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YAP-mediated induction of monoacylglycerol lipase restrains oncogenic transformation

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Abstract

The Hippo pathway is an evolutionarily conserved regulator of normal and oncogenic growth. Engagement of Hippo pathway signaling results in the inactivation of the transcriptional coactivator YAP by preventing its nuclear entry. The mechanisms underlying the oncogenic properties of YAP remain incompletely understood. Here we find that although the transactivation (TA) domain of YAP mediates YAP-dependent gene expression, it serves as an inhibitor of YAP-mediated anchorage-independent growth. We identify monoacylglycerol lipase (MAGL) as a YAP transcriptional target and an inhibitor of anchorage-dependent cell growth. Significantly, knockdown of MAGL expression leads to the augmentation of YAP-dependent cell transformation. Our results identify MAGL as a transcriptional target of YAP that restrains YAP-mediated cellular transformation.

Keywords

YAP; transcriptional activation; cell transformation; cancer

1. Introduction

The Hippo pathway was first discovered in *Drosophila* and is an evolutionarily conserved regulator of normal and oncogenic growth [1–4]. At the central core of this pathway is the transcriptional coactivator YAP. There are two main mechanisms by which YAP is regulated by upstream Hippo pathway components. First, YAP can be phosphorylated by the LATS protein kinase, resulting in inhibition of YAP function by promoting its cytoplasmic localization and degradation by the proteasome. Also, upstream Hippo pathway components can form physical complexes with YAP, thereby sequestering it at cell-cell junctions and preventing its nuclear access [1–4]. Through the negative regulation of YAP, the Hippo pathway has been shown to regulate multiple cancer cell properties that are important for tumorigenesis. These properties include excess proliferation, cell survival, cell competition,

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and the maintenance of a stem cell phenotype [4]. Loss-of-function mutations of upstream Hippo pathway components and gain-of-function mutations of YAP have been shown to promote tumor formation in mice and flies [1–4].

Here, we find that even though the transactivation (TA) domain of YAP is essential for the induction of target genes such as CTGF, removal of the TA domain enhances YAP-dependent cell transformation. We identify monoacylglycerol lipase (MAGL) as an additional transcriptional target of YAP, which is dependent on an intact TA domain. MAGL is a serine hydrolase that hydrolyzes monoacylglycerols to glycerol and fatty acid [5]. We find that MAGL functions as an inhibitor of transformation when overexpressed in cancer cells. Also, MAGL knockdown enhances the YAP-mediated transformation of human mammary epithelial cells. Thus, MAGL is a transcriptional target of YAP that appears to mediate the negative effects of the TA domain on YAP-mediated cell transformation.

2. Materials and Methods

2.1 Cell Culture, transfections, and viral infections

HEK293T, MCF10A, Fadu, and SCC15 cells were obtained from ATCC. The HNSCC cell lines SCC1, SCC9, SCC23, SCC3, SCC6, SCC11B were derived from SCC of the head, neck, and oral cavity at the University of Michigan (Ann Arbor, MI) and have been described previously [6]. HN6, HN12, HN13, HN30 HNSCC cell lines were a gift of Dr. Gutkind (NIH). Human oral keratinocytes (HOK) were a gift of Dr. Kang (UCLA). HEK293T, SCC9, SCC23, and other HNSCC cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units penicillin/ml, and 50 mg streptomycin/ml (Invitrogen). MCF10A and HOK cells were grown in Mammary Epithelial Growth Media and Keratinocyte Growth Media (Lonza), respectively. Transient transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). For retroviral transductions, plasmids in pBabePuro or pQCXIH were transfected into the Phoenix Amphi cell line and retroviral particles were used to infect cells. In order to knockdown MAGL, pLKO.1-based plasmids were transfected along with the packaging plasmids psPAX2 and pMD2.G into HEK293T cells and lentiviral particles were used to infect cells. Two days after viral infections, puromycin (2 µg/ml) or hygromycin (200 µg/ml) was added as appropriate and cells were selected for two or seven days, respectively.

2.2 Reagents and plasmids

FLAG M2, β -actin, and α -tubulin mouse monoclonal antibodies are from Sigma. Akt and phospho-Akt (Ser 473) antibodies are from Cell Signaling. YAP rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (sc-15407). MAGL chicken polyclonal antibody from Novus (NB100-2493), and used in conjunction with an anti-chicken IgY HRP secondary antibody (Promega). pBabePuro-FLAG-YAP2 and YAP2 5SA constructs were constructed by PCR amplification from the previously described plasmids pQCXIH-Myc-YAP2 and pQCXIH-Myc-YAP2 5SA [7], and ligated into pBabePuro-FLAG. YAP TA, containing an internal deletion of codons 291 to 497, was constructed by overlapping PCR

subcloning. MAGL was amplified from a full-length cDNA (Open Biosystems, MGL clone ID#3163689) and subcloned into pBabe-Puro. The S132A mutation was introduced by using the Quikchange Site-Directed PCR Mutagenesis Kit (Agilent Technologies). MAGL shRNA lentiviral expression construct were constructed by subcloning oligo containing the target sequence 5'-TAAGACAGAGGTCGACATTTA-3' into pLKO.1 puro (Addgene #8453). The packaging vectors psPAX2 (#12260) and pMD2.G (#12259) were from Addgene.

2.3 Invasion and soft agar assays

BD Biocoat Matrigel Invasion Chambers (12 well) were used for cell invasion assays according to manufacturer's instructions. MCF10 cells (5×10^5) were seeded in normal growth media in the upper chamber and the lower chamber was filled with growth media supplemented with 0.1% FBS. Invaded cells were stained after incubation of chambers at 37°C after 24 hr. For soft agar assays, cells were seeded in 12 well plates onto a base agarose layer (0.75 ml) consisting of 1% low melt agarose (Life Technologies) mixed with regular growth media at a 1:1 ratio. Cells (5×10^5) were trypsinized and resuspended in the top agarose layer (0.75 ml) consisting of 1.5% low melt agarose mixed with regular growth media at a 1:3 ratio. Samples were prepared in quadruplicate. Plates were incubated at 37°C for 1–2 weeks. Regular growth media (0.375 ml) was added 1 day after cell seeding and every 3 days thereafter.

2.4 Total RNA extraction and real-time RT-PCR

Total RNA was recovered using TRIzol reagent (Life Technologies) according to the manufacturer's protocol, and cDNA was synthesized using oligo(dT) primers and SuperScript III (Life Technologies). Real-time RT-PCR analysis was performed using iQ SYBR Green supermix (Bio-Rad) on an iCycler iQ real-time PCR detection system (Bio-Rad). Human *GAPDH* was used as an internal control to calculate the relative expression. Sequences of the primer pairs used were as follows: human *MAGL* (5'-GGATGTGTTGCAGCATGTGG-3' and 5'-GCGAAATGAGTACCATGCCG-3') and human *CTGF* (5'-CCAATGACAACGCCTCCTG-3' and 5'-TGGTGCAGCCAGAAAGCTC-3') and human *GAPDH* (5'-AGCCACATCGCTCAGACACC-3' and 5'-CGCCCAATACGACCAAATCCG-3').

2.5 Immunoblotting

Supernatants were aspirated and cells were lysed in Triton X-100 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 30 mM NaF, 2 mM sodium pyrophosphate, 0.1 mM Na_3VO_4 , 10mM β -glycerophosphate, 1 mM dithiothreitol) and supplemented with complete EDTA-free protease inhibitor cocktail (Agilent Technologies). Proteins were separated by SDS-PAGE.

3. Results

3.1 The transactivation domain of YAP impedes cellular transformation

Previous studies have shown that the C-terminus of YAP contains a potent C-terminal transactivation (TA) domain [8]. We sought to determine the effect of removal of the TA domain on the oncogenic activity of YAP. A constitutively active and oncogenic form of

YAP, called YAP 5SA, has been previously characterized. YAP 5SA contains alanine substitutions at five LATS phosphorylation sites, thereby preventing YAP proteasome degradation and cytoplasmic sequestration [7,9]. In order to study the effect of removal of the TA domain from aa 291 to 497 on the oncogenic activity of YAP 5SA, we introduced a deletion of the TA domain into constitutively active YAP (YAP 5SA TA). This deletion removes the TA domain without interrupting the C-terminal PDZ-binding motif, which is important of nuclear entry [10,11]. We stably expressed YAP 5SA and YAP 5SA TA in the nontransformed mammary epithelial cell line MCF10A by retroviral transduction. We will refer to these stably transduced cells as MCF10A/5SA and MCF10A/5SA TA from here on out for convenience.

We tested our stably transduced cells in both soft agar and cell invasion assays. The soft agar assay measures anchorage-independent growth, one of the hallmark characteristics of cellular transformation and uncontrolled cell growth. Consistent with previous studies, MCF10A/5SA cells demonstrated anchorage-independent growth and enhanced cell invasion, when compared to vector control cells [9] (Figure 1A). Interestingly, we found that MCF10A/5SA TA cells were able to induce significantly a greater number of soft agar colonies than MCF10A/5SA cells. However MCF10A/5SA TA cells demonstrated lower cell invasion than MCF10A/5SA cells (Figure 1B). These data suggest that the TA domain impedes YAP 5SA-dependent anchorage-independent growth, but is necessary for YAP 5SA-dependent cell invasion.

To examine the importance of the TA domain to transcriptional activation, we measured the levels of transcripts of the known YAP target gene *CTGF*. We purified RNA from both confluent and nonconfluent cells. Growing cells to confluency induces cell-cell contact, a known inhibitor of YAP [7]. As expected, confluent vector control MCF10A cells displayed less *CTGF* mRNA than nonconfluent cells (Figure 1C). Also, expression of YAP 5SA elevated *CTGF* mRNA in both confluent and nonconfluent cells. However, the YAP 5SA TA mutant was not able to enhance *CTGF* mRNA levels in confluent or nonconfluent conditions, demonstrating that it is not transcriptionally active (Figure 1C). Therefore, although deletion of the TA domain renders YAP 5SA transcriptionally inactive and inhibits cell invasion, this deletion enhances YAP 5SA-induced anchorage-independent growth. Our results suggest that the TA domain restrains YAP 5SA-mediated cell transformation.

3.2 Monoacylglycerol lipase is a transcriptional target of YAP

In our efforts to determine the mechanism behind the negative effect of the TA domain on cell transformation, we looked for candidate “tumor-suppressive” genes that were transcriptionally downregulated by the stable knockdown of YAP expression MCF10A cells by microarray analysis in a previously published data set [12]. In this data set, we focused our attention on the gene encoding for monoacylglycerol lipase (MAGL) whose expression was diminished in MCF10A cells with stable knockdown of YAP. MAGL is a serine hydrolase that breaks down monoacylglycerol to glycerol and fatty acid [5]. The role of MAGL in cancer remains completely understood. Previously, MAGL was shown to mediate cell invasion in highly invasive cancer cell lines [13]. On the other hand, MAGL was also

found to be reduced in a majority of primary colorectal cancers and to suppress colony formation in colorectal cancer cells when overexpressed [14].

In order to first determine whether *MAGL* was a target gene of YAP, we examined the expression of *MAGL* mRNA and protein in both nonconfluent and confluent MCF10A/5SA cells. We found that vector control cells displayed lower levels of *MAGL* mRNA levels when confluent than when nonconfluent, consistent with negative regulation by the Hippo pathway activation (Figure 2A). Significantly, MCF10A/5SA cells displayed elevated levels of *MAGL* mRNA under both confluent and nonconfluent conditions. Similar results were observed when examining *MAGL* protein levels from lysates of the same cells (Figure 2B). These results indicate that *MAGL* expression is negatively regulated by contact inhibition in which YAP is inactive and induced by the expression of active YAP. To determine whether this transcriptional regulation is dependent on the transcriptional activation function of YAP, we also examined cells stably expressing the YAP 5SA TA deletion mutant. We found that MCF10A/5SA TA cells failed to induce *MAGL* mRNA or protein, a result consistent with the idea that *MAGL* is a transcriptional target of YAP (Figures 2A and B).

3.3 Monoacylglycerol lipase is a suppressor of cell transformation

We found that *MAGL* expression at both the mRNA and protein level was diminished in a subset of head and neck squamous cell lines we examined when compared to normal human oral keratinocytes (Figures 3A and B). To determine whether *MAGL* could affect cell transformation, we stably transduced *MAGL* into both SCC23 and SCC9 cells, both of which display relatively low levels of *MAGL* at both the mRNA and protein levels. Interestingly, YAP protein levels in these two cell lines were elevated, suggesting that *MAGL* expression was uncoupled from regulation by YAP. We found that stable expression of *MAGL* was a potent inhibitor of anchorage independent growth in both cell lines (Figures 3C and D). Interestingly, a catalytically inactive version of *MAGL* in which the active site serine nucleophile is mutated to alanine (S132A) was also able to inhibit soft agar colony formation as well as wild-type *MAGL* (Figures 3C and D). Previous data had suggested that overexpression of *MAGL* could inhibit Akt activation in colorectal cancer cells [14]. We noted a slight decrease in Akt phosphorylation in SCC23 cells stably expressing *MAGL* but not with the S132A mutant (Figure 3E). These data together suggest that *MAGL* can inhibit cellular transformation when overexpressed in cancer cells, likely through mechanisms independent of Akt.

3.4 Monoacylglycerol lipase restrains YAP-mediated cell transformation

Given that deletion of the TA domain of YAP 5SA prevented *MAGL* induction while enhancing anchorage-independent growth, we sought to determine the effect of *MAGL* knockdown on YAP 5SA-mediated cell transformation. MCF10A/5SA cells were stably infected with lentivirus expressing control shRNA or shRNA targeting *MAGL*. Cells stably transduced with *MAGL* shRNA demonstrated diminished protein expression (Figure 4A). In soft agar assays, we found that knockdown of *MAGL* in MCF10A/5SA cells significantly enhanced anchorage-dependent growth (Figure 4B). On the other hand, we found that knockdown of *MAGL* inhibited matrigel invasion of MCF10A/5SA cells (Figure 4C). Thus, the induction of *MAGL* of YAP restrains YAP-mediated cell transformation.

4. Discussion

In order to investigate the impact of the deletion of the TA domain of YAP on YAP-mediated cell transformation, we have made stable pools of MCF10A cells expressing the oncogenic YAP 5SA mutant or a deletion mutant lacking the TA domain. In our soft agar assays, we found that deletion of the TA domain enhances the ability of YAP 5SA to cause anchorage-independent growth. At the same time, removal of the TA domain inhibits the ability of YAP 5SA to cause matrigel invasion. In our search for transcriptional targets of YAP that may contribute inhibit cell transformation, we discovered that the lipid hydrolase MAGL as a candidate YAP target. MAGL mRNA and protein levels are repressed by confluency, which activates the negative-regulating Hippo pathway, and are induced by YAP 5SA in a TA domain-dependent manner. Significantly, MAGL expression was reduced in several HNSCC cell lines and stable expression of MAGL in two cell lines potentially inhibited anchorage-independent growth. Furthermore, knockdown of MAGL expression in YAP-transformed cells inhibited cell transformation while enhancing matrigel invasion. Our results together reveal MAGL as a YAP target gene that restrains cellular transformation.

Our findings suggest that the cell transformation by YAP in cancer cells may be facilitated or potentiated by the loss of MAGL protein. Previously, it was found that MAGL played a part in the cell invasive phenotype of some highly invasive cancer cell lines [13]. Our results confirm the role of MAGL in cell invasion, but identify a novel function of MAGL in inhibiting anchorage-independent growth. It will be of considerable interest to determine the mechanism by which MAGL inhibits cell transformation given that our data suggests that its serine hydrolase activity may not be required for this function. Also, it will be interesting to examine how MAGL expression is lost in cancer cells and whether the presence of activated YAP and low MAGL expression can predict the transformed phenotype.

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Abbreviations

YAP	Yes-associated protein
MAGL	monoacylglycerol lipase
CTGF	connective tissue growth factor
HOK	human oral keratinocyte

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Highlights

The YAP transactivation domain negatively regulates cell transformation.

MAGL is a transcriptional target of YAP.

MAGL is a negative regulator of cell transformation.

MAGL restrains YAP-mediated cell transformation

Figure 1. The YAP TA domain negatively regulates YAP 5SA-dependent cell transformation
A, MCF10A cells stably expressing YAP 5SA or YAP 5SA Δ TA were examined for anchorage-independent growth by performing soft agar assays. The number of soft agar colonies is indicated (mean $\pm$ standard deviation). *, *P* value of < 0.001 for comparison of YAP 5SA and YAP 5SA Δ TA (*n*=4). **B**, MCF10A/5SA or MCF10A/5SA Δ TA cells were tested for cell invasion by performing matrigel cell chamber assays. The number of invaded cells is indicated (mean $\pm$ standard deviation). **C**, Total RNA from MCF10A/5SA, MCF10A/5SA Δ TA, or vector (V) control cells at confluent (C) or nonconfluent (NC) conditions was prepared and real-time PCR was performed using specific primers for *CTGF*.

Figure 2. MAGL is a transcriptional target of YAP

A, Total RNA from MCF10A/5SA, MCF10A/5SA Δ TA, or vector (V) control cells at confluent (C) or nonconfluent (NC) conditions was prepared and real-time PCR was performed using specific primers for *MAGL*. B, Whole cell lysates from MCF10A/5SA, MCF10A/5SA Δ TA, or vector (V) control cells at confluent (C) or nonconfluent (NC) conditions was prepared and probed with antibodies to MAGL α -tubulin.

A **B**

E

Figure 3. MAGL is a suppressor of cell transformation

A, Total RNA from various HNSCC lines and normal human oral keratinocytes (HOK) were prepared and real-time PCR was performed using specific primers for *MAGL*. *GAPDH* was used as an internal control. (B) Whole cell lysates from various HNSCC lines and HOKs were analyzed by SDS-PAGE and probed with MAGL antibody. C, SCC23 cells stably expressing YAP 5SA, YAP 5SA TA, or vector (V) alone were examined for anchorage-independent growth by performing soft agar assays. D, SCC9 cells stably YAP 5SA, YAP 5SA TA, or vector (V) alone were examined for anchorage-independent growth. E, Whole cell lysates from SCC23 cells stably expressing YAP 5SA or YAP 5SA TA were probed for MAGL, phospho-Akt, Akt, or β-actin.

A **B**

Figure 4. MAGL restrains YAP-induced cell transformation

MCF10A/5SA cells or vector control cells were stably transduced with control shRNA (shScramb) or MAGL shRNA (shMAGL). *A*, Whole cell lysates were prepared and probed for MAGL, Myc, or β -actin expression. *B*, Cells were examined for anchorage-independent growth by performing soft agar assays. The number of soft agar colonies is indicated (mean $\pm$ standard deviation). *C*, Cells were tested for cell invasion by performing matrigel cell invasion chamber assays. The number of invaded cells is indicated (mean $\pm$ standard deviation).