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Supplemental Information

In Silico Analysis of Kinase Expression Identifies WEE1 as a Gatekeeper against Mitotic Catastrophe in Glioblastoma

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INVENTORY OF SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

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SUPPLEMENTAL FIGURES

Figure S1, related to Figure 1. Full kinase expression profiles of cancer datasets. Heatmap of percentile fold change as in Figure 1A for kinases in datasets including kinase gene names and Entrez GeneIDs as row names and kinase groups. Vertical color bar indicates kinase cluster classes as calculated by Matlab code and supported by red color-coded meta-gene analysis on the left of the kinase gene cluster class color bar. Horizontal color bar indicates meta-dataset cluster classes as calculated by Matlab code and supported by red color-coded meta-dataset analysis above the dataset cluster class color bar. Number of normal tissue samples (N) and cancer samples (C) per dataset are shown. Percentile fold change values as in color legend, missing values in white. Nsclc: non-small cell lung carcinoma; GIST: gastrointestinal stroma tumor; ca: carcinoma.
Supplied as Image File

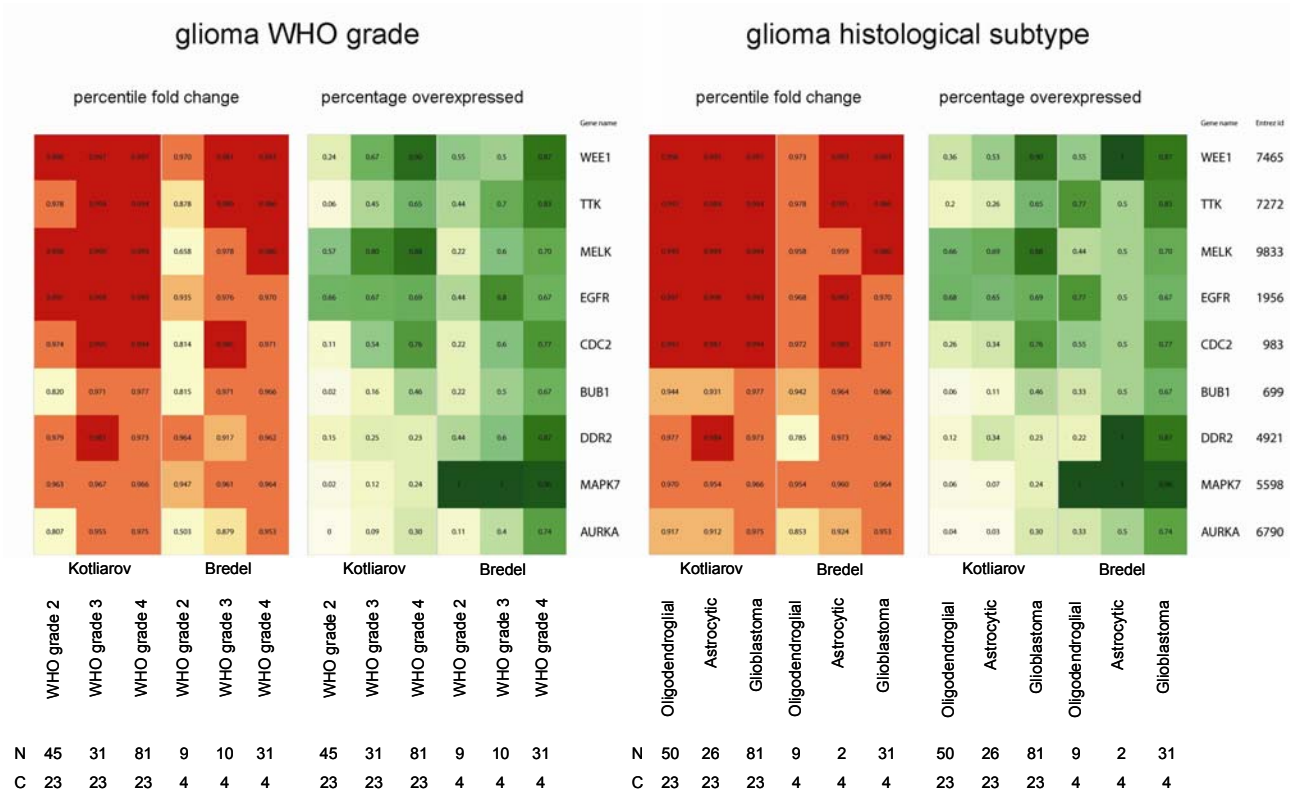
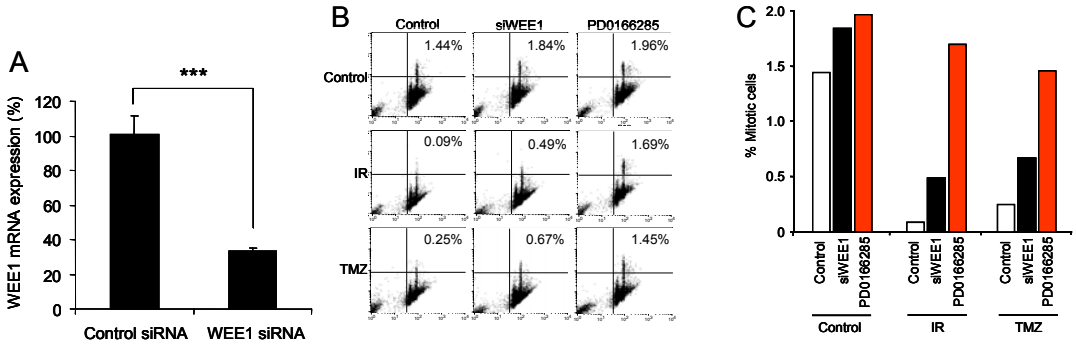


Figure S2, related to Figure 3. Heatmaps of percentile fold change gene expression and frequency of overexpression of 9 kinases in glioma samples of different grades (WHO-2, WHO-3 and WHO-4) and in histological subtypes (oligodendroglial and astrocytic) from 2 glioma datasets. Number of normal tissue samples (N) and cancer samples (C) per dataset are shown Actual values printed.

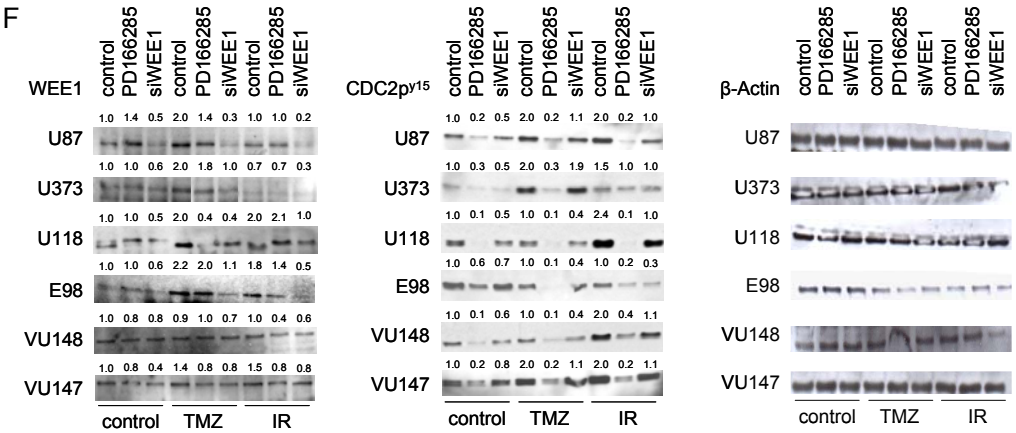


D

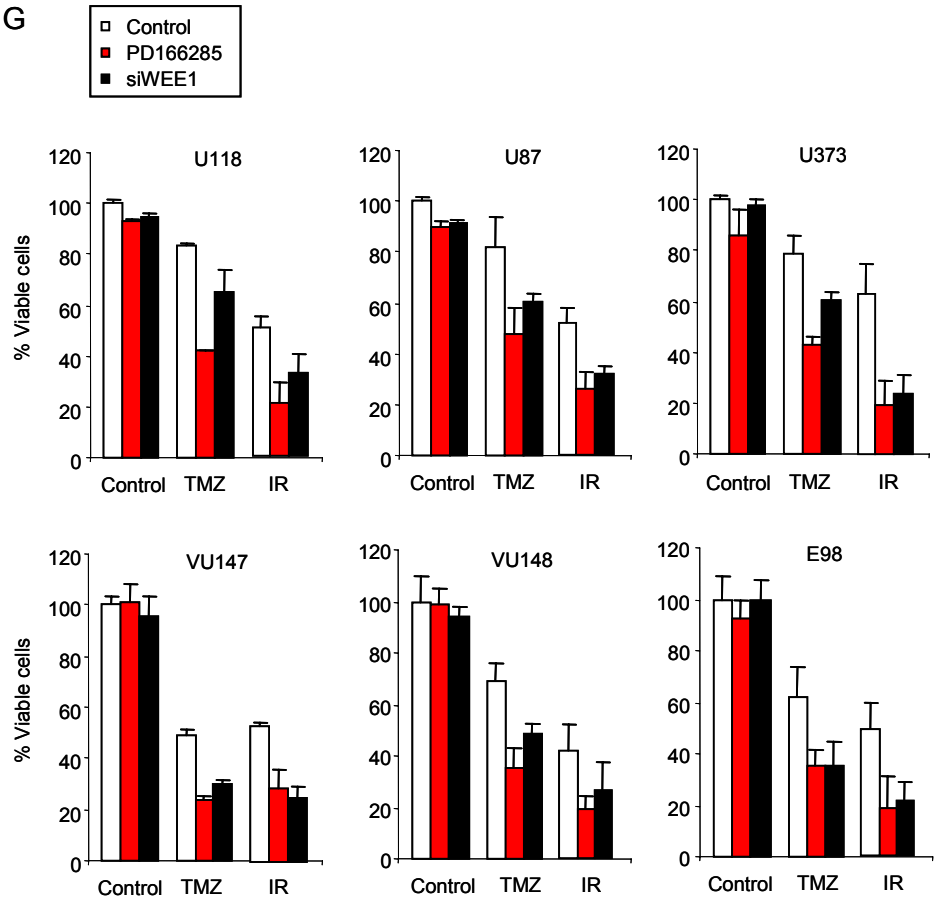
PI - G2/M fraction (%) Cell line	Control Control	Control siWEE1	Control PD0166285	IR Control	IR siWEE1	IR PD0166285	TMZ Control	TMZ siWEE1	TMZ PD0166285
U251MG	16	14	12	82	25	19	59	26	21
U87MG	19	16	14	54	29	21	48	25	17
U373MG	24	26	21	86	37	26	74	35	31
U118MG	16	14	13	59	21	19	51	16	14
E98	26	21	18	89	34	21	78	24	17
VU148	12	14	11	68	31	24	52	21	20
VU147	21	22	25	84	19	27	64	17	19
Primary Human Fibroblasts	11	13	9	16	14	14	15	13	10
Primary Human Astrocytes	17	16	13	21	19	14	19	14	16

E

pHH3 - Mitotic fraction (%) Cell line	Control Control	Control siWEE1	Control PD0166285	IR Control	IR siWEE1	IR PD0166285	TMZ Control	TMZ siWEE1	TMZ PD0166285
U251MG	1.44	1.84	1.96	0.09	0.49	1.69	0.25	0.67	1.45
U87MG	1.64	1.69	1.87	0.16	0.94	1.24	0.36	0.87	1.54
U373MG	1.98	2.09	2.17	0.23	1.15	1.67	0.29	1.45	1.85
U118MG	1.15	1.19	1.36	0.06	0.36	1.12	0.13	0.67	1.16
E98	2.13	2.21	2.29	0.62	1.84	2.15	0.92	1.73	1.94
VU148	0.84	0.89	1.13	0.07	0.56	0.69	0.16	0.63	0.76
VU147	1.36	1.54	1.58	0.51	1.13	1.26	0.66	0.84	1.25
Primary Human Fibroblasts	0.56	0.64	0.85	0.04	0.04	0.08	0.12	0.16	0.14
Primary Human Astrocytes	0.89	0.94	1.13	0.05	0.08	0.06	0.21	0.26	0.31

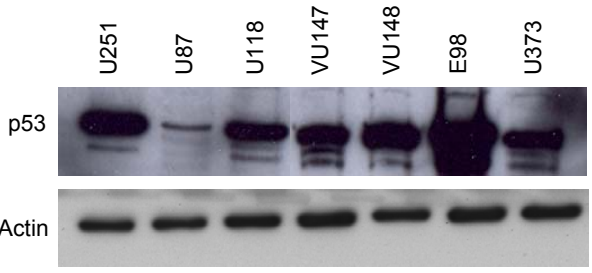


G



H

p53 status	mut (1)	wt (1)	mut (1)	mut (n.a.)	mut (n.a.)	mut (2)	mut (1)



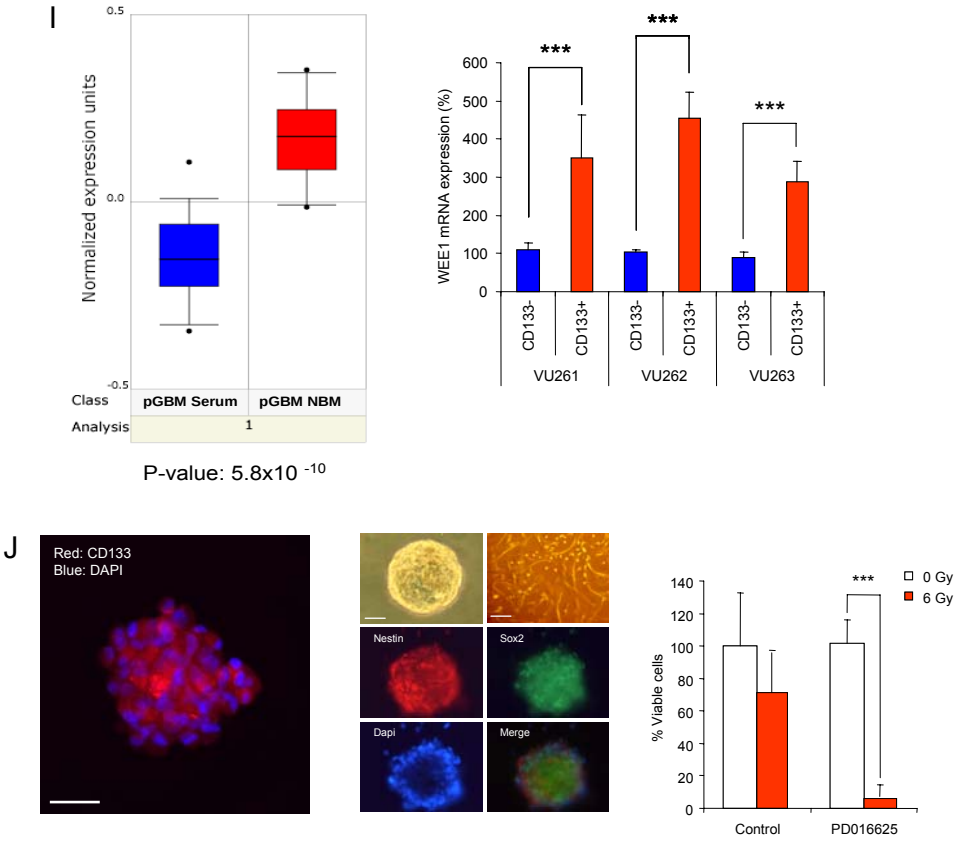


Figure S3, related to Figure 4. (A) Confirmation of WEE1 silencing by siRNAs using quantitative RT-PCR. U251MG cells were transfected with 50 nM WEE1 siRNA (Qiagen) or control siRNA (Qiagen), using Lipofectamine2000, according the manufacturer's protocol. After 48 hr the RNA was isolated and subjected to WEE1 and GAPDH quantitative RT-PCR. The data presented is an average of 3 independent experiments, and normalized to GAPDH expression levels. (B) Monitoring of cell cycle distribution by FACS. DNA content was monitored in U251MG cells by PI staining, and PHH3 staining was used as an indicator of mitotic entry. The percentage mitotic cells are depicted in (A), and quantitated in (C). (D) PI analysis of various treated GBM cell lines and non-neoplastic controls. (E) PHH3 analysis of various treated GBM cell lines and non-neoplastic controls. (F) WEE1, CDC2p^{y15}, and β-Actin protein analysis by Western blot on various GBM cell lines. (G) Cell viability analysis of various GBM cell lines treated with WEE1 siRNA or 0.5 μM PD0166285 in the presence or absence of 6 Gy IR or 100 μM TMZ. (H) Analysis of p53 and β-Actin protein expression by Western blot on various GBM cell lines. (1) (Ishii et al., 1999), (2) (Claes et al., 2008), n.a. = not available, and p53 status, wt = wildtype, mut = mutant. (I) WEE1 expression analysis in primary GBM cells cultured in standard medium (serum) versus stem cell media (NBM) (Lee et al., 2006), (left graph). WEE1 was found to be significantly overexpressed in primary GBM cells grown in stem cell media, as determined using ONCOMINE (www.oncomine.org). WEE1 mRNA expression in CD133 positive and negative populations of primary GBM cells, normalized to GAPDH mRNA expression (right graph). (J) CD133 expression analysis on GBM stem-like spheres (Left). Characterization of primary GBM stem-like neurosphere cultures (Middle). WST-1 viability analysis of primary GBM stem-like neurospheres at 4 days after treatment with 6 Gy of IR and/or 0.5 μM PD0166285 (Right). Size bars indicate 20 μm. Averages are shown from an experiment performed in triplicate. Error bars indicate S. D., ***p < 0.001, *t* test.

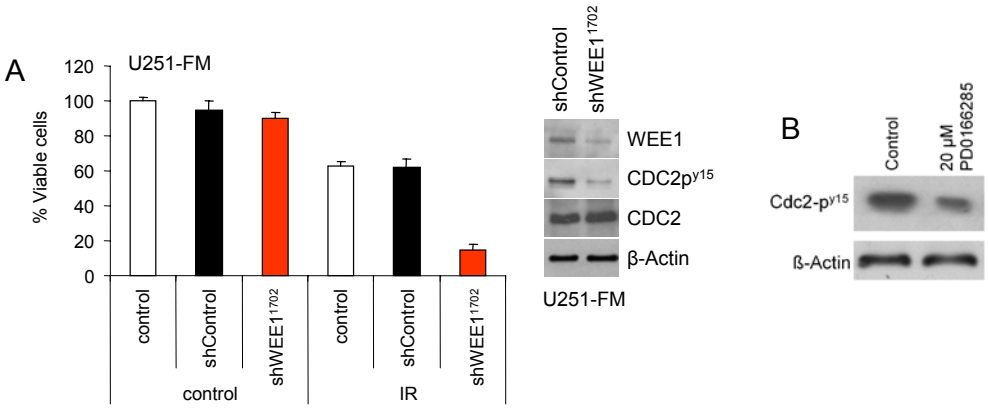


Figure S4, related to Figure 6. Analysis of WEE1 knock down in U251-FM cells. (A) U251-FM-shControl and U251-FM-shWEE1^{1702} cells were exposed to IR and TMZ and analyzed for cell viability using WST-1. U251-FM-shControl and U251-FM-shWEE1^{1702} cells were analyzed for WEE1, CDCp^{y15}, CDC2, and β-Actin protein expression by Western blot. Averages are shown from an experiment performed in triplicate. Error bars indicate S. D. (B) GBM tumors grown s.c. and injected i.v. with 100 µl of 20 µM of the WEE1 inhibitor PD0166285. At 24 hr after drug injection the tumor tissue was analyzed by Western blot for loss of WEE1-mediated CDC2 phosphorylation.

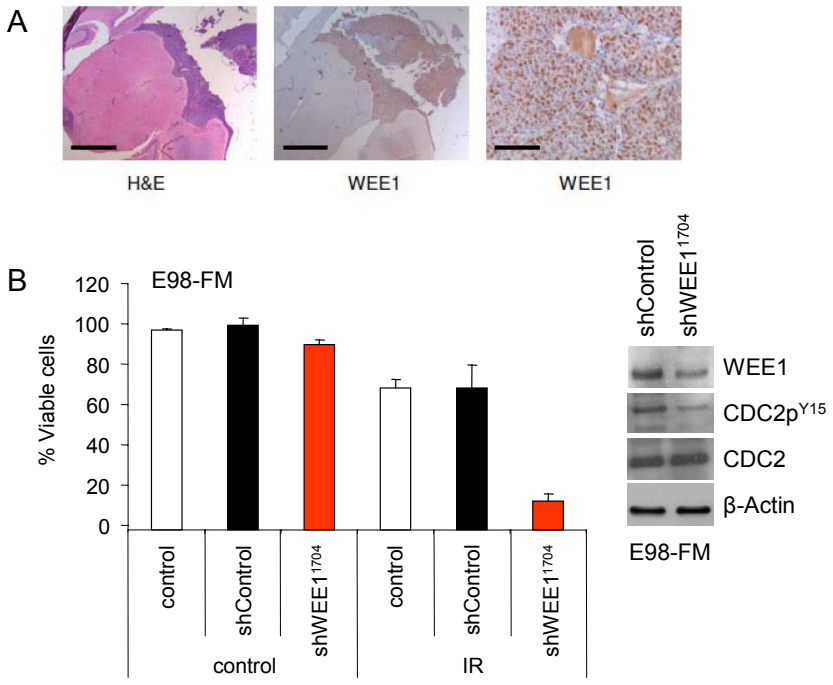


Figure S5, related to Figure 7. (A) WEE1 expression was determined by immunohistochemistry of E98 GBM tumor sections [size bars are 200 µm and 80 µm]. (B) Analysis of WEE1 knock down in E98-FM cells. Similar analysis as in Figure S4A for E98-FM cells using independent shWEE1^{1704} instead. Averages are shown from an experiment performed in triplicate. Error bars indicate S. D.

SUPPLEMENTAL TABLES

Table S1, related to Figure 1. Characteristics of microarray datasets.
Supplied as Excel file

Table S2, related to Figure 1. List of kinases analyzed in this study.
Supplied as Excel file

Table S3, related to Figure 2. Percentile fold change of kinase gene expressions of cancer versus normal samples in all datasets.
Supplied as Excel file

Table S4, related to Figure 2. Frequency of overexpression in cancer samples in all datasets.
Supplied as Excel file

Table S5, related to Figure 3. Characteristics of human samples for quantitative RT-PCR validation.

ID	age	gender	grade	pathology	localization
AA1	48	F	3	anaplastic astrocytoma	frontal L
AA2	37	M	3	anaplastic astrocytoma	parieto-occipital L
AA3	75	F	3	anaplastic astrocytoma	temporo-parietal L
AA4	35	M	3	anaplastic astrocytoma	frontal L
AO1	42	F	3	anaplastic oligodendroglioma	frontal R
GBM1	53	M	4	glioblastoma	temporo-parietal R
GBM2	41	F	4	glioblastoma	temporal L
GBM3	37	M	4	glioblastoma	temporal L
GBM4	61	M	4	glioblastoma	temporo-parietal L
GBM5	58	M	4	glioblastoma	parietal R
GBM6	60	F	4	glioblastoma	frontal R
GBM7	44	M	4	glioblastoma	unavailable
GBM8	61	M	4	glioblastoma	temporo-parieto-occipital R
GBM9	66	F	4	glioblastoma	unavailable
LGG1	47	M	2	low grade astrocytoma	unavailable
LGG2	44	F	2	low grade astrocytoma	frontal L
LGG3	30	F	2	low grade oligoastrocytoma	fronto-temporal R
LGG4	52	F	2	low grade astrocytoma	frontal R
N1	31	M	-	normal	
N2	48	M	-	normal	
N3	57	M	-	normal	
N4	66	M	-	normal	
N5	78	M	-	normal	
N6	29	M	-	normal	

Table S5, related to Figure 3. Characteristics of human samples and cell lines used for quantitative RT-PCR validation.

Table S6, related to Figure 3. Ordering of the primary samples and GBM cell lines.								
WEE1	TTK	MELK	EGFR	CDC2	BUB1	DDR2	MAPK7	AURKA
N5	1	5	4	5	4	3	6	1
N1	3	1	3	6	5	5	1	6
N4	5	3	1	1	1	4	3	5
N2	4	4	2	4	3	1	5	3
N3	2	2	5	3	6	2	4	4
N6	6	6	6	2	2	6	2	2
LGG4	4	4	3	1	4	4	4	1
LGG3	1	3	2	3	1	3	3	3
LGG1	3	1	4	2	3	1	1	2
LGG2	2	2	1	4	2	2	2	4
AA3	AO1	2	1	1	1	2	3	2
AA4	1	1	4	2	2	3	2	AO1
AA2	3	4	2	4	3	1	1	3
AA1	2	3	AO1	3	4	4	4	4
AO1	4	AO1	3	AO1	AO1	AO1	AO1	1
GBM7	7	7	3	7	7	7	7	7
GBM6	8	8	8	1	3	8	3	3
GBM8	6	3	7	6	6	1	6	6
GBM3	3	6	2	8	1	3	8	1
GBM1	1	1	9	3	8	6	1	8
GBM9	9	5	5	2	2	2	9	5
GBM2	5	2	1	9	5	5	2	2
GBM5	2	9	6	5	9	9	5	9
GBM4	4	4	4	4	4	4	4	4
U87MG	IGRG121	IGRG121	IGRG121	IGRG121	IGRG121	SF763	A60	IGRG121
Gli-06	Gli-06	SF763	D384MG	Gli-06	SKMG3	U251MG	IGRG121	Gli-06
SKMG3	D384MG	Gli-06	A60	U118MG	A60	D384MG	U251MG	U251MG
IGRG121	U251MG	U118MG	U87MG	U251MG	Gli-06	SF126	SF763	A60
A60	CCF-STTG	A60	T98G	SF126	SF126	IGRG121	D384MG	U118MG
T98G	SF763	D384MG	CCF-STTG	A60	SF763	T98G	CCF-STTG	D384MG
D384MG	SF126	SKMG3	U251MG	T98G	U118MG	GAMG	T98G	CCF-STTG
U251MG	A60	SF126	U118MG	SKMG3	CCF-STTG	SKMG3	SKMG3	SF763
U118MG	T98G	T98G	Gli-06	D384MG	D384MG	Hs683	U118MG	SKMG3
CCF-STTG	Hs683	U251MG	Hs683	Hs683	U251MG	U118MG	Gli-06	SF126
Hs683	U118MG	CCF-STTG	SF763	CCF-STTG	T98G	U373MG	GAMG	U87MG
SF126	U87MG	U87MG	SF126	SF763	U87MG	CCF-STTG	Hs683	T98G
SF763	SKMG3	Hs683	A58	U87MG	Hs683	Gli-06	A58	A58
GAMG	GAMG	A58	GAMG	A58	A58	U87MG	SF126	GAMG
A58	A58	U373MG	SKMG3	U373MG	U373MG	A58	U87MG	Hs683
U373MG	U373MG	GAMG	U373MG	GAMG	GAMG	A60	U373MG	U373MG

Table S6, related to Figure 3. Kinase ordering of human samples and cell lines used for quantitative RT-PCR validation.

Table S7, related to Figure 3. Multivariate Cox proportional-hazards regression analysis in 267 glioblastoma patients.			
	Hazard Ratio	95% CI	P-value
WEE1 low vs high	0.689	0.532 - 0.892	0.0047
age >55 vs <=55	1.887	1.425 - 2.500	0.0000
sex male vs female	1.222	0.921 - 1.622	0.1700
IR	0.482	0.169 - 1.369	0.1700
TMZ	1.728	0.804 - 3.711	0.1600
any chemotherapy	1.247	0.512 - 3.035	0.3600
Tumor recurrent vs initial	2.167	1.023 - 4.593	0.0430

Table S7, related to Figure 3. Multivariate Cox proportional-hazards regression analysis of WEE1 expression in glioblastoma patients.

SUPPLEMENTAL MOVIES

Movie S1, related to Figure 5. Visualization of mitotic catastrophe in irradiated U251MG glioblastoma cells in the presence of WEE1 inhibitor, as compared to U251MG cells irradiated only.
Supplied as Movie file

Movie S2, related to Figure 8. Visualization of mitotic catastrophe after inhibition of various kinases in irradiated U251MG glioblastoma cells.
Supplied as Movie file

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Selection of in silico analysis datasets

The Gene Expression Omnibus and ArrayExpress collection were searched to retrieve the vast majority of published microarray gene expression datasets comparing cancer with normal tissue samples. References in publications based on the retrieved datasets were screened to expand on available datasets. Of the 63 datasets obtained, 34 were suitable for further analysis (Table S1). One dataset (on seminoma and non-seminoma) provided two cancer type subgroups for analysis. Twenty-nine datasets were excluded for various reasons, such as less than four or only pooled normal tissue samples, normal tissue samples consisting of cells in culture, cancer and normal tissue samples hybridized to the same array instead of compared to a common reference or single channel measurements, data not publicly available, only data of selected lists of significant genes publicly available or unavailable identification of samples.

Selection of kinases

In order to comprehensively define the human kinase gene family, Entrez GeneIDs were first derived for the protein kinase complement of the human genome by basic local alignment. A total of 518 protein kinase sequences were identified in the human genome after exclusion of pseudogenes and secondary kinase domains in genes. Three of 518 protein kinases could not be translated to Entrez GeneIDs (SK592, SK707 and SK723). Furthermore, 33 genes in the lipid kinase class from the Human Protein Reference Database were added to this collection summing up to a total of 551 human kinases (Table S2).

Analysis of datasets

For all datasets $\log_2$ transformed intensity measurements were available for each probe in every sample. The original method of spot qualification and data normalization was maintained for each dataset. A new annotation of probes to Entrez GeneIDs for every array platform was accomplished either according to the latest Affymetrix's annotation file or by conversion from clone ID or GenBank accession using the SOURCE web application (Table S1). When multiple probes were converted to the same Entrez GeneID, signal intensities were averaged to obtain single values for each Entrez GeneID. In order to quantify the level of expression, the significance algorithm for microarrays analysis was performed using the *samr* package (version 1.24 by B. Narasimhan and R. Tibshirani) in R, A Language and Environment for Statistical Computing (release 2.4.1; Vienna, Austria; http://www.R-project.org) based on randomization. Unpaired two class comparison for cancer versus normal tissue samples was performed, unless paired samples were involved, in which case a paired analysis was performed. The selection of the delta parameter was based on a median false discovery rate less than 0.05. The default number of 100 permutations was used. This resulted in fold change values for each Entrez GeneID. Using the empirical cumulative distribution function in the *stats* package in R, the corresponding percentiles for the fold change values were obtained within each dataset. These percentiles of fold change can be directly compared between datasets. In order to quantify the frequency of overexpression, moderated t-statistics were calculated for each gene in every sample while taking account of small numbers of arrays. The hyper-parameters for the statistical model, consisting of prior variance s_0^2 and degrees of freedom d_0, were estimated from the normal tissue array data. The threshold of the t-statistics that determined substantial overexpression for each dataset was calculated using a maximum false discovery rate of 0.05. The code for use in R can be obtained from the authors upon request. The number of cancer samples with a t-statistic higher than the threshold divided by the overall number of cancer samples provided the frequency of cancer samples with overexpression for a particular gene.

Clustering

In order to classify the datasets and kinases according to percentile of fold change values in Figure 1A and S1, non-negative matrix factorization was used. The MatLab code was used to calculate 3 cluster classes of meta-genes and 13 cluster classes of meta-datasets. Because non-negative matrix factorization cannot handle missing values, values were imputed using ten nearest neighbors imputation from the *impute* library of R. The optimal number of dataset classes was 13 as determined by the maximum of the cophenetic correlation plot, which was

0.987 at rank 13 for the meta-gene analysis (data not shown). For correlation of the 9 glioblastoma-relevant kinases in Figure 2, the absolute value of the Pearson correlation coefficient was used. The coefficients were hierarchically clustered resulting in the cluster tree for rows in Figure 2.

Quantitative RT-PCR

Total RNA extraction was performed using the Phase Lock protocol according to the manufacturer's instructions (Qiagen). cDNA was prepared using random hexamers, RNAse inhibitor (Roche Applied Science, Indianapolis, IN) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Samples were incubated for 60 min at 37°C and 10 min at 95°C. The cDNA concentrations were normalized to yield equivalent PCR products. Quantitative RT-PCR was then performed using an iCycler (Bio-Rad, Hercules, CA) with SYBR green detection (Roche Applied Science) and Taq DNA Polymerase (Promega, Madison, WI). Primers for the 9 kinases were based on the Universal Probe Library from Roche. RNA integrity of the examined samples was confirmed using -2 microglobin. Reaction conditions were denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s. The cycle threshold (Ct) was determined in triplicate for each sample. The average of triplicates was used for analysis. To quantify the results obtained by quantitative RT-PCR for -2 microglobin, the standard curve method was used. Amplification plots for 4 dilutions of control template were used to determine the Ct value. A standard curve was generated by plotting the Ct values against the log of known input DNA copy numbers. To determine the quantity of the target gene-specific transcripts present in cancer samples relative to normal brain, we used the 2–••Ct method.

Cell lines

Sixteen glioblastoma cell lines were used in this study. The commercially available cell lines CCF-STTG, Hs683, U87MG, U118MG, U251MG, U373MG, T98G (ATCC, Middlesex, United Kingdom), SKMG3 (a gift of Dr C. Y. Thomas, University of Virginia Division of Hematology/Oncology, Charlottesville, VA), GAMG (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), D384MG, SF763, SF126 (a gift of Dr C. Van Bree, University of Amsterdam, Laboratory for Experimental Oncology and Radiation Biology, Amsterdam, the Netherlands), and the xenograft cell line IGRG121 (a gift of Dr B. Geoerger, Institut Gustave Roussy, Villejuif, France), and the cell lines Gli-06, A58 and A60, were cultured

in DMEM medium (Gibco, Breda, the Netherlands) containing 10% FBS (Gibco) and antibiotics (penicillin-streptomycin) (Gibco). The primary low passage glioblastoma lines VU147 and VU148 were derived directly from surgical specimens from the VU University Medical Center. E98 cells were derived from a subcutaneously growing tumor. The primary cells were cultured in DMEM medium containing 10% FBS and antibiotics (penicillin-streptomycin).

Magnetic cell separation

We isolated the CD133 positive and negative cells from primary GBM neurospheres by MACS sorting. GBM spheres were dissociated and resuspended in PBS containing 0.5% bovine serum albumin and 2 mmol/L EDTA. For magnetic labeling, CD133/1 Micro Beads were used (Miltenyi Biotech). Positive magnetic cell separation (MACS) was performed using several MACS columns in series.

FACS analysis

Cells were transfected and treated as described. After treatment cells were fixed in 70% ethanol. Prior to analysis by flow cytometry (Becton-Dickinson, Breda, the Netherlands), the cells were washed with PBS, incubated with 0.75 µg of anti-phospho-histone H3 (PHH3) (Upstate) per 10^6 cells for 2 hr at RT. Following washing, cells were incubated with FITC-conjugated secondary antibody at 1:50 for 30 min at RT. Samples were washed and resuspended in 0.15 mg/ml RNAse A for 20 min and 50 µg/µl of propidium iodide (PI) for 30 min at 37ºC. Cell cycle distribution assessment was performed using CellQuest software.

Cell counts and WST-1 cell viability assay

Cell counts were performed by trypsin treatment of the cells, inactivation of the trypsin, and subsequent Casy counter (Innovatis, Bielefeld, Germany) analysis. Cell viability and proliferation was analyzed using WST-1 (Roche Diagnostics GmbH, Mannheim, Germany). Cells were plated in a 96-well plate at $3x10^4$ cells per well. After 48 hr, WST-1 reagent was added (1/10 of the culture volume) and cells were incubated at 37°C for 2 hr before measuring the A_{450} using an ELISA plate reader (Wallac 1420 VICTOR^3™ V multilabel counter, Perkin Elmer, MA).

Clonogenic assay

Cells were plated in six-well plates with 150-6,000 cells per well depending on the used dose of irradiation and drug concentration. Cells were treated and subsequently incubated for 10-14 days to allow colony formation. Groups consisting of 50 cells or more were defined as a colony.

The cell cultures were washed twice with PBS and fixed with 3.7% formaldehyde and stained with Giemsa solution. The colony counts using light microscopy were performed independently by at least two investigators.

SUPPLEMENTAL REFERENCES

Ishii, N., Maier, D., Merlo, A., Tada, M., Sawamura, Y., Diserens, A. C., Van Meir, E. G. (1999). Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. Brain Pathol. 9(3), 469-79.