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A Putative Dual Role for Yorkie in Hippo pathway Signaling

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Caroline Hall

Senior Honors Project

A Putative Dual Role for Yorkie in Hippo pathway Signaling

Abstract

The Hippo pathway inactivates genes involved in organ size and when aberrant, can lead to cancer. To control organ size, the Hippo pathway inhibits Yorkie (Yki), a transcriptional co-activator that works with Scalloped (Sd), a DNA binding protein. When active, Yki translocates into the nucleus and initiates transcription. Conversely, when inactive, Yki remains in the cytoplasm. However, my work shows that cytoplasmic, inactive Yki interacts with other proteins in the Hippo pathway by recruiting them to the plasma membrane. Accordingly, this study challenges the notion that cytoplasmic Yki is inactive and instead, may play a dual role in the Hippo pathway.

Introduction

Significance

More than any other time in our history, Americans feel the effects of cancer, with every 1 in 4 men and 1 in 5 women dying from cancer annually¹⁰. Whether an individual suffered from cancer personally, or knows someone close who suffered, cancer has touched almost all of our lives. So, it is no surprise that cancer research in the scientific community has become a burgeoning field of study.

As an undergraduate student, I began my research on cancer through several lecture courses, which led me into Dr. Pam Vanderzalm's fruit fly, or *Drosophila melanogaster*, lab at John Carroll University (JCU). Dr. Vanderzalm's lab studies a cellular pathway that controls some of the biological mechanisms related to cancer development, including cell contact inhibition and organ size control¹⁴. This cellular pathway, called the Hippo pathway, employs

several proteins involved in cellular signaling for regulation of cell proliferation and cell death, including the protein YAP (in humans) or yorkie, (Yki) in fruit flies (Figure 1). The Hippo pathway is highly evolutionarily conserved⁵, and thus, human Hippo pathway genes have homologs in *D. melanogaster*. Using *D. melanogaster* as a model organism, I conducted a series of experiments questioning the proposed relationship of Yki to other proteins within the Hippo pathway in order to postulate a new, putative role for Yki's cellular function.



Figure 1: Major Proteins in the Phosphorylation Cascade of the Hippo Signaling Pathway

Basic Cancer Biology

The ubiquitous and menacing presence of cancer within our society has created the need for basic research into the mechanisms by which cancer functions. This basic research exposes the aberrant nature of cancer cells and thereby the eight hallmarks of cancer, most of which present themselves in cancerous cells¹. The Hippo pathway functions to regulate both proliferative signaling and growth suppression¹⁴, and when aberrant, these functions become two of the eight hallmarks of cancer. Proliferative signaling may become abnormal when a cell

continuously divides, creating a large mass of cells called a tumor. These cells continue to divide despite efforts to suppress growth, like cell-contact inhibition. Normal cell-contact inhibition occurs when healthy cells stop dividing when the cells contact too many neighboring cells. This growth suppression response occurs in a healthy cell by cell-to-cell communication, facilitated by protein factors released from the cells when they grow and start to become too crowded. However, cancerous cells continue to divide despite this cell-contact inhibition, allowing for the growth of a tumor. Once the cells continuously proliferate and fail to respond to growth suppressors, the other six hallmarks of cancer may, in any order, occur in the cell¹. These six other hallmarks include: resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction¹. After the appearance of any combination of several hallmarks, the cell is considered cancerous.

The Hippo pathway's Role in Cancer

Recent studies implicate the Hippo pathway in both proliferative signaling and growth suppression, via regulation of organ size and role in contact inhibition, respectively¹⁴. In healthy cells, Hippo pathway signaling is off to allow the cells to divide until the cells reach the proper size of the organ that they compose, a liver, for example¹⁴. The Hippo pathway is then activated once the cells have a large proportion of neighboring cells (i.e. contact inhibition) so that the cells stop dividing once they reach the proper organ size¹⁴. Thus, when certain steps of the Hippo pathway fail to function normally, inappropriate growth and cancer can result¹⁴. Additionally, when the Hippo pathway fails to function completely or is lost, known cancers develop².

Canonical Hippo pathway Signaling

Normal Hippo pathway functioning occurs via specific signals, leading to a cascade of events, via proteins, within the cell (Figure 1). In *D. melanogaster*, the major proteins in the Hippo pathway include: Expanded (Ex), Kibra (Kib), Merlin (Mer), Tao-1, Hippo (Hpo), Salvador (Sav), Warts (Wts), Mats (Mats), and Yorkie (Yki). The Hippo pathway becomes activated through unknown mechanisms including known receptor protein Fat¹⁴, G-coupled protein receptor signaling (in vertebrates) and changing cytoskeletal tension². After activation, Ex, Kib, and Mer, which are all apically located proteins (located near the plasma membrane in the cell cortex), act as upstream regulators of the kinase cascade^{6,9}. The significance of the localization of these proteins at the apical membrane is still not understood³, but may prove important in the future. In the kinase cascade, a series of proteins act to either activate or inactivate, via phosphorylation, downstream proteins, transferring the signal throughout the cell's cytoplasm and into the nucleus¹. The Hippo pathway kinase cascade includes the proteins Tao-1, Hpo, Sav, Wts, Mats, and Yki^{3,5} (Figure 1). Hpo and Sav form a complex, and when Hpo is phosphorylated by Tao-1, Hpo in turn phosphorylates Sav^{3,5}. The active Hpo/Sav complex allows Hpo to phosphorylate Wts and Mats⁴. Wts and Mats form a complex that enhances the kinase activity of Wts¹¹, allowing Wts to phosphorylate Yki. Phosphorylated Yki renders Yki inactive⁴ via cytoplasmic retention¹⁴.

As the downstream target molecule of the Hippo signaling pathway, Yki has a primary function in transcription; more specifically, as a transcriptional co-activator^{7,12}. When the Hippo pathway is not functioning, Yki may enter the nucleus and carry out this canonical role. As a transcriptional co-activator, Yki requires a specific binding partner, the transcription factor Scalloped (Sd)^{7,12}, which is directly bound to DNA. Upon binding of Yki to Sd, transcription of

the DNA downstream of the Yki/Sd complex occurs, which includes genes that promote cell growth and inhibit cell death. For example, one Yki target gene encodes for a protein that inhibits cell death, called *diap1*^{12, 14}. When Yki is not functioning in the nucleus, Yki is generally considered to be “inactive” in the cytoplasm¹⁴.

The Importance of Localization in the Hippo pathway

While the Hippo pathway often does function according to the canonical model, recent studies suggest that Hippo pathway proteins may regulate Yki by methods other than phosphorylation, depending on the cellular localization of the proteins involved³. To start, upstream regulators of the Hippo pathway, which are located in the cell’s cytoplasm near the plasma membrane, may directly inactivate Yki by recruiting Yki to this area of the cell, called the cell cortex³. Ex has been shown to directly bind Yki² and form a complex with Hpo and Wts³. Thus, the Hippo pathway may control Yki activity through mechanisms other than phosphorylation, blurring the lines between the upstream and downstream designations proposed by the canonical pathway. However, the mechanism by which the cell regulates Yki activity depends upon the localization of Yki within the cell; the closer Yki is to the “upstream” proteins of the pathway (Ex, Mer), the more likely these proteins will directly control Yki, while the further Yki is from the “upstream” pathway proteins, the less likely Yki will be phosphorylated³. Furthermore, control of Yki in this secondary manner may result from the location of “upstream” pathway proteins near the adherens junction (AJ) in *D. melanogaster* epithelial cells³. Current research suggests that the AJ may receive signals from neighboring cells, such as signals for growth suppression via contact inhibition, that regulate proteins of the Hippo pathway³. In order for this regulation to occur, however, “upstream” Hippo pathway proteins must localize near the AJ, in the cell cortex³. Accordingly, the location of Hippo pathway proteins plays a role in the

regulation of Yki in the cell, and plays an important role in the signaling events of the pathway. In addition to the importance of localization of other Hippo pathway proteins, the location of Yki within the cell also impacts Hippo pathway activity. Canonically, active Yki maintains a role within the nucleus as a transcriptional co-activator, while inactive Yki stays within the cytoplasm, where Yki may or may not play a secondary role (P. Vanderzalm, unpublished results).

Dual Role for Cytoplasmic Yorkie

Whether Yki is truly inactive within the cytoplasm has recently been called into question. Dr. Vanderzalm's lab has postulated that cytoplasmic Yki might have another role in the cell, unrelated to its nuclear role. One piece of evidence that led to this hypothesis is that the upstream regulators of the pathway, namely, the proteins Ex, Kib, and Mer, are all located near the apical membrane of the cell^{3,9}. When Yki is cytoplasmic, a significant amount of it is also found in this apical region¹³. In order to understand the significance of this Yki localization at the cell membrane, Dr. Vanderzalm eliminated the transcriptional binding site of Yki, so that Yki failed to act in its role as a transcriptional co-activator but could still remain active in the cytoplasm. This "transcriptionally-dead" version of Yki seemed more tightly associated with the plasma membrane than wild-type Yki, and its overexpression led to increased levels of Ex and Diap1 at the apical membrane, post-translationally (unpublished results). Accordingly, I want to know if Yki is sufficient to recruit Ex, Diap1, and other Hippo pathway proteins to the apical plasma membrane, further elucidating Yki's cytoplasmic function.

Myristoylation and Experimental Procedure

To determine if Yki plays a dual role in the cytoplasm, I used a chemically modified version of Yki, myristoylated Yki (Myr-Yki). Myristoylation specifically tethers Yki to the inner leaflet of the cell's plasma membrane. This means I can locate Yki at any given time in the cell, and thereby, observe if Yki is sufficient to recruit other proteins of the pathway to its location. I transfected this version of Yki into *D. melanogaster* S2 cells, along with the DNA for a protein of interest (Ex, Mer, Hpo, Diap1, or Sd). Next, I analyzed the expression of the proteins produced by the transfected DNA by immunofluorescence on a confocal microscope. Surprisingly, I observed that Yki is sufficient to recruit not only Ex, Mer, and Hpo, but also Diap1 and Sd to the plasma membrane. Accordingly, I propose that Yki plays a dual role in the cytoplasm and most likely, this role involves stabilizing other proteins.

Methods

Acquiring DNA Constructs

The Vanderzalm lab already had created all of the DNA constructs when I arrived. The DNA constructs used encoded epitope-tagged fusion proteins to facilitate detection by immunostaining. Myr-Yki expression was under the control of a UAS promoter, and was driven by Ubiquitin-Gal4⁴, while other constructs were expressed under the control of an actin promoter. Constructs used included UAS-FLAG-myr-Yki, Ubiquitin-Gal4, empty FLAG vector, HA-Ex, HA-Mer, V5-Wts, Myc-Hpo, HA-Sd, and HA-Diap1. Briefly, 48 hours after transfection, I fixed and stained the cells with primary antibodies against the epitope tags, and used fluorescently labeled secondary antibodies to visualize the proteins.

Cell Transfection

D. melanogaster S2 cells were maintained in Schneider's Media (S2 media) with 10% FBS (Fetal Bovine Serum) and split 1:1 or 1:4 when 90-100% confluent. To prepare for transfection, I split the cells 1:1 the day prior. On the day of transfection, I made a mixture of Schneider's Media with 10% IMS (Insect Medium Supplement) and DDAB (Dimethyldioctadecylammonium bromide) to act as a lipofection agent in a 2:1 ratio. The lipofection agent allowed for introduction of exogenous DNAs into the cells⁸. While the S2 media-DDAB mixture incubated for 5 minutes, I counted the S2 cell density and calculated the volume of cells to add to Schneider's Media with 10% IMS to create a $\sim 2-4 \times 10^6$ cells/well solution. Next, I prepared separate microfuge tubes for the transfected, exogenous DNAs by adding ~ 150 uL of the S2 media-DDAB mixture. To this 0.5 ug of each DNA was added to each transfection. The S2 media-DDAB-DNA mixture incubated for 15 minutes, during which time I added the calculated volume of cells and media (to a total of 2 mL) to add to a 6-well plate. After 15 minutes, I added the whole volume of the S2 media-DDAB-DNA mixture to a well drop-wise. To mix, I gently tipped the plate to each side. The cells then incubated for 4-6 hours at 25°C, at which time I removed the media and cells from the 6-well plate and added them to a 15 mL conical vial. I centrifuged the cells at 1000rpm for 5 minutes at room temperature, removed the S2 media containing IMS (supernatant) via pipette, added 2 mL of S2 media containing 10% FBS, transferred the cells to 2 mL microfuge tubes, resuspended the cells, then added them to a fresh 6-well plate. The cells then incubated for ~ 2 days at 25°C.

Indirect Immunostaining

Suspension Staining

The transfected cells were harvested from 6-well plates into 2 mL microfuge tubes and were centrifuged at 3000 rpm for 1 minute at room temperature. The supernatant was removed via pipette, and the cells were resuspended in 400 uL of fresh 2% paraformaldehyde (PFA) to fix them. The cell-PFA mixture incubated at room temperature for 10-15 minutes. After the incubation, cells were centrifuged at 3000rpm for 1 minute, the PFA was removed via pipette, and the cells were washed twice with 1mL 1X PBS. A mixture of 250 uL PBS, 0.1% Triton-X-100, and 1% normal goat serum (PTN) plus the appropriate primary antibody at the appropriate dilution was added to the cells and allowed to stain for ~1 hour at room temperature on the rocker. The primary antibodies used included: 1:1000 rabbit anti-HA (Rockland), 1:20,000 mouse anti-Myc (Cell Signaling), 1:1000 mouse anti-V5 (Pierce), 1:50,000 mouse anti-Flag (Sigma), and/or 1:1000 rabbit anti-Flag (Rockland). After staining, the cells were rinsed once with 1 mL 1X PBS, and stained in secondary antibody with PTN and incubated on the rocker for ~1 hour in the dark. Secondary antibodies (Jackson Immunochemicals) were all used at 1:1000 and included donkey anti-mouse or donkey anti-rabbit Cy3 for Flag, and donkey anti-rabbit Alexa-488/FITC for HA, and donkey anti-mouse Alexa-488/FITC for V5 and Myc. DAPI at 1:5000 was added during the incubation in secondary antibody. After staining in secondary, the cells were rinsed with 1 mL 1X PBS and resuspended in 25 uL of Invitrogen ProLong AntiFade mounting media. The whole volume of cells and media was then pipetted onto a glass slide, covered with a coverslip, and stored at 4°C in the dark.

Staining Cells on Concanavalin A-Treated Coverslips

First, I diluted the Concanavalin A (ConA) to a working concentration of 0.5 ug/mL in water. I distributed the ConA in a thin layer (~25 uL per coverslip) onto each coverslip and allowed the coverslips to dry in the fume hood for 1 hour. Next, I spun down the transfected cells in the 2 mL microfuge tubes and resuspended the cells in 200 uL of S2 media containing 10% FBS. I pipetted this whole volume onto the coverslip with ConA to allow adherence for one hour and covered to prevent evaporation. I gently washed the cells twice with 1X PBS. To fix the cells, I added 200 uL of 2% PFA onto each coverslip and incubated 10-15 minutes at room temperature. I washed once more with 1X PBS. I stained the cells in primary with PTN and the appropriate primary antibody at the same dilution as the suspension staining protocol. I covered the coverslips and incubated the stained coverslips for ~1 hour at room temperature. I washed once more with 1X PBS and incubated the cells in secondary antibody with PTN as outlined in the suspension staining protocol for ~1 hour in the dark and at room temperature. After staining, I washed the cells twice with 1X PBS and placed the coverslips cell-side down onto slides with 25 uL Invitrogen ProLong AntiFade mounting media. The slides were stored at 4°C in the dark.

Fluorescence Microscopy

I qualitatively analyzed the cells at 1000X magnification under the Olympus BX-60 compound fluorescence microscope at JCU. I used the correct light filters to visualize DAPI, Cy3, and Alexa-488 or FITC fluorescence, thereby viewing the cell's DNA, myr-Yki, and the Hippo protein of interest, respectively. However, the compound fluorescence microscope does not have a camera apparatus for fluorescent light, so I performed imaging at the confocal microscope in the Neuroscience Department of Case Western Reserve University.

Confocal Microscopy

I performed further qualitative research at 1000X magnification using the Zeiss LSM 510 Meta laser-scanning confocal microscope at the Department of Neuroscience at Case Western Reserve University. I used optical sectioning imaging to view the cells on the z-axis and used ImageJ imaging software (NIH) to process the images. Optical sectioning allowed me to ascertain co-localization of two proteins within a single cell. I imaged two or three cells in each experimental condition for a representative sample of qualitative data.

Results

Upstream Hippo pathway Proteins Co-Localize with Myristoylated Yorkie at the Plasma Membrane

To determine if Myr-Yki was sufficient to recruit candidate proteins to the cell's plasma membrane, I transfected *D. melanogaster* S2 cells with DNA constructs corresponding to Myr-Yki and one candidate protein, for each protein in turn. I then used staining techniques and subsequent immunofluorescence to detect the expression patterns of the proteins, using DAPI as a marker for the nucleus. The upstream Hippo pathway proteins scrutinized included: Ex, Hpo, and Mer. To begin, Ex co-localizes with Myr-Yki at the plasma membrane of the cell, meaning that Yki is sufficient to recruit Ex to the plasma membrane (Fig. 1). Since Ex alone appears in punctae diffusely throughout the cytoplasm of the cell (Fig. 1A and B), and Ex in the presence of Myr-Yki re-locates to the cell's plasma membrane (Fig. 1D and E), it follows that Yki recruits Ex to the plasma membrane. Similarly, Hpo also co-localizes with Myr-Yki at the plasma membrane of the cell, meaning that Yki is also sufficient to recruit Hpo to the plasma membrane (Fig. 2). Without Myr-Yki, Hpo localizes throughout the cell's cytoplasm and is faintly seen in

the nucleus (Fig. 2A and B), but, when Myr-Yki and Hpo are introduced to the cell together, Hpo moves to the cell's plasma membrane (Fig 2D and E). Thus, Yki must be sufficient to recruit Hpo to the plasma membrane. In addition, Mer also co-localizes with Myr-Yki at the cell's plasma membrane, meaning that Yki is also sufficient to recruit Mer to the plasma membrane (Fig. 3). When introduced to the cell alone, Mer localizes in punctae diffusely in the cytoplasm (Fig. 3A and B), but when introduced with Myr-Yki, Mer moves to the plasma membrane, where Myr-Yki localizes (Fig. 3D and E). Accordingly, Yki must be pulling Mer from the cytoplasm to the cell's plasma membrane. Since Dr. Vanderzalm's lab had previously demonstrated that Yki can co-immunoprecipitate with Ex, Mer, and Hpo (unpublished data), Yki's ability to re-locate Ex, Hpo, and Mer to the plasma membrane was consistent with these results.

Associated Hippo pathway Proteins Co-Localize with Myristoylated Yorkie at the Plasma Membrane

Yki exhibited a similar ability to recruit Sd and Diap1, proteins associated with the Hippo pathway, which came as a surprise. Sd is a transcription factor bound to the promoter region of Yorkie target genes. Accordingly, I expected Sd to remain bound to the target DNA even in the presence of Myr-Yki, and was using Sd as a negative control. As expected, Sd localized to the cell's nucleus in the absence of Myr-Yki (Fig. 4A and B), but surprisingly relocates to the plasma membrane in the presence of Myr-Yki (Fig. 4D and E). Therefore, Sd must have a stronger binding affinity for Yki than for DNA, since Myr-Yki is clearly sufficient to recruit Sd to the cell's plasma membrane.

Expanded, in addition to being an upstream activator of the Hippo pathway, is also a well-established Yorkie target gene⁵. However, preliminary results from the Vanderzalm lab

suggested that Yki was able to promote Ex protein levels at the membrane post-transcriptionally. In order to ascertain if other Yki target genes could be similarly affected by Yki post-transcriptionally, I tested whether Myr-Yki was sufficient to recruit Diap1 to the plasma membrane. Diap1 is a Yki target gene that prevents apoptosis from occurring in cells¹¹. Since Diap1 is a transcriptional output, rather than a Hippo signaling pathway protein, I expected that Diap1 might not be recruited by Yki at all. Without Myr-Yki, Diap1 appears diffusely throughout the entire cell, nucleus and cytoplasm (Fig. 5A and B). When co-transfected with Myr-Yki, Diap1 moves to the cell's plasma membrane, co-localizing with Myr-Yki (Fig. 5D and E). Therefore, Yki is sufficient to recruit Diap1 to the cell's plasma membrane.

Discussion

Yki's Dual Role in the Cytoplasm

Yki's ability to recruit Ex, Mer, Hpo, Sd, and Diap1 to the plasma membrane while in its "inactive", unphosphorylated version in the cytoplasm suggests a second role for Yki, distinct from its nuclear role. While Dr. Vanderzalm previously showed that Yki binds Ex, Mer, Sd, and Hpo in co-immunoprecipitation experiments (unpublished data), there was no evidence to support if Yki would recruit Diap1. Also, it was uncertain whether Yki that was specifically found in the cytoplasm would be capable of binding Sd. In fact, since Sd and Diap1 do not participate in the Hippo pathway signaling cascade, I did not expect co-localization of Sd and Diap1 with Myr-Yki. However, I was surprised to discover that Myr-Yki was sufficient to recruit both Sd and Diap1 to the plasma membrane. Furthermore, Myr-Yki's ability to bind to Ex, Mer, Hpo, Sd, and Diap1 and recruit them to the plasma membrane suggests that Yki's cytoplasmic role may involve stabilizing these proteins or the complexes they form.

Significance

While the canonical model of Hippo pathway signaling designates cytoplasmic Yki as inactive, my findings suggest that cytoplasmic Yki plays a functional role with Hippo pathway and associated proteins. Other studies support my findings, proposing that the localization of Yki (via protein-protein interactions) to the cell cortex functions as a mechanism of control of the signaling pathway². Specifically, Yki directly binds Ex, and forms a complex with Hpo and Wts². Since Ex localizes near the cell cortex, the entire protein-protein complex is recruited to the cell cortex, rendering Yki “inactive” and turning off the Hippo pathway. Similarly, another well-studied signaling pathway, the Wnt signaling pathway, is also regulated by localization of one of its key proteins, beta-catenin, to the cell cortex¹. Thus, cellular localization of proteins in a signaling pathway commonly acts as a key mechanism of controlling the pathway itself. Accordingly, cellular localization of Yki and Yki’s ability to recruit other proteins to that location suggests that Yki might actually play a role not only in stabilizing these proteins, but also in regulating the pathway itself. Moreover, since the Hippo pathway can cause tumorigenesis when aberrant, Yki’s role and location in the Hippo pathway becomes especially crucial. Therefore, whether or not Yki binds Ex, Mer, Hpo, Sd, or Diap1 directly, or indirectly via another protein, is an important question. In order to further elucidate Yki’s cytoplasmic role, this question of direct or indirect binding would need an answer. Accordingly, in the future, Dr. Vanderzalm’s lab would need to perform GST pull down assays with Yki and Mer, Hpo, and Diap1 (Ex and Sd are already known as direct interactors^{2,7,12}). Whether or not Yki’s dual role in the cytoplasm could contribute to aberrant cell growth is also still unknown. However, given Yki’s role in the Hippo pathway and when malfunctioning, tumorigenesis, Yki’s role in the cytoplasm could contribute to this abnormal growth.

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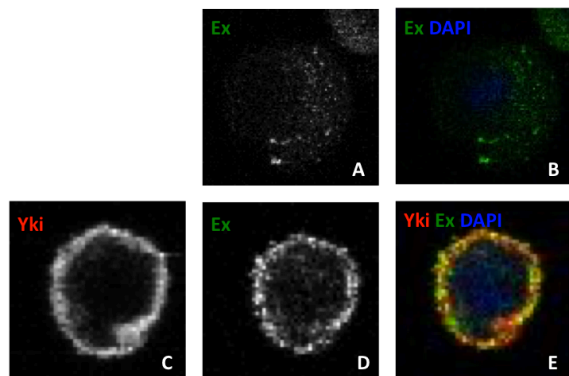


Figure 1. Myristoylated Yki Recruits Ex to the Plasma Membrane

A-B. Z-Stack of 630X Magnification of S2 cells expressing HA-Ex

A. HA-Ex localizes to the cytoplasm in punctae

B. DAPI stain indicates the nucleus, showing distinct cytoplasmic localization of Ex

C-E. Z-Stack of 630X Magnification of S2 cells expressing HA-Ex and Flag-Myr-Yki

C. Flag-Myr-Yki localizes to the plasma membrane

D. HA-Ex localizes to the plasma membrane in the presence of Myr-Yki

E. Merge of Flag-Myr-Yki channel and HA-Ex channel, together with DAPI, to show clear co-localization of Myr-Yki and Ex at the plasma membrane

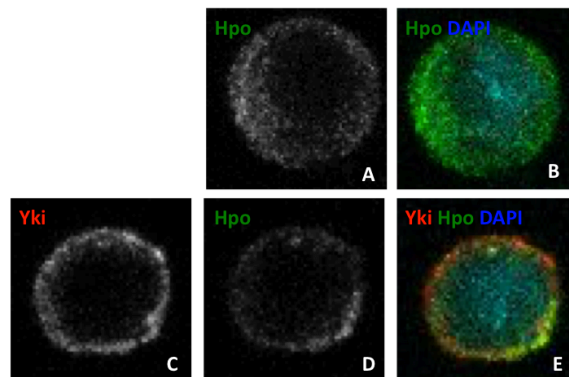


Figure 2. Myristoylated Yki Recruits Hpo to the Plasma Membrane

A-B. Z-Stack of 1000X Magnification of S2 cells expressing Myc-Hpo

A. Myc-Hpo localizes to the cytoplasm, and is faintly seen in the nucleus

B. DAPI stain indicates the nucleus, showing predominant cytoplasmic localization of Hpo

C-E. Z-Stack of 1000X Magnification of S2 cells expressing Myc-Hpo and Flag-Myr-Yki

C. Flag-Myr-Yki localizes to the plasma membrane

D. Myc-Hpo localizes to the plasma membrane in the presence of Myr-Yki

E. Merge of Flag-Myr-Yki channel and Myc-Hpo channel, together with DAPI, to show clear co-localization of Myr-Yki and Hpo at the plasma membrane

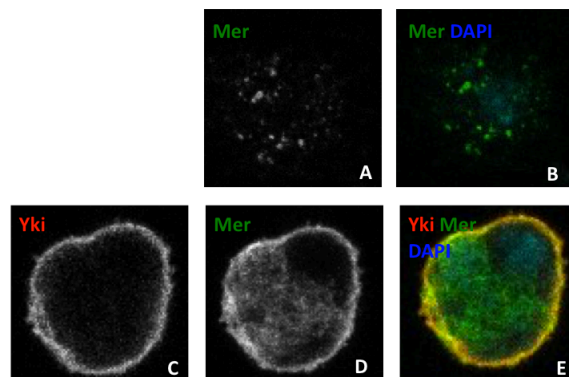


Figure 3. Myristoylated Yki Recruits Mer to the Plasma Membrane

A-B. Z-Stack of 1000X Magnification of S2 cells expressing HA-Mer

A. HA-Mer localizes to the cytoplasm in punctae

B. DAPI stain indicates the nucleus, showing distinct cytoplasmic localization of Mer

C-E. Z-Stack of 1000X Magnification of S2 cells expressing HA-Mer and Flag-Myr-Yki

C. Flag-Myr-Yki localizes to the plasma membrane

D. HA-Mer localizes to the plasma membrane (and cytoplasm) in the presence of Myr-Yki

E. Merge of Flag-Myr-Yki channel and HA-Mer channel, together with DAPI, to show clear co-localization of Myr-Yki and Mer at the plasma membrane

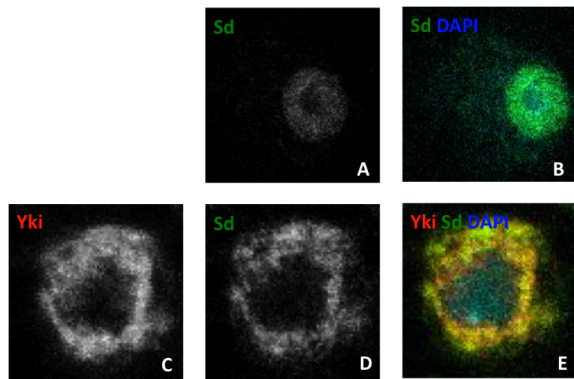


Figure 4. Myristoylated Yki Recruits Sd to the Plasma Membrane

A-B. Z-Stack of 1000X Magnification of S2 cells expressing HA-Sd

A. HA-Sd localizes to the nucleus

B. DAPI stain indicates the nucleus, showing distinct nuclear localization of Sd

C-E. Z-Stack of 1000X Magnification of S2 cells expressing HA-Sd and Flag-Myr-Yki

C. Flag-Myr-Yki localizes to the plasma membrane

D. HA-Sd localizes to the plasma membrane in the presence of Myr-Yki

E. Merge of Flag-Myr-Yki channel and HA-Sd channel, together with DAPI, to show clear co-localization of Myr-Yki and Sd at the plasma membrane

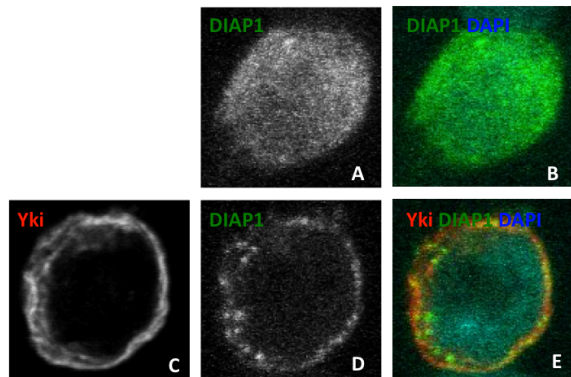


Figure 5. Myristoylated Yki Recruits Diap1 to the Plasma Membrane

A-B. Z-Stack of 1000X Magnification of S2 cells expressing HA-Diap1

A. HA-Diap1 localizes to the nucleus and cytoplasm

B. DAPI stain indicates the nucleus, showing diffuse localization of Diap1 throughout the cell

C-E. Z-Stack of 1000X Magnification of S2 cells expressing HA-Diap1 and Flag-Myr-Yki

C. Flag-Myr-Yki localizes to the plasma membrane

D. HA-Diap1 localizes to the plasma membrane in the presence of Myr-Yki

E. Merge of Flag-Myr-Yki channel and HA-Diap1 channel, together with DAPI, to show clear co-localization of Myr-Yki and Diap1 at the plasma membrane