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RESEARCH PAPER

The crucial role of DNA-dependent protein kinase and myelin transcription factor 1-like protein in the miR-141 tumor suppressor network

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ABSTRACT

Glioblastoma is the most aggressive brain tumor. Although miR-141 has been demonstrated to primarily function as a tumor suppressor in numerous malignancies, including glioblastoma, the mechanisms involved remain poorly understood. Here, it is shown that miR-141 is downregulated in glioblastoma cell lines and tissues and may exert its biological function via directly targeting *myelin transcription factor 1-like* (*MYT1L*). Using two glioblastoma cell lines that differ from each other by the functionality of DNA-dependent protein kinase (DNAPK), a functional involvement of DNAPK in the miR-141 tumor suppression network was observed. In M059K cells with a normal function of DNAPK, the enforced expression of miR-141 attenuated MYT1L expression and suppressed cell proliferation. Conversely, the inhibition of miR-141 expression promoted cell proliferation; however, in M059J cells with a loss-of-function DNAPK, miR-141 constitutively inhibited cell proliferation upon ectopic overexpression or inhibition. An overexpression of miR-141 suppressed M059J cell migration, while it had no effect on M059K. Furthermore, the ectopic expression of miR-141 induced an S-phase arrest in both cell lines, whereas the inhibition of miR-141 caused a G1 arrest in M059J and accelerated the S phase in M059K. An overexpression and suppression of miR-141 resulted in an aberrant expression of cell-cycle proteins, including p21. Moreover, MYT1L may be a transcription factor of p21 in p53-mutant cells, whereas DNAPK may function as a repressor of MYT1L. The findings revealed the crucial role of DNAPK in miR-141-mediated suppression of gliomagenesis and demonstrated that it may be a target molecule in miR-141-associated therapeutic interventions for glioblastoma.

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Introduction

The most common malignant primary brain tumors are gliomas, which account for 81% of malignant tumors [1]. Glioblastoma is the most common glioma and the most aggressive brain malignancy [2,3]. It is estimated that the age-standardized incidence rate for glioblastoma is 3.0 per 100,000 of the population in North America [3]. The prognosis of glioblastoma is poor with a two-year survival rate of 8.7% [3]. In general, glioblastomas can be classified into primary and secondary types, although it is difficult to distinguish them histologically. Primary glioblastoma is the most common type, accounting for 90% of cases, which manifests de novo and progresses rapidly in elderly patients without recognizable precursor lesions [2]. Secondary glioblastoma is less common, which develops primarily in younger patients from low-grade diffuse or anaplastic astrocytoma. Although the precise mechanism underlying glioblastomagenesis is poorly understood, epigenetic factors may be key players in the development of this disease because a deficiency in DNA repair has been demonstrated in 29–66% of glioblastomas by the epigenetic silencing of the *methylguanine-DNA methyltransferase* (*MGMT*) gene via DNA methylation [4,5].

Thousands of DNA-base lesions occur per cell per day due to various genotoxic stress factors. A deficiency in DNA repair could lead to the accumulation of deleterious mutations and the constitutive activation of DNA repair-associated proteins, resulting in tumorigenesis. DNA-dependent protein kinase (DNAPK, also known as DNA-PKcs), a key player in the non-homologous end joining (NHEJ) pathway, is primarily involved in repair of DNA

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double-strand breaks and has been linked to cancer. A large body of evidence has demonstrated that DNAPK was overexpressed in numerous human malignancies, such as nasopharyngeal, esophageal, gastric, colorectal, breast, cervical and non-small cell lung cancers, and the elevated expression was associated with higher tumor grade and poor survival [6]; however, the expression of DNAPK was also revealed to be downregulated in ovarian cancer [6], and its downregulation was correlated with tumor progression and metastasis.

Since a key role of microRNA lin-4 and let-7 in controlling developmental timing was first discovered in *Caenorhabditis elegans* in 2001 [7–9], this class of non-coding RNAs have been extensively studied. microRNAs (miRNAs/miRs) are abundant, small noncoding RNA molecules that negatively regulate about 60% of human protein-coding genes [10], and they are involved in all biological and pathological processes, including cancer. As demonstrated, miRNAs may act as an oncogene, a tumor suppressor or both. A large body of evidence has indicated that the activation of oncogenic miRNAs and/or loss-of-function of tumor suppressor miRNAs contribute to tumorigenesis. miR-141 is a member of the miR-200 family, which also includes miR-200a, miR-200b, miR-200c and miR-429. Growing evidence has demonstrated a dual role of miR-141 in the development of human malignancies. miR-141 has been reported to suppress cell proliferation and invasion and induce apoptosis and cell cycle arrest in numerous human cancers, such as breast cancer [11], gastric cancer [12,13], pancreatic cancer [14], prostate cancer [15], hepatocellular carcinoma [16], renal cell carcinoma [17], thyroid cancer [18], glioma [19,20] and head and neck squamous cell carcinoma [21]; however, recent studies have also indicated an oncogenic role of miR-141 in tumorigenesis. An overexpression of miR-141 significantly enhanced breast cancer cell migration/invasion and brain metastatic colonization [22,23]. miR-141 also promoted cell proliferation in nasopharyngeal carcinoma [24], ovarian cancer [25] and colorectal cancer [26].

Although miR-141 has been demonstrated to contribute to tumorigenesis, the direct targets through which the tumorigenesis is mediated remain largely unknown. The findings of this study showed that *MYT1L* is a novel direct target of miR-141. It was observed that MYT1L is overexpressed in both glioblastoma cell lines and glioma tissues, and that MYT1L expression is inversely correlated with miR-141 expression. Using two glioblastoma cell lines as a model system, a functional involvement of DNAPK in the miR-141 tumor suppression network was identified. In M059K cells with a normal function of DNAPK, an overexpression of miR-141 attenuates MYT1L expression and suppresses cell proliferation. Conversely, an inhibition of miR-141 promotes cell proliferation; however, in M059J cells with a loss-of-function of DNAPK, overexpression or inhibition of miR-141 constitutively suppresses cell proliferation. Furthermore, the overexpression or suppression of miR-141 leads to an aberrant expression of cell-cycle proteins, including p21, resulting in an alteration in the cell cycle. Moreover, MYT1L may function as a transcription factor of p21 in cells with a mutant p53, while DNAPK may act as a repressor of MYT1L. The results indicated a crucial role of DNAPK in the miR-141-mediated suppression of gliomagenesis.

Results

MYT1L is a direct target of miR-141

We were interested in studying the dualistic role of miR-141 in glioblastoma development. We focused on identification of potential targets of miR-141, paying specific attention to proteins that are essential for normal central nervous system development and cellular differentiation. A bioinformatics analysis using MiRGator v3.0 reported MYT1L as a predicted target for miR-141-3p in 4 miRNA target databases: targetScan, miRNAorg, PITA and miRDB (miRGator 3.0: R-squared of −0.5426). MYT1L is critical for nervous system development [27], but is rather under-investigated in its potential role in tumorigenesis.

Human brain tumor cell lines were then used as a model system to examine the relationship and functional interactions between miR-141 and MYT1L. The quantitative real-time RT-PCR (qRT-PCR) showed that miR-141 was downregulated in three of four brain tumor cell lines examined (Figure 1(a)). As expected, the Western blot analysis showed that MYT1L was upregulated in these cell lines (Figure 1(b)), which was negatively

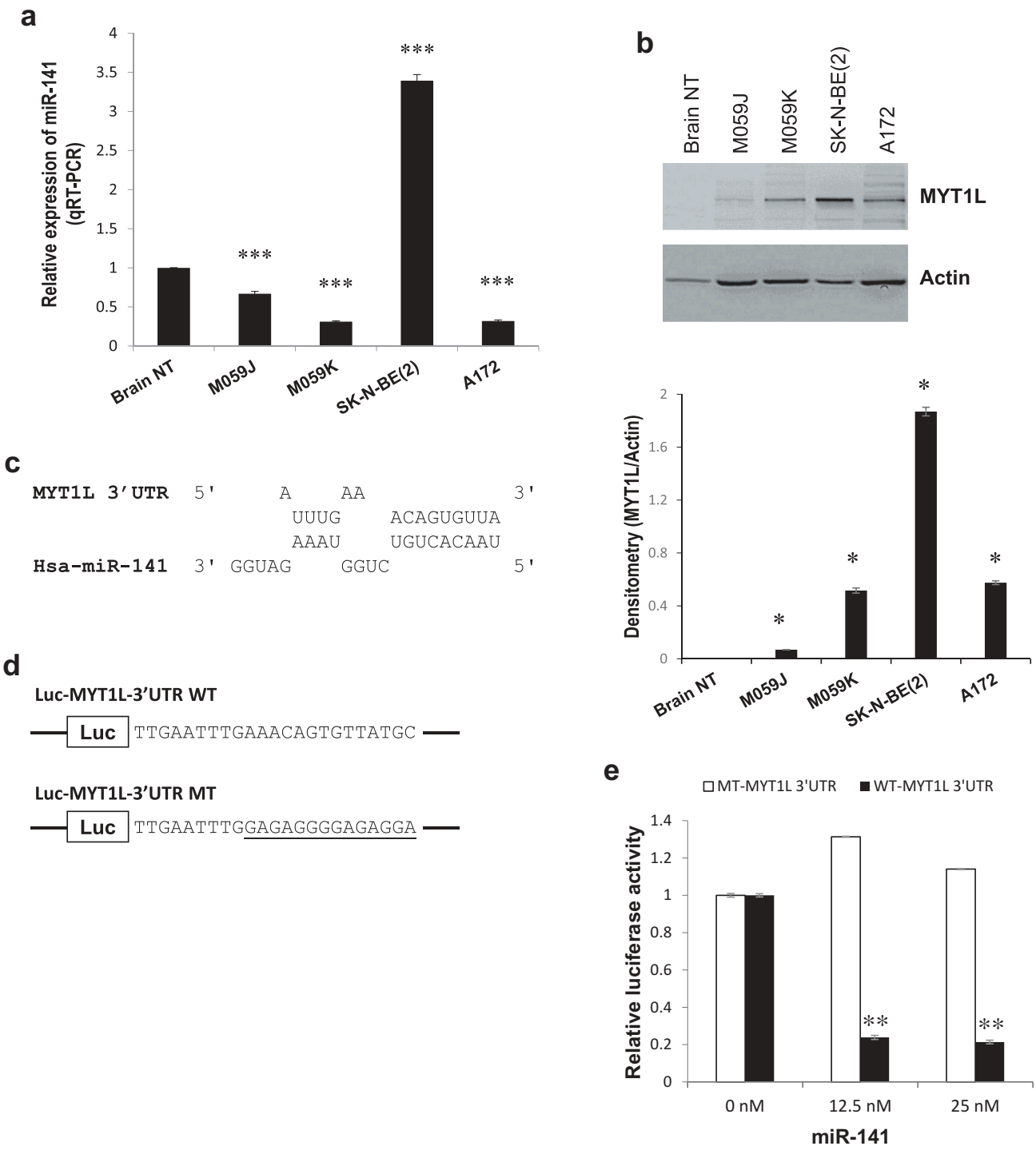


Figure 1. miR-141 directly targets MYT1L. (a) Total RNA isolated from the indicated cell lines and normal brain tissues (NT) was subjected to a qRT-PCR analysis using an hsa-miR-141 primer assay. (b) Whole cellular lysates prepared from the indicated cell lines and brain NT were subjected to a Western blot analysis using an antibody to MYT1L; the densitometry was performed using ImageJ. (c) Representative binding motif between miR-141 and MYT1L mRNA 3′UTR was predicted via four different methods. (d) Luciferase reporter bearing either wild-type or mutant MYT1L 3′UTR. (e) HEK293 cells were transiently transfected with either a wild-type or mutant MYT1L 3′UTR luciferase reporter in combination with the indicated concentration of miR-141; twenty-four hours after transfection, the luciferase activity was measured using a dual luciferase assay system. *indicates $p < 0.05$; **indicates $p < 0.01$; ***indicates $p < 0.0005$.

correlated with miR-141 expression. In contrast, in one specific line, SK-N-BE(2), a positive correlation (miR-141 and MYT1L were both upregulated) was found (Figure 1(a,b)). Taken together, it was hypothesized that miR-141 may directly target *MYT1L*. To test the hypothesis, a luciferase reporter

bearing either a wild-type or a mutant "seed sequence" of *MYT1L* 3′UTR was then generated according to the predicted binding motif (Figure 1(c,d)). The luciferase assay indicated that the luciferase activity of the wild-type construct was significantly attenuated by miR-141 (Figure 1(e), $p < 0.01$), and this reduction was completely abolished by the mutant construct, supporting the hypothesis.

miR-141 is downregulated, while MYT1L is upregulated in glioma tissues

To further establish the inverse correlation, the expression of miR-141 and MYT1L in a large cohort of glioma tissue samples was then determined using fluorescent in-situ hybridization (FISH) and immunohistochemistry (IHC). The FISH analysis showed that miR-141 was downregulated in 58.6% of benign (n = 140) and 56.7% of malignant (n = 30) glioma tissues examined (Figure 2(a–c)); however, IHC staining showed that MYT1L was upregulated in 31.4% of benign (n = 140) and 46.9% of malignant (n = 32) glioma tissue samples measured (Figure 2(a–c)). The upregulated MYT1L was positively correlated with the downregulated miR-141 (correlation r = 0.90644).

Ectopic expression of miR-141 suppressed glioblastoma cell proliferation and induced S-phase arrest

Next, the functional effect of ectopic miR-141 expression on cell biology was examined using two well-defined glioblastoma cell lines as a model system: M059J with a loss-of-function of DNAPK and M059K with normal DNAPK activity [28]. The qRT-PCR showed that miR-141 expression was extremely elevated, especially at 48 hours after transfection (Figure 3(a)), leading to a reduction in MYT1l expression (Figure 3(g)). Consequently, cell proliferation was significantly inhibited in both cell lines (Figure 3(b)). The enforced expression of miR-141 induced an S-phase arrest (Figure 3(c)), and suppressed apoptosis in both cell lines (Figure 3(d)). Interestingly, although the ectopic miR-141 suppressed the migration of M059J cells, it had no effect on M059K cells (Figure 3(e,f)). The Western blot analysis indicated that MYT1L and CDK2 were downregulated, whereas cyclin E1 was upregulated in both cell lines (Figure 3(g)). In addition, the overexpression of miR-141 attenuated the p21 expression in M059J cells, while it enhanced its expression in M059K cells (Figure 3(g)). We then looked at the contributing role of a demonstrated target of miR-141, zinc finger E box-binding homeobox 1 (ZEB1) [29,30] in miR-141-mediated suppression of M059K migration. Although the ectopic expression of miR-141 inhibited the ZEB1 expression in M059K cells, whereas enhancing its expression in M059J cells (Figure 3(g)). Taken together, these results suggest that miR-141 may act as a tumor suppressor, inhibiting cell proliferation and/or migration and inducing S-phase arrest in glioblastoma cells.

Phenotypic effect of miR-141 inhibition on glioma cells

To further validate the effect of miR-141 on cell biology, miR-141 was functionally inhibited in M059J and M059K cells using the miR-141 inhibitor. As expected, cell proliferation was significantly enhanced in M059K cells with a normal functional DNAPK (Figure 4(a), right panel), supporting the hypothesis that miR-141 functions as a tumor suppressor in glioblastoma. Surprisingly, the inhibition of mir-141 failed to restore the growth of DNAPK loss-of-function M059J cells, continuously suppressing cell proliferation (Figure 4(a), left panel). The inhibition of miR-141 induced apoptosis (Figure 4(b), right panel) and accelerated the S phase in M059K cells (Figure 4(c), lower panel), while it attenuated apoptosis (Figure 4(b), left panel) and induced a G1 arrest in M059J cells (Figure 4(c), upper panel). The Western blot analysis indicated that MYT1L, CDK2 and cyclin E1 were upregulated in both cell lines in response to the miR-141 inhibitor (Figure 4(d)). The inhibition of miR-141 also increased cyclin A2 expression in M059K cells, which was undetectable in M059J cells. Interestingly, p21 was overexpressed in M059J cells, while it was downregulated in M059K cells (Figure 4(d)). The expression of the cell cycle proteins examined was correlated with the expression of transcription factor MYT1L. To verify DNAPK function, the DNAPK was then

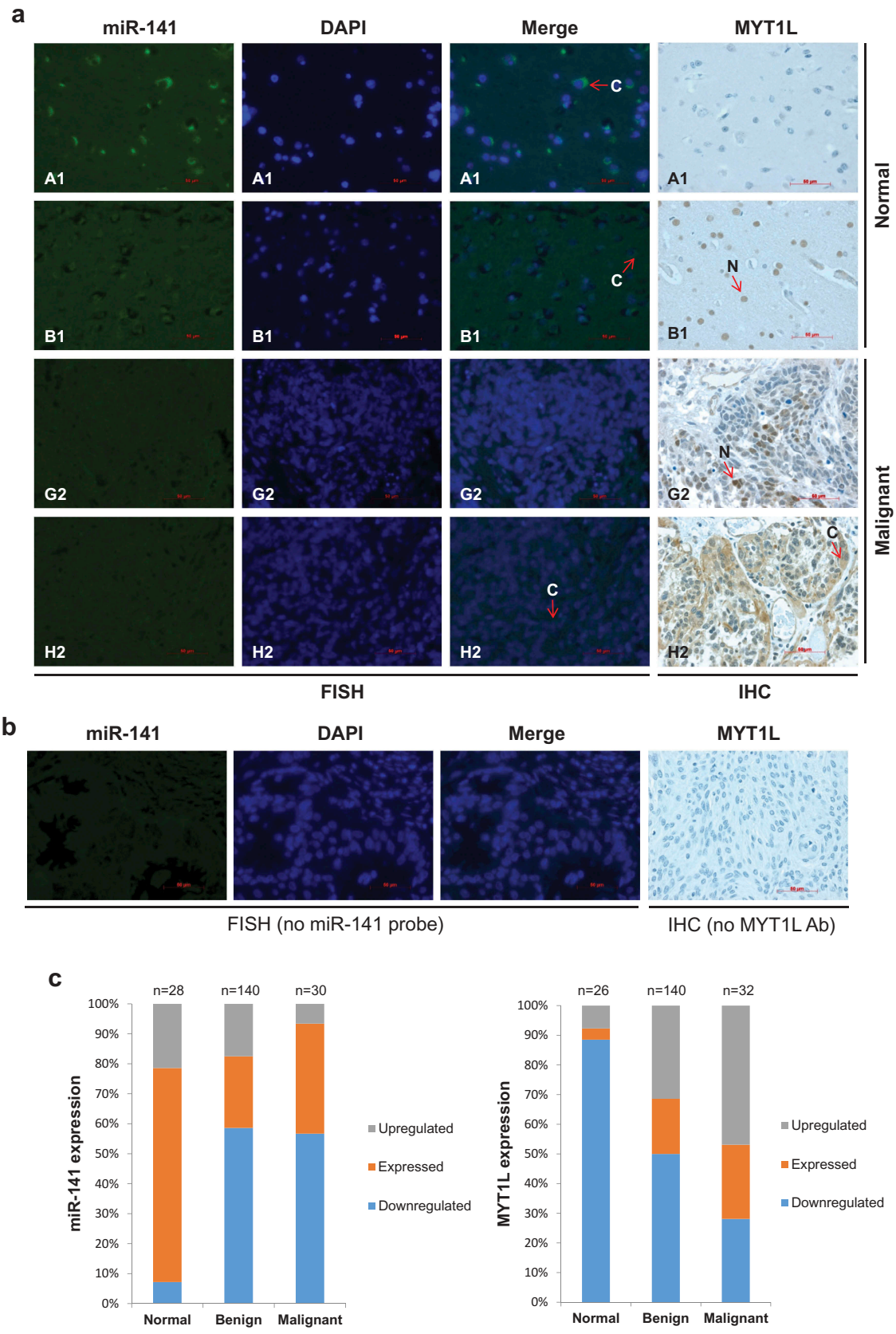
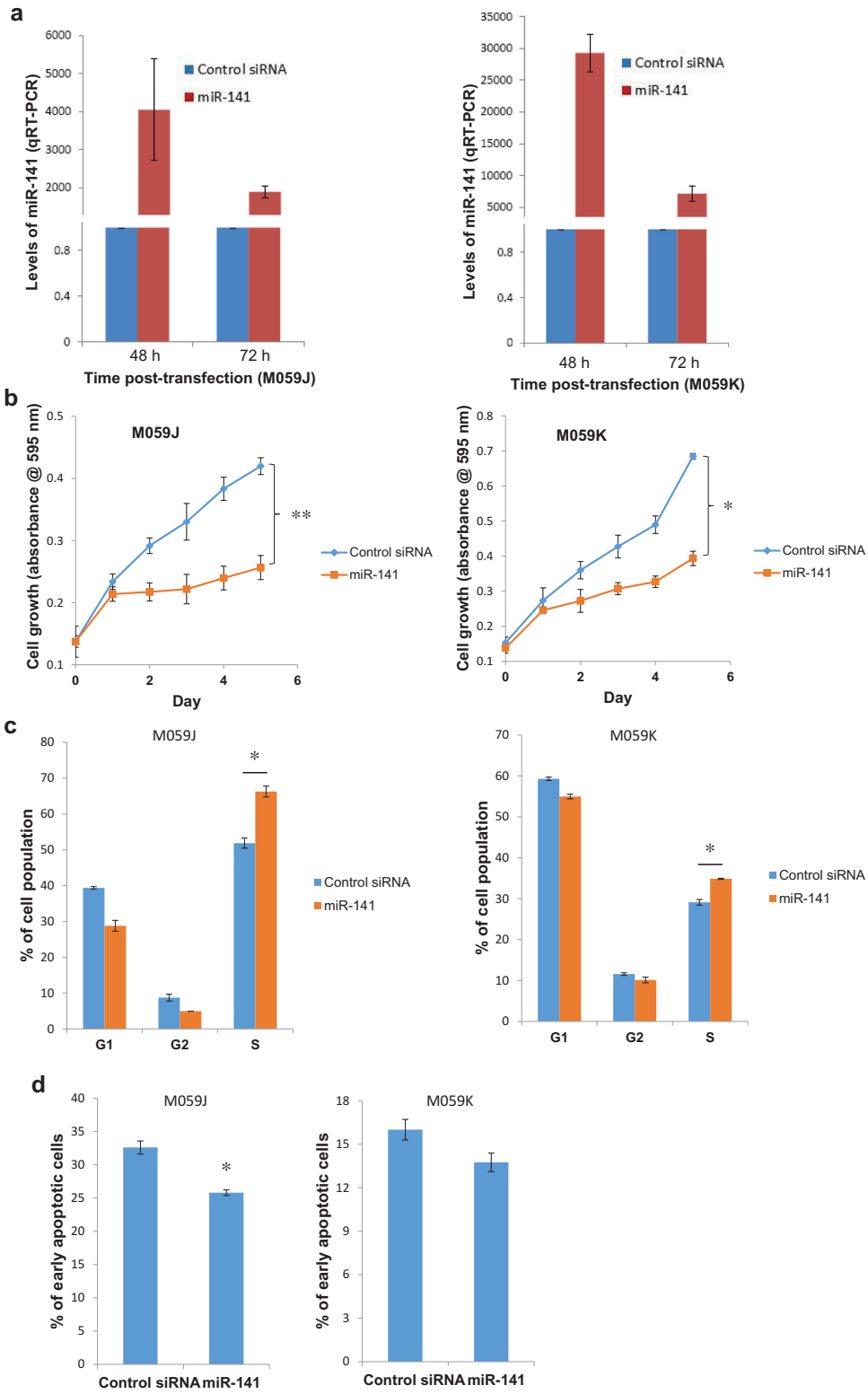


Figure 2. Expression of miR-141 and MYT1L in brain tumor tissues. (a) Representatives of miR-141 and MYT1L staining in the same sections of brain tumor tissue arrays. C, cytoplasm; N, nuclear; scale bar, 50 μm. (b) A representative panel of negative control staining; the tissue arrays/slides were incubated at 4ºC overnight without miR-141 probe or MYT1L antibody. (c) Statistical and correlation analyses of miR-141 and MYT1L expression in brain tumor and normal tissues.



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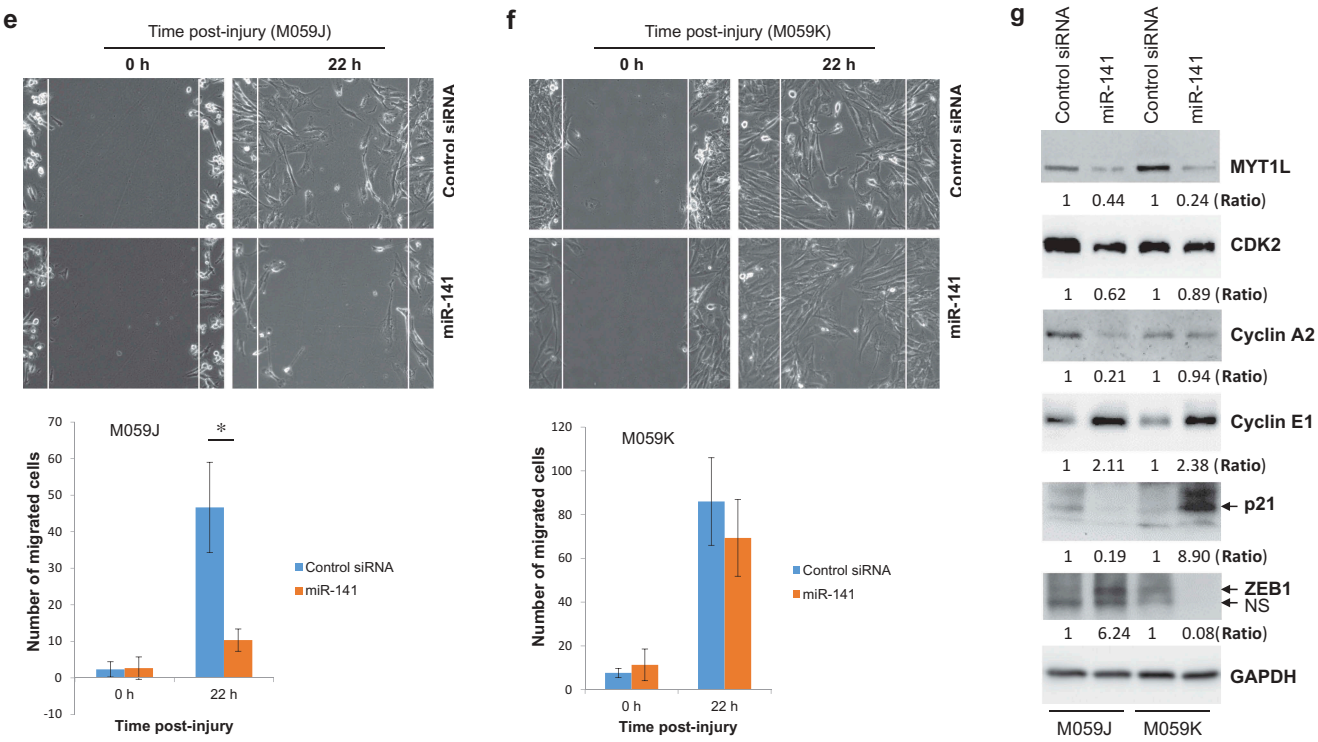

Figure 3. Ectopic expression of miR-141 suppressed proliferation and induced S-phase arrest in both normal function and loss-of-function DNAPK glioblastoma cell lines. (a) M059J and M059K cells were transfected with 20 nM of either miR-141 mimic or negative control siRNA; at 48 and 72 hours after transfection, total RNA isolated from these cells was subjected to a qRT-PCR analysis using an hsa-miR-141 primer assay. (b) At 24 hours after transfection, the cells were reseeded in 96-well plates, and the MTT assay was performed as described in the "Methods". (c) At 72 hours after transfection, the cells were harvested for cell cycle analysis using PI staining. (d) At 72 hours after transfection, the cells were harvested for apoptosis analysis using the V-FITC Annexin Apoptosis Detection Kit. (e-f) At 24 hours after transfection, the M059J (e) and M059K (f) cells were replated in 6-well plates, and the wound-healing assay was performed as described in the "Methods". (g) At 72 hours after transfection, whole cellular lysates prepared from the indicated cells were subjected to a Western blot analysis using antibodies against MYT1L, CDK2, cyclin A2, cyclin E1, p21, and ZEB1; GAPDH was used as a loading control; NS represents nonspecific. *indicates $p < 0.05$; **indicates $p < 0.02$.

knocked down in M059K cells using DNAPK siRNA. Knockdown of DNAPK significantly attenuated proliferation of M059K cells (Figure 4(e)). Interestingly, the DNAPK siRNA also caused a reduction in MYT1L expression (Figure 4(e), left panel). These results may suggest a role of MYT1L in the transcriptional regulation of cell cycle proteins and a functional dependance of miR-141 on DNAPK.

Transcriptional activation of p21 by MYT1L in p53 mutant cells

Although MYT1L is a putative transcription factor, its targets and consensus binding sites remain poorly understood. To explore the contributing role of MYT1L in controlling the transcription of cell cycle protein p21, cyclin-dependent kinase inhibitor 1A (CDKN1A), the p21 promoter region was analyzed. The bioinformatics analyses identified two candidate MYT1L consensus *cis*-acting elements in the p21 promoter: −729/-719 and −249/-239 (Figure 5(a), upper panel). Mutation of these two elements was then introduced using a mutagenesis kit (Figure 5(a), lower panel). Using HEK293 cells as a model system, the ectopic expression of MYT1L (via GFP fusion protein) caused an induction in luciferase activity in the reporter construct harboring the wild-type p21 promoter. When the $^{-724}$GG^{-723} motif in the promoter was mutated to $^{-724}$TT^{-723}, MYT1L responsiveness was attenuated. A more significant reduction in luciferase activity was found when $^{-246}$CC^{-245} in the motif was replaced with $^{-246}$AA^{-245} and when both elements −729/-719 and −249/-239 were mutated (Figure 5(b),

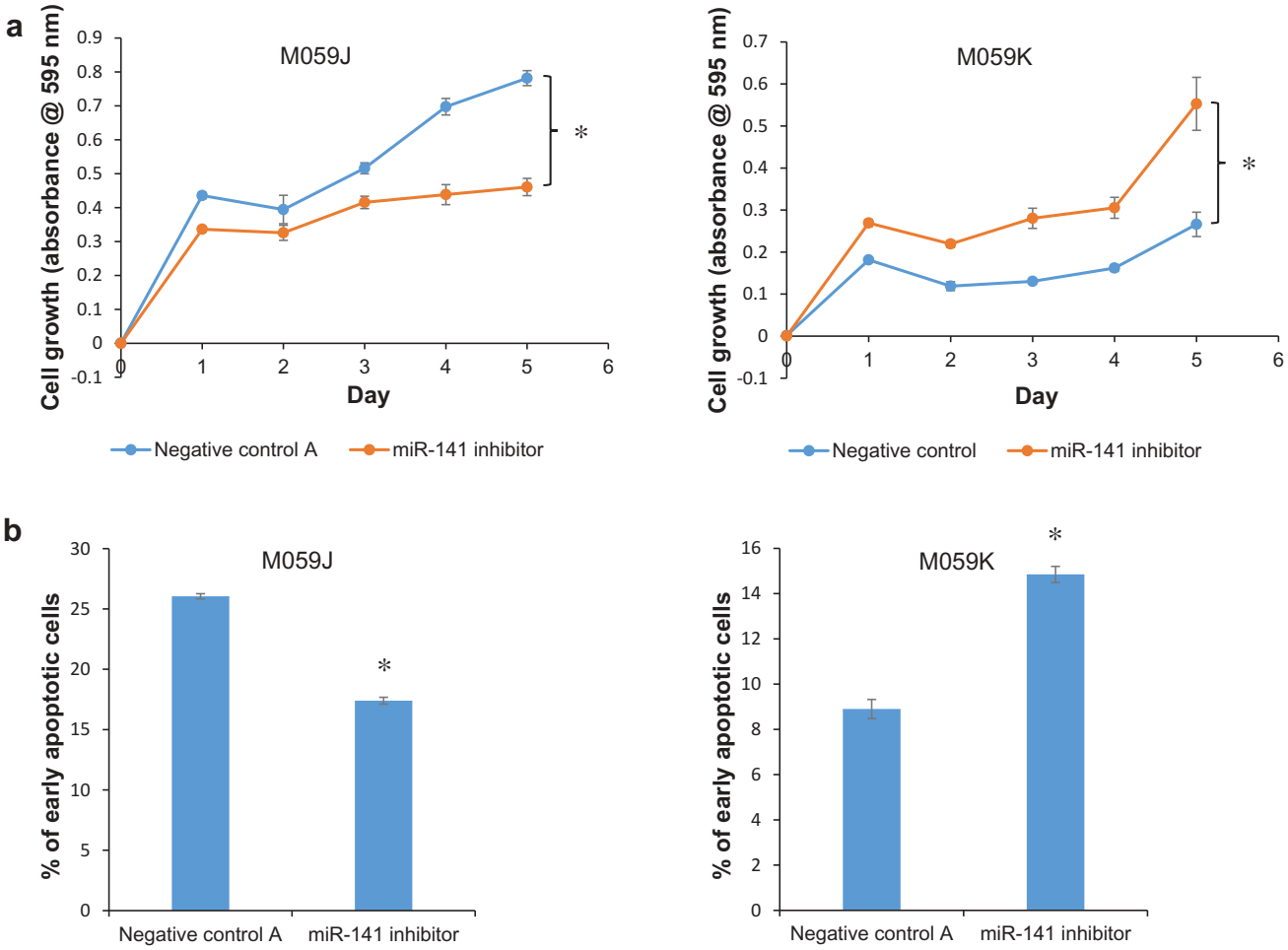

Figure 4. Continued.

upper panel); however, GFP alone also caused the same induction in luciferase activity. Similar results were also found in the CRISPR-triggered MYT1L expression system (Figure 5(b), lower panel). These results implicated that the induction of luciferase activity in HEK293 cells may be triggered by other key transcription factor(s), such as p53 [31]. MYT1L however may act as a transcriptional activator. To evaluate the potential effect of p53 and DNAPK on the MYT1L induction of p21 transcription, the luciferase assay was then repeated in M059J and M059K, which are two unique glioblastoma cell lines with a cancer-associated p53 mutation in exon 8 [32]. M059K cells express normal levels of DNAPK, while M059J cells lack DNAPK activity [28]. Interestingly, the MYT1L induction of luciferase activity was significantly attenuated by empty vector (GFP) in both cell lines, suggesting a key role of p53 in p21 transcription (Figure 5(c)). The induction of luciferase activity was globally reduced in the M059J line compared to the M059K line (Figure 5(c)), which may reflect a suppressive role of DNAPK in the MYT1L induction of p21 transcription. Taken together, the current data suggest that MYT1L may be an important transcription factor driving p21 transcription in p53-mutant cells, whereas DNAPK may function as a repressor of MYT1L.

Discussion

MYT1L is a neural-specific transcription factor [27], that belongs to the myelin transcription factor 1 family, which encodes zinc-finger-containing DNA-

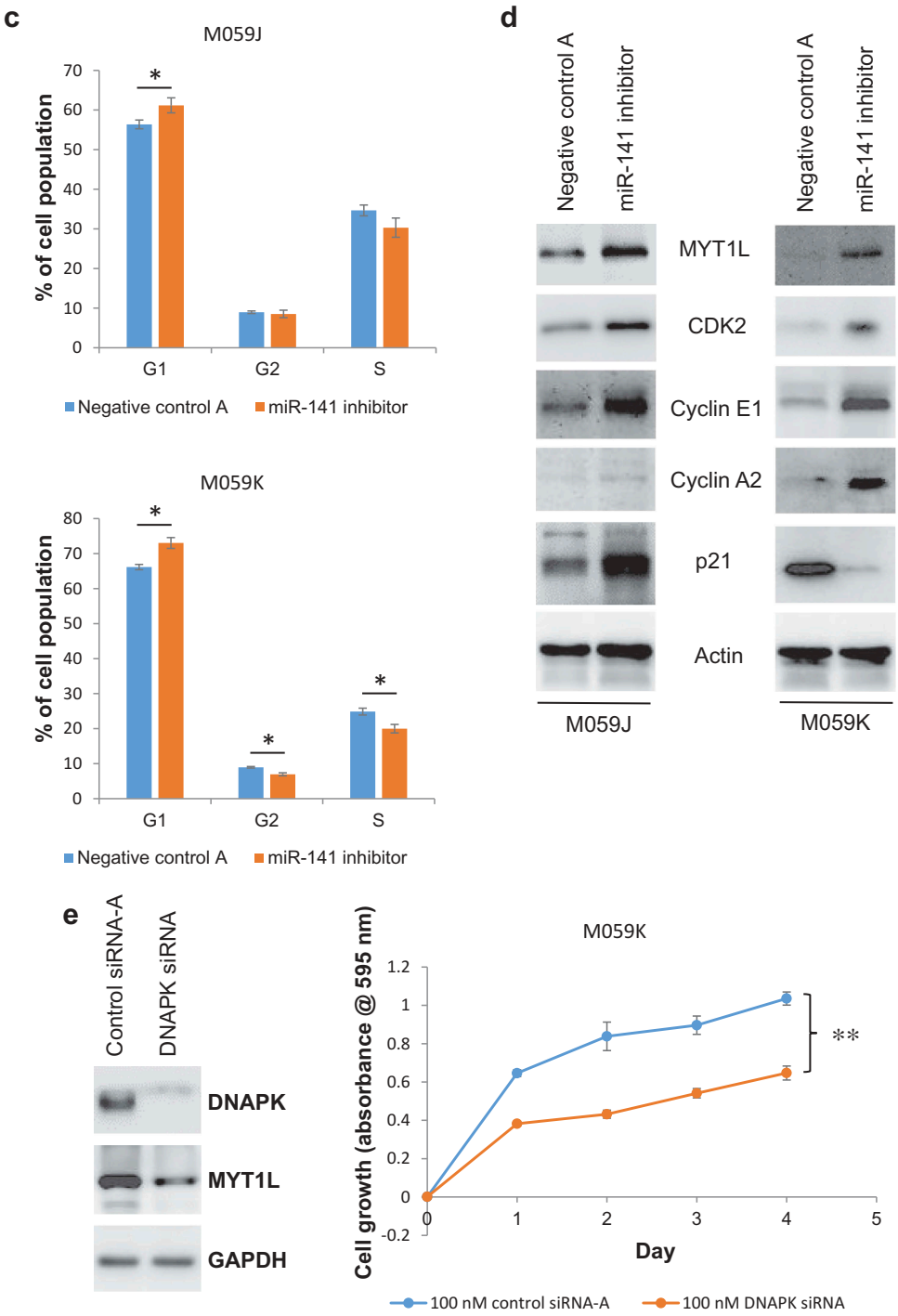

Figure 4. The effect of the inhibition of miR-141 on glioblastoma cell biology. (a) M059J and M059K cells were transfected with 40 nM of either an hsa-miR-141 inhibitor or negative control A; at 24 hours after transfection, the cells were reseeded in 96-well plates, and the MTT assay was performed as described in the "Methods". (b) At 72 hours after transfection, the cells were harvested for the apoptosis analysis using the V-FITC Annexin Apoptosis Detection Kit. (c) At 72 hours after transfection, the cells were harvested for cell cycle analysis using PI staining. (d) At 72 hours after transfection, whole cellular lysates were prepared and subjected to a Western blot analysis using antibodies to MYT1L, CDK2, cyclin A2, cyclin E1 and p21; actin was used as a loading control. (e) M059K cells were transfected with 100 nM of either DNA-PKcs siRNA or control siRNA-A; at 24 hours after transfection, the cells were reseeded in 96-well plates, and the MTT assay was performed as described in the "Methods"; at 72 hours after transfection, whole cellular lysates were prepared and subjected to a Western blot analysis using antibodies to DNA-PKcs and MYT1L; GAPDH was used as a loading control. * indicates $p < 0.05$; **indicates $p < 0.01$.

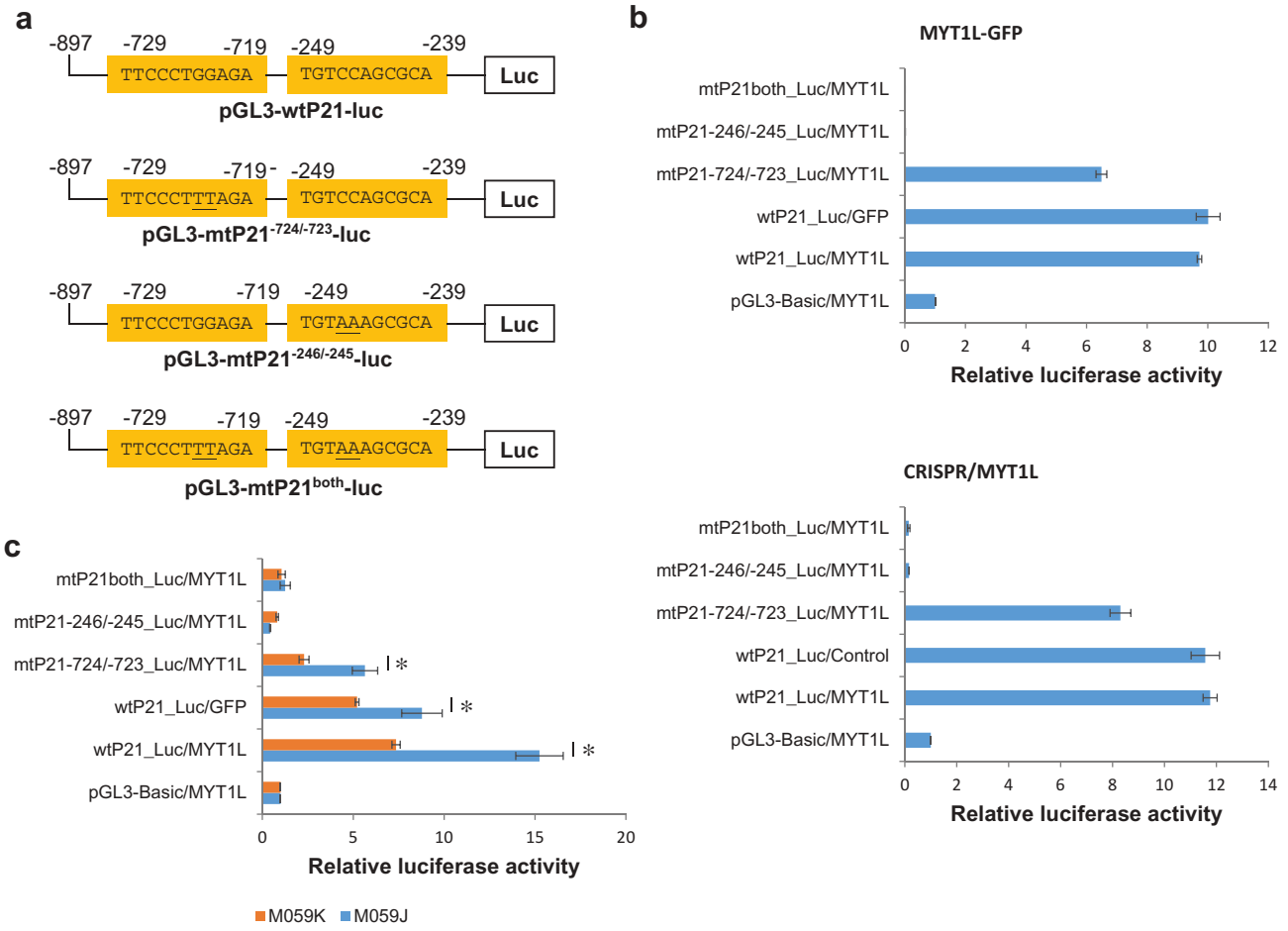

Figure 5. Myelin transcription factor 1-like (MYT1L) transactivates p21 transcription in p53-mutant glioblastoma cells. (a) Illustration of p21 promoter-luciferase reporter constructs bearing either wild-type or mutant MYT1L binding sites predicted using a DNA binding site predictor for zinc finger proteins. (b) HEK293 cells grown to 85% confluency were transfected with MYT1L expression vector (either pCMV6-MYT1L or MYT1L CRISPR activation plasmid) or empty vector, and p21 promoter-luciferase reporter construct containing either wild-type or mutant MYT1L binding site. The cells were incubated for a further 24 or 48 hours before determination of relative luciferase activity, as described in the "Methods" section. (c) M059J and M059K cells grown to 85% confluency were transfected with MYT1L expression vector (pCMV6-MYT1L) or empty vector (pCMV6-AC-GFP), and p21 promoter-luciferase reporter construct containing either wild-type or mutant MYT1L binding site. The cells were incubated for a further 24 hours before determination of the relative luciferase activity, as described in the "Methods" section. *indicates $p < 0.05$.

binding proteins [33]. A strong immunoreactivity of Myt1l has been found in both developing and differentiating neurons of embryonic rat brains, but not in oligodendrocytes [34]. A recent study also showed a predominant expression of MYT1L in human brains with notably high levels in fetal brains [35]. These findings may implicate that MYT1L plays a role in the development and differentiation of neurons. In recent years, one of the key advances in regenerative medicine is the discovery of the direct conversion of human non-neuronal cells to neurons driven by defined neural-specific transcription factors, including MYT1L [27,36,37]; however, as a transcription factor, the targets that are transcriptionally modulated by MYT1L and the contributing role of MYT1L in tumorigenesis remain poorly understood.

To the authors' knowledge, this study is the first to demonstrate that DNAPK communicates with MYT1L in the miR-141 tumor suppressor network, and it highlights the pivotal role of MYT1L in p21 transcription in p53-mutant glioblastoma cells. As the most common glioma and the most aggressive brain malignancy, glioblastoma has received considerable attention; however, the molecular biological and epigenetic mechanisms underlying the progression of

this disease remain largely unknown. The findings showed that miR-141 is downregulated in the human glioblastoma cell lines and glioma tissues examined, which is consistent with a previous report [19]. Although an oncogenic role has been suggested in some malignancies [24–26], miR-141 may primarily function as a tumor suppressor, and was found to be downregulated in numerous human cancers. MYT1L was elevated in several tested cell lines, including A172, M059J, M059K and SK-N-BE(2) and glioma tissues. MYT1L was directly regulated by miR-141, and its expression was inversely correlated with the levels of miR-141 in both cell line and tissue samples (Figure 1(a,b); Figure 2(a–c)). These findings may implicate a contributing role of miR-141/MYT1L axis in the development of glioblastoma. MYT1L is predominantly expressed in the central nervous system. Although accumulating evidence has demonstrated a crucial role of MYT1L in the direct conversion of human non-neuronal cells to neurons [27,36,37], its contributing role in tumorigenesis remains poorly understood. Using two unique glioblastoma cell lines as a model system, it was found that in M059K cells with normal DNAPK activity, the ectopic expression of miR-141 leads to a reduction in MYT1L expression, a suppression in cell proliferation, an S-phase arrest in cell cycles and a non-significant reduction in apoptosis. In contrast, the inhibition of miR-141 causes an induction in MYT1L expression, a promotion in cell proliferation and cell cycles and an induction in apoptosis (Figures 3, 4), suggesting a typical tumor suppressor role of miR-141 in gliomagenesis by targeting MYT1L. Interestingly, in M059J cells, with a loss-of-function of DNAPK, regardless of the ectopic expression or inhibition of miR-141, cell proliferation and apoptosis are unexpectedly attenuated consistently, although the cell cycle arrest varies in two responses (Figures 3, 4). This potentially suggests a dual role of miR-141 (tumor suppressor or oncogene) that consistently inhibits the growth of glioblastoma cells with a loss-of-function of DNAPK via targeting MYT1L. To the best of the authors' knowledge, these findings are novel because to date, none of miRNAs has been yet reported to possess a dual role in the same cell line. Although the precise mechanism underlying the dual role of miR-141 in cancer remains unknown, the aberrant expression of cell cycle regulators potentially targeted by miR-141 (Figures 3(g) and 4(d)) may contribute to the phenotypic alterations of glioblastoma cells. It was also found that the ectopic expression of miR-141 inhibits the migration of M059J cells, this is consistent with previous report [18], while it has no effect on M059K cells (Figures 3(e,f)). As a direct target of miR-141, ZEB1 has been reported to play a pivotal role on migration/invasion of malignant cells [29], including glioma [30]. However, it is not the case in our system, because the miR-141-induced upregulation of ZEB1 did not promote the migration of M059J cells (Figure 3(e)), and also the miR-141-mediated downregulation of ZEB1 had no effect on M059K cell migration (Figure 3(f)). Importantly, in recent years, accumulating evidence has strongly demonstrated a common inhibitory role of miR-141 in both proliferation and migration of many malignancies, such as glioma, thyroid, colorectal and prostatic cancers [18,19,38,39], suggesting that miR-141 may be common therapeutic target for different cancers. As a direct target of miR-141, although this is the first showing a dual role of MYT1L in tumorigenesis, a suppressive role of MYT1L has been demonstrated in human glioma cells as well, through transcriptional regulation of ataxin 2 binding protein 1 (A2BP1), both in vitro and in vivo [40]. These limited findings (both ours and others) may suggest a pivotal role of MYT1L in tumorigenesis and its functional dependance on cell context.

Although MYT1L is believed to act as a transcription factor, to date, only one transcriptional target has been reported (see ref. 40). It was found that the aberrant expression of p21 in both lines in response to either the overexpression or inhibition of miR-141 made p21 a likely target of MYT1L. This could better explain the phenotypic changes in both lines based on different DNAPK activities. It was revealed that MYT1L may be a key transcription factor that controls p21 expression through two novel *cis*-acting elements (−729/-719 and −249/-239) in p53-mutant cells, whereas DNAPK could communicate with MYT1L and may function as a repressor in MYT1L-mediated transcription (Figure 6). DNAPK (also known as PRKDC, DNA-PKcs and p350) is a nuclear serine/threonine protein kinase complex composed of a catalytic subunit of DNA-PKcs and a heterodimer of Ku proteins (Ku70/Ku80). Since its discovery, DNAPK has been extensively studied regarding its role in DNA-damage response. To date, one of the most well-characterized functions of

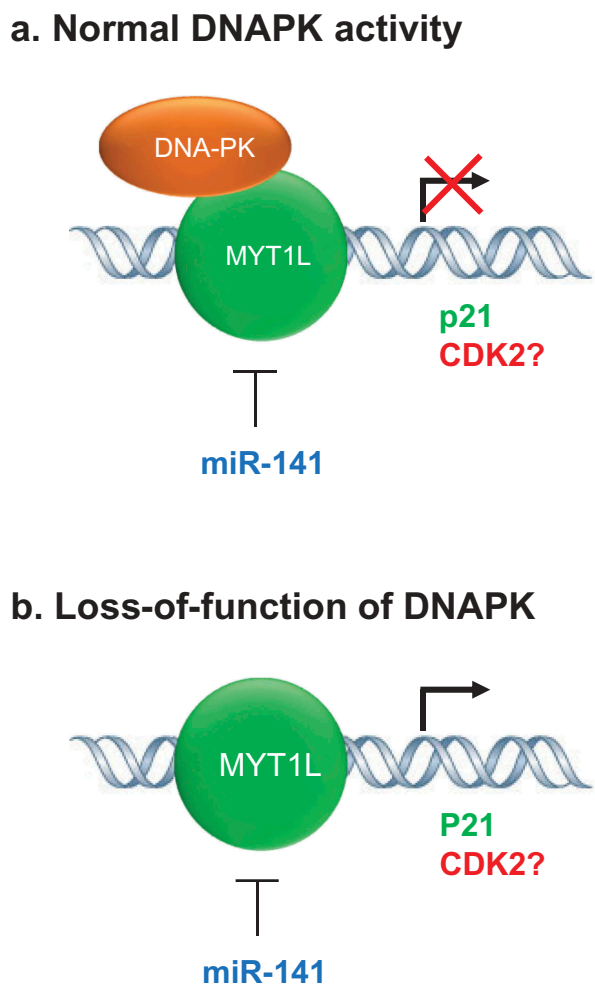

Figure 6. Involvement of DNAPK in MYT1L-mediated transcription in p53-mutant glioblastoma cells. (a) In the cells with normal DNAPK activity, DNAPK could bind to MYT1L and repress its transcription activity. (b) In the cells with a loss-of-function of DNAPK, MYT1L consistently activated the transcription of its target genes.

DNAPK is primarily to govern the repair of DNA double-strand breaks [41]. Other potential functions of DNAPK are poorly understood. DNAPK was originally characterized as a component of the Sp1 transcription complex in which it may modulate the transcriptional activity of the complex by phosphorylation of Sp1 [42]. For the first time, the findings revealed that DNAPK may be functionally involved in the miR-141 tumor suppressor network via communication with MYT1L, although this requires further validation using the chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) and electrophoretic mobility shift assay (EMSA) when a ChIP-grade anti-MYT1L antibody is commercially available. Although this is the first indication of potential interaction between DNAPK and transcription factor MYT1L, several lines of evidence have also demonstrated that DNAPK could bind to p53 on the p21 promoter and could attenuate p21 transcription, resulting in cell death [43]. We also provide evidence for the crucial role of DNAPK in driving glioblastoma cell proliferation (Figure 4(e)), which is consistent with previous reports [44,45]. However, how DNAPK knockdown causes a reduction of MYT1L is unclear.

The aberrant expression of both p21 and CDK2 induced by either miR-141 mimics or miR-141 inhibitors may contribute to the alterations in the cell cycles of either M059J or M059K cells. Interestingly, a dramatic difference was observed in the apoptotic response between M059J and M059K cells induced by either miR-141 mimics or miR-141 inhibitors (Figures 3(d) and 4(b)), and this apoptotic response is inversely correlated with changes in cell proliferation (Figure 3(b) and 4(a)). Whether and how the apoptotic response contributes to cell proliferation here is unclear. A possible explanation is so-called "apoptosis-induced compensatory proliferation" [46], which is an important mechanism involved in the maintenance of tissue homeostasis that was characterized by compensatory proliferation in surrounding cells triggered by apoptosis.

In conclusion, we showed that: MYT1L was upregulated and miR-141 downregulated in glioblastoma cell lines and glioblastoma tumor tissues. MYT1L is a direct target of miR-141 and may play a key role in governing p21 transcription in p53-mutant glioblastoma cells. DNAPK may function as a repressor that modulates MYT1L-governed p21 transcription, and potentially contributing to the miR-141-induced suppression of gliomagenesis.

Materials and methods

Cell culture

Human glioblastoma cell lines M059J, M059K and A172 and neuroblastoma cell line SK-N-BE(2) were purchased from ATCC (Manassas, VA, USA). M059J and M059K cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 medium with 2.5 mM L-glutamine adjusted to contain 15 mM HEPES, 0.5 mM sodium pyruvate and 1.2 g/L sodium bicarbonate supplemented with 0.05 mM non-essential amino acids, 10% fetal bovine

serum (FBS) and 1% penicillin/streptomycin (P/S). A172 cells were grown in an ATCC-formulated Dulbecco's Modified Eagle's Medium supplemented with 10% FBS and 1% P/S. SK-N-BE(2) cells were grown in a 1:1 mixture of ATCC-formulated Eagle's Minimum Essential Medium and F12 Medium supplemented with 10% FBS and 1% P/S. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA isolated from human brain normal tissues, the indicated cell lines, M059J and M059K cells that were transfected with either 20 nM miR-141 mimic or 20 nM AllStars negative control siRNA (QIAGEN) or 40 nM miR-141 inhibitor or 40 nM negative control A (Exiqon) was subjected to qRT-PCR using the miScript II RT Kit (QIAGEN), hsa-miR-141 miScript primer assays (QIAGEN) and the miScript SYBR® Green PCR Kit (QIAGEN) per the manufacturer's instructions. RNU6-2 was used as a loading control.

Bioinformatics

MirGator v3.0 (http://mirgator.kobic.re.kr/index.html) miRNA expression portal was used to evaluate the correlation between miR-141-3p (canonical form) and MYT1L in The Cancer Genome Atlas (TCGA) liver hepatocellular carcinoma dataset (TCGA-LIHC). Predicted target and miRNA-mRNA correlation search is available in miRGator v.3.0 under miRTarget & Expression tab. Normalized expression values were downloaded from miRGator site and Pearson's correlation coefficient between miR-141-3p and MYT1L expressions in TCGA-LIHC dataset was calculated in R programming enviroement.

The MYT1L binding site(s) in p21 promoter was predicted using Zinc Finger Protein-DNA Scoring Form, a DNA binding site predictor for Cys_2His_2 zinc finger proteins (http://zf.princeton.edu/form.php).

Generation of p21 promoter reporter plasmid

An 897-bp wild-type p21 promoter fragment was amplified by PCR using human genomic DNA and subsequently cloned into the pGEM-T easy vector (Promega). The fragment was released by digestion with *Hind* III and *Kpn* I and subcloned upstream of the luciferase gene in the pGL3-Basic vector (Promega) to produce the pGL3-wtP21-luc; the sequence identity was confirmed by automatic sequencing. The primers used for amplifying wild-type p21 promoter were p21 Prom-F, 5′-TTG GTA CCA ACC ACA GGG ATT TCT-3′, and p21 Prom-R, 5′-TTA AGC TTC TCT CAC CTC CTC TGA-3′.

Site-directed mutagenesis

Site-directed mutation of the predicted MYT1L-binding sites in p21 promoter was carried out using QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. The following primers were utilized to generate site-directed mutants. For the predicted motif at −729/-719 (ttccctggaga, complementary), p21-Prom MT1-F: 5′-GTC CAA TTC TTC TGT TTC CCT TTA GAT CAG GTT GCC CTT TTT TG-3′, p21-Prom MT1-R: 5′-CAA AAA AGG GCA ACC TGA TCT AAA GGG AAA CAG AAG AAT TGG AC-3′; for the predicted motif at −249/-239 (tgtccagcgca, primary), p21-Prom MT2-F: 5′-TTC GTG GGG AAA TGT GTA AAG CGC ACC AAC GCA GGC-3′, p21-Prom MT2-R: 5′-GCC TGC GTT GGT GCG CTT TAC ACA TTT CCC CAC GAA-3′. All mutants were confirmed by automated DNA sequencing.

The transient transfection and luciferase assay

A 144 bp full 3′UTR fragment of human MYT1L mRNA containing either a wild-type or mutant miR-141 binding sequence was cloned to a firefly/renilla dual luciferase reporter vector pEZX-MT01 to generate WT-MYT1L 3′UTR and MT-MYT1L 3′UTR reporter constructs, which was done by GeneCopoeia. HEK293 cells grown to 90% confluency in 12-well plates were transiently cotransfected with either a 0.3 μg of WT-MYT1L 3′UTR or a MT-MYT1L 3′UTR reporter and the indicated concentration of precursor hsa-miR-141 using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. HEK293 or M059J or M059K cells grown to 85% confluency in 6-well plates were transiently co-transfected with 1 μg of either

pGL3-wtP21-luc or pGL3-mtP21-luc reporter in combination with 0.5 μg of either pCMV6-MYT1L (MYT1L-GFP, OriGene) or pCMV6-AC-GFP (GFP, OriGene) or human MYT1L CRISPR activation plasmid (Santa Cruz) or control CRISPR activation plasmid (Santa Cruz) and 0.005 μg of pRL-TK (Promega) using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Twenty-four or forty-eight hours after transfection, the cells were lysed, and the relative luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) using a luminometer (FLUOstar Omega).

Western blot analysis

The indicated cells that were either transfected or untransfected were washed twice with ice-cold PBS and lysed in a radioimmunoprecipitation assay buffer (RIPA). Total protein lysate prepared from human adult brain normal tissues was purchased from BioChain and served as a normal control for the human cell lines. 50–100 μg of protein per sample was separated on either an 8% or 10% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond® P, GE Healthcare) at 4°C for 1.5 hours. Blots were incubated for 1 hour with 5% nonfat dry milk to block nonspecific binding sites, and then they were incubated overnight at 4°C with polyclonal/monoclonal antibodies specific to MYT1L (Abnova) or CDK2, DNA-PKcs, p21 (Abcam) or cyclin A2, cyclin E1 (Cell Signaling Technology) or ZEB1 (Santa Cruz Biotechnology). The immunoreactivity was detected using a peroxidase-conjugated antibody and was visualized by an ECL Plus Western Blotting Detection System (GE Healthcare). The blots were stripped before reprobing with an antibody against actin (Abcam) or GAPDH (Santa Cruz Biotechnology).

The transient transfection and MTT assay

M059J and M059K cells grown to 90% confluence were transiently transfected with either 20 nM hsa-miR-141 mimic (QIAGEN) or 20 nM AllStars negative control siRNA (QIAGEN) or 40 nM miRCURY LNA Power hsa-miR-141 inhibitor (EXIQON) or 40 nM miRCURY LNA Power Inhibitor negative control A (EXIQON) using Lipofectamine 3000 (Invitrogen) per the manufacturer's instructions. Twenty-four hours after transfection, 3.0×10^3 cells were plated in 96-well plates. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were performed using the Cell Proliferation Kit I (Roche Diagnostics GmbH) according to the manufacturer's instructions. The spectrophotometric absorbance of samples was measured at 595 nm using a microtiter plate reader (FLUOstar Omega).

Apoptosis and cell cycle analyses

M059J and M059K cells grown to 90% confluence were transiently transfected with either 20 nM hsa-miR-141 mimic (QIAGEN) or 20 nM AllStars negative control siRNA (QIAGEN) or 40 nM miRCURY LNA Power hsa-miR-141 inhibitor (EXIQON) or 40 nM miRCURY LNA Power Inhibitor negative control A (EXIQON) using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. At 72 hours after transfection, the cells were harvested for apoptosis and cell cycle analyses, which were performed using a BD FACSCanto™ II Flow Cytometer (BD Biosciences) with a propidium iodide staining solution and a BD Pharmingen™ V-FITC Annexin Apoptosis Detection Kit II (BD Biosciences) per the manufacturer's instructions.

Fluorescence in situ hybridization (FISH)

The expression of miR-141 in brain tumor specimens (NST961 and NGL961 tissue arrays; Pantomics) was detected by FISH as described previously [47]. Briefly, after deparaffinization, the sections were prehybridized for 20 minutes at 55°C followed by 1 hour of hybridization at the same temperature with a 1:1000 dilution of a miRCURY LNA™ hsa-miR-141 detection probe (Exiqon). After washing, the sections were blocked for 1 hour with a blocking solution and incubated with a 1:1000 dilution of anti-Digoxigenin-Fluorescein, Fab fragments (Roche) at 4°C overnight. Then, the research scientists and a pathologist independently analyzed the stained tissue sections.

Staining intensity was the criterion used for quantitating immunofluorescence staining. A range from 0 to 3 was used for classifying the intensity:

0 = absence of staining; 1 = weak staining; 2 = moderate staining; and 3 = intense staining.

Immunohistochemical analysis

The expression of MYT1L in the brain tumor specimens (NST961 and NGL961 tissue arrays; Pantomics) was determined by immunohistochemical staining using a rabbit polyclonal antibody to MYT1L (NOVUS Biologicals) according to the Biocare Medical instructions for immunohistochemistry. The stained tissue sections were analyzed independently by a pathologist and research scientists in a blind manner.

The criteria used for quantitating immunohistochemical staining included the staining intensity and percentage of cells stained. A range from 0 to 3 was used to classify the intensity of staining: 0 = absence of staining; 1 = weak staining; 2 = moderate staining; and 3 = intense staining. The number of cells stained was recorded according to the following classifications: a, <25% of cells stained; b, 25–50% of cells stained; c, 51–75% of cells stained; and d, >75% of cells stained.

Knockdown of DNAPK

M059K cells grown to 80% confluency were transiently transfected with either 100 nM DNA-PKcs siRNA (Santa Cruz Biotechnology) or 100 nM control siRNA-A (Santa Cruz Biotechnology). At 24 hours after transfection, 2×10^3 cells per well were plated in 96-well plates for MTT assay; the left cells were incubated for another 48 hours at 37°C in a humidified atmosphere of 5% CO_2; at 72 hours after transfection, the whole cellular lysates were prepare for western blotting.

Statistical analysis

The Student's *t*-test was used to determine the statistical significance of differences between the groups in hsa-miR-141 and MYT1L expression, cell growth, cell cycle and apoptosis. A one-way ANOVA in the SPSS Statistics19 software packages (IBM, NY) was used to examine the statistical significance in luciferase activity. The Pearson correlation was used to determine the statistical significance in has-miR-141 and MYT1L expressions between normal and tumor tissues. $p < 0.05$ was considered significant.

Article Highlights

(1) miR-141 is downregulated in glioblastoma cell lines and tissues.
(2) *MYT1L* is a direct target of miR-141.
(3) DNAPK may function as a repressor of MYT1L and play a crucial role in miR-141-mediated suppression of gliomagenesis.

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Author contributions

Experiments were conceived and designed by Bo Wang, Dongping Li, Youli Yao, Gerlinde A.S. Metz, Olga Kovalchuk, and Igor Kovalchuk. Experiments were performed by Bo Wang, Dongping Li, Youli Yao, Anna Kovalchuk, Mieke Heyns, and Rocio Rodriguez-Juarez. Data were analyzed by Bo Wang, Dongping Li, Igor kovalchuk and Olga Kovalchuk. Yaroslav Ilnytskyy provided bioinformatics and the TCGA analysis. Scoring of IHC and IF staining was done by Bo Wang and Roderick T. Bronson. The manuscript was prepared by Bo Wang. Igor Kovalchuk, Olga Kovalchuk, and Gerlinde A.S. Metz revised the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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