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Supplemental Information

Gravin Is a Transitory Effector of Polo-like Kinase 1 during Cell Division

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Inventory of Supplemental Information:

Supplemental Figure Legends

Figure S1, related to Figure 1: Gravin supresses tumor growth

Representative images of athymic (NU/J) mice implanted with U251 cells expressing (**A**) control shRNA (n=10) and (**B**) shRNA targeting Gravin (n=11). Lower panel shows the same tumors extracted 40 days post-injection.

Figure S2, related to Figure 2: Gravin is phosphorylated by CDK1/Cyclin B

(**A**) Extracted ion currents (XIC) depicting ion mass for the phospho-T766 parent ion from nocodazole arrested mitotic cells (top) and nocodazole arrested mitotic cells treated with roscovitine (bottom). Area under the curve for Gravin = 20,758,340 a.u.; area under the curve for Gravin + roscovitine = 6,965,429 a.u. (**B**) Flag-tagged Gravin or T766A mutant (indicated above each lane) were immunoprecipitated from cycling or mitotic HEK293 cells. Immune complexes were probed with phospho-T766 Gravin antisera (top) and anti-Flag antibodies (bottom).

Supplemental Table ST1, related to Figure 2: Phospho mass spectrometry data

Mass spectrometry report (Excel .xls file) of phospho-peptides identified from mitotic Gravin and mitotic Gravin treated with roscovitine, generated by Scaffold PTM (Proteome software).

Figure S3, related to Figure 3: T766 phosphorylation during mitosis

HEK293 cells were arrested at the G1/S boundary by double thymidine block. Cells were released into fresh DMEM containing nocodazole and harvested every two hours from time 0 to 14 hours (indicated above each lane). (**A**) Samples were immunoblotted with antibodies against (from top to bottom) phospho-T766 Gravin, total Gravin, phospho-Histone H3 and GAPDH (n=3). (**B**) Immunoblot analysis of the same samples using antibodies to the cell cycle

marker proteins (from top to bottom) phospho-T210 Plk1, total Plk1, Cyclin B1, Wee1, phospho-Y15 CDK1 and total CDK1 (n=3).

Figure S4, related to Figure 4: Gravin interacts with the mitotic kinase Plk1

(**A**) HeLa cells were treated either with or without nocodazole prior to Gravin immunoprecipitation and immunoblot analysis for associated Plk1. Immunoblot detection of endogenous Gravin (top) and Plk1 (bottom) show copurification of Plk1 from mitotic cells. Experiments with control (IgG) antibodies are included. (**B-Y**) Rat-2 fibroblasts grown on coverslips were fixed and stained with antibodies against Plk1 (left, green), phospho-T766 Gravin (mid-left, red) and DNA (mid-right, blue). Composite images (right) are included and stages of the cell cycle are indicated.

Figure S5, related to Figure 5: Gravin-Plk1 interaction facilitates cell cycle progression

(**A**) Gravin immune complexes phosphorylated with recombinant Plk1 or CDK1/Cyclin B1 were assessed for binding to a recombinant Plk1 polo-box domain fragment (PBD) harbouring H538A, K540M mutations that disrupt target peptide binding. (**B**) Quantitation of densitometry data in panel A (n=3 ± SEM). (**C**) Immunoblot analysis showing expression levels of wild type and mutant PBD fragments used in figure 5C and S5A. (**D**) Flag-tagged Gravin or various phospho-site mutants (indicated above each lane) were immunoprecipitated from mitotic cell lysates and incubated with recombinant PBD. Immunoblot detection of Gravin (top) and the PBD fragment (bottom) is shown. (**E**) Flag-tagged Gravin, Flag-T766D and Flag-T766E were immunoprecipitated from mitotic cell lysates and incubated with recombinant PBD. Immunoblot detection of phospho-T766 Gravin (top), Gravin (mid) and the PBD fragment (bottom) is shown. (**F**) HeLa cells stably expressing Gravin shRNA were transfected with a mCherry-tagged Gravin construct resistant to knockdown. Immunoblot detection of Gravin (top), mCherry (mid)

and GAPDH (bottom) is shown. (**G**) Representative shRNA-mediated knockdown of Gravin in PC-3 cells used for soft-agar proliferation assays. Immunoblot detection of Gravin (top) and GAPDH (bottom) is shown. (**H**) Stable PC-3 cell lines were generated expressing Flag-Gravin, Flag-T766D and Flag-T766E. Cells were plated in 96-well dishes and proliferation was analysed every 3 days by an MTS assay (n=4 ± SEM, quadruplicate determinations). (**I-R**) HEK293 cells transfected with control or Gravin shRNA were synchronized by double thymidine block, released into fresh media and fixed 8-9 hours later. Immunofluorescence of cells at defined stages of the cell cycle was performed with antibodies against Gravin (left), Plk1 (mid-left), γ-tubulin (mid-right), and composite images (right).

Movie S1, related for Figure 5:

Hela cells stably expressing control shRNA were released from a double-thymidine block into fresh DMEM for 6 hours. Cells were imaged every 20 seconds for 1h on a 63x 1.42 NA objective (Olympus) in a 37oC environmental chamber (Applied Precision/GE Healthcare). Real-time fluorescent detection of Histone 2B-GFP (green) and Plk1-Cherry (red) is shown.

Movie S2, related for Figure 5

Hela cells stably expressing Gravin shRNA were released from a double-thymidine block into fresh DMEM for 6 hours. Cells were imaged every 20 seconds for 1h on a 63x 1.42 NA objective (Olympus) in a 37oC environmental chamber (Applied Precision/GE Healthcare). Real-time fluorescent detection of Histone 2B-GFP (green) and Plk1-Cherry (red) is shown.

Movie S3, related for Figure 5

Hela cells stably expressing control shRNA were released from a double-thymidine block into fresh DMEM for 6 hours. Cells were imaged every 8 minutes for 12h on a 20x 0.75 NA

objective (Olympus) in a 37oC environmental chamber (Applied Precision/GE Healthcare). Real-time fluorescent detection of Histone 2B-GFP and Cherry-Tubulin is shown.

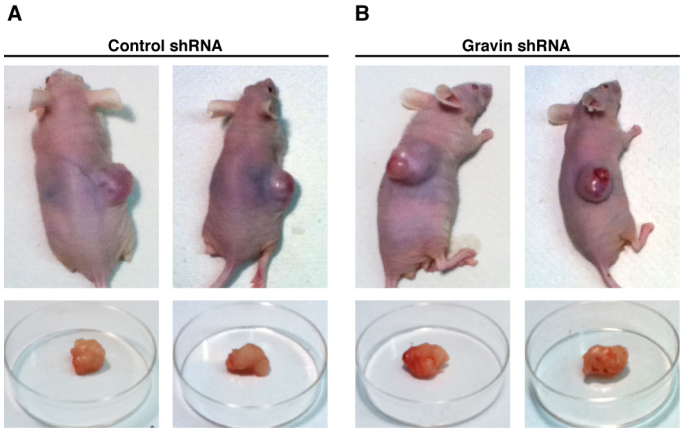
Movie S4, related for Figure 5

Hela cells stably expressing Gravin shRNA were released from a double-thymidine block into fresh DMEM for 6 hours. Cells were imaged every 8 minutes for 12h on a 20x 0.75 NA objective (Olympus) in a 37oC environmental chamber (Applied Precision/GE Healthcare). Real-time fluorescent detection of Histone 2B-GFP and Cherry-Tubulin is shown.

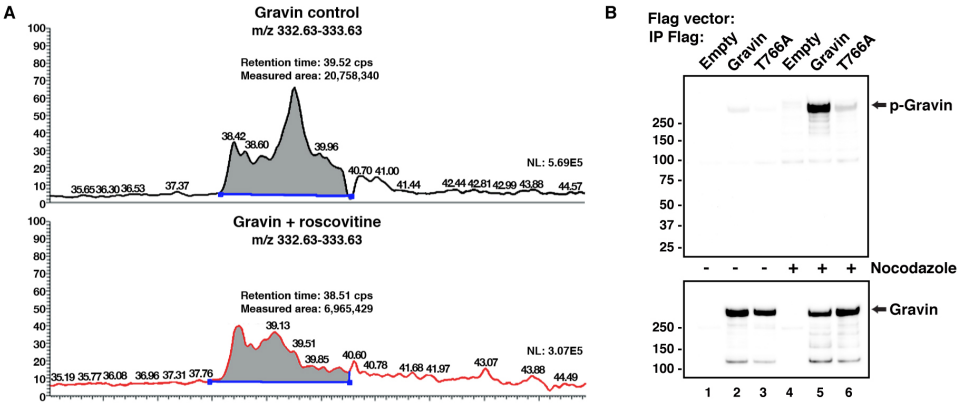
Figure S6, related to Figure 6: Phospho-T766 Gravin and glioblastoma multiforme

H&E stained histological sections from (**A**) control brain tissue and (**B**) glioblastoma showing hypercellularity and pseudopallisading necrosis. (**C & E**) Paraffin-embedded patient sections of healthy brain tissue stained with H&E. (**D**) Immunofluorescence detection of phospho-S10 Histone H3 (red), phospho-T766 Gravin (green) and DAPI (blue) followed by detection with secondary antibodies conjugated to Alexa Fluor dyes. (**F**) Sections co-stained with antibodies against total Gravin (red), phospho-T766 Gravin (green) and DAPI (blue). (**G & I**) Paraffin-embedded sections from glioblastoma tissue stained with H&E. (**H**) Equivalent pathological sections co-stained with antibodies against phospho-S10 Histone H3 (red), phospho-T766 Gravin (green) and DAPI (blue). (**J**) Additional sections co-stained with antibodies against total Gravin (red), phospho-T766 Gravin (green) and DAPI (blue).

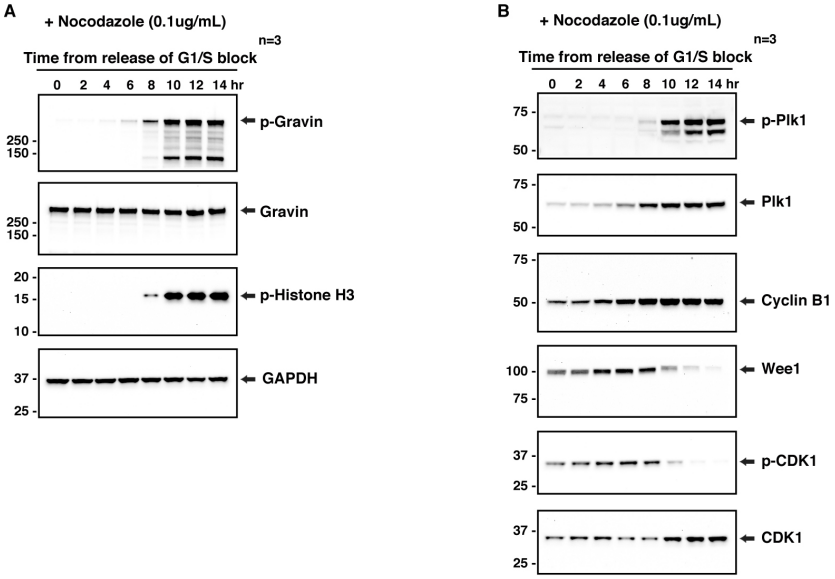
SUPPLEMENTAL FIGURE 1



SUPPLEMENTAL FIGURE 2



SUPPLEMENTAL FIGURE 3



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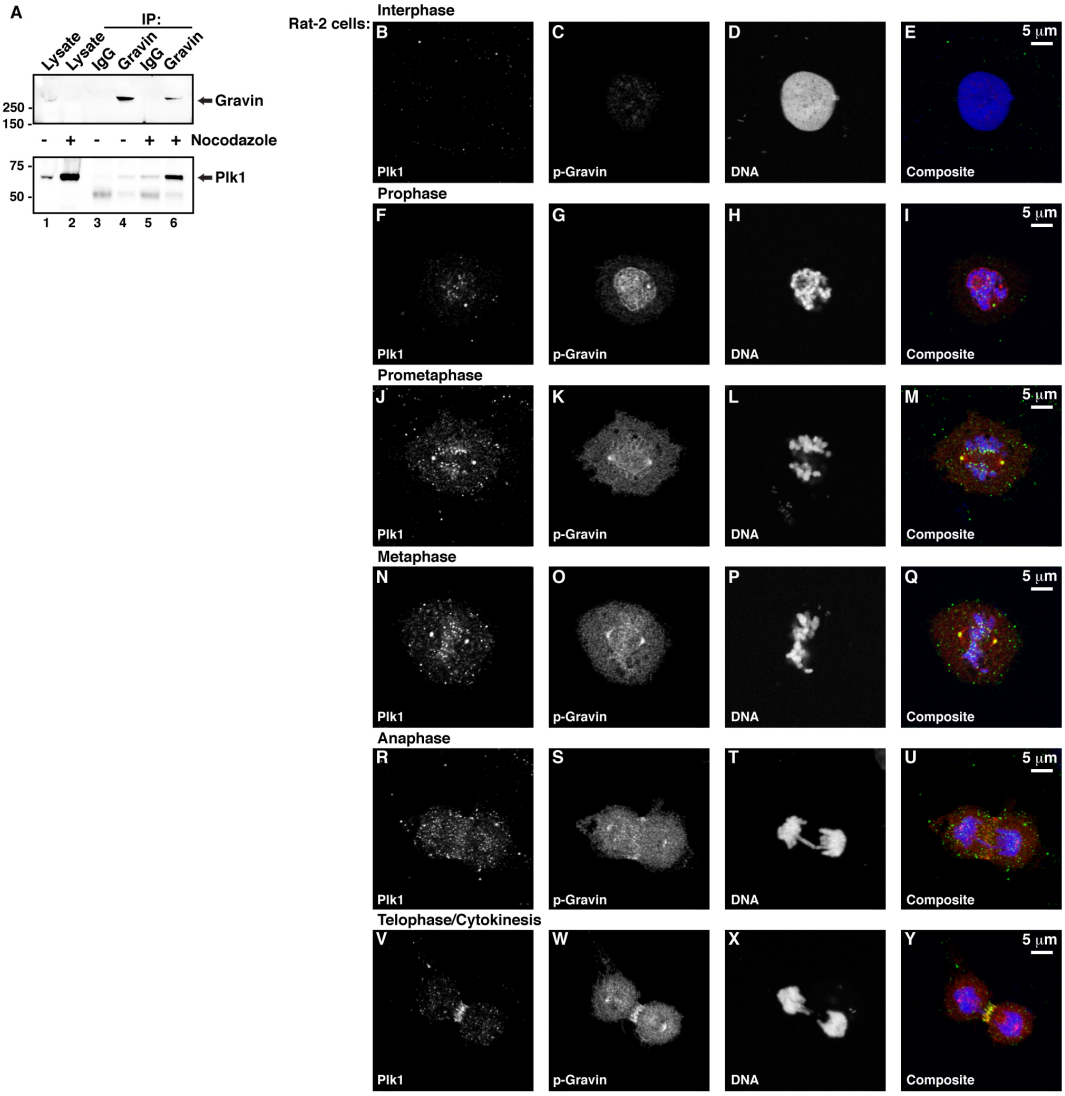
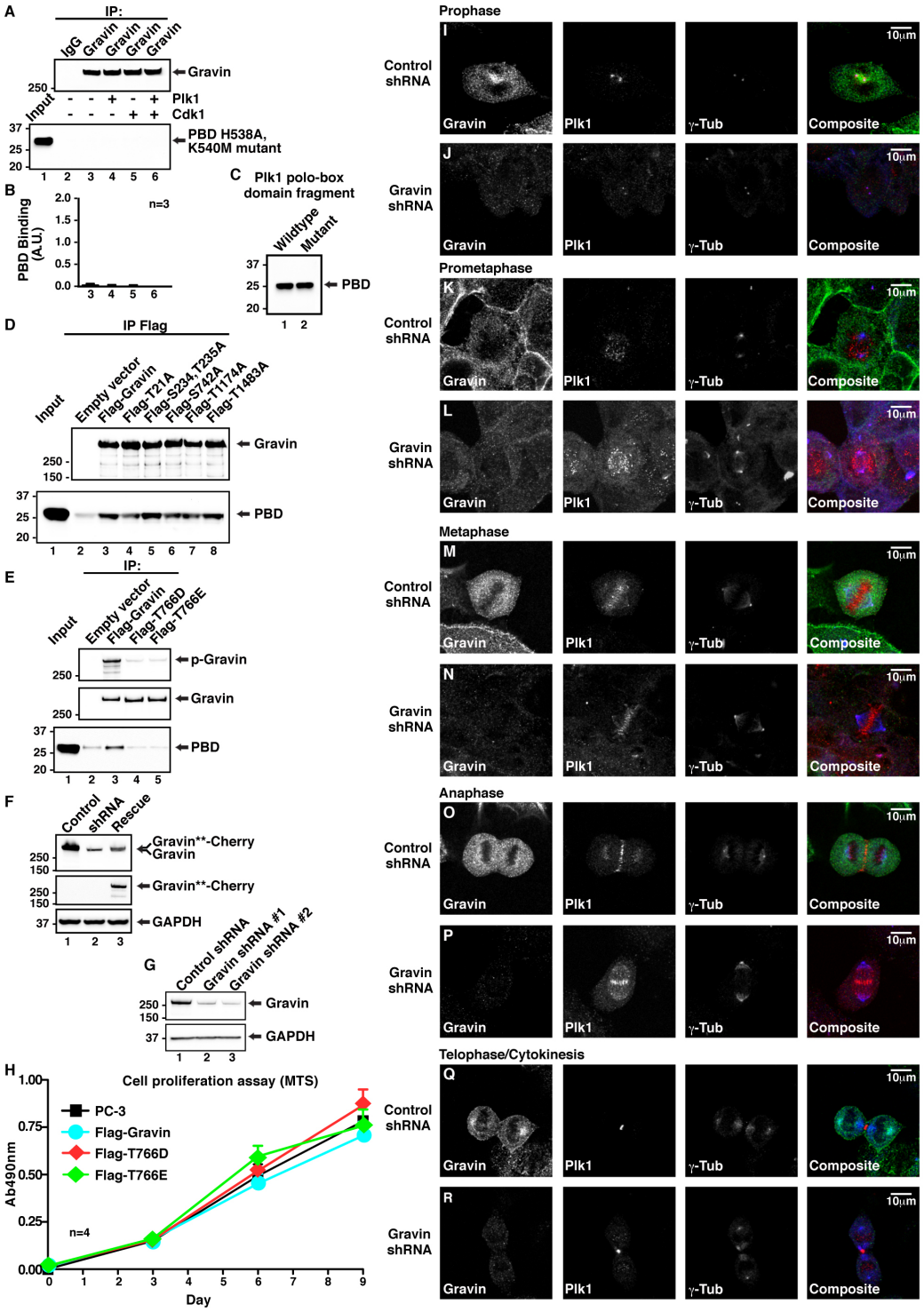
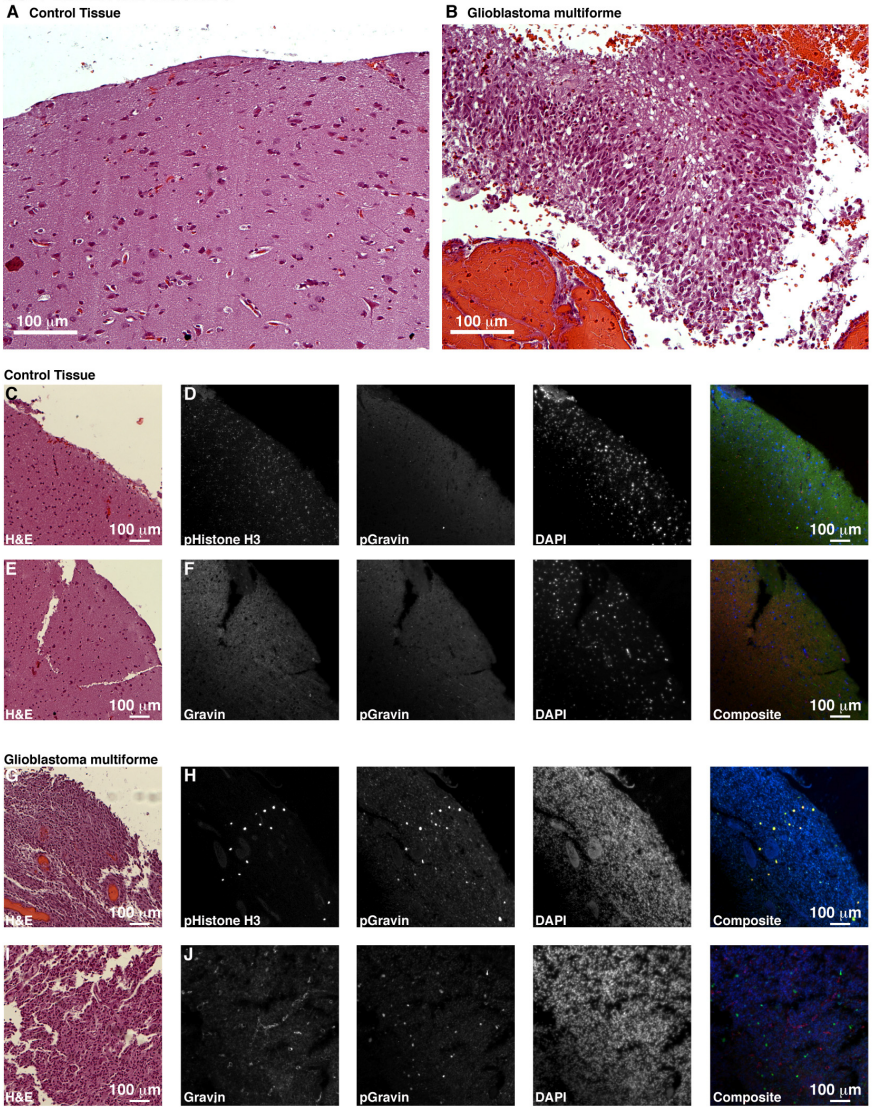


SUPPLEMENTAL FIGURE 5



SUPPLEMENTAL FIGURE 6



SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmid Constructs

Gravin was PCR amplified with an N-terminal Flag tag and subcloned into pcDNA3.1(+) (Invitrogen) or into pEGFP-N3 (Clontech). Plk1 cDNA was TOPO cloned into pcDNA3.1D/V5-His or subcloned into pEYFP-N1 expressing a C-terminal Cherry tag in place of YFP. The PBD of Plk1 (371-603aa) was subcloned into pGEX-6P1 (GE Healthcare). Site-directed mutagenesis was performed with a QuikChange II XL kit (Stratagene). All DNA constructs were verified by sequencing.

Experimental Animals and in vivo Tumorigenesis

Animals were housed at the University of Washington under a 12h light/dark cycle and provided free access to food and water. The institutional IACUC review committee approved all procedures used in this manuscript. Male mice homozygous for *Foxn1*^{nu} (Nu/J, Jackson Laboratories) were maintained in an ABSL-2 certified facility. U251 cells were resuspended in cold PBS at a density of 3 x 10^6 cells / 100μL and injected subcutaneously in the rear flank. From day 16, measurements of tumor width (w), length (l) and height (h) were taken every 2 days and converted to tumor volume by the formula w x l x h x 0.5236 = volume (Schmidt et al., 2004). Animals were sacrificed before tumors exceeded a volume of 1000mm^3. Tumors were flash frozen in liquid nitrogen for biochemistry or fixed overnight in 10% formalin for blocking in paraffin sections.

Cell Culture, Transfection and Generation of Stable Cell Lines

U251, HEK293, Hct116 and HeLa cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin / streptomycin (Invitrogen); PC-3 cells were grown in RPMI. For shRNA infection, cells were infected with control or Gravin shRNA lentiviral particles (Santa Cruz Biotech) in the presence of polybrene (5μg/mL) then selected with puromycin (2μg/mL). Transient transfections were performed using *Trans*IT-LT1 reagent (Mirus). Stable PC-3 cells

were generated by selection with G418 (300μg/mL). Transient transfections of siRNA targeting Gravin (Qiagen; Hs_AKAP12_5 & Santa Cruz Biotech; AKAP250 siRNA pool) were performed using Dharmafect-1 (Dharmacon).

Immunoprecipitation, Immunoblotting and Tissue Analysis

Cells were homogenized in HSE lysis buffer (20mM Hepes pH7.4, 150mM NaCl, 5mM EDTA and 1% Triton X-100) or RIPA buffer (50mM Tris pH7.4, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 0.5% Deoxycholate and 0.1% SDS) supplemented with protease and phosphatase inhibitors (1mM benzamidine, 1mM AEBSF, 2μg/mL leupeptin, 100nM okadaic acid, 1mM β-glycerophosphate and 20mM sodium fluoride). Tissue samples were pulverized under liquid nitrogen, homogenized in HSE buffer and rocked for 1hr. Extracts were centrifuged at 19,000 *g*. Protein concentration was determined using the *DC* Protein Assay kit (Biorad). Immunoprecipitations were rocked overnight with antibodies as indicated. Protein A- and G-agarose beads were added for 1hr, washed with lysis buffer and bound proteins were eluted in boiling sample buffer. Samples were resolved on 4-12% gradient NuPAGE gels (Invitrogen) and transferred to nitrocellulose for immunoblotting. Antibodies used in this report are: mouse anti-Gravin (Sigma; clone JP74), mouse anti-GAPDH (Sigma; GAPDH71.1), mouse anti-phospho-S10 Histone H3 (Millipore & Cell Signaling; clone 6G3), rabbit anti-phospho-T210 Plk1 (Santa Cruz Biotech), mouse anti-Plk1 (Millipore, clone 35-206), rabbit anti-Plk1 (Cell Signaling Technology), mouse anti-PBD (Zymed), mouse anti-Cyclin B1 (Cell Signaling Technology; V152), rabbit anti-Wee1 (Cell Signaling Technology), rabbit anti-phospho-Y15 CDK1 (Cell Signaling Technology), rabbit anti-CDK1 (Cell Signaling Technology), rabbit anti-PCM-1 (Cell Signaling Technology; G2000), mouse anti-α-tubulin (Sigma), mouse anti-β-actin (Sigma), rabbit anti-GFP (Invitrogen), mouse anti-V5 and V5-HRP (Invitrogen), mouse anti-Flag and Flag-HRP (Sigma) and rabbit anti-phospho-T766 Gravin (described below). Detection was with HRP-conjugated secondary antibodies (GE Healthcare) or with Mouse Trueblot HRP-

anti-mouse IgG (eBioscience) followed by enhanced chemiluminescence (Thermo Scientific). Densitometry was performed using NIH ImageJ software (v1.43u).

Generation of a polyclonal phospho-specific antibody against Gravin T766

New England Peptide (NEP) and SDIX were contracted to generate this antibody. Two rabbits were immunized with a KLH-conjugated Ac-CFKRLV(pT)PRKKS-amide peptide corresponding to the sequence surrounding Gravin T766. Serum from the two rabbits was pooled, pre-absorbed over a non-phospho Ac-CFKRLV(T)PRKKS-amide column and then affinity-purified against the phospho-T766 Gravin antigen to a concentration of 0.55mg/mL. The antibody was used at 1:1000 for immunoblotting, immunofluorescence and immunohistochemistry.

Fluorescence in situ Hybridization (FISH)

Hct116 cells plated on poly-l-lysine coated coverslips (18mm) were transfected with 25pmol control or Gravin shRNA on day 1 and 3. On day 6, cells were fixed in 4% paraformaldehyde / 1% glutaraldehyde, treated with sodium borohydride (5mg/mL; 30min at 37^{o}C) then digested with pepsin (4mg/mL; 15min at 37^{o}C). Following denaturation at 72^{o}C, the probe (CEP 18 SpectrumGreen; Abbott Molecular) and target DNA were hybridized overnight at 42^{o}C. Coverslips were imaged on a Deltavision system equipped with a Coolsnap HQ CCD camera (Photometrics), Softworx software (Applied Precision) using an Olympus 20x objective (NA = 0.75).

Soft Agar Proliferation Assay

PC-3 cells were transfected with control shRNA, or two shRNAs targeting Gravin. After 72 hours, cells were counted, resuspended in 2X DMEM/1.6% agar and plated in triplicate at densities of 5,000 and 10,000 cells in a 96-well dish containing a base layer of agar/DMEM. Cells were feed with 100uL RPMI and incubated for 6 days at 37^{o}C, 5% CO_2. Following solubilisation of agar and cell lysis, final cell number per well was calculated using a CyQuant green standard curve according to the manufacturer's recommendations (Cell Biolabs, Inc).

Mitotic Spindle Isolation

Spindles isolations from HEK293 cells were performed as described in (Sillje and Nigg, 2006).

In vitro Kinase Assays and Metabolic Labeling Experiments

Beads for immunokinase assays were washed with 1X kinase buffer (5mM MOPS pH7.2, 1mM EGTA, 4mM $MgCl_2$ and 0.05mM DTT) then resuspended in the same buffer. Reactions were started with 150ng recombinant Plk1 or CDK1/Cyclin B1 (Cell Signaling Technology) in 5X kinase buffer and incubated for 25 minutes at 30°C. Final concentrations of ATP were 5μM for Plk1 and 50μM for CDK1/Cyclin1 B1 plus 1μCi γ-^{32}P ATP. Kinase assays on spindle prep immunoprecipitations were performed in 1X kinase buffer with 5μM ATP, 1μCi γ-^{32} P ATP and 300μM substrate peptide (ISDELMDATFADQEAKKK) for 25 minutes at 30°C. Reactions were spotted on p81 paper, terminated with 75mM phosphoric acid and counted by liquid scintillation. For metabolic labeling experiments, mitotic cells were washed 2X and then resuspended in phosphate-free DMEM supplemented with dialyzed FBS and nocodazole. Roscovitine (75μM) or DMSO was added and cells were labelled for 4hr with 100μCi ^{32}-P orthophosphate. Cells were harvested and immunoprecipitated as described above.

Mass Spectrometry

Excised protein bands were digested according to standard protocol and analyzed by nano-scale liquid chromatography - tandem mass spectrometry (nLC-MS/MS). A 10cm 3.5μm Zorbax C18, 75μm ID analytical column with a gradient of 90 min from 2% acetonitrile, 0.1% formic acid to 35% acetonitrile, 0.1% formic acid was delivered by an Agilent 1100 nano-HPLC at 300nL/min. Data were acquired on a ThermoFisher LTQ linear ion trap, using a data-dependent method consisting of 1 MS scan ranges from 325-1600 m/z followed by 5 MS/MS scans. After peptide ions were selected for MS/MS, they were added to a dynamic exclusion

list for 20 sec. The resulting MS/MS spectra were searched using the MASCOT program version 2.2 against Ensembl Human Databases (Human Ensembl release 50).

Immunofluorescence and Immunohistochemistry

Cells grown on poly-l-lysine coated coverslips were fixed in ice-cold methanol for 20 minutes. Permeabilized cells were blocked in 10% donkey serum, 0.1% fish gelatin and incubated overnight with primary antibodies. Detection was with secondary antibodies conjugated to Alexa Fluor dyes (Invitrogen). For immunohistochemistry, deparaffinised sections were subjected to heat-induced antigen retrieval by submersion in 10mM Tris pH9.0, 1mM EDTA and 0.05% Tween-20 inside a pressure cooker. Imaging was performed on Zeiss 510 META confocal or Axio Observer.Z1 microscopes. Quantitation was performed with NIH ImageJ software (v1.43u) using particle analysis of thresholded, background-subtracted images.

Patient Samples

This study was approved by the University of Washington Institutional Review Board. Autopsy brain samples from a patient with glioblastoma were collected within 6 hours of death and flash frozen. Samples used for analysis were chosen to include viable glioblastoma (mitotically active hypercellular neoplasm with pseudopalisading necrosis and microvascular proliferation but no geographic necrosis or other treatment effects) and control (normocellular cerebral cortex with no evidence of neoplasia) tissues for comparison. Surgical resection specimens included controls (from lobectomy specimens for treatment of medically intractable epilepsy) and glioblastoma specimens. Control sections used for this study had no evidence of neoplasia, inflammation, cortical migration defects, or other abnormalities other than mild reactive gliosis. Glioblastoma sections used in this study were confirmed to contain pseudopalisading necrosis, microvascular proliferation, brisk mitotic activity, and marked hypercellularity. Appropriate measures for the protection of patient privacy were used.

Statistical Analysis

All values are reported as mean ± standard error (SEM) and the number of times the experiment was repeated (n) is shown on each figure. Data were graphed and analysed using GraphPad Prism software (v5.0c). Statistical significance was determined by Student's t-tests or ANOVA, with p=0.05 as the significance level.

Supplemental References

Schmidt, K.F., Ziu, M., Schmidt, N.O., Vaghasia, P., Cargioli, T.G., Doshi, S., Albert, M.S., Black, P.M., Carroll, R.S., and Sun, Y. (2004). Volume reconstruction techniques improve the correlation between histological and in vivo tumor volume measurements in mouse models of human gliomas. Journal of neuro-oncology *68*, 207-215.

Sillje, H.H., and Nigg, E.A. (2006). Purification of mitotic spindles from cultured human cells. Methods *38*, 25-28.