Clinical efficacy and predictive biomarkers of ONC201 in H3 K27M-mutant diffuse midline glioma

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Letter

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Abstract

Patients with diffuse midline glioma (DMG) harboring H3 K27M mutation have no proven therapies beyond radiation. ONC201, a DRD2 antagonist and mitochondrial ClpP agonist, has induced early responses in patients with H3 K27M-mutant DMG. We performed an integrated pre-clinical and clinical assessment of ONC201 treatment, in order to define response rates in H3 K27M-mutant DMG patients and to clarify predictors of response. ONC201 was effective in murine H3 K27M-mutant gliomas with excellent CNS penetration and survival benefit. H3 K27M-mutant DMG patients treated with ONC201 on active clinical trials (n=50) showed significant survival benefit in recurrent and non-recurrent settings, with multiple sustained responses. Tumor sequencing from treated patients demonstrates an EGFR/FOXG1-driven telencephalic gene regulatory network that imparts a critical resistance phenotype to ONC201. Genetic and pharmacologic knockdown of EGFR in H3 K27M-mutant cell cultures results in improved sensitivity to ONC201 and reduced FOXG1 enhancer binding, suggesting possible future combinatorial opportunities.

Main

The histone mutation H3 K27M in diffuse midline gliomas (DMG) is associated with aggressive clinical behavior and overall median survival of 12.0 months[1-3]. The H3 K27M mutation is most commonly found in midline central nervous system (CNS) structures in children and young adults[4], where the thalamus and brainstem account for the majority of patients carrying the mutation[1,5-7]. We assessed the blood-brain barrier (BBB) penetration of ONC201 in midline brain structures in non-tumor bearing mice. As previously shown[8,9], oral ONC201 achieves concentrations in brain tissue that greatly exceed plasma concentrations (Fig. 1a). At 30 minutes after treatment with 15 mg/kg ONC201, all midline structures demonstrated micromolar (μM) concentrations (Fig. 1a). To evaluate the in vivo efficacy of ONC201, we adopted a midline in utero electroporation (IUE) mouse model of H3 K27M- glioma[10,11], in which tumors harboring dominant negative *TP53*, *PDGFRA* D842V, and *H3F3A* (H3.3) K27M mutations ("PPK") are generated (Fig. 1b-c). Early passage PPK neurospheres and human H3 K27M- mutant DMG cell cultures SF7761 and QCTB-R059 demonstrate ONC201 sensitivity with IC50 of 0.5, 3 and 4 uM respectively (Fig. 1d); while H3 K27M-mutant DIPG007 cells remained relatively resistant. Weekly ONC201 treatment of mice bearing PPK tumors (125mg/kg once a week) significantly extended survival (p=0.02; median 107 vs. 77 days) with no observed toxicity (Fig. 1e).

In order to define response rates in H3 K27M DMG patients and to clarify the genomic and clinical predictors of response, we performed an integrated assessment of H3 K27M-mutant DMG patients treated with ONC201. The clinical analysis included patients with primary tumors in the thalamus or brainstem and confirmed H3 K27M mutation (inclusion/exclusion criteria in Extended Data Fig. 1). Overall survival (OS) is 28.1 months from diagnosis (median time from diagnosis to trial enrollment: 8.7 months) in a total of 50 H3 K27M-mutant DMG patients treated with ONC201 (27 thalamic and 23 brainstem), surpassing historical median OS of 12 months (n=274[12], p<0.0001) (Extended Data Fig. 2a).

In thalamic tumors, median OS has not been reached with a median follow-up of 18.5 months from diagnosis [n=27, historical median OS: 14.5 months (n=68[12], p=0.0001)] (Fig. 2a). Median OS is 17.1 months for patients with brainstem tumors [n=23; historical median OS: 11.9 months (n=206[12], p=0.0002)] (Fig. 2a). In patients who initiated ONC201 following radiation but prior to recurrence, median OS has not been reached with a median follow up of 16.8 months from diagnosis [8 thalamic, 8 brainstem; historical median OS: 12.0 months (n=274), p=0.0008] (Fig. 2b). In the recurrent setting, median OS is 7.9 months from recurrence (n=34), compared to historical H3 K27M median OS of 5.5 months from recurrence[13,14] (p=0.0029) (Fig. 2c).

In the post-radiation non-recurrent setting, median PFS is 20.1 months from diagnosis for thalamic patients (n=8; median time on drug without progression: 11.7 months, median time from diagnosis to trial enrollment: 3.7 months) and 13.0 months from diagnosis for brainstem patients (n=8; median time on drug without progression: 5.9 months, median time from diagnosis to trial enrollment: 3.7 months) (Fig. 2d). In the recurrent setting, median PFS for thalamic patients is 3.3 months from enrollment, similar to median PFS for brainstem patients of 3.9 months (Extended Data Fig. 2b). Median OS and PFS in ONC201-treated H3 K27M-mutant DMG patients are better than historical controls for all sub-groups, even when censoring patients who remain on-treatment (sensitivity analysis) upon follow- up (Extended Data Fig. 3).

These data show that H3 K27M-mutant patients have improved outcomes with ONC201 treatment, particularly those with thalamic and non-recurrent tumors at enrollment. Thalamic tumors with evaluable tumor imaging (n=21) demonstrated 1 complete response (CR) and 5 partial responses (PR) by RANO, with 4 additional (<50%) tumor regressions (Extended Data Fig. 4). Radiographic responses observed in thalamic patients on ONC201 treatment remain durable [median 10.7 (1.3-45.1) months] (Extended Data Fig. 5). Serial cell-free tumor DNA (tDNA) analysis for H3.3 K27M[15] in plasma and CSF demonstrated correlation with sustained clinical and radiographic response (Fig. 2e-f)

To explore the molecular attributes of H3 K27M-mutant DMG patients who respond to ONC201, we analyzed a single institution cohort (University of Michigan; n=13) where baseline tumor RNA-seq[16-18] was compared to best response (percentage reduction in tumor area). Analysis of known DMG driver genes revealed baseline expression of *EGFR* and the cortical developmental transcription factor *FOXG1* as the strongest biomarkers of resistance to ONC201 (Fig. 3a). Interestingly, EGFR has been shown to mediate oncogenic phenotypes in glioma through FOXG1 signaling in adult GBM cells[19]. Conversely, H3 K27M-mutant DMG patients with increased baseline expression of gene sets involved in mitochondrial function demonstrated improved radiographic response (Figure 3b); consistent with recent data showing that ONC201 binds and activates the mitochondrial protease ClpP in leukemia cells[20]. Gene co-expression, functional enrichment[21], and gene regulatory network analysis[22] revealed a telencephalic molecular phenotype and reduced mitochondrial function in *FOXG1*$^{+}$-H3 K27M-mutant DMGs (Extended Data Fig. 6). The latter association is consistent with FOXG1 regulation of mitochondrial bioenergetics in

mouse neural stem cells[23] and primary glial cells[24]; possibly introducing a glioma-specific resistance phenotype to ONC201.

Examining treatment responses in our cohort further, patients who had low expression of *EGFR* and *FOXG1* exhibited the largest reductions in tumor size on ONC201 treatment (Fig. 3c-d). No somatic alteration correlated with response (Fig. 3e). However, patients with co-occurring *EGFR* mutations did not respond to ONC201 (UMICH-006 and UMICH-017). UMICH-006, a nine-year-old patient with H3- K27M thalamic DMG with an activating EGFR pathway mutation ($EGFR^{A289V}$) and high *EGFR*/*FOXG1* tumor expression, never responded to ONC201 (Fig. 3f), and showed a spike in H3 K27M CSF tDNA variant allele fraction (VAF) at 6 months correlating with metastatic progression (Fig. 3g).

We next performed an analysis of human cancer cell lines from DepMap/PRISM[25] to assess correlation of FOXG1 and EGFR expression (and other reported predictive makers[26-28]) with sensitivity to ONC201. *CLPP* expression was identified as the strongest predictor of ONC201 sensitivity at the pan- cancer level[29]. A glioma-specific analysis (glioma cell lines) revealed high *FOXG1* expression levels at baseline as the third most significant predictor of sensitivity to ONC201 (Extended Data Fig. 7a-b). The other direct target of ONC201 response, DRD2[26-28], did not predict ONC201 response in either our patient cohort or DepMap/PRISM glioma cell atlas. Further analysis of the pan-cancer cell line atlas demonstrated that ONC201 resistance correlates with: (i) EGFR expression, (ii) EGFR dependency and (iii) EGFR inhibitor (EGFRi) sensitivity (Fig. 4a). Interestingly, previous data demonstrated that antipsychotics targeting DRD2 and EGFR inhibitors can work synergistically to target adult GBM cells (H3 K27M WT)[30].

Lentiviral-mediated shRNA knockdown of EGFR in the ONC201-resistant H3 K27M primary cell culture DIPG007 resulted in a transition from EGFR to PDGFRA activation (Fig. 4b) and marked improvement in response to ONC201 (Fig. 4c). This is consistent with positive correlation of *PDGFRA* expression with ONC201 treatment response in our clinical cohort (Fig. 3a). Two ONC201-sensitive H3 K27M cell cultures (SF7761 and QCTB-R059) displayed additive benefit to treatment with ONC201 and the EGFRi osimertinib (Fig. 4d and Extended Data Fig. 7c). Previous work has shown EGFR to drive oncogenesis in GBM through a FOXG1-dependent gene regulatory network[19]. Treatment of H3 K27M cells with osimertinib resulted in no change in FOXG1 protein levels (Extended Data Fig. 7c) but did reduce FOXG1 binding at established[19] enhancer loci (Fig. 4e,f).

We next explored the expression of EGFR and FOXG1 in non-tumor cells to see if they may influence ONC201 sensitivity either through the micro-environment or cell of origin. FOXG1 and EGFR is highly expressed in the developing and adult murine telencephalon (cortex) in comparison to midline structures (Extended Data Fig. 8). In human high-grade glioma single-cell[31] and bulk mRNA-seq[3], EGFR and FOXG1 expression is highest in adult H3 WT GBM tumor cells, and lowest in H3 K27M thalamic cells/tumors (Extended Data Fig. 9). This is consistent with the exceptional clinical responses seen in thalamic H3 K27M patients in our ONC201 cohort.

In our integrated preclinical and clinical analysis, ONC201 demonstrates unprecedented clinical activity in H3 K27M-mutant DMG, with favorable clinical and correlate responses. The most notable and durable responses are found in thalamic patients with therapy initiated following radiation and prior to recurrence. As a potential limitation of our analyses, patients included are clinical trial patients with a favorable baseline KPS (median KPS: 80), which introduces a potential selection bias when comparing patient outcomes with historical control. Nevertheless, the improvements in survival and sustained radiographic responses suggest a true treatment effect and establish ONC201 as the first therapy to improve outcomes in H3 K27M beyond radiation and re-irradiation.

Furthermore, we establish EGFR pathway activation and *FOXG1* expression as critical negative predictors of ONC201 response in H3 K27M-mutant DMG. Our data demonstrates that FOXG1 introduces a telencephalic gene regulatory network in H3 K27M-mutant DMGs that imparts a previously un-recognized resistance phenotype to ONC201. Future study will determine whether this phenotype is driven by reduced mitochondrial bioenergetics and whether ONC201-resistant H3 K27M-mutant DMGs (and H3 WT GBMs) will respond to combinatorial EGFR/FOXG1 targeting treatments.

Declarations

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AUTHORS CONTRIBUTIONS:

The methodology and supervision were carried out by **C.K; A.R.K, R.S.T, J.N, S.M.W, J.E.A, C.K** interpreted preclinical and clinical data; **A.R.K, S.M.W, C.K** wrote the manuscript and the manuscript was reviewed and edited by all co-authors; **S.G, A.C, S.C.K, P.Y.W, I.A, T.T.B, N.A.B, A.S, N.S, R.A.H, J.D, M.M, Y.O,**

M.D.H, D.D, T.F.C, B.M.E, M.M.K, Y.U, S.M, C.K carried out the clinical investigations. **R.S.T, G.L, J.E.A** provided and reviewed clinical data; **H.G, A.F, P.L.R, J.S, B.L.M, M.P.P, E.C, A.P, I.W, P.V, C.K-S, R.M, A.C, D.P, S.V** interpreted and curated clinical data, **C.T, R.C, V.N.Y, S.J** carried out in vitro and in vivo experiments and analyzed data; **S.M.W** performed and interpreted computational analyses; **T.N.P, Z.M, B.M, A.K.B, R.S, J.R.C, S.S, K.W M.D.D, J.E.C, L.J, M.G.F, A.R, J.N, S.M.W,** generated and interpreted

genomic and preclinical data.

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RST, GL, JEA are employees and shareholders of Oncoceutics Inc.

MM holds a BOD position on Oncoceutics with stock options, and has received honoraria for consulting relationships with Mevion, Karyopharm, Tocagen, Astra-Zeneca, Blue Earth Diagnostics, Celgene, and Abbvie.

DATA AVAILABILITY STATEMENT:

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

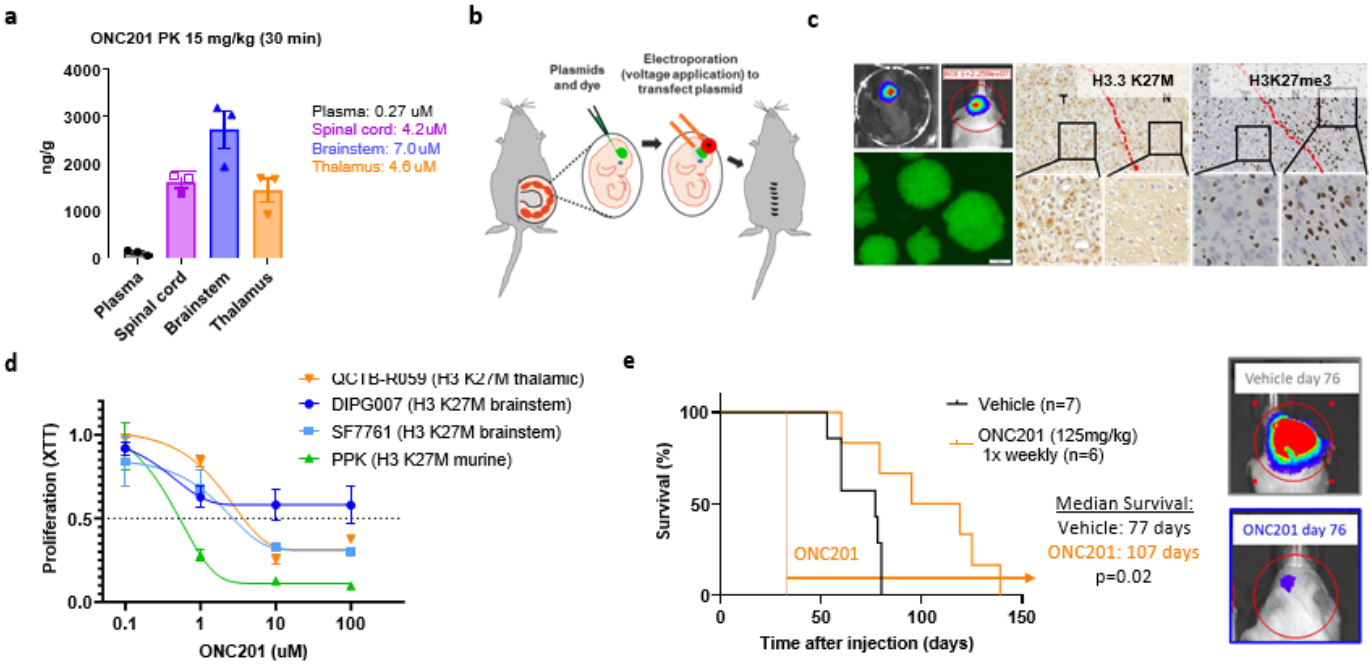

Figure 1

ONC201 demonstrates CNS penetration and survival benefit in an in utero electroporation (IUE) model. a, ONC201 preferentially accumulates in brain tissue following 15mg/kg treatment. All brain sites show higher concentration than plasma at 30 minutes. b, Schema of IUE model used to generate H3 K27M tumors in embryonic mouse brains. c, Plasmids of mutant TP53, PDGFRA and H3 K27M mutations (PPK)

are used to generate murine tumors that express GFP and luciferase for tumor monitoring purposes. IHC of brain tumor

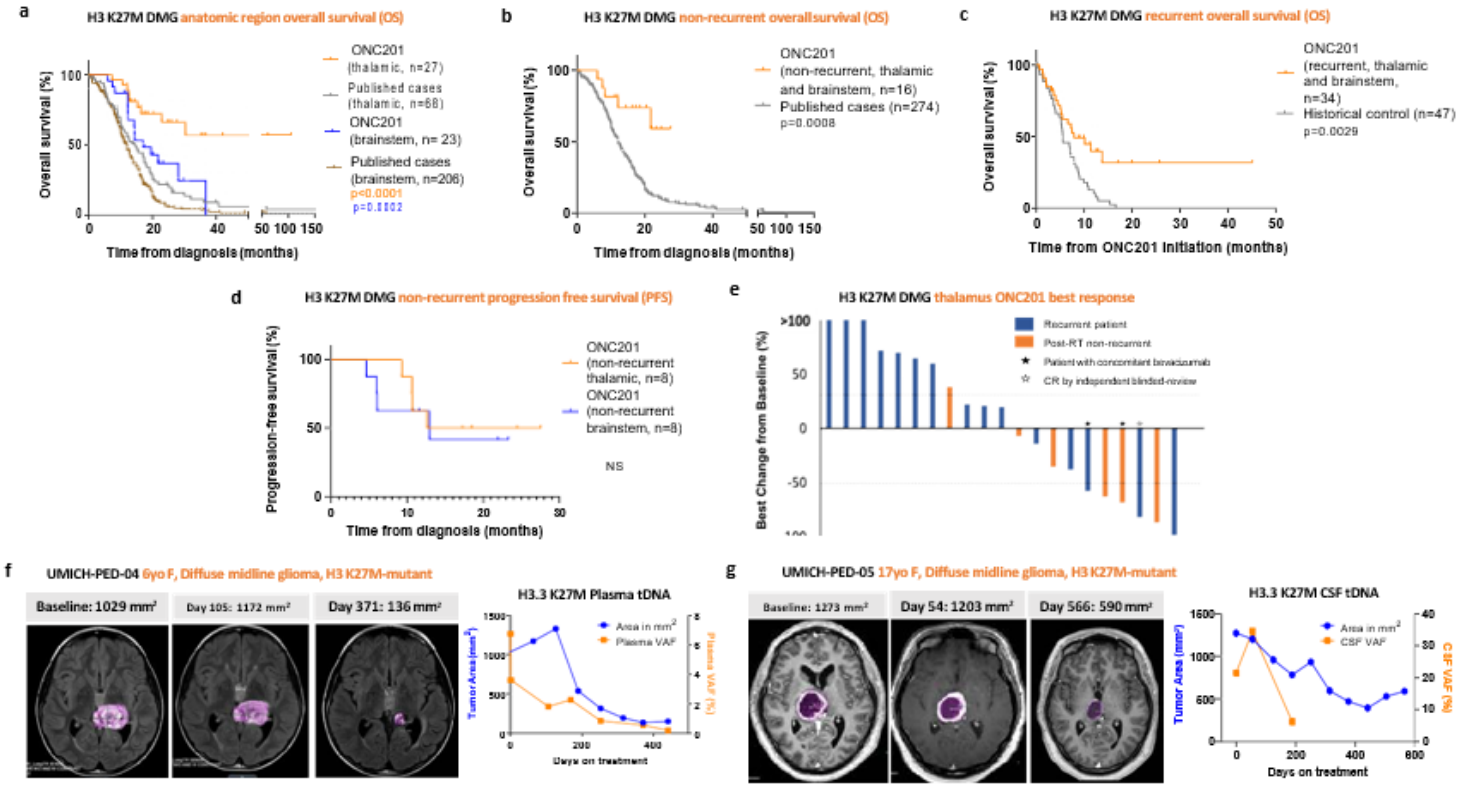

Figure 2

Survival of H3 K27M-mutant brainstem and thalamic DMG patients treated with ONC201 and H3F3A tDNA as a biomarker for ONC201 response. a, Median OS for thalamic patients has not been reached (median follow-up from diagnosis: 18.5 months). Thalamic historical median OS (n=68) is 14.5 months. Median survival for brainstem patients is 17.1 months and historical median OS (n=206, Pratt et al, Acta Neuropathologica, 2018) is 11.9 months. b, Median OS from diagnosis of non-recurrent thalamic and brainstem patients (n=16) has not been reached (median follow-up from diagnosis: 16.8 months, median time on drug: 10.6 months). Historical median OS (n=274, Pratt et al) is 12.0 months from diagnosis. c, Median OS from trial registration of recurrent thalamic and brainstem patients (n=34) is 7.9 months (median time on drug: 3.2 months). Historical median OS of H3 K27M thalamus and brainstem DMG, n=47 is 5.5 months from recurrence (Mackay et al, Cancer Cell, 2018 and Mueller et al, IJC, 2019). d, Median PFS is 20.1 months from diagnosis for thalamic and is 13.0 months from diagnosis for brainstem patients. e, Waterfall plot of best change from baseline in tumor burden by RANO. Twenty-one of 27 thalamic patients were evaluable and included in this analysis; dashed lines indicate 25% tumor increase (progressive disease) and 50% tumor reduction (partial response) in tumor burden from baseline. f, UMICH-PED-04 (post-radiation, non-recurrent) experienced 87% regression on ONC201. Serial plasma tDNA shows that H3F3A plasma tDNA correlates with radiographic response. g, For UMICH-PED-05 (post-radiation, non-recurrent) , reduction of 6-month H3F3A CSF tDNA correlated with the patient's sustained and ongoing clinical response. [p value by log-rank test for all panels].

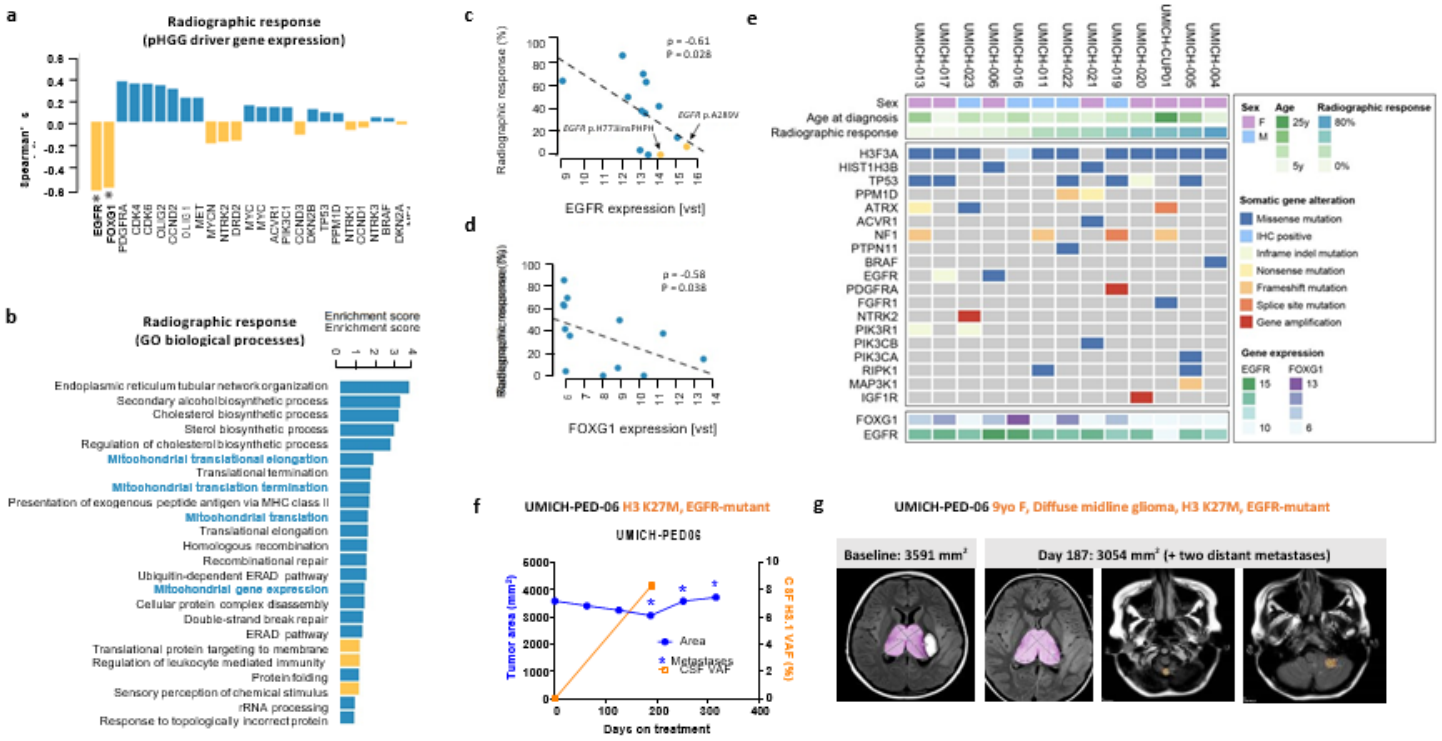

Figure 3

Elevated expression of EGFR and the cortical developmental transcription factor FOXG1 are critical negative predictors of ONC201 response in DMG. a, Correlation between best radiographic response in University of Michigan cohort (n=13) and driver gene expression levels in baseline tumor RNA-seq of H3 K27M-mutant DMGs (yellow=negative correlation with radiographic response; blue = positive correlation; *P<0.05 by algorithm AS 89). b, Gene set enrichment analysis for expression associated with radiographic response to ONC201 demonstrating positive correlation with multiple mitochondrial gene-sets (bold). c, Negative correlation between best radiographic response and EGFR gene expression levels in H3 K27M-mutant DMGs [variance stabilizing transformation (vst)]. d, Negative correlation between best radiographic response and FOXG1 gene expression levels in H3 K27M-mutant DMGs. e, Oncoplot for 13 thalamic and brainstem patients in UMICH cohort. f,g, UMICH-PED-06, a patient (post-radiation, non-recurrent) with thalamic EGFR V289V mutation experienced no tumor regression and an increase in CSF tDNA at 6 months (f) on ONC201, correlating with metastatic progression (g). [Spearman's ρ statistic is used to estimate a rank-based measure of association. P values are computed using algorithm AS 89].

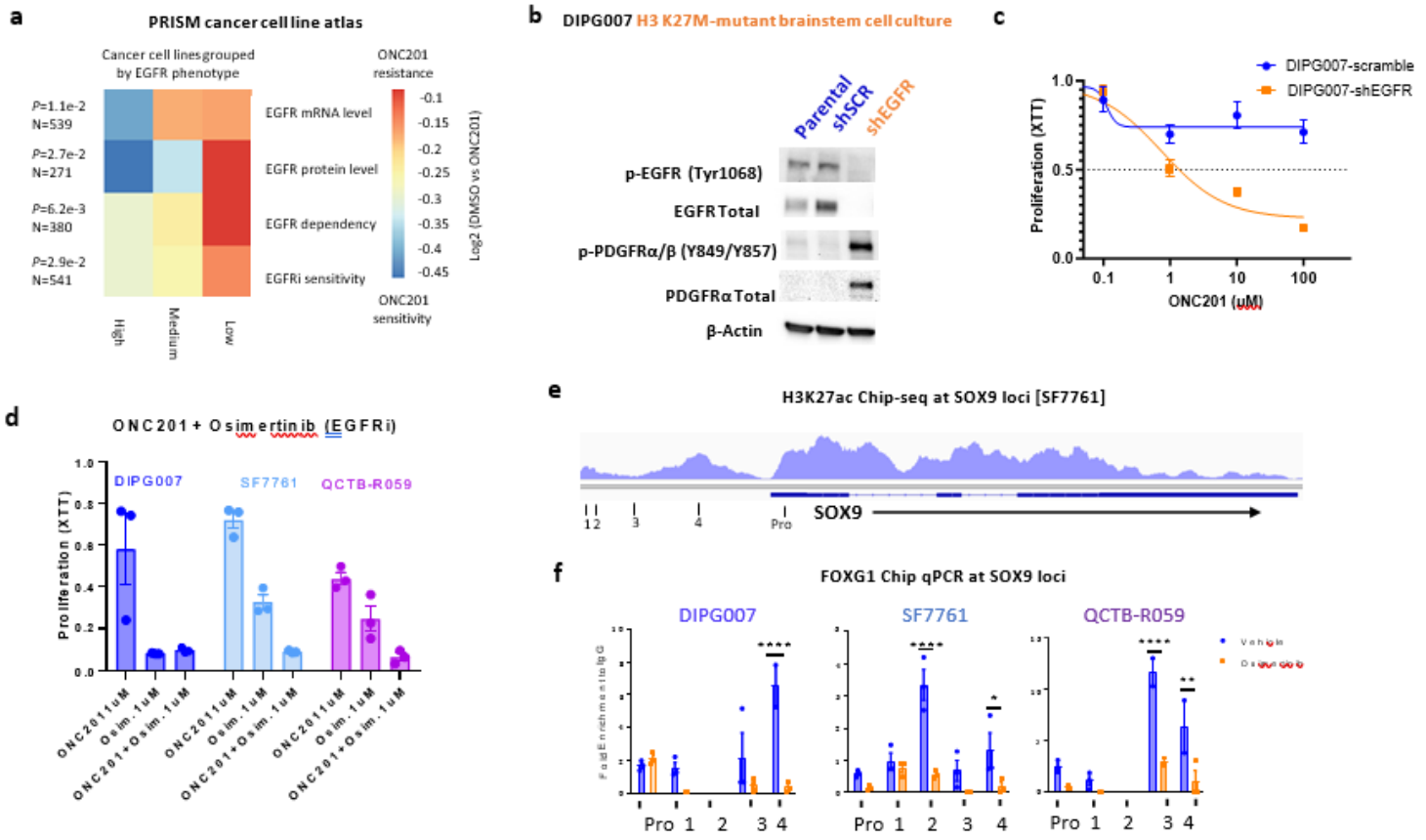

Figure 4

EGFR genetic and pharmacologic knockdown sensitizes cell lines to ONC201 treatment and reduces FOXG1 binding at established enhancer in H3 K27M cells. a, Correlation between EGFR phenotypes and ONC201 sensitivity in PRISM cancer cell lines (n=733), when organized by (i) EGFR mRNA level, (ii) EGFR protein level, (iii) EGFR CRISPR dependency, and (iv) EGFR inhibitor (EGFRi) sensitivity. b, Stable shRNA knockdown of EGFR in H3 K27M cells DIPG007 results in reduced EGFR activation and increase in PDGFRA activation. c, DIPG007-shEGFR cells demonstrate improved sensitivity to ONC201 compared to controls. d, Brainstem (SF7761) and thalamic (QCTB-R059) cell lines respond to ONC201 treatment in combination with EGFR inhibitor, osimertinib, better than either agent alone. EGFR inhibition is required to halt cell proliferation in ONC201-resistant cell line, DIPG007. e, H3K27ac ChIP-seq at SOX9 gene loci for H3 K27M SF7761 (GEO GSM2265662) displayed to denote potential enhancer (“1”- ”5”)/promoter sites (“PRO”). f, Chip qPCR shows reduction of FOXG1 binding (normalized to IgG) at enhancer loci with osimertinib (EGFR inhibitor) treatment across three H3 K27M primary cell cultures. [Data represent mean ± SEM; * $P \leq 0.05$, ** $P \leq 0.005$, **** $P \leq 0.00005$ by Welch’s t-test].

Supplementary Files

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