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CDK1 inhibition sensitizes normal cells to DNA damage in a cell cycle dependent manner.

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Abstract

CDK1 orchestrates the transition from the G2 phase into mitosis and as cancer cells often display enhanced CDK1 activity, it has been proposed as a tumor specific anti-cancer target. Here we show that the effects of CDK1 inhibition are not restricted to tumor cells but can also reduce viability in non-cancer cells and sensitize them to radiation in a cell cycle dependent manner.

Radiosensitization by the specific CDK1 inhibitor, RO-3306, was determined by colony formation assays in three tumor lines (HeLa, T24, SQ20B) and three non-cancer lines (HFL1, MRC-5, RPE). Initial results showed that CDK1 inhibition radiosensitized tumor cells, but did not sensitize normal fibroblasts and epithelial cells in colony formation assays despite effective inhibition of CDK1 signaling. Further investigation showed that normal cells were less sensitive to CDK1 inhibition because they remained predominantly in G1 for a prolonged period when plated in colony formation assays. In contrast, inhibiting CDK1 a day after plating, when the cells were going through G2/M phase, reduced their clonogenic survival both with and without radiation. Our finding that inhibition of CDK1 can damage normal cells in a cell cycle dependent manner suggests that targeting CDK1 in cancer patients may lead to toxicity in normal proliferating cells. Furthermore, our finding that cell cycle progression of non-cancer cells becomes easily stalled under normal culture conditions has general implications for testing anti-cancer agents in these cells.

Introduction.

Cyclin-dependent kinase 1 (CDK1), also known as cell division control protein 2 (cdc2) is required for the transition from the G2 phase into mitosis (reviewed in ref 1). The activity of CDK1 is tightly regulated by both cyclin levels and checkpoint kinases such as WEE1 and Chk1, ensuring the cell does not enter mitosis with incompletely replicated or damaged DNA.[2] CDK1 remains largely inactive until late G2, when the levels of cyclin B are sufficiently high to allow stable CDK1-cyclin B complex formation. This complex does not become activated until the removal of the CDK1 inhibitory phosphorylation sites Thr 14 and Tyr 15 by cdc25c phosphatases upon mitotic entry. This allows CDK1 to phosphorylate over 100 proteins, which then promote nuclear envelope breakdown, chromatin condensation and spindle assembly. Once the spindle assembly checkpoint is satisfied, the cell progresses from metaphase to anaphase, which requires the attenuation of CDK1 activity. This is mediated by the degradation of cyclin B1, promoted by the anaphase-promoting complex.[3]

Overexpression of CDK1 and cyclin D has been found in a number of tumors including breast, colorectal and lung tumors and is correlated with adverse prognosis (reviewed in ref 4). CDK1 inhibition has therefore been proposed to be an attractive anti-tumor strategy. Indeed, incubation with the specific CDK1 inhibitor RO-3306 was shown to be more pro-apoptotic in tumor than in normal cells.[5] Additional pre-clinical evidence suggests that CDK1 inhibition can also be used in combination therapy. It was shown that targeting CDK1 can specifically sensitize tumor cells to DNA-damaging agents without affecting the sensitivity of normal epithelial cells.[6] CDK1 inhibition can also act synergistically with PARP inhibitors in a tumor specific manner, and this combination was shown to prolong survival in a spontaneous mouse tumor model without apparent normal tissue toxicity.[7] Furthermore, it was recently shown that tumors carrying KRAS mutations could be particularly sensitive to CDK1 inhibition. Mutant KRAS colorectal cancer and pancreatic cell lines were significantly more sensitive to CDK1 inhibition than wild type KRAS tumor cell lines in long-term viability and colony formation assays.[8]

We recently screened a kinome siRNA library for novel radiosensitization targets using colony formation in HeLa cells after irradiation as an end point.[9] CDK1 was one of the potential radiosensitization targets identified in this screen. Although siRNA depletion of CDK1 has previously been shown to cause radiosensitization, the effect of pharmacological inhibition of CDK1 on radiosensitivity was only tested with compounds that target multiple CDKs.[10] In this paper, we investigate whether targeting CDK1 using the specific inhibitor RO-3306 [5] can radiosensitize tumor cells and whether the effect is indeed tumor specific.

Results

To validate CDK1 as a tumor specific radiosensitization target identified in our siRNA kinome library screen,[9] we tested the effect of the specific CDK1 inhibitor RO-3306, in three tumor lines and three normal lines that are commonly used for radiosensitivity studies [11, 12]. The tumor lines were the HeLa line used in the siRNA screen, the bladder transitional cell carcinoma line T24, and the head and neck squamous cell carcinoma SQ20B; the normal cell lines were HFL1 and MRC-5 fibroblasts, and RPE retinal epithelial cells. We found that pre-treatment with 5 μM RO-3306 for 20 hours sensitized all three tumor cell lines to irradiation (Fig. 1A). In addition, RO-3306 treatment had a stand-alone effect, also reducing colony formation in all three tumor cell lines in the absence of radiation (Fig. 1A). Shorter incubation times or lower concentrations of RO-3306 were less effective, whereas higher concentrations caused increased single agent toxicity (Supplementary Fig. 1). In contrast, when these conditions were tested in the three normal cell lines, we found that RO-3306 did not reduce colony formation in the presence or absence of irradiation (Fig. 1B).

These results confirmed CDK1 as a radiosensitization target and indicated that this effect was specific for tumor cells. In an attempt to identify the reason for the lack of a cytotoxic effect in normal cells, we first sought to determine whether the capacity of RO-3306 to suppress CDK1 function was comparable in both normal and tumor-derived cell lines. First we tested whether RO-3306 abrogated phosphorylation of CDK1 substrates in these cells using the MPM2 antibody, which recognizes the Ser/Thr-Pro motif in proteins phosphorylated by CDK1. To enhance assay sensitivity,

cultures were treated with nocodazole, arresting cells in pro-metaphase when CDK1 activity is highest, and then treated with RO-3306. As shown in Fig. 2A, RO-3306 treatment was equally effective in both tumor and normal cells in suppressing CDK1-mediated phosphorylation (Fig. 2A, Supplementary Fig. 2). As inhibition of CDK1 activity is expected to prevent cells from completing mitosis, we used flow cytometry to test whether RO-3306 treatment caused a G2/M arrest in normal cells. The cell cycle profile after 20 hours RO-3306 treatment showed that the inhibitor did cause a G2/M arrest in both tumor and normal cells (Fig. 2B). Although the percentage of cells in G2 after treatment was higher in tumor cell lines, these flow cytometry data indicate G2/M arrest was achieved for both tumor and normal cell lines following treatment. Together with the biochemical results, our tests were able to confirm that RO-3306 effectively inhibited CDK1 activity in normal cell lines.

We next investigated other potential explanations for differences in susceptibility to CDK1 inhibition observed between tumor and non-tumor cells. As the CDK1 inhibitor is expected to be mainly acting on cells going through G2, we decided to closely examine the cell cycle profile of normal cells plated in colony formation assays as performed in Fig. 1. For these assays, we routinely grow cells in standard culture flasks for 2-3 days and then reseed at low densities prior to treatment. We found that under these experimental conditions, normal cells appeared to be arrested in G1 for a prolonged period after plating. Cells then started to enter S-phase from 15 hours after seeding and reached G2 by 20-24 hours (Fig. 3A). This apparent arrest was not seen in tumor cells grown and plated in similar conditions (Supplementary Fig. 3). Interestingly, we found that those few normal cells that were still cycling at the point of cell seeding had cell cycle phase lengths that were not too dissimilar to that of tumor cells (Supplementary Fig 4). This suggests that proliferating normal cells progress through the cell cycle at a rate similar to that of tumor cells but that they are more prone to cell cycle arrest.

The findings described above could potentially explain the lack of CDK1 inhibition in in normal cells shown earlier (Fig. 1). Because of the experimental setup, which involved growing the cells for three days, it is conceivable that most of the normal cells were not going through G2 during RO-3306 exposure and thus less sensitive to CDK1 inhibition. To test this hypothesis, we compared colony formation capacity in normal cells after CDK1 inhibition (5 μM RO-3306, 20 hours) using either cells with

a low proportion of cells transiting G2 (by adding the compound 3 hours post-seeding), or with a higher proportion of cells transiting G2 (by adding the compound 24 hours post-seeding). When RO-3306 was added 3 hours after seeding, there was no effect on colony formation but when RO-3306 was added 24 hours after seeding, there was a significant decrease in clonogenic survival (Fig. 3B). These results show that normal cells are susceptible to CDK1 inhibition when they are passing through G2 phase and that the apparent lack of effect shown earlier was merely caused by the experimental setup. This was confirmed by studying the level of G2M arrest achieved by CDK1 inhibition comparing addition of inhibitor at 3 or 24 hours after seeding. When RO-3306 was added 3 hours after seeding, the G2M arrest in normal cells was minimal compared to the level of G2M arrest achieved when RO-3306 was added 24 hours after seeding (Supplementary Fig 5). In tumor cells in contrast, a high level of G2M arrest was achieved regardless of whether inhibitor was added at 3 or 24 hours after seeding (Supplementary Fig 5).

These results would predict that normal cells are more sensitive to continuous than short-term CDK1 inhibition when the inhibitor is added 3 h after plating. We compared the effects of short-term versus continuous RO-3306 treatment again assessing colony formation in the absence of irradiation. Interestingly, while there was a significant difference in IC50 between normal and tumor cells for the 20 hour treatment, this difference was lost for the continuous treatment, showing comparable IC50s for tumor and normal cells grouped together (Fig. 4). Together these results show that normal cells in these settings are less sensitive to short-term treatment because fewer cells pass through G2/M during the course of the incubation period. However, when cells are continuously exposed to the CDK1 inhibitor, the initial cell cycle distribution and growth rate are irrelevant and hence the differential sensitivity between tumor and normal cells is lost.

Following the finding that inhibiting CDK1 can kill normal cells going through G2/M, we next investigated whether this cell cycle dependency would also influence sensitization to radiation. This was again tested by adding the inhibitor at different time points after seeding and then irradiating after 20 hours of treatment. As shown earlier in Fig. 1, addition of RO-3306 3 hours after seeding, when the cells exhibited stalled cell cycle progression, did not cause any radiosensitization (Fig. 5). However,

when RO-3306 was added 24 hours after seeding, when many cells were entering G2 phase, significant radiosensitization could be seen in all three cell lines (Fig. 5). These results show that inhibiting CDK1 can sensitize normal cells to irradiation-induced DNA damage when they are actively progressing through the cell cycle. In contrast, when similar plating conditions were tested in tumor cells, we found comparable radiosensitization in cells treated with RO-3306 at 3 hours and 24 hours after seeding (Supplementary Fig. 6).

Discussion

In this paper we describe how targeting CDK1 using the specific inhibitor RO-3306 can reduce survival both as a single agent and in combination with irradiation in both tumor and normal cells. Our initial results suggested that the radiosensitization effect was restricted to tumor cells, implicating CDK1 as an ideal target for improving radiotherapy efficacy in patients without increasing normal tissue toxicity. The absence of normal cell radiosensitization with RO-3306 was noted despite the inhibitor abrogating downstream phosphorylation and inducing a cell cycle arrest in these cells. Careful investigation of the experimental setup subsequently revealed that when the normal cells were seeded from a near confluent flask in colony formation assays their cell cycle progression was stalled. This explained our earlier findings as it suggested that the normal cells in the initial clonogenic assays were effectively arrested at the time of treatment and thus less sensitive to CDK1 inhibition. It is important to note that it appears these cell cycle delay effects only become apparent when normal cells have been grown in culture for three days or more and reached a high level (> 70-80%) of confluency. This may explain the difference between results shown in Fig 2B and supplementary Fig 5. In contrast to the latter figure, the earlier figure showed a G2M arrest in normal cells treated with RO-3306 added only 4 hours after seeding. Assessment of the experimental procedures suggested that for the earlier experiment, the normal cells had not reached a high level of confluency at the point of seeding and were still actively cycling.

Subsequent experiments showed that treating normal cells enriched in S-G2 phase could uncover their apparent insensitivity to CDK1 inhibition. This cell cycle dependency was also evident for RO-3306 induced sensitization to radiation-induced DNA damage. Although the degree of radiosensitization was not as high as in tumor cells, our findings do show that normal cells are not insensitive to CDK1 inhibition.

Importantly, normal and tumor cells were equally sensitive to continuous CDK1 inhibition as a single agent. Our data suggests that the therapeutic window for CDK1 inhibition is therefore narrow and that prolonged CDK1 inhibition may aggravate normal tissue effects in patients.

CDK1 has been reported to function in G1 and S-phase (reviewed in [13]. Although we did not specifically look at the effect of CDK1 inhibition on G1 or S-phase cells, our finding that CDK1 inhibition did not affect normal cells that are predominantly arrested in G1 suggests that at least CDK1 inhibition in G1 phase does not affect viability.

Our findings are in contrast with the studies mentioned earlier that suggested that targeting CDK1 is an attractive anti-cancer strategy with potentially minimal normal tissue effects. Johnson and co-workers showed that CDK1 inhibition using shRNA or RO-3306 sensitized non-small-cell lung cancer cells to DNA-damaging agents without affecting the sensitivity of normal RPE epithelial cells.[6] Similar to our findings, their lack of effect on normal cells could perhaps be explained by the cells being stalled at the time of treatment. Several studies also showed that inhibition of CDK1 in vivo reduces xenograft growth but does not cause any normal tissue toxicity.[7, 8, 10] However, it is possible there were more subtle or long-term side effects, which are not easily detectable in a mouse model.

As RO-3306 is cleared very rapidly from the bloodstream[7], it is not suitable for in vivo assays or clinical application. The CDK inhibitors that are currently in clinical development are all targeting multiple CDKs. Many of these have reported adverse side effects in patients, including myelosuppression, anemia, diarrhea and nausea.[14] Dinaciclib (MK-7965), a CDK1, 2, 5, 9 inhibitor has been associated with leukopaenia and thrombocytopaenia.[15, 16] The clinical development of the CDK1, 2, 9 inhibitor AZD5438 was discontinued partly because of low tolerability.[17] Although it is not possible to identify the relative contribution of each CDK subtype to adverse outcomes in these studies, our results indicate it is probable these effects are, in part, attributable to CDK1 inhibition.

In summary our data shows that CDK1 inhibitor-induced toxicity and radiosensitization is not restricted to tumor cells and as such CDK1 inhibition would be expected to exacerbate normal tissue toxicity if used clinically. Furthermore, our

observation that an apparent lack of effect in normal cells can be attributed to experimental setup has general implications for in vitro testing for tumor specificity of novel anti-cancer compounds. Our finding that normal cells become easily arrested under standard culture conditions is particularly relevant for evaluation of compounds that target the cell cycle progression or are directed against targets only active in particular cell cycle phases.

Material & Methods

Cell culture

HeLa, T24, MRC-5, HFL1 and RPE (ARPE-19) cells were purchased from American Type Culture Collection (ATCC). SQ20B cells were kindly provided by Dr. Ralph Weichselbaum (University of Chicago). HeLa and SQ20B cells were cultured in DMEM, T24 in RPMI, MRC-5 cells in MEM and HFL1 cells were cultured in DMEM/F-12. Media (Sigma) were supplemented with 10% Fetal Bovine Serum (FBS; Pan Biotech) and cells were grown at 37°C and 5% CO2. Cells were regularly tested for mycoplasma using the MycoAlert kit (Lonza). Cell lines grown beyond four months after purchase were authenticated by short tandem repeat (STR) profiling by the DNA Diagnostics Centre.

Compounds and antibodies

RO-3306 was purchased from Sigma, dissolved at 10mM in DMSO and stored in aliquots at -80°C. Nocodazole (Sigma) was dissolved in 100 µg/ml in DMSO and stored in aliquots at -80°C. A final concentration of 100 ng/ml was used. Tumor cells were incubated for 16 h in nocodazole, normal cells for 20 h in order to allow sufficient accumulation of mitotic cells for both cell types. MPM-2 antibody was from Millipore, mouse anti-human beta-actin from Sigma, HRP-conjugated goat anti-mouse antibody was from Thermofisher (Pierce).

Colony formation assays

Single cell suspensions cells were plated at low densities in 6-well plates. For unirradiated wells, 200 cells and 500 cells per well were plated for tumor and normal cells, respectively; the seeding density was doubled for each 2 Gy increase in radiation dose to account for cell death. Cells were left to adhere for a minimum of 3 hours at 37°C prior to compound addition. Plates were irradiated at 2, 4 and 6 Gy

using a cesium-137 irradiator (GSR D1 from Gamma Service; dose rate 1.24 Gy/min). Colonies were grown for 8-14 days, stained with crystal violet and counted using the Gelcount automated colony counter (Oxford Optronics).

Immunoblotting

Protein lysates were prepared using RIPA lysis buffer (Thermo Scientific) and proteins were separated using SDS-PAGE electrophoresis followed by immunoblotting. Bound antibodies were detected by developing film exposed to nitrocellulose membrane incubated with chemiluminescence reagent (SuperSignal, Millipore).

Flow cytometry

Cells were seeded into 6-well plates (3 x 10^5 cells/well) or T175 flasks at a concentration of 2 x 10^5 cells/flask. At the appropriate time point, cells were lifted in trypsin, fixed in ice cold 70% ethanol and after a PBS wash stained in propidium iodide (PI) staining solution (50 µg/ml PI, 200 µg/ml RNase). Stained cells were run on a BD FACScan flow cytometer and plots analysed using ModFit software.

Cell cycle analysis through BrdU incorporation

Cells were grown for three days to 80% confluence and pulse-labeled with 10 µM BrdU for the last 30 min. After BrdU removal, cells were lifted, reseeded and grown for 0 h, 4 h 10 h or 17 h prior to lifting and fixing in ice-cold 70% ethanol. Staining for incorporated BrdU was performed as described [18]. Cell cycle phase length was determined similarly as described in reference 19. S-phase length was determined by calculating the relative movement (RM) at t = 0 and t = 4 h: $RM = (F_L - F_{G1})/(F_{G2M} - F_{G1})$, where F_L is the mean propidium iodide (DNA) fluorescence of undivided BrdU-labeled cells, F_{G1} is the mean propidium iodide fluorescence of the G1 cells, and F_{G2M} is the mean propidium iodide fluorescence of the G2M cells. RM values were plotted over time and after linear curve fitting, the time where RM =1 (i.e. length of S-phase) was determined. The length of the G2M phase was determined by calculating the proportion of BrdU labeled cells in G1 in the whole population, $P_{G1\text{-}BrdU}$, at time points 4 h and 10 h: $P_{G1\text{-}BrdU} = P_{BrdU\text{-}G1} \times P_{BrdU}/P_{G1}$, where $P_{BrdU\text{-}G1}$ is the percentage of BrdU labeled cells in G1, P_{BrdU} the percentage of BrdU labeled cells in the whole population, and P_{G1} the percentage of all cells in G1. $P_{G1\text{-}BrdU}$ values were plotted over

time and after linear curve fitting, the time where $P_{G1\text{-}BrdU} = 0$ (i.e. length of G2M phase) was determined. The cell cycle time was determined by calculating the RM of the BrdU labeled cells that had reentered S-phase ($RM_{divided}$) at 17 h. By using the slope of the increase in RM over time (determined in the S-phase calculation), the time where $RM_{divided}$ would again reach the RM measured at the beginning of the time course experiment ($RM_{t=0h}$), i.e. the cell cycle time, was determined. G1 length was calculated by subtracting S-phase and G2M lengths from the cell cycle length.

Statistics

Curve fitting and statistical analysis (two tailed t-tests and ANOVA) was performed in GraphPad Prism (version 6.0d). A p-value of < 0.05 was considered significant. Radiation survival data was fitted using non-linear regression with the linear quadratic equation: $S = \exp - (\alpha D + \beta D^2)$, S denotes survival probability, D (Gy) is radiation dose and α (Gy-1) and β (Gy-2) are parameter constants. The sensitization enhancement ratio at 10% surviving fraction (SER10) was calculated: SER10: $D_{untreated} / D_{treated}$, where $D_{untreated}$ and $D_{treated}$ are the radiation doses yielding 10% survival as calculated using the derived α and β values. To assess statistical difference between the two curves, a factorial 2-way ANOVA was performed with survival as the dependent variable and dose levels (2, 4 and 6 Gy) and treatment (RO-3306) as the two factors. Single agent IC50 values for RO-3306 were determined by four-parameter non-linear regression on survival fractions versus log-transformed compound concentrations.

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Disclosure of Interest

The authors report no conflict of interest

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Figure legends

Fig. 1. CDK1 inhibition sensitizes tumor cells but not normal cells to radiation.
A. Clonogenic survival assay of **(A)** tumor cells (HeLa, T24, and SQ20B) and **(B)** normal cells (HFL1, MRC-5 and RPE) treated with RO-3306 prior to radiation. Cells were seeded as single cells and treated with 5 µM RO-3306 from 3 h after seeding. Cells were irradiated 20 h after compound addition followed by a medium change. Colonies were stained at 7 - 13 days after seeding. Panels are representative of three independent experiments, data is presented as mean +/- SD from triplicate wells, p-values indicated were derived through two-way ANOVA testing whether the curves are statistically different.

Fig. 2. RO-3306 treatment causes inhibition of downstream signaling and cell cycle arrest in both tumor and normal cells.
A. Western blot stained with the MPM-2 antibody, recognizing phosphorylated CDK1 substrates. Cultures were enriched for mitotic cells by overnight nocodazole treatment followed by 2 h of 5 µM RO-3306 in the continuous presence of nocodazole. Both floating and adherent cells were lysed and subjected to immunoblotting. Image is representative of three independent experiments. For clarity, a longer exposure is shown for the normal cell lysates; a comparison of different exposure times is shown in supplementary Fig. 2.
B. Cell cycle profile of tumor and normal cells treated with RO-3306. Cells were seeded in 6-well plates and treated with 5 µM RO-3306 for 20 h. Cells were lifted, fixed in 70% ethanol and stained with propidium iodide followed by flow cytometry Representative histograms are shown (dark grey: G1, white: S; light grey: G2/M). Graph shows percentages in each cell cycle phase determined through curve fitting with ModFit and show the mean +/- sd from two independent experiments. Significant increases in G2/M proportion after RO-3306 treatment are indicated (* $p < 0.05$).

Fig. 3. Normal cells are sensitive to CDK1 inhibition in a cell cycle dependent manner.
A. Investigation of the cell cycle progression of untreated HFL1, MRC-5 and RPE cells plated for colony formation assays. Cultures were grown for three days to 80%

confluence, lifted and reseeded in the absence of drug. Cells were then fixed for cell cycle analysis at the time points indicated. Representative histograms are shown (dark grey: G1, white: S; light grey: G2/M). Graphs show percentages in each cell cycle phase over time determined through curve fitting with ModFit and show the mean +/- sd from three independent experiments. Significant differences in G2/M proportion from t=0 are indicated (* $p < 0.05$).

B. Clonogenic survival assay of HFL1, MRC-5 and RPE cells treated with RO-3306 at different time points. Cultures were grown for three days to 80% confluence, lifted and seeded as single cells and 5 µM RO-3306 was added at 3 or 24 h after seeding, followed by a medium change after 20 h of compound incubation. Colonies were stained after 12 days. Representative of three independent experiments, data is presented as mean +/- sd from triplicate wells. Statistical differences were confirmed by t-tests, *$p < 0.05$

Fig. 4. Normal and tumor cells are equally sensitive to continuous CDK1 inhibition.

A. Clonogenic survival assay of tumor and normal cells treated for 20 h or continuously with increasing concentration of RO-3306. Cultures were grown for three days to 80% confluence, lifted and seeded as single cells. RO-3306 was added 3 h after seeding and either removed after 20 h or left for the duration of the experiment. Colonies were stained after 12 days. Representative of three independent experiments, data is presented as mean +/- SD from triplicate wells.

B. Comparison of IC50 values for tumor and normal cells. IC50 values were determined for each cell line using the data in panel A. Mean IC50 was calculated for both tumor and normal cells grouped together. Statistical differences were confirmed by t-tests, ** $p < 0.01$

Fig. 5. CDK1 inhibition sensitizes normal cells to radiation in a cell cycle dependent manner.

A. Clonogenic survival assay of cells treated with RO-3306 at different time points followed by radiation. Cells were grown in T75 flasks for three days, lifted and plated as single cells in 6-well plates. RO-3306 (5 µM) was added either at 3 h after seeding (for stalled cells) or at 24 h after seeding (when many cells are entering G2), and cells were irradiated 20 h later followed by a medium change. Colonies were stained after

12 days. Representative of three independent experiments, data is presented as mean +/- SD from triplicate wells.

Fig 1

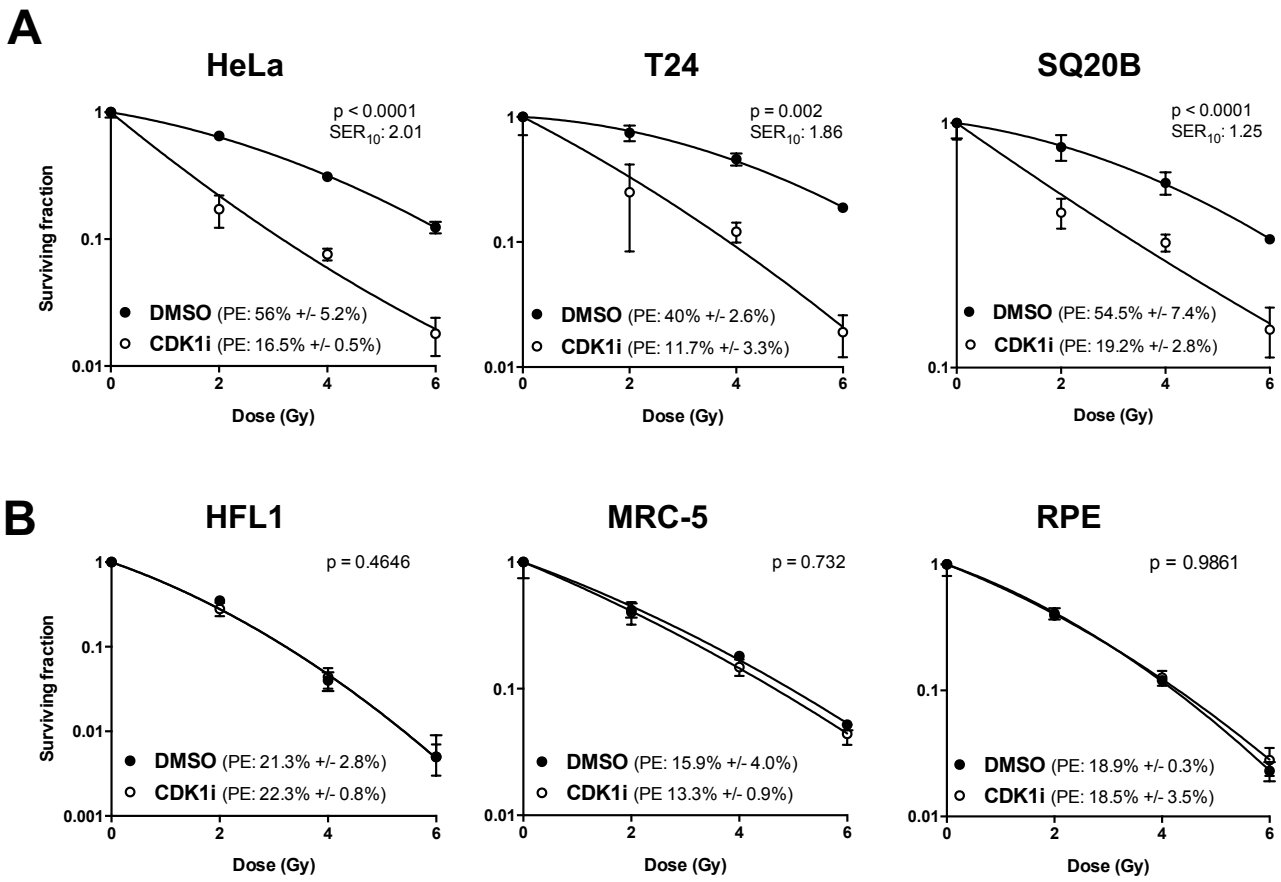
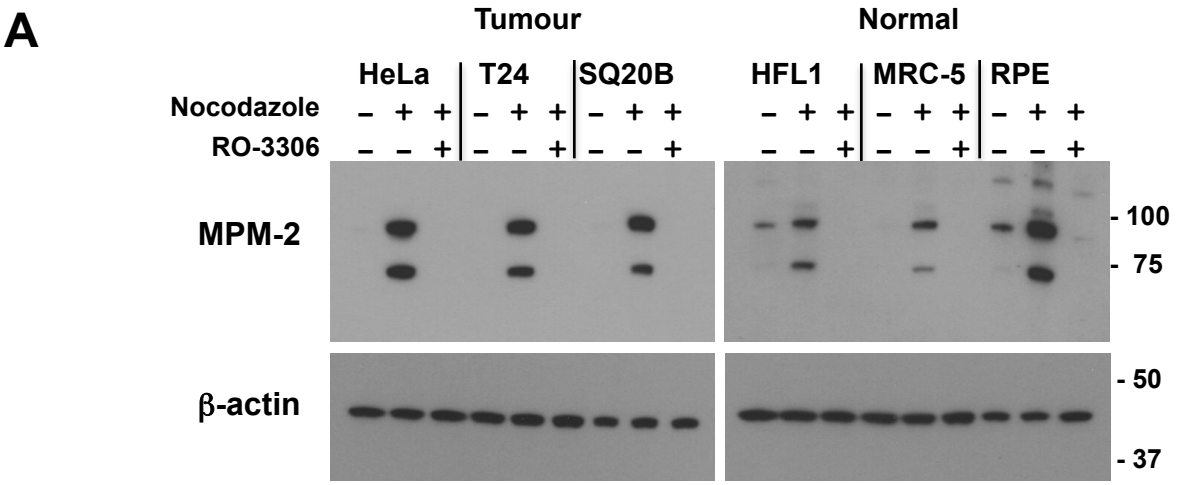

A
HeLa
p < 0.0001
SER10: 2.01
DMSO (PE: 56% +/- 5.2%)
CDK1i (PE: 16.5% +/- 0.5%)
T24
p = 0.002
SER10: 1.86
DMSO (PE: 40% +/- 2.6%)
CDK1i (PE: 11.7% +/- 3.3%)
SQ20B
p < 0.0001
SER10: 1.25
DMSO (PE: 54.5% +/- 7.4%)
CDK1i (PE: 19.2% +/- 2.8%)
Surviving fraction
Dose (Gy)
B
HFL1
p = 0.4646
DMSO (PE: 21.3% +/- 2.8%)
CDK1i (PE: 22.3% +/- 0.8%)
MRC-5
p = 0.732
DMSO (PE: 15.9% +/- 4.0%)
CDK1i (PE 13.3% +/- 0.9%)
RPE
p = 0.9861
DMSO (PE: 18.9% +/- 0.3%)
CDK1i (PE: 18.5% +/- 3.5%)

Fig 2


A
Tumour
Normal
HeLa
T24
SQ20B
HFL1
MRC-5
RPE
Nocodazole
RO-3306
MPM-2
β-actin
100
75
50
37

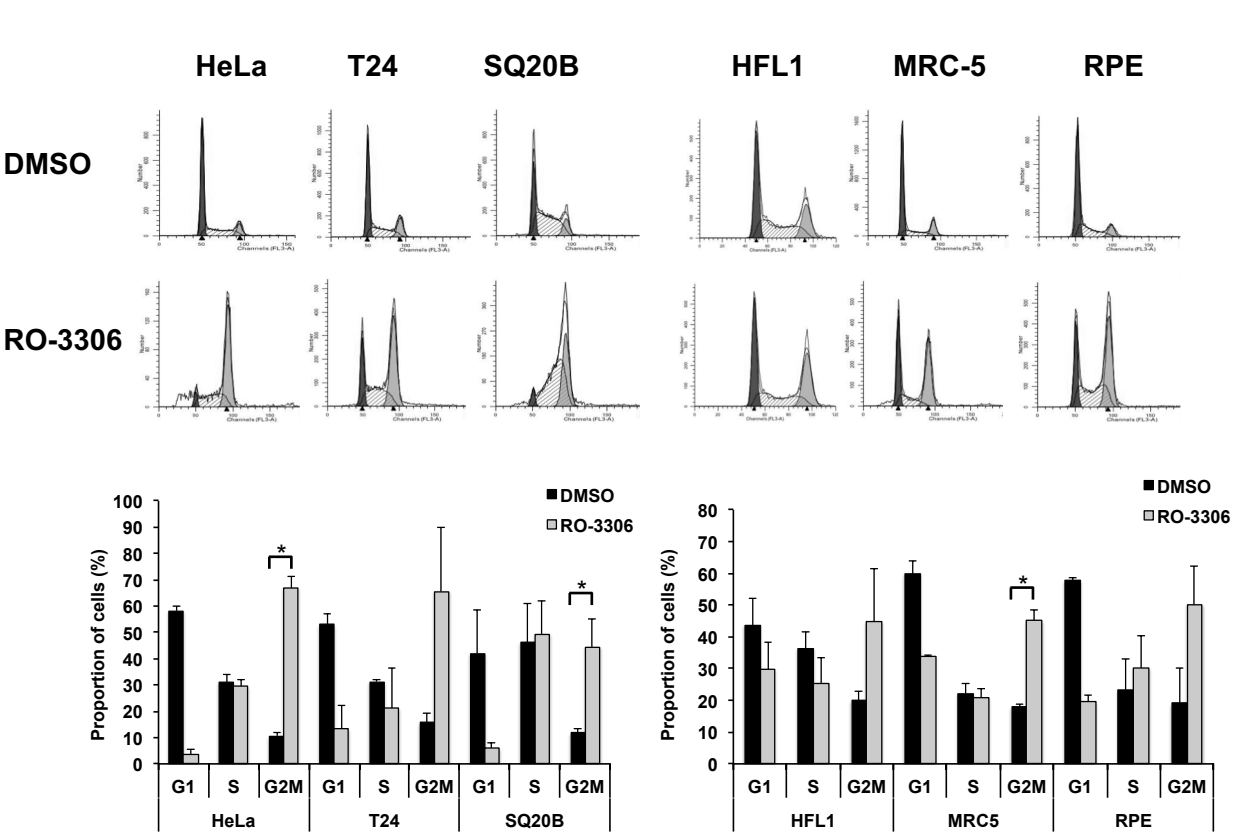

B
HeLa
T24
SQ20B
HFL1
MRC-5
RPE
DMSO
RO-3306
Proportion of cells (%)
G1
S
G2M
HeLa
T24
SQ20B
HFL1
MRC5
RPE

Fig 3

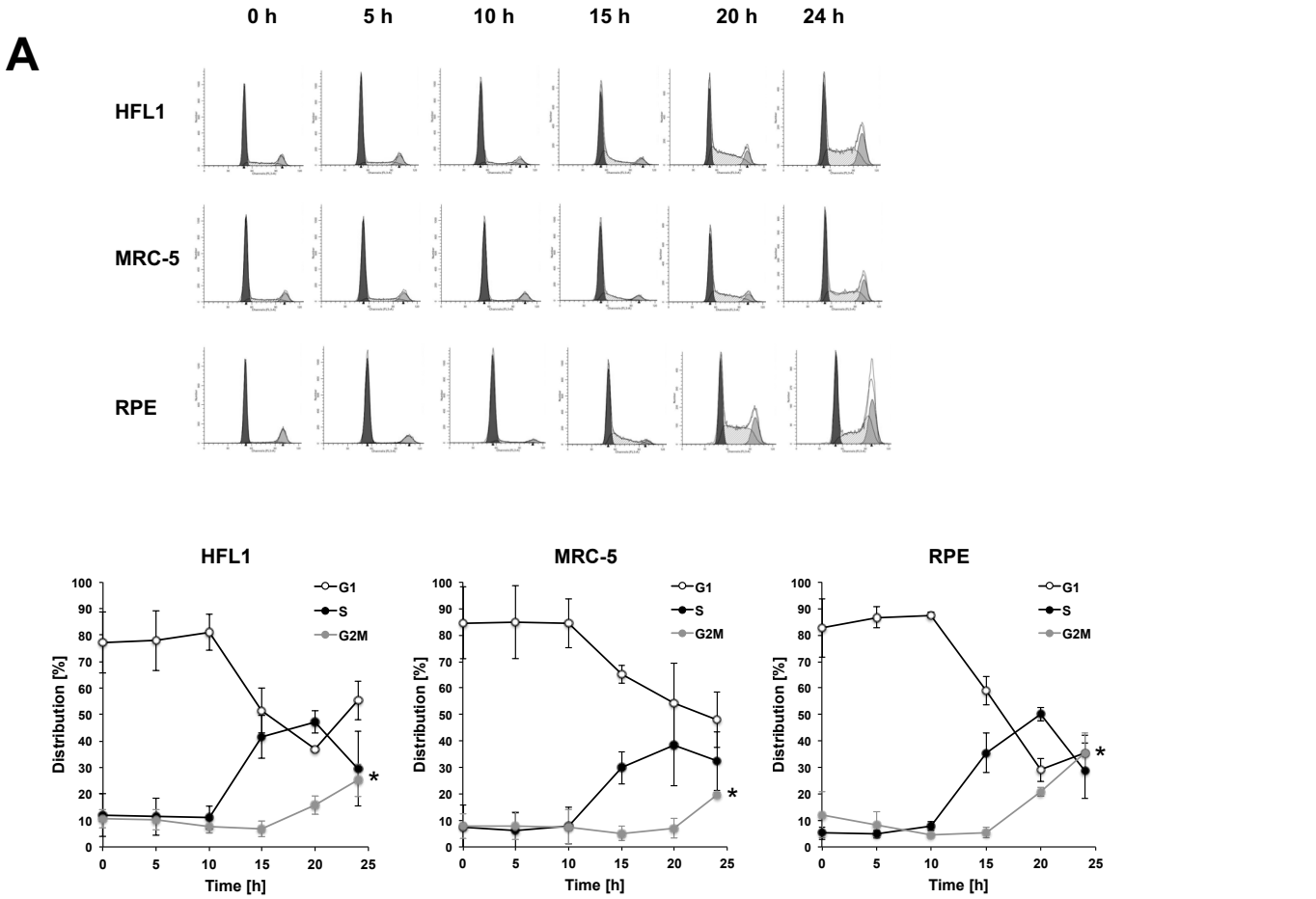
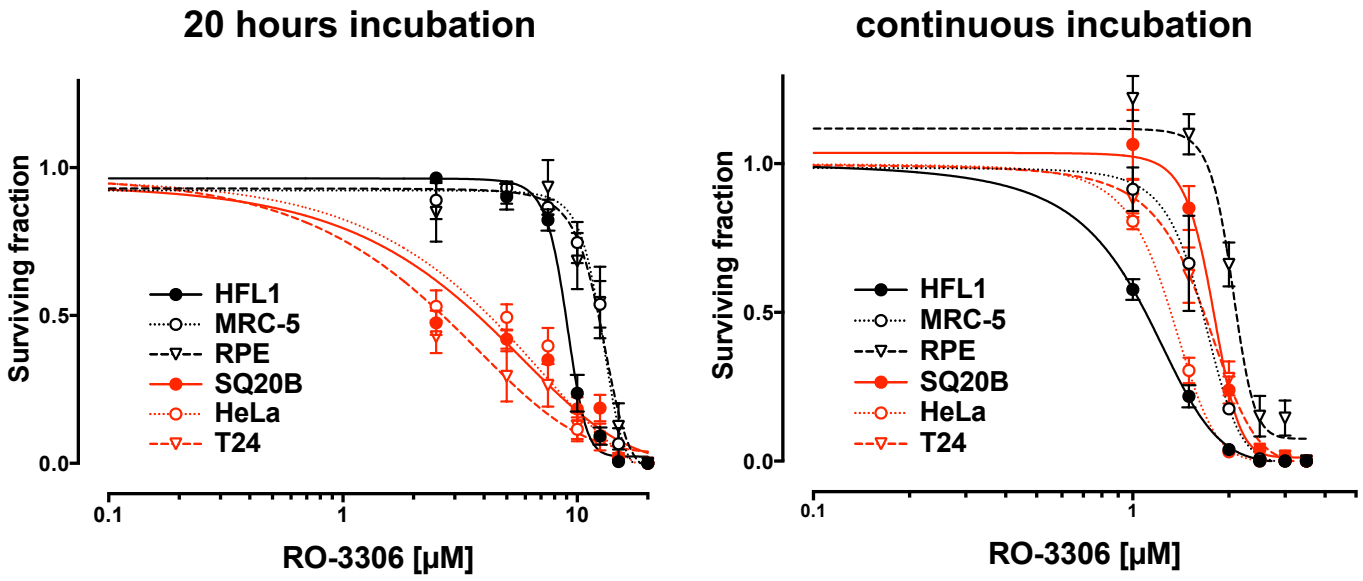

A
0 h
5 h
10 h
15 h
20 h
24 h
HFL1
MRC-5
RPE
HFL1
MRC-5
RPE
G1
S
G2M
Distribution [%]
Time [h]
*
B
3 h settling time
24 h settling time
Plating efficiency
DMSO
CDK1i
HFL1
MRC-5
RPE

Fig 4

A


20 hours incubation
continuous incubation
Surviving fraction
1.0
0.5
0.0
HFL1
MRC-5
RPE
SQ20B
HeLa
T24
0.1
1
10
RO-3306 [μM]

B

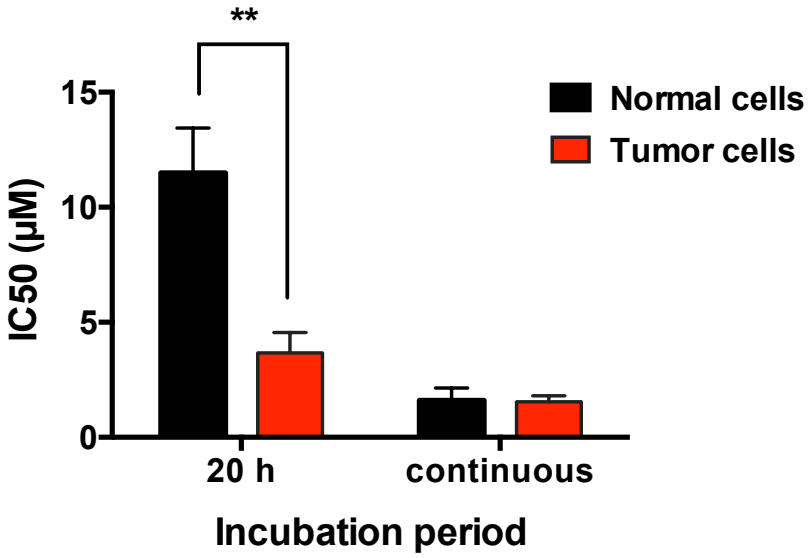
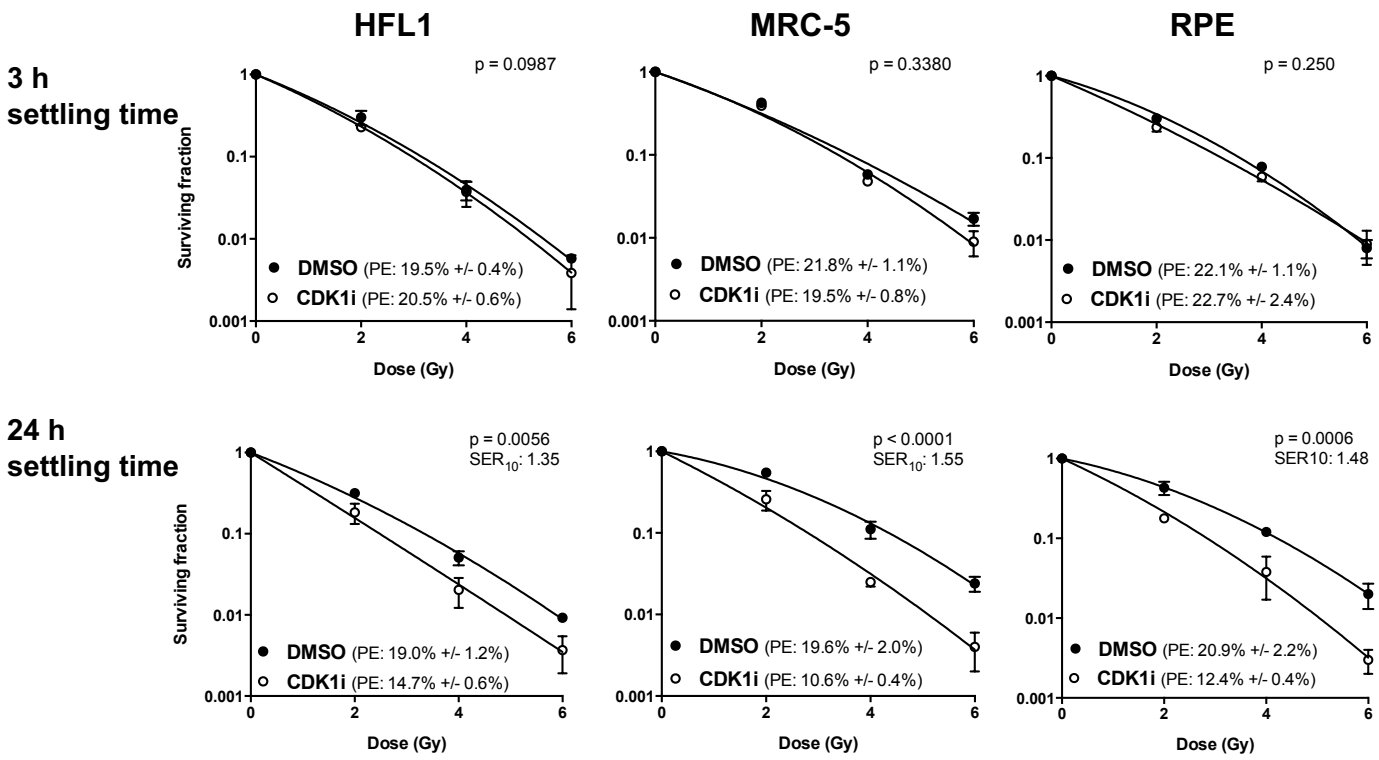
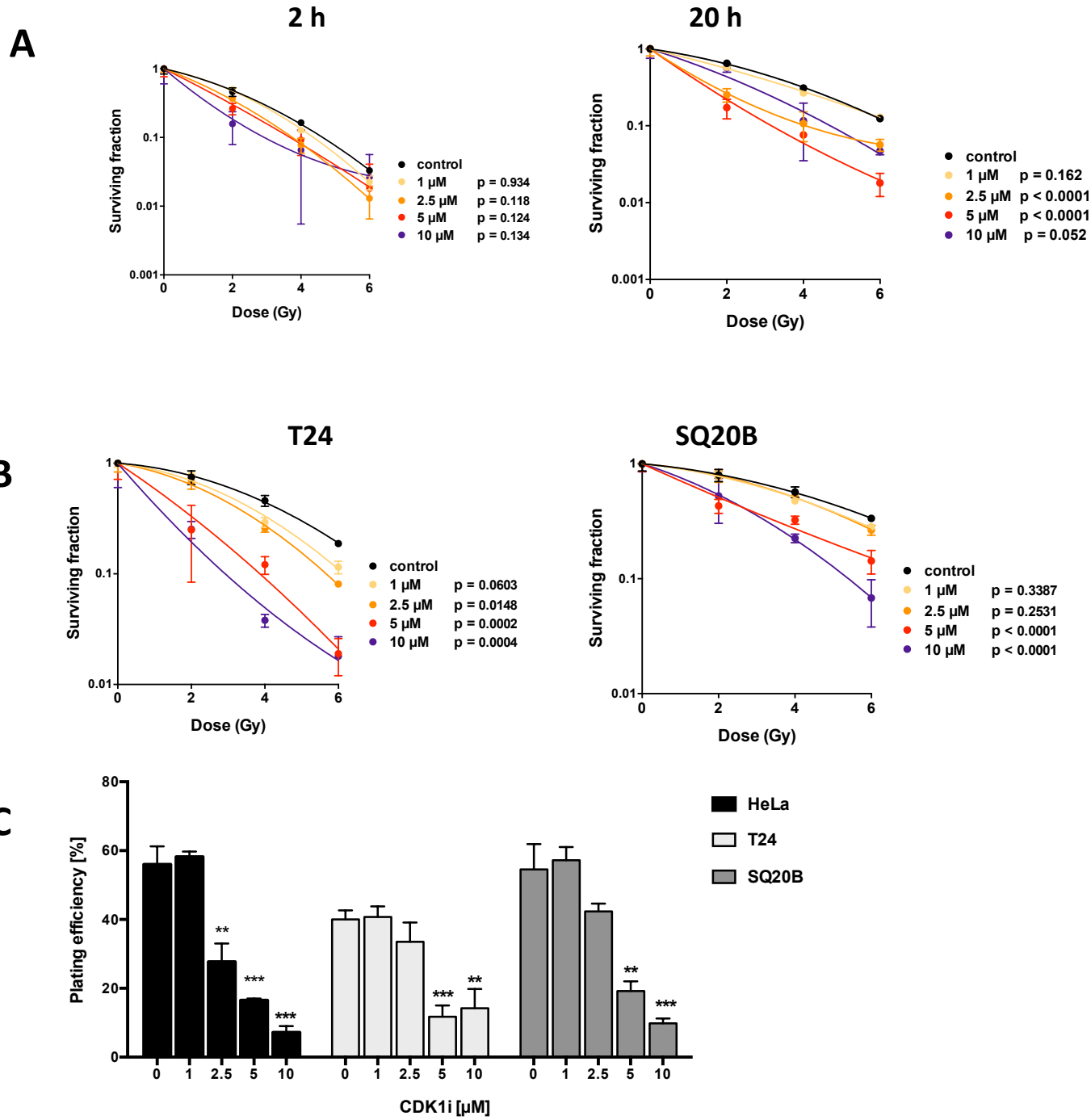

**
IC50 (μM)
15
10
5
0
Normal cells
Tumor cells
20 h
continuous
Incubation period

Fig 5


HFL1
MRC-5
RPE
3 h
settling time
24 h
settling time
Surviving fraction
Dose (Gy)
p = 0.0987
DMSO (PE: 19.5% +/- 0.4%)
CDK1i (PE: 20.5% +/- 0.6%)
p = 0.3380
DMSO (PE: 21.8% +/- 1.1%)
CDK1i (PE: 19.5% +/- 0.8%)
p = 0.250
DMSO (PE: 22.1% +/- 1.1%)
CDK1i (PE: 22.7% +/- 2.4%)
p = 0.0056
SER10: 1.35
DMSO (PE: 19.0% +/- 1.2%)
CDK1i (PE: 14.7% +/- 0.6%)
p < 0.0001
SER10: 1.55
DMSO (PE: 19.6% +/- 2.0%)
CDK1i (PE: 10.6% +/- 0.4%)
p = 0.0006
SER10: 1.48
DMSO (PE: 20.9% +/- 2.2%)
CDK1i (PE: 12.4% +/- 0.4%)

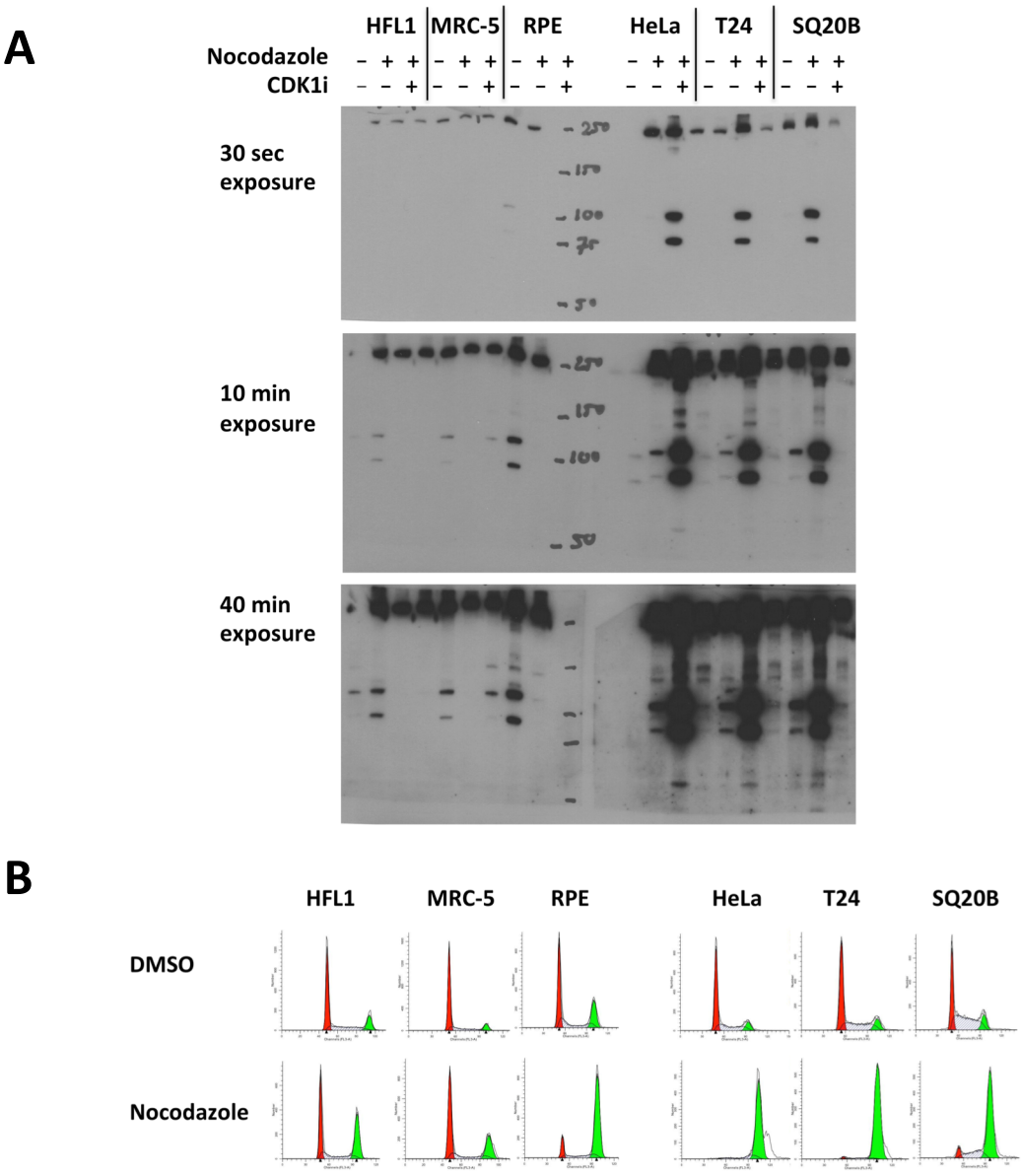


Suppl. Fig. 1: Optimization of RO-3306 concentration and timing.

A. Colony formation assay of HeLa cells treated with increasing concentrations of RO-3306 3 h after seeding. Cells were irradiated either 2 h after compound addition followed by a medium change 16 h later, or 20h after compound addition followed by a medium change immediately after irradiation. Data are presented as mean +/- SD from triplicate wells. P-values were derived by two-way ANOVA testing whether a treatment curve was statistically different from the control curve.

B. Colony formation assay of T24 and SQ20B cells treated with increasing concentrations of RO-3306 3 h after seeding followed by irradiation 20 h later. Medium was changed immediately after irradiation. Data are presented and analyzed as in (A).

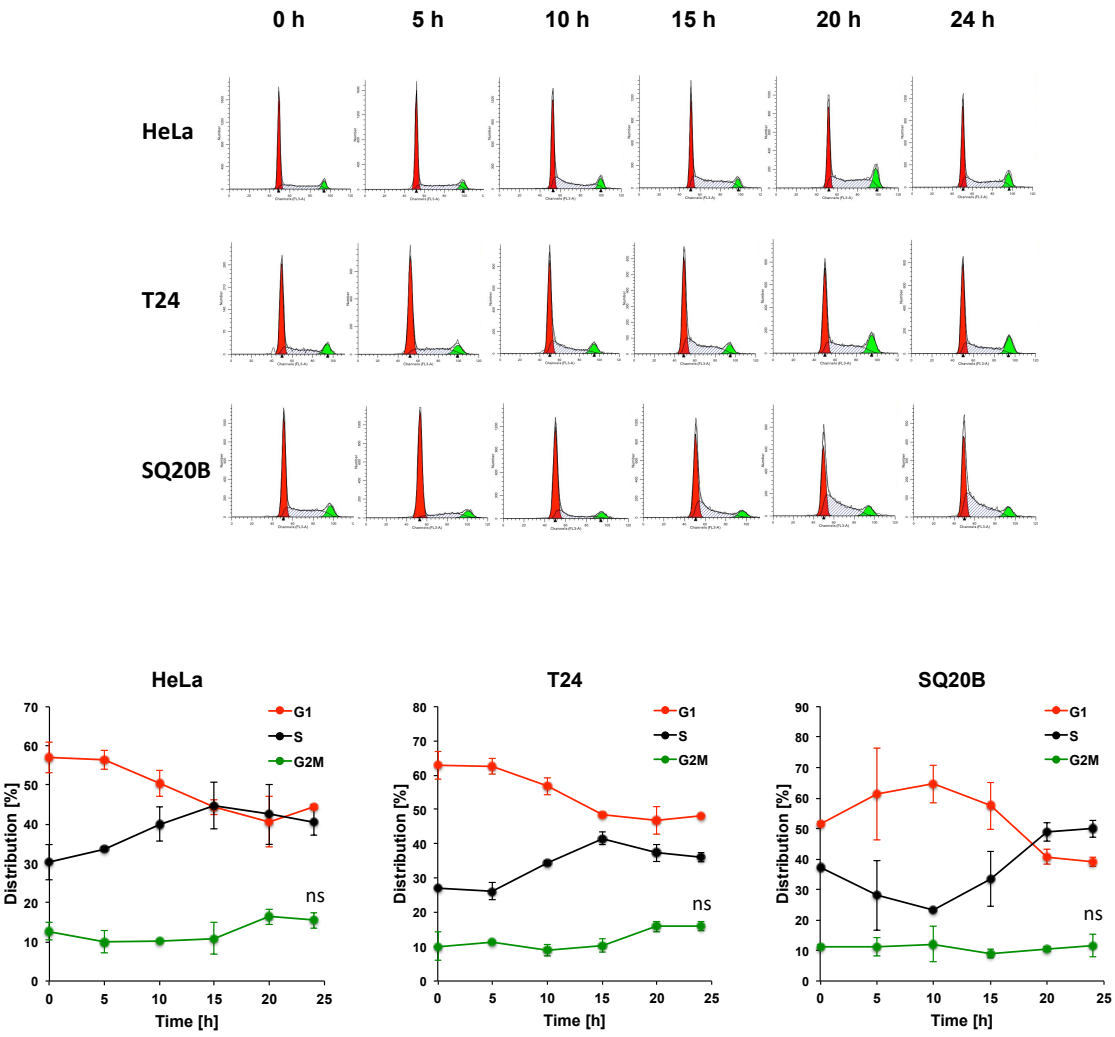
C. Plating efficiencies for HeLa, T24 and SQ20B cells treated with increasing concentrations of RO-3306 for 20 h. Values show mean +/- SD from triplicate wells and those that are significantly different from untreated cells are indicated (** $p < 0.01$; *** $p < 0.001$).



Suppl Fig. 2. Biochemical confirmation of RO-3306 activity.

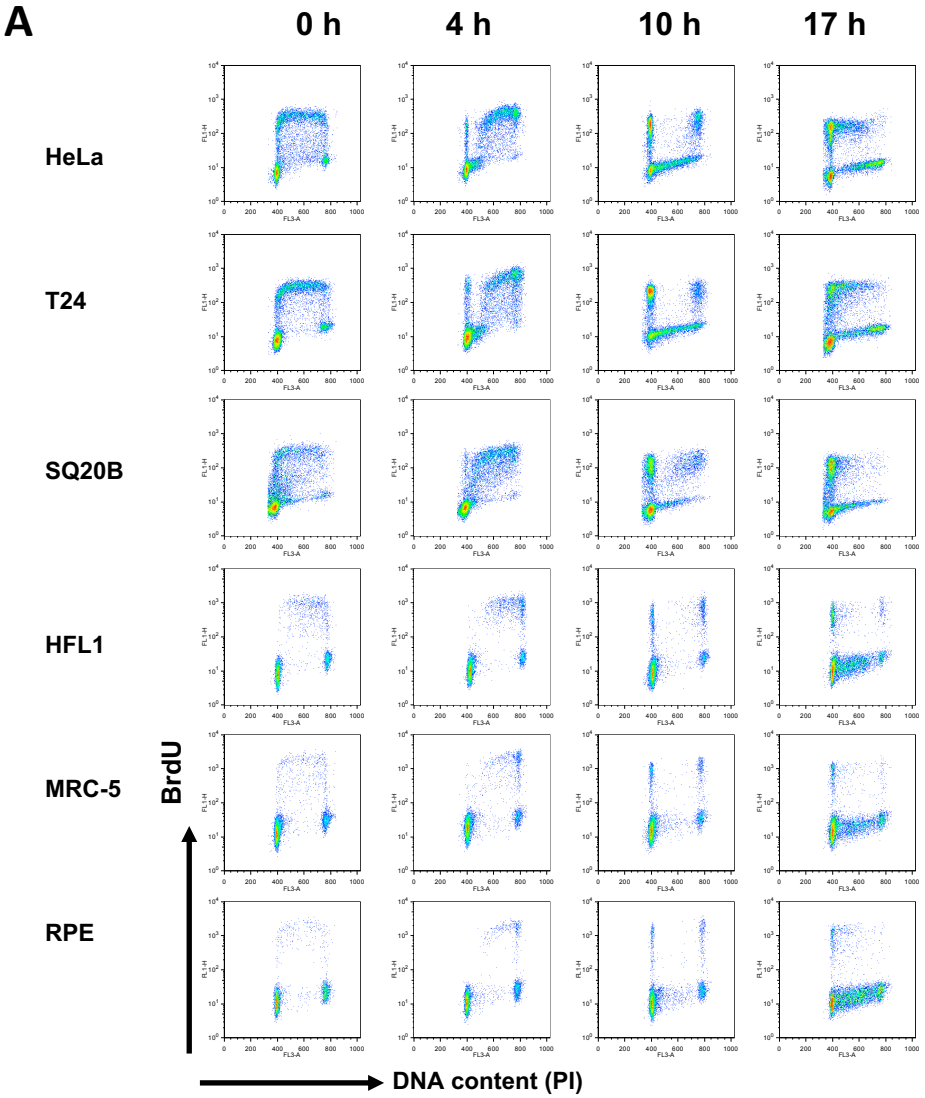
A. Western blots from Fig 2 in the main text, showing multiple exposure times. Cultures were enriched for mitotic cells by nocodazole (100 ng/ml) treatment (16 h for tumor cells, 20 h for normal cells) followed by 2 h of 5 µM RO-3306 in the continuous presence of nocodazole. Both floating and adherent cells were lysed and subjected to immunoblotting. Representative of three independent experiments.

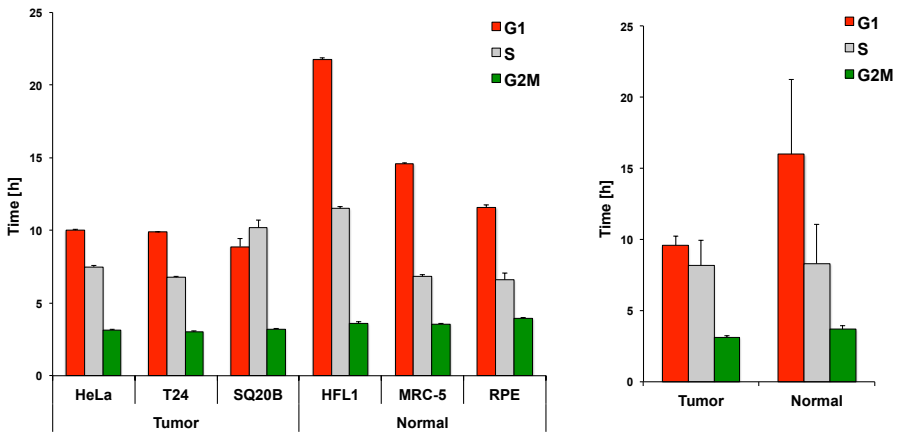
B. Confirmation of G2/M arrest in nocodazole treated cells used for the Western blot analysis in panel A. Cultures were enriched for mitotic cells by nocodazole (100 ng/ml) treatment (18 h for tumor cells, 22 h for normal cells). Cells were then lifted, fixed in 70% ethanol and stained with propidium iodide followed by flow cytometry and curve fitting with ModFit software (red: G1; white: S; green: G2/M).



Suppl. Fig. 3. Cell cycle progression in tumor cells

Investigation of the cell cycle progression of untreated HeLa, T24 and SQ20B cells plated for colony formation assays. Cultures were grown for three days to 80% confluence, lifted and reseeded. Cells were then fixed for cell cycle analysis at the time points indicated. Representative histograms are shown. Graphs show the percentages in G1, S and G2/M phase as determined through ModFit curve fit analysis and plotted as mean +/- sd (n=2). No significant difference was found between the fraction of G2M cells at t = 0 and at any of the subsequent time points (ns).

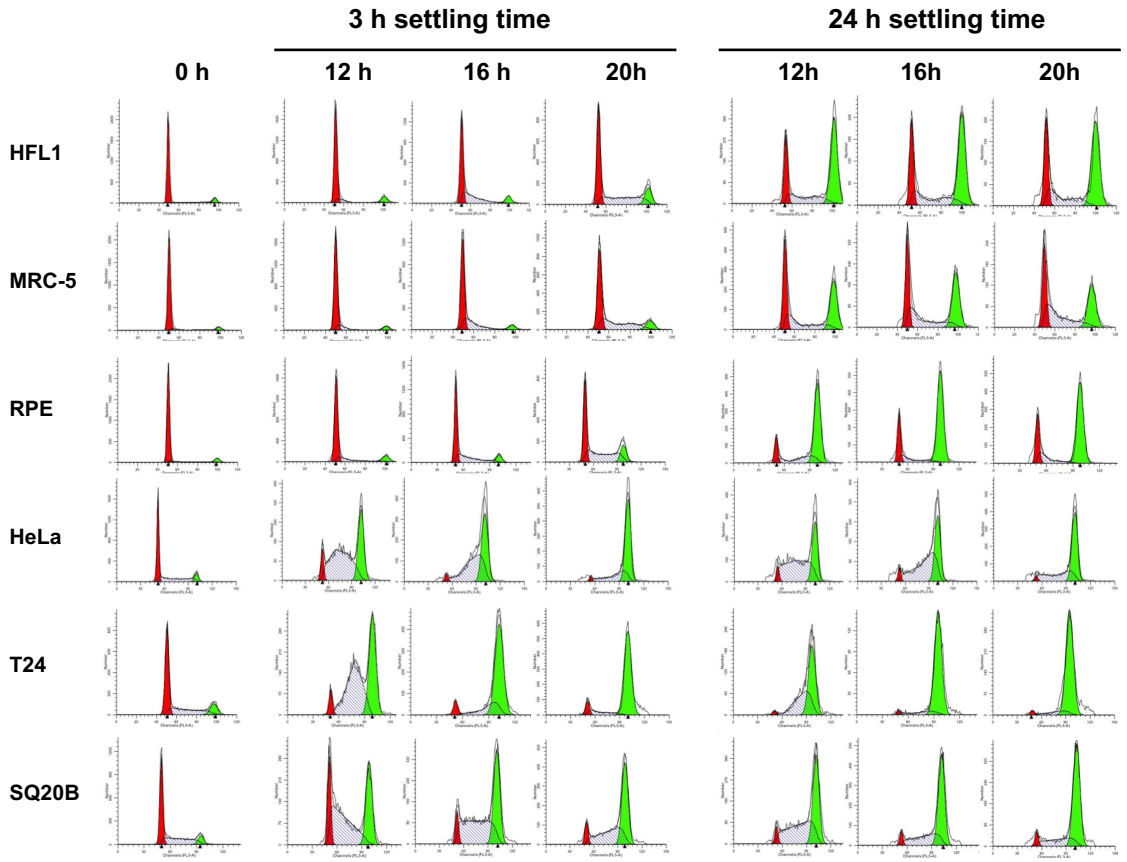


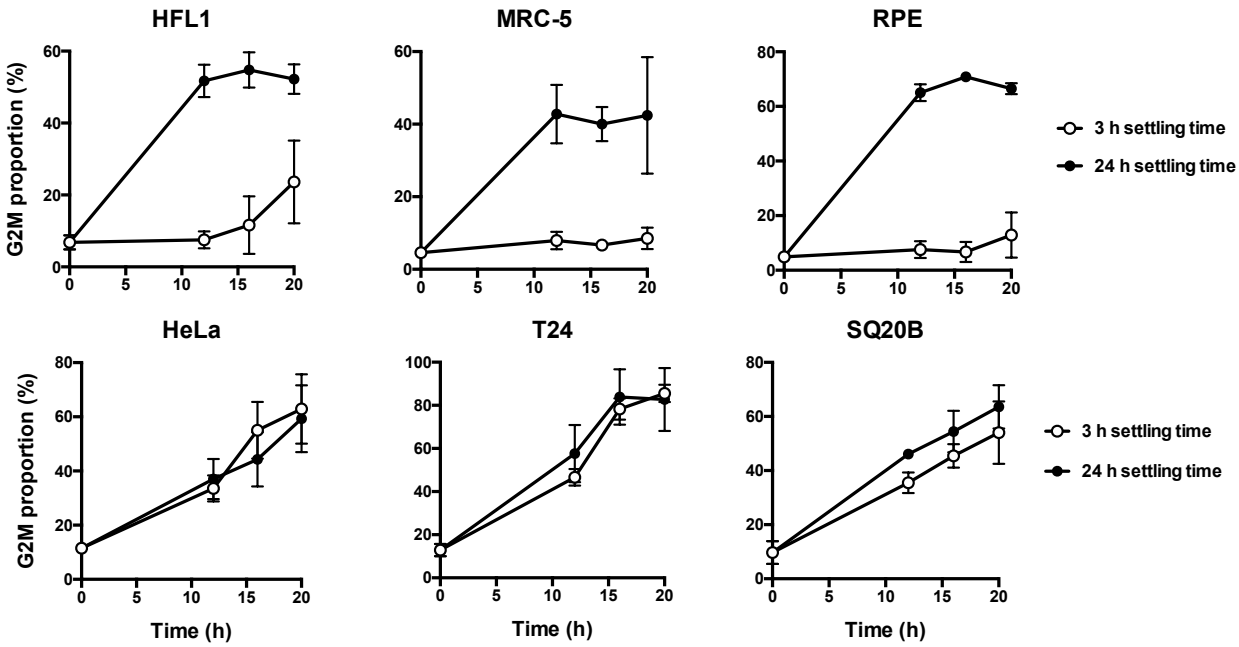


Suppl. Fig. 4. Cycling tumor and normal cells have comparable cell cycle phase lengths

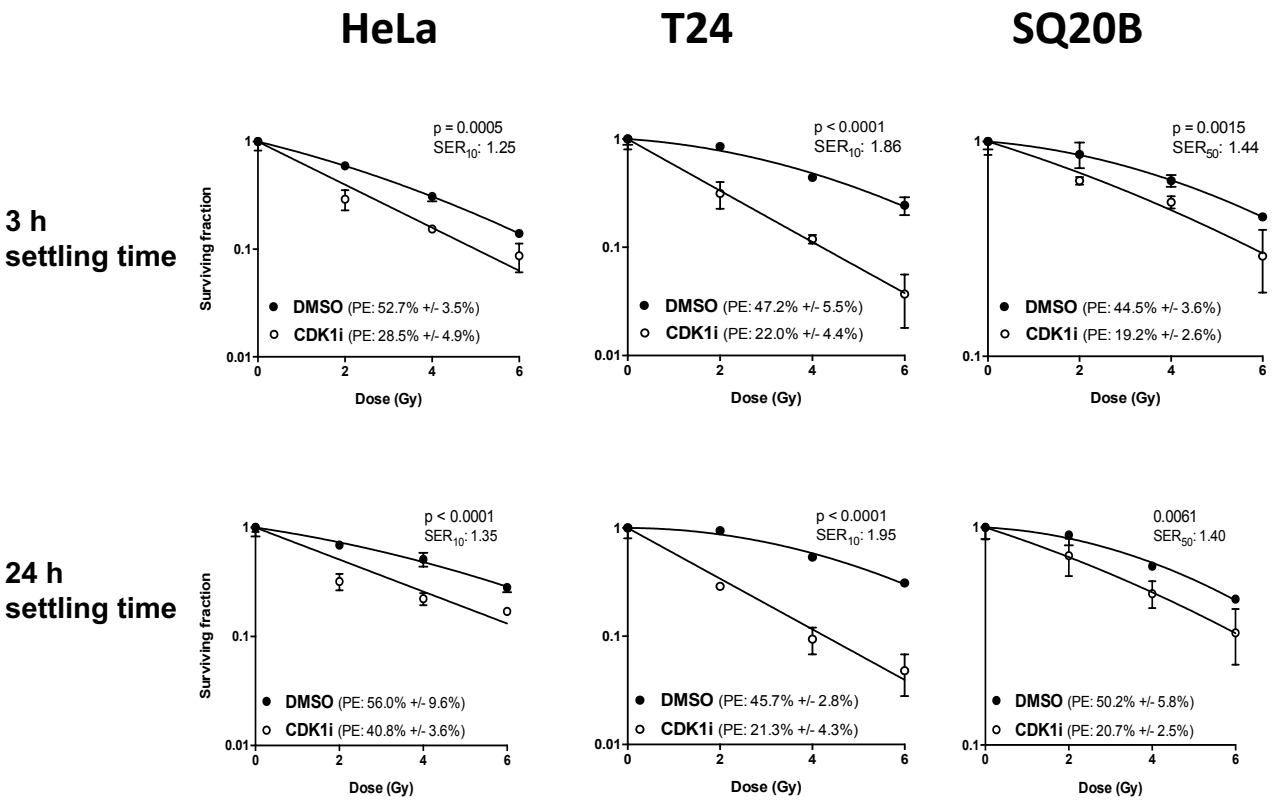
A) Pulse chase BrdU/PI profiles of normal cells analyzed at different time points after replating. Cells were grown for three days to 80% confluence and pulse-labeled with BrdU for 30 min just prior to replating. Reseeded cells were fixed at 0 h, 4 h 10 h and 17 h after replating and stained for BrdU and DNA content.

B) Cell cycle phase duration in tumor and normal cells determined through the BrdU pulse chase experiment shown in (A). Values show the means of duplicate acquisitions +/- sd.





Suppl. Fig. 5. CDK1 inhibitor-induced accumulation in G2M is affected by settling time in normal cells. Normal and tumor cell lines were grown for three days to 80% confluence, replated and treated with 5 µM RO-3306 added at 3 h or 24 h after seeding. Cell cycle profiles were analyzed at 12 h, 16 h and 20 h after RO-3306 addition. Graphs show the accumulation in the G2M phase over time as determined through ModFit curve fit analysis and plotted as mean +/- sd from two independent experiments.



Suppl. Fig. 6. CDK1 inhibition sensitizes tumor cells to independently of settling time. Clonogenic survival assay of tumor cells treated with RO-3306 at different time points after seeding followed by radiation. Cells were grown in T75 flasks for three days, lifted and plated as single cells in 6-well plates. RO-3306 (5 µM) was added either at 3 h after or 24 h after seeding, and cells were irradiated 20 h after compound addition followed by a medium change. Colonies were stained after 14 days. Representative of three independent experiments, data is presented as mean +/- SD from triplicate wells.