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Nuclear NAD+ synthase nicotinamide mononucleotide adenylyltransferase 1 contributes to nuclear atypia and promotes glioma growth

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Abstract

Background. Glioma is a malignant primary brain tumor with a poor prognosis and short survival. NAD+ is critical for cancer growth; however, clinical trials targeting NAD+ biosynthesis had limited success, indicating the need for mechanistic characterization. Nuclear atypia, aberrations in the size and shape of the nucleus, is widely observed in cancer and is often considered a distinctive feature in diagnosis; however, the molecular underpinnings are unclear.

Methods. We carried out high-resolution immunohistochemical analyses on glioma tissue samples from 19 patients to analyze the expression of NAD+ synthase nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1), and its correlation with nuclear atypia in gliomas. Utilizing a *Drosophila* model of glial neoplasia, we investigated the genetic role of nuclear NMNAT in glioma growth in vivo, elucidating the cellular mechanisms of NMNAT1 in promoting nuclear atypia and glioma growth.

Results. In low-grade glioma and glioblastoma, a higher transcription level of NMNAT1 is correlated with poorer disease-free survival. Samples of high-grade gliomas contained a higher percentage of glial cells enriched with NMNAT1 protein. We identified a specific correlation between nuclear NMNAT1 protein level with nuclear atypia. Mechanistic studies in human glioma cell lines and in vivo *Drosophila* model suggest that NMNAT1 disrupts the integrity of the nuclear lamina by altering the distribution of lamin A/C and promotes glioma growth.

Conclusions. Our study uncovers a novel functional connection between the NAD+ metabolic pathway and glioma growth, reveals the contribution of the NAD+ biosynthetic enzyme NMNAT1 to nuclear atypia, and underscores the role of nuclear NMNAT1 in exacerbating glioma pathology.

Key Points

- Higher nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) expression is correlated with severe glioma and poorer survival.
- Genetic requirement for nuclear NAD+ biosynthesis in glioma progression in vivo.
- Elevated NMNAT1 leads to nuclear atypia by disrupting nuclear lamina architecture.

Glioma is the most prevalent primary brain tumor arising from glial cells.[1] Gliomas are categorized into distinct types based on their cellular origins, such as astrocytoma, oligodendroglioma, ependymoma, and glioblastoma (GBM). According to the classification guidelines of the World Health Organization (WHO), gliomas are graded from 1 to 4, with higher grades indicating increasing malignancy.[2] Despite various available treatments, including surgery, radiotherapy, and

Importance to the Study

NAD+-related signaling pathways have been found to be dysregulated in gliomas. However, clinical trials targeting NAD+ biosynthetic pathway through nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme, have shown limited efficacy. Our study reveals that nicotinamide mononucleotide adenylyltransferase (NMNAT), the last enzyme in NAD+ biosynthesis, is elevated in gliomas and contributes to glioma growth, resulting in poor patient survival. We demonstrate that the expression level of nuclear NMNAT1 is correlated with the severity of nuclear atypia in patient tissues. Nicotinamide mononucleotide adenylyltransferase 1 disrupts the integrity of the nuclear lamina by altering the distribution of lamin A/C. Using an in vivo model of glial neoplasia, we show that upregulated nuclear NMNAT results in glioma growth, reduced nervous system function, and poor survival. Collectively, our work identified the nuclear NAD+ synthase NMNAT1 as an important predictor of prognosis and a potential therapeutic target for glioma treatment of high NMNAT1-expressing patients.

chemotherapy, the overall prognosis for GBM patients is poor, with an average survival of 15 months.[3] Mechanistic understandings of genetic and cellular alterations that promote glioma growth and progression are urgently needed to enhance diagnosis and prognosis.

NAD+ is an indispensable signaling cofactor that governs cancer metabolism through its coenzymatic involvement in numerous bioenergetic pathways, including glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation.[4] Dysregulation of NAD+-related signaling pathways has been implicated in cancer.[5] Nicotinamide mononucleotide adenylyltransferase, the final enzyme in the NAD+ salvage synthetic pathway, has emerged as a prospective candidate.[6] Nicotinamide mononucleotide adenylyltransferase is encoded by 3 genes in mammals, each with distinct subcellular localizations: NMNAT1 in the nucleus, NMNAT2 in the cytosol, and NMNAT3 in the mitochondria.[7] Dysregulations in both NMNAT1 and NMNAT2 have been linked to cancer. For instance, NMNAT1 is considered a marker for poor prognosis in renal cancer, as reduced NMNAT1 expression results in the epigenetic silencing of tumor suppressor genes, delays DNA repair, and enhances rRNA transcription.[8,9] In acute myeloid leukemia, NMNAT1-mediated NAD+ metabolism has been shown to regulate p53 acetylation, enabling cancer cells to evade apoptosis.[10] In colorectal cancer, NMNAT2 upregulation is correlated with the depth of cancer invasion.[11,12] In contrast, NMNAT2 depletion indirectly inhibits cell growth by reducing glucose availability in neuroblastoma cells.[13] Nicotinamide mononucleotide adenylyltransferase 2-mediated cytosolic NAD+ synthesis regulates ribosome adenosine diphosphate (ADP)-ribosylation to maintain protein homeostasis in ovarian cancer.[14] These findings underscore the regulatory role of compartmentalized NAD+ synthesis in cellular metabolism and rapid cancer cell growth and emphasize the potential of NMNAT as an alternative target within the NAD+ synthetic pathway, given their aberrant regulation and critical roles in cancer metabolism.

Nuclear atypia is widely observed in cancer cells and is often used in pathological examination as a key indicator of the aggressiveness and malignancy of cancer.[15] Cancer grading systems, which assess the degree of differentiation of cancer cells compared to normal cells, often incorporate the evaluation of nuclear atypia as a significant factor. This includes examining the size, shape, and chromatin pattern of nuclei, as well as the appearance of nucleoli.[16–18] Nuclear atypia is indicative of the genetic instability of cancer cells and their deviation from normal cell morphology and function.[19,20] Specifically, nuclear atypia is a critical factor in the grading of gliomas.[21,22] Glioma grading is based on the WHO classification system, which integrates various histological and molecular characteristics, to determine the glioma grade.[2] The current assessment of nuclear atypia is largely qualitative and impression based. The factors that contribute to the development of nuclear atypia in glioma remain unclear.

Our previous research using *Drosophila* and cellular models of glioma identified a genetic requirement for NMNAT to promote glioma growth through modulating NAD+-dependent posttranslational modifications of p53, thus inhibiting DNA damage-induced cell death.[23] In this study, to elucidate the compartment-specific functions of NMNAT in human glioma, we collected 19 glioma patient samples of various types and grades and carried out high-resolution immunohistochemistry studies. We discovered a strong 3-way correlation among nuclear NMNAT1 expression, nuclear atypia, and glioma severity. We further dissected the molecular and cellular mechanism of this correlation using an in vivo glial neoplasia model in *Drosophila* and human glioma cell lines. We identified nuclear NAD+ synthase NMNAT1 as a driving force for nuclear atypia and glioma aggressiveness. Our findings indicate NMNAT1 as a potential biomarker for glioma malignancy and a promising target for glioma therapy.

Methods

Ethics Statement

De-identified patient samples (formalin-fixed, paraffin-embedded, and precut) were obtained from the University of Miami Biospecimen Shared Resource (SCCC). This study uses *Drosophila* invertebrate models.

Fly Stocks and Culture

Flies were maintained at 21 °C room temperature with the standard medium. The following lines were used in this

study obtained from the Bloomington *Drosophila* Stock Center: (a) the driver used in all experiments: *repo-GAL4* and (b) *UAS-Egfr* (III); UAS-*Drosophila melanogaster* Nmnat (*UAS-nucNMNAT*, *UAS-nucNMNAT*^{WR}, and *UAS-cytNMNAT*) were generated in the laboratory.

Human Glioma Cell Line Culture

The human GBM cell line T98G was purchased from the American Type Culture Collection (ATCC, CRL-1609). Cells were maintained in Eagle's Minimum Essential Medium (EMEM, Sigma, M0325) supplemented with 10% Fetal Bovine Serum (FBS, ATCC, 30-2020). Cells were cultured at 37 °C, 5% CO_2.

Antibodies

The following commercially available antibodies were used: anti-Repo (1:250, DSHB, 8D12), anti-*Drosophila* Nmnat (1:3000), anti-NMNAT1 (1:1000, Abcam, ab45548), anti-NMNAT1 (1:1000, Santa Cruz, 271557), anti-NMNAT2 (1:500, Abcam, ab56980), and anti-Lamin A/C (1:250, Cell signaling, 41357). The secondary antibodies conjugated to Alexa 488/546/647 (1:250, Invitrogen), HRP-anti-Mouse, and HRP-anti-Rabbit (1:5000, Thermo Fisher).

Cells Transfection

Cells for transfection were seeded in a 6-well culture vessel (VWR) containing EMEM media with 10% FBS for 24 h. Plasmids were transfected with a transfection reagent (jetPRIME). Gene expression was measured by Western blot and real-time qPCR after cells were transfected at 48 h.

Immunocytochemistry of Cells

Cells were grown on 22-mm glass coverslips (VWR). After treatment, cells were rinsed 3 times with PBS, fixed for 15 min in 4% paraformaldehyde, washed 3 times with PBS, and permeabilized with 0.4% Triton X-100 in PBS for 5 min. After 3 times washing in PBS, blocking was performed by incubation in 5% normal goat serum in PBTX (PBS with 0.1% Triton X-100) at 37 °C for 30 min. Incubation with primary antibodies was performed in 5% goat serum in PBTX at 37 °C for 2 h. Next, cells were washed 3 times with PBS and incubated for 1 h at 37 °C with secondary antibodies in 5% goat serum in PBTX. Then, after 3 times washing with PBS, cells were stained with 4′,6diamidino-2-phenylindole (DAPI, 1:300, Invitrogen) at 37 °C for 5 min in PBTX solution. The cells were washed 3 times with PBS, and the coverslips were mounted on glass slides with VECTASHIELD Antifade Mounting Medium (Vector Laboratories) and kept at 4 °C before imaging.

Immunocytochemistry of Fly Brain

The larval brains were dissected in phosphate-buffered saline (PBS, pH 7.4), and fixed in PBS with 4% formaldehyde for 15 min. After the brains were washed in PBS containing 0.4% (v/v) Triton X-100 (PBTX) for 15 min 3 times, the brains were incubated with primary antibodies diluted in 0.4% PBTX with 5% normal goat serum overnight. Then, secondary antibodies were at room temperature for 1 h, followed by 4′,6diamidino-2-phenylindole (DAPI, 1:300, Invitrogen) staining for 10 min. Brains were mounted on glass slides with VECTASHIELD Antifade Mounting Medium (Vector Laboratories) and kept at 4 °C before imaging.

Immunohistochemistry of Glioma Patient Pathological Samples

Patient pathological samples of formalin-fixed paraffin-embedded tissues were performed with hematoxylin and eosin stain (H&E) staining and IHC staining for NMNAT1 (Santa Cruz sc-271557, dilution 1:500) and NMNAT2 (Abcam ab56980, dilution 1:500). Sections were prebaked at 60 °C for 30 min and loaded at automated stainer to perform the standard H&E and IHC protocol (Leica Bond RX). After staining, slides were automated dehydrated (Leica ST5020) and automated coverslip (Leica CV5030).

Confocal Image Acquisition and Image Analysis

Confocal microscopy was performed with an Olympus IX81 confocal microscope coupled with ×10, ×20 air lens or ×40, ×60 oil immersion objectives, and images were processed using FluoView 10-ASW (Olympus). Specifically, Figure 6L and M were analyzed using the Image J interactive 3D surface Plot plugin.

Drosophila *Negative Geotaxis Assay Using the AGM Platform*

The automated geotaxis monitor system is designed, and its experimental performance has been detailed in the referenced study.[24] Seven flies with the same genotype at 10 days old were preloaded in a plastic vial. The average speed is calculated over 10 s as a recording of the fly climbing to the final position (the maximum height is 14 cm).

Statistics

For each statistical test, biological sample size (n), and P value are indicated in the corresponding figure legends. All data in this manuscript are shown as mean ± SD or median ± quartiles (specified in figure legends). t-test was used to compare between 2 groups, and one-way ANOVA with Bonferroni's post hoc test was applied to compare among 3 or more groups. Data were analyzed with Prism (GraphPad Software). Specifically, nuclear circularity and nucleus size data were analyzed by the Wilcoxon rank sum test in R.

Results

Higher NMNAT1 mRNA Expression Predicts Poor Survival in Glioma Patients

To investigate the correlation between NMNAT mRNA expression levels and glioma prognosis, we analyzed how its

expression levels affect survival in low-grade glioma (LGG) and glioblastoma patients through the gene expression profiling interactive platform, GEPIA (http://gepia.cancer-pku.cn/) (Figure 1A and B). In the GEPIA datasets, a higher level of NMNAT1 mRNA expression was found in GBM ($n = 163$), compared to that of control (non-glioma patients) ($n = 207$) or LGG ($N = 518$) (Figure 1A). In contrast, the expression of NMNAT2 in GBM was lower than that of control or LGG (Figure 1B). Next, we compared the disease-free survival of glioma patients with high or low NMNAT expression levels. A strong negative correlation between NMNAT1 expression and patient survival can be seen in patients with LGG both when comparing survival in the median high-low (50-50) expression groups and the highest and lowest 10% expression groups (Figure 1C and G), suggesting that elevated NMNAT1 expression is significantly associated with a lower disease-free survival rate. Interestingly, NMNAT2 expression is positively correlated with LGG patient survival (Figure 1D). However, there is no significant difference in the survival of GBM patients with high or low NMNAT2 expression (Figure 1F and H). Collectively, these data reveal a specific correlation between NMNAT1 expression and patient survival, consistent with the role of NMNAT1 in driving glioma growth and further suggesting the possibility of higher NMNAT1 expression as a predictor of lower survival and poorer outcomes.

NMNAT1 Protein Level Was Elevated in GBM

The strong negative correlation between NMNAT1 mRNA expression and patient survival prompted us to examine the protein level in patient tissues with different types and grades of glioma. We collected a total of 19 glioma patient pathological samples with different glioma types and malignancies graded by pathological examination (Figure 2A) and carried out immunohistochemical analysis for human NMNAT1 and NMNAT2. Nicotinamide mononucleotide adenylyltransferase 1 staining showed an exclusive nuclear pattern consistent with NMNAT1 being the nuclear NAD+ synthase.[7] Both NMNAT1 positive nuclei (yellow arrowheads) and negative nuclei (white arrowheads) were observed in tissues of astrocytoma (Figure 2B) and GBM (Figure 2C). The percentage of NMNAT1-positive nuclei within the whole tissue was significantly higher in the high-grade glioma (GBM) tissues (Figure 2D). Nicotinamide mononucleotide adenylyltransferase 2 staining showed a diffused pattern consistent with the cytosolic localization of NMNAT2.[7] The average NMNAT2 intensity within whole tissue was analyzed for each sample and no significant difference was observed between LGG or GBM glioma samples (Supplementary Figure 2). In addition, 3 out of the 19 samples were found to contain adjacent non-glioma tissue, as confirmed by H&E staining, including 2 samples of GBM and 1 astrocytoma (Figure 3A and B). We carried out a comparative analysis between the glioma and adjacent nonglioma tissue within the same patient sample and observed a higher cell density in glioma tissues compared to the respective adjacent non-tumor tissues (Figure 3C). Comparative analysis of NMNAT1 staining revealed a slight elevation in the percentage of NMNAT1-positive nuclei (Figure 3D and E), although the statistical analysis did not reach significance, likely due to low sample size (Figure

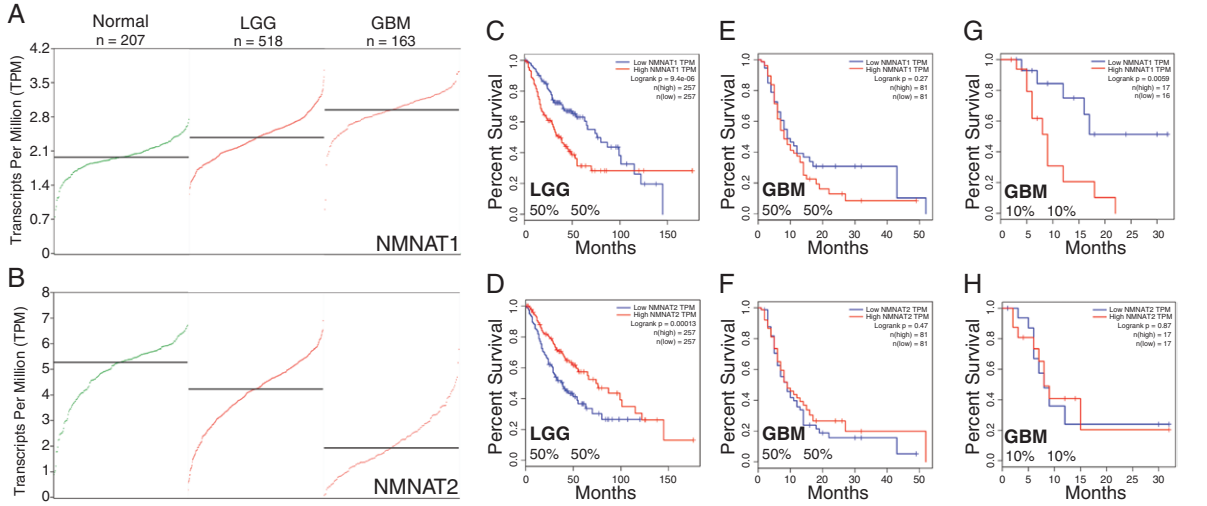

Figure 1. Correlation of nicotinamide mononucleotide adenylyltransferase (NMNAT) expression levels with patient survival in glioma. (A and B) Visualization of NMNAT1 and NMNAT2 mRNA expression in glioma from GEPIA datasets. The expression data are first $\log_2$ (TPM + 1) scaled and the $\log_2$FC is defined as median (tumor) – median (normal). Overexpressed genes as those passing the following thresholds: $\log_2$FC > 1, percentage > 0.9. Dot plot of expression of NMNAT in normal samples ($n = 207$), LGG (low-grade glioma) ($n = 518$), and GBM (glioblastoma) ($n = 163$). (C and D) Survival curves for patients with LGG expressing NMNA1 (C) and NMNAT2 (D) (top 50% versus bottom 50%). (E and F) Survival curves for patients with GBM expressing NMNA1 (E) and NMNAT2 (F) (top 50% versus bottom 50%). (G and H) Survival curves for patients with GBM are shown, with tumors expressing NMNA1 (G) and NMNAT2 (H) (top 10% versus bottom 10%). All associated log-rank *P*-values are shown. High NMNAT1-expressing tumors have extremely poor relapse-free rates compared to low NMNAT1-expressing tumors. In contrast, high NMNAT2 expression has been shown to better prognose in LGG but not GBM.

3F). Given that the adjacent non-tumor tissues likely include other cell types including neurons, the comparison of NMNAT1 level between tumor and non-tumor tissue needs to be considered with caution. Taken together, both mRNA and protein level analysis of NMNAT1 expression in glioma patient samples identified a strong association between a higher NMNAT1 expression level and a more severe (higher grade) glioma and poorer outcome in patient survival.

NMNAT1 Protein Level Is Positively Correlated With Nuclear Atypia in Glioma

Nuclear atypia is one of the pathological characteristics considered during pathological examination for glioma diagnoses and classification.[21,25] It refers to the abnormalities of cellular nuclei that exhibit irregular nuclear shape, larger nucleus size, and a higher ratio of nucleus to cytoplasm, which are used as a hallmark of cancer cell malignancy. However, the scoring of nuclear atypia has been mainly through qualitative, visual inspection of the pathological slides.[26] We next sought to develop an unbiased, quantitative method to assess nuclear atypia. The workflow uses Image J to first automatically select all nuclei in 3,3'-d iaminobenzidine (DAB)-stained images, and then quantitatively determine the size and circularity of each nucleus. The value of circularity ranges from 0 to 1 where 0 indicates a line and 1 indicates a circle. The circularity of all nuclei within each sample was plotted using violin plots (Figure 4A–D). All glioma samples were grouped by the glioma type and cellular origin and ordered by the median values of circularity. Given the shared cellular origin, astrocytoma, and GBM have been plotted together (Figure 4A). We carried out a pooled sample analysis and found that the nuclear circularity of GBM is significantly lower than that of astrocytoma (Figure 4B). The size of the nucleus was also analyzed in Image J and plotted in a similar manner as the circularity index (Figure 4E–H). Consistent with the observation of circularity, pooled sample analysis

A

Glioma type	Cell origin	Grade		
		2	3	4
Glioblastoma	Astrocyte			5
Astrocytoma		4	1	
Oligodendroglioma	Oligodendrocyte	3	2	
Ependymoma	Ependymal cell	4		
Total		14		5

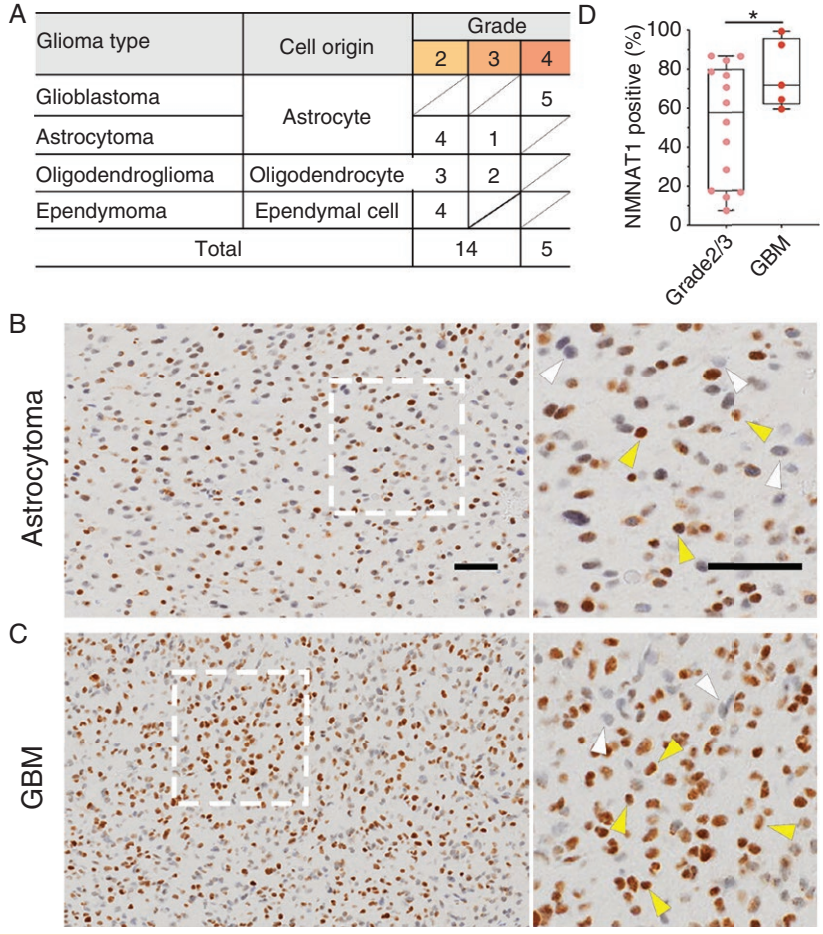

Figure 2. The expression of nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) protein is increased in GBM. (A) Collection of human glioma pathological samples with different grades and glioma types. (B and C) Immunohistochemistry detection of NMNAT1 in low-grade glioma (B) and GBM (C). The high magnification of the NMNAT1 staining nucleus shows the box area with the white dashed line on the left side. The white arrows indicate the negative staining nucleus and the yellow arrows indicate the nucleus with positive staining. Scale bar, 50 μm. (D) Quantification of the percentage of NMNAT1 positive staining in the nucleus in A. Data are presented as mean ± SD $n = 19$. The significance level was established by *t*-test. $^*P \leq .05$.

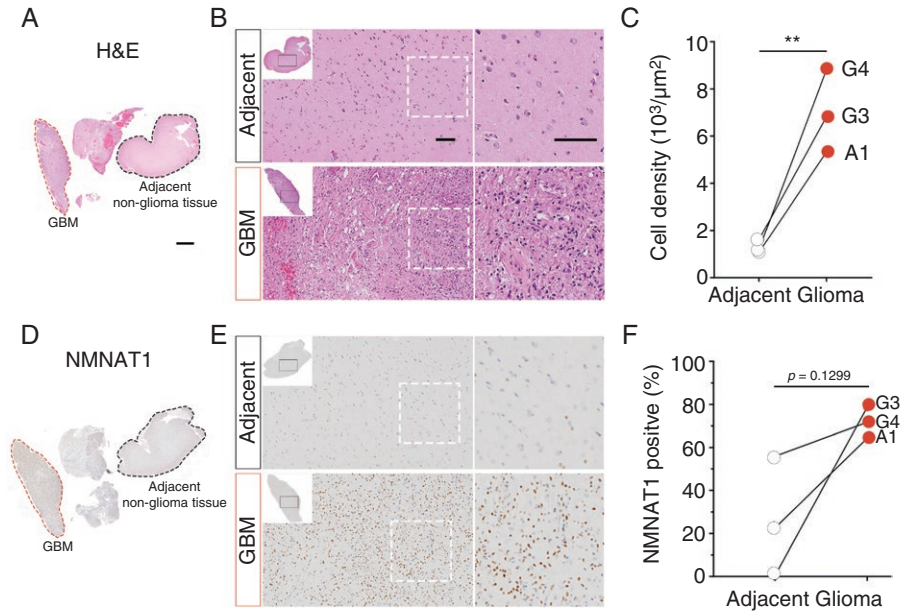

Figure 3. The expression of nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) increased in glioma compared to adjacent normal tissue. (A) Overview of H&E detection for human glioma sample with adjacent normal tissue. The normal tissue is marked with black dashed lines and tumor tissue is marked with red dashed lines, respectively. Scale bars, 1000 μm. (B) A high magnification area of H&E staining from the black box area in the first column. The second column shows the high magnification of the white box. Scale bars, 100 μm. (C) Quantification of the cell density of normal tissue and tumor tissue. Data are presented as paired, $n = 3$. (D) Overview of Immunohistochemistry detection of NMNAT1 in human glioma sample with adjacent normal tissue. The normal tissue is marked with black dashed lines and tumor tissue is marked with red dashed lines, respectively. Scale bars, 1000 μm. (E) A high magnification area of NMNAT1 staining from the black box area in the first column. The second column shows the high magnification of the white box. Scale bars, 100 μm. (F) Quantification of the percentage of NMNAT1 positive staining in adjacent tissue and GBM. $n = 3$. The significance level was established by *t*-test. $^{**}P \leq .01$.

showed that the average size of the nucleus of GBM is significantly larger than that of astrocytoma (Figure 4F). These results suggest that compared to lower-grade astrocytoma, GBM cells showed a more severe nuclear atypia, with a more irregular nuclear shape and larger size.

Given the observed higher NMNAT1 expression (Figure 2) and more severe nuclear atypia in GBM, we next assessed the correlation between NMNAT1 level and nuclear atypia. We first analyzed NMNAT1 protein level by IHC intensity in all NMNAT1 positive cells in each sample and plotted the distribution of NMNAT1 intensity next to the circularity of the same sample (Figure 4I–K). The samples were grouped by glioma type and cellular origin and ordered by median circularity. Next, we analyzed the nuclear circularity as a function of NMNAT1 intensity and found a significant inverse correlation between NMNAT1 expression and nuclear circularity in all samples combined (Figure 4L). When samples were plotted separately based on their cell type, a significant inverse correlation can be seen in astrocytoma and GBM of astrocyte origin (Figure 4M), and oligodendroglioma (Figure 4N). In ependymoma samples, the correlation was not significant, likely due to the limited number of samples (Figure 4O). Similarly, we analyzed the relationship between NMNAT1 intensity and nuclear size, however, no significant correlation between nucleus size and NMNAT1 expression was found (Supplementary Figure 4 [Supplement 1]), suggesting that nuclear size is regulated independently of NMNAT1. At the single-cell level, we also analyzed the relationship between NMNAT1 expression and nuclear atypia in all glioma cells of 5 GBM samples, ranging from 5969 to 344 922 cells in each sample, and observed a significant correlation between NMNAT1 expression and nuclear atypia in all GMB samples (Supplementary Figure 4 [Supplement 2]).

Collectively, these results reveal a strong correlation between NMNAT1 expression and nuclear atypia where higher NMNAT1-expressing cells possess severe nuclear irregularity, likely indicating higher glioma malignancy.

Nuclear NMNAT Is Required for Promoting Glial Neoplasia Growth in a Drosophila *Model of Glial Neoplasia*

To examine the effect of NMNAT1 in promoting glioma in vivo, we optimized a *Drosophila* glial neoplasia model by overexpressing constitutively active epidermal growth factor recepto (EGFR) in glial cells, driven by the pan-glial driver repo-GAL4.[27] EGFR is one of the most common genetic alterations in gliomas. Amplification of *EGFR* has been detected in GBM (57%),[28] and overexpression of EGFR has been used to model human glioma in *Drosophila*.[29] Glial neoplasia tissue in vivo can be detected and analyzed using high-resolution fluorescent imaging. The glial neoplasia tissue was marked by the accumulation of F-actin labeled by Phalloidin and glial cell marker Repo (Figure

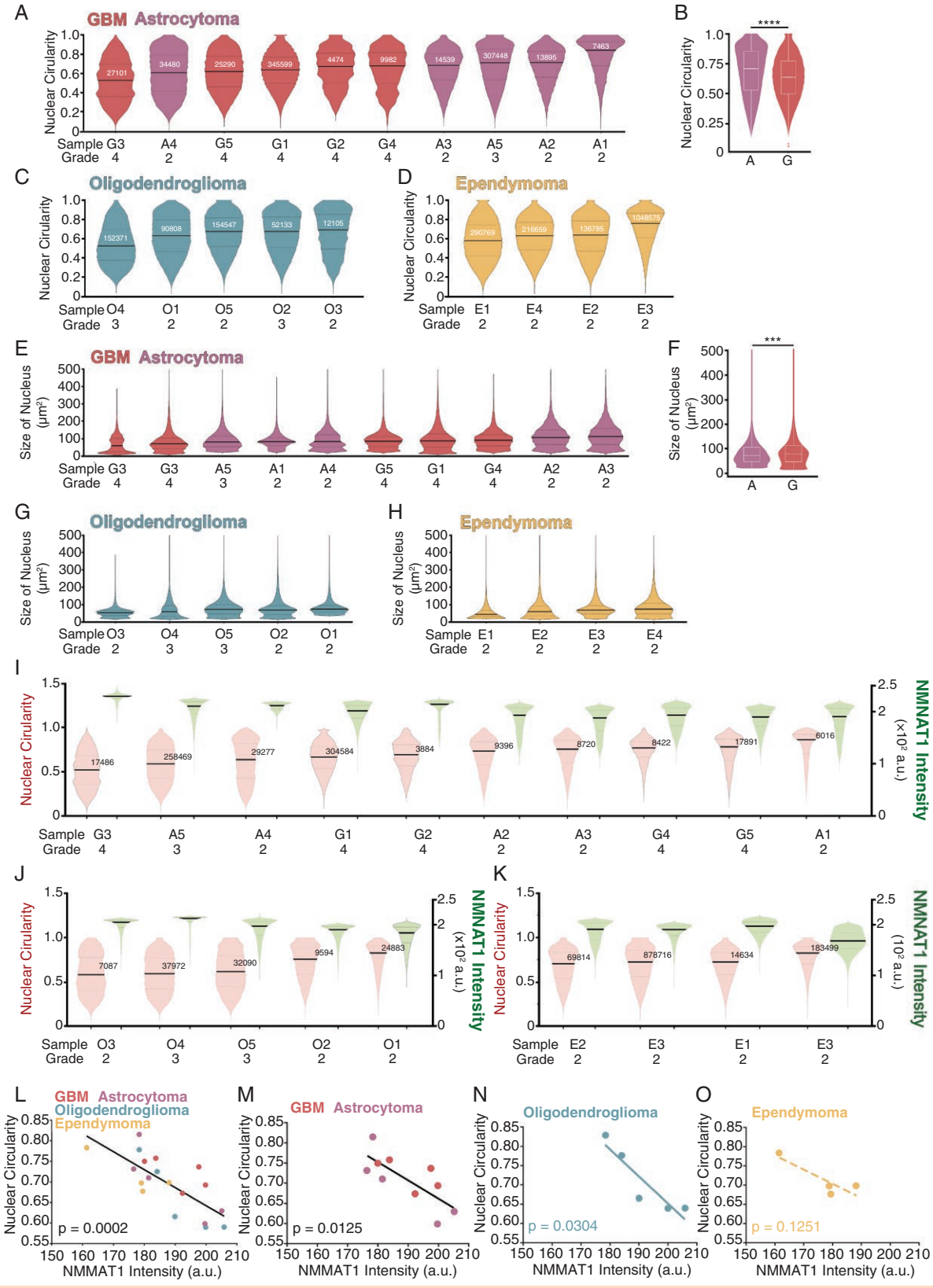

Figure 4. Reduced nuclear circularity and increased nucleus size are observed in GBM, and NMNAT1 expression is correlated with nuclear circularity in human gliomas. (A, C, and D) Quantification of nuclear circularity for gliomas in individual samples. Nucleus numbers are listed. Samples are aligned by median value and grouped into GBM and astrocytoma (A), oligodendroglioma (C), and ependymoma (D). (B) Quantification

of nuclear circularity for astrocytoma and GBM. The significance level was established by the Wilcoxon rank sum test. (E, G, and H) Quantification of the nucleus size in GBM and astrocytoma (E), oligodendroglioma (G), and ependymoma (H). (F) Quantification of nucleus size for astrocytoma and GBM. The significance level was established by the Wilcoxon rank sum test. (I–K) Quantification of nuclear circularity and NMNAT1 intensity for each nucleus in individual samples. Samples are aligned by the median value of circularity and grouped into GBM and astrocytoma (I), oligodendroglioma (J), and ependymoma (K). (L) The linear fit of NMNAT1 intensity and nuclear circularity of gliomas. (M) The linear regression of NMNAT1 intensity and nuclear circularity of GBM and astrocytoma. (N and O) The linear regression of NMNAT1 intensity and nuclear circularity of oligodendroglioma (N) and ependymoma (O). ***$P \leq .001$. ****$P \leq .0001$.

5A). To test the role of NMNAT in glioma growth in vivo, we overexpressed NMNAT isoforms. *Drosophila* has one Nmnat gene that expresses 2 protein isoforms through alternative splicing, a nuclear isoform nucNMNAT (PC) and a cytosolic isoform cytNMNAT (PD). The nuclear and cytoplasmic isoforms share similar enzymatic activity but are differentially regulated under stress conditions.[30] We also included an enzyme inactive nucNMNAT^{WR} isoform[23] to assess the requirement for enzyme activity. We generated flies expressing EGFR together with lacZ (control), nucNMNAT or cytNMNAT, and nucNMNAT^{WR} (Figure 5A). Expression of nuclear NMNAT significantly increased the total volumes of glial neoplasia, while expression of enzyme inactive nucNMNAT^{WR} remarkably decreased the volume of glial neoplasia (Figure 5B), suggesting that the nuclear NAD+ synthesis activity of NMNAT can drive glioma growth while enzyme inactive NMNAT may have dominant negative effects. The glial expression of EGFR caused lethality in the pupal stage and reduced eclosion rate.[31] The fly eclosion rate was correlated with glial neoplasia size. Consistent with the observation of glioma volume, expression of nucNMNAT significantly increased the lethality, while enzyme inactive nucNMNAT^{WR} decreased the pupal lethality and increased the total survival (Figure 5C). We also monitored the fly lifespan of eclosed adult flies and observed that nucNMNAT remarkably reduced the lifespan while nucNMNAT^{WR} expressing flies survived longer than the control (Figure 5D). These observations in glioma models are consistent with the findings in patient glioma samples. To analyze the overall health and mobility of adult flies, we carried out the negative geotaxis behavior analysis on a high-resolution automated geotaxis platform that we recently developed.[24] The platform consists of automated video capturing and tracking of behavior at a 30 Hz sampling rate, extraction of position information, and computation of speed and other parameters.[24] We analyzed over 70 tracks per genotype and found significantly reduced speed in nucNMNAT expression group compared to the control group, and an increased average speed in nucNMNAT^{WR} expression group (Figure 5E). These results suggest that nuclear NMNAT promoted glial neoplasia growth, reduced survival and life span, and impaired mobility.

Nuclear NMNAT Expression Induced Nuclear Atypia In Vivo in a Drosophila *Model of Glial Neoplasia.*

To assess the effect of NMNAT expression on nuclear atypia in vivo, we next analyzed nuclear size and circularity in the EGFR glial neoplasia model in *Drosophila*. Repo, the glial-specific transcription factor, was used as a marker for glial nuclei (Figure 6A). The size and circularity of all Repo-positive nuclei within the glial neoplasia area marked by the accumulation of F-actin (phalloidin positive) were analyzed and plotted (Figure 6B and C). Interestingly, overexpression of nuclear isoform of NMNAT, either wildtype (nucNMNAT) or enzymatically inactive (nucNMNAT^{WR}), increased nuclear size, while overexpression of cytoplasmic NMNAT (cytNMNAT) reduced nuclear size within glial neoplasia (Figure 6B). Next, we analyzed nuclear circularity and found a significant decrease in the circularity of cells overexpressing nuclear NMNAT (nucNMNAT), highlighting heightened nuclear atypia in nucNMNAT expressing cells (Figure 6C). Together, these results reveal a causal effect of overexpression of nuclear NMNAT on nuclear atypia in glioma. Specifically, the effect of nuclear NMNAT requires intact enzyme function suggesting a role for nuclear NAD+ synthesis in glioma genesis.

NMNAT1 Alters the Distribution of Lamin A/C

Lamins are a group of proteins that are the major constituent of nuclear membranes and are critical for supporting nuclear membrane integrity.[32] The expression and localization of lamins are essential for their proper function in the nucleus including regulation of gene expression, cell proliferation, and apoptosis; and aberrant localizations have been associated with many types of cancer and tumor invasiveness.[33] To probe for the underlying cellular mechanisms of NMNAT1 in nuclear atypia in glioma, we carried out immunofluorescent studies for lamin A/C in human GBM cells T98G expressing NMNAT1 (Figure 6D). T98G cells were transfected with human NMNAT1 and immunofluorescently labeled for lamin A/C, NMNAT1, and DAPI to mark the nuclei. Interestingly, compared to nontransfected cells where lamin A/C marks the nuclear envelope (Figure 6H and K), in cells with high levels of NMNAT1, lamin A/C localization was disrupted with fragmented membrane localization and internal accumulation and clustering within the nucleus (Figure 6G and J). The aberrant translocalization from the nuclear membrane and clustering within nucleoplasm in NMNAT1-expressing cells compared to neighboring control cells was better visualized by a fluorescence surface plot (Figure 6L and M). The intensity of lamin A/C within nucleoplasm is increased with NMNAT1 overexpressing (Figure 6P). Overexpression of NMNAT1 increased nucleus size and decreased nuclear circularity (Figure 6N and O). These results suggest NMNAT1 causes aberrant lamin protein localization and compromised nuclear envelop integrity, resulting in nuclear atypia and glioma genesis.

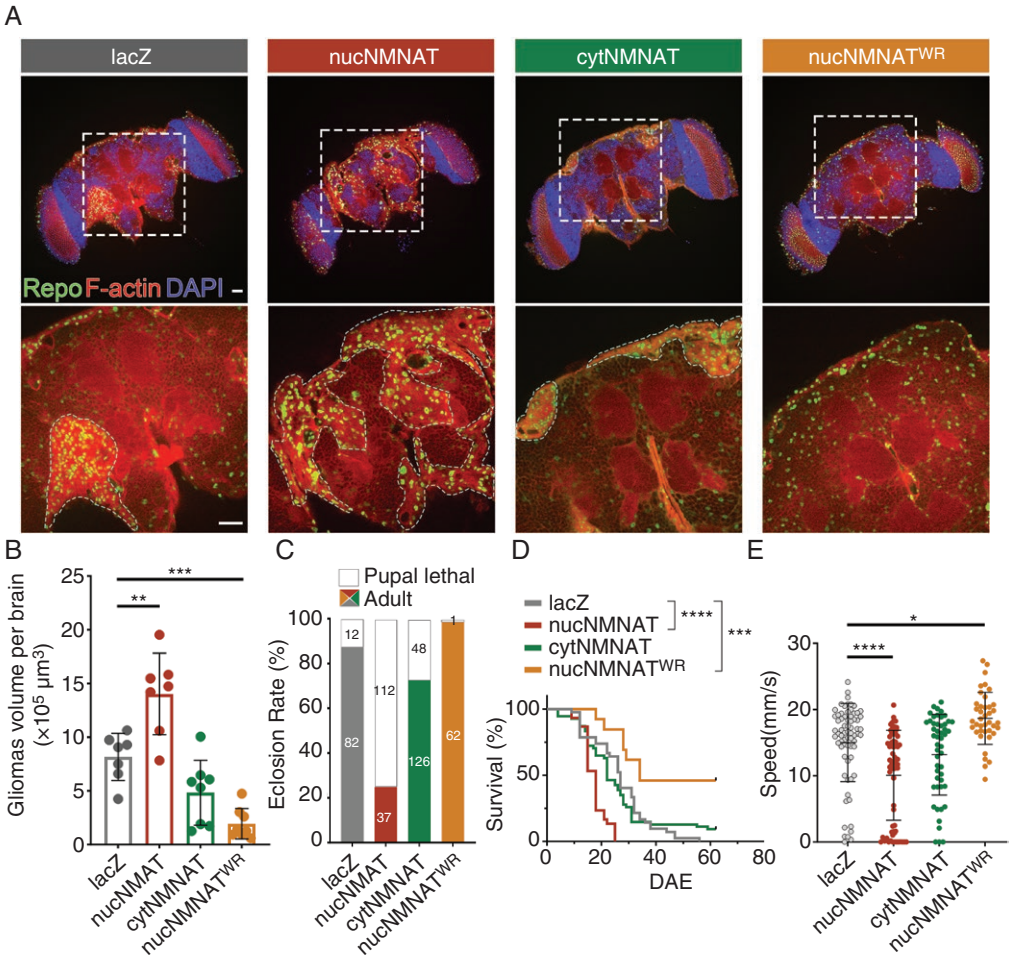

Figure 5. Overexpression of nuclear nicotinamide mononucleotide adenylyltransferase (NMNAT) promotes glial neoplasia growth in *Drosophila*. (A) Brains at 90% pupal stage with the glial expression of lacZ + Egfr, nucNMNAT + Egfr, cytNMNAT + Egfr, and nucNMNAT^{WR} + Egfr were probed for Repo (green), F-actin (red), and DAPI (blue). The second row shows the high magnification of white dashed box areas in the first row. The Glial neoplasia area is marked with cyan dashed lines. Scale bars, 30 μm. (B) Quantification of volumes of glial neoplasia with glial expression of lacZ + Egfr, nucNMNAT + Egfr, cytNMNAT + Egfr, and nucNMNAT^{WR} + Egfr. Data are presented as mean ± SD, $n \geq 7$. The significance level was established by one-way ANOVA post hoc Bonferroni test. (C) The eclosion rate of flies with glial neoplasia. (D) Lifespan for each group. The significance level was established by a log-rank test. (E) Quantification of average climbing speed for each fly. Data are presented as mean ± SD, $n = 7$. The significance level was established by one-way ANOVA post hoc Bonferroni test. *$P \leq .05$. **$P \leq .01$. ***$P \leq .001$. ****$P \leq .0001$.

Discussion

In this study, we have elucidated a pivotal role of compartment-specific NMNAT in driving glioma growth, where nuclear NAD+ synthase NMNAT promotes NAD+-dependent metabolic processes, induces lamin A/C mislocalization and nuclear atypia, leading to aggressive glioma growth and poor survival.

NAD+ serves as an essential cofactor in cellular energy metabolism, and its dysregulation has been implicated in various diseases, including cancer.[5,6] In the past decade, efforts to target the NAD+ synthetic pathway have primarily focused on the rate-limiting enzyme, NAMPT.[34,35] However, clinical trials targeting NAMPT have faced challenges, including low efficacy and high side effects.[36,37] Given the highly dynamic and compartmentalized function of NAD+, emerging evidence supports a model of local regulation of NAD+ concentration.[38] As the adenylyltransferase catalyzes the last step of NAD+ synthesis, NMNAT proteins are compartmentalized to support the local production of NAD+. Specifically, NMNAT1 is nuclear localized and has been shown to contribute to various types of cancer such as acute myeloid leukemia and breast cancer through interacting with NAD+ consuming enzyme SIRT6/7 or SIRT1 to support deacetylation activity.[10,39] Nicotinamide mononucleotide adenylyltransferase 2 is cytosolic and has been found to support ovarian cancer growth through enhancing NAD+-dependent mono ADP-ribosylation of the ribosome by PARP16[14] and promoting colorectal cancer development through regulating SIRT6-mediated deacetylation of p53.[11] These findings elucidate the

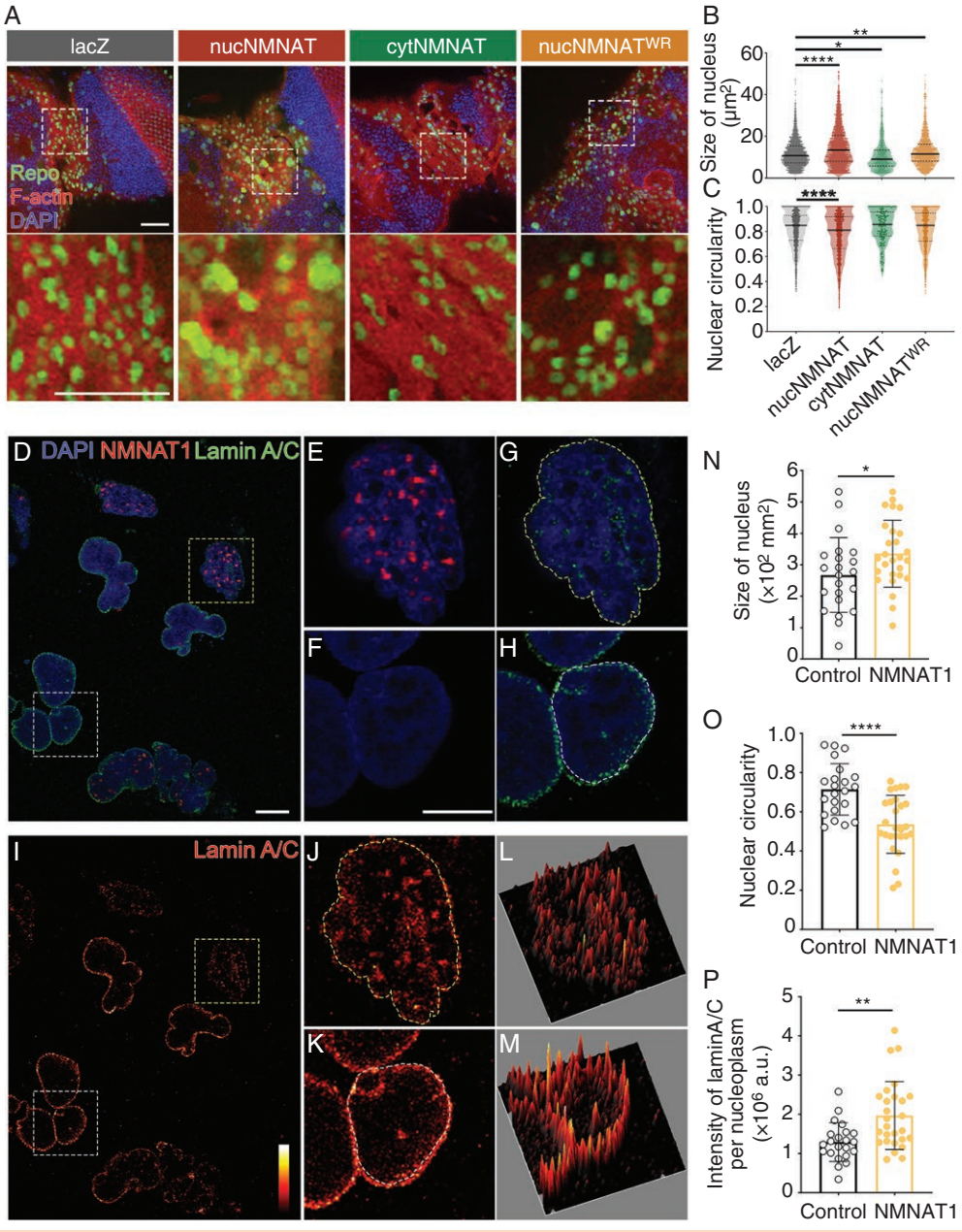

Figure 6. Overexpression of nuclear nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) leads to nuclear atypia by altering lamin A/C localization. (A) Brains at 90% pupal with the glial expression of lacZ + Egfr, nucNMNAT + Egfr, cytNMNAT + Egfr, and nucNMNAT^{WR} + Egfr were probed for Repo (green), F-actin (red), and DAPI (blue). The second row shows the high magnification of white box areas in the first row. Glial neoplasia is marked with dashed lines. The third row shows the high magnification of the glial neoplasia area in the second row. Scale bars, 30 μm. (B and C) Quantification of nucleus size (B) and nuclear circularity (C) for each cell in the glial neoplasia area with the glial expression of lacZ + Egfr, nucNMNAT + Egfr, cytNMNAT + Egfr, and nucNMNAT^{WR} + Egfr. Data are presented as median ± quarter, $n \geq 3$. The significance level was established by one-way ANOVA post hoc Bonferroni test. (D) Human GBM T98G cells transfected with DsRed-NMNAT1 were stained for DAPI (blue) and Lamin A/C (green). (E–H) The high magnification of the yellow boxed area (E and G) and white boxed area (F and H) in D. The intensity (0-4095) of lamin A/C is indicated in a heat map (I). (D–M) Scale bars, 10 μm. (J and K) The high magnification of the yellow (J) and white boxed (K) area in I. (L and M) The surface plot of J and K. Quantification of nucleus size (N), nuclear circularity (O), lamin A/C intensity (P). Data are presented as mean ± SD, $n \geq 20$. The significance level was established by *t*-test. *$P \leq .05$. **$P \leq .01$. ****$P \leq .0001$.

differential roles of NMNAT isoforms in the progression of tumors. Our studies in gliomas have uncovered that nuclear NMNAT contributes to glioma growth by regulating posttranslational modifications of proteins including PARylation and deacetylation, notably impacting the function and activity of crucial proteins like p53 in response to upstream signaling.[23] Specifically, we have observed that NMNAT1 with PARP1 and p53, forms a complex that locally

facilitates NAD+-dependent PARylation.[23] Cytoplasmic NMNAT2 plays a pivotal role in regulating cytosolic NAD+ levels. Our findings indicate that NMNAT2 expression facilitates the proliferation of GBM cells. Although the specific mechanisms by which NMNAT2 and NAD+-dependent post-translational modifications (PTMs) are regulated remain to be elucidated, we observed that NMNAT2 expression results in elevated levels of PARylation and upregulation of SIRT1 levels in GBM cells.[23] In our analysis, we examined NMNAT expression levels in datasets encompassing LGG and GBM, as well as tissue samples obtained from glioma patients. Our results have revealed a notably high expression of NMNAT1 in gliomas characterized by increased malignancy and poor prognoses. Nicotinamide mononucleotide adenylyltransferase 1 potentially serves as a predictor for glioma prognosis during grading. The NMNAT2 mRNA level is lower in GBM than in LGG. However, we didn't observe the reduced protein level of NMNAT2 in GBM samples. These findings have several implications. Firstly, the significance of the nuclear NAD+ pool maintained by NMNAT1 emerges as a principal driver of glioma proliferation. This role is delineated through in vitro studies in human GBM cells, in vivo experiments using the glial neoplasia model in *Drosophila*, and pathological analyses from glioma patients. Secondly, the effects of NMNAT2-mediated cytoplasmic NAD+ synthesis in glioma growth may involve distinct NAD+-dependent PTMs within cytosol facilitated by cytoplasmic PARPs and SIRTs, such as Mono(ADP)ylation of ribosomes.[14] Moreover, it is important to note that NMNAT1 and NMNAT2 can modulate each other's activity through the shared substrate NMN, highlighting a complex interplay in their regulation of subcellular NAD+ levels.[13] Further investigation into the distinct molecular mechanisms by which NMNAT1 and NMNAT2 regulate glioma growth would be very interesting. Glioma stem cells (GSCs) possess key properties that drive glioma initiation, therapeutic resistance, and recurrence. These cells are often under conditions of high replicative stress, which displays a significant demand for NAD+ to support NAD+-dependent processes such as DNA repair, metabolic regulation, and epigenetic modifications. Nicotinamide mononucleotide adenylyltransferase 1, as a critical enzyme in NAD+ biosynthesis, could play a pivotal role in maintaining the NAD+ pool within GSCs. Recent studies have suggested that NAD+ signaling is a core axis for GSC maintenance and that inhibition of NAD+ production can attenuate GSC characteristics, including self-renewal and tumorigenic potential.[40] Nicotinamide mononucleotide adenylyltransferase 1 may be involved in supporting the survival and function of GSCs through its role in NAD+ production. Investigating the correlation between NMNAT1 and established GSC markers in the future would be crucial for further understanding the potential link between NMNAT1 activity and stemness in glioma.

Nuclear architecture is maintained by nuclear matrix proteins, nuclear lamins, and nuclear pore complexes.[41] Altered nuclear architecture, or nuclear atypia, is a hallmark of cellular dysfunction. It has been observed in cells after viral infection, under stress, in aging, or cancer.[15,42–44] Although nuclear atypia is widely used in pathological examination, the grading is largely qualitative and visual inspection based.[26] Nuclear atypia is a well-documented feature in many cancers and is frequently associated with increased DNA damage, which promotes genomic instability—a hallmark of cancer progression. DNA damage can arise from various factors, including oxidative stress, replication errors, and environmental insults. Poly (ADP-ribose) polymerase (PARP) enzymes are activated to initiate DNA repair processes in response to this damage.[45] PARP plays a key role in DNA damage response and chromatin remodeling suggests a potential link to nuclear atypia. In our prior work,[23] we showed that overexpression of NMNAT1 in gliomas led to increased DNA damage and elevated PARP expression levels, indicating that DNA damage and subsequent PARP activation may contribute to the pathological features of gliomas. In the present study, we generated an unbiased, Image J-based batch process method to quantitatively determine circularity and size, 2 features of nuclear morphology, analysis of glioma patient tissue shows the correlation between NMNAT1 expression and nuclear circularity. We observed alterations in the nuclear shape associated with NMNAT1 expression. Nuclear atypia is associated with malignancy such as in thyroid nodules.[46] The grading of cancer based on nuclear atypia and other histological features helps in determining the prognosis and guiding treatment decisions. It is a fundamental aspect of cancer diagnosis and management, providing valuable information on the expected behavior of the tumor and the potential response to therapy.

Lamins are critical components in nuclear architecture, influencing various nuclear functions and signaling pathways.[47] Dysregulation of lamin A/C has been shown in cancer.[48,49] In addition, lamin A/C shows abnormal localization and structure in the nucleus.[50] Lamin proteins have been implicated in cancer progression by influencing genomic stability and chromosomal conformation, resulting in promoting cell proliferation and reducing sensitivity to apoptotic signals.[33] For example, lamin A/C proteins promote cell growth, migration, and invasion through the PI3K/AKT/PTEN pathway in prostate cancer.[51] Various studies have suggested that lamin A/C is a potential biomarker for prostate cancer therapy.[52,53] In ovarian cancer, lamin A interacts with heat shock protein HSP90 on DNA damage response to increase drug resistance.[54] Our study here revealed lamin A/C mislocalization as a direct consequence of elevated NMNAT1, further suggesting altered gene expression as a downstream effect of lamin A/C mislocalization, thereby contributing to NMNAT1-mediated glioma progression.

In conclusion, our findings support the working model that sustained nuclear NAD+ by higher NMNAT1 expression is able to accommodate the heightened demand for NAD+-dependent posttranslational modifications of key regulatory proteins including p53, and hence inhibit apoptosis and promote glioma growth. In addition, elevated NMNAT1 expression impairs nuclear lamina architecture and contributes to nuclear atypia. Taken together, our findings underscore the critical contribution of NMNAT-mediated compartmentalized NAD+ synthesis in glioma growth and development. These insights highlight the potential of NMNAT1 to serve as a predictive marker for assessing glioma prognosis and a target for glioma treatment. Glioblastoma is a highly heterogeneous disease influenced by a variety of genetic and epigenetic factors,

including mutations in oncogenes and tumor suppressor genes, alterations in signaling pathways, and changes in the tumor microenvironment. These confounding factors can significantly impact cellular behavior, including proliferation, survival, and morphology. Additional analyses on other key molecular markers and signaling pathways that are known to interact with or influence NMNAT1 activity would provide insights into the role of NMNAT and NAD+ metabolic pathways in maintaining the cellular environment and supporting the aggressive nature of GBM. This comprehensive approach will provide a more nuanced understanding of how NMNAT1 fits into the broader molecular landscape of GBM.

Supplementary material

Supplementary material is available online at *Neuro-Oncology Advances* (https://academic.oup.com/noa).

Keywords

Drosophila | EGFR | glioblastoma | lamin A/C | NAD+

Lay Summary

Gliomas are brain tumors that do not fully respond to current treatments. NAD+ is a molecule that plays an important role in cancer growth. The authors of this study wanted to understand how a protein called nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1), which is involved in the production of NAD+, might affect glioma growth. To do this they studied the proteins of tumors from 19 patients with gliomas and found that higher levels of NMNAT1 were linked to faster tumor growth. They also conducted laboratory experiments and discovered that the protein NMNAT1 disrupted the structure of the cells, which helped the tumors grow.

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Conflict of interest statement

None declared.

Authorship statement

R.G.Z., J.L., and Y.Z. designed experiments; Z.D.-P. generated critical reagents; J.L. performed the experiments and prepared figures; R.G.Z., J.L., Y.Z., T.C., and S.H.G. analyzed data. J.L., R.G.Z., and Y.Z. wrote and edited the manuscript.

Data availability

All data are included within the main text, figures, and Supplementary Materials. The patient mRNA expression profile and survival plots are accessible from the GEPIA database. Raw images of immunohistochemistry of patient pathological slides are available upon request.

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