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Splicing factor SRSF1 promotes gliomagenesis via oncogenic splice-switching of *MYO1B*

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24 Abstract

Abnormal alternative splicing (AS) caused by alterations of splicing factors contributes to tumor 25 progression. Serine/arginine splicing factor 1 (SRSF1) has emerged as a key oncodriver in 26 numerous solid tumors, leaving its roles and mechanisms largely obscure in glioma. Herein we 27 demonstrated that SRSF1 was increased in glioma tissues and cell lines. Moreover, its expression 28 was correlated positively with tumor grade and Ki-67 index, but inversely with patients' survival. 29 30 Using RNA-seq, we comprehensively screened and identified multiple SRSF1-affected AS events. Motif analysis revealed a position-dependent modulation of AS by SRSF1 in glioma. 31 Functionally, we verified that SRSF1 promoted cell proliferation, survival and invasion by 32 specifically switching the AS of myosin IB (MYO1B) gene and facilitating the expression of the 33 oncogenic and membrane-localized isoform, MYO1B-fl. Strikingly, MYO1B splicing was 34 dysregulated in parallel with SRSF1 expression in gliomas, and predicted the poor prognosis of 35 the patients. Further investigation revealed that SRSF1-guided AS of MYO1B gene increased the 36 tumorigenic potentials of glioma cells through the PDK1/AKT and PAK/LIMK pathways. 37 38 Taken together, we identify SRSF1 as an important oncodriver, which integrates the AS controlling of MYO1B into promotion of gliomagenesis, and represents a potential prognostic 39 biomarker and target for glioma therapy. 40

42 Introduction

Alternative splicing (AS) of pre-mRNA is a vital post-transcriptional process determining the 43 proteomic complexity of mammalians (1). Generally, AS regulation depends on the strength of 44 intrinsic cis-elements, including intronic and exonic enhancers and silencers which recruit 45 trans-acting splicing factors to promote or suppress the utilization of adjacent splice sites (2, 3). 46 Failure to accurately recognize spice sites due to either splice site mutation or splicing factor 47 dysregulation ultimately leads to the generation of abnormal mature mRNA variants encoding 48 for deleterious isoforms, and contributes substantially to tumor malignancy (1, 4, 5). The 49 "cancerous" splice variants of specific genes have turned into novel molecular biomarkers as 50 well as therapeutic targets to outwit cancer treatment (6). 51

Serine/arginine-rich splicing factors (SRSFs) are well-characterized for their roles in AS, 52 each comprising one or two RNA-recognition motifs (RRMs) and a serine/arginine-rich (RS) 53 domain (7). Among these SRSFs, SRSF1 is a prototypical splicing factor that specifically binds 54 to exonic enhancers and stimulates splicing (8). Increasing evidences demonstrate that, SRSF1, 55 56 predominately driven by the transcription factor MYC (9), is overexpressed in multiple human cancers, and exerts oncogenic roles via controlling AS of cancer-related genes (10-12). 57 Recently, genome-wide studies have extensively identified the AS targets of SRSF1 (13, 14), 58 and established its concentration/position-dependent splicing model (12, 15). The emerging 59 roles of SRSF1 through AS regulation in cancer are opening up a new therapeutic avenue. 60

Gliomas are the most frequent primary brain tumors (16, 17). Malignant glioma, especially glioblastoma (GBM), is associated with dismal prognosis, primarily due to its infiltrating properties and the emergence of chemo-resistance (18). Treatment options remain limited in part due to the still poor understanding of the basic biology of glioma. Given the complexity of splicing regulation in brain, abnormal splicing may be a significant but yet under-explored contributor to gliomagenesis. Indeed, functional studies have revealed several splicing factors as oncogenic candidates by switching the AS products toward the tumor-promoting isoforms
in glioma cells (19-21). However, whether SRSF1 participates in glioma onset and progression
remains unknown.

In the present study, we sought to elucidate the expression, clinical relevance, biological 70 function and underlying mechanism of SRSF1 in gliomas, focusing on the aspect of AS control. 71 Upregulation of SRSF1 was observed in gliomas and predicted patients' adverse prognoses. 72 Through RNA-sequencing (RNA-seq) and motif analyses, we systematically identified 73 hundreds of SRSF1-affected AS events, and described a position-dependent modulation of AS 74 by SRSF1 in glioma. We further verified that SRSF1 promotes the proliferation, survival and 75 invasion of glioma cells by switching the AS of myosin IB (MYO1B) gene. Taken together, our 76 study highlights a novel role of SRSF1 as a splicing regulator in glioma biology, which 77 contributes to multiple aspects of glioma phenotype. 78

80 **Results**

SRSF1 is increased in gliomas and its higher expression predicts worse prognosis. To 81 investigate whether SRSF1 was involved in gliomagenesis, we firstly explored its expression 82 alteration in glioma tissues. In silico analysis of the three published datasets from Oncomine 83 (http://www.oncomine.org) revealed a significant increase of SRSF1 mRNA content in GBM 84 tissues as compared with normal brain (NB) tissues (P<0.001; Supplemental Figure 1A). This 85 result was reinforced by the quantification of SRSF1 mRNA in 14 glioma tissues (including 6 86 lower grade gliomas (LGGs, WHO grade II-III) and 8 GBMs (WHO grade IV)) and 5 NBs 87 (Figure 1A). Western blot verified that human GBM tissues and cell lines showed significantly 88 higher levels of SRSF1 protein when compared with NBs and the human immortal astrocyte 89 cell line UC2, respectively (Figure 1, B and C, and Supplemental Figure 1, B and C). SRSF1 90 IHC confirmed its nuclear localization and a progressive increase of its labeling index (LI) with 91 the elevation of glioma grade (P<0.001; Figure 1D). Moreover, SRSF1 expression was 92 positively correlated with the proliferation index (Ki-67 LI; r=0.839, P<0.0001; Figure 1E, and 93 94 Supplemental Figure 1, D and E). Importantly, SRSF1 overexpression was obviously associated with older age (P < 0.0001), advanced grade (P < 0.0001), higher Ki-67 LI (P < 0.0001) and wild 95 type isocitrate dehydrogenase 1 and 2 (IDH1/2) status (P < 0.0001; Table 1). Kaplan-Meier 96 analyses showed that patients with higher levels of SRSF1 had shorter disease-free survival 97 (DFS; P<0.0001) and overall survival (OS; P<0.0001; Figure 1F). The prognostic value of 98 SRSF1 was further verified by The Cancer Genome Atlas (TCGA) data analysis (OS: P<0.0001; 99 Supplemental Figure 1F). Furthermore, even within the cohort of glioma patients with similar 100 ages (age \geq 50, age<50), identical *IDH1/2* gene type and similar Karnofsky Performance Status 101 (KPS; <90, ≥90), the association between high SRSF1 expression and poor prognosis remained 102 obvious (DFS: P<0.01~0.0001; OS: P<0.01~0.0001; Figure 1, G and H, and Supplemental 103 Figure 1G). Cox regression showed that SRSF1 LI was an independent predictor for DFS and 104

OS (Supplemental Table 1 and Supplemental Table 2). Taken together, these data strongly
 indicates that upregulation of SRSF1 is closely associated with glioma progression, and SRSF1
 is a potential prognostic biomarker for glioma patients.

SRSF1 increases tumorigenic potentials of glioma cells. Prompted by the above 108 findings, we examined whether SRSF1 exerted oncogenic functions in glioma. First of all, we 109 transiently silenced the endogenous SRSF1 expression with two independent siRNAs. Western 110 blot confirmed the efficient knockdown of SRSF1 in four GBM cell lines, including U87MG, 111 U251, LN229 and SNB19 (P<0.001; Supplemental Figure 2A). Compared with the control 112 siRNA, SRSF1 siRNAs significantly inhibited the growth of these cell lines as gauged by Cell 113 Counting Kit-8 (CCK-8) assays (P<0.001; Supplemental Figure 2B). Using the stable sub-cell 114 lines expressing control shRNA (sh-NC; WT) and SRSF1 shRNA (sh-SRSF1; KD), we found 115 that SRSF1 knockdown also severely impaired cell survival and invasion as proved by colony 116 formation (P<0.001; Supplementary Figure 2C) and transwell assays (P<0.001; Supplementary 117 Figure 2D), respectively. 118

To confirm whether SRSF1 is essential for SRSF1-mediated oncogenic roles in glioma, 119 we infected the KD sub-cell line with the lentivirus co-expressing luciferase plus the shRNA-120 resistant synonymous mutant of SRSF1 (SRSF1-mu; Supplemental Figure 2E) to restore 121 SRSF1 expression (KD+SRSF1-mu), and infected the WT and KD sub-cell lines with the 122 control lentivirus expressing luciferase alone (vec; WT+vec and KD+vec) as the controls 123 (Figure 2A). The three groups of stable sub-cell lines undergoing sequential lentivirus infection 124 and antibiotic selection were used in the following experiments both in vitro and in vivo. In 125 consistence with the previous results, SRSF1 knockdown severely impaired the proliferation, 126 survival and invasion abilities, while SRSF1 restoration significantly rescued the above defects 127 (P<0.001; Figure 2, B-D). In SW1088 cell line (a grade III astrocytoma cell line with lower 128 level of endogenous SRSF1; Figure 1C and Supplemental Figure 1C), we also observed the 129

promoting effects of SRSF1 on glioma cell proliferation and invasion (*P*<0.001; Supplemental
Figure 2, F-H). All these results demonstrate that SRSF1 is a potent promoter of glioma cell
proliferation, survival and invasion.

In addition, we also observed the changes in actin organization following SRSF1 knockdown in U87MG and U251 cells. Phalloidin labeling showed the appearance of diffused actin stress fibers with reduced fluorescence intensities in KD cells, whereas in control (WT) cells, F-actin was centralized mostly on cell cortex or lamellae (Figure 2E). Alterations in actin organization are accompanied with the formation of focal-adhesion at the edges of cells, consistent with a more spread cell morphology and larger cell area of KD cells (Figure 2E).

139 For animal experiments, the abovementioned three groups of U87MG stable sub-cell lines (WT+vec, KD+vec, KD+SRSF1-mu) were transplanted into nude mice. IHC results showed that 140 xenografts of the KD+vec group retained SRSF1 silencing, whilst those of the WT+vec and 141 KD+SRSF1-mu groups expressed high levels of SRSF1 (Supplemental Figure 3A). Our results 142 showed that SRSF1 knockdown (KD+vec) obviously inhibited the growth of the glioma 143 xenografts and increased the OS rates of the nude mice, and these effects were almost completely 144 reversed by replenishment with SRSF1-mu (KD+SRSF1-mu; Supplemental Figure 3, A-C). 145 Combined with our in vitro findings, these results demonstrate that SRSF1 increases the 146 147 tumorigenic potentials of glioma cells by facilitating their proliferation, survival and invasion.

Global landscape of the SRSF1-affected AS and gene expression in GBM cells. To screen SRSF1-regulated AS events involved in gliomagenesis, we conducted high throughout sequencing of RNA (RNA-seq) on the WT and KD sub-cell lines of U87MG and U251. With ~100 million 150-nt paired-end reads, we identified a total of 1348 and 1332 SRSF1-regulated AS events in U87MG and U251 cells, respectively, which could be classified into five AS categories (Figure 3A and Supplemental Table 3). The majority of these AS events belonged to skipped exon (SE). Subsequent analysis indicated the dual role of SRSF1 as a splicing activator and repressor, since it induced similar percentages of exon/intron inclusion (activation) and
exclusion (repression; Figure 3B and Supplemental Table 3). Among all the SRSF1-regulated
AS events, we found multiple ones shared by U87MG and U251 cells, most of which belonged
to the SE category (Figure 3C and Supplemental Table 3). Importantly, these overlapping
SRSF1-affected splicing targets were associated with tumor-related functions in the aspects of
cell cycle control, RNA splicing, cytoskeleton organization and focal adhesion (Figure 3, D and
E).

Apart from splicing control, we also observed the effects of SRSF1 on global gene 162 expression. Heatmap of the differentially expressed coding genes revealed cell-type specific 163 164 variations in U87MG and U251 cells (Supplemental Figure 4A). The overlapping ones were also involved in the tumor-related functions mentioned above (Supplemental Figure 4, B and 165 C), reiterating the roles of SRSF1 in gliomagenesis. Furthermore, we spotted the impacts of 166 SRSF1 on the expression of numerous non-coding RNAs, which also exhibited both generality 167 and specificity between U87MG and U251 cells (Supplemental Figure 4D). These results 168 propose that the glioma promoting roles of SRSF1 are closely associated with its modulating 169 effects on global AS and gene expression. 170

Validation and mechanistic exploration of SRSF1-guided AS in glioma. To verify the
accuracy of our RNA-seq results on AS, we subsequently validated the top 50 SRSF1-affected
AS events shared by U87MG and U251 cells (Supplemental Table 4). Representative results of
12 validated AS events were shown in Figure 4A and 4B. These results confirmed that SRSF1
either activated (Figure 4A) or repressed (Figure 4B) the splicing of the target exons/introns.

To determine whether the distribution of SRSF1 binding motif differs between SRSF1activated and -repressed cassette exons, we performed de novo discovery of the SRSF1 binding motif, using the sequences of 60 (30 activated and 30 repressed) validated SRSF1-regulated SE events shared by U87MG and U251 cells. The motifs derived from the SRSF1-activated

training set showed a predominant enrichment of GAGGGG within the cassette exons over the
flanking constitutive exons and introns (Figure 4C). However, SRSF1-repressed exons showed
the enrichment of the putative binding motifs in the flanking constitutive exons and introns
(Figure 4C).

Among the validated SRSF1-affected AS events, we focused on myosin IB (MYO1B) gene, 184 since its transcripts dramatically switched to the exon skipped isoform upon SRSF1 knockdown 185 (primer set 1; Figure 5A). Human MYO1B gene has 31 exons, in which exon 23 and 24 are 186 subjected to AS regulation. Therefore, this gene theoretically generates four different transcripts 187 depending on the inclusion/exclusion of the two alternative exons. Actually, we could only 188 189 distinguish three isoforms because exon 23 and 24 are of the same length. Reverse transcriptase PCR (RT-PCR) using primer set 2 that could amplify all the exon including or skipping 190 isoforms revealed that SRSF1 knockdown significantly resulted in skipping of the two adjacent 191 exons, and facilitated the expression of the truncated MYO1B isoform in KD sub-cell line 192 (MYO1B-t; Figure 5A). The splicing effect was quantified by determining the percentage ratio 193 of the full-length transcript containing exon 23 and 24 (MYO1B-fl) to total MYO1B transcripts 194 (fl%; Figure 5A). Meanwhile, exogenous HA tagged SRSF1-mu dose dependently increased 195 the fl%, and recovered the WT splicing pattern of MYO1B in KD sub-cell line (Figure 5B and 196 197 Supplemental Figure 5A). Similar results were also obtained in SW1088 cells (Supplemental Figure 5B). However, introduction of domain deletion mutants of SRSF1 (SRSF1-ARRM1, -198 Δ RRM2, - Δ RS) failed to recover or only weakly restored the WT splicing of *MYO1B* (Figure 199 5C), suggesting that all the three domains of SRSF1 are required for the efficient splicing of 200 201 MYO1B pre-mRNA.

To further examine whether SRSF1 binds to *MYO1B* exon 23 and 24 in vivo, we overexpressed HA tagged SRSF1-wt or its domain deletion mutants in U87MG cells (Figure 5D). To rule out the experimental artifacts caused by HA antigen, we also transfected the cells

with HA-vector (vec) or HA-SRSF2-wt (SRSF2-wt) plasmids as controls (Figure 5D). In vivo 205 crosslinking followed by immunoprecipitation (CLIP) and the following RT-PCR results 206 showed that SRSF1-wt bound to exon 23 and 24 with high affinity. However, the affinities of 207 the three SRSF1 domain deletion mutants and SRSF2-wt to exon 23 and 24 were low or even 208 negligible, similar to those to the flanking exon 22 and 25 (Figure 5E). This finding is in 209 consistence with the motif distribution feature of SRSF1-activated exons, which is 210 characterized by the predominant enrichment of SRSF1 binding motifs within the cassette 211 exons over the flanking constitutive exons. 212

To gain more mechanistic insights into SRSF1-regulated AS of MYO1B gene, we 213 214 constructed a minigene reporter spanning the genomic DNA fragment of MYO1B exon 22-25 (MYO1B-wt; Figure 5F). Splicing was assayed following transient transfection in WT and KD 215 sub-cell lines of U87MG. In accordance with the endogenous splicing pattern, exon 23 and 24 216 were nearly 40% included in the WT sub-cell line (lane 1), whereas SRSF1 knockdown 217 significantly inhibited the inclusion of the two exons (lane 2), indicating that the inclusion of 218 MYO1B exon 23 and 24 was SRSF1 dependent (Figure 5F). Sequence analysis revealed several 219 potential SRSF1 binding motifs in exon 23 and 24 of MYO1B mRNA (Supplemental Figure 5C). 220 We then examined the role of internal binding motifs in exon inclusion in detail. To this end, we 221 designed a series of motif deletion mutants of MYO1B minigene with the motif elements within 222 exon 23 and 24 deleted individually (MYO1B-del1 and -del2) or simultaneously (MYO1B-del3; 223 Figure 5F). Strikingly, MYO1B-del1 and -del2 displayed minor effects in exon exclusion, and 224 remained responsive to SRSF1 knockdown (lane 3-6 compared with lane 1-2; Figure 5F). 225 However, simultaneous deletion of the two motif elements (del3) almost abolished the inclusion 226 of exon 23 and 24, similar to the effect of SRSF1 deprivation (lane 7-8 compared with lane 1-2; 227 Figure 5F). Moreover, inserting three copies of GAGGGG (SRSF1 binding motif) into MYO1B-228 del2 (MYO1B-del2in) significantly restored the WT splicing pattern, and this effect was 229

completely abrogated by SRSF1 knockdown (lane 9-10 compared with lane 1-2; Figure 5F).
Collectively, these results prove that enrichment of SRSF1 binding motifs within the cassette
exon results in exon inclusion.

MYO1B-fl isoform increases the oncogenic capacities of GBM cells. Given that SRSF1 233 facilitated the expression of full-length MYO1B protein (MYO1B-fl, containing 58 unique 234 amino acids encoding two more IQ motifs in the C-terminal; Supplemental Figure 5, C and D), 235 we next investigated whether and how MYO1B-fl contributes to gliomagenesis. We designed 236 two different siRNAs targeting exon 23 and 24, and verified their isoform-specific silencing 237 effects on MYO1B-fl by Western blot (P<0.001; Figure 6A). CCK-8 assay showed that 238 MYO1B-fl knockdown efficiently suppressed the growth of GBM cells (P<0.001, Figure 6B). 239 Using the stable sub-cell lines expressing control shRNA (sh-NC) and MYO1B-fl shRNA (sh-240 MYO1B-fl), we found that MYO1B-fl knockdown considerably suppressed cell invasion 241 242 (P<0.001; Figure 6C). Moreover, MYO1B-fl knockdown (sh-MYO1B-fl) caused that MYO1B immunofluorescent signal faded obviously from cytomembrane, leaving only scattered 243 fluorescence diffusing in cytoplasm (Figure 6D). sh-MYO1B-fl cells also showed diffused actin 244 stress fibers with reduced intensities (cytoskeleton disorganization) and enlarged cell areas 245 (Figure 6D and Supplemental Figure 6A). 246

Prompted by the above results, we questioned whether the two MYO1B isoforms 247 (MYO1B-fl and -t) differ in subcellular localization. To this end, we expressed EGFP fused 248 MYO1B-fl or -t in U87MG and U251 cells (Supplemental Figure 6B), and found that MYO1B-249 fl-EGFP localized mainly on cytomembrane, while MYO1B-t-EGFP dispersed in cytoplasm 250 (Figure 6E, and Supplemental Figure 6, C and D). Furthermore, we found that SRSF1 251 knockdown induced a switch of MYO1B protein from cytomembrane aggregation to 252 cytoplasmic dispersion (Supplemental Figure 6E). All these results strongly indicate the 253 discrepancy of the subcellular localization among MYO1B isoforms. 254

To investigate whether subcellular localization determines the biologic functions of 255 MYO1B isoforms, we designed two shRNAs targeting the 3'-UTR of MYO1B mRNA (sh-256 MYO1B-total 1# and 2#) to simultaneously knockdown all the MYO1B isoforms (Supplemental 257 Figure 7A). We then established the sh-MYO1B-total sub-cell line by using the more efficient 258 one (Figure 7A). Thereafter, we infected the sub-cell line with the control lentivirus expressing 259 luciferase alone (vec; sh-MYO1B-total+vec), or the lentivirus co-expressing luciferase plus 260 fusion protein of MYO1B-fl-EGFP (MYO1B-fl; sh-MYO1B-total+MYO1B-fl) or MYO1B-t-261 EGFP (MYO1B-t; sh-MYO1B-total+MYO1B-t) to investigate the individual functions of 262 MYO1B isoforms (Figure 7A). Notably, knockdown of all MYO1B isoforms suppressed the 263 264 proliferation, survival and invasion abilities of U87MG and U251 cells. While MYO1B-fl markedly reversed the above defects, MYO1B-t exerted almost no rescue effects (Figure 7, B 265 and C, and Supplemental Figure 7B). Accordingly, MYO1B-fl overexpression significantly 266 increased the colony forming efficiency of U251 cells, while MYO1B-t had no obvious effect 267 (Supplemental Figure 7C). In animal experiments, glioma xenografts of the MYO1B-fl 268 overexpression group exhibited higher growth rate as compared with the control group 269 (Supplemental Figure 7, D and E). These results demonstrate that MYO1B-fl strongly promotes 270 the proliferation, survival and invasion of GBM cells, whereas MYO1B-t lacks the above 271 272 oncogenic properties.

Restoration of MYO1B-fl reverses the anti-glioma effects of SRSF1 knockdown. To 273 provide more evidence that SRSF1 promotes gliomagenesis by inducing MYO1B-fl expression, 274 we infected the U87MG WT and KD sub-cell lines with the control lentivirus expressing 275 luciferase alone (vec; WT+vec, KD+vec), and infected the KD sub-cell line with the lentivirus 276 co-expressing luciferase plus fusion protein of SRSF1-mu-HA (SRSF1-mu; KD+SRSF1-mu), 277 MYO1B-fl-EGFP (MYO1B-fl; KD+MYO1B-fl) or MYO1B-t-EGFP (MYO1B-t; 278 KD+MYO1B-t) to overexpress the corresponding protein (Figure 8A). Using the five groups of 279

stable sub-cell lines, we found that restoration of SRSF1-mu (KD+SRSF1-mu) and MYO1B-fl
(KD+MYO1B-fl), but not MYO1B-t (KD+MYO1B-t), efficiently reversed the adverse effects of
SRSF1 knockdown (KD+vec) on GBM cell proliferation, survival and invasion (Figure 8B, and
Supplemental Figure 8, A and B), underscoring the importance of SRSF1-regulated *MYO1B*splicing in gliomagenesis.

We then investigated the functional significance of MYO1B-fl in mediating the oncogenic 285 effects of SRSF1 in vivo. The abovementioned five groups of U87MG stable sub-cell lines 286 (WT+vec, KD+vec, KD+SRSF1-mu, KD+MYO1B-fl, KD+MYO1B-t) were transplanted into 287 nude mice. Consistent with the in vitro results, both SRSF1-mu and MYO1B-fl abrogated the 288 289 suppressive effects of SRSF1 silencing on xenograft growth and tumor cell proliferation (as assessed by Ki-67 index), and the two groups of nude mice showed lower survival rates (Figure 290 8, C-F, and Supplemental Figure 8C). SRSF1 silencing and restoration were confirmed by IHC 291 (Figure 8F). Most prominently, in contrast to the WT+vec controls, xenograft tumors formed by 292 SRSF1-silenced cells (KD+vec) showed no sign of invasion (Figure 8F). Although SRSF1 re-293 expression (KD+SRSF1-mu) completely restored the growth and invasion of the xenografts, 294 MYO1B-fl exerted only partial effects (Figure 8, C-F), suggesting the existence of other SRSF1-295 regulated AS targets. However, unlike MYO1B-fl, MYO1B-t failed to exert any "rescue" effects 296 on tumor growth and invasion (Figure 8, C-F, and Supplemental Figure 8C). Collectively, these 297 data indicate that SRSF1 facilitates GBM cell proliferation, survival and invasion at least 298 partially by switching MYO1B splicing pattern and favoring the expression of the full-length 299 MYO1B isoform. 300

Increased *MYO1B*-fl levels parallel with *SRSF1* expression and predict poor prognoses of glioma patients. We next examined the splicing pattern of *MYO1B* exon 23 and 24 in 14 gliomas and 5 NBs as mentioned above. Quite in agreement with the oncogenic potentials of MYO1B-fl, inclusion of exon 23 and 24 was more frequent in gliomas than in

NBs, and more frequent in GBMs than in LGGs (Figure 9A). Significantly, a positive 305 correlation was observed between the SRSF1 mRNA level and MYO1B-fl% in gliomas (P<0.01; 306 Figure 9B). These findings were further reinforced by analyzing RNA-seq data of a large cohort 307 of glioma patients obtained from the TCGA database: MYO1B-fl% was higher in GBMs than 308 in LGGs (P<0.001; Figure 9C), and positively correlated with SRSF1 level (P<0.0001; Figure 309 9D). Furthermore, higher MYO1B-fl% was closely associated with the worse OS of the patients 310 311 (P<0.0001; Figure 9E). Together, these results validate the mechanistic link between MYO1Bfl and SRSF1 overexpression in gliomas, and propose that MYO1B splicing can be used as a 312 novel independent prognosis factor for glioma patients. 313

314 SRSF1-guided MYO1B splicing determines cell fate through the PDK1/AKT and **PAK/LIMK pathways.** To better understand the intracellular signaling network underlying the 315 anti-glioma effects due to SRSF1 knockdown, we surveyed potential cancer-related signaling 316 pathways using phospho-antibody microarrays. We identified a spectrum of proteins whose 317 phosphorylation levels were increased (upregulation) or decreased (downregulation) by >15% 318 in SRSF1-silenced U87MG cells (Figure 10A). Many of these proteins in the phosphorylated 319 form, are of great importance for tumor cell proliferation and invasion. Analysis of the array 320 revealed the reduction in phosphorylation of several key components crucial for AKT signaling, 321 actin organization and MAPK signaling upon SRSF1 knockdown (Figure 10B). Among these 322 proteins, several could be re-phosphorylated by MYO1B-fl overexpression (Supplemental 323 Figure 9A), illustrating the importance of these molecules in mediating the oncogenic roles of 324 the SRSF1/MYO1B-fl axis in GBM cells. 325

Using Western blot, we screened out two groups of phospho-proteins whose levels were obviously impacted by SRSF1 knockdown and rescued by MYO1B-fl but not MYO1B-t. The first group included the key components and the downstream effectors of the pyruvate dehydrogenase kinase 1 (PDK1)/AKT pathway. Within this group, the levels of phospho-PDK1

(Ser241), phospho-AKT (Ser473), cyclin dependent kinase 2 (CDK2) and Cyclin E2 were
decreased whilst that of cyclin dependent kinase inhibitor 1A (p21^{WAF1}) was increased upon
SRSF1 knockdown and rescued by MYO1B-fl overexpression (Figure 10C). These changes were
perfectly simulated by Wortmannin, the specific PI3K inhibitor, which completely abrogated the
rescue effects of MYO1B-fl as well (Supplemental Figure 9B, upper).

The second group comprised phospho-p21 (RAC1) activated kinase 1/2/3 (PAK1/2/3; 335 Thr423/402/421), phospho-LIM domain kinase 1/2 (LIMK1/2; Thr508/505) and phospho-336 Cofilin (Ser3). All of them belonged to the PAK/LIMK pathway, and their levels were decreased 337 upon SRSF1 silencing and rescued by MYO1B-fl overexpression (Figure 10C). PAK inhibitor 338 339 IPA-3 efficiently mimicked the effects of SRSF1 silencing and abolished the recue effects of MYO1B-fl (Supplemental Figure 9B, bottom). We also observed that MYO1B-t overexpression 340 failed to reverse the activities of PDK1/AKT and PAK/LIMK pathways (Figure 10C). All these 341 findings clearly verify that PDK1/AKT and PAK/LIMK are key pathways mediating the 342 oncogenic functions of SRSF1 and its splicing target, MYO1B-fl in glioma. 343

As is known, membrane-localized PI3K catalyzes phosphatidylinositol-4, 5-bisphosphate 344 (PIP2) phosphorylated to phosphatidylinositol-3, 4, 5-trisphosphate (PIP3). PIP3 then recruits 345 effectors (e.g. PDK1, AKT) and induces their activation (22). Basing on the above finding of 346 MYO1B-fl cytomembrane localization, we wonder whether MYO1B-fl directly recruits PI3K 347 to cytomembrane to activate PDK1/AKT and PAK/LIMK signaling in glioma cells. Co-348 immunoprecipitation (Co-IP) assays demonstrated that exogenous MYO1B-fl bound to 349 endogenous p85, a regulatory subunit of PI3K, while MYO1B-t showed only very feeble 350 binding with p85 (Figure 10D). Immunoflourescence detection also verified that MYO1B-fl 351 and p85 co-localized on the cytomembrane in U87MG cells overexpressing MYO1B-fl-EGFP, 352 while MYO1B-t and p85 scattered severally in the cytoplasm in U87MG cells overexpressing 353 MYO1B-t-EGFP (Figure 10E and Supplemental Figure 9C). The membrane recruitment of 354

PI3K by MYO1B-fl ultimately resulted in the activation of PDK1/AKT and PAK/LIMK
signaling (Supplemental Figure 9D).

To understand the role of AKT activation in the tumor promoting effects of MYO1B-fl, 357 we introduced the constitutively active myristoylated AKT (myr-AKT) into sh-MYO1B-fl and 358 sh-MYO1B-total sub-cell lines. Strikingly, overexpression of myr-AKT effectively rescued the 359 expression of the downstream effectors (p21^{WAF1}, CDK2), as well as the proliferation and 360 survival of GBM cells (Figure 10, F and G, and Supplemental Figure 9, E and F). Therefore, 361 AKT activation is crucial for the functions of the tumor promoter MYO1B-fl. To conclude, all 362 these results demonstrate that PDK1/AKT and PAK/LIMK are important pathways linking 363 SRSF1-regulated AS of MYO1B and glioma progression. 364

366 **Discussion**

Our present work represents a comprehensive study of the splicing factor SRSF1 and its downstream AS landscape in glioma. During this study, we identified a key AS target, *MYO1B*, whose full-length isoform (MYO1B-fl) was closely associated with the onset and progression of glioma and the outcome of the patients. We concluded that SRSF1 promotes gliomagenesis by controlling the AS of tumor-associated genes, further highlighting the importance of AS as a crucial contributor to tumorigenesis.

Human SRSF1 gene is located on Chromosome 17q23. Amplification of this locus is 373 frequently seen in various kinds of tumors and correlated with poor prognoses (23, 24). Besides, 374 insightful reports from other scholars have demonstrated that SRSF1 is overexpressed in 375 extracranial tumors and plays oncogenic roles via controlling the AS of several tumor-related 376 377 genes (10-12). We herein provided solid evidences that SRSF1 was overexpressed in gliomas, and its overexpression was associated with a higher malignancy grade and a poorer survival. The 378 following function study verified that SRSF1 knockdown inhibited the proliferation, survival and 379 380 invasion in GBM cells in vitro, and suppressed the growth and infiltration of the intracranial 381 glioma xenografts in vivo. All these defects could be rescued by SRSF1 restoration. Additionally, SRSF1 overexpression promoted the proliferation and invasion of lower grade glioma cell line 382 (SW1088). These findings indicated that, SRSF1 is an important promoter for gliomagenesis, and 383 proposed its potential value as a prognostic biomarker for glioma patients. 384

Recently, genome-wide studies have extensively identified the endogenous AS targets of SRSF1 (13, 14), and established a concentration/position-dependent splicing model (12, 15). However, partly due to the context-dependent nature of SRSF1 in target selection, the key SRSF1-govened AS networks responsible for tumorigenesis usually differ greatly among different tumor types (13-15), and unfortunately, little is known about the SRSF1-affected AS in glioma up to now. In the present study, taking advantages of RNA-seq and other validating

methods, we identified hundreds of SRSF1-affected AS events in U87MG and U251, two GBM 391 392 cell lines. Most of these events belong to the skipped exon, and they participate in a wide range of tumor-related functions in the aspect of cell cycle control, RNA splicing, cytoskeleton 393 organization and focal adhesion. Among these events, the common ones shared by U87MG and 394 U251 represent only a relative small fraction, and this could be explained by the discrepancies 395 of the genomic and proteomic backgrounds of the two cell lines (25, 26). Nevertheless, the 396 "common" events still deserve further study, for they may reflect the generality of GBMs. In 397 the following mechanistic investigations including motif analyses, CLIP and minigene reporter 398 assays, we found that enrichment of the SRSF1-binding motifs within the cassette exons always 399 400 led to exon inclusion, whilst enrichment of the motifs in the flanking constitutive exons and introns always led to exon exclusion. These findings strongly indicate that, like several other 401 splicing factors (27, 28), SRSF1 determines exon inclusion/exclusion in a position-dependent 402 403 manner.

Among the numerous AS targets of SRSF1 identified in glioma, we focused on MYO1B 404 gene, for its mature mRNA products were dramatically switched to the exon skipped isoform 405 upon SRSF1 knockdown. In the following experiments, we found that SRSF1 participated in 406 the AS of MYO1B pre-mRNA by facilitating the inclusion of exon 23 and 24, and thereby 407 induced the expression of the full-length MYO1B isoform (MYO1B-fl; Figure 11). During this 408 process, the 2 RRM domains and the RS domain of SRSF1 were all required, since the 409 corresponding deletion mutants showed decreased affinities to the core elements within exon 410 23 and 24, and failed to recover the splicing pattern after SRSF1 knockdown. 411

MYO1B, a member of class I myosin, is a widely expressed, single-headed, actinassociated molecular motor which is associated with organelle trafficking, membrane tethering, Golgi organization, actin organization and actin polymerization (29). Previous studies have shown that MYO1B is highly expressed in metastatic prostate cancer and head and neck

squamous cell carcinoma (30, 31). Importantly, knockdown of MYO1B significantly inhibited 416 417 the migratory and invasive abilities of the tumor cells through influencing actin organization (30, 31). In agreement with these reports, we found that the full-length isoform of MYO1B 418 (MYO1B-fl) displayed glioma promoting activities by enhancing the proliferation, survival and 419 invasion of GBM cells. We also spotted that exogenous MYO1B-fl but not MYO1B-t (the 420 isoform preferentially expressed after SRSF1 knockdown) partially rescued the malignant 421 phenotypes and the tumorigenic abilities of the SRSF1-silenced GBM cell lines. Although these 422 results cannot exclude the existence of other mediators, they are still adequate to prove that 423 MYO1B-fl is a crucial "bridge" molecule that mediates the oncogenic effects of SRSF1 in 424 425 glioma. Since we have also proved in human glioma samples that the level of MYO1B-fl mRNA was increased in parallel with that of SRSF1 and the tumor grade, and occupied a considerable 426 proportion of the total MYO1B mRNA in GBM, we can safely conclude that SRSF1 participates 427 in glioma formation and malignant progression by inducing the expression of the full-length 428 MYO1B isoform. 429

Furthermore, we observed that the distribution of MYO1B altered from cytomembrane 430 aggregation to cytoplasmic dispersion upon SRSF1 knockdown. This is an interesting 431 phenomenon, since the localization of MYO1B is strictly controlled in mammalian cells. 432 433 Previous studies reported that MYO1B-fl concentrates mainly within the dynamic areas of the actin cytoskeleton, most notably in membrane ruffles, and the artificial truncated mutants 434 containing the motor or IQ tail domains show a partial overlapping cytoplasmic localization 435 with MYO1B-fl, but never concentrate in membrane ruffles (30, 32). Using EGFP fusion 436 proteins, we provided solid evidences that EGFP fused MYO1B-fl localized mainly on 437 cytomembrane, while EGFP fused MYO1B-t dispersed in cytoplasm. Thereby, the 438 redistribution of MYO1B upon SRSF1 knockdown could be perfectly explained by the switch 439 of RNA splicing and the decrease of the MYO1B-fl isoform. Our results provide an 440

interpretation for the diversity of MYO1B localization naturally seen in normal and tumor cells,
and inspire us to postulate that although encoded by the same gene, different isoforms of
MYO1B differ greatly in molecular features and biologic functions. Indeed, cellular
experiments demonstrated that exogenous MYO1B-fl, but not MYO1B-t, could fully reverse
the antineoplastic effects of MYO1B-total knockdown on GBM cells.

In the identification of the downstream pathways mediating the oncogenic effects of 446 SRSF1 and MYO1B-fl, we adopted the criteria that the phosphorylation profiles of the key 447 components are changed by SRSF1 knockdown and reversed by the replenishment of MYO1B-448 fl, but not MYO1B-t, for this criteria help to guarantee the specificity of the cause and effect. 449 450 Two pathways, i.e. PDK1/AKT and PAK/LIMK attracted our attention. Activated through a series of phosphorylation events including the ones mediated by PDK1, AKT signaling pathway 451 plays an important role in glioma progression and aggressiveness (33). Different from the 452 453 previous finding that SRSF1 bypasses the upstream AKT pathway to activate mTOR complex 1 (mTORC1) (34), we found that SRSF1 knockdown severely repressed the phosphorylation of 454 PDK1 and AKT, led to the increase of p21^{WAF1} and the decrease of CDK2, and thereby inhibited 455 the growth of glioma cells. 456

Downstream members of the Rho-GTPase pathway are critical intracellular mediators for 457 actin modeling. These factors control directional cell migration and are frequently dysregulated 458 in GBM (35). PAK-phosphorylated LIMK functions as a key promoter for mesenchymal and 459 amoeboid migration, largely through phosphorylating Cofilin at the Serine-3 (S3) residue and 460 blocking its actin binding ability (36). Screening of the invasion associated proteins found 461 upregulated LIMKs and phospho-Cofilin accumulating in the periphery of GBM (37). In the 462 present study, we observed that SRSF1 knockdown markedly repressed PAK/LIMK 463 phosphorylation and thereby blocked the phosphorylation of Cofilin. Moreover, MYO1B-fl 464 could rescue the activity of PDK1/AKT and PAK/LIMK signaling pathways in SRSF1-465

knockdown GBM cells, while MYO1B-t exerted no rescue effects. Combining with the results
that the specific inhibitors of the PI3K and PAK pathways abrogated the rescue effects of
MYO1B-fl, our findings confirmed that these two pathways are the key ones that mediate the
promoting effects of SRSF1 and MYO1B-fl on glioma cell growth and invasion.

As is known, PI3K is activated via membrane translocation induced by RTK, then 470 activated PI3K triggers AKT and PAK signaling by catalyzing PIP2 phosphorylation to produce 471 PIP3 in cytomembrane (38). Our Co-IP and immunofluorescence results identified that 472 MYO1B-fl, but not MYO1B-t, bound to p85 with high affinity, recruited p85 to cytomembrane 473 and subsequently activated PDK1/AKT and PAK/LIMK signaling (Figure 11). Moreover, myr-474 AKT overexpression effectively abolished the suppressions of MYO1B-fl or MYO1B-total 475 knockdown on the proliferation and survival of GBM cells, further demonstrating the 476 importance of AKT activation in mediating the oncogenic functions of MYO1B-fl in glioma. 477 478 These findings indicate that SRSF1-upregulated MYO1B-fl can activate PDK1/AKT and PAK/LIMK signaling pathways by directly binding and recruiting PI3K to cytomembrane, 479 therefore revealing a new mechanism by which the SRSF1/MYO1B axis promotes 480 gliomagenesis and highlighting the importance of PI3K in mediating the above MYO1B-fl 481 oncogenic effects. 482

In summary, in the present study, we demonstrated that unlike the truncated isoform of MYO1B, the full-length MYO1B serves as an oncogenic factor for glioma by increasing the proliferation, survival and invasion of glioma cells. The splicing factor SRSF1 promotes gliomagenesis and malignant progression by facilitating the inclusion of exon 23 and 24 in *MYO1B* pre-mRNA, and inducing the expression of the full-length isoform. SRSF1 and MYO1B-f1 represent novel prognostic biomarkers and potential targets for the therapy of malignant gliomas.

490 Methods

Tissue samples and clinicopathological data. The surgical specimens of 120 astrocytic 491 gliomas and 20 nontumoral brain tissues (control) were collected from Tianjin Medical 492 University General Hospital (TMUGH) with written consents. After surgical excision, 493 specimens were fixed immediately in 3.7% buffered formaldehyde solution and embedded in 494 paraffin. Then, 5 µm continuous sections were prepared for IHC of SRSF1 and Ki-67. 495 496 Histopathological diagnoses were independently made by two neuropathologists according to the 2016 WHO classification of central nervous system tumors (39). Sanger sequencing was 497 adopted to verify the gene type of IDH1/2 (39). The WHO grades, IDH1/2 statuses, KPS scores 498 and patients' clinical features were summarized in Supplemental Table 5. All the 120 glioma 499 patients had complete information and were followed from the date of operation till December 500 501 31, 2013, with the follow-up time ranging from 4.5 to 89 months.

502 Oncomine data and TCGA RNA-seq data analysis. Oncomine data analysis was 503 performed as previously described (28). RNA-seq data from a total of 240 human glioma 504 samples (120 cases of GBM and 120 cases of LGG) was downloaded from TCGA 505 (https://cancergenome.nih.gov/). Percentage ratio of *MYO1B-fl* transcript in all *MYO1B* 506 transcripts (*MYO1B*-fl%) was calculated based on the number of reads supporting inclusion and 507 exclusion events. *SRSF1* gene expression was calculated by counting the number of reads 508 falling into *SRSF1* gene. Linear analysis was performed between the two variables.

Immunohistochemistry (IHC). IHC was performed with mouse anti-human SRSF1
(catalog No. sc-33652) and mouse anti-human Ki-67 primary antibodies (catalog No. sc-23900;
Santa Cruz Biotechnology, USA) as previously described (40). PBS was used to substitute the
primary antibody as a negative control (only with bio-IgG and ABC complex). IHC images
were acquired under a DM6000B microscope (Leica, Germany).

514 Cell culture and reagents. Human GBM cell line U87MG and an anaplastic astrocytoma

(WHO grade III) cell line SW1088 were obtained from the American Type Culture Collection (ATCC). LN229, U251, U343 and SNB19 cells were purchased from the China Academia Sinica Cell Repository. The GBM cell line TJ905 from a Chinese patient was established and maintained by our lab. The human immortalized astrocyte cell line UC2 maintained by our lab was used as the nontumoral control. All the cells were cultured in DMEM (Gibco, USA) containing 10% FBS (Gibco) at 37°C in a humidified incubator with 5% CO₂. Wortmannin (PI3K inhibitor) and IPA-3 (PAK inhibitor) were ordered from Selleck (China).

siRNAs, plasmids and lentiviruses. The siRNAs targeting *SRSF1* or *MYO1B*-fl were
synthesized by Gene Pharma (China), and transfected with Lipofectamine RNAiMAX
(Invitrogen, USA). Their sequences were listed in Supplemental Table 6.

The plasmids expressing HA tagged wild type SRSF1 (SRSF1-wt), synonymous mutated 525 SRSF1 (SRSF1-mu) and SRSF1 domain deletion mutants (SRSF1- Δ RRM1, - Δ RRM2, - Δ RS) 526 were constructed using pCDNA3.0-HA vector. The plasmids expressing EGFP fused full-527 length MYO1B (MYO1B-fl) and truncated MYO1B (MYO1B-t) were constructed using 528 pEGFP-C1 vector. MYO1B minigene was constructed by amplifying the genomic sequence 529 spanning exons 22-25 of human MYO1B gene and cloned into pCMV-Tag2b vector, and its 530 531 mutants in which specific sequence was deleted or inserted were made based on the primary minigene. All the cloning primers were listed in Supplemental Table 7. The expression plasmid 532 for constitutively active AKT (myr-AKT) was obtained from Addgene (USA). 533

The knockdown lentiviruses expressing negative control shRNA (sh-NC) and shRNAs targeting *SRSF1* (sh-*SRSF1*), full-length *MYO1B* (sh-*MYO1B*-fl) or all isoforms of *MYO1B* (sh-*MYO1B*-total) were constructed using the PLKO.1-puro lentiviral vector and packaged in 293T cells (puromycin resistance). Their sequences were listed in Supplemental Table 8. The overexpression lentiviruses expressing luciferase alone (vec), and co-expressing luciferase plus fusion protein of SRSF1-mu-HA (SRSF1-mu), MYO1B-fl-EGFP (MYO1B-fl) or MYO1B-t-

EGFP (MYO1B-t) were constructed and packaged by Applied Biological Materials (Canada;
blasticidin resistance).

Stable sub-cell line. Firstly, four kinds of stable sub-cell lines from GBM cell lines were 542 individually established by infecting knockdown lentivirus expressing sh-NC, sh-SRSF1, sh-543 MYO1B-fl or sh-MYO1B-total, and selecting with puromycin. They included control group 544 (WT/sh-NC) and the knockdown groups of SRSF1 (KD), MYO1B-fl (sh-MYO1B-fl) or MYO1B-545 total (sh-MYO1B-total). Their pooled cultures were infected again with corresponding 546 lentiviruses to overexpress indicated proteins, and selected with blasticidin. The stable sub-cell 547 lines infected by dual lentiviruses included control group (WT+vec), knockdown groups 548 549 (KD+vec, sh-*MYO1B*-total+vec), rescue groups (KD+SRSF1-mu, KD+MYO1B-fl, KD+MYO1B-t, sh-MYO1B-total+MYO1B-fl, sh-MYO1B-total+MYO1B-t) and overexpression 550 group (WT+MYO1B-fl), and their pooled cultures were used for the present study. 551

552 **Cell growth, proliferation, survival and invasion assays.** Cell growth was quantified 553 with CCK-8 and the proliferating cells were labeled by 5-ethynyl-2'-deoxyuridine (EdU) 554 staining as described previously (39). The ability of anchorage-dependent growth (cell survival) 555 was assessed by colony formation assays as described previously (39). Transwell invasion assay 556 was performed as described previously (41).

Immunofluorescence staining. Cells grown on glass coverslips were fixed with 4% 557 paraformaldehyde (PFA, Sigma-Aldrich, USA), permeabilized with 0.2% Triton X-100, and 558 stained following the standard procedures (42). Rabbit anti-human MYO1B antibody (catalog 559 No. HPA013607) was purchased from Sigma-Aldrich (USA). Fluorescent Alexa Fluor 488 560 Phalloidin (catalog No. A-12379), rabbit anti-human p85 (PIK3R1) antibody (catalog No. 710400) 561 and TRITC-labeled goat anti-rabbit IgG antibody (catalog No. A-16101) were all purchased from 562 Thermo Fisher Scientific (USA). DAPI reagent was used to stain the cell nuclei. Cells were 563 imaged using an FV-1000 laser-scanning confocal microscope (Olympus, Japan). 564

Measurements of fluorescence labeling, cell area and protein colocalization. To 565 accurately identify the effects of SRSF1 and MYO1B-fl knockdowns on F-actin distribution in 566 cytoskeleton and cell morphology, we measured the line profile intensity of fluorescence 567 labeled by Phalloidin and cell area of the sub-cell lines in WT/sh-NC, KD and sh-MYO1B-fl 568 groups. To detect the difference of subcellular localizations between MYO1B-fl and MYO1B-569 t, we expressed the exogenous EGFP fusion proteins, MYO1B-fl-EGFP or MYO1B-t-EGFP, 570 571 in U251 cells, measured the fluorescence density (pixels) of the border (membrane location) and total in the single cell of each group, and calculated the percentage ratio (membrane index) 572 of the border pixels to total pixels. All the measuring analyses were finished with ImageJ 573 574 software (NIH, USA) as described previously (43, 44). The above experiments were repeated independently for 6 times. The mean \pm SD of cell area and membrane index was identified with 575 50 cells randomly selected from each of the 6 replicates (300 cells in total). The correlation of 576 subcellular localization between endogenous p85 (red) and exogenous MYO1B-fl-EGFP or 577 MYO1B-t-EGFP (green) was detected by the colocalization finder plugin of ImageJ software. 578

Intracranial xenograft assay in nude mice. The female BALB/C athymic nude mice at 579 the age of 4 weeks were purchased from the animal center of the Cancer Institute of Chinese 580 Academy of Medical Science (Beijing, China). The pooled cultures of stable sub-cell lines 581 sequentially infected with dual lentiviruses and selected with puromycin and blasticidin, i.e., 582 control group (WT+vec), SRSF1-KD group (KD+vec) and rescue groups (KD+SRSF1-mu, 583 +MYO1B-fl, +MYO1B-t) were transplanted intracranially $(3 \times 10^5 \text{ cells per mouse})$ under the 584 guidance of a stereotactic instrument as described previously (41). Bioluminescence imaging 585 was used to monitor the intracranial tumor growth and the survival situation of the animals was 586 recorded every day. After the sacrifice, the mouse brains were collected and subjected to IHC 587 and H&E staining. 588

589

RNA isolation, quantitative reverse transcriptase PCR (qRT-PCR), RT-PCR and in

vivo CLIP. Total RNA was isolated using the TRIzol reagent (Invitrogen, USA) following the 590 591 standard protocol. qRT-PCR of the mRNAs under study was performed using the SYBR Green PCR kit (Takara Bio, China). The specific primers were synthesized by Beijing Genomics 592 Institute (BGI, China) and their sequences were listed in Supplemental Table 9. The fold 593 changes of mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method. To discriminate the mRNA 594 variants of SRSF1-guided AS, RT-PCR was performed as described previously (27). In vivo 595 596 CLIP assay was performed as described previously (42). In brief, the plasmids coding for HA tagged SRSF1 and its domain deletion mutants as well as the empty vector were transiently 597 transfected into U87MG cells. After ultraviolet crosslinking, IP was performed using Magna 598 599 RIP kit (Millipore, USA). RNA enrichment was measured by RT-PCR. Specific primers for RT-PCR were synthesized by BGI (Supplemental Table 10). 600

601 **RNA-Seq and data analysis.** Total RNA isolated from stable WT and KD groups of 602 U87MG and U251 cells were subjected to paired-end RNA-Seq using Illumina HiSeq 2000 603 system according to the manufacturer's instruction. Reads mapping and data analysis for 604 differentially spliced exons/introns were carried out as described previously (27). The raw 605 sequence data has been submitted to Gene Expression Omnibus with accession number 109436.

Western blot and Co-IP. Western blot was carried out as described previously (39). The detailed information of commercially available primary antibodies was summarized in Supplemental Table 11. For Co-IP, cell extracts were incubated successively with GFP antibody (catalog No. T0005; Affinity Biosciences) for 4 to 6 h and with protein A/G agarose beads (catalog No. sc-2003; Santa Cruz Biotechnology) for 12 to 16 h. Bound proteins were then washed in lysis buffer (catalog No. P0013J; Beyotime Biotechnology, China), resuspended in protein sample buffer, separated by SDS-PAGE and detected by immunoblot.

613 **Motif analysis of SRSF1-regulated cassette exons.** Basing on the previously CLIP-614 identified (15) and RNA-seq predicted (13) consensus sequences for SRSF1, and also taking into account the degenerate nature of RNA binding sequences, we defined GA-rich 6-mers that
include at least one G and one A with the GA content ≥50% as potential SRSF1 binding sites.
Motif analysis for SRSF1 in U87MG and U251 cells was performed as described previously
(27).

619 **Cancer signaling phospho-protein profiling with antibody array.** Cell lysates were 620 applied to the Phospho Explorer Antibody Array, which was designed and manufactured by 621 Full Moon Biosystems (China). Data were collected and analyzed by Wayen Biotechnologies 622 (China). A ratio computation was used to measure the extent of protein phosphorylation: 623 phosphorylation ratio = phosphorylated value/unphosphorylated value.

624 Statistics. Statistical analyses were performed using SPSS 21.0 software (International Business Machines Corporation, USA). 2-tailed student's t test, Mann-Whitney test, 1-way 625 ANOVA, Pearson correlation analysis, Kaplan-Meier analysis, log-rank test, Cox's proportional 626 hazards regression model and χ^2 test were used to analyze the corresponding data. The medians 627 of the SRSF1 LIs, SRSF1 mRNA levels and MYO1B-f1% were used as the cutoffs in the survival 628 analyses of the corresponding cohort of glioma patients. A P value less than 0.05 was considered 629 statistically significant. All the experiments on cell lines were performed at least in triplicate. All 630 the quantitative data are presented as mean \pm SD. 631

632 **Study approval.** Human surgical specimens were collected from TMUGH with written 633 consent. qRT-PCR, Western blot and IHC on human samples were carried out in accordance 634 with the principles of the Helsinki Declaration and approved by the Ethics Committee of 635 TMUGH. The animal experiments were conducted strictly in accordance with a protocol 636 approved by the Institutional Animal Care and Use Committee of TMUGH.

638 Author contributions

Conception and design were performed by XZ, RW, XL and SY. Data acquisition was
performed by XZ, RW, XL, LY, DH, CS, CS, WL, CR and ZJ. Data analysis was performed
by XZ, RW, XL, QW and SY. Drafting of the manuscript was performed by XZ, YF, QW and

642 SY.

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Figure 1. SRSF1 overexpression is correlated with excessive glioma cell proliferation and 747 predicts poor prognoses of glioma patients. (A) Relative SRSF1 mRNA levels in glioma 748 tissues as detected by qRT-PCR. The mean of the normal brain (NB) group was arbitrarily set 749 to 1.0. Data are presented as mean \pm SD, n=3. (**B**, **C**) Western blot of SRSF1. The expression 750 levels of SRSF1 were compared between GBM tissues and NBs (B), as well as among the GBM 751 cell lines, UC2 (an immortal astrocyte cell line) and SW1088 (an anaplastic astrocytoma cell 752 line, WHO grade III) (C). β-actin was used as the loading control. (D) Left: IHC staining of 753 SRSF1 in control (nontumoral brain tissues) and the glioma tissues. The negative control was 754 established by using PBS to substitute the primary antibody. Scale bar, 20 µm. Right: 755 756 Comparison of SRSF1 expression levels among 20 normal brain tissues and 120 gliomas of various grades. The expression levels were represented by labeling indexes [LIs (%)] which 757 were calculated with Leica Image Pro Plus 5.0 software as the percentage ratio of positive cells 758 759 to total cells. Data are presented as box plots. Boxes represent the 25th and 75th percentiles, lines represent the median, and whiskers show the minimum and maximum points. ***P<0.001 760 by 1-way ANOVA with Tukey's post-test. (E) Pearson correlation analysis between the LIs of 761 SRSF1 and Ki-67 in the glioma samples (n=120). Pearson correlation test, r and P values are 762 shown. (F-H) Kaplan-Meier analyses of the DFS and OS of all the glioma patients (F) and the 763 patients with similar age (G) and identical IDH1/2 gene status (H). Patients were stratified into 764 high and low expression groups using the medians of SRSF1 LIs of the corresponding cohorts 765 as cutoffs. The *P* values of the log-rank (Mantel-Cox) tests are presented. 766



771	Figure 2. SRSF1 increases the tumorigenic abilities of GBM cells and induces the
772	reorganization of cytoskeleton. (A) Western blot of the endogenous SRSF1 and exogenous
773	SRSF1-mu (HA-tagged product of SRSF1 synonymous mutant) in the extracts of cells as
774	indicated. Loading control: β -actin. (B) Images of EdU staining (left) and the comparison of
775	EdU positive rates among the indicated cells (right). (C) Colony formation assay results. (D)
776	Transwell invasion assay results. (E) Left: Cytoskeleton was labeled with Phalloidin (green),
777	and cell nuclei were counter-stained with DAPI. Scale bar, 20 µm. Right: Green fluorescent
778	(Phalloidin) intensities were profiled along the red lines (upper and middle panels). Cell areas
779	were compared between the WT and the KD sub-cell lines. Data are presented as box plots,
780	n=300. *** P <0.001 by 2-tailed Student's <i>t</i> test (bottom panel). Data in (A-D) are presented as
781	mean \pm SD, n=3 for (A) and n=5 for (B-D). *** <i>P</i> <0.001 by 1-way ANOVA with Dunnett's
782	post-test. Representative images from biological triplicate experiments are shown for (B-E).



Figure 3. Global profiles of SRSF1-affected AS in GBM cells. (A) SRSF1-affected AS 786 events in U87MG (left) and U251 (right) cell lines. The AS events are classified into five 787 categories: skipped exon (SE), retained intron (RI), alternative 5' splice site (A5SS), alternative 788 3' splice site (A3SS) and mutually exclusive exon (MXE). (B) Relative fraction of AS events 789 affected positively (activation) or negatively (repression) by SRSF1 in each category. (C) 790 791 Overlapped AS events in each category of the activation/repression groups between U87MG and U251 cell lines. (D) Gene ontology of the common AS targets shared by U87MG and U251 792 cell lines. -Log₂ transformed fisher exact P values are plotted for each enriched functional 793 category. (E) Functional association network of the SRSF1-affected AS targets. Genes 794 incorporated in (**D**) were analyzed using the STRING database, and the subgroups are marked 795 according to their functions. 796



799

Figure 4. Validation and motif discovery of SRSF1-mediated AS in GBM cells. (A, B) RT-800 PCR validations of SRSF1-affected AS events. Representative images from three independent 801 experiments are presented. The structure of each PCR product is indicated schematically on the 802 right. Alternative exons/introns affected by SRSF1 are painted in orange. The four lanes for 803 GOLGA4 (B) are on the same gel but noncontiguous. The percentage ratios of inclusion 804 products (in%) of exon or intron to total products are provided below each gel. (C) Left: 805 806 Flowchart of the SRSF1-motif discovery from the RNA-seq data. Right: The sum of the Log₂ transformed fold change (FC) of the GA-rich 6-mers overrepresented within the five regions 807 around the regulated cassette exons is compared with that around the control cassette exons. 808 Red line represents SRSF1-mediated cassette exon activation, and blue line represents SRSF1-809 mediated cassette exon repression. Potential SRSF1 motifs derived from the 6-mers 810 overrepresented in the activated or repressed cassette exons are also given. 811



Figure 5. SRSF1 mediates exon 23-24 inclusion in MYO1B pre-mRNA. (A) Upper: Diagram 816 of the splicing variants of MYO1B mRNA and the primers for RT-PCR detection of exon 23 817 (primer set 1) and exon 23-24 (primer set 2) inclusion/exclusion. Bottom: AS of exon 23 (primer 818 set 1) and exon 23-24 (primer set 2) and expression of MYO1B isoforms were examined by 819 RT-PCR and Western blot, respectively. (B) Western blot of SRSF1 and the corresponding RT-820 PCR results of MYO1B mRNA fragments (primer set 2). (C) Upper: Schematic diagram of 821 SRSF1 domains and constructions of three SRSF1 mutants: $\Delta RRM1$ (deleting RRM1), $\Delta RRM2$ 822 (deleting RRM2), ΔRS (deleting RS). All the mutants were HA tagged. Bottom: Western blot 823 of the endogenous and exogenous SRSF1 with anti-HA and anti-SRSF1 antibodies. AS of exon 824 23-24 was detected by RT-PCR (primer set 2). (D) Western blot of exogenous SRSF1 and its 825 mutants using anti-HA tag antibody. (E) Direct binding between indicated proteins and 826 endogenous MYO1B RNA fragments verified by CLIP. (F) Left: Schematic diagram of 827 828 MYO1B minigene with the potential SRSF1-binding sites marked in red. MYO1B splicing reporters with the indicated deletion (del1-del3) or insertion (del2in) were generated. Right: 829 Splicing of MYO1B minigene and the reporters were verified by RT-PCR (primer set 2). The 830 percentages of MYO1B-fl to total MYO1B transcripts are presented using fl% in (A-C, F). 831



Figure 6. MYO1B-fl promotes GBM malignancy and MYO1B isoforms differ in 836 subcellular localization. (A) Endogenous MYO1B-fl was efficiently knocked down in 837 U87MG and U251 cells by MYO1B-fl siRNA (si-MYO1B-fl-1#, si-MYO1B-fl-2#) transfection 838 as verified by Western blot. Loading control: β-actin. (B) Growth curves of U87MG and U251 839 cells transfected with the siRNAs as indicated. (C) Transwell invasion assay results. (D) 840 Fluorescence images of the cells as indicated. MYO1B was stained by immunofluorescence 841 (red) and cytoskeleton was labeled by Phalloidin (green). Cell nuclei were counter-stained by 842 DAPI (blue). Scale bar, 20 µm. (E) Subcellular distribution of EGFP fused MYO1B. Cell nuclei 843 were counter-stained by DAPI (blue). Scale bar, 20 μ m. Data in (A-C) are presented as mean \pm 844 SD, n=3 for (A) and n=5 for (B, C). ***P<0.001 by 1-way ANOVA with Dunnett's post-test 845 for (A, B), 2-tailed Student's t test for (C). Representative images from biological triplicate 846 experiments are shown for (C, D). 847



850

851 Figure 7. MYO1B isoforms differ in biological functions. (A) Western blot of endogenous MYO1B and the exogenous MYO1B-fl/t expression. MYO1B was knocked down by the 852 specific shRNA (sh-MYO1B-total) in U87MG and U251 cells and the EGFP fused full-length 853 and truncated isoforms were overexpressed individually. Loading control: β -actin. (**B**, **C**) 854 Images of EdU staining and transwell invasion assays (**B**) and the statistical analysis results (**C**). 855 856 Representative images from biological triplicate experiments are shown for (B). Data in (C) are presented as mean ± SD, n=5. **P<0.01, ***P<0.001 by 1-way ANOVA with Dunnett's post-857 858 test.



Figure 8. MYO1B-fl partially recapitulates the SRSF1-mediated tumor promoting 863 phenotypes in GBM cells. (A) Western blot of endogenous and exogenous MYO1B and 864 SRSF1 in U87MG cells. Loading control: β-actin. (B) EdU staining and transwell invasion 865 assays of U87MG cells as indicated. Representative images from biological triplicate 866 experiments are shown. (C) Bioluminescence images of the intracranial glioma xenografts 867 formed by the indicated U87MG cells. Images of representative mice are shown. (D) 868 Bioluminescence quantification results at day 4, 11, 18 and 25 after implantation (n=8 for each 869 group). Data are presented as mean ± SD. **P<0.01, ***P<0.001 by 1-way ANOVA with 870 Dunnett's post-test. (E) Kaplan-Meier analysis of the OS of the glioma bearing nude mice. 871 ***P*<0.01 for the difference of WT+vec vs. KD+vec, KD+SRSF1-mu vs. KD+vec, WT+vec vs. 872 KD+MYO1B-t and KD+SRSF1-mu vs. KD+MYO1B-t; *P<0.05 for the difference of 873 KD+MYO1B-fl vs. KD+vec and KD+MYO1B-fl vs. KD+MYO1B-t by log-rank (Mantel-Cox) 874 test. (F) IHC of SRSF1 and Ki-67 in outgrowing tumor slices, and H&E staining images 875 showing the junctions between glioma xenografts and surrounding brain tissues. Red dotted 876 lines outline the boundaries of the tumors, and red double sided arrows indicate invasion 877 distances. Scale bar for IHC, 20 μ m. Scale bar for H&E, 100 μ m (100×) and 50 μ m (400×). 878 Images of representative tumors are shown. 879



Figure 9. AS of *MYO1B* is correlated with *SRSF1* levels and predicts poor prognoses. (A) 883 Splicing pattern of MYO1B in glioma tissues as detected by RT-PCR (primer set 2). MYO1B-884 fl% was presented as mean \pm SD, n=3. (**B**) Pearson correlation analysis between SRSF1 mRNA 885 level and *MYO1B*-fl% in tissue samples (n=19) as indicated in (A), r and P values by Pearson 886 correlation test are presented. (C) Comparisons of MYO1B-fl% between LGGs (n=120) and 887 GBMs (n=120) using TCGA RNA-seq data. ***P < 0.001 by 2-tailed Student's t test. (**D**) 888 889 Pearson correlation analysis between SRSF1 mRNA levels and MYO1B-f1% using the above TCGA data (n=240). Pearson correlation test, r and P values are presented. (E) Kaplan-Meier 890 analysis of the OS of the above patients in TCGA database. Patients were stratified into high 891 and low expression subgroups using the median of MYO1B-fl% as the cutoff. P<0.0001 by log-892 rank (Mantel-Cox) test. 893



Figure 10. SRSF1-guided MYO1B splicing determines cell fate through PDK1/AKT and 898 **PAK/LIMK pathways.** (A, B) Phosphoproteome array analysis of the expression changes of 899 the phosphoproteins upon SRSF1 knockdown in U87MG cells. The levels of the individual 900 proteins were normalized by total protein levels. Phosphoproteins with their levels increased 901 and decreased by more than 15% were labeled red and blue, respectively. (C) Western blot of 902 the indicated proteins in the extracts of U87MG cells. (D) Co-IP confirmation of the interaction 903 between EGFP fused MYO1B proteins (MYO1B-fl and MYO1B-t) and endogenous p85 PI3K 904 in U87MG and U251 cells. (E) Subcellular distribution of exogenous MYO1B-fl or MYO1B-t 905 (green) and endogenous p85 PI3K (red) in U87MG cells. Scale bar, 20 µm. Representative 906 images from biological triplicate experiments are shown. (F) Western blot of the indicated 907 proteins in U87MG cells. The lanes for MYO1B were on the same gel but noncontiguous. (G) 908 Representative images of EdU staining from biological triplicate experiments (left) and 909 910 quantification (right). Data are presented as mean \pm SD, n=5. ***P<0.001 by 1-way ANOVA with Tukey's post-test. 911



914

915 Figure 11. Schematic illustration of the molecular pathways by which SRSF1 promotes

916 gliomagenesis through regulating the AS of *MYO1B* pre-mRNA.

Feature	Non-increased (SRSF1 LI ≤ 51.5%) n=60	Increased (SRSF1 LI > 51.5%) n=60	Test of Significance
Gender			
Male	31	40	χ ² =2.7939
Female	29	20	<i>P</i> =0.0946
Age			
< 50	44	17	χ ² =24.3068
≥ 50	16	43	<i>P</i> <0.0001
Predominant side			
Left	26	31	
Right	30	26	
Middle	4	3	<i>P</i> =0.6936
Predominant location			
Frontal lobe	45	30	
Temporal lobe	11	16	
Parietal lobe	1	8	
Occipital lobe	1	2	
Others	2	4	<i>P</i> =0.0228
Grade			
II	40	0	
III	20	20	χ²=80.0000
IV	0	40	<i>P</i> <0.0001
Ki-67 LI			
≤ 16.7	50	9	χ²=56.0489
> 16.7	10	51	<i>P</i> <0.0001
IDH1/2 status			
Wild type (<i>IDH1/2</i>)	8	42	χ ² =39.6343
Mutant type (<i>IDH1</i> R132H)	52	18	<i>P</i> <0.0001
KPS score			
< 90	37	36	χ ² =0.0350
≥ 90	23	24	<i>P</i> =0.8516

918 Table 1. Relationship between SRSF1 expression and the clinicopathological 919 characteristics of the 120 glioma patients