TITLE: **YAP controls cell migration and invasion through a Rho-GTPase switch**

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SUMMARY:

Understanding the mechanisms controlling the invasive spread of non-diseased and transformed cells is central to understanding diverse processes including cancer progression. Here, we report that Yes-associated protein (YAP), a central transcriptional regulator implicated in controlling organ and body size, modulates a Rho-GTPase switch that drives cellular migration by transactivating the Rac1-GEF protein TRIO through direct modulation of its intronic enhancer. Additionally, YAP and TRIO may promote invasive behavior through putative crosstalk with STAT3 signaling, a potential downstream target. While we find this YAP-dependent infiltrative program in many cell types, it is particularly enhanced in a patient-specific way in the most common malignant brain tumor, glioblastoma (GBM), where hyperactivation of the YAP, TRIO, and STAT3 signatures also confers poor clinical outcome. Our analysis suggests that the YAP-TRIO-Rho-GTPase signaling network identified in this study is a ubiquitous regulator of invasive cell spread in both physiological and pathological contexts.

KEYWORDS:

YAP, GBM, glioblastoma, migration, invasion, Rho-GTPase, TRIO, Rac1, RhoA, STAT3, brain cancer

INTRODUCTION:

Cell migration is a tightly regulated process integrating multiple extracellular cues through intracellular signaling networks, which in turn defines the extent of cell polarization and the persistence and speed of locomotion. Extensive studies over the past few decades identified the Rho family of small GTPases, including Rac1 and RhoA, as key regulators of these processes. Ongoing analysis of the regulation of these GTPases and their interaction(*1*) suggests that they form a molecular circuit integrating the inputs from multiple other signaling networks. Insight into these mechanistic links can help identify important molecular components, whose activity can determine the outcome of cell migration-dependent events in development and disease. Ultimately, unraveling these converging pathways will clarify the determinants of cell migration, and may offer new therapeutic paradigms against the aberrant invasion seen in many cancers. Targeting migration pathways may be particularly effective against cancer where most deaths are caused by invasive and metastatic cells rather than the primary tumor mass.

Since many extracellular cues affecting cell migration also guide decisions such as cellular identity, it is likely that migratory behaviors are tightly integrated with transcriptional decision networks. Notably, Yes-associated protein (YAP) is known to integrate many inputs (Hippo signaling, E-Cadherin, mechanical cues, etc.) to orchestrate diverse cellular functions including growth, apoptosis, and migration, as well as cancer cell invasion and metastasis(*2–12*). From these studies, YAP has emerged as a proto-oncogene frequently overexpressed in a variety of cancers, including a particularly aggressive and infiltrative form of brain cancer, glioblastoma (GBM)(*7–9*, *13–19*). However, it is not fully understood how YAP expression and activity play a role in the regulation of the molecular networks controlling cell migration and invasion, and whether this role may enhance the progression of aggressive cancers. Here, we demonstrate that YAP binds a distal enhancer region to promote transcription of TRIO, a guanine nucleotide exchange factor (GEF) protein that regulates Rho GTPases function(*20*). YAP-mediated TRIO up-regulation activates Rac1, which in turn inhibits RhoA to increase migration speed. In addition, an increase in YAP-mediated TRIO signaling confers invasive potential, likely involving STAT3 as a

downstream target. These findings reveal YAP-TRIO signaling as a central regulator of cell dispersion that promotes motility and invasion through a Rho-GTPase switch. In addition, our work provides new insights into the clinical significance of YAP to the Mesenchymal subtype of GBM and the potential prognostic relevance of the connected YAP, TRIO, and STAT3 signaling signatures in GBM.

RESULTS:

YAP regulates migratory capacity

To analyze putative mechanisms of YAP-mediated control of migration and invasion, we used primary patient-derived cultures of human glioblastoma (GBM) cells due to their highly aggressive and invasive spread in vivo that may be associated with YAP hyperactivity(*13*, *21–24*). We also tested another commonly used GBM cell line, GBMA172. In addition, we explored whether these YAP-driven migratory and invasive mechanisms are conserved in non-pathological systems using cells from two different tissues of origin: adult human brain astrocytes (NHA) and adult human breast cells (MCF10A).

Using intraoperatively obtained primary GBM tissues from patients, we found that YAP protein expression was indeed elevated and transcriptionally active in a majority of GBMs examined (**Fig. 1A, Suppl Table 1, Fig. S1A-B**). From these primary GBM tissues, we derived primary cultures (JHGBM612, JHGBM640, JHGBM651, JHGBM1A) and confirmed that these cell lines had similarly high YAP protein levels as the primary GBM tissues from which they were derived (**Fig. S1C**). To test whether this elevated YAP expression contributes to changes in cell migration in these GBM cells, we used multiple shRNA constructs to specifically inhibit YAP expression (**Fig. S1D-F**). We then used this perturbation to investigate the migration of cells plated at low density on laminin- (NHA and GBM cell lines) or collagen-coated (MCF10A) 2D surfaces. We followed the migratory response at 10-minute intervals for 8 hours. Following the shRNA-mediated stable knockdown of YAP (shYAP) using two different targeting sequences (shYAP/shYAP-1, shYAP-2), we observed a significant decrease in cell displacement and migration speed when compared to the control non-targeting vector (shCtrl) in all GBM cell lines and

non-neoplastic NHA and MCF10A cells (**Fig. 1B-C, Fig. S1G-H, Movie S1**). Conversely, stable overexpression of a constitutively active YAP (YAP OE) versus control vector (CONT) in GBM (JHGBM612 and JHGBM1A) and NHA cells showed that YAP up-regulation was sufficient to increase migration speed (**Fig. 1D, Fig. S1I-M**). To determine whether YAP affects infiltrative cell migration and spread in vivo, we used a murine intracranial xenograft tumor model of human brain cancer(*25*, *26*), which recapitulated the cellular infiltration along the corpus callosum seen in the donor patient tumor (**Fig. S1N**). To do this, we injected 150,000 shCtrl or shYAP JHGBM612 cells into the striatum of the right hemisphere (at pre-determined coordinates from the bregma; see Materials and Methods) of immunocompromised mice and quantified the number of human GBM cells infiltrating along the corpus callosum into the left hemisphere 5 weeks post-injection. Firstly, we confirmed the presence of tumor after the xenograft transplantation of GBM cells by performing a standard H&E staining. The images indeed display features commonly seen in murine xenograft models of human GBM (**Fig. S1O**). More importantly, we observed significantly fewer shYAP cells infiltrating the contralateral hemisphere than shCtrl JHGBM612 cells (**Fig. 1E-F, Fig. S1P**). These findings, coupled with our in vitro observations, suggest that YAP can enhance infiltrative cell migration in different micro-environmental contexts.

We then sought to identify the mechanism by which YAP modulates migratory behavior in non-neoplastic and cancer cells. To do so, we examined the regulation of small Rho GTPases, which are implicated as central regulators of cell motility(*1*). Interestingly, we found that YAP knockdown increased the number and area of focal adhesions, strongly affecting the shape of the cells (**Fig. 1G-H, Fig. S1Q-R**) in a pattern reminiscent of the focal adhesion assembly phenotype observed upon RhoA hyperactivation(*27*). Consistent with pronounced focal adhesion assembly, an increase in absolute levels and ratio of phosphorylated and total LIM-kinase, a downstream target of Rho-associated protein kinase (ROCK), a major effector of RhoA(*1*), was observed in shYAP JHGBM651, JHGBM612, NHA, and MCF10A cells than their corresponding shCtrl cells (**Fig. 1I, Fig. S1S-U**). From these results, we conclude

that YAP regulates cytoskeletal organization and dynamics to potentiate cellular motility in vitro and in vivo.

YAP regulates migration through a Rho-GTPase switch

To better understand the regulation of RhoA by YAP, we measured its GTP load through G-LISA assays. We observed a significant increase in RhoA-GTP levels after YAP knockdown in JHGBM651 and NHA cells (**Fig. 2A, Fig. S2A**). Based on this data, we sought to test the relevance of RhoA-ROCK activity on cell speed. We found that pharmacologic inhibition of ROCK I/II using either Y27632 or H1152 restored the migration speed of shYAP-1 and -2 cells in a dose-dependent manner while having no effect on shCtrl cells (**Fig. 2B, Fig. S2B-L, Movie S2**). Furthermore, treatment with Y12732 or H1152 decreased the phosphorylation of Nonmuscle Myosin II (NMII), confirming the inhibition of ROCK activity (**Fig. S2M-N**). These results demonstrate that YAP can modulate RhoA-ROCK activity and that ROCK inhibition is sufficient to rescue the migratory speed of YAP knockdown cells. Thus, YAP promotes migration at least in part by suppressing RhoA-ROCK activity.

YAP can modulate the activity of RhoA either directly or indirectly. For instance, prior studies suggested that another small Rho-GTPase, Rac1, can antagonistically regulate RhoA(*28–30*), raising the possibility that modulation of RhoA activity by YAP might involve Rac1. We indeed found that Rac1-GTP levels were reduced after YAP knockdown (**Fig. 2C, Fig. S3A**). We also examined a downstream target of Rac1, p21-activated kinase (PAK), and found a pronounced decrease in phosphorylated PAK (p-PAK) level after normalizing to the total PAK protein level in shYAP cells compared to shCtrl cells (**Fig. S3B**). Thus, we inquired whether changes in Rac1 activity accounted for the decreased migratory speed of shYAP cells. We found that a pharmacological inhibitor of Rac1-GEFs, NSC23766, significantly decreased the migration speed of shCtrl cells in a dose-dependent manner, but not that of shYAP-1 or -2 cells (**Fig 2D, Fig. S3C-I, Movie S3**). Collectively, these findings demonstrate that YAP regulates the

small Rho-GTPase network governing cellular polarity and migration, leading to the activation of Rac1 and inhibition of RhoA in normal and cancer cells **(Fig. S3J**).

We next sought to clarify the precise regulatory mechanisms of YAP's interaction with Rac1 and RhoA given multiple scenarios of mutual regulation between the components. Our results suggest three possible hypotheses: 1) YAP activates Rac1 to inhibit RhoA, 2) YAP inhibits RhoA to activate Rac1, or 3) YAP independently modulates both Rac1 and RhoA. To test these hypotheses, we applied the logic of epistatic analysis by using pharmacological inhibitors of these small Rho-GTPases in lieu of dominant negative mutants. More specifically, we used sequential perturbation of the network components to deduce the regulatory hierarchy (further details and logic of the experiment are presented in **Fig. S4A**). Unlike the experiments presented in **Fig. 2B and 2D** which explored the dose dependence of the inhibitors on shCtrl and shYAP cells, our experiment here explores the time dependence of cell responses to either single or sequential inhibitor application at fixed concentrations, high enough to achieve the maximal effect (10μM Y27632 or 250μM NSC23766). In addition, the initiation time of the experiment is after the first inhibitor being used is already present long enough for the inhibitory effect to be achieved (1 hour). Thereafter, the migration speed of the cells can only change if the application of the second inhibitor has an added effect. Otherwise, the migratory speed should remain unchanged over time. Using this logic, we first verified that the migration speeds of shCtrl and shYAP-1or -2 cells were stable over a 6-hour period with or without treatment using Y27632 or NSC23766 (**Fig. 2E, panels I-III; Fig. S4B-C, panels I-III**). These results are consistent with our prior observations in **Fig. 2B and 2D**. Next, we used one or both pharmacological inhibitors of Rac1 and ROCK to determine how these signaling intermediates regulate each other in these cells. Specifically, we treated shCtrl and shYAP-1 or -2 cells with either the Rac1-GEF inhibitor or the ROCK inhibitor for 3 hours, followed by the addition of the other inhibitor for the next 3 hours (**Fig. 2E, panels IV-V; Fig. S4B-C, panels IV-V**). The order of inhibitor treatments was indispensable in this analysis to ascertain which one of the aforementioned hypotheses is valid (**Fig. S4A**). Remarkably, we found that in cells treated to inhibit ROCK, subsequent inhibition of Rac1-GEF did not

further alter the migration speed in either shCtrl or shYAP cells (**Fig. 2E, panel IV, Fig. S4B-C, panel IV**). However, ROCK inhibition after the initial Rac1-GEF inhibition rescued the cell migration speed of both shCtrl and shYAP cells (**Fig. 2E, Panel V, Fig. S4B-C, panel V**). Altogether, these results strongly favored the model (Hypothesis 1 in **Fig. S4A**) in which YAP activates Rac1 to inhibit ROCK signaling, resulting in the enhancement of cell migration.

We then investigated the effect of Rac1-GEF or ROCK inhibition on the activity of RhoA and Rac1 in shCtrl and shYAP cells. Our model predicts that Rac1 activity is not dependent on RhoA-ROCK signaling, and indeed we observed no change in Rac1-GTP or RhoA-GTP levels upon ROCK inhibition (**Fig. 2F, Fig. S4D-G**). Again, consistent with our model, we observed a significant increase in RhoA-GTP levels in both shCtrl and shYAP cells following Rac1 inhibition with NSC23766 (**Fig. 2G, Fig. S4H**). As expected, Rac1 inhibition also significantly reduced Rac1-GTP levels in both shCtrl and shYAP cells (**Fig. S4I**). Finally, we tested whether RhoA perturbation was sufficient to restore the migratory speed of shYAP cells. Treatment with two RhoA siRNAs or a dominant-negative RhoA (DN-RhoA) rescued the migratory speed of shYAP cells, with no significant effect on the migration speed of shCtrl cells (**Fig. 2H-J**). Likewise, introducing a constitutively active Rac1 construct yielded similar results (**Fig. 2K**). These findings demonstrate that YAP activates Rac1, resulting in the inhibition of RhoA-ROCK signaling, which altogether enhances cellular migration (**Fig. S4J**). Thus, YAP simultaneously and sequentially tunes Rac1 and RhoA activities to potentiate cell motility.

YAP directly transactivates the Rac1-GEF TRIO to modulate Rac1 activity

Next, we sought to determine how the transcriptional co-activator YAP regulates the activity of Rac1. Small GTPases can be regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs)(*31*). Since NSC23766 disrupts the interaction of Rac1 with two Rac1-GEFs, TRIO, and TIAM1(*32*), we sought to determine if YAP regulates one or both of these Rac1-GEFs. We observed a decrease in TRIO protein and gene expression after YAP knockdown in all cell lines tested

using two independent YAP-targeting shRNA sequences (**Fig. 3A-B, Fig. S5A-G**). Moreover, TRIO expression increased following YAP overexpression both in GBM and MCF10A cells (**Fig. 3C, Fig. S5H**). However, we did not observe any changes in TIAM1 gene expression in shYAP cells compared to shCtrl cells (**Fig. S5I**). Given that YAP, a transcriptional co-activator, cooperates with TEAD family transcription factors to directly modulate gene expression(*6*), we examined whether TRIO is transcriptionally regulated by YAP and whether YAP is found at known enhancer and/or promoter regions of TRIO. Using published chromatin immunoprecipitation (ChIP) sequencing data for YAP and TEAD4 in MDA-MB-231 cells(*33*), we found that both YAP and TEAD4 bind an intronic element of TRIO (Site 1: 14265617 to 14266192 base pairs on chromosome 5) that bears histone modification patterns indicative of active enhancers(*34*) (**Fig. 3D**). Specifically, this locus bears enhancer-associated H3K4 monomethylation (H3K4me1) and H3K27 acetylation (H3K27ac), with less promoter-associated H3K4 trimethylation (H3K4me3)(*35*). Notably, this enhancer region was independently reported to physically interact with TRIO's promoter(*36*). In addition, we found a weaker enrichment of YAP-TEAD4 binding at a second, intronic binding site (Site 2: 14268225 to 14268865 bp on chromosome 5) (**Fig. 3D**). We also scanned for the presence of TEAD binding motifs at these two TRIO enhancer loci in silico. Using previously reported TEAD motifs (*37*), we observed its presence at both TRIO enhancer sites. We also identified an additional TEAD4 consensus motif in the TRIO enhancer site 2 according to the JASPAR database(*38*) **(Suppl. File 1)**. By contrast, we found no evidence of YAP or TEAD4 binding at TIAM1 enhancer and promoter elements. Given these findings, we tested the MDA-MB-231 YAP binding sites by ChIP-PCR on both intronic TRIO enhancer sites in GBM cells. Indeed, significant YAP binding was observed at both enhancer sites of TRIO (**Fig. 3E, Fig. S5J**). To evaluate the functional consequence of this binding, we performed an enhancer luciferase assay using a 150 bp sequence (centered on the peak of YAP binding) paired with the promoter region of TRIO in HEK293 cells, with or without overexpression of YAP. We found that this sequence increased reporter activity compared to negative control and showed increased activity upon YAP overexpression (**Fig. 3F**). To further confirm that YAP only interacts with

the enhancer and not the promoter sequence of TRIO, we performed an additional control experiment using a 1470 bp promoter sequence of TRIO paired with a luciferase reporter, with or without overexpression of YAP. Since Zanconato *et al*, have previously shown that YAP's transcriptional regulation is largely restricted to direct enhancer binding with a few exceptions like CTGF where YAP occupies its promoter region, we also included a 612 bp sequence of CTGF's minimal promoter linked to a luciferase reporter as a positive control. Unlike CTGF's promoter, TRIO's full-length promoter sequence did not increase reporter activity and showed no enhancement in activity upon YAP overexpression (**Fig. S5K**). All these results suggest that YAP directly activates TRIO transcription by binding to its intragenic enhancer region.

Next, we examined whether YAP-TRIO signaling was sufficient to regulate Rac1 activity. We found an increase in Rac1-GTP levels in YAP OE cells compared to CONT cells (**Fig. 3G, Fig. S5L)**. Furthermore, stable overexpression of wildtype TRIO (TRIO OE) was sufficient to increase Rac1-GTP levels (**Fig. 3H, Fig. S5M)**. Consistent with these findings, shRNA-mediated stable knockdown of TRIO (shTRIO) decreased Rac1-GTP levels (**Fig. 3I, Fig. S5N-O)**. Given the antagonistic relationship between Rac1 and RhoA signaling, we assessed the activity of RhoA-GTP in these cells. We observed decreased RhoA-GTP levels in YAP OE and TRIO OE cells vs. control cells (**Fig. S5P-Q**), and increased RhoA-GTP levels in shTRIO cells vs. shCtrl cells **(Fig. S5R)**, paralleling our observations in shYAP cells. Collectively, these results demonstrate that YAP directly transactivates the Rac1-GEF TRIO and that TRIO is necessary and sufficient to activate Rac1 and inhibit RhoA.

Given that there has been evidence suggesting that TRIO-Rac1 signaling can inhibit YAP phosphorylation in HEK293 cells(*39*), we explored this potential feedback regulation in GBM cells. Interestingly, we did not observe any significant change in either phospho-YAP or YAP protein levels when we knocked down TRIO (**Fig. S5S**), indicating that no potential direct feedback regulation between YAP and TRIO-Rac1 signaling exists in the context of GBM cells.

To test whether our in vitro observations were consistent with data from clinical samples, we assessed TRIO expression in intraoperatively obtained primary GBM tissues from patients (**Fig. S5T**). We found that the full-length TRIO protein was indeed expressed in a majority of the GBM tissues analyzed. More importantly, its expression positively correlated with YAP expression in these clinical samples (**Fig. 3J**). Next, we surveyed gene expression profiles from the Repository for Molecular Brain Neoplasia Data (REMBRANDT) and The Cancer Genome Atlas (TCGA). Specifically, we explored whether there was a correlation between YAP and either TRIO or TIAM1 expression in these two GBM datasets. To enable this analysis, we first derived a list of genes significantly downregulated in shYAP compared to shCtrl mRNA samples from JHGBM651 and JHGBM612 cells (See Materials and Methods) (**Suppl Table 2**). We refer to these YAP-regulated genes as the GB-YAP signature. Gene signatures allow a more robust estimation of YAP activity from gene expression datasets than mRNA alone, since many transcriptional regulators, including YAP, undergo significant post-transcriptional regulation. We found that the GB-YAP signature of GBM cells correlated with YAP signatures derived from other cell types(*40*) (**Fig. S5U**). Furthermore, the REMBRANT data suggested that the patients with elevated GB-YAP signature genes also have significantly higher expression of TRIO, but not TIAM1 (**Fig. 3K**). Indeed, patients with TRIO overexpression were significantly overrepresented among patients with high GB-YAP signature expression in this dataset (comparing all observed patient group sizes to random expectation using Fisher's exact test (**Fig. 3L**). Taken together, these results derived from patient-derived tumor tissues support our in vitro observations that YAP modulates TRIO expression.

TRIO is necessary and sufficient for YAP-driven migration

Given our previous results, we sought to test whether TRIO was necessary and/or sufficient for YAP's control of cell migration. Consistent with its putative functional role, we found that silencing the expression of TRIO decreased the migration speed of GBM, NHA, and MCF10A cells (**Fig. 4A-B, Fig. S6A-F, Movie S5**). As a preliminary test, we used a specific pharmacological inhibitor of TRIO, ITX3 to

modulate its activity(*41*). We confirmed ITX3 specificity by treating shTRIO cells with this compound and observed no further change in Rac1-GTP levels or migration speed (**Fig. S6G-J**). We then found that ITX3 reduced the migration speed of shCtrl cells in a dose-dependent manner without affecting shYAP-1 or -2 cells (**Fig. S6K-M, Fig. S7A-G, Movie S4**). Furthermore, higher doses of ITX3 also decreased the migration speed of YAP OE GBM cells to match the speed of CONT cells (**Fig. S7H)**. Furthermore, ITX3 treatment significantly reduced Rac1-GTP levels in shCtrl and shYAP cells (**Fig. S7I**). Conversely, upon treatment with ITX3, a significant increase in RhoA-GTP level was observed in shCtrl, but not in shYAP cells (**Fig. S7J)**, demonstrating negative regulation of RhoA activity by TRIO-Rac1 signaling downstream of YAP. These results suggest that the effects of YAP on RhoA-GTPases and cell migration can be entirely explained by TRIO expression.

We further explored whether YAP-mediated Rac1 activation was largely driven by TRIO. Given that we found that, in contrast to TRIO, the gene expression of TIAM1 was not reduced in shYAP cells, combined TIAM1/TRIO inhibition using NSC23766 did not significantly alter the migration speed or Rac1-GTP level of shTRIO-1 or -2 cells (**Fig. S7K-P**). On the other hand, the introduction of constitutively active Rac1 (Rac1-CA) rescued the migration speed in shTRIO cells but not shCtrl cells (**Fig. S7Q**). Furthermore, we found no significant change in RhoA-GTP level in shTRIO cells treated with NSC23766, which contrasted with the increase seen in shCtrl cells treated with the inhibitor compared to vehicle (**Fig. S7R**). Additionally, the introduction of constitutively active Rac1 (Rac1-CA) significantly decreased RhoA-GTP levels in shTRIO cells but not in shCtrl cells (**Fig. S7S**). These results confirmed the specific role of TRIO but not TIAM1 signaling in mediating YAP's control of cell migration via activation of Rac1.

We then explored the effect of perturbations of TRIO on RhoA-dependent signaling and its phenotypic consequences. We found that ITX3 treatment significantly increased the RhoA-GTP level of shCtrl, but not shTRIO cells (**Fig. S8A**), providing additional evidence of the inhibitory effect of TRIO-dependent signaling on RhoA. Furthermore, the introduction of a dominant-negative RhoA construct

(RhoA-DN) rescued the migratory phenotype of shTRIO cells but not of shCtrl cells (**Fig. S8B**). Suppression of the downstream effector of RhoA with the ROCK inhibitors, Y27632 or H1152, rescued the migration speed of shTRIO cells, like our observations with shYAP cells (**Fig. S8C-H, Movie S6**). Consistent with our prior results, ROCK inhibitors did not significantly affect Rac1-GTP or RhoA-GTP levels in shTRIO cells (**Fig. S8I-L)**. However, the introduction of a dominant-negative RhoA construct (RhoA-DN) successfully rescued Rac1-GTP levels in shTRIO cells (**Fig. S8M**). These findings suggest that there exists a feedback regulation from RhoA, and not from downstream of RhoA (i.e. ROCK), to Rac1, which has been supported by other studies(*30*, *42*, *43*).

Next, we inquired whether TRIO was sufficient to rescue the migration speed of shYAP cells. First, we confirmed that overexpression of TRIO increases migratory speed (**Fig. 4C-D, Fig. S8N-O**). Remarkably, overexpression of a knockdown-resistant TRIO in shYAP-1 or -2 cells (shYAP-1 or -2 + TRIO OE) completely rescued migration speed to levels of shCtrl cells (**Fig. 4E, Fig. S8P)**. To further explore the molecular mechanisms of TRIO activity, we focused on its GEF function. TRIO has two distinct GEF domains, GEF1 and GEF2 that control Rac1 and RhoA activation, respectively. However, our findings indicate that TRIO activates Rac1 signaling while inhibiting RhoA to potentiate migration. Thus, we sought to determine whether one or both GEF domains are relevant for this TRIO-mediated migratory behavior seen in GBM cells. We monitored migration speed upon stable overexpression of wildtype TRIO (TRIO OE), TRIO with inactivating GEF1 (TRIO iGEF1 OE) or GEF2 (TRIO iGEF2 OE) mutations (**Fig. 4F-G**). Notably, we observed an increase in migration speed of TRIO iGEF2 cells, phenocopying the migratory behavior of TRIO OE cells (**Fig. 4F-G**). However, we observed no change in migratory speed of TRIO iGEF1 cells (**Fig. 4F-G**). These results demonstrate that the GEF1 domain of TRIO, responsible for activation of Rac1, is indispensable for TRIO's pro-migratory effect. Moreover, TRIO's RhoA-activating GEF2 domain appears dormant and is dispensable for YAP-TRIO-driven migration in GBM cells. Our results demonstrate that TRIO is necessary and sufficient to account for Rac1 activation by YAP, resulting in RhoA inhibition and increased cell migration.

YAP-TRIO signaling promotes cell invasion

In addition to modulation of migration speed, infiltration of the surrounding stroma requires enhanced invasive capacity, enabling cell navigation in structurally complex tissue environments. Interestingly, in both the TCGA and REMBRANDT tumor gene expression datasets, the GB-YAP signature is associated with higher expression of an invasive gene signature (IGS)(*44*) (**Fig. 5A, Fig. S9A**). In a reciprocal fashion, patients with elevated expression of the IGS were significantly overrepresented among patients with high GB-YAP signature expression (**Fig. S9B**). Consistent with these observations, we found that fewer shYAP and more YAP OE cells invaded through Matrigel-coated Boyden chambers than their respective control cells in vitro (**Fig. 5B-C, Fig. S9C-D**). This led us to explore if the YAP-TRIO signaling is also responsible for the regulation of cell invasion. Indeed, there was a significant decrease in the number of invaded shTRIO cells compared to shCtrl cells, similar to what we saw in shYAP cells (**Fig. 5D, Fig. S9E-F**). And there was a significant increase in the number of invaded TRIO OE cells than control cells (**Fig. 5E**). When applying the TRIO inhibitor ITX3, we observed reduced invasion of shCtrl cells but not in shYAP and shTRIO cells after treatment (**Fig. S9G-H**). As YAP-TRIO activates Rac1 and inhibits RhoA as illustrated earlier in this study, we also tested the involvement of RhoA in the regulation of cell invasion by applying the ROCK inhibitors, Y26732 and H1152, in shYAP and shTRIO cells. We found that ROCK inhibition rescued the invasive capacity of shYAP and shTRIO cells without significantly altering the invasion of shCtrl cells (**Fig. S9I-L**). Given the critical role of YAP-TRIO signaling in driving the migratory and invasive capacity of GBM cells in vitro, we sought to evaluate the relevance of this pathway in vivo using our murine intracranial xenograft model of GBM infiltration. To that end, we established control (CONT) and YAP overexpressing (YAP OE) JHGBM612 cells with or without TRIO knockdown. Next, we injected 50,000 CONT + shCtrl, CONT + shTRIO, YAP OE + shCtrl, or YAP OE + shTRIO JHGBM612 cells into the striatum (at pre-determined coordinates from the bregma; see Materials and Methods) of immunocompromised mice and quantified the number of human

GBM cells infiltrating along the corpus callosum 5-weeks post-injection. We observed that overexpression of YAP was sufficient to increase the invasion of JHGBM612 cells via the corpus callosum into the contralateral hemisphere (CONT + shCtrl vs. YAP OE + shCtrl; **Fig. 5F-G**). Moreover, knocking down TRIO significantly attenuated the invasive potential of these GBM cells (CONT + shCtrl vs. CONT + shTRIO; **Fig. 5F-G**). In addition, we found a significant reduction in the number of YAP OE + shTRIO cells vs. YAP OE + shCtrl cells migrating through the corpus callosum (**Fig. 5F-G**). Together, these results indicated that while YAP is sufficient to increase the invasive capacity of GBM cells, TRIO is necessary to promote YAP-driven cell dispersion in vivo.

STAT3 is potentially involved in YAP-mediated cell invasion

Next, we wanted to investigate a potential downstream target of YAP-TRIO signaling that might promote cell invasion. There is evidence suggesting that STAT3, a transcription factor known to promote invasion and metastasis(*45*), can be activated by Rac1(*46*). It is reported that Rac1 can directly bind to and regulate STAT3 activity(*47*, *48*). Rac1 can also indirectly induce STAT3 activity through the autocrine production of cytokine IL-6(*49*, *50*). Various biological processes involve activation of the Rac1-STAT3 signaling such as gland involution(*51*), chondrogenesis(*52*), and primordial follicle pool production(*53*). Additionally, studies have shown that the Rac1-STAT3 axis is important in promoting invasion and epithelial-mesenchymal transition in colorectal cancer(*48*) and gastric cancer(*54*). However, few efforts have been put into exploring this signaling axis in GBM and its potential crosstalk with YAP-TRIO. Interestingly, we found evidence of an association between elevated expression of the GB-YAP signature and a published GB-STAT3 signature(*55*), with significant overrepresentation of patients with elevated expression of the two signatures in GBM samples from both clinical datasets (**Fig. 6A-B, Fig. S10A**). We then surveyed primary GBM tissues for expression and phosphorylation of STAT3 to assess a possible clinical significance of this factor (**Fig. 6C**). We observed that the majority of specimens with elevated YAP expression also exhibited high STAT3 phosphorylation levels **(Fig. 6C-D)**. Conversely,

only a few tumor samples with low YAP levels had high STAT3 phosphorylation **(Fig. 6C-D)**. Given STAT3's presentation in clinical specimens and datasets, we sought to investigate its connection to the YAP-TRIO-Rac1 network. Firstly, we observed decreased phosphorylation of STAT3 on Tyr705 in shYAP-1 or -2 cells (**Fig. 6E, Fig. S10B**). Moreover, YAP OE cells expressed higher levels of phosphorylated STAT3 than CONT cells **(Fig. 6F, Fig. S10C)**. Functionally, pharmacological inhibition of STAT3 using LLL12 impaired both the invasive and migratory capacity of shCtrl cells more than that of shYAP cells (**Fig. 6G, Fig. S10D-F, Movie S7**). Similar to shYAP cells, we observed a decrease in STAT3 phosphorylation following the silencing of TRIO expression vs. shCtrl cells (**Fig. 6H, Fig. S10G-H**). Also, applying TRIO inhibitor ITX3 decreased STAT3 phosphorylation levels in a dose-dependent manner (**Fig. S10I**). Furthermore, TRIO OE GBM cells exhibited an increase in STAT3 phosphorylation compared to CONT cells (**Fig. 6I**). Remarkably, both stable overexpression of TRIO in shYAP cells (shYAP + TRIO OE) and overexpression of YAP in shTRIO cells (shTRIO + YAP OE) recovered the phosphorylation levels of STAT3 to shCtrl cell levels (**Fig. 6J-K**). To explore the link between Rac1 and STAT3, we treated the cells with the Rac1-GEF inhibitor, and we observed decreased phospho-STAT3 expression in a dose-dependent manner, mirroring our findings with TRIO inhibition (**Fig. S10J**). Collectively, these results suggest that STAT3 is involved and could be a potential candidate downstream of the YAP-TRIO-Rac1 axis to drive cell invasion.

YAP, TRIO, and STAT3 signatures predict poor clinical outcomes in glioblastoma

Our results suggest that YAP-driven migration and invasion are critical for the biology of aggressive cancers. We, therefore, explored the clinical implications of these findings for gliomas, of which GBM is the highest grade (Grade 4) tumor with a median survival of 14 months. We found that GB-YAP signature expression increased with glioma grade in the REMBRANDT dataset (**Fig. S11A**). Additionally, the percentage of glioma patients overexpressing the GB-YAP signature compared to non-cancer cortex increased with glioma grade (**Fig. 7A**). Having found that YAP is hyperactive in GBMs, we

focused on its prognostic value for these patients as determined by Kaplan-Meier analyses. Strikingly, higher expression of the GB-YAP signature predicted poor prognosis of GBM patients from both TCGA (**Fig. 7B**) and REMBRANDT cohorts (**Fig. S11B**). In addition, the YAP gene signature was more predictive than 91.5% of 1 million simulated, size-matched gene sets (empirical $p = 0.085$), suggesting it is one of the most predictive gene sets of its size.

Due to the molecular heterogeneity of GBMs, we explored if YAP activity was associated with one or more of the GBM subtypes including proneural, classical, mesenchymal, and CpG island methylator phenotype (G-CIMP) subtypes described previously(*56*). Notably, Mesenchymal GBMs are distinguished by the invasion of brain parenchyma coupled with pronounced angiogenesis(*57*). We found that there was a significant over-representation of GB-YAP-high patients among the Mesenchymal GBMs but not among other subclasses (**Fig. 7C**). Consistently, higher expression of the GB-YAP signature also corresponded to higher expression of Mesenchymal subclass genes (**Fig. 7D**). In vitro, we observed decreased expression levels of several Mesenchymal subclass-associated genes after YAP knockdown in three different GBM cell lines (**Fig. 7E**). On the other hand, we found that GB-YAP-low patients were significantly overrepresented among GBMs in the glioma G-CIMP subclass (**Fig. 7C**), which exhibits hypermethylation at CpG islands and is frequently associated with lower grade gliomas(*58*). To confirm its prognostic value, we evaluated progression-free survival along with GB-YAP signature expression using Kaplan-Meier analyses in each GBM subclass individually. Strikingly, despite its strong association with the Mesenchymal subclass in terms of signature gene expression, higher expression of the GB-YAP signature predicted significantly quicker recurrence in all the GBM subclasses (**Fig. S11C-E**).

We then investigated whether incorporating TRIO and STAT3 with GB-YAP signatures would maintain their predictive power considering TRIO and STAT3 expression have also been reported to increase with glioma grade(*59*) similarly to YAP. First, using the TCGA dataset, we determined the distribution of patient groups with hyperactivation of either one, two, or all three of the signaling components (i.e., YAP, TRIO, and STAT3). Hyperactivation was determined using the GB-YAP

signature, TRIO transcript, and GB-STAT3 signature (**Fig. 7F**). Indeed, we found a significant over-representation of GBM patients with hyperactivation of all the three members of YAP, TRIO and STAT3. Next, we stratified patients into 8 groups representing every possible combination of YAP, TRIO, and STAT3 co-activation, and performed Kaplan-Meier survival analyses. Notably, the groups that exhibited YAP co-activation with either TRIO or STAT3 or both had a significantly worse prognosis than the group without hyperactivation of any of these proteins (YAP^{LOW}, $TRIO^{LOW}$, $STAT3^{LOW}$) (**Fig. 7G-I, Fig. S11F-G, Fig. S12)**. Importantly, hyperactivation of TRIO or STAT3 or both without high YAP activity did not appear to be associated with significantly worse prognosis in this dataset (**Fig. S12**) similarly to the case when high YAP activity is combined with low level of both TRIO and STAT3, emphasizing the importance of YAP and also the rather strong interplay among YAP, TRIO and STAT3 in contributing to poor clinical outcome of GBM patients. These results should be viewed as preliminary, as tumor purity, mRNA degradation, and cohort size might be confounding factors in these analyses. Larger patient cohorts with matched expression data would help to validate these results. Nevertheless, collectively, our study provides important initial evidence of the clinical value of the YAP-driven pro-migratory and invasive signaling network in predicting patient outcomes (**Fig. 7J**).

DISCUSSION:

This study suggests a central and widespread role for YAP in controlling the migratory speed and invasiveness of cancer and normal cell types through the modulation of a Rho-GTPase switch. Previous work has illustrated the function of cytoplasmic YAP in regulating endothelial cell migration(*60*). Our work uncovers a YAP-mediated signaling network focusing on the role of nuclear YAP as a transcriptional coactivator. Specifically, we demonstrate that YAP increases the expression of the Rac1-GEF TRIO via direct modulation of its intronic enhancer, leading to activation of Rac1 and inhibition of RhoA small GTPases. This intricate cascade promotes not only cell motility but also invasive capacity, using both transcriptional and post-transcriptional mechanisms (**Fig. 7J**). Furthermore, we find that, although TRIO

harbors two GEF domains, its YAP-driven functions are mediated by the GEF1 domain's activation of Rac1. This agrees with studies in different systems that demonstrate Rac1 is the major target of TRIO(*20*). One known example of RhoA activation by TRIO is Gaq signaling, where Gaq binds to the PH2 module within the GEF2 domain, releasing PH2-mediated inhibition of DH2, thereby stimulating GEFD2 activity on RhoA(*61*). Nevertheless, the activity of both Rac1 and RhoA was found to be important in mediating the YAP-driven TRIO-dependent control of cell migration and invasion, suggesting the importance of the crosstalk between Rac1 and RhoA, downstream of TRIO activation.

Intriguingly, it has been reported that TRIO-mediated Rho-GTPase signaling can regulate YAP(*62–67*) which, coupled with our results, suggests that regulation between YAP and Rho-GTPases can be mutually enhancing, constituting a feedback loop that can stabilize migratory and invasive phenotypes. There have also been other reports suggesting alternative mechanisms of YAP regulating cell motility or Rac1 more specifically. For example, it has been shown that YAP regulates actin dynamics through the modulation of ARHGAP29 to promote metastasis of gastric cancer cells(*68*). Interestingly, unlike in GBM, YAP modulates Rac1 via transactivation of the Rac1-GEF, TIAM1, through an enhancer-mediated mechanism, during invadopodia formation in breast cancer, supporting the argument for disease-specific regulation(*69*).

In our study, we found that STAT3 is potentially involved in mediating YAP-driven infiltrative spread through an unknown mechanism. Since YAP expression and activity are controlled by a diverse range of stimuli and genetic alterations(*3*, *5*, *7*, *14*, *15*, *63*, *66*, *70*), the evidence of crosstalk between YAP, Rho-GTPases, and STAT3 establishes an even wider spectrum of inputs and mechanisms that regulate cell locomotion. A recent study reported the role of YAP signaling in the activation of the JAK-STAT3 pathway to regulate endothelial cell proliferation during angiogenesis(*71*), further establishing the significance of YAP-mediated activation of STAT3 in different phenotypic and cellular contexts. Likewise, additional studies have shown that STAT3 activation by YAP through other intermediatory regulators can be facilitated by direct interaction. For example, others have shown YAP-TEAD can co-

regulate with other partners (e.g. AP-1 family transcription factors such as JUNB) on a variety of target sites including STAT3(*72–74*). Thus, a detailed exploration of YAP-TRIO's mechanistic connection to STAT3 in the context of Rho-GTPase signaling is warranted. Altogether, these findings support a model of crosstalk between multiple shared signaling components in a cell-type and context-specific manner.

Together with our previous study by Park *et al*(*75*), our results suggest that YAP-controlled cell migration and invasion are fundamental functions present in many cell types. This pathway may be particularly important in developmental and physiological contexts involving episodes of large-scale migration such as wound healing and progenitor cell navigation during neural tissue development.

Given the widespread hyperactivation of YAP in many cancers(*11*), our study further clarifies the mechanisms controlling invasive cancer cell phenotypes. One of the hallmarks of tumor malignancy and progression to higher pathological grades is the ability of cancer cells to invade their surrounding parenchyma, intravasate surrounding blood, and lymphatic vessels, and eventually, seed distant tissues as metastatic tumors(*76*). Tumor recurrence and lethality can be greatly aided by the migratory and invasive capacity of cells(*77*). While the YAP-TEAD interaction has been shown to promote metastasis in breast cancer(*6*), the downstream signals responsible for increased metastatic potential have remained poorly understood. As with metastatic cancers, GBMs often evade eradication because individual cells spread from the primary bulk tumor, thus making complete resection and localized radiation treatment virtually impossible. Clinically, more than half of metastatic tumors display pronounced local infiltration(*24*) and can recur in as little as 3 months despite radical surgery, chemo- and radiotherapy(*21–23*). Thus, understanding the molecular basis for cell dispersal can inform patient prognosis and facilitate the development of improved treatment modalities(*77–80*). Importantly, our work highlights the predictive power of YAP, TRIO, and STAT3 signatures in the prognosis of GBM patients. Lastly, while tumor growth is another important aspect of aggressive cancers, our study is restricted to delineating the role of YAP in invasive spread only. While YAP has been implicated as a master regulator in cancers whereby its depletion can attenuate tumor growth in multiple cancers including breast cancer, gastric cancer,

etc.(*40*, *81*, *82*), very limited efforts have focused on studying the contributions of TRIO in the context of YAP signaling in this aspect(*83*). Intriguingly, in our in vivo invasive spread study, we observed a potential effect on tumor size upon YAP and TRIO modulation. However, our studies did not directly measure tumor growth in a controlled or detailed manner. Thus, further careful examination of YAP-TRIO signaling's role in cell proliferation and tumor growth would be interesting and clinically informative for the GBM field. Altogether, our study suggests that a network, systems perspective on the etiology of aggressive cancers can benefit from explicit analysis of molecular cascades that integrate numerous signaling pathways, inform the design of targeted and combination therapies, as well as augment our understanding of the drivers of transcriptionally distinct tumor subtypes.

ACKNOWLEDGEMENTS:

We thank the Department of Neurological Surgery and Oncology at Johns Hopkins Hospital for access to intraoperatively-obtained glioblastoma tissues; Lakesha Johnson and Liron Noiman for establishing primary cell cultures of these primary glioblastoma specimens; Colette Ap Rhys for designing and preparing lentiviruses; Conover Talbot Jr. for technical support and helpful discussions of microarray analysis; Tom Schaffer for technical support with the RhoA-GTP pull-down assay; and Kaisorn L. Chaichana for glioblastoma patient MRI images. This research was funded by NIH R01 NS070024 and NIH 5K08 NS055851 (A.Q.-H), and U54 CA209992 and U01 CA155758 (A.L). S.R.S. was supported by the National Science Foundation Graduate Research Fellowship. A.M. was supported by the Maryland Stem Cell Postdoctoral Research Fellowship.

AUTHOR CONTRIBUTIONS:

S.R.S. conceived and designed the project. S.R.S., N.D.T. performed the experiments. C.R. assisted with the experiments. N.D.T. performed bioinformatic analysis. J.P. tracked and analyzed migration time-lapse data. A.M. and G.V. assisted with early experiments. J.C.M-G assisted with YAP-TRIO in vitro rescue experiments. S.S. provided TRIO wildtype and mutant lentiviral and corresponding control constructs. S.W., and S. X. assisted with YAP-TRIO in vivo experiment. S.R.S., N.D.T., C.R., and J.P. interpreted the results. A.L. and A.Q.-H. supervised the project. S.R.S. and N.D.T. wrote the manuscript. S.R.S., C.R., S.M., A.L., and A.Q.-H. reviewed and revised the manuscript.

DECLARATION OF INTERESTS:

Microarray data of shCtrl and shYAP JHGBM651 and JHGBM612 cells have been deposited with NCBI Gene Expression Omnibus under accession code (*upon acceptance of manuscript*). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. S.R.S. is an equity holder and member of the scientific advisory board of NeuScience, Inc., and a consultant at Third Bridge Group Limited, which are not related to this work. Correspondence and requests for material should be addressed to A.L. (andre.levchenko@yale.edu) or A.Q.-H (Quinones-Hinojosa.Alfredo@mayo.edu).

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FIGURE LEGENDS:

Figure 1 | YAP regulates migratory capacity. A, Immunoblots of YAP expression in human non-cancer cortex and primary glioblastoma patient tissues. Blots are representative of 3 independent experiments. **B,** Migration pattern and average distance traveled by shCtrl or shYAP JHGBM651 cells in vitro on 2D surface. Trajectories are calculated from 20 cells for both shCtrol and shYAP JHGBM651 cells. **C, D,** Mean migration speed of shCtrl versus shYAP JHGBM651 cells or CONT versus YAP OE JHGBM612 cells. Mean migration speed is calculated from 30 cells in both C and D. * = $P<0.05$, Wilcoxon Rank-Sum test in both C and D. **E,** Left: Human Nuclei/DAPI immunofluorescent images of mouse brain coronal sections after injections of shCtrl or shYAP JHGBM612 cells (i.s. = injection site). Dashed lines outline the bulk tumor margins on the ipsilateral hemisphere. White boxes outline the area magnified in the right panel. Scale bar = 1mm. Right: magnified images of the corpus callosum in the right hemisphere (m.l. = midline, c.c. = corpus callosum, st = striatum, ctx = cortex, v = ventricle). Scale bar = 1mm. Images are representative of 5 mice per group. See also Supplemental Fig. 1P. **F,** Mean number of shCtrl or shYAP cells per mouse invading past the midline into the contralateral hemisphere. Quantifications are based on 5 mice per group. Student's t-test; * = $P<0.05$. **G,** Representative Paxillin/F-actin/DAPI immunofluorescent images of shCtrl or shYAP JHGBM651 cells of 3 independent experiments. Scale bar = 5μm. **H,** Mean number of focal adhesion (FA) per cell in shCtrl from 25 cells or shYAP JHGBM651 from 16 cells from 3 independent experiments. Student's t-test; * = $P<0.05$. **I,** Representative immunoblots of YAP, total and p-LIMK expression in shCtrl, shYAP JHGBM651 cells of 3 independent experiments. 2 independent shYAP constructs were used in JHGBM651 cells. **C-D, F, H,** Data shown is mean ± SEM.

Figure 2 | YAP regulates migration through a Rho-GTPase switch. A, G-LISA analysis of RhoA-GTP levels in shCtrl or shYAP JHGBM651 cells from 3 independent experiments. **B,** Mean migration speed

of shCtrl or shYAP JHGBM651 cells treated with increasing doses of the ROCK inhibitor Y27632. Wilcoxon Rank-Sum test; Red * = P<0.05, between corresponding doses of Y27632 and no drug treatment in shYAP cells; Red # = P<0.05, between shCtrl and shYAP cells at corresponding doses. **C,** G-LISA analysis of Rac1-GTP levels in shCtrl or shYAP JHGBM651 cells from 3 independent experiments. **A, C,** Student's t-test; * = P<0.05, between shCtrl and shYAP cells. **D,** Mean migration speed of shCtrl or shYAP JHGBM651 cells treated with increasing doses of the Rac1-GEF inhibitor NSC23766. Wilcoxon Rank-Sum test; Black * = P<0.05, between corresponding doses of NSC23766 and no drug treatment in shCtrl cells; Red # = P<0.05, between shCtrl and shYAP cells at corresponding doses. **E,** Mean migration speed of shCtrl and shYAP JHGBM651 cells treated with no drug, Y27632, or NSC23766 for 3 or 6 hours as indicated. Wilcoxon Rank-Sum test; Black * = P<0.05, between mean speed from hour1 to hour2 and that from hour4 to hour5 in shCtrl cells; Red * = P<0.05, between mean speed from hour1 to hour2 and that from hour4 to hour5 in shYAP cells; Red # = P<0.05, between mean speed in shYAP cells and that in shCtrl cells at corresponding time points. **F,** G-LISA analysis of Rac1-GTP levels in shCtrl or shYAP JHGBM651 cells treated with vehicle or Y27632 from 3 independent experiments. **G,** G-LISA analysis of RhoA-GTP levels in shCtrl or shYAP JHGBM651 cells treated with vehicle or NSC23766 from 3 independent experiments. **F-G,** Student's t-test; * = P<0.05, between drug and no drug treatment in shCtrl or shYAP cells; # = P<0.05, between shYAP and shCtrl cells with no drug treatment. **H,** Representative immunoblot of YAP and RhoA expression in shCtrl or shYAP JHGBM651 cells transfected with siCtrl or siRhoA of 3 independent experiments. **I,** Mean migration speed of shCtrl or shYAP JHGBM651 cells transfected with siCtrl or siRhoA. Wilcoxon Rank-Sum test; * = P<0.05, between siRhoA and siCtrl in shYAP cells; # = P<0.05, between siCtrl in shYAP and that in shCtrl cells. **J,** Mean migration speed of shCtrl or shYAP GBMA172 cells transfected with pCtrl or a dominant-negative RhoA vector (pRhoA-DN). Wilcoxon Rank-Sum test; * = P<0.05, between pRhoA-DN and pCtrl in shYAP cells; # = P<0.05, between pCtrl in shYAP and that in shCtrl cells. **K,** Mean migration speed of shCtrl or shYAP GBMA172 cells transfected with pCtrl or a constitutively active Rac1 vector (pRac1-CA). Wilcoxon Rank-Sum test;

* = P<0.05, between pRhoA-CA and pCtrl in shYAP cells; # = P<0.05, between pCtrl in shYAP and that in shCtrl cells. **B, D, E, I-K,** Mean migration speed is calculated from 30 cells. **A-G, I-K,** Data shown is mean ± SEM.

Figure 3 | YAP directly transactivates the Rac1-GEF TRIO. A, Representative immunoblot of TRIO expression in shCtrl or shYAP JHGBM651/JHGBM612 cells of 3 independent experiments. **B,** TRIO mRNA expression in shCtrl or shYAP JHGBM651/JHGBM612 cells from 3 independent experiments. Student's t-test; * = P<0.05, between shCtrl and shYAP. **C,** Representative immunoblot of TRIO expression in CONT or YAP OE JHGBM612 cells of 3 independent experiments. **D,** YAP/TEAD4 binding to an intronic region of TRIO as observed in a publicly available ChIP-sequencing dataset using MDA-MD-231 cells(*33*). Substantial H3K4 monomethylation (H3K4me1) and H3K27 acetylation (H3K27ac), without H3K4 trimethylation (H3K4me3), indicates an active enhancer element in this locus. **E,** ChIP-PCR querying across two enhancer elements of TRIO in JHGBM612 cells. Results are representative of 3 independent experiments. **F,** *TRIO* gene enhancer luciferase assay using TRIO enhancer segment in cells with or without YAP OE in JHGBM612 cells. **G-I,** G-LISA analysis of Rac1-GTP levels in CONT or YAP OE JHGBM612 cells, CONT or TRIO OE JHGBM612 cells, and shCtrl or shTRIO JHGBM651 cells, respectively, from 3 independent experiments. Student's t-test; * = P<0.05, between CONT and YAP/TRIO OE or between shCtrl and shTRIO. **J,** Correlation plot of relative YAP and TRIO protein expression (normalized to β-actin) in patient-derived primary GBM tissues based on densitometric quantification of immunoblots in Fig. 6C and Fig. S5T. **K,** TRIO and TIAM1 expression in GB-YAP high, intermediate, or low patient groups in the REMBRANDT GBM dataset. Student's t-test; * = P<0.05, between YAP^{High} and YAP^{Low}. **L,** Percentage of patients with elevated GB-YAP signature, TRIO transcript, or both in the REMBRANDT GBM dataset. P = Fisher's exact test using the sizes of the shown patient groups. **B, F-I, K,** Data shown is mean ± SEM.

Figure 4 | YAP-TRIO signaling regulates migration. A, Representative immunoblot of TRIO expression in shCtrl and shTRIO JHGBM651 cells with 2 independent shTRIO constructs of 3 independent experiments. **B,** Mean migration speed of shCtrl or shTRIO JHGBM651 cells. Wilcoxon Rank-Sum test; * = P<0.05, between shCtrl and shTRIO. **C,** Representative immunoblot of TRIO and YAP expression in CONT and TRIO OE JHGBM651 cells of 3 independent experiments. **D,** Mean migration speed of CONT and TRIO OE JHGBM651 cells. Wilcoxon Rank-Sum test; * = P<0.05, between CONT and TRIO OE. **E,** Mean migration speed of shCtrl, shYAP, and shYAP+TRIO OE JHGBM651 cells. Wilcoxon Rank-Sum test; * = P<0.05, between shYAP and shCtrl cells. **F,** Representative immunoblot of GFP and TRIO expression in CONT, TRIO OE (wildtype), TRIO iGEF1 OE, or TRIO iGEF2 OE JHGBM612 cells of 3 independent experiments. **G,** Mean migration speed of CONT, TRIO OE (wildtype TRIO), TRIO iGEF1 OE, or TRIO iGEF2 OE JHGBM612 cells. Wilcoxon Rank-Sum test; * = P<0.05, compared to CONT. **B, D, E, G,** Mean migration speed is calculated from 30 cells. Data shown is mean ± SEM.

Figure 5 | YAP-TRIO signaling promotes cell invasion. A, Invasive gene signature expression in GB-YAP high, intermediate, or low patient groups in the TCGA GBM dataset. Student's t-test; * = P<0.05, between YAP^{High} and YAP^{Low}. **B-C,** Matrigel Boyden invasion assay of shCtrl or shYAP JHGBM651 and CONT or YAP OE JHGBM612 cells, respectively, from 3 independent experiments. Student's t-test; * = P<0.05, between shCtrl and shYAP cells (B) and between CONT and YAP OE cells (C). **D,** Matrigel Boyden invasion assay of shCtrl, shYAP, or shTRIO JHGBM651 and JHGBM612 cells from 3 independent experiments. Student's t-test; * = P<0.05, compared to shCtrl cells. **E,** Matrigel Boyden invasion assay of CONT and TRIO OE JHGBM651 cells from 3 independent experiments. Student's t-test; * = P<0.05, between CONT and TRIO OE. **F,** Left: Human Nuclei/DAPI immunofluorescent images of mouse brain coronal sections after injections of CONT + shCtrl, CONT + shTRIO, YAP OE shCtrl, or YAP OE + shTRIO JHGBM612 cells (i.s. = injection site). Bulk tumor margins are indicated on the

ipsilateral hemisphere. White boxes outline the area magnified in the right panel. Scale bar = 1mm. Right: magnified images of the corpus callosum in the right hemisphere (m.l. = midline, c.c. = corpus callosum, st = striatum, ctx = cortex, v = ventricle). Scale bar = 500μm. Images are representative of 5 mice per group. **G,** Mean number of CONT + shCtrl, CONT + shTRIO, YAP OE shCtrl, or YAP OE + shTRIO cells per mouse invading corpus callosum into the contralateral hemisphere. Quantifications are based on 5 mice per group. Student's t-test; * = P<0.05, compared to shCtrl cells; # = P<0.05 between YAP OE shCtrl and CONT shCtrl cells. **A-E, G,** Data shown is mean ± SEM.

Figure 6 | STAT3 is potentially involved in YAP-mediated cell invasion. A, Expression of GB-STAT3 signatures in GB-YAP high, intermediate, or low patient samples in the TCGA GBM dataset. GB-STAT3 signatures were derived from BTIC or SNB19 GBM cells. Conserv = STAT3 signature conserved in both cell lines. Student's t-test; * = P<0.05, between YAP^{High} and YAP^{Low}. **B,** Percentage of patients with elevated GB-YAP signature, STAT3, or both in the REMBRANDT GBM dataset. P = Fisher's exact test using the sizes of the shown patient groups. **C,** Representative immunoblots of YAP and total and phospho-Tyr705 STAT3 expression in non-cancer cortex and primary glioblastoma patient tissues of 3 independent experiments. **D,** Percentage of High and Low YAP expressing patients with High or Low phospho-Tyr705/total STAT3 expression ratio from immunoblots in **C**. **E-F,** Representative immunoblot of YAP and total and phospho-Tyr705 STAT3 expression in shCtrl or shYAP JHGBM651/JHGBM612 and CONT or YAP OE JHGBM612 cells, respectively, of 3 independent experiments. **G,** Matrigel Boyden invasion assay of shCtrl and shYAP JHGBM651 cells treated with vehicle or the STAT3 inhibitor LLL12 from 3 independent experiments. Student's t-test; * = P<0.05, between drug and no drug treatment; # = P<0.05 between shYAP and shCtrl cells with no drug treatment. **H,** Representative immunoblot of total and phospho-Tyr705 STAT3 expression in shCtrl, shTRIO-1 JHGBM651 and JHGBM612 cells with 2 independent shTRIO constructs of 3 independent experiments. **I,** Representative immunoblot of total and phospho-Tyr705 STAT3 expression in CONT and TRIO OE JHGBM651 cells of 3 independent

experiments. **J,** Representative immunoblot of total and phospho-Tyr705 STAT3 expression in shCtrl and shYAP JHGBM651 cells with or without TRIO overexpression of 3 independent experiments. **K,** Representative immunoblot of total and phospho-Tyr705 STAT3 expression in shCtrl and shTRIO JHGB651 cells with or without YAP overexpression of 3 independent experiments. **A, G,** Data shown is mean ± SEM.

Figure 7 | YAP, TRIO, and STAT3 signaling predict poor clinical outcomes in glioblastoma. A, Percentage of patients with elevated GB-YAP signature expression. Patients are grouped by glioma grade in REMBRANDT and TCGA datasets. **B,** Kaplan-Meier graph of cumulative progression-free survival in patient groups defined by GB-YAP signature expression in TCGA dataset. P=Log-Rank test. **C,** Percentage of GBM patients with elevated GB-YAP signature expression in TCGA subtypes. P=Fisher's exact test. **D,** Expression of GBM subclass signatures in GB-YAP high, intermediate, or low patient samples in the TCGA dataset. GBM subclass signatures were derived from TCGA "Core" samples. Student's t-test; * = P<0.05, between YAP^{High} and YAP^{Low}. **E,** mRNA expression of Mesenchymal GBM subclass signature genes in shCtrl (black bars) or shYAP (red bars) in JHGB651, JHGB640, and JHGB612 cells. Student's t-test; * = P<0.05, between shYAP and shCtrl cells. **F,** Distribution of patient groups expressing either one, two, or all three of GB-YAP signature, TRIO transcript, and STAT3-conserved signature expression patterns. **G-I,** Kaplan-Meier graphs of cumulative progression-free survival in patient groups defined by the GB-YAP signature, TRIO transcript, and STAT3-conserved signature expression in the TCGA dataset. P=Log-Rank test. **J,** Schematic of the YAP-driven pro-migratory and invasive signaling pathway in a single cell on a layer of extracellular matrix. Thick red and purple arrows denote movement of the cell body during migration and invasion, respectively. Darker shading of the extracellular matrix around the invading edge of the cell represents the degradation of the surrounding tissue. D-E, Data shown is mean ± SEM.

Materials and Methods:

Cell culture. Patient primary glioblastoma tissue specimens were obtained at the Johns Hopkins Hospital under the approval of the Institutional Review Board (IRB). All primary cell lines were established from excess tumor tissue from patients undergoing surgical resection for glioblastoma as listed in **Suppl Table 1.** GBM cell line derivation and culture protocols were as previously described(*26*), without the addition of N2 supplement and cultured on laminin-coated (1μg/cm^2) plates. Specifically, DMEM/F12 (Invitrogen) was supplemented with B27 supplement (Invitrogen), antibiotic/antimycotic (Invitrogen), human EGF (Peprotech), and FGF (Peprotech). MCF10A cells were purchased from ATCC and maintained in complete MEGM media (Lonza) supplemented with 100 ng/mL cholera toxin (Sigma) as recommended. GBMA172 cells were purchased from ATCC and maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco). NHA cells were purchased from Lonza and cultured as recommended. All cell lines were tested and confirmed to be mycoplasm free using a PCR-based MycoDtect^{TM} kit from Greiner Bio-One.

Lentiviral transduction. Glycerol stocks of Human Mission TRC1 sequence-verified shRNA lentiviral plasmid vectors were obtained from the Johns Hopkins University High Throughput Biology Center (See below). Plasmids were isolated using a Spin Miniprep kit (Qiagen). Vesicular stomatitis virus glycoprotein-pseudotyped virus was produced by co-transfecting 293T cells using Lipofectamine 2000 (Invitrogen) with an shRNA transducing vector and 2 packaging vectors, psPAX2 and pMD2.G. On days 3 and 4 post-transfection, the virus was harvested and filtered through a 0.22-μm pore cellulose acetate filter before centrifugal concentration using a Centricon Plus-70 (Millipore). An empty TRC1 lentiviral construct was used as the Control virus (Sigma).

The TRIO (pCDH1-GFP TRIO), TRIO iGEF1 (TRIO QALE), TRIO iGEF2 (TRIO L2051E), and its corresponding control lentiviral plasmids (pCDH1) were generated by the Schmidt Lab. Vesicular stomatitis virus glycoprotein-pseudotyped virus was produced by co-transfecting 293T cells using

PureFection transfection reagent (Systems Biosciences) with the pCDH1-based lentiviral vector and pPACKH1 packaging plasmid mix (Systems Biosciences).

The YAP constitutively active overexpression lentiviral vector was generated using pCMV-Flag YAP127/128/131/381A plasmid (Addgene) and Duet011 lentiviral plasmid.

Primary GBM, GBMA172, MCF10A, and NHA cells were transduced with equal titers of concentrated virus in complete growth media supplemented with 1 μg/ml polybrene (Sigma) for 24 hours. Following transduction, cells were given 24 hours to recover before selection in 0.25 μg/ml (JHGBM651, JHGBM640, JHGBM1A) or 0.5 μg/ml (JHGBM612, GBMA172, MCF10A, and NHA) puromycin (Sigma) for a minimum of 6 days. Minimum effective puromycin or hygromycin concentrations were determined using kill curves of non-transduced cells (data not shown).

shRNA Plasmids

Target Gene	TRC Number	TRC Version
Human YAP Clone 1	TRCN0000107265	TRC 1
Human YAP Clone 2	TRCN0000107267	TRC 1
Mouse YAP/ Human Clone 3	TRCN0000107266	TRC 1
Human YAP Clone 4	TRCN0000107268	TRC 1
Human YAP Clone 5	TRCN0000107269	TRC 1
Human TRIO Clone 1	TRCN0000195292	TRC 1
Human TRIO Clone 2	TRCN0000196250	TRC 1
Human TRIO Clone 3	TRCN0000196751	TRC 1

Time-lapse microscopy, drug treatments, and analysis of single cell migration speed. GBM or MCF10A cells were seeded at a density of 2000-4000 cells/well in a 96-well tissue-culture polystyrene plate (BD Falcon) that was pretreated with 3 μg/cm^2 laminin for GBM cells (Sigma) or 5 μg/cm^2 collagen for MCF10A cells (Stem Cell Technologies). 48 hours after seeding, cells were fed with either control or treated complete growth medium (as indicated) and placed and imaged in a temperature- and gas-controlled environmental chamber (37 °C, 5% CO_2). Y27632 (Tocris), NSC23766 (Tocris), and ITX3 (Tocris) were resuspended in sterile PBS concentrations for the indicated times. Time-lapse microscopy was performed using a motorized inverted microscope (Olympus IX81) equipped with a Cascade 512B II CCD camera. Phase-contrast images were captured with a 10x objective (NA = 0.30) under the control of Slidebook 4.1 (Intelligent Imaging Innovations, Denver, CO) at 10–20 minute intervals for at least 6 hours. A single observer used a custom MATLAB script to view time-lapse images and manually mark cell body positions. (See **Supplemental Videos**). Due to the inhomogeneous seeding of cells throughout individual wells, care was taken to image 5-8 fields of view per well that were most comparable to other experimental conditions. Additionally, a minimum of 30 cells was tracked from 4 replicate wells for each experimental condition. Cell migration speed was computed as displacement (between each captured time point) over time. Individual cell speeds were summarized as the average speed within one-hour intervals. Since the speeds of many cell populations failed to pass normality testing, a Wilcoxon rank-sum test was used to compare experimental conditions. Error bars represent the standard error of the mean of the cell population in a given experimental condition.

Intracranial xenograft model and immunohistochemistry. All animal protocols were approved by the Johns Hopkins Animal Care and Use Committee. Briefly, JHGBM612 cells were resuspended at 75,000 cells/μl in complete growth media and placed on ice. 1-month old male NOD-SCID gamma-null (NSG) mice were anesthetized before intracranial injections following an established protocol(*84*). 2 μl of shCtrl

or shYAP cells were injected into the striatum; specifically (X: 1.5, Y: 1.34, Z: -3.5) mms from the bregma (n = 5 per group).

5 weeks post-injection, mice were anesthetized before trans-cardiac perfusion with saline followed by 10% formalin. Brains were then harvested and fixed overnight at 4 °C in 10% formalin. Fixed brains were placed in OCT compound (Tissue-Tek) overnight at 4 °C and subsequently flash-frozen. Embedded brains were cut into 10-micron coronal sections using a cryostat microtome.

Brain sections spaced every 500µm were stained with hematoxylin and eosin to identify landmark neuroanatomical structures and therefore identify comparable slides from each mouse for further analysis. Slides immediately adjacent to the identified slides were then selected for immunohistochemistry and quantification. Antigen retrieval was performed using sodium citrate (10 mM) for 30 minutes in a water bath at 95°C. Slides were rinsed in PBS and blocked in 10% normal goat serum for 1 hour. Primary antibody was diluted in PBS with 0.1% Triton X-100 and 2% normal goat serum. Slides were incubated with primary antibody overnight at 4°C. After rinsing with PBS, the slides were incubated with the appropriate anti–IgG secondary antibody (Molecular Probes, Invitrogen) conjugated with a fluorochrome for 1 hour and rinsed. Slides were then incubated with DAPI for 30 minutes and rinsed in PBS before visualization on an Olympus IX81 inverted microscope. Antibodies are listed at the end of this section.

Immunoblotting, antibodies, and immunocytochemistry. *Immunoblotting:* Tissues from the operating room were briefly rinsed in HBSS before being flash-frozen in liquid nitrogen. Proteins were extracted using T-PER (Pierce) tissue protein extraction reagent and a tissue homogenizer. GBM, MCF10A, and NHA cells were lysed on ice with a cell scraper and radioimmunoprecipitation assay lysis buffer (Pierce) supplemented with protease inhibitor tablets (Roche) and a phosphatase inhibitor cocktail (Pierce). Extracts were incubated for 45 minutes for complete lysis and centrifuged at 10,000 RPM for 10 minutes at 4°C to pellet cell debris. Supernatant protein concentrations were quantified using a BCA Protein Assay kit (Pierce) and a spectrophotometric plate reader (BioTek). Most protein lysates were separated by

running 30-100 µg of lysate on 10% Bis-Tris NuPage gels (Invitrogen) and subsequently, transferred to 0.2µm pore polyvinylidene fluoride (PVDF) membranes (BioRad). Because TRIO is an exceptionally large protein, some lysates were run on 4-12% Tris-Acetate NuPage gels and transferred to 0.45 µm pore PVDF membranes overnight. Primary antibody incubations were according to the manufacturer's recommendations in 0.1% Tween TBS supplemented with 5% non-fat dry milk or BSA, as recommended. Immunoreactive bands were visualized using the appropriate horseradish peroxidase-conjugated anti-IgG antibodies (Pierce). Bands were detected using enhanced chemiluminescence or prime detection reagent (GE Healthcare) whenever appropriate. Densitometric analysis was done with ImageJ. All immunoblotting experiments were performed in triplicate and repeated three times with similar results.

For *immunocytochemistry*, 160,000 GBM cells were seeded on laminin-coated 35-mm cell culture plates. 48 hours later, cells were washed in cold PBS and fixed in 10% formalin for 30-60 minutes. Cells were blocked in 10% normal goat serum for 1 hour. Primary antibody was diluted in PBS with 0.1% Triton X-100 and 2% normal goat serum. Cells were incubated with primary antibody overnight at 4°C. After rinsing with cold PBS, cells were incubated with the appropriate anti–IgG secondary antibody (Molecular Probes, Invitrogen) conjugated with a fluorochrome for 1 hour. Cells were rinsed in PBS before incubation with DAPI for 30 minutes. Finally, cells were rinsed in PBS before visualization on an Olympus IX81 inverted microscope.

For *focal adhesion analysis*, high-resolution images were captured using a fluorescence microscope at 20-40X. Quantification of area or size was conducted using ImageJ.

Antibodies used for these procedures are listed at the end of this section.

Pull-down of GTP-bound Small Rho GTPases. The Rac1-GTP Assay (Cell Bio Labs) and RhoA-GTP Assay (Millipore) kits were used in accordance with the manufacturer's instructions. Briefly, 1.2 x 10^6 MCF10A or GBM cells per dish were seeded into collagen- or laminin-coated 100 mm cell culture dishes (Corning). 48 hours later, cells were rinsed twice with ice-cold PBS and placed on ice. Extracts were

harvested quickly by scraping in ice-cold 1X assay buffer supplemented with protease and phosphatase inhibitors. Lysis was completed by incubation for 30 minutes on ice and vortexing every 10 minutes. Protein supernatants were isolated and quantified as above. 4 mg of shCtrl or shYAP MCF10A lysate was used as input for RhoA-GTP pull-down and 2 mg as input for Rac1-GTP pull-down. 1 mg of protein lysate was used as input for the Rac1-GTP pull-down of JHGBM651 cells. 50 µg of input lysates were kept for immunoblotting. Agarose beads conjugated with Rhotekin binding domain (RBD) were incubated with lysates for 4 hours, and p21-binding domain (PBD) agarose beads were incubated with lysates for 1 hour. Beads were rinsed three times with cold 1X assay buffer, resuspended in 2X SDS-PAGE sample buffer, and boiled for 10 minutes. Proteins were separated on 10% Bis-Tris NuPage gels, transferred to 0.2 µm pore PVDF membranes, and immunoblotted as described above. Mouse anti-RhoA antibody (26C4) was purchased from Santa Cruz. Refer to the end of this section for a listing of all antibodies used. In addition, for more quantitative analyses, G-LISA RhoA or Rac1 activation kits from Cytoskeleton were used according to the manufacturer's protocol.

Motif Search. MotifMap(*85*) was used to search 10 kilobase regions upstream of select transcription start sites in the hg19 reference genome. Significant motifs were called at a false discovery rate below 0.05.

Invasion assay. Matrigel-coated Boyden chambers (BD Biosciences) were used to assess the invasive capacity of cells. 100,000 cells were resuspended in their appropriate media supplemented with 0.5% FBS. Cell suspension aliquots were plated on the upper surface of the transwell plates with a porous PET membrane (8µm pores) pre-coated with a layer of growth factor reduced matrigel basement membrane matrix. 750 µl of 2% FBS containing DMEM media was placed in the lower chamber. Filters were incubated at 37C°, 5% CO2 for 48 hours. Invasion of cells was determined by fixing the membrane,

staining the cells using the Diff-Quik® staining kit, directly counting the number of invaded cells in 9 high-power fields at 10x using an Olympus 1X81 microscope system, and calculating the mean per well. The assays were run in triplicates and at least three independent experiments were performed.

RNA extraction and quantitative real-time PCR. Total RNA was extracted in TRIzol, phase-separated using chloroform, and precipitated overnight at 4°C in isopropanol. Further isolation of total RNA was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. 1 µg purified RNA was reverse transcribed using the Superscript III First-Strand cDNA Synthesis Kit (Invitrogen). Quantitative real-time PCR using SYBR Green (Applied Biosystems) was performed on a 7300 Cycler (Applied Biosystems) and the included data analysis software. GAPDH primers were used as a loading control. See the table below for all primer sequences used.

Primer pairs

Target Gene	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
YAP1	TAGCCCTGCGTAGCCAGTTA	TCATGCTTAGTCCACTGTCTGT
WWTR1	GTCCTACGACGTGACCGAC	CACGAGATTTGGCTGGGATAC
GAPDH	AAGGTGAAGGTCGGAGTCAAC	AAGGTGAAGGTCGGAGTCAAC
TRIO	CCAGGAGCGGACGATCCACCT	CCAGGAGCGGACGATCCACCT
TIAM1	GATCCACAGGAACTCCGAAGT	GCTCCCGAAGTCTTCTAGGGT
MCL-1 (Isoform 1)	TGCTTCGGAAACTGGACATCA	TAGCCACAAAGGCACCAAAAG

MMP9	GGGACGCAGACATCGTCATC	TCGTCATCGTCGAAATGGGC
CTGF	GGCAAAAAGTGCATCCGTACT	CCGTCGGTACATACTCCACA
MMP2	CTTCCAAGTCTGGAGCGATGT	TACCGTCAAAGGGGTATCCAT
FOSL2	TATCCCGGGAACTTTGACAC	TATCCCGGGAACTTTGACAC
RUNX1	CCCATCGCTTTCAAGGTG	CCCATCGCTTTCAAGGTG
CHI3L1/YKL 40	CCTGGGAGTGGAATGATGTGA	TCAGGTTGGGGTTCCTGTTCT
MET	AGCAATGGGGAGTGTAAAGAGG	CCCAGTCTTGTACTCAGCAAC
MERTK	CTCTGGCGTAGAGCTATCACT	AGGCTGGGTTGGTGAAAACA
CXCR4	ACTACACCGAGGAAATGGGCT	ACTACACCGAGGAAATGGGCT
CDH11	GTATCCTCGAAGGACAACCCT	GTATCCTCGAAGGACAACCCT
TNC	TCCCAGTGTTCGGTGGATCT	TCCCAGTGTTCGGTGGATCT
FN1	GAGAATAAGCTGTACCATCGCAA	GAGAATAAGCTGTACCATCGCAA
COL5A1	TACAACGAGCAGGGTATCCAG	TACAACGAGCAGGGTATCCAG

Biostatistical analysis. Statistical comparisons of experimental results from independent repeats across conditions were performed using unpaired two-tailed Student's t-test. The comparisons for cell migration data were analyzed using Wilcoxon rank-sum test due to its robustness to outliers. The significance of the association between two categories of patients for example, TCGA mesenchymal subtype and high YAP expression were analyzed using Fisher's exact test. The comparisons of survival data between different

patient groups were performed using Kaplan Meier log-rank test. To ensure reproducibility, all in vitro and in vivo experiments were repeated at least three times. The sample sizes, number of replicates and statistical test being used are described in each figure legend.

For Affymetrix data (GB-YAP, TCGA, and Rembrandt datasets), raw data files were processed in aroma.affymetrix (http://statistics.berkeley.edu/sites/default/files/tech-reports/745.pdf) using a custom CDF with updated probeset mappings(*86*) (BrainArray v15.1.0). Differential expression analysis was performed using the sam function of the siggenes package (version 1.28.0) in R. For Illumina beadchip data, signature probesets (GB-STAT3 and GB-C/EBPβ datasets(*55*)) were required to map to a single Entrez Gene ID. Redundant probesets mapping to the same gene were eliminated after differential expression analysis.

To derive gene signatures, each cell line was analyzed for differential expression independently (GB-YAP, GB-STAT3, and GB-C/EBPβ datasets). Genes with $q < .05$ and absolute fold-change > 1.5 were considered as differentially expressed genes, although $q < 0.1$ was used when no genes passed $q < 0.05$. This relaxed FDR was excused because the final signatures were taken as the genes significantly differentially expressed in both cell lines, at least one of which required $q < .05$.

To stratify patients into groups of low, intermediate, and high expression of a given gene signature, we used the gene set z-score(*87*). Briefly, RMA normalized data were quantile normalized, variance filtered, and log2 transformed. Gene sets were filtered to remove genes not present in the normalized, filtered data for each analysis. Each gene was converted into an expression z-score relative to other samples of the same class (e.g., other GBMs but not low-grade gliomas), and the gene set score (GSS) was computed as the sum of the member genes' z-scores divided by the square root of the size of the gene set. Samples with GSS < -0.5 were categorized as low, samples with GSS > 0.5 were high, and those between were intermediate. Since microarray data to derive a gene signature of TRIO was unavailable, patients with a TRIO gene z-score < -0.5 were called low and those with a TRIO gene z-score > 0.5 were called high. To compare expression level of a specific gene or gene signature between pre-stratified patient groups, the z

scores for all the genes in the signature were averaged. And the statistical comparison of the averaged z scores between patient groups were performed using unpaired two-tailed Student's t-test.

Antibodies

Target Protein	Company	Note
YAP p-S127	Cell Signaling	
YAP	Cell Signaling	
Beta-actin	Abcam	
Human Nuclei	Millipore	Clone 3E1.3
Paxillin	Sigma	Clone PXC10
F-actin	Sigma	
Rac1	Cell Biolabs	Included in kit
RhoA	Santa Cruz	Clone 26C4
ROCK1	Santa Cruz	Clone H-85
ROCK2	Santa Cruz	Clone C-20
LIMK1/2 p-T505/508	Cell Signaling	
LIMK1	Cell Signaling	
TRIO	Santa Cruz	Clones H-120 and D-20

STAT3 p-Y705	Cell Signaling	Clone D3A7
STAT3	Santa Cruz	Clone H-190
TEAD4	Aviva Labs	
WWTR1/TAZ	BD Pharmingen	Clone M2-303
PAK1/2 p-T423/T402	Cell Signaling	
PAK1/2	Cell Signaling	
NMIIa	Cell Signaling	
NMIIa p-S1943	Cell Signaling	

Accession codes. The microarray dataset of JHGBM651 and JHGBM612 cells treated with shCtrl or shYAP in triplicate will be made available on the Gene Expression Omnibus prior to publication.

SUPPLEMENTAL INFORMATION:

Supplemental Figures: The file named **"**Suppl Figs. 1-12" contains the following 12 supplemental figures with their associated legends listed below:

Supplemental Figure 1. YAP regulates migration. A, Densitometric analysis of YAP and YAP/β-actin expression in Fig. 1A. Student's t-test; * = P<0.05, between NCC (non-cancer cortex) and GBM. **B,** mRNA expression of YAP target genes, Mcl-1 and CTGF, in glioblastoma tissues compared to the non-cancer cortex. Student's t-test; * = P<0.05, comparing to NCC. **C,** Representative immunoblot of YAP and p-YAP expression in primary GBM cell cultures of 3 independent experiments. * = cells used in functional assays. Numbers correspond to Patient Sample ID listed in Suppl Table 1. **D,** Representative immunoblot of YAP expression in wildtype (WT), shCtrl, or shYAP JHGBM612 cells with 5 independent shYAP constructs of 3 independent experiments. s.e. = short exposure, l.e. = long exposure. **E,** Representative immunoblot of YAP and TAZ expression in shCtrl or shYAP JHGBM651 cells with 2 independent shYAP constructs of 3 independent experiments. **F,** Representative immunoblot of YAP expression in shCtrl or shYAP GBM A172 cells with 2 independent shYAP constructs of 3 independent experiments. **G-H,** Mean migration speed of shCtrl or shYAP MCF10A, NHA, JHGBM651/612/640/1A cells. Wilcoxon Rank-Sum test; * = P<0.05, comparing to shCtrl cells. **I, K-L,** Representative immunoblots of YAP expression in CONT or YAP OE NHA, JHGBM612, and JHGBM1A cells, respectively, of 3 independent experiments. **J, M,** Mean migration speed of CONT or YAP OE NHA, and JHGBM1A cells, respectively. Wilcoxon Rank-Sum test; * = P<0.05, between CONT and YAP OE. **H, J, M,** Mean migration speed is calculated from 30 cells. **N,** MRI images of Patient #30/JHGBM612. **O,** Representative H&E images showing the formation of solid tumors in the in vivo GBM xenograft model used in experiments presented in Fig.1E and Fig.5F after intracranial injection of JHGBM612 cells of 5 mice per group. Scale = 1mm. **P,** *Left:* Schematic of the murine intracranial xenograft model of GBM used to assess YAP's migratory and invasive capacity

in vivo. *Right:* Number of cells invading past the midline into the contralateral hemisphere. i.s. = injection site, c.c. = corpus callosum, st = striatum, ctx = cortex, v = ventricle, m.l. = midline, l.e. of brain = lateral edge of brain. Quantifications are based on 5 mice per group. Student's t-test; * = P<0.05, between shYAP and shCtrl cells at a corresponding distance from midline. **Q,** Analysis of focal adhesion (FA) in shCtrl or shYAP JHGBM651 cells. Student's t-test; * = P<0.05, between shCtrl and shYAP. **R,** Analysis of focal adhesion (FA) in shCtrl or shYAP GBMA172 cells. Scale = 5μm. Student's t-test; * = P<0.05, between shCtrl and shYAP. **S-U,** Representative immunoblots of YAP, total and phospho-Thr508 LIMK expression in shCtrl, shYAP JHGBM612, NHA, and MCF10A cells of 3 independent experiments. 2 independent shYAP constructs were used in JHGBM612 and NHA cells. **A-B, G-H, J, M, Q-R,** Data shown is mean ± SEM.

Supplemental Fig. 2. YAP regulates migration by inhibiting RhoA-ROCK signaling. A, G-LISA analysis of RhoA-GTP levels of shCtrl or shYAP NHA cells. Student's t-test; * = P<0.05, between shCtrl and shYAP. **B-L,** Mean migration speed of shCtrl, shYAP-1, or -2 JHGBM651/612/640/1A, NHA, and MCF10A cells treated with the ROCK inhibitors Y27632 or H1152. Mean migration speed is calculated from 30 cells. Wilcoxon Rank-Sum test; Red and black * = P<0.05, comparing to no drug treatment in shYAP-1 or -2 cells; Red # = P<0.05, comparing to shCtrl cells at corresponding drug concentration; Black # = P<0.05, comparing to shCtrl cells with no drug treatment. **M-N,** Representative immunoblots of NMII and phospho-Ser1943 NMII expression in JHGBM651 or JHGBM612 cells treated with vehicle or ROCK inhibitors Y27632 or H1152 of 3 independent experiments. **A-L,** Data shown is mean ± SEM.

Supplemental Fig. 3. YAP regulates migration by activating Rac1 signaling. A, G-LISA analysis of Rac1-GTP levels of shCtrl or shYAP NHA cells from 3 independent experiments. Student's t-test; * = P<0.05, between shCtrl and shYAP. **B,** Representative immunoblot of YAP, total and phospho-

T423/402 PAK in shCtrl and shYAP GBMA172 cells of 3 independent experiments. **C-I,** Mean migration speed of shCtrl, shYAP-1, or -2 JHGBM651/612/640, NHA, and MCF10A cells treated with the Rac1 inhibitor NSC23766. Mean migration speed is calculated from 30 cells. Wilcoxon Rank-Sum test; Black * = P<0.05, comparing to no drug treatment in shCtrl cells; Red # = P<0.05, between shYAP-1 or -2 and shCtrl cells at corresponding drug concentrations. **J,** Schematic representation of YAP modulating Rho-GTPases. **A, C-I,** Data shown is mean ± SEM.

Supplemental Fig. 4. YAP regulates migration by modulating a Rho-GTPase switch. A, Experimental paradigm to delineate the hierarchy of molecular events of YAP's modulation of a Rho-GTPase switch. **B-C,** Mean migration speed of shCtrl, shYAP-1, or -2 JHGBM651 and JHGBM612 cells treated with no drug/vehicle, Y27632, or NSC23766 for 3 or 6 hours as indicated. Mean migration speed is calculated from 30 cells. Wilcoxon Rank-Sum test; Black * = P<0.05, between mean speed from hour1 to hour2 and that from hour4 to hour5 in shCtrl cells; Red * = P<0.05, between mean speed from hour1 to hour2 and that from hour4 to hour5 in shYAP-1 or -2 cells; Red # = P<0.05, between mean speed in shYAP-1 or -2 cells and that in shCtrl cells at corresponding time points. **D-F, I,** G-LISA analysis of Rac1-GTP or RhoA-GTP levels of shCtrl or shYAP cells treated with vehicle, ROCK inhibitors Y27632 or H1152, or Rac1-GEF inhibitor NSC23766 from 3 independent experiments. Student's t-test; * = P<0.05, comparing to no drug treatment; # = P<0.05, comparing to shCtrl cells with no drug treatment. **G-H,** Rac1-GTP and RhoA-GTP pull-down assay of shCtrl or shYAP cells treated with vehicle, ROCK inhibitor Y27632, or Rac1-GEF inhibitor NSC23766 from 3 independent experiments. Student's t-test; * = P<0.05, comparing to no drug treatment; # = P<0.05, ## = P<0.01, comparing to shCtrl cells with no drug treatment. **J,** Schematic representation of the hierarchy of YAP's modulation of a Rho-GTPase switch. **B-I,** Data shown is mean ± SEM.

Supplemental Fig. 5. YAP transactivates TRIO signaling. A-F, Representative immunoblots of YAP and TRIO expression in shCtrl, shYAP JHGBM651/612/640/1A, MCF10A, and NHA cells of 3 independent experiments. 2 independent shYAP constructs are used in JHGBM651/612/1A, MCF10A and NHA cells. **G,** Relative TRIO mRNA expression in shCtrl or shYAP JHGBM651/612/640 and MCF10A cells from 3 independent experiments. Student's t-test; * = P<0.05, between shCtrl and shYAP. **H,** Representative immunoblot of YAP and TRIO expression in CONT or YAP OE MCF10A cells of 3 independent experiments. **I,** Relative TIAM1 mRNA expression in shCtrl or shYAP JHGBM651/612/640 cells from 3 independent experiments. **J,** ChIP-PCR querying across two enhancer elements of TRIO in JHGBM1A cells. Results are representative of 3 independent experiments. **K,** GFP-luciferase reporter assay with the promoter sequence of TRIO and CTGF (positive control) in CONT and YAP OE GBMA172 cells from 3 independent experiments. Student's t-test; * = P<0.05, between CONT and YAP OE. **L-N,** Representative immunoblots of pull-down analysis of Rac1-GTP levels in CONT and YAP OE JHGBM612, CONT and TRIO OE JHGBM612 cells or shCtrl and shTRIO JHGBM612 cells, respectively, of 3 independent experiments. **O,** G-LISA analysis of Rac1-GTP levels in shCtrl and shTRIO NHA cells from 3 independent experiments. Student's t-test; * = P<0.05, between shCtrl and shTRIO. **P-Q,** G-LISA analysis of RhoA-GTP levels in CONT, YAP OE, and TRIO OE JHGBM651 cells from 3 independent experiments. Student's t-test; * = P<0.05, between CONT and YAP or TRIO OE. **R,** G-LISA analysis of RhoA-GTP levels in shCtrl and shTRIO JHGBM651 and NHA cells from 3 independent experiments. Student's t-test; * = P<0.05, between shCtrl and shTRIO. **S,** Representative immunoblots of total and phospho-Ser127 YAP expression in shCtrl, shYAP, and shTRIO GBMA172 cells of 3 independent experiments. **T,** Representative immunoblots of TRIO expression in non-cancer cortex and patient-derived primary glioblastoma tissues of 3 independent experiments. **U,** Expression of YAP/TAZ and YAP signatures, derived from a variety of other tissues, in GB-YAP high, intermediate, or low patient groups in the REMBRANDT and TCGA GBM datasets. **G, I, K, O-R, U,** Data shown is mean ± SEM.

Supplemental Fig. 6. YAP regulates migration via TRIO signaling. A-E, Representative immunoblots of TRIO expression in shCtrl, shTRIO JHGBM612/640, MCF10A, NHA and GBMA172 cells of 3 independent experiments. 2 independent shTRIO constructs are used in JHGBM612, MCF10A, and NHA cells. **F,** Mean migration speed of shCtrl, shTRIO-1, or -2 JHGBM651/612/1A, NHA, and MCF10A cells. Wilcoxon Rank-Sum test; * = P<0.05, comparing to shCtrl cells. **G,** G-LISA analysis of Rac1-GTP levels in shCtrl or shTRIO JHGBM651 cells treated with vehicle or the TRIO inhibitor of ITX3 from 3 independent experiments. Student's t-test; * = P<0.05, comparing to no drug treatment; # = P<0.05, comparing to shCtrl with no drug treatment. **H,** Mean migration speed of shCtrl, shTRIO-1, or -2 GBM1A cells treated with vehicle or TRIO inhibitor ITX3. Wilcoxon Rank-Sum test; * = P<0.05, comparing to no drug treatment; # = P<0.05, comparing to shCtrl with no drug treatment. **I-M,** Mean migration speed of shCtrl, shTRIO-1, or -2, or shYAP-1, or -2 JHGBM651/612, NHA, and MCF10A cells treated with increasing doses of the TRIO inhibitor ITX3. Wilcoxon Rank-Sum test; Black * = P<0.05, comparing to no drug treatment in shCtrl cells; Red # = P<0.05, between shYAP and shCtrl at corresponding drug concentration; Blue # = P<0.05, between shTRIO-1 or -2 and shCtrl at corresponding drug concentration. **F, H-M,** Mean migration speed is calculated from 30 cells. **F-M,** Data shown is mean ± SEM.

Supplemental Fig. 7. YAP-TRIO signaling modulates Rac1-RhoA to drive migration. A-G, Mean migration speed of shCtrl, shYAP-1, or -2 JHGBM651/612/640/1A, NHA, and MCF10A cells treated with increasing doses of the TRIO inhibitor ITX3. **H,** Mean migration speed of CONT or YAP OE JHGBM612 cells treated with vehicle or the TRIO inhibitor ITX3. **A-H,** Wilcoxon Rank-Sum test; Black * = P<0.05, comparing to no drug treatment in shCtrl or CONT cells; Red * = P<0.05, comparing to no drug treatment in shYAP or YAP OE cells; Red # = P<0.05, between YAP OE and CONT at corresponding drug concentration. **I-J,** G-LISA analysis of Rac1-GTP or RhoA-GTP levels

in shCtrl or shYAP JHGBM651 cells treated with vehicle or the TRIO inhibitor ITX3 from 3 independent experiments. Student's t-test; * = P<0.05, comparing to no drug treatment in shCtrl or shYAP cells; # = P<0.05, comparing to shCtrl cells with no drug treatment. **K-O,** Mean migration speed of shCtrl, shYAP, or shTRIO JHGBM651/612/640 and NHA cells treated with increasing doses of the Rac1-GEF inhibitor NSC23766. Wilcoxon Rank-Sum test; Black * = P<0.05, comparing to no drug treatment in shCtrl cells; Red # = P<0.05, between shYAP-1 or -2 and shCtrl cells at corresponding drug concentration; Blue # = P<0.05, between shTRIO-1 or -2 and shCtrl cells at corresponding drug concentration. **P, R,** G-LISA analysis of Rac1-GTP or RhoA-GTP levels in shCtrl or shTRIO JHGBM651 cells treated with vehicle or the Rac1-GEF inhibitor NSC23766 from 3 independent experiments. Student's t-test; * = P<0.05, comparing to no drug treatment in shCtrl cells; # = P<0.05, comparing to shCtrl cells with no drug treatment. **Q,** Mean migration speed of shCtrl and shTRIO GBMA172 cells transfected with pCtrl or a constitutively active Rac1 vector (pRac1-CA). Wilcoxon Rank-Sum test; * = P<0.05, comparing to pCtrl in shTRIO cells; # = P<0.05, comparing to pCtrl shCtrl cells. **S,** RhoA-GTP pull-down assay of shCtrl or shTRIO GBMA172 cells transfected with pCtrl or a constitutively active Rac1 vector (pRac1-CA). Student's t-test; * = P<0.05, comparing to shTRIO cells transfected with pCtrl; # = P<0.05, comparing to shCtrl cells transfected with pCtrl. **A-H, K-O, Q,** Mean migration speed is calculated from 30 cells. All data shown is mean ± SEM.

Supplemental Fig. 8. YAP-TRIO signaling regulates migration by modulating Rac1-RhoA crosstalk. A, G-LISA analysis of RhoA-GTP levels in shCtrl or shTRIO JHGBM651 cells treated with vehicle or the TRIO inhibitor ITX3 from 3 independent experiments. Student's t-test; * = P<0.05, comparing to no drug treatment in shCtrl cells; # = P<0.05, comparing to shCtrl cells with no drug treatment. **B,** Mean migration speed of shCtrl and shTRIO GBMA172 cells transfected with pCtrl or a dominant-negative RhoA vector (pRhoA-DN). Wilcoxon Rank-Sum test; * = P<0.05, comparing to pCtrl in shTRIO cells; # = P<0.05, comparing to pCtrl shCtrl cells. **C, E, G-H,** Mean migration speed

of shCtrl, shYAP, or shTRIO JHGBM651/612/1A, and NHA cells treated with increasing doses of the ROCK inhibitor Y27632 or H1152. Wilcoxon Rank-Sum test; Black * = P<0.05, comparing to no drug treatment in shCtrl or shTRIO cells; Red * = P<0.05, comparing to no drug treatment in shYAP cells; Blue * = P<0.05, comparing to no drug treatment in shTRIO cells; Red # = P<0.05, between shYAP and shCtrl cells at corresponding drug concentration; Blue # = P<0.05, between shTRIO and shCtrl cells at corresponding drug concentration. **D, F,** Mean migration speed of shCtrl, shTRIO-1 or -2 JHGBM651/1A and NHA cells treated with vehicle or the ROCK inhibitor Y26732. Wilcoxon Rank-Sum test; * = P<0.05, comparing to no drug treatment in shTRIO cells; # = P<0.05, comparing to no drug treatment in shCtrl cells. **I-L,** G-LISA analysis of Rac1-GTP or RhoA-GTP levels in shCtrl or shTRIO JHGBM651 cells treated with vehicle or the ROCK inhibitor Y27632 or H1152 from 3 independent experiments. Student's t-test; # = P<0.05, comparing to no drug treatment in shCtrl cells. **M,** Rac1-GTP pull-down assay of shCtrl or shTRIO GBMA172 cells transfected with pCtrl or a dominant-negative vector (pRhoA-DN). Student's t-test; * = P<0.05, comparing to shTRIO cells transfected with pCtrl; # = P<0.05, comparing to shCtrl cells transfected with pCtrl. **N**, Representative immunoblots of TRIO and YAP expression in CONT of TRIO OE MCF10A cells of 3 independent experiments. **O,** Mean migration speed of CONT or TRIO OE MCF10A cells. Wilcoxon Rank-Sum test, * = P<0.05, comparing to CONT cells. **P,** Mean migration speed of shCtrl, shYAP-2, and shYAP-2+TRIO OE JHGBM651 cells. Wilcoxon Rank-Sum test, * = P<0.05, comparing to shCtrl cells. **B-H, O-P,** Mean migration speed is calculated from 30 cells. **A-M, O-P,** Data shown is mean ± SEM.

Supplemental Fig. 9. YAP-TRIO signaling promotes cell invasion. A, Expression of the Invasive signatures in GB-YAP high, intermediate, or low patient groups in the REMBRANDT GBM dataset. Student's t-test; * = P<0.05, between YAP^{High} and YAP^{Low}. **B,** Percentage of patients with elevated GB-YAP signature, Invasive signature, or both in the REMBRANDT GBM dataset. P = Fisher's exact test using the sizes of the shown patient groups. **C-D,** Matrigel Boyden invasion assay of shCtrl,

shYAP-1, or -2 JHGBM651/612, NHA, MCF10A cells and CONT or YAP OE MCF10A cells, respectively, from 3 independent experiments. Student's t-test, * = P<0.05, comparing to shCtrl or CONT cells. **E,** Matrigel Boyden invasion assay of shCtrl, shTRIO, or -2 JHGBM651 and NHA cells from 3 independent experiments. Student's t-test; * = P<0.05, comparing to shCtrl cells. **F,** Matrigel Boyden invasion assay of shCtrl, shYAP and shTRIO JHGBM640 cells from 3 independent experiments. Student's t-test; * = P<0.05, comparing to shCtrl cells. **G-H,** Matrigel Boyden invasion assay of shCtrl, shYAP, or shTRIO JHGBM651/612/640 and NHA cells treated with the TRIO inhibitor ITX3 from 3 independent experiments. **I-L,** Matrigel Boyden invasion assay of shCtrl, shYAP-1/2, or shTRIO1/2 JHGBM651/612 and NHA cells treated with the ROCK inhibitors Y26732 or H1152 from 3 independent experiments. **G-L,** Student's t-test; * = P<0.05, comparing to no drug treatment in shCtrl cells; # = P<0.05, comparing to shCtrl cells with no drug treatment. **A, C-L,** Data shown is mean ± SEM.

Supplemental Fig. 10. STAT3 is potentially involved in YAP-mediated cell invasion. A, Left: Expression of different STAT3 signatures in GB-YAP high, intermediate, or low patient groups in the REMBRANDT GBM dataset. Student's t-test; * = P<0.05, between YAP^{High} and YAP^{Low}. Right: Percentage of patients with elevated GB-YAP or STAT3 signature in the REMBRANT GBM dataset. P = Fisher's exact test. **B-C,** Representative immunoblots of YAP, total and phospho-Tyr705 STAT3 expression in shCtrl, shYAP-1, or -2 JHGBM651/612/640/1A, NHA, and MCF10A cells and CONT or YAP OE MCF10A cells of 3 independent experiments. **D,** Matrigel Boyden invasion assay of shCtrl and shYAP JHGBM612 cells treated with vehicle or the STAT3 inhibitor LLL12 from 3 independent experiments. Student's t-test; * = P<0.05, comparing to no drug treatment in shCtrl cells; # = P<0.05, comparing to shCtrl cells with no drug treatment. **E-F,** Mean migration speed of shCtrl, shYAP-1, or -2 JHGBM651 cells treated with increasing doses of the STAT3 inhibitor LLL12. Mean migration speed is calculated from 30 cells. Wilcoxon Rank-Sum test; Black * = P<0.05, comparing to no drug

treatment in shCtrl cells; Red * = P<0.05, comparing to no drug treatment in shYAP cells; Red # = P<0.05, between shYAP and shCtrl cells at corresponding drug concentration. **G-H,** Representative immunoblots of TRIO, total and phosphor-Tyr705 STAT3 expression in shCtrl, shTRIO-1, or -2 JHGBM1A, NHA, and MCF10A cells from 3 independent experiments. **I-J,** Representative immunoblots of total and phosphor-Tyr705 STAT3 expression in JHGBM651/612 cells treated with the TRIO inhibitor ITX3 or Rac1-GEF inhibitor NSC23766 from 3 independent experiments. **A, D-F,** Data shown is mean ± SEM.

Supplemental Fig. 11. Elevated YAP signaling predicts poor clinical outcomes in all GBM subtypes. A, Expression of GB-YAP signature expression across different glioma grades in REMBRANDT dataset. Data shown is mean ± SEM. **B,** Kaplan-Meier graph of cumulative overall survival in patient groups defined by GB-YAP signature expression in REMBRANDT dataset. P = Log-Rank test. **C-E,** Kaplan-Meier graphs of cumulative overall survival in patient groups defined by GB-YAP signature expression in each indicated GBM subclass in the TCGA dataset. P=Log-Rank test. **F,** Kaplan-Meier graph of cumulative overall survival in patient groups defined by GB-STAT3 signature expression in REMBRANDT dataset. P=Log-Rank test. **G,** Kaplan-Meier graph of cumulative overall survival in patient groups defined by both the GB-YAP and STAT3 signature expression in the REMBRANDT dataset. P = Log-Rank test.

Supplemental Fig. 12. Each component of the YAP-TRIO-STAT3 network is indispensable to promoting poor clinical outcomes in GBM patients. Kaplan-Meir graphs of all combinations of mixed expression groups of YAP, TRIO, STAT3 in TCGA microarray dataset. Tables include p-value of comparisons between different combination groups. YAP-TRIO survival combinations were also evaluated in RNA-seq expression data from TCGA (table shown). P = Log-Rank test.

Supplemental Movies: Seven (7) movies show migration of shCtrl, shYAP, or shTRIO JHGBM651 cells with different treatments (i.e., inhibitors against ROCK, Rac1-GEF, TRIO, or STAT3). Each movie is listed and described below:

Supplemental Movie 1. Migration of shCtrl and shYAP GBM cells. A representative time-lapse video of shCtrl (left) and shYAP (right) JHGBM651 cells. Images captured at 20-minute intervals for 6 hours. Each colored line represents the migration pattern of each cell. Scale bar = 100 μm.

Supplemental Movie 2. Migration of shCtrl and shYAP GBM cells with vehicle or ROCK inhibitor treatment. A representative time-lapse video of shCtrl (left) and shYAP (right) JHGBM651 cells with vehicle (top) or treatment with the ROCK inhibitor Y27632 (bottom). Images are captured at 20-minute intervals for 6 hours. Each colored line represents the migration pattern of each cell. Scale bar = 100 μm.

Supplemental Movie 3. Migration of shCtrl and shYAP GBM cells with vehicle or Rac1-GEF inhibitor treatment. A representative time-lapse video of shCtrl (left) and shYAP (right) JHGBM651 cells with vehicle (top) or treatment with the Rac1-GEF inhibitor NSC23766 (bottom). Images are captured at 20-minute intervals for 6 hours. Each colored line represents the migration pattern of each cell. Scale bar = 100 μm.

Supplemental Movie 4. Migration of shCtrl and shYAP GBM cells with vehicle or TRIO inhibitor treatment. A representative time-lapse video of shCtrl (left) and shYAP (right) JHGBM651 cells with vehicle (top) or treatment with TRIO inhibitor ITX3 (bottom). Images are captured at 20-minute intervals for 6 hours. Each colored line represents the migration pattern of each cell. Scale bar = 100 μm.

Supplemental Movie 5. Migration of shCtrl and shTRIO GBM cells. A representative time-lapse video of shCtrl (left) and shTRIO (right) JHGBM651 cells. Images captured at 20-minute intervals for 6 hours. Each colored line represents the migration pattern of each cell. Scale bar = 100 μm.

Supplemental Movie 6. Migration of shCtrl and shTRIO GBM cells with vehicle or ROCK inhibitor treatment. A representative time-lapse video of shCtrl (left) and shTRIO (right) JHGBM651 cells with vehicle (top) or treatment with ROCK inhibitor Y27632 (bottom). Images are captured at 20-minute intervals for 6 hours. Each colored line represents the migration pattern of each cell. Scale bar = 100 μm.

Supplemental Movie 7. Migration of shCtrl and shYAP GBM cells with vehicle or STAT3 inhibitor treatment.

A representative time-lapse video of shCtrl (left) and shYAP (right) JHGBM651 cells with vehicle (top) or treatment with STAT3 inhibitor LLL12 (bottom). Images are captured at 20-minute intervals for 6 hours. Each colored line represents the migration pattern of each cell. Scale bar = 100 μm.

Supplemental Tables: Two tables are submitted separately. Both tables are MS Excel files. Descriptions/titles for each Table are listed below:

Supplemental Table 1. Annotation of intraoperatively obtained human GBM tissues and cells with corresponding clinical characteristics of donor patient.

This table contains GBM tissue and cell ID numbers with their corresponding de-identified patient IDs, age, gender, and final pathology diagnosis.

Supplemental Table 2. Microarray data of shCtrl or shYAP JHGBM651 and JHGBM612 cells.

Supplemental File 1. Sequence of TEAD and TEAD4 binding motifs on TRIO enhancer sites.

Figure 1| YAP regulates migratory capacity

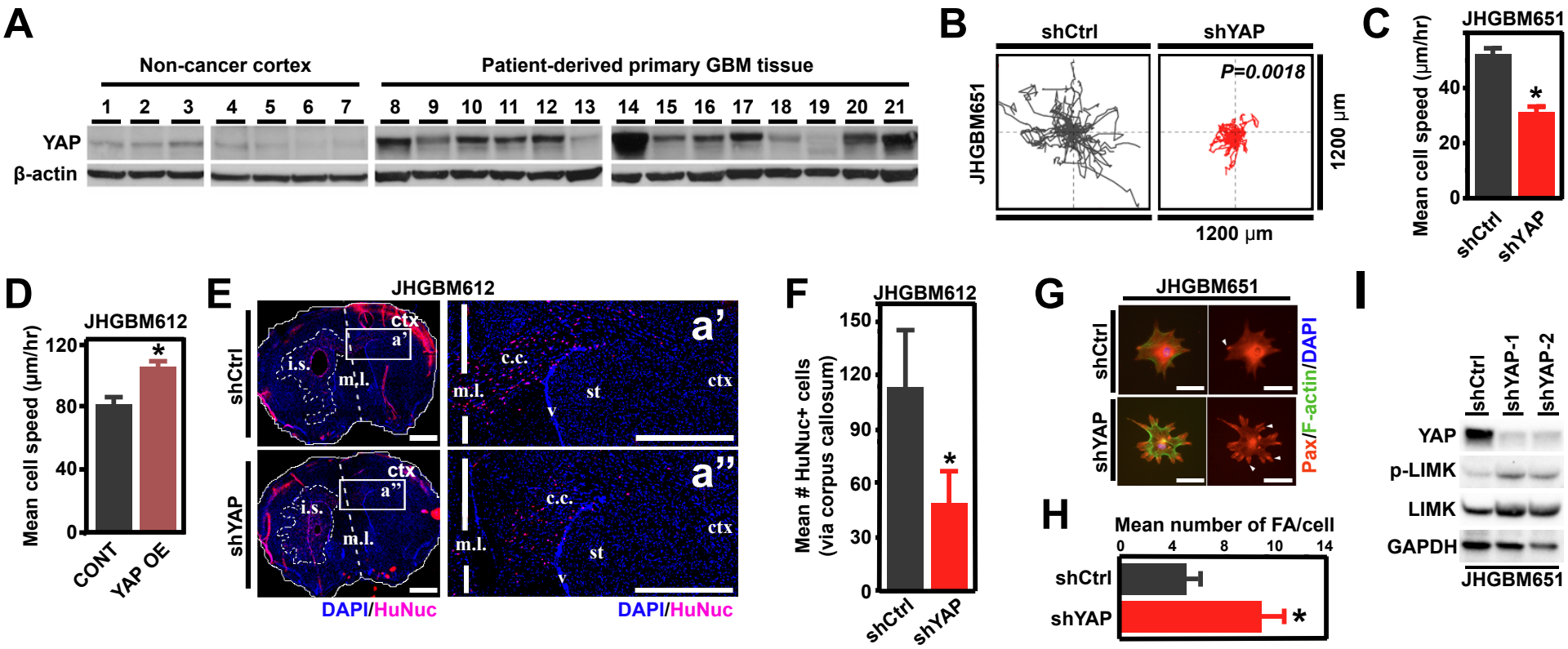


Figure 2| YAP regulates migration through a Rho-GTPase Switch

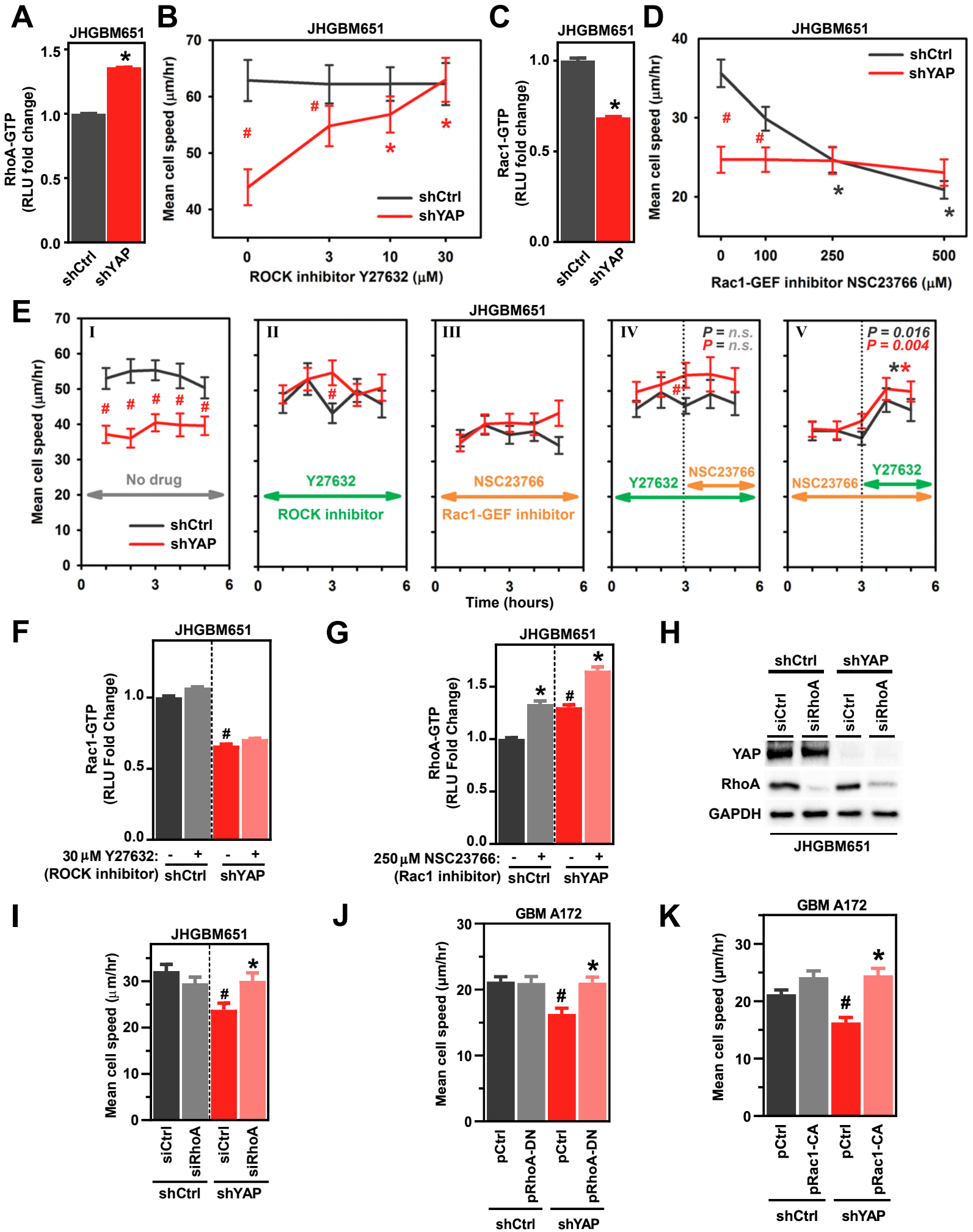


Figure 3| YAP directly transactivates the Rac1-GEF TRIO

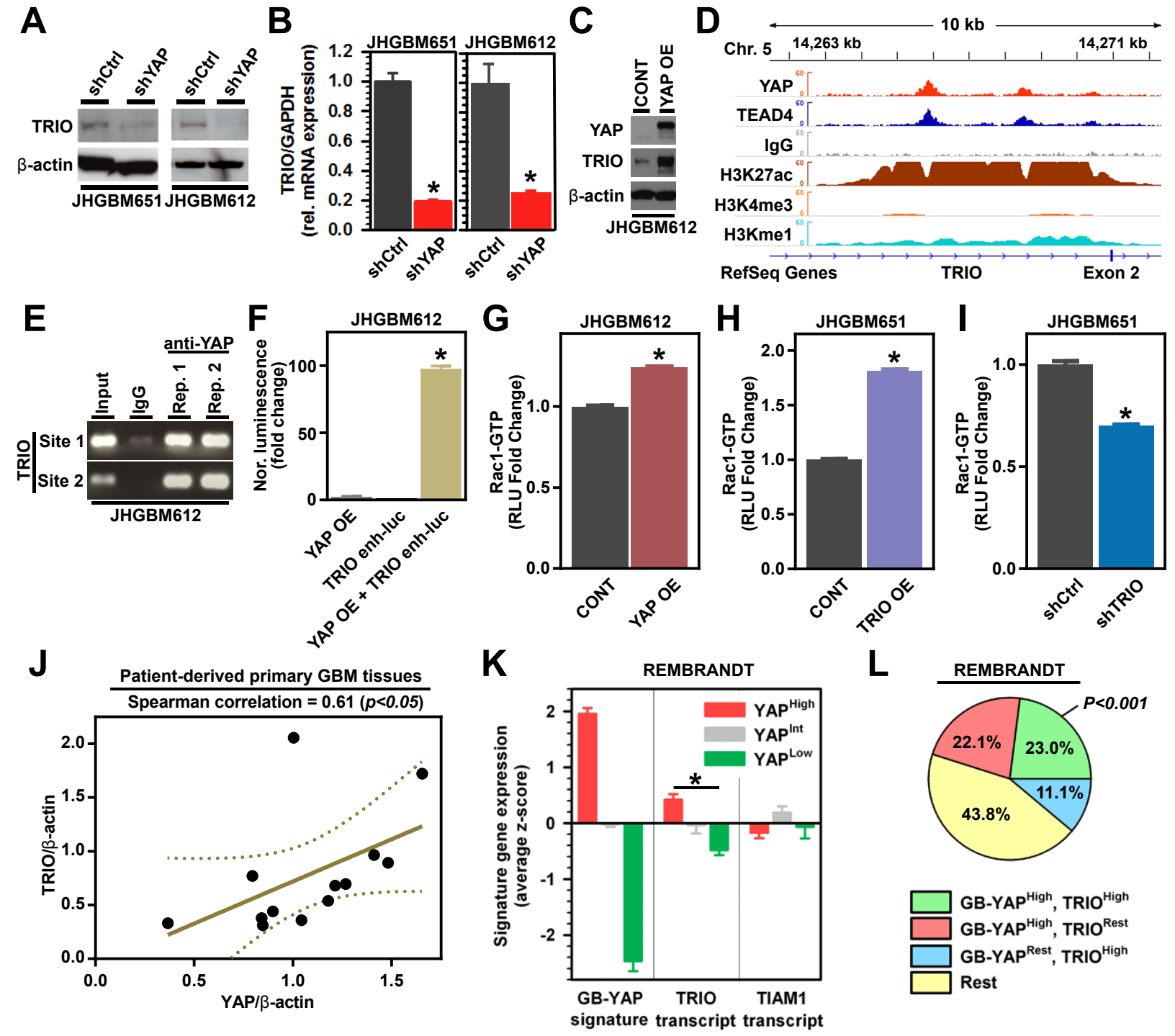


Figure 4| YAP-TRIO signaling regulates migration

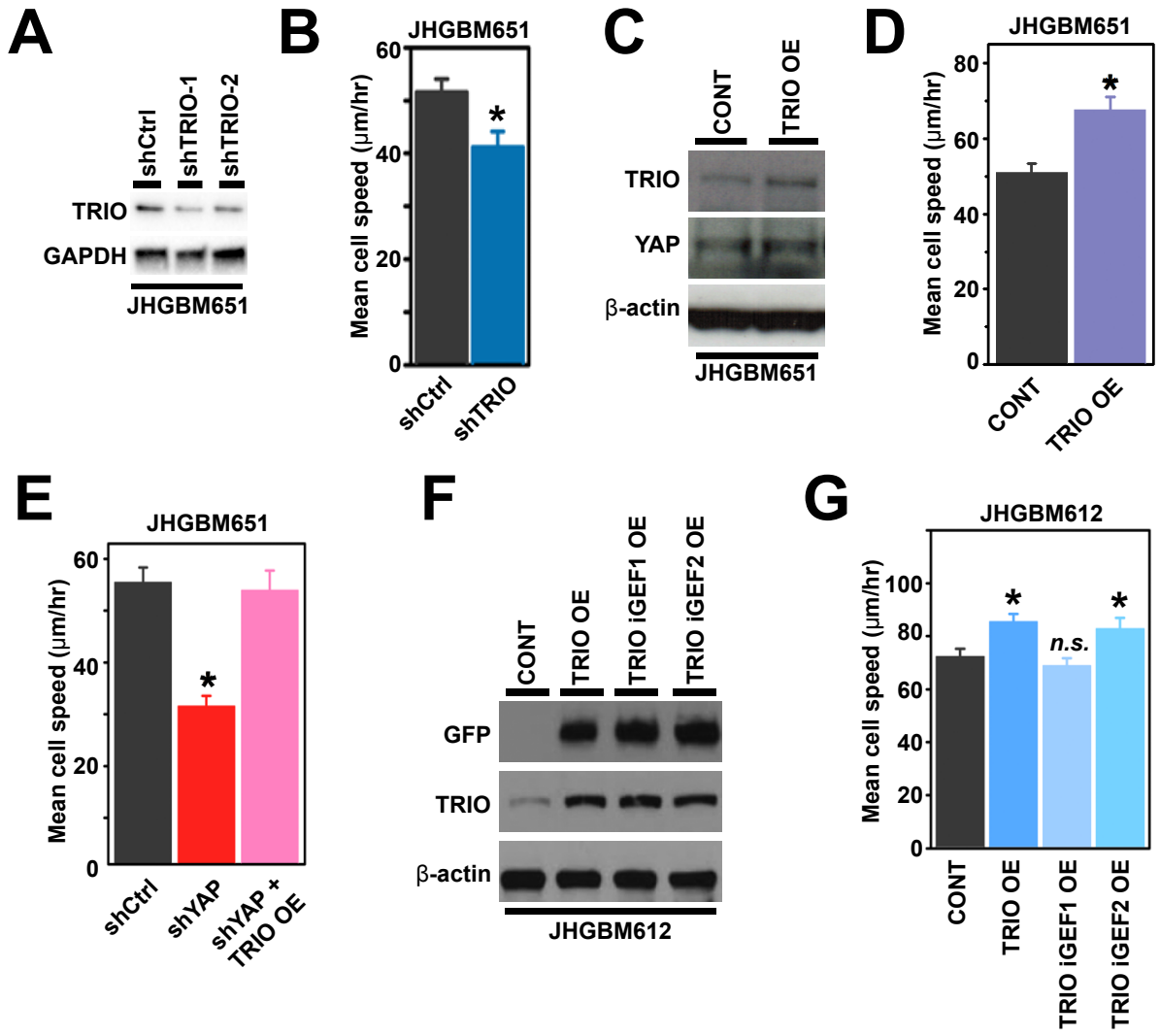


Figure 5| YAP-TRIO signaling promotes cell invasion

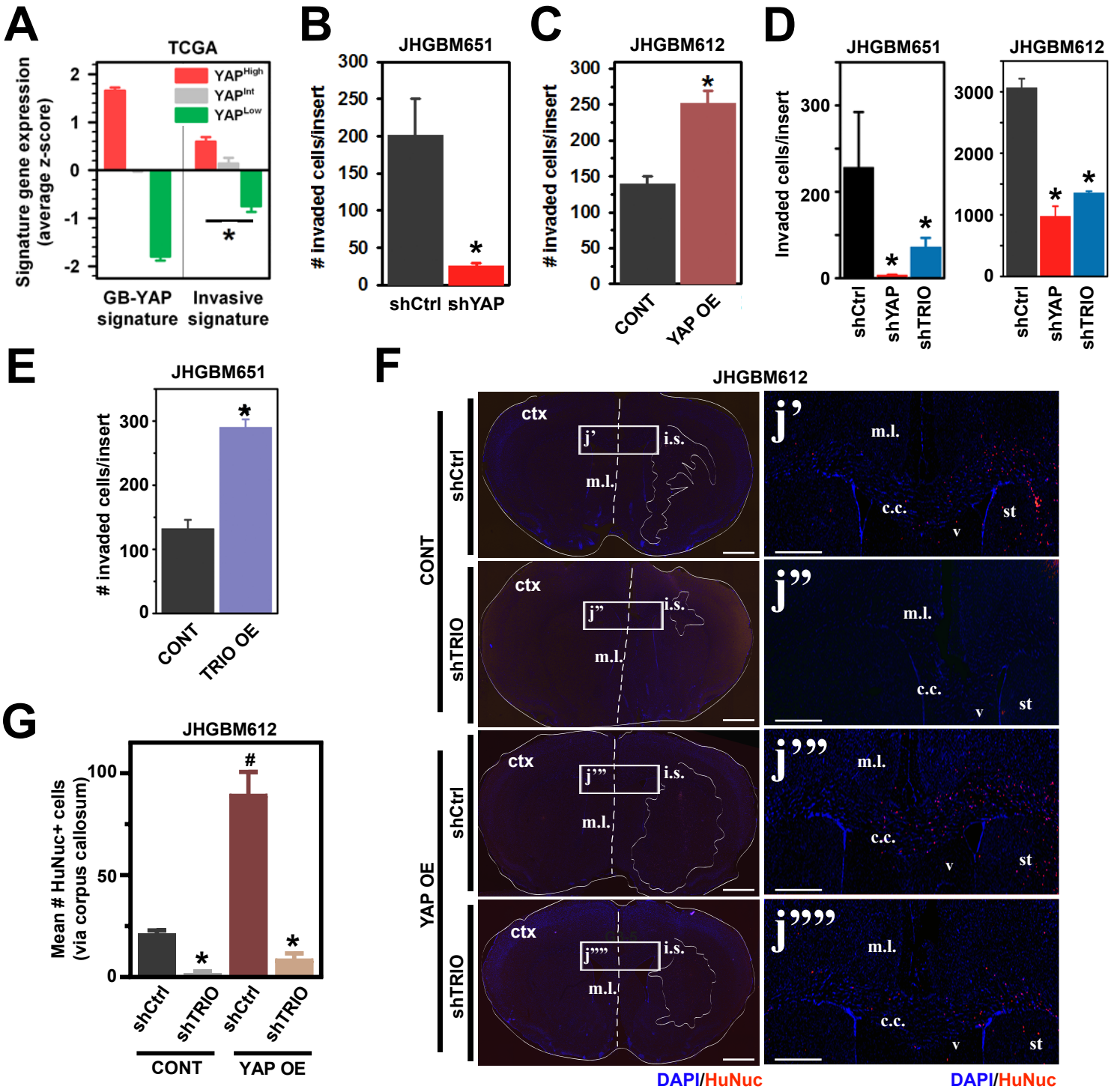


Figure 6| STAT3 is potentially involved in YAP-mediated cell invasion

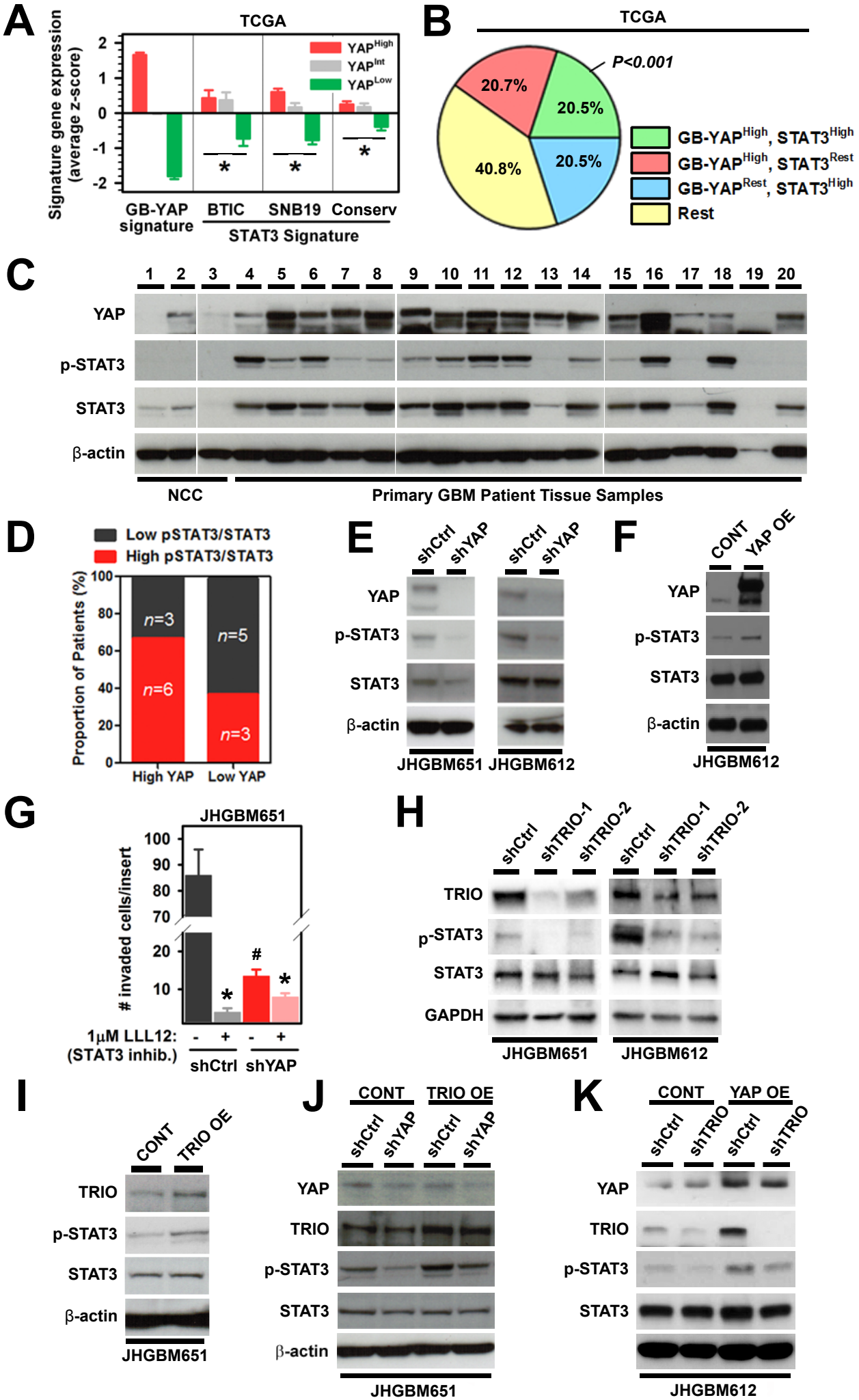
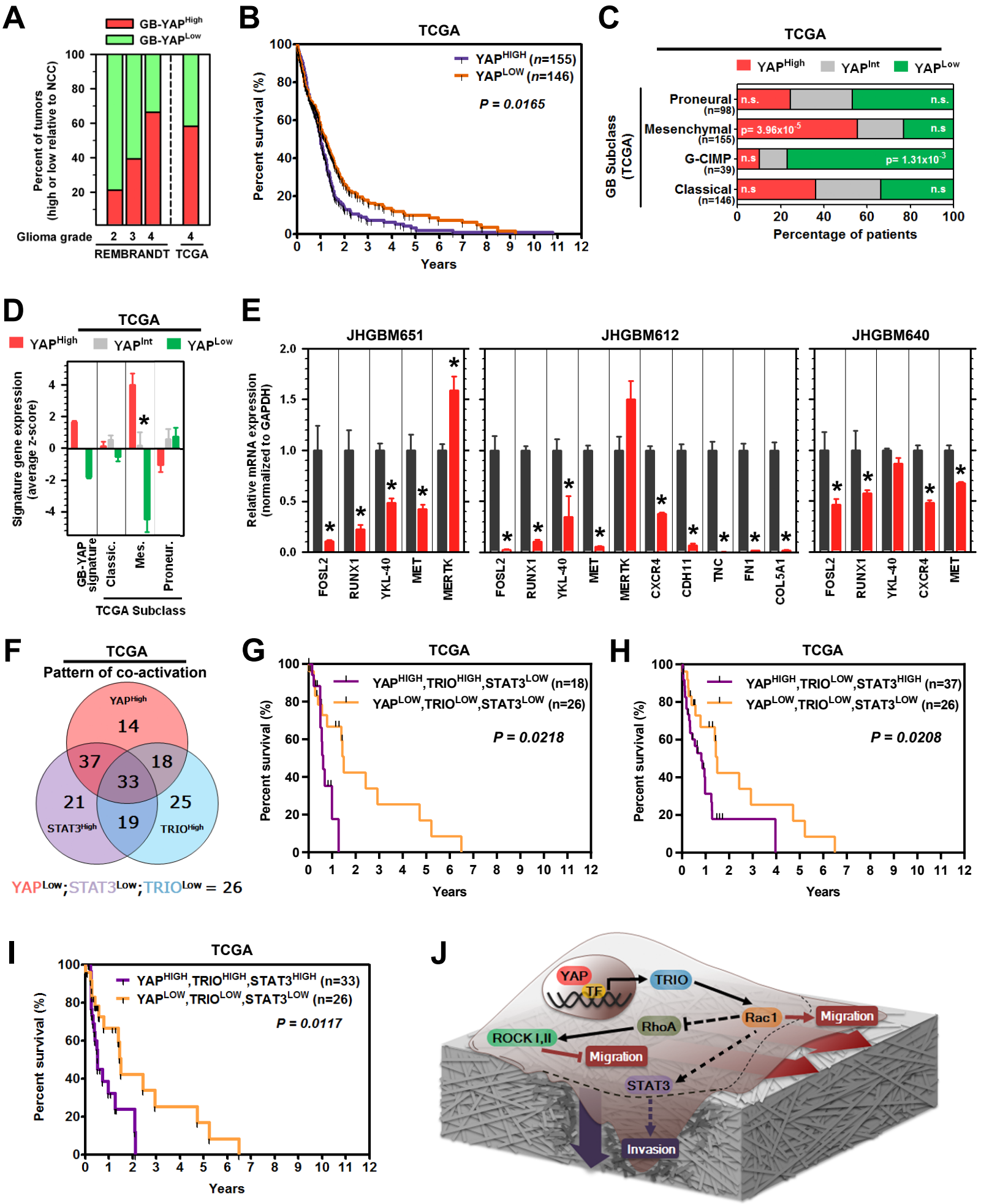
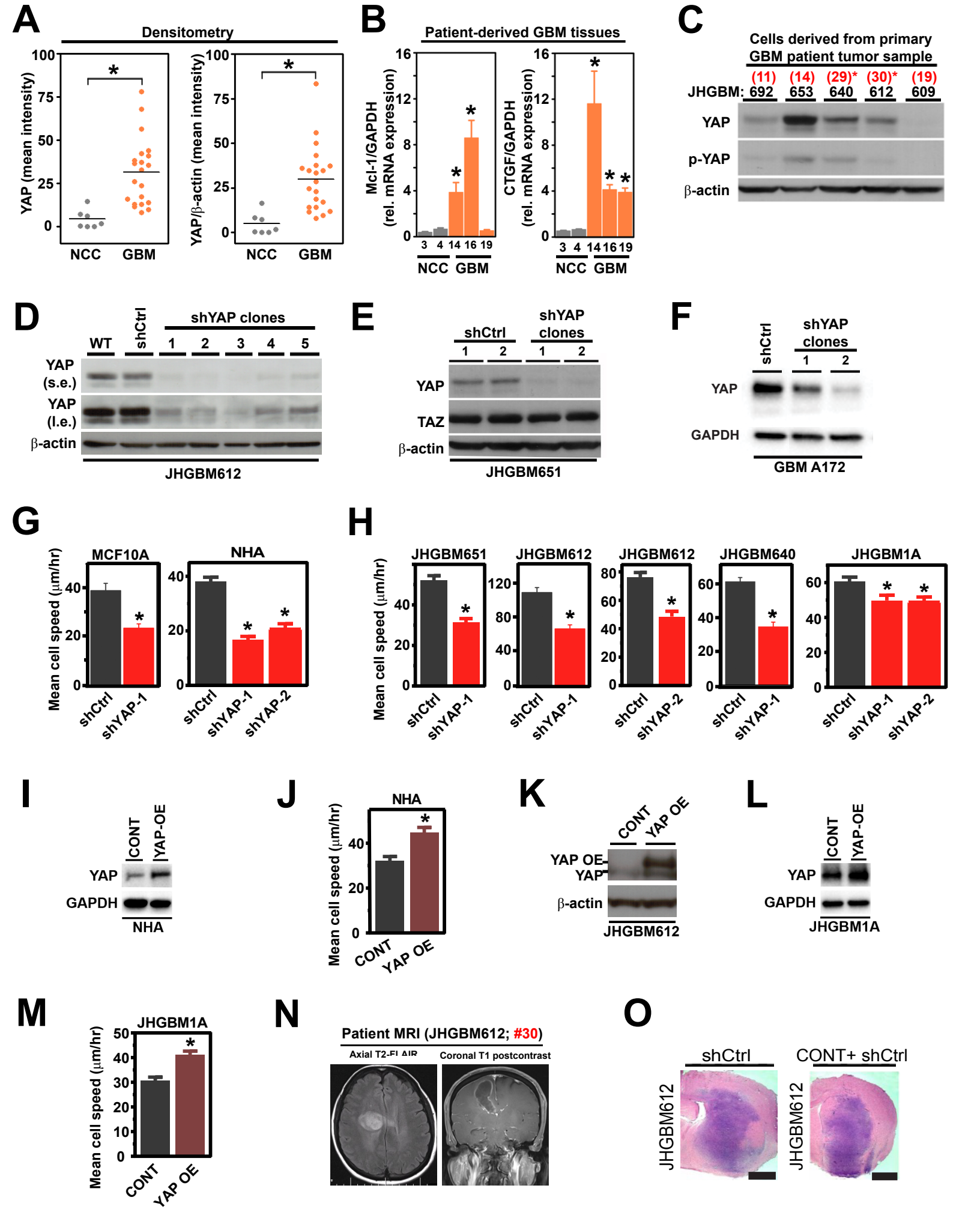


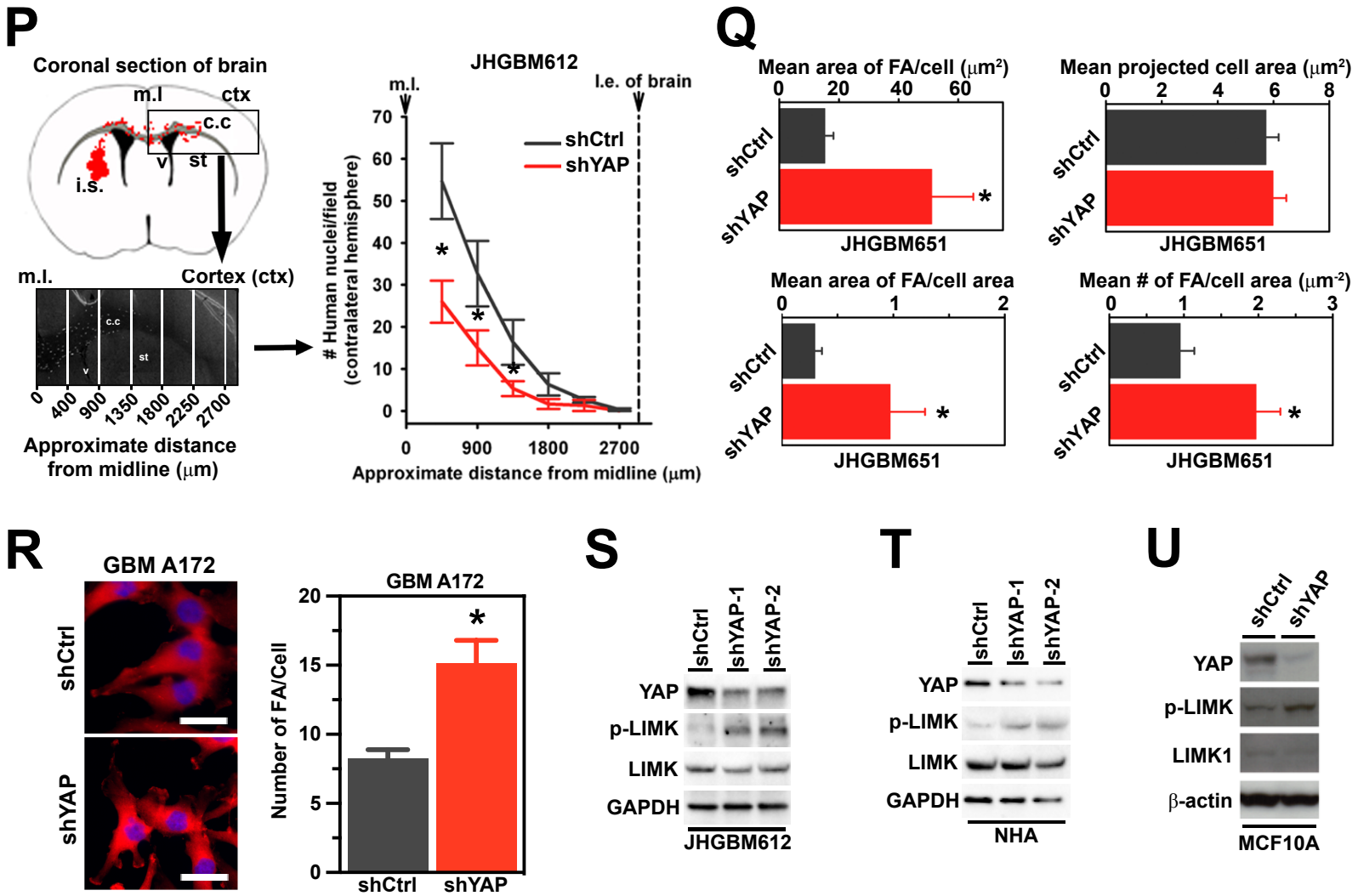
Figure 7| YAP, TRIO, and STAT3 signaling predict poor clinical outcomes in glioblastoma



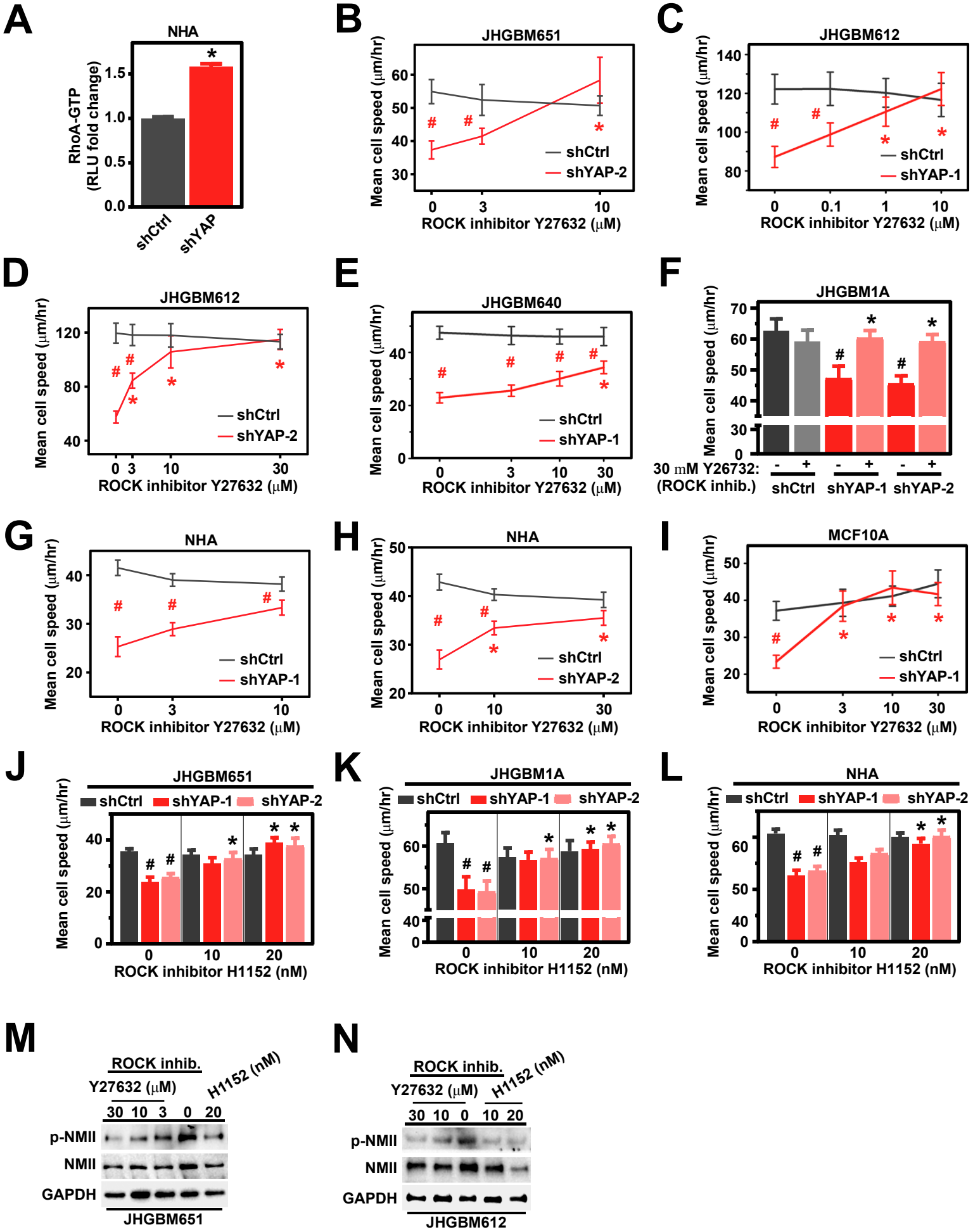
Supplementary Figure 1| YAP regulates migration



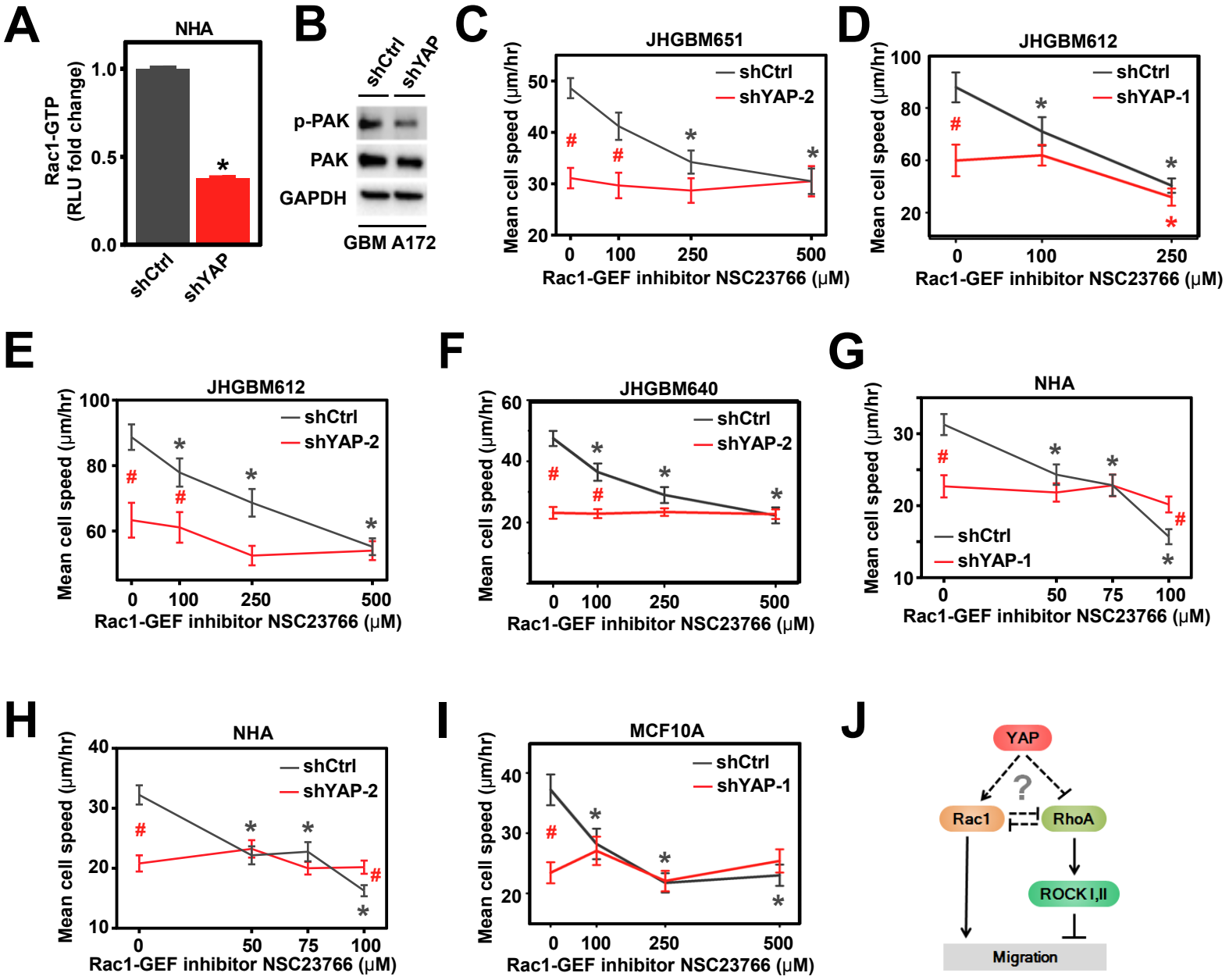
Supplementary Figure 1| YAP regulates migration



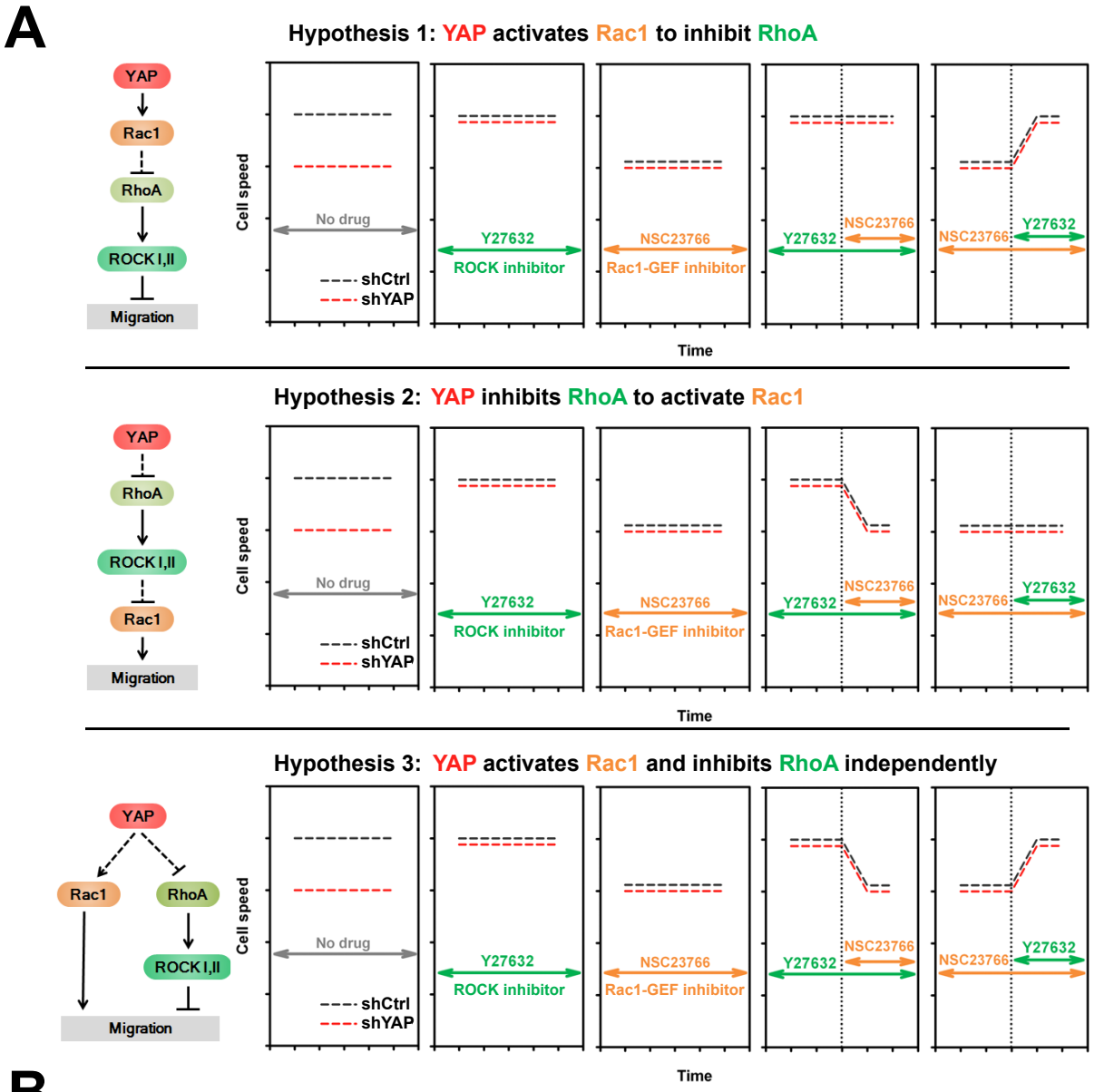
Supplementary Figure 2| YAP regulates migration by inhibiting RhoA-ROCK signaling

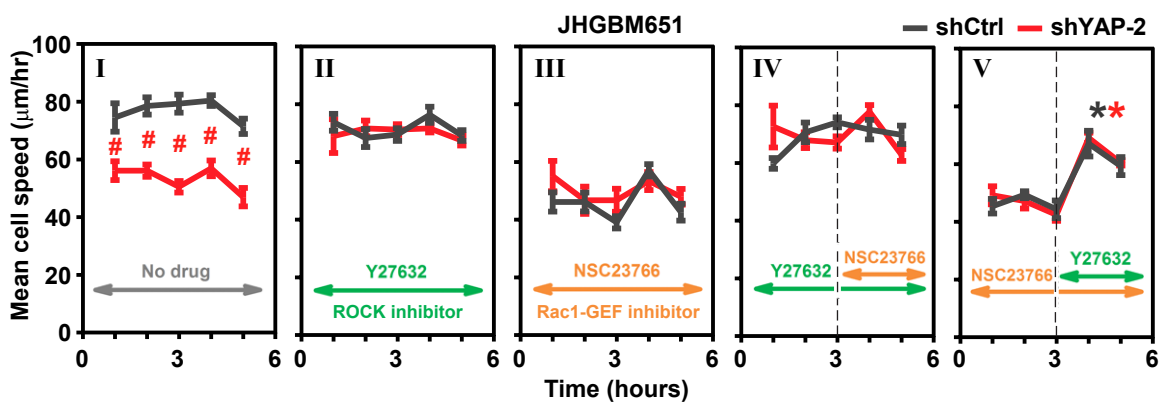


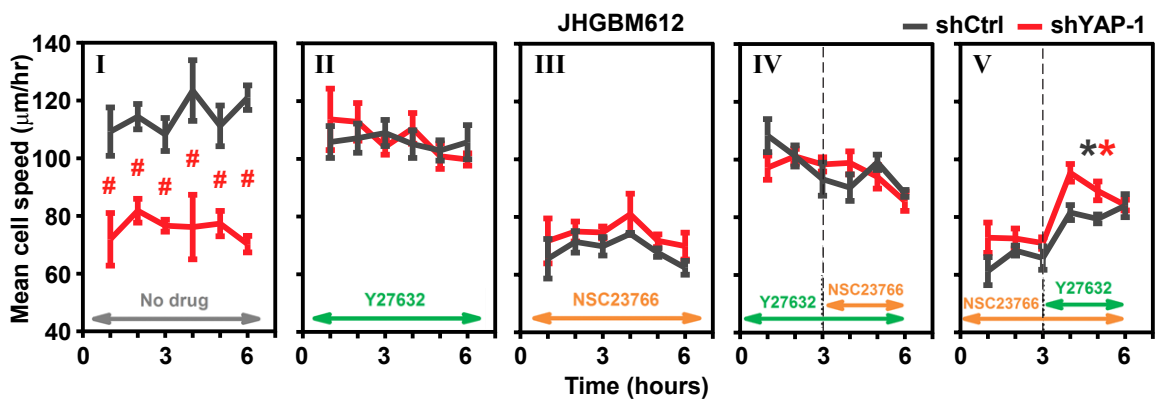
Supplementary Figure 3| YAP regulates migration by activating Rac1 signaling



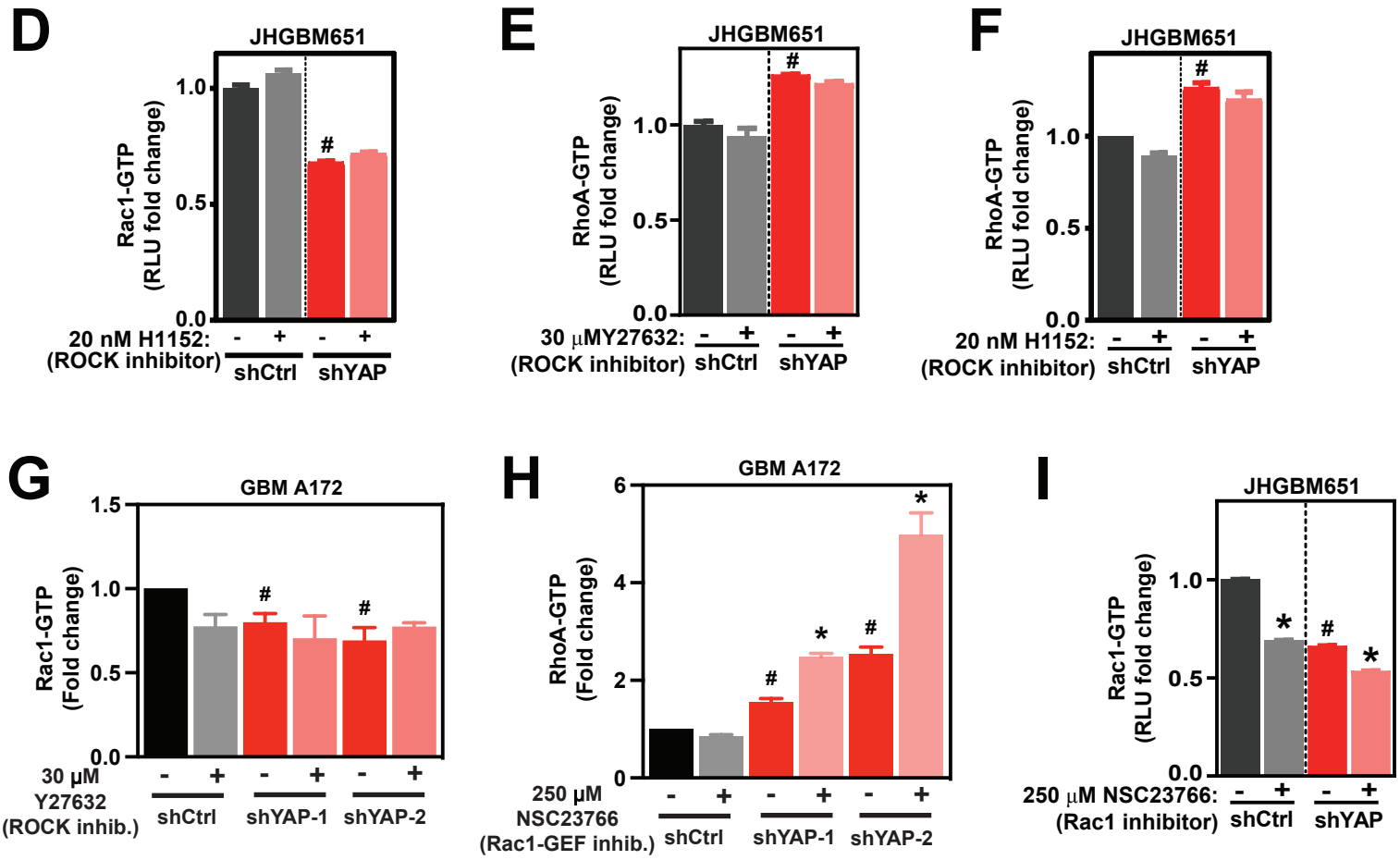
Supplementary Figure 4| YAP regulates migration by modulating a Rho-GTPase switch

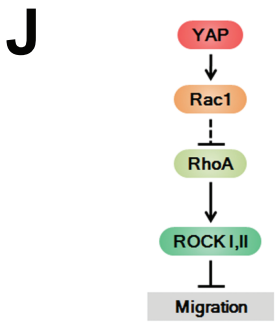




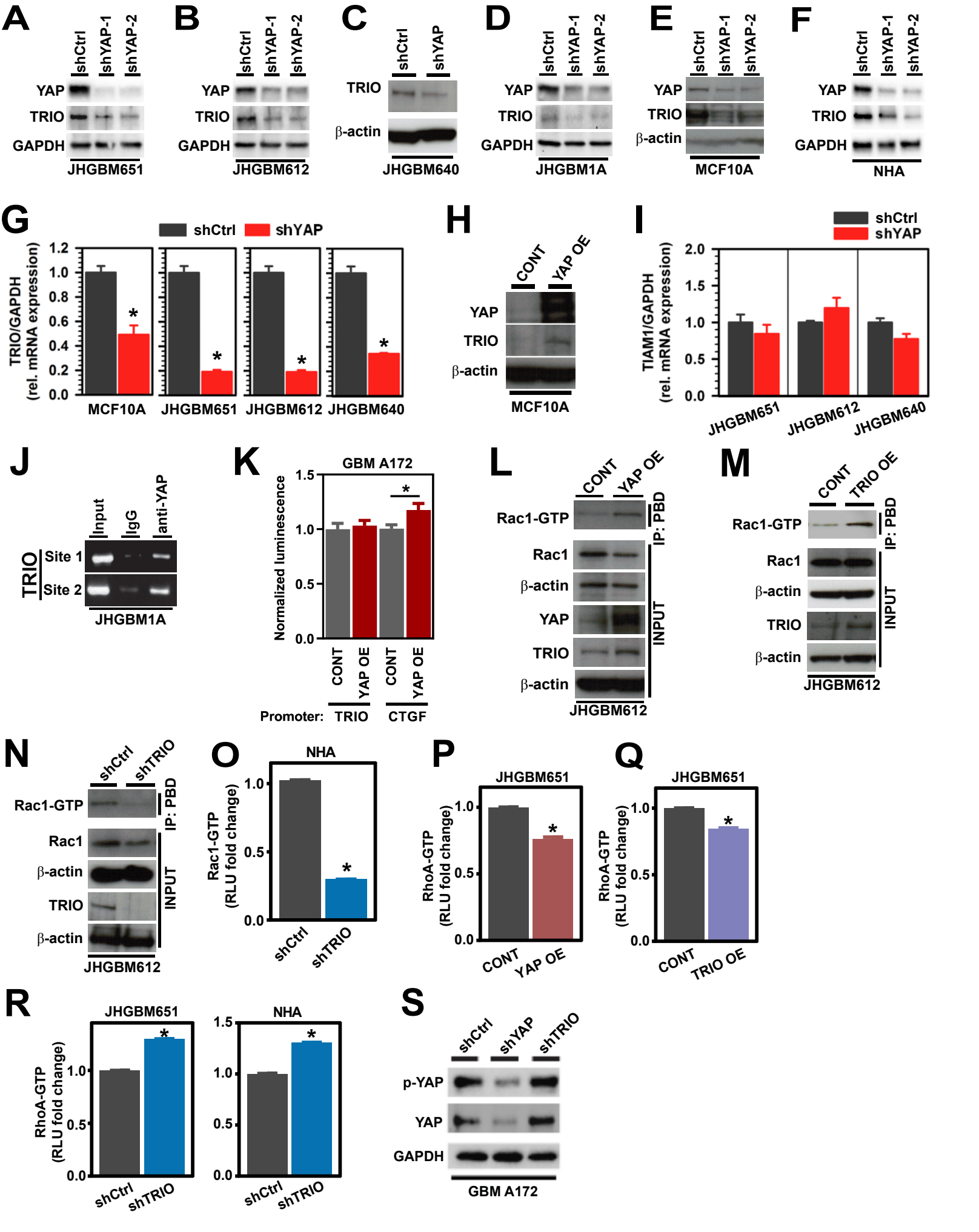


Supplementary Figure 4| YAP regulates migration by modulating a Rho-GTPase switch

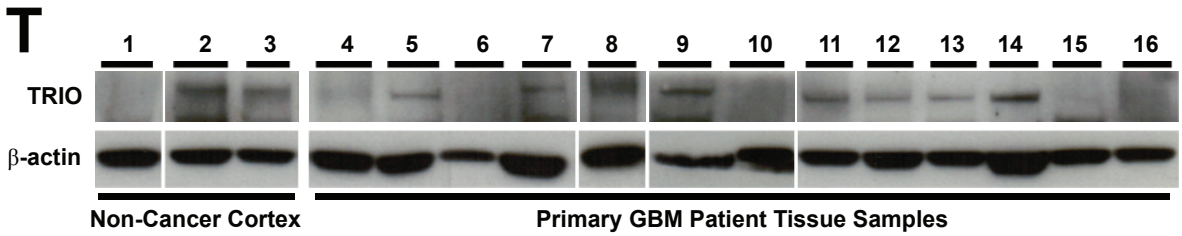


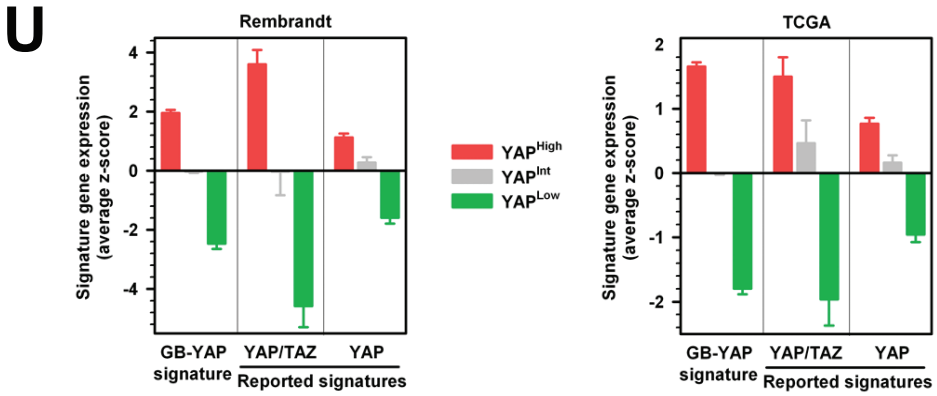


Supplementary Figure 5| YAP transactivates TRIO signaling

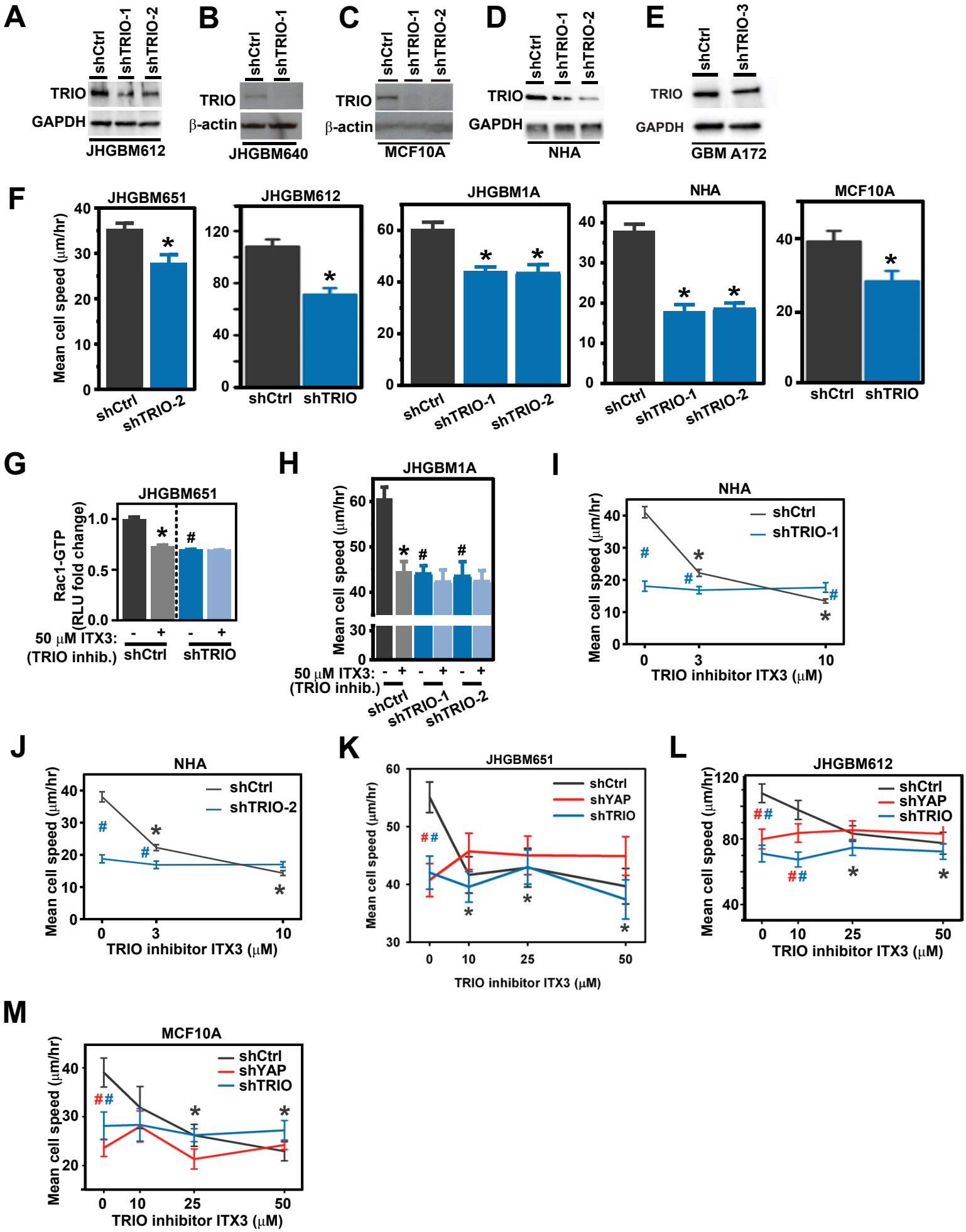


Supplementary Figure 5| YAP transactivates TRIO signaling

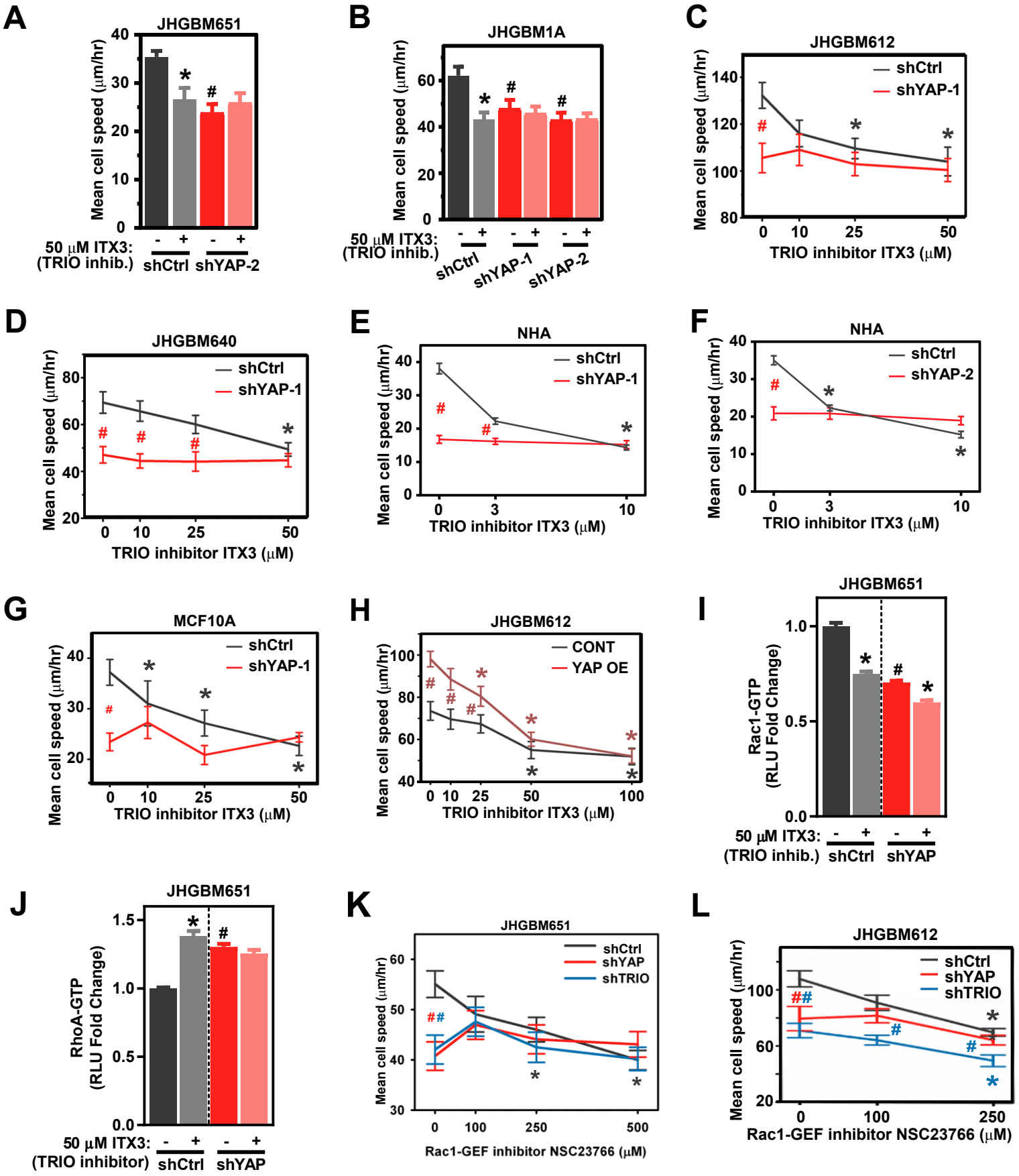




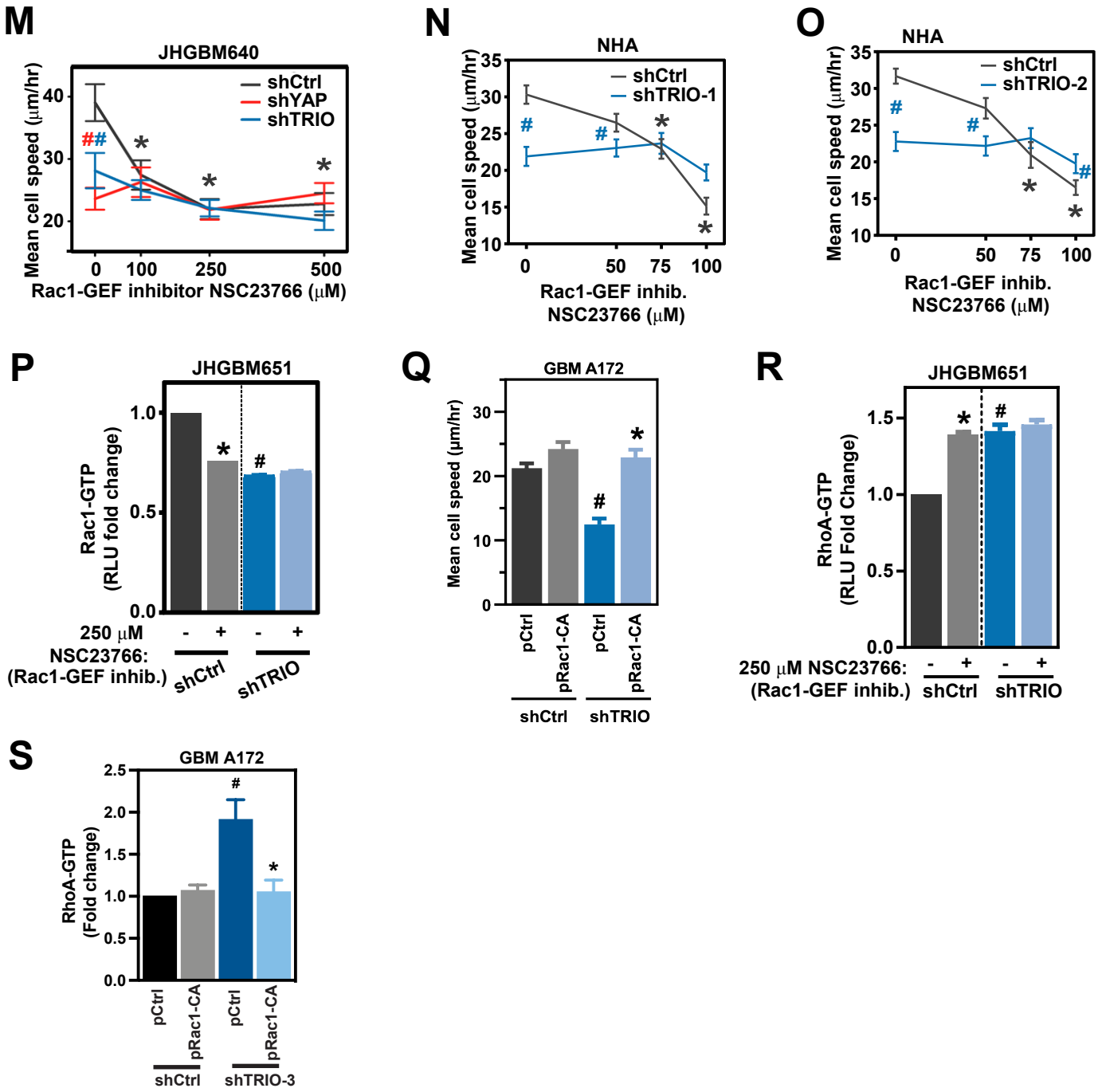
Supplementary Figure 6| YAP regulates migration via TRIO signaling



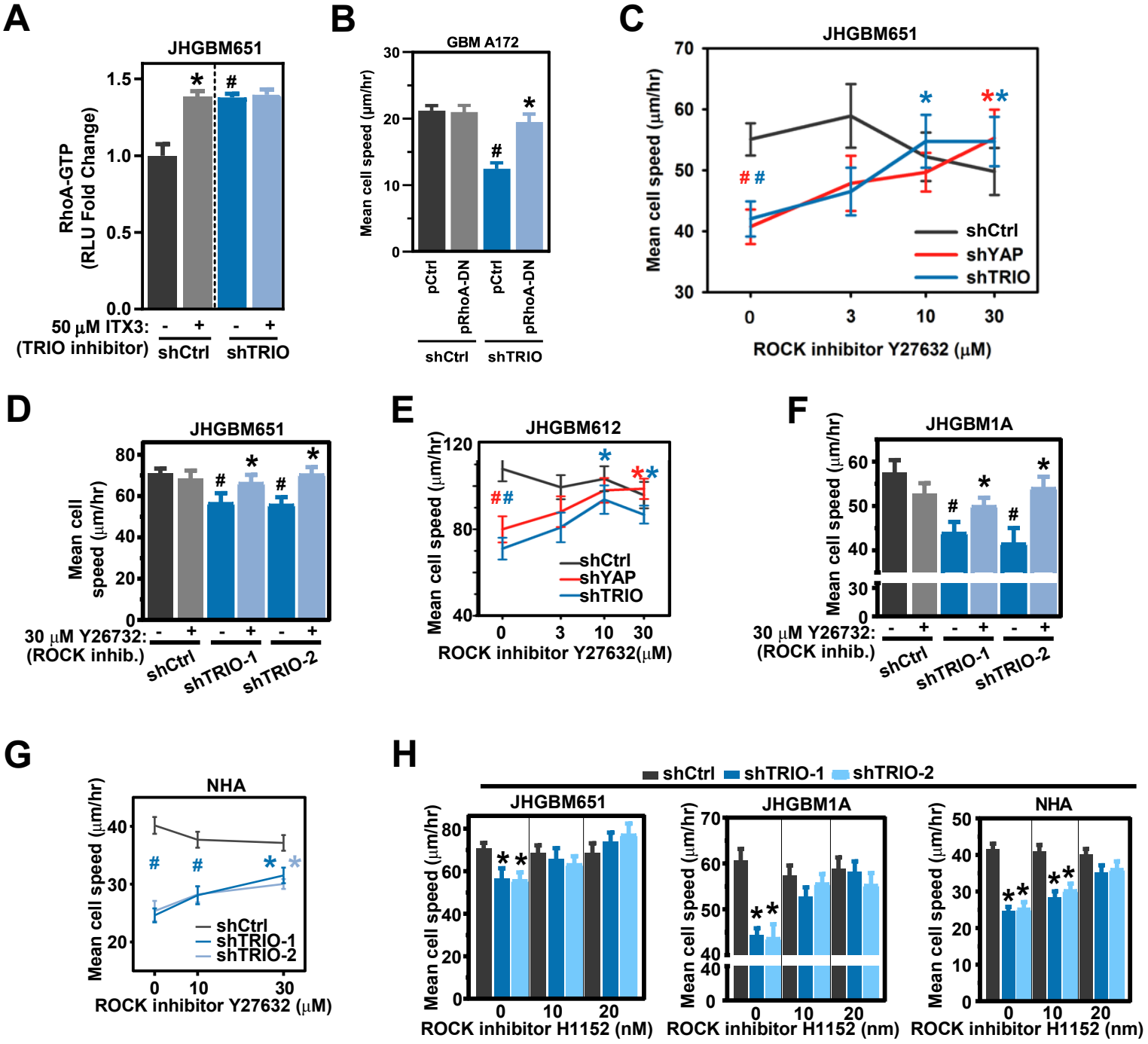
Supplementary Figure 7| YAP-TRIO signaling modulates Rac1-RhoA to drive migration



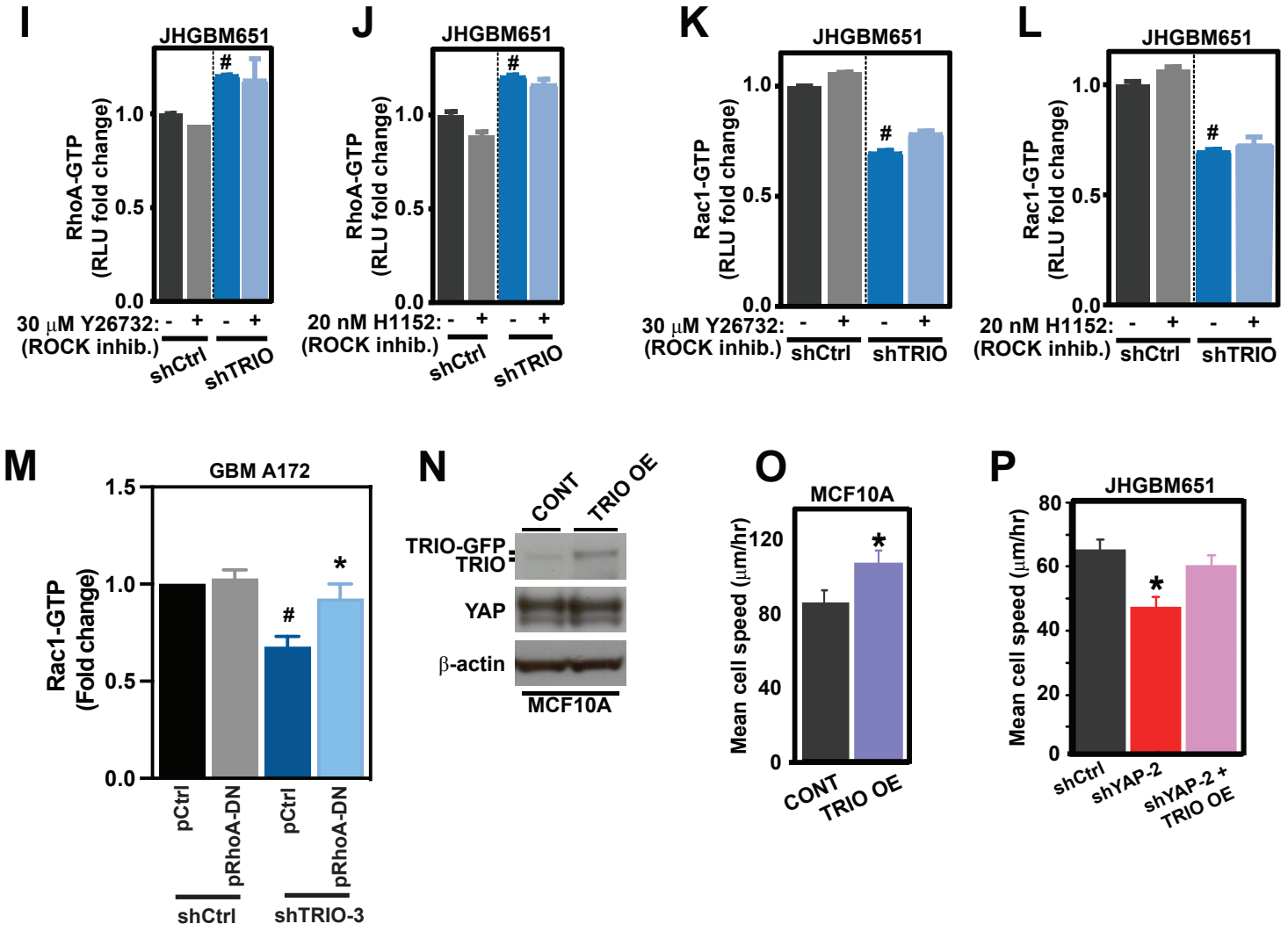
Supplementary Figure 7| YAP-TRIO signaling modulates Rac1-RhoA to drive migration



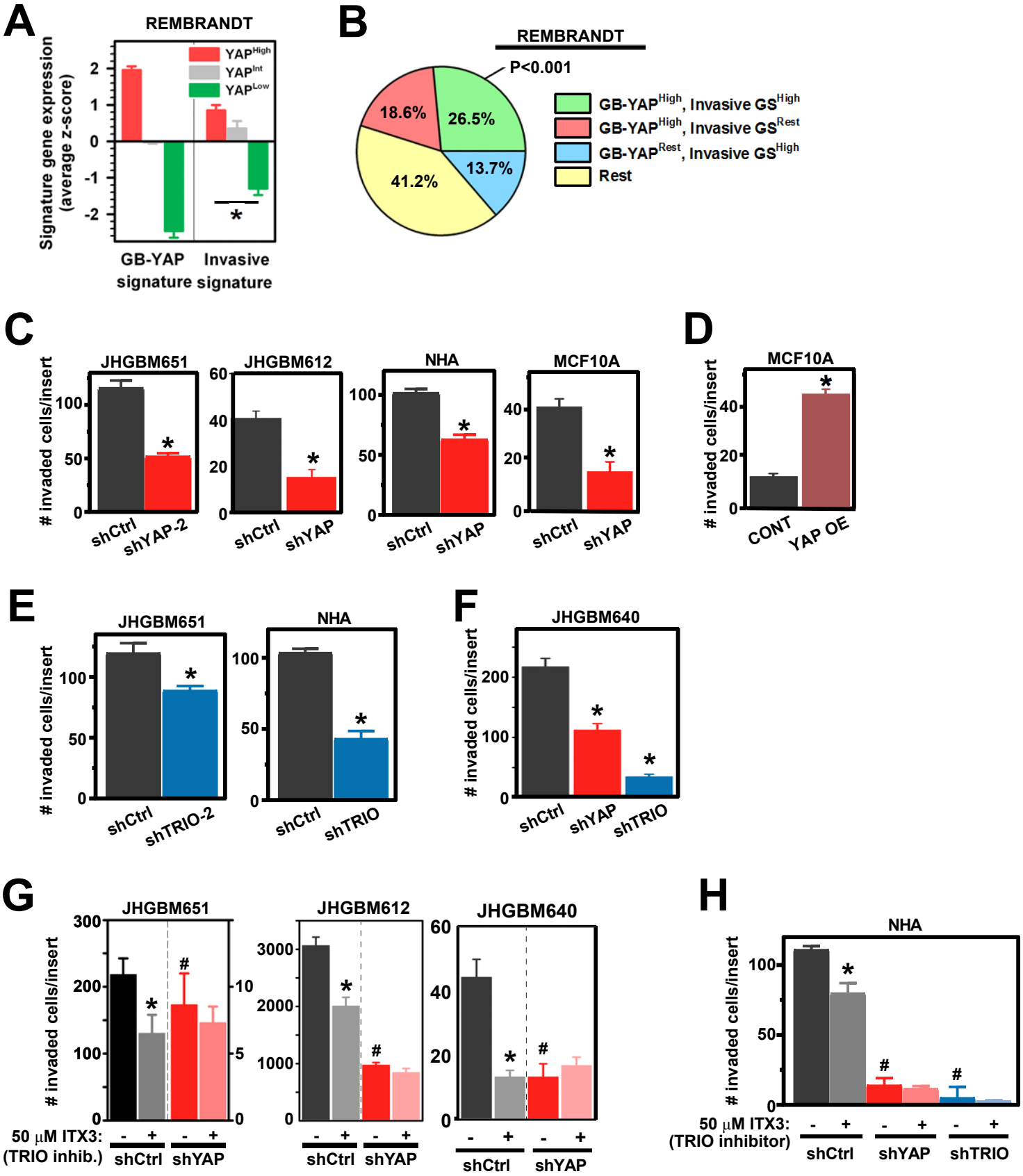
Supplementary Figure 8| YAP-TRIO signaling regulates migration by modulating Rac1-RhoA crosstalk



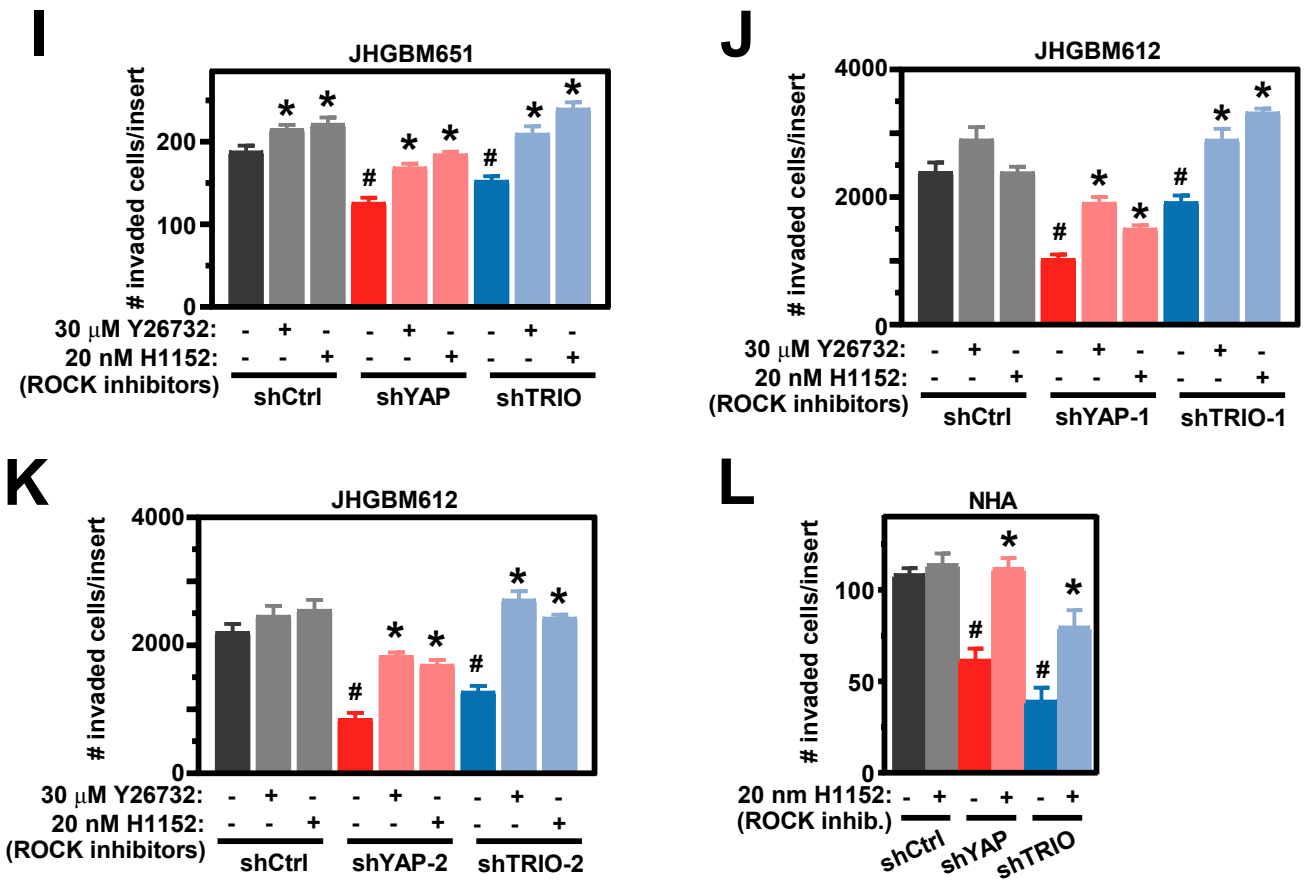
Supplementary Figure 8| YAP-TRIO signaling regulates migration by modulating Rac1-RhoA crosstalk



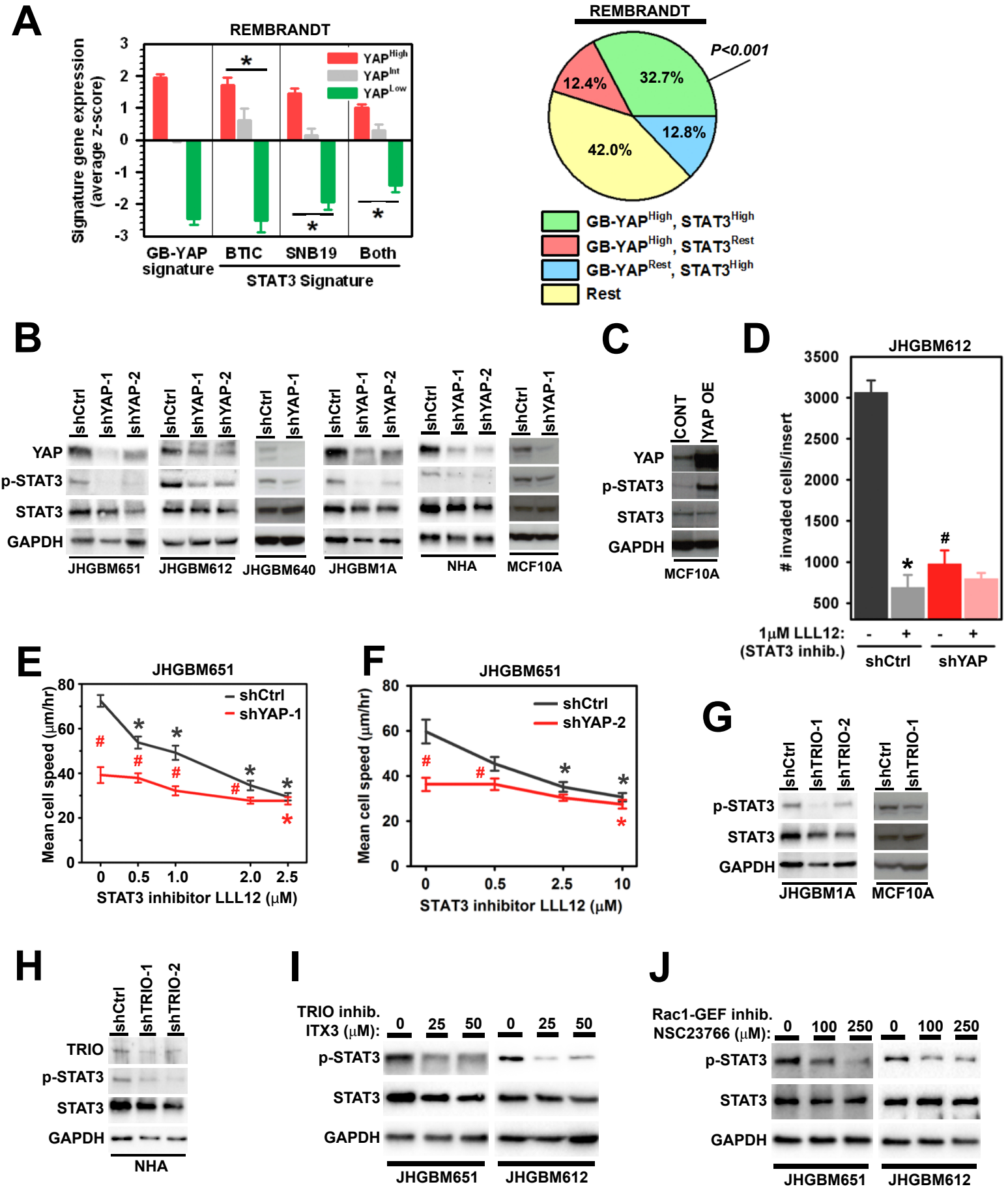
Supplementary Figure 9| YAP-TRIO signaling promotes cell invasion



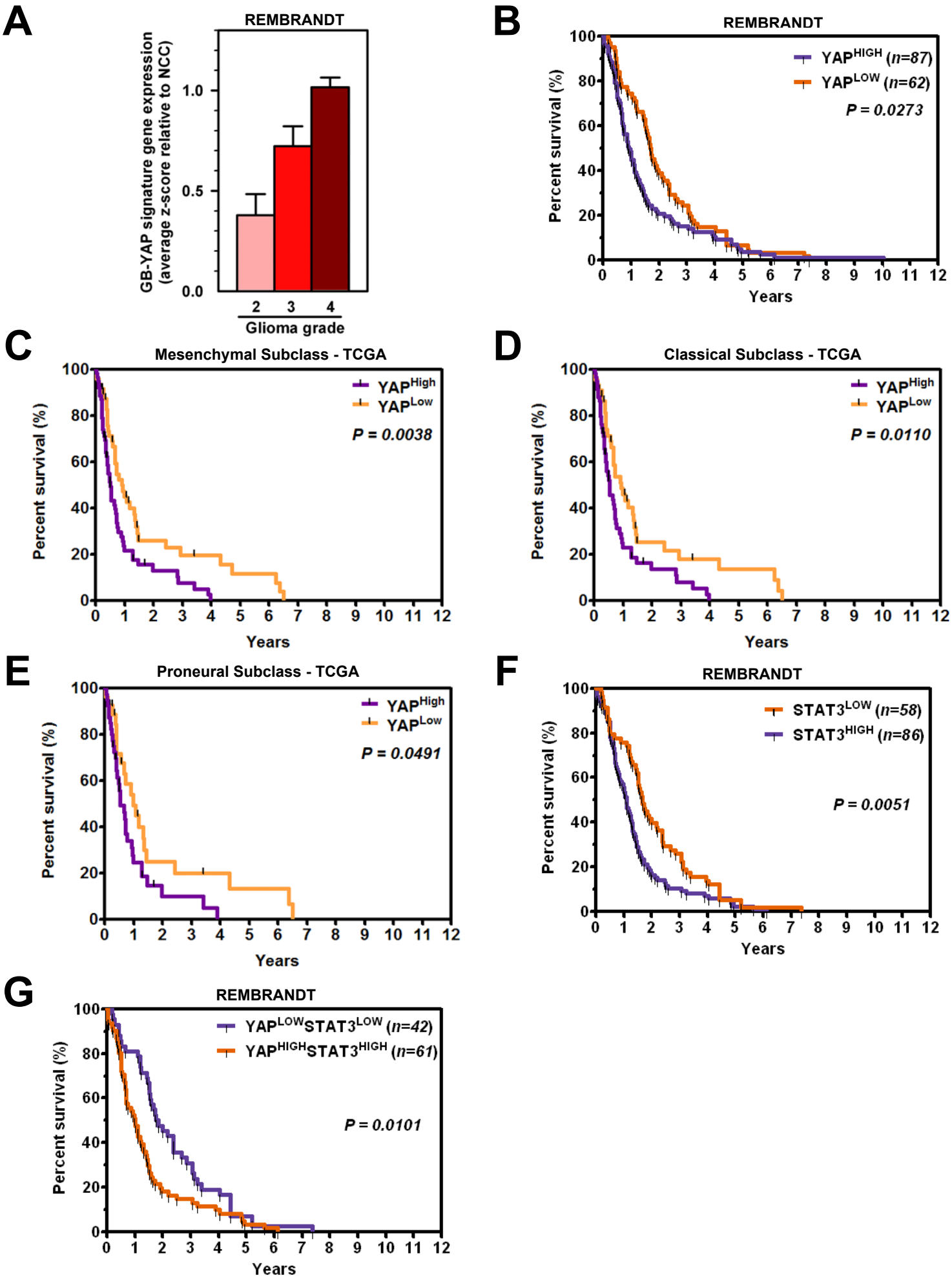
Supplementary Figure 9| YAP-TRIO signaling promotes cell invasion



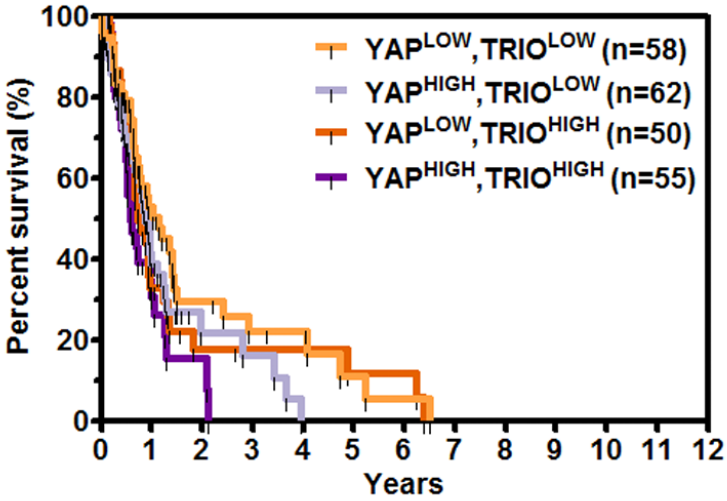
Supplementary Figure 10| STAT3 is potentially involved in YAP-mediated cell invasion

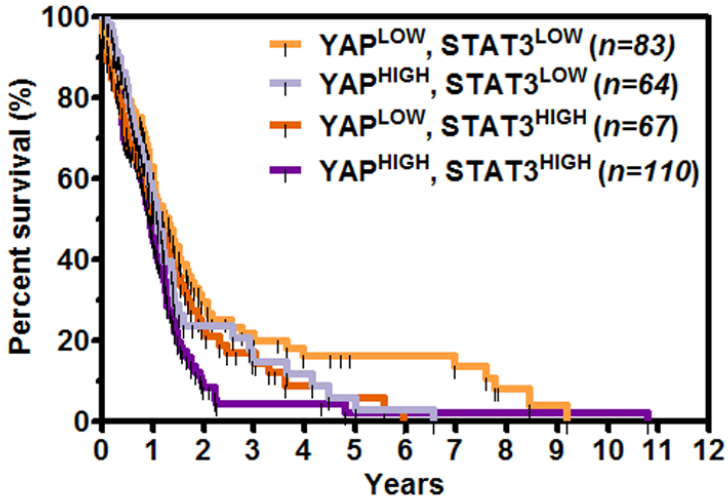


Supplementary Figure 11| Elevated YAP signaling predicts poor clinical outcomes in all GBM subtypes



Supplementary Figure 12| Each component of the YAP-TRIO-STAT3 network is indispensable to promoting poor clinical outcomes in GBM patients





YAP-TRIO (microarray)		
	Hi.Lo	Lo.Hi
Lo.Lo	0.151	0.32
Hi.Hi	0.17	0.184

YAP-TRIO (RNA-Seq)		
	Hi.Lo	Lo.Hi
Lo.Lo	0.08	0.191
Hi.Hi	0.1032	0.52

YAP-STAT3 (microarray)		
	Hi.Lo	Lo.Hi
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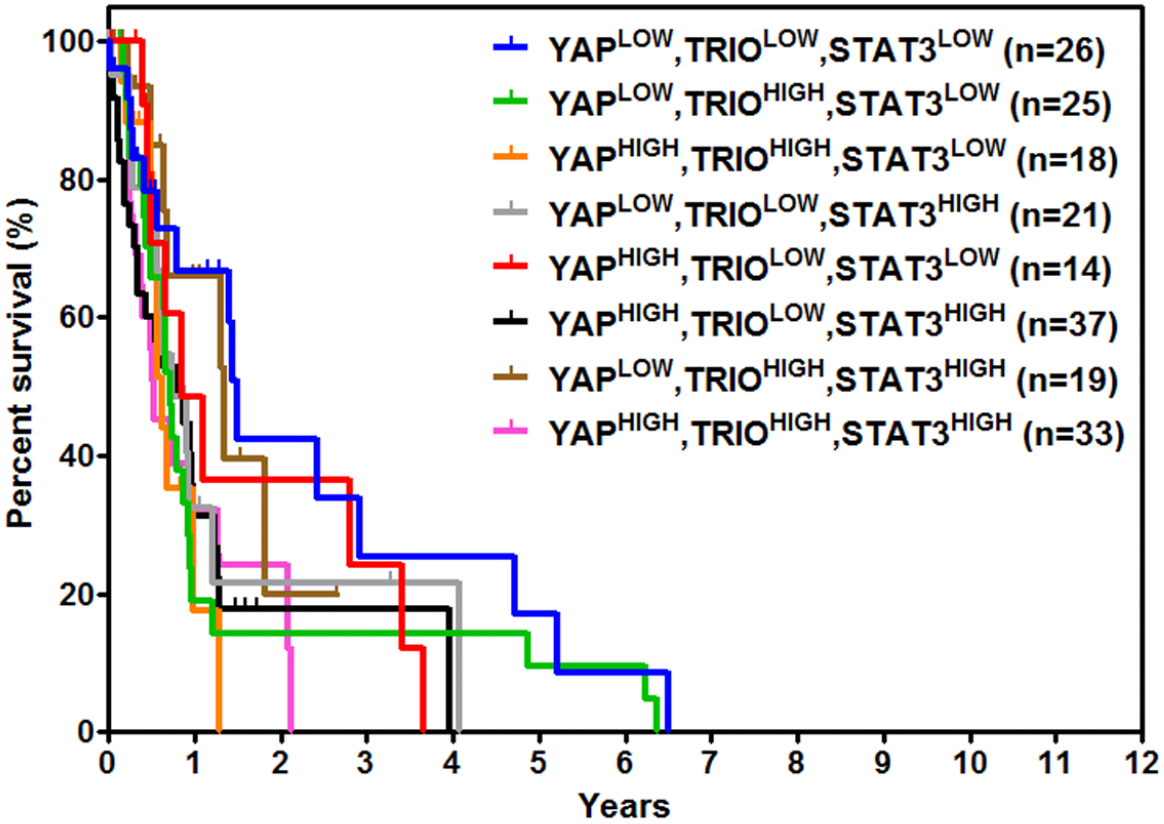
Hi.Hi	0.1019	0.1105



YAP-TRIO-STAT3 (microarray)							
	Hi.Lo.Lo.	Lo.Hi.Lo	Lo.Lo.Hi	Lo.Hi.Hi	Hi.Lo.Hi	Hi.Hi.Lo	
Lo.Lo.Lo	0.3869	0.0688	0.1452	0.7766	0.0208	0.0218	Lo.Lo.Lo
Hi. Hi. Hi	0.0823	0.6775	0.3479	0.058	0.9496	0.9977	Hi. Hi. Hi

Supplemental Table 1

Sample #	Identifier	Sex	Age	Pathology
1	JHNC693	F	57	Normal neocortex (meningioma; WHO grade I)
2	JHNC574	F	53	Normal neocortex (mesial temporal sclerosis; hippocampus)
3	JHNC557	M	29	Normal neocortex (mesial temporal sclerosis; hippocampus)
4	JHNC533	M	49	Normal neocortex (mesial temporal sclerosis; hippocampus)
5	JHNC503	M	28	Normal neocortex (mesial temporal sclerosis; hippocampus)
6	JHNC482	F	31	Normal neocortex (mesial temporal sclerosis; hippocampus)
7	JHNC399	F	48	Normal neocortex (mesial temporal sclerosis; hippocampus)
8	JHGB698	F	76	Glioblastoma
9	JHGB697	F	60	Glioblastoma (recurrent)
10	JHGB695	F	63	Glioblastoma
11	JHGB692	M	56	Glioblastoma
12	JHGB661	M	41	Glioblastoma (primitive neuroectodermal + oligodendroglial features)
13	JHGB658	F	58	Glioblastoma
14	JHGB653	M	74	Glioblastoma
15	JHGB652	M	34	Glioblastoma
16	JHGB651	M	49	Glioblastoma
17	JHGB620	F	73	Glioblastoma
18	JHGB615	F	76	Glioblastoma
19	JHGB609	M	42	Glioblastoma
20	JHGB608	M	69	Glioblastoma
21	JHGB579	M	73	Glioblastoma
22	JHGB577	F	57	Glioblastoma
23	JHGB576	M	80	Glioblastoma
24	JHGB567	M	48	Glioblastoma
25	JHGB549	F	83	Glioblastoma
26	JHGB547	M	64	Glioblastoma (recurrent)
27	JHGB544	M	70	Glioblastoma
28	JHGB529	M	86	Glioblastoma
29	JHGB640	F	49	Glioblastoma
30	JHGB612	F	56	Glioblastoma
31	JHGB531	F	77	Glioblastoma
32	JHGB496	F	53	Glioblastoma (recurrent, secondary gliosarcoma)

Supplemental Table 2

Entrez	Symbol	612-pval	651-pval	612-qval	651-qval	612-FoldChange	651-FoldChange	MeanFoldChange	612Ctrl1	612Ctrl2	612Ctrl3	612shYAP1	612shYAP2	612shYAP3	651Ctrl1	651Ctrl2	651Ctrl3	651shYAP1	651shYAP2	651shYAP3
399694	**SHC4**	0.00205	0.00010	0.07301	0.04276	-1.379	-3.106	-2.243	7.4839551	7.6177382	7.7319358	7.31080455	7.12908032	7.19332171	9.7397266	9.7441781	9.8435432	8.22370361	8.05828114	8.14009863
214	**ALCAM**	0.00704	0.00013	0.09841	0.04276	-1.304	-3.047	-2.176	9.8157206	9.7756539	9.9871523	9.55801542	9.43927087	9.49799493	9.9814406	9.9381418	10.084188	8.51608521	8.34151706	8.32344295
9341	**VAMP3**	0.00016	0.00024	0.06179	0.04276	-1.629	-2.660	-2.144	9.9855156	9.9817084	9.9368992	9.32605363	9.20766051	9.23286048	11.006444	10.939156	11.135149	9.63686576	9.58883951	9.62077401
79912	**PYROXD1**	0.00015	0.00028	0.06179	0.04276	-1.610	-2.557	-2.084	7.1071245	7.9997996	7.9442894	7.32265272	7.28724723	7.24441015	8.3042687	8.2966904	8.4197855	7.04325796	6.93140184	6.98281241
100507127	**LOC100507127**	0.00031	0.00039	0.06562	0.04276	-1.522	-2.583	-2.052	5.5913921	5.6240831	5.560639	4.98431997	5.04036732	4.93571711	9.5281243	9.5769733	9.8635548	8.29279189	8.16802368	8.40104863
54537	**FAM35A**	0.00054	0.00055	0.06562	0.04276	-1.722	-2.304	-2.013	9.0934122	9.3848512	9.4824813	8.88576041	8.55415917	8.50802298	8.890006	8.9405053	9.0434088	7.78195496	7.76169051	7.7172819
85465	**EPT1**	0.00161	0.00032	0.07166	0.04276	-1.444	-2.579	-2.012	8.7685883	8.744747	8.9095922	8.35711314	8.18615112	8.34773936	8.7658713	8.699322	8.8539976	7.49461685	7.41474162	7.30918086
9331	**B4GALT6**	0.00035	0.00058	0.06562	0.04276	-1.719	-2.304	-2.011	8.8737758	9.1588506	9.2433925	8.60671707	8.31619865	8.33682946	9.4204637	9.460288	9.5640861	8.34814582	8.23245108	8.2519923
88455	**ANKRD13A**	0.00109	0.00026	0.06926	0.04276	-1.344	-2.607	-1.975	9.0917385	9.3384377	9.2953219	8.92429604	8.8599007	8.88624629	10.549071	10.736302	10.641882	9.24570531	9.24299089	9.29220928
9926	**LPGAT1**	0.00024	0.00108	0.06179	0.04276	-1.744	-2.150	-1.947	9.5903735	9.6892349	9.859977	9.03855082	8.91369056	8.96483333	8.5765853	8.5497591	8.7095286	7.71000562	7.41787788	7.39459405
9582	**APOBEC3B**	0.00051	0.00115	0.06562	0.04276	-1.641	-2.214	-1.927	7.4284248	7.8658312	7.7211098	7.15560869	7.13966032	6.94267181	8.7359234	8.5639751	9.0460884	7.58147817	7.52651978	7.79725097
23625	**FAM89B**	0.00076	0.00083	0.06883	0.04276	-1.639	-2.178	-1.908	10.066334	10.331978	10.127884	9.60672215	9.37987263	9.5644063	10.913588	10.84968	10.943138	9.79073756	9.6482518	9.89894296
93487	**MAPK1IP1L**	0.00061	0.00053	0.06571	0.04276	-1.390	-2.346	-1.868	10.576874	10.675327	10.643927	10.2160741	10.167223	10.1714595	10.124633	10.274753	10.186438	9.01610316	8.96977584	8.90901281
84247	**LDOC1L**	0.00043	0.00076	0.06562	0.04276	-1.579	-2.146	-1.862	8.9256663	9.02934	8.9145271	8.3546138	8.23141632	8.35388164	9.2282188	9.1919293	9.263601	8.07897427	8.09670116	8.20238049
56951	**C5orf15**	0.00097	0.00073	0.06888	0.04276	-1.405	-2.307	-1.856	9.1348288	9.1011502	9.1289935	8.71504733	8.59970279	8.55904782	10.018111	9.9198543	10.201327	8.77481591	8.77390131	8.97276055
151246	**SGOL2**	0.00151	0.00066	0.07079	0.04276	-1.390	-2.306	-1.848	8.2303945	8.2294854	8.3384442	7.89330922	7.76211739	7.77009251	7.8455595	7.9532648	8.0516162	6.84315317	6.63511362	6.75680866
56062	**KLHL4**	0.00648	0.00037	0.09679	0.04276	-1.323	-2.345	-1.834	8.1508742	8.2871546	8.4883354	8.10881109	7.89682563	7.94571146	10.004036	10.029543	10.047863	8.81245134	8.78073439	8.79859095
345778	**MTX3**	0.00046	0.00109	0.06562	0.04276	-1.546	-2.114	-1.830	8.7691455	8.8098541	8.8578778	8.23152473	8.09156226	8.29226812	8.4885268	8.2979862	8.4789104	7.47541308	7.30847166	7.2418655
55076	**TMEM45A**	0.00153	0.00103	0.07079	0.04276	-1.408	-2.242	-1.825	7.3261748	7.6350792	7.7711607	7.25836547	7.13285269	7.23778487	7.8707265	7.7500605	8.0796724	6.92901599	6.48837268	6.78881221
80219	**COQ10B**	0.00038	0.00150	0.06562	0.04276	-1.617	-1.978	-1.798	9.3573365	9.4956854	9.5863705	8.92833665	8.72367292	8.88999081	10.156523	10.132899	10.390842	9.27215192	9.23122924	9.22445263
3037	**HAS2**	0.00087	0.00106	0.06883	0.04276	-1.522	-2.073	-1.798	9.2720524	9.585015	9.7254466	9.12963677	8.94897378	9.06896225	9.0056138	8.8125625	8.9135018	7.84221202	7.92147941	7.81227094
84061	**MAGT1**	0.00048	0.00092	0.06562	0.04276	-1.459	-2.120	-1.790	8.2508761	8.5052824	8.4784429	8.01796087	7.92145544	7.90120941	8.7751912	8.8560834	8.9445105	7.69976369	7.81950524	7.80366014
348995	**NUP43**	0.00100	0.00079	0.06920	0.04276	-1.369	-2.207	-1.788	9.7397359	9.7463318	9.810127	9.35298781	9.31001139	9.31286814	9.7927993	9.6243496	9.8319552	8.62616777	8.51565124	8.68050161
91947	**ARRDC4**	0.00056	0.00117	0.06562	0.04276	-1.547	-2.001	-1.774	6.9035405	7.3871558	7.3211491	6.68496815	6.63607926	6.85429886	10.250461	10.218934	10.252135	9.17280257	9.29225324	9.25441349
25923	**ATL3**	0.00073	0.00111	0.06783	0.04276	-1.400	-2.130	-1.765	9.0660034	9.3476683	9.3326617	8.89823722	8.79463222	8.87203783	9.7364654	9.9762267	10.104287	8.81558764	8.84514277	8.88277037
10413	**YAP1**	0.00075	0.00094	0.06883	0.04276	-1.445	-2.084	-1.765	9.3916559	9.3303555	9.2408421	8.7449183	8.80720976	8.71108402	10.190925	10.315098	10.298026	9.17305231	9.24257753	9.21052867
55872	**PBK**	0.00338	0.00100	0.08213	0.04276	-1.302	-2.212	-1.757	8.6720372	9.0516428	9.0756159	8.80214763	8.64020411	8.60756624	8.6595668	8.5870228	8.9539319	7.50259912	7.50687206	7.75490381
84002	**B3GNT5**	0.00318	0.00110	0.08109	0.04276	-1.328	-2.178	-1.753	9.4549366	9.5104457	9.5890234	9.21766667	9.0167782	9.18771316	8.8608708	8.7275344	9.1463796	7.87171088	7.80856144	7.68485893
84912	**SLC35B4**	0.00036	0.00143	0.06562	0.04276	-1.531	-1.971	-1.751	8.9523506	9.8527764	9.8738423	9.27790146	9.15237723	9.31553463	8.9405956	8.8864907	8.98928	8.06496358	7.87735267	7.93926946
127933	**UHMK1**	0.00198	0.00078	0.07301	0.04276	-1.361	-2.134	-1.747	8.8923281	8.9124736	8.9865117	8.58810361	8.39947026	8.52828765	9.5431163	9.4651403	9.442488	8.42406785	8.34552829	8.39995091
56990	**CDC42SE2**	0.00149	0.00108	0.07079	0.04276	-1.418	-2.046	-1.732	9.4535385	9.3786325	9.5125082	8.94546848	8.85821639	9.01994477	9.5680404	9.5128062	9.5604617	8.60128646	8.49550289	8.44696022
8803	**SUCLA2**	0.00092	0.00129	0.06883	0.04276	-1.452	-2.011	-1.731	9.310234	9.7375302	9.6437577	9.22987878	9.0785165	9.15027008	9.2304173	9.3134587	9.4319664	8.37048787	8.25171441	8.3297843
6884	**TAF13**	0.00188	0.00136	0.07232	0.04276	-1.380	-2.078	-1.729	8.9082147	9.2402039	9.1512289	8.82390402	8.61962592	8.74933957	9.1284024	9.2657939	9.5020033	8.33213614	8.19893162	8.19958893
7706	**TRIM25**	0.00049	0.00206	0.06562	0.04289	-1.524	-1.872	-1.698	9.0105973	9.2840377	9.1834203	8.66689122	8.5583594	8.65185076	8.77973	8.7521333	8.9292175	7.84488273	7.86589993	8.03599783
9221	**NOLC1**	0.00273	0.00118	0.07878	0.04276	-1.316	-2.053	-1.684	9.1531142	9.4079728	9.3739168	9.09291147	8.90102744	8.99096642	8.9861161	9.1440936	9.1124855	8.08620605	7.93718953	8.10678767
197021	**LCTL**	0.00119	0.00129	0.06926	0.04276	-1.343	-2.008	-1.676	6.4860431	6.4860282	6.4936377	6.0878414	6.10410441	6.00007051	7.4652952	7.4627247	7.6133636	6.47907022	6.45532722	6.58908282
2925	**GRPR**	0.00114	0.00684	0.06926	0.04386	-1.748	-1.577	-1.663	7.7096661	7.5919371	7.9634969	6.88562768	7.01508066	7.01545044	7.2269273	7.12793	7.054371	6.52804281	6.59028893	6.31829938
4644	**MYO5A**	0.00039	0.00247	0.06562	0.04289	-1.550	-1.772	-1.661	9.5039371	9.7922985	9.8214133	9.25271725	9.06421861	9.20688141	9.863231	9.9444041	9.980076	9.14795623	9.08245249	9.08033497
157567	**ANKRD46**	0.00045	0.00242	0.06562	0.04289	-1.514	-1.803	-1.658	8.1930777	8.487629	8.4838155	7.94806346	7.78360527	7.93179911	8.0558338	7.938472	8.1420813	7.20435218	7.2001913	7.18014545
6385	**SDC4**	0.00081	0.00291	0.06883	0.04289	-1.483	-1.781	-1.632	8.1901956	8.2734993	8.1551224	7.66547829	7.7025256	7.57022817	11.382157	11.362968	11.463579	10.4273415	10.5790773	10.7040162
64924	**SLC30A5**	0.00292	0.00166	0.08009	0.04276	-1.352	-1.903	-1.628	8.290301	8.406542	8.4454913	8.11253177	7.84195347	8.01905534	8.2491017	8.1678337	8.3331153	7.37158218	7.29556045	7.29719071
79188	**TMEM43**	0.00211	0.00127	0.07358	0.04276	-1.314	-1.939	-1.627	9.7728368	9.77829	9.7357266	9.44099611	9.31654864	9.3301715	10.027031	10.022538	10.024859	9.05755336	9.05300959	9.09702504
199857	**ALG14**	0.00041	0.00491	0.06562	0.04328	-1.601	-1.642	-1.622	8.1301503	8.1677882	8.1198946	7.57044546	7.3263332	7.49736556	7.3132617	7.1647389	7.3601485	6.55121047	6.61830966	6.52129839
196527	**ANO6**	0.00281	0.00177	0.07913	0.04276	-1.362	-1.874	-1.618	9.5453517	9.700377	9.8090154	9.44084117	9.25305051	9.23318987	10.198185	10.304437	10.351324	9.36042154	9.37707943	9.39801115
2013	**EMP2**	0.00295	0.00160	0.08009	0.04276	-1.305	-1.890	-1.597	8.2418603	8.272072	8.3583653	7.99853878	7.91137224	7.88396458	7.8983724	7.8533761	7.9104371	7.01474278	6.96483773	6.92856934
1948	**EFNB2**	0.00202	0.00239	0.07301	0.04289	-1.395	-1.783	-1.589	9.693586	10.02835	9.9697433	9.66102389	9.38402879	9.51093689	10.153686	10.244266	10.265109	9.41973739	9.38501345	9.35616648
7852	**CXCR4**	0.00065	0.00603	0.06571	0.04337	-1.546	-1.630	-1.588	7.9291132	7.8328654	7.9045929	7.38072448	7.17094003	7.16775011	8.8103058	8.5771344	8.8456445	8.15117947	8.09506263	7.8723726
10162	**LPCAT3**	0.00018	0.00365	0.06179	0.04319	-1.475	-1.689	-1.582	10.343602	10.421968	10.42531	9.87759775	9.86215264	9.85041664	10.633544	10.708349	10.758526	9.89393787	9.95037043	9.98815002
89782	**LMLN**	0.00127	0.00310	0.06999	0.04300	-1.364	-1.758	-1.561	8.7840778	8.8900735	8.9575244	8.45444138	8.43459514	8.53822766	8.3018001	8.2036467	8.2368068	7.57792964	7.38237389	7.33948809
5834	**PYGB**	0.00165	0.00198	0.07166	0.04289	-1.310	-1.810	-1.560	10.724798	10.810042	10.786993	10.4441417	10.344662	10.4366309	11.738603	11.749702	11.748031	10.8518825	10.8930859	10.9230264
4734	**NEDD4**	0.00245	0.00417	0.07578	0.04319	-1.426	-1.682	-1.554	6.9731822	7.4611237	7.528352	7.19197653	6.84554116	6.91000695	8.3241417	8.4527426	8.5474706	7.73935984	7.64614139	7.68904205
23545	**ATP6V0A2**	0.00162	0.00298	0.07166	0.04289	-1.344	-1.747	-1.545	9.6172218	9.8578311	9.8802349	9.51923707	9.35090951	9.4585533	8.8841845	8.9707284	8.9648275	8.0350365	8.15468262	8.21583305
5481	**PPID**	0.00256	0.00635	0.07659	0.04360	-1.442	-1.646	-1.544	7.2500425	7.5208774	7.6438237	7.17179359	6.83839434	7.15323472	7.3138349	7.3301274	7.7555142	6.84055666	6.7707144	6.63108234
51633	**OTUD6B**	0.00457	0.00399	0.08767	0.04319	-1.309	-1.757	-1.533	9.4284657	9.605622	9.6874723	9.27953719	9.11819003	9.3774842	9.1731045	9.0395127	9.2811612	8.57855985	8.24473808	8.23171177
36	**ACADSB**	0.00058	0.00798	0.06562	0.04426	-1.555	-1.507	-1.531	7.008503	7.6665594	7.7553504	7.162985	6.95191333	7.10696909	7.5846162	7.5991657	7.6803445	7.09427135	7.03837532	6.95596884
6605	**SMARCE1**	0.00023	0.00725	0.06179	0.04408	-1.533	-1.529	-1.531	10.372795	10.773426	10.773656	10.2271388	10.1064704	10.1385254	10.493414	10.487286	10.633775	9.91999796	9.93771361	9.91936228
9488	**PIGB**	0.00094	0.00624	0.06888	0.04354	-1.437	-1.614	-1.525	7.378349	7.711361	7.6508042	7.22941558	7.06113861	7.18412365	8.2951877	8.5391756	8.6687381	7.76809647	7.8289337	7.83461195
167153	**PAPD4**	0.00118	0.00358	0.06926	0.04319	-1.327	-1.710	-1.519	9.3268	9.4583565	9.4838268	9.08773734	9.02023089	9.07995038	9.6932873	9.6629132	9.8605518	9.00360901	8.93475152	8.95645442
394	**ARHGAP5**	0.00220	0.00416	0.07405	0.04319	-1.338	-1.691	-1.515	8.2384438	8.6385584	8.6834438	8.34616188	8.21545301	8.16048867	8.3227245	8.3233208	8.4899901	7.56086725	7.56592555	7.7349781
51762	**RAB8B**	0.00186	0.00411	0.07232	0.04319	-1.319	-1.681	-1.500	9.5381304	9.810321	9.8264435	9.50173006	9.37134714	9.38466559	10.694504	10.795227	10.868443	9.97046974	10.0339675	10.1062692
100506658	**OCLN**	0.00052	0.00809	0.06562	0.04433	-1.488	-1.511	-1.500	7.7301334	7.8970887	7.8736762	7.36085807	7.20961638	7.36586452	10.540653	10.554273	10.515033	9.94759101	9.83156481	10.0429385
10495	**ENOX2**	0.00101	0.00582	0.06920	0.04337	-1.421	-1.572	-1.497	8.2143673	8.4320182	8.3986179	7.97618843	7.79769467	7.9502233	8.2084923	8.2729222	8.3220969	7.639312	7.58636863	7.61963969
10282	**BET1**	0.00112	0.00460	0.06926	0.04323	-1.318	-1.670	-1.494	7.8822672	7.9899181	7.9885401	7.6161747	7.55875024	7.59879572	8.3764044	8.1820274	8.3701588	7.66695676	7.52480711	7.51825742
6388	**SDF2**	0.00155	0.00465	0.07079	0.04323	-1.347	-1.640	-1.494	8.9550953	9.1101071	9.0692449	8.66334332	8.57977617	8.73555148	9.1849944	9.1107602	9.1605855	8.3682917	8.42520963	8.52193836
23767	**FLRT3**	0.00079	0.00665	0.06883	0.04379	-1.386	-1.596	-1.491	9.6687216	9.8649663	9.8541902	9.4502457	9.36464032	9.35138222	9.3785663	9.3896446	9.5662498	8.64091535	8.72982744	8.93904253
25937	**WWTR1**	0.00279	0.00422	0.07913	0.04319	-1.315	-1.666	-1.490	8.6439291	8.4702743	8.5034901	8.16602958	7.9828614	8.12740244	11.505706	11.504408	11.638802	10.8096669	10.7536711	10.876933
113201	**CASC4**	0.00175	0.00529	0.07180	0.04337	-1.339	-1.626	-1.482	10.09057	10.586896	10.592008	10.2690035	10.1188388	10.1177954	10.60797	10.758757	10.831813	9.97113497	10.0613114	10.0629457
29982	**NRBF2**	0.00354	0.00408	0.08287	0.04319	-1.303	-1.661	-1.482	8.3957115	8.5334034	8.420791	8.15932058	8.07668824	8.05045799	8.6545258	8.5570882	8.6566313	7.92694771	7.83999405	7.90458088
79693	**YRDC**	0.00272	0.00514	0.07878	0.04334	-1.341	-1.608	-1.474	9.4903434	9.5385494	9.4334455	9.11475222	8.96282321	9.11020785	8.8966602	8.8428744	8.9386534	8.21269972	8.15263996	8.2578584
138151	**NACC2**	0.00098	0.00801	0.06888	0.04426	-1.436	-1.506	-1.471	9.4610497	9.5629954	9.4469986	8.96926838	8.95173942	9.02744675	9.558457	9.5835633	9.6070022	8.90323811	9.01415689	9.06017542
63877	**FAM204A**	0.00163	0.00767	0.07166	0.04410	-1.409	-1.524	-1.466	8.0738516	8.4551445	8.3521138	7.92315064	7.79007909	8.01445353	8.3873991	8.517363	8.5808799	7.9346449	7.88500694	7.84276859
53344	**CHIC1**	0.00332	0.00444	0.08203	0.04323	-1.304	-1.615	-1.460	8.0210211	8.2926947	8.1690942	7.84586328	7.84081825	7.85809542	8.7831226	8.8011779	8.7755021	8.09148107	8.08839861	8.10443447
8795	**TNFRSF10B**	0.00240	0.00655	0.07498	0.04369	-1.376	-1.537	-1.457	9.626713	9.8224705	9.7300569	9.42995629	9.18609701	9.32989664	10.389213	10.390515	10.44708	9.76630355	9.80014037	9.80068794
439949	**LOC439949**	0.00442	0.00727	0.08700	0.04408	-1.350	-1.553	-1.452	6.8404969	7.2425918	7.1381615	6.93836611	6.7281114	6.60436489	6.431959	6.2341532	6.2602647	5.77641938	5.59244517	5.65347763
10123	**ARL4C**	0.00288	0.00819	0.08009	0.04440	-1.371	-1.501	-1.436	9.3399578	9.3253554	9.3671136	9.06934782	8.81274343	8.7898715	10.872076	10.736221	10.857059	10.1891029	10.2290253	10.2880389
9303	**SNORD25**	0.00317	0.01236	0.08109	0.04548	-1.327	-1.509	-1.418	7.47757	8.1993953	8.2557619	7.84591916	7.69296449	7.92072993	6.1016079	6.4620837	6.4583613	5.73548083	6.10682025	5.40033224

Supplemental File 1

TRIO enhancer site 1:
>ref|NC_000005.9|:14265609-14266200 Homo sapiens chromosome 5, GRCh37.p13 Primary Assembly
ATTTTTAACATAATATGCTATATAAAAGCTTTTGCTCATTGTGAAAATATTTCTAGGGTGCTTTTTATTA
TAAATAAATCGGAGAGGGGCTTGTTGTTCCTGGACTATTTGTTATTGTTGTCTTAAATTTAATGCCACAG
AAGTGACCAAGTGAGCGAGCATTCTGGTCATCAGTGACTAGCTTAGAAAGGTATTTGTCTCAGTGCAGC
A
TCTCTTTGCAGGGTTTCAGGATGACAGATGAGTAATTGATTTGGTCAGTGGGGTATTAGATAGTTGAAG
T
TCCAGACTGTTGATTCAGTGGAAAGATATTTCACTGTTGAATGATAACTTGCAGAATATTCCTTGTTAGG
GCAGAGAGATTAATTTTCCATCTTATGAATATAAATCATACTATGATTGAAGGCTAAACTACCTAACATT
TCACAAAACAAAAATAGAAAGTACTAA**GGAAT**TTTGGGGTTCTTGGGTAGGGGTTCAGGGTCAAGATG
CT
GTGGAAATATATTTATTTGTTGGTTGAGTTCATTGATTGATTGATTCATTCATTCATCCATCCATCTTTC
TTTTCCTTTTCTTTTTTTTTGGAGACAGAGTC

TRIO enhancer site 2:
>ref|NC_000005.9|:14268225-14268865 Homo sapiens chromosome 5, GRCh37.p13 Primary Assembly
CAATAGGTTAACATAACTACAGCTCTGAGAAATGAAAGGTCACATGAGAGGGAGTCCATGACTCATAGT
G
GGACATAGGAAATTGAGACATTTATTGAGTTCTGTTTTCTGCACCCTACTTTCAAATCAATATGCTATTC
CTCCTGTGAGATGGCC**TGGAAT**ATGACAGCCCTCACTTCCATCTTCCACTCCATTGTGGGTATCAGTAAA
TAGGTGTGATTCAGAAAGTTAGCAGTAATTGTCAAAAATGCTT**TGGAATG**ATGACTCAGGTCACACAGC
T
CTCTATTGGGATATCAGCATTGGGATCAGGACATTCTTCTTGGTGATGGCTGTGTGACTCACAAGTGGG
G
CTCTGTCTCTCTAGTGTCGGAGCCAGGGCTGCAGTTGGTGGTGACATTTCTCTGTCTGAGGGCTTTGCTG
TGTCCTGGCCATGACGTGTGGGTGTGGACGTG**GGAATG**GTAGGAAGCATGCGTTTTCTCATCTGAGCA
AC
AGAAGGG**GGAAT**CCTCCCTTCAAACAGATAGGGATCTACAGCTGTCGCTTACTCCTTCCTAGTATTTGTC
TATCATGTGATCTGTCCCCTCCTCATTTGCCTGGGTCCCATAGCACTTCGTGCTGCCTATGAACATTTCA
TAATCTGCCTT

Motifs:
TEAD TGGAAT (Fig 2 from https://www.cell.com/cell-reports/pdf/S2211-1247(19)31169-6.pdf)
TEAD4 ACATTC (JASPAR2024: https://jaspar.elixir.no/matrix/MA0809.3/)
JUNB TGACTCA (JASPAR2024: https://jaspar.elixir.no/matrix/MA0490.1/)