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NEK2 promotes TP53 ubiquitination to enhance the proliferation and migration of TP53 wild-type glioblastoma cells

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The most common primary malignant tumor in the adult brain is glioblastoma multiforme (GBM); however, its underlying pathogenic mechanism remains elusive. The never in mitosis (NIMA)-related kinase 2 (NEK2) has been closely associated with the prognosis of various malignancies. Nevertheless, the complete elucidation of NEK2's potential clinical value, particularly in glioma prognosis and development, remains lacking. U87MG and A172 glioblastoma cells were infected with sh-NEK2 lentivirus or oe-NEK2 plasmid to investigate the effect of NEK2 on cell proliferation, migration, and invasion. Cell viability was measured using CCK-8 and colony formation assays, while Transwell assay was utilized to assess cell migration and invasion. Protein expression levels were determined through western blot analysis. Additionally, CGGA and TCGA databases were used for bioinformatics analysis in order to examine the NEK2 expression. Through comprehensive bioinformatics analysis, we identified elevated mRNA expression levels of NEK2 in gliomas compared to normal tissues, which correlated with poor prognosis among glioma patients. Moreover, functional experiments revealed that silencing NEK2 suppressed glioma cell proliferation while overexpression of NEK2 promoted migration and invasion capabilities. Finally, our study uncovered that NEK2 regulates the malignant progression of TP53 wild-type glioblastoma by facilitating TP53 ubiquitination.

Key words: the never in mitosis (NIMA)-related kinase 2 (NEK2); glioblastoma; TP53; ubiquitination; bioinformatics

The glioma, a malignant tumor that primarily occurs in the brain of adults, can be categorized into four different grades:1, 2, 3, and 4 [1, 2]. Despite the recent progress in therapeutic approaches, the outlook for individuals with glioma remains discouraging. The median survival time for grade 2 and 3 gliomas is approximately 11.6 years and 3 years respectively, the survival duration for individuals diagnosed with grade 4 gliomas is typically restricted to a mere 15 months [3–5]. Therefore, gaining an enhanced comprehension of the underlying mechanisms governing glioma occurrence crucial to discovering new diagnostic indicators and therapeutic strategies.

The NIMA-related kinase (NEK) 2, a serine/threonine kinase belonging to the NEK family, exhibits specific localization at the centrosome [6]. NEK2 is widely acknowledged as a versatile protein involved in the regulation of various aspects of the cell cycle, including replication and segregation of centrosomes, stabilization of microtubules, attachment of kinetochores, and activation of the spindle assembly checkpoint [7–10]. In the realm of scientific academia, there has been considerable focus on the significant involvement of NEK2 in the development of tumors in recent years. Multiple research studies have consistently revealed a robust association between its increased expression and unfavorable overall survival outcomes across various types of tumors [11–14]. More significantly, several studies have demonstrated an upregulation of NEK2 expression in glioma cells, thereby promoting the malignant progression of these cells. Furthermore, its association with a poor prognosis in patients further underscores its clinical relevance. For example, NEK2 promotes glioblastoma malignancy through the NIK/NF-κB signaling pathway [15]. However, the underlying mechanism responsible for the promotion of glioma progression by NEK2 has yet to be elucidated.

TP53 gene encodes the p53 protein, a pivotal regulator of cellular division and apoptosis, which undergoes activation and stabilization in response to diverse stressors including radiation, toxicants, hypoxia, excessive production of reactive oxygen species (ROS), and dysregulated cell cycle [16]. The TP53 tumor suppressor gene is the most frequently altered gene in cancer, and extensive research spanning several decades has unequivocally demonstrated its pivotal role in driving neoplastic processes, encompassing both initiation and progression of malignancies, such as acute myeloid leukemia [17], bone and soft tissue sarcomas [18], breast cancer [19], etc. Concurrently, studies have demonstrated that TP53 gene mutations are prevalent in glioma cells, thereby facilitating the malignant progression of this disease [20–23]. However, limited research has been conducted on the role and underlying mechanisms of wild-type TP53 in glioma.

In this study, we performed an initial examination of the contrasting levels of NEK2 expression in normal and tumor tissues within the database, along with its prognostic importance. Furthermore, we examined the influence of NEK2 removal and upregulation on the malignant advancement of glioma. Mechanistically, our findings additionally confirmed that NEK2 enhances the ubiquitination of TP53, resulting in augmentation of wild-type TP53 glioma cells. These results suggest that NEK2 promotes TP53 ubiquitination and facilitates the malignant progression of GBM tumors with wild-type TP53 status; thus, highlighting its potential as a promising therapeutic target.

Materials and methods

Bioinformatics analysis. The data derived from the Chinese Glioma Genome Atlas [24, 25] and The Cancer Genome Atlas were employed for conducting analyses on gene expression and survival. Gene ontology (GO) [26] and Kyoto Encyclopedia of Genes and Genomes (KEGG) [27] analyses were performed using the DAVID tool [28] with information obtained from the CGGA. The R software's ggplot2 package was employed for the execution of Analysis gene mapping.

Cell lines. Five human malignant glioma cell lines: U87MG (TP53 wild-type), LN18, A172(TP53 wild-type), T98G, U251, and NHA were acquired from the American Type Culture Collection. The DMEM (Corning, China) culture medium was used for culturing the cell lines, a modified Eagle medium, with the addition of 10% FBS (Procell, China), derived from fetal bovine serum. All cellular cultures were maintained in a humidified atmosphere with 5% CO_2 at a temperature of 37 °C.

Lentivirus and plasmid transfection. The NEK2 and control shRNA lentiviral vectors were acquired from Genchem, Inc. The target cells achieved a cell density of 70–80% following the transfection of 5 μl lentivirus. After a 12 h incubation period, the lentivirus-containing medium was substituted with a fresh DMEM medium supplemented with 10% FBS. After being infected, the cell lines were subjected to screening using a purinomycin solution (2 μg/ml, Solarbio, Inc.) and then maintained with a purinomycin solution (1 μg/ml) to establish stably infected cells for subsequent experiments. The subsequent experiments were conducted 48 h after the transfection process.

The NEK2 plasmid was synthesized by Gene Chemical Technology. An overexpression plasmid of NEK2 was obtained from Gene Chemical Technology. The plasmid concentration was 543 ng/μl and it was incubated at 37 °C for 48 h with Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) before conducting subsequent experiments.

Antibodies. The experiments employed antibodies against the following targets: NEK2 (WB: 1:1000, IP:1:100, ABclonal, A6811); TP53 (WB:1:1000, IP:1:100, ABclonal, A0263); GAPDH (1:10000, ABclonal, A19056), Ubiquitin (WB: 1:2000, Cell Signaling Technology, 3936).

Western blotting (WB), co-immunoprecipitation (co-IP), and ubiquitination assay. Cells were lysed in RIPA buffer (R0010; Solarbio, Inc.) supplemented with a protease inhibitor (P0100; Solarbio, Inc.), followed by the determination of cellular protein concentrations. The proteins that had been dissolved were then subjected to a process of denaturation by being exposed to high temperatures in a metal bath set at 98 °C for a duration of 10 min. Subsequently, equivalent quantities of protein were subjected to immunoblotting using the corresponding antibody. A gel imaging system was employed for the quantification of protein expression levels.

Cell samples were subjected to lysis using IP lysis buffer (R0010; Solarbio, Inc.). A mixture of Protein A/G agarose (sc2003; Santa Cruze, Inc.) and the appropriate antibody was prepared and added to the lysed cell samples. The mixture obtained was subjected to 16 h incubation at a temperature of 4 °C. Subsequently, immunoprecipitated proteins were detected by WB.

For the ubiquitination assay, cells were exposed to MG132 for a duration of 6 h and the cells were lysed in IP lysis buffer. The samples underwent gentle and constant shaking at 4 °C over 12 h while being incubated with anti-TP53 antibody or IgG. Finally, immunoprecipitation experiments using Ubiquitin antibody were conducted on the extracted proteins to determine the TP53 ubiquitination levels.

Cell counting kit-8 assay (CCK-8). The viability of glioma cells was assessed using the CCK-8 assay (CA1210; Solarbio, Inc.) to investigate the impact of NEK2 loss- and gain-of-function, as well as rescue experiments. The 96-well plates were utilized to culture cells at a density of 2,000 cells per well, and the incubation period lasted from 1 to 5 days. Afterward, following a 2 h incubation period with the CCK-8 solution, the microplate reader was utilized to measure and quantify the absorbance value (BioTek Instruments, Inc.).

Colony-formation assay. The 6-well plate was initially populated with a cell count of 500/well, followed by an incubation period of 14 days. Subsequently, the cells were

treated with a 4% paraformaldehyde solution for around 15 min at ambient temperature to fix them and then subjected to staining using a solution comprising of 2.5% crystal violet blue for approximately the same duration. The cell colonies obtained were visually examined, and a camera was employed to document photographic proof.

Transwell assay. The Cell Culture Insert (pore size: 3 μm; Corning, Inc.) was used for the Transwell assay, where cells (20,000 cells) were grown in DMEM medium without the addition of serum. The lower compartment was filled with a medium containing FBS as an attractant for chemotaxis. After a period of 48 h of incubation, the cells in the Transwell chambers were immobilized by treating them with a 4% paraformaldehyde solution, followed by staining using a 2.5% crystal violet staining solution. The quantity of infiltrating cells on the underside of the filter was subsequently measured.

Inhibitors of TP53 degradation. The TP53 degradation inhibitors (nutlin-3; cat. no. S1061) were procured from Selleck Chemicals. NEK2-overexpressing U87MG and A172 cells were subjected to a 24 h treatment with 2 μM nutlin-3 for reversion experiments.

Immunofluorescence assay. Cell slides were prepared in advance, and 2×10^4 cells were planted on each 24-well cell slide. After 24 h, the cells were fixed with 4% formaldehyde for 15 min, followed by the incubation with 0.5% Triton X-100 at room temperature for 20 min. A sufficient amount of diluted NEK2 and TP53 antibodies were then added to each well. The solution was placed in a cartridge and incubated overnight at 4 °C. A diluted fluorescent secondary antibody was added and incubated at 37 °C for 1 h without light. 100 μl DAPI working solution was added to each well for 10 min for staining. Fluorescent images were collected by applying an anti-fluorescence attenuating sealant (Sigma, USA) inverted onto a slide.

Statistical analysis. The mean ± SD was used to express all quantitative data. The analysis was conducted utilizing the software GraphPad Prism 8. The comparison between the two groups was conducted using the independent samples t-test, while for comparing multiple groups, the one-way analysis of variance (ANOVA) was employed. All statistical analyses were conducted using two-sided tests, and various threshold values ($p<0.05$, $p<0.01$, $p<0.001$) were deemed as evidence of statistically significant distinctions.

Results

The expression of NEK2 in glioblastoma surpasses that observed in low-grade glioma and astrocytoma, exhibiting a significant correlation with prognosis. To evaluate the disparities in NEK2 expression across different grades of glioma, we conducted an analysis on NEK2 expression data obtained from databases. The analysis of data from the CGGA database indicated a notable increase in NEK2 expression in WHO grade 4 gliomas when compared to WHO grade 2 and 3 gliomas (Figure 1A). The findings derived from TCGA database suggest a noteworthy decline in NEK2 expression in GBM when compared to low-grade gliomas. (Figure 1B). Importantly, the survival analysis demonstrated a notable increase in the survival rate of glioma patients exhibiting reduced NEK2 expression. (Figures 1C, 1D). Subsequently, the evaluation of NEK2 protein expression was conducted in cell lines of malignant glioma (A172, T98G, LN18, U251, and U87MG) as well as NHA cell line using the WB technique (Figures 1E, 1F). We decided to conduct further analysis on the cell lines U87MG and A172 due to their high levels of NEK2 expression along with being TP53 wild-type cell lines. In conclusion, our study demonstrates that compared to low-grade gliomas and astrocytomas, glioblastomas display notably elevated expression levels of NEK2, indicating a substantial correlation with prognosis.

NEK2 exerts inhibitory effects on the proliferation, migration, and invasion of glioma cells. To investigate the influence of NEK2 on glioma cells, we generated plasmids for upregulation and created two sets of shRNA lentiviruses to enhance or suppress NEK2 expression. The transfection efficiency of lentiviral overexpression and knockdown techniques, as well as the NEK2 expression level in U87MG and A172 cells, was evaluated using the WB method (Figures 2A, 2B; Supplementary Figures S1A, S1B). Subsequently, the CCK-8 assay and colony formation assay were employed to evaluate the proliferation capacity of U87MG and A172 cells. As illustrated in Figure 2C and Supplementary Figure S1C, receiving overexpressed plasmid transfection, the cells demonstrated a noticeable enhancement in cellular proliferation ($p<0.001$). Conversely, the lentiviral transfection of NEK2 knockdown led to a decrease in the cellular proliferation capacity ($p<0.001$). Repetitively, the enhanced expression of NEK2 led to an elevated capacity of glioma cells in colony formation (Figures 2D–2F; Supplementary Figures S1D–S1F), while downregulation of NEK2 inhibited their colony formation ability ($p<0.001$; Figures 2D–2F; Supplementary Figures S1D–S1F).

To investigate the impact of NEK2 on cellular migration and invasiveness, a Transwell assay was conducted. Initially, upregulation of NEK2 expression significantly enhanced glioma cell migration ability ($p<0.001$; Figures 2E, 2G; Supplementary Figures S1E, S1G), whereas downregulation of NEK2 expression markedly decreased their motility ($p<0.001$; Figures 2E, 2G; Supplementary Figures S1E, S1G). Following that, the lower chamber was supplemented with Matrigel to facilitate the execution of the Transwell invasion assay. Consistently, in glioma cells, overexpression of NEK2 augmented the invasive potential ($p<0.001$; Figures 2E, 2H; Supplementary Figures S1E, S1H), while knockdown of NEK2 using shRNA significantly inhibited their invasion ($p<0.001$; Figures 2E, 2H; Supplementary Figures S1E, S1H). These findings unequivocally demonstrate that elevated levels of NEK promote glioma cell motility.

Enrichment analyses of NEK2 were performed using KEGG and GO. The functionality and pathway enrichment

analyses of NEK2 were further explored by utilizing data from the CGGA database. The results of the GO analysis showed a notable enrichment of NEK2 biological processes (BP) in 'nuclear division' and 'organelle fission' (Figure 3A). Revealing the cellular component (CC) analysis, NEK2 was found to be predominantly enriched in the 'chromosomal region' and 'spindle' (Figure 3B). The enrichment of 'microtubule binding' and 'tubulin binding' was mainly observed in changes in molecular function (MF) (Figure 3C). The examination of KEGG data indicated a notable increase in NEK2 modifications in both TP53 and the cell cycle (Figure 3D).

NEK2 governs the regulation of TP53 and orchestrates its ubiquitination process. Taking into account the findings depicted in Figure 3, our study delved into the potential

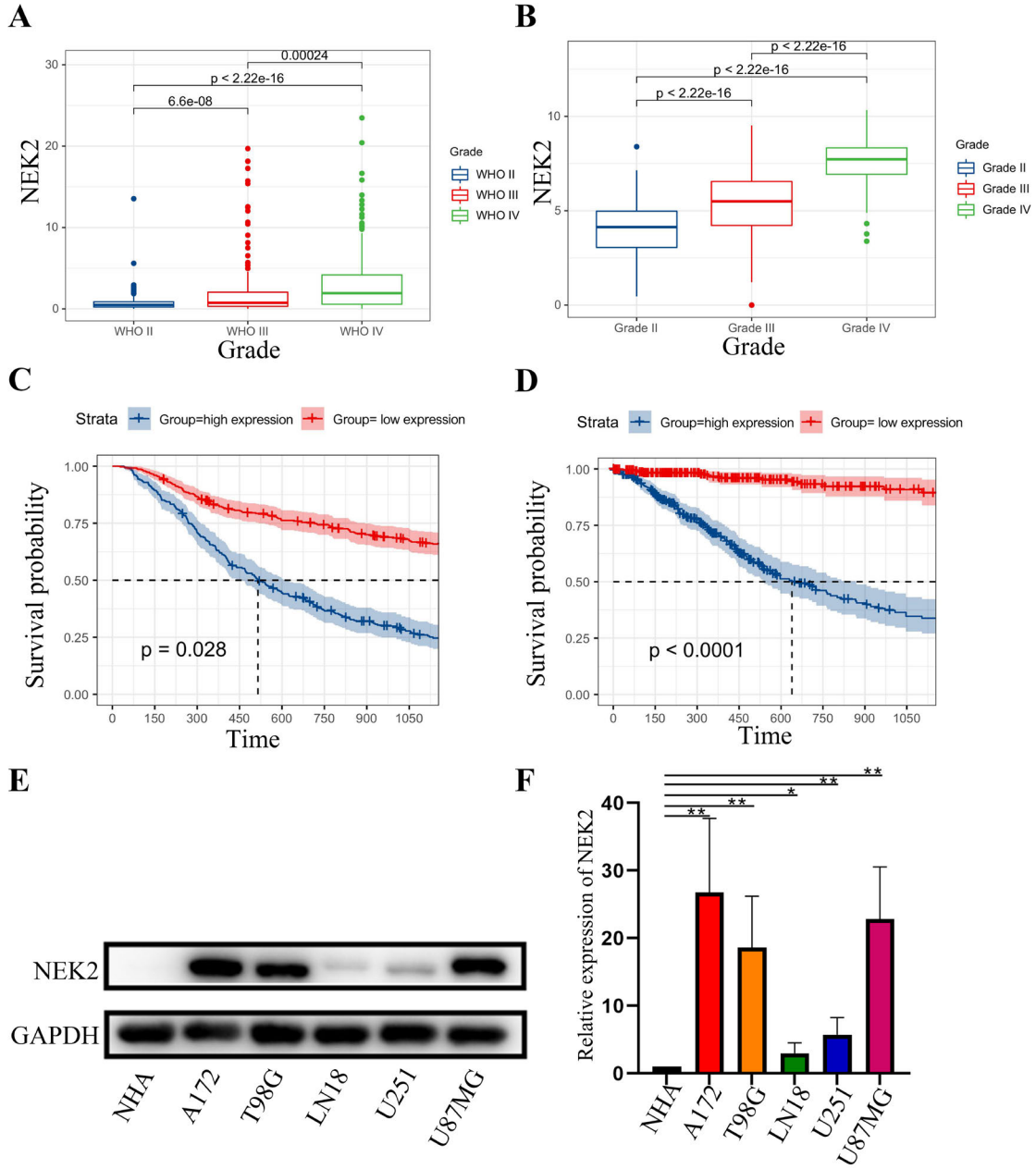

Figure 1. The expression of NEK2 in glioblastoma and astrocytoma, exhibiting a significant correlation with prognosis. A) The CGGA database was utilized for conducting gene expression analysis. B) The analysis of gene expression was conducted utilizing data obtained from TCGA database. C) Overall survival analysis was carried out employing data obtained from the CGGA database. D) Overall survival analysis was conducted utilizing data sourced from TCGA database. E, F) The Western blot analysis was employed to evaluate the protein expression levels of NEK2 in cell lines of astrocytoma and glioma. NEK2, NIMA-related kinase 2; GBM, glioblastoma; TCGA, The Cancer Genome Atlas; CGGA, Chinese Glioma Genome Atlas. Statistical significance was determined using *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

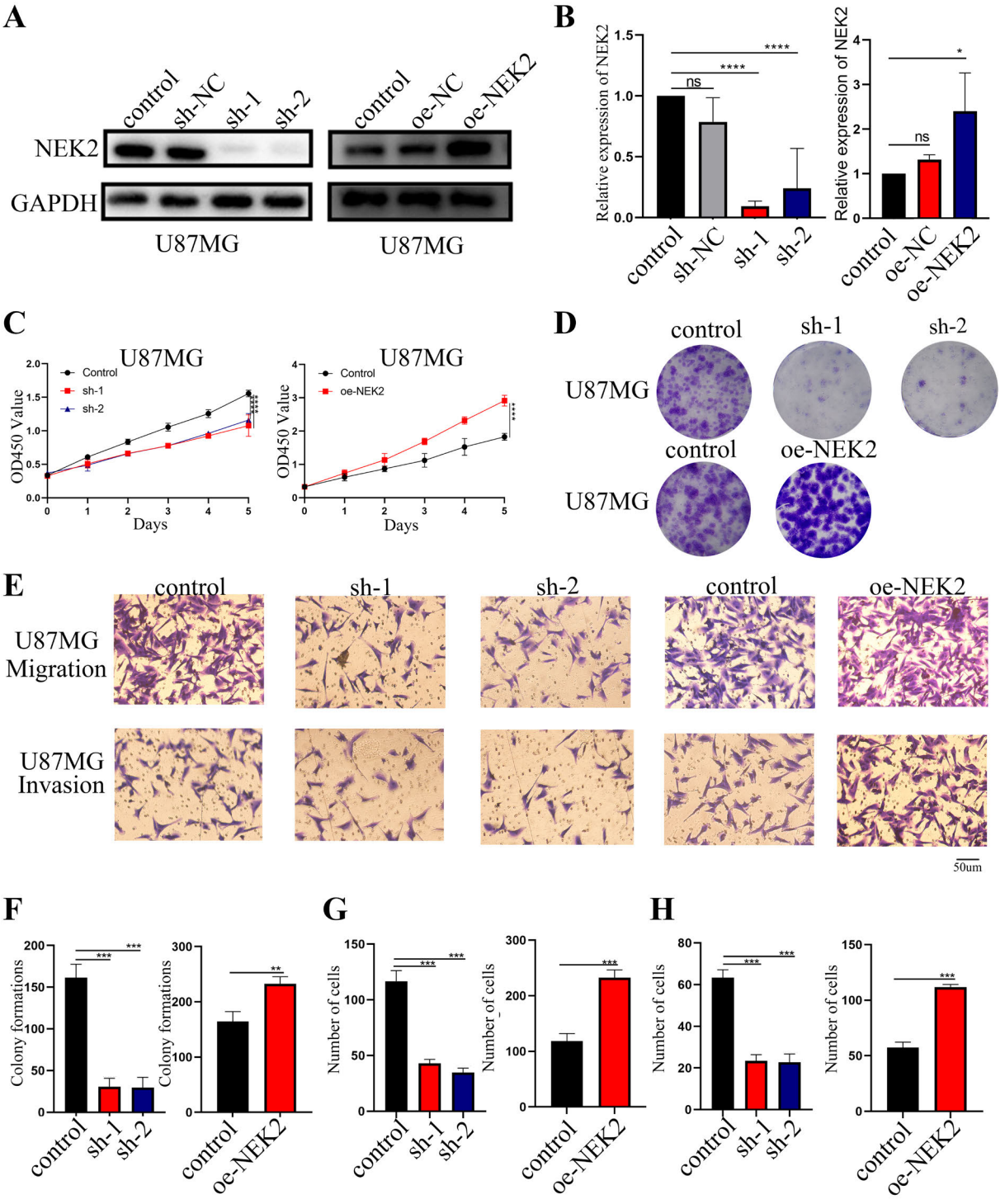

Figure 2. NEK2 exerts inhibitory effects on the proliferation, migration, and invasion of glioma cells. A, B) The transfection efficiency of knockdown (shRNA-NEK2-1, sh-1; shRNA-NEK2-2, sh-2) and plasmid overexpression (oe-NEK2), as well as the NEK2 expression in U87MG glioma cell line, was evaluated using WB analysis. C) Repercussions on the proliferation of U87MG glioma cells were observed in CCK-8 assay outcomes when NEK2 expression was downregulated or upregulated. D, F) The findings from the colony formation assay revealed how altering NEK2 expression levels affected the ability of U87MG glioma cells to form colonies. E, G, H) Transwell assays were conducted to assess the migratory and invasive potential of U87MG glioma cells following modulation of NEK2 expression levels. The data are presented as mean ± SD. Statistical significance was determined using *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Abbreviations: NEK2-NIMA-related kinase 2; NC-negative control; sh-short hairpin; oe-overexpression

role of NEK2 in modulating TP53 activity within gliomas characterized by wild-type TP53. WB demonstrated that knockdown or overexpression of NEK2 could influence the protein content of TP53 (Figures 4A, 4B; Supplementary Figures S2A, S2B). Subsequently, a co-IP experiment was conducted to investigate the interaction between NEK2 and TP53, revealing their mutual interaction (Figure 4C; Supplementary Figure S2C). At the same time, in order to verify the existence of co-localization between NEK2 and TP53, we conducted immunofluorescence experiments. The results show that NEK2 and TP53 are co-localized (Supplementary Figures S3A, S3B). NEK2 may contribute to the promotion of tumors through its direct interaction with TP53, supporting our initial hypothesis.

To elucidate the post-translational mechanism underlying NEK2-mediated promotion of TP53 expression, glioma cells were subjected to NEK2 knockdown or control conditions and subsequently treated with the protein synthesis inhibitor CHX at various time points. The findings indicated a decrease in the rate of TP53 degradation in the NEK2 knockdown cohort as compared to the control group (Figures 4D, 4E; Supplementary Figures S2D, S2E), suggesting that NEK2 enhances the breakdown of TP53 protein, thereby facilitating its degradation. Glioma cells were subjected to MG132 treatment, which effectively suppressed proteasomal degradation and led to a notable rise in the accumulation of ubiquitinated proteins. To analyze the levels of ubiquitination, immunoprecipitated TP53 samples from each group were adjusted for

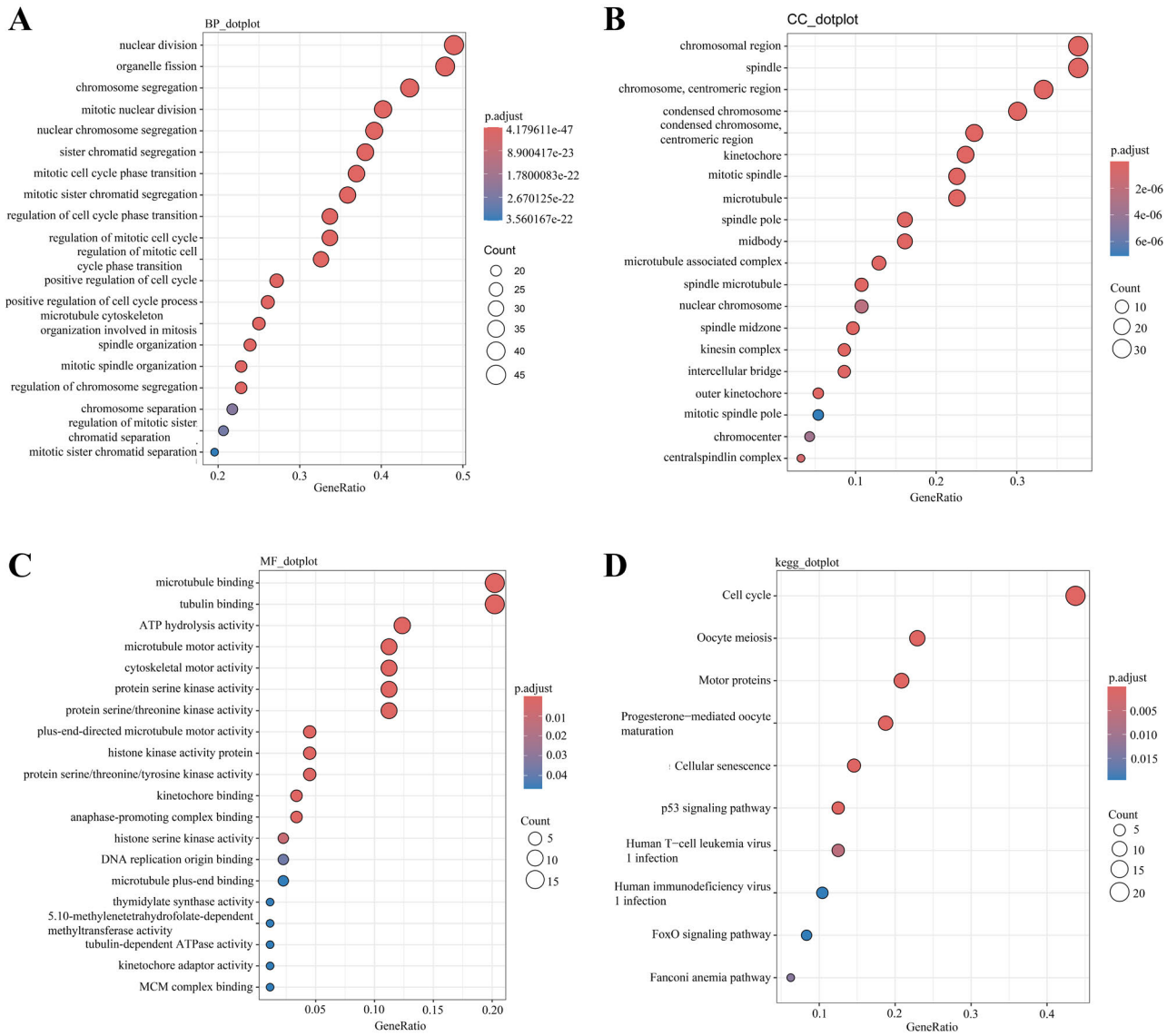

Figure 3. Enrichment analyses of NEK2 were performed using KEGG and GO databases. A) Biological process, B) cellular component, C) molecular function, and D) KEGG pathway. The R language package 'ggplots' was utilized to create the bubble diagram, aiming to enhance its professional and scholarly appearance. Abbreviations: GO-Gene Ontology; KEGG-Kyoto Encyclopedia of Genes and Genomes; NEK2-NIMA-related kinase 2

total protein content and subsequently examined using WB (Figure 4F; Supplementary Figure S2F). In summary, our research findings indicate that the promotion of the ubiquitination proteasomal degradation pathway by NEK2 plays a crucial role in facilitating glioblastoma development through its regulation of TP53 expression.

The inhibition of TP53 degradation can effectively counteract the proliferation, migration, and invasion induced by NEK2 overexpression in glioma. It remains unclear if NEK2's regulatory function in the malignant advancement of gliomas is associated with TP53 in TP53 wild-type gliomas. To investigate this, we overexpressed

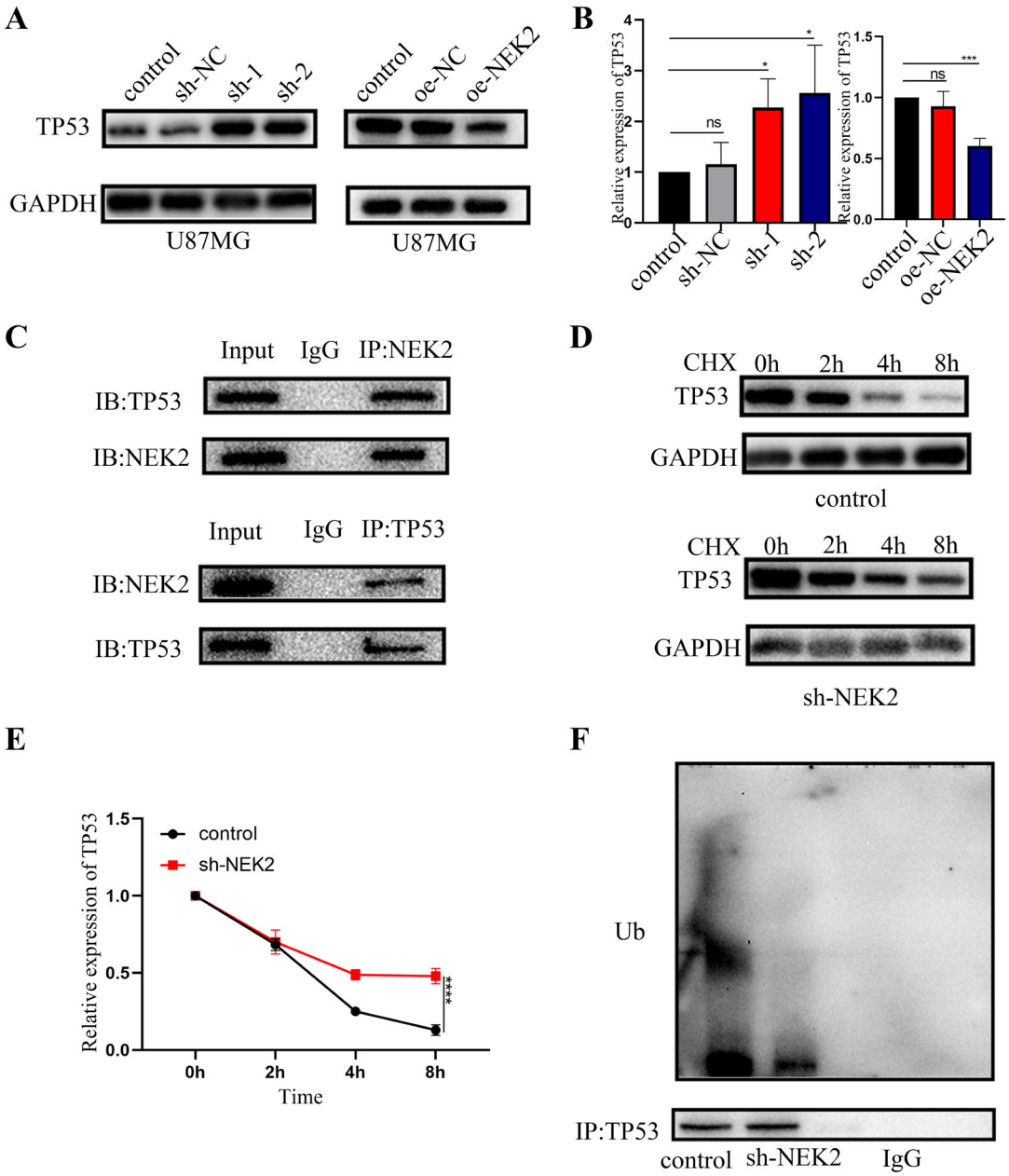

Figure 4. NEK2 governs the regulation of TP53 and orchestrates its ubiquitination process. A, B) The WB analysis was conducted to assess the expression of TP53 in the U87MG glioma cell line after knockdown (shRNA-NEK2-1, sh-1; shRNA-NEK2-2, sh-2) and plasmid overexpression (oe-NEK2). C) U87MG cellular extracts were subjected to lysis using IP lysis buffer, followed by incubation with either NEK2 antibody or control IgG. Immunoblotting analysis was conducted using TP53 and NEK2 antibodies for detection (upper images). To reciprocally immunoprecipitate the proteins, TP53 antibody or control IgG was employed, followed by immunoblotting with NEK2 and TP53 antibodies (lower images). D, E) The levels of TP53 protein were evaluated at different time points (0, 2, 4, and 8 h) after treating lysed U87MG cells with CHX to inhibit the synthesis of proteins F) The Ubi antibody was utilized for the WB analysis to assess the extent of TP53 ubiquitination. In U87MG cells, NEK2 knockdown led to a notable decrease in TP53 ubiquitination levels. Abbreviations: NEK2-NIMA-related kinase 2; NC-negative control; sh-short hairpin; IP-immunoprecipitation. The data are presented as mean ± SD. Statistical significance was determined using *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

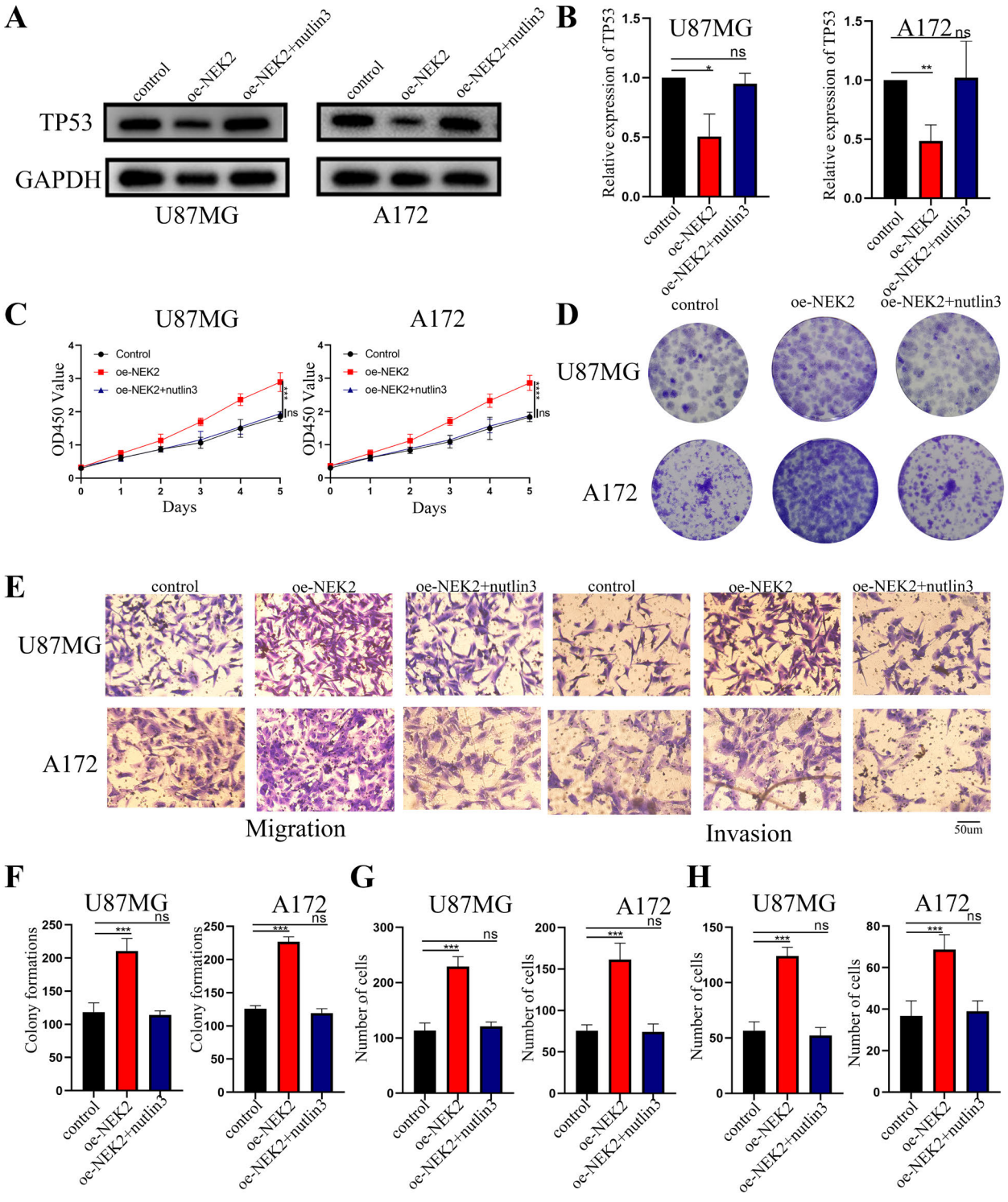

Figure 5. The inhibition of TP53 degradation can effectively counteract the proliferation, migration, and invasion induced by NEK2 overexpression in glioma. A, B) Plasmid transfection was used to induce NEK2 overexpression in U87MG and A172 cells, followed by a 24 h treatment with nutlin-3, an inhibitor of TP53 degradation. WB analysis was conducted to evaluate the impact of this treatment on inhibition. C) The growth of U87MG and A172 cells was assessed over a period of 1–5 days using the CCK-8 assay. D, F) The U87MG and A172 cell lines were subjected to colony formation assay. E, G, H) The migratory and invasive capacities of U87MG and A172 cells were assessed using Transwell migration and Matrigel® invasion assays. The data are presented as mean ± SD. Statistical significance was determined using *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Abbreviations: NEK2-NIMA-related kinase 2; NC-negative control; ns-not significant; oe-overexpression

NEK2 via plasmid transfection in U87MG and A172 cells and treated them with 2 μM nutlin-3, a TP53 pathway inhibitor of degradation, for 24 h. WB confirmed the efficacy of nutlin-3 treatment by showing significantly increased TP53 expression (Figures 5A, 5B). Importantly, the proliferation of NEK2-overexpressing cells was found to be decreased by nutlin-3, as indicated by the CCK-8 assay. (Figure 5C). Colony formation assays demonstrated that NEK2-induced increase in colony formation ability was attenuated following nutlin-3 treatment (Figures 5D, 5F). These findings suggest that nutlin-3 can reverse the proliferative effects induced by NEK2 overexpression in glioma cells via the TP53 pathway. Following that, rescue experiments were performed to evaluate the migratory and invasive capacities. Transwell assays with or without Matrigel® showed that nutlin-3 significantly decreased NEK2-induced increases in glioma cell migration and invasion (Figures 5E, 5G, 5H). In combination, the administration of nutlin-3 has the potential to alleviate the adverse effects of NEK2 overexpression on glioma's malignant advancement by regulating TP53.

Discussion

The NIMA-related kinase (NEK) 2, a serine/threonine kinase belonging to the NEK family, exhibits specific localization at the centrosome. It is widely acknowledged as a multifunctional protein involved in regulating various aspects of the cell cycle [6–10]. Multiple research studies have consistently observed its increased expression in different types of cancers and its correlation with poor overall survival results [11–14]. Hence, the exploration of NEK2's role development and its potential as a viable treatment target has gained substantial attention, resulting in noteworthy progress. Initially, NEK2's promotion of the cell cycle was identified as a major factor contributing to its pro-tumor effects. As a result, the objective of targeting NEK2 to achieve effective anti-tumor outcomes is centered around inhibiting the progression of cancer cells by slowing down their cell cycle during mitosis and cytokinesis [29, 30]. NEK2 is upregulated in cancer and has been linked to the initiation, advancement, spread, and resistance to treatment of tumors [31]. New findings have revealed the immunomodulatory impacts of NEK2, indicating that by targeting PD-L1, inhibiting NEK2 can trigger an immune response against pancreatic cancer [32]. Of great importance, a multitude of research studies have examined the crucial involvement of NEK2 in the advancement of glioma. Xiang and colleagues have provided evidence suggesting that the NEK2-induced activation of the NIK/NF-κB pathway plays a role in enhancing the malignancy levels observed in glioblastoma [15]. Guan *et al.* discovered that the suppression of circular RNA circPITX1 hinders glycolysis, thereby augmenting the sensitivity of glioma cells to radiation therapy through regulation of the miR-329-3p/NEK2 pathway [33]. Ye *et al.* discovered that NEK2 is directly targeted by MiR-128, leading to the induction of apoptosis in glioma cells [34]. Although the potential role of NEK2 in GBM and its associated pathways has been extensively discussed in these studies, further molecular mechanistic investigations are warranted to assess the clinical significance of NEK2 in GBM. In this ongoing research, we aim to shed light on a previously unidentified process by which NEK2 influences the progression of glioma.

The data from databases were utilized for conducting expression and survival analyses of glioma. The results unveiled a significant increase in NEK2 expression in GBM when compared to low-grade glioma, which was correlated with unfavorable disease progression. Experimental observations offer substantiating evidence for the involvement of NEK2 in promoting the advancement of glioma toward a malignant state. To enhance comprehension regarding the involvement of NEK2 in glioma, we conducted GO and KEGG analyses based on CGGA data. Interestingly, the findings revealed a notable enrichment of the TP53 signaling pathway as the primary pathway linked to NEK2.

The TP53 protein, a crucial suppressor of tumor growth, assumes a fundamental and diverse function in the progression and treatment of cancer [35, 36]. Despite more than 30 years of active research and a vast amount of literature, the complete understanding of the molecular mechanisms that drive TP53's tumor suppressor function is still lacking, continuing to be an area of ongoing investigation [37, 38]. Unraveling the tumor suppressive capacity of the TP53 gene not only holds profound implications for comprehending cancer biology but also offers potential avenues for enhancing cancer treatment through optimized utilization of TP53 function and identification of specific vulnerabilities associated with loss-of-function mutations in TP53. While exerting an effector role, impacting nearly all hallmark features of cancer, it remains unclear which among these functions are indispensable for its potent tumor suppressive activity and how these functions interplay. In the meantime, considerable investigations have been carried out regarding the involvement of TP53 in GBM. Despite unsuccessful attempts to develop drugs that facilitate refolding of mutant proteins into wild-type conformations, endeavors have been made to inhibit negative regulatory proteins MDM2 and MDM4 associated with TP53 [39]. These efforts aim to counteract the defective MDM2 and MDM4 generated by the amplification of their respective genes in GBM patients. During the trial, the administration of AMG 232, an inhibitor targeting MDM2, effectively impeded tumor progression [40]. Additionally, AZD1775, a Wee1 kinase inhibitor with superior brain tumor penetration capabilities, exhibited promising results; however further trials are required to establish its efficacy [41]. Furthermore, previous studies have confirmed that NEK2 can phosphorylate the Ser315 site of TP53, thereby affecting its stability and promoting degradation [42].

To elucidate the underlying mechanism by which NEK2 modulates TP53 in TP53 wild-type gliomas, we conducted co-IP and ubiquitination assays. Our findings unequivocally

validate the direct interaction between NEK2 and TP53, thereby facilitating its ubiquitination process.

To gain a deeper understanding of how NEK2 influences the regulatory effects on TP53 wild-type gliomas during their malignant progression through the TP53, we employed nutlin-3, a TP53 degradation inhibitor, to conduct phenotype rescue assays. Our findings demonstrated that nutlin-3 effectively reversed the NEK2-induced proliferation, migration, and invasion in TP53 wild-type glioma cells. Notably, NEK2 was found to exert its regulatory effects on the development of TP53 wild-type glioma cells via modulation of TP53.

In brief, this research offers valuable insights into how NEK2 regulates the malignant advancement of glioma cells with TP53 wild-type through its direct interaction with TP53 protein and modulation of the degradation pathway involving ubiquitylated proteasome. Furthermore, considering its involvement in the TP53 signaling pathway, NEK2 might potentially enhance the effectiveness of specific therapeutic interventions targeting this pathway. However, this study exclusively investigated the regulatory relationship between NEK2 and wild-type TP53 gliomas without considering mutant TP53 gliomas. Additionally, while our results indicate that NEK2 exerts its influence on glioma via the TP53 pathway at a cellular level, further validation in animal models is warranted. Furthermore, limitations exist regarding clinical translation in this study; hence further examinations are necessary to decipher the fundamental mechanisms and evaluate the clinical importance of NEK2.

Supplementary information is available in the online version of the paper.

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NEK2 promotes TP53 ubiquitination to enhance the proliferation and migration of TP53 wild-type glioblastoma cells

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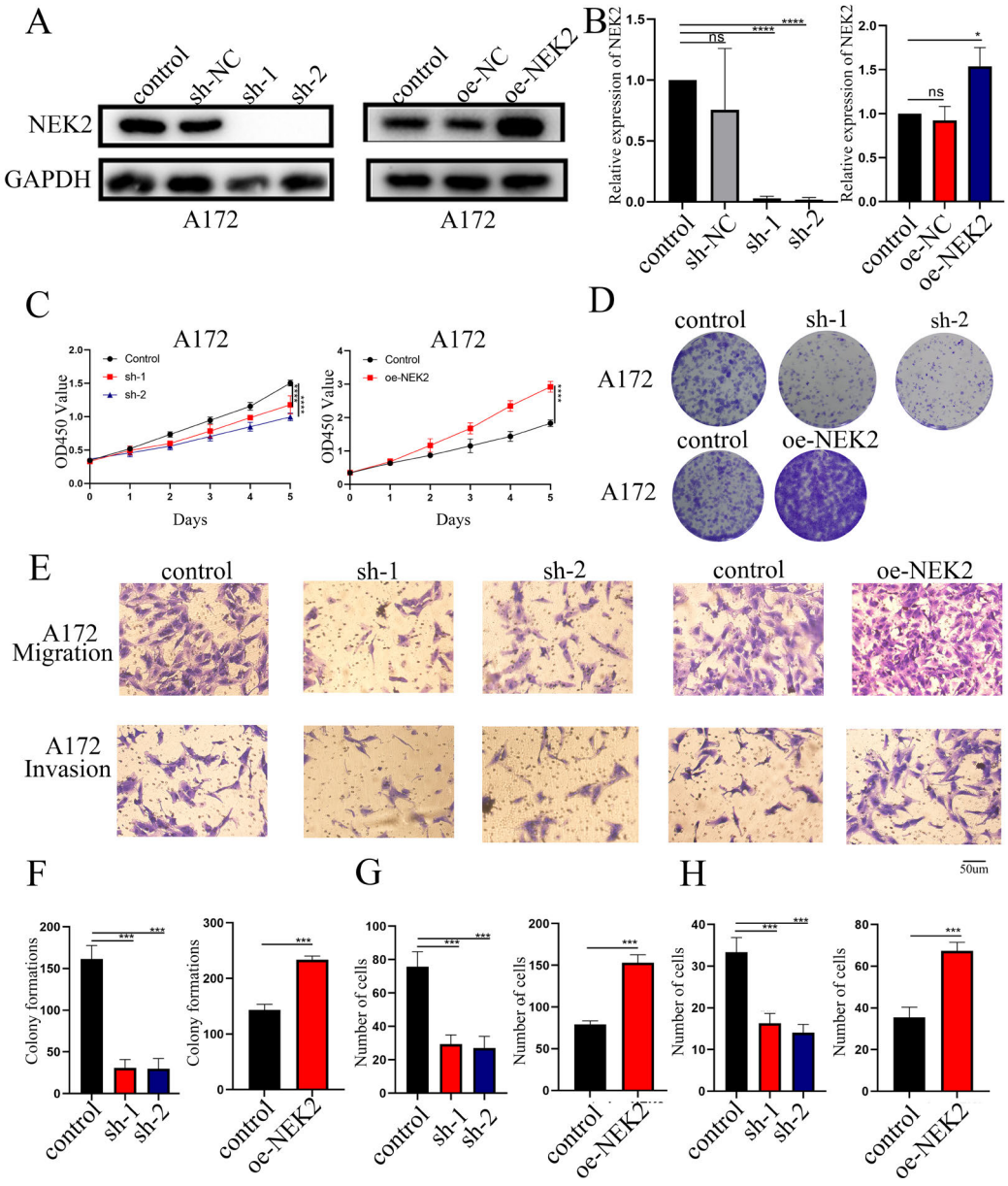
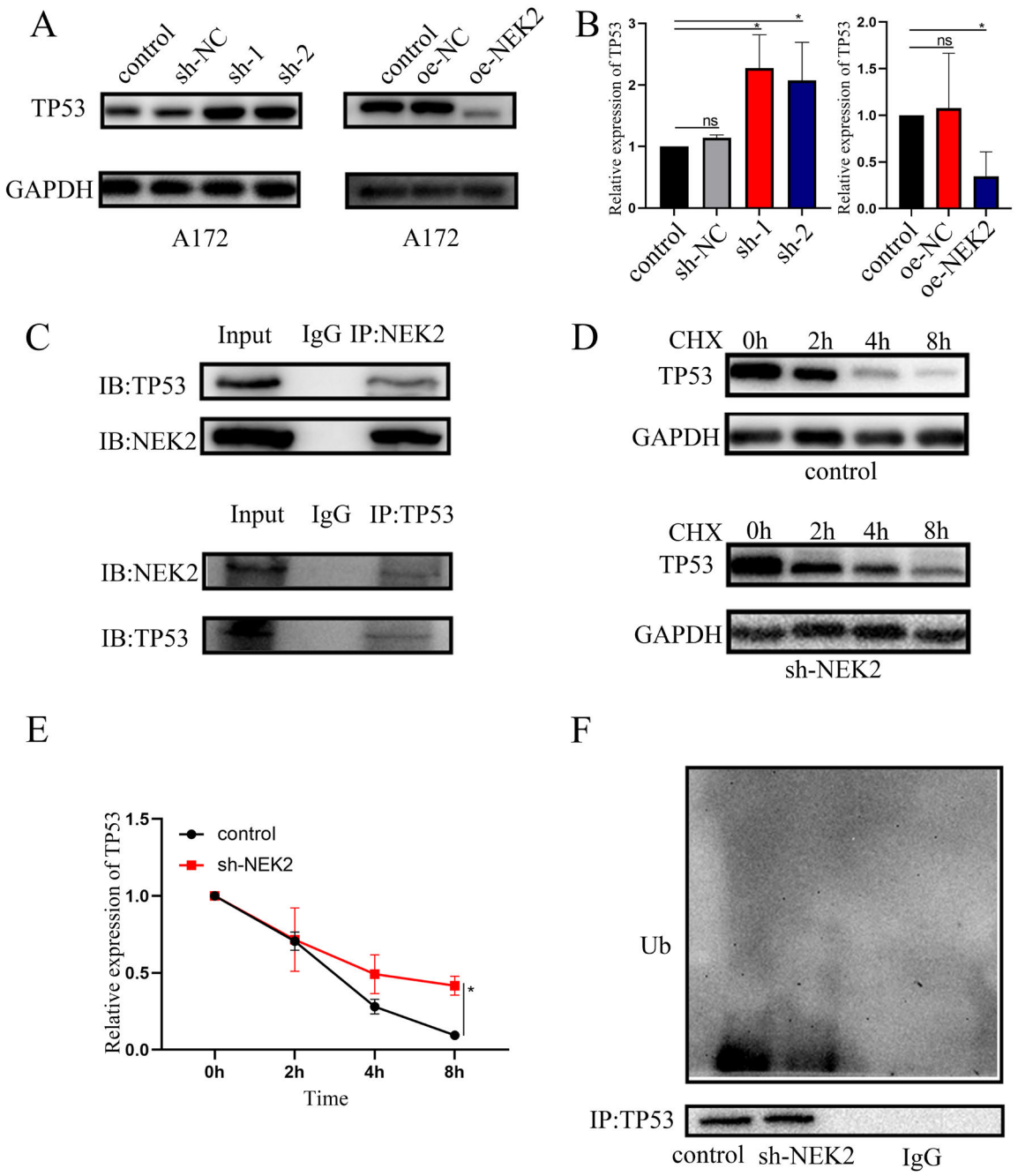
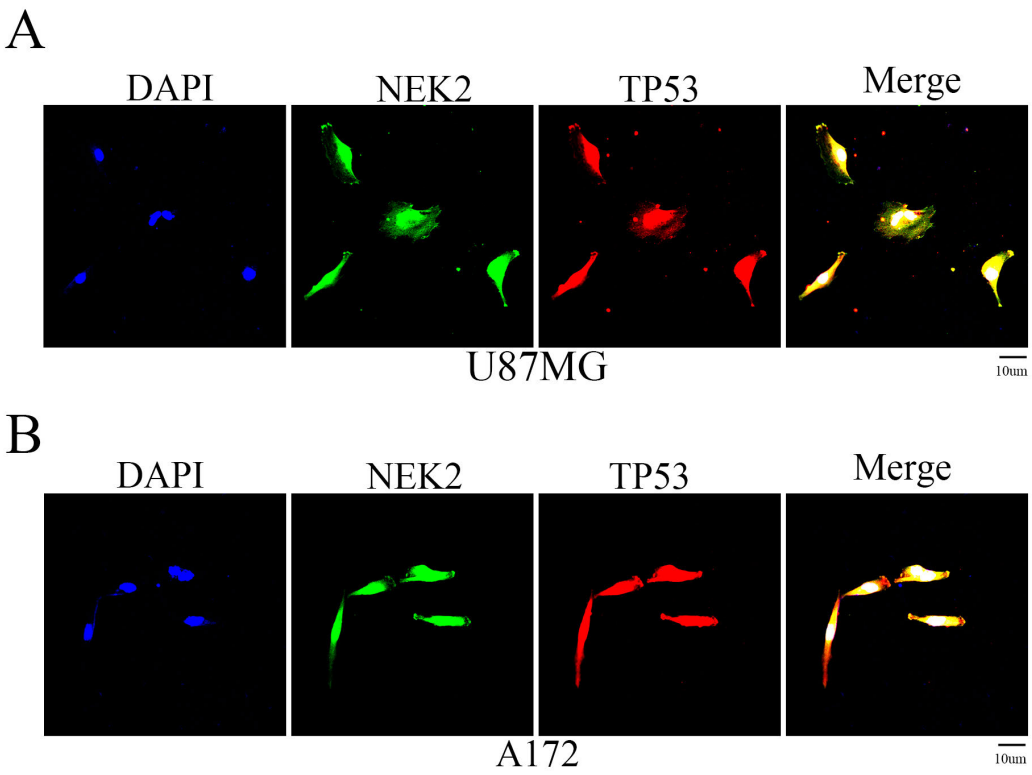
Supplementary Information



Supplementary Figure S1. NEK2 exerts inhibitory effects on the proliferation, migration, and invasion of glioma cells. A, B) The transfection efficiency of knockdown (shRNA-NEK2-1, sh-1; shRNA-NEK2-2, sh-2) and plasmid overexpression (oe-NEK2), as well as the NEK2 expression in A172 glioma cell line, was evaluated using Western blot analysis. C) Repercussions on the proliferation of A172 glioma cells were observed in CCK-8 assay outcomes when NEK2 expression was downregulated or upregulated. D, F) The findings from the colony formation assay revealed how altering NEK2 expression levels affected the ability of A172 glioma cells to form colonies. E, G, H) Transwell assays were conducted to assess the migratory and invasive potential of A172 glioma cells following modulation of NEK2 expression levels. The data are presented as mean±SD. Statistical significance was determined using *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Abbreviations: NEK2-NIMA-related kinase 2; NC-negative control; sh-short hairpin; oe-overexpression



Supplementary Figure S2. NEK2 governs the regulation of TP53 and orchestrates its ubiquitination process. A, B) The Western blot analysis was conducted to assess the expression of TP53 in A172 glioma cell line after knockdown (shRNA-NEK2-1, sh-1; shRNA-NEK2-2, sh-2) and plasmid overexpression (oe-NEK2). C) A172 cellular extracts were subjected to lysis using IP lysis buffer, followed by incubation with either NEK2 antibody or control IgG. Immunoblotting analysis was conducted using TP53 and NEK2 antibodies for detection (upper images). To reciprocally immunoprecipitate the proteins, TP53 antibody or control IgG was employed, followed by immunoblotting with NEK2 and TP53 antibodies (lower images). D, E) The levels of TP53 protein were evaluated at different time points (0, 2, 4, and 8 h) after treating lysed A172 cells with CHX to inhibit the synthesis of proteins F) The Ubi antibody was utilized for Western blot analysis to assess the extent of TP53 ubiquitination. In A172 cells, NEK2 knockdown led to a notable decrease in TP53 ubiquitination levels. Abbreviations: NEK2-NIMA-related kinase 2; NC-negative control; sh-short hairpin; IP-immunoprecipitation. The data are presented as mean±SD. Statistical significance was determined using *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.



Supplementary Figure S3. NEK2 and TP53 are co-expressed in glioma cells. A) Co-immunofluorescent staining of NEK2 (in green) and TP53 (in red) was performed in U87MG cells. B) Co-immunofluorescent staining of NEK2 (in green) and TP53 (in red) was performed in A172 cells.