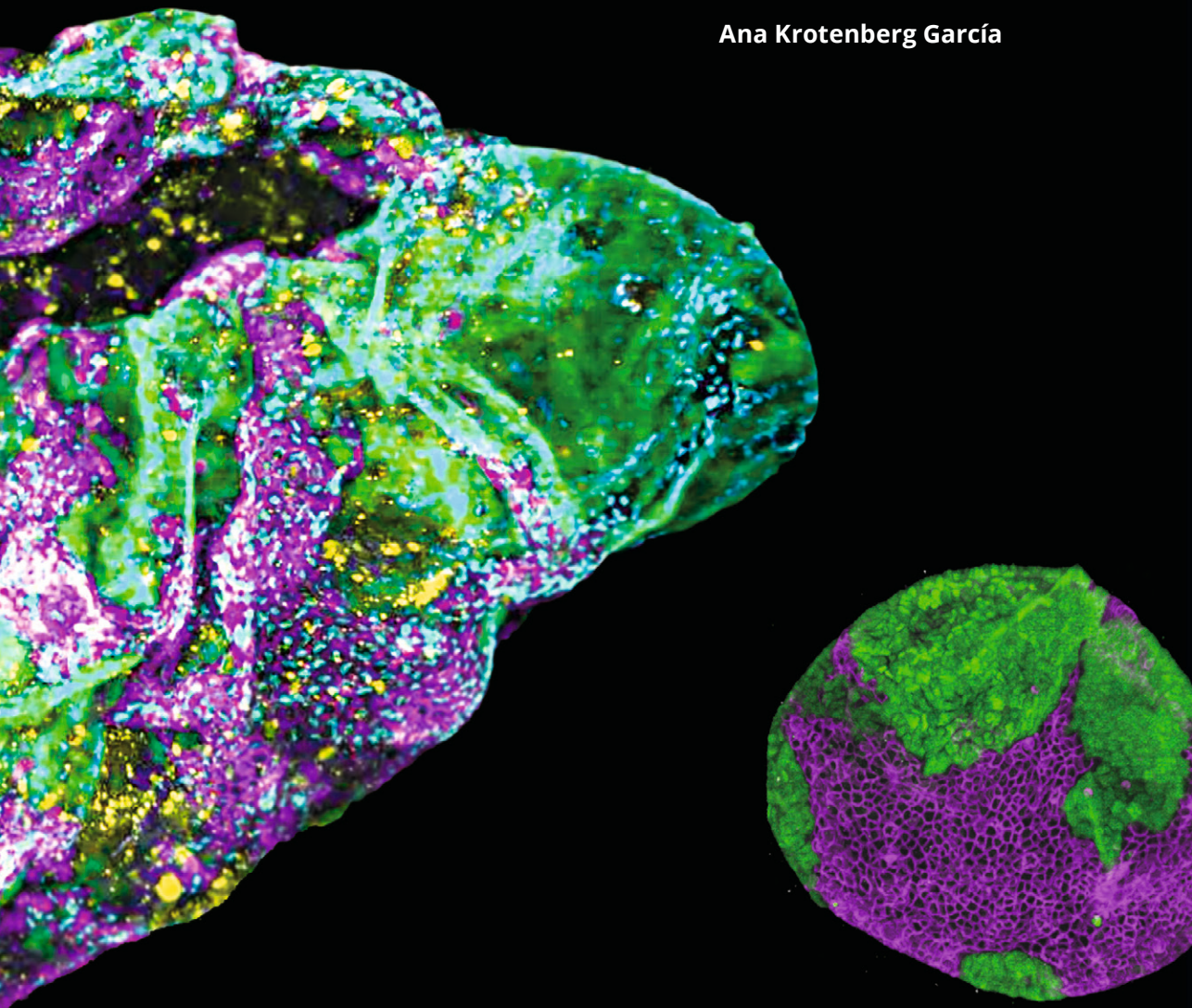
THE ROLE OF CELL COMPETITION IN INTESTINAL CANCER: FROM PRIMARY TUMOR TO LIVER METASTASIS

Ana Krotenberg García



THE ROLE OF CELL COMPETITION IN INTESTINAL CANCER: FROM PRIMARY TUMOR TO LIVER METASTASIS

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The role of cell competition in intestinal cancer: from primary tumor to liver metastasis

De rol van celcompetitie bij darmkanker: van primaire tumor tot levermetastase

(met een samenvatting in het Nederlands)

El papel de la competencia celular en el cáncer intestinal: del tumor primario a la metástasis hepática

(con un resumen en Español)

Proefschrift

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Quiero dedicar esta tesis a dos personas muy especiales que en los momentos de flaqueza en estos duros años, me han hecho recordar que la lucha por entender y combatir el cáncer merece la pena....

*La primera es **mi abuelo Hermán**, esté donde esté me encantaría que viera con orgullo que "sus ojitos" por fin se gradúa como doctora. Aunque ya no estés aquí, te llevamos siempre en nuestros corazones.*

*La segunda es mi queridísima amiga y prácticamente hermana, **Inge**. Tú me has demostrado lo que significa seguir adelante en los peores momentos y luchar contra cualquier adversidad, por muy dura que sea. Si este trabajo contribuye en algo, aunque sea mínimo, a ayudar a gente que pasa por la horrible experiencia que es un cáncer, ha merecido totalmente la pena. Gracias por estar de principio a fin ahí, independientemente de las circunstancias. Si no te hubiera tenido de ejemplo de superación tan cerca de mí, no sé si lo habría logrado. Ahora solo quiero decirte que, si yo he llegado al fin de este túnel, tú también lo harás, no te rindas nunca, ¡recuerda que eres todo un modelo a seguir y que indudablemente TU PUEDES!*

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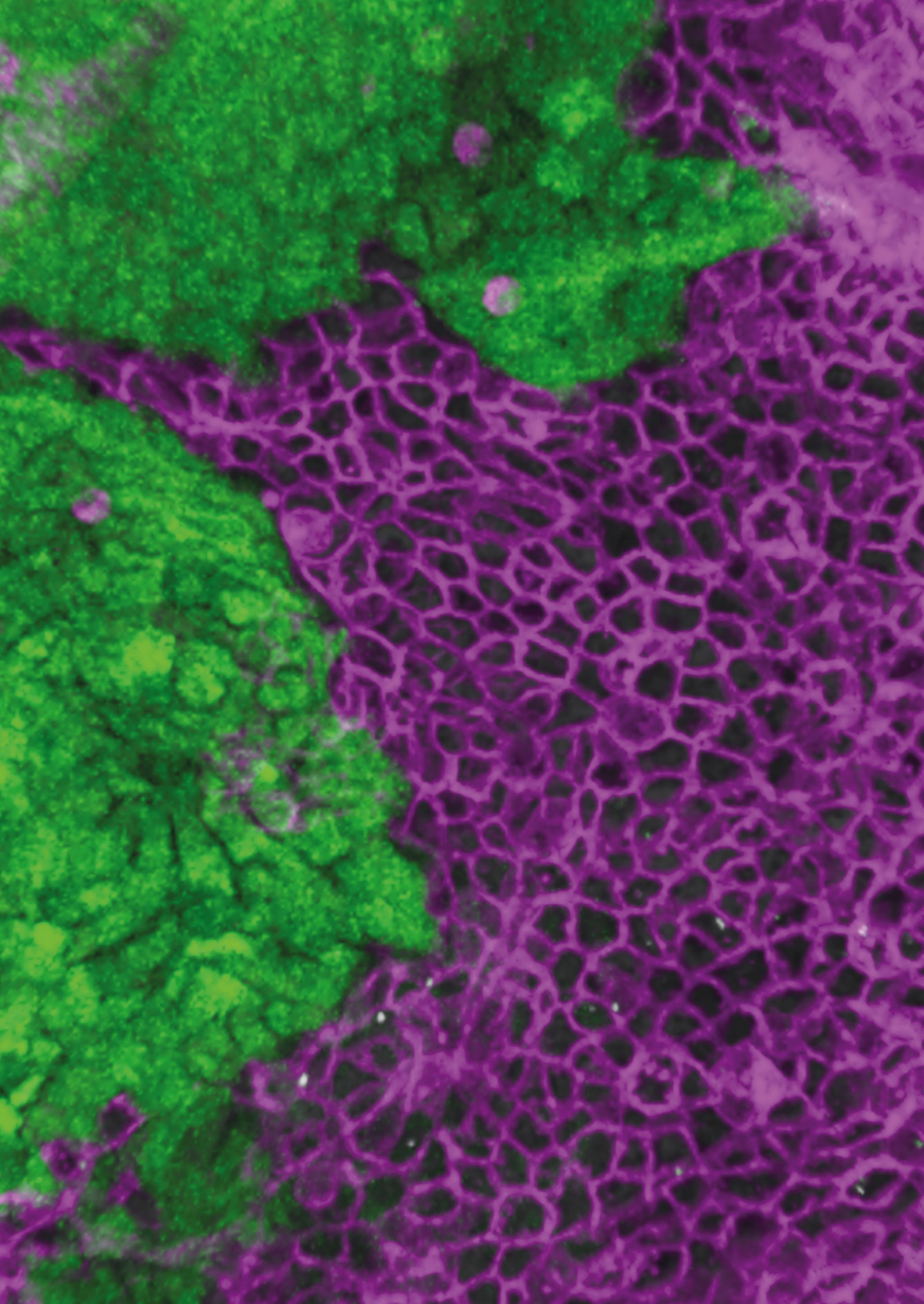

Cell competition in primary and metastatic colorectal cancer

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ABSTRACT

Adult tissues set the scene for a continuous battle between cells, where a comparison of cellular fitness results in the elimination of weaker "loser" cells. This phenomenon, named cell competition, is beneficial for tissue integrity and homeostasis. However, it is increasingly apparent that cell competition doubles as a tumor-promoting mechanism. The comparative nature of cell competition means that mutational background, proliferation rate and polarity all factor in to determine the outcome of this process. In this review, we explore the intricate and context-dependent involvement of cell competition in homeostasis and regeneration, as well as during initiation and progression of primary and metastasized colorectal cancer. We provide a comprehensive overview of molecular and cellular mechanisms governing cell competition and its parallels with regeneration.

CELL COMPETITION

During embryonic development, cells are subjected into a variety of pressures that may compromise their function and, subsequently, tissue integrity. Therefore, the effective elimination of abnormal cells is essential to ensure tissue fidelity and intact development of multicellular organisms. Upon cellular damage, cells activate a variety of innate stress response pathways that either promote cell survival or initiate programmed cell death [1]. Cell-based quality control mechanisms continuously guard the integrity of tissues and organs. These mechanisms are referred to as cell competition and consist of surveillance programs that can monitor relative cellular fitness levels among cells (Figure 1). During cell competition, those cells perceived as less fit ('losers') in a given microenvironment are eliminated, leading to the prevalence and expansion of the neighboring, fitter cells ('winners') [1]. Cell competition was originally described in *Drosophila melanogaster* almost 50 years ago. Morata and Ripoll studied the effect of *Minute* mutations, which affect genes encoding ribosomal proteins, on the development of wing imaginal discs [2]. Although homozygous mutations of *Minute* are lethal, heterozygous *Minute* flies are viable and show a minor general developmental delay due to reduction in cellular proliferation rate. Interestingly, studies in genetic mosaics showed that *Minute*$^{-/+}$ cells in a wild-type background cannot survive and are susceptible to apoptotic cell death while wild-type cells proliferate at the expense of the mutants [2,3]. These findings suggest that relative differences in the proliferation rates of the two cell populations leads to cell competition. A similar pattern was observed in mouse chimeras, where cells bearing heterozygous mutations in the *L24* ribosomal protein gene exhibit reduced levels of protein synthesis and proliferation, are outcompeted by wild-type cells [4]. The concept that cell competition depends on the relative rather than the absolute levels of fitness between two neighboring cells is reinforced by the phenomenon known as 'supercompetition'. According to this, wild-type cells can be eliminated if the surrounding cells pose a proliferative advantage over them [5]. This was first observed in the imaginal wing disc of *Drosophila,* where cells overexpressing the proto-oncogene *Myc* outcompete their normal wild-type neighbors that have a lower proliferation rate [6,7]. Furthermore, studies in mosaic murine tissues, such as the epiblast and heart, showed that *Myc* overexpression results in elimination of adjacent wild-type cells [8–10]. These findings support that cell competition is a context-dependent process that optimizes tissue fitness (extensively reviewed in: [11,12]). In this review, we describe the different modes of cell competition and highlight their role in primary and metastatic colorectal cancer.

Passive and active cell competition

Quality control of tissues plays a crucial role in maintaining the function of organs. Globally two distinct modes of cell competition can be distinguished. The first type passively influences the potential of cells to self-renew without affecting surrounding cells. This type of competition typically targets stem cells, a pool of undifferentiated cells found in various tissues that are responsible to replenish cells and ensure that damaged or aging tissues can be repaired or regenerated [13]. Tissue turnover often involves cells with a similar genetic profile and comparable fitness level. Therefore, tissue replenishment is driven by stochastic loss and replacement events between cells, which is known as neutral competition [14,15]. Even though genetically identical cells have an intrinsic comparable level of fitness cell-extrinsic factors, such as location within a stem cell niche and availability of growth factors, can still determine their maintenance within a tissue. This biased competition drives cell selection without direct interference of surrounding cells. In the second type of competition, the behavior of cells is directly influenced by neighbors with a differential fitness. This active cell competition can be considered as a stepwise process that requires cellular alterations that affect cellular fitness, the recognition of differences in relative fitness and cell selection. To decipher the factors that trigger cell competition, we should first unravel the key genetic alterations that can induce a gradient of fitness among cells. Such genetic alterations can globally be classified into three groups (Figure 1).

I Disparities in proliferation rates

Several studies have illustrated that two competing cell populations are often characterized by a differential proliferation rate where fitter cells grow faster than loser cells. This is highlighted in the wing disc of *Drosophila*, where slow-growing *Minute* mutants are outcompeted by fast-growing wild-type counterparts [2]. However, growth differences among adjacent cells are not determinant for promoting elimination of the hypo-proliferative clones. For instance, overexpression of the phosphoinositide3-kinase (PI3K) Dp110 subunit in the insulin growth pathway or of the pro-proliferative cell-cycle regulators Cyclin D and cyclin-dependent kinase 4 (CDK4) do not induce elimination of surrounding wild-type cells. Instead, overexpression of such genes results in larger organs [6,7]. Therefore, emphasis was given to regulators of cell growth as a potential trigger for cell competition. Initially, protein synthesis was thought to be a main driver of cell competition, based on the decreased ribosomal translation that characterizes *Minute* mutants in *Drosophila*. However, a recent study shows that proteotoxic stress is the main cause of outcompetition of *Minute* mutant cells [16]. *Myc* overexpression has been correlated with both enhanced protein synthesis and aerobic glycolysis [6,7] and in the mosaic wing disc of *Drosophila*, confrontation of *Myc*-overexpressing cells with wild-type cells enhances the metabolic switch of the former

to glycolysis [7,17]. Hence, *Myc*-overexpressing mutants acquire a 'supercompetitor' status by reprogramming their metabolism due to their interaction with wild-type cells. This suggests that differences in anabolic pathways and energy metabolism can induce competition among cells. Similarly, it could be argued that the metabolic reprogramming characterizing cancer cells help them to adopt a 'supercompetitor' behavior that favors their progression.

II Disparities in activation of signaling pathways

Several studies support that differences in signaling pathways regulating cell proliferation and growth, such as WNT, can trigger competition among those cells [18]. In the wing disc of *Drosophila*, cells with low levels of WNT/Wg are susceptible to elimination by wild-type counterparts. A second pathway that can trigger cell competition among cells with disparities in the signaling levels is the highly conserved JAK-STAT pathway. The effect of this pathway in cell competition was described in mosaic eyes and wing discs of *Drosophila*, consisting of *Stat92E*$^{-/-}$ and wild-type clones. Interestingly, *Stat92E*$^{-/-}$ clones are eliminated through apoptosis induced by wild-type cells that undergo compensatory proliferation [19]. Conversely, hyperactivation of JAK-STAT pathway allows cells to eliminate their wild-type counterparts [20]. The Hippo pathway, implicated in cell proliferation and maintenance of tissue homeostasis, is an additional example that stimulates competitive interactions among cells. Reduced TEAD activity in *in vitro* murine fibroblasts results in their elimination by surrounding wild-type cells. Furthermore, in *Drosophila* and mouse, overexpression of YAP/Yorkie or Tead4 with the subsequent Hippo inactivation can turn the cells into supercompetitors that enforce programmed cell death of wild-type neighbors [21,22].

III Loss of apico-basal cell polarity and architecture in epithelial tissues

Cell shape is fundamental in regulating the morphology, migration, and functionality of many cell types and therefore has a major impact on defining and maintain tissue architecture. There is a high correlation between cell shape and polarity, which is the asymmetric distribution of cellular components. Defects in cell polarity can compromise tissue homeostasis by affecting cellular response to signals, intercellular communication and intrinsic processes, such as survival, proliferation, and migration [23]. In epithelial tissues, every cell must form an apical surface, facing towards the outside of the tissue and a basal surface that is attached to the basal membrane. In *Drosophila*, homozygous mutations in any polarity-related genes result in excessive tissue overgrowth and loss of tissue organization. Notably, these mutants within a mosaic background are actively eliminated by the wild-type neighbors, which implies that disruption of cell polarity can render cells unfit and, therefore, induce cell competition [24].

Fitness-sensing mechanisms

Upon induction of cell competition, the second and most important step that allows competition is the ability of cells to sense differences in fitness (Figure 1). The transmembrane protein Flower constitutes a potential mechanism, by which cells could communicate their fitness status [25]. In fruit flies, *Flower (FWE)* gene encodes three isoforms, the 'ubiquitous' or FWE^{Ubi} that is expressed throughout the wing disc and another two $FWE^{Lose\text{-}A}$ and $FWE^{Lose\text{-}B}$ isoforms that are upregulated in cells with a 'loser' phenotype during cell competition [25]. Interestingly, this pattern was observed in mosaic tissues of *Myc*-overexpessing and *Scrib* mutants in *Drosophila*, where FWE^{Lose} expression was critical for 'loser' cell elimination [26,27]. Mammals have four FWE isoforms; FWE2 and FWE4 that act as super competitors and eliminate cells expressing FWE1 and FWE3 [28]. Furthermore, several tumor types express the 'winner' isoform of FWE, which provides a growth advantage over the surrounding stromal cells that express the 'loser' isoform [28]. Therefore, the 'Flower code' seems to play a crucial role in cell selection by informing neighboring cells about their relative fitness status and, consequently, promoting the elimination of a less fit population. However, the exact mechanisms by which cells express and recognize the 'Flower' proteins remain elusive.

In tissues, a high cell density can generate increased mechanical pressure through cell-to-cell and cell-to-extracellular matrix interactions, which can be released by balancing cell proliferation and elimination [29]. During mechanical cell competition, cells with superior mechanical properties force the elimination and delamination of inferior ones [30]. For instance, during notum development in *Drosophila*, oncogenic *Ras* mutations can cause cell overcrowding which is sensed by surrounding wild-type cells. Subsequently, these wild-type cells are eliminated via apoptosis and subsequent delamination through compaction-driven downregulation of the EGFR/ERK signaling [31]. Furthermore, *Scrib* knockdown in Madin-Darby Canine Kidney (MDCK) cells causes them to become highly sensitive to compaction and as a consequence, they are eliminated from co-cultures with wild-type cells [32]. These studies suggest that cells have developed several mechanisms by which they can eliminate less-fit cells based on their mechanical properties.

Execution of cell competition

After cells have evaluated their relative fitness, they should act according to their acquired 'winner' or 'loser' status (Figure 1). This entails the elimination of less-fit cells through apoptosis, extrusion or forced differentiation. In parallel, cells with higher fitness capacity can undergo compensatory proliferation and effectively fill the void left by eliminated cells.

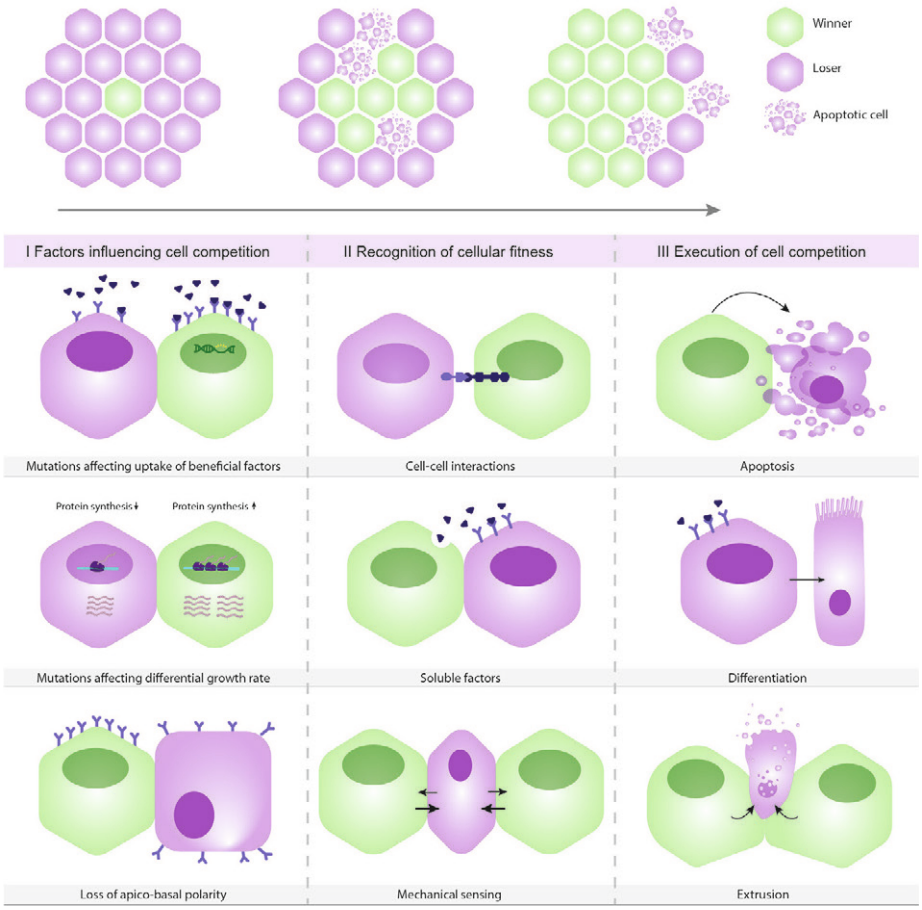

Figure 1: Schematic overview of the three stages of active cell competition. Cells acquire genetic alterations affecting their fitness levels. Relatively fitter cells become 'winners' that actively force elimination of less fit cells ('losers'). A) Induction of competition through mutations that can lead to differential uptake of beneficial factors from cells, mutations that can result in disparities of growth rates among cells and loss of apico-basal polarity that can compromise cell function and survival. B) Cells sense differences in cellular fitness of their neighbors through several distinct mechanisms, such as direct cell-to-cell interactions, soluble factors released from 'winner' cells and, the ability of cells to sense the mechanical pressure, under overcrowding conditions. C) Pathways involved in the execution of cell competition. 'Loser' cells can be either actively eliminated via apoptosis, forced to differentiation or extrusion from the epithelium.

Although the mechanisms driving compensatory proliferation of winner cells are less clear, some of the pathways related to the elimination of unfit cells have been identified, including the well-studied stress response pathways JNK and p53. C-Jun N-terminal kinase (JNK) signaling was the first pathway that was closely tied to cell competition. JNK signaling is increased in heterozygous *Minute* mutants in mosaic wing discs, as well as in wild-type cells that were surrounded by *Myc*-overexpressing cells in *Drosophila* [3,7]. In both cases, inhibition of the JNK pathway in cells that were regarded 'losers' reverted their elimination. In the fly midgut *Apc* mutations turns cells into 'supercompetitors' that eliminate the surrounding wild-type cell population in a JNK dependent manner [33]. However, the role of JNK signaling in elimination of unfit cells upon cell competition is less clear in other models of competition [6,22]. Therefore, it is suggested that although JNK signaling has an important role in active elimination of unfit cells, it might not be the global key regulator of inducing their programmed cell death. In cell competition induced upon loss of cell polarity, JNK signaling irrefutably plays a crucial role in promoting apoptosis in unfit cells in both MDCK cells and *Drosophila* models [34,35]. p53 constitutes a well-known stress response pathway that can enforce the elimination of unfit cells upon competition. In *Drosophila* mosaic tissues p53 senses the confrontation of two different cell populations and its upregulation can boost fitter cells to acquire a 'supercompetitor' status neighbors [6]. p53 signaling plays an important role in cell competition induced between MDCK cells with disrupted polarity and wild-type counterparts. *Scrib* knockdown cells show upregulation of p38 stress signaling leading to elevated p53 levels that serves as a sensor for damaged cells during mechanical competition. Interestingly, in wild-type cells, basal p53 levels can be sufficient to increase their sensitivity to cell crowding, and, eventually, result in their elimination [32]. Collectively, these are some of the mechanisms, proposed to initiate cell competition, sense the relative fitness levels among cells and execute the context-dependent elimination of unfit cell populations. Here, we will discuss those that influence primary and metastatic colorectal cancer.

CELL COMPETITION IN PRIMARY COLORECTAL CANCER

Intestinal homeostasis

Epithelial integrity is crucial for proper functioning of the intestinal tract. The epithelium acts as a selective barrier that allows absorption of nutrients and prevents pathogens from entering. To maintain tissue integrity, cells within the intestinal epithelium have a high turnover. Intestinal stem cells (ISCs) give rise to a variety of specialized cells that continuously replace the differentiated cells in the epithelium, which prevents accumulation of damaged and potentially harmful cells (Figure 2). ISCs reside at the

bottom of the crypt where they divide to give rise to Transit Amplifying (TA) cells. The progeny of TA cells undergo differentiation as they move along the crypt towards the villus. Most of the epithelium is occupied by enterocytes, which are responsible for selective absorption of ions, nutrients, and water from the intestinal lumen. They are characterized by their apical brush border of microvilli, maximizing their absorption surface. Enteroendocrine cells produce and secrete gastro-intestinal hormones such as secretins and gastrins to aid nutrient uptake, and e.g., ghrelin to signal to the enteric nervous system [36]. Tight junctions are crucial for maintenance of the epithelial barrier, as they are responsible for closely sealing adjacent epithelial cells and thus preventing leakage and pathogen entry [37]. This seal ensures that only selective transport can take place across the epithelial barrier. The epithelium itself is protected by a primary non-specific barrier that is formed by mucus produced by goblet cells under influence of STAT3 [38]. The intestinal mucus barrier is reinforced by a variety of enzymes and anti-microbial proteins such as α-defensins and lysozyme secreted by Paneth cells [38–41]. Paneth cells use this mechanism to defend ISCs and their location flanking ICSs at the bottom of crypts ensures that concentrations of anti-microbial proteins are highest near ISCs [42,43]. An extra layer of protection is provided by tuft cells. They act as chemo-sensors and were shown to play a role in secretion of endogenous intestinal opioids and anti-inflammatory prostanoids such as cyclooxygenase [44,45]. Besides their protective role, Paneth cells are required for development and maintenance of the stem cell niche [46]. The main additional components of this niche are CD34+ and Foxl-1 expressing mesenchymal cells [47,48]. Lastly, highly specialized cells like enteric glial cells and macrophages have been reported to play a role in the intestinal stem cell niche as well, but their roles are currently poorly understood [49,50].

Signaling during intestinal homeostasis

Communication between the different cellular components of the stem cell niche is crucial for maintenance of balanced proliferation in the crypt. WNT signaling is of vital importance for the maintenance of the ISC niche and through crosstalk with other signaling cascades plays a role in other processes such as crypt compartmentalization (Figure 2). WNT ligands are secreted by mesenchymal and epithelial cells and WNT levels peak in the bottom of intestinal crypts, where it stimulates ISCs proliferation [51]. As ISC progeny travels towards the villus, the received WNT concentration rapidly decreases, causing a switch from proliferation to differentiation [52]. Besides spatial regulation, WNT signaling is modulated through secretion of WNT antagonists by Paneth cells. Interestingly, during aging secretion of the WNT antagonist NOTUM is increased, causing a reduced proliferative potential of old ISCs [53]. WNT signaling also directly regulates Paneth cells, but they respond differently to the same ligands

due to differential expression of WNT receptors of the Frizzled family. Most ISCs express FZD5, but a subset of ISC progeny is primed to differentiate into Paneth cells through their expression of FZD6. This results in activation of the non-canonical WNT/PCP pathway, causing a cell-type specific response (Paneth- or ISC-specific) to WNT signaling [54,55]. In addition, Paneth cells use NOTCH signaling to communicate with ISCs. This is supported by the observation that NOTCH signaling from ISC-progeny, such as secretory enteroendocrine cells to ISCs is required for the maintenance of multipotency in flies [56]. Moreover, levels of NOTCH-ligand DELTA in fly ISCs determine daughter cell differentiation into enterocytes or enteroendocrine cells [57]. Genetic inhibition of NOTCH in mice showed its requirement for ISC proliferation and directs ISCs and TA cells towards a goblet cell fate [58]. Through inhibition of NOTCH signaling using γ-secretase inhibitors, which prevents cleavage of the NOTCH Intra-Cellular Domain (NICD) and subsequent downstream transcription regulation, the ISC marker *Olfm4* was identified as direct target of NOTCH activity [59]. Thus, NOTCH signaling is required for ISC proliferation and directs cell fate choice [60–62].

An additional stem cell niche-regulating pathway is EPH/Ephrin. The members of this protein family are transmembrane tyrosine kinase receptors that regulate cell migration and tissue integrity through bidirectional signaling. EPH/Ephrin signaling is most active on the border between complementary gene expression domains, and in zones where multiple gradients overlap. In other words, EPH/Ephrin signaling is highest on the border between the EPHA-high and EPHB-high populations, which is matched by an inverse presence of EFNA and EFNB. EPH-Ephrin signaling results in repulsion of cells of different cell types or cellular fitness, and adhesion of matching cells or cell types [63,64]. Moreover, EPHB signaling is required for niche maintenance and sorting of crypt and villus cells [65]. The genes encoding the EPHB2 and EPHB3 receptors are among the TCF-responsive genes, which means that intestinal EPH activity is indirectly regulated through WNT signaling [66]. Following the WNT gradient in the crypt, these two EPHB receptors are highly expressed in ISCs in the bottom of the crypt, while their ligand, EphrinB1, is exclusively expressed in the villus region. Thereby, the receptors and their ligands form opposing gradients that ensure crypt and villus cells remain segregated [67]. Paneth cells migrate towards the location with the highest WNT concentration using EPHB3 expression to move down the EphrinB1 gradient towards the bottom of the crypt, where they end up flanking ISCs [68].

Lastly, YAP/TAZ are crucial players in intestinal homeostasis. YAP/TAZ signaling is highly interconnected with WNT and EGF signaling. It directly regulates LGR5 expression in ISCs and controls self-renewal and progenitor expansion [69]. In fact, in YAP-deficient mice the ISC marker OLFM4 is downregulated, and crypt proliferation is dramatically reduced [70]. Moreover, deficiency of MST1/2 or LATS1/2, negative regulators of YAP/

TAZ activity, results in expansion of ISCs and loss of secretory cells, corresponding to a shift in cell fate to the absorptive lineage [71]. ISCs respond to mechanical forces corresponding to cell density through YAP/TAZ [72].

Intestinal regeneration

During homeostasis, the intestinal epithelium constantly regenerates to renew differentiated cell populations. In particular, enterocytes are short-lived (3-5 days) and need continuous replenishment. This high turnover is driven by the multipotent ISCs at the crypt bottom. However, during injury of the intestinal epithelium, homeostatic turnover is not sufficient and immediate repair and replacement of damaged cells is required to maintain tissue integrity.

Response to acute and chronic damage

Acute damage of intestinal epithelia can be mimicked by a variety of experimental strategies, such as ionizing radiation, and cell ablation using the Diphtheria Toxin Receptor (DTR) system. In the latter, the DTR is ectopically expressed in a cell type of interest, which renders them susceptible to Diphtheria Toxin (DT). For example, genetic engineering of the LGR5 locus was used to drive expression of the DTR in ISCs and treatment of animals with DT causes their ablation [73]. Exposure to ionizing radiation induces global damage in the intestinal epithelium, but it primarily affects proliferative cells. Paneth cells play a key role in this type of regeneration, by promoting proliferation in ISCs upon loss of enterocytes at the villus through NOTCH signaling. Besides the traditional LGR5+ ISC pool, additional reserve populations can contribute to intestinal regeneration. For example, a reserve pool of quiescent Tert+ stem cells are found around position +4 in the intestinal crypt. Upon exposure to ionizing radiation, these cells exit quiescence under the influence of WNT2b that is secreted by surrounding epithelial cells. Consequently, the Tert+ cells become mitotic and repopulate the damaged niche [74]. Interestingly, although the combination of radiation with DTR-driven ablation of LGR5+ cells reveals that radiation primarily affects LGR5- reserve stem cells, these cells hardly contribute to regeneration [75]. When damage to the ISCs is severe and these cells cannot repopulate the crypt, the tissue depends on alternative cell populations to sustain regeneration. Several different reserve stem cell pools have been described to fulfill this role. For example, quiescent p57+ tuft- and enteroendocrine precursors also dedifferentiate upon DTR-LGR5+ stem cell ablation [76]. Besides progenitors, Alpi+ enterocytes that reside inside the crypt region are reported to revert to highly proliferative stem cells that repopulate the niche [77]. Doxorubicin is a chemotherapeutic agent that inhibits topoisomerase 2 and specifically targets proliferative cells. Exposure to this drug causes a loss of ISCs,

causing a population of Defa4-expressing Paneth cell progenitors to dedifferentiate and repopulate the ISC niche [46].

In contrast to reversible damage, inflammation can be permanent and result in a chronic damage response. A commonly used experimental methods to mimic chronic diseases such as colitis and irritable bowel disease is prolonged treatment with dextran sulfate sodium (DSS). By combining induction of damage with lineage tracing experiments, it is possible to investigate the contribution of (reserve) progenitor pools, dedifferentiation and regeneration of the small intestine. For example, a progenitor pool destined to become Paneth cells and enteroendocrine cells can be activated to dedifferentiate upon exposure to epithelial damage or by receiving high NOTCH signaling, suggesting Paneth cells might be able to stimulate their progeny to alter their fate when required [78]. This population expresses stem cell markers like LGR5, but also enteroendocrine lineage markers such as MMP7 and Kit.

Signaling of the intestinal damage response

Both acute and chronic damage to the intestinal epithelium frequently results in reprogramming of epithelial cells to a dedifferentiated proliferative cell type. This reversion to a primitive state is often associated with an expression profile that largely overlaps with the transcriptome of spheroids generated from fetal stem cells [79,80]. The similarity between colitis-induced damage response and embryonic tissue has also been observed on single cell transcriptomics level, suggesting a common response of reactivation of embryonic gene programs upon occurrence of intestinal damage [80]. Upregulation of this embryonic signature is highly conserved between human and mouse. The precise signature may be variable, which could be caused by differences in age, across cell types and or even type of damage induced. However, several key players seem to be involved in this damage response regardless of damage context. The signature is characterized by expression of genes of the *Ly6* family, *annexins*, and *clusterin* [81,82]. It is also strongly associated with YAP activation [70]. In fact, YAP reprograms LGR5+ ISCs to an expanding LGR5- 'revival' stem cell pool upon damage induction [70,83]. This reprogramming causes inhibition of WNT-dependent proliferation and promotes cell survival of the epithelial cells. YAP/TAZ deficient intestines do not show this fetal-like damage response signature when exposed to damaging agents such as DSS, and subsequently do not regenerate properly [70,84]. In stromal cells surrounding the stem cell niche, damage induces secretion of EGF ligands NRG1, AREG, hbEGF and EREG, which bind EGF receptors on ISCs, resulting in increased proliferation [70,85]. Moreover, downregulation of the WNT pathway and upregulation of EGF can rescue ISC regeneration in YAP-deficient organoids, indicating that these two pathways downstream of YAP may act (partially) redundant in regeneration [70]. In most cases, the damage response is a temporary

state, with one notable exception. Persistent immune activation due to infection with parasitic helminths results in activation of a gene signature in LGR5+ cells that is characterized by expression of *Ly6a*, but not *annexin* or *clusterin* [86]. Interestingly, when the *in vivo* damaged ISCs are grown *in vitro* the spheroids remain cystic and show activation of this damage response gene signature long after isolation from the affected epithelium, indicating a long-lasting effect that extends beyond continued damage exposure. Taken together, intestinal regeneration is not just driven through proliferation of the stem cell compartment, but progenitors and even differentiated intestinal cells seem to be capable of repair, were it under the right circumstances. It has even been proposed that it is the sheer proximity to crypt bottom signals, that determines which cell type dedifferentiates and repopulates the crypt [83,87]. Regeneration in the intestine is fueled from a highly diverse variety of cell populations, showcasing the intestine's incredible plasticity (Figure 2). However, it is important to note that many of these damage induction models do not necessarily represent intestinal damage as it occurs *in vivo*. Adequate regeneration of the intestine is vital for intestinal functioning. It is vital to ensure that the regenerative proliferation program is abolished when the tissue has been replenished and when this inhibition does not happen at the correct moment, regeneration can eventually lead to tumor formation [88–91].

Cell competition in colorectal cancer

Colorectal cancer (CRC) is the third most common cancer type and the second leading cause of cancer-related deaths in the world. In 2020, more than 1,9 million people were diagnosed with CRC and over 930.000 deaths were recorded worldwide [92]. CRC is characterized by an increased heterogeneity on a molecular and genetic level, which is highly correlated with the observed differences in clinical outcome and treatment response of the patients [93]. Approximately 70-80% of CRCs occur sporadically, whereas 20-30% of the cases are familial. Among the familial CRC cases, only 5% are attributed to highly penetrant inherited mutations that have been well characterized and are known as hereditary CRC syndromes [94,95]. The cause of the remaining inherited CRCs, even though not yet fully understood, is likely linked to less penetrant alterations in multiple susceptibility genes regulated by environmental or other genetic factors [94]. In addition, chronic inflammation is associated with increased rates of CRC incidence. In fact, almost one fifth of patients with inflammatory bowel disease (IBD) have been reported to develop colitis-associated CRC [88,96]. Colorectal cancer is often associated with loss of heterozygosity of WNT regulator APC, causing hyperplasia of the epithelium [97]. Tumor progression is driven by a subsequent accumulation of oncogenic mutations, such as activating mutations in KRAS and loss tumor suppressor genes such as TGFβ and P53. During tumorigenesis, cancer cells are subjected to a remarkable array of intrinsic and extrinsic pressures

that result in genetic diversification in the cell population. Over time, subclones with a distinct genetic composition undergo a constant competition (Figure 2). Subclones, with features that increase their adaptation to the microenvironment, will survive and expand over unfit subclones leading to clonal selection [98,99]. This dynamic process drives tumor heterogeneity akin to the theory of Darwinian evolution. However, limitations in tracing early neoplasms hamper the investigation of the exact with competitive mechanisms driving clonal evolution [100]. It is proposed that since cancer cells tend to reactivate developmental pathways, they can co-opt active competitive mechanisms that take place in developing tissues [101]. In line with this, an increasing number of studies supports that cancer development, progression and evolution depend on discrepancies in the relative fitness among cancer cells, as well as between cancer cells and the microenvironment [102]. Here, we will specifically discuss those that impact primary and metastatic colorectal cancer.

Passive cell competition in primary colorectal cancer

Unlike during development, where apoptotic cell death is the main mechanism of cell competition, in adult tissues cell elimination is often mediated by inducing differentiation in stem cells through passive competition. In the intestine ISCs compete with their neighbors to prevail in a spatially confined niche. ISCs in the intestinal crypt continuously compete in a process called neutral drift, where stochastically arising neutral genetic variants spread through the stem cell compartment [103,104]. Eventually, random ISC clones gradually take over the crypt, reducing clonal diversity until the crypt is monoclonal. Importantly, winning clones have no cellular fitness advantage over their neighbors, they win by sheer chance. Even though ISCs are equipotent and mainly undergo neutral competition, local concentrations of niche factors can affect competition among ISCs [87]. The architecture of the intestinal crypt creates a bias that is unrelated to their genetic background or cellular fitness. It generates a slight advantage for ISCs that are closer to niche cells, and thus in better reach of growth factors, or ISCs in contact with more Paneth cells, as NOTCH signaling from the Paneth cells is contact dependent. Reversely, in aging intestinal epithelia, niche cell contacts may provide a disadvantage, as aged Paneth cells show enhanced secretion of the WNT inhibitor NOTUM, which promotes the differentiation of the surrounding ISCs [53]. Therefore, ISCs surrounded by fewer Paneth cells, or ISCs that are located on average further away from Paneth cells, are more likely to self-renew. Moreover, the subsequent reduction of functional ISCs can affect regenerative capacity of the tissue, which may cause alterations in the dynamics of competition within the crypt. This may create a bias towards a specific ISC clonal populations or even promote clonal expansion of mutant cells. Not just the location relative to Paneth cells, but also the location along the crypt axis is a key determinant of ISC self-renewal. ISCs at a further distance of the crypt bottom are at higher risk of being physically pushed out by their neighbors and thus forced to differentiate.

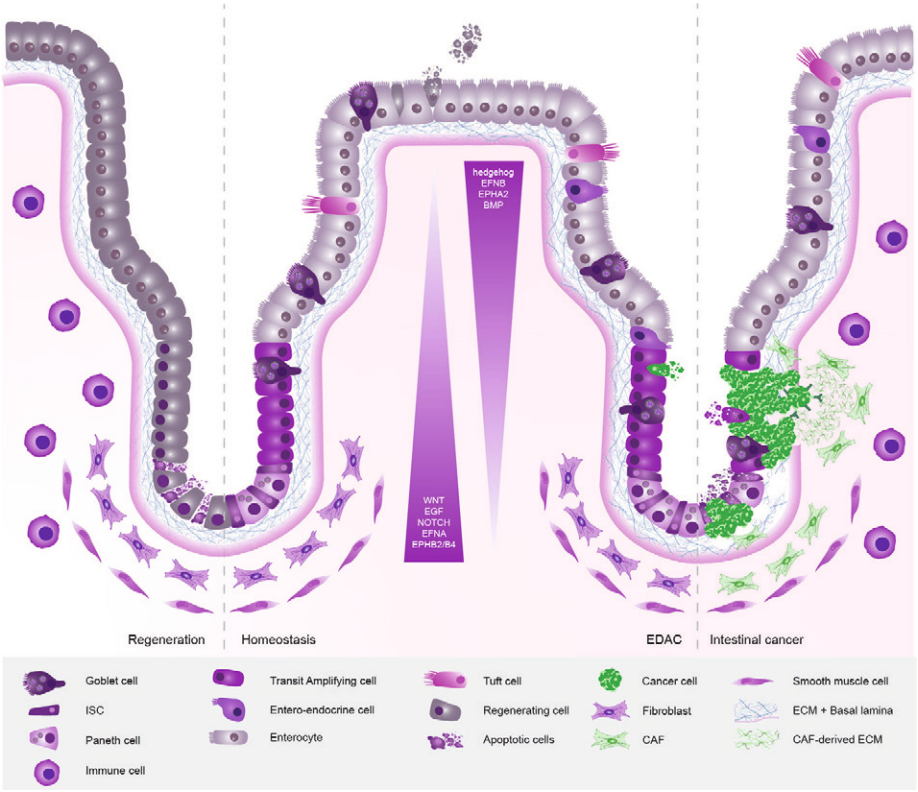

Figure 2: Schematic overview of the intestinal epithelium A) Upon damage, all cell types in the intestinal villus and crypt can regenerate and repopulate. Regenerating cells activate a damage-response associated with specific gene expression as well as loss of differentiated morphology. Moreover, immune cells enter the tissue to combat inflammation. B) During homeostasis, proliferation occurs in ISCs, and progeny differentiates as they move upwards along the villus. At the villus top, mature enterocytes are shed, maintaining a high cell turnover. In the center, gradients are shown as observed in homeostasis. C) During EDAC, early malignant cells are recognized by surrounding healthy epithelial cells and extruded locally from the tissue to prevent spread of cancer cells. D) When malignant cells are not recognized and extruded, cancer cells can progress. Upon gaining several mutations, their cellular fitness increases and eliminate surrounding wild-type epithelial cells.

Moreover, as the peak of the pro-proliferative WNT signaling gradient can be found in the crypt bottom, some ISCs inevitably contribute more progeny as they will proliferate more. So, the otherwise neutral competition between ISCs somewhat favors ISCs in lower positions within the crypt, increasing their likelihood of fixing an entire crypt. Importantly, as biased competition is still a stochastic process, this means that less fit ISCs can also end up dominating a crypt. If a specific clone does acquire a cellular fitness advantage through mutation, tissue fixation can occur in a faster and more efficient manner [105,106]. For example, gain of an oncogenic KRAS mutation by

LGR5+ cells in the mouse intestine results in faster cell division, which sets the scene for oncogenic crypt fixation [106]. After crypt fixation, monoclonal oncogenic KRAS crypts expand through the intestine by crypt fission. In some cases, this results in a hijack of neutral drift. For example, once a crypt is fixated by an oncogene-expressing transformed ISC, the now-oncogenic crypt takes over neighboring crypts through two distinct paracrine signaling mechanisms [107]. First, through secretion of BMP ligands, the neighboring crypt's ISCs are pushed towards differentiation by KRAS- or PI3K-expressing crypts. Second, PI3K-expressing crypts induce stromal cells to modify WNT levels at the bottom of the neighboring crypt. Together, these two mechanisms significantly speed up neutral drift.

Active cell competition in primary colorectal cancer

Epithelial defense against cancer (EDAC) is a process highlighting the impact of competition on suppressing tumorigenesis and is supported by the tendency of epithelial cells to suppress the outgrowth of mutant cells [108]. For instance, studies in flies have shown that cells carrying mutations in *Tp53* or *Src,* or overexpressing ERBB2 or YAP, could be extruded from different tissues [109–111]. In the murine intestinal epithelium, oncogenic HRAS expressing cells are rapidly eliminated through EDAC [112] (Figure 2). Despite the protective capacities of EDAC, cancer does arise in the intestine with high incidence. Mechanisms determining the outcome of cell competition between wild-type intestinal tissue and intestinal cancer cells are not well-understood. Some studies suggest that the outcome is largely a numbers game. During cell competition as EDAC, initial mixing ratios are essential for the outcome [27,112]. This is in stark contrast to mixed intestinal organoids, where mixing ratios, as well as degree of mosaicism, seem to determine the speed of competition, but not the outcome [113]. Turning of the proverbial tables can also be achieved when a cellular fitness discrepancy between wild-type tissue and transformed cells is decided in favor of cancer. This can occur either by lowering wild-type cell fitness or by increasing fitness of cancer cells. For example, a study in mice showed that diet affects the ability of the intestinal epithelium to extrude malignant cells [114]. In this study, mice were kept on a normal diet or a high-fat diet for three months, after which Ras^{V12} transformed cells were induced in the intestinal of pancreatic epithelium. In mice on a normal diet, the Ras^{V12} transformed cells were apically extruded through tumor-suppressive cell competition. In contrast, in mice on high-fat diet the metabolism of epithelial cells changed, as is their cellular fitness, abolishing their ability to outcompete malignant cells. As a result, apical extrusion does not occur, enabling the malignant cell to persist and proliferate. Moreover, chronic inflammation also impairs apical extrusion of Ras^{V12} cells [114]. Taken together, this demonstrates that maintaining a high cellular fitness level in normal epithelial cells is essential to eliminate malignant cells and prevent further tumor progression.

When intestinal cells fail to extrude transformed cells, the subsequent cancer cell expansion is not the result of the cancer cells adopting a winner status, rather a failure of the wild-type cells to do so. In contrast, during supercompetition, competitive interactions and mechanisms are specifically hijacked by cancer cells to eliminate healthy cells from the tissue. Cancer expansion can be promoted by eliciting release of growth-promoting factors from wild-type cells by forcing them to differentiate. This can be achieved in two different modes. First, cancer cells can acquire mutations rendering them independent from niche growth factors. Examples of this are mutations in WNT signaling components such as APC, resulting in WNT-ligand insensitivity [115,116]. These tumors no longer depend on pro-proliferative WNT signaling as in these cells, β-catenin is never degraded and WNT signaling is constitutively active. Second, loss of APC directly suppresses proliferation of surrounding wild-type ISCs through secretion of WNT antagonists such as NOTUM [117,118]. Third, in RNF43 tumors, a subset of cells are forced to functionally specify into niche cells, secreting growth factors and mucus, which the tumor uses to boost its own growth [119]. Often, supercompetition results in increased cancer expansion, for example because of increased space availability through wild-type elimination. Active supercompetition by tumors was first shown in adult tissues in the *Drosophila* gut, where APC-deficient adenoma cells eliminate neighboring wild-type cells through induction of apoptosis [120]. Protection of wild-type cells by expression of apoptosis inhibitors, prevents cancer cells expansion. This highlights the dependency of cancer cells on a growth-permissive environment for colonization of tissues. Similarly, in mixed murine intestinal organoids wild-type cells are actively eliminated by $APC^{-/-}$, $P53^{R172H}$, $KRAS^{G12D}$ mutated cancer cells in a JNK-dependent manner [113]. Remarkably, during competition ISCs are lost from competing wild-type organoids and the remaining wild-type cells revert to a primitive state that is very similar to the fetal-like damage response, marked by expression of annexins and Ly6 family proteins. It is however unclear if that is a direct consequence of cell competition, or a consequence of loss of ISCs through cell competition. Together, this shows that the mechanisms and responses that are inherent to the intestinal tissue can be used by cancer cells to drive their progression.

CELL COMPETITION IN THE LIVER AND DURING LIVER METASTASIS

Liver homeostasis

Approximately one-third of colorectal cancer patients develop metastases within the first three years after diagnosis. The liver is a main site of colorectal cancer metastasis and is responsible for most of the colorectal cancer-dependent lethality,

therefore we here focus on competitive interactions that occur in this organ. The liver is crucial for a plethora of processes that govern organismal physiology, such as metabolism, detoxification, regulation of blood clotting factors and bile synthesis [121]. This epithelial organ is predominantly quiescent, in comparison to other epithelial tissues such as intestine and skin [122]. However, upon damage such as acute injury or chronic inflammation, it shows a remarkable regenerative capacity [121,123,124]. The liver tissue comprises various cell types distributed around the hepatic architecture, including those surrounding endothelial ducts connecting the portal and central veins and those associated with bile ducts. Among the former are Kupffer cells, liver-resident macrophages, which form the liver's first line of defense against infections [125], and sinusoidal endothelial cells and stellate cells, which play crucial roles in liver regeneration and are responsible for synthesis of all extracellular matrix (ECM) components, as well as production of key signaling proteins [126–129]. The latter are primarily composed of hepatocytes and cholangiocytes, which together form the epithelial surface of liver tissue [124] (Figure 3).

Hepatocytes constitute 60% of the cells and 80% of the volume of the liver [124]. They display a characteristic distribution in the tissue, known as "zonation", and are typically divided in three zones. The division depends on their position relative to big vessels, and consequently oxygen availability in the environment [130,131]. Zone 1 hepatocytes are located close to the portal vein and hepatic arteries, which means they have easy access to highly oxygenated and nutrient-dense blood. Zone 1 hepatocytes express markers as urea cycle genes (*Cps1*), glutamine catabolism gene (*Gls2*), and E-cadherin and oversee important metabolic processes as gluconeogenesis, lipid metabolism and ureagenesis [132,133]. Hepatocytes in zone 3 are found near the central vein and express markers as glutamine synthase (*GS*) and members of the cytochrome P450 family such as *Cyp1a2*. These cells use glycolysis and the TCA cycle for energy production and are responsible for Glutamine synthesis and Xenobiotic metabolism. Zone 2 contains those hepatocytes that are located in between zones 1 and 3 (Figure 3). They display an intermediate expression profile and can be recognized by the lack of expression of markers of both zone 1 (E-cadherin) and zone 3 (GS). Additionally, zonation of hepatocytes is correlated to LGR5 expression and WNT signaling. Areas close to central vein (zone 3) express LGR5 together with activated β-catenin, while LGR5 is reduced around periportal veins (zone 1) overlapping with the expression of negative regulator *Apc*, demonstrating a gradient of WNT/ β-catenin signaling between zones [134]). Especially WNT2 and WNT9b are crucial in this distinction [121,130,132,133,135,136] (Figure 3). *Alb* and *Cyp3a11* are markers of mature hepatocytes, therefore, their expression level is a good predictor of functionality of the cells [137]. In addition, transcription factors of the HNF and C/ep families are required for liver function. For example, HNF4a one of the most common hepatocyte markers, is essential for hepatocyte differentiation and control of lipid homeostasis [138].

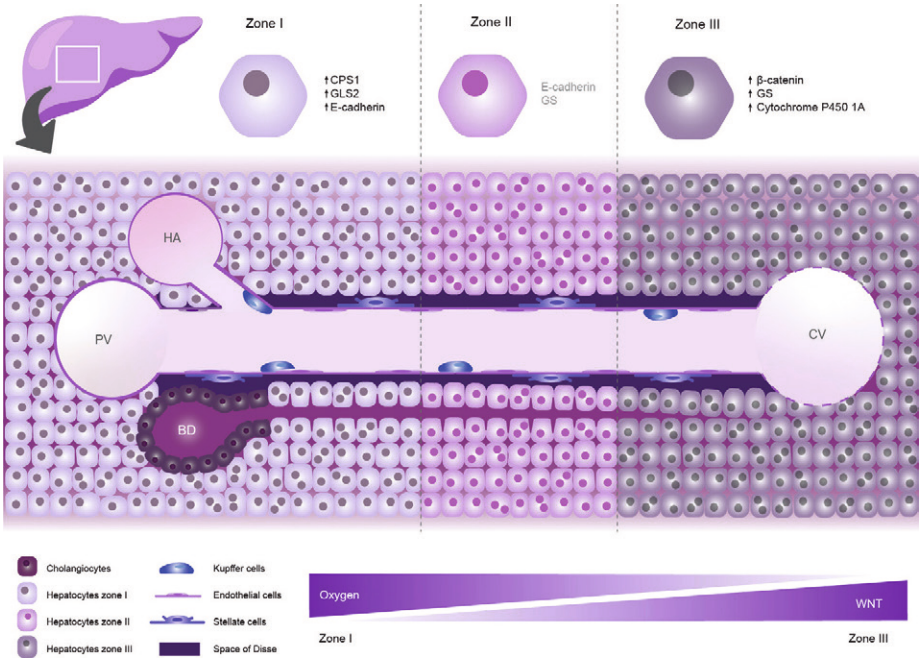

Figure 3: Schematic overview of the cellular composition and zonation of the liver in homeostasis The liver is divided in three zones; Hepatic arteries, the portal veins and bile ducts are found closest to Zone I. Hepatocytes in zone I in the highest oxygen but lowest WNT state. The transition area, Zone II, is where hepatocytes are characterized by absence of Zone I markers. Zone III hepatocytes surround the central vein and receive lower oxygen and nutrient input. Blood vessels are fenestrated, enabling exchange of nutrients, waste and oxygen. Liver resident macrophages (Kupffer cells) reside in the blood stream and stellate cells placed between the endothelium and hepatocytes in the space of Disse perform more general functions during development, regeneration and angiogenesis. Bile produced by hepatocytes is collected in the canaliculi between hepatocytes, and is accumulated in the bile ducts, which are surrounded by cholangiocytes.

Cholangiocytes encompass a smaller number of liver cells compared to hepatocytes and form 3-5% of the total liver cell population [139]. They are needed for bile formation, one of the main functions of the liver, which is required for digestion of lipids. There are different types of cholangiocytes that can be classified based on their location in the distinct biliary tracks (intrahepatic and extrahepatic) and unique marker expression patterns [140]. All cholangiocytes express high levels of the transcription factor SOX9 and cytokeratins KRT9 and KRT7 [141]. However, cholangiocytes from extrahepatic biliary tree, including gall blader cholangiocytes and common bile duct cholangiocytes, express aquaporins, mucins, FGF19 and SOX17, while intrahepatic bile duct-derived cholangiocytes express JAG11, TACSTD2, and several YAP target genes, corresponding with their localized functions [142]. Interestingly, organoid

cultures initially show expression patterns, functionality and expansion potential that is similar their area in the biliary tree of origin. However, specialization to other cholangiocyte types can be induced by addition of environmental cues to cultures. For instance, addition of bile to the cultures enhances specialization towards gallbladder cholangiocytes [142,143], illustrating the plasticity of the cell population. In addition, the morphology and size of murine cholangiocytes changes depending on their location in the biliary tree, size of the duct and state of the tissue [139,144,145]. For example, acute damage induces the appearance of small, less differentiated LGR5+ cholangiocytes that can differentiate into larger cholangiocytes[146], and can even act as hepatic progenitors [147].

Liver regeneration

The role of hepatocytes in regeneration

During liver damage, in particular acute injury caused by for instance viral infection, drugs and acute ischemia [148], the tissue switches from a quiescent to a regenerative state. At this stage regeneration is mostly driven by phenotypical fidelity, which means that each cell type is responsible for repopulation of their own cell type within the tissue [126,149]. Therefore, since hepatocytes are the most abundant cell type in the liver, they play a critical role in acute damage regeneration. Acute liver injury is frequently experimentally modeled by partial hepatectomy, where a large part of the liver is surgically removed.

Although the organ cannot reconstitute lost lobes, it will compensate by increasing the size of remaining lobes [122]. Lineage tracing experiments after partial hepatectomy showed increased proliferation of hepatocytes [121,136,150]. In addition, increase of hepatocyte size known as hypertrophy, can restore the mass of functional liver tissue during regeneration after acute injury [151]. Alternatively, acute liver injury can be induced by exposure to carbon tetrachloride (CCl4) or N-acetyl-para-amino phenol, which both cause liver toxicity. Interestingly, CCL4 administration causes loss of regenerative-prone pericentral vein LGR5-expressing hepatocytes. This damage is resolved by repopulation driven by LGR5- hepatocytes that switch to a LGR5+ population [152]. Lastly, HNF4a, crucial not only for hepatocyte function but also for liver development [138] is required to terminate liver regeneration. The expression of this transcription factor is downregulated in the first hours after damage and re-expressed at the end of regeneration to complete the process [121,153]. After regeneration, zonation is restored by zone 2 hepatocytes that give rise to zone 1 hepatocytes when there is periportal injury and in zone 3 hepatocytes during pericentral injury [131]. Collectively, hepatocytes assume a key role in liver regeneration following acute liver injury. They undergo reactivation from their quiescent state,

acquire proliferative capacity and increase in size. This underscores the significance of environmental signals in modulating the fitness of a particular cell type within a population of damaged cells.

The role of cholangiocytes in regeneration

Cholangiocytes play a vital role in regeneration when the hepatocyte-driven injury-response is impaired, for instance, when there is continuous tissue damage caused by chronic liver disease or severe liver injury. During such conditions, hepatocyte-fueled regeneration is compromised and proliferation and subsequent differentiation of cholangiocytes becomes increasingly important. For example, the loss of integrin β1 in damaged hepatocytes initiates a ductal reaction with cholangiocyte origin, resulting in non-hepatocyte derived hepatocytes [140,154]. In addition, lineage tracing studies have shown that experimental induction of chronic damage by repetitive administration of CCl4 and thioacetamide, or diets such as 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) [121,155] cause reactivation of cholangiocytes and subsequent differentiation into hepatocytes [154,156–158]. Importantly, *in vitro* differentiated cholangiocyte organoids can repopulate the mouse liver with hepatocytes upon transplantation [147,159]. It is still unclear whether cholangiocyte differentiation is sufficient to reconstitute the totality of lost tissue, but it is considered an important contributor, especially during chronic damage [122]. Interestingly, cholangiocytes can also undergo senescence upon chronical liver injury [160] and cholangitis [161]. This suggests that the regenerative response of these cells depends on the type and severity of the damage.

Bidirectionality of transdifferentiation

Transdifferentiation is not limited to the generation of hepatocytes from a cholangiocyte-origin, but instead is bidirectional: cholangiocytes can also arise from hepatocytes. Several studies show dedifferentiation of hepatocytes during liver injury such as non-alcoholic steatohepatitis [162], DDC-treatment [163] and alcoholic hepatitis [164]. Transdifferentiation coincides with an increased expression of cholangiocyte markers in the reprogrammed hepatocytes [163,164]. This hepatocyte to cholangiocyte reversion is not limited to chronic damage and has also been observed during *in vivo* regeneration [165] and *in vitro* assays [166,167]. Since this transdifferentiation can be bidirectional, cells in damaged mammalian livers can co-express both hepatocyte (e.g. HNF4a) and cholangiocyte (e.g. KRT19 and SOX9) markers simultaneously. Even if certain hepatocyte features remain, cells switch from a hepatocyte to cholangiocyte gene expression profile during transdifferentiation [128,158,163,168,169]. This illustrates the remarkable plasticity of the liver and the interconnected role of hepatocytes and cholangiocytes during liver damage. Furthermore, these observations emphasize that hepatocyte- and cholangiocyte-driven regeneration should not be considered as independent processes.

Molecular pathways involved in regeneration

To understand liver regeneration, insight into the involved molecular pathways and signaling is crucial. A major regulator of liver homeostasis and regeneration is the WNT/β-catenin pathway. There are 19 WNT proteins and many of them are produced in all liver cell types [127]. Since, WNT/β-catenin signaling is required for liver zonation [135,143], it also plays an important role in regeneration during early injury response. Activation of this pathway increases hepatocyte proliferation through ZNRF3 and RNF3 [170,171] and transdifferentiation of hepatocytes into cholangiocytes [121,127]. For instance, WNT expressing LRG5+, GS+ and axin2+ hepatocytes found in zone 3 [143] can self-maintain in homeostasis and are responsible of regeneration during hepatectomy. However, they are also more susceptible to transformation and induction of hepatocarcinoma due to misregulation of cell division [152]. Furthermore, activation of WNT signaling is not limited to hepatocytes [147] as damage induced LGR5+ cells, which can regenerate hepatocytes and bile ducts, can be found in areas near bile ducts upon damage. These LGR5+ cells, derived from murine or human damage induced livers, can form liver organoids *in vitro* when cultured in RSPO containing medium through activation of WNT signaling [147,159].

Another key controller of hepatocyte and cholangiocyte function is YAP/TAZ signaling. This pathway is a master regulator of cell proliferation and can integrate extra-cellular stimuli such as cell density and stiffness of the environment. YAP/TAZ signaling was originally identified by its role in regulation of organ size in *Drosophila* [172] and it fulfills a similar role in the mammalian liver [173]. During homeostasis, hepatocytes remain quiescent, keeping YAP expression low. Indeed, deletion of this pathway in adult hepatocyte does not cause a phenotype [174,175]. However, increased expression of YAP in hepatocytes leads to loss of hepatocyte identity and conversion to cholangiocytes [121,173]. The plasticity of this process is illustrated by the finding that reduction of this elevated YAP promotes redifferentiation into hepatocytes [176]. *In vitro*, hepatocyte YAP activation can be triggered by mechanical tension and formation of stress fibers, causing dedifferentiation and subsequent loss of hepatocyte function [173,177].

Moreover, YAP signaling is needed for liver regeneration both after acute and chronic liver injury. Downstream signaling of YAP such as NOTCH, induces hepatocyte to cholangiocyte transdifferentiation upon damage [173,176]. After acute injury such as partial hepatectomy, YAP activity is increased at the first day of regeneration and decreases afterwards [178]. TGFβ expression induced upon hepatectomy, induces YAP1 and consequently pSMAD2 activation, which promote proliferation and regeneration [179]. Alternatively, impairment of the YAP/TAZ upstream negative regulators LATS1/2 [180] or MST1/2 [181], induces hepatocyte proliferation and

transition of hepatocytes to cholangiocytes by transdifferentiation, which results in enhancement of liver regeneration. Importantly, liver specific deletion of both YAP and TAZ, through albumin-Cre-mediated recombination, causes major delays in liver regeneration post-hepatectomy [182]. Similarly, deletion of YAP/TAZ does not affect hepatocyte proliferation but impairs cholangiocyte dependent regeneration during acute [183] and chronic injury [171,173]. Together, this shows that YAP/TAZ activation is required for liver regeneration after acute injury, especially during cholangiocyte-dependent regeneration.

Cell competition in liver transplantation

Given the livers innate regenerative capacity, one of the investigated strategies for mitigating liver damage involves the transplantation of healthy hepatocytes into compromised tissue. Cell competition plays a key role, as the objective is to enable outcompetition of damaged cells by more robust counterparts, ultimately leading to functional tissue repopulation. Already in 1994 a study showed that transplanted adult liver cells into an adult damaged mouse liver replaced 80% of the damaged organ. To follow repopulation in liver, mature *myc*-expressing hepatocytes were injected in mice which overexpressed the hepatotoxic transgene, urokinase plasminogen activator (uPA). After 2-3 weeks the damaged livers showed a colonization of healthy infused cells, while they were absent in control mice lacking the Alb-uPa transgene [184]. After that, a plethora of publications supported the potential of liver transplantations in liver regeneration after damage [185–188]. Thus, fitter transplanted cells can outcompete damaged cells and repopulate the liver. A similar observation is made when a tissue fitness is reduced by radiotherapy post-surgical resection. Transplantation of healthy hepatocytes in such livers promotes the regeneration of damaged liver tissue and restores its functionality [189]. This phenomenon was substantiated by the combination of radiation with ischemia-reperfusion, where intra-splenic transplanted healthy hepatocytes facilitated the regeneration of the compromised tissue [190].

The competitive advantage exhibited by healthy liver cells over their defective counterparts can also be harnessed for gene therapy. For example, mouse and rat livers suffering from Hereditary Tyrosinemia Type I are repopulated by wild-type liver cells upon transplantation [186][188]. This suggests that transplantation of fitter cells in a less fit recipient liver will lead to competition that promotes repopulation to restore its functionality (Figure 4.B). Furthermore, when fetal liver cells from healthy rats were transplanted into DPPIV-F344 mutant livers via portal vein injection and partial hepatectomy, they exhibited higher proliferation compared to the host liver cells. Interestingly, the recipient liver cells display increased apoptosis in areas surrounding the repopulated tissue, hinting at the elimination of host cells

was triggered by transplanted fetal cells [191]. This elimination and repopulation is driven by a differential sensitivity to Activin A signaling, which induces apoptosis in older hepatocytes, while fetal cells are resistant and remain proliferative [192,193] and resembles active cell competition. These findings display the potential of utilizing cellular fitness to improve human liver transplantation, however, a major complication is potential tumorigenesis due to miss-regulation of cell competition during repopulation [194]. For instance, retrorsine, a proliferation blocking alkaloid, causes reduced fitness of liver tissue and a massive proliferation of transplanted normal hepatocytes that can result in development of hepatocellular carcinoma [187,195]. In contrast, no aberrant growth is observed after hepatocyte injection into non-retrorsine treated recipients. Similar tumor-promoting effects are observed in other models of liver repopulation reviewed in [196] Together, this shows that the host microenvironment during transplantation plays a deterministic role and, cell competition can control the growth of transplanted liver cells. This implies that an imbalance in cellular fitness can lead to a desired outcompetition of the less fit host tissue, but also facilitate overgrowth and subsequent tumor formation by fitter transplanted cells.

Cell competition in primary liver cancer

Overgrowth of fittest cells in the liver is not an exclusive consequence of transplantation. Competing interactions within the tissue also take place in homeostatic conditions without liver damage. However, when liver cells acquire mutations which confer them with a phenotypical advantage over neighboring tissue, such as higher proliferation, these mutated cells can overtake the tissue. If this repopulation takes place in an uncontrolled manner, it will result in liver tumor growth (Figure 4.C). Interestingly, there is a large overlap in mechanisms that are important for regulation of regeneration and those that are needed for growth restriction. A key example of this in hepatocellular Carcinoma, is YAP/TAZ signaling, which plays an important role in regulation of hepatocyte fate during regeneration [176]. Importantly, peritumoral hepatocytes activate YAP and TAZ, thereby inhibiting tumor growth. Conversely, when these mechanisms are disrupted or deleted, tumor progression is accelerated [197]. Therefore, YAP and TAZ show an important role in competition between tumor and healthy liver cells to control tumor growth.

Cell competition in liver metastasis

The liver is one of the most common sites of colorectal cancer metastasis. A major underlying cause is the location of the organ within the vascular system, as the blood circulation from the intestine directly drains into the portal vein which serves

as the principal entry point to the liver [198]. Moreover, the hepatic vasculature is characterized by fenestrations and a lack of subendothelial basement membrane, which allows cancer cells to escape from the blood circulation and colonize the liver niche [198–200]. The metastatic cascade globally occurs in four steps. First, during the "tumor infiltrated microvascular phase" cancer cells reach the microvasculature and get trapped in sinusoidal vessels. If these cells survive, they advance to the subsequent stage "extravascular or pre-angiogenic phase", which refers to extravasation of cancer cells. During this second step, cancer cells access the perisinusoidal space (or space of Disse) by endothelial transmigration. Here, they employ stellate cells for ECM deposition and release of angiogenic and growth factors, thereby setting the groundwork for tumor progression. Next, during the third step or "angiogenic phase", cancer cells increase expression of adhesion molecules and promote vascularization for sufficient oxygen and nutrients supply. Lastly, in the fourth step or "growth phase", tumor angiogenesis will facilitate expansion from micro-towards macrometastases [201].

In the liver, metastases exhibit diverse growth patterns, each distinguished by distinct histo-pathological traits (see [210] for a recent extensive overview). Notably, these distinct growth patterns engage various cellular interactions, and therefore some are more prone to be regulated by cell competition. Both the desmoplastic and pushing growth patterns, are characterized by a sharp well-defined edge of the metastasis, compression of surrounding liver cells and absence of liver mimicry. The difference between the growth patterns is the stromal barrier between cancer cells and liver tissue, which is only observed in desmoplastic metastases. Consequently, both growth patterns show tumor infiltration of immune cells, however other stromal cells are lacking in metastases with the pushing growth pattern. The replacement growth pattern, on the other hand, is very different from the other patterns as it is characterized by a high degree of cellular interactions between liver and cancer tissue. Cancer cells grow within plates of hepatocytes, display a high level of liver mimicry and preservation of stroma. In this pattern, cancer cells are in contact with and progressively replace the surrounding hepatocytes. By these means, replacement-type metastases can co-opt the existing sinusoidal vessels of the liver for blood supply, resulting in minimal hypoxia compared to desmoplastic liver metastases. The edge of metastasis of the replacement pattern lacks a sharp contour and is generally irregular. Importantly, clear contact between hepatocytes and tumor cells is observed. Some tumor infiltration can be found, but in general, the replacement pattern is characterized as immune desert both at tumor-liver interface and central part of the tumor. More recently, two new growth patterns of liver metastasis have been described: sinusoidal and portal. In the sinusoidal pattern, cancer cells grow in the sinusoidal vessel lumina or Disse space, which is adjacent to hepatocyte plates. Growth of metastases with a portal pattern happens within portal tracts and

septa and/or within lumen of biliary branches. In the landscape of colorectal cancer liver metastasis, the replacement pattern shows a poorer prognosis compared to desmoplastic or pushing patterns [211]. This reinforces the need to comprehend the intricate interactions between cancer and liver cells. These interactions are potentially influenced by processes akin to cell competition.

Passive competition in liver metastasis

Hepatocytes are a source of soluble factors that promote metastatic growth. They release growth factors like IGF-I and HGF-like protein/macrophage-stimulating protein (HGFL) which enhance tumor growth, motility, and invasiveness. For instance, liver-circulating HGF binds c-Met in cancer cells promoting growth of liver metastases by regulation of a wide range of downstream pro-metastatic pathways such as JAK2/STAT3 signaling, Ras-MAPPK/ERK and PI3K/AKT [212]. This pro-metastatic support can be further enhanced by hypoxia. Under hypoxic conditions, factors like HIFs, VEGFs, and G-CSF are induced, which support the colonization of cancer cells in pre-metastatic tissues [213,214]. Besides soluble factors, also modification of the amount and composition of ECM is crucial for metastatic growth, and it is often enhanced by resident immune cells. In the liver, Kupffer cells drive fibronectin synthesis and deposition by releasing Transforming Growth Factor β (TGFβ), which activates fibroblast-like hepatic stellate cells. Upon activation, HSCs can enrich the ECM in collagens I and IV, thereby promoting liver fibrosis which subsequently enhances tumor immune evasion and angiogenesis [215]. Liver fibrosis provides a supportive environment for metastatic colonization. For instance, patients with colorectal cancer and fibrotic livers exhibit higher metastatic incidence and worse survival rates [216,217]. Together, these findings highlight the pro-metastatic role of liver tissue and suggest potential passive mechanisms of cell competition that can be used by cancer cells to promote metastatic growth.

Active competition in liver metastasis

Besides passive mechanisms, active competitive interactions are at play between metastases and surrounding liver tissues. There are numerous examples of growth-promoting adaptations of cancer cells that are induced by liver tissue. For instance, PRL3 is specifically activated in CRC liver metastasis where it induces several pro-metastatic cascades. PRL3 promotes invasion through activation of the AKT pathway [218], enhances angiogenesis via the NK-κβ pathway, and leads to proliferation and invasiveness by activation of STAT3 signaling. This suggests that, upon their arrival in the liver, cancer cells receive cues from the surrounding tissue that enhance invasion and causes increased fitness through PRL3 activation [212]. Similarly, NOTCH activation is recurrently found in CRC liver metastasis, suggesting that liver microenvironment provides cues that activate this pathway.

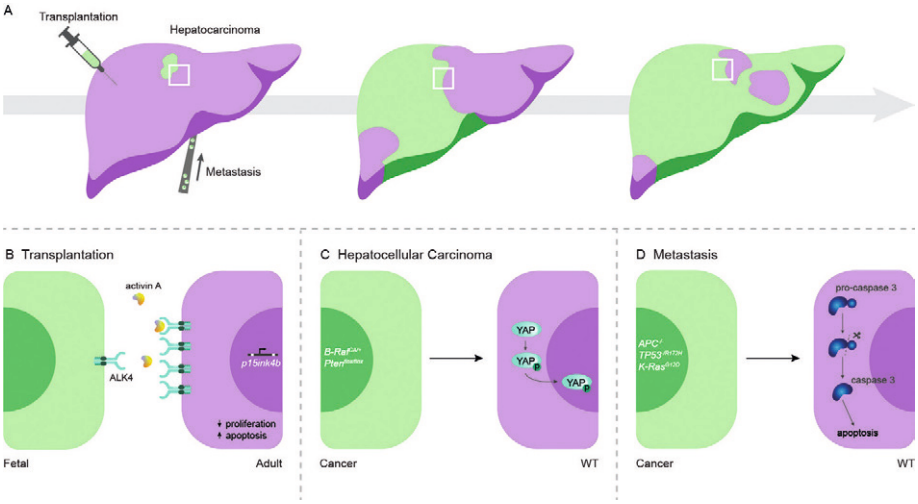

Figure 4: Schematic overview of competitive interactions in the liver
A) Examples of three types of cell competition that play a role in the liver, resulting in elimination of weaker cells (purple) and subsequent colonization of the liver by fitter cells (green). B) During transplantation adult hepatocytes are outcompeted by newly introduced hepatocytes that colonize the liver. Loss of regenerative capacity is associated with a rise in Activin A-mediated growth inhibition. C) Through accumulation of mutations, hepatocellular carcinoma cells gain a fitness advantage over non-mutated neighbors, resulting in tumor expansion. In response, wild-type peritumoral hepatocytes activate YAP signaling in attempt to control tumor progression [197]. D) Distant intestinal cancer cells reach the liver through the portal vein and interact with wild-type hepatocytes in the liver, resulting in induction of apoptosis, as well as promotion of their own expansion [222].

Interestingly, this has a dual effect as high expression of NOTCH 1 or 3 correlates with a poor prognosis while overexpression of NOTCH 2 or 4 has a protective function by blocking proliferation, invasion and migration [219]. This regulation by preferential expression of different forms of a membrane protein resembles the *Flower* code. Here, different isoforms of *Flower* would determine the fitness and elimination of competing cells [25,28]. Therefore, NOTCH could be a possible target to reduce metastasis through selective overexpression of specific NOTCH receptors. Recent studies have highlighted a role of hepatocytes in the establishment of a pro-metastatic microenvironment that resembles competitive interactions. In addition, cancer cells can use competitive interactions to impact surrounding liver tissue. A key example in colorectal cancer liver metastasis is vessel co-option, the utilization of pre-existing vessels by proliferating cancer cells [220]. This particularly prevalent metastases of the replacement growth pattern and is crucial for nutrient-supply and oxygenation. Furthermore, the CD95 (Fas-Ag/ Fas-L) pathway drives colorectal cancer tumor progression and invasiveness. Fas-L expressing cancer cells induce apoptosis in Fas Ag-bearing hepatocytes, creating a favorable niche for growth metastases in liver

tissue [221]. This, resembles active cell competition processes in where tumor cells eliminate surrounding healthy tissue [33,113] (Figure 4D). Lastly, YAP is activated in peritumoral hepatocytes surrounding metastatic lesions from melanoma and colorectal cancer in liver. As this is a tumor-preventive cell competition mechanism in primary hepatocellular carcinoma this suggests that similar mechanisms are important in liver metastasis [197]. Thus, a broad spectrum of pathways is activated in cancer cells within the liver microenvironment, facilitating the formation of metastases. Unraveling these pathways and the cues, factors, or ligands furnished by the liver environment - whether originating from cellular or non-cellular components - holds promise for discovering new therapeutic targets to impede CRC liver metastases.

DISCUSSION

The multifaceted role of cell competition in cancer underscores the need to further understand what determines winner or loser status. Generally, whether tumor or wild-type is outcompeted is context-dependent, illuminating the complexity of cellular interactions within distinct tissues. While cell competition acts as a safeguard against tumor initiation in the homeostatic intestine, its paradoxical role stimulating the growth of colorectal cancer highlights the intricate interplay between genetic and microenvironmental factors. For instance, loser populations share many characteristics with regenerating epithelia, which is an important aspect that is normally observed upon acute injury to protect the epithelium. The relationship between regeneration and supercompetition, is essential to comprehend the communication between cancer and the wild-type tissue. This allows a better understanding of the mechanisms involved in outcompetition of organ tissue and colonization by primary and metastatic tumors. Unraveling and targeting molecular pathways that enhance competitive interactions among normal cells may aid in protecting tissues against initiation of neoplastic transformation. Conversely, further understanding the tumor-promoting role of cell competition opens opportunities for targeted therapies aimed at disrupting or manipulating these competitive interactions. Precision medicine approaches that selectively inhibit key mediators of cell competition hold promise for impeding cancer progression, providing a promising direction for anti-tumor and anti-metastatic interventions.

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AUTHOR CONTRIBUTIONS

MEVL: Ideas, writing, proofreading, illustrations and approval of final paper. AKG: Ideas, writing, proofreading and approval of final paper. ML: Ideas, writing, proofreading and approval of final paper. SJES: Ideas, editing, proofreading and approval of final paper.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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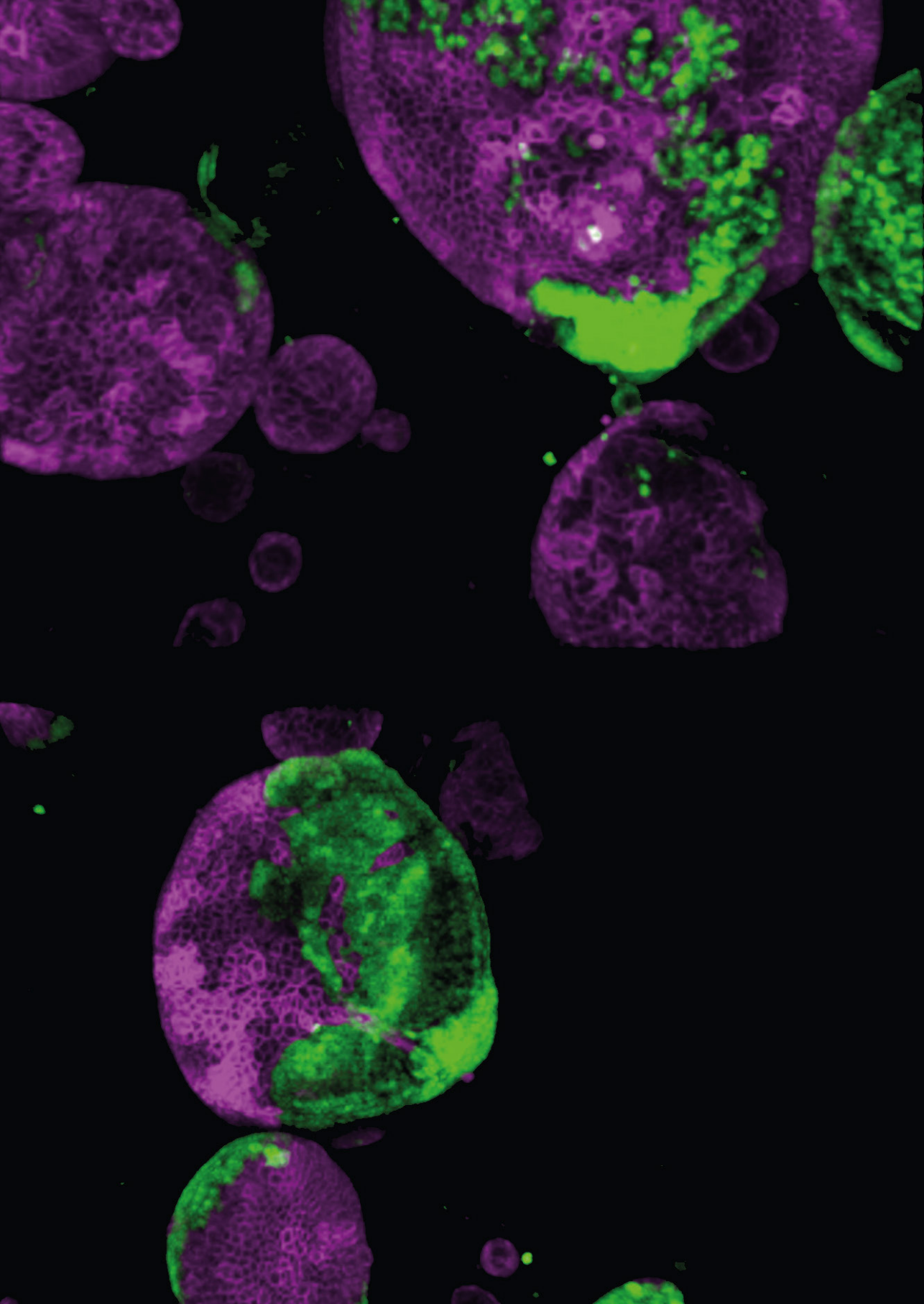
Outline of this thesis

Cell competition is crucial for quality control during development and homeostasis of tissues. Over the past years, the role of cell competition in progression of disease has emerged, in particular the impact on tumorigenesis. The central theme of this thesis is the remarkable versatility of cell competition throughout various stages of intestinal cancer tumorigenesis. Across the chapters, we explore the different competitive interactions that intestinal cancer cells engage during both primary tumor formation and metastasis. By understanding these interactions, we aim to develop tools to manipulate competition in each specific cell-to-cell interaction, with the ultimate aim of interfering with tumor growth. For this research we developed a variety of novel 3D culture models such as mixed organoids and microtissues. In **Chapter 2** we described the methodology for creating mixed organoids, composed of wild-type murine intestinal organoids and AKP ($APC^{-/-}$; $KRAS^{G12D}$; $p53^{fl/R172H}$) cancer organoids. Organoids have revolutionized the concept of mimicking tissue, and with this approach we try to go one step further by combining two different epithelial cell types within the same 3D organoid structure. In **Chapter 3**, we delve into the role of cell competition in primary intestinal tumor growth. This competition is studied by tracking the interaction between healthy and intestinal cancer cells within the same mixed organoid. In this chapter we elucidate the alterations that wild-type intestinal cells undergo when they are outcompeted by intestinal cancer cells. Additionally, in this chapter, we give mechanistic insight of the intercellular interactions between intestinal cancer and wild-type cells during competition by studying the activation of pathways, such as the stress-related JNK pathway. Using this knowledge, we also manipulate competition to revert the outcome of tumor growth. In **Chapter 4**, we study competition during liver metastasis. In this chapter, intestinal cancer cells with the same genetic background as those used in Chapter 3 face liver progenitors and hepatocytes in mixed organoids and microtissues, and we describe the outcome of these two interactions. This chapter shows how competition exhibits different outcomes depending on the relative fitness of the competing cells. In **Chapter 5,** we give insight into the molecular mechanisms driving competition in liver metastasis, with a focus on the mechano-transductors YAP/TAZ and stress-related JNK signaling. In **Chapter 6**, we examine organotropism of intestinal cancer cells. We monitor the adaptation and competitional advantage that these cells acquire in the liver micro-environment. Lastly, in **Chapter 7**, we discuss the findings described in this thesis in light of the current state of the field and highlight future directions of research.



Generation of mixed murine organoids to model cellular interactions

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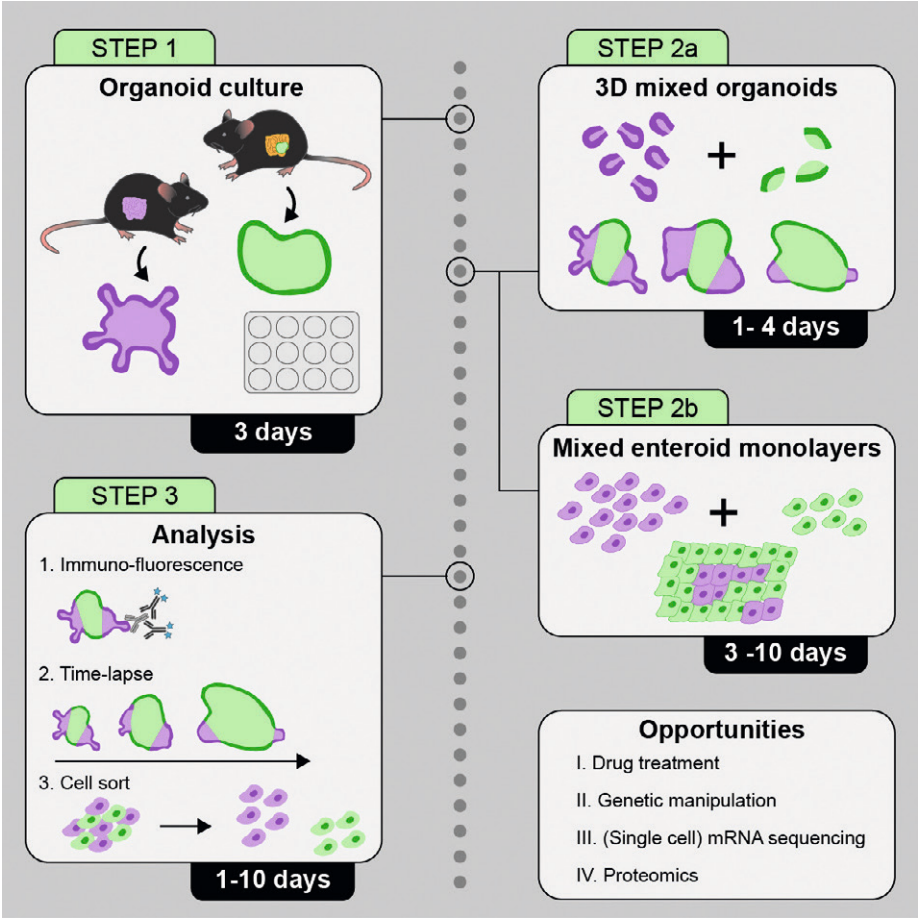
SUMMARY

Cell competition is a mechanism of interaction that dictates cell selection based on differences in cellular fitness. We designed a protocol to generate mixed murine organoids and enteroid monolayers used to study such complex cellular interactions in a mammalian system. This protocol is dedicated to follow the behavior of different cell populations over time, using (time-lapse) microscopy or transcriptome/proteome analysis.

For complete information on the generation and use of this protocol, please refer to (Krotenberg Garcia *et al.*, 2021).

GRAPHICAL ABSTRACT



BEFORE YOU BEGIN

In tissues, cellular interactions are essential for quality control, both during development and in homeostasis. In particular, competitive interactions can drive selection and thereby determine cell fate, growth and survival (Vishwakarma and Piddini, 2020). This mechanism was historically modeled in *Drosophila* (Simpson, 1979; Suijkerbuijk *et al.*, 2016), Madin–Darby canine kidney (MDCK) epithelial cells (Hogan *et al.*, 2009; Norman *et al.*, 2012) and more recently inducible mouse models (Kon *et al.*, 2017; Flanagan *et al.*, 2021; Hill *et al.*, 2021; van Neerven *et al.*, 2021). Here, we describe and readily adaptable method to generate mixed organoid cultures, which mimic the close interaction between different cell population.

This protocol describes the generation of mixed organoids and enteroid monolayers from two types of murine small intestine organoid cultures; wild-type cells (Krotenberg Garcia *et al.*, 2021) and *Apc*$^{-/-}$*Kras*$^{G12D/WT}$*Trp53*$^{-/R172H}$ cancer cells (Fumagalli *et al.*, 2017). Previously we have extended the use of the protocol to other colorectal cancer lines (Jackstadt *et al.*, 2019; Krotenberg Garcia *et al.*, 2021). Based on the flexibility of the protocol it can be adapted to generate mixed organoids from other cystic-growing cultures derived from different tissues. The individual organoid cultures described in this protocol were derived from different donor mice with a similar genetic background (C57BL/6JRj). However, we have previously generated mixed organoids from cultures with a different genetic background and did not observe aberrant behavior.

For successful execution of this protocol and subsequent analysis, labeling of both cell populations is essential. In particular, when loss (outcompetition) of one population is expected. Here we describe the use of wild-type intestine cells labeled with membrane-bound tdTomato (derived from mTmG transgenic mice (Muzumdar *et al.*, 2007)) and cancer cells labeled with Dendra2 (introduced by lentiviral transduction (Heinz, Oost and Snippert, 2020)).

Furthermore, the ratio of cells that are added to the mixing procedure is dependent on the characteristics of the original organoid cultures. For the intestinal model described here a 2:1 ratio (wild-type : cancer) is optimal. This ratio, was based on the difference in proliferation rate between the individual cell populations and validated experimentally, provides equal competition potential to both populations. In addition, other characteristics such as cell ratios that naturally occur in organ tissue can serve as a guideline.

This protocol has broad applications and can be used to study cellular interactions on an organoid and population basis. Involvement of specific molecular pathways can be revealed through a combination with chemical or genetic manipulation or downstream analysis such as (single cell) mRNA sequencing or proteomics.

Key resources table

REAGENT or RESOURCE	SOURCE
Antibodies	
anti-Phospho-c-Jun (Ser73) (D47G9) (Dilution 1:500)	Cell Signaling
anti-Cleaved Caspase-3 (Asp175) (Dilution 1:400)	Cell Signaling
anti-Aldolase B + Aldolase C antibody [EPR3138Y] (Dilution 1:300)	Abcam
anti-Lysozyme (EC 3.2.1.17) (Dilution 1:500)	Agilent
anti-Olfm4 (D6Y5A) (1:100)	Cell Signaling
Chicken anti-Rabbit, Alexa Fluor 647 (Dilution 1:500)	ThermoFisher Scientific
Chemicals, Peptides, and Recombinant Proteins	
Cultrex PathClear Reduced Growth Factor Basement Membrane Extract Type 2	R&D Systems
Advanced DMEM F/12	ThermoFisher Scientific
HEPES	Thermo Fisher Scientifc
Penicillin/streptomycin	Thermo Fisher Scientifc
GlutaMAX	Thermo Fisher Scientifc
R-spondin1	prepared in house
Noggin	prepared in house
B-27	Thermo Fisher Scientifc
hEGF	Peprotech
N-acetylcysteine	Sigma-Aldrich
Y-27632	Abmole
CHIR-99021	Tocris
TryplE	Thermo Fisher Scientific
DPBS, no calcium, no magnesium	Thermo Fisher Scientific
PBS tablets	Gibco
Paraformaldehyde 16% (w/v) in aqueous solution methanol-free	Alfa Aesar
BSA	Roche
4′,6-Diamidino-2-phenylindole Dihydrochloride (DAPI)	Toronto Research Chemicals
TX-100	Sigma-Aldrich
Deposited Data	
RNA-seq data	Krotenberg Garcia et al Cell Reports 2021
Experimental Models: Cell Lines	
Murine WT small intestine organoids Gt(ROSA) 26Sortm4(ACTB-tdTomato,-EGFP)Luo/J Mus	Krotenberg Garcia et al Cell Reports 2021

REAGENT or RESOURCE	SOURCE
Murine CRC organoids Villin-CreERT2Apcfl/flKrasG12D/WTTr53fl/R1	Fumagalli et al PNAS 2017
Software and Algorithms	
FIJI	https://imagej.net/
FlowJo 10.6.1	BD Biosciences
Imaris	Oxford Instruments
Other	
Cooled centrifuge	Eppendorf
Eppendorf centrifuge	Eppendorf
Tissue culture microscope equipped with fluorescent light source	Thermo Fisher Scientifc
Inverted confocal microscope	Leica
Spinning disk confocal microscope	Andor
Cell counter	Westburg
Pasteur Capillary Pipette, short size 150mm	VWR
5 mL Polystyrene Round-Bottom Tube with 35 µm Cell-Strainer Cap	Falcon
5 mL Polystyrene Round-Bottom Tube	Falcon
15 ml tubes	Sarstedt
1.5 mL Safe-Lock Tubes	Eppendorf
24 well CELLSTAR® plate, polystyrene	Greiner Bio-One
12 well CELLSTAR® plate, polystyrene	Greiner Bio-One
6 well CELLSTAR® plate, polystyrene	Greiner Bio-One
8 well chambered µ-Slide, glass-bottom	IBIDI
96 well µ-Plate, ibiTreat #1.5 polymer coverslip	IBIDI
96 well SensoPlate, glass-bottom	Greiner Bio-One
384 well microplate, tissue culture treated	Greiner Bio-One

MATERIALS AND EQUIPMENT

Culture media recipes

According to Sato et al. (Sato *et al.*, 2009) and Thorne et al (Thorne *et al.*, 2018)

Basic culture medium

Reagent	Stock concentration	Final concentration	Amount
Advanced DMEM/F-12	n/a	n/a	500 mL
HEPES	1 M	10 mM	5 mL
GlutaMAX	100X	1 %	5 mL
Penicillin/Streptomycin	10.000 U/mL	100 U/ml	5 mL
Total			**515 mL**

Prepare in a sterile environment, store at 4°C for up to 2 months

ENR culture medium (ENR: EGF, Noggin and RSPO1)

Reagent	Stock concentration	Final concentration	Amount
Basic culture medium	n/a	n/a	38.87 mL
B-27	50X	2 %	1 mL
N-Acetyl-L-Cysteine	500 mM	1.25 mM	125 µL
R-spondin1 Conditioned Medium (CM)	n/a	10 %	5 mL
Noggin Conditioned Medium (CM)	n/a	10 %	5 mL
human Epidermal Growth Factor (hEGF)	0.5 mg/ml	50 ng/ml	5 µL
Total			**50 mL**

Prepare in a sterile environment, store at 4°C for up to 2 weeks

Note: For in house production of conditioned media refer to Broutier et al (Broutier ***et al.***, 2016). Alternatively, Noggin-FC fusion protein and R-Spondin 3-FC fusion protein conditioned medium can be used (U-Protein Express BV, Cat. No.#N002 and #R001).

Enteroid plating medium

Reagent	Stock concentration	Final concentration	Amount
Basic culture medium	n/a	n/a	38.77 mL
B-27	50X	2 %	1 mL
N-Acetyl-L-Cysteine	500 mM	1.25 mM	125 µL
R-spondin1 CM	n/a	10 %	5 mL
Noggin CM	n/a	10 %	5 mL
hEGF	0.5 mg/ml	50 ng/ml	5 µL
CHIR-99021	3 mM	3 µM	50 µL
Y-27632	10 mM	10 µM	50 µL
Total			**50 mL**

Prepare in a sterile environment, store at 4°C for up to 2 weeks

Solution recipes

Fixation solution

Reagent	Stock concentration	Final concentration	Amount
Paraformaldehyde (methanol-free)	16 %	4 %	10 mL
PBS	2X from tablets	1X	20 mL
MilliQ water	n/a	n/a	10 mL
Total			**40 mL**

Store at -20°C for up to 1 month, avoid freeze thaw cycles.

CRITICAL: Paraformaldehyde is hazardous; wear gloves, work in chemical safety hood and dispose waste in accordance with local regulations.

Blocking solution

Reagent	Stock concentration	Final concentration	Amount
BSA	n/a	5 %	2.5 g
TX-100	10 %	0.2 %	1 mL
PBS	1X from tablets	1X	49 mL
Total			**50 mL**

Store at 4°C for up to 1 month.

Antibody incubation solution

Reagent	Stock concentration	Final concentration	Amount
BSA	n/a	2.5 %	1.25 g
TX-100	10 %	0.1 %	0.5 mL
PBS	1X from tablets	1X	49.5 mL
Total			**50. mL**

Store at 4°C for up to 1 month.

Washing solution

Reagent	Stock concentration	Final concentration	Amount
TX-100	10 %	0.1 %	0.5 mL
PBS	1X from tablets	1X	49.5 mL
Total			**50 mL**

Store at room temperature (18- 25 °C)for up to 6 months.

FACS buffer

Reagent	Stock concentration	Final concentration	Amount
dPBS	n/a	n/a	9.75 mL
B-27	50X	2 %	200 µL
N-Acetyl-L-Cysteine	500 mM	1.25 mM	25 µL
hEGF	0.5 mg/ml	50 ng/ml	1 µL
Y-27632	10 mM	10 µM	10 µL
DAPI	1 mg/ml	1 µg/mL	10 µL
Total			**10 mL**

Prepare in a sterile environment, store at 4°C for up to 2 weeks.

Media and reagent preparation

Timing: 2 days

- Thaw Reduced Growth Factor Basement Membrane Extract Type 2 (BME2) on ice or in a fridge overnight.
- Incubate unpackaged 6, 12 and 24 well plates (depending on the type of experiment) untreated polystyrene culture plates in a tissue culture incubator at 37 °C for at least 48h prior to use to promote formation of BME2 droplets during plating.
- Prepare all buffers and media according to the tables in the "Materials and equipment" section and store appropriately (see notes).
- Prepare glass Pasteur pipets by fire-polishing the tip of the pipets using a Bunsen burner (Figure 1). At least one pipet per individual culture should be prepared, spares are recommended.
- Cool centrifuges to 4°C.

Note: Instead of BME2 other culture matrices (e.g. Matrigel or Collagen) can be used. Select appropriate matrix depending on organoid culture of choice. Specific characteristics of certain matrices might interfere with downstream analysis. For example, Collagen does not dissolve well in the fixation solution (Step 33) and might need additional permeabilization steps, or Matrigel containing phenol-red might cause problems with auto-fluorescence in time-lapse experiments. Optimization is recommended when alternative matrices are used.

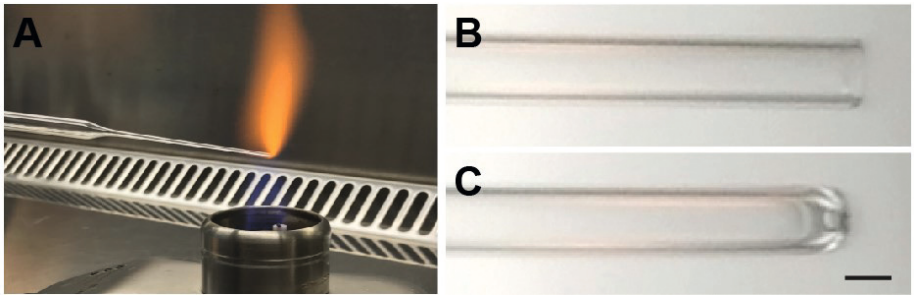


Figure 1: Preparation of glass Pasteur pipets. A) Fire-polish a glass Pasteur pipet by placing the tip in the flame of a Bunsen burner for a couple of seconds while continuously rolling the pipet. An example of a glass Pasteur pipet before (B) and after (C) fire-polishing. Scale bar = 1 mm.

STEP-BY-STEP METHOD DETAILS

Organoid culture

Timing: 7 days

This section describes the culture steps in preparation of small intestine wild-type and cancer organoids for generation of mixed cultures. For complete details on the isolation and culture of murine small intestine organoids please refer to (Sato *et al.*, 2009; Sato and Clevers, 2012; Andersson-Rolf *et al.*, 2014). For optimal and efficient execution of this protocol it is essential that the organoids have adapted to the experimental culture medium and are in a healthy state (Figure 2) prior to the start of the experiment.

1. Culture the organoids in 3D in BME2 for at least 7 days in ENR culture medium. There is no maximum duration of this adaptation time. Passage the cultures when the lumen fills with apoptotic cells, approximately once every 3-4 days.

Critical: It is essential that the experimental culture medium is uniform for all organoid cultures in the experiment and contains a combination of all factors that are required for the individual cultures. For example, the cancer population described in this protocol does not require hEGF or RSPO1, but is cultured in ENR medium, in order to avoid phenotypic changes during the experiment that could be caused by growth factors.

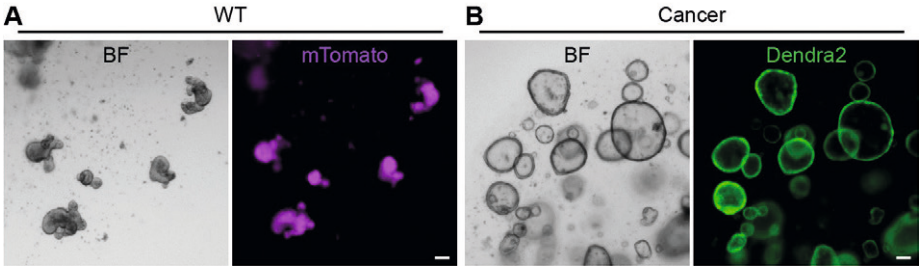


Figure 2: Input organoid cultures. Mature murine wild-type (A) and Cancer (B) organoid cultures passaged 3 days prior to the start of the experiment. Pay attention to the morphology of the organoids, which should have a smooth epithelium and lack apoptotic cells in their lumen. 2-6 crypt-like structures are optimal for wild-type small intestine cultures. A) Membrane-bound tdTomato labelled wild-type cells (Krotenberg Garcia ***et al.***, 2021) were derived from healthy small intestine tissue. B) Dendra2 labelled cancer cells were derived from ***Apc***$^{-/-}$***Kras***$^{G12D/WT}$***Trp53***$^{-/R172H}$ small intestinal tumors (Fumagalli ***et al.***, 2017). Scale bars = 100 µm

2. Passage organoid cultures 2-3 days prior to the start of the experiment until mature and healthy organoids are formed (Figure 2). Refer to Table 2 for guidance on input material.

Note: We have not observed differences in outcome between early and late passage cultures. However, depending on the organoid cultures of choice the use of early passages might be required.

Preparation of organoids

Timing: 15-30 minutes

This section describes the preparation of small intestine wild-type and cancer organoids for mixed cultures. After Step 7, organoids are ready to proceed to generation of 3D mixed organoids (Part A) or generation of mixed enteroid monolayers (Part B).

Note: Organoid cultures are very sensitive to fluctuations in temperature. Therefore, we recommend to use ice-cold reagents, keep cells on ice in between steps and use centrifuges that are cooled to 4 °C throughout the protocol.

3. Harvest organoids by scraping the bottom of the well and pipetting up and down with a p1000 pipette using ice-cold Basic culture medium.
4. Collect the organoid suspension in 15 mL centrifuge tubes and keep on ice.

Optional: To ensure harvest of all organoids, it is recommended to wash the well with another 1 mL of ice-cold Basic medium and collect leftovers.

5. Centrifuge at 300 x g at 4 °C for 5 minutes. Three clear layers should be formed; medium, BME2 and the pellet of organoids (top-to-bottom, Figure 3).
6. Carefully remove the medium and BME2 layers without disrupting the organoid pellet.
7. Resuspend the pellet in 1 mL of ice-cold Basic culture medium.

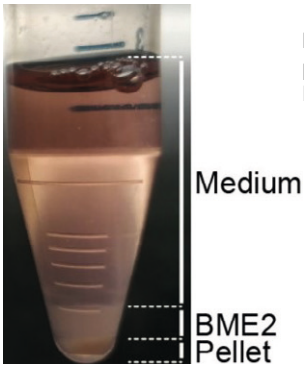


Figure 3: Preparation of organoids. Overview of the three phases formed by centrifugation of an organoid suspension. Medium (top), BME2 (middle) and organoid pellet (bottom).

Part A. Generation of 3D mixed organoids

Timing: 2-3 h

This section describes the main steps to generate 3D mixed organoids from small intestine wild-type and cancer cells. Once the 3D mixed organoids are formed, they can be used in different techniques such as cell sorting, immuno-fluorescence and time-lapse imaging, described later in this protocol.

8. Use a fire-polished glass Pasteur pipet to mechanically disrupt the organoids. Pipette up and down 10-20x while pressing the pipet tip against the bottom of the tube, until no clear structures are visible by eye. The suspension now contains clumps of 50-150 cells (Figure 4).

Note: Immediately before use, sterilize glass Pasteur pipets by briefly passing them through a flame and coat the inside walls of the pipet by aspiration of Basic culture medium to avoid attachment of the organoids.

Critical: Optimal breaking of the organoids is essential. Therefore, it is recommended to monitor the status at a microscope after every 5-10x of pipetting. Stop the breaking of the organoids before they are single cells (Figure 4).

9. Centrifuge at 300 x g at 4°C for 5 minutes.
10. Aspirate supernatant and resuspend the pellets in 300 µL ice-cold Basic culture medium.

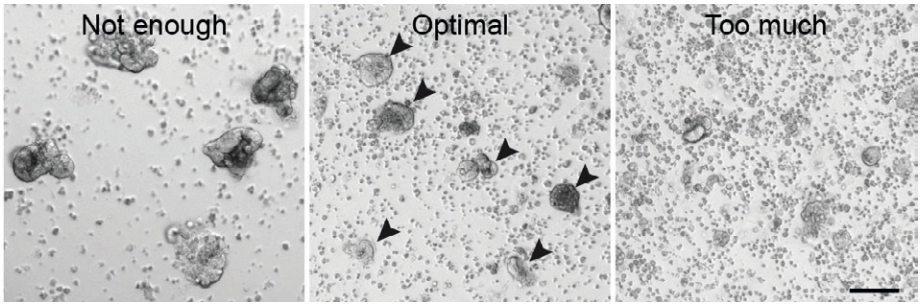


Figure 4: Generation of organoid clump suspension. Bright field examples of mechanically disrupted organoid cultures. Depicted are fragments that need more disruption (left), of optimal size (middle, indicated by arrow heads) or were disrupted too much (right). Scale bar = 100 µm.

Critical: The final ratio of cells in the mixed organoids is dependent on the input of the different cell populations (see Table 2). Therefore, it is important to compare the pellet size and adjust the amount of Basic culture medium that is added in Step 10 accordingly (add 300 µL to the smallest pellet, increase volume X times for a X times bigger pellet).

11. Prepare one Eppendorf tube for each of the conditions (Pure population A, Pure population B and Mix Population A : B) and add the correct amount of cell clump suspension (Table 1).

Note: The efficiency of generating mixed organoids is not 100%. Therefore, it is recommended to include duplicates of mixed conditions in order to generate equal numbers of pure and mixed organoids.

Note: The optimal ratio for mixing is dependent on the characteristics of the individual cultures, such as differences proliferation rate. It is recommended to include multiple ratios and validate the optimal mix conditions experimentally.

12. Centrifuge at 300 x g at 4°C for 5 minutes
13. Carefully aspirate the supernatant with a p200 pipette and resuspend the small pellet in 10 µL of ENR medium.
14. Incubate the Eppendorf vials containing the concentrated cell clump suspensions at 37 °C for 30 minutes to promote cell aggregation.

Table 1. Recommended volumes of cell clump suspension for mixing of 3D organoids

Condition	Ratio	Pure population A	Pure population B	Mix population A : B
1	1:1	**Vial 1A:** 100 µL	**Vial 1B:** 100 µL	**Vial 1AB:** 100 µL : 100 µL
2	2:1	**Vial 2A:** 100 µL	**Vial 2B:** 50 µL	**Vial 2AB:** 100 µL : 50 µL
3	3:1	**Vial 3A:** 100 µL	**Vial 3B:** 33 µL	**Vial 3AB:** 100 µL : 33 µL
4	4:1	**Vial 4A:** 100 µL	**Vial 4B:** 25 µL	**Vial 4AB:** 100 µL : 25 µL

Table 2. Recommended input and plating conditions for mixing of 3D organoids

Input per well for four conditions			Aggregation per condition			Plating per condition	
Plate type	**Size**	**No. organoids**	**Volume**	**+ENR Medium**	**+BME2**	**No. drops**	**Drop size**
24-well (Greiner)	1.9 cm^2	250	5 µL	10 µL	30 µL	3	12 µL
12-well (Greiner)	3.9 cm^2	500	10 µL	15 µL	50 µL	6	12 µL
6-well (Greiner)	9.6 cm^2	1000-1500	20 µL	50 µL	140 µL	15	12 µL

Critical: Aggregation of cells in a small volume is essential for efficient formation of mixed organoids. Do not increase the volume to more than 25 µL.

15. After the incubation time, dilute the aggregates with ENR medium (see Table 2 for volume).
16. Add double the volume of BME2 (compared to the diluted aggregates) to achieve a final ratio of 1:2 (see Table 2 for volume) and keep on ice.
17. Plate drops of 12 µL per well of an imaging-compatible plate. Output and drop size are based on use of a 96-well imaging plate (IBIDI). Adapt when different plates are used.

Note: For sorting of cells from 3D organoids it is recommended to plate mixed organoids in a 6 well format to increase yield.

Note: The input material is indicated per one well, this is sufficient for four conditions (1x each of pure populations and 2x mix)

Note: The volumes in Table 2 are guidelines. To avoid crowding it is recommended to plate one drop in a separate dish and monitor organoid density (Figure 5). Adjust the volume if needed.

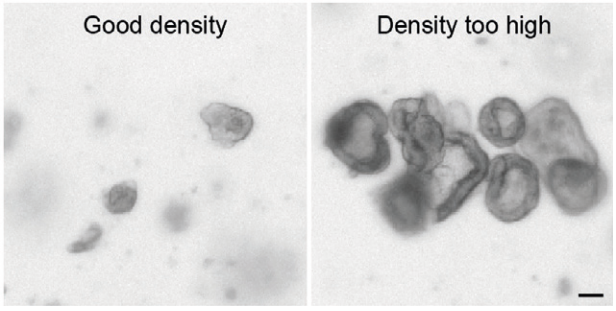


Figure 5: Plating density Bright field examples of cell aggregates that are plated at an optimal density (left) or a density that is too concentrated (right). Scale bar = 100 µm.

Note: The indicated drop size is optimized for plating of a single drop per well of a 96-well imaging plate (IBIDI). It is recommended to adjust the size when other plate types are used.

18. Invert the plate and let the drops solidify in a 37 °C incubator for 10-30 minutes.
19. Carefully add 150 µL room temperature ENR medium per well, do not disrupt the drops, and return the plate to the incubator (37 °C, 5% CO2).

Note: At this stage chemical manipulation can be started, for example by addition of small molecule inhibitors to the culture medium.

Part B. Generation of mixed enteroid monolayers

Timing: 2-3 h

This section describes the main steps to generate mixed enteroid monolayers (Thorne *et al.*, 2018) from individual small intestine wild-type and cancer cells. Once the monolayers are formed, they can be used in different techniques such as immuno-fluorescence and time-lapse imaging, described later in this protocol.

20. Coat imaging plates:
    a.Dilute BME2 to a final concentration of 0.8 mg/mL in ice-cold Basic culture medium and keep on ice.
    b.Add 50-150 µL of diluted BME2 per well (see Table 3 for appropriate volume) and incubate for 1h at 37 °C.
    c. Wash the coated wells twice with Basic culture medium and use the plate within 2h. Keep the plates at room temperature until use.
21. Continue with resuspended pellet (Step 7) and use a fire-polished glass Pasteur pipet to mechanically disrupt the organoids by briefly pipetting up and down.

Note: Immediately before use, sterilize glass Pasteur pipets by briefly passing them through a flame and coat the inside walls of the pipet by aspiration of Basic culture medium to avoid attachment of the organoids.

22. Centrifuge at 300 x g at 4°C for 5 minutes.
23. Aspirate the supernatant and add 1 mL of TrypLE at room temperature.
24. Dissociate the broken organoids by pipetting up and down using a fire-polished glass Pasteur pipet.
25. Once a single cell suspension is formed (after ± 3 minutes at room temperature) immediately add 10 mL of cold Basic culture medium and keep on ice.

Table 3. Recommended input and plating conditions for mixed enteroid monolayers

			Number of cells /well					
Well format			Ratio 1:1		Ratio 1:2		Ratio 1:4	
Type	Size	Volume	A	B	A	B	A	B
384 (Greiner)	0.10 cm^2	50 µL	3000	3000	3000	1500	3000	750
96 (Greiner)	0.34 cm^2	100 µL	10000	10000	10000	5000	10000	2500
96 (IBIDI)	0.56 cm^2	150 µL	15000	15000	15000	7500	15000	3750

Note: TrypLE is sufficiently inactivated by dilution. Additional inactivation steps (e.g. addition of serum or an inhibitor) might be required when other dissociation reagents are used. Check recommendations of the supplier.

Critical: Some organoid cultures are very sensitive to dissociation. It is recommended to reduce the incubation time in TrypLE to less than 5 minutes. This can be achieved by processing few samples simultaneously and repeated monitoring at a microscope until a single cell suspension is reached (similar to the right panel in Figure 4).

26. Centrifuge at 300 x g at 4°C for 5 minutes.
27. Aspirate the supernatant and add 200 µL of ice-cold Enteroid plating medium.
28. Count cells using a Bürker Türk Counting Chamber or automated cell counter
29. Dilute and plate cells in ice-cold Enteroid plating medium in an imaging compatible plate according to Table 3.
30. Let the cells adhere in an incubator (37 °C, 5% CO2).
31. Replace the medium with 37 °C ENR medium after 16-24h and refresh every 2-3 days.

Note: At this stage chemical manipulation can be started, for example by addition of small molecule inhibitors to the culture medium.

Immuno-fluorescence staining

Timing: 2 days

This section describes how to proceed with Immuno-fluorescence staining of mixed organoids or enteroid monolayers. The indicated volumes are based on use of a 96-well imaging plate (IBIDI) and can be adapted accordingly when different plates are used.

32. Carefully aspirate culture medium without disturbing the drop of BME2 or the monolayer.
33. Add 100 µL Fixation solution (see "Materials and equipment" section) and incubate at room temperature for 20 minutes.

Note: It is not required to include an additional wash step prior to fixation.

34. Aspirate the Fixation solution once the drop of BME2 is completely dissolved (Figure 6) and wash twice with 100 µL PBS.

Note: If the drop is not completely dissolved, the fixation can be prolonged for up to 30 minutes.

Note: The organoids are now attached to the bottom of the well.

Pause point: After fixation organoids can be stored in PBS for 2/3 weeks, protected from light at 4°C before continuing with immuno-staining.

35. Add 100 µL blocking solution (see "Materials and equipment" section) and incubate at room temperature for at least 30 minutes to block and permeabilize organoids.

Note: This step can be prolonged for several hours when convenient.

36. Add the appropriate primary antibodies diluted in Antibody incubation solution (50 µL per well) and incubate at 4°C protected from light overnight (8- 16 hours) or for up to several days.

Note: See "Expected outcome" section for examples of antibodies that can be used.

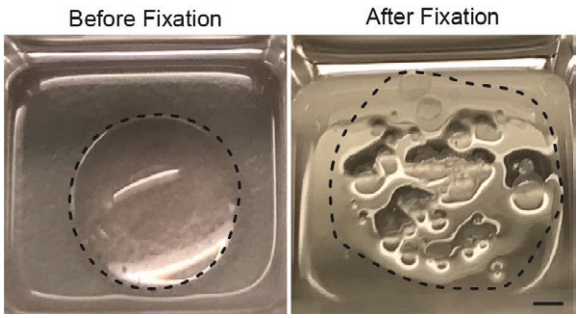


Figure 6: Fixation of mixed organoids. Drop of BME2 containing mixed 3D organoids before fixation (left) and after dissolving in fixation solution (right). The BME2 drop, before and after disruption, are outlined by a dashed line**.** Scale bar = 1 mm.

37. Wash three times for 5 minutes with 100 µL Washing solution at room temperature.
38. Add the appropriate secondary antibodies diluted in Antibody incubation solution (50 µL per well) and incubate at room temperature protected from light for at least one hour.

Note: At this step other dyes such as DAPI or phalloidin can be included. Selection of secondary antibodies should be based on the expression of fluorophores by the organoid cultures to avoid cross-talk.

Note: This step can be prolonged for up to several days. Incubate at 4°C when exceeding 5 hours.

39. Wash three times with 100 µL Washing solution for 5 minutes each at room temperature.
40. Rinse twice with 100 µL PBS and leave the organoids in PBS.

Pause point: Stained organoids can be can be stored in the dark at 4°C. To prevent loss of signal, we recommend proceeding with image analysis within one week. Ensure the organoids do not dry out by adding enough PBS and/or sealing plates with parafilm.

41. Proceed to imaging using a platform of choice. A confocal based platform is recommended, using a high NA 20-25x objective.
42. Acquire Z-stacks, see notes for recommendations.

Note: For 3D mixed organoids the thickness of Z-slices should be determined based on the detail of information that is needed for further analysis. For example, a thickness of 5 µm is sufficient for analysis at a cellular resolution. For optimal 3D reconstruction without loss of data, a system calculated optical slice thickness can be used.

Note: For enteroid monolayers, it is recommended to acquire Z-stacks covering the total thickness of the monolayer (approximately 20 µm) to avoid loss of 3D information. Data can be further analyzed via 3D reconstruction or Z-projection.
Time-lapse imaging of mixed organoids

Timing: 1-4 days

This section describes how to proceed with time-lapse imaging of mixed organoids or enteroid monolayers. Ensure cells are plated in an imaging-compatible plate at Step 17 or 29.

43. Refresh the medium 1 day after plating.
44. Add PBS to the surrounding empty wells to avoid evaporation.
45. Proceed to imaging using a platform of choice that is equipped with a CO2 and 37 °C incubator. A (spinning-disk) confocal based platform is recommended, using a high NA 20-25x objective.
46. Acquire images with a maximum time interval of one hour and a maximum Z-slice thickness of 5 µm for tracking of single cells in 3D.

Note: Organoids can be imaged for up to 72 hours. A change of medium is necessary for longer experiments.

Sorting cells from mixed organoids

Timing: 2-4 h

This section describes how to process previously mixed 3D organoids for cell sorting.

47. Harvest organoids by scraping the bottom of the well and pipetting up and down with a p1000 pipette using ice-cold Basic culture medium.
48. Collect the organoid suspension in 15 mL centrifuge tubes.
49. Centrifuge at 300 x g at 4 °C for 5 minutes. Three clear layers should be formed; medium, BME2 and the pellet of organoids (top-to-bottom, Figure 3).
50. Carefully remove the medium and BME2 layers without disrupting the organoid pellet.
51. Resuspend the pellet in 1 mL of ice-cold Basic culture medium.
52. Mechanically disrupt the organoids by briefly pipetting up and down using a fire-polished glass Pasteur pipet.
53. Centrifuge at 300 x g at 4 °C for 5 minutes.
54. Aspirate the supernatant and add 1 mL of TrypLE at room temperature.
55. Dissociate the broken organoids by pipetting up and down using a fire-polished glass Pasteur pipet.
56. Once a single cell suspension is formed (after ± 3 minutes) immediately add 10 mL of ice-cold Basic culture medium and keep on ice.

Note: TrypLE is sufficiently inactivated by dilution. Additional inactivation steps (e.g. addition of serum or an inhibitor) might be required when other dissociation reagents are used. Check recommendations of the supplier.

57. Centrifuge at 300 x g at 4°C for 5 minutes.
58. Resuspend pellet in 250 µL ice-cold FACS buffer and pass through a 35 µm cell strainer.
59. Keep cells on ice and immediately proceed to cell sorting using a platform of choice.

EXPECTED OUTCOMES

Our protocol describes the generation of mixed 3D organoids and enteroid monolayers from individual cell populations. We have not tried to derived mixed enteroid monolayers directly from mixed 3D organoids. During the course of the experiment growth and progression of the cultures can be monitored using a tissue culture microscope equipped with fluorescent light source. When generating mixed cultures of populations of different cellular fitness, a gradual loss of the weaker cell population is expected (Figure 7). The 3D cultures of wild-type small intestine and *Apc*$^{-/-}$*Kras*$^{G12D/WT}$*Trp53*$^{-/R172H}$ cancer cells mixed in a 2:1 ratio described here, consist of ± 40% wild-type cells 1 day after mixing and this decreases to ±10% on day 4 (Krotenberg Garcia *et al.*, 2021). The described enteroid monolayers mixed in a 2:1 ratio (wild-type : cancer) are composed of ±20% wild-type cells the moment a confluent monolayer has formed (Krotenberg Garcia *et al.*, 2021).

These mixed cultures can be used to study cellular interactions with a variety of methods. Immuno-fluorescence staining can reveal cell competition-induced changes in cellular behavior and morphology. We have previously observed that wild-type small intestine cells revert to a fetal-like state (Krotenberg Garcia *et al.*, 2021). This coincides which a diminished formation of crypt-like structures and reduced presence of cell types that are normally present in adult intestinal tissue, such as Paneth and intestinal stem cells (Figure 8A-8C). In addition, activation of signaling pathways can be studied using immuno-fluorescence. Wild-type cells are elimination via apoptosis and this is driven by active JNK signaling (Krotenberg Garcia *et al.*, 2021), which can be visualized using cl-CASP3 and cJUN-pS73 antibodies (Figure 8D-8E; Movie S1: 3D-reconstruction of confocal images of a mixed organoid, related to steps 32-42). Specific changes in cellular behavior can be tracked by time-lapse microscopy (Figure 9A-9B; Movies S2: Time-lapse series of control treated 3D reconstructed mixed intestinal organoid and S3: Time-lapse series of a competing enteroid monolayer, both related to steps 43-46). Cell sorting is used to detect changes on a population level (Figure 10A). Sorted cells can subsequently be used for further downstream analysis such as (mRNA) sequencing or proteomics. Such analysis has revealed a reversion of wild-type cells to a fetal-like state (Krotenberg Garcia *et al.*, 2021) and reduced expression of intestinal stem cell markers (Figure 10B).

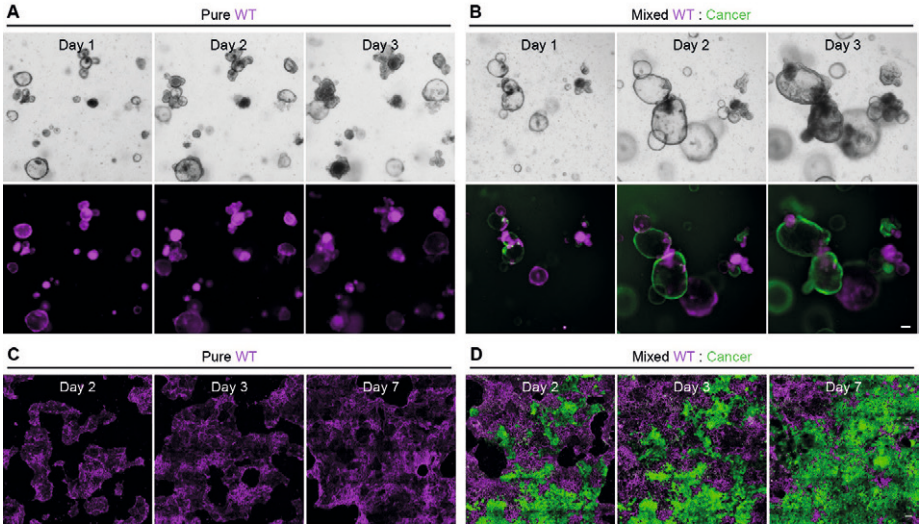


Figure 7: Progression of mixed 3D organoids and enteroid monolayers Monitoring growth of pure (A) and mixed (B) 3D organoids using a fluorescent tissue culture microscope and pure (C) and mixed (D) enteroid monolayers using a stage-calibrated confocal microscope for up to one week. Cancer cells are visualized in green. The wild-type population (magenta) is gradually lost from the cultures over time. Scale bars = 100 µm.

LIMITATIONS

Our protocol describes 3D mixed organoids and enteroid monolayers that are generated from adult stem cell derived cystic-growing organoid cultures. These cultures only contain epithelial cells and lack other cell types such as mesenchyme, neurons and immune cells. Therefore, the current protocol cannot be used to study the influence of a microenvironment. Extensive optimization is required to adapt this protocol for these purposes.

Currently, the maximum duration of the experiment is limited to the growth and passage requirements of the organoid cultures. The optimal duration for the mixed 3D cultures described in this protocol is three days and the maximum length is five days, by which all wild-type cells are eliminated. The mixed enteroid monolayers are viable for at least ten days, however most wild-type cells are lost within seven days after a full monolayer is formed. It is not possible to passage the organoids while preserving the mixed cellular interactions. Propagation of the long-term effects of cellular interactions can possibly be achieved by re-mixing of cells that were sorted from mixed populations.

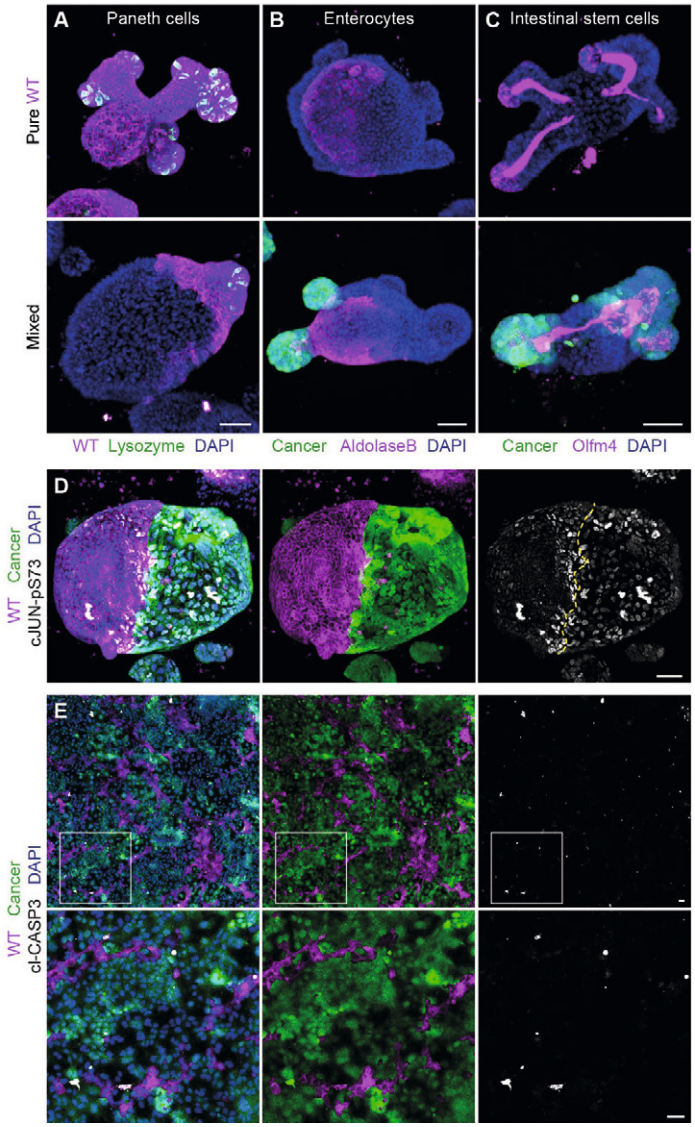


Figure 8: Analysis of cellular interactions using immuno-fluorescence Examples of analysis of interactions between cells in mixed 3D organoids and enteroid monolayers by immuno-fluorescence. A-C) Immuno-fluorescence staining of 3D-reconstructed pure (top) and mixed (bottom) organoids. Cancer (green) or wild-type (magenta) cells are visualized. The organoids were stained for lysozyme (A, green, Paneth cells), Aldolase B (B, magenta, enterocytes) or OLFM4 (C, magenta, intestinal stem cells), nuclei are visualized with DAPI (blue). D-E) Immuno-fluorescence staining of 3D-reconstructed mixed organoid (D) and mixed monolayer (E). Cancer (green) and wild-type (magenta) cells are visualized. The organoids were stained for cJUN-pS73 (D, grey) or cl-CASP3 (E, grey); nuclei are visualized with DAPI (blue). Also see Movie S1: 3D-reconstruction of confocal images of a mixed organoid, related to steps 32-42. Scale bars = 50 µm.

Generation of viable mixed organoids from cultures that have incompatible basic growth conditions can be problematic. For example, when one of the essential growth factors for one of the cell populations has a negative impact on the health of another cell population. In this instance, a shared experimental medium cannot easily be designed and redefinition of the minimal medium composition is required. In addition, extreme differences in the proliferation rate of the individual cultures will impair generation of sufficient mixed organoids. Here, adaptation of the experimental design is necessary. For example, by mixing cell clumps of the slow dividing population with fast growing single cells.

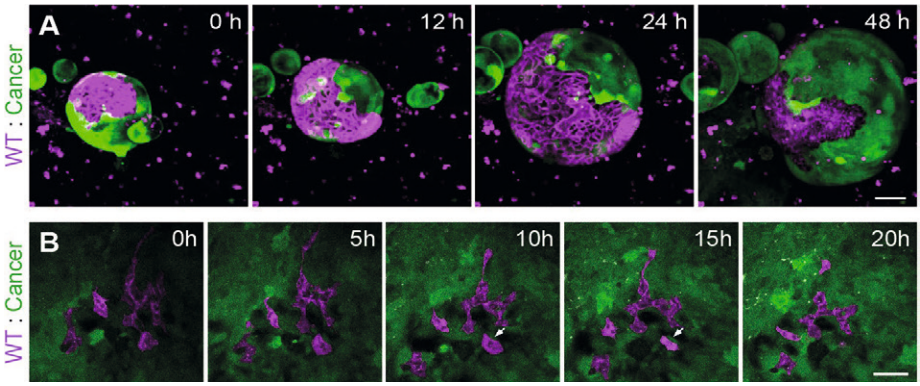


Figure 9: Analysis of cellular interactions using time-lapse microscopy A) Time-lapse series of a 3D-reconstructed mixed organoid, started one day after mixing. Cancer (green) and wild-type (magenta) cells are visualized. Also see Movie S2: Time-lapse series of control treated 3D reconstructed mixed intestinal organoid, related to steps 43-46. B) Time-lapse series of a mixed monolayer. Image acquisition was started after a full monolayer had formed, approximately three days after plating and the ratio shifted towards the cancer population. Cancer (green) and wild-type (magenta) cells are visualized. The arrow indicates an eliminated WT cell, which shrinks and is lost from the culture. Also see Movie S3: Time-lapse series of a competing enteroid monolayer, related to steps 43-46. Scale bars = 50 µm.

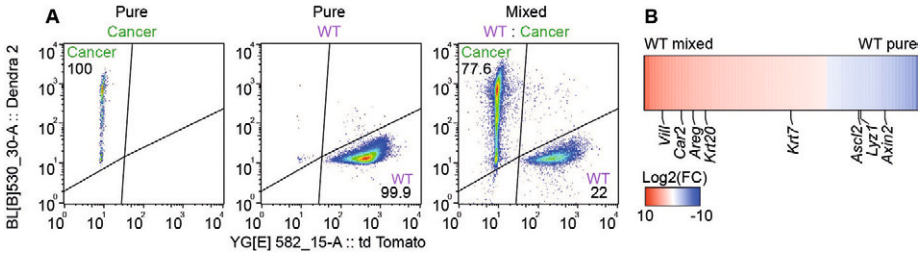


Figure 10: Analysis of cellular interactions using cell sorting A) Flow cytometry sorting of wild-type and cancer cells from pure and mixed cultures, the numbers in the corners display the percentage of sorted cells. B) Heatmap of the fold change of genes that are differentially expressed in wild-type cells upon mixing (Log2). Genes that are expressed by specific adult intestinal cell types are indicated.

TROUBLESHOOTING

Problem 1: Drops of BME2 attach to side of well (Step 17)

Potential solution:
Ensure unpackaged tissue culture plates are incubated in a heated CO2 incubator for at least 48 hours prior to use. Choose a smaller drop size if the problem persists.

Problem 2: (Mixed) organoids adhere to the bottom of the plate (Step 18).

Potential solution:
Invert the plate while drops are solidifying and/or use low-attachment/uncoated tissue culture plates

Problem 3: Only a low percentage of organoids are mixed.

Potential solution:
Reduce the volume of medium that is used during aggregation (Step 13). Ensure the organoids are optimally broken (Figure 4). Increase the amount of input material.

Problem 4: Mixed organoids fuse during the experiment.

Potential solution:
Plate less dense cultures after aggregation by adding higher volumes of medium and BME2 (Step 16, also see Figure 5).

Problem 5: It is difficult to distinguish the two different cell populations during FACS or microscopy analysis (see "Expected outcomes").

Potential solution:
Ensure both cell populations are differentially fluorescently labelled and this is bright enough for your analysis platform of choice (see "Before you begin"). In addition, a counterstain can be used in order to detect all cells in mixed organoids (e.g. DAPI or expression of a fluorescently-tagged histone H2B variant).

Problem 6: One of the cell populations is lost before the end of the experiment (Figure 7)

Potential solution:
Adapt the input ratio during mixing (Table 1) and/or ensure cells are mixed as clumps, not single cells (Figure 4).

Problem 7: Fluorescent signal is not equally distributed after immunofluorescence staining (see "Expected outcomes").

Potential solution:
Ensure BME2 drops are fully dissolved by fixation solution (Figure 6). Prolong permeabilization (Step 35) and/or increase the percentage of TX-100 in the Blocking solution.

Problem 8: Quality of images is reduced or lost during time-lapse image acquisition (Steps 43-46).

Potential solution:
Ensure the microscope is heated and incubate the plate in the system for at least 30 minutes before set-up the session to avoid drift. Carefully determine maximum Z-dimension before selecting positions to ensure the organoids stay within reach during acquisition and the objective is not damaged. Ensure the system is equipped with a C02 incubator and check flow rate. Use a dry objective for prolonged time-lapse imaging to prevent loss of water or oil immersion. Alternatively, a water pump can be installed to keep a stable water immersion at the objective. Ensure, photo-toxicity and photo-bleaching are kept at a minimum level by optimization of acquisition settings (e.g. laser power, scanning time).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Saskia J.E. Suijkerbuijk (s.suijkerbuijk@nki.nl).

Materials availability

This study did not generate new unique reagents, plasmids, or organoid lines

Data and code availability

This study did not generate new unique datasets or code.

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AUTHOR CONTRIBUTIONS

Methodology and Investigation, A.K.G and S.J.E.S. Writing – Original Draft, A.K.G and S.J.E.S. Writing – Review & Editing, A.K.G and S.J.E.S.; Funding Acquisition, S.J.E.S and J.v.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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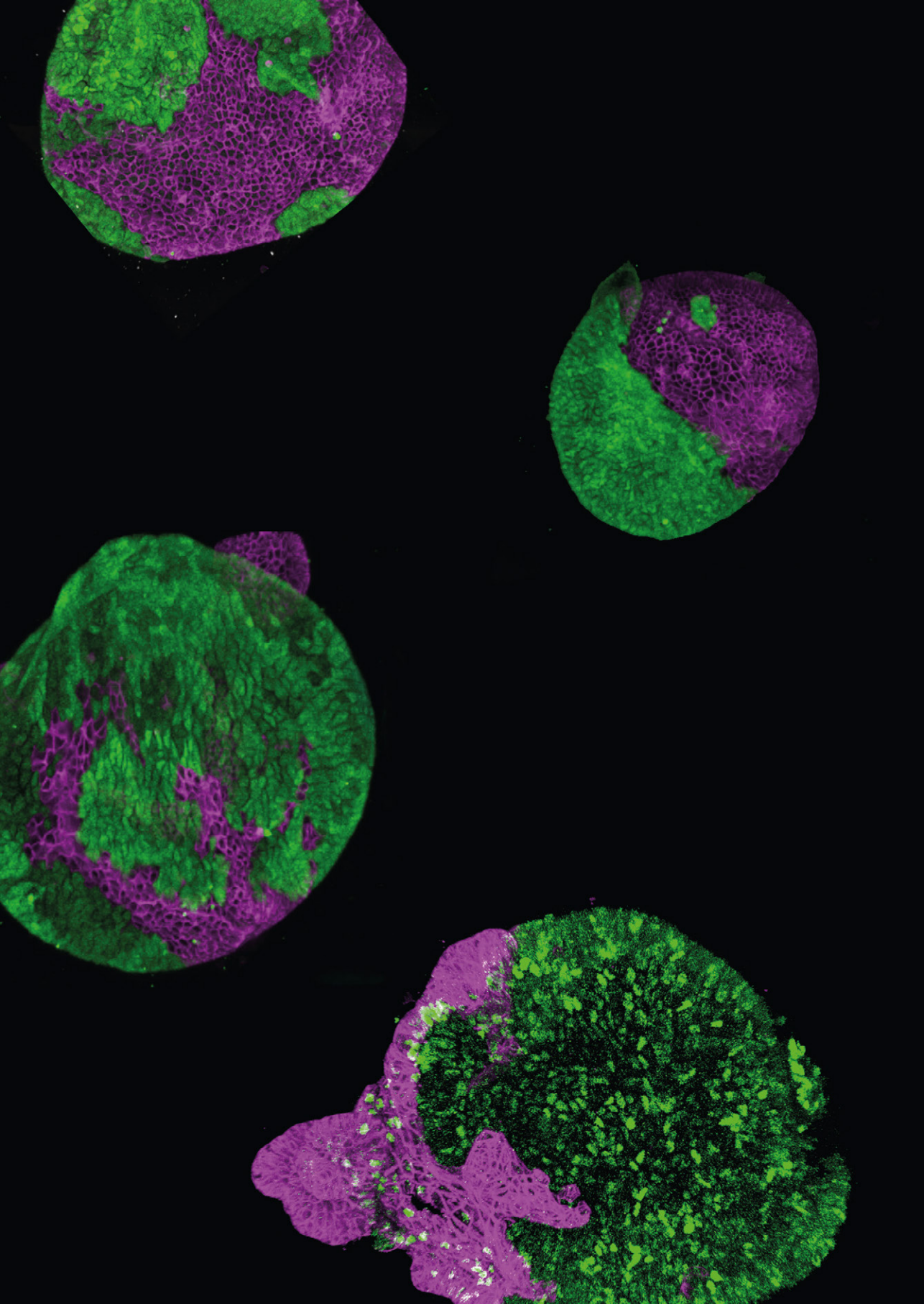
MOVIES LEGENDS

Movies are available at: https://doi.org/10.1016/j.xpro.2021.100997

Movie S1: 3D-reconstruction of confocal images of a mixed organoid. Related to Figure 8D and steps 32-42.
Cancer (green) and wild-type (magenta) cells are visualized. The organoid was stained for cJUN-pS73 (grey) and nuclei with DAPI (blue).

Movie S2: Time-lapse series of control treated 3D reconstructed mixed intestinal organoid. Related to Figure 9A and steps 43-46.
Cancer (green) and wild-type (magenta) cells are visualized.

Movie S3: Time-lapse series of a competing enteroid monolayer. Related to Figure 9B and steps 43-46.
Cancer (green) and wild-type (magenta) cells are visualized. Arrow indicates an example of a wild-type cells that shrinks and is eliminated.



3

Active elimination of intestinal cells drives oncogenic growth in organoids

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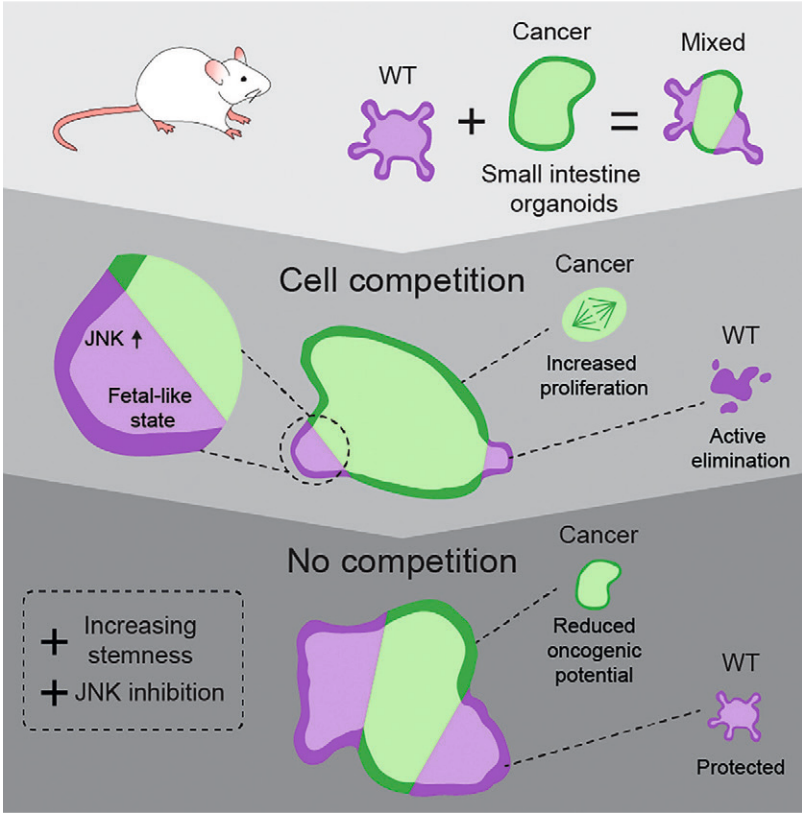
SUMMARY

Competitive cell-interactions play a crucial role in quality control during development and homeostasis. Here we show that cancer cells use such interactions to actively eliminate wild-type intestine cells in enteroid monolayers and organoids. This apoptosis-dependent process boosts proliferation of intestinal cancer cells. The remaining wild-type population activates markers of primitive epithelia and transits to a fetal-like state. Prevention of this cell state transition avoids elimination of wild-type cells and, importantly, limits the proliferation of cancer cells. JNK signaling is activated in competing cells and is required for cell state change and elimination of wild-type cells. Thus, cell competition drives growth of cancer cells by active outcompetition of wild-type cells through forced cell death and cell state change in a JNK dependent manner.

GRAPHICAL ABSTRACT



INTRODUCTION

Over the past years it became evident that the internal proliferative potential of tumor cells is not sufficient for their expansion. Instead, tumor cells need to acquire multiple hallmarks of cancer, including growth-supporting interplay of tumor cells and their environment in order to sustain their proliferation (Hanahan and Weinberg, 2011). The basis of this interaction is often formed by processes that are, in origin, essential for normal early development and homeostasis (Suijkerbuijk and van Rheenen, 2017). One of those processes, cell competition, regulates survival of cells based on their relative fitness. In a homotypic context, cells strive and form viable tissues. However, in tissues built by heterogenous populations weaker cells will be removed by surrounding stronger cells. These features provide a strong mechanism that controls overall tissue and organismal fitness (Bowling et al., 2019; Clavería and Torres, 2016). Indeed, quality control by cell competition starts in the early mouse embryo (Clavería et al., 2013; Sancho et al., 2013) and continuous to impact physiology up to late adulthood by determining the speed of aging (Merino et al., 2015).

In a tumor context, it has been shown that relative activation of YAP/TAZ in peritumoral hepatocytes can influence growth of liver tumors by a process akin to cell competition (Moya et al., 2019). Furthermore, entosis, a form of cancer-driven cell competition, is correlated with a poor prognosis in patients with pancreatic ductal adenocarcinoma (Hayashi et al., 2020). These are examples of competitive cell interactions that suggest that cell competition could influence oncogenic growth. In addition, cell competition enforced by differential expression of isoforms of the protein Flower, gives human cancer cells a competitive advantage over surrounding stromal tissue (Madan et al., 2019). We have shown that adenomas in the *Drosophila* midgut are dependent on active elimination of healthy surrounding tissue for their colonization (Suijkerbuijk et al., 2016). This illustrates that oncogenic growth can be driven by cell competition. However, the full potential and most of the mechanisms behind this process still need to be uncovered.

Here, we report that cell competition promotes growth of cancer cells in intestinal organoids. We show that cancer cells actively eradicate wild-type intestinal cells. Upon competition, JNK activation promotes a cell state transition in wild-type cells, which revert to a fetal-like state that is normally observed upon acute injury (Gregorieff *et al.*, 2015; Nusse *et al.*, 2018; Yui *et al.*, 2018). Together, these competitional processes result in an increased colonization potential of cancer cells.

RESULTS

Cancer cells outcompete wild-type small intestine cells

In order to investigate whether cell competition plays a role in mammalian intestinal cancer we exploited the 3D organoid system (Sato et al., 2009), which closely resembles the architecture of the intestinal tissue. This allowed study of the interaction between cancer cells and wild-type cells in near-native conditions. Two different types of organoid cultures were derived from mouse small intestines: membrane-bound tdTomato-labeled wild-type cells and Dendra2-labeled intestinal cancer cells derived from Villin-Cre^{ERT2}:*Apc*$^{fl/fl}$*Kras*$^{G12D/WT}$*Trp53*$^{fl/R172H}$ transgenic mice (Fumagalli *et al.*, 2017).

Wild-type and cancer cell cultures were dissociated into small clumps of cells and concentrated to enable formation of mixed organoids (Figure 1A). Using time-lapse imaging of these cultures we made two observations; 1) whereas pure wild-type cultures could expand over time (Figures 1B and 1D; Video S1), wild-type cells in mixed structures gradually disappeared (Figures 1B and 1D''; Video S3). 2) Tracking of the number of cancer cells by H2B-cerulian3 showed increased expansion in mixed compared to pure organoids (Figures 1C, 1D' and 1D''; Videos S2 and S3). In addition, we adapted the recently described enteroid monolayer culture system that recapitulates all key aspects of the intestinal epithelium (Thorne et al., 2018) to study cell competition. For this, dissociated single cells were plated separately or mixed together on matrix coated imaging plates and followed for up to 10 days (Figure S1A). Pure cultures gradually covered the surface until a stable enteroid monolayer was formed (Figures S1B-S1D). However, once a full monolayer developed in mixed culture conditions, the surface area taken up by wild-type cells was not maintained but instead gradually decreased over time (Figures S1B' and S1C). Furthermore, time-lapse imaging showed that wild-type cells reduced both in size and number when mixed with cancer cells (Figures S1E and S1E'; Video S4). Together, these data suggest that cancer cells can outcompete wild-type cells in mixed enteroid monolayer and organoid cultures.

Elimination of wild-type cells is driven by apoptosis

So far, we showed that the surface area of wild-type cells in mixed organoids declines over time, suggesting that they are eliminated by cancer cells. This was confirmed by a reduction in the percentage of wild-type cells in organoids from ±40% one day after mixing to ±10% on day four (Figures 2A and 2B). Additionally, we observed that the absolute number of wild-type cells that contributes to mixed organoids was lower four days after mixing while the number of total cells and of cancer cells increased (Figure 2C).

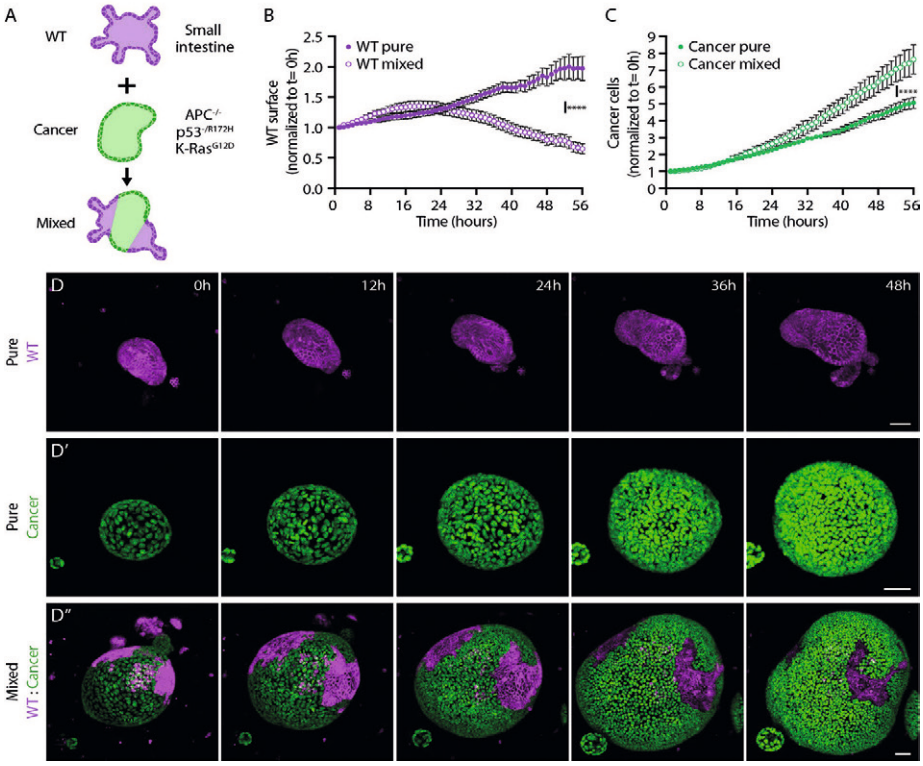


Figure 1. Cancer cells outcompete wild-type small intestine cells. A) Schematic depiction of a 3D model for cell competition in murine intestinal organoids. B-D) Analysis of wild-type and cancer organoid growth under pure and mixed conditions by live-imaging. Quantification of the wild-type cell surface (B) or number of cancer cells (C) within organoids normalized to the start of the time-lapse (Mean ±SEM, paired t-test, two-tailed; p<0.0001, n= 12 & 15 organoids (B); p<0.0001, n= 12 & 12 (C). D) Representative images of time-lapse series of pure wild-type (D), pure cancer (D') and mixed (D'') intestinal organoids, cancer cell nuclei are visualized by expression of H2B-Cerulian. Scale bars = 50μm. See also Figure S1

This indicates that, although expansion of the wild-type cell population is slower than that of the cancer cell population, a difference in proliferation rate cannot be the sole determinant of the loss of wild-type cells.

In order to characterize how wild-type cells are lost from mixed organoids we went back to time-lapse imaging. We observed two morphological changes that occurred to wild-type cells in mixed organoids compared to in pure organoids, the formation of typical crypt-villus structures was severely diminished and the extrusion of wild-type cells into the lumen of organoids increased (Figure 2D; Video S5). Interestingly, extrusion of wild-type cells was observed both at the interface with cancer cells

(Figure 2D, arrowhead) and within the wild-type cell mass (Figure 2D, asterisk). Even though elimination of wild-type cells can be induced at a distance from cancer cells, multiple lines of evidence suggest that cell competition is short-ranged; 1) wild-type and cancer cells are part of the same epithelium and these competing cell populations directly interact (Figures S2A). 2) Occasional organoids of pure wild-type origin persist nearby mixed organoids (Figure S2B), suggesting that elimination of wild-type cells depends on intra-organoid interactions with cancer cells. 3) Growth and behavior of pure wild-type organoids plated in the same well with pure cancer or mixed organoids was undistinguishable of solely wild-type cultures (Figures S2C and S2D), indicating that the presence of both cell populations within the same organoid is required for outcompetition of wild-type cells. It is important to note that this also shows that neither factors secreted by cancer cells nor depletion of components in the growth medium are sufficient to induce elimination of wild-type cells.

We next asked if programmed cell death is required for cancer-driven cell competition. We observed activation of Caspase 3, a marker of apoptosis, in extruding wild-type cells (Figure 2E). This activation occurred both in at the interface with cancer cells and further away and led to an overall increase in cell death in mixed wild-type cells (Figures 2E and 2F). Similarly, we observed increased rates of apoptotic wild-type cells in enteroid monolayers, while cancer cells are unaffected (Figures S1F-S1H). Surprisingly, treatment with the pan-Caspase inhibitor Z-VAD-FMK could not prevent elimination of wild-type cells (Figures S1I and S1J), suggesting that a redundant process can cause outcompetition of wild-type cells in these monolayers when apoptosis is inhibited. Potentially this can be mediated by live cell extrusion, a mechanism involved in maintenance of cell density under homeostatic conditions (Eisenhoffer *et al.*, 2012), but future work is required to determine involvement of this process. Importantly, time-lapse imaging of 3D cultures treated with Z-VAD-FMK showed increased maintenance of wild-type cells in mixed cultures (Figure 2G: Videos S6 and S7). Furthermore, quantification of the number of wild-type cells in mixed organoids showed that wild-type cells are not eliminated when apoptosis is inhibited (Figures 2H and 2I). It is interesting to note that, the average percentage of wild-type cells in Z-VAD-FMK treated mixed organoids is still lower (48.56%) than the expected percentage (66.67%) at the start of the experiment (based on the 2:1 starting ratio). This indicates that although wild-type cells are not eliminated under these conditions, they do not gain the potential to outcompete cancer cells. Together these data show that outcompetition of wild-type cells is active and dependent on programmed cell death in 3D cultures.

Cancer cells boost their growth by cell competition

Previously we observed increased expansion of competing cancer cells (Figure 1C) and therefore questioned whether cancer cells could be influenced by the presence of wild-type cells. First, we evaluated basal proliferation rates. By using markers that identify DNA replication (1-hour EdU pulse) and active cell division (pH3), we could distinguish three populations of cells; cells in S-phase (EdU^+), cells that proceeded from S-phase to mitosis ($EdU^+/pH3^+$) and mitotic cells ($pH3^+$). Whereas proliferation in pure wild-type organoids was restricted to crypt regions (Figure 3A), no obvious spatial organization of proliferating cells was observed in pure cancer organoids (Figure 3A'). Importantly, competing cancer cells showed increased proliferation throughout the cell cycle (Figures 3A-3D). This implies that intestinal cancer cells boost their own proliferation and benefit from competitive cell interactions with wild-type intestinal cells.

Cell competition induces a fetal-like state in WT cells

In order to characterize molecular mechanisms underlying cell competition driven by cancer cells we used bulk RNA sequencing to identify genes that are differentially expressed between pure and mixed organoids (Figures S3A-S3C). The transcriptome of competing cancer cells was very similar to that of pure cancer cells (Figure S3D), indicating that phenotypic changes in cancer cells induced by cell competition are not of a transcriptional nature. In contrast, the transcriptome of wild-type cells was dramatically changed upon cell competition (Figure S3D). Subsequent Gene Ontology analysis displayed enrichment of multiple cell death related pathways in competing wild-type cultures (Figure 4A) whereas processing of mRNA and cell proliferation were enriched in pure wild-type cultures (Figure 4B). These data confirm a negative impact of cell competition on wild-type cells. Interestingly, among the most highly upregulated genes were multiple members of the *Ly6* family (Figure 4C). The *Ly6* family genes are induced in intestinal epithelia after exposure to colitis (Flanagan et al., 2008) and one of its members, Stem Cell Antigen-1 (*Sca1/Ly6a*), has recently been shown to be a marker of regenerating colonic epithelia (Yui et al., 2018). Furthermore, *Sca1* expression is activated in small intestinal epithelia that have been challenged with parasitic helminths (Nusse et al., 2018). Importantly, both injury responses cause a reprogramming of the tissue and adoption of a undifferentiated fetal-like state (Nusse et al., 2018; Yui et al., 2018). This response, which is essential for maintenance of the epithelial barrier in the intestine, is also characterized by increased expression of genes of the Annexin family (Yui et al., 2018), which were also abundantly present amongst the highly upregulated genes in competing wild-type cells (Figure 4C). This prompted us to further investigate the exact transcriptional response induced in competing wild-type small intestinal cells and we observed enrichment of the previously reported fetal like and repair signatures (Figures 4D and S3E-S3G).

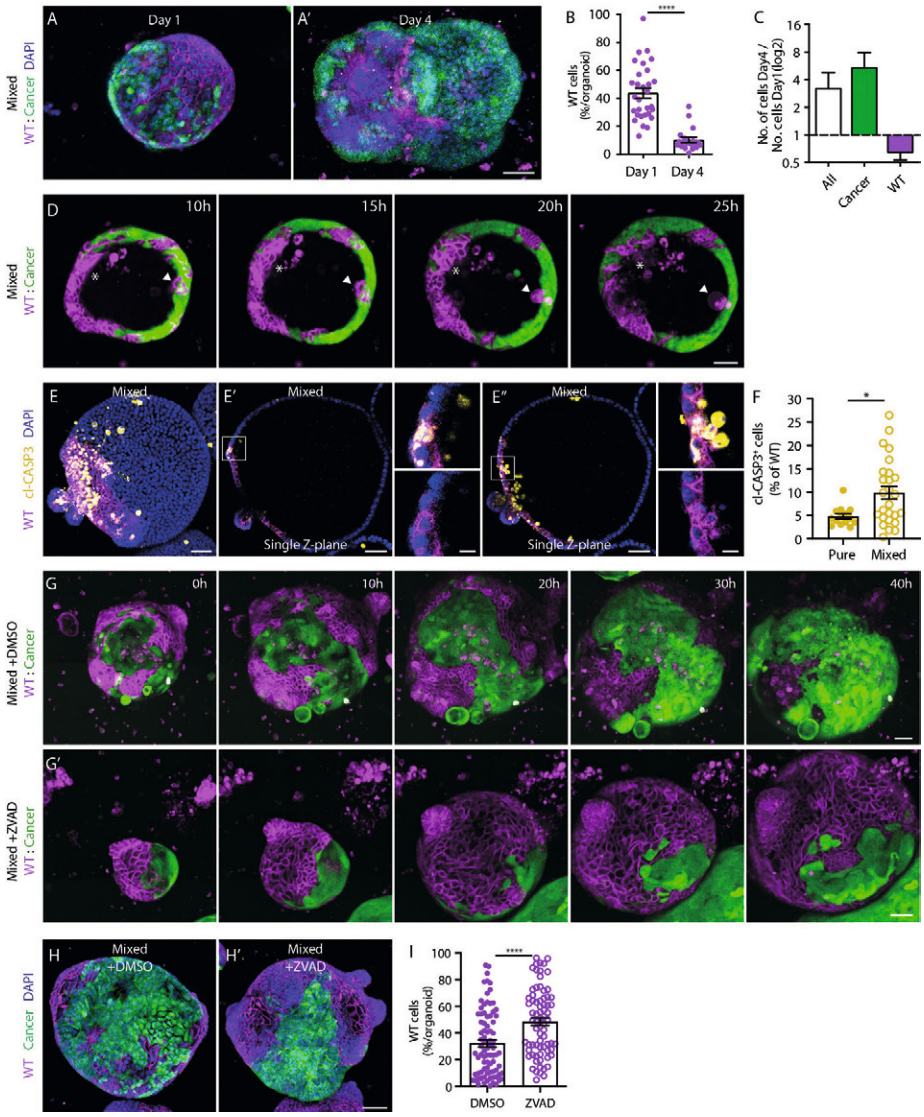

A
Day 1
A'
Day 4
Mixed
WT : Cancer DAPI
B
WT cells (%/organoid)
Day 1
Day 4
****
C
No. of cells Day4 / No. cells Day1(log2)
All
Cancer
WT
D
10h
15h
20h
25h
Mixed
WT : Cancer
E
E'
E''
Mixed
WT cl-CASP3 DAPI
Single Z-plane
F
cl-CASP3+ cells (% of WT)
Pure
Mixed
*
G
G'
0h
10h
20h
30h
40h
Mixed +DMSO
WT : Cancer
Mixed +ZVAD
WT : Cancer
H
H'
Mixed +DMSO
Mixed +ZVAD
WT Cancer DAPI
I
WT cells (%/organoid)
DMSO
ZVAD
****

Figure 2. Elimination of wild-type cells is driven by apoptosis A-C) Representative 3D-reconstructed confocal images of mixed organoids 1 day (A) and 4 days (A') after plating, nuclei are stained with DAPI (blue). B) The percentage of wild-type cells contributing to mixed organoids on Day 1 and Day 4 after plating is shown, each dot represents one organoid (Mean ±SEM, Mann-Whitney, two-tailed, p<0.0001, n= 30 &19 organoids). C) Displays the absolute number of cells in organoids shown in 'B', the ratio of 'day 4' over 'day 1' of all (white), cancer (green) and wild-type (magenta) cells are plotted on a Log2 scale (Mean ±SEM). D) Representative images of time-lapse series of mixed intestinal organoid, maximum projection six Z-stacks, extruding wild-type cells are indicated with an arrow head (at interface with cancer cell surface) and asterisk (within the wild-type cell population). E-F) Representative 3D-reconstructed I and single Z-plane (E' and E'') confocal images of a mixed organoid and quantification of the cl-CASP3+ cells relative to the total wild-type cell population (F). The organoids were stained for cl-CASP3 (yellow), nuclei are visualized with DAPI (blue). The insets display a 3.5x magnification of the area in the white box. Each dot in (F) represents one organoid (Mean ±SEM, unpaired t-test, two-tailed, p=0.0120, n=14 & 26 organoids). G) Representative 3D-reconstructed confocal images of time-lapse series of control (G) and apoptosis inhibited (G') mixed intestinal organoids. H-I) Representative 3D-reconstructed confocal image of control (H) and apoptosis inhibited (H') mixed organoids, nuclei are stained with DAPI (blue), and quantification of the percentage of wild-type cells contributing to mixed organoids (I), each dot represents one organoid (Mean ±SEM, unpaired t-test, two-tailed, p<0.0001, n=82 & 73 organoids). Scale bars = 50μm, excluding magnifications in I where scale bar = 10μm. See also Figures S1, S2, S4 and S5.

This indicates that cancer cells actively damage the surrounding wild-type epithelium, which activates a response resembling an epithelium that is recovering from DSS-induced colitis. Next, we validated the observed activation of the fetal-like response by immune-fluorescence staining of SCA1. A heterogenous expression of SCA1 was detected in cancer cells, which was unaltered in mixed compared to pure cancer (Figures 4E and 4F), thereby reflecting the results of the transcriptional analysis. On the other hand, wild-type cells showed a homogenous low expression of SCA1 in unchallenged conditions, which was dramatically increased in competing cells (Figures 4E and 4G). Thus, together these data show that wild-type small intestine cells revert to a fetal-like state when challenged by competing cancer cells.

Multiple types of intestinal cancer compete with WT cells

Over the past years it became apparent that colorectal cancer can be stratified in multiple subtypes based on transcriptional profiling (Guinney *et al.*, 2015). Each of these types display different molecular characteristics, which coincide with a different clinical progression of disease. So far, the here described cell competition models were based on classical adenocarcinoma cells, resembling colorectal subtype 2. We next wondered whether other colorectal cancer subtypes can drive cell competition in intestinal organoids. Recently, epithelial NOTCH1 signaling has been shown to induce tumor microenvironment similar to human CRC subtype 4 (Jackstadt *et al.*, 2019) and this is associated with an overall poor prognosis for patients. Organoids derived from these highly-metastatic small intestinal tumors, induced by activation of Kras, together with deletion of p53, and overexpression of NOTCH1 intracellular domain (KPN), were mixed with wild-type small intestine cells.

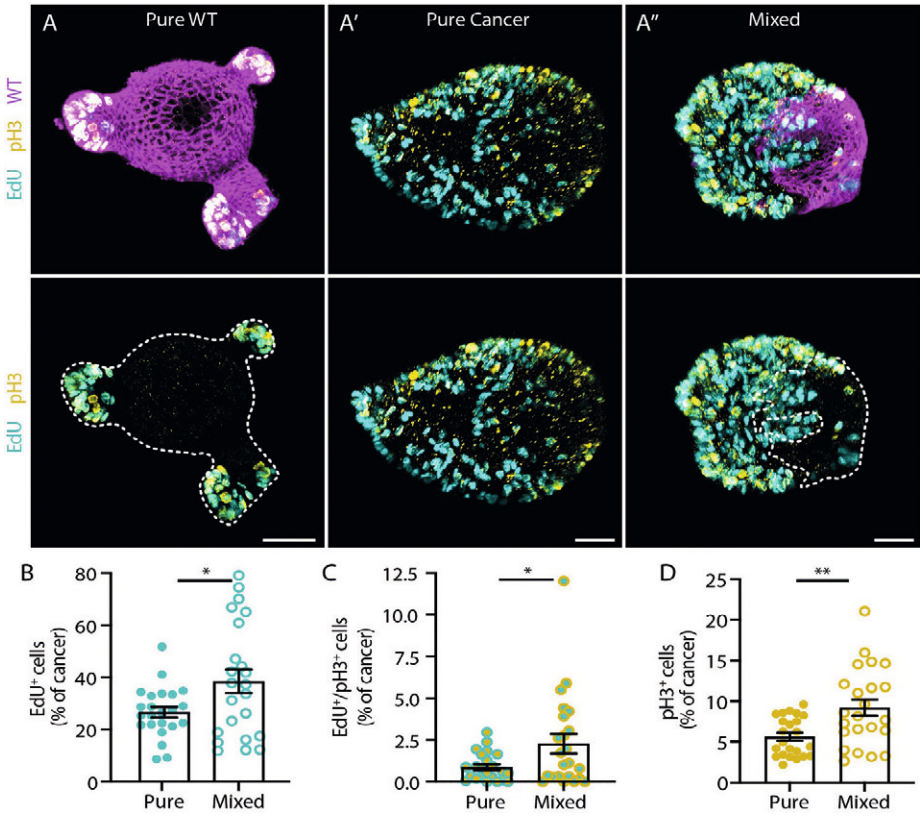


Figure 3. Cancer cells boost their growth by cell competition A) Representative 3D-reconstructed confocal images of pure wild-type (A), pure cancer (A′) and mixed (A″) organoids. Cells in S-phase are labelled with EdU (cyan) and mitotic cells are marked by pH3 (yellow), cells that progressed from S-phase to mitosis within one hour are double positive. B-D) Quantification of cancer cell proliferation in pure and mixed organoids. The number of cells in S-phase (B), cells that progressed from S-phase to mitosis within one hour (C) and cells in mitosis (D) relative to the total cancer cell population is plotted. Each dot represents one organoid (Mean ±SEM, unpaired t-test, two-tailed, p=0.0225 (B), p=0.0263 (C) and p=0.0027 (D), n= 23 organoids for each condition). Scale bars = 50µm

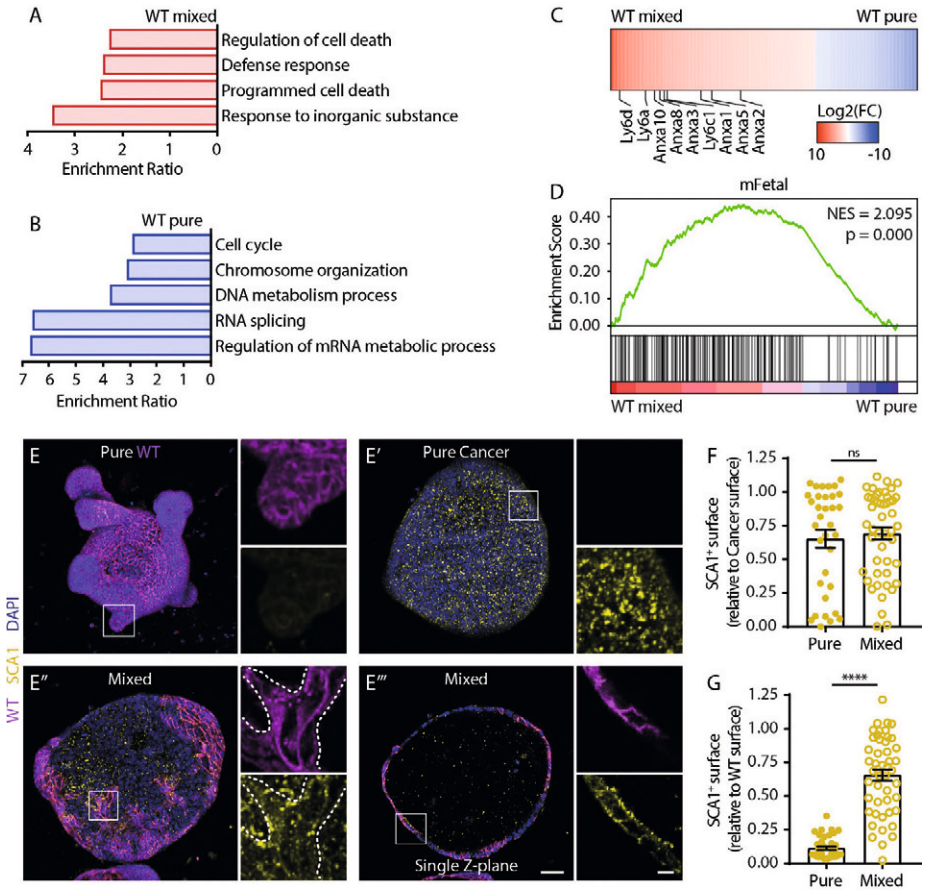


Figure 4 . Cell competition induces a fetal-like state in WT cells A, B) Gene Ontology analysis of differentially expressed genes ($p<0.05$) in wild-type populations that are enriched in mixed (A) and pure (B) cells. C) Heatmap of the fold change of genes that are differentially expressed in wild-type cells upon mixing (Log2). Genes of the ***Ly6*** and ***Anxa*** families are indicated. D) Gene Set Enrichment Analysis showing enrichment of a fetal signature (Yui ***et al.***, 2018) in mixed wild-type cells E-G) Representative 3D-reconstructed confocal images of pure WT I, pure cancer (E'), mixed (E'') organoids, and a single Z-plane of E' (E''') and quantification of the SCA1+ surface relative to the total cancer (F) or wild-type (G) surface area. The organoids were stained for SCA1 (yellow), nuclei are visualized with DAPI (blue). The insets display a 3.5x magnification of the area in the white box. Each dot in (F) and (G) represents one organoid (Mean ±SEM, Non-parametric, ANOVA, multiple comparisons: $p> 0.9999$, n=34 & 48 organoids (F); $p<0.0001$, n=39 & 48 organoids (G)). Scale bars = 50µm, excluding magnifications in I where scale bar = 10µm. See also Figure S3 and S4.

We found that KPN cancer cells can eliminate wild-type small intestine cells in an apoptosis dependent manner (Figures S4A and S4B). In addition, cancer organoids derived from less invasive *Apc*$^{fl/+}$*Trp53*$^{fl/fl}$ Rosa26$^{N1icd/+}$ (APN) small intestine tumors outcompeted wild-type cells in mixed organoids (Figures S4C and S4D). Importantly, both types of intestinal cancer organoids could, like classical adenocarcinoma cells, induce activation of SCA1 in wild-type cells (Figures S4E-S4J). Thus, together these data show that multiple types of intestinal cancer cells can induce a fetal-like state and eliminate wild-type cells.

The cell competition-induced fetal-like state induces a loss of LGR5^{+} stem cells

The induction of a fetal-like state in adult intestinal epithelia has been reported to coincide with loss of intestinal stem cell (ISC) markers and removal of their niche (Nusse et al., 2018). We therefore next questioned how intestinal stem cells are affected by cancer-driven cell competition. ISCs are marked by leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) (Barker et al., 2007). We next derived organoids from Lgr5^{DTR} transgenic mice (Tian et al., 2011), in which the first coding exon of *Lgr5* was replaced with enhanced green fluorescent protein (eGFP) and human diphtheria toxin receptor (DTR). With the use of these Lgr5-DTR-eGFP organoids we could detect ISCs localized in crypt regions of pure wild-type organoids (Figure 5A). Upon challenging these cells with competing cancer cells, we observed a marked decrease in the number of LGR5 positive cells (Figures 5A' and 5B). We observed direct interaction of LGR5^{+} stem cells with cancer cells (Figure 5A') and occasional extrusion of LGR5^{+} cells at this interface (Figure 5C). This suggests that the reduced population of LGR5^{+} cells is, at least in part, caused by elimination of these cells. However, since most of the wild-type population reverts to a fetal-like state (Figure 4G), the majority of eliminated wild-type cells are instead SCA1 positive (Figure S5A). Thus, cancer-driven cell competition induces a cell state transition in the surrounding wild-type epithelium that is characterized by loss of LGR5^{+} stem cells and adoption of a fetal-like state.

Increased stemness prevents cell competition

So far, we have shown that wild-type intestine cells undergo a cell state transition when exposed to cancer cells. We next wondered whether reversal of this process could disrupt cell competition. Therefore, we sought a way to interfere with the cell state of wild-type organoids and turned to the previously described treatment with CHIR99021 and valproic acid (CV) (Yin et al., 2014). This combined inhibition of glycogen synthase kinase 3β (GSK3β) and histone deacetylases (HDAC), reported to increase self-renewal of ISCs, indeed resulted in enrichment of LGR5-positive cells in cultures after three days of treatment (Figures S5B and S5C). This coincided with loss

of expression of SCA1 in mixed wild-type cells (Figures 5D and 5E), thus CV treatment can prevent the cell state change induced by cell competition. Furthermore, increased stemness prevented loss of wild-type cells from mixed organoids (Figures 5F and 5G). Interestingly, the increased competitive potential of wild-type cells after CV treatment prevented the over proliferation of cancer cells (Figures 5H and 5I). Since the mitotic index of pure cancer cultures was not decreased by CV treatment (Figure S5D), this was not a consequence of an autonomous effect of CV treatment on cancer cells. Thus, combined these data suggest that increasing stemness of wild-type cells increases their competitive potential and prevents elimination.

JNK signaling drives cell competition

Next, we questioned which signaling pathways could control elimination and cell state change of wild-type cells. Therefore, we performed a transcription factor target analysis on genes that were differentially expressed in bulk mRNA sequencing. This showed that AP-1 target sites were significantly enriched in genes that were higher expressed in competing wild-type cells (Figure 6A). AP-1 transcription factors are heterodimeric proteins that are activated upon exposure to numerous stressors, such as cytokines and hypoxia (Karin and Gallagher, 2005). To further characterize activation of AP-1 we analyzed phosphorylation of cJUN, an AP-1 family member (Minden et al., 1994). We observed increased nuclear signal of cJUN-pS73 in competing wild-type cells (Figures 6B and 6C), while activation was unchanged in cancer cells (Figures 6B and 6D). Interestingly, the distance of wild-type cJUN-pS73^{+} nuclei to cancer cells was smaller than the median distance of all wild-type nuclei (Figure 6E). Furthermore, there is an enrichment of wild-type cJUN-pS73^{+} nuclei at the interface with cancer cells (Figure 6F). Together, this suggests that short-ranged interactions with cancer cells promote activation of cJUN in wild-type cells. This phosphorylation site is a substrate of the Jun N-terminal kinase (JNK), which is activated in competing wild-type cells (Figure S6). JNK signaling plays a critical role in controlling both cell proliferation and death (Karin and Gallagher, 2005; Minden et al., 1994) and is a key regulator in many forms of cell competition (Tamori and Deng, 2011), including tumor-induced cell competition in the *Drosophila* adult intestine (Suijkerbuijk et al., 2016). We used treatment with the selective JNK inhibitor JNK-IN-8 (Zhang et al., 2012) to interfere with overall JNK signaling, which indeed prevented phosphorylation of cJUN in wild-type and cancer cells (Figures 6G-6I). Furthermore, treatment with JNK-in-8 significantly reduced SCA1 levels in wild-type cells (Figures 6J and 6K). Suggesting that active JNK signaling is required for the cell state transition that is enforced by cell competition. Importantly, we found that inhibition of JNK prevents elimination of wild-type cells (Figures 6L and 6M). Thus, JNK is activated in competing cells and is required for eradication and cell state transition of wild-type small intestine cells.

JNK activity in wild-type cells is required for cell competition

Next, in order to untangle where JNK activation is required, we sought a manner to specifically inhibit signaling in individual cell populations. Mitogen-activated protein kinase phosphatase 5 (MKP5, also known as DUSP10) is a member of the dual-specificity phosphatase family that inactivates JNK and p38 *in vitro* (Theodosiou *et al.*, no date; Tanoue, Moriguchi and Nishida, 1999) and regulates JNK activity in mouse cells (Zhang *et al.*, 2004). We observed that doxycycline inducible expression of MKP5, detected by co-expression of mTurquoise2 (Figure S7A), results in reduced activation of cJUN in wild-type and cancer cells (Figures 7A-7C). This confirms that MKP5 inactivates JNK signaling in mouse intestinal organoids. Expression of MKP5 solely in cancer cells did not affect SCA1 activation (Figures S7B and S7C) or prevent elimination of wild-type cells (Figures S7D and S7E). However, specific expression of the phosphatase in only wild-type cells inhibited SCA1 expression (Figures 7D and 7E) and, importantly, rescued wild-type cells (Figures 7F and 7G). Together, this shows that induction of a cell state transition and outcompetition of wild-type cells is dependent on JNK activity in wild-type cells.

DISCUSSION

Effects of competition on cell fate have been shown in many tissues. Classical examples are neutral drift in the intestinal stem cells niche (Lopez-Garcia et al., 2010; Snippert et al., 2010) and maintenance of stem cells in the *Drosophila* testis (Sheg et al., 2009). A dual effect of cell competition on weaker cell populations, through active elimination by cell death combined with reduced stem cell renewal, has been observed under homeostasis. Both in the adult *Drosophila* midgut and in developing mouse skin, weaker cells, induced by ribosome impairment or reduced expression of *mycn*, are removed from the tissue by stronger cells through apoptosis and forced differentiation (Ellis et al., 2019; Kolahgar et al., 2015). Here, we provide the first example of a combined mechanism of forced elimination and cell state transition in relation to cancer (Figure 7H).

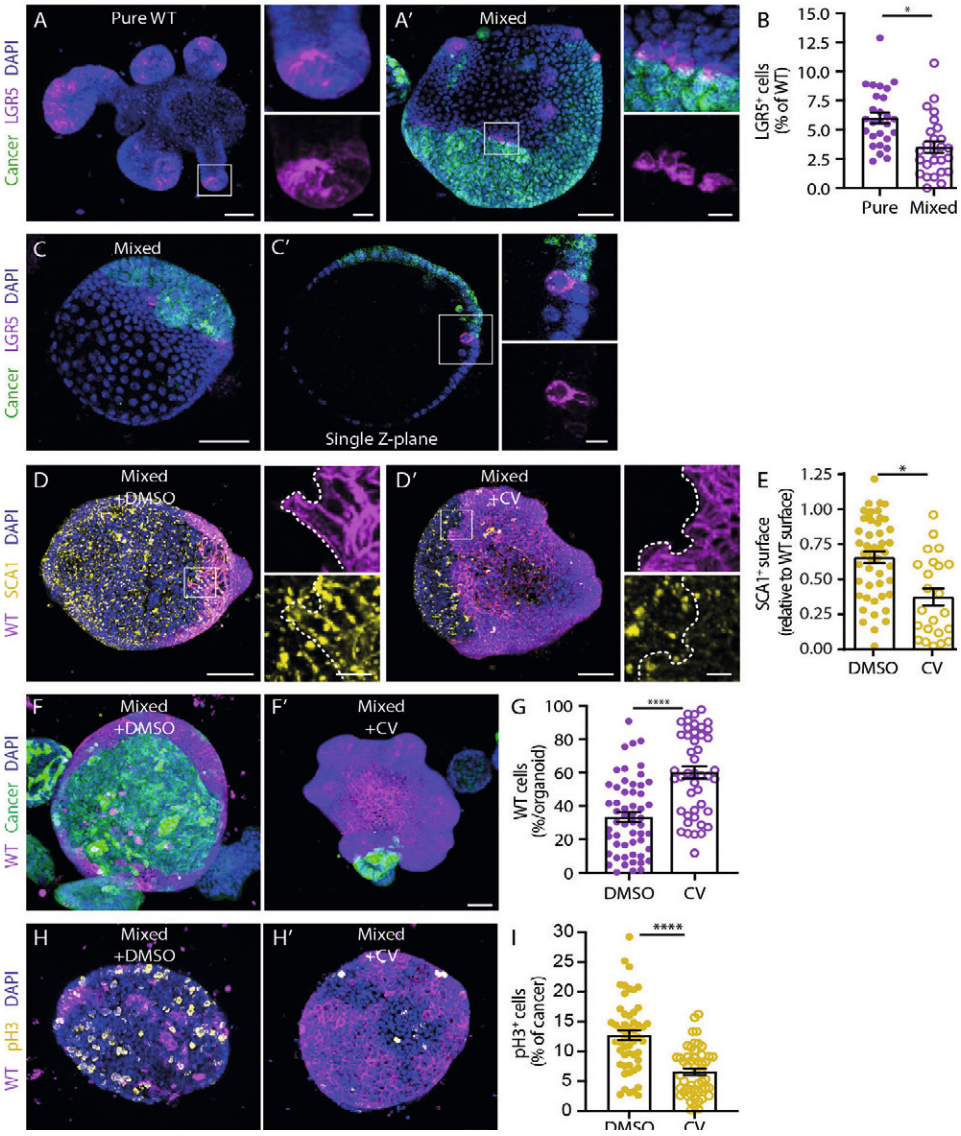
Many studies have reported that tumor growth is highly context dependent. In particular, a strong correlation exists between inflammation and intestinal cancer. For example, patients with Crohn's disease have 20-30 times higher risk of developing adenocarcinomas in the small intestine and inflammatory bowel disease is a strong risk factor for colorectal cancer (Beaugerie and Itzkowitz, 2015). Similarly, in mouse models of intestinal cancer, formation of colonic polyps is strongly enhanced by inflammation induced by infection with enterotoxigenic *Bacteroides fragilis* or treatment with dextran

sodium sulfate (Tanaka et al., 2006; Wu et al., 2009). Interestingly, these are conditions in which a fetal-like response is activated in the intestine (Gregorieff et al., 2015; Nusse et al., 2018; Yui et al., 2018), similar to the here reported primitive state induced upon cancer-driven cell competition. Future efforts should be directed towards increasing understanding of this connection of a fetal-like state and tumorigenesis.

Under normal circumstances, the fetal-like response promotes regeneration of the intestinal tissue. A recent study describing the kinetics of regeneration after removal of a damaging insult has shown that reformation of a homeostatic intestinal epithelium takes approximately three weeks (Wang et al., 2019). Therefore, during chronic exposure of the epithelium to an insult, such as close proximity of a tumor, healthy tissue will never be allowed to fully recover. This response is therefore counterproductive under the circumstances described here.

Interestingly, induction of a fetal-like state upon injury is not restricted to the intestinal epithelium and is also observed in multiple other tissues (Fernandez Vallone et al., 2016; Gadye et al., 2017; Lin et al., 2017). This suggests that our observation that tumors can push surrounding wild-type tissue in a primitive state could be more universal.

Cell competition can be tumor-suppressive, for example, cells expressing oncogenic H-Ras are eliminated from intestinal and pancreatic epithelia through apical extrusion (Kon et al., 2017; Sasaki et al., 2018). Furthermore, wild-type cells actively eliminate mutant aberrant foci in the skin (Brown et al., 2017). However, this effect is not solely determined by autonomous properties of the tumor but is highly context dependent. For instance, obesity induced by a high fat diet prevents cell competition-driven elimination of oncogenic cells (Sasaki et al., 2018). This illustrates how the surrounding environment dictates behavior of tumors and that tumor fitness, and thus its oncogenic potential, can be changed by external stimuli. Here, we report that JNK signaling is a major regulator of wild-type cell elimination and thus overall fitness of competing healthy cells. This opens up new options of treatment, where promoting fitness of the host tissue, through JNK inhibition, can help to tip the balance towards tumor-suppressive cell competition.


A
Pure WT
A'
Mixed
Cancer LGR5 DAPI
B
LGR5+ cells (% of WT)
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Pure
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C
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C'
Single Z-plane
Cancer LGR5 DAPI
D
Mixed +DMSO
D'
Mixed +CV
WT SCA1 DAPI
E
SCA1+ surface (relative to WT surface)
1.25
1.00
0.75
0.50
0.25
0.00
*
DMSO
CV
F
Mixed +DMSO
F'
Mixed +CV
WT Cancer DAPI
G
WT cells (%/organoid)
100
80
60
40
20
0
****
DMSO
CV
H
Mixed +DMSO
H'
Mixed +CV
WT pH3 DAPI
I
pH3+ cells (% of cancer)
30
25
20
15
10
5
0
****
DMSO
CV

Figure 5. Increased stemness prevents cell competition A-C) Representative 3D-reconstructed confocal images of pure WT (A) and mixed (A' and C) organoids and single Z-plane of C (C'), LGR5^{+} Intestinal stem cells (magenta) and nuclei (blue) are visualized. The insets display a 2x magnification of the area in the white box. B) Graph displays a quantification of the number of LGR5^{+} cells relative to the total number of wild-type cells, each dot represents one organoid (Mean ±SEM, one-way ANOVA, p=0.0219, n=27 & 26 organoids). D-E) Representative 3D-reconstructed confocal images of control (D) and CV (D') treated mixed organoids and quantification of the SCA1+ surface relative to the total wild-type surface area. The organoids were stained for SCA1 (yellow), nuclei are visualized with DAPI (blue). The insets display a 3.5x magnification of the area in the white box. Each dot in I represents one organoid (Mean ±SEM, Non-parametric, ANOVA, multiple comparisons: p= 0.0147, n=48 & 23 organoids). Displayed control organoids are from the same dataset used in panel 4G. F-G) Representative 3D-reconstructed confocal image of control (F) and CV treated (F') mixed organoids, nuclei are stained with DAPI (blue), and quantification of the percentage of wild-type cells contributing to mixed organoids (G), each dot represents one organoid (Mean ±SEM, unpaired t-test, two-tailed, p<0.0001, n=55 & 45 organoids). H-I) Representative 3D-reconstructed confocal images of control (H) and CV treated (H') mixed organoids. Mitotic cells are marked by pH3 (yellow) and nuclei with DAPI (blue). (I) Quantification of the number of pH3^{+} cells in mixed organoids relative to the total number of cancer cells, each dot represents one organoid (Mean ±SEM, one-way ANOVA, p<0.0001, n=53 & 50 organoids). Scale bars = 50μm, excluding magnifications in (A, C and D) where scale bar = 10μm. See also Figures S5

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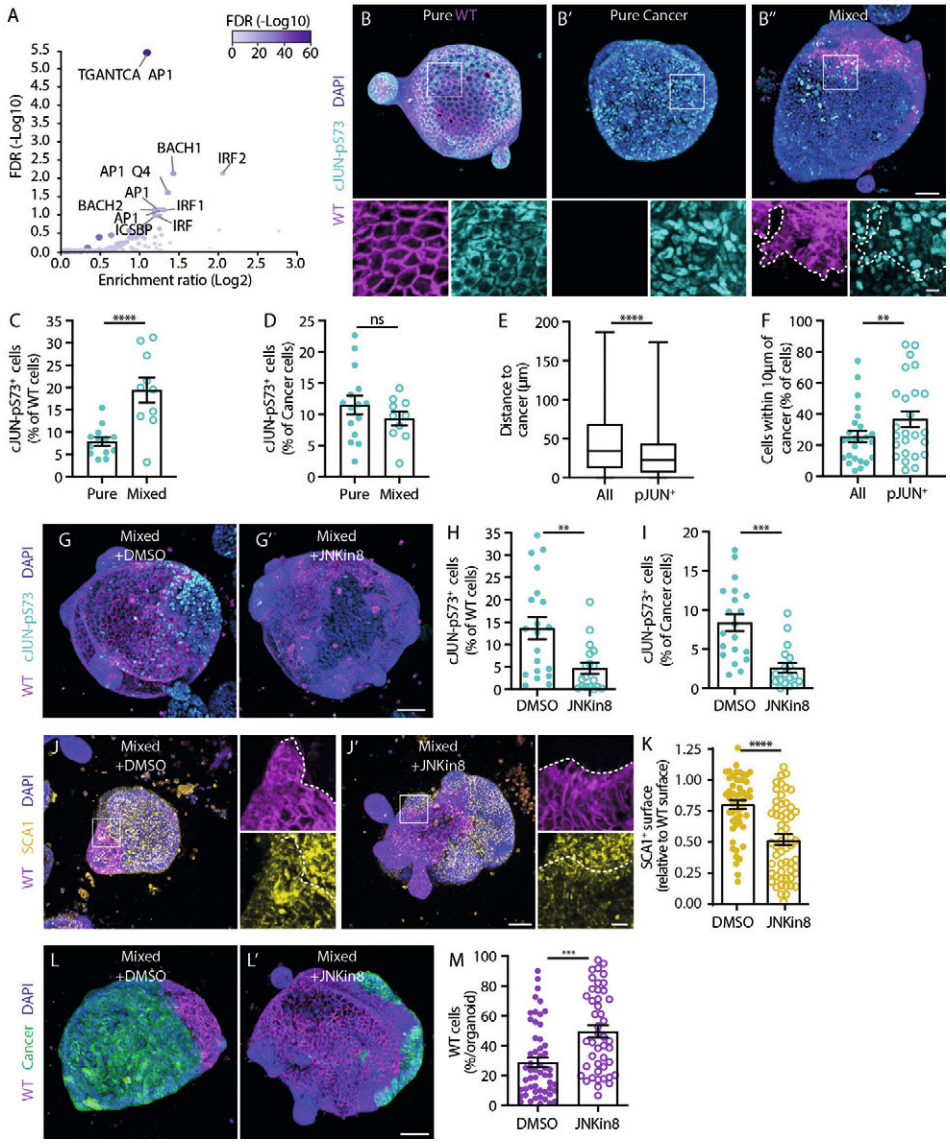
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TGANTCA AP1
BACH1
IRF2
AP1 Q4
AP1
BACH2
IRF1
AP1
IRF
ICSBP
FDR (-Log10)
Enrichment ratio (Log2)
B Pure WT
B′ Pure Cancer
B″ Mixed
DAPI
cJUN-pS73
WT
C
cJUN-pS73+ cells (% of WT cells)
****
Pure Mixed
D
cJUN-pS73+ cells (% of Cancer cells)
ns
Pure Mixed
E
Distance to cancer (µm)
****
All pJUN+
F
Cells within 10µm of cancer (% of cells)
**
All pJUN+
G Mixed +DMSO
G′ Mixed +JNKin8
H
cJUN-pS73+ cells (% of WT cells)
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DMSO JNKin8
I
cJUN-pS73+ cells (% of Cancer cells)
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DMSO JNKin8
J Mixed +DMSO
J′ Mixed +JNKin8
SCA1
K
SCA1+ surface (relative to WT surface)
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DMSO JNKin8
L Mixed +DMSO
L′ Mixed +JNKin8
Cancer
M
WT cells (%/organoid)
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DMSO JNKin8

Figure 6 – JNK signaling drives cell competition A) Transcription factor target analysis of differentially expressed genes (p<0.05) that are enriched in mixed wild-type cells. The graph displays enrichment (Log2) and FDR (-Log10) of gene sets, significantly enriched gene sets are indicated). B) Representative 3D-reconstructed confocal images of pure WT (B), pure cancer (B'), mixed (B'') organoids, stained for activated cJUN (cyan), nuclei are visualized with DAPI (blue). The insets display a 3x magnification of the area in the white box. C-D) Quantification of the number of cJUN-pS73^{+} cells relative to the total number of wild-type (C) and cancer (D) cells, each dot represents one organoid (Mean ±SEM, one-way ANOVA, multiple comparisons, p<0.0001, n=13 & 10 organoids (C) and p= 0.5740, n= 15 & 10 organoids (D)). E) Quantification of the distance of all (left) and cJUN-pS73+ (right) wild-type nuclei to the closest cancer cell surface in µm (median, 25th to 75th percentiles (box), min. to max. (whiskers), unpaired t-test, two-tailed, p<0.0001, n= 25 organoids). F) Quantification of wild-type cells within a one cell diameter (10 µm) of the closest cancer cell surface. The percentage of all (left) and cJUN-pS73^{+} (right) wild-type cells at the interface are displayed (Mean ±SEM, paired t-test, two-tailed, p=0.0093, n=25 organoids). G-I) Representative 3D-reconstructed confocal images of control (G) and JNKin8 treated (G') mixed organoids stained for activated cJUN (cyan), nuclei are visualized with DAPI (blue). H-I) Quantification of the number of cJUN-pS73^{+} cells relative to the total number of wild-type (H) and cancer (I) cells, each dot represents one organoid (Mean ±SEM, one-way ANOVA, multiple comparisons, p=0.0041, n=20 & 18 organoids (H) and p= 0.0001, n= 20 & 18 organoids (I)).J-K) Representative 3D-reconstructed confocal images of control (J) and JNKin8 treated (J') mixed organoids and quantification of the SCA1+ surface relative to the total wild-type surface area. The organoids were stained for SCA1 (yellow), nuclei are visualized with DAPI (blue). The insets display a 3.5x magnification of the area in the white box. Each dot in (K) represents one organoid (Mean ±SEM, Non-parametric, ANOVA, multiple comparisons: p< 0.0001, n=52 & 56 organoids). L-M) Representative 3D-reconstructed confocal image of control (L) and JNKin8 treated (L') mixed organoids, nuclei are stained with DAPI (blue), and quantification of the percentage of wild-type cells contributing to mixed organoids (M), each dot represents one organoid (Mean ±SEM, unpaired t-test, two-tailed, p<0.0001, n=55 & 45 organoids). Scale bars = 50µm, excluding magnifications in (B & J) where scale bar = 10µm. See also Figure S6

AUTHOR CONTRIBUTIONS

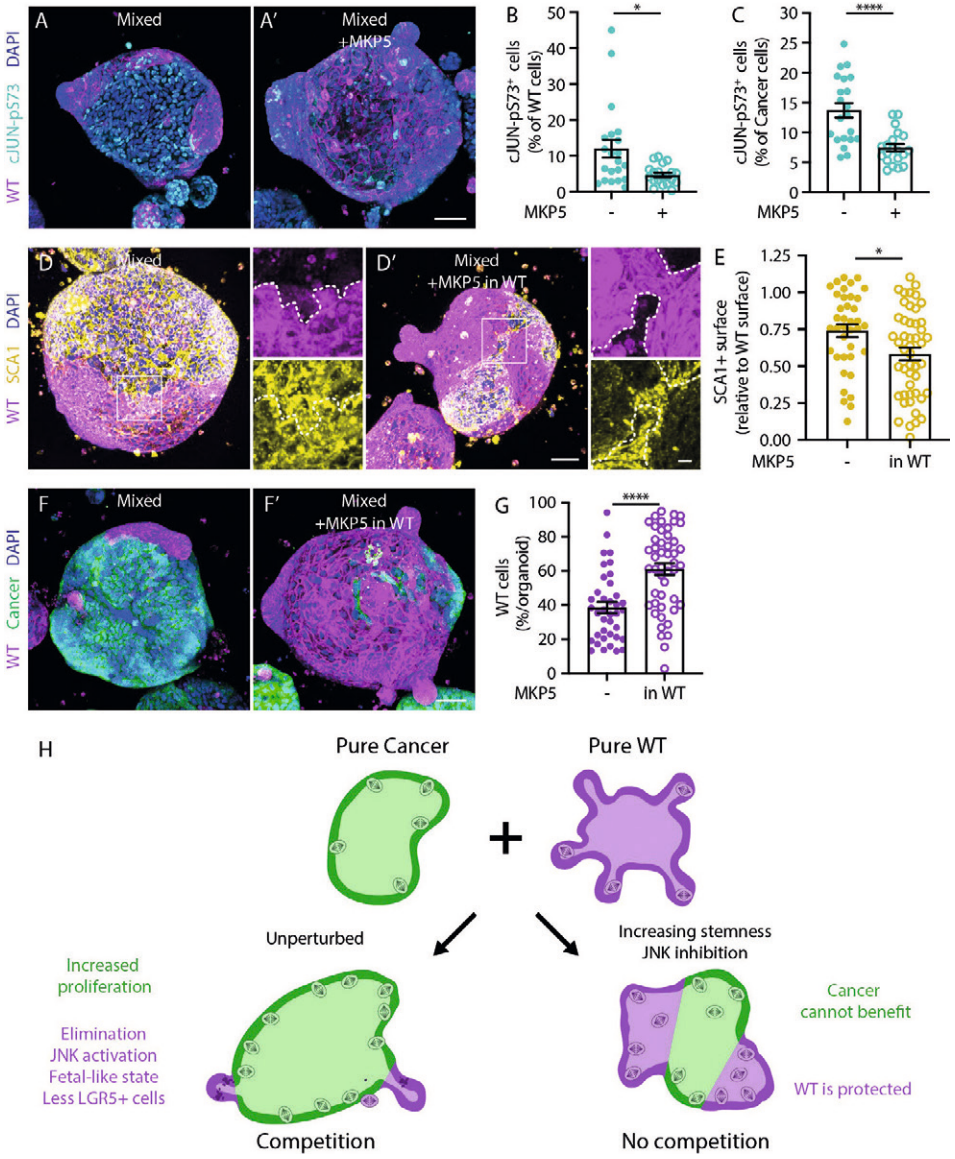
S.J.E.S and A.K.G. performed the experiments. H.L.Q. performed mRNA sequencing analysis, A.F., R.J., T.R.M.L., O.J.S and J.v.R. contributed knowledge and reagents. S.J.E.S. designed the experiments, supervised the study and wrote the manuscript together with J.v.R., and the manuscript was approved by all authors. Correspondence and requests for materials should be addressed to S.J.E.S (s.suijkerbuijk@nki.nl).

DECLARATION OF INTERESTS

H.Q.L. is employed by Boehringer Ingelheim Pharma GmbH & Co. KG.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community.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A'
Mixed
+MKP5
WT cJUN-pS73 DAPI
B
cJUN-pS73+ cells
(% of WT cells)
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C
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(% of Cancer cells)
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D
Mixed
D'
Mixed
+MKP5 in WT
WT SCA1 DAPI
E
SCA1+ surface
(relative to WT surface)
MKP5
in WT
F
Mixed
F'
Mixed
+MKP5 in WT
WT Cancer DAPI
G
WT cells
(%/organoid)
MKP5
in WT
H
Pure Cancer
Pure WT
Unperturbed
Increasing stemness
JNK inhibition
Increased
proliferation
Elimination
JNK activation
Fetal-like state
Less LGR5+ cells
Cancer
cannot benefit
WT is protected
Competition
No competition

Figure 7 – JNK activity in wild-type cells is required for cell competition A-C) Representative 3D-reconstructed confocal images of control (A) and MKP5 expressing (A') mixed organoids stained for activated cJUN (cyan), nuclei are visualized with DAPI (blue). B-C) Quantification of the number of cJUN-pS73^{+} cells relative to the total number of wild-type (B) and cancer (C) cells, each dot represents one organoid (Mean ±SEM, one-way ANOVA, multiple comparisons, p=0.0156, n=21 & 20 organoids (B) and p< 0.0001, n= 21 & 20 organoids (C)). D-E) Representative 3D-reconstructed confocal images of control (D) and doxycycline treated (D') mixed organoids formed by TET-inducible MKP5 wild-type and control cancer cells. The organoids were stained for SCA1 (yellow), nuclei are visualized with DAPI (blue). The insets display a 2.5x magnification of the area in the white box. E) Quantification of the SCA1+ surface relative to the total wild-type surface area, each dot represents one organoid (Mean ±SEM, Non-parametric, ANOVA, multiple comparisons: p=0.0129, n=38 & 48 organoids). F-G) Representative 3D-reconstructed confocal image of control (F) and doxycycline treated (F') mixed organoids formed by TET-inducible MKP5 wild-type and control cancer cells, nuclei are stained with DAPI (blue), and quantification of the percentage of wild-type cells contributing to mixed organoids (G), each dot represents one organoid (Mean ±SEM, unpaired t-test, two-tailed, p<0.0001, n=38 & 48 organoids). H) Schematic model depicting cell competition driven growth of intestinal tumor cells in murine organoids. Cancer cells induce active elimination and cell fate transition of wild-type cells, while fueling their oncogenic growth. Scale bars = 50µm, excluding magnifications in (D) where scale bar = 10µm. See also Figure S7.

STAR METHODS TEXT

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Saskia J.E. Suijkerbuijk (s.suijkerbuijk@nki.nl).

Materials availability

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability

All used software is listed in the Key Resources Table. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev and Lash, 2002) and are accessible through GEO Series accession number GSE176027 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE176027)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Isolation wild-type small intestine organoids

All experiments were performed in accordance with the Animal Welfare Committee of the Netherlands Cancer Institute, the Netherlands. Animals were kept were housed under standard laboratory conditions at the Netherlands Cancer Institute facility and received standard laboratory chow and water *ad libitum*. Wild-type small intestine organoids were derived as previously described (Sato et al., 2009), from Rosa26-Cre^{ERT2}::mT/mG (Muzumdar *et al.*, 2007) male and female animals age 9-30 weeks (C57BL/6J background), Lgr5^{DTR} transgenic mice (provided by the Genentech MTA program (Tian et al., 2011)) female animals age 9-19 weeks (C57BL/6J background) and eCadherin-mCFP (Snippert et al., 2010) female animals age 19-22 weeks (mixed background).

Isolation intestinal cancer organoids

Small intestine cancer organoids, derived from the small intestine of AKP: Villin-Cre^{ERT2}*Apc*$^{fl/fl}$*Kras*$^{G12D/WT}$*Tr53*$^{fl/R172H}$ mice were previously reported (Fumagalli *et al.*, 2017). NOTCH cancer organoids derived from KPN: Villin-Cre^{ERT2}*Kras*$^{G12D/WT}$*Trp53*$^{fl/fl}$Rosa26$^{N1icd/+}$ and APN: Villin-Cre^{ERT2}*Apc*$^{fl/+}$*Trp53*$^{fl/fl}$Rosa26$^{N1icd/+}$ small intestine primary tumors were previously reported (provided by the ACRCelerate Colorectal Cancer Stratified Medicine Network Consortium (Jackstadt *et al.*, 2019)). All experiments were performed according to UK Home Office regulations (Project License 70/8646), adhered to ARRIVE guidelines and were approved by local animal welfare and the ethical review committee at the University of Glasgow. Mice were housed in conventional cages in an animal room at constant temperature (19–23 °C) and humidity (55% ± 10%) under a 12-h light–dark cycle and were allowed access to standard diet and water *ad libitum*. Male mice (C57BL/6J background) of 7 to 16 weeks of age were induced with a single intraperitoneal injection of 2mg tamoxifen on D0 and either aged until clinical endpoint as evidenced by anaemia, hunching and/or weight loss to generate small intestinal tumour organoid lines (APN/KPN), or were sampled on D3 post-induction to generate transformed small intestinal organoids (AKP).

Culture of mouse organoids

All lines were cultured in drops of Cultrex PathClear Reduced Growth Factor Basement Membrane Extract Type 2 (Amsbio, 3533-005-02) in murine small intestinal organoids medium containing advanced DMEM/F12 medium (adDMEM/F12; Thermo Fisher Scientific, cat. No. 12634-010), GlutaMAX 1% (Thermo Fisher Scientific, cat. No. 35050-068), HEPES 10mM (Thermo Fisher Scientific, cat. No. 15630-056), 1x Penicillin/

streptomycin (10,000 U/ml; Thermo Fisher Scientific, cat. No. 15140-122). B27 2% (Thermo Fisher Scientific, cat. No. 17504-044), N-acetylcysteine 1.25 mM (Sigma-Aldrich, cat. No. A9165), mEGF 50ng/ml (Peprotech, cat. No. 315-09), Noggin and R-spondin1 both 10% (conditioned medium prepared in house).

Transduction of organoids

A dual lentiviral vector 3rd generation Tet-regulatory protein expression system was used for doxycycline-inducible expression of MKP5 (custom made by VectorBuilder Inc.). pLentiPGK Hygro DEST H2B-mCerulean3 was a gift from Markus Covert (Addgene plasmid #90234; http://n2t.net/addgene:90234; RRID: Addgene_90234) (Kudo *et al.*, 2018). Lentiviral transduction was preformed using standard procedures. In short, lentivirus was produced in HEK293T by co-transfection lentiviral plasmids with helper plasmids pMDLg/pRRE, pRSV-Rev and pMD2.G (gifts from Didier Trono, Addgene plasmids #12251, #12253 and #12259). Viral particles were harvested from cells four days after transfection and concentrated using 50kDa Amicon Ultra-15 Centrifugal Filter Units (Merck, cat#UFC905024). Organoids were dissociated by mechanical disruption and dissolved in 250µL ENR medium supplemented with 10µM Y-27632 and Polybrene (8mg/ml) together with concentrated virus. Cells were incubated at 32°C while spinning at 600xG for 1 hour followed by a 4-hour incubation at 37°C before plating in BME2. Selection was carried out from day 3 onwards with neomycin, blasticidin or hygromycin.

METHODS DETAILS

Generation of mixed enteroid monolayers

Enteroid monolayers were prepared as described previously (Thorne *et al.*, 2018), in short, single cell suspensions were generated from organoids by mechanical disruption and a digest with TrypLE Express (Thermo Fisher Scientific Cat# 12605-010). Approximately 4000 WT and/or 1000 cancer cells were seeded per well of BME2 coated (0.8mg/ml) 96-well plate in medium supplemented with CHIR-99021 and Y-27632. After 24hrs, cells were washed once and cultured in murine small intestinal organoid medium for the remainder of the experiment. For imaging purposes cells were plated in glass-bottom 96 well SensoPlates (Greiner Bio-One Cat#655892). Small molecule inhibitors were used in the following concentrations: Z-VAD-FMK (50µM, Bachem Cat# N-1510.0005), CHIR-99021 (3µM, Tocris Cat#4423), Y-2763 (10µM, Abmole Cat# M1817).

Generation of mixed 3D organoid cultures

3D mixed organoid cultures were prepared as follows; suspensions of small clumps of cells were generated from organoids by mechanical disruption and divided over Eppendorf vials in a 2:1 ratio (WT:cancer). Cells were concentrated by mild centrifugation, the pellet as resuspended in a small volume of murine small intestinal organoids medium and incubated at 37C for 30 minutes. Cell aggregates were plated in BME2 and cultured in murine small intestinal organoids medium. For imaging purposes cells were plated in ibiTreat #1.5 polymer coverslip 96 Well µ-Plate (IBIDI, cat# 89626) or µ-Slide 8 Well chambered slides (IBIDI, cat#80827). Small molecule inhibitors were used in the following concentrations: Z-VAD-FMK (50µM, Bachem Cat# N-1510.0005), CHIR-99021 (3µM, Tocris Cat#4423), Valproic acid (1mM, Sigma Cat# PHR1061-1G), Y-2763 (10µM, Abmole Cat# M1817), JNK-IN-8 (1µM, Sigma Aldrich, Cat#SML1246), Doxycycline (4.25µM, Sigma Aldrich, Cat#D9891).

Immuno-fluorescence

Enteroid monolayers and 3D organoids were fixed in 4% paraformaldehyde in PBS for 20 minutes followed by a block while permeabilizing in 5% BSA/ 0.2% Triton X100/ PBS for 30 minutes at room temperature. The stainings were performed overnight at 4°C with the following primary antibodies: anti-Cleaved Caspase-3 (Asp175) (Cell Signalling, #9661), anti-phospho-Histone H3 (Ser10) (Merck-Millipore, #06-570), anti-GFP (Abcam, #ab6673), anti-Phospho-c-Jun (Ser73) (D47G9) (Cell Signalling, #3270), anti-Phospho-JNK1+JNK2 (T183 + Y185) (Abcam, #ab4821) and anti-Ly-6A/E (Sca-1) (Biolegend, cat#108101). Appropriate Alexa Fluor labelled secondary antibodies (ThermoFischer Scientific) were combined with DAPI and/or Phalloidin Alexa Fluor 647 (ThermoFischer Scientific, cat# A22287). For labelling of cells in S-phase, a pulse of 10µm EdU (5-ethynyl-2'-deoxyuridine) was given one hour prior to fixation and detection was performed according to manufacturer's guidelines before starting the immunofluorescence staining using Click-iT EdU Cell Proliferation Kit for Imaging Alexa Fluor 647 (ThermoFischer Scientific, cat# C10340).

Microscopy

For fixed samples images were collected on an inverted Leica TCS SP8 confocal microscope (Mannheim, Germany) in 12 bit with 25X water immersion objective (HC FLUOTAR L N.A. 0.95 W VISIR 0.17 FWD 2.4 mm).

Sequential imaging of enteriod monolayers was done using the navigator function in LasX software (Leica) on an inverted Leica TCS SP8 confocal microscope (Mannheim,

Germany) in 12-bit with 25X water immersion (HC FLUOTAR L N.A. 0.95 W VISIR 0.17 FWD 2.4 mm). Merged images were rotated, aligned and cropped in Photoshop (Adobe).

Time-lapse microscopy of enteroid monolayers was performed on a Leica TCS SP5 confocal microscope (Mannheim, Germany) in 12-bit with 20X dry immersion objective (HCX PL APO CS 20.0x0.70 DRY UV).

Time-lapse microscopy of 3D organoids was done on an inverted Leica TCS SP8 confocal microscope (Figure 1), Leica-based spinning disk confocal microscope with an Andor Dragonfly system, with the Argon-laser of 488nm and the Diode-laser of 561nm using a 40mm pinhole and Andor sCMOS Zyla 4 2p camera (Figure 2), both equipped with a 20X dry immersion objective (HCX PL APO CS 20.0x0.70 DRY UV) or on an AxioObserver widefield microscope (Figures S3B-S3D) (Zeiss) equipped with an Orca FLASH 4.0 V3 grayscale sCMOS-camera (Hamamatsu) and using a 10X dry objective (N.A. 0.30 EC Plan-Neofluar Ph1). Whole drops of organoids were followed up to 90 hours and images were collected in 16-bit with a 6-hour time interval. ZEN software (Zeiss) was used to stitch mosaic images.

Flow Cytometry

For gene expression analysis, pure and mixed cells were cultured in 6-well plates and FACS sorted 3 days after mixing on a FACS Jazz system (BD). After a FSC/SSC gate doublets were excluded, live cells (DAPI negative) were sorted based on mTomato (wild-type cells) and Dendra2 (cancer cells) expression. Purity was determined by microscopy.

mRNA sequencing

Total RNA was extracted using the standard TRIzol (Invitrogen) protocol and used for library preparation and sequencing. mRNA was processed as described previously, following an adapted version of the single-cell mRNA seq protocol of CEL-Seq (Hashimshony *et al.*, 2012; Simmini *et al.*, 2014). In brief, samples were barcoded with CEL-seq primers during a reverse transcription protocol and pooled after second strand synthesis. The resulting cDNA was amplified with an overnight In vitro transcription reaction. From this amplified RNA, sequencing libraries were prepared with Illumina Truseq small RNA primers.

QUANTIFICATION AND STATISTICAL ANALYSIS

Flow cytometry

Data were manually analyzed using FlowJo 10.6.1 (https://www.flowjo.com/).

Microscopy

Imaris software (version 9.3.1, Oxford Instruments) was used for quantification and 3D reconstructions of fixed and time-lapse images. Quantification of cell number, surface and distance was all performed on 3D reconstructed images. In short, individual nuclei and markers (EdU, pH3, pJUN, LGR5) were segmented using the "spots" function and co-localization was determined when relevant (e.g. EdU & pH3). Next, the "surface" function was used to mask individual cell populations and determine the number predefined spots within these populations.

Images and movies were converted to RGB using FIJI, cropped and when necessary corrected for bleed through, smoothened, cropped, rotated and contrasted linearly.

Statistical analysis

Statistics were performed using GraphPad Prism. Paired or unpaired t-test was used when data showed normal distribution (verified with normality tests, provided by GraphPad Prism), whereas Mann-Whitney U test was used for data that did not display parametric distribution. Adoption of one statistical test or the other is indicated for each experiment in the Figure legend.

mRNA sequencing

From paired-end sequencing data read 1 was used to identify the Illumina library index and CEL-Seq sample barcode. After quality control and adaptor removal, read 2 was aligned to the MM10 RefSeq transcriptome using BWA (Li and Durbin, 2010). Reads that mapped equally well to multiple locations were discarded. Reads were quantified with featureCounts to generate read counts for each gene based on the gene annotation from Ensemble. Differential gene expression was analyzed, based on featureCounts results, using Deseq2 version 1.22.2 (Love, Huber and Anders, 2014).

Gene Ontology analysis was performed with Webgestalt (Liao *et al.*, 2019) (http://www.webgestalt.org/), with the parameters; Method: Over-representation Analysis (ORA); Organism: mmusculus; Enrichment Categories: geneontology_Biological_Process;

FDR Method: BH; Significance Level: Top 10; Redundancy reduction correction using Weighted set cover algorithm, FDR ≤ 0.05.

The heat map was generated using Morpheus developed by the Broad Institute (https://software.broadinstitute.org/morpheus) and scaled in Adobe Illustrator.

Gene Set Enrichment Analysis was performed with GSEA software developed by UC San Diego and Broad Institute (Mootha *et al.*, 2003; Subramanian *et al.*, 2005) (https://www.gsea-msigdb.org/gsea/index.jsp). Standard parameters were used with a pre-ranked dataset of differentially expressed genes in wild-type cells ($p<0.05$). Gene Sets that were used for comparison were the upregulated genes from the previously published 'mFetal' and 'mRepair' datasets (Yui *et al.*, 2018).

Transcription Factor target analysis was performed with Webgestalt (Liao *et al.*, 2019) (http://www.webgestalt.org/), with the parameters; Method: Over-representation Analysis (ORA); Organism: mmusculus; Enrichment Categories: network_Transcription_Factor_target; FDR Method: BH; Significance Level: Top 10.

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SUPPLEMENTARY FIGURES

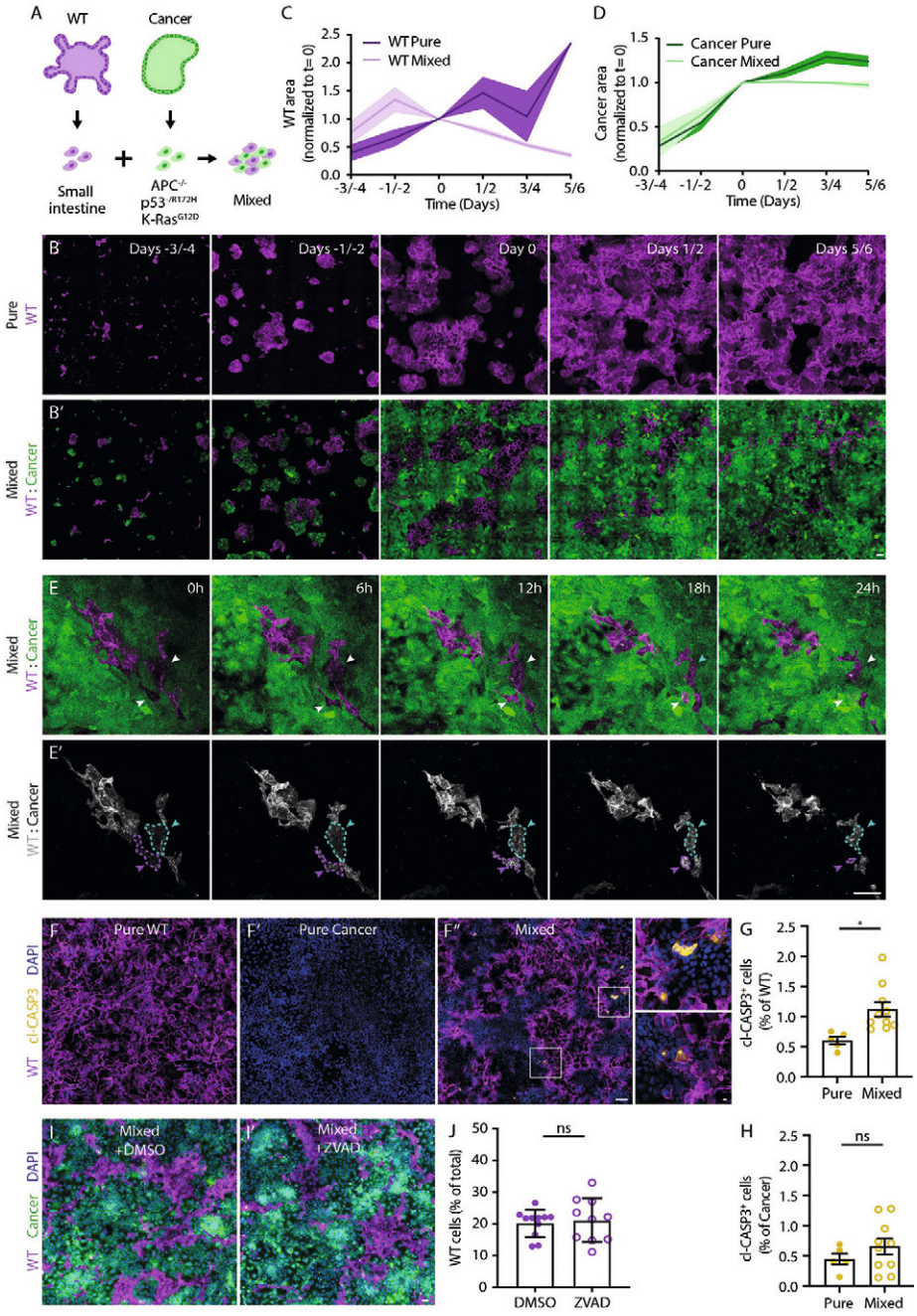


Figure S1 – Wild-type small intestine cells are eliminated by cancer cells in enteroid monolayers. Related to Figures 1 and 2.
A) Schematic representation of a model for cell competition in murine enteroid monolayers.
B-D) Representative pictures of sequential imaging of enteroid monolayers in pure (B) and mixed (B') conditions and quantification of the surface covered by wild-type (C) or cancer (D) populations over time normalized to Day 0 (Mean ±SEM). Day 0 is the moment a full monolayer is formed in mixed conditions.
E) Representative images of time-lapse series of a competing enteroid monolayer, arrow heads in (E') indicate examples of wild-type cells that are shrinking (cyan) and being eliminated (magenta).
F-H) Representative confocal images of pure wild-type (F) pure cancer (F') and mixed (F'') enteroid monolayers. Apoptotic cells are marked by cl-CASP3 (yellow). The insets display a 5.75x magnification of the area in the white box. G-H) Quantification of the cl-CASP3+ cells relative to the total wild-type (G) and cancer (H) cell population, each dot represents one imaged well (Mean ±SEM, unpaired t-test, two-tailed, p=0.0128 (G), p=0.3092, n=5 & 10 wells).
I-J) Representative confocal images of control (I) and Z-VAD-FMK (I') treated enteroid monolayers.
J) Quantification of the number of wild-type cells relative to the cell population, each dot represents one imaged well (Mean ±SEM, unpaired t-test, two-tailed, p=0.6926, n=11 & 10 wells).
Scale bars = 100μm, excluding magnifications in (F) where scale bar = 10μm.

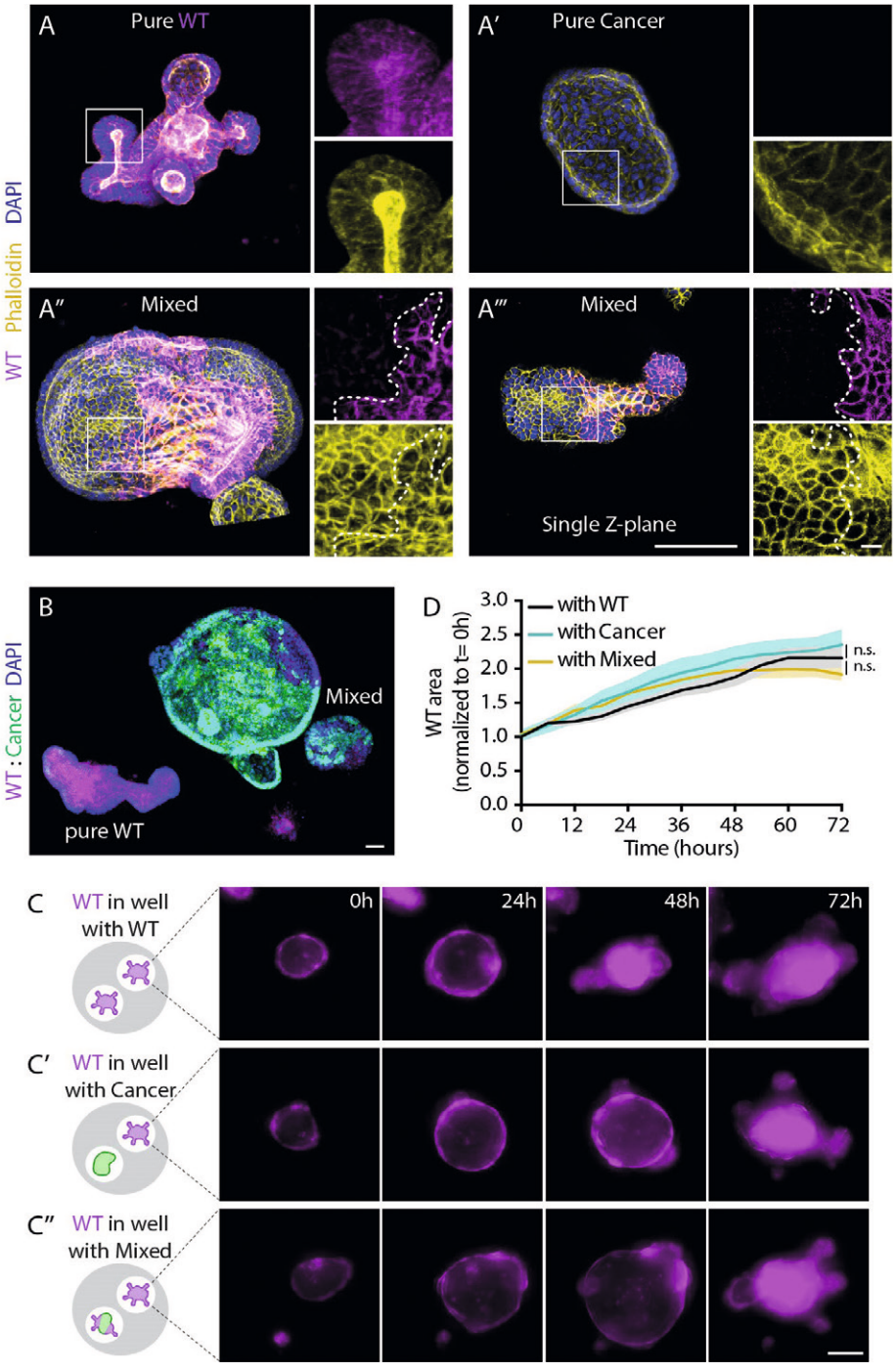
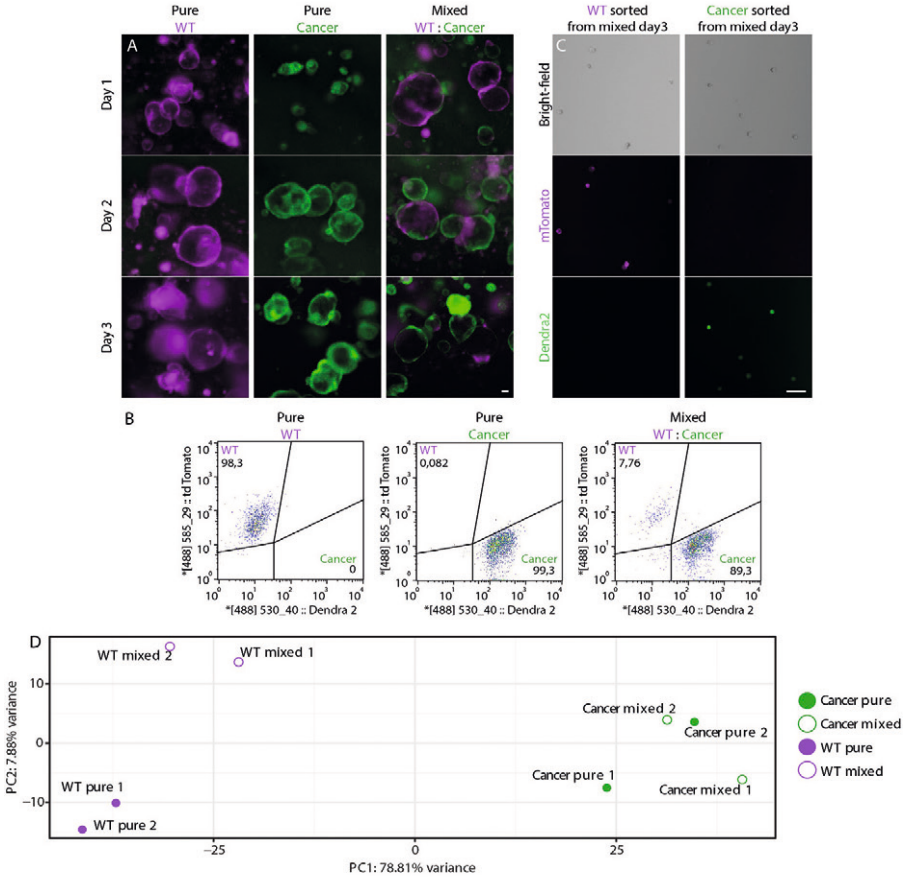

A
Pure WT
A′
Pure Cancer
A″
Mixed
A‴
Mixed
Single Z-plane
WT Phalloidin DAPI
B
Mixed
pure WT
WT : Cancer DAPI
D
with WT
with Cancer
with Mixed
n.s.
n.s.
WT area (normalized to t= 0h)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
12
24
36
48
60
72
Time (hours)
C
WT in well with WT
C′
WT in well with Cancer
C″
WT in well with Mixed
0h
24h
48h
72h

Figure S2 – Short-range communication is essential for cell competition. Related to Figure 2.
A) Representative 3D-reconstructed confocal images of pure WT (A), pure cancer (A') and mixed (A'') organoids, and a single Z-plane of A'' (A'''). The actin cytoskeleton is stained with Phalloidin (yellow), nuclei with DAPI (blue) and borders between wild-type and cancer cells are indicated by dashed lines. The insets display a 2.5x magnification of the area in the white box.
B) Representative confocal image of a mixed culture containing a pure WT organoid, nuclei are visualized with DAPI (blue).
C-D) Representative images from live-imaging of pure WT organoids co-cultured with pure WT I, pure cancer (C') or mixed (C'') organoids and quantification of the area covered by wild-type cells within indicated organoids (D) normalized to the start of the time-lapse (Mean ±SEM, 2-way ANOVA, multiple comparisons, n=18 organoids for each condition, 'WT in WT' vs. 'WT in cancer' p= 0.5453, 'WT in WT' vs. 'WT in Mix' p= 0.9689).
Scale bars = 100µm, excluding magnifications in (A) where scale bar = 10µm.



E

GeneSet	mFetal
Enrichment Score (ES)	0.4131525
Normalized Enrichment Score (NES)	2.0948782
Nominal p-value	0.000
FDR q-value	0.000
FWER p-Value	0.000

F

GeneSet	mRepair
Enrichment Score (ES)	0.43847787
Normalized Enrichment Score (NES)	1.8192958
Nominal p-value	0.0011933175
FDR q-value	5.030181E-4
FWER p-Value	0.001

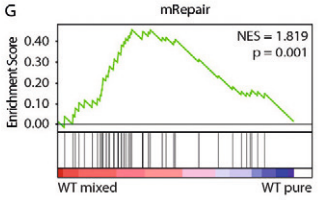


Figure S3 – Cell competition induces a fetal-like state in WT cells. Related to Figure 4.
A-C) Flow cytometry sorting of wild-type and cancer cells from pure and mixed cultures. A) Representative images of pure and mixed cultures 1, 2 and 3 days after plating. B-C) Analysis of cells after sorting, graphs in B show an analysis of 10.000 cells, numbers in the corners display the percentage of sorted cells. Representative images of sorted cells are shown in I Scale bars = 50µm.
D-G) Gene expression analysis of wild-type and cancer cells in pure and mixed conditions. D) displays a principal component analysis of all sample. D) Parameters of a gene Set Enrichment Analysis showing enrichment of a fetal signature (Yui et al., 2018) in mixed wild-type cells. F-G) Parameters and graph of a gene Set Enrichment Analysis showing enrichment of a repair signature (Yui et al., 2018) in mixed wild-type cells.

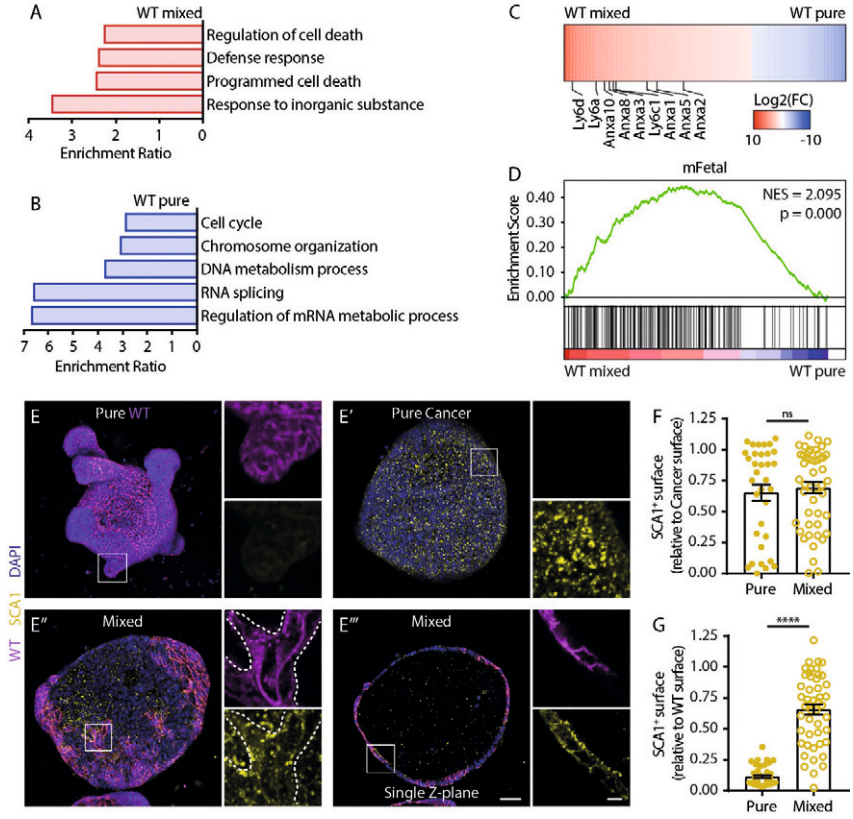


Figure S4 – Multiple types of intestinal cancer compete with WT cells. Related to Figures 2 and 4.

A-B) Representative 3D-reconstructed confocal image of control (A) and apoptosis inhibited (A') mixed organoids formed by KPN cancer and wild-type intestinal cells, nuclei are stained with DAPI (blue), and quantification of the percentage of wild-type cells contributing to mixed organoids (B), each dot represents one organoid (Mean ±SEM, unpaired t-test, two-tailed, p=0.019, n=36 & 30 organoids).
C-D) Representative 3D-reconstructed confocal image of control I and apoptosis inhibited (C') mixed organoids formed by APN cancer and wild-type intestinal cells, nuclei are stained with DAPI (blue), and quantification of the percentage of wild-type cells contributing to mixed organoids (D), each dot represents one organoid (Mean ±SEM, unpaired t-test, two-tailed, p<0.0001, n=38 & 44 organoids).
E-G) Representative 3D-reconstructed confocal images of pure WT I, pure KPN cancer (E'), mixed KPN (E'') organoids and quantification of the SCA1+ surface relative to the total KPN cancer (F) or wild-type (G) surface area. The organoids were stained for SCA1 (yellow), nuclei are visualized with DAPI (blue). The insets display a 2.5x magnification of the area in the white box. Each dot in (F) and (G) represent one organoid (Mean ±SEM, Non-parametric, ANOVA, multiple comparisons: p<0.0001, n=31 & 36 organoids (F); p<0.0001, n=28 & 36 organoids (G)).
H-J) Representative 3D-reconstructed confocal images of pure WT (H), pure APN cancer (H'), mixed APN (H'') organoids and quantification of the SCA1+ surface relative to the total APN cancer (I) or wild-type (J) surface area. The organoids were stained for SCA1 (yellow), nuclei are visualized with DAPI (blue). The insets display a 2.5x magnification of the area in the white box. Each dot in (I) and (J) represent one organoid (Mean ±SEM, Non-parametric, ANOVA, multiple comparisons: p=0.0939, n=54 & 58 organoids (I); p<0.0001, n=50 & 58 organoids (J)).
Scale bars = 50µm, excluding magnifications in (E and H) where scale bar = 10µm.

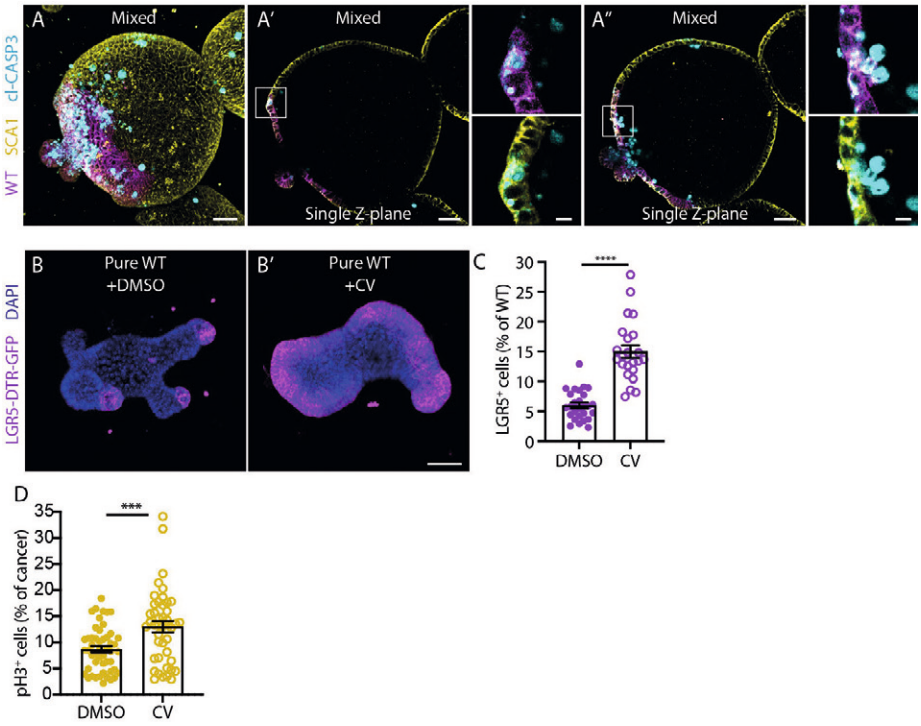


Figure S5 – Increased stemness prevents cell competition. Related to Figures 2 and 5.
A) Representative 3D-reconstructed (A) and single Z-plane (A' and A'') confocal images of a mixed organoid. The organoids were stained for cl-CASP3 (cyan) and SCA1 (yellow). The insets display a 3.5x magnification of the area in the white box. Organoid is from the same dataset used in panel 2E.
B) Representative 3D-reconstructed confocal images of control (B) and CV treated (B') pure WT organoids. LGR5+ Intestinal stem cells (magenta) and nuclei (blue) are visualized.
I Graph displays the number of LGR5+ cells relative to total number of wild-type cells, each dot represents one organoid (Mean ±SEM, unpaired T-test, two-tailed, p<0.0001, n=27 & 23 organoids).
Displayed DMSO control organoids are from the same dataset used in panel 5B.
D) Graph displays the number of pH3+ cells in pure organoids relative to the total number of cancer cells, each dot represents one organoid (Mean ±SEM, one-way ANOVA, multiple comparisons, p=0.0005, n=50 & 44 organoids).
Scale bars = 50µm

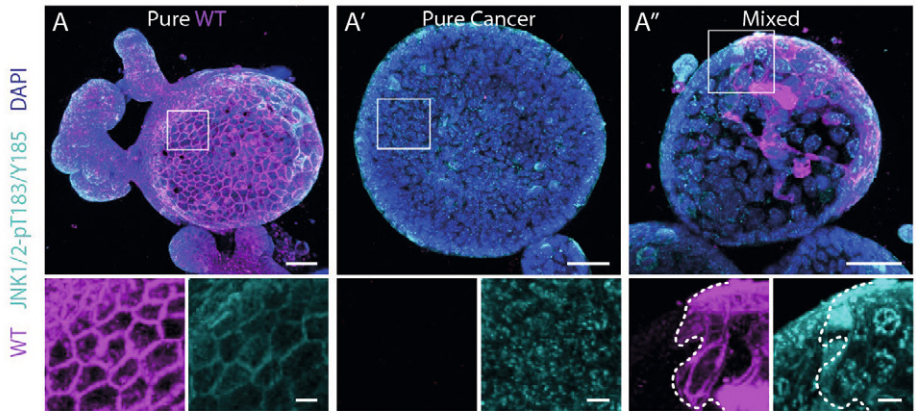


Figure S6 – JNK signaling drives cell competition. Related to Figure 6.
Representative 3D-reconstructed confocal images of pure WT (A), pure cancer (A'), mixed (A'') organoids, stained for activated JNK1/2-pT183/Y185 (cyan), nuclei are visualized with DAPI (blue). The insets display a 2.5x-3.5x magnification of the area in the white box.

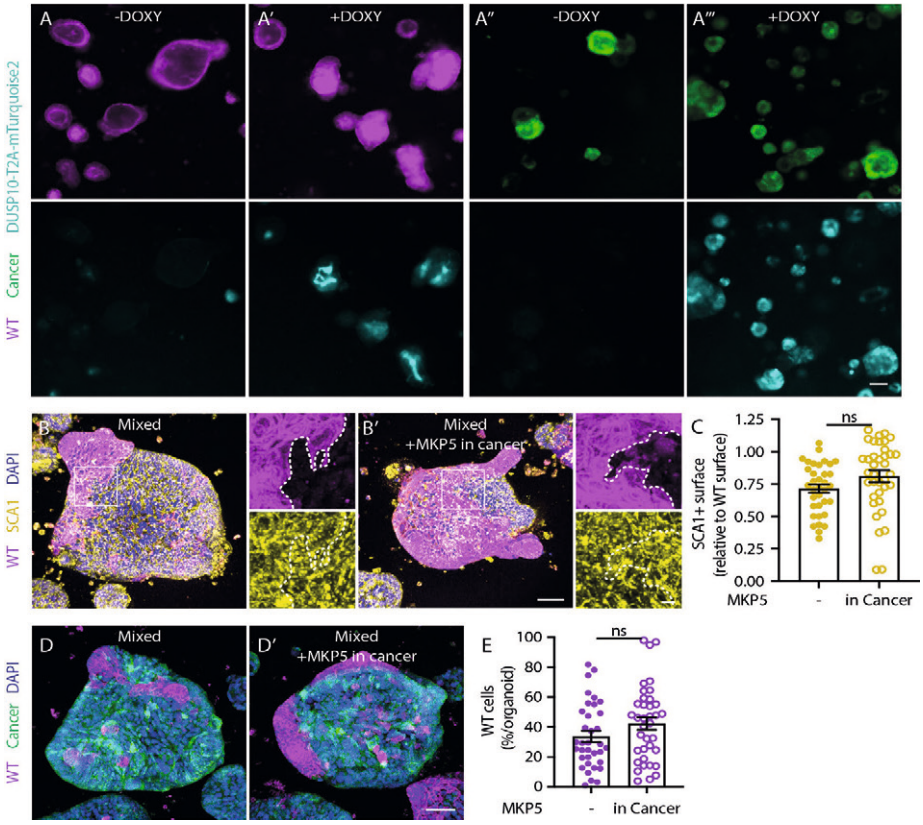


Figure S7 – JNK activity in wild-type cells is required for cell competition. Related to Figure 7.
A)Representative images of control (A) and doxycycline treated (A') organoids formed by TET-inducible MKP5 wild-type cells and control (A'') and doxycycline treated (A''') organoids formed by TET-inducible MKP5 cancer cells, co-expression of mTurquoise2 (Cyan) is shown.
B-C) Representative 3D-reconstructed confocal images of control (B) and doxycycline treated (B') mixed organoids formed by TET-inducible MKP5 cancer and control wild-type cells. The organoids were stained for SCA1 (yellow), nuclei are visualized with DAPI (blue). The insets display a 2.5x magnification of the area in the white box. C) Quantification of the SCA1+ surface relative to the total wild-type surface area, each dot represents one organoid (Mean ±SEM, ANOVA, multiple comparisons, p=0.2337, n=35 & 37 organoids).
D-E) Representative 3D-reconstructed confocal image of control (D) and doxycycline treated (E') mixed organoids formed by TET-inducible MKP5 cancer and control wild-type cells, nuclei are stained with DAPI (blue), and quantification of the percentage of wild-type cells contributing to mixed organoids I, each dot represents one organoid (Mean ±SEM, ANOVA, multiple comparisons, p=0.2892, n=35 & 37 organoids).
Scale bars = 50µm, excluding magnifications in (B) where scale bar = 10µm

SUPPLEMENTARY VIDEOS

Movies are available at: https://doi.org/10.1016/j.celrep.2021.109307

Video S1 – related to Figure 1D:
Time-lapse series of 3D reconstructed pure wild-type intestinal organoid.

Video S2 – related to Figure 1D':
Time-lapse series of 3D reconstructed pure cancer intestinal organoid.

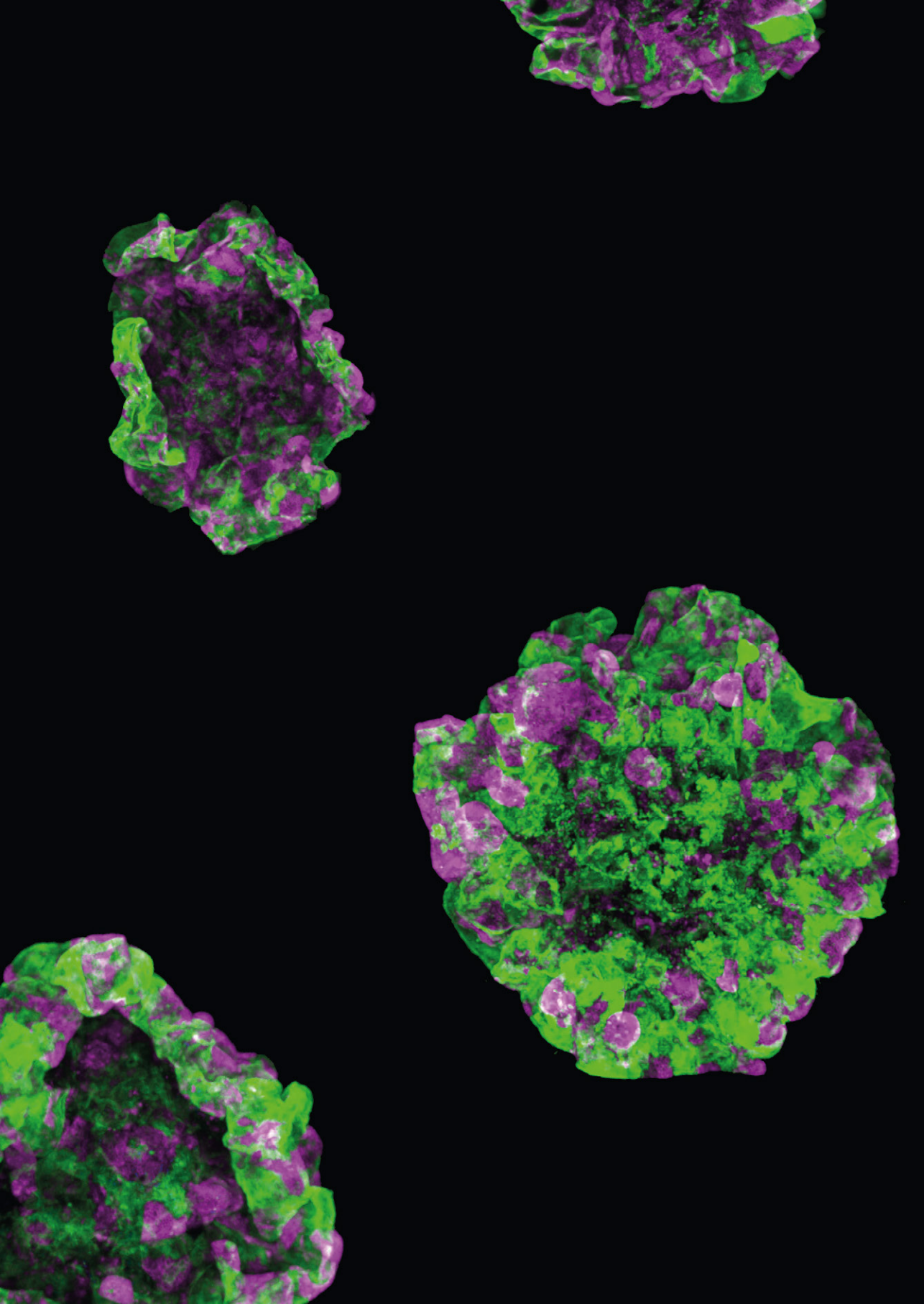
Video S3 – related to Figure 1D'':
Time-lapse series of 3D reconstructed mixed intestinal organoid.

Video S4 – related to Figure S1E:
Time-lapse series of a competing enteroid monolayer, arrow heads indicate examples of wild-type cells that are shrinking and being eliminated.

Video S5 – related to Figure 2D:
Time-lapse series of mixed intestinal organoid, 3D reconstructed on the left, maximum projection of 6 Z-stacks on the right. Cell extrusion is indicated with arrow heads.

Video S6 – related to Figure 2G:
Time-lapse series of control treated 3D reconstructed mixed intestinal organoid.

Video S7 – related to Figure 2G':
Time-lapse series of Z-VAD-FMK treated 3D reconstructed mixed intestinal organoid.



Cell competition promotes metastatic intestinal cancer through a multistage process

4

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Manuscript in revision at iScience

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SUMMARY

Cell competition plays an instrumental role in quality control during tissue development and homeostasis. Nevertheless, cancer cells can exploit this process for their own proliferative advantage. In our study, we generated mixed murine organoids and microtissues to explore the impact of cell competition on liver metastasis. Unlike competition at the primary site, the initial effect on liver progenitor cells does not involve the induction of apoptosis. Instead, metastatic competition manifests as a multistage process. Initially, liver progenitors undergo compaction, which is followed by cell cycle arrest, ultimately forcing differentiation. Subsequently, the newly differentiated liver cells exhibit reduced cellular fitness, rendering them more susceptible to outcompetion by intestinal cancer cells. Notably, cancer cells leverage different interactions with different epithelial populations in the liver, using them as scaffolds to facilitate their growth. Consequently, tissue-specific mechanisms of cell competition are fundamental in driving metastatic intestinal cancer.

Keywords
Cell Competition, intestinal cancer, differentiation, organoids, microtissues, metastasis, liver

INTRODUCTION

Worldwide, colorectal cancer is third most common cancer in males and second in females (International Agency for Research on Cancer, WHO). Approximately one-third of colorectal cancer patients develop metastases within the first three years after diagnosis[1–3]. The liver is a main site of colorectal cancer metastasis and is responsible for a large part of the colorectal cancer-dependent lethality.[1,3–6] Liver tissue is formed by several different cell types that all contribute to optimal liver function, such as detoxification, metabolism and bile production.[7] The two main epithelial cell types are cholangiocytes and hepatocytes. Cholangiocytes form the bile ducts and are an important source of progenitor cells.[8] During regeneration these cells can differentiate into hepatocytes as a response to chronic liver damage.[9,10] This process is characterized by the loss of expression of progenitor markers such as SOX9 and LGR5 and gain expression of typical hepatocyte markers like CYP and Albumin.[8] Most of the liver is formed by hepatocytes, which represent 60% of the cells and accounts for 80% of the volume of the tissue.[11] Under homeostatic conditions hepatocytes remain in a quiescent state. However, upon acute injury, hepatocytes can switch to a regenerative state which allows reconstitution of lost tissue through cell proliferation or increasing of cell size.[7,12–14] The colonization of a secondary organ by cancer cells requires cellular interactions with a novel microenvironment. In the liver this is reflected by various histological growth patterns of liver metastases.[15,16] Each of these patterns display distinct histological characteristics and often correlating to a specific prognosis. In particular, the replacement pattern, which is characterized by the direct interaction of cancer cells with the surrounding liver epithelium is associated with poor patient outcome.[15] The importance of interactions between liver cancer cells and surrounding liver tissue is further illustrated by the finding that peritumoral hepatocytes depend on YAP signaling to restrain tumor growth.[17] However, interactions of metastasis and healthy liver tissue are still poorly understood.

Cell competition is a vital mechanism that drives the continuous selection of cells based on their relative fitness. It serves as a mechanism for quality control during both development and adult tissue homeostasis by elimination of unfit cells.[18] For example, fluctuations in the expression levels of the transcription factor Myc play a crucial role in determining the survival of cells in the mouse epiblast and developing heart.[19,20] This mechanism ensures that only the fittest cells constitute these tissues. Additionally, cell competition is employed to remove early malignant cells, characterized by oncogenic Ras expression or a p53 mutation, from epithelial tissues like the pancreas and intestine.[21,22] It is important to note that cancer cells can exploit this process of cell competition to promote tumor growth.[18,23] A significant body of research has demonstrated the substantial contribution of cell

competition to various stages of primary colorectal cancer. During tumor initiation, APC mutant stem cells gain a crucial competitive advantage over wild-type stem cells through the secretion of the WNT antagonist NOTUM.[24,25] Additionally, the expression of oncogenic variants of KRas and PI3K leads to a similar competitive advantage.[26] At later stages of tumorigenesis, we have shown that intestinal cancer cells actively eliminate surrounding healthy epithelial tissue in both the *Drosophila* adult midgut and murine organoids.[27,28] In the latter, wild-type cells are eliminated in a JNK dependent manner and activate a fetal-like state, an injury-response that is often activated in damaged intestinal epithelia.[29,30] Importantly, the cancer population directly benefits from these competitive interactions by boosting their proliferation rate.

To understand the influence of surrounding tissue on growth of colorectal cancer liver metastasis it is crucial to identify the interactions between cancer cells and the epithelial cell populations. Here, we show that intestinal cancer cells outcompete wild-type liver cells in murine organoids and microtissues. When facing progenitor cells, cancer cells induce a cell cycle arrest and subsequent loss of the wild-type progenitor state. The interaction of cancer cells with differentiated, hepatocyte-like cells, causes a reduced cellular fitness and results in rapid outcompetion. Importantly, the cancer population uses wild-type tissue as a scaffold for tumor growth and benefits from these interactions through increased expansion.

RESULTS

Cancer cells outcompete wild-type liver cells

To investigate the role of cell competition in liver metastasis we adapted our previously developed 3D mixed organoid model.[31] For this, membrane-bound tdTomato-labeled wild-type liver cholangiocyte organoids were derived from liver tissue isolated from mTmG transgenic mice as described previously.[32] These cultures are formed by liver progenitor cells that can be differentiated into functional hepatocytes.[8] In addition, Dendra2-labeled small intestinal cancer organoids were derived from Villin-CreER^{T2} Apc$^{fl/fl}$ Kras$^{G12D/WT}$ Trp53$^{fl/R172H}$ transgenic mice.[27,33] To study cellular interactions during liver metastasis, clumps of wild-type liver and small intestinal cancer cells were aggregated to enforce formation of single mixed organoids (Figure 1A).

First, we followed the cellular behavior in organoids fixed on day 1, 3 and 4 after plating. Both wild-type and cancer populations increased over a period of four days in pure organoids (Figure 1B). However, the number of wild-type cells contributing to mixed organoids gradually decreased at day 3 (±30%) and 4 (±20%) compared to day 1 (±55%) after mixing (Figure 1C). Furthermore, the total number of cells per organoid showed a

7.5-fold expansion at day 3 compared to day 1, while the absolute number of wild-type cells only increased 2.5 times and the cancer population was responsible for most of the overall expansion (Figure 1D). This indicates that, even though wild-type cells can proliferate, they are outcompeted in mixed organoids.

To gain insight into the dynamics of this competitive behavior, individual nuclei were tracked based on expression of Histone H2B-Cerulean3 using time-lapse microscopy. Pure wild-type organoids showed exponential growth with a doubling time of approximately 24 hours (Figures 1E, 1F and S1A; Video S1). However, the growth rate of wild-type cells was dramatically increased to 33 hours in the presence of cancer cells (Figures 1E, F and S1A; Video S1). Importantly, towards the end of the experiment the average expansion of the wild-type population was reduced from 5.5 times in pure conditions to 3.2 times in mixed organoids (Figure 1G), confirming the outcompetition of wild-type cells. Interestingly, we observed a correlation between the wild-type expansion rate and the percentage of wild-type cells that contributed to the mixed organoid at the start of the experiment. Mixed organoids with a higher percentage of wild-type cells showed an expansion rate that approached pure conditions, while those with a low wild-type contribution also displayed little expansion (Figure 1H). This indicates that the initial number of wild-type cells determines the speed of competition. Together, these data show that wild-type liver cells are outcompeted by cancer cells in mixed organoids.

Increased expansion of competing cancer

Next, we questioned whether competition could also alter cancer cell behavior. Therefore, we went back to time-lapse microscopy of H2B-Cerulean3 expressing cells (Figure 2A and Video S2). Tracking of the absolute number of cells for up to 70 hours showed that, even though cancer cells proliferated more than wild-type cells in pure organoids (Figures 1F, 2B and S2), the proliferation rate of cancer cells was further increased in mixed organoids (Figures 2B and S2). Furthermore, the average expansion of cancer cells increased from 10.4 in pure to 16.7 times in mixed organoids towards the end of the experiment (Figure 2C). Interestingly, the expansion of cancer cells was inversely correlated to the percentage of cancer cells in the mixed organoid (Figure 2D), suggesting that cancer cells benefit from the presence of relative higher number of wild-type cells. Thus, these data show that wild-type cells provide a growth supportive role that benefits cancer cell expansion.

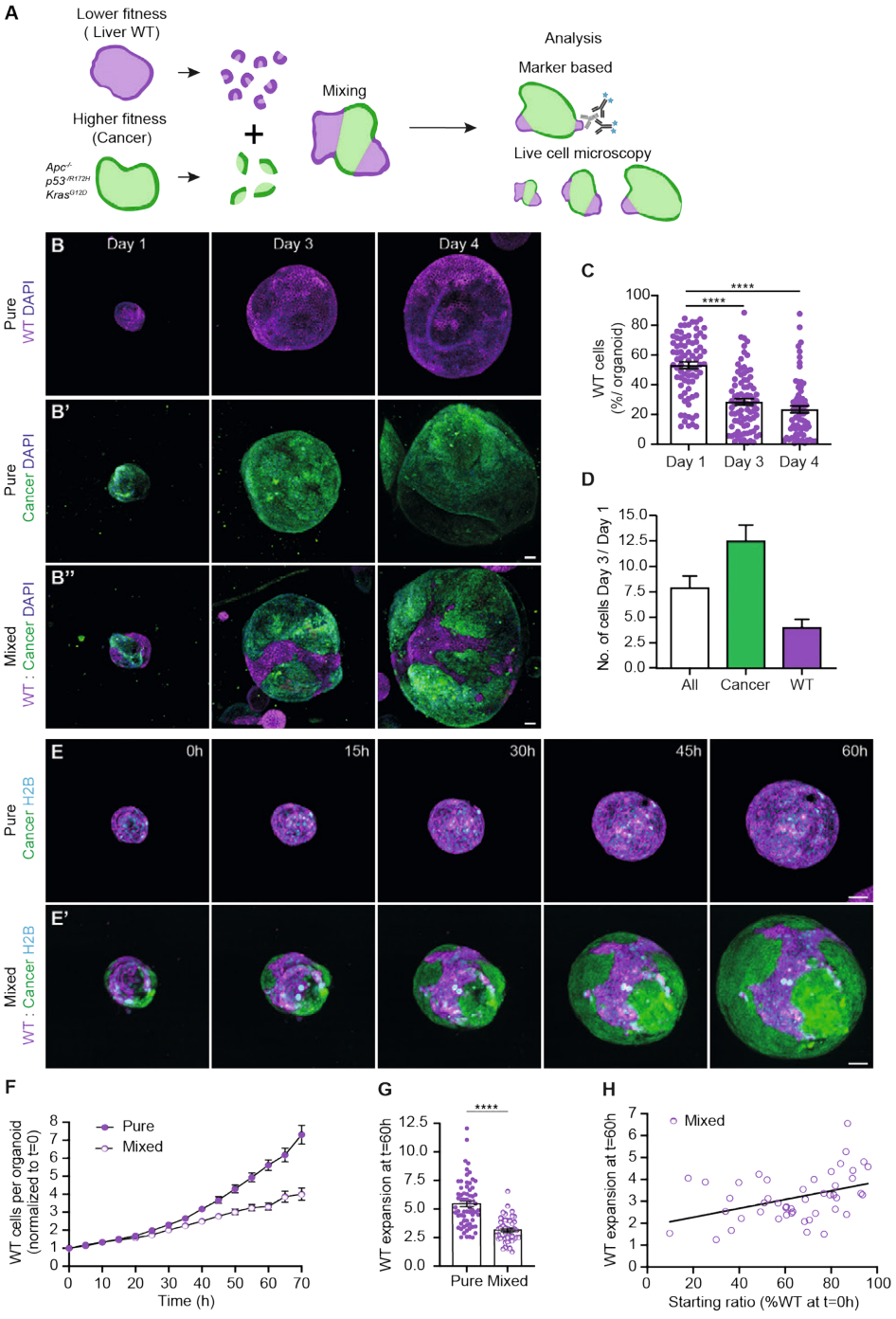

A
Lower fitness
(Liver WT)
Higher fitness
(Cancer)
Apc-/-
p53R172H
KrasG12D
Mixing
Analysis
Marker based
Live cell microscopy
B
Day 1
Day 3
Day 4
Pure
WT DAPI
B'
Pure
Cancer DAPI
B''
Mixed
WT : Cancer DAPI
C
WT cells
(%/ organoid)
****
****
Day 1
Day 3
Day 4
D
No. of cells Day 3/ Day 1
All
Cancer
WT
E
0h
15h
30h
45h
60h
Pure
Cancer H2B
E'
Mixed
WT : Cancer H2B
F
WT cells per organoid
(normalized to t=0)
Pure
Mixed
Time (h)
G
WT expansion at t=60h
****
Pure
Mixed
H
WT expansion at t=60h
Mixed
Starting ratio (%WT at t=0h)

Figure 1: Cancer cells outcompete wild-type liver cells (A) Schematic depiction of a 3D model for cell competition in murine liver and intestinal cancer organoids. (B) Representative maximum projections of 3D-confocal images of pure WT (B), cancer (B') and mixed (B'') organoids fixed at Day 1, 3 and 4 after plating and stained with DAPI (blue). (C) The percentage of WT nuclei contributing to mixed organoids 1, 3 and 4 days after mixing is shown; each dot represents one organoid (mean ± SEM; Kruskal-Wallis test; p<0.0001; n=78, 84 and 69 organoids). (D) Displays the absolute number of cells in organoids shown in (B); the ratio of 'day 3' over 'day 1' of all (white), cancer (green), and wild-type (magenta) cells are plotted (mean ± SEM; Ordinary one-way ANOVA). (E-H) Analysis of WT cells in pure and mixed H2B-Cerulean3 expressing organoids by live imaging. (E) Representative maximum projections of 3D-confocal images of time-lapse series of WT pure (E) and mixed (E') organoids. (F) Quantification of the number of WT nuclei in pure and mixed conditions per organoid normalized to the start of the time-lapse (mean ± SEM; paired t-test, two-tailed; p<0.0052; n= 65 and 47 organoids). (G) Quantification of WT expansion, the ratio of the number of WT nuclei at t=60h over t=0h is plotted; each dot represents one organoid (mean ± SEM; Mann-Whitney test, two-tailed; p<0.0001; n= 65 and 47 organoids). (H) Shows the WT expansion at t=60h plotted against the initial percentage of WT cells per mixed organoid (Simple linear regression; R^2=0.1639; p=0.0048, n=47 organoids). Scale bars represent 50µm. See also Figure S1 and Video S1.

Cancer induces compaction and cell cycle arrest of wild-type liver cells

So far, we showed a reduced expansion of the wild-type population in mixed organoids. To further understand the impact of competition on wild-type cells we closely examined interactions between the two cell populations. Two lines of evidence suggest that forces generated by cancer cells induce a morphological change in wild-type cells: 1) The nuclear shape of wild-type cells in mixed organoids appeared more elongated compared to wild-type nuclei in pure organoids (Figure 3A). 2) Measurement of the average distance between the five closest neighbors revealed a decreased inter-nuclear distance of competing wild-type cells (Figures 3B and C). Of note, competition did not affect the inter-nuclear distance of cancer cells in the same organoids (Figures 3B and S3A), indicating that these effects are not a consequence of the overall morphology of the tissue. Together, these data show that competition induces compaction of wild-type cells.

Cell compaction and tissue crowding have previously been described to cause active elimination of less fit cells.[34,35] However, in the time-lapse movies of mixed organoids we did not find evidence for increased apoptosis or extrusion of wild-type cells. This suggests that the lower expansion rate of competing wild-type cells is instead caused by an effect on cell proliferation. Therefore, we first used two complementary markers of cell proliferation to study the impact of competition on different phases of the cell cycle; DNA replication was visualized by incorporation of the thymidine analogue 5-Ethynyl-2'-deoxyuridine (EdU) and phosphorylation of Histone H3-Ser10 (pH3) was used to recognize mitotic chromatin.

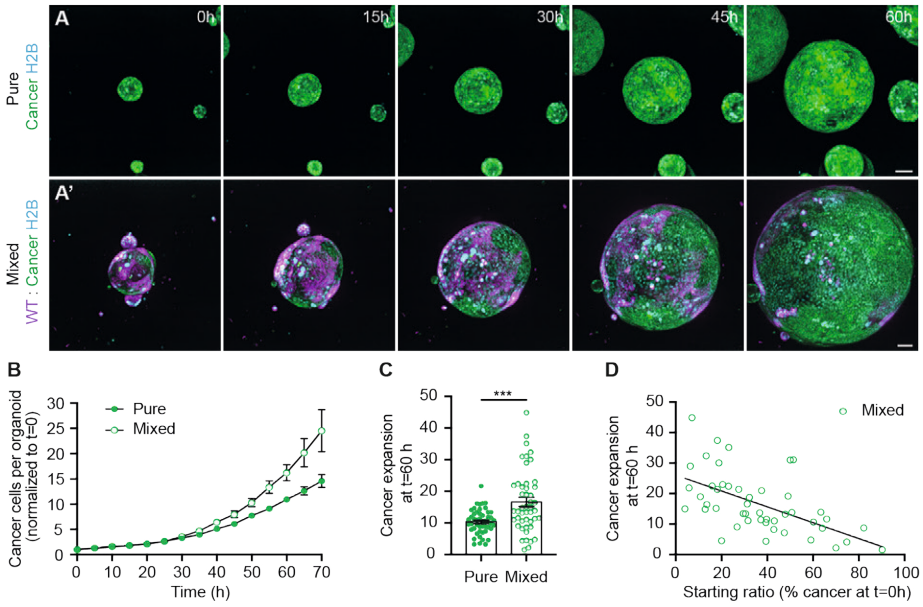


Figure 2: Increased expansion of competing cancer (A) Representative maximum projections of 3D-confocal images of time-lapse series of H2B-Cerulean3 expressing pure cancer (A) and mixed (A') organoids. (B) Quantification of the number of cancer nuclei in pure and mixed conditions per organoid normalized to the start of the time-lapse (mean ± SEM; paired t-test, two-tailed; $p<0.014$; n= 49 and 47 organoids). (C) Quantification of cancer expansion, the ratio of the number of cancer nuclei at t=60h over t=0h is plotted; each dot represents one organoid (mean ± SEM; Mann-Whitney test, two-tailed; $p<0.0004$; n= 49 and 47 organoids). (D) Shows the cancer expansion at t=60h plotted against the initial percentage of cancer cells per mixed organoid (Simple linear regression; $R^2=0.3229$; $p<0.0001$, n=47 organoids). Scale bars represent 50µm. See also Figure S2 and Video S2.

The combination of both markers allowed us to identify cells in S phase (EdU+), cells proceeding from S phase to mitosis (EdU+/pH3+) and cells in mitosis (pH3+). Importantly, for all three phases fewer wild-type cells were found in mixed organoids (Figures 3D-G), indicating that competition induces a decrease in wild-type proliferation throughout different phases of the cell cycle.

Next, as cell proliferation is a highly dynamic process, we aimed to visualize cell cycle progression in real-time. For this, wild-type organoids were derived from Fluorescent Ubiquitylation-based Cell Cycle Indicator-2 (FUCCI2) transgenic mice.[36] This allowed us to follow progression of wild-type cells from the G1 phase (based on hCDT1-mCherry) through S-G2-M phases (based on hGeminin-mVenus) of the cell cycle. Nuclei in pure wild-type organoids showed an alternating expression of hCDT1-mCherry and hGeminin-mVenus throughout the experiment (Figure 3H and Video S3), confirming continued active cell cycle progression of these cells. In contrast, where initially

cycling wild-type cells were detected in mixed organoids, this population rapidly decreased to almost absence towards the end of the experiment. Instead, there was an accumulation of the number of hCDT1-mCherry positive wild-type cells at later time-points (Figure 3H and Video S3). These finding were confirmed by quantification of expression of the FUCCI2 reporters in pure and mixed organoids fixed three days after plating (Figure S3D), which showed an increase of G1 and decrease of S-G2-M wild-type cells during competition (Figured 3I and 3J). Interestingly, we observed that a subpopulation of wild-type cells lost both FUCCI2 markers (Figures 3H insets and 3K). These cells arrested after degradation of hGeminin while CDT1 was not yet expressed and were in a putative G0 state. In addition, a similar growth arrested population was detected in the previously described cell proliferation experiment, where we found an increased percentage of wild-type cells that was negative for both EdU and pH3 in mixed organoids (Figure S3B). Even after extending the EdU pulse to 24 hours, ±30% of the competing wild-type population was EdU-/pH3- (Figure S3C), indicating a complete stop in the proliferation of a large population of competing wild-type cells. Together, these data show that cancer cells outcompete wild-type cells through compaction and a subsequent cell cycle arrest.

Increased competition through loss of WT progenitor state

A cell cycle arrest is typically linked to a high level of cell differentiation.[37] In addition, several types of cell competition are driven by forced differentiation of stem or progenitor cells. For example, in both the fly and mouse intestine increased differentiation causes loss of less fit cells.[24,25,38] Furthermore, stem cell displacement from the niche drives cell selection by forced differentiation in the mouse epidermis.[39,40] Therefore, we next wondered whether the cell cycle arrest of competing wild-type cells is correlated to an altered differentiation state. In adult liver tissue, progenitor cells can trans-differentiate into hepatocytes after chronic liver damage in a process known as ductal reaction and this can be mimicked in organoid cultures.[8,41] SOX9 is one of the main markers of the liver progenitor state and its expression strongly decreases during differentiation.[42–44] Therefore, we next focused on expression of this transcription factor. As expected, high nuclear SOX9 expression was found in pure liver progenitor cultures (Figures 4A and S4). Similarly, cancer cells showed heterogeneously high expression of SOX9, which was irrespective of the presence of wild-type cells (Figures 4A and S4). In contrast, competing wild-type cells showed a major reduction in average SOX9 expression (Figures 4A, 4B and S4).

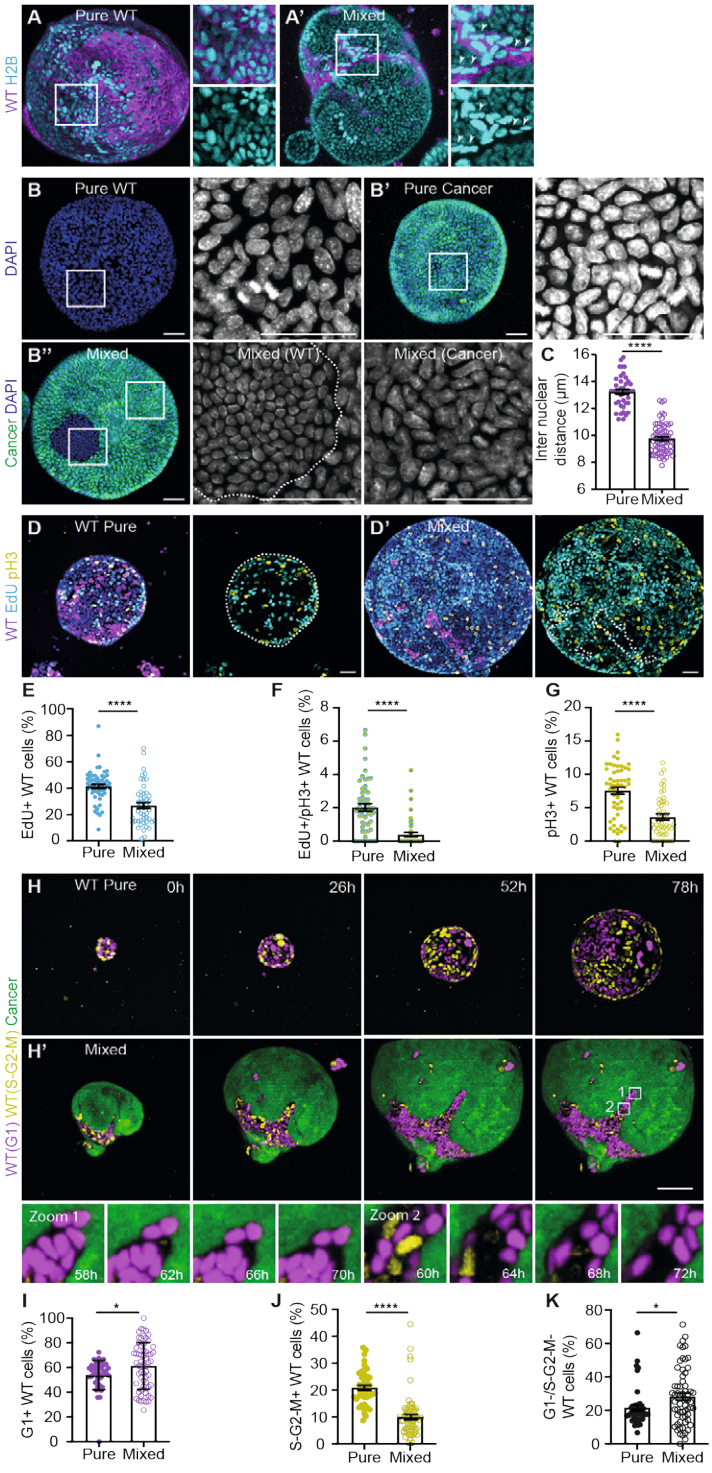

A
Pure WT
A'
Mixed
WT H2B
B
Pure WT
B'
Pure Cancer
DAPI
B''
Mixed
Mixed (WT)
Mixed (Cancer)
Cancer DAPI
C
Inter nuclear distance (µm)
Pure Mixed
D
WT Pure
D'
Mixed
WT EdU pH3
E
EdU+ WT cells (%)
F
EdU+/pH3+ WT cells (%)
G
pH3+ WT cells (%)
H
WT Pure
0h
26h
52h
78h
H'
Mixed
WT(G1) WT(S-G2-M) Cancer
Zoom 1
Zoom 2
I
G1+ WT cells (%)
J
S-G2-M+ WT cells (%)
K
G1-/S-G2-M- WT cells (%)

Figure 3: Cancer induces compaction and cell cycle arrest of WT liver cells (A) Representative maximum projections of 3D-confocal images of pure WT (A) and mixed (A') organoids. The insets display a 2x magnification of the area in the white box. Nuclei are visualized by expression of H2B-Cerulean3 (cyan). (B) Representative maximum projections of 3D-confocal images of pure WT (B), pure cancer (B') and mixed (B'') organoids. The insets display a 2.3x magnification of the area in the white box and white dotted line outlines the wild-type population. Nuclei are visualized with DAPI (grey). (C) Quantification of the inter-nuclear distance of WT cells in pure and mixed organoids; each dot represents one organoid (mean ± SEM; Ordinary one-way ANOVA, Sidak's multiple comparisons test; $p<0.0001$; n= 41 and 64 organoids). (D) Representative maximum projections of 3D-confocal images of pure WT (D) and mixed (D') organoids. Cells in S phase are labeled with EdU (cyan), and mitotic cells are marked by pH3 (yellow); cells that progressed from S phase to mitosis within 1.5h are double positive; the white dotted line outlines the wild-type population. The percentage of WT cells in S phase (E), that progressed from S phase to mitosis (F) and in mitosis (G) pure WT and mixed organoids are plotted; each dot represents one organoid (mean ± SEM; Ordinary one-way ANOVA, Sidak's multiple comparisons test; $p<0.0001$; n= 55 and 47 organoids). (H) Representative maximum projections of 3D-confocal images of time-lapse series of pure WT (H) and mixed (H') organoids. Cell cycle phase of WT cells is visualized by expression of hCDT1-mCherry (G1, magenta) and hGeminin-mVenus (S-G2-M, yellow). The insets display a 7.5x magnification of the area in the white box. (I-K) Quantification of WT cell proliferation in pure and mixed organoids. The percentage of WT cells in G1 (I), S-G2-M (J) and negative (K) are plotted; each dot represents one organoid (mean ± SEM; unpaired t test, two-tailed; $p<0.0275$, I; $p<0.0001$, J; $p<0.0318$, K; n=43 and 62 organoids). Scale bars represent 50µm. See also Figure S3 and S3.

Furthermore, the percentage of wild-type cells with high SOX9 expression was severely reduced in mixed organoids (Figure 4C), indicating that fewer progenitor cells remained in the competing wild-type population. On the other hand, the percentage of wild-type cells with low SOX9 expression was strongly increased (Figure 4D), suggesting that competition induces differentiation in most wild-type cells. Interestingly, we observed that organoids with fewer remaining wild-type cells also had more cells with low SOX9 expression (Figure 4E). Together, this suggests that more cancer cells can inflict a stronger response and that the strength of competition is determined by the relative contribution of cell populations. Thus, cell competition driven by intestinal cancer cells induces a loss of the progenitor state of wild-type liver cells.

Differentiated WT cells are effectively outcompeted

So far, our findings suggest that competition causes a cell cycle arrest and loss of progenitor state in wild-type cells. We next aimed to analyze the effect of differentiation on the outcome of competition. For this, wild-type liver progenitor cells were exposed to a well-characterized 15 days differentiation protocol (Figure 5A). This generated differentiated hepatocyte-like organoids that lack expression of SOX9 (Figure 5B). Furthermore, in contrast to wild-type progenitors, which showed a 4.5-fold expansion over de course of two days (Figures 5C and S5B), differentiated cells were non-proliferative (Figures 5C and 5D). This was confirmed using time-lapse microscopy (Figures S5A and Video S4). Importantly, the reduced proliferation was not caused by a general cytostatic effect of the differentiation medium as expansion of pure cancer organoids was not affected (Figures S5C-G and Video S5).

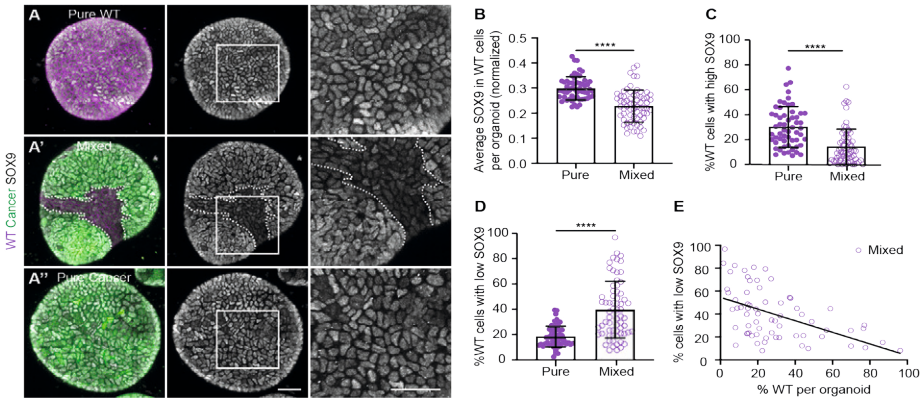


Figure 4: Increased competition through loss of WT progenitor state (A) Representative maximum projections of 3D-confocal images of WT pure (A), mixed (A') and cancer pure (A'') organoids fixed 3 days after plating. The organoids were stained for SOX9 (grey). The insets display a 2.5x magnification of the area in the white box and the white dotted line outlines the wild-type population. (B-E) SOX9 expression in WT cells. (B) Displays the average SOX9 intensity in WT cells in pure and mixed organoids; each dot represents one organoid (mean ± SEM; Mann-Whitney test, two-tailed; $p<0.0001$; n= 57 and 67 organoids). The percentage of WT cells with high (C) and low (D) SOX9 expression is plotted and each dot represents one organoid (mean ± SEM; Kruskal-Wallis test; $p<0.0001$; n= 57 and 67 organoids). (E) Shows the percentage of WT cells with low SOX9 levels plotted against the percentage of WT cells per organoid (Simple linear regression; $R^2=0.2511$; $p<0.0001$, n=67 organoids). Scale bars represent 50µm. See also Figure S4.

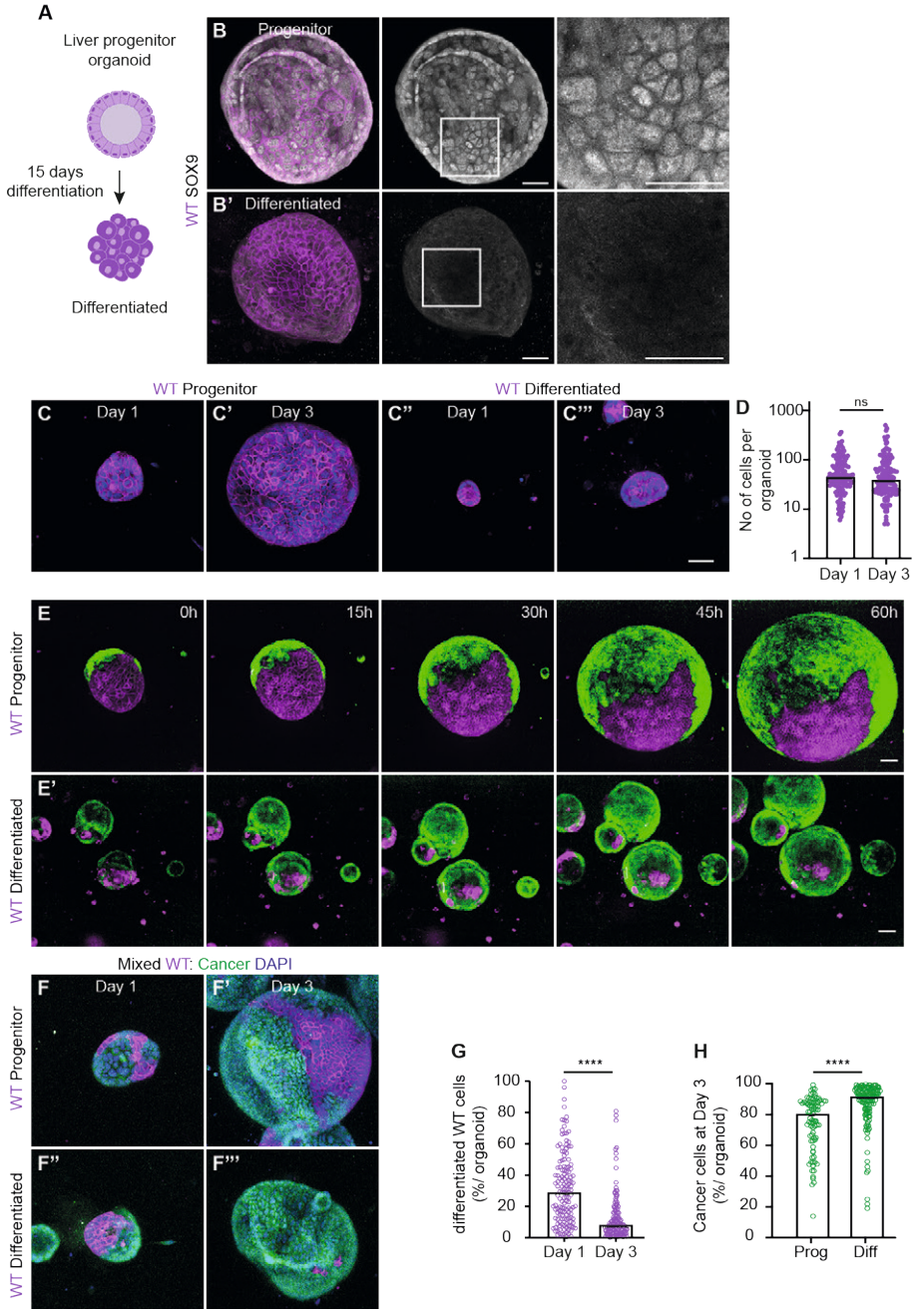
Next, differentiated wild-type organoid cultures were disrupted and aggregated with clumps of cancer cells to generate mixed organoids (Figure S5H). Using time-lapse microscopy, we observed a rapid takeover of the mixed organoids by the cancer population (Figure 5E and Video S6). This effect was obvious from early stages of the experiment, while the effects of competition on progenitor cells first start to appear after 30 hours (Figures 5E, 1F and Video S6). Together, this indicates that outcompetition of differentiated wild-type cells is much more efficient than that of progenitor cells. Indeed, the number of differentiated wild-type cells that contribute to mixed organoids reduced 3.5 times over the course of two days (Figures 5F and 5G) compared to a two-fold reduction of competing progenitor cells (Figures 5F and S5I). This led to mixed organoids that contained over 90% cancer cells three days after plating (Figures 5F and 5H). Thus, differentiated wild-type liver cells are non-proliferative and therefore more susceptible to outcompetition by intestinal cancer cells.

WT liver acts as a scaffold for tumor growth during competition.

Next, we aimed to develop a model which better resembles the overall organ structure of the liver and allows us to study liver-cancer interactions in a self-organizing matrix-free environment. We were inspired by reported microtissues from organs such as brain[45], heart[46] and liver[47–50] that were previously used to study the tissue development and response to injury. For this, pieces of hepatocyte-like differentiated organoids were aggregated in a U-bottom plate to obtain self-organizing liver microtissues with a size of 1-2mm (Figure 6A). To avoid cues coming from the extra-cellular matrix we withdrew the culture matrix during aggregation. These liver microtissues were devoid of SOX9 expression illustrating that the differentiation status of hepatocyte-like wild-type liver cells was conserved (Figure 6B).

To study competitive interactions, we added pieces of cancer organoids during aggregation (Figure 6A). This resulted in liver metastasis microtissues where patches of cancer cells are surrounded by liver tissue (Figure 6C). While characterizing these liver metastasis microtissues we made two observations: 1) pure cancer cells had difficulties to independently form coherent microtissues and had a size that was markedly smaller than that of pure wild-type microtissues (Figures 6C-E and S6). However, when surrounded by liver microtissue, cancer cells thrived (Figures 6C, 6E and S6). 2) In contrast, generation of pure wild-type microtissues was very efficient (Figure 6C and S6), while the volume of wild-type was much reduced in liver metastasis microtissues (Figures 6C, 6D and S6). This indicates that cancer tissue benefits from interactions with wild-type tissue and uses this as a scaffold for expansion. Furthermore, wild-type tissue suffers from the presence of cancer cells.

To understand the nature of the reduction in wild-type liver tissue volume we questioned whether this was caused by induction of cell death. Staining for cleaved-Caspase3 showed a higher number of apoptotic wild-type cells in mixed compared to pure microtissues (Figures 6F and 6G). Interestingly, in these liver metastasis microtissues, cancer and liver tissue intermingled and closely interacted. We observed that cell death was particularly prominent at the interface between the different cell populations (Figure 6F), suggesting that short-ranged interactions are important for elimination of wild-type cells. Thus, we developed a novel matrix-free model to study competition between liver and cancer tissue. Using these liver metastasis microtissues we show that wild-type liver tissue acts as a scaffold for intestinal cancer cells. This subsequently induces elimination of the wild-type cells, which further promotes colonization of liver tissue by cancer cells.


A
Liver progenitor organoid
15 days differentiation
Differentiated
B Progenitor
B' Differentiated
WT SOX9
WT Progenitor
WT Differentiated
C Day 1
C' Day 3
C'' Day 1
C''' Day 3
D
ns
No of cells per organoid
1000
100
10
1
Day 1 Day 3
E 0h
15h
30h
45h
60h
WT Progenitor
E'
WT Differentiated
Mixed WT: Cancer DAPI
F Day 1
F' Day 3
WT Progenitor
F''
F'''
WT Differentiated
G
****
differentiated WT cells (%/ organoid)
100
80
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40
20
0
Day 1 Day 3
H
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Cancer cells at Day 3 (% organoid)
100
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0
Prog Diff

Figure 5: Differentiated WT cells are effectively outcompeted (A) Schematic depiction of differentiation from progenitor cholangiocytes (top) to hepatocyte-like organoids (bottom) organoids. (B) Representative maximum projections of 3D-confocal images of SOX9 staining in progenitor (B) and differentiated (B´) WT organoids. The insets display a 3x magnification of the area in the white box. (C) Representative maximum projections of 3D-confocal images of progenitor (C and C') and differentiated (C'' and C''') WT organoids, fixed 1 day (C and C'') and 3 days (C' and C''') after plating; nuclei are visualized with DAPI (blue). (D) Quantification of the number of differentiated WT cells; each dot represents one organoid (Median; Kruskal-Wallis test, Dunn´s multiple comparison test; p>0.9999; n=132 and n=129). (E) Representative maximum projections of 3D-confocal images of time-lapse series of mixed organoids generated from WT progenitor (E) and WT differentiated (E') cells. (F) Representative maximum projections of 3D-confocal images of mixed organoids from WT progenitor (F and F') and WT differentiated (F'' and F''') organoids, fixed at day 1 (F and F'') and day 3 (F' and F''') after plating; nuclei are visualized with DAPI (blue). (G) shows the percentage of differentiated WT cells in mixed organoids at day 1 and day 3. (H) displays the percentage of cancer cells in mixed organoids generated from WT progenitor (left) or differentiated (right) cells; each dot represents one organoid (Median; Kruskal-Wallis test, Dunn´s multiple comparison test; p<0.0001; n=140 and n=128, G; n=81 and n=128, H). Scale bars represent 50µm. See also Figure S5 and Videos S4-S6.

DISCUSSION

Colonization of secondary organs is one of the most rate-limiting steps in the formation of metastasis and most cancer cells are eliminated by defense mechanisms of the host tissue before metastasis can form.[51] Upon arrival, cancer cells need to adapt to a novel microenvironment. This requires an intricate interplay between the metastatic cancer cells and a large variety of cell populations in the new organ. A large body of work has focused on interactions of cancer cells with non-epithelial cell populations. In the liver, for example, growth of colorectal cancer metastases is repressed by activated B cells.[52] In contrast, high levels of liver fibrosis correlate with a poor prognosis for patients with colorectal cancer, indicating a pro-metastatic effect of fibroblasts.[53] In addition, hepatic stellate cells, liver-specific mesenchymal cells, promotes the exit from dormancy of metastasized breast cancer through induction of a fibrotic injury response.[54] Even though hepatocytes can assist liver metastasis through a systemic response that facilitates formation of a pro-metastatic niche[55], direct interactions of metastatic cancer cells with the epithelial cells in the liver are less well-understood. Here, we identified multiple cellular interactions of intestinal cancer with the two main epithelial cell types of the liver. This multi-step process benefits the growth of cancer cells through outcompetition of healthy wild-type cells. Interestingly, such cellular interactions have previously been suggested based on histopathological growth patterns of liver metastasis.[15] Especially, the replacement pattern, which shows direct interactions and intermingling of liver tissue and cancer cells and is correlated with a poor outcome for patients. However, these studies are primarily based on end-point analysis of histopathological samples or imaging methods that do not reach cellular resolution. The culture models that we have developed here allow the dynamic

analysis of such competitive interactions with a subcellular and temporal resolution. Importantly, each dedicated model is optimized to study aspects of this multi-step competition process.

We previously showed that, at the primary site, competition driven by intestinal cancer cells can enforce a cell state transition of surrounding healthy small intestinal cells.[27] Interestingly, an analogous response is provoked in healthy liver tissue by the same intestinal cancer cells. However, during competition at the primary site, the neighboring intestinal wild-type cells dedifferentiate, while instead at the metastatic site, progenitor cells undergo cell cycle arrest and differentiation. Remarkably, in both tissues this is the natural response to tissue damage; In the intestine, injury causes reversion to a primitive fetal-like state[29,30,56] and chronic injury in the adult liver induces differentiation of cholangiocytes into hepatocytes through the so-called ductal reaction.[9,10] Together, this suggests that surrounding organ tissue reacts to cancer-driven competition by activation of an injury response that is embedded in the host tissue. In both tissues, activation of an injury response and the subsequent outcome of competition are similar and result in elimination of the wild-type population and increased growth of the cancer population. However, the steps that are taken to reach this outcome are tissue-specific. Where intestinal cells are rapidly forced to undergo apoptosis, the initial effect of competition on wild-type liver progenitors is compaction and initiation of a cell cycle arrest. Only differentiated cells are eventually eliminated via apoptosis. This has a major effect on the timing of events and the impact on the cancer population. Where inhibition of apoptosis is sufficient to block competition of primary intestinal cancer, this will unlikely prevent loss of liver tissue. In fact, the cancer population initially benefits from the presence of wild-type tissue. For example, competitive interactions with progenitor cells causes increased expansion of cancer cells and interactions with differentiated cells provide a scaffold for cancer cell colonization. Understanding how this feedback is regulated, in terms of the supply of niche factors, identification of involved paracrine signaling pathways or mechanical interactions is an important aim for further research.

The here described liver metastasis microtissues were inspired by microtissues from different organs such as brain[45] or heart[46], which have been instrumental to understand the function and response of the tissue of origin to different types of damage or drug response. Here, they were crucial to identify competitive interactions between differentiated liver tissue and cancer cells. In particular, the requirement of the wild-type tissue to provide a scaffold for colonization by cancer cells. Previously, liver microtissues with a functional biliary ductal network were generated, through aggregation of hepatocytes, cholangiocytes and fibroblasts[57].

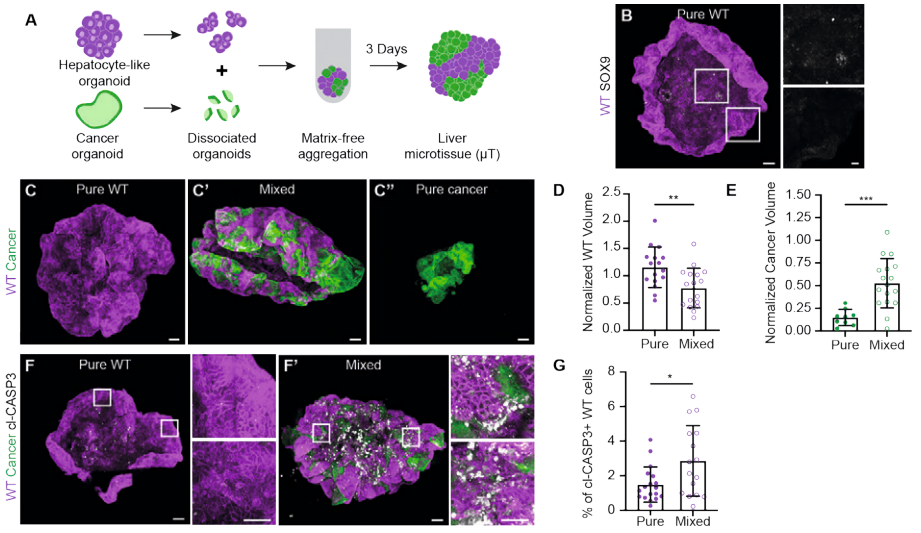

Figure 6: WT liver acts as a scaffold for tumor growth during competition. (A) Schematic depiction of generation of liver metastasis microtissues. (B) Representative 3D-reconstructed stitched confocal images of a pure WT microtissue stained for SOX9 (grey); nuclei are visualized with DAPI (blue). The insets display a 2.5x magnification of the area in the white box. (C) Representative 3D-reconstructed stitched confocal images of from pure WT (C), mixed (C') and pure cancer (C'') microtissues. (D-E) Quantification of the WT (D) and cancer (E) volume contributing to pure and mixed microtissues; each dot represents one microtissue (mean ± SEM; unpaired t-test, two-tailed; p<0.0057; n= 16 and 17 microtissues, D; p=0.005; n= 9 and 17 microtissues, E). (F) Representative 3D-reconstructed stitched confocal images of WT pure (F) and mixed (F') microtissues stained for Cleaved Caspase3 (grey); nuclei are visualized with DAPI (blue). The insets display a 7x magnification of the area in the white box. (G) Quantification of Cleaved Caspase3 positive WT cells within the epithelium of the pure and mixed microtissues; each dot represents one microtissue (mean ± SEM, Mann-Whitney test, two-tailed; p=0.0463; n= 18 and 16 organoids). Scale bars represent 100 µm excluding magnifications in, where scale bar represents 10 µm. See also Figure S6.

Combining these cell types with intestinal cancer cells would allow full recapitalization of the major cellular interactions during liver metastasis. Importantly, the exclusion of immune cells from this culture system allows us to untangle the complex cellular interplay without interference of the immune system. It would open the possibility to model other histopathological growth patterns of liver metastasis[15], such as the desmoplastic type, which is characterized by interactions with stromal cells. In addition, the models are easily adaptable to other cancers that are prone to metastasize to liver, such as breast and pancreatic cancer. Together, our findings can be used to create a true molecular understanding of how healthy cells and cancer cells interact and influence each other, which is a critical step forward towards competition-based cancer therapy.

ACKNOWLEDGMENTS

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METHODS

Culture of mouse organoids

Please refer to the tables (below) for the composition and recipes of all culture media and solution. Wild-type cholangiocytes were derived from Rosa26-CreERT2::mTmG[58] females 8-21 weeks, FUCCI2[36] males 8-21 weeks, Rosa26-rRtTA-H2BmCherry males 8-21 weeks as previously described[32]. In short, isolated liver tissue was minced on a petri dish and incubated in a digestion solution (Collagenase A and Dispase II). After digestion, several rounds of centrifugation were used to discard debris and isolate ducts. Isolated ducts were plated in Cultrex PathClear Reduced Growth Factor Basement Membrane Extract Type 2 (BME2) in mouse isolation medium. Small intestine cancer organoids, derived from the small intestine of Villin-Cre^{ERT2}*Apc*$^{fl/fl}$*Kras*$^{G12D/WT}$*Tr53*$^{fl/R172H}$ mice were previously reported.[33] All lines were cultured as described previously[31] in drops of BME2 in mouse liver expansion or isolation medium + Noggin.

Transduction of organoids

Lentiviral transduction was preformed using standard procedures. In short, lentivirus was produced in HEK293T by co-transfection of a dual lentiviral vector 3rd generation pLentiPGK Hygro DEST H2B-mCerulean3 with helper plasmids pMDLg/pRRE, pRSV-Rev and pMD2.G (gifts from Markus Covert and Didier Trono, Addgene plasmids #90234, #12251, #12253 and #12259). Viral particles were harvested from cells four days after transfection and concentrated using 50kDa Amicon Ultra-15 Centrifugal Filter Units (Merck, cat#UFC905024). Organoids were dissociated by mechanical disruption and dissolved in 250 µL ENR medium supplemented with Y-27632 and Polybrene together with the concentrated virus. Cells were incubated at 32°C while spinning at 600xG for 1 hour followed by a 4-hour incubation at 37°C before plating in BME2. Selection was carried out from day 3 onwards with hygromycin.

Generation of mixed organoids

Mixed organoids were generated as described previously.[31] In short, suspensions of small clumps of cells were generated from organoids by mechanical disruption and divided over Eppendorf vials in a 1:1 ratio (WT: cancer). Cells were concentrated by mild centrifugation and the pellet was resuspended in a small volume of mouse liver expansion medium and incubated at 37°C for 30 minutes. Cell aggregates were plated in BME2 and cultured in mouse liver expansion medium. For imaging purposes cells were plated in µ-Plate 96 well black uncoated plates or 15µ-Slide 8 well^{high} uncoated plates.

Differentiation

Cholangiocyte organoids were subjected to a 15-day differentiation protocol developed by STEM CELL Technologies ("Initiation, growth and differentiation of human organoids using HepatiCult™") following the suppliers' guidelines. In short, organoids were maintained in expansion medium for 5 days with a medium refreshment at day 3. On day 5 differentiation medium was added and refreshed every 3 days (on day 8, 11 and 14). At day 15 differentiated organoids were ready for use. For generation of mixed organoids the cultures were supplemented with Noggin at day 14. Cancer organoids were cultured in with differentiation medium + Noggin for at one day prior to mixing.

Generation of microtissues

Cancer organoids and hepatocyte-like organoids obtained by differentiation (described above) were kept in differentiation medium + Noggin for 24h prior to microtissue formation. Organoids were harvested and wash three times with basic medium to remove the culture matrix. After the last wash, the organoids were mechanically disrupted pipetting through a fire-polished glass Pasteur pipet. Cancer organoids were intensively disrupted to generate small pieces and wild-type organoids were treated with a gentle disruption to prevent induction of an injury response. The disrupted organoids were divided over Eppendorf vials in a 2:1 ratio (WT liver : cancer) and concentrated by gentle centrifugation. Cell pellets were dissolved in 5-8 µL differentiation medium +Noggin and incubated at 37°C for 30min. After aggregation, 100 µL /well of differentiation medium +Noggin was added and divided over 96w U bottom plates. Microtissues were allowed to mature at 37°C for 3 days.

Immuno-fluorescence

Immuno-fluorescence assays were performed as previously described[31], while protected them from light. In short, organoids were fixed with 4% Paraformaldehyde

(PFA) in PBS for 20-30 minutes. Microtissues were fixed by removal of 50 µL medium and addition of 50 µL of 8% PFA in PBS for 20min. Fixation was followed by a minimum of three washes with PBS0 and samples were stored in PBS0 at 4 °C until use. Samples were permeabilizated and blocked in PBS/0.5% BSA/ 0.5% TX-100 for at least 30 minutes and incubated with primary antibodies overnight at 4 °C. After washing three times in PBS/ 0.1% TX-100 samples were incubated with secondary antibodies and DAPI for from 1-3 hours at RT (organoids) up to overnight at 4 °C (organoids and microtissues)[5]. After three washes with PBS/ 0.1% TX-100 and twice with PBS the samples were kept in PBS for imaging. Microtissues, were mounted in RapiClear clearing solution on glass slides using iSpacers. For EdU detection, organoids were treated with EdU staining solution for 30 minutes prior to antibody incubation.

Microscopy

Imaging of fixed samples and time-lapse videos of FUCCI2 were acquired on a Carl Zeiss LSM880 Fast AiryScan Confocal Laser Scanning microscope (Axio Observer 7 SP with Definite Focus 2) equipped with a CO2 and 37 °C incubator. A Plan-Apochromat 20x/0.8 WD=0.55mm air objective was used to obtain 12bit images with a 1024 resolution, via bidirectional imaging, using a pinhole size of 1AU. A Z-slice thickness of 2.5 µm (fixed samples) and 5 µm (timelapse and microtissues) was used to cover the complete thickness of the sample (17 to 150 slices). For time-lapse imaging a time interval of 2 hours was used for a total duration of 80 hours. The following laser lines were used: 405nm, Laser Argon Multiline (445/488/514), 561nm and 633 nm.

Live-imaging of pure and mixed organoids containing mTmG-labeled wild-type cells were acquired with an Eclipse Ti2-E with PFS (Nikon, Japan) equipped with a Confocal Spinning Disc Unit CSU-W1-T1 (Yokogawa, Japan). A Plan Apo λD 20x / 0.80, WD=0.80, MRD70270 (Nikon, Japan) dry objective was used to obtain 16bit images with a 1024 resolution and 2x2 binning. A Z-slice thickness of 5 µm was used to cover the complete thickness of 300 µm. The time interval was 5 hours for a total duration of 60-70 hours. The following laser lines were used: Stradus 445 (441 nm / 80 mW, Vortran, USA), Stradus 488 (490 nm / 150 mW, Vortran, USA) and OBIS 561 (561 nm, 150 mW, Coherent, USA). Temperature control and CO2: STXG-PLAMX-SETZ21L (TokaiHit, Japan) were set at 37 °C and 5% CO2.

Image Analysis

Image processing and analysis were performed using the open-source platform FIJI[59] in combination with computational routines developed in the programming language Julia. For each raw image, program was developed that facilitated the selection of

individual organoids in the confocal and lateral planes of the raw image based on the DAPI channel. Next, a general computational pipeline was applied for each organoid; 1) kernel size definition, 2) image processing and 3) 3D object classification and analysis.

1) Kernel size definition. The kernel size, a theoretical value that is equal to the minimal size of the objects of interest inside of the image, was identified by measurement of the raw image after application of a rude processing pipeline, facilitating the automatic high-throughput processing of all the images. The processing pipeline was based on the usage of the average plus the standard deviation as the value to distinguish between foreground and background, and the application of a median filter (with a kernel size equal to the pixel resolution of the image). Lastly, the Euclidean Distance Transform was calculated and used to detect the maximum values. This correlates to the radius of the objects of interest (kernel size) in the 5% of confocal slices with the highest measurements of optical density, which corresponded to planes with the highest biological content. Finally, the kernel size was determined as the average of these values. For time-lapse data (Figures 1, 2 and 5), an additional step was added: harmonization of the kernel size for all the frames of the time-lapse by taking the average value of the kernel size calculated for the individual time frames.

2) Image processing. To extract and enhance the biological signal from the different channels of each image specific routines were applied. **DAPI** Intensities were homogenized, and background subtraction was performed using an adapted Top-Hat algorithm. This involved subtraction of a copy of the raw image after application of a Gaussian blur, minimum and maximum filters, based on the kernel radius calculated in the previous step. Next, a median filter was applied to preserve the shape of the nuclei. Background and foreground were distinguished by setting a cut-off value based on the average intensity of each pixel below the average intensity of the entire confocal slice histogram. The watershed algorithm in Fiji was used for segmentation of the objects in 2D. To avoid sharp borders and false object detection, two rounds of watershed algorithms were applied, with an intermediate step of median filtering (kernel size equal to the pixel resolution). The raw binary and segmented images were multiplied with the raw data to preserve the original pixel intensities. In order to detect the centroids, dimensions and orientation of each nuclei, the Object Segmentation and Counter Analytical Resources (OSCAR) model was applied using the parameters and measurements previously described.[46,60] **Dendra-2** (cancer) and **mTmG** (wild-type) were used to classify the different cell populations and therefore processed in parallel. Both channels were processed using a pipeline that included Gaussian blur, maximum, and minimum filters that were applied to each confocal slice. For the mTmG channel, this was followed by subtraction of the Dendra-2 channel. The resulting images were thresholded using the average intensity of each pixel that was

below of the average intensity of the whole confocal slice as a cut-off value. In EdU experiments (Figure 3) **H2B-mCherry**, expressed wild-type cells, was the sole marker that was used for classification of the cell populations. Therefore, a multi-step pipeline of filters was developed to validate correct classification. First, with a minimum filter was applied, followed by an adapted Top-Hat algorithm (kernel radius not multiplied by two), and a median filter with half the value of the kernel radius to smooth object borders. Outliers were removed to eliminate small patches of expression inside nuclei. The image was then binarized and multiplied by the raw data to preserve the original intensities. **EdU & pH3** processing was carried out in three steps. First, the previously explained advanced top-hap algorithm was applied to remove background and equalize the intensities of signal across the image. Then, Fiji routine was used to automatically apply the Li's Minimum Cross Entropy thresholding method based on the iterative version[61] over the intensity histogram formed by all confocal slices for each image. For EdU this was followed by application of an opening filter over the binary image to remove small artefacts while preserving the objects. For pH3, we applied a grayscale morphological opening and background subtraction based on a cut-off value that was the average of the intensities that were higher than the average intensity for the entire histogram. For **SOX9** the adapted top-hat algorithm was used, followed by application of a median filer and outlier detection in order to smoothen the signal.

3) 3D object classification and analysis. The last step of our analysis is used to extract 3D information from the processed images at a single cell level. To achieve this, the DAPI channel was subjected to an artificial intelligence routine that was specifically designed to detect objects in densely packed environments. Our routine has been thoroughly tested and optimized for the phenotypical characterization of 3D nuclei in multicellular samples of embryos and hearth microtissues.[46,60] The spatial dimensions of each 3D nucleus were used to quantify the volume of positive signal within each nucleus (referred to as marker volume) and calculation of the average intensity. For classification of cells (e.g. wild-type or cancer), the parameter alpha was introduced, which represents the ratio between the marker volume and the total volume. For the detection of wild-type cells using mTmG, EdU, and pH3 markers, an alpha value of 0.1 was used. For classification of wild-type cells based on H2B-mCherry alpha was set to 0.25. Classification of SOX9 expression was based on the relative intensity of SOX9 in all wild-type nuclei. For this, the individual experiments were first normalized based on the maximum intensity in the experiment. Next, the relative intensities of all wild-type nuclei were combined to determine the distribution of SOX9 expression within the whole cell population (See Figure S4). The 25 and 75 percentile values were used as threshold to respectively define SOX9-low and SOX9-high cells.

Imaris software was used for quantification of FUCCI2, compaction and microtissue experiments. Quantification of cell number and volume was performed on 3D reconstructed images. In short, individual nuclei were segmented using the "spots" function and classified as wild-type (Dendra2-) or cancer (Dendra2+). The average distance to the five closest neighbors was calculated for each nucleus as a proxy for internuclear distance. For FUCCI2 quantifications, the average intensity of hCDT1-mCherry and hGeminin-mVenus was measured. Thresholding was used to classify wild-type nuclei as G1 (mCherry+/mVenus-), S-G2-M (mCherry-/mVenus+) or G0-like (mCherry-/mVenus-).

3D reconstructions of microtissues from five independent differentiation experiments (Figure S6) were analyzed. The "surface" function was used to mask mTmG (wild-type) and Dendra2 (cancer) populations in pure and mixed microtissues and the total volume of the surfaces was calculated.

Quantification of Cleaved-Caspase3 was performed on 3D reconstructions of microtissues from four independent differentiations. For this, three crops with a dimension of 250 µm x 250 µm x 50 µm (X/Y/Z) were made in a blinded fashion. All quantifications were performed by a different person to avoid bias. First, wild-type nuclei were segmented and counted using the "spots" function in the DAPI channel. Next, in mixed conditions the "surface" function was used to classify wild-type cells. Lastly, the number of Cleaved-Caspase3+ cells within the wild-type epithelium was determined (excluding debris and extruded cells).

Visualization

Images were either 3D reconstructed in Imaris or a maximum projection was made in Fiji (indicated in the figure legends). For microtissues, raw images were first stitched using Zen software and colors were adjusted in Imaris prior snapshots were taken Images and movies were converted to RGB using FIJI, cropped and when necessary, smoothened, cropped, rotated and contrasted linearly.

Tables

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Sox9 (1:500)	Merck Millipore	AB5535
Anti-GFP (1:1000)	Abcam	ab6673
Anti-RFP (1:1000)	Chromo	5f8-150
Anti-Ki-67 (SolA15) (1:100)	ThermoFisher Scientific	14-5698-82
Anti-pH3 (1:500)	Merck Millipore	06-570
Anti-Cleaved Caspase 3 (Asp175) (1:200)	Cell Signaling Technology	9661S
Donkey anti-Goat, Alexa Fluor 488 (1:500)	ThermoFisher Scientific	A-32816
Donkey anti-Mouse Alexa Fluor 555 (1:500)	ThermoFisher Scientific	A-31570
Donkey anti-Mouse, Alexa Fluor 647 (1:500)	ThermoFisher Scientific	A-31571
Donkey anti-Rabbit, Alexa Fluor 555 (1:500)	ThermoFisher Scientific	A-31572
Donkey anti-Rabbit, Alexa Fluor Plus 647 (1:500)	ThermoFisher Scientific	A-31573
Donkey anti-Rat, Alexa Fluor 647 (1:500)	ThermoFisher Scientific	A-48272
4', 6-Diamidino-2-phenylindole Dihydrochloride (DAPI) (1:2000)	Toronto Research chemicals	D416050; CAS:28718-90-3
Chemicals, peptides, and recombinant proteins		
Cultrex PathClear Reduced Growth Factor Basement Membrane Extract Type 2	Bio-Techne	3533- 005-02
Doxycycline (0.425 mM)	Sigma Aldrich	D9891
Hygromycin (0.5mg/mL)	MedChemExpress	HY-B0490/CS-2609
Polybrene (8mg/ml)	Sigma Aldrich	TR-1003
Collagenase A (0.125mg/mL)	Roche	10103586001
Dispase II (0.125mg/mL)	Sigma-Aldrich	102441796
RapiClear® 1.49	SunJin Lab	RC149001
Advanced DMEM/F12	ThermoFisher Scientific	12634-010
HEPES	ThermoFisher Scientific	15630-056
GlutaMAX	ThermoFisher Scientific	35050-068
Penicillin/Streptomycin	ThermoFisher Scientific	15130-122
R-spondin1	Prepared in house	n/a
N21	R&D systems	AR008
Nicotinamide	Sigma Aldrich	N0636-100G
Noggin-Fc	Ipa therapeutics	N002
N-acetylcysteine	Sigma Aldrich	A9165
hFGF10	Peprotech	100-26
[Leu15]-gastrin I	Sigma Aldrich	G9145-.1MG
mEGF	Peprotech	315-09

REAGENT or RESOURCE	SOURCE	IDENTIFIER
hHGF	Peprotech	100-39
WNT Surrogate-Fc	Ipa therapeutics	N001
Y-27632	Abmole Bioscience	M1817
HepatiCult™ Organoid Basal Medium (Human)	STEMCELL Technologies	100-0387
HepatiCult™ Organoid Differentiation Supplement (Human)	STEMCELL Technologies	100-0388
Penicillin/Streptomycin	ThermoFisher Scientific	15130-122
Dexamethasone	Tokyo Chemical Industry	D1961
Tris (pH 8.5)	Merck Millipore	77861
Copper (II) sulfate hydrate	Sigma Aldrich	209201
647-Azide	Biotium	92084
Ascorbic acid	Sigma Aldrich	50817
Experimental models: Organisms/strains		
Rosa26-CreERT2; mTmG	The Jackson Laboratory	007676
Rosa26-rtTA::Col1a1-tetO-H2B-mCherry	The Jackson Laboratory	014602
Rosa26p-FUCCI2	RIKEN	CDB0203T
Villin-Cre^{ERT2}Apc$^{fl/fl}$Kras$^{G12D/WT}$Tr53$^{fl/R172H}$	Fumagalli et al, 2017 [33]	n/a
Recombinant DNA		
pLentiPGK Hygro DEST H2B-mCerulean3	Addgene	90234
pMDLg/pRRE	Addgene	12251
pRSV-Rev	Addgene	12253
pMD2.G	Addgene	12259
Software and algorithms		
Imaris	Oxford Instruments	9.9.1
FIJI	https://imagej.net	1.52p
Prism	GraphPad	9.5.1
ZEN Black	Zeiss	V2.3
MetaMorph	Molecular Devices, USA	7.8
Other		
12 well CELLSTAR® plate, polystyrene	Greiner Bio-one	665180
6 well CELLSTAR® plate, polystyrene	Greiner Bio-one	657160
U bottom 96 w plates	FaCellitate	F202003
µ-Plate 96 well black uncoated plates	IBIDI	89621
15µ-Slide 8 well^{high} uncoated plates	IBIDI	80801
iSpacers 0.05mm deep, 0.25mm deep	SunJin Lab and Co	#IS204
50kDa Amicon Ultra-15 Centrifugal Filter Units	Merck	UFC905024

Solution recipes

Basic Medium

Reagent	Stock	Final concentration	Amount (515mL total)
Advanced DMEM/F12	n/a	n/a	500 mL
HEPES	1 M	10 mM	5 mL
GlutaMAX	100x	1x	5 mL
Penicillin/ Streptomycin	10.000 U/mL	100 U/mL	5 mL

ENR Medium

Reagent	Stock	Final concentration	Amount (50mL total)
Basic medium	n/a	n/a	47.2mL
mEGF	500 µg/mL	50 ng/mL	5 µL
Noggin-Fc	100%	0.5%	250 µL
R-spondin1	100%	5%	2.5 mL
N21	50x	1x	1 mL
N-acetylcysteine	500 mM	1 mM	100 µL

Expansion medium

Reagent	Stock	Final concentration	Amount (50mL total)
Basic Medium	n/a	n/a	45.63 mL
R-spondin1	100%	5%	2.5 mL
N21	50x	1x	1 mL
Nicotinamide	1 M	10 mM	500 µL
Noggin-Fc	100%	0.5%	250 µL
N-acetylcysteine	500 mM	1 mM	100 µL
hFGF10	500 µg/mL	100 ng/mL	10 µL
[Leu15]-gastrin I	100 µg/mL	100 ng/mL	5 µL
mEGF	500 µg/mL	50 ng/mL	5 µL
hHGF	500 µg/mL	50 ng/mL	5 µL

Isolation medium

Reagent	Stock	Final concentration	Amount (50mL total)
Basic medium	n/a	n/a	49.95 mL
WNT Surrogate-Fc	5 µM	0.2 nM	2 µL
Y-27632	10 mM	10 µM	50 µL

Differentiation medium

Reagent	Stock	Final concentration	Amount (50mL total)
HepatiCult™ Organoid Basal Medium (Human)	n/a	93.9%	46.95 mL
HepatiCult™ Organoid Differentiation Supplement	n/a	5%	2.5 mL
Penicillin/ Streptomycin	10.000 U/mL	100 U/mL	0.5 mL
Dexamethasone	3 mM	3 uM	50 µL

EdU staining solution

Reagent	Stock	Final concentration	Amount (1mL total)
Tris (pH 8.5)	1 M	100 mM	100 µL
Copper (II) sulfate hydrate	100 mM	1 mM	10 µL
647-Azide	10 mM	10 µM	1 µL
Ascorbic acid	1 M	100 mM	100 µL
MilliQ	n/a	n/a	789 µL

Statistical analysis

Statistics were performed using GraphPad Prism. Paired or unpaired t test was used when data showed normal distribution (verified with a normality test), whereas Mann-Whitney test was used for data that did not display parametric distribution. For multiple comparisons we used one-way ANOVA for normal distribution data and Kruskal-Wallis test when data was not showing parametric distribution. Adoption of one statistical test or the other is indicated for each experiment in the figure legends.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used GPT-3.5/OpenAI in order to improve readability of the methods section. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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SUPPLEMENTARY FIGURES

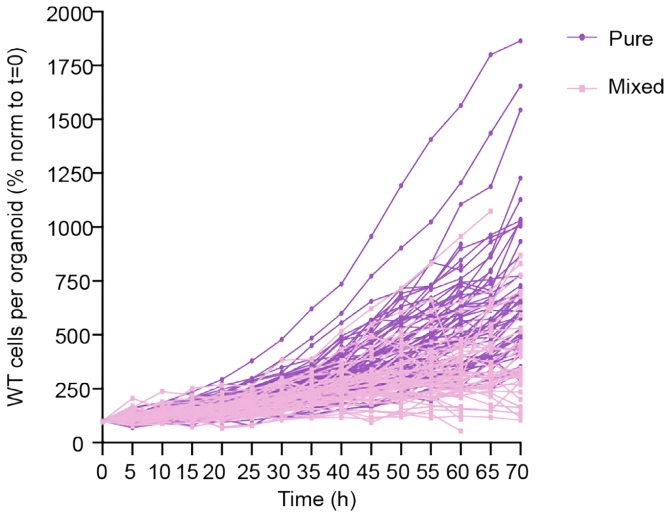

Figure S1: Cancer cells outcompete wild-type liver organoids. Related to Figure 1

Displays the wild-type cells in pure (dark purple) and mixed (light purple) that were followed during time-lapse imaging. The number of wild-type cells, normalized to T=0, is plotted against time. Each line represents an organoid

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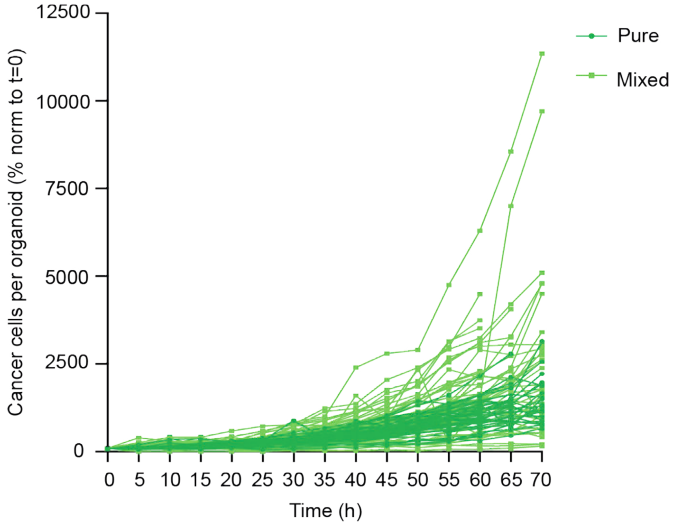


Figure S2: Increased expansion of competing cancer cells. Related to Figure 2

Displays the cancer cells in pure (dark green) and mixed (light green) that were followed during time-lapse imaging. The number of cancer cells, normalized to T=0, is plotted against time. Each line represents an organoid.

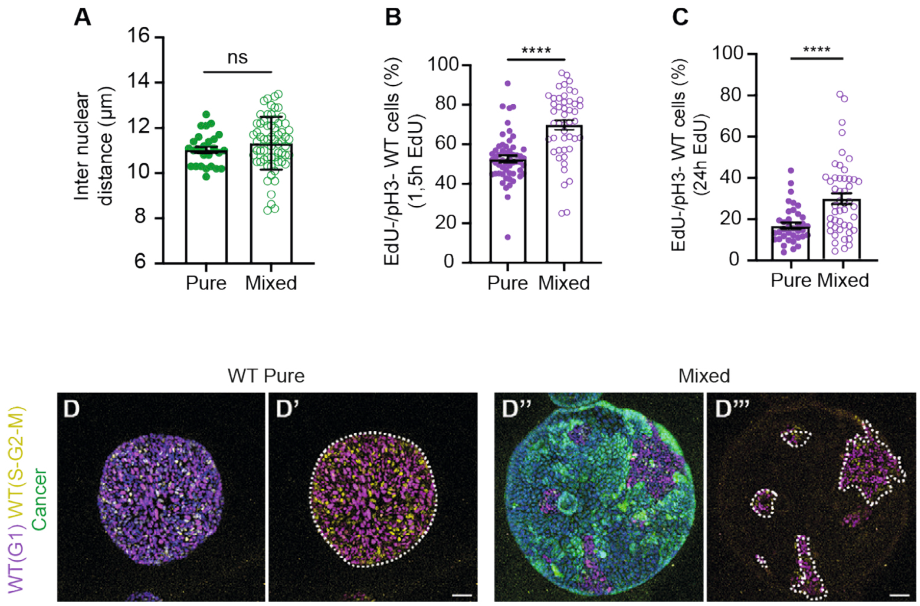


Figure S3: Cancer induces compaction and cell cycle arrest of wild-type liver cells. Related to Figure 3

(A) Quantification of the inter-nuclear distance of cancer cells in pure and mixed organoids; each dot represents one organoid (mean ± SEM; Ordinary one-way ANOVA, Sidak's multiple comparisons test; p<0.4681; n= 26 and 64 organoids). (B-C) Quantification of the percentage of EdU-/pH3- wild-type cells after 1.5 h (B) or 24 h (C) of EdU treatment; each dot represents one organoid (mean ± SEM; Ordinary one-way ANOVA, Sidak's multiple comparisons test; p<0.0001; n= 55 and 47 organoids in B; p<0.0001; n=37 and 48 organoids in C). (D) Z projection confocal images of pure wild-type (D and D') and Mixed (D'' and D''') FUCCI2 organoids fixed three days after planting. White dotted line indicates wild-type cells. Scale bars represent 50 µm.

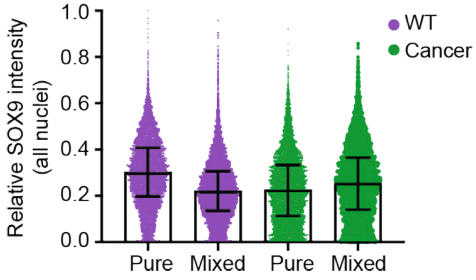


Figure S4: Increased competition through loss of WT progenitor state. Related to Figure 4

Displays the relative SOX9 intensities of all individual nuclei in pure and mixed organoids; each dot represents a nucleus (WT pure median =0.2475, n= 7998 nuclei; WT mixed median = 0.1870, n= 9083 nuclei; cancer pure median= 0.2686, n= 8093 nuclei; cancer mixed median= 0.2880, n= 66127).

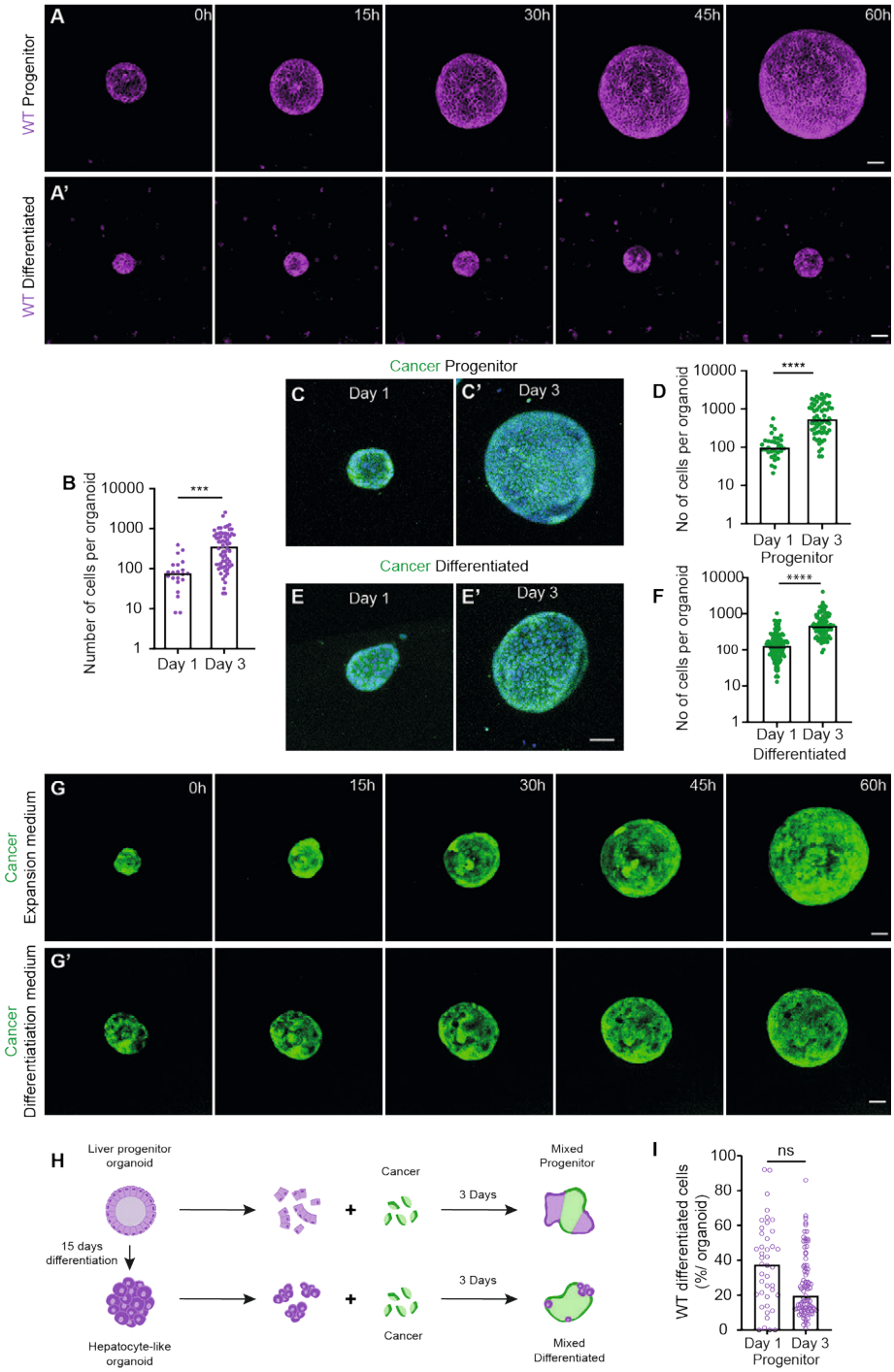

A
0h
15h
30h
45h
60h
WT Progenitor
A'
WT Differentiated
Cancer Progenitor
C
Day 1
C'
Day 3
B
Number of cells per organoid
10000
1000
100
10
1
***
Day 1 Day 3
D
No of cells per organoid
****
Day 1 Day 3
Progenitor
Cancer Differentiated
E
Day 1
E'
Day 3
F
No of cells per organoid
****
Day 1 Day 3
Differentiated
G
Cancer
Expansion medium
G'
Cancer
Differentiation medium
H
Liver progenitor organoid
15 days differentiation
Hepatocyte-like organoid
Cancer
3 Days
Mixed Progenitor
Mixed Differentiated
I
WT differentiated cells (%/ organoid)
ns
Day 1 Day 3
Progenitor

Figure S5 - Differentiated WT cells are effectively outcompeted. Related to Figure 5

(A) Representative maximum projections of 3D-confocal images of time-lapse series of WT progenitor (A) and WT differentiated (A') cells. (B) Quantification of the number of WT progenitor cells; each dot represents one organoid (Median; Kruskal-Wallis test, Dunn´s multiple comparison test; p=0.0008; n=21 and n=64). (C-F) Representative maximum projections of 3D-confocal images of pure cancer organoids cultured in expansion (C) and differentiation (E) medium, fixed at day 1 (C and E) and day 3 (C' and E') after plating; nuclei are visualized with DAPI (blue). Graphs display the number of cancer cells in pure cancer organoids cultured in expansion (D) and differentiation (F) medium; each dot represents one organoid (Median; Kruskal-Wallis test, Dunn´s multiple comparison test; p<0.0001; n=32 and n=61, D; n=124 and n=87, F). (G) Representative maximum projections of 3D-confocal images of time-lapse series of pure cancer organoids cultured in progenitor (G) and differentiation (G') medium. (H) Schematic depiction of the generation of mixed organoids from progenitor cholangiocyte (top) and differentiated hepatocyte-like (bottom) organoids. (I) shows the percentage of WT progenitor cells in mixed organoids at day 1 and day 3; each dot represents one organoid (Median; Kruskal-Wallis test, Dunn´s multiple comparison test; p=0.1671; n=42 and n=81). Scale bars represent 50 µm.

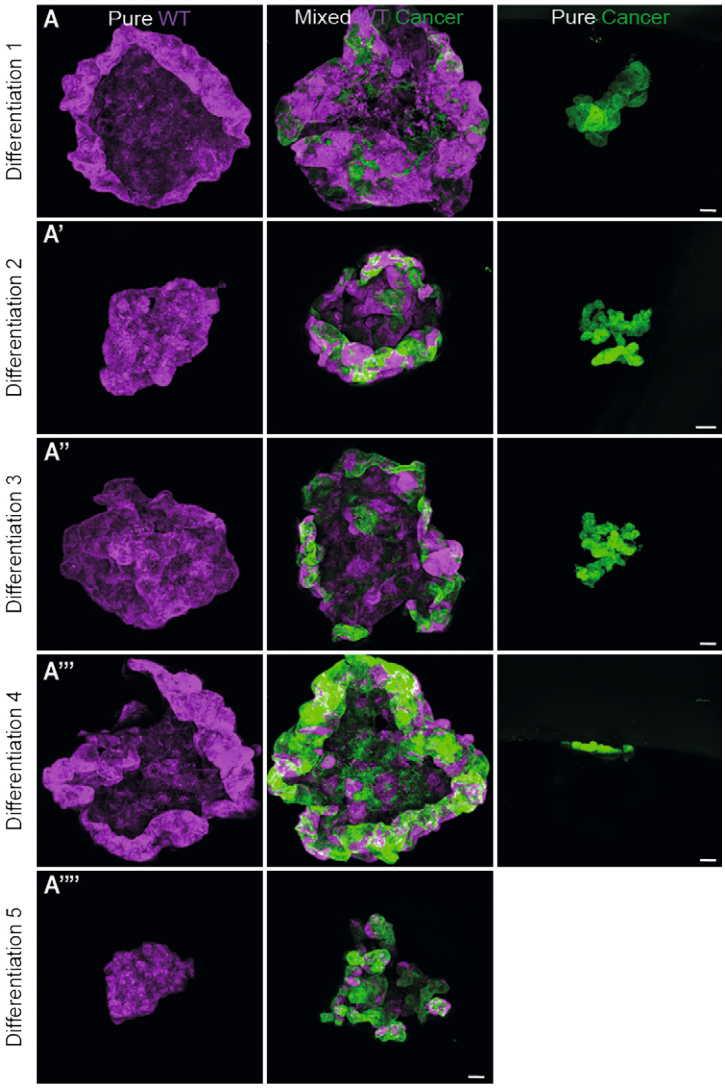


Figure S6 - WT liver acts as a scaffold for tumor growth during competition. Related to Figure 6

Representative 3D-reconstructed stitched confocal images of from pure WT (left), mixed (middle) and pure cancer (right) microtissues from each round of differentiation. Scale bars represent 100 μm.

SUPPLEMENTARY VIDEOS

Movies are available at: https://doi.org/10.1101/2023.09.14.557359

Video S1: Cancer cells outcompete wild-type liver cells
Time-lapse series of wild-type (left) and mixed (right) organoids.

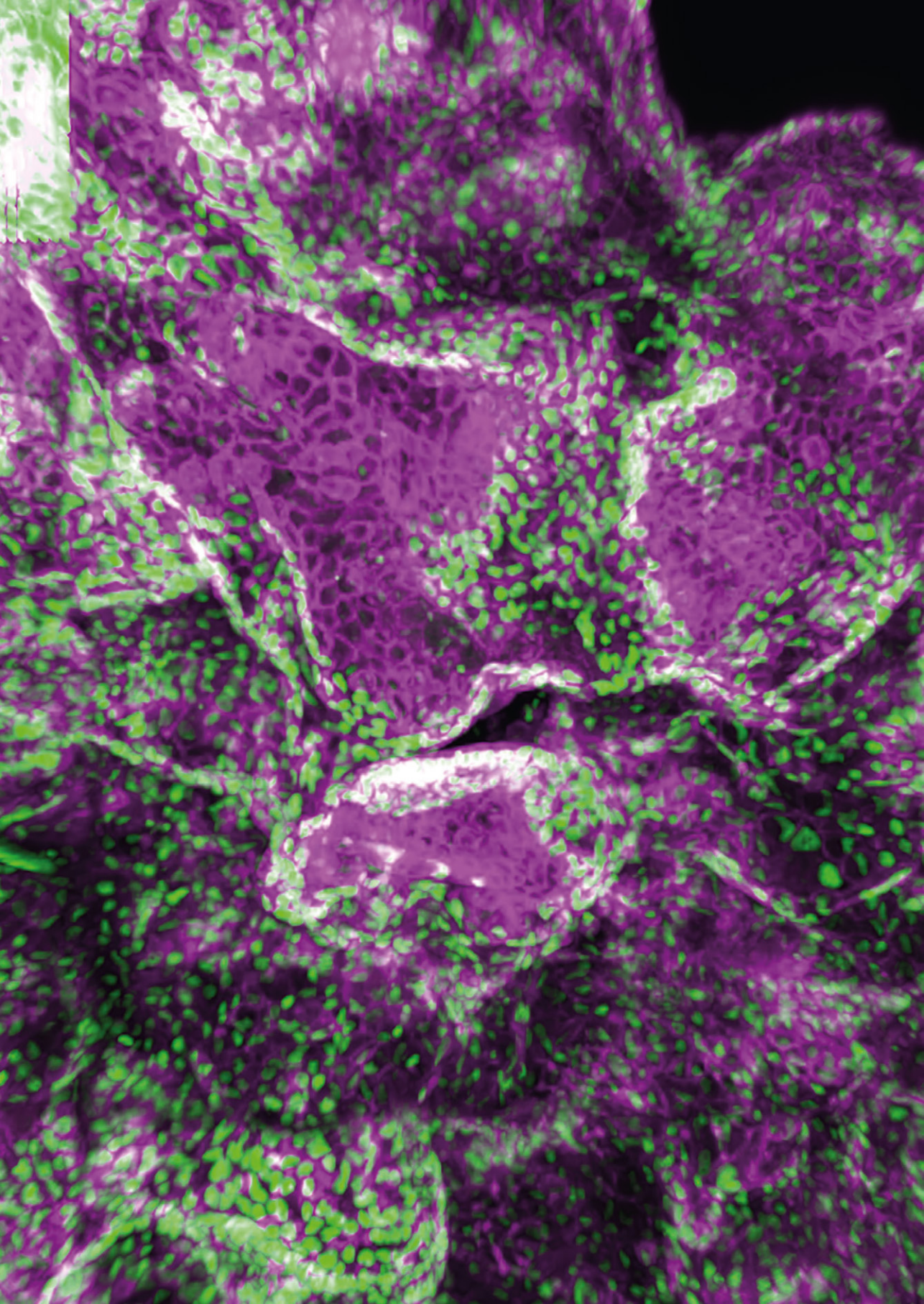
Video S2: Increased expansion of competing cancer cells
Time-lapse series of cancer (left) and mixed (right) organoids.

Video S3: Cancer induces compaction and cell cycle arrest of wild-type liver cells
Time-lapse series of wild-type (left) and mixed (right) FUCCI2 organoids.

Video S4: No proliferation of differentiated wild-type cells
Time-lapse series of wild-type progenitor (left) and differentiated (right) organoids.

Video S5: Differentiation medium does not affect cancer cell expansion
Time-lapse series of cancer organoids in expansion liver medium (left) and differentiation medium (right).

Video S6: Differentiated WT cells are effectively outcompeted
Time-lapse series of mixed organoids with progenitor (left) and differentiated (right) wild-type cells.



Molecular mechanisms of cell competition in liver metastasis

5

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ABSTRACT

JNK and YAP/TAZ signaling are key regulators of homeostasis, regeneration, and tumor formation. In addition, these pathways are crucial determinants of cell competition in a variety of tissues. Here, we dissect the role of JNK and YAP/TAZ signaling in cell competition driven by liver metastases of intestinal cancer. During competition, JNK activation is reduced in liver progenitor cells in mixed organoids. In contrast, this pathway is activated in hepatocytes in close proximity to metastatic lesions in liver tissue and microtissues, where most competition-induced cell death is observed. Progenitor liver cells intrinsically show high YAP as it is required to maintain their undifferentiated state. However, competition with cancer cells causes a reduction of YAP activation in liver progenitor cells, suggesting enforced differentiation towards hepatocytes. Importantly, constitutive activation of YAP protects liver progenitor cells from outcompetition by cancer cells. Together, these data highlight a dual role of JNK signaling during competition. Together with YAP/TAZ, JNK drives differentiation of liver progenitor cells, while JNK may trigger cell death in hepatocytes.

INTRODUCTION

Cell competition is crucial for tissue homeostasis and disease. During this process, cells constantly compare their fitness to that of neighboring cells, which ultimately causes elimination of less fit cells. These competitive interactions are important for the preservation of the most optimal cells during development and maintenance of tissues. However, this interplay can be copied by cells harboring pro-proliferative mutations, such as cancer cells. These, so-called super-competitors, use cell competition to eliminate surrounding wild-type cells and colonize tissues. The process of cell competition involves a cascade of events, including activation of pathways responsible for fitness sensing and execution of competition. Here, we focus on two key cell competition regulators, JNK and YAP/TAZ, which are both involved in the execution of cell competition and maintenance of liver tissue homeostasis.

Early work on cell competition in *Drosophila* revealed an important regulatory role for JNK signaling by orchestrating the elimination of less competitive or 'loser' cells. For example, in mosaic wing discs, ribosome-impaired *Minute* mutants activate the JNK signaling during competition with adjacent wild-type cells, which results in their elimination through apoptosis [1]. Similarly, in the wing disc, wild-type cells are eliminated by *Myc* overexpressing cells via JNK-dependent apoptosis [2]. Furthermore, *Minute* mutants in the midgut of flies, are also eliminated by wild-type cells in a JNK-dependent manner [3]. In the same organ, *Apc* mutant cells act as super-competitors and induce JNK-dependent elimination of wild-type cells [4]. These observations delineate the JNK pathway as a key mediator of apoptosis-dependent elimination of less fit cells, a concept that is also observed in mammalian models. For instance, knockdown of Mahjong in Madin-Darby canine kidney (MDCK) cells causes JNK activation and apoptosis in a competition-dependent manner. [5]. Lastly, wild-type intestine cells are actively eliminated by cancer cells via JNK pathway activation in mixed organoids [6].

JNK pathway has also been described as an enhancer of cell survival and proliferation. For instance, in the *Drosophila* midgut, wild-type cells near *Minute* mutants use competitive interactions to enhance their own proliferation potential through activated JNK signaling [3]. JNKs activate different substrates depending on specific stimuli, cell type and timing. In addition, phosphorylation by JNK can have different outcomes, such as protein activation, inactivation or targeting for degradation [7]. JNKs phosphorylate a number of activator protein 1 (AP-1) components, including cJUN [8], JunD, ATF2 [9], and beside AP-1 components, also cMyc [10], p53 [11], different histones [12,13] and several members of the BCL-2 family of apoptosis related proteins [14,15]. Interestingly, different JNKs can also have diverse regulatory effects, for instance, in human fibroblasts, inhibition of JNK2 results in a reduction

of p53 protein levels, whereas in these fibroblasts, inhibition of JNK1 increases p53 protein levels, suggesting JNK2 and JNK1 as a positive and negative regulator of p53 respectively [16]. The inverse was found for cJUN phosphorylation in UV-treated fibroblasts, where JNK2 is a negative regulator of cJUN and JNK1 a positive regulator [17]. Thus, the role of JNK in cell signaling is very broad, since its effect on protein phosphorylation is substrate and condition dependent.

JNK pathway also plays a role in carcinogenesis. JNK has been described both as a tumor-suppressor and oncokinase, reflecting its dual nature. For instance, tamoxifen-induced apoptosis in glioma cells is mediated by caspase 3 activation, and this effect is nullified when JNK1 is inhibited, highlighting the tumor-suppressive role of JNK [18]. In contrast, JNK activation and cJUN phosphorylation are required for transformation induced by oncogenic *Ras*, a mutation present in 30% of all human cancers [19]. Also, phosphorylation of cJUN by JNK is required for intestinal cancer development in $Apc^{min/-}$ mice [20]. Moreover, JNK has been described as promotor of colorectal cancer migration and invasion through CAMSAP2, via activation of JNK/cJUN/MMP-1 signaling [21] and MAPK signaling ERK and JNK dependent activation [22].

In the liver, the JNK pathway is involved in development of liver cancer and injury response. For instance, JNK1/2 are upregulated in hepatocellular carcinoma [23] and their deficiency promotes cholangiocarcinoma [24]. The JNK pathway is involved in protection against acute liver failure during multiple types on injury, such as treatment with Fluorofenidone [25], CCl_4 induced fibrosis [26] and lipotoxicity in Nonalcoholic Fatty Liver Disease (NAFLD) [27]. This indicates the wide variety of roles of JNK signaling upon different types of liver damage and suggests its potential involvement in responding to damage like metastasis. Together, this overview positions the JNK pathway as a key player in cell competition processes, tumor formation, the progression of colorectal cancer towards metastasis. However, the true role of the JNK or JNK-related pathways in cell competition during intestinal cancer liver metastasis remains poorly understood. Therefore, we here aim to dissect the role of JNK signaling in competition between intestinal cancer and liver cells.

Another important pathway in cell competition and colorectal liver metastasis, is YAP signaling. Analogous to the JNK pathway, YAP activation results in very different outcomes dependent on context, cells and timing. Canonical regulation of YAP is driven by the Hippo pathway and YAP activation leads to increased cell proliferation, survival and migration [28,29]. Moreover, YAP is one of the main mechano-transducers of external cues. This means that different matrix stiffness or mechanical forces from the environment regulate YAP activity and subsequent downstream signaling [30]. The role of YAP during competition has been described in early literature. For instance,

in *Drosophila*, activation of *YAP/Yorkie* or *Tead4*, caused by Hippo inactivation, turns cells into super-competitors that can induce programmed cell death in wild-type neighbors [31]. In murine fibroblast, activation of Hippo pathway and consecutive inactivation of YAP, results in elimination of mutant cells by surrounding wild-type cells [32]. In contrast, constitutive YAP activation of MDCK cells causes their apical extrusion when surrounded by wild-type cells [33–35]. However, when co-cultured with cells expressing *K-Ras* (G12V) or *v-Src* mutations, constitutive activation of YAP switches cells from loser to winners [33]. Therefore, this confirms that YAP activity within cells determines their fitness depending on fitness of their neighboring cells.

YAP also plays a substantial role in tumor growth [29]. For instance, AMPK which has been typically described as a tumor suppressor [36], phosphorylates YAP at multiple sites, inhibiting its activation and nuclear translocation [37]. In addition, elevated mRNA levels of YAP, TAZ, TEAD, and OCT4 are found in colorectal cancer tissues when compared to healthy tissues, indicating pathway upregulation in cancer [38]. The expression levels of Hippo pathway components MST1 and LAST2 in colorectal cancer (CRC) tissues are decreased compared to adenomas or non-tumor cells, indicating YAP activation in the tumors. Furthermore, YAP is not only associated with primary intestinal tumor growth but is also increased in liver metastasis, correlating with poor prognoses and relapse [39]. Moreover, it is required for formation of liver metastasis. For micrometastases to progress to macrometastases heterogeneous levels of YAP activation are required [40,41]. YAP is also involved in hepatocellular carcinoma, as misregulation of this pathway has been implicated in tumor growth in the liver [42]. Interestingly, in tumor bearing livers, YAP activation was found in healthy peritumoral hepatocytes, indicating a reaction of healthy tissue against tumor growth. This high activation of YAP/TAZ hampers tumor growth, which suggests that YAP signaling in healthy liver tissue is needed for tumor-suppression [43,44]. Whether such processes are at play during intestinal metastatic growth are unclear. Therefore, we here aim to investigate the role of YAP during competition between liver progenitor and intestinal cancer cells in organoid models.

RESULTS

Competition decreases JNK pathway activation in liver progenitor cells

Given the previously described role of JNK signaling in driving cell competition in intestinal primary tumors [6] [45], we questioned whether this pathway is also involved in competition during liver metastasis. To evaluate potential involvement, we first determined pathway activation in both progenitor and hepatocyte-like wild-type liver

cells during competition with intestinal cancer cells. For this, we used phosphorylation of cJUN at serine 73 (cJUN-pS73) as a proxy for pathway activation. This transcription factor is a downstream regulator of the pathway and a direct substrate of the JNK kinase. Cancer cells showed heterogeneous high activation of cJUN (Figures 1B and C), which was independent of competition (Figures 1D-F). In contrast, whereas pure wild-type cells showed high levels of nuclear cJUN-pS73, this was severely reduced during competition (Figures 1A, B and G). This was supported by a major decrease in the percentage of wild-type cells with high levels of cJUN activation in mixed organoids (Figure 1H) while the percentage of wild-type cells with low cJUN activation was strongly increased (Figure 1I). Together, this indicates that wild-type cells respond to competition by reduction of JNK pathway activation.

JNK pathway activation in peri-tumoral hepatocytes

Next, we wondered whether JNK activity is altered by competition in differentiated cells in the liver. Similar to JNK activation in cancer cells in organoids, we observed high levels of nuclear cJUN-pS73 in cancer cells in mixed microtissues and liver metastases (Figures 2B and C). Interestingly, differentiated wild-type cells display a different pattern. Low numbers of cJUN-pS73 positive nuclei were found in non-competing liver cells in pure microtissues and hepatocytes at a distance of metastases (Figures 2A and C, zoom 1). In contrast, high activation was observed in differentiated cells in close proximity to cancer cells (Figures 2A and C, zooms 2 and 3). Thus, in differentiated liver cells such as hepatocyte-like cells in *in vitro* microtissues and hepatocytes in *ex-vivo* liver tissue, JNK activation is increased by competition with cancer cells.

Competition decreases YAP activity in liver progenitor cells

We have previously described that wild-type liver progenitors undergo compaction during competition with cancer cells and this might trigger their forced differentiation [46]. This underscores a key role of mechanical forces in competition and suggests a potential involvement of YAP as regulator of mechano-transduction and differentiation [30,47–49]. To explore a role of this regulator in competition we analyzed nuclear YAP as a readout of pathway activation. Cancer cells showed a heterogenous pattern of YAP activation (Figures 3C), which is not altered by competition (Figures 3B, D, E and F). In pure conditions, wild-type liver progenitor cells showed high levels of nuclear YAP (Figure 3A). In contrast to the cancer population, YAP activation in wild-type cells is reduced by competition (Figures 3B and G). This is reflected by a severe reduction in the percentage wild-type cells with high levels of nuclear YAP in mixed organoids (Figure 3H), while the percentage of wild-type cells with low YAP levels was increased (Figure 3I). Thus, competition leads to a reduction of YAP activation in wild-type liver progenitors.

YAP activity alters competition

To investigate whether the lower levels of nuclear YAP in competing wild-type cells could affect the outcome of competition, we next aimed to alter YAP activity. Therefore, YAP signaling was genetically manipulated in liver wild-type progenitor cells by introduction of doxycycline-inducible YAP-5SA [50–52]. This YAP-5SA mutant, harbors mutations in five serine residues, preventing YAP phosphorylation and subsequent inactivation, resulting constitutive activation of YAP. Induction of YAP-S5A in liver progenitor cells caused an increase in the expression of YAP target genes, such as *Ankrd1*, *Ctgf,* and *Cyr61* (Figure 4A, B and C respectively), confirming activation of YAP signaling. Next, using time-lapse microscopy we tracked the behavior of cells in pure and mixed conditions. No difference in expansion was observed in pure wild-type organoids (Figure 4D, D' and F), indicating that the levels of YAP activity are already saturated, and an increased activation does not promote their proliferation rate. A small reduction in cancer cell proliferation was observed upon addition of doxycycline (Figure 4E, E' and G). This suggests that cancer cells are more sensitive to treatment with this antibiotic, which is in line with previous reports [53]. Interestingly, in mixed organoids constitutive activation of YAP in wild-type cells caused a lower rate of outcompetition compared to that of control wild-type cells (Figure 5A and A'). This was supported by a higher percentage of remaining YAP-S5A expressing wild-type cells in mixed organoids at the last time-points of the experiment (Figure 5A'). The absolute number of wild-type cells contributing to mixed organoids is only marginally increased in YAP-S5A conditions (Figure 5C), while instead, the absolute number of cancer cells in mixed organoids is decreased in these conditions (Figure 5D). This indicates that the different dynamics of competition in YAP-S5A organoids are mostly caused by reduced expansion of cancer cells. Furthermore, it implies that the growth benefit that cancer cells gain by competition [46] is dependent on decreased YAP activity in wild-type progenitor cells.

DISCUSSION

During the last decades, cell competition has emerged as an important area of research, shedding light on how cells interact with each other and sense the different fitness of their neighbors, a phenomenon that is not only crucial during homeostasis but also in pathological conditions. Cell competition dynamics are very dependent on the environment and involved competing cells. Thus, cell competition events are highly tissue-specific, indicating that mechanisms identified in one tissue may not be universally applicable. Therefore, here we aim to study the interactions between wild-type liver cells and intestinal cancer to unravel those mechanisms that promote metastatic growth.

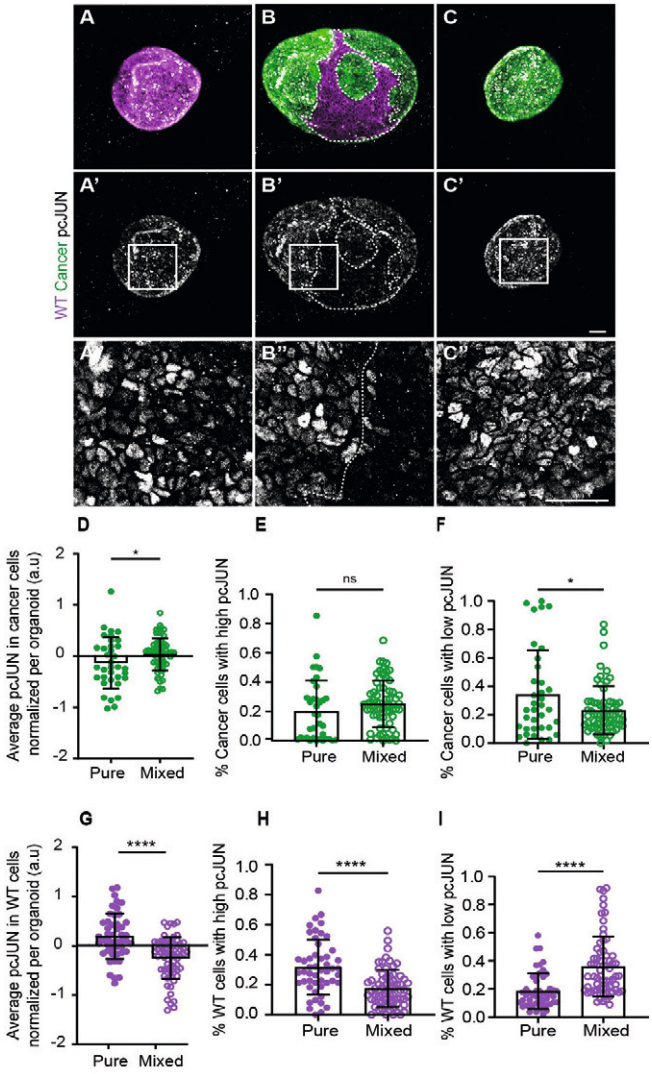

Figure 1. Competition decreases JNK pathway activation in liver progenitor cells (A-C) Representative maximum projections of 3D-confocal images of WT pure (A), mixed (B) and pure cancer (C) organoids fixed 3 days after plating. The organoids were stained for cJUN-pS73 (grey). The insets display a 3,5x magnification of the area in the white box and the white dotted line outlines the wild-type population. (D-I) cJUN-pS7 expression in cancer (D-F) and WT cells (G-I). (D) Displays the average cJUN-pS73 intensity in cancer cells in pure and mixed organoids (unpaired t-test, two tailed, p=0.044, N=35 and 66 respectively). (E-F) Show the percentage of cancer cells with high (E) and low (F) cJUN-pS73 expression (unpaired t-test, two tailed, p=0.1696; N=36 and 66, p=0.0219; N=36 and 66 respectively). (G) Displays the average cJUN-pS73 intensity in wild-type cells in pure and mixed organoids (unpaired t-test, two tailed, p<0.0001, N=48 and 66 respectively). (H-I) Show the percentage of wild-type cells with high (H) and low (I) cJUN-pS73 expression (unpaired t-test, two tailed, p<0.0001; N=50 and 66, p<0.0001; N=50 and 66 respectively). Scale bars represent 50µm.

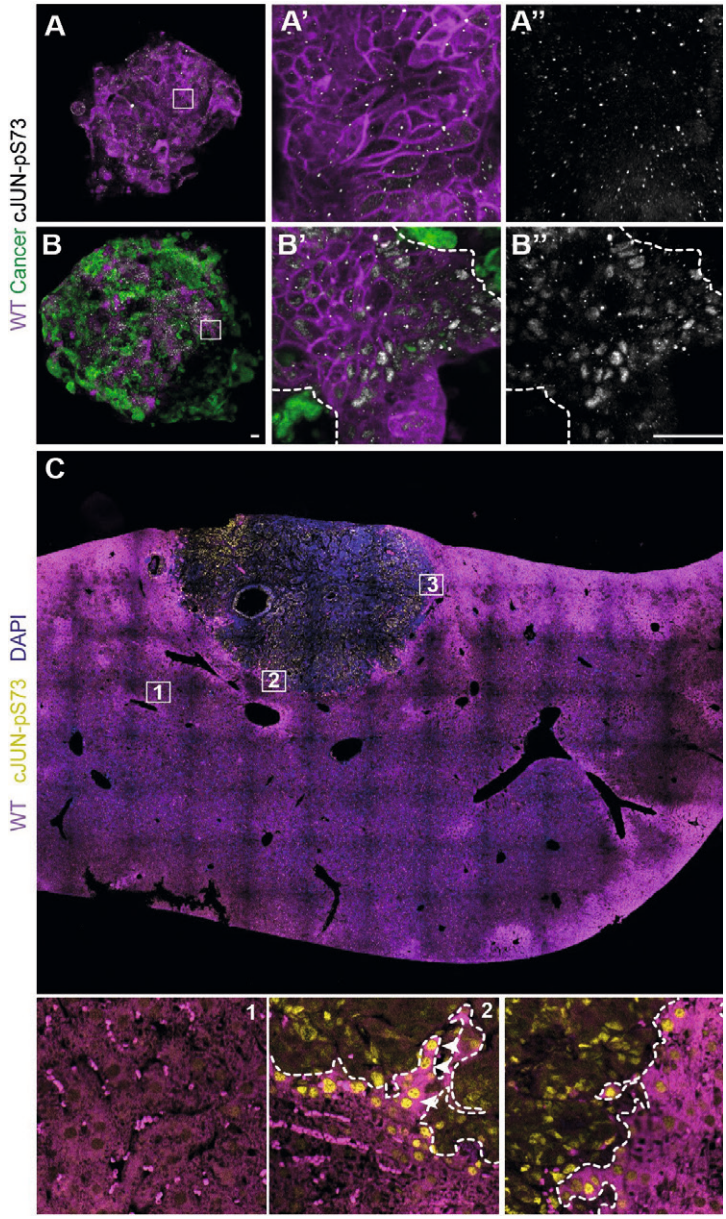

Figure 2. JNK pathway activation in peritumoral hepatocytes (A) Representative 3D-reconstructed stitched confocal images of a mixed microtissue stained for cJUN-pS73 (grey). The insets display a 9x magnification of the area in the white box and the white dotted line outlines the wild-type population. (C) Overview of liver tissue with metastasis (absence of magenta areas) stained for cJUN-pS73 (yellow). The insets display a 9,9x magnification of the areas in the white boxes, representing non-competing (zoom 1) and competing areas (zoom 2 and 3). Scale bars represent 50µm.

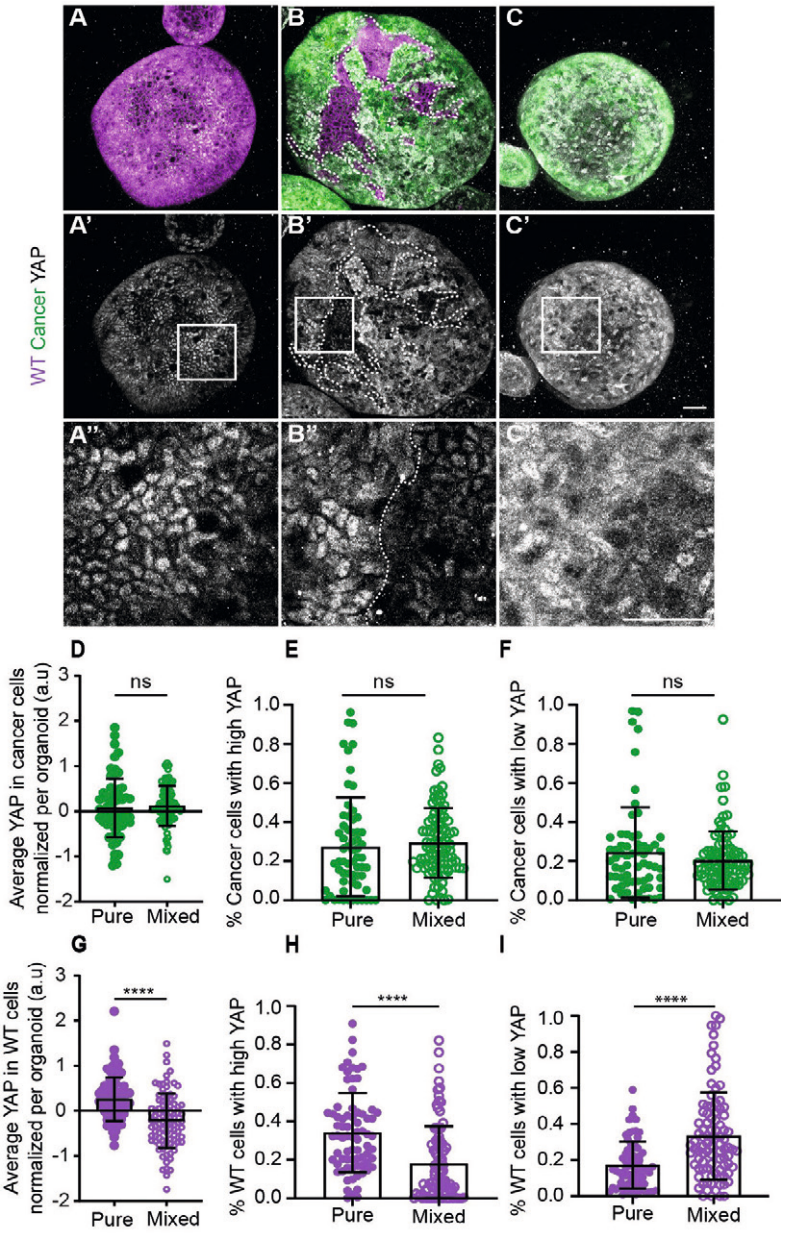

Figure 3. Competition decreases YAP activity in liver progenitor cells
(A-C) Representative maximum projections of 3D-confocal images of WT pure (A), mixed (B) and pure cancer (C) organoids fixed 3 days after plating. The organoids were stained for YAP (grey). The insets display a 3,75x magnification of the area in the white box and the white dotted line outlines the wild-type population. (D-I) YAP expression in cancer (D-F) and WT cells (G-I). (D) Displays the average YAP intensity in cancer cells in pure and mixed organoids (unpaired t-test, two tailed, p=0.631, N=63 and 92 respectively). (E-F) Show the percentage of cancer cells with high (E) and low (F) YAP expression (unpaired t-test, two tailed, p=0.5445; N=63 and 92, p=0.1855;

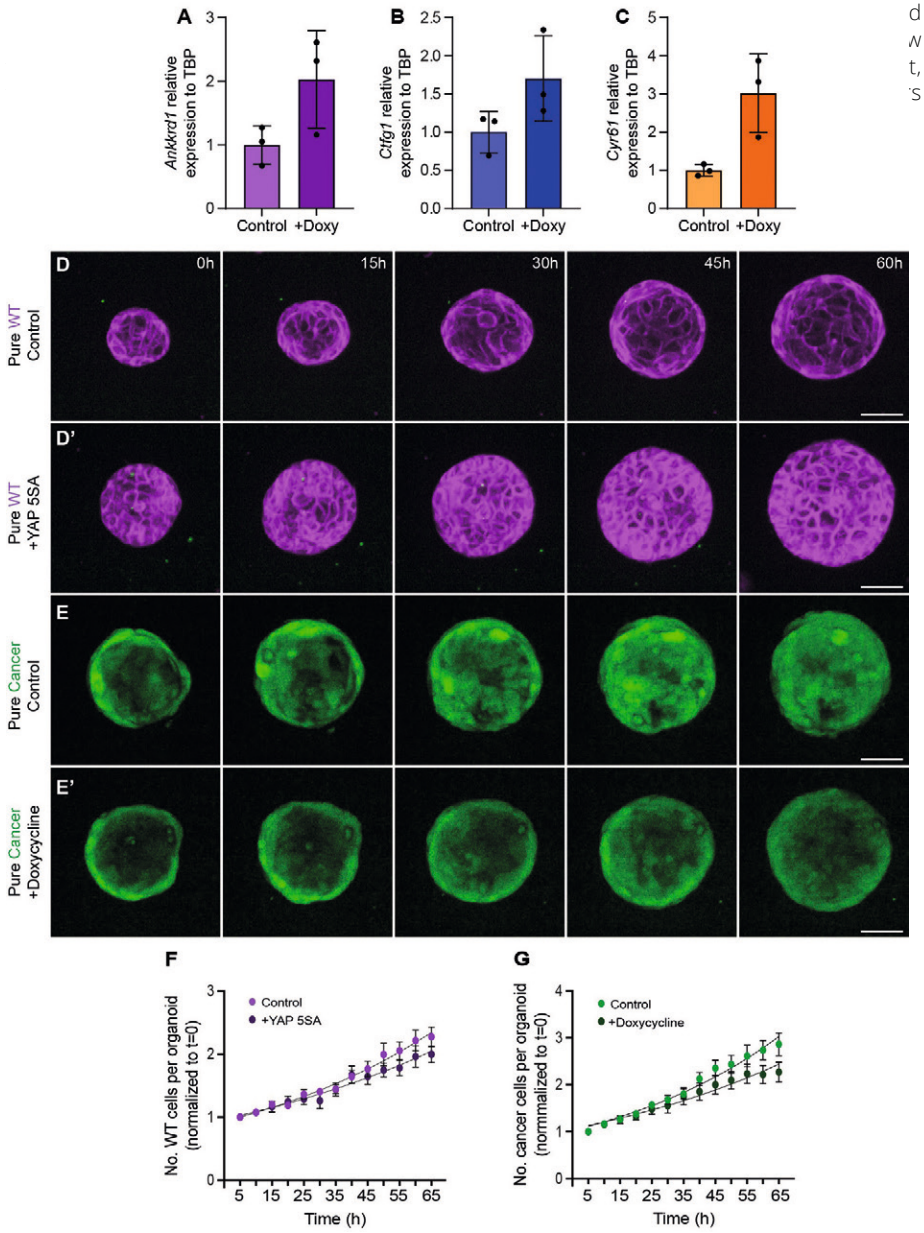

Figure 4. Constitutive YAP activation in pure liver progenitor cells (A-C) Display the relative expression of YAP target genes ***Ankrd1*** (A), ***Ctfg1*** (B) and ***Cyr61*** (C) in control and YAP-S5A expressing wild-type organoids 3 days after induction and relative to the housekeeping gene ***TBP***. (D-E) Representative maximum projections of 3D-confocal images of time-lapse series of control (D) and YAP-5SA expressing wild-type liver organoids (D'), and control (E) and doxycycline treated (E') cancer organoids. (F-G) Quantification of the number of wild-type (F) or cancer (G) cells per organoid, normalized to t=0 (Best fit values line; N=10 and 7; N=14 and 8 respectively). Scale bars represent 50µm.

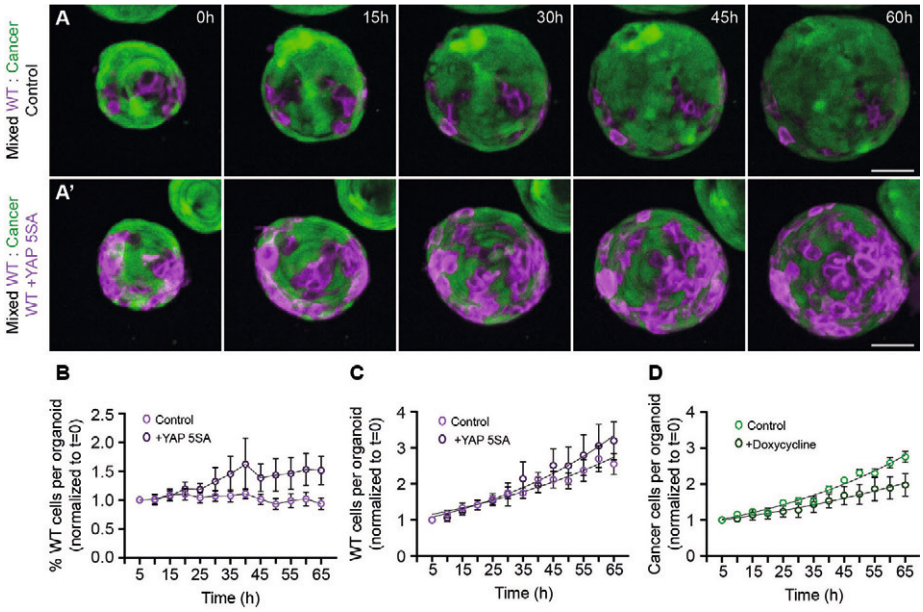

Figure 5. YAP activity alters competition. (A) Representative maximum projections of 3D-confocal images of time-lapse series of control (A) and YAP-5SA expressing wild-type liver (A') mixed organoids. (B) Quantification of the percentage of control and YAP-5SA expressing wild-type liver mixed organoids, per organoid, normalized to t=0. (paired t-test, two-tailed; P=0.0005; N=15 and 8 respectively). (C-D) Quantification of the number of wild-type (C) or cancer (D) cells per mixed organoid, normalized to t=0 (Best fit values line; N=15 and 8). Scale bars represent 50µm.

In our previous work, we found that JNK signaling is crucial for driving cell competition during intestinal primary tumor development through elimination of wild-type cells [6]. Here, we observed differential activation of JNK, dependent on the differentiation status of competing cells. Liver progenitor cells have high levels of JNK signaling in unperturbed conditions, which is decreased by competition (Figure 1). Differentiated hepatocytes, on the other hand, show low baseline activation that is increased by competition. We speculate that, in progenitor cells, JNK has a pro-proliferative role that is dampened by competition and ultimately results in forced differentiation. While in differentiated cells, such as hepatocytes in microtissues and liver tissue, JNK mediates a stress response as reaction to the insult provided by nearby cancer cells (Figure 2). Therefore, the role of JNK varies depending on the cell type and/or the differentiation status of the competing cells. Further research is required to unravel the mechanism of action of JNK pathway as driver of competition in liver. For instance, hyper-activation of JNK signaling in progenitor liver cells could potentially protect them and prevent their outcompetition. Conversely, inhibition of JNK in peritumoral hepatocytes could reduce their elimination and limit metastatic growth.

YAP is a major regulator of cell fate in the liver. It is highly expressed in progenitor cells where it maintains their proliferation and differentiation potential. In hepatocytes, YAP activity is less important in homeostasis but plays a crucial role during injury, when it is needed to induce their proliferation or trans-differentiation into progenitors [54–56]. Interestingly, previous work by Moya et al. highlighted the need of peritumoral YAP activation in the regulation of hepatocellular carcinoma [44]. However, whether this is a result of a damage response that is induced by the cancer cells and whether YAP plays a similar role in liver metastasis is unknown. Similar to JNK activity, we also observed a cell type dependent response of YAP to competition. The loss of nuclear YAP in competing progenitor cells likely reflects an increased differentiation state of these cells, which is supported by the finding that these cells also show decreased expression of the progenitor marker SOX9 [57–59]. However, the order of events during this forced differentiation remains unknown as our analysis of SOX9 and YAP expression levels does not allow us to assess whether the loss of SOX9 induces a reduction of YAP activation or *vice versa*. Another possibility is that the lower levels of these proteins is independent from each other. Simultaneous analysis of both SOX9 and YAP in pure and mixed organoids over time could reveal any relationship between these events. Similar information could be obtained by evaluation of the effect of constitutive YAP activation on SOX9 expression. We describe an intriguing effect of constitutive activation of YAP on competition. We encountered some technical difficulties in analysis of the time-lapse data that were caused by crowded organoid cultures. For instance, we were biased in the selection of organoids for analysis towards less crowded regions with smaller organoids, which resulted in an overall lower expansion rate of organoids compared to previous studies [46]. However, the competition-dependent over-proliferation of cancer cells is inhibited by constitutive activation of YAP in wild-type cells. This suggests that preventing the loss of YAP activity in competing wild-type cells can provide a competitive advantage that is sufficient to avoid their outcompetition. The mechanisms by which this is regulated remain currently unknown, but this finding opens up an exciting route of further investigation. In particular, the identification of molecules that can execute the communication of wild-type to cancer cells for the regulation of proliferation is an interesting path to explore.

MATERIALS AND METHODS

Culture of mouse organoids

Small intestinal cancer organoids derived from Villin-CreERT2::APC$^{fl/fl}$::KRAS$^{G12D/WT}$::P53$^{fl/R172H}$ transgenic were previously described in [60] and maintained as described in [61,62]. Wild-type cholangiocytes organoids from Rosa26-CreERT2::mTmG mice [63] were isolated and maintained as described in [62] [46].

Transduction of organoids

Lentiviral transduction was preformed using standard procedures. In short, lentivirus was produced in HEK293T by co-transfection of a dual lentiviral vector 3rd generation pLentiPGK Hygro DEST H2B-mCerulean3 to mark nuclei and wild-type liver progenitors were transduced with constitutively active YAP1 (YAP1-5SA). For this, Myc-YAP1-5SA from the pQCXIH-Myc-YAP1-5SA plasmid (Addgene #33093) which was cloned in a pDONR221 backbone and the Myc-tag was replaced with a Flag-tag (Epoch Life Science). The sequence 5'-CAATGCGGAATATCAATCCCAGCACAGCAAATTCTCCAAAATGTCAGG-3' was added between base pairs 982 and 983 of the YAP1 gene, similar to the previously described YAP1 constructs (Panciera et al., 2016). The Flag-YAP1-5SA construct was inserted into a pInducer20 backbone with blasticidine resistance cassette to generate pInducer20-FLAG416 YAP1-S5A-Ubc-rtTA-IRES-Blast x pLV. Transduction was performed using helper plasmids pMDLg/pRRE, pRSV-Rev and pMD2.G (gifts from Markus Covert and Didier Trono, Addgene plasmids #90234, #12251, #12253 and #12259). Viral particles were harvested from cells four days after transfection and concentrated using 50kDa Amicon Ultra-15 Centrifugal Filter Units (Merck, cat#UFC905024). Organoids were dissociated by mechanical disruption and dissolved in 250 µL ENR medium supplemented with Y-27632 and Polybrene together with the concentrated virus. Cells were incubated at 32°C while spinning at 600xG for 1 hour followed by a 4-hour incubation at 37°C before plating in BME2. Selection was carried out from day 3 onwards with hygromycin (0.5mg/mL) for H2B-mCerulean and Blasticidine (6.67uL/mL) for YAP-5SA.

RNA isolation, cDNA generation and qPCR

Doxycycline (4.25 µM) induced and non-induced YAP-5SA cholangiocyte organoids were isolated three days after plating. After one wash with PBS0, they were resuspended in 1mL of Trizol (Life technologies, Ref: 15596018) and kept at -80 degrees until RNA extraction. For RNA extraction, Trizol samples were thawed on ice for 5 minutes and then 200µL of chloroform were added and tubes were

inverted vigorously, followed by a centrifugation step at 4 degrees, 12000rpm for 5minutes. Afterwards, the supernatant was isolated and 550 µL of isopropanol 100% were added. Tubes were inverted vigorously and centrifugated for 12000rpm and 15 minutes at 4 degrees, to precipitate a pellet of RNA. The pellet was washed for 3 times with ethanol 70%, 5 minutes centrifugation at 4 degrees, 7500 rpm. Finally, the pellet was dissolved in 30 µL of nucleases-free water (Invitrogen, Ref: 10977-035). Afterwards, cDNA was generated using RT master mix kit according to manufacturer instructions (Thermo Fisher Scientific, High Capacity cDNA Reverse Transcription Kit, Ref: 4368814) but using 2uM of Oligo dT primers (Invitrogen, ref: 18418020). Finally, 20 ng of cDNA per well was used for qPCR, together with 5 µL / well of SYBR Green (Thermo Fisher Scientific, ref: 4367659), forward and reverse solution primers (0.6 µL /well of the mixed solution, 10uM) and 0.4 µL /well nuclease-free water. The primers used to analyze YAP overexpression were: Ankrd1 mouse (FWD: GCTGGTAACAGGCAAAAAGAAC; RV: CCTCTCGCAGTTTCTCGCT), Cnn2/Ctgf mouse (FWD: CCACCCGAGTTACCAATGAC; RV: GTGCAGCCAGAAAGCTCA) and Ccn1/Cyr61 mouse (FWD: CTGCGCTAAACAACTCAACGA; RV: GCAGATCCCTTTCAGAGCGG) and house-keeping gene TBP (FWD: ACCGTGAATCTTGGCTGTAAAC; RV: TCGGGTCCTAGACCAGTGTTC).

Liver tissues

Liver metastases were induced by injection of AKP cancer organoid cells in a mesenteric vein of male C557BL/6JRj acceptor mice. Tissues were extracted and treated after termination of animals. For detailed information, see Methods section of Chapter 6.

Generation of mixed organoids

Mixed organoids were generated as described previously [64]. In short, suspensions of small clumps of cells were generated from organoids by mechanical disruption and divided over Eppendorf vials in a 1:1 ratio (WT: cancer). Cells were concentrated by mild centrifugation and the pellet was resuspended in a small volume of mouse liver expansion medium and incubated at 37°C for 30 minutes. Cell aggregates were plated in BME2 and cultured in mouse liver expansion medium. For imaging purposes cells were plated in µ-Plate 96 well black uncoated plates (IBIDI, ref: 89621).

Generation of microtissues

Cancer organoids and hepatocyte-like organoids obtained by differentiation (described in [46]) were kept in differentiation medium + Noggin for 24h prior to microtissue formation. Organoids were harvested and washed three times with

basic medium to remove the culture matrix. After the last wash, the organoids were mechanically disrupted by pipetting through a fire-polished glass Pasteur pipet. Cancer organoids were intensively disrupted to generate small pieces and wild-type organoids were treated with a gentle disruption to prevent induction of an injury response. The disrupted organoids were divided over Eppendorf vials in a 2:1 ratio (WT liver:cancer) and concentrated by gentle centrifugation. Cell pellets were dissolved in 5-8 µL differentiation medium +Noggin and incubated at 37°C for 30min. After aggregation, 100 µL /well of differentiation medium +Noggin was added and divided over 96w U bottom plates (FaCellitate, ref: F202003). Microtissues were allowed to mature at 37°C for 3 days.

Immunofluorescence

Tissues frozen in OCT were cut with microtome in 0.18 µm slides and keep at -20 degrees until staining. The protocol for staining tissue slides is the same as followed for staining of organoids and microtissues described in Chapter 4 [46] with the addition of a short (5-20 min) PBS wash before start of the staining to rehydrate the tissue. Mixed organoids they were fixed and blocked as described in [46]. Microtissues were fixed by removal of 50 µL medium and addition of 50 µL of 8% PFA in PBS for 20min. Fixation was followed by a minimum of three washes with PBS0 and samples were stored in PBS0 at 4 °C until use. Samples were permeabilizated and blocked in PBS/0.5% BSA/ 0.5% TX-100 for at least 30 minutes and incubated with primary antibodies overnight at 4 °C. Used antibodies are anti-cJUN-pS73 primary antibody (1:200, Cell Signaling, #3270), anti-YAP1 (1:200, Santa Cruz, sc-101199), Donkey anti-Rabbit, Alexa Fluor 647 (1:500, ThermoFisher Scientific, A-31573) and Donkey anti-Mouse, Alexa Fluor 647 (1:500, ThermoFisher Scientific, A-31571). DAPI (1:2000, Toronto Research Chemicals, D416050; CAS: 28718-90-3) was used to visualize nuclei. Microtissues, were mounted in RapiClear clearing solution (SunJin Lab. RC149001) on glass slides using iSpacers (0.05mm deep, 025mm deep, SunJin Lab and Co, #IS204).

Microscopy

Images of liver tissues and fixed organoids were acquired on a Carl Zeiss LSM880 Fast AiryScan Confocal Laser Scanning microscope (Axio Observer 7 SP with Definite Focus 2). A Plan-Apochromat 20x/0.8 WD=0.55mm air objective was used to obtain 12bit images with a 1024 resolution, via bidirectional imaging, using a pinhole size of 0.5AU for tissues and 1AU for organoids. A Z-slice thickness of 5 µm (tissue and microtissues) and 2.5 µm (organoids) was used to cover the complete thickness of the sample (17 to 150 slices). The following laser lines were used: 405nm, Laser Argon

Multiline (445/488/514), 561nm and 633 nm. For tissues and microtissues the tile option was used to cover the the size of the sample.

Live-imaging of pure and mixed organoids containing mTmG-labeled wild-type cells was performed on an Eclipse Ti2-E with PFS (Nikon, Japan) equipped with a Confocal Spinning Disc Unit CSU-W1-T1 (Yokogawa, Japan). A Plan Apo λD 20x / 0.80, WD=0.80, MRD70270 (Nikon, Japan) dry objective was used to obtain 16bit images with a 1024 resolution and 2x2 binning. A Z-slice thickness of 5 µm was used to cover the complete thickness of 300 µm. The time interval was 5 hours for a total duration of 60-70 hours. The following laser lines were used: Stradus 445 (441 nm / 80 mW, Vortran, USA), Stradus 488 (490 nm / 150 mW, Vortran, USA) and OBIS 561 (561 nm, 150 mW, Coherent, USA). Temperature control and CO2: STXG-PLAMX-SETZ21L (TokaiHit, Japan) were set at 37 °C and 5% CO2.

Analysis

Image processing and analysis were performed as described in [46], Chapter 4. In summary, the open-source platform FIJI [65] was used in combination with computational routines developed in the programming language Julia. For each raw image, a program was developed that facilitated the selection of individual organoids in the confocal and lateral planes of the raw image based on the DAPI channel. Next, a general computational pipeline was applied for each organoid; 1) kernel size definition, 2) image processing and 3) 3D object classification and analysis, as described in [46]. Kernel size (1) definition was not modified. Image processing (2) and 3D object classification and analysis (3) were applied for DAPI, Dendra-2 and mTmG in the same way as described in Chapter 4. YAP and cJUN-pS73 followed the analysis described for SOX9, with one adaptation; since cJUN-pS73 and YAP histograms revealed a higher presence of outliers than organoids stained for SOX9, we used a less sensitive alternative to standardize these two staining. Next, to address the expression of cJUN-pS73 and YAP, we standardized the intensities by centering the points to an average of 0 and a standard deviation of 1.

Visualization

Images were either 3D reconstructed in Imaris software (Andor) or a maximum projection was made in Fiji (indicated in the figure legends). For liver tissues, raw images were first stitched using Zen software (Zeiss) and colors were adjusted in Imaris. Next, snapshots were taken with imaris and also converted to RGB using FIJI, cropped and when necessary, smoothened, cropped, rotated and contrasted linearly.

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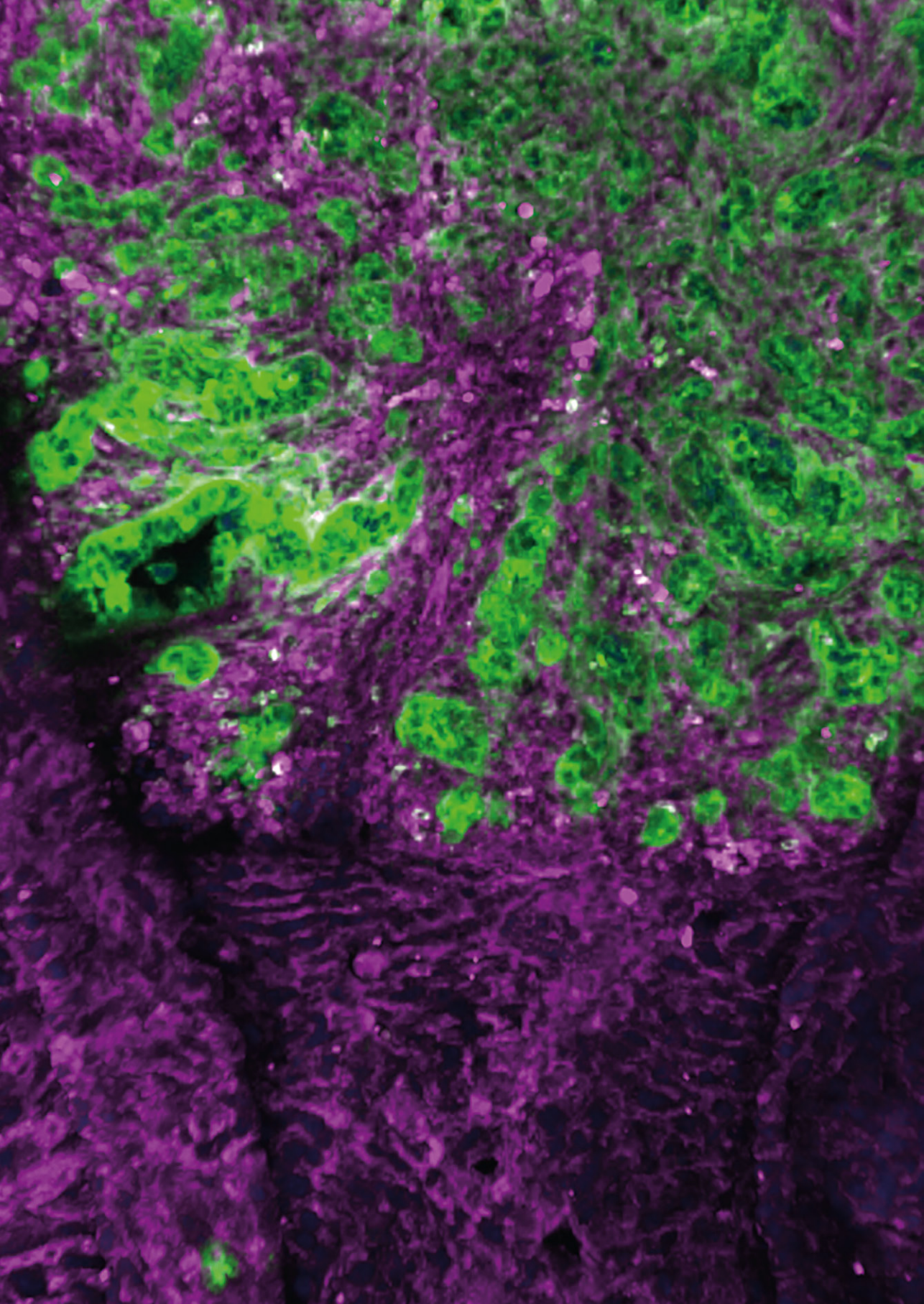

Enhanced metastasis-forming potential of intestinal cancer by exposure to the liver micro-environment

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ABSTRACT

Cancer dissemination and metastasis cause 90% of cancer-related deaths. Cell competition, a process that drives cell selection based on relative differences in cellular fitness, potentially plays a key role in metastasis. During the formation of metastases, invasive cancer cells engage interactions with tissue-specific cells from the microenvironment to adapt and enhance their fitness. Competition immediately starts when cells escape from the primary tumor and encounter a myriad of challenges during the metastatic cascade. It continuous to play a role upon arrival in the liver where invasive cancer cells interact with both cellular and non-cellular components of the liver tissue and adapt to the new environment. Here, we study how the colonization potential of intestinal cancer cells is affected by the repeated exposure to liver microenvironment. Interestingly, subsequent exposure and growth of intestinal cancer cells in the liver increases their invasive potential. This is characterized by an earlier onset of symptoms of liver malfunction and a more rapid and extensive colonization of the tissue. However, previous adaptation to the liver environment does not provide cancer cells with a competitive advantage over liver progenitor organoids *in vitro*. This indicates that a gain in cellular fitness in a certain environment does not guarantee an increased fitness in another environment and highlights the comparative nature of cell competition.

INTRODUCTION

Cancer cells that escape from a primary tumor and reach distant tissues to form metastases must adapt to a new environment. The metastatic cascade is a complex process influenced by several factors, including the tissue of origin of the primary tumor, inherent traits of the cancer cells, the stage of the tumor, and the unique characteristics of the secondary site [1]. The initial phase of metastasis requires local invasion, which means the detachment of cancer cells from the primary tumor and their infiltration into the surrounding stroma. For this, cancer cells lose cell-cell adhesion molecules such as E-cadherin and catenins [2] and modify molecular components of the tumor micro-environment. These early adaptations can result in distinct cancer phenotypes that precede distant dissemination [1]. To reach the secondary organ, cancer cells intravasate into the bloodstream to become Circulating Tumor Cells (CTCs). During this journey, they encounter multiple insults such as high shear forces of the blood circulation, anoikis resulting from cellular detachment and the attack by immune cells. These challenges force adaptation of CTCs by different mechanisms. For instance, CTC clustering promotes stemness via expression of markers such as NANOG, SOX2 and OCT4 [3]. Association of CTCs with platelets, helps the CTC adhesion, commonly referred to as a 'platelet cloak,' enabling evasion from immune surveillance [4]. Furthermore, CTCs may associate with neutrophils, particularly through a VCAM-1-dependent mechanism, which also induces cancer proliferation during circulation in the bloodstream [5]. Additionally, the evasion of CTCs from cytotoxic immune cells enhances their survival. Cell competition is a process based on continuous comparison of fitness between neighboring cells. It typically acts as a quality control mechanism to assure that only the fittest cells remain in the tissue. Therefore, it is expected that cell competition plays an important role during the transition from the primary to secondary site, initiating the adaptation of cancer cells towards invasion.

If CTCs survive in circulation, the next rate-limiting step of metastasis is the extravasation towards the secondary organ. This is highly dependent on the organotropism of a primary tumor. Each type of cancer preferably invades some organs rather than others. This concept, referred to as the 'seed and soil' hypothesis, was first proposed by Paget in 1880 [6]. Chemokines, metabolites and EVs released by the cells residing in the organ of secondary growth are crucial for this organotropism [7,8]. Extravasation starts by adhesion of CTCs and attachment in the endothelial lumen. This attachment is facilitated by cell adhesion and ligands, integrins and the Extracellular Matrix (ECM) [9]. Subsequently, cancer cells encounter additional vascular layers (e. g pericytes, smooth muscle cells) and the ECM before gaining access to the parenchyma of the new organ [1]. To facilitate the penetration in the new secondary niche, cancer cells release proteases and degradative enzymes or they induce their

release from non-cancerous cells, such as tissue-resident macrophages [10,11]. Alternatively, cancer cells can also extravasate via non-proteolytic mechanisms such as a diapedesis (mechanical deformation to squeeze between endothelial cells), but it is more typical for circulation through the lymphatic system [12].

In the next step of the metastatic cascade, once the secondary organ is reached, the now disseminated tumor cells (DTCs) face a new range of challenges in the new microenvironment. Host defense mechanisms are responsible for the elimination of majority of DTCs [13]. In some cases, and as a protective mechanism, DTCs enter a state of dormancy to avoid elimination while residing in the secondary tissue. During dormancy, DTCs acquire a quiescent state and stop dividing. However, upon environmental cues, DTCs can exit dormancy and initiate the formation of a secondary tumor [1]. In general, early secondary lesions are located close to vasculature to receive cues and nutrients. In more advance stages, tumor cells promote angiogenesis to foster their growth within the secondary organ [2,14]. Thus, a plethora of genetic and molecular changes take place in the cancer cells to overcome the array of challenges encountered in this journey. This suggests that cancer cells that can grow in secondary organs must have significantly adapted compared to the primary tumor cells to survive and colonize the new niche.

The liver stands out as one of the most prevalent organs for metastasis due to its extensive vascularization and relatively slow blood flow rate. Moreover, the hepatic vasculature is characterized by fenestrations and a lack of a subendothelial basement membrane, facilitating CTC extravasation [15,16]. The portal vein and hepatic artery serve as principal entry points to the liver [17] and are responsible for the 25% and 75% of the blood flow respectively [16]. The type of metastatic growth can be classified in five different growth patterns (recently reviewed by Lactacz et al.[18]) Each pattern is characterized by a distinct interaction between cancer cells and surrounding tissue. The most common and best characterized patterns are the desmoplastic, pushing and replacement patterns. Desmoplastic and pushing patterns show a clear boundary between cancer and liver tissue, either limiting the metastatic area with a fibrotic rim (in desmoplastic pattern) or without a rim, but mechanically pushing the liver tissue away from the metastasis (pushing pattern). Liver and cancer cells in the replacement pattern show a close contact and direct interaction and the edges of metastases with this growth pattern cannot be defined. Furthermore, this growth pattern is correlated with the worst prognosis for patients. Two new patterns were recently described: sinusoidal and portal, which show metastatic growth in ducts and vessels and cancer cells do not reach the liver parenchyma. Among the five different patterns, the type of metastatic growth that we observed in the experimental model used in this study closely resembles the replacement pattern. The direct interaction between cancer and liver cells that is associated with this pattern allows the study of competitive

interactions that occur during the final stages of the metastatic cascade [18]. In this chapter, we question whether adaptative changes take place in intestinal cancer cells during circulation, dissemination and liver colonization. We show these changes cause long-term adaptations that gives these cancer cells a competitive advantage when they are forced to undergo the metastatic cascade.

RESULTS

Subsequent liver enrichment enhances growth of intestinal cancer metastases

To assess if there is organotropism of intestinal cancer cells towards the liver, we experimentally mimicked the final stages of the metastatic cascade. For this, cancer cells from Villin-CreERT2::APC$^{fl/fl}$::KRAS$^{G12D/WT}$::P53$^{fl/R172H}$; Dendra2 organoids, derived from primary intestinal tumors, were introduced in the hepatic blood circulation of C557BL/6JRj mice by injection in a mesenteric vein (Figure 1A). Next, isolation of organoids derived from cells that colonized the liver and re-injection of those, took place consecutively, until intestinal cancer cells completed three rounds of liver exposure. Parental tumor cells were able to colonize the liver within three weeks (Figure 1.B, green) and form micrometastases (Figure 1D). At the endpoint of the experiment, mice were euthanized without displaying any signs of discomfort or illness, suggesting that metastasis growth did not severely compromise liver function. Additionally, macroscopic examination of livers from Round 1 injections (Figure 1D and D' and Supplementary 1A-C) revealed no substantial alterations in color, stiffness, or structural changes when compared to control livers (Figure 1C). This collectively demonstrates that parental tumor cells can reach and colonize in the liver. However, they were not sufficiently aggressive to cause a life-threatening symptoms in the mice after three weeks.

Micrometastases were isolated, and organoids were derived (Figure 1.D). To study the effect of the exposure to liver microenvironment, cells of these enriched organoids were introduced in the hepatic blood circulation and their colonization potential was followed, referred as Round 2 of injections. Injected mice showed severe discomfort two weeks after surgery. Consequently, this group of mice had to be terminated at day 15 post-surgery (Figure 1.B, Round 2). The one-time liver-enriched cancer cells aggressively colonized the liver, giving rise to macrometastases. Macroscopically, a notable alteration of tissue integrity was observed. The pale and rigid tumor mass significantly invaded the organ, leaving less than 50% of healthy tissue unaffected (Figure 1E and Supplementary 1D-E). Microscopically, metastases showed replacement pattern type growth; no clear metastatic borders were found in liver tissue and patches of liver cells could be found within the Dendra2-positive cancer mass (Figures 1 E" and E"'). Taken together, the combination of the significant morphological change of the liver and the severe discomfort of the mice

indicates severe impairment of liver function. Thus, the metastatic potential of intestinal tumor cells is increased upon exposure to liver environment.

Next, macrometastases from those mice were isolated and organoids were derived. Mice were injected with these macrometastases derived organoids, named as Round 3 of injections. These mice showed important severe discomfort and it was reached faster, one week after surgery (Figure 1B, Round 3). Colonization and growth of these cancer cells was markedly enhanced, occupying nearly the entire organ tissue (Figure 1F, Supplementary 1G-I). The little remaining liver tissue appeared intercalated with the metastatic mass, showing a replacement pattern (Figures 1F″ and F‴). Thus, repeated exposure to the liver microenvironment causes organotropism and boosts the colonization capacity of intestinal cancer cells.

Liver enrichment does not provide a competitive advantage *in vitro*

The observed differences between parental and liver-enriched cancer cells in their interactions with liver tissue suggest a change in cellular fitness. Therefore, we sought to investigate whether the increased level of colonization observed *in vivo* provides cancer cells with a competitive advantage. To study competition between liver and intestinal cancer cells (both parental and liver-enriched) we used a previously developed mixed organoid model [19]. For this, cancer cells isolated from parental and liver-enriched intestinal cancer organoids were mixed with wild-type liver progenitor organoids. After three days of competition, mixed and pure conditions were compared by immunofluorescence imaging. Pure parental and liver-enriched cancer organoids did not show major differences in growth and morphology (Figure 2A and D). Furthermore, in mixed organoids both parental and liver-enriched cancer cells showed an equal capacity to outcompete wild-type liver progenitors (Figures 2B, C, E and F). This suggests that, *in vitro*, there is no significant increase in the proliferative and competitive capacity of liver-enriched cancer cells. Therefore, the competitive advantage demonstrated by liver-enriched cancer cells within liver tissue *in vivo* is not recapitulated *in vitro*.

DISCUSSION

The aim of this study is to assess whether exposure to liver environment has an impact on the colonization potential of primary intestinal cancer cells. Previous work has highlighted that there is an initial selection of cancer cells that can reach the liver and form metastases after multiple challenges [1]. However, it remains unclear whether those cancer cells which successfully adapt and thrive in the liver microenvironment have undergone specific adaptations that enhance their potential to colonize the liver.

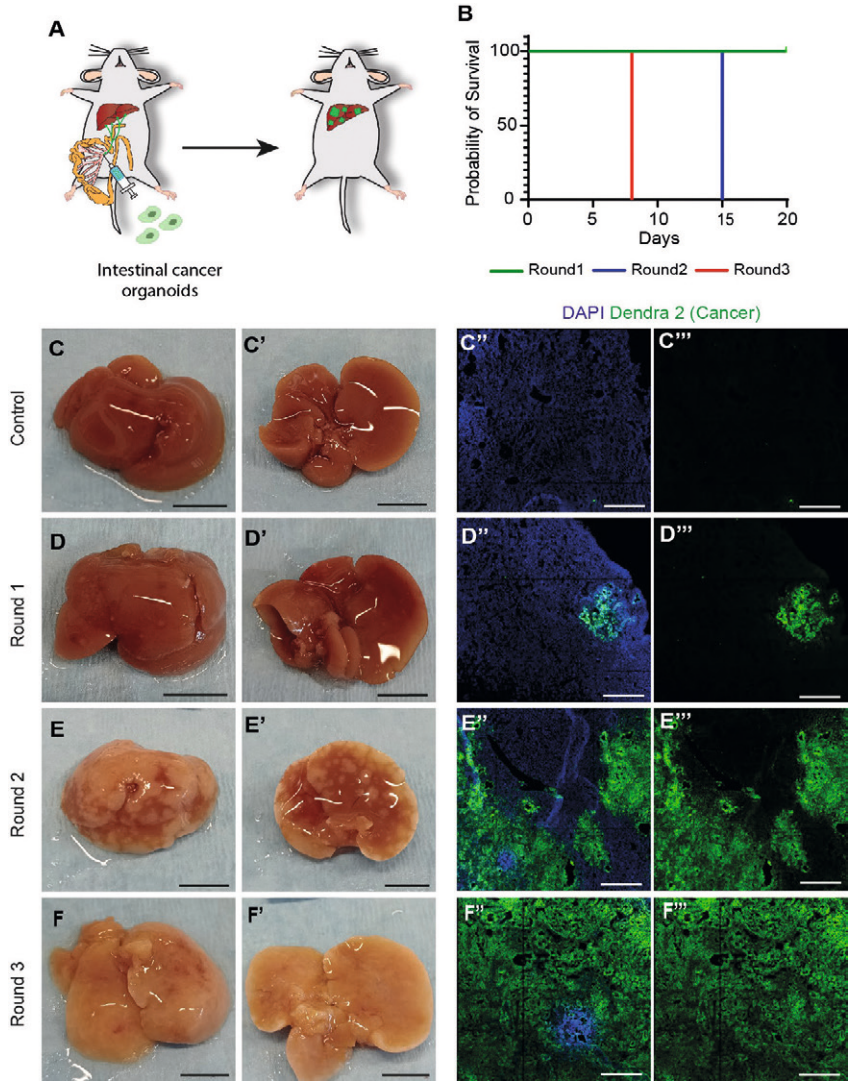

Figure 1. Subsequent liver enrichment enhances growth of intestinal cancer metastases. (A) Illustration of the mesenteric vein injection procedure. (B) Kaplan-Meier survival analysis showing a progressive reduction in survival across multiple rounds of liver enrichment. Each line displays an individual cohort; Round 1 (green) are animals injected with cells derived from the parental organoid line, Round 2 (blue) represents mice injected with cancer cells derived from micrometastases of Round 1, reaching the humane endpoint at 15 days post-surgery. Round 3 (red) represents mice injected with cancer cells derived from macrometastases of Round 2, reaching the human endpoint at 8 days post-surgery (Log-rank (Mantel-Cox) test, Chi square = 10.73, df = 2, P value = 0.0047; N= 3 mice per group). (C-F) Macroscopic images of top (') and bottom ('') of livers isolated from control (C), Round 1 (D), Round 2 (E) and Round 3 (F) animals. Immunofluorescent images of the same tissues stained with DAPI (blue) and Dendra2 (green). Scare bar represent 1cm in the macroscopic pictures and 500 µm in immunofluorescence images.

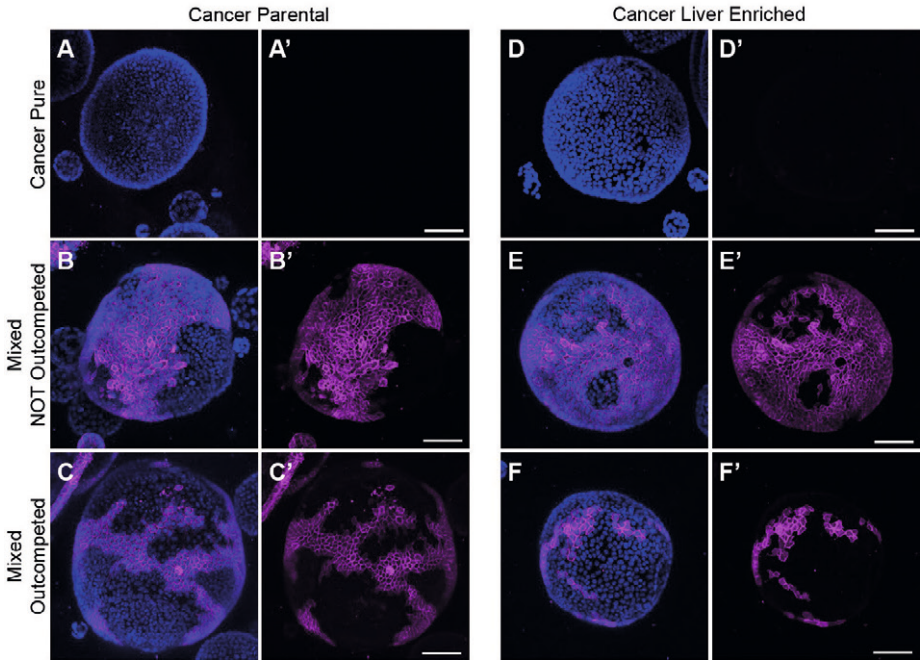

Figure 2. Liver enrichment does not provide a competitive advantage *in vitro*. Representative 3D-reconstructed confocal images of pure cancer (A and D) and mixed organoids (B-C and E-F), derived from parental (A-C) or liver-enriched (D-F) cancer organoids. Scale bar represents 100 µm, DAPI (blue) and liver membrane expressing tdTomato (purple).

In this study, the colonization, invasiveness, and disease progression of intestinal cancer metastasis was correlated to the number of passages in the liver.

To faithfully recapitulate the intricate journey of cancer cells to invade the liver, we employed mesenteric vein injections (Figure 1A). This approach skips the initial stages of the metastatic cascade, wherein cancer cells extravasate from the primary tumor into the bloodstream. Instead, this model is highly valuable to recapitulate the later stages of the metastatic cascade: dissemination from the portal vein and outgrowth in the liver. Therefore, this model allows us to gain insight into the mechanisms by which cancer cells colonize and adapt to the liver environment.

The $APC^{fl/fl}$::$KRAS^{G12D/WT}$::$P53^{fl/R172H}$ cancer cells exhibit a replacement pattern in the liver [18]. This means that there is no barrier between metastases and surrounding liver tissue, and cancer cells intermingle with liver cells (see Chapter 1). Thus, due to the absence of barriers between cells, this growth pattern allows competitive interactions between cancer and liver cells that are mediated by direct contact. Here, we provide evidence that that colonization and invasiveness of cancer cells in liver tissue is increased by previous exposure to the liver microenvironment (Figure 1).

This suggests that during previous contact with liver tissue cancer cells acquired a competitive advantage. The genetic and cellular adaptations of cancer cells that are induced by the liver environment remain unclear. Therefore, further functional and transcriptomic analysis, where parental and liver-enriched cancer organoids are compared, is required to address this question. For instance, single-cell RNA sequencing of parental and metastasis-derived cancer organoids may uncover gene signatures attributable to liver enrichment.

A limitation of this experimental setup is the lack of information about the early events of colonization. Since we examine the tissue at the experimental endpoint or once humane endpoint is reached, it remains unknown whether the reason of the increased colonization arises from a greater number of DTCs, a higher proliferation rate and/or enhanced survival of the cells, or a combination of these factors. To address that limitation, time-course experiments are needed. For instance, extracting the tissue at sequential time points or tracking liver colonization after injections by MRI or IVIS could offer valuable insights. Interestingly, the increased potential to metastasize acquired by cancer cells after liver enrichment had a direct effect on liver function. Mice subjected to the second and third rounds of injections displayed an accelerated onset of discomfort and reduced survival. Consequently, the robust colonization of the liver in these injection rounds is indicative of a pronounced impairment in liver function, leading to the appearance of clinical symptoms. Likely, caused of a reduction of remaining healthy liver tissue in rounds 2 and 3.

The adaptations and increased colonization capacity of liver-enriched cancer cells did not provide them with a competitive advantage in mixed organoids. We initially hypothesized that the use of organoids derived from heavily colonized livers would yield a more aggressive organoid line that could outcompete wild-type liver progenitors more rapidly than parental cancer organoids. However, the analysis of mixed organoids following a three-day competition assay revealed no disparity between the three-time liver-enriched and parental cancer lines. It is unlikely that adaptations of liver-enriched cancer cells are lost by *in vitro* propagation as the colonization potential of these cells *in vivo,* was further enhanced by each round of enrichment, while the cells were maintained *in vitro* between injections. However, there are multiple explanations for the absence of a competitive advantage *in vitro*: 1) in mixed organoids the cancer cells are exclusively confronted by liver progenitor cells, whereas *in vivo* cancer cells face a plethora of cell types, including hepatocytes, cholangiocytes, endothelial cells and immune cells. 2) The extracellular matrix components of the liver, which could be crucial for cancer proliferation and survival, are absent in organoids. 3) For *in vitro* culture, organoids are maintained in pro-proliferative conditions with an unlimited supply of growth factors. These conditions might obscure competitive differences,

such as independence of niche-factors, between parental and liver-enriched cancer cells. Our future aim is to investigate differences in competition potential between parental and liver-enriched cancer cells in a model system that better recapitulates the liver microenvironment. We recently developed liver metastasis microtissues containing hepatocyte-like cells, differentiated from liver progenitors, and cancer cells [20]. Using these microtissues we will study the cellular interactions of the different cancer cell populations with the main cell type of the liver (hepatocytes). Importantly, no exogenous extra-cellular matrix is supplemented to these cultures and the culture medium is developed to promote differentiation rather than proliferation. Therefore, these conditions are more like the liver-microenvironment. In addition, if necessary other cell types can be included in these microtissues.

In conclusion, primary intestinal cancer cells which can colonize the liver and repeated exposure to the liver microenvironment enhances their colonization potential. Notably, this enhanced colonization results in impaired liver function. Nonetheless, it is essential to faithfully recapitulate the liver microenvironment, as liver-enrichment does not provide an increased competitive advantage over progenitor liver cells in mixed organoids.

MATERIAL AND METHODS

Mice

All experiments were performed in accordance with the Animal Welfare Committee of the Netherlands Cancer Institute, the Netherlands. Animals were kept were housed under standard laboratory conditions at the Netherlands Cancer Institute facility and received standard laboratory chow and water ad libitum. Mice used as acceptors of mesenteric vein injections were C557BL/6JRj (3082) purchased from Janvier, males from 8-21 weeks of age.

Mesenteric vein injection

To mimic liver metastasis, mice underwent mesenteric vein injection of single cells (0.5-1 million cells in 100uL PBS) derived from cancer organoids. For a detailed description of the surgical procedure, please refer to [21]. The injected cancer organoids were derived from parental organoids (Round 1), or organoids derived from micrometastases (Round 2) and macrometastases (Round 3) from subsequent rounds of injections. All organoid lines were maintained in culture between injections. Mice were monitored closely and weighed daily during first three days after surgery

and twice per week after that until the end of the experiment or the humane endpoint was reached.

Tissue extraction, fixation and freezing

Mice were euthanized by CO2 and livers and lungs were extracted. Tissues were kept in PBS on ice until fixation in PLP buffer (25% of PFA 4%; 0,212% $NaIO_4$; 37,5% L-Lysine 0,2M in P-buffer; 37,5% P-buffer 0.1M Ph7,4) for 24h. Next, the organs were washed twice with P-buffer (40,5% Na_2HPO_4 0,2M; 9,5% NaH_2PO_4 0,2M; 50% H_2O ; pH= 7,4) and for 24h-72h in Sucrose solution (30% in P-buffer). Lastly, the organs were frozen in OCT (TissueTek ®) and kept at -80 degrees.

Organoids isolation

Metastases isolated from mouse livers were dissected from the tissue and minced on ice using a sterile scalpel. The tumor mass was digested at 37°C for 1 hour in 5mg/mL of collagenase (Roche, 10269638001) and 20µg/mL hyaluronidase (Sigma-Aldrich, 37326-33-3) dissolved in advanced DMEM/F12 medium (Thermo Fisher Scientific, 12634-010) containing 100µM Y-27632 (Abmole Bioscience, M1817), 4µg/mL DNAse I (Sigma-Aldrich, 10104159001). After digestion, the solution was filtered using a 100µm strainer, centrifuged at 300xg for 3minutes and plated in BME (see Chapter 2 for details on organoid plating). Organoids were maintained in ENR medium (see Chapter 2, 3 and 4 for composition) supplemented with 100µg/ml Primocin (InvivoGen, ant-pm-1) and 100µM Y-27632.

Tissue and organoids staining

Tissues frozen in OCT were cut using a microtome in 0.18 µm slides and kept at -20 degrees until use. The protocol for staining tissue slides is similar to the procedure used for staining of organoids and microtissues as described in Chapter 4 [20] with the small adaptation of a short (5-20 min) PBS wash before start of the staining to rehydrate the tissue. Cancer cells were stained with anti-Dendra2 primary antibody (1:300, Antibodies-Online, ABIN361314) and Donkey anti-Rabbit, Alexa Fluor 488 (1:500, ThermoFisher Scientific, A-21206) as secondary antibody together with DAPI (1:2000, Toronto Research Chemicals, D416050; CAS: 28718-90-3). Mixed organoids were fixed and blocked as described in [19] and stained with DAPI.

Culture of mouse organoids

Small intestinal organoids were derived from Villin-CreERT2::APC$^{fl/fl}$::KRAS$^{G12D/WT}$::P53$^{fl/R172H}$ transgenic mice 8-20 weeks old, as described in [22]. Maintenance of cancer organoid culture can be found in [19,21]. Wild-type cholangiocytes were derived from Rosa26-CreERT2::mTmG mice [23] female 8-21 weeks and maintenance as described in [20].

Generation of mixed organoids

Mixed organoids were generated from liver progenitor organoids and either parental or liver-enriched (Round 3) cancer organoids in a 1:1 (WT:Cancer) ratio. Prior to mixing, liver organoids were disrupted into clumps of cells while cancer organoids were dissociated into single cell suspensions. For this, cancer organoids were subjected to a first step of mechanical disruption and second step of TrypLE (Gibco) enzymatic disruption. Next, the same number of cells for both cancer lines (parental and liver-enriched) was added to generate mixed organoids. The protocol followed as described in [19] and details of media compositions and reagents are described in [20].

Microscopy

Image acquisition of liver tissues and fixed organoids were acquired on a Carl Zeiss LSM880 Fast AiryScan Confocal Laser Scanning microscope (Axio Observer 7 SP with Definite Focus 2). A Plan-Apochromat 20x/0.8 WD=0.55mm air objective was used to obtain 12bit images with a 1024 resolution, via bidirectional imaging, using a pinhole size of 0.5AU for tissues and 1AU for organoids. A Z-slice thickness of 5 µm (tissue) and 2.5 µm (fixed samples) was used to cover the complete thickness of the sample (17 to 150 slices). The following laser lines were used: 405nm, Laser Argon Multiline (445/488/514), 561nm and 633 nm. For tissues the tile option was used taking 7x7 tile imagines.

Visualization

Images were either 3D reconstructed in Imaris or a maximum projection was made in Fiji (indicated in the figure legends). For liver tissues, raw images were first stitched using Zen software and colors were adjusted in Imaris. Next, snapshots were taken with imaris and also converted to RGB using FIJI, cropped and when necessary, smoothened, cropped, rotated and contrasted linearly.

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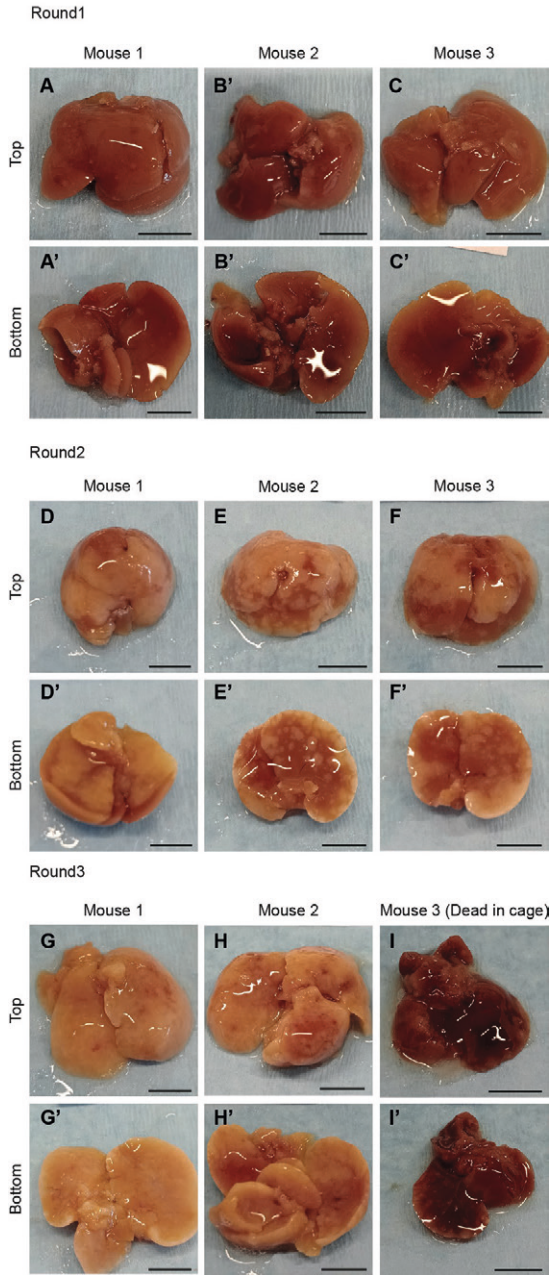
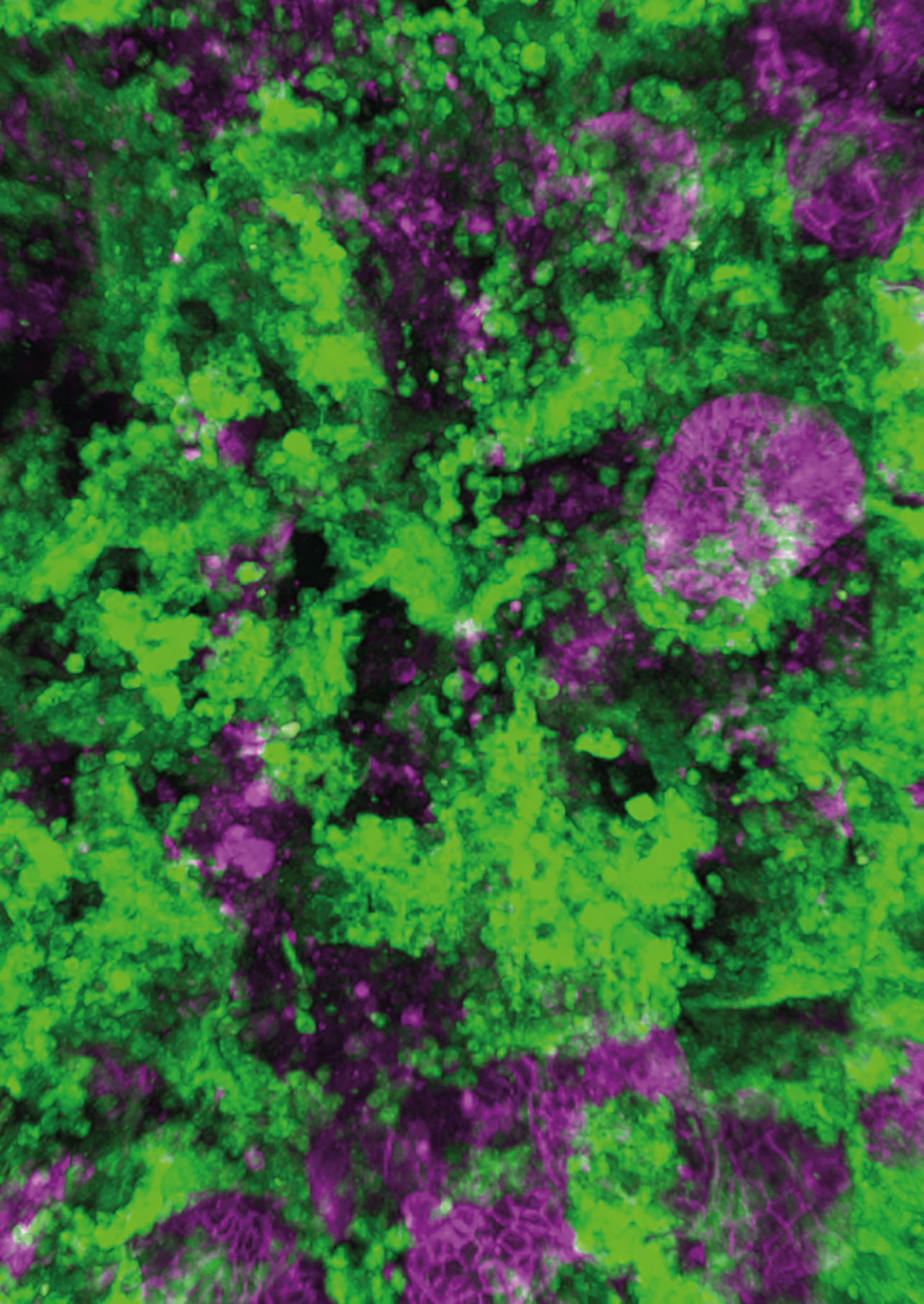
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Macroscopic overview of liver tissues. Overview of the livers isolated from all experimental mice. Top and bottom views of livers isolated from Round 1 (A-C), Round 2 (D-F) and Round 3 (G-I) of injections. Scale bars represent 1cm.

SUMMARY

The significance of cell competition in disease, particularly in cancer progression, has become increasingly apparent. Throughout tumorigenesis, cell-to-cell interactions play a key role in enabling cells to assess the fitness of their neighboring cells. This leads to the establishment of whether mutated cells will be outcompeted by healthy tissue, employing competition as a tumor suppression mechanism. Conversely, aberrant cells may become "supercompetitors," and win the battle with healthy cells. In this thesis, we delve into the interactions between intestinal wild-type and cancer cells during primary tumor and metastasis formation. **Chapter 2** outlines the development of a co-culture system that enables the investigation of direct cellular interactions between two epithelial cell types within the same organoid. This model allows the dissection of interactions between cells with a differential fitness based on both direct contact and short-range paracrine signaling. Our focus lies specifically on the interaction between intestinal wild-type and cancer cells, and it excludes the influence of non-epithelial cells such as those of the immune system. In **Chapter 3**, we demonstrate that the presence of intestinal cancer cells induces wild-type intestinal cells to revert to a fetal-like state. This reversion is accompanied by increased JNK activation and results in the active elimination of the wild-type population. Cancer cells exploit this competition to enhance their own growth. **Chapter 4** explores cell competition in a metastatic environment. We observe that intestinal cancer cells cause compaction of liver progenitor cells, leading to forced differentiation that is characterized by a cell-cycle arrest. While active elimination of liver progenitors is not observed, cancer cells can exploit these competitive interactions to increase their proliferation. In microtissues, competitive behavior of cancer cells manifests differently, here wild-type hepatocyte-like cells are used as a growth scaffold by cancer cells and subsequently actively eliminated. In **Chapter 5**, we investigate the dual role of the JNK pathway in cell competition in liver metastasis. We find that JNK activation is reduced in liver progenitors during competition, suggesting a pro-proliferative role that supports the maintenance of a progenitor state. However, JNK activation is increased in wild-type hepatocytes near cancer cells, indicating activation of this pathway during outcompetition of these differentiated cells. Additionally, we explore the role of YAP/TAZ in cell competition, observing a significant reduction in YAP activation in competing liver progenitors. Enhanced activation of YAP is sufficient to protect liver progenitors against outcompetition by intestinal cancer cells. Lastly, **Chapter 6** demonstrates that the *in vivo* liver microenvironment, promotes the colonization potential of intestinal cancer cells. This suggests that competition between cancer and liver cells induces adaptation of cancer cells to the liver environment, enhancing their ability to disseminate and colonize this tissue.



Discussion 7

Cell competition: from safeguarding tissues to disease development and new roles to discover

Cell competition is a concept which refers to cell-cell interactions based on fitness sensing of neighbor cells, leading to identification of the fittest (winners) which remain and the weakest (losers) which are eliminated by different mechanisms. The bases of cell competition were stablished during the first clonal analysis studies in *Drosophila* performed by Bryant and Schenderman (1969) and Garcia-Bellido and Meriam (1971) [1–3]. This process has been understood for years as a quality control mechanism to ensure that only the best cells persist in the tissue during development and homeostasis. In this thesis, we study the role of competition in growth of primary intestinal cancer (**Chapter 3**) and liver metastasis (**Chapter 4**) using mammalian models. We unravel how the interaction of healthy tissue with a fitter neighbor such as AKP ($APC^{-/-}$; $KRAS^{G12D}$; $p53^{fl/R172H}$) cancer cells, results in elimination (intestine and hepatocytes) or forced differentiation (liver progenitors) of the weakest cells. We show that the execution of the outcompetition relies on different pathways such as JNK or YAP/TAZ (**Chapter 3 and 5**), which are altered in the loser cells by competition. Therefore, our work provides support that in tumorigenesis, in different environments, cancer cells are super-competitors which, by mimicking cell competition processes, can promote their survival and growth at cost of affected wild-type.

Our findings lead to potential novel roles of cell competition. For instance, in **Chapter 1** of this thesis, we explain how cell competition can influence liver regeneration and transplantation. When healthy liver cells are transplanted in a chronically damaged liver, fitness recognition starts that leads to re-population of the tissue by the fittest cells, while simultaneously the damaged and weaker resident cells are eliminated [4–8]. Transplantation of fetal liver cells in an aged liver results in similar outcome. Aged tissue does not necessarily imply that the tissue is sick or malfunctioning, but aged tissue is less fit than young tissue [9]. Identifying how the recognition of fitness and execution of competition takes place during transplantation, which means understanding the competition cascade, can give us powerful tools to improve transplantation and regeneration. For instance, it is known that liver fetal cells are more resistant to Activin A signaling, which lead to apoptosis, while aged cells show higher number of receptors and therefore more sensitivity. Therefore, during transplantation of fetal cells the aged liver is eliminated through Activin A [10,11]. Thus, manipulation of Activin A sensitivity could be used to improve transplantation efficiency through cell competition.

Interestingly, cell competition has also been described in adult tissue with a low regenerative capacity such as heart and brain [12,13]. Understanding the mechanisms

of fitness sensing and competition execution in those tissues in homeostasis, could give important insight into improvement of transplantation efficiency. For instance, in mammalian heart, *Myc* overexpressing cardiomyocytes eliminate and replace wild-type cardiomyocytes [12]. Therefore, if there is damage to heart tissue, transplantation of *Myc* healthy cardiomyocytes should repopulate the heart in a more efficient manner than wild-type counterparts, putatively improving the outcome of the transplantation. Similarly, in the brain of *Drosophila*, there is a fitness sensing based on detecting amyloid-β deposition in neurons during the development of Alzheimer´s disease. Healthy neurons identify these amyloid-defective neurons through *Flower* sensing and execute competition, by elimination via *azot* expression [13]. Important advances could be made in brain regeneration by promoting the sensing of defective neurons during disease.

Lastly, cell competition can also play a role during bacterial infection. Upon infection, bacteria-bearing cells usually show a competitional disadvantage against their healthy counterparts, and therefore the outcome of competition results in their elimination [14]. Moreover, the stiffness of the Extracelullar Matrix (ECM) regulates motility and physical forces which can modulate the output of cellular competitors. It has been described that increasing ECM stiffness promotes extrusion of infected cells, reducing the infection size [14]. Therefore, unraveling the processes of cell competition which take place during different pathogen infections, could be a useful mechanism to eliminate infected cells during the early stages of the infectious disease and avoid future complications due to antibiotic, antiviral or antifungal resistance. As an example, if elimination of infectious cells is more efficient by higher ECM stiffness, administration of antibiotics could be complemented with factors and cues that promote ECM deposition such as hypoxia factors or TGFβ [15], to increase the stiffness of the area where the treatment should have an effect.

Taken together, the role of cell competition has evolved enormously during the years. From its originally described function in development and tissue homeostasis, this process became rapidly important for tumorigenesis. However, the co-existence of fitter and weaker cells is applicable to many other circumstances. Here, through elimination of less fit cells, cell competition has major potential influence on processes like transplantation, regeneration and infection. Therefore, cell competition-based therapies are an innovative new route to improve treatment of disease.

Innovative models of mammalian cell competition: mixed organoids and microtissues

The research presented in this thesis underscores the significance of cell-cell interactions in cell competition. To properly delve into these interactions, suitable

models are needed. In this thesis, two novel models are presented: mixed organoids and microtissues. The generation of mixed organoids is extensively described in **Chapter 2**. Organoids have revolutionized the understanding of modeling disease in the last decade. This approach has significantly improved the fidelity of disease models mimicking the actual tissue architecture and function. Until now, organoids from different tissues such intestine, breast and pancreas have combined different cell types from the same tissue within the same organoid [16–19], but organoid models which combine cells from different epithelial origin are limited. In **Chapter 3**, we describe how generation of mixed intestinal organoids can recapitulate the cellular interactions that take place during primary tumor formation. This model offers the great advantage of showing interactions between only epithelial cells without masking interactions by stroma or immune cells. The adaptation of this model to study liver metastasis is described in **Chapter 4**. Although liver progenitor organoid cultures have been previously described [20,21], our approach uniquely simulates the competitive environment of metastatic growth, wherein liver progenitors and intestinal cancer cells compete for space within the organoid. Importantly, these models can be extrapolated to many other cancer types. For this, a key challenge is the medium formulation, which must support the growth and viability of diverse cell types. Additionally, while direct cell contact is established between competing cells within the organoids, the model is constrained by some limitations. For instance, the maximum duration of experiments is limited, once mixed organoids are formed, they cannot be expanded without disturbing the competition process. The use of microtissue models has evolved enormously during the last years [22,23]. They have previously been used to mimic various organs such as brain [24] and heart [25], and were adapted in our study to liver metastasis. As described in **Chapter 4**, we used differentiated liver progenitor and intestinal cancer organoids to model metastatic competitive interactions in the liver in microtissues. The absence of an artificial matrix in microtissues, allows the formation of a more realistic tissue environment and avoids external cues, which is a notable advantage of this model.

There are unique characteristics of these models which allow the discovery of important aspects of competition. The nature of the models, where epithelial cells are exclusively in close contact with other epithelial cells from a different origin, assure that the observed effects are only caused by the interaction of these two cell types. For instance, in **Chapter 5**, we describe the decreased activation of YAP and JNK during competitive interactions between liver progenitors and intestinal cancer cells, while in competing hepatocytes in microtissues we observed increased JNK activation. Where, previous studies indicate that liver damage induces JNK activation [26] and activation of YAP in liver cells during primary liver cancer driven competition [27], their findings are based on complex tissues. Therefore, in these studies it is not possible

to distinguish the influence of the individual cell populations. In contrast, our models allow the separation of effects, which led to the finding that competition has a different impact on liver progenitors than hepatocytes. In addition, these models allow the dynamic analysis of behavioral changes of the individual cell populations in space and time. For instance, we could define that active elimination of wild-type intestinal cells begins after 24h of competition and that this is highest near cancer cells, while all liver progenitor cells stop proliferating between 24h and 72h of competition. Such findings are crucial for our understanding both competition and tumor growth and are impossible to discern in complex tissues [28–30], or minimalistic monolayer cultures [31,32]. Moreover, mixed organoids and microtissues allow us to determine the influence of wild-type cells on the behavior of cancer cells. The detrimental effect of tumors on surrounding organ tissue have been widely studied [27,33]. However, by direct comparison to the behavior of cancer cells grown in pure conditions we can dissect the direct effects of competitive interactions also in cancer and not only in wild-type cells. This is particularly striking in microtissues (**Chapter 4**), where we observed that pure cancer shows poor survival, instead these cells fully depend on hepatocyte-like cells for viability, using them as scaffold to grow. This specific outcome would unlikely have been elucidated with a different competition model.

In summary, this thesis makes a substantial contribution to the development of novel models of cell competition. Mixed organoids and microtissues facilitate the investigation of direct interactions between different cell types, including cancer cells. These models can easily be adapted to other organs, such as lung, breast, brain, pancreas, and uterus. The exclusive focus on epithelial cells in these models allows us to delve into the direct cell interactions from the main players in tumor development which in many occasions is masked by the influence of tumor microenvironment such as the immune system and stroma.

Cell competition induces tissue reprogramming

When a tissue is challenged by injury, the response of the unaffected surrounding tissue often results in an adaptation of its differentiation status. This response does not always go in the same direction. In some cases, the tissue responds by de-differentiation to a primitive state, while in other cases, damage instead induces differentiation. Interestingly, cancer-driven cell competition shows a lot of similarities to the damage response of tissues. In this section, we discuss how these two types of reprogramming are triggered by cell competition, depending on the environment and cell types involved in the process.

In **Chapter 3** of this thesis, we describe how competition with intestinal cancer causes wild-type intestinal cells to revert to a primitive state [34]. These cells start to express

a “fetal-like” gene signature, which is also found during colitis [35,36] and helminthic parasite infection [37]. In addition, TGFB-1 dependent intestinal regeneration causes activation of this signature, independent of cancer lesions [38]. Therefore, in the intestine the reaction to competition during primary tumor growth overlaps with a response to damage and regeneration. Moreover, the same year as our publication of **Chapter 3** [39], paracrine activation of fetal signatures in intestinal organoids adjacent to cancer was reported. This is dependent on regenerative YAP-associated signaling and supports the hypothesis of regeneration-driven fetal signature expression [40]. Together this indicates that, sensing -either by direct contact or by paracrine signaling- of the presence of primary cancer cells by wild-type intestinal tissue, triggers a competitive response which, at least initially, shares many similarities to other types of tissue damage and is based on reverting to a less differentiated state.

The expression of fetal genes in the tissue during cancer driven competition is not exclusive to the intestine. Similar to primary intestinal tumors, lung adenocarcinoma (LUAD) parallels injury repair mechanisms. In this tissue, expression of p53 promotes differentiation of alveolar type 2 cells towards alveolar type 1 thereby avoiding LUAD, which mimics the natural injury repair response in the lungs. Loss of p53 leads to an accumulation of alveolar type 1 transitional cells, a process also observed in fibrosis [41]. This indicates that lung cells sense p53 mutant cells as fitter, initiating a competition response in which, lung cells are reprogrammed into a less differentiated stage. Moreover, the reversion to an undifferentiated stage is also found in different components of the tumor microenvironment. Recent reviews defined the concept of “onco-fetal reprogramming” referring to a cancer-driven fetal reprogramming within the tumor microenvironment. This suggests that such reprogramming confers plasticity and resistance against cancer growth in both epithelial and non-epithelial cells, but it is especially found in tumor-associated macrophages, cancer-associated fibroblasts, and endothelial cells [42,43]. In summary, the reversion of peritumoral cells to a primitive state has been described in different tissues and in various cells of the tumor microenvironment. This suggests that reprogramming of competing tissue, could be a universal competition-driven response to damage. The purpose of this response could be to avoid elimination by cancer or to communicate the state of damage to cells at a distance of cancer lesions. However, if the damage persists, the reverted wild-type epithelial tissue is ultimately eliminated. Future experiments, designed to block reversion to a fetal-like state can unravel whether this prevent outcompetition of wild-type cells, and if that is sufficient to impair growth of cancer cells.

Injury can also have an opposite effect on the surrounding tissue and cause differentiation instead of reverting to less differentiated state. For instance, in the liver chronic damage induces differentiation of progenitor cells to overcome the loss of hepatocytes, in a response called “ductal reaction” [44,45]. **Chapter 4** of this thesis

explores how cancer-induced competition evokes a similar reaction in liver progenitor cells. Our study shows that liver progenitors undergo compaction, which leads to forced differentiation, when mixed with intestinal cancer cells. Therefore, in our model of liver metastasis, we have shown that liver progenitor cells sense metastasis as a source of chronic damage and respond to it with a "ductal reaction" *in vitro*. This forced differentiation of peritumoral cells has been also found in the skin. In this tissue, basal cell carcinoma, induced by overexpression of the oncogene SmoM2 leads to terminal differentiation of neighboring wild-type cells. Interestingly, this forced differentiation is dependent on the stiffness of the micro-environment and was only found in areas of the skin with low collagen deposition [46]. Thus, competition drives reprograming of neighboring wild-type cells, but it can manifest in different manners. The reason of this different outcome of competition remains unknown. However, we speculate that cancer cells induce differentiation of their competitors to reduce their proliferation rate and thereby facilitate colonization of the tissue by cancer cells.

In microtissues hepatocyte-like cells show increased apoptosis during competition with intestinal cancer, which is very similar to the response of hepatocytes to acute damage in the liver. Therefore, microtissues may recapitulate a response to acute damage that is driven by metastases. Whether this also induces reprogramming of hepatocyte-like cells within microtissues remains elusive. Therefore, fetal liver signatures and damage response signatures should be studied in competing wild-type cells in microtissues [47,48]. Thus, cancer cells trigger a competitive reaction which resembles a damage response, leading to cell-fate reprograming in surrounding healthy tissue. Whether this reprogramming induces a progenitor state or induces differentiation, depends on the involved tissue, environment and cell types.

Targeting cell competition to treat cancer

In **Chapter 3** of this thesis, we investigate the intricate competitive interactions between tumor and wild-type cells during primary intestinal tumor growth. Understanding these interactions is crucial not only for gaining fundamental knowledge about cancer biology but also for developing novel cancer treatments. Historically, cancer treatment strategies have primarily focused on targeting and weakening cancer cells. This approach is often insufficient because of several reasons. For instance, cancer cells can adapt to the treatment and become resistant. Also, they can enter dormancy and become inactive for days, months or even years, until their reactivation causes secondary growth and relapse. Moreover, radio- and chemotherapies have a negative impact on the surrounding tissue. As discussed in **Chapter 1** and throughout the thesis, the outcome of cellular competition is context-dependent, suggesting that, if weakening the stronger competitor is ineffective, an alternative strategy could be

to strengthen the weaker competitor. Therefore, enhancing the fitness of wild-type cells during competition, could lead to a reversion of roles within the competitive landscape. Thus, by empowering the wild-type to protect them from outcompetition might limit tumor growth.

In support of this concept, **Chapter 3** presents three examples where altering competition between wild-type and cancer intestinal cells favored the protection of wild-type cells. For example, we observed that blocking apoptosis prevented outcompetition of surrounding wild-type cells. Similarly, enhancing stemness of wild-type cells, not only promotes their proliferation, but also prevents reversion to a fetal-like state and elimination. Furthermore, we demonstrated that modulating the JNK pathway in wild-type intestinal cells alters their susceptibility to cancer-driven elimination. Chemical or genetic downregulation of this pathway increased the resistance of wild-type cells against cancer. Together, this underlines the potential of targeted interventions to strengthen wild-type cells which enables them to resist cancer outcompetition. In the patients, this would probably not eliminate the cancer cells but would lead to control and restriction of tumor growth.

This innovative approach is already being explored in a clinical trial by Vermeulen laboratory. One of the most important discoveries in the field of colorectal cancer in the last years has been the fixation of APC mutant Intestinal Stem Cells (ISC) by outcompetition of their wild-type counterparts. APC mutant ISCs have a dual competitive advantage. On one hand, the dependence of WNT signaling is lost in mutant ISCs, leading to unrestricted proliferation. On the other hand, APC mutants secrete WNT antagonists, such as NOTUM, to which mutant ISCs are unsensitive but which induce differentiation of neighboring wild-type ISCs. This results in the replacement of wild-type ISCs by APC mutant ISCs in crypts and ultimately, adenoma formation [49–52]. Lithium is used as a GSK-3β inhibitor which boosts WNT signaling in wild-type cells, counteracting the effects of WNT antagonists coming from APC mutants. This leads to a supportive effect for wild-type ISCs and protection from outcompetition, this increases their chances of crypt fixation and reduces the risk of adenomas formation [53]. Exploiting this knowledge, Vermeulen laboratory, is currently conducting a Phase II clinical trial using lithium to prevent outcompetition of wild-type tissue nearby adenomas in Familial Adenomatous Polyposis (FAP) patients [54].

In summary, the role of cell competition in tumor formation and growth extends beyond theoretical relevance to practical applications in cancer therapy. The context-dependent nature of cellular competition presents a unique advantage for identifying new treatment targets. We have described how cancer can exploit cell competition mechanisms to its advantage. Now, by exploiting our understanding of these

interactions, we can aim to enhance the competitive ability of wild-type cells when they encounter cancer, transforming wild-type cells into the 'winners' of this cellular battle.

Mechanistic regulation of competition in primary and metastatic intestinal cancer

In **Chapter 1**, we outlined a conceptual framework for cellular competition, which is built on three steps: (1) the coexistence of cells with varying fitness levels within the same tissue, (2) the sensing of differential fitness among these cells, and subsequently (3) the execution of a competitive response [55]. Current research within our group is focused on elucidating the molecular mediators of cell-to-cell interactions that facilitate the recognition of differential fitness. Modulating these early stages of fitness sensing could lead to significant alterations in the competitive dynamics. The research presented in this thesis, however, concentrates primarily on the effectors of competition, specifically the pathways activated once the roles of 'winner' and 'loser' cells have been established. **Chapter 5** delves into two pathways – JNK and YAP/TAZ – which our findings suggest to play crucial roles in mediating cellular competition. Nevertheless, a comprehensive mechanistic understanding of their involvement remains elusive, and it is likely that other important pathways contributing to competition execution have yet to be discovered. One of the main take-home-messages of this thesis is that competition cascades are not uniform and highly depend on the environment and competing cells. Also, both winner and losers are subjected to modifications conditioned by how competition develops. In this thesis, we describe various competitive interactions between epithelial cells that are responsible for primary and metastatic intestinal tumor growth. We consider that these interactions can be classified in two types of competition: a "molecular-JNK-driven competition", causing elimination of loser cells, and a "mechanical-YAP-dependent competition", which results in forced differentiation of loser cells. In this section, we discuss the mechanistic meaning for each of them.

"Molecular-JNK-driven competition", is described in **Chapter 3, 4 and 5** and is found in primary intestinal cancer and peritumoral hepatocytes (1). This type of interaction is characterized by competition of cells with a high degree of fitness mismatch. Wild-type intestinal cells and hepatocytes, show a low proliferation rate and high differentiation state, while cancer cells show high proliferation and less differentiation. These inherent disparities likely confer a competitive advantage to the cancer cells, enabling them to be recognized as the fittest within the tissue. (2) Subsequent fitness sensing between the two cell populations, sharing an epithelial layer in mixed organoids or a common space within the same microtissue, is crucial. Unfortunately, our research has yet to investigate these recognition interactions, leaving a gap for future studies. (3) Lastly, once the two populations are recognized as winner and loser, competition is executed by starting

a damage response reaction in the wild-type cells, which in intestinal cells is reflected by reversion to a fetal-like state. This triggers to activation of stress pathways and JNK upregulation, a phenomenon that we have observed in both mixed intestinal organoids and liver microtissues. A critical question is whether the general damage response causes stress pathway activation, or conversely, the stress response is an initial reaction to competition, subsequently causing a broader damage response. To clarify this, a time-course study examining markers of both responses is needed. Regardless of the sequence of events, the stress indicated by JNK activation results in the apoptosis of loser cells (wild-type cells). Consequently, cancer cells sense the elimination of losers and react by boosting their growth. Further research is required to determine the precise timing and nature of the signals that trigger this enhanced cancer growth.

"Mechanical-YAP-dependent competition" is described in **Chapter 4 and 5** and is found during the reaction of liver progenitors to metastases (1). Cells interacting in this type of competition have a more comparable level of fitness; both intestinal cancer organoids and liver progenitor organoids show high proliferation rates and contain mostly undifferentiated cells. Here, the effect of competition, which manifest as a decreased proliferation rate, becomes apparent only after 24h to 72h meaning that the step of fitness sensing requires a longer time (2). The precise mechanisms governing this final decision are not yet understood. However, changes of cellular and nuclear shape of liver progenitors suggest that cancer cells exert a mechanical force on liver progenitors. This constrains the proliferative space of wild-type liver cells causing their compaction. Liver progenitors potentially interpret this force as tissue damage, triggering a response that leads to differentiation – the execution phase of competition (3). We speculate that the damage-response initiated by compaction mimics the "ductal reaction" found when there is chronic liver damage [44,45], and therefore YAP inactivation-driven initiation of differentiation. The nature of the interaction between cancer cells and liver progenitors – whether purely mechanical or involving additional signaling modalities remains an area for future exploration. Actually, JNK signaling, which is involved in stress-signaling in molecular competition models, shows a reduced activation in competing liver progenitors. This indicates that JNK also plays a role in this type of competition, however, probably not as stress-signaling but as a regulator of liver progenitor proliferation. Therefore, we propose that this type of competition is a combination of mechanical and molecular competition. The ultimate outcome of "mechanical-YAP-dependent competition" is forced differentiation. Due to technical limitations, we are unable to track this competition for longer than five days, which is not sufficient for liver progenitor cells to complete the differentiation process to a mature hepatocyte state. Nevertheless, we hypothesize that, if liver progenitor cells could complete differentiation, these competing hepatocytes become susceptible to "molecular-JNK-driven competition". Competing cancer cells, take advantage of the loss

of proliferation and increase differentiation of their liver neighbors to enhance their growth. Interestingly, intestinal cancer cells show a major growth increase when there is a higher representation of liver progenitor cells at the start point of competition. This suggests that intestinal cancer cells benefit from either physical support or molecular cues provided by liver progenitor cells to improve their fitness and increase their growth.

Lastly, besides JNK and YAP/TAZ, additional signaling molecules and pathways must be also relevant for competition in primary and metastatic intestinal cancer. For instance, the fitness sensing mechanisms are unknown for the different competition models described in this thesis. Therefore, investigating ligand-receptor could shed light on the fitness-defining interactions, in particular pairs such as EPH/EPHRIN, WNT [56–58] and EGF/ERBB signaling are of high interest. Additionally, we wonder about the requirement of mechano-transduction and besides YAP exploring other mechano-transducers like Piezo1 could yield valuable insights [59–61]. Similar to JNK pathway, JAK/STAT signaling has been described as a regulator of competition in *Drosophila*, controlling the imaginal disc size [62] and the elimination of less fit *Minute* cells in the midgut [63]. Additionally, in liver, the inhibition of this pathway suppresses hepatocellular carcinoma initiation [64]. Therefore, JAK/STAT is a potential executor of competition in primary and metastatic intestinal tumor growth. As we have described, competition leads to damage response activation in both wild-type liver and intestinal cells during competition, therefore studying key molecules expressed during damage, such as TNF [65] could be instrumental in understanding the outcome of competition.

In summary, here, we describe potential mechanistic regulation of primary and metastatic intestinal cancer. However, further research is required to unravel the details of the competition cascade. Nonetheless, the discoveries presented in this thesis lay a foundational groundwork, setting the stage for subsequent in-depth investigations into this complex and promising area of research.

The impact of cell competition on liver metastasis

In this research, we have mainly focused on response of wild-type cells to competition. In **Chapter 6**, we highlight the influence of competition on the cancer population, and in particular, on adaptations when they transition to a new environment, a process that is essential for metastasis. Cancer cells which leave the primary tumor face numerous challenges, including immune surveillance and physical barriers. This requires adaptive modifications to achieve successful metastatic colonization. Upon entering the liver, cancer cells are subjected to a competitive environment, marked by interactions with diverse cell types, extracellular matrices, immune responses, and various soluble factors. Previous sections of this thesis have described the reactions

of liver progenitor cells and hepatocytes to the invasion of intestinal cancer. **Chapter 6** provides a different insight of metastatic interactions and competition between intestinal cancer and liver cells, revealing that increased exposure of cancer cells to the liver microenvironment correlates with enhanced aggressive traits in these cells. A critical observation is that only the fittest cancer cells, those capable of surviving the journey and colonizing the liver, contribute to metastasis formation. This exemplifies the ongoing fitness assessment and intrapopulation competition among cancer cells. Consequently, metastases in the liver are formed predominantly by cancer cells that have demonstrated a superior adaptability to the liver environment. This is demonstrated by the increased number and size of metastatic foci observed in subsequent liver transplantations. Particularly, after the third round of transplantation, a significantly more pronounced effect was observed, where the organ was almost completely colonized by tumor tissue. The progressively increased repopulation of liver tissue by intestinal cancer cells among the subsequent transplantations suggests a natural selection process within the cancer cell population, favoring cells with enhanced adaptability and competitive advantage in the new environment. Since our readout of colonization is based on tissue examination at the endpoint of the experiment, whether the adaptations of cancer cells are manifested as increased proliferation or improved grafting capabilities remain unknown. However, due to the clear effect of liver microenvironment in cancer colonization, it is suggested that cancer cells activate specific pathways and markers which promote the colonization and growth in subsequent exposure to liver. Further investigation is required to identify those pathways and markers increased in liver enriched intestinal cancer cells. For instance, single-cell RNA sequencing of samples from primary tumor and intestinal cancer cells that were subsequently exposed to liver, would provide important information about differentially expressed genes upon adaptation to liver microenvironment. Targeting the pathways where those genes play a role could be a valid approach to reduce metastasis formation. Furthermore, we wondered if these adaptations of cancer cells are intrinsic to intestinal cancer cells or driven by tissue-specific competitive interactions. To investigate this, we must examine whether intestinal cancer cells undergo similar adaptations in different environments, such as other secondary metastatic sites like the lungs. Alternatively, if the changes present in the liver environment are unique to interactions with liver tissue and not replicated in other tissues, this would open a promising line of research, where cancer metastasis could be modulated by manipulation of competition cascades in different tissues. Therefore, subsequent grafting in other organs together with comparison of the differentially activated pathways in liver and other tissues, could reveal how tissue-specific this adaptation is. In conclusion, cancer cells also experience cell competition processes during metastatic growth. This assures that only the fittest cancer cells remain within the lesions and allows adaptation to liver microenvironment.

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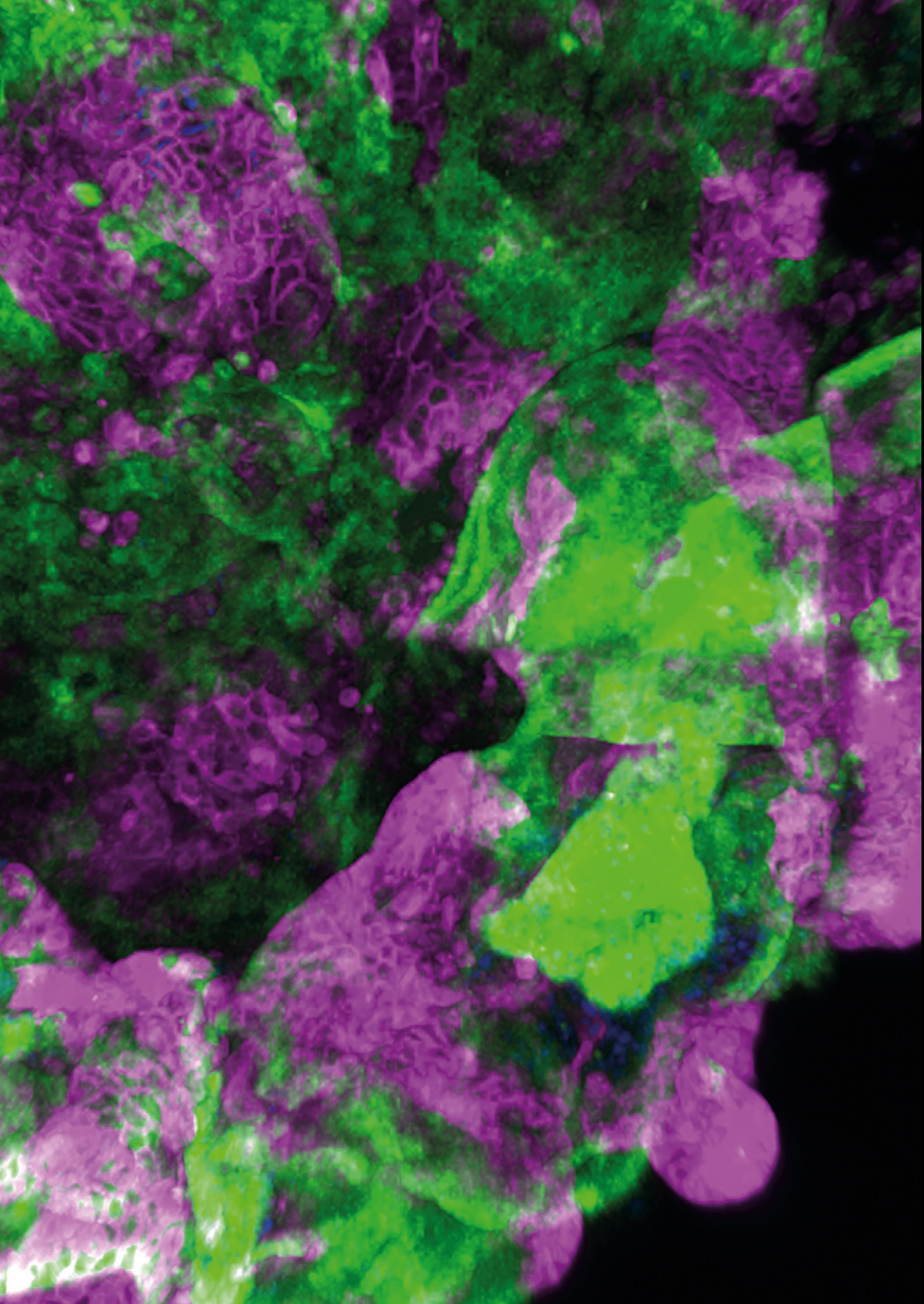

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ENGLISH LAYMAN SUMMARY

All our tissues are formed by a highly organized collection of living units called cells. To ensure the proper functioning of these tissues, continuous communication between cells is essential. A crucial type of information that is shared between cells is their fitness status. Fitness is a characteristic of cells that make them more adaptable, stronger, and prone to survive within a tissue. There is a continuous comparison of cellular fitness to ensure that the fittest cells, which thus means those with the best characteristics, remain in the tissue, while less fit cells are eliminated. This process is known as cell competition and serves as a quality control system to ensure that our tissues are primarily composed of the healthiest and strongest cells. However, some cell competition mechanisms can be exploited by cancer cells and help them to survive and proliferate. For example, when a non-mutated healthy cell, known as a wild-type cell, acquires an oncogenic mutation that promotes its proliferation, its fitness also increases. This cancer cell can use mechanisms of cell competition to colonize a tissue. In this thesis, we study cell competition that take place in intestinal cancer. We focus both on the intestine, where primary tumors initially arise and, on the liver, a secondary site where tumors grow after escaping from the primary site during metastasis. We want to understand the battle of intestinal cancer cells with wild-type intestinal cells and wild-type liver cells. To achieve this, we use lab-grown mouse-derived mini-organs and mini-tissues, also known as organoids and microtissues. These are 3D cell-cultures that resemble the tissues they originate from. We use these two models to mix cancer cells with wild-type intestinal or liver cells and study their interactions.

In **Chapter 1**, we provide an overview of what cell competition is and which cell competition processes occur in intestinal and liver tissues. **Chapter 2** describes a detailed technical protocol for the generation of mixed organoids that contain cancer and wild-type intestinal cells. It also shows various examples of assays that can be performed with these mixed organoids. In **Chapter 3**, we show that cell competition occurs between intestinal wild-type and cancer cells in mixed organoids. We discovered that cancer cells cause wild-type cells to activate signals of damage and stress. This puts them in danger and results in their cell death. Additionally, we found that cancer cells use these signals to enhance their own growth. Importantly, blocking these signals can protect wild-type cells against elimination and prevent the increased proliferation of cancer cells. In **Chapter 4**, we focus on interactions between intestinal cancer cells and two types of liver cells: cells that form the tubes that transport bile (bile duct cells) and cells that form most of the liver and are responsible for digestion and detoxification (hepatocytes). Cell competition causes bile duct cells to change their identity and become more similar to hepatocytes. Hepatocytes, on the other hand, are

forced to die by neighboring cancer cells. Cancer cells benefit from the interactions with both liver cell types. They use cell competition to increase their survival and proliferation. In **Chapter 5**, we describe how liver cells respond to mechanisms of cell competition. We explain that cancer cells cause different types of signaling in the different cell types of the liver. Bile duct liver cells reduce signals of stress, while these signals are increased in hepatocytes. On the other hand, cell competition causes bile duct liver cells to respond to mechanical forces. Manipulation of the signals involved in this response protect wild-type cells from losing the battle against cancer. Lastly, in **Chapter 6**, we study cell competition between intestinal cancer cells and liver tissue in mice. We observe a significant increase in growth of cancer cells in the liver after exposure to the liver environment. This suggests that cancer cells can adapt to this new environment which makes them more aggressive.

In conclusion, throughout this thesis, we focus on the process of cell competition at different levels of intestinal cancer evolution. We provide understanding of the consequences of competition to primary and metastasized tumors. We also demonstrate the importance of the context where competition takes place. We show that the outcome of competition can vary, depending on the involved tissue microenvironment and cells. Together, the research of this thesis shows that the fitness of a cell is very dependent on their surroundings. This has important implications in developing treatments for cancer and other diseases, since new therapies could be based on strengthening healthy cells and transform them in the winners in the battle against cancer growth.

DUTCH LAYMAN SUMMARY (NEDERLANDSE SAMENVATTING)

Al onze weefsels worden gevormd door een georganiseerde verzameling levende eenheden die cellen worden genoemd. Om ervoor te zorgen dat deze weefsels goed functioneren, is continue communicatie tussen deze cellen essentieel. Een cruciaal stuk informatie die de cellen met elkaar delen, is hun fitnessstatus. Fitness is een kenmerk van cellen dat hen meer aanpasbaar, sterker en kansrijker maakt om te overleven binnen een weefsel. Er vindt een voortdurende vergelijking tussen de cellen plaats van de cellulaire fitness, om ervoor te zorgen dat de meest fitte cellen, oftewel met de beste kenmerken, in het weefsel overleven, terwijl minder fitte cellen worden geëlimineerd. Dit proces staat bekend als celcompetitie en dient als een kwaliteitscontrolesysteem om ervoor te zorgen dat onze weefsels voornamelijk bestaan uit de gezondste en sterkste cellen. Sommige mechanismen van celcompetitie kunnen echter worden misbruikt door kankercellen en hen helpen te overleven en te prolifereren. Bijvoorbeeld, wanneer een niet-gemuteerde gezonde cel, bekend als een wildtype cel, een oncogene mutatie verwerft die de proliferatie bevordert, neemt ook zijn fitness toe. Deze kankercel kan mechanismen van celcompetitie gebruiken om een weefsel te koloniseren. In dit proefschrift bestuderen we celcompetitie die plaatsvindt bij darmkanker. We richten ons zowel op de darm, waar primaire tumoren in eerste instantie ontstaan, en op de lever, een secundaire locatie waar darmtumoren groeien nadat ze zijn ontsnapt aan de primaire locatie tijdens uitzaaiing. We willen de strijd tussen darmkankercellen en wildtype darm- en levercellen begrijpen. Om dit te bereiken, gebruiken we in het laboratorium gekweekte mini-organen en -weefsels afkomstig van muizen, ook wel organoïden en microtissues genoemd. Dit zijn 3D-celculturen die lijken op hun oorspronkelijke weefsels. We gebruiken deze twee modellen om kankercellen te mengen met wildtype darm- of levercellen en hun interacties te bestuderen.

In **Hoofdstuk 1** geven we een overzicht van wat celcompetitie is en welke processen van celcompetitie plaatsvinden in darm- en leverweefsels. **Hoofdstuk 2** beschrijft een gedetailleerd technisch protocol voor het maken van gemengde organoïden die kanker- en wildtype darmcellen bevatten. Het toont ook verschillende voorbeelden van proeven die kunnen worden uitgevoerd met deze gemengde organoïden. In **Hoofdstuk 3** laten we zien dat celcompetitie plaatsvindt tussen kanker- en wildtype darmcellen in gemengde organoïden. We ontdekten dat kankercellen ervoor zorgen dat wildtype cellen signalen van schade en stress activeren. Dit brengt de wildtype cellen in gevaar en resulteert in hun celdood. Daarnaast hebben we ontdekt dat kankercellen deze signalen gebruiken om hun eigen groei te bevorderen. Bovendien kunnen wildtype cellen beschermt worden tegen eliminatie en kan de toegenomen

proliferatie van kankercellen voorkomen worden door het blokkeren van deze signalen. In **Hoofdstuk 4** richten we ons op interacties tussen darmkankercellen en twee soorten levercellen: cellen die de buizen vormen die gal transporteren (galbuiscellen) en cellen die het grootste deel van de lever vormen en verantwoordelijk zijn voor de spijsvertering en het verwijderen van schadelijke stoffen (hepatocyten). Celcompetitie zorgt ervoor dat galbuiscellen hun identiteit veranderen en vergelijkbaar worden met hepatocyten. Hepatocyten daarentegen worden gedwongen om te sterven in de aanwezigheid van kankercellen. Kankercellen profiteren van de interacties met beide soorten levercellen, omdat ze celcompetitie gebruiken om hun overleving en proliferatie te vergroten. In **Hoofdstuk 5** beschrijven we hoe levercellen reageren op mechanismen van celcompetitie. We leggen uit dat kankercellen verschillende soorten signalering veroorzaken in de verschillende celtypen van de lever. Galbuislevercellen verminderen signalen van stress, terwijl deze signalen worden verhoogd in hepatocyten. Aan de andere kant zorgt celcompetitie ervoor dat galbuislevercellen reageren op mechanische krachten. Manipulatie van de signalen die betrokken zijn bij deze veranderingen helpt de wildtype cellen tegen de strijd tegen kanker. Tot slot bestuderen we in **Hoofdstuk 6** celcompetitie tussen darmkankercellen en leverweefsel bij muizen. We observeren een significante toename van de groei van kankercellen in de lever na blootstelling aan de leveromgeving. Dit suggereert dat kankercellen zich kunnen aanpassen aan deze nieuwe omgeving, waardoor ze agressiever worden.

Ter conclusie, in dit proefschrift richten we ons op het proces van celcompetitie op verschillende niveaus van de evolutie van darmkanker. We bieden inzicht in de gevolgen van competitie voor primaire en uitgezaaide tumoren. We demonstreren ook het belang van de context waarin de competitie plaatsvindt. We tonen aan dat de uitkomst van celcompetitie kan variëren, afhankelijk van de betrokken cellen en micro-omgeving van het weefsel. Collectief tonen de onderzoeken in dit proefschrift aan dat de fitheid van een cel sterk afhankelijk is van hun omgeving. Dit heeft belangrijke implicaties voor de ontwikkeling van behandelingen voor kanker en andere ziekten, aangezien nieuwe therapieën gebaseerd kunnen worden op het versterken van gezonde cellen en hen veranderen in de winnaars in de strijd tegen de groei van kanker.

SPANISH LAYMAN SUMMARY

Todos nuestros tejidos están formados por un conjunto de unidades vivas y altamente organizadas llamadas células. Para garantizar el funcionamiento adecuado de estos tejidos, es esencial la comunicación continua entre las células. Un tipo de información crucial que se comparte entre las células es el estado de su condición física o "fitness". El "fitness" es una característica de las células que las hace más adaptables, más fuertes y favorece su supervivencia dentro de un tejido. Entre células, existe una comparación continua del "fitness" celular para asegurar que las células más aptas, es decir, aquellas con las mejores características, permanezcan en el tejido, mientras que las células menos aptas sean eliminadas. Este proceso se conoce como competición celular y sirve como un sistema de control de calidad para garantizar que nuestros tejidos estén compuestos principalmente por las células más saludables y fuertes. Sin embargo, algunos mecanismos de competición celular pueden ser copiados por las células cancerosas, ayudándoles a sobrevivir y proliferar. Por ejemplo, cuando una célula sana, no mutada, conocida como célula "wild-type", adquiere una mutación oncogénica que promueve su proliferación, su "fitness" también mejora. Esta célula cancerosa puede utilizar mecanismos de competición celular para colonizar un tejido. En esta tesis, estudiamos la competición celular que tiene lugar en el cáncer intestinal. Nos enfocamos tanto en el intestino, donde inicialmente surgen los tumores primarios, como en el hígado, el sitio secundario donde los tumores crecen después de escapar de su localización primaria durante la metástasis. En este estudio queremos comprender la batalla entre las células cancerosas intestinales con las células sanas tanto intestinales como hepáticas. Para lograr esto, utilizamos mini órganos y tejidos derivados de ratones cultivados en laboratorio, también conocidos como organoides y microtejidos. Éstos son cultivos celulares en 3D que se asemejan a los tejidos de los que provienen. Usamos estos dos modelos para mezclar células cancerosas con células sanas intestinales o hepáticas y estudiar sus interacciones.

En el **Capítulo 1**, proporcionamos una visión general sobre qué es la competición celular y qué procesos de competición celular ocurren en los tejidos intestinales y hepáticos. El **Capítulo 2** describe un protocolo técnico detallado para la generación de organoides mixtos que contienen células de cáncer intestinal y células intestinales sanas. También muestra varios ejemplos de ensayos que se pueden realizar con estos organoides mixtos. En el **Capítulo 3**, mostramos que la competición celular ocurre entre células intestinales sanas y células de cáncer en organoides mixtos. Descubrimos que las células cancerosas hacen que las células "wild-type" o sanas activen señales de daño celular y estrés. Esto las pone en peligro y las conduce a su muerte celular. Además, encontramos que las células cancerosas utilizan estas señales para inducir su propio crecimiento. Es importante destacar que bloquear

estas señales de daño y estrés en las células "wild-type", puede protegerlas del efecto de las células de cáncer en ellas y por lo tanto de su eliminación, así como prevenir el aumento de proliferación en células cancerosas. En el **Capítulo 4**, nos enfocamos en las interacciones entre células de cáncer intestinal y dos tipos de células hepáticas: las células que forman los conductos que transportan la bilis (células de los conductos biliares) y las células que forman la mayor parte del hígado y son responsables de la digestión y desintoxicación (hepatocitos). La competición celular hace que las células de los conductos biliares cambien su identidad y se vuelvan más similares a los hepatocitos. Por otro lado, en los hepatocitos se induce la muerte celular por la presencia de células cancerosas vecinas. Las células cancerosas, sin embargo, se benefician de las interacciones con ambos tipos de células hepáticas, utilizando la competición celular para aumentar su supervivencia y proliferación. En el **Capítulo 5**, describimos cómo responden las células hepáticas a los mecanismos de competición celular. Explicamos que las células cancerosas causan señalizaciones diferentes en los distintos tipos celulares del hígado. Las células de los conductos biliares hepáticos reducen las señales de estrés, mientras que estas señales se aumentan en los hepatocitos. Por otro lado, la competición celular hace que las células de los conductos biliares hepáticos respondan a fuerzas mecánicas que las células de cáncer ejercen sobre ellas. La manipulación de las señales involucradas en esta respuesta protege a las células no cancerosas de perder la batalla contra el cáncer. Por último, en el **Capítulo 6**, estudiamos la competición celular entre las células de cáncer intestinal cuando crecen en el hígado de ratones. Observamos un aumento significativo en el crecimiento de las células de cáncer en el hígado después de la exposición al ambiente hepático. Esto sugiere que las células cancerosas pueden adaptarse a este nuevo ambiente, haciéndolas más agresivas.

En conclusión, a lo largo de esta tesis, nos centramos en el proceso de competición celular en diferentes niveles de la evolución del cáncer intestinal. Explicamos las consecuencias de la competición celular para los tumores primarios y metastásicos. También demostramos la importancia del contexto donde tiene lugar esta competición. Mostramos que el resultado de la competición celular puede variar, dependiendo del microambiente del tejido y de las células involucradas que compiten entre sí. En definitiva, los estudios de esta tesis muestran que el "fitness" de cada célula depende mucho de su entorno. Esto tiene importantes implicaciones en el desarrollo de tratamientos para el cáncer y otras enfermedades, ya que las nuevas terapias podrían basarse en fortalecer las células sanas y transformarlas en las ganadoras en la batalla contra el crecimiento del cáncer.

CURRICULUM VITAE

Ana Krotenberg García was born in Valladolid, Spain, on December 2nd, 1992. She completed her pre-university education at I.E.S Julián Marías in Valladolid, specializing in the science track. Subsequently, she pursued her bachelor's studies in Biotechnology at the University of Salamanca, graduating in 2014. During her undergraduate studies, she undertook internships at the Applied Ophthalmology Institute and the Immunology Laboratory (ImmunoLab) in Valladolid during the summer of 2013. It was during her bachelor's thesis that she developed a keen interest in cancer research, specifically focusing on breast cancer. Her project, titled "Identification and validation of molecular targets differentially expressed in positive Her2 tumors," was conducted at the Cancer Research Center of Salamanca as part of the group of Prof. Dr. Jesús Pérez Losada. After completing her bachelor's degree, Ana was awarded a scholarship from the Spanish Association Against Cancer for the summer of 2014 to continue her research from her bachelor's thesis. Following this, she pursued a Master's degree in Biology of Disease in the Netherlands, at Utrecht University, from 2015 to 2017. During her master's studies, Ana furthered her interest in cancer research through major internships at the Hubrecht Institute in Utrecht, in the group of Dr. Daniele Guardavaccaro. Her research focused on the "Roles of FBXW4 in mitotic progression and their possible implications in cancer development". Additionally, she conducted a Summer Research Project at the Hubrecht Institute, investigating the role of membrane trafficking in cytokinesis using lipophilic dyes and confocal microscopy. Furthermore, Ana completed a literature review for her master's writing assignment titled "Extracellular granzymes in matrix remodeling and endothelial function" at the Utrecht Medical Center (UMCU) under the supervision of Prof. Dr. Niels Bovenschen in the Pathology Department. Her minor internships during her master's program were at the Netherlands Cancer Institute (NKI) in Amsterdam, where she worked on the project "Role of integrin α3β1 in hair bulge population: the impact on hair follicle dynamic and α3β1-mediated skin tumorigenesis," in the group of Prof. Dr. Arnoud Sonnenberg. After obtaining her Master's degree, Ana worked for two years until the end of 2018 at Leiden University Medical Center (LUMC) in Leiden, as a research technician in the group of Prof. Dr. Christine Mummery. During these years, she contributed to several multidisciplinary projects involving hiPSC-derived cardiac microtissues, triple transient measurement of cardiomyocytes monolayers, and the utilization of chicken embryonic models to study vascular development using iPSCs-derived endothelial cells. In 2019, Ana commenced her PhD at the Netherlands Cancer Institute (NKI) in Amsterdam, in the group of Prof. Dr. Jacco van Rheenen, under the supervision of Dr. Saskia Suijkerbuijk. Together, they established a team focused on the study of cell competition in intestinal cancer. In 2021, when Dr. Suijkerbuijk established her own laboratory at Utrecht University (UU), Ana joined her to continue

their cell competition studies as an independent group. Throughout her PhD research, Ana concentrated on investigating cellular interactions between wild-type and cancer cells in primary tumors and liver metastasis of intestinal cancer, utilizing organoids and mice as models.

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en la que confío tanto, que estoy segura que donde sea que acabemos, haremos por vernos y seguir cultivando esta relación. Por el momento, Londres es el siguiente destino y espero que en unos años estemos más cerquita 😊. Por último, no me gustaría acabar sin decirte que eres una persona impresionante Rebeca, una de las personas más justas, claras, leales y empáticas que he conocido, no sabes todo lo que he aprendido de tí. Tenerte a mi lado estos años ha sido un verdadero placer, pero que formes parte de mi vida, eso sí que es un tesoro. Muchisimas gracias por todo, incluyendo estar a mi lado como paranymph en este día tan importante. Te quiero muchísimo amiga.

Jorian, OMG Joriancitoo it´s happening!! It is incredible that we have been more than a year talking about our defenses and within four months, both of us experienced this... And now, I have the chance to tell you how important you are and have been for me. First of all, I would like to admit that when I was told about my "forced" move from the NKI to UU I would never think I could find such a good friend as you are. Obviously, I was wrong. I still remember my first months in the Dev Bio department, or maybe better said "the *C. elegans* world" xD, where I was completely ultra lost, trying to introduce my organoids in a "worm" environment. Even now, that we have been more than two years there, there are people that confuse which organs we are working with and confuse liver with pancreas... Right Joriancito? ¬¬ But anyway, coming to the origins, it was very difficult to find in such a "Dutch" environment (sorry, really, there were no other internationals but Olga at that point), someone with my late work schedule, and even less during corona times. But there you were, in my office, to make me feel in company in those scary corridors of the Kruyt building. Even if we barely spoke at the beginning, I was stupid enough to not know how to open the incubator-shaker of the lab, and there you were to recue me... The start point of many many many situations where you would save me :P. I think from the evenings when you were preparing the movie for Mike, I could realize how nice Olga and you were and how funny you were together. More and more and more and even more talking in the office was happening in the next weeks. I must say that the talking is not very surprising knowing MY talking skills... But my friend, you were also a super easy person to talk to, and day by day I could discover that you were actually a very loyal, trustworthy, empathetic and kind person to share my problems with. Jorian, you cannot imagine how important and what an enormous support you were for me in those months where I was still landing in the department. Basically, how grateful I felt to find a friend, a very good and especial friend, in the new department. And like this, our relationship evolved more and more until the point of behaving as a real 80-year-old couple because we reached a very especial degree of confidence with each other... That kind of confidence where you understand what the other is thinking with only looking into his/her eyes... Therefore, if this real connection was so good in the work environment, we definitely had to exploit it also outside the lab... Especially if we were two very party animals & beer-lovers and you needed a house were to stay in

Utrecht, because otherwise you had to be Cinderella and go to your loved Grugru at midnight xD. Then, we started joking about having your "survival kit" in the lab for nights in Utrecht that could get longer... And just like that, I turned into your major hostess and you started having a secondary house in Utrecht :P , so the survival kit became a real secondary life set hahaha. I actually think, this was one of the best ideas we ever had, you know I loved to have you around and that you were always welcomed at my place, especially because every time you stayed that was meaning an amazing party night together. We shared a whole list of really nice experiences and memories together, which include not only night parties, but great dinners in B&B, long post-dinners (normally post Chupitos or Back and Forth, of course), great brown beers, birthdays, King Days, macumbas, festivals and many other festivities... I think these experiences that many times were shared with my Spanish friends stated to show you two things: (1) that you really fit in a latino environment and (2) that to fit even better, learning the language would be a plus... And there you go! You turned into a very good Spanish student that I was always happy to help (or better say, to correct by punching when a mistake and/or a Southamerican-non-Spanish-from-Spain word appeared xD). I am sorry for your poor arm, but being honest, you cannot deny that it was an efficient learning technique :P . And I even brought you to Spain! So you could have the whole immersion ahaha. But we didn´t stop there, and this especial friendship continued evolving and we started to be there also for emotional support in really hard moments of the life, like the last year and months of a PhD. We started to meet for not only partying but also working... In those mentally tiring days were you really need to stay at home to write a thesis, having a personal pilar, an advisor, a cheerleader and a good friend is really needed, and you were definitely mine. It is very difficult to explain how important you were for me, in general, but especially in those moments where the mind is really challenged... It is something I will never be able to forget and something that only us can understand. I just hope I could be there for you at the same level as you were for me. And again, you continued saving me in those times and after, because when I decided to come back to Spain at the end of my PhD, there you were to offering me a house where to stay (and even where to live! Ahahh). Who would tell me that after making so much fun about Grugru I was going to become a citizen too?? xD. But this only shows how amazing friend and kind person you are. If the decision to come back to Spain was already difficult, leaving behind very important friends like you, made it even harder. To finish this whole novel, I think it is very clear that I feel super grateful for having you as a friend, you are a real treasure, and I just can say THANK YOU Jorian, for being always there, for supporting me and making my life funnier and easier, for listening and understanding me always, for sending me nephews pictures to cheer me up!, for helping me with EVERYTHING, for being my pilar, being with you made me feel at home in many senses, and that is very difficult to find... Also thank you for being my paranymph and share with me this important moment of my life, you definitely had to be part of it... I am convinced that

this friendship is so pure and strong that we deserve to maintain it alive for the rest of our lives, wherever we are, I trust us! I love you sooo much Joriancito!

And now it´s time to say thank you to a very broad group of people that were important for me during my PhD, so I say thanks to my NKI colleagues and friends: **Arianna,** thanks for your contribution to our first paper and your lovely high energy and Italian recipes in the group activities :P . **Colinda,** it was so nice to share the office with you and have the real proof that hard work has the deserved outcome. I am super impressed by your career trajectory and even more by how kind and nice you are even being under so much research pressure. Really, very proud and happy for you and I see you as a great example for the women in the field. Hopefully our ways cross again! **Evelyne,** the superwoman of the lab, you release this kind of good vibe that makes people want you around, please never change that positivity! **Laura,** another super woman and hard-worker member of the lab that deserves all the success of the world. Thanks for being always there for help, advice, input and support even after we left the NKI. **Jeroen,** it was very nice to have you around, not only you were super helpful in the lab but also a super caring person with whom have a casual conversation and of course, have some beers :P. **Jessica,** I will always remember our shared complains in the office, at the end we were feeling so comfortable there that our office was our little confessionary. Thanks for making that happen and for part of this "releasing moments". **Kerstin,** I will always admire your motivation and your drive for science, not even that cutie little one stop you from checking some histology slides when needed. You were always so kind and willing to listen and give advice, that we felt very sad to say goodbye to you. In any case, I really hope it was for the best and you are enjoying your life in Switzerland. **Koen,** my Dutch with latin soul friend! It is incredible how even if you were in the lab for a short period, we connected so good! I really enjoyed our continuously joking lunches! Please never change! **Lennart,** definitely one of the kindest and smart people I´ve ever met, it was a pleasure to meet you and enjoy your company and your nice input. **Linda,** also if it was short, such a nice time with you and super grateful for your help with the microscope and for microscope analysis. **Lotte,** the master of the fusion and fission crypts. I will always remember you as one of the nicest people that I met at the beginning of my PhD, always willing to help with a smile. **Saskia E,** you definitely were the engine of the lab when I started my PhD, the person who to ask for everything and that was very much appreciated. However, I don´t only say thanks just for that, but also for being an example of how personal choices are important in this life of only work and for being brave enough to follow your heart to reach a happier life. **Tim,** my dear roomie, always there to help me with lab and non-lab problems, for instance with my Dutch calls, or anything related to the Dutch system xD, but also such a creative and resolutive technician. **Hendrink**, your input was enormously appreciated by us, but I

must confess I also loved the Hendrik from outside the lab, willing to have some beers and share a nice conversation.

To the amazing NKI microscopy and FACs people **Lenny, Marjolijn, Amalie, Bram** always there to save me with any problem with the microscope, but I have to say that the winners are Lenny with her patient while setting up the SP8 over the weekend and undoubtedly Marjolijn, with whom I spent looooong hours in the dark room of the Spinning Disk. Either remotely by phone or in person, there you were to set up every over weekend experiment, which possibly were 90% failing for every time a different random reason and only a 10% succeeding, but giving us amazing good movies... So we can say we learn a lot about handling that microscope and also frustration hahaha. **And Martijn,** our amazing FACS expert always there to help you with your experiments and also to make jokes and make you laugh 😊.

To **Ellen, Suzanne and Rosalinda** who were our amazing organizers of everything! That office in the middle of the corridor is totally the center of activity and control of Molecular Pathology, as if we were talking about a surveillance tower. Without these three women nothing would work properly in the C2 department, I´m sure! Please keep on taking care of all of us, as you have been doing until now, you are totally indispensable!

To my NKI students **Tibe and Maeve. Tibe,** you were the first student I had so basically, you introduced me in the "supervising" world... And I have to say that with students as sweet as you this is a pleasure. I remember with a lot of love our long times in the animal facility that came together with long talks about life and also very interesting conversations about your culture, I really learnt a lot from you! **Maeve,** you were my first master student and as such you demonstrated to be very smart and independent. However, I really liked our connection, definitely we had the same style of understanding and enjoy the life. I am still waiting for those drinks together! :P

To my Jacquitos **Maria, Dimitris, Guillaume,** definitely you were the responsible ones to make Jacco´s lab the super nice environment that I was very sad to leave behind when Saskia and I moved to Utrecht. **Maria,** I was always amazed by, not only your incredibly intelligence, but also how skilled and smart you are in the lab, how patient and kind you are with everyone (especially Dimitris xD), even with the crazy workload that you always had. You were always doing thousands of things at the same time, supervising thousands of people and even if we were going to ask you any stupid thing about the lab, there you were with the right answer and a super warm smile. In conclusion, you are another superwoman met in the story of the PhD. **Dimitrikiiiiiiiis,** my lovely Greek in the lab... I remember having a bad day in the lab and then going to talk to you and suddenly you turned my day in a better one. You are the kind of person that

release happiness and stability and positivity, one of the very few optimistic people that you can meet in science, please never change that! Having you around can cheer up anyone! **Guillaume,** how convenient it is that you could speak perfect Spanish... Not only because you understood the culture much better than the rest, but also because it made it super easy to gossip around together with Rebeca and other Spanish crew in the department (the maximun gossipers team xD), but also... to complain about our lives together... Somehow it felt much better to share it with you and just release the pain together. Obviously, I have to remark another well-known quality of yours... Your food knowledge! I think I´ve never found someone with better abilities to evaluate and choose dishes as well as giving gastronomic recommendations.

To my Bonitas Malakas **Chris, Julia, Mariana, Claire, Andy, Ana, Lou** and malakos **Dario and Daniel. Chris,** my very first friend in The Netherlands... I can't imagine that the very cold-piece of ice girl that I met at the Utrecht University introduction days, would actually be such a sweet and valuable friend for me, for years and years after that moment. Sorry, I had to mention that, because you know, it kind of takes a bit of time and work to reach that heart inside you... But that is because it is one of the most precious treasures that a person can find, so, indeed, you are doing great in protecting it :P. What I could say about you and us, Christinaki mou, that you don´t know yet? And I am being very literal with this question... I think you already know me very very well and also everything about me... You are the kind of person that is able to look at me and know exactly if I am ok or something is wrong, and that is only achieved by real and pure friends. We have a long story in our backs, with ups and downs, but at the end of the day, we were, we are and I´m sure we will be there for each other. We experienced very nice and happy moments together, trips, borrels, birthdays, parties, excursions, dinners... But we were also there to be the shoulder where to cry when the other needed it. I know this is true, because you are still, one of the first people that come into my head when I have important news or a major problem, and this is like this because you always responded as a real good friend. No matter if it was to give me super smart and adult advice or to just listen to my pains and give me a big hug or to make a joke about my story and laugh together... I could not have found a better companion for all those nine years in the Netherlands... And as we said many times, no matter where we end up from now on, this friendship has become strong enough to survive any distance! So let´s look forward to the many many nice moments and memories to create together that are still to come! I love you so so much Christinaki mou. **Juliaaa!** O major dicho... Flooooor!!! Jajajaj si tuviera que escoger alguien para representar a la expresión "ser un amor de persona" serías indudablemente tú. No sé cómo lo haces para ser tan sumamente dulce y "caring" con todo el mundo. Desde que nos conocimos en el C2 retreat después de una o dos semanas de empezar mi doctorado en el C2 department, supe que seríamos amigas... Y es que nuestra amistad empezó ya con muy buen pie: en el baño hablando

de Macumba y reggaetón xD. De ahí con alguna ayudita de cenas de españoles y o fiestas del NKI, pero sobre todo gracias a los borrels de los viernes, empezamos a compartir muuuuchas noches de bailoteo y drinks... Y es que será difícil olvidar el típico plan de viernes de: borrel, nachos y ribs en radion y después al centro a buscar bares de reggaetón!! Pero nuestra amistad no quedó ahí... Seguimos evolucionando hacia amigas de mucho más que salir y empezamos a compartir nuestros secretos, llorarnos nuestras penas, quedar a solas y apoyarnos infinitamente... Y, aunque lo negaré ante un juez, es lo que más valoro de haberte tenido todo este tiempo ahí en el departamento. Eres una persona única, con la que se puede contar siempre y en la que poder confiar. No sabes lo que me alegro de que hayas formado parte de mi vida estos duros años del PhD y lo que me alegro que hayamos mantenido esto incluso aunque me mudase a Utrecht. También, tengo que decirte que me duele muchísimo estar en otro país en momentos tan importantes como el que estás viviendo ahora, porque me encantaría estar a tu lado día a día. No obstante, confío en que las amistades de verdad como la nuestra, sobreviven la distancia y el tiempo, y confío en que el destino nos volverá a juntar en el futuro. Por último, quiero darte las gracias por estar ahí a mi lado como un apoyo incondicional, SIEMPRE, te quiero amiga. **Marianita,** it is impressive how a relationship can evolve between friends. I must admit that at the beginning, me, as many other people in the NKI felt a bit intimidated by your strong personality, with the super directness that could be comparable to a pure Dutch person and you ultra-mega fast talking. However, it didn´t took long to take out that mask and discover the real Mariana. Honestly, what a blessing that this happened, because you are actually a very very good friend, the kind of person that knows how to listen and how to advice people, the kind of person that is super willing to help, the kind of person that always have a stupid-smart joke to make you laugh and therefore, the kind of person that you would like to have around. Even if you had to divide your time in many different groups and had a complex agenda, we shared many many nice moments in and outside the lab during the PhD life, and I really hope we can keep on building moments together in our "post-PhD" time, because I´m sure our friendship story is not done yet 😊. **Claire,** the amazing Claire... What a super scientist you are, I always say that if I have to think about two role models in science those are Saskia and you. Definitely, one of the smartest people I´ve ever met, but not only that, what an efficiency! Whatever task you have to do, I don´t know how, but you manage to do it good and super fast! And not only that... You also like to enjoy life and you are continuously traveling, meeting friends and enjoying life... I think that you are just amazing! I loved sharing with you the office and also that even if it was difficult to find proper quality time for us, whenever we found it, with you, I was feeling like talking with family. I could share any of my problems and you were always replying with a very honest and super useful and wise advice or opinion. The only thing I could regret about us in the last years is to have not spend even more time together! Rember that you are a great scientist, colleague, confident, friend and mother! **Andy**, I always

admired your dedication and your commitment towards many aspects of the life. This holds true both for science and for relationships. As a scientist, you are one of the most hard-worker people I´ve ever met. Incredibly, even if things failed again and again and again, there you are, never giving up and holding on... I really learnt a lot from you. Similarly, you are a great friend, because your commitment with people is enormous, also how caring and sweet you are with the rest, please never change that! **Ana**, fue super genial tener a otra hispanoparlante con la que desahogar todos los problemas del día a día. Desde el principio me sentí super acogida por ti, fuiste de las primeras caras y conversaciones amables que encontré en el departamento, y ahí seguiste hasta hoy. Gracias por hacerme pasar los días del PhD más llevaderos 😊. **Lou**, it was short but very intense. You arrived to the department and perfectly fitted with us!! You are such a funny and honest person with so incredible and surrealistic family and friends' stories that having a lunch with you was never boring. It is a pity that you are so far away now, but I believe (and hope) that our paths will find each other somewhen in the future. **Dario and Daniel**, our two men of the group... We are sorry-not-sorry about all the gossiping around you, but we know that you also enjoyed it guys :P. Thanks for being part of our group and make our borrels and dinners funnier with your smart Swiss sense of humor 😊 .

Veronika and **Alba,** I place you here at the NKI people section, but actually I could put you in many other sections since we have shared experiences at the NKI but also in Utrecht and now more and more in Leiden! And this only means that wherever we go, we are able to keep this meaningful friendship strong and active. Both of you were there as a big support in good and bad situations of my life and I could always ask you for a SOS meeting to share my problems, this really means a lot to me. Even during COVID we manage to enjoy together and do fun trips like Texel one. Also, I really appreciate your effort in ticking the things in my "to do list" before leaving the Netherlands... I could not go to a naked spa with all of my friends xD But there were you girls, helping me to open myself to new experiences hahahah. And of course, it meant a lot to me to be next to both of you as paranymph in your defences... Since one repetitive topic in our meetings was how much shit you have to deal with during the PhD xD. **Veronika,** we met as a supervisor-master student, but I think we both found out that we were a perfect match. I will never thank you enough how much I learnt from you, not only scientifically, which definitely was a looooot, and also how funny was working those long hours if you do it supervised by someone that is a friend; but also personally, along all these years, when you always gave me very good advice and were able to reason very clearly situations that in my mind were within a dark cloud and I could not see properly. You were always the calm, wise, objective and sensible voice that I needed to hear in the dark moments. **Alba**, you also have been amazingly supportive during all these years, I was always surprised by how good you could understand my problems and worries and

give opinion and advice. You know we missed you a lot in the months that you were at the boat, right? But here you are, back and nothing changed between us! I really hope we can keep on hearing about each other´s adventures even if we are physically far away, as good friends do! 😊

My Spanish NKI team **Carmen, Alberto** y **Eric. Carmencitaaa**, quién nos iba a decir aquel NKI retreat en el que nos conocimos que íbamos a llegar a ser tan cercanas, pero yo agradezco mil aquel momento en el que te acercaste a Rebeca y a mí y nos preguntaste si éramos españolas... Y a mí que me reconocieras por Union! Pero he de decir que fue un grandísimo descubrimiento en mi vida del que todavía doy gracias. Los múltiples planes en Utrecht con nuestros picoteos en las casas, la experiencia en el Willem (xD), los viajes en bus, las mil veces que te intentaba convencer para ir a Macumba y luego nada (xD cero rencor!), largos paseos durante el COVID, planes con Nilo, Inge, Irene, Inno! Y las infinitas charlas de confesiones en tu office del NKI... Incluso te quedaste en mi casa en Valladolid! (donde ya sabes que eres super bienvenida), muchas experiencias que sin duda hicieron más llevaderas las frustraciones del PhD (y es que, qué sería una conversación nuestra sin mentar a Raimond o a Saskia xD). Yo sé que los últimos años han sido muy duros para ti y no sólo por el PhD, pero has demostrado con creces lo fuerte y lo luchadora que eres, y yo siento que es un gran privilegio contar con alguien así como amiga. Tu vales mucho y superarás todo, cómo lo hiciste hasta ahora! **Alberto,** simplemente eres la hostia! Me encanta tu actitud despreocupada, alegre y fiestera... Encontrarme contigo por los pasillos y tener una conversación me alegraba siempre el día, porque siempre tenías alguna historia loca que compartir... Los borrels, las cenas, los viajes y en general cualquier tipo de plan no es lo mismo sin ti! Me encantas, en serio! **Eric,** aunque nos conocíamos desde antes del PhD y ya desde entonces me parecías un tío estupendísimo. Pero desde el momento en que empecé con las entrevistas y casi casi somos compañeros de laboratorio, confirmé la evidencia. Muchísimas gracias por ser tan honesto conmigo y dar tan buenos consejos sobre el PhD. Muchísimas gracias por estar pendiente de que estuviera bien y por intentar aprovechar tu experiencia personal para ayudarme. Eres de verdad una bellísima persona que se merece todo lo mejor del mundo.

Dèji, my amazing Caribbean friend... We can't deny that our connection is extremely powerful. I can't believe how many moments we experienced together after that first Macumba where we met very randomly. You were definitely great company to drink beers and dance reggaeton (OMG your dancing moves!! 😁), but even better to share thoughts, worries, and give advice. I know we both experienced difficult moments during the last year, and I really wish things had been different and easier, but what stays in my mind are all the good moments we enjoyed, all the dancing, drinks, laughs, and deep stories we were able to share with each other... We really managed to open up

our hearts, huh? I will never regret what we have experienced; you were an amazing support for me and I learned a lot from you... And who knows if our paths will cross again in the future?

My UU colleagues and friends: **Vincent,** the major savior of the lab, the absolutely handyman, the encyclopedia of the lab... You are the person where to go when you cannot find something, need advice for a new protocol, need to order anything, have questions about paperwork... In conclusion, the soul of the lab. I am super amazed of all the knowledge but also kindness that can fit in that ginger (and once, in the past, long haired) head. But you are not only this, you are also (at least for me) the person who to share frustrations with, because you always had some nice words of understanding and advice to give. I am very glad that you were my roomie and I feel super grateful for all the help you gave to Saskia and me during these two years. We really miss you in the department! **Ruben,** our heavy metal guy (second if we count Vincent historically hahah) of the lab! You are amazingly resolutive and your help during my last chapters of the PhD was very very much appreciated. I am very happy we met and we shared nice experiences inside and outside the lab. **Suzan,** the sweetest PI of the department and possibly of all The Netherlands. Your kindness and caring personality show that it is still possible to be successful in science and have a great soul. I appreciate every single input comment you gave about my project. I have to apologize for all the moments that I took the office with Saskia to discuss about my PhD, I know we were very annoying and long in our meetings, but having you as the neighbor of the boss made the visit to Saskia more appealing, because you would be there with your warm and sweet smile to welcome everyone to the office. **Mike,** you are undoubtedly one of the funniest group leaders I´ve ever met. It is impressive your mental speed to make quick, smart and very funny jokes in every situation! Almost as impressive as your speed in appearing in the office when someone sends an email about sweets brought from somewhere else to be shared in the department hahahah... Such a sweet tooth! But these things make you a very unique character and definitely representant of the department. Also, I enormously appreciate all your comments, input and questions about my project, because it really meant to me that a *C.elengans* king was really making an effort in understanding the organoids world. Not even understanding but also collaborating... I really loved the great connection between Saskia and you and how you were always willing to find projects that could overlap between our labs, even if the research field could seem not overlapping at all. It was a real pleasure to meet you and share the las two years of the PhD with you. **Alex,** even our short overlap I always admire how intelligent you are, you input in the discussions was always super useful. It is a pity that for healthy reason we could not spend more time together, but I wish that situation improves soon. **Tessa,** it was great to meet you and share so many Sunday evenings at the Kruyt hahah, always nice to know that a familiar face would be in the department in the same weird times as

me xD, also, you are the real proof that scientist can be cool Zumba dancers! :P **Olga,** thanks for being there in many many reasons. Initially, you were my other buddy as internationals in the department. But afterwards we became much more... You were there in very hard moments for me, and I will never forget it. Also, a great company to bring and show my country 😊 . **Mo,** what a great addition to Mike´s lab! And I mean it both inside and outside the lab. When you arrived, we were all very impressed by your sharp questions and we could see your undoubtable scientific skills. However, what I like the most is your kindness with everyone and your great partying vibe! Come on, we really needed another party animal to join Jorian and me from this department! I just regret we did not go out even more in my time there :P. **Joren,** also another great addition for the department, always super approachable and social. I really loved having you around with your proactivity to make social plans and your smart jokes and teasing... Including your continuous ability of forgetting the exact dates of MY events xD xD. **Elise,** our bubble paper girl! You are such a sweet and smart person that for sure will achieve anything that you propose in life. I wish you totally the best in the future and really hope we keep on meeting every now and then 😊. **Loes,** I always liked having another dancer in the department, at least I was not the only one running away to a dancing class after an experiment that was running late :P . I love your energy and good vibe and your party soul too... We could be twins with very different music and dancing taste... But at the end what matters is just dancing! Also, I need to pay you at some point all the times that you were there in the laboratory and save me switching on the Imaris computer ahhaah. **Stefanaki mou,** how lucky I was having you as my roomie, your ironic style of humor was always able to take some laughs from us as well as your smart jokes, please never change that! **Kaila,** the sweetest girl of the department! It was lovely to share the office with you, always there willing to listen to my crazy problems and my crazy life and to give a super warm and comforting answer that made me feel better. Thanks for being like this! **Ben,** definitely the coolest teacher, it was very nice to have met you, your presence in the meetings and the social activities always improved the quality of the experience 😊 **Joao, Tiago and Lyn,** we overlap very few time, but I´m happy we did a bit and wish you the best in your future time in at Dev Bio. **Savvas,** Savvasito... You know I really really like you since your student times with Jorian, I could feel you like a little brother 😊. I think you have one of the biggest hearts that I could ever find and every time we did something together inside or outside the lab we had a great time! Also, you have a great taste for important things in your life... Always go for the Spanish people! They are good! :P (I´m not in bias at all eh) I hope your Spanish gets better and better every time we meet haahh :P. You are simply amazing and definitely one of the big treasures that moving to DevBio gave me, please never change, you have a very pure soul! **Boris,** I think all started in tissue culture obviously, with hours and hours stuck in a hood... You were there making your funny-ironic comments and making me laugh continuously. Since then, I realized I wanted you as a friend because you were genuinely

nice! And there you go! Super good friends 😊 I really liked finding you in the corridors and talking about life and you showing me your nephews' photos and videos... So cute!!... Thank you for that! You always knew how to cheer me up in the hard moments of the PhD! **Ilya,** our amazing imaging guy... Thank you so much for all the support during the hours and hours that we spend in those dark microscope rooms... You were always there to save us from any microscopy problem, during and after the experiment and your input was absolutely needed for stablishing the lab and allow the type of very very very long imaging experiments that we needed to do. Undoubtedly, Suijkerbuijk lab owe you a lot, and we know it! But also, thanks for your very funny comments and your continuous presence in the borrels... It is always nice to have you around! And ey, as you told me... Here I am, taking care about the acknowledgments and thinking a lot about the dress that I will wear in the defense day, as you said, the two only things that people will care about xD.

To my UU Student **Joyce,** I also need to thank you a lot for helping so much with the project, even if we passed some personal dramas in the way, you were always there ready to take care of the organoids and take amazing images and movies that finally, really helped for our last publication. Thank you so much for this support, because without that the last paper and this thesis would not have happened.

To my amazing group mates: **Merel, Mario, Maria.** Thank you all for making our closer work environment so nice and enjoyable... Having you as a close workmate made the idea of going to the lab every day very appealing! **Merel,** you know you are super especial for me GUAPI. I still cannot believe that I told Saskia that we could work very good but never be friends, I was so super wrong!! Since you arrived, you brought to the lab a wind of good vibe and very good organization too. Having you there not only meant to have someone else to discuss my results, explain my scientific and technical problems and share my frustrations of the project, it also meant to have a good friend that I was meeting daily and with whom I could count with in the goods and the bads. It is true that the tissue culture experienced many of our tears, but also, we created such a good connection between us that we could really talk about and share everything, making us feeling at home while working. Not only this, but also, we know each other so well that with only looking at each other´s eyes, we knew perfectly what the other was thinking and that is very complex to find. You were ALWAYS there for me, ALWAYS... Either to have a walk because I had a stupid lab day or to talk about Inge or to bring me in a SOS situation some bubble paper for packing my moving (xD)... Thank you for every single moment you dedicated to me, I am aware they were many, but I promise you that here you have a friend forever! I hope you know that... I love you so much Merelcita. **Marieteeeee,** madreee mia la de on line OSCAR meetings que hemos tenido, o llamadas por Teams or whatsapp voice messages para hablar de malditos análisis de

organoides... Aunque soy consciente de que nos hemos podido quemar mucho por este proyecto, tengo que decirte que alegro muchísimo que accedieras a colaborar con nosotras, que eres un tío muy muy genial y que me alegro un montón de haberte conocido tanto profesional como personalmente. Espero que siempre te hayas sentido arropado por nosotros en tus visitas a Utrecht y sabes que siempre podrás contar conmigo para tomar algo donde sea que coincidamos. Eres una persona super especial e interesante y como digo, me alegro mucho de que desde este proyecto formes parte de mi vida también. Gracias, gracias, gracias por todo... Sin ti, esta tesis nunca podría haberse escrito. **Maria,** or better, Maraki mou!!! The sweetest successor I could ever have... Why you did not join earlier to the lab ehhh??? You know how much I like you?!?!?!? The answer is: super a loooot!!! I am so happy that this exciting project that I started is now in your hands... I completely trust you as a scientist to make it shine and discover amazing things in the intestinal liver metastasis cell competition field. Please, never ever doubt about your skills because you completely deserve this position. You learnt so fast and for me it was a real pleasure to "teach" you everything of the project (I am NOT going to say "supervise" you, you know, right?). Thanks to your passion for the project and your understanding about my situation (you know... Crazy times of finishing the thesis), you made the task of transferring you the knowledge very funny and pleasant, and you were super helpful for me. Not only this, Maraki mou, we connected super well, and immediately while talking with you I was feeling as talking with a friend of ages, someone I could be completely myself, someone with whom to laugh and someone who I definitely want in my life forever. Really Maria, thanks for improving considerably my last months in the department and for enjoy with me outside the lab too, you are amazing and please believe it because it´s true! Now face this exciting PhD with that sweet smile that characterize you, YOU CAN DO IT!! And you know... If you need me, I´m here eh! 😊.

And now that I finished with the colleague part... Of course, there are many other people that were important and supporting from outside a lab and who also deserve to be part of this section:

For instance, my lovely Penguins team! **Dan,** you have been part of all my story in The Netherlands, from the beginning to the end and became one of my closest friends there, you know that, right? Who could tell us when we were neighbors in Cambridgelaan that we would share so many moments over years and years?? I have to say that I feel that you are one of my best friends and we have been there for each other in the best and the worst. Our Bilstraat parties, beers and confessions in Gist, travels with the other penguins, house dinners during COVID, casual beers around Utrecht... Many nice moments for which you made my experience in Utrecht memorable. Thanks for being there Dan! **Veronica,** the mami of the team :P . Ay my Verito, it is so nice that still after so many years we keep on having loooong calls/video-calls to update each other, and

this is because, as you said once, after our experience in Utrecht we became family. Actually, I think your real family could adopt us at this point, after so many visits to you and them and after getting so close with them too. I just want to say that you are lovely, a great friend and now also a great mother of our youngest penguin 😊. Love you and miss you a lot Verito. **Marcos,** aunque siempre fuiste, eres y serás el bully oficial del grupo y Ele Mere y yo tus principales targets, sé perfectamente que lo haces porque nos quieres un montón, y ya sabes que es totalmente recíproco. Muchísimas gracias por alegrarnos los viajes con tu inigualable rapidez mental para hacer bromas y chistes y por cuidarme como si fueras un hermano mayor... En los momentos donde era necesario (y más durante el PhD), has estado ahí como un apoyo enorme, como un amigo de verdad. **Clara,** Clariiiii lo mismo digo de ti, como me has acogido siempre y cómo me has cuidado, en los momentos en los que he estado vulnerable para apoyarme, para animarme y darme consejo. No sabes lo que le agradezco a Andrés que nos pusiera en contacto! **Ele Mele,** mi super compi de fiesta... Lo que unió La Choufle que no lo separe el hombre! Jajaj Desde las noches locas en Utrecht empezando en Bilstraat hasta el Sonorama, mil experiencias compartidas... Pero no sólo de fiesta eh! No se me olvida que viniste a mi graduación del máster y que yo no volaría prácticamente para un día en Barcelona desde Holanda por cualquier 30 cumpleaños :P. Pero te lo mereces, porque siempre siempre has estado ahí cuando te he necesitado, para escucharme aunque fuera por whatsapp y responderme con las palabras que necesitaba en ese momento... Así por ello y mucho más, gracias amiga, por acompañarme todos estos años... te quiero un montón! **Maria,** otro gran descubrimiento que me dio Utrecht, siempre tan generosa y amable, tan pendiente de todo y todos, espero que pronto vivamos cerquita y podamos seguir aumentando los planes y las experiencias juntas porque cada vez que nos encontramos me sabe muy a poco! **Pancho,** tu también eres la hostia xD, cada vez que nos reunimos descubro un poco más lo genial que eres y lo que me alegro de que formemos parte de este grupo. Y además de tu indudable gracia innata y tus inmensas habilidades de hacer bromas y animarnos la existencia, ya sabes que te necesito para movilizar a estos penguins tranquilotes y sacarles (casi casi sin forzarles) de fiesta un poquillo :P, gracias por ser mi aliado 😊 y si ellos no se animan... Ya nos quedamos nosotros xD. **Iratita,** esa segunda madre del grupi! Aunque nuestra relación se empezó a estrechar más en los últimos años, he de decir que me ha encantado que pasará. Llegar al kryt building y echarnos una buena parlada o quedar los findes se convirtió en una recurrente vía de escape para las tensiones del PhD**.** Me encantó recuperar el contacto porque eres genial y me lo paso super bien contigo!

Also, my unforgatable Leiden friends deserve some words in this section... Well I call you Leiden friends because everything started in the LUMC but actually you are and come from all over the world by now... **Duncan,** from the beginning, sharing the office, we had so much fun! We really got into the same humor and vibe. It doesn't matter if

it's just sharing a beer in a bar or walking around the amazing streets of Berlin while you take very artistic photos… Every experience together is magical and unique. I really love that we try to update each other every now and then because this means that we really care about each other, and that is genuine. Thanks for being part of my life, and please, never leave. **Rubencitoo!** I still remember when I started working at the LUMC and you came to pick me up to tell me we will be stuck with each other for all the trainings… That was an easy start! Super nice Dutch guy coming to me to guide me all around xD. Afterwards, a lot of joking around about my "verbal diarrhea" or about your amazing skills to make fun of me ("Ruben, don´t make me wet!" xD) started. Years and years, we got to know each other more and more and we found out how amazing we are together. I really love every single talk that we have either on the phone, video call or in person (although I prefer in person since I can hug you :P). If I look backwards, you were there always for every good or bad moment that life brought me, as a real support. I also know I can always count on you and please don't doubt that you also have me there ok? Love you Rubencito! **Karinilla,** you are just amazing… My first contact with you was in a meeting when you almost broke the chair, and then I realized how special and funny you are. It is a real pity to have you so far away, but I think our friendship became much stronger than that, and we will keep on trying to be in touch, wherever destiny brings us… Please let´s really work in that because I want you so much in my life. Also, please take care of yours, try to avoid the ER as much as possible hahah. **Olehcito,** my dear husband, unfortunately in the distance… You cannot imagine how much I appreciate our friendship and how much I miss you and I want to hug you. I am truly sorry that we have to share experiences in the distance but I have the hope that one day, we will be able to meet again and enjoy the years that this situation is taking away from you/us. Stay strong and hold on, because whenever is possible we are going to celebrate that I got this PhD together. **Giulia,** I really admire how you can do everything, having a successful scientific life, a family and a personal life. Elena and you are such an example of being able of overcome everything when there is love. I really love your personality and every time we meet, I have the feeling I can be totally open and share everything with you, I just feel at home… Thanks for all the support in these years! **Elena,** you are absolutely one of the most caring people in the world. Thanks for being there in every reunion and make it especial, always giving small personalized details for each of us, what a wonderful person you are, really! **Amanda!** I know you are very young to understand this, but since you arrived, a shiny light came into my heart, and I cannot describe why, but every time I see you a strong happiness fills me… I really would like to follow your growth and that you remember who I am when you get older even if this means continuous trips to The Netherlands 😊. **Maria,** we saw each other in many scenarios of the life, good and bad in all the years, we shared the office, the pain of daily work, the work as technicians at the LUMC and afterwords more fun thing such as trips, nights, drinks. In all these situations, we really enjoy together and could open up like

family, thanks for making me feel like this and with that, helping me overcome the PhD. **Amy,** the pumpkin queen :P, you are totally amazing with your abilities to spontaneously organize drinks, dinners and other different events and also not spontaneously like big traditions as the Christmas dinners (I will never forget those!)... Always with an idea in mind to gather the people and make us enjoy and being a great super great hostess! Also, you have been great advisor giver in all the crucial and hard situations of my life... Thanks a lot for that! **Loukia,** the dater of the group... Now I cannot imagine a gathering with these people without talking about your dates or later guys hahah but I hope you understand that we do this because we love you and wish the best for you. I really appreciated the quality time that we spent together during COVID and in our Italy trip, because I could get to know you much better and understand the wonderful person that you are and how lucky I am for having you as a friend. **Vivi,** another superwoman in science, you are amazing and I know you will achieve anything that you want in your life. Already since your arrival in the department I was impressed by your professionality, but luckily, I was also impressed by how approachable and easy going you are. This made it super easy to be super open and talk for hours and hours in tissue culture until become a real friendship, cheers to that! **Marc,** cuantísimo me alegro de que, a pesar de haberme ido hayamos seguido quedando todo este grupete, porque esto me ha dado la oportunidad de conocerte más y más y darme cuenta lo genial que eres. Espero seguir en contacto porque cada vez que estamos juntos me siento realmente bien y conectado contigo. Eres genialísimo! **Conal,** you are undoubtedly one of the funniest guys I´ve ever met! Being with you means hours and hours of laugh and fun and this even without understanding 30% of the content because of the accent xD (ey I´m getting better, before I was missing the 50 % xD). Thanks for definitely make my life longer with your jokes and funny comments! **Berendillo,** or I should call you Robot 1? I remember our CRACK-IT experience and our long-lasting experiments with a lot of love, even if I had to be a bit bossy with the timer hahaha. Thanks for show me so many things during my technician experience and make me evolve as a scientist! Since then, every time I listen to Sean Paul, I have deja vues with the triple measurements xD. **Elisita,** my older sister, I know that our time together was before the PhD, but I think this pre-time, where I learnt many things from you, shaped my mind in a way to be ready to face the PhD. First of all, I must say I really enjoyed the time we spent together at LUMC and I really admired your hard-work and dedication. However, the best thing I remark from that time is that we became very close friends, something that we can be proud to extend until now. I would really love to share this milestone in my life with you, even if I am thousands of Km far away. I am sure we can keep on fighting the distance and be part of each other lives for many years more.

Of couse, I cannot forget my dear Utrecht friends... **Inge,** mi hermana, ya sabes todo lo que hemos pasado juntas, en las buenas y en las malas, pero siempre ahí sal y pimienta

como amigas de verdad. Con poquísimas personas puedo decir que siento esta cercana, cercanísima conexión, y es que de verdad sabes que eres muy muy importante para mí. Desde que nos conocimos en salsa, empezamos una bonita amistad que se iría completando con más y más experiencias que no me voy a poner a enumerar, porque entonces ocupo tres hojas más. Vivir contigo fue de las mejores cosas que me han pasado en la vida, de verdad, aprendimos muchísimo la una de la otra y entramos en nuestras familias como una más, tanto yo en la tuya como tú en la mía. Sé que los últimos años no han sido los mejores, yo con el estrés del PhD y tu bueno... Con los problemas de salud que han venido... Pero sabes qué? Que esto sólo nos enseña a hacernos más fuerte y valorar más a las personas que han estado ahí a nuestro lado. Tú, definitivamente, has estado en el mío. Aunque no puedas estar presente en este día tan especial, sabes que te llevo en el corazón y que siento tu apoyo. Recuerda lo fuerte que eres y que de todo se sale, lo harás tú sola, pero recuerda que hay mucha gente que te quiere para apoyarte en este camino. Ojalá podamos volver a disfrutar de estar juntas muy pronto. Te quiero, hermana. **Sergio,** ese cubanito que vino a la vida de mi querida amiga y que nos ha conquistado a todos. Eres una persona impresionante, super fuerte, super empática, super cuidadosa y también muy muy divertida. No podría pensar en nadie mejor como "hermano" postizo :P. Aunque sigas intentando quitarme las ganas de ir a Cuba en cada conversación que tenemos, pasar tiempo contigo es genial, y no sólo porque en ese tiempo probablemente estemos disfrutando de una buena cerveza (tripple tú o una Golden Carouls... Y yo una dubble por supuesto), sino porque sabes perfectamente escuchar y aconsejar. En fin, la perfecta mezcla entre alguien que te apoya y que puede salir contigo. Gracias por estar pendiente siempre de cómo me iba el doctorado, de verdad que se agradece el interés. Espero que pronto podamos celebrar todos juntos este acontecimiento 😊. **María,** mi queridíiisima María queso... Quién nos lo iba a decir desde el Willem, la fiesta Mexicana de Bilstraat y el botellón, hasta ahora. Eres mi compañera de viaje, de fiesta, de brunch, de planes diurnos, de cervecitas y de concietos... En definitiva, mi compañera de vida casi... No sé qué habría hecho sin ti estos últimos años en Utrecht, porque semana tras semana nos acercamos más y más hasta llegar a un ponto de amistad que con poca gente he llegado. Nuestros padres que nos conocen, amigos en común y ahora eres hasta mi casera cada vez que voy a Utrecht, son esas cosas que denotan lo mucho que hemos compartido. Me encanta como eres y cómo piensas, de verdad que vas muy avanzada para tu edad, pero es que las experiencias vitales que has tenido y tu forma de afrontar la vida son impresionantes. Bueno, María, tú eres impresionante en general. Tengo esa sensación de que contigo me podría ir o podría superar cualquier cosa, y Colombia no ha sido más que la confirmación de eso. No sabes lo contenta que me siento de tenerte y lo mucho muchísimo que me has ayudado desde que apareciste en mi vida. GRACIAS por estar, AMIGA. Te quiero mucho mucho mucho. **Maciek,** mi segundo otro casero en Holanda jajajaj, que contenta me siento de haberte conocido y que hayamos pasado

tantos findes juntos. Eres una persona muy muy interesante y confío en que cuidarás mucho de Maria, porque si algo se ve claramente en ti es que eres muy buena persona. Espero que sigamos compartiendo experiencias, sea donde sea. **Flurinita** how lucky I was to find you at Bilstraat! Since we met, I understood we could be a great match... And there we were! 2 years living together with a lot of situations along the way that we could overcome. Thanks for being so supportive and listen to all my dramas the last two years of my PhD, you really became not only a flat mate but a real friend, and I hope to maintain this friendship for many years more. **Patrickitooooo** the best ultra best huger ever! How lucky I that I took the decision to start dancing at Touchee and find you there. You showed me how therapeutic dancing can be. You were not only an amazing teacher and dancer, but by knowing you more and more I realize the beautiful soul that I had in front of me in every class. You cared about me so much and I feel so grateful for that... I really appreciate every talk we had outside the classes, every Soul Power, every time we managed to share a bit of time and of course, every hug! Thanks for becoming part of my life Dancing Machine, you are amazing! **Evaggelikaki mou** from years and years in the past until now, I know that you are such an amazing person to have in my life, and even if the distance made a bit more difficult to keep us updated, every time we managed it feels like time did not pass. Being at your wedding was super special for me, and now I want to share this milestone of mine with you too. Thanks for being part of my life even in the distance. **Thirza,** I know that we got to know each other more in depth later in time, but what a great finding! I am super super happy we spend some nights together and the trip to Switzerland because I could discover how nice person you are 😊.

And still more people that were important during these PhD experience and special mention in this thesis: **Robertini,** you were my main support in The Netherlands during the first years and since then, you have been present in my life as a very very good friend. Even if we don't talk every day, I still have a special place for you in my heart and I wish you could be sharing this important day with me. **Dani,** tú estuviste ahí cuando toda esta aventura empezó, de hecho, me animaste mucho a enfrentarme a ello y me apoyaste como el que más. Me diste muchísima fuerza para seguir adelante en los momentos complicados del principio donde no me sentía suficiente para esta responsabilidad. Así que, tengo que agradecerte mucho ese primer empujón, quizá sin él, no estaría aquí ahora. **Davide,** we are both very aware that things were not easy for us, and one important burden that we carried were our respective PhDs. Understanding this pain and effort was undoubtedly the best way to support each other, and now I can only say thank you for being there for a big part of the PhD, listening to me and giving me the right words at the right moment. **Javi,** llegaste un poquito tarde a mi experiencia holandesa, pero desde que llegaste has sido un añadido genial en mi vida. Ojalá puedas estar ahí este día tan especial y podamos compartir muchos muchos

momentos más en el fututo. **Yader,** tú también llegaste muy al final de esta experiencia, literalmente cuando prácticamente había terminada. Sin embargo, te mereces sin duda unas palabras de agradecimiento, ya que, en estos últimos meses, en los que acabar la tesis se me ha hecho muy muy pesado y no llegaba a ver el final, has estado ahí prácticamente día a día para sacarme una sonrisa y recordarme que siempre hay que seguir adelante. Aunque desgraciadamente estos ánimos llegaban por teléfono, espero que muy pronto podamos vernos de nuevo y celebrar este gran acontecimiento de mi vida en persona.

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mucho en un mundo donde la gente de nuestra edad cada vez se amuerma más (y los fiesteros por naturaleza como nosotros necesitamos nuestra dosis de salseo ajjajaj), sino también mi compañero de aventuras indudable. Ya te he dicho muchas veces lo especial e importante que eres para mí y lo feliz que soy de que formes parte de mi vida, pero te lo repetiré las veces que haga falta. Siempre que te he necesitado has estado ahí y deseo que sigamos compartiendo aventuras y experiencias por muchos muchos muchos muhos años más.

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el mundo quiere y disfruta el salir, y que hay que aceptarlo porque se puede ser feliz aun así. Es decir, muchas gracias de verdad por abrirme los ojos y ayudarme a aceptar lo diferente a mí, de verdad que gracias a ti he aprendido mucho acerca del respeto y la aceptación, algo que sin duda me ha sido muy útil a lo largo del PhD. Y por último ten claro que tu hermana te quiere muchísimo**.** Y finalmente, a **mis padres**, quienes se merecen de los que más un eterno párrafo en este libro y en esta sección. Mis padres que han sido los pilares de mi vida hasta ahora y que incondicionalmente han apoyado mis decisiones laborales y personales. Aquellos que me han inculcado unos valores de valor y perseverancia, que han hecho que me enfrente a esta experiencia de PhD y haya llegado hasta aquí hoy. Esas personas a las que no solo les debo la vida, sino también una educación y una evolución como persona, es decir, a aquellos que me han guiado para ser lo que soy yo ahora. Mamá, papá, creo que nunca podré expresar con palabras la tremenda gratitud que siento hacia vosotros, lo muchísimo que valoro el amor que hay entre vosotros y el que nos habéis dado a nosotras, vuestras hijas; lo afortunada que me siento de formar parte de esta familia, que, por supuesto, no es perfecta, pero es mi casa, mi hogar, a donde correr y resguardarme cuando todo se desmorona. Como ya sabéis, estos 5 años de doctorado han sido muy intensos en lo bueno y en lo malo. Pero cuando he tenido una situación difícil ahí estabais vosotros… Ese núcleo duro para mostrarme la luz y ayudarme a salir del túnel. O también para mostrar orgullo y felicidad por las pequeñas cosas que iba consiguiendo a lo largo de este duro camino. **Papá,** gracias por ser esa persona tan estable y tan segura de sí misma en la que todas nos apoyamos. Tu visión analítica y objetiva de las cosas siempre me ha ayudado a poner un punto de cordura y lógica en mis pensamientos cuando los sentimientos me sobrepasaban. Pero a la vez, gracias por hacer todo esto desde la cercanía y el cariño. Ya sé que no puedo esperar una conversación eterna que salga de ti, pero siempre has tenido ese cálido abrazo para mí, que instantáneamente me llevaba a sentirme segura y reconfortada, o esas palabras de necesitaba oír en ese momento específico. **Mamá,** tu y yo sabemos que somos dos volcanes en erupción, y que como tú dices, nos mordemos a la mínima. Sin embargo, soy super consciente de que eso es porque somos muy parecidas, y la verdad, no siento otra cosa más que orgullo si eso es verdad. Orgullo de parecerme a la persona con el corazón más grande que conozco, una persona capaz de mover montañas por sus seres queridos y mucho más por sus hijas. Mamá, no te creas que no sé todo lo que has luchado por nosotras y los quebraderos de cabeza que te hemos dado y te seguimos dando. Pero ese puro amor que te compone ahí te mantiene a nuestro lado, apoyándonos más y más sin nunca defraudarnos. Para mí, eres admirable, una superwoman. Si a alguien tengo que agradecer todas las experiencias que he vivido estos años y la cantidad de amigos que he hecho, es sin duda a ti, porque la capacidad de relación y socialización es sin duda tuya. Pero no vamos a centrarnos sólo en la genética, tú me has enseñado a ser empática, a luchar por mis objetivos y a no rendirme. Cuando tu madre (bueno, en este caso ambos padres) son

luchadores natos y alguien que trabaja muy duro, creces absolviendo estos valores y estas conductas y puedes conseguir logros como un PhD. Así que mamá, muchas gracias. En definitiva, para los dos... Gracias por cuidarme y quererme, especialmente a lo largo de este duro camino que ha sido el PhD. De momento no sabemos qué me deparará el futuro, pero estoy segura de que, con vuestro apoyo, todo saldrá bien. Desde mi corazón sólo puedo decir: GRACIAS y OS QUIERO.