Discussion

Although other brain tumors have adopted molecular definitions in the most recent WHO classification[1], meningiomas continue to be classified by microscopic assessment of histopathological features alone into three grades. While this classification has some advantages in that it can be widely applied in conventional neuropathology laboratories, the clinical behaviour of meningiomas does not consistently conform to their WHO grade[2,3]. Some histologically benign meningiomas recur rapidly despite adequate resection and conversely a subset of higher grade meningiomas remain stable after surgery without evidence of early disease recurrence. Additionally, options for medical therapies are limited for patients with aggressive tumors that recur relentlessly despite multiple rounds of surgery and radiation. Easily adopted molecular classifications of meningioma that reliably reflect tumor behaviour and inform on novel medical therapies are greatly needed.

Landmark genetic and epigenetic studies on meningioma have provided important insights into the possibility of refining meningioma classification using molecular data. These studies have principally performed unsupervised analyses of a single molecular datatype, most commonly DNA methylation or mutational analyses, with correlations to other datatypes, based on availability[4–15]. In other cancers, formal integration of multiple molecular datatypes has led to the discovery of clinically and biologically relevant consensus groups that cannot be identified from any single datatype alone[16–18]. However, integration of molecular platforms has not been explored yet in meningiomas, or other non-malignant tumor types, in part due to the very few number of samples that have concurrent genome-wide data across the primary platforms of DNA methylation, whole-genome or whole-exome sequencing, and RNA sequencing[7,11,13,14]. Here, we assembled a large cohort enriched for higher-grade meningiomas with extended longitudinal clinical data (up to 15-year follow-up) and integrated matched DNA somatic copy number, DNA point mutation, DNA methylation, transcriptome, and proteomic data in order to robustly subtype meningiomas into four novel consensus molecular groups. We demonstrated that meningiomas in each molecular group had distinctive clinical outcomes that superseded existing WHO and other molecular classifications in two independent cohorts, and showed that the novel, prototypical biology of each group could inform selection of novel medical therapies (see **Extended Data Fig. 12**)

Building on the known distinction between NF2-mutant and non-NF2 mutant meningiomas[5,8] we comprehensively characterized two novel consensus groups of benign meningiomas. The first

(MG1) is composed of NF2-mutant meningiomas with concurrent loss of chromosome 22q that are characterized by predominantly myeloid immune cell infiltration which we confirmed using single cell RNA sequencing, deconvolution of bulk RNA profiles, and protein data. These immunogenic meningiomas showed benign behaviour with low recurrence rates, suggesting that immunogenicity may prevent rapid tumor growth in this consensus group. Elegant studies have recently shown that the meninges are a hub for homeostatic immunity, with the bone marrow of the adjacent skull being the primary source of myeloid cells in healthy meninges and in the setting of an inflamed CNS. As meningiomas arise from the meninges and can frequently invade the adjacent skull, it is possible that immune cells are recruited through similar mechanisms in immunogenic meningiomas[19,20].

The second benign molecular group (MG2) with favourable outcomes were comprised of meningiomas with either driver mutations known to be mutually exclusive with NF2 (TRAF7, AKT1, KLF4, SMO) or meningiomas with several polysomies, including those previously reported in angiomatous and microcystic meningiomas[21,22]. Concordant with the copy number findings, vasculature and angiogenic pathways were upregulated in MG2 meningiomas by gene expression and protein data. Deconvolution of bulk RNA data using single cell signatures showed enrichment of endothelial cells in these tumors. Two of the MG2 tumors in our study harbored NF2 mutations and CNA that were atypical of MG2 meningiomas, suggesting there could be room for refinement of automated assignments from unsupervised clustering using mutational data.

Although previous methylation-based classifications have identified groups of meningiomas with poor outcomes[4,23,24], very little has been discovered with respect to the drivers of aggressiveness in these tumors, despite their overwhelming contribution to the morbidity of disease in these patients. The robust representation of these less common, but more aggressive meningioma phenotypes, together with the comprehensive molecular analyses we have undertaken enabled important discoveries in this regard. Specifically, we defined two distinct groups of aggressive meningiomas, that were driven by either a hypermetabolic (MG3) or proliferative biological phenotype (MG4). These tumors harbored novel statistically significant and recurrent driver mutations in the epigenetic regulator gene *KDM6A* and tumor suppressor gene *PTEN*. Point mutations in *PTEN* and *KDM6A* have each been previously reported in only one of over 1000 tumors profiled by unbiased mutational sequencing in the literature[8,25]. The fact that these mutations occur at rate similar to other known meningioma driver genes in our

study likely reflects the enrichment of the more clinically aggressive tumors in our cohort[5,8,10]. The identification of *PTEN* as a driver mutation implicates a divergence in the PIK3/AKT/mTOR pathway that separates meningiomas with more aggressive behavior from meningiomas with downstream AKT1 mutations that generally have a more indolent course. Additional subthreshold mutational hits were identified suggesting that more focused mutational analyses of more aggressive meningiomas may reveal additional driver mutations yet to be discovered. In addition to the above, we found that MG3 tumors, and to a greater extent MG4 tumors, showed significant genomic instability that was not observed in either immunogenic (MG1) or benign NF2-wildtype (MG2) meningiomas and were associated with patterns of aberrant DNA methylation and enrichment for critical master regulators of cell proliferation in MG4 tumors. Altogether, these findings highlight convergence of multiple distinct but related alterations, as opposed to one alteration in isolation, contribute to aggressive behavior in meningiomas.

Importantly, the establishment of molecular groups facilitated the discovery of novel therapeutic vulnerabilities. Vorinostat, a histone deacetylase inhibitor approved for the treatment of other cancers[26], targeted genes that were uniquely enriched in proliferative (MG4) meningiomas. By developing faithful models of meningiomas that align to the molecular groups we described here, we showed that Vorinostat had viable anti-proliferative effects on only MG4 meningioma cells *in-vitro* and *in-vivo*. Although the molecular mechanisms behind Vorinostat's therapeutic effect need to be deciphered, these promising results warrant further investigation in human studies that would be most informative in a setting where patient's meningiomas can be stratified according to their MGs.

The multiplicity of molecular features that were specific for each group also facilitates implementation of these findings clinically, even in centers without comprehensive molecular testing. For example, we show that loss of chr 1p is almost universally seen in MG3 and MG4 meningiomas and essentially absent in MG1 and MG2 meningiomas. As well, gain of chr 1q was highgly specific to MG4 tumors. Similarly, the novel mutations we describe in *PTEN* and *KDM6A* were exclusive to MG3 and MG4 meningiomas. As most conventional neuropathology labs are familiar with chr 1p analyses and mutational analyses for *PTEN* as part of the diagnostic work-up for gliomas, these approaches can be immediately applied to identify higher risk meningiomas and their MGs. We also identified protein marks that were enriched in each MG using proteomic data, and validated one protein-mark for each MG by blinded immunohistochemical, further demonstrating the potential for these novel MGs to be

distinguished using conventional techniques familiar to all neuropathology labs. It is possible that staining for multiple group-specific proteins may further improve the discrimination of MG groups beyond what was reported in our study. With further careful validation and standardization of staining, scoring, and reporting, these immunohistochemical markers, as well as the common molecular alterations we defined for each group, may be considered for inclusion in future iterations of recognized grading systems.

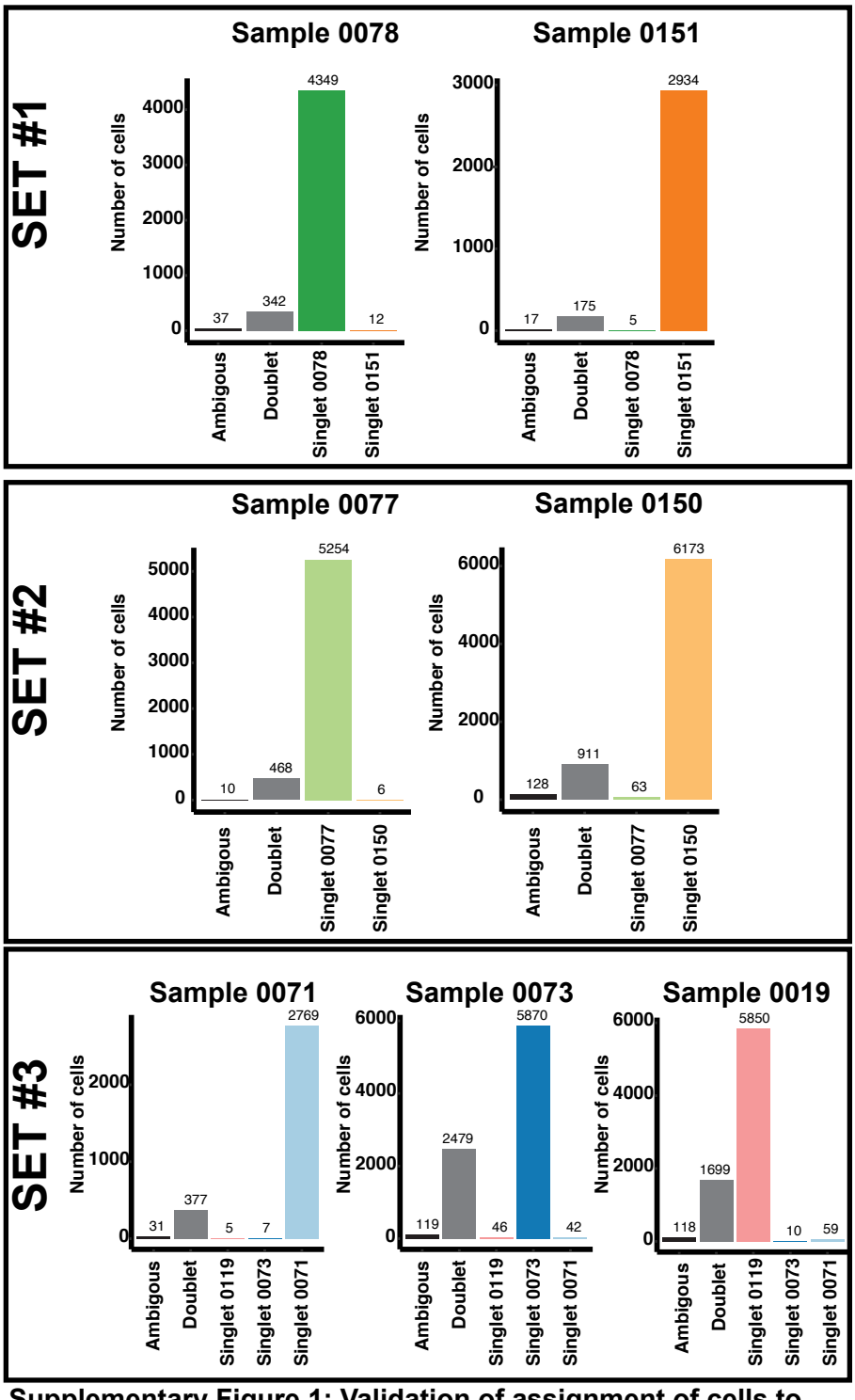
Lastly, we reported on the first single cellular RNA sequencing analysis of human meningioma tumors. In line with other cancers, we found that the variation between neoplastic cells of different tumors was more prominent than variation within the same tumor[27–31]. Most tumors harbored a single dominant cluster of cells. In some cases, we found a smaller second cluster of cells that generally reflected cycling neoplastic cells. In addition to this, clonality assessments of bulk mutational profiles revealed that most mutations in meningioma were clonal. We did, however, identify four recurrent meta-expression programs (cell cycle, metabolism, inflammatory, mesenchymal) from our single cell data. With the exception of the dominant cell cycle program, these represented subtle continuous patterns of variation in gene expression, with differences in the distribution of activation of these programs across MGs. For example, the inflammatory program was generally inactive in MG4 tumors compared to the others. The genes in this inflammatory program notably were shown to also be repressed in a general oncogenic program of synovial sarcoma[32]. This may reflect neoplastic-immune cell crosstalk that merits further and more focused investigation in larger cohorts.

In summary, we have generated a key TCGA-style resource for meningiomas, the most common primary intracranial tumor. Integration of multiple comprehensive datatypes in a single analysis yielded novel findings that had biological, prognostic and therapeutic relevance. We described patterns of intra- and intertumoral heterogeneity in meningioma by coupling single cell and bulk analyses, providing orthogonal support for our proposed consensus molecular classification.

References (Discussion)

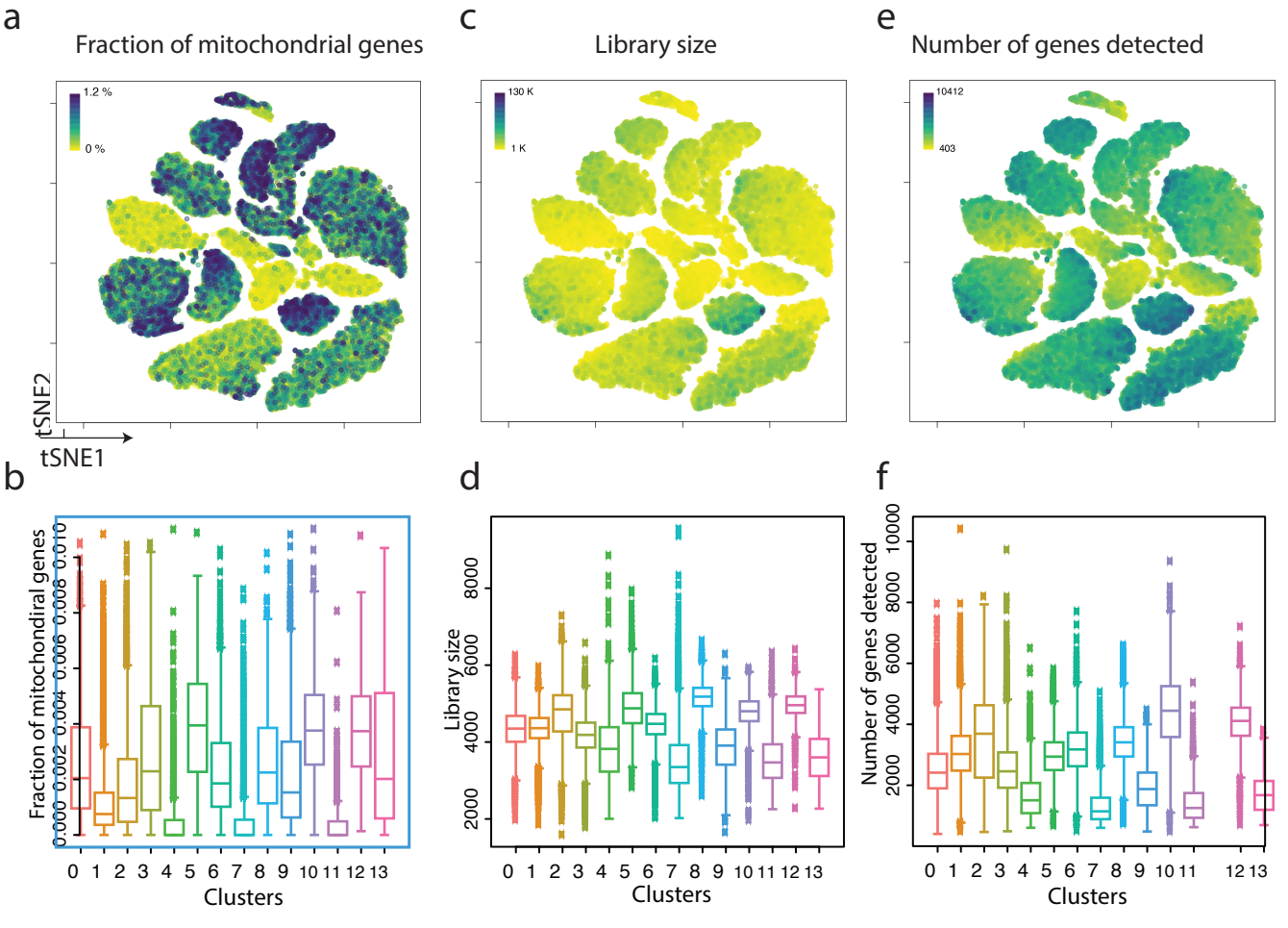
1. Louis, D. N. *et al.* The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol.* **131**, 803–20 (2016).
2. Goldbrunner, R. *et al.* EANO guidelines for the diagnosis and treatment of meningiomas. *Lancet. Oncol.* **17**, e383-91 (2016).
3. Nassiri, F., Tabatabai, G., Aldape, K. & Zadeh, G. Challenges and opportunities in meningiomas: recommendations from the International Consortium on Meningiomas. *Neuro. Oncol.* **21**, i2–i3 (2019).
4. Sahm, F. *et al.* DNA methylation-based classification and grading system for meningioma: a multicentre, retrospective analysis. *Lancet Oncol.* **18**, 682–694 (2017).
5. Clark, V. E. *et al.* Genomic analysis of non-NF2 meningiomas reveals mutations in TRAF7, KLF4, AKT1, and SMO. *Science (80-.).* **339**, 1077–1080 (2013).
6. Patel, A. J. *et al.* Molecular profiling predicts meningioma recurrence and reveals loss of DREAM complex repression in aggressive tumors. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 21715–21726 (2019).
7. Collord, G. *et al.* An integrated genomic analysis of anaplastic meningioma identifies prognostic molecular signatures. *Sci. Rep.* **8**, 13537 (2018).
8. Brastianos, P. K. *et al.* Genomic sequencing of meningiomas identifies oncogenic SMO and AKT1 mutations. *Nat. Genet.* **45**, 285–289 (2013).
9. Reuss, D. E. *et al.* Secretory meningiomas are defined by combined KLF4 K409Q and TRAF7 mutations. *Acta Neuropathol.* **125**, 351–358 (2013).
10. Clark, V. E. *et al.* Recurrent somatic mutations in POLR2A define a distinct subset of meningiomas. *Nat. Genet.* **48**, 1253–1259 (2016).
11. Harmancl, A. S. *et al.* Integrated genomic analyses of de novo pathways underlying atypical meningiomas. *Nat. Commun.* **8**, 14433 (2017).
12. Bi, W. L. *et al.* Genomic landscape of high-grade meningiomas. *NPJ genomic Med.* **2**, (2017).
13. Vasudevan, H. N. *et al.* Comprehensive Molecular Profiling Identifies FOXM1 as a Key Transcription Factor for Meningioma Proliferation. *Cell Rep.* **22**, 3672–3683 (2018).
14. Paramasivam, N. *et al.* Mutational patterns and regulatory networks in epigenetic subgroups of meningioma. *Acta Neuropathol.* **138**, 295–308 (2019).
15. Youngblood, M. W. *et al.* Correlations between genomic subgroup and clinical features in a cohort of more than 3000 meningiomas. *J. Neurosurg.* 1–10 (2019) doi:10.3171/2019.8.JNS191266.
16. Hoadley, K. A. *et al.* Cell-of-Origin Patterns Dominate the Molecular Classification of 10,000 Tumors from 33 Types of Cancer. *Cell* **173**, 291-304.e6 (2018).
17. Koboldt, D. C. *et al.* Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61–70 (2012).
18. Cancer Genome Atlas Research Network *et al.* Comprehensive, Integrative Genomic Analysis of Diffuse Lower-Grade Gliomas. *N. Engl. J. Med.* **372**, 2481–98 (2015).
19. Rustenhoven, J. *et al.* Functional characterization of the dural sinuses as a neuroimmune interface. *Cell* **184**, 1000-1016.e27 (2021).
20. Cugurra, A. *et al.* Skull and vertebral bone marrow are myeloid cell reservoirs for the meninges and CNS parenchyma. *Science (80-.).* eabf7844 (2021) doi:10.1126/science.abf7844.
21. Abedalthagafi, M. S. *et al.* Angiomatous meningiomas have a distinct genetic profile with multiple chromosomal polysomies including polysomy of chromosome 5. *Oncotarget* **5**, 10596–606 (2014).
22. Kuroi, Y. *et al.* Identification of shared genomic aberrations between angiomatous and microcystic meningiomas. *Neuro-Oncology Adv.* **1**, (2019).
23. Nassiri, F. *et al.* DNA methylation profiling to predict recurrence risk in meningioma:

development and validation of a nomogram to optimize clinical management. *Neuro. Oncol.* **21**, 901–910 (2019).
24. Olar, A. *et al.* Global epigenetic profiling identifies methylation subgroups associated with recurrence-free survival in meningioma. *Acta Neuropathol.* **133**, 431–444 (2017).
25. Peters, N. *et al.* Analysis of the PTEN gene in human meningiomas. *Neuropathol. Appl. Neurobiol.* **24**, 3–8 (1998).
26. Reardon, D. A. *et al.* Phase II study of Gleevec® plus hydroxyurea (HU) in adults with progressive or recurrent meningioma. *J. Neurooncol.* **106**, 409–15 (2012).
27. Venteicher, A. S. *et al.* Decoupling genetics, lineages, and microenvironment in IDH-mutant gliomas by single-cell RNA-seq. *Science (80-.).* **355**, eaai8478 (2017).
28. Izar, B. *et al.* A single-cell landscape of high-grade serous ovarian cancer. *Nat. Med.* **26**, 1271–1279 (2020).
29. Tirosh, I. *et al.* Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science (80-.).* **352**, 189–196 (2016).
30. Patel, A. P. *et al.* Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science (80-.).* **344**, 1396–1401 (2014).
31. Kinker, G. S. *et al.* Pan-cancer single-cell RNA-seq identifies recurring programs of cellular heterogeneity. *Nat. Genet.* **52**, 1208–1218 (2020).
32. Lawrence, M. S. *et al.* Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* **499**, 214–218 (2013).



Supplementary Figure 1: Validation of assignment of cells to patients

Shown are barplots of cellular genotypes derived from comparing germline SNPs from snRNA-seq data to SNPs derived from bulk RNA-seq data for each sample. Samples that were processed at similar times were considered as "Sets". Overall, there were very few cells with inconsistent genotypes to the sample of origin (0.1 to 0.9% of all cells). For example, in Set 1, only 12 cells (0.2%) from sample CAM_0078 were considered singlets of another sample in this Set (CAM_0151). Cells with genotypes that aligned to the expected sample were retained and reported on in this manuscript.



Supplementary Figure 2: High-quality data from filtered cells are retained for analyses.
a,c,e, Uniform Manifold Approximation and Projection of snRNA-seq data as in Fig. 4a. The fraction of mitochondrial genes (a), library size (b), and number of genes detected (c) are shown as color gradients for each cluster.
b,d,f, Boxplots of quality control metrics by cluster assignments from Fig. 5b for fraction of mitochondrial genes (b), library size (d), and number of genes detected (f).