Supplementary files



**Figure S1. Phenotypic and functional characterization of sedimentation field-flow fractionation (SdFFF)-sorted cell subpopulations from colorectal cancer (CRC) cell lines.** (A) Specificity of fluorescence emitted by flow cytometry was checked using isotype controls. The histograms obtained were used to position the positivity thresholds of our markers of interest. (B-C) The proportion of S-phase and G2/M cells in the cell cycle was analyzed by flow cytometry for each sorted cell subpopulation and presented in the bar plot from at least three biological replicates. F3 subpopulation has significantly fewer cells in S phase for WiDr and SW620 cell lines (B), and in G2/M phase for all cell lines (C) compared to the other subpopulations. (D-E) Tumor volume was measured throughout the in vivo tumor initiation assay using a caliper. (D) Fifty days after injection, the average tumor volume obtained in the five mice in each cell concentration group is summarized in the bar plot and reveals that it is highest in the F3 condition at 1000 cells. (E) The appearance of a tumor with a volume higher than 100 mm3 was obtained between 36 and 39 days for F3 and TP, whereas it was reached only after 46 days for F1, as shown in the tumor growth curves. (F) Collected tumors larger than 100 mm3 were photographed 50 days after injection. Scale in centimeters. All these results are represented as means ± SEM and statistical differences with \*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001 and \*alone for significant results compared to TP using One-way ANOVA test.



**Figure S2. Response of SdFFF-sorted cell subpopulations to oxaliplatin and irinotecan from CRC cell lines.** (A-F) Response to oxaliplatin and irinotecan was assessed in 2D culture. (A) After three days of oxaliplatin treatment, IC50 values were obtained by MTT assay from at least three biological replicates. F3 subpopulation has a significantly higher IC50 than F1 for the WiDr cell line and this trend is also observed for the SW480 and SW620 cell lines. (B) Cell proliferation rate after oxaliplatin treatment was measured by BrdU assay and presented in the bar plot as a ratio between treated and untreated conditions. After treatment, proliferation decreases in all cell subpopulations compared to the untreated condition (dashed line), but the rate appears to be higher in the F3 subpopulation for all cell lines. (C) Using the ELISA cell death assay, apoptosis rate after treatment was measured and compared to the untreated condition (dashed line). Apoptosis significantly increased in F3 compared to F1 for the T84 cell line. (D) The IC50 values obtained after three days of irinotecan treatment appear to be slightly higher in F3 for SW480, SW620, and T84 cell lines, with a significant difference between F3 and TP for T84, which is the most resistant of the four cell lines. (E) As with oxaliplatin, cell proliferation was assessed after irinotecan treatment and decreased in all cell lines compared with the untreated condition, but the rate remained higher in F3 versus F1 for WiDr, SW480 and T84. (F) Apoptosis was also assessed after irinotecan treatment and the rate is significantly decreased in F1 and F3 versus TP for T84, with a similar trend for SW480 and SW620. (G-H) Response to oxaliplatin and irinotecan was also investigated in 3D culture from colonospheres. (G) After oxaliplatin treatment, the number of colonospheres formed was significantly enhanced in F3 compared to TP for WiDr, with a similar trend for SW480 and SW620. (H) Colonospheres was significantly increased after irinotecan treatment in F3 compared to F1 for the SW480 cell line, with the same trend observed for SW620 and T84. All these results are represented as means ± SEM and statistical differences with ns for not significant, \*p-value < 0.05, \*\*p-value < 0.01 and \*alone for significant results compared to TP using One-way ANOVA test.



**Figure S3. Phenotypic and functional characterization of SdFFF-sorted cell subpopulations from CRC primary cultures.** (A) The expression level of CSC markers, CD44, LGR5, BMI1, and CD133, was assessed by flow cytometry and plotted as a bar plot from three biological replicates. The percentage of positive cells varied very slightly between the sorted cell subpopulations, with an expression level that appeared to be higher in F1 compared with F3, except for LGR5 in the tumor-invaded peritoneum primary culture. (B) G0/G1-phase cells, quantified by flow cytometry, are significantly higher in F3 compared to other subpopulations for the tumor-invaded peritoneum primary culture, with a similar trend for the early stage primary culture. (C) For both primary cultures, the percentage of cells in G2/M phase decreases significantly in F3 compared to F1 and F2. (D-E) Cell clonogenicity was assessed by a soft agar assay. Images of the colonies formed (D) as well as the bar plot (E) show that F3 forms more and larger colonies compared to the other subpopulations for both primary cultures. Scale bar 1 mm. All these results are represented as means ± SEM and statistical differences with ns for not significant, \*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001 and \*alone for significant results compared to TP using One-way ANOVA test for analysis of CSC marker expression and cell cycle distribution, and Kruskal-Wallis test for clonogenicity.



**Figure S4. Response of SdFFF-sorted cell subpopulations to oxaliplatin and irinotecan from CRC primary cultures.** (A-F) Response to oxaliplatin and irinotecan was assessed in 2D culture. (A) After treatment, the IC50 values obtained for oxaliplatin were comparable between the sorted cell subpopulations, but CPP35 was the more resistant of the two primary cultures. (B-C) Cell proliferation and apoptosis induced after oxaliplatin treatment were measured and presented as a ratio between treated and untreated conditions. Proliferation rate was significantly increased in F3 compared to TP for CPP35 primary culture (B) and apoptosis rate appeared slightly decreased in F3 compared to TP and F1 (C). For CPP14, proliferation and apoptosis rates were comparable between cell subpopulations. (D-F) Cell viability with IC50 values, proliferation and apoptosis were also evaluated after irinotecan treatment. (D) IC50 values are comparable between the sorted cell subpopulations for both primary cultures, with an IC50 that appears slightly higher in F3. No significant differences are observed for proliferation and apoptosis after irinotecan treatment; however it appears that the proliferation rate is slightly higher in F3 compared to F1 for CPP35 (E) and the apoptosis rate is slightly lower in F3 versus F1 for both primary cultures (F). (G-H) Response to oxaliplatin and irinotecan was also explored in 3D culture from colonospheres. Colonospheres are equivalent in number in CPP14 cell subpopulations after oxaliplatin (G) and irinotecan (H) treatment. For CPP35, the number of colonospheres seemed to be slightly higher in F3 compared to F1 for both chemotherapies. All these results are represented as means ± SEM and statistical differences with ns for not significant, \*p-value < 0.05 and \*alone for significant results compared to TP using One-way ANOVA test.

**Table S1*.*** Lists of antibodies used for the analysis of CSC marker expression by flow cytometry.

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| **Antibodies** | **Fluorescent dyes** | **References** | **Manufacturers** |
| Anti-CD44 (G44-26) | FITC | 555478 | BD Pharmingen™ |
| Anti-LGR5 (DA03-22H2.8) | PE-Vio 770 | 130-100-847 | Miltenyi Biotec |
| Anti-CD133/1 (AC133) | PE-Vio 615 | 130-113-671 | Miltenyi Biotec |
| Anti-BMI-1 (F-9) | PE | sc-390443 | Santa Cruz |
| Viobility™ 405/452 |  | 130-109-816 | Miltenyi Biotec |
| Anti-IgG2bκ (27-35) | FITC | 555742 | BD Pharmingen™ |
| Anti-IgG2bκ (ES26-5E12.4) | PE-Vio 770 | 130-102-656 | Miltenyi Biotec |
| Anti-IgG1 (IS5-21F5) | PE-Vio 615 | 130-113-201 | Miltenyi Biotec |
| Anti-IgG1κ (MOPC-21) | PE | 400111 | Biolegend |