BIDIRECTIONAL REGULATION OF YAP AND ALDH1A1

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DEDICATION

This project could not have been completed without the love and support of my family and friends. Specifically, I would like to dedicate this work to my parents, Amy and Greg Martien, my brother Patrick Martien, and most importantly, my soon-to-be wife, Lindsey Weber. Each of you has been by my side throughout my successes and failures, and for that, I sincerely thank you.

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Matthew F. Martien

BIDIRECTIONAL REGULATION OF YAP AND ALDH1A1

Breast cancer is the second leading cause of cancer death for women in the United States. Approximately, 1 in 5 women will recur with cancer within 10 years of completing treatment and recent publications have suggested that breast cancer stem cells confer resistance to therapy. These reports highlight aldehyde dehydrogenase 1A1 (ALDH1A1) and Yes-associated protein (YAP) as a biomarker and key mediator of the stem cell phenotype respectively. To further understand how YAP and ALDH1A1 facilitate chemoresistance, this study investigated how ALDH1A1 specific inhibition affected YAP activity and growth of basal-like breast cancer cells, which are enriched in cancer stem cells. Intriguingly, attenuation of growth by ALDH1A1 inhibition was observed when cells were plated on a reconstituted basement membrane. Further, the inhibition of cell growth correlated with cytosolic retention of YAP and a reduction in YAP signaling. In a complementary analysis, the overexpression of YAP correlated with an increased level of ALDH1A1 transcript. Results from this study indicate a novel mechanism by which basal-like breast cancer cells utilize YAP to maintain the stem cell phenotype and also suggest ALDH1A1 as a potential therapeutic target for breast cancer therapy.

Clark Wells, PhD, Chair

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ABBREVIATIONS

ALDH	Aldehyde Dehydrogenase
ALDH1A1	Aldehyde Dehydrogenase 1A1
AMPK	AMP Activated Protein Kinase
aVEGF	Anti-vascular endothelial growth factor
BCA	Bicinchoninic acid
CD	Cluster of Differentiation
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin Immunoprecipitation
CM037	Cynthia Morgan 037
CTGF	Connective Tissue Growth Factor
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECAR	Extracellular Acidification Rate
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-to-mesenchymal Transition
ER	Estrogen Receptor
ER-	Estrogen Receptor Negative
FACS	Fluorescent-activated Cell Sorting
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
HER-	Human Epidermal Growth Factor Receptor Negative
HER2	Human Epidermal Growth Factor Receptor 2

LATS 1/2	Large Tumor Suppressor 1/2
LKB1	Liver Kinase B1
MaSC	Mammary Tissue Stem Cell
mTOR	Mammalian Target of Rapamycin
ME	Mammary Epithelia
MST 1/2	Mammalian STE20-like Protein Kinase 1/2
OCR	Oxygen Consumption Rate
PFA	Paraformaldehyde
PBS	Phosphate Buffered Saline
PEI	Polyethylenimine
PPAR	Peroxisome Proliferator-activated Receptor
PR	Progesterone Receptor
PR-	Progesterone Receptor Negative
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RXR	Retinoid X Receptor
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine
Thr	Threonine

TAZ	Tafazzin
TBST	Tris-buffered saline with Tween-20
TEAD	TEA domain transcription factor
UCSC	University of California Santa Cruz
YAP	Yes-associated Protein

INTRODUCTION

Breast cancer is the second leading cause of cancer death for women in the United States (American Cancer Society, 2013). Currently, around 60,000 women develop non-invasive tumors per year and the rate of incidence increases until the age of 50 where then a decline is observed (Table 1 and Figure 1) (American Cancer Society, 2013). Sadly however, nearly 240,000 women develop invasive breast cancer per year and the rate of mortality steadily increases with age (Table 1 and Figure 1) (American Cancer Society, 2013). Treatments include surgery, radiation therapy, and chemotherapy (American Cancer Society, 2013), but one in five women will have recurrence of breast cancer within 10 years of completing treatment (A. M. Brewster et al., 2008). While the biological mechanism underlying cancer recurrence is very poorly understood, recent research suggests that subpopulations of breast cancer stem cells survive therapy and rapidly promote metastasis and invasion (A. Grosse-Wilde et al., 2015; M. Luo et al., 2015; M. Wang, Y. Wang, & J. Zhong, 2015).

Recent studies have linked cells that have undergone an epithelial to mesenchymal transitions (EMT) to having a stem cell identity (A. Grosse-Wilde et al., 2015). During EMT, epithelial markers such as E-cadherin, Claudins and Occludins are lost while proteins that mediate the mesenchymal cell phenotype such as Snail, Twist, and Slug are upregulated (Figure 2) (G. Barriere, P. Fici, G. Gallerani, F. Fabbri, & M. Rigaud, 2015). Claudins and Occludins are critical for maintaining cellular tight junctions while E-Cadherin is the main strand protein at adherence junctions (Berk A Lodish H, Zipursky SL, et al. , 2000). Together these proteins are essential for maintaining intercellular attachments to make up an epithelial sheet or duct while maintaining a

physical barrier between the internal and external environment. Conversely, Snail, Twist, and Slug function as transcription factors that suppress the expression of cadherin (H. Peinado, F. Portillo, & A. Cano, 2004) and facilitate the molecular events involved in cellular motility.

The molecular events that promote cell proliferation *in situ* must also be considered when attempting breast cancer therapies. Considerable effort has gone into understanding and developing drugs against the human epidermal growth factor receptor 2 (EGFR2) (J. Zekri et al., 2015) and the estrogen receptor (ER) (M. Yamamoto-Ibusuki, M. Arnedos, & F. Andre, 2015). Unfortunately, while coupling these targeted approaches with standard chemotherapy and radiation improves outcomes, the high frequency of recurrence of the cancer still remains. This justifies new approaches where the role of breast cancer stem cells is one of the most promising avenues for developing new therapeutics.

Stem cells are generally defined as self-renewing cells that have the pluripotent potential to regenerate any particular tissue (Berk A Lodish H, Zipursky SL, et al., 2000b). For almost six decades, the concept was that mammary tissue stem cells (MaSCs) reside within the heterogeneous architecture of the tissue and facilitate the cycles of regeneration and regression with successive pregnancies. (A. Skibinski & C. Kuperwasser, 2015). This theory was validated in 2006 when Shackleton *et al.* and Stingl *et al.* demonstrated in a murine model that MaSCs, isolated by fluorescent-activated cell sorting (FACS), are essential for lobule outgrowth. While their markers of CD49 and CD24 for isolating stem cells have recently been replaced by CD44 and CD24, the original work illustrates the significance of MaSCs in the initiation of the breast cancer.

Subsequent analyses have identified aldehyde dehydrogenase 1A1 (ALDH1A1) as an additional high value biomarker for cancer stem cells (Y. Liu et al., 2014; Y. Luo et al., 2012; I. Olmez, W. Shen, H. McDonald, & B. Ozpolat, 2015; S. Wu et al., 2015). High expression of ALDH1A1 is also correlated with a poor clinical outcomes as evidenced by one report which found a 26% increase in risk per Kaplan-Meir curve analysis (S. Wu et al., 2015). ALDH1A1 belongs to the aldehyde dehydrogenase (ALDH) superfamily of enzymes which collectively catalyze the oxidation of aldehydes to their corresponding carboxylic acids (B. Jackson et al., 2011). ALDH1A1 converts retinaldehyde to retinoic acid (RA), the key ligand for the retinoic acid receptor (RAR). Because RAR is important for eukaryotic gene expression (Berk A Lodish H, Zipursky SL, et al., 2000a) and promotes the differentiation of embryonic stem cells (J. H. Chuang, L. C. Tung, & Y. Lin, 2015), ALDH1A1 has been proposed to inhibit differentiation by depleting RA. All translational efforts have focused on inhibiting ALDH1A1 to combat alcohol dependence (A. Yoshida, V. Dave, R. J. Ward, & T. J. Peters, 1989) but last year, inhibition of ALDH1A1 was shown to prevent the formation ovarian spheroids *in vitro* (S. Condello et al., 2014). This strongly implicated ALDH1A1 in the function of ovarian cancer stem cells. Thus, the importance of ALDH1A1 in other cancer types, such as breast, that rely on stem cells for growth and recurrence needs to be considered.

Two related factors that have been implicated in cancer stem cell renewal are the Yes-associated protein (YAP) and Tafazzin (TAZ). These structurally similar transcriptional co-activators are tightly regulated by the HIPPO Tumor Suppressor Pathway (M. Cordenonsi et al., 2011) in growth-arrested cells. Canonically, HIPPO signaling is characterized by the activation of the mammalian STE20-like (MST) 1/2

protein kinases that phosphorylate the large tumor suppressors (LATS) 1/2 whose downstream target is YAP (Y. Hao, A. Chun, K. Cheung, B. Rashidi, & X. Yang, 2008), the transcription co-activator of key pro-growth factors (J. Avruch et al., 2012). Interestingly, the HIPPO pathway responds to anti-growth cellular signals including: intercellular contacts, lack of growth factors, and metabolic stress (S. Piccolo, S. Dupont, & M. Cordenonsi, 2014). More recently in the context of stem cells, it has been suggested that YAP is regulated by metabolic states such as aerobic glycolysis (E. Enzo et al., 2015), but the mechanism by which this occurs remains to be determined.

Several cell lines have been developed for studying different subtypes of breast cancer. For example, cell lines such as MCF7 (ER+/PR+/HER-) and SKBR3 (ER-/PR-/HER+) are of luminal origin, whereas MDA-MB-468 (ER-/PR-/HER-) cells are derived from the basal epithelia (R. M. Neve et al., 2006). These cell lines are an essential component of drug discovery, especially in recent work focusing on the response to chemotherapy of basal-like triple negative cell lines (B. T. Hennessy et al., 2009).

Although, ALDH1A1 expression has been implicated as a cancer stem cell marker (Y. Liu et al., 2014; Y. Luo et al., 2012; I. Olmez et al., 2015; S. Wu et al., 2015), its functional significance in this context remains to be determined. Given that triple-negative breast cancers are a disease without a molecular target (F. Tomao et al., 2015), strategies for targeting the disease are needed as evidenced by Table 1 and Figure 1. This investigation directly addresses this point by investigating the impact of selective inhibition of ALDH1A1 on basal like breast cancer growth and simultaneously the role of YAP inducing and mediating ALDH1A1 function.

Table 1. **Estimated Number of Breast Cancer Cases and Deaths for 2013**. Approximately 230,000 invasive cases and nearly 40,000 deaths were predicted for 2013. These staggering numbers illustrate the need to further develop therapeutic strategies for the disease. (Table was adapted from American Cancer Society, 2013)

Age (Yrs)	In Situ Cases	Invasive Cases	Deaths
<40	1,900	10,980	1,020
<50	15,650	48,910	4,780
50-64	26,770	84,210	11,970
65+	22,220	99,220	22,870
All Ages	64,640	232,340	39,620

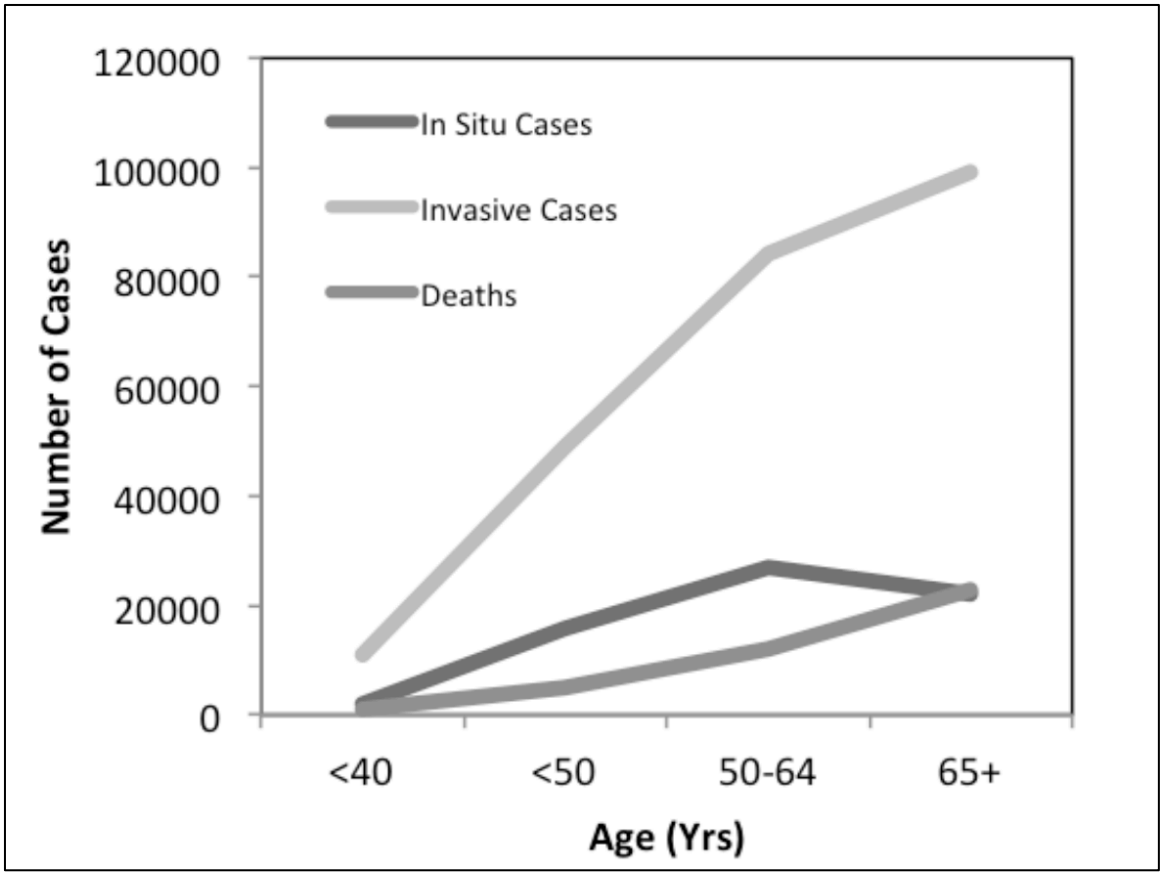

Figure 1. **The Number of Invasive Breast Cancer Cases and Mortality Increases with Age.** The number of *in situ* cases increases with age until between the ages of 50 and 64. Conversely, there is a decline in the number of cases between the ages of 50 and 64. Importantly however, there is an increase in the number of invasive cases and number of deaths with age. (Figure was adapted from American Cancer Society, 2013)

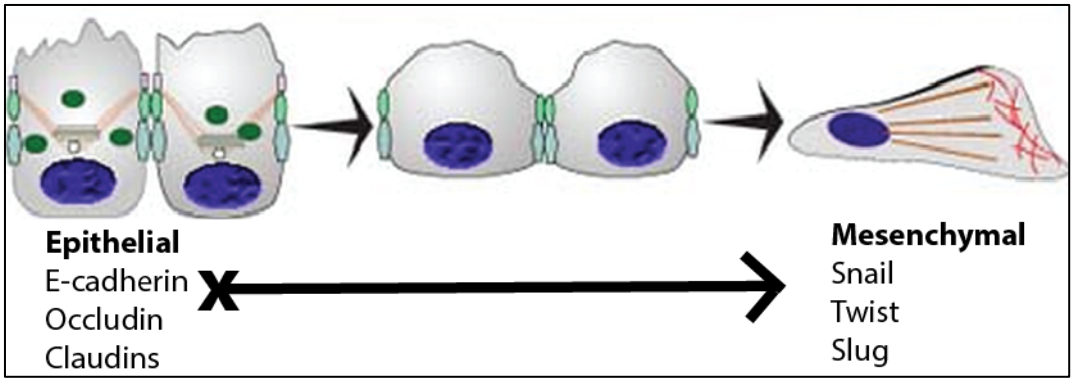

Figure 2. **The Epithelial to Mesenchymal Transition**. The EMT has been suggested to drive metastasis and stem cell renewal. Molecularly, EMT is characterized by the loss of epithelial markers such as e-cadherin, occludin, and claudin, and instead replaced by mesenchymal markers such as Snail, Twist, and Slug. (Figure was adapted from C.D. Wells, 2015).

MATERIALS AND METHODS

Cell line

MDA-MB-468 cells were passaged at 37^{o} C with 5% CO_2 on 10 cm^2 dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 5 ml of Penicillin/Streptomycin. Once plates were confluent, growth media was aspirated and cells were detached from the plate by adding 1 ml of trypsin supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA). After 15 minutes of incubation at 37^{o}C, adding 3 ml of complete DMEM quenched the reaction. Cells were then split into assay after being spun down at 0.4 x g for 1.5 minutes, re-suspended in complete DMEM, and counted on a hematocytometer.

Growth assays

Growth assays were completed by plating 300,000 cells with, or without, 150 µl of a reconstituted, lamin- rich, basement membrane (Matrigel©). Images were acquired on the 1500Z Nikon Stereoscope and subjected to colony number and surface area analysis using ImageJ64 software.

Viral Transfection

In 1 ml of serum-free media the following transfection mixture was prepared: 20 µg of target cDNA,10 µg pRRE and pRSV-REV, and 6 µg VSVG. 25 µl of polyethylenimine (PEI) (2 µg/mL) was then added drop wise to the mixture while being vortexed at Setting 7. Mixtures were incubated at room temperature for 5 minutes and added drop wise to a 10 cm^2 plate that was seeded with 4 million 293T cells. Finally,

viral media was collected every 24 hours, spun down, and transferred to a new 15 ml Falcon© tube. Collection was completed for a total of 48 hours.

Viral Infection

24 hours prior to viral infection, MDA-MB-468 cells, that were approximately 95% confluent on a 10 cm^2 plate, were trypsinized, spun down at 0.4 x g, and re-seeded on 6 cm dishes at a count of 300,000 cells/dish. After 24 hours, growth medium was aspirated and dishes were incubated for 8 hours with viral media that was diluted 1:2 in complete DMEM. Finally, viral media was aspirated and cells were again cultured in complete media for 24 hours at 37^oC.

CM037 and Verterporfin Treatment

MDA-MB-468 cells were split for assay into 6 cm (500,000 cells/dish) or 35 mm (300,000 cell/dish) dishes and were treated with Verteporfin, a non-specific YAP-TEAD inhibitor, or an ALDH1A1 specific inhibitor, Cynthia Morgan 037 (CM037). Verteporfin was obtained from the laboratory of Dr. Clark Wells and CM037 was a gift from Dr. Thomas Hurley. Stock solutions of Verteporfin (1 mM) and CM037 (5 mM) were initially diluted in dimethyl sulfoxide (DMSO) and stored at -20^oC. To standardize DMSO concentration (v/v), stock solutions were warmed at room temperature for one hour before cell treatment, diluted in DMSO to a target concentration, and finally diluted in complete DMEM. For example, Matrigel© growth assays at 20 μM were completed as follows. In 35 mm dishes (2 ml assay media/dish), the 5 mM CM037 stock was first diluted to 4 mM in DMSO where then 10 μl of the 4 mM solution was raised to 2 ml in

complete DMEM and applied to the plate. To control for the concentration of DMSO, a separate plate was treated with 10 µl of DMSO standard (Sigma Aldrich) and raised up in 2 ml of complete DMEM.

Western Blotting

Protein was purified via RIPA extraction (1 ml/10cm dish) after cells were washed 2x with phosphate buffered saline (PBS). After protein extraction, samples were incubated on ice for 10 minutes and spun down at 10,000 RPM for 10 minutes at 4^0C, where the supernatant was transferred to a new microcentrifuge tube. Next, a bicinchoninic acid (BCA) assay was used to determine the protein concentration of each sample. Normalization was completed by diluting each sample in RIPA and 6X loading dye. Finally, samples were boiled for 5 min at 95^0C and 30 µg of protein was loaded into each well of a 10% (w/v) sodium dodecyl sulphate-polyacrylamide gel and separated by electrophoresis (SDS-PAGE) for 60 minutes at 110V.

Next, protein was transferred to a nitrocellulose membrane for 1 hour at 24V. After transfer was complete, the membrane was blocked in 5% (w/v) milk, washed with tris-buffered saline with 0.05% (v/v) Tween-20 (TBST), and incubated overnight in the following primary antibodies diluted in TBST: mouse anti-YAP (Abnova, H00010413-MO1) 1:1000, rabbit anti-pYAP (Cell Signaling, 4911S) 1:1000, and mouse anti-GAPDH (Millipore, MAB374) 1:10,000. After primary incubation, the membrane was washed 3x for 5 min with TBST and then incubated with a secondary antibody that was either goat anti-rabbit Dylight 680 (Thermo-Scientific) or goat anti-mouse Dylight 800 (Thermo-

Scientific) (1:20,000 in TBST) for 30 minutes where it was then visualized on the LiCor©.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Following assay media aspiration and 1x PBS wash, cells were lysed in 0.5 ml of Tri Reagent LS, collected in a 1.7 ml microcentrifuge tube, and incubated at room temperature for 5 minutes. Ribonucleic acid (RNA) was then extracted by adding 100 µl of chloroform and spinning samples down at 13,000 RPM for 10 min at 4^0C. The aqueous supernatant was transferred to a new 1.7 ml microcentrifuge tube and RNA was precipitated with an equivalent volume of isopropanol and spun down at 13,000 RPM for 10 min at 4^0C. Supernatant was removed and RNA was washed with 500 µl of 75% ethanol. Samples were finally spun down at 13,000 RPM for 10 min at 4^0C, the supernatant was removed, and pellets were re-suspended in 20 µl of H_2O

Next, 5 µl of RNA was incubated with: 2 µl of oligo dT (50 uM), 4 µl dNTPs (10mM), and 10 µl H_2O. After incubation for 5 minutes at 65^0C, complimentary deoxyribonucleic acid (cDNA) was synthesized by incubating the above mixture with: 10 µl of H_2O, 2 µl of reverse transcriptase (RT), and 4 µl of RT Buffer. After 1 hr. at 50^0C, sample concentrations were determined by the NanoDrop© and standardized to 300 ng/µl in H_2O.

Finally, 5 µl (300 ng/µl) of cDNA, 10 µl of SYBRGreen, 1 µl (10 uM) of the target forward primer, 1 µl (10 uM) of the target reverse primer, and 10 µl of H_2O was added to each well of the 96-well ABI-FastOpti plate and assayed per the Eppendorf RealPlex ABI-FastOpti Protocol. Forward and reverse primers used for this study are as

follows: CTGF (AGGAGTGGGTGTGTGACGA/CCAGGCAGTTGGCTCTAATC), ALDH1A1 (AGATTGGATCCCCGTGGCGT/TTGACTCCATTGTCGCCAGCAG), and GAPDH (GCTCTCTGCTCCTCCTGTTC/ACGACCAAATCCGTTGACTC).

Immunofluorescence

MDA-MB-468 cells were seeded on 100 µl of Matrigel© in 35 mm MatTek© dishes and incubated in assay media per cell culture conditions as described previously. After 72 hours of treatment, assay media was aspirated and cells were fixed with 400µl of 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Cells were then washed 3x with 0.5 ml of 1X PBS and permeabilized with 0.5 ml of Triton X-100 (0.5% in PBS) for 10 minutes at 4^0C. After allowing Matrigel© to come to room temperature, cells were washed 3x with 0.5 ml of 1X PBS and incubated with 400 µl of blocking buffer (0.2g Saponin, 10g BSA, 200 ml 1X PBS) for 60 minutes at room temperature. Blocking buffer was then aspirated and samples were incubated with 1 ml of primary antibody (mouse anti-YAP, 1:1000 in blocking buffer) overnight at 4^0C. The following day, Matrigel© was again warmed to room temperature and washed 3x with 0.5 ml blocking buffer for 5 minutes. Then, samples were incubated with 1 ml of secondary antibody (Goat anti-Mouse, Alexa 488, 1:500 in blocking buffer) for 1 hr. in the dark. After 3x wash with 0.5 ml blocking buffer, samples were then incubated with 1ml of phalloidin toxin covalently coupled to the cruzfluor 560 (1:4000 in blocking buffer) for 20 min in the dark. Finally, samples were rinsed 3x with 0.5 ml of blocking buffer and then incubated with 1 ml of DAPI (Hoechst 1:3000 in blocking buffer) for 7 min in the dark, and again rinsed 1x with blocking buffer for 5 minutes.

Statistical Analysis

All statistical analyses were completed using an Independent Samples T test using SPSS software Version 22.

RESULTS

The MDA-MB-468 Cell Line is Sensitive to ALDH1A1 Specific Inhibition in 3D culture but not when cultured on plastic.

Work by the Hurley Laboratory (Indiana University School of Medicine, Indianapolis) identified CM037 as a compound that specifically inhibited ALDH1A1, as compared to nine ALDH family members, from a high-throughput esterase activity screen (C. A. Morgan & T. D. Hurley, 2015). With a K_i value of approximately 0.23 μM, and an IC50 value of 4.6 μM, competitive inhibition of ALDH1A1 is achieved by the tricyclic ring occupying the hydrophobic pocket formed by Phe171, Val460, and Phe466 of ALDH1A1. Additionally, this interaction may involve a weak hydrogen bond between the carbonyl of the core structure and Cys302 of ALDH1A1.

The characterization of CM037 provided a platform for the intracellular analysis of ALDH1A1 function and Condello *et al*. was first to demonstrate that selective inhibition of ALDH1A1 significantly prevented ovarian spheroid formation (S. Condello et al., 2014). Because breast cancer and ovarian cancer have overlapping molecular signatures (J. M. Jonsson et al., 2014), we investigated how CM037 would affect the phenotype of a triple-negative breast cancer cell line, MDA-MB-468.

MDA-MB-468 cells were originally derived in 1977 through a pleural effusion and are a basal-like, metastatic cell line that lack the estrogen receptor (ER-), progesterone receptor (PR-), and the human epidermal growth factor receptor (HER-) (R. Cailleau, M. Olive, & Q. V. Cruciger, 1978). The cell line also models therapeutic challenges and has been shown to have stem-like properties (S. Samanta et al., 2015).

Despite the proliferative potential of the cell line, we were able to effectively attenuate cell growth, and do so in a manner that more closely resembles an *in vivo* status.

To determine whether inhibition of ALDH1A1 affects the growth of MDA-MB-468 cells, these cells were plated and cultured as a monolayer on plastic or on Matrigel®, before and during treatment with a dose range of CM037. As a monolayer, MDA-MB-468 cell growth was increasingly inhibited at 50 and 70 μM of CM037 but were unaffected by lower concentrations of 10, 20, and 30 μM (Figure 3). However, cells plated on Matrigel® showed attenuated growth (>50%) at 10 μM or great concentrations of CM037 (Figure 4). This suggested that a concentration of 20 μM of CM037 would be effective for subsequent cell based experiments.

Unpublished data by the Wells lab has shown that when MCF10A cells are seeded on Matrigel®, growth is enhanced by endogenous expression of YAP. Further, Verteporfin, a compound that disrupts the interaction of YAP with the TEA domain (TEAD) transcription factor (E. Felley-Bosco & R. Stahel, 2014), has been shown to inhibit the growth of multiple breast cancer cell types including MDA-MB-468 cells. This strongly suggests that YAP activity is required for the growth of these cells. As summarized in Figure 5A, sensitivity to Verteporfin and CM037 was observed when cells were plated on Matrigel® and the combination of the two compounds had a greater effect than Verteporfin or CM037 alone (Figure 5B). Additionally, by measuring and plotting colony size, or colony number against the time in days, a linear decrease in the number of colonies and an exponential increase in colony size can be observed (Figure 5B). Based on the results from these growth assays, further investigation into the link between ALDH1A1 function and YAP activity was undertaken.

Inhibition of ALDH1A1 Affects YAP Signaling in 3D

The transcription of connective tissue growth factor (CTGF) is directly mediated by TEAD and is therefore tightly coupled to YAP activity (J. Avruch et al., 2012). Consequently, any effects of ALDH1A1 activity on YAP activity could be measured indirectly by measuring the levels of CTGF mRNA transcript. The levels of CTGF transcript were therefore monitored in cells treated with CM037 for three days using qRT-PCR (See Materials and Methods). As expected, MDA-MB-468 cells cultured as a monolayer or in Matrigel® and treated with Verteporfin (1 μM) showed a significant decrease in CTGF levels (Figure 6B). However, no significant effect on CTGF transcript was observed in MDA-MB-468 cells cultured in 2D, or 3D, upon treatment with CM037 (Figure 6B). While not statistically significant, cells cultured in Matrigel® showed a decrease in mean levels of CTGF transcript (Figure 6B).

Inhibition of ALDH1A1 Promotes Cytosolic Retention of YAP

Previous work has described that YAP is targeted for degradation in cells undergoing stress (D. Pan, 2010). Here, MDA-MB-468 cells cultured as a monolayer and treated with CM037 did not show a similar effect (Figure 6A). However, the decrease in CTGF transcript in 3D (Figure 6B), while not statistically significant, suggested that inhibition of ALDH1A1 might be impacting YAP activity. To determine whether inhibition of ALDH1A1 was impacting YAP cellular localization, MDA-MB-468 cells were treated for 3 days with CM037 (20 uM) or Verteporfin (0.25 uM) and then fixed for analysis by immunofluorescence (See Materials and Methods). As observed in Figure 7**,** cells treated with CM037, showed YAP staining in the cytosol in peri-nuclear punctae,

whereas control cells showed a diffuse stain for YAP that was equally distributed between the nucleus and the cytosol. These results highlight a potential link between YAP inhibition and metabolic stress induced by the inhibition of ALDH1A1.

YAP Activity Correlates with Increased Transcription of ALDH1A1

Recent work has suggested that the expression of ALDH1A1 (H. Q. Duong et al., 2012), and the binding of YAP to TEAD4, is important for the chemoresistant phenotype (Y. Xia, Y. L. Zhang, C. Yu, T. Chang, & H. Y. Fan, 2014). Based on this information, and data acquired from our qRT-PCR analyses, we wondered if the transcription of ALDH1A1 is regulated by YAP activated TEAD4. I therefore analyzed whether any TEAD family transcription factors had been reported to bind in the promoter of any of the ALDH genes in the chromatin immunoprecipitation (ChIP) data from the University of California Santa Cruz (UCSC). This revealed that TEAD4 has been shown to precipitate multiple fragments of the promoter of the ALDH1A1 gene (Table 1 and Table 2). While TEAD was also observed to bind five other ALDH genes, generally it was highly enriched for binding ALDH1A1. This strongly suggested that YAP activation might promote the expression of ALDH1A1.

To explore the possibility that YAP may induce ALDH1A1 transcription, MDA-MB-468 cells were stably infected (See Materials and Methods) with either wild-type YAP, or red fluorescent protein (RFP) and treated with CM037 (20 μM) or Verteporfin (0.25 μM). As shown in Figure 8, overexpression of wild-type YAP led to a significant ($p < 0.001$) increase in ALDH1A1 expression, which was significantly ($p < 0.001$) reduced when cells were treated CM037, or Verteporfin. Based on these results, it is concluded

that YAP activity drives ALDH1A1 expression whereas ALDH1A1 function is required for YAP activity.

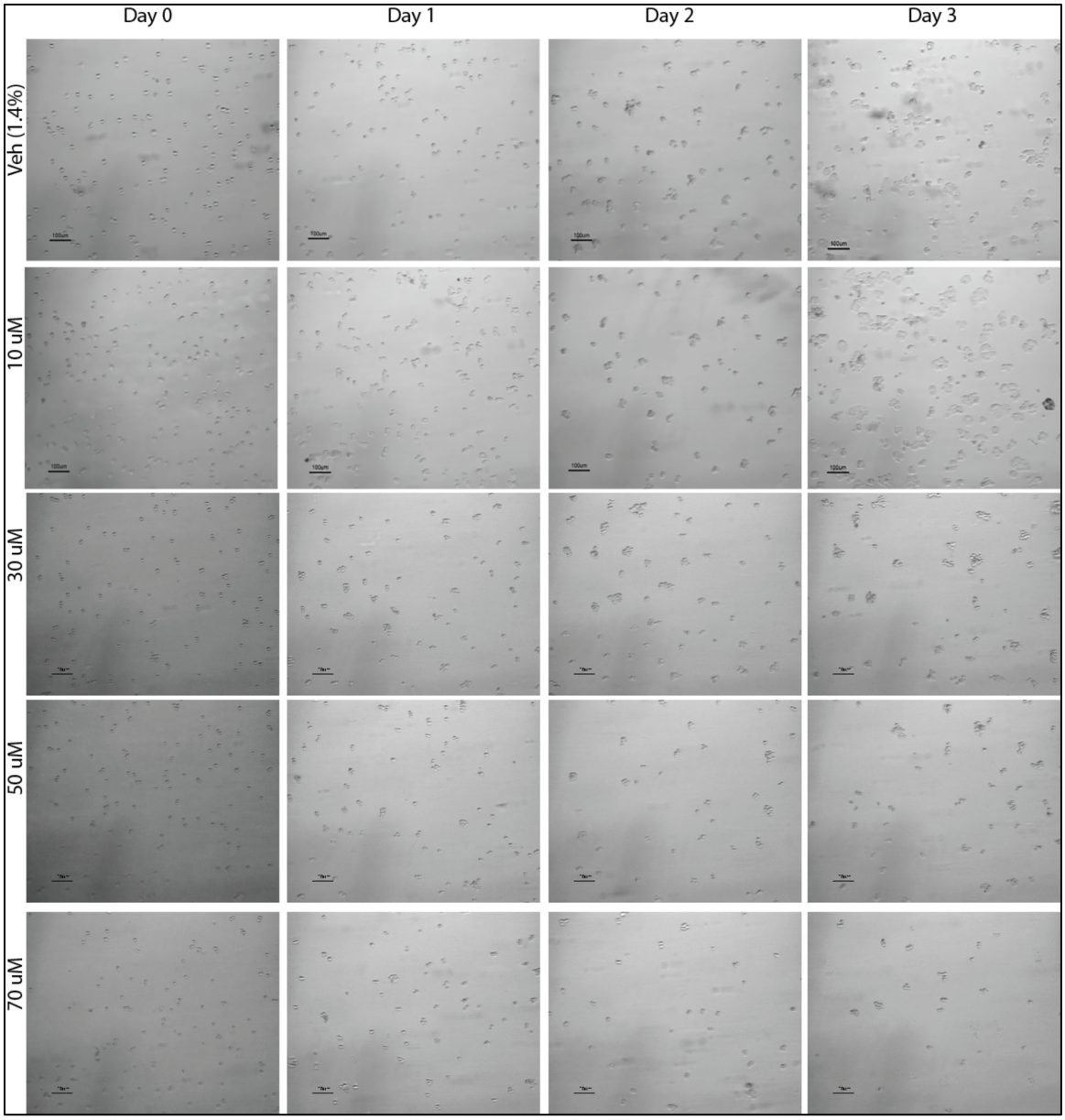

Figure 3. **Specific Inhibition of ALDH1A1 Does Not Affect Cell Growth in 2D**. Specifically inhibiting ALDH1A1 did not dramatically affect cell growth when MDA-MB-468 cells are cultured as a monolayer. Based on this result, it was reasonable to conclude that ALDH1A1 is not required for growth in 2D.

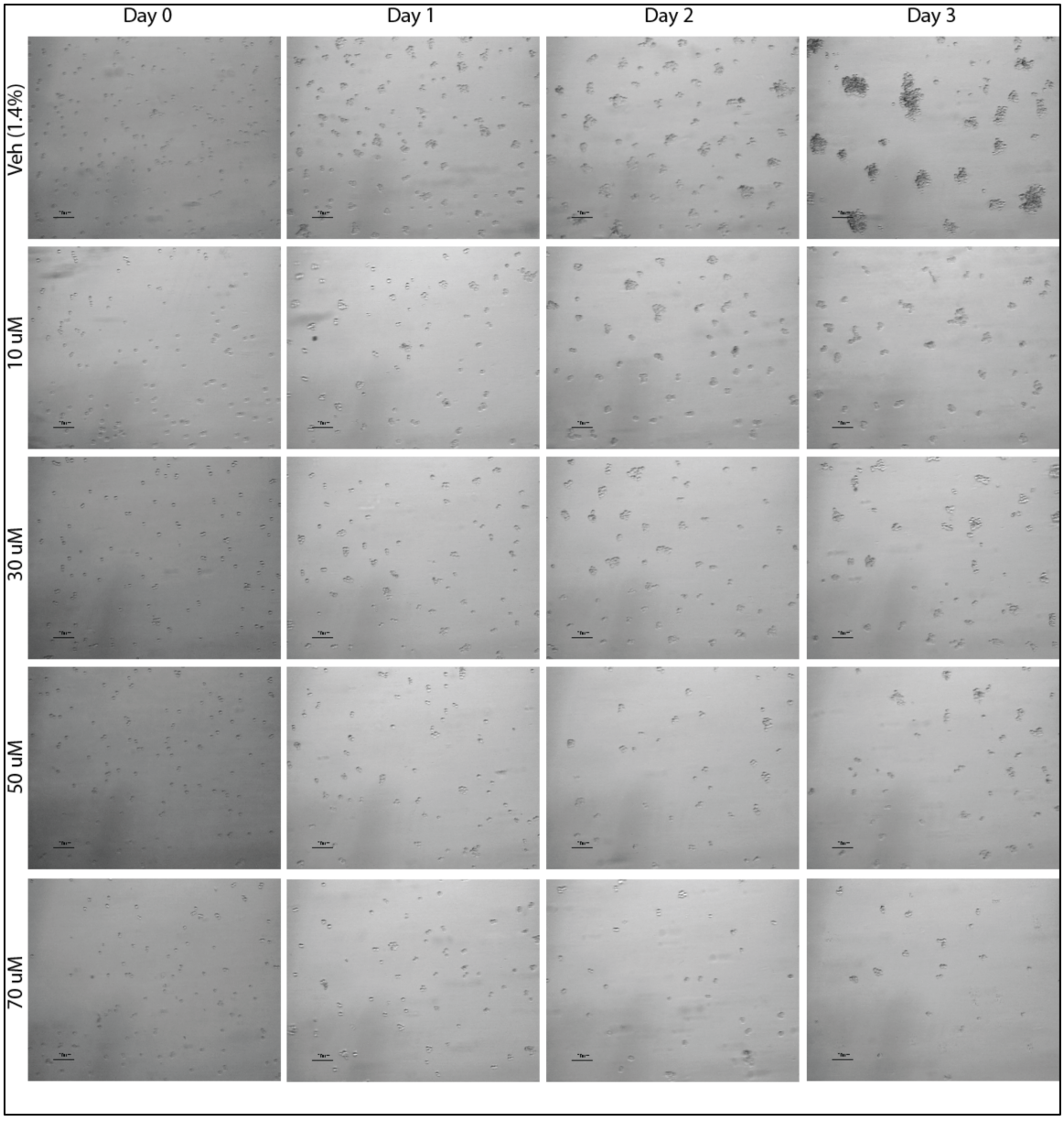

Figure 4. **MDA-MB-468 Cells are Sensitive to Inhibition of ALDH1A1 in 3D Culture.** When MDA-MB-468 cells were cultured on a lamin-rich reconstituted basement membrane (Matrigel®), and treated with CM037, the ALDH1A1 specific inhibitor, growth attenuation was observed. From this, it was postulated that ALDH1A1 activity was tuned by a mechansim sensitive to microenvironment changes, such as YAP.

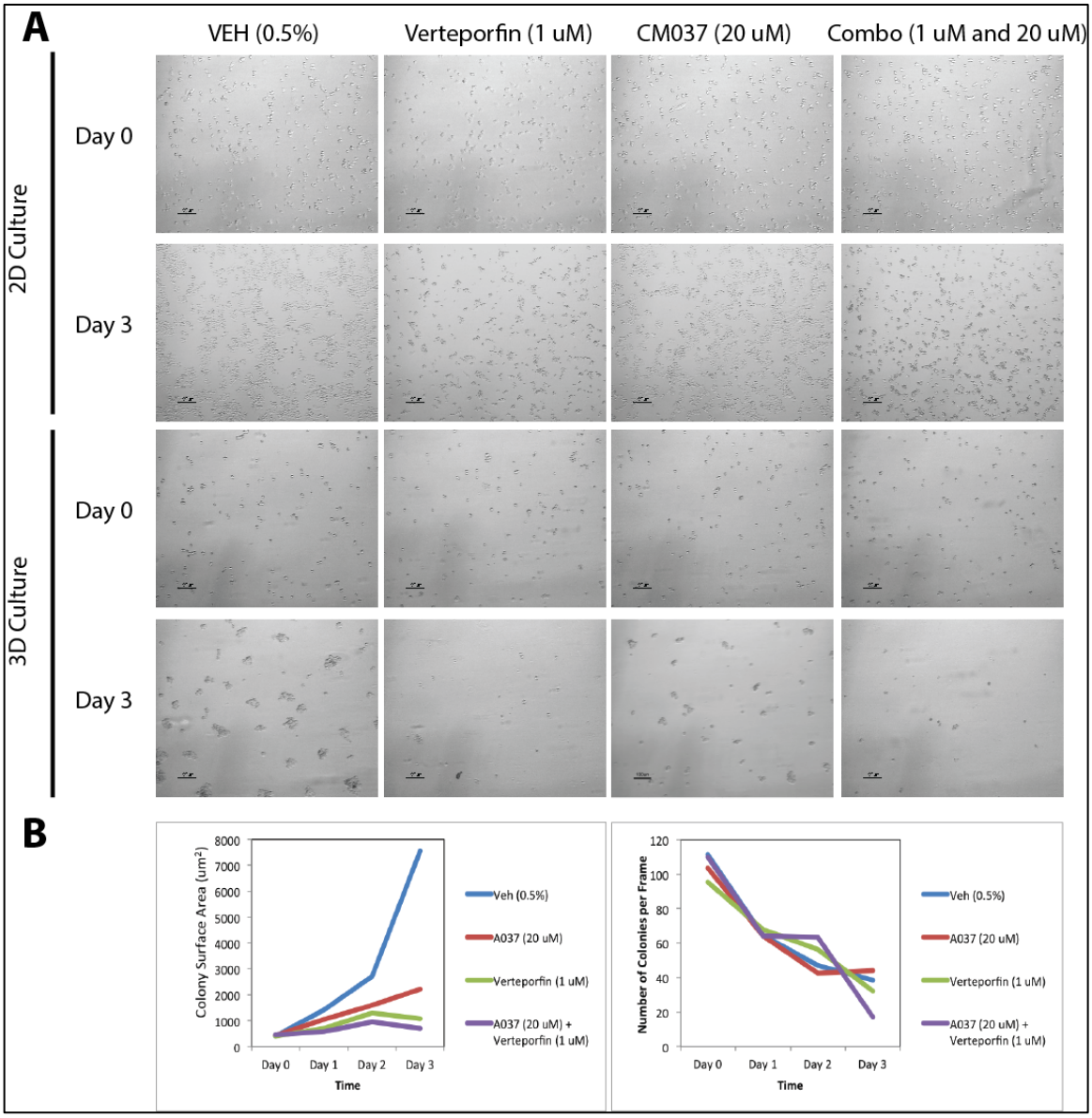

Figure 5. **Inhibition of ALDH1A1 Attenuates MDA-MB-468 Growth in 3D Culture**. A) Response to ALDH1A1 inhibition was unremarkable when MDA-MB-468 cells were cultured as a monolayer (upper panel). However, growth inhibition was acquired when cells were cultured in Matrigel®, a lamin-rich, basement membrane. This result indicated that ALDH1A1 activity could be correlated with signaling cascades sensitive to the extracellular environment. B) To delineate whether ALDH1A1 inhibition affected growth, or migration, pixel density analysis of colony number and size was used (See Materials and Methods). As shown above, the exponential growth in colony size was attenuated when cells were treated with CM037 (20 uM), Verteporfin (1 uM), or both. Compared to colony number however, the differences between treatment groups were unremarkable, suggesting that ALDH1A1 inhibition was affecting growth and not migration.

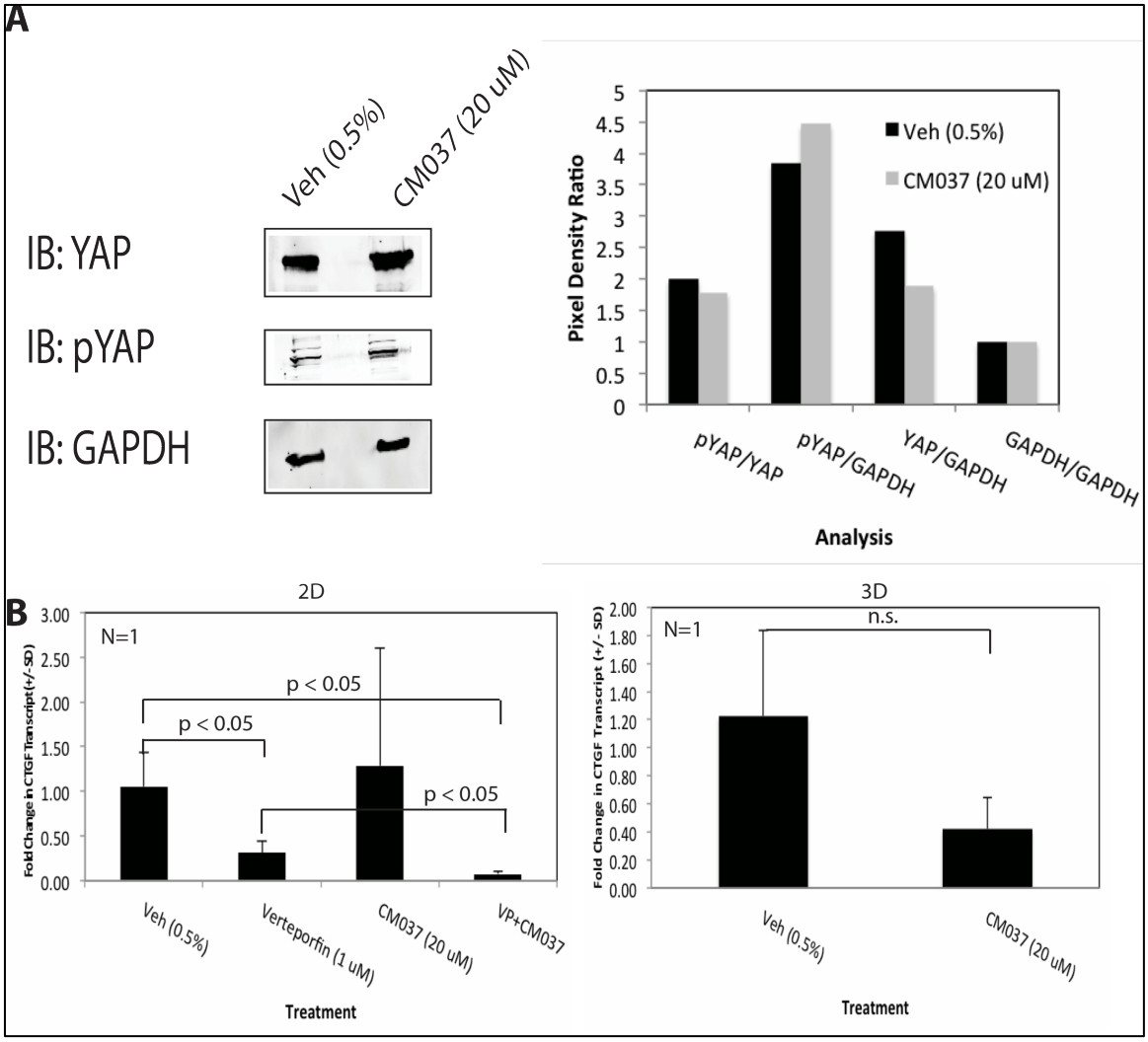

Figure 6. **Inhibition of ALDH1A1 Affects YAP Signaling**. A) Based on Western blot and pixel density analysis, it is reasonable to propose that as a monolayer, MDA-MB-468 cells do not respond to ALDH1A1 inhibition by phosphorylating YAP or modifying the amount of YAP protein. B) Conversely, when MDA-MB-468 cell are cultured in Matrigel®, there is a significant reduction in YAP nuclear activity, as measured by the fold change in CTGF transcript. Together, these results suggest that YAP activity and ALDH1A1 function are not mutually exclusive.

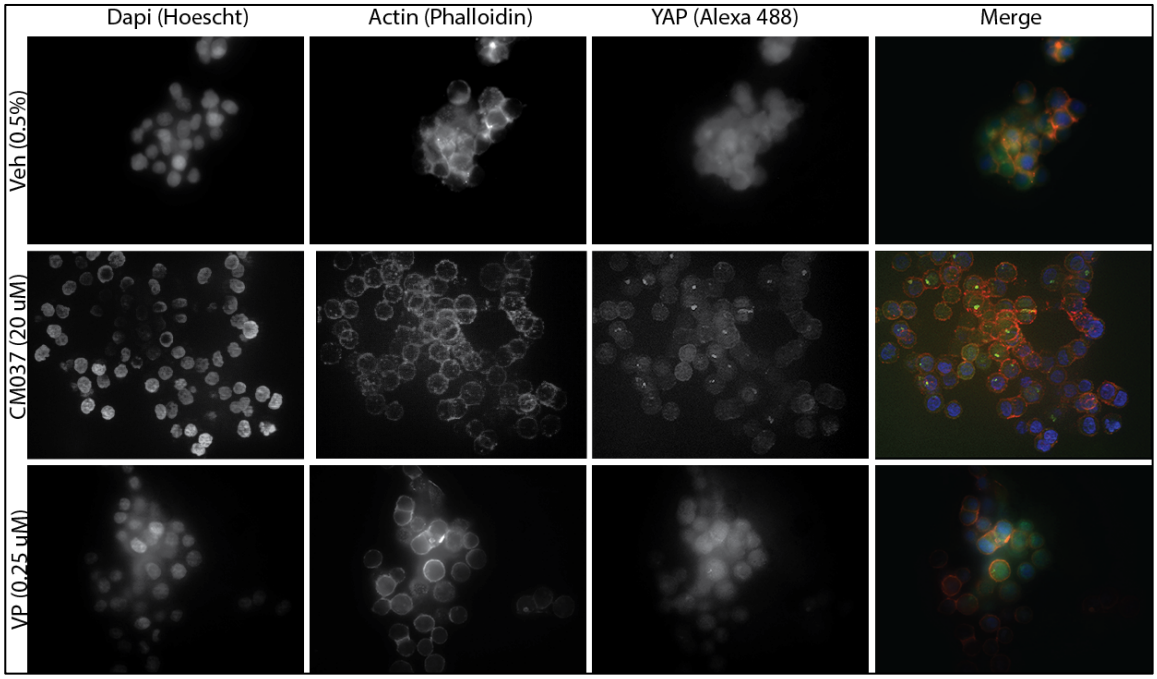

Figure 7. **Inhibition of ALDH1A1 facilitates cytosolic retention of YAP**. Previous work has demonstrated that YAP can be retained in the cytosol and targeted for degradation under metabolic stress. In this investigation, when MDA-MB-468 cells were cultured in Marigel© and treated with CM037, YAP was localized in the cytosol. These findings correlate with the growth assays and changes in CTGF, and ALDH1A1, transcription, indicating that the inhibition of ALDH1A1 is a form of cellular stress that prevents YAP nuclear localization.

Table 2. **ALDH Isoenzyme Characterization.** The ALDH isoenzyme family consists of 19 isoenzymes, each with a distinct coding region, function and cellular localization. Expression of ALDH1A1, or ALDH3A1, has been implicated as a marker for cancer stem cells but the functionality of the enzymes remains to be elucidated. TEADs have been more thoroughly defined in regards to embryonic development and bind to coding regions of ALDH1A1, ALDH1A2, ALDH2, ALDH4A1, and ALDH5A1. Based on this information, it is reasonable to conclude that YAP could drive expression of the TEAD-containing ALDHs.

Human Gene	Coding Region Position	Implicated in Stem Cells	TEAD Element
ALDH1A1	chr9:75,516,124-75,567,916	Yes	YES
ALDH1A2	chr15:58,247,395-58,357,848	No	YES
ALDH1A3	chr15:101,420,113-101,454,978	No	No
ALDH1B1	chr9:38,395,746-38,397,299	No	No
ALDH1L1	chr3:125,822,634-125,899,811	No	No
ALDH1L2	chr12:105,418,202-105,478,214	No	No
ALDH2	chr12:112,204,787-112,247,379	No	YES
ALDH3A1	chr17:19,641,534-19,648,442	Yes	No
ALDH3A2	chr17:19,552,285-19,576,547	No	YES
ALDH3B1	chr11:67,782,768-67,795,406	No	No
ALDH3B2	chr11:67,430,686-67,434,406	No	No
ALDH4A1	chr1:19,199,339-19,229,017	No	YES
ALDH5A1	chr6:24,495,225-24,533,940	No	YES
ALDH6A1	chr14:74,527,345-74,551,097	No	No
ALDH7A1	chr5:125,880,657-125,930,890	No	No
ALDH8A1	chr6:135,239,553-135,271,191	No	No
ALDH9A1	chr1:165,632,287-165,667,795	No	No
ALDH16A1	chr19:49,963,102-49,973,724	No	No
ALDH18A1	chr10:97,366,519-97,397,163	No	No

Table 3. **TEAD Response Elements in the ALDH Isoenzymes.** Further analysis of the ALDH genes indicates that TEAD binds to multiple regions of particular ALDH genes, suggesting that the expression of TEAD-containing ALDHs is differentially regulated.

Human Gene	TEAD Position 1	TEAD Position 2	TEAD Position 3
ALDH1A1	chr9:75562768-75563207	chr9:75549119-75549558	
ALDH1A2	chr15:58306274-58306588	chr15:58357920-58358283	
ALDH2	chr12:112204502-112204751	chr12:112212050-112212252	
ALDH3A2	chr17:19563703-19564009		
ALDH4A1	chr1:19228972-19229221	chr1:19221937-19222300	chr1:19210636-19210999
ALDH5A1	chr6:24504872-24505510		

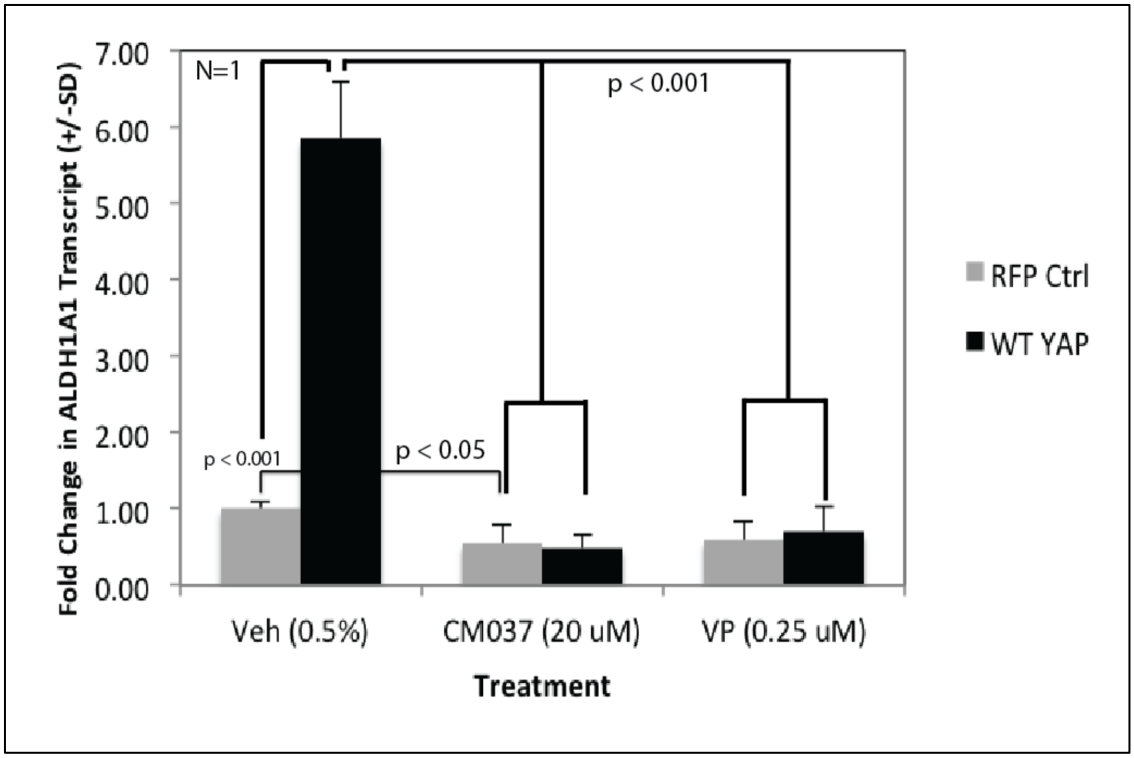

Figure 8. **Overexpression of YAP Correlates with an Increase in ALDH1A1 Expression.** By overexpressing YAP and promoting cell growth, the expression of ALDH1A1 increased significantly. Additionally, by treating cells with CM037 or Verteporfin, the increase in transcription of ALDH1A1 was attenuated. Together, these results demonstrate a novel link between YAP activity and the expression of ALDH1A1.

DISCUSSION

Anatomically, the highly specialized epithelia, specifically the luminal and myoepithelial cells, maintain a highly ordered architecture that dictates the function of the tissue (T. Gudjonsson, M. C. Adriance, M. D. Sternlicht, O. W. Petersen, & M. J. Bissell, 2005). These cells have been shown to be sensitive to changes in the composition of the underlying stroma and are implicated as structural tumor suppressors of the breast tissue (T. Gudjonsson et al., 2002). However, cancerous cells have altered responses to said regulation (D. Hanahan & R. A. Weinberg, 2011) which facilitates their aberrant growth and progression (M. W. Pickup, J. K. Mouw, & V. M. Weaver, 2014). Furthermore, pro-growth signaling can be altered by culture conditions that more closely reflect an *in vivo* status (M. W. Pickup et al., 2014). Consistently, we observed a dramatic increase in the potency of inhibition of the rapid proliferation of MDA-MB-468 cells by CM037 when cells were cultured in Matrigel© versus on plastic (Figure 4). Thus, the Matrigel© microenvironment is proposed to increase the dependence of these cells on YAP for their growth, which in turn makes them more sensitive to metabolic stresses induced by inhibiting ALDH1A1. In regards to tumor progression and applicability to patient outcomes, this suggests that as breast cancer progress into a more invasive form that presumably has a higher population of mesenchymal cells, they may become more sensitive to treatment with CM037.

As tumors progress and occupy more space, vasculature and other means of nutrient acquisition become limited, and cells sustain limitless replicative potential by alternative means (D. Hanahan & R. A. Weinberg, 2011). In this regard, Otto Warburg was first to postulate that cancer cells preferentially metabolize glucose through

glycolysis instead of the more efficient pathway, oxidative phosphorylation, even in the presence of oxygen (C. R. Justus, E. J. Sanderlin, & L. V. Yang, 2015; O. Warburg, 1956a, 1956b; O. Warburg, F. Wind, & E. Negelein, 1927). However, the by-products, mainly aldehydes and reactive oxygen species (ROS), of such a stressed metabolic state are toxic to the cells and therefore must be metabolized appropriately. ALDHs have already been characterized as mediators of cell detoxification (G. Muzio, M. Maggiora, E. Paiuzzi, M. Oraldi, & R. A. Canuto, 2012) and in particular, ALDH1A1 has been summarized to mediate the detoxification of oxazaphosphorine anticancer drugs as well as lipid peroxidation products (D. P. Agarwal, U. von Eitzen, D. Meier-Tackmann, & H. W. Goedde, 1995; N. E. Sladek, 1999). Based on our results, and the assumption that YAP is inducing a vulnerability to metabolic stress, it can be theorized that therapeutic intervention of CM037 exacerbates metabolic stress beyond a capacity that cells can handle and thus prevents breast cancer stem cells from utilizing stromal changes to facilitate YAP-mediated growth and transcription of stem-like genes, such as ALDH1A1. Additional experimentation would be needed to validate this idea and to define the mechanism by which this occurs. However, an analysis of the literature suggests a model described in the proceeding paragraph.

The AMP-activated protein kinase (AMPK) is a serine/threonine kinase (Ser/Thr) that is sensitive to cellular energy levels and is activated by changes in AMP:ATP, and ADP:ATP, ratios (D. Carling & B. Viollet, 2015). AMPK phosphorylates several substrates and recent work has shown that AMPK phosphorylates YAP in response to cellular energy stress (J. S. Mo et al., 2015). Additionally, liver kinase B1 (LKB1), which independently regulates AMPK activity via phosphorylation of Thr172 (D. Carling & B.

Viollet, 2015), also has been shown to regulate YAP through cytosolic retention post phosphorylation at Ser127 (H. B. Nguyen, J. T. Babcock, C. D. Wells, & L. A. Quilliam, 2013). Based on this information, one could postulate that the decrease in YAP activity (Figure 6 and Figure 8), and YAP cellular localization (Figure 7), is facilitated by YAP phosphorylation by either AMPK or LKB1. In order to test this hypothesis, co-immunoprecipitation assays of YAP and AMPK, or LKB1, and immunoblotting of phosphorylated YAP would be completed. Additionally, one should consider the potential impact of conversion of RA by ALDH1A1 and the consequent reduction in its levels that are available to promote RAR dependent gene expression that promotes cellular differentiation.

Canonically, RA promotes embryonic gene expression by binding to the nuclear retinoic acid receptor (RAR) (M. Petkovich, N. J. Brand, A. Krust, & P. Chambon, 1987) and facilitating the formation of the retinoid X receptor (RXR) and RAR heterodimer. The RAR-RXR complex then recognizes RA response elements (RARE) within the genome to coordinate gene expression (G. Duester, 2008). Independently of ligand presence (P. Germain et al., 2006), the RXR has also been shown to form a heterodimer with the peroxisome proliferator-activated receptor (PPAR) (S. A. Kliewer, K. Umesono, D. J. Noonan, R. A. Heyman, & R. M. Evans, 1992), which results in transcription of genes involved in cell metabolism (T. Varga, Z. Czimmerer, & L. Nagy, 2011; J. M. Way et al., 2001). In this context, it could be hypothesized that CM037 treatment, which inhibits formation of RA, triggers a nutrient recycling event where a metabolic flux is enhanced by RXR binding to PPAR, instead of RAR. It would therefore be potentially interesting to test whether ALDH1A1 inhibition or YAP activation in MDA-MB-468

cells alters the formation of the RXR-PPAR complex. If so, this would suggest a new mechanism by which the basal-like phenotype in MDA-MB-468 cells is driven by their expression of ALDH1A1

As previously stated, YAP could be inactivated through phosphorylation by AMPK or LKB1, in response to ALDH1A1 inhibition. However, this claim could be invalid if we were unable to clearly demonstrate that CM037 treatment is a metabolic stress. To test the degree to which this compound induces metabolic stress, one could measure the ratio of NAD/NADH in control cells or cells treated with CM037. If cells are highly stressed it would be predicted that NAD^+ will accumulate in excess over NADH. This could be further explored through a more complete characterization of the metabolic profile of these cells using the Seahorse assay, which would allow the determination of whether treatment with CM037 altered the extracellular acidification rate (ECAR) and/or oxygen consumption rate (OCR). Basal metabolism would be predicted to be glycolytic, whereas cells treated with CM037 and/or Verteporfin may have reduced glycolytic potential. If the NAD/NADPH and Seahorse assays demonstrate that metabolic stress is induced by CM037 treatment, then a novel mechanism by which YAP and ALDH1A1 are regulated could be proposed. Furthermore, results from these assays could provide novel support for directing therapies towards cancer metabolism.

Overall, there is a lack of demonstrated efficacy of new therapies against triple-negative breast cancer (E. Andreopoulou, S. J. Schweber, J. A. Sparano, & H. M. McDaid, 2015). For instance, the ineffectiveness of adjuvant therapies still remains a concern. For example, the inhibitor of the mammalian target of rapamycin (mTOR), everolimus (M. Yunokawa et al., 2012), and the anti-vascular endothelial growth factor

(aVEGF) monoclonal antibody, bevacizumab (K. Miller et al., 2007) failed to improve primary clinical outcomes such as progression-free survival. However, the work in this thesis, suggests a novel paradigm for treatments centered around the YAP/ALDH1A1 axis (Figures 5 and 8) that might be effective in the least treatable phase of the disease involving metastatic breast cancer. To this end, a xenograft transplant model could be proposed, where mice would be injected with MDA-MB-468, or patient-derived cells, and treated with, or without, CM037. To assess the efficacy of the compound overall survival, tumor grade, and rate of metastasis would be measured. This might extend our observations (Figures 3, 4, and 5) by showing that inhibition of ALDH1A1 impacts real tumor development

Overall, several outstanding questions remain. For instance, because cancer cells utilize various biochemical and molecular alternatives to promote growth and proliferation, there is the potential that the phenotype observed upon ALDH1A1 inhibition was propagated by another mechanism, or pathway, separate from inactivation of YAP such as through PPAR-RXR formation. Further, because we did not directly test total ALDH activity, it is not clear whether overall metabolism of aldehydes is being impacted by treatment with CM037 or whether there is a specific impact of ALDH1A1 inhibition. Another question is how the overexpression of YAP drives the transcription of the ALDH genes that have been shown to have TEAD4 binding within the gene. Furthermore, what is the role of activating a profile of ALDH1 genes versus ALDH1A1? Given our specific effects with CM037, it seems likely that ALDH1A1 is playing a critical role that may be complemented by expression of the other ALDH isoforms. Further, how important is the induction of the ALDH isoforms by YAP attributed to

establishing the metabolic profile of cancer stem cells? The answers to these questions will ultimately indicate whether treatment with CM037 will be a viable alternative for clinical studies testing its real therapeutic potential (Figure 9).

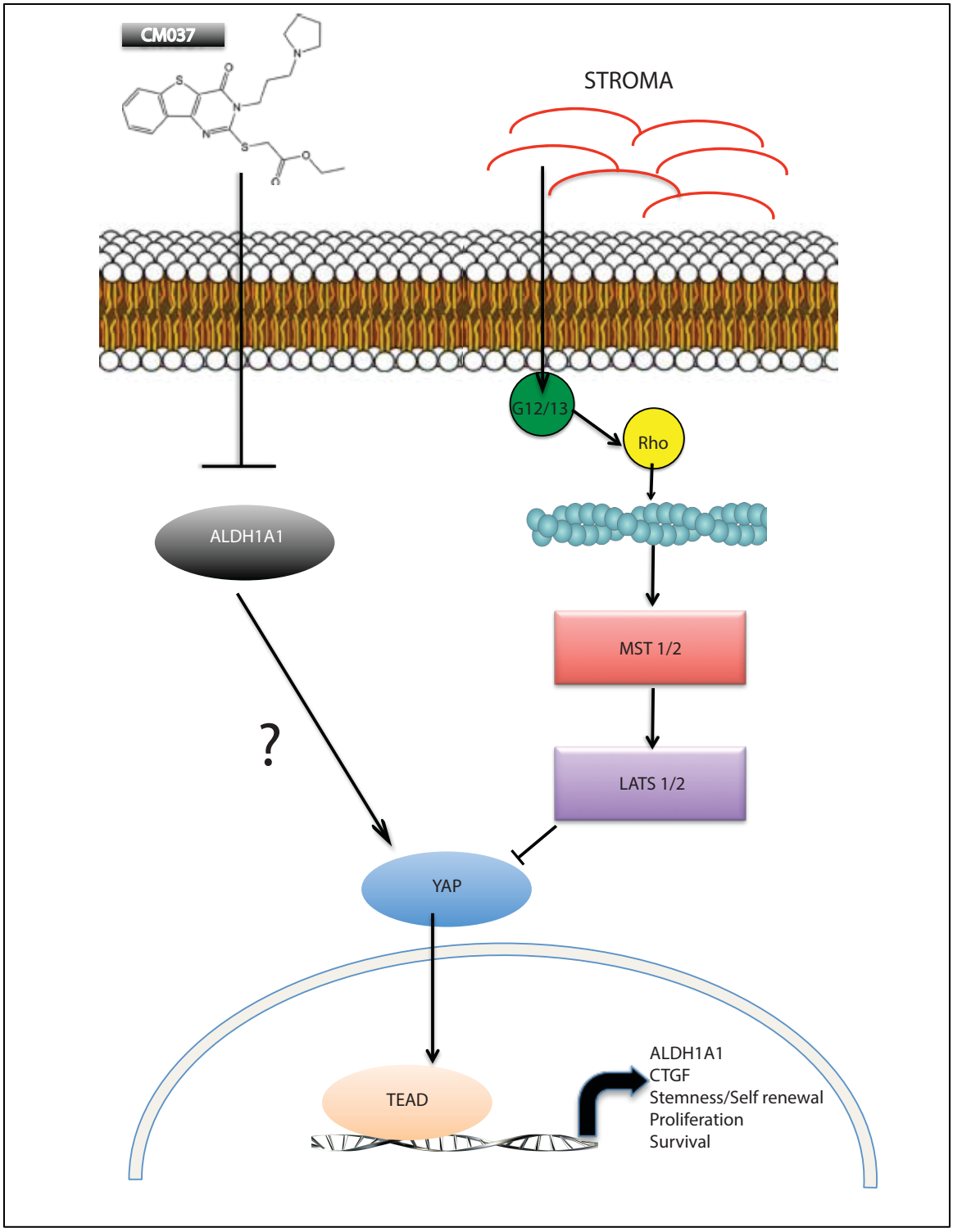

Figure 9. **Proposed Model for CM037 Inhibition of the YAP-Mediated Stem Cell Phenotype.** Based on the data collected in this study, there is reasonable evidence to conclude that stem-cell phenotype is facilitated by YAP nuclear localization where genes such as ALDH1A1 are up regulated. However, the stem-cell phenotype, which is thought to contribute to metastasis and recurrence, could be targeted by therapeutic administration of CM037.

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CURRICULUM VITAE

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Education

2015 M.S., Biochemistry and Molecular Biology, Indiana University, Indianapolis, IN

2014 B.S., Biology and Psychology, Magna Cum Laude, University of Colorado Denver, Denver, CO

Minor, Spanish Language and Chemistry, University of Colorado Denver, Denver, CO

Academic Honors

2014 Magna Cum Laude, University of Colorado Denver, Denver, CO

2011-2014 Reisher Scholar, University of Colorado Denver, Denver, CO

2011-2014 Dean's List, University of Colorado Denver, Denver, CO

2010 President's Scholarship, Saint John's University, Collegeville, MN

2010 Saint John's Abbey Grant, Saint John's University, Collegeville, MN

2010 Recognition Scholarship, Saint John's University, Collegeville, MN

2010 Saints Scholarship, Saint John's University, Collegeville, MN

Experience

2014 Molecular Biology and Biochemical Research, Laboratory of Clark Wells, Indiana University School of Medicine, Indianapolis, IN

2011-2014 Clinical Research, Student Research Assistant, Department of Neurology University of Colorado School of Medicine, Aurora, CO

2014 Pediatric Healthcare, Department of Neurology, Children's Hospital Colorado, Aurora, CO

2011-2014 Outpatient Healthcare, Department of Neurology, University of Colorado School of Medicine, Aurora, CO

2012 Leadership, organized *Kickin' it for a Cause*, suicide awareness kickball fundraiser, Longmont, CO

2012 Family Medicine Healthcare, Longs Peak Family Practice, Longmont, CO

2012 Inpatient Healthcare, Department of Neurology University of Colorado School of Medicine, Aurora, CO

Organizations and Active Memberships

2014 Morning Dove Therapeutic Riding Volunteer,

2013 Colorado Therapeutic Riding Center Volunteer, Longmont, CO

2011 Psi Chi Honors Society, University of Colorado Denver, Denver, CO

2011 Longmont United Hospital Volunteer, Longmont, CO

Certifications and Trainings

2014 Basic Life Support

2014 CITI Biomedical Responsible Conduct of Research

2014 IATA Hazardous Material Shipping

2014 Blood Borne Pathogens

2013 Multiple Sclerosis Functional Composite (MSFC) Administration

2013 Sloan Visual Acuity Assessment Administration

2011 CITI Health Information Privacy and Security (HIPS)

2011 CITI Group 1 Biomedical Investigators

Conferences Attended and Presentations Given

2015 Literature Review, Hypoxia regulates Hippo signalling through the SIAH2 ubiquitin E3 Ligase, Indiana University School of Medicine, Indianapolis, IN

2014 A Comparative Analysis of Gilenya™ and Tecfidera™, Neurology Research Retreat, Department of Neurology, University of Colorado School of Medicine

2014 Neurology Grand Rounds, Department of Neurology, University of Colorado School of Medicine, Aurora CO

2014 Pediatric Grand Rounds, Department of Pediatrics, University of Colorado, Anschutz Medical Campus

2013 Alzheimer's Research Symposium, Department of Neurology, University of Colorado School of Medicine Poster Presentation

2013 BIOL 4070, Human Reproductive Biology, Sex and Spinal Cord Injuries in Males

Professional History

2014 Patient Service Assistant, Pre/Post-Operative Care Unit, Indiana University Health, Riley Children's Hospital, Indianapolis, IN

2011-2014 Student Research Assistant, Department of Neurology, University of Colorado School of Medicine, Aurora, CO

2009-2011 Information Technology Assistant, Longmont, CO

2011 Service Clerk, Subway, Longmont, CO

2010 Grounds Crew Assistant, St. John's University, Collegeville, MN

2009-2010 Personal Assistant, Canyon Personal Training and Fitness, Longmont, CO

2007-2008 Youth Sports Official, Ed & Ruth Lehman YMCA, Longmont, CO