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# Comprehensive Molecular Profiling Identifies FOXM1 as a Key Transcription Factor for Meningioma Proliferation

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## SUMMARY

Meningioma is the most common primary intracranial tumor, but the molecular drivers of aggressive meningioma are incompletely understood. Using 280 human meningioma samples and RNA sequencing, immunohistochemistry, whole-exome sequencing, DNA methylation arrays, and targeted gene expression profiling, we comprehensively define the molecular profile of aggressive meningioma. Transcriptomic analyses identify FOXM1 as a key transcription factor for meningioma proliferation and a marker of poor clinical outcomes. Consistently, we discover genomic and epigenomic factors associated with FOXM1 activation in aggressive meningiomas. Finally, we define a FOXM1/Wnt signaling axis in meningioma that is associated with a mitotic gene expression program, poor clinical outcomes, and proliferation of primary meningioma cells.



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AUTHOR CONTRIBUTIONS

H.N.V., S.M.C., P.K.S., M.W.M., A.P., and D.R.R. designed the study. S.E.B., A.W., G.F.R., S.T.M., and D.R.R. collected clinical data and constructed the database. J.J.P., M.P., and A.P. performed pathologic assessments. H.N.V., J.J.P., M.P., B.A.T., G.F.R., and D.R.R. performed experiments and data analysis. H.N.V., S.E.B., J.J.P., M.P., and D.R.R. interpreted molecular data. H.N.V., S.E.B., J.J.P., M.P., A.W., S.T.M., F.Y.F., S.M.C., P.K.S., M.W.M., M.S.B., A.P., and D.R.R. designed and critically revised the figures. H.N.V. and D.R.R. wrote the manuscript. H.N.V., S.E.B., J.J.P., M.P., B.A.T., A.W., G.F.R., S.T.M., J.Z., F.Y.F., T.N., S.M.C., P.K.S., M.W.M., M.S.B., A.P., and D.R.R. read, edited, and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND SOFTWARE AVAILABILITY

The accession number for the large-scale sequencing data reported in this paper is GEO: GSE101638.

SUPPLEMENTAL INFORMATION

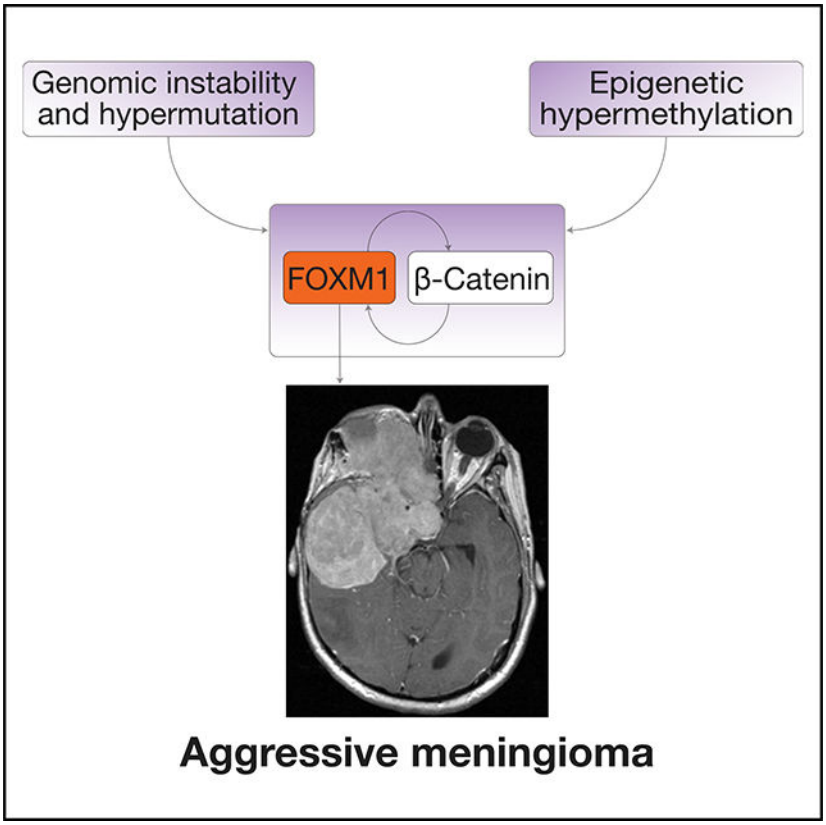
Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.013.

In summary, we find that multiple molecular mechanisms converge on a FOXM1/Wnt signaling axis in aggressive meningioma.

## In Brief

Using multiplatform molecular profiling, Vasudevan et al. comprehensively define the molecular profile of aggressive meningioma. They identify genomic, epigenomic, and transcriptomic mechanisms that converge on a FOXM1/Wnt signaling axis in aggressive meningioma that is associated with meningioma cell proliferation and is a marker of poor clinical outcomes across molecular subgroups.

## Graphical Abstract




## INTRODUCTION

Meningioma, a cancer of the cerebral and spinal meninges, is the most common primary CNS tumor in the United States (Ostrom et al., 2016). Surgery and radiation are the mainstays of meningioma treatment but are insufficient to control 40%–75% of high-grade tumors (Rogers et al., 2015). There are no curative therapies or effective molecular treatments for aggressive meningiomas (Wen et al., 2010). Thus, understanding meningioma biology is essential for identifying biomarkers and developing novel therapies to improve outcomes.

Extent of resection and tumor grade are the most important clinical factors for meningioma outcome (Rogers et al., 2015). With respect to the latter, the World Health Organization (WHO) categorizes meningiomas into grade I, grade II (atypical), and grade III (anaplastic) tumors on the basis of mitotic activity and histopathologic features (Louis et al., 2016). Outcomes are worst for WHO grade III meningiomas, but a subset of low-grade meningiomas are also prone to local recurrence and poor survival despite otherwise reassuring histopathology and optimal therapy (Rogers et al., 2015). Thus, clinically aggressive meningiomas are defined by high grade, local recurrence, or both.

Patients with neurofibromatosis type II (NF2), a genetic condition associated with inactivating mutations in the *NF2* gene, have an elevated risk for developing meningiomas of all grades. Similarly, targeted sequencing reveals recurrent *NF2* mutations in non-syndromic meningiomas (Lekanne Deprez et al., 1994; Ruttledge et al., 1994). Whole-genome and whole-exome sequencing identifies other mutated genes in meningioma, such as *SMO, AKT1, KLF4, TRAF7*, and *POLR2A* (Brastianos et al., 2013; Clark et al., 2013, 2016; Reuss et al., 2013). Yet beyond *NF2*, recurrent mutations in meningioma are rare (Bi et al., 2016). Furthermore, genomic techniques have not identified associations between specific mutations and clinical outcomes. Thus, to improve understanding of meningioma biology, recent work has focused on multiplatform characterization using genomic, epigenomic, and gene expression techniques (Harmanci et al., 2017; Sahm et al., 2017), and a classification system based on DNA methylation pattern identifies meningioma subgroups with differences in tumor location and progression-free survival (Sahm et al., 2017). Yet despite these advances, targetable pathways associated with poor meningioma outcomes remain elusive.

To elucidate the molecular drivers of meningioma, we developed an integrated database containing comprehensive clinical data and tissue from 261 patients who underwent surgical resection of 280 meningiomas (Table S1). The database was enriched for high-grade and recurrent meningiomas, which define patients who stand to benefit most from improved understanding of meningioma biology and novel therapeutic strategies. We performed RNA sequencing (RNA-seq), immunohistochemistry (IHC), whole-exome sequencing (WES), DNA methylation profiling, and targeted gene expression profiling to systematically characterize the transcriptomic, genomic, and epigenomic features of meningioma in relation to key clinical parameters and outcomes (Figure 1). We discovered two distinct transcriptomic clusters of meningiomas and demonstrated that aggressive tumors from either cluster were marked by elevated Forkhead Box M1 (*FOXM1*) mRNA expression.

FOXM1 is a pro-mitotic transcription factor (Fu et al., 2008; Laoukili et al., 2005) that is required for cell proliferation during development (Korver et al., 1998; Ye et al., 1997). In cancer, FOXM1 is implicated in the proliferation of hepatocellular carcinoma (Kalinichenko et al., 2004), prostate cancer (Kalin et al., 2006), basal cell carcinoma (Teh et al., 2002), and glioma (Liu et al., 2006). In meningioma, FOXM1 expression has previously been shown to be highly expressed in tumors compared with normal tissue and particularly enriched in invasive tumors (Laurendeau et al., 2010). Consistently, we found that FOXM1 protein expression in meningioma was associated with increased cell proliferation and poor clinical outcomes. Furthermore, we found that meningiomas from patients with poor outcomes were

characterized by high somatic mutation burden and genome-wide hypermethylation of genes that otherwise inhibit FOXM1 activity. Finally, we defined a FOXM1/Wnt signaling axis in meningioma that was associated with a mitotic gene expression program, poor clinical outcomes, and proliferation of primary meningioma cells. In summary, our data support a paradigm in which increased FOXM1 activity cooperates with dysregulated Wnt signaling to drive meningioma proliferation and tumor growth.

## RESULTS

To understand meningioma biology, we retrospectively identified human meningioma samples from the University of California, San Francisco, Brain Tumor SPORE Biospecimen Core from 1990–2015 for multiplatform molecular characterization (Figure 1). Only meningiomas from patients with available clinical follow-up data were included. Likewise, only meningiomas with sufficient tissue for re-grading according to current histopathologic criteria were included (Louis et al., 2016). In all cases, diagnostic imaging was re-reviewed to define meningioma location, extent of resection, and local recurrence. Fresh frozen meningiomas were selected for RNA-seq. Meningiomas with paired normal tissue were selected for WES and DNA methylation profiling. Formalin-fixed, paraffin-embedded meningiomas were selected for NanoString targeted gene expression profiling, tissue microarrays (TMAs) and IHC. The study population was intentionally enriched for high-grade and recurrent meningiomas, which define patients with aggressive tumors who stand to benefit most from improved understanding of meningioma biology. Toward that end, data from each sequencing modality were stratified according to clinical parameters such as meningioma grade, setting (i.e., primary versus recurrent), prior treatment, location, radiographic and histopathologic characteristics, local recurrence, and survival (Table S1).

### FOXM1 Expression Unifies Aggressive Meningiomas from Distinct Molecular Subgroups

Genomic analyses of meningioma reveals mutations that are associated with tumor location and histologic subtype (Clark et al., 2013, 2016; Reuss et al., 2013), and epigenomic features identify meningioma subgroups with differences in tumor location and progression-free survival (Sahm et al., 2017). In contrast to genomic and epigenomic features, the meningioma transcriptome is poorly understood. To identify targetable pathways associated with poor meningioma outcomes, we investigated the transcriptomic landscape of 42 aggressive meningiomas using RNA-seq (Figure 1; Table S1). Unsupervised hierarchical clustering of the top 2,000 most variable genes identified two distinct clusters (Figures 2A and S1A). From a clinical perspective, these transcriptomic clusters were associated with differences in patient sex, and meningioma location and grade, but not patient age, prior cranial radiation, or any other clinical parameters (ANOVA $p > 0.05$) (Figures S1C–S1F; Table S1). To identify molecular signatures enriched in each cluster, we performed differential expression (Love et al., 2014) and Gene Ontology (GO) analyses (Huang et al., 2009) (Table S2). We found that the transcriptomic cluster associated with female sex and low-grade meningiomas of the skull base was enriched for focal adhesion genes (Figure S1G). The transcriptomic cluster associated with male sex and high-grade meningiomas was enriched for metabolic and oxidative phosphorylation genes (Figure S1H). Despite these differences, there were no differences in meningioma local-recurrence free survival (LRFS)

or overall survival (OS) according to transcriptomic cluster assignment (Figures S1I and S1J).

The discovery of meningioma transcriptomic clusters without associations to clinical outcomes suggests that aggressive meningiomas may be unified by a small subset of genes critical for tumor cell proliferation. To test that hypothesis, we performed differential expression analysis on the basis of meningioma grade to identify genes enriched in high-grade tumors, which classically define aggressive meningiomas. We found a total of 2,012 genes across all comparisons (Figure 2B; Table S3), with the greatest number of differences between WHO grade I and WHO grade III meningiomas. GO analysis of differentially expressed genes revealed that cell division and mitosis genes were the most significantly enriched transcripts in WHO grade III meningioma relative to WHO grade I meningioma (Figure 2C), which provided a mechanistic foundation toward understanding meningioma proliferation. We therefore performed chromatin enrichment analysis (ChEA) to identify candidate transcription factors regulating the genes within WHO grade III tumors (Lachmann et al., 2010) and found that published FOXM1 chromatin immunoprecipitation sequencing (ChIP-seq) targets (Chen et al., 2013; Wiseman et al., 2015) constituted the largest percentage of WHO grade III meningioma genes (Figure 2D). Indeed, FOXM1 targets accounted for 11% of genes enriched in WHO grade III meningiomas, compared with only 3% of genes enriched in WHO grade I meningiomas (Figure S1K), and *FOXM1* itself was enriched in high-grade meningiomas (Figure 2E).

FOX transcription factors are implicated in meningeal development (Zarbalis et al., 2012), and prior analysis of atypical meningioma revealed enrichment of genes from the FOXM1 transcriptional network (Harmanci et al., 2017). To visualize the biologic impact of the FOXM1 transcriptional network in meningioma, we compared how target genes from published FOXM1 ChIP-seq datasets were distributed among transcriptomic clusters. Although WHO grade III meningiomas were over-represented in cluster 2 (Figure 2A), neither *FOXM1* mRNA expression nor FOXM1 target gene distribution was significantly different between transcriptomic clusters (Figures S1L and S1M). Given that our database was enriched for meningiomas with aggressive behavior across all grades, one explanation of these data is that FOXM1 also mediates cell proliferation in the subset of low-grade meningiomas that are prone to recurrence (Louis et al., 2016). In support of that hypothesis, we segregated meningiomas of all grades according to *FOXM1* mRNA expression and found that increased *FOXM1* expression was associated with worse LRFS and OS (Figures 2F and 2G). In summary, WHO grade III meningiomas are enriched in a FOXM1 target gene network associated with mitosis and cell division, and increased *FOXM1* expression serves as a marker for meningiomas with poor clinical outcomes.

## FOXM1 Expression Is Associated with Meningioma Proliferation and Poor Clinical Outcomes

High-grade meningiomas are characterized by elevated cell proliferation (Louis et al., 2016). To determine if FOXM1 was associated with meningioma proliferation, we quantified FOXM1 protein in a total of 52 tumors constituting both indolent and aggressive meningiomas (Table S1). IHC for FOXM1 revealed significant intra-meningioma

heterogeneity that was restricted to meningioma cells, as denoted by co-staining for SSTR2A (Figures 3A and 3B) (Menke et al., 2015). Co-staining for the cell proliferation marker MIB1 demonstrated a strong association between FOXM1 and mitotic activity in meningioma (Figures 3C and 3D). In agreement with previous data (Rogers et al., 2015), meningioma proliferation, as defined by MIB1 labeling index ≥ 6%, was associated with decreased LRFS and decreased OS (Figures 3E and 3F). Consistently, we found that high FOXM1 protein expression, as defined by ≥75 nuclei/mm$^2$, was also associated with decreased LRFS and a strong trend toward decreased OS (Figures 3G and 3H). Thus, high FOXM1 protein expression in meningioma is associated with increased cell proliferation and poor clinical outcomes.

## Aggressive Meningiomas Are Characterized by High Somatic Mutation Burden

To determine if genomic mutations underlie FOXM1 expression in meningioma, we performed WES of 24 aggressive tumors with matched control tissue (Figure 1; Table S1). Consistent with previous WES data (Bi et al., 2016; Brastianos et al., 2013; Harmanci et al., 2017; Sahm et al., 2017), we found that *NF2* was the most commonly mutated gene in meningioma (Figure 4A; Table S4). We did not identify mutations in *SMO, AKT1, KLF4, TRAF7*, or *POLR2A*, nor any recurrent mutations in cell proliferation genes. Rather, there was a wide range of somatic mutations and large-scale copy number variations across samples (Figures 4B and 4C), with more large-scale copy number variations in meningiomas with *NF2* mutations (Figure S2) (Bi et al., 2016; Harmanci et al., 2017). We identified a strong association between patient age and meningioma somatic mutation burden, with tumors from older patients containing a greater number of somatic mutations (Figure 4D). Furthermore, the number of somatic mutations was significantly increased in high-grade meningiomas (Figure S3A), convexity meningiomas (Figure S3B), and meningiomas in patients with histories of prior cranial radiation (Figure S3C) but not across any other clinical parameters (ANOVA $p > 0.05$) (Table S1). As there were no differences in meningioma recurrence or patient survival according to any individual mutations, we analyzed outcomes according to meningioma somatic mutation burden and discovered that an increased number of somatic mutations was associated with worse disease-specific survival (DSS) and a trend for worse LRFS (Figures 4E and S3D). Although we did not identify any mutations in FOXM1, *NF2* is a negative regulator of FOXM1, and loss of NF2 is known to induce FOXM1 activity to promote cancer cell growth (Quan et al., 2015). In that regard, our data recapitulate earlier studies identifying *NF2* as the most commonly mutated gene in aggressive meningioma and demonstrate a relationship between meningioma hypermutation and poor clinical outcomes.

## Epigenomic Hypermethylation Is Associated with High Somatic Mutation Burden in Aggressive Meningioma

Epigenomic methylation is implicated in meningioma progression and has been proposed as an additional criteria to stratify meningioma patients (Sahm et al., 2017). To identify epigenomic factors that might induce FOXM1 expression in meningioma, we performed 850K DNA methylation array profiling of 26 aggressive meningiomas (Figure 1; Table S1). Unsupervised hierarchical clustering of the top 2,000 most variable probes identified three distinct methylation clusters corresponding to high, medium, and low DNA methylation

levels (Figures 5A and S4A). Meningiomas within the high-methylation cluster were from older patients (Figure 5B) and displayed increased somatic mutation burden compared with meningiomas within the medium- and low-methylation clusters (Figures 5C and S4B). In addition, meningioma hypermethylation was associated with increased tumor grade (Figure S4C), convexity location (Figure S4C), and *NF2* mutations (Figure S4E) but not with any other clinical parameters or specifically mutated genes (ANOVA $p > 0.05$) (Table S1). Furthermore, patients with high-methylation tumors exhibited worse DSS compared with those with medium- and low-methylation meningiomas (Figures 5D and S4F). In concert with our WES results and previous studies of DNA methylation in meningioma (Harmanci et al., 2017; Sahm et al., 2017), these data suggest that DNA hypermethylation and hypermutation delineate aggressive meningiomas.

To understand the biological significance of DNA methylation in aggressive meningioma, we identified differentially methylated probes (DMPs) between clusters (Figure S5A; Table S5) (Ritchie et al., 2015) and performed Genomic Regions Enrichment of Annotations Tool (GREAT) analysis to identify enriched processes (McLean et al., 2010). Consistent with previous results, hypermethylated sites in the high-methylation cluster were over-represented for Homeobox genes (Figure S5B) (Harmanci et al., 2017). We analyzed the distribution of DMPs according to clinical parameters, and only subgroups based on meningioma grade (1,523 DMPs) resulted in significant DMP sets. Sites of hypermethylation in high-grade tumors showed enrichment for focal adhesion and cell polarity genes (Figures S5C and S5D; Table S5). In addition, hypermethylated DMPs in the high-methylation cluster with poor DSS (Figure 5D) were enriched for H3K27me3 and PRC targets (Figure 5E), each of which leads to a functional switch toward FOXM1-dependent transcription in cancer (Mahara et al., 2016). Consistently, the expression of many published PRC targets by RNA-seq (Bracken et al., 2006), such as *BMP2, HOXD4, ATF3, HOXA13*, and *HOXC5*, was decreased in meningiomas with increased *FOXM1* expression (Figures 5F, 5G, and S5E–S5G).

## A FOXM1/Wnt Signaling Axis Promotes Cell Proliferation Gene Expression in Aggressive Meningioma

To confirm the associations between elevated *FOXM1* mRNA expression, FOXM1 target gene expression, and poor clinical outcomes in meningioma, we performed NanoString targeted gene expression profiling within a validation cohort of 96 meningiomas (Figure 1; Tables S1 and S6). Consistent with our RNA-seq results, increased *FOXM1* mRNA expression within the validation cohort identified meningioma patients with decreased LRFS and OS (Figures 6A and 6B). FOXM1 transcript and protein expression was more common in high-grade (Figures 6C and S6A) and recurrent meningiomas (Figure 6D and S6B) but was not restricted to WHO grade III meningiomas. Indeed, we found that increased *FOXM1* expression also delineated a subgroup of WHO grade II meningiomas with poor LRFS (Figure S6C). To further analyze the relationship between *FOXM1* expression and clinical markers of aggressive meningioma behavior, we constructed multivariate Cox proportional-hazard models accounting for independent variables of patient age, meningioma grade, setting (i.e., primary versus recurrent), extent of resection, adjuvant radiotherapy, and *FOXM1* expression. Accordingly, we found that high *FOXM1* expression was the most

important factor for tumor recurrence (HR 2.06, robust SE [RSE] 0.64, 95% confidence interval (CI) 1.12–3.80, p = 0.020) or death (HR 3.13, RSE 1.39, 95% CI 1.31–7.48, p = 0.010) when accounting for competing variables of meningioma behavior (Table S7).

FOXM1 directly interacts with β-catenin to transduce Wnt signals and regulate cell proliferation (Chen et al., 2016; Gong and Huang, 2012; Zhang et al., 2011), and dysregulated Wnt signaling is implicated in meningioma (Fèvre-Montange et al., 2009; Pérez-Magán et al., 2010). To determine if Wnt pathway activation was associated with poor meningioma outcomes, we assayed β-catenin localization using IHC and TMAs containing 232 meningiomas (Figure 1; Table S1). We identified abundant cytoplasmic β-catenin staining in meningioma and rare nuclear staining (Figure 6E), with occasional co-localized of nuclear β-catenin and nuclear FOXM1 by confocal microscopy (Figure 6F). Meningiomas with nuclear β-catenin staining by IHC showed significantly decreased LRFS (Figure 6G), and multivariate Cox proportional-hazard modeling revealed that nuclear β-catenin staining remained significant for meningioma recurrence when accounting for competing variables of meningioma behavior (HR 2.69, RSE 0.74, 95% CI 1.57–4.60, p < 0.001) (Table S7). In addition to nuclear localization of β-catenin, the Wnt pathway can be activated in cancer through epigenomic silencing of secreted Wnt antagonists such as SFRP family members (Fukui et al., 2005; Suzuki et al., 2004). Consistently, we found that methylation of the *SFRP1* promoter was also associated with decreased LRFS (Figure 6H). Furthermore, *SFRP1* mRNA was suppressed in tumors with high *FOXM1* mRNA expression (Figure 6I). Thus, dysregulated Wnt signaling, as evidenced by (1) increased *FOXM1* expression, (2) nuclear β-catenin localization, (3) *SFRP1* promoter hypermethylation, and (4) decreased *SFRP1* expression in tumors enriched in *FOXM1*, is associated with poor clinical outcomes for meningioma.

To elucidate the downstream transcriptional network that transduces FOXM1/Wnt-mediated cell proliferation, we selected genes enriched in WHO grade III meningioma from our RNA-seq analysis that corresponded to FOXM1 targets from published ChIP-seq datasets (Table S6) (Chen et al., 2013; Wiseman et al., 2015). Unsupervised hierarchical clustering of RNA-seq samples on the basis of FOXM1 target genes revealed two distinct clusters (Figure 6J), with decreased LRFS and OS in the cluster associated with high FOXM1 target gene expression (Figures 6K and 6L). To visualize the biologic impact of FOXM1 target gene expression in meningioma, we constructed a protein-protein interaction network (Figure 6M). Consistent with the relationship between FOXM1 expression and meningioma proliferation (Figures 2 and 3), many of the genes within the FOXM1 target protein-protein interaction network were critical for cell proliferation. Among those, *TOP2A, CCNA2*, and *CKS2* were included in our targeted validation dataset (Figure 1; Table S1), and high expression of each was associated with decreased LRFS (Figures S6D–S6F). In summary, data across multiple molecular platforms and patient cohorts identify a FOXM1/Wnt signaling axis that promotes cell proliferation gene expression in aggressive meningioma.

## FOXM1/Wnt Signaling Drives Primary Meningioma Cell Proliferation

To test the mechanistic relationships between FOXM1/Wnt signaling and meningioma proliferation, we used primary meningioma cells derived from fresh patient samples (Table

S8) and previously validated primary meningioma cells (Mei et al., 2017). We identified nuclear foci of FOXM1 and β-catenin in primary meningioma cells by confocal microscopy and discovered that the intensity of cellular SFRP1 staining was inversely correlated with nuclear FOXM1 expression (Figures 7A–7D). Real-time qPCR showed that increased *FOXM1* expression was associated with increased expression of the pro-mitotic FOXM1 target genes *CCNA2* and *CCNB2* in primary meningioma cells (Figure 7E). Consistently, we found that primary meningioma cells with higher levels of *FOXM1* and FOXM1 target genes had higher rates of proliferation (Figure 7F).

To determine if FOXM1 or Wnt signaling was sufficient to drive primary meningioma cell proliferation, we overexpressed FOXM1 or β-catenin and found evidence of increased cell proliferation through (1) nuclear Ki-67 staining (Figure 7G), (2) expression of pro-mitotic FOXM1 target genes (Figure 7H), and (3) increased cell proliferation (Figure 7I). Conversely, to determine if FOXM1 was necessary for primary meningioma cell proliferation, we transduced cells with *FOXM1* short hairpin RNAs (shRNAs) or treated cells with the FOXM1 antagonist FDI-6 and measured primary meningioma cell proliferation. We found that both genetic and pharmacologic inhibition of *FOXM1* decreased proliferation of primary meningioma cells (Figures 7J and 7K). In summary, these data demonstrate that FOXM1/Wnt signaling is associated with pro-mitotic gene expression and proliferation in primary meningioma cells and, furthermore, that FOXM1 is both necessary and sufficient for primary meningioma cell proliferation.

## DISCUSSION

Approximately 20% of meningiomas are high grade and characterized by elevated tumor cell proliferation, leading to local recurrence despite optimal therapy (Louis et al., 2016; Rogers et al., 2015). As there are no effective systemic or molecular therapies for meningioma, patients with high-grade tumors often require serial craniotomy and re-irradiation, both of which are associated with significant morbidity and offer little hope for cure (Wen et al., 2010). Moreover, the molecular drivers of meningioma are incompletely understood, and targetable pathways associated with poor meningioma outcomes remain elusive. Here, we use RNA-seq, IHC, WES, DNA methylation, targeted gene expression profiling, and primary meningioma cells to comprehensively define the molecular landscape of aggressive meningioma. In addition to illuminating biomarkers for meningioma stratification, our data shed light on targets for molecular therapy that may improve outcomes for meningioma patients (Laurendeau et al., 2010)

Integrating our findings with previous molecular analyses of meningioma, we propose a model that highlights interactions between transcriptomic, protein level, genomic, and epigenomic features that converge to drive meningioma proliferation through a FOXM1/Wnt signaling axis (Figure 7L) (Laurendeau et al., 2010). We find that expression of *FOXM1* mRNA (Figures 2H, 2I, 6A, and 6B), FOXM1 protein (Figures 3G and 3H), and FOXM1 target genes (Figures 6K and 6L), as well as nuclear β-catenin (Figure 6G) and suppression of Wnt antagonists (Figure 6H) in the context of elevated *FOXM1* mRNA expression (Figure 6I), delineate meningiomas with poor survival and high rates of local recurrence. On a genomic level, *NF2* is a negative regulator of FOXM1/Wnt signaling in cancer (Quan et

al., 2015) that is recurrently mutated in aggressive meningioma (Figure 4A) (Bi et al., 2016; Harmanci et al., 2017). Therefore, loss of *NF2* may induce meningioma proliferation by stabilizing FOXM1, although a direct relationship between *NF2* loss and FOXM1 protein levels has not been demonstrated in meningioma. Epigenomically, aggressive meningiomas are characterized by DNA hypermethylation with enrichment of H3K27me3 and suppression of PRC targets and Wnt antagonists (Figures 5, 6H, and 6I) (Harmanci et al., 2017; Sahm et al., 2017). H3K27me3 hypermethylation and PRC inhibition are associated with a functional switch toward the FOXM1 transcriptional program (Mahara et al., 2016), which cooperates with β-catenin to potentiate Wnt signaling and cancer cell growth (Chen et al., 2016; Gong and Huang, 2012; Zhang et al., 2011). Mechanistically, FOXM1 expression is associated with meningioma proliferation (Figures 3C and 3D) and induction of a transcriptional network (Figures 6J and 6M) that is characterized by cell proliferation genes (Figures S6D–S6F), each of which is associated with poor clinical outcomes. In support of that hypothesis, we find that FOXM1 is both necessary and sufficient to drive primary meningioma cell proliferation (Figures 7I–7K). In summary, we propose that multiple molecular mechanisms converge on a FOXM1/Wnt signaling axis which underlies cell proliferation in aggressive meningioma (Figure 7L).

We identify meningioma molecular subgroups on the basis of transcriptomic profile (Figure 2). Akin to recurrent mutations (Clark et al., 2013, 2016), meningioma transcriptomic clusters are associated with clinical parameters such as tumor location but not local control or survival. Rather, we find that elevated FOXM1 expression delineates aggressive meningiomas from distinct transcriptomic subgroups, further supporting the hypothesis that molecular alterations across multiple regulatory levels converge on a unified cell proliferation program in meningioma.

Although we found that high-grade meningiomas with poor clinical outcomes are characterized by increased somatic mutation burden (Figure 4) (Bi et al., 2016), we did not identify mutations in *SMO, AKT1, KLF4, TRAF7*, or *POLR2A*, each of which is implicated in primary skull base meningiomas (Clark et al., 2013, 2016). We did identify mutations in other genes that are known to be mutated in meningioma, including *SMARCB1, PI3K* family members, *APC, CDKN2A, KDM53*, and *TP53* (Table S4). One possible explanation for these differences is patient selection. Whereas previous genomic investigations of meningioma focused primarily on WHO grade I tumors, our study focused on high-grade and recurrent meningiomas, which define a high-risk subset of patients who stand to benefit most from improved understanding of meningioma biology and novel therapeutic strategies. Consequently, many of our patients had histories of prior surgery or cranial radiation (Table S1). Predictably, we discovered more somatic mutations in meningiomas from patients with histories of cranial radiation (Figure S3C). However, we did not identify any transcriptomic differences in meningiomas from patients with and those without histories of cranial radiotherapy (Figure S2C).

Our data corroborate the existence of meningioma molecular subgroups with distinct clinical outcomes according to DNA methylation profile (Harmanci et al., 2017; Sahm et al., 2017). Of note, we performed epigenomic analyses on fewer meningioma samples than previous investigations (Sahm et al., 2017). Thus, it is perhaps unsurprising that we identified fewer

DNA methylation clusters than previously reported. Nevertheless, we confirm that meningiomas with high DNA methylation are associated with poor clinical outcomes. Although our data do not provide a direct relationship between DNA methylation alterations and FOXM1 expression, prior work in head and neck cancer suggests a role for FOXM1 in inducing global methylation changes, and a similar mechanism may be present in meningioma (Teh et al., 2012).

The identification of FOXM1 as a master transcription factor for meningioma proliferation provides a potential molecular target with prognostic and therapeutic significance. Robust biomarkers to predict meningioma recurrence are lacking, particularly for WHO grade II meningiomas, which follow a variable course that cannot be predicted solely on the basis of histopathologic features. We find that FOXM1 expression differentiates aggressive and indolent WHO grade II meningiomas, suggesting that FOXM1 can be used to select patients for adjuvant therapy. Furthermore, FOXM1 IHC is a useful and expedient biomarker for outcome that can be implemented in sundry clinical settings, thereby obviating the need for expensive and time-consuming genomic or epigenomic sequencing. With respect to advancing a paradigm of molecular therapy for meningioma, FOXM1 has been postulated as a therapeutic target in multiple tumors, and small molecular antagonists such as FDI-6 have demonstrated preclinical promise (Gormally et al., 2014). Given the interaction between FOXM1 and Wnt signaling, concurrent or sequential Wnt antagonism may also be an effective therapy for meningioma patients if the technical challenges associated with Wnt pathway inhibition can be overcome (Kahn, 2014). Regardless, developing tractable *in vivo* models that recapitulate the molecular characteristics of human meningioma and preclinical pharmacologic studies to shed light on the efficacy and toxicity of these novel molecular agents are essential prior to initiating trials in human meningioma patients.

## EXPERIMENTAL PROCEDURES

### Database Design and Development

Patients treated with surgical resection for meningioma at the University of California, San Francisco, from 1990 to 2015 were retrospectively identified from a prospective tissue biorepository. Both male and female patients of all ages with sufficient tissue for re-grading were included. For all cases, meningioma grade was re-evaluated on the basis of current histopathologic criteria (Louis et al., 2016), and diagnostic imaging was re-reviewed to define meningioma location, extent of resection, and date of local recurrence. Local recurrence after gross total resection was defined as a local recurrence of any size on subsequent brain imaging. After subtotal resection, the Response Evaluation Criteria in Solid Tumors (RECIST) were adapted to define local recurrence as interval growth of ≥20% along any dimension. Demographic, clinical, and histopathologic variables, including MIB1 labeling index, were extracted from the electronic medical record, pathology databases, radiology archives, and institutional cancer center. Survival status of patients was obtained from a combined search of the electronic medical record, institutional cancer registry, and Surveillance, Epidemiology and End Results (SEER), Department of Motor Vehicles (DMV), Social Security, and nationwide hospital databases, as well as publicly available obituaries. LRFS, DSS, and OS were quantified from the date of meningioma resection until

the last date of contact or the date of meningioma recurrence, date of death from tumor recurrence, or date of death from any cause, respectively. A summary of the demographic, clinical, histopathologic and radiographic parameters that were assembled and investigated with respect to meningioma molecular data is available in Tables S1 and S2–S4. This study was approved by the Institutional Review Board, Human Research Protection Program Committee on Human Research, protocol 10–03204.

### Nucleic Acid Isolation and Real-Time qPCR

Nucleic acids were isolated for sequencing in the Brain Tumor SPORE Biospecimen and Pathology Core, and the Raleigh laboratory, at the University of California, San Francisco. DNA and RNA were isolated from flash-frozen meningiomas containing >70% tumor cells as determined by H&E staining of frozen sections. For WES and DNA methylation profiling, DNA was isolated using standard techniques. For RNA-seq and real-time qPCR, RNA was isolated from meningiomas and primary meningioma cells using the RNeasy Mini Kit (QIAGEN, Valencia, CA). cDNA was synthesized from cell culture samples using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time qPCR primers are described in Table S8. Real-time qPCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA) and a Life Technologies (Grand Island, NY) QuantStuio 6 Flex Real Time PCR system using the ΔΔCt method relative to *GAPDH* expression.

### Immunofluorescence

Cells on glass coverslips were washed in PBS and fixed in 4% paraformaldehyde for 8 min. Following incubation in blocking buffer for 30 min at room temperature (2.5% BSA, 0.1% Triton X-100, and 0.03% $NaN_3$), cells were incubated with primary antibodies in blocking buffer overnight at 4°C. The next day, cells were washed three times in PBS and incubated with secondary antibodies and DNA dyes in blocking buffer at room temperature for 1 hr. In addition to those listed above, the following antibodies and fluorescent molecules were used: Alexa-conjugated secondary antibodies (Life Technologies), DAPI (Thermo Fisher Scientific), Hoechst 3342 (Life Technologies), antiKi67 rabbit polyclonal (ab15580; Abcam), and SFRP1 rabbit monoclonal (EPR7003). Following three final washes in PBS, coverslips were mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific). Fluorescent microscopy was performed using an SP5 confocal microscope (Leica, Wetzlar, DE). Image processing was completed using ImageJ (Schneider et al., 2012). For fluorescence quantifications, regions of interest were selected and quantified with normalization to background fluorescence.

### Cell Culture

M3, M6, M8, M10, and M12 primary meningioma cells were cultured in Neurobasal Medium (Thermo Fisher Scientific) supplemented with EGF and FGF (Sigma-Aldrich), B27 and N2 (Thermo Fisher Scientific), glutamine, and 5% fetal bovine serum (Table S8). BEN-MEN-1 and HBL-52 primary meningioma cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% newborn calf serum and glutamine (Mei et al., 2017). FOXM1 and CTNNB1 constructs in the pCMV6-Entry vector were obtained from OriGene (Rockville, MD) and transfected using Lipofectamine LTX

with Plus Reagent (Thermo Fisher Scientific). Mission shRNAs in the pLKO.1 vector were obtained from Sigma-Aldrich and transduced using lentivirus particles (Table S8). FDI-6 was obtained from Sigma-Aldrich and reconstituted in DMSO. All experiments were performed 72 hr after transfection, transduction, or initiation of pharmacologic treatment. Proliferation assays were performed using the Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit and a GloMax Discovery Multimode Microplate Reader (Promega).

### Statistical Analysis

All IHC and sequencing data were de-identified and processed irrespective of clinical parameters and outcomes. Scatterplots show median ± 95% CI. Student's unpaired t test, chi-square test, Fisher's exact test, and ANOVA were used to compare among groups. LRFS, DSS, and OS were estimated using the Kaplan-Meier method and compared using log rank tests. Statistical significance was defined as $p \leq 0.05$. Data were dichotomized at the mean for outcome analyses according to MIB1 labeling index, FOXM1 protein expression, somatic mutation burden, and NanoString targeted gene expression profiling. Hazard ratios (HR) were estimated via maximum likelihood using the Cox proportional-hazard model with RSEs via Stata version 13.1 (StataCorp). WHO grade II and grade III meningiomas were aggregated to create a composite independent variable for Cox models. Breslow's method was used to correct for ties.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS

We thank David Solomon, MD, PhD, for providing Ben-Men-1 and HBL-52 primary meningioma cell lines. The University of California, San Francisco, Brain Tumor SPORE Biospecimen and Pathology Core provided tissue, TMAs, and histologic services (NIH grant P50CA097257). This work was supported by grants from the Linda Wolfe Meningioma Research Fund, the Goddard Foundation, and the University of California, San Francisco, Physician Scientist Scholar Program to D.R.R. and from the NIH (1F32CA213944-01) to S.T.M.

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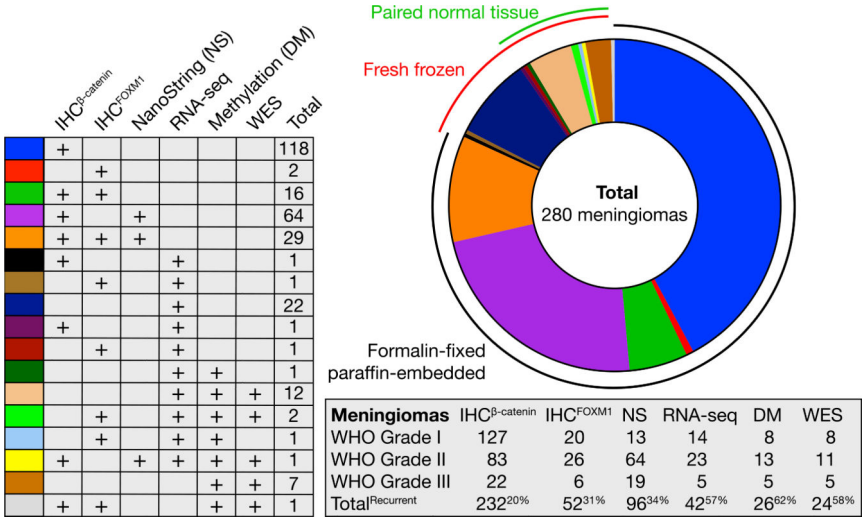
## Highlights

- Genomic, epigenomic, and transcriptomic factors identify meningioma molecular subgroups
- FOXM1 expression delineates aggressive meningiomas across molecular subgroups
- FOXM1/Wnt signaling is associated with mitotic gene expression in aggressive meningioma
- FOXM1 signaling drives primary meningioma cell proliferation




| Meningiomas | IHCβ-catenin | IHCFOXM1 | NS | RNA-seq | DM | WES |
|---|---|---|---|---|---|---|
| WHO Grade I | 127 | 20 | 13 | 14 | 8 | 8 |
| WHO Grade II | 83 | 26 | 64 | 23 | 13 | 11 |
| WHO Grade III | 22 | 6 | 19 | 5 | 5 | 5 |
| TotalRecurrent | 23220% | 5231% | 9634% | 4257% | 2662% | 2458% |

**Figure 1. Study Design**

Comprehensive molecular profiling strategy of meningioma ($IHC^{\beta\text{-catenin}}$, β-catenin IHC; $IHC^{FOXM}$, FOXM1 IHC; IHC, immunohistochemistry; DM, 850K DNA methylation profiling; NS, NanoString targeted gene expression profiling; RNA-seq, RNA sequencing; WES, whole-exome sequencing).

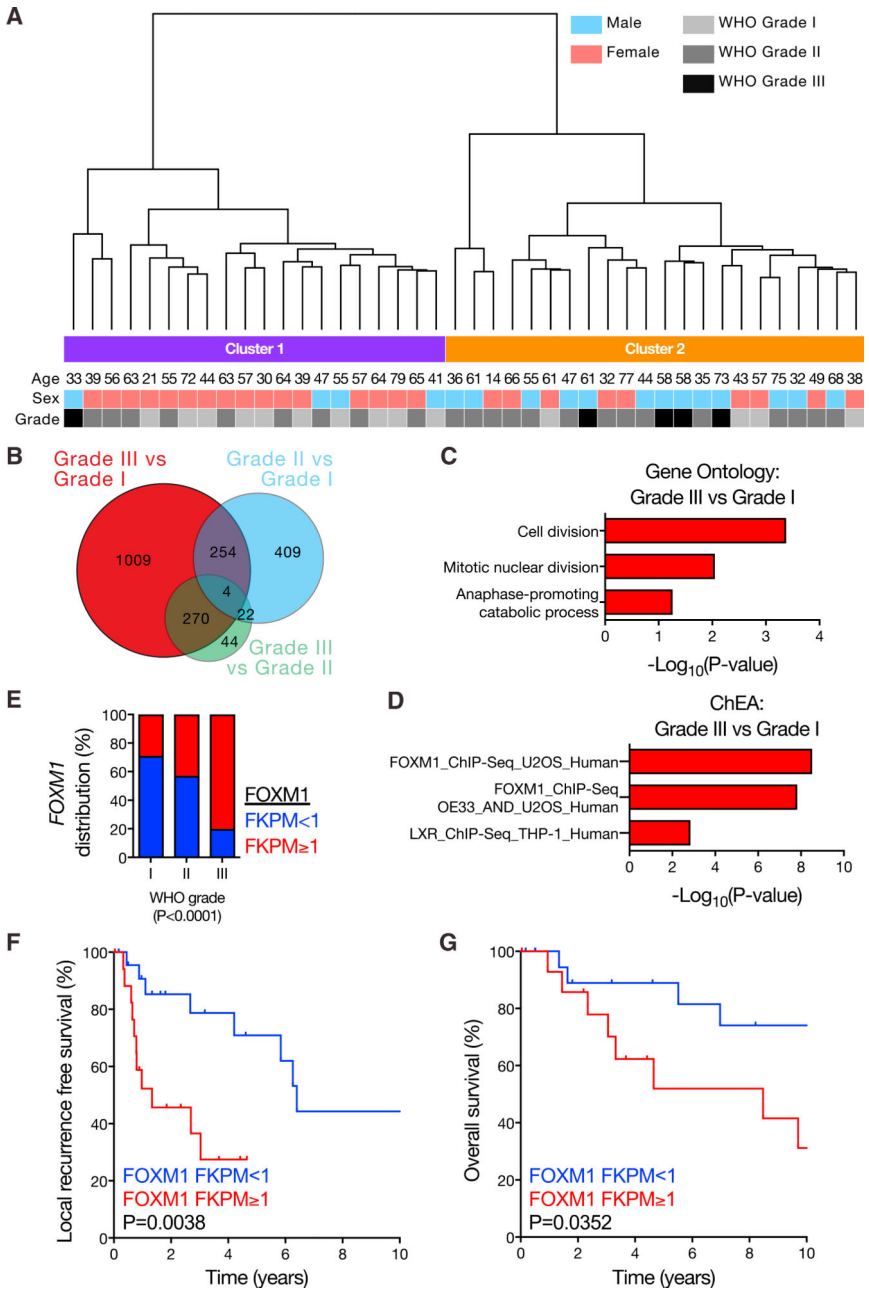



**Figure 2. RNA Sequencing Identifies Distinct Transcriptomic Clusters of Meningiomas and a Mitotic Signature Associated with FOXM1 Activity in Aggressive Tumors**

(A) Unsupervised hierarchical clustering on the basis of the top 2,000 most variable genes segregates meningiomas into two transcriptomic clusters (n = 42).

(B) Differential expression analysis of RNA-seq data on the basis of meningioma grade reveals a total of 2,012 significant genes ($q < 0.1$) with the greatest differences between WHO grade I and grade III meningiomas.

(C) GO analysis for biological processes shows enrichment for cell division genes in WHO grade III meningiomas compared with WHO grade I meningiomas.

(D) ChEA identifies FOXM1 as a putative regulator of the transcriptomic signature in WHO grade III meningiomas.
(E) High-grade meningiomas are enriched in *FOXM1* mRNA by RNA-seq (fragments per kilobase of transcript per million reads, FKPM).
(F and G) Meningiomas with *FOXM1* FKPM ≥ 1 are associated with poor LRFS (F) and OS (G) relative to meningiomas with *FOXM1* FKPM < 1.

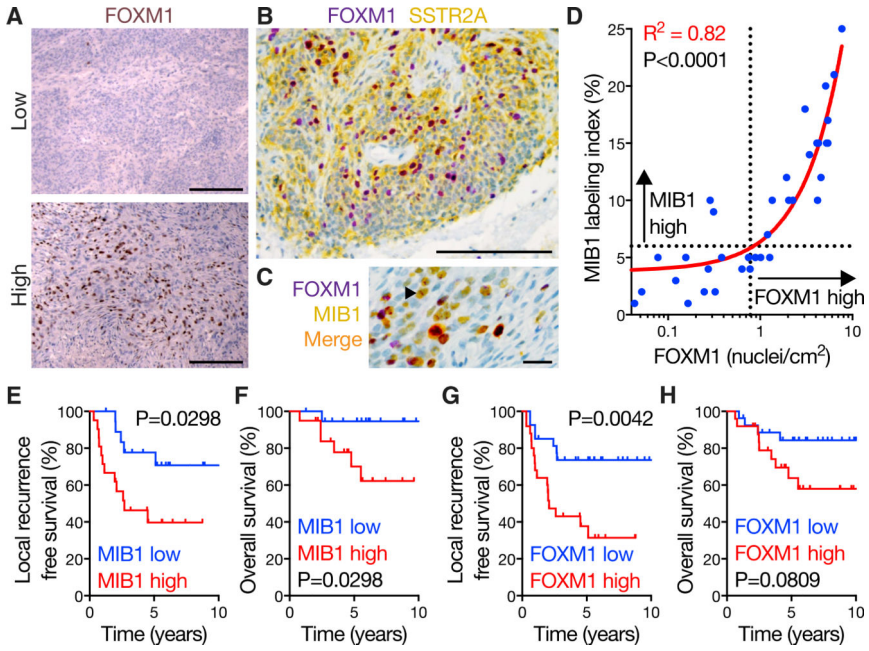



**Figure 3. FOXM1 Protein Expression Is Associated with Meningioma Proliferation and Poor Clinical Outcomes**

(A) Hematoxylin staining with IHC for FOXM1 from two meningiomas reveals significant intra- and inter-tumor heterogeneity of FOXM1 expression. Scale bars, 100 μm.

(B) Co-IHC for FOXM1 and the meningioma cell marker SSTR2A demonstrates that FOXM1 expression is restricted to a subpopulation of meningioma cells. Scale bar, 100 μm.

(C) Co-IHC for FOXM1 and the cell proliferation marker Ki-67, denoted by the MIB1 clone, shows that proliferating meningioma cells are characterized by nuclear FOXM1 which decorates mitotic spindles (arrowhead). Scale bar, 25 μm.

(D) Meningioma proliferation, denoted by MIB1 labeling index of the cell proliferation marker Ki-67, is associated with FOXM1 expression ($p < 0.0001$, $n = 38$ meningiomas). Dashed lines denote thresholds for subsequent outcomes analyses according to MIB1 labeling index and FOXM1 expression levels.

(E and F) Elevated meningioma MIB1 labeling index is associated with poor LRFS (E) and OS (F) relative to meningiomas with low MIB1 labeling index ($n = 40$).

(G and H) Elevated meningioma FOXM1 protein expression is associated with poor LRFS (G) and OS (H) relative to meningiomas with low FOXM1 protein expression ($N = 52$).

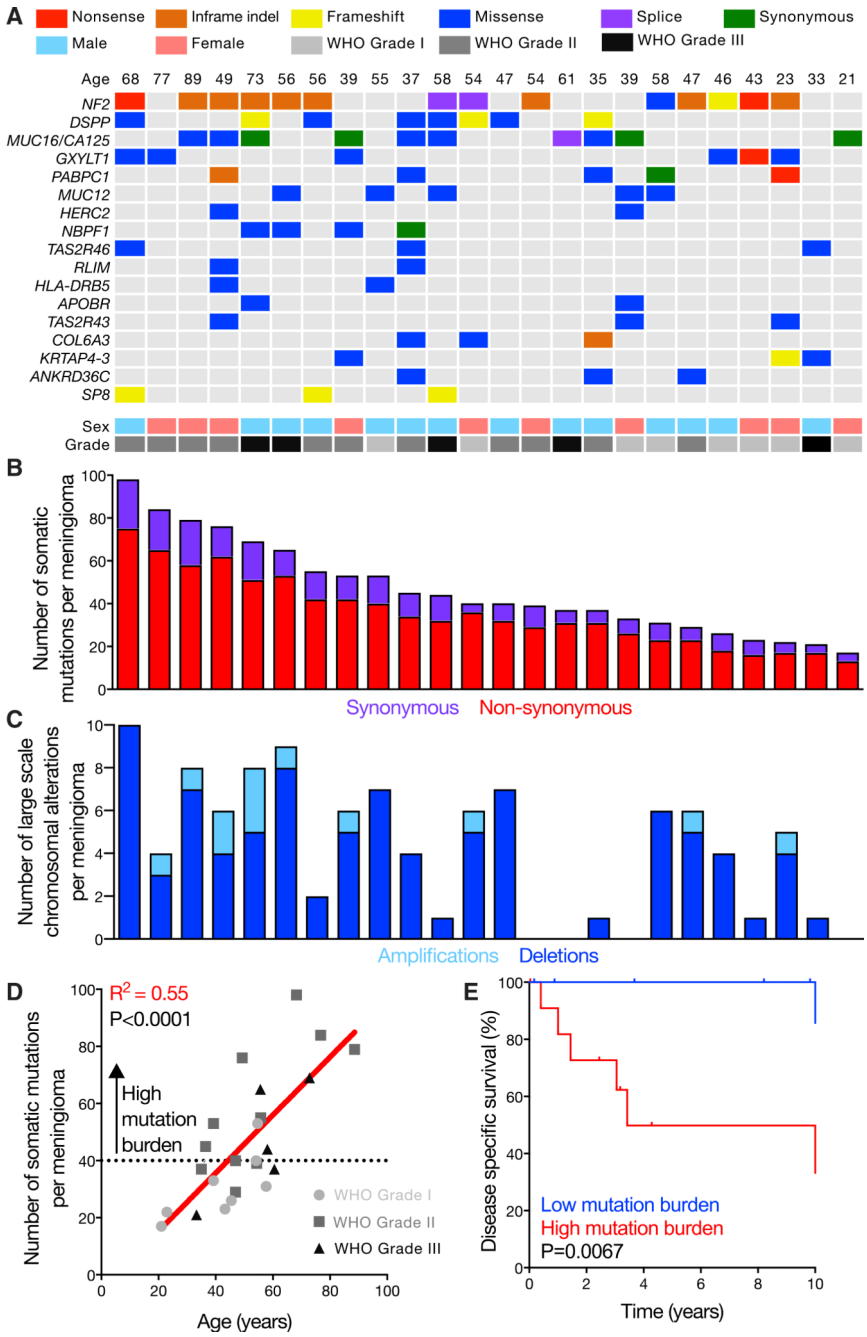



**Figure 4. Whole-Exome Sequencing Reveals High Somatic Mutation Burden in Aggressive Meningiomas**

(A) Analysis of recurrent somatic mutations occurring in at least three tumors confirms that *NF2* is the most commonly mutated gene in aggressive meningioma (n = 24).

(B) The number of somatic mutations per meningioma ranges from 17 to 98, with 4–23 synonymous mutations and 13–75 nonsynonymous mutations.

(C) The number of large-scale chromosomal alterations per meningioma, defined as comprising greater than one-third of a chromosomal arm, ranges from 0 to 10, with 0–3 amplifications and 0–10 deletions.

(D) The number of somatic mutations per meningioma is associated with patient age for all meningioma grades ($p < 0.0001$). Dashed line denotes median somatic mutation count used as a cutoff for subsequent outcomes analyses according to mutation burden.
(E) High meningioma somatic mutation burden is associated with poor DSS relative to low meningioma somatic mutation burden.

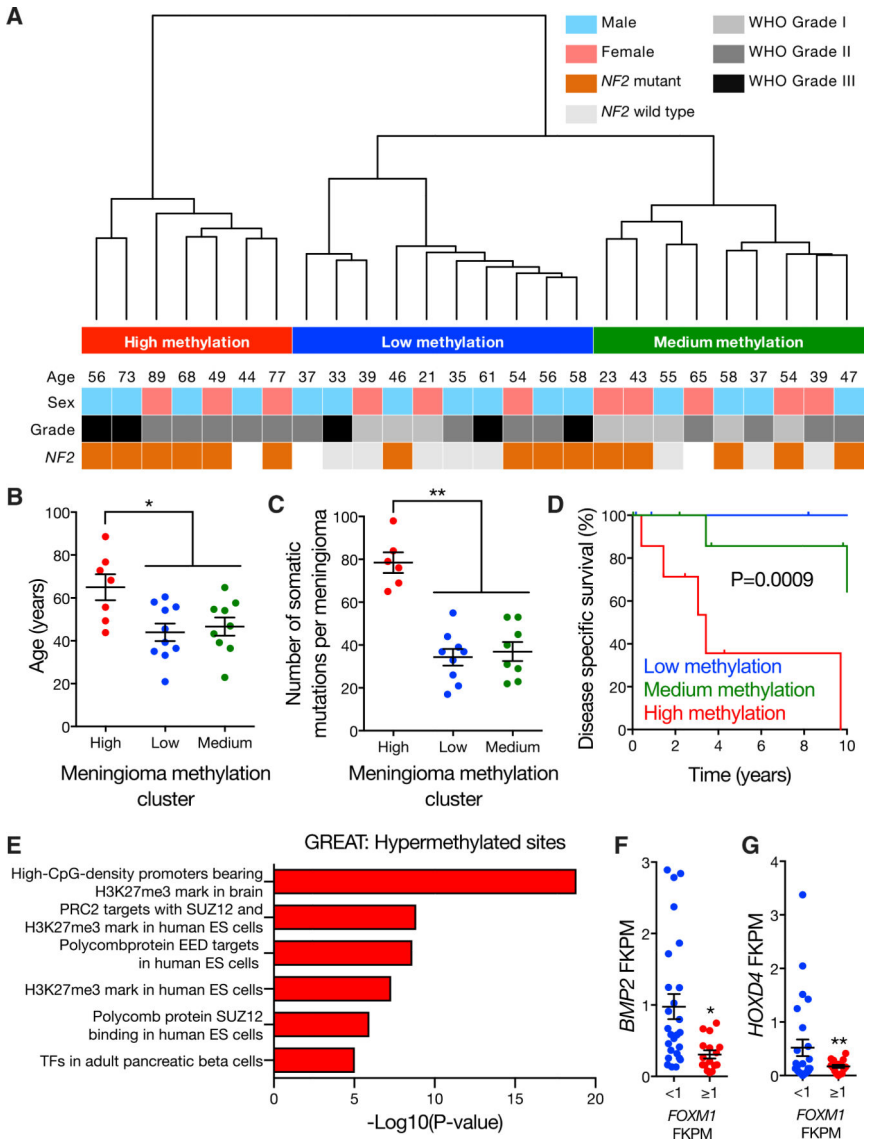



**Figure 5. DNA Methylation Profiling Identifies Distinct Epigenomic Clusters that Are Associated with Meningioma Somatic Mutation Burden and Clinical Outcomes**

(A) Unsupervised hierarchical clustering on the basis of the top 2,000 most variable probes segregates meningiomas into three clusters according to high, low, and medium DNA methylation (n = 26). (B and C) High meningioma DNA methylation is associated with increased patient age (B) and elevated somatic mutation burden (C) relative to low and medium meningioma DNA methylation clusters (*p = 0.0123, **p < 0.0001).

(D) High meningioma DNA methylation is associated with poor DSS relative to medium and low meningioma methylation clusters.

(E) GREAT results for differentially methylated sites between high and low meningioma DNA methylation clusters identifies hypermethylation of H3K27me3 and PRC targets in the high-methylation cluster.

(F and G) Meningiomas with increased *FOXM1* mRNA expression by RNA-seq display decreased expression of PRC target genes *BMP2* (F) and *HOXD4* (G) (*p = 0.0010, **p = 0.0393).

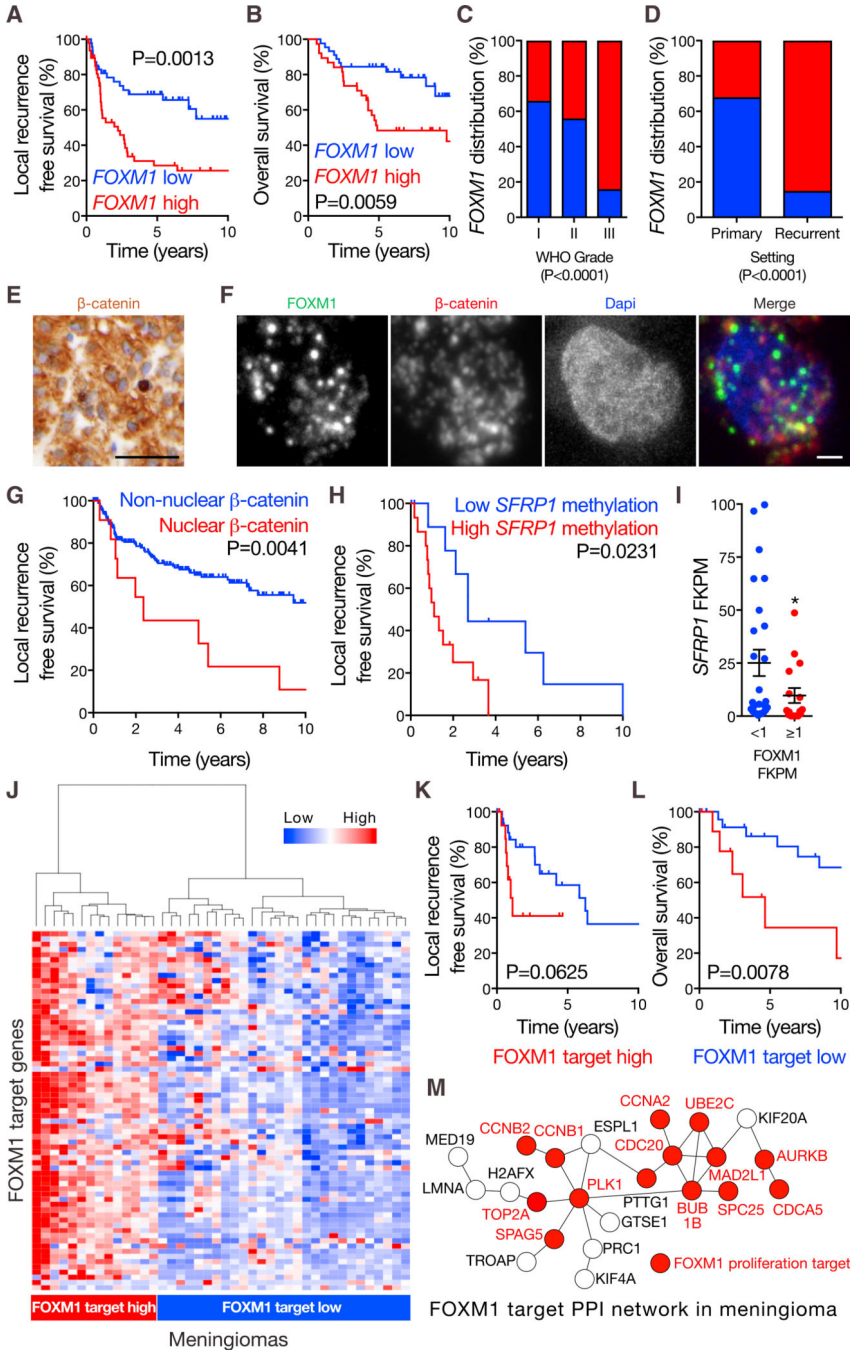



**Figure 6. A FOXM1/Wnt Signaling Axis Promotes Cell Proliferation Gene Expression in Aggressive Meningioma**

(A and B) Elevated meningioma *FOXM1* mRNA expression by NanoString targeted gene expression profiling is associated with poor LRFS (A) and OS (B) relative to meningiomas with low *FOXM1* mRNA expression (n = 96).

(C and D) High-grade and recurrent meningiomas are enriched in *FOXM1* mRNA by NanoString gene expression profiling. Red denotes *FOMX1*-high tumors, and blue denotes *FOXM1*-low tumors.

(E) Meningioma β-catenin staining by IHC shows abundant cytoplasmic and occasional nuclear staining. Scale bar, 20 μm.
(F) Meningioma FOXM1 and β-catenin nuclear staining and co-localization by immunofluorescence. Scale bar, 2 μm.
(G) Meningioma nuclear β-catenin staining by IHC (n = 11) is associated with poor LRFS relative to meningiomas without nuclear β-catenin staining (n = 221).
(H) Hypermethylation of the *SFRP1* promoter by DNA methylation profiling is associated with poor LRFS relative to meningiomas without *SFRP1* promoter hypomethylation (n = 24).
(I) Meningiomas with elevated *FOXM1* mRNA expression by RNA-seq display decreased *SFRP1* expression (*p = 0.0378).
(J) Unsupervised hierarchical clustering of meningiomas according to FOXM1 target gene expression (n = 69) by RNA-seq segregates tumors into two clusters (n = 42).
(K and L) Elevated meningioma FOXM1 target gene expression is associated with poor LRFS (K) and OS (L) relative to meningiomas with low FOXM1 target gene expression.
(M) A FOXM1 target protein-protein interaction (PPI) network constructed from genes enriched in high-grade meningiomas by RNA-seq highlights a cell proliferation program.

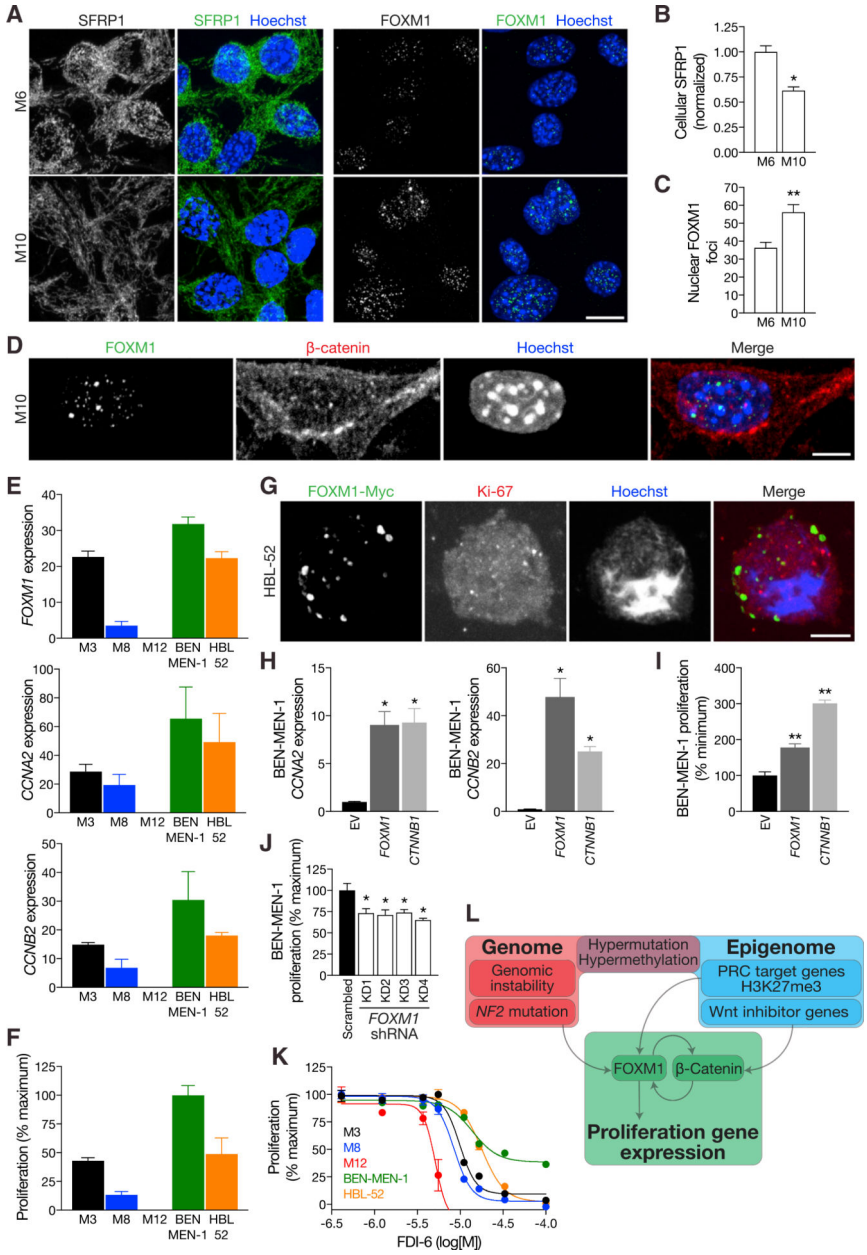



**Figure 7. FOXM1/Wnt Signaling Drives Primary Meningioma Cell Proliferation**

(A–C) Immunofluorescence reveals an inverse relationship between SFRP1 and FOXM1 expression in M6 and M10 primary meningioma cells. Scale bar, 10 μm (*p < 0.0001, **p = 0.0008).

(D) Immunofluorescence shows cytoplasmic and nuclear β-catenin staining in M10 primary meningioma cells. Scale bar, 5 μm.

(E and F) Real-time qPCR and tetrazolium assays show a strong association between expression of *FOXM1* (E), expression of FOXM1 target genes *CCNA2* and *CCNB2* (E), and proliferation of M3, M8, M12, BEN-MEN-1, and HBL-52 primary meningioma cells (F).

(G) Overexpression of FOXM1-Myc induces HBL-52 primary meningioma cell proliferation as demonstrated by nuclear Ki-67 staining. Scale bar, 5 μm.

(H and I) Real-time qPCR and tetrazolium assays demonstrate that overexpression of *FOXM1* or *CCNB1* induces expression of FOXM1 target genes *CCNA2* and *CCNB2* (H) and proliferation of BEN-MEN-1 primary meningioma cells (I) (*$p < 0.002$, **$p < 0.02$).
(J and K) Tetrazolium assays in primary meningioma cells transduced with *FOXM1* shRNAs, or treated with the FOXM1 antagonist FDI-6, show that *FOXM1* knockdown (KD) and FOXM1 pharmacologic inhibition blocks primary meningioma cell proliferation (*$p < 0.03$).
(L) An integrated molecular model of aggressive meningiomas, which are characterized by DNA hypermutation and hypermethylation. *NF2* mutation stabilizes FOXM1 protein, and hypermethylation of H3K27me3 and PRC target genes facilitates a functional switch toward a FOXM1 transcriptional program. Hypermethylation of Wnt antagonists, such as *SFRP1*, activates β-catenin to cooperate with FOXM1 to drive expression of cell proliferation genes.