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Journal

Oncogene, 37(41)

ISSN

0950-9232

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Publication Date

2018-10-11

DOI

10.1038/s41388-018-0301-5

Peer reviewed



YAP and MRTF-A, transcriptional co-activators of RhoA-mediated gene expression, are critical for glioblastoma tumorigenicity

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Received: 12 June 2017 / Revised: 28 February 2018 / Accepted: 13 April 2018
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Abstract

The role of YAP (Yes-associated protein 1) and MRTF-A (myocardin-related transcription factor A), two transcriptional co-activators regulated downstream of GPCRs (G protein-coupled receptors) and RhoA, in the growth of glioblastoma cells and in vivo glioblastoma multiforme (GBM) tumor development was explored using human glioblastoma cell lines and tumor-initiating cells derived from patient-derived xenografts (PDX). Knockdown of these co-activators in GSC-23 PDX cells using short hairpin RNA significantly attenuated in vitro self-renewal capability assessed by limiting dilution, oncogene expression, and neurosphere formation. Orthotopic xenografts of the MRTF-A and YAP knockdown PDX cells formed significantly smaller tumors and were of lower morbidity than wild-type cells. In vitro studies used PDX and 1321N1 glioblastoma cells to examine functional responses to sphingosine 1-phosphate (S1P), a GPCR agonist that activates RhoA signaling, demonstrated that YAP signaling was required for cell migration and invasion, whereas MRTF-A was required for cell adhesion; both YAP and MRTF-A were required for proliferation. Gene expression analysis by RNA-sequencing of S1P-treated MRTF-A or YAP knockout cells identified 44 genes that were induced through RhoA and highly dependent on YAP, MRTF-A, or both. Knockdown of *F3* (tissue factor (TF)), a target gene regulated selectively through YAP, blocked cell invasion and migration, whereas knockdown of *HBEGF* (heparin-binding epidermal growth factor-like growth factor), a gene selectively induced through MRTF-A, prevented cell adhesion in response to S1P. Proliferation was sensitive to knockdown of target genes regulated through either or both YAP and MRTF-A. Expression of TF and HBEGF was also selectively decreased in tumors from PDX cells lacking YAP or MRTF-A, indicating that these transcriptional pathways are regulated in preclinical GBM models and suggesting that their activation through GPCRs and RhoA contributes to growth and maintenance of human GBM.

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Electronic supplementary material The online version of this article (<https://doi.org/10.1038/s41388-018-0301-5>) contains supplementary material, which is available to authorized users.

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Introduction

G protein-coupled receptors (GPCRs) are well-accepted targets in drug therapy. Their dysregulated activation in disease generally occurs through upregulation or increased availability of their ligands. GPCRs that couple to $G\alpha_{12}$ and

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$G\alpha_{13}$ proteins regulate guanine nucleotide exchange factors (GEFs) for RhoA activation. The $G\alpha_{12}$ and $G\alpha_{13}$ proteins, as well as the Rho GEFs, are oncogenic and there is considerable evidence implicating RhoA signaling in aberrant cell growth [1–8]. While several mechanisms through which RhoA signaling contributes to cell transformation and proliferation have been suggested [9–15], there is limited evidence for their importance in vivo.

RhoA signaling was determined more than two decades ago to mediate serum-induced gene transcription. Notably, RhoA-mediated transcriptional responses to serum, while involving serum response factor (SRF), did not require the canonical transcriptional co-activator, ternary complex factor (TCF) [16, 17]. Instead, another transcriptional co-activator, MRTF-A, a member of the myocardin gene family, was determined to serve this function [18, 19]. Under basal conditions MRTF-A is bound to G-actin and this prevents it from translocating to the nucleus. Serum or GPCRs that activate RhoA increase the polymerization of actin, releasing MRTF-A and allowing it to accumulate in the nucleus [18–21]. Yes-associated protein (YAP) is another transcriptional co-activator which was recently shown to be regulated in response to activation of GPCRs that couple to $G\alpha_{12/13}$ to activate RhoA [13, 22–25]. The dephosphorylation of YAP in response to RhoA-mediated signals leads to its nuclear translocation. YAP does not serve as a co-activator of SRF but rather of the TEA domain containing transcription factor family [23, 25–29]. Thus, there are parallel pathways involving activation of both MRTF-A and YAP through which GPCRs that activate RhoA can induce transcriptional gene programs.

Previous work from our laboratory demonstrated that both thrombin and sphingosine 1-phosphate (S1P) agonists elicit mitogenic responses in 1321N1 cells [10, 30] (a subclone of the U118-MG cells isolated from human cerebral glioblastoma multiforme (GBM) [31]) through coupling of their cognate GPCRs (PAR-1 and $S1P_{2/3}$) to RhoA activation. We also reported that YAP and MRTF-A are concomitantly activated in response to S1P-induced and thrombin-induced RhoA signaling in these cells [30]. Cyclic stretch has also been shown to coordinately activate YAP and MRTF-A [32]. Remarkably, we showed that both transcriptional co-activators were required for S1P to induce expression of the cell matrix protein Cyr61 (CCN1), as well as three other common target genes [30]. Both co-activators are also required for S1P-mediated proliferation as well as for stretch-mediated proliferation [30, 32]. Our work and that of others suggests that MRTF-A and YAP can interact to co-regulate common gene targets [30, 33]. Here we explored whether distinct, as well as common, functions of MRTF-A-mediated and YAP-mediated transcriptional programs contribute to cellular responses and in vivo tumor growth of human GBM.

GBM, a highly malignant and deadly tumor, is known to be driven through genomic alterations in three core pathways: Tp53 (86%), Rb (79%), and receptor tyrosine kinase (RTK)/Ras/phosphoinositide 3 (PI3)-kinase signaling (90%) [34, 35]. Notably, RhoA has been shown to synergize with Ras in inducing transformation [4, 36–38]. GBM tumors and cell lines both overexpress sphingosine kinase which, in turn, generates S1P [39–41], as well as thrombin [42, 43] and autotaxin, the enzyme responsible for lysophosphatidic acid (LPA) formation [44, 45]. Thus, we postulated that activation of GPCRs by these ligands in the tumor environment leads to RhoA-mediated transcriptional responses that complement the effects of Ras activation on GBM progression. Here, we carried out studies using both 1321N1 glioblastoma cells and tumor-initiating cells from patient-derived xenografts (PDX) to demonstrate that YAP and MRTF-A and their target genes play critical roles in functional responses to GPCR ligands in vitro and GBM tumor growth in vivo.

Results

YAP and MRTF-A in glioblastoma and PDX cells

Our previous work demonstrated synergistic effects of YAP and MRTF-A on expression of CCN1 and other cancer-associated genes, as well as on proliferation elicited by RhoA activation in the human 1321N1 glioblastoma cell line. We postulated that this pathway contributes to progression of GBM and first sought evidence for this by interrogation of Project Betastasis, a repository for molecular brain neoplasia data. Elevated levels of $G\alpha_{12}$ and RhoA, upstream activators of YAP and MRTF-A, and of CCN1, a canonical YAP-regulated and MRTF-A-regulated target gene, were documented in astrocytoma and in GBM of the mesenchymal subtype (Supplemental Fig. 1A). Data from The Cancer Genome Atlas (TCGA) were also analyzed and revealed that the mRNA for $G\alpha_{12}$, an upstream activator of RhoA, is elevated in 26% of GBM patient tumors (Supplemental Fig. 1B), the highest of all tumor types surveyed (Supplemental Fig. 1C). The $G\alpha_{12}$ homolog $G\alpha_{13}$ is also upregulated, as are RhoA and several GPCRs that regulate its activation (e.g., $S1P_2$, $S1P_3$ and PAR-1).

PDX are tumor explants grown at low passage under conditions that conserve their original tumor characteristics. Surveying multiple previously studied GBM PDX lines [46–48], we determined the levels of mRNA expression of genes involved in the signaling pathway of interest, in particular $G\alpha_{12}$, RhoA, YAP, MRTF-A, and CCN1 (Supplemental Table 1). The mRNA level of the lowest expressing PDX line for any given gene was set at 1, and

expression of that gene in the other lines determined relative to that value. We selected GSC-23 from amongst these lines for further study because this line had relatively high levels of expression of all of the genes of interest, as well as a highly aggressive nature [49].

To examine involvement of YAP and MRTF-A in GSC-23 cell responses, we made stable knockdowns of these transcriptional co-activators using short hairpin RNA (shRNA), achieving 65 and 75% knockdown for YAP and MRTF-A, respectively (Supplemental Fig. 2A, B). The expression of TAZ, a YAP paralog, and of MRTF-B, an MRTF-A paralog was also examined and found to be unchanged (Supplemental Fig. 2A, B). Two sets of cells with different shRNA sequences (but similar levels of knockdown) were analyzed to eliminate off-target effects. To examine the role of YAP and MRTF-A in the self-renewal capability of the cancer stem cells (GSC-23), we carried out an *in vitro* extreme limiting dilution assay in which cells were serially diluted, plated, and frequency of sphere formation determined (Fig. 1a). This assay assesses the fraction of cancer stem cells in a tissue culture capable of forming spheres, an indicator of cancer stem cell self-renewal capacity [50]. The number of cells needed to form neurospheres was more than doubled when either YAP or MRTF-A were deleted (1/ stem cell frequency increased from 20.9 to between 48.5 and 51.6 for YAP and MRTF-A knockdown, respectively). To further assess effects of these transcriptional co-activators on the stem-like properties of GSC-23 cells, we analyzed neurosphere formation and size, plating cells in microwells to ensure that proliferation and sphere formation result from single cells. Single sphere formation was reduced by more than 60% in both YAP and MRTF-A knockdown cells compared to control cells (Fig. 1b). Finally, we examined a number of canonical stem cell genes (e.g., CCND1, MYC, NANOG, OCT4, PAX6, SOX2, and NESTIN) [50] and demonstrated that their expression was diminished in response to downregulation of MRTF-A or YAP (Fig. 1c). These results indicate that YAP and MRTF-A are required to maintain stem cell properties of these GSC cells.

In vivo growth of orthotopic GSC-23 xenografts

The GSC-23 cells with shRNA knockdown of YAP or MRTF-A were then injected intracranially into immunocompromised mice and their growth properties compared to those of shRNA control cells. There was more than a 50% reduction in tumor size in mice injected with cells with reduced YAP and a 60% decrease in cells with reduced MRTF-A relative to control shRNA cells (Fig. 2a–c). Brains from these mice were sectioned and stained with hematoxylin and eosin (H&E). Knockdown of either

YAP or MRTF-A reduced the number of mitotic figures, an indicator of tumor proliferation (Fig. 2d–f). Mice injected with GSC-23 cells lacking YAP or MRTF-A lived significantly longer than did mice injected with control shRNA cells (50% mortality at 15 days compared to 22 days) (Fig. 2g). Thus, inhibition of YAP or MRTF-A signaling reduces tumor growth and extends overall survival. Tumors from different groups were also isolated at the time that control group showed neurological signs to demonstrate that both YAP and MRTF-A mRNA levels remained downregulated (Supplemental Fig. 3). Expression of YAP and MRTF-A target genes discovered below were also determined in the tumors, and are discussed later (Fig. 5).

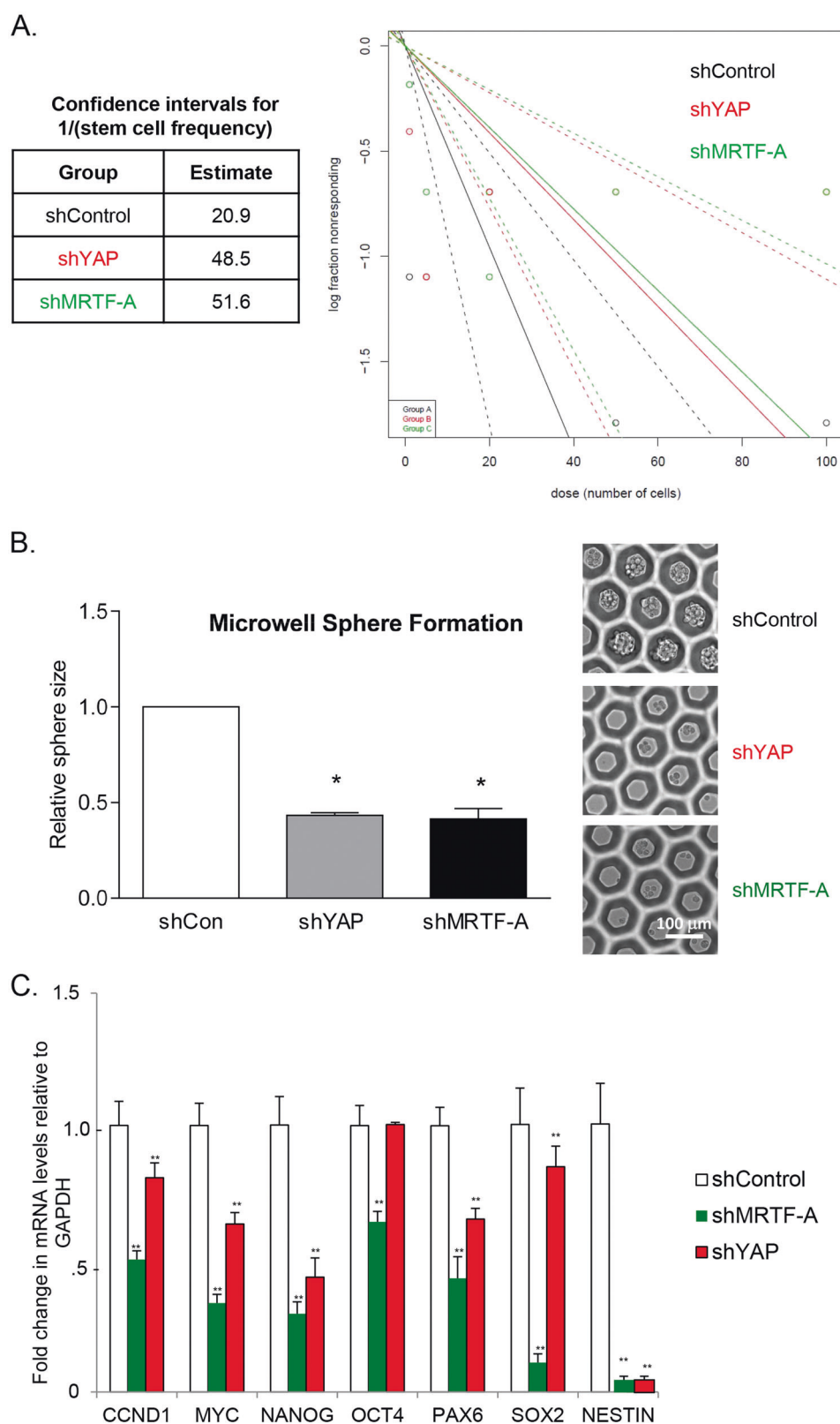
YAP and MRTF-A involvement in adhesion, migration, and invasion in GSC-23 cells

To determine if YAP and MRTF-A differentially contribute to cellular responses relevant to tumor progression, we examined cell adhesion, migration, and invasion of GSC-23 GBM cells. Adhesion to gelatin was stimulated by S1P treatment and blocked by knockdown of MRTF-A, but this response was remarkably unaffected by knockdown of YAP (Fig. 3a, b). Conversely, reducing YAP significantly reduced S1P-stimulated invasion of GSC-23 cells through Matrigel-coated transwells (Fig. 3c, d). Invasion also reflects the ability of the cells to migrate; thus, we also examined migration through transwells in the absence of Matrigel and determined that this was blocked by knockdown of YAP but not MRTF-A (Fig. 3e, f). These data indicate that YAP signaling plays a critical role in regulating cellular invasion and migration in response to S1P, while MRTF-A controls S1P-regulated adhesion of GSC-23 cells.

YAP and MRTF-A involvement in adhesion, migration, and invasion of 1321N1 glioblastoma cells

We then turned to the 1321N1 human glioblastoma cell line, in which we previously demonstrated co-ordinate and synergistic agonist signaling to YAP and MRTF-A [30], for *in vitro* loss of function studies. YAP and MRTF-A gene deletion were achieved by CRISPR/Cas9 gene editing (Supplemental Fig. 4A, B). Two distinct clones, each generated with two separate guide RNAs, were used to confirm all observations and eliminate errors due to off-target effects. We also determined that TAZ and MRTF-B, paralogs that could serve redundant functions with YAP and MRTF-A, respectively [51, 52], were not transcriptionally upregulated in the CRISPR knockout cells (Supplemental Fig. 4C, D).

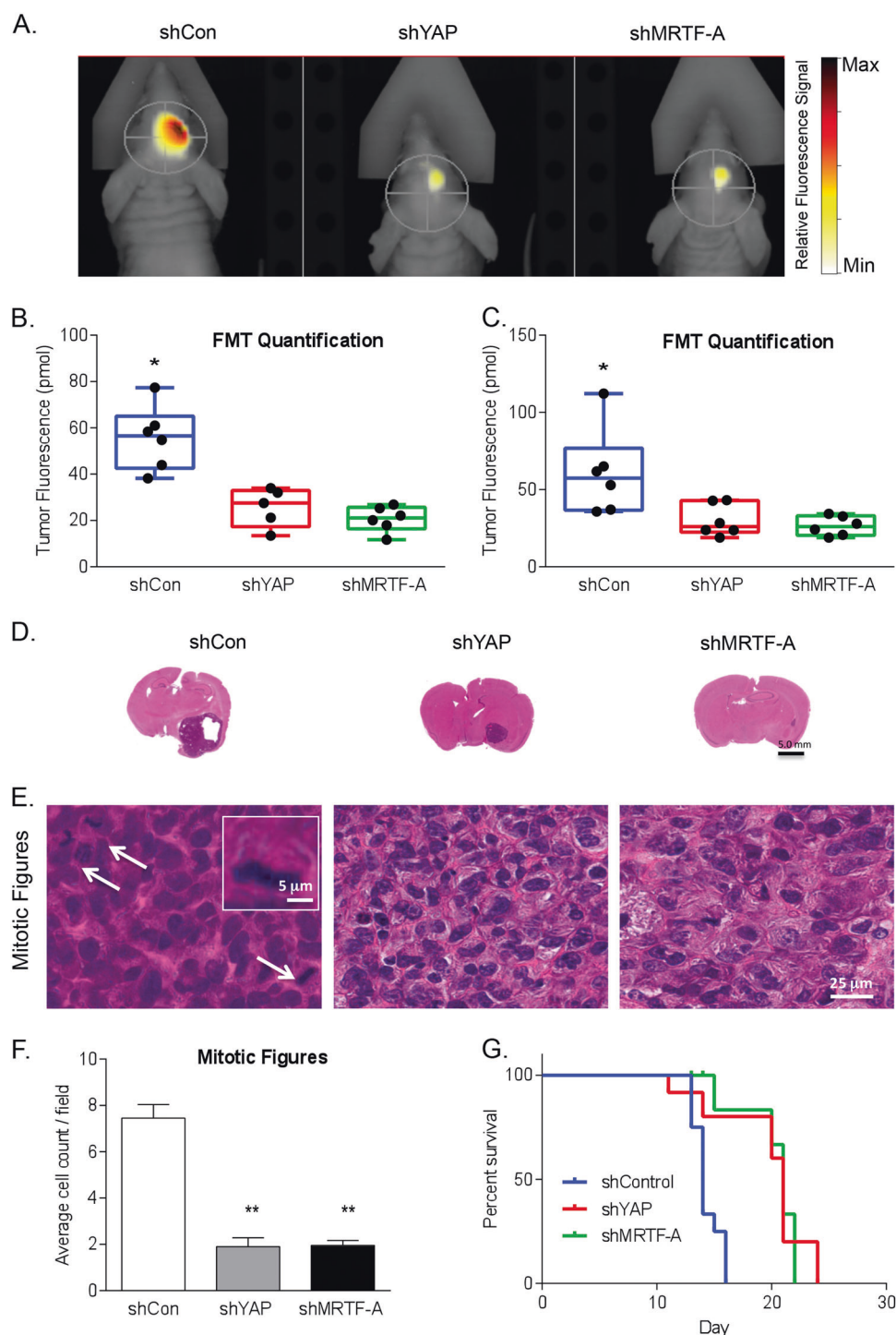
Fig. 1 YAP and MRTF-A are both required for maintenance of stem cell properties in GSC-23. **a** shControl, shYAP, or shMRTF-A knockdown GSC-23 cells were seeded at different doses into 96-well plates. The total number of spheres per well per dose per replicate per group was quantified at 14 days in culture and analyzed using the extreme limiting dilution analysis (ELDA) using at 0.95 confidence interval. Left panel, the estimated stem cell frequency in each shRNA group determined by ELDA. Right panel, plot of sphere-forming frequencies using ELDA analysis. **b** shControl, shYAP, or shMRTF-A GSC-23 cells were dissociated and single cells plated into 24-well plates coated with hydrogel microwells. The size of the sphere in each microwell was quantified after 14 days in culture. Left panel, bar plot quantification. * $P < 0.05$ vs. shControl ($n = 5$). Right panel, representative brightfield sphere images in microwells. **c** mRNA expression analysis of cancer-associated stem cell genes by qPCR in GSC-23 shControl, shYAP, and shMRTF-A knockdown cells ($n = 3$ biological samples with three replicates each, ** $p < 0.01$ vs. shControl, two-way ANOVA)



As shown above in studies with GSC-23 cells, S1P increases wild-type 1321N1 cell adhesion to extracellular matrix (Fig. 4a, b). The ability of S1P to increase adhesion

was abrogated in MRTF-A CRISPR knockout cells but not in YAP CRISPR knockout cells (Fig. 4a, b). S1P also significantly increased invasion of wild-type cells through

Fig. 2 Growth, proliferation, and lethality are dependent on YAP and MRTF-A. shRNA control or knockdown YAP or MRTF-A GSC-23 cells labeled with IRFP720 were intracranially injected into syngeneic nu/nu mice. **a–c** Brain tumor growth was monitored by measuring fluorescence emission at 720 nm using an FMT 2500 Fluorescence Tomography System (Perkin Elmer); $*p < 0.05$ vs. shControl ($n = 6$). **a** Representative fluorescence molecular tomography images of mice engrafted with GSC-23 shControl, shYAP, and shMRTF-A. **b, c** Relative fluorescence quantification from separate experiments using different shRNA knockdown cells per group. **d** Representative images of mouse brain cross sections showing the effect of shYAP and shMRTF-A on brain tumor compared with shControl at 15 days post intracranial injection (H&E, hematoxylin and eosin stained). **e** Representative H&E images of mitotic figures from different brain tumor xenograft conditions at high power magnification (HPM, $\times 40$). **f** Mitotic figures quantification of five fields from two brain cross sections per xenograft condition ($**p < 0.01$ vs. control, $n = 4$ animals per group). **g** Kaplan–Meier survival curve. Control and knockdown survival curves were significantly different using the Mantel–Cox test ($n = 6$)



Matrigel-coated transwells (Fig. 4c, d) and this response was abolished when YAP was deleted, whereas knockout of MRTF-A had no effect (Fig. 4c, d). S1P-stimulated migration was also dependent on YAP but not MRTF-A (Fig. 4e, f). Thus, 1321N1 glioblastoma cells and GSC-23 cells show the same specificity for MRTF-A-mediated signaling vs. YAP-mediated signaling in the regulation of agonist-mediated cellular responses.

YAP-dependent and MRTF-A-dependent target gene analysis in 1321N1 cells

The differential requirements for the two transcriptional co-activators in mediating adhesion vs. migration/invasion suggests that they effect expression of distinct genes involved in these processes. We performed RNA-sequencing (RNA-seq) to examine gene expression

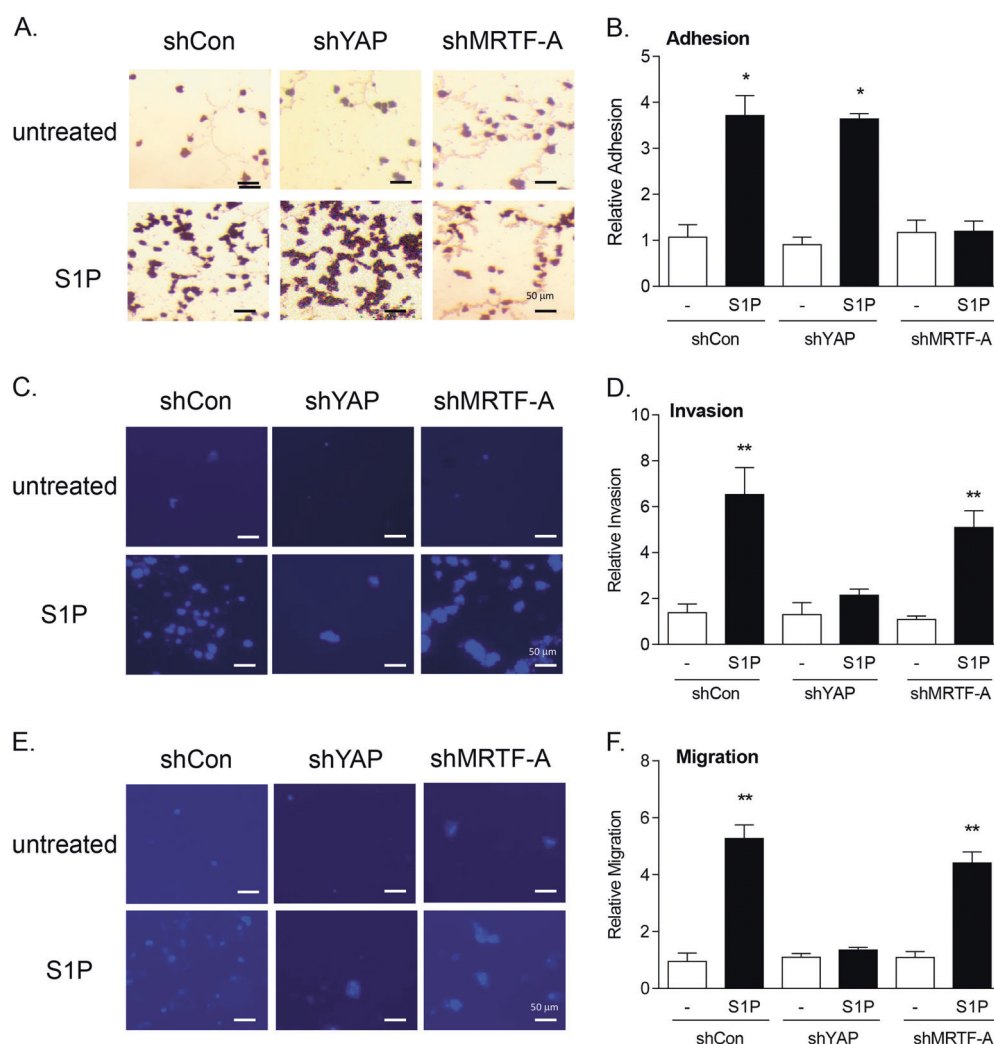


Fig. 3 S1P-induced adhesion is dependent on MRTF-A, while S1P-induced migration and invasion are dependent on YAP in GSC-23 cells. Stable knockdown of YAP and MRTF-A in GSC-23 cells was achieved using lentivirus transduction with shRNA constructs. **a, b** Twenty-four hours growth factor-starved control, YAP, or MRTF-A shRNA knockdown GSC-23 cells were pretreated with 0.3 μ M S1P for 2 h before being dissociated and plated on gelatin-coated plates overnight. Unbound cells were aspirated, and cells remaining adherent were stained and fixed with crystal violet. Cell-bound dye was quantified by measuring absorbance; * $p < 0.05$ vs. shControl untreated (n

= 9 of three separate experiments done in triplicate). **c–f** Twenty-four hours growth factor-starved shRNA control, YAP, or MRTF-A knockdown GSC-23 cells were dissociated and resuspended in serum-free medium. Cell suspensions were added to the upper chamber of Matrigel-coated (**c, d**) or Matrigel-uncoated (**e, f**) microwells and vehicle or 0.3 μ M S1P was added into the lower chamber. Live cells invaded to the lower surface of the membrane after 16 h were stained with Hoechst and invasion or migration quantified; ** $p < 0.01$ vs. shControl untreated ($n = 9$, of three separate experiments done in triplicate)

changes, comparing YAP and MRTF-A knockout 1321N1 cells with wild-type cells. We also examined RhoA CRISPR knockout cells to establish which of the S1P-regulated genes were RhoA-dependent. Cells were treated with S1P for 1 h and RNA was prepared for analysis by RNA-seq. The list of genes generated from the RNA-seq analysis was first analyzed for genes that were upregulated by S1P in wild-type cells but not in RhoA knockout cells. A set of 276 genes was determined with high confidence ($p < 0.01$) to be S1P-induced and RhoA-dependent. Nearly all of these (250) required either YAP or MRTF-A for their induction by S1P (using a probability value (p value) of

< 0.05). A more stringent cut-off value of $p < 0.01$ was used to narrow to 44 the number of genes for further interrogation (Table 1). Of these genes, 25% were found to be dependent only on YAP for their induction by S1P, 39% were dependent only on MRTF-A, and 36% were genes that showed attenuated expression when either YAP or MRTF-A were knocked out (Table 1A–C). The RNA-seq data was verified by quantitative PCR (qPCR) using independent sets of wild-type and knockout cells. Two genes of interest, tissue factor (*TF*) and sprouty RTK signaling antagonist 2 (*SPRY2*), were verified as YAP-dependent, and were shown to be unaffected by knockout of MRTF-A (Supplemental

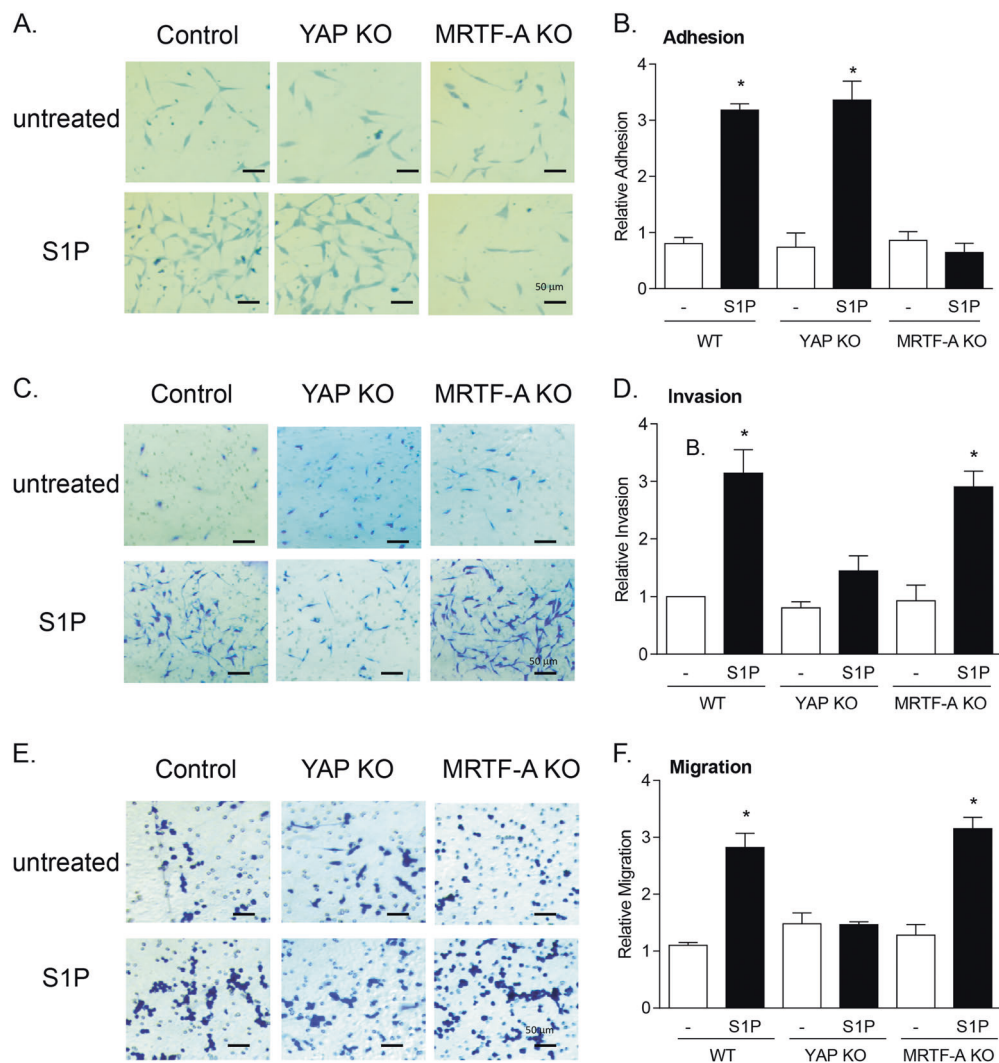


Fig. 4 S1P-induced adhesion is dependent on MRTF-A, while S1P-induced migration and invasion are dependent on YAP in 1321N1 cells. **a, b** Twenty-four hours of serum-starved wild-type, YAP, or MRTF-A CRISPR/Cas9 knockout 1321N1 glioblastoma cells were pretreated with for 2 h with 0.3 μ M S1P before being trypsinized and plated on fibronectin-coated plates overnight. Unbound cells were aspirated, and cells remaining adherent were stained and fixed with crystal violet. Cell-bound dye was quantified by measuring absorbance. * $p < 0.05$ vs. wild-type untreated ($n = 9$ of three separate

experiments done in triplicate). **c–f** Twenty-four hours of serum-starved wild-type, YAP, or MRTF-A knockout 1321N1 glioblastoma cells were trypsinized and resuspended in serum-free DMEM medium. The cell suspension was added into the upper chamber of Matrigel-coated (**c, d**) or Matrigel-uncoated (**e, f**) microwells, and vehicle or 0.3 μ M S1P was added into the lower chamber for 16 h. Cells invaded to the lower surface of the membrane after 16 h were stained with crystal violet and invasion or migration quantitated; * $p < 0.05$ vs. wild-type untreated ($n = 9$, of three separate experiments done in triplicate)

Fig. 5A). Heparin-binding epidermal growth factor-like growth factor (*HBEGF*) and dual specificity phosphatase-1 (*DUSP1*) were verified as MRTF-A-dependent, which were not dependent on YAP (Supplemental Fig. 5B). Genes shown in our previous work [30] to be dependent on both YAP and MRTF-A, including *CCN1* (Cyr61), *CCN2* (CTGF), *ANKRD1*, *ACTA2*, and *MYC*, were also identified by RNA-seq (Table 1) and confirmed by qPCR to lose responsiveness for S1P when either YAP or MRTF-A were deleted (Supplemental Fig. 5C). We also tested the effects of TAZ and MRTF-B on S1P-mediated gene expression.

When these transcriptional co-activators were 50–75% downregulated by siRNA, S1P-regulated expression of TF, *HBEGF*, *CCN1*, *CTGF*, or *MYC* were unaffected (Supplemental Fig. 6).

YAP-dependent and MRTF-A-dependent target gene analysis in GSC-23 cells and tumors

A similar profile of target gene regulation was also observed in GSC-23 shControl, shYAP, and shMRTF-A knockdown cells treated with S1P. Specifically TF mRNA was

Table 1 RhoA-dependent genes upregulated by S1P and dependent on YAP, MRTF-A, or both YAP and MRTF-A. Data obtained from RNA-seq analysis of CRISPR-Cas9 knockout 1321N1 cells stimulated with S1P for 1 h. Values are expressed as S1P-induced increase (fold over untreated) in the three cell lines. Genes in *italics* are transcription factors/transcriptional regulators. Genes in **bold** were selected for further study. The expression of genes in A was decreased at $p < 0.01$ compared to WT in YAP knockout cells; genes in B were decreased at $p < 0.01$ compared to WT in MRTF-A knockout cells; genes in C were decreased at $p < 0.01$ compared to WT by either YAP or MRTF-A deletion. Venn diagram shows transcriptional co-activator dependence of the 44 most highly downregulated genes

A. Regulated only by YAP

	WT	YAP KO ($p < 0.01$ vs WT)	MRTFA KO
<i>EGR3</i>	108.66	13.19	116.11
<i>EGR2</i>	24.54	10.04	16.15
<i>EGR1</i>	17.88	6.82	18.05
<i>IER3</i>	14.13	6.43	18.27
<i>JUNB</i>	6.08	1.75	6.28
<i>FOS</i>	5.47	2.19	7.30
F3	4.13	2.49	4.05
THBS1	2.98	1.66	3.94
<i>KLF10</i>	2.69	1.60	3.39
<i>BCL6</i>	2.21	1.41	2.06
SPRY2	2.02	1.62	2.13

B. Regulated only by MRTF-A

	WT	YAP KO	MRTFA KO ($p < 0.01$ vs WT)
<i>TNFAIP3</i>	20.42	11.09	2.74
<i>IL8</i>	13.32	9.39	2.31
<i>NR4A3</i>	11.95	20.17	7.44
<i>NFKBIZ</i>	11.14	7.74	3.86
<i>NR4A1</i>	8.76	22.09	4.52
<i>CXCL3</i>	8.49	7.89	2.51
<i>NR4A2</i>	6.45	12.00	3.19
<i>PER1</i>	5.86	4.64	2.08
<i>ATF3</i>	5.80	3.99	2.64
DUSP1	5.58	6.43	2.50
DUSP5	3.29	3.54	1.34
<i>BHLHE40</i>	3.17	4.66	2.55
HBEGF	3.13	3.61	1.42
<i>ZFP36</i>	3.12	3.15	1.33
<i>ERRFI1</i>	2.50	3.78	1.45
<i>C14orf43</i>	2.42	3.06	1.45
<i>KDM6B</i>	2.15	2.57	1.66

C. Regulated by both YAP and MRTF-A

	WT	YAP KO ($p < 0.01$ vs WT)	MRTFA KO ($p < 0.01$ vs WT)
<i>FOSB</i>	11.90	1.91	2.57
CTGF	7.37	1.15	1.36
CYR61	6.66	1.07	1.74
LIF	5.32	2.10	2.22
<i>ZC3H12A</i>	4.56	2.23	2.04
<i>CSRNP1</i>	4.47	1.39	1.11
GBP1	4.26	3.07	2.37
ANKRD1	3.99	1.64	1.55
PTGER4	3.70	2.04	2.06
<i>MAFF</i>	3.16	2.10	2.07
ACTA2	2.75	1.61	1.18
TUFT1	2.53	1.25	1.40
<i>IER2</i>	2.49	1.40	1.46
MYC	2.47	1.10	1.23
DLX2	2.42	1.14	1.01
PPP1R15A	2.23	1.80	1.95

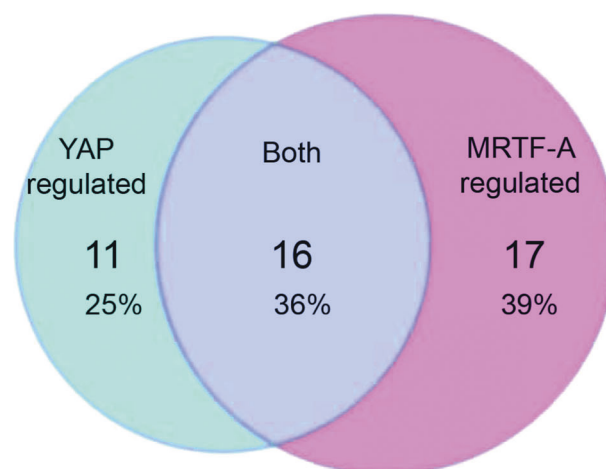
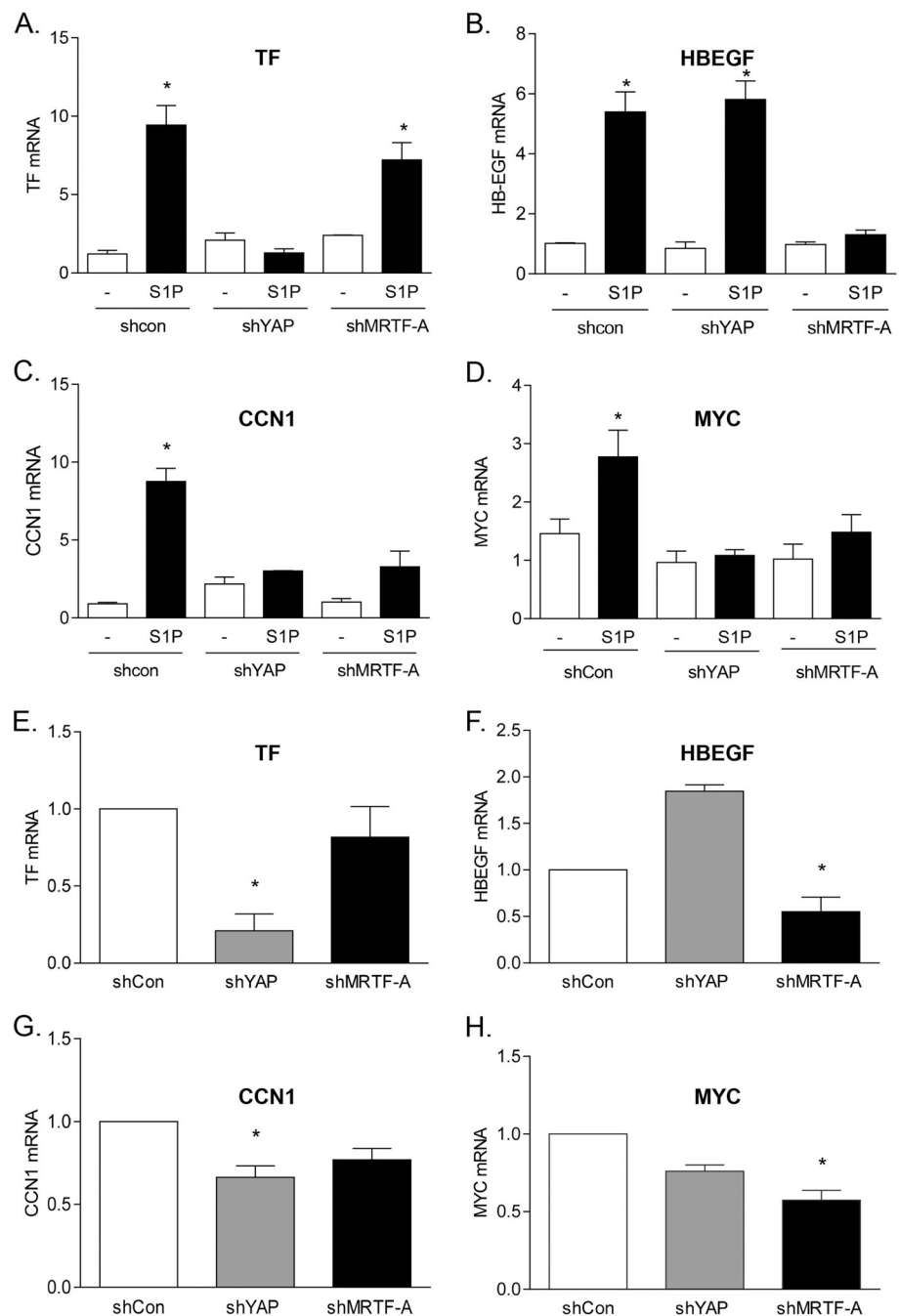


Fig. 5 YAP-dependent or MRTF-A-dependent gene expression in GSC-23 cells and in GSC-23-derived tumors. **a–d** Control, YAP, or MRTF-A shRNA knockdown GSC-23 cells were growth factor starved for 24 h and then stimulated for 1 h with 0.3 μ M S1P. Lysates were subject to quantitative PCR analysis of mRNA levels (qPCR) for *TF*, *HBEGF*, *CCN1*, or *MYC* genes; * p < 0.05 vs. shControl untreated (n = 3). **e–h** Tumors from mice injected with shControl, shYAP, or shMRTF-A knockdown GSC-23 cells were removed at the time that the control group showed neurological signs, total RNA isolated, and mRNA levels analyzed by qPCR for *TF*, *HBEGF*, *CCN1*, and *MYC* genes; * p < 0.05 vs. shControl (n = 6 from two separate experiments done in triplicate)



increased in control cells and this response was lost in cells in which YAP was downregulated (Fig. 5a), while HBEGF was increased by S1P in control cells but not in cells harboring shRNA for MRTF-A (Fig. 5b). CCN1 and MYC induction were both prevented when either YAP or MRTF-A were down-regulated (Fig. 5c, d). Expression of these genes was also analyzed in the GSC-23 cell tumors described in Fig. 1. In tumors from YAP knockdown cells, TF mRNA was down-regulated while that for HBEGF was not (Fig. 5e, f), while in tumors from MRTF-A knockdown

cells HBEGF mRNA was significantly lower while that for TF was not (Fig. 5e, f). Finally, CCN1 and MYC mRNA were found to be diminished in tumors from GSC-23 cells in which either YAP or MRTF-A were knocked down (Fig. 5g, h). In addition, we further interrogated TCGA for these and other genes identified through RNA-seq to be RhoA and YAP or MRTF-A regulated. This analysis revealed upregulation in mRNA for a number of candidate genes including *F3* (TF), *HBEGF*, *LIF*, and *DUSP5* (Supplemental Fig. 7).

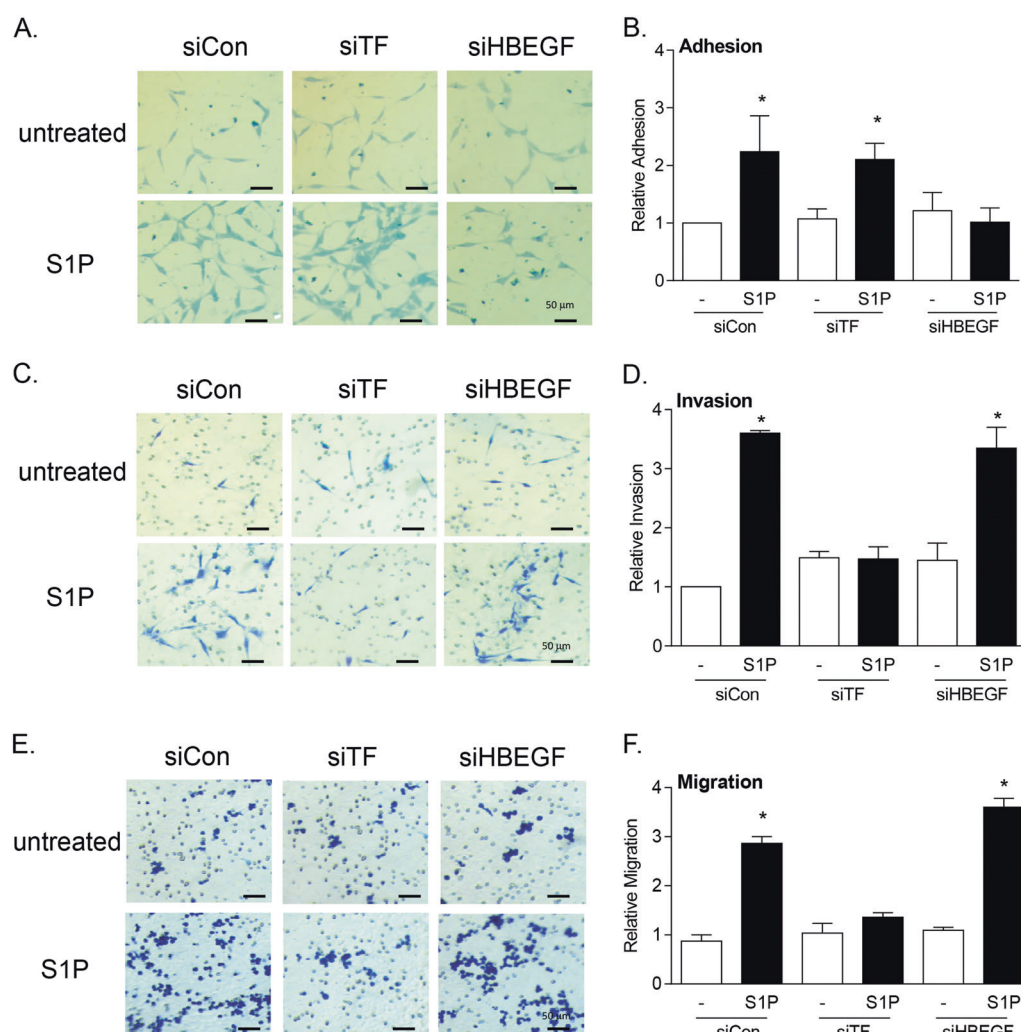


Fig. 6 S1P-induced adhesion is dependent on an MRTF-A target gene and invasion/migration is dependent on a YAP downstream target gene. **a, b** Twenty-four hours of serum-starved control, HBEGF, or TF siRNA knockdown 1321N1 glioblastoma cells were pretreated with for 2 h with 0.3 μ M S1P before being trypsinized and plated on fibronectin-coated plates overnight. Unbound cells were aspirated, and cells remaining adherent were stained and fixed with crystal violet. Cell-bound dye was quantified by measuring absorbance. * $P < 0.05$ vs. siControl untreated ($n = 9$ of three separate experiments done in

triplicate). **c–f** Twenty-four hours of serum-starved control, HBEGF, or TF knockdown 1321N1 glioblastoma cells were trypsinized and resuspended in serum-free DMEM medium. Subsequently, the cell suspension was added into the upper chamber of Matrigel-coated (**c, d**) or Matrigel-uno-coated (**e, f**) microwells and vehicle or 0.3 μ M S1P was added into the lower chamber. Cells invaded to the lower surface of the membrane after 16 h were stained with crystal violet and invasion or migration quantitated; * $p < 0.05$ vs. siControl untreated ($n = 9$, of three separate experiments done in triplicate)

MRTF-A and YAP target genes involved in adhesion, migration, invasion, and proliferation

Finally, we asked whether the selected MRTF-A-regulated and YAP-regulated target genes had effects on cellular responses to S1P that mirrored those of their transcriptional co-activator. We used siRNA to knockdown HBEGF, which has been implicated in an autocrine loop that acts on EGFR and drives signaling through constitutively activated EGFR in GBM [53–55]. Knockdown of HBEGF reduced cell adhesion (Fig. 6a, b) without affecting invasion or migration of cells (Fig. 6c–f). We used siRNA to knockdown TF based on evidence that it is upregulated and

correlates with malignancy in glioblastoma [56], as well as its involvement in metastasis and invasiveness [57, 58]. Knockdown of TF prevented cell migration and invasion (Fig. 6c–f) but not adhesion (Fig. 6a, b). Thus, the cellular effects of knocking down genes we identified as MRTF-A or YAP targets (HBEGF and TF, respectively) paralleled those seen when their specific transcriptional co-activators were deleted (Figs. 3 and 4).

Our previous studies demonstrated that GPCR activation by thrombin or S1P-stimulated proliferation of 1321N1 cells through RhoA-regulated pathways [9, 10] and that YAP and MRTF-A were both required for this response [30]. Using the CRISPR knockout cells generated for the

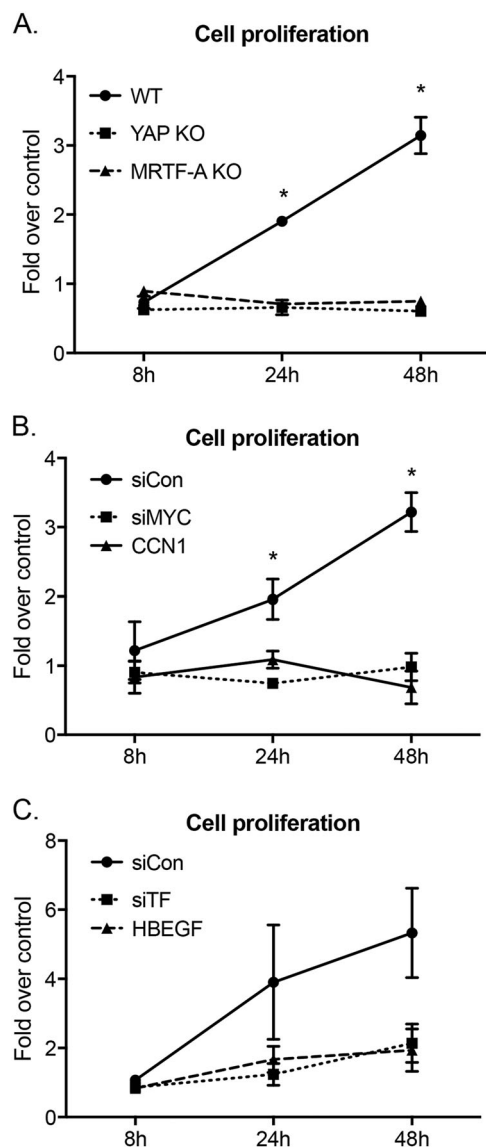


Fig. 7 S1P-induced cell proliferation is dependent on both YAP and MRTF-A and their downstream target genes. Twenty-four hours of serum-starved (a) wild-type, YAP, or MRTF-A knockout 1321N1 glioblastoma cells or cells transfected for 48 h with (b) siMYC or siCCN1, and (c) with siTF or siHBEGF, were treated with 0.3 μ M S1P and cell number determined at 8, 24, or 48 h using a cell counter or microscopic methods. Data are expressed relative to untreated control at each time point; * $p < 0.05$ vs. siCon ($n = 9$, of three separate experiments done in triplicate)

current studies, we confirmed that deletion of either YAP or MRTF-A abolished 1321N1 proliferation in response to S1P (Fig. 7a). Two of the downstream gene targets dually regulated by YAP and MRTF-A (*CCN1* and *MYC*) with established involvement in growth of tumor cells including glioblastoma [59, 60] were then tested. Their knockdown was shown to also significantly reduce S1P-dependent proliferation (Fig. 7b). Finally, S1P-stimulated cell proliferation was found to be reduced by either HBEGF or TF knockdown (Fig. 7c).

Discussion

We investigated two transcriptional pathways that are concomitantly activated by GPCR coupling to RhoA and which have not been previously examined for their requirement in GBM tumor growth and progression. Our studies used a PDX tumor-initiating cell line, GSC-23, for in vivo work examining the role of YAP and MRTF-A in tumor formation, and the human glioblastoma-derived 1321N1 cell line to identify YAP and MRTF-A target genes and their role in cellular responses.

The findings presented here demonstrate that both YAP and MRTF-A contribute significantly to in vivo tumor formation. Specifically, knockdown of either YAP or MRTF-A in GSC-23 PDX cells reduces intracranial tumor formation, with concomitant reductions in tumor cell proliferation and a slower onset of mortality. This observation is consistent with our demonstration that knockdown of either YAP or MRTF-A diminishes the self-renewal capacity of GSC-23 cells in vitro, as indicated by limiting dilution, and their stem-like properties as indicated by diminished neurosphere formation. We also show here, as previously [30], that YAP and MRTF-A are both required for proliferation of 1321N1 glioblastoma cells in response to S1P.

We identified a number of genes regulated by GPCRs through RhoA signaling that are induced by the co-ordinate activation of YAP and MRTF-A (Table 1C). We focused largely on upregulated genes that encode molecules which could serve signaling functions, rather than on the numerous transcription factors that were also upregulated and would initiate further cascades of gene expression. Knockdown of *CCN1* markedly attenuated S1P-stimulated cell proliferation, as did knockdown of the selectively regulated YAP and MRTF-A target genes, *TF* and *HBEGF* (Fig. 7). These observations gain further relevance in light of our in vivo studies showing that expression of these genes was decreased in YAP and MRTF-A down-regulated GSC-23-derived tumors. *CCN1* is highly regulated at the transcriptional level in response to GPCRs and other growth factors. Its expression has been linked to growth of GBM tumors [61–63] in part through activation of integrin signaling [64]; thus, its attenuated expression by YAP and MRTF-A knockdown may contribute to diminished in vivo tumor growth of GSC-23 knockdown cells. We also demonstrated requirements for *MYC*, a transcription factor which was of particular interest because c-Myc is highly expressed in glioma cancer stem cells [65] and required for glioma cell proliferation [60]. Regardless of whether decreased expression of these particular genes is responsible for diminished GSC-23 cell tumorigenesis, our findings are noteworthy in demonstrating that these genes are regulated in the in vivo tumor cell environment. Furthermore, their

expression requires YAP and MRTF-A, transcriptional co-activators that are not expected to be basally active but to respond to signals from GPCRs and RhoA. This suggests that pathways using these upstream regulators are actively engaged in the tumor cell environment. Signaling to YAP and MRTF-A could occur through glioma cell interactions with components of the extracellular matrix, as they are regulated by cyclic stretch [32], but the primary pathways for YAP and MRTF-A activation are through GPCR ligands (e.g., S1P, LPA, and thrombin) which also are major components in the serum [52, 66]. Notably, GPCR ligands such as S1P, thrombin, or LPA are generated at or have access to sites of tumor growth [39–45], underscoring the potential for GPCR activation to contribute to in vivo growth of glioblastoma cells.

We established here that there also are sets of genes, the induction of which requires only YAP or MRTF-A. We do not fully understand the temporal or mechanistic determinants of whether or when YAP or MRTF-A would be independently activated through GPCRs and RhoA, although there is evidence for their distinct temporal regulation in response to stretch [32], and this could be a significant factor in determining GBM phenotype. The potential significance of activating only one of the two transcriptional pathways is emphasized by the cellular studies carried out here. We show that S1P-stimulated cell invasion and migration of 1321N1 or GSC-23 cells is reduced when YAP is downregulated, but unaffected by knockdown of MRTF-A. This is of interest in light of the finding that YAP can regulate actin cytoskeletal dynamics through its effect on transcription of a GTPase-activating protein (GAP) for Rho [67]. Another link is with the YAP target gene, *TF*, the loss of which we found to prevent S1P-stimulated cell migration and invasion of 1321N1 cells. Notably, YAP has been linked to glioma cell invasion [27, 68, 69], as has TF [70, 71]. Remarkably, these two pathways have not been previously related to one another, nor shown to be coordinately regulated by GPCR activation and RhoA signaling. TF, once considered only a member of the coagulation cascade, is now known to signal both through generation of thrombin and direct activation of the protease-activated receptors PAR-1 and PAR-2 [72]. Thus, early activation and expression of this gene may amplify and sustain RhoA signaling pathways which have well established role in cell invasion and migration.

Our studies also delineated a selective effect of MRTF-A and its target gene HBEGF on adhesion of either 1321N1 or GSC-23 cells to fibronectin or gelatin. Further work will be needed to understand the significance of this finding, but MRTF-A signaling has been shown to regulate numerous targets involved in cytoskeletal dynamics [52], which may in turn affect the ability of cells to adhere to matrix. In addition HBEGF interacts with integrins and could

contribute to cell adhesion to fibronectin [73]. Thus, in addition to the dual involvement of YAP and MRTF-A in control of cell proliferation, we suggest that YAP, which contributes to tumor cell invasion and migration, and MRTF-A, which contributes to adhesion, are also both required for their unique effects on these processes.

The work presented here is the first to comprehensively link signaling to GPCRs, through YAP and/or MRTF-A activation, to expression of specific target genes, in vitro cellular responses, and in vivo tumor growth. Our working hypothesis is that activation of RhoA, as would occur through increases in ligands that stimulate specific GPCRs (or in response to mutations leading to constitutive activation of G $\alpha_{12/13}$ or Rho GEFs), leads to activation of YAP and MRTF-A and to transcriptional upregulation of genes that contribute to GBM formation and progression. A comprehensive analysis of cancer-associated genes across 21 tumor types identified 33 genes not previously shown to be significantly mutated in cancer, including not only RhoA and a RhoA GAP, but also myocardin (with mutations in the region of putative interaction with YAP) substantiating the role of this signaling pathway in cancer [74]. Targeting the known driver pathways in GBM (i.e., EGFR mutations, Ras activation, and increased PI3 kinase signaling) has shown limited clinical efficacy. Clearly, other molecular mechanisms contribute to escape from therapy and continue to support progression of the disease. Enhanced and amplified signaling through GPCRs and RhoA could engage novel pathways, such as those defined here, which complement the established drivers of GBM progression. In addition, activation of RhoA/YAP/MRTF-A signaling and downstream gene expression may play a critical role in GBM stem cell self-renewal. Accordingly, blocking GPCRs that activate YAP and MRTF-A, or critical downstream gene targets delineated here could, in combination with traditional therapies, provide a new modality for treatment of GBM.

Materials and methods

Antibodies and other materials

Anti-YAP (Catalog# sc-15407), MRTF-A (Catalog# sc-21558), RhoA (Catalog# sc-418), HBEGF (Catalog# sc-28908), and TF (Catalog# sc-377187) antibodies were purchased from Santa Cruz Biotechnology. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Cell Signaling Technology (Catalog# 2118). S1P was obtained from Avanti Polar Lipids (Catalog# 860662). Control scrambled siRNA and constructs targeting HBEGF (Catalog# SI00030303), TF (Catalog# SI03058209), and MYC (Catalog# SI00300902) were purchased from Qiagen.

shRNA constructs were obtained from the La Jolla Institute of Allergy and Immunology. One hundred and thirty microns of hydrogel wells in 24-well plate format were purchased from MuWells Incorporated.

RNA-sequencing

Ten micrograms of total RNA extracted from cultured cells was used for RNA-seq library preparation according to the Illumina-provided protocol. Six libraries were mixed for multiplexed pair-end sequencing using the Illumina HiSeq 2000 platform. Single-end reads that passed through Illumina filters were filtered for reads aligning to transfer RNA, ribosomal RNA, adapter sequences, and spike-in controls. Genes were considered differentially expressed between two groups of samples when the DESeq2 analysis demonstrated an adjusted *p* value of <0.05 with a twofold change in gene expression. Cluster analyses including principal component analysis and hierarchical clustering were performed using standard algorithms and metrics. RNA-seq data has been submitted to the Gene Expression Omnibus and an accession number will be provided when available.

CRISPR/Cas9-mediated generation of knockout cells

Guide RNA sequences targeting the YAP, MRTF-A, or RhoA exons were cloned into the pX330 plasmid [75]. Constructs along with a puromycin selection vector were transfected into 1321N1 cells with polyethylenimine. After 24 h of transfection, cells were selected with 1.5 µg/ml puromycin for 72 h, and single cells were obtained by flow cytometry. Clones were screened by immunoblotting with anti-YAP, MRTF-A, or RhoA antibodies.

Cell culture and transfection

A human glioblastoma cell line termed 1321N1 cells (purchased from Sigma-Aldrich, Catalog# 86030402) has been used extensively in our previous work. These cells are routinely grown in Dulbecco's modified Eagle's medium (DMEM) at 37°C in a 10% CO₂ incubator. The medium was supplemented with fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (4 mM), purchased from Invitrogen. Dharmafect 4 (GE Dharmacon) was used for siRNA transfection in 1321N1 cells. GSC-23 cells (from collaborator F Furnari) were cultured in DMEM/F12 (Invitrogen). DMEM/F12 was supplemented with 50 × B27 (Invitrogen), 1% penicillin and streptomycin (Invitrogen), 5 µg/ml heparin (Sigma-Aldrich), 20 ng/ml EGF, and 20 ng/ml basic FGF (Pepro-tech). GSC-23 cells were grown at 37°C and 5% CO₂ in suspension plates or flasks with filter caps. For knockdown

in GSC-23 cells, we used lentiviral shRNA and selected for knockdown cells using puromycin.

Quantitative PCR

Trizol was used to extract total RNA from glioblastoma cells. TaqMan Universal Master Mix was used to amplify cDNA in the presence of primers (Applied Biosystems) for *YAP*, *MRTF-A*, *TF*, *HBEGF*, *MYC*, *CCN1*, *CTGF*, *ACTA2*, and *ANKRD1* genes. GAPDH mRNA was used to normalize data, with fold change calculated, as previously reported [76]. Analysis of mRNA for stem cell genes was performed using SYBR Green (BioRad) and primers were purchased from Integrated DNA Technologies.

Cell proliferation assay

To assay cell proliferation in control, knockout, or knock-down 1321N1 cells, cells at 60% confluence were plated in 6-well plates and cultured in serum-free media in the presence or absence of 0.3 µM S1P. Cell numbers were determined using a cell counter or microscopic counting methods. Two separate experiments with three wells for each condition was used. Data are expressed as fold increase with S1P, compared to control untreated cells assayed at the same time point.

Cell adhesion assay

Forty-eight well plates were coated with 4.8 µg/ml fibronectin or 0.01% gelatin overnight at 4°C. PDX cell adhesion was studied using gelatin, rather than fibronectin, which can elicit PDX cell differentiation [77]. Plates were washed with PBS and blocked with 1% bovine serum albumin in PBS for 1 h at 37°C. 1321N1 or GSC-23 cells (2.5 × 10⁴ cells per ml, 200 µl per well) were added to the plate and allowed to attach for 16 h at 37°C and 10% CO₂. Unbound cells were aspirated, and cells remaining adherent were stained and fixed with 0.1% crystal violet in 20% methanol solution with PBS for 20 min. Unbound dye was removed by washing with water, and the plate was dried overnight at room temperature. Cell-bound dye was reconstituted with 300 µl of 100% methanol and quantitated by measuring absorbance at 600 nm.

Cell migration/invasion assay

We conducted transwell migration and invasion assays using transwell chambers (24-well, 8 µm pore size). Uncoated or Matrigel-coated membranes were used for migration and invasion assays, respectively. Cells were digested with 0.25% trypsin containing EDTA and resuspended in serum-free DMEM. Two hundred microliters of

cell suspension was added into the upper chamber, while 600 μ l medium with 0.3 μ M S1P was added into the lower chamber. After incubation for 16 h, wet cotton swabs were used to wipe off cells that did not migrate through the pores from the upper face of the filters. Cells attached to the lower surface of the inserts were fixed with cold methanol for 10 min and then stained with 0.01% crystal violet. Filters were washed in water and observed using an inverted microscope.

Intracranial Injections

Seven-week-old athymic nu/nu mice from Harlan Sprague Dawley Inc. were utilized for all in vivo experiments. A total of 5×10^5 GSC-23 control or knockdown cells, tagged with near-infrared IRFP720, were intracranially injected into the mouse brain using a stereotactic system (1.0 mm anteroposterior and 2.0 lateral from Bregma suture and 3 mm below the pial surface). Tumor growth was monitored using an FMT 2500 Fluorescence Tomography System (Perkin Elmer). Tumor fluorescence emission, at 720 nm, was collected. Survival until the onset of neurologic sequelae in the control group was used to determine the time of sacrifice. All procedures have been reviewed and approved by the Institutional Animal Use and Care Committee (IACUC) at the University of California San Diego, protocol# S00192M.

Limiting dilution and microwell sphere formation assays

Glioma spheres were dissociated into single cells and 1, 5, 10, 20, 50, and 100 cells per well were plated in 96-well plates. Five replicates were used for each experimental condition. The total number of spheres, per well and per treatment, were quantified after 14 days in culture. Data were analyzed by extreme limiting dilution analysis (ELDA, <http://bioinf.wehi.edu.au/software/elda/>). For the microwell sphere formation assay, glioma spheres were dissociated into single cells and 200 cells per well were plated into 24-well plates. Three replicates for each experimental condition were used. The size of each sphere in each microwell was quantified after 14 days in culture. Both assays are used routinely to assess cancer stem cells' characteristics in glioblastoma [78].

Statistical analysis

Means $\pm$ SEM (standard error of mean) are reported for all results. The Student's *t* test was used to compare two groups with one characteristic. Two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to analyze data from two groups with multiple

characteristics. One-way ANOVA and Tukey's multiple comparison test were used to analyze data from experiments with a single characteristic but more than two groups. The normal distribution of data points was determined using the D'Agostino and Pearson Omnibus test. *P* values <0.05 were considered significant and are represented by one asterisk in all figures. *P* values <0.01 are represented by two asterisks in all figures.

Acknowledgements We thank Jacqueline Sayyah for initial help with culture and analysis of 1321N1 cells, and Silvio Gutkind for assistance with interrogation and analysis of TCGA data

Author contributions OMY completed much of the experimental work, data analysis, and figure preparation; JAB advised on PDX cell experiments and performed orthotopic xenograft injections; FBF contributed PDX cells and advice on data interpretation; SWP generated the CRISPR knockout cells; DR and AK carried out experiments examining TAZ and MRTF-B involvement; JS generated lentiviral shRNA constructs; KLG advised on manuscript preparation and YAP signaling; BD and JG assisted with RNA-seq and analysis under the direction of AR; OMC contributed studies on cell proliferation and finalized manuscript preparation. SM advised on design of the experiments and analysis of data and contributed to the manuscript preparation; JHB provided overall study supervision, data interpretation, and manuscript preparation.

Funding This work was supported by NIH Grants GM036927, HL028143, HL085577, and CA170682 to JHB; HL097037 to SM; NS080939 to FBF and JAB; AI40127 and AI109842 to AR. OMY and SWP were supported by T32 GM007752; OMY also received support from T32 DK00754; BD is an HHMI postdoctoral fellow from the Jane Coffin Childs Fund; OMC is a PDE fellow from The Brazilian National Council for Scientific and Technological Development.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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