Optimization of Techniques for Visualizing Protein Functionality in Cell Culture

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Abstract

The Hippo pathway is a highly conserved signaling pathway responsible for the regulation of cellular growth, proliferation and apoptosis. The *Drosophila* pathway is made up of four key proteins Salvador, Hippo, Warts and Mats (Salvador, MST1/2, Lats1/2 and Mob in mammals, respectively). At the heart of the signaling cascade are the two core kinases Hippo(MST) and Warts(Lats). In order for the pathway to function both of these kinases must be phosphorylated and active. Ultimately, Hippo(MST) will phosphorylate Warts(Lats). Once phosphorylated, Warts(Lats) is active and proceeds to phosphorylate its target Yorkie(YAP). Phosphorylation of Yorkie(Yap) sequesters the transcriptional co-activator from the nucleus leading to a downregulation of pro-growth genes. The function of the Hippo pathway core proteins in cultured cells can be monitored using luciferase-based reporter assay where luciferase signal is a proxy for Yorkie/YAP localization. I have adapted existing protocols to use this assay in both human (HEK293T) and insect (S2) cell lines in the Kavran Lab. Once established, I used the luciferase assay to help elucidate the role of the linker region and phosphorylation sites of MST2 in kinase activity, define the minimal functional unit of Lats2, validate the biological significance of cysteine 624 in Hippo, and learn about the role of SARAH domains in protein binding and activity. Additionally, preliminary work has been started to investigate the "hierarchy" of SARAH domains and to validate the SARAH domain interactions of dSalvador required for homodimer formation.

Primary Reader: Dr. Jennifer Kavran. Secondary

Reader: Dr. Barry Zirkin

Preface

Optimization of Techniques for Visualizing Protein Functionality in Cell Culture has been written to fulfill the graduation requirements for a Masters in Science from the department of Biochemistry and Molecular Biology at the Johns Hopkins Bloomberg School of Public Health. The research basis of this thesis was conducted in Dr. Jennifer Kavran's lab between July of 2016 to April of 2018.

My work was undertaken under the supervision and instruction of Dr. Kavran in order to establish cell culture and protocols for various cell based assays. The ultimate goal of these protocols is to provide experiments that can be used to recapitulate *in vitro* protein and biophysical data in the context of a cellular environment. Through Dr. Kavran's continual support and teaching I was ultimately able to optimize an easily used and reproducible luciferase reporter assay to examine Hippo pathway proteins in cell culture. I am incredibly grateful for all that Dr. Kavran has done for me and owe her a great debt of gratitude.

Additionally, I would like to thank all of the members of the Kavran lab for their help in establishing assays, their continual insights and knowledge, and for creating a wonderful and hospitable lab environment. The last two years have been fun and exciting because of Dr. Thao Tran, Leah Cairns, Thomas Koehler, Yoo Jin Kim and all of the multiple graduate rotations students that have come through the lab. Last, but certainly not least, I would also like to thank Dr. Barry Zirkin for his insights throughout the last two years and for agreeing to be the second reader of this thesis.

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The Hippo Pathway

The Hippo pathway has emerged as a critical regulator of cellular maintenance, proliferation and apoptosis. However, dysregulation of the pathway leads to tumorigenesis. Recently, multiple studies have demonstrated that the Hippo pathway is a potent driver of oncogenesis in lung, breast, colorectal and liver cancers [1-5]. It is important to note that there is very little evidence that somatic mutations within the Hippo pathway are common causes of tumor formation. Rather, it appears that within the cancer environment, the Hippo pathway becomes dysregulated and drives further progression of disease. To better understand why this occurs, more needs to be known about the biochemical interactions of Hippo pathway proteins.

First discovered in *Drosophila*, and named for the hippopotamus looking overgrowth phenotype, the Hippo pathway is now known to be highly conserved across multiple species [6-8]. At the center of the *Drosophila* pathway are four key proteins, Salvador, Hippo, Warts and Mats (Salvador, MST1/2, Lats1/2 and Mob). These proteins form the core of the kinase cascade that regulates the cellular localization of Yorkie (YAP in mammals) [1,6]. Phosphorylation of the transcriptional co-activator, Yorkie (YAP), prevents translocation to the nucleus. In the absence of phosphorylation, Yorkie (YAP) translocate to the nucleus and interact with their binding partners SCALLOPED(TEAD). Interaction with these transcription factors leads to the propagation of pro-growth and proliferation signals [5,9-11].

At the heart of the *Drosophila* Hippo pathway is the regulatory kinases, Hippo and Warts. These two kinases make up the regulatory cascade that ultimately phosphorylates Yorkie and prevents it from binding with its transcription factor, SCALLOPED [6,7,11]. The activity of Hippo and Warts is modulated by additional adapter proteins that associate with the kinase proteins. The first adapter protein of particular interest to our lab is the *Drosophila* form of Salvador

(dSalvador) which acts to stimulate the activity of Hippo. While the mechanism by which dSalvador stimulates Hippo kinase activity is not exactly known, it is hypothesized it does this through stabilizing the complex of phosphorylated Hippo with Warts leading to increased pathway activity [7,12]. The second protein of interest to our lab is *Drosophila* RassF (dRassF). dRassF has the opposite effect as dSalvador. dRassF preferentially binds un-phosphorylated, inactive, Hippo and prevents it from forming the homodimer required for activation [13]. Interestingly, when you look at the interaction of the mammalian orthologues of Hippo (MST) and RassF(RassF5), RassF5 appears to have the reverse effect, stimulating MST activity [14,15]. Our lab is interested in better understanding more about these interactions between Hippo, dSalvador, and RassF. Additionally, the protein structure of dSalvador is still not well understood and we are working to obtain a protein structure and crystallographic studies.

In the mammalian Hippo pathway, the central cassette is made up of the two kinases, MST1/2 and Lats1/2 [16-19]. Like the *Drosophila* pathway, these two kinases are modulated by adapter proteins in particular human Salvador and Mob1 [20,21]. MST1/2 responds to upstream signals and begins the kinase cascade. To become activated, MST must form a homodimer through its SARAH domain and auto-phosphorylate itself [12,15,22]. Once activated, MST undergoes conformational changes that allow for Mob1 binding and phosphorylation [23]. Active MST additionally, phosphorylates Salvador. Once phosphorylated both Salvador and Mob1 help in propagating the kinase cascade by promoting association of MST and Lats [23,24]. MST then phosphorylates Lats. Once phosphorylated, Lats is active and can phosphorylate its target YAP, sequestering the transcriptional co-factor and marking it for degradation [8,10,19,25]. While the physiological activity of the mammalian Hippo pathway has been thoroughly studied, the biophysical interaction and protein structures of the pathway are less well understood. In

particular, our lab is interested in determining protocols for protein crystallization of the core kinases, Lats and MST. Additionally, we hope to discover more about the interactions between the adapter protein Mob1 and these core kinases.

Optimization of luciferase reporter assay

Introduction

Background of the luciferase reporter assay

In order to test the functionality, of different variants of Hippo pathway components in cells, The Kavran Lab was interested in optimizing a Dual-Luciferase® Reporter Assay System (Promega Corporation) protocol [26,27]. By comparing luciferase results between wild type and mutated proteins, we could establish a reliable readout of their kinase activity in either *Drosophila* and mammalian cells.

The luciferase assay is an ideal tool for examining protein function in cells because it is a relatively simple experiment and can be easily adapted to any signaling pathway that ultimately controls transcription. This assay uses the bioluminescence of the firefly luciferase protein as a reporter for protein expression levels. The level of luciferase luminescence is proportional to its level of protein expression [28-30]. There are multiple forms of the luciferase protein. In our reporter assay we use the forms isolated from the firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*). Because these two proteins are distinct, this allows us to use one protein, firefly luciferase, as a reporter for transcriptional regulation and the other, *Renilla* luciferase, as the internal control for the experiment [30]. For ease of explanation, throughout the rest of this paper, firefly luciferase will be referred to as "luciferase" and *Renilla* luciferase as "*Renilla."* More explanation will follow on the need for the *Renilla* internal control.

Expression of firefly and Renilla luciferase

The luciferase gene can be put under the control of a promoter that is responsive to a gene, or genes, of interest. In our system, we use a Gal-4-upstream activating sequence (UAS) promoter fused to the luciferase gene and Gal-4 DNA binding domain (Dbd) fused to Yorkie

(*Drosophila* system) or TEAD (mammalian system). This level of control over the expression of the luciferase gene allows for easy visualization of the activity of the protein, or proteins, of interest [29].

Since there is variability in transfection from well to well, an internal control must be added to the assay to account for this variation. A plasmid encoding a second luminescent protein that will be under the control of a constitutive promoter is also co-transfected into the cells. The luminescence from this protein, *Renilla,* reports on the transfection efficiency and can then be used to normalize signal between different wells by accounting for any variation resulting from differing transfection efficiency, cell number, or cell lysis efficiency. Classically, the *Renilla* form of luciferase is used as this internal control [30,31]. In our protocol, to achieve constitutive activity, *Renilla* was cloned into an expression vector with a SV40 promoter. Ultimately, there are a lot of factors influencing variation with in a single replicate of a luciferase assay let alone across multiple experimental replicates performed on separate days. Without this internal *Renilla* control, it would be much harder to interpret trends and results.

Luciferase procedure

A typical luciferase experiment consists of three stages, transient transfection of cells, cell harvesting followed by cells lysis, and reading the luciferase levels of the cell lysates with a luminometer. While the first two steps of the assay can be tricky, they are not unique to a luciferase assay. Indeed, transient transfections and harvesting cells for their cell lysate are used in a multitude of molecular biology experiments. For that reason, I will spend less time dedicated to the explanation of the underlying principles of those techniques. However, the detection of luminescence for a luciferase assay is pretty unique to this kind of experiment and does require further explanation.

As previously mentioned, having two distinct luminescent signals is the foundation of the luciferase assay. Because the firefly and *Renilla* forms of luciferase are derived from different species, they require different substrates to illuminate [30,31]. This distinction makes it possible to discriminate between each protein's bioluminescence within the same sample. To create luminescence, both versions of the luciferase protein used in our protocol must be reacted with their specific substrate. This procedure is done following the protocol set out by the manufacturer of the substrates, Promega Corporation. First, Luciferase Assay Reagent II (LARII) (Promega Corporation) will be added to the cell lysate. This substrate will react with the firefly form of luciferase, releasing light. The luminometer will record the intensity of this light and give it a numerical value. The reaction will then be quenched. Second, Stop & Glo® Reagent (Promega Corporation) will react with the *Renilla* form of luciferase and again the luminescent intensity will be recorded [30].

Mammalian and Drosophila Luciferase

Our mammalian protocol for the luciferase reporter assay focuses on four components of the Hippo pathway, mammalian Ste20-like kinase (MST1/2), hSalvador, large tumor suppressor kinase (Lats1/2) and Mob1. These proteins make up the central regulatory cassette in the pathway [22,24,32]. MST activation leads to the phosphorylation of Lats. This turns on Lats activity, allowing it to phosphorylate its target Yes-associated protein (Yap). Phosphorylation of Yap prevents its translocation into the nucleus and marks it for degradation. Therefore, it is unable to interact with its transcriptional binding partner TEAD [1,7,25]. However, in the absence of Lats phosphorylation, YAP is able to translocate to the nucleus and bind to TEAD, turning on a whole host of pro-growth genes [1,5].

In the context of our luciferase assay, the results of Hippo pathway activity are seen through it phosphorylation state of YAP. When the Hippo pathway is on, Lats will phosphorylate YAP. This prevents YAP from interacting with TEAD. As previously described, TEAD has been fused to a Gal-4-Dbd. Without the interaction between YAP and TEAD, the Gal-4-Dbd does not bind to the Gal-4-UAS and luciferase is not transcribed. When Hippo pathway activity is off, YAP is able to associate with TEAD, leading to the Gal-4-Dbd binding to the Gal4-UAS and transcription of luciferase is activated. What this will ultimately mean is we expect to see low luciferase luminescence values when the Hippo pathway is on and high values when the pathway is off.

The *Drosophila* and mammalian luciferase reporter assays are based upon the same principle. They both use the core Hippo pathway components to ultimately phosphorylate their downstream targets, in *Drosophila,* Yorkie and in mammals, YAP [6,7]. For the *Drosophila* reporter assay, a Gal-4-DNA binding domain (Gal-4-Dbd) is fused to Yorkie. In the absence of Hippo pathway proteins, Yorkie is not phosphorylated, allowing the Gal-4-Dbd to interact with the Gal-4 promoter fused the luciferase gene and promoting gene transcription. Conversely, when all of the *Drosophila* pathway components, Hippo, Warts, Mats and dSalvador are transfected into the cells, you get a representation of Hippo pathway activity turned on. In this case, the kinase cascade ultimately phosphorylates Yorkie and prevents the Gal-4-Dbd from binding to the Gal-4-UAS linked to luciferase [7,33,34].

Research Question

Previous experiments have shown that co-transfecting members of the Hippo pathway into a luciferase system as described above does ultimately lead to the phosphorylation Yorkie/YAP and prevents the luciferase protein from being transcribed [10,35,36]. These results

suggest it is possible to adapt this assay to report on the function of variants of dSalvador, Hippo, MST and Lats. Additionally, previous work showed that MST alone could reduce luciferase expression levels by approximately sixty-fold and Lats (Warts) alone could reduce luciferase expression levels approximately one hundred-fold [9,10,35].

Currently in our lab we have projects examining protein interactions involving dSalvador, Hippo and dRassF, the minimal functional unit of Lats2 and the linker region of MST2. However, the bulk of this work has been using *in vitro* studies with purified protein. In order to confirm our *in vitro* findings in a cellular context and show the biological significance of our work, we wanted to establish protocols for cell based assays. We proposed adapting the luciferase assay to both mammalian and *Drosophila* cells as a tool in our ongoing projects to examine protein function. We were especially interested in the results showing that a single Hippo pathway component was sufficient enough to significantly reduce luciferase expression compared to the positive control. Based on these results, using a luciferase assay, we would be able to study individual proteins instead of having to test them in the context of all the Hippo pathway components [10,35,36]. However, at the inception of this project there was not a protocol for cell culture in the lab, let alone a luciferase assay. Because of this, the entire protocol from cell culture to luminescence detection had to be optimized to our reagents, plasmids, equipment and ability.

Results

Mammalian Luciferase

To begin experiments, we needed to establish the linear detection range of the luminometer. We used purified luciferase protein (Promega Corporation) in decreasing ten-fold

dilutions reacted with stock manufacturer LAR substrate at 1x concentration (Promega Corporation) (Figure 1A). We also wanted to determine the optimal concentration to use of the commercial substrates. During my previous work in the Camargo lab I had been successful in using stock manufacturer substrate diluted 3-fold with PBS. To evaluate the efficacy of diluted substrate, the same ten-fold dilution of purified luciferase protein was reacted with 1:3 diluted substrate. There was no difference in detection efficiency between diluted and undiluted substrate (Figure 1A). Using Graphpad Prism7, a line was fit to all the data points and we determined that the linear range of detection for the luminometer was between 100 and 10^6 units (Figure 1A).

Optimal transfection reagent and cell type

To find the transfection reagent most suitable for our assay we ran a test luciferase assay comparing the efficiency of each of the transfection reagents present in the lab on HEK293 cells (American Type Culture Collection (ATCC)). Cells were transiently transfected with either luciferase and *Renilla* or luciferase, *Renilla* and YAP using either Effectene (Qiagen), Fugene 6 (Promega Corporation), Lipofectamine LTX (Thermo Fisher Scientific) and PolyJet (SignaGen Laboratories) each according to the manufacturer's instructions. Each transfection reagent has an increase in luciferase expression in the positive control (with YAP) compared to the luciferase and *Renilla* alone (Figure 1B.). However, the luciferase activity from cells transfected with PolyJet, Effectene and Fugene 6 is ten to twenty-fold higher than the cells transfected with Lipofectamine LTX, indicating those were superior transfection reagents for this assay (Figure 1B).

To identify the optimal cell type for our protocol we used a luciferase assay to compare HEK293 cells (ATCC) and HEK293T cells (ATCC). HEK293T are a derivative of HEK293

cells that are "highly transfectable and contain the SV40 t-antigen (ATCC)." HEK293T cells have ten-fold more luciferase expression levels compared to HEK293 cells using PolyJet transfection reagent (Figure 2B).

Using fluorescence activated cell sorting (FACS), with the assistance of the Holland lab, we checked the transfection efficiency of HEK293T and HEK293FT cells with a reporter green fluorescent protein (GFP) plasmid using PolyJet (Figure 3). Additionally, as a transfection control, replicates of our experiment were performed by the Holland lab using their normal transfection reagent, Transfection Grade Linear Polyethylenimine Hydrochloride Max (PEI) (Thermo Fisher Scientific). When tested, there was no significant difference in GFP expression between HEK293T and HEK293FT cells (Figure 3). Interestingly, there was less than a fold change in efficiency between PolyJet and PEI (Figure 3).

Testing frozen cell lysate

In my previously lab work in the Camargo Lab we had frozen luciferase assay cell lysate in order to accommodate scheduling. When we compared assay results between fresh and frozen cell lysate we never saw any difference. However, before adopting this procedure, we wanted to validate that in our hands we would also not see a difference. To do this, a luciferase assay was run using lysate from cells transfected with either luciferase and *Renilla,* luciferase, *Renilla*, and YAP, or luciferase, *Renilla*, YAP and Hippo pathway proteins using PolyJet transfection reagent that was either fresh or previously frozen (Figure 4). When comparing the exact same experimental samples, fresh or previously frozen, no difference in luciferase activity was noted. This confirmed that samples could be frozen and luminescence recorded at a later time (Figure 4).

Optimization of plasmid ratios and incubation times

After performing these initial luciferase assays we noticed that when Hippo pathway components were transfected, we did not see the previously published reduction in luciferase expression (Figure 2C) [10,36]. In order to try to rectify this issue, we tested different incubation times post transfection (Figure 5). Cells were transfected with plasmids encoding luciferase and *Renilla,* luciferase, *Renilla*, and YAP, or luciferase, *Renilla*, YAP and Hippo pathway proteins using PolyJet transfection reagent. They were then harvested and lysed at either 48 or 72 hours. Unfortunately, there was no change between these time points (Figure 3A). Additionally, we tested cells transfected with either luciferase and *Renilla,* luciferase, *Renilla*, and YAP, or luciferase, *Renilla*, YAP and MST or Lats using PolyJet at 24, 48, 72 or 96 hours. Again, there was no change in luciferase expression between different time points (Figure 5C). Lastly, an experiment was run at two different time points in which the amount of YAP was changed compared to our original protocol (Figure 5B). Cells were transfected with either luciferase, *Renilla*, and YAP (14ng or 70ng), or luciferase, *Renilla*, YAP (14ng or 70ng) and Hippo pathway proteins using PolyJet transfection reagent. After either 24 or 48 hours cells were harvested and lysed. By reducing the amount of YAP, there appears to be a half-fold reduction in relative luciferase activity in the samples with Hippo pathway components added (Figure 5B).

Based upon Zhao et. al. 2007, the major players in the luciferase reporter assay are the core Hippo kinases, MST and Lats [10]. With that in mind, we tested to see either kinase alone would be sufficient to inhibit luciferase activation. Cells were transfected with either luciferase and *Renilla,* luciferase, *Renilla*, and YAP, or luciferase, *Renilla*, YAP and MST or Lats using PolyJet. In order to try and saturate the system the amount of each kinase was significantly increased from the original 50ng protocol to either 250ng or 500ng. As previously mentioned, the cells were harvested at four time points, 24, 48, 72 and 96 hours. Increasing MST or Lats did

not inhibit relative luciferase activity. When compared to the positive control (with YAP) there was less than a fold reduction in expression levels (Figure 5C).

Continued optimization of cell lines and transfection reagents

Because we had not been able to reproduce previously published results with the cells and reagents in lab, we bought new HEK293T cells (ATCC) and a new transfection reagent Lipofectamine 2000 (Thermo Fisher). I had worked previously with Lipofectamine 2000 in the Camargo Lab and had never had transfection issues when using it. We compared newly purchased cells to the cells already in lab, transfecting them with either luciferase and *Renilla,* luciferase, *Renilla*, and YAP, or luciferase, *Renilla*, YAP and Hippo components using either PEI or Lipofectamine 2000. Cells transfected with luciferase, *Renilla*, YAP using Lipofectamine 2000 have a two hundred-fold increase in relative luciferase compared to cells transfected using PEI (Figure 6A and 6B). Additionally, when comparing the cells purchased from ATCC to the HEK293T cells already in culture in the lab, there is a seventy-fold greater reduction in luciferase expression when Hippo components are added to luciferase, *Renilla* and YAP, (Figure 6A and 6B).

Optimal luciferase conditions

Through optimizing the amount of each plasmid being transfected for our luciferase assay, we were ultimately able to achieve between a sixty and two hundred-fold reduction in relative luciferase activity when Hippo components are added (Figure 7A and 7B). This was the result of adjusting the amount of Gal-4-UAS-Luciferase that we transfected. By increasing the amount of plasmid encoding for the luciferase protein and reducing the amount of plasmid encoding TEAD, our results began to reproduce previously published data. We were also able to replicate the sixty-fold reduction in luciferase expression by MST when compared to luciferase,

Renilla, and YAP. However, this difference in not statistically significant (Figure 7) [10]. Unfortunately, we could not replicate the previously published results of a one hundred-fold reduction of relative luciferase activity by Lats alone (Figure 7) [10].

Drosophila Luciferase

To test the *Drosophila* reporter assay, S2 cells were transfected with luciferase and *Renilla* (null control)*,* luciferase, *Renilla* and Yorkie (positive control) using Fugene6. The two core kinases Hippo and Warts were also transfected, either alone or together. Experiments with Hippo alone, Warts alone, and Hippo with Warts all reduced the relative luciferase activity to levels within error of the null control (Figure 8A). The reduction of luciferase expression to the null control signifies that Hippo and Warts' kinase activity is enough to completely prevent Yorkie from driving luciferase transcription. This result matches previously published work [7,35]. While our results for Hippo and Warts activity are similar to published work, relative luciferase activity in the positive control was about six-fold lower than previously published. While this difference is not large in context of the mammalian luciferase assay, in the *Drosophila* assay, the relative luciferase activity seen in the positive control maxes out around fifteen to twenty-fold higher than the null control.

Optimization of plasmid ratio

In order to increase the relative luciferase activity for the positive control, the amount of Yorkie used in our standard protocol was increased in two-fold amounts of Yorkie, from 25ng to 200ng. To make sure that the amount of Yorkie would not saturate the system, experiments with luciferase, *Renilla,* increasing amounts of Yorkie and constant amounts, 50ng, of Hippo and Warts were run alongside the experiments with luciferase, *Renilla* and the increasing amounts of Yorkie. The increased amounts of Yorkie (50ng, 100ng, or 200ng) have approximately seven-

fold higher luciferase expression levels than the 25ng used in the original protocol (Figure 8B). These expression levels match previously published results [7,35] Complete reduction of luciferase expression by Hippo and Warts was seen in experiments conducted with 25ng, 50ng and 100ng of Yorkie with Hippo and Warts (Figure 8B). However, when 200ng of Yorkie were transfected into S2 cells, there was no longer a significant reduction in relative luciferase activity when compared to the positive control (Figure 8B). This signifies that at 200ng of plasmid encoding Yorkie, the system becomes saturated and Hippo and Wart's kinase activity is no longer great enough to inhibit luciferase activation.

Optimization for dSalvador

One of our research questions in the Kavran Lab is how dSalvador, in particular the extended N-terminal region of dSalvador's SARAH domain impacts Hippo signaling. Therefore, during *Drosophila* assay optimization it was important to make sure that our protocol was optimized to examine dSalvador function. To establish a protocol for monitoring the effects of dSalvador on Hippo pathway signaling, S2 cells were transfected with the null control, positive control, luciferase, *Renilla,* Yorkie and dSalvador alone or with dSalvador co-expressed with Hippo, or Hippo and Warts using Fugen6. When dSalvador was added alone it resulted in a two hundred-fold reduction of luciferase expression compared to the positive control but did not result in the complete reduction of relative luciferase activity seen in previous experiments with Hippo or Warts (Figure 9A). To try and get the complete reduction of relative luciferase activity, the experiment was repeated but with 200ng of plasmid encoding for dSalvador instead of the 50ng previously used. Again, there was a significant, two hundred-fold reduction in activity but there was not a complete reduction of luciferase expression (Figure 9B).

Methods

Cell Culture

Mammalian cells, HEK 293, 293T and 293FT were cultured in Dulbecco's Modified Eagle Medium (Gibco) with 2mM L-Glutamine (Gibco) and 5% heat inactivated Fetal Bovine Serum (FBS) (VWR). After research and additional testing, we determined that a synthetic form of FBS could be used in culture media and switched the lab over to FBEssence (FBE) (VWR). Culture chamber was maintained at 37ºC with 5% CO_2. All mammalian Luciferase transfections were performed in 12 well cell culture plates seeded with 5*10^4 cells in 1mL of culture media. Cells were seeded and transfected on the same day for all mammalian reactions. After 48 hours cells were harvested and lysed. Cell lysate was then used for the luciferase assay.

Drosophila S2 cells were cultured in Schneider's Insect Medium (Gibco) with 10% heat-inactivated FBS. S2 cells cannot be grown in media with FBE. The culture chamber was maintained at 28ºC without CO_2. All *Drosophila* luciferase experiments were performed in 12 well cell culture plates seeded with 1*10^5 cells in 1mL of culture media. Cells were seeded on day 0 and transfected on day 2. 72 hours after transfection cells were harvested and lysed.

Mammalian and Drosophila Luciferase Protocol:

Transfections for both mammalian and *Drosophila* cells were performed following a standard protocol (Appendix 1). For each luciferase experimental condition, two or three wells (depending upon well availability) were transfected in replicate from the same master mix of transfection reagent and DNA. These experimental replicates are in order to prevent any significant transfection variation that could confound results. In order to obtain publication level results, multiple biological replicates across multiple days are needed but for many of the early test luciferase assays only the two experimental replicates were obtained.

After transfection, cells were cultured according to a standard protocol (Appendix 1). At the appropriate time, cells were harvested and lysed. Luciferase assay was then performed using the appropriate commercially available substrates and a H1 Synergy (Biotek) luminometer for detection of luminescence (Appendix 1).

The results from the luminometer are raw intensity values and do not immediate give us much information about our proteins of interest. In order to better understand the data, we first divide the raw "luciferase" value by the corresponding "*Renilla"* value for each replicate which will be called the "ratio". As previous mentioned, this takes into account any variations across wells. Next, the ratio values for the positive control and our experimental wells will be divided by the ratio value of the null control. In our protocol, the null control will be wells transfected with only luciferase and *Renilla*. The ratio value from the first well of the null control duplicate will be used to divide the ratio value for the first well of the positive control duplicate and continuing to the experimental wells. The ratio value of the second well of the null control will be used in the same manner. The results of this should give the null control a value of one with the positive control and experiments with a positive number. That number is plotted as relative luciferase activity.

Graphs were plotted using Graphpad Prism7 using either an XY, column or grouped graph depending on the experimental parameters. The Y-axis correlates to the relative luciferase expression given in units, as previously described. The X-axis represents the experimental conditions of the results in that column of the graph. To calculate p values, the appropriate statistical test (t-test, one-way ANOVA, two-way ANOVA, etc.) was performed using the analysis tool in Graphpad Prism7.

Fluorescence-activated Cell Sorting

6 well plates were seeded with 3*10^5 mammalian cells in 2mL of culture media. Cells were transfected with 2ug of empty pcDNA or pcDNA-GFP (plasmid code 112, Kavran Lab) plasmid. After 48 hours, the transfected cells were harvested from their plates. Due to the adherent nature of HEK293T and HEK293FT and in order to be as gentle as possible so not to lyse the cells, 0.05% Trypsin-EDTA (Gibco) was used to remove the adherent cells instead of mechanical force. To do this Trypsin was pre-warmed to 37ºC in a water bath, media was removed from the cells and 0.5mL of Trypsin was added to each well. The plate was then returned to the incubator for 2 minutes, or until cells easily lifted off the plate. 1mL of DMEMF12 with 5% FBE and 2mM L-glutamine was then added to each well in order to neutralize the Trypsin. Then the cell containing media was transferred to 1.5mL Eppendorf tubes. Tubes were then centrifuged at 1200*g for 5 minutes. The supernatant was removed and the cell pellet was gently washed with 1mL of Phosphate buffered Saline (PBS). This was then spun down again and supernatant aspirated. Finally, the cells were re-suspended in 2mL of PBS, transferred to appropriate FACs tubes and sorted by a Fluorescence-activated cell sorting machine. GFP gates were set against cells transfected with an empty plasmid.

Transfection reagents

Effectene (Qiagen), Fugene 6 (Promega Corporation), Lipofectamine 2000 (Thermo Fisher Scientific), Lipofectamine LTX (Thermo Fisher Scientific), Transfection Grade Linear Polyethylenimine Hydrochloride (PEI) Max (Thermo Fisher Scientific) and PolyJet (SignaGen Laboratories).

Mammalian Plasmids

pcDNA-GFP (MacroLab), pcDNA3-Myc-Lats, pcDNA3-HA-Mob, pcDNA3-HA-Mst2, pcDNA3-HA-hSalvador, pcDNA3-CMV-Yap2, pCMX-Gal4-Tead4, pGL4.31-Gal4-UAS-

Luciferase, pRL-SV40-*Renilla.* All mammalian luciferase plasmids were obtained from the Addgene repository. Plasmid codes can be found in the plasmid table for the final protocol.

Drosophila Plasmids

pAc-Gal4Dbd-YkiV5 (Irvine Lab), pUAST-Gal4-Luciferase (Irvine Lab), pRL-SV40-*Renilla* (Irvine Lab), pAWF-dHpo (Tapon Lab), pAWF-dMats (Tapon Lab), pAMN-dSav (Tapon Lab), pAWF-dWts (Tapon Lab) and pAWF-dRassF (Tapon Lab). Plasmid codes can be found in the plasmid table for the final protocol.

Discussion

Mammalian Luciferase

There was not very much cell culture to speak of in lab when protocol optimization began. Thanks to Dr. Kavran's previous lab we had many of the needed reagents to make media and maintain the cell cultures. While there were not many issues with the reagents, there were some challenges when it came to the equipment like determining the detection limits of the luminometer (Figure 1A).

The two biggest challenges to optimization though turned out to finding the optimal cell line and transfection reagent. Originally, we had HEK293 cells in culture but we quickly discovered their transfection efficiency was not good enough (Figure 2B). This left us using HEK293T and HEK293FT cells. Both these cells are optimized for transfections over the normal HEK293 cells. When they were compared to each other there was no difference in transfection efficiency of HEK293T and HEK293FT cells (FACS DATA). Ultimately though because we could not replicate previously published result, we purchased brand new HEK 293T cells from ATCC. The new HEK293T cells proved to be able to be transfected efficiently and with

optimization of other variables ultimately produced the results we were hoping for (Figure 5B and 6).

Besides determining the right cell line, finding the optimal transfection reagent was the biggest road block to our experiments. In particular, the results of the FACS experiment (Figure 3) inadvertently caused a significant challenge in optimizing the protocol. As a positive control in the FACS experiment, we used the transfection reagent that the Holland Lab uses, PEI. PEI is significantly cheaper and has an even easier protocol than PolyJet, the transfection reagent we were using. As a transfection reagent, it worked great for single plasmids as seen in the FACS data and it was assumed that it would work just as well for the luciferase assay (Figure 3). For a long time, challenges with cells hid the issue with using PEI for multiple plasmids. Ultimately though, through research and reading online science forums it was determined that PEI was not efficient at transfecting more than three of four plasmids. This was why we believe there was not significant inhibition of luciferase activity when PEI was being used to transfect in all of the Hippo pathway components (Figures 6). The same science forums that I had used to find out about PEI also gave suggestions about alternative transfection reagents but ultimately, I turned the transfection reagent I had used while working in the Camargo lab and I was comfortable working with, Lipofectamine 2000 (Thermo Fisher). Immediately, we saw large increases in the expression of our proteins following transfection with two to three hundred-fold increases in luciferase expression in experiments transfected with Lipofectamine 200 compared to PEI (Figure 6). After a few trial runs, we were able to see results similar to what was previously demonstrated (Figure 6 and 7) [10].

The final part of the puzzle in getting all of our data to match previously published results, was finding the right ratios of plasmids to transfect. Our original protocol used a 1:1 ratio

of all the plasmids, except for *Renilla*. From previous work with luciferase assays in the Camargo Lab, I knew that *Renilla* had very good expression and at a 1:1 ratio would not fall within the luminometer's detection range. With that in mind, the original protocol used only 14ng of *Renilla* DNA in all of the transfections. It was quickly discovered that the 1:1 DNA ratio was maxing out the luminometer detection limits (Figure 2). In Figure 2B it should be noted that an experimental value is missing for the HEK293T value in column "2" this a because the luciferase value exceeded the detection limit of the luminometer, resulting in an "overflow" instead of a numerical value. Additionally, because there was little to no luciferase inhibition from the Hippo components, it was hypothesized that the system was being saturated with YAP (Figure 2B). To prevent this, the amount of YAP was reduced to a third of the amount of the rest of the plasmids. This allowed for sufficient levels of activity of the Hippo kinases to phosphorylate and sequester YAP (Figure 7). In the end, the biggest change to the transfection protocol came from a suggestion from a friend of the lab, Chris Cho. He suggested that we increase the amount of Gal4-Luciferase and Hippo components being transfected in and reduce the amount of Gal4-TEAD. This was based off of his previous experience with luciferase assay [37]. This change finally made it possible to partially reproduce the Zhao et. al. 2007 results (Figure 7).

Our protocol was ultimately able to reproduce the one hundred to one hundred twenty-fold luciferase expression reduction seen with all the Hippo pathway components as well as the fifty to sixty-fold reduction with MST alone, but we were not able to reproduce the one hundred-fold reduction from Lats alone that Zhao et. al. 2007 show. With all the Hippo pathway components, we can significantly inhibit luciferase expression. Individual proteins show either not reduction in luciferase expression or only weak inhibition that is not significant. This means

that we do have a protocol where we can test the functionality of our Lats and MST mutants, but only in the context of all the other Hippo pathway components. Unfortunately, the dynamic range of our results doesn't provide the space to judge relative activity level. Instead, our protocol can only be used to give a binary answer of either the mutant is functional or it is not. In the future, optimizing a protocol that allows examination of individual proteins would be ideal. Finding a system that relied upon fewer plasmid could further increase our expression levels and creating a dynamic range where a single protein could be examined. Additionally, recent unpublished work in our lab suggests that our HEK293T cells have a relatively high background level of Hippo pathway activity. This could prove to be confounding in our system as we have seen that the luciferase assay is fairly sensitive to change and can have wide variation between experiments. Switching to an alternative cell line might help to address this problem and further optimize our protocol.

Drosophila Luciferase

Unlike the mammalian luciferase reporter assay, our protocol was able to replicate previously published result using S2 cells on the first attempt [7]. The only major difficulty was establishing a culture protocol for our S2 cells. Originally, the media, Grace's insect media (Thermo Fisher) supplemented with 10% FBS, that we used did not work. However, we quickly realized that this was not the appropriate media for S3 cells. Upon suggestion from another lab, and researching preferred media for S2 cells, we switched our media to Schneider's *Drosophila* media (Gibco) with 10% FBS. *Drosophila* culture media must be supplemented with FBS and not FBE. When the synthetic form of FBS, FBE, that our lab uses in mammalian media was used instead, the S2 cells died.

Preliminary S2 luciferase data showed that our protocol resulted in six-fold (about 50% lower) than published data for luciferase expression in the positive control. In order to rectify this issue, we tested the amount of Yorkie that could raise our luciferase expression while still responding to Hippo and Warts activity. Figure 8B. shows that there was no change in the level of luciferase expression between different Yorkie amounts except for when 200ng of Yorkie was used. The previous experiment was conducted with 25ng of Yorkie. Since our reduction of luciferase expression was complete, we hypothesized that levels of Hippo and Warts were saturating the system. As hypothesized, Hippo and Warts were saturating the system. By increasing the amount of plasmid encoding Yorkie transfected to 50ng we were able to increase our luciferase expression in the positive control almost eight-fold, similar to that of previous work [7]. We did note that between the 50ng, 100ng, 200ng of plasmid encoding for Yorkie there was not change in luciferase expression. This suggests that 50ng of Yorkie was enough to saturate the Gal-4 binding site on luciferase and that Yorkie was no longer the limiting factor in the system. Due to these results, the protocol was amended to use 50ng of plasmid encoding for Yorkie.

Since dSalvador alone did not reduce luciferase expression to the desired level, it could not be used alone in our protocol however, this was not unexpected. Instead, dSalvador must be co-transfected with Hippo in order to reduce luciferase expression to the desired result. This would later prove to be a confounding issue. The amount of luciferase reduction seen between experiments with Hippo and dSalvador and Hippo alone provides a very small dynamic range. It is important to note that in experiments with dSalvador, the luciferase expression level is much higher than in other S2 experiments (Figure 8 and 9). This allows for a much larger fold

reduction than usual however, it is extremely variable. The elevated level of expression is seen in some later experiments but not others.

A.

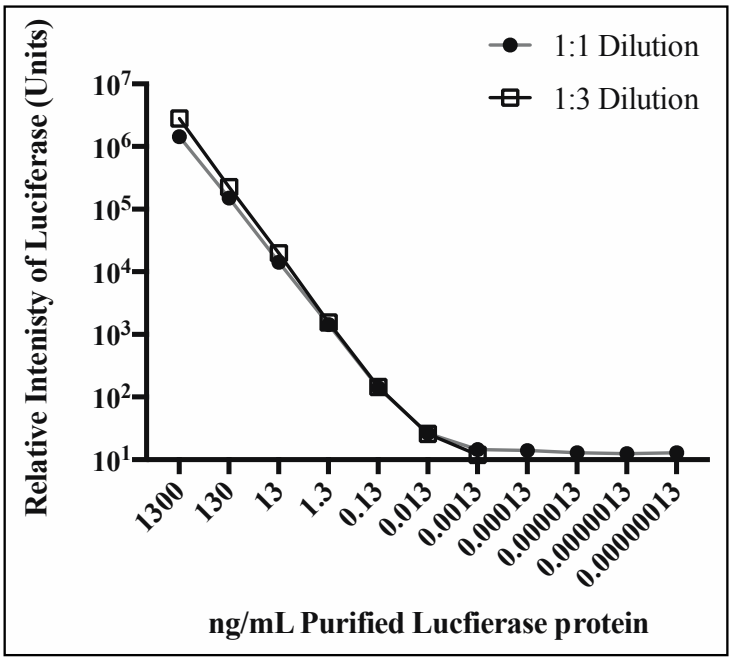


Figure 1A. The dynamic range of luciferase detection

Purified luciferase protein was diluted in a ten-fold manner with PBS and then reacted with substrate. Using a luminometer, luminescent emission was detected and unit readout was provided. Additionally, 1:3 diluted substrate was tested for efficacy in comparison with manufacturer's stock. Luminescent values were then plotted against the known value of purified protein to obtain a standard curve. A line was then fit to the data to demonstrate the linear range of detection of the luminometer.

B.

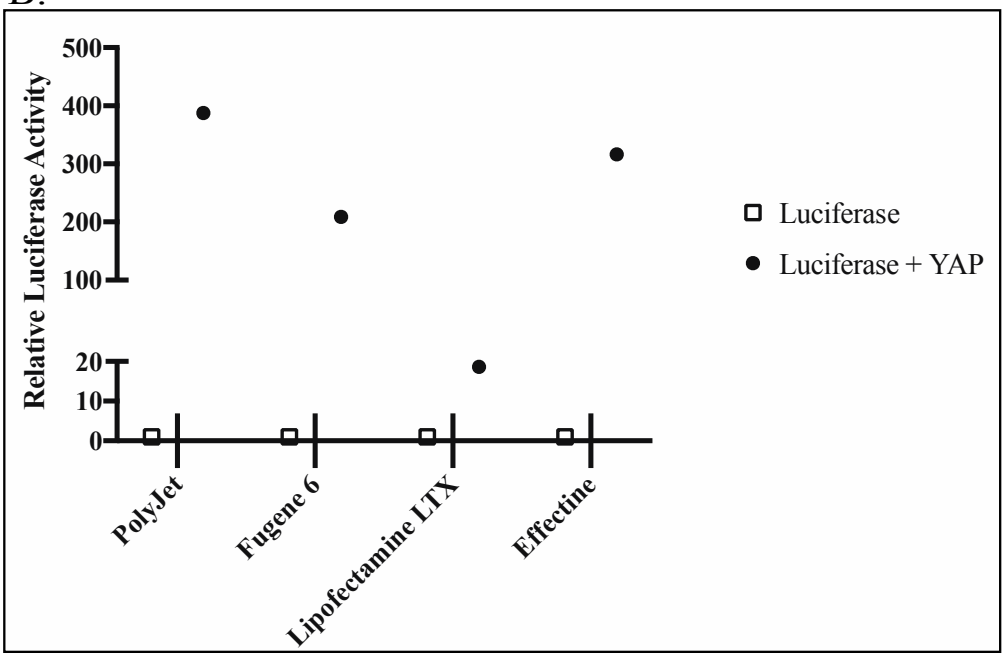
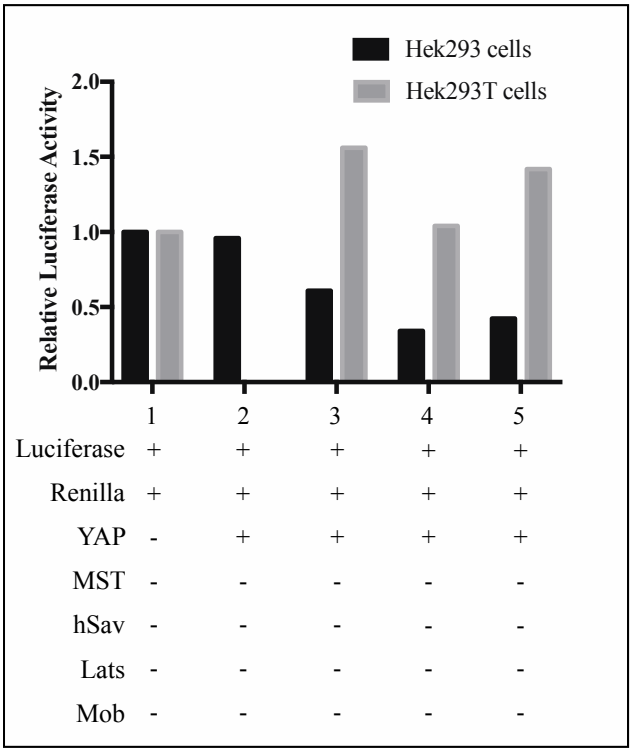


Figure 1B. Optimal transfection reagent for luciferase reporter assay

Different transfection reagents were used to transfect in 10ng of Renilla, 70 ng of Gal4-Luciferase, 70ng Gal4-TEAD, and 70ng of Yap1 using either PolyJet, Fugene 6, Lipofectamine or Effectine. After 48 hours cells were lysed and luciferase assay was performed using cell lysate.

A.



B.

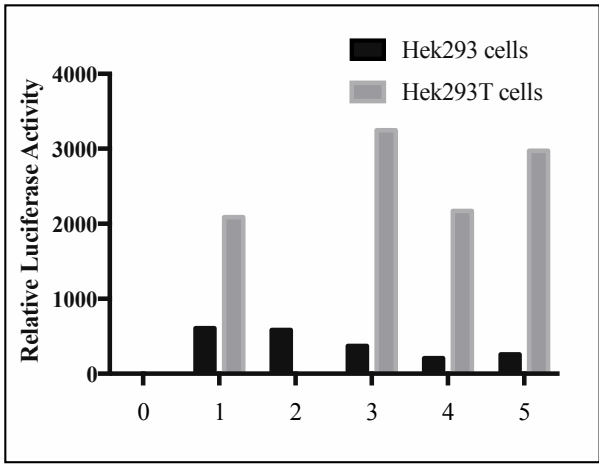


Figure 2. Optimization of luciferase reporter assay in Hek293 cells

A. Using PolyJet transfection reagent, Hek293 and Hek293T cells were transfected with Gal-4-Dbd-TEAD, Gal-4-UAS- luciferase, Renilla, Yap and Hippo pathway components. After 48 hours cells were lysed and luciferase assay was run using cell lysate. Luciferase expression was normalized to the positive control (1. Luciferase, Renilla, and YAP). The black bars represent results from Hek293 cells. The grey bars represent results from Hek293T cells.
B. Instead of normalizing to the positive control, the same data as A. was normalized to the null control (0. Luciferase and Renilla). The null control in our system takes into account any endogenous YAP that could interact with Gal-4-Dbd-TEAD leading to luciferase expression. Of note, column 2 is lacking a value for Hek293T cells because the value for the expression level exceeded the detection limits of our system.

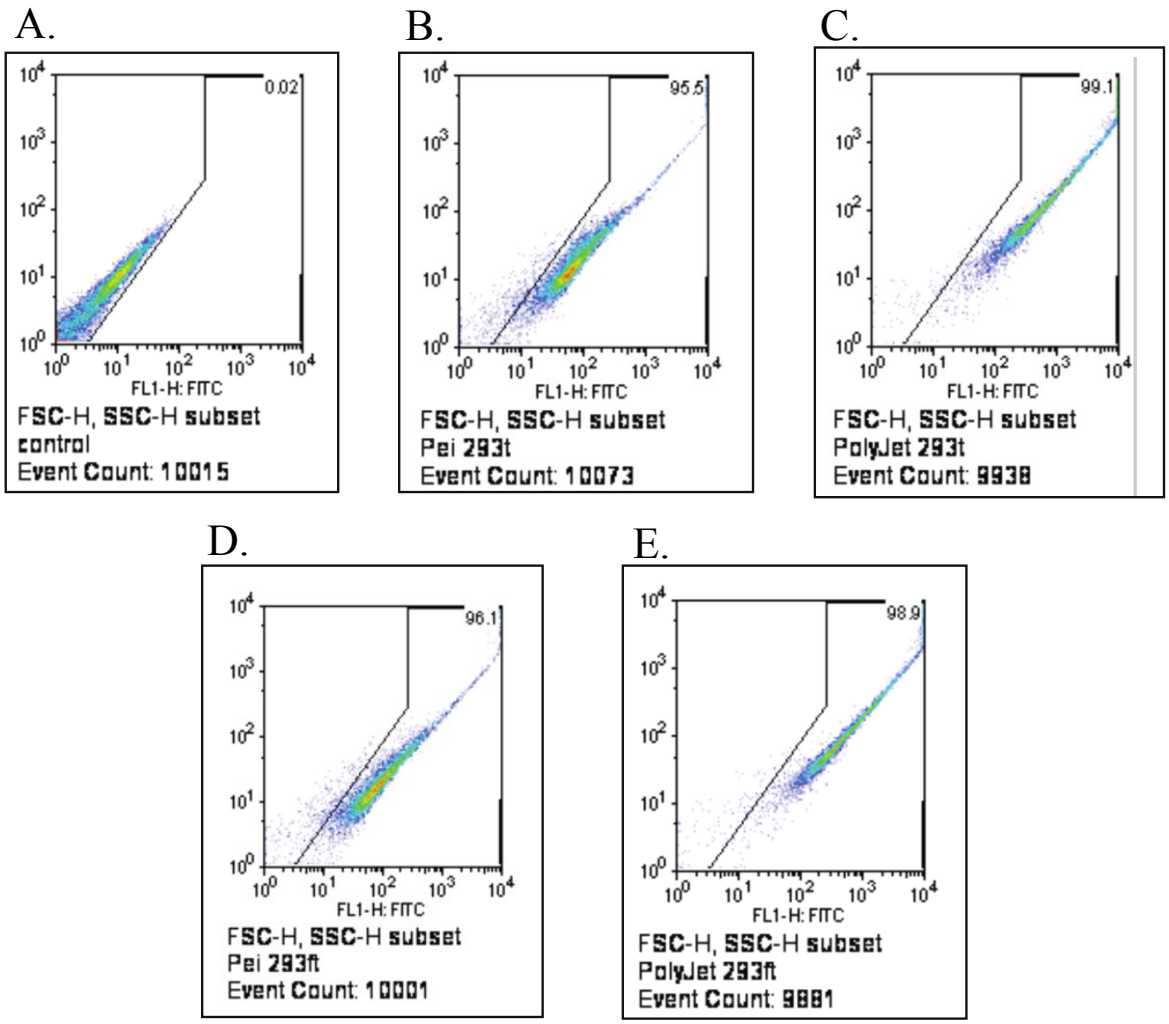


Figure 3. Fluorescence Activated Cell Sorting (FACS) of Hek293T and Hek293FT cells

Hek293T cells transfected with empty plasmid encoding nothing (panel A). Panels B-E were transfected with a plasmid that encodes green fluorescent protein (GFP) (plasmid 115, Kavran Lab). Panels B and C are Hek293t cells. Panels D and E are Hek293FT cells. For panels B and D GFP encoding plasmid was transfected using PEI. For panels C and E GFP encoding plasmid was transfected using PolyJet.

Cells in the blank controls (Panel A) were used to set sorting gates. All cells to the left of the middle line are green fluorescent protein (GFP) negative. A total of 10,000 cells were sorted.).02% of null cells were positive for GFP using the gate setting we chose. Heat map corresponds to the number of cells that are present at that fluorescent intensity (red is high cell number, blue is low cell number). For all panels, the Y-axis is side scatter and the X-axis the FL1-H:FITC channel which captures green fluorescence.

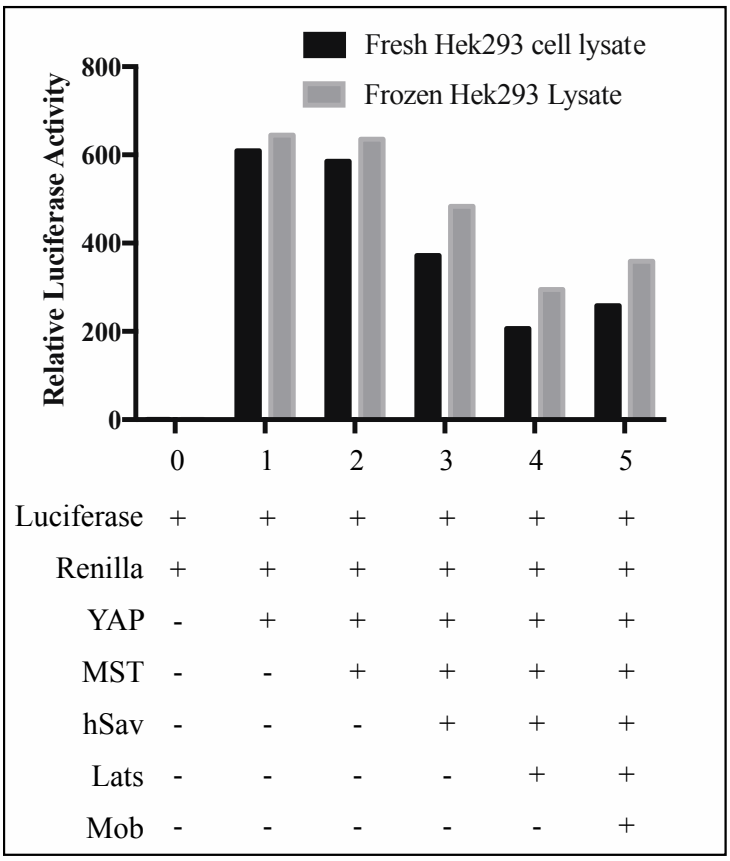
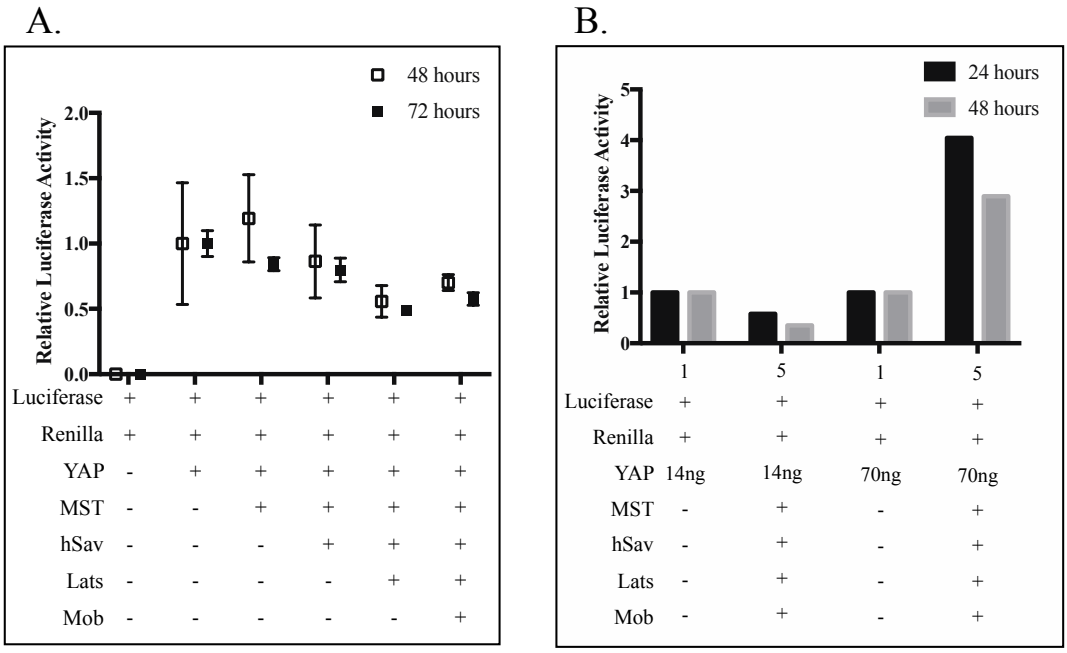


Figure 4. Comparison of luciferase assays performed on fresh or previously frozen cell lysate

Using PolyJet transfection reagent, Hek293 cells were transfected with Gal-4-Dbd-TEAD, Gal-4-UAS- luciferase, Renilla, Yap and Hippo pathway components. After 48 hours cells were lysed and luciferase assay was run using cell lysate. Luciferase expression was normalized to the null control (0. Luciferase and Renilla). The null control in our system takes into account any endogenous YAP that could interact with Gal-4-Dbd-TEAD leading to luciferase expression. Lysate was either previously frozen or fresh. Black bars represent results from fresh lysate. Grey bars represent results from previously frozen lysate.



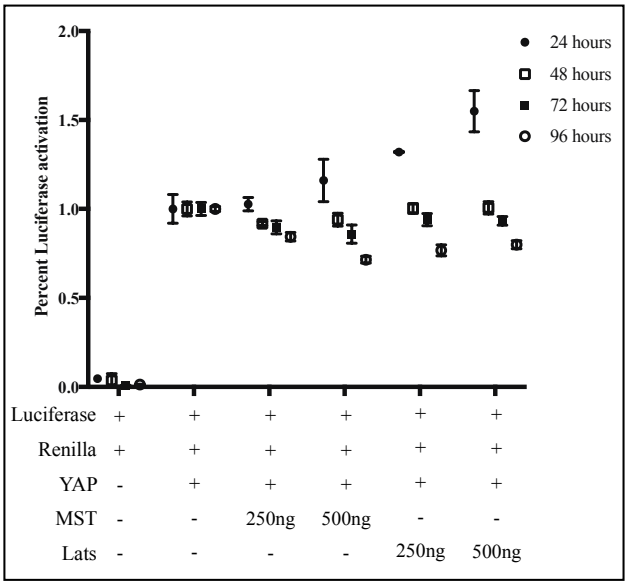


Figure 5. Optimal post transfection incubation period and determination of ideal plasmid ratios

A. Using PolyJet transfection reagent, Hek293 and Hek293T cells were transfected with Gal-4-Dbd-TEAD, Gal-4-UAS- luciferase, Renilla, Yap and Hippo pathway components. After 24 and 48 hours cells were lysed and luciferase assay was run using cell lysate. Luciferase expression was normal - ized to the positive control (1. Luciferase, Renilla, and YAP). The black boxes represent results from 24 hours. The empty boxes bars represent results from 48 hours cells. Error bars represent the standard deviation, as calculated by Graphpad Prism7, between the two well replicates.

B. To try and find optimal induction of luciferase activity, the amount of YAP transfected was varied. Cells were then harvested at either 24 or 48 hours following transfection. Results were normalized to the positive control (luciferase, Renilla and Yap). Black boxes represent results from 24 hours. Grey boxes represent result from 48 hours.

C. Increasing amount, 250ng or 500ng, of the two core kinases, MST and Lats, were transfected. After 24, 48, 72 and 96 hours cells were lysed and luciferase assay was run using cell lysate. Luciferase expression was norm alized to the positive control (1. Luciferase, Renilla, and YAP). Black circle represent data from 24 hours. Empty boxes re present data from 48 hours. Black boxes represent data from 72 hours. Empty circles represent data from 96 hours. Error b ars represent the standard deviation, as calculated by Graphpad Prism7, between the two well replicates.

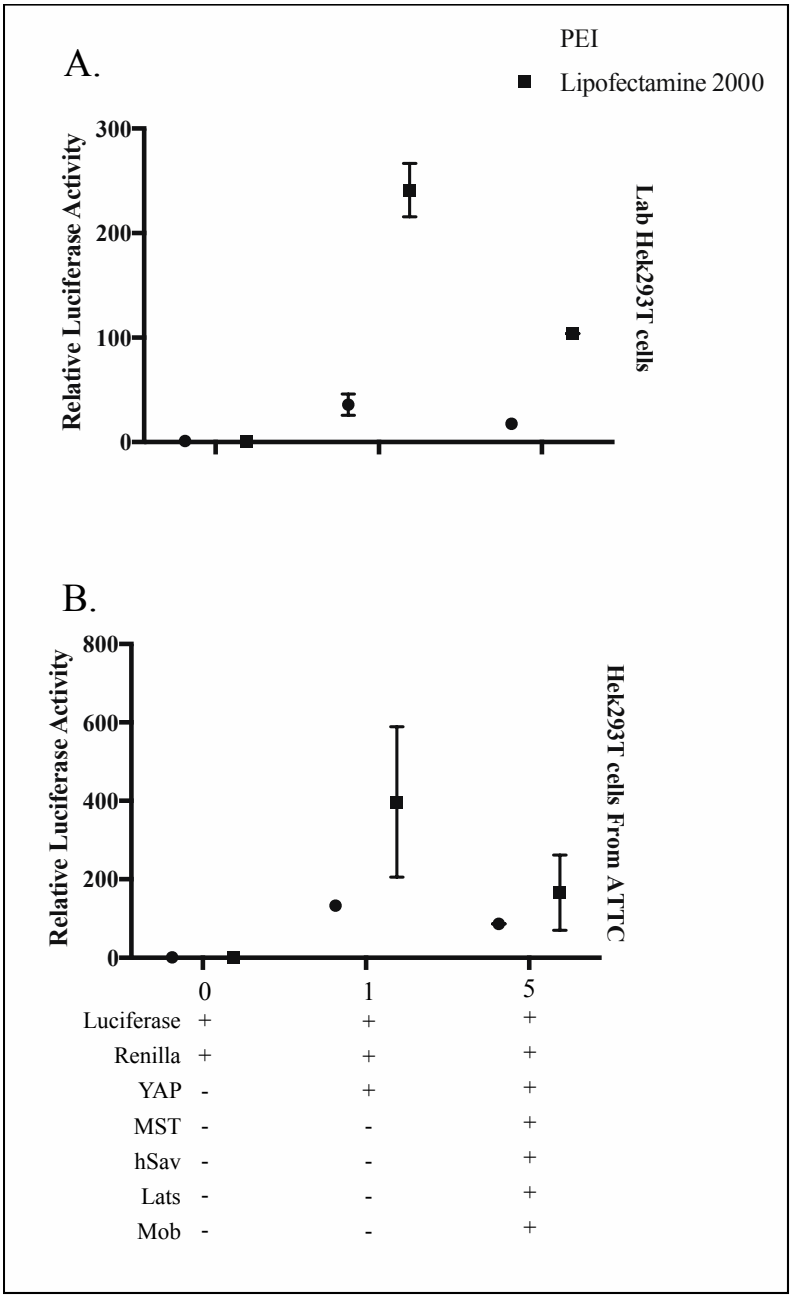


Figure 6. Hek293T cells purchased from ATTC and the alternative transfection reagent Lipofectamine 2000 increase luciferase expression

A. Hek293T cells previously cultured in lab were transfected with Gal-4-Dbd-TEAD, Gal-4-UAS-luciferase, Renilla, YAP and Hippo components using either PEI or Lipofectamine 2000. After 48 hours cells were harvested. Results were normalized to the null control (luciferase and Renilla). Using GraphPad Prism, standard deviation was calculated. Error bars represent the standard deviation between replicates. Black circles represent results from cells transfected with PEI. Black boxes represent results from cells transfected with Lipofectamine 2000.
B. Hek293T cells purchased from ATCC were transfected with Gal-4-Dbd-TEAD, Gal-4-UAS-luciferase, Renilla, YAP and Hippo components using either PEI or Lipofectamine 2000. After 48 hours cells were harvested. Results were normalized to the positive control (luciferase, Renilla, and YAP). Using GraphPad Prism, standard deviation was calculated. Error bars represent the standard deviation between replicates. Black circles represent results from cells transfected with PEI. Black boxes represent results from cells transfected with Lipofectamine 2000.

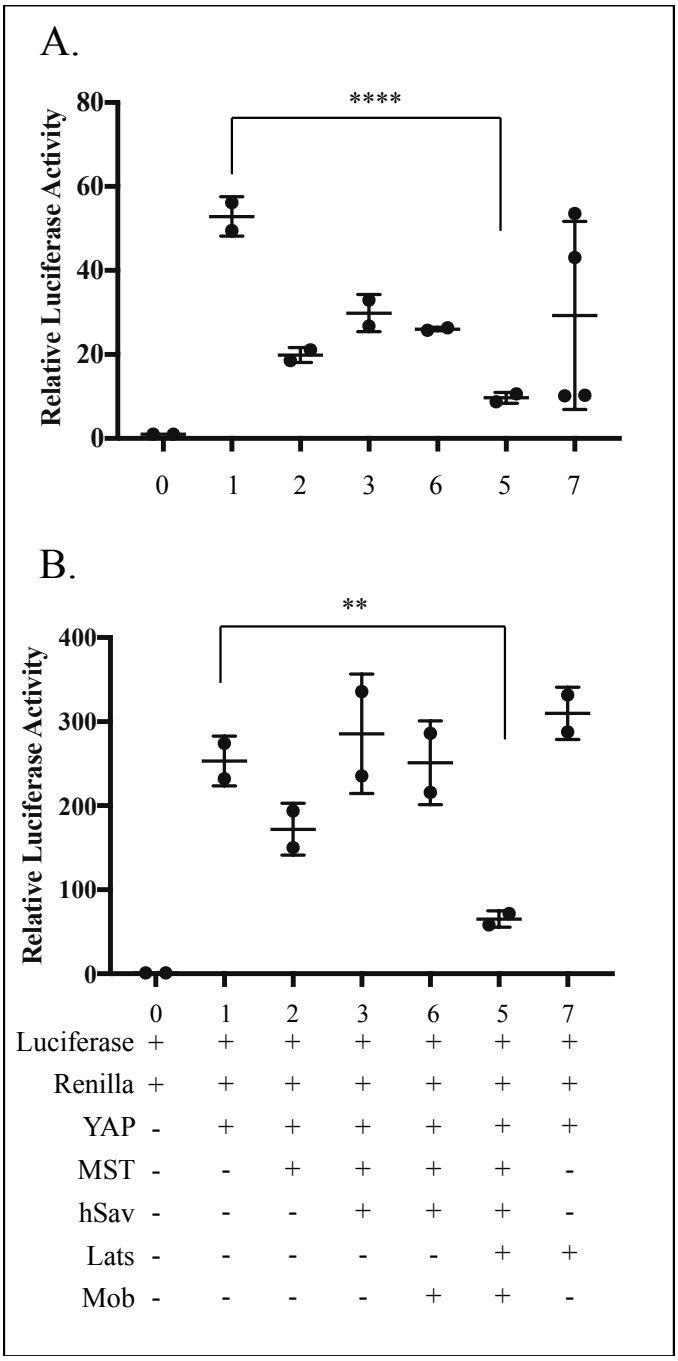


Figure 7. Hippo pathway components inhibit luciferase expression

A. First run of new protocol using Lipofectamine 2000, new Hek293t cells and plasmid ratios. Hek293T cells previously cultured in lab were transfected with Gal-4-Dbd-TEAD, Gal-4-UAS-luciferase, Renilla, YAP and Hippo components using Lipofectamine 2000. Results were normalized to the null control (luciferase and Renilla). Using Graphpad Prism7, luciferase expression values were plotted. Error bars represent the standard deviation between the two replicates of each experimental condition. Statistics calculated in Graphpad Prism (p value 0.001, n=2).
B. Second run repeating previous experiment to confirm results. (p value 0.005, and 0.009 n=2).

A.

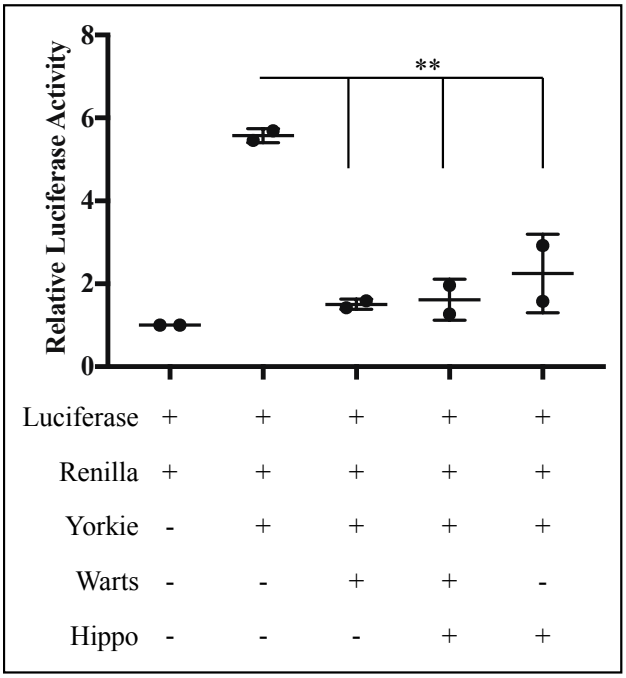
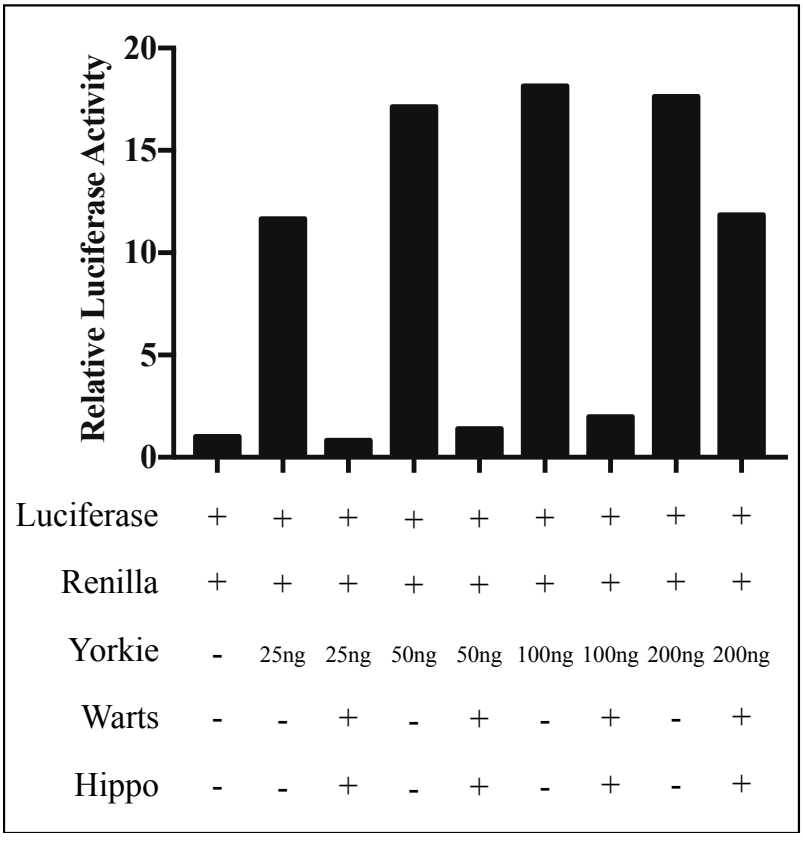


Figure 8. Optimization of Drosophila luciferase plasmids and plasmid concentration

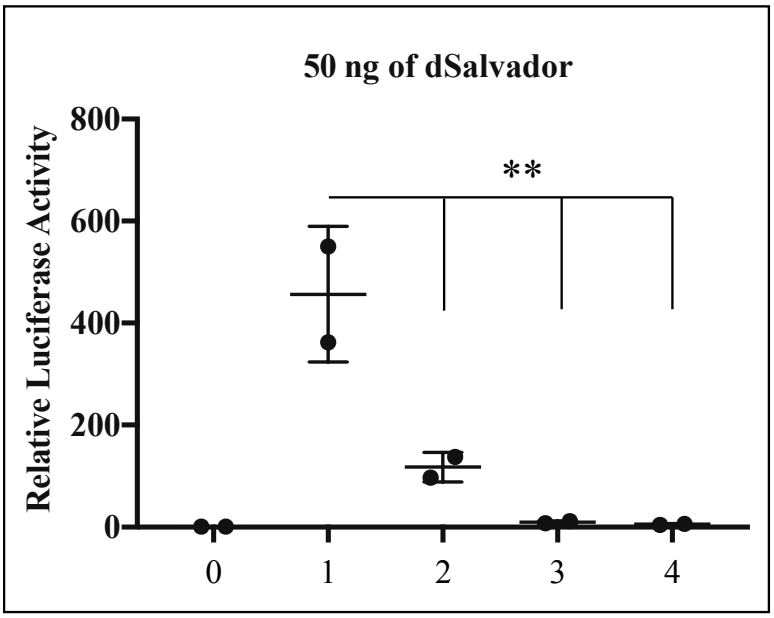
A. S2 cells were transfected with plasmids encoding for Gal-4-UAS-luciferase, Renilla, Gal-4-Dbd-Yorkie, Warts and Hippo using Fugene 6. Two replicates of each sample were run. Data was normalized to the luciferase expression of the null control (luciferase and Renilla). Normalized data was then plotted and standard deviation determined using Graphpad Prism7. Statistical analysis was performed comparing experiments to the positive control (luciferase, Renilla and Yorkie) (p value 0.001, 0.003, 0.001, n=2).

B.



B.
Using the same protocol as above, increasing amounts of Yorkie were added to samples with or without Hippo and Warts. Experiments run with 25ng, 50ng, 100ng and 200ng of Yorkie. Two replicates of each sample were run. Data was normalized to the luciferase expression of the null control (luciferase and Renilla). Normalized data was then plotted and standard deviation determined using Graphpad Prism7.

A.



B.

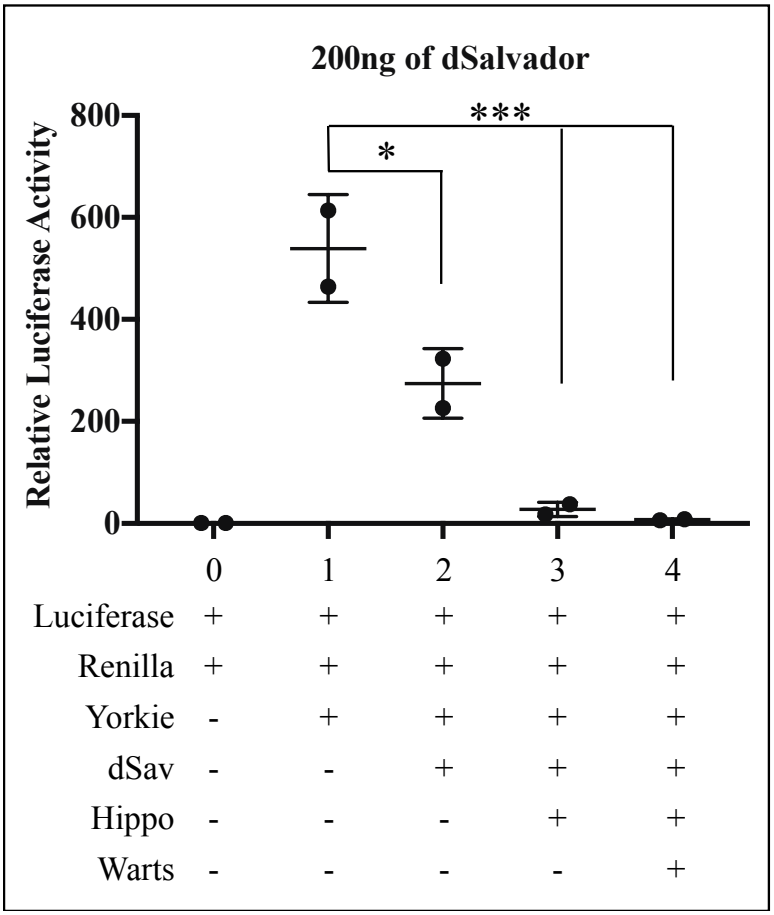


Figure 9. Optimization of dSalvador in Drosophila luciferase

A. S2 cells were transfected with plasmids encoding Renilla, a Gal4-luciferase, Gal4Dbd-Yorkie, Hippo, Warts and 50ng of dSav using Fugene6. Two replicates of each experiment were obtained. Experimental values were normalized to the null control (luciferase and Renilla). Normalized values were plotted using Graphpad Prism. Error bars represent the standard error as determined by Prism. Statistical analysis was performed comparing experiments to the positive control (luciferase, Renilla and Yorkie) (p value 0.007, 0.002, 0.002, n=2).
B. In the same way as above, S2 cells were transfected with plasmids encoding Renilla, a Gal4-luciferase, Gal4DBD-Yorkie, Hippo, Warts and 200ng of dSav using Fugene6. (p value 0.01, 0.0008, 0.0007, n=2).

Table1. Original mammalian Plasmid Protocol

Final DNA concentration of 1μg of DNA per well. In order to reach 1μg of total DNA empty pcDNA was transfected in addition to our experimental plasmids. Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid	Concentration
90	Gal4-UAS-Luciferase	70ng
91	pRL-SV40-*Renilla*	14ng
92	p2xFlagCMV2-Yap2	70ng
93	Gal4-Dbd-TEAD4	70ng
94	pcDNA-HA-Mst2	70ng
95	pcDNA-HA-Sav	70ng
96	pcDNA-HA-Mob	70ng
97	pcDNA-Myc-Lats	70ng

Table 2. Final mammalian Plasmid Protocol

Final concentration of 1μg of DNA per well. In order to reach 1μg of total DNA empty pcDNA was transfected in addition to our experimental plasmids. Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid	Concentration
90	Gal4-UAS-Luciferase	100ng
91	pRL-SV40-*Renilla*	10ng
122	p2xFlagCMV2-Yap2	25ng
93	Gal4-Dbd-TEAD4	75ng
94	pcDNA-HA-Mst2	75ng
95	pcDNA-HA-Sav	75ng
96	pcDNA-HA-Mob	75ng
97	pcDNA-Myc-Lats	75ng

Table 3. Original Drosophila Plasmid Protocol

Final concentration of 0.5µg of DNA per well. In order to reach 0.5µg of total DNA empty pAc5.1 vector was transfected in addition to our experimental plasmids. Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid	Concentration
75	Gal4-Dbd-Ykiv5	25ng
76	Gal-4-pUAST-Luciferase	75ng
77	pRL-SV40-*Renilla*	10ng
66	pAWF-dHpo	50ng
68	pAWF-dMats	50ng
69	pAMN-dSav	50ng
70	pAWF-dWts	50ng
71	pAHF-dRassF	50ng

Table 4. Final Drosophila Plasmid Protocol

Final concentration of 0.5µg of DNA per well. In order to reach 0.5µg of total DNA empty pAc5.1 vector was transfected in addition to our experimental plasmids. Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid	Concentration
75	Gal4-Dbd-Ykiv5	50ng
76	Gal-4-pUAST-Luciferase	75ng
77	pRL-SV40-*Renilla*	10ng
66	pAWF-dHpo	50ng
68	pAWF-dMats	50ng
69	pAMN-dSav	50ng
70	pAWF-dWts	50ng
71	pAHF-dRassF	50ng

Understanding SARAH domains and the novel structure of dSalvador

Introduction

SARAH Domains

At the heart of the *Drosophila* Hippo pathway lies Hippo, one of the pathways core kinases, and two adaptor proteins that help modulate Hippo's activity. These three proteins, Hippo, dSalvador, and dRassF all associate through C-terminal SARAH domains (**Sa**lvador-**Ra**ssF-**H**ippo). A SARAH domain is a C-terminal alpha helix that facilitates protein binding through anti-parallel coiled-coil interactions [38-40]. Not only do these SARAH domains facilitate the heterodimer formation between Hippo and dRassF, as well as. Hippo and Salvador, they also allow the respective proteins to form homodimers. Importantly, the Hippo homodimer formation that is required for activation is mediated by its SARAH domain [15,38].

Human Salvador and MST hetero- and homodimer formation

Drosophila Salvador (dSalvador), a scaffolding protein, is known to play a role in the activation of Warts by facilitating the association of Hippo and Warts [41]. However, there is far less understood about the role of human Salvador [42]. Recent work has shown that MST (Hippo) and the human orthologue of Salvador (also known as WW45) associate with each other leading to the phosphorylation of Salvador [12]. Like in the SARAH domain interaction of Hippo and dSalvador, the association of MST and Salvador is dependent upon the C-terminal coiled-coil domains of each protein interacting to form a heterodimer [12]. Despite this requirement to form a heterodimer between MST and Salvador, the same research showed that the homodimer formation of Salvador could be achieved in mutants lacking the C-terminal coiled-coil region. The authors hypothesized that the mediator of dimer formation was the tryptophan residues in the WW domains interacting with proline rich areas of dSalvador [12,43]. However, due to the

instability of their protein constructs, the exact interactions that allow for the dSalvador homodimer formation were not determined [12].

Structure of dSalvador and the discovery of the "seatbelt" region

There has been a lot of work studying the structures of Hippo (MST) and dRassF (RassF) and how they interact, but less is known about the structure of dSalvador and the complex it forms with Hippo [14,15,39,40]. Therefore, our lab endeavored to characterize the protein interactions of dSalvador and determine a structure of the protein. Ultimately, work by Leah Cairns, a Ph.D. candidate in the lab, revealed a novel structure for dSalvador. Critical to her success was the discovery of an extended N-terminal "seatbelt" region in the SARAH domain [38]. This approximately 20 amino acid residue region was shown to increase the stability of the complex between dSalvador and Hippo *in vitro*, as well as, help stabilize the dSalvador protein, allowing for crystallization studies [38].

Research Questions

Previous studies have focused on characterizing the Hippo and dRassF interactions of the Hippo pathway. However, now that the structure of dSalvador and its SARAH domain can be crystalized and the structure examined, our lab wanted to explore dSalvador and its homodimer and heterodimer interactions. As previously mentioned, work has shown that the mammalian homologue does not require the C-terminal coiled-coil domain to facilitate homodimers of Salvador forming [12]. Since this has yet to be demonstrated in the *Drosophila* protein and because the authors were never able to confirm their hypothesis that binding could result from the tryptophan in the WW domains interacting with proline rich areas in dSalvador, we are interested in examining the requirement of the SARAH and WW domains of dSalvador for dimer formation [12]. We propose to use binding assays to examine variants of dSalvador lack its

SARAH domain, its WW domain, or both to better understand how dSalvador forms its homodimer (Figure 13C).

In the process of determining the structure of the dSalvador/Hippo complex, it was noted that the dSalvador/Hippo dimer associates with another dSalvador/Hippo dimer resulting in the formation of a dimer of dimers. Facilitating this dimerization was a disulfide bond between Hippo and Hippo at cysteine 624 [38]. Due to the fact that this cysteine was not conserved and the relatively small size of the bond, we hypothesized that it was not biologically relevant. Instead, we believed that this was an artifact of crystal packing. However, we wanted to confirm, using cell based studies, that it did not play a role in activity.

Some of Leah Cairns' unpublished data from her Ph.D. work on SARAH domains suggests that there is a wide variation in the bond strength of homo and heterodimers formed by dRassF, dSalvador and Hippo. In particular, the dimer between dSalvador and Hippo is stronger than any of the other hetero- or homo-dimers formed between dRassF, dSalvador and Hippo. Knowing this, we wondered if the strength of the interaction between SARAH domains changes the regulatory potential of a protein? To test this, we propose interchanging the SARAH domains between dRassF, Hippo and dSalvador. To do this, the protein body will be attached to either its own SARAH domain or the SARAH domain of one of the other two proteins. A general schematic of this plan can be seen in Figure 13B. Ultimately, we hope to perform cell based assays to see if you swap a SARAH domain with a different protein, for example the SARAH domain of dSalvador with the core protein domain of dRassF, do you alter the strength of its dimer or change its regulatory activity?

Results

Validating the role of the N-terminal extension of Salvador

In order to validate the significance of the N-terminal extension (seatbelt) region of dSalvador's SARAH domain, a mutant without the seatbelt was compared to the wild type protein in a *Drosophila* based luciferase reporter assay. Using Fugene 6, Gal-4-UAS luciferase, *Renilla,* Gal-4-Dbd-Yki and Hippo were transfected into S2 cells. Either wild type dSalvador including the seatbelt or a variant (plasmid code MV, Kavran lab), lacking the seatbelt region, were also transfected. In an attempt to improve detection and increase the dynamic range of the experiment two concentrations plasmid encoding for Gal-4-Dbd-Yorkie, 50ng or 200ng, were used. Following incubation, cells were harvested and cell lysate was used for a luciferase assay. A total of five replicates were performed for each experimental treatment. Using Graphpad Prism7, normalized luciferase values were plotted and statistical analysis comparing wild type dSalvador to the variant without a seatbelt region was performed. In both of the experimental set-ups using 50ng or 200ng of Yorkie, there was no significant change in luciferase expression (p value 0.82, 0.99) (Figure 10). This signifies that in the context of the luciferase assay, the seatbelt region does not play a role in regulation Hippo kinase activity.

Validating the oligomeric state of Hippo:dSalvador

To confirm that the disulfide bond between Hippo was indeed an artifact of crystal packing and not biologically significant, Gal-4-UAS luciferase, *Renilla,* Gal-4-Dbd-Yki and either wild type Hippo or a C624S variant (plasmid code MT, Kavran lab) were transfected into S2 cells using Fugene 6. A total of nine replicates were performed across multiple days. Using Graphpad Prism7, data normalized in excel were plotted and statistical analysis comparing luciferase values for wild type or C624S Hippo were compared to each other. The C624S mutant eliminated the potential for the disulfide bond between Hippo dimers. There was no difference in

relative luciferase activity between the wild type Hippo and C624S Hippo variant (p value 0.91) (Figure 11). This confirms our hypothesis that the disulfide bond is not biologically relevant and just an artifact of crystal packing.

Role of SARAH domains interactions

First, we wanted to reproduce published results and confirm the role of dRassF as an inhibitor of Hippo activity and establish conditions for a luciferase assay to report on that inhibition. Using Fugene 6, Gal-4-UAS luciferase, *Renilla,* Gal-4-Dbd-Yki, Hippo and dRassF were transfected into S2 cells. Three separate experiments were performed for a total of seven replicates. In hopes of saturating Hippo, four replicates were run where the amount of plasmid encoding dRassf transfected was increased to from 50ng to 200ng. In order to see the inhibitory trend, the first four replicates of the experiment have been combined in Figure 12A. and the last three replicates in Figure 12B. This was done because of the wide variation in luciferase expression levels between experiments. Data normalized in excel were plotted and statistical analysis comparing the activity of Hippo in the presence or absence of dRassf was performed using Graphpad Prism7. When the experiments from different days were separated as described above, the results are statistically significant (p value 0.02, 0.003) but when values across all experiments are combined, the range of luciferase expression creates a standard deviation that makes all results statistically insignificant (p value 0.27). Increasing the amount of plasmid encoding for dRassF did not result in a statistically significant difference between with or without dRassF (p value 0.28) (Figure 12C). These results signify that there is an inhibitory effect by dRassF on Hippo activity seen through an increase in relative luciferase expression. However, further optimization is needed to enhance the dynamic range of the difference.

We are just in the beginning stages of the experiments to test the "hierarchy" of SARAH domains. Issues finding the optimal cell based system to express our experimental plasmid constructs has proven challenging. However, by transferring our *Drosophila* genes into a p6a mammalian expression vector and expressing the proteins in HEK293T cells we can get sufficient protein expression for our studies (Figure 13B). Now that our plasmids do indeed express, the proposed experimental set up involves a series of co-immunoprecipitations between plasmids that have had their endogenous SARAH domain replaced by a SARAH domain from one of the other two proteins. Because we do not know that our constructs will still enable normal interactions, we want to validate that the SARAH domains in the variants still facilitate binding. Following that, experiments will be conducted to examine binding strength and activity levels of our variants through *in vitro* and cell based assays.

dSalvador dimerization

As previously discussed, the work showing that human Salvador does not require its C-terminal coiled-coil domain for dimerization has not been demonstrated with the *Drosophila* protein. Additionally, the mechanism by which dimer formation is facilitated in the absence of coiled-coil domains was never discovered [12]. In order to replicate the experiment with dSalvador and determine the exact mechanism of dimer formation, variants lacking the SARAH domain, a WW binding domain, or variants lacking both have been cloned into a mammalian, p6a, expression vector (Figure 13C). Just as with the SARAH swap, S2 cells could not produce suitable protein expression for our experiments so we had to switch to using a mammalian expression vector in HEK293T cells. We have confirmed that our plasmids express and the proposed experimental setup is outlined in Figure 13C (Figure 13A). Using co-

immunoprecipitation, we will check to see if the results from human Salvador can be reproduced using the *Drosophila* form of the protein.

Methods

Luciferase assay

We used our standard luciferase assay protocol for S2 cells—see Appendix 1 and "Optimization of luciferase reporter assay."

Western Blot Analysis

Protocol adapted from Cairns et. al 2018. HEK293T cells (ATCC) were cultured in DMEMF12 (Gibco) supplemented with 5% FBE (VWR) and 2mM L-glutamine at 37 °C and 5% CO2. Cells were transfected with a total of 1µg of total DNA (p6a plasmids) (Thermo Fisher) encoding the indicated proteins using PEI DNA Transfection Reagent (Thermo Fisher) according to the manufacturer's protocol. Cells were harvested at 24h following transfection in ice-cold RIPA buffer supplemented with 50mM NaF, 1mM Na_3VO_4, 0.5mM phenylmethylsulfonyl fluoride, and Universal Nuclease (Thermo Fisher). Total protein of each experimental condition was determined using a bicinchoninic acid assay (BCA assay) (Thermo Fisher). Concentrations were calculated by comparing concentrations to a standard curve made from samples of a known concentration. Each sample was then standardized to 2.5µg per µL. 9.5µL of cell lysate were loaded onto a gel and analyzed by Western blotting using 1:800 dilution of Myc (Santa Cruz, number sc40) followed by 1:10,000 dilution of goat α-mouse 800RD (LICOR, number 925-32210), 1:1000 dilution of HA (Roche, number 11867423001) followed by 1:10,000 dilution of goat α-rat 800RD (LICOR, number 925-32219), 1:1000 dilution of FLAG (Sigma, number

F1804) followed by 1:10,000 dilution of goat α-mouse 800RD. Blots were scanned on an Odyssey IR Imaging System (LI-COR).

Discussion

The N-terminal extension of dSalvador

One of the continued challenges we have faced with the luciferase assay has been its inability to tell us anything more than a binary answer. This issue continued to come up with our efforts to show that the extended "seatbelt" domain enhances dSalvador/Hippo binding. While we were able to demonstrate the increased binding affinity using purified protein, in the context of the luciferase assay the difference between wild type and mutant was not pronounced enough to make any conclusions. Theoretically, without the "seatbelt" binding between dSalvador and Hippo should be weakened and the kinase activity of Hippo should go down. As a result, we expected to see an increase in luciferase expression in the no "seatbelt" mutant. There may be a slight increase however, again there is not a distinct enough range to parse out the subtle change (Figure 11).

Biological relevance of cysteine 624 in Hippo

As noted before, during crystallographic studies of the complex between dSalvador and Hippo, Leah Cairns noticed that a disulfide bond was being formed between two Hippo proteins in dimers at the cysteine 624 residue. Because of this, we needed to validate that this disulfide bond did not play a role in Hippo function. *In vitro* data with purified protein from the lab already showed that the removal of the disulfide bond did not interrupt Hippo and dSalvador binding, however we needed to confirm that in a cell based setting, that the C624S variant would not impact Hippo's kinase activity. Since there was no change between wild type and variant it

was concluded that the disulfide bond that forms between the two Hippo dimers is a result of simple crystal packing [38].

dRassF inhibits Hippo activity

It is known that dRassF inhibits Hippo's kinase activity so it was not unexpected that we would be able to see this through the luciferase assay and the result provides a foundation to pursue additional experiments with dRassF. Previous work has shown through co-immunoprecipitation, activity assays and fly based experiments that dRassF preferentially binds un-phosphorylated Hippo. This in turn, prevents Hippo from auto-phosphylating itself and becoming active [13]. Moving forward it will be interesting to see if we can change the ability of dRassF to inhibit Hippo activity by attaching a stronger SARAH domain. By attaching a SARAH domain with a higher binding strength, for example Salvador's SARAH domain, we may be able to increase the inhibitory effect.

Future dSalvador and SARAH swap experiments

By far the biggest challenge we faced in trying to get the SARAH swap project off the ground was finding a way to express our plasmids in *Drosophila* cells. We had previously noticed that raw expression levels for firefly luciferase were 50 to 100-fold lower in S2 cells compared to HEK293T cells. While there was still enough signal for the luciferase assay, it suggests that in S2 cells there is lower overall protein expression levels and that could explain why we were unable to detect protein expression in S2 cells by Western blot. Ultimately, we decided to transfer the *Drosophila* genes into mammalian expression vectors. As soon as we did this we were able to get expression at high enough levels to be visualized through Western blot (Figure 13). Now that we have expression we can proceed to experiments that examine the "hierarchy" of SARAH domains and dSalvador dimerization.

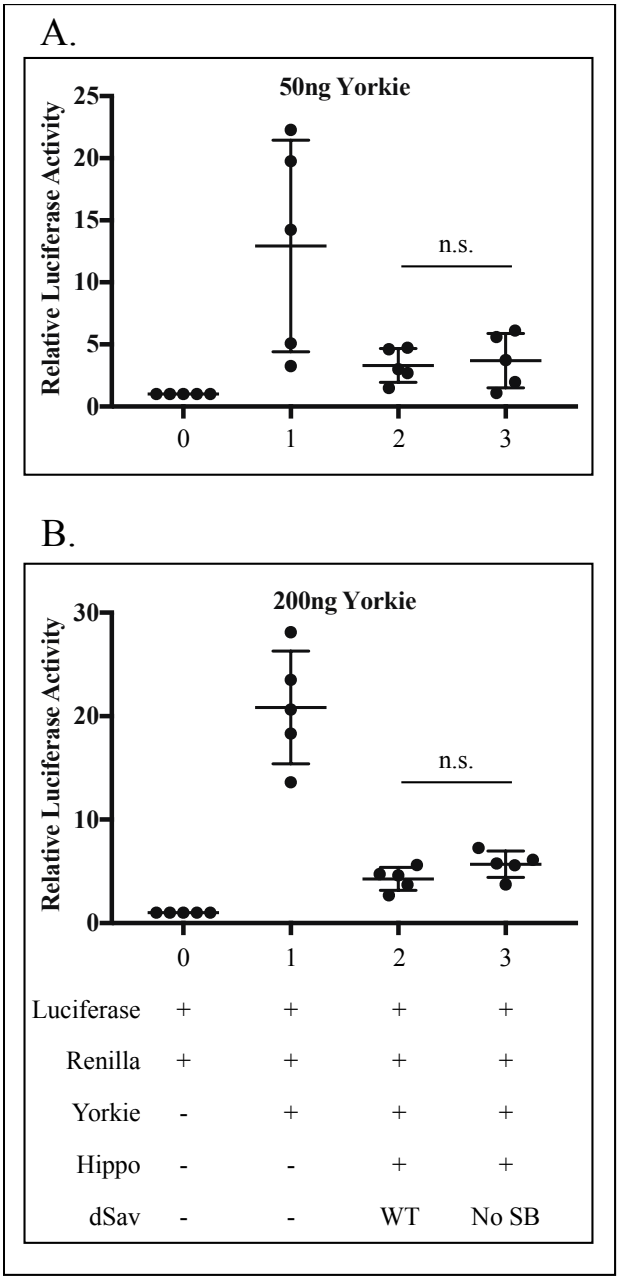


Figure 10. Comparison of dSalvador activity with or withou t its “seatbelt” (SB) region.

A. S2 cells were transfected with plasmids encoding Renilla , a Gal4-luciferase, Gal4DBD-Yorkie, Hippo and either wild type (wt) dSalvador or a no SB dSalvador variant usin g Fugene 6. A total of five replicates over two experiments were obtained. Luciferase values were normalized to the nu ll control (luciferase and Renilla). Using Graphpad Prism, values were plotted as relative luciferase expression, standa rd error was calculated and statistical analysis comparing wt to no SB was performed (p value 0.82, n=5). Error bars repr esent the standard deviations as calculated in Prism.
B. Experiment was run as above except, in an attempt to inc rease the dynamic range of the experiment and better visual - ize a difference between wt and no SB mutants, the amount of Yorkie transfected was increased to 200ng (p value 0.99, n=5).

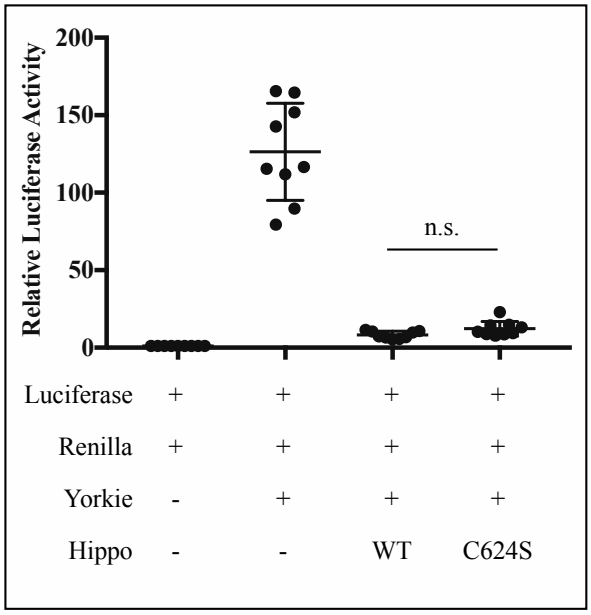


Figure 11. Biological significance of Hippo Cyste - ine 624.

S2 cells were transfected with plasmids encoding Renilla, a Gal4-luciferase, Gal4DBD-Yorkie, and either wild type or C624S Hippo. Nine experimen - tal replicates over four experiments were obtained. Luciferase values were normalized to the null control (luciferase and Renilla). Using Graphpad Prism, values were plotted as relative luciferase expression, standard error was calculated and statistical analysis comparing wild type to C624S was performed (p value 0.91, n=9). Error bars represent the standard deviations as calculated in Prism.

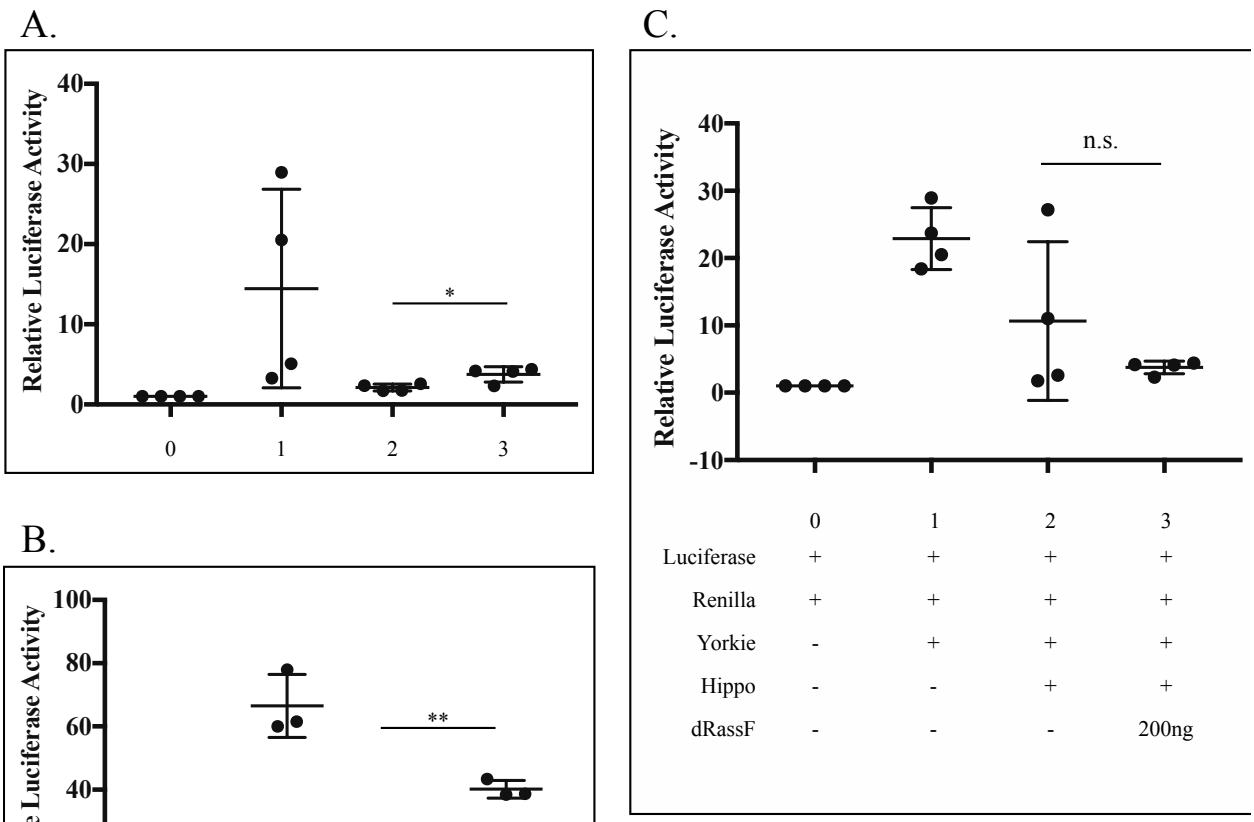


Figure 12. dRassF inhibits Hippo kinase activity

A. S2 cells were transfected with plasmids encoding Renilla, a Gal4-luciferase, Gal4DBD-Yorkie, Hippo and dRassF using Fugene6. After 3 days, the levels of luciferase were measured. Four replicates over two experiments were obtained. Luciferase values were normalized to the null control (luciferase and Renilla). Using Graphpad Prism, values were plotted as relative luciferase expression, standard error was calculated and statistical analysis comparing Hippo with or without dRassF (p value 0.02, n=4). Error bars represent the standard deviations as calculated in Prism.

B. To confirm the previous findings, three additional replicates were conducted. The same protocol as above was used (p value 0.003, n=3).

C. Using the protocol as in A., in an attempt to increase the effect of dRassF, the amount of dRassF transfected was increased from 50ng to 200ng (p value 0.28).

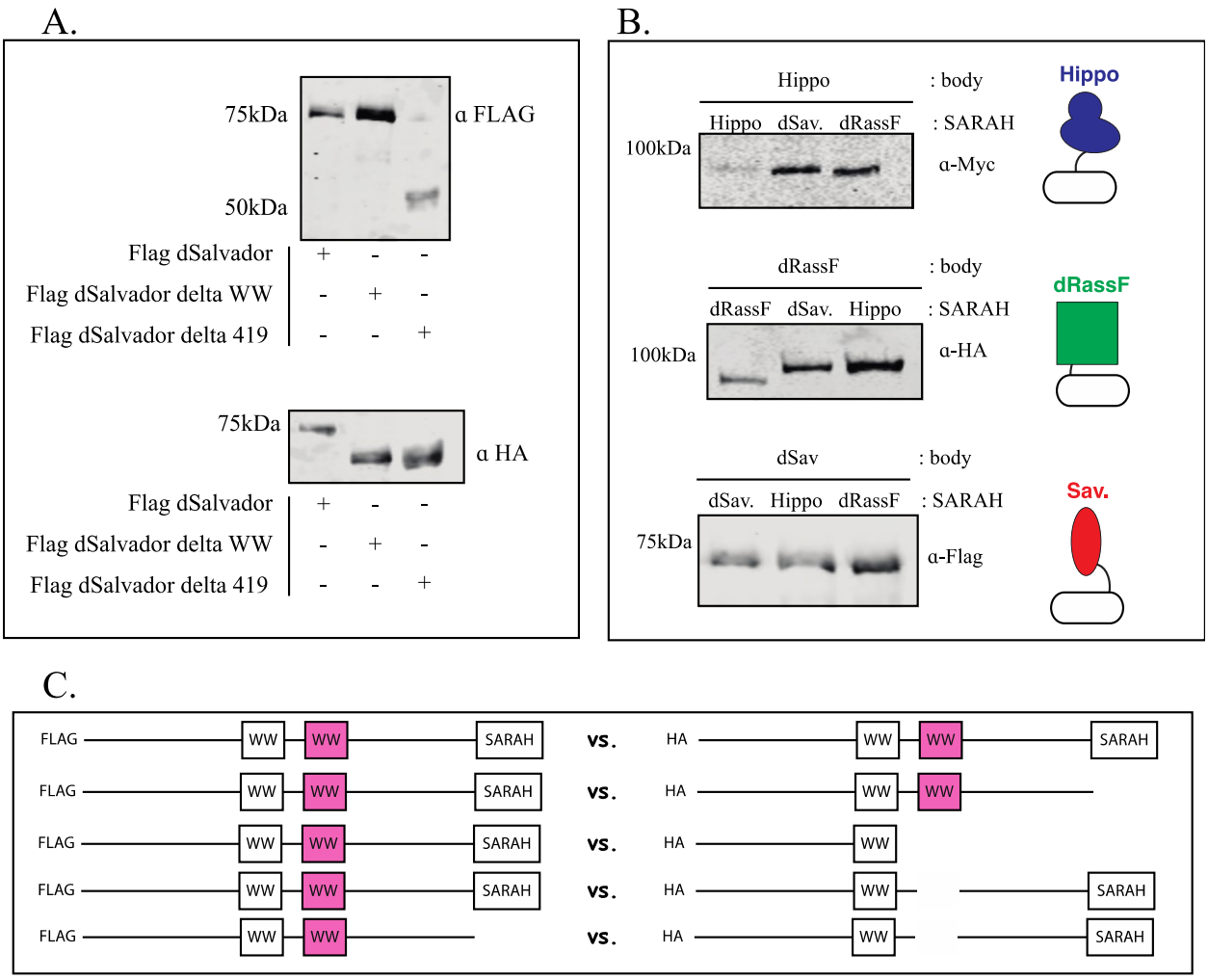


Figure 13. SARAH domain dimerization and swaps

A. dSalvador variants were cloned into the mammalian expression vector, p6a. Hek293T cells were then transfected with plasmids encoding for the various dSalvador constructs using PEI max. After 24 hours cells were harvested and lysed using RIPA buffer. Cell lysates were analyzed by Western Blot analysis using antibodies that recognized the appropriate epitope tags (HA and Flag).
B. SARAH swap variants were cloned into the mammalian expression vector, p6a. The core protein body of either Hippo, dRassF or dSalvador was fused to all three different SARAH domains. Hek293T cells were then transfected with plasmids encoding for the various dSalvador constructs using PEI max. After 24 hours cells were harvested and lysed using RIPA buffer. Cell lysates were analyzed by Western Blot analysis using antibodies that recognized the appropriate epitope tags (HA, Myc and Flag). Cartoons represent the general design of the SARAH swaps. Colored areas represent the protein body. Empty white ovals represent the potential different SARAH domains.
C. Proposed experimental design for examining the SARAH dimerization of dSalvador. Cartoon representations correspond to the dSalvador variant the are expressed in A.

Table 5. dSalvador luciferase plasmids

For the luciferase assays the following plasmids and concentration were used. Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid	Concentration
75	Gal4-Dbd-Ykiv5	50ng
76	Gal-4-pUAST-Luciferase	75ng
77	pRL-SV40-*Renilla*	10ng
66	pAWF-dHpo	50ng
68	pAWF-dMats	50ng
69	pAMN-dSav	50ng
70	pAWF-dWts	50ng
MT	pAc5.2-C624S-Hippo	50ng
MV	pAc2.5-dSav-deltaSB	50ng or 200ng
71	pAHW-dRassF	50ng or 200ng

Table 6. SARAH swaps plasmids

The following plasmids were used in the expression test for SARAH swaps. All are based in the mammalian p6a expression vector. Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid (Protein Body/SARAH domain)
NN	Myc-Hippo
NO	Flag-dSalvador
PA	HA-dRassF
PB	Myc Hippo/Ssb
PC	HA dRassF/Ssb
PD	Flag dSav no sb/ Hippo
PE	Flag dSav no sb/ dRassF
PF	Myc Hippo/dRassF
PI	HA RassF/Hpo

Table 7. dSalvador dimerization plasmids

The following plasmids were used in the expression test for dSalvador dimerization. All are based in the mammalian p6a expression vector. Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid
NO	Flag-dSalvador
OZ	HA-dSalvador
OV	Flag-dSalvador-delta419
OW	HA-dSalvador-noWW
OX	Flag-dSalvador-deltaWW
PG	HA-dSalvador-delta528

Optimization of Mammalian Ste20 (MST) variants for crystallographic studies

Introduction

Mammalian Ste20 (MST1/2)

Mammalian Ste20 (MST1/2), is a core kinase within the Hippo pathway. It is the homologue to the *Drosophila* protein, Hippo, and works in tandem with the other core Hippo pathway kinase Lats [6,16]. Constitutive MST activation and cleavage is critical for maintaining regulation of cell growth and proliferation. Loss of MST function has been shown in *in vitro* to cause rapid and massive cellular overgrowth [17,44]. Interestingly, reintroduction of functional MST ameliorates this phenotype [17]. The exact mechanism by which this cancer phenotype is sustained is not yet known. However, there is some work indicating that in the absence of functional MST, tumor necrosis factor alpha does not induce apoptosis possibly leading to this proliferative phenotype [16].

Role of Mob1 in Hippo pathway signaling

Recently, it has been demonstrated that there is an adapter protein, Mob1, that helps to facilitate the signaling cascade between MST and Lats [23]. Auto-phosphorylation of MST is required for Mob1 to be recruited to the kinase, but once MST has been phosphorylated, there are multiple Mob binding motifs (MBM) on the active kinase that Mob1 can associate with [23,24]. Mob1 will ultimately bind both MST and Lats. The active MST will phosphorylate Mob1, which in turn, enhances Mob1's ability to activate Lats [23]. Activated Lats then regulates YAP through phosphorylation and sequestration of the transcriptional co-activator [16,18].

The structure of MST1/2

There are five isoforms of MST but for the purpose of our studies, MST2 was used [44]. The protein is made up of three main parts, a core kinase domain (amino acids (a.a.) 26–327), a

flexible linker region (a.a.328–429), and a SARAH domain (a.a 430-480) [15,23,24]. Within the core kinase domain of MST2 there is an activation loop with a phosphorylation site at threonine 180 [45]. Phosphorylation of this threonine leads to the activation of MST2. The linker region is a fairly unstructured region of the protein, approximately 100 amino acids in size. Within this region there are multiple phosphorylation sites and MBM, of particular interest to us is the MBM at threonine 378 [24]. This motif has been shown to facilitate binding between MST and Mob1 and MST's subsequent phosphorylation of Mob1 [24].

Research Question

As demonstrated by previous work, there are multiple sites within the linker region that support binding between active MST and Mob1[23,24]. However, the relative affinity and importance of the different sites of Mob1 binding is still to be elucidated. Certainly, while there have been recent advancements in our understanding of the role of Mob1 and the interaction between it and MST, there is still much to be discovered. For that reason, our lab is interested in establishing conditions that make it possible to crystalize MST and Mob1 proteins in complex. A significant roadblock to this is the linker region attaching the SARAH domain to the core kinase domain of MST. The relatively long unstructured region inhibits protein crystallization. Therefore, in collaboration with Dr. Thao Tran, I have been working to determine variants of MST2 that have a reduced linker region, allowing for crystallization, but that still maintain normal function. These variations include removing the linker altogether, removing certain phosphorylation sites, and removing the previously published MBM at threonine 378 [24]. Using the optimized luciferase assay we hope to determine the best possible construct of MST for crystallization experiments.

Results

Validating the importance of the MST linker region

Our study of MST began by confirming that linker was indeed required for MST functionality. Plasmids encoding for Gal-4-Dbd-TEAD, Gal-4-UAS-luciferas, *Renilla,* YAP and Hippo pathway components were transfected into HEK293T cells using Lipofectamine 2000. Within the context of our experiment, wild type MST or MST no linker (plasmid code GY, Kavran lab), were transfected in addition to Salvador, Mob1 and Lats. As a negative control for MST activity we used a kinase inhibited version, D146N, of MST (plasmid code IH, Kavran lab). A total of four replicates were obtained from two separate experiments. Using Graphpad Prism7, values were plotted and statistical analysis comparing the MST variant without a linker region to wild type MST was performed (p value 0.0001) (Figure 14B). In the absence of the linker, MST losses all function, with luciferase expression levels returning to the same levels as the positive control, luciferase, *Renilla* and YAP (Figure 14B).

Next, in an attempt to find a variant where the linker region was reduced instead of removed completely, we tested two MST variants with parts of the linkers removed. A schematic of these two constructs can be seen in Figure 14A. Following the same procedure as before, we tested to truncated version of MST, one with the amino acids from NKS...PDN (plasmid code EZ, Kavran lab) the other with amino acids from SHT...VIN and from NKS...PDN (plasmid code FA, Kavran lab) removed. Statistical analysis using Graphpad Prism7 revealed that there was no statistically significant difference in luciferase expression between wild type MST and the two variants (Figure 14B) (p value 0.99, 0.99).

Validating the importance of MBM at threonine 378

Interested in understanding the importance of the MBM described by Ni et al. we tested a MST variant with the published MBM removed (plasmid code GX, Kavran lab) against wild type MST. Again, following the same procedure, we performed a luciferase assay to test MST function. Similar to the two linker variants, statistical analysis performed using Graphpad Prism7 showed not difference in function between wild type and MST variant (p value 0.99) (Figure 14B).

Methods

Luciferase

Standard mammalian luciferase protocol was followed (Appendix 1).

Discussion

A minimal MST linker region is required for function

As previously discussed, the luciferase reporter can only give a binary answer. This means that in the context of our MST2 experiments the only information that we obtained was whether or not a mutant was functional. Unfortunately, the luciferase assay was not able to parse out more muted changes in levels of activity. The linker region contains multiple phosphorylation sites within it and is believed to play a role in the recruitment and binding of Mob1 [15,23,24]. The results of the luciferase assay further support the idea that at least a minimal region of the linker is required to maintain normal kinase activity (Figure 14B). The two experimental mutants of MST2 that had phosphorylation sites within the linker region removed proved to retain wilt type function (Figure 14B). This might suggest that the specific phosphorylation sites are not critical for function. However, the more likely explanation is that

since there are so many phosphorylation sites within the linker region, there is a certain level of redundancy that allows for the kinase to retain functionality.

The MBM at threonine 378 is not required for function

While Ni et al. only highlight the MBM at threonine 378, later work by Couzens et. al. show that in fact, there are many MBMs on active MST. Therefore, it is not unexpected that the MBM variant retained its wild type activity (Figure 14B). Here, the limitation of the luciferase assay may be a confounding factor as MST2 activity might be partially reduced by loss of the MBM. However, the other MBMs more than certainly provide a redundancy that allows for Mob1 and MST to still bind [23]. Further experiments are needed to examine other MBMs and potentially eliminate more than one at a time.

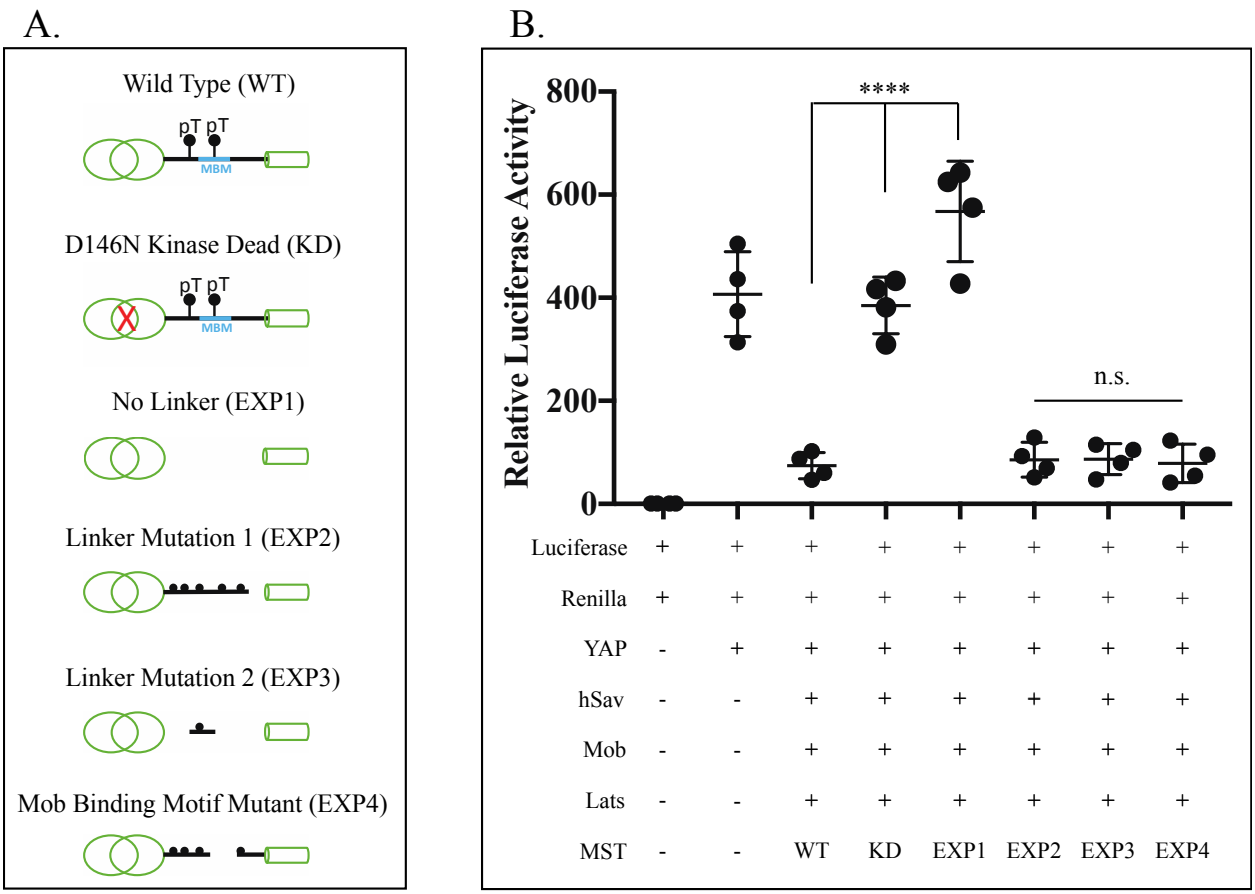


Figure 14. Determining the minimal functional unit of MST

A. Schematic of wild type and experimental variants of MST2. Each of the schematics correlates to the plasmid used in the luciferase assay. Green ovals represent the core kinase domain of MST. The green rectangle represents the SARAH domain. The black line represents the linker region with black dots representing phosphorylation sites. Mon binding motifs are represented by MBM in blue below the linker region. Red "x" represents inhibition of kinase activity.
B. Using Lipofectamine 2000, Gal4Dbd-Tead, Gal4-Luciferase, Renilla, YAP, hSav, Mob, Lats, wild type (wt) and mutant MST2 variants were transfected into Hek293T cells. After 48 hours cells were harvested and lysed. A total of four replicates from two experiments were obtained. Luciferase data was normalized to the null control (luciferase and Renilla). Using Graphpad Prism, values were plotted as relative luciferase expression, standard error was calculated and statistical analysis comparing wild type to experimental MST variants (p value 0.0001, n=4). Error bars represent the standard error as calculat - ed by Prism.

Table 8. MST variant plasmids

For the luciferase assays the following plasmids and concentration were used. Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid	Concentration
90	Gal4-UAS-Luciferase	100ng
91	pRL-SV40-*Renilla*	10ng
122	p2xFlagCMV2-Yap2	25ng
93	Gal4-Dbd-TEAD4	75ng
94	pcDNA-HA-Mst2	75ng
95	pcDNA-HA-Sav	75ng
96	pcDNA-HA-Mob	75ng
97	pcDNA-Myc-Lats	75ng
EZ	pcDNA-MST-deltaL1	75ng
FA	pcDNA-MST-deltaL2	75ng
GY	pcDNA-MST-noL	75ng
GX	pcDNA-MST-noMBM	75ng
IH	pcDNA-MST-D146N	75ng

Minimal Functional Unit of Large tumor suppressor (Lats)

Introduction

Large tumor suppressor

Along with MST, Lats makes up the central cassette of the Hippo pathway. It plays a critical role in the regulation of proliferation and growth. This is because its kinase activity is responsible for phosphorylating the target of the Hippo pathway, YAP [8,19,46]. Loss of Lats function is disastrous and can lead to a severe overgrowth phenotype, carcinogenesis and death in mice [3,32]. While there is no evidence yet that Lats drives any human carcinomas, its clear role in the progression of cancer in mice demonstrates the importance of understanding the kinase, its activity, and the mechanism by which it is activated better.

Recent studies support that Lats complexes with both Mob1 and MST and these events are required for its activiation[24]. When Lats associates with active MST, MST phosphorylates Lats on threonine 1079 located in the hydrophobic motif of Lats. The phosphorylation of threonine 1079 subsequently stimulates the auto-phosphorylation of serine 909 in the activation loop [19,46]. Additional work has elucidated a Mob binding motif within the N-terminal region of Lats [21,47]. In order for Mob1 to bind to this motif it must undergo phosphorylation by MST. The exact mechanism by which phosphorylation of Mob1 occurs and then promotes Lats binding is not known. However once Mob1 interacts with binding motif on the N-terminal region of Lats, it assists in regulating Lats activation [21,24]. Ultimately, once Lats is activated it can proceed to phosphorylate its target, YAP. This in turn inhibits YAP's ability to translocate to the nucleus and prevents the transcription of pro-growth genes [47].

Research Question

While recent works have shed light on the mechanism by which Lats is activated, there is still much more to learn. Our lab is particularly interested in studying Lats as both an individual protein and in a complex with its binding partner, Mob1. To this point crystallographic studies have focused on the N-terminal Mob binding domain and not the entire protein [21,47]. This may be due to the fact that Lats is predicted to be relatively large and unstructured. In particular, outside of the Mob binding domain, the N terminal region is believed to be relatively unordered, making the entire protein hard to isolate. Our lab hypothesized that because of the lack of homology on the N- and C-terminal region of Lats, significant amounts of amino acid residues could be removed without changing its kinase activity. By doing this, the protein could more easily be isolated. The protocol that a graduate student in the lab, TJ Koehler, has proposed to use in order to purify Lats relies on the cleavage of a C-terminal intein tag. This type of cleavage results in a cysteine scar. In order to validate the kinase activity of these smaller versions of Lats, luciferase reporter assays were used to compare activity against wild type. Additionally, mutants were made that had a cysteine inserted, similar to the product of intein cleavage, to check if the addition would have any biological impact.

Results

N-Terminal Truncations

The N-terminal region of Lats, as previously discussed, is predicted to be large and unstructured. This ultimately plays a large role in the challenges of crystalizing the entire Lats protein. With that in mind we compared variants of Lats with truncations to the N-terminus that still retained the N-terminal Mob binding domain with wild type Lats. Using Lipofectamine 2000, HEK293T cells were transfected with Gal-4-Dbd-TEAD, Gal-4-UAS-luciferas, *Renilla,*

YAP and Hippo pathway components. A kinase dead, D827N, version of Lats was used as a negative control (plasmid code KE, Kavran Lab). A total of four replicates from two distinct experiments were collected. Values were normalized to the null control, luciferase and *Renilla.* Using Graphpad Prism7, luciferase expression values were plotted and appropriate statistical analysis comparing variants to wild type Lats performed. Following the kinase dead column, the N-terminal length of the variants increases from left to right (Figure 15A). None of the N-terminal truncations show a significant difference in luciferase expression compared to wild type. However, the most severe truncation does show a much higher degree of variation between replicates.

C-Terminal Truncations

Compared to the N-terminus, the length of the C-terminus before the conserved amino acid sequence of the kinase domain of Lats is reached, is much smaller. Therefore, truncations of the C-terminus were far less aggressive than the N-terminus. In the same way as described above variants of the C-terminus were tested against wild type Lats. Following the column for kinase dead, the experimental variants increase in C-terminal length from left to right (Figure 15B). Statistical analysis using Graphpad Prism, comparing wild type to C-terminal variants shows not significant difference between any column. Similar to the N-terminal truncations, the most aggressive truncation does show a higher degree of variation and is trending towards luciferase expression similar to that of the kinase dead control.

Cysteine Addition

Because the protocol for intein tag cleave leaves a cysteine scar, we wanted to determine if the insertion of a cysteine would have any impact on the activity of Lats. Within the C-terminus we looked for regions where the insertion of the cysteine would be far enough away

from the threonine that gets phosphorylated that it would not interfere with activation. Additionally, candidates for areas of insertion had to be in sequence areas that did not share homology with the *Drosophila* orthologue, Warts, as those areas are presumed to be part of the core kinase domain. Based upon these parameters, two variants were made one, H1071C (plasmid code HC, Kavran lab) and the other, A1075C (plasmid code IB, Kavran lab). Following the standard protocol as described above these two variants were compared to wild type Lats. Statistical analysis revealed that there was no difference in luciferase expression between either variant and wild type (Figure 15C) (p value 0.99, 0.99). These results imply that the addition of a cysteine scar from intein cleavage will not impact the activity of Lats.

Methods

Luciferase

Standard mammalian luciferase assay protocol was followed for all experiments (Appendix 1).

Discussion

Determination of Lats minimal functional unit

Ultimately, the results of our luciferase assays indicated that from the whole 1130 amino acid Lats protein, the region from amino acids 621 to 1085 are critical for activity and was defined as the minimal function unit (MFU). Using the data from the luciferase assay combined with activity assays performed by another master's student, Yoo Jin Kim, TJ has selected various forms of the MFU to clone into expression vectors. He will use these clones to express Lats and hopefully purify a well-behaved protein that can be used for crystallographic studies.

The cysteine scar from intein cleavage will not change Lats activity

Since the cysteine residue proved to have no impact on Lats function, TJ decided to proceed with the intein tag protocol in order to isolated functional and well behaved Lats protein. Now that Lats has been purified on its own, further work to crystalize it can proceed. This project is in its beginning stages and there is still much to be done but the isolation of Lats protein is a huge step forward.

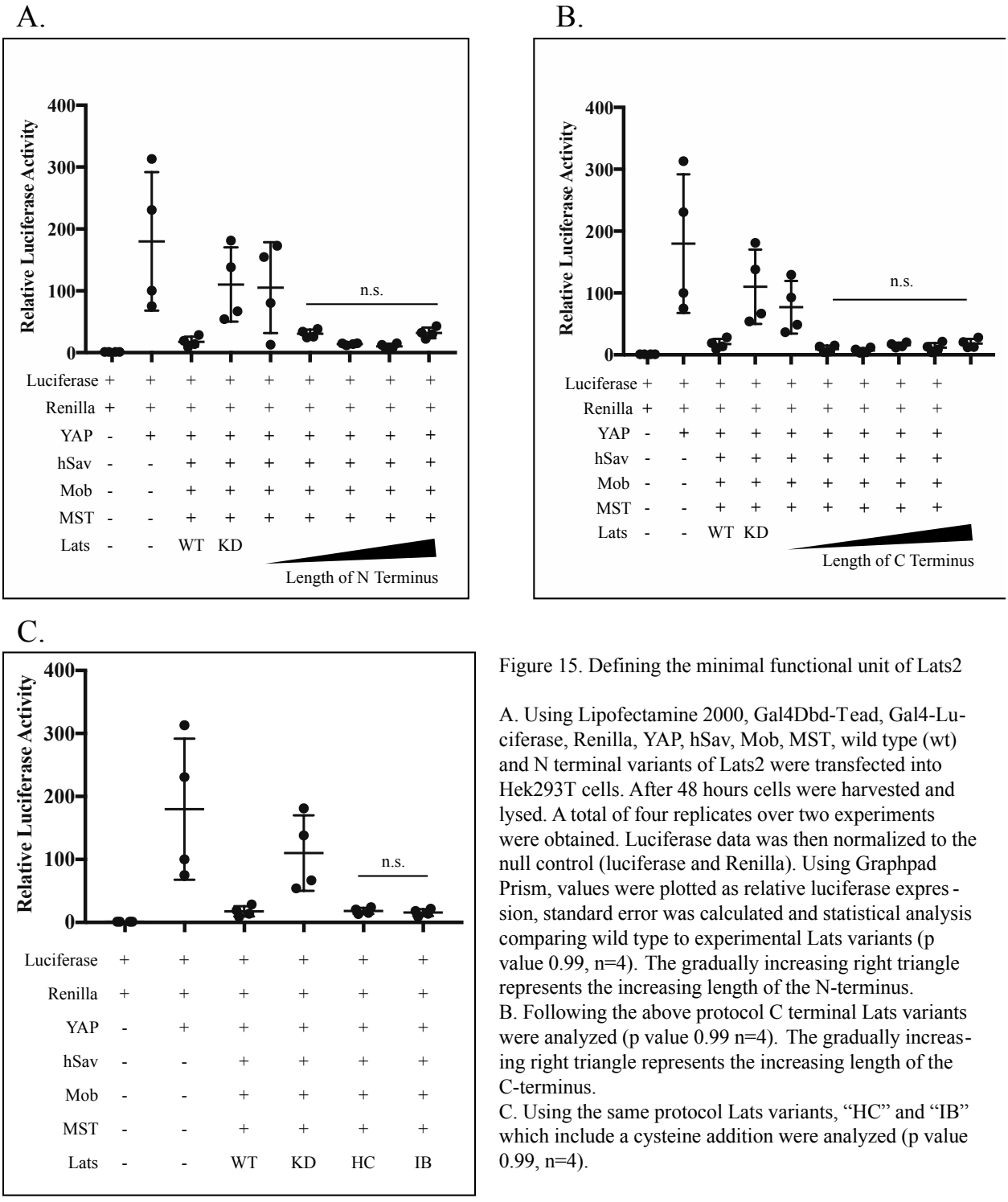


Figure 15. Defining the minimal functional unit of Lats2

A. Using Lipofectamine 2000, Gal4Dbd-Tead, Gal4-Luciferase, Renilla, YAP, hSav, Mob, MST, wild type (wt) and N terminal variants of Lats2 were transfected into Hek293T cells. After 48 hours cells were harvested and lysed. A total of four replicates over two experiments were obtained. Luciferase data was then normalized to the null control (luciferase and Renilla). Using Graphpad Prism, values were plotted as relative luciferase expression, standard error was calculated and statistical analysis comparing wild type to experimental Lats variants (p value 0.99, n=4). The gradually increasing right triangle represents the increasing length of the N-terminus.
B. Following the above protocol C terminal Lats variants were analyzed (p value 0.99 n=4). The gradually increasing right triangle represents the increasing length of the C-terminus.
C. Using the same protocol Lats variants, "HC" and "IB" which include a cysteine addition were analyzed (p value 0.99, n=4).

Table 9. C-terminal variant plasmids

For the luciferase assays the following plasmids and concentration were used. C-terminal lengths are listed from shortest at the top to longest at the bottom Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid	Concentration
90	Gal4-UAS-Luciferase	100ng
91	pRL-SV40-*Renilla*	10ng
122	p2xFlagCMV2-Yap2	25ng
93	Gal4-Dbd-TEAD4	75ng
94	pcDNA-HA-Mst2	75ng
95	pcDNA-HA-Sav	75ng
96	pcDNA-HA-Mob	75ng
97	pcDNA-Myc-Lats	75ng
KE	pcDNA-Lats-D827N	75ng
HE	pcDNA-Lats-Ctermtrunc	75ng
HB	pcDNA-Lats-Ctermtrunc	75ng
FW	pcDNA-Lats-Ctermtrunc	75ng
FV	pcDNA-Lats-Ctermtrunc	75ng
FU	pcDNA-Lats-Ctermtrunc	75ng
FT	pcDNA-Lats-Ctermtrunc	75ng

Table 10. N-Terminal variant plasmids

For the luciferase assays the following plasmids and concentration were used. N-terminal lengths are listed from shortest at the top to longest at the bottom. Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid	Concentration
90	Gal4-UAS-Luciferase	100ng
91	pRL-SV40-*Renilla*	10ng
122	p2xFlagCMV2-Yap2	25ng
93	Gal4-Dbd-TEAD4	75ng
94	pcDNA-HA-Mst2	75ng
95	pcDNA-HA-Sav	75ng
96	pcDNA-HA-Mob	75ng
97	pcDNA-Myc-Lats	75ng
KE	pcDNA-Lats-D827N	75ng
FZ	pcDNA-Lats-Ntermtrunc	75ng
GJ	pcDNA-Lats-Ntermtrunc	75ng
GN	pcDNA-Lats-Ntermtrunc	75ng
GI	pcDNA-Lats-Ntermtrunc	75ng
FS	pcDNA-Lats-Ntermtrunc	75ng

Table 11. cysteine addition plasmids

For the luciferase assays the following plasmids and concentration were used. Code refers to the code that the Kavran Lab has assigned to the plasmid. Complete list of Kavran Lab plasmids can be found on the Kavran Lab Google Drive.

Code	Plasmid	Concentration
90	Gal4-UAS-Luciferase	100ng
91	pRL-SV40-*Renilla*	10ng
122	p2xFlagCMV2-Yap2	25ng
93	Gal4-Dbd-TEAD4	75ng
94	pcDNA-HA-Mst2	75ng
95	pcDNA-HA-Sav	75ng
96	pcDNA-HA-Mob	75ng
97	pcDNA-Myc-Lats	75ng
KE	pcDNA-Lats-D827N	75ng
HC	pcDNA-Lats-H1071C	75ng
IB	pcDNA-Lats-A1075C	75ng

Conclusion

Ultimately, I was able to establish a very reliable and reproducible protocol for using a luciferase reporter assay to test different protein constructs that our lab was interested in studying. However, there are a few limitations to our protocol. Most notably, all of the Hippo pathways core proteins and adapters must be transfected into our system. This means that it is impossible to study an individual protein's activity through luciferase. Rather our protocol allows us to see how it functions within the context of the entire pathway. Additionally, over the optimization process we came to realize that as a result of our dynamic range and the high level of variability between assays, our protocol cannot describe a range of activity levels, instead it can only provide a binary result.

We were able to get some great preliminary results in our Lats and MST studies and published our work on Hippo. This was really exciting but moving forward there is a lot more work to do. There are still aspects of our protocol that can be further refined and exploring alternative cell types may be beneficial in helping answer our research questions. Recently, we discovered that basal levels of Hippo pathway expression in our HEK293T cells is relatively high. This has confounded some experiments and may contribute to the overall variability in our luciferase assays.

In addition to challenges with our mammalian cells, our *Drosophila* cells have continued to be unreliable in producing results. We have explored different *Drosophila* cell types to no avail. This has proved to be one of the biggest roadblocks to pushing *Drosophila* protein projects forward. While we have worked around this by transferring proteins to mammalian expression vectors, this method comes with its own confounding factors. To best study our proteins of

interest it will be a priority of upcoming work to optimize a *Drosophila* cell based system that is both function for luciferase reporter assays and immunoprecipitation studies.

Originally, we planned to have the SARAH domain studies as the largest part of this master's thesis. We were really excited about learning more about the hierarchy of SARAH domains and following up on previous studies of dSalvador's SARAH domain. However, due to the unforeseen issues setting up the luciferase assay and continued struggles with *Drosophila* cell work, we are only now ready to proceed with these experiments. In the last month of my time here, I will work on the dSalvador SARAH dimerization studies but following students will have to complete the SARAH swap work.

The last two years of work has been an incredible learning experience and helped tremendously in reinforcing biochemical and molecular biology principles and techniques. I am incredibly grateful for the opportunity to continue to pursue research and in my next steps I hope to stay connected to oncology studies and bench work. Enough cannot be said for the environment that Dr. Kavran has created in her lab. The collaboration and comradery between master's students, doctoral students and post docs is second to none and made the last two years that much more enjoyable. Dr. Kavran has challenged me intellectually and academically, pushed me to be more successful and most importantly, has been the best mentor I could have asked for. I am excited to continue to follow the growth of her lab and wish her all the best in the coming years.

Appendix 1.

Complete protocol for luciferase reporter assay

The following is the basic protocol for the luciferase reporter assay in both Drosophila and mammalian cells.

Preparation of Luciferase Reaction Substrate

Thaw Luciferase Assay Reagent II (LARII) (Promega Corporation) Stop & Glo® Reagent (Promega Corporation) and add appropriate substrate to each buffer following manufacturer's directions. Dissolve all of the dry LAR II substrate in 10mL of the LAR II buffer provided. Mix the entire Eppendorf of Stop & Glo® substrate with the 10mL bottle of the Stop & Glo® buffer. Make sure not to cross-contaminate the LARII and the Stop & Glo® reagents as this will render them useless. Once mixed, aliquot out 1mL aliquots of each diluted substrate mix into Eppendorf tubes and freeze at -80ºC.

Cell culture and preparation of cell lysate

General information

In order to account for transfection variability each experiment should be done in either duplicate or triplicated depending on the number of wells you have available. These replicates should be all transfected from the same master mix of experimental DNA and transfection reagent to maintain consistency. In order to be able to use the statistical analysis tool in Prism a minimum of two replicated from two separate experiments, giving you an n=4 is needed. However, for publication, two or three replicates from four separate experiments, giving you an n=8-12 is generally accepted. This amount off data points allows for a high degree of certainty when drawing any conclusions from the data.

Cell Culture

Mammalian cells, HEK 293, 293T and 293FT were cultured in Dulbecco's Modified Eagle Medium (Gibco) with 2mM L-Glutamine (Gibco) and 5% heat inactivated Fetal Bovine Serum (FBS) (VWR). After research and additional testing, we determined that a synthetic form of FBS could be used in culture media and switched the lab over to FBEssence (FBE) (VWR). Culture chamber was maintained at 37ºC with 5% CO_2. All mammalian Luciferase transfections were performed in 12 well cell culture plates seeded with 5*10^4 cells in 1mL of culture media. Cells were seeded and transfected on the same day for all mammalian reactions. After 48 hours cells were harvested and lysed. Cell lysate was then used for the luciferase assay.

Drosophila S2 cells were cultured in Schneider's Insect Medium (Gibco) with 10% heat-inactivated FBS. S2 cells cannot be grown in media with FBE. The culture chamber was maintained at 28ºC without CO_2. All *Drosophila* luciferase experiments were performed in 12 well cell culture plates seeded with 1*10^5 cells in 1mL of culture media. Cells were seeded on day 0 and transfected on day 2. 72 hours after transfection cells were harvested and lysed.

For more information about cell culture see the protocols for both mammalian and *Drosophila* cell culture on the Kavran Lab Google drive.

Mammalian cell transfection, incubation and harvesting protocol

Day 1.

Plate 5*10^4 HEK293T cells per well in a twelve well plate in 1mL of DMEMF12 with 5%FBE and 2mM L-glutamine. In Eppendorf tube mix 25µL of OptiMEM (Gibco) reagent with 1µg of total DNA per well. In a separate Eppendorf tube mix 25µL of OptiMEM with 2µL of Lipofectamine 2000 reagent. The DNA: Lipofectamine ratio should always remain 1:2. Mix DNA complex with Lipofectamine complex vigorously using the vortex and incubate at room

temperature for 5-10 minutes. Add dropwise the 50μL of the reaction mixture to each well. Swirl plate to make sure transfection mixture disperses evenly in well. Return to incubator.

DNA amounts (per well) for optimal Luciferase assay: Refer to Table 2 on page 34.

Day 2.

Remove media with transfection mixture. Lipofectamine 2000 is highly toxic to the cells so media must be replaced on the cells no later than 24 hours post transfection. Replace with 1mL of fresh supplemented DMEM media. This must be done gently as cells have a tendency to lift off the plate if added in too harsh a matter. Under the microscope the wells should be approximately 40-50% confluent.

Day 3.

Each well should be 80-100% confluent under the microscope. However, transfecting the cells does make them clump so confluency may appear lower or cells may have lifted off while changing media. As long as the wells are over 50% confluency there should be enough cells to detect luciferase signal. Aspirate media from each well and gently wash with 1mL of Phosphate Buffered Saline (PBS). Aspirate PBS making sure not to disrupt cells. While washing with PBS, thaw 5x Passive Lysis Buffer (PLB) (Promega Corporation) and 1mL aliquots of LARII and Stop & Glo® substrates (Promega Corporation). Dilute 5x PLB to 1x concentration in PBS. Add 150μL of 1x PLB to each experimental well. Lyse cells at room temperature on a rocker for 10 min. While cells are lysing, dilute each 1mL Eppendorf of LARII and Stop & Glo® reagents 1:3 with PBS in to their own 15mL tubes. Once cells are lysed, you can either perform the experiment or freeze lysate at negative twenty degrees. To run luciferase assay load 10μL of lysate into the wells of a ninety-six well white plate making sure to skip the first two wells of the

first row in order to prime injectors on luminometer. At this point you are ready to move to the luminometer and detect luciferase signal.

Drosophila cell transfection, incubation and harvesting protocol

Day 0.

Plate 10^5 S2 cells per well in a twelve well plate in 1mL of Schneider's *Drosophila* media with 10% FBS.

Day 2.

In Eppendorf tube mix 25µL of serum free Schneider's *Drosophila* media 0.5µg of total DNA per well. In a separate Eppendorf tube mix 25µL of serum free media with 2µL Fugene6 reagent. The DNA: Fugene6 ratio should always remain 1:2. Mix DNA complex with Fugene 6 complex vigorously using the vortex and incubate at room temperature for 15-20 minutes. Add the 50µL of the reaction mixture dropwise to each well. Swirl plate to make sure transfection mixture disperses evenly in well.

DNA amounts (per well) for optimal Luciferase assay: Refer to Table 4. Page 36

Day 5.

Remove media and cells from each well and spin down in an Eppendorf tube at 1200*g's for 5 minutes at room temperature. Gently wash pellet with 1mL of Phosphate Buffered Saline (PBS) and spin again. Aspirate PBS making sure not to disrupt cells. While washing with PBS, thaw 5x Passive Lysis Buffer (PLB) (Promega Corporation) and 1mL aliquots of LARII and Stop & Glo® substrates (Promega Corporation). Dilute 5x PLB to 1x concentration in PBS. Add 150µL of PLB to each experimental Eppendorf. Lyse cells at room temperature on a rotating shaker that fits 1.5mL Eppendorf tubes for 10 min. While cells are lysing, dilute 1mL aliquots of LARII and Stop & Glo® reagents 1:3 with PBS in their own 15mL tubes. Once cells are lysed, you can

either perform the experiment or freeze lysate at negative twenty degrees. To run luciferase assay load 10μL of lysate into the wells of a white ninety-six well plate making sure to skip the first two wells of the first row in order to prime injectors on luminometer. At this point you are ready to move to the luminometer and detect luciferase signal.

Luminometer Protocol

1. On the home-screen of HP laptop attached to the "Synergy H1" luminometer open the "Gene 5 2.0" program.
2. Under the "read now" tab open "Kavran Lab Brendan" protocol
3. When the protocol is opened it will automatically take you to the well selection pop-up. Exit the pop-up.
4. Open the "Synergy H1" instrument icon on the top middle of the page (it is a picture of the luminometer).
5. In the instrument icon, select the dispenser tab
6. Put each injector into the bottles of water provided (yellow tape with injector 1, red tape with injector 2)
7. In the dispenser tab, set dispense rate to 300 μL/sec, and dispense amount to 1000μL.
8. Prime 1mL of water in both dispenser 1 and 2, making sure that you put the priming plate in the plate holder to catch any excess liquid.
9. Replace the water bottles with the tubes of LARII and Stop & Glo®. Firefly should be put with dispenser 1 and Stop-and-Glow with dispenser 2.
10. Prime 1mL of reagent into each dispenser
11. Once primed, replace priming plate with you 96 well experimental plate. Make sure that the "A1" corner is aligned properly in the plate holder.

12. Exit instrument reader control pop-up
13. Select the green circle for “read now” in the top icon bar
14. Using you mouse highlight all the wells that you want to be read and press start
15. After run save experiment to an external hard drive
16. To export click the export icon on the top icon bar and save the excel spreadsheet to and external drive. DO NOT save to the computer, the Wang Lab regularly deletes saved results for some reason.
17. Remove your 96 well plate and replace it with priming plate. Also remove any leftover substrate reagent and save at negative twenty degrees for your next experiment.
18. Follow the instructions on the luminometer for cleaning using the provided water and ethanol.

Luciferase Normalization

The results from the luminometer are raw intensity values and do not immediately give us much information about our proteins of interest. In general, for HEK293T cells you should expect to see *Renilla* values in the mid 10^5 and firefly values from the high 10^4 to the low 10^5. For S2 cells, both firefly and *Renilla* values should fall between the mid 10^3 to the mid 10^4. If wells give an overflow value you will either have to re-run the entire plate with all the samples equally diluted or, if you have enough replicates, you can choose to exclude the well. In order to better understand the data, we first divide the raw firefly luciferase value by the corresponding *Renilla* luciferase value for each replicate. This will be called the “ratio”.

Next, the ratio values for the positive control and our experiments will be divided by the ratio value of the null control, which in our protocol will be cells transfected with luciferase and *Renilla* alone. The ratio value from the first well of the null control duplicate will be used to

divide the ratio value for the first well of the positive control duplicate and continuing to the experimental wells. The ratio value of the second well of the null control will be used in the same manner. The results of this should give the null control a value of one with the positive control and experiments with some positive number which is the relative luciferase activity.

To plot the data, copy the fold values from excel into the appropriate graph setup, either XY, column, or grouped in Prism. I prefer column graphs and they are relative easy to work with once in Prism. In Prism, the program will automatically make a graph. You can edit the graph to make it look like how you prefer i.e. columns, dot plot etc. Prism will also automatically calculate the standard deviation and use that to make error bars. To obtain p values use the Analyze tool and select the appropriate statistical test.

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Curriculum Vitae

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Education

Johns Hopkins University Bloomberg School of Public Health -Baltimore, MD (2016-present)

Masters of Science **-**Anticipated 2018

- Graduate research with Dr. Jennifer Kavran
- Department of Biochemistry and Molecular Biology, focus: Cancer Biology

Carleton College -Northfield, MN

Bachelor of the Arts **-**2014

- Major: Biology

School for International Training -Kenya (Fall 2012)

Experience

Graduate Research -Kavran Lab, Johns Hopkins Bloomberg School of Public Health (2017-present)
Clinical Research Assistant -Soda Tax Study, Johns Hopkins Bloomberg School of Public Health (summer 2017)
Research Assistant II -Camargo Lab, Harvard University/Boston Children's Hospital (2015-2016)
Research Assistant I -Camargo Lab, Harvard University/Boston Children's Hospital (2014-2015)
Research Assistant -Albuquerque Lab, University of Maryland School of Medicine (Summer 2013)
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Publications, Abstracts and Posters

- Cairns, L., Tran, T., **Fowl**, **B**., Patterson, A., Kim, YJ., Bothner, B., Kavran, J., Salvador has an extended SARAH domain that mediates binding to Hippo. *Journal of Biological Chemistry.* March 8, 2018.
- Yimlamai, D., **Fowl, B**., Softic, S., Camargo, F., Tu1696 Yap/Taz Activity Drive Liver Fibrosis. *Gastroenterology* , Volume 150 , Issue 4 , S1165
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- Yimlamai, D., **Fowl, B.,** Camargo, F. Yap Taz Drive Fibrosis. Poster presented at: *Boston Children's Hospital Stem Cell Day*; 2015 December, 14; Boston, MA.
- Yimlamai, D., **Fowl, B.,** Camargo, F. Yap Taz Drive Fibrosis. Poster presented at: *Digestive Disease Week,* 2016 May, 21-24; San Diego, CA.