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## Supplemental Information

# Plasticity of Lgr5-Negative Cancer Cells Drives Metastasis in Colorectal Cancer

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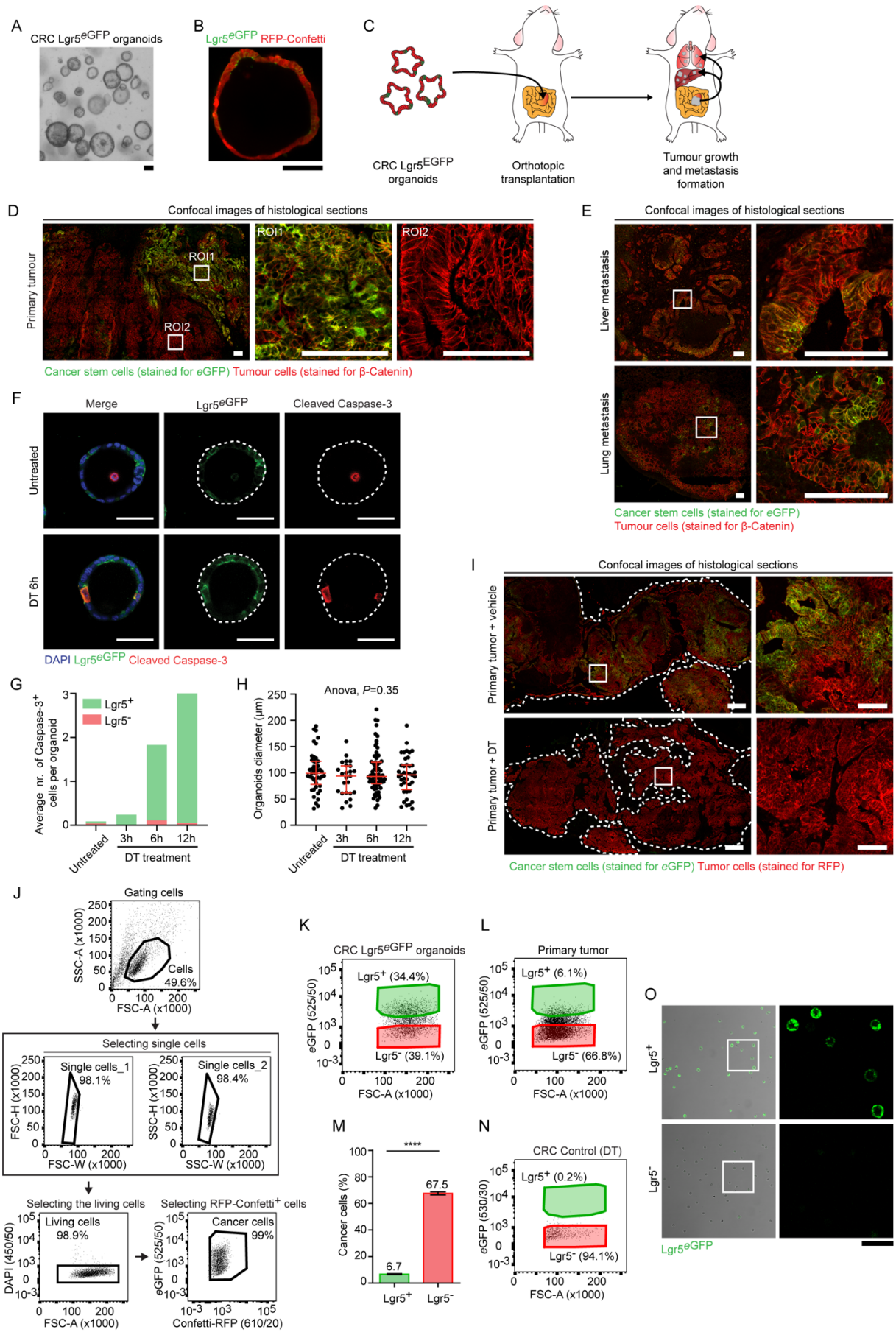

A
CRC Lgr5eGFP organoids
B
Lgr5eGFP RFP-Confetti
C
CRC Lgr5EGFP organoids
Orthotopic transplantation
Tumour growth and metastasis formation
D
Confocal images of histological sections
Primary tumour
ROI1
ROI2
Cancer stem cells (stained for eGFP) Tumour cells (stained for β-Catenin)
E
Confocal images of histological sections
Liver metastasis
Lung metastasis
Cancer stem cells (stained for eGFP)
Tumour cells (stained for β-Catenin)
F
Merge
Lgr5eGFP
Cleaved Caspase-3
Untreated
DT 6h
DAPI Lgr5eGFP Cleaved Caspase-3
G
Average nr. of Caspase-3+ cells per organoid
Lgr5+
Lgr5-
Untreated
3h
6h
12h
DT treatment
H
Anova, P=0.35
Organoids diameter (µm)
Untreated
3h
6h
12h
DT treatment
I
Confocal images of histological sections
Primary tumor + vehicle
Primary tumor + DT
Cancer stem cells (stained for eGFP) Tumor cells (stained for RFP)
J
Gating cells
SSC-A (x1000)
FSC-A (x1000)
Cells 49.6%
Selecting single cells
Single cells_1 98.1%
Single cells_2 98.4%
FSC-H (x1000)
FSC-W (x1000)
SSC-H (x1000)
SSC-W (x1000)
Selecting the living cells
Selecting RFP-Confetti+ cells
Living cells 98.9%
Cancer cells 99%
DAPI (450/50)
FSC-A (x1000)
eGFP (525/50)
Confetti-RFP (610/20)
K
CRC Lgr5eGFP organoids
Lgr5+ (34.4%)
Lgr5- (39.1%)
eGFP (525/50)
FSC-A (x1000)
L
Primary tumor
Lgr5+ (6.1%)
Lgr5- (66.8%)
eGFP (525/50)
FSC-A (x1000)
M
****
Cancer cells (%)
6.7
67.5
Lgr5+
Lgr5-
N
CRC Control (DT)
Lgr5+ (0.2%)
Lgr5- (94.1%)
eGFP (530/30)
FSC-A (x1000)
O
Lgr5+
Lgr5-
Lgr5eGFP

**Figure S1 related to Figure 1. (A)** Representative example of a CRC organoid culture generated from VillinCre-ER$^{T2}$; *APC*$^{fl/fl}$; *KRAS*$^{LSL\text{-}G12D/+}$; *P53*$^{KO/KO}$; R26R-Confetti; Lgr5$^{DTR\text{-}eGFP}$ transgenic mice. **(B)** Representative example of a VillinCre-ER$^{T2}$; *APC*$^{fl/fl}$; *KRAS*$^{LSL\text{-}G12D/+}$; *P53*$^{KO/KO}$; R26R-Confetti; Lgr5$^{DTR\text{-}eGFP}$ endogenously expressing RFP-Confetti, in which CSCs are additionally labelled with membrane-bound *e*GFP (Lgr5$^{DTR\text{-}eGFP}$). **(C)** Experimental setup: VillinCre-ER$^{T2}$; *APC*$^{fl/fl}$; *KRAS*$^{LSL\text{-}G12D/+}$; *P53*$^{KO/KO}$; R26R-Confetti; Lgr5$^{DTR\text{-}eGFP}$ were orthotopically transplanted in recipient mice. Over time, organoids grow into primary intestinal tumors that spontaneously metastasize to liver and lungs. **(D)** Representative confocal images of orthotopic primary CRC. ROI = region of interest. **(E)** Representative confocal images of spontaneous liver (upper panel) and lung (lower panel) metastases derived from orthotopic CRC. **(F)** Representative confocal images of CRC Lgr5$^{eGFP}$ organoids either untreated or treated with diphtheria toxin (DT) for 6 hours, stained with cleaved-Caspase-3 (red). Scales bars in A-F are all 100 µm. (**G)** Average number of cleaved Caspase-3 positive Lgr5$^{+}$ or Lgr5$^{-}$ cancer cells per CRC Lgr5$^{eGFP}$ organoid, analyzed in different experimental conditions (untreated, DT-treated for either 3, 6 or 12 hours). **(H)** Diameter of CRC Lgr5$^{eGFP}$ organoids included in the cleaved-Caspase-3 analysis. Red lines represent medians ± interquartile range. *P*-value calculated with one-way Anova test. **(I)** Representative examples of primary tumors either treated with vehicle (upper panel) or diphtheria toxin (DT) (lower panel). Dashed lines highlight tumor edges. Scale bars 500 µm (overview), 100 µm (zoom-in). (**J)** FACS strategy enabling the identification and sorting of Lgr5$^{+}$ CSCs and Lgr5$^{-}$ cancer cells in organoids and orthotopic primary tumors. **(K)** Representative example of FACS profile of CRC Lgr5$^{eGFP}$ organoids. **(L)** Representative example of FACS profile of Lgr5$^{+}$ CSCs and Lgr5$^{-}$ cancer cells in primary orthotopic CRC. **(M)** Quantification of Lgr5$^{+}$ CSCs and Lgr5$^{-}$ cancer cells observed in orthotopic CRC by FACS ($n$=8). Data are presented as mean ± SEM, *P*-value ****< 0.0001, calculated with unpaired t-test with Welch's correction. **(N)** Representative example of FACS profile of CRC Lgr5$^{eGFP}$ organoids subjected to selective ablation of Lgr5$^{+}$ CSCs by diphtheria toxin treatment (DT), used to set the Lgr5$^{-}$ gating. **(O)** Representative examples of sorted Lgr5$^{+}$ CSCs and Lgr5$^{-}$ cancer cells. Scales bars are all 100 µm.

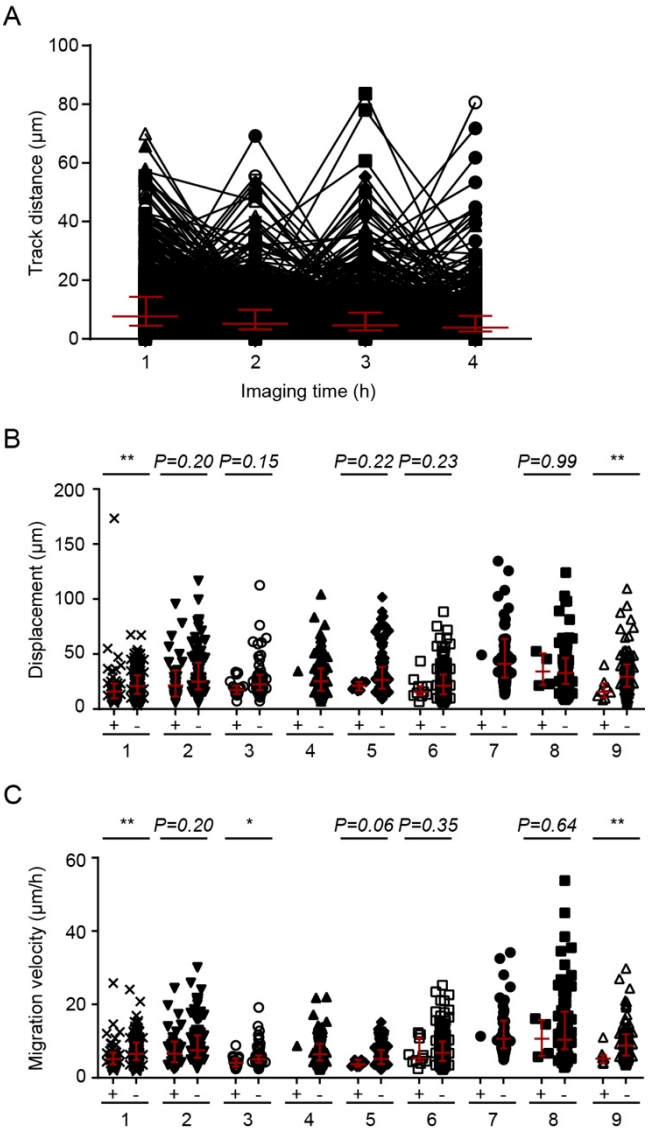
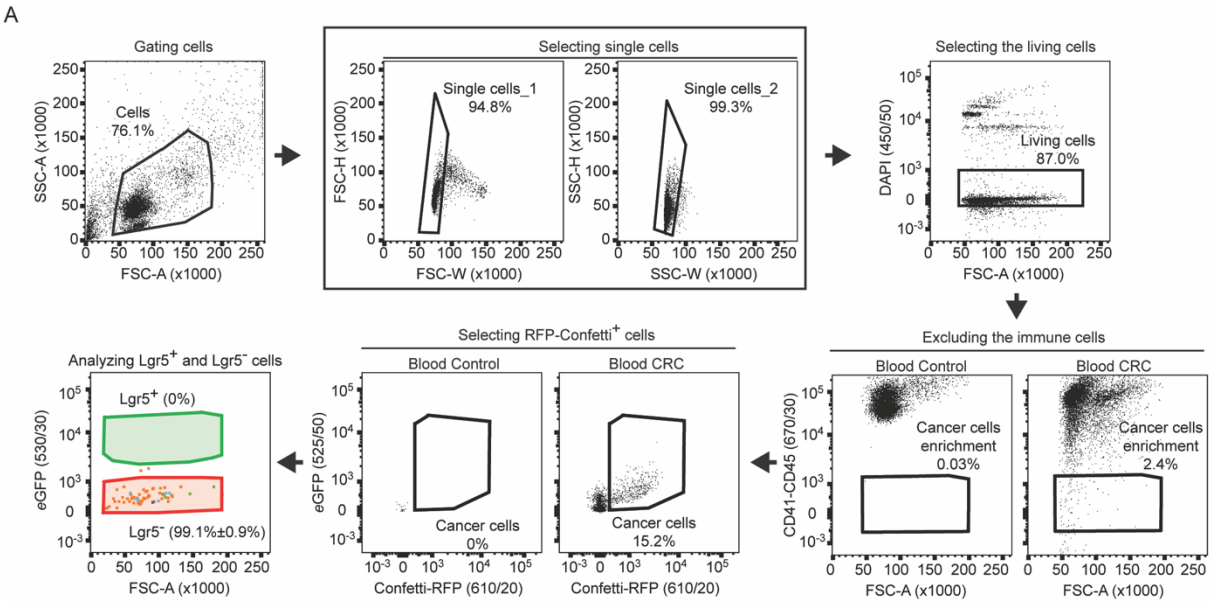
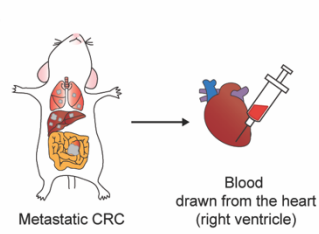


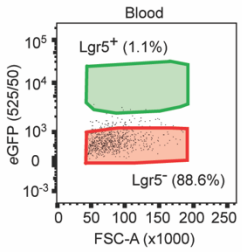

**Figure S2 related to Figure 2. (A)** Track distance of individual migratory cells followed by intravital imaging over the course of 4 hours. Each line represents an individual cell. Red lines indicate median (per time point) ± interquartile range. **(B-C)** *In vivo* displacement (B) and velocity (C) of escaping Lgr5$^{+}$ (+) CSCs and Lgr5$^{-}$ (-) cancer cells imaged in individual mice (n=9). Each data point represents a cell. *P*-value *< 0.05, **< 0.001. Red lines indicate median ± interquartile range. *P*-values calculated with Mann-Whitney U-test.

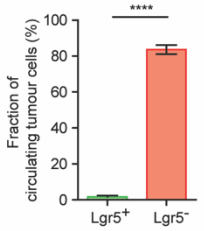


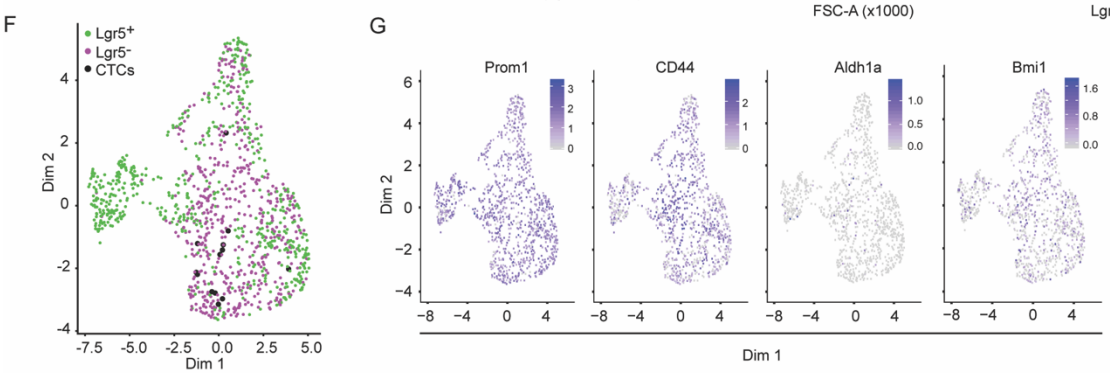

B

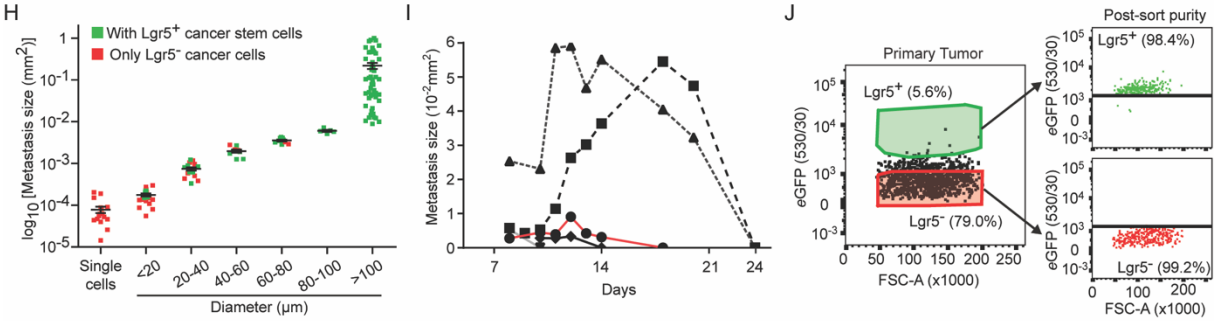
| | Total count | Lgr5+ | Lgr5- |
|---|---|---|---|
| Sample 1 | 5 | 0 | 5 |
| Sample 2 | 1 | 0 | 1 |
| Sample 3 | 6 | 0 | 6 |
| Sample 4 | 56 | 0 | 54 |




K

| Replicate | Lgr5- post-sort purity (%) | Nr. of Lgr5+ cells injected as purity control | Nr. of metastasis in purity control | Nr. of metastasis in Lgr5+ sample | Nr. of metastasis in Lgr5- sample |
|---|---|---|---|---|---|
| 1 | 100% | - | 0 | 105 | 76 |
| 2 | 99.2% | 48 | 0 | 52 | 17 |
| 3 | 100% | - | 0 | 90 | 25 |
| 4 | 100% | - | 0 | 77 | 24 |

**Figure S3 related to Figure 3. (A)** FACS strategy enabling distinctive sorting of circulating $Lgr5^+$ CSCs and $Lgr5^-$ cancer cells. Blood control = blood sample collected from a control NOD.Cg-Prkdc$^{scid}$ Il2rg$^{tm1Wjl}$/SzJ (NSG) mouse. **(B)** Table illustrating the total count of circulating $Lgr5^+$ CSCs and $Lgr5^-$ cancer cells in each blood sample obtained from the portal vein of mice bearing metastatic CRC tumors (see Figure 3A). The colour-coding links individual circulating tumor cells shown in Figure 3B to the corresponding blood sample. **(C)** Experimental setup: A metastatic intestinal tumor model was used to sample blood from the right ventricle of the heart. Blood was analyzed by FACS for the presence of circulating tumor cells. **(D)** Representative example of FACS profile of circulating $Lgr5^+$ CSCs and $Lgr5^-$ cancer cells detected in the systemic blood**. (E)** Quantification of circulating tumor cells found in the systemic blood ($n$=5). Data are presented as mean ± SEM, $P$-value ****< 0.0001, calculated with unpaired t-test with Welch's correction. **(F)** tSNE plot representing unbiased clustering of $Lgr5^+$ (green), $Lgr5^-$ (purple) in primary tumor, liver metastasis and circulating tumor cells (CTCs, black). **(G)** tSNE plots showing expression level of selected cancer stem cell marker genes in $Lgr5^+$, $Lgr5^-$ cancer cells of primary tumor, liver metastasis and circulating tumor cells clustered in (F). **(H)** Analysis of spontaneous liver metastases found in mice bearing orthotopic CRC tumors. Metastases are grouped per diameter range and plotted according to size (i.e. area represented in $mm^2$). Each individual point represents a metastatic lesion ($n$=132). The colour-coding identifies metastases composed of only $Lgr5^-$ cancer cells (red) and metastases containing $Lgr5^+$ CSCs (green). Black lines represent means ± SEM. **(I)** Diphtheria toxin (DT) treated metastasis plotted by their actual size ($\mu m^2$), followed over time by intravital imaging. The red line in represents the example shown in Fig. 3G. **(J-K)** Post-sort purity analysis on $Lgr5^+$ and $Lgr5^-$ sorted populations. (K) $Lgr5^-$ post-sort purity controls calculated in each experiment ($n$=4) that were performed for the analysis shown in Figure 3J to 3K. The table illustrates the equivalent number of $Lgr5^+$ cells injected in the purity control acceptor mouse and the number of metastases observed in purity control, $Lgr5^+$ and $Lgr5^-$ injected mice in each experimental replicate ($n$=4). Red highlights the example depicted in (J).

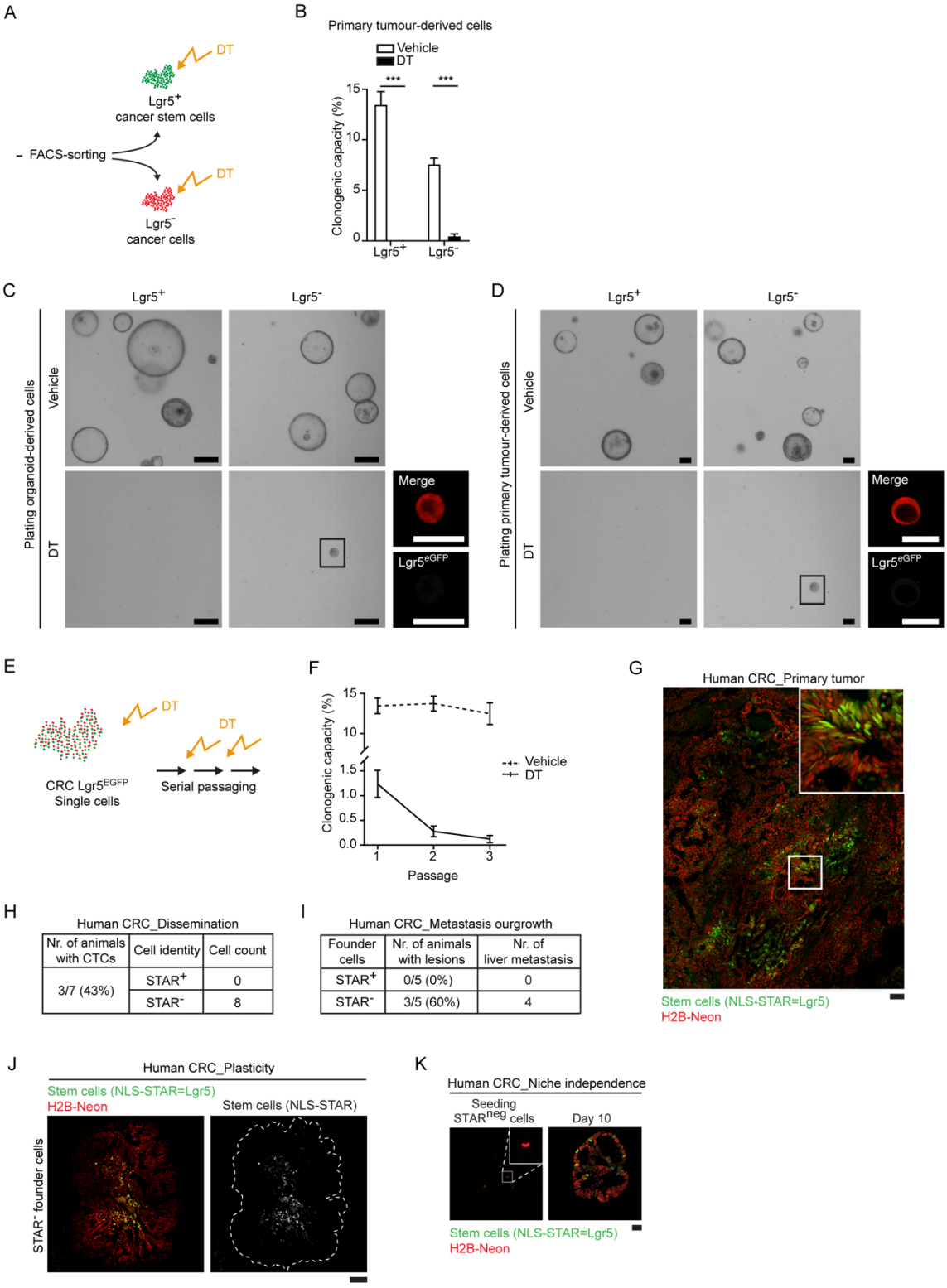

A
DT
Lgr5+
cancer stem cells
FACS-sorting
DT
Lgr5-
cancer cells
B
Primary tumour-derived cells
Vehicle
DT
***
***
Clonogenic capacity (%)
15
10
5
0
Lgr5+
Lgr5-
C
Lgr5+
Lgr5-
Plating organoid-derived cells
Vehicle
DT
Merge
Lgr5eGFP
D
Lgr5+
Lgr5-
Plating primary tumour-derived cells
Vehicle
DT
Merge
Lgr5eGFP
E
DT
DT
CRC Lgr5EGFP
Single cells
Serial passaging
F
Clonogenic capacity (%)
15
10
1.5
1.0
0.5
0.0
1
2
3
Passage
Vehicle
DT
G
Human CRC_Primary tumor
Stem cells (NLS-STAR=Lgr5)
H2B-Neon
H
Human CRC_Dissemination
Nr. of animals with CTCs
Cell identity
Cell count
3/7 (43%)
STAR+
0
STAR-
8
I
Human CRC_Metastasis ourgrowth
Founder cells
Nr. of animals with lesions
Nr. of liver metastasis
STAR+
0/5 (0%)
0
STAR-
3/5 (60%)
4
J
Human CRC_Plasticity
Stem cells (NLS-STAR=Lgr5)
H2B-Neon
Stem cells (NLS-STAR)
STAR- founder cells
K
Human CRC_Niche independence
Seeding
STARneg cells
Day 10
Stem cells (NLS-STAR=Lgr5)
H2B-Neon

**Figure S4 related to Figure 4. (A-B)** Clonogenicity assay of sorted Lgr5$^{+}$ CSCs and Lgr5$^{-}$ cancer cells derived from colorectal orthotopic primary tumors. Single cells were subjected to either vehicle or diphtheria toxin (DT) treatment (*n*=3 independent experiments). Data were collected 6 days after plating. Values are presented as mean ± SEM, *P*-value ***< 0.0001, calculated with unpaired t-test with Welch's correction. **(C-D)** Representative example of organoid formation assay of sorted Lgr5$^{+}$ CSCs and Lgr5$^{-}$ cancer cells derived from either CRC organoid cultures (C) and orthotopic colorectal primary tumors 6 days after plating (D). Single cells were subjected to either vehicle or diphtheria toxin (DT) treatment. Scale bars, 100 µm. **(E-F)** Clonogenicity assay of CRC Lgr5$^{eGFP}$ organoid-derived cells. Single cells underwent serial passaging (every 6 days) while kept under constant vehicle or diphtheria toxin (DT) treatment (*n*=3 independent experiments). **(G)** Representative confocal image of a human orthotopic primary tumor (mutated in P53, PIK3CA, BRAF, ERBB3, RNF43). DsRed-expressing Lgr5$^{+}$ cells (using an ASCL2-responsive minigene (STAR) that labels Lgr5$^{+}$ CSCs with DsRed, Oost et al., 2018) are shown with a green lookup table for consistency with the images of our mouse model. All cancer cells express nuclear mNeon (red lookup table). **(H)** Total count of circulating STAR$^{+}$ CSCs and STAR$^{-}$ cancer cells in blood samples obtained from mice bearing human metastatic CRC tumors. **(I)** Number of metastatic foci derived from mesenteric vein injection of STAR$^{+}$ CSCs or STAR$^{-}$ cancer cells (*n*=5 biological replicates). **(J)** Representative confocal images of metastases generated from human Lgr5$^{-}$ cancer cells. **(K)** Representative example of human organoid formation assay of sorted STAR$^{-}$ cancer cells and cultured in minimum CRC medium (i.e. not containing stem cell-inducing growth factors). Scale bars, 100 µm.