**Oncogenic EGFR signaling activates an mTORC2-NF-κB pathway that promotes chemotherapy resistance**

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**SUPPLEMENTAL INFORMATION**

## Supplemental Experimental Procedures

### Antibodies and reagents

Antibodies obtained were directed against the following: mTOR, Rictor, Raptor, EGFR, p-EGFR Tyr1068, Akt, Akt1, Akt2, Akt3, p-Akt Ser473, p-Akt Thr308, Akt, PKCα, p-NDRG1 Thr 346, p-p65 Ser536, p65, p-IκBα Ser32/36, IκBα, p-S6 Ser235/236, S6, Bcl-xL, CyclinD1, PARP, cleaved PARP (Cell Signaling); β-actin (Ambion); p-PKCα Ser657, Myc Tag clone 9E10 (Millpore); NDRG1 (Sigma); EGFR/EGFRvIII cocktail antibody (Upstate); PTEN clone 6H2.1 (Cascade BioScience). Reagents used are cisplatin, temozolomide, etoposide, rapamycin, polybrene (Sigma), Akti-1/2, BMS-345541 (Calbiochem), and PP242 (Chemdea).

### Cell Proliferation and death Assays

Cells were placed in 96-well plates at $2x10^3$cells/well in 100μl of growth medium and then incubated for 48 hours in each condition of treatment. Cell proliferation was examined with Cell Proliferation Assay Kit (Millpore) according to the manufacturer's instructions. The absorbance of the treated and untreated cells was measured with a microplate reader (Bio-Rad) at 420 to 480nm. Cell death was assessed by trypan blue exclusion (Invitrogen).

### Western blotting

Cultured cells or snapfrozen tissue samples were lysed and homogenized with a Lysis buffer AM1 and phosphatase inhibitor and protease inhibitor cocktail (Active Motif).

Equal amounts of protein extracts were separated by electrophoresis on 4-12% NuPAGE Bis-Tris Mini Gel (Invitrogen), and then transferred to a nitrocellulose membrane (GE Healthcare) with XCell II Blot Module (Invitrogen). The membrane was blocked for 1 hour in Tris-buffered saline containing 0.1% Tween20 and 5% nonfat milk and then probed with various primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase (HRP). The immunoreactivity was revealed with Super Signal West Pico Chemiluminescent Substrate or West Femto Trial kit (Thermo Scientific).

**Cell cycle assays**

Cells were fixed in 70% ethanol diluted in PBS, and the samples were stored at -20 ℃. The fixed cells were resuspended in PBS containing 20μg/ml propidium iodide (PI) and 10μg/ml RNase A, and incubated for 10minutes at 37℃. Flow cytometric analysis was performed using Coulter EPICS Flow Cytometer (Beckman-Coulter).

**Electrophoretic mobility shift assay (EMSA)**

Nuclear extracts were made using a kit according to the manufacturer's instructions (Active Motif). EMSA were carried out using EMSA "Gel-Shift" Kit (Panomics) according to the manufacturer's instructions. Nuclear extracts were incubated with a biotin-labeled oligonucleotide containing the consensus binding sequence for NF-κB (5'-CATCGGAAATTTCCGGAAATTTCCGGAAATTTCCGGC-3') for 30min at 15°C, and transcription factor-bound oligonucleotide was separated from unbound oligonucleotide by electrophoresis on a 6% polyacrylamide gel. After transfer to nylon membrane (GE Healthcare) using a semidry blot module (Bio-Rad), the biotin-labeled bands were

visualized using horseradish peroxidase (HRP)-based chemiluminescence. The specificity of binding was verified using positive control and unlabeled consensus oligonucleotide corresponding to NF-κB binding sequence as a competitor in the binding reaction.

**Luciferase reporter assays**

Cells were seeded in six-well plates and were transfected with 900ng of total DNA using Fugene 6 (Roche) following the manufacturer's instructions. In all, 270ng of pNFκB-MetLuc2-luciferase reporter and 30ng of pSEAP2-control vector were cotransfected. Cells were harvested after 48hr of transfection, and luciferase reporter assays were performed using the Dual Secreted Luciferase Reporter System (Clontech). All transfections were performed in triplicate.

**mTORC2 *in vitro* kinase assay**

Kinase assays on endogenous mTORC2 were performed as described previously (1). Near-confluent 100-mm plates of the given cell line were lysed in 1 ml mTORC lysis buffer, and mTORC2 was immunoprecipitated with 1.5 μg anti-Rictor antibodies, as described above. Inactive Akt1/PKBα (Upstate Biotechnology) was used as the substrate for these reactions, and phosphorylation was detected by immunoblotting. Immunoblots will be used to determine the relative levels of mTORC2 components (mTOR and Rictor) coimmunoprecipitated with Rictor, as an indication of complex stability. All appropriate positive and negative controls for the *in vitro* kinase assay will be performed.

**Immunohistochemical and immunofluorescent staining**

Paraffin-embedded tissue blocks were sectioned using the UCLA Pathology Histology and Tissue Core Facility. Immunohistochemical staining was performed as previously described (2). Slides were counterstained with hematoxylin to visualize nuclei. Paraffin-embedded tissue sections underwent immunohistochemical analysis in which the results were scored independently by two pathologists who were unaware of the findings of the molecular analyses. Quantitative image analysis to confirm the pathologists' scoring was also performed with Soft Imaging System software (3). We have previously demonstrated the utility of this quantitative method for measuring drug-specific effects in paraffin-embedded tissue samples from GBM patients enrolled in clinical trials with targeted agents (2, 4).

**TUNEL staining**

Cells were placed in 8-well chamber slides at $2x10^4$cells/well in 500 µl of growth medium and then incubated for 48 hours in each condition of treatment. Apoptotic cells were evaluated with the In Situ Cell Death Detection Kit, Fluorescein and following the manufacturer's protocol (Roche). Nuclei are stained blue by 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). TUNEL-positive cells were visualized with a fluorescein microscope (Nikon ECLIPSE 90i). The percentage of apoptosis was calculated as the percentage of TUNEL-positive cells out of 400 cells from each group using NIH images.

**Plasmid and siRNA transfection**

Myc-Rictor expression construct (plasmid 11367) was obtained from Addgene. Transient transfections were performed using Fugene 6 (Roche) in full serum, with medium change after 24 hours, and cells were typically harvested 48hr post-transfection. Transfection of small interfering RNAs (siRNA) into GBM cell lines was carried out using Lipofectamine RNAiMAX (Invitrogen) in full serum, with medium change after 24 hours. On-TARGETplus SMARTpool siRNAs (Thermo Scientific, Dharmacon Division) specifically targeting Rictor (catalog #L-016984-00-0005), Raptor (catalog #L-004107-00-0005), SGK (catalog #L-003027-00-0005) and nontargeting control pools of siRNAs were used at 10nM, and cells were harvested 48hr post-transfection.

**shRNA infection**

shRNA Rictor-1 (plasmid 1853) and Scramble (plasmid 1864) constructs were obtained from Addgene. Lentivirus-mediated delivery of shRNA directed against Rictor was performed as described previously (5). Cells were infected in the presence of 6 μg/ml protamine sulfate, selected for puromycin resistance, and analyzed on the seventh day after infection.

**Adenoviral infection**

Recombinant replication-deficient adenovirus type 5 encoding a dominant negative mutant of human IκBα (Ad-CMV-IκBα(DN)) and adenovirus encoding β-galactosidase (LacZ) (Ad5-CMV-LacZ) for control was obtained from Vector Biolabs. GBM cells were incubated for 24hr on 12-well plates at 2 x $10^5$cells/well in 10% serum medium. Then, cells were washed and were incubated at a multiplicity of infection of 100 plaque-

forming units per cell in 0.5ml 1% serum medium. After 12 h of infection, the culture volume was adjusted to 1ml with 10% serum medium, and cells were harvested 48hr post-transfection.

**Xenograft model**

U87-EGFRvIII cells were implanted into immunodeficient SCID/Beige mice for subcutaneous xenograft studies. SCID/Beige mice were bred and kept under defined-flora pathogen-free conditions at the Association for Assessment of Laboratory Animal Care–approved Animal Facility of the Division of Experimental Radiation Oncology, UCLA. For subcutaneous implantation, exponentially growing tumor cells in culture were trypsinized, enumerated by trypan blue exclusion, and resuspended at $3\times10^6$ cells/ml in a solution of Dulbecco's phosphate-buffered saline (dPBS) and Matrigel (BD Biosciences). Tumor growth was monitored with calipers by measuring the perpendicular diameter of each subcutaneous tumor. Tumor was treated with cisplatin (3 or 5mg/kg; Sigma) and normal saline every day. Mice were euthanized if tumors reached 14mm in maximum diameter or animals showed signs of illness. All experiments were conducted after approval by the Chancellor's Animal Research Committee of UCLA.

**References**

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