10 YEARS OF BREAKTHROUGHS
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Non-Canonical Hedgehog Signaling is a Positive Regulator of the WNT Pathway and is Required for the Survival of Colon Cancer Stem Cells

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CHALLENGE

Colon cancer is a heterogeneous tumor that represents the third most common cancer and fourth most common cause of cancer deaths worldwide.

Recent data supports the existence of a subpopulation of cancer stem cells (CSCs) as both the drivers of tumor growth and the source of relapse following treatment.

Elucidation of the molecular pathways that regulate CSC survival and contribute to tumor heterogeneity may therefore lead to more effective treatments. **(Figure 1)**

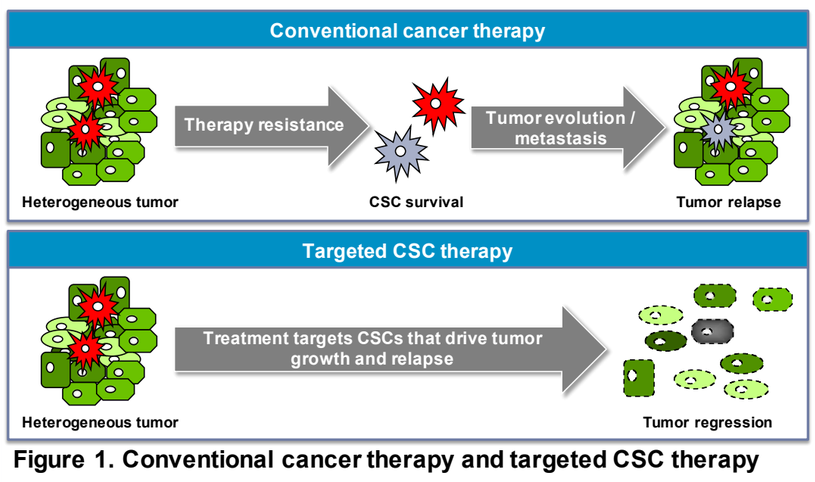

Figure 1. Conventional cancer therapy and targeted CSC therapy

Wnt and Hedgehog signaling frequently cooperate to control cell growth, homeostasis and cancer. In the intestine, Wnt signaling drives crypt-base stem cell self-renewal. Conversely, GLI-dependent Hedgehog signaling antagonizes Wnt signaling in differentiated cells at the top of the crypt.

Activating mutations in Wnt signaling are found in 90% of colon cancers. Hedgehog genes, while rarely mutated, are upregulated in colon cancer. However, therapeutic strategies that directly target Wnt or canonical (SMO-dependent) Hedgehog signaling have been unsuccessful.

Here, as part of the OncoTrack consortium, we used patient-derived organoids (PDOs) and xenograft models of colon cancer to demonstrate the survival of colon CSCs is dependent on non-canonical (SMO-independent) Hedgehog signaling, which acts as a positive regulator of Wnt signaling to regulate CSC differentiation and survival.

THE ONCOTRACK CONSORTIUM

- **Goal**: To develop and assess novel approaches for the identification of new biomarkers for colon cancer
- **Approach**: Detailed characterization of colon cancers combining novel *in vitro* and *in vivo* models, high-throughput sequencing, and systems biology

Start date: 01.01.2011	IMI funding:	€16 757 282
End date: 31.12.2016	EFPIA in kind:	€10 976 557
IMI1 - Call 2	Other:	€ 3 346 480
www.oncotrack.eu	Total Cost:	€31 080 319

Key publications:

- Regan JL, *et al.* Non-Canonical Hedgehog signaling is a positive regulator of the WNT pathway and is required for the survival of colon cancer stem cells. Cell Rep. 2017
- Schütte M, *et al.* Molecular dissection of colorectal cancer in pre-clinical models identifies biomarkers predicting sensitivity to EGFR inhibitors. Nat Commun. 2017

- OncoTrack members

CHARITÉ, BAYER, MPIMG, Lilly, Boehringer Ingelheim, Pfizer, AstraZeneca, MERCK, Roche, Janssen, VHIO Vall d'Hebron Institute of Oncology, UCL, Medizinische Universität Graz, Technische Universität Dresden, iPRI, Université Paris Sud, Uppsala Universitet, epo Experimental Pharmacology & Oncology Berlin-Buch, ALACRIS Theranostics GmbH

APPROACH & METHODOLOGY

- PDOs were generated in Matrigel™ culture from freshly isolated primary tumors and metastases
- The frequency of CSCs was determined by limiting dilution (LD) transplantation **(Figure 2A)**
- Increased aldehyde dehydrogenase (ALDH) activity is a hallmark of CSCs. ALDH^{Positive} and ALDH^{Negative} cells were isolated by fluorescence assisted cell sorting (FACS) and tested for CSC frequency by serial xenotransplantation at LD **(Figure 2B)**
- ALDH^{Positive} and ALDH^{Negative} cells were subjected to whole-transcriptome analysis **(Figure 2C)**
- Differentially expressed genes were selected for functional analysis by RNA interference and small molecule inhibition

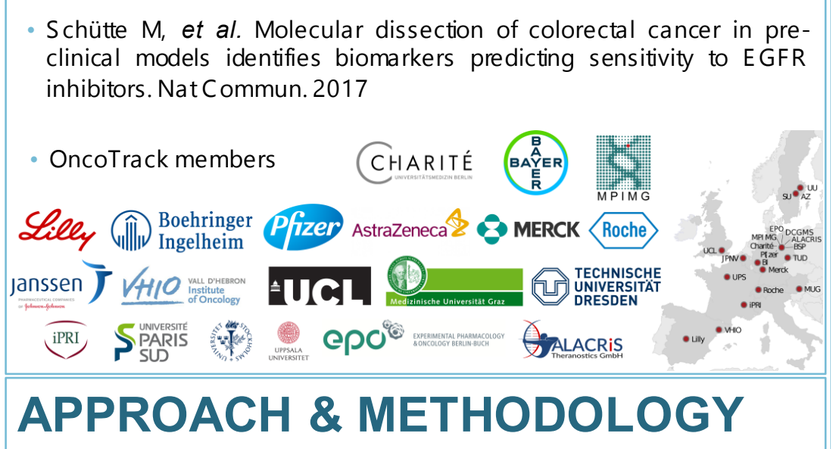

Figure 2. Summary of study methodology

RESULTS

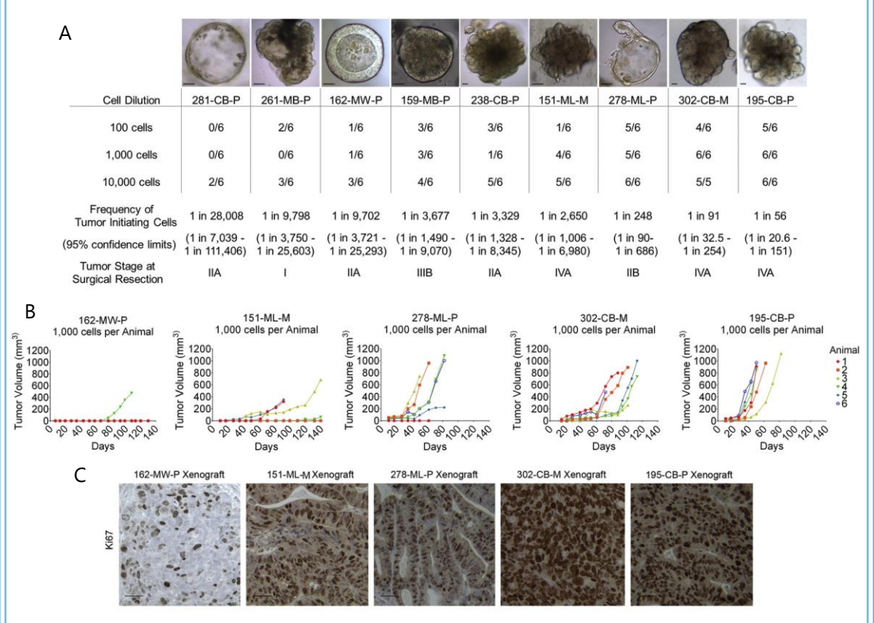

Figure 3. CSCs in colon cancer PDOs vary in frequency and are enriched in more advanced tumors (A) LD transplantation of PDO cells. Growth curves (B) and Ki67 staining (C) for five xenograft models.

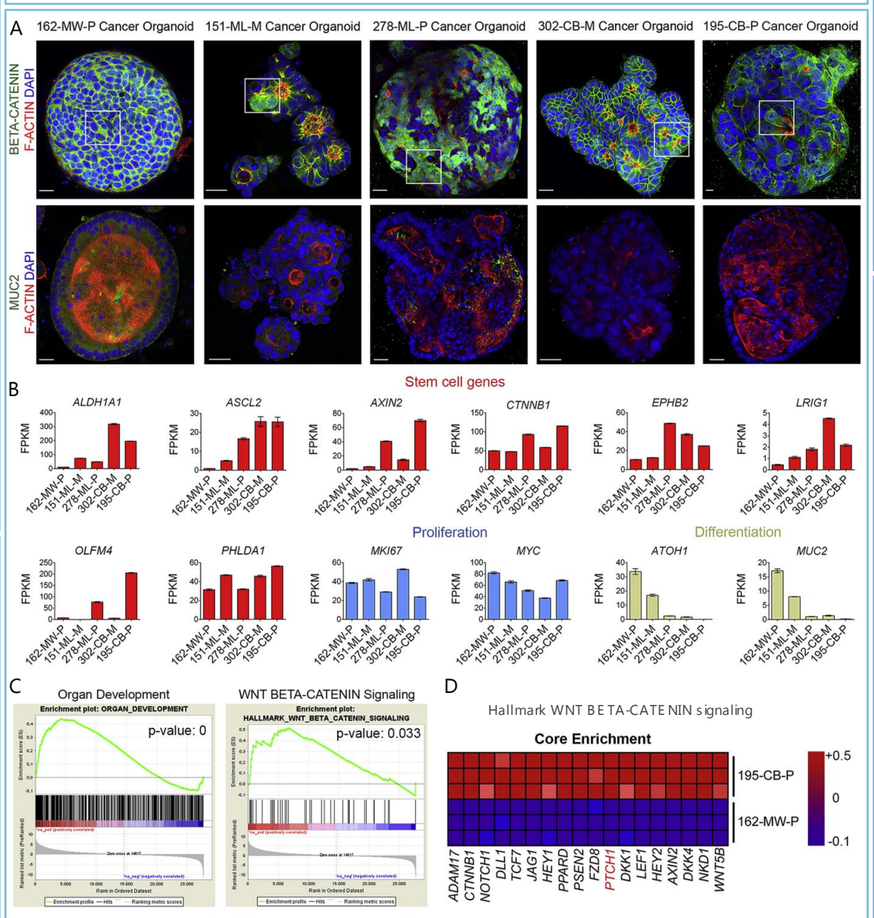

Figure 4. CSC-Enriched PDOs are heterogeneous, poorly differentiated and enriched for Wnt signaling genes (A) Immunofluorescence staining of PDOs. (B) RNA-seq generated FPKM values. (C) GSEA and (D) heatmap showing enrichment for Wnt signaling genes

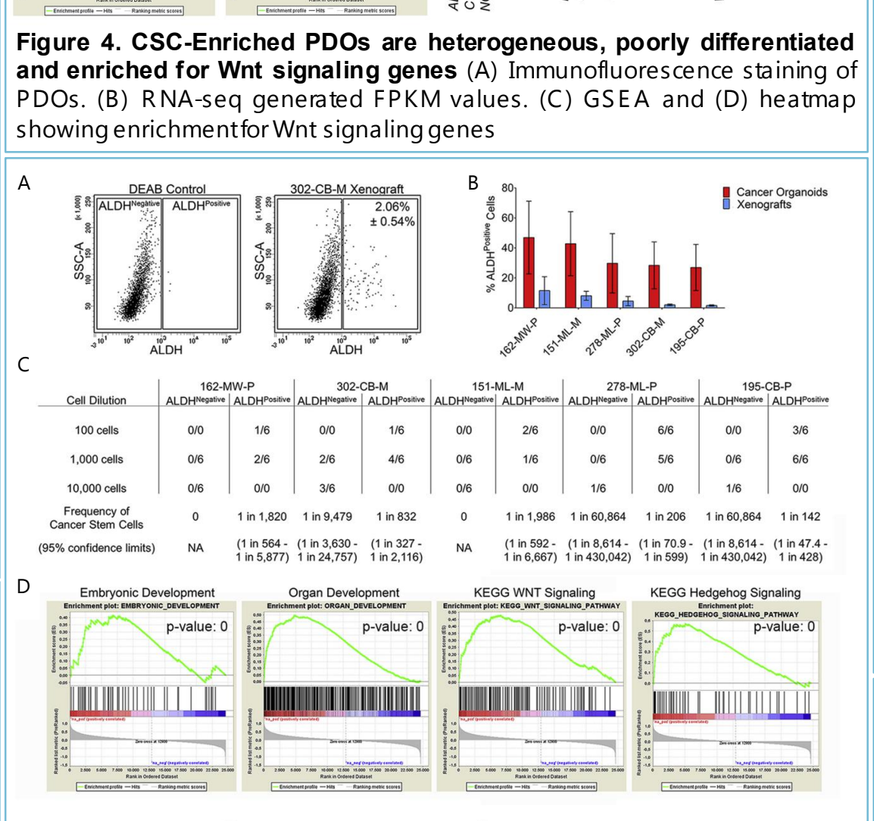

Figure 5. PDOs are enriched for ALDH^{Positive} CSCs and non-canonical Hedgehog signaling (A) Aldefluor assay FACS plots. (B) Frequency of ALDH^{Positive} cells in PDOs and xenografts. (C) LD serial transplantation table. (D) GSEA and (E) gene expression analysis of ALDH^{Positive} CSCs. Immunofluorescence staining of (F) PDOs and (G) "crypt-like" structures in frozen xenograft sections.

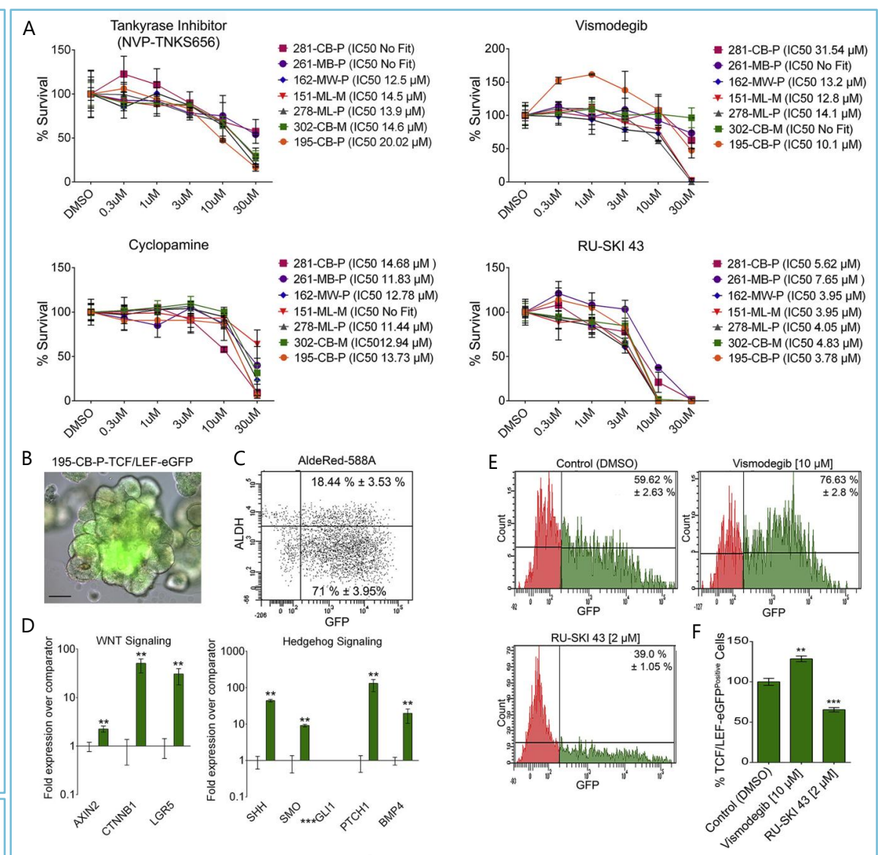

Figure 6. Non-canonical PTCH1-dependent Hedgehog signaling is a positive regulator of Wnt signaling (A) PDO survival 72 h after treatment with Tankyrase inhibitor, SMO inhibitors vismodegib and cyclopamine, and the HHAT (SHH signaling/PTCH1) inhibitor RU-SKI 43. (B) TCF/LEF-eGFP Wnt signaling reporter PDOs. (C) Aldefluor assay FACS plots. (D) TCF/LEF-eGFP^{Positive} gene expression analysis. FACS plots (E) and frequency (F) of TCF/LEF-eGFP^{Positive} cells treated with vismodegib or RU-SKI 43.

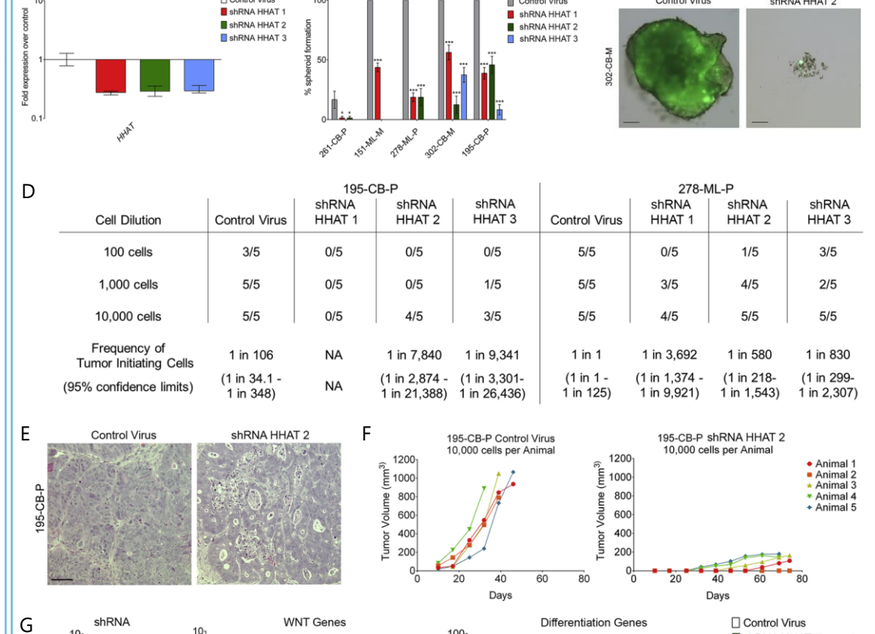

Figure 7. Non-canonical Hedgehog signaling is required for CSC survival and regulates differentiation *in vivo* (A) shRNA HHAT knockdown. (B and C) Effect of shRNA HHAT on spheroid formation. (D) LD shRNA HHAT transplants. (E) H&E staining and (F) growth curves of shRNA HHAT xenografts. (G) Gene expression analysis of shRNA HHAT xenografts. (H) Pairwise correlation of Hedgehog genes in clinical samples. (I) *PTCH1* expression in colon cancer patients across different tumor stages.

CONCLUSIONS & IMPACT

- Colon cancer PDOs are enriched for CSCs and Wnt signaling genes
- Hedgehog signaling in CSCs is non-canonical, SHH-dependent and PTCH1-dependent
- Non-canonical Hedgehog signaling is a positive regulator of Wnt signaling (Figure 8)
- CSC survival depends on HHAT-mediated palmitoylation of SHH
- HHAT is a possible therapeutic target in colon cancer
- PTCH1 is a potential biomarker for colon cancer prognosis

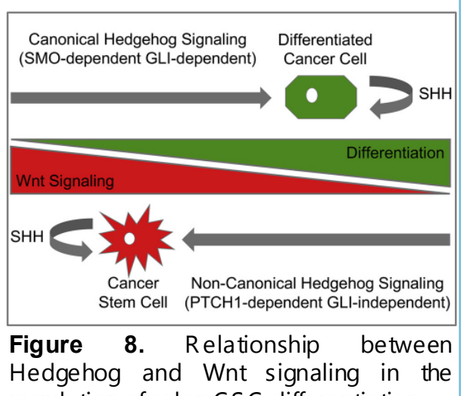

Figure 8. Relationship between Hedgehog and Wnt signaling in the regulation of colon CSC differentiation

VALUE OF IMI COLLABORATION

- Patient recruitment and tissue collection by public partners
- Access to novel patient-derived tumor models, primary tissue samples, sequencing and clinical data from public and private consortium partners
- Access to small molecule inhibitors from EFPIA partners
- Organization of collaborators into workgroups based on expertise facilitated specialization and ease of interdisciplinary and international collaboration
- Invaluable contributions and insights gained from regularly presenting and discussing work with OncoTrack consortium partners at IMI meetings

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