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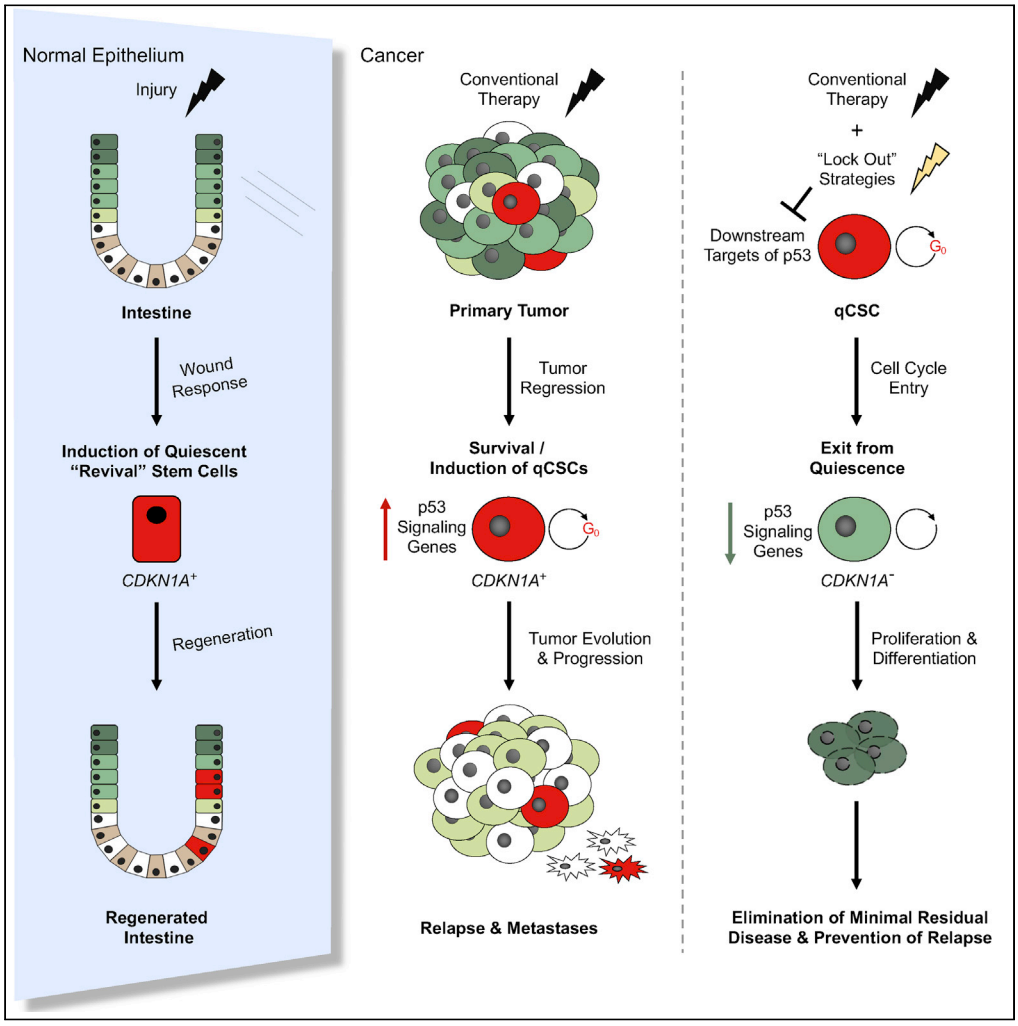

Joseph L. Regan, Dirk Schumacher, Stephanie Staudte, ..., Ulrich Keilholz, Reinhold Schäfer, Martin Lange

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Highlights

Colon tumors contain therapy-resistant quiescent cancer stem cells (qCSCs)

qCSC gene expression mirrors that of quiescent stem cells of the regenerating gut

qCSCs are enriched for p53 signaling genes

qCSC elimination may be achieved by inhibiting downstream targets of p53 signaling

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RNA sequencing of long-term label-retaining colon cancer stem cells identifies novel regulators of quiescence

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SUMMARY

Recent data suggest that therapy-resistant quiescent cancer stem cells (qCSCs) are the source of relapse in colon cancer. Here, using colon cancer patient-derived organoids and xenografts, we identify rare long-term label-retaining qCSCs that can re-enter the cell cycle to generate new tumors. RNA sequencing analyses demonstrated that these cells display the molecular hallmarks of quiescent tissue stem cells, including expression of p53 signaling genes, and are enriched for transcripts common to damage-induced quiescent revival stem cells of the regenerating intestine. In addition, we identify negative regulators of cell cycle, downstream of p53, that we show are indicators of poor prognosis and may be targeted for qCSC abolition in both p53 wild-type and mutant tumors. These data support the temporal inhibition of downstream targets of p53 signaling, in combination with standard-of-care treatments, for the elimination of qCSCs and prevention of relapse in colon cancer.

INTRODUCTION

Molecular and functional intratumoral heterogeneity contributes to differences in treatment outcomes between patients with colon cancer of similar mutational profiles (Kreso et al., 2013). Studies of functional heterogeneity, as defined by phenotypic differences between cells, suggest that cancer stem cells (CSCs) are responsible for tumor growth, metastasis, and therapy resistance (De Angelis et al., 2019; Barker et al., 2008; Brock and Huang, 2017; Moore et al., 2011; O'Brien et al., 2007; Shackleton et al., 2009). CSCs share many of the characteristics of normal tissue stem cells, including unlimited self-renewal, the ability to generate differentiated daughter cells, and chemoresistance (Reya et al., 2001; Sell, 2004).

The normal intestine is maintained by highly clonogenic crypt base LGR5^{Positive} stem cells and also contains a population of rare quiescent (G0 phase) stem cells that act as a clonogenic reserve capable of re-entering the cell cycle upon perturbation of tissue homeostasis, e.g., after injury, leading to loss of the cycling crypt base stem cells (Ayyaz et al., 2019; Barker et al., 2007; Buczacki et al., 2013; Cheng and Leblond, 1974; Clevers, 2013; Potten, 1977). Cancer often recapitulates the cellular hierarchy of the tissue in which it arises and recent evidence suggests that many tumor types contain rare slow cycling/quiescent cancer stem cells (qCSCs) (Chen et al., 2012; Dembinski and Krauss, 2009; Ebinger et al., 2016; Gao et al., 2010; Kabraji et al., 2017; Lagadinou et al., 2013; Lin et al., 2013; Pece et al., 2010; Roesch et al., 2010; Saito et al., 2010; Vanner et al., 2014; Zeuner et al., 2014). Conventional chemotherapies and radiotherapies target proliferating cells and require active cycling for induction of apoptosis (Kreso et al., 2013). In addition, cellular quiescence has been shown to facilitate immune evasion (Malladi et al., 2016). Thus, non-dividing qCSCs may escape conventional therapeutic strategies and represent the source of disease relapse after treatment (De Angelis et al., 2019; Chen et al., 2016; Moore and Lyle, 2011; Shen et al., 2020).

Cell cycle activation in qCSCs has been proposed as a therapeutic strategy to sensitize qCSCs to treatment and lead to long-term disease-free survival without relapse (Chen et al., 2016; Moore and Lyle, 2011). However, the molecular profiling of qCSCs for the identification of novel cell cycle regulators that do not also perturb cellular homeostasis in healthy tissues has been limited by both the rarity of qCSCs and the small

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number of suitable experimental assays available for their detection. Patient-derived organoids (PDOs) echo the morphological, differentiation, intratumor mutational, and drug sensitivity status of the original tumor (Fujii and Sato, 2021; Roerink et al., 2018; Vlachogiannis et al., 2018) and thus provide an excellent model for the prospective isolation and profiling of qCSCs.

Strategies for the identification of quiescent cells employ pulse-chase approaches, including label retention (e.g., bromodeoxyuridine, PKH26, carboxyfluorescein succinimidyl ester [CFSE]), wherein dividing cells lose the label and quiescent or slow cycling cells retain the label for an extended period of time, or the dilution of histone 2B-GFP (H2B-GFP) (Blanpain and Simons, 2013). In contrast to the H2B-GFP approach (Puig et al., 2018), which can identify transient quiescent cells, label retention allows for the identification of cells that remain quiescent from the early stages of tumorigenesis. This is important because cells selectively surviving chemotherapy have been shown to be the same cells that are quiescent/slow cycling in untreated tumors and not cells that became quiescent upon drug treatment (Francescangeli et al., 2020). Such label-retaining cells (LRCs) have previously been reported in colon cancer cell lines, xenografts, and, more recently, in PDOs (Buczacki et al., 2018; Francescangeli et al., 2012, 2020; Moore et al., 2011).

However, to date, the transcriptomic profiling of qCSCs in patients with colon cancer has been limited to microarray analyses of transiently slow-cycling H2B-GFP^{Positive} cells from a single patient with colorectal cancer by Puig et al., (2018) and of PKH26^{Positive} LRCs from two colon cancer patient-derived (via spheroid culture) xenograft models by Francescangeli et al., (2020). In addition, the LRCs reported in the latter study were not directly tested for proliferative or self-renewal capacity before molecular profiling and instead relied on expression of CD133 (AC133) as evidence of a stem cell phenotype.

Here, we report the identification and first whole-transcriptome RNA sequencing analyses of label-retaining qCSCs in a panel of PDOs encompassing primary colon tumors and metastases. These cells maintain a large proliferative capacity, persist long term *in vivo,* and display the molecular hallmarks of quiescent tissue stem cells (Cheung and Rando, 2013), including enrichment for p53 pathway and developmental gene sets alongside downregulation of cell cycle, transcription, biosynthesis, and metabolism genes. In addition, we show that qCSCs are enriched for p53-interacting negative regulators of cell cycle that we propose may be targeted for cell cycle activation and the elimination of qCSCs in both wild-type and p53 mutant cancers. These data provide a valuable resource for the development of novel therapeutic strategies geared toward the elimination of minimal residual disease and the prevention of relapse.

RESULTS

Colon cancer PDOs contain rare label-retaining qCSCs that persist long term *in vivo*

PDOs faithfully recapitulate the cellular heterogeneity, architecture, somatic copy number, and mutation spectra of the original tumor and predict treatment response (Amaral et al., 2020; Choi et al., 2019; Dijkstra et al., 2021; Ganesh et al., 2019; Maru et al., 2019; Nagle et al., 2018; Ooft et al., 2019; Sachs et al., 2018; Shannon and Pitelka, 1981; Tiriac et al., 2018; Vlachogiannis et al., 2018; Weeber et al., 2015; Yao et al., 2020). They are thus widely and increasingly used as preclinical models and in translational studies, including personalized therapy design (Wensink et al., 2021). To determine whether PDOs contain non-cycling LRCs, we performed an initial 72-h pulse-chase experiment using CM-DiL dye. PDOs were established as previously described (Regan et al., 2017; Schütte et al., 2017), processed to single cells, uniformly labeled with CM-DiL dye, and seeded in Matrigel culture. CM-DiL is diluted with each cell division, halving its fluorescence between each daughter cell until it becomes undetectable. Non-cycling cells can thus be identified by their label retention. After 72 h the majority of PDO cells had lost the CM-DiL dye but some PDOs contained non-cycling LRCs (Figure 1A). To determine the frequency of these non-cycling (G0) cells we performed 5-ethynyl-2′-deoxyuridine (EdU) cell cycle analysis on a panel of colon cancer PDOs (Table S1). This analysis demonstrated that PDOs contain non-cycling cells that do not proliferate and remain in G0 within a 72 h period (Figures 1B and 1C).

To determine the long-term proliferative capacity of these non-cycling cells, we labeled cells with the lipophilic fluorescent dye PKH26. Unlike CM-DiL, which is suitable for short-term label retention studies, PKH26 labeling can be used to identify non-cycling cells for up to 6 months (*in vitro* and *in vivo*) (Cicalese et al., 2009; Horan et al., 1990). PDOs were dissociated to single cells, labeled with PKH26, and replated in Matrigel culture. After 12 days, PDOs were re-processed to single cells and analyzed by fluorescence-assisted cell sorting (FACS). These data demonstrated that PDOs contain rare, non-cycling, long-term LRCs (Figures

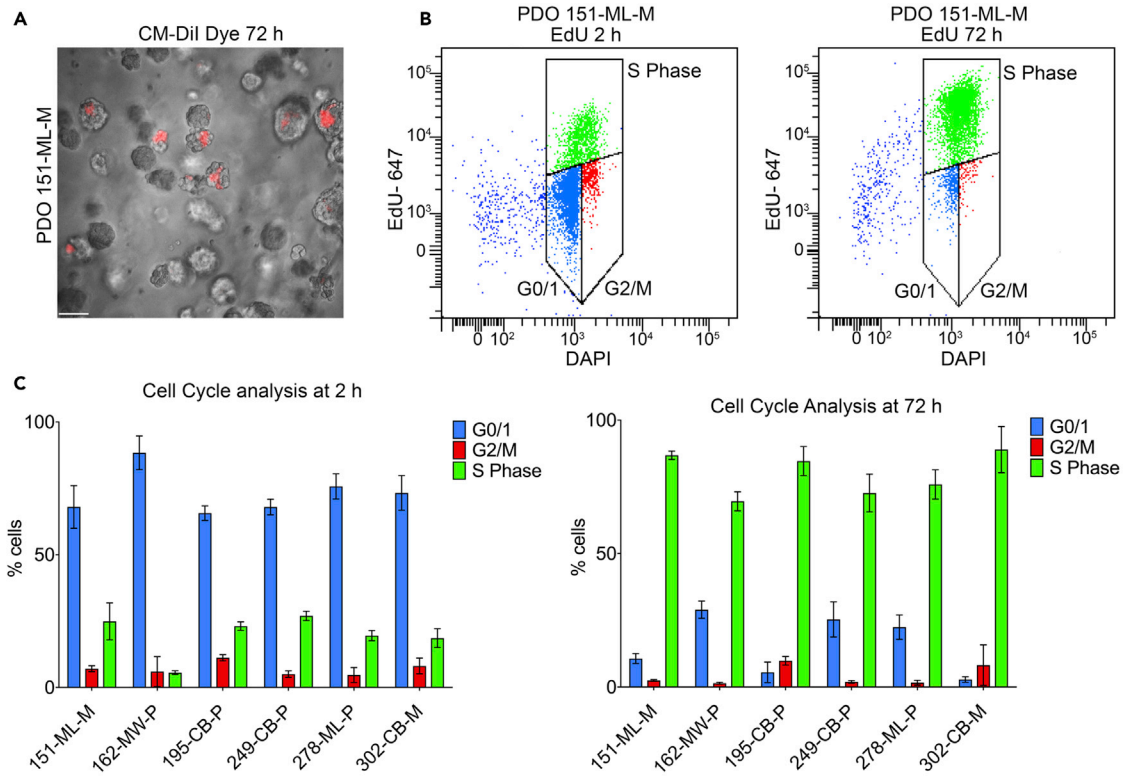


Figure 1. Colon cancer PDOs contain a subpopulation of non-cycling cells

(A) Phase contrast image of colon cancer PDOs labeled with cell-tracker dye CM-DiL after 72 h (scale bar, 75 μm) (see also Table S1).

(B) Representative FACS plots of EdU cell cycle analysis of 151-ML-M PDO cells at 2 h (left hand side) and 72 h (right hand side) after labeling.

(C) Percentage of cells in G0/1, G2/M, and S phase at 2 and 72 h post EdU labeling in PDO models 151-ML-M, 162-MW-P, 195-CB-P, 249-CB-P, 278-ML-P, and 302-CB-M (±SD; data from three independent experiments).

2A and 2B). Crucially, FACS isolation and replating of PKH26^{Positive} DAPI^{Negative} (live) cells from 12-day cultures demonstrated that they are not label retaining due to terminal differentiation or senescence but can re-enter the cell cycle to generate organoids and have a large proliferative capacity (Figures 2C–2F). In addition, non-adherent spheroid formation assays, the gold standard assay for testing stem cell function *in vitro* (Ricci-Vitiani et al., 2007; Weiswald et al., 2015), showed that PKH26^{Positive} cells are enriched for self-renewing CSCs (Figure 2G).

To test whether these cells also persisted long term *in vivo* we generated xenografts by transplanting PKH26-labeled cells. Long-term tracking of LRCs in xenografts requires the slow growth of the tumor. Cells were therefore transplanted at a low cell number based on knowledge of tumor growth rates from previous limiting dilution xenotransplantation assays, in which xenografts were generated from 1,000 PDO cells (Regan et al., 2017).

Unlabeled cells, lacking the burden of carrying a fluorescent dye, may be at a competitive advantage over labeled cells. Therefore, immediately before transplantation, PKH26-labeled cells were processed by FACS to exclude unlabeled cells and thus ensure that only live (DAPI^{Negative}) PKH26-labeled cells would give rise to tumors. Significantly, analysis of xenograft tissue demonstrated the presence of PKH26^{Positive} LRCs for up to 80 days after transplantation (Figure 2H). Previous studies have observed quiescence to be a transient state (Puig et al., 2018). However, these data demonstrate that quiescence can be stable and persist long-term from the initial stages of tumor development.

RNA sequencing of PKH26^{Positive} cells reveals the molecular signature of qCSCs

To generate a molecular profile of qCSCs we carried out RNA sequencing analyses of PKH26^{Negative} (cycling) cells and PKH26^{Positive} (non-cycling) qCSCs isolated from a panel of six different PDO models

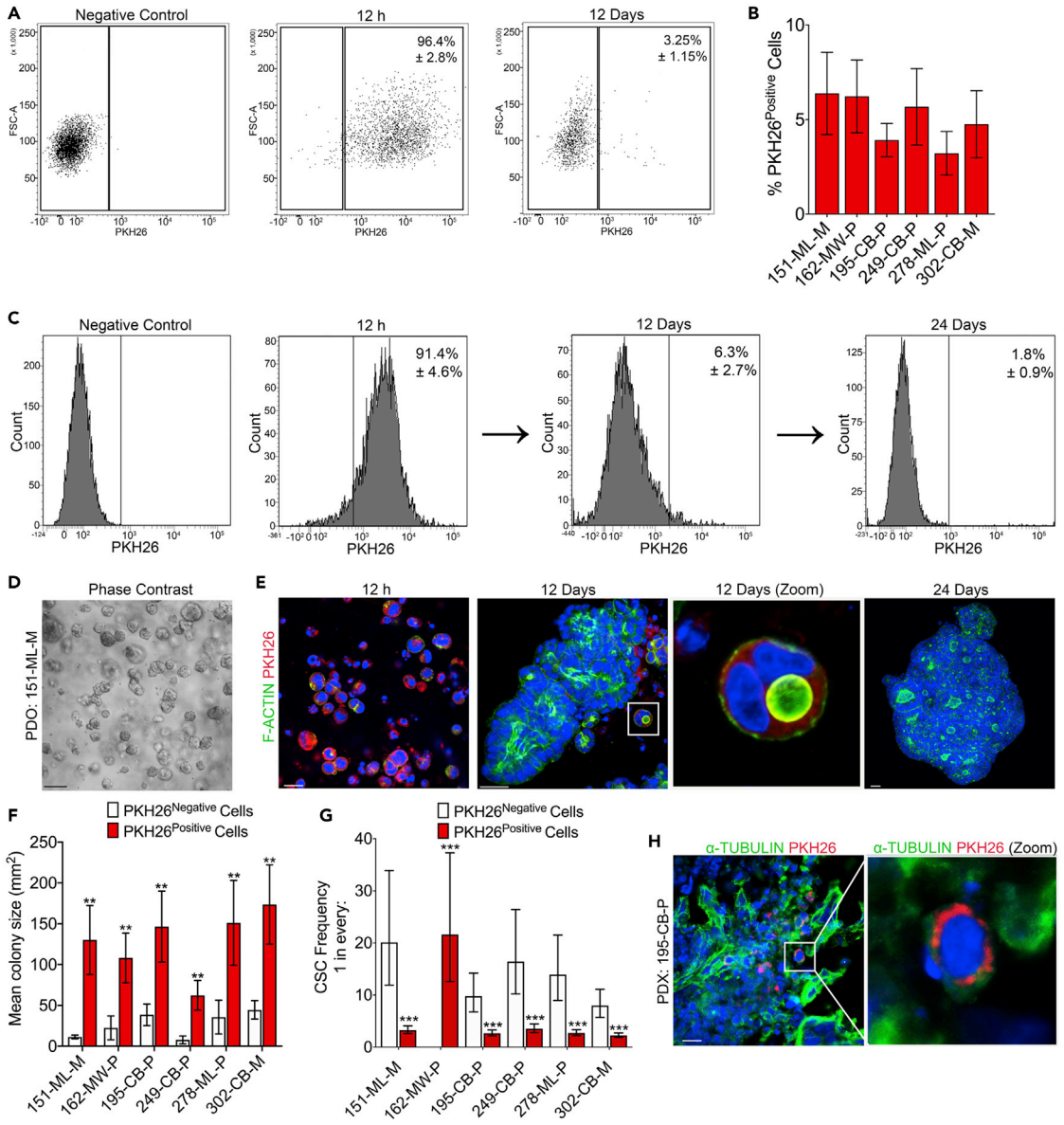


Figure 2. Non-cycling PDO cells are quiescent CSCs that can re-enter cell cycle and persist long term *in vivo*

(A) Representative FACS plots of PKH26-labeled 278-ML-P PDO cells after 12 h (middle panel) and 12 days (right side panel) compared with non-labeled control (left side panel).

(B) Frequency of PKH26^{Positive} LRCs in PDO models after 12 days (±SD; data from 5 independent experiments).

(C) FACS histograms demonstrating frequency of PKH26^{Positive} cells in 151-ML-M PDOs at 12 h (left side panel) and 12 days (middle panel) after staining and 24 days (right side panel) after FACS isolation and serial replating of PKH26^{Positive} cells from 12-day cultures.

(D and E) (D) Phase contrast of unlabeled PDOs (negative control) (scale bar, 100 μm) and (E) immunofluorescence images of PKH26-labeled PDOs at 12 h and 12 days (middle panels) and 24 days after FACS isolation and serial re-plating of PKH26^{Positive} LRCs from 12-day cultures (right side panel). Cells are stained for F-ACTIN (green), and nuclei are counterstained with DAPI (blue) (scale bars, 20 μm).

(F) Mean colony size of PKH26^{Negative} and PKH26^{Positive} cell-derived PDOs in Matrigel culture (±SD; data from three independent experiments). **p value: < 0.01 (t test).

(G) Limiting dilution spheroid formation assay of PKH26^{Negative} and PKH26^{Positive} cells (data from three independent experiments). The p values for pairwise tests of differences in CSC frequencies between PKH26^{Negative} and PKH26^{Positive} cells in 151-ML-M, 162-MW-P, 195-CB-P, 249-CB-P, 278-ML-P, and 302-CB-M tumors are 1.27×10^{-13}, 1.87×10^{-5}, 6.42×10^{-11}, 1.12×10^{-10}, 3.5×10^{-14}, 6.14×10^{-12}, respectively. ***p < 0.001 (t test).

(H) Immunofluorescence image of a frozen PDX section derived from 1,000 PKH26-labeled 195-CB-P PDO cells 80 days post transplantation. Magnified region indicates a long-term label-retaining PKH26^{Positive} cell. Cells are stained for α-tubulin (green), and nuclei are counterstained with DAPI (blue) (scale bar, 100 μm).

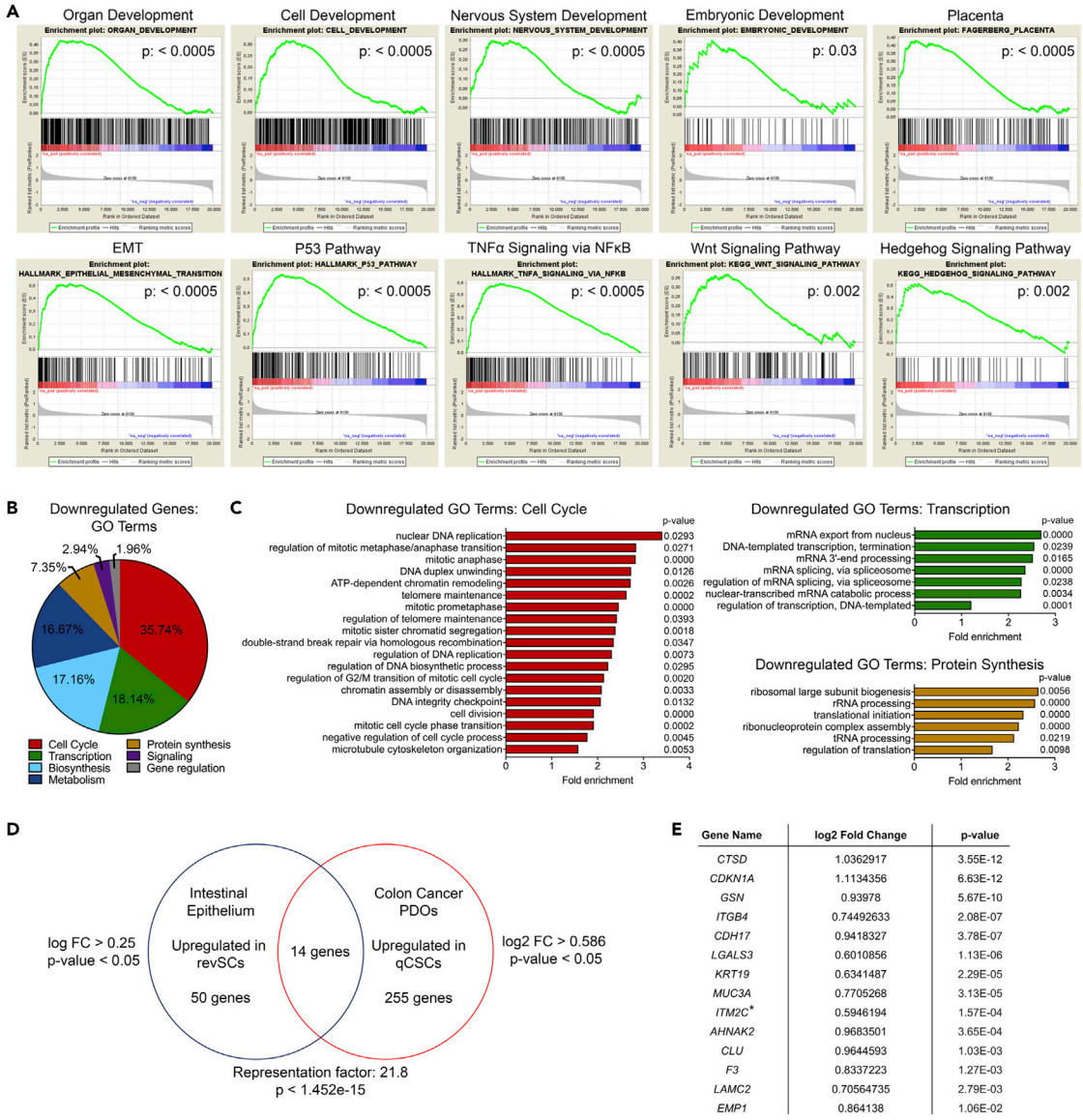
(Table S1) after 12 days in Matrigel culture. These data demonstrated that PKH26^{Positive} qCSCs are enriched for stem cell-associated gene sets, such as embryonic development, organ development, placenta, nervous system development, epithelial-mesenchymal transition, Wnt, and hedgehog signaling (Figure 3A).

At the same time as showing enrichment for genes associated with growth and development, PKH26^{Positive} qCSCs have downregulated cell cycle, transcription, protein synthesis, metabolism, and biosynthesis genes (Figures 3B and 3C). These data are in agreement with the transcriptional profiles of slow cycling/qCSCs reported in previous studies (Buczacki et al., 2018; Francescangeli et al., 2020; Puig et al., 2018) and demonstrate a common molecular signature of qCSCs.

The normal intestine contains quiescent stem cells that can regenerate the damaged intestine upon loss of crypt base stem cells following injury, although whether cellular plasticity or distinct cell types are responsible for this remains unclear. *Bmi1* (Yan et al., 2012), *Hopx* (Takeda et al., 2011), *Lrig1* (Powell et al., 2012), and *Tert* (Montgomery et al., 2011) have previously been reported as markers of quiescent "+4" stem cells, although subsequent studies have shown that actively cycling crypt base stem cells also express these markers at equivalent levels (Muñoz et al., 2012). Similarly, we did not detect enhanced expression of these markers in qCSCs (Data S1). This is also in agreement with a recent single-cell RNA sequencing (scRNA-seq) analyses of the regenerating mouse intestine that identified a damage-induced quiescent cell type, termed revival stem cells (revSCs) (Ayyaz et al., 2019). These cells, required for the regeneration of a functional intestine, are extremely rare during normal homeostasis and are characterized by enhanced expression of the pro-survival stress response gene *Clu* (Zhang et al., 2014). Interestingly, we find that many of the genes that make up the molecular signature of these quiescent revSCs are also enriched in qCSCs and have been found to regulate therapy resistance in various types of cancer. These common genes include *CLU* (Koltai, 2014); *CTSD* (Mahajan et al., 2020; Oliveira et al., 2015); *CDKN1A* (Koster et al., 2010; Liu et al., 2013; Maiuthed et al., 2018; Morris-Hanon et al., 2017; Xia et al., 2011); *EMP1* (Ariës et al., 2014; Jain et al., 2005); *MUC3* (Lesuffleur et al., 1993); *LAMC2* (Huang et al., 2017); *KRT19* (Asfaha et al., 2015); *LGALS3* (Wang et al., 2019); *F3*, *ITM2B*, and *ITGB4* (Folgiero et al., 2008; Stewart and O'Connor, 2015); *CDH17* (Atukorala and Mathivanan, 2018; Qiu et al., 2013); and *GSN* (Chung et al., 2015; Ilmer et al., 2016) (Figures 3D, 3E, and S1 and Data S1). Considering that colon cancer is a heterogeneous tumor that recapitulates the cellular hierarchy of the intestine, these data suggest that the qCSCs identified here may be the tumor equivalent of revSCs. However, in contrast to revSCs and previous studies on qCSCs, our data demonstrate that qCSCs are enriched for p53 signaling (Figures 3A and 4A).

Negative cell cycle regulators downstream of p53 are indicators of poor prognosis and required for qCSC maintenance

Loss of p53 signaling in hematopoietic stem cells (HSCs) and neural stem cells (NSCs) causes these cells to exit quiescence and enter the cell cycle (Itahana et al., 2002; Liu et al., 2009; Meletis et al., 2006). Targeting p53 signaling may have the same effect in qCSCs but is complicated by the role of p53 as a tumor suppressor and guardian of homeostasis (Lane, 1992). However, targeting negative cell cycle regulators downstream of p53 may provide novel strategies for qCSC elimination without affecting the role of p53 in healthy cells. In addition, such strategies may also enable cell cycle activation of qCSCs in p53 mutant tumors. Differential gene expression analysis, comparing PKH26^{Negative} and PKH26^{Positive} cells, identified the negative cell cycle regulators *AKAP12* (Gelman, 2010; Lin et al., 2000; Liu et al., 2011; Reggi and Diviani, 2017), *CD82* (Hur et al., 2016), *CDKN1A* (El-Deiry, 1993; Vogelstein et al., 2000; Wade Harper, 1993; Xiong et al., 1993), *FHL2* (Hellerbrand, 2010; Labalette et al., 2008; Lee et al., 2006; Martin et al., 2007), *GPX3* (An et al., 2018; Barrett et al., 2013; Wang et al., 2012), *KIAA0247* (Huang et al., 2011; Polato et al., 2014), *LCN2* (Chakraborty et al., 2012; Chiang et al., 2016; Kim et al., 2017), *TFF2* (Bossenmeyer-Pourie et al., 2002; Dubeykovskaya et al., 2016, 2019; Thim, 1997; Tu et al., 2009), *UNC5B* (Huang et al., 2020; Kong et al., 2016; Okazaki, 2011), and *ZMAT3* (Bersani et al., 2014; Hellborg et al., 2001) to be enriched in qCSCs (Figure 4A and Data S1). Significantly, each of these genes is a target of p53 (Bersani et al., 2014; Fischer, 2017; Gelman, 2010; Lane, 1992; Lee et al., 2006; Liu et al., 2011; Marreiros et al., 2005; Miyamoto et al., 2016; Polato et al., 2014; Rouillard et al., 2016; Soutto et al., 2014), and with the exceptions of *LCN2* and *ZMAT3*, is associated with reduced survival in colorectal cancer (CRC) (Figure 4B). Interestingly, CD82, KIAA0247, and UNC5B proteins localize to the cell surface and may therefore have potential as new markers for the prospective isolation of qCSCs in CRC. Indeed, CD82 has previously been identified as a marker for prospectively isolating stem cells from human fetal and adult skeletal muscle and is a functional surface marker of long-term HSCs (Alexander et al., 2016; Hur et al., 2016).



Gene Name	log2 Fold Change	p-value
CTSD	1.0362917	3.55E-12
CDKN1A	1.1134356	6.63E-12
GSN	0.93978	5.67E-10
ITGB4	0.74492633	2.08E-07
CDH17	0.9418327	3.78E-07
LGALS3	0.6010856	1.13E-06
KRT19	0.6341487	2.29E-05
MUC3A	0.7705268	3.13E-05
*ITM2C**	0.5946194	1.57E-04
AHNAK2	0.9683501	3.65E-04
CLU	0.9644593	1.03E-03
F3	0.8337223	1.27E-03
LAMC2	0.70564735	2.79E-03
EMP1	0.864138	1.06E-02

Figure 3. qCSCs display the molecular hallmarks of quiescent tissue stem cells, including enrichment for p53 pathway and genes common to damage-induced quiescent revSCs of the regenerating intestine

(A) RNA sequencing-generated gene set enrichment analysis for organ development (nominal p value = < 0.0005), cell development (nominal p value = < 0.0005), nervous system development (nominal p value = < 0.0005), embryonic development (nominal p value = 0.03), placenta (nominal p value = < 0.0005), epithelial-mesenchymal transition (nominal p value = < 0.0005), p53 pathway (nominal p value = < 0.0005), TNFα signaling via NF-κB (nominal p value = < 0.0005), Wnt signaling pathway (nominal p value = 0.002), and hedgehog signaling pathway (nominal p value = 0.002) in 12-day PKH26^{Positive} LRCs (compared with PKH26^{Negative} cells) from PDO models 151-ML-M, 162-MW-P, 195-CB-P, 249-CB-P, 278-ML-P, and 302-CB-M (n = 4 separate cell preparations).

(B) Gene ontology (GO) groups downregulated in PKH26^{Positive} LRCs.

(C) Cell cycle, transcription, and protein synthesis GO terms downregulated in PKH26^{Positive} LRCs.

(D) Venn diagram shows the number of upregulated RNA sequencing-generated transcripts identified in intestinal revSCs (50 genes; log fold change >0.25, p value < 0.05) by Ayyaz et al. (2019) and in PKH26^{Positive} qCSCs (255 genes; log2 fold change >0.586, p value < 0.05) (see also Data S1) and upregulated in both revSCs and PKH26^{Positive} qCSCs (14 genes; representation factor 21.8, p value < 1.452 × 10^{-15}). The representation factor is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups. A representation factor >1 indicates more overlap than expected of the two independent groups.

(E) Table shows the 14 genes upregulated in both revSCs and PKH26^{Positive} qCSCs. *ITM2C is a paralog of revSC-enriched Itm2b (see also Figure S1 and Data S1).

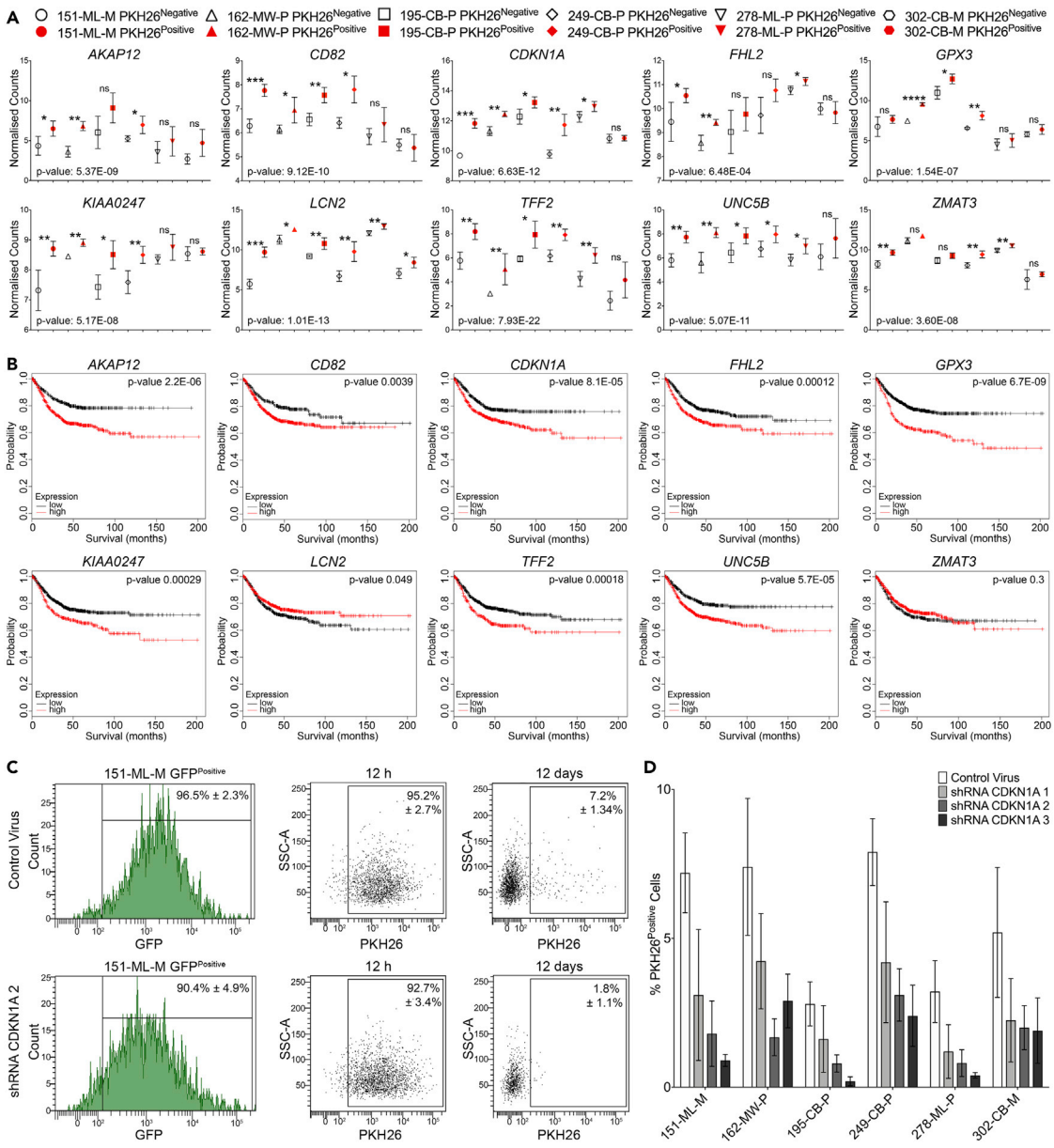


Figure 4. qCSCs are enriched for p53-interacting negative regulators of cell cycle that are indicators of poor prognosis and may be targeted for qCSC elimination

(A) RNA sequencing-generated normalized counts for negative cell cycle regulator and p53 target genes *AKAP12*, *CD82*, *CDKN1A*, *FHL2*, *GPX3*, *KIAA0247*, *LCN2*, *TFF2*, *UNC5B*, and *ZMAT3* in PKH26^{Negative} and PKH26^{Positive} cells (±SD; n = 4). ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (t test). See also Data S1.

(B) Kaplan-Meier survival curves for *AKAP12*, *CD82*, *CDKN1A*, *FHL2*, *GPX3*, *KIAA0247*, *LCN2*, *TFF2*, *UNC5B*, and *ZMAT3* in patients with colorectal cancer comparing lower quartile to upper quartile (log rank p values = 2.2×10^{-6}, 0.004, 8.1×10^{-5}, 0.00012, 0.0003, 0.049, 0.00018, 5.7×10^{-5} and 0.3, respectively). Of these, higher *AKAP12*, *CD82*, *CDKN1A*, *FHL2*, *GPX3*, *KIAA0247*, *LCN2*, *TFF2*, and *UNC5B* are significant at FDR <10%. Results based on data generated by the Kaplan-Meier Plotter (kmplot.com) (Nagy et al., 2018).

(C) Representative FACS plot of PKH26-labeled 151-ML-M control-GFP cells (top row) and shRNA CDKN1A-GFP cells (bottom row) after 12 h and 12 days.

(D) Frequency of PKH26^{Positive} LRCs in shRNA CDKN1A PDO models after 12 days compared with control virus-transduced cells (±SD; n = 3) (see also Figure S2).

Deletion of *CDKN1A* (p21), which is the downstream mediator of p53 induced cell-cycle arrest (El-Deiry, 1993; Georgakilas et al., 2017), leads to cell cycle activation and exhaustion of quiescent HSCs and NSCs (Cheng, 2000; Kippin et al., 2005). In addition, *CDKN1A* is highly expressed in noncycling intestinal

crypt base stem cells (Powell et al., 2012) and revSCs (Ayyaz et al., 2019). We therefore selected *CDKN1A* as a candidate gene to determine whether targeting the p53 pathway would eliminate qCSCs. Significantly, short hairpin RNA (shRNA)-mediated knockdown of *CDKN1A* (Figure S2) in PKH26-labeled qCSCs resulted in the elimination of PKH26^{Positive} label-retaining qCSCs (Figures 4C and 4D).

DISCUSSION

Colon cancer is a heterogeneous tumor entity containing a subpopulation of qCSCs that may promote tumor cell heterogeneity, plasticity, and resistance to various types of stress, including resistance to conventional treatments (Moore and Lyle, 2011). However, the rarity and plasticity of qCSCs has made them an elusive and challenging cell state to define and target. Here, we provide the first whole-transcriptome analyses of a population of colon cancer patient-derived long-term label-retaining qCSCs and identify genes that may provide novel targets for their elimination.

Label retention has previously been used as a strategy for the isolation of both healthy quiescent tissue stem cells and qCSCs from a variety of cancer types (Buczacki et al., 2013; Cotsarelis et al., 1990; Dembinski and Krauss, 2009; Moore and Lyle, 2011; Pece et al., 2010; Roesch et al., 2010; Smith, 2005; Wilson et al., 2008). In agreement with these studies, we show that PKH26^{Positive} LRCs isolated from colon cancer PDOs are qCSCs capable of entering the cell cycle and self-renewing after replating in adherent and non-adherent cell culture conditions and maintain long-term quiescence in xenograft models. Interestingly, *in vivo* these cells were located at the tumor border, suggesting that quiescence may be induced at the invasive tumor front where such cells may be primed for metastatic dissemination. This is in agreement with previous studies showing cell-cycle arrest/decreased proliferation and increased levels of Wnt signaling at the invasive front of colorectal tumors (Dawson et al., 2014; Harbaum et al., 2011; Jung et al., 2001; Rubio, 2008; De Smedt et al., 2017).

RNA sequencing of qCSCs demonstrated that they display the molecular hallmarks of quiescence (Cho et al., 2019) while also being enriched for the same developmental and stem cell-associated gene sets previously described for actively cycling ALDH^{Positive} CSCs (Regan et al., 2017), which unlike PKH26^{Positive} LRCs are enriched in PDOs. We previously reported that hedgehog signaling in active colon CSCs is non-canonical (SHH-dependent, PTCH-dependent, SMO-independent, GLI-independent) and acts as a positive regulator of Wnt signaling for CSC survival (Regan et al., 2017). In agreement with our work, a subsequent study from (Buczacki et al., 2018) demonstrated that qCSC survival in CRC is also dependent on non-canonical hedgehog signaling-mediated regulation of Wnt signaling (Buczacki et al., 2018). In addition, several of the genes common to both the revSCs reported by Ayyaz et al. (2019) and qCSCs, namely, *CLU* (Schepeler et al., 2007), *CTSD* (Basu et al., 2019), *CDKN1A* (Xu et al., 2016), *EMP1* (Yao et al., 2011), *MUC3* (Pai et al., 2016), *LAMC2* (Sánchez-Tilló et al., 2011), *KRT19* (Saha et al., 2017), *LGALS3* (Korkmaz et al., 2016), F3 (Camps et al., 2020; Kinchen et al., 2018), *ITGB4* (Avvisato et al., 2007), *CDH17* (Wang et al., 2013), and *GSN* (Shimura et al., 2004), are targets and/or regulators of Wnt signaling. Overall, these data demonstrate that both cycling and non-cycling CSCs share overlapping molecular profiles and further support the targeting of non-canonical hedgehog signaling to prevent disease relapse (Buczacki et al., 2018; Regan, 2018; Regan et al., 2017).

However, the molecular mechanisms that distinguish non-cycling qCSCs from cycling CSCs required further elucidation. p53 signaling plays a crucial role in regulating cellular stress responses such as DNA damage repair, senescence, apoptosis, and cell-cycle arrest in virtually all cell types (Vogelstein et al., 2000; Vousden and Lane, 2007). In addition, it is an important regulator of stem cell self-renewal and differentiation in embryonic and adult tissue stem cells (Jain and Barton, 2018; Meletis et al., 2006; Tosoni et al., 2015) and CSCs (Freed-Pastor et al., 2012; Tschaharganeh et al., 2014; Zhao et al., 2010). Significantly, it has also been demonstrated to be essential for the maintenance of quiescence in HSCs, NSCs, muscle stem cells, and lung progenitor cells (Flamini et al., 2018; Itahana et al., 2002; Liu et al., 2009; McConnell et al., 2016; Zheng et al., 2008).

Here we show that qCSCs, in contrast to cycling ALDH^{Positive} CSCs (Regan et al., 2017), are enriched for p53 signaling genes (Figures 3A and 3D). p53 is mutated in 40%– 50% of CRCs. Reflecting this, half the tumors included in our study contain a p53 mutation (Table S1). However, regardless of mutation status, all PDO models expressed *CDKN1A*, the downstream mediator of p53-induced cell-cycle arrest (El-Deiry, 1993; Georgakilas et al., 2017), which we demonstrate is enriched in and required for the maintenance of qCSCs.

This is in agreement with other studies showing *CDKN1A* expression to be independent of p53 mutation status (van Oijen et al., 1998; Perez et al., 2016; Phalke et al., 2012; Sato et al., 1997; Zhang et al., 2015). For example, in head and neck squamous cell carcinoma *CDKN1A* is also overexpressed in non-proliferating cells in both p53 wild-type and p53 mutant tumors (van Oijen et al., 1998). The mechanisms regulating the enrichment of p53 signaling genes in p53 mutant qCSCs are unclear but undoubtedly reflect the complexity of the p53 pathway, in which cellular context (cell state, epigenetic status, microenvironment, activating signal) may be the predominant determinant of signaling activity and biological outcome (Kastenhuber and Lowe, 2017). These data suggest that targeting negative regulators of cell cycle downstream of p53, in both wild-type and mutant p53 tumors, may provide novel therapeutic "lock-out" strategies to induce the proliferation of qCSCs and thereby sensitize them to chemotherapeutics and prevent relapse (Cho et al., 2019; Kobayashi et al., 2011; Takeishi et al., 2013).

Considering the role of p53 as a tumor suppressor and guardian of homeostasis in healthy tissues, as well as its inactivation in many cancers, most strategies to date have focused on the development of p53 activators (Levine, 2019). However, our data, and others, suggest that strategies that activate p53 may lead to therapy resistance (Kim et al., 2009). For example, in glioma there is evidence that p53 DNA repair activities contribute to overall survival potential and drug resistance (Batista et al., 2007) and in breast cancer p53 induces senescence, drives resistance to therapy, and is associated with poor therapeutic response and overall survival (Jackson et al., 2012; Ungerleider et al., 2018).

Inhibiting p53 could interfere with its role in normal tissue homeostasis or lead to the activation of senescent cancer cells in other tissues. However, healthy cells have lower p53 expression levels than cancer cells (Rogel et al., 1985) and single-dose treatments, that avoid the unwanted consequences of sustained p53 inhibition, may be sufficient to eliminate qCSCs. This was recently demonstrated by Webster et al. (2020) in melanoma, where a single dose of p53 inhibitor during the early stage of BRAF/MEK inhibitor treatment resulted in improved response to therapy (Webster et al., 2020).

In addition, targeting negative cell cycle regulators downstream of p53, such as those identified here (*AKAP12*, *CD82*, *CDKN1A*, *FHL2*, *GPX3*, *KIAA0247*, *LCN2*, *TFF2*, *UNC5B*, and *ZMAT3*), may provide novel strategies for activating cell cycle in qCSCs without affecting the role of p53 in healthy cells. For example, p53-dependent activation of p21 (*CDKN1A*) is an important axis in senescence-dependent tumor suppression. However, despite p21 playing an important role in mediating the p53-dependent cellular response to stress, lack of p21 does not promote tumor development (Choudhury et al., 2007). Furthermore, p21 maintains CSC self-renewal, limits proliferation, and confers therapy resistance in numerous cancer types in which its temporal inhibition has been proposed as a strategy to overcome resistance to DNA-damaging chemotherapy and radiation (El-Deiry, 2016; Tian et al., 2000; Kokunai et al., 2001; Koster et al., 2010; Maiuthed et al., 2018; Morris-Hanon et al., 2017; Viale et al., 2009; Weiss, 2003; Xia et al., 2011). Indeed, several small molecule inhibitors of p21 have been reported, including butyrolactone I (Joanna et al., 2002), LLW10 (Park et al., 2008), sorafenib (Inoue et al., 2011), and UC2288 (Wettersten et al., 2013), that could serve as novel drugs for the elimination of therapy-resistant qCSCs. Significantly, these p21 inhibitors have been shown to be p53 independent and so may be effective in both wild-type and p53 mutant cancers without perturbing p53 function in healthy cells (Liu et al., 2013).

These data demonstrate the existence of long-term p53 signaling-dependent qCSCs in colon cancer and provide evidence supporting the temporal inhibition of p53 signaling, in combination with standard-of-care treatments, for the elimination of qCSCs and prevention of disease relapse. The p53 target genes identified here, along with the publication of our qCSC whole-transcriptome data, will provide a valuable resource for the development of such therapeutic strategies in the future.

Limitations of the study

Our data suggest that qCSCs may be the tumor equivalent of revSCs, which were recently identified by scRNA-seq of the normal mouse intestine during homeostasis and injury (Ayyaz et al., 2019). However, revSCs have not yet been identified in the human intestine and their potential role in carcinogenesis and tumor heterogeneity remain to be determined. In addition, xenotransplantation of $PKH26^{Positive}$ cells demonstrated that they maintain quiescence *in vivo* for up to 80 days. Unfortunately, due to the rarity of these cells *in vivo*, we were unable to isolate sufficient numbers for RNA sequencing. Future scRNA-seq studies can be used to overcome the obstacle of qCSC rarity *in vivo* and will be required to determine

lineage relationships, e.g., between revSCs and qCSCs, and cell-state heterogeneity within colon cancer (Giraddi et al., 2018; Wahl and Spike, 2017). Furthermore, the molecular mechanisms that maintain high levels of p53 signaling genes in p53 mutant qCSCs remain unclear, but are likely influenced by cellular context, including cell state and epigenetic factors. The elucidation of such mechanisms may be facilitated through future single-nucleus transposase-accessible chromatin sequencing (snATAC-seq), which examines global chromatin accessibility and has been shown to be a better indicator of cell state than scRNA-seq (Chung et al., 2019; Regan and Smalley, 2020). The transcriptome data of functionally tested qCSCs presented here will help to inform cell state designations in such future large-scale single-cell profiling endeavors.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, J.L.R.; methodology, J.L.R.; investigation, J.L.R., D.S., S.S., A.S., R.L., J.T., T.J., J.H., and M.L.; writing – original draft, J.L.R.; writing – review & editing, J.L.R, R.S., and M.L; visualization, J.L.R; data curation, A.S. and J.T.; resources, J.H., U.K., C.R.A.R., and B.G.; supervision, J.L.R., D.M., D.H., R.S., and M.L.

DECLARATION OF INTERESTS

A.S., T.J., D.M., and. D.H. are employees of Bayer AG. R.L., J.T., and M.L. are employees of Nuvisan ICB GmbH. C.R.A.R. is a founder of CELLphenomics GmbH.

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REFERENCES

Alexander, M.S., Rozkalne, A., Colletta, A., Spinazzola, J.M., Johnson, S., Rahimov, F., Meng, H., Lawlor, M.W., Estrella, E., Kunkel, L.M., and Gussoni, E. (2016). CD82 is a marker for prospective isolation of human muscle satellite cells and is linked to muscular dystrophies. Cell Stem Cell *19*, 800–807.

Amaral, R., Zimmermann, M., Ma, A.-H., Zhang, H., Swiech, K., and Pan, C.-X. (2020). A simple three-dimensional in vitro culture mimicking the in vivo-like cell behavior of bladder patient-derived xenograft models. Cancers *12*, 1304.

An, B.C., Choi, Y.-D., Oh, I.-J., Kim, J.H., Park, J.-I., and Lee, S.-W. (2018). GPx3-mediated redox signaling arrests the cell cycle and acts as a tumor suppressor in lung cancer cell lines. PLoS One *13*, e0204170.

Ariës, I.M., Jerchel, I.S., van den Dungen, R.E.S.R., van den Berk, L.C.J., Boer, J.M., Horstmann, M.A., Escherich, G., Pieters, R., and den Boer, M.L. (2014). EMP1, a novel poor prognostic factor in pediatric leukemia regulates prednisolone resistance, cell proliferation, migration and adhesion. Leukemia *28*, 1828–1837.

Asfaha, S., Hayakawa, Y., Muley, A., Stokes, S., Graham, T.A., Ericksen, R.E., Westphalen, C.B., von Burstin, J., Mastracci, T.L., Worthley, D.L., et al. (2015). Krt19(+)/Lgr5(-) cells are radioresistant cancer-initiating stem cells in the colon and intestine. Cell Stem Cell *16*, 627–638.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., et al. (2000). Gene Ontology: tool for the unification of biology. Nat. Genet. *25*, 25–29.

Atukorala, I., and Mathivanan, S. (2018). PO-066 Knockdown of cadherin 17 inactivates WNT signalling pathway and induces apoptosis in colorectal cancer cells. ESMO Open *3*, A47.

Avvisato, C.L., Yang, X., Shah, S., Hoxter, B., Li, W., Gaynor, R., Pestell, R., Tozeren, A., and Byers, S.W. (2007). Mechanical force modulates global gene expression and β-catenin signaling in colon cancer cells. J. Cell Sci. *120*, 2672 LP–2682.

Ayyaz, A., Kumar, S., Sangiorgi, B., Ghoshal, B., Gosio, J., Ouladan, S., Fink, M., Barutcu, S., Trcka, D., Shen, J., et al. (2019). Single-cell transcriptomes of the regenerating intestine reveal a revival stem cell. Nature *569*, 121–125.

Barker, N., Ridgway, R.A., van Es, J.H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A.R., Sansom, O.J., and Clevers, H. (2008). Crypt stem cells as the cells-of-origin of intestinal cancer. Nature *457*, 608.

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., and Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature *449*, 1003–1007.

Barrett, C.W., Ning, W., Chen, X., Smith, J.J., Washington, M.K., Hill, K.E., Coburn, L.A., Peek, R.M., Chaturvedi, R., Wilson, K.T., et al. (2013). Tumor suppressor function of the plasma glutathione peroxidase gpx3 in colitis-associated carcinoma. Cancer Res. *73*, 1245–1255.

Basu, S., Cheriyamundath, S., Gavert, N., Brabletz, T., Haase, G., and Ben-Ze'ev, A. (2019). Increased expression of cathepsin D is required for L1-mediated colon cancer progression. Oncotarget *10*, 5217–5228.

Batista, L.F.Z., Roos, W.P., Christmann, M., Menck, C.F.M., and Kaina, B. (2007). Differential sensitivity of malignant glioma cells to methylating and chloroethylating anticancer drugs: p53 determines the switch by regulating xpc, ddb2, and DNA double-strand breaks. Cancer Res. *67*, 11886 LP–11895.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Statist Soc. B *57*, 289–300.

Bersani, C., Xu, L.-D., Vilborg, A., Lui, W.-O., and Wiman, K.G. (2014). Wig-1 regulates cell cycle arrest and cell death through the p53 targets FAS and 14-3-3σ. Oncogene *33*, 4407–4417.

Blanpain, C., and Simons, B.D. (2013). Unravelling stem cell dynamics by lineage tracing. Nat. Rev. Mol. Cell Biol. *14*, 489–502.

Bossenmeyer-Pourie, C., Kannan, R., Ribieras, S., Wendling, C., Stoll, I., Thim, L., Tomasetto, C., and Rio, M.-C. (2002). The trefoil factor 1 participates in gastrointestinal cell differentiation by delaying G1-S phase transition and reducing apoptosis. J. Cell Biol. *157*, 761–770.

Brock, A., and Huang, S. (2017). Precision oncology: between vaguely right and precisely wrong. Cancer Res. *77*, 6473 LP–6479.

Buczacki, S.J.A., Popova, S., Biggs, E., Koukorava, C., Buzzelli, J., Vermeulen, L., Hazelwood, L., Francies, H., Garnett, M.J., and Winton, D.J. (2018). Itraconazole targets cell cycle heterogeneity in colorectal cancer. J. Exp. Med. *215*, 1891 LP–1912.

Buczacki, S.J.A., Zecchini, H.I., Nicholson, A.M., Russell, R., Vermeulen, L., Kemp, R., and Winton, D.J. (2013). Intestinal label-retaining cells are secretory precursors expressing Lgr5. Nature *495*, 65–69.

Camps, J., Breuls, N., Sifrim, A., Giarratana, N., Corvelyn, M., Danti, L., Grosemans, H., Vanuytven, S., Thiry, I., Belicchi, M., et al. (2020). Interstitial cell remodeling promotes aberrant adipogenesis in dystrophic muscles. Cell Rep. *31*. https://doi.org/10.1016/j.celrep.2020.107597.

Chakraborty, S., Kaur, S., Guha, S., and Batra, S.K. (2012). The multifaceted roles of neutrophil gelatinase associated lipocalin (NGAL) in inflammation and cancer. Biochim. Biophys. Acta *1826*, 129–169.

Chen, J., Li, Y., Yu, T.-S., McKay, R.M., Burns, D.K., Kernie, S.G., and Parada, L.F. (2012). A restricted cell population propagates glioblastoma growth after chemotherapy. Nature *488*, 522–526.

Chen, W., Dong, J., Haiech, J., Kilhoffer, M.-C., and Zeniou, M. (2016). Cancer stem cell quiescence and plasticity as major challenges in cancer therapy. Stem Cells Int. *2016*, 1740936.

Cheng, T. (2000). Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science *287*, 1804–1808.

Cheng, H., and Leblond, C.P. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine V. Unitarian theory of the origin of the four epithelial cell types. Am. J. Anat. *141*, 537–561.

Cheung, T.H., and Rando, T.A. (2013). Molecular regulation of stem cell quiescence. Nat. Rev. Mol. Cell Biol. *14*, 329–340.

Chiang, K.-C., Yeh, T.-S., Wu, R.-C., Pang, J.-H.S., Cheng, C.-T., Wang, S.-Y., Juang, H.-H., and Yeh, C.-N. (2016). Lipocalin 2 (LCN2) is a promising target for cholangiocarcinoma treatment and bile LCN2 level is a potential cholangiocarcinoma diagnostic marker. Sci. Rep. *6*, 36138.

Cho, I.J., Lui, P.P., Obajdin, J., Riccio, F., Stroukov, W., Willis, T.L., Spagnoli, F., and Watt, F.M. (2019). Mechanisms, hallmarks, and implications of stem cell quiescence. Stem Cell Rep. *12*, 1190–1200.

Choi, S.I., Jeon, A.-R., Kim, M.K., Lee, Y.-S., Im, J.E., Koh, J.-W., Han, S.-S., Kong, S.-Y., Yoon, K.-A., Koh, Y.-H., et al. (2019). Development of patient-derived preclinical platform for metastatic pancreatic cancer: PDOX and a subsequent organoid model system using percutaneous biopsy samples. Front. Oncol. *9*, 875.

Chung, C.Y., Ma, Z., Dravis, C., Preissl, S., Poirion, O., Luna, G., Hou, X., Giraddi, R.R., Ren, B., and Wahl, G.M. (2019). Single-cell chromatin analysis of mammary gland development reveals cell-state transcriptional regulators and lineage relationships. Cell Rep. *29*, 495–510.e6.

Chung, L.-Y., Tang, S.-J., Wu, Y.-C., Sun, G.-H., Liu, H.-Y., and Sun, K.-H. (2015). Galectin-3 augments tumor initiating property and tumorigenicity of lung cancer through interaction with β-catenin. Oncotarget *6*, 4936–4952.

Choudhury, A.R., Ju, Z., Djojosubroto, M.W., Schienke, A., Lechel, A., Schaetzlein, S., Jiang, H., Stepczynska, A., Wang, C., Buer, J., et al. (2007). Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. Nat. Genet. *39*, 99–105.

Cicalese, A., Bonizzi, G., Pasi, C.E., Faretta, M., Ronzoni, S., Giulini, B., Brisken, C., Minucci, S., Di Fiore, P.P., and Pelicci, P.G. (2009). The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell *138*, 1083–1095.

Clevers, H. (2013). A unifying theory for the crypt. Nature *495*, 53–54.

Cotsarelis, G., Sun, T.-T., and Lavker, R.M. (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell *61*, 1329–1337.

Cumming, G., Fidler, F., and Vaux, D.L. (2007). Error bars in experimental biology. J. Cell Biol *177*, 7–11.

Dawson, H., Koelzer, V.H., Karamitopoulou, E., Economou, M., Hammer, C., Muller, D.-E., Lugli, A., and Zlobec, I. (2014). The apoptotic and proliferation rate of tumour budding cells in colorectal cancer outlines a heterogeneous population of cells with various impacts on clinical outcome. Histopathology *64*, 577–584.

De Angelis, M.L., Francescangeli, F., La Torre, F., and Zeuner, A. (2019). Stem cell plasticity and dormancy in the development of cancer therapy resistance. Front. Oncol. *9*, 626.

De Smedt, L., Palmans, S., Andel, D., Govaere, O., Boeckx, B., Smeets, D., Galle, E., Wouters, J., Barras, D., Suffiotti, M., et al. (2017). Expression profiling of budding cells in colorectal cancer reveals an EMT-like phenotype and molecular subtype switching. Br. J. Cancer *116*, 58–65.

Dembinski, J.L., and Krauss, S. (2009). Characterization and functional analysis of a slow cycling stem cell-like subpopulation in pancreas adenocarcinoma. Clin. Exp. Metastasis *26*, 611.

Dijkstra, K.K., van den Berg, J.G., Weeber, F., van de Haar, J., Velds, A., Kaing, S., Peters, D.D.G.C., Eskens, F.A.L.M., de Groot, D.-J.A., Tesselaar, M.E.T., and Voest, E.E. (2021). Patient-derived organoid models of human neuroendocrine carcinoma. Front. Endocrinol. *12*, 627819.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15–21.

Dubeykovskaya, Z.A., Duddempudi, P.K., Deng, H., Valenti, G., Cuti, K.L., Nagar, K., Tailor, Y., Guha, C., Kitajewski, J., and Wang, T.C. (2019). Therapeutic potential of adenovirus-mediated TFF2-CTP-Flag peptide for treatment of colorectal cancer. Cancer Gene Ther. *26*, 48–57.

Dubeykovskaya, Z., Si, Y., Chen, X., Worthley, D.L., Renz, B.W., Urbanska, A.M., Hayakawa, Y., Hu, T., Westphalen, C.B., Dubeykovskiy, A., et al. (2016). Neural innervation stimulates splenic TFF2 to arrest myeloid cell expansion and cancer. Nat. Commun. *7*, 10517.

Ebinger, S., Özdemir, E.Z., Ziegenhain, C., Tiedt, S., Castro Alves, C., Grunert, M., Dworzak, M., Lutz, C., Turati, V.A., Enver, T., et al. (2016). Characterization of rare, dormant, and therapy-resistant cells in acute lymphoblastic leukemia. Cancer Cell *30*, 849–862.

El-Deiry, W. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817–825.

El-Deiry, W.S. (2016). p21(WAF1) mediates cell-cycle inhibition, Relevant to cancer suppression and therapy. Cancer Res. *76*, 5189–5191.

Fischer, M. (2017). Census and evaluation of p53 target genes. Oncogene *36*, 3943–3956.

Flamini, V., Ghadiali, R.S., Antczak, P., Rothwell, A., Turnbull, J.E., and Pisconti, A. (2018). The satellite cell niche regulates the balance between myoblast differentiation and self-renewal via p53. Stem Cell Rep. *10*, 970–983.

Folgiero, V., Avetrani, P., Bon, G., Di Carlo, S.E., Fabi, A., Nisticò, C., Vici, P., Melucci, E., Buglioni, S., Perracchio, L., et al. (2008). Induction of ErbB-3 expression by α6β4 integrin contributes to tamoxifen resistance in ERβ1-negative breast carcinomas. PLoS One *3*, e1592.

Francescangeli, F., Contavalli, P., De Angelis, M.L., Careccia, S., Signore, M., Haas, T.L., Salaris, F., Baiocchi, M., Boe, A., Giuliani, A., et al. (2020). A pre-existing population of ZEB2+ quiescent cells with stemness and mesenchymal features dictate chemoresistance in colorectal cancer. J. Exp. Clin. Cancer Res. *39*, 2.

Francescangeli, F., Patrizii, M., Signore, M., Federici, G., Di Franco, S., Pagliuca, A., Baiocchi, M., Biffoni, M., Ricci Vitiani, L., Todaro, M., et al. (2012). Proliferation state and Polo-like Kinase1 dependence of Tumorigenic colon cancer cells. Stem Cells *30*, 1819–1830.

Freed-Pastor, W.A., Mizuno, H., Zhao, X., Langerød, A., Moon, S.-H., Rodriguez-Barrueco, R., Barsotti, A., Chicas, A., Li, W., Polotskaia, A., et al. (2012). Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway. Cell *148*, 244–258.

Fujii, M., and Sato, T. (2021). Somatic cell-derived organoids as prototypes of human epithelial tissues and diseases. Nat. Mater. *20*, 156–169.

Ganesh, K., Wu, C., O'Rourke, K.P., Szeglin, B.C., Zheng, Y., Sauvé, C.-E.G., Adileh, M., Wasserman, I., Marco, M.R., Kim, A.S., et al. (2019). A rectal cancer organoid platform to study individual responses to chemoradiation. Nat. Med. *25*, 1607–1614.

Gao, M.-Q., Choi, Y.-P., Kang, S., Youn, J.H., and Cho, N.-H. (2010). CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. Oncogene *29*, 2672–2680.

Gelman, I.H. (2010). Emerging roles for SSeCKS/Gravin/AKAP12 in the control of cell proliferation, cancer malignancy, and barriergenesis. Genes Cancer *1*, 1147–1156.

Georgakilas, A.G., Martin, O.A., and Bonner, W.M. (2017). p21: a two-Faced genome guardian. Trends Mol. Med. *23*, 310–319.

Giraddi, R.R., Chung, C.-Y., Heinz, R.E., Balcioglu, O., Novotny, M., Trejo, C.L., Dravis, C., Hagos, B.M., Mehrabad, E.M., Rodewald, L.W., et al. (2018). Single-cell transcriptomes distinguish stem cell state changes and lineage specification programs in early mammary gland development. Cell Rep. *24*, 1653–1666.e7.

Harbaum, L., Pollheimer, M.J., Kornprat, P., Lindtner, R.A., Schlemmer, A., Rehak, P., and Langner, C. (2011). Keratin 7 expression in colorectal cancer – freak of nature or significant finding? Histopathology *59*, 225–234.

Hellborg, F., Qian, W., Mendez-Vidal, C., Asker, C., Kost-Alimova, M., Wilhelm, M., Imreh, S., and Wiman, K.G. (2001). Human wig-1, a p53 target gene that encodes a growth inhibitory zinc finger protein. Oncogene *20*, 5466–5474.

Hellerbrand. (2010). FHL2 suppresses growth and differentiation of the colon cancer cell line HT-29. Oncol. Rep. *23*. https://doi.org/10.3892/or_00000810.

Zheng, H., Ying, H., Yan, H., Kimmelman, A.C., Hiller, D.J., Chen, A.-J., Perry, S.R., Tonon, G., Chu, G.C., Ding, Z., et al. (2008). p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. Nature *455*, 1129–1133.

Horan, P.K., Melnicoff, M.J., Jensen, B.D., and Slezak, S.E. (1990). Fluorescent cell labeling for in vivo and in vitro cell tracking. Methods Cell Biol. *33*, 469–490.

Hu, Y., and Smith, G.K. (2009). ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. Journal of Immunological Methods *347*, 70–78. https://doi.org/10.1016/j.jim.2009.06.008.

Huang, D., Du, C., Ji, D., Xi, J., and Gu, J. (2017). Overexpression of LAMC2 predicts poor prognosis in colorectal cancer patients and promotes cancer cell proliferation, migration, and invasion. Tumor Biol. *39*. https://doi.org/10.1177/1010428317705849.

Huang, C.-J., Yang, S.-H., Huang, S.-M., Lin, C.-M., Chien, C.-C., Chen, Y.-C., Lee, C.-L., Wu, H.-H., and Chang, C.-C. (2011). A predicted protein, KIAA0247, is a cell cycle modulator in colorectal cancer cells under 5-FU treatment, Journal of Translational Medicine. Biomed. Cent. *9*, 82.

Huang, Y., Zhu, Y., Zhang, Z., Li, Z., and Kong, C. (2020). UNC5B mediates G2/M phase arrest of bladder cancer cells by binding to CDC14A and P53. Cancer Gene Ther. https://doi.org/10.1038/s41417-020-0175-x.

Hur, J., Choi, J.-I., Lee, H., Nham, P., Kim, T.-W., Chae, C.-W., Yun, J.-Y., Kang, J.-A., Kang, J., Lee, S.E., et al. (2016). CD82/KAI1 maintains the dormancy of long-term hematopoietic stem cells through interaction with DARC-expressing macrophages. Cell Stem Cell *18*, 508–521.

Ilmer, M., Mazurek, N., Byrd, J.C., Ramirez, K., Hafley, M., Alt, E., Vykoukal, J., and Bresalier, R.S. (2016). Cell surface galectin-3 defines a subset of chemoresistant gastrointestinal tumor-initiating cancer cells with heightened stem cell characteristics. Cell Death Dis. *7*, e2337.

Inoue, H., Hwang, S.H., Wecksler, A.T., Hammock, B.D., and Weiss, R.H. (2011). Sorafenib attenuates p21 in kidney cancer cells and augments cell death in combination with DNA-damaging chemotherapy. Cancer Biol. Ther. *12*, 827–836.

Itahana, K., Dimri, G.P., Hara, E., Itahana, Y., Zou, Y., Desprez, P.-Y., and Campisi, J. (2002). A role for p53 in maintaining and establishing the quiescence growth arrest in human cells. J. Biol. Chem. *277*, 18206–18214.

Jackson, J.G., Pant, V., Li, Q., Chang, L.L., Quintás-Cardama, A., Garza, D., Tavana, O., Yang, P., Manshouri, T., Li, Y., et al. (2012). p53-Mediated senescence Impairs the apoptotic response to chemotherapy and clinical outcome in breast cancer. Cancer Cell *21*, 793–806.

Jain, A.K., and Barton, M.C. (2018). p53: emerging roles in stem cells, development and beyond. Development *145*, dev158360.

Jain, A., Tindell, C.A., Laux, I., Hunter, J.B., Curran, J., Galkin, A., Afar, D.E., Aronson, N., Shak, S., Natale, R.B., et al. (2005). Epithelial membrane protein-1 is a biomarker of gefitinib resistance. Proc. Natl. Acad. Sci. U S A *102*, 11858 LP–11863.

Joanna, K.S., Bipin, C.D., Rui Hong, D.T., and Dicker, W.S.E.-D. (2002). The cyclin-dependent kinase inhibitor butyrolactone is a potent inhibitor of p21 WAF1/CIP1 expression. Cell Cycle *1*, 87–93.

Jung, A., Schrauder, M., Oswald, U., Knoll, C., Sellberg, P., Palmqvist, R., Niedobitek, G., Brabletz, T., and Kirchner, T. (2001). The invasion front of human colorectal Adenocarcinomas shows Co-Localization of nuclear β-catenin, cyclin D_1, and $p16^{INK4A}$ and is a region of low proliferation. Am. J. Pathol. *159*, 1613–1617.

Kabraji, S., Solé, X., Huang, Y., Bango, C., Bowden, M., Bardia, A., Sgroi, D., Loda, M., and Ramaswamy, S. (2017). AKT1low quiescent cancer cells persist after neoadjuvant chemotherapy in triple negative breast cancer. Breast Cancer Res. *19*, 88.

Kastenhuber, E.R., and Lowe, S.W. (2017). Putting p53 in context. Cell *170*, 1062–1078.

Kim, E., Giese, A., and Deppert, W. (2009). Wild-type p53 in cancer cells: when a guardian turns into a blackguard. Biochem. Pharmacol. *77*, 11–20.

Kim, S.-L., Lee, S.T., Min, I.S., Park, Y.R., Lee, J.H., Kim, D.-G., and Kim, S.-W. (2017). Lipocalin 2 negatively regulates cell proliferation and epithelial to mesenchymal transition through changing metabolic gene expression in colorectal cancer. Cancer Science *108*, 2176–2186.

Kinchen, J., Chen, H.H., Parikh, K., Antanaviciute, A., Jagielowicz, M., Fawkner-Corbett, D., Ashley, N., Cubitt, L., Mellado-Gomez, E., Attar, M., et al. (2018). Structural remodeling of the human colonic mesenchyme in inflammatory bowel disease. Cell *175*, 372–386.e17.

Kippin, T.E., Martens, D.J., and van der Kooy, D. (2005). p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. Genes Dev. *19*, 756–767.

Kobayashi, A., Okuda, H., Xing, F., Pandey, P.R., Watabe, M., Hirota, S., Pai, S.K., Liu, W., Fukuda, K., Chambers, C., et al. (2011). Bone morphogenetic protein 7 in dormancy and metastasis of prostate cancer stem-like cells in bone. J. Exp. Med. *208*, 2641–2655.

Kokunai, T., Urui, S., Tomita, H., and Tamaki, N. (2001). Overcoming of radioresistance in human gliomas by p21WAF1/CIP1 antisense oligonucleotide. J. Neuro Oncol. *51*, 111–119.

Koltai, T. (2014). Clusterin: a key player in cancer chemoresistance and its inhibition. OncoTargets Ther. *Vol. 7*, 447–456.

Kong, C., Zhan, B., Piao, C., Zhang, Z., Zhu, Y., and Li, Q. (2016). Overexpression of UNC5B in bladder cancer cells inhibits proliferation and reduces the volume of transplantation tumors in nude mice. BMC Cancer *16*, 892.

Korkmaz, G., Horozoglu, C., Arıkan, S., Gural, Z., Sağlam, E.K., Turan, S., Özkan, N.E., Kahraman, O.T., Yenilmez, E.N., Düzköylü, Y., et al. (2016). LGALS3 and AXIN1 gene variants playing role in the Wnt/β-catenin signaling pathway are associated with mucinous component and tumor size in colorectal cancer, Bosnian Journal of Basic Medical Sciences. Assoc. Basic Med. Sci. Fed. Bosnia Herzegovina *16*, 108–113.

Koster, R., di Pietro, A., Timmer-Bosscha, H., Gibcus, J.H., van den Berg, A., Suurmeijer, A.J., Bischoff, R., Gietema, J.A., and de Jong, S. (2010). Cytoplasmic p21 expression levels determine cisplatin resistance in human testicular cancer, the Journal of Clinical Investigation. Am. Soc. Clin. Invest. *120*, 3594–3605.

Kreso, A., O'Brien, C.A., van Galen, P., Gan, O.I., Notta, F., Brown, A.M.K., Ng, K., Ma, J., Wienholds, E., Dunant, C., et al. (2013). Variable clonal Repopulation dynamics influence chemotherapy response in colorectal cancer. Science *339*, 543–548.

Labalette, C., Nouët, Y., Sobczak-Thepot, J., Armengol, C., Levillayer, F., Gendron, M.-C., Renard, C.-A., Regnault, B., Chen, J., Buendia, M.-A., and Wei, Y. (2008). The LIM-only protein FHL2 regulates cyclin D1 expression and cell proliferation, the Journal of Biological Chemistry. Am. Soc. Biochem. Mol. Biol. *283*, 15201–15208.

Lagadinou, E.D., Sach, A., Callahan, K., Rossi, R.M., Neering, S.J., Minhajuddin, M., Ashton, J.M., Pei, S., Grose, V., O'Dwyer, K.M., et al. (2013). BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. Cell Stem Cell *12*, 329–341.

Lane, D.P. (1992). p53, guardian of the genome. Nature *358*, 15–16.

Lee, S.-W., Kim, E.-J., and Um, S.-J. (2006). FHL2 mediates p53-induced transcriptional activation through a direct association with HIPK2. Biochem. Biophysical Res. Commun. *339*, 1056–1062.

Lesuffleur, T., Porchet, N., Aubert, J.P., Swallow, D., Gum, J.R., Kim, Y.S., Real, F.X., and Zweibaum, A. (1993). Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. J. Cell Sci. *106*, 771–783.

Levine, A.J. (2019). Targeting therapies for the p53 protein in cancer treatments. Annu. Rev. Cancer Biol. *3*, 21–34.

Liberzon, A., Birger, C., Thorvaldsdóttir, H., Ghandi, M., Mesirov, J.P., and Tamayo, P. (2015). The molecular signatures database hallmark gene set collection. Cell Syst. *1*, 417–425.

Lin, X., Nelson, P., and Gelman, I.H. (2000). SSeCKS, a major protein kinase C substrate with tumor suppressor activity, regulates G(1)–>S progression by controlling the expression and cellular compartmentalization of cyclin D, Molecular and Cellular Biology. Am. Soc. Microbiol. *20*, 7259–7272.

Lin, W., Rajbhandari, N., Liu, C., Sakamoto, K., Zhang, Q., Triplett, A.A., Batra, S.K., Opavsky, R., Felsher, D.W., DiMaio, D.J., et al. (2013). Dormant cancer cells contribute to residual disease in a model of reversible pancreatic cancer. Cancer Res. *73*, 1821 LP–1830.

Liu, Y., Elf, S.E., Miyata, Y., Sashida, G., Liu, Y., Huang, G., Di Giandomenico, S., Lee, J.M., Deblasio, A., Menendez, S., et al. (2009). p53 regulates hematopoietic stem cell quiescence. Cell Stem Cell *4*, 37–48.

Liu, W., Guan, M., Hu, T., Gu, X., and Lu, Y. (2011). Re-expression of AKAP12 inhibits progression and metastasis potential of colorectal carcinoma in vivo and in vitro. PLoS One *6*, e24015.

Liu, R., Wettersten, H.I., Park, S.-H., and Weiss, R.H. (2013). Small-molecule inhibitors of p21 as novel therapeutics for chemotherapy-resistant kidney cancer. Future Med. Chem. *5*, 991–994.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550.

Mahajan, U.M., Goni, E., Langhoff, E., Li, Q., Costello, E., Greenhalf, W., Kruger, S., Ormanns, S., Halloran, C., Ganeh, P., et al. (2020). Cathepsin D expression and gemcitabine resistance in pancreatic cancer. JNCI Cancer Spectr. *4*. https://doi.org/10.1093/jncics/pkz060.

Maiuthed, A., Ninsontia, C., Erlenbach-Wuensch, K., Ndreshkjana, B., Muenzner, J.K., Caliskan, A., Husayn, A.P., Chaotham, C., Hartmann, A., Vial Roehe, A., et al. (2018). Cytoplasmic p21 mediates 5-fluorouracil resistance by inhibiting pro-apoptotic Chk2. Cancers *10*, 373.

Malladi, S., Macalinao, D.G., Jin, X., He, L., Basnet, H., Zou, Y., de Stanchina, E., and Massagué, J. (2016). Metastatic latency and immune evasion through autocrine inhibition of WNT. Cell *165*, 45–60.

Marreiros, A., Dudgeon, K., Dao, V., Grimm, M.-O., Czolij, R., Crossley, M., and Jackson, P. (2005). KAI1 promoter activity is dependent on p53, junB and AP2: evidence for a possible mechanism underlying loss of KAI1 expression in cancer cells. Oncogene *24*, 637–649.

Martin, B.T., Kleiber, K., Wixler, V., Raab, M., Zimmer, B., Kaufmann, M., and Strebhardt, K. (2007). FHL2 regulates cell cycle-dependent and doxorubicin-induced p21Cip1/waf1 expression in breast cancer cells. Cell Cycle *6*, 1779–1788.

Maru, Y., Tanaka, N., Itami, M., and Hippo, Y. (2019). Efficient use of patient-derived organoids as a preclinical model for gynecologic tumors. Gynecol. Oncol. *154*, 189–198.

McConnell, A.M., Yao, C., Yeckes, A.R., Wang, Y., Selvaggio, A.S., Tang, J., Kirsch, D.G., and Stripp, B.R. (2016). p53 regulates progenitor cell quiescence and differentiation in the airway. Cell Rep. *17*, 2173–2182.

Meletis, K., Wirta, V., Hede, S.-M., Nistér, M., Lundeberg, J., and Frisén, J. (2006). p53 suppresses the self-renewal of adult neural stem cells. Development *133*, 363–369.

Miyamoto, T., Kashima, H., Yamada, Y., Kobara, H., Asaka, R., Ando, H., Higuchi, S., Ida, K., Mvunta, D.H., and Shiozawa, T. (2016). Lipocalin 2 enhances migration and resistance against cisplatin in endometrial carcinoma cells. PLoS One *11*, e0155220.

Moore, N., and Lyle, S. (2011). Quiescent, slow-cycling stem cell populations in cancer: a review of the evidence and discussion of significance. J. Oncol. *2011*, 396076.

Montgomery, R.K., Carlone, D.L., Richmond, C.A., Farilla, L., Kranendonk, M.E.G., Henderson, D.E., Baffour-Awuah, N.Y., Ambruzs, D.M., Fogli, L.K., Algra, S., and Breault, D.T. (2011). Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells. Proc. Natl. Acad. Sci. U S A *108*, 179–184.

Moore, N., Houghton, J., and Lyle, S. (2011). Slow-cycling therapy-resistant cancer cells. Stem Cells Dev. *21*, 1822–1830.

Morris-Hanon, O., Furmento, V.A., Rodríguez-Varela, M.S., Mucci, S., Fernandez-Espinosa, D.D., Romorini, L., Sevlever, G.E., Scassa, M.E., and Videla-Richardson, G.A. (2017). The cell cycle inhibitors p21(cip1) and p27(Kip1) control proliferation but enhance DNA damage resistance of glioma stem cells. Neoplasia *19*, 519–529.

Muñoz, J., Stange, D.E., Schepers, A.G., van de Wetering, M., Koo, B.-K., Itzkovitz, S., Volckmann, R., Kung, K.S., Koster, J., Radulescu, S., et al. (2012). The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent '+4' cell markers. EMBO J. *31*, 3079–3091.

Nagle, P.W., Plukker, J.T.M., Muijs, C.T., van Luijk, P., and Coppes, R.P. (2018). Patient-derived tumor organoids for prediction of cancer treatment response. Semin. Cancer Biol. *53*, 258–264.

Nagy, Á., Lánczky, A., Menyhárt, O., and Győrffy, B. (2018). Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. Sci. Rep. *8*, 9227.

van Oijen, M.G.C.T., Tilanus, M.G.J., Medema, R.H., and Slootweg, P.J. (1998). Expression of p21 (Waf1/Cip1) in head and neck cancer in relation to proliferation, differentiation, p53 status and cyclin D1 expression. J. Oral Pathol. Med. *27*, 367–375.

Okazaki, S. (2011). Clinical significance of UNC5B expression in colorectal cancer. Int. J. Oncol. https://doi.org/10.3892/ijo.2011.1201.

Oliveira, C.S.F., Pereira, H., Alves, S., Castro, L., Baltazar, F., Chaves, S.R., Preto, A., and Côrte-Real, M. (2015). Cathepsin D protects colorectal cancer cells from acetate-induced apoptosis through autophagy-independent degradation of damaged mitochondria. Cell Death Dis. *6*, e1788.

O'Brien, C.A., Pollett, A., Gallinger, S., and Dick, J.E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature *445*, 106–110.

Ooft, S.N., Weeber, F., Dijkstra, K.K., McLean, C.M., Kaing, S., van Werkhoven, E., Schipper, L., Hoes, L., Vis, D.J., van de Haar, J., et al. (2019). Patient-derived organoids can predict response to chemotherapy in metastatic colorectal cancer patients. Sci. Transl. Med. *11*, eaay2574.

Pai, P., Rachagani, S., Dhawan, P., and Batra, S.K. (2016). Mucins and Wnt/β-catenin signaling in gastrointestinal cancers: an unholy nexus. Carcinogenesis *37*, 223–232.

Park, S.-H., Wang, X., Liu, R., Lam, K.S., and Weiss, R.H. (2008). High throughput screening of a small molecule one-bead-one-compound combinatorial library to identify attenuators of p21 as chemotherapy sensitizers. Cancer Biol. Ther. *7*, 2015–2022.

Pece, S., Tosoni, D., Confalonieri, S., Mazzarol, G., Vecchi, M., Ronzoni, S., Bernard, L., Viale, G., Pelicci, P.G., and Di Fiore, P.P. (2010). Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell *140*, 62–73.

Perez, R.E., Shen, H., Duan, L., Kim, R.H., Kim, T., Park, N.-H., and Maki, C.G. (2016). Modeling the etiology of p53-mutated cancer cells*. J. Biol. Chem. *291*, 10131–10147.

Phalke, S., Mzoughi, S., Bezzi, M., Jennifer, N., Mok, W.C., Low, D.H.P., Thike, A.A., Kuznetsov, V.A., Tan, P.H., Voorhoeve, P.M., and Guccione, E. (2012). p53-Independent regulation of p21Waf1/Cip1 expression and senescence by PRMT6. Nucleic Acids Res. *40*, 9534–9542.

Polato, F., Rusconi, P., Zangrossi, S., Morelli, F., Boeri, M., Musi, A., Marchini, S., Castiglioni, V., Scanziani, E., Torri, V., and Broggini, M. (2014). DRAGO (KIAA0247), a new DNA damage-responsive, p53-inducible gene that cooperates with p53 as oncosuppressor. [Corrected]. J. Natl. Cancer Inst. *106*, dju053.

Potten, C.S. (1977). Extreme sensitivity of some intestinal crypt cells to X and γ irradiation. Nature *269*, 518–521.

Powell, A.E., Wang, Y., Li, Y., Poulin, E.J., Means, A.L., Washington, M.K., Higginbotham, J.N., Juchheim, A., Prasad, N., Levy, S.E., et al. (2012). The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. Cell *149*, 146–158.

Puig, I., Tenbaum, S.P., Chicote, I., Arqués, O., Martínez-Quintanilla, J., Cuesta-Borrás, E., Ramírez, L., Gonzalo, P., Soto, A., Aguilar, S., et al. (2018). TET2 controls chemoresistant slow-cycling cancer cell survival and tumor recurrence. J. Clin. Invest. *128*, 3887–3905.

Qiu, H., Zhang, L., Ren, C., Zeng, Z., Wu, W., Luo, H., Zhou, Z., and Xu, R. (2013). Targeting CDH17 suppresses tumor progression in gastric cancer by downregulating Wnt/β-catenin signaling. PLoS One *8*, e56959.

Regan, J.L. (2018). Cell fate in colon cancer stem cells: to GLI or not to GLI? Mol. Cell Oncol. *5*, e1445940.

Regan, J.L., Schumacher, D., Staudte, S., Steffen, A., Haybaeck, J., Keilholz, U., Schweiger, C., Golob-Schwarzl, N., Mumberg, D., Henderson, D., et al. (2017). Non-canonical hedgehog signaling is a positive regulator of the WNT pathway and is required for the survival of colon cancer stem cells. Cell Rep. *21*, 2813–2828.

Regan, J.L., and Smalley, M.J. (2020). Integrating single-cell RNA-sequencing and functional assays to decipher mammary cell states and lineage hierarchies. NPJ Breast Cancer *6*, 32.

Reggi, E., and Diviani, D. (2017). The role of A-kinase anchoring proteins in cancer development. Cell Signal. *40*, 143–155.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105–111.

Ricci-Vitiani, L., Lombardi, D.G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., and De Maria, R. (2007). Identification and expansion of human colon-cancer-initiating cells. Nature *445*, 111–115.

Roerink, S.F., Sasaki, N., Lee-Six, H., Young, M.D., Alexandrov, L.B., Behjati, S., Mitchell, T.J., Grossmann, S., Lightfoot, H., Egan, D.A., et al. (2018). Intra-tumour diversification in colorectal cancer at the single-cell level. Nature *556*, 457–462.

Roesch, A., Fukunaga-Kalabis, M., Schmidt, E.C., Zabierowski, S.E., Brafford, P.A., Vultur, A., Basu, D., Gimotty, P., Vogt, T., and Herlyn, M. (2010). A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. Cell *141*, 583–594.

Rogel, A., Popliker, M., Webb, C.G., and Oren, M. (1985). p53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos, and tumors. Molecular and Cellular Biology *5*, 2851 LP–2855.

Rouillard, A.D., Gundersen, G.W., Fernandez, N.F., Wang, Z., Monteiro, C.D., McDermott, M.G., and Ma'ayan, A. (2016). The harmonizome: a collection of processed datasets gathered to serve and mine knowledge about genes and proteins. Database *2016*. https://doi.org/10.1093/database/baw100.

Rubio, C.A. (2008). Arrest of cell proliferation in budding tumor cells ahead of the invading edge of colonic carcinomas. A preliminary report. Anticancer Res. *28*, 2417–2420.

Sachs, N., de Ligt, J., Kopper, O., Gogola, E., Bounova, G., Weeber, F., Balgobind, A.V., Wind, K., Gracanin, A., Begthel, H., et al. (2018). A living biobank of breast cancer organoids captures disease heterogeneity. Cell *172*, 373–386.e10.

Saha, S.K., Choi, H.Y., Kim, B.W., Dayem, A.A., Yang, G.-M., Kim, K.S., Yin, Y.F., and Cho, S.-G. (2017). KRT19 directly interacts with β-catenin/RAC1 complex to regulate NUMB-dependent NOTCH signaling pathway and breast cancer properties. Oncogene *36*, 332–349.

Saito, Y., Uchida, N., Tanaka, S., Suzuki, N., Tomizawa-Murasawa, M., Sone, A., Najima, Y., Takagi, S., Aoki, Y., Wake, A., et al. (2010). Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. Nat. Biotechnol. *28*, 275–280.

Sánchez-Tilló, E., de Barrios, O., Siles, L., Cuatrecasas, M., Castells, A., and Postigo, A. (2011). β-catenin/TCF4 complex induces the epithelial-to-mesenchymal transition (EMT)-activator ZEB1 to regulate tumor invasiveness. Proc. Natl. Acad. Sci. *108*, 19204 LP–19209.

Sato, M., Kawamata, H., Harada, K., Nakashiro, K., Ikeda, Y., Gohda, H., Yoshida, H., Nishida, T., Ono, K., Kinoshita, M., and Adachi, M. (1997). Induction of cyclin-dependent kinase inhibitor, p21WAF1, by treatment with 3,4-dihydro-6-[4-(3,4)-dimethoxybenzoyl)-1-piperazinyl]-2(1H)-quinoline (vesnarinone) in a human salivary cancer cell line with mutant p53 gene. Cancer Lett. *112*, 181–189.

Sato, T., Stange, D.E., Ferrante, M., Vries, R.G.J., van Es, J.H., van den Brink, S., van Houdt, W.J., Pronk, A., van Gorp, J., Siersema, P.D., and Clevers, H. (2011). Long-term expansion of epithelial organoids from

human colon, Adenoma, adenocarcinoma, and Barrett's Epithelium. Gastroenterology *141*, 1762–1772.

Schepeler, T., Mansilla, F., Christensen, L.L., Orntoft, T.F., and Andersen, C.L. (2007). Clusterin expression can be modulated by changes in TCF1-mediated Wnt signaling. J. Mol. Signal. *2*, 6.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods *9*, 671–675. https://doi.org/10.1038/nmeth.2089.

Schütte, M., Risch, T., Abdavi-Azar, N., Boehnke, K., Schumacher, D., Keil, M., Yildiriman, R., Jandrasits, C., Borodina, T., Amstislavskiy, V., et al. (2017). Molecular dissection of colorectal cancer in pre-clinical models identifies biomarkers predicting sensitivity to EGFR inhibitors. Nat. Commun. *8*. https://doi.org/10.1038/ncomms14262.

Sell, S. (2004). Stem cell origin of cancer and differentiation therapy *51*, 1–28.

Shackleton, M., Quintana, E., Fearon, E.R., and Morrison, S.J. (2009). Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell *138*, 822–829.

Shannon, J.M., and Pitelka, D.R. (1981). The influence of cell shape on the induction of functional differentiation in mouse mammary cells in vitro. In Vitro *17*, 1016–1028.

Shen, S., Vagner, S., and Robert, C. (2020). Persistent cancer cells: the deadly survivors. Cell *183*, 860–874.

Shimura, T., Takenaka, Y., Tsutsumi, S., Hogan, V., Kikuchi, A., and Raz, A. (2004). Galectin-3, a novel binding partner of β-catenin. Cancer Res. *64*, 6363 LP–6367.

Smith, G.H. (2005). Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands. Development *132*, 681–687.

Soutto, M., Chen, Z., Saleh, M.A., Katsha, A., Zhu, S., Zaika, A., Belkhiri, A., and El-Rifai, W. (2014). TFF1 activates p53 through down-regulation of miR-504 in gastric cancer. Oncotarget *5*, 5663–5673.

Stewart, R.L., and O'Connor, K.L. (2015). Clinical significance of the integrin α6β4 in human malignancies. Lab. Invest. *95*, 976–986.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., and Mesirov, J.P. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U S A *10*, 15545–15550.

Takeda, N., Jain, R., LeBoeuf, M.R., Wang, Q., Lu, M.M., and Epstein, J.A. (2011). Interconversion between intestinal stem cell populations in distinct niches. Science *334*, 1420–1424.

Takeishi, S., Matsumoto, A., Onoyama, I., Naka, K., Hirao, A., and Nakayama, K.I. (2013). Ablation of Fbxw7 eliminates leukemia-initiating cells by preventing quiescence. Cancer Cell *23*, 347–361.

The Gene Ontology Consortium (2018). The gene ontology resource: 20 years and still GOing strong. Nucleic Acids Res. *47*, D330–D338.

Thim, L. (1997). Trefoil peptides: from structure to function. Cell Mol. Life Sci. *53*, 888–903.

Tian, H., Wittmack, E.K., and Jorgensen, T.J. (2000). p21WAF1/CIP1 antisense therapy radiosensitizes human colon cancer by converting growth arrest to apoptosis. Cancer Res. *60*, 679–684.

Tiriac, H., Belleau, P., Engle, D.D., Plenker, D., Deschênes, A., Somerville, T.D.D., Froeling, F.E.M., Burkhart, R.A., Denroche, R.E., Jang, G.-H., et al. (2018). Organoid profiling identifies common responders to chemotherapy in pancreatic cancer. Cancer Discovery *8*, 1112–1129.

Tosoni, D., Zecchini, S., Coazzoli, M., Colaluca, I., Mazzarol, G., Rubio, A., Caccia, M., Villa, E., Zilian, O., Di Fiore, P.P., and Pece, S. (2015). The Numb/p53 circuitry couples replicative self-renewal and tumor suppression in mammary epithelial cells. J. Cell Biol. *211*, 845–862.

Tschaharganeh, D.F., Xue, W., Calvisi, D.F., Evert, M., Michurina, T.V., Dow, L.E., Banito, A., Katz, S.F., Kastenhuber, E.R., Weissmueller, S., et al. (2014). p53-Dependent nestin regulation links tumor suppression to cellular plasticity in liver cancer. Cell *158*, 579–592.

Tu, S.P., Chi, A.L., Ai, W., Takaishi, S., Dubeykovskaya, Z., Quante, M., Fox, J.G., and Wang, T.C. (2009). p53 inhibition of AP1-dependent TFF2 expression induces apoptosis and inhibits cell migration in gastric cancer cells, American Journal of Physiology-Gastrointestinal and Liver Physiology. Am. Physiol. Soc. *297*, G385–G396.

Ungerleider, N.A., Rao, S.G., Shahbandi, A., Yee, D., Niu, T., Frey, W.D., and Jackson, J.G. (2018). Breast cancer survival predicted by TP53 mutation status differs markedly depending on treatment. Breast Cancer Res. *20*, 115.

Vanner, R.J., Remke, M., Gallo, M., Selvadurai, H.J., Coutinho, F., Lee, L., Kushida, M., Head, R., Morrissy, S., Zhu, X., et al. (2014). Quiescent $Sox2^{+}$ cells drive hierarchical growth and relapse in sonic hedgehog subgroup medulloblastoma. Cancer Cell *26*, 33–47.

Viale, A., De Franco, F., Orleth, A., Cambiaghi, V., Giuliani, V., Bossi, D., Ronchini, C., Ronzoni, S., Muradore, I., Monestiroli, S., et al. (2009). Cell-cycle restriction limits DNA damage and maintains self-renewal of leukaemia stem cells. Nature *457*, 51–56.

Vlachogiannis, G., Hedayat, S., Vatsiou, A., Jamin, Y., Fernández-Mateos, J., Khan, K., Lampis, A., Eason, K., Huntingford, I., Burke, R., et al. (2018). Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. Sci. *359*, 920–926.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307–310.

Vousden, K.H., and Lane, D.P. (2007). p53 in health and disease. Nat. Rev. Mol. Cell Biol. *8*, 275–283.

Wade Harper, J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805–816.

Wahl, G.M., and Spike, B.T. (2017). Cell state plasticity, stem cells, EMT, and the generation of intra-tumoral heterogeneity. NPJ Breast Cancer *3*, 14.

Wang, H., Luo, K., Tan, L.-Z., Ren, B.-G., Gu, L.-Q., Michalopoulos, G., Luo, J.-H., and Yu, Y.P. (2012). p53-induced gene 3 mediates cell death induced by glutathione peroxidase 3, the Journal of Biological Chemistry. Am. Soc. Biochem. Mol. Biol. *287*, 16890–16902.

Wang, Y., Shek, F.H., Wong, K.F., Liu, L.X., Zhang, X.Q., Yuan, Y., Khin, E., Hu, M., Wang, J.H., Poon, R.T.P., et al. (2013). Anti-Cadherin-17 antibody modulates beta-catenin signaling and tumorigenicity of hepatocellular carcinoma. PLoS One *8*, e72386.

Wang, H., Song, X., Huang, Q., Xu, T., Yun, D., Wang, Y., Hu, L., Yan, Y., Chen, H., Lu, D., and Chen, J. (2019). LGALS3 promotes treatment resistance in glioblastoma and is associated with tumor risk and prognosis. Cancer Epidemiol. Biomarkers Prevent. *28*, 760 LP–769.

Webster, M.R., Fane, M.E., Alicea, G.M., Basu, S., Kossenkov, A.V., Marino, G.E., Douglass, S.M., Kaur, A., Ecker, B.L., Gnanapradeepan, K., et al. (2020). Paradoxical role for wild-type p53 in driving therapy resistance in melanoma. Mol. Cell *77*, 633–644.e5.

Weeber, F., van de Wetering, M., Hoogstraat, M., Dijkstra, K.K., Krijgsman, O., Kuilman, T., Gadellaa-van Hooijdonk, C.G.M., van der Velden, D.L., Peeper, D.S., Cuppen, E.P.J.G., et al. (2015). Preserved genetic diversity in organoids cultured from biopsies of human colorectal cancer metastases, Proceedings of the National Academy of Sciences of the United States of America. Natl. Acad. Sci. U S A *112*, 13308–13311.

Weiss, R.H. (2003). $p21^{Waf1/Cip1}$ as a therapeutic target in breast and other cancers. Cancer Cell *4*, 425–429.

Weiswald, L.-B., Bellet, D., and Dangles-Marie, V. (2015). Spherical cancer models in tumor biology. Neoplasia *17*, 1–15.

Wensink, G.E., Elias, S.G., Mullenders, J., Koopman, M., Boj, S.F., Kranenburg, O.W., and Roodhart, J.M.L. (2021). Patient-derived organoids as a predictive biomarker for treatment response in cancer patients. NPJ Precision Oncol. *5*, 30.

Wettersten, H.I., Hee Hwang, S., Li, C., Shiu, E.Y., Wecksler, A.T., Hammock, B.D., and Weiss, R.H. (2013). A novel p21 attenuator which is structurally related to sorafenib, Cancer Biology & Therapy. Landes Biosci. *14*, 278–285.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E., et al. (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118–1129.

Xia, X., Ma, Q., Li, X., Ji, T., Chen, P., Xu, H., Li, K., Fang, Y., Weng, D., Weng, Y., et al. (2011). Cytoplasmic p21 is a potential predictor for cisplatin sensitivity in ovarian cancer. BMC Cancer *11*, 399.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. Nature *366*, 701–704.

Xu, J., Chen, Y., Huo, D., Khramtsov, A., Khramtsova, G., Zhang, C., Goss, K.H., and Olopade, O.I. (2016). β-catenin regulates c-Myc and CDKN1A expression in breast cancer cells. Mol. Carcinogenesis *55*, 431–439.

Yan, K.S., Chia, L.A., Li, X., Ootani, A., Su, J., Lee, J.Y., Su, N., Lee, J.Y., Su, N., Luo, Y., et al. (2012). The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. Proc. Natl. Acad. Sci. U S A *109*, 466 LP–471.

Yao, H., Ashihara, E., Strovel, J.W., Nakagawa, Y., Kuroda, J., Nagao, R., Tanaka, R., Yokota, A., Takeuchi, M., Hayashi, Y., et al. (2011). AV-65, a novel Wnt/β-catenin signal inhibitor, successfully suppresses progression of multiple myeloma in a mouse model. Blood Cancer J. *1*, e43.

Yao, Y., Xu, X., Yang, L., Zhu, J., Wan, J., Shen, L., Xia, F., Fu, G., Deng, Y., Pan, M., et al. (2020). Patient-derived organoids predict chemoradiation responses of locally advanced rectal cancer. Cell Stem Cell *26*, 17–26.e6.

Zeuner, A., Francescangeli, F., Contavalli, P., Zapparelli, G., Apuzzo, T., Eramo, A., Baiocchi, M., De Angelis, M.L., Biffoni, M., Sette, G., et al. (2014). Elimination of quiescent/slow-proliferating cancer stem cells by Bcl-XL inhibition in non-small cell lung cancer. Cell Death Differ. *21*, 1877–1888.

Zhang, F., Kumano, M., Beraldi, E., Fazli, L., Du, C., Moore, S., Sorensen, P., Zoubeidi, A., and Gleave, M.E. (2014). Clusterin facilitates stress-induced lipidation of LC3 and autophagosome biogenesis to enhance cancer cell survival. Nat. Commun. *5*, 5775.

Zhang, Q., Song, Y., Chen, W., Wang, X., Miao, Z., Cao, L., Li, F., and Wang, G. (2015). By recruiting HDAC1, MORC2 suppresses p21 Waf1/Cip1 in gastric cancer. Oncotarget *6*, 16461–16470.

Zhao, Z., Zuber, J., Diaz-Flores, E., Lintault, L., Kogan, S.C., Shannon, K., and Lowe, S.W. (2010). p53 loss promotes acute myeloid leukemia by enabling aberrant self-renewal. Genes Dev.t *24*, 1389–1402.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-α-Tubulin	Sigma	T5168; RRID: AB_477579
Alexa Fluor® 647 Phalloidin	Thermo Fisher	#A22287; RRID: AB_2620155
Biological samples		
Patient-derived organoids (PDOs)	Charité Universitätsmedizin Berlin, Germany	OncoTrack
Chemicals, peptides, and recombinant proteins		
CellTracker™ CM-DiI fluorescent dye	Thermo Fisher	C7000
PKH26 Red Fluorescent Cell Linker	Sigma-Aldrich	PKH26GL
Critical commercial assays		
Click-iT EdU assay	Invitrogen	#C10337
RNeasy Mini Plus RNA extraction kit	Qiagen	ID:74136
Sensiscript RT kit	Qiagen	ID:205213
Ovation RNA-Seq System V2	NuGEN	7102-A01
Ultralow V2 Library System	NuGEN	0344NB-A01
Deposited data		
Array data	This paper; www.ebi.ac.uk/arrayexpress	E-MTAB-8924
Oligonucleotides		
CDKN1A MISSION shRNA Lentiviral Transduction Particles: TRCN0000040123, TRCN0000287021, TRCN0000287091	Sigma-Aldrich	SHCLNV-NM_000389
CDKN1A (Hs00355782_m1)	Thermo Fisher	#4331182
GAPDH (Hs02758991_g1)	Thermo Fisher	#4331182
Software and algorithms		
ELDA software	Hu and Smith, 2009	http://bioinf.wehi.edu.au/software/elda/index.html
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/
DESeq2	Love et al., 2014	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
STAR aligner (version 2.4.2a).	Dobin et al., 2013	https://github.com/alexdobin/STAR
Kaplan-Meier Plotter	Nagy et al., 2018	www.kmplot.com/analysis
Gene Ontology Resource	Ashburner et al., 2000	(www.geneontology.org)
GSEA software	Liberzon et al., 2015; Subramanian et al., 2005	https://www.gsea-msigdb.org/gsea/index.jsp
Prism 8.0	GraphPad	https://www.graphpad.com/scientific-software/prism/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact Joseph L. Regan (joseph.regan@charite.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The accession number for the RNA-sequencing data reported in this paper is ArrayExpress: E-MTAB-8924.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human tissue samples

Tumor material was obtained with informed consent from colon cancer patients (3 male and 3 female; detailed in Table S1) under approval from the local Institutional Review Board of Charité University Medicine (Charité Ethics Cie: Charitéplatz 1, 10117 Berlin, Germany) (EA 1/069/11) and the ethics committee of the Medical University of Graz and the ethics committee of the St John of God Hospital Graz (23-015 ex 10/11). Tumor staging was carried out by experienced and board-certified pathologists (Table S1). Age of subjects can be inquired through info@oncotrack.eu.

Patient-derived organoids

Cancer organoid cultures were established and propagated as previously described(Sato et al., 2011; Schütte et al., 2017). Briefly, resected tumor samples were enzymatically digested with Collagenase IV (C9407, Sigma-Aldrich), DNaseI (A3778,0050, AppliChem) and Dispase (07913, Stem Cell Technologies) at 37°C for 60 min. Suspensions were washed, filtered, and depleted of red blood cells using Red Blood Cell Lysis Solution (00-4333-57, Invitrogen). Cells were mixed with phenol-red free growth factor-reduced Matrigel (356231, Corning) and seeded into 24-well plates. Solidified droplets were overlaid with culture medium consisting of Advanced DMEM/F12 (12634-010, Gibco) supplemented with 1% penicillin/streptomycin, 1% HEPES buffer (1064859, Fisher Scientific), 1% Glutamax, 1x N2 (#17502-048, Invitrogen), 1x B27 (17504-044, Invitrogen), 50 ng/ml EGF (E9644, Sigma), and 1mM N-acetylcysteine (A9165-5G, Sigma) and maintained at 37°C. Organoids were released from Matrigel and passaged by adding 5 ml Advanced DMEM/F12 followed by centrifugation and digestion of pellets with TrypLE Express (12604-013, Gibco).

Xenotransplantation

Housing and handling of animals followed European and German Guidelines for Laboratory Animal Welfare. Animal experiments were conducted in accordance with animal welfare law, approved by local authorities, and in accordance with the ethical guidelines of Bayer AG. PDOs were processed to single cells and labelled with PKH26 (PKH26GL, Sigma-Aldrich) following manufacturer's instructions and DAPI (to exclude dead cells). $PKH26^{Positive}$ $DAPI^{Negative}$ cells were collected by FACS and immediately transplanted by injecting subcutaneously in PBS and Matrigel (1:1 ratio) into female 8 – 10-week-old $nude^{-/-}$ mice at 1000 cells per animal. The purity of the sorted $PKH26^{Positive}$ cell population was confirmed by post-sort FACS analysis.

METHOD DETAILS

Cell cycle analysis and colony forming assays

Cell cycle analysis was carried using the Click-iT EdU assay (Invitrogen, #C10337) and assessed by FACS on a BD LSR II analyzer. For colony forming assays, PDOs were processed to single cells and labelled with CellTracker™ CM-DiI fluorescent dye (C7000, Thermo Fisher) or PKH26 following manufacturer's instructions and DAPI (to exclude dead cells). $PKH26^{Positive}$ $DAPI^{Negative}$ (live) cells were sorted by FACS (BD FACS Aria II) into Matrigel culture. After 12 days, PDOs were once again processed to single cells and sorted by FACS, seeding $PKH26^{Positive}$ $DAPI^{Negative}$ cells and $PKH26^{Negative}$ $DAPI^{Negative}$ cells separately at limiting dilution into 96-well adherent Matrigel and 384-well non-adherent ultra-low attachment plates at a frequency of 100 and 1 cell per well, respectively. The purity of the sorted $PKH26^{Positive}$ cell population was confirmed by post-sort FACS analysis. PDO sizes were determined by ImageJ software analysis. Ultra-low attachment wells containing spheroids were counted and used to calculate the CSC frequency using ELDA software (http://bioinf.wehi.edu.au/software/elda/index.html)(Hu and Smith, 2009).

Immunofluorescence staining

Tumors were fixed in 4% paraformaldehyde overnight and cryopreserved in OCT compound. Immunohistochemistry of frozen sections was carried out via standard techniques with α-Tubulin (T5168, mouse monoclonal, Sigma; diluted 1:1000) and a secondary conjugated antibody at room temperature for 2 hours. For immunofluorescence imaging of PDOs, cultures were fixed in 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton X-100 for 30 min and blocked in phosphate-buffered saline (PBS) with 10% bovine serum albumin (BSA). F-actin was stained with Alexa Fluor® 647 Phalloidin

(#A22287, Thermo Fisher; diluted 1:20) for 30 min at room temperature. Nuclei were counterstained with DAPI. Negative controls were performed using the same protocol with substitution of the primary antibody with IgG-matched controls. Cancer organoids were then transferred to microscope slides for examination using a Zeiss LSM 700 Laser Scanning Microscope.

RNA sequencing

$PKH26^{Negative}$ $DAPI^{Negative}$ and $PKH26^{Positive}$ $DAPI^{Negative}$ cells from four different biological replicates were isolated by FACS. Cells were lysed in RLT buffer and processed for RNA using the RNeasy Mini Plus RNA extraction kit (Qiagen). Samples were processed using NuGEN's Ovation RNA-Seq System V2 and Ultralow V2 Library System and sequenced on an Illumina HiSeq 2500 machine as 2x125nt paired-end reads. The raw data in Fastq format were checked for sample quality using our internal NGS QC pipeline. Reads were mapped to the human reference genome (assembly hg19) using the STAR aligner (version 2.4.2a) (Dobin et al., 2013). Total read counts per gene were computed using the program featureCounts (version 1.4.6-p2) in the subread package, with the gene annotation taken from Gencode (version 19). Variance-stabilizing transformation from the Bioconductor package DESeq2 (Love et al., 2014) was used for normalization and differential-expression analysis.

Viral transduction

Cells were seeded in 100 µl volumes of antibiotic free culture media at 1.0 $x10^5$ cells per well in ultra-low attachment 96-well plates. Control and shRNA lentiviruses were purchased from Sigma-Aldrich (Table S2). Viral particles were added at a multiplicity of infection of 1. Cells were transduced for up to 96 h or until GFP positive cells were observed before being embedded in Matrigel for the establishment of lentiviral transduced cancer organoid cultures. Puromycin (2 µg/ml) was used to keep the cells under selection.

Gene expression analysis

For quantitative real-time RT-PCR analysis RNA was isolated using the RNeasy Mini Plus RNA extraction kit (Qiagen). cDNA synthesis was carried out using a Sensiscript RT kit (Qiagen). RNA was transcribed into cDNA using an oligo dTn primer (Promega) per reaction. Gene expression analysis was performed using TaqMan® Gene Expression Assays (Applied Biosystems) (Table S3) on an ABI Prism 7900HT sequence detection system (Applied Biosystems). GAPDH was used as an endogenous control and results were calculated using the Δ-ΔCt method. Data were expressed as the mean fold gene expression difference in three independently isolated cell preparations over a comparator sample with 95% confidence intervals. Survival curves were generated using the Kaplan-Meier Plotter (www.kmplot.com/analysis) (Nagy et al., 2018). Gene ontology enrichment analysis was carried out using the Gene Ontology Resource (www.geneontology.org)(Ashburner et al., 2000; The Gene Ontology Consortium, 2018).

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 8.0 was used for data analysis and imaging. All data are presented as the mean ± SD, followed by determining significant differences using the two-tailed t test. Significance of RT-PCR data was determined by inspection of error bars as described by Cumming et al. (2007) (Cumming et al., 2007). Gene set enrichment analysis was carried out using pre-ranked feature of the Broad Institute GSEA software version 2 using MSigDB v5.1 gene sets (Liberzon et al., 2015; Subramanian et al., 2005). The ranking list was derived from the fold changes calculated from the differential gene expression calculation and nominal p-values. P-values <0.05 were considered as statistically significant. The representation factor and the associated probability of finding an overlap were calculated using http://nemates.org/MA/progs/representation.stats.html. Survival curves were generated using the Kaplan-Meier Plotter (www.kmplot.com/analysis)(Nagy et al., 2018). For the final list of significant genes, False Discovery Rate (FDR) was computed using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).