


# Nuclear IMPDH Filaments in Human Gliomas

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## Abstract

The analysis of nuclear morphology plays an important role in glioma diagnosis and grading. We previously described intranuclear rods (rods) labeled with the SDL.3D10 monoclonal antibody against class III beta-tubulin (TUBB3) in human ependymomas. In a cohort of adult diffuse gliomas, we identified nuclear rods in 71.1% of IDH mutant lower-grade gliomas and 13.7% of IDH wild-type glioblastomas (GBMs). The presence of nuclear rods was associated with significantly longer postoperative survival in younger ( $\leq 65$ ) GBM patients. Consistent with this, nuclear rods were mutually exclusive with Ki67 staining and their prevalence in cell nuclei inversely correlated with the Ki67 proliferation index. In addition, rod-containing nuclei showed a relative depletion of lamin B1, suggesting a possible association with senescence. To gain insight into their functional significance, we addressed their antigenic properties. Using a TUBB3-null mouse model, we demonstrate that the SDL.3D10 antibody does not bind TUBB3 in rods but recognizes an unknown antigen. In the present study, we show that rods show immunoreactivity for the nucleotide synthesizing enzymes inosine monophosphate dehydrogenase (IMPDH) and cytidine triphosphate synthetase. By analogy with the IMPDH filaments that have been described previously, we postulate that rods regulate the activity of nucleotide-synthesizing enzymes in the nucleus by sequestration, with important implications for glioma behavior.

**Key Words:** Cytoophidia, Glioblastoma, Glioma, IMPDH filaments, Nuclear rods.

## INTRODUCTION

Despite significant progress in elucidating the molecular mechanisms underlying gliomagenesis, clinical progression with malignant transformation continue to define the natural history of gliomas (1). Therapeutic targeting of class-defining molecular changes is a rational approach to treatment but has produced limited success in terms of improving survival and preventing recurrence. There is recent evidence that nongenetic features, including histopathological ones, segregate with certain epigenetically defined tumor subgroups with distinct prognostic trajectories (2). Thus, the identification of additional morphological markers which can supplement molecular genetics may contribute to the evolution of a more granular brain tumor classification. Alterations in nuclear morphology have contributed substantially to our understanding of the biology of tumor behavior. Indeed, the assessment of nuclear dysmorphia is commonly employed in the pathological grading of tumors (3). In addition, structural and numeric alterations in a variety of membraneless nuclear organelles, including nucleoli, Cajal bodies, promyelocytic leukemia protein nuclear bodies, and others, provide information regarding tumor behavior (4).

We have described rod-shaped intranuclear bodies (rods) immunoreactive for class III beta-tubulin (TUBB3) in human neurons (5), ependymal cells (5), and pancreatic beta cells (6), as well as in human ependymomas (7). We also identified them in astrocytomas and oligodendrogliomas. The latter finding was surprising because, unlike non-neoplastic ependymal cells, rods are not present in non-neoplastic astrocytes and oligodendrocytes (5). Thus, their presence in these gliomas implies a specific role for these structures in a neoplastic context in these cell types. Understanding the biological significance of these structures is predicated on elucidating their biochemical composition. Although rods show intense staining for the neuron-specific tubulin isoform class III  $\beta$ -tubulin (TUBB3) using the SDL.3D10 monoclonal antibody (SDL), they do not stain for other antibodies generated against TUBB3, nor for antibodies against pan- $\beta$ -tubulin, suggesting that they are composed of an unrelated antigen. There is precedence for cross-reactivity of the SDL.3D10 antibody with an unknown antigen in an unusual cellular structure similar to rods. “Loukoumasomes” are large annular or linear cytoplasmic structures that were originally described in the cytoplasm of rat sympathetic ganglion neurons (8). Like rods, loukoumasomes

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stain intensely for SDL3D10, but not for other antibodies against TUBB3 or pan  $\beta$ -tubulin. Loukoumasomes, in turn, are morphologically identical to cellular structures variably referred to as “cytoophidia” or “rods and rings” (reviewed in [9, 10]). These filamentous structures consist of polymeric assemblies of the nucleotide-synthesizing enzymes inosine monophosphate dehydrogenase (IMPDH) or cytidine triphosphate synthetase (CTPS). On the basis of these inter-relationships, we reasoned that rods might be antigenically related to these structures and possibly immunoreactive for IMPDH and CTPS.

In the present study, we investigate the presence of nuclear rods in a cohort of human gliomas and compare their prevalence among oligodendrogliomas, astrocytomas, and glioblastomas (GBMs). We correlate their presence with cellular markers of proliferation and, in GBMs, examine their relationship with postoperative survival. In order to gain mechanistic insight, we address the antigenic profile of rods with respect to TUBB3, IMPDH, and CTPS. The results of these studies reveal a novel morphological feature of gliomas with important implications for understanding nongenetic determinants of their biological and clinical behavior.

## MATERIALS AND METHODS

### Study Cohort

All studies were performed in accordance with the ethical guidelines of the Ottawa Hospital Research Institute and St. Michael's Hospital, respectively. Tissue microarrays (TMAs) were prepared using a manual tissue microarrayer (Leica Microsystems, Inc., Wetzlar, Germany) from representative formalin-fixed, paraffin-embedded blocks of glioma tissue recovered from the tissue archives in the Departments of Pathology of The Ottawa Hospital and St. Michael's Hospital. The cohort consisted of 37 IDH mutant, 1p/19q codeleted oligodendrogliomas (16 grade 2, 21 grade 3), 22 IDH mutant, ATRX mutant astrocytomas (12 grade 2, 10 grade 3), and 87 IDH wild-type GBMs.

### *TUBB3*<sup>-/-</sup> Mouse Brain Processing

*TUBB3*<sup>-/-</sup> mice were previously generated and kindly provided by Drs Elizabeth Engle and Long Cheng, Boston Children's Hospital and Harvard Medical School, Boston, MA (11). Adult *TUBB3*<sup>-/-</sup> mice were intracardially perfused with 10% neutral formalin, the skull removed, and the brains carefully dissected. Brains were then fixed in 10% neutral formalin for 24–48 hours and processed for embedding in paraffin. Serial sections at 5 mm thickness were collected and mounted on superfrost-coated slides (Fisher Scientific, Pittsburgh, PA).

### Immunostaining

The primary antibodies employed are listed in the Table. For double-labeling immunofluorescence studies, following incubation in the primary antibodies, TMAs were incubated in a cocktail of goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to Alexafluor 488 and 594 fluorescent tags (ThermoFisher, Waltham, MA). TMAs were

coverslipped using aqueous mounting media containing 1.5  $\mu$ g/ml 4'-6-diamidino-2-phenylindole as a nuclear counterstain (Vector Laboratories, Burlingame, CA).

### Microscopy

Microscopic imaging was performed in the Cell Biology and Image Acquisition core facility at The University of Ottawa. High-resolution microscopic imaging was performed using a Zeiss 880 Confocal Microscope with Airyscan Fast (Zeiss, Oberkochen, Germany). TMAs stained for SDL, SDL/Ki67, SDL/Lamin B1, and SDL/Lamin A/C were scanned using a Zeiss M1 Slide Scanner with fluorescence capability (Zeiss).

### Electron Microscopy

Tissue was fixed in sodium cacodylate buffered 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA), dehydrated through graded ethanol and acetone, and embedded in Spurr's resin (Ted Pella, Inc., Mountain Lakes, CA). Eighty nanometer thin sections were mounted onto copper grids (Ted Pella, Inc.) and examined with a Hitachi H 7100 transmission electron microscope (Hitachi, Tokyo, Japan).

### Data Analysis

Image analysis was performed on digital images using Zeiss Zen (Zeiss) and Fiji (12) software. For quantitative analysis of rod frequency and Ki67 index in double-stained images for SDL and Ki67, an area of 200  $\mu$ m  $\times$  200  $\mu$ m was selected randomly in each image. The total number of nuclei in the selected area was calculated by Fiji software and the number of nuclei containing rods was counted manually. Rod frequency was defined as the proportion of nuclei with rods. The same procedure was used for Ki67 index calculation.

Nuclear and cytoplasmic intensity of Lamin B1 and Lamin A/C staining were analyzed using Fiji software. For this analysis, 15 nuclei with rods and 15 nuclei without rods were selected randomly in each image.

All the statistical analyses were done using SPSS Statistics 24.0 statistical software (IBM, Chicago, IL). The  $\chi^2$  test was used for categorical variables. Because the Kolmogorov-Smirnov test of normality was statistically significant, non-parametric tests were used for analysis. The Mann-Whitney U test, Spearman's rank correlation, and linear regression analysis were used for continuous data. The significance level was set at  $\alpha = 0.05$ . Kaplan-Meier survival analysis was performed to investigate the effect of nuclear rods on postoperative survival among 83 of 87 GBM patients with available survival data. Further survival analysis was also done on stratified data based on age and sex to analyze the effect of these factors. All Kaplan-Meier survival curves were reported with a p-value obtained from the log-rank test. All statistical analyses were conducted using an  $\alpha = 0.05$  significance.

**TABLE.** Primary Antibodies Used in the Study

Antibody	Clone	Host	Dilution	Source	Product #
TUBB3	SDL.3D10	Mouse monoclonal	1:100	Millipore Sigma, Burlington, MA	T8660
TUBB3	TUJ1	mouse monoclonal	1:200	BioLegend, San Diego, CA	801213
TUBB3	TU20	Mouse monoclonal	1:500	Millipore Sigma	MAB1637
TUBB3	N/A	Rabbit polyclonal	1:2000	BioLegend	802001
TUBB (pan)	EPR16774	Rabbit monoclonal	1:200	Abcam, Cambridge, UK	ab179513
TUBA	EP1332Y	Rabbit monoclonal	1:250	Abcam	ab52866
IMPDH1	N/A	Rabbit polyclonal	1:50	Proteintech, Rosemont, IL	22092-1-AP
IMPDH1	F-6	Mouse monoclonal	1:50	Santa Cruz, Dallas, TX	sc-166551
IMPDH2	N/A	Rabbit polyclonal	1:200	Proteintech	12948-1-AP
CTPS1	N/A	Rabbit polyclonal	1:200	Abcam	ab244492
CTPS2	EPR16735	Rabbit monoclonal	1:400	Abcam	ab196016
Ki67	SP6	Rabbit monoclonal	1:100	Abcam	ab16667
Lamin B1	N/A	Rabbit polyclonal	1:200	Abcam	ab16048
Lamin A/C	EPR4100	Rabbit monoclonal	1:250	Abcam	ab108595

## RESULTS

### Morphology

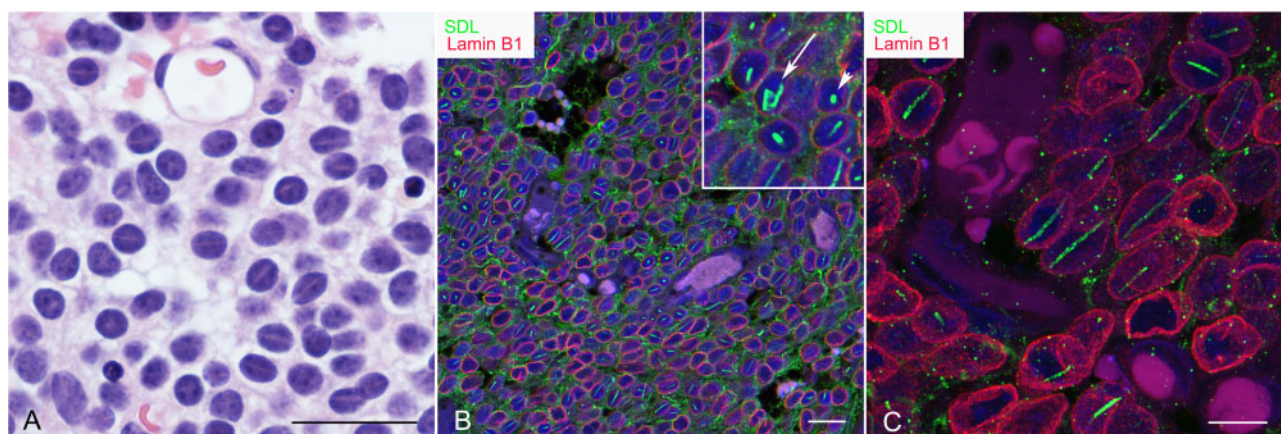
Rod-shaped structures were identified within the nuclei of tumor cells in a large proportion of gliomas. A small number could be seen in hematoxylin and eosin-stained sections where they appeared as a linear “gap” in hematoxylin staining containing a slightly eosinophilic “blush” (Fig. 1A). As reported previously, these rods stained intensely using the SDL.3D10 (SDL) monoclonal antibody generated against TUBB3 (5, 7, 13–15) (Fig. 1B, C). Rods varied in morphology with the majority consisting of long, thread-like structures, in many cases spanning the entire nuclear diameter (Fig. 1B, C). Most rod-bearing nuclei contained a solitary rod although rare nuclei showed 2 or even 3 rods. Longer rods were oriented parallel to the long axis of nuclei (Fig. 1C). In addition to the more common straight linear forms, curved linear structures, dot-shaped structures (Fig. 1B, arrowhead and inset), and sheets (Fig. 1B, arrow and inset) were observed. Many

appeared to be “hollow” with respect to SDL staining; showing a “bilaminar” appearance in longitudinal, and annular in transverse sectional planes.

By electron microscopy, when viewed in the longitudinal plane (Fig. 2A–C) rods consisted of compact, parallel arrays of slightly wavy electron dense filaments measuring from 6 to 10 nm in diameter. When viewed in the transverse plane, they appeared as circular arrays of short, slightly wavy filaments (Fig. 2D–F).

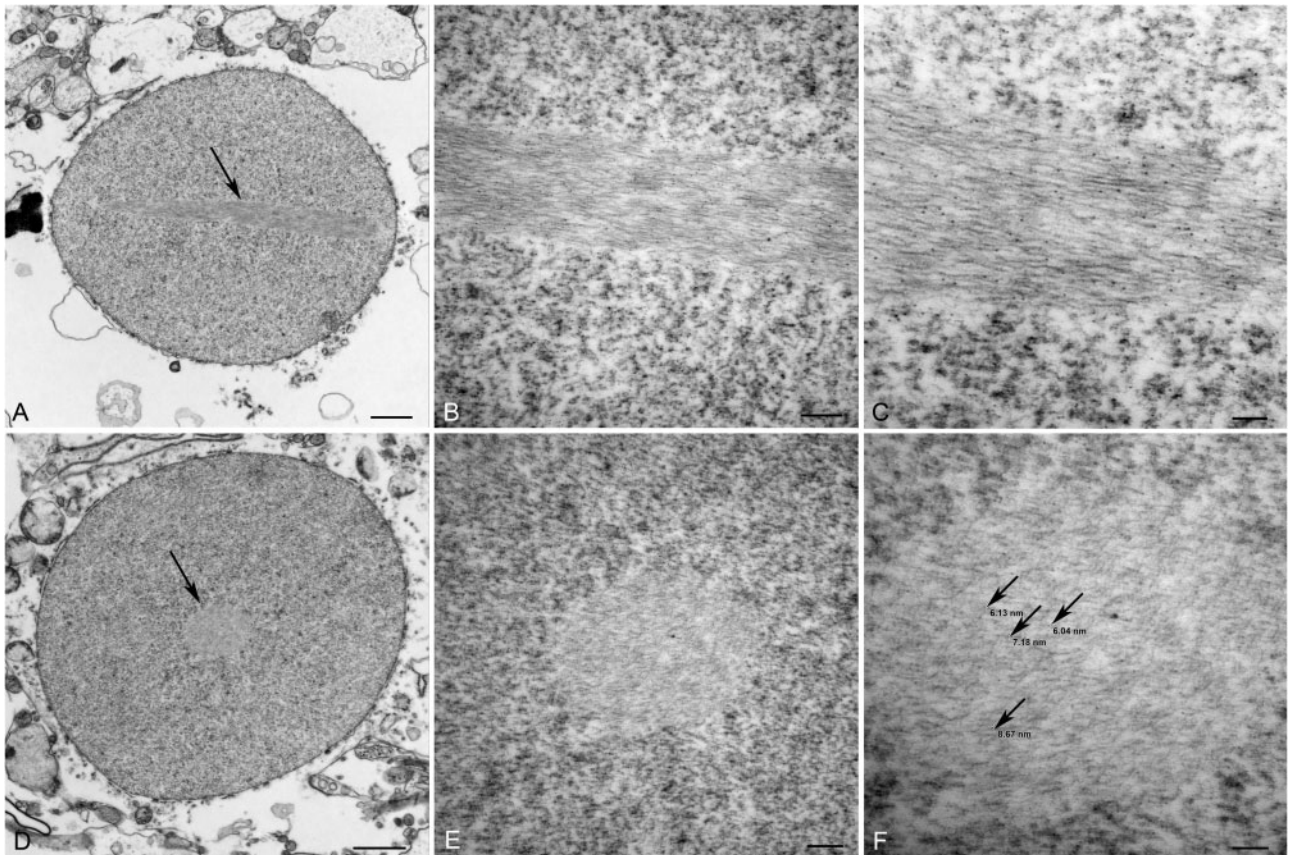
### Rods Are Prevalent in IDH Mutant Oligodendrogliomas and Astrocytomas, but Not IDH Wild-Type GBMs

Overall, rods were present at various frequencies (0.4%–37% of tumor cells) in 71.1% of IDH mutant lower-grade gliomas and 13.7% of IDH wild-type gliomas (all GBMs). We employed a semiquantitative scale to assess the frequency of nuclear rods using 5% and 10% to define 4 categories with 0,



**FIGURE 1.** Rod morphology. **(A)** Rods in this oligodendroglioma are visible as hematoxylin-poor, slightly eosinophilic linear zones in hematoxylin and eosin-stained sections of some tumors. **(B)** Confocal image showing double immunofluorescence staining for SDL (green) and lamin B1 (red) in an oligodendroglioma. The majority of rods are linear. Occasional paracrystalline sheets (arrow shows a curved sheet) and dot-shaped (arrowhead) morphologies are seen (see inset). **(C)** Three-dimensional reconstruction of a confocal z-stack showing rods in an oligodendroglioma at higher magnification. Rods are oriented parallel to the long axis of nuclei. Scale bars: **A** = 25 μm; **B** = 20 μm; **C** = 10 μm.





**FIGURE 2.** Rod ultrastructure. Electron micrographs showing rods in longitudinal (**A–C**; arrow in **(A)**) and transverse (**D–F**; arrow in **(D)**) planes within nuclei of an oligodendrogloma. The rods consist of parallel arrays of slightly wavy electron dense filaments. Scale bars: **A, D** = 1  $\mu$ m; **B, E** = 200 nm; **C, F** = 100 nm.

low (0–5%), moderate (5.1%–10%), and high (>10%) prevalence. The results for the entire cohort are summarized in [Figure 3A and B](#). Rods were significantly more prevalent in oligodendroglomas (78.3%; 81% of grade 2, 76.2% of grade 3) than astrocytomas (59%; 66.7% of grade 2, 50.0% of grade 3) ([Fig. 3](#)). Among grades 2 and 3 oligodendroglomas and astrocytomas, there was no significant correlation between the presence or frequency of rods and tumor grade, sex, or tumor site. There was a significant negative correlation between the frequency of rods and age in oligodendroglomas but no relationship with age in astrocytomas or GBMs. With respect to molecular genetic profile, 1 tumor with an R172K IDH2 mutation had the highest frequency of rods ([Fig. 3C](#)). However, overall, tumors with IDH2 (R172K, R172G) or non-canonical IDH1 (R132C, R132G) mutations did not show significant differences in terms of rod frequency ([Fig. 3C](#)).

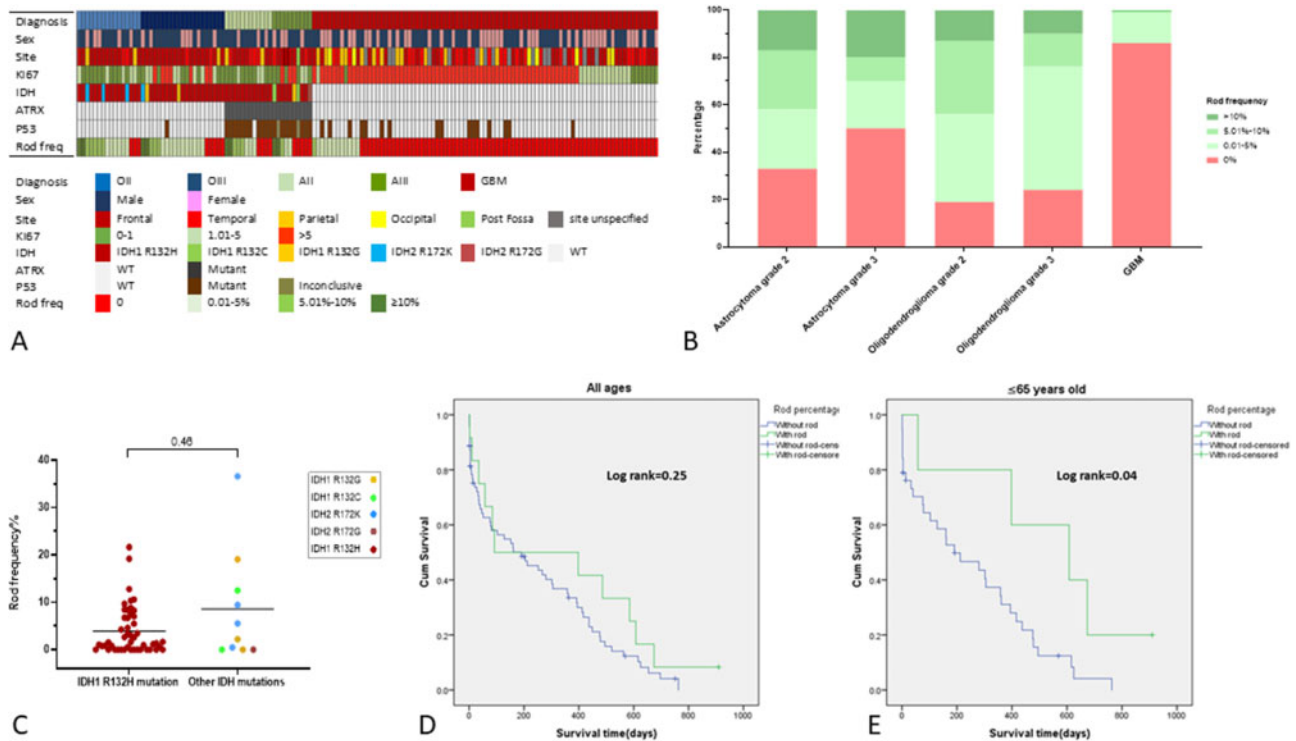
To determine whether the presence of rods conferred any prognostic information, we analyzed postoperative survival and recurrence in 83 IDH wild-type GBM patients with available data. The postoperative follow-up period ranged from 1 to 910 days. The average postoperative follow-up period was 328 days for rod-positive and 210 days for rod-negative patients. The duration of postoperative follow-up did not differ significantly among the 2 groups ( $p = 0.15$ ). Across all age groups, there was no significant difference in postoper-

ative survival among patients with rod-positive and rod-negative tumors ([Fig. 3D](#)). However, when patients were stratified by age, the presence of rods conferred significantly improved postoperative survival in those 65 years of age and below ([Fig. 3E](#)). Stratification by sex revealed no significant effect for rods on postoperative survival.

### Rods Are a Marker of Proliferative Quiescence in Glioma Cells

In light of the above findings, we next investigated the relationship between the presence of rods and glioma cell proliferation. Double immunostaining revealed mutual exclusion of rods and the proliferation marker Ki67 ([Fig. 4A–C](#)). In addition, there was a significant inverse correlation between the frequency of rods and the Ki67 proliferation index in both oligodendroglomas ([Fig. 4D](#)) and astrocytomas ([Fig. 4E](#)). This suggested that rod formation correlates with cellular proliferative quiescence in glioma cells.

Senescence, the irreversible arrest of cell proliferation ([16](#)), represents an important stage in the malignant evolution of glioma cells. To begin to investigate a possible relationship between rod formation and senescence, we performed double labeling for rods using SDL and lamin B1. Lamin B1 is an intermediate filament protein which, along with lamin A/C,



**FIGURE 3. (A)** Oncoprint summarizing demographic features, tumor localization, Ki67 proliferation index, IDH/ATRX/p53 mutation status, and rod frequency (freq) for our entire adult diffuse glioma cohort. **(B)** Bar graph showing the relative proportion of oligodendrogliomas, astrocytomas, and GBMs containing rods. Rod frequency is illustrated semiquantitatively using 5% and 10% cutoffs into 3 categories represented by increasingly darker shades of green (pink = no rods). **(C)** Dot plot comparing rod frequencies among gliomas with IDH1R132H mutations and those with IDH2 and noncanonical IDH1 mutations. **(D, E)** Kaplan-Meier analyses comparing postoperative survival among subjects with rod-positive (green) versus rod-negative (blue) IDH wild-type glioblastomas. There was no significant difference in survival among subjects of all ages whereas those 65 years of age and younger with rods exhibited significantly improved survival **(E)**.

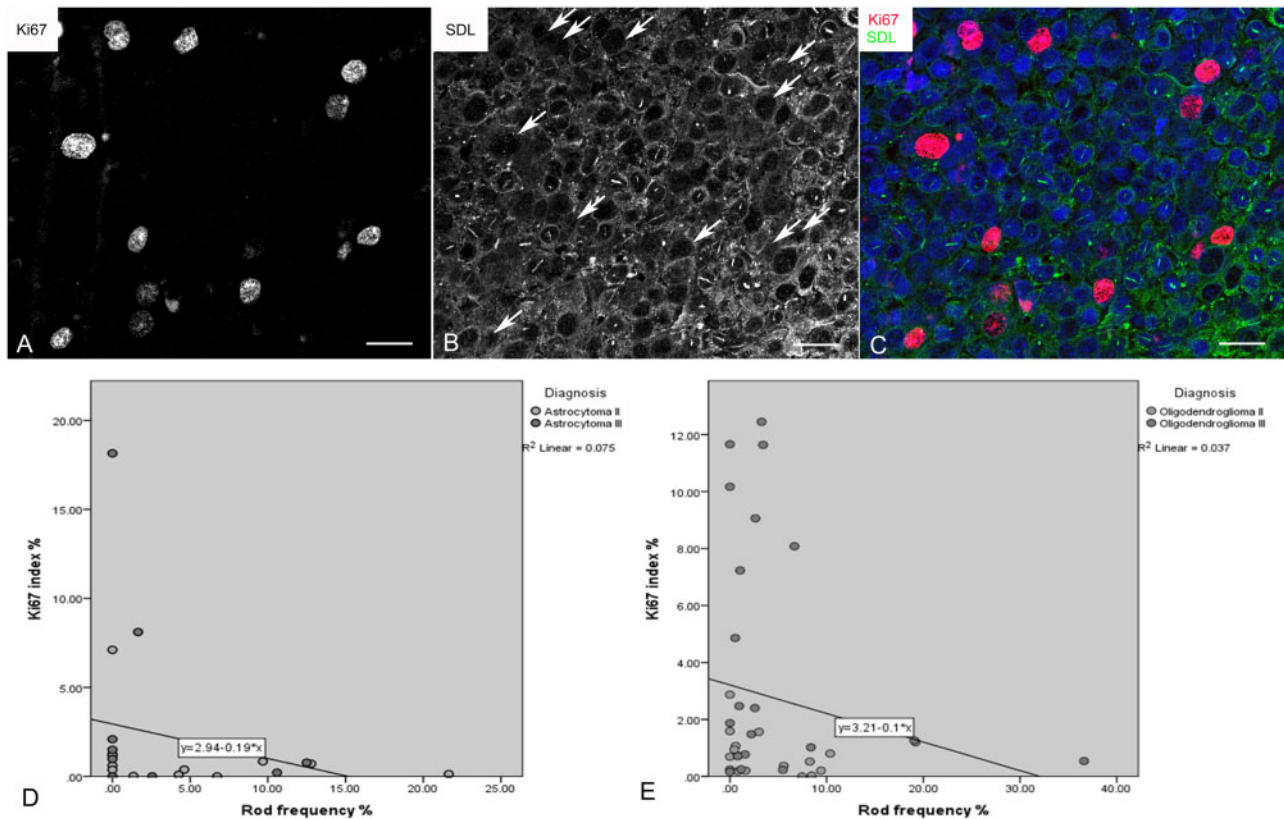
forms a meshwork lining the inner nuclear membrane. Reduced nuclear membrane staining for lamin B1 has become an established marker of senescence (17). The intensity of nuclear membrane lamin B1 staining was significantly reduced in rod-containing nuclei relative to their negative counterparts (Fig. 5A, B). This phenomenon was selective for lamin B1 as there was no similar reduction for lamin A/C (Fig. 5C, D).

### Antigenicity: Rods Are Related to IMPDH Filaments

In order to gain insight into the functional significance of rods in glioma cells, we investigated their antigenic properties, focusing first on the specificity of SDL staining for TUBB3. The SDL monoclonal antibody was generated against a synthetic peptide (CESESQGPK) corresponding to the c-terminal tail of TUBB3 (18). However, rods did not stain for other anti-TUBB3 monoclonal antibodies, including the TUI-1 and TU-20 clones, which were generated against an almost identical C-terminal isotype-defining epitope of the protein. They also failed to stain with a polyclonal anti-TUBB3 antibody (Fig. 6A-C) as well as an antibody to pan  $\beta$ -tubulin (Fig. 6D-F). These results suggested that SDL recognizes something other than TUBB3 in rods. To investigate this, we

performed immunostaining studies on the brains of *Tubb3*-null mice. These mice were generated by deleting exon 4, which represents over 80% of the TUBB3 gene and contains the isotype-defining c-terminus, including the SDL epitope (11). Not only were rods detected in neurons of *Tubb3*-null brains using SDL, but the rods were more intensely stained than those in control brains (Fig. 6G, H). These results indicate that the SDL antibody recognizes something other than TUBB3 in rods.

There are 2 isoforms of IMPDH (1 and 2) that share 84% sequence homology, both of which have been described in IMPDH filaments. SDL-immunoreactive intranuclear rods stained for IMPDH using 3 different anti-IMPDH antibodies including a rabbit polyclonal antibody targeting amino acids 170–235 of IMPDH1 (Fig. 7A-C; specificity knockdown validated in [19]) and a rabbit polyclonal antibody targeting amino acids 1–350 of IMPDH2 (specificity knockdown validated in [19, 20]). Lighter staining was observed with a mouse monoclonal antibody targeting amino acids 380–410 and recognizing both IMPDH1 and 2. Due to the significant sequence homology among the 2 isoforms, cross-reactivity of the antibodies with the alternate isoforms could not be excluded. Consequently, it was not possible to determine whether IMPDH filaments were comprised exclusively of IMPDH1, 2, or were



**FIGURE 4.** Inverse relationship between rods and proliferation. **(A–C)** Confocal multichannel image showing Ki67-immunoreactive nuclei **(A)** lacking rods **(B; arrows indicate Ki67-positive nuclei shown in A)**. The merged pseudocolored image is shown in **C**. Scale bars = 20  $\mu$ m. **(D, E)** Regression analyses showing inverse correlation between Ki67 proliferation index and rod prevalence in astrocytomas **(D)** and oligodendrogliomas **(E)**.

a mixture of both. Consistent with reports of a nuclear localization of IMPDH, a proportion of glioma cell nuclei also showed diffuse nuclear staining (21). This was especially strong using the rabbit polyclonal anti-IMPDH2 antibody. IMPDH filaments have been described in close association with independent filaments comprised of the pyrimidine synthesizing enzyme CTPS (22). Like IMPDH, CTPS exists in 2 isoforms which share 74% sequence homology. A proportion of SDL-immunoreactive rods showed immunostaining for CTPS using a rabbit monoclonal antibody generated against a 13 amino acid synthetic peptide between amino acids 50 and 100 of CTPS2 (Fig. 7D–F). CTPS staining appeared to be confined to larger rods. These results suggest that rods are related to nuclear forms of IMPDH and mixed IMPDH and CTPS filaments.

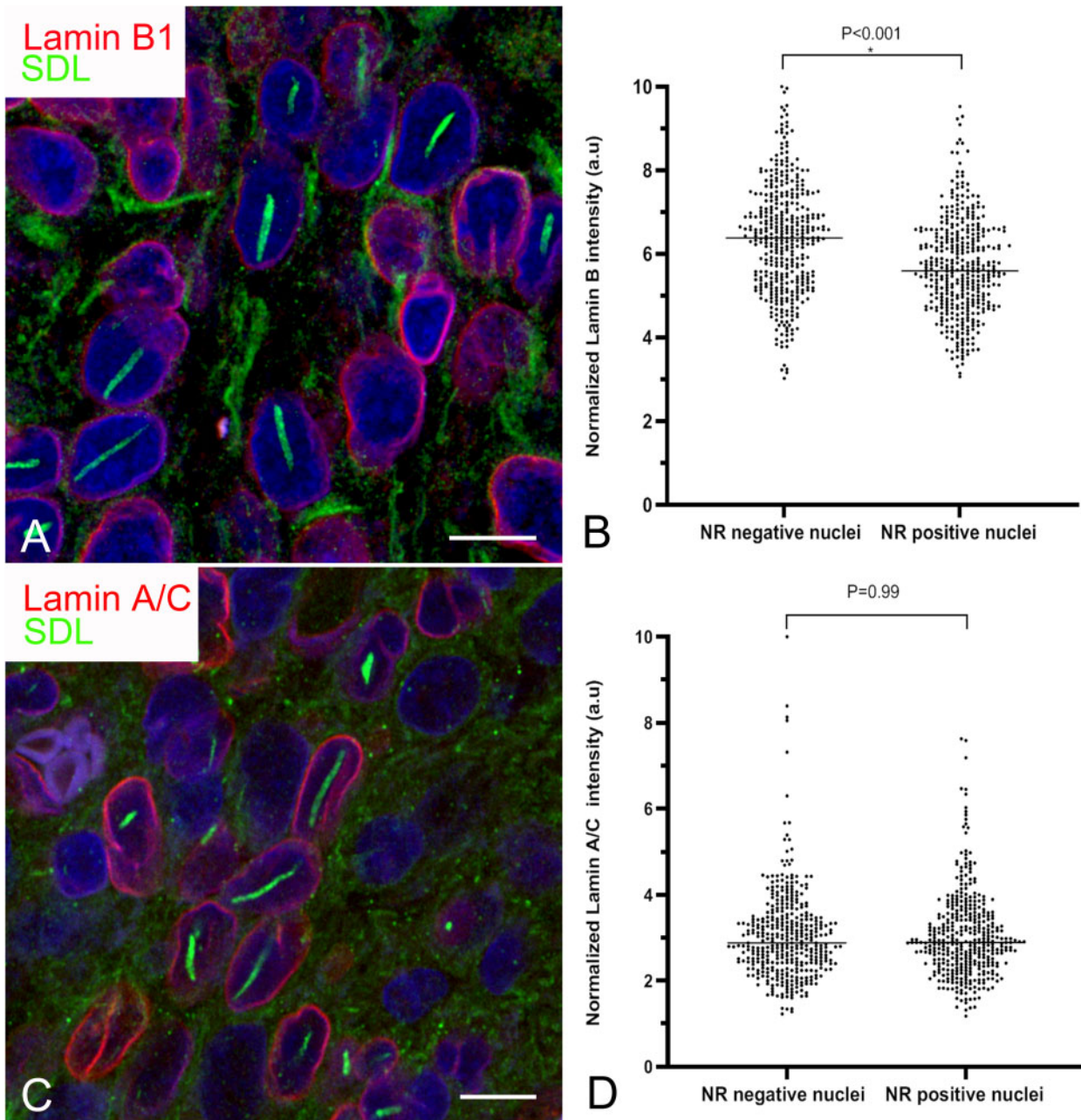
## DISCUSSION

In the present study, we extend our previous observations in ependymomas to demonstrate the presence of intranuclear SDL-immunoreactive rods in adult diffuse gliomas. In our original study of these structures in the human brain, rods were not present in astrocytes or oligodendrocytes. Thus, their presence in the neoplastic counterparts of these cells indicates that they may have an important role in gliomas.

Using a well-characterized TUBB3-null mouse model, we confirmed that the SDL-immunoreactive moiety in rods is not TUBB3. Instead, rods are immunoreactive for IMPDH, the rate-limiting enzyme in the *de novo* synthesis of GTP and ATP nucleotides. IMPDH catalyzes the NAD-positive-dependent oxidation of IMP to xanthine monophosphate (23). This pivotal step in GMP synthesis controls the size of the guanine nucleotide pool. Thus, IMPDH is a key enzyme for genome replication and cell proliferation. IMPDH activity and GTP abundance are transformation and progression linked in cancer cells (24, 25). Indeed, GTP levels and IMPDH activity are markedly increased in GBM (24, 25). In addition to their role as building blocks to support DNA replication and cell division, purine nucleotides are involved in myriad metabolic and cell signaling functions.

Metabolic regulation via filamentation has been described for an increasing number of enzymes (26). IMPDH filament formation has been implicated as a mechanism to regulate its activity, the direction of which is influenced by substrate availability, allosteric feedback inhibition by nucleotide products, and the identity of the enzyme isoform (27). At least 1 study has linked filament formation to increased enzymatic activity (28). IMPDH filaments have been described predominantly in the cytoplasm where their formation can be potently induced by a variety of stimuli, including pharmaco-



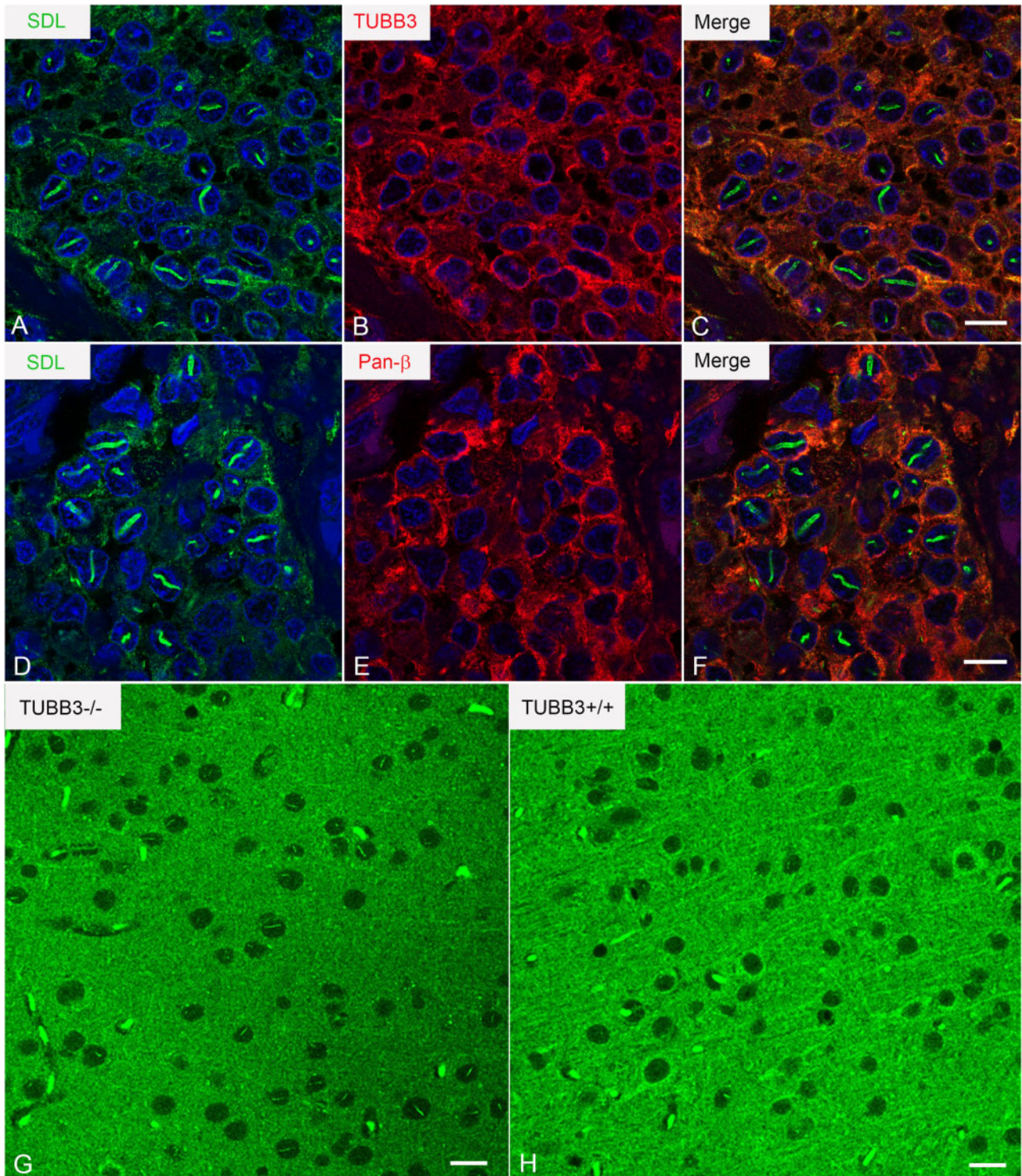


**FIGURE 5.** Rods are associated with selective depletion of lamin B1. **(A, C)** Confocal images of an oligodendrogloma showing visibly reduced lamin B1 **(A;** red) but not lamin A/C **(C;** red) staining intensity in cells with rods (green). Scale bars = 10  $\mu$ m. **(B, D)** Grouped scatter plots of pooled data from astrocytomas and oligodendrogliomas showing significantly reduced staining intensity for lamin B1 **(B)** but not lamin A/C in **(D)** cells with nuclear rods (NR).

logical inhibitors (reviewed in [9, 10]). Nuclear forms have also been described, although their functional significance is less well studied. Based on their morphological similarity as well as their immunoreactivity for IMPDH and CTPS, we postulate that nuclear rods in glioma cells are related in some way to nuclear IMPDH filaments. It will be important to identify the SDL-immunoreactive antigen in this context as an increasing number of proteins have been identified in IMPDH filaments, including the small GTPase ARL2 complexed with the

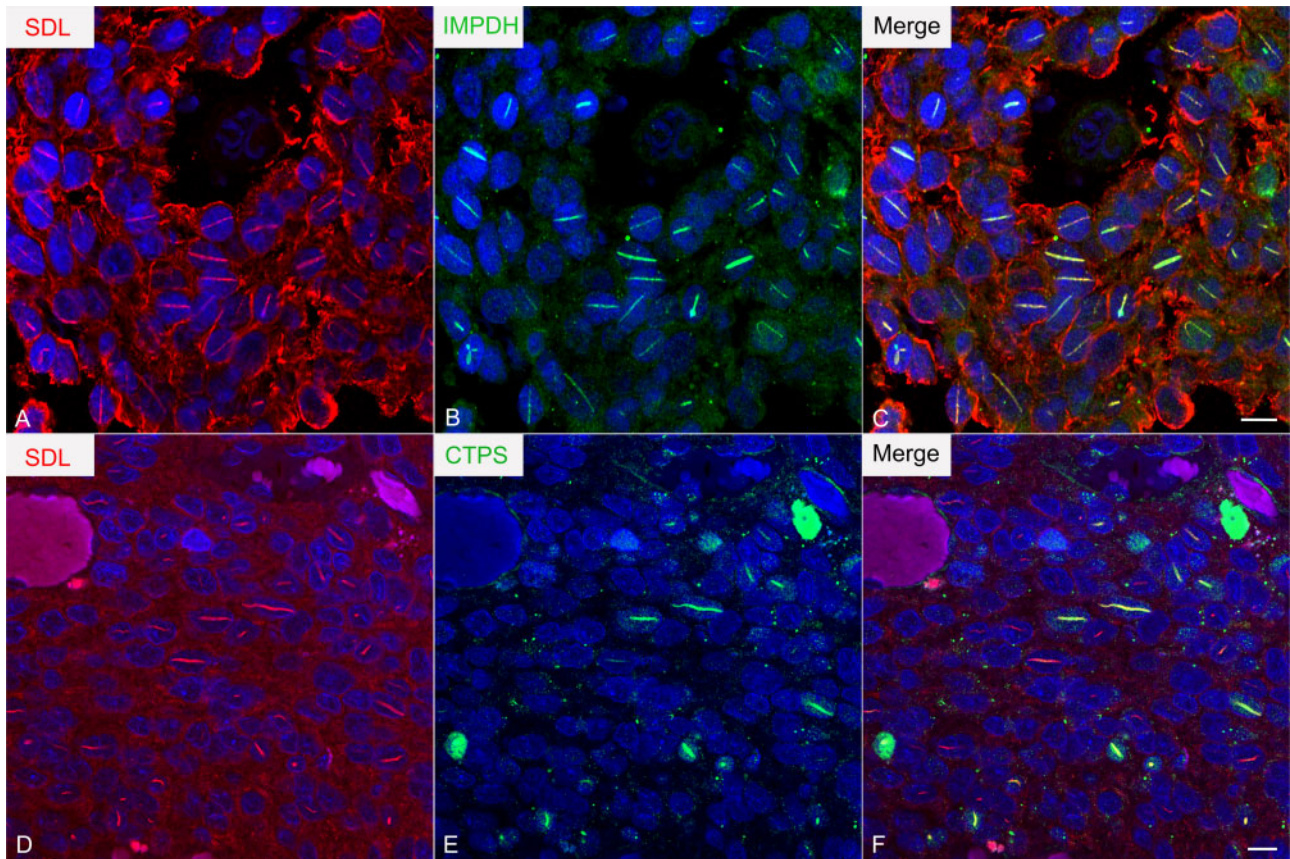
tubulin-binding cofactor D, suggesting possible roles for these structures beyond nucleotide regulation, including microtubule dynamics (29). It is also possible that IMPDH- and SDL-based filaments are independent entities but are physically juxtaposed. This has been described for IMPDH- and CTPS-based filaments and may reflect a mechanism for the coordinated regulation of purine and pyrimidine synthesis (30). The presence of IMPDH in rods has important implications for understanding the associations we have demonstrated in this





**FIGURE 6.** Rod antigenicity: SDL does not recognize TUBB3 in rods. **(A–F)** SDL-immunoreactive rods **(A, D;** green) show no staining for TUBB3 using an anti-TUBB3 polyclonal antibody **(B;** red) or pan- $\beta$ -tubulin using a rabbit monoclonal antibody **(E,** red). Merged images in **C** and **F**. **(G, H)** SDL stains rods in *Tubb3*-null mice. Confocal images of the mesial frontal cortex of *Tubb3*<sup>-/-</sup> **(G)** and *Tubb3*<sup>+/+</sup> **(H)** mice immunostained using SDL.3D10. Scale bars: **C, F** = 10  $\mu$ m; **G, H** = 15  $\mu$ m.





**FIGURE 7.** Rods are immunoreactive for IMPDH and CTPS. Double immunofluorescence of an oligodendrogloma showing colocalization of IMPDH (**A**; green) and CTPS (**D**; green) with SDL (**B**, **E**; red) in rods. Merged images in **C** and **F**. Scale bars = 10  $\mu$ m.

study, including that between rods and IDH mutant gliomas, reduced proliferation, and increased survival in a subset of GBM patients.

The prevalence of rods in oligodendrogliomas versus astrocytomas and GBMs may be related to differential metabolic profiles. Specifically, as a critical substrate in the *de novo* purine nucleotide synthetic pathway, it is not surprising that changes in the concentration of glutamine markedly influence IMPDH filamentation, with glutamine depletion and supplementation inducing and reversing filament formation, respectively (31). This is compelling in the context of our previous studies showing rods in human pancreatic beta cells which were significantly reduced in diabetic subjects (32) as glutamine is present in high concentrations in  $\beta$  cells and is a powerful insulin secretagogue (33). Interestingly, this effect is dependent on IDH (34). Glutamine is also essential for cancer cells to fuel nucleotide and nucleic acid synthesis for proliferation. Indeed, the reliance of malignant cells on glutamine (glutamine “addiction”) has been implicated as a therapeutic target (35). In this context, rod formation may represent a glutamine-related metabolic signature in gliomas. Consistent with this, the ordinal prevalence of rods (oligodendrogliomas > astrocytomas > GBMs) correlates with relative glutamine levels among these tumor types. Relative to IDH wild-type GBMs, IDH mutant gliomas show suppressed levels of gluta-

mate and in turn are more reliant on glutaminase, which catalyzes the production of glutamate at the expense of intracellular glutamine (36). In addition, there are significant differences in the metabolic signatures among oligodendrogliomas and astrocytomas, including glutamine concentrations (37). Significant metabolic differences have also been described among IDH mutant gliomas with R132H versus non-canonical or IDH2 mutations (38). With the evolution of an increasingly granular subclassification of adult diffuse gliomas, it will be interesting to determine whether the presence of rods characterizes novel metabolic subclasses across molecular groups. Along the same lines, determining whether rod-positive and -negative gliomas form distinct subclusters within individual glioma groups using epigenetic (methylation) profiling may be informative.

Our study provides evidence for an inverse relationship between nuclear rod formation and glioma cell proliferation. That rod prevalence decreases along the established hierarchical scale of increasingly aggressive tumor behavior (oligodendrogloma > astrocytoma > GBM) is consistent with this. Although the significance of nuclear IMPDH filaments is unknown, IMPDH has been shown to accumulate in the nucleus in a cell cycle-dependent manner where it “moonlights” as a transcription factor to influence proliferation in either direction (21). Interestingly, nuclear IMPDH attenuates expression

of histone genes and E2F, a key driver of cell proliferation (21). E2F plays a central role in the induction of senescence (39–41). By influencing IMPDH activity in the nucleus through physical sequestration, rod formation may reflect an intranuclear regulatory mechanism impacting cell proliferation. The relationship between rods and lamin B1 depletion, an established morphological marker of senescence (42), is interesting in this regard and suggests a possible role for nuclear rod formation in senescence. These findings also have implications for glioma prognosis. Consistent with their correlation with reduced proliferation, the presence of rods portended a significantly more favorable prognosis in terms of postoperative survival for patients with IDH wild-type GBM who were under 65 years of age at the time of surgery. The absence of an association in elderly patients may reflect the presence of age-associated comorbidities contributing to mortality.

This study has potential therapeutic implications. IMPDH has a long history as an attractive antineoplastic target (43). Notably, IMPDH-targeting drugs have been demonstrated to influence IMPDH filament formation (9). The development of next generation, more efficacious IMPDH-targeting drugs is ongoing and is predicated on a more nuanced understanding of IMPDH regulation in glioma cells (43). A salient example is the recent study by Shireman et al (44) who demonstrated an interaction between IMPDH and the ciliary GTPase ARL13B, a negative regulator of purine salvage. Inhibition of this interaction using the FDA approved drug mycophenolate mofetil (MMF) enhanced temozolomide-induced DNA damage by forcing cells to rely on purine salvage. The authors suggested that MMF and temodol may be effective as a combination therapy. In addition to providing insight regarding mechanisms of action, assessment of rod prevalence in resected tissue specimens may provide information regarding the response to treatment. Our findings may also have implications for the development of anti-glioma immunotherapies. In hepatitis patients treated with the antiviral drug ribavirin, IMPDH filaments form in peripheral blood mononuclear cells. Remarkably, drug-induced filament formation in these patients initiates a robust autoimmune response with the development of autoantibodies targeting IMPDH (45). It will be interesting to determine whether patients with rod-bearing gliomas harbor such autoantibodies and whether this autoantigenic property can be leveraged for the development of novel anti-glioma immunotherapies.

We have described a novel morphological feature in human adult diffuse gliomas. Future studies will provide mechanistic insight regarding the functional significance of rods and their role in IMPDH regulation and glioma behavior. In addition to informing future fundamental studies, the results of our studies have clinical relevance. Our study indicates that rods represent a morphological marker of reduced glioma cell proliferation and that their presence in glioma specimens has prognostic value in patients with GBM. Thus, evaluation of rods in resected glioma tissue may supplement the diagnostic arsenal available to neuropathologists to convey prognostic information to treating clinicians. Further understanding of the protein composition and functional significance of rods in

gliomas, as well as the immune response to these structures, could foster the development of novel therapeutic approaches.

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