Elucidating the role of YAP in directing mesenchymal stem cell fate

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Thesis

ELUCIDATING THE ROLE OF YAP IN DIRECTING MESENCHYMAL STEM CELL FATE

by

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LISA BRENAN

ABSTRACT

Mesenchymal stem cells (MSCs) are a type of multipotent stem cell capable of differentiating into several cell types, including fat and bone cells. The Hippo pathway effectors TAZ and YAP have been implicated as important regulators of MSC fate, but their roles in this process are poorly understood. The goal of my thesis work was to illuminate the roles of YAP and TAZ in MSC differentiation. I examined how depleting YAP and/or TAZ, or expressing YAP mutants affect the differentiation of C3H10T1/2 cells, which are a multipotent mouse embryo fibroblast cell line capable of forming bone, fat and cartilage.

Interestingly, knocking down either YAP or TAZ had different effects on C3H10T1/2 differentiation. YAP knockdown cells that underwent a brown/beige adipogenic protocol showed a significant increase in the amount of lipids produced as compared to control, suggesting that YAP has a role in inhibiting adipogenesis in these cells. YAP knockdown also increased alkaline phosphatase activity in cells subjected to an osteogenic protocol, while simultaneously producing lipid droplets. In contrast, knockdown of TAZ resulted in a decrease in both lipogenesis (in adipogenic differentiation) and alkaline phosphatase activity (in osteogenic differentiation). These observations indicate that depletion of YAP or TAZ leads to defective MSC
differentiation, and that without proper YAP and TAZ signaling MSC cells may arrest in an immature state.

Bone Morphogenic Proteins (BMPs) are known to play important roles in MSC differentiation, and YAP/TAZ have been implicated in BMP regulation in other systems. I therefore hypothesized that YAP/TAZ may affect MSC differentiation in part by altering BMP signaling. Analysis of activated phosphorylated Smad1 (p-Smad1), a key transcriptional effector of BMP signaling, showed that TAZ and YAP are required to promote p-Smad1 levels. Depletion of TAZ and YAP showed an increase in the expression of Gremlin1 (GREM1), an inhibitor of BMP signaling, and an increase in the expression of BMP4. These observations suggest that TAZ and YAP promote BMP signaling by controlling BMP ligand activity.

Given that BMP signaling has important roles early in MSC differentiation, I hypothesized that YAP and TAZ may direct these early processes. To test this, I generated dox-inducible C3H10T1/2 cells capable of expressing wild type YAP or two mutant forms of YAP: a nuclear localized YAP mutant (YAP-5SA), and a transcriptionally inactive YAP mutant (YAP-5SA,S94A). Adipogenic differentiation with these cells showed a reduced level of lipogenesis in the constitutively active nuclear YAP mutant. Analysis of adipogenic markers by qPCR showed a reduction in both PPARγ and UCP1. Osteogenic differentiation experiments in these cells showed that transient expression of all forms of YAP increased the amount of alkaline phosphatase activity, with the 5SA form showing the greatest activity. RNA expression analysis showed that the early marker Runx 2 was highly upregulated in cells overexpressing wild type YAP,
while the late markers osteocalcin and osteopontin were greatly reduced in comparison to control. As alkaline phosphatase activity is a mid-stage marker for bone differentiation, the staining results and qPCR data indicate that overexpression of YAP may result in arrest of the differentiation process.

A microarray was performed using isolates from 10T1/2 cells transduced with TAZ, YAP or TAZ/YAP siRNA to further evaluate the mechanism through which TAZ and YAP may be acting. The TAZ and YAP double knockdown showed a large change in global gene expression as compared to both the control and the single knockdowns. Among the genes that showed a large change from control was BMP4, the expression of which was reduced more than two-fold over control. Additionally, GREM1 was up-regulated almost two-fold by the double knockdown. Also upregulated was the gene periostin which encodes a protein that enhances BMP incorporation into connective tissues, and may play a role in the mineralization of the extracellular matrix of bone.

Based on my observations, along with those of others, I propose that TAZ and YAP are major factors in controlling early cell fate determination of MSCs. There appear to be multiple levels of regulation, and thus the next steps should include clarifying the roles of YAP and TAZ in vivo, and placing their activity chronologically in the differentiation timeline. Such studies will offer insight into stem cell differentiation and may eventually allow for therapeutic opportunities.
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LIST OF ABBREVIATIONS

ALP ......................................................................................... Alkaline Phosphatase
AMOT ................................................................................... Angiomotin
AMOTL2 ............................................................................... Angiomotin Like 2
APS ......................................................................................... Ammonium Persulfate
Areg ......................................................................................... Amphiregulin
BCA ......................................................................................... Bicinchoninic Acid
Birc ...................................................................................... Baculoviral IAP Repeat Containing
BMP ....................................................................................... Bone Morphogenic Protein
BSA ......................................................................................... Bovine Serum Albumin
βTrCP .................................................................................. Beta-Transducin Repeat Containing
Ca ......................................................................................... Calcium
cDNA .................................................................................. Complementary DNA
CFU-F .............................................................................. Colony Forming Unit - Fibroblast
CO₂ ....................................................................................... Carbon Dioxide
Col2a1 ............................................................................. Collagen 2a1
Col10a1 ........................................................................... Collagen 10a1
Crb ......................................................................................... Crumbs
CTGF .................................................................................. Connective Tissue Growth Factor
cycE .................................................................................. Cyclophiillin E
DAPI .................................................................................. 4',6-Diamidino-2-Phenylindole
dH$_2$O .......................................................................................... Distilled Water
DMEM .......................................................................................... Dulbecco’s Modified Eagle Medium
DMSO .............................................................................................. Dimethyl Sulfoxide
DNA ........................................................................................... Deoxyribonucleic Acid
dNTPs ......................................................................................... Deoxyribonucleotide Triphosphates
DTT .................................................................................................. DL-dithiothreitol
DVL .................................................................................................. Disheveled
ECM .............................................................................................. Extra Cellular Matrix
EDTA ............................................................................................. Ethylenediaminetetraacetic Acid
EGF ............................................................................................... Epidermal Growth Factor
EGFR ............................................................................................. Epidermal Growth Factor Receptor
EGR1 ............................................................................................. Early Growth Response
Ex ..................................................................................................... Expanded
FABP4 ............................................................................................ Fatty Acid Binding Protein 4
FABPαP2 ....................................................................................... Fatty Acid Binding Protein αP2
FBS ............................................................................................... Fetal Bovine Serum
FERM .............................................................................................. 4.1 protein, Ezrin, Radixin, Moesin
FGF ............................................................................................... Fibroblast Growth Factor
FOXH1 .......................................................................................... Forkhead Box H1
FOXO ............................................................................................. Forkhead Box O
GAPDH ........................................................................................... Glyceraldehyde 3-Phosphate Dehydrogenase
GPCR ............................................................................................. G-Protein-Coupled Receptor
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<tr>
<td>Hpo</td>
<td>Hippo</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<tr>
<td>IAP</td>
<td>Inhibitor of Apoptosis</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
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<td>JNK</td>
<td>c-Jun N-Terminal Kinase</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium Phosphate</td>
</tr>
<tr>
<td>Kibra</td>
<td>Kidney and Brain Expressed Protein</td>
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<tr>
<td>Lats</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>LPA</td>
<td>Lysophosphatidic Acid</td>
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<td>LV</td>
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<td>Mg</td>
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MgCl$_2$ .................................................. Magnesium Chloride
Mob .................................................. Mps One Binder Kinase Activator-Like
mRNA .................................................. Messenger RNA
MSC .................................................. Mesenchymal Stem Cell
Mst .................................................. Mammalian Sterile 20–Like Kinase
NaCl .................................................. Sodium Chloride
NaF .................................................. Sodium Fluoride
Na$_2$HPO$_4$ ........................................ Sodium Phosphate
NaOH .................................................. Sodium Hydroxide
Na$_3$Vo ........................................ Sodium Orthovanadate
NCBI .................................................. National Center for Biotechnology Information
NDR .................................................. Nuclear Dbf-2-Related
NF2 .................................................. Neurofibromatosis type-2
NFAT5 .................................................. Nuclear Factor of Activated T Cells 5
OCN .................................................. Osteocalcin
OCT4 .................................................. Octamer Binding Protein
OPN .................................................. Osteopontin
PAGE ........................................ Polyacrylamide Gel Electrophoresis
PAX .................................................. Paired-Box
PBS .................................................. Phosphate Buffered Saline
PCR .................................................. Polymerase Chain Reaction
PFA .................................................. Paraformaldehyde
PGC1α.............Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Alpha
PPARγ..................................................Peroxisome Proliferator-Activated Receptor Gamma
qPCR..........................................................Quantitative Real-Time PCR
RhoA..........................................................Ras Homolog Family Member A
RNA ..........................................................Ribonucleic Acid
ROCK ..........................................................Rho-Kinase
RT ..............................................................Room Temperature
RTK ..........................................................Receptor Tyrosine Kinase
RUNX ..........................................................Runt-Related Transcription Factor
S1P ..............................................................Sphingosine 1-Phosphate
SAPK ..........................................................Stress-Activated Protein Kinase
Sav ...............................................................Salvador
SCF ...........................................................Skp, Cullin, F-box Containing Complex
Sd ...............................................................Scalloped
SDS ............................................................Sodium Dodecyl Sulphate
Ser ..............................................................Serine
SH3 .............................................................Src Homology Domain 3
Sox9 ...........................................................SRY (Sex Determining Region Y)-Box 9
SRF ...........................................................Serum Response Factor
STAT ..........................................................Signal Transducer and Activator of Transcription
T3 .................................................................Triiodothyronine
TAD ..............................................................Transcriptional Activation Domain
TAE.......................................................... Tris-Acetic Acid- Ethylenediaminetetraacetic acid
TAZ.......................................................... Transcriptional Coactivator with PDZ-Binding Motif
TB .................................................................................................. Transfer Buffer
TBS .................................................................................................. Tris Buffered Saline
TBST............................................................................................. Tris Buffered Saline – Tween
TEAD............................................................................................ TEA Domain
TEMED............................................................................................ Tetramethylethylenediamine
TGFβ................................................................. Transforming Growth Factor β
UCP1.......................................................... Uncoupling Protein 1
Wnt........................................................ Wingless-Type MMTV Integration Site Family
WT .................................................................................................. Wildtype
Wts.................................................................................................. Warts
WWC1 .......................................................... WW domain-containing protein 1
WWTR1........................................ WW Domain Containing Transcription Regulator 1
YAP......................................................................................... Yes-Associated Protein
Yes ............................................................................................. Yamaguchi 73 and Esh Avian Sarcoma Virus
Yki................................................................................................. Yorkie
INTRODUCTION

Stem Cells

Stem cells are non-specialized cells that are capable of long-term self-renewal as well as differentiating into other, more specialized types of cells. They may be derived from embryos (e.g. embryonic stem cells derived from the inner cell mass of an embryo) or adult tissues (known as somatic or adult stem cells). Cells that exhibit stem cell properties can be generated by expressing certain genes in somatic cells (known as induced pluripotent stem cells or iPSCs). Stem cells may be totipotent, pluripotent or multipotent. Totipotent stem cells are capable of differentiating into any cell in the developing embryo or the extra-embryonic material. Pluripotent stem cells are able to differentiate into any cell in the body, but not extra-embryonic cells. Multipotent stem cells can become cells of several lineages, but do not have the differentiation potential of pluripotent cells.

Mesenchymal stem cells (MSCs) are a type of multipotent stem cell. They are capable of differentiating into a variety of mesodermal tissues, the best studied of which are cells of the bone, fat and cartilage lineages (59). There is preliminary data indicating that MSCs are also able to differentiate into other tissues such as neurons, myocytes and endothelial cells, but these results have yet to be confirmed (5). MSCs were first identified in bone marrow, but have since been isolated from a number of tissues (13,74). There is evidence that MSCs from different sources have varying differentiation potentials (1,29).
MSCs have a fibroblast-like morphology. They were originally identified by A.J. Friedenstein and partners from bone marrow isolates as colony-forming-units-fibroblast (CFU-F) (20) due to their ability to proliferate into colonies from a single cell. Other studies have identified cell surface markers that are usually present on MSCs and determined that they lack most of the markers of hematopoietic cells commonly found with them in the bone marrow (9,14,59,65,70). However, despite a large body of work since that time, there is still variation among the criteria that researchers use to determine the identity of MSCs in culture. Additionally, nearly all work done with MSCs has been in vitro, due in part to their low abundance in tissues and in part to a lack of cell surface markers specific to multipotent MSCs. This means that the in vivo niche inhabited by MSCs is still poorly understood, particularly as MSCs have been isolated from so many different tissues where the microenvironments may be quite different. Still, the International Society for Cellular Therapy has proposed three criteria for the positive identification of MSCs, which they term “multipotent mesenchymal stromal cells:” 1) they must adhere to standard tissue culture plastic in culture, 2) they must express the surface markers CD105, CD79 and CD90 but not express hematopoietic markers and 3) they must differentiate into osteogenic, adipogenic and chondrogenic cells (14).

Other names for MSCs include mesenchymal stromal cells, bone marrow stromal cells and osteogenic stem cells and there is some debate as to whether “mesenchymal stem cell” is an appropriate name for this group of progenitor cells (5). The term “mesenchymal stem cell” was originally founded on the supposition that MSCs could
generate a number of tissues beyond those associated with skeletal lineages. This supposition has yet to be confirmed.

**Mechanotransduction and Stem Cell Differentiation**

Cells detect and respond to numerous chemical and mechanical signals from both within and outside the cell. The cell is able to integrate these signals from its environment and respond accordingly. Chemical signaling via cellular receptors and protein interaction cascades has long been studied in cells, but it is only recently that methods of testing mechanical signaling have become available. Study of the transduction of signals from cell-cell or cell-extracellular matrix (ECM) junctions is a field still in its infancy, but some of the mechanisms by which cellular mechanotransduction takes place are beginning to be understood.

The term “mechanotransduction” describes the process of cells converting mechanical stimuli from outside the cell to intracellular signals (80). Mechanosensing can be achieved through specialized cellular structures that respond to mechanical stimuli with specific biochemical reactions, as is typical of sensory cells, or through a response of the protein complexes found at focal adhesions or cellular junctions. Much of the work done on the signaling ability of cells at focal adhesions has looked at the specific molecular composition of these complexes. There have been almost 200 proteins identified as components of focal adhesions to date, with approximately half acting as binding proteins and the other half acting as regulators of the binding proteins (23). The main component of focal adhesions are integrins, which act as both inside-out and outside-in signalers. Some of the proteins associated with the integrins are signaling
molecules which initiate signaling cascades. These cascades lead to local changes in the cytoskeleton and may eventually lead to global changes in the cell, but the cellular response may take some time.

Cells are able to directly transfer mechanical cues from the cell surface to the nucleus via the cellular cytoskeleton (14). This phenomenon is explained by a cellular model called the “tensegrity” model. The tensegrity model states that a cell, rather than being a homogenous “bag” of viscous cytoplasm surrounded by a plasma membrane, has a prestiffened cytoskeletal structure, relying mainly on actin and myosin, which connects focal adhesions at the plasma membrane to the nuclear lamina by various adapter proteins. Because of the rigid state of the cytoskeleton, mechanical signals received at focal adhesions can be transmitted directly to the nucleus far more quickly than is able to be achieved by chemical signaling (80).

Direct nuclear signaling may be able to effect cellular changes more quickly than chemical signaling because the direct contact with the nucleus allows nearly instant transduction of signals. The lamins of the nucleus not only give the nucleus structure, but also interact with chromatin and DNA (80), either on their own or through binding to other effector proteins. The result is that transduction of a mechanical signal from the plasma membrane to the nucleus could have an immediate effect on chromatin modification and transcriptional regulation. A mechanical tugging on the nuclear lamins could also effect changes via the distortion of nuclear pore complexes. This distortion could allow or exclude proteins from the nucleus in a selective manner, providing another pathway for signal transduction. Mediating the nucleocytoplasmic shuttling of
developmentally important transcriptional regulators is one mechanism by which mechanical signals direct cellular processes. The Myocardin-related transcriptional regulators, Serum Response Factor (SRF), YAP/TAZ, β-catenin and SMAD signaling molecules have all been implicated in mechanotransduction (68). Interestingly, these transcriptional regulators have important roles in directing stem cell differentiation and thus may mediate stem cell activity in response to mechanical signals.

Differentiation is the process whereby unspecialized cells become more specialized. This occurs during development as well as in tissue regeneration and wound healing. The end result of this process is a cell dedicated to performing a particular function. It is well documented that MSCs are capable of differentiating into cells of the osteogenic, adipogenic and chondrogenic lineages. The possibility of MSCs differentiating into other cell types, such as neurons and myocytes, has been suggested but not definitively proven. MSC differentiation can be induced in vitro using either chemical or mechanical means. MSCs plated and grown to confluence can be induced to differentiate with specific cocktails of chemicals (7,40,55,89). For osteogenesis, confluent MSCs are incubated with a mix of ascorbic acid, β-glycerophosphate, and dexamethasone for 18-21 days. Adipogenic differentiation is induced with a cocktail of dexamethasone, insulin, isobutyl methyl xanthine and indomethacin (9). Chondrogenesis requires centrifugation of the MSCs, followed by culture of the resulting micropellet with transforming growth factor β (TGFβ) 1 or 3 and dexamethasone (9,38).

Alternatively, many mechanical stimuli have been shown to induce differentiation in MSCs (figure 1). Differences in the stiffness of the matrix that MSCs are plated on
can induce them to differentiate, with softer matrices directing cells toward adipogenesis (0.7-1kPa), chondrogenesis (~4kPa) or myogenesis (~10kPa) and a harder matrix directing cells toward osteogenesis (~40kPa) (18,19,41,97). The ability of MSCs to detect matrix stiffness has been linked to myosin II at focal adhesions (18,69). Shearing forces have also been shown to effect MSC differentiation, with greater force steering cells toward bone formation and less force directing them toward fat formation (98). More recently, it has been shown that MSC differentiation programs respond as much to changes in shearing forces as to the force itself (47,50). The amount of cell spreading and cell shape likewise appears to impact MSC cell fate decisions, with a lesser amount of spreading inducing adipogenesis while greater spreading promotes osteogenesis (18,21). The effects of cell shape on MSC differentiation appears to be modulated by RhoA, with greater RhoA activity driving osteogenesis and RhoA inhibition leading to adipogenesis (53). Recent evidence also describes a role for the focal adhesion protein vinculin as an initiator of mechanotransduction via a MAPK1 binding site that can be exposed by stress from the ECM (22,32).

**Figure 1:** Localization of YAP and TAZ in relation to cell shape and matrix stiffness in mesenchymal stem cells. ECM stiffness can drive localization of YAP and TAZ, which in turn can be a determinant of cellular differentiation.
MSCs from diverse sources appear to have varying differentiation potentials. In general, differentiation of tissue-specific stem cells are more effective when differentiating toward the tissues from which they originated (60). The greatest adipogenic potential was seen in MSCs derived from adipose tissue, while the greatest osteogenic or chondrogenic potential was seen in MSCs derived from bone marrow or synovial tissue, respectively.

The Hippo Pathway

A prerequisite for the development of multicellularity is the ability to control cell proliferation, differentiation and apoptosis. These processes must be coordinated during development and adult tissue maintenance in order to ensure proper functioning of the organism. Dysregulation of these processes lead to a number of diseases, notably developmental syndromes and cancer.

Despite the wide variety of cell types and tissue structures present across the animal kingdom, the developmental processes that cultivate these differences are controlled by relatively few signaling pathways (58). These pathways include Hedgehog, TGFβ, Wnt, JAK/STAT, RTK, Notch and Hippo.

The Hippo pathway was originally elucidated in Drosophila melanogaster in screens for tumor suppressor genes. Components of the pathway were identified by mosaic screens for tissue overgrowth phenotypes. The pathway was later characterized as a major regulator of organ size in development, with defects in the various pathway
constituents resulting in tissue overgrowth due to increased cellular proliferation. Further studies identified the pathway in mammals and confirmed that the pathway is conserved in most multicellular organisms. That the pathway has an important and longstanding place in evolutionary history is implied by the identification of pathway constituents in some of the earliest multicellular creatures (31) and in the unicellular ancestors of these animals (66).

The Hippo pathway is a highly conserved kinase cascade signaling pathway regulated by cell-cell and cell-ECM interactions and cell polarity. In Drosophila, activation of the pathway results in phosphorylation and nuclear export of Yorkie (Yki), a transcriptional regulator. Pathway stimulation occurs when the Ste20 family protein kinase Hippo (Hpo) is activated; activated Hpo then phosphorylates the adaptor proteins Salvador (Sav) and Mats, which bind to Hpo and the nuclear Dbf-2-related (NDR) family protein kinase, Warts (Wts), respectively. These complexes facilitate the phosphorylation and activation of Wts. Wts is then able to phosphorylate Yki, sequestering it in the cytoplasm and preventing its interaction with Scalloped (Sd), a transcription factor that requires Yki as a coactivator. The end result of Hippo pathway activation is inhibition of cell proliferation and promotion of apoptosis.

Upstream activators of the Drosophila Hippo kinase pathway include Neurofibromatosis type-2 (NF2)/Moesin-Ezrin-Radixin-like protein (Merlin; Mer) and Expanded (Ex). Both are FERM-domain containing adaptor proteins; these domains are known in proteins that act as links between transmembrane proteins and the cellular cytoskeleton (10). Investigations into the cellular location of these proteins indicate that
they do, indeed, localize to the cell membrane at adherens junctions (6,44). Another protein associated with the Mer/Ex complex is Kidney and Brain Expressed Protein (Kibra), also called WW domain-containing protein 1 (WWC1), which appears to promote the Mer/Ex interaction. Interestingly, Kibra has also been reported to interact directly with Wts and to be required for phosphorylation of Yki on S168, though it is not required for Wts/Yki binding. (24). A further component of the upstream regulation for the Hippo pathway in *Drosophila* is the protocadherin Fat, which localizes to the apical cell membrane, is known to be required for the stability of Ex and may act in parallel to Mer (83).

The core components of the *Drosophila* Hippo pathway are highly conserved in mammals: mammalian sterile 20–like kinase-1 and 2 (Mst1 and Mst2) are redundant homologs of Hpo; Salvador homolog 1 (Sav1 or WW45) is the Sav homolog; Mps One Binder kinase activator-like 1A and 1B (Mob1A and Mob1B, jointly known as Mob1) are homologs of Mats; Large Tumor Suppressor Kinase 1 and 2 (Lats1 and Lats2) are Wts homologs; and Yamaguchi 73 and Esh avian sarcoma virus (Yes) – associated protein (YAP, also called YAP1) and its paralog Transcriptional Co-activator with PDZ-binding motif (TAZ, also known as WW domain-containing transcription regulator 1, referred to as WWTR1) are Yki homologs. The upstream regulators Mer and Kibra are also found in mammalian cells. Several of these mammalian pathway components (Mst2, Mob1, Lats1 and YAP) are capable of functionally rescuing the knockout of their homolog in *Drosophila* (17).
Hippo pathway regulation in cells is complex. Cell-contact inhibition of proliferation was one of the first activators of the Hippo pathway to be recognized. This is accomplished through the protein components of tight junctions and adherens junctions. Complexes that regulate apicobasal cell polarity, such as the Crumbs (Crb) complex, also seem to have an effect on the Hippo pathway (77), as do the dynamics of the actin cytoskeleton. Additionally, Mer and Kibra have been linked to the Hippo pathway as upstream effectors, though the exact mechanism remains unclear. Recently identified regulators of the Hippo pathway are G-protein-coupled receptors (GPCRs). Various GPCRs have been shown to modulate Hippo signaling (90), with diverse stimuli resulting in activation of differing GPCRs that either inhibit or activate the Hippo pathway.

Gene targets of the mammalian Hippo pathway differ from those of the Drosophila pathway. In Drosophila, cycE, diap1 and bantam are well-characterized genes that are directly induced by Yki (93). These genes contribute to the tissue overgrowth phenotype by which the pathway was originally identified. However, in the mammalian pathway, cycE is not induced by YAP/TAZ and the diap1 homologs, Bric5 and Bric2 are only induced depending on the cellular context. A homolog of bantam has not been identified in mammals. The best characterized gene targets of the mammalian Hippo pathway are those whose transcription is controlled by the TEA domain (TEAD) family of transcription factors, among them Connective Tissue Growth Factor (CTGF), β2 Integrin, Amphiregulin (Areg) and Baculoviral IAP Repeat Containing 5 (Birc5) (52).
*Drosophila* Sd belongs to this family, and in mammals, the transcription factors activated are TEADs 1 through 4.

The mammalian Hippo pathway (figure 2) has largely been characterized in terms of its role in cell proliferation and apoptosis. Mammalian MST1/2 has been shown to be activated by Caspase 3 cleavage in response to pro-apoptotic stimuli (26,45,62), with the cleaved fragment translocating to the nucleus and phosphorylating histone H2B and FOXO3 (46). The activation of histone H2B results in condensation of the chromatin, DNA fragmentation and finally apoptosis. This cleavage was shown to be a general feature of the apoptotic response in many cell types. Curiously, the *Drosophila* Hpo kinase lacks the Caspase cleavage site identified in MST1, indicating this kinase is an unlikely target for Caspases (84).

The activated Hippo pathway results in the phosphorylation, and thus inactivation and cytoplasmic retention, of the Hippo pathway effectors, TAZ and YAP. When active, these effectors act as transcriptional co-activators, binding to transcription factors such as the TEAD family and initiating transcription of pro-proliferation genes. In this way, TAZ and YAP promote cell proliferation and direct cells away from apoptosis.
Figure 2: The Mammalian Hippo Pathway. The core pathway is highly conserved and consists of MST1/2, LATS1/2 and TAZ/YAP. Inputs from multiple sources are capable of stimulating the pathway, which results in phosphorylation and inhibition of TAZ and YAP.

In addition to its role in cell proliferation and apoptosis, the Hippo pathway appears to crosstalk with many other signaling pathways, including Wnt (2,37,43,76), TGFβ/SMAD (77), Notch, Hedgehog (93) and c-Jun N-terminal kinase (JNK) stress-activated protein kinase (SAPK) (12). Crosstalk with the Wnt pathway is achieved via
the Hippo pathway effector TAZ, which, when phosphorylated, is able to bind to the Wnt pathway protein Disheveled (DVL), preventing its phosphorylation and inhibiting the downstream activation of the Wnt pathway effector β-catenin, a transcriptional co-activator for Wnt target genes (76).

MST2 colocalizes with filamentous actin in the cellular cytoskeleton. Disruption of the actin cytoskeleton causes activation of MST1/2, which subsequently activates the JNK SAPK pathway (12), coupling Hippo pathway activation to the dynamics of the actin cytoskeleton. Moreover, Mer, a known upstream regulator of the Hippo pathway, has been found to bind to F-actin, which sequesters it from interaction with Wts (88). In turn, this interaction prevents Mer from activating Wts and inhibits the phosphorylation of TAZ and YAP. Mer binding to F-actin may provide another mechanism for relaying signals into the cell via the Hippo pathway in response to cytoskeletal alterations.

A recently described upstream regulator of the Hippo pathway is G-protein-coupled receptors (GPCRs). GPCRs are activated by binding of extracellular ligands to the receptor, which then relay signals into the cell via their associated G proteins. It has recently been demonstrated that stimulation with serum-borne lysophosphatidic acid (LPA) or sphingosine 1-phosphate (S1P) leads to activation of Gα12/13 GTP-binding proteins, which then inhibit LATS1/2, instigating nuclear localization of the Hippo pathway effectors TAZ and YAP (90). In contrast, stimulation with the hormones glucagon or epinephrine caused an increase in phosphorylation of TAZ and YAP by LATS1/2, decreasing their transcriptional coactivator activity (90).
Hippo Pathway Effectors: TAZ and YAP

YAP and TAZ are homologs of the Drosophila Hippo pathway effector Yki. Sudol originally identified YAP in the chicken via its interaction with the Src homology domain 3 (SH3) of Yamaguchi 73 and Esh avian sarcoma virus (Yes) (71). The following year, Sudol et al. reported the discovery of YAP in both humans and mice (72). It was later reported that humans and mice harbor multiple isoforms of YAP.

TAZ was first identified via a screen for 14-3-3 protein binding partners (42). It was characterized as a paralog of YAP that also functioned as a transcriptional co-activator. TAZ was also described as binding to a PDZ domain containing protein, NHERF-2, that couples plasma membrane proteins to actin in the cytoskeleton, implicating a role for TAZ in mechanotransduction.

TAZ and YAP share certain structural domains (figure 3), among them a TEAD binding site, a transcriptional activation domain (TAD), a PDZ-binding domain at the C-terminus, a 14-3-3 binding site and WW domains, as well as a coiled coil region. TAZ is the smaller of the two proteins, though both proteins encompass isoforms having either one or two WW domains (82). Additionally, YAP contains a proline rich region at the N-terminus and an SH3 binding domain that TAZ lacks.
Figure 3: The Hippo Pathway Effectors YAP and TAZ. Homologous transcriptional regulators YAP and TAZ share many features, including WW domains, TEAD binding domains, a PDZ-binding site and a transcriptional activation domain encompassing the coiled-coil region of the protein. Additionally, YAP has an N-terminal proline-rich region, an SH3 binding site and a PPXΦP motif. Both proteins contain multiple regulatory phosphorylation sites.

The various protein binding domains present in TAZ and YAP provide a mechanism for regulation of their localization and activity. The WW domains of TAZ and YAP bind to a PPxY (proline/proline/any amino acid/tyrosine) motif; many of the proteins containing this PPxY motif are known to be interactors of TAZ and YAP. In addition to the WW domains, TAZ and YAP may be regulated by binding to other proteins through the PDZ-binding motif or the 14-3-3 binding site. PDZ motif-containing proteins are frequently transmembrane proteins or associated with the cytoskeleton. This motif and the WW domains mediate binding to cell polarity complexes such as Crumbs (77), coupling cell density and cell polarity to the Hippo pathway. 14-3-3 binding is induced by phosphorylation of TAZ or YAP on Ser89 (in human TAZ) or Ser127 (in human YAP) and results in cytoplasmic retention of TAZ and YAP. This phosphorylation is carried out by LATS1/2 through activation of the Hippo
pathway; 14-3-3 binding is one of the major regulatory mechanisms for TAZ and YAP. Cytoplasmic retention of TAZ and YAP prevents their interaction with nuclear transcription factors.

LATS1/2 phosphorylates TAZ at four sites (Ser66, Ser89, Ser117 and Ser311) and YAP at five sites (Ser61, Ser109, Ser127, Ser164 and Ser397). Ser89 (for TAZ) and Ser127 (for YAP) are the most critical of these residues for inhibition and cytoplasmic retention (28,95). LATS1/2 phosphorylation of TAZ and YAP on Ser311 and Ser397, respectively, promotes further phosphorylation by CK1ε/δ kinases on Ser300 in TAZ and Ser400 or Ser403 in YAP (75). Modification of the proteins in this way recruits the βTrCP/SCF ubiquitin ligase. This leads to ubiquitination and proteasomal degradation of TAZ and YAP (75,93). TAZ and YAP are also phosphorylated by c-ABL on Tyr321 and Tyr 407, respectively. These modifications result in changed nuclear activity for each: YAP phosphorylation by c-ABL in response to DNA damage stabilizes YAP and stimulates the expression of proapoptotic genes, whereas c-ABL phosphorylation of TAZ in response to hyperosmotic stress results in a physical interaction with nuclear factor of activated T cells 5 (NFAT5), and a resultant inhibition of the activity of this transcription factor.

Unshared mechanisms of regulation for TAZ and YAP also exist. TAZ is reported to be phosphorylated on Ser58 and Ser62 by glycogen synthase kinase 3β (GSK3β), also targeting TAZ for degradation, while no interaction of GSK3β with YAP has been described (36). Likewise, homeodomain-interacting protein kinase (HIPK2) has
been identified as a YAP stabilizer, promoting its nuclear activity (61) while interactions with TAZ have not been defined.

The TEAD family of transcription factors are major targets of TAZ and YAP. They are evolutionarily conserved proteins and orthologs of TEAD transcription factors can be found in all eukaryotes, from single-celled organisms to vertebrates. TEADs play a crucial role in development, from early embryonic development to regulation of organ size and tissue growth in the later embryo. They do this through transcription of genes that control cell proliferation, cell fate, cell migration and adhesion and apoptosis. Both TAZ and YAP contain a TEAD-binding domain at the N-terminus of the proteins, though YAP’s TEAD-binding domain contains a PxxΦP (Φ is a hydrophobic residue) that is lacking in the TEAD-binding domain of TAZ. This lack may be the basis for differential activation of TEAD target genes by TAZ and YAP.

Aside from the TEADs, numerous other transcription factors have been identified as binding partners of TAZ and/or YAP. They share binding to a few transcription factors, including SMAD1/2/3 and RUNX1/2, but they also have binding partners that are unique to each of them (52). YAP binds p63, p73 and EGR1, while binding partners unique to TAZ include PPARγ, SMAD4 and PAX3/8. Additionally, TAZ and YAP are able to form dimers with themselves and each other to interact with other transcription factors.

Though TAZ and YAP have largely been characterized for their roles as coactivators, there is evidence that they may also act as transcriptional repressors. YAP may be recruited by RUNX2 to inhibit transcription of osteocalcin (OCN) (91), while
TAZ is known to bind to PPARγ to inhibit expression of fatty-acid binding protein αP2 (FABPαP2) (33).

Transcriptionally active TAZ and YAP are nuclear, while the phosphorylated proteins are cytoplasmic. However, there is a role for cytoplasmic TAZ and YAP as regulators of other signaling pathways. An example of this is the aforementioned part that phosphorylated TAZ plays in inhibiting the Wnt pathway through interaction with DVL. Phosphorylated TAZ and YAP are also available to interact with the effectors of the TGFβ pathway: cytoplasmic YAP acts as a corepressor with SMAD7 to inhibit SMAD signaling, while YAP and TAZ binding to SMAD2/3/4 control cytoplasmic shuttling of these transcription factors.

It has recently been shown that TAZ and YAP regulate expression of secreted proteins, indicating a role for Hippo signaling in non-cell autonomous functions (28,92). YAP was shown to induce the expression of AREG, leading to the activation of epidermal growth factor receptor (EGFR) signaling. EGFR signaling induces cell proliferation and migration. Interestingly, YAP can also induce expression of negative regulators of apoptosis, including BIRC5/surviving and BIRC2/cIAP1, which are members of the inhibitor of apoptosis (IAP) family and homologs of the Drosophila diap1 gene (15).

Cell-contact inhibition was one of the first regulators of TAZ and YAP to be identified. Zhao et al. (95) showed that cell-cell adhesion powerfully restricts TAZ and YAP from the cell nucleus. Cells that express TAZ and YAP mutants that cannot be phosphorylated do not respond to cell-cell contacts and continue to proliferate.
Establishment of cell polarity is also regulated by cell-cell contacts. Cellular components that control epithelial cell polarity have recently been shown to have interactions with Hippo pathway components, including TAZ and YAP. The Crumbs complex of proteins is a major factor in establishing the apical domain of epithelial cells, while Scribble establishes the basal-lateral domain. Members of the Crumbs complex, in particular the AMOT family of proteins, have been shown to interact with TAZ and YAP and to direct their cellular localization (77). AMOTL2 inhibits translocation of YAP to the nucleus, possibly by stabilizing complexes of MST1/2, LATS1/2 and YAP and increasing YAP phosphorylation (81,94). In contrast, the p130-AMOT isoform is able to bind to YAP in the cytoplasm and blocks its phosphorylation by LATS1/2. Additionally, p130-AMOT appears to act as a cofactor with the YAP-TEAD complex in the nucleus, helping to regulate a subset of YAP target genes (87). Scribble appears to inhibit TAZ nuclear localization in much the same way that AMOTL2 inhibits YAP (11).

Along with cell-contact inhibition, more recent evidence linking TAZ and YAP localization to changes in the actin cytoskeleton and matrix stiffness indicate a role for TAZ and YAP in the transduction of mechanical signals from outside the cell to the cell nucleus. There is some evidence that the signals from GPCRs are transmitted to TAZ and YAP via Rho-GTPases (90). Rho-GTPases are a group of GTPases that effect the actin cytoskeleton. They induce the activation of Rho-kinase (ROCK), which promotes nuclear TAZ and YAP.

Dupont et al. (16) showed that a soft matrix of 0.7 to 1kPa reduced cell spreading and promoted cytoplasmic retention of TAZ and YAP, while a stiff 40kPa matrix
promoted cell spreading and induced translocation of TAZ and YAP to the nucleus. These differences in relation to the stiffness of the ECM influence cell fate decisions in mesenchymal stem cells (18), with a stiff matrix and nuclear TAZ and YAP leading to osteogenesis and a soft matrix and cytoplasmic TAZ and YAP leading to adipogenesis (33). Chondrogenesis also occurs in response to a softer matrix (approximately 4kPa) and cytoplasmic TAZ and YAP (97). Myogenesis is directed by a slightly firmer matrix of approximately 10kPa, though TAZ and YAP localization has not been characterized for this line of MSC differentiation. Interestingly, regulation of localization of TAZ and YAP via mechanical cues seems to be independent of the LATS1/2 kinases and the canonical Hippo pathway (16).

The Hippo pathway and TAZ and YAP have been shown to have important roles in embryonic development and stem cell regulation. It appears that changes in TAZ and YAP localization are required for the first cell fate decisions that occur in the blastocyst. At the onset of cell compaction and the formation of junctions between cells at the 8-cell stage of the embryo, the innermost cells, being more compacted, lose polarity. This change causes a re-localization of TAZ and YAP from the nucleus to the cytoplasm and results in differing cell fates, with the outer cells, having nuclear TAZ/YAP, becoming the trophectoderm, while the inner cells, with cytoplasmic TAZ/YAP, become the cells of the inner cell mass. TAZ/YAP knockout results in defects of cell fate determination and embryonic death prior to implantation (57); however, deletion of either TAZ or YAP alone does not show the same phenotype. YAP-null mouse embryos show defects in yolk sac vasculogenesis, chorioallantoic fusion, and elongation of the body, resulting in
embryonic lethality at day ~8.5 (56). TAZ-null mice, however, are characterized by renal
cysts and kidney and lung defects and minor skeletal defects (34,51). TAZ-null mice
survive birth, though only about one in five live to adulthood. Interestingly, YAP
knockout in the zebrafish (Danio rerio) also results in defects of axis formation and
elongation and a reduction in the size of the head as well as a delay in key developmental
events (35).

TAZ and YAP also have important roles in stem cells and YAP, in particular, has
been shown to be highly expressed in mouse embryonic, neural and hematopoietic stem
cells (63). Studies have shown that TAZ/YAP activity is required to maintain the
pluripotent state of stem cells. Stimulation of the TGFβ pathway activates the
transcription factors SMAD2/3, which form complexes with TAZ and YAP (77). These
complexes bind to TEADs and OCT4 (a core regulator of stem cell pluripotency) to
maintain the pluripotent state (3). When the cell receives signals for differentiation, the
complexes are disassembled with the assistance of the transcription factor Forkhead Box
H1 (FOXH1) (4).
METHODS

Cell culture and cell sources

The mouse embryonic fibroblast cell line, C3H/10T1/2 and human embryonic kidney 293T (HEK 293T) cells were obtained from American Type Culture Collection.

Mouse C3H/10T1/2 cell culture

Mouse C3H/10T1/2 cells were cultured in high-glucose Dulbecco’s Modified Eagle Medium (DMEM; Corning CellGro), supplemented with 10% fetal bovine serum (FBS; Thermo Scientific) and 1% penicillin/streptomycin (GE Lifesciences) in a humidified incubator, in the presence of 5% carbon dioxide (CO₂) at 37°C. Aliquots of the cells were also cryopreserved in freezing medium by the protocol described below for later use.

Cells were passaged at sub-confluence. To passage, medium was aspirated from the tissue culture plates, cells were rinsed once with PBS and were detached from the plate via incubation with trypsin (0.25 % trypsin, 1 mM EDTA; GE Lifesciences) for 3 to 5 minutes at room temperature in the tissue culture hood. Cells were then collected by the addition of complete culture medium and passaged. Passage of the cells was performed every 2-3 days.

Cells were counted as described below and plated as described in the differentiation protocols. The cell concentration was determined by counting the number of cells/1mm² in a hemacytometer. To improve accuracy, a minimum of 100 cells were counted in 3 to 5 1mm² squares and the number of cells were averaged per 1mm². This
number was then multiplied by $10^4$ to obtain the number of cells/ml of the original sample. Finally, the total number of cells was determined by multiplying the number of cells/ml with the volume of the medium (in ml) in which cells were resuspended.

**Human embryonic kidney 293T (HEK 293T) cell culture**

The human embryonic kidney 293T (HEK 293T) cell line was used for the packaging of the lentiviral vectors listed previously: pLVX-TP-Ctrl (vector with control insert), pLVX-TP-3F YAP2A WT (wildtype YAP2A), pLVX-TP-3F YAP2A 5SA (constitutively nuclear 5 serine-to-alanine phosphorylation mutant YAP2A), pLVX-TP-3F YAP2A 5SA/S94A (constitutively nuclear 5 serine-to-alanine phosphorylation mutant with mutation in the TEAD-binding domain YAP2A), pLKO1-puro shCtrl (vector with control hairpin), pLKO1-puro shTAZ m (mouse TAZ), pLKO1-puro shYAP m67 (mouse YAP) (see below for packaging of lentiviruses).

Cells were grown in high-glucose DMEM (Corning CellGro) supplemented with 10% FBS (Thermo Fisher), and 1% penicillin/streptomycin (GE Lifesciences) and incubated in a humidified incubator at 37°C in the presence of 5% CO$_2$. Cells were passaged every 2-3 days.

**Cell cryopreservation and defrosting**

Cells were collected by trypsin treatment as described above and placed in 15ml Falcon tubes. They were then centrifuged at 900 rpm for 5 minutes at 4°C. The medium was then aspirated and the cell pellet was resuspended in freezing medium consisting of culture medium containing 20% FBS (Thermo Fisher) and 10% DMSO (American
Bioanalytical. This cell suspension was then aliquoted into cryotubes (Corning Bioscience), and stored at -80°C in a styrofoam rack to allow a cooling rate of 1°C per minute.

Cells were thawed rapidly in a waterbath set at 37°C. Immediately after defrosting, cells were transferred to a tissue culture plate containing 9.5 ml of pre-warmed cell culture medium and incubated in a humidified incubator at 37°C in the presence of 5% CO₂.

**Mouse YAP2A overexpression.**

**Plasmids.**

Plasmids used for YAP overexpression in the C3H/10T1/2 cell line were as following: pLVX-TP-Ctrl, pLVX-TP-3F YAP2A WT, pLVX-TP-3F YAP2A 5SA, pLVX-TP-3F YAP2A 5SA/S94A.

**Mouse TAZ and YAP knockdown.**

Plasmids used for TAZ and YAP knockdown in the C3H/10T1/2 cell line were as following: pLKO1-puro shCtrl, pLKO1-puro shTAZ m, pLKO1-puro shYAP m67

All plasmids were made by previous members of the lab.

**Transformation of plasmids to NEB5α competent cells.**

Transformation of plasmids into NEB5α competent cells (New England Biolabs) by heat-shock was performed as follows.
Competent cells were removed from the -80°C freezer, placed immediately on ice, and allowed to thaw for 2 to 5 minutes. The cells were gently pipetted and 25 μl aliquots were placed in chilled microfuge tubes. One microliter of purified plasmid DNA of any concentration (20 to 100 ng/μl) was added to the competent cells, gently mixed with the tip of the pipette, and incubated on ice for 10 minutes. The tubes were then heated to 42°C for 50 seconds in a water bath, and were subsequently placed on ice for 2 minutes. The full volume of the transformation mixture was then plated on pre-warmed (at 37°C) Luria-Bertani (LB) agar plates with appropriate antibiotic selection, and incubated overnight at 37°C. The following morning, single colonies were picked and inoculated to a starter culture of 5 ml of LB plus the appropriate antibiotic, which was incubated for 8 hours at 37°C in a horizontal shaking incubator at 270 rpm.

Plasmid DNA purification.

Plasmid purification from transformed cells was performed using the Zymo Research ZR Plasmid Miniprep™ - Classic kit (Zymo Research), according to the recommendations of the manufacturer.

After bacterial transformation as described above, the 5ml cultures were centrifuged at 3,000 x g for 10 min at 4°C, and the supernatant was aspirated from the pellet. The pellet was then resuspended in 200 μl of Buffer P1 by vortexing and moved to a microfuge tube. Two hundred microliters of Buffer P2 were then added, mixed thoroughly by vigorously inverting the sealed tube 4 to 6 times, and incubated at room temperature (RT) for 1-3 min. Then, 400 μl of chilled Buffer P3 were added to the lysate,
mixed thoroughly by vigorously inverting 4 to 6 times, and incubated at room
temperature for 1 min. The tubes were then centrifuged at 15,000 rpm for 3 min. And the
supernatant removed to a spin column. The spin column was centrifuged at 15,000 rpm for
30 sec. and the flow through was discarded. The column was washed with 200 µl of
Endo-wash buffer and spun again at 15,000 rpm for 30 sec. The flow through was again
discarded and the column washed again with 500 µl of wash buffer. After an additional
spin at 15,000 rpm for 2 min., the column was removed to a new microfuge tube. One
hundred microliters of dH₂O was added to the column. The column was incubated at
room temperature for 1 min., then the DNA was eluted from the column by centrifugation
at 15,000 rpm for 30 sec. The quantity and quality of each plasmid preparation was
determined using a NanoDrop 2000C spectrophotometer (Thermo Scientific).

**Transfections.**

**Transient transfection of mouse C3H/10T1/2 cells for the study of mouse TAZ and
YAP2A knockdown.**

Mouse C3H/10T1/2 cells were transiently transfected with siRNA using
Dharmafect 1 Transfection Reagent (GE Lifesciences) according to the recommendations
of the manufacturer.

Cells were plated in 6–well plates, containing antibiotic-free medium (2 ml/well),
at a density of approximately 100,000 cells per well, and allowed to attach. At the time of
the transfection, the cells were approximately 30 to 50% confluent. For each well, 1 µM
of siRNA was mixed with 3 µl of Dharmafect reagent, with the mixture diluted to 200 µl
total in Opti-mem (Life Technologies), and mixed by pipetting. Samples were incubated for 20 min at room temperature and the full 200 µl of the transfection mix was added to each well. Cell culture medium was changed after 24 h.

**Lentiviral (LV) production**

HEK293T cells were used to package the lentivirus in order to infect the C3H/10T1/2 cells.

293T cells were plated in 6-well plates at a density of approximately 100,000/well in regular cell culture medium composed of high glucose DMEM (Corning CellGro) supplemented with 10% FBS (Thermo Scientific) and 1% of penicillin/streptomycin (GE Lifesciences). Cells were allowed to attach and were subsequently transfected with TurboFect transfection reagent (GE Lifesciences) according to the recommendations of the manufacturer.

Twenty-four hours post-transfection, the medium in the wells was replaced with 2 ml of fresh virus medium containing high glucose DMEM (Corning CellGro) supplemented with 10% heat-inactivated FBS (Thermo Scientific). Forty-eight hours after this media change, the medium containing LV particles was collected. Harvested media was filtered through a 0.45 µm syringe filter (EMD Millipore) to remove cell debris. Viral supernatant was then aliquoted and stored at -80°C, until needed. Virus was made using the previously described plasmids as well as the packaging plasmids ps-PAX2 and pCMV-VSV-G.
**Lentiviral (LV) transduction of C3H/10T1/2 cells.**

C3H/10T1/2 cells were transduced with lentiviruses (LVs). First, a TET-ON virus was transduced to create a stable cell line with a doxycycline inducible-expression cassette, then virus for overexpression of wildtype and mutant forms of mouse YAP2A (as described above) was transduced into the stable TET-ON cells.

Cells were plated in 6-well tissue culture plates (Falcon™ Becton Dickinson), at a density of 100,000 cells/well, in a medium (2 ml/well) composed of DMEM (Corning CellGro) supplemented with 10% FBS (Thermo Scientific) and 1% penicillin/streptomycin (GE Lifesciences) and were allowed 2 to 4 h to attach. Aliquots of viral supernatants (described previously) were thawed and the entire aliquot was added to the well. Twenty-four hours after infection, cells were trypsinized and moved to 10 cm dishes (BD Falcon). Forty-eight hours after infection, media was replaced with selection media. Selection ran until all cells in a control plate had died. Transduced cells were maintained in selection media. Cells were also expanded and frozen down as above for stocks.

**Cell differentiation assays**

Cells used for these experiments had been stably transduced with YAP2A mutant overexpressing or TAZ and YAP2A knockdown lentiviruses, or had been transiently transduced with TAZ and YAP knockdown lentiviruses or siRNA.
In vitro adipogenesis assay of C3H/10T1/2 cells.

The in vitro adipogenesis assay with C3H/10T1/2 cells for brown fat differentiation was performed as described by McDonald, et. al. 2015(54). Cells were collected as for passaging and counted as described above. Cells were then plated at a concentration of $1.64 \times 10^5$ cells per well in 6-well plates and allowed to attach for 1 hour. Cells were then treated with BMP7 (Peprotech) at a concentration of 1.25pM for three days. Doxycycline-inducible cells were also treated with doxycycline (Clontech) at 0.5 μg/ml at the same time. After treatment with BMP for 72 h, the medium was changed to an adipogenic induction medium, consisting of growth medium supplemented with 5 μM dexamethasone (Sigma), 52 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma), 5 μg/ml insulin (Sigma, USA), 2 mM T3 (Sigma) and 12.5 mM indomethacin (Sigma).

After 24 h, the adipogenic induction medium was replaced with adipogenic maintenance medium, consisting of growth medium supplemented with 2.5 μg/ml insulin (Sigma, USA) and 2 mM T3 (Sigma). The cells were maintained in this media for a total of 7 days, with media being refreshed every 2-3 days. On day 8, cells were harvested for RNA, protein and staining.

In vitro osteogenesis assay of mouse C3H/10T1/2 cells.

The in vitro osteogenesis assay with C3H/10T1/2 cells was adapted from the protocol described by Carroll et al. 2012(8). Cells used for this experiment were previously transduced with the plasmids described above. Cells were collected as for passaging and counted as described above. Cells were then plated at a concentration of $1.64 \times 10^5$ cells per well in 6-well plates and allowed to attach for 1 hour. Cells were then
treated with BMP7 (Peprotech) at a concentration of 1.25pM for three days. Doxycycline inducible cells were also treated with doxycycline (Clontech) at 0.5 μg/ml at the same time.

After 72 h of BMP treatment, the media was aspirated from the plates and cells were washed one time with PBS, then the osteogeneic differentiation media was added. This media consisted of growth medium supplemented with L-ascorbic acid (Sigma) at 70 ng/ml, β-glycerophosphate (Sigma) at 8 mM and dexamethasone (Sigma) at 10 ng/ml. Osteogenic differentiation was allowed to run for 18 days, with media being refreshed every 2-3 days. On day 18, cells were harvested for RNA, protein and staining.

**Histological staining.**

**Oil red O staining.**

Oil red O staining protocol was adapted from Wu, et. al. 1998(85). Briefly, staining was performed to stain intracellular lipid vacuoles following the *in vitro* adipogenesis assay of C3H/10T1/2 cells as described previously. After 8 days of differentiation, cells were washed twice with Ca++- and Mg++-free PBS and were fixed with 1 % PFA (Electron Microscopy Sciences) in PBS for 30 min at room temperature. Fixing solution was removed, plates were washed two times with PBS and incubated with Oil red O working solution (preparation described in appendix 1) for 60 min at room temperature. Stain solution was removed. Plates were then rinsed three times with PBS, then left in PBS for imaging. Images were taken using a Nikon Coolpix 4300 digital camera modified for use with a microscope by a Zarf Enterprise LNS-23D/CP4300
microscope adapter lens. Above steps were performed in three empty wells of a 6-well plate to obtain blanks for quantitation.

Oil Red O dye extraction and quantitation protocol was adapted from the protocol described by Ramirez-Zacarias et al. 1992(64). The PBS was aspirated from the plates and the plates were allowed to dry completely. Oil Red O was extracted using 3.6 ml of 100% isopropanol per well of a 6-well plate and incubated at room temperature for 10 min. Extractions were stored in microfuge tubes at 4°C. The absorbance was measured at 500 nm by spectrophotometry (NanoDrop).

**Staining for alkaline phosphatase activity**

Staining for alkaline phosphatase (ALP) activity was performed using the Leukocyte Alkaline Phosphatase kit (Sigma) according to the manufacturer’s instructions. Cells were washed twice with diH\textsubscript{2}O, then fixed for 30 s with fixative solution prepared as per the manufacturer’s instructions from the supplied concentrated citrate solution. After fixing, cells were again washed for 45 s with diH\textsubscript{2}O, then were incubated with the alkaline phosphatase staining solution prepared from dissolved Fast Blue RR Salt and Napthol AS-MX Phosphate Alkaline Solution. Cells were incubated in the dark for 30 m in this solution, then washed for 2 m with diH\textsubscript{2}O. Cells were then counter-stained with hematoxylin for 10 m. The final step was washing of the cells in diH\textsubscript{2}O for 3 m. Cells were then imaged using an inverted, phase contrast light microscope (Nikon TMS) in conjunction with a digital camera (Nikon Coolpix 4300) fitted with a microscope adapter lens (Zarf Enterprise LNS-23D/CP4300). For quantitation of staining, 5 fields were imaged at 20x magnification.
Protein extraction and Bicinchoninic protein assay (BCA).

Cells were rinsed twice with PBS and incubated in 1x protein lysis buffer (described in appendix 1) for 10 min at 4 ºC. Cells were lysed by pipetting and the lysate was transferred to microfuge tubes. Lysate was spun in a refrigerated centrifuge for 10 min at 15,000 rpm and 4 ºC. The lysate was removed from the cell debris and protein was quantified using the Pierce® BCA Protein Assay kit (Thermo Scientific), according to the recommendations of the manufacturer. Pierce® BCA Protein Assay Reagent A was diluted 1:50 in Pierce® BCA Protein Assay Reagent B. Four hundred microliters per sample of this solution was placed in a disposable cuvette (Denville). Twenty microliters of sample was added to the cuvette and the mixture was briefly vortexed. Twenty microliters of IP wash buffer was used as a blank. The cuvettes were then incubated for 20 min at 37°C, and the absorbance was measured at 562 nm by spectrophotometry (NanoDrop). The protein samples were then normalized to the lowest protein concentration to ensure even loading on the gel.

Western blotting.

For Western blotting, samples were prepared such that the amount of protein in each sample was equal to that in 100 µl of the lowest sample concentration. These samples were mixed with 30 µl of 5x sample buffer (see appendix 1), and boiled at 95°C. Samples were allowed to cool and then briefly centrifuged to recover evaporated buffer.

Samples were electrophoresed on either 8% or 10% polyacrylamide gels in 1x running buffer (preparation described in appendix 1) at 200 V for 1 h 30 min. Six microliters of BLUEStain 3 protein ladder (Gold Biotechnology) were included.
Proteins were transferred to nitrocellulose membrane (BioRad) for 1 h 30 min at 100 V, using a wet method in the presence of transfer buffer (described in appendix 1). Membranes were then blocked in TBST (see appendix 1) containing 5% powdered dry milk (Lab Scientific, Inc.) or 5% bovine serum albumin (BSA; Fisher Scientific) (for the pSMAD1/5 antibody) for 1 h at room temperature (RT) on a benchtop shaker.

Membranes were incubated with the primary antibody overnight, on a nutator placed at 4°C. Primary antibody was dissolved in TBST containing either 5% powdered milk or BSA. The following day, membranes were washed three times for 10 min each with TBST. The membranes were then probed with a secondary antibody, which was conjugated to horseradish peroxidase (HRP), for 1 hour at RT. After incubation with the secondary antibody, membranes were washed again three times for 10 min each wash with TBST.

The antibody signal was detected by chemiluminescence, using Super Signal ® West Dura or Femto (both Thermo Scientific) as a substrate, using a ChemiDox XRS+ imager (BioRad) and Image Lab analysis software (BioRad) Version 5.1.

**Gene expression analysis.**

**Ribonucleic acid (RNA) extraction.**

RNA from cells in monolayers was extracted with the RNeasy® Mini kit (Qiagen) according to the manufacturer’s instructions. Flow through was discarded at each step until the final elution. The medium was removed and cells were washed once with PBS. Cells were then lysed in 350 µl of Buffer RLT. The cell and buffer RLT
mixture was passed 4 to 6 times through a 23 G needle to assist lysis. This lysate was then spun in a centrifuge at 15,000 rpm for 3 min to clear cell debris. The supernatant was mixed with 350 µl of 70% ethanol (Pharmaco-Aaper) to precipitate RNA. The mixture was then placed on a spin column and spun at 15,000 rpm for 15 sec. Cells were washed with 700 µl of Buffer RW1, then centrifuged again at 15,000 rpm for 15 sec. The column was washed with 500 µl of Buffer RPE. Following another 15 sec spin at 15,000 rpm, the column was washed a final time with 500 µl of Buffer RPE and spun at 15,000 rpm for 2 min to dry the column. Finally, RNA was eluted from the column with 30 µl of RNase-free water and a 15 sec spin at 15,000 rpm. RNA purity and concentration was checked with the NanoDrop 2000C (Thermo Scientific).

**Reverse transcription - complementary deoxyribonucleic acid (cDNA) synthesis.**

For cDNA synthesis, the iScript Reverse Transcriptase kit (BioRad) was used according to the manufacturer’s protocol. Briefly, 1 µg of RNA was mixed in 8 strip PCR tubes (Fisher Scientific) with 4 µl iScript Reaction Mix and 1 µl iScript Reverse Transcriptase, then the final volume was brought to 20 µl with dH2O. Tubes were placed in a MyCycler Thermal Cycler (BioRad) set with the following program: 5 min at 25°C, 30 min at 42°C and 5 mi at 85°C. The cDNA was then allowed to cool, and the volume brought to 200 µl by adding 180 µl of dH2O. Samples were stored at -20°C. All samples within a single experiment were subjected to cDNA synthesis simultaneously.
Quantitative real time polymerase chain reaction (qPCR).

Quantitative real time polymerase chain reaction (qPCR) was performed in a total reaction volume of 10 μl. Each reaction consisted of 5 μl Fast SYBR-green (Applied Biosystems), 2 μl (1 μM) each of sense and antisense primers and 1 μl complementary deoxyribonucleic acid (cDNA), that was produced as described in section above. The primers used for qPCR were designed with NCBI PrimerBlast software and were all intron-spanning. Each sample was plated in duplicate in wells of a 96-well plate qPCR plate (Life Technologies), which was sealed with clear TempPlate RT Optical Film (USA Scientific) to prevent evaporation or contamination. The plate was quick-spun to concentrate all reagents in the bottom of each well. qPCR was performed in a ViiA7 qPCR thermal cycler (Applied Biosystems) with Viia7 Ruo software, and cyclic conditions were as follows: a pre-incubation step at 95°C for 20 sec, followed by 40 cycles of amplification (95°C for 1 sec, 60°C for 20 sec), and a final melt curve cycle of 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. The relative expression ratio was calculated using the ΔΔC_T method. DNA primers used for Q-RT-PCR are listed in Appendix 2.

Statistical analysis.

Statistical analysis was performed with Microsoft Excel 2013 using the Analysis ToolPak Add-In. Results have been normalized to control. All results are expressed in graphs as mean ± standard deviation from repeats.
RESULTS

INTRODUCTION

TAZ in Mesenchymal Stem Cells

TAZ is known to inhibit adipogenesis, and this role has been proposed to be mediated via interaction and inhibition of the adipogenic master regulator, PPARγ (7); however, all investigations to date have studied TAZ in differentiation of white adipocytes. TAZ’s role in differentiation of brown or beige adipocytes has not yet been clarified. TAZ has also been shown to promote osteoblastic differentiation by binding to and activating Runx2, (40,79). Consistent with a role in osteogenesis, overexpression of TAZ increases bone density in mice (86).

YAP in Mesenchymal Stem Cells

Descriptions of a role for YAP in mesenchymal stem cells have been somewhat contradictory. Roles for YAP in the inhibition of osteogenesis in mesenchymal stem cells have been described. One group suggested that tyrosine phosphorylation of YAP promotes binding to Runx2 on the osteocalcin (OCN) promoter, preventing OCN transcription, and consequently inhibition of osteogenesis (91). Another group proposes that YAP is required in moderate amounts for adipogenesis, with either overexpression or knockdown resulting in inhibited adipocyte differentiation (67). They propose that YAP acts downstream of the Sox2 transcription factors in this context (67). However, others
have found that inhibition of YAP is required for adipogenesis (89). YAP has also been described as being required for the maintenance of “stemness” in stem cells (48,67).

In addition to the specific binding to transcriptional regulators of differentiation, roles have been described for both TAZ and YAP as integrators of other signaling pathways that have been shown to impact differentiation (75,78). These pathways include Wnt, TGF-β, BMP and ECM signaling.

In this chapter, I describe work that was aimed at understanding the roles of YAP and TAZ in mesenchymal stem cell differentiation. While I examined both YAP and TAZ, the role of TAZ in mesenchymal stem cell differentiation is somewhat better understood than that of YAP, and thus many of my experiments focused primarily on YAP.

**YAP knockdown in Mesenchymal Stem Cell Differentiation**

To evaluate the role of YAP in MSC fat differentiation, I performed adipogenic differentiations using a protocol that induces the differentiation of C3H10T1/2 cells into brown fat cells. C3H10T1/2 cells are a multipotent mouse embryo fibroblast cell line capable of forming bone, fat and cartilage, among other tissues (25).

C3H10T1/2 cells that had been stably transfected with shRNA targeting YAP or TAZ were subjected to brown fat differentiation. The YAP knockdown cells showed a significant increase in the amount of lipids produced as compared to control (Figure 4), while TAZ knockdown showed a decrease in lipids. The staining results were confirmed with qPCR to measure expression of adipogenic marker mRNA (Figure 5). These results imply that YAP is inhibiting brown fat differentiation in these cells.
Figure 4 Oil Red O staining of intracellular lipids in knockdown cells. A) Western blot for knockdown of TAZ and YAP. B) Representative images of Oil Red O staining of lipids in shRNA transduced TAZ and YAP knockdown 10T1/2 cells. C) qPCR for mRNA expression of TAZ and YAP. D) Quantification by spectrophotometry of Oil Red O staining of cells in A. *P < .05, **P < .01, ***P < .001

Figure 5 Expression of brown adipogenic markers in knockdown cells. Quantification of mRNA expression of established brown fat markers in the differentiation of shRNA transduced TAZ and YAP knockdown 10T1/2 cells. *P < .05, **P < .01, ***P < .001

I also looked at the osteogenic differentiation capacity of the C3H10T1/2 stable knockdown cells. Knockdown of TAZ in C3H10T1/2 cells exposed to an osteogenic
differentiation protocol reduced the amount of differentiation as assayed by staining for alkaline phosphatase activity (figure 6). In contrast, YAP knockdown increased both the number of cells expressing alkaline phosphatase and the intensity of the staining. qPCR analysis showed no change in the early osteogenic marker, Runx 2, but the YAP knockdown showed a large induction of the later marker, osteocalcin (OCN) (figure 7). YAP appears to inhibit bone differentiation in C3H10T1/2 cells, though this needs to be confirmed as the YAP knockdown was not very strong and this experiment has only been performed once.

Figure 6 Staining for alkaline phosphatase activity in knockdown cells. A) Representative images of staining for alkaline phosphatase activity in shRNA transduced TAZ and YAP knockdown 10T1/2 cells. B) qPCR for mRNA expression of TAZ and YAP. *P < .05, **P < .01, ***P<.001
Interestingly, there was also conspicuous formation of lipid droplets in the YAP knockdown cells subjected to the bone differentiation protocol. There was little to no lipid formation seen in either the control or the TAZ knockdown cells, indicating this effect was due to the knockdown of YAP, rather than an issue with the differentiation protocol. This effect was also seen in all repeats of the experiment, though the lipid formation was only quantitated in one repeat (figure 8). This implies that YAP plays a role in determining stem cell fate and that removal of YAP leads to a confusion of differentiation signals.

Figure 7 Expression of osteogenic markers in knockdown cells. Quantification of mRNA expression of established bone differentiation markers in shRNA transduced TAZ and YAP knockdown 10T1/2 cells.

Figure 8 Oil Red O staining of intracellular lipids in knockdown bone differentiation. A) Representative images of Oil Red O staining of lipids in shRNA transduced TAZ and YAP knockdown 10T1/2 cells subjected to bone differentiation. B) Quantification by spectrophotometry of Oil Red O staining of cells in A.
Bone Morphogenic Protein in Mesenchymal Stem Cell Differentiation

Bone Morphogenic Proteins (BMPs) are known to play a role in MSC differentiation. Interestingly, BMPs, through activation of SMAD signaling, appear to have both pro-adipogenic and pro-osteogenic activities (39) and some studies have shown that BMP signaling specifically promotes adipogenesis (73). However, other studies have shown that BMP signaling must be modulated for the induction of white fat differentiation (27). TAZ and YAP are known to be involved in regulation of BMP signaling.

With this in mind, I hypothesized that observed effects of YAP knockdown in MSC differentiation may be due to altered BMP signaling. I performed Western blots to determine if there were changes in BMP signaling with TAZ and YAP knockdown. I transfected 10T1/2 cells with TAZ and YAP siRNA, then induced the cells with BMP 7. I probed for pSMAD1/5 as an indicator of activation of BMP signaling. pSMAD induction is greatly reduced in the TAZ/YAP double knockdown, while little difference is seen in the single knockdowns (Figure 9). This suggests that TAZ and YAP promote BMP signaling through activation of SMADs.

Figure 9 pSMAD changes with YAP knockdown. A) Western blot for pSMAD1/5 in TAZ and YAP knockdown. B) qPCR for expression of GREM1 in TAZ and YAP knockdown. *P < .05, **P < .01, ***P < .001
Knowing that TAZ and YAP knockdown impedes BMP signaling, as shown, I hypothesized that TAZ and YAP may inhibit expression of proteins that inhibit BMP. A known inhibitor of BMPs is Gremlin (GREM1) (27). I postulated that TAZ and YAP were regulating expression of GREM1. This proposition was confirmed with qPCR (Figure 9). These two experiments demonstrate that TAZ and YAP are promoting BMP signaling by inhibiting expression of GREM1.

**YAP overexpression in Mesenchymal Stem Cell Differentiation**

BMPs act both early and late in differentiation. Knowing this, I generated stably transfected CH310T1/2 cells expressing several forms of YAP (Figure 10). These constructs are under the control of a doxycycline-inducible promoter, allowing me to control when these YAP forms are expressed.

![Diagram of YAP mutants](image)

**Figure 10 YAP mutants used for overexpression studies.** A) Schematic diagrams showing YAP wildtype construct and mutated sites in the YAP mutants. B) Western Blot showing inducibility of YAP overexpression.
During the differentiation process, cells were induced at day -3 with doxycycline during BMP induction (figure 11). Induction with doxycycline ran for three days, and then both BMP and doxycycline were removed prior to changing to differentiation media on day 0. This produced expression of the YAP constructs that peaked on day 0 and was substantially reduced by day 3 (figure 12), limiting high expression to the early time points of differentiation.

**Figure 11 Timeline of C3H10T1/2 differentiation protocol.** Cells were plated and induced with doxycycline and BMP at day -3. At day 0, doxycycline and BMP were removed and differentiation induction media was added. Terminal differentiation was achieved by day 8 for fat and day 18 for bone.

**Figure 12 YAP expression decreases after withdrawal of doxycycline.** Western blot for Flag-YAP 5SA shows expression fading after removal of dox on day 0.

Adipogenic differentiation was performed with early BMP and doxycycline induction as described above. Overexpression of wildtype YAP and the nuclear/TEAD-binding mutant (5SA/S94A) modestly increased the amount of lipid from control levels (Figure 13), while the constitutively active nuclear mutant (5SA) showed reduced levels of lipid accumulation. These results were also confirmed with qPCR for adipogenic markers (Figure 14). Wildtype YAP and the 5SA/S94A mutant both showed an induction of the brown fat marker UCP1, and wildtype also showed increased levels of the general
fat marker PPARγ. In contrast, the 5SA mutant showed reductions in these two markers and a moderate increase in the brown fat marker PGC1α. These results imply that nuclear YAP may inhibit adipogenesis.

![Figure 13](image1)

**Figure 13 Oil Red O staining of intracellular lipids in overexpressing cells.** A) Representative images of Oil Red O staining of lipids in 10T1/2 cells overexpressing wildtype and mutant forms of YAP. B) Quantification by spectrophotometry of Oil Red O staining of cells in A.

![Figure 14](image2)

**Figure 14 Expression of adipogenic markers in overexpressing cells.** Quantification of mRNA expression of established adipogenic markers in the differentiation of 10T1/2 cells overexpressing wildtype and mutant forms of YAP. Also shown are markers that specify brown fat. *P < .05, **P < .01, ***P < .001

I also performed osteogenic differentiation experiments in these stably transfected C3H10T1/2. All forms of YAP appeared to increase the amount of alkaline phosphatase activity, a marker for bone differentiation, with the constitutively active 5SA form showing the greatest activity (Figure 15).
Figure 15 Staining for alkaline phosphatase activity in overexpressing cells. Representative images of staining for alkaline phosphatase activity in 10T1/2 cells overexpressing wildtype and mutant forms of YAP.

Additionally, qPCR was run to identify changes in mRNA expression of osteogenic markers (Figure 16). The early marker Runx 2 was highly upregulated in cells overexpressing wild type YAP, while the late markers osteocalcin and osteopontin were greatly reduced in comparison to control. In contrast, Runx 2 expression in the 5SA and 5SA/S94A mutants was not obviously changed from control. Additionally, osteopontin levels in the 5SA/S94A mutant were increased more than two-fold, while the 5SA mutant showed a reduction in osteopontin and no change in osteocalcin. Alkaline phosphatase activity is a mid-stage marker for bone differentiation. The staining results combined with the qPCR data indicate that overexpression of YAP may result in arrest of the differentiation process, with mutations in YAP allowing more or less progress through the process but still impeding advancement to full differentiation.
Figure 16 Expression of osteogenic markers in overexpressing cells. Quantification of mRNA expression of established osteogenic markers in the differentiation of 10T1/2 cells overexpressing wildtype and mutant forms of YAP.

Remarkably, as in the YAP knockdown cells, the YAP overexpression constructs also showed lipid accumulation in the bone differentiations (figure 17). The extent of lipid accumulation was in fact greater in the YAP overexpression bone differentiations than in the YAP knockdown bone differentiations. The nuclear YAP mutant showed the greatest amount of lipid accumulation, while the wildtype and nuclear/TEAD-binding mutant showed slightly elevated levels. These results taken together imply that nuclear YAP is needed early in differentiation for both osteogenesis and brown adipogenesis, but signaling of YAP needs to be modified later for correct response to differentiation signals.
Figure 17 Oil Red O staining of intracellular lipids in YAP overexpression bone differentiation. A) Representative images of Oil Red O staining of lipids in YAP wildtype and mutant transduced 10T1/2 cells subjected to bone differentiation. B) Quantification by spectrophotometry of Oil Red O staining of cells in A.

**Genome-wide Gene Expression in TAZ/YAP knockdown**

To further evaluate the mechanism through which TAZ and YAP may be acting, a microarray was performed using isolates from 10T1/2 cells transduced with TAZ and YAP siRNA. While there were some moderate differences in gene expression between the single knockdowns and control, the double knockdown showed a large change in global gene expression as compared to both the control and the single knockdowns (figure 17). These data indicate that there is some redundancy in the roles of TAZ and YAP. The homology between the two proteins (figure 3) and the number of shared binding partners that have been identified (52) reinforce this idea.

Interestingly, BMP 4 expression was reduced more than two-fold over control in the double TAZ/YAP knockdown. Additionally, GREM1 was up-regulated almost two-fold by the double TAZ/YAP knockdown, consistent with our prior observations.
Two integrin subunits (β6 and β7) were also downregulated in the double knockdown by at least two-fold, as were a number of other genes associated with cytoskeletal arrangements in the cell.

Among the genes upregulated at least two-fold by the double knockdown were a large number of genes associated with apoptosis, including caspases and apolipoprotein L family members. Notably, also upregulated was the gene periostin. The protein product of this gene enhances BMP1 incorporation into connective tissues and may play a role in the mineralization of the extracellular matrix of bone. These data demonstrate further support for the idea that TAZ and YAP have a function in BMP signaling and MSC differentiation.

Figure 18 Heatmap of gene expression for TAZ and YAP knockdown. A heat map from the DNA microarray performed using 10T1/2 cells transduced with control, TAZ, YAP or TAZ and YAP siRNA. Results for TAZ/YAP double knockdown cells are shown inside the yellow box.
DISCUSSION

The role of YAP and TAZ in mesenchymal stem cells appears to be complex. Contradictory descriptions of its activity in differentiation, particularly in the case of YAP, indicate that we have only scratched the surface of the role of this protein in the regulation of MSCs. What can be appreciated is that, among their many functions in the cell, YAP and its paralog TAZ are molecular switches that impact MSC cell fate.

The results from my differentiation experiments suggest that YAP acts as a positive regulator of BMP signaling. Knockdown of TAZ and YAP reduced SMAD1 phosphorylation, indicating that BMP-mediated SMAD activation is inhibited under these conditions. Our microarray data suggest that YAP and TAZ promote the expression of BMP4, and repress the expression of the BMP inhibitor Gremlin 1 (GREM1). Indeed, our qPCR analysis of GREM showed that it is upregulated upon YAP knockdown, and further upregulated with YAP/TAZ knockdown. Additionally, other regulators of BMP activity, such as periostin, were shown by our microarray to be effected by YAP/TAZ knockdown.

The induction of a brown adipogenic differentiation program in TAZ and YAP knockdown cells showed an increase in lipid accumulation in YAP knockdown cells, suggesting that the presence of YAP may inhibit fat production in these differentiating cells. This is in agreement with published findings. In this case, YAP appears to perform a similar function to TAZ, which has been shown to inhibit white fat adipogenesis. That the TAZ knockdown showed a decrease in lipid accumulation from control may indicate a role for TAZ in promoting brown fat differentiation, or alternatively may suggest
inadequate knockdown in the experiments performed. Further experiments are needed for clarification.

Osteogenic differentiation in TAZ knockdown cells showed decreased alkaline phosphatase activity, a marker for bone differentiation. This is in agreement with the established role of TAZ in promoting osteogenesis. There was low induction of the late marker osteocalcin in the TAZ knockdown cells as compared with control, though there was also no downregulation of RUNX2. In contrast, the YAP knockdown cells showed a marked increase in alkaline phosphatase activity and a large induction of OCN. This is also in agreement with an inhibitory role for YAP in osteogenesis by blocking transcription of OCN by RUNX2. That the YAP knockdown cells displayed lipid accumulation, even when exposed to a bone differentiation protocol, indicate that nuclear YAP may be needed early in the differentiation process to make cell fate determinations.

My YAP overexpression studies indicate that active YAP may inhibit lipogenesis. Notably, expression of the YAP nuclear mutant showed a reduction in Oil Red O staining and little induction of brown fat-specific genes. In contrast, the wildtype and nuclear/TEAD binding mutant showed small increases in staining for lipids. qPCR for general and brown fat markers confirmed these findings, showing a reduction in expression of PPARγ, the fat differentiation master regulator, as well as a reduction in the brown fat marker UCP1 with the 5SA mutant. Interestingly, the 5SA mutant also showed upregulation of the brown fat marker PGC1α, which is a coactivator of PPARγ. PGC1α is a major regulator of mitochondrial biogenesis. These results imply that YAP may be a
factor in mitochondrial biogenesis independent of adipogenesis. This idea needs further investigation.

YAP and YAP mutants in the overexpression osteogenic differentiations showed an increase in alkaline phosphatase activity. The YAP 5SA mutant displayed the greatest activity, followed by the 5SA/S94A mutant and the wild type. Surprisingly, wild type YAP, which showed the least alkaline phosphatase activity of the overexpressing YAPs, showed a more than 4-fold induction of Runx2, an early osteogenic marker, in qPCR studies, with little to no induction of the later markers, osteopontin and osteocalcin. The two mutant forms of YAP showed minimal changes in Runx 2. Furthermore, the 5SA/S94A mutant shows a greater than 2-fold induction of the later marker osteopontin.

It is known that osteopontin appears earlier than osteocalcin in the differentiation of bone (49), so it may be that the 5SA/S94A mutant allows differentiation to progress further but the process still arrests at a later timepoint. In addition, alkaline phosphatase activity occurs earlier in osteogenesis than induction of either osteopontin or osteocalcin, so the staining results may simply represent that the cells are progressing to this point and then being arrested at some stage just beyond alkaline phosphatase induction. Such intermediate differentiation of stem cells has been described by expression of nuclear-localized YAP in other systems (96). As in the knockdown bone differentiations, overexpression of all forms of YAP also showed lipid accumulation, the nuclear YAP mutant showing a very large induction of lipids. Because these constructs were only expressed in the cells at early timepoints, this is further evidence that YAP’s action in
differentiation may be at an early fate-determining step. Additional studies are needed to confirm these results and to define a mechanism for YAP’s regulation of osteogenesis.

The roles for YAP in differentiation may be related to the crosstalk that occurs between the Hippo pathway and other signaling pathways. Wnt and BMP signaling both have described roles in development and cellular differentiation, and TAZ and YAP are known to interact with key effectors of both of these pathways in several cellular contexts. In differentiation, Wnt is thought to be pro-osteogenic and anti-adipogenic, while BMP has been described as promoting both osteogenic and adipogenic differentiation in MSCs (39). Having demonstrated that YAP effects BMP signaling during differentiation, follow-up on how YAP-BMP crosstalk affects MSC fate will be of interest. Additionally, there is evidence for TAZ and YAP having opposing roles in regulation of Wnt signaling, and it may be that their interactions with Wnt in the context of MSC differentiation are important for determining cell fate. Investigating the role of TAZ and YAP in Wnt regulation during differentiation may prove to be important to our understanding of how these decisions are made.

Based on my observations, YAP appears to be factor involved in defining MSC differentiation. It also appears that TAZ and YAP localization may be a major factor in the initial cell fate determination of MSCs. A model can be generated based on my findings (Figure 18). There appear to be multiple mechanisms and levels of regulation and there is evidence for YAP’s action at several different steps. The complexity of YAP’s interactions with other molecules in the differentiation pathways means that there is a great deal of work still to be done. Confirmation of the above results with repetition
of the experiments is the crucial first step. Other steps include clarifying YAP’s interaction partners and placing them chronologically in the differentiation timeline. YAP’s behavior in *in vivo* differentiation is another important line of investigation. An additional area that needs further study is the proposition that YAP is a “stemness” gene needed to maintain the potentiality and proliferative ability of stem cells. These studies can offer promising insight into stem cell differentiation and may eventually allow for therapeutic opportunities.

![Proposed model for YAP's role in mesenchymal stem cell differentiation.](image)

*Figure 19 Proposed model for YAP’s role in mesenchymal stem cell differentiation.*
## APPENDIX 1

### Reagents and Equipment

#### Cell culture reagents and materials

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Company Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM (with 4.5g/L glucose, L-glutamine and sodium pyruvate)</td>
<td>Corning CellGro</td>
</tr>
<tr>
<td>OPTI-MEM\textsuperscript{®} +GlutaMax (reduced serum)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>FBS</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Trypsin – EDTA [1x, (0.25% Trypsin, 1mM EDTA-4Na in HBSS without Ca++ and Mg++)]</td>
<td>GE LifeSciences</td>
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<tr>
<td>Penicillin/Streptomycin (with 10,000 units/mL penicillin and 10,000μg/mL streptomycin)</td>
<td>GE LifeSciences</td>
</tr>
<tr>
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</tr>
<tr>
<td>Agarose LE</td>
<td>Gold Biotechnology</td>
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<tr>
<td>Puromycin</td>
<td>InvivoGen</td>
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<tr>
<td>G418</td>
<td>Gold Biotechnology</td>
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<td>American Bioanalytical</td>
</tr>
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<td>Fisher Scientific</td>
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<tr>
<td>Potassium phosphate (KH\textsubscript{2}PO\textsubscript{4})</td>
<td>American Bioanalytical</td>
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<tr>
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<td>Corning Bioscience</td>
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<td>BD Falcon\textsuperscript{TM}, Becton Dickinson</td>
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<td>15 ml Falcon tubes</td>
<td>BD Falcon\textsuperscript{TM}</td>
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<td>Haussser Scientific</td>
</tr>
<tr>
<td>C3H10T1/2, mouse embryo fibroblast cell line</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>HEK293T, human embryonic kidney cell line</td>
<td>American Type Culture Collection</td>
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</table>

**In vitro cell differentiation assay reagents**

<table>
<thead>
<tr>
<th>Product Name</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Recombinant human bone morphogenic protein 7 (BMP-7)</td>
<td>Peprotech</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Glycerol 2-phosphate disodium salt hydrate (β-glycerophosphate)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Triiodothyronine (T3)</td>
<td>Sigma</td>
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<tr>
<td>Dexamethasone</td>
<td>Sigma</td>
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<tr>
<td>3-isobutyl-1-methylxanthine (IBMX)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Insulin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Clontech</td>
</tr>
<tr>
<td>TAZ and YAP siRNA</td>
<td>Sigma</td>
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**Protein assay reagents and materials**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Company Name</th>
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<tbody>
<tr>
<td>Sodium Fluoride (NaF)</td>
<td>Fisher Scientific</td>
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<td>Sodium orthovanadate (Na₃Vo)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tween® 20</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>BLUEStain 3 protein ladder</td>
<td>Gold Biotechnology</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>BioRad</td>
</tr>
<tr>
<td>Protease inhibitor cocktail, 100x</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Aprotonin</td>
<td>Pierce Biotechnology</td>
</tr>
<tr>
<td>PEPA</td>
<td>Pierce Biotechnology</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS), 20% in water</td>
<td>American Bioanalytical</td>
</tr>
<tr>
<td>DL-dithiothreitol (DTT)</td>
<td>Gold Biotechnology</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Fisher Scientific</td>
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<tr>
<td>Triton X-100</td>
<td>American Bioanalytical</td>
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<tr>
<td>3',3'',5',5''-tetrabromophenolsulfophthalein sodium salt (Bromophenol Blue)</td>
<td>Sigma</td>
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<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Butanol</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Ammonium persulfate (APS)</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED)</td>
<td>Thermo Fisher</td>
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</tbody>
</table>
Powdered dry milk | Lab Scientific, Inc.
Tris base | Fisher Scientific
Glycine | Fisher Scientific
Methanol | Fisher Scientific
Whatman paper | Fisher Scientific
Pierce® BCA Protein Assay kit | Thermo Scientific
Disposable Cuvette | Denville

**Thermo Scientific Pierce® BCA Protein Assay kit components**

- Pierce® BCA Protein Assay Reagent A
- Pierce® BCA Protein Assay Reagent B
- Albumin Standard

**Staining reagents**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Company Name</th>
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<tbody>
<tr>
<td>Ethanol</td>
<td>Pharmaco-Aaper</td>
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<tr>
<td>Oil Red O</td>
<td>Alfa Aesar</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>American Bioanalytical</td>
</tr>
<tr>
<td>Leukocyte Alkaline Phosphatase kit</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA), 16%</td>
<td>Electron Microscopy Sciences</td>
</tr>
</tbody>
</table>

**Sigma-Aldrich Leukocyte Alkaline Phosphatase kit components**

- Fast Blue RR Salt capsules
- Mayer’s Hematoxylin Solution
- Citrate Concentrated Solution
- Napthol AS-MX Phosphate Alkaline Solution

**List of antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalog Number</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal antiTAZ/YAP</td>
<td>8418</td>
<td>Cell Signaling</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Rabbit antiFLAG-HRP</td>
<td>A8592</td>
<td>Sigma</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Rabbit polyclonal antiphospho-SMAD1/5</td>
<td>13820S</td>
<td>Cell Signaling</td>
<td>1:1,000</td>
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<tr>
<td>Rabbit antiGAPDH</td>
<td>G9545</td>
<td>Sigma</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Rabbit antiGREM1</td>
<td>4383</td>
<td>Cell Signaling</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Rabbit antiSMAD (total SMAD)</td>
<td>1649-1</td>
<td>Epitomics</td>
<td>1:1,000</td>
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<tr>
<td>Donkey antiRabbit – HRP</td>
<td>NA934V</td>
<td>GE Healthcare</td>
<td>1:1,000</td>
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</tbody>
</table>

Includes primary and secondary antibodies used for immunoblotting with optimal dilutions.
Cell transformation reagents and materials

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Company Name</th>
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</thead>
<tbody>
<tr>
<td>NEB5α competent cells</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>Polypropylene, round-bottom tubes</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>American Bioanalytical</td>
</tr>
<tr>
<td>Tryptone</td>
<td>Fisher Scientific</td>
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<tr>
<td>Sodium chloride (NaCl)</td>
<td>Fisher Scientific</td>
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<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>American Bioanalytical</td>
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<tr>
<td>Magnesium chloride (MgCl2)</td>
<td>Fisher Scientific</td>
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<tr>
<td>100 x 20 mm cell culture dishes</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>LB Agar</td>
<td>Remel</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>Gold Biotechnology</td>
</tr>
<tr>
<td>Spectinomyocin</td>
<td>Gold Biotechnology</td>
</tr>
<tr>
<td>ZR Plasmid Miniprep™ - Classic kit</td>
<td>Zymo Research</td>
</tr>
</tbody>
</table>

Zymo Research ZR Plasmid Miniprep™ - Classic kit components

- Collection tubes
- Zymo-Spin™ IIN Columns
- Buffer P1
- Buffer P2
- Buffer P3
- Endo-Wash Buffer
- Plasmid Wash Buffer
- DNA Elution Buffer

Transfection reagents

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Company Name</th>
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</thead>
<tbody>
<tr>
<td>TurboFect</td>
<td>GE Lifesciences</td>
</tr>
<tr>
<td>Dharmafect</td>
<td>GE Lifesciences</td>
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</table>

qPCR reagents and materials

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Company Name</th>
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</thead>
<tbody>
<tr>
<td>8 strip PCR tubes</td>
<td>Fisher Scientific</td>
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<tr>
<td>deoxyribonucleotide triphosphates</td>
<td>American Bioanalytical</td>
</tr>
<tr>
<td>(dNTPs)</td>
<td></td>
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<tr>
<td>iScript Reverse Transcriptase kit</td>
<td>Bio Rad</td>
</tr>
<tr>
<td>qPCR primers</td>
<td>Sigma</td>
</tr>
<tr>
<td>96-well qPCR plates</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>TempPlate RT Optical Film</td>
<td>USA Scientific</td>
</tr>
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</table>
Fast SYBR-green | Applied Biosystems  
|----------------|------------------|----------------|
| RNeasy® Mini kit | Qiagen           | Ethylenediaminetetraacetic acid (EDTA) | American Bioanalytical

**Qiagen RNeasy® Mini kit components**

- RNeasy® Mini Spin columns
- Collection tubes
- Buffer RLT
- Buffer RW1
- Buffer RPE
- RNase-free water

**iScript Reverse Transcriptase kit components**

- iScript Reverse Transcriptase
- iScript Reaction Mix, 5x
- Magnesium chloride (MgCl)
- Nuclease-Free Water

**Equipment**

<table>
<thead>
<tr>
<th>Item</th>
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<tbody>
<tr>
<td>Inverted, phase contrast light microscope [Nikon TMS]</td>
</tr>
<tr>
<td>Nikon Coolpix 4300 digital camera</td>
</tr>
<tr>
<td>Zarf Enterprise LNS-23D/CP4300 microscope adapter lens for Nikon Coolpix 4300 digital camera</td>
</tr>
<tr>
<td>Olympus Camedia D-535 Zoom digital camera</td>
</tr>
<tr>
<td>Image J image processing software</td>
</tr>
<tr>
<td>BioRad ChemiDox XRS+ imager and Image Lab software</td>
</tr>
<tr>
<td>Thermo Scientific NanoDrop 2000C spectrophotometer</td>
</tr>
<tr>
<td>BioRad MyCycler Thermal Cycler PCR machine</td>
</tr>
<tr>
<td>Applied Biosystems ViiA7 qPCR cycler and ViiA7 Ruo software</td>
</tr>
<tr>
<td>UVP Visi-Blue transilluminator</td>
</tr>
<tr>
<td>Eppendorf Centrifuge 5424</td>
</tr>
<tr>
<td>Eppendorf Centrifuge 5424R</td>
</tr>
<tr>
<td>Thermo Scientific Multi-Purpose Rotator</td>
</tr>
<tr>
<td>Fisher Scientific Nutator</td>
</tr>
</tbody>
</table>
Reagents

Luria – Bertani (LB) medium

Luria - Bertani (LB) medium was composed of 1% (w/v) tryptone (Fisher Scientific), 0.5% (w/v) yeast extract (American Bioanalytical), and 1% NaCl (Fisher Scientific). The pH of the solution was adjusted to 7.5 with sodium hydroxide (American Bioanalytical). For the preparation of agar plates, 40g LB Agar (Remel) was dissolved in 1L dH2O. The solution was subsequently autoclaved, and the appropriate antibiotics were added for each plasmid, at a concentration of 50 mg/ml. The LB, agar and antibiotic solution was poured into 100 x 15 mm dishes (Fisher Scientific) and allowed to set at room temperature. LB was stored at room temperature, while agar plates were preserved at 4 to 6°C.

Paraformaldehyde (PFA), 10%

Twenty milliliters of 16% PFA solution (Electron Microscopy Sciences) was diluted in 12 ml of phosphate buffered solution (PBS) and stored at room temperature.

Phosphate buffered saline (PBS), 10x

For use in cell culture and staining, PBS was prepared as a 10X concentrated stock, which was diluted accordingly. Final dilution was filtered through a 0.2µm bottle-top filter for use. PBS for cell culture was stored at 4°C, while PBS for staining was stored at room temperature. The 10X solution contained the following final concentrations: 1.37 M NaCl (Fisher Scientific); 27 mM KCl (American Bioanalytical); 100 mM Na2HPO4 (Fisher Scientific); and 18 mM KH2PO4 (American Bioanalytical).
The pH of the solution was adjusted to 7.4 with concentrated hydrochloric acid (American Bioanalytical) and the volume was brought up to 1 L.

**Protein lysis buffer, 10x**

For use in Western blotting, 10x lysis buffer was prepared to the following final concentrations: 500 mM Tris-HCl (pH 7.4; Fisher Scientific), 1500 mM NaCl (Fisher Scientific), 10 mM EDTA (American Bioanalytical) and 5% Triton X-100 (American Bioanalytical).

For a working solution, buffer was diluted to 1X in distilled water and protease inhibitors were added. Working solution was kept on ice and used the same day.

**Transfer buffer (TB), 10x**

Transfer buffer was prepared as a 10x stock and diluted accordingly for use. The stock solution was prepared from 30.3 g Tris base (Fisher Scientific), and 144.1 g glycine (Fisher Scientific) dissolved in 800 ml of distilled water, and then made up to 1 L. For Western blotting, TB was diluted with distilled water and methanol (Fisher Scientific) to 1X concentration. Final methanol concentration was 20%.

**Tris buffered saline (TBS), 10x**

TBS was prepared as a 10x concentrated stock solution which was diluted for use accordingly. In 800 ml of distilled water, 24.2 g of Tris base (Fisher Scientific), and 87.7 g of NaCl (Fisher Scientific) were dissolved and the final volume was brought up to 1 L.
**Tris buffered saline – tween (TBST).**

TBST contains Tris-Buffered saline (TBS) (see previous for preparation) and 0.1% polyoxyethylenesorbitan monolaurate (Tween®-20) (Fisher Scientific).

**Western Blot sample loading buffer, 5x**

Reducing 5x sample buffer was prepared by mixing 20 ml 0.5 M Tris-HCl (pH 6.8; Fisher Scientific), 3.9 g DL-dithiothreitol (DTT; Gold Biotechnology), and 5 g sodium dodecyl sulphate (American Bioanalytical). Subsequently, 25 ml glycerol (Fisher Scientific) and 0.025 g Bromophenol blue sodium salt (Sigma) were added to the solution. The solution was mixed and the final volume brought to 50 ml with 0.5 M Tris-HCl (pH 6.8). The solution was then divided into 1 ml aliquots and were stored at -20°C. Aliquots were warmed at room temperature prior to use.

**SDS-PAGE gel resolving buffer**

SDS-PAGE gel resolving buffer consisted of 1.5 M Tris base (Fisher Scientific).

**SDS-PAGE gel stacking buffer**

SDS-PAGE gel stacking buffer consisted of 0.5 M Tris base (Fisher Scientific).

**SDS-PAGE running buffer, 10x**

Running buffer was prepared as a 10x stock and diluted accordingly. For the stock, 121.2 g Tris base (Fisher Scientific), 576 g glycine (Fisher Scientific) and 40 g sodium dodecyl sulfate (American Bioanalytical) were dissolved in 3.7 L of dH2O. Once dissolved, the volume of the buffer was brought to 4 L and stored at room temperature.
**Tris-Acetic acid- Ethylenediaminetetraacetic acid (TAE), 50x**

TAE was prepared as a 50x stock and diluted accordingly for use. A solution was made of 242 g of Tris base (Fisher Scientific) and 600 ml of dH₂O. Once the Tris was dissolved, 57.1 ml Glacial Acetic Acid (American Bioanalytical) and 18.6 g of EDTA (American Bioanalytical) were added to the solution and the volume was brought to 1 L with dH₂O. Solution was stored at room temperature.

**Immunoprecipitation (IP) Wash Buffer, 1x**

IP Wash buffer was prepared to the following reagent concentrations: 50 mM Tris (pH 7.4; Fisher Scientific), 150 mM NaCl (Fisher Scientific), 1 mM EDTA (American Bioanalytical) and 0.1% Triton X-100 (American Bioanalytical).

**Oil Red O Stock Solution**

Oil red O stock solution was prepared by dissolving 0.5 g of Oil Red O (Alfa Aesar, UK) in 100 ml isopropanol (American Bioanalytical). Solution was stirred overnight, then filtered through two layers of Whatman paper (Fisher Scientific).

For a working solution, 6 ml of the stock solution were diluted with 4 ml of distilled water, mixed and filtered with a 0.22µm syringe filter. Working solution was used immediately.
## APPENDIX 2

List of PCR primers

Quantitative real time polymerase chain reaction (qPCR) primers specific to mouse cDNA.

<table>
<thead>
<tr>
<th>Gene/Transcript</th>
<th>Gene bank accession number</th>
<th>PCR product size (bp)</th>
<th>Primer sequence (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>TAZ</td>
<td>NM_001168281</td>
<td>127</td>
<td>CAGCCTCTGAATCATGTGAACCTC</td>
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<td></td>
<td>NM_133784</td>
<td></td>
<td>GGCTAGTGCCACAGCTTGCTG</td>
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<tr>
<td>YAP</td>
<td>NM_009534.3</td>
<td>106</td>
<td>AATGTGGACCTTGGGCACACT</td>
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<tr>
<td></td>
<td>NM_001171147.1</td>
<td></td>
<td>ACTCCACGTCAGATTTCG</td>
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<tr>
<td>PPARγ</td>
<td>NM_011146.3</td>
<td>134</td>
<td>TGGTGCCCTCGCTGATGC</td>
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<td>NM_001127330.1</td>
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<td>AATGCGAGTGCTCTGGCTAC</td>
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<td>Col10a1</td>
<td>NM_009925.4</td>
<td>100</td>
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<td></td>
<td></td>
<td>GAAAGGGTATTTGAGGAGCAGCA</td>
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<td>Col2a1</td>
<td>NM_001113515.2</td>
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<td>CTTATACCTACCTGCTCCCAT</td>
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<td>Sox9</td>
<td>NM_011448.4</td>
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<td>Runx2</td>
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<td>UCP1</td>
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<td>PGC1α</td>
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<td></td>
<td>NM_177113</td>
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<td>TCAAGAAAGGTCAAGTTCAGGAAGA</td>
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</tbody>
</table>

All original primers were designed with NCBI primer blast tool software. Primers for UCP1 and PGC1α were kindly donated by members of the Farmer lab.
APPENDIX 3

List of plasmids

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>TET-ON (G418)</td>
<td>Hiemer, et. al. 2014 (30)</td>
</tr>
<tr>
<td>pLVX-TP-Ctrl</td>
<td>Hiemer, et. al. 2014 (30)</td>
</tr>
<tr>
<td>pLVX-TP-3F YAP2A WT</td>
<td>Hiemer, et. al. 2014 (30)</td>
</tr>
<tr>
<td>pLVX-TP-3F YAP2A 5SA</td>
<td>Hiemer, et. al. 2014 (30)</td>
</tr>
<tr>
<td>pLVX-TP-3F YAP2A 5SA/S94A</td>
<td>Hiemer, et. al. 2014 (30)</td>
</tr>
<tr>
<td>pLKO1-puro shCtrl</td>
<td>Hiemer, et. al. 2014 (30)</td>
</tr>
<tr>
<td>pLKO1-puro shTAZ m</td>
<td>Hiemer, et. al. 2014 (30)</td>
</tr>
<tr>
<td>pLKO1-puro shYAP m67</td>
<td>Hiemer, et. al. 2014 (30)</td>
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<td>psPAX2</td>
<td>Hiemer, et. al. 2014 (30)</td>
</tr>
<tr>
<td>pCMV-VSV-G</td>
<td>Hiemer, et. al. 2014 (30)</td>
</tr>
</tbody>
</table>
REFERENCES


VITA

LISA BRENAN

nanerb@bu.edu • 781-439-1435 • YOB: 1980
Permanent Address: 42 Tillotson Rd, Apt. #1 • Needham, MA 02494

EDUCATION

Boston University School of Medicine, Boston, MA
Master of Science in Biochemistry, expected 2015

Boston University, Boston, MA
Bachelor of Arts in Biology 2012

University of Illinois at Urbana-Champaign, Urbana, IL
Master of Fine Arts in Stage Management 2003

Florida Southern College, Lakeland, FL
Bachelor of Arts in Theater 2001

EXPERIENCE

Boston University School of Medicine Biochemistry Department, Boston, MA
Graduate Research Student 2014
• Engineer a research project for thesis project
• Conduct experiments using the following techniques: mammalian and bacterial cell culture, bacterial transformation, cloning, DNA digests, DNA gel electrophoresis, qPCR, Western blots, stem cell differentiation, creation of stable cell lines, mouse work, lentivirus production

Boston University School of Medicine Biochemistry Department, Boston, MA
Laboratory Research Technician 2012
• Conduct experiments at the direction of the principal investigator
• Techniques used: creation of fusion proteins, lentivirus production, production of DNA chimeras for proteins, tissue culture, transformation, cloning, Western blotting, DNA gel electrophoresis
• Maintain laboratory stocks and equipment

Boston University School of Medicine Microbiology Department, Boston, MA
Undergraduate Research Assistant 2011
• Develop and perform assay for high-throughput evaluation of effectiveness of novel chemicals as inhibitors of vesicular stomatitis virus
- Maintain communication with group responsible for synthesizing compounds
- Compile and present results in lab meetings.

Boston University School of Medicine Department of Physiology and Biophysics, Boston, MA

**Undergraduate Research Assistant** 2011
- Transform *E. coli* with prepared plasmids, culture and induce bacteria to express study proteins
- Purify expressed proteins in preparation for NMR study

New England Veterinary Oncology Group LLP, Waltham, MA

**Receptionist** 2008
- Greet clients, schedule appointments, answer phones, enter lab work into computers, take payments, obtain records from referring veterinarians, coordinate visits among different departments, office organization, office equipment management, support clinical staff

The Boston Conservatory, Boston, MA

**Assistant Stage Manager** 2007
- Rehearsal scheduling, running of rehearsals, maintain production records, coordinate various aspects of rehearsals, coordinate actors, maintain accurate running script, supervise running crew, assist with various scenic and lighting elements, maintain properties and costume lists

Yarn, LLC, New Haven, CT

**Sales Associate** 2006
- Customer assistance, teaching lessons, maintaining store and inventory, placing orders for clients

Advanced Placement, Milford, CT

**Temporary Worker** 2006
- Secretarial, administrative and reception work
- Data entry, database creation, scheduling, client contact

Long Wharf Theatre, New Haven, CT

**Production Intern** 2006
- Run crew, theatre prep, actor care and transportation

Z Space Theatre, San Francisco, CA

**Production Intern /Assistant Stage Manager** 2005
- Run crew, manage rehearsals and facilities, actor care
Hampshire Shakespeare Company, Amherst, MA

Stage Manager/Assistant Director 1999

- Run rehearsals, manage properties and actors, coordinate production meetings, maintain book for show, consult in design process

SKILLS

Technical: Windows, Mac and Linux Operating Systems, Microsoft Office Suite (Word, Excel, PowerPoint), Adobe Acrobat Reader, Image J, Data entry and Database creation and management, C++, graphic design

Laboratory: Protein purification, fluorescence microscopy, Western blotting, microbiology, SDS-PAGE, mammalian cell culture, pipetting, handling of infectious agents, plaque assay, experimental design, staining, centrifugation, biosafety cabinet use, gel electrophoresis, bacterial transformation, spectrophotometry, Bradford assay, BCA assay, microplate reader, expression cloning, PCR, primer design, Co-IP