Nobiletin and Xanthohumol Sensitize Colorectal Cancer Stem Cells to Standard Chemotherapy

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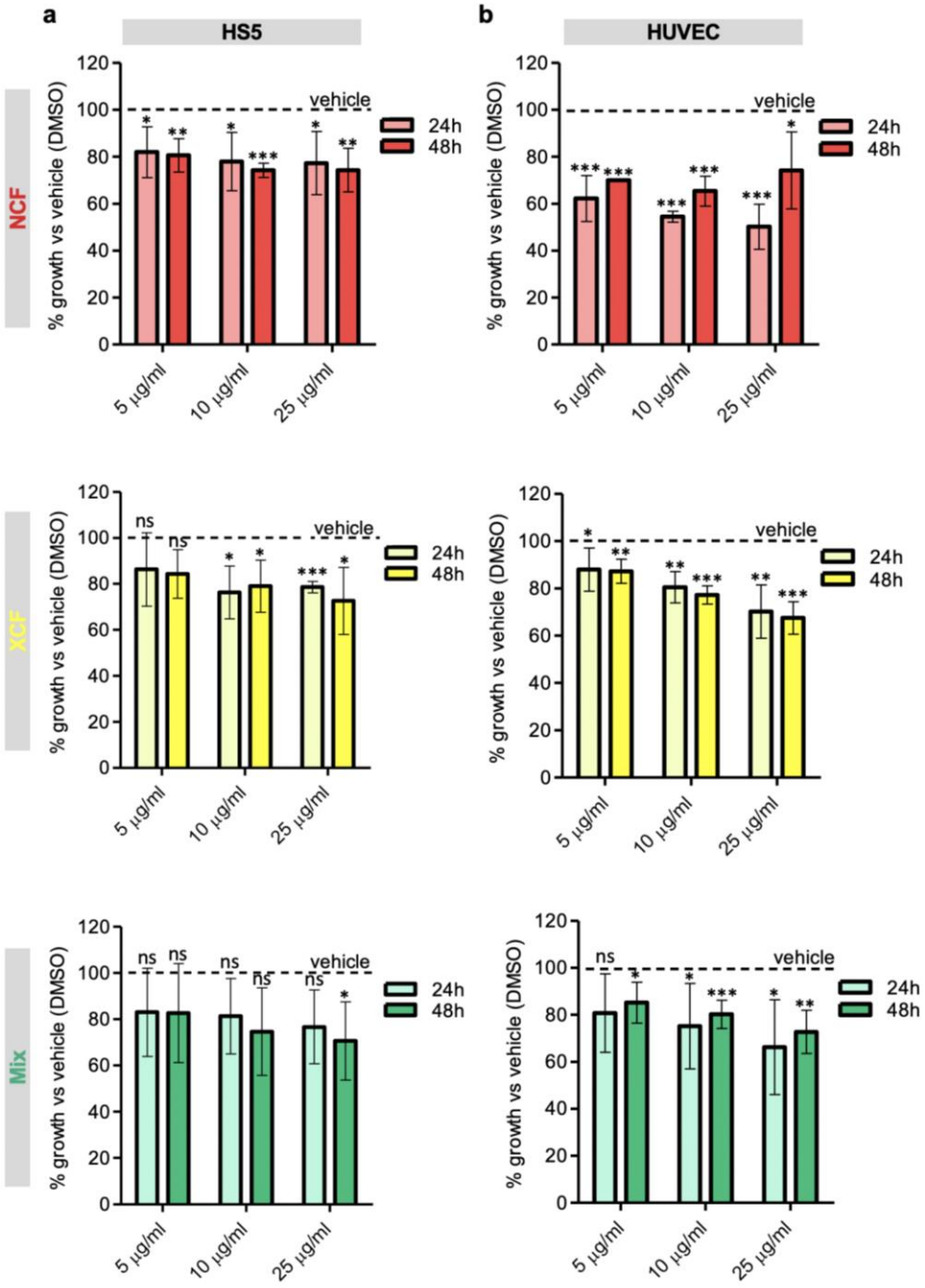
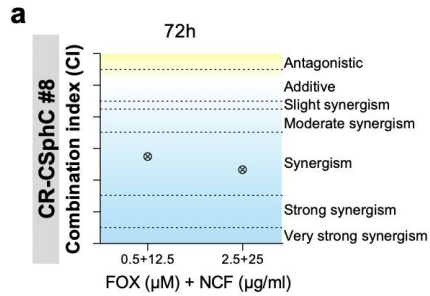
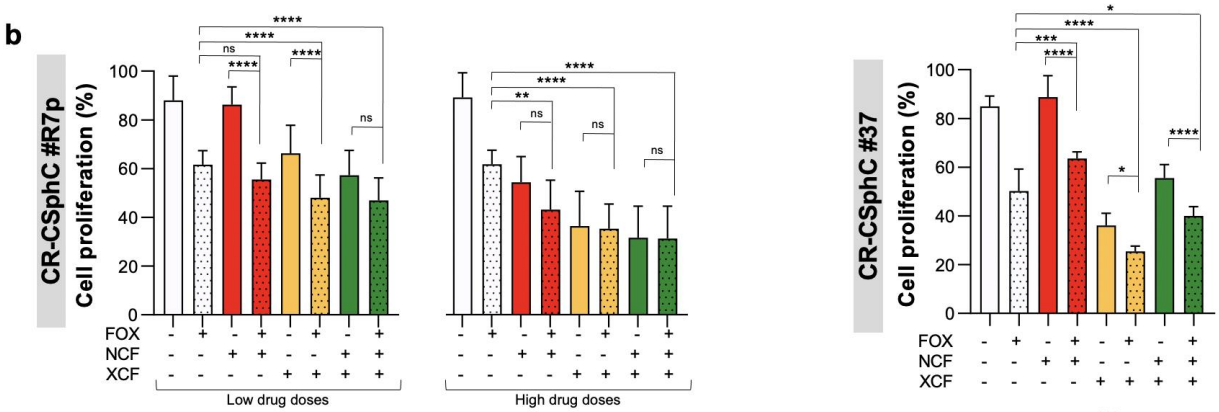
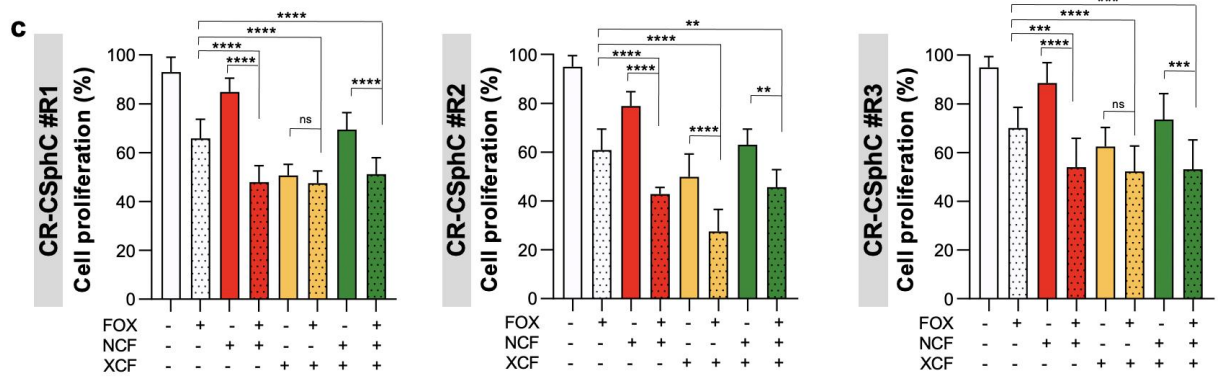
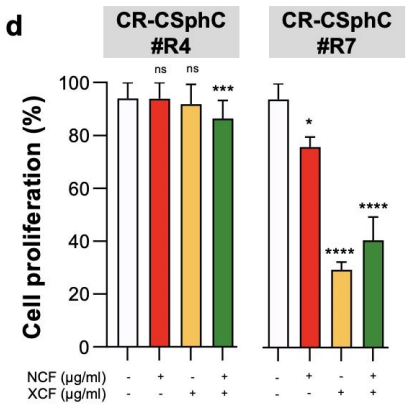


Figure S1. NCF and XCF do not affect non-transformed cells. Percentage of growth of HS5 (**a**) and HUVEC (**b**) cell lines treated with 5, 10, and 25 µg/ml of NCF, XCF, or the Mix of extracts for 24 and 48 hours. Values are plotted as the percentage of 5 growth versus the vehicle (DMSO, dotted line). Data are represented as means ± SD. Comparisons between two groups (cells treated with the extracts vs cells treated with the vehicle) were made using a two-tailed Student's t-test: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.


a
72h
CR-CSphC #8
Combination index (CI)
Antagonistic
Additive
Slight synergism
Moderate synergism
Synergism
Strong synergism
Very strong synergism
0.5+12.5
2.5+25
FOX (μM) + NCF (μg/ml)


b
CR-CSphC #R7p
Cell proliferation (%)
FOX
NCF
XCF
Low drug doses
High drug doses
CR-CSphC #37


c
CR-CSphC #R1
CR-CSphC #R2
CR-CSphC #R3
CR-CSphC #R4
Cell proliferation (%)
FOX
NCF
XCF


d
CR-CSphC #R4
CR-CSphC #R7
Cell proliferation (%)
NCF (μg/ml)
XCF (μg/ml)

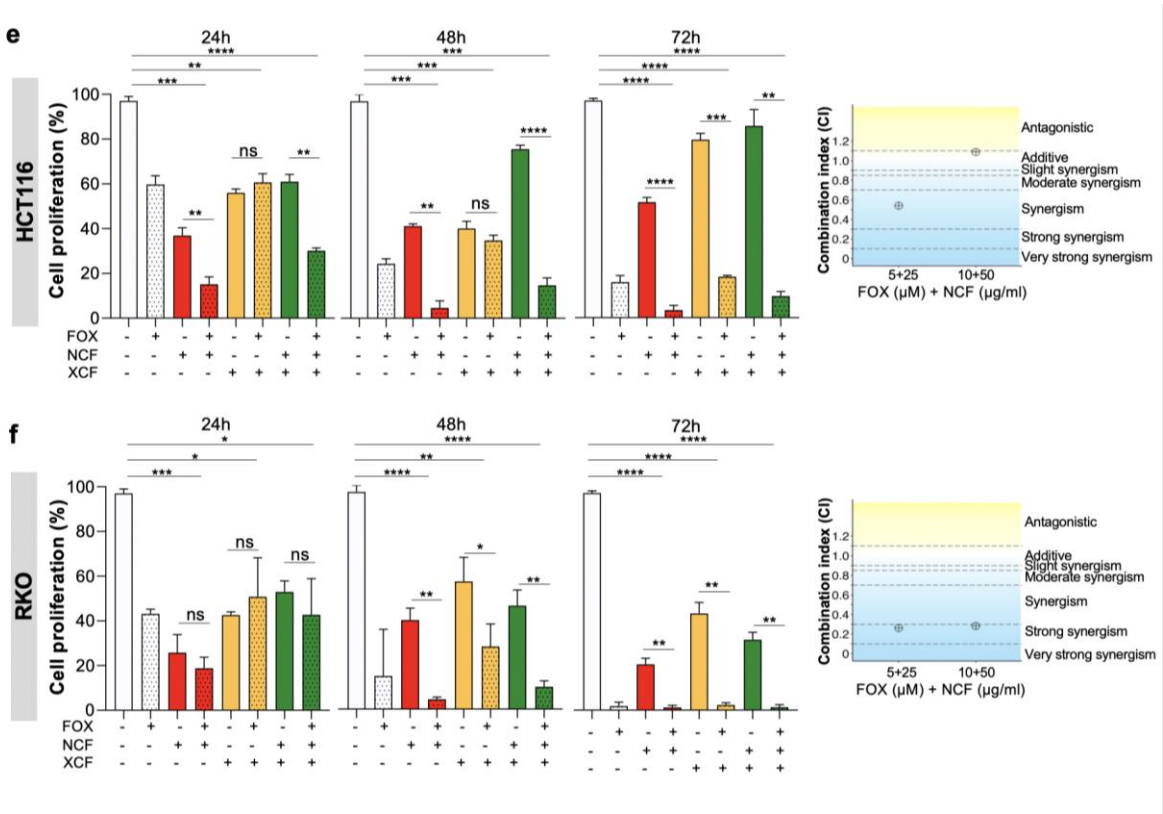


Figure S2. NCF and XCF sensitize cancer cells to chemotherapy (**a**) Synergy plot representing the combination index (CI), computed in CompuSyn by using Chou-Talalay method, calculated from cell proliferation data of CR-CSphCs (#8) treated with different FOX and NCF dose pair at 72 hours; (**b**) Cell proliferation percentage of primary CR-CSphCs (#R7p) treated with 25 and 40 μg/ml of NCF, XCF or Mix extracts alone or in combination with 5 μM FOX for 72 hours (left panel) and CR-CSphCs (#37) treated with 25 μg/ml of NCF, XCF or Mix extracts alone or in combination with 1.25 μM FOX for 72 hours (right panel); (**c**) Cell proliferation percentage of CR-CSC #R1, #R2, #R3, #R4 treated with 40 μg/ml of NCF, XCF or Mix extracts alone or in combination with 5 μM FOX for 72 hours; (**d**) Cell proliferation percentage of CR-CSC #R4 and #R7 treated with 25 μg/ml of NCF, XCF or Mix extracts for 72 hours; (**e,f**) Cell viability of CRC cell lines (HCT116 and RKO) treated with 25 μg/ml of NCF, XCF or Mix extracts alone or in combination with 5 μM FOX at the indicated time points (left panel). Synergy plot representing the combination index (CI), computed in CompuSyn by using Chou-Talalay method, calculated from cell proliferation data of CRC cell lines treated with different FOX and NCF dose pair at 48 hours (right panel). Data are represented as mean ± SD of three independent experiments. Percentage of untreated control (vehicle) is shown. Comparisons between two groups were made using a two-tailed Student's t-test: ns, not significant, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

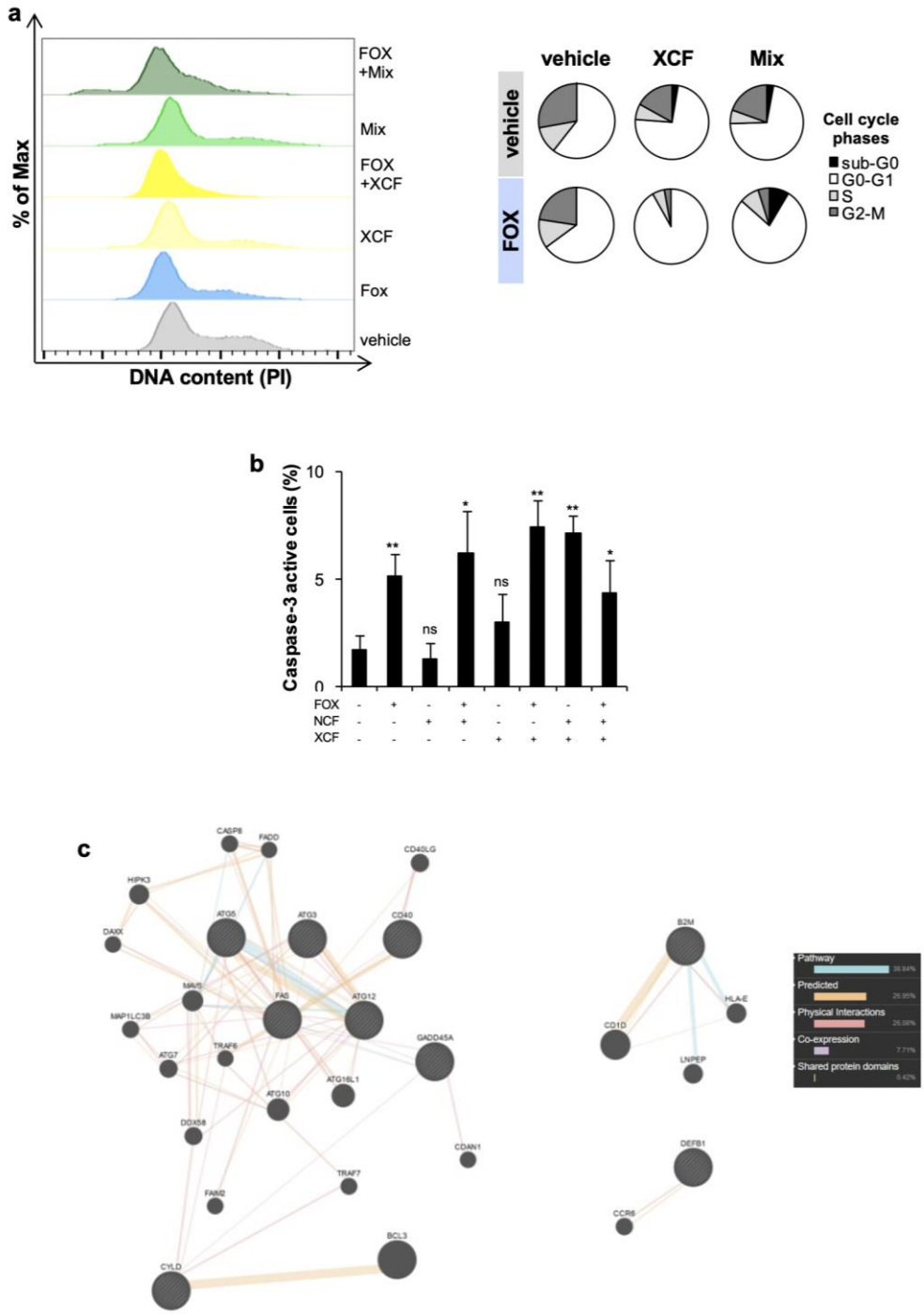


Figure S3. NCF and XCF plus chemotherapy induce apoptosis in CR-CSphCs (**a**) Representative flow cytometry analysis of cell cycle phases distribution in CR-CSphCs (#8) exposed to 0.5 μM FOX and 12.5 μg/ml Xanthohumol or Mix, alone or in combination, for 48 hours. DNA content was assessed by propidium iodide (PI) staining; (**b**) Percentage of cells treated as in (a) showing caspase-3 activity assessed by flow cytometry analysis; (**c**) Network integration of multiple genes showed in Figure 3C calculated by geneMANIA software.

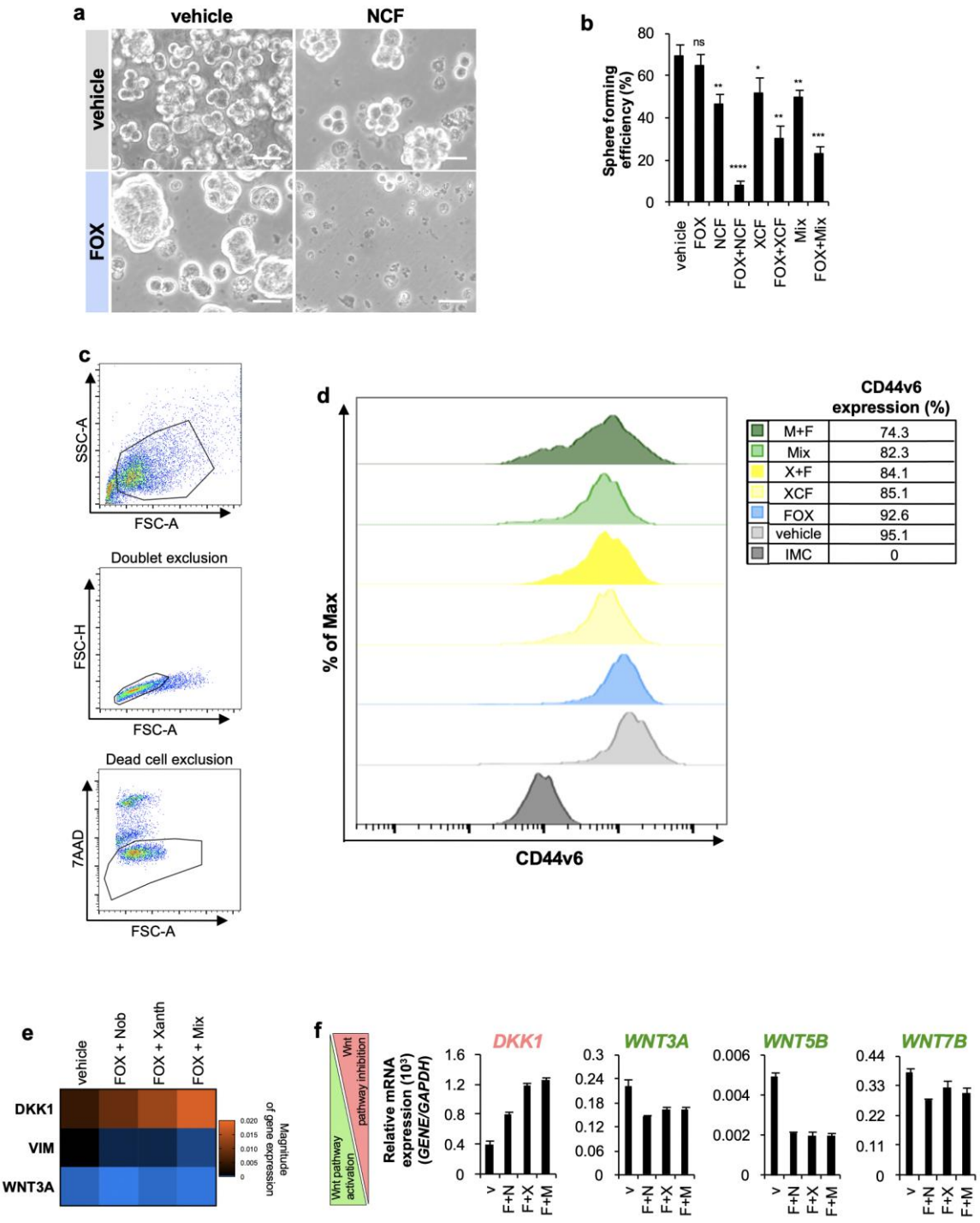


Figure S4. NCF and XCF plus chemotherapy counteract the stemness potential of CR-CSCs (**a**) Phase contrast image showing the sphere forming capability of CR-CSCs, cultured at low cell density, treated with 0.5 μM FOX and 12.5 μg/ml NCF, alone or in combination, for 48 hours, scale bar represents 25μm; (**b**) Sphere forming efficiency of CR-CSCs treated with 0.5 μM FOX and 12.5 μg/ml NCF, alone or in combination, for 48 hours (**c**) Representative flow cytometry analysis showing the gating strategies of CR-CSphC shown in Figure 3D; (**d**) Representative flow cytometry analysis of CD44v6 expression on CR-CSCs following 48 hours treatment with 12.5 μg/ml of NCF, XCF or Mix extracts alone or in combination with 0.5 μM FOX for 72 hours; (**e**) Gene expression analysis of genes involved in stemness and EMT in CR-CSphCs after exposure to 0.5 μM FOX and 12.5 μg/ml NCF, XCF or Mix, as compared to control (vehicle) for 48 hours. Data are expressed as $2^{-\Delta\Delta Ct}$ expression values normalized to *GAPDH* and *HPRT* genes; (**f**) Relative mRNA expression levels of genes involved in Wnt pathway activation (*DKK1*, *AXIN1*, *AXIN2*, *CK1*) and inhibition (*WNT3A*, *WNT5B*, *WNT7B*). *GAPDH* was used as control.

	#	Age	Gender	Site	Stage	Grading	TNM classification	MSI clinical relevant	CD44v6 (%)	*KRAS*	*BRAF*	*APC*	*PIK3CA*
CR-CSphC	3	85	F	colon (right site)	IIIC	G3	T3N2M0	MSI	33	*wt*	*mut*	*wt*	*mut*
	8	57	F	colon (right site)	IV	G3	T3N2M1	MSS	92.3	*mut*	*wt*	*mut*	*mut*
	24	51	F	colon (right site)	IIA	G2	T3N0M0	MSI	9.96	*wt*	*wt*	*mut*	*wt*
	37	82	M	colon (right site)	IIIC	G3	T3N2M0	MSI	46	*wt*	*wt*	*mut*	*wt*
	59	78	M	colon	IIIB	G3	T3N1M0	MSS	85.4	*mut*	*wt*	*wt*	*wt*
	R7p	70	M	colon (left side)	NA	NA	NA	NA	NA	*wt*	*mut*	NA	*wt*
Chemoresistant CR-CSphC	R1	65	M	liver metastasis of colon cancer (sigmoid)	IIA	NA	T3N0M0	MSS	13.7	*wt*	*wt*	NA	NA
	R2	61	F	liver metastasis of colon cancer (rectum)	IV	NA	T3N1M1	MSS	19.1	*wt*	*wt*	NA	NA
	R3	62	F	liver metastasis of colon cancer (rectum)	IIIB	NA	T3N1M0	MSS	22.8	*mut*	*wt*	NA	NA
	R4	38	M	liver metastasis of colon cancer (rectum)	IV	NA	T4N1M1	MSS	93	*wt*	*mut*	NA	NA
	R6	69	F	liver metastasis of colon cancer (left site)	NA	NA	NA	NA	NA	*wt*	*wt*	NA	*wt*
	R7	70	M	liver metastasis of colon cancer (left site)	NA	NA	NA	NA	NA	*wt*	*mut*	NA	*wt*

Table S1. CR-CSphCs characterization, CD44v6 expression, MSI profile, and *KRAS*, *BRAF*, *APC* and *PIK3CA* gene mutational profile. TNM classification is referred to the time of initial diagnosis. Wt, wild-type; mut, mutated; NA, data not available.