Supplemental Data

Journal: Journal of Clinical Investigation

EGFR Phosphorylation of DCBLD2 Recruits TRAF6 and Stimulates Akt-promoted Tumorigenesis


Supplemental Figures 1-24 and Supplemental Tables 1-2
Supplementary Figure 1. Genomic expression of DCBLD2 gene is up-regulated in clinical GBMs. 

(A) Digital karyotyping reveals a focal region on chromosome 3q12.1 displaying high copy numbers that indicate gene amplification. This amplified region includes two genes, ST3GAL6 and DCBLD2. 

(B) Quantitative real time reverse transcription PCR (Q-PCR) identifies increased expression of DCBLD2 but not ST3GAL6 in 14 out of 28 clinical GBM tumors. 

(C) Serial analysis of gene expression (SAGE) of DCBLD2 and ST3GAL6 in GBMs. Box, 25th-75th percentile with median. Whiskers, minimum and maximum values. 

(D) SAGE of DCBLD2 in subsets of gliomas. Normal vs. GBM, P<0.05. Box, 25th-75th percentile with median. Whiskers, minimum and maximum values. 

(E) Genomic expression of DCBLD2 among GBM subtypes (n=116). Number of GBM tumors, neural, 19, proneural, 37, classical, 22 and mesenchymal, 38. P<0.001 for mesenchymal vs. neural, mesenchymal vs. proneural, mesenchymal vs. classical. 

(F) Interphase FISH in a clinical GBM tumor TB2580 identifies tumor cells with amplification of DCBLD2 (green, FITC) compared to a chromosome 3 reference control (red, TRITC). Nuclei are stained blue (DAPI). Red arrows, DCBLD2 probe. Yellow arrows, Chromosome 3 reference probe. Overlap of DCBLD2 and chromosome 3 probes produces white-dish color indicated by yellow arrows. 

Data are derived from The Cancer Genome Anatomy Project (C and D) or The Cancer Genome Atlas (TCGA, E).
Supplementary Figure 2. Expression of EGFR, DCBLD2, and TRAF6 in cell lines derived from human glioma, lung cancer, HNC and melanoma.

A. Expression of EGFR, DCBLD2 and TRAF6 in nine glioma cell lines.
B. Expression of EGFR, DCBLD2 and TRAF6 in four HNC lines.
C. Expression of EGFR, DCBLD2 and TRAF6 in six lung cancer cell lines.
D. Expression of EGFR, DCBLD2 and TRAF6 in four melanoma cell lines.

β-actin was used as a loading control. Data are representative of three independent experiments.
Supplementary Figure 3. Knockdown of endogenous DCBLD2 showed minimal effect on glioma cell proliferation in vitro and a modest impact on glioma tumor growth in the brain.

A. Knockdown of endogenous DCBLD2 by two different shRNAs (shD2#1, shD2#2) in glioma U87, SNB19 and LN444 cells. IB analysis. β-actin was used as a loading control.

B. Depletion of endogenous DCBLD2 did not affect in vitro cell proliferation of glioma cells. Cell proliferation of indicated glioma cells was determined by WST-1 assays using serum-starved glioma cells from panel A. Cells were seeded in 6 replicates. Error bars, ± SD.

C. Inhibition of DCBLD2 by shRNA knockdown only displayed a modest impact on tumorigenesis of glioma U87 cells in the brain of mice. Representative images of H&E stained brain sections with indicated U87 gliomas from 5 mice per group of two independent experiments. Scale bars, 100 µm. Data are representative of two to three independent experiments.
**Supplementary Figure 4.** DCBLD2 is required for EGFR-driven glioma tumorigenesis.

**(A)** Effect of DCBLD2 knockdown by shD2#1, shD2#2 or shC on glioma cell colony formation. Cells were seeded on soft agar in triplicate. Scale bars, 1 mm.

**(B) & (C)** Analyses of gene expression of WT EGFR or EGFRvIII in various patient-derived glioma stem cells (GSC) by RT-PCR using primers that specifically distinguish gene products of WT or EGFRvIII (1) (B) or by IB using an anti-EGFR antibody (C).

WT EGFR were detected in GSC528, JK18, JK42 and JK 83 whereas EGFRvIII is expressed in GSC83, 1123 and JK92 cells. Data are representative of two independent experiments.
Supplementary Figure 5. EGF stimulates p-Y-DCBLD2 in cell lines derived from lung cancer, HNC and melanoma.
IP-IB assays. EGF stimulates p-Y-DCBLD2 in lung cancer 343T, HNC PCI-15B and melanoma A375 cells. Immunoprecipitated p-Y-DCBLD2 was detected with a pan anti-tyrosine antibody, 4G10. p-Y-EGFR was detected with an anti-p-EGFRY1045 antibody. DCBLD2 and β-actin were used as loading controls. Data are representative of three independent experiments.

Supplementary Figure 6. Reciprocal IP-IB analyses failed to detect DCBLD2 association to EGFR in glioma cells.  IP-IB assays. Immunoprecipitated DCBLD2 (upper panels) or EGFR (lower panels) was subjected to IB analyses of EGFR and DCBLD2 from U87/EGFR WT cells stimulated with EGF (50 ng/ml, 10 min) with or without pretreatment of EGFR inhibitor, Erlotinib (10 μM, 1h). Data are representative of three independent experiments.
Supplementary Figure 7. Mutant forms of DCBLD2\textsuperscript{F621}, DCBLD2\textsuperscript{F750}, and DCBLD2\textsuperscript{F621/F750} have no effect on EGFRvIII-stimulated Erk1/2 activity.

IB analyses of Erk1/2 activity. Re-expression of Flag-DCBLD2 shRNA-resistant DCBLD2\textsuperscript{WT} (WT) or indicated mutants or a vector control does not affect p-Erk1/2 in U87/EGFRvIII/shD2 cells. Erk1/2 and β-actin were used as loading controls. Data are representative of three independent experiments.

Supplementary Figure 8. Re-expression of Flag-DCBLD2 shRNA-resistant DCBLD2\textsuperscript{WT} or DCBLD2\textsuperscript{F621} but not DCBLD2\textsuperscript{F750}, DCBLD2\textsuperscript{F621/F750} mutant, or a vector control (C), rescues EGFRvIII-stimulated soft agar colony formation \textit{in vitro}.

A. Soft agar colony formation assay for U87/EGFRvIII/shD2 that separately re-express shRNA-resistant WT or mutant forms of DCBLD2. Representative images of three independent experiments. Scale bars, 1 mm.

B. Quantifications of soft agar colonies. Error bars from 3 replicates, ± SD. Compared to shControl, **, \( P < 0.01 \). Data are representative of three independent experiments.
Supplementary Figure 9. Validation of the specificity of the anti-p-DCBLD2\textsuperscript{Y750} antibody.

A. IB assays. EGFR\textsuperscript{vIII} activates p-DCBLD2\textsuperscript{Y750} that is inhibited by shRNA knockdown of DCBLD2. The EGFR\textsuperscript{vIII}-stimulated p-DCBLD2\textsuperscript{Y750} was detected with a rabbit anti-p-DCBLD2\textsuperscript{Y750} antibody generated against a specific phospho-peptide containing p-Y750 and surrounding amino acids. β-actin was used as a loading control.

B. Re-expression of shRNA-resistant DCBLD2\textsuperscript{WT} and mutant DCBLD2\textsuperscript{F621}, but not DCBLD2\textsuperscript{F750} or DCBLD2\textsuperscript{F621/F750}, rescues the EGFR\textsuperscript{vIII}-stimulated p-DCBLD2\textsuperscript{Y750} in glioma U87/EGFR\textsuperscript{vIII}/shD\#2 cells. β-actin was used as a loading control.

C. IHC assays of human a clinical GBM tumor tissue with the specific anti-p-DCBLD2\textsuperscript{Y750} antibody in the presence or absence of a specific blocking peptide synthesized with identical amino acid sequence of an immunoactive peptide for generating this specific anti-p-DCBLD2\textsuperscript{Y750} antibody including the Y750 residue. IHC was performed twice on the GBM sample with the blocking peptide with similar results. Scale bars, 50 µm. Data are representative of two to three independent experiments.
Supplementary Figure 10. EGFR phosphorylates DCBLD2 at Y750 in vitro.

In vitro kinase phosphorylation assay. A Flag-tagged DCBLD2\textsuperscript{WT} (WT) or a DCBLD2\textsuperscript{F750} mutant was separately expressed in HEK293T cells and immunoprecipitated with an anti-Flag antibody. The immunoprecipitated DCBLD2 proteins were pre-treated with a recombinant protein tyrosine phosphatase (PTP) and then incubated with or without a recombinant active EGFR. The reaction mixtures were examined by IB analysis using the specific anti-p-DCBLD2\textsuperscript{Y750} antibody. Flag-DCBLD2 was used as a loading control. Data are representative of two independent experiments.
Supplementary Figure 11. p-DCBLD2<sup>Y750</sup> is stimulated by EGF but not HGF or PDGF-A in various types of human cancer cells.

Glioma SNB19, lung cancer 343T, HNC PCI-15B and melanoma A375 cells were serum-starved for 24 h and treated with or without 50 ng/ml PDGF-A, 100 ng/ml EGF, or 40 ng/ml HGF for 5 min. A specific anti-p-DCBLD2<sup>Y750</sup> antibody was used to detect p-Y of endogenous DCBLD2 in these cells; anti-c-Met (p-Y1230/Y1234/Y1235), anti-p-PDGFR<sub>α</sub> (p-Y754) and anti-p-EGFR (p-Y1045) antibodies were used to examine p-Y of c-Met, PDGFR<sub>α</sub> and EGFR, respectively. DCBLD2, PDGFR<sub>α</sub>, EGFR, and β-actin were used as loading controls. Data are representative of three independent experiments.
Supplementary Figure 12. Association of DCBLD2 with TRAF6 and effect of knockdown of DCBLD2 on cell proliferation in primary GBM cells with high levels of endogenous EGFRvIII expression.

A. IP-IB analyses of association of DCBLD2 with TRAF6. Human primary short-term cultured glioma GBM6 and GBM39 cells express endogenous EGFRvIII at high levels. GBM14 has non-detectable EGFR WT or EGFRvIII proteins (2, 3). β-actin was used as a loading control.

B. Knockdown of DCBLD2 by shRNA (shD2) inhibits EGFRvIII-stimulated Akt activity in primary GBM cells with endogenous EGFRvIII overexpression. Akt and β-actin were used as loading controls.

C. Knockdown of DCBLD2 attenuates cell proliferation of GBM6 and GBM39 cells with high levels of endogenous EGFRvIII expression. Cells were seeded in 6 replicates.

D. Knockdown of DCBLD2 inhibits soft-agar colony formation of GBM6 and GBM39 cells. Cells were seeded on soft agar in three replicates.

In panels C and D, error bars, ± SD. Compared with shC-treated cells, *, P<0.05. Data are representative of three independent experiments.
Supplementary Figure 13. EGFRvIII stimulates TRAF6 E3 ligase activity.

IP-IB analyses. HA-TRAF6 and His-Ub were co-expressed with or without EGFRvIII in HEK293T cells. His-Ub was precipitated by Ni-nitrilotriacetic acid (NTA). β-actin was used as a loading control. Data are representative of three independent experiments.
Supplementary Figure 14. Loss of TRAF6 E3 ligase activity has no effect on EGFRvIII-induced association of TRAF6 with DCBLD2.

IP-IB analyses. Flag-DCBLD2 and HA-TRAF6 WT or a C70A mutant was co-expressed with or without EGFRvIII in HEK293T cells. HA-TRAF6, Flag-DCBLD2, and β-actin were used as loading controls. Data are representative of three independent experiments.
Supplementary Figure 15. Expression of DCBLD2, Skp2, and TRAF6 in human breast cancer and glioma cell lines.

IB analyses. Cell lysates of human cancer cell lines were examined for expression of DCBLD2, TRAF6 and Skp2 with corresponding antibodies. DCBLD2 was detected at low levels in four of five breast cancer cell lines examined. In contrast, DCBLD2 is expressed at high levels in all four glioma cell lines examined here and nine glioma cell lines (including these four) shown in Supplemental Figure 2A. β-actin was used as a loading control. Data are representative of two independent experiments.

<table>
<thead>
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<th>TRAF6</th>
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<th>β-actin</th>
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<td>Glioma</td>
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**Table:**

- DCBLD2
- TRAF6
- Skp2
- β-actin

**Breast Cancer:** MDA-MB-231, BT474, MDA-MB-468, SUM149, U87

**Glioma:** U87, SNB19, T98G, LN444
Supplementary Figure 16. Inhibition of TRAF6 expression decreases DCBLD2 expression in mouse embryonic fibroblasts (MEF) and glioma cells. IB analyses of expression of DCBLD2 and TRAF6.

A. Expression of endogenous DCBLD2 was at very low levels in TRAF6 null (TRAF6\textsuperscript{−/−}) MEF but at high levels in TRAF6 wild type (TRAF6\textsuperscript{WT}) MEF.

B. Expression of endogenous DCBLD2 was significantly decreased when endogenous TRAF6 was knocked down by siRNAs in glioma SNB19 and U87 cells.

C. Expression of endogenous DCBLD2 was markedly decreased when endogenous TRAF6 was knocked down by two separate shRNAs, #1 and #3, but not shRNAs #2 and #4, or a control shRNA in glioma U87/EGFRvIII cells.

β-actin was used as a loading control. Data are representative of two independent experiments.
Supplementary Figure 17. Re-expression of DCBLD2 WT or DY750 mutant in glioma U87 cells or patient-derived glioma stem cells (GSC#83) that endogenous DCBLD2 was knocked down by shRNA for DCBLD2 (shD2).

IB analyses. Cell lysates of various U87 or patient-derived glioma stem cells (GSC#83) were examined for expression of DCBLD2 WT or DCBLD2 D750 mutant. shC, cells expressed a scrambled control shRNA. shD2, cells expressed a shRNA for DCBLD2. GFP, cells expressed GFP, WT, cells express DCBLD2 WT and D750, cells expressed DCBLD2 D750 mutant. β-actin was used as a loading control. Data are representative of two independent experiments.
Supplementary Figure 18. Examples of a clinical GBM sample showing co-expression of EGFR, p-EGFR\textsuperscript{Y1172}, p-DCBLD2\textsuperscript{Y750}, TRAF6, and p-Akt\textsuperscript{T308} and a GBM sample with negative staining for these proteins. Representative images of two GBM specimens that were IHC stained positive (J213) or negative (J45) by indicated antibodies. Scale bars, 50 μm. IHC analyses on these two GBM samples using these five antibodies were performed twice. Data are representative of two independent experiments.
Supplementary Figure 19. Examples of two clinical HNC samples showing co-expression of EGFR, p-EGFR<sup>Y1172</sup>, p-DCBLD2<sup>Y750</sup>, TRAF6, and p-Akt<sup>T308</sup> and two HNC samples with negative staining for these proteins. Representative images of two HNC specimens that were IHC stained positive (P32-1AFS and HN11-6062) and two HNC tissues that showed negative staining (P7 and HN11-6026-3J) by indicated antibodies. Scale bars, 100 µm. IHC analyses on these HNC samples in TMAs using these five antibodies were performed twice. Data are representative of two independent experiments.
Supplementary Figure 20. Expression of EGFR, p-DCBLD2\textsuperscript{Y750}, TRAF6 and p-Akt in separate cohorts of 19 GBM specimens, 15 HNC samples and their matched normal tissues. IB analyses of expression of EGFR, p-DCBLD2\textsuperscript{Y750}, TRAF6 and p-Akt\textsuperscript{T308} in 19 snap-frozen clinical GBM specimens (A), 15 snap-frozen HNC samples (T) and their matched normal tissues (N) (B). β-actin was used as a loading control. Data are representative of 2 independent experiments.
Supplementary Figure 21. High expression of either p-EGFR$^{Y1172}$ or p-DCBLD2$^{Y750}$, or co-expression of p-EGFR$^{Y1172}$ with TRAF6, correlates with worse survival of patients with gliomas.

Kaplan-Meier analyses of patients with high p-EGFR$^{Y1172}$, p-DCBLD2$^{Y750}$-expressing, or co-expression of p-EGFR$^{Y1172}$-TRAF6 tumors (blue line) versus low p-EGFR$^{Y1172}$, p-DCBLD2$^{Y750}$-expressing or low co-expression of p-EGFR$^{Y1172}$-TRAF6 tumors (red line) in IHC-stained WHO grades II-IV glioma specimens showed in Figure 8A and Supplemental Table 1. Expression of TRAF6 does not correlate with clinical outcomes. $P$ values were calculated by using log-rank test. Black bars, censored data.
Supplementary Figure 22. High expression of p-EGFR\textsuperscript{Y1172} or co-expression of EGFR with TRAF6 correlates with worse survival of patients with HNCs.

Kaplan-Meier analyses of patients with high expression of p-EGFR\textsuperscript{Y1172}, p-DCBLD2\textsuperscript{Y750} or co-expression of p-EGFR\textsuperscript{Y1172}-TRAF6 tumors (blue line) versus low p-EGFR\textsuperscript{Y1172} p-DCBLD2\textsuperscript{Y750} or p-EGFR\textsuperscript{Y1172}-TRAF6-expressing tumors (red line) in IHC-stained TMA of HNCs showed in Figure 8C and Supplemental Table 2. Expression of p-DCBLD2\textsuperscript{Y750} does not correlate with clinical outcomes. \(P\) values were calculated by using log-rank test. Black bars, censored data.
Supplementary Figure 23. EGFRvIII, p-DCBLD2Y750, TRAF6, and p-AktT308 are co-expressed in clinical gliomas and co-expression of EGFRvIII with p-DCBLD2Y750 correlates with poor survival of patients with gliomas

A. A total of 148 clinical primary glioma specimens including WHO grade I to IV tumors were analyzed by IHC for p-DCBLD2Y750, TRAF6, p-AktT308 and EGFRvIII. Representative images of serial sections of a grade IV GBM tissue using anti-EGFRvIII (clone 8.3), anti-p-DCBLD2Y750, anti-TRAF6, and anti-p-AktT308 antibodies are shown. Inserts, isotype-matched IgG controls of the same area in adjacent sections. Arrows, positive staining. Scale bars, 50 µm. IHC analyses on these glioma tumor samples using these four antibodies were performed twice. Data are representative of two independent experiments.

B. Kaplan-Meier analyses of patients with high EGFRvIII/p-DCBLD2Y750-expressing tumors (blue line) versus low EGFRvIII/p-DCBLD2Y750-expressing tumors (red line) in IHC staining assays (A) of WHO grades I-IV gliomas. P values were calculated by using log-rank test. Black bars, censored data.
Supplementary Figure 24. The levels of expression of p-DCBLD2\textsuperscript{Y750} and p-EGFR\textsuperscript{Y1172} are increased in WHO tumor grade II to IV clinical gliomas compared with normal brain tissues.

IHC staining of 31 WHO grade II, 23 grade III and 78 grade IV (GBM) glioma specimens using anti-EGFR\textsuperscript{Y1172} or anti-p-DCBLD2\textsuperscript{Y750} antibodies with validated specificities. The data were compared with four IHC-stained normal brains that had no detectable pathological lesions. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$. 
Supplementary Table 1. Spearman’s rank correlation analysis of expression level of p-EGF$^Y1172$, p-DCBLD2$^Y750$, TRAF6 and p-Akt$^T308$ in human clinical glioma specimens by IHC staining.

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Note: a. $P<0.05$; b. $P<0.01$ and c. $P<0.001$
Supplementary Table 2. Spearman’s rank correlation analysis of expression level of p-EGFR$^{Y1172}$, p-DCBLD2$^{Y750}$, TRAF6 and p-Akt$^{T308}$ in human clinical HNC specimens by IHC staining.

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Note: a. $P<0.05$; b. $P<0.01$ and c. $P<0.001$

References:


Full unedited gels for Figure 1

A

anti-DCBLD2
anti-p-EGFR
anti-EGFR
anti-β-actin

D

anti-DCBLD2
anti-p-EGFR
anti-EGFR
anti-β-actin

Full unedited gels for Figure 2

A

anti-EGFR
anti-DCBLD2
anti-β-actin

G

anti-EGFR
anti-DCBLD2
anti-β-actin

Rectangles indicate cropped areas used in indicated Figures
Rectangles indicate cropped areas used in indicated Figures

Full unedited gels for Figure 3
Rectangles indicate cropped areas used in indicated Figures.
Rectangles indicate cropped areas used in indicated Figures
Full unedited gels for Figure 6

A

- anti-TRAF6
- anti-β-actin

C

- anti-p-Akt_T308
- anti-Akt
- anti-β-actin

D

- anti-Akt
- anti-p-Akt_T308
- anti-β-actin

E

- anti-HA
- anti-Akt
- anti-p-Akt_T308
- anti-β-actin
Full unedited gels for Figure 7

**A**
- anti-p-Y-EGFR
- anti-EGFR
- anti-Flag (DCBLD2)
- anti-p-AktT308
- anti-Akt
- anti-TRAF6
- anti-p-Y-EGFR

**C**
- anti-p-Y-EGFR
- anti-EGFR
- anti-Flag (DCBLD2)
- anti-p-AktT308
- anti-Akt
- anti-TRAF6
- IP: anti-Flag D2
- IP: anti-TRAF6
Full unedited gels for Supplemental Figure 2

Rectangles indicate cropped areas used in indicated Figures
Full unedited gels for Supplemental Figure 3

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Supplemental Figure 4C

Supplemental Figure 5

Supplemental Figure 6

Supplemental Figure 7

Supplemental Figure 9

Rectangles indicate cropped areas used in indicated Figures
Full unedited gels for the indicated Figures

Supplemental Figure 10

- anti-p-Y750
- anti-Flag
- anti-p-Y750
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- anti-p-Y750
- anti-DCBLD2
- anti-p-Y750
- anti-DCBLD2
- anti-p-Y750
- anti-DCBLD2
- anti-p-Y750

Supplemental Figure 11

- anti-p-Y-PDGFRα
- anti-PDGFRα
- anti-p-Y-MET
- anti-MET
- anti-p-Y-EGFR
- anti-EGFR
- anti-β-actin

Rectangles indicate cropped areas used in indicated Figures
Supplemental Figure 12

A

anti-EGFR

anti-DCBLD2

anti-TRAF6

anti-β-actin

Supplemental Figure 13

B

anti-DCBLD2

anti-p-Akt\textsuperscript{T308}

anti-p-Akt

anti-β-actin

Supplemental Figure 14

anti-DCBLD2

anti-Y-DCBLD2\textsuperscript{Y750}

anti-TRAF6

Supplemental Figure 15

anti-TRAF6

anti-Spk2

Rectangles indicate cropped areas used in indicated Figures
Full unedited gels for the indicated Figures

Supplemental Figure 16

A

anti-DCBLD2

anti-TRAF6

anti-β-actin

B

anti-DCBLD2

anti-TRAF6

anti-β-actin

C

anti-DCBLD2

anti-TRAF6

anti-β-actin

Supplemental Figure 17

A

anti-DCBLD2

anti-β-actin

B

anti-DCBLD2

anti-β-actin

Rectangles indicate cropped areas used in indicated Figures
Full unedited gels for Supplemental Figure 20

A

- anti-EGFR
- anti-p-DCBLD2\(^{Y750}\)
- anti-TRAF6
- anti-p-Akt\(^{T308}\)
- anti-\(\beta\)-actin

B

- anti-EGFR
- anti-p-DCBLD2\(^{Y750}\)
- anti-TRAF6
- anti-p-Akt\(^{T308}\)
- anti-\(\beta\)-actin

Rectangles indicate cropped areas used in the indicated Figures.