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EGFR suppresses p53 function by promoting p53 binding to DNA-PKcs: a noncanonical regulatory axis between EGFR and wild-type p53 in glioblastoma

Jie Ding[†], Xiaolong Li[†], Sabbir Khan[†], Chen Zhang, Feng Gao, Shayak Sen, Amanda R. Wasylishen, Yang Zhao, Guillermina Lozano, Dimpy Koul, and WK Alfred Yung

Department of Neuro-Oncology, Brain Tumor Center, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA (J.D., X.L., S.K., C.Z., F.G., S.S., D.K., W.K.A.Y.); Department of Genetics, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA (A.R.W., G.L.); UTHealth Graduate School of Biomedical Sciences, Houston, Texas, USA (G.L.); Department of Dermatology, Stanford University School of Medicine, Stanford, California, USA (Y.Z.); Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA (Y.Z.)

[†]These authors contributed equally to this work.

Corresponding Author: WK Alfred Yung, Department of Neuro-Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA (wyung@mdanderson.org).

Abstract

Background. Epidermal growth factor receptor (*EGFR*) amplification and *TP53* mutation are the two most common genetic alterations in glioblastoma multiforme (GBM). A comprehensive analysis of the TCGA GBM database revealed a subgroup with near mutual exclusivity of *EGFR* amplification and *TP53* mutations indicative of a role of EGFR in regulating wild-type-p53 (wt-p53) function. The relationship between EGFR amplification and wt-p53 function remains undefined and this study describes the biological significance of this interaction in GBM.

Methods. Mass spectrometry was used to identify EGFR-dependent p53-interacting proteins. The p53 and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) interaction was detected by co-immunoprecipitation. We used CRISPR-Cas9 gene editing to knockout EGFR and DNA-PKcs and the Edit-R CRIPSR-Cas9 system for conditional knockout of EGFR. ROS activity was measured with a CM-H_2DCFDA probe, and real-time PCR was used to quantify expression of p53 target genes.

Results. Using glioma sphere-forming cells (GSCs), we identified, DNA-PKcs as a p53 interacting protein that functionally inhibits p53 activity. We demonstrate that EGFR knockdown increased wt-p53 transcriptional activity, which was associated with decreased binding between p53 and DNA-PKcs. We further show that inhibition of DNA-PKcs either by siRNA or an inhibitor (nedisertib) increased wt-p53 transcriptional activity, which was not enhanced further by EGFR knockdown, indicating that EGFR suppressed wt-p53 activity through DNA-PKcs binding with p53. Finally, using conditional EGFR-knockout GSCs, we show that depleting EGFR increased animal survival in mice transplanted with wt-p53 GSCs.

Conclusion. This study demonstrates that EGFR signaling inhibits wt-p53 function in GBM by promoting an interaction between p53 and DNA-PKcs.

Key Points

1. EGFR signaling inhibits wt-p53 function in GBM
2. EGFR functions by enhancing the interaction between DNA-PKcs and p53 and functionally inhibits p53 activity
3. EGFR plays an essential role in tumor maintenance in wt-p53 glioma.

Importance of the Study

EGFR is the most frequently amplified gene in GBM and plays a crucial role in maintaining tumor growth. Around 30% of GBM patients harbor p53 mutation. In malignant tumors with wt-p53, the normal function of p53 is suppressed by Mouse double minute 2 homolog (MDM2) and Mouse double minute 4 homolog (MDM4). Remarkably, like *MDM2*, *EGFR* amplification shows mutual exclusivity with *TP53* mutation in GBM. This result raises the question of whether EGFR plays a role in suppressing wt-p53 activity in GBM. Here, we report the functional relationship between *EGFR* and *p53* in GBM. We showed that EGFR inhibited wt-p53 function by enhancing the interaction between DNA-PKcs and p53. Knockdown of either EGFR or DNA-PKc increased wt-p53 transcriptional activity due to decreased binding between p53 and DNA-PKcs. These findings provided a new noncanonical regulatory axis between EGFR and wt-p53 in GBM for novel biological roles.

Glioblastoma multiforme (GBM) is the most aggressive and common primary brain tumor in adults. The current standard treatment includes surgical resection, radiotherapy, and adjuvant chemotherapy with temozolomide; however, the outcome of GBM patients remains poor, with a median survival duration of 15–17 months.[1,2] Novel therapeutic approaches are desperately needed to improve the outcome of these patients. A comprehensive analysis of The Cancer Genome Atlas (TCGA) GBM database led to the identification of three relevant subgroups (proneural, classical, and mesenchymal).[3–5] Based on the genome-wide DNA, RNA, and epigenome analyses of TCGA GBM samples, translational studies are needed to bring these research results closer to the clinical routine and evaluate markers that may define the main molecular subgroups, predict prognosis and identify potential future treatment targets.

GBM arises through the accumulation of genetic alterations, including 1p/19q co-deletion, isocitrate dehydrogenase1/2 (*IDH1/2)* gene mutation, *EGFR* amplification, and mutation, phosphatase and tensin homolog (*PTEN*) alterations, *TP53* (which encodes the p53 tumor suppressor protein) mutation, and telomerase reverse transcriptase (*TERT*) promoter gene mutations.[6] Of these genetic changes, *EGFR* amplification and *TP53* mutation are the two most common in GBM. About 57% of GBM patients have *EGFR* amplification, and 50% have *TP53* mutation.[7–9] Although *EGFR* amplification and/or overexpression and *TP53* mutation are 2 genetic events that seem to molecularly differentiate these clinical subtypes of GBM, while the association of these 2 hallmarks has been considered to be almost mutually exclusive, studies have shown coexistence of *p53* mutation and *EGFR* amplification in subsets of primary GBM tumors[10–13] and the prognostic significance of these concurrent alterations has not been studied. GBM subgroup showing simultaneous alteration of EGFR and p53 pathways are characterized by a worse clinical outcome and simultaneous deregulation of the EGFR and p53 pathways may indicate a relevant cell cycle deregulation that leads to more aggressive GBM formation.[9]

The signaling pathway cascades activated by *EGFR* amplification, include activation of cyclooxygenase-2,[14,15] KRAS and AKT signaling, and mammalian target of rapamycin (MTOR), together with phosphatidyl-inositol-3-kinase (PI3K) pathways.[16–19] Mutations of *TP53* can disrupt normal p53 functions, and missense mutations can have a gain of function (GOF) which promotes tumor proliferation and metastasis.[20] In the remaining malignant tumors with wt-p53, the normal function of p53 is usually suppressed so that the tumors can avoid apoptosis and maintain high proliferation. MDM2 and MDM4 are the main suppressors of p53. In most cases, the levels of MDM2 and MDM4 are overproduced to suppress the normal pro-apoptotic function of p53.[21] A pan-cancer study from TCGA reveals that *MDM2* and *MDM4* amplification shows mutual exclusivity with TP53 mutation in GBM.[22] In the present study, we also observed a mutually exclusive relationship between EGFR and *TP53* mutation, raising the question as to whether EGFR plays a direct role in suppressing wt-p53 activity in GBM. The molecular interactions between EGFR and p53 have yet to be described and the prognostic significance has not been studied. Therefore, this study was undertaken to evaluate the concurrent expression of wt-p53 and EGFR in GBM to understand the mechanism of tumor maintenance.

Using GSCs as the model system,[23–27] we demonstrate that EGFR plays an essential role in tumor maintenance in wt-p53 GSCs. We further showed that EGFR inhibits wt-p53 function by enhancing the interaction between DNA-PKcs and p53. These findings provided a new noncanonical regulatory axis between EGFR and wt-p53 in GBM.

Materials and Methods

Cell Lines and Reagents

Patient-derived glioma sphere-forming cell (GSC) lines with varying EGFR and p53 status were used in this study. The GSCs were established by isolating neurosphere-forming cells from fresh surgical specimens of GBM between 2005 and 2008, as described previously.[28] Cells were authenticated by testing short tandem repeats using the Applied Biosystems AmpFISTR Identifier kit (Foster City, CA). The most recent authentication was performed on July 31, 2017. This study was approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center. The GSC lines were cultured in DMEM/F12 medium containing B27 supplement (Invitrogen, Carlsbad, CA), basic fibroblast growth factor (20 ng/mL), and epidermal growth factor (20 ng/mL). Erlotinib and nedisertib were purchased from Selleckchem (Houston, TX).

Cell Growth Curve

GSC lines were treated in triplicate, with or without doxycycline (1 μg/mL), for 3, 6, and 10 days. Cell growth was tested using the CellTiter-glo (Promega, Madison, WI) viability assay.

Immunoblotting Analysis

Cell lysates were analyzed by western blot analysis as previously described.[29] The membranes were probed with the following primary antibodies: anti-EGFR, anti-p53, anti-DNA-PKcs, anti-phosphorylated p53, anti-PUMA (all from Cell Signaling, Boston, MA, USA), anti-MDM2, and anti-MDM4 (Abcam, Cambridge, MA, USA), and anti-p21 (BD, San Jose, CA, USA). Anti-β-actin or anti-GAPDH antibodies were purchased from Sigma (St. Louis, MO, USA) and used as the loading control. Densitometric quantification of immunoblots was done using NIH open-source software Image J (version 1.53, https://imagej.nih.gov/ij/). The protein expression was normalized to actin/GAPDH and presented as a fold change in the untreated or control group.

Immunoprecipitation

Cells were lysed in lysis buffer (50 mM Tris–HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, and 1 mM phenylmethylsulfonyl fluoride. Cell lysate (200 to 1000 μg of protein) was immunoprecipitated with specific antibodies and Pierce Protein A/G UltraLink Resin (Thermo Fisher) for 18 h at 4°C and eluted using 2× SDS buffer and followed by Western blot detection.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from GSCs using the RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Real-time quantitative PCR was performed with the SuperScript III One-Step RT-PCR System and Sybrgreen DNA polymerase (Invitrogen, Grand Island, NY, USA). The sequences of primers for quantitative PCR are GAPDH forward 5′-GGAGCGAGATCCCTCCAAAAT-3′, GAPDH reverse 5′-GGCTGTTGTCATACTTCTCATGG-3′, PUMA forward 5′- GACCTCAACGCACAGTACGAG-3′, PUMA reverse 5′- AGGAGTCCCATGATGAGATTGT-3′, p21 forward 5′-TGGAGACTCTCAGGGTCGAAA-3′, and p21 reverse 5′-GGCGTTTGGAGTGGTAGAAATC-3′.

RNA Interference

EGFR and DNA-PKcs were silenced in GSCs by transfecting 40 nM siRNA against human EGFR (cat. no. SI00300104 and SI02660147, QIAGEN, Germantown, MD, USA), DNA-PKcs (cat. no. SI02224236 and SI02224229, QIAGEN) and non-silencing negative control siRNA (cat. no. 1027415, QIAGEN) using Lipofectamine 2000 (Invitrogen Life Technologies). The knockdown efficiency was measured by immunoblotting.

For the CRISPR-Cas9-mediated EGFR knockout, a double nickase plasmid (sc-400015) and control double nickase plasmid (sc-437281, Santa Cruz) were transfected with Lipofectamine 2000 (Invitrogen, Grand Island, NY) for 48 hours. Cells with green fluorescent protein were sorted onto 96-well plates to form single colonies. After 4–5 weeks, single colonies were transferred to 24-well plates to determine cell proliferation. A reverse transcription-polymerase chain reaction was performed, followed by sequencing to confirm complete allelic knockout.

Inducible Knockout Using the Edit-R CRISPR-Cas9 System for Genome Engineering

EGFR editing in GSC11 cells with Edit-R-inducible lentiviral Cas9 (CAS11229, Dharmacon, Lafayette, CO, USA) and EGFR sgRNA (GSGH11838-246991992, Dharmacon) was performed by inducing Cas9 expression with doxycycline using isolated clonal cells according to the manufacturer's instructions (Dharmacon). Edit-R Lentiviral sgRNA Non-targeting Control also from Dharmacon (GSG11811) was used as a control.

Mass Spectrometry

Protein samples of control and EGFR knockout GSC262 cells were separated by 5% SDS-PAGE. Proteins bands were Visualized by Coomassie Brilliant Blue G Colloidal (Sigma) and a band of interest was subjected to mass spectrometry analysis at Clinical and Translational Proteomics Service Centre, University of Texas Health Science Center, Houston, Texas. The Exponentially Modified Protein Abundance Index (emPAI) was used to show the relative quantitation of the proteins in a mixture on the basis of protein coverage by the peptide matches in a database search result.[30] The formula is emPAI = $10\exp(N_{observed}/N_{observable}) - 1$, where $N_{observed}$ is the number of experimentally observed peptides and $N_{observable}$ is the calculated number of observable peptides for each protein.

ROS Measurement

To measure ROS activity, GSCs were stained with a CM-H_2DCFDA probe, according to the manufacturer's instructions (Molecular Probes, Invitrogen). Cells were suspended in prewarmed HBSS containing a 5–10 μmol/L CM-H2DCFDA probe and incubated at 37°C for 30 minutes. ROS activity was acquired with FACS (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Intracranial Tumor Studies

All mouse experiments were performed in compliance with the National Institutes of Health guidelines for animal research and approved by the MD Anderson Cancer Center Institutional Animal Care and Use Committee. For intracranial studies, animals were randomly divided into three groups ($n = 16$). GSC11-control and GSC11-EGFRKO35 cells

(5 x 10^5) were implanted in 6- to 8-week-old nude mice as described previously.[31] Two days after cell implantation, half of mice were given a normal diet and the other half were given a doxycycline diet for conditional EGFR knockout. Tumor growth was visualized and quantified using the IVIS system. Mice were monitored daily and euthanized when moribund. Whole brains were extracted, rapidly frozen in liquid nitrogen, and stored at –70°C or stored in formalin.

Statistical Analysis

The statistical analysis was performed using Student's *t*-test. The results are presented as the mean of three independent experiments. We used Kaplan–Meier method to plot survival curves and used log-rank tests to compare survival curves between groups. Differences were considered statistically significant at $P < .05$ for all comparisons.

Results

EGFR Amplification and TP53 Alterations Are Mutually Exclusive in GBM

Inactivation of p53 in most cancers results from the loss or mutation of *TP53* or amplification of *MDM2* or *MDM4* which encode negative regulators of p53.[21] To identify additional candidate negative regulators of the p53 pathway, we evaluated sequencing data from 543 GBM patients from TGCA.[5] The analysis showed that in addition to a mutual exclusive relationship between amplification of *MDM2* and *TP53* alterations, the amplification of *EGFR* and *TP53* alterations were also mutually exclusive in GBM patients indicating a potential role of EGFR in wt-p53 inhibition (Figure 1A and Supplementary Table S1). To explore if this relationship could be generalized to other tumor types, we evaluated a lung cancer study (as *EGFR* alterations are also recurrent in lung adenocarcinomas) and a pan-caner study.[22,32] We find that in the 1144 patients with lung cancer and the pan-cancer study of 10945 patients, *EGFR* amplification co-exists with *TP53* alterations and is not associated with wt-p53 co-occurrence (Figure 1B, C and Supplementary Table S1). Thus, the relationship between *EGFR* and *TP53* is unique to GBM. To evaluate this relationship in GBM, we selected five GSC lines and divided them into two groups on the basis of *TP53* status: wild-type and *TP53* with alterations (Figure 1D). *EGFR* amplification was evaluated by copy number variation, as determined by an OncoScan array, and stratified according to a threshold of copy number variation ≥2.[33] Western blot analysis further confirmed EGFR and p53 protein levels in the GSCs with EGFR amplification (Figure 1E).

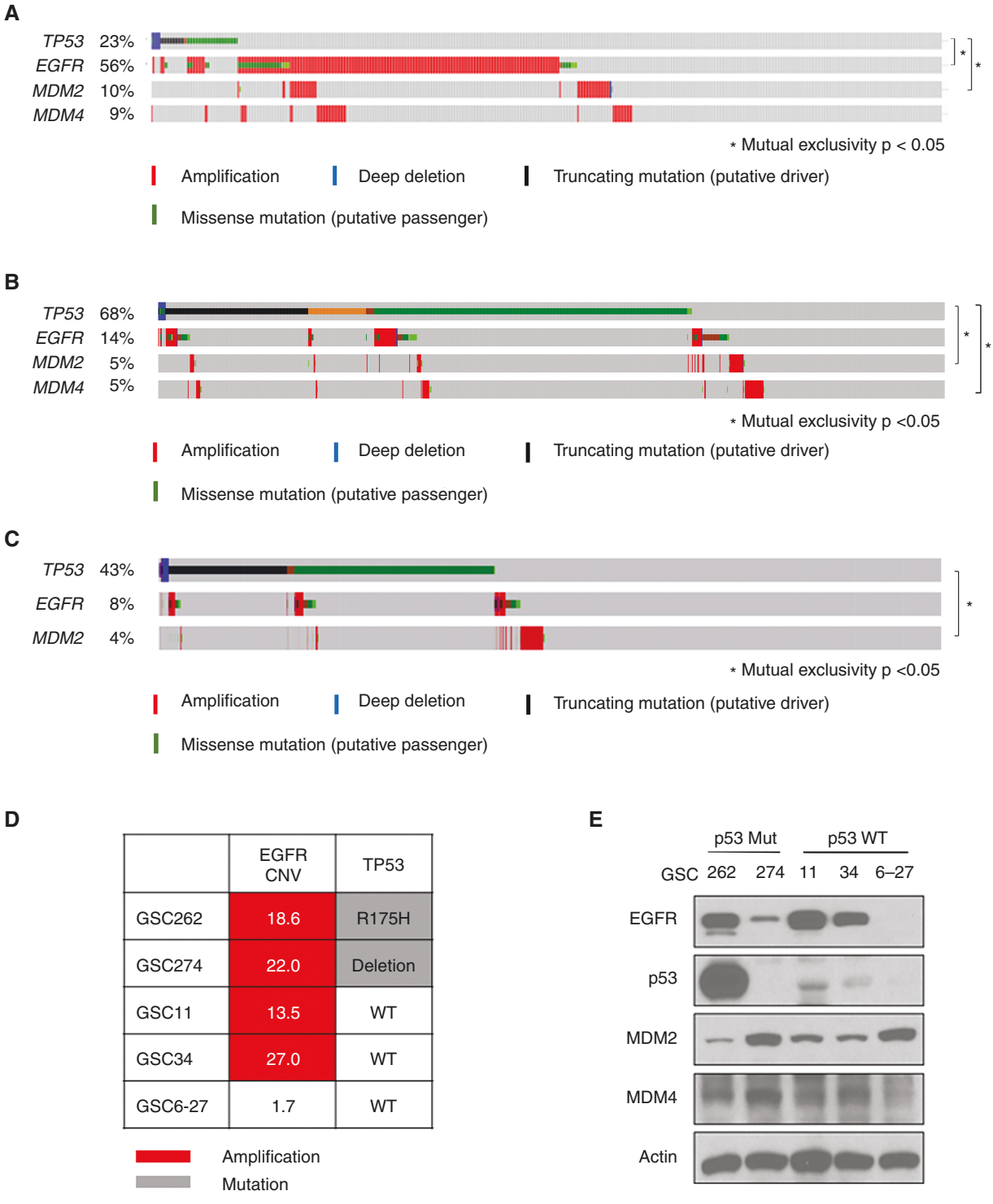
EGFR Suppresses p53 Activity in GSCs With wt-p53 Status

To study the role of EGFR in regulating p53 function, we knocked down *EGFR* expression using siRNA in both wt-p53 and mut-p53 GSCs. In wt-p53 GSCs (GSC11 and GSC34), p53 protein levels and activity were increased after EGFR knockdown, as indicated by increased p21 and PUMA protein expression and mRNA expression (Figure 2A, B, E–H). PUMA and p21 are among the most important downstream effectors of p53, and increased p21 and PUMA at both the protein and mRNA levels after EGFR knockdown indicated that EGFR plays a role in inhibiting p53 activity (Figure 2A, B, E–H). In comparison, EGFR knockdown in mut-p53 cells (GSC 274 and GSC 262) had no effect on p21 and PUMA levels both at the protein as well as mRNA level (Figure 2C, D, I–L). These data indicate the EGFR regulated p53 activity in GSCs harboring wt-p53 but not mut-p53 GSCs. Of note, attempts to delete EGFR was lethal to cells with *EGFR* amplification, indicating an essential role of EGFR in cell survival.

Further, we used erlotinib, an EGFR inhibitor to study the suppression of EGFR function and its effects on p53 regulation. We show that *PUMA* mRNA levels were increased in both wt-p53 and mut-p53 GSCs with erlotinib treatment (Figure 2M–P). However, we did not observe a tight regulation of p21 with erlotinib treatment (Supplementary Figure S1), which may be due to other signaling mechanism regulating p21other than EGFR.

EGFR Induces Physical Binding Between DNA-PKcs and p53 In Vitro

p53 associates with many co-factors or binding partners that selectively alter its transcriptional activity. In addition, many proteins such as MDM2, bind both wt-p53 and mut-p53. To determine how EGFR regulates p53 activity and to identify binding partners of p53 that are EGFR dependent, we performed a p53 immunoprecipitation experiments followed by coomassie blue staining of samples from EGFR deleted mut-p53 and control cells. We used mut-p53 GSC262 cells to knockout EGFR since EGFR deletion in wt-p53 cells killed cells due to essential role of EGFR for cell survival. The immunoprecipitation results revealed a band of 400 KD in control cells; this 400 KD band was significantly reduced in EGFR knockout cell immunoprecipitates, indicating that EGFR alters the binding affinity of p53-binding partners to p53 (Figure 3A). The other bands in the immunoprecipitates were non-specific bands as they appeared in both samples. To further identify the binding protein, we analyzed the 400 KD band using mass spectrometry. A total ion chromatogram of the immunoprecipitated band identified DNA-PKcs in the presence of intact EGFR (Supplementary Table S2 and Figure S2). DNA-PKcs refers to DNA-dependent protein kinase catalytic subunit (cs) for which *PRKDC* (Protein Kinase, DNA-Activated, Catalytic Subunit) is a protein-coding gene. We next wanted to validate the interaction and confirm that DNA-PKcs is indeed a binding partner of p53. For that, we performed p53 immunoprecipitation followed by Western blot analysis with a DNA-PKcs antibody. The results showed that DNA-PKcs clearly forms a complex with wt and mut-p53 (Figure 3B). To validate the EGFR-dependence of the wt-p53 and DNA-PKcs interaction we used short-term knockdown of EGFR in wt-p53 cells. Clear interactions were present in scrambled treated cells; however, the interaction between DNA-PKcs and p53 was



	EGFR CNV	TP53
GSC262	18.6	R175H
GSC274	22.0	Deletion
GSC11	13.5	WT
GSC34	27.0	WT
GSC6-27	1.7	WT

Amplification
Mutation

Fig. 1 *EGFR* amplification and *TP53* alteration are mutually exclusive in GBM. (A) Mutual exclusivity analysis of TCGA GBM dataset (Cell 2013[5]). (B) Mutual exclusivity analysis of TCGA pan-lung cancer dataset (Nat Genet 2016[32]). (C) Mutual exclusivity analysis of MSKCC pan-cancer dataset (Nat Med 2017[22]). (D) *EGFR* and *TP53* status in a series of GSCs. (E) Western blot (WB) analysis with the indicated antibodies of protein lysates from a series of GSCs.

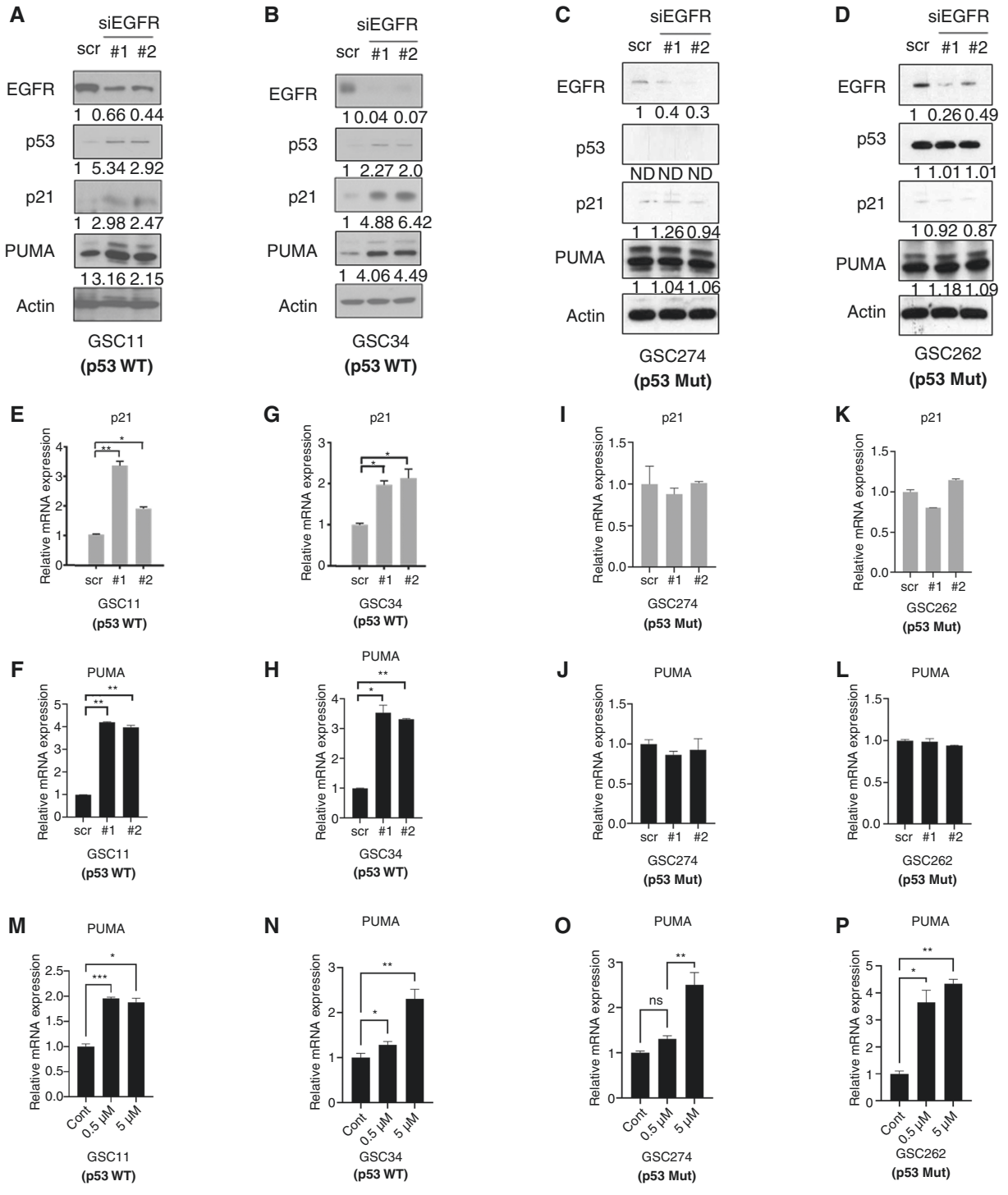


Fig. 2 EGFR knockdown enhanced wt-p53 activity in GSCs. (A-D) WB analysis with the indicated proteins in whole cell lysates of GSC11, GSC34, GSC274 and GSC 262 with EGFR knockdown. scr, small interfering control (scrambled); #1 and #2 siRNA targeting EGFR, (E-L) qPCR results showing relative mRNA expression of p21 and PUMA after siRNA knockdown. (M-P) qPCR results showing relative mRNA expression of PUMA with erlotinib treatment at indicated doses for 24h. Quantification of proteins is shown at the bottom of each band. Not detected (ND). $^{*}P < .05$; $^{**}P < .01$ and $^{***}P < .001$ as compared to untreated condition.

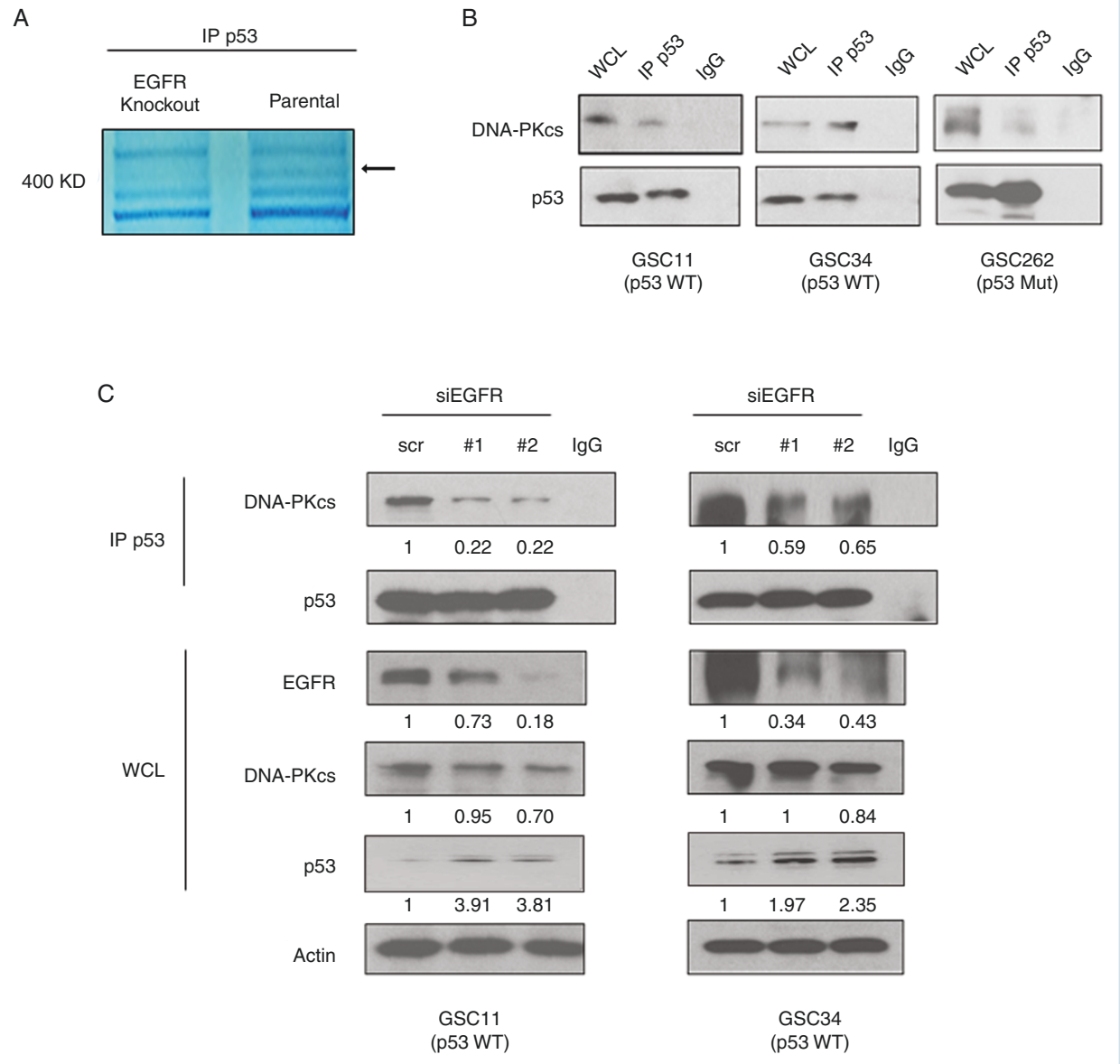


Fig. 3 EGFR knockdown attenuated physical binding between DNA-PKcs and p53. (A) Coomassie Brilliant Blue staining of the gel after co-immunoprecipitation (co-IP) of p53 in GSC262 parental and EGFR knockout cells. (B) Co-IP of p53 in GSCs, and (C) EGFR knockdown by two siRNA followed by WB analysis with the indicated antibodies. WCL, whole-cell lysates. IgG, control immunoglobulin G. Quantification of DNA-PKcs and p53 was shown at the bottom.

attenuated in knockdown EGFR cells (Figure 3C). These data demonstrate the interaction between p53 and DNA-PKcs is EGFR dependent.

Inhibition of DNA-PKcs Restores wt-p53 Activity in GSCs

It is well known that binding of p53 with its partners modulates p53 activity. For that, we knocked down DNA-PKcs using siRNA in wt-p53 GSCs. We demonstrated that DNA-PKcs knockdown increased p53 activity in wt-p53 GSCs, as shown by increased p21 and PUMA both at the protein and at mRNA levels (Figure 4A–C). We also evaluated if treatment with the DNA-PKcs inhibitor nedisertib would also modulate p53 activity. We showed that treatment with the DNA-PKcs inhibitor nedisertib activated p53, as shown by an increased level of p21 and PUMA. Increased p53 activity coincided with increased pp53(S15) phosphorylation after nedisertib treatment in wt-p53 GSCs (Figure 4D and E. Further, a modest increase in total DNA-PKcs expression was observed at higher doses of nedisertib treatment (Figure 4F). These data indicate that DNA-PKcs binds p53 and inhibits its activity in wt-p53 GSCs.

To rule out the possibility of the increased p21 and PUMA due to DNA damage accumulation response under

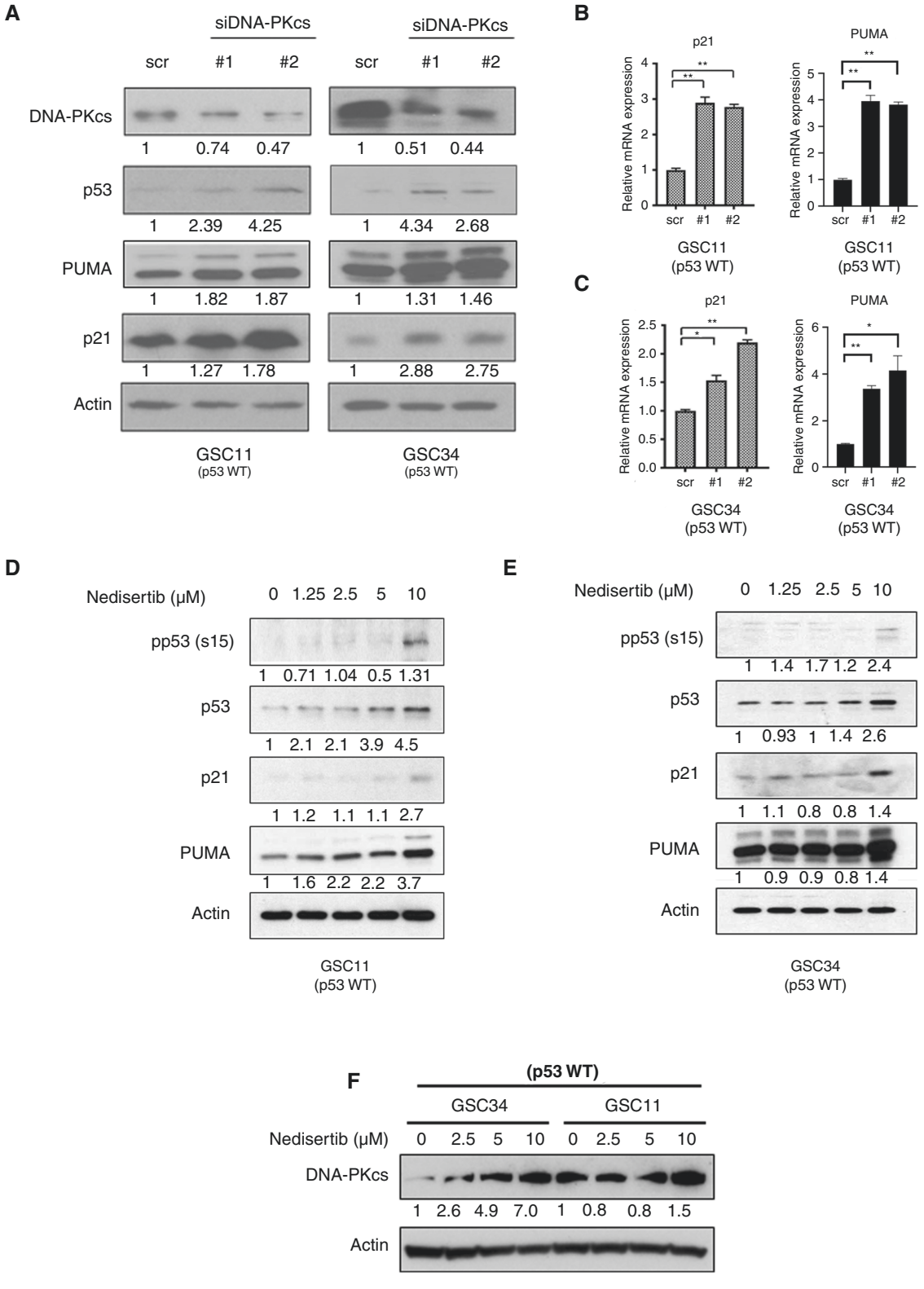


Fig. 4 Inhibition of DNA-PKcs promoted wt-p53 activity in GSCs. (A) WB analysis of protein of interest after DNA-PKcs knockdown. scr, and #1 and #2 siRNA targeting DNA-PKcs. (B and C) Data showing relative mRNA expression of p21 and PUMA. $^{*}P < .05$ and $^{**}P < .01$ as compared to scr siRNA control. (D and F) WB analysis protein of interest after 72 hr nedisertib treatment at indicated doses. Quantification of DNA-PKcs, PUMA, p21 and p53 is shown at the bottom of respective blot.

DNA-PKcs knockdown, we examined accumulation of DNA damage after treating cells with nedisertib in two each of wt-p53 and mut-p53 cells. We used both cleaved-PARP and γ-H2AX expression by WB to assess DNA damage and apoptosis (Supplementary Figure S3). Our data revealed no clear trend in accumulation of DNA damage and induction of p21 and PUMA expression in the context of either wild-type or mutant p53 cells. Thus, induction of p21 and PUMA with either EGFR or DNA-PKcs knockdown in wt-p53 cells are primarily associated with p53 regulation by EGFR.

EGFR Inhibits p53 Activity by Affecting the Binding Between DNA-PKcs and p53

To determine whether DNA-PKcs is required for EGFR-mediated p53 inhibition, we first generated a DNA-PKcs knockout (KO) GSC with wt-p53 from a single clone, followed by second knockdown of EGFR in the DNA-PKcs KO cells. p53 was activated following loss of DNA-PKcs alone and not further increased with EGFR knockdown; indicating that EGFR inhibition of p53 requires DNA-PKcs (Figure 5A).

ROS Regulates EGFR-Mediated DNA-PKc and p53 Binding

In previous studies, we showed the role of ROS in regulating EGFR-mediated DNA damage response.[33] Elevated ROS levels were reported to be associated with EGFR amplification.[34] Therefore, we next determined whether ROS is involved in regulation of p53 function by EGFR. We measured ROS levels in EGFR knockdown cells by flow cytometry. Knockdown of EGFR by siRNA did not lead to a significant decrease in ROS levels (Supplementary Figure S4). Further, to determine whether ROS regulates p53 activity in GSCs, we treated GSC11 and GSC34 cells with YCG063, ROS inhibitor, and showed that both wt-p53 cell lines decreased ROS production by DCFDA staining and flow cytometry (Figure 5B and C). To further understand how ROS suppresses p53 activity in GSCs, we checked the binding between DNA-PKcs and p53 after ROS inhibitor treatment. We found that ROS inhibitor did not affect either the levels of DNA-PKcs or the levels of p53 in GSC11; however, we observed a decreased binding between DNA-PKcs to p53 and increased p53 activity (Figure 5D). In addition, we showed that in two wt-p53, ROS inhibition by YCG063 increased p53 targets genes *p21* and *PUMA* expression, clearly showing that ROS regulates p53 activity in GSCs (Figure 5D–H). Further, it has been reported that pharmacological inhibitor of EGFR erlotinib decreases ROS levels in GBM, non-small cell lung cancer, LPS-induced inflammation, and adenine-induced kidney injury.[35–38] These findings indicate that ROS is involved as a signaling mediator in the EGFR-mediated regulation of p53 through the binding of DNA-PKcs and p53.

EGFR Functions as the Driver of Tumor Maintenance in wt-p53 GSCs

To study whether EGFR plays an essential role in tumor maintenance in wt-p53 GSCs, we generated a doxycycline-inducible, Edit-R CRISPR-Cas9 system for the conditional knockout of EGFR in GSC11 cells (Figure 6A). EGFR knockout by the Edit-R CRISPR-Cas9 in clone 35 (EGFRKO35) was confirmed by western blot analysis (Figure 6A). EGFR depletion in GSC11 (GSC11-EGFRKO35) cells led to cell death with 1 μg/mL doxycycline treatment, whereas GSC11 control cells proliferated, demonstrating that EGFR was essential for the cell survival (Figure 6B). Further, GSC11 control and GSC11 EGFRKO35 cells were treated with doxycycline (1 μg/mL) for 10 days and cell growth curve showed that EGFR knockout significantly reduced cell number (Figure 6C).

We then implanted the inducible EGFR knockout cells in the brain. On day 2 after implantation, half mice ($n = 8$) were given a normal diet and remaining half ($n = 8$) were fed doxycycline diet to delete EGFR. Consistent with in vitro results, tumor volume in the GSC11-EGFRKO35 group with a doxycycline diet was significantly reduced compared with the mice fed with normal diet (Figure 6D, E). Furthermore, the GSC11-EGFRKO35 group with the doxycycline diet showed an extended median survival duration compared to GSC11-EGFRKO35 group with a normal diet (Figure 6F). Total EGFR protein levels in bulk tumor samples from mice after EGFR knockout showed a significant decrease in protein levels compared with the respective control groups (Figure 6G). These findings indicate that EGFR plays an essential role in tumor maintenance in wt-p53 GSCs and overrides the tumor suppressor function of p53.

Discussion

There is increasing evidence that both the inactivation of tumor suppressor genes and the activation of oncogenes are involved in the evolution of GBM. The most common genetic alterations in GBM include loss of heterozygosity on chromosome 10, mutations in *TP53*, amplification and rearrangements of *EGFR* gene, amplification of *MDM2*, mutations of *PTEN*, loss of tumor suppressor genes $p16^{INK4a}/p14^{ARF}$, and mutations of retinoblastoma gene.[10] Furthermore, a comprehensive genomic-based classification of GBM TCGA database led to the identification of three relevant subgroups (proneural, classic, and mesenchymal).[5,39,40] It also revealed a subgroup with near mutual exclusivity of *EGFR* amplification and *TP53* mutations. Despite the high frequency of *EGFR* and *TP53* genetic alterations in gliomas, little is known about their crosstalk during tumor progression. Therefore, this study was undertaken to investigate the functional relationship between EGFR and p53 in the context of tumorigenesis in GBM. Our results demonstrated that wt-p53 activity is attenuated downstream of EGFR via the passenger partner DNA-PKcs.

The p53 (tumor suppressor) is a principal mediator of cell cycle arrest, senescence, and apoptosis in response to stress and cellular damage. Approximately 30% of patients with GBM harbor *TP53* mutations or deletion. Amplification of *MDM2* and *MDM4* can be identified in 25% of patients with wt-p53 GBM. MDM2 and MDM4 are two critical negative regulators of p53, and amplification of *MDM2* or *MDM4* is mutually exclusive with *TP53* mutation in various malignant tumors.[21,41] Interestingly, amplification of *EGFR* is also

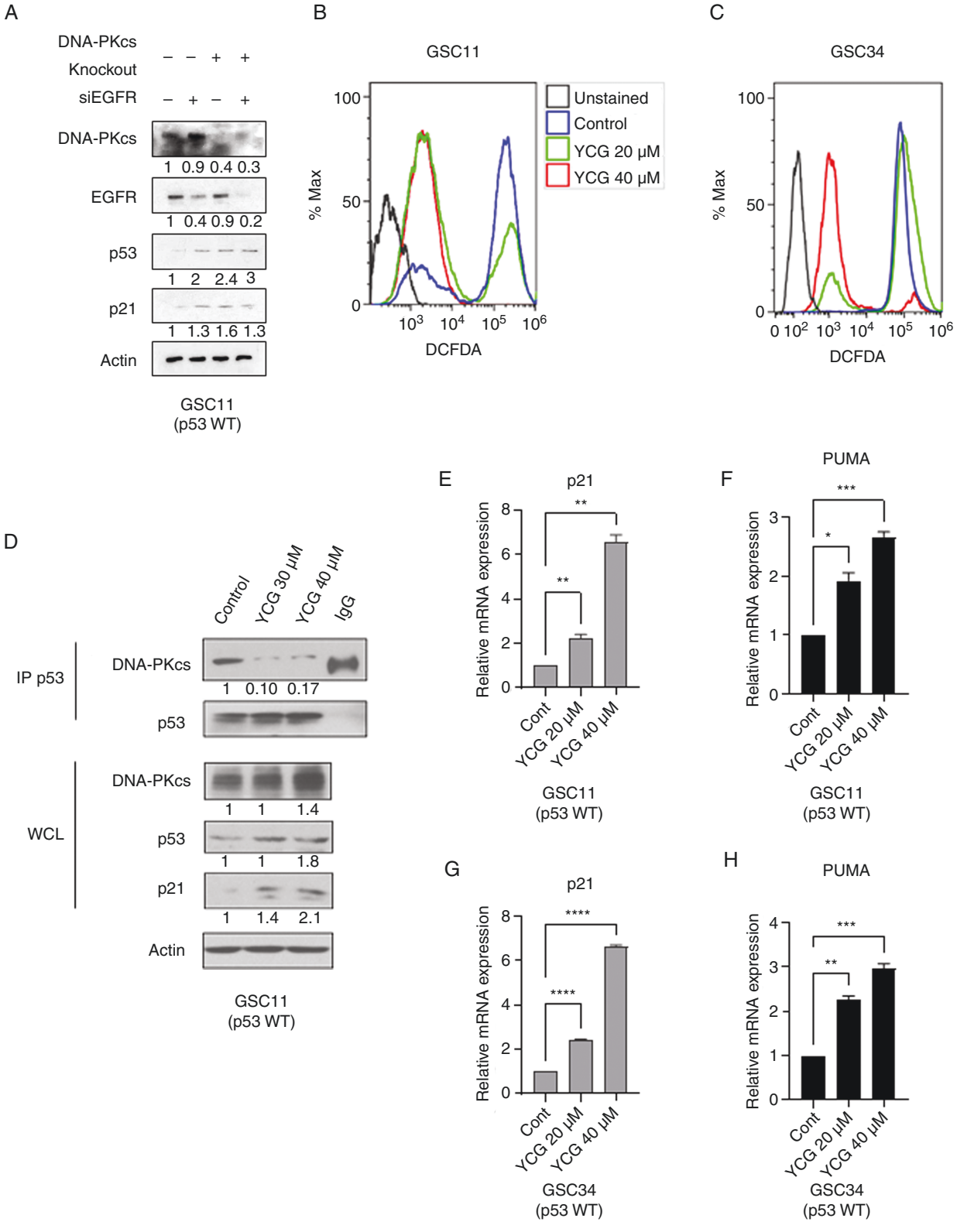


Fig. 5 EGFR inhibited p53 activity by inducing the binding between DNA-PKcs and p53. (A) WB analysis of protein of interest from GSC11 parental and DNA-PKcs knockout cells with or without EGFR knockdown. (B and C) ROS activity in GSC11 and GSC34 treated with YCG063, at indicated doses for 72hr. (D) Co-IP of p53 in GSC11 with or without YCG063 treatment followed by WB analysis. (E-H) Data showing relative mRNA expression of p21 and PUMA in GSCs with or without YCG063 treatment at indicated doses for 72 he treatment. *$P < .05$,**$P < 0.01$, ***$P < .001$ and ****$P < .0001$. Quantification of DNA-PKcs, p21 and p53 was shown at the bottom.

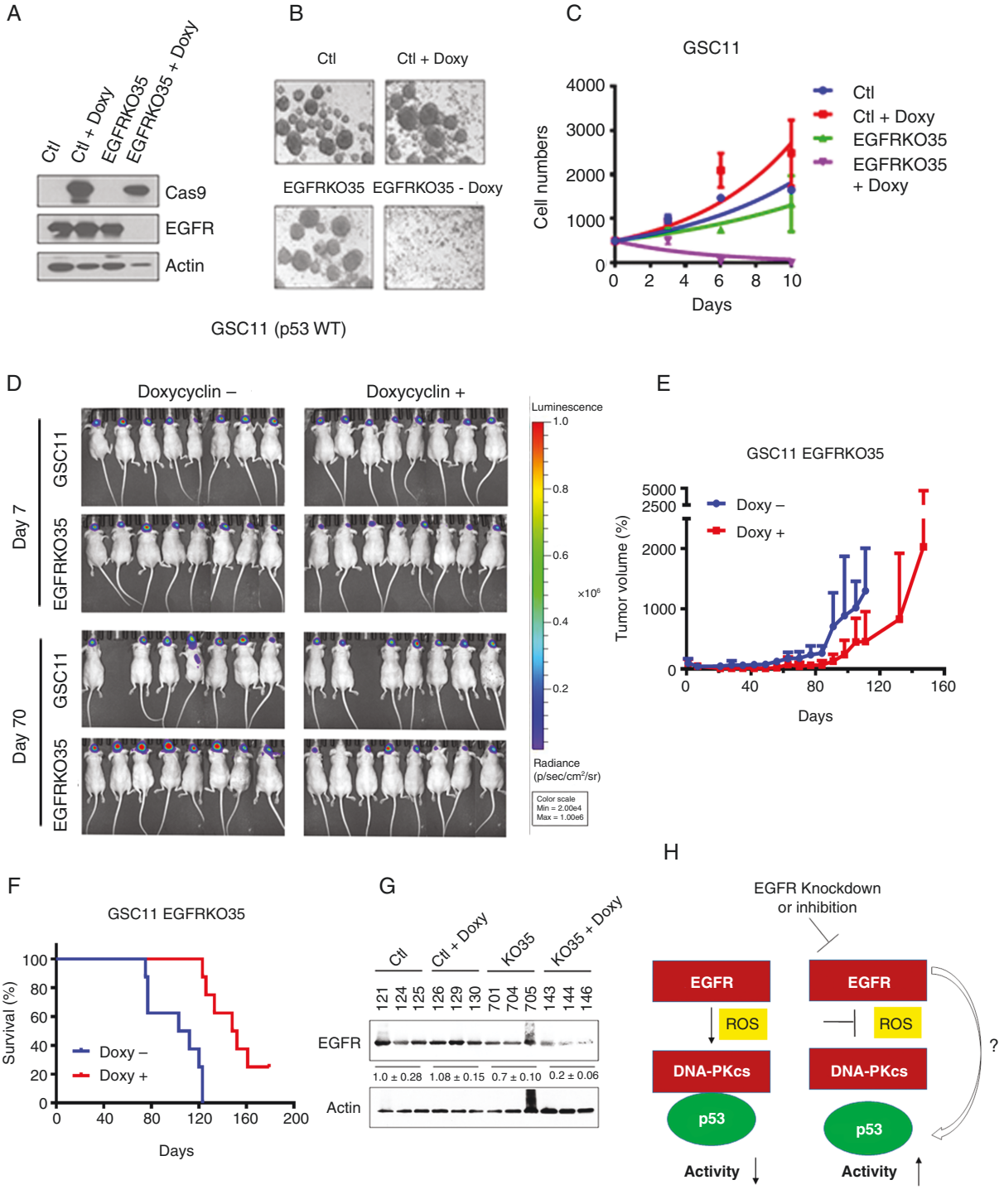


Fig. 6 EGFR knockout suppressed tumor growth and prolonged mice survival in vivo. (A) Western blot analysis with the indicated antibodies of protein lysates from GSC11 control and EGFRKO35 cells with or without doxycycline treatment. Cells were collected on day 7 after adding doxycycline. (B) Morphological images of the parental GSC11 (Ctl) and doxycycline-inducible *EGFR* knockout GSC11 (EGFRKO35) cells with or without doxycycline (100 ng/mL) in the culture media. Images were taken 7 days after doxycycline incubation. (C) Cell growth curve of GSC11 control and EGFRKO35 cells with or without doxycycline treatment. (D) GSC11 control and EGFRKO35 cells were implanted in nude mice (16 mice in each group) to establish intracranial models. Half of the mice in each group was given doxycycline diet. Representative bioluminescent imaging of tumor on day 7 and day 70 was shown. (E) Tumor growth was evaluated by normalizing to bioluminescence at the treatment start. $^{*}P < .05$. (F) Kaplan–Meier survival analysis of EGFRKO35 bearing mice after the indicated treatments. (G) Western blot analysis with the indicated antibodies of tumor samples from each group. Quantification of EGFR is shown at the bottom. (H) Schematic model summarizing the proposed mechanism of EGFR regulating the interaction between DNA-PKcs and p53.

mutually exclusive with *TP53* mutation in GBM, which is rare in other cancers. *EGFR* amplification occurs in about 60% of primary GBMs, indicating that EGFR has a significant role as a driver of tumorigenesis in GBM. The main function of EGFR amplification is to stimulate tumor growth by activating its downstream signaling pathways, such as the PI3K/AKT and MAPK/ERK signaling pathways. However, whether EGFR functions to promote tumor proliferation by directly or indirectly suppressing tumor suppressors, such as p53, was unclear.[42] Our data showed that EGFR expression overrides wt-p53 function, as knockdown of EGFR in GSCs with wt-p53 restored p53 activity, indicating that alterations of EGFR expression can affect p53 activity and may play an important role in glioma tumorigenesis. Because the cellular localization of EGFR and p53 are mainly distant, we explored the possibility that EGFR interacts with p53, either directly or indirectly, via an intermediate that acts as a bridge between EGFR and p53. Previous studies have shown the existence of a nuclear EGFR signaling pathway and gene activation in glioma.[14,42,43] Thus, our current study raised the possibility of the existence of overlapping mechanisms that might be influencing the subcellular localization of EGFR and regulating p53 function.

The finding that EGFR depletion in wt-p53 cells restored p53 activity and function raises the possibility of crosstalk between EGFR and p53 in the cytoplasmic compartment, since no binding between EGFR and p53 was observed in the nucleus (unpublished data). Interestingly, a critical kinase in DNA repair pathways (DNA-PKcs) was among the candidates that were observed to be p53 binding partners.[44] As a key enzyme in the nonhomologous end-joining pathway of DSB repair, DNA-PKcs recruits repair proteins by phosphorylating its substrates. Activated DNA-PKcs expression is significantly higher in human glioma and correlates with malignant development and poor prognosis in glioma patients.[45] A recent study identified DNA-PKcs as a key DNA repair enzyme in GSCs, which drives radiation resistance in GBM.[46] While in the nucleus, EGFR interacts with and stimulates the kinase activity of DNA-PKcs, which results in the proficient repair of DSBs and provides an explanation for the radio-resistance conferred by EGFR.[47] Further prior studies have shown that DNA-PKcs and p53 can form a stable complex in cells; however, the exact regulation of the interaction between DNA-PKcs and p53 is still unclear and is an important area of investigation.[48]

In our study, the binding between DNA-PKcs and p53 was significantly reduced with EGFR knockdown, which demonstrates that EGFR signaling, and DNA repair pathways are closely related in GBM. p53 function is context-dependent, which can either cause cell cycle arrest or induce apoptosis and cell senescence. Therefore, p53 plays a crucial role in controlling cellular fate, and downregulation of EGFR in wt-p53 cells led to the restoration of p53 activity and tumor suppressor function of p53. In this aspect, we showed that EGFR induces the interaction between p53 and DNA-PKcs; this binding suppresses p53 phosphorylation at Ser-15 residue. Further inhibition of DNA-PKcs by knockdown or nedisertib restores wt-p53 anti-tumor function.

In conclusion, our finding demonstrates that EGFR regulates wt-p53 transcriptional activity, mainly by promoting an interaction between p53 and DNA-PKcs, thereby maintaining tumor growth in GBM. Further, DNA-PKcs also promotes cell survival by facilitating DNA repair under various DNA damaging conditions. Future investigations are needed to elucidate the precise mechanism by which EGFR mediates the DNA-PKc and p53 interaction. Considering the role of DNA-PKc in complex formation with p53, the use of DNA-PKcs inhibitors in conjunction with EGFR inhibitors could be a promising therapeutic strategy for a subgroup of GBM patients with EGFR amplification and wt-p53 status.

Supplementary material

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

Keywords

DNA-PKcs | EGFR | glioblastoma | wt-p53

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