, and wrap b the dissection plates in parafilm to prevent drying out. Wrap the tissue with

foil if the tissues have fluorescent protein expressed. The primary antibody needs to be diluted in

blocking buffer (0.1-0.3%PBST with 5%NGS or FBS). Adjust the concentration of TX-100

according to the target protein. If the target protein is located on the membrane, a lower

concentration of TX-100 should be used, while if the target protein is located in the nucleus, a

higher concentration of TX-100 should be provided. Wash the tissues with 0.1%PBST three times

after incubation.

Secondary staining: Incubate the tissues in a secondary antibody for 2 hours under RT with the

dissection plate wrapped with parafilm and foil. Dilute the secondary antibody in 0.3% blocking

buffer. Wash the tissues three times with 0.3%PBST after incubation.

Mounting: Spread about 10ul of glycerol on the slides and carefully remove the wing discs in the

glycerol. Carefully cover the tissues with a cover slip and prevent gas bubble and tissues

overlapping. Seal the slides with nail polish. Images are collected with Olympus FV300 or

Olympus FV1000 confocal Laser Scanning Microscopy.

Table 2-13 Antibodies used in immunostaining in this study

Antibody	Species	Dilutions	Source
p-PH3	rabbit	1:100	Cell Signaling and Dr. Yanming Wang
DE-Cadherin	rat	1:50	Developmental Studies Hybridoma Bank at University of Iowa
Dlg	mouse	1:50	Developmental Studies Hybridoma Bank at University of Iowa

HA	mouse	1:100	Sigma
Hpo	rat	1:200	Dr. Nicolas Tapon
YAP	rabbit	1:200	Santa Cruze
Alex Fluor 2nd Ab	rat/mouse/rabbit	1:200	Invitrogen
DAPI		1:2000	Invitrogen
Draq5		1:200	Invitrogen
Phallotoxins		1:200	Invitrogen

2.5.5 Adult Wing Size Measurement and Statistical Analysis

Raise flies of wild-type and experimental groups together under the same condition. Collect adult male fly wings under dissection microscope. Align the wings on the slides and mount them under cover slides with nail polish. Take wing images under dissection microscope via SPOT Basic Imaging software. Measure the wing area in pixels by ImageJ. Do statistical analysis and normalize the wing size to wild-type group.

Chapter 3

Hippo is activated by dimerization in living tissue

3.1 Introduction

The discovery of Hippo (Hpo) signaling has significantly advanced understanding of the mechanisms and diseases related to growth control, a key issue in developmental biology. Through restricting cell proliferation and promoting apoptosis, Hpo signaling plays a vital role in restricting tissue growth and organ size in both invertebrate and vertebrate animals (Pan, 2010; Halder and Johnson, 2011; Zhao et al., 2011; Staley and Irvine, 2012). Among the primary components of Hpo signaling, Hpo is the key kinase, which phosphorylates other components to activate the pathway when it is activated (as reviewed in Chapter 1.3). However, the regulation method of the Hpo kinase activity was not well understood. It is known that Hpo is activated by phosphorylation on the Thr 195 site (Wu et al., 2003); however, the way in which Hpo is phosphorylated and how Hpo phosphorylation is regulated during tissue development and disease formation remains to be discovered. Mammalian homologs of Hpo protein Mst1 and Mst2 are studied to be activated by autophosphorylation via forming a homo-dimer (reviewed by de Souza and Lindsay, 2004). Thus, I proposed that *Drosophila* Hpo might be activated by dimerization as well. In this study I explored the Hpo dimerization mechanism both *in vivo* and *in vitro*.

[Data presented in chapters 3 and 4 are taken from "*Deng, Y., Matsui Y., Zhang Y., Lai Z.-C., 2013. Hippo activation through homodimerization and membrane association for growth inhibition and organ size control. Dev.Biol.15;375(2):152-9*". Data from Figure 3-5 C, Figure 3-6 and Figure 4-7 were generated by Yurika Matsui, while the rest data were generated by Yaoting Deng]

3.2 Set up BiFC system in *Drosophila* for visualizing Hpo dimer formation

The Hpo kinase plays an important role in growth inhibition during animal development; however, the way in which Hpo is activated during development is still a mystery. It has been shown that mammalian Hpo homologs Mst1 and Mst2 are activated through auto-phosphorylation by forming a homo-dimer. Thus in this project, I propose that Hpo is activated via self-dimerization and auto-phosphorylation as well.

To investigate Hpo dimerization in living cells, I applied the Bimolecular Fluorescence Complementation (BiFC) technique to directly observe protein-protein interaction in living cells and developing tissues. By BiFC, the fluorescent protein Venus is split into Venus N-terminus (VN) and Venus C-terminus (VC) components (Kerppola, 2006; Hu, 2009). VN and VC alone cannot produce a fluorescent signal. However, when fused to two proteins A and B respectively, they may complement each other and generate fluorescence once proteins A and B interact (Figure3-1).

In my project, in order to study Hpo dimerization, I first fused full-length Hpo with HA-VN or Flag-VC tag (HA-HpoVC and Flag-HpoVN). HA-SavVC and Flag-SavVN were made to serve as controls. These fusion proteins were then put into pUAST-attB vector, which expresses the transgene under the control of UAS-binding sites.

To examine the expression level of each construct, each pUAST-attB vector was transfected into S2R+ cells together with pAC-Gal4, which expresses the Gal4 protein under the control of an actin promoter. A western blot against HA or Flag tag was performed to confirm that each protein had an appropriate expression level.

In order to study the protein-protein interaction in vivo, I transformed the pUAST vector into *Drosophila* and established stable transgenic lines. To generate site-directed transgenic flies (Bischof et al., 2007; Fish et al., 2007), phiC31flies were used to make transgenic lines with

insertions on either the second chromosome (51C) or on the third chromosome (86F). Together

with Yifan Zhang, we established transgenic lines after DNA injection performed by Rainbow

Transgenic Flies Inc. or by ourselves. All HA-VC tagged genes were inserted on the second

chromosome, and all Flag-VN tagged genes were inserted on the third chromosome. Each

transgenic line was kept as either a homozygote or a heterozygote with a balancer chromosome.

Genetic crosses were set up to combine all UAS-Gene-VC and UAS-Gene-VN pairs as needed.

To drive the expression of the transgenes, each UAS line was crossed with Gal4 drivers as needed.
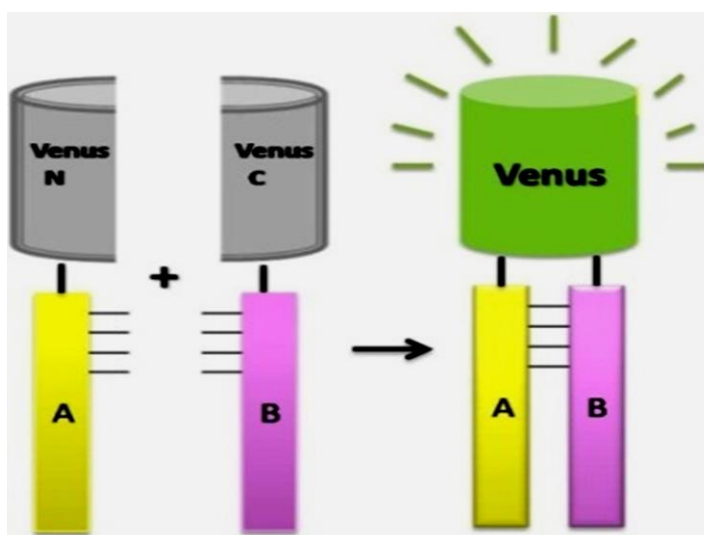
Figure 3-1 Overview of BiFC assay. When proteins A and B form a complex, it may allow their fusion partners Venus N- and C-terminal polypeptides to complement to exhibit fluorescence (Deng et al., 2013).

3.3 Verify Hpo kinase function in BiFC system

In order to investigate whether the VN or VC tag disrupts Hpo protein function, the adult

fly wing analysis was utilized to test Hpo protein activity in restricting tissue growth. HpoVC or

HpoVN was expressed specifically in the *Drosophila* wing when driven by a wing-specific Gal4

driver, *MS1096-Gal4*. The result showed that the wings of transgenic flies carrying HpoVC or

HpoVN have smaller wings compared with wild-type flies (Figure 3-2). This phenotype is similar

to that of full-length Hpo transgenic flies (e.g. Wu et al., 2003). Thus, VN or VC tags do not

disrupt Hpo protein activity in inhibiting tissue growth.

Figure 3-2 Adult fly wing analysis shows the modification of BiFC to Hpo protein has no obvious influence on their function in growth inhibition. Expression of the transgenes was driven by a wing-specific Gal4 driver, MS1096 (Deng et al., 2013).

3.4 Visualize Hpo kinase dimerization directly in living cells

In this project, I proposed that Hpo might get activated via auto-phosphorylation in a

homo-dimer. In order to examine the Hpo dimerization, I fused the full-length Hpo with VN and

VC to perform the BiFC assay. The Hpo dimerization was observed directly in living cells.

HpoVC and HpoVN were transfected into S2R+ cells or were expressed in transgenic

flies together to examine the Hpo-Hpo interaction. The results showed that HpoVC could interact

with HpoVN and give out positive BiFC signals both in *Drosophila* S2R+ cells and third-instar

larva wing discs (Figure 3-3 A&C). By contrast, bFosVC could not interact with HpoVN, and

bFosVC and HpoVN gave out negative BiFC signals (Figure 3-3 B&D), indicating the specificity

of the BiFC assay. These results support the hypothesis that Hpo can form a homo-dimer.
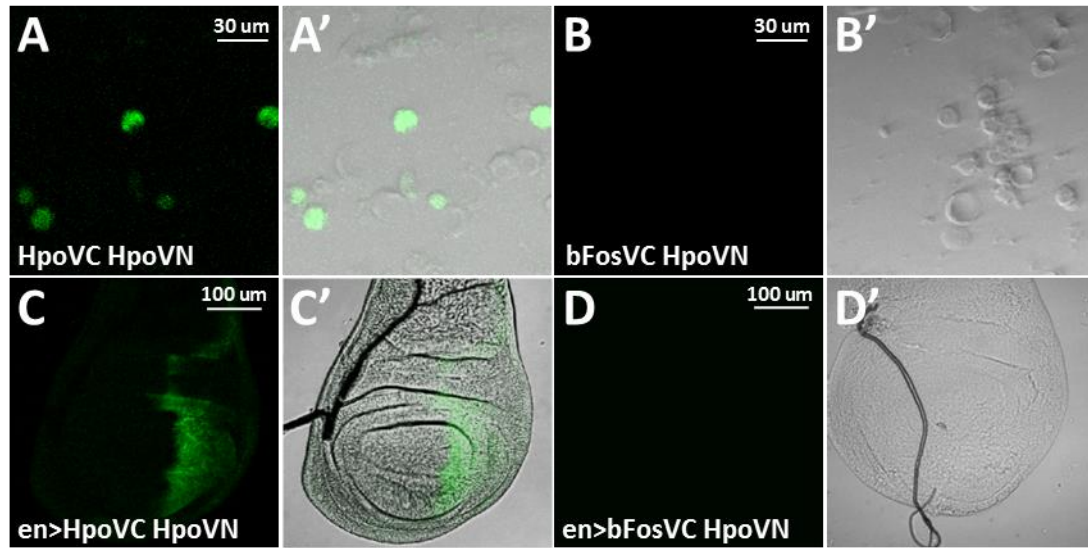
Figure 3-3 Visualize Hpo dimerization in living cells (A-A') Direct visualization of Hpo homo-dimer in transiently transfected S2R+ cells. (B-B') bFosVC/HpoVN was used as a negative control. (C-C') Direct visualization of Hpo homo-dimer in third-instar larval wing disc with en-Gal4/UAS-HpoVC/HpoVN genotype. (D-D') en-Gal4/UAS-bFosVC/HpoVN wing discs were used for a negative control (Deng et al., 2013).

3.5 Hpo kinase-dead mutant acts dominant-negatively

A previous study shows that the kinase-dead version of Hpo has a dominant negative effect (Wu et al., 2003). I observed the same phenomenon in my study. I fused kinase-dead Hpo (Hpo^{KD}) with VC or VN. Hpo^{KD}VC and Hpo ^{KD}VN were then expressed specifically in the fly wings when driven by the wing specific Gal4 driver, MS1096-Gal4. The result showed that the transgenic flies carrying Hpo^{KD}VC or Hpo ^{KD}VN in the wings had significantly larger wings compared to wild-type flies (Figure3-4 E-H). The average wing size from wild-type (WT) flies was normalized to 1, and the wing size from Hpo^{KD}VC or Hpo ^{KD}VN flies was significantly larger (Figure 3-4 E, compare Hpo^{KD}VC or Hpo ^{KD}VN group with WT group, ** marked significant differences, examined by student t-test, $p<0.01$). Thirty wings were collected and analyzed in each group.

Interestingly, when the Hpo^{KD}VC and Hpo ^{KD}VN pair was co-transfected into S2R+ cells, it was unable to give out a BiFC signal, indicating either that Hpo^{KD}VC and Hpo ^{KD}VN failed to interact with each other or that they did interact but the protein structure was changed, resulting in the failure of VN and VC to complement to each other (Figure 3-4 B). However, Hpo^{KD} can interact with wild-type Hpo (Figure 3-4 C-D). This result suggests that Hpo^{KD} might interact with wild-type Hpo and form an Hpo^{KD}-Hpo^{WT} complex, which explains the dominant-negative effect of the Hpo^{KD} protein.
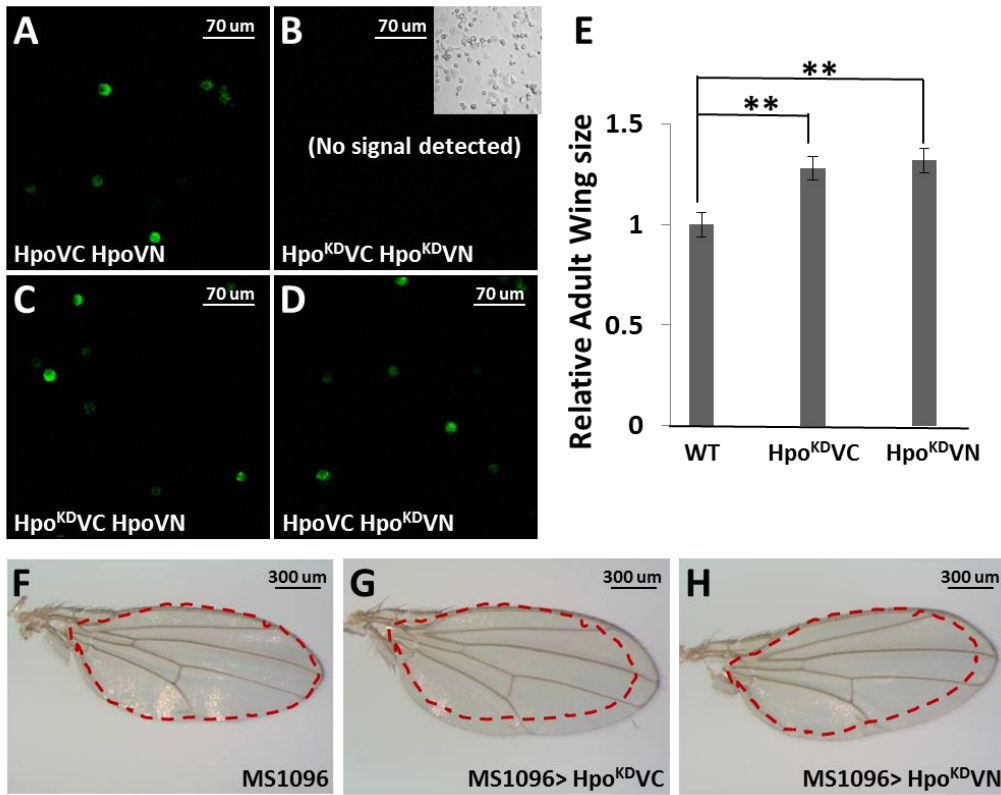
Figure 3-4 Hpo kinase-dead mutant protein is still able to associate with wild-type Hpo protein. (A-D) BiFC assay revealed that HpoKD fails to allow VC/VN complementation, but is able to bind wild-type Hpo. (E) Adult wing analysis shows that Hpo^{KD} has a dominant-negative effect to increase the wing size. N>30 wings for each genotype. Data show mean $\pm$ SD, ** indicates p<0.01 by student t-test. (F-H) Adult wing analysis shows dominant-negative effect of Hpo^{KD} (Deng et al., 2013).

3.6 Hpo dimerization and its trans-phosphorylation

3.6.1 Hpo dimerization is necessary for its phosphorylation

My previous work has shown that Hpo protein can interact with each other and form homo-dimers. During the previous stage of my project, the Jin group (2012) showed the same result. They discovered that different from Hpo mammalian homologs Mst1 and Mst2, which form homo-dimers by SARAH domain interaction, Hpo dimerization is mediated by two sites: the M242 site in the N-terminal kinase domain and the I634 site in the C-termial SARAH domain (Jin et al., 2012). I also observed that Hpo N-terminus alone without the SARAH domain can form a homo-dimer and generate a positive BiFC signal (data not shown).

In order to verify that dimerization is crucial for Hpo to become phosphorylated, Hpo^{M242E,I634D} was generated to abolish two Hpo dimerization sites. The Hpo^{M242E,I634D} was then fused with a VC or VN tag and expressed in *Drosophila* S2R+ cells. In Jin's work, they verified that Hpo^{M242E,I634D} indeed failed to dimerize (Jin et al., 2012), and in my study Hpo^{M242E,I634D} VC and Hpo^{M242E,I634D} VN showed a negative BiFC signal (Figure 3-5 A-B). Importantly, my western blot analysis indicates that Hpo^{M242E,I634D} is unable to be phosphorylated (Figure 3-5 C), which is consistent with the work of Jin's group (Jin et al., 2012). Mutagenesis and western blot were done by Yurika Matsui.

To further verify whether Hpo could only be phosphorylated in a homo-dimer, a cell sorting experiment was applied to separate BiFC positive cells with BiFC negative cells. In this experiment, HpoVC and HpoVN were co-transfected into S2R+ cells and, after 40 hours' incubation, the cells were sorted by their BiFC fluorescence (Figure 3-5 D). Western blot was then applied to these two groups of cells. The result showed that BiFC-positive cells are more phosphorylated than BiFC negative cells (Figure 3-5 E, right panel shows western blot

quantitative result). Together with the work of Jin's group, these results support that Hpo

dimerization facilitates its auto-phosphorylation.
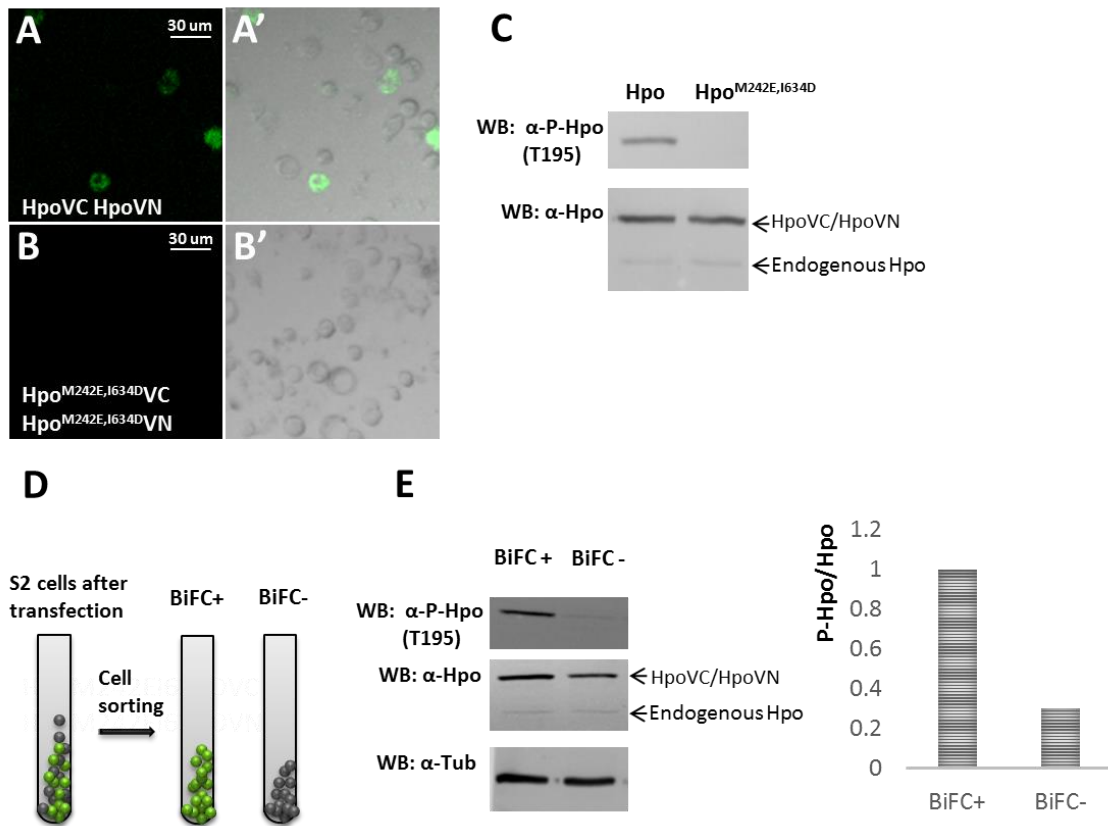
Figure 3-5 Hpo dimerization is crucial for its phosphorylation. (A-B') Mutations of Met242 and Ile634 abolished Hpo dimerization in S2R+ cells. (C) Hpo autophosphorylation at Thr195 cannot occur without dimerization. (D) Overview of cell sorting experiment. BiFC signal is shown in green. (E) Western blot result shows that BiFC-positive cells had more phosphorylated Hpo compared to BiFC-negative cells. Right panel shows western blot quantitative result by Scan Storm. Relative expression level of p-Hpo to total Hpo in BiFC+ group was normalized to 1 (Deng et al., 2013). Figure C was generated by Yurika Matsui.

3.6.2 Hpo is activated by trans-phosphorylation within the homo-dimer

Furthermore, I examined Hpo inter-molecular phosphorylation. In this experiment, I co-

transfected kinase-dead Hpo (Hpo^{KD}) with Hpo^{T195E} or Hpo^{T195A}. Hpo^{T195E} or Hpo^{T195A} carried

mutations on the phosphorylation sites and thus could not be detected by the phosphor-specific

Hpo antibody targeting the Thr195 site. Hpo^{T195E} has been shown to have partial kinase activity, while Hpo^{T195A} has no kinase activity (Jin et al., 2012). Western blot data showed that Hpo^{KD} could be phosphorylated by Hpo^{T195E}, but not by Hpo^{T195A} (Figure 3-6). This result indicates that Hpo is trans-phosphorylated by its binding partner within the homo-dimer. Mutagenesis and western blot were done by with Yurika Matsui.
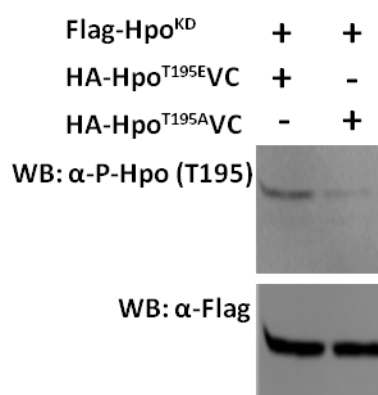
Figure 3-6 Hpo was activated by trans-phosphorylation. Hpo^{KD} was trans-phosphorylated by Hpo^{T195E} and endogenous Hpo proteins (Deng et al., 2013). This experiment was done by Yurika Matsui.

3.6.3 Phosphorylation is not required for Hpo dimer formation

I further explored whether Hpo phosphorylation is crucial for Hpo dimerization. In this experiment, two evolutionarily Hpo phosphorylation sites (Thr 189 and Thr 195) were mutated to Ala or Glu (Glantschnig et al., 2002). These Hpo variants were then fused with VC or VN and their interactions were analyzed by BiFC assay. The result shows that none of these mutations had obvious influence on the BiFC signal (Figure 3-7 A-G), indicating that phosphorylation is not required for Hpo to be dimerized.
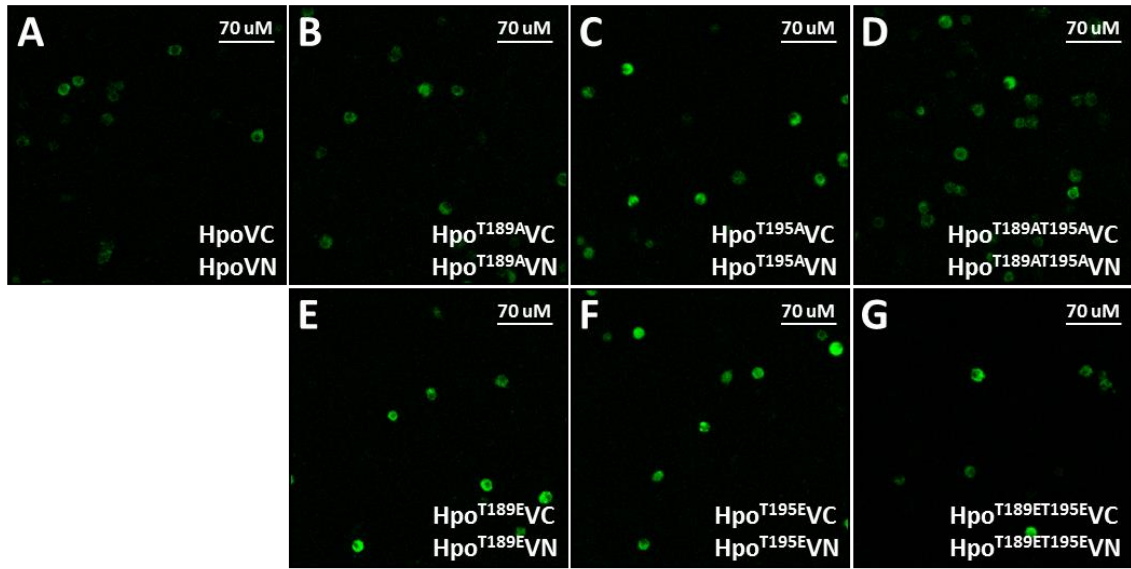
Figure 3-7 Phosphorylation is not required for Hpo to form a homo-dimer. (A) Hpo VC can form a dimer with HpoVN (B-G) Mutation of Thr189 and Thr195 auto-phosphorylation sites to Ala or Glu does not influence the Hpo BiFC signal (Deng et al., 2013).

3.7 Chapter summary

In this section, I explored the Hpo activity regulation mechanism by taking advantage of observing protein-protein interaction directly using the BiFC technique in *Drosophila* living tissues. This work demonstrated that Hpo kinase could be activated through trans-phosphorylation by forming a homo-dimer, while phosphorylation is not necessary for Hpo kinase to be dimerized. Importantly, this mechanism is conserved from *Drosophila* to mammals. Mst1 and Mst2, which are mammalian homologs of *Drosophila* Hpo protein, are also activated through dimerization and thus become auto-phosphorylated in response to upstream signaling (reviewed by de Souza and Lindsay, 2004). Moreover, my observation indicates that a kinase-dead version of Hpo could interact with a wild-type Hpo to form a Hpo^{KD}-Hpo^{WT} dimer, and this explains the dominant-negative effect of the Hpo kinase dead protein.

Chapter 4

Regulation of Hpo kinase by upstream molecules

4.1 Introduction

In the previous section, I demonstrated the mechanism by which Hpo kinase is activated through auto-phosphorylation via forming a homo-dimer. However, crucial questions remain about how Hpo activity and its dimerization are regulated by upstream signals.

Kibra, Expanded (Ex) and Merlin (Mer) have been identified as Hpo upstream molecules genetically, which transmit signals from the extracellular to the core components of Hpo signaling (reviewed in Grusche et al., 2010). These three proteins form a complex located in the sub-apical region of epithelia cells. Hpo kinase appears to be activated by the Kibra/Ex/Mer (KEM) complex, although by which method among these three proteins transmits the signal was still unclear. In addition, previous studies suggest that to recruit the core complex to the plasma membrane is important for activating the Hpo pathway (Grusche et al., 2010), and multiple interactions have been found between the KEM complex and the core components of the Hpo pathway (reviewed in Pan et al., 2013). By taking these evidences into account, I propose that the KEM complex might regulate Hpo kinase activity by recruiting Hpo to the plasma membrane. Moreover, since none of the Kibra/Ex/Mer proteins are kinases, the membrane recruitment might regulate Hpo kinase activity by influencing the protein's own dimerization and auto-phosphorylation.

4.2 Detect subcellular localization of Hpo and Hpo dimer in developing tissues

In order to study the influence of the KEM complex on Hpo and Hpo dimer sub-cellular localization, I first investigated the localization of Hpo and Hpo dimer under normal conditions.

Total Hpo localization was detected by immunostaining third-instar larva wing discs from wild-type flies and transgenic flies carrying over-expressed full-length Hpo. Both endogenous Hpo and Flag-tagged Hpo showed a portion of protein associated with the plasma membrane, while most of the proteins were found in the nucleus and cytoplasma (Figure 4-2 A-F, white arrow indicates one example of membrane-associated Hpo). The membrane association is indicated by co-localization with the adherent junction marker E-cadherin (E-cad). Furthermore, the Hpo dimer localization was detected by BiFC assay *in vivo* in third-instar larva wing discs. Similar to the total Hpo protein, the Hpo dimer (BiFC signal) was observed to be broadly distributed as well (Figure 4-1 A-I). Membrane association is indicated by co-localization with the F-actin staining marker (phallotoxins), and nucleus localization is indicated by co-localization with the nucleus marker (DAPI). However, compared with the total Hpo protein, the Hpo dimer showed more peripheral localizeation (Figure 4-2 G-N, white arrow indicates one example).

In order to illustrate how Hpo dimer activity is influenced by its subcellular localization, I highlighted the proliferating cells by immunostaining with phosphorylated-Histone 3 (PH3). The result showed that in all the PH3-positive cells (n=89), the Hpo dimer (BiFC signal) is found in the nucleus, suggesting that nucleus-localized Hpo dimer is not able to inhibit cell proliferation (Figure 4-1 D-I, white arrow shows one example).
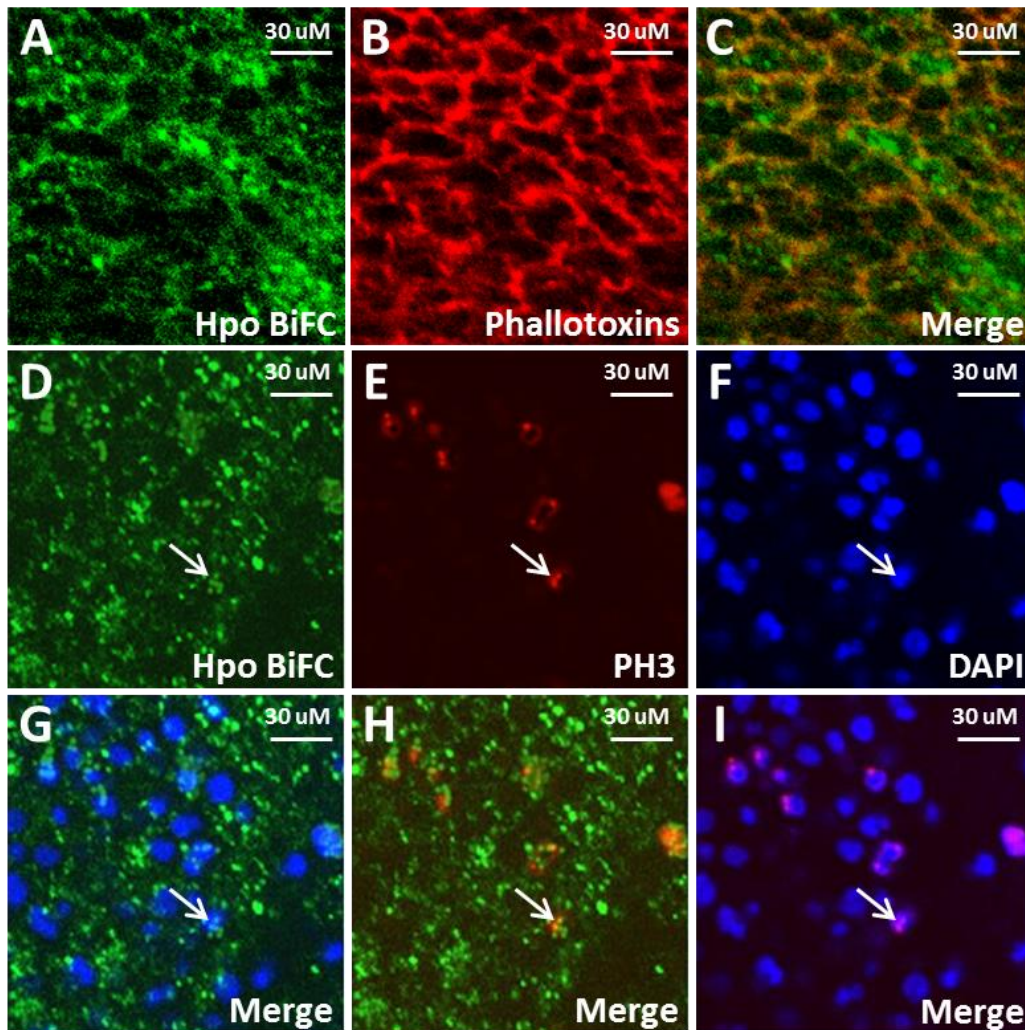
Figure 4-1 Subcellular localization of Hpo homo-dimer in developing tissues (A-C) Hpo dimer localization is shown by Hpo-Hpo BiFC (green) at the apical region of cells in larval wing discs. Phallotoxin was used to highlight the cell membrane region (red). (D-I) In the proliferating cells labeled by PH3 staining (red), Hpo BiFC signal (green) was always found in the nucleus. DAPI staining highlighted the nucleus (blue). Arrows point out one example. Expression of tagged *hpo* transgenes was driven by *en-Gal4* in the posterior compartment of late third-instar larval wing discs (Deng et al., 2013).
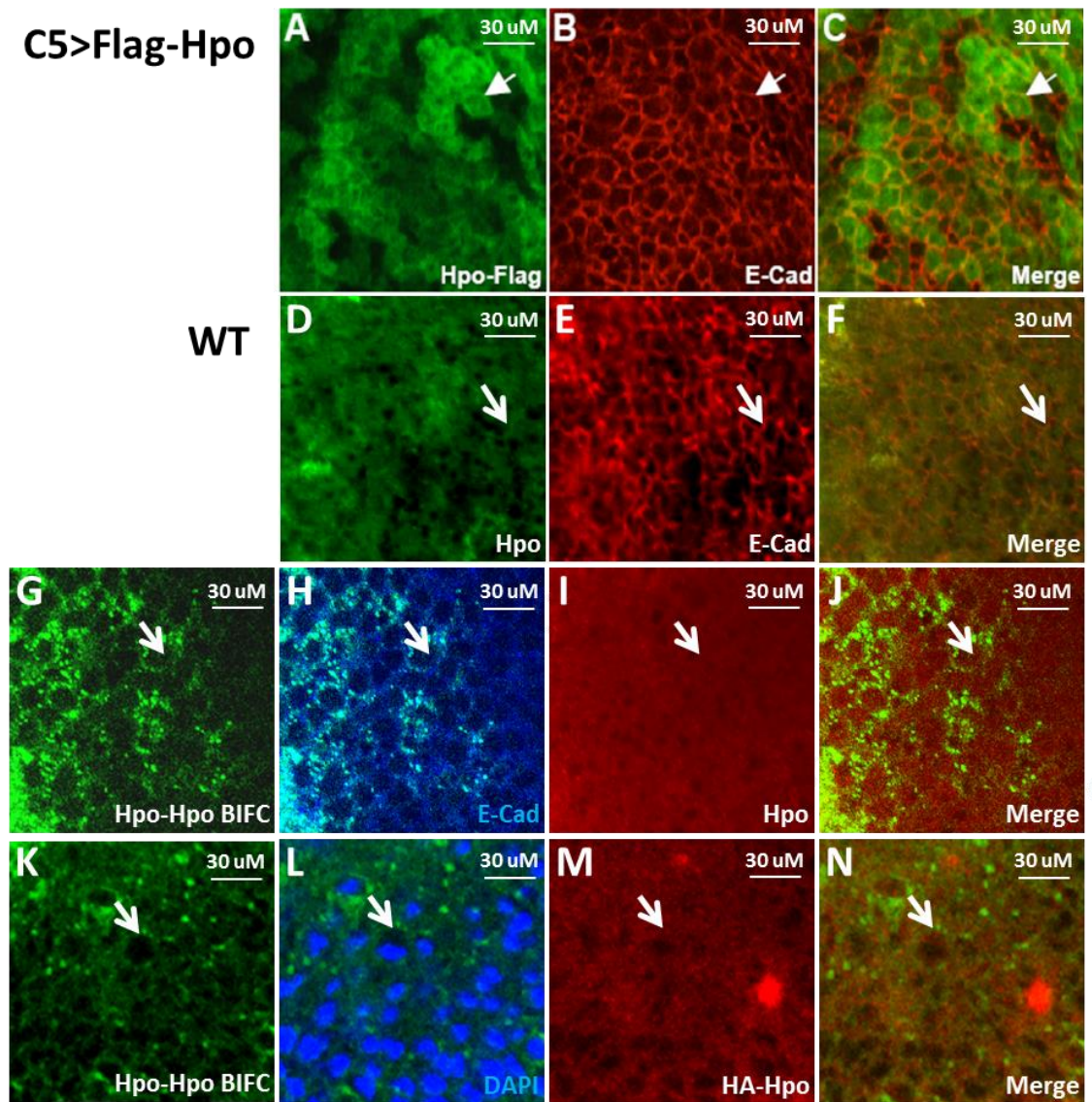
Figure 4-2 Detection of Hpo and Hpo dimer in developing wing tissues. (A-C) Flag-tagged Hpo (green) was stained in C5-Gal4/UAS-Flag-hpo third-instar larval wing discs. Arrows identify a single cell. (D-F) Endogenous Hpo (green) was detected in wild-type larval wing discs. Arrows identify a single cell. DE-cadherin (red) in (A-F) was a marker for the plasma membrane. (G-N) While endogenous Hpo and HA-Hpo (red) are usually distributed throughout the cell, Hpo dimer (green) shows a more membrane-associated pattern in many cells. The BiFC signal was generated in *en-Gal4/UAS-hpoVC hpoVN* larval wing discs. DE-cadherin and the nucleus were detected in blue in (H) and (L), respectively. Arrows identify some specific examples of more membrane-associated pattern for Hpo dimer compared to total Hpo protein (Deng et al., 2013).

4.3 Membrane targeting is crucial for Hpo kinase phosphorylation and activity

My previous experiment indicated that Hpo dimer activity is influenced by its sub-cellular localization. In addition, previous work by our lab has shown that the plasma membrane is a crucial site for Mats (one of the Hpo pathway's core components) activation (Ho et al., 2010). Thus, I proposed that membrane-targeting is also important for Hpo to be activated.

To test this idea, I generated HpoVN/HpoVC containing a myristoylation (Myr) signal sequence in the Hpo N-terminus for membrane localization. Myristoylation is a co-translational protein modification, which leads the modified protein to the plasma membrane (Ho et al., 2010). In this study, I added the peptide MGNCLTTQKGEPDKPA at the N-terminus of target proteins to facilitate their myristoylation.

The Hpo kinase activity was then detected by western blot against phosphorylated Hpo. The result shows that membrane-targed Hpo (Myr-HpoVN) was more phosphorylated compared with wild-type Hpo (Figure 4-3 A, right panel shows quantification of the western blot result). Furthermore, a luciferase reporter assay shows that membrane-targeted Hpo (Myr-HpoVN) was more potent at repressing the transcription induced by Yki/Sd than unmyritoylated Hpo (HpoVN) (Figure 4-3 B, ** indicates significant differences, $p < 0.01$ by student t-test). These data support the idea that cell membrane association is crucial for Hpo to be phosphorylated and activated.
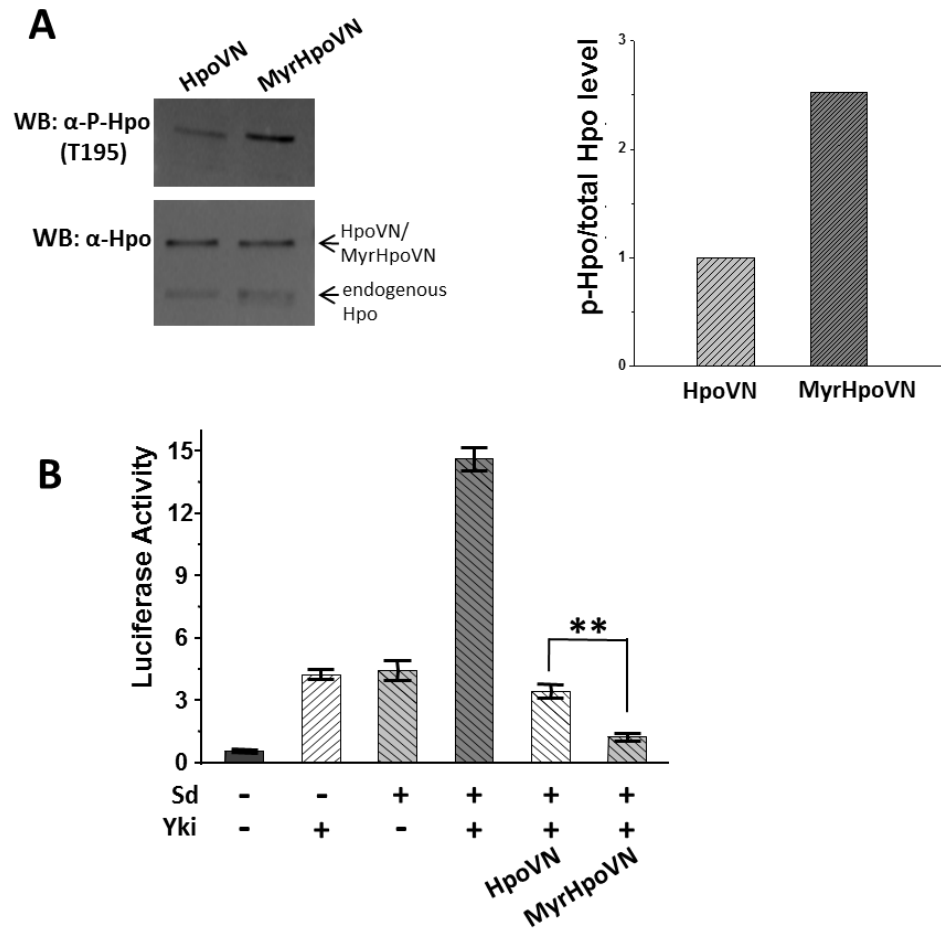
Figure 4-3 Membrane targeting facilitates Hpo phosphorylation and activation (A) MyrHpoVN was more phosphorylated at Thr195 than non-myristoylated Hpo. Right panel shows western blot quantification by ScanStorm software. (B) A luciferase reporter assay indicates that myristoylated Hpo was more potent for reducing Yki/Sd transcription activity than non-myristoylated Hpo. All the experiments were done with S2R+ cells. Data show mean $\pm$ SD of three independent experiments, ** indicates p<0.01 by student t-test (Deng et al., 2013).

4.4 Membrane-targeting facilitates Hpo dimerization

I have demonstrated in the previous chapter that Hpo kinase is activated through auto-

phosphorylation by forming a homo-dimer. Thus, I propose that membrane targeting could

facilitate Hpo activation by influencing its dimerization. A BiFC assay was utilized to indicate

Hpo dimerization efficiency. Interestingly, Myr-HpoVC and Myr-HpoVN failed to give out BiFC

signals (data not shown), suggesting either that they failed to associate with each other when both

are forced to the membrane with a designed space structure, or that they did associate but VN and

VC failed to complement. However, Myr-Hpo did form a dimer with unmyritoylated Hpo (Figure

4-4 A, HpoVC and Myr-HpoVN was used in the experiment). Importantly, the BiFC assay

showed that Myr-HpoVN/HpoVC had a higher efficiency at forming Hpo dimer than

HpoVN/HpoVC pair (Figure 4-4 B, compare HpoVC/HpoVN with HpoVC/MyrHpoVN, **

indicates p<0.01 by student t-test). The Hpo dimerization efficiency was indicated by BiFC signal

measured by flow cytometry. The result indicates that membrane-targeting facilitates Hpo
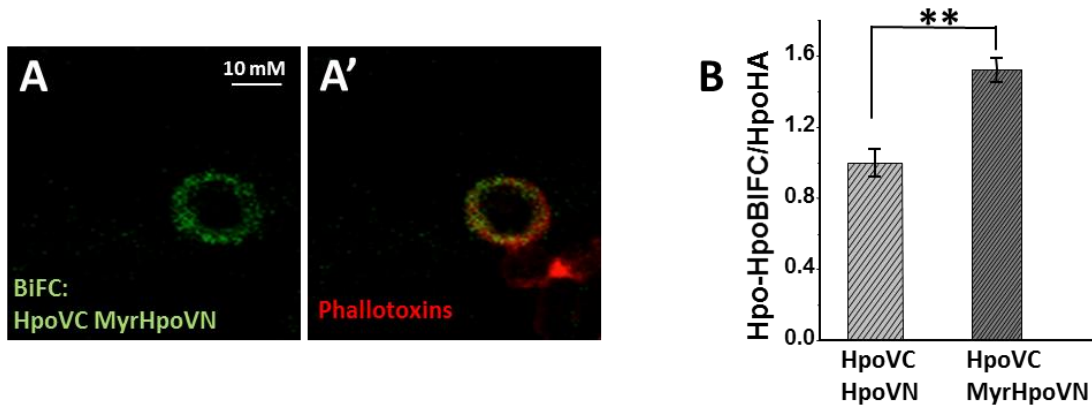
dimerization.

Figure 4-4 Membrane targeting facilitates Hpo dimerization (A) Myristoylation (Myr)-tagged HpoVN can interact with wild-type HpoVC and generate BiFC signal in the cell membrane region (green) of transfected *Drosophila* S2R+ cells. (B) MyrHpoVN was more effective at forming Hpo dimer compared with HpoVN. Through flow cytometry, Hpo dimer formation was measured by BiFC levels, and the BiFC signal was normalized with HA-Hpo expression levels. Data show mean $\pm$ SD of three independent experiments, ** indicates p<0.01 by student t-test (Deng et al., 2013).

4.5 Kibra, Ex and Mer act together to regulate Hpo dimer formation

Kibra, Ex and Mer were demonstrated to function together in regulating Hpo signaling in a partially redundant manner (Hamaratoglu et al., 2006; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). However, none of these proteins is kinase, which is able to phosphorylate Hpo directly. Therefore, I proposed that Kibra, Ex and Mer might regulate Hpo activity by influencing its own dimerization.

To test this idea, RNA interference (RNAi) was used to knock down endogenous Kibra, Ex and Mer in S2R+ cells. Western blot results showed that the RNAi was effective in knocking down these genes (Figure 4-6). mCherry RNA (not *Drosophila* derived) was used as the control RNA. This western blot analysis was done by Yurika Matsui.
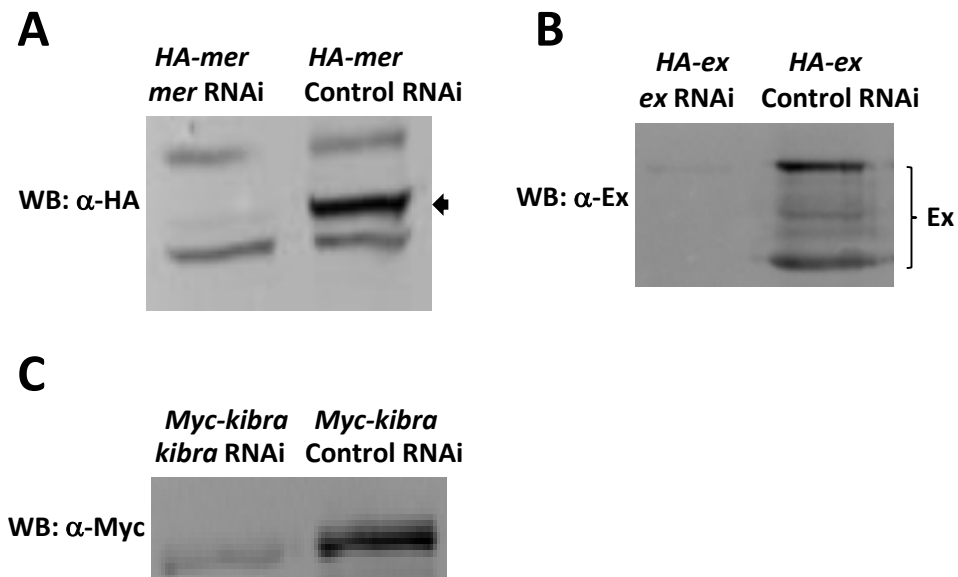
Figure 4-6 RNAi efficiency test for Kibra, Ex and Mer. *Drosophila* **S2R+ cells were transfected with** *HA-mer*, *HA-ex*, **or** *Myc-kibra*. **Specific RNAi treatment was done in order to abolish or reduce expression of these genes. The results show that the RNAi treatment can be effective in decreasing Mer, Ex, and Kibra expression levels in S2R+ cells. mCherry RNAi was used as the control RNAi (Deng et al., 2013). This data was done by Yurika Matsui.**

The Hpo dimerization was then tested by BiFC assay. The efficiency of Hpo dimerization was indicated by BiFC signal measured by flow cytometry. The result showed that both Mer/Ex double knocking out and Mer/Ex/Kibra triple knocking out could reduce the Hpo-Hpo BiFC signal significantly (Figure 4-7 A, left panel, student t-test shows significant differences among each group, ** indicates $p<0.01$ by student t-test). In contrast, the Hpo-Sav BiFC signal was not influenced by Kibra/Ex/Mer triple knocking down (Figure 4-7 A, right panel, student t-test shows insignificant differences, $p>0.5$). mCherry RNA was used as control RNA in Figure 4-7 A. On the other hand, when overexpressing Ex and Mer, the Hpo-Hpo BiFC signal was increased significantly (Figure 4-7 B, left panel, student t-test shows significant differences between Ctrl and Mer, Ex overexpression, ** indicates $p<0.01$ by student t-test), whereas the Hpo-Sav interaction was not changed significantly under the condition of Mer, Ex overexpression (Figure 4-7 B, right panel, student t-test shows insignificant differences between Ctrl and Mer, Ex overexpression, $p>0.05$). An empty vector was used as control DNA for Figure 4-7 B. There data support the idea that Kibra, Ex and Mer can regulate Hpo dimer formation.
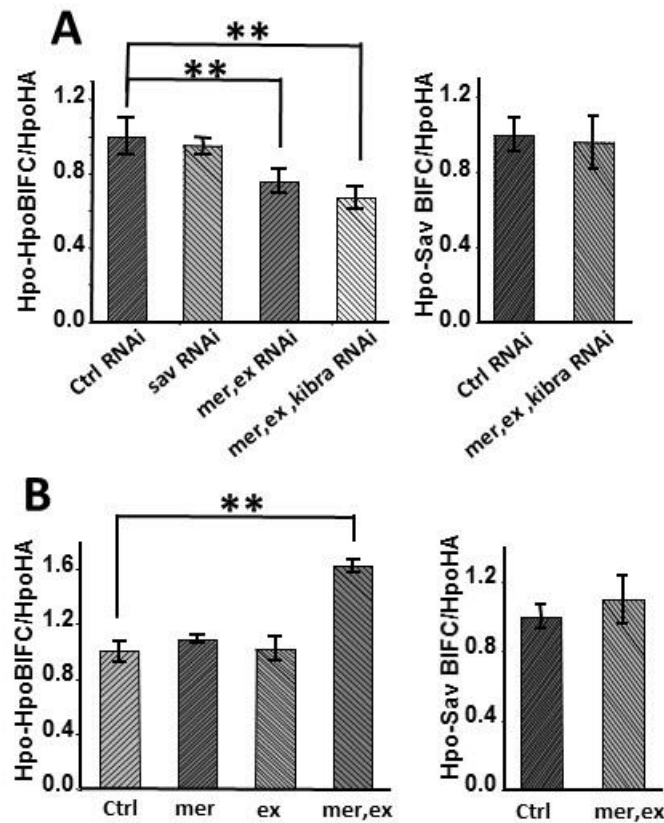
Figure 4-7 Mer, Ex and Kibra can regulate Hpo dimerization (A) Knocking down Mer, Ex and Kibra decreased Hpo dimerization but had no obvious effect on Hpo-Sav complex formation in *Drosophila* S2R+ cells. BiFC signal was measured by flow cytometry, and BiFC levels were normalized with HA-Hpo expression levels. Data show mean $\pm$ SD of three independent experiments, ** indicates p<0.01 by student t-test. (B) Over-expression of Mer and Ex together in S2R+ cells significantly increased Hpo homo-dimer but not Hpo-Sav complex formation. Data show mean $\pm$ SD of three independent experiments, ** indicates p<0.01 by student t-test. (Deng et al., 2013)

4.6 Kibra, Ex and Mer act together to regulate Hpo dimer sub-cellular localization

My previous experiment shows that Hpo dimerization efficiency and Hpo activity can be regulated by Hpo sub-cellular localization. However, my preliminary data shows that Ex and Mer do not have obvious effects in regulating total Hpo protein localization (data not shown). Thus I proposed that Ex, Mer and Kibra might regulate Hpo dimer sub-cellular localization.

To test this idea, I arbitrarily classified the distribution of Hpo dimer (indicated by BiFC signal) into three patterns: membrane-associated, cytoplasm, and cytoplasm + nucleus (Figure 4-8 A), and I examined the Hpo dimer localization in S2R+ cells under different conditions. The results indicated that the portion of membrane-associated Hpo dimer decreased when endogenous Kibra, Ex and Mer were knocked down, while the cytoplasm + nucleus localized Hpo dimer increased (Figure 4-8 B, n=432). On the other hand, when Kibra, Ex and Mer were over-expressed, the membrane-associated Hpo dimer increased, while the cytoplasm + nucleus localized Hpo dimer decreased (Figure 4-8 C, n=523). I further examined the Hpo dimer localization in wing discs of third-instar larva. The result showed that Hpo dimers were more nucleus localized under Ex and Mer knocking down compared with normal conditions. Ex and Mer knocking down was achieved by expressing ExRNAi and MerRNAi specifically in wing discs, while the same amount of dsRed was expressed in the control group. Nucleus-localized Hpo dimer was indicated by co-localization of the BiFC signal with the nucleus marker (DAPI). In the previous section, I demonstrated that Hpo dimer has lower activity in restricting tissue growth when it is nucleus-localized. Thus these results together support the idea that Kibra, Ex and Mer can recruit Hpo dimer to the plasma membrane and thus facilitate its activation.
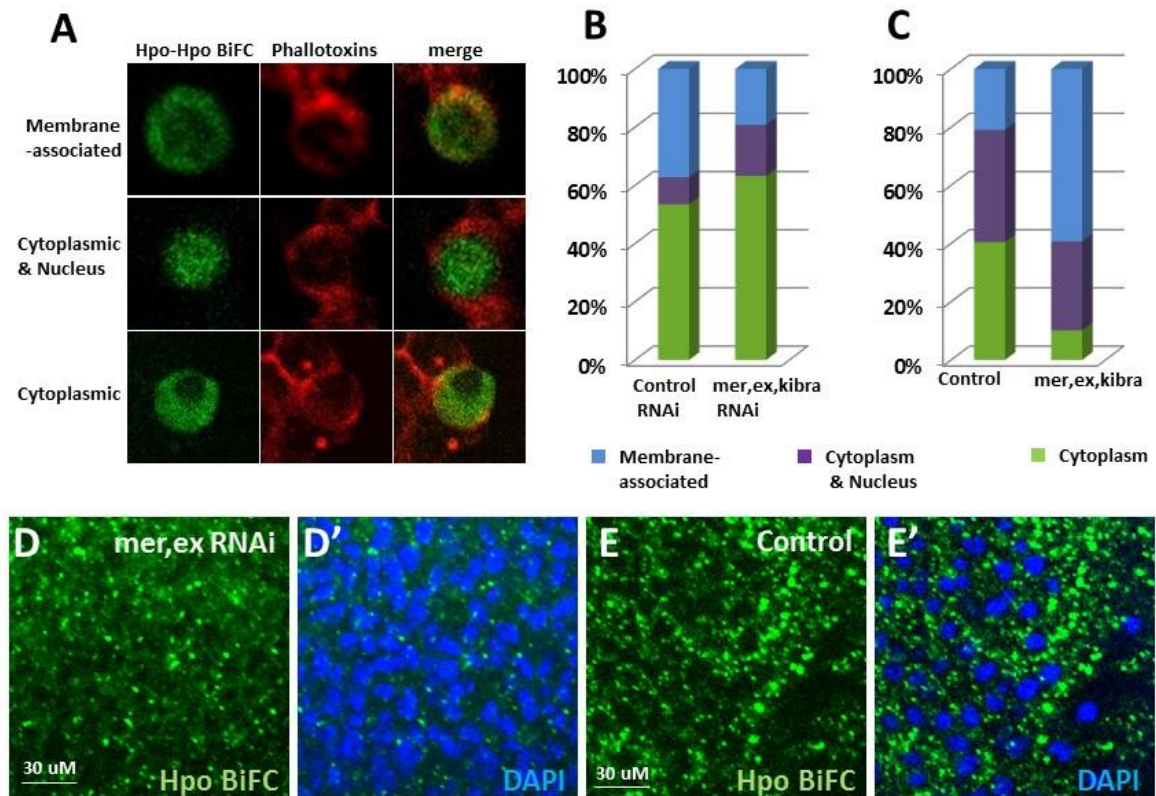
Figure 4-8 Mer, Ex and Kibra act together to regulate Hpo dimer subcellular localization (A) Classification of Hpo dimer subcellular localization patterns in S2R+ cells. (B) Statistical analysis to illustrate subcellular localization patterns of Hpo homodimer in S2R+ cells. Knocking down Ex, Mer and Kibra decreased membrane-associated Hpo dimer, whereas over-expression of Mer/Ex/Kibra increased Hpo dimerization efficiency in S2R+ cells (C). (D-D') Knocking down *ex* and *mer* by RNAi moderately increased nucleus-associated Hpo dimer (green) in larval wing discs. (E-E') As a control, BiFC signal (green) was found to be less associated with the nucleus in *C5-Gal4/UAS-HpoVC HpoVN* larval wing discs. Hpo dimer in the nucleus was identified by co-localization with DAPI (blue) (Deng et al., 2013).

4.7 Chapter summary

In this section, I demonstrated the regulation mechanism by which the Kibra/Ex/Mer complex influences Hpo activity. I found that Hpo kinase activity is affected by its sub-cellular localization, and the Kibra/Ex/Mer complex regulates Hpo activity by recruiting Hpo to the plasma membrane.

I discovered that membrane association increases Hpo's activity, and membrane-targeted Hpo shows higher efficiency in forming a dimer compared with wild-type Hpo. Interestingly, knocking down of Ex, Mer and Kibra suppresses Hpo membrane association, while overexpression of Ex, Mer and Kibra promotes Hpo membrane association. Consistently, Ex, Mer and Kibra work together to positively regulate Hpo dimerization efficiency as well. These observations support the mechanism in which membrane association increases Hpo dimerization efficiency, thus promoting Hpo activation, and the kibra/Ex/Mer complex regulates Hpo dimerization via influencing Hpo membrane association to promote Hpo kinase activity.

In sum, in this project, I conclude that homo-dimerization and membrane association are both crucial for the activation of Hpo kinase. My discoveries support the model wherein the Kibra/Ex/Mer complex recruits Hpo to the plasma membrane, which facilitates its dimerization, and the membrane association promotes Hpo kinase activation (Figure 4-9). This model brings significant insights to understanding how upstream molecules transduce extracellular signals to the Hpo pathway via the Kibra/Ex/Mer complex.
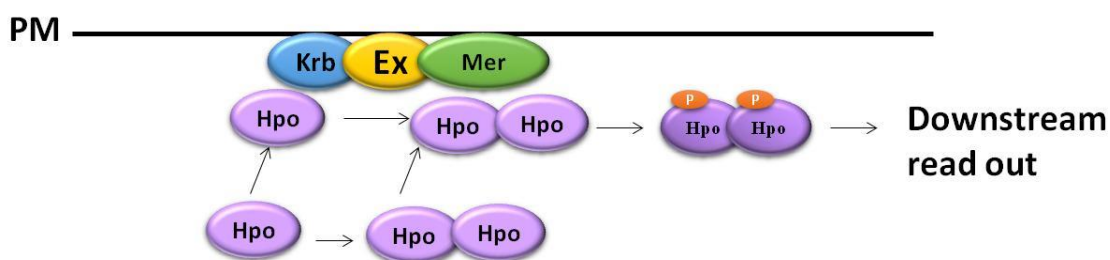
Figure 4-9 Hpo kinase activity regulation model. Hpo is activated by trans-phosphorylation during homo-dimer formation. Homo-dimerization and membrane association are both crucial for Hpo kinase to be activated. The Kibra/Ex/Mer complex recruits Hpo to the plasma membrane, which facilitates its dimerization, and the membrane association promotes Hpo kinase activation.

Chapter 5

Yap1 inhibits FFA-induced beta cell apoptosis

5.1 Introduction

β-cells in the mammalian pancreas are responsible for insulin homeostasis. Chronically increased concentration of plasma free fatty acid (FFA) might contribute to type 2 diabetes by causing both insulin resistance and β-cell failure (Boden, 2008; Boden and Shulman, 2002). Under diabetic conditions, β-cells fail to secrete enough insulin to compensate for insulin resistance in peripheral cells due to β-cell dysfunction (Haber et al., 2003; Lupi et al., 2002) and β-cell death (Shimabukuro et al., 1998; Lupi et al., 2002). However, the mechanism of FFA-induced β-cell apoptosis is still not clear. Studies of FFA receptors have brought a new perspective to address FFA-induced dysfunction and apoptosis of β-cells. GPR40, which belongs to the G-protein-coupled receptor (GPCR) family, has been identified as a long chain FFA receptor expressed preferentially in β-cells and is related to glucose homeostasis regulation and insulin secretion (Itoh and Hinuma, 2005; Tomita et al., 2006). GPR40 is also responsible for FFA-induced β-cell apoptosis and in cultured human and mouse cells, and GPR40 knockdown can rescue FFA-induced apoptosis (Itoh et al., 2003; Salehi et al., 2005; Tomita e al., 2006). However, FFA-initiated GPCR signaling still needs to be further characterized. YAP has been identified as a GPCR effector through F-actin dynamics under specific conditions in mammalian systems recently (reviewed in Yu et al., 2013). Thus, in this study I explored the role of Yap1 and F-actin in regulating β-cell survival under FFA-treated conditions.

[All the data in Chapter 5 were generated by Yaoting Deng]

5.2 Palmitate can induce apoptosis in rat β-cells

Previous studies have shown that high concentrations of saturated FFAs (such as palmitate) cause more marked apoptosis, while the toxicity of unsaturated FFAs (such as oleate) is much less (Karaskov et al., 2006; Eitel et al., 2002). In this project, in order to study FFA-induced apoptosis, I examined the toxicity of high concentrations of palmitate in rat β-cell INS-1 832/13.

INS-1 832/13 cells were starved from the serum for 12 hours, following different concentrations of palmitate treatment. After 24 hours of palmitate treatment, the cells were harvested and apoptosis was measured through an Annexin V based assay. Annexin V binds to phosphatidylserine (PS) presented on the outer leaflet of the plasma membrane of apoptotic and dead cells, whereas propidium iodide (PI) is a nucleic acid dye and can only stain dead cells due to their disrupted membrane. Therefore, apoptotic cells can be identified as Annexin V-positive and PI-negative cells. Each experiment was repeated at least three times. One hundred thousand cells were analyzed by flow cytometry for each sample. Within one experiment, each group had four parallel repeats, and the average of the four parallel samples was used as final data.

The rat β-cells were treated with plamitate from 0.2mM to 0.5mM. Ethanol (palmitate solvent) was used as the palmitate control. The result indicated that palmitate showed slight toxicity under 0.2mM; however, palmitate induced obvious apoptosis when its concentration was higher than 0.3mM (Figure 5-1).
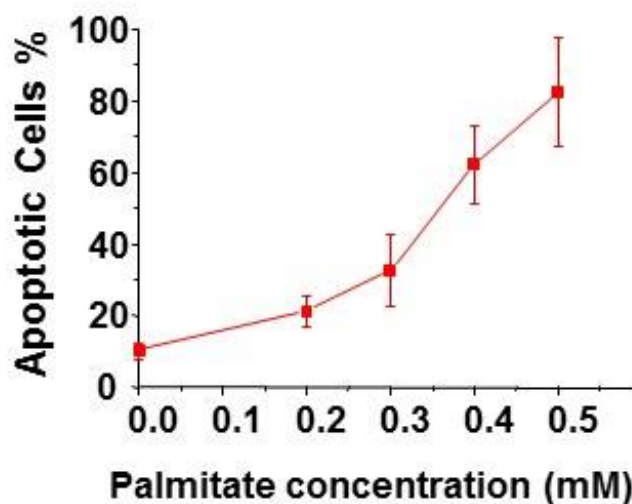
Figure 5-1 Palmitate can induce apoptosis in rat β-cells. INS-1 832/13 cells were treated with different concentrations of palmitate for 24 hours after 12 hours serum starvation. The apoptotic cells were measured by flow cytometry after Annexin V staining. Apoptotic cells were recognized as Annexin V positive and PI negative cells. Data shows the mean $\pm$ SD of four independent experiments. Ethanol was used as negative control.

5.3 F-actin dynamics are critical for rat β-cells viability under palmitate treatment

F-actin appears to be regulated by FFA and glucose, and is critical for maintaining β-cell metabolic function (Kalwat and Thurmond, 2013). The long-chain FFA receptor GPR40 might regulate F-actin remodeling through Gαq/11 to promote glucose-stimulated insulin secretion in normal β-cells (Ferdaoussi, et al., 2012). However the role of F-actin during FFA-induced apoptosis in β-cells is still unclear. In order to test whether F-actin plays a role in mediating the impact of palmitate on β-cell viability, I examined the F-actin level under different conditions.

Rat β-cells were treated with 0.4mM palmitate for 24 hours, and ethanol was used as palmitate control. The immunostaining against F-actin (phallotoxin) showed that, compared with the control group, the F-actin staining intensity was increased after 24 hours of palmitate treatment (Figure 5-2 A). Then the quantification of F-actin level by flow cytometry was carried out to confirm this result. Immunostaining using phallotoxin was performed to different groups of

cells, and the F-actin level (indicated by the intensity of phallotoxin) was measured by flow cytometry. The F-actin level in the control group was normalized to 1 (Figure 5-2 B). The result showed that F-actin had accumulated significantly after palmitate treatment (Figure 5-2 B, compare the column in palmitate group with the control group. ** indicates P<0.01 by student t-test). In contrast, F-actin polymerization inhibitor Cytochalasin D (CytoD) suppressed the F-actin signal (Figure 5-2 B, compare the column in CytoD group with the control group, ** indicates p<0.01 by student t-test). 0.5 uM of CytoD was used as the literature recommended (Yu et al., 2013). Cell images were taken by Olympus FV-300 confocal microscopy. One hundred thousand cells were measured for each experiment. Three independent experiments were performed within each group.

I further studied whether F-actin dynamics are crucial for palmitate-induced apoptosis. CytoD was used to disrupt F-actin polymerization, and β-cell apoptosis was analyzed after different treatments. By Annexin V analysis, I found that CytoD could indeed inhibit palmitate-induced apoptosis of INS-1 832/13 cells when I treated the cells with CytoD together with palmitate for 24 hours (Figure 5-2 C shows 24 hr treatment, Figure 5-2 D shows Annexin V analysis under 24 hr treatment condition, ** indicates p<0.01 by student t-test). A western blot against activated Caspase 3 was performed to confirm this result (Figure 5-2 E). Therefore, F-actin plays a critical role in FFA-induced β-cell apoptosis. Interestingly, after a prolonged treatment with CytoD subsequent to palmitate induction (Figure 5-2 F shows 48 hr treatment), CytoD was no longer effective for this inhibition. Instead, it even enhanced the palmitate-induced apoptosis somewhat (Figure 5-2 G, compare palmitate group with palmitate + CytoD group, * indicates p<0.05 by student t-test). Analysis of activated Caspase 3 provides consistent results (Figure 5-2 H). Therefore, it is possible that a delayed response might provoke a protective mechanism to enhance β-cell viability after the initiation of palmitate-induced apoptosis, and this

mechanism might be also under the regulation of F-actin dynamics. For the Annexin V assay,

four independent experiments were performed in each group.
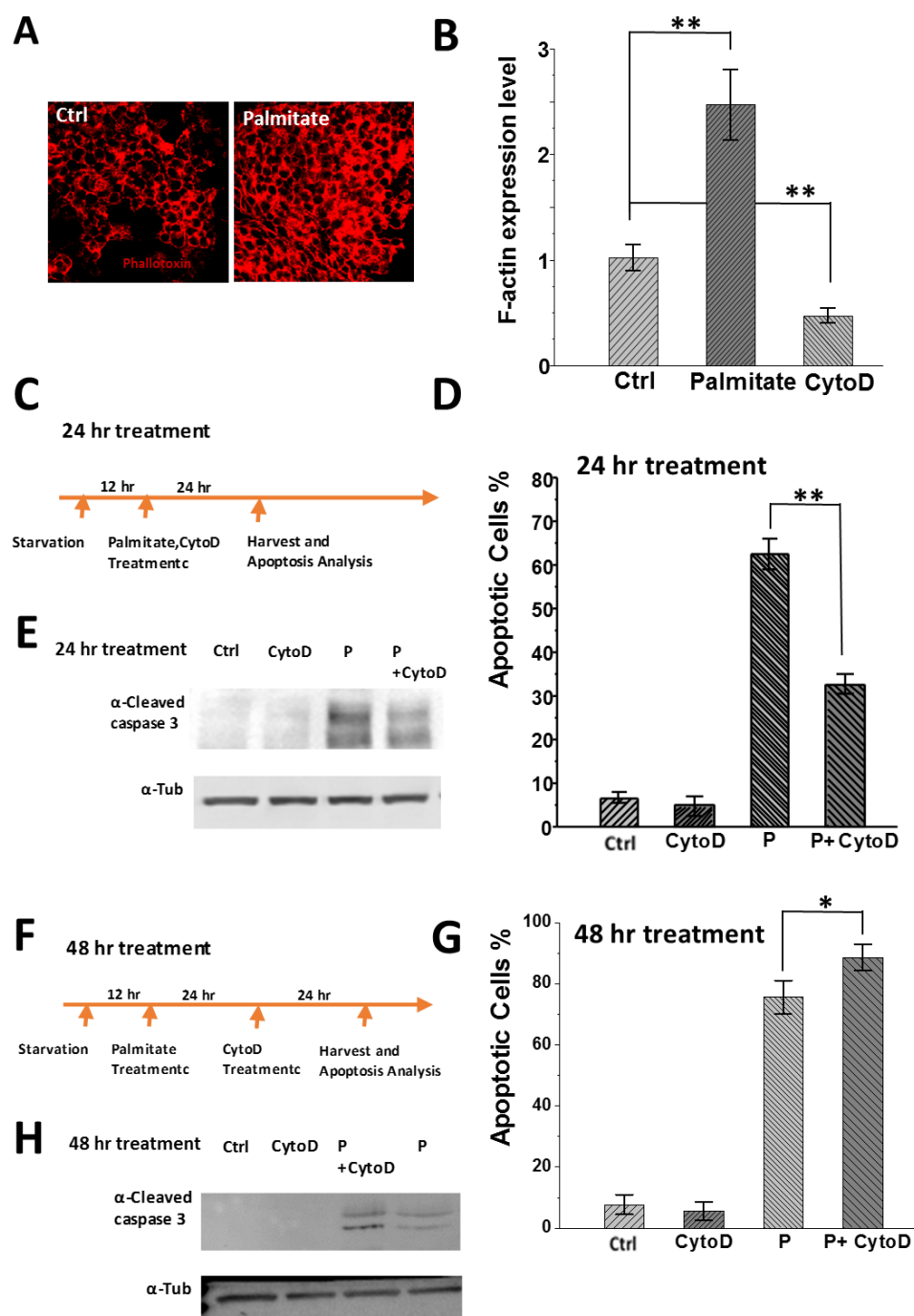
Figure 5-2 F-actin is critical for palmitate induced apoptosis. (A) Immunostaining data shows that F-actin accumulated after 24 hr palmitate treatment. (B) Quantification of F-actin shows that F-actin signal increased after 24 hr palmitate treatment, while CytoD repressed F-actin signal. F-actin quantification was measured by Flow cytometry after phallotoxin staining. Data shows the mean ± SD of three independent experiments. ** indicates p<0.01 by student t-test. (C) Time schedule for 24 hr treatment. (D) CytoD can inhibit palmitate-induced apoptosis under 24 hr treatment in Annexin V anaylsis. Data shows the mean ± SD of four independent experiments. ** indicates p<0.01 by student t-test. (E) Western blot targeting cleaved caspase 3 shows that CytoD inhibits palmitate-induced apoptosis under 24 hr treatment. (F) Time schedule for 48 hr treatment. (G) CytoD can slightly enhance palmitate-induced apoptosis under 48 hr treatment in Annexin V anaylsis. Data shows the mean ± SD of four independent experiments. * indicates p<0.05 by student t-test. (H) Western blot targeting cleaved caspase 3 shows that CytoD amplifies palmitate-induced apoptosis signal under 48 hr treatment. P: palmitate; CytoD: Cytochalasin D. Equals amounts of palmitate solvent ethanol and CytoD solvent DMSO were used as the control.

5.4 Yap1 is activated during palmitate-induced apoptosis in both rat and mouse β-cells

Previous experiments have shown that F-actin is involved in palmitate-induced apoptosis, and there might be a delayed protective mechanism preventing β-cell from palmitate-induced apoptosis, which is regulated by F-actin dynamics as well. Therefore, we want to explore the mechanism downstream of F-actin under palmitate treatment. Yap has been shown to be regulated by GPCRs through F-actin modulation under multiple conditions (reviewed in Yu et al., 2013 and Chapter 1.7-1.8). GPCRs might activate or inhibit Yap activity under different conditions depending on which Gα protein is involved (Codella and Irvine, 2012). Thus, I propose that Yap1 (rat Yap) might be involved during palmitate-induced apoptosis through FFA receptors. In order to test this idea, I have analyzed Yap1 activity regulation under palmitate treatment.

Rat β-cells INS-1 832/13 were treated with 0.3mM palmitate. After palmitate treatment, the β-cells were observed for apoptosis and Yap1 localization after 2, 4, 6, 8, 10, 12 and 24 hours. I found that the apoptosis signal began to increase gradually after 10 hours of palmitate treatment, and the apoptosis signal increased significantly after 24 hours (Figure 5-3 A). Yap1 was observed

to begin translocating from cytosol to the nucleus after 12 hours of treatment (data not shown). After 24 hours of palmitate treatment, Yap1 was observed to translocate into the nucleus in most β-cells (Figure 5-3 C-C'), whereas Yap1 was predominantly localized in the cytoplasm of untreated cells (Figure. 5-3 B-B'). These results indicate that Yap1 was activated after the initiation of palmitate-induced apoptosis. Moreover, a western blot against p-Yap1 (at the conserved Ser 127 site) also indicated that Yap1 was activated by dephosphorylation after 24 hours of palmitate treatment (Figure 5-5 C). Furthermore, Yap1 was observed to translocate to the nucleus under the 48-hour treatment conditions as well (data not shown). Rat β-cell apoptosis was analyzed by Annexin V assay. Yap1 nucleus localization was characterized by Yap1 co-localization with the nucleus marker Draq5 via immunostaining.

Similar observations were made with mouse MIN 6 β-cells (Figure 5-5 E-F'), indicating that it might be a general response for Yap1 to be activated after palmitate-induced apoptosis in mammalian β-cells. MIN 6 cells were treated with 0.5mM palmitate for 48 hours after 12 hours of serum starvation, in accordance with the literature (Zhang et al., 2007) to induce significant apoptosis (Figure 5-3 D, ** indicates $p<0.01$ by student t-test). The same amount of ethanol was used as a control.
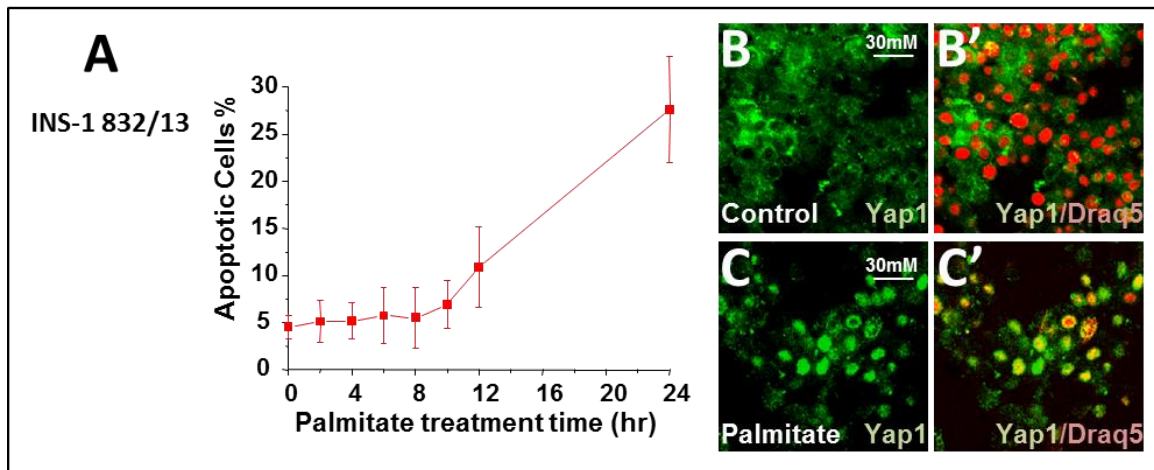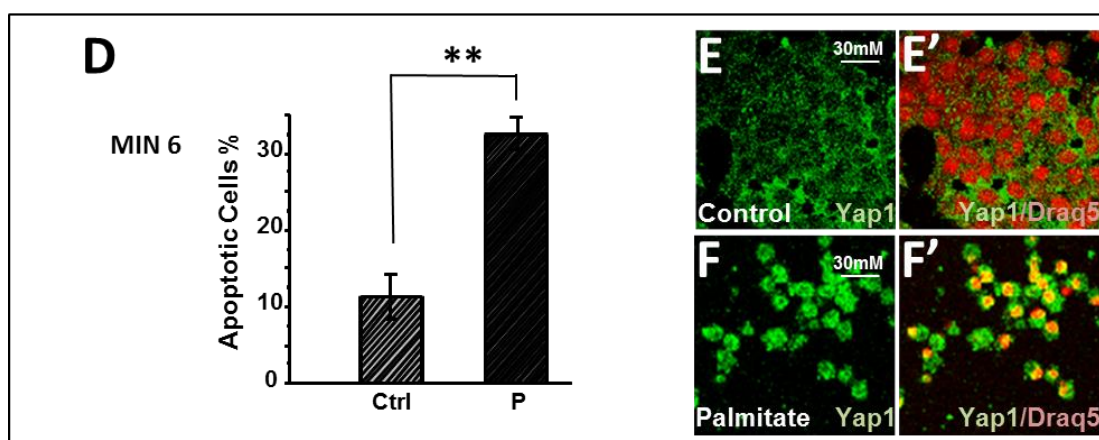
Figure 5-3 Yap1 is activated after palmitate treatment. (A) In rat β-cell INS-1 832/13, apoptosis signal begins to increase after 10 hr palmitate treatment. Apoptosis was measured at indicated time point via Annexin V assay. Data shows the mean ± SD of three independent experiments. (B-C) Immunostaining data shows that Yap1 translocates to the nucleus in most cells after 24 hr palmitate treatment. Draq 5 indicates nucleus staining. (D) In mouse β-cell MIN6, significant apoptosis is induced by 0.5 mM palmitate after 48 hr treatment. Apoptosis was measured by Annexin V assay. Data shows the mean ± SD of four independent experiments. ** indicates $p<0.01$ by student t-test. (E-F) In MIN6, Yap1 translocates to the nucleus in most cells after 48 hr palmitate treatment. Draq 5 indicates nucleus staining. Palmitate solvent ethanol was used as control for experiments shown in B-F. P: palmitate.

5.5 Yap1 positively regulates β-cell viability during palmitate-induced apoptosis

I showed in previous experiments that Yap1 is activated upon palmitate treatment. However, there remains the important question as to the role of Yap1 during palmitate-induced apoptosis. Yap1 has two main functions during development and the formation of some diseases. In most studies, people regard Yap1 as a positive regulator of tissue growth when it associates with the DNA transcription activator TEAD1, promoting cell proliferation and inhibiting apoptosis (Pan et al., 2007). However, under some disease conditions, Yap1 is observed to promote apoptosis when associating with the DNA coactivator P73 (Polesello et al., 2006; Hwang et al., 2007; Guo et al., 2007). In order to study Yap1 function during palmitate-induced β-cell

apoptosis, I knocked down Yap1 by Lentiviral-induced shRNA in INS-1 832/13 cells (Figure 5-4 A). The lentiviral vector carrying scribble RNA was used as a control.

The rat β-cells were treated with lentivirus for 4 days, and then Annexin V analysis was performed to detect apoptosis in different groups. The result showed that knocking down Yap1 induced apoptosis in INS-1 832/13 cells even without any other treatment (Figure 5-4 B, compared Yap1i group with Ctrli group, ** indicates p<0.01 by student t-test). Control shRNA showed that the apoptosis was not due to lentiviral infection (Figure 5-4 B, left column). Therefore, Yap1 plays a positive role and is required for β-cell survival. Using a phospho-Histone 3 (PH3)-specific antibody to recognize mitotic cells, I found that the reduction of Yap1 function had no significant effect on cell proliferation (Figure 5-4 C, compared Yap1i column with Ctrli column, student t-test shows statistic insignificant, p>0.05). I also performed a cell number counting experiment. The same number of cells were seeded for a Yap1 RNAi group and a control RNAi group in low density. After viral infection, the cells were counted every day to produce an increasing curve for cell number increase. The result showed that Yap1 prevented cell number increase compared to the control experiment (Figure 5-4 D). Thus, the lack of cell number increase in the absence of Yap1 function was likely due primarily to increased apoptosis. Importantly, when cells were treated with palmitate under 0.2mM for 24 hours, Yap1 reduction enhanced β-cell apoptosis significantly, further indicating that Yap1 indeed plays a protective role in β-cells treated with palmitate (Figure 5-4 E, compare Yap1i group with Ctrli group in palmitate column, ** indicates p<0.01 by student t-test). The cells in Figure 5-4 E were treated with virus for 2 days before palmitate treatment.
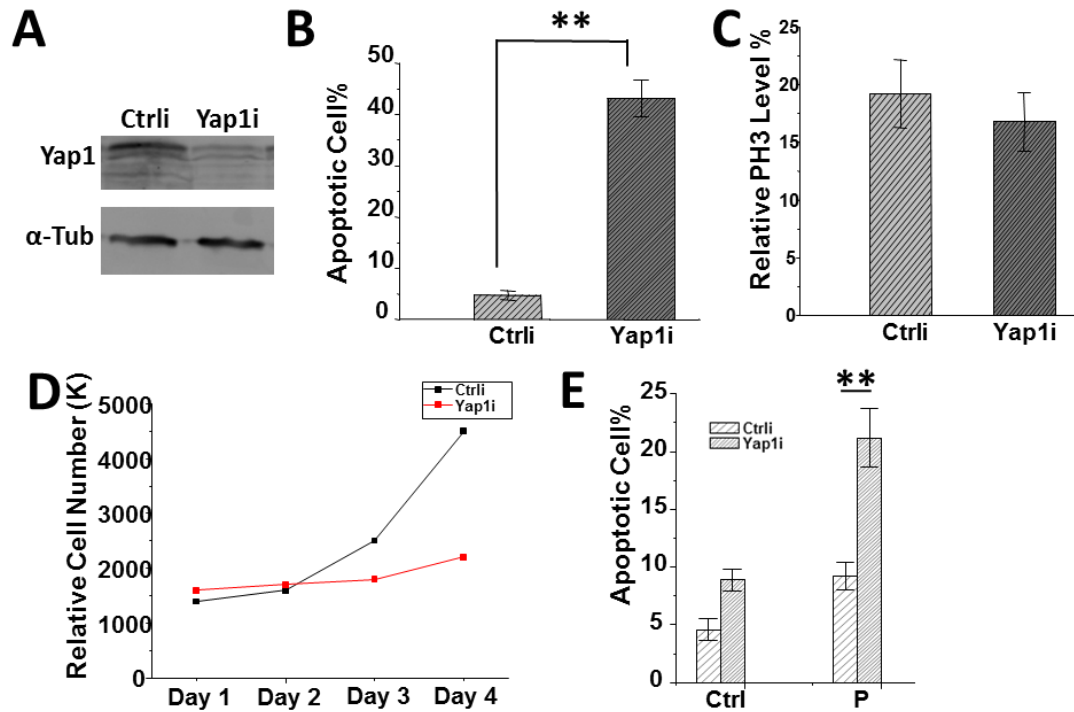
Figure 5-4 Yap1 positively regulates rat β-cell viability. (A) Western blot against Yap1 shows shRNA knocking down efficiency. **(B)** Yap1 knocking down induced β-cell apoptosis. The apoptosis was measured by Annexin V assay. Data shows the mean ± SD of three independent experiments. ** indicates $p<0.01$ by student t-test. **(C)** Knocking down of Yap1 does not obviously influence β-cell proliferation (student t-test shows statistic insignificance, $p>0.05$). PH3 quantification was measured by flow cytometry after staining with PH3 antibody. **(D)** Cell counting experiment shows that the increase of cell population was inhibited by knocking down Yap1. Cell numbers were counted every day after viral induction. **(E)** Yap1 knocking down amplifies apoptosis signal after 24 hr of palmitate treatment. The apoptosis was measured by Annexin V assay. Data shows the mean ± SD of three independent experiments. ** indicates $p<0.01$ by student t-test. For (A-E) Yap1i: Yap1 shRNA, Ctrli: scribble RNA, ethanol was used as palmitate control.

5.6 F-actin modulation regulates Yap1 activity

Lipid ligands might regulate Yap/Taz activity through the GPCR pathway in many cases (Yu et al., 2012; Yu et al., 2013; Codella and Irvine, 2012). F-actin polymerization was discovered to increase Yap/Taz activity downstream of GPCR (Yu et al., 2013). Therefore, I propose that Yap1 might be regulated by F-actin dynamics under palmitate treatment. To test

whether Yap1 responds to FFA signaling through F-actin, I used CytoD to block F-actin polymerization.

To do so, rat β-cells INS-1 832/13 were exposed to palmitate and CytoD using 24 hours of treatment, as described in Figure 5-2 C. The cells were harvested after 24 hours, and Yap1 localization was observed by immunostaining. The results showed that CytoD inhibited Yap1 translocation to the nucleus after palmitate treatment (Figure 5-5 A, B). Yap1 nucleus localization was indicated by co-localization with the nucleus marker (Draq5). A western blot against phosphorylated Yap1 (Ser27) also indicated that CytoD inhibited Yap1 activation by phosphorylation (Figure 5-5 C, right panel shows quantification of western blot). The western blot quantification was done by the software StormScan. These observations suggest that palmitate might regulate Yap1 activity through F-actin modulation.

Previous studies have shown that under certain circumstances F-actin dynamics can be regulated by Hpo signaling in mammalian cells, and in *Drosophila* it was also observed that Yki could regulate F-actin by controlling the expression of F-actin regulators (Fernández et al., 2011; reviewed in Matsui and Lai, 2013). To test whether Yap1 can also regulate F-actin dynamics and form a feedback loop in β-cells, I tested F-actin polymerization under the condition of knocking down Yap1. Yap1 knocking down was achieved by Lentiviral shRNA infection. The result showed that knocking down Yap1 did not influence F-actin dynamics significantly (Figure 5-5 D, compare Yap1i group with Ctrli, F-actin expression level of Ctrli group is normalized to 1, $p > 0.1$ by student t-test). Therefore, Yap1 does not appear to be used a feedback mechanism to influence the activity of F-actin in β-cells.
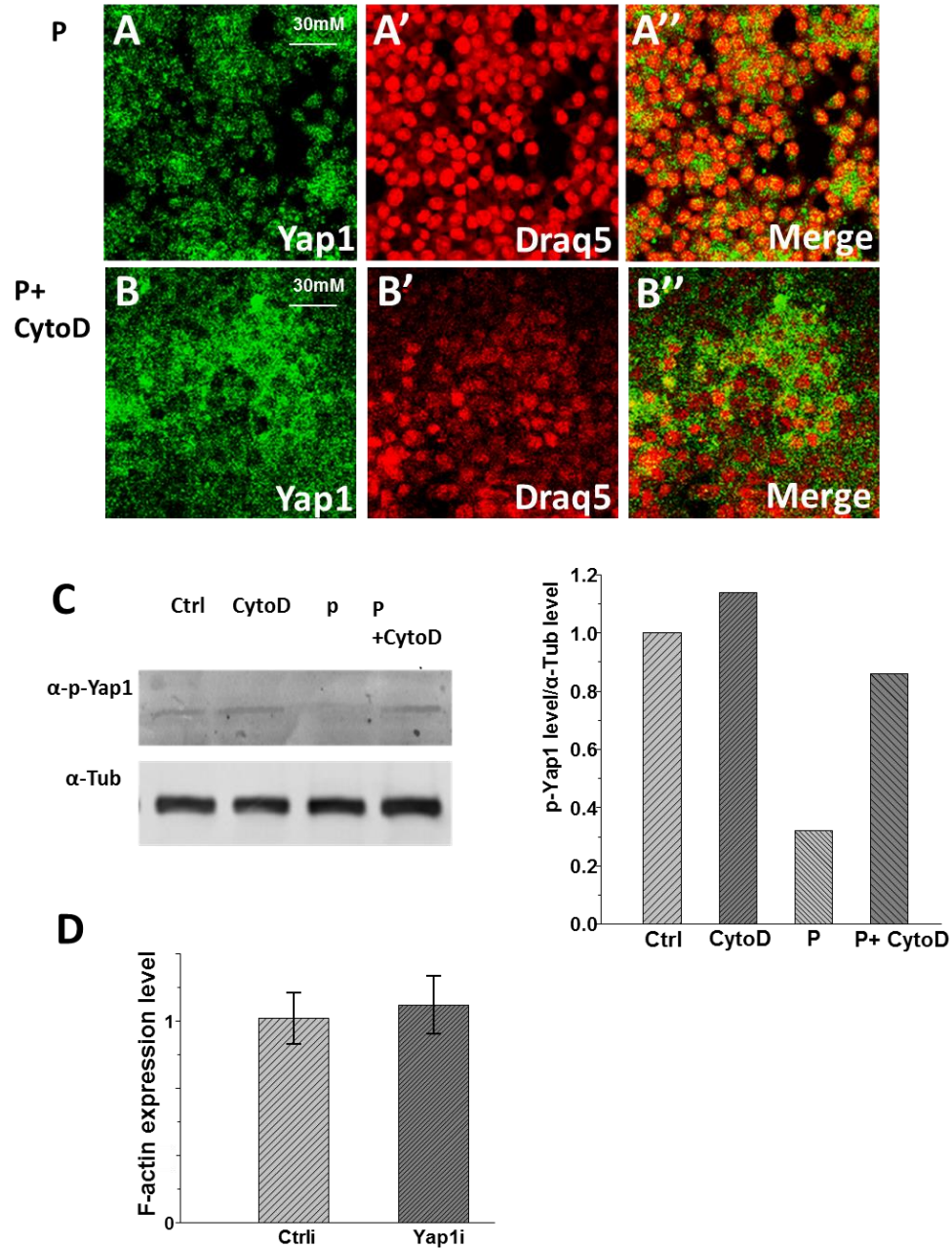
Figure 5-5 Yap1 activity is regulated by F-actin modulation. (A-B) CytoD inhibits Yap1 nucleus translocation under 24 hr palmitate treatment. Draq 5 indicates nucleus. **(C)** Western blot result shows that Yap1 is dephosphorylated under 24 hr palmitate treatment; however; this dephosphorylation is inhibited by CytoD. Right panel shows quantification of western blot by software ScanStorm. Tublin level is used as loading control. P: palmitate **(D)** Yap1 has no obvious influence on F-actin dynamics. F-actin was quantified by Flow Cytometry after phillotoxin staining. Ctrli: scribble RNA Yap1i: Yap1 shRNA. Data shows mean $\pm$ SD of four independent experiments. Student t-test shows insignificant differences (p>0.1). Ethanol and DMSO was used as control in the data (A-D).

5.7 The regulation of other upstream molecules under palmitate treatment

While the way in which GPCR signaling regulates Yap1 activity is not fully understood, several studies have revealed that it works differently from the canonical mammalian Hpo pathway (reviewed in chapter 1.6). It is studied that Lats1, rather than Mst1/2, is regulated by F-actin and is responsible for Yap1 activation under conditions such as cellular attachment and physical stretch (Codella and Irvine, 2012; Yu et al., 2012). Therefore, I propose that Lats1 but not Mst1/2 is involved in palmitate-mediated apoptosis.

INS-1 832/13 cells were treated with palmitate or with CytoD using a 24 hours treatment as described in Figure 5-2 C. Then western blots were performed to detect phosphorylated Lats1 (Ser909) and phosphorylated Mst1 (Thr193)/Mst2 (Thr180) under different treatment conditions. I found that Lats1 was dephosphorylated after palmitate treatment, and this dephosphorylation could be blocked by F-actin depolymerization (Figure 5-6 A). This Lats1 dephosphorylation pattern was similar to that of Yap1 phosphorylation (Figure 5-5 C), supporting a model that Lats1 kinase might be responsible for the phosphorylation of Yap1. Further experiments are needed to confirm the role of Lats1 in Yap1 activity regulation. However, phosphorylated Mst1/2 was not influenced by palmitate or CytoD or both treatments (Figure 5-6 B). Therefore, palmitate appears to regulate Yap1 activity independently of Mst1/2 in the rat β-cells.
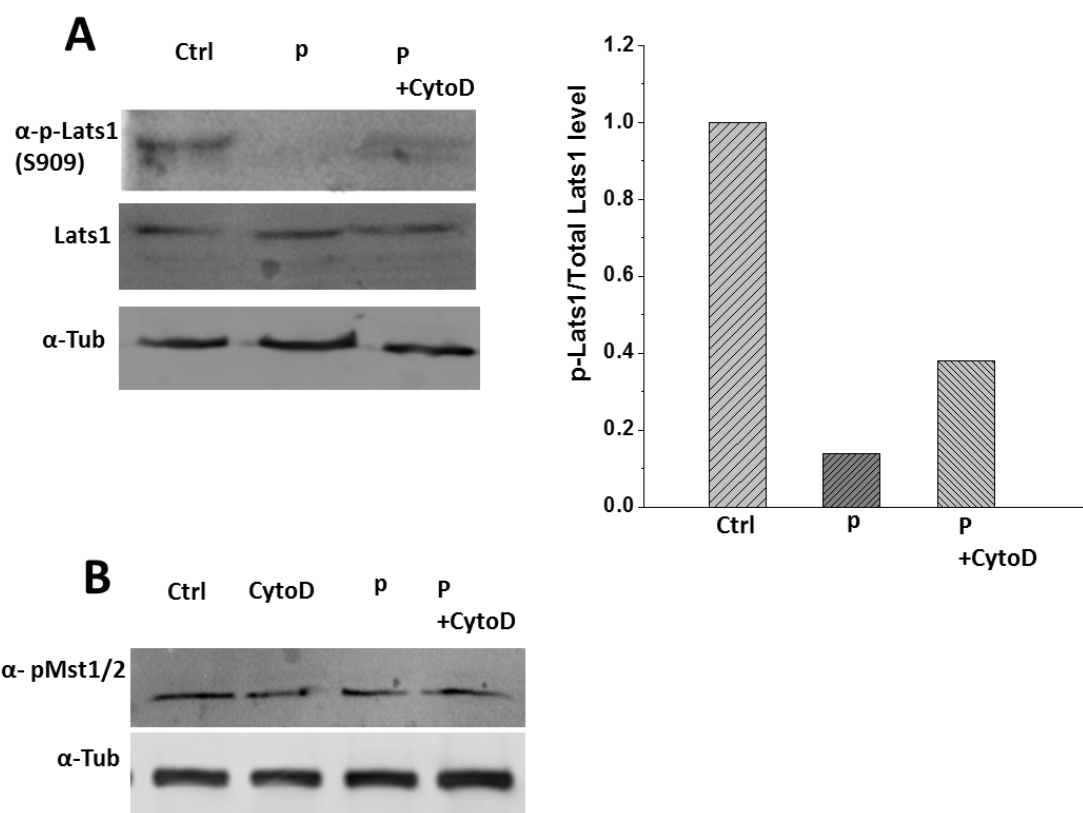
Figure 5-6 Lats1, but not Mst1/2 is dephosphorylated under palmitate treatment in INS-1 832/13 cells. (A) Palmitate dephosphorylates Lats1 via F-actin. INS-1 832/13 cells were treated with palmitate or with CytoD for 24 hours. Western blot against p-lats1 shows Lats1 is dephosphorylated by palmitate treatment, and this dephosphorylation is inhibited by CytoD. Right panel shows quantification of p-Lats1/ total Lats1 level by software ScanStorm. (B) Western blot against p-Mst1/2 shows that phosphorylated Mst level is not influenced by palmitate or CytoD treatment. P: palmitate. Ethanol and DMSO were used as control.

5.8 Expression of CTGF, a Yap1 downstream target, is activated by palmitate treatment in an F-actin dependent manner

My further task is to explore the downstream targets of Yap1 responding to palmitate

treatment. Under most conditions, Yap1 functions as a transcription co-activator by interacting

with DNA-binding proteins such as TEA-domain proteins (TEAD) to promote proliferation and

inhibit apoptosis (Pan, 2007). However, p73 transcription factor associates with Yap1 under

certain disease conditions to promote apoptosis (Polesello et al., 2006; Hwang et al., 2007; Guo et al., 2007). To begin to identify downstream target genes of Yap1 in β-cells, a number of known targets of Yap1 identified in other cell types were examined through quantitative reverse transcription-polymerase chain reaction (RT-PCR) for their expression pattern under palmitate treatment.

Rat β-cells were treated with palmitate and CytoD using both 24 hours treatment and 48 hours treatment, as described in Figure 5-2 C&F. Expression levels of several Yap1/p73 and Yap1/TEAD1 target genes were determined by quantitative RT-PCR. The expression of each gene was normalized to the expression of α-Tublin. It turned out that P73 target gene Bax, which is a pro-apoptosis response gene, had no obvious change after 24 hours treatment; however, its expression was increased after 48 hours treatment. Bax expression level was not influenced by CytoD (Figure 5-7 A-B). The expression of Pml, which is also an apoptosis related gene, was upregulated after 24 hours of palmitate treatment, yet dropped to normal levels after 48 hours (Figure 5-7 A-B). Although expression levels of Bax and Pml were influenced by palmitate treatment, their expression patterns were not tightly correlated with Yap1 activity.

Expression of several TEAD1 target genes that include connective tissue growth factor (CTGF), Cyr61 and Axl, was also examined. Cyr61 and Axl expression levels had no significant change after palmitate treatment. However, the expression level of CTGF strictly followed the activation pattern of Yap1 upon palmitate treatment. Under both 24 hour and 48 hour treatments, CTGF's expression level was upregulated by palmitate. This upregulation was inhibited by CytoD in both cases (Figure 5-7 C-D). The CTGF regulation pattern is consistent with Yap1 activity, which may be activated by palmitate, yet inhibited by CytoD.

To further confirm whether CTGF acts as Yap1 downstream under palmitate treatment, CTGF's expression pattern was examined under the condition of knocking down Yap1. Cells were treated with lentivirus carrying Yap1 RNAi sequence or control RNA sequence for 2 days,

and then 0.2mM palmitate (or with CytoD) was utilized for both 24 hour and 48 hour treatment

(described in Figure 5-2 C&F). The result showed that under both conditions, Yap1 knocking

down inhibited the increase of CTGF expression after palmitate treatment (Figure 5-7 E, compare

Yap1i group with Ctrli group in palmitate column, ** indicates p<0.01 by student t-test). These

data suggest that CTGF is a Yap1 downstream target in β-cells under palmitate treatment.
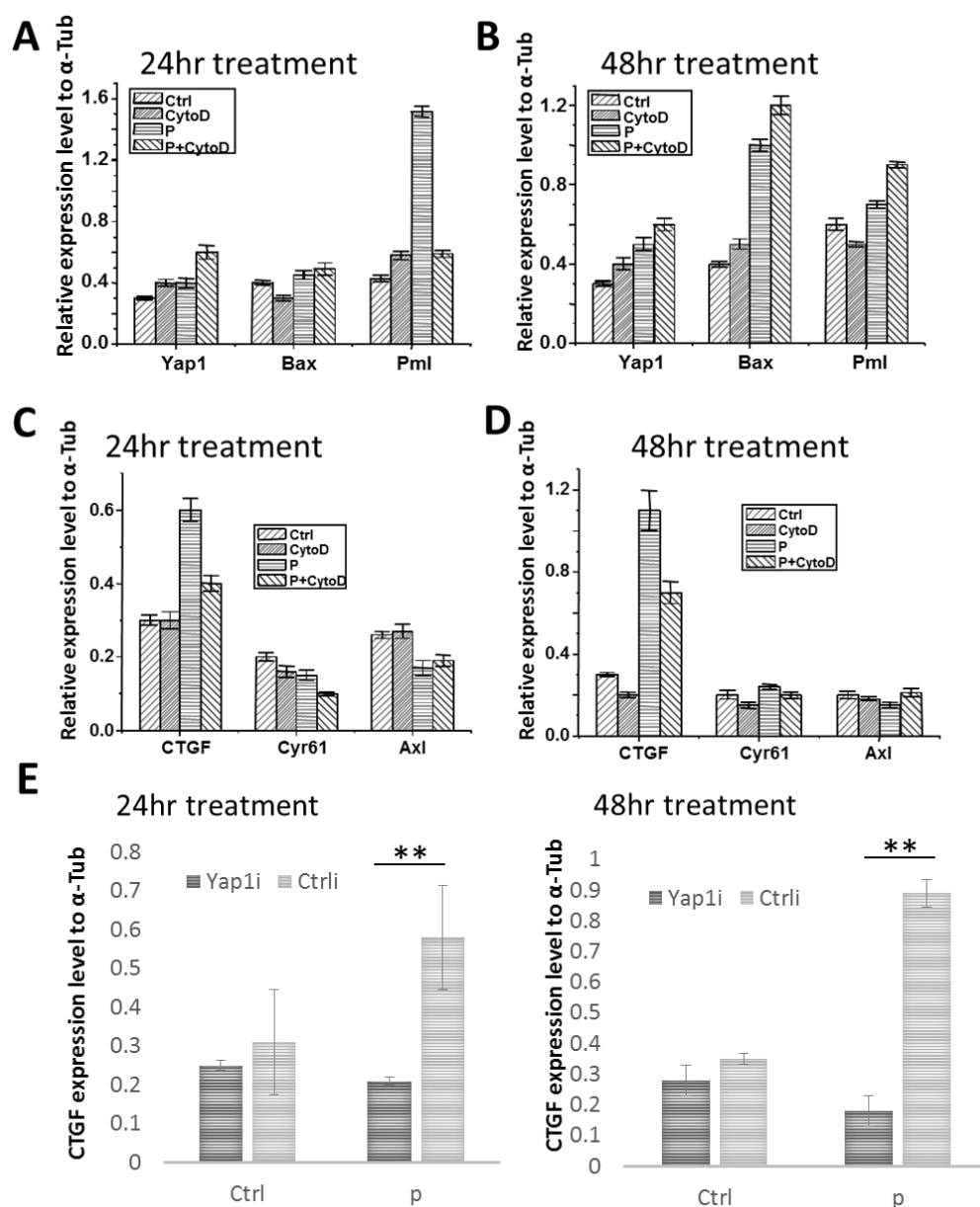
Figure 5-7 CTGF acts as a downstream target of Yap1 in response to palmitate treatment. (A-B) P73/Yap1 downstream genes Bax and PML show no correlation with Yap1 activity regulation by palmitate and CytoD treatment. (C-D) TEAD1/Yap1 targeting gene CTGF shows a consistent expression level with Yap1 activity regulation by palmitate and CytoD treatment. Gene expression level is measured by quantitative-RT-PCR. Rat β-cells were treated with palmitate and CytoD using both 24 hour treatment and 48 hour treatment, as described in Figure 5-2 C&F. Data shows mean ± SD of three independent experiments. (E) Yap1 knocking down repressed CTGF overexpression under palmitate treatment using both 24 hour and 48 hour treatment. Data shows mean ± SD of three independent experiments. ** indicates p<0.01 by student t-test. For (A-E), ethanol was used as palmitate control. P: palmiate, Yap1i: Yap1 shRNA, Ctrli: scribble RNA.

5.9 CTGF might inhibit palmitate-induced apoptosis in rat β-cells

CTGF is highly expressed in mouse embryonic β-cells and functions to promote cell proliferation (Gunasekaran et al., 2012). In adult islet, CTGF expression levels decrease dramatically, and CTGF no longer promotes cell proliferation (Gunasekaran et al., 2012). However, high levels of CTGF expression are still detected in adult ducts or the vasculature, suggesting that CTGF might still be involved in the maintenance of islet function in the adult pancreas (discussed in Crawford et al., 2009). Because CTGF turns out to be the best candidate target gene of Yap1 in β-cells in my previous experiment, my next task is to test whether CTGF is able to enhance β-cell viability under palmitate treatment.

Rat β-cells were treated with palmitate and CytoD by both 24 hours treatment and 48 hours treatment methods, as described in Figure 5-2 C&F, and human CTGF was added to the culture medium together with palmitate. 1ug/ml of human CTGF was used as the final concentration, in accordance with the manufacturer's recommendation. 5mM NaOAC (CTGF solvent) was used as the control. The β-cells were harvested and analyzed for apoptosis via Annexin V assay after treatment. The results indicated that CTGF could inhibit palmitate-induced apoptosis in β-cells under both 24 hours and 48 hours treatment conditions (Figure 5-8 A-B,

compare palmitate column with palmitate + CTGF column, ** indicates p<0.01 by student t-test).

Under 48 hours treatment, CTGF could also inhibit apoptosis even in the presence of F-actin

inhibitor CytoD (compare Figure 5-8 A with Figure 5-2 G). Therefore, CTGF enhances β-cell

survival under palmitate treatment and acts downstream of F-actin modulation. To test whether

CTGF exhibits any effect on β-cell growth under the condition without palmitate treatment, β-cell

number was counted over a period of four days. Interestingly, CTGF incubation did not

significantly influence the growth profile of β-cells (Figure 5-8 C). Therefore, CTGF does not

appear to dramatically affect the survival and proliferation of β-cells under normal conditions.
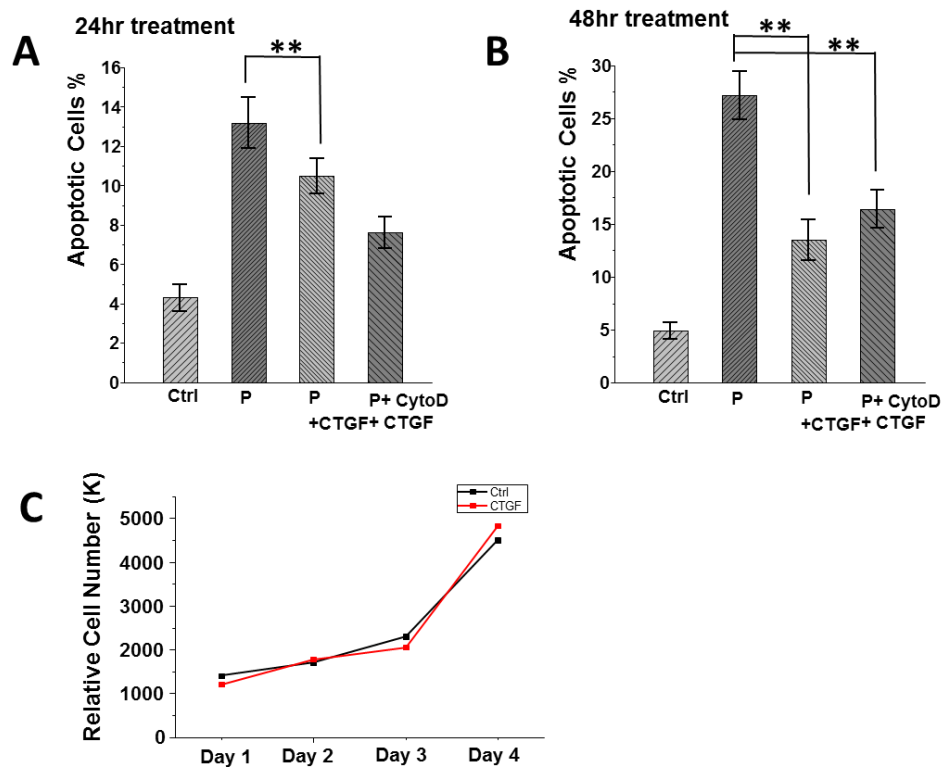
Figure 5-8 CTGF inhibits palmitate-induced apoptosis in rat β-cells. (A-B) Human CTGF could inhibit palmitate-induced apoptosis under both 24 hr and 48 hr treatment. Under 48 hr treatment, CTGF could also inhibit apoptosis enhancement trigged by CytoD. Apoptosis was measured via Annexin V assay. Ethanol, DMSO and 5mM NaOAC were used as palmitate, CytoD and human CTGF control respectively. Data shows mean ± SD of three independent experiments. ** indicates p<0.01 by student t-test. (C) Cell counting experiment shows that CTGF has no obvious influence on β-cell proliferation. Cells were counted every day after CTGF treatment. 5mM NaOAC was used as CTGF control. P: palmitate.

5.10 Chapter summary

In this study, I have investigated apoptosis induced by FFA palmitate in β-cells and explored the apoptosis mechanism. I have discovered a potential protection mechanism for maintaining β-cell viability by Yap1 activation, and CTGF has been identified as Yap1 downstream that improves β-cell viability under palmitate treatment.

In my study, high concentration of saturated FFA (palmitate) shows toxicity in rat β-cells. In addition, I found that F-actin accumulates under palmitate treatment, and palmitate-induced apoptosis is F-actin dynamics dependent. Disruption of F-actin polymerization would repress the palmitate-stimulated apoptosis if the disruption is performed before apoptosis initiation. However, the disruption of F-actin might amplify the palmitate-induced apoptosis signal when the disruption is performed after apoptosis initiation. This suggests that there might be some potential protection mechanisms responding to palmitate treatment through F-actin as well, yet in a time-delayed manner.

Yap1 has been discovered to respond to palmitate treatment in my study. After palmitate treatment, Yap1 is translocated from cytosol to the nucleus and activated by dephosphorylation. This response is found both in rat and mouse β-cells. Yap1 knocking down experiments show that Yap1 plays a positive role in regulating β-cell viability under palmitate treatment. Knocking down Yap1 would amplify the palmitate-induced apoptosis signal. However, Yap1 shows no significant influence on β-cell proliferation.

Similar to Hpo signaling regulation by GPCRs in other studies (e.g. Codella and Irvine, 2012; Yu et al., 2013), the disruption of F-actin polymerization would inhibit Yap1 translocation after palmitate treatment. Interestingly, Lats1 is inactivated by palmitate treatment in rat β-cells, but Mst1/2 seems not involved, suggesting that Lats1 might be responsible for Yap1 activation during the process. However, further investigation is needed to support this idea. On the other

hand, Yap1 activity has no obvious influence on F-actin dynamics, indicating that F-actin's regulation of Yap1 activity might not generate a feedback loop.

CTGF has been identified as a Yap1 downstream target that responds to palmitate treatment in β-cells. CTGF is known to promote β-cell proliferation during embryonic development. In adult β-cells, CTGF expression is decreased. However, high CTGF levels are suggested to maintain islet function (discussed in Gunasckaran et al. 2012). In this study, I show that under palmitate treatment, CTGF expression is upregulated by Yap1 activation to improve β-cell viability. Treatment of human CTGF to cultured β-cells can rescue the β-cells from palmitate-induced apoptosis. Based on these studies, I propose a model for an FFA-induced β-cell apoptosis mechanism. High concentrations of FFA might induce apoptosis through cytoskeleton F-actin modulation (signaling indicated by black arrows in Figure 5-9). However, FFA could also activate Yap1 activity through F-actin dynamics, in a time-delayed manner. Yap1 is activated by F-actin through Lats1 inactivation, bypassing Mst1/2, to modulate CTGF expression level. Upregulated CTGF can enhance β-cell viability and rescue β-cells from palmitate-triggered apoptosis (signaling indicated by yellow arrows in Figure 5-9). This model provides significant views to understand FFA-induced β-cell apoptosis, and the Yap1-induced protection mechanism might bring new insights for diabetes researches.
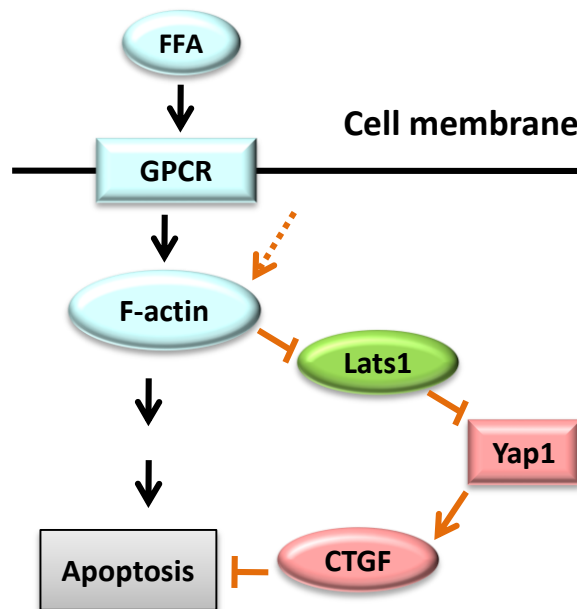
Figure 5-9 A working model to highlight the relationship between FFA-induced apoptosis and Yap1 activation. Results from this study support that F-actin plays a critical role in FFA-induced apoptosis. I propose that Yap1 is activated in response to FFA directly or indirectly. Yap1 activation is also dependent on F-actin remodeling, which blocks the activity of a negative regulator of Yap1, the Lats1 kinase. Expression of Yap1 target genes such as CTGF antagonizes apoptosis and promotes cell survival.

Chapter 6

Conclusions and Perspectives

6.1 Conclusions

In this thesis, I mainly described two projects: the investigation of the Hpo activation mechanism in *Drosophila* developing tissue and the exploration of palmitate-induced apoptosis in mammalian pancreatic β-cells.

In the first project, I studied how Hpo kinase is activated and regulated by its upstream molecules. By using a Bimolecular Fluorescence Complementation (BiFC) assay, I investigated the mechanism that Hpo could be activated by autophosphorylation through forming homo-dimer, and the membrane association appears to be crucial for Hpo activity regulation as well. I further demonstrated that upstream molecules Kibra/Ex/Mer could promote Hpo activity by facilitating its membrane association, and the membrance association increases Hpo dimerization efficiency. Thus, dimerization and membrane association are both important for Hpo kinase activation in growth control.

In the second project, I explored the mechanism by which palmitate induces apoptosis in mammalian pancreatic β-cells. I discovered that palmitate induces apoptosis in a mechanism that involves F-actin modulation. The disruption of F-actin polymerization before apoptosis initiation represses palmitate-induced apoptosis. However, the disruption of the F-actin structure after apoptosis initiation might enhance the apoptosis phenotype, which suggests that some potential protection mechanism is involved through F-actin, but in a time-delayed manner. I further discovered that Yap1 could respond to palmitate treatment and positively regulate β-cell viability. Moreover, Yap1 is activated through F-actin polymerization and Lats1 inactivation, bypassing

Mst1/2. CTGF has been identified as a Yap1 downstream target that enhances β-cell viability under palmitate treatment.

6.2 Perspectives

6.2.1 Hpo activity regulation

Hpo kinase plays a crucial role in tissue development and tumorigenesis. However, how Hpo kinase is regulated was less understood. In this study, by directly observing protein-protein interaction, I demonstrated the Hpo kinase activation mechanism by which Hpo can be activated by trans-phosphorylation via forming a homo-dimer. Importantly, the same mechanism was discovered in the mammalian systems as well. Kinase Mst1 and Mst2, which are mammalian homologs of Hpo protein, are also activated through autophosphorylation in homo dimer to inhibit tissue growth (reviewed in Souza and Lindsay, 2004). However, different from *Drosophila* Hpo, in which only N-terminus dimerization is crucial for its kinase activity, the mammalian MST dimerization occurs only in the C-terminals SARAH domain, and the C-terminals dimerization reinforces MST1/2 in the cytoplasm (Lee et al., 2002). Similarly, *Drosophila* Hpo dimer is more cytoplasmic localized compared with total Hpo (Figure 4-2 G-N); however, how Hpo dimerization regulates its sub-cellular localization needs to be further explored.

Moreover, in this study I discovered that besides dimerization, membrane association is also critical for Hpo to become activated. Hpo dimer is widely distributed in the nucleus, cytoplasm and plasma membrane. However the nuclear localized Hpo and Hpo dimer have shown low activity to inhibit cell proliferation (Figure 4-1 and Jin et al., 2012), while the membrane-targeted Hpo shows higher capability to inhibit Yki/Sd transcription activity (Figure4-3). On the

contrary, cleaved Mst1/2 translocates into the nucleus to promote apoptosis (reviewed in Souza and Lindsay, 2004). Although using different mechanisms, the activity of both *Drosophila* Hpo and mammalian Mst1/2 are regulated by sub-cellular distribution. Two nucleus export signals (NES) and one nucleus localization signal (NLS) have been identified in the SARAH domain of Mst1/2 (Lee et al., 2002), and the NES is conserved from *Drosophila* to mammals (Jin et al., 2012). Interestingly cancer genomic sequencing programs show that multiple mutations in Mst1/2 NES and NLS have been found in human cancer patients (cBioPortal and COSMIC data), indicating that the regulation of sub-cellular localization might play important roles in human cancer formation. To further study whether mutations in NES/NLS are crucial for human STK4 (Mst1)/STK3 (Mst2) kinase activity might bring critical insights into carcinogenesis mechanisms.

My research provides a model that membrane recruitment is crucial for Hpo to be activated (Figure 4-9). Besides Hpo, other Hpo pathway components also show higher activity by membrane recruitment, such as Mats (Ho et al., 2010). Moreover, this mechanism is conserved from *Drosophila* to mammals. Membrane-targeted Mst1 shows higher kinase activity as well (Praskova et al., 2004), suggesting that the plasma membrane could be an evolutionarily conserved location for the Hpo pathway regulation. Although the membrane recruitment mechanism is not fully understood, multiple interactions among Hpo pathway core components and membrane associated upstream molecules have been discovered (Table 6-1). It is possible that multiple interactions between the Kibra/Ex/Mer complex and Hpo/Sav help Hpo/Sav membrane association, and the cell membrane association might locally increase Hpo/Sav concentration to induce Hpo dimerization and Hpo-Sav interaction.

Table 6-1 Multiple interactions between Kibra/Ex/Mer complex and Hpo/Sav complex (reviewed in Pan et al., 2010)

Hpo pathway components	Physical interaction partners
Hpo	Sav, Ex, Kibra, Hpo
Sav	Hpo, Mer, Kibra, Ex (putative)
Kibra	Mer, Ex, Sav, Hpo
Ex	Kibra, Mer, Hpo, Sav (putative)
Mer	Kibra, Ex, Sav

Furthermore, my study showed that *Drosophila* Hpo activity is regulated by the upstream molecules Kibra/Mer/Ex via plasma membrane recruitment (discussed in Figure 4-10). In my model, Kibra/Mer/Ex work together to recruit Hpo protein to the plasma membrane, thereby increasing its dimerization efficiency and activity, while Mer or Ex alone is not sufficient to regulate Hpo dimerization (Figure 4-9). However, other studies have shown that Ex or Mer mutation alone can induce increased Yki activity and produce an over-growth phenotype in specific tissue or during specific developmental stages, although *ex, mer* double mutations produce a much stronger phenotype (McCartney et al., 2000; Hamaratoglu et al., 2006; Maitra et tal., 2006). These observations suggest that Ex and Mer could regulate Yki activity by bypassing Hpo regulation under particular conditions. Ex was studied to suppress Yki activity by directly interacting with Yki and thus restricting Yki on the plasma membrane during eye development (Badouel et al., 2009). Therefore, Hpo pathway regulation shows complexity and specificity. Further studies need to be done to discover Hpo dimerization regulation's specificity during different developmental stages and in different tissue types in order to understand the tissue growth mechanisms regulated by Hpo signaling.

6.2.2 Yap1 might increase β-cell viability through CTGF under FFA treatment

Type 2 diabetes are strongly associated with obesity, which in turn is associated with elevated levels of FFAs in the blood (Boden, 1997; Tataranni et al., 2005; Moller and Kaufman, 2005). Chronically elevated FFA promotes both insulin resistance and β-cell death (Prentki and Madiraju, 2011; Morgan, 2009). Therefore, a better understanding of how FFA signaling leads to β-cell dysfunction and death would contribute to the development of strategies for management and treatment of diabetes. In this study, I have investigated FFA-induced β-cell apoptosis and discovered a protective role of Hpo signaling to improve β-cell survival. While it has been discovered that saturated FFA such as palmitate induces β-cell apoptosis, I found that F-actin accumulates under palmitate treatment, and palmitate-induced apoptosis is F-actin dynamics dependent. Thus, F-actin plays crucial role in regulating FFA-induced apoptosis. However, further study is needed to demonstrate the downstream apoptosis signaling regulated by F-actin.

As Yap1 is critical in regulating cell proliferation and apoptosis in many mammalian cell types, I have investigated whether Yap1's activity is required in β-cells. In my study, through immunostaining, the expression of Yap1 was observed in rodent β-cell lines like INS-1 832/13 and MIN6. Compared to my work, a previous study has shown that Yap1 expression was lost following endocrine specification in developing mouse embryos and Yap1 was hardly detected in mouse adult β-cells through immunohistochemical analysis (George et al., 2012). It is possible that the level of Yap1 protein in mouse embryonic and adult β-cells was too low to be detectable compared to other cells surrounding the islet. It is also possible that Yap1 expression has been positively selected in the β-cell lines. Therefore, it is crucial to repeat this work *in vivo* or at least in primary cell lines to support this mechanism.

Besides the Yap1 expression data, three other lines of evidence from this study also support that Yap1 plays a positive role in regulating β-cell number. First, loss-of-function

analysis of Yap1 has shown that Yap1 is required to antagonize apoptosis (Figure 5-4 B), although reduction of Yap1 does not appear to have a significant influence on β-cell proliferation (Figure 5-4 C). Second, upon FFA treatment, Yap1 is translocated from cytoplasm to the nucleus and activated by dephosphorylation due to inactivation of its upstream inhibitor, the Lats1 kinase (Figure 5-3; Figure 5-5). Third, expression of a well-established Yap1 downstream target gene, CTGF, is increased upon Yap1 activation (Figure 5-7C and 5-7D). CTGF is effective in suppressing β-cell apoptosis induced by FFA (Figure 5-8A and 5-8B). In a model, I propose that Yap1 activation is a critical part of cellular response to FFA signaling and activated Yap1 suppresses apoptosis through its downstream target genes such as CTGF (Figure 5-9).

The CTGF gene was identified as a direct YAP/TEAD target required for cell growth (Zhao et al., 2008). In the mouse embryonic pancreas, CTGF functions in both endothelial cells and β-cells, and is both required and sufficient to promote β-cell proliferation (Guney et al., 2011). Similar to Yap1 expression level, in adult β-cells, CTGF expression decreases over the course of development as well. However, high CTGF levels are found in ducts or the vasculature, suggesting that CTGF might be involved in the maintenance of islet function in adult pancreas (Gunasekaran et al., 2012). While a role of CTGF in promoting proliferation and viability of β-cells in adult pancreas will need to be further investigated, it is also important to elucidate the molecular mechanism by which CTGF suppresses apoptosis. The discovery that CTGF promotes β-cell viability under FFA treatment without inducing extra proliferation makes CTGF a good candidate to study β-cell survival mechanism and for potential diabete treatment in the future.

It is intriguing that the disruption of F-actin polymerization was no longer effective in suppressing FFA-induced apoptosis if the F-actin inhibitor CytoD is added at a later stage, a day after FFA treatment. Although it is possible that F-actin function is no longer required for apoptosis to occur at a later stage, my results support an idea that FFA-mediated stress leads to the activation of a protective mechanism that promotes β-cell survival in an F-actin dependent

manner. Yap1 activation provides the molecular basis of this protective mechanism. Animal experiments might be needed in the future study to support this mechanism.

Similar to Yap1 regulation by GPCRs in other studies (Yu et al., 2013; Codelia et al., 2012), FFA-induced Yap1 activation in β-cells is also dependent on Lats1, but not Mst1/2, inactivation. Furthermore, this regulation is mediated through F-actin remodeling. Although FFA signaling might directly lead to Yap1 activation, I cannot exclude the possibility that the effect of FFA on Yap1 activation is indirect; other signaling pathways might be deployed to cause Yap1 activation due to the initial FFA signaling in β-cells. Supporting the latter model, Yap1 activation in β-cells appears to occur after apoptosis has been initiated. Moreover, apoptotic cells are known to be able to send out proliferative and survival signals to influence other cells through paracrine as well as autocrine signaling (Fan et al., 2008; Sun et al., 2011). Therefore, further investigation is needed to uncover this potential mechanism in detail.

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Appendix

A.1 Plamids generated in this study

Plasmid	Note
pBiFC_HA_Hpo_VC	Mammalian cell expression vectors. pBIFC and bjun-bFos control vectors are from Addgene. pBIFC_VC155 Sequencing primers:pCMV_F; VC155_R pBIFC_VN173 Sequencing primers:CMV_F; VN173_R Sequence information for the vectors can be found : http://people.pharmacy.purdue.edu/~hu1/
pBiFC_Flag_Hpo_VN	
pBiFC_HA_HpoN_VC	
pBiFC_Flag_HpoN_VN	
pBiFC_HA_Hpo^{KD}_VC	
pBiFC_Flag_Hpo^{KD}_VN	
pBiFC_HA_Hpo^{KD}N_VC	
pBiFC_Flag_Hpo^{KD}N_VN	
pBiFC_HA_Mer_VC	
pBiFC_Flag_Ex_VN	
pBiFC_HA_Sav_VC	
pBiFC_Flag_Sav_VN	
pUAST_HA_Hpo_VC	Gal4 drived UAS expression vectors. Can be used to generate site-directed transgenic flies directly. Need to be co-transfect with pAC-Gal4 is want to express the protein in cultured S2R+ cells. Sequencing primers: 212, 213
pUAST_Flag_Hpo_VN	
pUAST_HA_Hpo^{KD}_VC	
pUAST_Flag_Hpo^{KD}_VN	
pUAST_HA_Mer_VC	
pUAST_Flag_Ex_VN	
pUAST_HA_Sav_VC	

pUAST_Flag_Sav_VN	
pUAST_HA_Hpo^{M292EI634D}_VC	
pUAST_Flag_Hpo^{M292EI634D}_VN	
pUAST_HA_Hpo^{T189A}_VC	
pUAST_Flag_Hpo^{T189A}_VN	
pUAST_HA_Hpo^{T189E}_VC	
pUAST_Flag_Hpo^{T189E}_VN	
pUAST_HA_Hpo^{T195A}_VC	
pUAST_Flag_Hpo^{T195A}_VN	
pUAST_HA_Hpo^{T195E}_VC	
pUAST_Flag_Hpo^{T195E}_VN	
pUAST_HA_Hpo^{T189AT195A}_VC	
pUAST_Flag_Hpo^{T189AT195A}_VN	
pUAST_HA_Hpo^{T189ET195E}_VC	
pUAST_Flag_Hpo^{T189ET195E}_VN	
pUAST_HA_bfos_VC	
pUAST_Flag_bjun_VN	
pH_diapI_HRE	Reporter vectors which can be expressed in *Drosophila* cells.
pH_Akt_HRE1	
pH_Akt-HRE2	

A.2 Other plasmids used in these studies

Plasmid	Note
pAc5.1_Yki-V5	
pAc5.1_FlagHpo	
pAc5.1_FlagHpo^{KD}	
pAc_HAMer	Dr. George Halder
pAc_HAEx	
pAc_HASav	Dr. Nicolas Tapon
pAc_HAKibra	
pUAST_HASd	Dr. Jiang Jin
3xSd_Luc	
pAc-Gal4	Addgene
pMD2.G	Dr. Guan Kunliang
psPAX2	
pLKO.1_YAP shRNA	
pLKO.1_Control shRNA	

A.3 *Drosophila* lines generated in these studies

Fly lines	Note
UAS_HpoVC	51C site on second chromosome.
UAS_Hpo^{KD}VC	Normal expression level
UAS_bFosVC	

UAS_HpoVN	86F site on third chromosome.
UAS_Hpo^{KD}VN	High expression level
UAS_bJunVN	
UAS_SavVN	
UAS_HpoVC; UAS_HpoVN/ST	
UAS_Hpo^{KD}VC; UAS_Hpo^{KD}VN/ST	
UAS_bFosVC; UAS_bJunVN/ST	
UAS_bFosVC; UAS_HpoVN/ST	
UAS_HpoVC; UAS_SavVN/ST	
UAS_HpoVC; C5Gal4, UAS_HpoVN/ST	

A.4 Other *Drosophila* lines used in these studies

Fly lines	Note
dpp-Gal4	Gal4 driver lines
en-Gal4	
UAS_dicer; *en*-Gal4, UAS_GFP	
en-Gal4, UAS_RFP	
C5-Gal4	
ms1096-Gal4	
actin-Gal4	
Catons	Wild type
w-	whilte eye

attp (51C)	Microinjection Tool lines
attp (86Fb)	
UAS_Mer	
UAS_Ex	
Adv/SM1; UAS_Mer, UAS-Ex/TM6	
UAS_MerRNAi	
UAS_ExRNAi	
UAS_MerRNAi; UAS_ExRNAi/ST	
Mer^4; FRT40Ap[Mer$^+$GFP]/CyoGFP;eyeFLP/TM6B	Dr. Ken Irvine Ex,Mer double mutant lines
FRT40A; Ex^{el}/CyoGFP;hs FLP/TH6B	
UAS-FlagHpo	Dr. Jin Jiang

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Ye,X., **Y. Deng**, and Z,-C. Lai. (2012)."Akt is negatively regulated by Hippo signaling for growth inhibition in *Drosophila.", Developmental Biology 369:115-123*

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