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1 **IDHwt and IDHmut adult-type diffuse gliomas display distinct alterations in ribo-**
2 **somal biogenesis and 2'O-methylation of ribosomal RNA**

3
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44

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47 tors

48

49 **Running title**

50 Ribosome alterations in high-grade gliomas

51

52 **Issue section**

53 Basic and translational investigation

54

55 **Key points (3)**

56 • rRNA 2'Ome profiling distinguishes IDHwt and IDHmut adult-type diffuse glioma-
57 mas

58 • Elevated expression of ribosome biogenesis factors is correlated with IDH mu-
59 tational status

60 • High grade adult-type diffuse gliomas differentially respond to the RNA Pol I
61 inhibitors BMH-21 and CX5461

62

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64

65 **Abstract**

66

67 **Background:** High-grade adult-type diffuse gliomas (HGGs) constitute a heterogene-
68 ous group of aggressive tumors that are mostly incurable. Recent advances highlight-
69 ing the contribution of ribosomes to cancer development have offered new clinical per-
70 spectives. Here, we uncovered that IDHwt and IDHmut HGGs display distinct altera-
71 tions of ribosome biology, in terms of rRNA epitranscriptomics and ribosome biogene-
72 sis, which could constitute novel hallmarks that can be exploited for the management
73 of these pathologies.

74 **Methods:** We analyzed (i) the ribosomal RNA 2'O-ribose methylation (rRNA 2'Ome)
75 using RiboMethSeq and in-house developed bioinformatics tools
76 (<https://github.com/RibosomeCRCL/ribomethseq-nf> and [rRMSAnalyzer](#)) on three inde-
77 pendent cohorts compiling 71 HGGs (IDHwt n=30, IDHmut n=41) and 9 non-neoplastic
78 samples, (ii) the expression of ribosome biogenesis factors using medium throughput
79 RT-qPCR as a readout of ribosome biogenesis, and (iii) the sensitivity of 5 HGG cell
80 lines to RNA Pol I inhibitors (CX5461, BMH21).

81 **Results:** Unsupervised analysis demonstrated that HGGs could be distinguished
82 based on their rRNA 2'Ome epitranscriptomic profile, with IDHwt glioblastomas dis-
83 playing the most significant alterations of rRNA 2'Ome at specific sites. In contrast,
84 IDHmut HGGs are largely characterized by an overexpression of ribosome biogenesis
85 factors compared to non-neoplastic tissues or IDHwt glioblastomas. Finally, IDHmut
86 HGG-derived spheroids display higher cytotoxicity to CX5461 than IDHwt glioblas-
87 toma, while all HGG spheroids display a similar cytotoxicity to BMH-21.

88 **Conclusion:** In HGGs, IDH mutational status is associated with specific alterations of
89 the ribosome biology and with distinct sensitivities to RNA Pol I inhibitors.

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92

93 **Importance of the study (146 words)**

94

95 Consistent multi-omics studies have shown that high-grade adult-type diffuse gliomas
96 (HGGs) can be classified into three main groups, *i.e.*, IDHmut and 1p/19q codeleted
97 oligodendrogliomas, IDHmut astrocytomas and IDHwt glioblastomas, based on their
98 genetic, transcriptomic and DNA methylation profiles. Recent advances have high-
99 lighted the contribution of ribosomes to cancer development and have offered new
100 clinical perspectives. **Herein, we show that ribosomal RNA (rRNA) epitranscriptomic
101 and ribosome biogenesis are different in distinct HGG types. We uncovered that IDHwt
102 glioblastomas display the most prominent defects in rRNA epitranscriptomics, whereas
103 IDHmut astrocytomas and oligodendrogliomas exhibit enhanced expression of ribo-
104 some biogenesis factors compared to IDHwt glioblastomas.** Moreover, based on their
105 IDH mutational status, HGG-derived cell lines displayed distinct responses to CX5461
106 and BMH-21, two clinically-evaluated inhibitors of the RNA Pol I that transcribes
107 rDNAs. This study identifies a connection between HGG oncogenesis and the ribo-
108 some biology, and highlights new therapeutic strategies.

109

110

111 Introduction

112 High-grade adult-type diffuse gliomas (HGGs) are brain tumors resembling glial cells
113 that display highly heterogeneous prognoses and treatment responses. HGGs com-
114 prise three main histomolecular types, astrocytomas, oligodendrogliomas and glioblas-
115 tomas, based notably on the mutational status of *isocitrate dehydrogenase (IDH) 1* and
116 2^{1,2}. Indeed, glioblastomas are IDHwt, whereas astrocytomas and oligodendrogliomas
117 are IDHmut, and can be further discriminated by the heterozygous 1p19q co-deletion
118 occurring in oligodendrogliomas. IDHwt and IDHmut HGGs are associated with distinct
119 epigenetic and transcriptomic dysregulations, leading to cancer-specific features.
120 Thus, despite important advances in their histomolecular classification and under-
121 standing of their oncogenesis, HGGs remain mostly incurable. For instance, grade 4
122 IDHwt glioblastoma patients treated with the conventional combination of surgery, ra-
123 diotherapy and temozolomide (TMZ) chemotherapy, display a median survival of only
124 15 months. Grade 3 IDHmut astrocytoma and oligodendroglioma patients treated with
125 radiotherapy and chemotherapy exhibit a much better outcome with a median survival
126 of 10 and 15 years, respectively, nevertheless 20 to 30% of the patients die within the
127 first five years after diagnosis^{3,4}. Therefore, the identification of novel molecular mech-
128 anisms dysregulated in distinct HGG histomolecular types may significantly improve
129 current therapeutic options.

130 Several studies highlighted that ribosome biogenesis (RiBi) and functions are altered
131 in cancer cells and that ribosomes can support oncogenic functions⁵. For instance, the
132 c-MYC oncogenic activity is in part supported by a dysregulation of genes implicated
133 in RiBi and global protein synthesis⁶. In addition, levels of RiBi are generally increased
134 in cancer cells to support the high protein synthesis demand caused by their exacer-
135 bated proliferation rate^{7,8} and therefore, the inhibition of rRNA synthesis specifically
136 kills cancer cells without affecting normal cells^{9,10}. Such observations led to the devel-
137 opment of molecules specifically inhibiting RiBi that showed objective responses in
138 clinical trials, such as CX5461¹¹⁻¹³.

139 In addition to alteration of RiBi, recent observations suggest that variations of ribosome
140 composition could also occur in cancer and be involved in disease etiology¹⁴⁻¹⁶. The
141 ribosome is composed of 80 ribosomal proteins and 4 ribosomal RNAs (rRNAs), the
142 latter supporting the enzymatic activity of the peptidyl-bond formation during the trans-
143 lation of mRNAs into proteins. For many decades, the ribosome was considered as a
144 monolithic entity displaying a similar composition in all cells constituting an organism.

145 However, it appears now that the ribosome composition can display some degree of
146 variations, both at the level of ribosomal proteins and rRNA chemical modifications,
147 which contributes to modulate intrinsic translational activities that could shape particu-
148 lar phenotypes^{17,18}. Variations of the ribosome composition at ribosomal protein levels
149 have been reported in HGGs^{19–22}. In IDHwt glioblastomas, the overexpression of the
150 ribosomal protein RPS6 was shown to promote acquisition of glioma stem cell proper-
151 ties, a hallmark of the most aggressive IDHwt glioblastomas^{20–22}. In addition, RPL22L1
152 isoforms are expressed in distinct regions of IDHwt glioblastomas through alternative
153 splicing and induce the production of ribosomes with specific compositions, which pro-
154 mote translational bias towards specific mRNA subsets^{15,19,23–30}. In addition to riboso-
155 mal proteins, the chemical modifications of rRNA represent one of the major contribu-
156 tors to ribosome heterogeneity and led to the emergence of the notion of rRNA epi-
157 transcriptomics¹⁵. One of the main modifications, the methylation of the rRNA 2'O-ri-
158 bose (rRNA 2'Ome), occurs at 106 known rRNA sites in humans and the 2'Ome at
159 specific positions are essential for rRNA activity. The catalysis of rRNA 2'Ome is per-
160 formed by an rRNA methylation complex composed of the methyl-transferase fibrillar
161 (FBL) and a single non-coding C/D box small nucleolar RNA (snoRNA or snoRD),
162 which guides FBL at specific sites by base-pairing¹⁵. Hence, modulations of *FBL* or
163 *snoRD* expression are sufficient to affect rRNA 2'Ome^{23,24,26}. Interestingly, alterations
164 of 2'Ome have been observed in cancer and we recently demonstrated by profiling 195
165 primary mammary tumors using the RiboMethSeq approach, that only 40% of the
166 known 2'O-methylated sites are altered, suggesting that only few rRNA sites can tol-
167 erate a lack of 2'Ome. Moreover, rRNA 2'Ome alterations are not random since rRNA
168 2'Ome profiles were associated with breast cancer subtypes and tumor grades²⁵. Sim-
169 ilarly, alterations of rRNA 2'Ome were described in a cohort of 17 diffuse large B-cell
170 lymphoma samples²⁷ and of 94 acute myeloid leukemia samples²⁸. Importantly, alter-
171 ations of rRNA 2'Ome at some specific sites can affect both the translation of particular
172 mRNA subsets and cell proliferation^{15,23,24,26 29,30}. To date, whether alterations of rRNA
173 epitranscriptomics occurs in gliomas and contributes to disease etiology remains un-
174 explored. Here, we investigated whether IDHwt and IDHmut HGGs display alterations
175 in ribosome biology, in terms of rRNA epitranscriptomics and ribosome biogenesis, to
176 exploit these features as novel therapeutic targets of these diseases.

177

178

179 **Materials and methods**

180

181 **Human grade 3-4 adult-type diffuse glioma and non-neoplastic samples**

182 Three cohorts were built: a technical cohort (8 grade 4 IDHwt glioblastomas, 3 non-
183 tumoral samples); a test cohort detailed in Table 1 (13 IDHwt glioblastomas, 13 IDHmut
184 astrocytomas, 14 IDHmut and 1p/19q codeleted oligodendrogliomas, 6 non-tumoral
185 samples); a validation cohort (9 IDHwt glioblastomas, 6 IDHmut astrocytomas, 8
186 IDHmut and 1p/19q co-deleted oligodendrogliomas). The percentage of tumoral cells
187 was estimated by a neuropathologist as described in Fig.S1. Additional details are pro-
188 vided in Supplementary Information.

189

190 **Cell culture**

191 Human IDHwt glioblastoma (5706, N131520), IDHmut astrocytoma (LGG85) and
192 IDHmut and 1p/19q codeleted oligodendroglioma (BT138, BT237) cells were cultured
193 as spheres as described in Supplementary Information.

194

195 **Reverse Transcription and real time quantitative PCR**

196 cDNA synthesis was performed using the Prime Script RT Reagent kit (Takara). Me-
197 dium throughput qPCR was performed using the Biomark HD system (Fluidigm) as
198 previously described³¹ (Table S1). The median Ct value of 5 housekeeping mRNAs
199 was used for normalization.

200

201 **RiboMethSeq**

202 RiboMeth-seq was performed as previously described using the Illumina sequencing
203 technology^{25,32}. To process the sequencing data, a novel nextflow pipeline Ribo-
204 MethSeq-nf was developed and is currently available ([https://github.com/Ri-
205 bosomeCRCL/ribomethseq-nf](https://github.com/RibosomeCRCL/ribomethseq-nf)). This pipeline processes sequencing data as previ-
206 ously described^{25,32,33}. To calculate the C-score, which reflects the rRNA 2'Ome level,
207 the novel R package rRMSAnalyzer was developed ([https://github.com/Ri-
208 bosomeCRCL/rRMSAnalyzer](https://github.com/RibosomeCRCL/rRMSAnalyzer)). The identification of significant alterations in rRNA
209 2'Ome levels between groups was performed by applying two consecutive thresholds:
210 the adjusted p-value < 0.05 (Kruskal-Wallis with FDR adjustment); and the mean Δ C-
211 score (*i.e.*, difference between the highest and lowest mean C-score of the groups of
212 interest) > 0.05.

213 **IC₅₀ assay**

214 3.10³ cell spheroids were treated with CX5461 (Sigma-Aldrich)³⁴ or BMH-21 (Sigma-
215 Aldrich)¹⁰. Cell cytotoxicity was assessed by CellTox™ Green Cytotoxicity Assay
216 (Promega) and by CellTiter-Glo3D® luminescent cell viability assay (Promega). Cell
217 viability was expressed as a percentage of the signal intensity normalized against
218 DMSO (1%).

219

220

221 **Results**

222

223 **A standardized approach for large-scale analyses of human samples using Ri-** 224 **boMethSeq**

225 Before investigating alterations of rRNA 2'Ome in the three main histomolecular HGGs,
226 we first optimized the recently described RiboMethSeq approach^{25,32,35} to determine
227 reliable quantifications of 2'Ome levels at 106 rRNA sites from patient tumor samples.
228 First, based on **the technical** cohort (n=11) of IDHwt glioblastomas and non-neoplastic
229 tissues, we observed that the C-score, which reflects rRNA 2'Ome levels at specific
230 sites, was similar using either a manual or an automated RNA extraction protocol
231 (Fig.S2). Second, we used the NovaSeq Illumina sequencing platform (up to 10 billion
232 reads) to increase the total number of **useful** reads (Fig.S3A) and the number of sam-
233 ples sequenced in a single flowcell.

234 We then randomly separated RNA samples of **the test** cohort and prepared two inde-
235 pendent libraries of 23 samples (40 HGGs and 6 non-neoplastic samples), each library
236 also contained a commercially-available "reference" total RNA. Unsupervised analysis
237 of the entire **test** cohort using PCA based on C-scores of either all rRNA positions
238 (7055 sites) or the 106 positions corresponding to known rRNA 2'Ome sites, clearly
239 distinguished samples depending on the library of origin, as illustrated by the lack of
240 clustering of reference RNA (**Fig. S3B, left panels**). We evaluated adjustment of Ribo-
241 MethSeq data using the ComBat-seq algorithm, one of the most routinely used tools
242 to adjust RNA-seq data³⁶. Upon ComBat-seq adjustment, no distinction between sam-
243 ples based on their library of origin was observed in C-scores, **including for the two**
244 **reference RNA (Fig.S3B, right panels)**. These data demonstrate the efficacy of the
245 ComBat-seq algorithm at removing batch effect from RiboMethSeq data.

246 Based on these results, we developed bioinformatics tools to perform reproducible
247 analyses of RiboMethSeq data arising from large-scale cohorts ([https://github.com/Ri-
249 bosomeCRCL](https://github.com/Ri-
248 bosomeCRCL)).

250 **rRNA 2'Ome profiles discriminate IDHwt from IDHmut adult-type diffuse gliomas**

251 Using the optimized approach selected above, we then investigated whether altera-
252 tions of rRNA 2'Ome differentially occur in the three main histomolecular HGGs **using**
253 **the test cohort**: IDHwt glioblastoma (G, n=13), IDHmut astrocytoma (A, n=13), IDHmut
254 and 1p19q codeleted oligodendroglioma (O, n=14) and non-neoplastic cerebral cortex
255 (NT, n=6) (Table 1). Using unsupervised hierarchical clustering analysis (HCA), we
256 first evaluated rRNA 2'Ome levels at the 106 known sites in the 46 non-neoplastic and
257 glioma samples (Fig.1). The C-score reflects the rRNA 2'Ome levels as it corresponds
258 to the ratio of the 5' read-end counts at a nucleotide position to the local 5' read-end
259 count coverage; and when close to 1, C-score indicates that all rRNA molecules of the
260 sample are 2'O-methylated at this specific site; whereas a C-score below 0.9 reflects
261 a mix of 2'O-unmethylated and 2'O-methylated rRNA molecules. Here, most rRNA
262 2'Ome sites had a score close to 1, albeit some sites were below 0.9, substantiating
263 recent results in human samples from diffuse large B cell lymphoma, acute myeloid
264 leukemia and breast cancer^{25,27,28}. These data further confirmed observations by us
265 and others that some rRNA molecules exist without 2'Ome at some specific sites, in-
266 cluding in non-neoplastic tissue.

267 Interestingly, all IDHwt glioblastoma samples formed a separate branch (left-hand side
268 of the HCA dendrogram), suggesting that their rRNA 2'Ome profile was clearly different
269 from IDHmut astrocytoma and oligodendroglioma, as well as from non-neoplastic tis-
270 sue (Fig.1). Principal Component Analyses (PCAs) based on the rRNA 2'Ome profile
271 indicated that the PC2 axis (variance=14.1%) strongly differentiated glioblastomas
272 from other samples (Fig.S4A). **To ensure consistency between the results of our clas-
273 sification and the expected outcome of the patients, we correlated PCA axes with sur-
274 vivals and mitotic index, as an internal control of tumor sample classification. Con-
275 sistent with the known differences regarding glioblastomas and IDHmt HGGs charac-
276 teristics, PC2 was significantly correlated with the *IDH1/2* mutational status, the mitotic
277 index, overall survival (OS) and progression-free survival (PFS) (Fig.S4B-D). To vali-
278 date these observations, we analyzed rRNA 2'Ome levels using RiboMethSeq on the**

279 **technical cohort, corresponding to an independent cohort composed of 8 IDHwt glioblastomas and 3 non-neoplastic tissues.** Unsupervised PCA based on the rRNA 2'Ome profile showed that IDHwt glioblastomas could once again be clearly separated from non-neoplastic tissues (Fig.S4E). Altogether, our data revealed that rRNA 2'Ome levels vary in HGGs as well as in non-neoplastic cerebral cortex, and that rRNA 2'Ome profiles discriminate IDHwt glioblastomas not only from non-neoplastic tissues but also from IDHmut HGG types.

286

287 **Most variable rRNA 2'Ome sites are sufficient to discriminate IDHwt glioblastoma from IDHmut adult-type diffuse gliomas**

289 To better characterize differences in rRNA 2'Ome profiles between histomolecular HGG types, we then focused on the most variable rRNA 2'Ome sites. To identify such sites, we compared the variability of C-scores at each single site among all HGG samples of the test cohort (n=40) using the distribution of the inter-quartile range (IQR). A set of 19 sites showed an IQR higher than median + 2 × median absolute deviation (mad) and were considered as the most variable rRNA 2'Ome sites among HGGs (red, Fig.2A), in agreement with our recent findings that only a subset of rRNA sites displays variability in 2'Ome levels²⁵. Interestingly, PCAs indicated that rRNA 2'Ome profiles based on this set of 19 sites are sufficient to discriminate IDHwt glioblastomas, IDHmut astrocytoma and oligodendrogliomas (Fig.2B).

299 **To validate these observations, we used a second, independent validation cohort of 23 HGG samples (9 IDHwt glioblastomas, 6 IDHmut astrocytomas and 8 IDHmut, 1p/19q codeleted oligodendrogliomas) (Fig.S1B). We performed an unsupervised analysis using the 19 most variable rRNA 2'Ome sites identified using the test cohort (Fig.2A). As for the test cohort, unsupervised analyses of the validation cohort distinguished the three histomolecular subtypes of HGGs (Fig.2C). In addition, the projection of the validation cohort samples onto the PCA constructed from the test cohort enabled us to identify the histomolecular subtypes of each sample, suggesting that *de novo* profiling of rRNA 2'Ome could help in the classification of HGGs (Fig.2D). In addition, comparison of test and validation cohorts in term of percentage of tumor cells suggests that the tumor heterogeneity did not affect our conclusions (Fig. S1B). Indeed, in the validation cohort, the mean percentage of tumor cells is significantly lower in astrocytomas (38%) compared to oligodendrogliomas (69%), while no difference was observed with glioblastomas (9%) (Kruskal-Wallis: p=0.045*; Mann-Whitney: A vs O**

313 $p=0.024^*$). In contrast, in the test cohort, the mean percentage of tumor cells is signif-
314 icantly lower in astrocytomas (38%) and oligodendrogliomas (39%) compared to glioblastomas (55%) (Kruskal-Wallis: $p=0.034^*$; Mann-Whitney: A vs G $p=0.0172^*$; O vs G
315 $p=0.0348^*$).
316

317 These data further emphasize the specificity of IDHwt glioblastoma rRNA 2'Ome pro-
318 files compared to IDHmut HGGs and suggest that IDHwt glioblastomas display strong
319 alterations of rRNA 2'Ome levels at some specific sites.
320

321 **Glioblastomas display the most frequent site-specific rRNA 2'Ome alterations**

322 To identify rRNA sites whose 2'Ome levels significantly differed between HGG histo-
323 molecular types and non-neoplastic tissues, we performed a systematic analysis of
324 each of the 106 rRNA 2'Ome sites using the test cohort and applied two consecutive
325 thresholds, a Kruskal-Wallis test with adjusted p-values < 0.05 and a cut-off value for
326 mean $\Delta C\text{-scores}_{\text{max-min}} > 0.05$. From this screen, only 16 rRNA 2'Ome sites displayed
327 a significant variation in their level of methylation in at least one HGG or non-neoplastic
328 tissue (Fig.3). Pairwise comparisons for these 16 sites revealed that 4 and 6 sites dis-
329 played significant alterations in 2'Ome levels in high-grade astrocytomas and high-
330 grade oligodendrogliomas, respectively, compared to non-neoplastic tissues (Fig.3B).
331 In addition, rRNA 2'Ome levels appeared to be significantly increased on 6 sites in
332 high-grade astrocytomas compared to high-grade oligodendrogliomas. **Similar altera-**
333 **tions in rRNA 2'Ome levels between IDHwt and IDHmut tumours were observed using**
334 **the validation cohort (Fig.S5A)**. Finally, the main differences could be attributed to gli-
335 oblastomas that displayed 12 and 14 of the 16 rRNA sites significantly altered com-
336 pared to non-neoplastic samples or high-grade astrocytoma and oligodendrogliomas,
337 respectively. Strikingly, rRNA 2'Ome levels were mostly lower in glioblastomas com-
338 pared to non-neoplastic tissues, high-grade astrocytoma or high-grade oligodendrogli-
339 omas (Fig.3A). The same overall decrease in rRNA 2'Ome levels was confirmed in
340 glioblastoma compared to non-neoplastic tissues using the technical cohort (Fig.S5B).
341 Altogether, these results indicate that 2'Ome levels are altered at specific rRNA sites
342 in HGGs, while glioblastomas display the highest frequency of alterations in rRNA
343 2'Ome levels compared to non-neoplastic cerebral cortex and other HGG histomolec-
344 ular types.
345

346 **Changes in C/D box snoRNA expression levels only partially explain alterations**
347 **of rRNA 2'Ome levels**

348 To investigate the origin of alterations of rRNA 2'Ome in HGGs, we focused on the
349 expression of C/D snoRNAs (snoRDs), which guide in a sequence-specific manner the
350 methyl-transferase FBL toward the rRNA nucleotide to methylate. We thus examined
351 the correlation between levels of rRNA 2'Ome and related snoRDs. It was reported
352 that snoRNA expression levels can be inferred from RiboMethSeq data^{37,38}. Thus, we
353 used RiboMethSeq raw data from the NovaSeq platform and applied an in-house pipe-
354 line to overcome limitations of snoRNA analysis by next-generation sequencing (see
355 Supplementary methods)^{39,40}. A strong and significant correlation (about 92%) be-
356 tween snoRNA levels either determined from RiboMethSeq or measured by RT-qPCR
357 was observed in 9 glioma samples for 11 out of 12 selected snoRDs, thus validating
358 our approach (Fig.S6A). Given that 2'Ome at a single rRNA site is catalyzed either by
359 one or several snoRD^{15,41}, we restricted our analysis to the rRNA 2'Ome sites (n=46)
360 guided by a single snoRD. No significant correlation was observed between levels of
361 rRNA 2'Ome and of corresponding snoRDs among 37 sites (Fig.S6B). In contrast, a
362 significant albeit relatively low positive correlation was observed for 9 pairs of
363 snoRD/rRNA sites, the best correlation being that of the 18S-Am576 site and its asso-
364 ciated SNORD93 (p_{adj}=9.10⁻⁶, r=0.68) (Fig.S6B-C). Thus, alterations of rRNA 2'Ome
365 levels cannot be exclusively attributed to changes in C/D box snoRNA expression lev-
366 els.

367

368 **The expression profile of ribosome biogenesis factors discriminates IDHmut and**
369 **IDHwt HGGs**

370 Since variations in snoRD expression levels cannot explain most of the changes ob-
371 served in rRNA 2'Ome levels, we tested a novel hypothesis. Indeed, in cancer, varia-
372 tions of 2'Ome levels in rRNAs are believed to passively arise from the link between
373 ribosome biogenesis (RiBi) and concomitant rRNA chemical modifications, whereby
374 changes in ribosome synthesis impact the rate-limiting rRNA 2'Ome process and
375 therefore influence 2'Ome profiles^{15,25,27}. Therefore, an overall decrease in rRNA
376 2'Ome levels observed in different cancer tissues may be caused by RiBi hyperactiva-
377 tion to sustain a high demand in protein synthesis necessary to support the highly pro-
378 liferative cancer cells. As IDHwt glioblastomas possess a higher proliferative rate than
379 IDHmut astrocytoma and oligodendrogliomas, including in our test cohort (based on

380 the mitotic index) (Fig.S4C-D), we hypothesized that the decrease in rRNA 2'Ome lev-
381 els at specific positions in IDHwt glioblastomas could be due to a rate-limiting 2'Ome
382 process caused by an exacerbated RiBi.

383 To investigate RiBi dysregulations across HGGs, we initially applied a gold-standard
384 approach by analyzing levels of the 47S rRNA precursor (pre-rRNA), using Northern
385 blot (Fig.S7A). However, this approach using tumor samples was challenging, notably
386 due to the need for a large quantity of biological materials. Nevertheless, in a panel of
387 9 analyzable samples, IDHmut astrocytoma and oligodendrogliomas surprisingly
388 seemed to express more pre-47S rRNAs than IDHwt glioblastomas. However, only two
389 IDHwt glioblastoma samples were analyzed, therefore preventing us to draw any con-
390 clusion. To bypass this technical issue, we then measured the expression of a set of
391 20 genes involved in the early RiBi stages (referred to as "RiBi-gene set"). To reflect
392 as much as possible this multistep process involving more than 200 factors⁷, we se-
393 lected the RiBi-gene set implicated in the main RiBi process, including rRNA transcrip-
394 tion (*NCL*, *NPM*, *POLR1A*, *TAF1A*, *TAF1B*, *TAF1C* and *UBTF*), rRNA maturation
395 (*BOP1*, *PES1* and *WDR12*), snoRNA biogenesis (*RUVBL1*, *PIDH1D1* and *RUVBL2*),
396 and H/ACA (*DKC1*, *GAR1*, *NHP2* and *NOP10*) and C/D box (*NOP56*, *SNU13* and *FBL*)
397 snoRNP complexes (Fig.4A). Of note, three genes (*PIH1D1*, *RUVBL2* and *FBL*) are
398 located on the long arm of chromosome 19 (19q), which undergoes a heterozygous
399 deletion in high-grade oligodendrogliomas. A readout for RiBi at steady-state was de-
400 termined by quantifying mRNA expression levels of these selected genes by medium
401 throughput RT-qPCR in our validation series. mRNA levels were normalized against
402 the median mRNA expression of 5 housekeeping genes, which did not significantly
403 vary among the four groups (Fig.S7B).

404 To examine the association between expression levels of RiBi factors and HGGs, we
405 first performed an unsupervised approach using a PCA based on the RiBi-gene set
406 profile (Fig.4B). Interestingly, three main clusters were observed. A first large cluster,
407 composed of both non-neoplastic tissues and IDHwt glioblastomas (NT/G cluster), was
408 distinct from two other clusters corresponding to IDHmut oligodendrogliomas (O) and
409 astrocytoma (A). PC1 (variance: 67.8%), in particular, separated the NT/G cluster from
410 A/O clusters, while the PC2 (variance: 9%) distinguished the O cluster from others.
411 These data suggest that the expression profile of only 20 RiBi factors discriminate
412 HGGs.

413 To ensure consistency between the results of our classification and the expected out-
414 come of the patients, we first calculated Pearson's correlation coefficients between the
415 first 5 PC dimensions and OS or PFS as an internal control (Fig.4C and Fig.S7C).
416 Consistently, we observed a significant association exclusively for PC1 and PC2, indi-
417 cating that the clustering based on RiBi-gene set profiles provided by these two axes
418 is sufficient to recapitulate all clinical data of interest. OS and PFS were significantly
419 correlated with both PC1 and PC2 in a negative manner, indicating that samples clus-
420 tering at the right-hand side of the PC1 and top of PC2, *i.e.*, IDHwt glioblastomas,
421 display lower OS and PFS. Indeed, IDHwt glioblastoma patients exhibit the poorest
422 OS (less than 30 months) and PFS (under 30 months), followed by IDHmut astrocy-
423 toma and oligodendroglioma patients that tend to cluster at the left part of PC1 and the
424 top or bottom part of PC2, respectively.

425 Strikingly, we also identified a strong correlation between PC1 and PC2 axes, and the
426 *IDH1/2* mutational and 1p/19q co-deletion status, respectively (Fig.S7D-E). Indeed,
427 PC1 (G vs. A/O clusters) was significantly correlated with the *IDH1/2* mutational status
428 while PC2 (A/G vs. O clusters) segregated HGG tumors based on the 1p/19q co-dele-
429 tion status. Thus, expression profiling of the RiBi-gene set was strongly correlated with
430 both clinical features and distinct genomic alterations of the HGG test cohort. Alto-
431 gether, these results suggest that the expression signature of only 20 genes involved
432 in ribosome biogenesis is sufficient to discriminate IDHwt from IDHmut HGG histomo-
433 lecular types and that RiBi displays IDH mutational status-dependent alterations.

434

435 **Ribosome biogenesis factors are highly expressed in IDHmut adult-type diffuse** 436 **gliomas**

437 Next, we individually compared mRNA expression levels of the 20 RiBi factors among
438 the different HGGs (Fig.5 and Fig.S8A). Significant differences were observed in the
439 expression of all tested RiBi factors between different groups (Mann Whitney test,
440 Fig.S8A). Surprisingly, only a few RiBi genes were significantly differentially expressed
441 in IDHwt glioblastomas compared to non-neoplastic samples (9 out of 20), with very
442 moderate changes (≤ 2 -fold change). In contrast, IDHmut astrocytoma and oligoden-
443 drogliomas significantly overexpressed most of these RiBi genes (19 and 16 out of 20
444 RiBi genes, respectively). For instance, expression of *NCL*, which encodes a key factor
445 in rRNA synthesis, increased by 2- and 3-fold in IDHmut astrocytoma and oligoden-
446 drogliomas, respectively ($p < 0.001$), while *NCL* expression levels in IDHwt samples

447 only slightly increased (Fig.5A and Fig.S8A). Likewise, the use of a second distinct set
448 of primers to analyze *NCL* expression provided the exact same trend (Fig.S8B), there-
449 fore ruling out a potential technical caveat. These results suggest that ribosome bio-
450 genesis could be enhanced in IDHmut astrocytoma and oligodendrogliomas compared
451 to IDHwt glioblastomas and non-neoplastic tissues.

452 Interestingly, genes located on chromosome 19q (*FBL*, *PIH1D1*, *RUVBL2*) displayed
453 a particular expression pattern (Fig.5C, 5E and Fig.S7E). Like other genes involved in
454 RiBi, *FBL*, *PIH1D1* and *RUVBL2* were significantly overexpressed in IDHmut astrocy-
455 tomas compared to both IDHwt glioblastomas and non-neoplastic samples. However,
456 mRNA expression levels in IDHmut oligodendrogliomas were lower than in IDHmut
457 astrocytomas and displayed expression profiles resembling those of IDHwt glioblasto-
458 mas. Thus, *FBL* was highly expressed exclusively in IDHmut astrocytomas, whereas
459 its expression levels were equivalent in IDHmut oligodendrogliomas and IDHwt glioblastomas,
460 as confirmed by a second set of primers (Fig.S8B). Considering that
461 IDHmut oligodendroglioma samples exhibited a heterozygous deletion of *FBL*, *PIH1D1*
462 and *RUVBL2* genes located on 1p/19q chromosomes, these data indicate that the spe-
463 cific expression profile observed for these genes in IDHmut oligodendrogliomas is
464 likely due to a haploinsufficiency caused by genetic alterations. Interestingly, removal
465 of these three genes in the RiBi-genes set still allowed us to distinguish IDHwt from
466 IDHmut HGGs (data not shown), suggesting that copy number variation (CNVs) affect-
467 ing RiBi gene expression does not impact the distinction between IDHwt and IDHmut,
468 as expected. Therefore, the ribosome biogenesis pathway is strongly enhanced in
469 IDHmut gliomas, *i.e.*, high-grade astrocytoma and oligodendroglioma, but remains
470 moderately affected in IDHwt glioblastoma.

471
472 **HGGs display distinct cytotoxicity to RNA pol I inhibitors CX5461 and BMH-21**
473 Having identified an IDH mutational status-dependent alteration of RiBi in HGGs, we
474 hypothesized that IDHmut and IDHwt HGGs display distinct cytotoxicity to RNA Pol I
475 inhibitors, BMH-21 and CX5461, which inhibit the transcription of the 47S pre-rRNA
476 and are promising new cancer treatments. We used a panel of 5 representative HGG
477 cell lines, comprising IDHmut astrocytoma and IDHmut and 1p/19q codeleted oli-
478 godendroglioma cell lines (LGG85 and BT138/237, respectively) and IDHwt glioblas-
479 toma cell lines (5706 and N131520). Analyses of HGG spheroids in response to 72-

480 hour treatments with RNA pol I inhibitors showed that all spheroids were similarly sen-
481 sitive to BMH-21 with an IC_{50} ranging from 1.06 to 1.56 μ M (Fig.6A-C), as recently
482 observed ⁴². However, CX5461 strongly impacted the viability of IDHmut astrocytoma
483 and oligodendroglioma spheroids (IC_{50} ranging from 5.92 to 7.55 μ M), but not of IDHwt
484 glioblastoma spheroids (Fig.6D-F). Hence, these results are consistent with our previ-
485 ous findings that IDHmut astrocytomas and oligodendrogliomas may be addicted to an
486 enhanced RiBi pathway, therefore potentially sensitizing these AGD histomolecular
487 types to clinically available RiBi inhibitors. Altogether, our results highlight the potency
488 of RNA Pol I inhibitor usage as potential HGG therapy and further support an over-
489 activation of the RiBi pathway in IDHmut astrocytomas and oligodendrogliomas com-
490 pared to IDHwt glioblastomas and non-neoplastic tissues.

491

492

493 **Discussion**

494 High-grade adult-type diffuse gliomas (HGGs) are heterogeneous tumors associated
495 with distinct, albeit poor, survival rates due to the lack of effective targeted therapies,
496 in particular for the most aggressive histomolecular type, the IDHwt glioblastoma.
497 Here, by performing the first concomitant analysis of rRNA 2'Ome and ribosome bio-
498 genesis in primary tumors, we report distinct, uncoupled alterations of rRNA epitran-
499 scriptomics and ribosome biogenesis in IDHmut and IDHwt HGGs, therefore revealing
500 specific dysregulations of the ribosome biology that constitute new IDH mutational sta-
501 tus-associated hallmarks of HGGs.

502 **In the last 7 years, alterations of rRNA 2'Ome profiles have been reported using a**
503 **newly developed approach RiboMethSeq in numerous cellular models and only in**
504 **three types of cancers, namely breast cancer, acute myeloid leukemia and diffuse**
505 **large-B cell lymphoma (DLBCL)^{15,25,27,28}. Such alterations have been shown to be re-**
506 **stricted to only 40% of the known rRNA 2'Ome sites, suggesting that only one third of**
507 **the sites may possess regulatory functions on ribosome activity.** We now show that
508 rRNA 2'Ome profiles also vary in HGGs. As previously observed in other cancers, only
509 a small subset of known rRNA 2'Ome sites display variability in their 2'Ome levels in
510 HGGs, suggesting these positions can tolerate absence of 2'Ome. The 19 most varia-
511 ble rRNA 2'Ome sites were randomly distributed on the ribosome structure, suggesting
512 no coordinated effects on functional domains of the ribosome. Notably, similar in-
513 creases in rRNA 2'Ome levels at 18S_Am576 and decreases at 18S_Gm1447 were

514 observed in the most aggressive HGG type, glioblastoma, and breast cancer subtypes
515 (triple negative or TNBC), suggesting a link between these sites and cancer aggres-
516 siveness²⁵. A recent study demonstrated that alterations of 2'Ome levels at a single
517 rRNA site are sufficient to affect cell proliferation, a hallmark of cancer aggressive-
518 ness²⁶. Moreover, rRNA 2'Ome at 18S_Gm1447 was recently shown to support leuke-
519 mic stem cell functions by modulating translation²⁸. Hence, whether these rRNA 2'Ome
520 sites common to both HGGs and breast cancer contribute to the acquisition of cancer
521 cell characteristics remains to be deciphered and could potentially represent new tar-
522 getable vulnerabilities.

523 **In IDHwt glioblastomas, alterations in rRNA 2'Ome levels mostly correspond to a de-**
524 **crease as observed in both the test and validation cohorts.** In DLBCL, the global de-
525 crease in rRNA 2'Ome levels was correlated with the Ki67-estimated high proliferative
526 rate of tumors. One hypothesis was that low rRNA 2'Ome levels indirectly resulted from
527 an increase in rRNA synthesis associated with the hyperproliferative rate of cancer
528 cells, which rendered components of the rRNA 2'Ome machinery limiting, although
529 rRNA synthesis was not analyzed²⁷. Consistently, we observed specific alterations of
530 rRNA 2'Ome in IDHwt glioblastomas, which are the most proliferative tumors and dis-
531 play the highest mitotic index in our test cohort. However, we surprisingly observed an
532 elevated ribosome biogenesis in IDHmut astrocytomas and oligodendrogliomas com-
533 pared to glioblastomas, suggesting that RiBi levels are not correlated with the prolifer-
534 ative rate, at least in HGGs, and cannot solely explain alterations of rRNA epitran-
535 scriptomics in HGGs. Even though the rate of ribosome biogenesis may contribute to
536 regulating rRNA 2'Ome through a passive effect, additional molecular mechanisms
537 should be further explored to identify the origin of rRNA 2'Ome alterations in cancer
538 and understand the observed rRNA site- and cancer type-specificity. The mechanisms
539 may include alterations of expressions and/or activities of RNA-binding proteins, such
540 as DDX21 and FMRP, which contribute to the formation of *bona fide* functional snoRNP
541 complexes^{29,43}. Here, we report that alterations of C/D box snoRNA expression could
542 be sufficient to explain alterations of rRNA 2'Ome levels at some, but not all, rRNA
543 sites. The evolution of annotation and/or knowledge in biology of C/D box snoRNAs
544 may fill the gap to better understand causes of rRNA 2'Ome alterations in cancer. Al-
545 together, our findings that main HGG histomolecular types are associated with altera-
546 tions in either ribosome quantity or quality, challenge the hypothesis that the decrease

547 in rRNA 2'Ome levels mainly results from a passive effect caused by an exacerbated
548 ribosome biogenesis.

549 Our data reveal that IDHmut HGGs, including both high-grade astrocytoma and oli-
550 godendroglioma, display the highest expression of ribosome biogenesis factors, sug-
551 gesting an increase in ribosome biogenesis. Whether dysregulations of *IDH1/2* func-
552 tions, notably through the production of the oncometabolite D-2-hydroxyglutarate,
553 could directly impact the regulation of ribosome biogenesis would need to be further
554 explored. Nevertheless, the specificity of ribosome biogenesis alterations in HGGs of-
555 fers novel perspectives for clinical applications. Building on our observations, we found
556 that HGGs are sensitive to the newly developed RNA pol I inhibitors, CX5461 and
557 BMH-21, the former being successfully evaluated in clinical trials in advanced solid and
558 hematological cancers⁷. Sensitivity of HGGs to RNA pol I inhibitors CX5461 and BMH-
559 21 has already been reported^{42,44,45}, even though discrepancies regarding the sensi-
560 tivity of glioblastomas to CX5461 exist between our data and previous ones, possibly
561 due to differences in experimental settings and genetic backgrounds of tested cell
562 lines. In particular, the 3D culture conditions could decrease drug sensitivity compared
563 to 2D culture conditions, as already reported⁴⁶. In addition, the differential sensitivity of
564 glioblastomas to CX5461 and BMH-21 could be attributed to off-target effects of these
565 molecules as these RNA Pol I inhibitors act through distinct mechanisms and are
566 known to affect several cellular pathways, including DNA repair^{10,34,47}. Thus, whether
567 the activity of these compounds may also rely on the genetic background of tested
568 models impacting the different sensitivities of HGG types to CX5461 and BMH-21,
569 should be further investigated.

570 Altogether, our data indicate that alterations of the ribosome biology in HGGs are de-
571 pendent on the IDH mutational status and could represent targetable features in clinic.
572 Thus, recent discoveries in the field of ribosomes have opened new avenues not only
573 for a better understanding of cellular processes that contribute to HGG development
574 and aggressiveness but also for designing future HGG type-specific therapeutic strat-
575 egies.

576 **Conflict of interest**

577 The authors have no conflicts of interest to declare.

578

579 **Ethical Approval**

580 All the experiment protocol for involving human was in accordance with the guidelines
581 of French regulation. Written informed consent was obtained from all patients.

582

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591

592 **Author contribution**

593 HP, NEHM, SB, JH, FB, VA, MC, JP, DB, LCV, SD performed and analysed experi-
594 ments. HP, NEHM, JK, ET, DB performed statistical descriptions and bioinformatic
595 analyses. HP, TC, AF, JK, ET, LT, AV developed bioinformatic tools. LCV, EH, MS
596 provided cell lines. DM, FD provided human samples and clinical data. AF, JK, ET, VA,
597 SG, MSS, EH, MS, DM, FC, VM, SB supervised experimental process. HP, NEHM,
598 SB, JK, SG, FD, VM and SD interpreted the data. VM and SD shaped the clinical and
599 research question and supervised the project coordination. AV, MSS, EH, MS, MG,
600 JJD, FD, VM and SD provided financial supports. HP, NEHM, VM and SD wrote the
601 first draft of the manuscript. All authors have read and approved the manuscript.

602

603 **Availability of datasets**

604 The generated RiboMethSeq data are available in the GEO profile (GSE224104, token
605 for reviewer access: qhabqswmfzotlwh). The datasets include the fastq of each sam-
606 ple, a C-score matrix for each cohort after batch effect adjustment using ComBat-seq
607 and a matrix summarizing the clinical data.

608

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770
771

772 **Figure Legends**

773

774 **Figure 1. rRNA 2'Ome levels vary in high-grade adult-type diffuse gliomas.** An
775 unsupervised hierarchical clustering of C-scores at the 106 known rRNA 2'O-ribose
776 methylated (2'Ome) sites was performed in a test cohort of 40 high-grade (3-4) adult-
777 type diffuse glioma (HGG) samples and 6 non-tumoral, non-neoplastic cerebral cortex
778 (NT) samples. C-scores are represented by a color scale from 0 (black) to 1 (yellow).
779 IDHwt glioblastomas (G), high-grade astrocytomas (A), high-grade oligodendrogl-
780 omas (O) and non-neoplastic (NT) samples are depicted in pink, green, purple, and
781 grey, respectively. The mean C-score for each site across the 46 samples is shown on
782 the right-hand side of the graph. 86 sites have a mean C-score higher than 0.9 (black)
783 and 20 sites lower than 0.9 (red).

784

785 **Figure 2. The most variable rRNA 2'Ome sites are sufficient to distinguish differ-**
786 **ent HGG types. (A)** Distribution of the interquartile range (IQR) of the C-score to de-
787 termine the C-score variability across HGG samples of the test cohort (n=40). rRNA
788 2'Ome sites are ranked by increasing IQR value. The IQR distribution curve is plotted
789 at the right-hand side of the graph. The "most variable sites" correspond to those with
790 an IQR higher than median + 2 × median absolute deviation (mad) and were colored
791 in red (19 sites). **(B-D)** Unsupervised Principal Component Analysis (PCA) based on
792 C-scores of the 19 most variable sites as identified in (A). **Independent PCA was per-**
793 **formed on IDHwt glioblastoma (G, pink circle), high-grade astrocytoma (A, green trian-**
794 **gle) and high-grade oligodendrogloma (O, purple diamond) samples of both test (B,**
795 **n=40) and validation (n=23) cohorts. Validation cohort samples were projected on PCA**
796 **of the test cohort (D).** Percentage of variance explained by PC1 and PC2 are indicated.
797 95 % confidence ellipsoids around the centroid of each group (larger pink circle, green
798 triangle and purple diamond) are indicated.

799

800 **Figure 3. rRNA 2'Ome levels are differently altered in HGG types. (A)** **Box plots**
801 **showing the distribution of C-score in HGG and non-neoplastic samples of the test**
802 **cohort for 16 rRNA 2'O-ribose methylated (2'Ome) sites that exhibited both statistically**
803 **and biologically significant alterations between groups. These sites were identified us-**
804 **ing both Kruskal-Wallis statistical tests (with an adjusted p-value threshold of < 0.05)**

805 and a mean $\Delta C\text{-score}_{\text{max-min}} > 0.05$ (absolute difference between the highest and low-
 806 est mean C-score). The adjusted p-values corresponding to the statistical tests are
 807 indicated on the bottom left-hand side of each panel and median C-scores are repre-
 808 sented by a black line within the box plots. **(B)** Pairwise comparison of mean C-score
 809 groups for the 16 significantly deregulated rRNA 2'Ome sites in the test cohort. Positive
 810 (UP) and negative (DOWN) $\Delta C\text{-score}_{\text{group1-group2}}$ are shown in blue and red, respec-
 811 tively. Adjusted p-values: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns,
 812 not significant.

813

814 **Figure 4. Expression profiles of RiBi factors distinguish HGG types. (A)** Panel of
 815 genes involved in ribosome biogenesis constituting the 20 RiBi-gene set analyzed in
 816 HGG and non-neoplastic samples. Genes located on the short arm of chromosome 1
 817 (1p) or long arm of chromosome 19 (19q), which are heterozygously deleted in high-
 818 grade oligodendrogliomas, are indicated. **(B)** A principal component analysis (PCA)
 819 based on the mRNA expression profile of the RiBi-gene set. Each dot represents a
 820 non-neoplastic (NT, grey square), IDHwt glioblastoma (G, pink circle), high-grade as-
 821 trocytoma (A, green triangle) or high-grade oligodendroglioma (O, purple diamond)
 822 sample. Ellipsoids shows 90% confidence interval around the centroid (larger grey
 823 square, pink circle, green triangle, and purple diamond) of each group. Percentage of
 824 variance explained by PC1 and PC2 are indicated. **(C)** A heatmap showing Pearson's
 825 correlation coefficients of PC1 to PC5 axes with the overall survival (OS), progression-
 826 free survival (PFS) and mitotic index. R-values are depicted by different colors from -
 827 0.6 (red, negative correlation) to 0.6 (blue, positive correlation). Significant correlations
 828 are indicated by an asterisk: ** $p < 0.01$; *** $p < 0.001$.

829

830 **Figure 5. High-grade astrocytomas and oligodendrogliomas display the highest**
 831 **increased expression in ribosome biogenesis factors.** Box plots showing relative
 832 mRNA expression levels determined by RT-qPCR analysis of the RiBi genes impli-
 833 cated in rRNA **(A)** transcription and **(B)** maturation, **(C)** snoRNA biogenesis or associ-
 834 ated to **(D)** H/ACA box and **(E)** C/D box snoRNAs in IDHwt glioblastomas (G, pink),
 835 high-grade astrocytomas (A, green), high-grade oligodendrogliomas (O, purple) and
 836 non-neoplastic (NT, grey) samples. *FBL*, *PIH1D1* and *RUVBL2* are located on chro-
 837 mosomes 1p or 19q.

838 **Figure 6. Glioma spheroids reveal distinct histomolecular type-dependent sen-**
839 **sitivity to ribosome biogenesis inhibitors. (A)** Representative high-content screen-
840 ing microscopy images of 5706, N131520, BT138, BT237 and LGG85 cell line spher-
841 oids treated or not (DMSO) with 10 μ M of BMH-21 for 72 h. Hoechst and CellTox
842 labelling are depicted in blue and green, respectively. Cell lines representative of gli-
843 blastomas, high-grade oligodendrogliomas and astrocytomas are shown in pink, pur-
844 ple and green, respectively. **(B)** Representative graphs indicating the viability percent-
845 age in response to increasing BMH-21 concentrations in 5706, N131520, BT138,
846 BT237 and LGG85 cell line spheroids. Cell lines representative of IDHwt glioblasto-
847 mas, IDHmut oligodendrogliomas and astrocytomas are framed in pink, purple and
848 green, respectively. **(C)** A table indicating means and standard deviations (SD) of
849 BMH-21 IC₅₀ calculated from graphs displayed in (B) (n=7). (D), (E) and (F) as in (A)
850 (B) and (C), respectively, for CX5461 (n=3).
851

1 **IDHwt and IDHmut adult-type diffuse gliomas display distinct alterations in ribo-**
2 **somal biogenesis and 2'O-methylation of ribosomal RNA**

3
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44

45 **Keywords**

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47 tors

48

49 **Running title**

50 Ribosome alterations in high-grade gliomas

51

52 **Issue section**

53 Basic and translational investigation

54

55 **Key points (3)**

56 • rRNA 2'Ome profiling distinguishes IDHwt and IDHmut adult-type diffuse glioma-
57 mas

58 • Elevated expression of ribosome biogenesis factors is correlated with IDH mu-
59 tational status

60 • High grade adult-type diffuse gliomas differentially respond to the RNA Pol I
61 inhibitors BMH-21 and CX5461

62

63 **Word count:** 4991

64

65 **Abstract**

66

67 **Background:** High-grade adult-type diffuse gliomas (HGGs) constitute a heterogene-
68 ous group of aggressive tumors that are mostly incurable. Recent advances highlight-
69 ing the contribution of ribosomes to cancer development have offered new clinical per-
70 spectives. Here, we uncovered that IDHwt and IDHmut HGGs display distinct altera-
71 tions of ribosome biology, in terms of rRNA epitranscriptomics and ribosome biogene-
72 sis, which could constitute novel hallmarks that can be exploited for the management
73 of these pathologies.

74 **Methods:** We analyzed (i) the ribosomal RNA 2'O-ribose methylation (rRNA 2'Ome)
75 using RiboMethSeq and in-house developed bioinformatics tools
76 (<https://github.com/RibosomeCRCL/ribomethseq-nf> and [rRMSAnalyzer](#)) on three inde-
77 pendent cohorts compiling 71 HGGs (IDHwt n=30, IDHmut n=41) and 9 non-neoplastic
78 samples, (ii) the expression of ribosome biogenesis factors using medium throughput
79 RT-qPCR as a readout of ribosome biogenesis, and (iii) the sensitivity of 5 HGG cell
80 lines to RNA Pol I inhibitors (CX5461, BMH21).

81 **Results:** Unsupervised analysis demonstrated that HGGs could be distinguished
82 based on their rRNA 2'Ome epitranscriptomic profile, with IDHwt glioblastomas dis-
83 playing the most significant alterations of rRNA 2'Ome at specific sites. In contrast,
84 IDHmut HGGs are largely characterized by an overexpression of ribosome biogenesis
85 factors compared to non-neoplastic tissues or IDHwt glioblastomas. Finally, IDHmut
86 HGG-derived spheroids display higher cytotoxicity to CX5461 than IDHwt glioblas-
87 toma, while all HGG spheroids display a similar cytotoxicity to BMH-21.

88 **Conclusion:** In HGGs, IDH mutational status is associated with specific alterations of
89 the ribosome biology and with distinct sensitivities to RNA Pol I inhibitors.

90

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92

93 **Importance of the study (146 words)**

94

95 Consistent multi-omics studies have shown that high-grade adult-type diffuse gliomas
96 (HGGs) can be classified into three main groups, *i.e.*, IDHmut and 1p/19q codeleted
97 oligodendrogliomas, IDHmut astrocytomas and IDHwt glioblastomas, based on their
98 genetic, transcriptomic and DNA methylation profiles. Recent advances have high-
99 lighted the contribution of ribosomes to cancer development and have offered new
100 clinical perspectives. Herein, we show that ribosomal RNA (rRNA) epitranscriptomic
101 and ribosome biogenesis are different in distinct HGG types. We uncovered that IDHwt
102 glioblastomas display the most prominent defects in rRNA epitranscriptomics, whereas
103 IDHmut astrocytomas and oligodendrogliomas exhibit enhanced expression of ribo-
104 some biogenesis factors compared to IDHwt glioblastomas. Moreover, based on their
105 IDH mutational status, HGG-derived cell lines displayed distinct responses to CX5461
106 and BMH-21, two clinically-evaluated inhibitors of the RNA Pol I that transcribes
107 rDNAs. This study identifies a connection between HGG oncogenesis and the ribo-
108 some biology, and highlights new therapeutic strategies.

109

110

111 **Introduction**

112 High-grade adult-type diffuse gliomas (HGGs) are brain tumors resembling glial cells
113 that display highly heterogeneous prognoses and treatment responses. HGGs com-
114 prise three main histomolecular types, astrocytomas, oligodendrogliomas and glioblas-
115 tomas, based notably on the mutational status of *isocitrate dehydrogenase (IDH) 1* and
116 2^{1,2}. Indeed, glioblastomas are IDHwt, whereas astrocytomas and oligodendrogliomas
117 are IDHmut, and can be further discriminated by the heterozygous 1p19q co-deletion
118 occurring in oligodendrogliomas. IDHwt and IDHmut HGGs are associated with distinct
119 epigenetic and transcriptomic dysregulations, leading to cancer-specific features.
120 Thus, despite important advances in their histomolecular classification and under-
121 standing of their oncogenesis, HGGs remain mostly incurable. For instance, grade 4
122 IDHwt glioblastoma patients treated with the conventional combination of surgery, ra-
123 diotherapy and temozolomide (TMZ) chemotherapy, display a median survival of only
124 15 months. Grade 3 IDHmut astrocytoma and oligodendroglioma patients treated with
125 radiotherapy and chemotherapy exhibit a much better outcome with a median survival
126 of 10 and 15 years, respectively, nevertheless 20 to 30% of the patients die within the
127 first five years after diagnosis^{3,4}. Therefore, the identification of novel molecular mech-
128 anisms dysregulated in distinct HGG histomolecular types may significantly improve
129 current therapeutic options.

130 Several studies highlighted that ribosome biogenesis (RiBi) and functions are altered
131 in cancer cells and that ribosomes can support oncogenic functions⁵. For instance, the
132 c-MYC oncogenic activity is in part supported by a dysregulation of genes implicated
133 in RiBi and global protein synthesis⁶. In addition, levels of RiBi are generally increased
134 in cancer cells to support the high protein synthesis demand caused by their exacer-
135 bated proliferation rate^{7,8} and therefore, the inhibition of rRNA synthesis specifically
136 kills cancer cells without affecting normal cells^{9,10}. Such observations led to the devel-
137 opment of molecules specifically inhibiting RiBi that showed objective responses in
138 clinical trials, such as CX5461¹¹⁻¹³.

139 In addition to alteration of RiBi, recent observations suggest that variations of ribosome
140 composition could also occur in cancer and be involved in disease etiology¹⁴⁻¹⁶. The
141 ribosome is composed of 80 ribosomal proteins and 4 ribosomal RNAs (rRNAs), the
142 latter supporting the enzymatic activity of the peptidyl-bond formation during the trans-
143 lation of mRNAs into proteins. For many decades, the ribosome was considered as a
144 monolithic entity displaying a similar composition in all cells constituting an organism.

145 However, it appears now that the ribosome composition can display some degree of
146 variations, both at the level of ribosomal proteins and rRNA chemical modifications,
147 which contributes to modulate intrinsic translational activities that could shape particu-
148 lar phenotypes^{17,18}. Variations of the ribosome composition at ribosomal protein levels
149 have been reported in HGGs^{19–22}. In IDHwt glioblastomas, the overexpression of the
150 ribosomal protein RPS6 was shown to promote acquisition of glioma stem cell proper-
151 ties, a hallmark of the most aggressive IDHwt glioblastomas^{20–22}. In addition, RPL22L1
152 isoforms are expressed in distinct regions of IDHwt glioblastomas through alternative
153 splicing and induce the production of ribosomes with specific compositions, which pro-
154 mote translational bias towards specific mRNA subsets^{15,19,23–30}. In addition to riboso-
155 mal proteins, the chemical modifications of rRNA represent one of the major contribu-
156 tors to ribosome heterogeneity and led to the emergence of the notion of rRNA epi-
157 transcriptomics¹⁵. One of the main modifications, the methylation of the rRNA 2'O-ri-
158 bose (rRNA 2'Ome), occurs at 106 known rRNA sites in humans and the 2'Ome at
159 specific positions are essential for rRNA activity. The catalysis of rRNA 2'Ome is per-
160 formed by an rRNA methylation complex composed of the methyl-transferase fibrillar
161 (FBL) and a single non-coding C/D box small nucleolar RNA (snoRNA or snoRD),
162 which guides FBL at specific sites by base-pairing¹⁵. Hence, modulations of *FBL* or
163 snoRD expression are sufficient to affect rRNA 2'Ome^{23,24,26}. Interestingly, alterations
164 of 2'Ome have been observed in cancer and we recently demonstrated by profiling 195
165 primary mammary tumors using the RiboMethSeq approach, that only 40% of the
166 known 2'O-methylated sites are altered, suggesting that only few rRNA sites can tol-
167 erate a lack of 2'Ome. Moreover, rRNA 2'Ome alterations are not random since rRNA
168 2'Ome profiles were associated with breast cancer subtypes and tumor grades²⁵. Sim-
169 ilarly, alterations of rRNA 2'Ome were described in a cohort of 17 diffuse large B-cell
170 lymphoma samples²⁷ and of 94 acute myeloid leukemia samples²⁸. Importantly, alter-
171 ations of rRNA 2'Ome at some specific sites can affect both the translation of particular
172 mRNA subsets and cell proliferation^{15,23,24,26 29,30}. To date, whether alterations of rRNA
173 epitranscriptomics occurs in gliomas and contributes to disease etiology remains un-
174 explored. Here, we investigated whether IDHwt and IDHmut HGGs display alterations
175 in ribosome biology, in terms of rRNA epitranscriptomics and ribosome biogenesis, to
176 exploit these features as novel therapeutic targets of these diseases.

177

178

179 **Materials and methods**

180

181 **Human grade 3-4 adult-type diffuse glioma and non-neoplastic samples**

182 Three cohorts were built: a technical cohort (8 grade 4 IDHwt glioblastomas, 3 non-
183 tumoral samples); a test cohort detailed in Table 1 (13 IDHwt glioblastomas, 13 IDHmut
184 astrocytomas, 14 IDHmut and 1p/19q codeleted oligodendrogliomas, 6 non-tumoral
185 samples); a validation cohort (9 IDHwt glioblastomas, 6 IDHmut astrocytomas, 8
186 IDHmut and 1p/19q co-deleted oligodendrogliomas). The percentage of tumoral cells
187 was estimated by a neuropathologist as described in Fig.S1. Additional details are pro-
188 vided in Supplementary Information.

189

190 **Cell culture**

191 Human IDHwt glioblastoma (5706, N131520), IDHmut astrocytoma (LGG85) and
192 IDHmut and 1p/19q codeleted oligodendroglioma (BT138, BT237) cells were cultured
193 as spheres as described in Supplementary Information.

194

195 **Reverse Transcription and real time quantitative PCR**

196 cDNA synthesis was performed using the Prime Script RT Reagent kit (Takara). Me-
197 dium throughput qPCR was performed using the Biomark HD system (Fluidigm) as
198 previously described³¹ (Table S1). The median Ct value of 5 housekeeping mRNAs
199 was used for normalization.

200

201 **RiboMethSeq**

202 RiboMeth-seq was performed as previously described using the Illumina sequencing
203 technology^{25,32}. To process the sequencing data, a novel nextflow pipeline Ribo-
204 MethSeq-nf was developed and is currently available ([https://github.com/Ri-
205 bosomeCRCL/ribomethseq-nf](https://github.com/RibosomeCRCL/ribomethseq-nf)). This pipeline processes sequencing data as previ-
206 ously described^{25,32,33}. To calculate the C-score, which reflects the rRNA 2'Ome level,
207 the novel R package rRMSAnalyzer was developed ([https://github.com/Ri-
208 bosomeCRCL/rRMSAnalyzer](https://github.com/RibosomeCRCL/rRMSAnalyzer)). The identification of significant alterations in rRNA
209 2'Ome levels between groups was performed by applying two consecutive thresholds:
210 the adjusted p-value < 0.05 (Kruskal-Wallis with FDR adjustment); and the mean Δ C-
211 score (*i.e.*, difference between the highest and lowest mean C-score of the groups of
212 interest) > 0.05.

213 IC₅₀ assay

214 3.10³ cell spheroids were treated with CX5461 (Sigma-Aldrich)³⁴ or BMH-21 (Sigma-
215 Aldrich)¹⁰. Cell cytotoxicity was assessed by CellTox™ Green Cytotoxicity Assay
216 (Promega) and by CellTiter-Glo3D® luminescent cell viability assay (Promega). Cell
217 viability was expressed as a percentage of the signal intensity normalized against
218 DMSO (1%).

219

220

221 Results

222

**223 A standardized approach for large-scale analyses of human samples using Ri-
224 boMethSeq**

225 Before investigating alterations of rRNA 2'Ome in the three main histomolecular HGGs,
226 we first optimized the recently described RiboMethSeq approach^{25,32,35} to determine
227 reliable quantifications of 2'Ome levels at 106 rRNA sites from patient tumor samples.
228 First, based on the technical cohort (n=11) of IDHwt glioblastomas and non-neoplastic
229 tissues, we observed that the C-score, which reflects rRNA 2'Ome levels at specific
230 sites, was similar using either a manual or an automated RNA extraction protocol
231 (Fig.S2). Second, we used the NovaSeq Illumina sequencing platform (up to 10 billion
232 reads) to increase the total number of useful reads (Fig.S3A) and the number of sam-
233 ples sequenced in a single flowcell.

234 We then randomly separated RNA samples of the test cohort and prepared two inde-
235 pendent libraries of 23 samples (40 HGGs and 6 non-neoplastic samples), each library
236 also contained a commercially-available "reference" total RNA. Unsupervised analysis
237 of the entire test cohort using PCA based on C-scores of either all rRNA positions
238 (7055 sites) or the 106 positions corresponding to known rRNA 2'Ome sites, clearly
239 distinguished samples depending on the library of origin, as illustrated by the lack of
240 clustering of reference RNA (Fig. S3B, left panels). We evaluated adjustment of Ribo-
241 MethSeq data using the ComBat-seq algorithm, one of the most routinely used tools
242 to adjust RNA-seq data³⁶. Upon ComBat-seq adjustment, no distinction between sam-
243 ples based on their library of origin was observed in C-scores, including for the two
244 reference RNA (Fig.S3B, right panels). These data demonstrate the efficacy of the
245 ComBat-seq algorithm at removing batch effect from RiboMethSeq data.

246 Based on these results, we developed bioinformatics tools to perform reproducible
247 analyses of RiboMethSeq data arising from large-scale cohorts ([https://github.com/Ri-
249 bosomeCRCL](https://github.com/Ri-
248 bosomeCRCL)).

250 **rRNA 2'Ome profiles discriminate IDHwt from IDHmut adult-type diffuse gliomas**

251 Using the optimized approach selected above, we then investigated whether altera-
252 tions of rRNA 2'Ome differentially occur in the three main histomolecular HGGs using
253 the test cohort: IDHwt glioblastoma (G, n=13), IDHmut astrocytoma (A, n=13), IDHmut
254 and 1p19q codeleted oligodendroglioma (O, n=14) and non-neoplastic cerebral cortex
255 (NT, n=6) (Table 1). Using unsupervised hierarchical clustering analysis (HCA), we
256 first evaluated rRNA 2'Ome levels at the 106 known sites in the 46 non-neoplastic and
257 glioma samples (Fig.1). The C-score reflects the rRNA 2'Ome levels as it corresponds
258 to the ratio of the 5' read-end counts at a nucleotide position to the local 5' read-end
259 count coverage; and when close to 1, C-score indicates that all rRNA molecules of the
260 sample are 2'O-methylated at this specific site; whereas a C-score below 0.9 reflects
261 a mix of 2'O-unmethylated and 2'O-methylated rRNA molecules. Here, most rRNA
262 2'Ome sites had a score close to 1, albeit some sites were below 0.9, substantiating
263 recent results in human samples from diffuse large B cell lymphoma, acute myeloid
264 leukemia and breast cancer^{25,27,28}. These data further confirmed observations by us
265 and others that some rRNA molecules exist without 2'Ome at some specific sites, in-
266 cluding in non-neoplastic tissue.

267 Interestingly, all IDHwt glioblastoma samples formed a separate branch (left-hand side
268 of the HCA dendrogram), suggesting that their rRNA 2'Ome profile was clearly different
269 from IDHmut astrocytoma and oligodendroglioma, as well as from non-neoplastic tis-
270 sue (Fig.1). Principal Component Analyses (PCAs) based on the rRNA 2'Ome profile
271 indicated that the PC2 axis (variance=14.1%) strongly differentiated glioblastomas
272 from other samples (Fig.S4A). To ensure consistency between the results of our clas-
273 sification and the expected outcome of the patients, we correlated PCA axes with sur-
274 vivals and mitotic index, as an internal control of tumor sample classification. Con-
275 sistent with the known differences regarding glioblastomas and IDHmt HGGs charac-
276 teristics, PC2 was significantly correlated with the *IDH1/2* mutational status, the mitotic
277 index, overall survival (OS) and progression-free survival (PFS) (Fig.S4B-D). To vali-
278 date these observations, we analyzed rRNA 2'Ome levels using RiboMethSeq on the

279 technical cohort, corresponding to an independent cohort composed of 8 IDHwt glioblastomas and 3 non-neoplastic tissues. Unsupervised PCA based on the rRNA 2'Ome profile showed that IDHwt glioblastomas could once again be clearly separated from non-neoplastic tissues (Fig.S4E). Altogether, our data revealed that rRNA 2'Ome levels vary in HGGs as well as in non-neoplastic cerebral cortex, and that rRNA 2'Ome profiles discriminate IDHwt glioblastomas not only from non-neoplastic tissues but also from IDHmut HGG types.

286

287 **Most variable rRNA 2'Ome sites are sufficient to discriminate IDHwt glioblastoma from IDHmut adult-type diffuse gliomas**

289 To better characterize differences in rRNA 2'Ome profiles between histomolecular HGG types, we then focused on the most variable rRNA 2'Ome sites. To identify such sites, we compared the variability of C-scores at each single site among all HGG samples of the test cohort (n=40) using the distribution of the inter-quartile range (IQR). A set of 19 sites showed an IQR higher than median + 2 × median absolute deviation (mad) and were considered as the most variable rRNA 2'Ome sites among HGGs (red, Fig.2A), in agreement with our recent findings that only a subset of rRNA sites displays variability in 2'Ome levels²⁵. Interestingly, PCAs indicated that rRNA 2'Ome profiles based on this set of 19 sites are sufficient to discriminate IDHwt glioblastomas, IDHmut astrocytoma and oligodendrogliomas (Fig.2B).

299 To validate these observations, we used a second, independent validation cohort of 300 23 HGG samples (9 IDHwt glioblastomas, 6 IDHmut astrocytomas and 8 IDHmut, 301 1p/19q codeleted oligodendrogliomas) (Fig.S1B). We performed an unsupervised analysis using the 19 most variable rRNA 2'Ome sites identified using the test cohort (Fig.2A). As for the test cohort, unsupervised analyses of the validation cohort distinguished the three histomolecular subtypes of HGGs (Fig.2C). In addition, the projection of the validation cohort samples onto the PCA constructed from the test cohort enabled us to identify the histomolecular subtypes of each sample, suggesting that *de novo* profiling of rRNA 2'Ome could help in the classification of HGGs (Fig.2D). In addition, comparison of test and validation cohorts in term of percentage of tumor cells suggests that the tumor heterogeneity did not affect our conclusions (Fig. S1B). Indeed, in the validation cohort, the mean percentage of tumor cells is significantly lower in astrocytomas (38%) compared to oligodendrogliomas (69%), while no difference was observed with glioblastomas (9%) (Kruskal-Wallis: p=0.045*; Mann-Whitney: A vs O

313 p=0.024*). In contrast, in the test cohort, the mean percentage of tumor cells is signif-
314 icantly lower in astrocytomas (38%) and oligodendrogliomas (39%) compared to glioblastomas (55%) (Kruskal-Wallis: p=0.034*; Mann-Whitney: A vs G p=0.0172*; O vs G
315 p=0.0348*).

317 These data further emphasize the specificity of IDHwt glioblastoma rRNA 2'Ome pro-
318 files compared to IDHmut HGGs and suggest that IDHwt glioblastomas display strong
319 alterations of rRNA 2'Ome levels at some specific sites.

320

321 **Glioblastomas display the most frequent site-specific rRNA 2'Ome alterations**

322 To identify rRNA sites whose 2'Ome levels significantly differed between HGG histo-
323 molecular types and non-neoplastic tissues, we performed a systematic analysis of
324 each of the 106 rRNA 2'Ome sites using the test cohort and applied two consecutive
325 thresholds, a Kruskal-Wallis test with adjusted p-values < 0.05 and a cut-off value for
326 mean ΔC -scores_{max-min} > 0.05. From this screen, only 16 rRNA 2'Ome sites displayed
327 a significant variation in their level of methylation in at least one HGG or non-neoplastic
328 tissue (Fig.3). Pairwise comparisons for these 16 sites revealed that 4 and 6 sites dis-
329 played significant alterations in 2'Ome levels in high-grade astrocytomas and high-
330 grade oligodendrogliomas, respectively, compared to non-neoplastic tissues (Fig.3B).
331 In addition, rRNA 2'Ome levels appeared to be significantly increased on 6 sites in
332 high-grade astrocytomas compared to high-grade oligodendrogliomas. Similar altera-
333 tions in rRNA 2'Ome levels between IDHwt and IDHmut tumours were observed using
334 the validation cohort (Fig.S5A). Finally, the main differences could be attributed to gli-
335 oblastomas that displayed 12 and 14 of the 16 rRNA sites significantly altered com-
336 pared to non-neoplastic samples or high-grade astrocytoma and oligodendrogliomas,
337 respectively. Strikingly, rRNA 2'Ome levels were mostly lower in glioblastomas com-
338 pared to non-neoplastic tissues, high-grade astrocytoma or high-grade oligodendrogli-
339 omas (Fig.3A). The same overall decrease in rRNA 2'Ome levels was confirmed in
340 glioblastoma compared to non-neoplastic tissues using the technical cohort (Fig.S5B).
341 Altogether, these results indicate that 2'Ome levels are altered at specific rRNA sites
342 in HGGs, while glioblastomas display the highest frequency of alterations in rRNA
343 2'Ome levels compared to non-neoplastic cerebral cortex and other HGG histomolec-
344 ular types.

345

346 **Changes in C/D box snoRNA expression levels only partially explain alterations**
347 **of rRNA 2'Ome levels**

348 To investigate the origin of alterations of rRNA 2'Ome in HGGs, we focused on the
349 expression of C/D snoRNAs (snoRDs), which guide in a sequence-specific manner the
350 methyl-transferase FBL toward the rRNA nucleotide to methylate. We thus examined
351 the correlation between levels of rRNA 2'Ome and related snoRDs. It was reported
352 that snoRNA expression levels can be inferred from RiboMethSeq data^{37,38}. Thus, we
353 used RiboMethSeq raw data from the NovaSeq platform and applied an in-house pipe-
354 line to overcome limitations of snoRNA analysis by next-generation sequencing (see
355 Supplementary methods)^{39,40}. A strong and significant correlation (about 92%) be-
356 tween snoRNA levels either determined from RiboMethSeq or measured by RT-qPCR
357 was observed in 9 glioma samples for 11 out of 12 selected snoRDs, thus validating
358 our approach (Fig.S6A). Given that 2'Ome at a single rRNA site is catalyzed either by
359 one or several snoRD^{15,41}, we restricted our analysis to the rRNA 2'Ome sites (n=46)
360 guided by a single snoRD. No significant correlation was observed between levels of
361 rRNA 2'Ome and of corresponding snoRDs among 37 sites (Fig.S6B). In contrast, a
362 significant albeit relatively low positive correlation was observed for 9 pairs of
363 snoRD/rRNA sites, the best correlation being that of the 18S-Am576 site and its asso-
364 ciated SNORD93 (p_{adj}=9.10⁻⁶, r=0.68) (Fig.S6B-C). Thus, alterations of rRNA 2'Ome
365 levels cannot be exclusively attributed to changes in C/D box snoRNA expression lev-
366 els.

367

368 **The expression profile of ribosome biogenesis factors discriminates IDHmut and**
369 **IDHwt HGGs**

370 Since variations in snoRD expression levels cannot explain most of the changes ob-
371 served in rRNA 2'Ome levels, we tested a novel hypothesis. Indeed, in cancer, varia-
372 tions of 2'Ome levels in rRNAs are believed to passively arise from the link between
373 ribosome biogenesis (RiBi) and concomitant rRNA chemical modifications, whereby
374 changes in ribosome synthesis impact the rate-limiting rRNA 2'Ome process and
375 therefore influence 2'Ome profiles^{15,25,27}. Therefore, an overall decrease in rRNA
376 2'Ome levels observed in different cancer tissues may be caused by RiBi hyperactiva-
377 tion to sustain a high demand in protein synthesis necessary to support the highly pro-
378 liferative cancer cells. As IDHwt glioblastomas possess a higher proliferative rate than
379 IDHmut astrocytoma and oligodendrogliomas, including in our test cohort (based on

380 the mitotic index) (Fig.S4C-D), we hypothesized that the decrease in rRNA 2'Ome lev-
381 els at specific positions in IDHwt glioblastomas could be due to a rate-limiting 2'Ome
382 process caused by an exacerbated RiBi.

383 To investigate RiBi dysregulations across HGGs, we initially applied a gold-standard
384 approach by analyzing levels of the 47S rRNA precursor (pre-rRNA), using Northern
385 blot (Fig.S7A). However, this approach using tumor samples was challenging, notably
386 due to the need for a large quantity of biological materials. Nevertheless, in a panel of
387 9 analyzable samples, IDHmut astrocytoma and oligodendrogliomas surprisingly
388 seemed to express more pre-47S rRNAs than IDHwt glioblastomas. However, only two
389 IDHwt glioblastoma samples were analyzed, therefore preventing us to draw any con-
390 clusion. To bypass this technical issue, we then measured the expression of a set of
391 20 genes involved in the early RiBi stages (referred to as "RiBi-gene set"). To reflect
392 as much as possible this multistep process involving more than 200 factors⁷, we se-
393 lected the RiBi-gene set implicated in the main RiBi process, including rRNA transcrip-
394 tion (*NCL*, *NPM*, *POLR1A*, *TAF1A*, *TAF1B*, *TAF1C* and *UBTF*), rRNA maturation
395 (*BOP1*, *PES1* and *WDR12*), snoRNA biogenesis (*RUVBL1*, *PIDH1D1* and *RUVBL2*),
396 and H/ACA (*DKC1*, *GAR1*, *NHP2* and *NOP10*) and C/D box (*NOP56*, *SNU13* and *FBL*)
397 snoRNP complexes (Fig.4A). Of note, three genes (*PIH1D1*, *RUVBL2* and *FBL*) are
398 located on the long arm of chromosome 19 (19q), which undergoes a heterozygous
399 deletion in high-grade oligodendrogliomas. A readout for RiBi at steady-state was de-
400 termined by quantifying mRNA expression levels of these selected genes by medium
401 throughput RT-qPCR in our validation series. mRNA levels were normalized against
402 the median mRNA expression of 5 housekeeping genes, which did not significantly
403 vary among the four groups (Fig.S7B).

404 To examine the association between expression levels of RiBi factors and HGGs, we
405 first performed an unsupervised approach using a PCA based on the RiBi-gene set
406 profile (Fig.4B). Interestingly, three main clusters were observed. A first large cluster,
407 composed of both non-neoplastic tissues and IDHwt glioblastomas (NT/G cluster), was
408 distinct from two other clusters corresponding to IDHmut oligodendrogliomas (O) and
409 astrocytoma (A). PC1 (variance: 67.8%), in particular, separated the NT/G cluster from
410 A/O clusters, while the PC2 (variance: 9%) distinguished the O cluster from others.
411 These data suggest that the expression profile of only 20 RiBi factors discriminate
412 HGGs.

413 To ensure consistency between the results of our classification and the expected out-
414 come of the patients, we first calculated Pearson's correlation coefficients between the
415 first 5 PC dimensions and OS or PFS as an internal control (Fig.4C and Fig.S7C).
416 Consistently, we observed a significant association exclusively for PC1 and PC2, indi-
417 cating that the clustering based on RiBi-gene set profiles provided by these two axes
418 is sufficient to recapitulate all clinical data of interest. OS and PFS were significantly
419 correlated with both PC1 and PC2 in a negative manner, indicating that samples clus-
420 tering at the right-hand side of the PC1 and top of PC2, *i.e.*, IDHwt glioblastomas,
421 display lower OS and PFS. Indeed, IDHwt glioblastoma patients exhibit the poorest
422 OS (less than 30 months) and PFS (under 30 months), followed by IDHmut astrocy-
423 toma and oligodendroglioma patients that tend to cluster at the left part of PC1 and the
424 top or bottom part of PC2, respectively.

425 Strikingly, we also identified a strong correlation between PC1 and PC2 axes, and the
426 *IDH1/2* mutational and 1p/19q co-deletion status, respectively (Fig.S7D-E). Indeed,
427 PC1 (G vs. A/O clusters) was significantly correlated with the *IDH1/2* mutational status
428 while PC2 (A/G vs. O clusters) segregated HGG tumors based on the 1p/19q co-dele-
429 tion status. Thus, expression profiling of the RiBi-gene set was strongly correlated with
430 both clinical features and distinct genomic alterations of the HGG test cohort. Alto-
431 gether, these results suggest that the expression signature of only 20 genes involved
432 in ribosome biogenesis is sufficient to discriminate IDHwt from IDHmut HGG histomo-
433 lecular types and that RiBi displays IDH mutational status-dependent alterations.

434

435 **Ribosome biogenesis factors are highly expressed in IDHmut adult-type diffuse** 436 **gliomas**

437 Next, we individually compared mRNA expression levels of the 20 RiBi factors among
438 the different HGGs (Fig.5 and Fig.S8A). Significant differences were observed in the
439 expression of all tested RiBi factors between different groups (Mann Whitney test,
440 Fig.S8A). Surprisingly, only a few RiBi genes were significantly differentially expressed
441 in IDHwt glioblastomas compared to non-neoplastic samples (9 out of 20), with very
442 moderate changes (≤ 2 -fold change). In contrast, IDHmut astrocytoma and oligoden-
443 drogliomas significantly overexpressed most of these RiBi genes (19 and 16 out of 20
444 RiBi genes, respectively). For instance, expression of *NCL*, which encodes a key factor
445 in rRNA synthesis, increased by 2- and 3-fold in IDHmut astrocytoma and oligoden-
446 drogliomas, respectively ($p < 0.001$), while *NCL* expression levels in IDHwt samples

447 only slightly increased (Fig.5A and Fig.S8A). Likewise, the use of a second distinct set
448 of primers to analyze *NCL* expression provided the exact same trend (Fig.S8B), there-
449 fore ruling out a potential technical caveat. These results suggest that ribosome bio-
450 genesis could be enhanced in IDHmut astrocytoma and oligodendrogliomas compared
451 to IDHwt glioblastomas and non-neoplastic tissues.

452 Interestingly, genes located on chromosome 19q (*FBL*, *PIH1D1*, *RUVBL2*) displayed
453 a particular expression pattern (Fig.5C, 5E and Fig.S7E). Like other genes involved in
454 RiBi, *FBL*, *PIH1D1* and *RUVBL2* were significantly overexpressed in IDHmut astrocy-
455 tomas compared to both IDHwt glioblastomas and non-neoplastic samples. However,
456 mRNA expression levels in IDHmut oligodendrogliomas were lower than in IDHmut
457 astrocytomas and displayed expression profiles resembling those of IDHwt glioblasto-
458 mas. Thus, *FBL* was highly expressed exclusively in IDHmut astrocytomas, whereas
459 its expression levels were equivalent in IDHmut oligodendrogliomas and IDHwt glioblastomas,
460 as confirmed by a second set of primers (Fig.S8B). Considering that
461 IDHmut oligodendroglioma samples exhibited a heterozygous deletion of *FBL*, *PIH1D1*
462 and *RUVBL2* genes located on 1p/19q chromosomes, these data indicate that the spe-
463 cific expression profile observed for these genes in IDHmut oligodendrogliomas is
464 likely due to a haploinsufficiency caused by genetic alterations. Interestingly, removal
465 of these three genes in the RiBi-genes set still allowed us to distinguish IDHwt from
466 IDHmut HGGs (data not shown), suggesting that copy number variation (CNVs) affect-
467 ing RiBi gene expression does not impact the distinction between IDHwt and IDHmut,
468 as expected. Therefore, the ribosome biogenesis pathway is strongly enhanced in
469 IDHmut gliomas, *i.e.*, high-grade astrocytoma and oligodendroglioma, but remains
470 moderately affected in IDHwt glioblastoma.

471
472 **HGGs display distinct cytotoxicity to RNA pol I inhibitors CX5461 and BMH-21**
473 Having identified an IDH mutational status-dependent alteration of RiBi in HGGs, we
474 hypothesized that IDHmut and IDHwt HGGs display distinct cytotoxicity to RNA Pol I
475 inhibitors, BMH-21 and CX5461, which inhibit the transcription of the 47S pre-rRNA
476 and are promising new cancer treatments. We used a panel of 5 representative HGG
477 cell lines, comprising IDHmut astrocytoma and IDHmut and 1p/19q codeleted oli-
478 godendroglioma cell lines (LGG85 and BT138/237, respectively) and IDHwt glioblas-
479 toma cell lines (5706 and N131520). Analyses of HGG spheroids in response to 72-

480 hour treatments with RNA pol I inhibitors showed that all spheroids were similarly sen-
481 sitive to BMH-21 with an IC₅₀ ranging from 1.06 to 1.56 μM (Fig.6A-C), as recently
482 observed ⁴². However, CX5461 strongly impacted the viability of IDHmut astrocytoma
483 and oligodendroglioma spheroids (IC₅₀ ranging from 5.92 to 7.55μM), but not of IDHwt
484 glioblastoma spheroids (Fig.6D-F). Hence, these results are consistent with our previ-
485 ous findings that IDHmut astrocytomas and oligodendrogliomas may be addicted to an
486 enhanced RiBi pathway, therefore potentially sensitizing these AGD histomolecular
487 types to clinically available RiBi inhibitors. Altogether, our results highlight the potency
488 of RNA Pol I inhibitor usage as potential HGG therapy and further support an over-
489 activation of the RiBi pathway in IDHmut astrocytomas and oligodendrogliomas com-
490 pared to IDHwt glioblastomas and non-neoplastic tissues.

491

492

493 **Discussion**

494 High-grade adult-type diffuse gliomas (HGGs) are heterogeneous tumors associated
495 with distinct, albeit poor, survival rates due to the lack of effective targeted therapies,
496 in particular for the most aggressive histomolecular type, the IDHwt glioblastoma.
497 Here, by performing the first concomitant analysis of rRNA 2'Ome and ribosome bio-
498 genesis in primary tumors, we report distinct, uncoupled alterations of rRNA epitran-
499 scriptomics and ribosome biogenesis in IDHmut and IDHwt HGGs, therefore revealing
500 specific dysregulations of the ribosome biology that constitute new IDH mutational sta-
501 tus-associated hallmarks of HGGs.

502 In the last 7 years, alterations of rRNA 2'Ome profiles have been reported using a
503 newly developed approach RiboMethSeq in numerous cellular models and only in
504 three types of cancers, namely breast cancer, acute myeloid leukemia and diffuse
505 large-B cell lymphoma (DLBCL)^{15,25,27,28}. Such alterations have been shown to be re-
506 stricted to only 40% of the known rRNA 2'Ome sites, suggesting that only one third of
507 the sites may possess regulatory functions on ribosome activity. We now show that
508 rRNA 2'Ome profiles also vary in HGGs. As previously observed in other cancers, only
509 a small subset of known rRNA 2'Ome sites display variability in their 2'Ome levels in
510 HGGs, suggesting these positions can tolerate absence of 2'Ome. The 19 most varia-
511 ble rRNA 2'Ome sites were randomly distributed on the ribosome structure, suggesting
512 no coordinated effects on functional domains of the ribosome. Notably, similar in-
513 creases in rRNA 2'Ome levels at 18S_Am576 and decreases at 18S_Gm1447 were

514 observed in the most aggressive HGG type, glioblastoma, and breast cancer subtypes
515 (triple negative or TNBC), suggesting a link between these sites and cancer aggres-
516 siveness²⁵. A recent study demonstrated that alterations of 2'Ome levels at a single
517 rRNA site are sufficient to affect cell proliferation, a hallmark of cancer aggressive-
518 ness²⁶. Moreover, rRNA 2'Ome at 18S_Gm1447 was recently shown to support leuke-
519 mic stem cell functions by modulating translation²⁸. Hence, whether these rRNA 2'Ome
520 sites common to both HGGs and breast cancer contribute to the acquisition of cancer
521 cell characteristics remains to be deciphered and could potentially represent new tar-
522 getable vulnerabilities.

523 In IDHwt glioblastomas, alterations in rRNA 2'Ome levels mostly correspond to a de-
524 crease as observed in both the test and validation cohorts. In DLBCL, the global de-
525 crease in rRNA 2'Ome levels was correlated with the Ki67-estimated high proliferative
526 rate of tumors. One hypothesis was that low rRNA 2'Ome levels indirectly resulted from
527 an increase in rRNA synthesis associated with the hyperproliferative rate of cancer
528 cells, which rendered components of the rRNA 2'Ome machinery limiting, although
529 rRNA synthesis was not analyzed²⁷. Consistently, we observed specific alterations of
530 rRNA 2'Ome in IDHwt glioblastomas, which are the most proliferative tumors and dis-
531 play the highest mitotic index in our test cohort. However, we surprisingly observed an
532 elevated ribosome biogenesis in IDHmut astrocytomas and oligodendrogliomas com-
533 pared to glioblastomas, suggesting that RiBi levels are not correlated with the prolifer-
534 ative rate, at least in HGGs, and cannot solely explain alterations of rRNA epitran-
535 scriptomics in HGGs. Even though the rate of ribosome biogenesis may contribute to
536 regulating rRNA 2'Ome through a passive effect, additional molecular mechanisms
537 should be further explored to identify the origin of rRNA 2'Ome alterations in cancer
538 and understand the observed rRNA site- and cancer type-specificity. The mechanisms
539 may include alterations of expressions and/or activities of RNA-binding proteins, such
540 as DDX21 and FMRP, which contribute to the formation of *bona fide* functional snoRNP
541 complexes^{29,43}. Here, we report that alterations of C/D box snoRNA expression could
542 be sufficient to explain alterations of rRNA 2'Ome levels at some, but not all, rRNA
543 sites. The evolution of annotation and/or knowledge in biology of C/D box snoRNAs
544 may fill the gap to better understand causes of rRNA 2'Ome alterations in cancer. Al-
545 together, our findings that main HGG histomolecular types are associated with altera-
546 tions in either ribosome quantity or quality, challenge the hypothesis that the decrease

547 in rRNA 2'Ome levels mainly results from a passive effect caused by an exacerbated
548 ribosome biogenesis.

549 Our data reveal that IDHmut HGGs, including both high-grade astrocytoma and oli-
550 godendroglioma, display the highest expression of ribosome biogenesis factors, sug-
551 gesting an increase in ribosome biogenesis. Whether dysregulations of *IDH1/2* func-
552 tions, notably through the production of the oncometabolite D-2-hydroxyglutarate,
553 could directly impact the regulation of ribosome biogenesis would need to be further
554 explored. Nevertheless, the specificity of ribosome biogenesis alterations in HGGs of-
555 fers novel perspectives for clinical applications. Building on our observations, we found
556 that HGGs are sensitive to the newly developed RNA pol I inhibitors, CX5461 and
557 BMH-21, the former being successfully evaluated in clinical trials in advanced solid and
558 hematological cancers⁷. Sensitivity of HGGs to RNA pol I inhibitors CX5461 and BMH-
559 21 has already been reported^{42,44,45}, even though discrepancies regarding the sensi-
560 tivity of glioblastomas to CX5461 exist between our data and previous ones, possibly
561 due to differences in experimental settings and genetic backgrounds of tested cell
562 lines. In particular, the 3D culture conditions could decrease drug sensitivity compared
563 to 2D culture conditions, as already reported⁴⁶. In addition, the differential sensitivity of
564 glioblastomas to CX5461 and BMH-21 could be attributed to off-target effects of these
565 molecules as these RNA Pol I inhibitors act through distinct mechanisms and are
566 known to affect several cellular pathways, including DNA repair^{10,34,47}. Thus, whether
567 the activity of these compounds may also rely on the genetic background of tested
568 models impacting the different sensitivities of HGG types to CX5461 and BMH-21,
569 should be further investigated.

570 Altogether, our data indicate that alterations of the ribosome biology in HGGs are de-
571 pendent on the IDH mutational status and could represent targetable features in clinic.
572 Thus, recent discoveries in the field of ribosomes have opened new avenues not only
573 for a better understanding of cellular processes that contribute to HGG development
574 and aggressiveness but also for designing future HGG type-specific therapeutic strat-
575 egies.

576 **Conflict of interest**

577 The authors have no conflicts of interest to declare.

578

579 **Ethical Approval**

580 All the experiment protocol for involving human was in accordance with the guidelines
581 of French regulation. Written informed consent was obtained from all patients.

582

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591

592 **Author contribution**

593 HP, NEHM, SB, JH, FB, VA, MC, JP, DB, LCV, SD performed and analysed experi-
594 ments. HP, NEHM, JK, ET, DB performed statistical descriptions and bioinformatic
595 analyses. HP, TC, AF, JK, ET, LT, AV developed bioinformatic tools. LCV, EH, MS
596 provided cell lines. DM, FD provided human samples and clinical data. AF, JK, ET, VA,
597 SG, MSS, EH, MS, DM, FC, VM, SB supervised experimental process. HP, NEHM,
598 SB, JK, SG, FD, VM and SD interpreted the data. VM and SD shaped the clinical and
599 research question and supervised the project coordination. AV, MSS, EH, MS, MG,
600 JJD, FD, VM and SD provided financial supports. HP, NEHM, VM and SD wrote the
601 first draft of the manuscript. All authors have read and approved the manuscript.

602

603 **Availability of datasets**

604 The generated RiboMethSeq data are available in the GEO profile (GSE224104, token
605 for reviewer access: qhabqswmfzotlwh). The datasets include the fastq of each sam-
606 ple, a C-score matrix for each cohort after batch effect adjustment using ComBat-seq
607 and a matrix summarizing the clinical data.

608

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618

619

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772 **Figure Legends**

773

774 **Figure 1. rRNA 2'Ome levels vary in high-grade adult-type diffuse gliomas.** An
775 unsupervised hierarchical clustering of C-scores at the 106 known rRNA 2'O-ribose
776 methylated (2'Ome) sites was performed in a test cohort of 40 high-grade (3-4) adult-
777 type diffuse glioma (HGG) samples and 6 non-tumoral, non-neoplastic cerebral cortex
778 (NT) samples. C-scores are represented by a color scale from 0 (black) to 1 (yellow).
779 IDHwt glioblastomas (G), high-grade astrocytomas (A), high-grade oligodendrogl-
780 omas (O) and non-neoplastic (NT) samples are depicted in pink, green, purple, and
781 grey, respectively. The mean C-score for each site across the 46 samples is shown on
782 the right-hand side of the graph. 86 sites have a mean C-score higher than 0.9 (black)
783 and 20 sites lower than 0.9 (red).

784

785 **Figure 2. The most variable rRNA 2'Ome sites are sufficient to distinguish differ-**
786 **ent HGG types. (A)** Distribution of the interquartile range (IQR) of the C-score to de-
787 termine the C-score variability across HGG samples of the test cohort (n=40). rRNA
788 2'Ome sites are ranked by increasing IQR value. The IQR distribution curve is plotted
789 at the right-hand side of the graph. The "most variable sites" correspond to those with
790 an IQR higher than median + 2 × median absolute deviation (mad) and were colored
791 in red (19 sites). **(B-D)** Unsupervised Principal Component Analysis (PCA) based on
792 C-scores of the 19 most variable sites as identified in (A). Independent PCA was per-
793 formed on IDHwt glioblastoma (G, pink circle), high-grade astrocytoma (A, green trian-
794 gle) and high-grade oligodendrogloma (O, purple diamond) samples of both test (B,
795 n=40) and validation (n=23) cohorts. Validation cohort samples were projected on PCA
796 of the test cohort (D). Percentage of variance explained by PC1 and PC2 are indicated.
797 95 % confidence ellipsoids around the centroid of each group (larger pink circle, green
798 triangle and purple diamond) are indicated.

799

800 **Figure 3. rRNA 2'Ome levels are differently altered in HGG types. (A)** Box plots
801 showing the distribution of C-score in HGG and non-neoplastic samples of the test
802 cohort for 16 rRNA 2'O-ribose methylated (2'Ome) sites that exhibited both statistically
803 and biologically significant alterations between groups. These sites were identified us-
804 ing both Kruskal-Wallis statistical tests (with an adjusted p-value threshold of < 0.05)

805 and a mean $\Delta C\text{-score}_{\text{max-min}} > 0.05$ (absolute difference between the highest and low-
 806 est mean C-score). The adjusted p-values corresponding to the statistical tests are
 807 indicated on the bottom left-hand side of each panel and median C-scores are repre-
 808 sented by a black line within the box plots. **(B)** Pairwise comparison of mean C-score
 809 groups for the 16 significantly deregulated rRNA 2'Ome sites in the test cohort. Positive
 810 (UP) and negative (DOWN) $\Delta C\text{-score}_{\text{group1-group2}}$ are shown in blue and red, respec-
 811 tively. Adjusted p-values: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns,
 812 not significant.

813

814 **Figure 4. Expression profiles of RiBi factors distinguish HGG types. (A)** Panel of
 815 genes involved in ribosome biogenesis constituting the 20 RiBi-gene set analyzed in
 816 HGG and non-neoplastic samples. Genes located on the short arm of chromosome 1
 817 (1p) or long arm of chromosome 19 (19q), which are heterozygously deleted in high-
 818 grade oligodendrogliomas, are indicated. **(B)** A principal component analysis (PCA)
 819 based on the mRNA expression profile of the RiBi-gene set. Each dot represents a
 820 non-neoplastic (NT, grey square), IDHwt glioblastoma (G, pink circle), high-grade as-
 821 trocytoma (A, green triangle) or high-grade oligodendroglioma (O, purple diamond)
 822 sample. Ellipsoids shows 90% confidence interval around the centroid (larger grey
 823 square, pink circle, green triangle, and purple diamond) of each group. Percentage of
 824 variance explained by PC1 and PC2 are indicated. **(C)** A heatmap showing Pearson's
 825 correlation coefficients of PC1 to PC5 axes with the overall survival (OS), progression-
 826 free survival (PFS) and mitotic index. R-values are depicted by different colors from -
 827 0.6 (red, negative correlation) to 0.6 (blue, positive correlation). Significant correlations
 828 are indicated by an asterisk: ** $p < 0.01$; *** $p < 0.001$.

829

830 **Figure 5. High-grade astrocytomas and oligodendrogliomas display the highest**
 831 **increased expression in ribosome biogenesis factors.** Box plots showing relative
 832 mRNA expression levels determined by RT-qPCR analysis of the RiBi genes impli-
 833 cated in rRNA **(A)** transcription and **(B)** maturation, **(C)** snoRNA biogenesis or associ-
 834 ated to **(D)** H/ACA box and **(E)** C/D box snoRNAs in IDHwt glioblastomas (G, pink),
 835 high-grade astrocytomas (A, green), high-grade oligodendrogliomas (O, purple) and
 836 non-neoplastic (NT, grey) samples. *FBL*, *PIH1D1* and *RUVBL2* are located on chro-
 837 mosomes 1p or 19q.

838 **Figure 6. Glioma spheroids reveal distinct histomolecular type-dependent sen-**
839 **sitivity to ribosome biogenesis inhibitors. (A)** Representative high-content screen-
840 ing microscopy images of 5706, N131520, BT138, BT237 and LGG85 cell line spher-
841 oids treated or not (DMSO) with 10 μ M of BMH-21 for 72 h. Hoechst and CellTox
842 labelling are depicted in blue and green, respectively. Cell lines representative of glioblastomas, high-grade oligodendrogliomas and astrocytomas are shown in pink, purple and green, respectively. **(B)** Representative graphs indicating the viability percentage in response to increasing BMH-21 concentrations in 5706, N131520, BT138, BT237 and LGG85 cell line spheroids. Cell lines representative of IDHwt glioblastomas, IDHmut oligodendrogliomas and astrocytomas are framed in pink, purple and green, respectively. **(C)** A table indicating means and standard deviations (SD) of BMH-21 IC₅₀ calculated from graphs displayed in (B) (n=7). (D), (E) and (F) as in (A) (B) and (C), respectively, for CX5461 (n=3).
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851

Table 1. Clinical characteristics of patients with high-grade primary diffuse gliomas constituting the test cohort

	Glioblastoma (n=13)	Astrocytoma grade 3/4 (n=13)	Oligodendroglioma grade 3 (n=14)
Age (years) mean [min-max]	60 [52-73]	36 [25-52]	53 [35-66]
Gender			
Female	4 (31%)	4 (31%)	5 (36%)
Male	9 (69%)	9 (69%)	9 (64%)
Tumor location			
Temporal	3 (23.1%)	3 (23.1%)	2 (14.3%)
Frontal	7 (53.8 %)	10 (76.9%)	12 (85.7%)
Occipital	1 (7.7%)	0 (0%)	0 (0%)
Parietal	2 (15.4%)	0 (0%)	0 (0%)
Genetic alteration			
mutation <i>IDH1/2</i>	0 (0%)	13 (100%)	14 (100%)
del 1p/19q	0 (0%)	0 (0%)	14 (100%)
ATRX-	NA	12 (92.3%)	0 (0%)
Progression			
Yes	12 (92.3%)	5 (38.5%)	3 (21.4%)
No	0 (0%)	8 (61.5%)	11 (78.6%)
NA	1 (7.7%)	0 (0%)	0 (0%)
Tumor cells (%) mean [min-max]	55 [30-85]	38 [20-60]	40 [20-70]

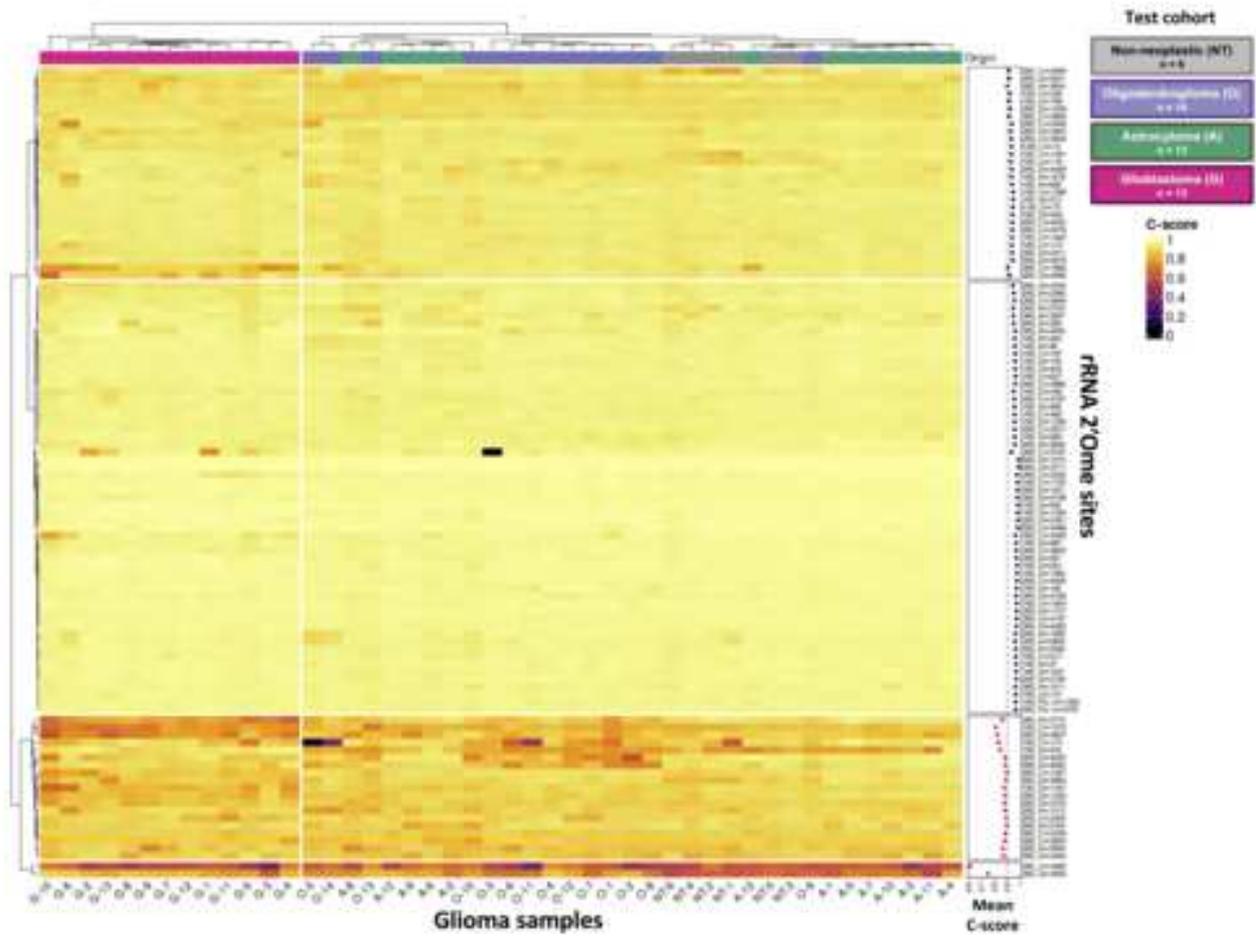


Figure 1

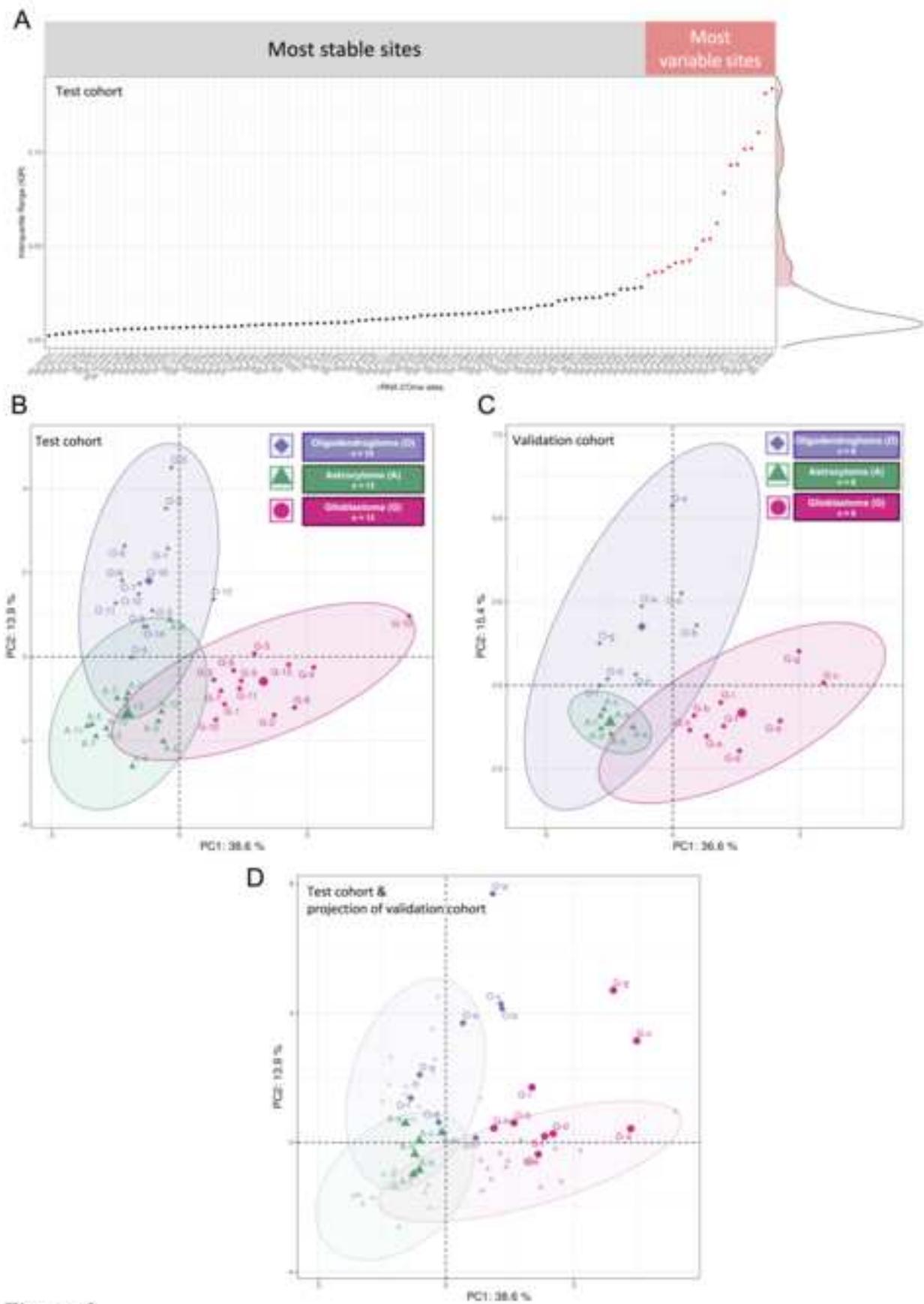
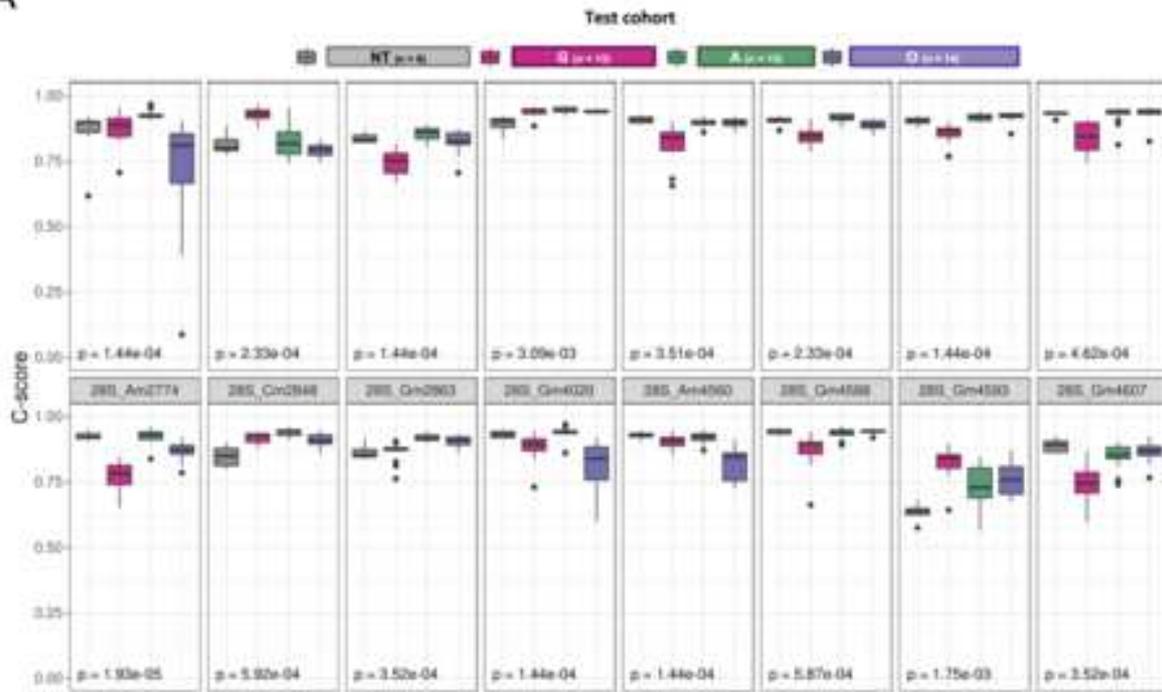


Figure 2

A



B

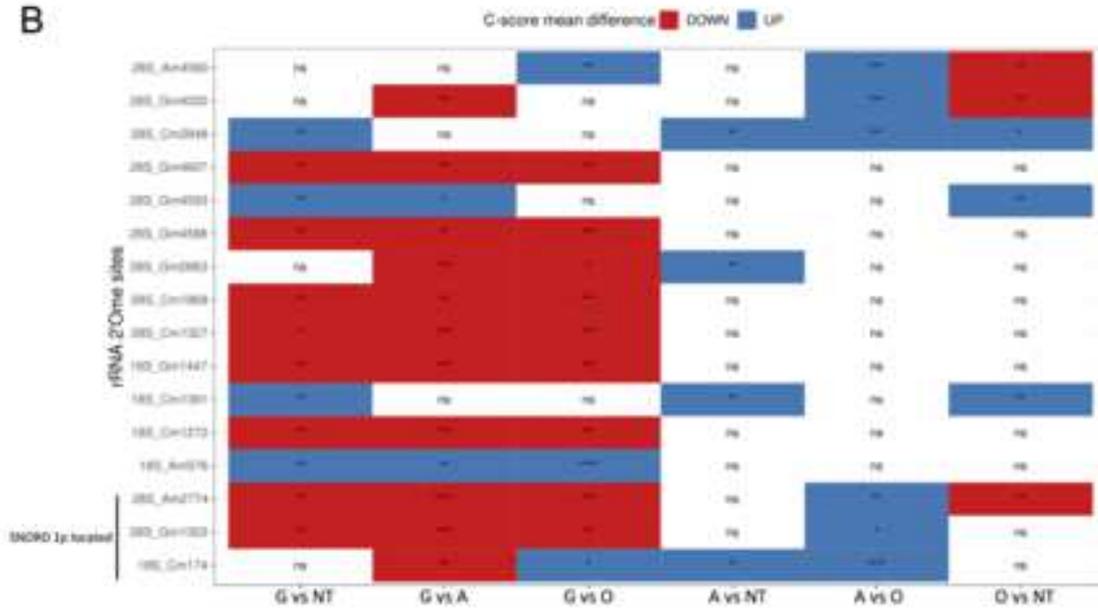
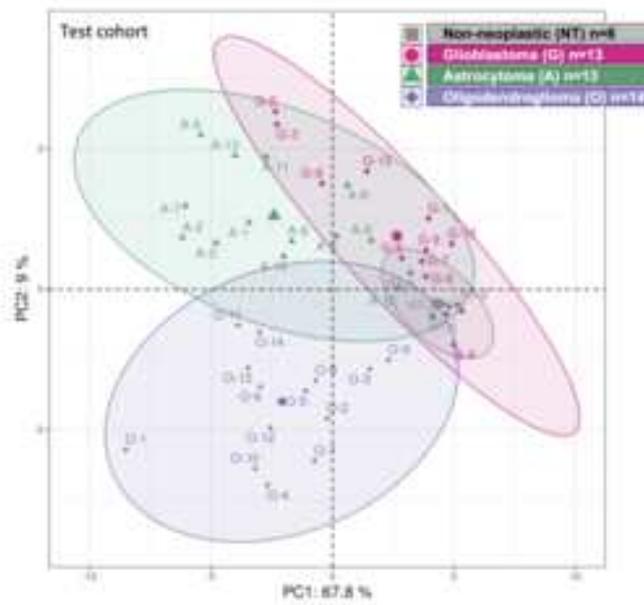


Figure 3

A



B



C

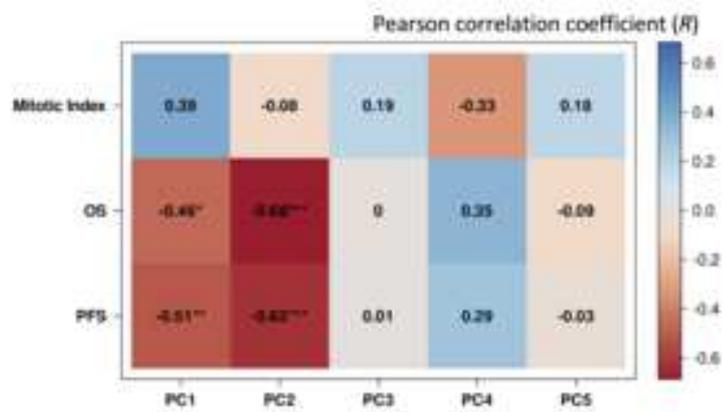


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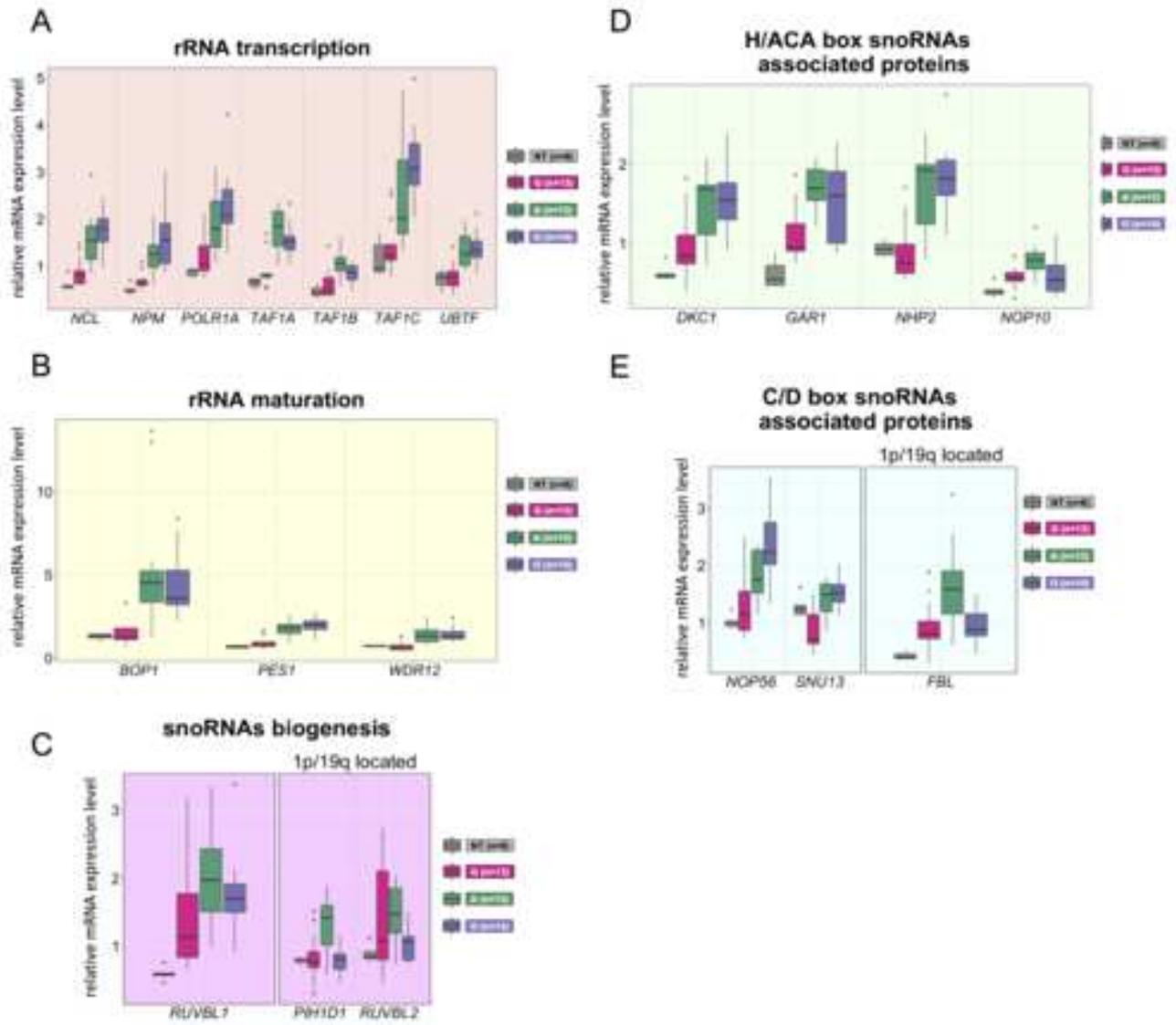


Figure 5

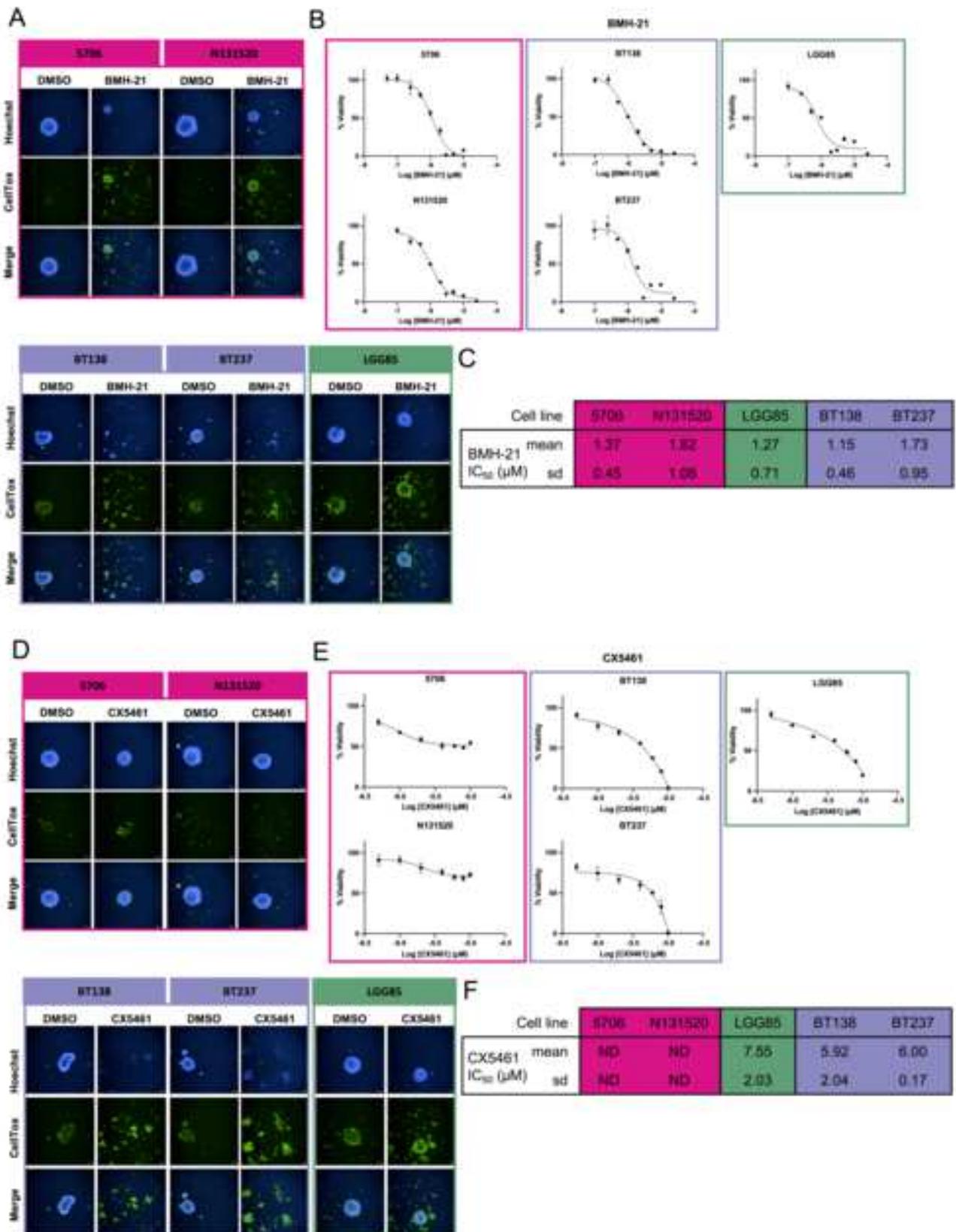
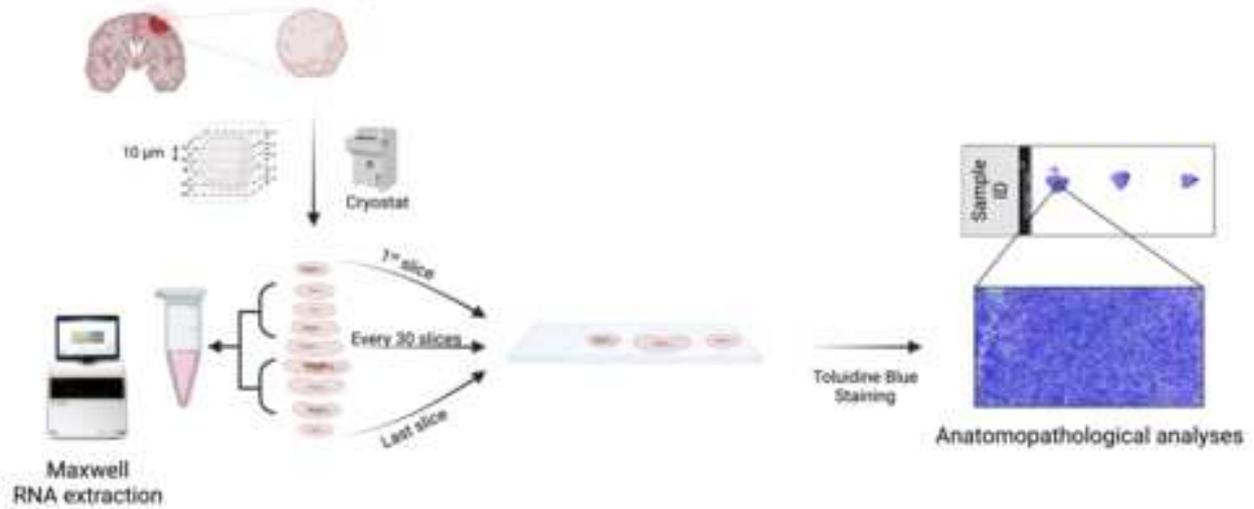


Figure 6

A

Frozen HGG and non tumoral samples



B

Test Cohort

	Sample ID	% tumoral cells	Sample ID	% tumoral cells	
Oligodendrogliomas	O-1	60	Glioblastomas	G-1	30
	O-2	40		G-2	80
	O-3	20		G-3	50
	O-4	50		G-4	60
	O-5	20		G-5	40
	O-6	40		G-6	65
	O-7	60		G-7	60
	O-8	50		G-8	70
	O-9	20		G-9	30
	O-10	50		G-10	50
	O-11	20		G-11	30
	O-12	30		G-12	70
	O-13	70		G-13	70
	Astrocytomas	A-1	20		
A-2		20			
A-3		30			
A-4		30			
A-5		20			
A-6		60			
A-7		30			
A-8		40			
A-9		60			
A-10		30			
A-11		40			
A-12		60			
A-13		50			

Validation Cohort

	Sample ID	% tumoral cells
Oligodendrogliomas	O-a	70
	O-b	90
	O-c	80
	O-d	80
	O-e	40
	O-f	60
	O-g	90
	O-h	40
Astrocytomas	A-a	30
	A-b	20
	A-c	20
	A-d	30
	A-e	80
	A-f	50
Glioblastomas	G-a	50
	G-b	60
	G-c	70
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	G-e	40
	G-f	70
	G-g	70
	G-h	30
	G-i	60

Figure S1

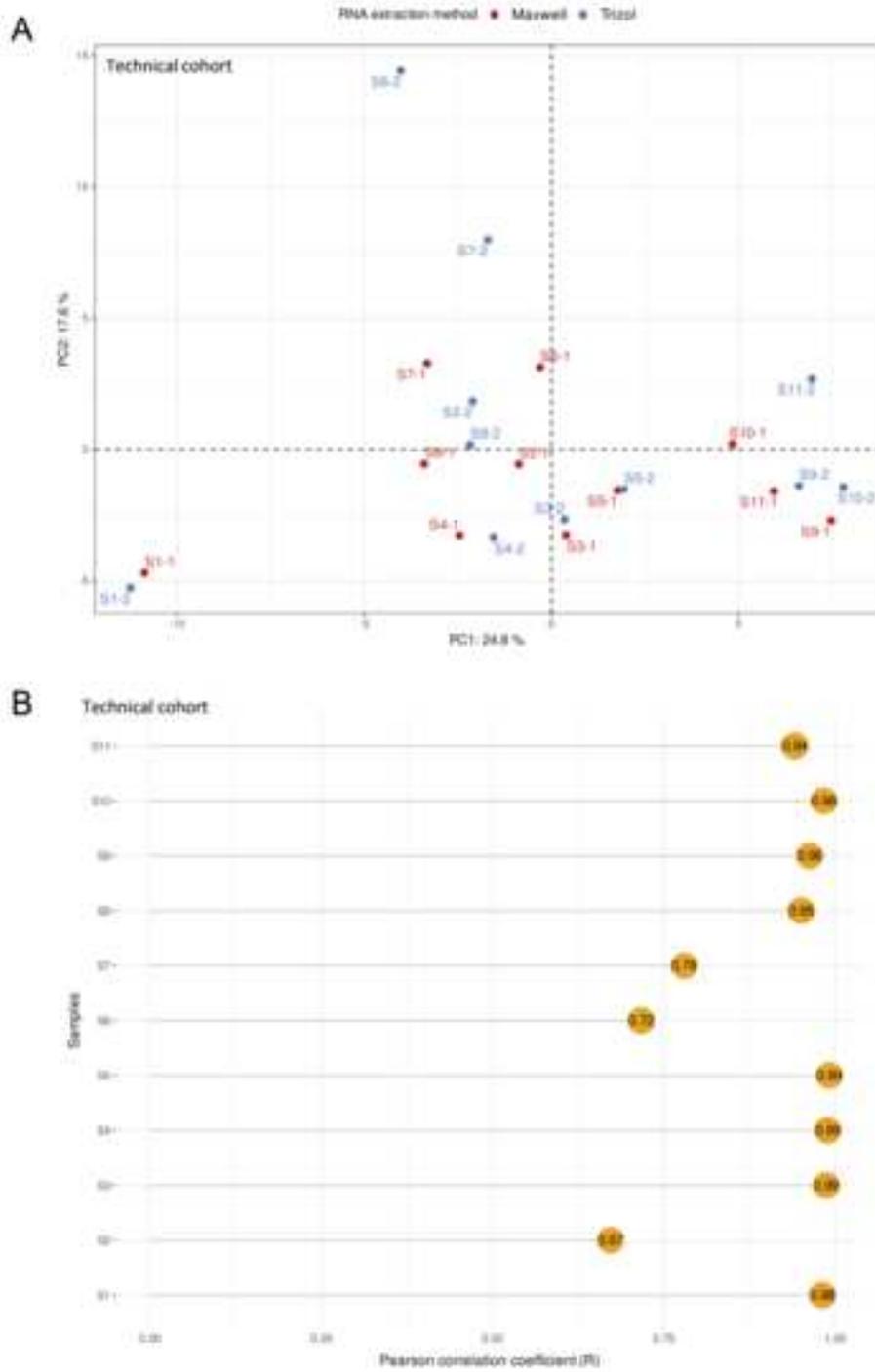


Figure S2

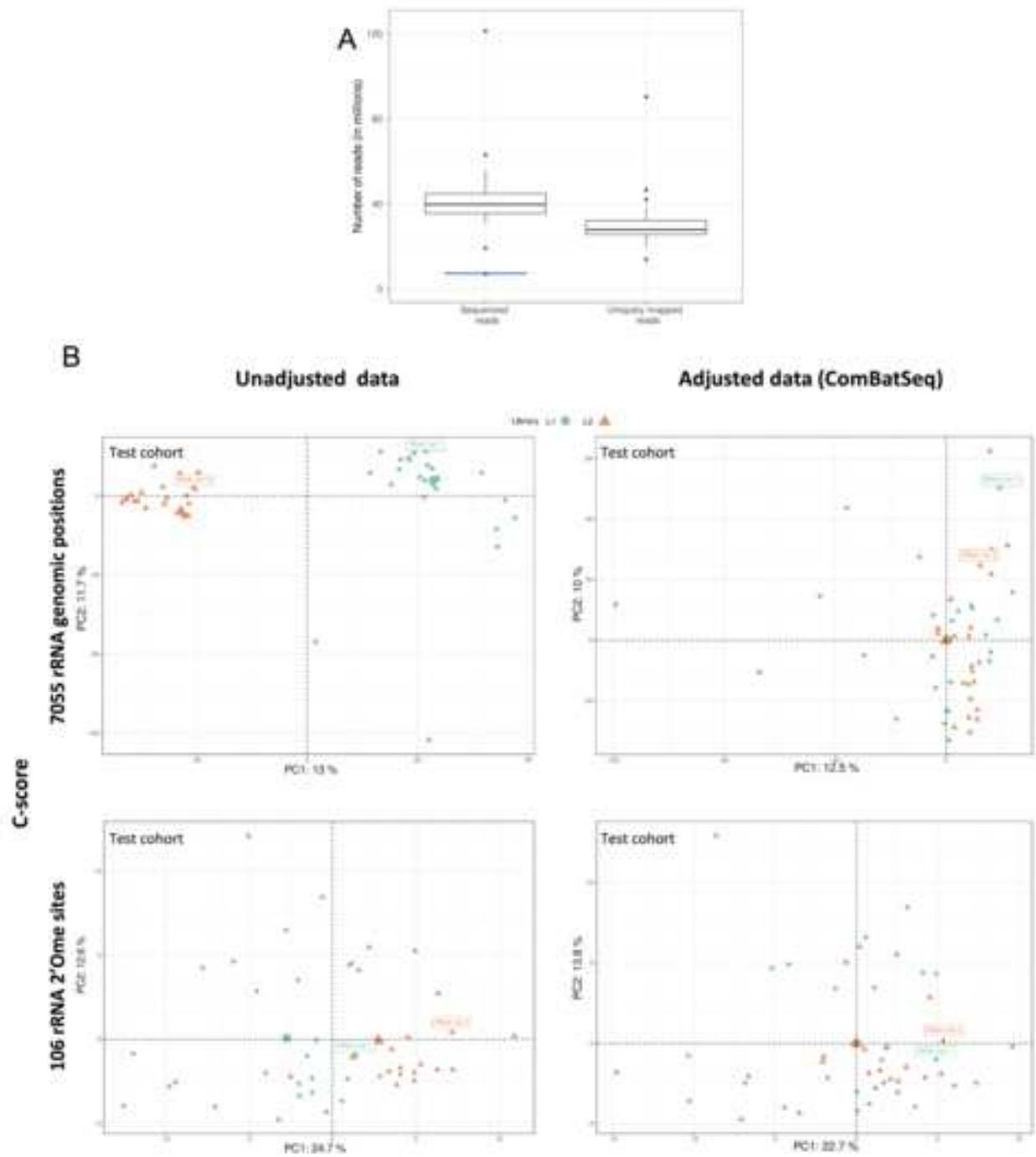


Figure S3

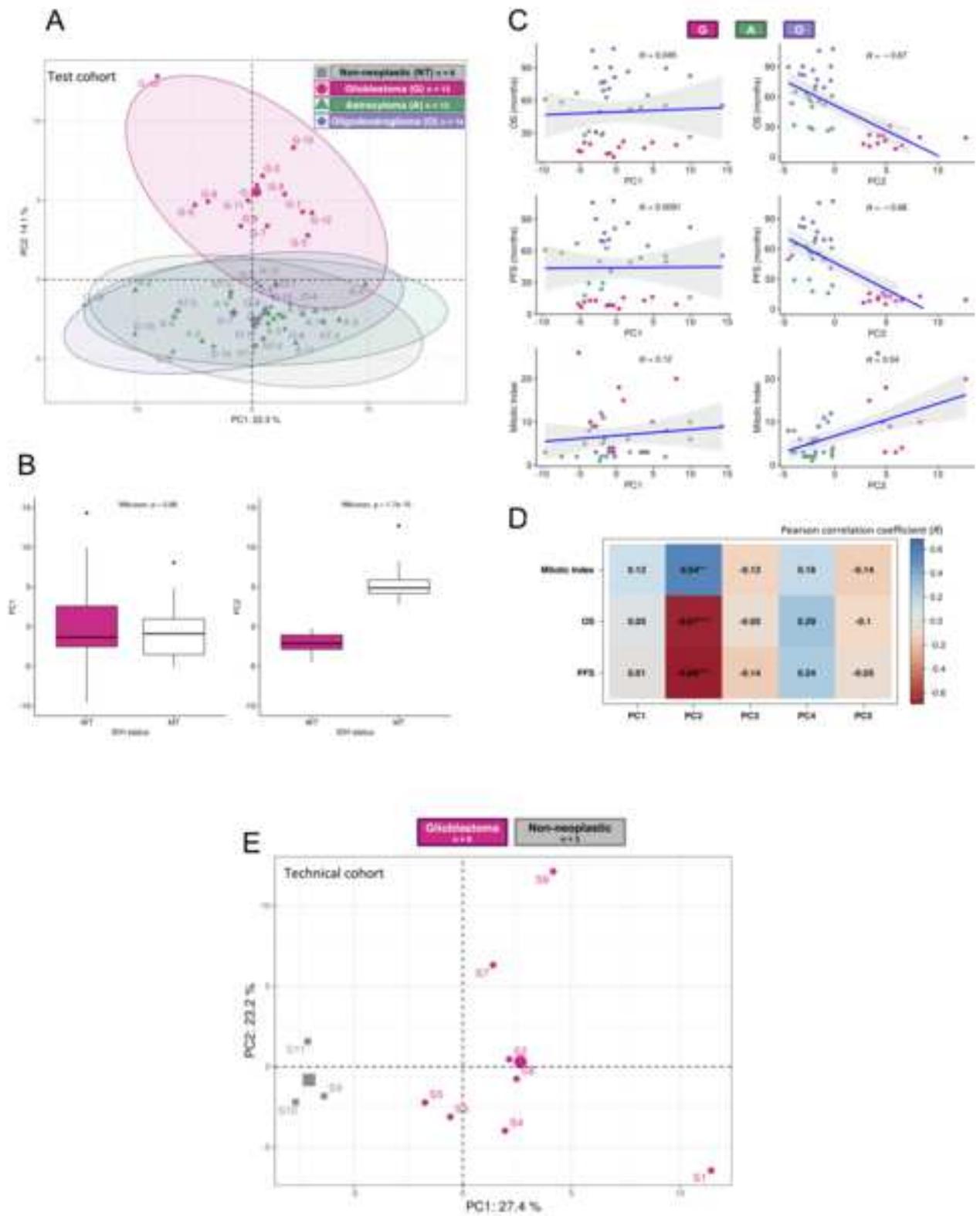


Figure S4

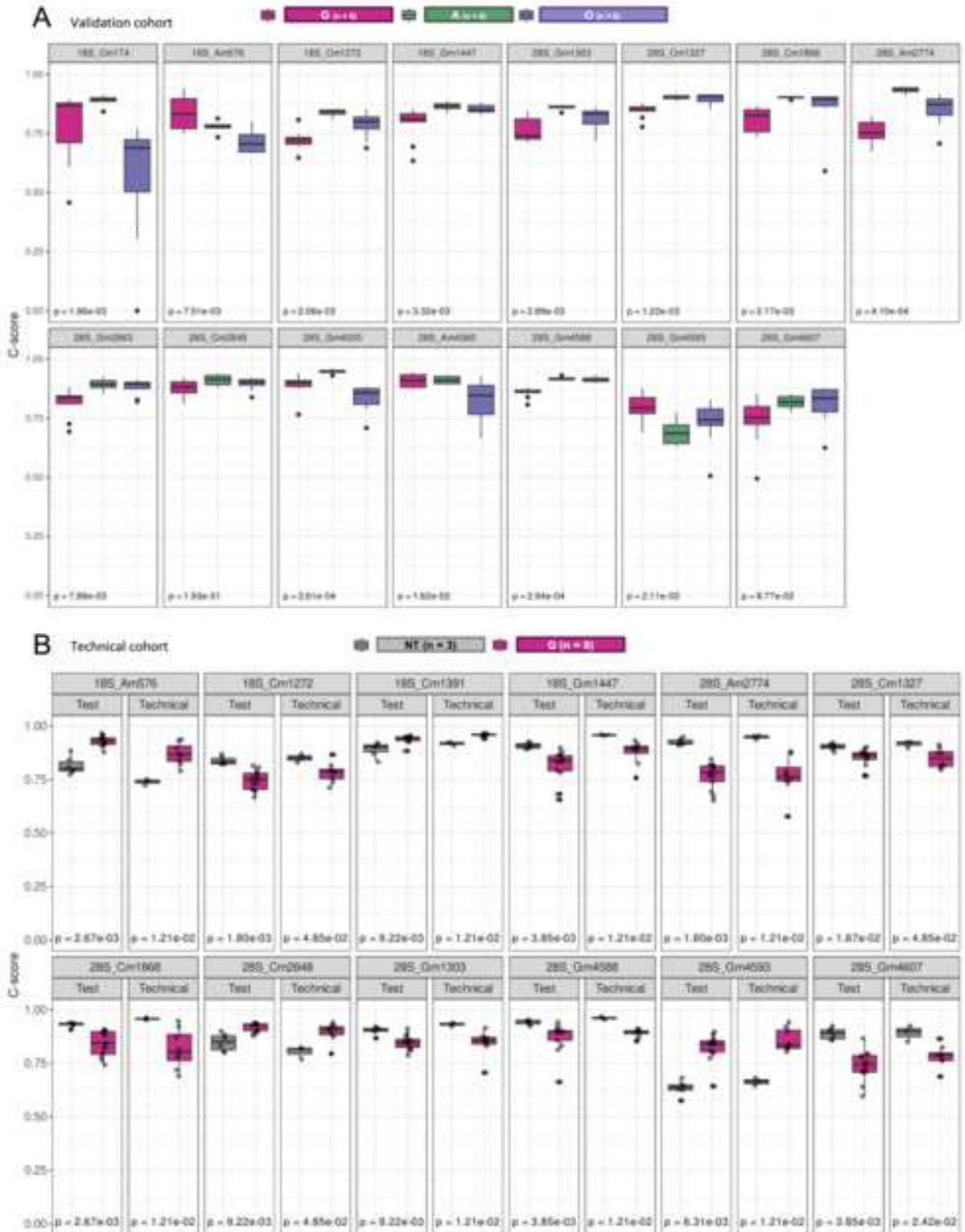


Figure S5

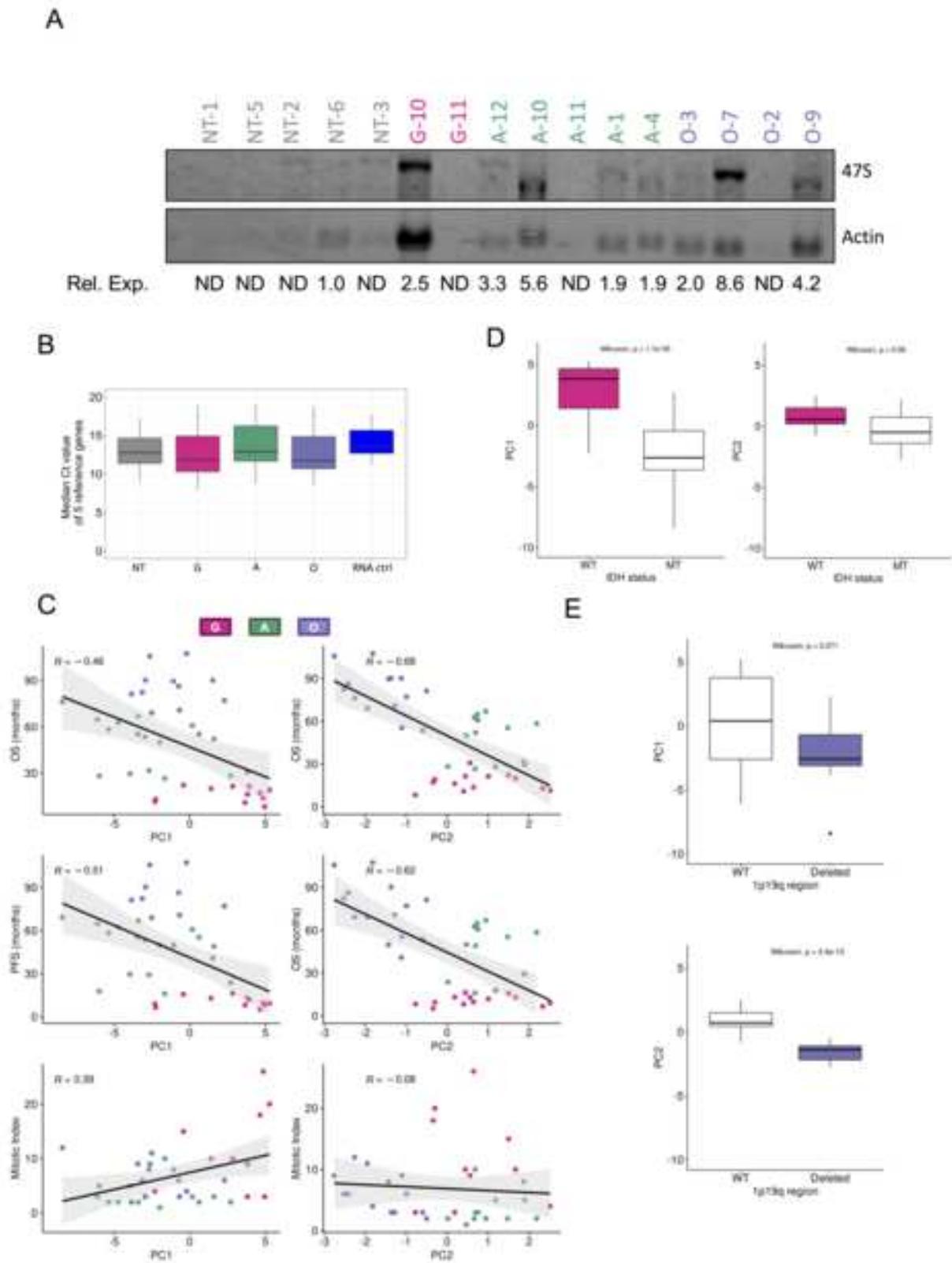


Figure S7

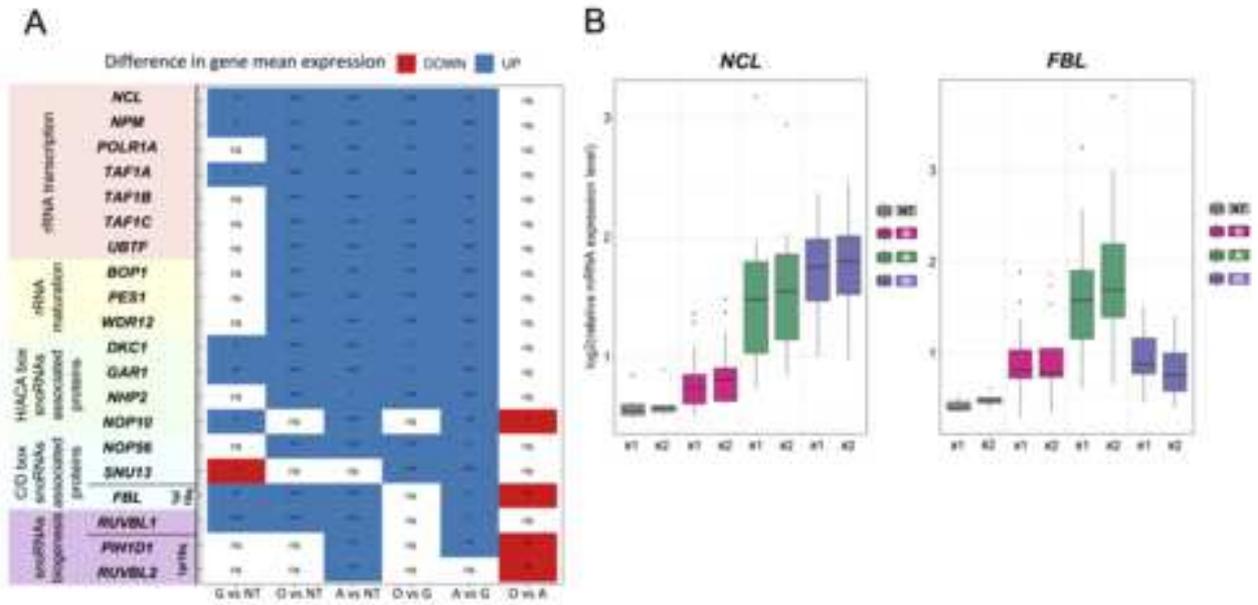


Figure S8

1 **Supplementary Information**

2

3 **IDHwt and IDHmut adult-type diffuse gliomas display distinct alterations in** 4 **ribosome biogenesis and 2'O-methylation of ribosomal RNA**

5

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47 **Supplementary Materials and Methods**

48

49 **Cell culture**

50 Human IDHwt glioblastoma 5706 and N131520 (from Dr A Idbaih), IDHmut
51 astrocytoma LGG85 (from Dr JP Hugnot), and IDHmut and 1p/19q codeleted
52 oligodendroglioma BT138 and BT237 (from Dr K Ligon) cell lines were cultured as
53 spheres in Ultra Low Attachment 6-well plates (Dutscher) to inhibit cell adhesion and
54 promote sphere formation. Spheroids were passaged at most 30 times by enzymatic
55 dissociation (Accumax, Sigma-Aldrich,). Glioblastoma and astrocytoma spheroids
56 were cultured in DMEM/F12 medium (Gibco) supplemented with 1X B27 and 1X N2
57 supplement (Fisher Scientific), 20 ng/mL bFGF (Miltenyi), 20 ng/mL EGF (Miltenyi) and
58 1X Penicillin/Streptomycin (Gibco). Oligodendroglioma spheroids were cultured in
59 NeuroCult NS-A Proliferation medium (STEMCELL technologies) supplemented with
60 20 ng/mL bFGF, 20 ng/mL EGF and 1X Penicillin/Streptomycin.

61

62 **Human grade 3-4 adult-type diffuse glioma and non-neoplastic samples**

63 Three cohorts were build using tumoral and non-neoplastic samples collected between
64 2010-2016 (NeuroBioTec, CRB HCL, Lyon, France, Biobank BB-0033-00046). First, a
65 technical cohort containing 11 samples, including 8 grade 4 IDHwt glioblastoma (G)
66 and 3 non-tumoral (NT) samples (epilepsy surgery), was selected. Second, a test
67 cohort was composed of 40 high-grade (3-4) primary adult-type diffuse glioma samples
68 and comprised 13 IDHwt glioblastomas (G, annotated G1 to G13), 13 IDHmut
69 astrocytomas (grade 3 n=9, grade 4 n=4) (A, A1 to A13) and 14 IDHmut and 1p/19q
70 codeleted oligodendrogliomas (grade 3) (O, A1 to A14) (WHO 2021 classification)
71 (Table 1). Six non-tumoral (NT) control samples were provided by patients without
72 diagnosed brain neoplastic events between 2003-2015 (epilepsy surgery for tuberous
73 sclerosis complex n=3 or for other cause n=2, autopsy n=1). Finally, a validation cohort
74 was composed of 23 high-grade (3-4) gliomas, including 9 IDHwt glioblastomas (G,
75 annotated Ga to Gi), 6 IDHmut astrocytomas (grade 3 n=5, grade 4 n=1) (A, Aa to Af)
76 and 8 IDHmut and 1p/19q co-deleted oligodendrogliomas (grade 3) (O, Oa to Oh).
77 The percentage of tumoral cells was estimated by a neuropathologist using Carbolec
78 Toluidine Blue staining (RAL Diagnostics) during RNA preparation (Fig.S1). HGGs
79 were classified using an integrated histomolecular algorithm according to WHO 2021

80 classification, e.g., IDH-R132H immunostaining followed by *IDH1/2* targeted Next
81 Generation Sequencing. In the test cohort, tumor samples were associated with clinical
82 data, including the *IDH1/2* mutational status, the mitotic index and patient survival
83 (Table 1). Overall survival (OS) was defined as the survival duration from the date of
84 surgery (biopsy or surgery) to either the date of death or last follow-up. Progression-
85 free survival (PFS) corresponded to the survival duration from the date of surgery
86 (biopsy or surgery) to either the date of radiological tumor progression, death, or last
87 follow-up. Patients were informed and written consent of all participants was obtained
88 in accordance with French regulations.

89

90 **Sample preparation and RNA purification**

91 Approximately 15 to 30 frozen 10 µm sections were cut on a cryostat (Leica Biosystems
92 CM3050S) (Supplementary Figure S1A). The first, last and every 30th section of each
93 sample were stained with Carboic Toluidine Blue (RAL Diagnostics) to assess sample
94 homogeneity and, when required, to estimate the percentage of tumoral cells before
95 RNA purification (Supplementary Figure S1B). Total RNA was extracted using either
96 the Maxwell RSC simplyRNA Tissue Kit (Promega) on a Maxwell RSC Instrument
97 (Promega) configured with Low Elution Volume (LEV) hardware according to the
98 manufacturer's protocol or TRIzol (Invitrogen) according to manufacturer's instructions.
99 RNA quality and concentrations were measured using a Nanodrop 2000
100 Spectrophotometer (ThermoScientific).

101

102 **RiboMethSeq**

103 RiboMeth-seq was performed as previously described using the Illumina sequencing
104 technology^{25,32}. Briefly, total RNA was fragmented under alkaline conditions prior to
105 the preparation of 24 sample libraries using the NEBNext Multiplex Small RNA Library
106 kit (New England Biolabs). A calibrated source of total RNA (Human Xpress Ref
107 Universal Total RNA, Qiagen) was introduced in each library. In contrast to previous
108 studies, sequencing was performed using an Illumina NovaSeq sequencer in single-
109 end mode (SR50), in order to sequence up to 48 samples (equivalent to 2 libraries)
110 per run with a median sequencing depth of 40 million reads per sample.

111 To process the sequencing data, a novel nextflow pipeline RiboMethSeq-nf was
112 developed and is currently available ([https://github.com/RibosomeCRCL/ribomethseq-
113 nf](https://github.com/RibosomeCRCL/ribomethseq-nf)). This pipeline processes sequencing data as previously described^{25,32,33}. Briefly,

114 fastQC was used to verify sequencing quality and adapter removal was performed
115 using Trimmomatic. The trimmed reads were aligned by Bowtie2 on the 7.2 kb-long
116 rRNA sequence of reference (NR_046235) and the 5' read-ends were computed using
117 bedtool genomcov utility. To calculate the C-score, which reflects the rRNA 2'Ome
118 level, the novel R package rRMSAnalyzer was developed
119 (<https://github.com/RibosomeCRCL/rRMSAnalyzer>). The C-score was calculated
120 using a normalization against the median raw counts of neighboring +/- 6 nucleotide
121 window²⁵.

122 The C-scores of the 106 admitted rRNA 2'Ome sites were extracted for further
123 analysis, either as a rRNA 2'Ome profile or as a site-by-site comparison. The most
124 variable rRNA 2'Ome sites were identified using the distribution of the inter quartile
125 range (IQR) of each site across the test cohort samples²⁵, the sites having an IQR
126 higher than median + 2 × median absolute deviation (mad) being defined as the most
127 variable ones. The identification of significant alterations in rRNA 2'Ome levels
128 between groups was performed by applying two consecutive thresholds: the adjusted
129 p-value < 0.05 (Kruskal-Wallis with FDR adjustment); and the mean ΔC-score (*i.e.*,
130 difference between the highest and lowest mean C-score of the groups of interest) >
131 0.05.

132

133 **Batch effect adjustment of RiboMethSeq data using ComBat-Seq**

134 Batch effect adjustment of RiboMethSeq data was performed using 5' read-end count
135 matrix as input for ComBat-seq¹. As currently reported for RNA-seq data, a batch effect
136 between two libraries may be observed using RiboMethSeq. ComBat-seq tool was
137 developed to adjust batch effect in RNA-seq data following a negative binomial
138 distribution and both its input and output are integer counts. ComBat-seq was used
139 with default parameter settings. This process produces an adjusted 5' read-end count
140 matrix that is then used to calculate the C-score as described above. Adjustment of
141 RiboMethSeq data using ComBat-seq has been included in the rRMSAnalyser R
142 package as an optional function (<https://github.com/RibosomeCRCL/rRMSAnalyzer>).

143

144 **Reverse Transcription and real time quantitative PCR**

145 cDNA synthesis was performed using the Prime Script RT Reagent kit (Takara) from
146 500 ng or 200 ng of total RNA for real time and medium throughput qPCR, respectively.
147 Gene expression was evaluated by medium throughput qPCR using the Biomark HD

148 system (Fluidigm), as previously described²¹, and according to the manufacturer's
149 instructions. For each sample, two independent reverse transcription reactions were
150 performed. A PCR multiplex was first carried out to amplify genes of interest and
151 facilitate their detection (Table S1). After exonuclease I treatment, qPCR reactions
152 were performed on a 96.96 Dynamic Array™ IFC (Fluidigm) using the Master Mix 2X
153 EvaGreen (Biorad) according to manufacturer's recommendations, in technical
154 triplicates. The Fluidigm Real Time PCR Analysis software (v 4.5.2) was used for the
155 calculation of relative fold-changes by applying the $2^{-\Delta CT}$ method based on a total of 6
156 Ct values per sample. The median Ct value of 5 housekeeping mRNAs (*ACTIN*,
157 *GAPDH*, *HPRT1*, *PGK1*, *PPIA*) was used for normalization. Data were then normalized
158 against Human Xpress Ref Universal Total RNA (Qiagen) as a standard RNA.
159 Real time qPCR was performed using SYBR Green (Roche, Applied Biosystem)
160 according to the manufacturer's protocol. Serial dilutions were systematically included
161 to calculate qPCR efficacy, verify amplification linearity, and calculate relative cDNA
162 concentrations. Relative fold-change was calculated as described above using *PPIA*
163 mRNA levels to normalize SNORD levels.

164

165 **Northern blot**

166 RNA samples were analyzed by northern blot as previously described². Briefly, total
167 RNAs were resolved by electrophoresis on 1.2% agarose, 6% formaldehyde, 0.02 M
168 3-(*N*-morpholino)propanesulfonic acid (MOPS) gels. Transfers were performed by
169 capillarity on a nylon membrane (Nytran SuperCharge, Whatman) with 10X SSC (1.5
170 M sodium chloride, 0.15 M sodium citrate, pH 7.0). Membranes were incubated
171 overnight at 42°C with hybridization buffer (ULTRAhyb™-Oligo, Invitrogen) containing
172 Dy682-conjugated human β -actin (10 nM) and Dy782-conjugated 47S pre-rRNA (50
173 nM) DNA probes. Membranes were washed four times with 0.1X SSC and 0.1% SDS
174 before signal exposure on ChemiDoc MP (Bio-Rad). Quantification was performed
175 using Image Lab software (Bio-Rad) and relative 47S pre-rRNA levels were
176 determined by normalization against β -actin mRNA levels.

177 β -actin probe 1: 5'-Dy682-TTGACATGCCGGAGCCGTTGTGACGAC-3'

178 β -actin probe 2: 5'-Dy682-CACACGCAGCTCATTGTAGAAGGTGTGGTGCC-3'

179 β -actin probe 3 5'-Dy682-CGTACATGGCTGGGGTGTGAAGGTCTCAAACAT-3'

180 47S ribosomal RNA probe:

181 5'-Dy782-
182 CGGAGGCCCAACCTCTCCGACGACAGGTCGCCAGAGGACAGCGTGTTCAGC-3'

183

184 **snoRNA quantification using RiboMethSeq data**

185 SnoRNAs were quantified from RiboMethSeq datasets as previously described ³.
186 Briefly, Trimmomatic was used to remove adaptors and low-quality reads ⁴. Trimmed
187 reads were aligned with STAR ⁵ to the human genome assembly (Ensembl hg38 V101)
188 using our custom annotation (based on Ensembl hg38 V101) containing added
189 snoRNAs

190 (https://zenodo.org/record/4570182/files/hg38_Ensembl_V101_Scottlab_2020.gtf) ⁶.

191 Counts were attributed to genomic features using CoCo ⁷ and our custom annotation.

192

193 **IC₅₀ assay**

194 Approximately 3.10^3 cells per well were seeded onto 96-well microplates Ultra Low
195 Attachment (Corning, 4515) in triplicate to form spheroids. After overnight incubation,
196 spheroids were treated with seven or nine doses of CX5461 (Sigma-Aldrich)²⁴ or BMH-
197 21 (Sigma-Aldrich)²⁵, respectively, at constant final DMSO concentrations (1%). After
198 72 h, cell cytotoxicity was assessed by CellTox™ Green Cytotoxicity Assay (Promega),
199 according to the manufacturer's instructions, followed by imaging using Opera
200 Phoenix® Plus High-Content Screening System, and by CellTiter-Glo3D® luminescent
201 cell viability assay (Promega) quantified with Spark® microplate to determine the IC₅₀.
202 Cell viability was expressed as a percentage of the signal intensity normalized against
203 DMSO (1%).

204

205 **Statistical methods**

206 Data and statistical analyses of both RT-qPCR and RiboMethSeq as well as graphical
207 representation, were performed using R (v 4.1.2). Unsupervised data analysis was
208 achieved by hierarchical clustering and principal component analysis (PCA).
209 Hierarchical clustering was generated with the ComplexHeatmap package using
210 Manhattan distance and Ward's linkage method. PCA was computed using ade4
211 package with the default parameters and visualized via factoextra package. To
212 correlate the first 5 principal components (PCs) of the PCA on gene expression or
213 rRNA 2'Ome profiles with the clinical features (OS, PFS, mitotic index), the

214 eigencorplot function of PCAtools package was used. Pearson's correlation coefficient
215 and the False Discovery Rate (FDR) method for the adjusted p-values were applied.
216 Pairwise comparison between groups was performed using non-parametric Wilcoxon
217 rank-sum test, or Kruskal-Wallis test when more than 2 groups were identified. The
218 FDR method was used for p-value adjustment when multiple tests were undertaken. A
219 p-value < 0.05 was considered to be statistically significant. Regarding the rRNA
220 2'Ome, in addition to a significant adjusted p-value, a mean Δ C-score greater than 0.05
221 was considered for identifying differentially methylated 2'Ome rRNA 2'Ome sites, the
222 Δ C-score corresponding to the difference between the highest and the lowest mean C-
223 score of the groups of interest.
224 Cell viability and IC₅₀ were determined using the log(inhibitor) vs. response variable
225 slope sigmoidal (four PL) function of GraphPad Prism (v 9.5.0 (525)).

226

227

228

229 **Supplementary References**

230

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254

255

256

257

258 **Supplementary Table 1. RT-qPCR primer sequences**

Genes	Forward primer	Reverse primer	Genomic location
<i>*Actin</i>	5'-CCAACCGCGAGAAGATGA-3'	5'-TCCATCACGATGCCAGTG-3'	7p22.1
<i>BOP1</i>	5'-GACGATCCTGACTACTGGCG-3'	5'-ACCTGCTCATCCGTCACTCT-3'	8q24.3
<i>DKC1</i>	5'-CCCTTTGGAAAAGCTGTTGA-3'	5'-TAATCTTGGCCCCATAGCAG-3'	Xq28
<i>FBL #1</i>	5'-CCTGGGGAATCAGTTTATGG-3'	5'-CCAGGCTCGGTACTCAATTTT-3'	19q13.2
<i>FBL #2</i>	5'-CCTGCGTAATGGAGGACACT-3'	5'-GCTGAGGCTGTGGAGTCAAT-3'	
<i>*GAPDH</i>	5'-AGCCACATCGCTCAGACAC-3'	5'-GCCCAATACGACCAAATCC-3'	12p13.31
<i>GAR1</i>	5'-CAAGACCAAGGACCTCCAGA-3'	5'-TCATCTTCACAGGGATGCAG-3'	4q25
<i>*HPRT1</i>	5'-TGACACTGGCAAAACAATGCA-3'	5'-GGTCCTTTTACCAGCAAGCT-3'	Xq26.2
<i>NCL #1</i>	5'-CCAGAACCAAATGGCAAAT-3'	5'-CTGATTGCTCTGCCCTCAAT-3'	2q37.1
<i>NCL #2</i>	5'-GTCAGCAAGGATGGGAAAAG-3'	5'-TAGATCGCCCATCGATCTCT-3'	
<i>NHP2</i>	5'-GGTCAACCAGAACCCCATC-3'	5'-TTCTGCTTCACCGCTTTCTT-3'	5q35.3
<i>NOP10</i>	5'-GAAGAAATTTGACCCGATGG-3'	5'-TGAAGCGTTTCTTGATGGTG-3'	15q14
<i>NOP56</i>	5'-AGGCTATTCTGGATGCCTCA-3'	5'-GTAGGCTCTGGCGGTATTCA-3'	20p13
<i>NPM</i>	5'-TTGTTGAAGCAGAGGCAATG-3'	5'-TATTTCAAAGCCCCCAAGG-3'	5q35.1
<i>PES1</i>	5'-GGCAAGAGGCGAAAAATCCG-3'	5'-TTCTTCTCAGACCTCACCGC-3'	22q12.2
<i>*PGK1</i>	5'-AAGTGAAGCTCGGAAAGCTTCTAT-3'	5'-AGGGAAAAGATGCTTCTGGG-3'	Xq21.1
<i>PIH1D1</i>	5'-TGAGTCTGGGAGAGCCTCAT-3'	5'-AAATCGCTGTTCTGCATCCT-3'	19q13.33
<i>POLR1A</i>	5'-CTGAGCCCCTGGGAATTGAG-3'	5'-CCTTCATTCTTCCACAGGGCA-3'	2p11.2
<i>*PPIA</i>	5'-GTCAACCCACCGTGTCTT-3'	5'-CTGCTGTCTTTGGGACCTTGT-3'	7p13
<i>RUVBL1</i>	5'-AAGGGGATGTGC ACA AAA AG	5'-CACATCCAAGTCATGCAAGG-3'	3q21.3
<i>RUVBL2</i>	5'-CGCTCTTCTCAGGTGACACA-3'	5'-CAGCACTCCAGGGATGATCT-3'	19q13.33
<i>SNU13</i>	5'-GCTACTGGACCTCGTTCAGC-3'	5'-ACTCAGAGATGCCCTGTTG-3'	22q13.2
<i>TAF1A</i>	5'-TCCTGGAGTTTGGGACCCTT-3'	5'-TGGTGAGTACCTCTTGGGCT-3'	1q41
<i>TAF1B</i>	5'-CGAGGAGGCGGAAGAGTTTA-3'	5'-TCTCTCTGTAACATTGTGGCAAGA-3'	2p25.1
<i>TAF1C</i>	5'-CGGAGTGAAGATGCTGGACA-3'	5'-GCCCCCAAACGAAAAAGCAA-3'	16q24.1
<i>UBTF</i>	5'-TGTGGAACGACCTGTCTGAG-3'	5'-CTCTCCGACTGAGCCTTGAG-3'	17q21.31
<i>WDR12</i>	5'-TAAAGGGGCAGAGGAATGGAT-3'	5'-CAACATCCGTATGTCCACAA-3'	2q33.2
<i>SNORD31</i>	5'- ACCAGTGATGAGTTGAATACCG-3'	5'- CACAGCTCAGAAAATACCTTTCA-3'	11q12.3
<i>SNORD104</i>	5'-GCCTGCTGTGATGACATTCC-3'	5'-TCAGACTCCAGTTCGCATCA-3'	17q23.3
<i>SNORD144</i>	5'-TTCATATCCAGTGATTAACCTTTTC-3'	5'-ATTCAATAATCACAACGGTTCA-3'	4q21.22
<i>SNORD127</i>	5'-GCAACTGTGATGAAAGATTTGGT-3'	5'-GGCAACATCAGTTTAGAGGGA-3'	14q21.2
<i>SNORD46</i>	5'-AGAATCCTTAGGCGTGGTTG-3'	5'-ATGACAAGTCCTTGCAATTGG-3'	1p34.1
<i>SNORD93</i>	5'-GCCAAGGATGAGAACTCTAATCTGA-3'	5'-GCCTCAGGTAATCCTTTAATCCA-3'	7p15.3
<i>SNORD69</i>	5'-TGAAGCAAATGATGATAAACTGG-3'	5'-AACATGAAGCTCAGGGTTGG-3'	3p21.1
<i>SNORD119</i>	5'-AACCTTGACTGAAGCTGATGA-3'	5'-CTGGATCTCAGAGTAATCCTGCT-3'	20p13
<i>SNORD65</i>	5'-AAATCACCCAAAATAGCTGGAA-3'	5'-TCAGAAAACCATAGTTCCACCA-3'	17p11.2
<i>SNORD91A</i>	5'-CGTCTGAACCTGTCTGAAGCA-3'	5'-GGAGAAGTCTCAGAACCACACA-3'	17p13.3
<i>SNORD91B</i>	5'-AGAGCCAATGATGTTTTTATTCAA-3'	5'-ACACAGAAGTTGCATCACTGG-3'	17p13.3
<i>SNORD80</i>	5'-ACAATGATGATAACATAGTTCAGCAG-3'	5'-ATCAGATAGGAGCGAAAGACTTAATA-3'	1q25.1

259 **Housekeeping genes*

260

261 **Supplementary Figure Legends**

262

263 **Figure S1. Sample processing and anatomopathological analyses. (A)** Diagram
264 presenting the workflow of sample processing. Briefly, snap frozen tissues of the test
265 and validation cohorts were cut into 10 μm -slices using a cryostat. The first, the last as
266 well as one slice every 30 sections were placed on glass slides and stained with
267 Toluidine Blue for analysis by an anatomopathologist to estimate the number of slices
268 required for further analyses, to verify the tumor integrity in the course of tumor cutting
269 and to collect tumoral features (percentage of tumoral cells, necrosis). Other slices
270 were pooled and RNA extraction was performed on a Maxwell® apparatus. For the
271 technical cohort, pooled slices were separated in half and RNA was extracted using
272 either Trizol or Maxwell®. Created with Biorender.com. **(B)** A table indicating the
273 percentage of tumor cells in each sample from the test and the validation cohorts.

274

275 **Figure S2. Reproducibility of RiboMethSeq using two different methods of RNA**
276 **extraction. (A)** An unsupervised Principal Component Analysis (PCA) based on C-
277 scores of the 106 known rRNA 2'Ome sites of the technical cohort composed of 11
278 samples. Total RNA was extracted either by Maxwell (S1-1 to S11-1, red) or Trizol (S1-
279 2 to S11-2, blue) methods. Percentage of variance explained by PC1 and PC2 are
280 indicated. **(B)** Summary of Pearson's correlation coefficient (R) of C-scores obtained
281 with the two different RNA extraction methods. In the technical cohort, a strong
282 correlation between C-scores was observed within the same sample, regardless of the
283 method used for RNA purification.

284

285 **Figure S3. RiboMethSeq optimization using Illumina NovaSeq device and**
286 **ComBat-seq algorithm. (A)** Distribution of the numbers of either sequence reads or
287 uniquely mapped reads using the Illumina NovaSeq device in the 48 samples of the
288 test cohort (40 HGGs, 6 non-neoplastic and 2 reference RNAs). A blue line indicates
289 the mean of sequence reads obtained in a previous work using an Illumina HiSeq
290 device (Marcel et al. NAR cancer 2021). **(B)** Unsupervised Principal Component
291 Analysis (PCA) based on either C-scores at 7055 rRNA sites (top) or C-scores at the
292 106 known rRNA 2'Ome sites (bottom) before (left) and after (right) adjustment of 5'-

293 end read counts using ComBat-seq algorithm. The 48 samples of the test cohort were
294 prepared as two distinct libraries (L1, green; L2, red). RNA control samples included
295 in each library are indicated.

296

297 **Figure S4. IDHwt glioblastomas have a distinct rRNA 2'Ome profile, which is**
298 **correlated with clinical features. (A)** Unsupervised Principal Component Analysis
299 (PCA) on adjusted C-scores at the 106 known rRNA 2'Ome sites in the test cohort
300 composed of IDHwt glioblastoma (G, pink circle), high-grade astrocytoma (A, green
301 triangle), high-grade oligodendroglioma (O, purple diamond) and non-neoplastic (NT,
302 grey square) samples. Percentage of variance explained by PC1 and PC2 are
303 indicated. The 95 % confidence ellipsoids around the centroid (larger pink circle, green
304 triangle, purple diamond and grey square) of each group are drawn. **(B)** Box plots
305 showing the distribution of IDHwt and IDHmut HGG samples of the test cohort on PC1
306 (left panel) or PC2 (right panel) axes. P-values using non-parametric Wilcoxon rank-
307 sum test are indicated on top. **(C)** Correlations between the clinical features (OS:
308 overall survival; PFS: progression-free survival; and mitotic index) and projections on
309 either PC1 (left) and PC2 (right) are displayed for the HGG types of the test cohort.
310 Pearson's correlation coefficients (*R*) are indicated on top. **(D)** A heatmap showing
311 Pearson's correlation coefficients (*R*), as calculated in (C), of PC1 to PC5 axes with
312 sample clinical features for the test cohort. *R* values are depicted by different colors
313 from -0.6 (red, negative correlation) to 0.6 (blue, positive correlation), Significant
314 correlations are indicated by an asterisk: ***p* < 0.01; ****p* < 0.001. **(E)** Unsupervised
315 PCA based on the C-score of the 106 known rRNA 2'Ome sites of the technical cohort
316 composed of 3 non-neoplastic (grey square) and 8 IDHwt glioblastoma (pink circle)
317 samples.

318

319 **Figure S5. Comparison of rRNA 2'Ome sites significantly altered between HGG**
320 **subtypes (A) or glioblastoma and non-neoplastic samples (B) in independent**
321 **cohorts.** Box plots showing the C-score distribution in HGG subtypes of the validation
322 cohort (A) or in IDHwt glioblastoma (pink) and non-neoplastic (grey) samples of the
323 technical cohort (B) for the 15 and 12 sites identified in the test cohort shown in Figure
324 3, respectively. P-values determined by Kruskal-Wallis or Mann-Whitney statistical

325 tests are indicated at the bottom of each panel and median C-scores are drawn as
326 black lines.

327

328 **Figure S6. Comparison of rRNA 2'Ome levels and expression of related C/D box**
329 **snoRNAs. (A)** Correlations of the expression of 12 C/D box snoRNAs (SNORDs) in a
330 panel of 9 HGG samples of the test cohort either determined from RiboMethSeq data
331 (TPM, y-axis) or measured by RT-qPCR (relative mRNA expression, x-axis).
332 Spearman's correlation coefficients (R) are indicated with corresponding p-values. **(B)**
333 Summary of Spearman's correlation coefficient (R) between C-scores at 46 rRNA sites
334 and associated-SNORD expressions both determined by RiboMethSeq in the test
335 cohort. The most variable sites identified in Figure 2 are indicated. Significant
336 correlations are indicated with asterisk: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p <$
337 0.0001 . **(C)** Correlation of C-scores at 18S_Am576 and corresponding expression
338 levels of the related SNORD93 in the test cohort. R and p-value are indicated on the
339 top left-hand side of the graph.

340

341 **Figure S7. HGG histomolecular types display IDH mutational status-associated**
342 **disparities in RiBi. (A)** Northern blots using probes targeting the 47S pre-rRNA and
343 actin mRNAs on IDHwt glioblastoma (G, pink), high-grade astrocytoma (A, green),
344 high-grade oligodendroglioma (O, purple) and non-neoplastic (NT, grey) samples. The
345 47S pre-rRNA expression is normalized against actin mRNA signals and expression
346 levels depending on "NT-6" samples are indicated below ("Rel. Exp"). ND: 47S pre-
347 rRNA expression levels could not be determined due to undetectable actin mRNA
348 levels. **(B)** Distribution of median Ct values of 5 reference genes (*ACTIN*, *GAPDH*,
349 *HPRT1*, *PGK1*, *PPIA*) in the HGG subtypes of the test cohort: IDHwt glioblastomas (G,
350 pink); high-grade astrocytomas (A, green); high-grade oligodendrogliomas (O, purple);
351 non-neoplastic (NT, grey) samples; and a reference RNA (RNA ctrl, blue) **(C)**
352 Correlation between the clinical features (OS: overall survival; PFS: progression-free
353 survival; and mitotic index) of HGG samples and projections on either PC1 (left) or PC2
354 (right) axes from PCA in Figure 4B. Pearson's correlation coefficients (R) are indicated
355 on top left-hand side. Each dot corresponds to a sample colored as in (B). **(D)**
356 Distribution of IDHwt and IDHmut HGG samples on PC1 (left panel) or PC2 (right
357 panel) axes from PCA in Figure 4B. P-values using non-parametric Wilcoxon rank-sum

358 test are indicated on top. **(E)** Distribution of 1p19q deleted or non-deleted (“WT”) samples on PC1 (top panel) or PC2 (bottom panel) axes from PCA in Figure 4B. P-values using non-parametric Wilcoxon rank-sum test are indicated on top.

361

362 **Figure S8. RiBi is elevated in high-grade astrocytomas and oligodendrogliomas.**

363 **(A)** Summary of pairwise comparisons of relative mRNA expression levels of individual
364 RiBi genes from the RiBi-gene set. Positive and negative fold-changes are shown in
365 blue and red, respectively. Adjusted p-value using Wilcoxon Mann Whitney statistical
366 test are indicated (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns, not
367 significant). Genes located in the region 1p/19q are indicated. **(B)** Relative expression
368 of *NCL* (left) and *FBL* (right) mRNAs in IDHwt glioblastoma (G, pink), high-grade
369 astrocytomas (A, green), high-grade oligodendrogliomas (O, purple), non-neoplastic
370 (NT, grey) samples determined by RT-qPCR using 2 different primer sets (#1 and #2).