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RAPID COMMUNICATION

Single-cell analyses identify anaphase-promoting complex subunit 11 as a switch controlling neuronal differentiation of glioblastoma cells

Glioblastoma (GBM) is the most common and lethal malignancy in the central nervous system.[1] One of the major difficulties in treatment is that the initial clinical diagnosis of GBM is already WHO grade IV, without recognizable lower-grade precursor lesions. Copy number variations (CNVs) were found to appear in malignant cells several years before the initial diagnosis of GBM.[2] Less differentiation and more aggressive phenotypes were observed in GBM cells with a higher degree of CNVs.[3] Additionally, CNVs provide more accurate stratification of clinical outcomes than does the WHO grade system.[4] Therefore, we reasoned that differentially expressed genes (DEGs) among GBM cells with different CNV statuses would be significant for the aggressiveness of GBM. Here we leveraged the single-cell RNA-sequencing (scRNA-seq) to construct the CNV profile of GBM at single-cell resolution, divided GBM cells into different clusters according to their CNV statuses, and investigated the molecular functions of DEGs among GBM clusters. Through a series of experiments, we identified anaphase-promoting complex subunit 11 (ANAPC11) as a switch controlling the neuronal differentiation of GBM cells, providing a novel alternative for the development of differentiation-inducing therapy to overcome GBM.

To get a single cell-resolution landscape of GBM's CNV, we analyzed the CNV status of GBM through scRNA-seq. By non-supervised clustering, GBM cells were divided into different CNV groups with distinct CNV patterns (Fig. S1A). We then analyzed the DEGs among different CNV groups. To scale up cell numbers and reduce potential biases, five independent scRNA-seq datasets were analyzed. The interested DEGs list was narrowed down to the mutually shared genes (Fig. S1B and Table S1) that were found to be mainly involved in the cell cycle, protein synthesis, and energy metabolism (Table S2). Because CNV is directly associated with the replication and distribution of chromosomes, we focused on genes in the cell cycle processes, which led us to *EGFR*, *PTN*, *CLU*, *EEF2*, *MT3*, *NPM1*, *PSMA7*, *UBB*, and *ANAPC11* (Fig. S1C and Table S2). Interestingly, the former eight genes were reported to be associated with glioma aggressiveness, while the function of *ANAPC11* in glioma remains unknown. Therefore, we investigated *ANAPC11* in this study.

Bulk RNA-seq, scRNA-seq, and Western blot analyses revealed that *ANAPC11* was up-regulated in GBM compared with normal cells (Fig. 1A, B; Fig. S2A, B, S3J). Higher expression of *ANAPC11* was correlated with higher grades of glioma (Fig. S2C). Immunohistochemistry and survival analysis showed that higher expression of ANAPC11 protein was positively associated with a worse outcome in GBM patients (Fig. 1C; Fig. S2D).

After confirmation of the up-regulated expression pattern of ANAPC11 in GBM, we sought to analyze the impact of ANAPC11 knockdown on GBM cells. In the National Center for Biotechnology Information Reference Sequences database, 12 transcript variants of *ANAPC11* were recorded, encoding for 3 isoforms of ANAPC11 protein (Fig. S3A). However, little is known about the expression profile of *ANAPC11* transcript variants in GBM. Based on the encoded proteins, transcript variants of *ANAPC11* were divided into three groups, transcript variant 1 (group 1) for isoform 1, transcript variants 2 to 11 (group 2) for isoform 2, and transcript variant 14 (group 3) for isoform 4. We designed primers that can specifically detect the three groups of *ANAPC11* transcript variants respectively (Table S3, 4). Quantitative polymerase change reaction (qPCR) results demonstrated that transcript variants 2 to 11 dominated in glioma tissues (Fig. S3B), primary GBM cells (Fig. 1D), and classic GBM cell lines (Fig. S3C). Furthermore,

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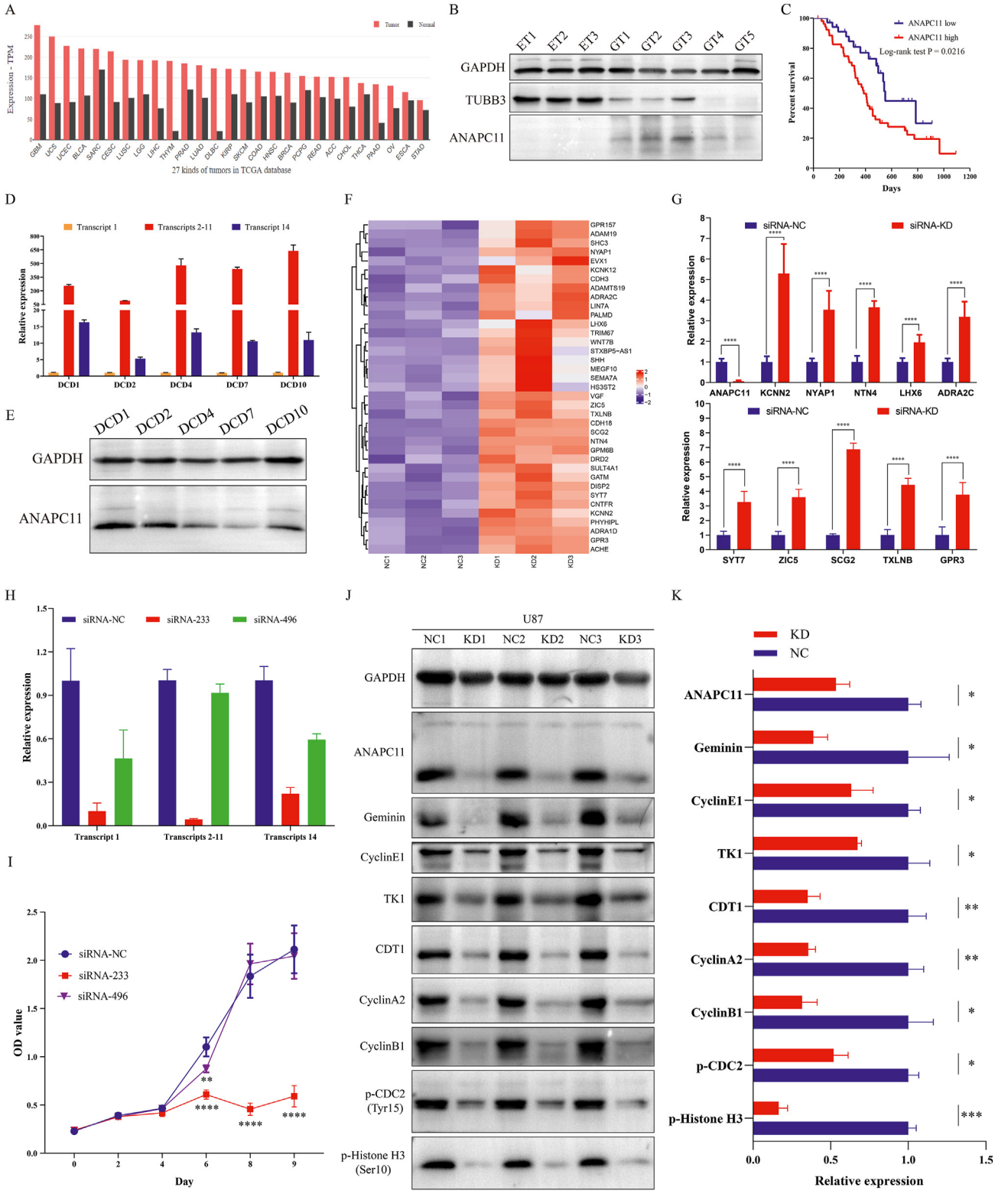

Figure 1 Knockdown of ANAPC11 in GBM cells promotes neuronal differentiation and reduces proliferation. **(A)** Compared to the corresponding normal samples, *ANAPC11* mRNA was up-regulated in 27 kinds of tumors in the Cancer Genome Atlas database. **(B)** ANAPC11 protein was up-regulated in GBM tissues (GT) compared to epilepsy brain tissues (ET). **(C)** Kaplan-Meier survival analysis of GBM patients in ANAPC11 high expression group (n = 53) and low expression group (n = 35) (Log-rank test, P = 0.0216). **(D)** Expression of transcript variants 2 to 11 was higher than that of transcript variants 1 and 14 in five primary GBM cell lines. **(E)** ANAPC11 protein isoforms expression profile in five primary GBM cell lines. The ANAPC11 antibody from Cell Signaling Technology (#14090) was used in this figure. **(F)** The heatmap showing the differentially expressed neuron-associated genes between siRNA-NC

isoform 2 was found to be the dominant isoform of ANAPC11 in GBM cells (Fig. 1E; Fig. S3D—I).

To distinguish the effects of different *ANAPC11* transcript variants, two kinds of small interfering RNAs (siRNAs) were designed. The first kind of siRNA (siRNA-233) targeted the common sequence of all *ANAPC11* transcript variants, while the second kind (siRNA-454 and siRNA-496) spared group 2 (transcript variants 2 to 11) (Fig. S4A). Interestingly, GBM cells showed longer processes only in the siRNA-233 group, in which all the *ANAPC11* transcript variants were decreased (Fig. S4A—C). We used siRNA-233 to repeat the *ANAPC11* knockdown assay and confirmed that the morphology changes of GBM cells could last to at least the 5th day after siRNA transfection (Fig. S4D—H). These results indicated that the knockdown of *ANAPC11* led to longer processes of GBM cells and the decrease of transcript variants 2 to 11 was necessary for GBM cell morphology changes.

To uncover the underlying biological meanings of the morphology changes induced by *ANAPC11* knockdown, we analyzed the genes negatively correlated with *ANAPC11* expression in GBM RNA-seq datasets from the Cancer Genome Atlas and Chinese Glioma Genome Atlas. In the results of gene function enrichment analyses, we were intrigued by the consistent enrichment in items associated with the axon and synapse (Fig. S5A, B). We inferred that the elongated processes of GBM cells might result from the elevated expression of neuron-associated genes after *ANAPC11* knockdown. To examine this hypothesis, we performed RNA-seq analyses of the control group and *ANAPC11*-knockdown group in U87MG and a primary GBM cell line DCD10. Gene ontology enrichment analysis revealed that many up-regulated genes in *ANAPC11*-knockdown groups were significantly enriched in the synapse, axon, and neuronal differentiation (Table S5). We further reviewed the functions of all up-regulated genes in *ANAPC11*-knockdown groups based on published studies, which also led us to the discovery of documented neuron-associated genes involved in axonogenesis and synapse formation (Fig. 1F; Fig. S6A—C). The elevated expression of some neuron-associated genes in *ANAPC11*-knockdown groups was validated by qPCR (Fig. 1G; Fig. S6D). Furthermore, immunofluorescence assays demonstrated the higher expression of a neuronal marker Tubulin Beta 3 Class III in *ANAPC11*-knockdown groups compared with control groups (Fig. S6E). Collectively, these results revealed that the knockdown of *ANAPC11* in GBM cells induced neuronal differentiation.

Next, we examined whether the greater extent of differentiation was at the expense of proliferation. CCK8 colorimeter analysis showed a lower proliferation speed of GBM cells in the *ANAPC11*-knockdown group compared to the control group (Fig. 1H, I). Interestingly, the reduction of GBM cell proliferation was mediated by siRNA-233 rather than by siRNA-496 that spared *ANAPC11* transcript variants 2 to 11 (Fig. 1H, I). Cell counting and EdU assay further demonstrated that siRNA-233 inhibited GBM cell proliferation (Fig. S7A—E).

ANAPC11 is the catalytic core of the anaphase-promoting complex, contributing to the transition from the G1 phase to the S phase and the separation of sister chromatids during cell cycles.[5] We hypothesized that the neuronal differentiation and proliferation inhibition in the *ANAPC11*-knockdown group were associated with disrupted cell cycle homeostasis. scRNA-seq analysis showed that *ANAPC11* was enriched in G1-phase in oligodendrocytes while in S and G2/M phases in GBM cells (Fig. S8A). GBM cells with lower expression of *ANAPC11* showed a higher percentage in the G1 phase (Fig. S8B). To analyze the cell cycle phase changes in the *ANAPC11*-knockdown group, several proteins vital for different phases were examined by Western blot. Interestingly, geminin, chromatin licensing and DNA replication factor 1 (CDT1), cyclin E1, TK1, cyclin A2, cyclin B1, p-Histone H3 (Ser10), and phosphorated cell division cycle 2 (p-CDC2) (Tyr15) were decreased in GBM cells of the *ANAPC11*-knockdown group compared to the control group (Fig. 1J, K), which indicated the exit from cell cycle after *ANAPC11*-knockdown.

In summary, this study demonstrated that *ANAPC11* was heterogeneously expressed in GBM cells with different CNV statuses. Higher expression of ANAPC11 was correlated with worse outcomes in GBM patients. Transcript variants 2 to 11 encoding for isoform 2 dominated the pool of *ANAPC11* transcript variants in GBM. Knockdown of ANAPC11 in GBM cells promoted exit from the cell cycle, inhibited proliferation, and induced neuronal differentiation, which can be leveraged to develop a differentiation-inducing treatment for GBM.

Author contributions

Supervision: YWL, STQ, and GLH. Conception and design: RWY, YWL, and ZYW. Acquisition, analysis, and interpretation of data: RWY, ZYW, STQ, BWN, JLG, KSL, HMS, SDX, YXZ, XRW, CMH, GLH, and YWL. Drafting and revising the manuscript: RWY, ZYW, and YWL, with inputs from other authors.

Conflict of interests

The authors declare no conflict of interests.

and siRNA-KD groups of GBM cells DCD10. **(G)** qPCR validation of ten neuron-associated genes up-regulated in GBM cells DCD10 of the siRNA-KD group compared to the NC group. Unpaired *t*-test, ****$P < 0.0001$. **(H)** qPCR examination of the *ANAPC11* knockdown effects mediated by siRNA-233 and siRNA-496. **(I)** Results of CCK8 assay showing inhibited proliferation of GBM cells in the siRNA-233 group. Two-way ANOVA test: **, $0.001 \leq P < 0.01$; ****, $P < 0.0001$. **(J)** Western blot detection of cell cycle-associated proteins in GBM cells of siRNA-NC and siRNA-KD groups, with three biological repeats in each group. ANAPC11 knockdown effect was achieved by siRNA-233. **(K)** Statistical analysis of protein band signal intensity in Figure 1J. Unpaired *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.12.017.

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