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The Central Nervous System-Restricted Transcription Factor Olig2 Opposes p53 Responses to Genotoxic Damage in Neural Progenitors and Malignant Glioma

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SUMMARY

High-grade gliomas are notoriously insensitive to radiation and genotoxic drugs. Paradoxically, the p53 gene is structurally intact in the majority of these tumors. Resistance to genotoxic modalities in p53-positive gliomas is generally attributed to attenuation of p53 functions by mutations of other components within the p53 signaling axis, such as *p14*^{Arf}, *MDM2*, and *ATM*, but this explanation is not entirely satisfactory. We show here that the central nervous system (CNS)-restricted transcription factor Olig2 affects a key post-translational modification of p53 in both normal and malignant neural progenitors and thereby antagonizes the interaction of p53 with promoter elements of multiple target genes. In the absence of Olig2 function, even attenuated levels of p53 are adequate for biological responses to genotoxic damage.

INTRODUCTION

The *p53* tumor suppressor gene and its downstream effectors play a multifaceted role in protection from cellular stress, genotoxic damage, and inappropriate mitogenic cues (Brugarolas et al., 1995; el-Deiry et al., 1993; Prives and Hall, 1999). Activation of p53 transcriptional functions by any of these biological events results in transient growth arrest, permanent growth arrest or programmed cell death (Brugarolas et al., 1995; el-Deiry et al., 1993; Prives and Hall, 1999; Wynford-Thomas, 1999; Zilfou and Lowe, 2009). One key effector of p53 biological responses is the cell cycle inhibitor protein p21$^{WAF1/CIP1}$ (hereafter called "p21") encoded by *CDKN1A*. *CDKN1A* is a direct transcriptional target of p53 and ablation of *CDKN1A* can phenocopy some, though not all, aspects of *p53* loss of function (Sherr and Roberts, 1999).

Recent studies on the production of induced pluripotent stem (iPS) cells highlight a hitherto unappreciated oppositional relationship between p53, p21 and the stem cell phenotype (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009). Ablation of *p53* greatly enhances the efficiency of iPS formation from normal fibroblast cells and p21 is an important component of this outcome (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009). The iPS work resonates with earlier studies showing that targeted disruption of either *p53* (Meletis et al., 2006) or *Cdkn1a* (Kippin et al., 2005) compromises the relative quiescence of neural progenitors and accelerates self-renewal in the mouse.

If the p53:p21 regulatory axis suppresses self-renewal, how do stem cells in the general, and neural stem cells in the particular, suppress these functions during development and tissue repair? The molecular mechanism of p53:p21 suppression would likely involve proteins that are unique to uncommitted progenitor cell types because global disruption of p53 functions gives rise to an oncogenic phenotype (Donehower et al., 1992; Harvey et al., 1993). For neural progenitors, the

Significance

The data show how a molecular mechanism for sustaining the replication competent state of neural progenitors at early times in CNS development has been co-opted by high-grade glioma. The bHLH transcription factor Olig2 opposes p53 functions in both normal and malignant neural progenitor cells via modulation of posttranslational modifications of p53. The findings shed light on developmental origins of these tumors and may have practical overtones for targeted therapy.

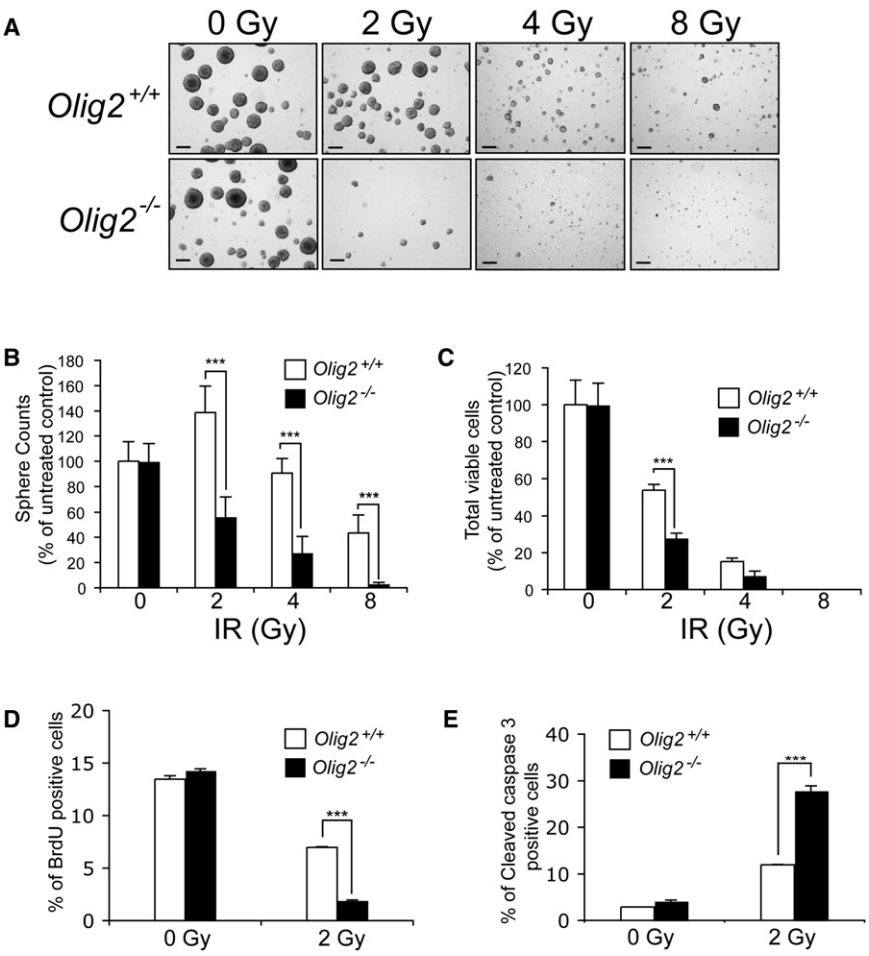

Figure 1. Olig2 Promotes Survival in Irradiated Neural Progenitor Cells

(A) Differential response of *Olig2*$^{+/+}$ and *Olig2*$^{-/-}$ neural progenitors to ionizing radiation. Secondary neurosphere assays were counted at day 5 following treatment of *Olig2*$^{+/+}$ or *Olig2*$^{-/-}$ cells with 2, 4, or 8 Gy of ionizing radiation. Scale bars = 100 μm.

(B) Quantitation of data in (A). The bars in the histogram represent the percentage of secondary neurospheres formed in irradiated samples relative to the untreated control samples.

(C) Quantitation of percentage of viable cells in the irradiated samples relative to untreated control in the sample sets from (A) using Trypan blue exclusion.

(D) BrdU uptake (*Olig2*$^{+/+}$ and *Olig2*$^{-/-}$) in cells either untreated or treated with 2 Gy of IR. Cells were treated with 2 Gy of IR; 24 hr postradiation, they were pulsed with 10 μM BrdU for 1 hr and then analyzed by FACS. Data shown here represents percentage uptake in irradiated samples relative to untreated samples.

(E) Detection of apoptosis in *Olig2*$^{+/+}$ and *Olig2*$^{-/-}$ cells after radiation treatment. Cells were treated with 2 Gy of IR and 24 hr posttreatment analyzed for activated caspase 3 by FACS analysis. The bar graph represents percentage of cleaved caspase 3-positive cells present in each sample.

For all graphs, error bars indicate SEM. The data are representative of three independent experiments. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. See also Figure S1.

bHLH transcription factor Olig2 is a plausible candidate, based on expression and functional studies.

During CNS development, Olig2 is expressed in progenitor cells that give rise to neurons and to myelinating oligodendrocytes (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). In the postnatal brain, Olig2 is expressed in transit amplifying type C neural progenitors of the subventricular zone that give rise to olfactory neurons and oligodendrocytes (Hack et al., 2004; Menn et al., 2006). Olig2 is also expressed in NG2-positive glia, which is the most prevalent cycling progenitor cell type in the postnatal brain (Jackson et al., 2006; Ligon et al., 2006; Magnus et al., 2007). A pathological correlate of Olig2 expression in healthy neural progenitors is seen in primary cancers of the CNS where OLIG2 is expressed in 100% of diffuse gliomas irrespective of grade (Ligon et al., 2004). Beyond merely marking malignant astrocytomas, Olig2 expression is required for tumor formation in a genetically relevant murine model of high-grade human glioma (Ligon et al., 2007).

Malignant gliomas are notoriously resistant to radiation and genotoxic drugs. Paradoxically, the *p53* gene is structurally intact in the majority of adult high-grade primary gliomas (Cancer Genome Atlas Research Network, 2008). Resistance to genotoxic modalities in p53-positive gliomas has been attributed to attenuation of p53 functions by genetic or epigenetic changes within a p53 signaling axis that includes the *p14*^{Arf}, *MDM2*, and *ATM* gene products (Nakamura et al., 2001; Cancer Genome Atlas Research Network, 2008; Parsons et al., 2008; Reifenberger et al., 1993). However, CNS lineage-specific factors that inhibit p53 pathways have not been reported. In this study, we investigated the potential function for Olig2 as an antagonist of p53 function in normal and malignant neural progenitors.

RESULTS

Olig2 Suppresses Biological Responses to Genotoxic Damage

As shown in Figure 1A, normal neural progenitors (wild-type for both *Olig2* and *p53*) can form secondary neurospheres within 5 days after exposure to radiation. The *Olig2* null counterparts of these cells are significantly more sensitive to radiation. The same differential sensitivity to genotoxic damage is observed in cells treated with Temozolomide, a genotoxic drug that is now a standard-of-care chemotherapeutic agent for adult patients with recurrent high-grade glioma (see Figure S1 available online). Loss of Olig2 impacts both the number of secondary neurospheres and the total number of cells in these secondary neurosphere assays (Figures 1B and 1C). Cell cycle arrest (as monitored by BrdU uptake) and to a lesser extent programmed

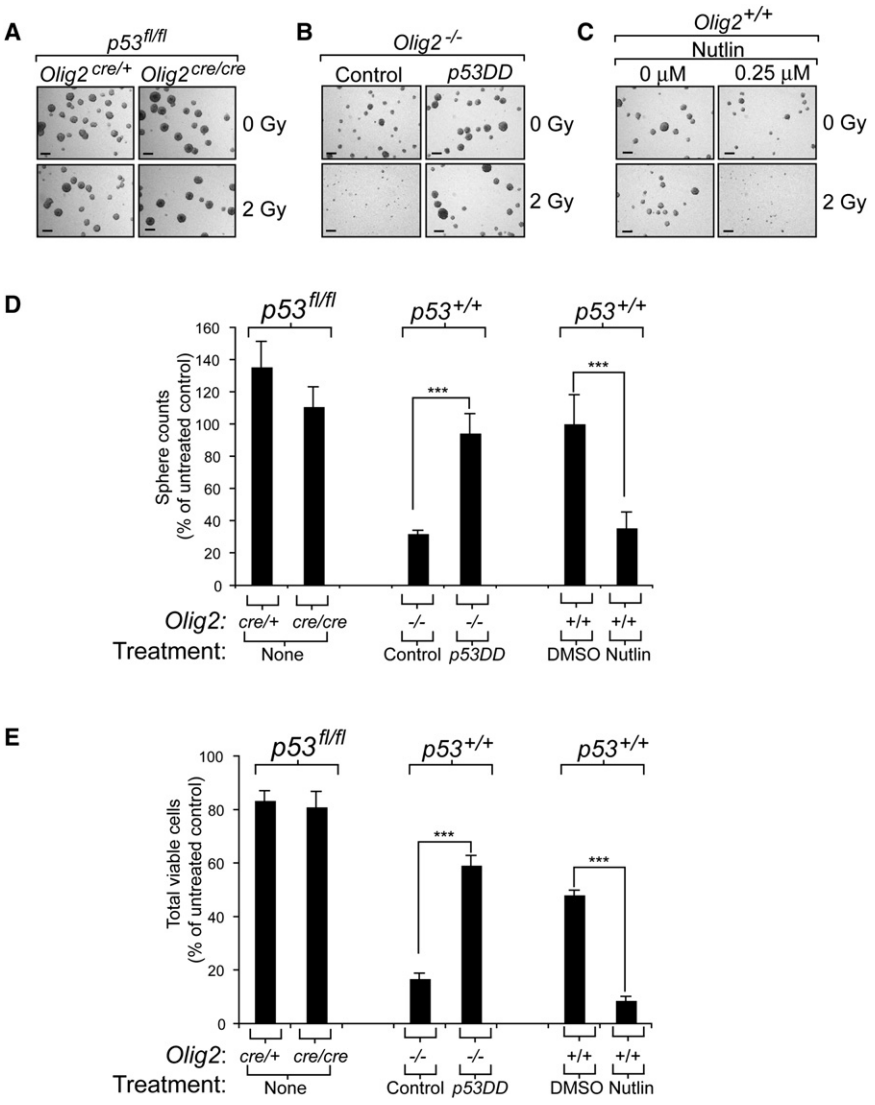

Figure 2. Olig2-Mediated Radiation Resistance Depends on p53 Status

(A) *Olig2-tva-cre*$^{+/-}$ driver mice (Schuller et al., 2008) and *p53* conditional null (*p53*$^{fl/fl}$) mice were crossed to obtain neural progenitors that were null for *p53* and either null or heterozygous for Olig2 function. The cells (neurosphere cultures) were then dissociated, treated with 2 Gy of IR, and allowed to form secondary neurospheres for 5 days after treatment. Scale bars = 100 μm.

(B) *Olig2*$^{-/-}$ cells were transduced with an expression vector encoding a dominant negative mutation of *p53* (*p53DD*) or with a vector control. The cells were then irradiated and secondary neurosphere assays were conducted as per (A) above. Scale bars = 100 μm.

(C) Stabilizing p53 can radiosensitive *Olig2* wild-type cells. *Olig2*$^{+/+}$ cells were treated with 0.25 μM Nutlin or DMSO alone for 16 hr and then exposed to 2 Gy of IR. The cells were grown for 5 days to allow secondary sphere formation.

(D) Quantitation of the percentage of neurospheres formed after radiation as compared with untreated control samples. Scale bars = 100 μm.

(E) Quantitation of percentage of viable cells after radiation treatment as compared with untreated controls.

For all graphs, the data are representative of three independent experiments. ***$p < 0.001$. Error bars indicate SEM.

cell death (as monitored by cleaved caspase 3) contribute to the differential sensitivity of *Olig2* null cells (Figures 1D and 1E).

An Oppositional Relationship between Olig2 and p53

Experiments summarized in Figure 2 document an oppositional relationship between Olig2 and p53. In one set of studies, we intercrossed *Olig2-tva-cre*$^{+/-}$ driver mice (Schuller et al., 2008) and *p53* conditional null (*p53*$^{fl/fl}$) mice to obtain neural progenitors that were null for *p53* and either null or heterozygous for Olig2 function. As shown (Figures 2A, 2D, and 2E) *Olig2* status is irrelevant to radiation sensitivity in cells wherein *p53* has been genetically ablated. In a second set of experiments, we used the carboxy-terminal dominant-negative fragment of (*p53DD*) to block p53 transcriptional functions. As indicated (Figures 2B, 2D, and 2E), ablation of *Olig2* does not rescue radiosensitivity in the presence of dominant-negative p53. In a third set of experiments, we treated *Olig2*-positive cells with a p53 agonist (Nutlin, an Mdm2 inhibitor) (Vassilev et al., 2004). As indicated (Figures 2C–2E), we show that Nutlin-mediated stabilization of p53 promotes radiosensitivity in the presence of Olig2. Thus, the oppositional relationship between *Olig2* and *p53* is symmetrical.

An Olig2:p53 Oppositional Relationship in Malignant Glioma

To explore biological functions of Olig2 in p53-positive gliomas, we began with genetically accessible murine models. We prepared neurosphere cultures from *p16/p19*$^{-/-}$ mice (*p19* being the mouse ortholog of human *p14*) and then transduced these cells with a constitutively active mutation of the epidermal growth factor receptor (*EGFRvIII*) identified originally in human glioblastoma. At a genetic level, these murine "tumor neurospheres" emulate the "classic" group of human gliomas (*p16/p14*$^{-/-}$;mutant *EGFR*; wild-type *p53*) (Verhaak et al., 2010) and form invasive tumors that recapitulate the histopathology of high-grade human gliomas when engrafted into the brains of SCID mice (Bachoo et al., 2002; Ligon et al., 2007).

As expected, *p16/p19*$^{-/-}$ neurospheres are attenuated relative to wild-type neurospheres with respect to the level of activated p53 protein that can be detected following gamma irradiation (Figure 3A). However, in secondary neurosphere assays, *p16/p19*$^{-/-}$ neurospheres and wild-type neurospheres are equally tolerant of gamma irradiation. Ablation of *Olig2* function unmasks a radiosensitive phenotype (Figures 3B–3E). Thus, the major contributor to the radioresistance phenotype of normal and malignant neural progenitors is *Olig2* status rather than

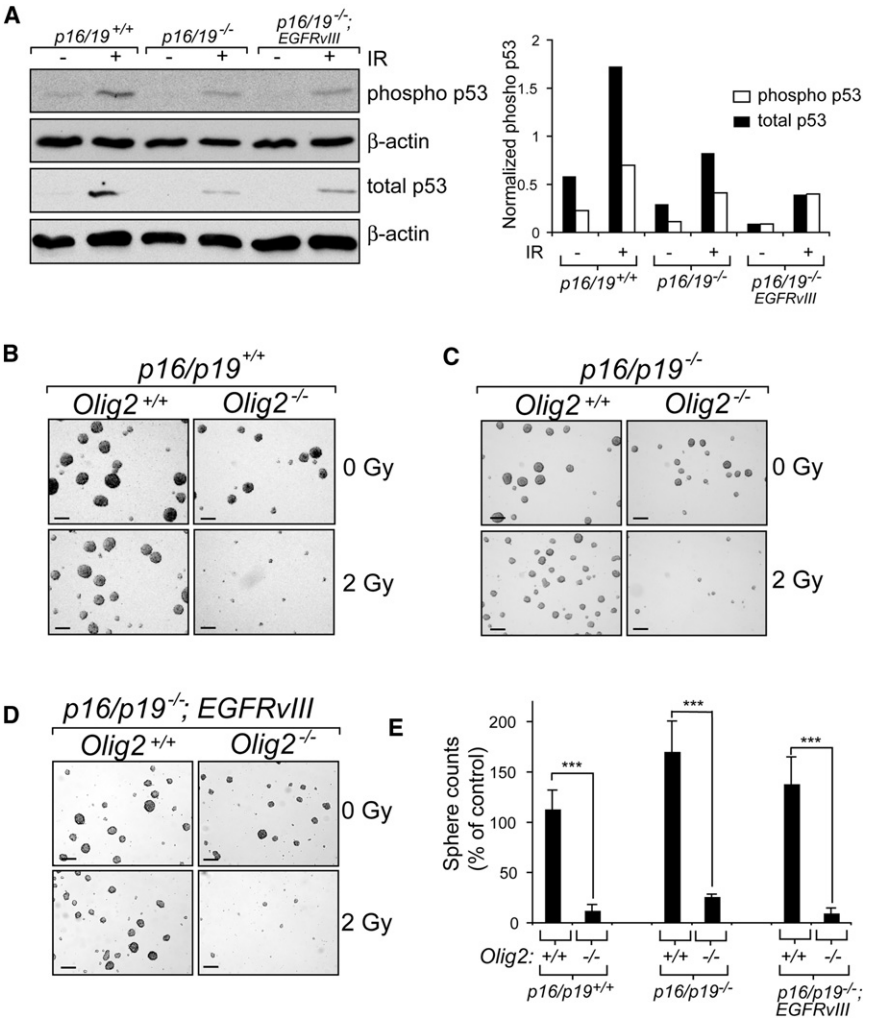

Figure 3. Olig2 Opposes Cellular Responses to Radiation in Normal and Malignant Neural Progenitors

(A) Ablation of *p19* attenuates the amount of activated p53 that is produced in response to radiation. Neurosphere cultures of the indicated genotypes were exposed to 2 Gy of gamma irradiation. Cell lysates obtained 6 hr posttreatment were analyzed by immunoblotting with antibodies recognizing phosphorylated p53 (Ser15), total p53 and β-actin; the quantification results are shown at right.

(B–D) Neurosphere cultures of the indicated genotypes were exposed to 2 Gy of gamma irradiation. Secondary neurosphere assays were counted at day 5 posttreatment. Scale bars = 100 μm.

(E) Quantification of data in (B)–(D). The data shown are the percentage of secondary neurospheres formed in irradiated samples relative to untreated control samples. The results shown are compiled from three independent experiments with three independent cell lines. ***$p < 0.001$. Error bars indicate SEM.

$p16^{Ink4a}/p19^{Arf}$. Additional experiments with the murine tumor neurospheres recapitulate the oppositional relationship between Olig2 and p53. The expression of p53DD restores radiation resistance in the absence of *Olig2* whereas stabilization of p53 with Nutlin promotes radiosensitivity in the presence of *Olig2* (Figure 4).

All primary human gliomas express OLIG2 protein (Ligon et al., 2004, 2007). Accordingly, for human gliomas, we conducted shRNA knockdown experiments to extend our observation of Olig2:p53 cross-antagonistic interactions. As shown in Figure 5, OLIG2 promotes survival of two radiation-treated human glioma neurosphere lines that are wild-type for *p53* (the BT37 and BT112 lines). However, OLIG2 knockdown is without effect in human glioma cells wherein p53 function has been genetically ablated (BT70 line). Notably, OLIG2 knockdown does not impair survival of any of the human cells in the absence of radiation treatment.

Olig2 Requirement for Glioma Formation Is p53 Dependent

Previous studies have shown that *Olig* gene function is required in order for murine tumor neurospheres to form intracranial tumors in SCID mice (Ligon et al., 2007). In the absence of *Olig1/2*, these cells engraft and survive for at least 70 days, but they do not proliferate to form tumors (Ligon et al., 2007). Experiments summarized in Figure 6 indicate that the *Olig2* requirement for proliferation in vivo is another manifestation of the Olig2:p53 oppositional relationship for both mouse and human glioma cell types.

In the murine tumor neurospheres, even a single copy of the *p53* gene is sufficient to suppress tumor formation in the absence of *Olig2*. The requirement for *Olig2* is overcome by removing both copies of *p53* (Figure 6A). We do note, however, that *Olig2* heterozygous, *p53* null tumors develop more quickly than their *Olig2* null counterparts ($p < 0.003$) raising the possibility of some *p53*-independent functions that contribute to tumor growth (Figure 6A). Predictably, the histopathology of *p53* null tumors that form in the absence of *Olig2* is quite different from that of the Olig2-positive/p53-positive tumors that more closely emulate the human disease. In particular, the *Olig2* null tumors have a much greater proportion of GFAP-positive cells and a near complete absence of cells that express oligodendrocyte (PDGFRα) and neuronal (Tuj1) progenitor-associated markers (Figure 6B).

Intracranial growth of p53-positive human gliomas is likewise promoted by *OLIG2*. As indicated in Figure 6C, shRNA knockdown of OLIG2 reduces the penetrance and significantly extends the latency of two different p53-positive glioma neurosphere lines. Moreover, the tumors that eventually do arise from implants of the p53-positive lines show expression of endogenous OLIG2 protein (Figure 6D). By contrast, penetrance and latency of a *p53* null human glioma line show no significant responses to *OLIG2* knockdown (Figure 6C). Moreover, the *p53* mutant tumors proliferate in the absence of OLIG2

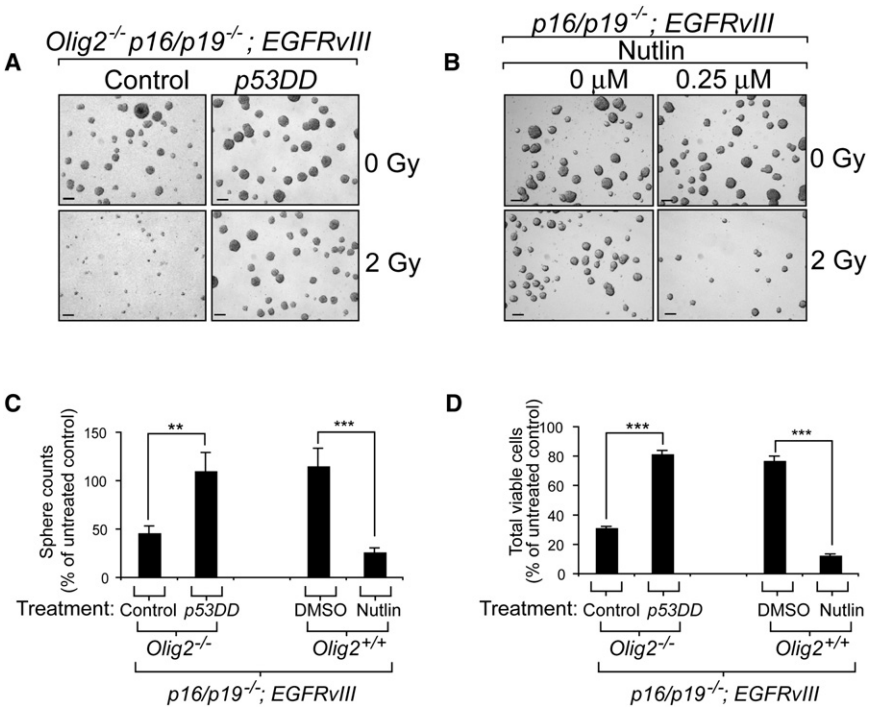

Figure 4. Olig2-Mediated Radiation Resistance in Tumor Progenitor Cells Is Dependent on p53 Status

(A) Suppression of *p53* function. An expression vector encoding a dominant negative mutant of *p53 (p53DD)* was transduced into *Olig2*$^{-/-}$ *p16/p19*$^{-/-}$; *EGFRvIII* tumor neurospheres as described in the text. These cells, together with vector controls were irradiated as shown. Secondary neurosphere assays were counted at day 5 posttreatment. Scale bars = 100 μm.

(B) Enhancement of *p53* function. *Olig2*$^{+/+}$ tumor neurospheres were treated with 0.25 μM Nutlin (an Mdm2 inhibitor) or DMSO control for 16 hr and then exposed to 2 Gy of radiation. Secondary neurosphere assays were counted at day 5 post-irradiation. Scale bars = 100 μm.

(C) Quantitation of percentage of secondary neurospheres formed in treated samples as compared with untreated samples.

(D) Quantitation of percentage of viable cells after radiation treatment as compared with untreated control samples. For both graphs, the data are compiled from three independent experiments. ***p < 0.001, **p < 0.01. Error bars indicate SEM.

expression (Figure 6E). As noted with the genetically defined mouse tumor neurospheres, there is a trend (not rising to statistical significance) for OLIG2 to accelerate tumor development from p53 mutant human glioma cells suggesting additional p53-independent functions for growth in vivo (Figure 6C).

Olig2 Suppresses p53 Acetylation and DNA Targeting

How might Olig2 oppose p53 biological functions? As indicated in Figure 7A and Figure S2, Olig2 does not affect the overall abundance of p53 protein in either control or irradiated cells. Likewise, Olig2 status is irrelevant to basal or radiation-induced p53 phosphorylation in any of these cell types. However, as shown in Figure 7B, Olig2 suppresses the acetylation of p53 in both normal and malignant murine neural progenitors and in human glioma neurosphere lines. Coincident with p53 hypoacetylation, Olig2 suppresses both basal and radiation-induced interactions of p53 with several well-characterized p53 target genes (*Cdkn1a*, *Wig1*, *Bax*, and *Mdm2*) as shown by chromatin immune precipitation experiments. Again, this Olig2-mediated suppression of p53 gene targeting is seen in both normal and malignant murine neural progenitors and in human glioma neurosphere cells (Figure 7C). In summary, Olig2 acts upon a key posttranslational modification of p53 protein itself to suppress downstream genetic and biological responses (Figure 7D).

The Oppositional Relationship between Olig2 and p53 Is Channeled through p21 in Some but Not All of the Cell Types

The mechanisms that regulate p53-mediated biological responses to DNA damage are complex (for reviews, see Hollstein and Hainaut, 2010; Riley et al., 2008; Vogelstein et al., 2000; Vousden and Prives, 2009). Activation of p53 stimulates the expression of some genes and suppresses the expression of others depending upon cell type, time point of measurement and extent of DNA damage (Fei et al., 2002; Kannan et al., 2001; Wei et al., 2006). The biological impact of diminished DNA targeting was examined in detail for one p53 target gene, namely, *CDKN1A.*

As indicated in Figures 8A, 8B, and Figure S3, Olig2 suppresses radiation-induced expression of p21 in some, but not all of the cell types studied here. The expression of p21 is regulated by a wide range of cellular cues including growth factors and activated oncogenes (Gartel and Tyner, 1999; Olson et al., 1998). Although the sample size is small, the ability of Olig2 to oppose radiation-induced expression of p21 appears to correlate with *EGFR* status. In the two mouse lines and one human line that express wild-type EGFR at physiologic levels, Olig2 opposes the induction of p21. In the mouse line and human line (BT112) that express mutated or amplified *EGFR*, the relationship between Olig2 expression and p21 expression is not seen.

Functional relationships between p21 induction and growth arrest were probed using genetically defined murine progenitor cell lines. As shown in Figure 8C knockdown or knockout of *p21/CDKN1A* rescues radiation-induced growth arrest in the two cell types wherein *Olig2* status dictates the response of p21 to irradiation (wild-type and *p16/p19*$^{-/-}$ neurospheres) but not in the one cell type wherein *Olig2* status is irrelevant to p21 induction (the *p16/p19*$^{-/-}$;*EGFRvIII* mouse tumor neurospheres). Notwithstanding the apparent irrelevance of *CDKN1A* in this latter cell type, the *p16/p19*$^{-/-}$;*EGFRvIII* mouse neurospheres undergo radiation-induced growth arrest in an Olig2-dependent fashion as noted for the other cell types above (Figures 3 and 4). Together, these findings indicate that Olig2-dependent radioprotection employs additional mechanisms besides p21 repression, depending on the oncogenic context.

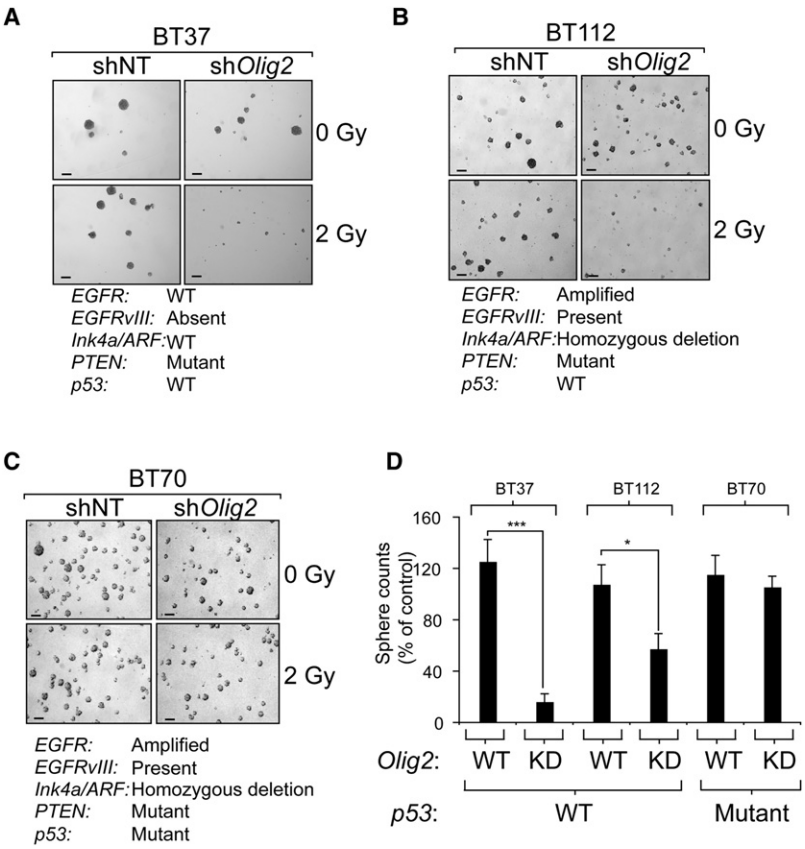

Figure 5. Olig2 Promotes Radioresistance in p53-Positive Human Glioma Cells

(A) BT37, a *p53* wild-type human cell line, was infected with either control or sh*OLIG2* containing lentivirus. After 48 hr, the cells were dissociated, exposed to 2 Gy of IR, and allowed to form secondary neurospheres for 5 days after treatment. Untreated cells served as control. Scale bars = 100 μm.

(B) Same as (A) except that the line used (BT112) has amplified EGFR locus.

(C) Same as (A) except that the line used (BT70) carries a mutant *p53* (Arg273Cys).

(D) Quantitation of percentage of secondary neurospheres formed in treated samples as compared with untreated controls. The difference in secondary neurosphere formation between the $Olig2^{+/+}$ (WT) and knockdown (KD) is significant to ***$p < 0.001$ (BT37) and *$p < 0.05$ (BT112) for the *p53* wild-type cells. Error bars indicate SEM.

DISCUSSION

Data summarized here document an intrinsic oppositional relationship between Olig2 and p53 in normal neural progenitors and in human high-grade glioma cells. The *p53*-positive murine and human glioma cell lines tested here differ from each other with respect to *EGFR* status and Ink4a/Arf expression. In addition, all of the human cell lines differ from the murine models with regard to PTEN function (ablated in the human lines and wild-type in the murine lines) (see details in Supplemental Experimental Procedures). Despite the different signaling pathways that are operative in these multiple murine and human cell lines, the fundamental relationship between Olig2 expression and p53 function is maintained: When Olig2 is expressed, p53-mediated responses to genotoxic damage are suppressed. When Olig2 is ablated (mouse cells) or suppressed by shRNA (human cells) even attenuated levels of p53 function (as seen, for example, in $p16/p19^{-/-}$ neurospheres) are sufficient to trigger radiation induced growth arrest and apoptosis.

The intrinsic oppositional relationship between p53 and Olig2 documented here may contribute to the notorious resistance of p53-positive gliomas to radiation and chemotherapy. The majority (~75%) of human high-grade gliomas have a structurally intact *p53* gene and retain at least some level of p53 function (Nakamura et al., 2001; Cancer Genome Atlas Research Network, 2008; Parsons et al., 2008). In the fullness of time, the ability to disrupt Olig2:p53 interactions in glioma cells may have practical applications in oncology. We recently describe a potential strategy for developing small molecule inhibitors of Olig2 functions specifically those involved in proliferation and tumorigenesis (Sun et al., 2011).

Generality of the Olig2:p53 Oppositional Relationship

The experiments summarized here are focused upon high-grade human glioma cells and a "genetically relevant" murine model of high-grade glioma. However, the high-grade gliomas are only one component of a group of tumors known collectively as "diffuse gliomas" that includes all classes of astrocytomas (WHO grades I through IV), mixed gliomas, and oligodendrogliomas (Kleihues and Cavenee, 2007). In addition, the high-grade gliomas as defined by conventional histopathological criterion can be further subclassified into at least four distinct tumor types as indicated by integrated genomic analysis (Verhaak et al., 2010). In the face of this genetic and histopathological diversity, OLIG2 is expressed in 100% of the human diffuse gliomas irrespective of grade (Ligon et al., 2004).

The pervasive expression of OLIG2 in this group of human tumors invites generalizations with respect to biological function. However, a paper by Sun et al. (2011) suggests an important qualifier on OLIG2 function within other members of the diffuse glioma group. Sun et al. show that Olig2 is a phosphoprotein and the phosphorylation state is developmentally regulated. Phosphorylated Olig2, found in proliferating neural progenitors and p53-positive human glioma cells, displays promitotic and anti-p53 functions, whereas nonphosphorylated Olig2 is associated with nonproliferating, myelinating oligodendrocytes in mature white matter of the brain and is devoid of anti-p53 activity. The observations of Sun et al. may reconcile the pervasive expression of OLIG2 in diffuse gliomas with the practical clinical experience that tumor grade, rather than OLIG2 expression, is the most important prognostic indicator for human gliomas.

The percentage of OLIG2-positive cells in oligodendrogliomas, for example, generally exceeds the fraction of OLIG2 positive cells in high-grade gliomas (Lu et al., 2001; Marie

et al., 2001), and yet many oligodendrogliomas respond well to radiation and chemotherapy (Cairncross and Macdonald, 1988; Fortin et al., 1999; Macdonald et al., 1990). Likewise, pediatric low-grade astrocytomas are responsive to radiation and/or chemotherapy (Kortmann et al., 2003; Packer et al., 1997; Prados et al., 1997) even though they too are positive for OLIG2 expression (Ligon et al., 2004). The observations of Sun et al. raise the interesting possibility that OLIG2 in oligodendrogliomas and pediatric astrocytomas may consist mainly of the unphosphorylated protein that marks noncycling cells in mature white matter. By contrast OLIG2 in the high-grade gliomas as studied and modeled here is mainly the phosphorylated protein that is specifically associated with anti-p53 function.

Inhibition of p53 Acetylation

How does a bHLH transcription factor inhibit a key posttranslational modification of p53? Thus far, chromatin immune precipitation assays (data not shown) and expression profiling studies (Ligon et al., 2007) reveal no direct or indirect genetic targets of Olig2 that would influence the acetylation state of p53. Likewise, antibody pull-down experiments have thus far failed to reveal any interaction between the Olig2 and p53 proteins. Preliminary yeast two-hybrid trapping screens and antibody pull down experiments have revealed a direct interaction between Olig2 and a protein known as Brd7 (data not shown). The Olig2:Brd7 interaction is provocative because Brd7 facilitates the interaction of p300 with p53 and thus promotes p53 acetylation (Drost et al., 2010). The observations suggest the testable hypothesis that Olig2 competes with p53 for an important coregulator protein that facilitates acetylation; however, considerably more work will be needed to test this hypothesis.

Critical p53 Target Genes

Further studies are also required to identify the entire repertoire of p53 genetic targets that are impacted by OLIG2, and how these contribute to suppression of p53-dependent responses to genotoxic damage. In normal neural progenitors, the p21 cell cycle inhibitor is clearly a major component of the Olig2:p53 oppositional relationship (Figure 8), and the Olig2-mediated suppression of p53 responses to genotoxic damage is identical in normal and malignant neural progenitors. However, the role of p21 in the Olig2:p53 relationship is clearly less pronounced or even irrelevant in some of the malignant lines (e.g., those expressing *EGFRvIII)*.

As indicated in Figure 7, Olig2 likewise suppresses the interactions of p53 with several other well-characterized p53 target genes. The additional impacted p53 target genes include *Wig1*, *Bax*, and *Mdm2* encoding, respectively, a cell cycle inhibitor, a proapoptotic protein and a p53 antagonist. Expression profiling studies show that two of these genes (*Wig1* and *Mdm2*) are repressed by Olig2 in normal neural progenitor cells (Ligon et al., 2007). Work in progress is aimed at determining whether the diminished p53 targeting interactions noted in Figure 7 are reflected at the level of gene expression in malignant neural progenitors. To date, we have not detected any effect of Olig2 on radiation-induced expression of p53-regulated proapoptotic genes such as *Puma*, *Bax*, and *DR5* but this negative result could reflect limitations of our assay conditions.

In addition, it should be noted that Olig2 might directly regulate the expression of genes that control cell growth and/or responses to genotoxic damage. Olig2 is known to function as a transcriptional repressor during spinal cord development (Mizuguchi et al., 2001; Novitch et al., 2001). Chromatin immune precipitation studies have shown that Olig2 itself binds to promoter/enhancer elements adjacent to the proximal p53 binding site on *CDKN1A* and that Olig2 can suppress expression of a luciferase reporter gene driven by this region of the *CDKN1A* promoter (Ligon et al., 2007). Conceivably, direct repression of p21 expression by Olig2 could augment the antagonistic action of Olig2 on p53 acetylation/DNA targeting noted in Figure 7. Still other preliminary studies show that Olig2 promotes the expression of certain DNA repair genes (e.g., *Mre11a*, *Hus1*, *Fancl*, and *Rad51l3*) (data not shown). Enhanced repair of DNA damage could oppose p53-mediated growth arrest/apoptosis by removing the genotoxic stimulus to p53 function.

Cell of Origin for Malignant Glioma

Mouse modeling studies highlight postnatal neural progenitors of the subventricular zone as a plausible cell of origin for at least some of the human high-grade gliomas (for review, see Stiles and Rowitch, 2008). Using one of these models, Wang et al. (2009) have shown that the initial signs of malignant transformation are seen in Olig2-positive transit amplifying cells of the subventricular zone. Early loss of p53 function within these cells appears to provide no selective advantage (Wang et al., 2009). The Olig2:p53 oppositional relationship described here could explain why Olig2-positive neural progenitors might be a permissive cell of origin for malignant glioma. Our data suggest that Olig2 expression in these transit-amplifying cells is already tantamount to p53 loss of function, and so true genetic loss of *p53* in such cells might have only a nuanced additive effect.

Why would normal neural progenitors express a transcription factor that opposes the guardian functions of p53 to genotoxic damage? A compelling incentive is provided by studies showing that p53 and p21 oppose the self-renewal of adult neural stem cells (Kippin et al., 2005; Meletis et al., 2006). Olig2-mediated vulnerability to malignant transformation might be the price paid by the adult CNS to maintain replication competence in these cells and sustain a reserve of neural progenitors for response to injury and for normal turnover of certain neural populations.

Therapeutic Opportunities

The Olig2:p53 signaling axis described here highlights conserved regulatory relationships between normal development and neoplastic disease that are implicit within the so called "cancer stem cell" hypothesis (Tan et al., 2006). Certain aspects of this hypothesis (e.g., the frequency of stem-like "tumor initiating cells" within solid tumors) have been contended (Boiko et al., 2010; Eaves, 2008; Quintana et al., 2008; Shackleton et al., 2009). Nevertheless, therapeutic opportunities for cancer might be embedded within molecular mechanisms that control growth and survival of normal progenitor cells during development. The results shown in Figures 5 and 6 indicate that small molecule inhibitors of OLIG2 function capable of crossing the blood-brain barrier might serve as highly selective therapeutics for malignant glioma, perhaps as adjuvants to conventional radiation and chemotherapy. Moreover, this strategy is not dependent on

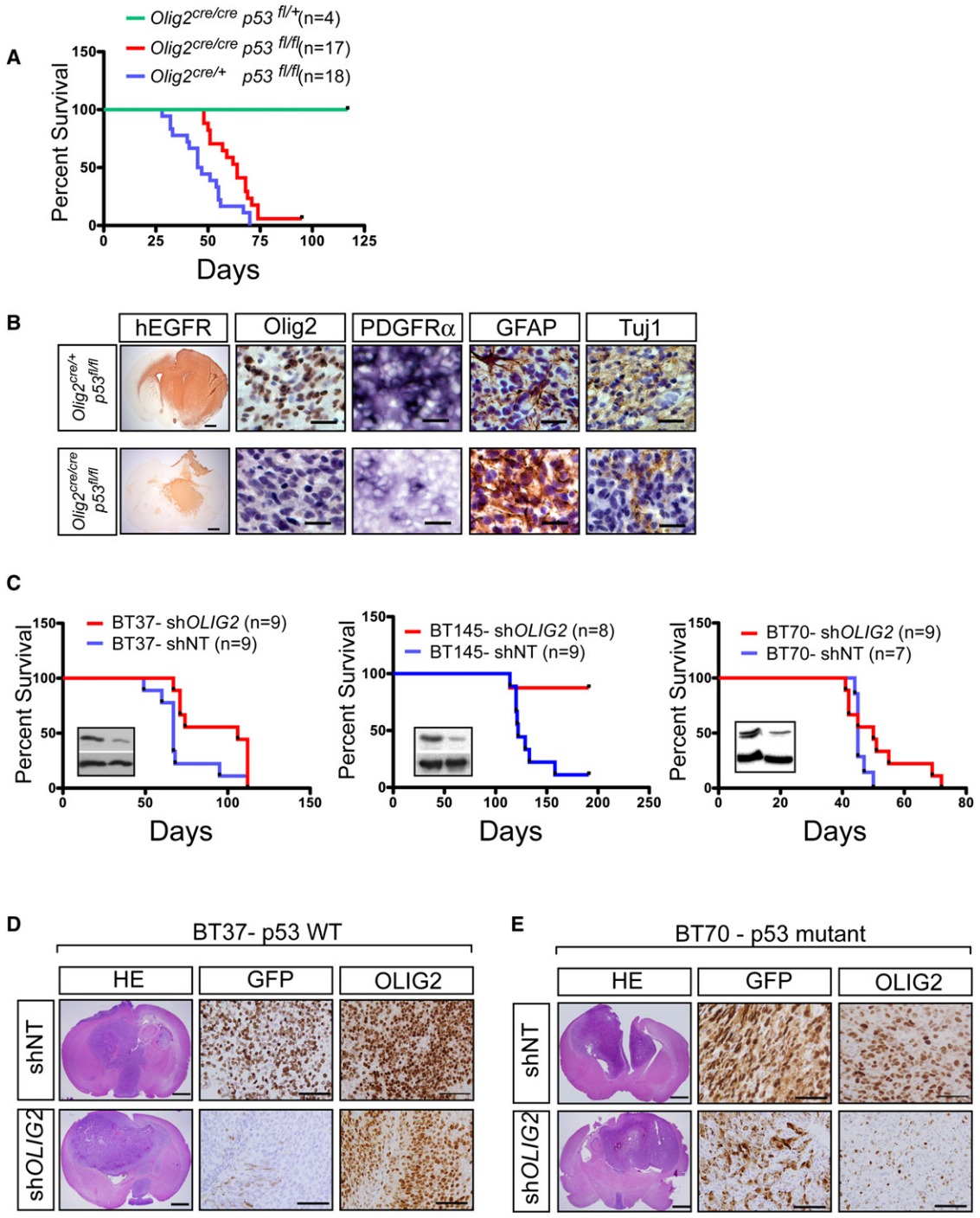

Figure 6. Olig2 Requirement for Tumorigenesis Is p53 Dependent

(A) Murine neural progenitors with the indicated *Olig2* and *p53* genotypes were transduced with *EGFRvIII* and injected into the brains of SCID mice. As indicated by the survival plots, mice injected with $Olig2^{cre/cre}$ $p53^{fl/+}$ cells fail to form tumors in contrast to $Olig2^{cre/cre}$ $p53^{fl/fl}$ cells ($p < 0.0003$). Note also that $Olig2^{cre/+}$ $p53^{fl/fl}$ cells can form tumors with significantly shorter latency period than $Olig2^{cre/cre}$ $p53^{fl/fl}$ cells (median survival 46 days and 64 days, respectively, $p < 0.003$).

(B) Comparison of tumors derived from $Olig2^{cre/+}$;$p53^{fl/fl}$ and $Olig2^{cre/cre}$;$p53^{fl/fl}$ cells. Note the greater proportion of GFAP-positive (astrocyte-like) cells and a near complete absence of PDGFRα positive (oligodendrocyte progenitor-like) and Tuj1-positive (neuron-like) cells in the *Olig2* null tumors. Scale bars: the hEGFR panels 1 mm and others 25 μm.

(C) Kaplan-Meier survival analysis of SCID mice intracranially implanted with human glioma cell lines transduced with *OLIG2* shRNA or nontarget shRNA (shNT). The differences in survival between two corresponding groups are $p < 0.013$ and 0.001 for p53 wild-type BT37 and BT145 lines, respectively, and $p < 0.51$ for the p53 mutant BT70 line.

lineage predictions of the "cancer stem cell" hypothesis, as it targets a transient amplifying Olig2-positive cell during tumorigenesis irrespective of the nature of its cellular precursor.

Transcription factors are generally considered to be poor targets for drug development because their interactions with DNA and coregulator proteins involve large areas of surface contact. However, efficacious surrogate targets for OLIG2-based drug development might emerge in the form of coregulator proteins, functionally essential downstream genetic targets or key posttranslational modifying enzymes. We recently identified a set of three closely spaced serine residues in the amino terminus of Olig2 that, when phosphorylated, promote the growth of neural progenitors in cell culture and in the brain (Sun et al., 2011). Small molecule inhibitors of the protein kinases that regulate Olig2 phosphorylation state might have practical applications in glioma therapy.

Finally, it should also be noted that p53 and p21 play generic roles in suppressing the self-renewal of stem cells (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009). It is thus conceivable that oppositional relationships between p53 and progenitor-specific transcription factors analogous to the one described here, will be observed in a broader range of cancer types.

EXPERIMENTAL PROCEDURES

Mouse Procedures

Animal husbandry was performed according to DFCI and UCSF guidelines under IACUC approved protocols. *Olig2-tva-cre*$^{+/-}$ mouse line (Schuller et al., 2008) was crossed to the conditional *p53* knockout line (FVB.129-Trp53tm1Brn, NCI mouse repository). Five- to 6-week-old immunocompromised SCID mice were obtained from Taconic (ICRSC-M). For orthotopic transplants, neurospheres were dissociated and resuspended in HBSS at a density of 100,000 cells/μl. Two μl was injected in the right striatum 2 mm lateral to the bregma. Animals were euthanized at the onset of neurological symptoms or once moribund.

Human Materials

All human subjects work was reviewed by the Institutional Review Board Committees of the Brigham and Women's Hospital and Dana-Farber Cancer Institute for appropriate use, that informed consent was obtained from all subjects when required, and appropriate waiver of consent requirements was obtained for minimal risk studies.

Neurosphere Cultures

Murine neural progenitor cells were isolated using techniques previously described (Ligon et al., 2007) with the modification that cells were cultured in the presence of EGF (20 ng/ml) and bFGF (20 ng/ml).

Olig2$^{cre/+}$ *p53*$^{fl/fl}$, *Olig2*$^{cre/cre}$*p53*$^{fl/fl}$ and *Olig2*$^{cre/cre}$*p53*$^{fl/+}$ cells were generated by crossing *Olig2*$^{cre/+}$*p53*$^{fl/fl}$ to *Olig2*$^{cre/+}$*p53*$^{fl/+}$ mice. Neurosphere cultures were established as previously described (Ligon et al., 2007). Cultures were infected at first passage with retrovirus expressing *EGFRvIII* (Bachoo et al., 2002) and treated with puromycin (2 μg/ml) 48 hr after infection. Cultures were orthotopically transplanted at different passage numbers (P4 to P8).

The human glioma cell lines (BT37 and BT112) were derived from Brigham and Women's Hospital patients undergoing surgery according to IRB approved protocols. The BT70 (also referred to as GBM6) line was received from C. David James (Pandita et al., 2004) (UCSF). The cells derived from glioblastoma (GBM) biopsies were implanted into immunocompromised mice. Dissected xenografts were processed as described earlier (Ligon et al., 2007). For adherent conditions, cells were cultured as previously described by Pollard et al. (2009).

The relevant genotype of these cell cultures is detailed in Supplemental Experimental Procedures.

Secondary Neurosphere Assays

The frequency of secondary neurospheres formed upon replating of disaggregated primary neurospheres has been used as a surrogate measure of self-renewal in replication-competent neural progenitor cells (Bao et al., 2006; Molofsky et al., 2005; Reynolds and Weiss, 1996). Cultures were performed as described previously (Ligon et al., 2007). Details are provided in the Supplemental Experimental Procedures.

Histology Analysis and Immunohistochemistry

Histological screening of tumors and immunohistochemistry procedures were performed as previously described (Ligon et al., 2007). In situ hybridization was performed for PDGFRα using antisense digoxigenin-labeled *PDGFRα* riboprobe as described previously (Lu et al., 2000).

Cleaved Caspase 3 Detection

Radiation induced apoptosis was measured by quantitating cleaved caspase 3-positive cells 24 hr post radiation treatment as compared with untreated samples. In brief, the neurospheres were dissociated and fixed with 4% paraformaldehyde for 10 min at 37°C and then permeabilzed by incubation in 90% methanol for 30 min on ice. The cells were stained with anti cleaved Caspase 3 (Cell Signaling Technology 1:100). The cells were analyzed by flow cytometry. At least 10,000 gated events were counted for each sample. The data were then analyzed by FlowJo Software.

Chromatin Immunoprecipitation

Chromatin immunoprecipitations were performed as described earlier (Ligon et al., 2007). In brief, neurosphere cultures were treated with 2 Gy ionizing radiation. Untreated cells served as controls. The cells were collected 3 hr postradiation and fixed with 1% formaldehyde for 15 min at room temperature and processed for chromatin immunoprecipitations with p53 antibody (FL-393, Santa Cruz).

The sequences for primers used for quantitative ChIP analysis are provided in Supplemental Experimental Procedures.

Virus Production and Packaging

Construction of retroviral vector encoding the constitutively active mutant of *EGFR* (*EGFRvIII*) has been described (Bachoo et al., 2002). Lentiviral stocks were produced as previously described (Moffat et al., 2006). Details are provided in the Supplemental Experimental Procedures.

Statistical Analysis

Two-way ANOVA with Bonferroni posttests were carried out to confirm there was interaction between genotype and treatment (radiation or cytotoxic drugs). All data presented showed significant interaction between genotype and treatment. One-way ANOVA with Newman Kleus multiple comparison test was used to analyze significant difference between treated samples. Z-test was used to analyze fold enrichment of p53 to its target promoters in Olig2 null cells as compared with wild-type cells.

(D) Immunohistochemistry of tumors derived from injections of *p53*-positive BT37 human glioma cell line. The tumors formed by cells infected with sh*OLIG2* vector no longer express the GFP marker and express OLIG2 at levels comparable to that seen in tumors that arise from control cells (shNT). Scale bars for H&E staining are 1.25 mm and all others 50 μm.

(E) Immunohistochemistry of tumors derived from injections of *p53* mutant BT70 human glioma cell line. The tumors arising in these mice injected with sh*OLIG2* continue to express the GFP marker and show a significant knockdown of OLIG2 relative to tumors that arise from control cells (shNT). Scale bars for H&E staining are 1.25 mm and all others 50 μm.

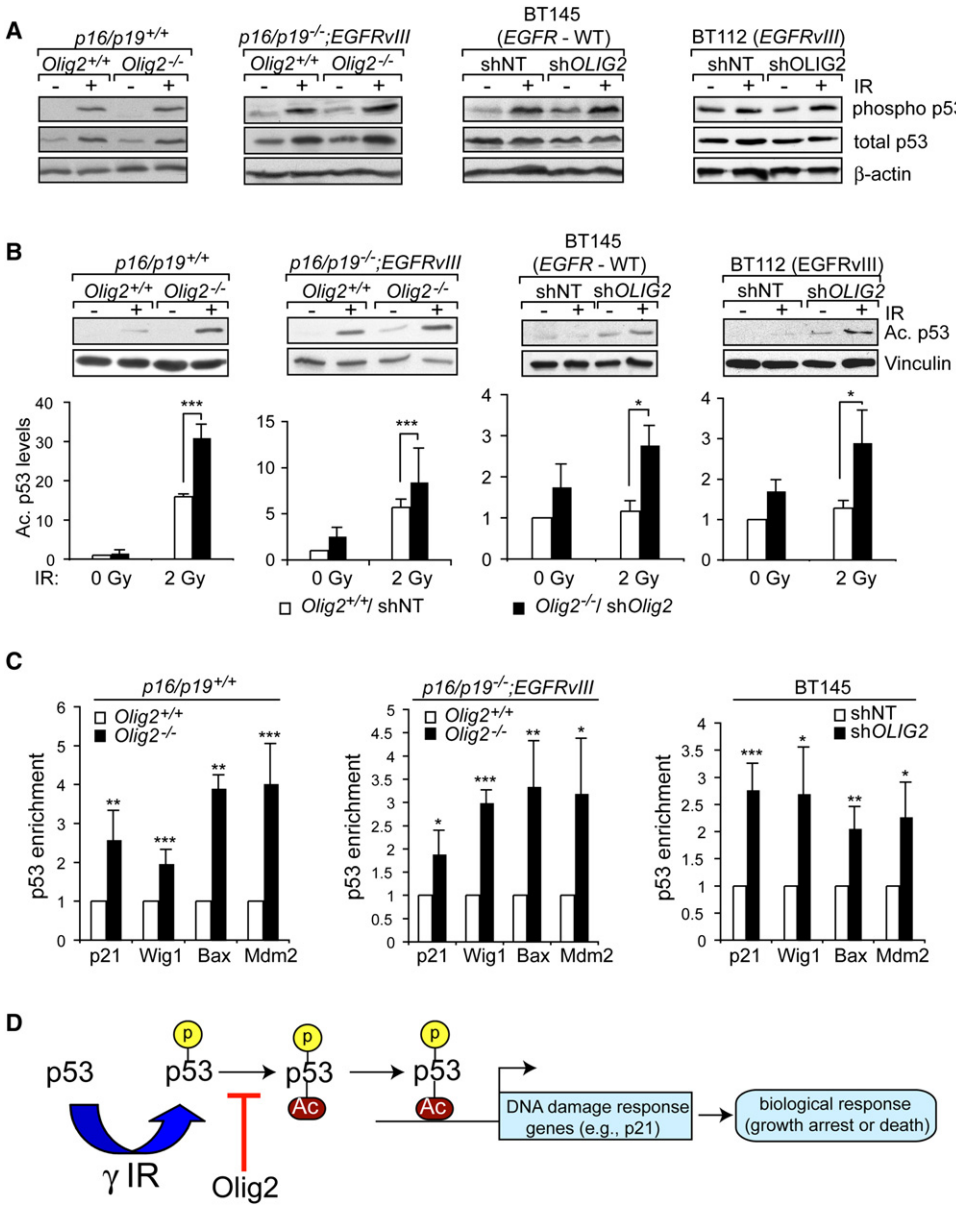

Figure 7. Olig2 Suppresses Acetylation and DNA Binding of p53

(A) Ablation of *Olig2* does not affect basal expression of p53 or its phosphorylation upon DNA damage. Cell lysates from cultures either untreated (–) or treated (+) with 2 Gy of IR were obtained 6 hr posttreatment and analyzed by immunoblotting with antibodies recognizing phosphorylated p53 (Ser15), total p53, and β-actin.

(B) Olig2 suppresses DNA damage induced acetylation of p53. Cell lysates from cultures either untreated (–) or treated (+) with 2 Gy of IR in the presence of HDAC inhibitor were obtained 6 hr posttreatment and analyzed by immunoblotting with antibodies recognizing acetylated p53 (lys379) or vinculin. Immunoblots from five independent experiments were quantitated. The bar graphs represent acetylated p53 levels after radiation in the indicated cell lines. *$p < 0.05$, ***$p < 0.001$.

(C) Quantitative ChIP analysis of p53 bound to its target promoters (*Cdkn1a*, *Wig1*, *Bax*, *and Mdm2*). The bar graphs represent ratio of fold enrichment of p53 at target sites in *Olig2*$^{-/-}$ cells over *Olig2*$^{+/+}$ cells. For all graphs the data is compiled from three independent experiments. For (B) and (C), error bars represent SEM and *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(D) Model for Olig2-mediated negative regulation of p53 signaling pathway. DNA damage leads to stabilization and activation of p53 through posttranslational modifications (phosphorylation and acetylation). Activated p53 transactivates its downstream targets, which leads to either growth arrest or cell death (Barlev et al., 2001; Dornan et al., 2003; Horn and Vousden, 2007; Riley et al., 2008). As indicated, Olig2 suppresses p53 acetylation and thereby affects p53 association with target promoters.

See also Figure S2.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and Supplemental Experimental Procedures and can be found online at doi:10.1016/j.ccr.2011.01.035.

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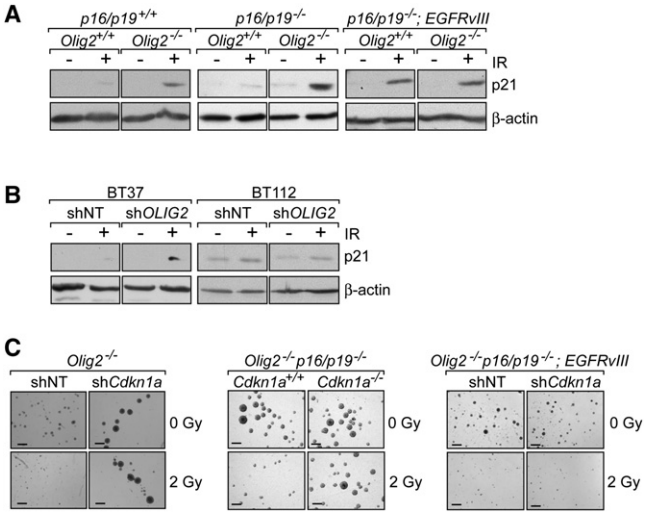

Figure 8. Olig2 Promotes Radiation Resistance in Part through Suppression of p21

(A) Cell lysates from indicated murine cell lines either untreated (–) or treated (+) with 2 Gy of gamma radiation were obtained 6 hr posttreatment and analyzed by immunoblotting with antibodies recognizing p21 and β-actin.

(B) Human glioma cell lines (BT37 and BT112) were transduced with a control hairpin (NT) or sh*Olig2* and treated with 2 Gy of gamma radiation. Cell lysates from untreated (–) or treated (+) samples were obtained 6 hr posttreatment and analyzed by immunoblotting with antibodies recognizing p21 and β-actin.

(C) Ablation of *Cdkn1a* in *Olig2*$^{-/-}$ cells with wild-type *EGFR* restores radiation resistance. *Olig2*$^{-/-}$ (left panels) or *Olig2*$^{-/-}$ *p16/p19*$^{-/-}$; *EGFRvIII* (right panel) cells were infected with retrovirus expressing control shNT or sh*Cdkn1a*. After 48 hr cells were dissociated, exposed to 2 Gy of IR and allowed to form spheres for 5 days after treatment. Untreated cells served as control. *Olig2*$^{-/-}$ *p16/p19*$^{-/-}$*Cdkn1a*$^{-/-}$ cells were treated with 2 Gy of IR and allowed to form spheres for 5 days after treatment (middle panel). Untreated cells served as control. Scale bars = 100 μm.

See also Figure S3.

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