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Modulation of transcriptional activity in brain lower grade glioma by alternative splicing

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Proteins that modify the activity of transcription factor (TF), often called modulators, play a vital role in gene transcriptional regulation. Alternative splicing is a critical step of gene processing and it can modulate gene function by adding or removing certain protein domains, and therefore influences the activity of a protein. The objective of this study is to investigate the role of alternative splicing in modulating the transcriptional regulation in brain lower grade glioma (LGG), especially transcription factor ELK1, which is closely related to various diseases, including Alzheimer's disease and down syndrome. Results showed that changes in the exon inclusion ratio of proteins APP and STK16 are associated with changes in the expression correlation between ELK1 and its targets. Meanwhile, the structural features of the two modulators are strongly associated with the pathological impact of exon inclusion. Our analysis suggests, protein in different splicing level could play different functions on transcription factors, hence induces multiple genes dysregulation.

1 **Modulation of transcriptional activity in brain lower grade glioma by**
2 **alternative splicing**

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10 **Abstract**

11 Proteins that modify the activity of transcription factor (TF), often called modulators, play a vital
12 role in gene transcriptional regulation. Alternative splicing is a critical step of gene processing and
13 it can modulate gene function by adding or removing certain protein domains, and therefore
14 influences the activity of a protein. The objective of this study is to investigate the role of
15 alternative splicing in modulating the transcriptional regulation in brain lower grade glioma (LGG),
16 especially transcription factor ELK1, which is closely related to various diseases, including
17 Alzheimer's disease and down syndrome. Results showed that changes in the exon inclusion ratio
18 of proteins APP and STK16 are associated with changes in the expression correlation between
19 ELK1 and its targets. Meanwhile, the structural features of the two modulators are strongly
20 associated with the pathological impact of exon inclusion. Our analysis suggests, protein in
21 different splicing level could play different functions on transcription factors, hence induces
22 multiple genes dysregulation.

23 **Keywords**

24 alternative splicing, amyloid precursor protein, EST domain-containing protein Elk-1, lower grade
25 glioma, modulator, serine/threonine kinase 16

26 **Introduction**

27 Alternative splicing (AS) is a key regulator of gene expression as it generates numerous
28 transcripts from a single protein-coding gene. In humans, over 95% of multi-exonic protein-coding
29 genes undergo AS(Wang, Sandberg et al. 2008), and AS plays an important role in cellular
30 differentiation and organism development(Castle, Zhang et al. 2008, Wang, Sandberg et al. 2008).
31 As AS affects numerous genes and is highly important for regulating a given gene's normal

32 expression and tissue specificity, it is not surprising that changes in AS are frequently associated
33 with human diseases, such as cancers(Kozlovski, Siegfried et al. 2017) and neurodegenerative
34 diseases(Scotti and Swanson 2016). Recent genome-wide analyses of cancer transcriptomes have
35 demonstrated that splicing changes are often global rather than gene specific(Jung, Lee et al. 2015).
36 Undoubtedly, widespread splicing changes, such as altered cassette exon inclusion ratios of
37 proteins, influence the expression of numerous genes and consequently cause aberrant gene
38 regulation.

39 Lower grade glioma (LGG) is a type of cancer that develops in the glial cells of the brain.
40 Tumors are classified into grades I, II, III or IV based on standards set by the World Health
41 Organization(Ostrom, Gittleman et al. 2013). Regardless of tumor grade, tumors compress normal
42 brain tissue as they grow, frequently causing disabling or fatal effects. The Cancer Genome Atlas
43 (TCGA) consortium has produced a comprehensive somatic landscape of glioblastoma by
44 combining molecular and clinical data, and TCGA has become a valuable resource for studying
45 gene deregulation in LGG.

46 Modulators are proteins that modify the activity of transcription factors (TFs) and influence the
47 expression of their target genes. Our current knowledge of TF modulation mainly comes from
48 experimental studies that measure the expression levels of a few target genes(Lachmann, Xu et al.
49 2010). The objective of this study is to explore the role of AS in modulating the transcriptional
50 activities of TFs in LGG. The modulated relationships among TF-modulator-targets are inferred
51 using a known probabilistic model, named GEM(Babur, Demir et al. 2010). EST domain-
52 containing protein Elk-1 (ELK1) is one of the TFs whose regulation activity is most influenced by
53 162 splicing events, corresponding to 123 AS modulator proteins. Finally, amyloid precursor

54 protein (APP) and serine/threonine kinase 16 (STK16), modulators whose exon inclusion ratios
55 are associated with the activity of ELK1, are analyzed in detail.

56 **Materials & Methods**

57 *Construction of triplets*

58 We implemented a known algorithm named GEM(Babur, Demir et al. 2010) to predict (splicing
59 modulator-TF-target) triplets. There are four input types: gene expression profiles, gene splicing
60 profiles, modulator list and transcription factor-target relations. The modulator hypothesis predicts
61 that the correlation between the expression levels of the TF and the target must change as the
62 splicing level of the modulator changes. The percentage of exon inclusion ratio (PSI) is used to
63 estimate the splicing level of a candidate modulator in LGG. We established a 5% false discovery
64 rate as the threshold to call the triplets.

65 *Data processing and selection*

66 RNA-Seq data were download from the TCGA-LGG data portal as bam files. STAR aligner
67 (version 2.3.0) was used to uniquely align each file uniquely to the hg19 human genome. We kept
68 uniquely aligned reads with a minimum splice junction overhang of five nucleotides using default
69 parameters. Gene expression level is estimated using a tool named NGSUtils (version 0.5.9)
70 (Breese and Liu 2013) with the default parameters for calling gene expression. The splicing level
71 (PSI) is estimated using a probabilistic model called Mixture of Isoforms (MISO)(Katz, Wang et
72 al. 2010). The TF-target relations are derived from the ENCODE (The Encyclopedia of DNA
73 Elements) project. The workflow of data processing and selection is described in Figure1.

74 For the candidate modulators, we keep the splicing events where over 95% samples have
75 confidence interval (CI) less than 0.25 and only analyze predicted cassette exons that have at least

76 10 reads supporting exon inclusion or exclusion in at least one sample. We fill the missing PSI
77 value of a sample with the median PSI value of that splicing event. Finally, AS events were
78 selected based on candidate modulators whose PSI IQR (interquartile range) were larger than 0.1
79 As the input data require sufficient variability, we filtered out genes whose gene expression
80 coefficient variation (CV) was less than 50% and keep genes in which over 95% of samples had
81 expression values.

82 *Database and related software*

83 The implementation of GEM is available through SourceForge
84 (<https://sourceforge.net/projects/modulators>). Statistical analysis and data processing were
85 performed using R version 3.0.1 (www.r-project.org). DAVID(Dennis, Sherman et al. 2003) and
86 IPA (Ingenuity Pathway Analysis) were used to perform gene function and pathway analysis.
87 Protein-protein interactions were predicted by the STRING database (<http://string-db.org>).

88 **Results and Discussion**

89 *Global inferring modulators of all TFs*

90 We assume that all TFs have the potential ability to interact with their modulator candidates.
91 Seven hundred and sixty-five AS events were considered as putative modulators, and 173,598 TF-
92 target pairs composed of 74 TFs and 17,425 targets were used to infer modulated triplets. The
93 number of inferred splicing modulators varied across all TFs, and the percent of influenced targets
94 ranged from 0 to 33.5% for each TF (Figure 2).

95 Figure 3 summarizes the number of modulators of 26 TFs whose influence targets over 10%.
96 The number of inferred modulators ranges from 1 to 262. EST domain-containing protein Elk-1
97 (ELK1) was one of the 26 TFs that had the greatest number of predicted modulators. A total of

98 262 splicing events corresponding to 187 proteins were identified as ELK1 modulators because
99 their splicing outcome highly correlated with changes in ELK1's transcriptional activity.

100 *Gene function analysis of ELK1 modulators in LGG*

101 ELK1 is a member of the ETS transcription factor family, which is closely related to various
102 diseases, including Alzheimer's disease, down syndrome and breast cancer, in a dose-dependent
103 manner(Peng, Yang et al. 2017). It can significantly regulate the expression of c-Fos, which is a
104 key gene for cell proliferation and differentiation(Chambard, Lefloch et al. 2007). In this study,
105 we inferred five hundred and forty splicing events as ELK1 modulators.

106 Figure 4A summarizes the distribution of inferred modulators of ELK1. Two hundred and sixty-
107 two modulators influence over 10% of ELK1's targets, 49 modulators influence at least 20% of its
108 targets, and 5 modulators influence more than 30% of its targets, including
109 'chr2:39931221:39931334: +@chr2:39934189:39934326: +@chr2:39944150:39945104: +' (TMEM178A, Transmembrane protein 178A precursor), 'chr2:74685527:74685798: +@chr2:74686565:74686689: +@chr2:74686770:74686872: +' (WBP1, WW domain binding protein 1), 'chr2:36805740:36806008: -@chr2:36787928:36788008: -@chr2:36785581:36785656: -' (FEZ2, Fasciculation and elongation protein zeta-2), 'chr5:175788605:175788809: -@chr5:175786484:175786570: -@chr5:175782574:175782752: ' (KIAA1191, Putative monooxygenase p33MONOX), 'chr2:74685527:74685798: +@chr2:74686565:74686679: +@chr2:74686770:74686872: +' (WBP1).

117 As many inferred modulators may have similar protein functions or function related, we
118 performed pathway and function enrichment analysis to explore the functions of these modulated
119 genes. We filtered modulators whose influenced targets less than 10% and finally 262 splicing
120 events as modulators corresponding to 129 proteins are remained as ELK1 final modulators. After

121 removing duplicated gene symbols and unannotated genes, 126 proteins can be mapped to the
122 Ingenuity Knowledge Base that are subject to core analysis.

123 Results show that over 80% of these splicing proteins related to cancer are enriched and most
124 of the enriched canonical pathways are overlapped with certain genes. As Table 1 summarized,
125 these modulators are enriched in three types of diseases, including neurological disease,
126 organismal injury and abnormalities disease, and cancer, respectively. Molecular and cellular
127 function enrichment analysis showed that over 20% of the modulators were associated with
128 cellular movement (28/123), cellular assembly and organization (32/123), and cellular function
129 and maintenance (26/123); 11% and 8% of the modulators were highly enriched in cell
130 morphology (14/123), and cell-to-cell signaling and interaction process (10/123), respectively.
131 Top5 modulator-enriched pathways (Figure 4B) are highly ($p < 0.05$) associated with signaling
132 processes, including clathrin-mediated endocytosis signaling, CTLA4 signaling in the cytotoxic T
133 lymphocyte pathway, nNOS signaling in neurons and calcium signaling pathways.

134 *APP modulates ELK1 transcriptional activity*

135 Amyloid precursor protein (APP) was one of modulators of interest and its analysis is described
136 in detail here. An interaction between APP and ELK1 is mentioned in the STRING database.
137 Several AS isoforms of APP have been observed in humans. The isoforms range in length from
138 639 to 770 amino acids, and certain isoforms are preferentially expressed in neurons; changes in
139 the neuronal ratio of these isoforms have been associated with Alzheimer's disease (Matsui,
140 Ingelsson et al. 2007).

141 One splicing event of APP detected as a modulator was “chr21:27354657:27354790:-@
142 chr21:27372330:27372497:-@chr21:27394156:27394358:-”. Different inclusion ratios of the
143 alternatively spliced exon in APP protein influence 18.6% of the targets of ELK1, and the 7th exon,

144 which contains a vital domain named BPT/Kunitz inhibitor (BPTI) (residues 291-341), is the
145 alternatively spliced exon. The splice isoforms that contain the BPTI domain possess protease
146 inhibitor activity.

147 According to GEM algorithm, unmodulated ELK1 activity was classified into three categories
148 according to α_f : activation if positive, inhibition if negative, and inactive if zero. Similarly, by
149 comparing α and β coefficients, modulators are classified into three classes: enhance, attenuate or
150 invert the activity of the ELK1. Hence, there are six possible categories of action. The APP
151 modulation categories and their interpretations are listed in supplement file Table S1.

152 As Table 2 summarized, without APP modulation of APP, unmodulated ELK1 inhibits 172
153 targets, and activates 31 targets. However, when APP interacts with ELK1 as a modulator, the
154 original transcriptional activity of ELK1 becomes different: APP attenuates ELK1 inhibition roles
155 on 164 targets, inverts inhibition activity on 8 targets, and enhances activation on 14 targets.
156 Meanwhile, APP also inverts or attenuate ELK1 activity on 31 targets, including 1 targets activity
157 is inverted and 30 targets activity is attenuated.

158 We randomly selected four targets (ANKRD34A, DDX27, DVL3 and HEATR1) of ELK1 to
159 explore the different activities of ELK1 under the modulation of differential inclusion levels of
160 APP protein. Ideally, the inclusion level of the splicing modulator and expression of ELK1 should
161 have high variance and low correlation in the samples. We divided rank-ordered PSI values of
162 APP splicing modulators, extracting ELK1 and its target samples that were consistent with APP
163 splicing modulator samples in upper/lower tertile, and estimated the differences in correlation
164 between ELK1 and its target using Spearman's correlation.

165 Figure 5A shows the examples of APP-modulated ELK1 target genes and the corresponding
166 action modes. As shown in Figure 5A, when the exon inclusion level of APP was in lower tertile,

167 an increase in the gene expression level of ELK1 resulted in a significant increase in the gene
168 expression of its target ANKRD34A. Spearman's correlation of gene expression between ELK1
169 and ANKRD34A was 0.71 ($p < 2.2e-16$), which means that in this condition, ELK1 plays an
170 enhancement role on its target. However, when the PSI value of the APP modulator is in upper
171 tertile, the correlation decreased into 0.30 ($p = 0.0085$). For the other two targets, DDX27 and
172 DVL3, the correlations changed from -0.47 and -0.53, respectively, to non-significant ($p > 0.1$).
173 For these three cases, the APP modulator attenuates the activation of ELK1. The opposite
174 modulation occurs on target HEATR1. When the exon is spliced out of the protein, ELK1 negative
175 regulates the expression of HEATR1 with a correlation as -0.38 ($p = 0.0005$); however, when the
176 exon is excluded from the mature mRNA, the APP modulator inverts the activation of ELK1 on
177 its target with a correlation of 0.70 ($p < 2.2-16$).

178 We evaluated the exon's impact on APP protein using ExonImpact (Li, Feng et al. 2017). The
179 results showed that the alternatively spliced exons of APP protein that we detected have a high
180 probability (0.57 and 0.48) of being associated with disease. This result indicates that changes in
181 the inclusion or exclusion level of spliced exons can lead to significant changes with respect to
182 APP protein function.

183 Figure 5B visualized the global effect of changing the inclusion ratios of alternately spliced
184 exons in APP and influences on the relationship between ELK1 and its target. The two groups of
185 samples are selected based on the 7th exon inclusion ratio of APP. The high and low inclusion
186 groups contain samples with the top and bottom 30% of PSI values. The correlation patterns
187 between ELK1 and its targets in the two groups are different, clearly showing that different splicing
188 levels of APP can modulate the transcriptional activity of ELK1.

189 *STK16 modulates ELK1 transcriptional activity*

190 The AS event
191 “chr2:220111379:220111598:+@chr2:220111835:220111968:+@chr2:220112137:220112257:+”
192 for protein serine/threonine kinase 16 (STK16) is another interesting modulator that we identified.
193 Inferred STK16 modulated triplets and their modulation categories are listed in Table S2. STK16
194 is a membrane-associated protein kinase that phosphorylates on serine and threonine residues. An
195 interaction between STK16 and ELK1 is inferred from the biochemical effect of one protein upon
196 another in the BioGrid database. The alternatively spliced exon that acts as a modulator of SKT16
197 is the 4th exon and it locates in a region that encodes for a kinase domain named Pkinase that is
198 associated with the protein’s proton acceptor.

199 Figure 6A shows the modulating effect of STK16 on ELK1, and TMEM60 is one of targets we
200 randomly detected. The samples in the two groups are selected using the same method mentioned
201 above. A negative correlation (-0.37 , $p = 0.0004$) is only shown when the exon is included in the
202 final product. The exon’s impact in protein function analysis (Li, Feng et al. 2017) shows that this
203 alternatively spliced exon has a high disease probability of 0.67, which indicates that changes in
204 the exon inclusion or exclusion ratio might cause a gain or loss in protein function. We know that
205 the specific alternatively spliced exon of STK16 encodes a kinase domain, so it is not surprising
206 that the loss of this exon will cause a change in protein function and may ultimately influence
207 numerous normal gene functions.

208 Figure 6B shows the global modulating effect of STK16 on ELK1. The low and high inclusion
209 groups contain samples with the top and bottom 30% of PSI values, which indicate exon exclusion
210 and inclusion in the final protein. A positive correlation between ELK1 and its targets is clearly
211 shown when the exon is excluded, whereas this correlation becomes negative when the exon is
212 included. This result suggests that the 4th cassette exon in STK16 is important to final protein

213 function and that changes in the splicing level of STK16 are associated with the differential
214 transcriptional activity of ELK1.

215 **Conclusions**

216 We globally dissected the role of AS in regulating the transcriptional activity of TFs in LGG
217 using TCGA-LGG data. ELK1 was one of TFs with the greatest number of inferred modulators,
218 e.g., APP and STK16. The results show that changes in the AS of APP and STK16 proteins are
219 associated with changes in the transcriptional activity of ELK1 and that the structural features of
220 the two proteins are strongly associated with the pathological impact of exon inclusion. The
221 presented results provide important insights on the modulating role of AS on transcription
222 regulation in LGG.

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225 improved the early version of this manuscript.

226

227 **Competing interests**

228 The authors declare that they have no competing interests.

229

230

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- 263

264 **Additional files**

265 Additional file 1: Table S1. Inferred APP modulated triplets and their modulation categories.

266 Additional file 2: Table S2. Inferred STK16 modulated triplets and their modulation categories

267

Table 1 (on next page)

ELK1 modulators protein function and disease enrichment ($p < 0.001$)

Each number in the table indicates the account of ELK1 modulators enriched in specific function or disease. The statistical threshold is $p < 0.001$.

Molecular and Cellular Functions

Cellular assembly and organization	32	Cellular movement	28
Cellular function and maintenance	26	Cell morphology	14
Cell-to-cell signaling and interaction	10		

Diseases and Disorders

Neurological disease	37	Organismal injury and abnormalities disease	113
Cancer	110		

Table 2 (on next page)

Interpretation of the categories of APP modulation, and the inequality constraints that the category should satisfy

Each number in the table indicates the number of triplets in each classification. '+' and '-' signs in the columns indicate significantly positive and negative values, respectively.

Modulation classification	Explanation	# triplets	γ	α_f	β_f	β_m	$\alpha_f + \beta_m$
Attenuates inhibition	F, alone, inhibits T – M attenuates F activity	164	+	-			
Enhances inhibition	Modulated F inhibits T	0	-		-	-	-
Inverts inhibition	F, alone, inhibits T - M inverts F activity	8	+	-	+	+	+
Inverts activation	F, alone, activates T – M inverts F activity	1	-	+	-	-	-
Enhances activation	Modulated F activates T	14	+		+	+	+
Attenuates activation	F, alone, activates T – M attenuates F activity	30	-	+			

‘+’ and ‘-’ signs in the columns indicate significantly positive and negative values, respectively.

Figure 1(on next page)

Workflow for data processing and selection.

The whole workflow including three parts: obtain the transcriptional profile, expression and splicing calling and construct the modulated triplets.

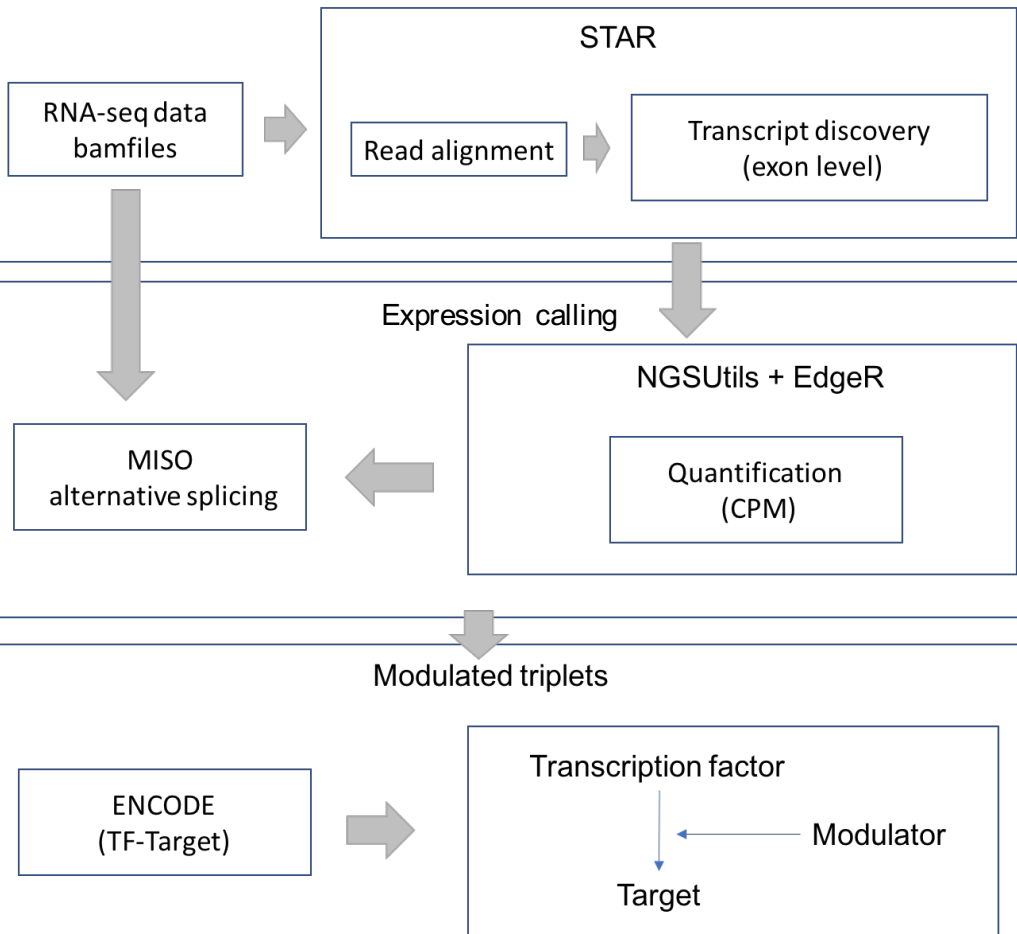
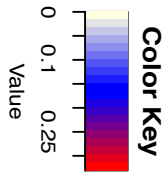


Figure 2(on next page)

The effect of transcription factors activity regulated by splice modulator proteins.

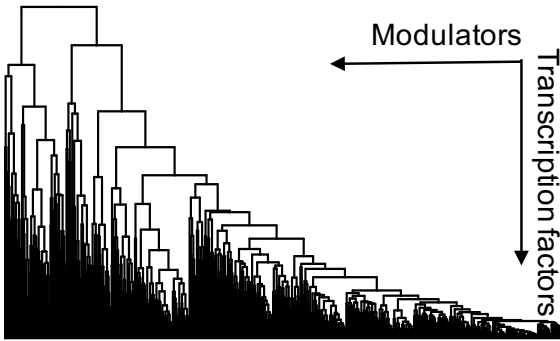
Each row represents a candidate modulator and each column indicates a transcription factor.

The color much darker means a much higher percent targets of TF is influenced.



Modulators

Transcription factors



Panel 1

K1
DLHE40
DLHE1
EST
ABPA
EAD1
ES1
ES2
CF12
NF217
STAT5A
FAP2C
CF4
AX5
F1
NF143
YC
MAZ
CTCF
NF263
K4
FX5
TF3C2
BTF33
BTF33
NFKB1
R4A1
MAX
NFYA
MAZ
CF3
RF1
FOX2
BFD1
RE1
SF2
AP2A
EAD4
GAT1
YY1
JUN
FOSL1
NFYB281
NR3C1
NR2C2
CEBPB
CEBPA
EGR1
MAFF
NFYB
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PSF1
PRDM1
TAF
TCF7L2
GF11
GATA3
RUNX3

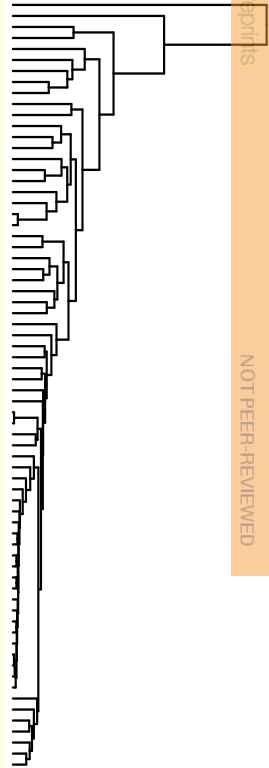


Figure 3(on next page)

Summarized counts of inferred modulators of TFs.

The x-axis represents the transcription factor list, and the y-axis represents the counts of inferred modulators. The number on each TF indicates the number of modulators of each TF that influence more than 10% of its targets.

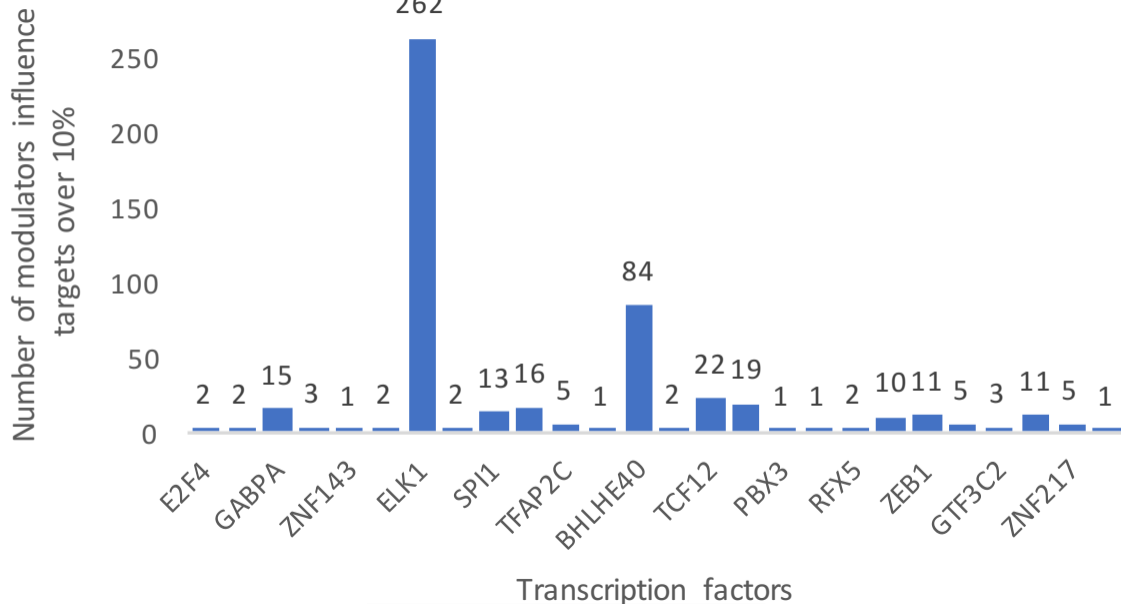


Figure 4(on next page)

Statistical analysis of the modulators of ELK1.

(A) Distribution of the number of ELK1 modulators. The x-axis represents the percentage of targets influenced by modulator proteins. The y-axis indicates the percent of modulators of ELK1. The number noted on each column indicates the percent of modulators in each classification. (B) IPA of ELK1 modulators that influenced over 10% of its targets. The x-axis is the $-\log_{10}$ transformed p of each enriched pathway (y-axis).

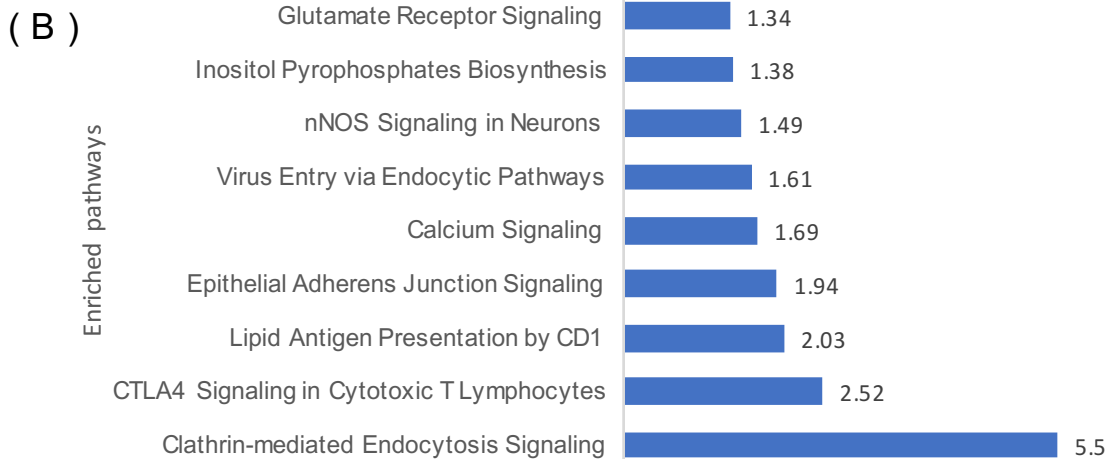
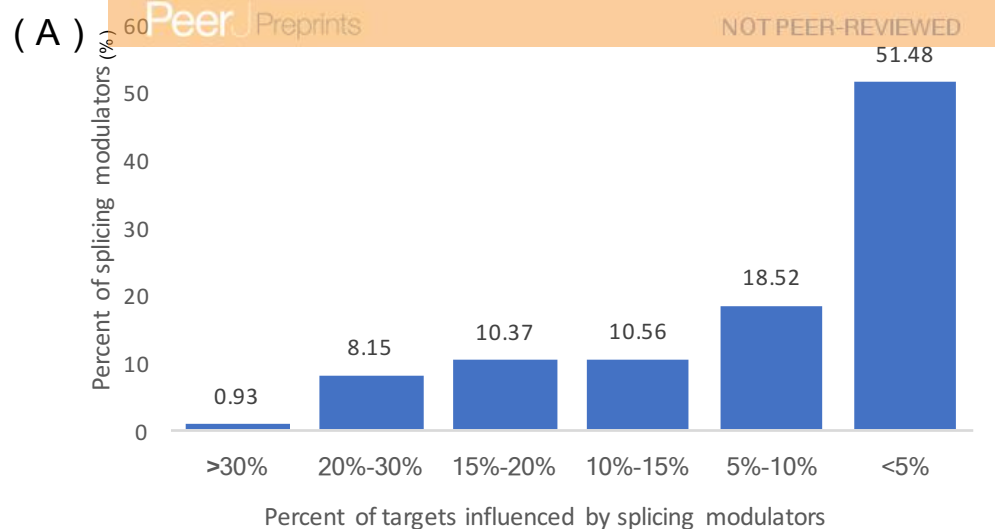
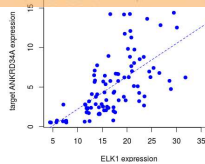


Figure 5(on next page)

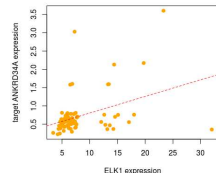
APP is a modulator that influences the activity of ELK1 in LGG.

(A) Examples of different correlations between ELK1 and its targets under the modulation of APP with differential splicing levels. (B) Visualization of how APP regulates the stability of ELK1 protein. Gene expression profiles are displayed with genes in rows and samples in columns. Expression values of each gene are rank transformed, median centered and rescaled between $[-0.5, 0.5]$. Samples were partitioned based on the alternatively spliced exon inclusion level of APP and sorted by the expression levels of ELK1 within each partition.

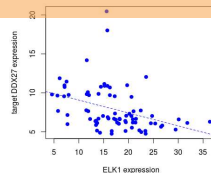
(A)

Peer Preprints
ANKRD3A

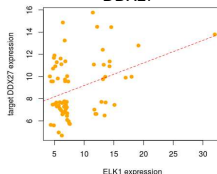
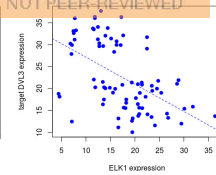
ANKRD3A



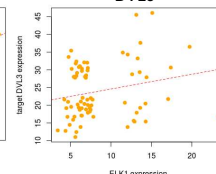
DDX27



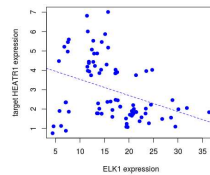
DDX27

NOT PEER-REVIEWED
DVL3

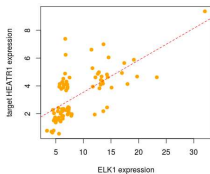
DVL3



HEATR1



HEATR1



(B)

Splicing PSI low

chr21:27394156:27394358:-
@chr21:27372330:27372497:-
@chr21:27354657:27354790:-

Splicing PSI high

ELK1

ELK1

ELK1 targets

ELK1 targets

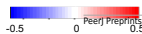
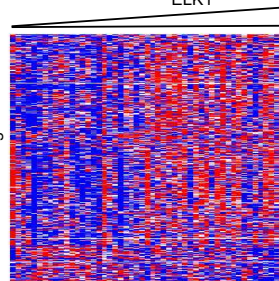
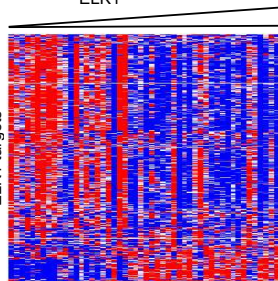
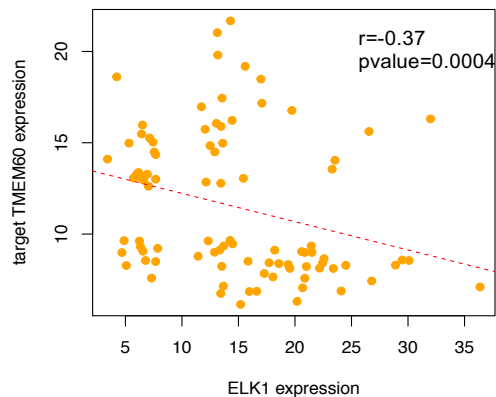
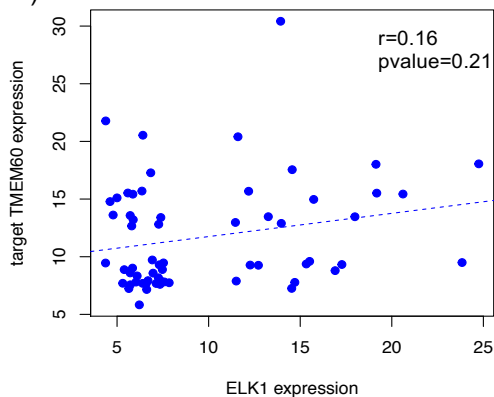


Figure 6(on next page)

STK16 is a modulator that affects the transcriptional activity of ELK1 in LGG.

(A) Examples of differential regulation activities of ELK1 on its target under the modulation of STK16 with differential splicing levels. The spliced exon is excluded in the final production of STK16 in the first scenario, and the exon is included in the final production of STK16 in the second scenario. (B) STK16 regulates the stability of ELK1. See Figure 3B for interpretation of this graph.

(A)



(B)

Splicing PSI low

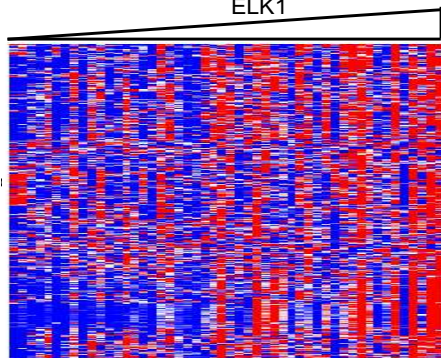
chr2:220111379:220111598:+
 @chr2:220111835:220111968:+
 @chr2:220112137:220112257:+

Splicing PSI high

ELK1

ELK1

ELK1 targets



ELK1 targets

