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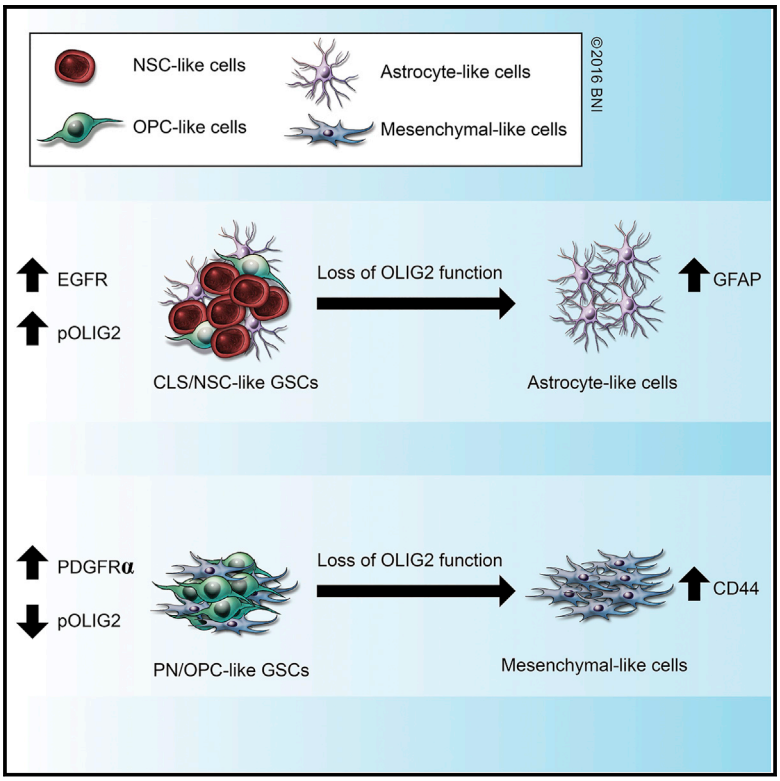
Lineage-Restricted OLIG2-RTK Signaling Governs the Molecular Subtype of Glioma Stem-like Cells

Graphical Abstract



Highlights

- OLIG2 forms a feedforward loop with mitogenic signaling in neural progenitors
- Inhibition of EGFR results in depletion of OLIG2 protein
- Phosphorylation state of OLIG2 alters expression of RTKs
- Loss of OLIG2 function in GSCs results in mesenchymal transformation

Authors

Robert Kupp, Lior Shtayer, An-Chi Tien, Emily Szeto, Nader Sanai, David H. Rowitch, Shwetal Mehta

Correspondence

shwetal.mehta@dignityhealth.org

In Brief

Kupp et al. find that the CNS-restricted transcription factor OLIG2 acts as a critical mediator of growth factor signaling in normal and malignant neural progenitors. Abrogation of OLIG2 function impairs expression of EGFR and PDGFRα, the two most commonly dysregulated growth factor receptors in malignant gliomas.

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Lineage-Restricted OLIG2-RTK Signaling Governs the Molecular Subtype of Glioma Stem-like Cells

Robert Kupp,[1] Lior Shtayer,[1,4] An-Chi Tien,[2] Emily Szeto,[1] Nader Sanai,[1] David H. Rowitch,[2,3] and Shwetal Mehta[1,5,*]
[1]Division of Neurobiology, Barrow Brain Tumor Research Center, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ 85013, USA
[2]Departments of Pediatrics and Neurological Surgery, University of California, San Francisco, San Francisco, CA 94143, USA
[3]Wellcome Trust-MRC Stem Cell Institute and Department of Pediatrics, University of Cambridge, Cambridge CB2 0AH, UK
[4]Present address: Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, USA
[5]Lead Contact
*Correspondence: shwetal.mehta@dignityhealth.org
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SUMMARY

The basic helix-loop-helix (bHLH) transcription factor OLIG2 is a master regulator of oligodendroglial fate decisions and tumorigenic competence of glioma stem-like cells (GSCs). However, the molecular mechanisms underlying dysregulation of OLIG2 function during gliomagenesis remains poorly understood. Here, we show that OLIG2 modulates growth factor signaling in two distinct populations of GSCs, characterized by expression of either the epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor alpha (PDGFRα). Biochemical analyses of OLIG2 function in normal and malignant neural progenitors reveal a positive feedforward loop between OLIG2 and EGFR to sustain co-expression. Furthermore, loss of OLIG2 function results in mesenchymal transformation in PDGFRα^{HIGH} GSCs, a phenomenon that appears to be circumscribed in EGFR^{HIGH} GSCs. Exploitation of OLIG2′s dual and antithetical, pro-mitotic (EGFR-driven), and lineage-specifying (PDGFRα-driven) functions by glioma cells appears to be critical for sustaining growth factor signaling and GSC molecular subtype.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor in adults, and despite profound molecular and genetic analyses, patient outcome following standard-of-care therapies remains dismal, with an average life expectancy of 12–15 months (Cloughesy et al., 2014). Intratumoral heterogeneity, diffuse infiltration throughout the brain parenchyma, and resistance to traditional therapies pose a major clinical challenge, which inevitably leads to tumor recurrence and the demise of the patient (Cloughesy et al., 2014). Recent work has highlighted the epigenetic and transcriptional networks of glioma stem-like cells (GSCs) in human GBM (Suvà et al., 2014) and their resemblance to niche-restricted progenitors of the adult mammalian brain (Alcantara Llaguno et al., 2015). Due to their unique ability to self-renew and differentiate into the neuro-glial lineages of the CNS, GSCs present a therapeutic burden by sustaining intratumoral heterogeneity and resistance to traditional therapies (Lathia et al., 2015).

In the developing CNS, the balance between progenitor proliferation and subtype specification is regulated by a diverse set of proteins and extracellular cues. At the apex of this process are transcription factors (TFs), such as the basic-helix-loop-helix (bHLH) proteins, which harbor contradicting pro-neurogenic and anti-neurogenic functions (Imayoshi and Kageyama, 2014a). The dynamic expression of these proteins at various developmental stages reflects their bi-functional nature—where constitutive expression drives cell-cycle exit and differentiation, and oscillatory expression sustains multipotency (Imayoshi and Kageyama, 2014b). During embryogenesis, the bHLH TF Olig2 is essential for expansion of the progenitor pools and for specification of the oligodendroglial lineage (Meijer et al., 2012). Universal expression of OLIG2 in almost all cases of diffuse pediatric and adult glioma suggests overlapping mechanisms between CNS development and gliomagenesis (Ligon et al., 2004; Otero et al., 2011). In this study, we set out to address the molecular mechanisms that sustain OLIG2 expression and its pro-mitotic functions in cycling neural progenitors. Cross-species investigative analysis suggests that OLIG2 forms a positive feedforward loop with the receptor tyrosine kinases (RTKs) epidermal growth factor receptor (EGFR) and the lineage-restricted platelet-derived growth factor receptor alpha (PDGFRα). We describe an OLIG2-dependent cell-autonomous nuclear mechanism for regulation of growth factor signaling in normal and malignant neural progenitors.

RESULTS

EGFR Signaling Sustains Olig2 Protein in Cycling Neural Progenitors

To address the mechanisms that sustain Olig2 expression in neural progenitors, we utilized the protein biosynthesis inhibitor cycloheximide (CHX) to determine the half-life of Olig2 protein. We observed a half-life between 4–8 hr for Olig2 protein in normal

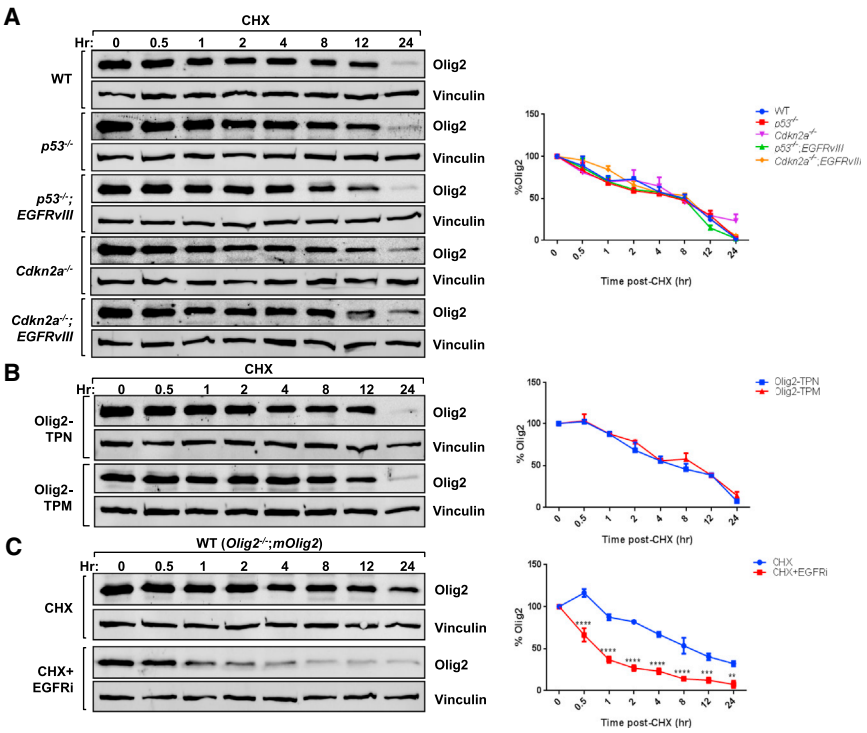

Figure 1. EGFR Signaling Regulates Olig2 Protein Turnover in Cycling Neural Progenitors

(A) Murine neural progenitors were challenged with cycloheximide (25 μg/ml CHX). Lysates were collected at indicated time points and immunoblotted with antibodies directed against Olig2 and Vinculin.

(B) *Olig2* null cells expressing either phospho mutants (TPN or TPM) were treated with CHX and lysates were collected at indicated time points. Immunoblotting was performed with antibodies directed against Olig2 and Vinculin.

(C) Neural progenitors were treated with CHX alone or in combination with EGFRi (10 μM erlotinib). Immunoblotting was performed similarly to above. Quantifications are shown on the right. Error bar indicates mean ± SEM ($n \geq 3$). ****$p < 0.0001$. See also Figure S1.

(wild-type; $p53^{-/-}$; $Cdkn2a^{-/-}$) and malignant ($Cdkn2a^{-/-}$; *EGFRvIII* and $p53^{-/-}$;*EGFRvIII*) neural progenitors, irrespective of the genetic background (Figure 1A). Importantly, Olig2 levels (mRNA and protein) were similar, with minimal deviation, across selected genotypes (Figure S1A). When neural progenitors were plated in factor-free media (i.e., depleted of exogenous EGF/FGF2), phosphorylation of Olig2 at its amino-terminus ($Ser_{10,13,14}$) rapidly declined (Figure S1B) (Sun et al., 2011). We observe no significant difference in the turnover time of either triple-phospho-null (TPN; Ser to Ala) or triple-phospho-mimetic (TPM; Ser to Glu/Asp) phospho mutant of Olig2 (Figure 1B).

Previous reports have implicated mitogenic signaling as important regulator of $Olig2^{+}$ cells during CNS development and gliomagenesis (Gonzalez-Perez et al., 2009; Persson et al., 2010). To address the direct role of active EGF signaling in regulating Olig2 protein expression, we utilized a known EGFR inhibitor, erlotinib (henceforth referred to as EGFRi). Treatment of neural progenitors expressing Olig2 ($Olig2^{-/-}$;*mOlig2*) with EGFRi in the presence of CHX over a 24-hr time course resulted in rapid depletion of the Olig2 protein ($T_{1/2}$ = 1 hr) (Figure 1C). Consistent with this observation, treatment of murine neural progenitors with EGFRi resulted in depletion of the Olig2 protein, irrespective of the genetic background and phosphorylation status (Figures S1C and S1D). These data suggest that EGFR signaling mediates the stability of Olig2 in neural progenitors and is independent of amino-terminal phosphorylation and genetic background.

Phosphorylation of Olig2 Alters Expression of Growth Factor Receptor Tyrosine Kinases

We and others have shown the *Egfr* is a direct genetic target of Olig2 in neural progenitors in chromatin immunoprecipitation sequencing (ChIP-seq) and RNA sequencing (RNA-seq) studies (Mateo et al., 2015; Meijer et al., 2014). Qualitative analysis of Olig2 binding sites reveals its localization to intronic and distal upstream regions of the *Egfr* loci, marked by deposition of H3K4me1 and H3K27ac, indicating these sites as putative enhancers (Figure S2A). To validate these ChIP-seq findings on whether *Egfr* is a direct genetic target of Olig2, we analyzed *Olig2* null neural progenitors expressing control GFP (knockout [KO]), wild-type (WT), TPN, or TPM forms of murine Olig2. Immunoblot analysis suggests that germline ablation (KO) or loss of amino-terminal phosphorylation (TPN) of Olig2 reduces the expression of Egfr in normal (WT) and malignant ($Cdkn2a^{-/-}$;*EGFRvIII*) neural progenitors (Figure 2A). We have previously shown that the phosphorylation state of Olig2 alters the mRNA levels of various RTKs (Meijer et al., 2014); however, these cell lines were engineered to express either phospho mutant under a constitutive viral promoter against an *Olig2* null genetic background. We extend these observations to knockin (KI) neural progenitors, wherein the endogenous *Olig2* locus was replaced with either the TPN or TPM coding sequences ($Olig2^{TPN/TPN}$, $Olig2^{TPM/TPM}$). Consistent with the overexpression system, we observed a significant upregulation of the RTKs *Egfr*, *Fgfr3*, and *Pdgfrα* mRNA and repression of the cell-cycle inhibitor *Cdkn1a* and astrocytic marker *Gfap* mRNA in TPM- versus TPN-expressing cells (Figure S2B).

We have previously reported that Olig2 phosphorylation is dispensable for progenitor proliferation and tumor initiation when *p53* is genetically inactivated. However, the rate of tumor formation is significantly increased in the presence of Olig2 (i.e., $Olig2^{cre/+}$ versus $Olig2^{cre/cre}$) in a murine model of malignant glioma harboring deletion of the *p53* locus ($p53^{flox/flox}$;*EGFRvIII*) (Mehta et al., 2011). To assess if Olig2-mediated regulation of *Egfr* is dependent on the tumor suppressor protein p53, we attenuated p53 function in phospho mutant KI cells. As shown in Figure 2B, ectopic expression of dominant-negative p53 (p53DD) in neural progenitors expressing TPN mutant, was

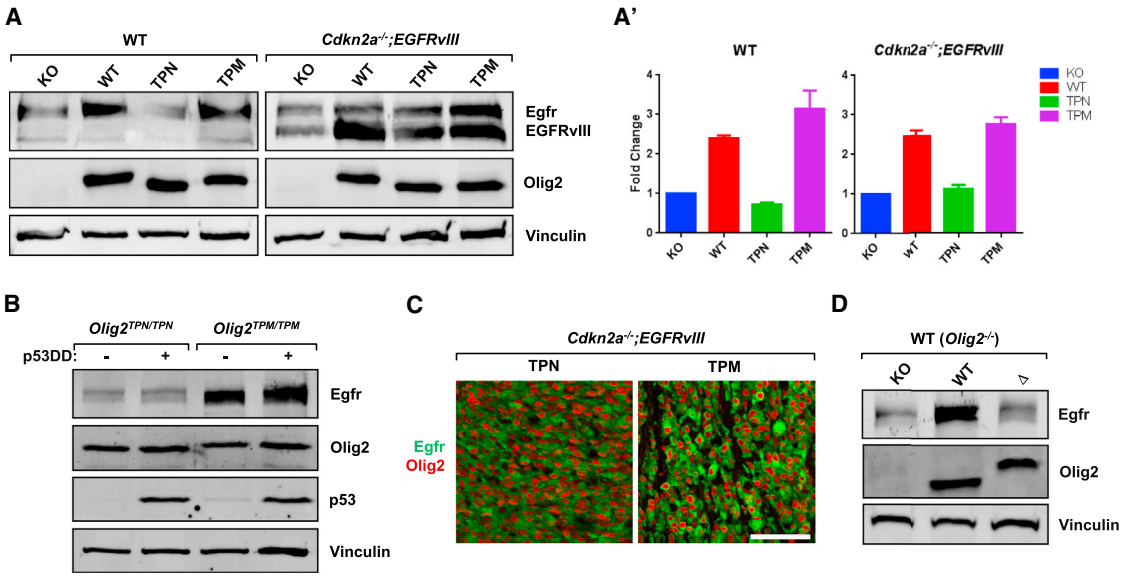

Figure 2. Olig2 Regulates Expression of *Egfr* in Murine Neural Progenitors

(A) *Olig2* null cells expressing control GFP, wild-type (WT), or phospho mutants (TPN or TPM) of Olig2 were assayed for Egfr and Olig2 protein levels. Quantifications of Egfr (and EGFRvIII) protein levels are shown on the right (A'). Error bar indicates mean ± SEM ($n \geq 3$).

(B) Knockin neural progenitors expressing the TPN or TPM mutant of Olig2 (*Olig2*$^{TPN/TPN}$ and *Olig2*$^{TPM/TPM}$) expressing either control GFP or dominant-negative p53 (*p53DD*). Protein levels of Egfr, Olig2, and p53 were assayed as above.

(C) Comparison of intracranial xenografts derived from murine glioma cells expressing TPN or TPM mutants of Olig2 (V5 tagged). Sections were stained with Egfr (green) and V5 (red) antibody to visualize tumor cells. Scale bar, 100 μM.

(D) *Olig2* null cells expressing control GFP, wild-type (WT), or FLAG/HA-tagged DNA binding mutant (Δ) of Olig2 were assayed for Egfr and Olig2 protein levels ($n \geq 3$).

See also Figure S2.

insufficient to induce Egfr expression suggesting that Olig2-mediated regulation of Egfr is independent of p53 status.

To confirm phospho-dependent upregulation of Egfr in vivo, we orthotopically transplanted murine glioma cells (*Cdkn2a*$^{-/-}$; *EGFRvIII*) expressing either of the Olig2 phospho mutants into the brains of immunocompromised mice. This murine glioma model recapitulates the hallmark genetic lesions of the "classical" GBM molecular subtype: constitutive EGFR signaling (*EGFRvIII*), homozygous deletion of the *Cdkn2a* locus, and an intact *p53* gene (Verhaak et al., 2010). *Olig2* function is required to initiate tumors in this glioma model, and amino-terminal phosphorylation regulates its tumorigenic competence (Ligon et al., 2007; Sun et al., 2011). Consistent with our in vitro findings, we observed stronger immunoreactivity of Egfr protein in TPM-expressing tumors compared to TPN-expressing tumors (Figure 2C).

To confirm if Olig2 DNA-binding is required to promote expression of Egfr, we analyzed *Olig2* null cells expressing control GFP (KO), WT-Olig2, or a DNA-binding mutant (ΔOlig2) that harbors a point mutation (N114H) in the basic region that abrogates canonical E-Box recognition (Meijer et al., 2014). As seen in Figure 2D, germline ablation of *Olig2* or loss of DNA-binding function led to diminished expression of Egfr in wild-type murine neural progenitors. Finally, we tested whether ΔOlig2 can antagonize endogenous Olig2 protein function. Ectopic expression of ΔOlig2 in malignant neural progenitors (*Cdkn2a*$^{-/-}$; *EGFRvIII* and *p53*$^{-/-}$;*EGFRvIII*) led to substantial repression of Egfr protein levels (Figure S2C). These findings suggest that Olig2 promotes Egfr expression through activity of the amino-terminal phosphorylation motif in a DNA-binding-dependent manner.

OLIG2 Regulates Expression of EGFR and PDGFRα in hGSCs

To address whether the cross-regulatory loop between OLIG2 and RTKs persists in human GSCs (hGSCs), we isolated and enriched for neurosphere-forming cells from patient-derived GBM tissues (Table S1). Immunoblot analysis showed that hGSCs with higher levels of pOLIG2 shared expression of the EGFR (BT112, BT286, GB82, BT145), with varying levels of the PDGFRα. In contrast, pOLIG2 levels are largely diminished in EGFR^{LOW} (BT187, BT142, GB3, GB16, GB80) hGSCs, which often displayed high expression of PDGFRα, reminiscent of the proneural GBM subtype (Figure 3A). To simplify the nomenclature, we have termed hGSCs displaying EGFR^{HIGH} expression as classical (CLS) and EGFR^{LOW}/PDGFRα^{HIGH} expression as proneural (PN). To confirm a role for active EGFR signaling in sustaining OLIG2 protein in hGSCs (BT145, BT112), we treated the cells with EGFRi + CHX and observed a rapid depletion of the OLIG2 protein, compared to CHX alone (Figure S3A). These findings suggest that, similar to murine neural progenitors, EGFR signaling sustains the OLIG2 protein in CLS hGSCs.

The expression of OLIG2 protein in both PN and CLS hGSCs led us to inquire if these developmentally regulated pro-neural

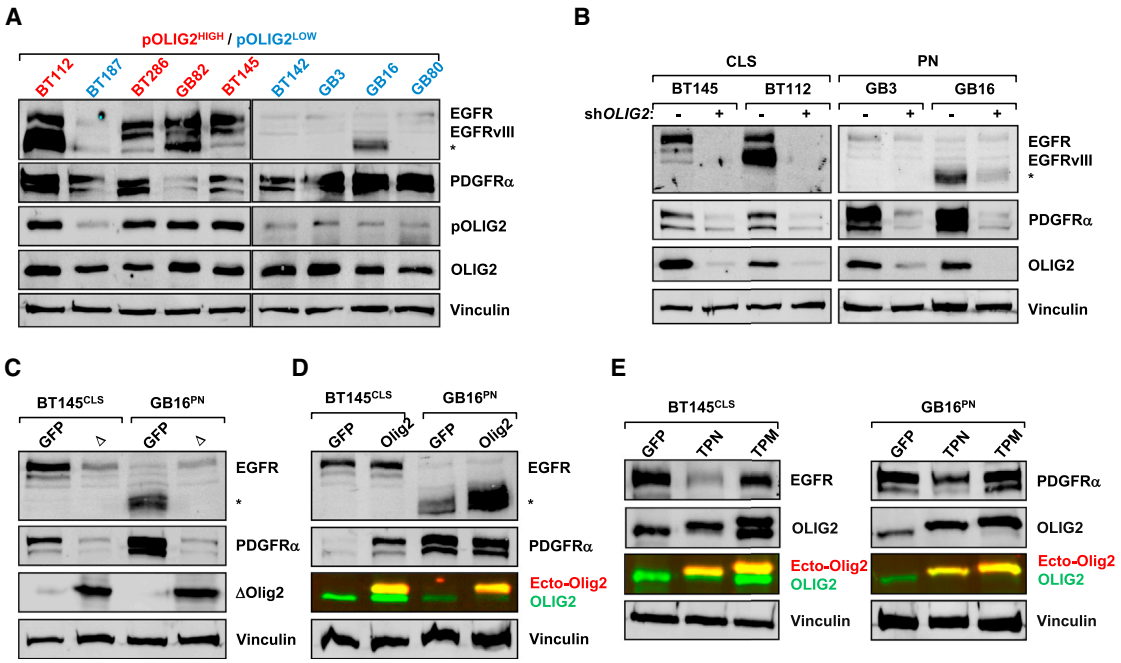

Figure 3. OLIG2 Regulates Expression of RTKs in hGSCs

(A) Lysates derived from hGSCs were immunoblotted with antibodies to pOLIG2, OLIG2, EGFR, PDGFRα, and Vinculin.

(B) hGSCs (BT145, BT112, GB3, GB16) were transduced with shNT (non-target control) or sh*OLIG2*. Lysates were collected 48 hr post-infection and immunoblotted for EGFR, PDGFRα, OLIG2, and Vinculin.

(C) hGSCs expressing GFP or d Olig2 (FLAG tag), and the levels of EGFR and PDGFRα were assayed as above.

(D) hGSCs were transduced with retrovirus expressing GFP or WT-Olig2 (V5 tag), and the levels of EGFR and PDGFRα were assayed using standard immunoblotting techniques.

(E) Lysates from hGSCs expressing GFP or Olig2 phospho mutants (TPN and TPM) were collected to assay protein levels of EGFR and PDGFRα, ectopic (V5 tag), and endogenous OLIG2 ($n \geq 3$). *Denotes 90 kDa EGFR band, uniquely expressed in GB16.

See also Figure S3.

(lineage-specifying) and anti-neural (proliferative) functions of OLIG2 were co-opted in hGSCs. After acute silencing of *OLIG2* in both CLS (BT145 and BT112) and PN (GB3 and GB16) hGSCs, we observed a significant downregulation of both RTKs, independent of the molecular subtype (Figure 3B). We confirmed specificity and effects of small hairpin RNA (shRNA)-mediated knockdown by utilizing three alternate hairpins targeting the human *OLIG2* ORF (Figure S3B). In contrast to recent findings (Lu et al., 2016), we did not observe a parallel upregulation of EGFR protein following acute silencing of *OLIG2* in PDGFRα^{HIGH} hGSCs (Figures 3B and S3C). Furthermore, analysis of published OLIG2/H3K27ac ChIP-seq data in hGSCs (Suvà et al., 2014) confirms OLIG2 binding at both distal and intronic enhancers at both the *EGFR* and *PDGFRA* loci (data not shown). We also observed a significant downregulation of both RTK transcripts (*EGFR* and *PDGFRA*) following acute *OLIG2* silencing (Figure S3D).

We next addressed whether ΔOlig2 can assert itself in a dominant-negative manner in hGSCs. We assessed protein levels of EGFR and PDGFRα from two CLS (BT145, BT112) and two PN (GB3, GB16) lines where we have chronically overexpressed ΔOlig2. We observed a substantial downregulation of the RTKs in all hGSCs surveyed (Figures 3C and S3E), indicating an OLIG2 loss-of-function phenotype mediated by the DNA-binding mutant (Δ). qRT-PCR analysis confirmed the downregulation of RTK transcripts (i.e., *EGFR*, *PDGFRA*) in presence of ΔOlig2 (Figure S3F). To further confirm OLIG2-mediated regulation of RTKs, we ectopically introduced wild-type Olig2 or control GFP into either the BT145^{CLS} or GB16^{PN} hGSC line. Surprisingly, following overexpression of WT-Olig2 we observed an induction of PDGFRα protein in our CLS/EGFR^{HIGH} line (BT145) and induction of EGFR protein in our PN/PDGFRα^{HIGH} line (GB16) (Figure 3D). Finally, we asked if the phosphorylation status of OLIG2 is critical for sustaining the expression of RTKs in human GSCs. We utilized retroviral expression vectors to introduce control GFP or the phospho mutants (TPN, TPM) in either the BT145^{CLS} or GB16^{PN} hGSC lines. We observed a reduction in the protein levels of both the EGFR and PDGFRα (albeit more modest) protein following overexpression of TPN-Olig2, but not TPM-Olig2 (Figure 3E). These data suggest that OLIG2 regulates the expression of both the EGFR and PDGFRα in hGSCs.

OLIG2 Is the Nuclear Gateway for Proneural-Mesenchymal Transition

We next asked whether loss of *OLIG2* leads to a shift in GBM molecular subtype. To investigate the functional outcomes of *OLIG2* knockdown in hGSCs, we utilized shRNA to ablate the OLIG2 protein in both CLS/EGFR^{HIGH} and PN/PDGFRα^{HIGH} hGSCs.

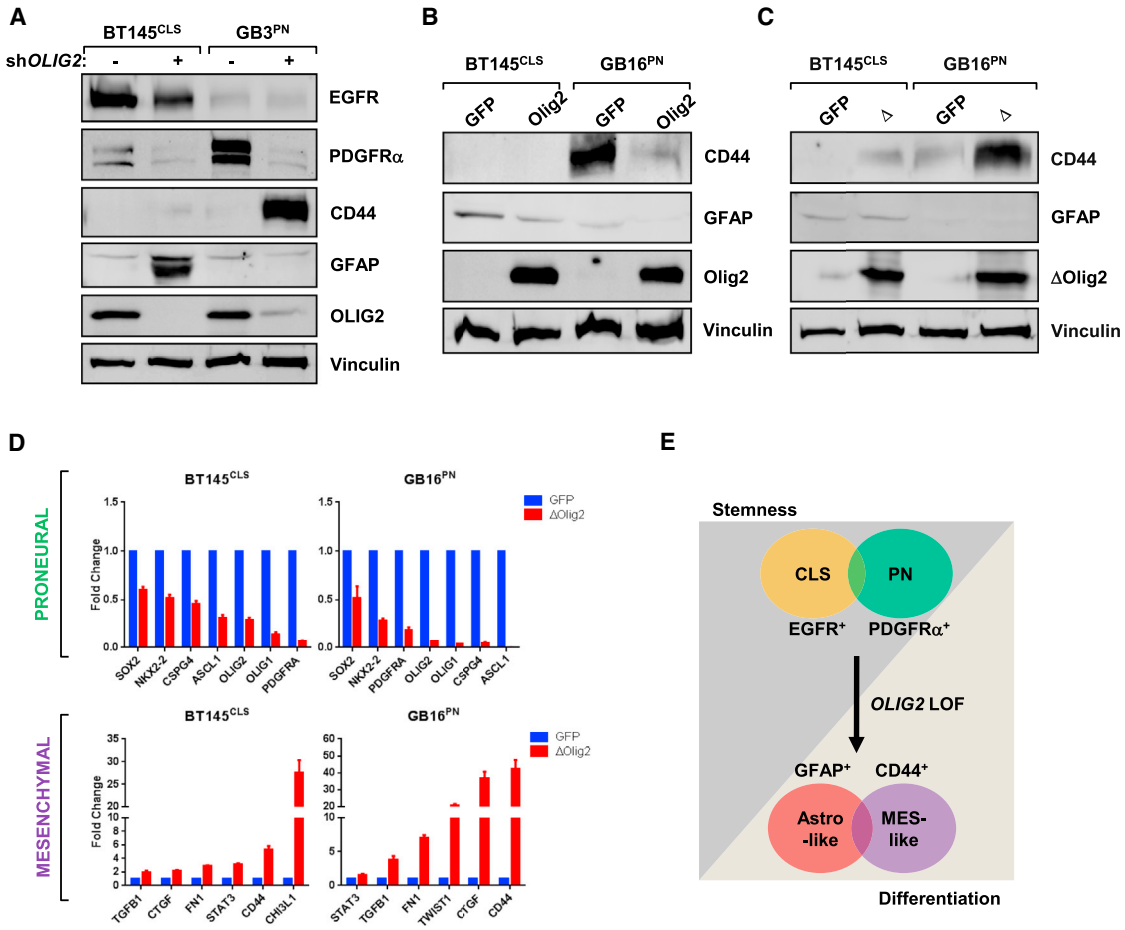

Figure 4. OLIG2 Is the Nuclear Gateway for Proneural-Mesenchymal Transition

(A) hGSCs (BT145, GB3) were transduced with shNT (non-target control) or sh*OLIG2*. Lysates were collected 48 hr post-transduction and immunoblotted for the indicated proteins.

(B and C) Lysates from hGSCs expressing control GFP, WT-Olig2 (V5 tag), or ΔOlig2 (FLAG tag) were collected and immunoblotted for CD44, GFAP, and ectopic Olig2. Note: blots presented here were re-probed from Figures 3C and 3D.

(D) qRT-PCR analysis of hGSCs (BT145, GB16) expressing control GFP or ΔOlig2 for indicated subclass genes. *HPRT* and *18 s* were utilized as housekeeping genes. Error bar indicates mean ± SEM (n ≥ 3).

(E) Proposed model of OLIG2-mediated regulation of growth factor signaling.

See also Figure S4.

We observed a robust induction of the astrocytic marker GFAP in our CLS lines (BT145, GB71, GB82), whereas the mesenchymal marker CD44 is induced in PN lines (GB3, GB16, GB80) (Figures 4A and S4A). However, this "mutually exclusive" upregulation of the GFAP and CD44 proteins does not fully extend to all hGSC lines examined (e.g., GB71^{CLS}): lines that express appreciable GFAP protein prior to *OLIG2* knockdown, appear to upregulate both GFAP and CD44 (Figure S4A). Consistent with a loss of "stemness," we observed a substantial downregulation of the glioma master transcription factor SOX2 following *OLIG2* knockdown in all CLS/PN lines examined (Figure S4A). These data suggest that acute loss of *OLIG2* collapses the "stem" state in hGSCs, with a subsequent shift to a more differentiated state.

To further assess OLIG2-dependent regulation of GFAP and CD44, we utilized hGSC lines (BT145^{CLS} and GB16^{PN}) to ectopically express WT-Olig2 or the DNA-binding mutant ΔOlig2. We observed repression of both the CD44 and GFAP proteins in lines where we have overexpressed WT-Olig2 (Figure 4B). In striking contrast, chronic overexpression of dominant-negative Olig2 (ΔOlig2) resulted in upregulation of the CD44 protein (but not GFAP)—suggesting mesenchymal (but not astroglial) conversion (Figure 4C). To assess if loss of *OLIG2* function triggers a proneural-mesenchymal transition (PMT), we performed expression analysis for GBM molecular subtype-specific genes in four independent hGSCs lines expressing ΔOlig2 (BT145, BT112, GB3, GB16). We observed a catastrophic loss of proneural class genes and upregulation of mesenchymal class genes in our ΔOlig2-expressing hGSCs compared to GFP-expressing controls, independent of the original molecular subtype (i.e., CLS versus PN) (Figures 4D and S4B). Conversely, overexpression of WT-Olig2 results in upregulation of proneural class genes and downregulation of mesenchymal class genes (Figure S4C).

We further extend these "PMT" expression shift observations to murine glioma lines of defined genetic backgrounds (*Cdkn2a*$^{-/-}$*;EGFRvIII* and *p53*$^{-/-}$*;EGFRvIII*) expressing ΔOlig2 (Figure S4D). Thus, our data support a model in which OLIG2 serves as a critical mediator of growth factor signaling (i.e., RTK expression) and GSC identity (Figure 4E).

DISCUSSION

A key determinant of intratumoral heterogeneity in malignant gliomas is likely to be glioma stem-like cells, which promote tumor growth, recurrence, and therapeutic resistance (Lathia et al., 2015). Identification of proteins that drive GSC biology is of special interest in neuro-oncology. One such protein that is expressed in GSCs, glial-restricted progenitors and their respective progeny is the bHLH TF Olig2 (Ligon et al., 2007). During the early stages of CNS development, Olig2 serves to prevent premature cell-cycle exit in developmentally uncommitted neural progenitors, and at later time points, promote differentiation into oligodendrocytes (Meijer et al., 2012). Previous work has coupled the pro-mitotic functions of Olig2 to phosphorylation of a cluster of serines at its amino-terminus (Ser^{10,13,14}), also known as the phospho-serine-motif. Mutation of this motif into a "null" state (Ser to Ala) impedes neural progenitor proliferation and the tumorigenic capacity of murine GSCs (Sun et al., 2011). Further biochemical analyses of these mutants suggest little to no alteration in genomic targeting, but rather a difference in intranuclear localization and co-regulator association (Meijer et al., 2014). Here, we investigated the mechanisms that sustain OLIG2 expression and its proliferative functions in neural progenitors and assessed whether these signals are co-opted in malignant gliomas. We have identified an "OLIG2-RTK" signaling axis, which functions in a feedforward manner to sustain co-expression. This loop appears to be conserved in both normal and malignant neural progenitors, highlighting a previously unappreciated relationship between OLIG2 and EGFR signaling (Aguirre et al., 2007; Persson et al., 2010).

Constitutive growth factor signaling is a hallmark of diffuse gliomas, propagated by activating genetic lesions in RTKs, most commonly within the *EGFR* and *PDGFRA* locus (Verhaak et al., 2010), which appear to be mutually exclusive oncogenic events (Snuderl et al., 2011). Mutations at the *EGFR* locus occur in the classical subtype and mutations in the *PDGFRA* locus occur primarily in the proneural subtype (Verhaak et al., 2010). While these two molecular subtypes appear similar on a histopathological level, their expression profiles suggest that they might have disparate cellular origins. Recent elegant mouse modeling studies (Alcantara Llaguno et al., 2015) suggests that both multipotent neural stem cells (NSCs) and lineage-restricted oligodendrocyte precursor cells (OPCs) are capable of giving rise to anatomically and molecularly unique types of GBM when transformed with identical mutations (i.e., *Tp53*, *Pten*, *Nf1* deletion) using cell-specific Cre-drivers (*Nestin*^{creER} for NSCs, *Ascl1*^{creER} for NSC/OPCs, *Cspg4*^{creER} for OPCs). These authors observed expression of Olig2 in both type I ("NSC"-like) and type II ("OPC"-like) tumors in their genetically engineered mouse models. In our patient-derived GSCs, we observe universal expression of OLIG2 and two distinct states of phosphorylated OLIG2 (low versus high), which appear to correlate with dominant expression of the RTKs EGFR or PDGFRα (CLS versus PN) (Figure 3A). As the developmental functions of Olig2 amino-terminal phosphorylation are presumably complete following the formation of mature white matter—its presence in PDGFRα-expressing (PN) hGSCs presents a paradox. Is the phospho-serine-motif performing an identical function in these hGSCs (compared to their CLS counterparts)? Our biochemical analyses of *OLIG2* function in both hGSC populations (CLS and PN) suggest a requirement for intact phosphorylation to sustain expression of the EGFR, while having only a modest impact on PDGFRα expression (Figure 3E). This finding is consistent with previous observations that indicated amino-terminal phosphorylation is not critically required for oligodendroglial lineage specification (Sun et al., 2011). However, expression of both RTKs is repressed upon introduction of a DNA-binding mutant (ΔOlig2), which appears to function in a dominant-negative manner (Figures 3C and 4D). This suggests that while phospho-serine-motif functions might persist in PN hGSCs, the larger pool of un-phosphorylated OLIG2 may be performing a secondary function.

We have previously reported that the pro-mitotic functions of Olig2 indirectly promote radio-resistance through antagonization of p53 transactivation (Mehta et al., 2011). Recent work (Bhat et al., 2013; Carro et al., 2010) has proposed a phenomenon termed "proneural-mesenchymal transition" as mediating radio-resistance and aggressive recurrence in malignant gliomas. In this model of progressive resistance, glioma cells acquire mesenchymal traits and lose proneural gene expression. In this study, we attempted to reconcile these results through cross-species investigative analysis of OLIG2 molecular function in normal and malignant neural progenitors. We observe that CLS hGSCs resist mesenchymal transformation and shift toward the astroglial lineage (i.e., GFAP versus CD44 upregulation) following ablation of *OLIG2*. In contrast, PN hGSCs strongly upregulate the CD44 protein (Figures 4A and S4A). Notably, mesenchymal transformation does not appear to be entirely exclusive to astrocytic differentiation, as we observe a parallel upregulation of GFAP in some lines following *OLIG2* loss (Figure S4A). This does not, however, exclude fate conversion—as during development the CD44 protein has been shown to label astroglial-restricted progenitors that do not yet express GFAP (Liu et al., 2004; Gonzalez et al., 2014).

Thus, OLIG2 that is uniquely CNS-restricted among a quartet of glioma master TFs (Suvà et al., 2014), appears to function as a "nuclear gateway" for proneural gene and RTK expression in hGSCs (Figures 4D and 4E). Similar molecular subtype conversion findings were recently presented (Lu et al., 2016), however, these authors observed a "proneural-classical" subtype shift following *Olig2* deletion. These authors employed a murine glioma model that initiates tumors in a very specific cell type (white matter OPCs) bearing mutations unique to the proneural subtype (*Tp53;Pten* deletion; *Pdgfb* overexpression), excluding the possibility of transformation in more naive cells (i.e., neural stem/progenitor cells) and Olig2 functions therein (i.e., sustaining *Egfr* expression; Figure 2). Similar to our findings, Lu et al. (2016) also observed a global collapse of the proneural gene expression program following chronic *Olig2* deletion, consistent

with a loss of OPC character. Unlike our observed PMT, these authors observed an upregulation of the *Egfr* and classical subtype genes. However, it is important to note that the EGFR can be expressed in a plethora of normal and neoplastic cells, many of which do not express OLIG2 (e.g., astrocytes, immortalized glioma cell lines) and lack cancer stem cell identity (Carén et al., 2015; Suvà et al., 2014). Thus, this observation by Lu et al. (2016) of *Egfr* upregulation following chronic *Olig2* deletion is possibly a result of astroglial restriction (and dissimilar to our acute silencing experiments). Importantly, a functional role for Egfr in oligodendroglial lineage cells during CNS development and gliomagenesis has been well documented (Aguirre et al., 2007; Persson et al., 2010), and a role for Olig2 in repression of the *Egfr* locus is difficult to reconcile with this pre-existing data (as postulated in Lu et al., 2016). Consistent with our murine data and unlike Lu et al. (2016) study, we observed a decrease in EGFR protein after loss of OLIG2 function in hGSCs. It is possible that differences in other genomic alterations apart from PDGFRα amplifications also play a role in subtype specification (Ozawa et al., 2014). Further analysis of the micro-environmental cues and intracellular mediators of this proliferation-differentiation (EGFR-PDGFRα) switch will provide additional mechanistic insight into the process of subtype conversion and may present novel therapies for patients with malignant gliomas (Singh et al., 2016). We therein propose a model where OLIG2 resides at the intersection of two cross regulatory loops in normal and malignant neural progenitors, forming a compulsory link between cell-cycle regulation and sub-type specification, dictated through spatiotemporal protein modifications that promote self-renewal or fate choice.

EXPERIMENTAL PROCEDURES

Animal Procedures, Tissue Harvest, and Cell Culture

Animal husbandry was performed according to Barrow Neurological Institute (BNI) guidelines under IACUC-approved protocols. Derivation and culture of neural progenitor cell lines used have been described previously (Mehta et al., 2011). Generation of murine *Olig2* mutant constructs have been previously described (Meijer et al., 2014).

Immunoblotting and qRT-PCR

Immunoblotting was performed using standard protocols previously described (Meijer et al., 2014). A full list of antibodies used is provided in the Supplemental Information. RNA and cDNA was prepared using previously described methods (Meijer et al., 2014).

Statistical Analysis

For each experiment, data were collected from at least three biological repeats and analyzed by one-way or two-way ANOVA with Bonferroni multiple comparisons test as indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.040.

AUTHOR CONTRIBUTIONS

R.K. and S.M. conceived and designed the experiments. R.K. performed all experiments except for the following: L.S. performed IF staining, A.-C.T. generated phospho-mutant knockin neural progenitors, and E.S. generated patient-derived lines. A.-C.T., N.S., and D.H.R. contributed unpublished reagents and materials. R.K. and S.M. analyzed data. R.K. and S.M. wrote the manuscript.

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