GM2 mediated YAP/TAZ regulation: elucidation of critical involvement of the master regulator LATS kinases.

A Summer Project Report under RISE Programme, 2016-17, Department of Biotechnology, Government of West Bengal.

Submitted by:

Avinandan Kar

Under the supervision of **Dr. Kaushik Biswas**, **Bose Institute**, Kolkata.

Roll Number: **MBIO194022**

Registration Number: **138349 of 2016-17**

MSBT2/7 Project

Department of Life Science and Biotechnology, Jadavpur University.

Declaration

I hereby declare that the project report entitled “GM2 mediated YAP/TAZ regulation: elucidation of critical involvement of the master regulator LATS kinases” , is a faithful record of bonafide and original research work carried out by me under the supervision of Dr. Kaushik Biswas, Associate Professor, Division of Molecular Medicine, Bose Institute, Kolkata. No part of this project work has been submitted by any other research fellow or student till date.

AVINANDAN KAR

Acknowledgement

I would like to express my heartfelt gratitude to my guide Dr. Kaushik Biswas for his expert advice and unwavering support throughout the course of this project.

I would like to thank Mr. Barun Mahata for his support and guidance during the course of my project.

I sincerely thank Dr. (Mrs) Manjari Kundu, Mr. Avisek Banerjee, Mr. Pravat Kumar Parida, Mr. Shibjyoti Debnath, Mr. Abhishek Sarkar and Ms. Elora Khamrui for their constant support and for making me feel at home during the project duration.

I would also like to present my gratitude to Department of Biotechnology, Government of West Bengal, for selecting and allowing me to conduct this Summer Project under RISE (Biotechnology Based Research Internships for Science & Engineering) Programme, 2016-17.

Index

Abstract

Hippo signaling is an evolutionarily conserved regulator of tissue growth and cell fate. LATS1/2 is the upstream kinase of the Hippo signaling pathway which regulates phosphorylation and dephosphorylation of YAP/TAZ to modulate nuclear-cytoplasmic shuttling. To define the role of GM2 in regulation of Hippo pathway, here we aimed to generate LATS2 knockout cell line. CRISPR-Cas9 genome engineering technology has been applied to achieve this goal. Here, we have designed and constructed CRISPR-Cas9 plasmid against coding sequence of LATS2. The sequence verified CRISPR construct against human LATS2 were then transfected into HeLa cells to assess the functionality and to generate LATS2 knockout Hela cell Line. Further, LATS2 CRISPR construct transfected Hela cells were selected and clonal selection strategy was undertaken to select, expand and clonally purify LATS2 knockout Hela cells. Here, we have shown that CRISPR-Cas9 construct against LATS2 is functional and we were also able to generate LATS2 knockout Hela cell line. This LATS2 knockout cell will help us to understand the role GM2 in regulation of Hippo signaling in future studies.

Introduction

Hippo tumor suppressor pathway which controls cell growth, tissue homeostasis, and organ size has been found to be inactivated in many cancers [1]. YAP (Yes Associated Protein) and its paralog TAZ (Tafazzin/WWTR1) are transcription modulators (co-activator / co-repressor), regulated by this well characterized signaling pathway. LATS1 and LATS2 are master regulators serine / threonine kinases which are involved in phosphorylation and inactivation of YAP and TAZ by means of their cytoplasmic retardation and proteasomal degradation [2]. Mostly, upstream MST1 / MST2 kinases through LATS1 and LATS2 kinases determine the fate of YAP/TAZ in canonical Hippo signaling pathway but this is not the ultimate. Many other proteins such as AMOT (angiomotin) family of proteins also determine the YAP/TAZ localization and transcriptional activity through direct interaction in non-canonical way in a context dependent manner [3].

In *Drosophila*, where the Hippo pathway have originally been described, loss of the tumour suppressor Warts (the fly counterpart of mammalian LATS1/2) is larval lethal [4]. In contrast, LATS1 knock-out (KO) mice are viable and develop tumors [5], while LATS2 knock-out mice die during embryonic development, most likely due to defective cytokinesis resulting in genomic instability. Immortalized LATS2 null MEFs display loss of contact inhibition indicating that LATS1/2 might function as tumor suppressors in mammals [6-8]. Many studies suggest that MST1/2 does not always signal through LATS1/2 to YAP and MST/LATS signaling are dispensable for YAP regulation in many specific cell types [9, 10]. These findings demand further investigations for reassessment of LATS1/ LATS2 to establish them as master regulators of YAP/TAZ.

Gangliosides (sialic acid containing glycosphingolipids) which are ubiquitous in mammalian cells and predominant in the outer leaflet of the lipid bilayer of the cell membrane plays many roles including but not limited to intercellular communication, modulating cell signaling, cell cycle and cellular motility [11]. During the past few years, gangliosides have emerged as one of the major players in mediating tumor-induced immune suppression. Several of these gangliosides are

not only found to be over-expressed in various tumors but also actively shed from tumor cell surface into the surrounding tumor microenvironment, thereby modulating host immune response[12-14]. Tumor-derived gangliosides (GM1, GM2, GD3) have already been documented to cause immune cell dysfunction through their ability to kill T cells by apoptosis or by impairing antigen presentation by dendritic cells [15-17]. These tumor-shed gangliosides modulate the function of adjacent tumor cells either by promoting or inhibiting their invasion, migration or angiogenic property or by suppressing and interfering with host cellular immune response thus acting as a double edged sword in cancer progression. Among the various complex gangliosides, GM2 is one of the key members of GSLs involved in various intercellular cross talks modulating signal transduction, cell cycle events, and cell adhesion and cell motility [18]. A recent publication from our laboratory has shown ganglioside GM2 to enhance tumor cell migration and invasion by directly interacting with cell surface integrins [19]. In yet another publication from our lab, TALEN mediated disruption of GM2-synthase have reduced anchorage independent growth (AIG) and anoikis resistance property of cancer cell thereby indicating that GM2 may be involved in epithelial to mesenchymal transition (EMT), a key process governing the transformation to a malignant phenotype [20].

Despite recent findings from our lab strongly supporting the functional significance of ganglioside GM2 in tumor growth, progression and metastasis, the precise molecular mechanism by which GM2 promotes tumor growth, progression and metastasis is yet unknown. Preliminary studies show GM2 regulates YAP signaling. Knockdown of GM2 from GM2 expressing cells shows downregulation of YAP activated genes and upregulation of YAP repressed genes whereas exogenous addition of GM2 in GM2-deficient cells shows upregulation of YAP activated genes and downregulation of YAP repressed genes. Further, exogenous addition of GM2 results in nuclear localization of YAP. All these data drive us to dissect out the entire molecular mechanism by which GM2 modulates YAP and the Hippo pathway in mediating EMT.

On the basis of these preliminary studies we aim to dissect out the component of the Hippo signaling pathway which is critically involved in this GM2 mediated YAP regulation. Hence we first want to known the involvement of LATS1/LATS2 kinases in GM2 mediated YAP regulation through the following objectives:-

Review of Literature:

The CRISPR-Cas9 Genome Editing Technology

The CRISPR-Cas9 technique is a novel genome engineering tool which is being widely used in different systems due to its technical simplicity, time economy and specificity. After the advent of designer nuclease mediated genome editing, like ZFNs (Zinc Finger Nucleases) and TALEN (Transcription Activator-like Effector Nuclease), the CRISPR-Cas9 system is the popular method of choice . The acronym CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. The CRISPR-Cas system is essentially a microbial adaptive immune system which protects against invasion by viruses, mediated by RNA guided cleavage of viral DNA by Cas nuclease. It has been discovered among a wide variety of bacterial and archeal systems. The system essentially consists of CRISPR associated (Cas) genes, some non-coding RNAs, and an array of short direct repeat sequences separated by spacers with unique sequences (protospacers), obtained from invading viral DNA, which forms the CRISPR array. Within the DNA target, each protospacer is associated with a protospacer adjacent motif (PAM) sequence which varies from system to system[21].

There are three features to CRISPR-Cas immunity- 1) Adaptation: insertion of new spacers into the CRISPR locus obtained from prior viral attacks; 2) Expression: Transcription of non-coding crRNA from these inserted spacer sequences and there processing; 3) Interference: detection and degradation of cognate foreign DNA in a RNA dependent manner by Cas protein(s). The general mechanism of this system is simple. In response to injection of foreign DNA by the mobile genetic element, the bacterial cell responds by transcribing 2 sets of non-coding RNA molecules- the crRNA (CRISPR RNA) and the trRNA (tracer RNA). The two RNA molecules form a tertiary complex with the Cas protein(s). The trRNA serves as a scaffold for the crRNA while the crRNA contains the cognate sequence of the foreign DNA and guides the Cas nuclease to the target DNA [22].

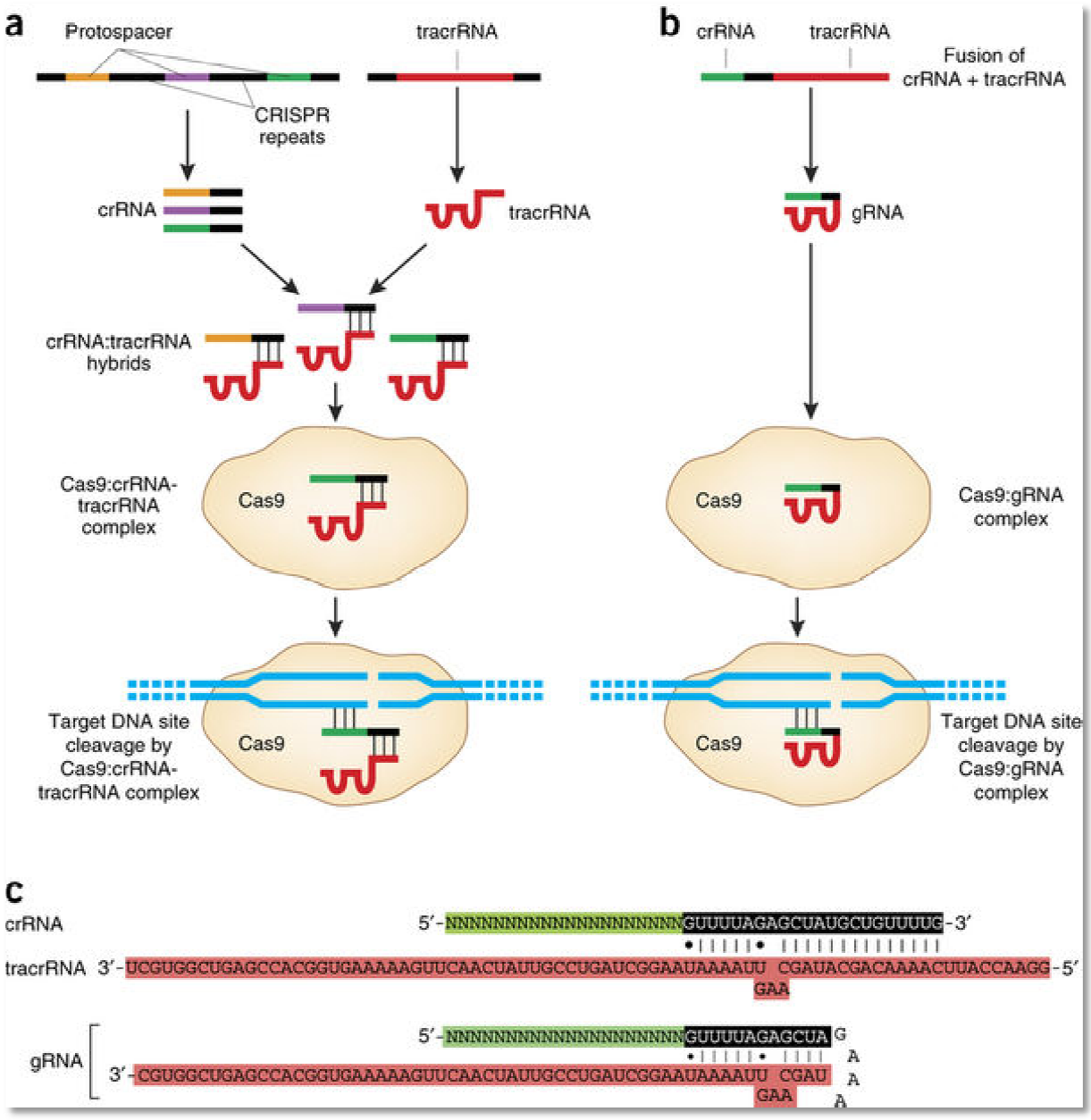

Figure 1- Naturally occurring and Engineered CRISPR-Cas systems.

There are 3 types of CRISPR systems- Type I, Type II, and Type III. Of these, the Type II system is the best characterised. Cas9 is the endonuclease in this system with a 20nt crRNA unit which guides the nuclease to the target. In *Streptomyces pyogenes*, the target region must be 5' to the NGG PAM sequence. This system is the most commonly used system for genome engineering.The system we have used

in the present study makes use of a Cas9 nuclease which is codon-optimised for expression in mammalian systems along with the requisite RNAs. The crRNA and the trRNA are fused together to form a single guide RNA (sgRNA). Any sequence adjacent to a PAM sequence can be chosen to design a sgRNA to guide the Cas9 nuclease to the target DNA loci. The Cas9 nuclease makes a double-stranded break (DSB) 3bp 5' to PAM sequence. The rationale here for mutagenesis at the target loci is the cells repair mechanism. Non-homologous end joining (NHEJ) is commonly used for repair of DSBs. It involves non-specific addition or loss of nucleotides at the cut site. This leads to indel mutations at the target loci which might lead to frameshifting and even premature stop codon formation. Homology dependent repair (HDR) may also be used for insertion of desired sequence. In this case, a template(donor) DNA containing the desired sequence needs to be co-transfected. Cas9 and the sgRNA are generally introduced into the cell by plasmid mediated transfection where they can be transcribed and Cas9 can also betranslated. Direct injection of Cas9 protein and mRNA has also been used in embryos [23].

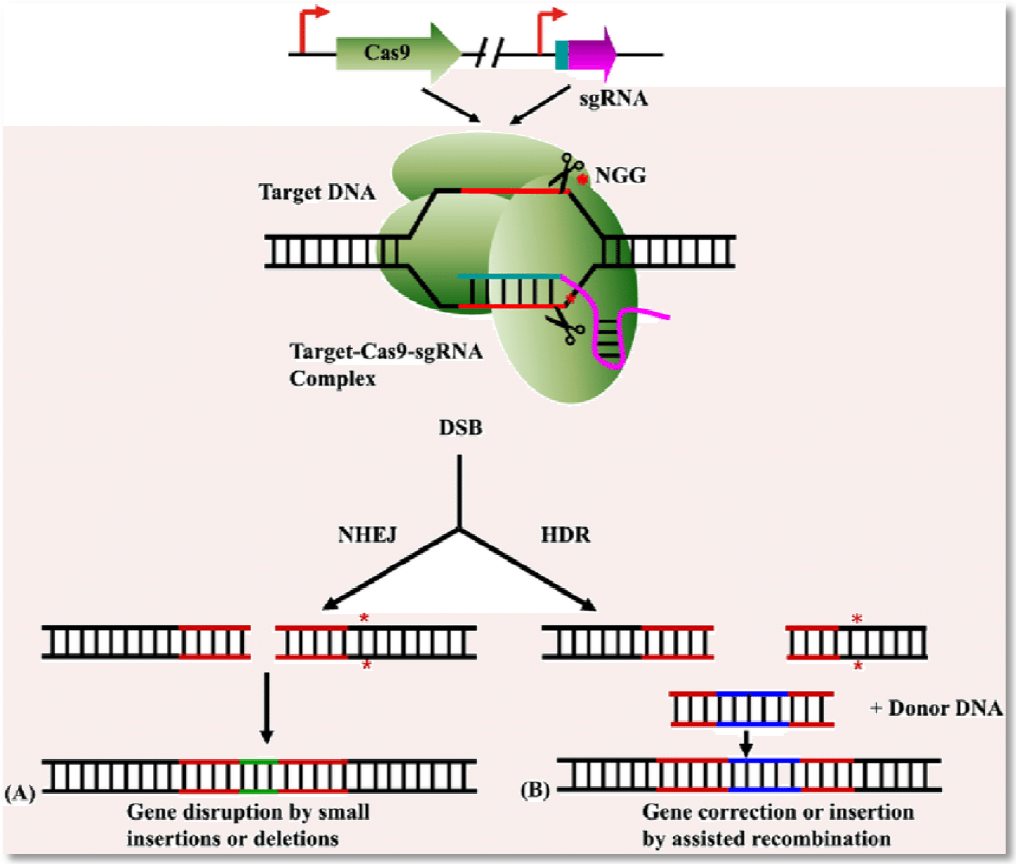

Figure 2- The two possible mechanisms of Double Stranded Break Repair: Non Homologous End Joining (NHEJ) and Homology Dependent Repair (HDR).

The Hippo Signaling Pathway

Hippo pathway is very well known and important signalling pathway known to modulate cell proliferation, differentiation and death; therefore, the hippo pathway functions as a key node to regulate these biological processes to control tissue homeostasis and organ size control. Dysregulation of the hippo pathway has been identified in broad range of cancer including liver, kidney, lung, brain, prostate and colorectal cancer. Studies have shown that YAP/TAZ (key components of the Hippo signalling pathway) activity is increased as a result of increased expression and nuclear localization in human tumor samples. This is consistent with inactivation of the Hippo pathway which is known to inhibit YAP and TAZ activity mainly by promoting cytoplasmic retardation of YAP/TAZ through signaling cascade dependent phosphorylation and ubiquitin proteasome-mediated degradation. As compared with the other oncogenic signaling pathway only few cancers are associated with direct mutational activation/inactivation of hippo component proteins; rather, wide range of extracellular or secreted factors are known to regulate hippo pathway most robustly, for example, Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are two amphipathic, non protein secretory molecule which regulate YAP/TAZ activity through G- protein coupled receptor [24].

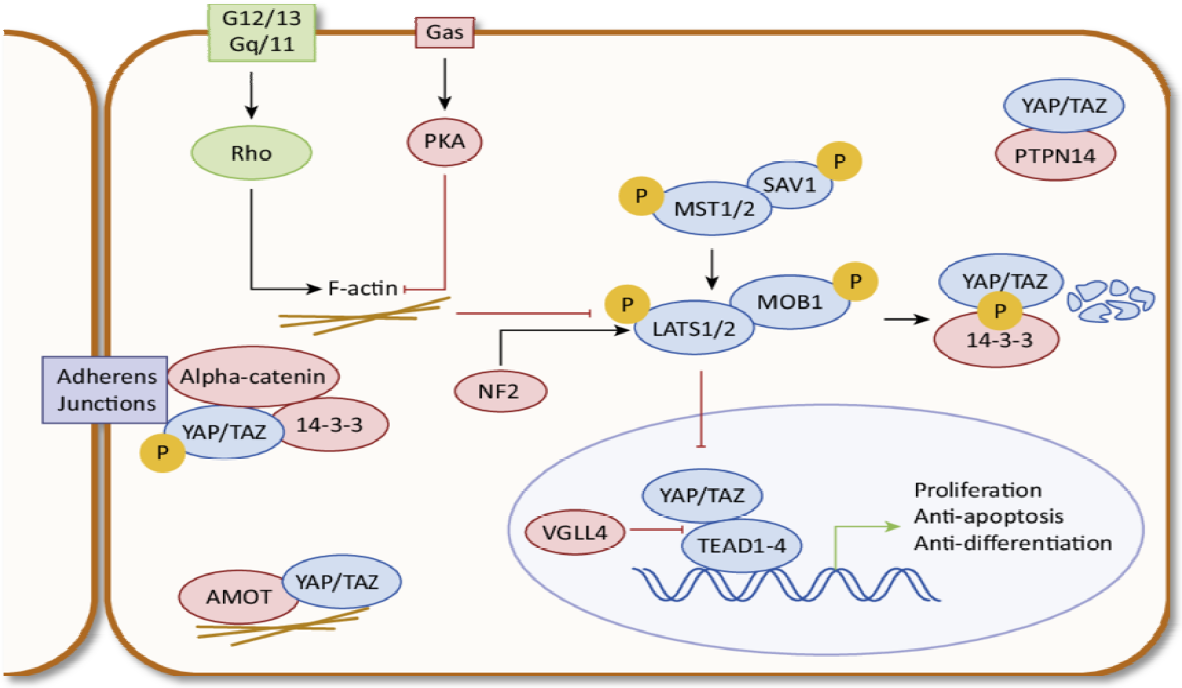

Figure 3- The Hippo Signaling Pathway.

Core to the Hippo pathway is a kinase cascade, wherein Mst1/2 (ortholog of Drosophila Hippo) kinases and SAV1 form a complex to phosphorylate and activate LATS1/2. LATS1/2 kinases in turn phosphorylate and inhibit the transcription co-activators YAP and TAZ, two major downstream effectors of the Hippo pathway. When dephosphorylated, YAP/TAZ translocate into the nucleus and interact with TEAD1-4 and other transcription factors to induce expression of genes that promote cell proliferation and inhibit apoptosis. The Hippo pathway is involved in cell contact inhibition, and its activity is regulated at multiple levels: Mst1/2 and YAP/TAZ phosphorylation and activity are modulated by phosphatases; Lats1/2 and YAP/TAZ stability are regulated by protein ubiquitination; and LATS1/2 activity is also regulated by the cytoskeleton [9].

Gangliosides

Gangliosides are sialic-acid containing glycosphingolipids which are abundantly found in the nervous system of vertebrates. They are also found to be expressed on the outer leaflet of the plasma membrane in neuronal cells. They are generally associated with sphingomyelin and cholesterol in lipid rafts which are associated with cell-cell communication, cell-cell adhesion and signal transduction. Gangliosides are known to play important physiological functions in cell growth, differentiation and embryogenesis. However, aberrant expression and shedding of gangliosides is associated with disease conditions like cancer. Many tumor cells are known to shed

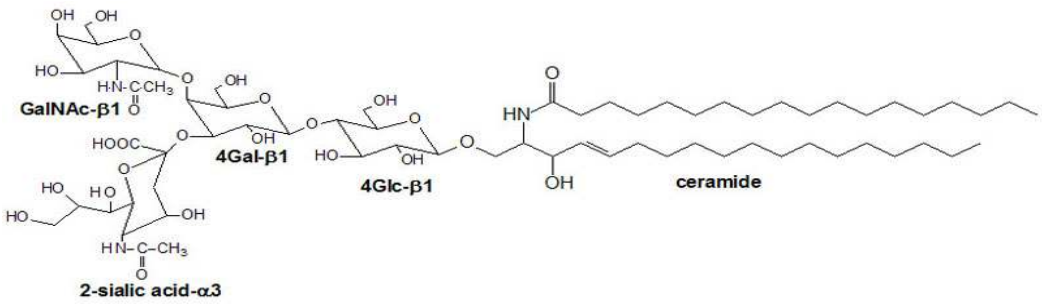

Figure 4- Structure of GM2.

gangliosides into the tumor microenvironment which can enhance tumor malignancy by altering local signaling. Gangliosides like GM2 have been reported to support anchorage independent growth of tumor cells as well as enhance tumor cell

migration. Gangliosides also help in immune-evasion of tumors by causing dysfunction or apoptosis of immune effector cells in the tumor microenvironment [25].

Significance of LATS2 Kinase in Hippo Signaling Cascade

By controlling the YAP1 proto-oncoprotein Hippo signalling plays important roles in cancer-associated processes. Current evidence suggests that the Hippo kinases MST1/2 together with the MOB1 scaffold protein promote the formation of active MOB1/LATS complexes which phosphorylate and thereby inhibit YAP1. However, the regulatory mechanisms of MST1/2-MOB1-LATS signalling are currently under investigation. The Ser872 T-loop and Thr1041 hydrophobic motif (HM) phosphorylation of LATS2 is essential for LATS2 activation. MST1/2 phosphorylate LATS2 on Thr1041, but not Ser872, while MOB1 binding to LATS2 supports both phosphorylation events .Therefore, LATS2 variants carrying specific modifications that mimic gain or loss of phosphorylation and/or abolish MOB1/LATS2 interactions are under study. Taken together, LATS2 kinase plays acritical role in Hippo signaling [26].

Aims and Objectives:

1. **Design, construction and validation of LATS2 CRISPR.**
   Guide oligos (20 mers) against LATS2 CDS will be designed just after the translation initiation site to completely disrupt the protein using *http://crispr.mit.edu* and the sgRNA (guide oligo) will be chosen on the basis of high “on-target score” and low “off-target score” in the whole exome. Guide oligo will then be cloned into sgRNA and Cas9 expression vector with appropriate selection marker. Sequence verified LATS2 CRISPR plasmid will be transfected into HEK-293T cells to check the functionality of the CRISPR plasmids within the cells. Genomic DNA will be isolated from transfected cells and mismatch sensitive/heteroduplex sensing T7E1 assay will be performed.
2. **Generation and characterization of LATS2 knockout cell line.**
   After assessing the DNA cleavage activity at targeted locus of the designed LATS2 CRISPR, Hela cells will be transfected with the human LATS2

CRISPR plasmid. 24 hours post transfection, cells will be shortly selected (for 4-5 days) against selection marker. Then, cells will be grown to form colonies from single cell and each colony will be expanded. Expression of target protein will be validated by western blot. Further, in/del DNA mutations in the targeted locus will be validated by PCR amplification, TA-cloning and sequencing of CRISPR targeted locus.

Successful fulfilment of the above objectives will generate stable LATS2-KO cell line, which will be used to define the involvement of these master regulator in GM2 mediated Hippo-YAP/TAZ signaling in cancer.

Materials and Methods:

Culture and maintenance of cell lines

Hela cells were maintained in DMEM medium containing 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, non-essential amino acids, 100 units/ml penicillin, 100mg/ml streptomycin and 50mg/ml gentamycin sulphate.

CRISPR Oligomer Designing and Construction

CRISPR oligo pairs were designed for human LATS2. First, all the transcript variants were identified for LATS2 searching in the Gene database of NCBI. There is only one transcript variant for LATS2. The exonic sequences of the 2nd exon within the CDS of LATS2 were chosen. The sequences were submitted to the online CRISPR design tool (http://crispr.mit.edu) for guide RNA selection and assessment of off-target binding probability. After analysis, CRISPR guide oligomers were chosen, based on high on-target score and low off-target score.

Phosphorylation and annealing of Designed CRISPR Oligomers

The synthesized sense and antisense CRISPR oligomers were annealed with the addition of 5’ phosphate group. T4 polynucleotide kinase (PNK) was used. Reaction contained 100µM sense and antisense oligomers and 5 units of T4 PNK. Reaction

conditions are shown in **Figure 2**. After reaction, the annealed DNA were diluted in the raio 1:50 for ligation reaction.

Restriction Digestion and Alkaline Phosphatase Treatment

pSpCas9(BB)-2A-Puro (Addgene) vector was digested with BbsI restriction endonuclease. 3µg plasmid DNA was digested in a 30µl reaction with 20 units of BbsI. Digestion was carried out overnight at 37°C.The digested products were then subjected to alkaline phosphatase treatment. 4 units of FastAP (Fermentas) was used. Reaction was carried out at 37°C for 30 minutes. Gel extraction was performed after running the digested plasmid in an 0.75% agarose gel. Concentration of gel extracted Bbs I digested pSpCas9(BB)-2A-Puro was 87.76 ng/µl.

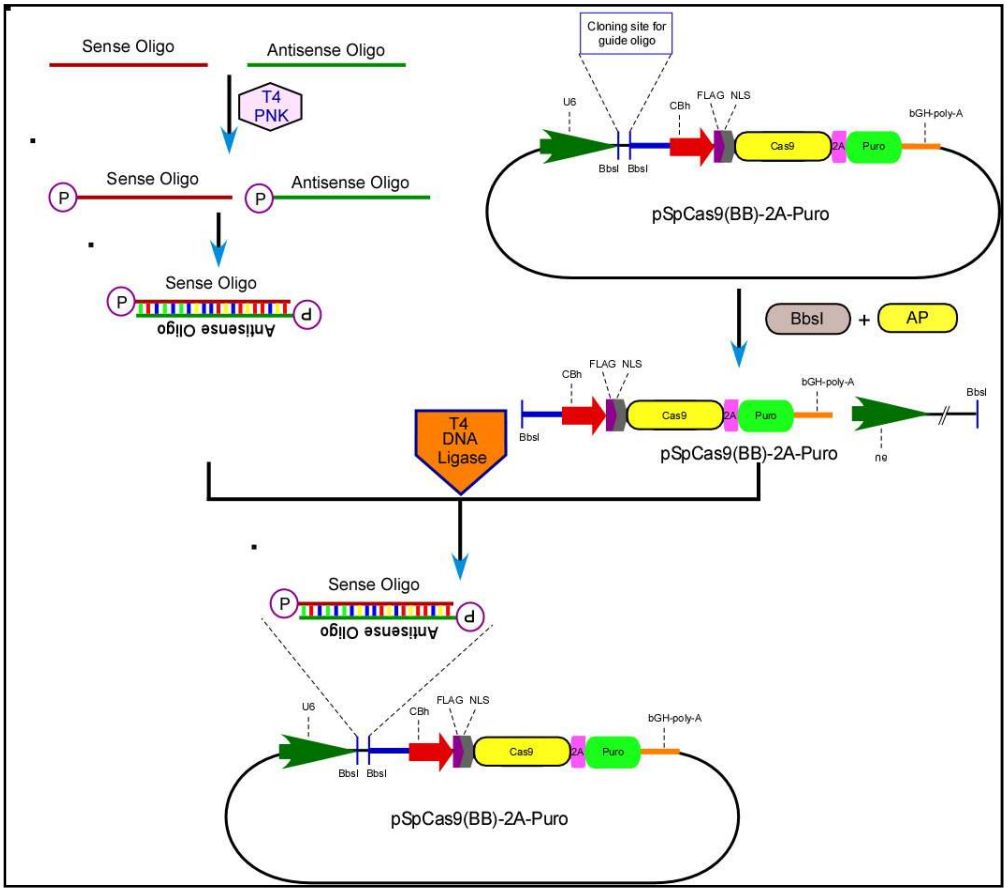

Figure 5. Schematic depicting cloning of CRISPR oligomer pair into pSpCas9(BB)-2A-Puro plasmid.

Ligation of Annealed CRISPR Oligomers into Bbs I digested and AP treated pSpCas9(BB)-2A-Puro

A ligation reaction was set up using 88ng digested pSpCas9 vector and 2µM annealed DNA. Ligation was carried out using QuickLigase (New England BioLabs Inc.). Reaction was carried out at 25°C for 15 minutes.

Preparation of Competent Cells

XL-1 Blue strain of *Escherichia coli* was first streaked onto a LB agar plate and incubated overnight at 37°C. A single colony was picked and 10ml of LB broth media was inoculated. This was then placed overnight at 37°C in shaking conditions. The following day 200ml of LB broth media was inoculated with 200µl of this overnight culture. OD was monitored every hour (at 600nm). When the OD reached 0.4-0.5, the conical flask was placed in ice for 30 minutes, following which cells were centrifuged at 5000rpm for 10 minutes at 4°C. Pellets obtained post centrifugation were washed with 100mM $CaCl_2$ and placed in 4°C overnight in 5ml of 100mM $CaCl_2$. The next day, cells were added carefully to 20% glycerol, aliquoted and snap frozen in liquid nitrogen. Fresh aliquot was stored at -80°C.

Transformation

Competent XL1 Blue cells stored at -80°C were used for transformation. 5µl DNA from the ligation reaction was added to about 65µl competent cells. The competent cells were then forced to take up the ligated CRISPR constructs by giving a heat shock at 42°C for 75 seconds followed by snap chilling in ice. 1000µl of SOC (Super Optimal Broth with catabolite repression) medium was added and incubated at 37°C for 1 hour for recovery. The cells were then pelleted and 900µl supernatant was removed. The pellet was suspended in remaining 100µl SOC and spread onto LB-Amp plates. Plates were incubated overnight at 37°C. Next day, single colonies were picked and introduced into 5ml LB with ampicillin. The tubes were incubated at 37°C under shaking condition.

Plasmid DNA Isolation

Overnight transformed XL1 Blue cells in liquid cultures were harvested and plasmid isolation was carried out using HiPurA^{TM} Plasmid DNA Miniprep Purification Kit (HiMedia Laboratories). Harvested cells were resuspended in resuspension buffer HP1 (with RNase A) and mixed by gentle pipetting and vortexing. Resuspended cells were lysed using lysis solution HP2. Lysis was stopped by neutralization solution HN3. Debris was pulled down by centrifugation. Supernatant was loaded onto plasmid binding column and flowthrough discarded. Bound plasmid was washed with wash buffers HPB and HPE (ethanol added) followed by elution in elution buffer EB (QIAGEN).

Expansion of the sequenced plasmids

Competent XL1 Blue cells stored at -80°C were used for transformation. 1µl DNA from the sequenced construct was added to about 65µl competent cells. The competent cells were then forced to take up the ligated CRISPR constructs by giving a heat shock at 42°C for 75 seconds followed by snap chilling in ice. 1000µl of SOC (Super Optimal Broth with catabolite repression) medium was added and incubated at 37°C for 1 hour for recovery. The cells were then pelleted and 950µl supernatant was removed. The pellet was suspended in remaining 50µl SOC and spread onto LB-Amp plates. Plates were incubated overnight at 37°C. Next day, single colonies were picked and introduced into 150ml LB with ampicillin. The tubes were incubated at 37°C under shaking condition. Overnight transformed XL1 Blue cells in liquid cultures were harvested and plasmid isolation was carried out using HiPurA^{TM} Endotoxin free Plasmid DNA Midiprep Purification Kit (HiMedia Laboratories).

Genomic DNA Isolation

Cells were lysed with RIPA buffer followed by removal of debris. Supernatant was treated with equal volume phenol:chloroform:isoamyl alohol (25:24:1) to separate protein. Aqueous phase was subjected to ethanol precipitation. Aqueous phase was mixed with 2.5 volumes chilled ethanol and 1/10th volume 3M sodium acetate (pH 5.2), mixed and stored at -80°C for 20 minutes. Then the precipitated DNA was pulled down at 13000rpm at 4°C for 15 minutes. The pellet was washed twice with 70% ethanol and subjected to drying. Dried pellet was resuspended in TE buffer.

T7E1 Assay

The target loci for the CRISPR constructs were PCR amplified from the genomic DNA isolated from the Hela cells using the T7E1 primers as shown in **Figure**. Approximately 100ng DNA was used per 20µl reaction in 5 sets. PCR conditions are as shown in **Figure**. Following PCR amplification of target loci, the amplicons were subjected to PCR purification in a column based method to remove Taq polymerase, dNTPs, salts. The purified PCR product was then used to set up a denaturation-renaturation reaction. 600ng of DNA was denatured at 95°C for 10minutes and slowly renatured by gradually decreasing the temperature at the rate of 0.1°C/sec till 25°C to form heteroduplexes. The amplicons were then digested with 5U of T7E1 endonuclease for 30 minutes at 37°C. Reaction was stopped by addition of EDTA and was analysed by 2% agarose gel electrophoresis. The expected fragment lengths are shown in the **Figure**.

Transfection into Hela cells and puromycin selection

Hela cells were transfected with the CRISPR constructs using Lipofectamine LTX (ThermoFisher Scientific). The transfection was carried out in 6 well plate at 60% confluency with 4µg plasmid DNA per well.After transfection, cells were allowed to recover for 48 hours before starting selection using puromycin with a final concentration of 1µg/ml.

Clonal Population Selection

After removal of puromycin selection, Hela cells that survived the selection pressure, were allowed to proliferate and increase in number. The cells were then counted, diluted and seeded into 96 well plates such that there is 1 cell per 200µl i.e. 1 cell per well. The cells were then allowed to expand and each clonal population have been analyzed by Western Blot to check the expression of LATS2.

Western Immunoblot

Cells were lysed using RIPA buffer (Thermo Scientific) containing Halt Protease Inhibitor (Thermo Scientific) and EDTA. Total protein concentration was estimated using BCA (Bicinchonic Acid) assay. Cell lysates were then boiled in the presence of

Laemmli's buffer for 5 minutes and loaded onto an 8% SDS PAGE gel. After gel electrophoresis, the proteins were transferred onto a methanol activated PVDF membrane by wet transfer method. The membrane was blocked with 5% BSA dissolved in Tris-buffered saline containing 0.1% Tween20 (TBST) for 1 hour. After blocking, membranes were probed overnight at 4°C with mouse anti-FLAG primary antibody in antibody dilution buffer (5% bovine serum albumin in TBST). After washing, the membrane was probed with HRP tagged anti-rabbit IgG secondary antibody for 90 minutes. The protein bands were detected by developing the blot in ECL solution and viewed in BioRad ChemiDoc. Hsp90 was also developed on the same membrane as a loading control.

RESULTS

Design, construction and validation of LATS2 specific CRISPR.

To design LATS2 specific CRISPR plasmid which can specifically target all transcript variants of LATS2, a genomic region within the LATS2 loci was chosen which is present in the coding sequence (CDS) of LATS2 mRNA, so that we can generate complete LATS2 knockout cell line. The 2nd exonic sequence within the coding sequence (CDS) in the transcript was chosen for LATS2 as shown in **Figure 6a**. Then 20nt guide sequence was designed by submitting these sequences to the online CRISPR Design tool (http://crispr.mit.edu). Guide oligomers were chosen based on a high on-target score and a low off-target score. The oligomers were then annealed and phosphorylated using T4 polunucleotide kinase. Annealed guide oligomer pairs containing 5' overhangs complementary to BbsI digested overhangs were then cloned into BbsI digested sgRNA and Cas9 expression vector pSpCas9(BB)-2A-Puro. Vetor map and annealed LATS2 oligos were shown in **Figure 6b.** LATS2 specific CRISPR plasmids were then sequence verified. So, to validate the genome editing potential of designed and constructed LATS2 specific CRISPR constructs, equal amounts of LATS2 specific constructs in pSpCas9(BB)-2A-Puro backbone and empty pSpCas9(BB)-2A-Puro were transfected into Hela cells using Lipofectamine LTX in 6 well format. To check whether the designed sgRNAs actually targeted the Cas9 nuclease to the desired loci and created insertion

or deletion (indel) mutations, a T7E1 assay was performed. The target loci for LATS2 was amplified using the T7E1 primers. The amplified fragments were denatured and renatured. This would lead to the formation of heteroduplexes due to random in/del mutations created by NHEJ during repair after targeted cleavage by guide RNA directed Cas9 within the cell population. The targeted cleavage sites for the T7E1 amplicon was represented schematically in **Figure 6c**. The renatured heteroduplexes were then digested with T7E1 endonuclease which cleaves at sites of mismatched base-pairing. On analysing the resulting fragments in a 2% agarose gel, cleavage were found in LATS2 CRISPR transfected cells but not in the control vector transfected cells as shown in **Figure 6d**. This result confirmed that the designed CRISPR against LATS2 was functional.

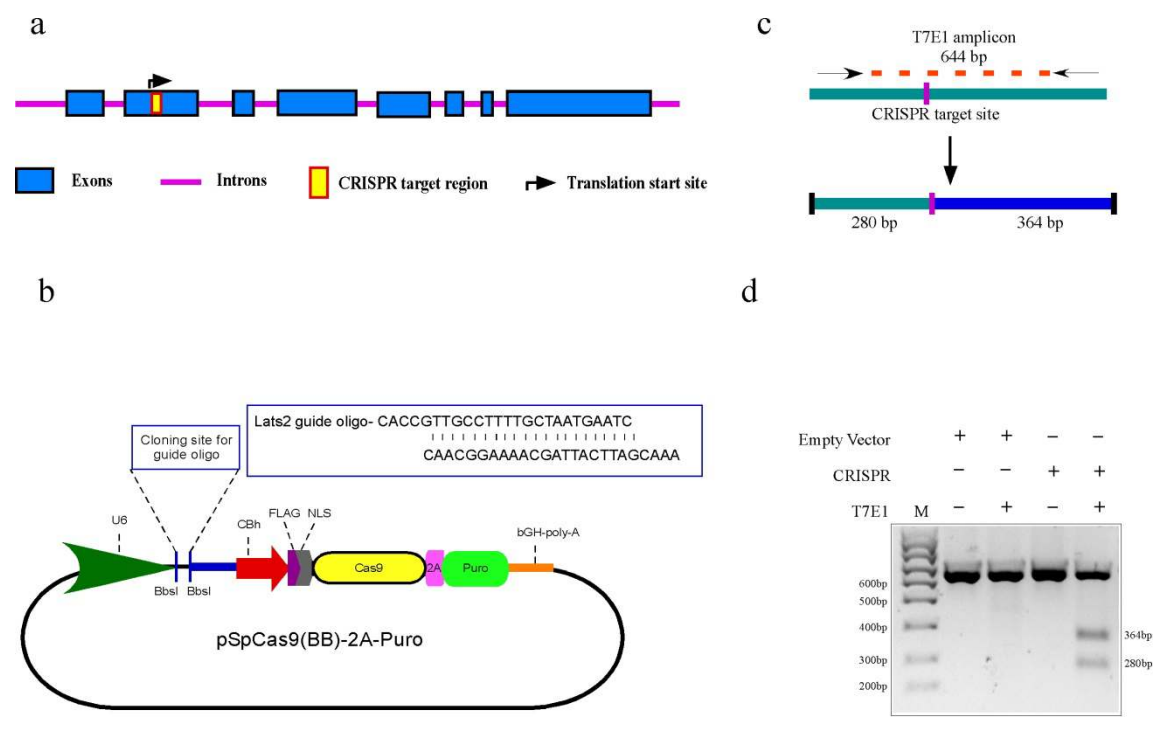

Figure 6. Constuction and validation of CRISPR-Cas9 targeted against human LATS2 locus. **Fig. 6a** shows transcript of human LATS2 gene. Blue boxes indicate exons, purple line indicate adjacent introns, yellow box with red outline indicates CRISPR target region and black arrows indicate translation start site. **Fig. 6b** shows the vector map of CRISP-Cas9 cassette and the cloning site and sequence for "Guide" oligo targeted against LATS2. Guide oligos were designed using http://crispr.mit.edu and the single guide RNA (sgRNA) was then cloned into sgRNA and Cas9 (yellow) expression vector containing a puromycin selection cassette to obtain a CRISPR construct. Sequence verified LATS2 was then transfected into Hela cells using lipofectamine LTX reagent to check the functionality of CRISPR plasmids within the cells. Following initial selection with puromycin over the week, genomic DNA was isolated from transfected cells and T7E1 assay was performed. For this, the genomic region covering the Cas9 target region (**Fig. 6c**) was PCR amplified followed by digestion with T7E1 endonuclease. **Fig. 6d** shows 2 bands

corresponding to the LATS2 target site indicative of successful CRISPR.

Generation of LATS2 knockout Hela cell line.

Since the specific aim of the project was to generate LATS2 knockout in Hela cell line, the functional CRISPR constructs were transfected into the Hela cells using Lipofectamine LTX. The transfected Hela cells were selected against puromycin (1µg/ml) for 10 days and then 200 cells were plated in 100mm dish to form colony as shown in **Figure 7a**. Then each of the single colonies were expanded in 24 well plate formats and then transferred to 6 well plate formats. Each individual clonal population were then harvested for western blot analysis. Western blot analysis shows that LATS2 was knocked out from several clones, as shown in **Figure 7b.** Clones # 1, 3, 5 show complete LATS2 KO, whereas clones # 2, 4, 9 and 10 show partial KO.

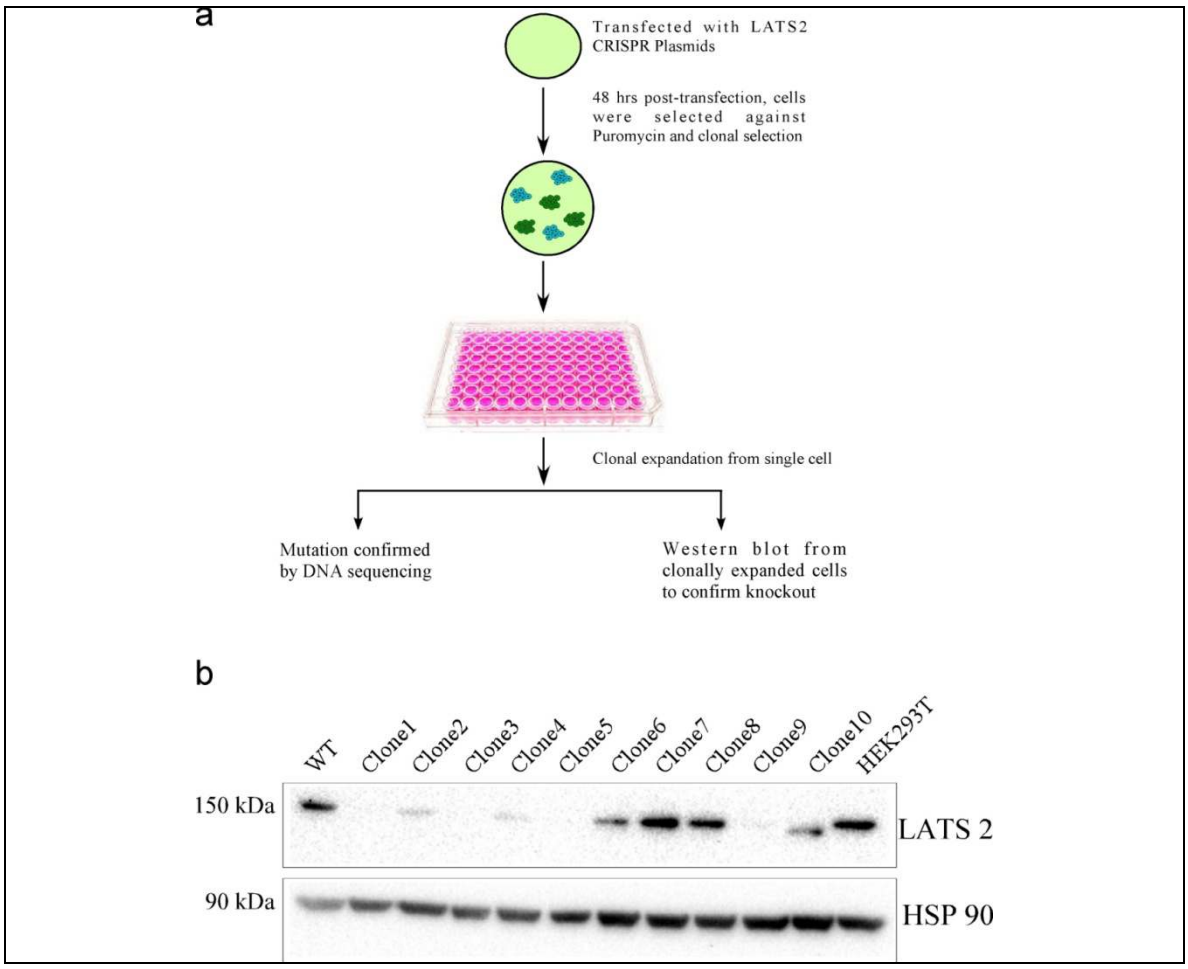

Figure 7. **Fig 7a** shows schematic representation of clonal selection strategy for LATS2 knockout Hela cell generation. **Fig 7b** shows western blot analysis of 10 clonally selected cells to assess LATS2 expression. Clone 1, 3,5 and 9 shows no LATS2 expression suggesting CRISPR-Cas9 mediated LATS2 knockout.

Conclusion:

Here we aimed to generate a LATS2 knockout Hela cells using the CRISPR-Cas9 genome editing technology. From our data, it is evident that we have been able to successfully design functional CRISPR constructs for knocking out LATS2. We have confirmed cloning of CRISPR guide oligomers into pSpCas9(BB)-2A-Puro vector by sequencing and genome editing potential of designed CRISPR by T7E1 assay. Finally, clonal selection strategy was undertaken to establish LATS2 knockout Hela cell line and western blot analysis shows that LATS2 have been knocked out from several clonally selected Hela cells.

References

1. Yu, F.X., B. Zhao, and K.L. Guan, *Hippo Pathway in Organ Size Control, Tissue Homeostasis, and Cancer.* Cell, 2015. **163**(4): p. 811-28.
2. Gomez, M., V. Gomez, and A. Hergovich, *The Hippo pathway in disease and therapy: cancer and beyond.* Clin Transl Med, 2014. **3**: p. 22.
3. Paramasivam, M., et al., *Angiomotin family proteins are novel activators of the LATS2 kinase tumor suppressor.* Mol Biol Cell, 2011. **22**(19): p. 3725-33.
4. Xu, T., et al., *Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase.* Development, 1995. **121**(4): p. 1053-63.
5. St John, M.A., et al., *Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction.* Nat Genet, 1999. **21**(2): p. 182-6.
6. McPherson, J.P., et al., *Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity.* EMBO J, 2004. **23**(18): p. 3677-88.
7. Yabuta, N., et al., *Lats2 is an essential mitotic regulator required for the coordination of cell division.* J Biol Chem, 2007. **282**(26): p. 19259-71.
8. Harvey, K.F., X. Zhang, and D.M. Thomas, *The Hippo pathway and human cancer.* Nat Rev Cancer, 2013. **13**(4): p. 246-57.
9. Meng, Z., et al., *MAP4K family kinases act in parallel to MST1/2 to activate LATS1/2 in the Hippo pathway.* Nat Commun, 2015. **6**: p. 8357.
10. Yu, F.X., et al., *Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling.* Cell, 2012. **150**(4): p. 780-91.
11. Allende, M.L. and R.L. Proia, *Lubricating cell signaling pathways with gangliosides.* Curr Opin Struct Biol, 2002. **12**(5): p. 587-92.
12. Buggins, A.G., et al., *Microenvironment produced by acute myeloid leukemia cells prevents T cell activation and proliferation by inhibition of NF-kappaB, c-Myc, and pRb pathways.* J Immunol, 2001. **167**(10): p. 6021-30.
13. Floutsis, G., L. Ulsh, and S. Ladisch, *Immunosuppressive activity of human neuroblastoma tumor gangliosides.* Int J Cancer, 1989. **43**(1): p. 6-9.
14. Shurin, G.V., et al., *Neuroblastoma-derived gangliosides inhibit dendritic cell generation and function.* Cancer Res, 2001. **61**(1): p. 363-9.
15. Biswas, K., et al., *GM2 expression in renal cell carcinoma: potential role in tumor-induced T-cell dysfunction.* Cancer Res, 2006. **66**(13): p. 6816-25.
16. Das, T., et al., *GM1 and tumor necrosis factor-alpha, overexpressed in renal cell carcinoma, synergize to induce T-cell apoptosis.* Cancer Res, 2008. **68**(6): p. 2014-23.
17. Jales, A., et al., *Ganglioside-exposed dendritic cells inhibit T-cell effector function by promoting regulatory cell activity.* Immunology, 2011. **132**(1): p. 134-43.
18. Todeschini, A.R., et al., *Ganglioside GM2-tetraspanin CD82 complex inhibits met and its cross-talk with integrins, providing a basis for control of cell motility through glycosynapse.* J Biol Chem, 2007. **282**(11): p. 8123-33.
19. Kundu, M., et al., *Ganglioside GM2 mediates migration of tumor cells by interacting with integrin and modulating the downstream signaling pathway.* Biochim Biophys Acta, 2016. **1863**(7 Pt A): p. 1472-89.
20. Mahata, B., et al., *TALEN mediated targeted editing of GM2/GD2-synthase gene modulates anchorage independent growth by reducing anoikis resistance in mouse tumor cells.* Sci Rep, 2015. **5**: p. 9048.

21. Nemudryi, A.A., et al., *TALEN and CRISPR/Cas Genome Editing Systems: Tools of Discovery.* Acta Naturae, 2014. **6**(3): p. 19-40.
22. Rath, D., et al., *The CRISPR-Cas immune system: biology, mechanisms and applications.* Biochimie, 2015. **117**: p. 119-28.
23. Ran, F.A., et al., *Genome engineering using the CRISPR-Cas9 system.* Nature Protocols, 2013. **8**(11): p. 2281-2308.
24. Yu, F.X. and K.L. Guan, *The Hippo pathway: regulators and regulations.* Genes Dev, 2013. **27**(4): p. 355-71.
25. Schnaar, R.L., R. Gerardy-Schahn, and H. Hildebrandt, *Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration.* Physiol Rev, 2014. **94**(2): p. 461-518.
26. Hoa, L., et al., *The characterisation of LATS2 kinase regulation in Hippo-YAP signalling.* Cell Signal, 2016. **28**(5): p. 488-497.