Supplementary Information

Figure S1: *Conservation of the DSL motif in fibulin-3 and canonical Notch ligands*. A) Representation and alignment of canonical Notch ligands of the Delta and Jagged families together with fibulin-3 (*Fib-3*). B) Alignment (using ClustalW software) of the N-terminal sequences containing the Delta-Serrate-Lag (*DSL*) motif involved in Notch activation. Fibulin-3 contains 8 of the 11 aminoacids that form the DSL consensus. This sequence is highly conserved in fibulin-3 across species. *b*: basic aminoacid; *a*: acidic aminoacid. Aminoacids in red indicate conservation of the DSL residues between fibulin-3 and the Notch ligands while those in green show divergence from the consensus in fibulin-3 or in the Notch ligands themselves. *VWFC:* vonWillebrand Factor consensus sequence; *EGF:* epidermal growth factor; *aa:* aminoacid.

Figure S2: *Specificity and efficacy of siRNAs against fibulin-3 and Notch-1*. SiRNA sequences against human and rat fibulin-3 have been previously described (Hu et. al., 2009). Notch-1 (*N1*) validated siRNAs were purchased from Qiagen. A-B) Targeting siRNAs (two per target gene) were tested alone or combined. Knockdown of each target gene was quantified by qRT-PCR (A) and Western blotting (B) in rat (*CNS1*) and human (*G8*) cells. The non-targeting sequence *AllStars* siRNA (Qiagen) was used as negative control.

Figure S3: *Conditional knockdown of fibulin-3*. A) Lentiviral design used to induce fibulin-3 shRNA. The chimeric repressor TetR-KRAB targets the hybrid promoter TetOp-U6 and causes epigenetic silencing of a 2-3 kilobase region, preventing expression of shRNA and eGFP. This repressor is inactivated by Doxycycline (*Dox*). B) U251MG cells stably transduced with a control lentivirus were exposed to 20 µg/ml Dox (or vehicle) for 48h, fixed and stained with propidium iodide (red). Results show the efficient induction of the system. Bar: 100 µm. C)

Glioma cells stably transduced with control or fibulin-3-targeted lentiviruses were cultured in presence or absence of Dox during 48 h. Results from qRT-PCR demonstrate efficient knockdown of fibulin-3 mRNA (mean ± SD: *** $p<0.001$, two-way ANOVA for each cell type). D) Western blotting of serum-free conditioned medium from U251MG and CNS1 cells show efficient reduction of secreted fibulin-3 after shRNA induction. *BSA*: bovine serum albumin was added as loading control in the conditioned medium.

Figure S4: *Fibulin-3 is not a Notch target in glioma cells*. Glioma cells (U251 and CNS1) transfected with Notch-1 intracellular domain (NICD), Notch-1 siRNA, or their respective controls were processed for qRT-PCR. Results show that genes typically regulated by Notch (Hes1 and Hes5) responded to upregulation or downregulation of Notch signaling. However, fibulin-3 was not affected by these treatments (mean ± SD: ** $p<0.01$, *** $p< 0.01$, one-way ANOVA for each gene).

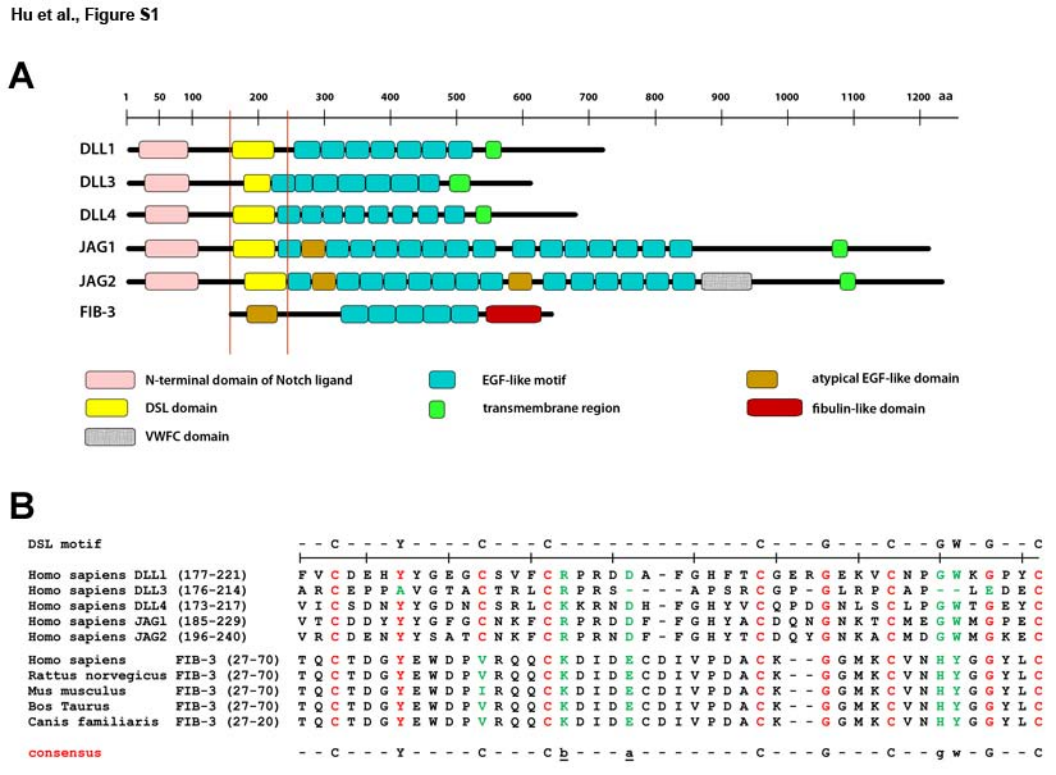
Figure S5: *Fibulin-3 knockdown slows cell growth and increases cell death*. A) CNS1 cells transfected with fibulin-3 or control siRNAs were serum-starved for 24 h, fixed, stained with propidium iodide and analyzed by flow cytometry. Results from a representative experiment (repeated in triplicate) show that fibulin-3 knockdown increased cell death (sub-G1 fraction) in serum-starved glioma cells. B-C) U251MG glioma cells were stably transfected with fibulin-3 or control shRNAs and subjected to antibiotic selection for two weeks. Once selection was completed, cells with stable fibulin-3 knockdown showed reduced viability (B) and clumped morphology over time (C), which prevented the use of these cells for long-term experiments (mean ± SD: ** $p<0.01$, two-way ANOVA).

Figure S6: *Knockdown of fibulin-3 in the tumor downregulates Notch-dependent genes and increases apoptosis*. A) Representative images of a G8-derived tumor transduced with control lentivirus. Nuclei were stained with DAPI and false-colored in red. Notice the invasive tumor cells (arrows) past the bulk of the tumor (dashed line) and the efficient activation of the lentiviral system (eGFP expression). Bar: 50 μm. B) Control and fibulin-3-targeted G8 tumors (N=3/condition) were microdissected and processed for qRT-PCR. Results show significant knockdown of fibulin-3 mRNA in the tumor tissue as well as downregulation of the Notch-dependent genes Hes1 and Hes5 (mean ± SD: * $p<0.05$; ** $p<0.01$ by two-tailed Student's t-test for each gene). C) Cell proliferation (Ki67-positive cells) quantified in CNS1- and G8-derived tumors did not show significant changes after fibulin-3 knockdown. Bar: 100 μm. D) In contrast, TUNEL staining in the same tumors showed a 3- to 4-fold increase in the number of apoptotic figures (arrowheads) after induction of fibulin-3 shRNA. Bar: 50 μm (***$p<0.001$, two-tailed t-test).

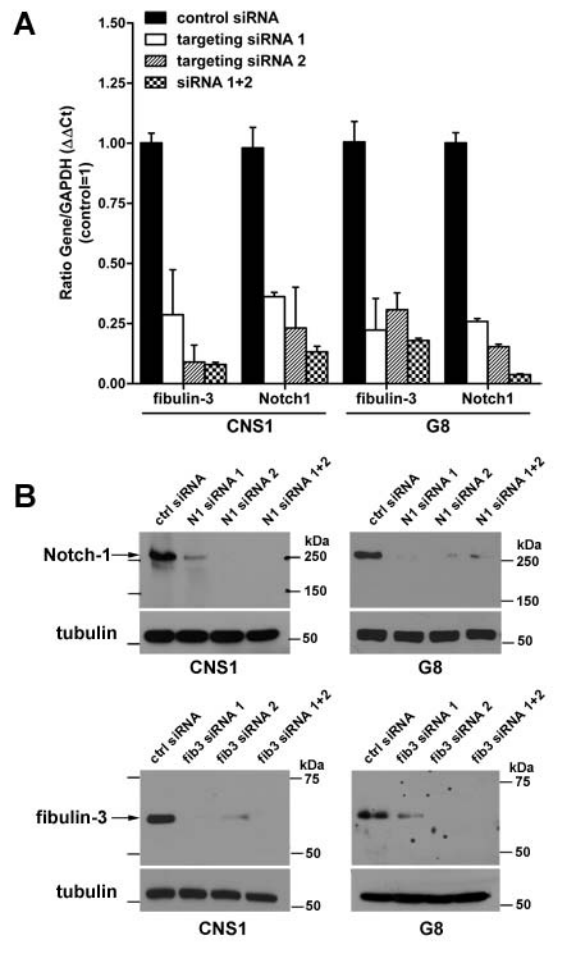
Figure S7: *Fibulin-3 is highly upregulated in high-grade gliomas and associates with the mesenchymal phenotype*. A) Normalized microarray results showing expression of fibulin-3 in glioblastoma, collected from the databases The Cancer Genome Atlas (TCGA) and Repository for Molecular Brain Neoplasia Data (REMBRANDT). Frequency analysis shows that fibulin-3 was upregulated 2-fold or higher over control levels in more than 80% of the samples from both databases. B) Normalized microarray results showing expression of fibulin-3 (probeset 201842_s_at) and fibulin-4 (206580_s_at) in grade III and grade IV gliomas, collected from the Gene Expression Omnibus dataset GSE4271 (49). Tumor groups are 1: WHO grade III primary; 2: WHO grade III recurrent; 3: WHO grade IV primary without necrosis; 4: WHO grade IV primary with necrosis; 5: WHO grade IV recurrent. The center of the bubbles shows the average

expression value in each group; the size of the bubbles represents the number of samples in each group. The results show that, for all tumor groups, fibulin-3 levels were higher in samples matching the mesenchymal signature compared with samples matching the proneural signature. Results were almost identical for fibulin-4.

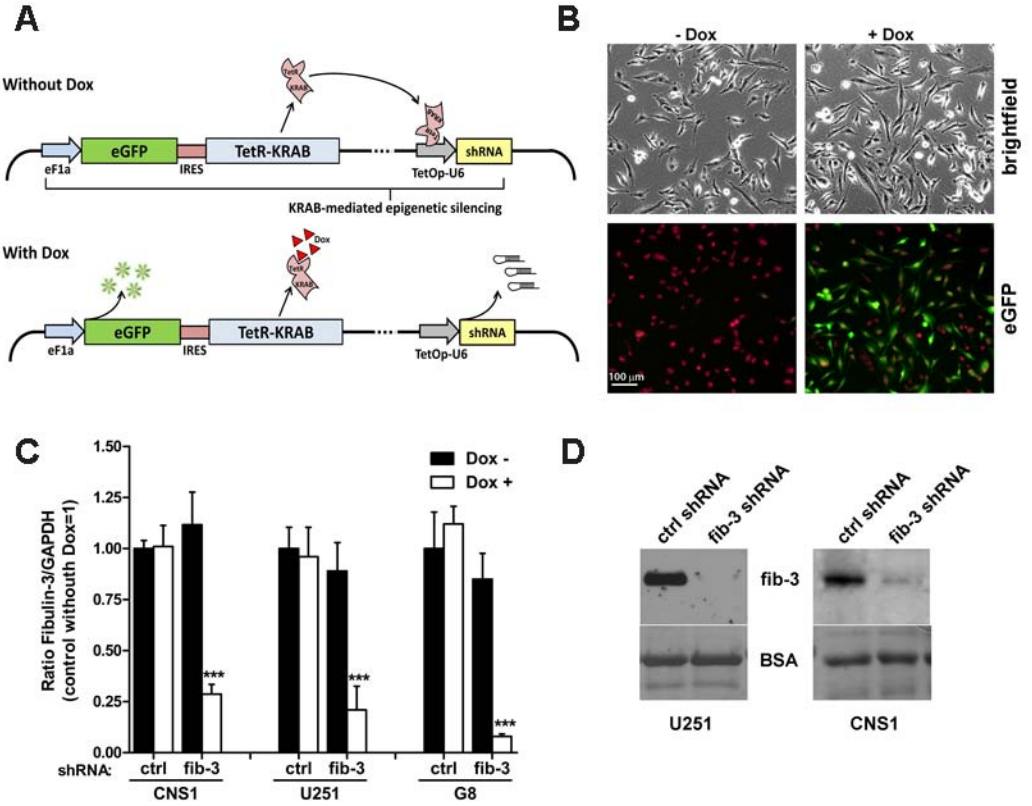
Hu et al., Figure S1



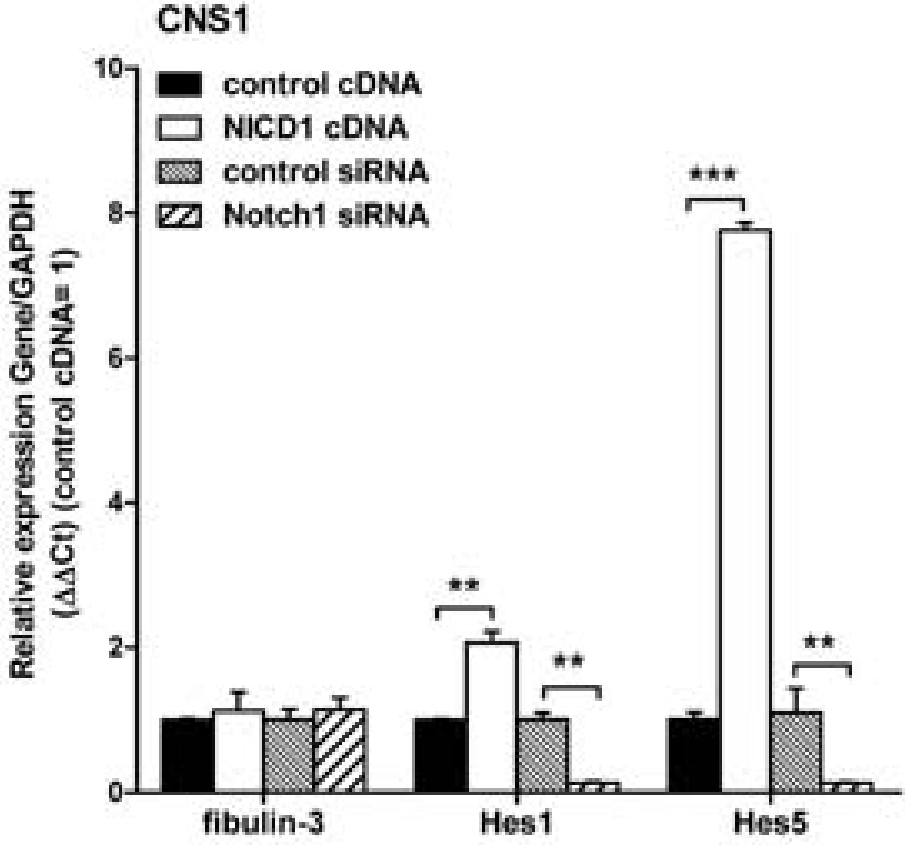
Hu et al., Figure S2

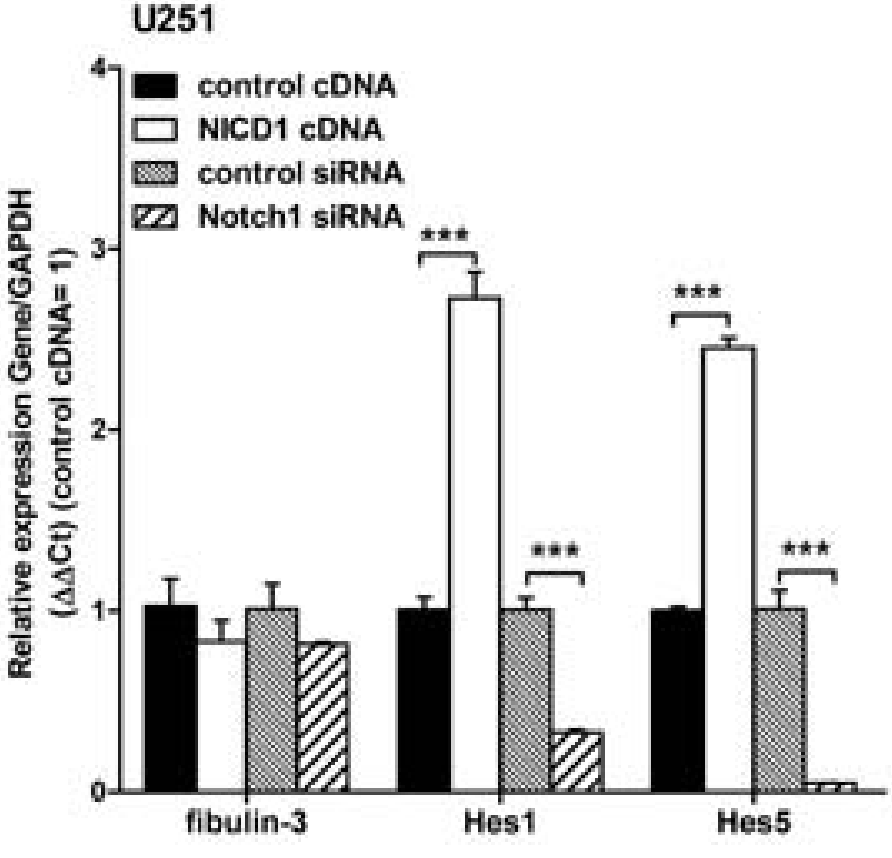


Hu et al., Figure S3

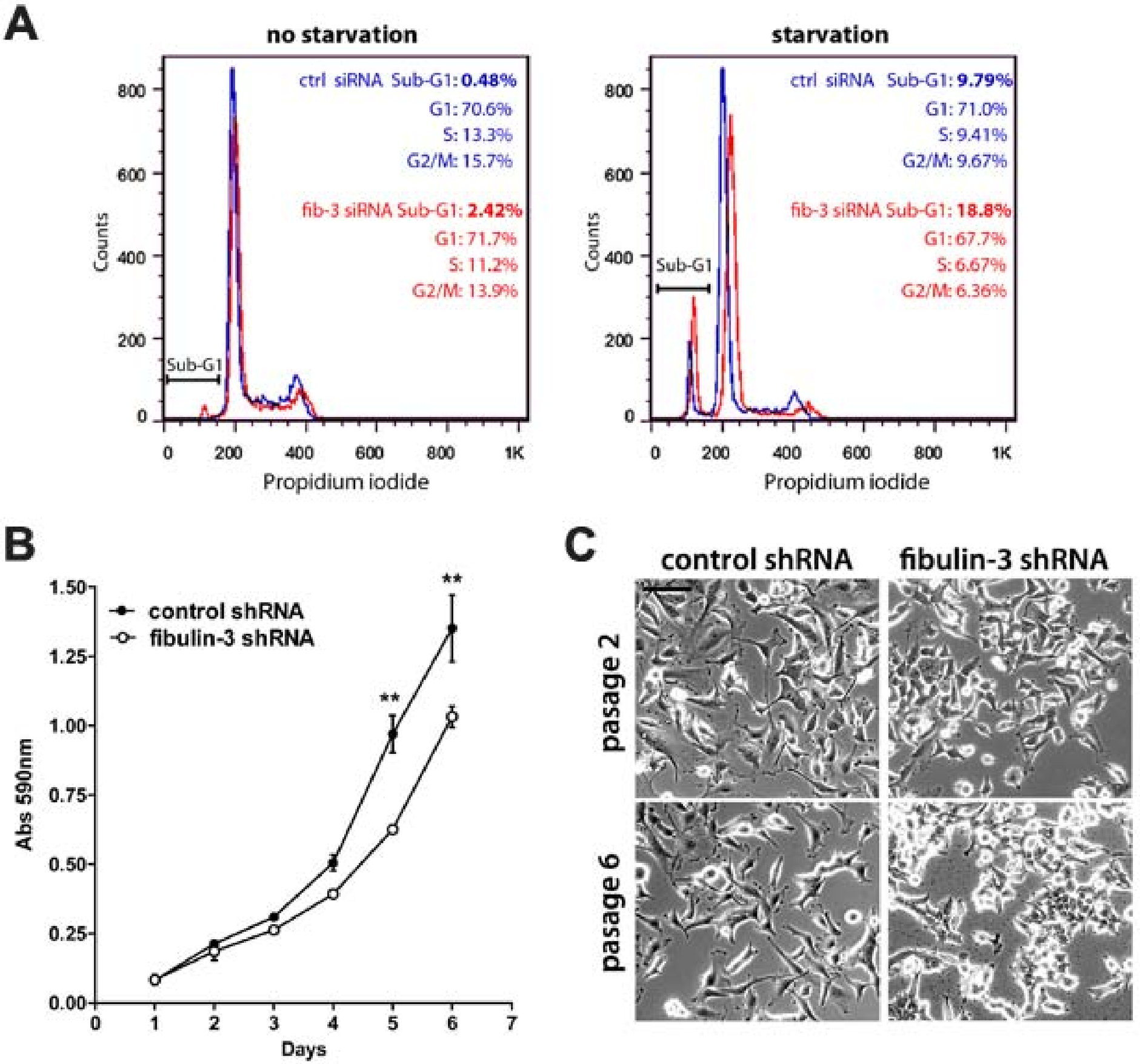


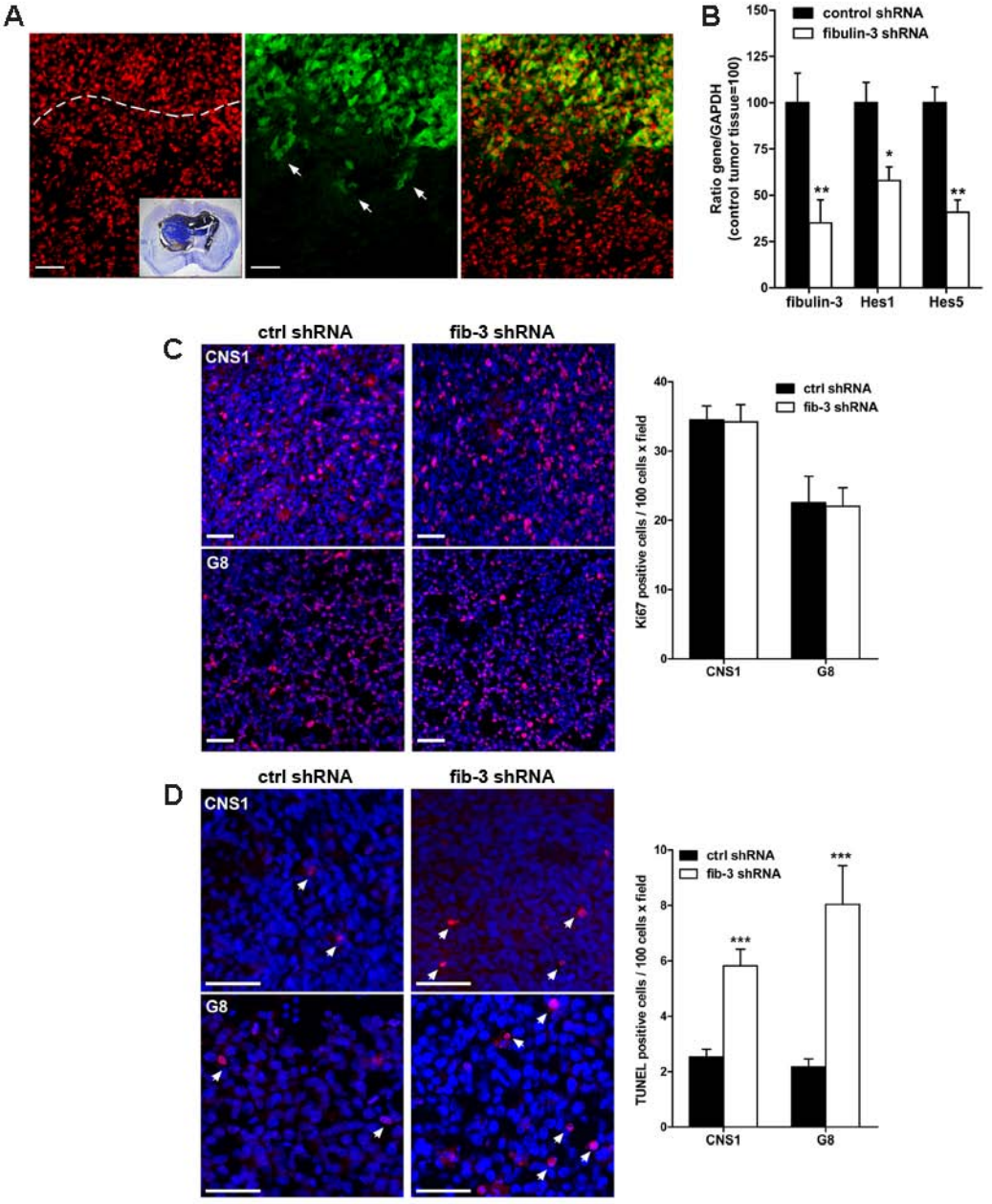
Hu et al., Figure S4





Hu et al., Figure S5




A
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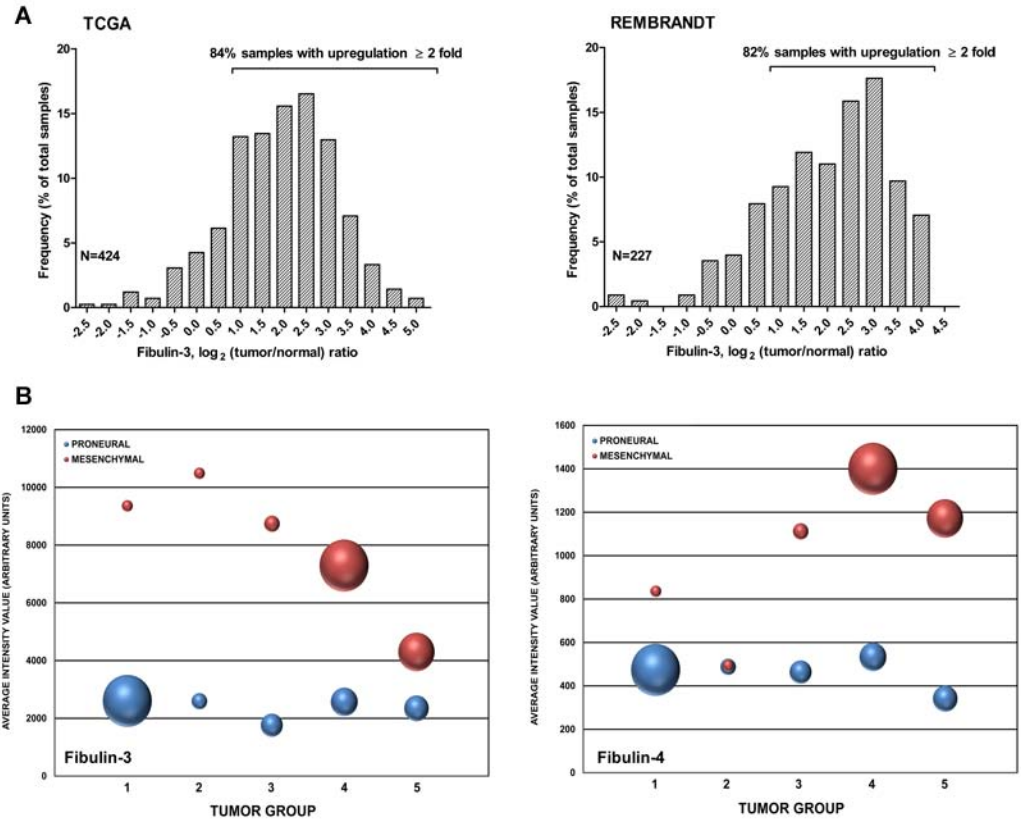
Hu et al., Figure S7



Supplemental Table I:

Antibodies used for Western blotting and immunohistochemistry

Antigen	Source	Catalog number
EGF Receptor	Cell Signaling Technology	2232
$pTyr^{1173}$ EGFR	Cell Signaling Technology	4407
P42/P44 MAPK (Erk 1/2)	Santa Cruz Biotechnology	SC-94
$pThr^{202}/pTyr^{204}$ Erk 1/2	Cell Signaling Technology	4377
Akt	Cell Signaling Technology	9272
$pSer^{473}$ Akt	Cell Signaling Technology	4058
Notch-1 (human)	Cell Signaling Technology, #D6F11	4380
Notch-1 (rat)	Santa Cruz Biotechnology, #8G10	SC-32756
neoepitope Val^{1744}-Notch-1 (NICD)	Cell Signaling Technology	2421 and 4147
Hes1	Santa Cruz Biotechnology	SC-25392
caspase-3	Cell Signaling Technology	9662
Ki67	Thermo Scientific	RM-9106
Fibulin-3 (human)	Santa Cruz Technology, #mab3-5	SC-33722
Fibulin-3 (rat)	Santa Cruz Technology, N-16	SC-46100
alpha/beta tubulin	Cell Signaling Technology	2148