Between cancer and therapy: Studies of the colon
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CHAPTER 7

ER-stress induced differentiation sensitizes colon cancer stem cells to chemotherapy

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HIGHLIGHTS
- Colon-CSCs are more resistant to chemotherapy than differentiated cancer cells
- Activation of the unfolded protein response causes differentiation of colon-CSCs
- UPR-induced differentiation enhances response to chemotherapy in vitro and in vivo

ETOBLURB
Colon cancer stem cells (colon-CSCs) are more resistant to chemotherapy than differentiated cancer cells. Wielenga and Colak et al. show that activation of the Unfolded Protein Response (UPR) forces colon-CSCs into differentiation, which augments their sensitivity to conventional chemotherapy.

SUMMARY
Colon cancer stem cells (colon-CSCs) are more resistant to conventional chemotherapy than differentiated cancer cells. This subset of therapy refractory cells is therefore believed to play an important role in post-therapeutic tumor relapse. In order to improve the rate of sustained response to conventional chemotherapy, development of approaches is warranted that specifically sensitize colon-CSCs to treatment. Here we report that ER-stress induced activation of the Unfolded Protein Response (UPR) forces colon-CSCs to differentiate, resulting in their enhanced sensitivity to chemotherapy in vitro and in vivo. Our data suggest that agents that induce activation of the UPR may be used to specifically increase sensitivity of colon-CSCs to the effects of conventional chemotherapy.

Graphical abstract
INTRODUCTION

In many cancers, a small subpopulation of cells is responsible for tumor-initiation, -growth and metastasis. In the colon, these so-called colon cancer stem cells (colon-CSCs) are characterized by the expression of cell surface markers such as CD133, LGR5 and CD166. Furthermore, these cells display high levels of ALDH1 enzyme activity and Wnt signaling activity. Importantly, colon-CSCs show increased resistance to conventional chemotherapies and are believed to be responsible for tumor regeneration after initial response to chemotherapy. Therefore, therapeutic outcomes after chemotherapy may be improved with therapies that specifically target the eradication of colon-CSCs.

Mechanisms that regulate stem cell dynamics in the healthy intestinal epithelium may give fundamental insights into the biology of their malignant counterparts. An important organelle that regulates the homeostasis of normal intestinal stem cells is the endoplasmic reticulum (ER). Novel proteins that are synthesized in the ER are assisted by chaperones for their proper folding. The major ER chaperone GRP78 is in a dynamic equilibrium between folding proteins and ER transmembrane receptors. An increased load of folding proteins shifts GRP78 away from the transmembrane receptors, a situation termed ER-stress that results in the activation of the unfolded protein response (UPR). We have recently shown that activation of the UPR forces normal intestinal epithelial stem cells into differentiation.

RESULTS

Activation of the Unfolded Protein Response reduces stemness of colon-CSCs

We hypothesized that if the differentiating effects of the Unfolded Protein Response (UPR) would be conserved between normal intestinal stem cells and colon-CSCs, this may be exploited to sensitize colon-CSCs to chemotherapy. To specifically investigate the effects of the UPR on colon-CSCs, we used patient derived spheroid cultures of colon cancer cells with Wnt driven GFP expression. In these cultures colon-CSCs are marked by high Wnt pathway activity (Wnt-high) whereas more differentiated cancer cells have lower Wnt pathway activity (Wnt-low). We have previously established that Wnt-high cells exhibit a higher clonogenic potential and are more resistant to chemotherapy than Wnt-low cells, indicating that the Wnt-driven GFP reporter efficiently distinguishes between CSCs and more differentiated cancer cells.

The UPR can be activated in vitro with subtilase cytotoxin AB (SubAB), a bacterium derived protease that specifically cleaves ER chaperone GRP78. Gene ontology analysis of SubAB treated Wnt-high cells showed that the top three upregulated genesets were: unfolded protein response (p=1.3x10^{-9}), ER associated catabolic process (p=3.9x10^{-7}) and endoplasmic reticulum lumen (p=2.4x10^{-7}). This confirmed the validity of using SubAB to induce ER-stress and activate the UPR. Interestingly, SubAB treatment resulted in a significant downregulation of several established intestinal stem cell associated genes such as OLFM4 and LGR5 in FACS-sorted colon-CSCs (Wnt-GFP-high) 24 hours post-treatment. (Figure 1a, Figure S1a). Gene set enrichment analysis (GSEA) using a previously described
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colon-CSC geneset confirmed the loss of expression of genes that define colon-CSCs\(^{17}\) (Figure 1b). This suggests that ER-stress results in loss of the colon-CSC phenotype. Indeed, a FACS based analysis showed that treatment with SubAB reduced expression of CD133 and almost completely abrogated expression of LGR5 (Figure 1c). Similar to SubAB, treatment with the UPR-inducing agent thapsigargin, also resulted in downregulation of stem cell markers, corroborating the results obtained with SubAB (Figure S1b). In contrast to thapsigargin and SubAB, salubrinal inhibits the phosphatase of eIF2α and thereby only activates the PERK-eIF2α branch of the UPR\(^{18}\). Interestingly, salubrinal was equally effective in downregulating stem cell markers (Figure S1c), suggesting that activation of the PERK-eIF2α branch is sufficient for UPR induced differentiation of colon-CSCs, as previously described for normal intestinal stem cells\(^{14}\).

To exclude the possibility that LGR5 positive cells disappeared from the spheroid cultures by a process of cell death of the LGR5 positive colon-CSCs, we now FACS-sorted colon-CSCs (Wnt-GFP\(^{\text{hi}}\)) before start of the treatment (Figure 1d). Sorted colon-CSCs did not show increased propidium iodide (PI) uptake upon treatment with SubAB (Figure 1e) and survived in culture. As previously observed in the unsorted spheroid cultures, LGR5 expression was lost upon treatment (Figure 1f). Together these experiments establish that Wnt-GFP\(^{\text{hi}}\) cells not only lose the transcriptional profile of colon-CSCs but also lose protein expression of the stem cell marker LGR5 when exposed to ER-stress.

Self-renewal capacity is a hallmark of stemness and can be determined by limiting dilution analysis. To test the functional consequences of the ER-stress induced loss of the colon-CSC profile and LGR5 protein expression we performed a limiting dilution assay. Intriguingly, SubAB treatment resulted in a loss of self-renewal capacity \textit{in vitro} and a decrease in potential to form xenografts \textit{in vivo} (Figure 1g,h), demonstrating that activation of UPR differentiates colon-CSCs both phenotypically as well as functionally.

**UPR activation results in differentiation of colon-CSCs towards an enterocyte phenotype**

In line with the hypothesis that ER-stress may induce differentiation of colon-CSCs, loss of expression of stem cell markers concurred with increased expression of the master inhibitor of intestinal stem cell cycle progression \(P21^{\text{cip1/waf1}}\) or cyclin-dependent kinase inhibitor 1 (\(CDKN1\))\(^{19}\) at 24 hours post-treatment. This was followed by upregulation of enterocyte markers CK20, VIL2, SI and \(FABP2\) after 48-72 hours treatment with SubAB (Figure 2a). Intriguingly \(MUC2\), a marker of secretory goblet cells was downregulated, arguing that ER-stress may differentiate intestinal epithelial stem cells towards an absorptive phenotype rather than a secretory phenotype.

When colon-CSCs were grown in 3D matrigel culture, SubAB treatment dramatically increased the percentage of differentiated spheres (90% vs 11%, \(p<0.001\), Figure 2b) with increased cellular polarization and the formation of a central lumen. Taken together these findings indicate that UPR activation results in phenotypic differentiation of colon-CSCs.
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Figure 1. Activation of the Unfolded Protein Response in colon-CSCs results in loss of stemness. (A) Gene expression analysis of colon-CSCs (Wnt-GFP<sup>hi</sup>) cells treated with SubAB or protease-dead SubA<sub>272B</sub> control (24 hours, 1µg/ml) in three independent experiments. Treatment with SubAB resulted in upregulation of UPR target genes and loss of several intestinal stem cell markers. These findings were validated on quantitative RT-PCR (Figure S1A) and were confirmed by treatment with other UPR-activating agents thapsigargin and salubrinal (Figure S1B-C) and on other primary spheroid cultures derived from different patients with colon cancer (Figure S1D). (B) Gene Set Enrichment Analysis of SubAB treated colon-CSCs revealed profound loss of a previously described colon-CSC signature (de Sousa et al., 2011). (C) Flow cytometry analysis of 24 hour SubAB treatment (1µg/ml) resulted in downregulation of colon-CSC markers CD133 and LGR5. Data are representative of three independent experiments. (D) Experimental setup for (E-F). In these experiments colon-CSCs (Wnt-GFP<sup>hi</sup>) were first sorted before treatment for 24 hours with SubAB or SubA<sub>272B</sub> control (1µg/ml). (E) SubAB treatment did not result in increased cell death in colon-CSCs as assessed by propidium iodide uptake. (F) Similar to spheroid culture (C), sorted colon-CSCs lost expression of LGR5 upon UPR activation by SubAB. (G) Limiting-dilution assay performed on colon-CSCs (Wnt- GFP<sup>hi</sup>) and differentiated cancer cells (Wnt-GFP<sup>lo</sup>). Cells were pre-treated for 24 hours with SubAB or SubA<sub>272B</sub> control (1µg/ml) and plated out in normal CSC-medium without treatment. Exclusion of dead cells was performed with propidium iodide (PI). (F) In vivo limiting-dilution assay. Colon-CSCs were pre-treated for 24 hours with SubAB or SubA<sub>272B</sub> (1µg/ml) and sorted in matrigel with indicated quantities and subcutaneously injected into nude mice. Exclusion of dead cells was performed with propidium iodide (PI). Values in (E) are mean ± SEM, values in (G, H) are mean with 95% CI, *p<0.05, **p < 0.01, ***p < 0.001
Figure 2. Differentiation of colon-CSCs occurs towards an enterocyte phenotype and loss of stemness can be reversed. (A) Quantitative RT-PCR for differentiation markers relative to GAPDH at different time points after start of treatment with SubAB or protease-dead SubA<sub>272B</sub> control (1µg/ml). Data are representative of three independent experiments. (B) 3D matrigel culture showed phenotypic differentiation by SubAB treatment as seen by enhanced polarization, smoothening of the outer layer and development of a central lumen. Data are representative of three independent experiments. (C) Experimental setup for (C-D). Spheroid cultures were harvested for further analysis directly after 24 hour treatment with SubAB or protease-dead SubA<sub>272B</sub> control (1µg/ml) or 24-48 after replacement of the treatment with normal CSC-medium. Quantitative RT-PCR for stem cell markers LGR5 and OLFM4 relative to GAPDH reveals that loss of expression of stem cell markers can be reversed after therapy withdrawal. (D) Limiting dilution analysis at indicated times after treatment withdrawal demonstrating that the loss of self-renewal capacity by UPR activation is completely lost 48 hours after treatment withdrawal. Transient salubrinal treatment resulted in similar reversible effects of UPR induced differentiation (Figure S2). Values in (A-C) are mean ± SEM, values in (D) are mean with 95% CI, *p<0.05, **p < 0.01, ***p < 0.001.
UPR induced loss of stemness is reversed by treatment withdrawal
To investigate to what extent UPR induced differentiation is irreversible, we transiently treated spheroid cultures for 24 hours with subAB and performed further analyses directly after the treatment or 24-48 after replacement of the treatment with normal CSC-medium (Figure 2c). After 48 hours of treatment withdrawal, the spheroid cultures had almost completely regained their OLFM4 expression (Figure 2c). In concordance, UPR induced loss of self-renewal capacity was completely reversed at this timepoint (Figure 2d). The same observation was made in salubrinal treated colon-CSCs (Figure S2a-c). These data clearly indicate that the effects of UPR activation are transient and are reversed when colon-CSCs are given the opportunity to recover in the absence of ER-stress. Surprisingly, the expression of LGR5 was even increased at 48 hours post SubAB and salubrinal treatment (Figure 2c and S2c), suggesting that transient UPR activation may ultimately elicit a regenerative response in these cells resulting in expansion of the stem cell pool.

UPR induced differentiation sensitizes colon-CSCs to chemotherapy
Colon-CSCs are suggested to be more resistant to conventional chemotherapy and thereby to drive recurrence of the tumor after initial response to therapy. Indeed we found colon-CSCs to be more resistant to oxaliplatin than differentiated cancer cells (Figure 3a, Figure S3) confirming previous reports15. The specific eradication of these therapy refractory cells may therefore provide a window of opportunity to improve outcomes of chemotherapy. We hypothesized that ER-stress induced colon-CSC differentiation could be an attractive option to achieve this goal. To test this hypothesis, spheroid cultures of colon-CSC were treated with SubAB or salubrinal, followed by conventional chemotherapeutic regimens. UPR activation sensitized colon-CSCs towards oxaliplatin and 5-FU but also for chemotherapy regimens including, FOLFOX and FOLFIRI in vitro (Figure 3a,b, Figure S3). In vivo UPR activation was obtained by treating mice with salubrinal, because SubAB causes hemolytic uremic syndrome20. In line with the in vitro observations of a compensatory response elicited by transient UPR activation, treatment with salubrinal alone resulted increased growth of xenografts derived from colon-CSCs. In combination with oxaliplatin however, salubrinal suppressed growth of subcutaneous xenografts (Figure 3c,d). This indicates that UPR activation sensitizes colon-CSCs towards chemotherapy in vitro and in vivo.

DISCUSSION
Forced differentiation of colon-CSCs is an attractive and feasible avenue in the development of new strategies to achieve a more sustained response to chemotherapy. Previously, bone morphogenetic protein (BMP) signaling was shown to induce differentiation of colon-CSCs by the inhibition of Wnt signaling, which increased their sensitivity to chemotherapy21. This effect however was only found in colon-CSCs that did not have simultaneous mutations in SMAD4 and constitutive activation of PI3K. A more broad effect appears to be exerted by inhibition of the Notch pathway, which strongly suppresses cancer stemness22, and in addition neutralizing antibodies against DLL4 were shown to decrease tumor-initiating cell frequency and sensitize colon-CSCs to chemotherapy23.
Figure 3. UPR induced differentiation sensitizes colon-CSCs to chemotherapy in vitro and in vivo.
(A) Percentages of apoptotic cells, measured by caspase3 activity (CaspGlow) for colon-CSCs (Wnt-
GFPhigh) and differentiated cancer cells (Wnt-GFPlow) after indicated treatments. Colon-CSCs were 
more resistant to oxaliplatin (24 hours, 50µM) compared to differentiated cancer cells. Pre-treatment 
with SubAB (24 hours 1µg/ml) or salubrinal (Figure S3) resulted in enhanced sensitivity to oxaliplatin 
induced apoptosis. (B) Sensitization of colon-CSCs was confirmed for other chemotherapeutic 
agents 5-FU (200µg/ml), FOLFOX (1,25µM oxaliplatin and 50µM 5-FU) and FOLFIRI (1µM irinotecan 
and 50µM 5-FU). (C) Illustration of experimental setup for in vivo experiments. (D) Survival curves 
for xenotransplanted mice, treated weekly with salubrinal (1mg/kg), oxaliplatin (1mg/kg), or a 
combination of both for four weeks. Values in (A,B) are mean ± SEM. Significance was measured 
by two-way anova (A), one way anova (B) or LogRank (Mantel Cox) test (D), followed by Bonferroni 
posttest for multiple comparisons. Significance was defined as *p<0.05, **p < 0.01, ***p < 0.001.

Our findings now show that induction of ER-stress and subsequent activation of the UPR is an effective 
means to induce differentiation of colon-CSCs. UPR activation results from various stimuli that are 
known to impair cellular integrity such as protein misfolding or aggregation, oxidative injury, and 
viral infection. In normal physiology UPR-mediated stem cell differentiation may therefore act as a 
mechanism to guarantee integrity of the stem cell pool by forcing the differentiation of damaged stem 
cells. Indeed, we recently showed that activation of the UPR in healthy intestine by genetic deletion
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of ER-chaperone Grp78 resulted in loss of stem cells by differentiation and quick repopulation of healthy stem cells that were not recombined44. This protective function may be preserved for other healthy and cancerous tissues as well. Likewise, van Galen et al. have shown that the integrity of the hematopoietic stem cell pool is governed by the UPR by clearance of individual HSCs after stress in order to prevent propagation of damaged stem cells24.

Our in vitro findings show that ER-stress induced differentiation of CSCs results in their enhanced sensitivity to chemotherapy. Our in vivo experiments established that treatment with salubrinal enhanced the efficacy of oxaliplatin in inhibiting tumor growth but it should be noted that this experiment did not formally examine if this was due to CSC differentiation. The compensatory regenerative response we observed in our in vitro experiments within 48 hours after therapy withdrawal indicates that chemotherapy should be administered in a critical time window after UPR activation. Together these data identify the UPR as a pathway that may be targeted to optimize the sensitivity of colon-CSCs for chemotherapy and improve outcome in patients with colon cancer.

EXPERIMENTAL PROCEDURES

Cell culture

Spheroid cultures were isolated from different patients with colon cancer in accordance with the rules of the medical ethical committee of the AMC. Spheroid cultures with a TCF/LEF driven GFP reporter for Wnt signaling activity were previously described 8. The 10% highest Wnt-GFP expressing cells represent the colon-CSC population whereas the 10% lowest Wnt-GFP expressing cells represent differentiated colon cancer cells. Colon-CSCs were cultured under ultralow-adherent condition as described previously25 in CSC-medium. All treatments were performed after overnight adherence in 12 wells plates (50,000 cells per well). For composition of CSC medium and reagents, see supplemental information.

RNA extraction, quantitative RT-PCR and microarray

Spheroid cultures were adhered overnight and treated for 24 hours with SubAB or protease-dead SubA272B (1µg/ml). Colon-CSCs (Wnt-GFP+high) were sorted and lysed in 350 µl RLT buffer (RNeasy, QIAGEN). RNA extraction was performed according to manufacturer’s instructions. For cDNA synthesis, 1µg of RNA was transcribed using Revertaid (Fermentas). Quantitative RT-PCR was performed using SybrGreen (QIAGEN) according to manufacturers’ protocol on a BioRad iCycler using specific primers for the mRNA of interest (see below). For microarray, RNA was labeled using cRNA labeling kit for Illumina arrays (Ambion) and hybridized with Illumina HT12 Arrays. The array data were analyzed on the R2 bioinformatic platform (http://r2.amc.nl). Differentially expressed genes were extracted using ANOVA test (p < 0.05) and FDR post-analysis correction. GSEA were done using GSEA software from the Broad Institute (http://www.broadinstitute.org/gsea). The gene sets used were previously described47. Heatmaps were generated using TreeView software generated by the Eisen lab (http://www.eisenlab.org/eisen).
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Quantitative RT-PCR primers
See supplemental information

Flow cytometry and FACS sorting
Spheroid cultures were adhered overnight and treated for 24 hours with SubAB or protease-dead SubA_{272B} (1µg/ml). Flow cytometry was performed on trypsin-dissociated spheroid cultures with anti-LGRS-biotin antibody (4D11F, 1:100, BD Biosciences) and AC133/CD133-APC antibody (1:25, Miltenyi Biotec). Dead cells were excluded with PI (1:1000).

3D matrigel differentiation assay
For 3D matrigel cultures, trypsin-dissociated spheroid cultures were dissolved in ice cold, liquid matrigel and allowed to solidify at 37° before addition of CSC-medium. Treatment were added to the 3D matrigel cultures two days thereafter and continued for four days. Quantification of differentiated spheres was performed by three independent observers that were blinded for the treatment.

Limiting-dilution Assays
For in vitro limiting-dilutions, spheroid cultures were pre-treated for 24 hours with SubAB or protease-dead SubA_{272B} control (1µg/ml). Colon-CSC (Wnt-GFP\textsuperscript{high}) and differentiated colon cancer cells (Wnt-GFP\textsuperscript{low}) were sorted into a 96 wells plate containing normal CSC-medium without treatment at 1, 2, 4, 8, 16, 32, 64, 128, 256 cells per well. Dead cells were excluded with PI.

In vitro cell death assays
Spheroid cultures were pre-treated for 24 hours with SubAB or protease-dead SubA_{272B} (1µg/ml) followed by 24 hour treatment with chemotherapeutic regimens (see reagent section); oxaliplatin (50µM), 5-FU (200µg/ml) FOLFOX (1,25µM oxaliplatin followed after 90 minutes by 50µM 5-FU) and FOLFIRI (irinotecan 1µM, followed after 90 minutes by 50µM 5-FU). To compare colon-CSCs (Wnt-GFP\textsuperscript{high}) to differentiated cancer cells (Wnt-GFP\textsuperscript{low}) apoptosis was measured at the single-cell level by caspase 3 activity using CaspGlow active staining kit (Red-DEVD-FMK) according to the manufacturer's instructions (BioVision, Milpitas, CA, USA). In short, after treatment, spheroid cultures were made single cells using trypsin-EDTA. 50 000 cells were washed with CSC-medium and stained with RED-DEVD-FMK for 1 h at 37 °C. Subsequently, cells were washed twice with wash buffer.

Animal experiments
The protocol of this study was approved by the animal ethics committee of the University of Amsterdam (permit number ALC102862). For xenograft studies, 7.000 FACS-sorted Wnt-GFP\textsuperscript{high} cells were suspended in 100µl of PBS/BSA mixed with Matrigel at a 1:1 ratio and injected subcutaneously into nude mice (Hsd:Athymic Nude/Nude) (Harlan). After 3–8 weeks visible tumors arose and therapy started when tumors size reached 50-100 mm\textsuperscript{3}. Salubrinal (1mg/kg) oxaliplatin (1mg/kg), a combination of both or DMSO control was injected intraperitoneally once a week for four weeks. Tumor growth was measured once a week and all mice were sacrificed when the tumor size reached 1
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For *in vivo* limiting-dilution assay spheroid cultures were treated with SubAB or SubA<sub>272B</sub> control for 24 hours and Wnt<sup>GFP<sup>™</sup></sup> cells were sorted directly into matrigel and injected subcutaneously into nude mice (Hsd:Athymic Nude/Nude) (Harlan) as described previously. Tumor development was assessed weekly until 10 weeks after injection.

**Statistical Analysis**

Data are expressed as mean ± SEM. Unless otherwise indicated, statistical significance was determined by ANOVA (one way or two way) with a Bonferroni post-test. For statistical analysis of Kaplan-Meier survival curves with Log-rank (Mantel-Cox) test was performed followed by Bonferroni post-test for multiple comparisons. For Limiting Dilution Assays, clonal frequency and statistical significance were evaluated with the Extreme Limiting-dilution Analysis (ELDA) ‘limdil’ function (http://bioinf.wehi.edu.au/software/elda/index.html). All significance was defined as *p < 0.05, **p < 0.01, ***p < 0.001.

**Accession numbers**

Microarray data have been deposited in the Gene Expression Omnibus Database with the accession number GSE65879.

**Author contributions**

MCBW, SC, JH, LV, JPM, GRvdB designed the experiments. MCBW, SC, JFvLdJ conducted the experiments. MCBW, SC, and HMR performed the *in vivo* experiments. JCP and AWP provided SubAB and SubA<sub>272B</sub>. MCBW, SC, JH, LV, JPM and GRvdB discussed data and commented on results and on the manuscript. MCBW, SC, JPM and GRvdB wrote the manuscript. JPM and GRvdB supervised all the experiments.

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Figure S1, related to Figure 1. Validation of the UPR induced differentiation by different agents and on different spheroid cultures. (A-C) Quantitative RT-PCR relative to GAPDH at indicated doses of (A) SubAB, (B) thapsigargin and (C) salubrinal. (D) Quantitative RT-PCR relative to GAPDH on indicated primary spheroid cultures after treatment with SubAB or SubA272B control (24 hours, 1µg/ml). Data are representative of three independent experiments. Mean ± SEM are depicted, *p<0.05, **p < 0.01, ***p < 0.001
Figure S2, related to Figure 2. Loss of stemness by salubrinal is reverted after therapy withdrawal. (A) Experimental setup for (B-C). Spheroid cultures were harvested for further analysis directly after 24 hour treatment with salubrinal (25µM) or 24-48 after replacement of the treatment with normal CSC-medium. (B) Limiting dilution analysis at indicated times after salubrinal, demonstrating that the loss of self-renewal capacity by salubrinal treatment is gradually lost 24-48 hours after treatment withdrawal. (C) FACS analysis of colon-CSC markers CD133 and LGR5 after indicated timepoints. Values in (A-C) are mean ± SEM, values in (D) are mean with 95% CI, *p<0.05, **p < 0.01, ***p < 0.001.
Figure S3, related to Figure 3. Salubrinal induced differentiation sensitizes colon-CSCs to oxaliplatin. Percentages of apoptotic cells, measured by caspase3 activity (CaspGlow) for colon-CSCs (Wnt-GFP$^{high}$) and differentiated cancer cells (Wnt-GFP$^{low}$) after indicated treatments. Colon-CSCs were more resistant to oxaliplatin (24 hours, 50µM) compared to differentiated cancer cells. Pre-treatment with salubrinal (24 hours 25µM) resulted in enhanced sensitivity to oxaliplatin induced apoptosis. Mean ± SEM depicted. Significance was measured by two-way anova, followed by Bonferroni posttest for multiple comparisons. Significance was defined as *p<0.05, **p < 0.01, ***p < 0.001.