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Brachyury-YAP regulatory axis drives stemness and growth in cancer

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SUMMARY

Molecular factors that define stem cell identity have recently emerged as oncogenic drivers. For instance, brachyury, a key developmental transcriptional factor, is also implicated in carcinogenesis, most notably of chordoma, through mechanisms that remain elusive. Here, we show that brachyury is a crucial regulator of stemness in chordoma and in more common aggressive cancers. Furthermore, this effect of brachyury is mediated by control of synthesis and stability of Yes-associated protein (YAP), a key regulator of tissue growth and homeostasis, providing an unexpected mechanism of control of YAP expression. We further demonstrate that the Brachyury-YAP regulatory pathway is associated with tumor aggressiveness. These results

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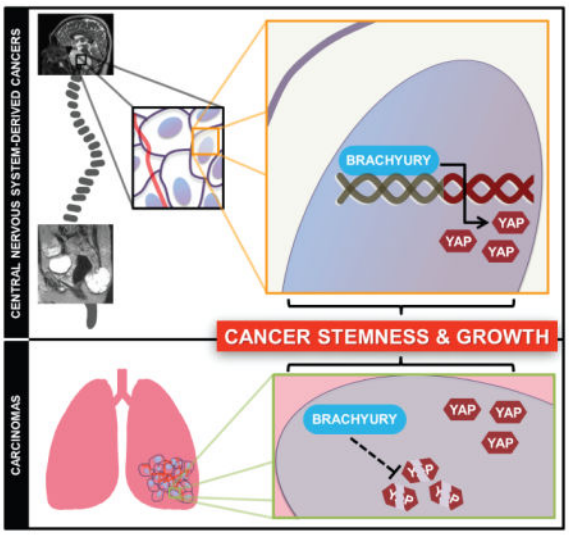
AUTHOR CONTRIBUTIONS

All authors contributed extensively to the work presented in this paper. SRS and AM conceived the initial study. SRS designed the experiments and overall study direction. SRS, JMD, NDT, AM, JCM, SG, and DHH conducted the experiments and performed data analyses. SRS wrote the manuscript. All authors edited the manuscript. CP, AL, and AQH supervised the work.

elucidate a mechanism of controlling both tumor stemness and aggressiveness through regulatory coupling of two developmental factors.

eTOC BLURB

Malignant neoplasms exhibit uninhibited and dysregulated growth coupled with acquisition of stem-like properties that are integral to the development and progression of disease. Shah et al demonstrate a critical role of brachyury in regulating stemness and growth by activating YAP through direct transcriptional and post-transcriptional mechanisms in various cancers.



INTRODUCTION

Accumulating evidence suggests malignant neoplasms contain a distinct subset of cells that hijack stem cell transcriptional programs to attain tumor initiating and propagating capability (Ben-Porath et al., 2008; Kreso and Dick, 2014; Mani et al., 2008; Reya et al., 2001). These tumor initiating cells (TIC) have the capacity to self-renew, develop into all tumor cell subtypes, and seed new lesions. In addition, numerous studies have shown that these TICs are particularly chemo- and radio-resistant, providing a source for tumor recurrence after therapy (Frank et al., 2010; Holohan et al., 2013; Hsu et al., 2012). Together, these findings suggest that TICs are necessary and sufficient to sustain prolonged oncogenic growth and feed the progression of tumor malignancy (Beck and Blanpain, 2013). However, the regulatory pathways that confer the TIC phenotype remain poorly understood.

Given that TIC exhibit properties similar to normal stem cells, transcriptional programs coordinating early embryogenesis have recently emerged as drivers of oncogenesis and potential therapeutic targets (Ben-Porath et al., 2008; Kim and Orkin, 2011; Wong et al., 2008). Brachyury, a core T-box transcription factor, plays a vital role during development in early embryonic gastrulation events and notochord formation (Edwards et al., 1996; Herrmann et al., 1990; Herrmann and Lehrach, 1988; Kavka and Green, 1997; Kispert and Herrmann, 1993; Morrison et al., 1996; Showell et al., 2004; Smith, 1997; Smith et al., 1997; Wilkinson et al., 1990). Post-developmentally, brachyury is expressed in the testes and

some thyroid tissues, but is undetectable in all other non-neoplastic adult tissues (Edwards et al., 1996; Hamilton et al., 2015). Interestingly, recent studies have reported the expression of brachyury in several epithelial cancers where it promotes growth, confers resistance to chemo- and radiotherapy, and drives epithelial-to-mesenchymal transition (EMT) (Cho et al., 2010; Fernando et al., 2010; Haro et al., 2013; Huang et al., 2013; Imajyo et al., 2012; Jezkova et al., 2016; Kobayashi et al., 2014; Larocca et al., 2013; Li et al., 2016; Miettinen et al., 2015; Palena et al., 2014; Park et al., 2008; Pinto et al., 2015; Pinto et al., 2014; Pires and Aaronson, 2014; Roselli et al., 2012; Sarkar et al., 2012; Shao et al., 2015; Shimoda et al., 2012; Vujovic et al., 2006; Xu et al., 2015; Yoshihama et al., 2016); however, the mechanistic details of how brachyury mediates these features of tumor progression have not been fully elucidated. Furthermore, the lack of brachyury expression in most adult non-neoplastic tissues and exclusive tumor-specific expression underscores its value as a potential diagnostic and therapeutic target in cancer. These observations provide a strong impetus to better understand the transcriptional network driven by brachyury in cancer.

Chordomas are rare tumors of the osseous spine and skull base that may serve as an ideal model system to understand brachyury-driven networks in cancer (Sarabia-Estrada et al., 2017). These tumors are believed to arise from remnants of the notochord, a mesoderm-derived embryonic structure that is critical for neurulation and embryonic tissue organization (Chugh et al., 2007). Interestingly, familial cases of these neoplasms contain a genomic amplification of the locus harboring brachyury, and it is nearly ubiquitously expressed in both familial and sporadic chordomas (Barresi et al., 2014; Hsu et al., 2011; Hu et al., 2014; Jambhekar et al., 2010; Mathios et al., 2015; Miettinen et al., 2015; Nelson et al., 2012; Oakley et al., 2008; Presneau et al., 2011; Yang et al., 2009). However, our understanding of the role played by brachyury in this neoplasm is limited.

Three lines of evidence suggest that chordomas may harbor a cancer stem cell population that drives their progression. First, cancer stem cells are known to exhibit radio-resistance due to their enhanced DNA-repair capacity and reactive oxygen species (ROS) defenses, and their self-renewal potential (Bao et al., 2006; Rycaj and Tang, 2014), and chordomas demonstrate a remarkable amount of radio- and chemo-therapy resistance clinically (Walcott et al., 2012). Second, chordomas exhibit a predilection to seed new tumors in patients via iatrogenic spread of cells along the surgical route particularly when the tumor capsule is violated, a hallmark of TICs (Arnautovic and Al-Mefty, 2001; Dieter et al., 2011). Lastly, although not a prerequisite, it has been proposed that cancer stem cells may arise from transformed stem cell niches (Kreso and Dick, 2014; Sanai et al., 2005) and chordomas arise from transformed primordial remnants of the notochord (Salisbury et al., 1993). Furthermore, brachyury is predominantly a transcriptional regulator of early mesoderm development (Kispert et al., 1995a) and has been shown to be highly expressed in chordoma due to a gene duplication event (Kelley et al., 2014) and more recently has been implicated in orchestrating EMT in various metastatic carcinoma cell lines (Roselli et al., 2012). EMT is crucial for the acquisition of stem-like properties and malignant transformation (Caixeiro et al., 2014; Mani et al., 2008; Roselli et al., 2012). In summary, these considerations led us to explore the hypothesis that chordomas harbor a stem-like population of cells which is governed by brachyury signaling.

In this report, we demonstrate that brachyury drives cell cycle progression, stemness, and tumor growth in chordoma through direct transcriptional activation of Yes-associated protein (YAP), an effector of the Hippo pathway and a master regulator of organ development (Dong et al., 2007; Pan, 2010). By binding to the proximal region of the YAP promoter, brachyury was found to transactivate YAP-dependent signaling in chordoma. Furthermore, we find that brachyury expression is elevated in glioblastomas (GBM), the most common and aggressive type of primary brain cancer (Chaichana et al., 2013a; Chaichana et al., 2013b; Smith et al., 2015; Stupp et al., 2005), and in a majority of brain metastases derived from various carcinomas. Surprisingly, we also observed that brachyury enhanced YAP signaling in lung cancer cells by increasing protein stability instead of transcriptional activation, suggesting a dual role of brachyury in YAP expression. In addition, we find that this regulatory axis correlates with differentiation status of lung carcinomas, an indicator of tumor pluripotency and aggressiveness. Overall, our work provides molecular insights into brachyury-driven gene regulation in cancer and identifies it as a positive regulator of YAP. Moreover, our data provides important insights into how developmental programs are co-opted by cancer.

RESULTS

Brachyury regulates cancer stemness and tumor initiating capacity in chordoma

To delineate brachyury-driven mechanisms of cancer stemness and growth, we began our studies by utilizing patient-derived primary and recurrent chordoma cells due to their high and stable endogenous expression of brachyury. Using our previously established patient-derived primary sacral chordoma cell line JHC7 (Hsu et al., 2011), we sought to uncover the transcriptional network under the control of brachyury in cancer. To this end, we first generated lentiviral-based shRNA constructs that specifically target brachyury expression (shT) as well as a control non-targeting vector (shCtrl) (Fig. S1a). Next, using a genome-wide expression microarray analysis, we profiled RNA samples from JHC7 cells transduced with shT and shCtrl, in triplicate. Genes with greater than 2-fold decrease in RNA abundance relative to shCtrl were considered to be potential targets of brachyury and together defined the “T gene signature” (Table S1). We then performed functional annotation of this gene set using Gene Ontology (GO) to identify biological processes regulated by brachyury; significant enrichment of cell cycle/proliferation (44.2%) and organ development/homeostasis (16.2%) categorized biological functions was associated with brachyury positive gene targets (Fig. 1a). Consistent with this data, we observed a significant decrease in mRNA levels of cell cycle regulator genes following brachyury knockdown in two independent chordoma lines (JHC7 and UCH1, a recurrent chordoma cell line (Scheil et al., 2001)) (Fig. 1b and Fig. S1b). Also in agreement with these findings, we observed a decrease in proliferation in JHC7- and UCH2-shT cells (Fig. S1c, d) and augmented G_1 phase accumulation in JHC7-shT cells (Fig. 1c). These results were consistent with a previous report (Presneau et al., 2011) demonstrating reduced proliferation of UCH1 cells silenced for brachyury expression. Taken together, these results demonstrate that brachyury regulates cell cycle progression and proliferation in chordoma.

To investigate the possibility that chordomas may harbor a cancer stem cell population that drives their progression, JHC7 cells were evaluated for expression of stem cell markers

frequently exhibited by multipotent mesenchymal stem cells. JHC7 cells and xenografts derived from this line exhibited strong expression of vimentin, CD90, CD105, Oct4 and nestin (Fig. S1e, f, g). *In vitro*, these cells demonstrated transdifferentiation capacity by readily differentiating into oil red-positive adipocytes and GFAP- and Tuj1-positive neuroglial cells (Fig. S1h, i), a quality that often distinguishes multipotent mesenchymal stem cells. Furthermore, a subpopulation of chordoma cells exhibited increased aldehyde dehydrogenase activity (ALDH), a functional marker of cancer stem cells, and this population was significantly reduced when the cells were grown in adherent conditions that promote differentiation (Fig. S2a.) Chordoma xenografts also exhibited the ability to undergo serial tumor formation in mice, which is a hallmark of cancer stem cells (Fig. S2b). Serial passaging of chordoma xenografts enriched for the cancer stem cell population as determined by side population analysis (Fig. S2c). Finally, brachyury knockdown resulted in a significant drop in the enriched cancer stem cell population, with a decrease in side population frequency (28.95% vs. 4.31%; Fig. S2d). Collectively, these findings suggest that chordomas harbor a putative stem-like cell population.

Given the role of brachyury in stem cell identity and fate determination during development, we explored whether brachyury modulates any of the core stem-cell regulatory networks. Using the T signature for gene set enrichment analysis (Irizarry et al., 2009), we found a significant enrichment of signatures specific for embryonic stem cells (ES1, ES2), Nanog, Oct4, Sox2, NOS (Nanog, Oct4, Sox2) targets, and the polycomb repressive complex-2 (PRC2) (Fig. 1d). Moreover, we observed a significant decrease in the mRNA levels of stemness-related genes including ABCG2, OCT4, SOX2, and ABCB1 following brachyury knockdown in JHC7 cells (Fig. S2e, f). Based on this data, we investigated the functional relevance of brachyury in self-renewal capacity through an extreme limiting dilution assay (ELDA) using previously described methods (Hu and Smyth, 2009). Interestingly, we found a significant reduction in self-renewing capacity and stem cell frequency in shT cells as compared to the shCtrl JHC7 cells (Fig. 1e). To determine whether brachyury, through its control of stemness, also affects tumor-initiating capacity *in vivo*, we used a murine subcutaneous xenograft model of chordoma. We subcutaneously injected JHC7 cells at limiting dilutions (10^3–10^6 cells) into immunocompromised mice and quantified tumor-initiation frequency 12 months following tumor implantation. Consistent with brachyury's role in modulation of stemness properties *in vitro*, we observed a significant decrease in tumor-initiating capacity with decreasing cell dilutions implanted, demonstrating that implantation of 10^6 JHC7 cells is necessary to successfully form tumors with 100% frequency (Fig. 1f). Based on this data, we investigated whether brachyury regulates tumor-initiating capacity *in vivo* by injecting 10^6 shCtrl or shT JHC7 cells and monitoring tumor formation using the same protocol. While none of the JHC7 shT-injected mice developed tumors (0/6), nearly all (5/6) of the JHC7 shCtrl-injected mice formed tumors (Fig. 1g). It is important to point out that there was significant size variability among the tumors in the JHC7 shCtrl group; it remains to be investigated whether these differences correlated with the level of brachyury expression in the various xenografts. Overall, these results demonstrate that chordomas harbor a putative cancer stem cell population, and that brachyury regulates cancer stemness and tumor-initiating capacity.

Brachyury regulates proliferation and stemness in glioblastoma

Given our results showing brachyury as a potent regulator of chordoma cell proliferation, stemness, and tumor-initiating capacity, we inquired whether brachyury plays a role in other central nervous system (CNS) derived cancers. While chordomas are characteristically slow-growing tumors with a median patient survival of 7 years (McMaster et al., 2001), GBM are the most common and aggressive type of primary brain cancer, and have a dismal patient prognosis with an overall median survival of only 14.6 months (Chaichana et al., 2013a; Chaichana et al., 2013b; Smith et al., 2015; Stupp et al., 2005). Thus, it was of interest to investigate the potential role of brachyury in driving stemness and growth in this highly proliferative and aggressive CNS-derived cancer. We found that in a fraction of intraoperatively obtained primary GBM tissues from patients, the brachyury protein expression was significantly elevated compared to non-cancer cortex tissue (Fig. 2a, b). Next, we surveyed RNA-sequencing profiles of patient-derived GBM tissues from The Cancer Genome Atlas (TCGA) to determine whether our observations using intraoperatively-obtained patient tissues were consistent with a larger cohort of clinical samples. As shown in Fig. 2c, 24% of GBM patient tissues exhibited detectable brachyury mRNA (T) in the TCGA dataset, suggesting that brachyury may play a role in a subset of GBM. Given these observations and the recent stratification of GBM to account for its molecular heterogeneity (Brennan et al., 2013; Verhaak et al., 2010), we examined the prognostic value of T mRNA and whether its expression is associated with one or more genetic subclasses of GBM. While T-transcript expression does not predict progression-free survival (Fig. S3a), we found a significant over-representation of T-positive patients among the Classical GBM compared with the other subclasses (Fig. 2d). These observations warrant further studies to evaluate the potential clinical value of T expression in GBM.

The expression of brachyury was then evaluated in a panel of patient-derived primary cultures of GBM cells (Fig. S3b), where it was found to be upregulated in 2/4 cell lines. Using the brachyury-positive cell lines, GBM1A and GBM1049, we then sought to determine whether brachyury plays a role in glioblastoma proliferation and stemness. With both cell lines, we found a significant decrease in proliferation in shT- compared to shCtrl-GBM cells (Fig. 2e–g). Next, we inquired whether brachyury expression was enhanced in stem cell-enriched GBM spheroids compared to adherent cultures. Our results demonstrated that brachyury was significantly higher in GBM1A spheres than in adherent cultures, which was accompanied by elevated nestin expression, a marker of GBM stemness, and reduced expression of the differentiation markers GFAP and TUJ1 in spheroid vs. adherent cultures (Fig. S3c). Congruent with these results, nestin expression was significantly decreased after brachyury-knockdown in GBM1A and GBM1049 cells (Fig. S3d, e). Moreover, a significant reduction in self-renewing capacity and in stem cell frequency was observed in both cell lines following knockdown of brachyury (shT) versus shCtrl cells using ELDA (Fig. 2h, i). In addition, shT GBM1A cells formed significantly smaller spheroids than the corresponding shCtrl GBM1A cells (Fig. S3f). Taken together, our studies demonstrate that a subset of GBMs express brachyury and that it promotes tumor cell proliferation and stemness in this malignancy.

Brachyury regulates YAP in CNS-derived cancers

In cancer, transcription factors interact with oncogenic signaling pathways to synergistically drive disease progression. Thus, we sought to identify effectors of brachyury-driven regulatory networks in cancer. Using the T gene signature, we performed gene set enrichment analysis to check for higher-than-randomly-expected representation of individual signal transduction pathways. Notably, only a small subset of pro-oncogenic and pro-growth signatures were significantly associated with the T gene signature, of which the YAP/TAZ signature (canonically associated with activity of the Hippo pathway) registered as the strongest association (Fig. 3a). YAP, a transcriptional co-activator, is implicated in the regulation of the organ size during development, and has recently been reported to regulate cancer stemness and growth in numerous studies. However, the transcriptional regulators and drivers of YAP expression are poorly defined. Thus, we sought to identify a potential regulatory connection between YAP and brachyury. Using JHC7, UCH1, and UCH2 chordoma cell lines (Bruderlein et al., 2010), we observed a dramatic decrease in the expression of YAP protein and mRNA following brachyury silencing (Fig. 3b, c and Fig. S4a, b). Furthermore, the expression of MCL-1, a well-known YAP target, was also reduced in shT- compared to shCtrl-cells (Fig. 3d). Likewise, expression of Cyclin D1, CTGF, CYR61, ANKRD1 and c-MYC, indicators of YAP's co-transcriptional activity, was significantly decreased in shT JHC7 cells while no significant change in AXL-1 was observed (Fig. S4c). Given these findings, we then evaluated the expression of brachyury and YAP in intraoperatively obtained primary chordoma tissues. As shown in Fig. 3e, f, the majority of chordoma tissues expressed high levels of both brachyury and YAP proteins, and their expression was restricted to tumor cells (Fig. 3e and Fig. S5). Moreover, a positive correlation was found between brachyury and YAP protein levels in these chordoma tissues (Fig. 3g), consistent with our findings in the chordoma cell lines (Fig. S4d). The expression of T and YAP mRNA levels also positively correlated in a subset of chordoma tissues analyzed (Fig. 3h). In addition, both T and YAP mRNA levels positively correlated with the expression of downstream targets of YAP such as MCL-1, Cyclin D1, and CTGF (Fig S6a–c, e–g). However, neither T nor YAP mRNA levels correlated with AXL-1 expression (Fig S6d, h). Altogether, these results demonstrate that brachyury regulates the expression and activity of YAP in chordoma.

Since our previous work and that of others have demonstrated that GBMs have an elevated expression of YAP (Artinian et al., 2015; Orr et al., 2011; Xu et al., 2010), we also investigated whether a brachyury-driven mechanism of YAP regulation might be co-opted in the primary cultures of GBM cells. A significant decrease in YAP protein and mRNA expression were observed in primary GBM cells silenced for brachyury expression (Fig. S3g–k). Collectively, our data suggest that brachyury may regulate YAP expression and activity in CNS-derived tumors.

Brachyury directly activates the transcription of YAP

Next, we sought to determine the molecular mechanism of the brachyury-YAP regulatory network. Analysis of chordoma cells silenced for the expression of brachyury demonstrated reduced levels of pLATS1 expression compared to the corresponding shCtrl cells, thus ruling out the role of canonical Hippo signaling pathway in the control of YAP (Fig. 4a). Given

brachyury's function as a transcription factor, and our findings that brachyury regulates YAP transcript levels, we posited that brachyury may modulate YAP through direct transcriptional regulation. For this reason, a publicly available chromatin immunoprecipitation (ChIP) sequencing dataset of brachyury in UCH1 cells was analyzed. Interestingly, brachyury was found to be associated with the proximal promoter region of YAP, corresponding to a region spanning −1 kb directly upstream of the transcriptional start site (TSS) (Fig. 4b). This binding pattern was then validated by performing a ChIP-PCR querying across the −1.5 kb region upstream from the TSS of the YAP promoter in JHC7 cells. Utilizing two independent antibodies specific for epitopes on the C- and N-terminus of brachyury, a significant enrichment of brachyury binding was observed in the −1 kb region upstream of the TTS of the YAP promoter, suggesting a conserved chromatin occupancy pattern in JHC7 cells (Fig. 4c). To evaluate the functional consequence of this binding, we conducted a YAP gene promoter luciferase assay using the −105bp and −608bp regions upstream of the TSS in JHC7 cells +/− siRNA-mediated knockdown of brachyury. As expected, a significantly enhanced signal was observed with the luciferase reporter vector containing the longer segment of the YAP promoter region (−608bp to TSS), compared to the shorter promoter sequence (−105bp to TSS) (Fig. 4d, e). Furthermore, the luminescence signal of the −608bp YAP-luciferase reporter was significantly decreased after brachyury knockdown (siT, Fig. 4e). These findings led us to investigate whether YAP's paralog, TAZ, is similarly regulated by brachyury. While TAZ expression was lower in JHC7 cells silenced for brachyury expression (Fig. S4e), we observed no significant enrichment of brachyury in the promoter region of TAZ (Fig. S4f). Taken together, these results demonstrate that brachyury directly activates the transcription of the YAP gene in chordoma cells.

Brachyury regulates stemness and proliferation through YAP

To further investigate whether brachyury potentiates cancer stemness and proliferation in chordoma through the activation of YAP, lentiviral-based shRNA constructs that specifically target YAP expression were used (Fig. S6i) with JHC7 cells. A G1 phase accumulation was observed in shYAP JHC7 versus shCtrl cells (Fig. 5a), which phenocopied the effect of brachyury knockdown (Fig. 1c). Similarly, YAP knockdown significantly reduced the self-renewing capacity and stem cell frequency based on ELDA (Fig. 5b), and decreased the side population and expression of the stemness marker ABCG2 (Fig. S6j,k) in JHC7 cells. In addition, the proliferation of shYAP JHC7 cells was markedly decreased compared to shCtrl cells (Fig. 5c, Fig. S6i), indicating that YAP is a critical regulator of stemness and growth in chordoma.

Next, we inquired whether YAP is sufficient to rescue the self-renewal and proliferative capacity of chordoma cells silenced for brachyury expression. To this end, we stably overexpressed a constitutively hyperactive form of YAP, bearing mutations at S127/128/131/381A (YAP-OE), or an empty control vector (CONT), in shT and shCtrl JHC7 cells. YAP overexpression rescued the expression of various critical cell cycle regulators in shT JHC7 cells (Fig. 5d, e, and Fig. S6m). Moreover, reintroduction of YAP reversed the cell cycle arrest observed in shT cells (shT YAP-OE cells), restoring the G_1 phase levels to levels similar to those of shCtrl CONT cells (Fig. 5f). More importantly, overexpression of YAP was also sufficient to rescue self-renewal capacity in shT JHC7 cells

based on ELDA (Fig. 5g). We also observed higher proliferation in shCtrl YAP-OE cells compared to all of the other groups tested relative percent decrease in proliferative capacity in shT YAP-OE versus shCtrl YAP-OE cells was not as pronounced as that seen in shT CONT than shCtrl CONT cells (Fig. 5h). Thus, these experiments demonstrated that YAP is an effector of brachyury-mediated growth and stemness in cancer.

Brachyury-YAP regulatory axis is evident in non-CNS-derived carcinomas

Brachyury has recently been implicated in potentiating tumor stemness and growth in epithelial cancers (Cho et al., 2010; Fernando et al., 2010; Haro et al., 2013; Hung et al., 2013; Imajyo et al., 2012; Jezkova et al., 2016; Kobayashi et al., 2014; Larocca et al., 2013; Li et al., 2016; Miettinen et al., 2015; Palena et al., 2014; Park et al., 2008; Pinto et al., 2015; Pinto et al., 2014; Pires and Aaronson, 2014; Roselli et al., 2012; Sarkar et al., 2012; Shao et al., 2015; Shimoda et al., 2012; Vujovic et al., 2006; Xu et al., 2015; Yoshihama et al., 2016); however, the molecular details are poorly defined and it is not known whether the brachyury-YAP signaling is also co-opted by non-CNS-derived carcinomas. Hence, we first examined the expression of brachyury and YAP in a panel of patient-derived primary metastases to the brain from different types of carcinomas. As shown in Fig. S7a, elevated expression of brachyury and YAP were observed in multiple cases of metastases to the brain, as compared to non-cancer cortex tissues (Fig. 2a). The analysis of expression levels in these clinical specimens also revealed a significant positive association between brachyury and YAP proteins (Fig. S8a). In particular, we found a significant positive correlation between these two proteins in brain metastatic lesions originating from lung carcinoma (Fig. S8b, c), which is one of the most common sources of brain metastases encountered clinically. Congruent with the results at the protein level, elevated expression of the T gene signature was also found to be associated with higher expression of genes regulated by YAP (termed YAP gene signature) in the TCGA lung cancer database (Fig. S7b), although a direct correlation between T and YAP mRNA was not observed in these samples.

Given the above findings, we next explored whether brachyury regulates YAP in lung carcinoma cells by establishing single clonal populations of H460 cells with different levels of brachyury (high, intermediate, and low T-expressing clones) generated via the CRISPR/Cas9 methodology, and subsequently evaluating YAP expression. As shown in Fig. S7c, the levels of YAP protein directly correlated with those of brachyury in the H460 cells, and the expression of TAZ protein (Fig. S8d) and various downstream target genes of YAP (Fig. S8f) were all decreased in the brachyury low clones. Like the results with chordoma cell lines, T-low H460 cells exhibited lower levels of pLATS1 and pMST1/2 expression than T-high cells, thus ruling out the role of canonical Hippo signaling pathway in the modulation of YAP in this system (Fig. S8e). Interestingly, no significant changes were observed in YAP mRNA levels among these clones (Fig. S7d), suggesting that unlike CNS-derived cancers, brachyury might regulate YAP through a non-transcriptional mechanism in lung carcinoma cells. In agreement with this idea, the analysis of YAP protein stability in the high and intermediate T-expressing H460 clones treated with cycloheximide for blockade of protein translation demonstrated a pronounced reduction in the apparent half-life of YAP protein in the intermediate clone (~43min) versus the high clone (>4hr) (Fig. S7e). To confirm this result, the high and intermediate T-expressing H460 clones were treated with the

proteasomal inhibitor MG132, which increased YAP protein levels in the intermediate but not the high T-expressing clone (Fig. S7f). A similar experiment conducted with JHC7 chordoma cells treated with MG132 (Fig. S4g), however, showed no changes in YAP expression, thus ruling out protein stabilization as a mechanism of YAP control in chordoma cells.

To ascertain the role of brachyury in the control of YAP expression in H460 cells, a rescue experiment was conducted where the T-low expressing clone was transfected with a vector encoding for brachyury (pT) vs. an empty control (pCMV). The results are shown in Fig. S8g, h, demonstrating that re-introduction of brachyury in the low-T H460 clone is sufficient to reconstitute the expression of YAP and TAZ. Collectively, these experiments demonstrate that brachyury is necessary and sufficient to regulate YAP expression in these cancer cells.

Given the above results, the lung adenocarcinoma TCGA database was interrogated to elucidate a potential association between brachyury and stemness-related gene signatures (ES1, ES2, NOS) in primary lung cancer tissues. Elevated expression of the T gene signature in lung tumor samples was associated with higher expression of gene signatures registering the presence of embryonic stem cells, e.g., Nanog, Oct4, and Sox2 (Fig. S7g). Validating these correlations, we observed a significant decrease in *in vitro* anchorage-independent colony formation, a measure of cancer stemness, in shT H460 cells versus shCtrl cells (Fig. S7h) as well as in shYAP vs. shCtrl H460 cells (Fig. S8i). In agreement with these results, H460 clones with various levels of brachyury also demonstrated a positive association between brachyury levels and the expression of the stemness markers, Oct4 and ABCB1 (Fig. S8j).

Since lung adenocarcinomas are classified by differentiation status as a correlate of aggressiveness (Sun et al., 2006), we hypothesized that the transcriptional activity of brachyury would be higher in the poorly differentiated subtype. As shown in Fig. S7i, patient samples corresponding to poorly differentiated lung cancer had a significantly higher T signature expression than those with a well-differentiated subtype. Since tumor-initiating cells are thought to be enriched in the poorly differentiated and more aggressive subtypes of cancer, this clinical data lends support to the role of brachyury in governing cancer stemness. Thus, the brachyury-YAP regulatory network in lung cancer may serve as a potent driver of aggressive behavior.

To expand our observations to other non-CNS carcinoma models, lung H1299 cells were transfected to overexpress brachyury. In this model system where basal YAP expression is endogenously high, however, the addition of brachyury had no effect on the levels of YAP (Fig. S8k). This result suggested that expression of YAP expression may be independent of brachyury in certain brachyury-negative cells. However, other model systems, including pancreatic PANC-1, prostate ONYCAP23, Colon SW480 and SW620 cells, also demonstrated a positive correlation between brachyury and YAP protein levels when brachyury was either overexpressed or silenced (Fig. S9a–c). In agreement with a non-transcriptional mechanism of control, all these cell lines also showed modulation of YAP protein without an effect on the expression of YAP mRNA levels (Fig. S9d–g) in response to manipulations of brachyury expression. Collectively, our study demonstrates that brachyury-

based regulation of YAP can occur through a transcriptional and/or post-transcriptional mechanism in different tumors, leading to enhanced YAP-dependent oncogenic activity.

DISCUSSION

Our work suggests an important role for the embryonic transcription factor brachyury in driving cancer stemness and growth. During development, brachyury maintains stem and progenitor cells during vertebrate neuro-axis formation (Kispert et al., 1995b), suggestive of a role for brachyury in the transformation of notochordal remnants and subsequent progression of chordoma. Our studies demonstrate that brachyury controls cell cycle progression, proliferation, and cancer stemness to regulate tumor initiating capacity in chordoma and other, more common cancers. Furthermore, surprisingly, we find that this function of brachyury is achieved through control of expression of another developmental factor, the transcriptional co-activator YAP (Dong et al., 2007). Given that YAP is overexpressed and hyperactive in numerous cancers (Zanconato et al., 2016), it is of vital interest to identify critical regulators of YAP. Thus, our study identifies a transcriptional regulator of this proto-oncogene YAP (Wang et al., 2013; Wu et al., 2013; Zhu et al., 2015). Furthermore, we demonstrate the conservation of this brachyury-YAP regulatory mechanism in another type of aggressive CNS-derived cancer, glioblastoma, suggesting a common presence of this regulatory linkage across different tissues of origin, and making this regulatory interaction a potential new biomarker of cancer stemness and aggressiveness.

Unlike with chordomas, the present study also demonstrates that brachyury-driven YAP expression can also be controlled by enhancing protein stability in lung carcinomas, highlighting different regulatory scenarios leading to equivalent functional outcomes. It is possible that both transcriptional and translational regulation of YAP expression by brachyury co-exist in any tissue of origin, but to different relative degrees, subject to additional regulatory constraints.

This analysis also highlights the importance of co-opting developmental factors in carcinogenesis. Both brachyury and YAP are key controllers of developmental patterning and growth and homeostasis of normal tissues. As such, their roles in controlling stemness of both normal and cancerous tissues is perhaps not surprising. However, the regulatory linkage of these factors is not completely understood, making it of interest to examine its role in both normal development and across a larger set of different cancer types. Rosenbluh J. et al. reported that YAP binds to TBX5, a T-box family transcription factor, along with other components to form a transcriptional complex to regulate synthesis of antiapoptotic genes (Rosenbluh et al., 2012). More recently, Mohamed et al showed that overexpression of constitutively active YAP increased brachyury-promoter reporter activity, suggesting that YAP positively regulates expression of brachyury (Mohamed et al., 2016). However, it remained unclear whether brachyury's pro-oncogeneic function was mediated by YAP signaling. Thus, the unexpected role of brachyury as a regulator of YAP expression uncovered by our study can reveal further details of regulation of YAP both in normal and pathological conditions, suggesting a putative mechanism for the common observation of YAP overexpression across multiple cancers.

Given that our findings indicate that brachyury-YAP signaling is co-opted by a variety of cancer types, from some of the rarest and slow-growing but lethal tumors to the most prevalent and aggressive types, this brachyury-YAP regulatory cascade may serve as potential potent therapeutic target. Clinically, with the advent of vaccines and small molecule inhibitors against brachyury and YAP (Hamilton et al., 2013), respectively, targeting this hyperactive regulatory network may offer prognostic benefit to cancer patients. Hence, our study underscores the clinical value of identifying and delineating aberrant regulatory networks in cancer.

EXPERIMENTAL PROCEDURE

Cell culture

The patient-derived primary chordoma cell line, JHC7, was established from tissue obtained intraoperatively and processed for cell culture as described in *Hsu, Mohyeldin, Shah et al., 2011*. UCH1 and UCH2, were obtained from The Chordoma Foundation. Primary patient-derived glioblastoma tissue samples 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.

Lentiviral transduction

Cells were transduced with equal titers of virus in growth media supplemented with polybrene (Sigma) for 24 hours. After transduction, cells were cultured in normal media for 24 hours prior to selection.

In vivo experiments: subcutaneous xenografts

Animal protocols were approved by the Johns Hopkins School of Medicine Animal Care and Use Committee. Subcutaneous tumor cell implantation into mice was conducted according to the protocol as described previously (Hsu et al., 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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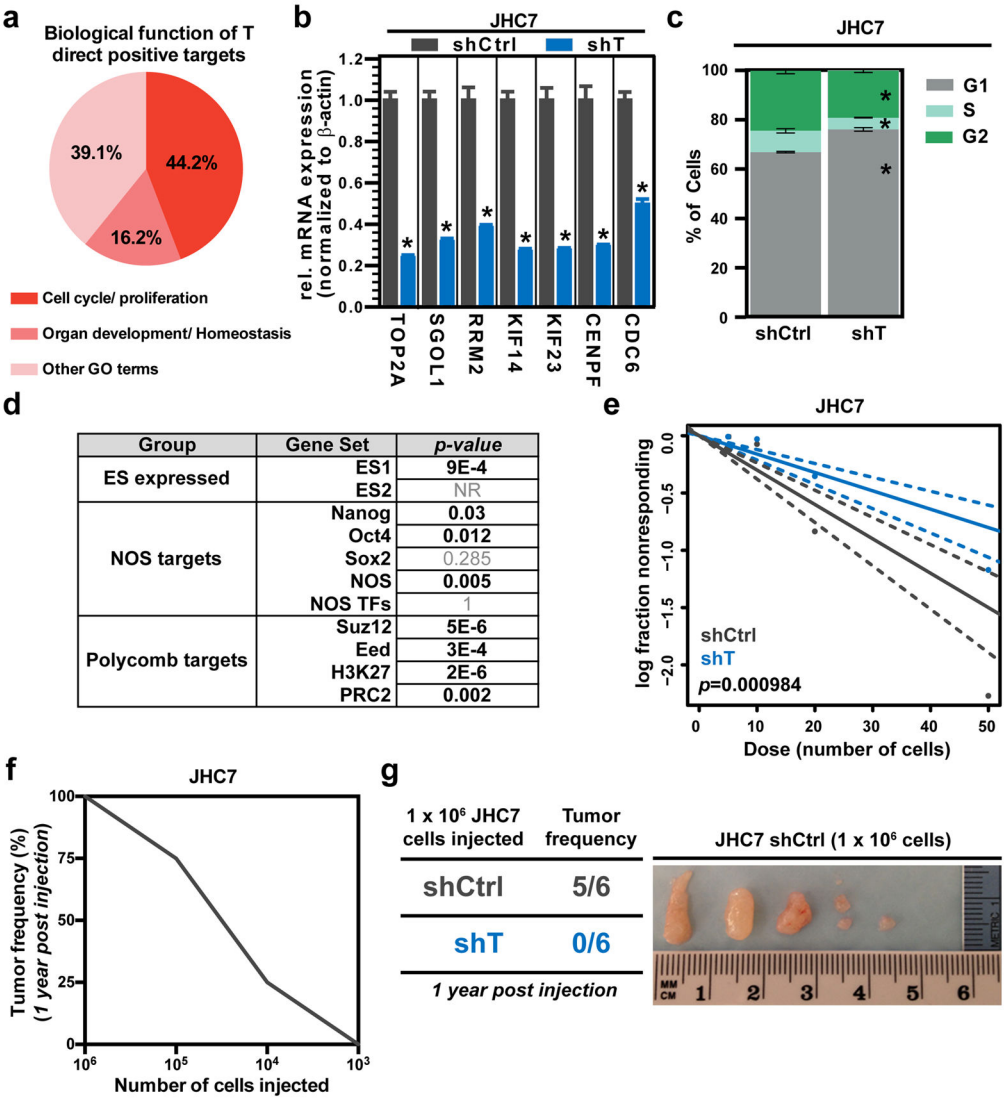
HIGHLIGHTS

- Chordomas harbor a putative cancer stem cell population which is driven by brachyury
- Brachyury-YAP regulatory axis: Identification of a direct transcriptional regulator of YAP
- Brachyury can enhance YAP activity through a post-transcriptional mechanism in carcinomas
- Brachyury-YAP activity is tied to tumor aggressiveness in several types of cancers



Group	Gene Set	*p-value*
ES expressed	ES1	9E-4
	ES2	NR
NOS targets	Nanog	0.03
	Oct4	0.012
	Sox2	0.285
	NOS	0.005
	NOS TFs	1
Polycomb targets	Suz12	5E-6
	Eed	3E-4
	H3K27	2E-6
	PRC2	0.002

Figure 1. Brachyury regulates cancer stemness and tumor-initiating capacity in chordoma

a, Distribution of biological functions associated to brachyury (T) direct positive targets, identified by GO terms. **b,** mRNA expression of cell cycle regulator genes in shCtrl or shT JHC7 cells. **c,** Average percentage of cells in each cell cycle phase in shCtrl or shT JHC7 cells. **d,** Gene set enrichment analysis (GSEA) of T signature for signatures specific for embryonic stem cells (ES expressed), Nanog, Oct4, and Sox2 (NOS) targets, and polycomb targets. **e,** Representative *in vitro* extreme limiting dilution assay plating decreasing numbers of shCtrl or shT JHC7 cells. Solid lines: mean; dotted lines: 95% confidence interval; circles: values obtained in each cell dilution. **f,** *In vivo* limiting dilution assay showing relationship between number of JHC7 cells injected subcutaneously in mice and tumor formation capacity after a year (as assessed by tumor frequency in %). **g,** *Left:* Table showing tumor frequency in mice injected subcutaneously with the 1×10^6 shCtrl or shT JHC7 cells after 1 year. *Right:* Images of isolated JHC7 tumor mass from mice injected

subcutaneously with 1×10^6 shCtrl cells after 1 year. All error bars are s.e.m. * = $P<0.05$. See also Figures S1, S2, and S3.

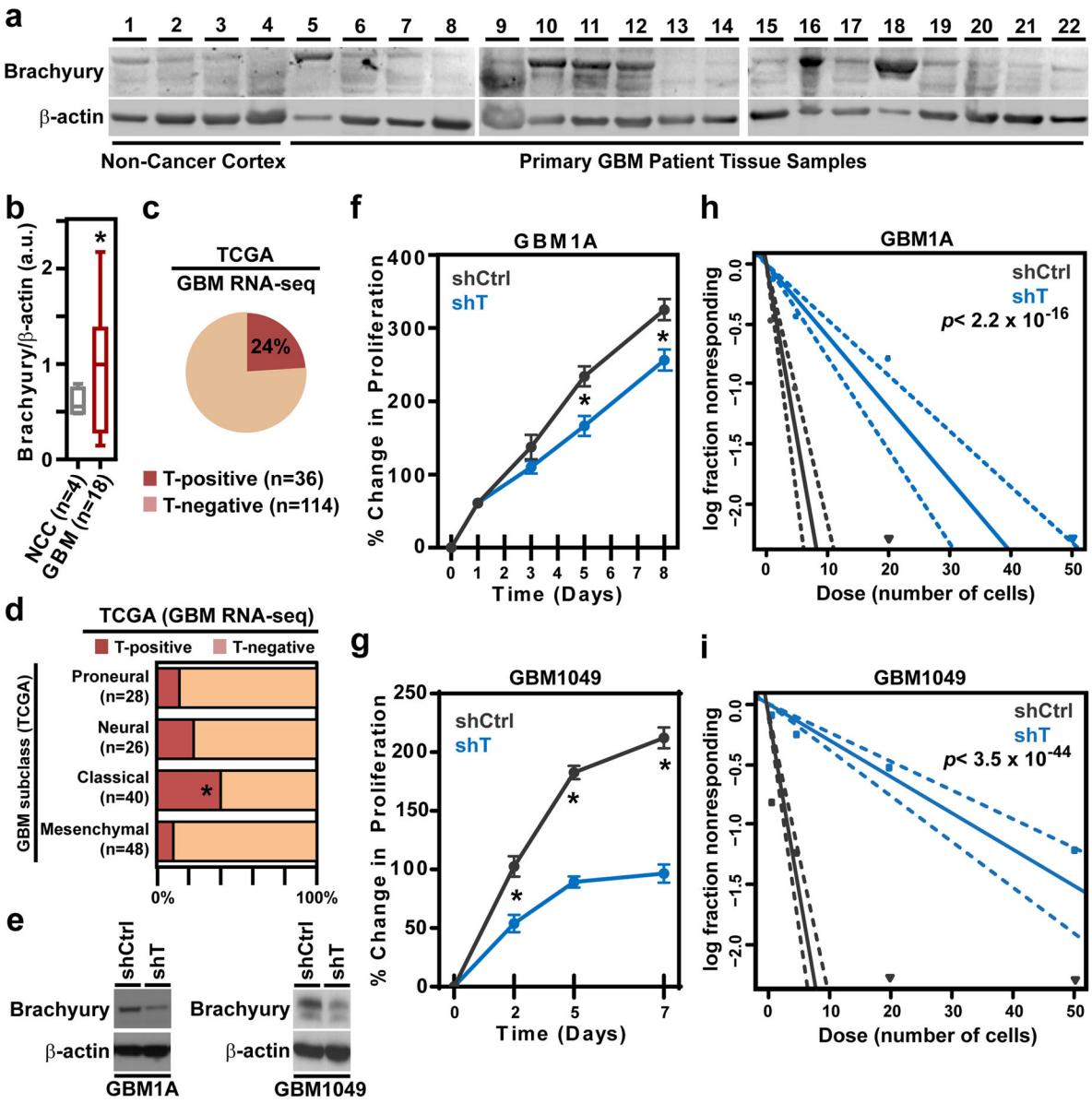


Figure 2. Brachyury regulates proliferation and stemness in glioblastoma

a, Immunoblots of brachyury expression in non-cancer cortex and primary glioblastoma patient tissues. **b,** Densitometric quantification of brachyury expression (normalized to β-actin) from the immunoblots shown in a. a.u. = arbitrary units. **c,** Percentage of T-positive or T-negative expression in patient-derived glioblastoma tissues from the RNA-seq dataset of TCGA. **d,** Percentage of T-positive or T-negative GBM patients in each TCGA subtype. Fisher's exact test. **e,** Representative immunoblot of brachyury expression in shCtrl or shT GBM1A and GBM1049 cells. **f, g,** Representative long-term MTT proliferation assay of shCtrl or shT GBM1A and GBM1049 cells. **h, i,** Representative *in vitro* extreme limiting dilution assay plating decreasing numbers of shCtrl or shT GBM1A and GBM1049 cells. Solid lines: mean; dotted lines: 95% confidence interval; circles: values obtained in each cell dilution. All error bars are s.e.m. * = $P<0.05$. See also Figure S4.

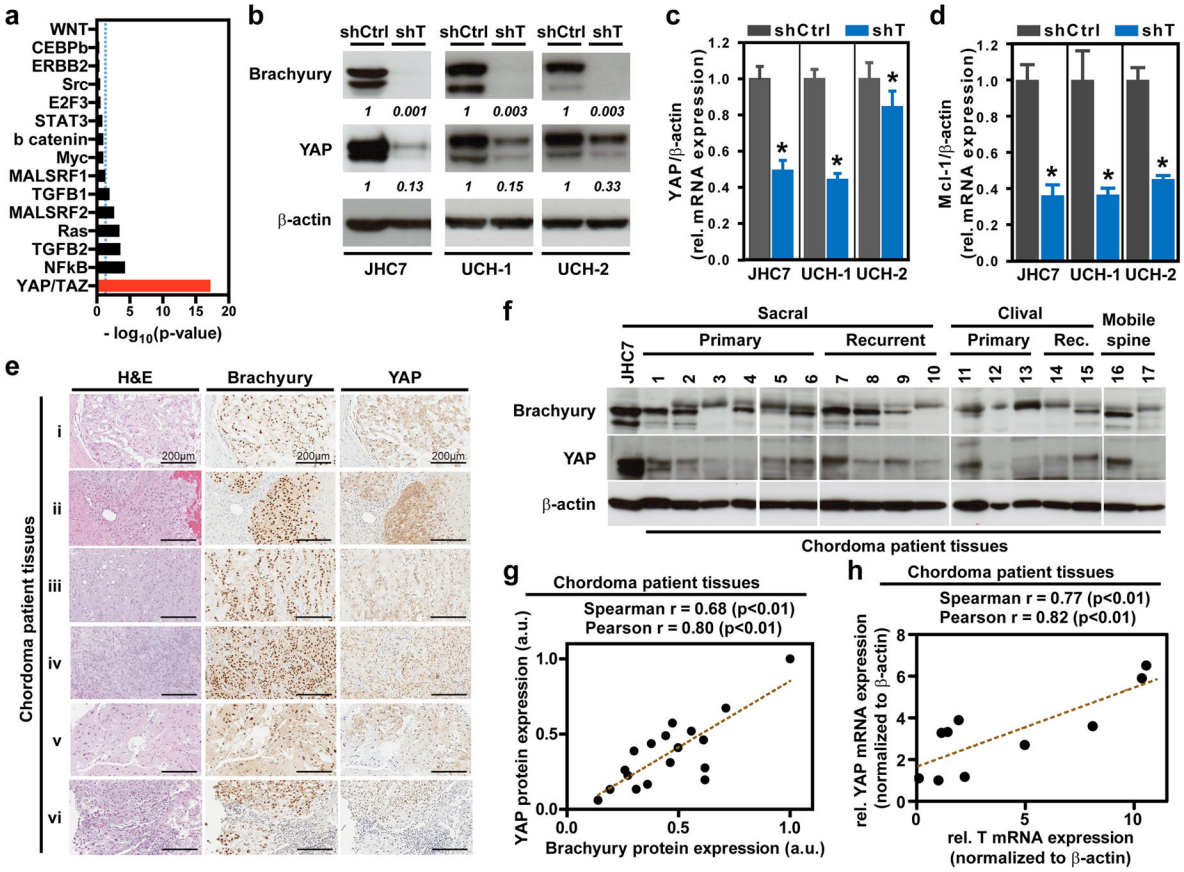


Figure 3. Brachyury regulates YAP in CNS-derived cancers

a, Statistical enrichment of the T signature genes among other pathway-specific signatures (Fisher's exact test). **b,** Representative immunoblot of brachyury and YAP expression in shCtrl or shT JHC7, UCH1, and UCH2 cells. **c,** YAP mRNA expression in shCtrl vs. shT JHC7, UCH1, and UCH2 cells. **d,** Mcl-1 mRNA expression in shCtrl vs. shT JHC7, UCH1, and UCH2 cells. **e,** Representative images of immunohistochemical staining of Brachyury, YAP, and H&E in patient-derived chordoma tissues. Scale bar = 200μm. **f,** Immunoblots of brachyury and YAP expression in primary and recurrent sacral, clival, or mobile spine chordoma patient tissues. **g,** Correlation plot of relative brachyury and YAP protein expression (normalized to β-actin) based on densitometric quantification of immunoblots in e; a.u. = arbitrary units. **h,** Correlation plot of relative brachyury and YAP mRNA expression (normalized to β-actin) in a subset of patient-derived chordoma tissues (n=10). All error bars are s.e.m. * = P<0.05. See also Figure S4, S5, S6, and S7.

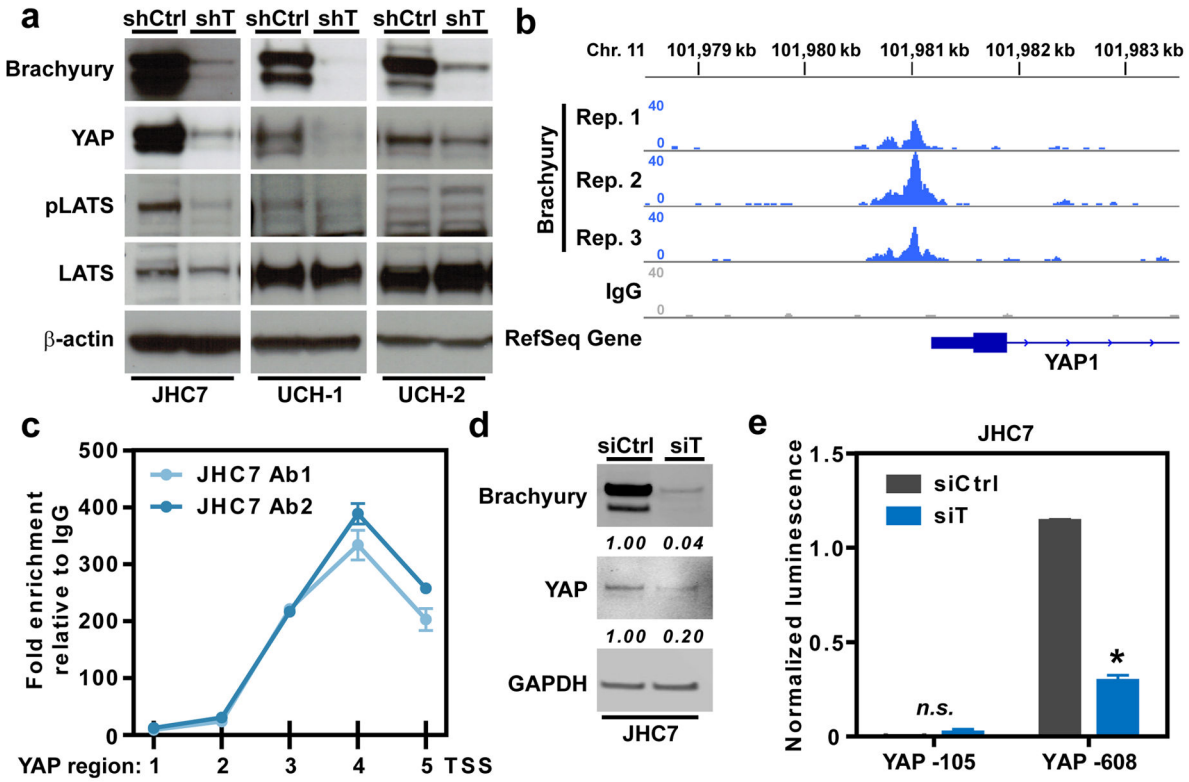
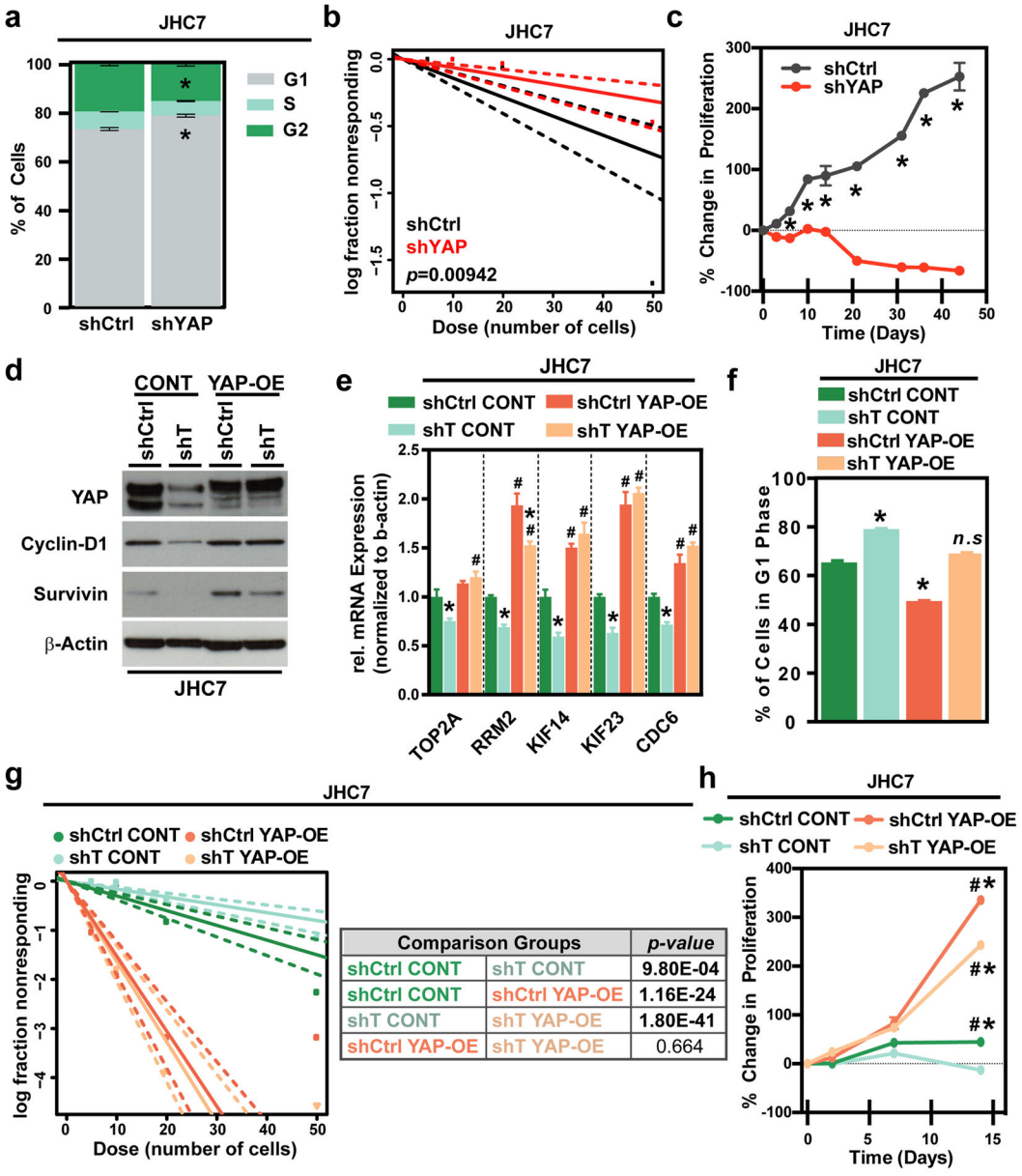


Figure 4. Brachyury directly activates transcription of YAP

a, Brachyury binding to the proximal promoter region of YAP as observed in a publicly available ChIP-sequencing dataset using UCH1 cells. **b,** ChIP-PCR querying across the −1.5 kb region upstream of the transcriptional start site (TSS) of the YAP promoter in JHC7 cells using two independent antibodies specific for epitopes on the C- (Ab1) and N-terminus (Ab2) of brachyury. **c,** Representative immunoblot of brachyury and YAP expression in siCtrl or siT JHC7 cells. **d,** *YAP* gene promoter luciferase assay using the −105bp and −608bp regions upstream of the TSS in siCtrl or siT JHC7 cells. **e,** Schematic of transcriptional regulation of YAP by brachyury. All error bars are s.e.m. * = P<0.05. See also Figure S5.



Comparison Groups		*p-value*
shCtrl CONT	shT CONT	9.80E-04
shCtrl CONT	shCtrl YAP-OE	1.16E-24
shT CONT	shT YAP-OE	1.80E-41
shCtrl YAP-OE	shT YAP-OE	0.664

Figure 5. Brachyury regulates stemness and proliferation through YAP

a, Average percentage of cells in each cell cycle phase in shCtrl or shYAP JHC7 cells. **b,** Representative *in vitro* extreme limiting dilution assay plating decreasing numbers of shCtrl or shYAP JHC7 cells. **c,** Representative long-term MTT proliferation assay of shCtrl or shYAP JHC7 cells. **d,** Representative immunoblot of YAP, Cyclin-D1, and Survivin expression in shCtrl or shT JHC7 cells transfected with empty vector (CONT) or YAP-overexpression (YAP-OE) plasmids. **e,** mRNA expression of cell cycle regulator genes in shCtrl or shT JHC7 cells with CONT or YAP-OE overexpression. **f,** Average percentage of cells in G1 phase in shCtrl or shYAP JHC7 cells with CONT or YAP-OE overexpression. **g,** Representative *in vitro* extreme limiting dilution assay plating decreasing numbers of shCtrl or shYAP JHC7 cells with CONT or YAP-OE overexpression. Solid lines: mean; dotted

lines: 95% confidence interval; circles: values obtained in each cell dilution. **h,** Representative long-term MTT proliferation assay of shCtrl or shYAP JHC7 cells with CONT or YAP-OE overexpression. In h, * = significant versus Day 0, # = significant versus shCtrl CONT group for the same day. All error bars are s.e.m. *, # = $P<0.05$. See also Figure S6.