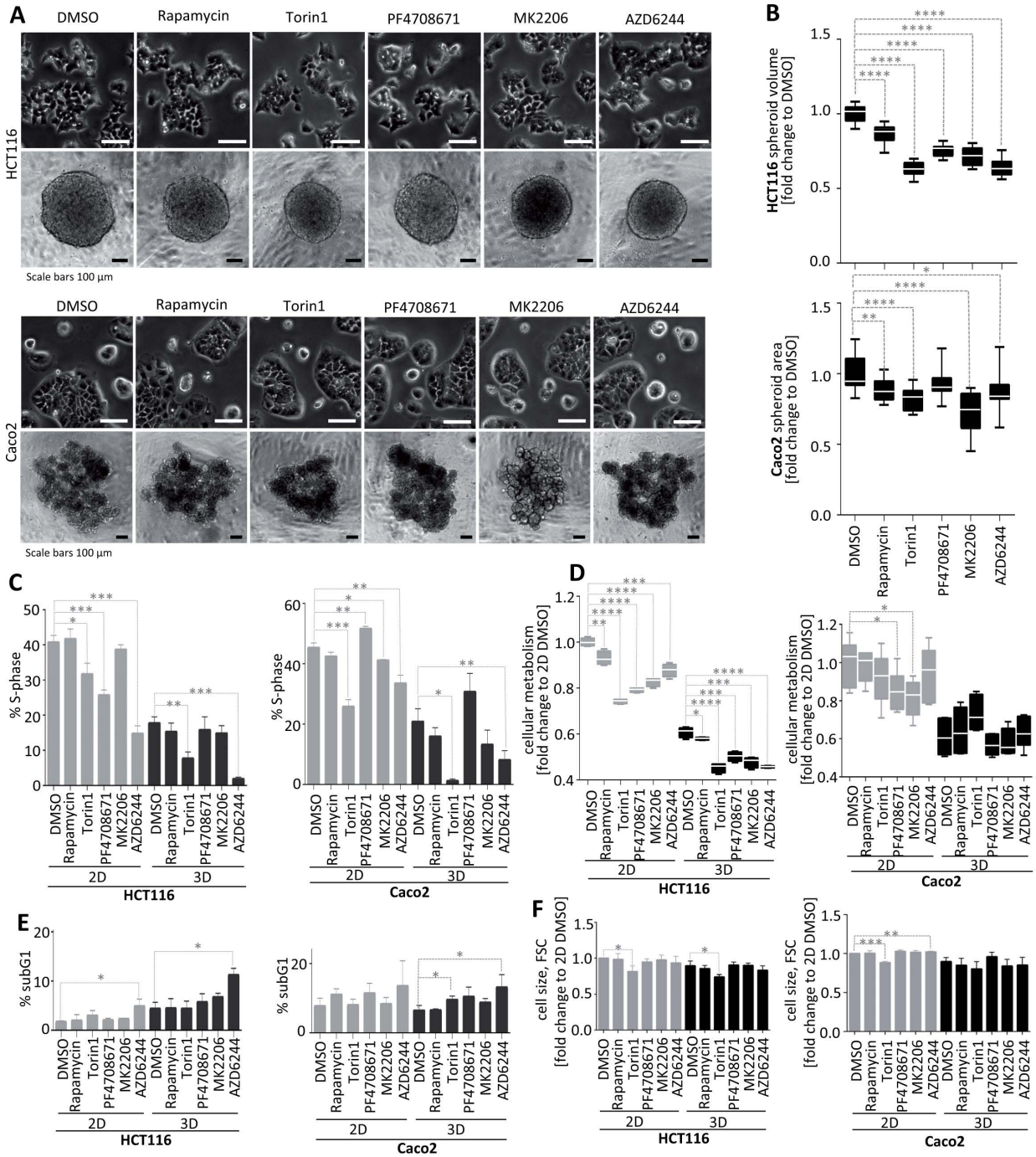
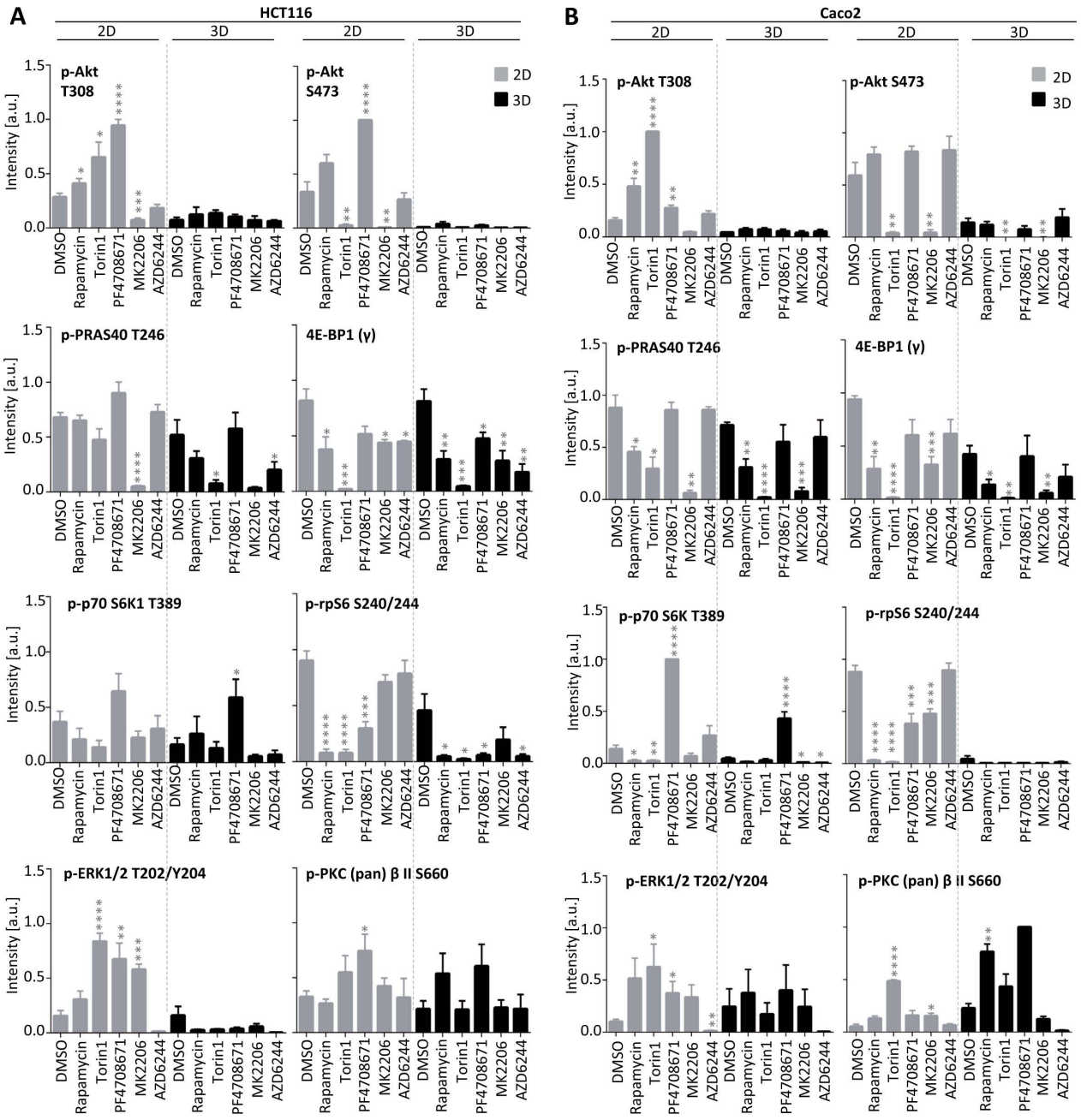
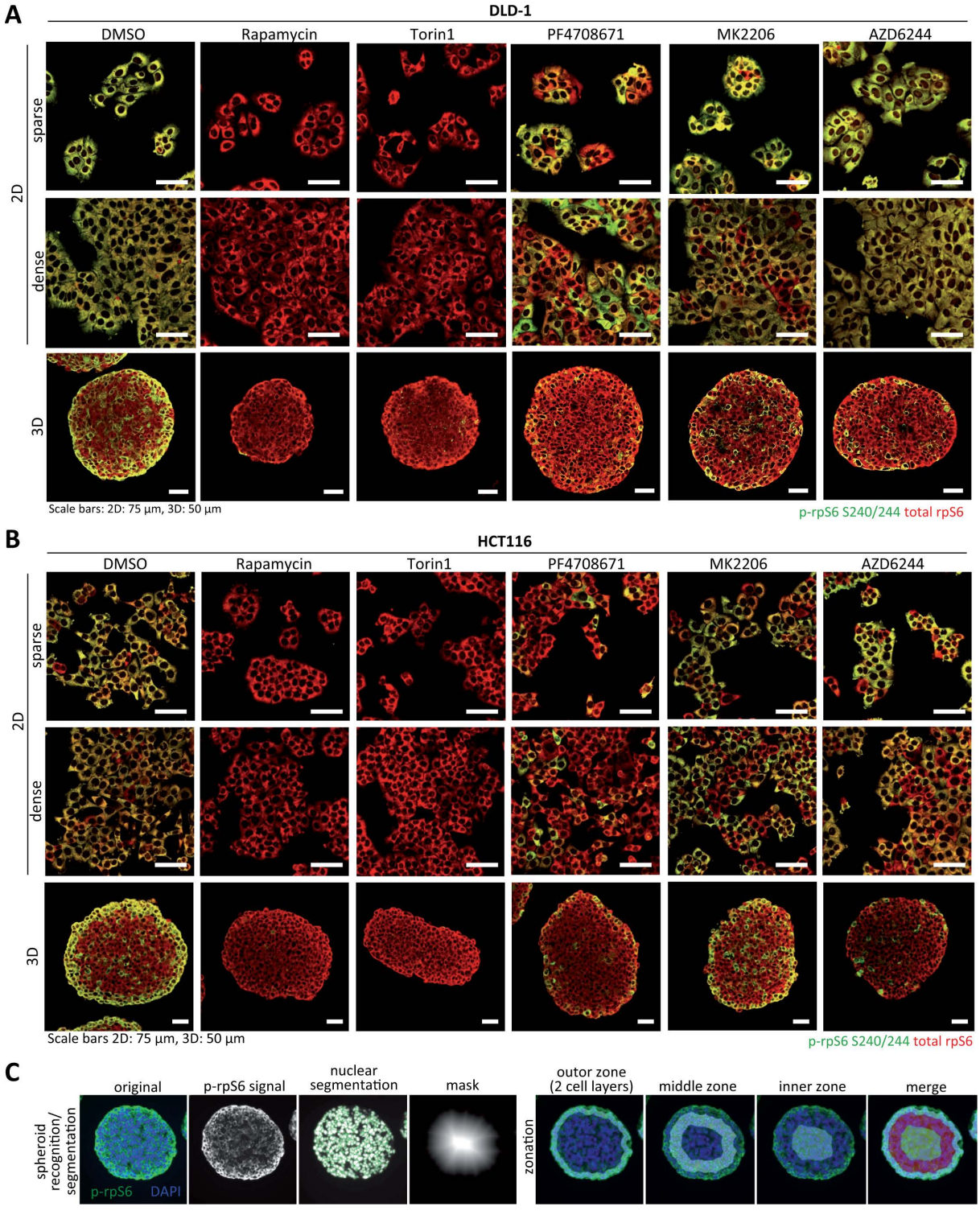


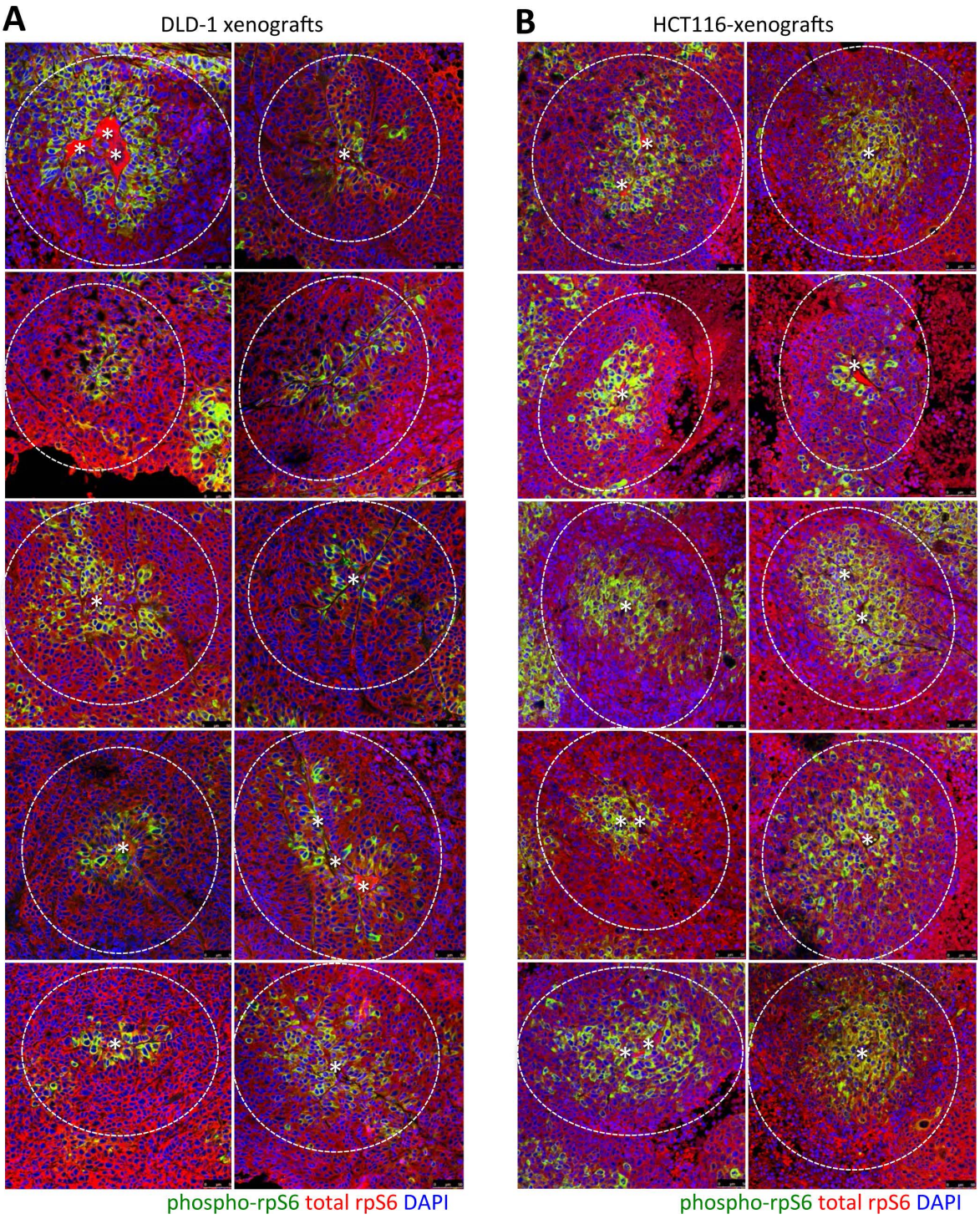
Supplementary Figure S1 – Morphologic and phenotypic analysis of colon cancer spheroids compared to monolayer cultures. HCT116 and Caco2 were grown in 2D and 3D for 24h and treated with Rapamycin (100 nM), the mTOR inhibitor Torin1 (250 nM), the S6K1 inhibitor PF4708671 (10 µM), the AKT inhibitor MK2206 (1 µM) and the MEK1 inhibitor AZD6244 (1 µM) for another 24h. DMSO served as control. Representative brightfield microscopic images of HCT116 and Caco2 (**A**) are shown. Scale bars represent 100 µm. For HCT116 (n=15 per condition) and Caco2 (n=15 per condition), the spheroid volume and the spheroid area (**B**), respectively, were calculated. The percentage of HCT116 and Caco2 in 2D and 3D cultures in S-phase (**C**) and subG1-phase (**E**) was determined using the Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Invitrogen, Thermo Scientific Inc., Waltham, MA). Metabolic activity (**D**) of HCT116 and Caco2 cells cultured as monolayers and spheroids ± inhibitors was evaluated using the CellTiter-Glo luminescence assay (Promega, Madison, WI) by analyzing the level of ATP present within the cells (n = 6 per condition). Evaluation of the forward scatter (FSC) was used for determining cell size of HCT116 and Caco2 (**F**) 2D and 3D cultures. Bar graphs represent the mean ± standard deviation (SD). Boxplots were created in GraphPad Prism 4. The box represents the middle 50% of the data ranging from the lower quartile (Q1) to the upper quartile (Q3). The line within the box indicates the median (Q2) and the whiskers represent the lower and upper 25% of the data - the minimum and maximum values respectively. Detailed data are available in supporting figure 2.



Supplementary Figure S2 – PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in the presence or absence of inhibitory compounds. HCT116 and Caco2 colon cancer cells, grown in untreated conditions in 2D and 3D (3,000 cells per spheroid) for 24h, were treated with DMSO, 100 nM Rapamycin, 250 nM Torin1, 10 µM PF4708671, 1 µM MK2206 and 1 µM AZD6244 for another 24h. 2D and 3D cultures were harvested and subjected to Western blot analysis. The bands in Western blots from two or three independent experiments were evaluated densitometrically with ImageJ (n=2-8 per condition). Quantification is shown for (**A**) HCT116 and (**B**) for Caco2 cells. Bars are mean integrated density ±SEM. For statistical analysis Student's t-test (unpaired, two-tailed**)** was performed. p-values are indicated. Detection of phospho-proteins is indicated by a prefixed "p-". α-tubulin and GAPDH served as loading control. Western blots used for this quantification can be viewed in Supporting Figure SF5 for HCT116 and in Supporting Figure SF6 on fig**share**.



Supplementary Figure S3 – Phopsho-rpS6 and total rpS6 expression in human colon cancer spheroids. **A** DLD-1 and **B** HCT116 colon cancer cell spheroids were formed for 24h and subsequently treated with DMSO, 100 nM Rapamycin, 250 nM Torin1, 10 µM PF4708671, 1 µM MK2206 and 1 µM AZD6244 for another 24h. Spheroids were subjected to FFPE and sections stained with antibodies against phospho-rpS6 (green) and total rpS6 (red). Representative samples are shown. **C** StrataQuest software was used for automated spheroid recognition and subsequent zonation by generating a mask followed by distance transformation. This allowed automatic generation of three zones. Nuclear segmentation was performed to measure cytoplasmic p-rpS6 fluorescence intensity around individual DAPI stained nuclei in the different zones.



Supplementary Figure S4 – Phopsho-rpS6 and total rpS6 expression in human colon cancer xenografts.
A DLD-1 and **B** HCT116 colon cancer cells were subcutaneously injected into SCID mice and xenograft tumors were removed after reaching a size of 1cm diameter. Tumors were subjected to FFPE and sections stained with antibodies against phospho-rpS6 (green) and total rpS6 (red). Nuclei are counterstained with DAPI (blue). Analyzed areas for phopsho-rpS6 intensity quantification are shown (white dotted circles). The position of cut blood vessels perpendicular to the section plane are indicated (white stars). Scale bars (black): 50 µm.

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Supporting Figure legends for Figures on figshare

(https://figshare.com/s/c80fdda565dc5e338f42)

Supporting Figure SF1 (figshare) - Analysis of PI3K/AKT/mTOR- and RAS/RAF/MAPK-singaling in 2D vs 3D cell culture. The colon cancer cells (LS174T, HT29, SW620, HCT116, Caco2 and DLD-1) were cultured in 2D and 3D for 48h. Cells and spheroids were harvested and Western blot analysis of different proteins of the PI3K/AKT/mTOR- and RAS/RAF/MAPK-pathway was carried out. Detection of phosphorylated proteins is indicated by p- prior to the protein name and the phosphorylated amino acid is indicated behind the protein name. α-tubulin and GAPDH served as loading control. Two independent biological replicates are shown (**AB**).

Supporting Figure SF2 (figshare) - Analysis of cell cycle and PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in 2D vs 3D cell culture. The colon cancer cells (LS174T, HT29, SW620, HCT116, Caco2 and DLD-1) were cultured in 2D and 3D for 48h. Cells and spheroids were harvested and cell cycle analysis (**A**) was performed using the Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Invitrogen, Thermo Scientific Inc., Waltham, MA) (LS174T: n = 4, HT29: n = 3, SW620: n = 6, HCT116: n = 8, Caco2: n = 4, DLD-1: n = 9 per condition).

Supporting Figure SF3 (figshare) - Apoptosis detection in HCT116 cells to inhibitor treatment in 2D vs 3D. HCT116 cells were grown as monolayer and spheroids (3,000 cells per spheroid) for 24h. 2D and 3D samples were cultured in the presence or absence of Rapamycin, Torin1, PF4708671, MK2206 and AZD6244 for another 24h. DMSO served as control. Apoptotic cells were identified by cleaved caspase 3 staining (green) in 2D and in spheroid sections. Apoptotic cells are indicated by white arrowheads. Nuclei are counterstained with DAPI (blue).

Supporting Figure SF4 (figshare) - Molecular evaluation of PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in the presence or absence of inhibitory compounds. DLD-1 colon cancer cells, grown in 2D and 3D for 24h, were treated with DMSO, which served as control, Rapamycin, the pan mTOR inhibitor Torin1, the S6K1 inhibitor PF4708671, the AKT inhibitor MK2206 and the MEK1 inhibitor AZD4266 for another 24h. Afterwards, 2D and 3D cultures were harvested and whole protein extracts were subjected for Western blot analysis of different proteins of the PI3K/AKT/mTOR- and RAS/RAF/MAPK-pathway. **A** biological replicate 2, **B** biological replicate 3. Detection of phospho-proteins is indicated by p- prior to and the phosphorylated amino acid is indicated behind the protein name. α-tubulin and GAPDH served as loading control.

Supporting Figure SF5 (figshare) - Molecular evaluation of PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in the presence or absence of inhibitory compounds.

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HCT116 colon cancer cells, grown in 2D and 3D for 24h, were treated with DMSO, which served as control, Rapamycin, the pan mTOR inhibitor Torin1, the S6K1 inhibitor PF4708671, the AKT inhibitor MK2206 and the MEK1 inhibitor AZD4266 for another 24h. Afterwards, 2D and 3D cultures were harvested and whole protein extracts were subjected for Western blot analysis of different proteins of the PI3K/AKT/mTOR- and RAS/RAF/MAPK-pathway. **A** biological replicate 1, **B** biological replicate 2. Detection of phospho-proteins is indicated by p- prior to and the phosphorylated amino acid is indicated behind the protein name. α-tubulin and GAPDH served as loading control.

Supporting Figure SF6 (figshare) - Molecular evaluation of PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in the presence or absence of inhibitory compounds. Caco-2 colon cancer cells, grown in 2D and 3D for 24h, were treated with DMSO, which served as control, Rapamycin, the pan mTOR inhibitor Torin1, the S6K1 inhibitor PF4708671, the AKT inhibitor MK2206 and the MEK1 inhibitor AZD4266 for another 24h. Afterwards, 2D and 3D cultures were harvested and whole protein extracts were subjected for Western blot analysis of different proteins of the PI3K/AKT/mTOR- and RAS/RAF/MAPK-pathway. **A** biological replicate 1, **B** biological replicate 2. Detection of phospho-proteins is indicated by p- prior to and the phosphorylated amino acid is indicated behind the protein name. α-tubulin and GAPDH served as loading control.

Supporting Figure SF7 (figshare) - Gradual decrease of rpS6 phosphorylation from the outside to the inner core of DLD-1 spheroids. DLD-1 colon cancer cells were grown in 2D and 3D conditions for 24h and treated with DMSO, Rapamycin, Torin1, PF4708671, MK2206 and AZD6244 for another 24h. Representative images of p-rpS6 S240/244 immunohistochemistry staining (chromogen: diaminobenzidine, brown) of DLD-1 cells cultured on filter membranes and as spheroids (cross sections, 5 µm). Scale bars: 20 µm.

Supporting Figure SF8 (figshare) - Different spheroid formation time does not alter phenotypes in 3D. **A** DLD-1 colon cancer cells were seeded at different cell numbers for spheroid formation (1000, 1500, 2000, 3000) and these spheroids were for different time periods in order to reach the same spheroid size prior to experimentation (e.g. 3000 cells were incubated for 24 hrs, whereas 1000 cells were incubated for 96 hrs to reach the same sphere size). The initial differences of spheroid sizes are shown below in A after 24 hrs. After 24, 48 and 72 hrs of further incubation spheroids formed by 2000, 1500 and 1000 cells, respectively, reached the same size as 3000 cells after 24 hrs. **B** These spheroids of the same size but with different spheroid formation periods were then subjected to cell cycle analysis (EdU incorporation) and DNA staining (7-AAD) and displayed no apparent differences in cell cycle distribution. **C** To generalize these observations the same experimental approach was repeated with HCT116 cells and showed the same results.

Supporting Figure SF9 (figshare) - The presence of methylcellulose does not affect treatment response. DLD-1 colon cancer cells, grown in 2D and 3D for 24h, were treated with DMSO, which served as control, Rapamycin, the pan mTOR inhibitor Torin

and the AKT inhibitor MK2206 for further 24h in the presence or absence of 0.3% methylcellulose. Spheroid formation was carried out in ultra low attachment plates (96 well U-shaped, Thermo Scientific) to avoid cell attachment in the absence of methylcellulose. 2D and 3D cultures were harvested and whole protein extracts were subjected to Western blot analysis of different proteins of the PI3K/AKT/mTOR pathway. No difference in Akt and S6 phosphorylation was detected in methylcellulose containing culture conditions versus methylcellulose free incubation in DMSO controls and in Torin1 or MK2206 treated cells.

Supplementary Table S1 - Mutational status

	CaCo-2	LS174T	DLD-1	HT29	HCT116	SW620	
MS status	MSS	**MSI**	**MSI**	MSS	**MSI**	MSS	27
CRC transcriptional subtype	Stem	Goblet	Stem	Goblet	Stem	Transit amplifying	27, 28
APC	**p.Q1367*** [1]	wt [2,3]	**p.I1417fs*2** [4]	**1)p.E853*** [1], **2)p.T1556fs*** [1,2], **3) p.E853*** [2]	wt [1,2]	**p.Q1338*** [4]	
CTNNB1	**1) -** [7], **2) p.G245A** [8]	**p.S45F** [2,3]	wt [8]	wt [2,8]	**p.S45del** [2]	wt [7]	
KRAS	wt [5,6]	**p.G12D** [2,3]	**p.G13D** [21]	wt [2,5]	**p.G12D** [2, 24]	**p.G12V** [25]	
HRAS	wt	wt	wt	wt	wt	wt	28
NRAS	wt	wt	wt	wt	wt	wt	28
BRAF	wt [5,6]	wt [2,3]	wt [17]	**p.V600E** [2,5,6]	wt [2]	wt [3]	
MAP2K4	-	-	-	wt [2]	wt	p.?	
EGFR	wt [9]	wt [2,3]	wt [18]	wt [2, 9,18]	wt	wt [9]	
EP300	wt [10]	-	**p.E1014*** [19]	**p.M1470fs*3** [10]	**1) p.M1470fs*3** [10], **2) p.N1700fs*9** [10]	wt [10]	
H3K27 demethylase	-	**p.E1316fs** [2,3]	-	-	wt [2]	-	
IDH1	-	-	**p.G97D** [20]	**wt** [2]	-	**wt** [20]	
PTEN	positive	positive	positive	positive	positive	positive	28
PIK3CA	wt [12]	**p.H1047R** [2,3]	**E545K** [22]	**p.P449T** [2]	**p.H1047R** [2]	wt [12]	
MLH1	-	wt [2,3]	-	wt [2]	**p.S252*** [2]	wt [2]	
MLL3	wt [11]	-	**p.G3438D** [11]	-	-	wt [11]	
CDKN2A(p16Ink4, p19 ARF)	-	wt [2,3]	-	-	**1) p.R24fs*20** [2], **2) p.G23fs/?** [24], **3) p.E74fs*15** [2]	wt	
SMAD4	**p.D351H** [13]	wt [2,3]	wt [13]	**p.Q311*** [2,13]	wt [2, 13]	**?**	
SMO	wt [14]	-	**p.T640A** [14]	wt [2, 14]	-	wt [14]	
TP53	**1) p.Glu204X** [15], **2) -** [16]	-	wt [23]	**p.R273H** [2]	wt [2]	**1) p.R273H, 2) p.P309S**	

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Supplementary Table S2 - Primary and secondary antibodies

Name	Company	Cat#	Clone#	Dilution	
Primary antibody				*Immunoblot*	*IF, IHC*
p-Akt T308-XP®	Cell Signaling Technology	13038	D25E6	1:1000	-
p-Akt S473-XP®	Cell Signaling Technology	4060	D9E	1:1000	1:100
Akt pan	Cell Signaling Technology	2920	40D4	1:2000	-
p-PRAS40 T246	Cell Signaling Technology	2640	-	1:1000	-
4E-BP1	Cell Signaling Technology	9452	-	1:1000	-
p-PKC (pan) β S660	Cell Signaling Technology	9371	-	1:1000	-
p-ERK1/2 T202/Y204-XP®	Cell Signaling Technology	4370	D13.14.4E	1:1000	1:300
ERK1/2	Cell Signaling Technology	4695	137F5	1:1000	-
p-p70 S6K T389	Cell Signaling Technology	9234	108D2	1:1000	-
p70 S6K c-term	Cell Signaling Technology	9202	-	1:1000	-
p-rpS6 S240/244	Cell Signaling Technology	2215	-	1:1000	-
p-rpS6 S240/244-XP®	Cell Signaling Technology	5364	D68F8	-	1:800
rpS6	Cell Signaling Technology	2317	54D2	1:1000	-
p-Rb S807/811	Cell Signaling Technology	9308	-	1:1000	-
KI67	Cell Signaling Technology	9449	8D5	-	1:400
IRS1	Cell Signaling Technology	2382	-	1:1000	-
Cytokeratin 18	Dako	M7010	DC10	-	1:50
CD31	Dako	M0823	JC70A	-	1:100
E-Cadherin	abcam	ab1416	HECD-1	1:1000	1:100
α Tubulin	Calbiochem	CP06	DM1A	1:5000	-
GAPDH	Trevigen	2275-PC-100	-	1:10,000	-
Secondary antibody					
anti-mouse IgG-heavy and light chain antibody, HRP conjugate	Bethyl Laboratories Inc.	A90-116P	-	1:10,000	-
anti-rabbit IgG-heavy and light chain antibody, HRP conjugate	Bethyl Laboratories Inc.	A120-101P		1:10,000	-
anti-rabbit IgG (H+L) antibody, AlexaFluor® 488 conjugate	Thermo Scientific Lab.	A-11034		-	1:500
anti-mouse IgG (H+L) antibody, AlexaFluor® 546 conjugate	Thermo Scientific Lab.	A-11030		-	1:500
anti-rabbit IgG antibody, biotinylated	Vector Laboratories	BA-1100		-	1:500

Supplementary Table S3

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