Supplementary Material and Methods and Figure Legends for

Cyclin D1 and cdk4 mediate development of neurologically destructive oligodendroglioma

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MATERIAL AND METHODS:

Antibodies used in these studies.

The following antibodies were used for immunohistochemistry: cdk4 (1mg/ml; H22; Santa Cruz); cyclin D1 (6mg/ml; H295; Santa Cruz); OLIG2 (5mg/ml; Millipore); NeuN (4mg/ml; A60, Millipore); GFAP (1mg/ml; Dako); Iba1 (0.5mg/ml; Wako Chemicals); Ki-67 (0.01mg/ml; Vector); cathepsin X (1/1000; R & D Systems); cathepsin H (1/1000; R & D Systems); cathepsin S (1/1000; R & D Systems); CSF1R (1/1000; C20; Santa Cruz); PDGFR (1/100; Cell Signaling; #3164); FLAG-M2 (Sigma; 1mg/ml); nestin (0.5mg/ml; BD Pharmingen); CD34 (5mg/ml; EBiosciences).

The following antibodies were used for western blotting:OLIG2 (1/2000; Millipore); cyclinD1 (1/1000; Ab3; Neomarkers); cdk4 (1/500; H22; Santa Cruz); cdk2(1/500; M2; Santa Cruz); Iba1 (1/500; Wako); cathepsin X (1/1000; R & D Systems); cathepsin H (1/1000; R & D Systems); cathepsin S (1/1000; R & D Systems); CSF1R (1/1000; C20; Santa Cruz); p-p70S6K (1/1000; Cell Signaling #9234); pERK1/2 (1/1000; Cell Signaling #4377).

Flow cytometry for detection of nestin-positive cells

Whole brains were removed from neonatal mice and enzymatically and mechanically dissociated by treatment with papain (Worthington Biochemicals) and extensive pipetting. The papain was inactivated using ovomucoid (Worthington Biochemicals) and the cells collected by centrifugation (1100 rpm; 5 minutes). The cells were washed three time in ovomucoid and Dulbecco's Modified Eagles Medium (DMEM) by centrifugation (600 rpm, 10 minutes) to remove cellular debris. The cells were then resuspended in 500ml DMEM and counted by trypan blue exclusion with a haemocytometer. $1x10^6$ viable cells were fixed in 4% paraformaldehyde and permeabilised overnight at 4°C with 70% ethanol. The next day, cells were blocked in 5% goat serum in 1%BSA/PBS prior to treatment with the nestin antibody. Cells were washed in 1%BSA/PBS and then a secondary antibody conjugated to AlexaFluor-488 (1/60; BD Pharmingen) added. After incubation, cells were washed as previous and then propidium iodide added. Stained cells were analyzed on a FACScan machine (BD Biosciences).

FIGURE LEGENDS:

Supplementary Figure 1. CyclinD1 and cdk4 are ovexpressed in oligodendroglial tumors. (A) Cyclin D1 mRNA expression profiles. Whisker plots of cyclin D1 mRNA in normal brain, oligodendroglioma, and Glioblastoma multiformae were assembled from microarray data in the Oncomine database (66). **(B)** Cyclin D1 and cdk4 protein expression was assessed immunohistochemically in normal and PDGF-transformed wild-type mouse brains. Note the marked absence of cyclin D1 or cdk4 positive cells in normal brain sections. H&E staining is shown of representative normal brain and oligodendroglioma which develops in *nestin-tvA* transgenic wild type mice following the

introduction of RCAS vectors expressing PDGF. **(C)** Whole brain lysates were prepared from an unchallenged mouse (normal) and a wild type mouse challenged with RCAS-HA-PDGF and symptomatic for glioma. Proteins were resolved by SDS-PAGE and blotted for cyclinD1, cdk4 and OLIG2. The asterisk marks the cdk4 band. The background non-specific band was seen in extracts from *cdk4*$^{-/-}$ animals as well (data not shown).

Supplementary Figure 2. Histological criteria for tumor grading. The histological landmarks described in the panels below were used to stratify tumors into low (equivalent to WHO grade II), moderate (equivalent to WHO grade III) and high (equivalent to WHO grade IV) grade tumors. These criteria are derived from Kleihues and Cavanee (2000). **(A)** Normal brain cortex (*left*) and cortex harboring a glial tumor (*right*). The glioma is identified as a cluster of densely-packed, hyperchromatic nuclei, with cytoplasmic clearing (nuclear halo). Magnification 100x. **(B)** Histological features denoting a low grade doligodendroglioma, including nuclear halo (cytoplasmic clearing, *left*), white matter tracking (also intrafasicular queuing through the corpus collosum, *center*) and perineuronal satellitosis (*right, yellow arrows*). These hallmarks were found in all tumors, but the absence of criteria from (C) or (D) identified these tumors as low grade. Magnification 100x (left and center panels); 400x (right panel). **(C)** Histological features denoting a moderate grade oligodendroglioma, including microvascular proliferation (*left, yellow arrow*) and brisk mitotic figures (*right, yellow arrows*). These features, along with the absence of criteria from (D) identified these tumors as moderate grade. Magnification 100x (left and center panels); 400x (right panel). **(D)** Necrosis and pseudopalisading regions (*yellow arrow*) marked tumors as high grade. Magnification 400x.

Supplementary Figure 3. cdk2 expression is dispensable for glioma formation. The absence of cdk2 did not affect survival (A), tumor incidence (B), or tumor grade (C) when nestin-positive progenitor cells were infected with RCAS-HA-PDGF. This is exactly as described in the legend to figures 1A, 1B and 1C. Tumor formation was initiated as previous in neonate wild type (n=13) and cdk2 knockout (n=13) nestin-tvA mice and the mice followed for symptoms of morbidity. Glial tumors from wild-type (n=11) and cdk2 knockout (n=12) were graded histologically as per Figure 1C. The others could not be graded due to autolysis of the material following death and prior to harvest of the tissue.

Supplementary Figure 4. PDGFR-α expression, downstream PDGF signaling and vasculature are not affected by the absence of cyclinD1. **(A)** PDGFR-α receptor expression is unaffected by deletion of cyclinD1. A representative wild type and cyclinD1 knockout animal infected with PDGF and symptomatic for tumor formation was sacrificed, the brains removed and fixed. H & E staining was used to determine gross tumor formation and cyclinD1 and PDGFR-α expression by immunohistochemistry using anti-cyclinD1 and PDGFR-α antibodies. **(B)** Downstream PDGF signaling is unaffected by the absence of cyclinD1. Whole-brain lysates were generated from morbid wild type and cyclinD1 knockout animals and probed for expression of OLIG2 (to normalize for tumor size), cyclinD1 and phosphorylated ERK1/2 and p70S6K. **(C)** Tumor vessel formation and vasculature is not changed by deletion of cyclinD1. Brains from morbid wild type and cyclinD1 knockout animals were fixed and stained for the vessel marker, CD34. Images are shown at both 100x and 400x magnifications.

Supplementary Figure 5. Microglia activation and progression of PDGF-expressing Ink4a/Arf tumor cells and microglia is hindered in cyclin D1 deficient stroma. **(A)** Schematic of the transplant experiments. Glial tumors derived from

Ink4a-Arf$^{-/-}$*;tvA*$^{+}$ mice challenged with PDGF were dissociated into single-cell suspensions and stereotactically injected into the brains of 5-8-week-old wild type and *cyclin D1* knockout recipients. Mice were then followed for symptoms of glioma. **(B and C)** Morbidity and tumor growth was followed as described in the legend to figures 1A and 1B. **(D)** Tumor grade was assessed in transplant recipients as described in the legend to figure 1C. **(E)** Iba1 and cathpesin X staining to look at the activation of microglia is as described in the legend to figure 5B.