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Therapeutic inhibition of PI3K/AKT and WNT/ β -catenin pathways in colorectal cancer

Oriol Arqués Casamitjana

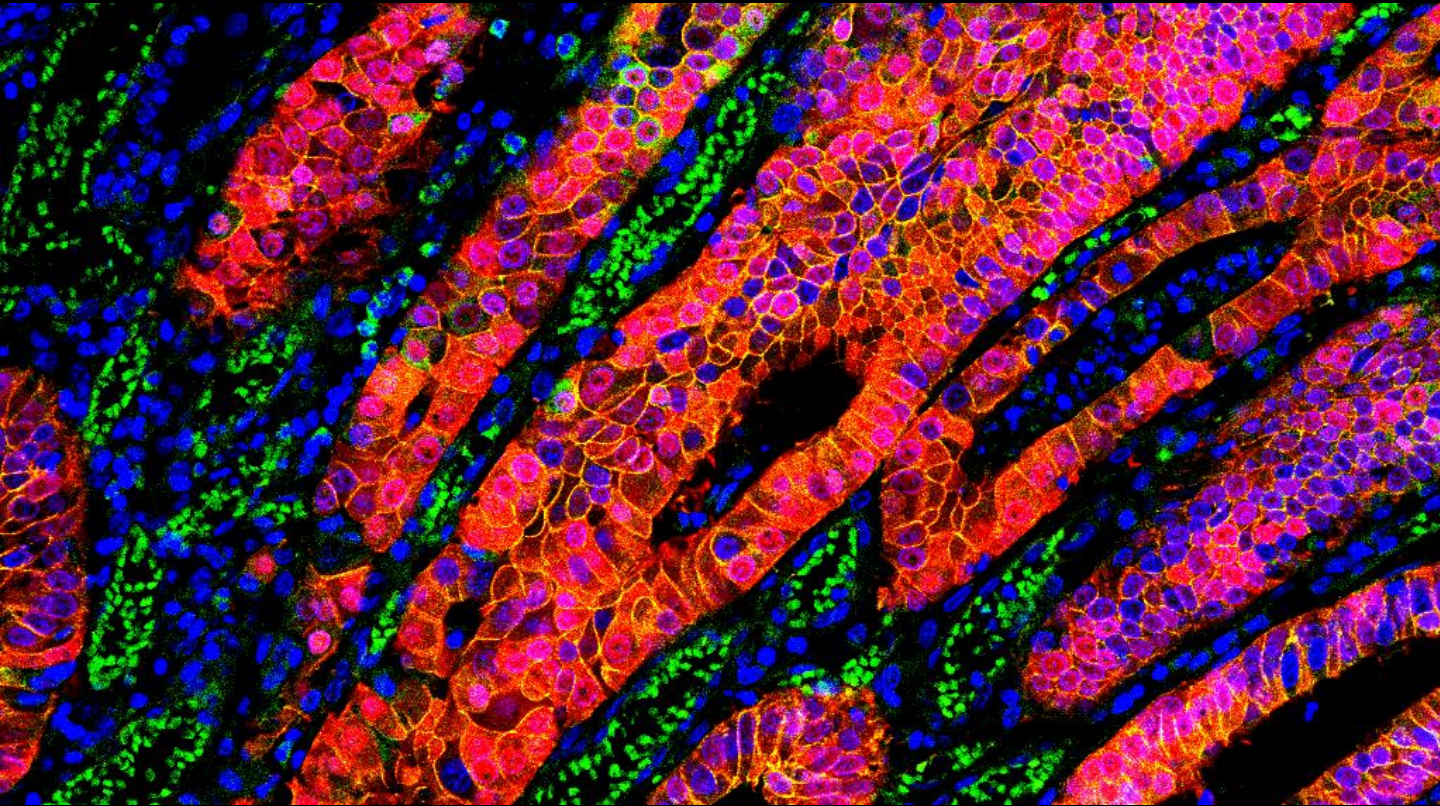
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DOCTORAL THESIS

THERAPEUTIC INHIBITION OF PI3K/AKT AND WNT/ β -CATENIN PATHWAYS IN COLORECTAL CANCER



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THERAPEUTIC INHIBITION OF PI3K/AKT AND WNT/ β -CATENIN PATHWAYS IN COLORECTAL CANCER

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Per optar al grau de Doctor per la Universitat de Barcelona

Treball realitzat sota la direcció del Dr. Héctor García Palmer i tutelat pel Dr. Joan Seoane Suárez en el laboratori de Cèl·lules Mare i Càncer dins el programa de Recerca Translacional de l'Institut d'Oncologia de la Vall d'Hebron (VHIO).

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SUMMARY

Colorectal cancer (CRC) is a leading cause of death worldwide, especially in the late stages of the disease when relapse and metastasis occur. Despite the improvements achieved recently in terms of treatment and diagnosis, current therapies for advanced CRC are still not curative. Among the new therapeutic strategies evaluated in the clinic, PI3K/AKT signaling pathway inhibitors are widely studied and are promising candidates. However, in the case of CRC, resistance to such drugs is very frequent. Alterations in the Wnt signaling pathway are thought to be one of the earliest steps in the initiation of CRC. This pathway controls the stability of its main effector, β -catenin, which has been shown to interact with FOXO3a, a tumor suppressor controlled by the PI3K/AKT signaling pathway. In this work we studied the molecular crosstalk between both factors and its implications in CRC. We showed that β -catenin is able to induce resistance to FOXO3a-mediated apoptosis by modulating its transcriptional activity. Instead of promoting apoptosis, simultaneous activation of both factors induced cell cycle arrest and imposed a metastatic phenotype. Furthermore, FOXO3a activation by PI3K or AKT inhibitors induced metastasis of tumors with high nuclear β -catenin content. Such activated β -catenin also conferred resistance to these treatments in CRC patient-derived xenografts (PDXs). We proved that inhibition of the Wnt pathway, using tankyrase inhibitors, could reduce nuclear β -catenin amounts and sensitize CRC cells and PDX models to the treatment with PI3K and AKT inhibitors. In accordance, we have shown that nuclear β -catenin could be used as a biomarker of resistance to PI3K/AKT pathway inhibitors while FOXO3a could constitute a biomarker of sensitivity to Wnt/tankyrase inhibitors. Altogether, our results could help improving the molecular selection of CRC patients and their stratification for the treatment with PI3K/AKT and Wnt pathway inhibitors.

RESUM

El càncer colorectal (CRC) és una malaltia amb una gran mortalitat a nivell mundial, especialment en estadis avançats en els quals apareixen les recaigudes i/o metàstasis. Tot i les millores aconseguides en els darrers anys a nivell diagnòstic les teràpies actuals no curen la malaltia. Entre les noves teràpies experimentals que s'estan avaluant en la clínica, els inhibidors de la via de PI3K/AKT són uns candidats molt prometedors. Malgrat això, en el cas del CRC, la resistència a aquests fàrmacs és molt freqüent. S'ha descrit que la principal causa d'inici d'aquesta malaltia són alteracions en la via de Wnt. Aquesta via de senyalització controla l'estabilitat del seu principal efector, la β -catenina, proteïna amb capacitat d'interaccionar amb FOXO3a, un supressor tumoral controlat per la via de PI3K/AKT. En aquesta tesi hem estudiat les implicacions moleculars d'aquesta interacció en el context del CRC. Hem mostrat que la β -catenina és capaç de causar resistència a l'apoptosi induïda per FOXO3a modificant la seva activitat transcripcional. En comptes de promoure apoptosi, l'activació simultània dels dos factors indueix parada del cicle cel·lular i promou un fenotip metastàtic. A més, l'activació de FOXO3a mitjançant l'ús d'inhibidors de PI3K o AKT promou un fenotip metastàtic en els tumors que tenen un alt contingut de β -catenina nuclear. Aquesta β -catenina activa també confereix resistència a aquests tractaments en xenògrafs derivats de pacients de CRC (PDXs). Hem demostrat que la inhibició de la via de Wnt, utilitzant inhibidors de tankyrases, pot reduir els nivells nuclears de β -catenina i sensibilitzar les línies cel·lulars i els PDXs de CRC als tractaments amb inhibidors de PI3K i AKT. D'aquesta manera hem demostrat que la β -catenina nuclear pot ser utilitzada com a biomarcador de resistència als inhibidors de la via de PI3K/AKT mentre que FOXO3a pot constituir un biomarcador de sensibilitat als inhibidors de la via de Wnt. En conjunt els nostres resultats poden ajudar millorar la selecció molecular dels pacients de CRC i la seva estratificació pel tractament amb inhibidors de les vies de PI3K/AKT i Wnt.

ABBREVIATIONS

AA	Amino acid
AKT	V-akt murine thymoma viral oncogene homolog
APC	Adenomatous polyposis coli
AR	Androgen receptor
BRAF	V-raf murine sarcoma viral oncogene homolog B1
CBP	CREB-binding protein
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CK1	Casein kinase 1
CPAP	Centrosomal P4.1-associated protein
CRC	Colorectal cancer
mCRC	Metastatic colorectal cancer
CTID	Clinical trial ID
DVL	Dishevelled
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FAP	Familial adenomatous polyposis
FOLFOX	5-Fluorouracil/leucovorin/oxaliplatin
FOLFIRI	5-Fluorouracil/leucovorin/irinotecan
5-FU	5-Fluorouracil
FOXO	Forkhead box O family of transcription factors
FZD	Frizzled
FFPE	Formalin-fixed paraffin-embedded
GEMM	Genetically engineered mouse model
GPCR	G-protein-coupled receptor
GSK3	Glycogen synthase kinase 3
GSV	GLUT4 storage vesicle
HNPCC	Hereditary non-polyposis colorectal cancer
HIF1α	Hypoxia Induced Factor 1 α
IHC	Immunohistochemistry
JNK	Jun N-terminal kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog

LEF	Lymphoid transcription factors
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
MAPK	Mitogen activate protein kinase
MMR	Mismatch repair
MSI	Microsatellite instability
NES	Nuclear export signal
NHEJ	Non-homologous end-joining
NLS	Nuclear localization signal
4OHT	4-hydroxy-tamoxifen
PARP	Poly (ADP-ribose) polymerase
PKD1	Phosphoinositide-dependent kinase 1
PDX	Patient-derived xenograft
PH	Pleckstrin homology domain
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4,5-biphosphate
PIP 3	Phosphatidylinositol-3,4,5-triphosphate
PORCN	O-acyltransferase enzyme Porcupine
S6K1	Ribosomal protein S6 kinase 1
PTEN	Phosphatase and tensin homologue
RTK	Receptor tyrosine kinase
TAZ	Transcriptional co-activator with PDZ binding motif
TCF	T-cell transcription factor
TGFβ	Transforming growth factor β
mTOR	Mammalian target of rapamycin
VEGF	Vascular endothelial growth factor
VDR	Vitamin D receptor
YAP	Yes-associated protein

INTRODUCTION

1. Colorectal cancer

1.1. Overview

Colon and rectum comprise the final part of the digestive tract. Their function is to reabsorb water, ions or unabsorbed nutrients and to eliminate waste from the body. In anatomical terms, the colon is divided in proximal colon (ascending and transverse colon) and the distal colon (descending and sigmoid colon). To perform their functions, their walls contain the four characteristic layers of the digestive system: mucosa, sub-mucosa, muscularis and serosa (American Cancer Society, 2013).

Colorectal cancer (CRC) is a leading cause of death related to cancer. It is the third most common cancer in men and the second in women, with 1.4 million new cases per year worldwide and 694.000 deaths in 2012 (Ferlay et al., 2015). Incidence clearly increases with age, being the median age at diagnosis 68 years for males and 72 years for females (Siegel et al., 2012). It is estimated that 25% of the patients present metastasis at the time of diagnosis and almost an additional 25% will develop metastasis at some point. Overall, around 50% of CRC patients are affected by metastasis, which is the main cause of mortality (Van Cutsem et al., 2014).

From a pathological point of view CRC tumors are classified in stages using the TNM system that evaluates tumor invasion into the wall of the organ (T), lymph node affection (N) and presence of metastases (M) (**Table 1**). The combination of these factors provides the basis for therapeutic decisions. In this sense, the prognosis of CRC depends on tumor location and stage at diagnosis. Overall, the management of the disease has improved in the past decades reaching a 5-year relative survival rate of 64.3%. Localized-low staged tumors present around 90% of 5-year relative survival rate, decreasing to 69.2% for regionally spread tumors and dropping dramatically to 11.7% for distant spread tumors (Brenner et al., 2014; Siegel et al., 2012).

1.2. Molecular pathogenesis

Cancer can be considered a genetic disease. CRC is a tumor type for which much is known about the driver mutations that promote tumor initiation and progression (Fearon, 2011). Its origin can be sporadic or be caused by inherited predispositions. Around a quarter of all CRC cases have a familial predisposition, but only 5% are caused by highly penetrant hereditary syndromes with well defined genetic defects. The most common autosomal dominant disorders are familial adenomatous polyposis (FAP), causing 1% of the cases, and hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, accounting for 2-4% of all CRC cases (Bogaert and Prenen, 2014; Castells et al., 2009; Kinzler and Vogelstein, 1996; Rustgi, 2007).

The majority of CRC tumors arise from benign adenomatous polyps in the mucosa. With time, cells acquire more genetic defects progressing to carcinomas, which finally can spread and metastasize. This process has been termed the adenoma-carcinoma sequence (Fearon, 2011).

Stage	T	N	M
0	Tis	N0	M0
I	T1-2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T2	N1	M0
	T1	N2a	M0
IIIB	T3-T4a	N1	M0
	T2-T3	N2a	M0
	T1-T2	N2b	M0
IIIC	T4a	N2a	M0
	T3-T4a	N2b	M0
	T4b	N1-N2	M0
IVA	T1-4	N0-2	M1a
IVB	T1-4	N0-2	M1b

Description

Primary tumor (T)	
Tis	Tumor restricted to mucosa (Carcinoma <i>in situ</i>)
T1	Tumor invades submucosa
T2	Tumor invades muscularis propia
T3	Tumor invades through the muscularis propia into the subserosa
T4a	Tumor invades the serosa
T4b	Tumor directly invades neighbouring tissues or organs
Regional lymph nodes (N)	
N0	No cancer cells in regional lymph nodes
N1	Cancer cells detectable in 1-3 regional lymph nodes
N2a	Cancer cells detectable in 4-6 regional lymph nodes
N2b	Cancer cells detectable in 7 or more regional lymph nodes
Distant metastases (M)	
M0	No distant metastases detected
M1a	Metastasis to 1 distant organ or distant lymph node
M1b	Metastasis to more than 1 distant organ or distant lymph nodes

Table 1. TNM staging system for colorectal cancer. Adapted from (Brenner et al., 2014; Wolpin and Mayer, 2008).

In 1990, Fearon and Vogelstein proposed a multistep genetic model in which sequential accumulation of genetic mutations (inactivation of tumor suppressor genes or activation of oncogenes) leads to a selective growth advantage for normal cells (Fearon and Vogelstein, 1990). As depicted in **Figure 1**, the inactivation of the *adenomatous polyposis coli* gene (*APC*) is thought to be one of the earliest events in the formation of the initial lesions. Further accumulation of mutations in key genes such as *KRAS*, *TP53*, *BRAF* or *PIK3CA* or chromosomal losses such as 18q, allow tumors to evolve from adenomas to carcinomas. Additional mutations promote tumors to advance to metastatic stages.

A subpopulation of tumors, affecting 15-20 % of all CRC patients, presents a different genetic profile characterized by the accumulation of thousands of mutations in coding genomic regions (Bogaert and Prenen, 2014; Fearon, 2011). Such genotype is consequence of initiating mutations or epigenetic silencing of genes of the mismatch repair (MMR) system (i.e. *MSH2*, *MSH3*, *MSH6* or *PMS2*). MMR system is responsible for the repair of replication errors that DNA polymerases preferentially make in repetitive sequences of the genome known as microsatellites. In fact, Perucho and colleagues described an additional model to that from Fearon and Vogelstein's, in which MMR-deficient tumors acquire a "mutator phenotype" due to the inability of tumor cells to repair such replication errors (Ionov et al., 1993). Consequently, such tumors develop microsatellite instability (MSI) and are known as hypermutant/MSI (Boland and Goel, 2010).

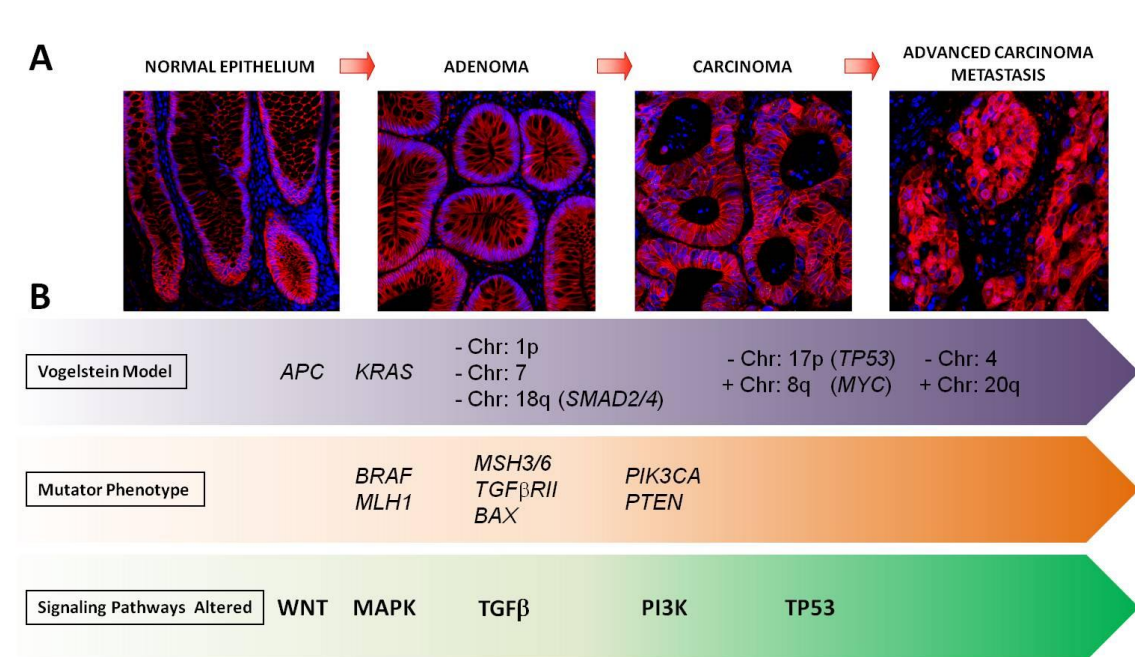


Figure 1. Colorectal cancer progression and most frequent molecular alterations. (A) The panel shows the Vogelstein model for CRC progression. For each stage, a representative β -catenin immunohistochemical staining shows the structure of the colon epithelia or the tumor tissue. (B) Schematic representation of the chronological order of appearance of genes and molecular signaling pathways most frequently altered in CRC. Adapted from (Danielsen et al., 2015).

Using next generation sequencing, the Cancer Genome Atlas Network (TCGA, 2012) and the Broad Institute (Lawrence et al., 2014) have widened our knowledge about the molecular alterations of CRC. Both studies confirmed that those key genes identified many years ago as drivers of the disease are mutated in a high percentage of CRC cases (i.e. *APC*, *KRAS* or *TP53*) (**Figure 2**). Interestingly, they also identified many other genes that appear to be mutated in significantly higher rate than expected. Among these they found genes such as *PIK3CA* and *BRAF* that, as it will be discussed below, may be important in the diagnosis and prognosis of the disease.

Those studies also helped confirming the existence of hypermutant tumors with hundreds of mutations. Interestingly the frequencies of driver mutations in key oncogenes and tumor suppressor genes were significantly different when compared with non-hypermutant tumors (TCGA, 2012). In addition, some mutated genes are distinctive of hypermutant tumors, what helps to better understand the genetic nature of such patient sub-population.

In CRC there are other genetic or epigenetic alterations, beyond oncogenic mutations, that are responsible for the defects observed in tumor cells. Those processes include chromosomal instability (CIN) and the CpG island methylator phenotype (CIMP). CIN is the most common cause of genomic instability, which can cause an imbalance in chromosome numbers and high frequency of loss-of-heterozygosity (LOH) (Pino and Chung, 2010). CIMP is defined by an increased gene silencing due to a global hypermethylation of CpG islands in promoter regions. One of the genes most commonly inhibited by CIMP is the member of the mismatch repair machinery MLH1. For this reason MSI and CIMP are often linked (Mojarad et al., 2013; Rashid et al., 2001; Sideris and Papagrigoriadis, 2014).

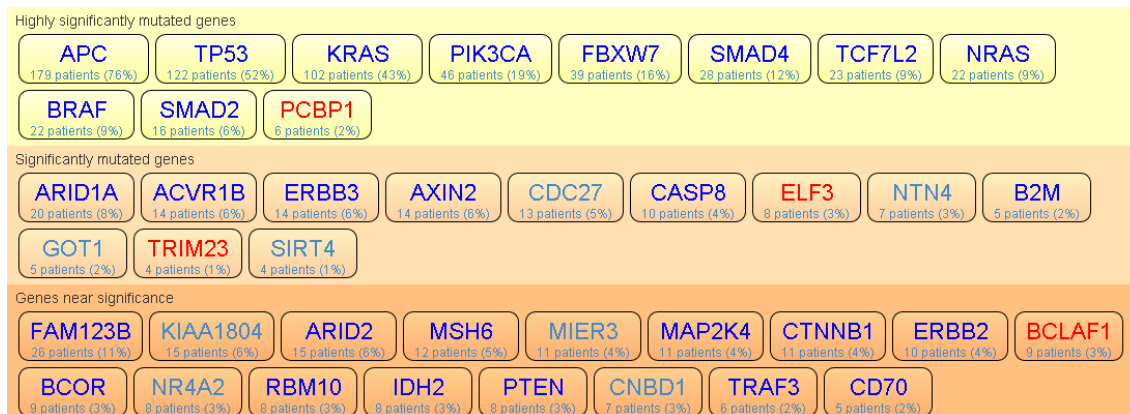


Figure 2. Genes significantly mutated in colorectal cancer. Genes mutated in CRC identified by whole-exome sequencing comparing tumor and normal samples. Color of gene names indicates whether the gene was previously known to be implicated in cancer (dark blue), it is a novel gene with clear connection to cancer (red) or it is an additional novel gene in colorectal cancer (light blue). Adapted from (Lawrence et al., 2014). Available online: http://www.tumorportal.org/tumor_types?type=CRC.

1.3. Clinical management of colorectal cancer

1.3.1. Conventional chemotherapeutic agents

As mentioned before, the outcome of the disease and therefore the therapeutic strategy against CRC mainly depends on tumor stage. The most effective treatment option is surgery, although is not always possible to perform. For patients with localized disease (stage I and II) (**Table 1**) surgical resection of the tumor and the adjacent area or total resection of the segment affected is sufficient. However, high-risk stage II and stage III patients require adjuvant chemotherapy (Van Cutsem et al., 2013). High-risk in stage II is determined by the presence of at least one of the following characteristics: lymph node sampling <12; poorly differentiated tumor; vascular, lymphatic or perineural invasion; tumor presentation with obstruction or tumor perforation; T4 stage (Labianca et al., 2013).

The backbone of the adjuvant chemotherapeutic regimens currently used in the clinic includes the use cytotoxic agents that affect DNA replication inducing cell death especially in dividing cells (**Table 2**). Until 1996, 5-Fluorouracil (5-FU) was the only therapeutic agent available for the treatment of CRC (Wolpin and Mayer, 2008). 5-FU inhibits thymidylate synthetase, the rate-limiting enzyme in pyrimidine nucleotide synthesis. Nowadays it is administered together with leucovorin, a reduced form of folic acid, that helps increasing the effect of 5-FU. Later, three other molecules were approved by the FDA: capecitabine, irinotecan and oxaliplatin, widening the therapeutic options available for CRC patients. Capecitabine is pro-drug that when absorbed is metabolized to 5-FU and can be administered orally, in contrast to 5-FU that is normally given intravenously (Meropol, 1998). Irinotecan is a topoisomerase I inhibitor that blocks DNA replication and induces apoptosis (Garcia-carbonero and Supko, 2002). Oxaliplatin is a platinum analog that forms DNA adducts blocking DNA replication and transcription, which finally induces apoptosis (Raymond et al., 1998).

Compared to 5-FU monotherapy, its combination with oxaliplatin (FOLFOX) or with irinotecan (FOLFIRI) increases survival of the patients. These combinatory treatments have a similar effects but present different toxicity profiles (Van Cutsem et al., 2014). In this sense, there are additional combinations available and the election can vary depending on the case. For that reason, consensus guidelines for the treatment of CRC were described by the European Society for Medical Oncology (ESMO) in 2012 (Schmoll et al., 2012).

1.3.2. Target-directed therapies for advanced and metastatic colorectal cancer

The introduction of the so-called target-directed or biological agents has caused a positive impact in the management of CRC. Studies show that combinatorial treatments of conventional chemotherapy with target-directed agents have increased the median overall survival (Brenner et al., 2014). However, although therapeutic options have augmented in recent years, surgery is still the most effective treatment. For this reason, in the cases of patients with unresectable metastases the main aim of the treatment should be reduction of tumor size to make them resectable.

Biological agents currently used in the clinic are drugs designed to target molecular processes essential for tumor growth such as angiogenesis or to block proliferative signals (**Table 2**) (Curtin, 2013). Monoclonal antibodies against vascular endothelial growth factor (VEGF), bevacizumab (Kim et al., 1993), or the epidermal growth factor receptor (EGFR), cetuximab (Goldstein et al., 1995) and panitumumab (Yang et al., 1999, 2001), are now regularly included in combination with chemotherapy (FOLFOX, FOLFIRI, etc.) for the treatment of advanced and metastatic colorectal cancer (mCRC). There are two drugs that are not antibodies, aflibercept (VEGF signaling inhibitor) (Holash et al., 2002) and regorafenib (Tyrosine kinase inhibitor) (Wilhelm et al., 2011), which are also currently used when initial treatments fail (Van Cutsem et al., 2014).

Table 2. Current chemotherapies for colorectal cancer		
Drug	Commercial Name	Description
Chemical agents		
5-Fluorouracil /Leucovorin (5-FU)	Adrucil®	Pyrimidine analog. Cytotoxic agent that when metabolized, inhibits DNA synthesis by blocking thymidylate synthase. It is administered together with leucovorin (reduced folic acid) to increase 5-FU half-life.
Capecitabine	Xeloda®	Pro-drug that is metabolized to 5-FU. It can be administered orally in contrast to 5-FU that is usually given intravenously (IV).
Oxaliplatin	Eloxatin™	Cytotoxic platinum analog that forms DNA adducts and induces apoptosis. It can also inhibit DNA replication and mRNA synthesis.
Irinotecan	Camptosar®	Topoisomerase I inhibitor that exerts its cytotoxicity by inhibiting DNA replication and transcription.
Biological agents		
Bevacizumab	Avastin®	Humanized monoclonal antibody (IgG1) against circulating VEGF-A preventing its binding to VEGF receptors. It functions as an anti-angiogenic agent.
Aflibercept	Zaltrap®	Recombinant fusion protein consisting of the extracellular domains of human VEGFR-1 and -2 and the Fc portion of human IgG1. It prevents VEGF-A, -B and PlGF from binding to their receptors.
Regorafenib	Stivarga®	Is an orally available multi tyrosine kinase inhibitor that target VEGFR1, VEGFR2 and VEGFR3 as well as PDGFRβ, Tie-2, c-KIT, FGFR1, RET and BRAF
Cetuximab	Erbix®	Chimeric murine/human IgG1 monoclonal antibody that target the extracellular domain of EGFR causing its internalization and degradation.
Panitumumab	Vectibix®	Fully human monoclonal antibody (IgG2) against the extracellular domain of EGFR preventing ligand binding, dimerization of the receptor and signaling.

1.3.3. Predictive biomarkers of drug response

Despite having improved the therapeutic options available, the results are not optimal. Still, most of the advanced and mCRC patients do not benefit from treatments showing drug-resistance and later relapse. Only few patients with the appropriate molecular characteristics can benefit from selected target-directed therapies. It is therefore mandatory to find robust molecular biomarkers for selecting the best targeted-therapy for each patient.

A paradigmatic example of predictive biomarker is the activating mutation of the oncogene *KRAS* that confer resistance to anti-EGFR therapies such as cetuximab. More recently, mutations of other members of the RAS family such as *NRAS* or *HRAS* have also demonstrated to confer resistance to such therapies. Since RAS proteins are downstream effectors of the EGFR signaling, their oncogenic activation abrogates the activity of drugs inhibiting upstream components of the pathway. The mutational status of RAS genes is currently evaluated in the clinic and almost 60% of all advanced CRC patients are consequently excluded from anti-EGFR therapies (Van Cutsem et al., 2014).

To date, there are no other available biomarkers in CRC that had demonstrated a robust prediction of drug-response beyond RAS mutations (Van Cutsem et al., 2013). To overcome this matter, several groups have tried to catalogue CRC tumors in different molecular subtypes, using complex molecular profiles, to identify patients sensitive or resistant to particular treatments. Different classifications of CRC tumors have shown prognostic value and some even predict drug-response (Bogaert and Prenen, 2014). Initial characterization segregated patients in five groups depending on their MSI, CIMP and *BRAF* mutational status (Jass, 2007). Differences due to anatomical position of the tumor are also important. Tumors from proximal colon seem to have higher rate of MSI which can have an impact on the treatment (Albuquerque et al., 2011). More recently, two studies on gene expression profiling provided different signatures and CRC classifications (Sadanandam et al., 2013; De Sousa E Melo et al., 2013). Both studies demonstrated that each tumor subtype showed different sensitivity or resistance to chemotherapy or anti-EGFR drugs. Yet, a unified classification is still missing; however, it is clear that the molecular alterations in cellular signaling pathways are the cause of the resistance to target-directed drugs.

2. Main altered pathways in colorectal cancer and their components

Next generation sequencing has permitted to identify some of the molecular signaling pathways that are more frequently altered in CRC including the Wnt/ β -catenin, TGF- β , RTK/RAS/PI3K and TP53 pathways (**Figure 3**).

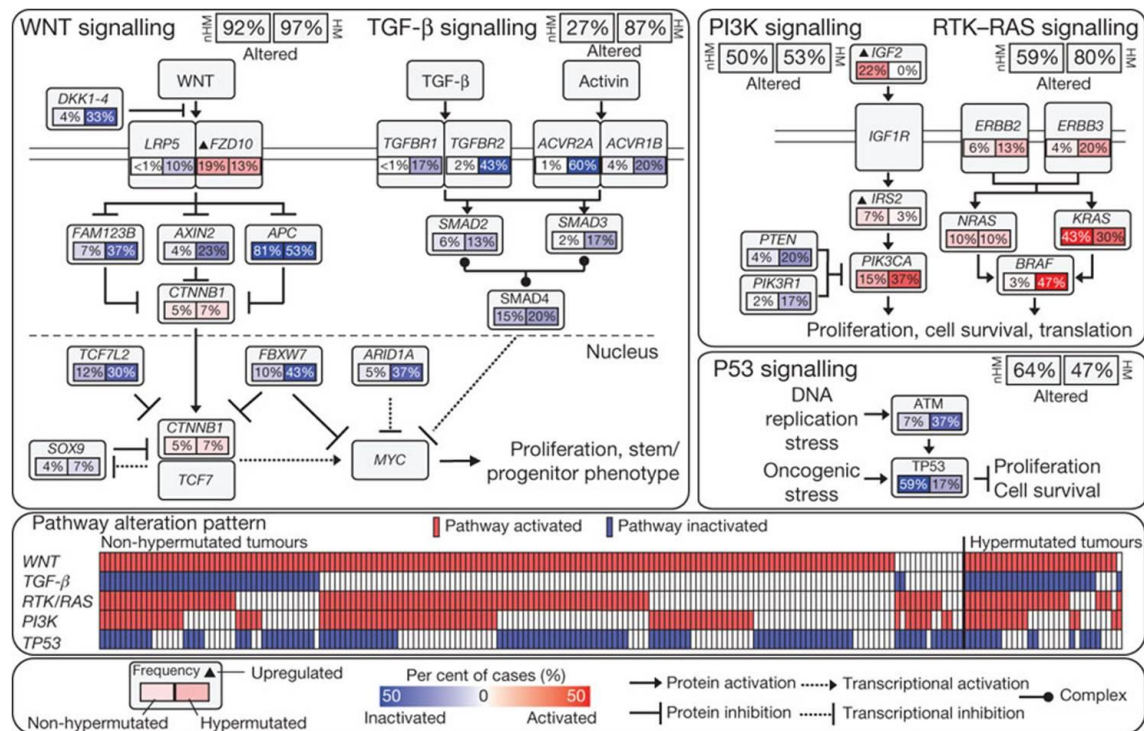


Figure 3. Frequency of alterations in molecular signaling pathways in colorectal cancer. Upper panels show a representation of the genes that participate in the most frequently altered signaling pathways in CRC. For each gene, its function in the pathway (activating or inhibiting) and also its frequency of alteration in either hypermutant (HM) or non-hypermutant (nHM) tumors are indicated. Red color symbolizes activation whereas blue color denotes inactivation of the genes. Middle panel shows the frequency of alterations in hypermutant or non-hypermutant tumors of the different signaling pathways altered in colorectal cancer: Wnt, TGF- β , RTK/RAS, PI3K and TP53. Bottom panel shows the figure legend. Adapted from (TCGA, 2012).

2.1. RTK/RAS/ERK-MAPK signaling pathway

2.1.1. The epidermal growth factor receptor (EGFR)

EGFR is a transmembrane receptor tyrosine kinase (RTK) that belongs to the erythroblastic leukemia viral oncogene homolog (ERBB) family of cell membrane receptors. Apart from EGFR (also known as HER1) this family includes three more members, ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). All of them are single pass membrane receptors that contain an extracellular ligand-binding domain and a cytoplasmic tyrosine kinase domain (Krasinskas, 2011).

Ligand binding induces dimerization of the receptors forming homo- or hetero-dimers between the different receptors of the family. Then the tyrosine kinase domains gets autophosphorylated, creating docking sites for adaptor proteins that will activate two main downstream signaling pathways related to cellular growth and proliferation (**Figure 4**): the ERK mitogen activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathways (Citri and Yarden, 2006; Tabernero, 2007).

As mentioned before, cetuximab and panitumumab are two monoclonal antibodies against EGFR used in the clinical management of CRC. Both bind to the receptor on the extracellular domain and block its ligand binding and dimerization, inhibiting the activation of downstream signaling pathways. Endogenous mutations in EGFR are not very common in CRC (Krasinskas, 2011). However, it has been described that there is an acquired resistance to the treatment due to mutations in the extracellular domain of EGFR that prevent cetuximab binding beyond the above mentioned RAS mutations (Arena et al., 2015; Montagut et al., 2012).

2.1.2. KRAS and the ERK mitogen activated protein kinases (MAPK)

Kirsten rat sarcoma viral oncogene homolog (KRAS) belongs to the RAS family of small GTPases formed by KRAS, HRAS and NRAS (Samatar and Poulikakos, 2014). They switch between inactive and active state by exchanging GDP for GTP (Prior et al., 2012). Upon RTK phosphorylation, adaptor proteins such as GRB2 lead to activation of RAS proteins which in turn activate the ERK-mitogen activated protein kinase (MAPK) cascade (RAF-MEK-ERK) (**Figure 4**). RAS proteins can also activate the PI3K signaling pathway (Fruman and Rommel, 2014).

KRAS is the member of the family most frequently mutated in CRC (30-40%). Most of the cases are missense mutations in codons 12, 13 or 61 (TCGA, 2012) that lead to a constitutive activation of KRAS and the consequent hyperactivation of the downstream signaling pathways. As pointed out before, *KRAS* mutations cause resistance to anti-EGFR therapies. Therefore, determination of the mutational status of *KRAS* is a key step in the election of the therapeutic regimen for advanced CRC patients.

2.1.3. BRAF

V-raf murine sarcoma viral oncogene homolog B1 (BRAF) is a member of the RAF family of serine-threonine protein kinases composed by ARAF, BRAF and CRAF. They are the first kinases in the ERK-MAPK pathway. Upon RAS activation, they are recruited to the membrane, becoming activated and able to phosphorylate the kinase MEK, which in turn phosphorylate ERK (**Figure 4**) (Samatar and Poulikakos, 2014).

BRAF is the most frequent altered member of the RAF family in cancer. It usually presents the missense activation mutation V600E. In CRC, around 5-10% of cases present BRAF^{V600E} (Lawrence et al., 2014), however, this frequency can increase in the group of patients with MSI to more than 40% of the cases (Thiel and Ristimäki, 2013). Mutations in *BRAF* are associated

with poor prognosis and are mutually exclusive with *KRAS* mutations (Clarke et al., 2013). It seems that patients that harbor *BRAF* mutations neither benefit from anti-EGFR therapies (Roock et al., 2011). The development of BRAF inhibitors is an attractive option. For melanoma patients, that present a high frequency of BRAF^{V600E} mutants, these inhibitors are routinely used in the clinic. In mCRC these inhibitors are currently being tested in clinical trials with promising results (personal communication).

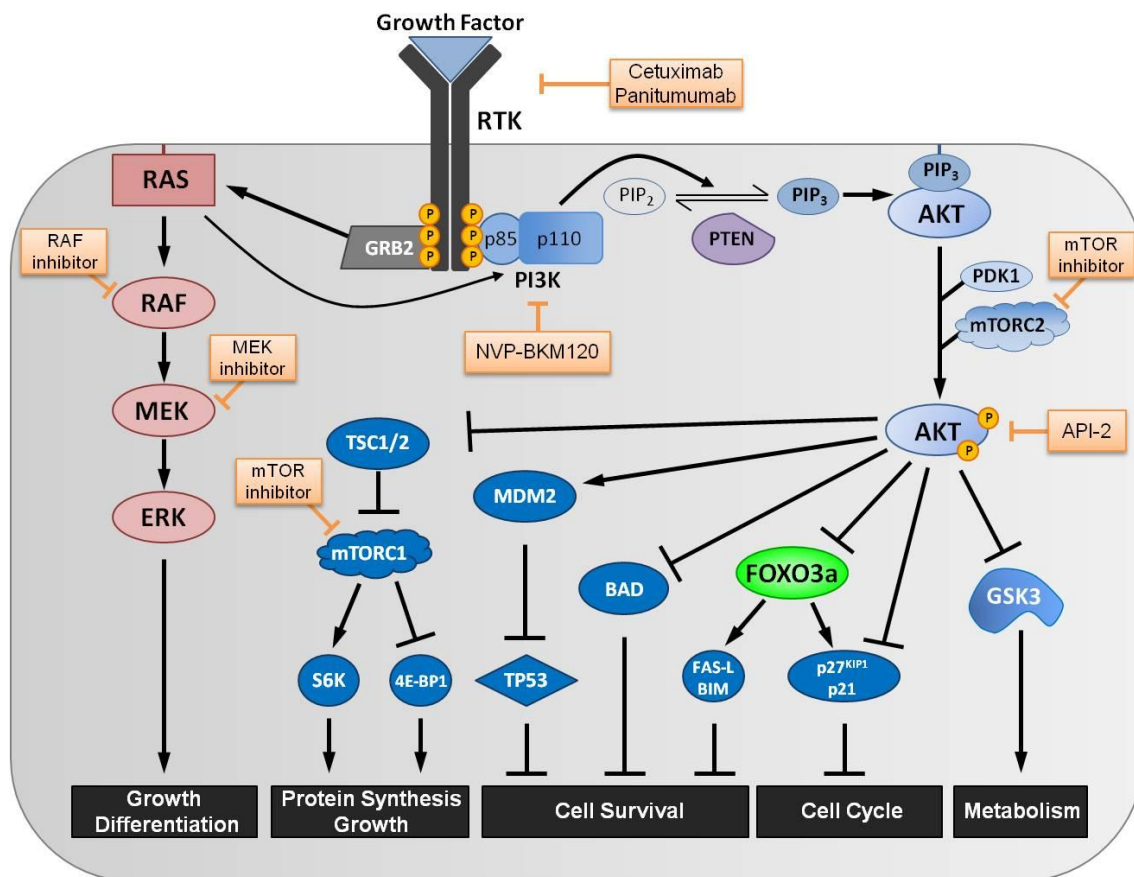


Figure 4. Overview of the RTK/RAS/PI3K/AKT signaling pathways. Schematic representation of the RAS and PI3K pathways. Both are induced by the activation of receptor tyrosine kinases (RTK) such as EGFR. RAS activates the ERK-MAPK pathway. PI3K activates its downstream effector AKT, which in turn modulates many proteins, including the mTORC1 complex and FOXO proteins. Therapeutic inhibitors described in this thesis are indicated in orange boxes. Activating (Arrows) or inhibitory (T-lines) functions are indicated. PI3K is composed of two subunits (p85 and p110). RAS and PIP₃ are bound to the membrane. Phosphorylation events are indicated by a circle containing the letter P. Molecular outcomes are indicated in dark boxes. Adapted from (Markman et al., 2009).

2.2. PI3K/AKT/mTOR signaling pathway

Input signals from RTKs, G-protein-coupled receptors (GPCRs) or RAS proteins activate PI3K. When active, it converts membrane phosphatidylinositol-4,5-bisphosphate (PIP₂) to phosphatidylinositol-3,4,5-trisphosphate (PIP₃), creating docking sites to activate downstream effectors (**Figure 4**). The most relevant is the serine-threonine protein kinase AKT. For a full activation, AKT needs to be phosphorylated by the phosphoinositide-dependent kinase 1

(PDK1) and mTOR complex 2 (mTORC2). Then, AKT modifies the activity of several proteins promoting cell growth and proliferation by activating the mTOR complex 1 (mTORC1) and its downstream effectors, such as the ribosomal protein S6 kinase 1 (S6K1). AKT also promotes cell survival, by inhibiting pro-apoptotic factors such as forkhead box (FOXO) proteins and cell cycle inhibitors such as p27^{KIP1} (*CDKN1B*) and p21 (*CDKN1A*) (Fruman and Rommel, 2014; Liu et al., 2009; Markman et al., 2009). The major negative regulator of PI3K pathway is the tumor suppressor phosphatase and tensin homologue (PTEN), which converts PIP₃ back to PIP₂ blocking AKT activation.

2.2.1. PI3K

Phosphatidylinositol 3-kinases (PI3K) form a family of lipid kinases whose primary biochemical function is to phosphorylate hydroxyl groups of phosphoinositides. They are divided into three classes (I-III) according to their structural characteristics and substrate specificity. Class IA members are heterodimers consisting of a regulatory subunit (p85) and a catalytic subunit (p110). There are five p85 isoforms that can associate with the three p110 isoforms (p110 α , p110 β , p110 δ). Class IB forms heterodimers between the catalytic subunit p110 γ and the regulatory subunits p101, p84 or p87 (Liu et al., 2009). Class I is associated with glucose homeostasis, metabolism, growth, proliferation and survival. Class II members possess only a single catalytic subunit (C2 α , C2 β , C2 γ) and are involved in the regulation of membrane trafficking. Class III PI3K has a single catalytic subunit also (VPS32) and participates in the regulation of autophagy (Markman et al., 2009).

The PI3K signaling pathway has a very relevant role in cancer biology since *PIK3CA* gene, which encodes the catalytic isoform p110 α , and *PTEN* are respectively the second and third most frequently mutated genes in cancer patients (Lawrence et al., 2014). In CRC, around 15% of the cases present activating mutations in the *PIK3CA* gene and between 2-10% present *PTEN* inactivation (higher in MSI tumors). Mutations in *PIK3CA* often occur in hotspots in the helical domain, on exon 9 (E542K or E545K), or in the kinase domain, on exon 20 (H1047R). In all the cases, these mutations lead to a hyperactivation of the pathway (Danielsen et al., 2015). p110 α subunit presenting mutations in exon 9 still require for its activation the interaction with RAS proteins (RAS-GTP) but not its binding to the p85 subunits. In contrast, mutations in exon 20 turn p110 α active independently of RAS but requiring interaction with p85 (Liu et al., 2014). *PIK3CA* mutations are suggested to predict poor prognosis (Clarke et al., 2013): Mutations in exon 20 are associated with a lack of cetuximab activity in *KRAS*-WT tumors while exon 9 mutations are usually associated with *KRAS* mutations (Roock et al., 2011).

2.2.2. AKT

V-akt murine thymoma viral oncogene homolog (AKT), also known as protein kinase B (PKB), belongs to a family of serine-threonine protein kinases that acts downstream of PI3K. There are three isoforms, AKT1, AKT2 and AKT3 (encoded by genes *AKT1*, *AKT2* and *AKT3*) that share a similar structure containing an amino-terminal pleckstrin homology domain (PH), required

for binding to phosphatidylinositol groups, a central serine-threonine catalytic domain, and a small carboxy-terminal regulatory domain (Liu et al., 2009).

The action of PI3K creates multiple PIP₃ at the cytoplasmic face of the plasma membrane. AKT is able to bind to PIP₃ through its PH domain, which induces a conformational change that exposes two residues, T308 and S473. Their phosphorylation by PDK1 and mTORC2 activate AKT that in turn, can phosphorylate many other proteins, including tuberous sclerosis proteins (TSC1/2), glycogen synthase kinase 3 (GSK3) and FOXO transcription factors (**Figure 4**) (Liu et al., 2009). Hence, AKT regulates many cellular processes promoting cell growth and proliferation and preventing apoptosis and cell cycle arrest.

AKT is not frequently mutated in cancer, however, it is an interesting candidate for target directed therapies because of its central position downstream of PI3K in the RTK/RAS/PI3K pathway.

2.2.3. mTOR complexes

Mammalian target of rapamycin (mTOR) is a serine-threonine protein kinase belonging to the PI3K superfamily. It is essential in the regulation of cellular growth and proliferation by monitoring nutrient availability, cellular energy, oxygen levels, and mitogenic signals (Liu et al., 2009). mTOR forms two complexes interacting with different proteins, mTORC1 (mTOR-Raptor) and mTORC2 (mTOR-Rictor). Both complexes have different subcellular localizations and functions.

mTORC1 is a downstream effector of PI3K signaling (**Figure 4**). Active AKT phosphorylates and inhibit TSC1/2, a repressor of mTORC1 complex. When activated, mTORC1 modulates its downstream targets, S6K1 and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1). mTORC2 is able to phosphorylate and activate AKT. Activating mTOR mutations or inactivating mutations of the repressor TSC1/2, although not very frequent, have been described (Lawrence et al., 2014; Sato et al., 2010). For this reason and because mTOR is controlled by the PI3K signaling pathway it is a candidate for target-directed therapies.

2.2.4. PI3K/AKT/mTOR signaling pathway inhibitors

Because of its central role in cellular biology, being a downstream effector of RTK and *KRAS*, PI3K signaling pathway is an interesting candidate for the development of new target-directed agents. The main rationale for inhibiting PI3K signaling, which is hyperactivated in many tumors, is the restoration of cell cycle arrest and apoptotic functions of downstream factors (i. e. FOXOs) that are blocked by this pathway.

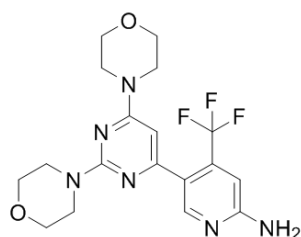
Since the proteins that participate in PI3K signaling are kinases, they are potential targets for small molecule inhibitors. The first compounds targeting this pathway approved for cancer treatment were the mTOR inhibitors (rapamycin analogs) everolimus and temsirolimus. Since then, many efforts have been made in developing specific PI3K and also lately AKT inhibitors

(Bauer et al., 2015). At this moment, there are five different classes of agents in clinical trials targeting the PI3K/AKT/mTOR signaling pathway: Pan-PI3K inhibitors (inhibiting the four class I members), isoform specific PI3K inhibitors, mTOR inhibitors, dual PI3K/mTOR inhibitors and AKT inhibitors.

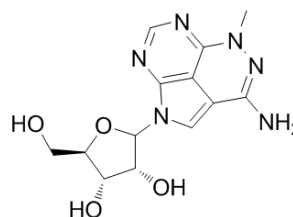
Despite the fact that the rational of inhibiting this signaling pathway is very promising, results in the clinic are not as satisfactory as expected. This is the case for CRC, where PI3K signaling inhibition has shown limited responses (Papadatos-pastos et al., 2015). In the particular case of PI3K inhibitors, patients were enrolled in most clinical trials when presented oncogenic mutations in exons 9 and/or 20 in the *PIK3CA* gene or *PTEN* loss. Although such mutations may confer sensitivity to PI3K inhibitors in preclinical models, no significant correlation has been observed in cancer patients. Therefore, no predictive biomarkers of sensitivity or resistance have been characterized for these types of treatments up to now. Their identification will be crucial to select patients that could benefit the most from these therapies (Dienstmann et al., 2014). Overall, results from clinical trials suggest that PI3K signaling pathway inhibitors could improve their therapeutic potential if used in combination with other targeted directed drugs.

In the context of this thesis we used two different inhibitors, a pan-PI3K inhibitor, NVP-BKM120, and a pan-AKT inhibitor, API-2:

- **NVP-BKM120:** Also known as buparlisip is a pan-Class I PI3K inhibitor developed by Novartis (Maira et al., 2012) (**Figure 5**). In the context of CRC, phase II clinical trials in combination with panitumumab (clinical trial ID (CTID): NCT01591421) or irinotecan (CTID: NCT01304602) are ongoing. This drug has also reached phase III for the treatment of breast cancer patients (CTID: NCT01633060).
- **API-2:** Also known as triciribine, is a pan-AKT inhibitor that binds to their PH domain and blocks their recruitment to the plasma membrane (Berndt et al., 2010; Yang et al., 2004) (**Figure 5**). Although limited efficacy has been observed so far (Dienstmann et al., 2011), API-2 is currently used in phase II clinical trials for patients with breast cancer (CTID: NCT01697293) and ovarian cancer (CTID: NCT01690468).



NVP-BKM120



API-2

Figure 5. Chemical structures of two PI3K pathway inhibitors. Structures of the pan-Class I PI3K inhibitor NVP-BKM120 (left) and the pan-AKT inhibitor API-2 (right).

2.3. FOXO3a

Forkhead box O (FOXO) proteins belong to a subclass of the forkhead box (FOX) family of transcription factors, defined by a common DNA-binding domain (Myatt and Lam, 2007). Their function is to regulate cell homeostasis in response to stress (Eijkelenboom and Burgering, 2013). The FOXO subgroup consists of four members: FOXO1, FOXO3a (also known as FOXO3), FOXO4 and FOXO6. The best studied member is FOXO3a. All FOXOs contain four domains, a highly conserved forkhead DNA-binding domain followed by a nuclear localization signal (NLS), a nuclear export signal (NES) and a C-terminal transactivation domain (Obsil and Obsilova, 2008).

FOXO proteins have shown to possess redundant and isoform specific functions (Paik et al., 2007). Tuning of their activity is achieved through post-translational modifications that alter their subcellular localization. FOXOs can be modified by phosphorylation, acetylation, methylation and ubiquitination. The best described events are phosphorylation by AKT and the Jun N-terminal kinase (JNK) (**Figure 6**). In the presence of oxidative stress, phosphorylation by JNK activates FOXOs favoring their shuttle into the nucleus, where they can exert their functions as transcription factors (Zhao et al., 2011). The presence of growth factors blocks FOXO activity through the PI3K signaling pathway. Activated AKT enters the nucleus phosphorylating FOXO proteins and inducing their nuclear export through interaction with 14-3-3 proteins and exportins (Brunet et al., 1999). Additionally, other molecular pathways can also contribute to regulate FOXOs transcriptional activity including factors such as p38, AMPK or sirtuins (Eijkelenboom and Burgering, 2013).

FOXOs can regulate several target genes and cellular processes including cell cycle arrest, resistance to oxidative stress, maintenance of cell homeostasis and apoptosis (**Table 3**) (Eijkelenboom and Burgering, 2013; van der Vos and Coffey, 2011). Their capacity to induce apoptosis and the fact that their function is counteracted by the oncogenic PI3K/AKT signaling pathway have been the main reasons to consider FOXO proteins as tumor suppressors (Paik et al., 2007). Abnormal activation of RAS and PI3K/AKT signaling are one of the most frequent alterations in cancer that promote FOXO translocation to the cytoplasm and consequent evasion of apoptosis. Therefore, part of the pro-apoptotic activity achieved by several PI3K signaling inhibitors depends on nuclear FOXO relocation.

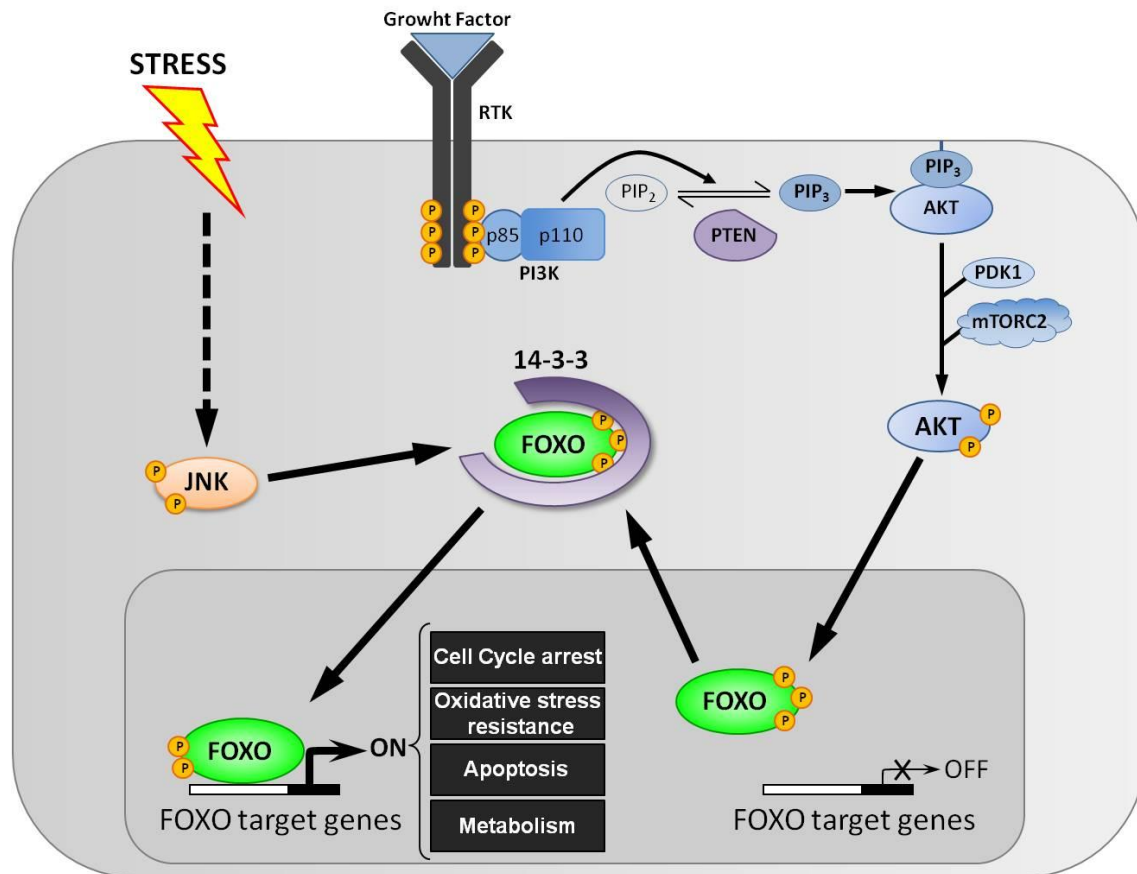


Figure 6. Regulation of FOXO signaling. Activity of FOXO proteins is negatively regulated by the PI3K signaling pathway through phosphorylation by AKT in the nucleus. This phosphorylation event increases the affinity of 14-3-3 proteins for FOXOs and the consequent nuclear export. Cellular stress positively regulates FOXO proteins through the phosphorylation by JNK that induces FOXOs nuclear translocation. Arrows indicate phosphorylation events highlighted by circles containing the letter P. The dotted line represents indirect activation. Molecular outcomes induced by FOXO proteins are indicated in dark boxes.

FOXO proteins can induce or repress the expression of many different genes by interacting with several transcription factors and transcriptional co-activators, what explains their cell context specific effects. Several well characterized partners of FOXO proteins include downstream effectors of other signaling pathways (SMADs or CCAAT/enhancer-binding protein- α/β , C/EBP- α/β) or nuclear receptors such as the retinoic acid receptor or the androgen receptor (van der Vos and Coffer, 2008). However, the most relevant FOXO partner in the context of CRC is β -catenin. The interaction of FOXO proteins with β -catenin is evolutionally conserved and is detected from *Caenorhabditis elegans* to mammalian cells (Essers et al., 2005). In *C. elegans* it has been shown that the interaction between DAF-16 (FOXO ortholog) and BAR-1 (β -catenin ortholog) is necessary for oxidative stress resistance and lifespan regulation. DAF-16/FOXO is regulated by insulin signaling, through PI3K pathway, which provided a direct link between the PI3K/AKT and the Wnt signaling pathways in controlling organism homeostasis.

Table 3. FOXO-regulated processes and target genes	
Regulated Processes	Target Genes
Cell fate decisions	
Cell cycle arrest	CDKN1B; CDKN1A; CDKN2B; CDKN2A; CCND1; RBL2
Apoptosis	BCL2L11; BCL6; PMAIP1; BBC3; FASLG; TNFSF10; TNF
Metabolism	
Gluconeogenesis	PCK1; G6PC; APOC3 AGRP;
Redox balance, stress resistance	SOD2; CAT; PRDX3; SENP
Protein homeostasis	
Mitophagy	PINK1
Proteosomal breakdown	FBXO32; TRIM63
Autophagy	GABARAPL1; ATG12; BNIP3; BCEN1
Signaling	
Increased PI3K signaling	PI3K; IRS2
Increased EGF signaling	EGFR
Decreased Myc signaling	MXI1
Extracellular matrix	
Extracellular matrix degradation	MMP1; MMP9
Cell type-specific functions	
Pluripotency maintenance	OCT4, SOX2
Immune system	IL7R; CCR7; SELL; RAG1; RAG2; FOXP3; IFNG; IRF7

Adapted from (Eijkelenboom and Burgering, 2013) and (van der Vos and Coffey, 2011).

2.4. The Wnt/ β -catenin signaling pathway

Wnt signaling is one of the fundamental mechanisms that direct cell proliferation, polarity and fate determination during embryonic development and maintenance of stem cells and tissue homeostasis in adults. Because of that, alterations in Wnt pathway components can have a potent detrimental outcome and are linked to several diseases including cancer (Clevers, 2006; Clevers and Nusse, 2012). Indeed, mutations in *APC* are one of the most frequent and well characterized events leading to oncogenic Wnt/ β -catenin signaling activation and CRC initiation.

Canonical or Wnt/ β -catenin pathway activation induces the stability of its main effector, β -catenin. β -catenin can be found at different cellular pools: In adherent junctions bound to E-cadherin, free in the cytoplasm and in the nucleus (Valenta et al., 2012). In the absence of Wnt ligands, free cytoplasmic β -catenin is degraded by the action of a destruction complex formed by the scaffolding proteins AXIN1/2, adenomatous polyposis coli (APC) and the serine/threonine kinases glycogen synthase kinase 3 (GSK3) and casein kinase 1 α (CK1 α). β -catenin is sequentially phosphorylated by CK1 α and GSK3 creating binding sites for the E3 ubiquitin ligase β -TrCP leading to β -catenin ubiquitination and further degradation by the proteasome (**Figure 7**) (MacDonald et al., 2009).

Binding of secreted Wnt ligands to their receptors, the seven-pass transmembrane Frizzled receptor (FZD) and its co-receptors low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), blocks the activity of the β -catenin degradation complex. Wnt-FZD-LRP5/6 complex

leads to phosphorylation of LRP5/6, binding of the protein Dishevelled (DVL) and the recruitment of the degradation complex to the cell membrane (MacDonald et al., 2009). At this stage, newly synthesized β -catenin can be accumulated in the cytoplasm and in turn enters the nucleus, where it acts as a transcriptional co-activator interacting with several transcription factors. The best defined β -catenin transcription partners are the T-cell factor/lymphoid enhancer factors (TCF/LEF) that allow the expression of canonical Wnt/ β -catenin target genes.

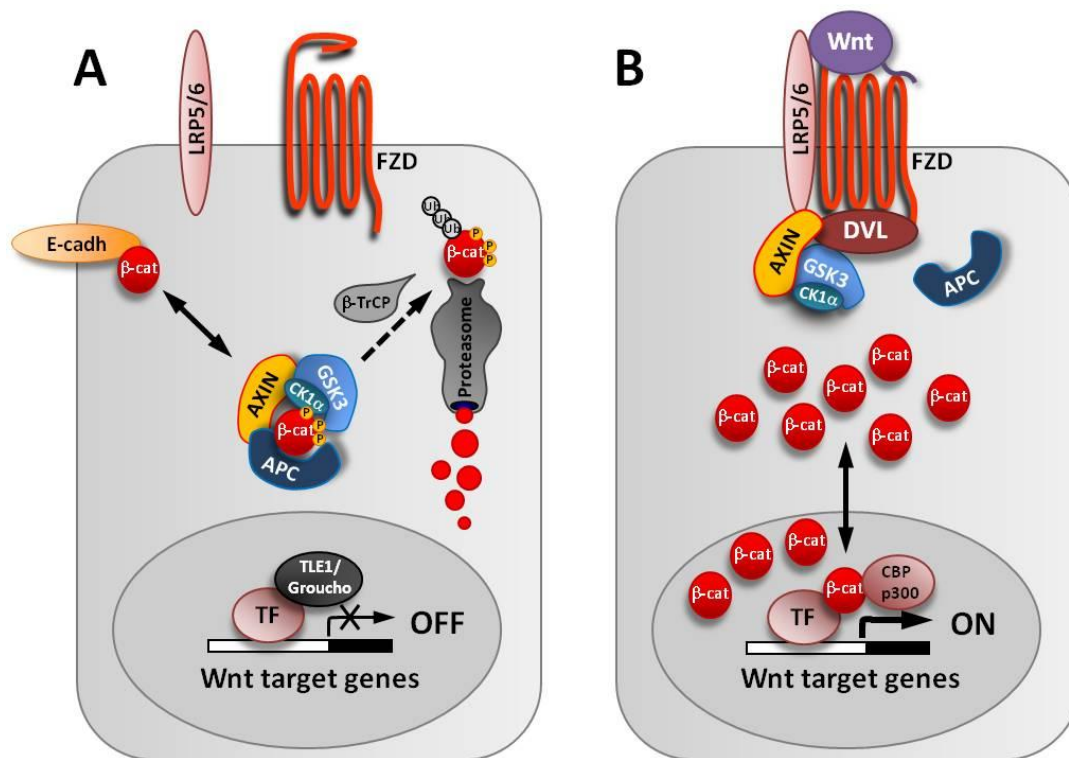


Figure 7. Overview of the Wnt/ β -catenin signaling pathway. (A) In the absence of Wnt ligands, β -catenin binds to E-cadherin or is released in the cytoplasm, where it is degraded by the action of the destruction complex formed by AXIN, APC and the kinases CK1 α and GSK3. Both kinases sequentially phosphorylate β -catenin which is then ubiquitinated by the E3 ubiquitin ligase β -TrCP and degraded by the proteasome. The best characterized transcription factors that interact with β -catenin, TCF/LEFs, are inactive since they are bound to the transcriptional repressor TLE1/Groucho. **(B)** In the presence of Wnt ligands the receptor complex formed by FZD and LRP5/6 recruit DVL and disrupts the degradation complex allowing the accumulation of β -catenin in the cytoplasm and its translocation to the nucleus. There, it acts as a transcriptional co-activator interacting with several transcription factors and additional co-activators including CBP and p300.

Wnt ligands can signal through other mechanisms independent of β -catenin, these are the so called non-canonical or β -catenin independent Wnt pathways (Kikuchi et al., 2009). These processes are less understood but control planar cell polarity (PCP) and calcium signaling (Kikuchi et al., 2009; Veeman et al., 2003). Apparently, specific Wnt ligands (such as Wnt5a or Wnt11) trigger such non-canonical processes interacting with specific FZD proteins or other receptors such as Ror or Ryk receptors (Stamos et al., 2012).

2.4.1. Components of the β -catenin degradation complex

2.4.1.1. AXIN1/2

AXIN1/2 serve as scaffold proteins for the other components of the destruction complex. There are two AXIN proteins encoded by two different genes, *AXIN1* and *AXIN2* (also called conductin) and each gene encodes two isoforms created by alternative splicing. Both proteins have redundant functions in Wnt signaling, however, *AXIN1* is constitutively expressed and is essential for the maintenance of low Wnt signaling activity in basal state (Zeng et al., 1997), whereas *AXIN2* is a direct Wnt target gene and acts as a negative feedback loop (Lustig et al., 2002). Both AXIN1/2 possess a DIX (Dishevelled/AXIN homologous domain) that allows its homodimerization and heterodimerization with DVL. They also have domains to interact with the other components of the degradation complex (APC, GSK3 and CK1 α) as well as with β -catenin (Salahshor and Woodgett, 2005). Some studies suggest that AXIN1/2 levels are low in cells, meaning that they may be a rate-limiting factor in the formation of β -catenin degradation complexes (Lee et al., 2003).

Post-translational modifications alter AXIN1/2 functions. On the one hand, phosphorylation by GSK3 and CK1 α increases its stability and affinity for β -catenin. On the other hand, AXIN1/2 stability and turnover are regulated by tankyrases, proteins that poly (ADP-ribosyl)ate AXIN1/2, which further leads to their ubiquitination by the E3-ubiquitin ligase RNF146 and their subsequent degradation by the proteasome (Callow et al., 2011; Zhang et al., 2011). In CRC, somatic mutations that affect AXIN1/2 functions have been described (**Figure 3**) (TCGA, 2012).

2.4.1.2. APC

Adenomatous polyposis coli (APC) is the largest protein of the destruction complex and has many different protein domains. The N-terminal region contains an oligomerization domain, responsible for homodimerization and an armadillo repeat domain that enables APC to interact with different cytoskeleton regulators. The C-terminal region contains domains for the interaction with microtubules and microtubule-associated proteins. These domains allow APC to participate in other cellular processes related to spindle formation, kinetochore attachment and maintenance of microtubule stability, however, it is not known if interactions with cytoskeleton regulators are important for its role in the Wnt destruction complex (Fodde et al., 2001). It is in the central region where APC harbors several domains that allow its interaction with AXIN1/2 and β -catenin. It contains three 15 amino acid (aa) repeats for β -catenin binding and seven 20 aa repeats for β -catenin release after its phosphorylation. Interspersed between the 20 aa β -catenin binding sites, there are three AXINs binding sites (SAMP) (Xing et al., 2004; Xu and Kimelman, 2007). APC also possesses nuclear localization sequences (NLS) and nuclear exporting signals (NES). Apparently, APC can enter the nucleus and affect β -catenin mediated transcription (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000).

The most frequent initiating mutations in CRC are truncating mutations in APC that eliminate some β -catenin binding sites making the degradation complex less efficient. For this reason

APC is considered a tumor suppressor gene. Besides, in most of the cases, *APC* truncating mutations do not eliminate all β -catenin binding sites, making cells harboring those *APC* truncations still sensitive Wnt signaling and partially proficient for β -catenin degradation (Voloshanenko et al., 2013). Beyond abnormal activation of the Wnt pathway, some truncating mutations in *APC* also impair its binding to microtubules and are therefore associated to CIN observed in CRC cells (Fodde et al., 2001).

2.4.1.3. GSK3

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase with two isoforms, GSK3- α and GSK3- β , encoded by two different genes *GSK3A* and *GSK3B*. They regulate many cellular processes such as the synthesis of glycogen, microtubule stability, cell cycle and also the Wnt signaling pathway where both isoforms have a redundant function (Doble and Woodgett, 2003; Doble et al., 2007).

GSK3 has been described to be a negative and a positive regulator of the Wnt signaling pathway. In the degradation complex GSK3 phosphorylates β -catenin enhancing its degradation. However, GSK3 needs a priming phosphorylation event in β -catenin at serine 45 carried out by CK1 α . Then GSK3 phosphorylates β -catenin at serine 33, serine 37 and threonine 41, which will be then recognized by the E3-ubiquitin ligase β -TrCP (Hart et al., 1999). Furthermore, GSK3 is able to phosphorylate AXIN1/2 and APC, increasing their affinity for β -catenin (Rubinfeld et al., 1996; Willert et al., 1999; Yamamoto et al., 1999). When Wnt ligands induce the recruitment of the destruction complex to the membrane, GSK3 is able to phosphorylate the co-receptor LRP5/6 enhancing its affinity for AXINs, increasing the inhibition of the destruction complex and promoting stabilization of free cytoplasmic β -catenin (Zeng et al., 2005).

2.4.1.4. CK1 α

Casein kinase 1 family of serine/threonine kinases contains different isoforms α , β , γ 1, γ 2, γ 3, δ and ϵ encoded by distinct genes (Price, 2006). They have a role in several cellular processes including Wnt signaling (Del Valle-Pérez et al., 2011). CK1 ϵ and γ are able to phosphorylate DVL and LRP6 (Casagolda et al., 2010; Davidson et al., 2005). CK1 α participates in the destruction complex by priming β -catenin for its degradation phosphorylating its serine 45 (Verheyen and Gottardi, 2010).

2.4.2. β -catenin

2.4.2.1. Structure and regulation

β -catenin is the central member of the canonical Wnt pathway apart from being a structural component of adherens junctions (Valenta et al., 2012). This variety of functions is given for its characteristic structure. Like the related members of the armadillo superfamily of proteins, β -catenin has a central region formed by 12 armadillo repeats flanked by flexible N- and C-terminal domains (Xing et al., 2008).

The armadillo domain forms a rigid platform that allows β -catenin to interact with many proteins such as APC, AXIN1/2 or several transcription factors. Since many proteins share overlapping binding sites in the armadillo region, simultaneous binding of β -catenin with more than one of its partners is not possible (Xu and Kimelman, 2007). This creates a competition for β -catenin binding that explains its functions and its tight regulation in cells. β -catenin N-terminal domain is important for cell-to-cell adhesion since it binds to α -catenin (Pokutta and Weis, 2000). This domain also controls β -catenin stability because CK1 α and GSK3 sequentially phosphorylate serine 45, threonine 41, serine 37 and serine 33. Serines 33 and 37 are recognized by the E3 ubiquitin ligase β -TrCP, which ubiquitinates β -catenin committing it to degradation by the proteasome (Hart et al., 1999). Importantly, around 5% of CRC cases present mutations that impair phosphorylation in these residues (TCGA, 2012). Such alterations are oncogenic because they increase β -catenin stability and are mutually exclusive with APC mutations (Morin et al., 1997; TCGA, 2012). The C-terminal domain is essential for β -catenin transcriptional activity (Mosimann et al., 2009).

In basal conditions β -catenin is located at the cytoplasmic side of the membrane bound to cadherins (E- or N-cadherin) and to α -catenin forming adherens junctions. These interactions increase β -catenin stability. Post-translational modifications such as phosphorylation of several residues reduce β -catenin affinity for its partners and leads to its release from adherens junctions (Piedra et al., 2001; Valenta et al., 2012). Free cytoplasmic β -catenin has low stability due to the action of the Wnt degradation complex.

Once the activity of the destruction complex is impaired, cytoplasmic β -catenin is gradually stabilized and can enter the nucleus. This process is complex and not fully characterized. β -catenin does not have any NLS or NES suggesting that it might not translocate to the nucleus using a classical import pathway. Indeed, β -catenin is able to interact with several proteins of the nuclear pore complex allowing its pass to the nucleus (Sharma et al., 2012). Furthermore, different modifications affect the ability of β -catenin to enter the nucleus. For instance the phosphorylation by AKT (Fang et al., 2007) or by Rac1 through JNK signaling (Wu et al., 2008) are two modifications that favor β -catenin nuclear translocation and transcriptional activity, indicating that stability and activity of β -catenin can be regulated by other pathways like PI3K signaling. Once in the nucleus, β -catenin can be exported by interaction with proteins such as AXIN1/2 or APC. These two members of the destruction complex are able to enter the nucleus and force β -catenin export (Cong and Varmus, 2004; Neufeld et al., 2000).

2.4.2.2. Transcriptional functions

In the nucleus β -catenin acts as a transcriptional co-activator and promotes the expression of many genes by binding to transcription factors. The first transcription factors described to interact with β -catenin were the T-cell factor/lymphoid enhancer factors (TCF/LEF) family of transcription factors (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996), hereafter referred to as TCFs. In absence of β -catenin, TCFs interact with transcriptional repressors such as TLE1/Groucho. β -catenin displaces co-repressors from TCFs and promotes transcription of Wnt target genes (TCF/ β -catenin targets) recruiting further transcriptional co-activators through its C-terminal domain such as histone acetyl transferases CREB-binding protein (CBP) and p300 or the SWI/SNF factor BRG1 (**Figure 7**) (Mosimann et al., 2009; Valenta et al., 2011; Van de Wetering et al., 1997).

Despite TCFs are considered the canonical partner of β -catenin, growing evidences describe that it performs multiple TCF/LEF independent roles, suggesting that β -catenin interacts with other transcription factors and induces alternative sets of genes in a context dependent manner (Valenta et al., 2012) (**Figure 8**). Structure of β -catenin does not allow interaction with more than one transcription factor simultaneously. For this reason there is a tightly regulated competition between many transcription factors to bind to β -catenin.

Nuclear receptors such as the androgen receptor (AR) or vitamin D receptor (VDR), in the presence of their ligands, can bind to β -catenin and inhibit TCF signaling. AR can also bind to β -catenin in the cytoplasm allowing its nuclear translocation (Mulholland et al., 2005). VDR interacting with β -catenin can induce differentiation of colon cancer cell lines by increasing expression of E-cadherin (Pálmer et al., 2001). Alternatively, β -catenin reinforces pluripotency in mouse embryonic stem cells increasing transcription mediated by Oct-4 (Kelly et al., 2010). Hypoxia Induced Factor 1 α (HIF1 α) is able to sequester β -catenin under hypoxia conditions, increasing cell survival and adaptation to low oxygen conditions (Kaidi et al., 2007). YAP (Yes-associated protein) and its homolog TAZ (transcriptional co-activator with PDZ binding motif) are transcriptional effectors of the Hippo pathway. They are degraded by the Wnt destruction complex together with β -catenin and their interaction is necessary for CRC cell survival (Azzolin et al., 2012; Rosenbluh et al., 2012). Similarly, FOXO transcription factors can compete with TCF for β -catenin under oxidative stress conditions to promote their specific-transcriptional program (Essers et al., 2005; Hoogeboom et al., 2008).

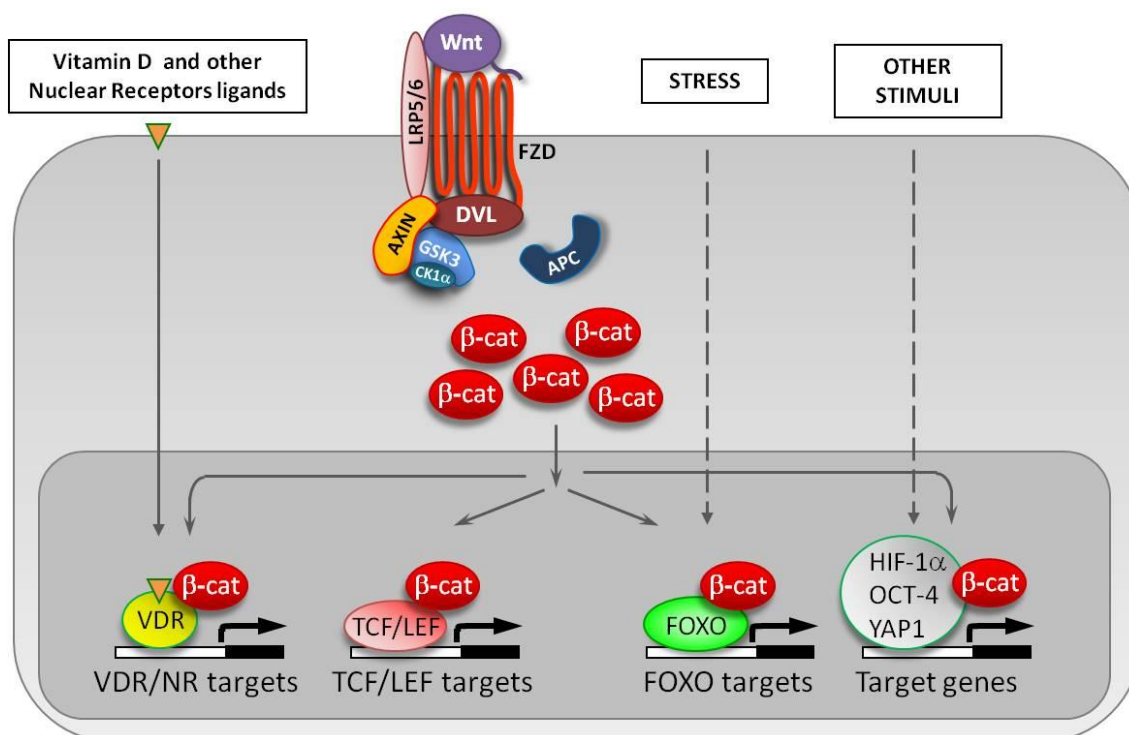


Figure 8. Transcription factors interacting with β -catenin. Upon their specific stimuli, several transcription factors compete to interact with β -catenin in the nucleus. These include vitamin D receptor or additional nuclear receptors (i.e. Estrogen or retinoic acid receptor), TCF/LEF family of transcription factors, FOXO proteins, HIF-1 α , OCT-4 or YAP1 among others. β -catenin interaction with those transcription factors modulate their transcriptional output.

2.4.3. Inhibiting the Wnt signaling pathway

Since the Wnt signaling pathway was linked to cancer in the 1990s, many efforts have been made to develop effective Wnt inhibitors. Several molecules have been identified with good *in vitro* and *in vivo* activity (Barker and Clevers, 2006; Voronkov and Krauss, 2013), however, there is none approved for clinical use (Kahn, 2014). Targeting Wnt signaling has been difficult because most players exert their functions by protein-protein interactions and only few are enzymes with a catalytic activity prone to be inhibited by small molecules. Furthermore, many of those players have redundant functions, what increases the difficulty to reach a complete pathway inhibition (Anastas and Moon, 2013). Recently, the discovery of new agents and characterization of their mechanism of action have led to the entrance of Wnt inhibitors in clinical trials for the first time.

The Wnt pathway can be blocked at three different levels, at the level of the receptor complex and their ligands, at transcriptional level in the nucleus or at the level of Wnt degradation complex in the cytoplasm (**Figure 9**).

2.4.3.1. Upstream inhibition of the pathway

Therapies designed to block upstream the pathway include blocking antibodies or peptides against Wnt receptors (FZDs or LRP5/6) or against ligands. Three antibodies have reached phase I clinical trials, a fully humanized monoclonal antibody against several FZDs (OMP18R5, vantictumab (Smith et al., 2013), a radiolabelled chimeric monoclonal antibody against FZD10 (OTSA101-DTPA-90Y (Fukukawa et al., 2008)) and a chimeric antibody fusing Fc-IgG and FZD8 that binds all Wnt ligands (OMP54F28 (DeAlmeida et al., 2007)).

In the case of small molecules, the most promising strategy consists in blocking the maturation of Wnt ligands by inhibiting the enzymatic activity of the O-acyltransferase Porcupine (PORCN) (Chen et al., 2009; Liu et al., 2013) (**Figure 9**). Wnts are cysteine-rich glycoproteins. To fully function as ligands they undergo several post-translational modifications in the endoplasmic reticulum (ER) and golgi apparatus (Herr et al., 2012). The most critical modification is the palmitoylation by PORCN in the ER (MacDonald et al., 2009). Alterations in PORCN lead to immature Wnt ligands that are retained inside the ER (Takada et al., 2006). The PORCN inhibitor NVP-WNT974 (Novartis), formerly known as NVP-LGK974, is already in phase I clinical trials, being the first small molecule to inhibit Wnt signaling tested in clinical trials designed for mCRC patients (CTIDs: NCT01351103 and NCT02278133). The potential disadvantages of these kinds of therapies are that downstream alterations in Wnt pathway components such as *APC* truncating mutations may cause innate treatment resistance.

2.4.3.2. Inhibition of transcriptional activity

Targeting the interaction between β -catenin and their partners in the nucleus is challenging. β -catenin/TCFs interaction involves large regions of both proteins making difficult the use of small molecules to block it (Clevers and Nusse, 2012). However, some are under investigation (Kahn, 2014) (**Figure 9**). Better results have been obtained by blocking the interaction between β -catenin and the transcriptional machinery. PRI-724 (PRISM Pharma), blocking β -catenin/CBP interaction, has reached phase I clinical trials for myeloid malignancies and advanced solid tumors. Currently, another trial is planned for mCRC patients (CTID: NCT02413853) (El-Khoueiry et al., 2013; Emami et al., 2004).

2.4.3.3. Inhibition in the cytoplasm

Targeting Wnt signaling at the cytoplasmic level has experienced a big boom in the recent years. Some approaches focused on targeting DVL or the degradation complex kinases GSK3 and CK1 α (Pyrvinium) (Reviewed in (Clevers and Nusse, 2012; Kahn, 2014)) (**Figure 9**). However, these targets participate in many cellular processes, as described before, and also some can act as either positive or negative regulators of the Wnt pathway. At this moment, one of the most promising strategies is the inhibition of tankyrases, enzymes that control AXIN1/2 stability. In this work we have used tankyrase inhibitors as Wnt signaling inhibitors.

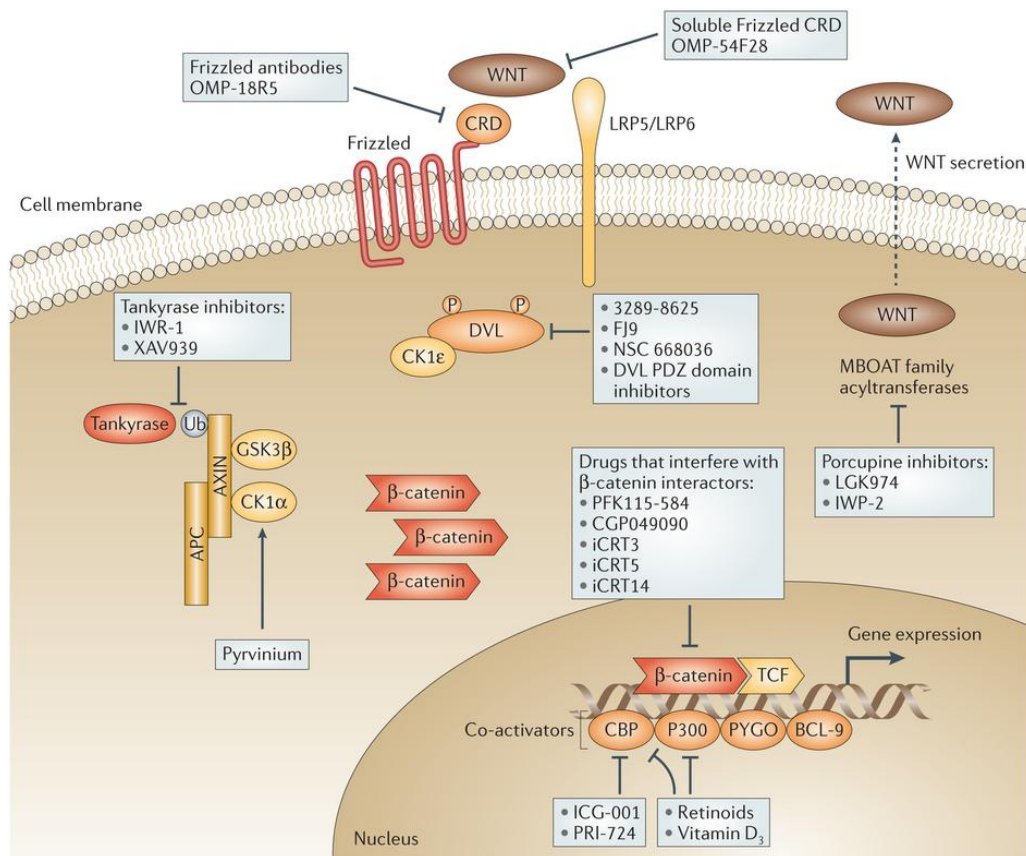


Figure 9. Wnt signaling pathway inhibitors. Overview of the currently available inhibitory molecules targeting components of the Wnt pathway. Adapted from (Kahn, 2014)

2.4.4. Tankyrases

2.4.4.1. Overview and structure

Tankyrases belong to the poly (ADP-ribose) polymerase (PARP) superfamily of proteins also known as ADP-ribosyltransferase diphtheria toxin-like (ARTD) family that comprises 17 members. Their function is to post-translationally modify acceptor proteins adding polymers of ADP-ribose monomers using NAD^+ as substrate, a process known as PARsylation (Lehtiö et al., 2013; Riffell et al., 2012) (**Figure 10**).

There are two tankyrases, tankyrase 1 (TNKS1 or PARP5a) and tankyrase 2 (TNKS2 or PARP5b) that form a subgroup in the PARP superfamily with a characteristic structure. They both possess an ankyrin repeat domain, a sterile alpha motif (SAM) and the catalytic PARP domain (Haikarainen et al., 2014). The ankyrin repeat domain is divided in five repeat clusters that provide binding sites for protein-protein interaction, while the SAM domain allows homo- and hetero-dimerization. The catalytic domain, responsible for PARsylation, has two binding sites, an acceptor site, which accommodates the target protein and a donor site. The donor site binds and hydrolyses NAD^+ and it is divided in two binding pockets to include both products generated by NAD^+ hydrolysis, the nicotinamide and the adenosine (ADP-ribose) molecule that will be bound to the acceptor protein.

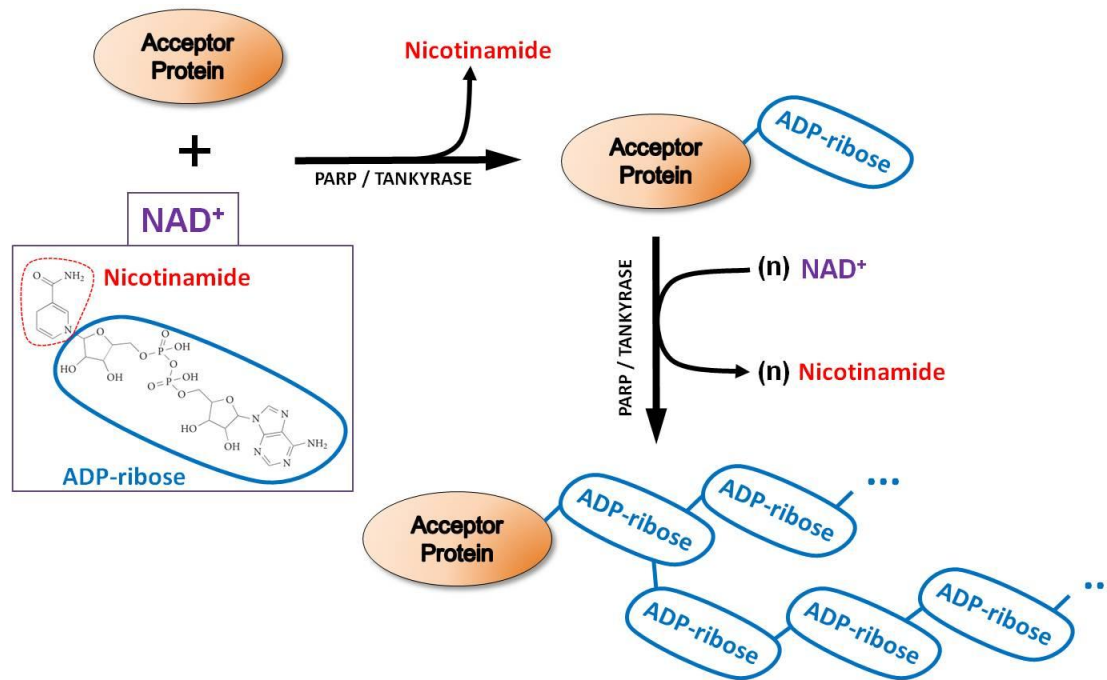


Figure 10. The process of protein PARsylation. Poly (ADP-ribosyl)ation or PARsylation is a post-translational modification carried out by the proteins belonging to the PARP family, including tankyrases. This process involves the cleavage of NAD⁺ into one molecule of nicotinamide and one of ADP-ribose. ADP-ribose is then covalently linked to an acceptor protein by the action of PARP/Tankyrases. Using additional molecules of NAD⁺, these enzymes can elongate the ADP-ribose chain creating polymers that are commonly branched.

2.4.4.2. Cellular functions

TNKS1 and TNKS2 double knockout is lethal in mice while the knockouts of both proteins separately are not. This indicates that a functional redundancy exist between TNKS1 and TNKS2 (Chiang et al., 2008). Tankyrase-dependent post-translational modification of target proteins affects their function or location, commonly inducing their ubiquitination and proteasomal degradation (**Figure 11**). Tankyrases can be found in many different cellular locations allowing them to participate in many different cellular processes.

2.4.4.2.1. Telomere maintenance and DNA repair

Tankyrases were discovered for their role in telomere homeostasis since they bind TRF1 (Telomere repeat factor 1), a member of the shelterin complex that protect telomeres from degradation and their fusion with other chromosomes (De Lange, 2005). Tankyrases bind and PARsylate TRF1 reducing its affinity for the complex and promoting its disassembly. Consequently tankyrases are considered to promote telomere length by increasing the accessibility of telomeres to the elongating enzyme telomerase (Smith and De Lange, 2000; Smith et al., 1998) (**Figure 11A**). Additionally, tankyrases are also involved in DNA repair by stabilizing the non-homologous end-joining (NHEJ) protein DNA-PK (Dregalla et al., 2010).

Reduced levels of TNKS1 sensitizes cells to ionizing radiation-induced cell death, chromosome aberration and telomere fusion (**Figure 11B**).

2.4.4.2.2. Mitosis

During mitosis, tankyrases can be found in the spindle poles and in the centrosomes, promoting centriole elongation and mitotic spindle formation. Tankyrases inhibits the function of the centrosomal P4.1-associated protein (CPAP) during the G1 phase by PARsylation, leading to its degradation (Kim et al., 2012). Furthermore, tankyrases also promote mitosis by PARsylation of other proteins such as the mitotic kinetics regulator MIK1 (Ozaki et al., 2012) or NuMA (Chang et al., 2009), directing a change in their subcellular localization to the centrosome and spindle poles respectively. Finally, tankyrases are necessary for the fully resolution of sister chromatids and avoid fusion in telomeres during anaphase (Hsiao and Smith, 2009) (**Figure 11C**).

2.4.4.2.3. Insulin-mediated glucose uptake

Tankyrases associate with GLUT4 storage vesicles (GSVs) to transport glucose into the cells upon insulin stimulation. Some results suggest that tankyrases interact with GSVs forming a complex with AXINs and KIF3A. Tankyrase activity leads AXINs to degradation and disassembly of the complex. Insulin signaling inhibits tankyrases through phosphorylation mediated by AKT, promoting the stability of Tankyrase/AXIN/KIF3A complex and the membrane translocation of GLUT4 storage vesicles (Guo et al., 2012). Furthermore, tankyrases also interact with IRAP, a GSV cargo protein. It has been suggested that IRAP PARsylation is necessary for a correct trafficking of GSVs to the membrane (Yeh et al., 2007) (**Figure 11D**).

2.4.4.2.4. Proteasome regulation

A recent study identified tankyrases as regulators of proteasomal activity. Tankyrases promote the assembly and activity of the 26S proteasome complex by PARsylation of proteins that prevent the formation of the proteasomal complex (Cho-Park and Steller, 2013).

2.4.4.2.5. Wnt signaling pathway

As commented before, tankyrases were recently characterized as modulators of Wnt signaling by controlling the stability of AXIN1/2 (**Figure 11E**). Tankyrases lead AXINs to degradation, altering the function of the β -catenin degradation complex and in turn increasing β -catenin stability in cells (Huang et al., 2009). Tankyrases can bind and PARsylate AXIN1/2 which are then recognized by the E3 ubiquitin ligase RNF146 through its WWE domain. RNF146 ubiquitinates AXIN1/2 leading to their degradation by the proteasome. However, during the formation of the ternary complex between tankyrase/AXIN/RNF146, tankyrases are also able

to PARsylate RNF146 and themselves, causing the simultaneous ubiquitination and degradation of the three proteins (Callow et al., 2011; Zhang et al., 2011). Because tankyrases can modulate Wnt signaling they are promising targets for the development of new Wnt inhibitors.

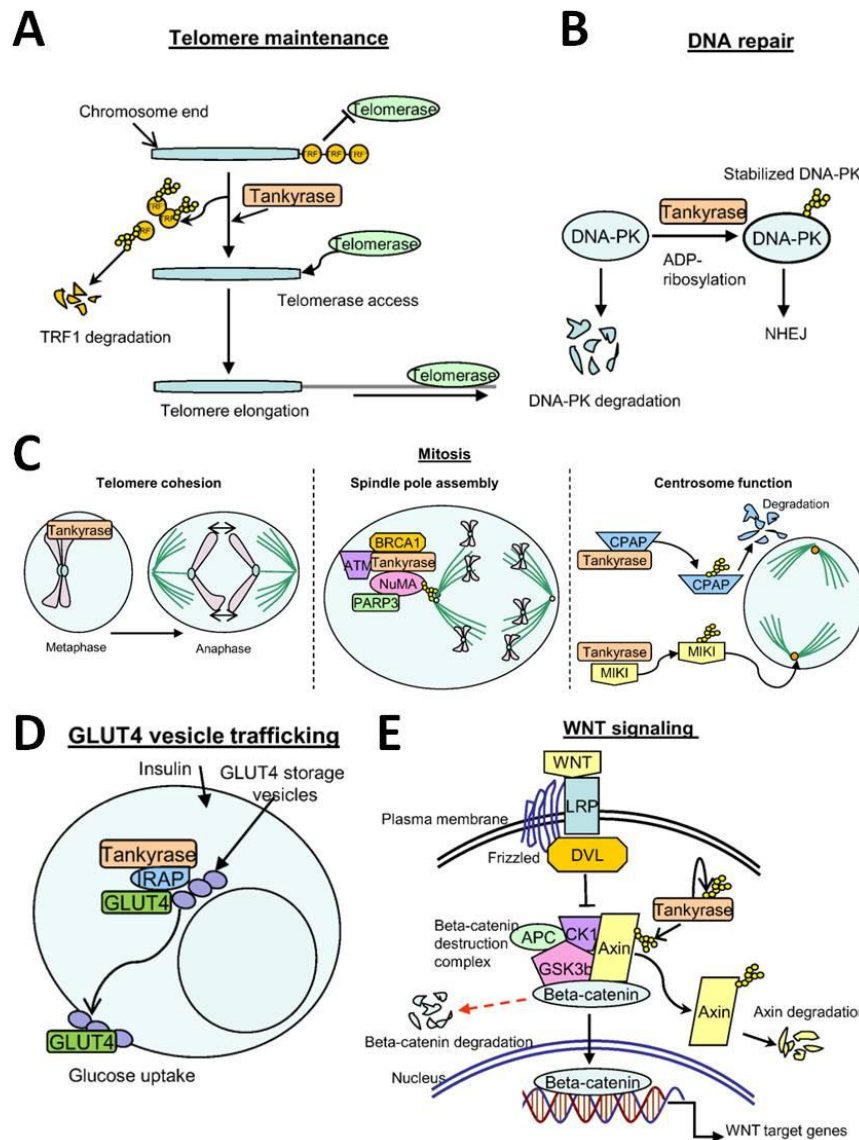


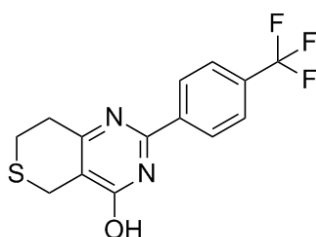
Figure 11. Tankyrases functions. (A) Tankyrases PARsylate and displace TRF1 from telomeres promoting their elongation by enabling telomerase function. (B) DNA-PK is a key player in NHEJ-mediated DNA repair. Tankyrases PARsylation leads to its stabilization. (C) Tankyrases display different roles in mitosis. They promote sister chromatid resolution before anaphase (left panel). They PARsylate NuMA ensuring spindle pole formation (middle panel). Finally, tankyrases regulate centrosome function by increasing degradation of the centrosome-associated protein CPAP and stabilizing MIK1. (D) Tankyrases regulate exocytosis of GLUT4-storage vesicles upon insulin stimulation through interaction with IRAP and also forming a ternary complex with AXIN/KIF3A. (E) Tankyrases promote Wnt signaling since they enhance AXIN1/2 degradation, which decreases the amount of Wnt degradation complexes that can be formed in the cell. For this reason, tankyrases increase β -catenin stability and transcription. Adapted from (Lupo and Trusolino, 2014)

2.4.4.3. Tankyrase inhibitors

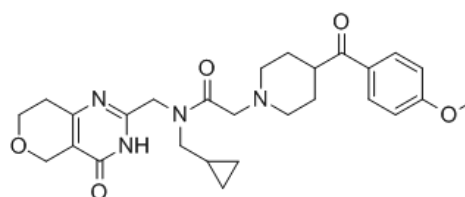
Since tankyrases were linked to the Wnt signaling pathway many groups have successfully developed effective inhibitors (Haikarainen et al., 2014). They can be classified in three categories depending on the pocket they bind: the nicotinamide subsite, the adenosine subsite or both (dual binders).

2.4.4.3.1. Inhibitors targeting the nicotinamide subsite

Tankyrase inhibitors targeting the nicotinamide pocket mimic the structure of nicotinamide with an additional scaffold to increase specificity. This pocket is actually poorly conserved in the PARP protein family but tankyrases possess a more hydrophobic pocket compared to other PARPs, which makes feasible the design of specific tankyrase inhibitors belonging to this subtype. The first molecule of this class described was XAV939, developed by Novartis (Huang et al., 2009) (**Figure 12**). In fact, the discovery XAV939 allowed establishing the link between tankyrases and Wnt signaling. Despite being a selective tankyrase inhibitor, it still presents some activity against PARP1 and PARP2. Several other compounds have been synthesized after XAV939 with better selectivity and bioavailability, reviewed in (Haikarainen et al., 2014).



XAV939



NVP-TNKS656

Figure 12. Chemical structures of two tankyrase inhibitors. XAV939 (left) and NVP-TNKS656 (right).

2.4.4.3.2. Inhibitors targeting the adenosine subsite

The adenosine subsite is a targetable pocket in the catalytic domain of all PARPs, however, inhibitors described so far are specific for tankyrases but not for other PARP members (Haikarainen et al., 2014). The first described molecules of this class were IWRs (Chen et al., 2009). Lately other compounds such as WIKI4 (James et al., 2012), JW74 (Waalder et al., 2011) or its derivate G007-LK (Lau et al., 2013) have been synthesized, being the latter a compound with a very interesting profile *in vivo* because of its high stability and good pharmacokinetics in mice.

2.4.4.3.3. Dual binders

The last category of tankyrase inhibitors comprises molecules that simultaneously bind both the nicotinamide and adenosine subsites. The first example described was a quinazolinone that showed enhanced activity towards tankyrases than PARP2 (Bregman et al., 2013). Novartis optimized the structure of XAV939, targeting the nicotinamide subsite, to obtain NVP-TNKS656 which works as a dual binder (**Figure 12**). NVP-TNKS656 demonstrated to be a highly potent and specific tankyrase inhibitor capable to inhibit Wnt-driven tumors *in vivo* and presenting good pharmacokinetic properties (Shultz et al., 2013).

For the purpose of this thesis we used the two tankyrase inhibitors developed by Novartis, XAV939 and NVP-TNKS656, to inhibit the Wnt signaling pathway.

3. Patient-derived xenografts in translational medicine

Many compounds in advanced clinical trials fail to progress because of a lack of efficacy that could have been predicted with better pre-clinical studies (Hidalgo et al., 2014; Johnson et al., 2001). Furthermore there is a need of using appropriate biomarkers for patient selection and to monitor response (Hidalgo et al., 2014). The most used and well studied systems are conventional cell lines that can be used *in vitro* or transplanted *in vivo* to generate xenografts. Cell lines were originally derived from cancer patient tumors and cultured in plastic dishes. This process is known to select clones that better adapt to grow in such artificial environment, leading to the expansion of the most aggressive cells and loss of cell heterogeneity present in the original tumor. Deep genetic characterization has shown that the panel of available CRC cell lines represents the majority of alterations found in CRC patients (Mouradov et al., 2014). However, despite they possess such genetic alterations present in the clinical setting, cell lines lack most of the potential to predict drug-response due to their homogeneity (Hidalgo et al., 2014).

Genetically engineered mouse models (GEMMs) have been an interesting approach to overcome some problems that cell lines present. They offer the possibility to study the role of the microenvironment and also that of the immune system in the development of the disease (McIntyre et al., 2015). However, mouse models for colorectal cancer do not fully recapitulate the human disease. One example is the APC^{min/+} model, which carries a truncating mutation in APC that causes the development of hundreds of low-grade adenomas. Although some adenomas progress to carcinomas, tumors are predominantly restricted to the small intestine being not frequent in the colon or rectum as observed in humans (Zeineldin and Neufeld, 2013). Such differences may be due to the fact that the APC mutation in APC^{min/+} model is a very severe C-terminal truncation not very frequent in CRC patients. Furthermore it is difficult that CRC GEMM develop metastasis, which limit the possibility to study late stages of the disease (McIntyre et al., 2015).

Patient-derived xenograft (PDX) models are the closest to the clinical setting and overcome many of the problems that conventional cell lines and GEMMs have. PDXs are derived from cancer patient tumors and implanted directly into immunodeficient mice, most commonly

subcutaneously or orthotopically. They are able to retain key characteristics of the original patient's tumor, such as its histopathologic architecture and genetic features. Moreover, they recapitulate the original tumor cell heterogeneity. For all these reasons, PDXs are nowadays the most suitable models for drug screening and biomarker identification, providing results that can be better translated to the clinic (Bertotti et al., 2011; Julien et al., 2012; Puig et al., 2013).

OBJECTIVES

Colorectal cancer is a disease with a relatively good prognosis when detected at early stages. In contrast, advanced and mCRC patients present a worse outcome because their tumors are typically highly resistant to available therapies. Hence, there is an urgent need to improve predictive biomarkers of drug-response and to develop better therapeutic agents. Furthermore, mechanisms of drug-resistance are poorly understood, what complicates even more the selection of effective treatments.

The goal of this project was to describe the determinants of resistance to PI3K/AKT pathway inhibitors in advanced mCRC and testing the efficacy of novel drug combinations for its overcoming.

The specific objectives were as follows:

- Study the role of the β -catenin and FOXO3a crosstalk on drug-resistance and its clinical implications in the context of CRC.
- Characterize the efficacy and determinants of response to treatment with PI3K, AKT and tankyrase inhibitors in CRC.
- Identify new predictive and pharmacodynamic biomarkers of response to such target-directed drugs.

Directors Report

The PhD student Oriol Arqués Casamitjana will present his doctoral thesis dissertation by scientific publications. The complete references, impact factors and his contribution for each article are pointed out next.

- **Article 1**

β -catenin confers resistance to PI3K and AKT inhibitors and subverts FOXO3a to promote metastasis in colon cancer.

Stephan P. Tenbaum, Paloma Ordóñez-Morán, Isabel Puig, Irene Chicote, **Oriol Arqués**, Stefania Landolfi, Yolanda Fernández, José Raúl Herance, Juan D. Gispert, Leire Mendizabal, Susana Aguilar, Santiago Ramón y Cajal, *Simó Schwartz Jr*, Ana Vivancos, Eloy Espín, Santiago Rojas, José Baselga, Josep Tabernero, Alberto Muñoz, Héctor G. Palmer.

Reference: Nature Medicine. 2012 Jun; 18(6):892-901. doi: 10.1038/nm.2772.

Journal Impact factor: 27.363

Contribution: Oriol Arqués participated in the experimental work of the article. In particular, he participated in the characterization of IQGAP2 role in colorectal cancer as a target gene of FOXO3a and β -catenin. He contributed to the establishment of IQGAP2 knockdown cells and their posterior analyses.

- **Article 2**

Tankyrase inhibition blocks Wnt/ β -catenin pathway and reverts resistance to PI3K and AKT inhibitors in the treatment of colorectal cancer

Oriol Arqués, Irene Chicote, Isabel Puig, Stephan P. Tenbaum, Guillem Argilés, Rodrigo Dienstmann, Natalia Fernández, Ginevra Caratù, Judit Matito, Daniel Silberschmidt, Jordi Rodon, Stefania Landolfi, Aleix Prat, Eloy Espín, Ramón Charco, Paolo Nuciforo, Ana Vivancos, Wenlin Shao, Josep Tabernero, Héctor G. Palmer

Reference: Clinical Cancer Research. 2015 Jul; 29. doi: 10.1158/1078-0432.CCR-14-3081. Pii: 3081.2014. [Epub ahead of print]

Journal Impact factor: 8.722

Contribution: Oriol Arqués was the responsible and performed most of the experiments. He also performed the analysis and helped in the interpretation of the data. He participated in the writing process of the manuscript and the posterior revisions.

Thesis Director

Dr. Héctor G. Palmer

RESULTS

1. ARTICLE 1

β -catenin confers resistance to PI3K and AKT inhibitors and subverts FOXO3a to promote metastasis in colon cancer.

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β -catenin confers resistance to PI3K and AKT inhibitors and subverts FOXO3a to promote metastasis in colon cancer

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The Wnt– β -catenin and PI3K–AKT–FOXO3a pathways have a central role in cancer. AKT phosphorylates FOXO3a, relocating it from the cell nucleus to the cytoplasm, an effect that is reversed by PI3K and AKT inhibitors. Simultaneous hyperactivation of the Wnt– β -catenin pathway and inhibition of PI3K–AKT signaling promote nuclear accumulation of β -catenin and FOXO3a, respectively, promoting cell scattering and metastasis by regulating a defined set of target genes. Indeed, the anti-tumoral AKT inhibitor API-2 promotes nuclear FOXO3a accumulation and metastasis of cells with high nuclear β -catenin content. Nuclear β -catenin confers resistance to the FOXO3a-mediated apoptosis induced by PI3K and AKT inhibitors in patient-derived primary cultures and in corresponding xenograft tumors in mice. This resistance is reversed by XAV-939, an inhibitor of Wnt– β -catenin signaling. In the presence of high nuclear β -catenin content, activation of FOXO3a by PI3K or AKT inhibitors makes it behave as a metastasis inductor rather than a proapoptotic tumor suppressor. We show that it is possible to evaluate the β -catenin status of patients' carcinomas and the response of patient-derived cells to target-directed drugs that accumulate FOXO3a in the nucleus before deciding on a course of treatment. We propose that this evaluation could be essential to the provision of a safer and more effective personalized treatment.

Colon cancer is a leading cause of death worldwide¹. Although surgical resection combined with adjuvant therapy is efficient at the early stages of disease, subsequent relapse and metastasis often occur. At these advanced stages, resistance to conventional therapies is frequent and treatments are ineffective². To overcome this resistance, a new generation of target-directed drugs is being tested in clinical trials. Therefore, a better understanding of the molecular mechanisms responsible for patients' responses to these drugs is crucial to identify new predictive biomarkers and facilitate treatment decisions.

Abnormal activation of the wingless related MMTV integration site (Wnt)– β -catenin pathway by mutations is responsible for the initiation of more than 90% of colon cancers³. As a result, β -catenin accumulates in the nucleus, binds the T cell factor (TCF) or lymphoid enhancer factor (LEF) transcription factors and

induces the expression of Wnt target genes that have key roles in tumor progression⁴. We and others have reported that binding of β -catenin to different transcription factors enhances the expression of alternative sets of target genes^{5–7}. Forkhead box O3 (FOXO3a) is one of those transcription factors for which β -catenin acts as a transcriptional coactivator, enhancing the expression of common target genes⁸. However, the possible physiological relevance of the crosstalk between FOXO3a and β -catenin in cancer has not been addressed.

FOXO proteins control multiple processes in eukaryotic organisms by regulating the expression of specific sets of genes involved in stem-cell maintenance, stress resistance, longevity, differentiation or metabolism^{9,10}. In cancer, FOXO transcription factors are considered to be tumor suppressors because they induce cell-cycle arrest and apoptosis¹¹.

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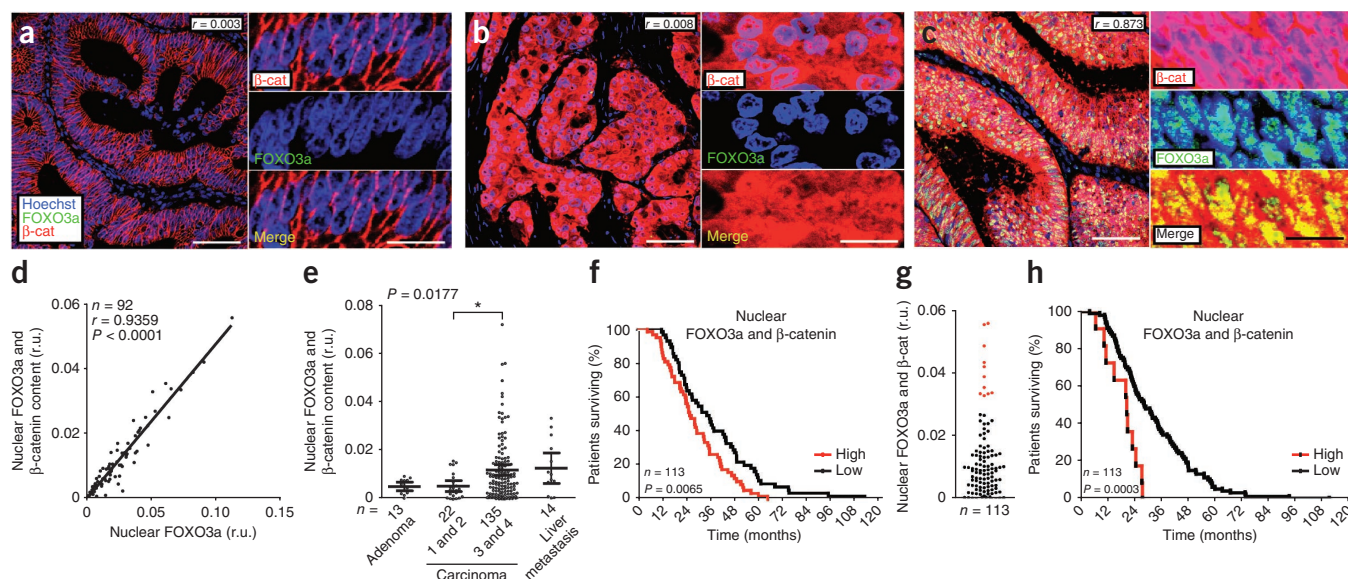


Figure 1 High nuclear concentrations of FOXO3a and β -catenin correlate with metastatic stage and shorter survival in patients with colon cancer. (a–c) Representative immunostained sections (left, scale bars, 100 μ m) and the respective magnifications (right, scale bars, 20 μ m) of tumors with low β -catenin (β -cat) and low FOXO3a expression (a), high β -catenin and low FOXO3a expression (b) and high β -catenin and high FOXO3a expression (c). FOXO3a is shown in green, β -catenin is shown in red, and Hoechst 33342 is shown in blue. The Spearman's coefficient of nuclear colocalization (r) is indicated (b,c). The merged magnified images lack blue channels, and yellow color indicates FOXO3a and β -catenin colocalization. (d) Plot depicting nuclear FOXO3a and β -catenin immunofluorescent signal intensity in human colon carcinomas. Correlation was evaluated by nonparametric Spearman test. The number of cases (n), coefficient of correlation (r) and P values are indicated. Relative units (r.u.) are calculated as described in the Online Methods. (e) Column scatter plot showing the amount of FOXO3a and β -catenin colocalization in tumors at different stages of colon-cancer progression. Horizontal lines indicate arithmetic mean values, and error bars show the 95% CI. The number of cases (n) and P values from nonparametric analysis of variance (ANOVA) analysis with Kruskal-Wallis test are indicated. Asterisk indicates a significant difference ($P < 0.05$) between groups as quantified by Dunn's multiple comparison test. (f) Kaplan-Meier survival analysis of patients with colon cancer separated into two groups by the median for the nuclear FOXO3a and β -catenin colocalization signal. The higher signal is shown in red ($n = 58$), and the lower signal is shown in black ($n = 55$). P values were calculated by log-rank (Mantel-Cox) test. (g) Plot representing the nuclear immunofluorescent signal for FOXO3a and β -catenin colocalization. Dots of the highest decile values are shown in red. (h) Kaplan-Meier survival analysis of patients with colon cancer separated into two groups by the highest decile for the nuclear FOXO3a and β -catenin colocalization signal. The higher signal is shown in red ($n = 11$), and the lower signal is shown in black ($n = 102$). P values were calculated by log-rank (Mantel-Cox) test.

Phosphorylation of FOXO proteins by activated v-akt murine thymoma viral oncogene homolog (AKT) or serum/glucocorticoid regulated kinase 1 (SGK1) induces their sequestration in the cytoplasm and the consequent inhibition of their transcriptional activity^{12,13}. AKT is part of the rat sarcoma (RAS)-phosphoinositide-3-kinase (PI3K)-phosphatase and tensin homolog (PTEN)-AKT-mammalian target of rapamycin (mTOR) oncogenic pathway, which is frequently altered by activating mutations in colon and many other cancers¹⁴. Concerted efforts have been made in both academia and industry to identify specific anti-tumoral inhibitors of PI3K or AKT that relocate FOXO proteins back into the nucleus and thus restore their capacity to induce cell-cycle arrest and apoptosis¹⁵. In this study, we used the PI3K inhibitor NVP-BKM120 and the AKT signaling inhibitor-2 (API-2), which inhibit cell growth and induce apoptosis in human cancer cells in culture and in tumors growing in mice^{16,17}. Phase 1 and 2 clinical trials with both compounds have been conducted in patients with solid metastatic cancers with promising results^{18–20}.

Considering the role of the Wnt- β -catenin and PI3K-AKT-FOXO3a pathways in tumorigenesis and the interaction of FOXO3a and β -catenin, we studied their crosstalk in colon cancer. We found that the joint action of the two transcription factors induced metastasis and that β -catenin conferred resistance to FOXO-induced apoptosis. Inhibitors of PI3K and AKT not only did not induce apoptosis *in vitro* or *in vivo*, but they also promoted metastasis when the concentration of nuclear β -catenin was high in colon-cancer cells.

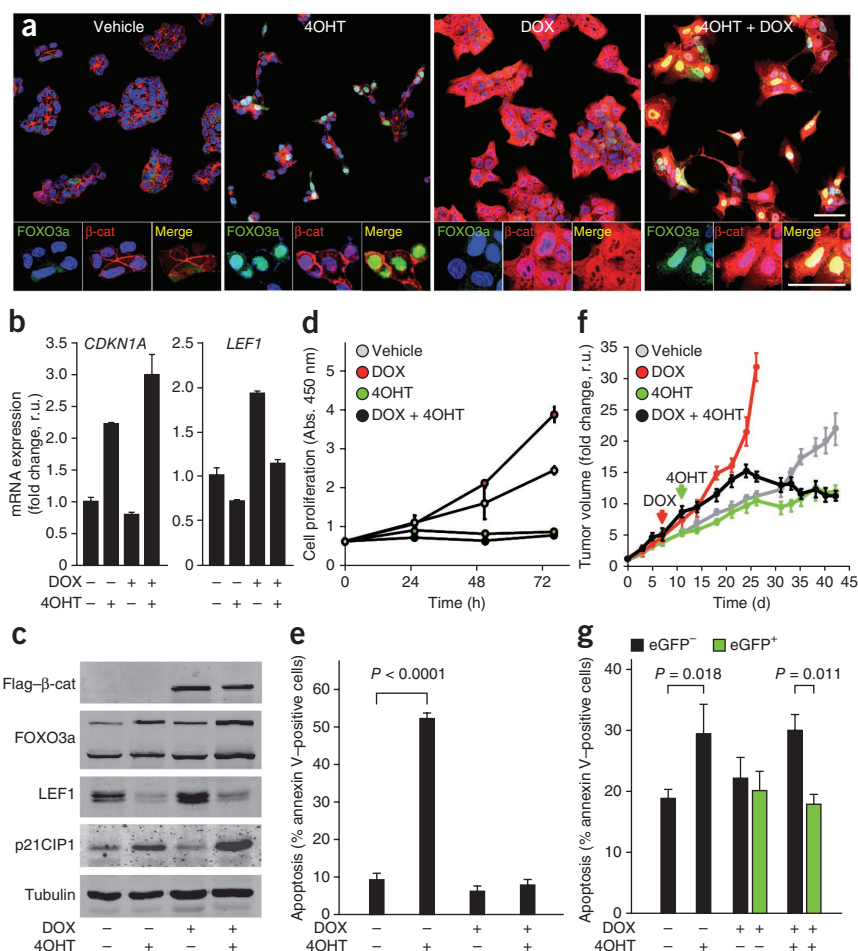
By studying the response of patient-derived colon-cancer-initiating cells to such target-directed drugs, we highlight the clinical relevance of our findings.

RESULTS

Nuclear FOXO3a and β -catenin concentrations in colon cancer

To assess the amount of nuclear FOXO3a and β -catenin in human colon cancer, we performed double immunofluorescent staining on paraffin sections of tumors at different stages. Nuclear FOXO3a accumulated alone in nonmalignant adenomas but always colocalized with β -catenin in the nuclei of tumoral cells in carcinomas and liver metastasis. We also detected carcinomas with high concentrations of nuclear β -catenin but low concentrations of nuclear FOXO3a. We found the highest nuclear concentrations of both factors in a subset of primary carcinomas at stages 3 or 4 from patients with metastatic disease (Fig. 1a–e and Supplementary Fig. 1a–c). Patients with high nuclear FOXO3a and β -catenin colocalization signals had, on average, an 8-month shorter survival time than patients with low colocalization signals. The median survival time was 33.7 months for patients with low nuclear FOXO3a and β -catenin signals and was 25.7 months for patients with high nuclear FOXO3a and β -catenin signals (hazard ratio (HR) 1.724, 95% CI 1.164–2.552). When we were more restrictive and analyzed the group of patients with the top 10% highest colocalization signals, the median survival was even shorter, 21.03 months, whereas it was 28.9 months for

Figure 2 FOXO3a and β -catenin modulate colon cancer cell proliferation and apoptosis. (a) Immunofluorescence analysis of FOXO3a (green) and β -catenin (red) expression in DLD-1F β -C7 cells treated as indicated. Scale bar, top images, 50 μ m. Below are magnifications of the upper images. Scale bar, bottom images, 20 μ m. Nuclei were stained with Hoechst 33342 (blue). The merged magnification images lack the blue channel, and yellow color indicates FOXO3a and β -catenin colocalization. DOX, doxycycline. (b) Normalized relative *CDKN1A* and *LEF1* RNA expression levels in DLD-1F β -C7 cells treated with doxycycline, 4OHT or both measured by real-time RT-PCR. (c) The amounts of the indicated proteins in DLD-1F β -C7 cells as assessed by western blot. Flag-tag antibody detects exogenous Flag-tagged S33Y β -catenin. FOXO3a antibody recognizes endogenous protein (lower band) and FOXO3a3A-ERTM fusion protein (upper band). Tubulin served as the loading control. (d) Graphic showing the proliferation of DLD-1F β -C7 cells treated as indicated. Abs., absorbance. (e) Chart depicting the percentage of apoptotic DLD-1F β -C7 cells in response to the indicated treatments as measured by flow cytometry. The *P* value corresponds to an unpaired *t* test. Error bars show $\pm$ s.d. of triplicates from three independent experiments (b,d,e). (f) Tumor growth in nude mice (*n* = 8, two tumors per mouse) injected subcutaneously with DLD-1F β cells and treated as indicated. Error bars show means $\pm$ s.d. of the volume of 16 tumors per treatment. (g) Graphic representing the percentage of apoptotic subpopulations positive (green bars) or negative for S33Y β -catenin-eGFP (black bars) of DLD-1F β cells in tumors (*n* = 6) growing in nude mice treated as indicated. Error bars show means $\pm$ s.d. of the volume of six tumors per treatment. *P* values correspond to unpaired *t* tests.



the other 90% of individuals who had lower colocalization signals (HR 6.392, 95% CI 2.322–17.6). Nuclear FOXO3a alone also correlated with metastatic stage and shorter median survival time (low FOXO3a content, 32.57 months, compared to high FOXO3a content, 25.37 months, HR 1.684, 95% CI 1.137–2.493). In contrast, β -catenin alone did not predict metastasis stage or survival time (HR 1.212, 95% CI 0.8331–1.765) (Fig. 1f–h and Supplementary Fig. 1c–f).

β -catenin diverts FOXO3a-induced apoptosis to metastasis

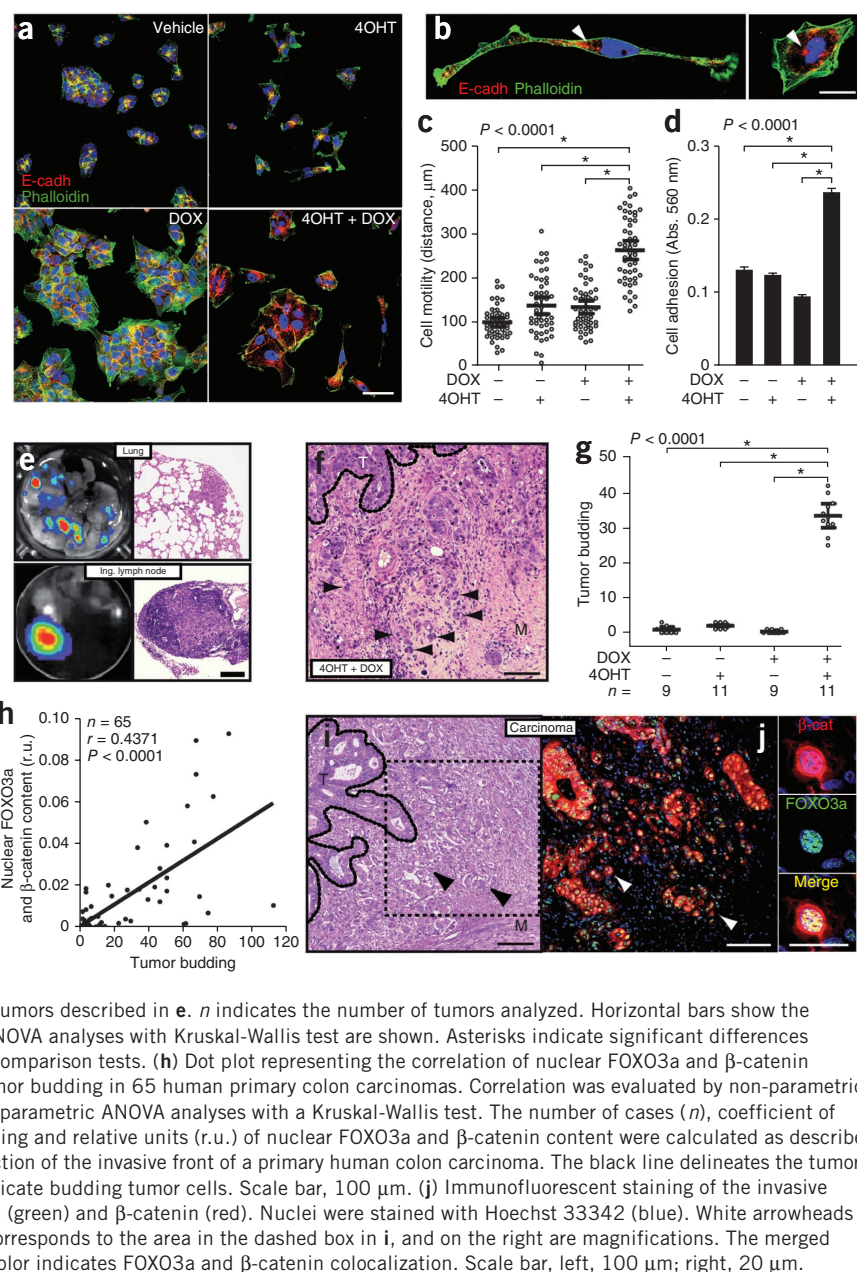
We expressed a tamoxifen-inducible (4-hydroxy-tamoxifen, 4OHT) AKT-insensitive mutant of FOXO3a (FOXO3a3A-ERTM)²¹ and a stable β -catenin S33Y mutant that is inducible by doxycycline in a clone of DLD-1 human colon cancer cells (DLD-1F β -C7). The mutant β -catenin was expressed with a lentivirus, together with enhanced GFP (eGFP) using an internal ribosome entry site (IRES) (Online Methods). Exogenous FOXO3a and β -catenin proteins accumulated in the nucleus after the corresponding 4OHT or doxycycline treatments (Fig. 2a and Supplementary Fig. 2), reaching concentrations in the nucleus that were comparable to those caused by hydrogen peroxide, in the case of FOXO3a (Supplementary Fig. 2a,b,d), or, in the case of β -catenin, to those present in cancer cell lines with naturally occurring β -catenin accumulation, such as the HCT116 or SW480 human colon cancer cell lines (Supplementary Fig. 2a,c,d). β -catenin enhanced the induction of the known FOXO3a target gene *CDKN1A*

(also known as p21CIP1) that resulted from treatment with 4OHT. Likewise, nuclear β -catenin accumulation resulting from doxycycline treatment induced the TCF target gene *LEF1*, whose expression was reduced by FOXO3a (4OHT) (Fig. 2b,c)^{22,23}. FOXO3a alone reduced cell proliferation by accumulating cells in G1 phase of the cell cycle²⁴, whereas β -catenin alone increased cell proliferation. When both factors were expressed simultaneously in the nucleus, the cells had a reduced proliferation, similar to that seen with FOXO3a alone, indicating its dominant effect over β -catenin (Fig. 2d and Supplementary Fig. 3a,b). Separately, FOXO3a induced apoptosis, whereas β -catenin did not have a major effect. Nuclear accumulation of both factors resulted in a complete rescue of FOXO3a-induced apoptosis by β -catenin independently of proliferation, as this effect also occurred in cells arrested in G1 phase using pretreatment with mitomycin C (Fig. 2e and Supplementary Fig. 3c–e).

We injected a pool of DLD-1F β cells subcutaneously into nude mice that were all positive for FOXO3a3A-ERTM, although only 62% of the mice expressed S33Y β -catenin and eGFP. This mixed pool of cells recapitulated a heterogeneity in the nuclear concentrations of β -catenin that was similar to that detected in human colon carcinomas (Supplementary Figs. 4a–d and 5a,b). In mice treated with doxycycline, tumors accumulated high amounts of nuclear β -catenin and grew more rapidly, whereas those treated with 4OHT had increased nuclear FOXO3a content and grew more slowly compared to control

Figure 3 Nuclear FOXO3a and β -catenin drive cell scattering and metastasis.

(a) Immunofluorescence analysis of DLD-1F β -C7 cells treated as indicated to detect E-cadherin (E-cadh) (red) and phalloidin-FITC-stained (phalloidin) actin filaments (green). Scale bar, 100 μ m. (b) Immunofluorescence analysis of E-cadherin in DLD-1F β -C7 cells treated with 4OHT and doxycycline. Arrowheads indicate E-cadherin cytosolic aggregates. Scale bar, 10 μ m. Nuclei were stained with Hoechst 33342 (blue; a,b). (c) Scatter plot representing the motility of DLD-1F β -C7 cells treated as indicated showing the distance covered by 50 individual cells in a time-lapse video recording one image every 20 min during 120 h. Horizontal bars indicate means, and error bars show the 95% CI. (d) Graphic representing adhesion to the substrate of DLD-1F β -C7 cells treated as indicated 5 min after seeding. Error bars show means $\pm$ s.d. of six replicates from two independent experiments. *P* values for non-parametric ANOVA analyses with Kruskal-Wallis test are shown. Asterisks indicate significant differences ($P < 0.0001$) between groups using Dunn's multiple comparison tests (c,d). (e) Bioluminescence imaging of organs obtained from necropsy of nude mice bearing orthotopic tumors from HT29F β cells injected in the cecum walls of the mice. Images overlaid with a gradual bioluminescent signal (blue to red) that originated from luciferase-expressing metastatic cells (left). H&E staining of the corresponding histological sections. Ing., inguinal. Scale bar, 100 μ m. (f) H&E-stained histological section of the invasive front of a primary tumor xenograft from HT29F β cells growing in the cecum walls of nude mice treated with 4OHT and doxycycline described in e. Scale bar, 100 μ m. (g) Column scatter plot presenting tumor budding in the primary tumors described in e. *n* indicates the number of tumors analyzed. Horizontal bars show the mean and the 95% CI. *P* values for non-parametric ANOVA analyses with Kruskal-Wallis test are shown. Asterisks indicate significant differences ($P < 0.0001$) between groups using Dunn's multiple comparison tests. (h) Dot plot representing the correlation of nuclear FOXO3a and β -catenin immunofluorescence signals with the incidence of tumor budding in 65 human primary colon carcinomas. Correlation was evaluated by non-parametric Spearman test, and *P* values were determined by non-parametric ANOVA analyses with a Kruskal-Wallis test. The number of cases (*n*), coefficient of correlation (*r*) and *P* values are indicated. Tumor budding and relative units (r.u.) of nuclear FOXO3a and β -catenin content were calculated as described in the Online Methods. (i) H&E-stained histological section of the invasive front of a primary human colon carcinoma. The black line delineates the tumor edge. T, tumor bulk; M, muscle. Black arrowheads indicate budding tumor cells. Scale bar, 100 μ m. (j) Immunofluorescent staining of the invasive front of a human primary colon carcinoma for FOXO3a (green) and β -catenin (red). Nuclei were stained with Hoechst 33342 (blue). White arrowheads indicate budding tumor cells. The image on the left corresponds to the area in the dashed box in i, and on the right are magnifications. The merged magnified image lacks the blue channel, and yellow color indicates FOXO3a and β -catenin colocalization. Scale bar, left, 100 μ m; right, 20 μ m.

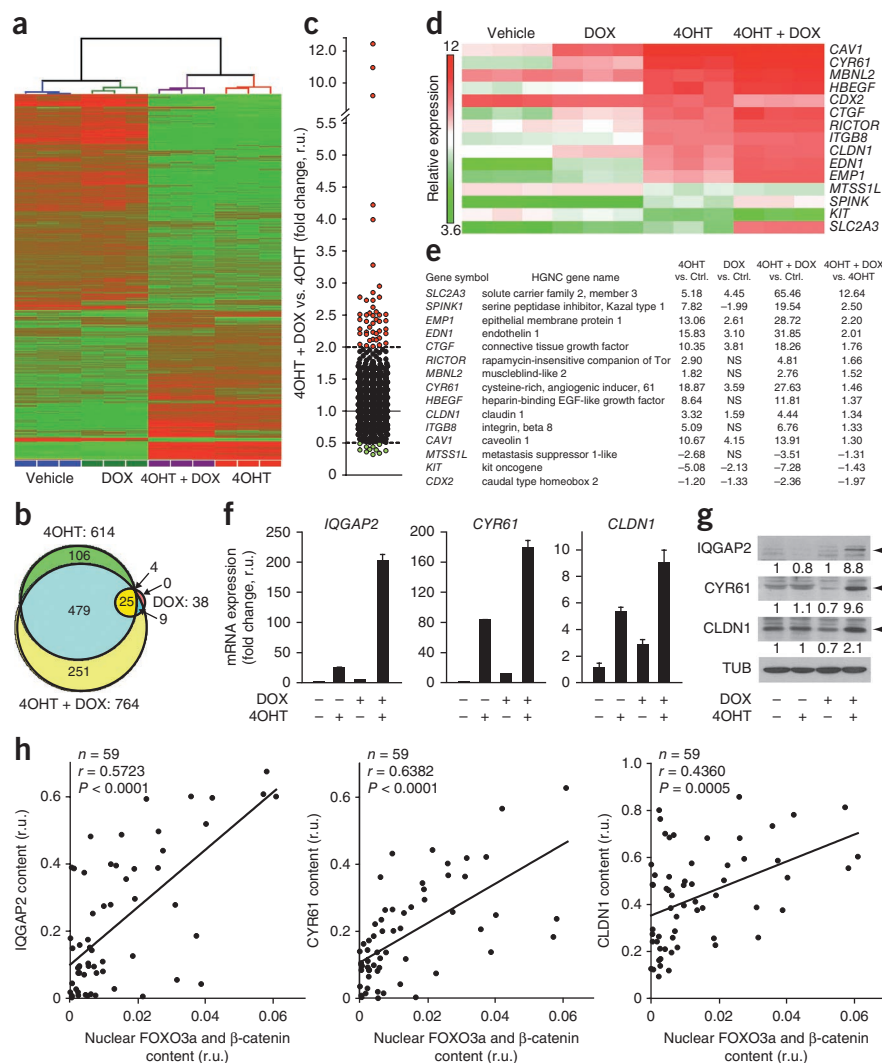


mice treated with vehicle (Fig. 2f). Combined treatment with doxycycline and 4OHT resulted in tumors with similar sizes at the end of the experiment as those treated with 4OHT alone. We observed that FOXO3a induced apoptosis only in DLD-1F β cells lacking β -catenin expression (eGFP⁻ cells) both in culture and in xenograft tumors (Fig. 2g and Supplementary Fig. 4e). Within the tumor tissue, this resulted in high amounts of cell death, which was partially rescued by β -catenin expression (Supplementary Fig. 5c,d). We confirmed that nuclear accumulation of FOXO3a and β -catenin was significantly ($P < 0.0001$) increased as a result of treatment with 4OHT and doxycycline, respectively (Supplementary Fig. 5e,f).

Cells accumulating high amounts of both FOXO3a and β -catenin scattered and lost their epithelial phenotype in subconfluent cell cultures and *in vivo*. Nuclear FOXO3a alone also promoted a change in cell shape, but this phenotype is typical of the cell detachment associated with apoptosis (Fig. 2a and Supplementary Figs. 5f and 6a).

We detected relocation of E-cadherin protein from cell-to-cell contacts to cytoplasmic aggregates in cells in which FOXO3a and β -catenin induced this scattering phenotype (Figs. 2a and 3a,b and Supplementary Figs. 5f and 6a,b,e,f). We did not detect E-cadherin protein fragments, a repression of E-cadherin expression or an increase in the concentrations of Snail1, zinc finger E-box binding homeobox 1 (Zeb1) or Slug in these cells as a result of accumulation of FOXO3a, β -catenin or both. This ruled out E-cadherin proteolysis or the canonical epithelial-to-mesenchymal transition as mechanisms of the cell-to-cell detachment (Supplementary Fig. 6c,d). Further, these morphological changes correlated with increased cell motility and adhesion to substrate (Fig. 3c,d and Supplementary Fig. 6e-g). Although a small proportion of cells developed an increased size and extended cytoplasm that resembled the morphology of senescent cells, they were negative for senescence-associated β -galactosidase activity (Fig. 2a and Supplementary Fig. 6b,f,h).

Figure 4 FOXO3a and β -catenin regulate a defined gene expression program. **(a)** Gene clustering diagram calculated using robust-multiarray-normalized expression values from the genome-wide microarray analysis. Triplicates of each treatment are indicated as colored rectangles at the bottom. Significance was calculated using two-tailed ANOVA with a significance cutoff of $P \leq 0.05$. Gene expression scaling, low (green) to high (red). **(b)** Venn diagram showing the number of genes regulated by the indicated treatments using a high-stringency analysis (with a Bonferroni cutoff $P \leq 0.05$) and having a twofold change compared to control in at least one of the conditions (**Supplementary Table 2**). **(c)** Plot representing changes in the relative expression of regulated genes comparing 4OHT and doxycycline with 4OHT alone. Red, higher than twofold induction; green, lower than twofold repression. **(d)** Metastasis signature. Heat map of robust-multiarray-normalized expression values for the genes involved in metastasis. Triplicates for each treatment are shown. Green, low expression; red, high expression. **(e)** Gene expression values of metastasis signature genes shown as a fold change compared to control vehicle-treated cells (Ctrl.) for the indicated treatments. The official gene symbols and HGNC gene names are listed. NS, not significant. **(f)** Mean relative mRNA expression levels of *IQGAP2*, *CYR61* and *CLDN1* transcripts measured by real-time RT-PCR in DLD-1F β -C7 cells after the indicated treatments. Error bars, triplicate values $\pm$ s.d. obtained in three independent experiments. **(g)** Western blot analysis of the indicated proteins in DLD-1F β -C7 cells. Numbers below the blot indicate the relative amount of each protein compared to the amount of the protein in vehicle-treated cells. All values are corrected by the respective tubulin signal that served as the loading control. **(h)** Dot plot showing the immunofluorescent signal intensity for each indicated protein correlated to the nuclear FOXO3a and β -catenin colocalization signal intensity in 59 human colon carcinomas. Correlation was evaluated by non-parametric Spearman test. The number of patients (n), coefficient of correlation (r) and P values are indicated. Relative units (r.u.) were calculated as described in the Online Methods.



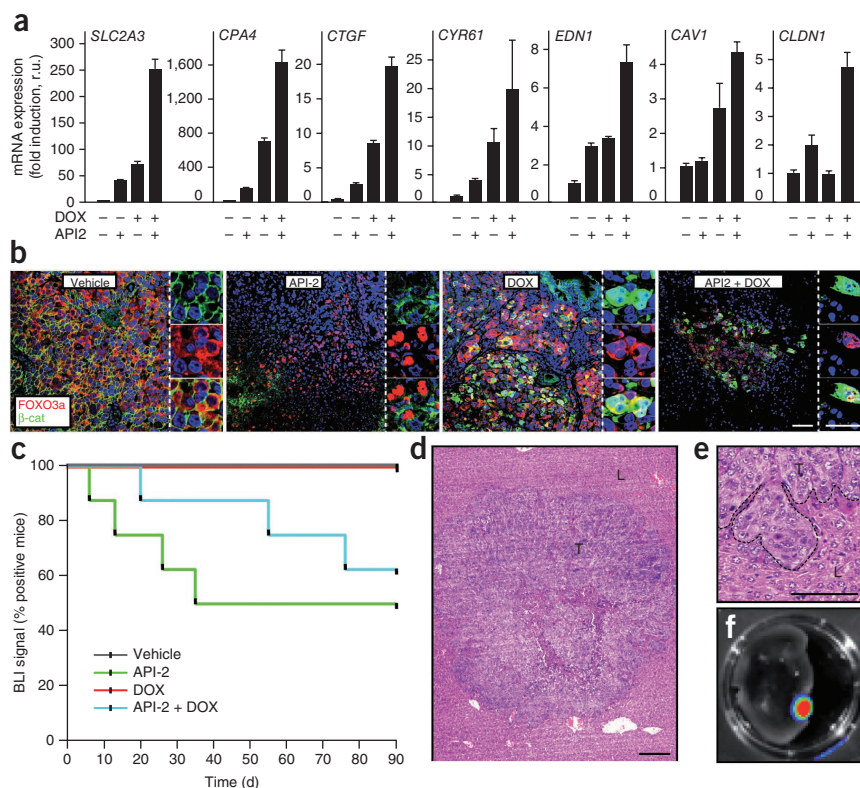
We then tested the metastatic potential of cells expressing high amounts of nuclear FOXO3a and β -catenin, but no tumor was formed when we injected 1×10^6 DLD1-F β cells in the cecum walls of 50 nude mice. Thus, we engineered the same inducible systems to express exogenous FOXO3a and β -catenin as those used in DLD1-F β cells in human HT29 colon cancer cells (HT29F β). Treatment with doxycycline and 4OHT promoted equivalent effects on proliferation, apoptosis and cell scattering in HT29F β cells as those previously observed in DLD1-F β cells (**Supplementary Fig. 7**). We then injected HT29F β cells into the cecum walls of immunodeficient nude mice to assess whether the scattering phenotype induced by FOXO3a and β -catenin was indicative of an enhanced metastatic potential. We used a pool of these cells, all of which expressed FOXO3a3A-ERTM and a luciferase reporter but only 41% of which contained the S33Y β -catenin-IRES-eGFP construct (Online Methods).

We observed an increase in tumor bioluminescent signal in immunodeficient nude mice treated with doxycycline that decreased or even disappeared in mice treated with 4OHT and that was weak in mice treated with both drugs together (**Supplementary Fig. 8a,b**).

We performed exhaustive necropsies and *ex vivo* analyses of the internal organs of these mice using luminometry. We used histology to analyze tissues positive for bioluminescence to confirm metastases. We saw metastases in significantly more mice that had combined nuclear accumulation of FOXO3a and β -catenin than in any other group of mice in the experiment ($P = 0.022$), with the lungs being the most significant dissemination site of the metastases ($P = 0.038$) (**Fig. 3e** and **Supplementary Fig. 8c,d**). A control experiment conducted with parental luciferase-expressing HT29 cells that did not express exogenous FOXO3a or β -catenin did not show any such differences in metastasis frequencies after analogous 4OHT or doxycycline treatments (**Supplementary Fig. 8e**). Treatment with 4OHT induced nuclear FOXO3a accumulation, leading to marked intratumoral cell death, which was rescued by β -catenin (**Supplementary Fig. 9a,b**).

E-cadherin relocation, loss of epithelial organization and cell scattering occurred only in the cell population that was positive for the S33Y β -catenin-eGFP mutant in mice treated with both 4OHT and doxycycline (**Supplementary Fig. 9b-d**). Tumor budding was significantly increased in primary carcinomas growing in

Figure 5 AKT inhibition by API-2 promotes metastasis of cells with high nuclear β -catenin content. **(a)** Relative mRNA levels of the indicated genes measured by real-time RT-PCR in DLD-1F β -C7 cells treated with API-2, doxycycline or both, as indicated. Error bars, $\pm$ s.d. of triplicates from three independent experiments. **(b)** Immunofluorescence analysis of FOXO3a (red) and β -catenin (green) expression in primary tumors growing in the cecum walls of nude mice. Scale bar, left images, 100 μ m. Magnification of cells within the primary tumors (right). Scale bar, right images, 25 μ m. Nuclei were stained with Hoechst 33342 (blue). **(c)** Kaplan-Meier analysis of bioluminescence (BLI) showing the percentage of nude mice injected in the cecum wall with HT29F β cells and treated as indicated that retained the signal over time. The number of mice used is listed in **Supplementary Table 5**. **(d)** H&E staining of liver metastasis from a cecum-injected mouse treated with API-2 and doxycycline. T, tumor; L, liver tissue. **(e)** A magnification of the image in **d** (top). The dashed line indicates the tumor-liver edge. *Ex vivo* image of infiltrated liver overlaid with gradual false-colored bioluminescent signal (blue, low; red, high) (bottom). Scale bar, **d**, 200 μ m; **e**, 100 μ m.



the cecum walls of mice treated with both 4OHT and doxycycline (**Fig. 3f,g**). We also found nuclear colocalization of FOXO3a and β -catenin in scattered cells at the invasive fronts of human colon carcinomas (**Fig. 3h–j**). By measuring the FOXO3a and β -catenin colocalization signal in the tumor bulk, we found that it had a positive correlation with tumor budding at the invasive front. Only a minor proportion of carcinomas had an increased accumulation of these factors at the invasive front, as has been previously described for β -catenin (**Supplementary Fig. 10**)²⁵.

FOXO3a and β -catenin co-regulate metastasis-relevant genes

Transcriptomic analyses showed that FOXO3a alone provoked a substantial change in the gene expression pattern of DLD-1F β cells²⁶, whereas β -catenin alone produced only a minor effect in these cells (**Fig. 4a,b** and **Supplementary Tables 1** and **2**). However, coexpression of β -catenin and FOXO3a enhanced the expression of many known or undescribed FOXO3a-induced target genes (**Fig. 4c**, **Supplementary Fig. 11a,b** and **Supplementary Tables 1** and **2**). In contrast, other genes involved in cell differentiation, such as *MUC13*, *VIL1* or *CDX2*, were repressed by nuclear FOXO3a, and the concomitant presence of nuclear β -catenin further enhanced such inhibition.

β -catenin enhances the expression of many FOXO3a target genes that are involved in cytoskeleton remodeling, cell shape and motility (**Supplementary Table 3**). The products of some of these genes are part of the protein complexes that control the stability of cell-to-cell contacts (**Supplementary Fig. 11c,d**)²⁷. *IQGAP2* is one of these target genes that is involved in the stability of E-cadherin at adherent junctions^{28,29}. The knockdown of *IQGAP2* expression by shRNAi partially preserved the amount of E-cadherin that was associated with the actin cortex at cell-to-cell contacts in DLD1-F β -C7 cells treated with 4OHT and doxycycline. The induction of cell motility that was promoted by nuclear FOXO3a and β -catenin was also significantly reduced when we knocked down *IQGAP2* ($P = 0.0467$). We also

observed that *IQGAP2* expression correlated with tumor budding in a set of 59 human colon carcinomas (**Supplementary Fig. 12**). We found *IQGAP2* in association with E-cadherin aggregates in the cytoplasm of scattering DLD1-F β -C7 cells in culture or in subcutaneous xenografts as a result of FOXO3a and β -catenin nuclear colocalization and in cancer cells with disrupted epithelial organization in human colon carcinomas (**Supplementary Fig. 13**).

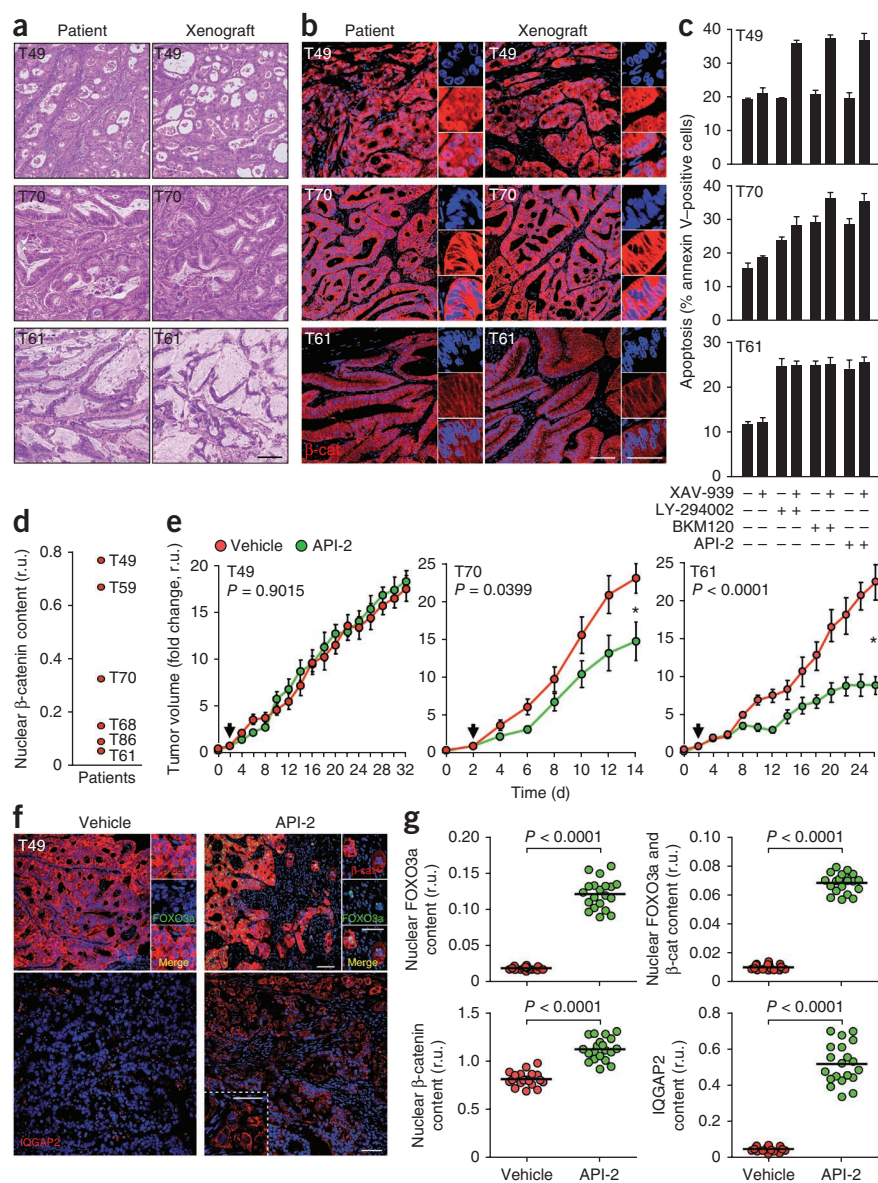
FOXO3a and β -catenin regulated a defined set of metastasis-relevant genes (**Fig. 4d,e** and **Supplementary Table 4**). In addition to *IQGAP2*, we also studied *CYR61* and *CLDN1*, which have both been reported to be associated with cell-to-cell disassembly and cell scattering, motility and metastasis^{30,31}. The concentrations of nuclear FOXO3a alone or FOXO3a and β -catenin together correlated with the amount of protein expression of each of these three target genes in a set of 59 primary human colon carcinomas. The amount of nuclear β -catenin did not have any significant correlation with the protein expression of these genes (**Fig. 4f–h** and **Supplementary Figs. 14** and **15**).

A gene set enrichment analysis showed that FOXO3a regulated genes involved in apoptosis, whereas its coexpression with β -catenin also modulated genes controlling cell morphology, adhesion and motility. Nuclear β -catenin attenuated the upregulation of the proapoptotic genes *TNFSF10* and *BCL2L11*, and it synergized with FOXO3a to induce the upregulation of *FASLG* and the anti-apoptotic gene *HIPK3*. Moreover, β -catenin caused nuclear translocation of FAS-associated adaptor protein (FADD) and inhibited the poly (ADP-ribose) polymerase 1 (PARP1) cleavage that was induced by FOXO3a (**Supplementary Figs. 11a** and **16** and **Supplementary Table 3**).

AKT inhibition induces metastasis cooperating with β -catenin

The phosphorylation of FOXO3a by AKT translocates it from the nucleus to the cytoplasm. We therefore used the anti-tumoral drug API-2, which specifically inhibits AKT, to relocate FOXO3a back to

Figure 6 High nuclear β -catenin concentrations confer resistance to PI3K and AKT inhibitors in colon cancer patient-derived cells. **(a)** H&E staining of the primary carcinomas of the patients (left) and subcutaneous tumors (right, xenograft) generated in NOD-SCID mice from the corresponding patient-derived cells. Scale bar, 100 μ m. **(b)** Immunofluorescent staining for β -catenin (red) in the primary carcinomas of the patients (left) and the corresponding subcutaneous xenograft tumors in mice (right). The smaller images on the right correspond to magnifications that show the amount of nuclear β -catenin. Nuclei were stained with Hoechst 33342 (blue). Scale bar, left images, 100 μ m; right images, 50 μ m. **(c)** Percentages of apoptotic cells in patient-derived sphere cultures treated as indicated. Apoptosis was measured by flow cytometry. Bars represent the mean of three independent experiments. Error bars, $\pm$ s.d. Cells were treated with the indicated compounds as described in the Online Methods. **(d)** Graph showing the amount of nuclear β -catenin measured by immunofluorescence in the primary colon carcinomas of each indicated patient. Relative units (r.u.) were calculated as described in the Online Methods. **(e)** Cancer cells derived from tumors from the three indicated patients were injected subcutaneously in NOD-SCID mice and treated with API-2 or vehicle ($n = 5$, two flanks per mouse). Arrows mark the initiation of treatment. The represented fold change was calculated by comparing the tumor volume at each given time point to the volume at the first day of treatment. Relative units (r.u.) were calculated as described in the Online Methods. Error bars, $\pm$ s.d. of ten tumors per treatment. P values correspond to non-parametric Mann-Whitney test. Asterisks indicate significant differences (with the P values indicated on the graphs) between the volumes of the tumors growing in mice treated with vehicle compared to mice treated with API-2. **(f)** Immunofluorescent staining for FOXO3a (green) and β -catenin (red) in histological sections of subcutaneous xenografted tumors from patient T49 treated as indicated (top images). The images on the right are magnifications that show the amounts of nuclear FOXO3a and β -catenin. Scale bar, left images, 100 μ m; right images, 50 μ m. Immunofluorescent staining for IQGAP2 (red) in the same xenograft tumors (bottom images). The insert shows a magnification of the cells with IQGAP2 accumulated in their cytoplasmic aggregates. Scale bar, main images, 100 μ m; insert, 50 μ m. Nuclei were stained with Hoechst 33342 (blue). **(g)** Column scatter plots showing the expression of each indicated protein in xenografted tumors from patient T49 treated as labeled. Five images were quantified for each of the four tumors treated. P values correspond to non-parametric Mann-Whitney test comparing the values of protein expression in each quantified picture. Relative units (r.u.) were calculated as described in the Online Methods.



the nucleus. Nuclear FOXO3a accumulation resulting from treatment with 4OHT or the AKT inhibitor API-2 promoted the scattering of DLD-1F β cells when concomitant high nuclear concentrations of β -catenin were induced by doxycycline. Some of the FOXO3a and β -catenin target genes defined in our metastasis signature were induced by API-2 and were further enhanced by β -catenin in DLD1-F β -C7 cells (Figs. 4d,e and 5a and Supplementary Fig. 17b,c).

In tumors of HT29F β cells growing in the cecum walls of nude mice, treatment with API-2 and doxycycline induced the nuclear accumulation of FOXO3a and β -catenin, respectively, as well as the loss of the epithelial phenotype and the promotion of cell scattering (Fig. 5b and Supplementary Fig. 17a). Most crucially, although

API-2 treatment reduced the primary tumor bioluminescence signal, it simultaneously promoted the development of metastasis when nuclear β -catenin was expressed. A significantly higher number of mice had metastases when treated with both API-2 and doxycycline compared to mice treated with vehicle ($P = 0.015$) (Fig. 5c and Supplementary Table 5). We found more mice affected in the lungs or lymph nodes or that had carcinomatosis as a result of treatment with both API-2 and doxycycline than as a result of vehicle treatment. Notably, API-2 treatment produced liver metastasis when β -catenin concentrations were high. We never detected this dissemination site previously in our HT29F β -cell model, and this site recapitulated the main organ affected in human patients with colon cancer (Fig. 5d-f).

β -catenin confers resistance to PI3K and AKT inhibitors

We observed a dose-dependent resistance to API-2-induced apoptosis that correlated with nuclear β -catenin content in DLD-1F β -C7 cells. HCT-116F cells with high endogenous amounts of mutant nuclear β -catenin or HT29F β cells with medium amounts were more resistant to NVP-BKM120- and API-2-induced apoptosis than DLD-1F β cells with any amount of nuclear β -catenin (**Supplementary Figs. 17b–h and 18a,b and Supplementary Table 6**). In L8 colon cancer cells that are heterogeneous with respect to nuclear β -catenin concentrations and transcriptional activity, LY-249002, a pharmacological inhibitor of PI3K, preferentially induced apoptosis in the cell population with low expression of β -catenin (**Supplementary Fig. 19a–c**). Notably, the tankyrase inhibitor XAV-939, which reduces nuclear β -catenin concentrations and transcriptional activity, increased the sensitivity of HCT-116F cells to NVP-BKM120- and API-2-induced apoptosis (**Supplementary Figs. 17d,e, 18b and 19d–f**)³².

We also analyzed the response to treatment of patient-derived colon cancer cells. Corresponding sphere cultures can regenerate carcinomas that preserve the same amounts of nuclear β -catenin and are histologically indistinguishable from the primary tumors of the patients after subcutaneous injection in nonobese diabetic severe combined immunodeficient (NOD-SCID) mice (**Fig. 6a,b and Supplementary Fig. 18c,d**). Treatment of sphere cultures with LY-294002 or NVP-BKM120 and API-2 induced the nuclear accumulation of FOXO3a and apoptosis in cells derived from patients T61, T68, T70 and T86, who originally presented with low nuclear β -catenin concentrations in their primary carcinomas. In contrast, sphere cultures derived from patients T49 and T59, who had high nuclear β -catenin concentrations, were innately resistant to the cell death mediated by these drugs. Notably, reduction of β -catenin concentrations by XAV-939 reverted such resistance (**Fig. 6c,d and Supplementary Fig. 18e–g**). In contrast, the sphere cultures were resistant to conventional chemotherapeutic agents such as 5-fluorouracil (5-FU) or oxaliplatin, and a combination treatment with these agents and XAV-939 did not sensitize the cultures to apoptosis (**Supplementary Fig. 18h**).

In vivo, treatment with API-2 inhibited the growth of the subcutaneous tumors generated in NOD-SCID mice when they were injected with cells derived from patient T61 (low β -catenin concentration), whereas mice injected with cells derived from patient T70 (intermediate β -catenin concentration) had only a partial response to API-2, and mice injected with cells derived from patient T49 (high β -catenin concentration) were resistant to API-2 treatment (**Fig. 6d,e**). The reduction of S6 ribosomal protein phosphorylation confirmed the efficacy of API-2 treatment in inhibiting PI3K-AKT pathway signaling *in vivo* (**Supplementary Fig. 20**). An analysis of the xenograft tumor derived from patient T49 revealed that API-2 treatment induced nuclear FOXO3a accumulation and increased cytoplasmatic IQGAP2 concentrations in tumor cell populations with disrupted epithelial organization. We also observed an enrichment of cells with high nuclear β -catenin concentrations in API-2-treated xenograft tumors from patient T49 compared to vehicle-treated control tumors (**Fig. 6f,g**).

DISCUSSION

Metastases are responsible for over 90% of deaths in patients with cancer, but the molecular mechanisms that drive this process are not well understood. Our data reveal previously unknown mechanisms by which FOXO3a and β -catenin can induce metastasis independently of the corresponding upstream pathways that control their nuclear accumulation^{13,33}.

Whereas some findings have related an abnormal activation of the Wnt- β -catenin pathway to metastasis³⁴, the contribution of PI3K-AKT-FOXO signaling to this process is not clear. On the one hand, mutational activation of PI3K and induction of AKT activity mediate metastasis³⁵, which might encourage the use of therapeutic inhibitors of PI3K and AKT. On the other hand, AKT can inhibit cell motility by blocking the transcriptional activity of nuclear factor of activated T cells (NFAT)³⁶. Our results, which show that AKT inhibitors promote metastasis resulting from nuclear FOXO3a accumulation and cooperation with β -catenin, support the second notion.

Few studies have correlated FOXO3a function with metastasis or poor prognosis in cancer^{37,38}. On the contrary, most studies have defined FOXO proteins as tumor suppressors, as they induce cell-cycle arrest and apoptosis^{9,11}. Indeed, the combined knockdown of FOXO1, FOXO3 and FOXO4 in mice is responsible for tumor initiation³⁹. However, our results reveal a previously unknown role for FOXO3a as an inducer of metastasis when acting in concert with β -catenin. We observed that the simultaneous nuclear accumulation of FOXO3a and β -catenin in primary tumors correlated with stages 3 and 4 of local or distant metastases and significantly shorter overall survival time of patients with colon cancer.

We show that to acquire such metastatic capacity, FOXO3a and β -catenin regulate functional sets of common target genes that are involved in reorganizing the cytoskeleton, destabilizing cell-to-cell contacts and increasing cell motility and migration, angiogenesis or evasion of the immune system. In particular, we revealed *IQGAP2* as a new target gene of FOXO3a- β -catenin complex that is required for destabilizing E-cadherin-containing adherens junctions, cell scattering and motility in colon carcinomas, indicating its role in metastasis.

AKT inhibition by API-2 induces nuclear FOXO3a accumulation and, in concert with nuclear β -catenin, promotes the expression of the aforementioned metastasis-related target genes and metastasis in mice. In fact, the nuclear accumulation of both factors correlates with tumor budding in human colon carcinomas.

Drug resistance is another crucial clinical feature that determines tumor relapse and patient survival. In particular, the mechanisms underlying resistance to new PI3K or AKT inhibitors are largely unknown^{18,40,41}. Part of the anti-tumoral action of PI3K and AKT inhibitors is based on nuclear FOXO accumulation and subsequent cell-cycle arrest and apoptosis⁴². We show that nuclear β -catenin activity acts as a new mechanism of resistance to the FOXO3a-induced apoptosis promoted by inhibitors of PI3K or AKT. Thus, the induction of FOXO3a proapoptotic target genes such as *TNFSF10* (encoding TRAIL) or *BCL2L1* (encoding BIM)⁴³ is reduced by nuclear β -catenin, whereas FOXO3a and β -catenin cooperate synergistically to induce the anti-apoptotic kinase homeodomain interacting protein kinase 3 (HIPK3), which is responsible for FADD phosphorylation and confers resistance to death-receptor-mediated apoptosis^{44,45}.

Colon carcinomas are heterogeneous tissues containing cell populations with diverse biological properties and various amounts of nuclear β -catenin^{3,25,46,47}. Cells with high nuclear β -catenin content behave as cancer stem cells^{48,49} and are resistant to the FOXO3a-induced apoptosis that is promoted by inhibitors of PI3K or AKT. This resistant behavior leads to relapse and metastasis by inducing FOXO3a and β -catenin common target genes, supporting the concept of metastatic cancer stem cells (**Supplementary Fig. 21**)^{49–52}. Indeed, sphere cultures derived from patients with colon cancer, which are enriched in cancer stem cells and which originated from tumors with high nuclear β -catenin content, were resistant to PI3K and AKT inhibitors *in vitro* and in subcutaneous xenograft tumors.

The information provided by these sphere cultures highlights the validity of studying target-directed therapies in cells derived from colon tumors immediately after surgical resection, as patients could therefore benefit from a personalized, experiment-based decision on the optimal treatment method. In the particular case of PI3K or AKT inhibitors, undesirable induction of FOXO3a and β -catenin target genes and promotion of metastasis could be predicted and avoided.

We show that the tankyrase inhibitor XAV-939 (ref. 32) sensitizes resistant cells specifically to the FOXO3a-induced apoptosis that is promoted by PI3K or AKT inhibitors. In contrast, XAV-939 does not have any benefit when combined with conventional chemotherapeutic agents such as 5-FU or oxaliplatin in cultures of colon-cancer-initiating cells. This points to a valid strategy to overcome drug resistance and metastasis by combining PI3K or AKT and Wnt- β -catenin inhibitory drugs. Currently used Wnt signaling inhibitors have reduced activity *in vivo*, and a new generation of tankyrase inhibitors with satisfactory *in vivo* activity must be developed to translate our results to clinical practice.

The benefit of some PI3K and AKT inhibitors is currently being tested in clinical trials in patients with colon cancer with advanced metastatic disease¹⁵, and these inhibitors may be tested as part of adjuvant therapy in the future. Our findings call for the measurement of nuclear β -catenin concentrations as a biomarker of response to treatments that provoke FOXO3a nuclear accumulation to predict apoptosis or metastasis. This would be advisable for PI3K and AKT inhibitors and may be extended to other drugs such as cetuximab or receptor tyrosine kinase inhibitors that act upstream of a common oncogenic pathway.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.P.T., P.O.-M. and I.P. contributed equally to the experiments. S.P.T. also contributed to writing the manuscript, and I.C. also performed experiments. O.A. helped to perform IQGAP2 knockdown experiments. S.L. classified human tumors according to histopathological criteria and built the tissue arrays. Y.F. performed and analyzed live imaging assays (IVIS), which were supervised by S.S. J.R.H., S.R. and J.D.G. participated in live imaging experiments. L.M. and

A.V. performed mutational analysis of human colon carcinomas. S.A. cloned and tested lentiviral constructs. S.R.y.C. provided human specimens. E.E. performed surgery on patients with colon cancer. J.B. supervised the project. J.T. supervised the project and provided clinical follow up on all the patients included in the study. A.M. and H.G.P. wrote the manuscript and supervised the project. H.G.P. was responsible for designing all the experiments and analyzing and interpreting all data.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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- Jemal, A. *et al.* Global cancer statistics. *CA Cancer J. Clin.* **61**, 69–90 (2011).
- Jänne, P.A. & Mayer, R.J. Chemoprevention of colorectal cancer. *N. Engl. J. Med.* **342**, 1960–1968 (2000).
- Clevers, H. Wnt/ β -catenin signaling in development and disease. *Cell* **127**, 469–480 (2006).
- Reya, T. & Clevers, H. Wnt signalling in stem cells and cancer. *Nature* **434**, 843–850 (2005).
- Pálmer, H.G. *et al.* Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β -catenin signaling. *J. Cell Biol.* **154**, 369–387 (2001).
- Olson, L.E. *et al.* Homeodomain-mediated β -catenin-dependent switching events dictate cell-lineage determination. *Cell* **125**, 593–605 (2006).
- Pálmer, H.G., Anjos-Afonso, F., Carmeliet, G., Takeda, H. & Watt, F.M. The vitamin D receptor is a Wnt effector that controls hair follicle differentiation and specifies tumor type in adult epidermis. *PLoS ONE* **3**, e1483 (2008).
- Essers, M.A. *et al.* Functional interaction between β -catenin and FOXO in oxidative stress signaling. *Science* **308**, 1181–1184 (2005).
- Calnan, D.R. & Brunet, A. The FoxO code. *Oncogene* **27**, 2276–2288 (2008).
- van der Horst, A. & Burgering, B.M. Stressing the role of FoxO proteins in lifespan and disease. *Nat. Rev. Mol. Cell Biol.* **8**, 440–450 (2007).
- Myatt, S.S. & Lam, E.W. The emerging roles of forkhead box (Fox) proteins in cancer. *Nat. Rev. Cancer* **7**, 847–859 (2007).
- Brunet, A. *et al.* Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857–868 (1999).
- Dehner, M., Hadjihannas, M., Weiske, J., Huber, O. & Behrens, J. Wnt signaling inhibits Forkhead box O3a-induced transcription and apoptosis through up-regulation of serum- and glucocorticoid-inducible kinase 1. *J. Biol. Chem.* **283**, 19201–19210 (2008).
- Walther, A. *et al.* Genetic prognostic and predictive markers in colorectal cancer. *Nat. Rev. Cancer* **9**, 489–499 (2009).
- Dienstmann, R., Rodon, J., Markman, B. & Tabernero, J. Recent developments in anti-cancer agents targeting PI3K, Akt and mTORC1/2. *Recent Pat. Anticancer. Drug Discov.* **6**, 210–236 (2011).
- Yang, L. *et al.* Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. *Cancer Res.* **64**, 4394–4399 (2004).
- Zheng, Y. *et al.* Novel phosphatidylinositol 3-kinase inhibitor NVP-BKM120 induces apoptosis in myeloma cells and shows synergistic anti-myeloma activity with dexamethasone. *J. Mol. Med. (Berl)* (2011).
- Liu, P., Cheng, H., Roberts, T.M. & Zhao, J.J. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat. Rev. Drug Discov.* **8**, 627–644 (2009).
- Hoffman, K. *et al.* Phase I–II study: tricyclic nucleoside phosphate) for metastatic breast cancer. *Cancer Chemother. Pharmacol.* **37**, 254–258 (1996).
- Bendell, J.C. *et al.* Phase I, dose-escalation study of BKM120, an oral pan-class I PI3K inhibitor, in patients with advanced solid tumors. *J. Clin. Oncol.* **30**, 282–290 (2012).
- Dijkers, P.F., Medema, R.H., Lammers, J.W., Koenderman, L. & Coffey, P.J. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr. Biol.* **10**, 1201–1204 (2000).
- Seoane, J., Le, H.V., Shen, L., Anderson, S.A. & Massague, J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* **117**, 211–223 (2004).
- Hoogbeem, D. *et al.* Interaction of FOXO with β -catenin inhibits β -catenin/T cell factor activity. *J. Biol. Chem.* **283**, 9224–9230 (2008).
- Kops, G.J. *et al.* Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* **419**, 316–321 (2002).
- Brabletz, T. *et al.* Variable β -catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc. Natl. Acad. Sci. USA* **98**, 10356–10361 (2001).
- Delpuech, O. *et al.* Induction of Mxi1-SR α by FOXO3a contributes to repression of Myc-dependent gene expression. *Mol. Cell. Biol.* **27**, 4917–4930 (2007).
- Wells, C.D. *et al.* A Rich1/Amot complex regulates the Cdc42 GTPase and apical-polarity proteins in epithelial cells. *Cell* **125**, 535–548 (2006).
- Yamashiro, S., Abe, H. & Mabuchi, I. IQGAP2 is required for the cadherin-mediated cell-to-cell adhesion in *Xenopus laevis* embryos. *Dev. Biol.* **308**, 485–493 (2007).

29. Natale, D.R. & Watson, A.J. Rac-1 and IQGAP are potential regulators of E-cadherin-catenin interactions during murine preimplantation development. *Gene Expr. Patterns* **2**, 17–22 (2002).
30. Tsai, M.S., Hornby, A.E., Lakins, J. & Lupu, R. Expression and function of CYR61, an angiogenic factor, in breast cancer cell lines and tumor biopsies. *Cancer Res.* **60**, 5603–5607 (2000).
31. Dhawan, P. *et al.* Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. *J. Clin. Invest.* **115**, 1765–1776 (2005).
32. Huang, S.M. *et al.* Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* **461**, 614–620 (2009).
33. Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M. & Hemmings, B.A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785–789 (1995).
34. Brabletz, T., Jung, A., Dag, S., Hlubek, F. & Kirchner, T. β -catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am. J. Pathol.* **155**, 1033–1038 (1999).
35. Samuels, Y. *et al.* Mutant PIK3CA promotes cell growth and invasion of human cancer cells. *Cancer Cell* **7**, 561–573 (2005).
36. Yoeli-Lerner, M. *et al.* Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Mol. Cell* **20**, 539–550 (2005).
37. Chen, J. *et al.* Constitutively nuclear FOXO3a localization predicts poor survival and promotes Akt phosphorylation in breast cancer. *PLoS ONE* **5**, e12293 (2010).
38. Storz, P., Doppler, H., Copland, J.A., Simpson, K.J. & Toker, A. FOXO3a promotes tumor cell invasion through the induction of matrix metalloproteinases. *Mol. Cell. Biol.* **29**, 4906–4917 (2009).
39. Paik, J.H. *et al.* FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell* **128**, 309–323 (2007).
40. Jun, T., Gjoerup, O. & Roberts, T.M. Tangled webs: evidence of cross-talk between c-Raf-1 and Akt. *Sci. STKE* **1999**, PE1 (1999).
41. Zunder, E.R., Knight, Z.A., Houseman, B.T., Apsel, B. & Shokat, K.M. Discovery of drug-resistant and drug-sensitizing mutations in the oncogenic PI3K isoform p110 α . *Cancer Cell* **14**, 180–192 (2008).
42. Gomes, A.R., Brosens, J.J. & Lam, E.W. Resist or die: FOXO transcription factors determine the cellular response to chemotherapy. *Cell Cycle* **7**, 3133–3136 (2008).
43. Obexer, P., Geiger, K., Ambros, P.F., Meister, B. & Ausserlechner, M.J. FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells. *Cell Death Differ.* **14**, 534–547 (2007).
44. Rochat-Steiner, V. *et al.* FIST/HIPK3: a Fas/FADD-interacting serine/threonine kinase that induces FADD phosphorylation and inhibits fas-mediated Jun NH(2)-terminal kinase activation. *J. Exp. Med.* **192**, 1165–1174 (2000).
45. Tourneur, L. & Chiocchia, G. FADD: a regulator of life and death. *Trends Immunol.* **31**, 260–269 (2010).
46. Shackleton, M., Quintana, E., Fearon, E.R. & Morrison, S.J. Heterogeneity in cancer: cancer stem cells versus clonal evolution. *Cell* **138**, 822–829 (2009).
47. Fodde, R. & Brabletz, T. Wnt/ β -catenin signaling in cancer stemness and malignant behavior. *Curr. Opin. Cell Biol.* **19**, 150–158 (2007).
48. Clevers, H. The cancer stem cell: premises, promises and challenges. *Nat. Med.* **17**, 313–319 (2011).
49. Vermeulen, L. *et al.* Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat. Cell Biol.* **12**, 468–476 (2010).
50. Brabletz, T., Jung, A., Spaderna, S., Hlubek, F. & Kirchner, T. Opinion: migrating cancer stem cells—an integrated concept of malignant tumour progression. *Nat. Rev. Cancer* **5**, 744–749 (2005).
51. Todaro, M. *et al.* Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* **1**, 389–402 (2007).
52. Van der Flier, L.G. *et al.* The intestinal Wnt/TCF signature. *Gastroenterology* **132**, 628–632 (2007).

ONLINE METHODS

Cell culture. Cell lines were cultured under standard conditions. DLD-1F cells are DLD-1 derivatives expressing pcDNA-FOXO3a(3A):ERTM (ref. 21). HT29F and HCT-116F cells express pLHCX-HA-FOXO3a(3A):ERTM. HT29F cells additionally carry pGL4.51(luc2/CMV/Neo) (Promega). pSIN-TRE-Flag- β -catenin^{S33Y}-IRES-GFP-inducible lentivirus was used to generate DLD-1F β and HT29F β cells. Cell migration was quantified using time-lapse microscopy (3 frames per h for 120 h) using the cell-tracking plugin (ImageJ software). All parental cell lines were originally obtained from American Type Culture Collection.

Patient-derived cells. Primary colon-cancer cells were obtained from the adenocarcinomas of the patients immediately after surgery, as described⁵³. Written informed consent was signed by all patients. The project was approved by the Research Ethics Committee of the Vall d'Hebron University Hospital, Barcelona, Spain (approval ID, PR(IR)79/2009).

Mice, xenotransplantation and bioluminescence. Experiments were conducted following the animal care directive of the European Union (86/609/EEC) and were approved by the Ethical Committee of Animal Experimentation of Vall d'Hebron Institute of Research (approval ID, 40/08 CEEA and 47/08/10 CEEA). Colon-cancer cells were orthotopically transplanted in the cecum walls of female NOD-SCID mice as previously described⁵⁴. Doxycycline (2 mg/ml; Sigma-Aldrich) was administered *ad libitum* in drinking water containing 5% sucrose (Sigma-Aldrich). 4OHT (100 μ g per dose, 1 μ g/ μ l in sesame oil; Sigma-Aldrich) and API-2 (1 mg/kg in PBS and 2% DMSO; Tocris Bioscience) were injected intraperitoneally every other day. Bioluminescence imaging was performed with the IVIS Spectrum Imaging System (Caliper Life Sciences).

Mutational status of patient-derived cells and cancer cell lines. Samples were genotyped for 740 cancer-related mutations in 57 genes. OncoCarta (Sequenom), CLIA (MD Anderson Cancer Center, Houston, Texas) and VHIO-Card panels of somatic mutations (Supplementary Table 7) were analyzed with a MassARRAY spectrometer (Sequenom). Mutation data were from the COSMIC database (www.sanger.ac.uk/cosmic).

Western blot, histology and immunofluorescence. Tumor budding was quantified as described⁵⁵. Western blot and immunofluorescence were performed as described⁵⁶. The antibodies used are listed in Supplementary Table 8. The fluorescence images were analyzed with the MBF ImageJ for Microscopy program (www.macbiophotonics.ca) using criteria described earlier^{56,57}.

Flow cytometry. The proportions of apoptotic cells were determined using the annexin V-eGFP (GenScript), annexin V-phycoerythrin (PE) (BD Pharmingen) or annexin V-allophycocyanin (APC) (Bender MedSystems) kits. Anti-human TRA-85-APC staining (R&D Systems) discriminated cells between the species when analyzing human xenograft tumors growing in mice. Dead cells were detected by propidium iodide or DAPI staining.

Quantitative RT-PCR and transcriptomics. Quantitative RT-PCR was done using the comparative C_T method as described using SYBR Green (Applied Biosystems) and specific primer pairs (Supplementary Table 9a), applying geNorm algorithms to select reference transcripts (*SDHA* and *PPIA*) to use the geometric means for normalization and to calculate normalized s.d. values⁵⁸. The transcriptome of the DLD-1F β -C7 cells was determined on an Affymetrix Human Gene 1.1-ST Array. Raw and normalized Minimum Information About a Microarray Experiment (MIAME)-compliant microarray data were deposited at ARRAYEXPRESS (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-3262.

Plasmids. pSIN-TRE-Flag- β -catenin^{S33Y}-IRES-GFP is a derivative⁵⁹ of the pRRL-cPPT-hPGK-TMPrtTA-WPRE plasmid⁶⁰ that has human S33Y Flag- β -catenin cds introduced. pLHCX-HA-FOXO3a(A3):ERTM is a derivative of the

FOXO3a(A3):ERTM (ref. 21) and pLHCX plasmid (Clontech). 7TGP was previously described⁶¹. pGIPZ-shRNAmir constructs were from Open Biosystems (Supplementary Table 9b).

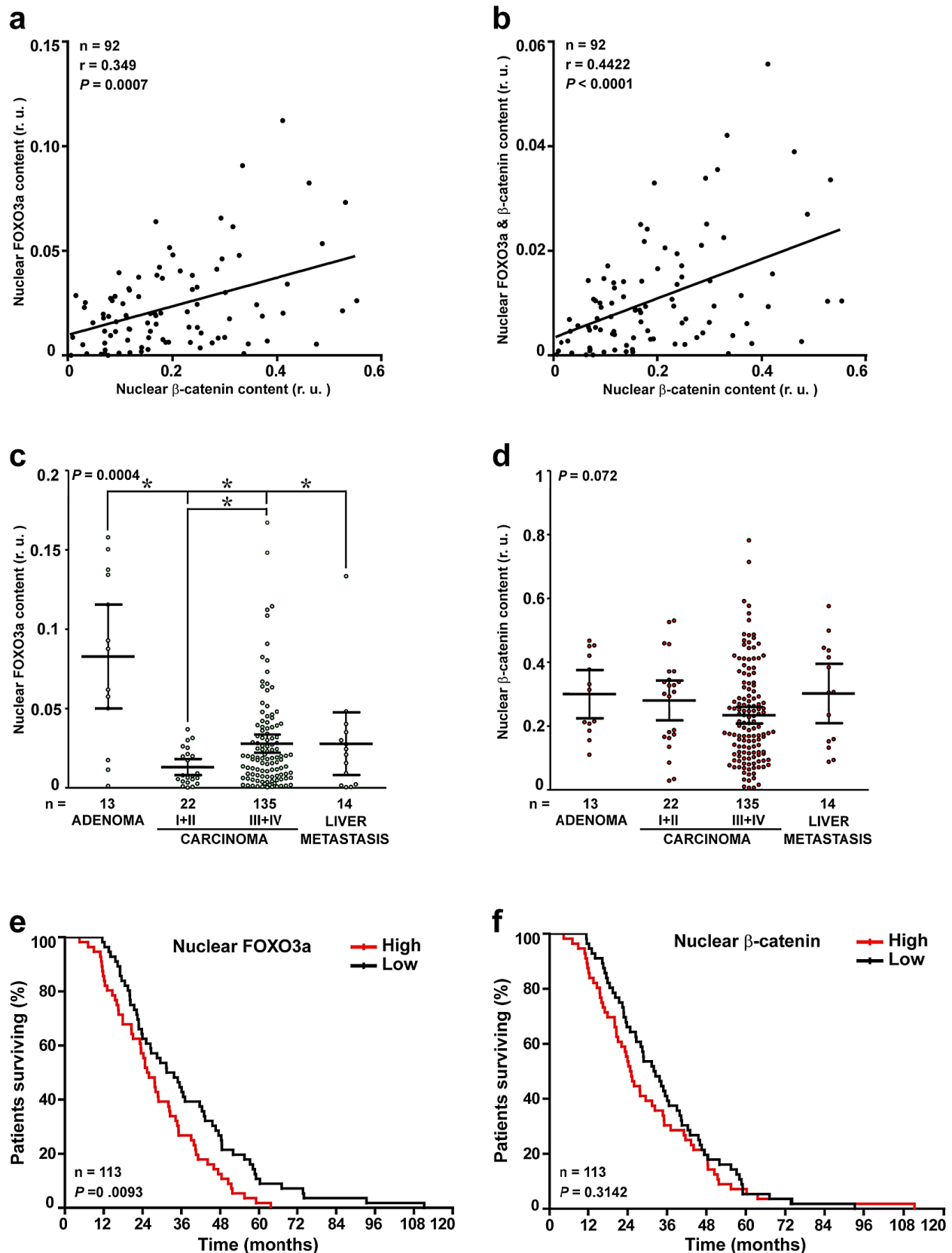
Reagents and treatments. Unless specified otherwise, cells were treated as follows: 2.5 μ g/ml doxycycline for 36 h (Sigma-Aldrich), followed 12 h later with 50 nM 4OHT for 24 h (Sigma-Aldrich). Cells were also treated with 3.3 μ M XAV-939 or LDW-643 (Novartis) for 120 h, followed 48 h later with 20 μ M LY-294002 (Merck), 20 μ M API-2 (Tocris Bioscience), 0.8 μ M NVP-BKM120 (Selleck Chemicals), 50 μ g/ml 5-FU (Sigma-Aldrich) or 100 μ M oxaliplatin (Sigma-Aldrich) for 72 h. LDW-643 is an analog of XAV-939 with no activity and was used as a control. Cells were treated with 10 μ g/ml mitomycin C for 3 h (Sigma-Aldrich), before 4OHT or doxycycline addition. Primary fibroblasts were treated with 0.1 μ M doxorubicin (Sigma-Aldrich) for 24 h to induce senescence. Hoechst 33342 was used at a dose of 5 μ g/ml (Sigma-Aldrich).

Viral infection. Lentiviral or retroviral particles were produced in 293T cells using standard procedures and psPAX2 and pMD2.G (Addgene) or pCL-Ampho (Imgenex Corp.) packaging vectors, respectively.

Statistics. Any $P < 0.05$ were considered statistically significant. We calculated correlation coefficients (r) using two-tailed nonparametric Spearman tests, which make no assumption about the distribution of the values, to compare the fluorescent signal values of different proteins, subcellular protein colocalization or tumor budding in tissue sections of human carcinomas. We used the nonparametric Kruskal-Wallis test to compare three or more groups of unpaired values that did not follow any particular distribution. This applied, for example, to measurements of protein colocalization in human carcinomas at different tumor stages and in comparing different treatments in cell motility and adhesion assays, as well as in quantifying tumor budding in orthotopically growing tumors. Then, Dunn's multiple comparison post-test was used to identify differences in the sum of ranks between each pair of groups of values. We analyzed the survival of the patients using the Kaplan-Meier method and compared the curves using a log-rank (Mantel-Cox) test assuming that the hazard function was the same at all times. We used the nonparametric Mann-Whitney test to compare unmatched groups of values corresponding to xenograft tumor volumes or luminescence signals, tissue protein expression and cell motility. Differences in apoptosis were analyzed by an unpaired t test comparing the means of two groups of values. We used Fisher's exact test to analyze the differences in incidence of metastasis compared to control.

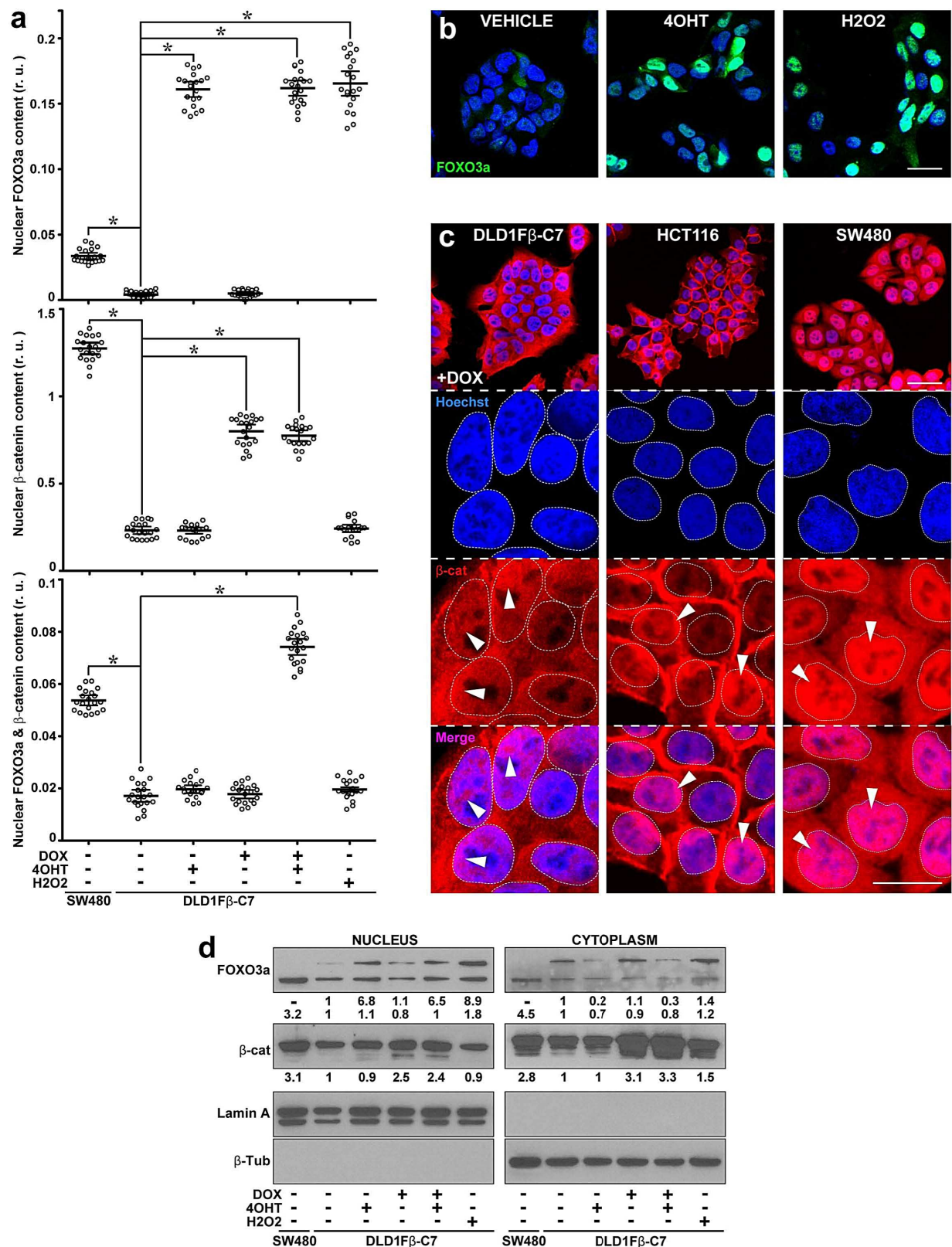
53. Kreso, A. & O'Brien, C.A. Colon cancer stem cells. *Curr. Protoc. Stem Cell Biol.* **7**, 3.1.1–3.1.12 (2008).
54. Céspedes, M.V. *et al.* Orthotopic microinjection of human colon cancer cells in nude mice induces tumor foci in all clinically relevant metastatic sites. *Am. J. Pathol.* **170**, 1077–1085 (2007).
55. Ueno, H., Murphy, J., Jass, J.R., Mochizuki, H. & Talbot, I.C. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology* **40**, 127–132 (2002).
56. Barbáchano, A. *et al.* SPROUTY-2 and E-cadherin regulate reciprocally and dictate colon cancer cell tumorigenicity. *Oncogene* **29**, 4800–4813 (2010).
57. Arques, O., Chicote, I., Tenbaum, S., Puig, I. & Palmer, H.G. Standardized relative quantification of immunofluorescence tissue staining. *Protoc. Exchange* published online, doi:10.1038/protex.2012.008 (2 April 2012).
58. Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034 (2002).
59. Aguilar, S. *et al.* Bone marrow stem cells expressing keratinocyte growth factor via an inducible lentivirus protects against bleomycin-induced pulmonary fibrosis. *PLoS ONE* **4**, e8013 (2009).
60. Barde, I. *et al.* Efficient control of gene expression in the hematopoietic system using a single Tet-on inducible lentiviral vector. *Mol. Ther.* **13**, 382–390 (2006).
61. Fuerer, C. & Nusse, R. Lentiviral vectors to probe and manipulate the Wnt signaling pathway. *PLoS ONE* **5**, e9370 (2010).

SUPPLEMENTARY FIGURE 1



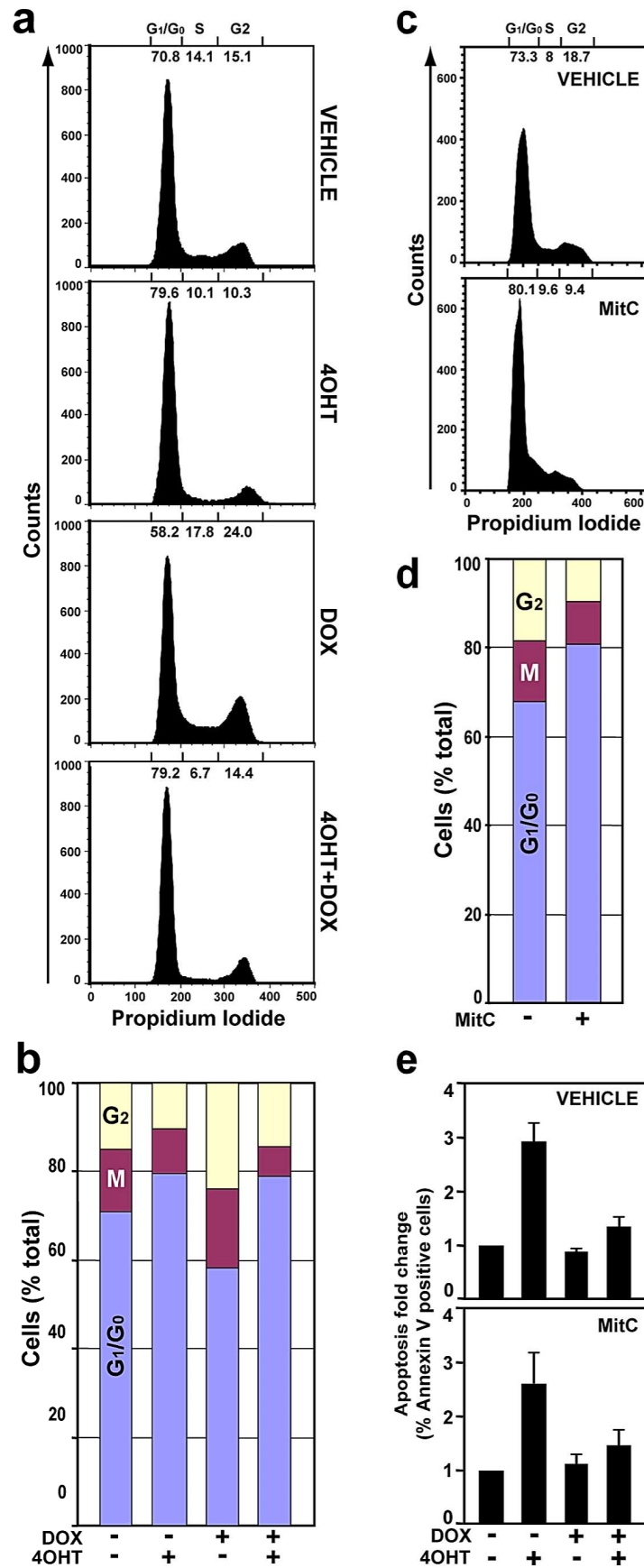
Supplementary Figure 1. Correlation of nuclear FOXO3a and β -catenin expression levels with tumor stage and patient survival in colon cancer. (a-b) Dot plot correlating nuclear β -catenin signal with FOXO3a or their colocalization signal in human colon carcinoma. Relative units (r. u.) were calculated as explained in Methods section. Number of cases (n), Spearman coefficient of correlation (r) and P value are indicated. (c-d) Column scatter plots showing the intensity of nuclear signal for each protein in tumors at different stages of progression. Horizontal bars mark the mean, error bars indicate the 95% CI. P value for non-parametric variation was analysed by Kruskal-Wallis test. Asterisks indicate significant differences between groups upon Dunn's multiple comparison test. (e, f) Kaplan-Meier survival analysis of colon cancer patients separated into two groups according to the mean level of FOXO3a (e) or β -catenin (f) expression: Higher than the mean (red line; $n = 58$ in e and $n = 56$ in f) and lower than the mean (black line; $n = 55$ in e and $n = 57$ in f). P values by Log-rank (Mantel-Cox) test are shown. HR = 1.212; 95% CI, 0.8331-1.765 for (e) and HR = 1.684; 95% CI, 1.137-2.493 for (f).

SUPPLEMENTARY FIGURE 2



Supplementary Figure 2. Exogenous FOXO3a and β-catenin expression reach physiological levels. (a) Column scatter plot representing nuclear levels of FOXO3a, β-catenin or their colocalization in SW480 and DLD1Fβ-C7 cells treated as indicated. 20 random fields and a minimum of 200 immunostained cells were quantified for each condition. The represented values correspond to the signal measured in each individual field. Asterisks mark statistically significant differences as calculated by a non-parametric Mann Whitney test comparing all experimental conditions with vehicle-treated DLD1Fβ-C7 cells. (b) Representative confocal microscopy analysis of FOXO3a expression (green) in DLD1Fβ-C7 cells treated for six hours as indicated. Scale bar, 25 μm. Nuclear staining, Hoechst 33342 (blue). (c) Upper panels: Confocal immunofluorescence pictures to visualize protein amounts and location in DOX-treated DLD1Fβ-C7 cells and in untreated HCT116 or SW480 colon cancer cells stained for β-catenin (red). Scale bar 25 μm. Lower panels: Corresponding magnifications showing nuclear β-catenin. Nuclei are delineated by dashed lines. Arrowheads point nuclear β-catenin. Blue (Hoechst 33342) and red (β-catenin) channels are shown separately and merged to visualize nuclear β-catenin. Scale bar, 10 μm. (d) Protein expression of FOXO3a (lower band), FOXO3aA3:ER™ (upper band) and β-catenin in SW480 and DLD1Fβ-C7 cells fractionated for nuclear and cytoplasmic compartments as assessed by Western blot. Numbers below the blot represent relative amounts of protein in each experimental condition versus the amount in DLD1Fβ-C7 treated with vehicle. All values are corrected to Laminin A or β-Tubulin levels, that serve as controls for gel loading and purity of fractions. Cells were treated with vehicle or with 4OHT (6 h), DOX (18 h) or H₂O₂ (6 h).

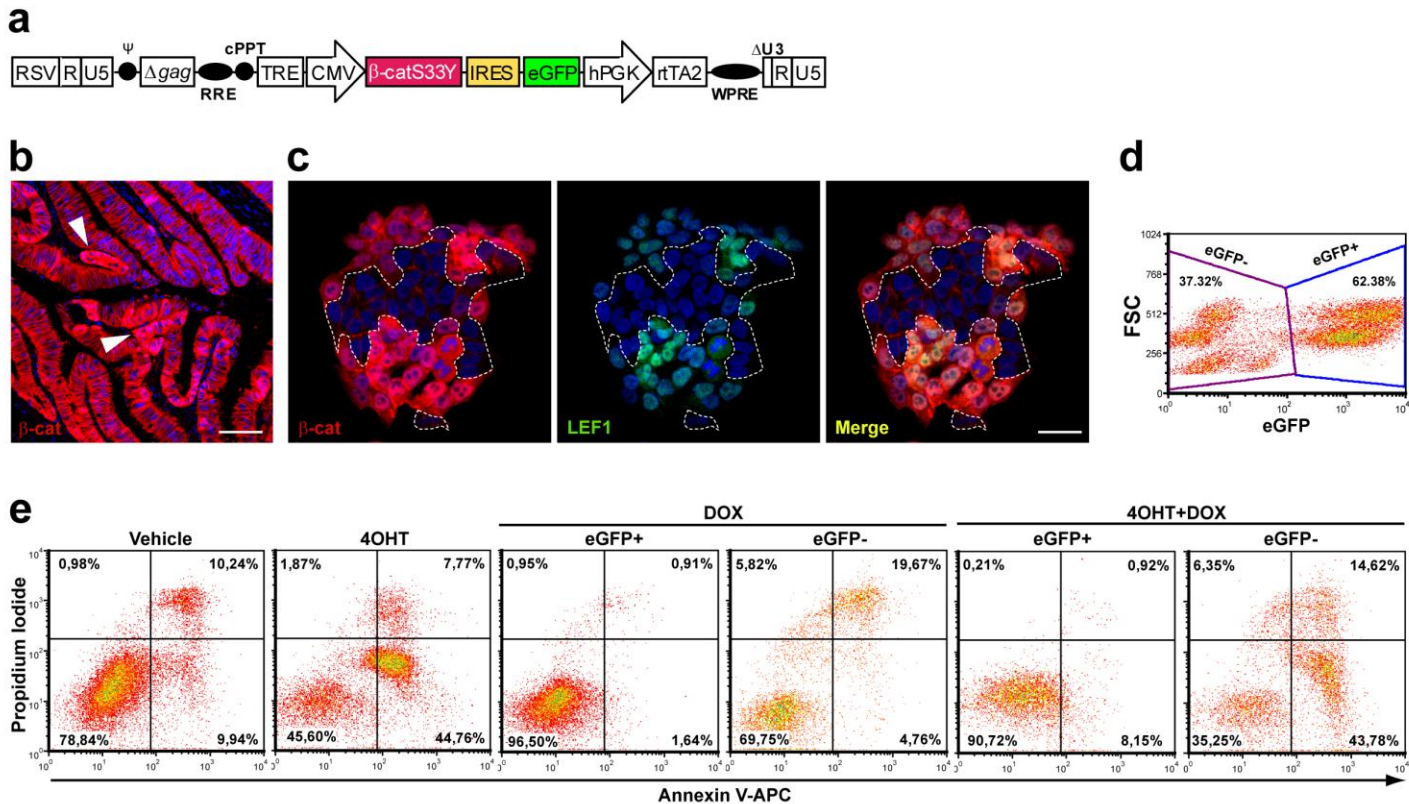
SUPPLEMENTARY FIGURE 3



Supplementary Figure 3. Cell cycle arrest induced by FOXO3a is dominant over nuclear β-catenin effects.

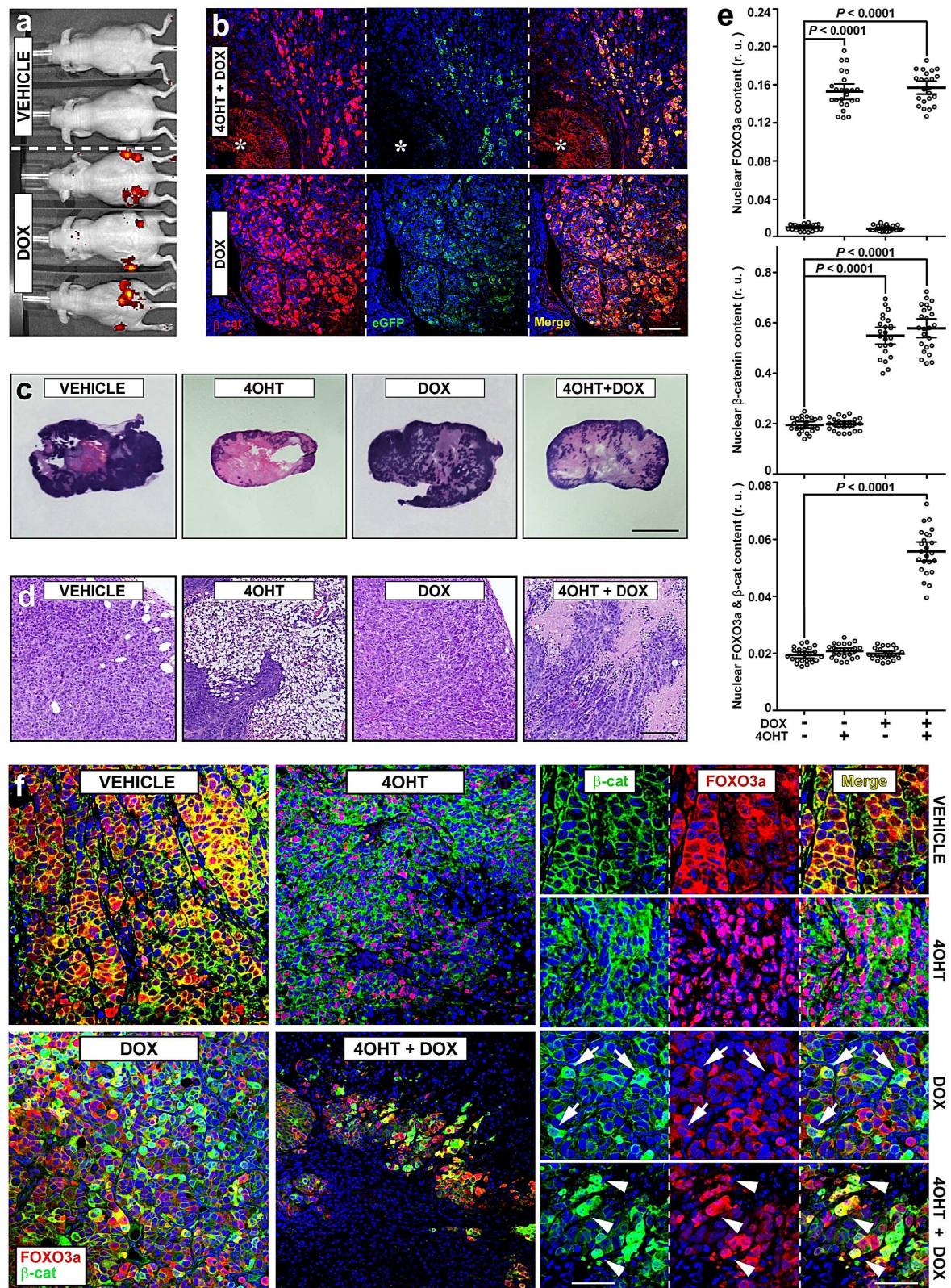
(a,c) Cell cycle profile of DLD1Fβ-C7 cells treated as indicated. Ethanol-fixed cells were analyzed by flow cytometry using propidium iodide staining. Numbers indicate the percentage of cells at different cell cycle phases. Apoptotic (sub-G₀) populations were gated out. MitC = mitomycin C (b,d) Graph depicting the percentage of DLD1Fβ-C7 cells in each cell cycle phase upon the indicated treatments. (e) Chart representing the proportion of apoptotic DLD1Fβ-C7 cells upon the indicated treatments. Bars show the mean of three independent experiments, errors are +/- s.d.

SUPPLEMENTARY FIGURE 4



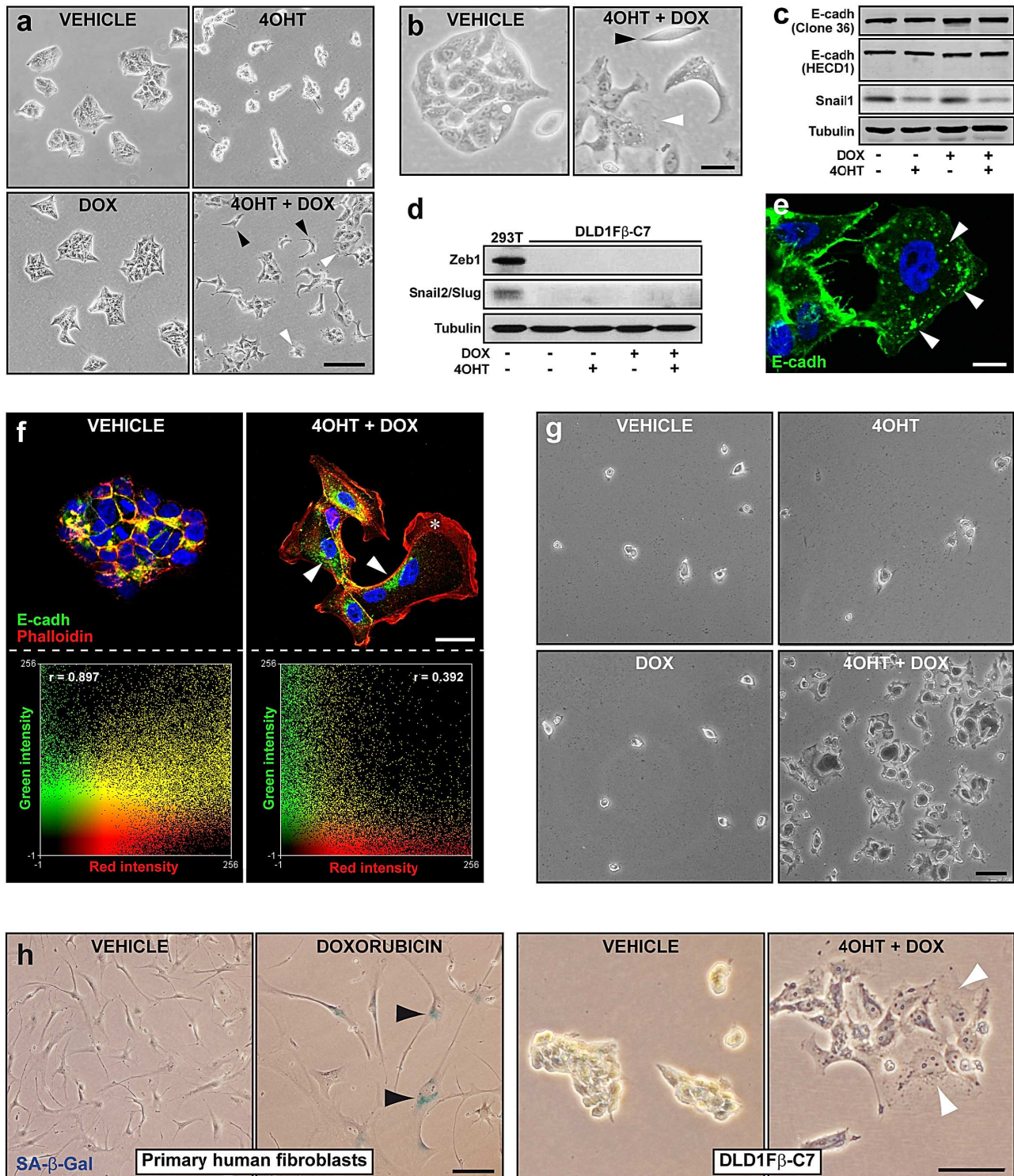
Supplementary Figure 4. DLD1Fβ cell system recapitulates β-catenin heterogeneity. (a) Scheme of the DOX-inducible lentiviral pSIN-TRE-flag-β-Catenin^{S33Y}-IRES-eGFP construct. TRE, tetracyclin (tet) response element; CMV, cytomegalovirus minimal promoter; β-catS33Y, human flag-tagged β-catenin^{S33Y} coding sequence; IRES, internal ribosome entry site; eGFP, enhanced green fluorescent protein; hPGK, human phosphoglycerate kinase promoter; rtTA2, reverse tet-activator. Additional lentiviral regulatory elements of the backbone are described elsewhere (Barde et al., 2006). (b) Immunofluorescence analysis of β-catenin expression (red) in a human primary colon carcinoma. Arrowheads indicate cells with distinctive high levels of nuclear β-catenin. Scale bar, 100 μm. (c) Immunofluorescence analysis of β-catenin (red) and LEF1 (green) in DLD1Fβ cells. Dashed lines mark the edge between cells expressing high (infected) or low (uninfected) levels of nuclear β-catenin. Scale bar, 25 μm. (b,c) Nuclei were stained with Hoechst 33342 (blue). (d) Discrimination of β-catenin positive (eGFP+) and negative (eGFP-) subpopulations of DLD1Fβ cell cultures by flow cytometry. (e) Measurement of apoptotic eGFP+ and eGFP- DLD1Fβ cell subpopulations upon the indicated treatments by Annexin V staining and flow cytometry. Percentage of cells per each quadrant are indicated.

SUPPLEMENTARY FIGURE 5



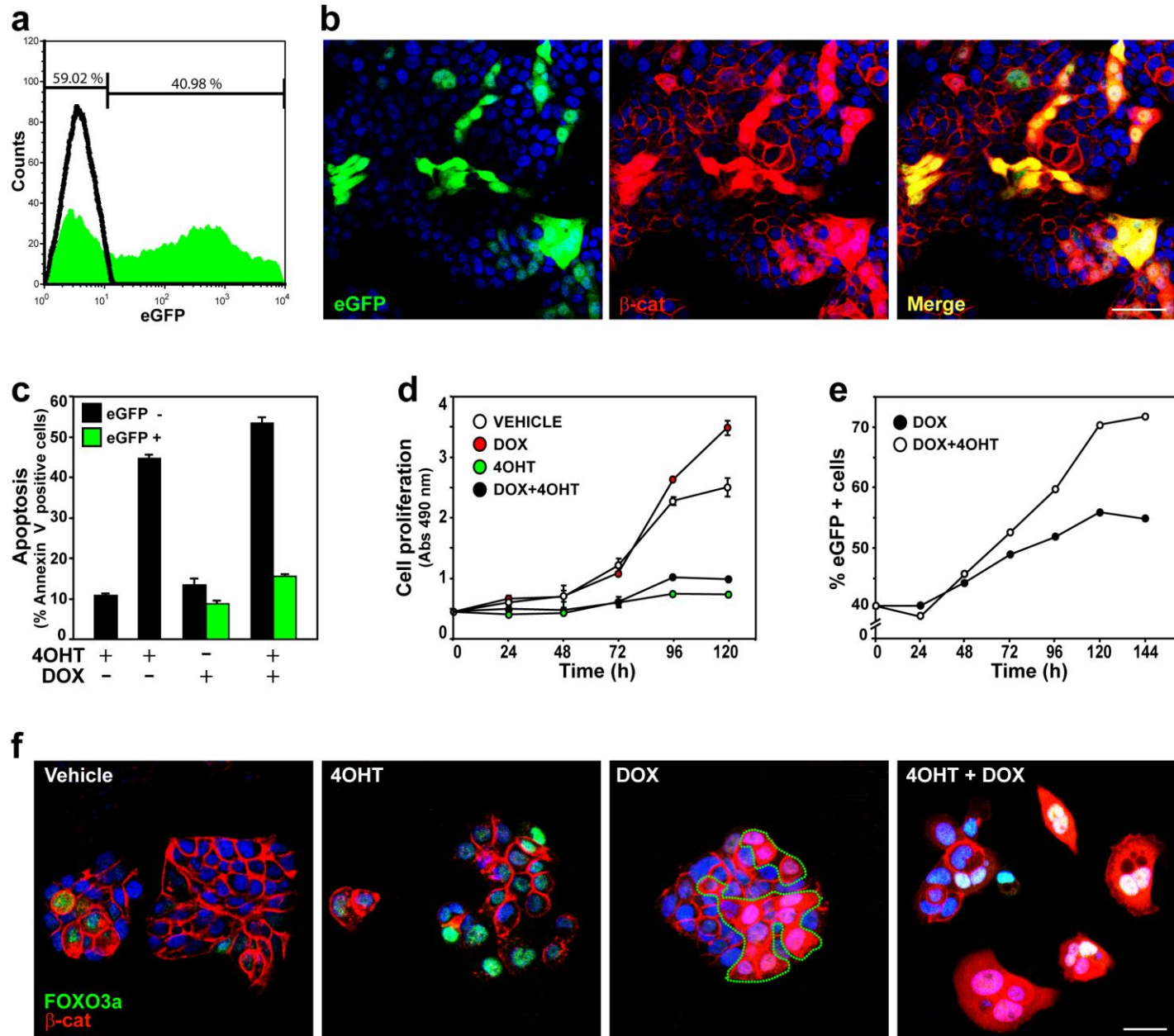
Supplementary Figure 5. DLD1F β -derived xenograft tumors recapitulate β -catenin heterogeneity in vivo. (a) Life fluorescence imaging (IVIS[®]) of anesthetized nude mice injected with DLD1F β cells five days after treatment with vehicle or DOX. eGFP fluorescent signal of the tumors is visualized in false colors (intensity: red < yellow). (b) Immunofluorescence analysis of eGFP (green) and β -catenin (red) in subcutaneous DLD1F β cell tumors growing in nude mice treated as indicated. The asterisk marks a eGFP-negative area where DLD1F β cells do not express exogenous β -catenin^{S33Y} and retain an epithelial phenotype. Scale bar, 100 μ m. (c) Macroscopic view of hematoxylin & eosin stained subcutaneous tumors from nude mice xenografted with DLD1F β cells and treated as indicated. Scale bar, 1 cm. (d) Representative sections of hematoxylin & eosin stained DLD1F β xenograft tumors of the indicated experimental cohorts. Scale bar, 200 μ m. (e) Column scatter plot representing nuclear levels of FOXO3a, β -catenin and their colocalization in subcutaneous tumor xenografts of DLD1F β cells growing in nude mice and treated as indicated (a-c). Three random fields were quantified per eight xenografts corresponding to every group of treatment. The 24 values correspond to the relative protein level in each field quantified. Differences between groups are calculated by non-parametric Mann Whitney test and significant *P* values are indicated. (f) Immunofluorescence analysis of β -catenin (green) and FOXO3a (red) in the same tumors shown in (c,d). Large left panels show merged signals. Small right panels show the fluorescence in each channel separately at high magnification. Scale bars, 100 μ m. Arrows and arrowheads indicate cells with eGFP- β -catenin^{S33Y} expression alone or together with nuclear FOXO3a, respectively. Scale bar, 100 μ m. (b,f) Nuclei were stained using Hoechst 33342 (blue).

SUPPLEMENTARY FIGURE 6



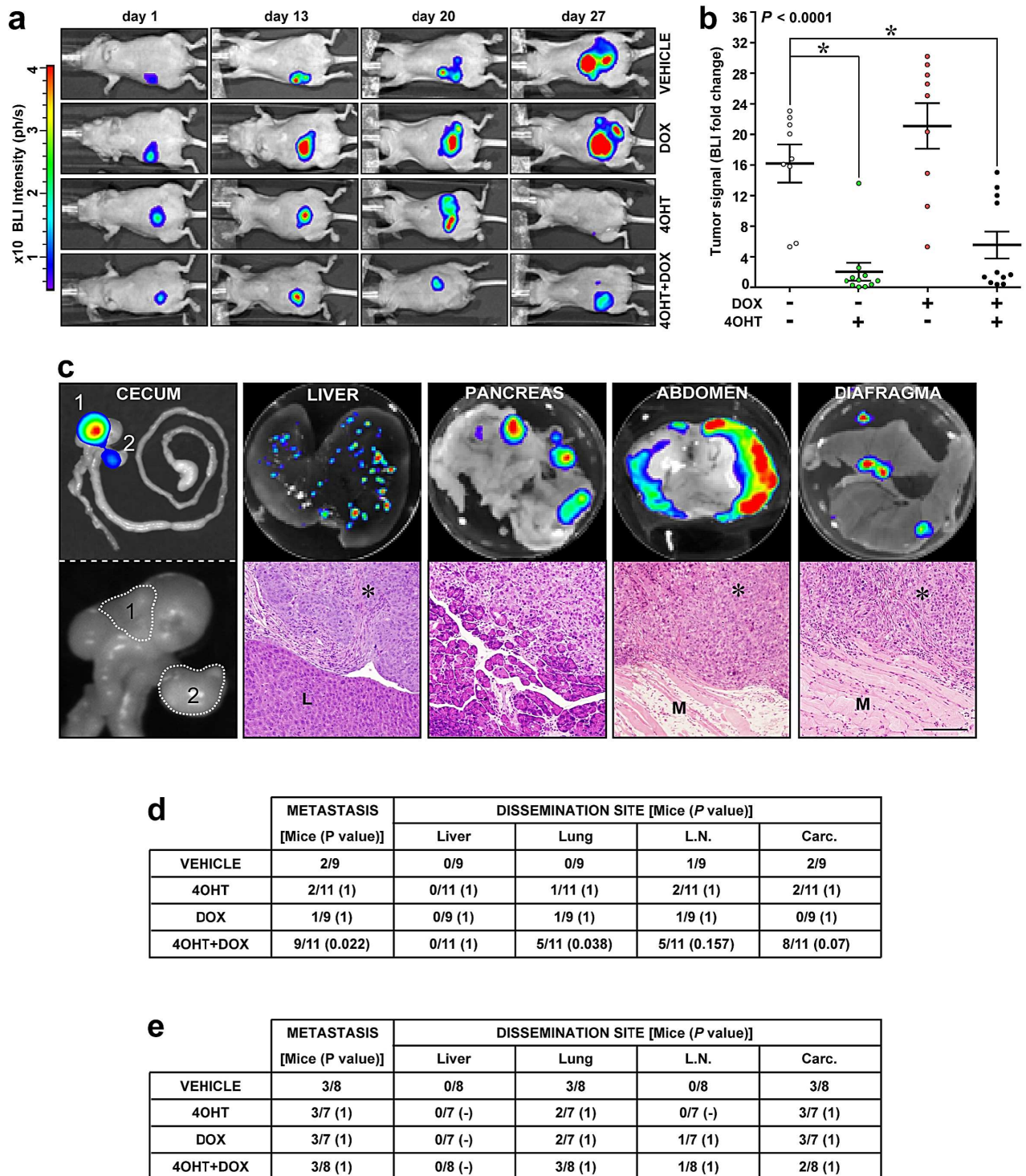
Supplementary Figure 6. Nuclear FOXO3a and β-catenin cooperate to promote E-cadherin cytosolic accumulation and to impose a mesenchymal phenotype. (a) Representative phase-contrast images of cultured DLD1Fβ-C7 cells treated as indicated. Scale bar, 100 μm. (b) Magnifications showing epithelial, mesenchymal (black arrowhead) and flat shape (white arrowhead) cell phenotypes. Scale bar, 25 μm. (c,d) Protein expression in DLD1Fβ-C7 cells treated as indicated and analyzed by Western blot. Tubulin served as loading control. (c) Two different E-cadherin antibodies (clone 36 and HECD1) were used to occasionally detect E-cadherin cleavage products. (d) 293T cells transfected with ZEB1 and SLUG expression vectors, respectively, served as positive controls. (e) Immunofluorescence analysis of E-cadherin (green) showing cytosolic aggregates (arrowheads). Scale bar, 10 μm. (f) Colocalization of immunostained E-cadherin (green) and Phalloidin-FITC-bound actin (red) in DLD1Fβ-C7 cells treated as indicated (upper panels). White arrowheads point E-cadherin cytosolic aggregates separated from the actin cytoskeleton. Asterisk indicates actin cortex typical of lamellipodia. Scale bar, 25 μm. Lower panels: Color scatter plots showing the corresponding intensity correlation analysis of green and red channels of the upper confocal pictures. Yellow dots represent colocalized E-cadherin and actin. Pearson's correlation coefficient (r) is indicated. (g) Adhesion to the substrate. Phase-contrast images of differently treated DLD1Fβ-C7 cells adhered to cell culture dishes five minutes post-seeding. Scale Bar, 25 μm. (h) Phase-contrast images of human primary skin fibroblasts (left panels) and DLD1Fβ-C7 cells (right panels) treated as indicated and stained for acidic senescence-associated (SA) β-galactosidase activity (blue). Black arrowheads show positively-stained cells. White arrows point at flat shaped cells negative for SA-β-gal staining. Scale Bar, 25 μm.

SUPPLEMENTARY FIGURE 7



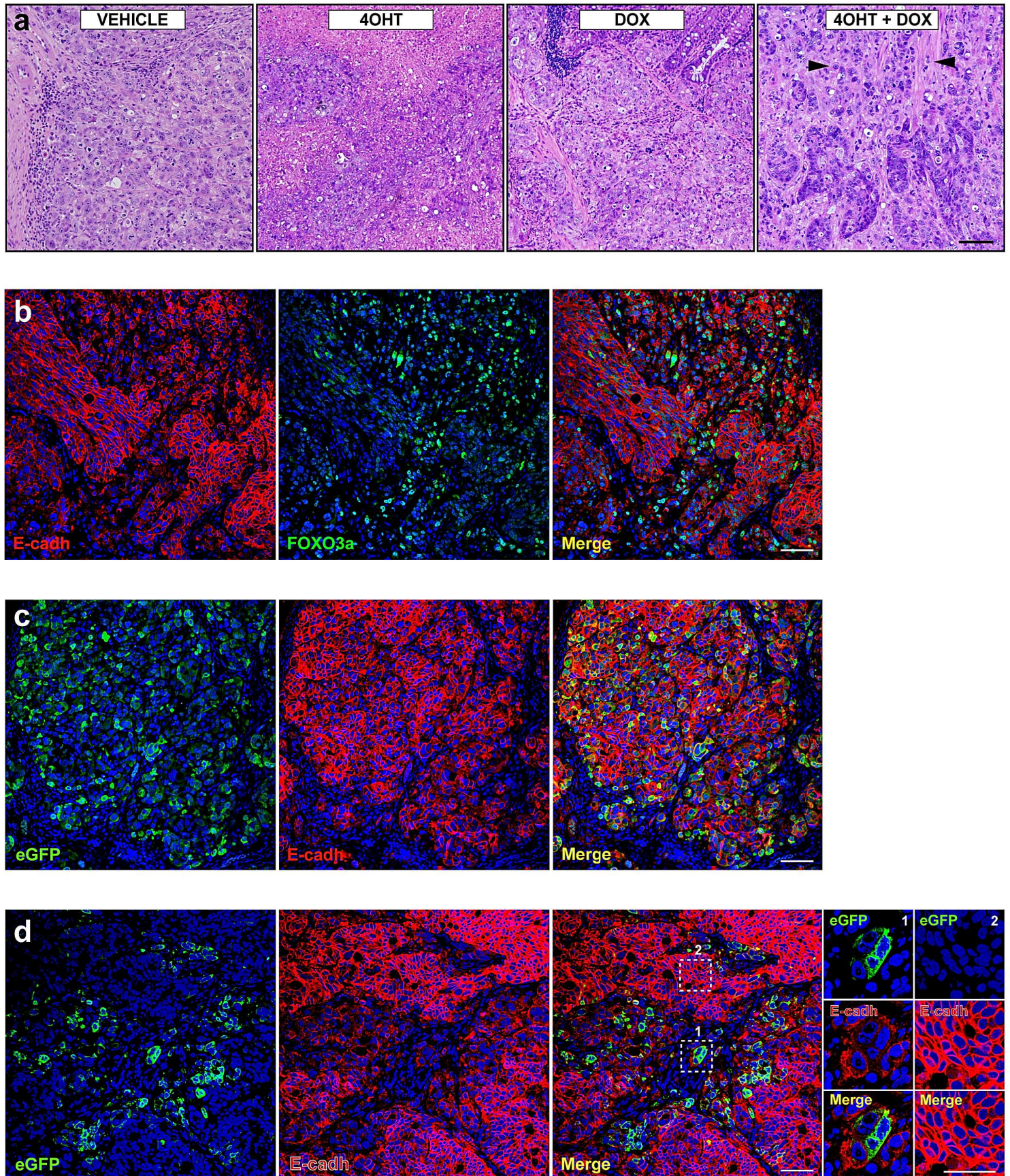
Supplementary Figure 7. Nuclear FOXO3a and β -catenin control the fate of HT29F β colon cancer cells. (a) eGFP expression in HT29F β cells treated with vehicle (black line) or DOX (green) measured by flow cytometry. Percentages of each population (eGFP+ and eGFP-) are indicated. (b) Immunofluorescence and confocal microscopy analysis of β -catenin (red) and eGFP (green area) in HT29F β cells treated with DOX. Scale bar, 50 μ m. (c) Proportion of apoptotic HT29F β cells, analyzed by flow cytometry, expressing or not eGFP and β -catenin^{S33Y} in response to different treatments. Error bars show $\pm$ s.d. of triplicates from three independent experiments. (d) Proliferation of HT29F β cells treated as indicated. Error bars show $\pm$ s.d. of triplicates from three independent experiments. (e) Proportion of eGFP/ β -catenin^{S33Y} positive and negative cell population by flow cytometry in cultures of HT29F β cells treated as indicated. (f) Immunofluorescence and confocal microscopy analysis of FOXO3a (green) and β -catenin (red) in HT29F β cells. Dashed line delineate the population of β -catenin-expressing cells. Scale bar, 25 μ m. (b,f) Nuclei were stained with Hoechst 33342 (blue).

SUPPLEMENTARY FIGURE 8



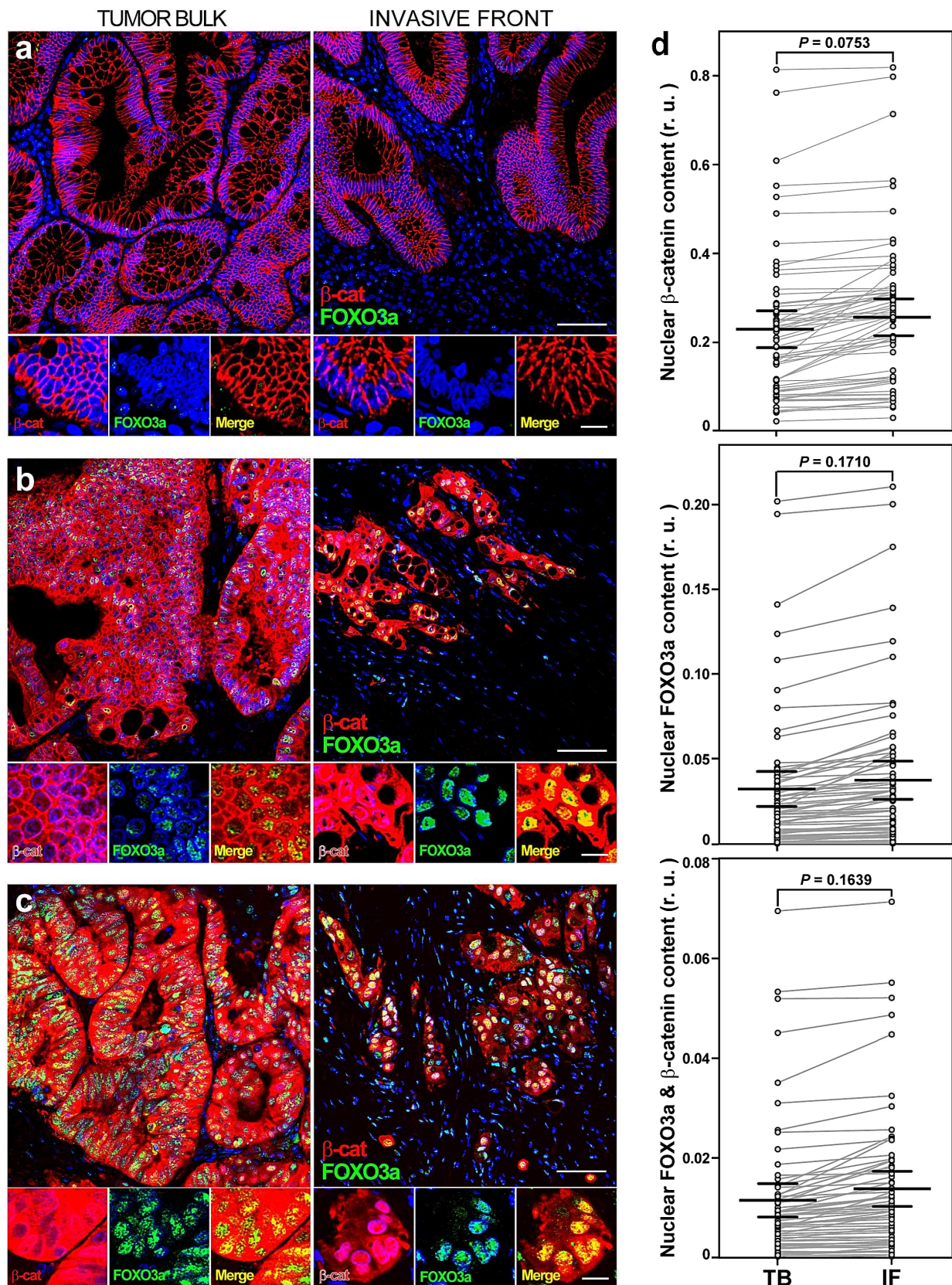
Supplementary Figure 8. Generation of distant metastases by HT29Fβ cells injected in the cecum of nude mice. (a) Representative example of bioluminescence images (IVIS®) of luciferin-injected nude mice bearing orthotopic tumors from cecum-injected luciferase-expressing HT29Fβ cells. Time after initiation of treatment is indicated in days. Light signal intensity (BLI) is expressed as photons per second (ph/s) in a false-color code (blue < red). (b) Column scatter plot presenting the fold change in bioluminescent signal intensity emitted by each tumor after comparing day 0 versus day 27 post treatment. Bars and errors show the mean and the 95% CI, respectively. *P* value was calculated comparing all groups of values with those obtained in vehicle-treated mice. A non-parametric Mann Whitney test was used and asterisks label significant differences. (c) Images of infiltrated organs obtained after necropsy. Upper panels: Images of primary tumor (cecum) or carcinomatosis affecting different organs and overlaid with gradual false-colored bioluminescent signal exemplified in (a) (blue < red). The left lower panel shows a magnification of primary tumor located in the cecum (1) and affected lymph node (2) delimited by dotted lines. The right lower panels show hematoxylin & eosin-stained carcinomatosis adhered to the indicated tissues. Asterisks indicate tumoral HT29Fβ cells. Muscle tissue in abdomen or diaphragm (M) and liver tissue (L) are indicated. Scale bar, 200 μm. (d,e) Incidence of metastasis in mice injected in the cecum with luciferase-expressing HT29Fβ (d) or parental HT29 (e) cells and treated as indicated. L.N. = lymph node, Carc. = carcinomatosis, including any tumor mass growing in the abdominal cavity eventually affecting adjacent organs. Ratios indicate the numbers of animals with affected tissue per total number in each group of treatment. *P* values listed in brackets were calculated versus vehicle-treated mice using a Fisher's exact test. Only metastases detected by luminometry and confirmed by histological exploration were counted.

SUPPLEMENTARY FIGURE 9



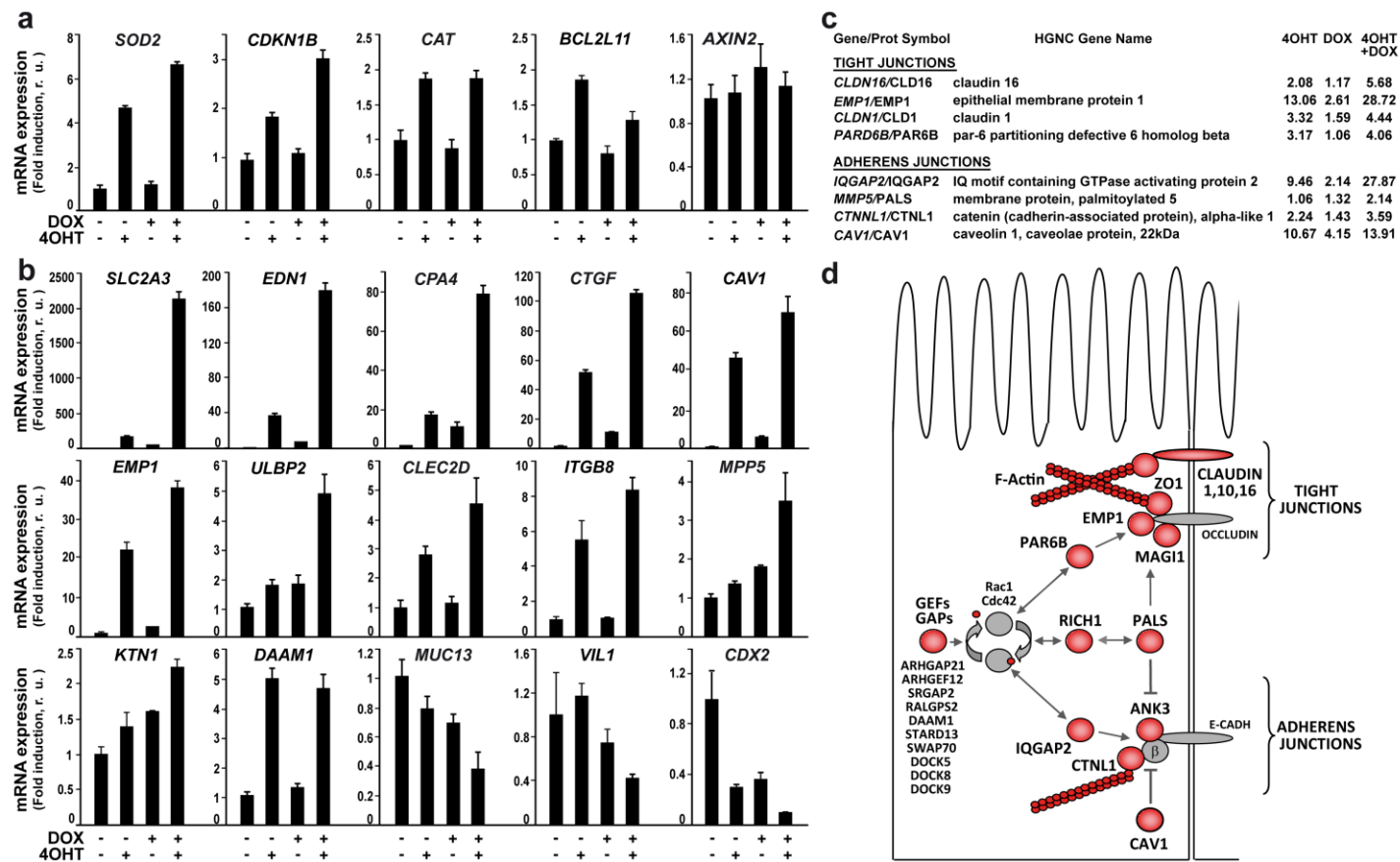
Supplementary Figure 9. FOXO3a and β -catenin control HT29F β cells fate in primary tumors growing in the cecum wall of nude mice. (a) Hematoxylin & eosin staining of primary tumors generated from HT29F β cells growing in the cecum of injected nude mice treated as indicated. Arrowheads point at budding tumor cells. Scale bar, 100 μ m. (b) Immunofluorescence staining of E-cadherin (red) and FOXO3a (green) of primary tumors of HT29F β cells growing in the cecum of nude mice treated with 4OHT+DOX. Scale bar, 100 μ m. (c) Immunofluorescent staining of eGFP (green) and E-cadherin (red) of primary tumors of HT29F β cells growing in the cecum of nude mice treated with doxycyclin. Scale bar, 100 μ m. (d) Immunofluorescent staining to detect eGFP (green) and E-cadherin (red) in sections of tumors growing in the cecum wall of mice treated with DOX+4OHT. Scale bar, 100 μ m. Dashed squares 1 and 2 indicate regions magnified in right panels. Scale bar, 50 μ m. (b,c,d) Nuclear staining, Hoechst 33342 (blue).

SUPPLEMENTARY FIGURE 10



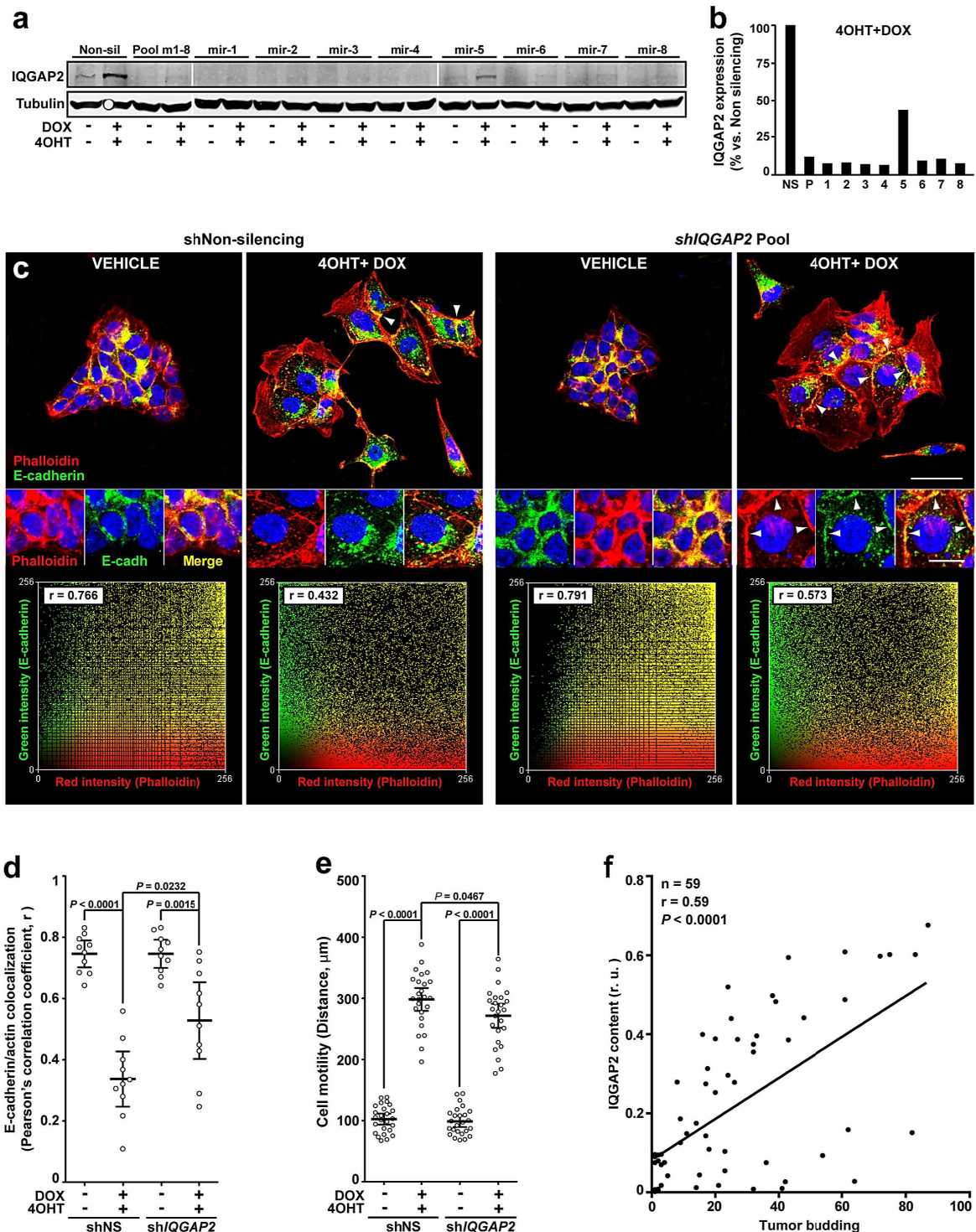
Supplementary Figure 10. The amounts of nuclear FOXO3a and β -catenin are comparable between tumor bulk and the corresponding invasive front in human primary colon carcinomas. (a-c): Immunofluorescent staining of tumor bulk (left panels) and the corresponding invasive front (right panels) of human primary colon carcinomas. (a) Carcinoma with low levels of both proteins at both tumor locations. (b) Carcinoma with increased amounts of both nuclear proteins at the invasive front. (c) Carcinoma presenting general high levels of both proteins at the tumor bulk and the invasive front. Samples were stained for FOXO3a (green) and beta-catenin (red). Nuclei were stained with Hoechst 33342 (blue). Scale Bar, 100 μ m. Lower inserts correspond to magnifications showing split or merged channels. The magnified merged image lacks the blue color channel and yellow color indicates FOXO3a and β -catenin colocalization. Scale Bar, 25 μ m. (d) Column scatter plot representing nuclear levels of the indicated proteins in tumor bulks (TB) and the corresponding invasive fronts (IF) of 65 primary human colon carcinomas. Lines connecting dots label values of pair wise analyzed samples. Horizontal bars show means and 95% CI. P values of Mann Whitney test are indicated.

SUPPLEMENTARY FIGURE 11



Supplementary Figure 11. FOXO3a and β -catenin regulate a common set of target genes.
(a,b) Relative expression levels of several mRNAs analyzed by real-time RT-PCR in DLD1Fj-C7 cells treated as indicated for 24 h. Data is represented as fold induction over control. Bars show mean $\pm$ s.d. of triplicates from three independent experiments. (a) Known FOXO3a and β -catenin target genes analyzed as quality control of the microarray analysis. (b) Validation of candidate target genes identified in the transcriptomic analysis. (c) Expression values of genes known to be involved in formation of tight and adherens junctions. Values are shown as fold change for the indicated treatments. Official gene and protein (prot) symbols and HGNC Gene Names are listed. (d) Schematic representation of tight and adherens junctions between two epithelial cells including their main components and regulators. Gene products found to be co-regulated by FOXO3a and β -catenin in our system are represented with red symbols.

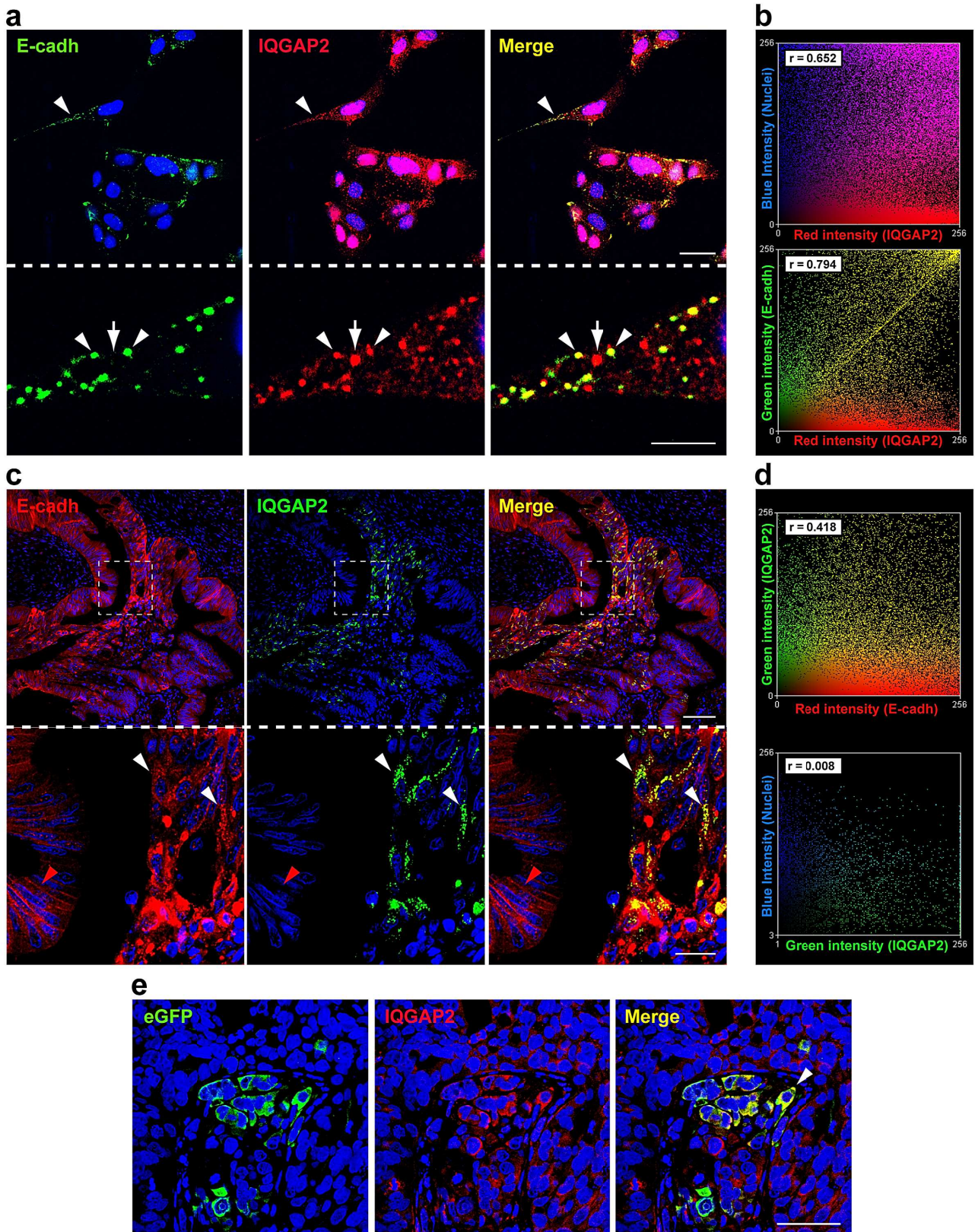
SUPPLEMENTARY FIGURE 12



Supplementary Figure 12. IQGAP2 plays a role in E-cadherin relocation, cell motility and correlates with tumor budding in human colon carcinomas.

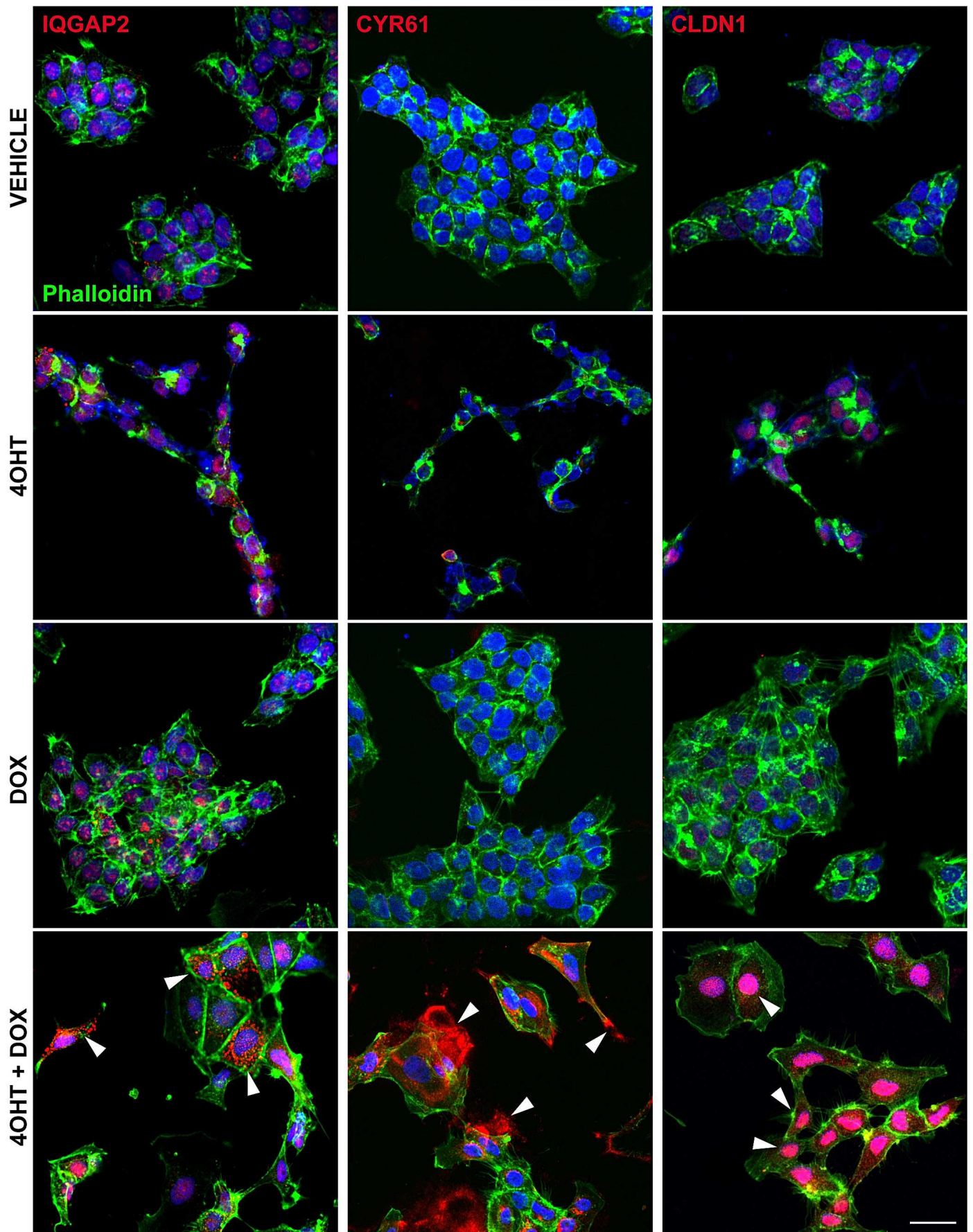
(a) Analysis of IQGAP2 protein levels by western blot in DLD1F β -C7 cells infected with lentivirus expressing non-silencing shRNA (Non-sil), a pool of eight different shRNAs against IQGAP2 or each shRNA separately and treated as indicated. White vertical bars indicate that samples were run in separated gels. (b) Quantification of IQGAP2 expression detected by Western blot and shown in panel (a). Values correspond to the percentage of IQGAP2 expression in each culture versus cells infected with lentivirus coding for a non-silencing shRNA and treated with 4OHT + DOX. (c) Colocalization of immunostained E-cadherin (green) and Phalloidin-FITC-bound actin (red) in DLD1F β -C7 cells treated as indicated (upper panels). Scale bar, 25 μ m. Lower pictures show magnifications to better visualize E-cadherin subcellular localization. Scale bar, 10 μ m. White arrowheads point E-cadherin bound to actin cytoskeleton. Lower panels: Color scatter plots showing the corresponding intensity correlation analysis of green and red channels of the upper confocal pictures. Yellow dots represent colocalized E-cadherin and actin. Pearson's correlation coefficient (r) is indicated. (d) Dot plot representing the values of E-cadherin/actin colocalization coefficients calculated for ten different pictures randomly taken from DLD1F β -C7 cells treated as indicated. Using a non-parametric Mann Whitney test we analyzed the differences between treatments or IQGAP2 knock down with cells expressing non-silencing shRNA (shNS) as controls. P values are indicated for each compared pair of values. (e) Scatter plot representing motility of DLD1F β -C7 cells treated as indicated and expressing a non-silencing shRNA (shNS) or a pool of shRNA knocking down IQGAP2. Values correspond to the distance covered by 25 individual cells in a time-lapse video, recording one picture every 20 min during 120 h. (d,e) Horizontal bars indicate means, errors show the 95% CI. (f) Dot plot representing the correlation of IQGAP2 immunofluorescent signal with the incidence of tumor budding in 59 human primary colon carcinomas. Correlation was evaluated by non-parametric Spearman test. Number of cases (n), coefficient of correlation (r), and P values are indicated. Tumor budding and relative units (r. u.) of IQGAP2 content are calculated as explained in Methods section.

SUPPLEMENTARY FIGURE 13



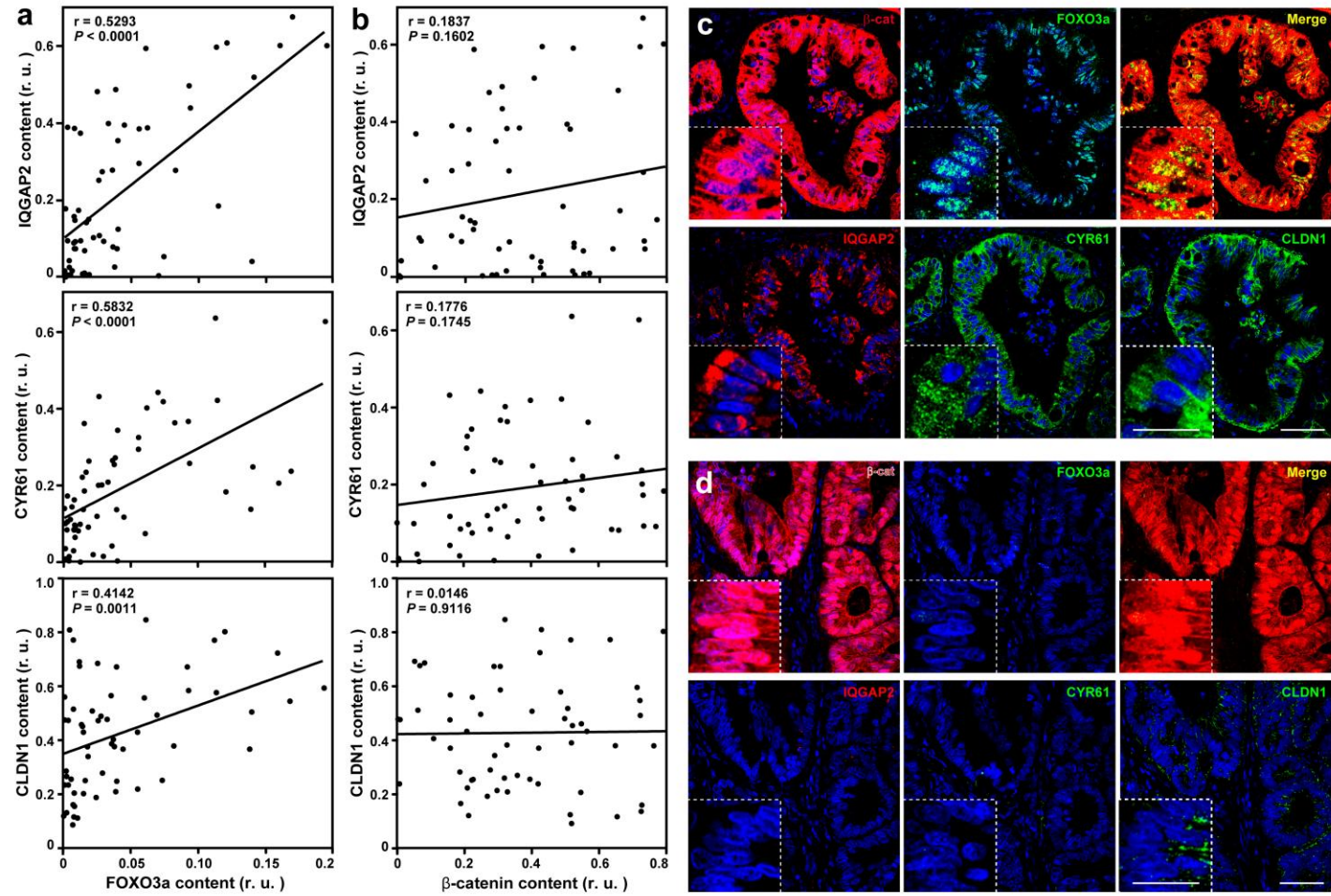
Supplementary Figure 13. IQGAP2 and E-cadherin colocalize in cytosolic aggregates in colon cancer cells with lost epithelial phenotype. (a) Immunofluorescence and confocal microscopy analysis of E-cadherin (green) and IQGAP2 (red) in DLD1F β -C7 cells treated with 4OHT + DOX. Upper and lower panels show different magnifications. Scale bars, 25 μ m. Arrowheads indicate cytoplasmic aggregates of colocalization of both proteins detected. White arrows show IQGAP2 aggregates with absent E-cadherin. (b,d) Color scatter plots showing the corresponding intensity colocalization analysis of green (E-cadherin), red (IQGAP2) and blue (nucleus) channels of the confocal pictures shown in (a) and (c). Yellow or violet dots represent colocalized E-cadherin and IQGAP2 or nuclear IQGAP2, respectively. Pearson's correlation coefficient of colocalization is indicated (r). (c) Upper panels: Representative confocal picture of double immunofluorescence to detect IQGAP2 (green) and E-cadherin (red) in a human primary colon carcinoma. Dashed-lined squares in upper panels mark the areas shown at higher magnification in lower panels. Red arrowhead indicates E-cadherin located at cell-to-cell contacts. Scale bars: upper panels, 100 μ m; lower panels, 25 μ m. (e) Representative confocal picture of double immunofluorescence to detect eGFP (green) and IQGAP2 (red) in subcutaneous tumors generated by DLD1F β cells in nude mice treated with 4OHT + DOX (Fig. 2f). Scale bar, 100 μ m. (a-c) White arrowheads indicate cytosolic aggregates where E-cadherin and IQGAP2 colocalize. Nuclei were stained with Hoechst 33342 (blue).

SUPPLEMENTARY FIGURE 14



Supplementary Figure 14. FOXO3a and β -catenin induce the expression of proteins involved in cell scattering. Immunofluorescence and confocal microscopy analysis of IQGAP2, CYR61 and CLDN1 proteins (red) in DLD1F β -C7 cells treated as indicated. Nuclei were stained with Hoechst 33342 (blue) and the actin cytoskeleton with Phalloidin-FITC (green). Double treatment (4OHT + DOX) of DLD1F β -C7 cells promotes IQGAP2 accumulation in cytosolic aggregates and nucleus, CYR61 secretion to the extracellular matrix and CLDN1 accumulation within the cell nucleus (respective white arrowheads). Scale bar, 25 μ m.

SUPPLEMENTARY FIGURE 15



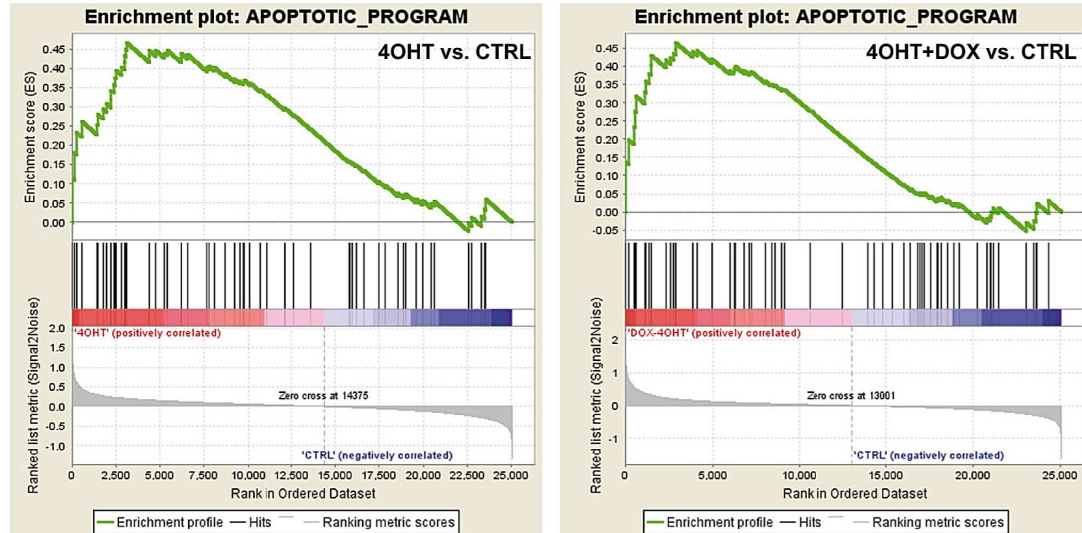
Supplementary Figure 15. Correlation of nuclear FOXO3a and β-catenin accumulation with the expression levels of their target proteins IQGAP2, CYR61 and CLDN1 in human primary colon carcinomas. (a,b) Dot plot representing the correlation between the amounts of the indicated target proteins to nuclear FOXO3a (a) or beta-catenin (b) immunofluorescent signal in 59 human primary colon carcinomas. Correlation was evaluated by non-parametric Spearman test. Coefficient of correlation (r) and P values are indicated. Relative units (r. u.) are calculated as explained in Methods section. **(c,d)** Confocal pictures of immunofluorescence staining to detect FOXO3a (green) and β-catenin (red) colocalization as well as IQGAP2 (red), CYR61 or CLDN1 (green) proteins in consecutive histological sections. Representative carcinomas with low (c) or high (d) nuclear FOXO3a and β-catenin colocalization signal are shown. Nuclei were stained with Hoechst 33342 (blue). Inserts show magnifications. Merged pictures lack the blue color channel and yellow color indicates FOXO3a and β-catenin colocalization. Scale bars: main panels, 100 μm; inserts panels, 25 μm.

SUPPLEMENTARY FIGURE 16

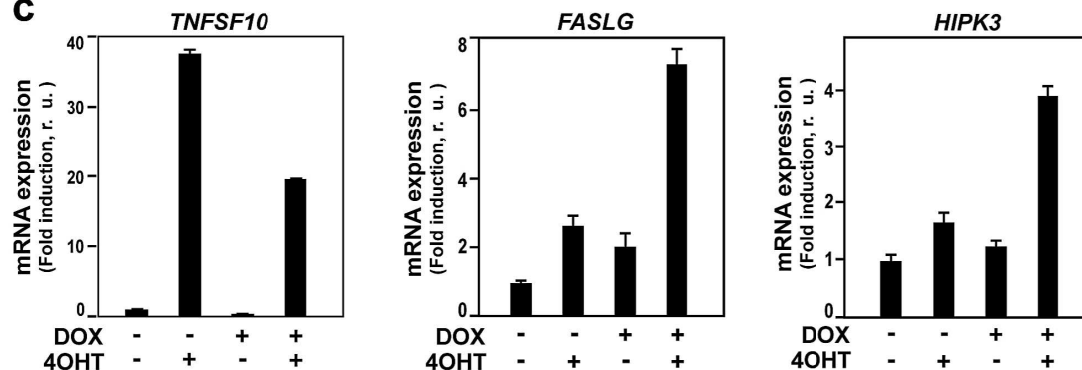
a

4OHT vs. CTRL				4OHT+DOX vs. CTRL			
Gene sets most significantly enriched	GO term ID	NES	NOM p-val	Gene sets most significantly enriched	GO term ID	NES	NOM p-val
BLOOD HOMEOSTASIS				CELL MORPHOLOGY AND ADHESION			
COAGULATION	GO:0050817	1.760	0.002	BASOLATERAL PLASMA MEMBRANE	GO:0016323	1.804	< 0.0001
BLOOD COAGULATION	GO:0007596	1.741	0.002	ADHERENS JUNCTION	GO:0005912	1.512	0.061
HEMOSTASIS	GO:0007599	1.654	0.004	CYTOPLASM ORGANIZATION	GO:0007028	1.595	0.018
IMMUNE SYSTEM				IMMUNE SYSTEM			
REGULATION OF DEFENSE RESPONSE	GO:0031347	1.732	0.011	REGULATION OF DEFENSE RESPONSE	GO:0031347	1.623	0.016
APOPTOSIS				B CELL ACTIVATION	GO:0042113	1.556	0.031
APOPTOTIC PROGRAM	GO:0006915	1.681	0.007	METALLOPROTEASES			
CELL STRUCTURE DISASSEMBLY DURING APOPTOSIS	GO:0006921	1.597	0.021	METALLOENDOPEPTIDASE ACTIVITY	GO:0004222	1.559	0.034
CELL MORPHOLOGY AND ADHESION				APOPTOSIS			
HOMOPHILIC CELL ADHESION	GO:0007156	1.581	0.028	APOPTOTIC PROGRAM	GO:0006915	1.523	0.017
PROLIFERATION				PROLIFERATION			
CELL DIVISION	GO:0051301	-1.678	0.009	POSITIVE REGULATION OF CELL CYCLE	GO:0045787	-1.529	0.030
				CELL DIVISION	GO:0051301	-1.490	0.046

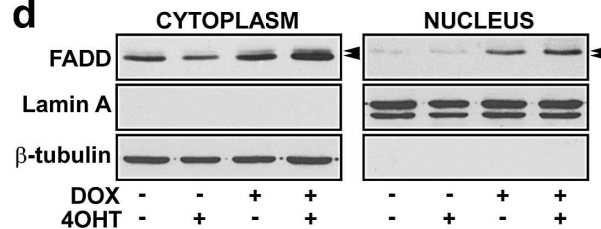
b



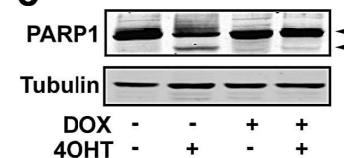
c



d

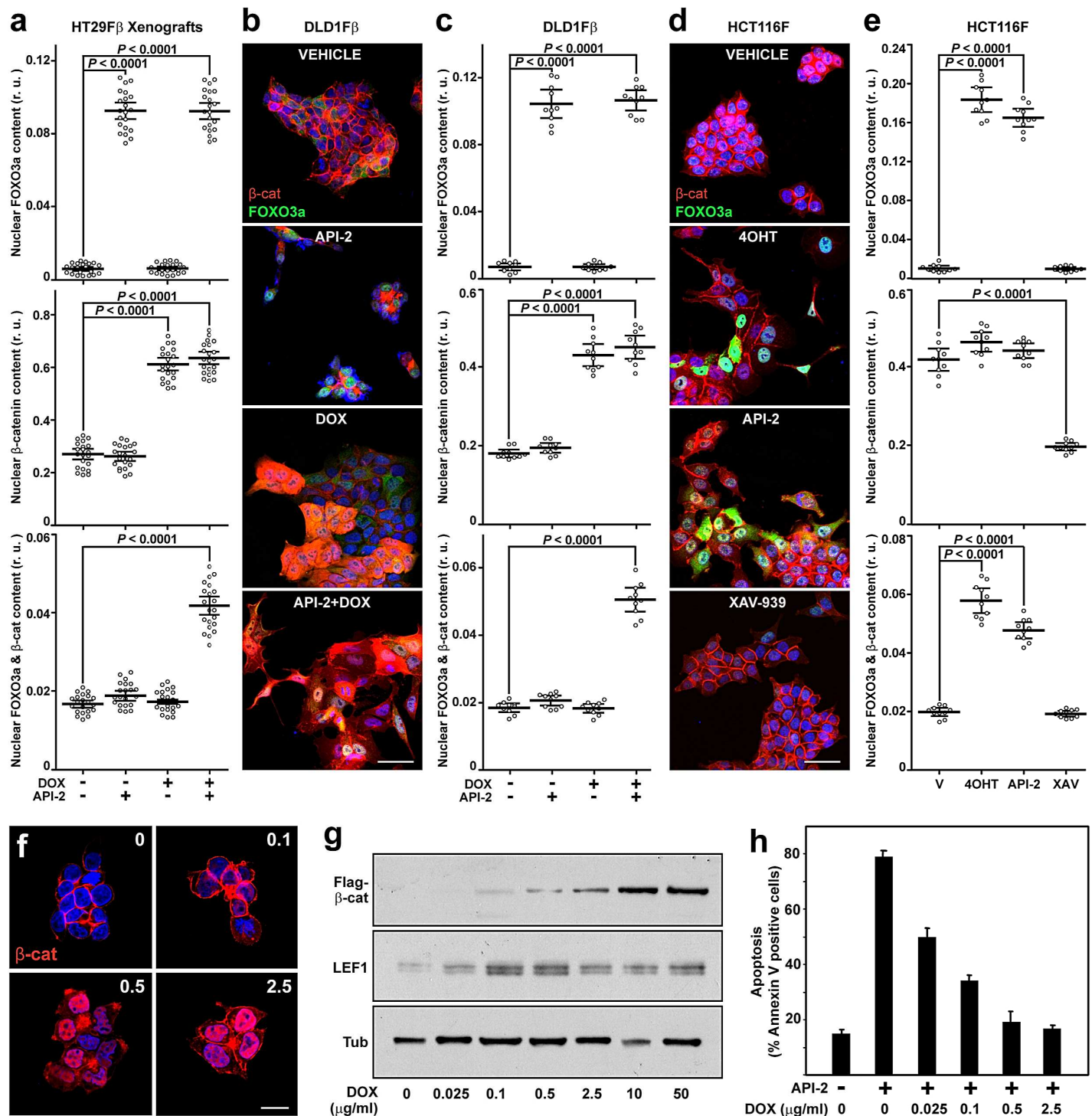


e



Supplementary Figure 16. β-catenin rescues from FOXO-induced apoptosis by modulating the expression of a defined set of target genes. (a) Gene Set Enrichment Analysis (GSEA, www.broadinstitute.org) of RMA-normalized microarray data from DLD1Fβ-C7 cells treated as indicated, comparing to molecular signature gene sets (Gene Ontology Consortium). The GO term ID (Gene Ontology Identification; www.geneontology.org) of the respective cellular process, component or molecular function is shown. NES, normalized enrichment factor; NOM, corresponding nominal *P* value as provided by the software. Underrepresented terms (negative correlation) are shown in italics. (b) Charts representing the GSEA result for enrichment of the “Apoptotic_Program” gene set obtained by comparing the gene expression values of the indicated treatments of DLD1Fβ-C7 cells. (c) Real-time RT-PCR analysis of three apoptosis-related transcripts in DLD1Fβ-C7 cells treated as indicated for 24 h. Data are represented as fold induction over the values obtained from cells treated with vehicle. Error bars show $\pm$ s.d. of triplicates from three independent experiments. (d) Western blot detecting FADD protein in nuclear and cytosolic fractions of DLD1Fβ-C7 cells treated as indicated. Arrowheads indicate FADD protein doublets. Laminin A and β-Tubulin served as controls for gel loading and purity of fractions. (e) Western blot detecting PARP1 protein in DLD1Fβ-C7 cells treated as indicated. Arrowheads mark full-length and cleaved PARP1 polypeptides. α-Tubulin levels were detected to control gel loading.

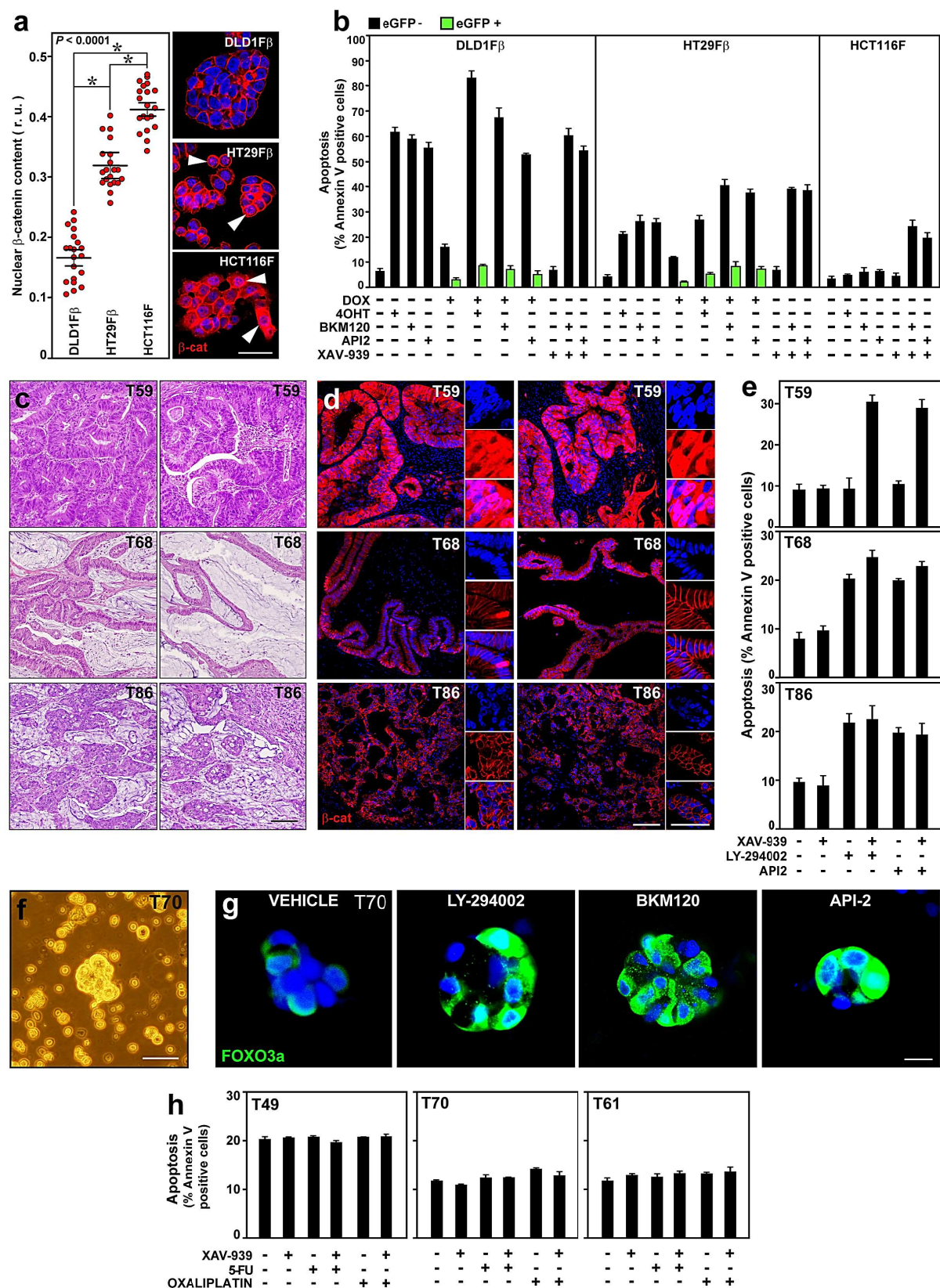
SUPPLEMENTARY FIGURE 17



Supplementary Figure 17. Nuclear β -catenin confers resistance to the AKT inhibitor API-2 that promotes FOXO3a nuclear accumulation and apoptosis.

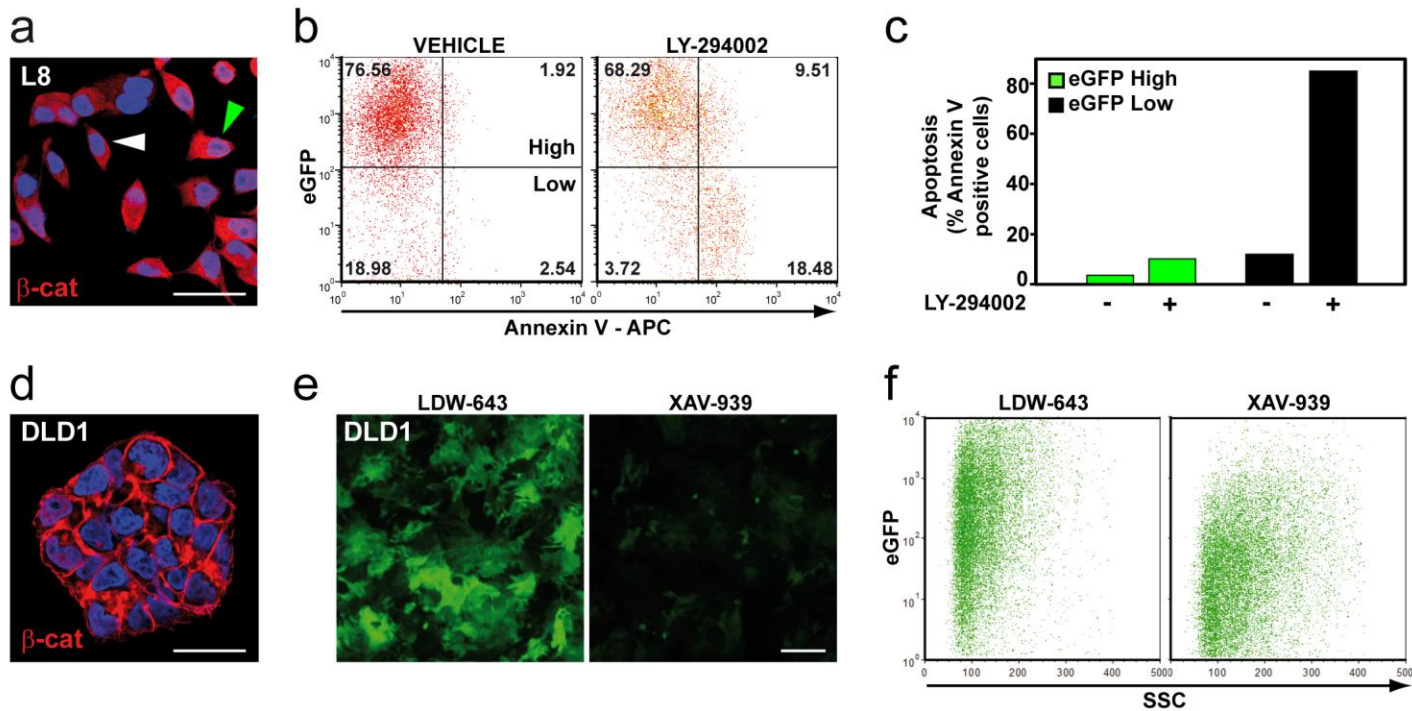
(a,c,e) Column scatter plots representing quantification of nuclear FOXO3a, β -catenin or their colocalization levels in tumor xenografts of HT29F β cells growing in the cecum wall of nude mice (Fig. 5) (a), in DLD1F β -C7 (c) or HCT116F (e) cells treated as indicated. (a) Three random fields were quantified per seven xenografts corresponding to every group of treatment. The 21 values correspond to the relative protein level in each field quantified. Differences between vehicle-treated mice and the rest of the groups were analyzed by a non-parametric Mann Whitney test and significant P values are indicated. (c,e) 10 random fields and a minimum of 100 immunostained cells were quantified for each condition. The represented values correspond to the signal measured in each individual field. Significant differences were calculated by a non-parametric Mann Whitney test comparing all experimental conditions with vehicle-treated DLD1F β -C7 cells and significant P values are shown. (b,d) Immunofluorescence and confocal microscopy analysis of β -catenin (red) and FOXO3a (green) in DLD1F β -C7 (b) or HCT116F (d) cells treated as indicated. Scale bar, 50 μ m. (f) Immunofluorescence and confocal analysis to detect nuclear β -catenin levels (red) in DLD1F β -C7 cells treated with DOX. Numbers indicate the concentration in μ g/ml of doxycycline used. Scale bar, 25 μ m. (b,d,f) Nuclei were stained with Hoechst 33342 (blue). (g) Analysis by Western blot of the exogenous Flag- β -catenin^{S33Y} (Flag antibody) and endogenous LEF1 protein expression in DLD1F β -C7 cells treated with the concentrations of DOX indicated. Tubulin was used as loading control. (h) Proportion of apoptotic DLD1F β -C7 cells treated as indicated and analyzed by Annexin V binding and flow cytometry. Error bars show $\pm$ s.d. of triplicates from three independent experiments.

SUPPLEMENTARY FIGURE 18



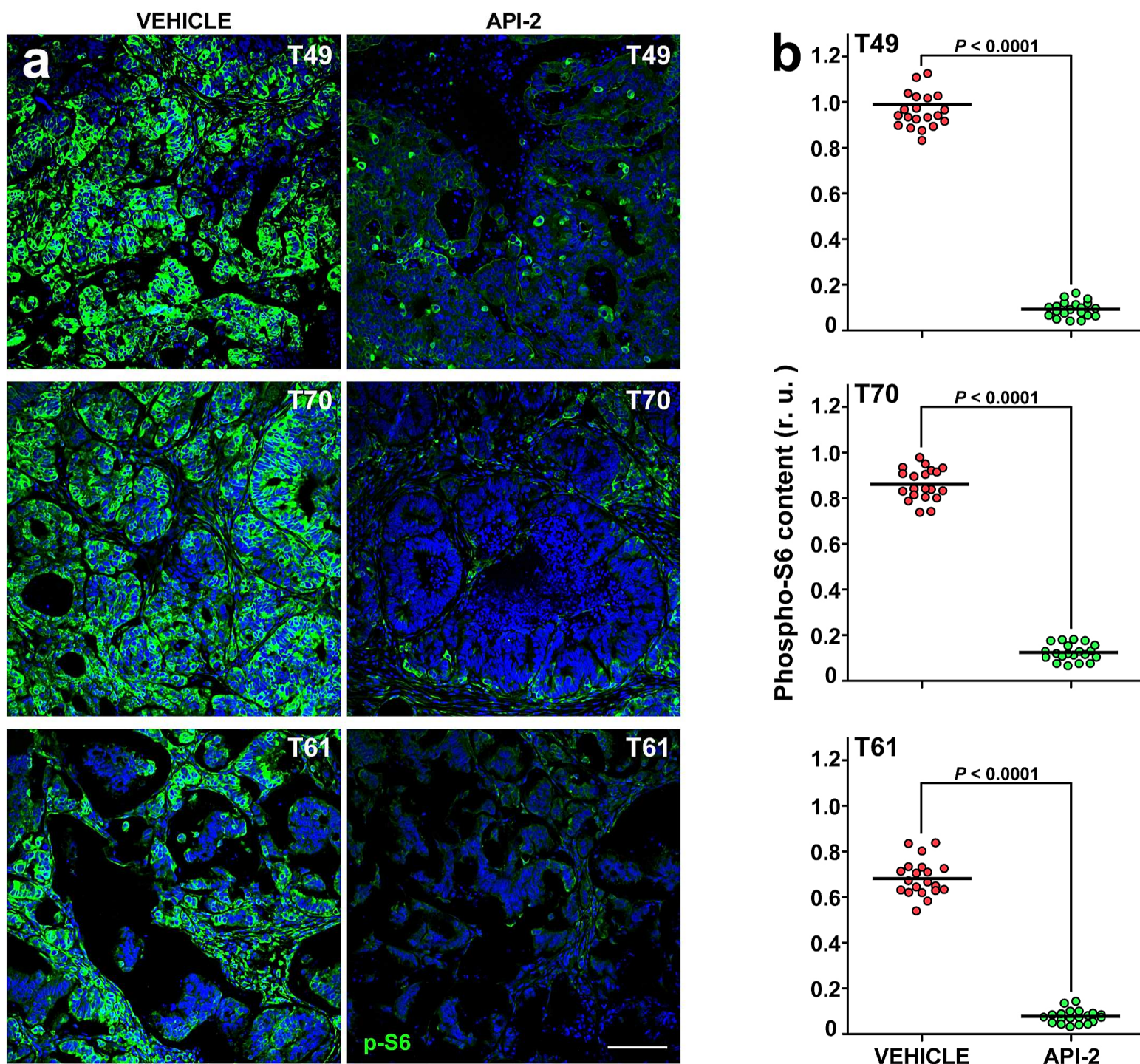
Supplementary Figure 18. Nuclear β -catenin confers resistance to FOXO3a-induced apoptosis promoted by PI3K and AKT inhibitors. (a) Immunofluorescence and confocal microscopy analysis of β -catenin levels (red) in three indicated colon cancer cell lines. White arrowhead indicate cells with nuclear β -catenin. Scale bar, 25 μ m. Column scatter plot representing the quantification of nuclear β -catenin amounts. Indicated P value was calculated by non-parametric ANOVA analysis with Kruskal-Wallis test. Asterisk marks significant difference between groups quantified by Dunn's multiple comparison test. Bars and errors indicate the mean and the 95% CI. (b,e,h) Percentage of apoptotic cells measured by flow cytometry in the same colon cancer cell lines shown in (a) or in patient-derived cells from indicated primary colon carcinomas (e,h) upon treatment. Error bars show $\pm$ s.d. of triplicates from three independent experiments. (c) Images of hematoxylin & eosin stained histological sections of patients' primary carcinomas (left panels) and subcutaneous xenograft tumors (right panels) generated from the respective patient-derived cells in NOD-SCID mice. Scale bar, 100 μ m. (d) Immunofluorescence and confocal microscopy analysis of β -catenin (red) in patients' primary carcinomas (left panels) and respective subcutaneous tumors xenografts grown in NOD-SCID mice (right panels). Insets: Magnifications showing nuclear β -catenin expression. Nuclei were stained with Hoechst 33342 (blue). Scale bars, 100 μ m; inserts, 50 μ m. (f) Representative phase-contrast image of sphere cultures of cells derived from the tumor of patient T70. Scale bar, 100 μ m. (g) Immunofluorescence and confocal microscopy analysis of FOXO3a (green) in patient-derived sphere cultures (patient T70) treated with the indicated compounds. Scale bar, 20 μ m. (a,d,g) Nuclei were stained with Hoechst 33342 (blue).

SUPPLEMENTARY FIGURE 19



Supplementary Figure 19. Colon cancer cells with high TCF/LEF- β -catenin-dependent transcription are more resistant to apoptosis induced by PI3K inhibition. (a) Immunofluorescence and confocal microscopy analysis of β -catenin (red) in L8 colon cancer cell cultures showing two coexisting cell populations with high (green arrowhead) or low (white arrowhead) nuclear β -catenin levels. Scale bar, 25 μ m. (b) Analysis by flow cytometry of the proportion of apoptosis in a pool of L8 cells infected with a lentiviral eGFP-reporter for TCF/LEF- β -catenin transcriptional activity (7TGP) upon treatment with the PI3K inhibitor LY294002. Percentages of cells per quadrant are indicated. (c) Quantification of apoptotic cells in the same L8-7TGP cell cultures differentiating between eGFP high- and low-expressing subpopulations treated as indicated. (d) Immunofluorescence and confocal microscopy analysis of β -catenin levels (red) in parental DLD1 colon cancer cells. Scale bar, 25 μ m. (a,d) Nuclei were stained with Hoechst 33342 (blue). (e) Representative images of eGFP fluorescent signal from DLD1 cells infected with the β -catenin-responsive 7TGP lentiviral reporter treated with either LDW643 (inactive analogue) or XAV-939 tankyrase inhibitor. Scale bar, 100 μ m. (f) Chart showing flow cytometry analysis to quantify the intensity of eGFP fluorescent signal in the same DLD1-7TGP cells treated as indicated. Side Scatter (SSC).

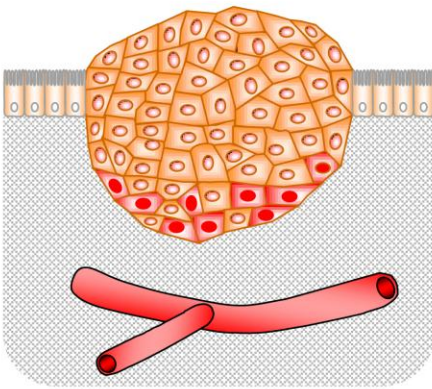
SUPPLEMENTARY FIGURE 20



Supplementary Figure 20. API-2 treatment inhibits AKT signaling in xenograft tumors grown from patient-derived cells. (a) Immunofluorescence and confocal microscopy analysis of phospho-S6 protein (green) in representative histological sections of patient-derived xenograft tumors from mice treated with vehicle or API-2. Samples correspond to xenografts growing in mice from the experiment endpoint shown in Figure 6e . Scale bar, 200 μ m. Nuclei were stained with Hoechst 33342 (blue). (b) Dot plots representing quantification of phospho-S6 protein content in 4 random taken pictures from 5 different xenograft tumors corresponding to the in vivo experiment shown in Fig. 6e (red dots, vehicle; green dots, API-2 treatment). Bars indicate the means. Indicated P values were calculated by unpaired t-test.

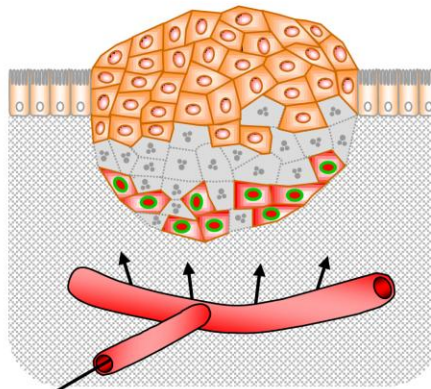
SUPPLEMENTARY FIGURE 21

Heterogeneous
Carcinoma



Cell growth

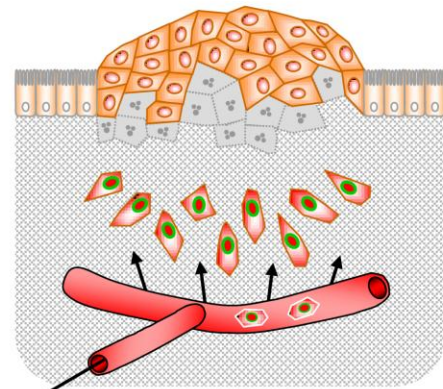
Responding
Carcinoma



PI3K or AKT inhibitors

Selective apoptosis

Metastatic
Carcinoma



PI3K or AKT inhibitors

Cell scattering & Invasion

-  Apoptotic colon cancer cell
-  Colon cancer cell with low nuclear beta-catenin
-  Colon cancer cell with high nuclear beta-catenin
-  Colon cancer cell with high nuclear beta-catenin and FOXO3a

Supplementary Figure 21. Model summarizing colon carcinoma progression driven by nuclear FOXO3a and β -catenin in presence of PI3K or AKT inhibitory drugs. In absence of treatment, a primary colon carcinoma is built of proliferative cancer cells with heterogeneous levels of nuclear β -catenin. Specific drugs, such as PI3K and AKT inhibitors, that provoke nuclear FOXO3a accumulation, induce FOXO3a-mediated apoptosis selectively in those cancer cells with low nuclear β -catenin content, whereas cells with high nuclear β -catenin levels remain viable and reduce proliferation. In these resistant cancer cells, nuclear FOXO3a and β -catenin act in concert regulating a defined set of target genes responsible for cell scattering and metastasis. Although the tumor partially responds to treatment, the resistant cell population disseminates and contributes to treatment-induced metastasis.

Supplementary Table 1: FOXO3a and/or beta-catenin regulated genes (12 h treatment)

Gene Symbol	FC 4OHT		FC DOX		FC 4OHT+DOX		FC 4OHT+DOX vs. 4OHT	Known Target
	vs. Vehicle	P-value*	vs. Vehicle	P-value*	vs. Vehicle	P-value*		
<i>SLC2A3</i>	2.5	1.0	2.4	1.0	13.7	0.002	5.56	
<i>ANKRD1</i>	26.6	3.81E-06	9.2	8.61E-05	121.6	1.84E-07	4.56	
<i>FSIP2</i>	n.s.	---	n.s.	---	4.2	0.023	4.36	
<i>SLC1A2</i>	8.4	2.80E-04	n.s.	---	34.7	5.00E-06	4.11	
<i>C10orf118</i>	1.4	1.0	n.s.	---	5.3	0.008	3.74	
<i>MCOLN3</i>	1.8	1.0	1.8	1.0	5.0	0.008	2.80	
<i>IQGAP2</i>	16.5	3.33E-06	2.3	0.042	44.4	2.98E-07	2.69	
<i>TMPRSS11F</i>	4.0	0.232	n.s.	---	10.8	0.004	2.68	
<i>LCA5</i>	2.4	0.273	1.4	1.0	6.4	0.001	2.65	
<i>CPA4</i>	n.s.	---	5.3	0.023	3.0	0.528	2.65	
<i>CYP24A1</i>	3.2	1.0	n.s.	---	8.3	0.034	2.62	
<i>KTN1</i>	1.4	1.0	1.3	1.0	3.4	0.002	2.54	
<i>RB1CC1</i>	1.9	1.0	1.4	1.0	4.9	0.032	2.54	
<i>CD2AP</i>	1.5	1.0	1.5	1.0	3.7	0.012	2.54	
<i>LOC100128868</i>	3.3	0.493	2.0	1.0	8.1	0.007	2.47	
<i>RIMKLB</i>	14.2	0.005	1.7	1.0	35.2	0.001	2.47	
<i>FRMD6</i>	n.s.	---	1.8	1.0	2.8	0.019	2.43	
<i>KRT40</i>	6.0	0.244	n.s.	---	14.4	0.012	2.42	
<i>JMJD1C</i>	3.2	0.196	n.s.	---	7.8	0.003	2.41	
<i>NIPBL</i>	1.4	1.0	1.3	1.0	3.4	0.006	2.40	
<i>HSPA4L</i>	-5.2	4.40E-04	1.3	1.0	-2.2	0.139	2.40	
<i>CLEC2B</i>	2.6	0.463	n.s.	---	6.3	0.004	2.38	
<i>DAB2</i>	1.9	1.0	2.1	1.0	4.5	0.007	2.34	
<i>EMP1</i>	4.7	0.486	1.7	1.0	10.9	0.019	2.33	(b)
<i>NAV3</i>	1.9	1.0	1.7	1.0	4.2	0.007	2.28	
<i>ARHGAP29</i>	3.9	4.80E-04	1.6	1.0	8.8	1.13E-05	2.27	
<i>BMPR1B</i>	2.7	0.109	1.6	1.0	6.0	0.001	2.25	
<i>CCDC68</i>	3.4	0.106	1.9	1.0	7.2	0.003	2.12	
<i>KLHL28</i>	1.6	1.0	n.s.	---	3.4	0.020	2.11	
<i>GPR52</i>	1.7	0.571	2.2	0.032	3.5	0.001	2.05	
<i>RARB</i>	4.3	0.218	n.s.	---	8.6	0.011	2.03	
<i>INPP4B</i>	1.7	1.0	1.3	1.0	3.4	0.002	2.02	
<i>RECQL</i>	1.4	1.0	1.7	1.0	2.7	0.017	1.99	
<i>HIST1H2AL</i>	-6.6	0.007	n.s.	---	-3.3	0.207	1.98	
<i>PIK3CA</i>	4.1	0.002	n.s.	---	8.1	9.33E-05	1.98	(b)
<i>GBP3</i>	n.s.	---	1.5	1.0	2.0	0.027	1.98	
<i>MPP5</i>	n.s.	---	1.6	0.287	2.2	0.007	1.97	
<i>GOLGB1</i>	1.7	1.0	n.s.	---	3.3	0.027	1.95	
<i>NCRNA00189</i>	4.8	0.097	n.s.	---	9.3	0.006	1.95	
<i>ACTA2</i>	1.9	1.0	1.5	1.0	3.6	0.042	1.88	
<i>PNPLA8</i>	1.7	1.0	n.s.	---	3.2	0.011	1.86	
<i>NSAP11</i>	18.4	9.48E-06	n.s.	---	34.0	2.07E-06	1.85	
<i>PRDM1</i>	10.8	0.001	2.3	1.0	19.9	1.79E-04	1.83	
<i>KLHL5</i>	1.2	1.0	1.4	1.0	2.2	0.015	1.82	
<i>KRTAP9-2</i>	6.4	0.392	2.0	1.0	11.7	0.050	1.82	
<i>NT5E</i>	1.7	0.090	n.s.	---	3.1	2.47E-04	1.81	
<i>C13orf15</i>	1.7	1.0	1.5	1.0	3.1	0.008	1.81	
<i>LPHN3</i>	15.6	2.02E-06	n.s.	---	28.2	4.27E-07	1.81	
<i>WWC2</i>	1.6	1.0	1.8	1.0	2.9	0.040	1.80	
<i>CTNNAL1</i>	1.5	1.0	1.6	1.0	2.6	0.045	1.79	
<i>FRYL</i>	3.0	0.120	n.s.	---	5.4	0.005	1.79	
<i>GOPC</i>	1.5	0.304	n.s.	---	2.6	2.70E-04	1.79	
<i>ATP13A3</i>	1.4	1.0	1.8	0.018	2.5	0.001	1.76	
<i>PLK2</i>	2.2	0.005	1.7	0.078	3.8	7.34E-05	1.74	
<i>CLDN1</i>	4.5	0.001	1.8	0.530	7.8	4.24E-05	1.74	
<i>DCP2</i>	1.3	1.0	1.2	1.0	2.2	0.023	1.72	
<i>HNMT</i>	4.2	0.004	n.s.	---	7.1	3.56E-04	1.71	
<i>RICTOR</i>	4.2	0.022	n.s.	---	7.2	0.002	1.71	
<i>ARHGAP42</i>	2.0	1.0	n.s.	---	3.3	0.049	1.69	

STAMBPL1	1.7	1.0	1.2	1.0	2.9	0.022	1.69
CFTR	1.2	1.0	1.1	1.0	2.0	0.011	1.67
FCHO2	1.9	0.277	n.s.	---	3.2	0.003	1.67
HIPK3	1.4	0.447	1.2	1.0	2.3	2.95E-04	1.67
KRTAP9-8	2.9	0.214	1.4	1.0	4.7	0.012	1.63
SGTB	2.3	0.148	n.s.	---	3.7	0.004	1.63
L3MBTL3	4.3	4.13E-05	1.2	1.0	7.0	4.21E-06	1.63
SLC7A11	5.8	1.52E-04	1.4	1.0	9.4	2.28E-05	1.61
CLDN16	5.4	0.059	n.s.	---	8.6	0.009	1.61
DAAM1	4.2	0.197	n.s.	---	6.7	0.023	1.60
RASA1	1.8	1.0	n.s.	---	2.9	0.028	1.59
VSNL1	-5.4	0.048	n.s.	---	-3.4	0.461	1.56
CTGF	6.3	4.13E-05	3.2	0.001	9.8	7.40E-06	1.56
RAB3B	2.8	0.003	n.s.	---	4.3	2.15E-04	1.53
NEIL3	-3.2	0.025	n.s.	---	-2.1	0.650	1.52
HBEGF	7.7	0.001	n.s.	---	11.7	1.46E-04	1.52
MBNL2	2.3	0.558	n.s.	---	3.4	0.027	1.51
TMEM133	1.9	1.0	1.3	1.0	2.9	0.035	1.51
ALS2CR12	5.1	0.077	n.s.	---	7.7	0.014	1.51
YOD1	1.7	1.0	1.3	1.0	2.6	0.048	1.50
WDR19	1.6	1.0	n.s.	---	2.5	0.033	1.50
XRN1	2.2	0.777	n.s.	---	3.2	0.035	1.49
TFPI	21.9	7.40E-06	-1.2	1.0	32.5	2.84E-06	1.48
PFDN4	1.6	0.303	n.s.	---	2.3	0.003	1.47
TBC1D15	2.7	0.028	1.3	1.0	4.0	0.002	1.47
PDLIM5	1.7	1.0	1.4	1.0	2.5	0.028	1.45
STARD13	2.8	0.046	n.s.	---	4.0	0.004	1.44
SPTLC3	-3.9	2.12E-04	1.2	1.0	-2.7	0.002	1.44
CRIM1	1.5	0.569	n.s.	---	2.1	0.004	1.44
RAB30	2.4	0.135	1.5	1.0	3.4	0.010	1.43
KRTAP9-4	3.1	0.111	1.4	1.0	4.4	0.014	1.43
TEAD1	1.8	0.361	1.4	1.0	2.5	0.009	1.43
GCNT1	3.2	1.36E-04	1.4	1.0	4.5	1.82E-05	1.40
UTRN	3.5	0.003	1.5	1.0	4.8	4.40E-04	1.39
HAPLN3	3.9	0.045	n.s.	---	5.5	0.009	1.39
PCMTD1	4.1	0.022	-1.5	1.0	5.7	0.004	1.39
HEG1	4.5	0.002	1.8	1.0	6.2	3.76E-04	1.37
POC1B	1.5	1.0	1.2	1.0	2.0	0.030	1.36
FMNL2	3.4	0.019	1.5	1.0	4.7	0.003	1.36
IMPA1	1.6	0.451	1.2	1.0	2.2	0.013	1.34
FBXO30	3.8	0.006	n.s.	---	5.2	0.001	1.34
HEATR5B	2.1	0.439	n.s.	---	2.8	0.037	1.34
ITGA2	2.2	0.006	1.1	1.0	3.0	0.001	1.34
ARHGAP21	2.4	0.013	1.3	1.0	3.2	0.001	1.34
NFE2L2	2.0	0.070	n.s.	---	2.7	0.005	1.34
NDFIP2	1.5	1.0	n.s.	---	2.0	0.029	1.33
CAV2	2.5	0.084	1.6	1.0	3.3	0.011	1.33
OSGIN2	2.5	0.004	1.3	1.0	3.3	4.91E-04	1.32
CDC14A	5.5	7.34E-07	1.4	0.288	7.2	2.19E-07	1.32
STK17A	2.0	0.136	1.5	1.0	2.6	0.010	1.32
CRIP1	1.8	0.559	n.s.	---	2.3	0.034	1.31
FEM1C	2.2	0.004	1.2	1.0	2.9	4.02E-04	1.31
SEPP1	5.0	1.55E-04	-2.3	0.031	6.5	4.70E-05	1.30
TSPAN8	1.8	0.831	n.s.	---	2.3	0.050	1.30
PLEKHA1	1.9	0.226	1.2	1.0	2.5	0.019	1.30
LGR5	1.7	0.040	n.s.	---	2.2	0.002	1.29
E2F8	-3.6	0.011	n.s.	---	-2.8	0.054	1.28
KRTAP3-1	n.s.	---	2.9	1.02E-04	1.3	1.0	1.28
RP2	1.9	0.285	n.s.	---	2.5	0.026	1.28
IL18	3.2	0.027	n.s.	---	4.1	0.006	1.27
SNORD73A	-2.8	0.030	n.s.	---	-2.2	0.228	1.27
CD55	5.7	0.086	n.s.	---	7.3	0.032	1.27
GDA	2.9	0.001	n.s.	---	3.6	1.84E-04	1.27

(b)

(a)

CHIC2	3.2	0.047	n.s.	---	4.0	0.011	1.27	
SYNE2	3.4	0.047	n.s.	---	4.4	0.012	1.27	
ZNF192	3.4	0.003	n.s.	---	4.4	0.001	1.27	
SLC28A3	5.4	1.76E-04	n.s.	---	6.8	6.32E-05	1.26	
UBE2D1	4.7	2.61E-04	n.s.	---	5.9	8.74E-05	1.26	
CAV1	4.1	2.01E-04	2.0	0.060	5.2	6.12E-05	1.26	(b)
RALGPS2	6.2	3.75E-04	n.s.	---	7.8	1.49E-04	1.25	
CYP39A1	5.5	0.008	n.s.	---	6.9	0.003	1.25	
YPEL5	5.7	0.009	-1.5	1.0	7.1	0.004	1.25	
ABCA4	21.5	5.53E-09	n.s.	---	26.8	3.17E-09	1.25	
CRY1	1.7	0.122	1.2	1.0	2.1	0.008	1.25	
TJP1	1.7	0.070	1.2	1.0	2.1	0.005	1.24	
JPH1	-4.3	0.039	n.s.	---	-3.4	0.132	1.24	
PTPN14	2.5	0.003	1.3	1.0	3.1	0.001	1.24	
ERV3	2.7	0.087	n.s.	---	3.3	0.020	1.24	
DOCK9	2.0	0.051	1.2	1.0	2.4	0.006	1.23	
TOR1AIP2	2.7	0.020	1.3	1.0	3.3	0.005	1.23	
LGALS8	1.8	0.002	n.s.	---	2.2	1.89E-04	1.23	
CHMP4C	2.0	0.012	n.s.	---	2.5	0.002	1.22	
CCDC50	1.7	0.334	1.3	1.0	2.1	0.031	1.22	
RBMS2	2.1	0.235	n.s.	---	2.6	0.040	1.22	
RIOK3	5.1	7.47E-06	1.5	0.233	6.2	2.96E-06	1.22	
RAET1L	6.8	0.001	n.s.	---	8.3	0.001	1.22	
SERPINB8	2.7	0.042	n.s.	---	3.3	0.011	1.21	(b)
TIMP2	2.9	0.020	1.3	1.0	3.6	0.006	1.21	(b)
TES	2.5	3.05E-04	1.3	1.0	3.0	6.59E-05	1.21	
RNF217	3.0	0.162	1.4	1.0	3.6	0.049	1.21	
FAM134B	6.8	3.67E-06	n.s.	---	8.2	1.77E-06	1.20	
UBN2	2.6	5.06E-05	1.2	1.0	3.2	1.29E-05	1.20	
TMX4	2.6	0.023	n.s.	---	3.1	0.006	1.20	
SESN3	6.0	2.25E-05	-1.2	1.0	7.2	1.06E-05	1.20	
CLK1	1.9	0.194	1.2	1.0	2.3	0.033	1.20	
USP33	2.5	0.037	1.2	1.0	3.0	0.010	1.19	
RGL1	2.5	0.016	n.s.	---	2.9	0.004	1.19	
PMAIP1	13.1	2.28E-04	1.4	1.0	15.6	1.36E-04	1.19	
WHAMM	2.0	0.051	n.s.	---	2.3	0.009	1.19	
PLCE1	-3.5	0.032	n.s.	---	-2.9	0.092	1.18	
ODZ3	-3.1	0.003	n.s.	---	-2.6	0.011	1.18	
CYBRD1	5.7	6.10E-06	1.1	1.0	6.7	2.97E-06	1.18	
SAV1	1.8	0.264	n.s.	---	2.1	0.041	1.18	
GFPT2	2.0	0.047	-1.6	1.0	2.4	0.010	1.18	
DOCK5	1.8	0.085	1.3	1.0	2.1	0.014	1.17	
SNAP23	3.0	0.004	n.s.	---	3.5	0.001	1.17	
HIST1H2BM	-4.3	0.024	n.s.	---	-3.7	0.058	1.17	
XK	-2.4	0.025	1.4	1.0	-2.1	0.111	1.17	
ZFAND5	2.1	0.012	n.s.	---	2.4	0.003	1.17	
ITGB8	5.2	0.008	n.s.	---	6.1	0.004	1.17	
ARNTL	2.2	0.067	1.2	1.0	2.6	0.017	1.16	
PPFIBP1	2.6	0.002	1.4	1.0	3.0	0.001	1.16	
NETO2	-2.5	0.002	n.s.	---	-2.1	0.008	1.16	
TTPA	-3.2	0.016	1.4	1.0	-2.8	0.044	1.16	
DUSP6	3.5	0.008	2.1	0.400	4.0	0.004	1.16	(b)
BRD8	2.5	0.068	n.s.	---	2.9	0.022	1.16	
GPR137B	2.1	0.003	n.s.	---	2.4	0.001	1.15	
GAB1	2.6	0.048	1.2	1.0	2.9	0.017	1.15	
TLR6	2.8	0.077	n.s.	---	3.2	0.030	1.14	
BAG2	-2.4	0.030	1.2	1.0	-2.1	0.098	1.14	(a)
SWAP70	2.6	0.020	n.s.	---	3.0	0.008	1.14	
PARD6B	3.5	0.001	n.s.	---	4.0	2.81E-04	1.14	
DOCK8	2.4	0.001	n.s.	---	2.7	3.22E-04	1.13	
UBL3	2.6	0.008	n.s.	---	3.0	0.003	1.13	
PAWR	1.9	0.008	1.3	1.0	2.1	0.002	1.13	
TGFBR2	2.6	0.007	1.3	1.0	2.9	0.003	1.12	

ERO1LB	2.6	0.065	n.s.	---	3.0	0.028	1.12	
TGFA	3.1	0.012	n.s.	---	3.4	0.006	1.12	(b)
SLC1A1	3.3	3.96E-05	1.6	0.074	3.6	1.93E-05	1.12	
SOS2	2.0	0.125	n.s.	---	2.3	0.042	1.12	
PIM1	2.8	0.008	n.s.	---	3.2	0.004	1.11	
KLF7	2.8	0.041	n.s.	---	3.2	0.019	1.11	
TNFRSF21	3.4	0.006	n.s.	---	3.8	0.003	1.11	(b)
AFF4	3.2	1.51E-04	n.s.	---	3.5	7.72E-05	1.11	
AVIL	4.1	0.002	n.s.	---	4.5	0.001	1.11	
SNORD28	-2.2	0.030	1.4	1.0	-2.0	0.081	1.10	
BMP2	3.7	0.026	1.9	1.0	4.1	0.015	1.10	
ANK3	2.9	0.011	n.s.	---	3.2	0.006	1.10	
RRS1	-2.3	0.014	n.s.	---	-2.1	0.034	1.09	
TM4SF1	3.0	0.001	1.1	1.0	3.3	3.25E-04	1.09	
TRIP12	1.8	0.038	n.s.	---	2.0	0.014	1.09	
POLR1B	-2.3	9.00E-05	1.2	1.0	-2.2	2.03E-04	1.09	(a)
THBS1	2.5	0.001	2.1	0.008	2.8	0.001	1.09	
SEMA3C	4.3	0.066	n.s.	---	4.7	0.044	1.09	(b)
RTN3	3.1	0.009	n.s.	---	3.4	0.005	1.08	
CLTB	2.0	0.017	1.2	1.0	2.2	0.007	1.08	
ZFAND6	2.4	0.059	-1.2	1.0	2.6	0.031	1.08	
IL22RA1	2.6	0.028	n.s.	---	2.8	0.016	1.08	
C1orf163	-2.6	0.009	n.s.	---	-2.4	0.017	1.08	
KLF5	2.7	2.48E-04	n.s.	---	2.9	1.41E-04	1.08	(b)
HSD17B11	2.6	4.62E-04	-1.1	1.0	2.7	2.55E-04	1.08	
NEDD4L	4.5	0.009	n.s.	---	4.9	0.006	1.07	(b)
CDKN1B	2.1	0.001	n.s.	---	2.2	0.001	1.07	(c)
ARID5B	3.9	0.001	-1.3	1.0	4.2	0.001	1.07	(a)
RNF39	3.7	0.029	-1.3	1.0	4.0	0.020	1.07	
MAGI1	5.1	0.002	n.s.	---	5.4	0.002	1.07	
SLC31A2	5.3	4.17E-05	n.s.	---	5.7	3.07E-05	1.07	(b)
GADD45B	2.1	0.051	1.3	1.0	2.3	0.028	1.06	
TRAK2	4.6	0.006	n.s.	---	4.8	0.004	1.06	
CORO1A	3.4	0.030	-1.6	1.0	3.6	0.021	1.06	(b)
KLF6	6.5	0.009	n.s.	---	6.9	0.007	1.06	
GPR125	2.9	0.032	1.3	1.0	3.1	0.021	1.06	
TGFBR3	1.9	0.063	n.s.	---	2.0	0.033	1.06	
ABLIM1	2.5	0.057	n.s.	---	2.6	0.036	1.06	
JAK1	2.7	0.002	-1.2	1.0	2.8	0.001	1.06	
CYR61	19.6	1.18E-04	4.0	0.043	20.6	1.04E-04	1.05	(b)
CCNG2	6.2	0.022	-1.9	1.0	6.5	0.018	1.05	(b)(c)
C18orf45	-2.1	0.003	n.s.	---	-2.0	0.004	1.05	
KLK10	3.2	0.003	-1.4	1.0	3.3	0.002	1.05	(b)
CDKN2B	3.2	0.015	n.s.	---	3.3	0.012	1.04	
FOXP1	18.9	8.57E-07	-1.4	1.0	19.7	7.62E-07	1.04	
LIPG	-3.2	0.001	-1.5	1.0	-3.1	0.001	1.04	
GATS	5.8	0.001	-1.6	1.0	6.0	0.001	1.04	
SNORD78	-2.7	0.010	1.3	1.0	-2.6	0.014	1.04	
MTNR1A	2.2	0.005	1.1	1.0	2.3	0.004	1.04	
CDCA7	-3.7	0.002	1.4	1.0	-3.6	0.002	1.03	(a)
LDLR	2.9	2.59E-04	n.s.	---	3.0	2.16E-04	1.03	
ABHD3	2.4	0.037	n.s.	---	2.5	0.030	1.02	
SAT1	2.7	0.001	-1.5	0.556	2.7	0.001	1.02	
TSC1	2.0	0.058	n.s.	---	2.1	0.047	1.02	
ERRFI1	2.2	0.001	n.s.	---	2.2	0.001	1.02	
NPIPL3	3.9	0.007	n.s.	---	4.0	0.007	1.02	
NCS1	-2.1	0.014	1.2	1.0	-2.0	0.017	1.02	
ATXN1	5.9	6.89E-05	1.4	1.0	5.9	6.49E-05	1.01	
TCF12	2.2	0.003	n.s.	---	2.3	0.003	1.01	
ATL1	6.5	4.20E-04	-1.3	1.0	6.5	4.00E-04	1.01	
FAM27E3	-2.5	0.015	n.s.	---	-2.5	0.016	1.01	
KIT	-3.2	0.012	-1.4	1.0	-3.2	0.012	1.00	
SRGAP2	3.5	0.002	n.s.	---	3.5	0.002	1.00	

<i>CENPP</i>	-2.0	0.042	1.2	1.0	-2.0	0.043	1.00	
<i>TNS4</i>	2.1	0.001	n.s.	---	2.1	0.001	1.00	
<i>GLUL</i>	8.3	0.026	n.s.	---	8.3	0.026	1.00	
<i>SH3TC2</i>	3.4	0.046	n.s.	---	3.4	0.046	1.00	
<i>SMPDL3A</i>	4.8	0.001	-1.2	1.0	4.8	0.001	1.00	(b)
<i>ZRANB1</i>	2.6	0.008	1.2	1.0	2.6	0.008	1.00	
<i>CITED2</i>	3.2	4.78E-06	1.1	1.0	3.1	4.92E-06	1.00	
<i>HECA</i>	3.1	0.001	n.s.	---	3.1	0.001	1.00	
<i>STX11</i>	3.0	0.007	1.6	1.0	3.0	0.007	0.99	
<i>UBE2H</i>	2.7	0.013	-1.4	1.0	2.7	0.014	0.99	(b)
<i>ATP8A1</i>	7.3	1.06E-04	n.s.	---	7.2	1.09E-04	0.99	
<i>EGR2</i>	4.0	2.47E-04	2.0	0.052	4.0	2.58E-04	0.99	
<i>SNX9</i>	3.0	0.035	n.s.	---	3.0	0.037	0.99	
<i>EDN1</i>	13.4	0.003	2.0	1.0	13.3	0.003	0.99	(b)
<i>PIGM</i>	-2.0	0.047	n.s.	---	-2.1	0.042	0.99	
<i>PDIA6</i>	2.3	0.027	-1.5	1.0	2.2	0.030	0.99	
<i>DDI2</i>	4.3	0.001	n.s.	---	4.3	0.001	0.98	
<i>EIF2C4</i>	4.3	0.016	n.s.	---	4.2	0.018	0.98	
<i>ARHGEF12</i>	2.8	2.46E-04	n.s.	---	2.8	2.93E-04	0.98	
<i>NT5C2</i>	2.3	0.004	n.s.	---	2.2	0.005	0.98	
<i>DPYSL2</i>	2.1	0.001	-1.2	1.0	2.0	0.001	0.98	
<i>IL1F7</i>	19.4	9.79E-05	-2.1	1.0	18.8	1.07E-04	0.97	
<i>LIPH</i>	3.7	0.001	-2.0	0.089	3.5	0.001	0.96	
<i>FMO5</i>	5.0	0.005	-1.3	1.0	4.8	0.007	0.96	
<i>TMEM173</i>	4.9	1.02E-04	n.s.	---	4.7	1.25E-04	0.96	
<i>CYB5RL</i>	-3.2	0.017	n.s.	---	-3.3	0.013	0.96	
<i>TSR1</i>	-2.3	0.012	1.2	1.0	-2.4	0.008	0.96	
<i>SLC12A6</i>	4.0	3.64E-04	n.s.	---	3.8	4.74E-04	0.96	
<i>DUSP5</i>	9.5	7.74E-06	1.3	1.0	9.0	9.43E-06	-1.06	(b)
<i>GEMIN4</i>	-3.0	0.004	n.s.	---	-3.1	0.003	-1.06	(a)
<i>BRAP</i>	2.1	0.001	n.s.	---	2.0	0.001	-1.06	
<i>TMEM200A</i>	-2.6	0.030	n.s.	---	-2.8	0.018	-1.07	
<i>WIPI2</i>	2.4	0.005	-1.2	1.0	2.3	0.010	-1.07	
<i>RRP1B</i>	-2.2	0.046	n.s.	---	-2.4	0.024	-1.07	
<i>FCHSD1</i>	2.8	0.037	n.s.	---	2.6	0.063	-1.08	
<i>C10orf2</i>	-2.5	0.085	n.s.	---	-2.7	0.047	-1.08	
<i>PTPRJ</i>	3.3	3.95E-05	n.s.	---	3.0	6.64E-05	-1.08	
<i>NOP2</i>	-2.1	0.048	n.s.	---	-2.3	0.023	-1.08	
<i>BCAS1</i>	3.5	0.003	-1.7	1.0	3.2	0.005	-1.08	
<i>WDFY3</i>	3.4	0.003	n.s.	---	3.1	0.005	-1.08	
<i>ACPP</i>	11.7	0.001	-1.7	1.0	10.8	0.001	-1.08	
<i>TMEM25</i>	-2.4	0.087	n.s.	---	-2.6	0.044	-1.08	
<i>EFNA5</i>	-2.7	0.002	n.s.	---	-3.0	0.001	-1.09	
<i>NPC1</i>	2.1	0.015	n.s.	---	1.9	0.039	-1.09	
<i>RRP12</i>	-2.1	0.040	n.s.	---	-2.3	0.017	-1.09	
<i>NDP</i>	-2.4	0.014	1.5	1.0	-2.6	0.007	-1.09	
<i>MACF1</i>	2.6	0.002	n.s.	---	2.4	0.004	-1.09	
<i>TDRD7</i>	2.8	0.010	1.2	1.0	2.6	0.020	-1.10	
<i>OPTN</i>	3.9	0.002	-1.2	1.0	3.5	0.004	-1.10	
<i>RASGRP3</i>	10.1	0.010	1.5	1.0	9.2	0.014	-1.10	
<i>CDCA7L</i>	2.1	0.006	1.2	1.0	1.9	0.018	-1.10	
<i>FKBP4</i>	-2.0	0.003	1.1	1.0	-2.2	0.001	-1.10	
<i>POLR1A</i>	-2.3	0.025	n.s.	---	-2.5	0.011	-1.10	
<i>BYSL</i>	-1.9	0.002	n.s.	---	-2.1	0.001	-1.10	(a)
<i>CTH</i>	-3.1	0.054	-1.9	1.0	-3.5	0.029	-1.10	
<i>USP2</i>	-2.0	0.078	-1.3	1.0	-2.2	0.028	-1.10	
<i>ARL2BP</i>	-2.0	0.015	-1.2	1.0	-2.2	0.005	-1.10	
<i>SLC5A3</i>	2.5	0.010	n.s.	---	2.3	0.025	-1.10	
<i>TMEM86A</i>	3.9	0.001	-1.6	1.0	3.5	0.002	-1.11	
<i>MXI1</i>	5.0	1.02E-04	n.s.	---	4.5	1.75E-04	-1.11	(b)
<i>SASH1</i>	3.9	0.010	n.s.	---	3.5	0.018	-1.11	
<i>SCIN</i>	38.6	6.34E-07	n.s.	---	34.7	8.04E-07	-1.11	
<i>RAB5B</i>	2.3	0.014	-1.3	1.0	2.1	0.041	-1.12	

NPNT	-2.0	0.021	n.s.	---	-2.2	0.007	-1.12	
FAM59A	3.2	0.023	n.s.	---	2.8	0.050	-1.12	
LRP10	2.7	5.46E-05	-1.3	1.0	2.4	1.44E-04	-1.12	
CTSB	3.0	3.30E-04	-1.4	1.0	2.7	0.001	-1.12	(b)
S100A13	2.8	0.045	n.s.	---	2.5	0.110	-1.12	(b)
OR7E87P	-1.9	0.141	-1.6	1.0	-2.1	0.039	-1.13	
PSMG3	-2.0	0.089	n.s.	---	-2.3	0.028	-1.13	
GRIK4	-2.6	0.008	-1.3	1.0	-3.0	0.003	-1.13	
NTAN1	3.5	2.15E-04	n.s.	---	3.1	4.92E-04	-1.13	
SQLE	-2.4	0.056	-1.2	1.0	-2.7	0.019	-1.14	
TIMM8B	-1.9	0.002	n.s.	---	-2.2	3.53E-04	-1.14	
SORL1	2.6	4.27E-05	-1.2	1.0	2.3	1.42E-04	-1.14	
PPP1R15A	2.1	0.006	1.2	1.0	1.9	0.027	-1.15	
MRPL16	-1.8	0.113	n.s.	---	-2.1	0.023	-1.15	
B4GALNT3	13.7	0.001	n.s.	---	11.9	0.002	-1.15	
FOXO1	3.6	0.012	n.s.	---	3.1	0.030	-1.15	
MAML3	8.6	0.001	-1.5	1.0	7.5	0.001	-1.16	
PYCR1	-3.3	0.004	n.s.	---	-3.8	0.001	-1.16	
C10orf26	6.4	0.002	n.s.	---	5.5	0.004	-1.16	
MRPL49	-1.9	0.020	n.s.	---	-2.3	0.004	-1.16	
DSP	2.5	0.004	-1.2	1.0	2.2	0.015	-1.16	
NME4	-1.9	0.208	n.s.	---	-2.3	0.045	-1.16	
UCA1	2.7	0.043	-1.4	1.0	2.4	0.148	-1.17	
RAB9A	3.1	0.026	n.s.	---	2.7	0.077	-1.17	
NRM	-2.0	0.201	-1.2	1.0	-2.3	0.043	-1.17	
GPLD1	2.2	0.036	n.s.	---	1.9	0.188	-1.17	
PELI2	3.5	0.002	-1.3	1.0	3.0	0.007	-1.17	
MAOB	5.4	1.23E-04	-1.5	1.0	4.6	2.73E-04	-1.17	
SLC4A11	2.4	0.042	-1.5	1.0	2.0	0.197	-1.18	
FOSL2	3.1	1.17E-04	n.s.	---	2.6	4.00E-04	-1.18	
TBC1D8	4.1	2.33E-05	1.2	1.0	3.5	6.24E-05	-1.18	
CHMP4B	2.4	5.90E-05	n.s.	---	2.0	3.21E-04	-1.18	
GATSL1	4.6	0.001	-2.2	0.190	3.9	0.003	-1.18	
KIF13B	3.0	0.015	n.s.	---	2.5	0.053	-1.18	
CTDSP2	2.7	0.003	-1.2	1.0	2.3	0.014	-1.19	
ATP9A	4.2	0.001	-1.3	1.0	3.6	0.004	-1.19	
NECAP2	2.7	0.021	n.s.	---	2.3	0.090	-1.19	
RERE	2.5	1.36E-04	-1.2	1.0	2.1	0.001	-1.19	(b)
LGR4	8.9	1.76E-04	n.s.	---	7.4	3.42E-04	-1.19	
EGLN3	3.1	0.029	n.s.	---	2.6	0.105	-1.19	
SUN1	3.0	0.017	n.s.	---	2.5	0.067	-1.20	
ARRDC3	8.9	6.37E-07	-1.7	0.045	7.5	1.26E-06	-1.20	
TRIM14	-2.6	0.020	n.s.	---	-3.2	0.005	-1.20	
AIMP2	-2.5	0.002	-1.2	1.0	-3.0	4.47E-04	-1.20	
FGF20	-3.6	3.88E-04	n.s.	---	-4.3	1.36E-04	-1.20	
ZFHX2	2.2	0.014	-1.4	1.0	1.8	0.099	-1.20	
LY6G6C	19.9	0.005	n.s.	---	16.6	0.008	-1.20	
PRR15	2.9	0.006	-2.2	0.053	2.4	0.026	-1.20	
MFSD6	2.3	0.004	-1.1	1.0	1.9	0.026	-1.20	
SLC29A1	-2.2	0.030	n.s.	---	-2.7	0.006	-1.20	
C8orf55	-2.0	0.087	n.s.	---	-2.4	0.014	-1.21	
MTSS1L	-2.2	0.041	1.3	1.0	-2.7	0.008	-1.21	
TGFB3	-4.0	0.119	-1.7	1.0	-4.8	0.045	-1.21	
PAQR4	-1.7	0.085	n.s.	---	-2.0	0.008	-1.21	
S100P	2.3	0.016	n.s.	---	1.9	0.123	-1.21	(b)
IRS2	9.7	1.14E-06	n.s.	---	8.0	2.31E-06	-1.21	
EPHX4	3.1	0.039	n.s.	---	2.6	0.157	-1.21	
REXO2	3.6	7.15E-05	n.s.	---	3.0	2.64E-04	-1.22	
MAST3	2.2	0.001	-1.2	1.0	1.8	0.007	-1.22	
DNMBP	2.2	0.021	-1.3	1.0	1.8	0.170	-1.22	
SCD	-2.2	0.084	n.s.	---	-2.7	0.015	-1.22	(a)
SLC2A10	3.4	0.041	n.s.	---	2.8	0.159	-1.22	
CCDC86	-2.9	0.004	-1.3	1.0	-3.6	0.001	-1.22	

<i>BTG1</i>	4.3	0.004	n.s.	---	3.5	0.014	-1.23	(c)
<i>ANP32A</i>	-1.9	0.168	n.s.	---	-2.4	0.022	-1.23	
<i>PLXNB1</i>	2.1	0.034	n.s.	---	1.7	0.401	-1.23	
<i>MBTPS1</i>	2.1	0.008	-1.2	1.0	1.7	0.095	-1.23	
<i>SPINK1</i>	11.8	4.06E-04	-2.2	1.0	9.6	0.001	-1.23	
<i>PDK4</i>	6.5	0.006	-1.4	1.0	5.3	0.014	-1.23	
<i>MEGF9</i>	3.3	3.14E-04	n.s.	---	2.7	0.001	-1.23	
<i>TST</i>	2.7	0.027	-1.4	1.0	2.2	0.167	-1.24	(b)
<i>HBP1</i>	5.3	0.005	-1.7	1.0	4.2	0.015	-1.24	
<i>BCL6</i>	8.6	9.18E-08	-1.5	0.034	6.9	2.14E-07	-1.24	(b)(c)
<i>AKAP1</i>	-2.0	0.076	n.s.	---	-2.5	0.010	-1.24	
<i>BTG2</i>	2.6	0.016	n.s.	---	2.1	0.111	-1.24	
<i>NFIC</i>	-2.1	0.018	n.s.	---	-2.7	0.003	-1.24	
<i>NDRG1</i>	13.9	4.24E-06	-1.5	1.0	11.2	8.45E-06	-1.24	
<i>IPO4</i>	-2.2	0.266	n.s.	---	-2.7	0.040	-1.25	
<i>BACH2</i>	10.9	1.07E-05	n.s.	---	8.7	2.35E-05	-1.26	
<i>IGF1R</i>	7.0	0.001	n.s.	---	5.6	0.003	-1.26	(b)
<i>SLC16A3</i>	4.2	0.020	n.s.	---	3.3	0.075	-1.26	
<i>KIF3C</i>	4.5	0.003	-1.8	1.0	3.6	0.012	-1.26	(b)
<i>C17orf103</i>	4.2	0.007	n.s.	---	3.3	0.029	-1.26	
<i>SLC6A9</i>	-2.5	0.011	-1.2	1.0	-3.2	0.002	-1.27	
<i>TMEM161A</i>	-1.9	0.074	-1.3	1.0	-2.5	0.007	-1.27	
<i>TNFSF10</i>	40.0	4.23E-06	-2.0	1.0	31.5	7.22E-06	-1.27	(c)
<i>GYLTL1B</i>	-2.3	0.229	n.s.	---	-3.0	0.035	-1.27	
<i>BOD1</i>	-1.6	0.308	n.s.	---	-2.1	0.014	-1.27	
<i>KPNA7</i>	40.9	9.86E-05	n.s.	---	32.0	1.69E-04	-1.28	
<i>SNORD75</i>	-2.0	0.128	1.5	1.0	-2.5	0.013	-1.28	
<i>CARS2</i>	-2.3	0.027	n.s.	---	-2.9	0.004	-1.28	
<i>TMEM201</i>	-2.7	0.002	1.2	1.0	-3.5	3.62E-04	-1.28	
<i>EPB41L1</i>	-1.9	0.440	n.s.	---	-2.5	0.041	-1.28	
<i>TTLL12</i>	-2.1	0.358	n.s.	---	-2.7	0.041	-1.28	
<i>FOXO4</i>	3.5	0.008	-1.4	1.0	2.8	0.042	-1.28	
<i>TEP1</i>	2.9	0.010	n.s.	---	2.3	0.078	-1.29	
<i>CYTH2</i>	2.4	0.007	-1.3	1.0	1.9	0.088	-1.29	
<i>C19orf48</i>	-1.9	0.119	n.s.	---	-2.5	0.010	-1.29	
<i>ZFYVE1</i>	5.0	4.34E-04	n.s.	---	3.8	0.002	-1.29	
<i>GMPR</i>	2.2	0.001	-1.3	1.0	1.7	0.028	-1.29	
<i>GAB2</i>	3.0	3.74E-04	1.2	1.0	2.3	0.003	-1.30	
<i>CHD6</i>	2.4	0.001	n.s.	---	1.8	0.017	-1.30	
<i>SLC19A1</i>	-1.8	0.115	n.s.	---	-2.3	0.006	-1.31	(a)
<i>NDUFV3</i>	-1.7	0.109	-1.2	1.0	-2.2	0.004	-1.31	
<i>ERBB3</i>	8.3	2.19E-05	-1.3	1.0	6.3	6.65E-05	-1.32	(b)
<i>RNF208</i>	-2.4	0.106	-1.3	1.0	-3.2	0.013	-1.32	
<i>LNX1</i>	3.7	4.45E-04	-1.4	1.0	2.8	0.003	-1.33	
<i>IGFL4</i>	-3.8	0.002	-1.4	1.0	-5.1	4.31E-04	-1.33	
<i>WDR74</i>	-1.7	0.058	n.s.	---	-2.3	0.002	-1.33	(a)
<i>TNRC18</i>	2.2	0.034	-1.4	1.0	1.6	1.0	-1.34	
<i>SLC16A2</i>	-1.6	0.093	-1.2	1.0	-2.1	0.002	-1.34	
<i>GTPBP3</i>	-1.5	1.0	n.s.	---	-2.0	0.023	-1.34	
<i>TUBA1A</i>	5.9	0.001	n.s.	---	4.4	0.003	-1.34	
<i>IER3</i>	2.4	1.97E-05	n.s.	---	1.8	0.001	-1.35	
<i>CBLB</i>	5.5	0.002	-1.5	1.0	4.1	0.007	-1.35	
<i>TMEM92</i>	6.5	0.005	n.s.	---	4.8	0.018	-1.35	
<i>AMPD2</i>	-1.8	0.868	-1.4	1.0	-2.5	0.046	-1.35	(a)
<i>GRB7</i>	6.4	3.67E-04	-2.1	0.416	4.7	0.002	-1.36	(b)
<i>HES1</i>	-2.1	0.213	-1.5	1.0	-2.8	0.015	-1.36	
<i>SLC2A12</i>	11.8	0.001	n.s.	---	8.6	0.003	-1.36	
<i>RNF122</i>	4.1	0.001	n.s.	---	3.0	0.006	-1.36	
<i>TMEM140</i>	11.9	3.92E-06	n.s.	---	8.7	1.15E-05	-1.37	
<i>SLC44A2</i>	-1.5	1.0	-1.2	1.0	-2.1	0.020	-1.38	
<i>ATG2A</i>	3.0	0.016	-1.3	1.0	2.2	0.208	-1.38	
<i>TIGD5</i>	-1.7	1.0	n.s.	---	-2.3	0.038	-1.38	
<i>TRERF1</i>	-1.9	0.014	-1.3	1.0	-2.6	0.001	-1.39	

<i>SLC45A1</i>	9.9	0.017	n.s.	---	7.1	0.057	-1.39	
<i>SOBP</i>	-2.1	0.028	n.s.	---	-2.9	0.002	-1.39	
<i>PPAP2B</i>	2.8	1.36E-05	-1.5	0.013	2.0	2.94E-04	-1.40	
<i>RNF187</i>	-1.4	0.981	-1.4	1.0	-2.0	0.007	-1.41	
<i>IL10RA</i>	5.9	0.002	n.s.	---	4.2	0.009	-1.41	
<i>CCL20</i>	3.8	0.004	-1.9	0.962	2.7	0.041	-1.42	
<i>PNPLA3</i>	-2.5	0.392	-1.3	1.0	-3.6	0.035	-1.43	
<i>INSIG1</i>	-2.1	0.006	-1.3	1.0	-3.0	2.70E-04	-1.43	
<i>ARHGAP17</i>	2.2	0.005	n.s.	---	1.5	0.476	-1.43	
<i>GTF2I</i>	-1.9	0.882	n.s.	---	-2.7	0.031	-1.44	(b)
<i>VAMP5</i>	2.5	0.029	-1.3	1.0	1.7	1.0	-1.44	
<i>LAD1</i>	-1.9	0.412	n.s.	---	-2.8	0.016	-1.44	
<i>PIK3IP1</i>	9.5	1.66E-04	n.s.	---	6.6	0.001	-1.44	
<i>SH3RF2</i>	-2.6	0.075	n.s.	---	-3.8	0.006	-1.45	
<i>INSR</i>	5.0	3.37E-04	n.s.	---	3.5	0.003	-1.46	
<i>ZNRF3</i>	-1.8	0.518	n.s.	---	-2.7	0.014	-1.46	(a)
<i>RAB43</i>	5.3	0.003	-1.6	1.0	3.6	0.022	-1.46	
<i>MPI</i>	-2.0	0.395	-1.4	1.0	-3.0	0.015	-1.47	
<i>TCP11L2</i>	10.7	1.17E-04	-1.6	1.0	7.2	4.79E-04	-1.47	
<i>CARHSP1</i>	2.3	2.27E-04	-1.2	1.0	1.5	0.036	-1.48	
<i>MID1IP1</i>	-1.8	0.006	-1.1	1.0	-2.7	1.09E-04	-1.48	
<i>YPEL1</i>	3.8	0.002	-1.6	1.0	2.5	0.032	-1.48	
<i>CABLES1</i>	2.6	0.026	n.s.	---	1.7	1.0	-1.48	
<i>EPN3</i>	-2.2	0.406	-1.8	1.0	-3.4	0.020	-1.49	
<i>ULK1</i>	2.6	0.009	-1.5	1.0	1.8	0.538	-1.50	
<i>SLC43A2</i>	11.5	3.84E-04	-1.6	1.0	7.7	0.002	-1.50	
<i>ZNF827</i>	-2.7	0.278	n.s.	---	-4.0	0.021	-1.50	
<i>GSN</i>	2.5	0.028	-1.4	1.0	1.6	1.0	-1.52	(b)
<i>PPFIBP2</i>	-1.5	1.0	-1.4	1.0	-2.3	0.024	-1.52	
<i>TNS3</i>	-1.6	1.0	n.s.	---	-2.5	0.019	-1.53	
<i>MED22</i>	-1.8	0.092	n.s.	---	-2.8	0.001	-1.53	
<i>FASN</i>	-1.7	1.0	-1.2	1.0	-2.6	0.021	-1.53	
<i>CALCOCO1</i>	5.3	0.002	-1.5	1.0	3.5	0.017	-1.53	
<i>LAMC2</i>	2.7	0.003	-1.4	1.0	1.8	0.221	-1.53	
<i>REPS2</i>	4.4	0.010	-1.4	1.0	2.9	0.129	-1.54	
<i>SCNN1B</i>	6.7	0.001	n.s.	---	4.3	0.005	-1.55	
<i>TMCC3</i>	7.3	0.001	-1.4	1.0	4.7	0.005	-1.55	
<i>REG4</i>	3.4	0.032	n.s.	---	2.2	0.812	-1.55	
<i>THOC6</i>	-1.9	0.073	-1.3	1.0	-2.9	0.001	-1.55	
<i>CA5B</i>	9.0	0.008	n.s.	---	5.8	0.042	-1.55	
<i>TLCD1</i>	-2.0	0.094	-1.4	1.0	-3.1	0.002	-1.56	
<i>DBP</i>	-2.9	0.001	-1.7	0.114	-4.6	3.25E-05	-1.58	
<i>PLCG2</i>	18.2	7.77E-05	n.s.	---	11.5	3.03E-04	-1.58	(b)
<i>PIK3C2B</i>	-2.2	0.193	-1.6	1.0	-3.4	0.005	-1.59	
<i>C10orf10</i>	35.1	3.28E-05	n.s.	---	21.9	1.00E-04	-1.60	
<i>SGSH</i>	-1.8	0.747	-1.5	1.0	-2.8	0.008	-1.60	
<i>NDUFB10</i>	-1.4	1.0	-1.2	1.0	-2.2	0.015	-1.60	
<i>FBXO32</i>	31.4	1.27E-04	n.s.	---	19.6	4.05E-04	-1.60	(c)
<i>PDZK1IP1</i>	2.6	0.025	-1.4	1.0	1.6	1.0	-1.63	
<i>TP53INP2</i>	4.8	0.002	-1.4	1.0	2.9	0.028	-1.64	
<i>SULT2B1</i>	-2.4	0.246	-1.9	1.0	-4.0	0.008	-1.65	
<i>ID3</i>	2.7	0.006	-2.1	0.050	1.6	1.0	-1.66	(a)
<i>STARD10</i>	-1.2	1.0	-1.5	1.0	-2.1	0.037	-1.67	
<i>ZNF608</i>	2.9	0.003	n.s.	---	1.7	0.513	-1.68	
<i>SLC1A4</i>	-2.2	0.132	-1.3	1.0	-3.6	0.003	-1.68	(b)
<i>LFNG</i>	7.1	0.005	n.s.	---	4.2	0.048	-1.68	
<i>TNFRSF9</i>	-3.6	0.245	-3.1	0.578	-6.1	0.018	-1.69	
<i>EFNA4</i>	-1.4	1.0	-1.4	1.0	-2.5	0.044	-1.70	
<i>DGCR2</i>	-1.3	1.0	-1.2	1.0	-2.2	0.005	-1.70	
<i>ZNF618</i>	-2.1	0.067	n.s.	---	-3.7	0.001	-1.74	
<i>DAPK1</i>	2.2	4.37E-04	-1.6	0.022	1.3	1.0	-1.76	(b)
<i>TNFSF15</i>	3.2	0.001	-1.7	0.299	1.8	0.189	-1.77	
<i>SERPIND1</i>	6.0	0.001	-1.9	1.0	3.3	0.015	-1.83	

GPR120	-2.2	0.795	-1.4	1.0	-4.0	0.010	-1.85	(c)
SOD2	3.9	0.007	-1.4	1.0	2.1	0.708	-1.87	
SLC22A23	5.4	0.003	n.s.	---	2.9	0.095	-1.88	
HSD17B2	11.8	0.024	n.s.	---	6.2	0.236	-1.91	
PRR15L	-3.0	0.101	-2.0	1.0	-5.7	0.003	-1.91	(a)
ASCL2	-4.1	0.008	-1.4	1.0	-7.9	4.32E-04	-1.93	
KRTAP5-6	-2.5	0.218	-2.8	0.107	-4.9	0.003	-1.97	
CDX2	-1.6	1.0	-1.4	1.0	-3.5	0.002	-2.11	
SULT1C4	-3.6	0.002	-3.9	0.001	-7.8	5.04E-05	-2.18	(a)
TXNIP	-1.2	1.0	-2.1	0.146	-2.7	0.014	-2.27	
TRIM31	-1.7	1.0	-3.5	0.010	-4.1	0.004	-2.37	
RND1	6.8	3.52E-04	-1.4	1.0	2.8	0.044	-2.43	
DHRS3	4.5	0.005	-1.4	1.0	1.8	1.0	-2.46	(a)
TMOD1	20.1	0.001	n.s.	---	7.9	0.011	-2.54	
PLA2G2F	4.5	0.022	n.s.	---	1.5	1.0	-2.95	

Supplementary Table 1. High Stringency Gene List of Genes Regulated at 12 h Treatment. The results of the microarray analysis were cut-off at a significance level of Bonferroni adjusted P -value (asterisk) ≤ 0.05 (corresponding to a false discovery rate (FDR) P -value ≤ 0.00005) and a fold change (FC) of at least two-fold in at least one of the treatments. The fold change is displayed as value in treated cells versus value in control cells (vehicle). n.s. = not significant in a two-tailed ANOVA analysis (P -value > 0.05). The list is sorted indicating first the highest fold change value for double treatment (4OHT+DOX) versus 4OHT. Known target genes were previously described: (a) gene found to be regulated by Δ N-TCF (van der Flier et al., 2007); (b) FoxO3a target gene described by Delpuech et al., 2007 and (c) summarized by Greer and Brunet, 2005.

Supplementary Table 2: FOXO3a and/or beta-catenin regulated genes (24 h treatment)

Gene Symbol	4OHT		DOX		4OHT+DOX		FC 4OHT+DOX vs. 4OHT	Known Target
	FC	P-value*	FC	P-value*	FC	P-value*		
<i>SLC2A3</i>	5.2	0.004	4.4	0.007	65.5	2.24E-06	12.63	
<i>SLC1A2</i>	1.7	1.0	n.s.	---	19.1	0.006	10.96	
<i>KRTAP9-2</i>	1.9	1.0	n.s.	---	17.7	0.006	9.49	
<i>CPA4</i>	5.3	9.90E-05	4.3	2.85E-04	22.2	6.94E-07	4.22	
<i>ANKRD1</i>	49.5	1.88E-06	14.7	3.62E-05	197.6	1.67E-07	3.99	
<i>DAB2</i>	2.3	0.226	2.2	0.322	7.6	2.62E-04	3.28	
<i>IQGAP2</i>	9.5	7.80E-05	2.1	0.322	27.9	3.46E-06	2.95	
<i>RTKN2</i>	-3.4	0.001	1.3	1.0	-1.2	1.0	2.91	
<i>KRTAP9-4</i>	2.3	1.0	n.s.	---	6.3	0.009	2.78	
<i>CLDN16</i>	2.1	1.0	n.s.	---	5.7	0.005	2.73	
<i>WWC2</i>	1.8	1.0	2.4	0.369	4.8	0.004	2.62	
<i>DEPDC1</i>	-5.9	0.025	n.s.	---	-2.3	1.0	2.60	
<i>KIF18A</i>	-5.6	0.034	n.s.	---	-2.1	1.0	2.60	
<i>DIAPH3</i>	-2.9	0.016	1.3	1.0	-1.1	1.0	2.56	
<i>PRDM1</i>	7.6	0.001	2.2	1.0	19.4	6.05E-05	2.55	
<i>SEMA5A</i>	1.3	1.0	3.1	0.001	3.3	0.001	2.53	
<i>LPHN3</i>	4.7	0.984	n.s.	---	11.7	0.032	2.51	
<i>MS4A15</i>	6.1	0.031	n.s.	---	15.3	0.001	2.51	
<i>SPINK1</i>	7.8	0.006	-2.0	1.0	19.5	3.65E-04	2.50	
<i>ULBP2</i>	1.7	1.0	n.s.	---	4.3	0.002	2.50	
<i>CLDN10</i>	3.0	0.714	n.s.	---	7.4	0.007	2.49	
<i>ARHGAP11A</i>	-5.0	0.008	n.s.	---	-2.0	1.0	2.48	
<i>KRTAP2-4</i>	1.4	1.0	2.7	0.161	3.5	0.031	2.43	
<i>C13orf15</i>	1.9	1.0	1.4	1.0	4.6	0.002	2.43	
<i>CLEC2D</i>	1.9	1.0	n.s.	---	4.5	0.018	2.40	
<i>SLC31A2</i>	3.6	0.179	1.6	1.0	8.3	0.004	2.31	(b)
<i>STARD13</i>	3.5	0.521	1.5	1.0	8.0	0.012	2.28	
<i>PHLDB2</i>	1.3	1.0	1.5	1.0	3.0	0.040	2.28	
<i>BRCA1</i>	-3.9	0.013	n.s.	---	-1.7	1.0	2.25	
<i>SLC28A3</i>	2.6	0.029	n.s.	---	5.8	2.47E-04	2.25	
<i>ATAD5</i>	-5.4	0.007	n.s.	---	-2.4	0.948	2.25	
<i>NSAP11</i>	9.9	0.040	n.s.	---	22.1	0.004	2.23	
<i>SLC45A1</i>	18.0	1.12E-04	n.s.	---	39.8	1.64E-05	2.21	
<i>SDR16C5</i>	2.8	0.957	n.s.	---	6.2	0.014	2.21	
<i>EMP1</i>	13.1	0.004	2.6	1.0	28.7	4.68E-04	2.20	(b)
<i>PSG9</i>	3.4	0.950	n.s.	---	7.2	0.026	2.14	
<i>JMJD1C</i>	2.6	0.510	n.s.	---	5.5	0.006	2.12	
<i>HEG1</i>	5.6	0.001	2.0	0.631	11.9	3.95E-05	2.11	
<i>CP110</i>	1.7	1.0	n.s.	---	3.5	0.043	2.10	
<i>SELP</i>	5.2	1.70E-04	n.s.	---	10.8	9.63E-06	2.06	
<i>AMOTL2</i>	2.3	0.003	2.2	0.005	4.6	2.54E-05	2.02	
<i>EDN1</i>	15.8	1.42E-05	3.1	0.016	31.9	2.36E-06	2.01	(b)
<i>PUS7L</i>	-2.8	0.047	n.s.	---	-1.5	1.0	1.96	
<i>ZBED2</i>	1.7	1.0	n.s.	---	3.3	0.047	1.95	
<i>AXL</i>	3.0	0.272	n.s.	---	5.8	0.008	1.94	
<i>NAV3</i>	1.3	1.0	2.0	0.291	2.5	0.030	1.93	
<i>REEP1</i>	1.8	1.0	n.s.	---	3.5	0.030	1.93	
<i>VSIG1</i>	2.7	0.627	-1.6	1.0	5.2	0.015	1.91	
<i>PIK3CA</i>	3.0	0.009	n.s.	---	5.7	2.34E-04	1.91	(b)
<i>C4orf21</i>	-4.4	0.035	n.s.	---	-2.3	1.0	1.91	
<i>RIMKLB</i>	21.2	0.004	1.8	1.0	40.1	0.001	1.89	
<i>SKIL</i>	1.7	1.0	n.s.	---	3.1	0.027	1.86	
<i>NPIPL3</i>	2.6	0.012	n.s.	---	4.7	2.45E-04	1.85	
<i>DUT</i>	-4.0	0.007	1.3	1.0	-2.2	0.576	1.85	
<i>MAGI3</i>	-2.5	0.019	n.s.	---	-1.4	1.0	1.82	
<i>TNC</i>	-3.9	0.046	2.7	0.459	-2.1	1.0	1.81	
<i>GCNT1</i>	1.9	0.017	1.2	1.0	3.5	1.18E-04	1.81	
<i>RAB3B</i>	3.5	0.042	n.s.	---	6.3	0.002	1.80	
<i>ANLN</i>	-2.8	0.036	1.3	1.0	-1.6	1.0	1.79	

HAUS6	-3.3	0.046	n.s.	---	-1.8	1.0	1.79
MIR222	2.3	1.0	1.7	1.0	4.1	0.023	1.79
RAD51AP1	-3.4	0.041	n.s.	---	-1.9	1.0	1.79
CTGF	10.3	2.28E-05	3.8	0.002	18.3	4.04E-06	1.77
COL10A1	2.3	0.503	n.s.	---	4.1	0.012	1.75
DSCC1	-3.1	0.046	1.4	1.0	-1.8	1.0	1.75
ABCA4	17.4	2.11E-05	n.s.	---	30.3	5.15E-06	1.74
STX11	2.0	0.255	1.5	1.0	3.5	0.003	1.73
RARB	4.0	0.008	n.s.	---	6.8	0.001	1.73
FCER1A	4.9	0.141	n.s.	---	8.3	0.016	1.71
NBEAL1	1.2	1.0	-1.2	1.0	2.1	0.042	1.70
PSG7	3.1	0.217	n.s.	---	5.3	0.013	1.69
XG	3.7	0.411	n.s.	---	6.2	0.034	1.68
WDHD1	-3.5	1.78E-04	n.s.	---	-2.1	0.011	1.67
SASH1	2.1	1.0	n.s.	---	3.4	0.029	1.67
PLK2	2.8	0.001	2.0	0.008	4.6	2.07E-05	1.66
MMP3	4.4	0.080	n.s.	---	7.2	0.008	1.66
RICTOR	2.9	0.056	n.s.	---	4.8	0.003	1.66
CCDC99	-3.8	0.049	n.s.	---	-2.3	1.0	1.63
IL1F7	13.0	2.33E-06	-1.9	0.100	21.1	5.80E-07	1.63
GADD45B	2.3	0.117	1.4	1.0	3.7	0.003	1.63
NTAN1	2.5	0.030	n.s.	---	4.0	0.001	1.62
MALT1	1.8	1.0	1.6	1.0	2.9	0.031	1.62
KIF15	-3.4	0.024	1.3	1.0	-2.1	1.0	1.62
LAMA2	-2.7	0.007	n.s.	---	-1.7	1.0	1.62
DEPDC1B	-3.4	0.027	n.s.	---	-2.1	1.0	1.62
GPRC5A	2.7	0.099	1.8	1.0	4.4	0.005	1.62
MAP2	1.6	0.018	-1.2	1.0	2.5	6.27E-05	1.62
TINAGL1	3.0	0.006	2.1	0.121	4.8	3.43E-04	1.62
CLIC5	2.7	0.153	n.s.	---	4.3	0.007	1.62
TBC1D19	1.6	0.271	-1.3	1.0	2.6	0.001	1.62
YOD1	1.9	1.0	1.3	1.0	3.1	0.029	1.61
KPNA7	12.0	1.36E-04	-1.9	1.0	19.3	3.42E-05	1.61
C21orf7	4.1	0.002	n.s.	---	6.6	2.55E-04	1.60
CTNNAL1	2.2	0.068	1.4	1.0	3.6	0.002	1.60
RAB30	2.0	1.0	n.s.	---	3.2	0.021	1.60
SEMA3A	-2.4	0.050	n.s.	---	-1.5	1.0	1.59
TIMELESS	-2.5	0.033	n.s.	---	-1.6	1.0	1.59
GREB1	4.1	0.001	n.s.	---	6.5	8.25E-05	1.59
FGD4	1.5	1.0	n.s.	---	2.3	0.046	1.59
ARHGAP29	4.9	0.008	1.3	1.0	7.7	0.001	1.57
FBXO32	13.4	0.006	-1.6	1.0	21.0	0.002	1.57
WDR36	-2.9	0.040	n.s.	---	-1.8	1.0	1.56
ZDHHC21	1.5	0.439	1.2	1.0	2.4	0.002	1.55
NTN4	3.0	0.147	n.s.	---	4.7	0.012	1.55
C6orf15	3.1	0.045	n.s.	---	4.7	0.004	1.55
SCML1	-2.8	0.036	1.3	1.0	-1.8	1.0	1.54
ZSWIM4	2.6	0.364	n.s.	---	4.0	0.023	1.54
TNFAIP1	2.2	0.037	1.6	1.0	3.4	0.001	1.54
TFPI	22.3	0.002	n.s.	---	34.3	0.001	1.54
CSRNP2	1.5	0.125	1.2	1.0	2.3	0.001	1.53
LY6G6E	2.7	0.146	n.s.	---	4.1	0.010	1.53
MPHOSPH9	-5.0	0.007	n.s.	---	-3.3	0.071	1.52
MBNL2	1.8	0.874	n.s.	---	2.8	0.017	1.52
FOS	2.1	0.839	1.4	1.0	3.2	0.031	1.52
CCDC68	3.4	0.111	1.6	1.0	5.2	0.012	1.52
CHN2	1.9	0.373	n.s.	---	2.9	0.009	1.51
HELLS	-3.5	0.002	1.3	1.0	-2.3	0.044	1.51
CDKN2B	3.3	0.384	1.6	1.0	5.0	0.043	1.51
EIF1AY	1.9	0.135	n.s.	---	2.9	0.003	1.51
MAML3	3.2	0.012	-1.7	1.0	4.8	0.001	1.51
FCHSD1	2.3	0.031	n.s.	---	3.5	0.001	1.51
TUFT1	2.8	0.072	n.s.	---	4.2	0.006	1.50

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<i>TBC1D15</i>	2.0	0.003	n.s.	---	3.0	7.68E-05	1.50	
<i>TMEM173</i>	5.3	1.88E-04	-1.3	1.0	8.0	3.40E-05	1.50	
<i>CGA</i>	7.9	0.002	n.s.	---	11.8	0.001	1.50	
<i>TEK</i>	6.2	0.149	n.s.	---	9.2	0.033	1.49	
<i>SLC7A11</i>	5.3	4.15E-04	1.5	1.0	7.9	7.72E-05	1.49	
<i>RAD51</i>	-2.5	0.001	n.s.	---	-1.7	0.069	1.48	
<i>CSGALNACT2</i>	3.9	0.159	n.s.	---	5.7	0.024	1.47	
<i>SYNE2</i>	2.9	0.371	-1.4	1.0	4.2	0.036	1.47	
<i>NEBL</i>	3.1	0.046	n.s.	---	4.6	0.005	1.47	
<i>PTPRR</i>	2.1	0.204	n.s.	---	3.1	0.009	1.47	
<i>FYN</i>	2.0	0.011	-1.3	1.0	3.0	3.69E-04	1.47	
<i>GTSE1</i>	-4.0	0.003	n.s.	---	-2.8	0.036	1.46	
<i>TMSB4X</i>	1.6	1.0	n.s.	---	2.3	0.022	1.46	
<i>CYR61</i>	18.9	2.90E-05	3.6	0.019	27.6	1.10E-05	1.46	(b)
<i>FRYL</i>	2.0	0.045	n.s.	---	2.9	0.002	1.46	
<i>RGL1</i>	1.6	0.035	-1.3	1.0	2.4	3.95E-04	1.46	
<i>AMIGO2</i>	3.4	0.258	1.5	1.0	4.9	0.035	1.46	
<i>SLC1A3</i>	-3.9	0.001	1.2	1.0	-2.7	0.013	1.46	
<i>TTC37</i>	-2.9	0.033	n.s.	---	-2.0	0.764	1.45	
<i>NT5E</i>	2.7	0.026	n.s.	---	3.9	0.002	1.45	
<i>DOCK5</i>	2.0	0.026	1.4	1.0	2.8	0.001	1.45	
<i>SERPINB8</i>	2.9	0.096	1.3	1.0	4.1	0.010	1.45	(b)
<i>PYGL</i>	2.3	0.085	-1.4	1.0	3.3	0.005	1.44	
<i>CRIM1</i>	2.0	0.002	1.2	1.0	2.9	5.70E-05	1.43	
<i>SECISBP2L</i>	2.4	0.158	n.s.	---	3.4	0.012	1.43	
<i>FBXO30</i>	3.2	0.002	n.s.	---	4.6	2.11E-04	1.43	
<i>FANCA</i>	-2.7	0.026	n.s.	---	-1.9	0.726	1.43	
<i>XRN1</i>	1.9	0.382	n.s.	---	2.7	0.014	1.43	
<i>RALGPS2</i>	5.6	2.69E-04	1.2	1.0	8.0	6.11E-05	1.43	
<i>ALS2CR12</i>	5.8	0.005	-1.3	1.0	8.2	0.001	1.42	
<i>PLOD2</i>	3.2	1.51E-04	1.2	1.0	4.6	1.89E-05	1.42	
<i>CEP76</i>	2.1	0.357	1.4	1.0	3.0	0.020	1.42	
<i>NOC3L</i>	-2.9	3.86E-04	1.3	1.0	-2.1	0.009	1.42	(a)
<i>TEAD1</i>	1.9	0.258	1.3	1.0	2.6	0.009	1.42	
<i>BLM</i>	-3.1	0.033	n.s.	---	-2.2	0.500	1.42	
<i>MCTP1</i>	13.4	1.45E-05	1.4	1.0	19.0	5.35E-06	1.42	
<i>C5orf41</i>	2.7	0.001	-1.6	0.280	3.8	1.05E-04	1.42	
<i>BACH1</i>	4.5	0.019	1.5	1.0	6.4	0.004	1.41	
<i>OSGIN2</i>	2.5	0.253	n.s.	---	3.5	0.024	1.40	
<i>DAAM1</i>	4.1	0.019	n.s.	---	5.8	0.004	1.40	
<i>PRC1</i>	-2.8	0.029	n.s.	---	-2.0	0.541	1.40	(b)
<i>CLTB</i>	2.7	0.215	1.4	1.0	3.8	0.025	1.40	
<i>ELOVL6</i>	-2.8	0.033	n.s.	---	-2.0	0.528	1.39	
<i>CEP78</i>	-3.2	0.040	n.s.	---	-2.3	0.471	1.38	
<i>GULP1</i>	3.3	0.012	n.s.	---	4.6	0.002	1.38	
<i>VILL</i>	1.7	1.0	n.s.	---	2.4	0.049	1.37	
<i>SLC30A5</i>	-2.4	0.039	n.s.	---	-1.7	1.0	1.37	
<i>DUSP1</i>	2.7	0.127	n.s.	---	3.7	0.016	1.37	
<i>HBEGF</i>	8.6	0.004	n.s.	---	11.8	0.001	1.37	
<i>CYTL1</i>	4.8	0.018	n.s.	---	6.6	0.005	1.36	
<i>UBE2B</i>	1.7	1.0	n.s.	---	2.4	0.045	1.36	
<i>SYNJ1</i>	-2.0	0.013	n.s.	---	-1.5	0.978	1.36	
<i>RAD18</i>	-2.2	0.013	1.2	1.0	-1.6	0.479	1.36	
<i>L3MBTL3</i>	4.9	0.001	n.s.	---	6.6	1.61E-04	1.36	
<i>ABTB1</i>	4.3	0.001	n.s.	---	5.8	2.96E-04	1.36	
<i>TIMP2</i>	3.4	0.018	n.s.	---	4.7	0.003	1.35	(b)
<i>PMAIP1</i>	9.7	0.002	n.s.	---	13.2	0.001	1.35	
<i>PHACTR2</i>	2.0	0.158	n.s.	---	2.7	0.010	1.35	
<i>BAMBI</i>	-2.1	0.005	n.s.	---	-1.6	0.253	1.35	
<i>ATL1</i>	4.1	0.210	-1.5	1.0	5.5	0.050	1.35	
<i>PBK</i>	-3.3	0.011	n.s.	---	-2.4	0.095	1.34	
<i>RAB5A</i>	1.6	0.353	n.s.	---	2.2	0.010	1.34	
<i>PEX3</i>	-4.9	0.012	n.s.	---	-3.6	0.058	1.34	

<i>CLDN1</i>	3.3	0.052	1.6	1.0	4.4	0.010	1.34	
<i>FCHO2</i>	2.1	0.201	-1.4	1.0	2.8	0.018	1.33	
<i>HIST1H1B</i>	-2.7	0.014	n.s.	---	-2.0	0.187	1.33	
<i>ZNF185</i>	4.0	3.59E-04	n.s.	---	5.3	8.16E-05	1.33	
<i>ITGB8</i>	5.1	0.009	n.s.	---	6.8	0.003	1.33	
<i>WDFY3</i>	2.7	0.001	-1.2	1.0	3.6	1.43E-04	1.33	
<i>FABP5</i>	-2.3	0.032	1.5	1.0	-1.7	0.722	1.33	
<i>SCIN</i>	23.6	1.44E-04	n.s.	---	31.3	7.34E-05	1.32	
<i>MARCH3</i>	4.1	0.027	n.s.	---	5.4	0.007	1.32	
<i>HECA</i>	2.5	0.107	n.s.	---	3.3	0.014	1.32	
<i>GTF2B</i>	1.7	0.531	n.s.	---	2.2	0.024	1.32	
<i>PIK3AP1</i>	4.4	0.042	1.4	1.0	5.7	0.011	1.32	
<i>CTSL1</i>	1.7	0.137	1.2	1.0	2.2	0.005	1.32	
<i>POC1B</i>	1.5	0.409	1.2	1.0	2.0	0.010	1.31	
<i>TMSL3</i>	1.6	1.0	n.s.	---	2.1	0.034	1.31	
<i>ATP6V1E1</i>	2.8	0.167	n.s.	---	3.7	0.029	1.31	
<i>CYB5R2</i>	-2.3	0.009	1.3	1.0	-1.7	0.193	1.31	
<i>KIF22</i>	-3.3	0.001	n.s.	---	-2.5	0.006	1.31	(b)
<i>CYTH3</i>	2.3	0.390	n.s.	---	3.0	0.048	1.31	
<i>AURKA</i>	-3.0	0.002	n.s.	---	-2.3	0.014	1.31	(b)
<i>RAB4B</i>	2.9	0.003	n.s.	---	3.7	4.72E-04	1.31	
<i>INPP4B</i>	3.0	0.005	n.s.	---	3.9	0.001	1.31	
<i>DDX11</i>	-2.4	0.031	1.2	1.0	-1.8	0.462	1.31	
<i>CAV1</i>	10.7	6.38E-05	4.1	0.003	13.9	2.75E-05	1.30	(b)
<i>POLH</i>	-3.8	0.043	n.s.	---	-2.9	0.218	1.30	
<i>MFGE8</i>	4.2	4.34E-04	n.s.	---	5.4	1.16E-04	1.30	
<i>RASA1</i>	1.9	0.341	n.s.	---	2.5	0.028	1.30	
<i>TRAM1</i>	1.7	0.490	n.s.	---	2.1	0.022	1.30	
<i>SLC7A1</i>	-2.4	1.01E-04	1.7	0.008	-1.9	0.002	1.30	(b)
<i>NUSAP1</i>	-2.7	0.016	n.s.	---	-2.1	0.164	1.30	
<i>IL18</i>	3.3	0.001	n.s.	---	4.3	3.00E-04	1.29	
<i>CA5B</i>	3.4	0.001	-1.4	1.0	4.4	1.34E-04	1.29	
<i>XPO4</i>	-2.8	0.013	n.s.	---	-2.1	0.122	1.29	
<i>PLEKHA1</i>	1.7	0.777	n.s.	---	2.2	0.040	1.29	
<i>MMD</i>	1.8	0.221	1.2	1.0	2.4	0.016	1.29	
<i>HIST2H3A</i>	-2.5	0.007	n.s.	---	-2.0	0.083	1.29	
<i>DNAJB6</i>	2.3	0.034	1.4	1.0	3.0	0.004	1.29	
<i>PPP2CB</i>	1.8	0.104	1.5	1.0	2.3	0.006	1.29	
<i>CAV2</i>	5.3	0.002	1.5	1.0	6.8	0.001	1.29	
<i>HIST1H4K</i>	-2.2	1.10E-04	1.2	1.0	-1.7	0.002	1.29	
<i>PHGDH</i>	-2.6	0.003	1.3	1.0	-2.1	0.025	1.28	
<i>C11orf82</i>	-3.1	0.032	n.s.	---	-2.4	0.208	1.28	
<i>APLF</i>	1.8	0.125	n.s.	---	2.3	0.009	1.28	
<i>PARD6B</i>	3.2	0.039	n.s.	---	4.1	0.009	1.28	
<i>SESTD1</i>	5.4	0.047	n.s.	---	6.9	0.017	1.28	
<i>TIMP3</i>	11.5	0.003	n.s.	---	14.7	0.001	1.28	
<i>GSG2</i>	-2.5	0.017	1.3	1.0	-2.0	0.171	1.28	
<i>BUB1</i>	-2.6	4.88E-04	n.s.	---	-2.0	0.005	1.28	
<i>CHEK1</i>	-2.0	0.014	1.2	1.0	-1.6	0.351	1.28	
<i>NKD1</i>	-2.5	0.001	1.4	1.0	-2.0	0.009	1.28	
<i>SLC7A5</i>	-2.3	2.50E-04	1.4	0.198	-1.8	0.004	1.28	(a)
<i>HAPLN3</i>	8.6	0.013	n.s.	---	11.0	0.006	1.28	
<i>FANK1</i>	5.5	0.019	n.s.	---	7.0	0.007	1.28	
<i>EZH2</i>	-2.6	0.008	1.2	1.0	-2.1	0.073	1.27	
<i>MSH6</i>	-2.3	0.034	1.2	1.0	-1.8	0.432	1.27	
<i>SYTL4</i>	1.7	0.365	n.s.	---	2.2	0.025	1.27	
<i>RNF13</i>	2.1	0.412	n.s.	---	2.6	0.050	1.27	
<i>CHMP4C</i>	1.9	0.016	n.s.	---	2.5	0.001	1.27	
<i>SLC2A12</i>	7.7	0.001	-1.5	1.0	9.8	0.001	1.27	
<i>MXI1</i>	3.4	0.060	n.s.	---	4.4	0.016	1.27	(b)
<i>PAWR</i>	1.7	0.079	n.s.	---	2.2	0.005	1.27	
<i>TCF19</i>	-2.4	0.007	1.3	1.0	-1.9	0.076	1.27	
<i>AURKB</i>	-2.6	0.014	1.4	1.0	-2.0	0.124	1.27	(a)

SKA3	-3.2	0.012	n.s.	---	-2.6	0.063	1.27	
PIK3IP1	5.7	0.002	n.s.	---	7.3	0.001	1.26	
SHCBP1	-2.9	0.001	n.s.	---	-2.3	0.010	1.26	
OBFC2A	2.1	0.129	n.s.	---	2.7	0.016	1.26	
ODZ3	-3.5	3.52E-05	1.2	1.0	-2.8	1.81E-04	1.26	
RFC2	-2.6	0.033	n.s.	---	-2.1	0.259	1.26	
SNHG12	-2.9	0.027	n.s.	---	-2.3	0.170	1.26	
STXBP5	1.6	0.445	1.2	1.0	2.1	0.025	1.26	
UBE2C	-2.4	0.004	1.2	1.0	-1.9	0.047	1.26	(b)
EIF2C4	3.5	0.009	-1.4	1.0	4.4	0.002	1.26	
UPK3B	2.1	0.109	1.6	1.0	2.7	0.015	1.26	
SUV39H2	-2.9	0.015	n.s.	---	-2.3	0.095	1.26	
WDR45	3.2	0.047	n.s.	---	4.0	0.012	1.26	(b)
PDLIM5	2.2	0.012	1.5	1.0	2.7	0.002	1.26	
MRPS25	-3.8	0.031	n.s.	---	-3.1	0.125	1.26	
SESN3	3.7	0.019	-1.8	1.0	4.7	0.006	1.25	
ATG2A	1.8	0.123	-1.1	1.0	2.2	0.010	1.25	
NEK2	-3.2	0.017	n.s.	---	-2.5	0.087	1.25	(b)
BACH2	8.3	3.62E-04	n.s.	---	10.4	1.64E-04	1.25	
TSPAN6	1.7	0.219	-1.2	1.0	2.1	0.016	1.25	
UBE2D1	4.7	0.002	n.s.	---	5.9	0.001	1.25	
CDC14A	5.0	1.83E-05	1.3	1.0	6.3	6.67E-06	1.25	(b)
INTS7	-2.6	0.017	n.s.	---	-2.1	0.121	1.25	
CDKN1A	3.0	0.051	n.s.	---	3.8	0.013	1.24	(c)
PCMTD1	4.9	0.057	-1.9	1.0	6.1	0.022	1.24	
MXRA7	3.0	0.160	n.s.	---	3.7	0.041	1.24	
SCARA3	3.7	0.007	3.1	0.021	4.5	0.002	1.24	
SSFA2	2.0	0.034	n.s.	---	2.5	0.005	1.24	
TRAK2	3.9	0.006	-1.6	1.0	4.8	0.002	1.24	
CHRNA1	2.2	0.063	1.3	1.0	2.8	0.011	1.24	
RAD54L	-3.3	0.031	n.s.	---	-2.6	0.136	1.24	
RIOK3	5.0	0.002	n.s.	---	6.2	0.001	1.24	
RAD51C	-2.5	0.004	n.s.	---	-2.0	0.033	1.24	
SNORD4A	-2.7	0.006	n.s.	---	-2.2	0.036	1.23	
UTP20	-2.7	0.022	1.4	1.0	-2.2	0.130	1.23	
RTN3	3.5	0.021	n.s.	---	4.4	0.006	1.23	
TDRD7	2.1	0.136	-1.3	1.0	2.6	0.021	1.23	
UCHL5	-2.9	3.15E-04	1.2	1.0	-2.4	0.002	1.23	
RAET1L	8.5	0.002	1.6	1.0	10.5	0.001	1.23	
DDX21	-2.1	0.003	1.5	0.425	-1.7	0.038	1.23	(a)
LMO7	2.5	0.177	n.s.	---	3.0	0.040	1.22	
CYTH1	2.4	0.165	n.s.	---	3.0	0.036	1.22	
UCK1	-2.4	0.007	n.s.	---	-2.0	0.048	1.22	
NAV2	2.0	0.200	-1.7	1.0	2.4	0.029	1.22	
GFPT2	2.8	0.110	n.s.	---	3.4	0.030	1.22	
RNF122	2.3	0.196	n.s.	---	2.8	0.042	1.21	
SNAP23	2.9	7.50E-05	n.s.	---	3.5	1.99E-05	1.21	
IRF6	1.9	0.039	-1.3	1.0	2.3	0.005	1.21	
CHIC2	3.6	4.87E-04	n.s.	---	4.4	1.62E-04	1.21	
RSPH3	2.2	0.091	n.s.	---	2.6	0.017	1.21	
TES	3.5	2.47E-04	1.3	1.0	4.3	8.07E-05	1.21	
PPIH	-2.9	0.023	1.2	1.0	-2.4	0.098	1.21	
NOL6	-3.7	0.001	1.3	1.0	-3.0	0.002	1.21	
NUDT4	6.5	1.77E-04	1.3	1.0	7.9	8.28E-05	1.21	
SPAG5	-3.6	0.010	n.s.	---	-3.0	0.032	1.21	
XPR1	1.7	0.023	n.s.	---	2.1	0.002	1.21	
SWAP70	2.9	0.003	n.s.	---	3.5	0.001	1.21	
SEC14L1	3.3	0.106	n.s.	---	3.9	0.035	1.21	
AFF4	3.1	6.71E-05	n.s.	---	3.7	1.99E-05	1.20	
ZYX	2.2	0.110	2.4	0.046	2.7	0.022	1.20	
HIST1H2BM	-4.3	0.045	n.s.	---	-3.6	0.123	1.20	
OSGEPL1	-3.3	0.032	n.s.	---	-2.8	0.111	1.20	
RFC5	-3.1	0.001	1.2	1.0	-2.5	0.005	1.20	

<i>HIVEP2</i>	2.4	0.002	1.4	1.0	2.8	4.21E-04	1.20	
<i>C15orf23</i>	-2.1	0.002	1.2	1.0	-1.8	0.016	1.20	
<i>SP100</i>	1.8	0.333	-1.3	1.0	2.2	0.047	1.20	
<i>KLF7</i>	2.3	0.069	n.s.	---	2.7	0.015	1.20	
<i>ZFH2</i>	2.3	0.189	-1.5	1.0	2.7	0.044	1.19	
<i>TJP1</i>	1.7	0.021	n.s.	---	2.0	0.002	1.19	
<i>TMOD2</i>	3.1	0.042	n.s.	---	3.7	0.014	1.19	
<i>MBNL3</i>	-2.8	0.015	n.s.	---	-2.3	0.062	1.19	
<i>RANBP1</i>	-2.3	0.001	1.3	1.0	-1.9	0.008	1.19	
<i>B4GALNT3</i>	10.2	0.002	1.5	1.0	12.1	0.001	1.19	
<i>CCNG2</i>	6.2	0.046	-2.0	1.0	7.3	0.023	1.19	(b)(c)
<i>PTPN14</i>	3.0	1.04E-04	1.2	1.0	3.6	3.31E-05	1.19	
<i>EPS8L2</i>	2.0	0.078	n.s.	---	2.4	0.015	1.19	
<i>TROAP</i>	-3.4	0.005	1.3	1.0	-2.9	0.015	1.18	
<i>MAP1LC3B2</i>	2.7	0.001	1.2	1.0	3.2	3.50E-04	1.18	
<i>PTPN12</i>	2.5	0.001	n.s.	---	2.9	1.40E-04	1.18	
<i>CDCA5</i>	-2.4	0.004	1.3	1.0	-2.1	0.020	1.18	
<i>CYP39A1</i>	5.6	2.75E-04	-1.5	1.0	6.6	1.33E-04	1.18	
<i>NDRG1</i>	10.0	4.53E-06	-1.8	0.231	11.8	2.61E-06	1.18	
<i>SMURF2</i>	1.8	0.243	n.s.	---	2.1	0.036	1.18	
<i>OR2A20P</i>	2.9	3.96E-05	n.s.	---	3.4	1.26E-05	1.18	
<i>KDEL2</i>	-3.1	0.014	n.s.	---	-2.6	0.046	1.18	
<i>AGR2</i>	2.7	0.001	-1.4	1.0	3.1	1.59E-04	1.18	
<i>SLC29A3</i>	2.2	0.093	n.s.	---	2.6	0.022	1.18	
<i>FLJ44874</i>	3.1	0.013	1.7	1.0	3.6	0.005	1.18	
<i>DOCK9</i>	1.8	0.165	n.s.	---	2.2	0.029	1.17	
<i>SNORD28</i>	-2.8	0.017	1.2	1.0	-2.4	0.062	1.17	
<i>ZFYVE1</i>	4.0	4.68E-05	-1.4	1.0	4.7	2.00E-05	1.17	
<i>WDR1</i>	2.4	0.016	-1.3	1.0	2.8	0.004	1.17	
<i>ACVR1C</i>	-2.4	0.025	n.s.	---	-2.0	0.114	1.17	
<i>NUP50</i>	4.6	0.018	n.s.	---	5.4	0.008	1.17	
<i>CR2</i>	-2.7	0.010	1.2	1.0	-2.3	0.036	1.17	
<i>SEMA3C</i>	5.0	0.001	-1.5	1.0	5.9	0.001	1.17	(b)
<i>ZBTB38</i>	2.0	0.010	n.s.	---	2.3	0.002	1.17	
<i>FANCC</i>	-2.4	0.018	n.s.	---	-2.0	0.078	1.17	
<i>FOXM1</i>	-2.6	0.001	1.2	1.0	-2.3	0.002	1.16	(b)
<i>NECAP2</i>	2.3	0.009	n.s.	---	2.7	0.002	1.16	
<i>WWTR1</i>	4.1	0.019	n.s.	---	4.7	0.009	1.16	
<i>SLC1A1</i>	2.7	0.007	n.s.	---	3.2	0.003	1.16	
<i>CCDC50</i>	2.0	0.187	1.3	1.0	2.3	0.046	1.16	
<i>PTPN21</i>	2.7	0.018	1.4	1.0	3.1	0.007	1.15	
<i>CHMP5</i>	1.9	0.044	n.s.	---	2.1	0.009	1.15	
<i>PPFIBP1</i>	2.8	0.028	n.s.	---	3.2	0.010	1.15	
<i>ILDR2</i>	-2.2	0.034	1.3	1.0	-1.9	0.149	1.15	
<i>ETHE1</i>	2.0	0.023	-1.2	1.0	2.3	0.005	1.15	
<i>ATP8A1</i>	6.3	0.001	-1.5	1.0	7.2	0.001	1.15	
<i>NCAPD3</i>	-3.3	4.16E-04	n.s.	---	-2.9	0.001	1.15	
<i>GATA6</i>	1.9	0.016	n.s.	---	2.2	0.004	1.15	
<i>PDGFRL</i>	-3.4	0.001	-1.4	1.0	-3.0	0.003	1.15	
<i>TCP11L2</i>	9.7	0.001	-1.6	1.0	11.2	0.001	1.15	
<i>TMEM140</i>	10.1	5.83E-06	n.s.	---	11.5	3.71E-06	1.14	
<i>TNS1</i>	2.0	0.051	n.s.	---	2.3	0.014	1.14	
<i>SMC1A</i>	-2.0	2.30E-04	1.1	1.0	-1.8	0.001	1.14	
<i>SHROOM3</i>	2.5	2.28E-04	1.3	1.0	2.9	7.87E-05	1.14	
<i>RAB9A</i>	3.2	0.014	n.s.	---	3.7	0.006	1.14	
<i>CD99</i>	3.8	1.67E-04	n.s.	---	4.3	7.98E-05	1.14	
<i>IL2RG</i>	4.6	1.23E-04	n.s.	---	5.2	6.49E-05	1.14	
<i>LDLR</i>	3.7	0.087	1.8	1.0	4.2	0.043	1.13	
<i>ARID5B</i>	4.1	0.001	-1.5	1.0	4.6	3.61E-04	1.13	(a)
<i>TCF12</i>	2.2	0.067	n.s.	---	2.5	0.022	1.13	
<i>GATSL1</i>	3.7	4.87E-04	-2.0	0.079	4.2	2.38E-04	1.13	
<i>ITGA3</i>	2.1	0.021	n.s.	---	2.3	0.006	1.13	
<i>CD59</i>	2.2	0.011	-1.1	1.0	2.5	0.003	1.13	(b)

<i>TMPO</i>	-2.5	0.001	n.s.	---	-2.2	0.002	1.13	
<i>RACGAP1</i>	-3.2	0.004	n.s.	---	-2.8	0.010	1.13	
<i>FANCG</i>	-2.0	0.002	1.3	1.0	-1.8	0.008	1.13	
<i>MCM2</i>	-3.2	3.72E-04	1.3	1.0	-2.9	0.001	1.12	
<i>ANXA1</i>	2.2	0.005	1.4	1.0	2.4	0.002	1.12	
<i>ABLIM1</i>	1.9	0.148	n.s.	---	2.2	0.045	1.12	
<i>PIK3C3</i>	1.9	0.072	n.s.	---	2.1	0.021	1.12	
<i>SLC12A6</i>	3.2	0.059	-1.3	1.0	3.6	0.030	1.12	
<i>SUN1</i>	2.4	6.79E-05	n.s.	---	2.7	2.66E-05	1.12	
<i>BACE1</i>	-2.2	0.003	1.1	1.0	-2.0	0.010	1.12	
<i>POLB</i>	2.0	0.002	1.1	1.0	2.2	4.92E-04	1.12	
<i>KLHL24</i>	3.3	0.018	-2.0	0.977	3.7	0.009	1.11	
<i>SNX9</i>	2.8	0.027	n.s.	---	3.1	0.013	1.11	
<i>NAPG</i>	2.5	0.004	1.2	1.0	2.7	0.002	1.11	
<i>POLD1</i>	-2.3	0.040	1.2	1.0	-2.1	0.114	1.11	
<i>MEGF9</i>	2.6	0.019	-1.2	1.0	2.9	0.008	1.11	
<i>GBE1</i>	2.0	0.046	n.s.	---	2.3	0.016	1.11	
<i>GM2A</i>	2.2	0.036	-1.2	1.0	2.4	0.013	1.11	(b)
<i>SLC4A11</i>	2.4	0.033	n.s.	---	2.7	0.014	1.11	
<i>DCAF6</i>	1.9	0.012	-1.6	0.210	2.1	0.004	1.11	
<i>CRIP1</i>	2.7	0.075	n.s.	---	3.0	0.035	1.11	
<i>WDSUB1</i>	3.5	0.060	n.s.	---	3.8	0.033	1.11	
<i>NSUN5P2</i>	-2.3	0.023	-1.2	1.0	-2.1	0.062	1.11	
<i>ZFAND5</i>	2.0	0.061	n.s.	---	2.2	0.022	1.11	
<i>HIST1H3I</i>	-2.7	0.004	n.s.	---	-2.4	0.008	1.11	
<i>REEP3</i>	2.1	0.022	n.s.	---	2.3	0.008	1.11	
<i>KIF18B</i>	-3.0	1.18E-04	1.1	1.0	-2.7	2.54E-04	1.11	
<i>C9orf100</i>	-3.0	0.013	n.s.	---	-2.7	0.027	1.11	
<i>ERV3</i>	4.2	0.003	-1.4	1.0	4.6	0.001	1.11	
<i>HADH</i>	-3.5	0.009	n.s.	---	-3.1	0.018	1.11	
<i>QPCTL</i>	-3.2	0.001	n.s.	---	-2.9	0.001	1.11	
<i>SKP2</i>	-2.9	0.018	n.s.	---	-2.6	0.039	1.11	
<i>RRM1</i>	-2.1	0.025	n.s.	---	-1.9	0.072	1.11	
<i>CBLB</i>	4.2	0.008	-1.4	1.0	4.6	0.005	1.10	
<i>CALCOCO1</i>	7.4	0.005	-1.6	1.0	8.2	0.003	1.10	
<i>SPTB</i>	2.0	0.042	1.2	1.0	2.2	0.015	1.10	
<i>ZFAND6</i>	2.5	4.69E-04	-1.2	1.0	2.7	2.10E-04	1.10	
<i>STX7</i>	2.7	0.001	-1.2	1.0	3.0	0.001	1.10	
<i>ICK</i>	2.1	1.52E-05	-1.2	1.0	2.3	5.65E-06	1.10	
<i>RCCD1</i>	-2.8	0.009	-1.2	1.0	-2.5	0.020	1.10	
<i>ARHGEF2</i>	2.5	0.070	n.s.	---	2.8	0.034	1.10	
<i>REPS2</i>	3.7	0.041	-1.4	1.0	4.1	0.024	1.10	
<i>CALHM2</i>	3.7	0.017	n.s.	---	4.0	0.010	1.10	
<i>RBMS2</i>	3.1	0.077	1.5	1.0	3.5	0.042	1.10	
<i>MTO1</i>	-2.7	0.006	1.2	1.0	-2.5	0.013	1.10	
<i>SPTAN1</i>	1.8	0.019	1.2	1.0	2.0	0.007	1.10	
<i>TP73</i>	-2.5	0.022	n.s.	---	-2.3	0.049	1.10	
<i>TSR1</i>	-2.6	3.90E-04	1.4	1.0	-2.4	0.001	1.09	
<i>PC</i>	-2.8	0.015	n.s.	---	-2.6	0.029	1.09	
<i>CXCL5</i>	-3.0	0.045	n.s.	---	-2.7	0.084	1.09	(a)
<i>LASS6</i>	-2.1	0.008	1.2	1.0	-1.9	0.021	1.09	
<i>C1orf116</i>	3.2	0.010	1.3	1.0	3.5	0.006	1.09	
<i>KIF3C</i>	4.5	0.003	-1.5	1.0	4.9	0.002	1.09	(b)
<i>TMEM86A</i>	4.1	0.001	-2.1	0.136	4.5	0.001	1.09	
<i>KLF6</i>	6.2	0.003	1.4	1.0	6.7	0.002	1.09	
<i>NDUFV1</i>	-2.5	0.024	n.s.	---	-2.3	0.050	1.09	
<i>GAB1</i>	2.3	0.006	n.s.	---	2.5	0.003	1.09	
<i>TYMS</i>	-2.4	0.004	n.s.	---	-2.2	0.008	1.09	
<i>TNFRSF21</i>	4.3	6.38E-05	1.6	0.428	4.7	4.13E-05	1.09	(b)
<i>TEP1</i>	2.2	0.008	-1.3	1.0	2.3	0.004	1.09	
<i>CCDC21</i>	-3.3	0.013	n.s.	---	-3.0	0.022	1.09	
<i>E2F8</i>	-4.0	0.013	n.s.	---	-3.7	0.021	1.08	
<i>ANAPC1</i>	-2.8	0.011	n.s.	---	-2.6	0.020	1.08	

SEPP1	3.9	3.95E-04	-3.1	0.002	4.2	2.53E-04	1.08
ACPP	4.7	0.001	-2.5	0.037	5.1	4.65E-04	1.08
OPTN	4.2	0.017	n.s.	---	4.5	0.011	1.08
BCL6	7.3	0.001	-1.6	1.0	7.9	0.001	1.08
TGFBR3	2.1	0.048	n.s.	---	2.3	0.023	1.08
FEN1	-2.9	0.001	n.s.	---	-2.7	0.002	1.08
RCAN3	2.4	0.003	-1.4	1.0	2.6	0.002	1.08
TUBA1A	7.7	0.022	n.s.	---	8.3	0.017	1.08
STRADB	2.2	9.54E-05	-1.2	1.0	2.4	4.71E-05	1.08
LASS5	2.0	0.063	n.s.	---	2.1	0.029	1.08
GDA	2.6	0.001	n.s.	---	2.8	0.001	1.08
SAT1	3.9	2.34E-05	-1.6	0.098	4.2	1.54E-05	1.08
TOR1AIP2	2.8	0.020	n.s.	---	3.0	0.012	1.08
NAF1	-2.3	0.004	1.4	1.0	-2.1	0.007	1.07
LAMC2	2.4	0.028	-1.4	1.0	2.6	0.016	1.07
RYBP	2.0	0.031	n.s.	---	2.1	0.015	1.07
YIPF1	1.9	0.011	-1.2	1.0	2.0	0.005	1.07
FRMD5	-2.2	0.043	n.s.	---	-2.1	0.084	1.07
OTUD1	2.2	0.092	n.s.	---	2.3	0.049	1.07
PTPRG	2.4	2.74E-05	1.4	0.073	2.5	1.50E-05	1.07
MAGI1	4.7	0.002	n.s.	---	5.0	0.001	1.07
NT5C2	2.8	0.004	n.s.	---	3.0	0.002	1.07
JAK1	2.9	2.62E-05	-1.2	1.0	3.1	1.62E-05	1.07
GMDS	-2.6	0.001	n.s.	---	-2.4	0.001	1.07
NUP93	-2.1	0.023	n.s.	---	-2.0	0.046	1.07
LY6G6C	55.0	6.21E-06	1.4	1.0	58.5	5.49E-06	1.07
FOXP1	14.2	8.93E-06	-1.7	1.0	15.1	7.46E-06	1.06
C7orf68	-3.7	0.035	n.s.	---	-3.5	0.050	1.06
CYBRD1	7.3	1.16E-04	n.s.	---	7.7	9.19E-05	1.06
ARHGEF12	2.7	2.23E-04	n.s.	---	2.9	1.41E-04	1.06
IPO9	-2.2	0.013	n.s.	---	-2.0	0.024	1.06
PRSS23	-2.5	0.005	n.s.	---	-2.3	0.008	1.06
TTF2	-3.8	0.003	n.s.	---	-3.5	0.005	1.06
PPAT	-2.5	0.010	1.2	1.0	-2.4	0.017	1.06
TRMU	-2.2	0.036	n.s.	---	-2.1	0.064	1.06
XRCC6BP1	-3.0	0.017	n.s.	---	-2.8	0.025	1.06
LRRC8A	2.0	0.016	1.2	1.0	2.1	0.009	1.06
PINX1	-3.0	0.046	n.s.	---	-2.8	0.069	1.06
NCAPH	-2.4	0.001	1.2	1.0	-2.2	0.002	1.06
ESPL1	-3.4	0.005	1.3	1.0	-3.2	0.007	1.06
POU5F1	-2.6	0.017	n.s.	---	-2.5	0.026	1.06
FMNL2	6.0	0.002	n.s.	---	6.3	0.001	1.05
NHLRC3	-2.0	0.014	-1.3	1.0	-1.9	0.026	1.05
PNRC1	2.4	0.046	-1.2	1.0	2.5	0.030	1.05
YPEL5	5.7	0.001	-1.7	1.0	5.9	0.001	1.05
URB1	-2.5	0.034	n.s.	---	-2.3	0.051	1.05
UCA1	8.9	0.002	n.s.	---	9.4	0.001	1.05
DDI2	3.2	0.008	-1.3	1.0	3.4	0.006	1.05
KIAA0101	-2.2	0.022	n.s.	---	-2.1	0.035	1.05
ATXN1	5.3	0.001	n.s.	---	5.5	0.001	1.05
BTG1	3.8	0.058	n.s.	---	3.9	0.045	1.04
TIPARP	2.8	0.018	n.s.	---	3.0	0.013	1.04
CYTH2	2.8	0.052	n.s.	---	3.0	0.039	1.04
RAB43	4.4	0.011	-1.5	1.0	4.6	0.009	1.04
ANGPT2	13.7	1.25E-04	n.s.	---	14.3	1.10E-04	1.04
BMPR2	2.1	0.002	-1.2	1.0	2.2	0.002	1.04
VEPH1	7.8	0.024	n.s.	---	8.2	0.020	1.04
ARHGAP21	2.7	0.020	n.s.	---	2.8	0.015	1.04
HSD17B11	2.8	0.007	n.s.	---	2.9	0.005	1.04
FOXO1	4.9	4.97E-04	1.7	1.0	5.1	4.11E-04	1.04
RHOF	2.1	0.001	1.3	1.0	2.2	0.001	1.04
IGFBP4	2.9	0.002	1.4	1.0	3.1	0.001	1.04
GEMIN5	-3.1	0.008	1.3	1.0	-3.0	0.010	1.04

(b)(c)

(c)

(a)

ABHD4	3.4	0.001	n.s.	---	3.5	0.001	1.04	
TPRA1	2.6	2.63E-04	n.s.	---	2.7	1.98E-04	1.04	
FADS3	2.8	0.009	1.2	1.0	2.9	0.007	1.04	
HSPE1	-2.2	0.005	n.s.	---	-2.2	0.007	1.04	
FLNB	2.5	0.001	n.s.	---	2.5	3.96E-04	1.03	
TUBG1	-2.1	0.013	n.s.	---	-2.1	0.018	1.03	
SLC5A3	2.7	0.054	n.s.	---	2.8	0.043	1.03	
BUB1B	-2.9	0.002	n.s.	---	-2.8	0.003	1.03	
VAMP5	3.7	0.034	n.s.	---	3.8	0.028	1.03	
PDSS1	-2.5	0.016	1.2	1.0	-2.5	0.021	1.03	
KLF5	2.6	0.003	n.s.	---	2.7	0.002	1.03	(b)
PRR15	2.4	0.047	-1.9	0.541	2.5	0.036	1.03	
MACF1	2.7	2.62E-04	n.s.	---	2.8	2.10E-04	1.03	
DNMT1	-2.0	0.007	n.s.	---	-2.0	0.009	1.03	
NOP56	-2.2	0.033	n.s.	---	-2.2	0.042	1.02	
RNFT2	-3.2	0.021	n.s.	---	-3.1	0.025	1.02	
RND1	3.1	0.021	n.s.	---	3.2	0.019	1.02	
NEDD4L	4.3	0.001	n.s.	---	4.3	0.001	1.02	(b)
PIGW	-2.7	0.011	n.s.	---	-2.6	0.012	1.01	(a)
DTL	-2.4	0.022	n.s.	---	-2.3	0.024	1.01	
GPR137B	2.6	0.001	n.s.	---	2.6	0.001	-1.00	
CDC34	2.2	0.001	n.s.	---	2.2	0.001	-1.00	
RRP12	-2.2	0.046	1.4	1.0	-2.1	0.048	-1.00	
SERINC2	2.6	0.006	n.s.	---	2.6	0.007	-1.00	
PHF21A	2.7	0.012	-1.7	1.0	2.7	0.012	-1.00	
PIK3CB	2.0	0.030	n.s.	---	2.0	0.031	-1.00	
PDZK1IP1	5.3	0.018	-1.4	1.0	5.3	0.018	-1.00	
PPP1R15A	3.3	0.040	1.5	1.0	3.3	0.040	-1.00	
PDIA6	2.8	1.56E-04	-1.4	0.350	2.8	1.55E-04	-1.00	
HSPA4L	-4.6	0.015	n.s.	---	-4.6	0.015	-1.00	
MAP4K4	2.2	0.021	n.s.	---	2.2	0.021	-1.00	
CCNDBP1	2.9	0.003	-1.4	1.0	2.9	0.003	-1.00	
GATS	6.0	0.001	-1.7	1.0	6.1	0.001	-1.00	
LGR4	5.0	0.006	n.s.	---	5.0	0.006	-1.01	
SOD2	4.1	0.003	n.s.	---	4.1	0.003	-1.01	(c)
TRIM29	3.4	0.040	-1.3	1.0	3.4	0.042	-1.01	
FOSL2	3.3	1.76E-04	n.s.	---	3.3	1.69E-04	-1.01	
KIF20A	-3.8	4.28E-04	1.2	1.0	-3.8	4.11E-04	-1.01	
TSPAN8	3.1	3.56E-05	1.2	1.0	3.2	3.38E-05	-1.01	
FGF20	-5.2	1.33E-04	n.s.	---	-5.2	1.28E-04	-1.01	
MCM3	-3.0	0.036	n.s.	---	-3.0	0.034	-1.01	
CITED2	3.8	0.049	n.s.	---	3.8	0.047	-1.01	
C10orf26	7.1	0.024	n.s.	---	7.0	0.025	-1.01	
LRP10	2.8	0.001	-1.2	1.0	2.8	0.001	-1.01	
SLC2A10	3.2	4.23E-05	n.s.	---	3.2	4.54E-05	-1.01	
SLC35D2	2.7	0.042	-1.9	1.0	2.8	0.039	-1.01	
ITGAX	3.1	0.030	-1.8	1.0	3.1	0.028	-1.01	
TCOF1	-2.1	0.023	1.4	1.0	-2.2	0.020	-1.01	
ERBB3	5.9	2.47E-04	-1.4	1.0	5.8	2.61E-04	-1.01	(b)
ARRDC3	8.0	1.48E-05	-1.5	1.0	7.9	1.55E-05	-1.01	
DPYSL2	2.5	9.07E-05	n.s.	---	2.4	1.02E-04	-1.01	
CENPM	-2.6	1.60E-05	n.s.	---	-2.7	1.44E-05	-1.01	
RERE	2.5	0.007	n.s.	---	2.4	0.008	-1.01	(b)
TGFA	3.2	0.008	n.s.	---	3.2	0.007	-1.01	(b)
FAM64A	-2.9	0.029	n.s.	---	-2.9	0.026	-1.01	
EPRS	-2.3	0.035	n.s.	---	-2.3	0.030	-1.02	
LGMN	2.3	0.032	-1.3	1.0	2.3	0.028	-1.02	
TMCC3	6.8	4.68E-04	-1.8	1.0	6.7	0.001	-1.02	
SRM	-2.0	0.004	1.1	1.0	-2.0	0.004	-1.02	(a)
ABI1	2.4	0.002	n.s.	---	2.4	0.002	-1.02	
GPX8	-3.2	0.012	n.s.	---	-3.3	0.011	-1.02	
LGR5	2.2	0.004	n.s.	---	2.3	0.003	-1.02	(a)
DUSP5	9.8	4.70E-04	1.5	1.0	9.6	0.001	-1.02	(b)

<i>PLK1</i>	-2.7	0.003	1.2	1.0	-2.8	0.003	-1.02	
<i>SFMBT1</i>	-2.8	0.027	n.s.	---	-2.9	0.023	-1.02	
<i>FARSB</i>	-3.1	0.008	n.s.	---	-3.2	0.006	-1.03	
<i>IARS</i>	-2.2	0.027	n.s.	---	-2.3	0.021	-1.03	(b)
<i>CD55</i>	9.8	0.006	1.5	1.0	9.5	0.007	-1.03	
<i>OR2A7</i>	3.7	0.007	n.s.	---	3.6	0.009	-1.03	
<i>GALNT4</i>	2.1	0.003	n.s.	---	2.0	0.004	-1.03	
<i>TM4SF1</i>	4.1	3.88E-05	1.6	0.237	4.0	4.72E-05	-1.04	
<i>TTC13</i>	-3.7	0.013	-1.5	1.0	-3.8	0.011	-1.04	
<i>SUPT16H</i>	-2.3	0.055	n.s.	---	-2.3	0.039	-1.04	
<i>PTTG1IP</i>	2.3	0.030	n.s.	---	2.2	0.043	-1.04	
<i>OR2A4</i>	3.7	0.006	n.s.	---	3.6	0.008	-1.04	
<i>PSRC1</i>	-2.3	0.009	1.2	1.0	-2.4	0.006	-1.04	
<i>E2F2</i>	-2.4	0.053	n.s.	---	-2.5	0.038	-1.04	
<i>SHMT1</i>	-2.5	0.047	n.s.	---	-2.6	0.034	-1.04	
<i>RBMX</i>	-2.0	0.011	n.s.	---	-2.1	0.007	-1.04	
<i>ZNF692</i>	-2.2	0.015	n.s.	---	-2.3	0.010	-1.04	
<i>SPAG9</i>	2.3	0.050	1.2	1.0	2.2	0.075	-1.04	
<i>SNORA45</i>	-2.3	0.012	1.6	1.0	-2.4	0.008	-1.05	
<i>CRTAM</i>	6.0	0.001	2.4	0.197	5.7	0.001	-1.05	
<i>TGFBR2</i>	3.2	0.028	1.5	1.0	3.0	0.038	-1.05	
<i>TBC1D8</i>	2.4	0.037	n.s.	---	2.3	0.056	-1.05	
<i>XPO5</i>	-2.0	0.016	1.2	1.0	-2.1	0.010	-1.05	
<i>TRNP1</i>	2.2	0.048	n.s.	---	2.1	0.079	-1.05	
<i>ITGB6</i>	2.0	0.029	-2.0	0.021	1.9	0.052	-1.05	
<i>CAD</i>	-3.1	0.048	n.s.	---	-3.2	0.034	-1.05	(a)
<i>PAK1IP1</i>	-2.8	0.007	1.2	1.0	-2.9	0.005	-1.06	
<i>DUSP6</i>	5.2	1.92E-04	2.8	0.008	4.9	2.53E-04	-1.06	(b)
<i>CTSB</i>	5.0	1.46E-04	n.s.	---	4.7	1.94E-04	-1.06	(b)
<i>IRS2</i>	9.0	3.08E-05	n.s.	---	8.5	3.82E-05	-1.06	
<i>DHRS11</i>	-2.5	0.051	n.s.	---	-2.6	0.032	-1.06	
<i>ERRFI1</i>	3.4	7.13E-08	1.2	0.069	3.2	1.08E-07	-1.06	
<i>EGLN3</i>	4.6	0.021	-1.5	1.0	4.3	0.029	-1.06	
<i>SCFD2</i>	-3.2	0.035	n.s.	---	-3.4	0.024	-1.07	
<i>ACY3</i>	-2.3	0.088	-1.7	1.0	-2.5	0.049	-1.07	
<i>COL17A1</i>	2.2	0.001	-1.1	1.0	2.1	0.002	-1.07	
<i>C10orf2</i>	-2.4	0.021	1.4	1.0	-2.6	0.012	-1.07	
<i>APCDD1</i>	-3.0	0.022	n.s.	---	-3.3	0.014	-1.07	
<i>UCP2</i>	3.2	0.038	-1.4	1.0	3.0	0.061	-1.07	
<i>CYP2S1</i>	3.1	4.00E-05	-1.1	1.0	2.9	6.67E-05	-1.07	
<i>PPIF</i>	-2.3	0.001	1.2	1.0	-2.5	4.10E-04	-1.07	(a)
<i>TNS4</i>	2.3	1.18E-04	1.2	1.0	2.1	2.46E-04	-1.07	
<i>CGREF1</i>	-2.0	0.007	n.s.	---	-2.2	0.003	-1.08	
<i>HLA-E</i>	2.3	0.004	n.s.	---	2.1	0.009	-1.08	
<i>UBE2H</i>	3.3	0.011	n.s.	---	3.1	0.018	-1.08	(b)
<i>GPRC6A</i>	-3.0	0.053	-2.4	0.284	-3.2	0.032	-1.08	
<i>KCTD21</i>	2.5	1.44E-04	-1.5	0.132	2.3	2.88E-04	-1.08	
<i>PPAP2B</i>	2.2	4.21E-04	-1.7	0.006	2.0	0.001	-1.08	
<i>SLC2A1</i>	2.2	0.001	1.4	0.371	2.0	0.002	-1.08	
<i>PSMG3</i>	-2.2	0.098	n.s.	---	-2.3	0.045	-1.09	
<i>BAG2</i>	-2.5	0.064	n.s.	---	-2.7	0.032	-1.09	(a)
<i>SLC43A2</i>	8.2	2.73E-04	n.s.	---	7.5	3.79E-04	-1.09	
<i>PYCR1</i>	-3.5	0.004	1.2	1.0	-3.8	0.002	-1.09	
<i>RPL27A</i>	-2.3	0.007	n.s.	---	-2.5	0.003	-1.09	
<i>GDF15</i>	2.3	0.004	-2.0	0.014	2.1	0.009	-1.09	
<i>MDC1</i>	-2.5	0.042	n.s.	---	-2.7	0.021	-1.09	
<i>PXK</i>	-2.2	0.029	n.s.	---	-2.4	0.013	-1.09	
<i>CASZ1</i>	2.1	0.040	-1.5	1.0	2.0	0.103	-1.09	
<i>HSPG2</i>	2.4	0.004	-1.4	1.0	2.2	0.010	-1.09	
<i>NUCKS1</i>	-1.8	0.004	n.s.	---	-2.0	0.001	-1.09	
<i>TMEM59</i>	2.0	0.013	-1.4	1.0	1.9	0.036	-1.10	
<i>NAT10</i>	-2.1	0.072	n.s.	---	-2.3	0.030	-1.10	
<i>GPR161</i>	2.4	0.004	-1.4	1.0	2.1	0.011	-1.10	

<i>KIFC1</i>	-2.4	0.005	n.s.	---	-2.6	0.002	-1.10	
<i>PLAT</i>	6.3	0.002	n.s.	---	5.7	0.003	-1.10	
<i>ATP5G1</i>	-1.9	0.028	n.s.	---	-2.1	0.010	-1.10	
<i>MAST3</i>	2.3	0.048	-1.2	1.0	2.1	0.121	-1.10	
<i>ACACA</i>	-2.3	0.016	n.s.	---	-2.5	0.007	-1.10	
<i>CAMK2N1</i>	2.3	0.020	-2.0	0.061	2.1	0.053	-1.10	
<i>MMACHC</i>	-2.7	0.051	n.s.	---	-3.0	0.025	-1.10	
<i>WRB</i>	2.4	0.036	n.s.	---	2.2	0.088	-1.11	
<i>AKAP13</i>	2.3	0.008	n.s.	---	2.1	0.022	-1.11	
<i>TOE1</i>	-2.3	0.001	n.s.	---	-2.6	2.45E-04	-1.11	
<i>USP2</i>	-3.0	0.009	-1.3	1.0	-3.4	0.005	-1.11	
<i>HNMT</i>	4.4	0.008	-1.7	1.0	3.9	0.014	-1.12	
<i>C5orf13</i>	-4.8	0.031	n.s.	---	-5.4	0.018	-1.12	
<i>LEF1</i>	-2.7	1.62E-04	n.s.	---	-3.1	7.07E-05	-1.12	(a)
<i>NR6A1</i>	2.2	0.020	n.s.	---	2.0	0.063	-1.12	
<i>TNRC18</i>	2.2	0.010	-1.2	1.0	2.0	0.031	-1.12	
<i>MRPS34</i>	-2.5	0.004	n.s.	---	-2.8	0.002	-1.12	
<i>SORL1</i>	3.3	0.003	-1.2	1.0	2.9	0.007	-1.12	
<i>TNFSF10</i>	20.8	1.15E-05	-2.6	0.092	18.6	1.56E-05	-1.12	(c)
<i>MRGPRX2</i>	18.2	1.88E-05	1.3	1.0	16.3	2.60E-05	-1.12	
<i>TARBP1</i>	-2.0	0.090	n.s.	---	-2.3	0.029	-1.12	
<i>C19orf48</i>	-2.3	0.074	n.s.	---	-2.6	0.027	-1.13	
<i>CARS2</i>	-2.5	0.052	n.s.	---	-2.9	0.021	-1.13	
<i>CLIC3</i>	3.9	0.025	-1.5	1.0	3.4	0.049	-1.13	
<i>TRIP13</i>	-2.4	0.028	n.s.	---	-2.7	0.011	-1.13	
<i>UCK2</i>	-2.1	0.062	n.s.	---	-2.4	0.020	-1.13	
<i>ANGEL1</i>	-2.7	0.010	n.s.	---	-3.0	0.004	-1.13	
<i>NUP210</i>	-2.1	0.141	n.s.	---	-2.4	0.047	-1.13	
<i>PCDH1</i>	2.3	0.038	-1.5	1.0	2.0	0.133	-1.14	
<i>PRPF19</i>	-2.2	0.020	n.s.	---	-2.6	0.006	-1.14	
<i>KLK10</i>	6.1	0.002	n.s.	---	5.4	0.003	-1.14	(b)
<i>SREBF1</i>	-1.8	0.110	-1.2	1.0	-2.1	0.025	-1.14	
<i>STEAP2</i>	-2.1	0.029	-1.2	1.0	-2.4	0.008	-1.14	
<i>SERPINE2</i>	3.3	5.46E-05	1.1	1.0	2.9	1.40E-04	-1.14	
<i>RRP1B</i>	-2.1	0.037	1.4	1.0	-2.4	0.010	-1.14	
<i>ATP9A</i>	4.8	5.72E-05	-1.4	1.0	4.2	1.17E-04	-1.15	
<i>EPHX4</i>	3.5	0.001	-1.3	1.0	3.0	0.003	-1.15	
<i>S100P</i>	7.6	1.09E-05	n.s.	---	6.6	1.89E-05	-1.15	(b)
<i>RAB11FIP5</i>	3.3	0.009	1.3	1.0	2.9	0.022	-1.15	
<i>GEMIN4</i>	-3.4	1.90E-04	1.2	1.0	-3.8	8.13E-05	-1.15	(a)
<i>CAB39L</i>	-2.0	0.140	-2.7	0.012	-2.3	0.037	-1.15	
<i>TP53INP2</i>	3.5	1.56E-04	-1.5	0.747	3.1	3.93E-04	-1.15	
<i>NLE1</i>	-3.1	0.042	n.s.	---	-3.5	0.017	-1.15	(a)
<i>ITLN2</i>	9.3	0.001	n.s.	---	8.1	0.002	-1.15	
<i>TOMM40L</i>	-2.1	0.108	-1.3	1.0	-2.5	0.030	-1.15	
<i>WDR46</i>	-2.2	0.146	n.s.	---	-2.5	0.042	-1.15	
<i>OAS3</i>	-2.2	0.087	-1.2	1.0	-2.6	0.025	-1.15	
<i>LIPH</i>	3.3	0.001	-2.3	0.012	2.8	0.002	-1.16	
<i>IL10RA</i>	12.0	7.86E-05	n.s.	---	10.4	1.26E-04	-1.16	
<i>MRPL12</i>	-2.0	0.073	n.s.	---	-2.3	0.016	-1.16	(a)
<i>GRB7</i>	5.9	0.002	-1.8	1.0	5.1	0.005	-1.16	(b)
<i>DKC1</i>	-2.3	0.169	n.s.	---	-2.7	0.050	-1.16	(a)
<i>ITM2B</i>	2.4	0.001	-1.3	1.0	2.1	0.006	-1.16	
<i>NFX1</i>	-2.0	0.087	n.s.	---	-2.3	0.019	-1.16	
<i>GLUL</i>	16.8	3.96E-05	1.5	1.0	14.4	6.13E-05	-1.16	
<i>DLEU1</i>	-3.7	0.007	n.s.	---	-4.3	0.003	-1.16	
<i>FSTL3</i>	8.3	1.31E-04	n.s.	---	7.1	2.37E-04	-1.16	
<i>MAOB</i>	20.8	6.05E-05	n.s.	---	17.8	9.10E-05	-1.17	
<i>PRMT3</i>	-2.6	0.154	n.s.	---	-3.0	0.050	-1.17	
<i>THBS1</i>	5.5	2.92E-05	2.5	0.004	4.7	6.16E-05	-1.17	
<i>ILF3</i>	-1.7	0.079	n.s.	---	-2.0	0.012	-1.17	
<i>WDR74</i>	-1.8	0.221	1.4	1.0	-2.1	0.037	-1.17	(a)
<i>SLC1A4</i>	-3.0	2.50E-04	-1.3	1.0	-3.5	8.56E-05	-1.17	(b)

NBEAL2	-2.5	0.035	n.s.	---	-2.9	0.010	-1.17	
HES7	-2.1	0.058	1.5	1.0	-2.5	0.013	-1.17	
KIF13B	3.2	0.042	n.s.	---	2.7	0.128	-1.17	
AADAT	-2.4	0.026	n.s.	---	-2.8	0.007	-1.18	
CCDC101	-2.0	0.020	n.s.	---	-2.3	0.004	-1.18	
WDR76	-2.8	0.026	n.s.	---	-3.3	0.008	-1.19	
CLU	3.0	0.001	n.s.	---	2.5	0.003	-1.19	(b)
TNFRSF19	-3.2	0.019	1.4	1.0	-3.8	0.006	-1.19	
EGFR	3.2	0.024	n.s.	---	2.7	0.084	-1.19	
SF3B3	-1.8	0.145	n.s.	---	-2.2	0.021	-1.20	
PLBD1	-1.8	0.068	-1.8	0.070	-2.2	0.009	-1.20	
AFF1	2.1	0.005	-1.3	1.0	1.7	0.045	-1.20	
ABHD3	3.4	0.005	-1.4	1.0	2.8	0.018	-1.20	
ASF1A	-1.9	0.064	n.s.	---	-2.3	0.009	-1.20	
KSR2	-3.0	0.029	n.s.	---	-3.6	0.009	-1.20	
CDC42EP2	2.7	0.034	n.s.	---	2.2	0.161	-1.20	(b)
GPI	-1.8	0.291	n.s.	---	-2.1	0.037	-1.20	
NRM	-2.5	0.001	n.s.	---	-3.0	3.18E-04	-1.21	
DCAKD	-2.4	0.006	n.s.	---	-2.9	0.001	-1.21	
RAG1AP1	-2.3	0.155	-1.2	1.0	-2.8	0.034	-1.21	
MAZ	-2.4	0.128	1.3	1.0	-2.9	0.029	-1.21	
ZMYM3	-2.0	0.184	-1.5	1.0	-2.4	0.028	-1.21	
SLC16A9	-6.5	0.021	n.s.	---	-7.9	0.010	-1.21	
MLEC	-1.7	0.212	n.s.	---	-2.1	0.020	-1.22	
TUBA4A	-2.0	0.015	n.s.	---	-2.4	0.002	-1.22	
AFAP1	-2.3	0.001	n.s.	---	-2.8	2.63E-04	-1.22	
SLCO4A1	-2.9	0.088	n.s.	---	-3.5	0.024	-1.22	
TSPAN1	4.6	2.62E-06	-4.1	4.73E-06	3.7	8.23E-06	-1.23	
KIF2C	-2.6	0.112	n.s.	---	-3.2	0.026	-1.23	
UTP18	-1.7	0.014	1.2	1.0	-2.0	0.001	-1.23	
FRRS1	-2.8	0.152	n.s.	---	-3.4	0.038	-1.23	
MT1X	-1.8	0.077	n.s.	---	-2.2	0.007	-1.23	
SLC44A2	-1.9	0.390	-1.4	1.0	-2.3	0.047	-1.23	
MAGT1	-1.8	0.044	-1.2	1.0	-2.2	0.004	-1.23	
MANEAL	-3.3	0.001	n.s.	---	-4.0	3.90E-04	-1.24	
PCYOX1L	-2.8	0.011	n.s.	---	-3.4	0.003	-1.24	
UTP14A	-1.7	0.161	1.5	1.0	-2.1	0.012	-1.24	
LRRC37A4	-2.2	0.032	-1.2	1.0	-2.7	0.005	-1.24	
LGALS3BP	-2.4	0.165	-1.7	1.0	-2.9	0.031	-1.24	
PA2G4	-1.8	4.78E-05	n.s.	---	-2.2	3.94E-06	-1.24	
GNL3	-2.2	0.005	n.s.	---	-2.7	0.001	-1.24	
AIMP2	-2.0	0.128	n.s.	---	-2.5	0.016	-1.24	
CDK4	-2.2	0.022	n.s.	---	-2.7	0.003	-1.25	(a)
MRPL16	-2.3	0.275	n.s.	---	-2.8	0.046	-1.25	
IGF1R	5.9	1.15E-05	-1.3	1.0	4.7	3.32E-05	-1.25	(b)
PLCE1	-4.1	0.001	n.s.	---	-5.1	2.41E-04	-1.25	
PRKAR2A	-1.7	0.281	1.2	1.0	-2.2	0.021	-1.26	
ARHGAP17	3.0	1.66E-04	n.s.	---	2.4	0.001	-1.26	
GSN	3.7	0.008	-1.9	1.0	2.9	0.038	-1.26	(b)
CTTNBP2	-2.5	0.158	n.s.	---	-3.1	0.028	-1.27	
CD9	2.2	0.012	-1.2	1.0	1.7	0.187	-1.27	(b)
HGSNAT	2.2	0.016	-1.7	0.321	1.7	0.234	-1.27	
CLDN2	-1.9	0.108	1.6	1.0	-2.4	0.010	-1.27	(a)
HSD17B10	-1.7	0.475	n.s.	---	-2.2	0.031	-1.27	
CRAT	-2.3	2.24E-04	-1.5	0.068	-2.9	2.89E-05	-1.27	
SCARB1	-1.8	0.098	n.s.	---	-2.3	0.008	-1.27	
TNS3	-1.7	0.474	-1.5	1.0	-2.1	0.027	-1.27	
COQ9	-1.6	0.562	-1.2	1.0	-2.0	0.025	-1.28	
MID1IP1	-2.0	0.449	n.s.	---	-2.5	0.047	-1.28	
ATP1B1	-1.6	0.058	-1.7	0.033	-2.0	0.002	-1.28	
LPHN1	-2.8	0.007	n.s.	---	-3.6	0.001	-1.28	
MOBK2B	-2.4	0.234	n.s.	---	-3.0	0.036	-1.28	
APRT	-2.4	0.192	-1.3	1.0	-3.0	0.030	-1.28	

<i>ELL3</i>	-3.0	0.155	n.s.	---	-3.8	0.034	-1.28	
<i>ANP32B</i>	-2.1	0.026	n.s.	---	-2.7	0.003	-1.28	
<i>KLRG1</i>	-3.5	0.055	-2.3	1.0	-4.5	0.014	-1.28	
<i>LCP1</i>	-4.8	0.050	-2.1	1.0	-6.2	0.016	-1.28	
<i>JPH1</i>	-3.3	0.029	n.s.	---	-4.2	0.007	-1.29	
<i>RPL23AP32</i>	3.0	0.010	n.s.	---	2.3	0.074	-1.29	
<i>MYC</i>	-1.8	0.439	n.s.	---	-2.4	0.034	-1.29	(a)
<i>HNRNP</i>	-2.2	0.008	n.s.	---	-2.9	0.001	-1.29	
<i>RTKN</i>	-2.1	0.172	n.s.	---	-2.7	0.018	-1.29	
<i>SLC6A9</i>	-2.2	0.265	1.3	1.0	-2.8	0.032	-1.29	
<i>GYLTL1B</i>	-2.6	0.114	n.s.	---	-3.3	0.019	-1.29	
<i>TRAK1</i>	2.4	0.041	n.s.	---	1.8	0.557	-1.29	
<i>ERAP2</i>	-2.0	0.143	-1.6	1.0	-2.6	0.013	-1.29	
<i>SDC1</i>	-2.6	0.005	n.s.	---	-3.4	0.001	-1.30	
<i>TMEM97</i>	-2.4	0.299	n.s.	---	-3.1	0.043	-1.30	
<i>SPTLC3</i>	-3.1	0.003	1.3	1.0	-4.1	0.001	-1.30	
<i>PFN2</i>	-2.2	0.058	n.s.	---	-2.9	0.006	-1.31	(b)
<i>MTSS1L</i>	-2.7	0.004	n.s.	---	-3.5	0.001	-1.31	
<i>TMOD1</i>	20.1	4.95E-06	-1.2	1.0	15.3	1.05E-05	-1.31	
<i>DHCR24</i>	-1.7	0.053	n.s.	---	-2.2	0.002	-1.32	
<i>VDR</i>	-1.6	0.266	n.s.	---	-2.1	0.008	-1.32	
<i>SH3RF2</i>	-2.8	2.95E-04	n.s.	---	-3.6	4.40E-05	-1.32	
<i>FAM3B</i>	-4.3	0.019	-1.4	1.0	-5.7	0.005	-1.32	
<i>SSBP2</i>	-3.1	0.001	-1.6	0.921	-4.2	2.50E-04	-1.32	
<i>PBXIP1</i>	4.5	0.014	-1.7	1.0	3.4	0.066	-1.33	
<i>POLR1A</i>	-2.1	0.007	n.s.	---	-2.9	0.001	-1.33	
<i>OSBPL1A</i>	-2.1	0.320	-1.5	1.0	-2.8	0.028	-1.33	
<i>ALDH3A1</i>	3.0	1.90E-06	-1.5	0.008	2.3	2.11E-05	-1.33	
<i>SLC12A2</i>	-1.6	0.024	-1.4	0.722	-2.2	0.001	-1.34	
<i>NPM3</i>	-2.5	0.068	n.s.	---	-3.3	0.008	-1.34	
<i>TRIM14</i>	-3.5	0.047	n.s.	---	-4.7	0.009	-1.35	
<i>ST6GALNAC2</i>	3.1	0.023	-1.9	1.0	2.3	0.237	-1.35	
<i>HIST1H2BC</i>	4.1	4.73E-07	-1.7	0.001	3.0	3.27E-06	-1.35	
<i>TRAP1</i>	-2.5	0.027	1.2	1.0	-3.4	0.003	-1.35	
<i>FASN</i>	-1.8	0.058	n.s.	---	-2.4	0.002	-1.36	
<i>ATP2C2</i>	-1.8	0.267	n.s.	---	-2.5	0.012	-1.36	
<i>TMEM161A</i>	-2.3	0.292	n.s.	---	-3.1	0.027	-1.36	
<i>PDCD2L</i>	-1.9	0.732	n.s.	---	-2.5	0.036	-1.37	
<i>PLCG2</i>	12.3	0.003	n.s.	---	8.9	0.007	-1.38	(b)
<i>LPCAT1</i>	-1.7	1.0	-1.3	1.0	-2.3	0.049	-1.38	
<i>ACSM3</i>	-3.5	0.172	-2.0	1.0	-4.9	0.031	-1.38	
<i>TGFB1</i>	2.4	0.003	n.s.	---	1.7	0.105	-1.39	
<i>VSNL1</i>	-4.6	0.003	1.4	1.0	-6.4	0.001	-1.39	
<i>ACOT13</i>	-1.8	0.441	-1.4	1.0	-2.5	0.014	-1.39	
<i>ALPP</i>	9.4	0.002	n.s.	---	6.7	0.009	-1.41	
<i>THOC6</i>	-2.6	0.001	-1.3	1.0	-3.7	4.88E-05	-1.42	
<i>UGT8</i>	-4.1	3.68E-05	n.s.	---	-5.9	6.38E-06	-1.42	
<i>FBXO31</i>	-1.8	0.476	1.2	1.0	-2.6	0.015	-1.42	
<i>PTPRU</i>	-2.3	0.186	n.s.	---	-3.3	0.013	-1.43	
<i>TMEM92</i>	7.5	0.007	n.s.	---	5.2	0.030	-1.43	
<i>SLC25A12</i>	-3.8	0.084	n.s.	---	-5.5	0.014	-1.43	
<i>MYRIP</i>	-3.4	0.233	n.s.	---	-4.9	0.034	-1.43	
<i>KIT</i>	-5.1	4.41E-04	-2.1	0.160	-7.3	9.09E-05	-1.43	
<i>ABHD14A</i>	-1.6	0.440	n.s.	---	-2.2	0.005	-1.43	
<i>SLC25A10</i>	-2.5	0.147	n.s.	---	-3.7	0.012	-1.44	
<i>GLTP</i>	-1.4	1.0	-1.2	1.0	-2.1	0.039	-1.45	
<i>PEMT</i>	-1.4	1.0	n.s.	---	-2.0	0.007	-1.45	
<i>CDCA7</i>	-3.0	0.155	n.s.	---	-4.4	0.018	-1.45	(a)
<i>ASCL2</i>	-4.1	0.041	-1.5	1.0	-6.0	0.007	-1.45	(a)
<i>IDH2</i>	-3.4	0.172	-1.7	1.0	-4.9	0.022	-1.46	
<i>ZNF618</i>	-1.9	0.048	-1.2	1.0	-2.8	0.001	-1.46	
<i>PDCD11</i>	-2.1	0.192	1.3	1.0	-3.0	0.008	-1.47	(a)
<i>MYBBP1A</i>	-2.2	0.089	1.3	1.0	-3.2	0.004	-1.48	(a)

<i>ANP32A</i>	-2.3	0.067	n.s.	---	-3.4	0.003	-1.49	
<i>MPI</i>	-1.4	1.0	-1.1	1.0	-2.1	0.007	-1.50	
<i>SULT1C4</i>	-11.2	2.32E-04	-6.9	0.001	-16.8	6.88E-05	-1.50	
<i>DHCR7</i>	-1.7	0.055	1.3	1.0	-2.6	0.001	-1.50	
<i>DPP4</i>	3.3	0.017	-1.7	1.0	2.2	0.397	-1.51	
<i>HIST1H1C</i>	2.7	0.014	n.s.	---	1.8	0.816	-1.51	
<i>OAF</i>	-1.4	1.0	1.3	1.0	-2.1	0.035	-1.52	
<i>NME4</i>	-2.2	0.055	n.s.	---	-3.4	0.002	-1.53	
<i>CEACAM1</i>	4.9	0.001	-2.6	0.045	3.2	0.009	-1.54	
<i>TTYH2</i>	3.6	0.008	n.s.	---	2.3	0.203	-1.55	
<i>APEX1</i>	-2.2	0.046	n.s.	---	-3.4	0.002	-1.56	(a)(b)
<i>BCL11A</i>	-3.5	0.079	n.s.	---	-5.5	0.008	-1.56	
<i>DAPK1</i>	2.5	0.002	-2.1	0.009	1.6	0.292	-1.56	(b)
<i>TTC39A</i>	-3.1	0.043	-2.3	0.399	-4.8	0.003	-1.56	
<i>GTF2I</i>	-1.5	1.0	-1.2	1.0	-2.3	0.034	-1.57	(b)
<i>ALPPL2</i>	4.3	0.024	-1.7	1.0	2.8	0.369	-1.57	
<i>CREB3L2</i>	-1.4	1.0	-1.4	1.0	-2.2	0.027	-1.57	
<i>CBX6</i>	-1.6	1.0	n.s.	---	-2.5	0.009	-1.58	
<i>MRGPRX1</i>	11.3	1.70E-04	n.s.	---	7.2	0.001	-1.58	
<i>SEPT3</i>	-2.0	1.0	n.s.	---	-3.2	0.041	-1.59	
<i>CPA6</i>	-4.4	0.212	-2.5	1.0	-6.9	0.027	-1.59	
<i>ATP8B1</i>	-1.9	1.0	-1.5	1.0	-3.0	0.027	-1.60	
<i>ZNF827</i>	-3.0	0.012	-1.6	1.0	-4.8	0.001	-1.60	
<i>KIAA1147</i>	-2.3	0.006	-1.4	1.0	-3.7	1.66E-04	-1.61	
<i>OAS1</i>	-1.6	1.0	-1.7	1.0	-2.6	0.045	-1.63	
<i>EPN3</i>	-2.4	0.130	-1.6	1.0	-3.9	0.004	-1.63	
<i>DBP</i>	-3.0	0.070	-2.3	0.577	-5.0	0.004	-1.65	
<i>SCD</i>	-1.8	0.168	n.s.	---	-2.9	0.001	-1.66	(a)
<i>AK2</i>	-2.1	1.0	1.5	1.0	-3.6	0.024	-1.67	
<i>ECH1</i>	-1.5	1.0	-1.5	1.0	-2.5	0.003	-1.68	
<i>KDM1A</i>	-1.3	1.0	-1.3	1.0	-2.1	0.013	-1.68	
<i>TNFRSF9</i>	-5.5	0.003	-2.6	0.266	-9.3	4.22E-04	-1.68	
<i>NFIC</i>	-1.8	0.374	n.s.	---	-3.1	0.003	-1.68	
<i>C20orf54</i>	2.2	0.040	n.s.	---	1.3	1.0	-1.70	
<i>KRTAP5-6</i>	-3.5	0.205	-3.9	0.111	-6.0	0.014	-1.70	
<i>LRIG3</i>	-2.5	0.736	n.s.	---	-4.4	0.023	-1.74	
<i>AKAP1</i>	-1.9	0.099	n.s.	---	-3.3	0.001	-1.75	
<i>GK</i>	3.0	0.030	n.s.	---	1.7	1.0	-1.76	
<i>TSPAN7</i>	-2.1	1.0	-1.7	1.0	-3.8	0.049	-1.81	
<i>UBTF</i>	-1.2	1.0	-1.3	1.0	-2.2	0.048	-1.83	
<i>CXXC5</i>	2.1	0.017	-1.9	0.043	1.1	1.0	-1.87	
<i>REG4</i>	50.1	4.11E-05	-1.6	1.0	26.5	1.67E-04	-1.89	
<i>SEMA3F</i>	-2.0	1.0	-3.2	0.054	-4.0	0.014	-1.96	
<i>CDX2</i>	-1.2	1.0	-1.3	1.0	-2.4	0.006	-1.96	
<i>CD177</i>	12.9	1.90E-05	-2.8	0.022	6.5	2.20E-04	-1.97	
<i>HSD17B2</i>	19.3	5.64E-06	n.s.	---	9.5	4.93E-05	-2.03	
<i>ZNRF3</i>	-1.4	1.0	1.2	1.0	-2.9	4.43E-04	-2.03	(a)
<i>SCNN1A</i>	-1.3	1.0	-2.1	0.004	-2.6	0.001	-2.04	
<i>SLC6A20</i>	2.3	0.003	-1.4	1.0	1.1	1.0	-2.07	
<i>MYB</i>	-3.4	0.143	n.s.	---	-7.4	0.003	-2.21	(a)
<i>PPFIBP2</i>	-1.3	1.0	-1.5	1.0	-2.9	0.003	-2.23	
<i>ERN2</i>	-1.2	1.0	-2.7	0.008	-2.9	0.006	-2.37	
<i>PRR15L</i>	-2.3	0.288	-3.6	0.012	-5.6	0.001	-2.40	
<i>TRIM31</i>	-3.6	0.269	-6.2	0.020	-8.8	0.005	-2.41	
<i>PLLP</i>	-1.6	1.0	-2.0	1.0	-4.3	0.050	-2.73	
<i>TNFSF15</i>	4.1	1.30E-04	-1.5	1.0	1.5	1.0	-2.77	
<i>VIL1</i>	-3.2	0.013	-2.7	0.049	-8.9	9.45E-05	-2.78	
<i>MUC13</i>	-1.7	1.0	-4.7	0.001	-4.9	0.001	-2.85	
<i>C3</i>	3.8	0.014	-3.7	0.015	-1.2	1.0	-4.61	

Supplementary Table 2. High Stringency Gene List of Genes Regulated at 24 h Treatment. The results of the microarray analysis were cut-off at a significance level of Bonferroni adjusted *P*-value (asterisk) ≤ 0.05 (corresponding to a false discovery rate (FDR) *P*-value ≤ 0.00005) and a fold change (FC) of at least two-fold in at least one of the treatments. The fold change is displayed as value in treated cells vs. value in control cells (vehicle). n.s. = not significant in a two-tailed ANOVA analysis (*P*-value > 0.05). The list is sorted indicating first the highest fold change value for double treatment (4OHT+DOX) versus 4OHT. Known target genes were previously described: (a) gene found to be regulated by Δ N-TCF (van der Flier et al., 2007); (b) FoxO3a target gene described by Delpuech et al., 2007 and (c) summarized by Greer and Brunet, 2005.

Supplementary Table 3:

FOXO3a and/or beta-Catenin target genes involved in cytoskeleton reorganization, cell morphology and motility

Gene Symbol	HGNC Gene Name	4OHT FC	DOX FC	4OHT+DOX FC	FC 4OHT+DOX vs. 4OHT	Reference (PMID)
<i>IQGAP2</i>	IQ motif containing GTPase activating protein 2	9.5	2.1	27.9	2.95	17617398
<i>STARD13</i>	StAR-related lipid transfer domain containing 13	3.5	1.5	8.0	2.28	16217026
<i>MACF1</i>	microtubule-actin crosslinking factor 1	1.9	n.s.	3.0	1.60	14636561
<i>CD2AP</i>	CD2-associated protein	1.5	n.s.	2.3	1.55	15910750
<i>AVIL</i>	advillin	2.3	n.s.	3.5	1.51	9664034
<i>DOCK5</i>	dedicator of cytokinesis 5	2.0	1.4	2.8	1.45	19004829
<i>RALGPS2</i>	Ral GEF with PH domain and SH3 binding motif 2	5.6	1.2	8.0	1.43	17462626
<i>DAAM1</i>	dishevelled associated activator of morphogenesis 1	4.1	n.s.	5.8	1.40	16630611
<i>UTRN</i>	utrophin	1.9	n.s.	2.6	1.35	7589238
<i>SCIN</i>	scinderin	23.6	n.s.	31.3	1.32	8780652
<i>PDLIM5</i>	PDZ and LIM domain 5	2.2	1.5	2.7	1.26	20091751
<i>SWAP70</i>	SWAP switching B-cell complex 70kDa subunit	2.9	n.s.	3.5	1.21	11961559
<i>SRGAP2</i>	SLIT-ROBO Rho GTPase activating protein 2	2.0	n.s.	2.3	1.20	19737524
<i>DOCK8</i>	dedicator of cytokinesis 8	1.7	n.s.	2.1	1.19	15304341
<i>DOCK9</i>	dedicator of cytokinesis 9	1.8	n.s.	2.2	1.17	12172552
<i>ABLIM1</i>	actin binding LIM protein 1	1.9	n.s.	2.2	1.12	9245787
<i>CORO1A</i>	coronin, actin binding protein, 1A	2.4	-1.4	2.6	1.08	17442961
<i>DNMBP</i>	dynamin binding protein	1.5	-1.2	1.6	1.08	16757518
<i>ARHGEF12</i>	Rho guanine nucleotide exchange factor (GEF) 12	2.7	n.s.	2.9	1.06	12196628
<i>ARHGAP21</i>	Rho GTPase activating protein 21	2.7	n.s.	2.8	1.04	16184169
<i>RND1</i>	Rho family GTPase 1	3.1	n.s.	3.2	1.02	17639085
<i>GSN</i>	gelsolin	3.7	-1.9	2.9	-1.26	15526166
<i>MID1IP1</i>	MID1 interacting protein 1	-2.0	n.s.	-2.5	-1.28	15070402
<i>TMOD1</i>	tropomodulin 1	20.1	-1.2	15.3	-1.31	10741420

Supplementary Table 3. FOXO3a and/or beta-Catenin target genes involved in cytoskeleton reorganization, cell morphology and motility. Genes were extracted from the list of genes regulated at 24 h treatment (Supplementary Table 2). The official gene symbol and gene names from Human Gene Nomenclature Committee (HGNC; www.genenames.org) are provided next to fold change values (FC) from gene expression analysis displayed as value of treated cells versus value of control cells (vehicle). The Pubmed unique identifier (PMID; www.ncbi.nlm.nih.gov/pubmed) refers to the corresponding relevant literature.

Supplementary Table 4:**Metastasis-related Genes**

Gene Symbol	Reference (PMID)
<i>SLC2A3</i>	12842083, 18401196
<i>EDN1</i>	11719468, 9607532
<i>EMP1</i>	20980531, 17389037
<i>CYR61</i>	11059746, 20635370
<i>SPINK1</i>	19384300
<i>CTGF</i>	12842083
<i>CAV1</i>	19015640, 9717814
<i>HBEGF</i>	19421193
<i>ITGB8</i>	19723662
<i>RICTOR</i>	20978191
<i>CLDN1</i>	15965503
<i>MBNL2</i>	19996206
<i>CDX2</i>	18581275, 19724852
<i>MTSS1L</i>	17497115
<i>KIT</i>	9687504, 8957075

Supplementary Table 4. Genes Known to be Involved in Metastasis. The Pubmed unique identifier (PMID; www.ncbi.nlm.nih.gov/pubmed) relates the genes of the metastasis signature provided in Figure 4d and e to the literature.

Supplementary Table 5. Metastasis induced by API-2 and β -catenin

	METASTASIS [Mice (<i>P</i> value)]	DISSEMINATION SITE [Mice (<i>P</i> value)]			
		Liver	Lung	L.N.	Carc.
VEHICLE	2/9	0/9	0/9	1/9	2/9
API-2	2/8 (1)	0/8 (1)	1/8 (0.47)	1/8 (1)	2/8 (1)
DOX	1/9 (1)	0/9 (1)	1/9 (1)	1/9 (1)	0/9 (1)
API-2+DOX	7/8 (0.015)	2/8 (0.206)	3/8 (0.082)	2/8 (0.576)	5/8 (0.15)

Supplementary Table 5. Metastasis induced by API-2 and β -catenin. Incidence of metastasis in mice injected in the cecum with HT29F β cells and treated as indicated. L.N. = lymph node, Carc. = carcinomatosis, including any tumor mass growing in the abdominal cavity eventually affecting adjacent organs. Ratios indicate the numbers of animals with affected tissue per total number in each group of treatment. P values listed in brackets were calculated vs. vehicle by Fisher’s exact test. Only metastases detected by luminometry and confirmed by histological exploration were taken into account.

Supplementary Table 6: Mutational Status of Patient-derived Cells and Colon Cancer Cell Lines

(a) Tumor sample	Genes mutated	MMR-deficiency	microsatellite instability (MSI)
T49	KRAS ^{G13D}	n.d.	n.d.
T59	KRAS ^{G12C} , PIK3CA ^{E542K}	n.d.	n.d.
T61	APC ^{T1556fs*3} , KRAS ^{A146T}	MSH1, PMS2	BAT25, BAT26, NR24, MONO27
T68	KRAS ^{G12D}	n.d.	n.d.
T70	---	n.d.	n.d.
T86	APC ^{E1408*} , BRAF ^{K601E}	n.d.	n.d.
(b) Cell line	Known mutations	MMR-deficiency	microsatellite instability (MSI)
DLD1	APC (truncation) (1), KRAS ^{G13D} , PIK3CA ^{E545K} , TP53 ^{S241F}	MSH6 (2)	---
HT29	APC ^{E853*} (3), APC ^{T1556fs*3} , BRAF ^{V600E} , PIK3CA ^{P449T} (3), PIK3CA ^{Q546K} , SMAD4 ^{Q311*} (3), TP53 ^{R273H}	---	---
HCT116	CDKN2A ^{R24fs*20} (3), CDKN2a(p14) ^{E74fs*15} (3), CTNNB1 ^{S45del} (3), KRAS ^{G13D} , PIK3CA ^{H1047R}	MSH6 ^{P1087fs*5} , MLH1 ^{S252*} (3)	BAT25, BAT26 (3)

Supplementary Table 6. Mutational Status of Patient-derived Cells and Colon Cancer Cell Lines. (a) Genomic DNA from patients' tumor samples was submitted to mutational analysis using the OncoCarta, CLIA and VHIO Card panels (Supplementary Table 7) with a MassARRAY spectrometer (Sequenom) detecting the indicated mutated genes. Mismatch repair-deficiency (MMR) was deduced from immunohistochemical staining for MSH2, MLH1, MSH6/GTBP and PMS2. Analysis of microsatellite instability (MSI) was done by MSI-Analysis System (Promega). (b) The indicated cell lines harbour the listed mutations. Mutational data was obtained by Sequenom analysis mentioned as for (a), from the literature (PMID: Pubmed unique identifier; www.ncbi.nlm.nih.gov/pubmed; (1) 9065402, (2) 10919711) or from the Sanger Institute's COSMIC database (3) (www.sanger.ac.uk/cosmic). --- = any detected, n.d. = not determined.

Supplementary Table 7: OncoCarta, CLIA and VHIO Card Panels of Analysis of Somatic Mutations

Gene	ONCOCARTA							
	Mutation							
ABL1	G250E Y253F	F317L E255K	M351T E255V	E355G D276G	F359V F311L	H396R T315I	Q252H	Y253H
AKT1	rs11555435	rs11555431	rs11555432	rs12881616	rs11555433	rs11555436	rs34409589	
AKT2	S302G	R371H						
BRAF	G464R V600E G466R D594V G	F595L V600K F468C	G596R V600R G469S	L597S V600L G469E	L597R K601N G469A	L597Q K601E G469V	L597V G464V G469R	T599I G464E G469R
CDK	R24C	R24H						
EGFR	R108K N771_P772>SVDNR T790M E709G E746_A750del L747_S752del, P753S E746_T751del, I ins S752_I759del	S768I P772_H773insV L858R E709V E746_T751del L747_T751del E746_A750del, T751A	V769_D770insASV H773>NPY L861Q G719S E746_A750del A750P E746_T751del, V ins	V769_D770insCV H773_V774insNPH A289V G719C E746_T751del, S752D T751A E746_A750del, V ins	D770_N771>AGG H773_V774insPH G598V G719A L747_E749del T751P L747_E749del, A750P	V769_D770insASV H773_V774insH E709K M766_A767insAI E746_T751del L747_T750del T751I L747_T750del, P ins	V769_D770insASV V774_C775insHV E709H E746_T751del L747_S752del S752I/F L747_S752del, Q ins	D770_N771insG T263P E709A E746_A750del L747_T751del L747_Q ins T751
ERBB2	L755P	G776S	G776LC	G776VC	A775_G776insYVMA	P780_Y781insGSP	P780_Y781insGSP	S779_P780insVGS
FGFR1	S125L							
FGFR3	P252T	G370C	Y373C	A391E	K650Q	K650E	K650T	K650M
FLT3	I836del	D835H	D835Y					
HRAS	G12V Q61P	G12D Q61K	G13C	G13R	G13S	Q61H	Q61L	Q61R
JAK2	V617F							
KIT	D52N L576P V825A V559A	V559del P585P E839K V559G	V559_V560del D579del M552L V559I	V560del Y503_F504insAY Y568D V560D	P551_V555del K642E F584S V560G	Y553_Q556del D816V W557R K550_K558del	Y570_L576del D816H W557G K558_V560del	E561K D816Y V559D K558_E562del
KRAS	G12V G13D	G12A A59T	G12D Q61E	G12C Q61K	G12S Q61L	G12R Q61R	G12F Q61P	G13V Q61H
MET	R970C	T992I	Y1230C	Y1235D	M1250T			
NRAS	G12V G13D	G12A G13C	G12D G13R	G12C G13S	G12R A18T	G12S Q61L	G13V	G13A
PDGFRA	V561D D842_H845del	I843_S847>T I843_D846del	D842V S566_E571>K	T674I	F808L	D846Y	N870S	D1071N
PIK3CA	R88Q P539R	H1047Y E542K	R38H E545K	C901F Q546K	M1043I H701P	M1043I H1047R	N345K H1047L	C420R
RET	C634R	C634W	C634Y	E632_L633del	M918T	A664D		

VHIO CARD

Gene	Mutation							
<i>ABL1</i>	D276G L248V	E255K M244V	E255V M351T	E355G Q252H	F317L T315I	F359V Y253F	G250E Y253H	H396R
<i>AKT2</i>	R371H	S302G						
<i>APC</i>	E1286* E1397* Q1429* S1341R	E1306* E1408* Q1447* S1465fs*3	1309* E1464* R1114* T1537K	E1309fs*4 Q1291* R1450* T1556fs3	E1309fs6 Q1328* R213* Y472S	E1322* Q1367* R283* Y472S	E1353* Q1378* R302* Y472S	E1379* Q1406* R876* Y472S
<i>BRAF</i>	D587A G596R S605F/N	D587E I592M T599I	D594E I592V V471F	E586K K601del V600A/D/E/G/K/L/M/R	F595L L597Q/S/V/R Y472S	F595S L618W Y472S	G464E/R/V R444W Y472S	G469R/V/A/S R603* Y472S
<i>CDH1</i>	IVS7-1009-G1A	Y380_K440del						
<i>CDKN2A</i>	D108N/Y S43I	D84Y W15*	E61*	E69*	E88*	P48L	R58*	R80*
<i>CSF1R</i>	L301S/*	Y969C/F/H/*						
<i>CTNNB1</i>	A13T W25_D32del	A21T	A5_A80del	G34R	S33C/Y/F	T41I	T42fs*7	V22_G38del
<i>EGFR</i>	A289V E746_A750V G719A/D L747_E749del L858M S752Y	D761N/Y E746_S752A G724S L747_P753Q L861Q T790M	D770_N771insG E746_S752D G735S A750P N771_P772SVDNR V742A	D770_N771insN E746_S752V G810D L747_P753S P733L W731*	E709A E746_T751A G810S L747_R748FP P753S	E709K E746_T751del H773R L747_T751del R108K	E734K E746K K745R L747_T751S/P R677H	E746_A750del G598V L730F L833V S752_I759del
<i>ERa</i>	K303R							
<i>ERBB2</i>	D769H	G776S/C	G776V	L755P	L755S	V777L		
<i>FBXW7</i>	R278*	R465C	R465H	R505C	S582L			
<i>FGFR1</i>	P252T	S125L						
<i>FGFR2</i>	K310R	N549K	S252W	S372C				
<i>FGFR3</i>	A391E Y373C	G697C	K650M	K650Q/E	L794fs*23	R248C	S249C	S371C
<i>FLT3</i>	D835H/Y/V/E							
<i>GATA1</i>	Q17*	V74I						
<i>GNA1</i>	Q209L							
<i>GNAQ</i>	Q209	Q209P/L/R						
<i>HRAS</i>	G12C/R/S/D/V	G13C/R/S/D	Q61K/L/P/R/H					
<i>JAK1</i>	K142fs*26	L431fs*22						
<i>JAK3</i>	A572V	P132T	V722I					
<i>KIT</i>	D52N V654A	D816Y W557_K558del	K558_E562del W557G	L576P W557R/G	N822K Y503_F504insAY	T670I Y823D	V559I	V560G

KRAS	A59T	L19F	Q22K					
MAG	L202M							
MEK1	P124L							
MLH1	V384D							
MPL	W515A/K/L/R							
MSH6	P1087fs*5							
MYC	A59V	N101T	P57S	S77F				
NF2	Q212*	Q337*	Q400*	R196*	R262*	R466*		
NF3	Q410*							
NOTCH4	E1977Q							
NRAS	A18T							
PDGFRA	D842_D846E S566_E571K	D842_D846G S566_E571R	D842_D846N	D842_M844del	D842_S847E/A	D842I	I843_D846del	I843_S847T
PI3KR1	T576del							
PIK3CA	G118D	P539R						
PIK3R5	R28C							
PTEN	A151T N323fs*21 R233*	E299* Q171* R335*	G129R Q214* W274*	G165R R130* Y68H	I101T R130fs*4	K267fs*9 R130G	K6fs*4 R173C	L42R R173H
RB1	E137* R556*	E748* R579*	L199*	L660fs*2	R320*	R358*	R455*	R552*
RET	A883F	C634R	C634W	C634Y	D631_L633E	D631G	E632_L633del	E768D
RUNX1	D198G	D198N	R107C	R166*	R204Q			
SMAD4	D351H	G386R	Q245*	R361H				
SMARCB1	R158*	R40*	Y47*					
SRC	Q531*							
STK11	D194Y Q170*	E199K/* Q37*	E57fs*7 R87K	F264fs*22 W332*	G135R Y49D	G196V	L282fs*3	P281fs*6
TP53	A159V H179Q R196* V173M	A161T H179Y R213* V272M	C141Y L194R R248Q W146*	C176F/Y P151S R249S Y163C	C242S P152L R273C Y236C	E285K P278L R273H	E298* P300L R282W	G266E R158H S241C
VHL	E160K W117*	I151S	L85P	L89H	Q96*	R161*	S65L	S68*

CLIA PANEL

Gene	Mutation							
AKT1	E17K	G173R	K179M					
AKT2	E17K	G175R						
AKT3	E17K	G171R						
BRAF	D594G/V	G464E	G466_V/E	G466R	G469R	K601E/N	L597R_1790TG	V600A/D/E/G/K/L/M/R
EGFR	L858M							
GNAQ	Q209H/L/P/R/Y							
GNAS	Q227L/R	R201H						
IDH1	R132C/G/H/L/S/V							
IDH2	R172G/K/M/S/W							
KRAS	A146T/P/V	G12A/V/D/S	G13C/S/D	Q61E/H/K/L/P/R				
MET	H1112L/R/Y	H1124D	M1268T	N375S	N848S	R988C	T1010I	Y1248C/H
	Y1253D							
NRAS	G12R/S/C/V/A/D/P/N/Y	G13R/S/C/V/A/D/N/Y	Q61E/H/K/L/P/R					
PIK3CA	A1046V	C420R	E110K	E418K	E453K	E542K/Q/V/G	E545A/D/G/K/Q/V	F909L
	G1049R/S	H1047R/L/Y	K111N	M1043I/V	N345K	P539R	Q060K	Q546E/H/K/L/P/R
	R088Q	S405F	T1025A/S	Y1021C/H/H				
RET	M918T							

Supplementary Table 7. OncoCarta, CLIA and VHIO Card Panels of Analysis of Somatic Mutations of 740 tumor-related mutations in 57 genes. OncoCarta, Thomas et al., 2007; VHIO Card, Cancer Genomics Group, VHIO, Barcelona Spain; CLIA PANEL, Molecular Diagnostics Laboratory, MD Anderson Cancer Center, Houston, Tx, USA

Supplementary Table 8: Antibodies**Primary antibodies**

Protein	Clone	Provider	Host
α -TUBULIN	B-5-1-2	Sigma Aldrich	mouse
β -Catenin	14	BD Transduction Laboratories	mouse
β -TUBULIN	TUB2.1	Sigma Aldrich	mouse
CLAUDIN1	JAY.8	Invitrogen	rabbit
CYR61	H-78	Santa Cruz Biotechnology	rabbit
E-Cadherin	36	BD Transduction Laboratories	mouse
E-Cadherin	ECCD-2	Invitrogen	rat
E-Cadherin	HECD1	Abcam	mouse
FADD	A66-2	BD Transduction Laboratories	mouse
FLAG	M2	Sigma Aldrich	mouse
FOXO3A	#9467	Cell Signaling Technology	rabbit
GFP	B2	Santa Cruz Biotechnology	mouse
GFP	FL	Santa Cruz Biotechnology	rabbit
IQGAP2	BB9	Millipore	mouse
LAMININ A/C	N-18	Santa Cruz Biotechnology	goat
LEF1	C18A7	Cell Signaling Technology	rabbit
MLH1	G168-728	BD PharMingen Biosciences	mouse
MSH2	G219-1129	BD PharMingen Biosciences	mouse
MSH6/GTBP	44	BD PharMingen Biosciences	mouse
p21-WAF-1	Ab-11; CP74	Thermo Scientific	mouse
PARP1	#9542	Cell Signaling Technology	rabbit
PMS2	A16-4	BD PharMingen Biosciences	mouse
P-S6 Ribosomal Protein (Ser240/244)	#2215	Cell Signaling Technology	rabbit
SLUG	G-18	Santa Cruz Biotechnology	goat
SNAI1	---	Dr. K.-F. Becker (TU München, Germany)	rat
TRA-1-85-APC (human)	TRA-1-85	R&D Systems	mouse
ZEB1	E-20	Santa Cruz Biotechnology	goat

Secondary antibodies

Antibody	Provider
Alexa Fluor 488 goat anti-mouse IgG	Invitrogen
Alexa Fluor 488 goat anti-rabbit IgG	Invitrogen
Alexa Fluor 488 goat anti-rat IgG	Invitrogen
Alexa Fluor 680 goat anti-mouse IgG	Invitrogen
Alexa Fluor 555 goat anti-mouse IgG	Invitrogen
Donkey anti-goat IgG HRP conjugated	Santa Cruz
Goat anti-mouse IgG HRP conjugated	ICN Biomedicals
Goat anti-rabbit IgG HRP conjugated	ICN Biomedicals
Immuno Pure goat anti-rat IgG HRP conjugated	Pierce

Supplementary Table 8. Antibodies. All primary and secondary antibodies against the indicated proteins used in the present study are listed next to the corresponding clone number, provider and host species.

Supplementary Table 9a: qPCR Primer sequences

Oligo name	Oligo sequence (5' to 3')	Oligo name	Oligo sequence (5' to 3')
<i>AXIN2-fw</i>	AGTCAGCAGAGGGACAGGAA	<i>AXIN2-rev</i>	GTGGACACCTGCCAGTTTCT
<i>BCL2L11-fw</i>	AGCACCCATGAGTTGTGACAAATC	<i>BCL2L11-rev</i>	CGTTAAACTCGTCTCCAATACGC
<i>CAT-fw</i>	TTTCCAGGAAGATCCTGAC	<i>CAT-rev</i>	ACCTTGGTGAGATCGAATGG
<i>CAV1-fw</i>	GGCAGTTGTACCATGCATTA	<i>CAV1-rev</i>	ATTTTCCCAACAGCTTCAAA
<i>CDH1-fw</i>	AGAACGCATTGCCACATACACTC	<i>CDH1-rev</i>	CATTCTGATCGGTTACCGTGATC
<i>CDKN1A-fw</i>	ACCCTAGTTCTACCTCAGGC	<i>CDKN1A-rev</i>	AAGATCTACTCCCCATCAT
<i>CDKN1B-fw</i>	CTGCAACCGACGATTCTTCTACT	<i>CDKN1B-rev</i>	GGGCGTCTGCTCCACAGA
<i>CDX2-fw</i>	ACAGTCGCTACATCACCATC	<i>CDX2-rev</i>	GATTTTCTCTCCTTTGCTC
<i>CLDN1-fw</i>	CCTATGACCCAGTCAATGC	<i>CLDN1-rev</i>	TCCCAGAAGGCAGAGAGAAG
<i>CLEC2D-fw</i>	AGTGTGTGGAATGGTTGCTG	<i>CLEC2D-rev</i>	TGGCTCTTGATGGCAGTTAG
<i>CPA4-fw</i>	CCAGTATCACCTCCATCTT	<i>CPA4-rev</i>	CGTCTTCTCCATAATCGGT
<i>CTGF-fw</i>	GCTACCACATTTCTACCTAGAAATCAG	<i>CTGF-rev</i>	GACAGTCCGTCAAAACAGATTGTT
<i>CYR61-fw</i>	ATTGTAGAAAGGAAGCCTTGCTCAT	<i>CYR61-rev</i>	TCCAATCGTGGCTGCATTAG
<i>DAAM1-fw</i>	GATCAGCTCAATTCCATGGC	<i>DAAM1-rev</i>	GGTTTGTCTCCTCAGTGCTGT
<i>EDN1-fw</i>	TCCTGCTCTTCCCTGATGGA	<i>EDN1-rev</i>	TGCTCAGGAGTGTTGACCCA
<i>EMP1-fw</i>	TAAGGCCGGACGAGTTCATG	<i>EMP1-rev</i>	ACAGCAACCTCCACTTCCCTC
<i>FASLG-fw</i>	CACTTTGGGATTCTTCCAT	<i>FASGL-rev</i>	GTGAGTTGAGGAGCTACAGA
<i>HIPK3-fw</i>	CATTGGGATGTGTGATTGCAG	<i>HIPK3-rev</i>	AACAAGTGTCTCTCTGGCAA
<i>IQGAP2-fw</i>	AGAGGAGATGGATGAGAGGA	<i>IQGAP2-rev</i>	TCAGTGGTTGGTGGCAATTC
<i>ITGB8-fw</i>	CCCATCTGTGCATGTTATAATACC	<i>ITGB8-rev</i>	CATAAAATTAGCTTCGGCTCC
<i>KTN1-fw</i>	GGATCAGGAAGAAGAAAGC	<i>KTN1-rev</i>	GTTGCATGAATAAGGGGTTC
<i>LEF1-fw</i>	CGAAGAGGAAGGCGATTTAG	<i>LEF1-rev</i>	GTCTGGCCACCTCGTGTC
<i>MPP5-fw</i>	GCCAGTATGGAGGAGAACT	<i>MPP5-rev</i>	CGGCTAATGATGACAGAGTC
<i>MUC13-fw</i>	TTTGCCTGTGTTTAGAAGGG	<i>MUC13-rev</i>	GAATGTTTCTTCTCTGGGTC
<i>PPIA/CF-fw</i>	CTCCTTTGAGCTGTTTGACAG	<i>PPIA/CF-rev</i>	CACCACATGCTTGCCATCC
<i>SDHA-fw</i>	TGGGAACAAGAGGGCATCTG	<i>SDHA-rev</i>	CCACCACTGCATCAAATTCATG
<i>SLC2A3-fw</i>	CTCTTCGTCACCGCTTT	<i>SLC2A3-rev</i>	AGTCCATAAAGCAGCCA
<i>SNAI1-fw</i>	CACTATGCCGCGCTCTTTC	<i>SNAI1-rev</i>	GGTCGTAGGGCTGCTGGAA
<i>SOD2-fw</i>	AAGGGAGATGTTACAGCCAGATA	<i>SOD2-rev</i>	TCCAGAAAATGCTATGATTGATATGAC
<i>TNFSF10-fw</i>	AAAGAGGTCCTCAGAGAGTAGCAGC	<i>TNFSF10-rev</i>	GCTCAGGAATGAATGCCCACTC
<i>ULBP2-fw</i>	TCAGCTGGAGAATTACACAC	<i>ULBP2-rev</i>	CAAAGAGGAGGAAGATCTGC
<i>VIL1-fw</i>	ACACAGGTGGAGGTGCAGAAAT	<i>VIL1-rev</i>	GGTTGGTCGCTGTCCACTTC

Supplementary Table 9b: shRNAmir constructs

shRNAmir construct	catalogue #
pGIPZ-shNonSil-miR	RHS-4346
pGIPZ-shIQGAP2-miR-1	V2LHS_90989
pGIPZ-shIQGAP2-miR-2	V2LHS_90988
pGIPZ-shIQGAP2-miR-3	V2LHS_257272
pGIPZ-shIQGAP2-miR-4	V2LHS_90993
pGIPZ-shIQGAP2-miR-5	V2LHS_90991
pGIPZ-shIQGAP2-miR-6	V3LHS_364274
pGIPZ-shIQGAP2-miR-7	V3LHS_364277
pGIPZ-shIQGAP2-miR-8	V3LHS_364276

Supplementary Table 9. (a) qPCR Primer sequences. The sequences (5' to 3') of primers used to amplify the indicated gene products by quantitative PCR are listed. fw = forward; rev = reverse **(b) shRNAmir constructs.** Relation of nomenclature of shRNAmir constructs used in this work and Open Biosystem's catalogue numbers.

2. ARTICLE 2

Tankyrase inhibition blocks Wnt/ β -catenin pathway and reverts resistance to PI3K and AKT inhibitors in the treatment of colorectal cancer

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ABSTRACT

Purpose

Oncogenic mutations in the KRAS/PI3K/AKT pathway are one of the most frequent alterations in cancer. Although PI3K or AKT inhibitors show promising results in clinical trials, drug-resistance frequently emerges. We previously revealed Wnt/ β -catenin signaling hyper-activation as responsible for such resistance in colorectal cancer (CRC). Here we investigate Wnt-mediated resistance in patients treated with PI3K or AKT inhibitors in clinical trials and evaluate the efficacy of a new Wnt/tankyrase inhibitor, NVP-TNKS656, to overcome such resistance.

Experimental designs

CRC patient-derived sphere cultures and mouse tumor xenografts were treated with NVP-TNKS656, in combination with PI3K or AKT inhibitors.

We analyzed progression-free survival of patients treated with different PI3K/AKT/mTOR inhibitors in correlation with Wnt/ β -catenin pathway activation, oncogenic mutations, clinico-pathological traits and gene expression patterns in 40 CRC baseline tumors.

Results

Combination with NVP-TNKS656 promoted apoptosis in PI3K or AKT inhibitor-resistant cells with high nuclear β -catenin content. High FOXO3A activity conferred sensitivity to NVP-TNKS656 treatment.

13 out of 40 patients presented high nuclear β -catenin content and progressed earlier upon PI3K/AKT/mTOR inhibition. Nuclear β -catenin levels predicted drug-response whereas clinico-pathological traits, gene expression profiles or frequent mutations (*KRAS*, *TP53* or *PIK3CA*) did not.

Conclusions

High nuclear β -catenin content independently predicts resistance to PI3K and AKT inhibitors. Combined treatment with a Wnt/tankyrase inhibitor reduces nuclear β -catenin,

reverts such resistance and represses tumor growth. FOXO3A content and activity predicts response to Wnt/ β -catenin inhibition and together with β -catenin may be predictive biomarkers of drug-response providing a rationale to stratify CRC patients to be treated with PI3K/AKT/mTOR and Wnt/ β -catenin inhibitors.

Translational relevance

To date PI3K and AKT inhibitors are showing limited clinical benefit mostly due to unknown resistance mechanisms and the lack of predictive biomarkers of drug response. We demonstrate that Wnt inhibitors can overcome β -catenin-induced resistance to PI3K and AKT inhibitors in CRC tumors. We also provide the experimental evidence for a rational stratification of patients to be treated with PI3K/AKT and/or Wnt/ β -catenin pathway inhibitors using β -catenin and FOXO3A as predictive biomarkers of drug response. Such refined molecular selection of patients could represent a significant improvement in response to treatment and an important step forward in advancing CRC therapy.

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of death worldwide (1), mostly because conventional treatments or new target-directed drugs are ineffective in patients presenting late-stage metastatic disease (2). It is therefore crucial to unmask the molecular mechanisms responsible for such resistance and to provide new predictive biomarkers of drug-response that could improve the selection of patients sensitive to treatment.

Activating mutations in genes encoding constituents of the KRAS/PI3K/AKT signaling pathway can be considered one of the most frequent cancer-causing genetic alterations in solid tumors, including CRC (3). Thus, a new generation of drugs targeting PI3K or AKT activity is being tested in numerous clinical trials with promising results in some tumor types. Unfortunately, CRC patients show an enhanced resistance to these drugs (4-8).

Active AKT phosphorylates FOXO proteins promoting their sequestration in the cytoplasm and blocking their capacity to induce the expression of target genes coding for proteins involved in cell cycle arrest and apoptosis (9). Therefore, the efficacy of PI3K and AKT inhibitors can be mediated in part through nuclear relocalization of FOXO proteins and consequent induction of apoptosis. We previously described that Wnt/ β -catenin oncogenic

signaling confers resistance to FOXO3A-dependent apoptosis promoted by PI3K or AKT inhibitory drugs (10). Such resistance was driven by nuclear β -catenin that impaired the capacity of FOXO3A to execute its apoptotic program. Thus, we hypothesized that reducing nuclear β -catenin content by Wnt inhibitors would overcome the resistance to PI3K or AKT inhibitors and combined treatments could be beneficial for treating CRC patients.

Abnormal activation of the Wnt/ β -catenin pathway by mutations in *APC*, *CTNNB1*/ β -catenin or *AXIN2* is responsible for the initiation and progression of almost all colorectal cancers (11). These mutations reduce the capacity of the Wnt pathway destruction complex, formed by APC, AXIN and GSK3 β , to commit β -catenin to degradation. As a result, β -catenin accumulates in the nucleus, binds the TCF/LEF transcription factors and induces the expression of Wnt target genes that play key roles in tumour progression (12). We and others have previously shown that binding of β -catenin to different transcription factors enhances the expression of alternative sets of target genes (13). FOXO3A is one of these transcription factors, for which β -catenin acts as a transcriptional coactivator (14).

Since inappropriate activation of the Wnt/ β -catenin pathway was first linked to colon cancer three decades ago, there has been intense interest in developing effective inhibitors (15, 16). It has been described that tankyrases promote AXIN1/2 polyubiquitination and degradation through the proteasome (17). Recently, a new family of tankyrase inhibitors was shown to stabilize AXIN1/2, enhancing the activity of the destruction complex and reducing free β -catenin. These inhibitors are showing promising preclinical results as Wnt/ β -catenin inhibitory drugs for the treatment of Wnt-addicted tumors (18-20).

Here, we present evidence that high nuclear β -catenin content is associated to resistance to PI3K and AKT inhibitors in the context of clinical trials, whereas frequent mutations or clinicopathological traits implicated in CRC progression do not. We demonstrate that combining these drugs with NVP-TNKS656, a new therapeutic small molecule inhibitor of Wnt/tankyrase pathway that reduces nuclear β -catenin (21), overcomes such resistance and represses tumor growth in CRC patient-derived xenograft (PDX) models. We also identified FOXO3A as a determinant of response to Wnt/ β -catenin inhibitors and FOXO3A/ β -catenin target genes as better pharmacodynamic markers than the canonical TCF/ β -catenin targets.

Our data indicates that nuclear FOXO3A and β -catenin content and activity could be valuable predictive biomarkers of drug-response and we propose an experimental-based

rationale to better guide the molecular selection of CRC patients entering new clinical trials with PI3K/AKT and/or Wnt/ β -catenin pathway inhibitors.

MATERIAL AND METHODS

Patients in clinical trials

Patients were enrolled in clinical trials with PI3K/AKT/mTOR inhibitors carried out in the Vall d'Hebron University Hospital, Barcelona, Spain (Clinical trial identifiers: 14-MC-JWAA, NCT01115751; B2151001, NCT00940498; CBEZ235A2101, NCT00620594; BKM120X2101, NCT01723800; CBYL719X2101, NCT01219699; INK1117-001, NCT01449370; PAM4743g, NCT01090960; XL765-00, NCT00485719). Tumor response was assessed according to RECIST 1.0 or 1.1 (22, 23). We analyzed formalin-fixed paraffin-embedded (FFPE) tumor samples from CRC patients at baseline before entering clinical trials with PI3K/AKT/mTOR inhibitors.

Patient-derived cells

Written informed consent was signed by all patients. The project was approved by the Research Ethics Committee of the Vall d'Hebron University Hospital, Barcelona, Spain (Approval ID: PR(IR)79/2009). Patient-derived cells were obtained as previously described (24). Cells were injected subcutaneously in NOD-SCID mice or were seeded as sphere cultures.

Animals, xenotransplantation and treatments

Experiments were conducted following the European Union's animal care directive (2010/63/EU) and were approved by the Ethical Committee of Animal Experimentation of VHIR - Vall d'Hebron Institute of Research (ID: 40/08 CEEA and 47/08/10 CEEA). Xenografts were obtained as described in (24). API2 (1 mg/kg in PBS-2% DMSO; Tocris Bioscience) was administered by intraperitoneal injection three times per week, NVP-TNKS656 (100 mg/kg) was injected subcutaneously bi-daily.

Gene expression

Gene expression of 292 selected genes was profiled in baseline tumors of patients treated with PI3K/ATK/mTOR inhibitors using the nCounter platform from Nanostring Technologies. Differentially expressed genes were identified in tumors presenting high or low nuclear β -catenin content using Partek Genomics Suite Software. Lists were cut-off at fold change of 1.2 and a P value < 0.075 (two-tailed one way ANOVA test). For microarray analyses, we used a genome wide Human Gene 1.0 ST Array (Affimetrix). Data was acquired using the Affimetrix GeneChip/GeneTitan platforms. Genes were considered differentially expressed in NVP-TNKS656 versus vehicle treated tumors at 1.5 fold change and $P < 0.05$ using a two-tailed one way ANOVA test. Microarray data is deposited at ArrayExpress database (E-MTAB-2446). To perform RT-qPCR, RNA from endpoint tumor xenografts was used to synthesize cDNA using Superscript-III reverse transcriptase with oligo-dT and random hexamer primers (Life Technologies). A 7900HT qPCR System was used with Power SYBR-green (Applied Biosystems) and specific primer pairs. Relative gene expression was determined by the comparative C_T method (25) and significance was determined using an unpaired t test with Welch-correction. See details in Supplementary Methods.

Genotyping

Tumor samples from CRC patients in clinical trials with PI3K/AKT/mTOR inhibitors were genotyped by sequencing the amplified product of a multiplexed PCR reaction (Amplicon sequencing) as described in Supplementary Methods. Frequent mutations in 57 oncogenes and tumor suppressor genes were interrogated (Supplementary Table S1). Microsatellite instability was analyzed using the MSI-Analysis System (Promega). Purified patient-derived cells were genotyped straight after surgical resection of patients' tumors by Sequenom or Haloplex platforms. Genotyping by Sequenom (CLIA panel) was performed as previously described (10). Haloplex Target Enrichment System (Agilent Technologies) was used to capture the complete coding regions of 388 oncogenes and tumor suppressor genes (Supplementary Table S2).

Three PDX models were genotyped by Exome sequencing. Patients provided written informed consent for somatic and germline DNA analysis. Mutations were called with VarScan2 software, either using the mpileup2snp or somatic commands, depending on the availability of normal tissue (26). Non-tumoral tissue was not available for PDX-P2, thus, common SNPs were filtered according to the 1000 genome catalogue (27). SIFT

and Polyphen-2 helped predicting functionality of the identified mutations. Complete Exome sequencing data from PDX-P2, P5 and P30 is available at SRA database at NCBI (BioProject ID: PRJNA242531).

Immunohistochemistry and immunofluorescence

Samples from paraffin-embedded tissues were stained as described in (24) using the following antibodies (Supplementary Table S3). Nuclei were stained with Hoechst 33342 (5 µg/ml; Sigma-Aldrich). Pictures of the immunofluorescent signal were captured using a NIKON C2+ confocal microscope and analyzed with MBF ImageJ software using criteria previously described (28, 29).

β-catenin immunohistochemistry was done using the Dako Autostainer Plus Staining System. For visualization, EnVision™ FLEX detection system (DAKO, Carpinteria, CA) was used. Sections were counterstained with hematoxylin, dehydrated, cleared, and mounted for examination. β-catenin staining was evaluated by a pathologist as described in Supplementary Methods.

DLD1F and HT29F cells were seeded on glass cover-slips and treated for six hours with 4OHT 100 nm (4-hydroxytamoxifen, Sigma-Aldrich). Cells were fixed in 4% para-formaldehyde (PFA) and immunofluorescent staining was performed as described previously (10).

Apoptosis assays

Patient-derived cells were seeded in suspension as sphere cultures on low attachment multiwell dishes, whereas cell lines were seeded in adherent multiwell cell culture dishes. Cells were pre-treated with NVP-TNKS656 (100 nM, Novartis) or DMSO for 48 h and then with API2 (20 µM, Tocris Bioscience) and/or NVP-BKM120 (2.4 µM, Selleck Chemicals) for another 48 h prior apoptosis analysis. Proportions of apoptotic cells were determined using the Annexin V-eGFP (BioVision) kit. Dead cells were detected as DAPI positive (1 µg/ml, Roche). Cells were analyzed by flow cytometry using a Navios Flow Cytometer (Beckman Coulter).

To measure apoptosis by immunofluorescence in sphere cultures, cells suspended in culture media were mixed 1:1 with Matrigel (BD Biosciences), fixed for 1 h in 4% PFA, permeabilized with PBS/1% Triton X-100 at room temperature for 3 h and blocked over

night at 4°C in PBS/1% Triton X-100/3% BSA. Samples were incubated for 24 h with primary antibodies (Supplementary Table S3). Secondary antibodies and Hoechst 33342 (5 µg/ml) were incubated over night at room temperature.

Cell Culture

Cell lines were cultured under standard conditions. DLD1F cells are DLD1 derivatives expressing pcDNA-FOXO3A(3A)ERTM (30). HT29F cells express pLHCX-HA-FOXO3A(3A):ERTM. All parental cell lines were originally obtained from American Type Culture Collection (ATCC). Cell lines were authenticated by short-tandem repeat analysis by the cell bank.

Western blot

The detailed protocol for protein extraction is described in Supplementary Methods. Western blot was performed as described in (10) using specific antibodies (Supplementary Table S3).

TCF/LEF1 reporter assays

DLD1 cell line was stably transfected with a vector (7TGP, obtained at Addgene) expressing eGFP controlled by a promoter containing seven TCF/LEF transcription factor binding sites (7xTOP) (31). Cells were treated with NVP-TNKS656 100 nM (Novartis) for seven days and eGFP accumulation was measured by flow cytometry using a Navios Flow Cytometer (Beckman Coulter).

Statistics

We analyzed progression free survival of patients by the Kaplan-Meier method and compared the curves using a Log-rank (Mantel-Cox) test. We used Pearson's correlation test to compare time on previous line of treatment versus time on treatment with PI3K/AKT/mTOR inhibitors of patients analyzed for nuclear β-catenin content and to correlate apoptosis versus nuclear β-catenin or FOXO3A content in sphere cell cultures treated with NVP-TNKS656, API2 and NVP-BKM120. Pearson's correlation test was also

used to compare nuclear FOXO3A content in patients' and corresponding PDX tumors, or in primary tumors versus liver metastases or to compare with *SLC2A3* mRNA levels.

Differences in apoptosis of treated sphere cell cultures; levels of AXIN1, β -catenin, phosphoS6, Ki67 and cleaved-CASPASE3 expression in tumor xenografts; mRNA expression of NVP-TNKS656 target genes by qRT-PCR; and apoptosis of DLD1F or HT29F cell lines, were analyzed by an unpaired *t* test comparing the means of two groups of values. Fisher's exact test served to analyze the differences in response among patients regarding nuclear β -catenin content, mutations affecting *PIK3CA*, *KRAS*, *TP53*, *APC* or tumor histological TNM status. *P* values lower than 0.05 were considered significant in all tests.

RESULTS

Effective pharmacological inhibition of Wnt/ β -catenin and PI3K/AKT pathways reduces tumor growth

We hypothesized that reducing nuclear β -catenin content could be sufficient to sensitize CRC tumors to the treatment with PI3K or AKT inhibitors. Consequently, we blocked the Wnt/ β -catenin signaling using the tankyrase inhibitor NVP-TNKS656 (21) in CRC PDX models. We selected five PDX models with high (P2, P7, P19, P22 and P30) and five with low nuclear β -catenin content (P5, P6, P31, P33 and P34) (Fig. 1A and B). Sphere cell cultures derived from xenograft tumors of each model were treated with API2 or NVP-BKM120, inhibiting AKT or PI3K activity respectively, alone or in combination with NVP-TNKS656 (Fig. 1C and Supplementary Table S4). API2 or NVP-BKM120 induced significantly less apoptosis in cells with high rather than low nuclear β -catenin content by measuring the proportion of Annexin V-positive cells. Combination with NVP-TNKS656 significantly increases apoptosis in cells with high as opposed to low nuclear β -catenin content. Similar results were observed by using NVP-XAV939, another inhibitor of tankyrase activity (Supplementary Fig. S1). Alternative measurement of apoptosis by cleaved-CASPASE3 showed equivalent results (Supplementary Fig. S2). Furthermore, apoptosis induced by API2 or NVP-BKM120 treatment showed a significant inverse correlation with nuclear β -catenin content (Fig. 1D). Such correlation was lost when API2 or NVP-BKM120 was combined with NVP-TNKS656. Mutations in *PIK3CA*, *KRAS* or *TP53* genes or tumor site did not condition a differential response of sphere cell cultures to PI3K or AKT inhibition (Supplementary Fig. S3). Only one out of the 10 PDX models

FIGURE 1

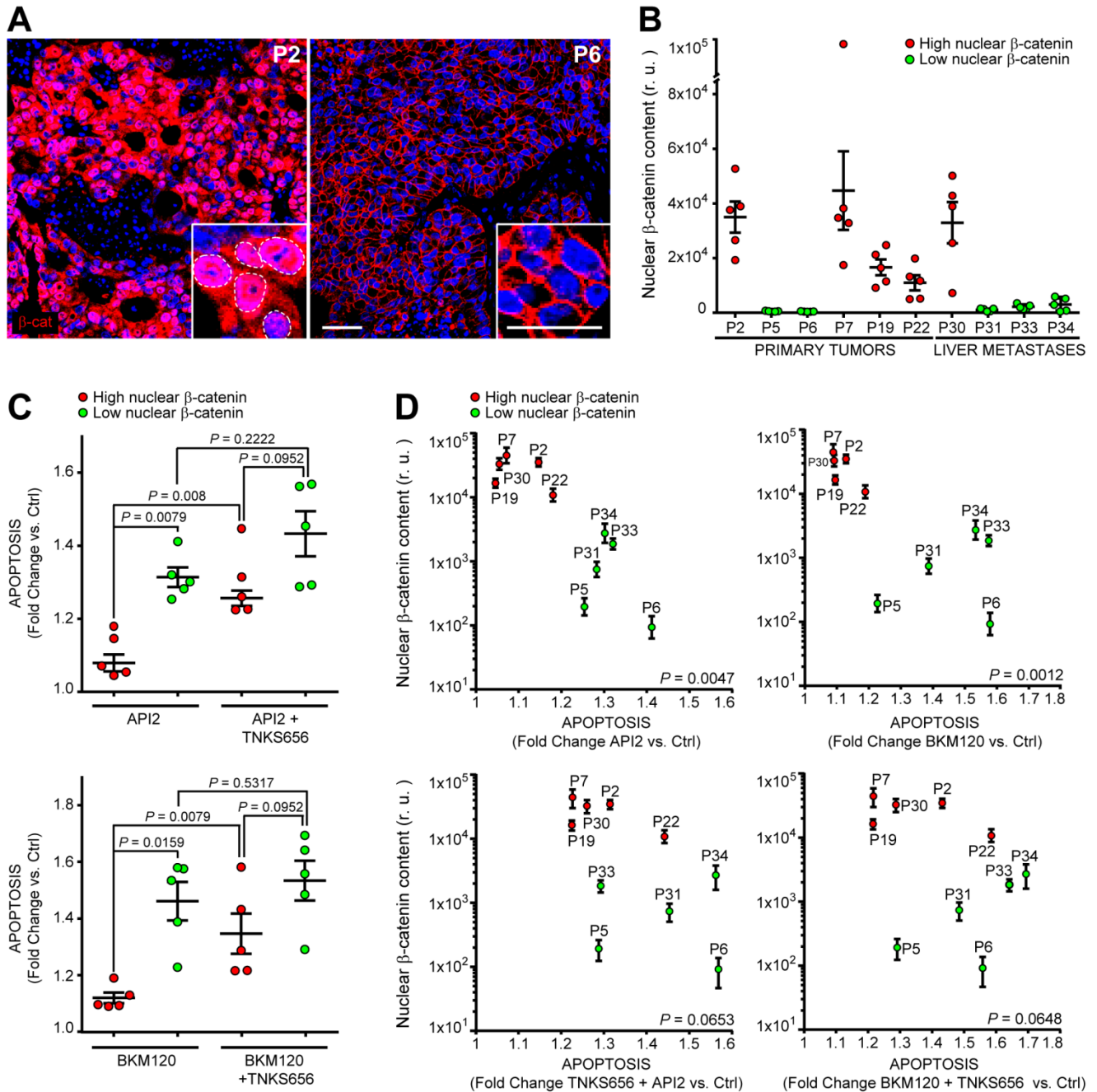


Figure. 1. CRC patient-derived cells with high amounts of nuclear β -catenin present high sensitivity to API-2 or NVP-BKM120 in combination with NVP-TNKS656. (A) Representative pictures of immunofluorescence and confocal microscopy of histological sections of the indicated PDX models with high (left) or low (right) nuclear β -catenin content. Inserts show magnification to better visualize β -catenin sub-cellular localization. Dashed lines delineate nuclei. Nuclei were stained with Hoechst 33342 (blue). Scale bars, 100 μ m; magnifications 50 μ m. **(B)** Column scatter plot showing the amount of nuclear β -catenin measured by immunofluorescence and confocal microscopy in 10 primary tumors and liver metastases from which sphere cell cultures were derived and used to test drug-response. Horizontal lines indicate arithmetic mean values, and error bars show standard deviation. **(C)** Column scatter plot showing the apoptosis induced in sphere cell cultures of these 10 patient-derived models treated as indicated. Data are represented as fold change of apoptotic cells induced by the treatment compared to cells treated with vehicle. Horizontal lines indicate arithmetic mean values, and error bars show Standard Error of the Mean (SEM). *P* values correspond to unpaired *t* tests. The original percentage of Annexin V positive cells is shown in Supplementary Table S4 **(D)** Scatter plots representing the apoptosis induced by API-2 (left upper panel) and NVP-BKM120 (right upper panel) or API-2 + NVP-TNKS656 (left lower panel) and NVP-BKM120 + NVP-TNKS656 (right lower panel) in sphere cell cultures of these 10 patient-derived models versus the histological amount of nuclear β -catenin in the original patient's tumors. Data are represented as fold change of apoptotic cells induced by the treatment compared to cells treated with vehicle. *P* values correspond to Pearson's correlation test. **(B, D)** β -catenin relative units (r. u.) were calculated as described in Methods.

presented microsatellite instability (MSI), preventing the possibility to evaluate its impact on drug response (Supplementary Table S5).

Our data indicates that nuclear β -catenin content conditions drug-response in patient-derived sphere cell cultures whereas frequent mutations in CRC do not (Fig. 1C and D, Supplementary Fig. S3, Supplementary Table S5).

We further investigated these results *in vivo*. Cells from three PDX models with known nuclear β -catenin and FOXO3A status and with limited response to AKT or PI3K inhibition *in vitro* (P2, P5 and P30), were injected subcutaneously into NOD-SCID mice (Fig. 1B, D and 2A). Treatment with NVP-TNKS656 caused a systemic reduction of nuclear β -catenin content and function in skin and intestine, tissues where Wnt pathway tightly controls homeostasis, but showed no major negative side effects (Supplementary Fig. S4).

API2 alone or in combination with NVP-TNKS656 did not repress tumor growth in PDX-P5, with low basal nuclear β -catenin and FOXO3A content, probably representing a case of a CRC tumor resistant to AKT inhibition due to mechanisms independent of nuclear β -catenin accumulation (Fig. 2A and B). Exome sequencing revealed a mutation in the *AKT2* gene that could explain the lack of reduction of phosphor-S6 or tumor growth upon API2 treatment in this model (Supplementary Fig. S5 and Supplementary Table S6).

PDX-P2 presented high nuclear β -catenin and low FOXO3A amounts, was resistant to API2 alone, and yet tumor growth rate was reduced upon combination with NVP-TNKS656 (Fig. 2A and B). Tankyrase inhibition significantly reduced nuclear β -catenin, API2 decreased phosphor-S6 content, and both diminished proliferation (Fig. 2C, Supplementary Fig. S5).

PDX-P30, derived from a liver metastasis, presented high amounts of both nuclear β -catenin and FOXO3A (Fig. 2A). It was also resistant to API2, but NVP-TNKS656 treatment alone reduced tumor growth rate equally to the drug combination (Fig. 2B). Tankyrase but not AKT inhibition promoted apoptosis, whereas the number of proliferative cells was not affected (Supplementary Fig. S5). This was the only model where NVP-TNKS656 alone showed an effect on tumor growth, probably due to high endogenous amounts of FOXO3A that might have induced apoptosis when nuclear β -catenin was reduced by NVP-TNKS656. NVP-TNKS656 increased AXIN1 protein levels in all subcutaneous tumors confirming its activity as a Wnt/tankyrase pathway inhibitor (21) (Fig. 2D).

FIGURE 2

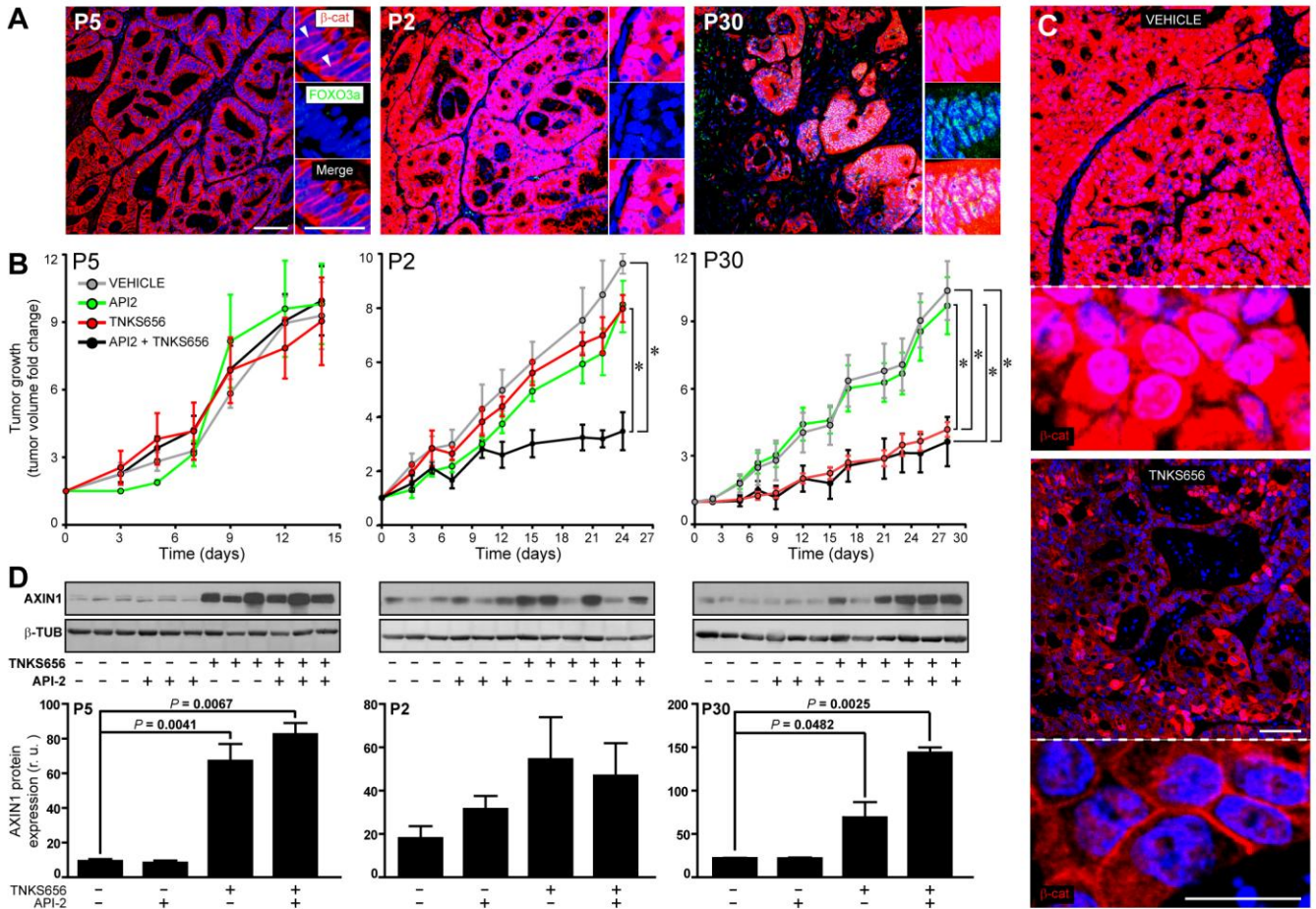


Figure 2. NVP-TNKS656 stabilizes AXIN1 and reduces both, nuclear β -catenin and tumor growth alone or in combination with the AKT inhibitor API-2 in CRC PDX models. (A) Representative pictures of double immunofluorescent staining and confocal microscopy to detect β -catenin (red) and FOXO3A (green) in histological sections of subcutaneous xenografted tumors from patients P2, P5 and P30. Right panels show magnifications to visualize the amounts of nuclear β -catenin and FOXO3A. Scale bar, 100 μ m; magnifications, 20 μ m. Nuclei were stained with Hoechst 33342 (blue). Arrowheads point β -catenin localized exclusively in cell membranes in tumors from P5 model. (B) Tumor cells derived from the three indicated patients were injected subcutaneously in NOD-SCID mice and treated as indicated. A minimum of 5 mice with tumors in both flanks was treated in each group. The graphs represent the fold change calculated by comparing the tumor volume at each given time point to the volume at the first day of treatment. Error bars and $\pm$ S.D. are shown for tumor volume fold change of all tumors. Unpaired t tests were used to compare the area under the curve generated for each growing tumor along the experiment and grouped by treatment. Asterisks indicate significant differences ($P < 0.05$). (C) Representative pictures showing immunofluorescent staining of β -catenin in tumor xenografts from PDX-P2 model growing in mice treated with vehicle or NVP-TNKS656. Inserts show magnifications to better visualize β -catenin reduction from cytoplasm and nuclei upon tankyrase inhibition. Scale bars, 100 μ m; inserts 50 μ m. (D) Three xenograft tumors per group of treated mice were processed for the analysis of AXIN1 by western blot (upper panels). β -tubulin was used as loading control. Blots were quantified using ImageJ software (lower panels). Protein expression levels are represented as relative units (r. u.) versus vehicle treated samples. Bars indicate standard deviation. P values correspond to unpaired t tests.

FOXO3A/ β -catenin target genes are pharmacodynamic markers of response to Wnt/tankyrase inhibitory drugs

Although NVP-TNKS656 reduced the high nuclear β -catenin content observed in both PDX-P2 and PDX-P30, treatment only reduced tumor growth rate in the latter model (Fig. 2 and Supplementary Fig. S5B). Contrarily, NVP-TNKS656 did not affect tumor growth in PDX-P5 model. We studied whether the high FOXO3A content observed in the metastatic PDX-P30 model (Fig. 2A) could determine the repression by NVP-TNKS656 of a distinct set of Wnt/ β -catenin target genes and its enhanced sensitivity to treatment. RNA from tumors of PDX-P2, PDX-P30 and PDX-P5 models was analyzed at the end point of the *in vivo* experiments (Fig. 2B). The three models showed a distinct gene expression pattern that was modified by NVP-TNKS656 treatment (Fig. 3A, Supplementary Tables S7, S8 and S9). The prometastatic *S100A4* gene was repressed in both PDX-P2 and PDX-P30 models (Fig. 3B). Four NVP-TNKS656-repressed genes in PDX-P2, two in PDX-P5 and yet none in PDX-P30, were direct TCF/ β -catenin targets (Supplementary Table S10). Instead, two genes in PDX-P2, one in PDX-P5 and nine in PDX-P30 were FOXO3A/ β -catenin targets (Supplementary Table S11), many of them formally associated with metastasis (10, 32-34). The regulation of some of these NVP-TNKS656-repressed genes was further confirmed by qRT-PCR in the same tumor xenograft samples (Fig. 3C). Any gene evaluated in PDX-P5 model was significantly regulated by NVP-TNKS656, a result in line with its low nuclear β -catenin content and its lack of tumor growth response (Fig. 2B). Our data suggests that FOXO3A/ β -catenin targets could be better pharmacodynamic markers than TCF/ β -catenin target genes for evaluating therapeutic Wnt/tankyrase pathway inhibition.

FOXO3A determines the response to Wnt/ β -catenin pathway inhibitors

NVP-TNKS656 treatment was particularly effective in a tumor with high endogenous nuclear β -catenin and FOXO3A content, promoting apoptosis, reducing tumor growth rate (Fig. 2) and preferentially repressing the expression of FOXO3A instead of TCF target genes (Fig. 3). The repression of Wnt/ β -catenin pathway by NVP-TNKS656 alone did not promote apoptosis in DLD1 or HT29 colon cancer cells but enhanced the apoptosis induced by exogenous nuclear FOXO3A-ER (Fig. 4). This data confirms the capacity of nuclear β -catenin to confer resistance to FOXO3A-induced apoptosis (10) and, therefore, the therapeutic value of reducing nuclear β -catenin by tankyrase inhibitors in FOXO3A-active cancer cells.

FIGURE 3

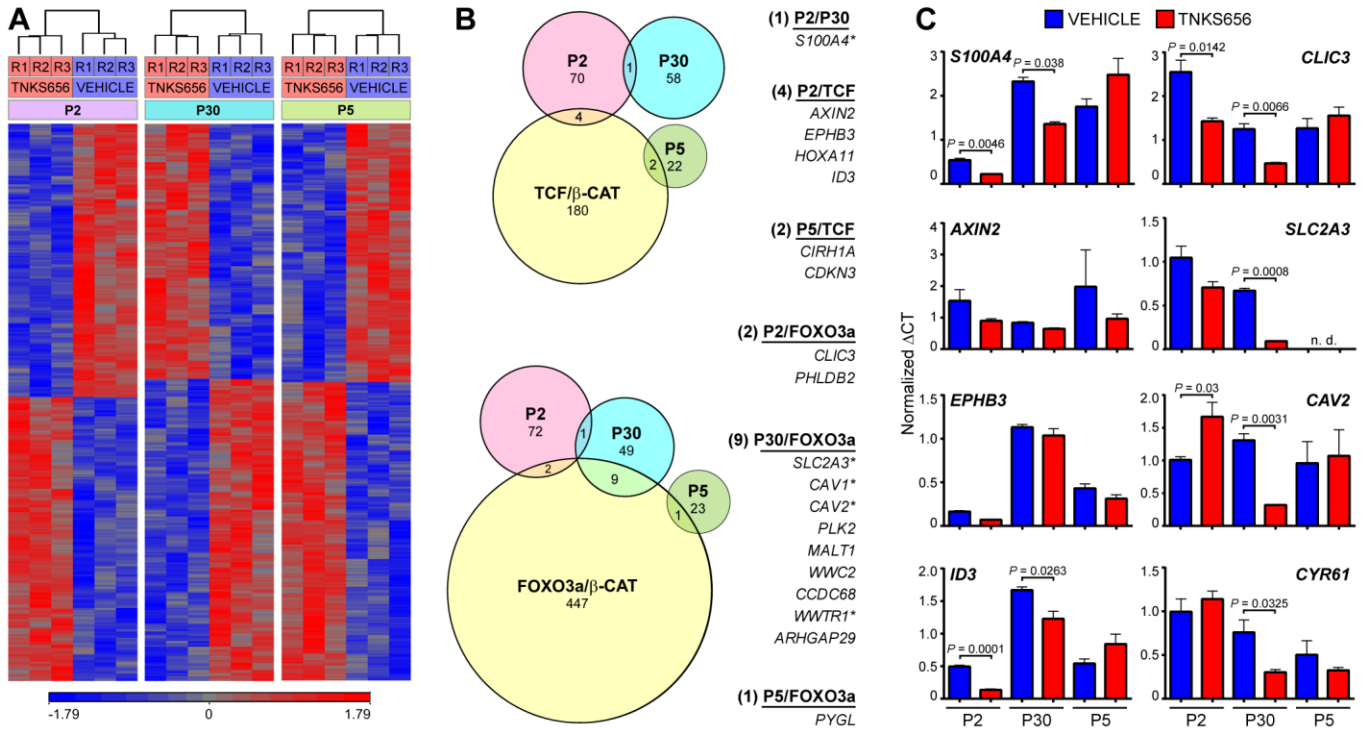


Figure. 3. Reduction of nuclear β -catenin content by NVP-TNKS656 regulates gene expression in CRC PDX models. (A) Three tumor xenografts from mice injected with PDX-P2, PDX-P30 or PDX-P5 treated with vehicle or NVP-TNKS656 were analyzed for gene expression at the end point of the experiments. Gene clustering diagrams shown were calculated using robust-multiarray-normalized expression values from genome-wide microarray analysis. Triplicates of each treatment are indicated as colored rectangles (R1, R2 and R3). Significance was calculated using two-tailed ANOVA with a significance cutoff of $P \leq 0.05$. Gene expression scaling was indicated from low (blue) to high (red). **(B)** Venn diagrams showing the number of genes down-regulated by NVP-TNKS656 in all PDX models and genes that belong to the TCF/ β -catenin (44) or FOXO3A/ β -catenin (10) transcriptional programs. Common genes are indicated on the right and enumerated in brackets. Asterisks indicate genes involved in metastasis **(C)** Relative mRNA expression levels of selected genes was confirmed by quantitative RT-PCR in the same xenograft tumors from the three PDX models treated with vehicle (blue bars) or NVP-TNKS656 (red bars). Expression values are shown as normalized Δ CT. Error bars $\pm$ S.D. of triplicate values obtained in three independent measurements are shown. P values correspond to unpaired t tests.

FIGURE 4

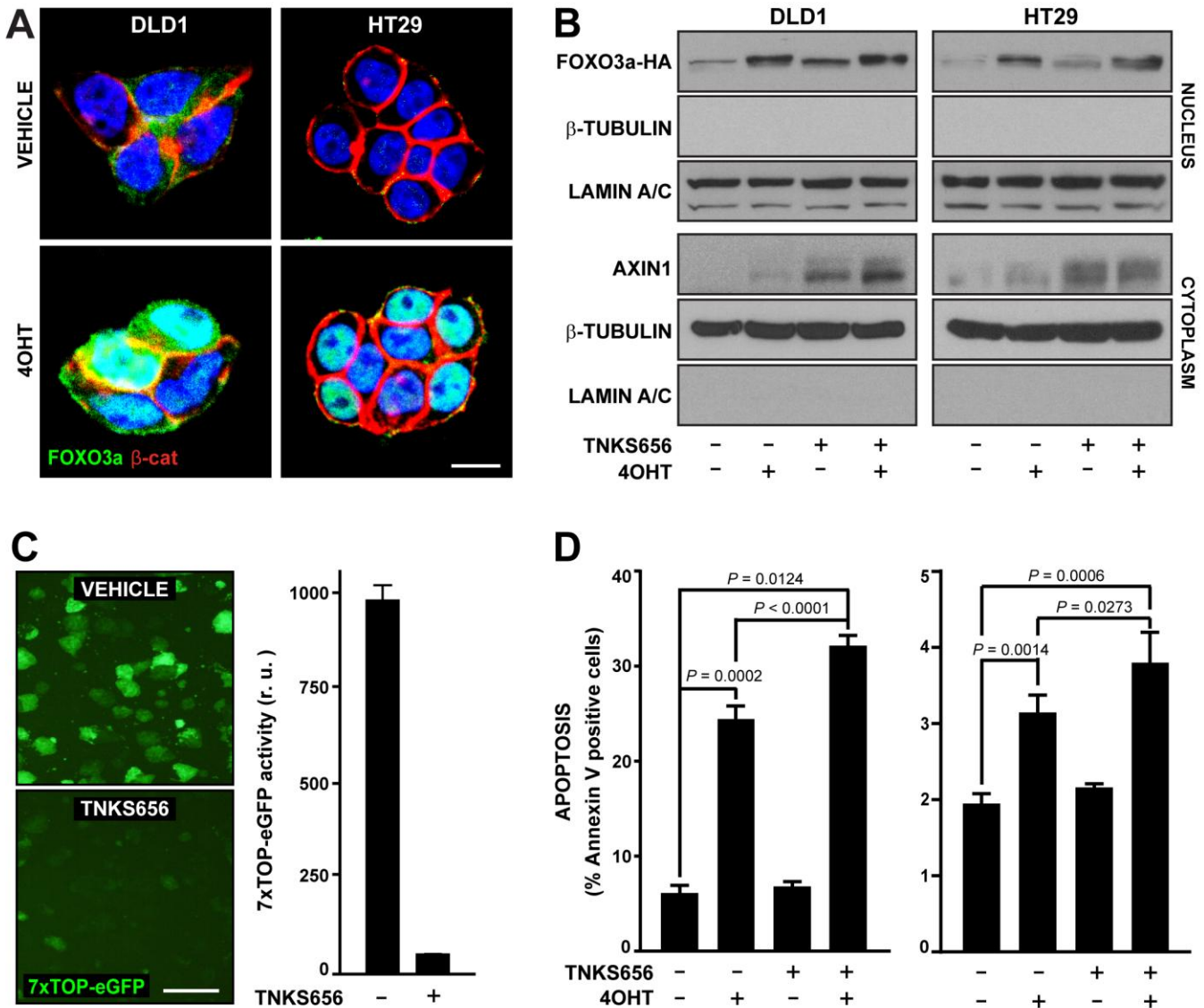


Figure. 4. NVP-TNKS656 sensitizes CRC cell lines to the apoptosis induced by FOXO3A. (A) Immunofluorescence and confocal microscopy to detect the translocation of exogenous FOXO3A(A3):ERTM from cytoplasm to nucleus of DLD1F and HT29F colon cancer cells upon tamoxifen (4OHT) treatment. Scale bar, 10 mm. (B) Western blots showing the amount of exogenous FOXO3A(A3):ERTM in the nucleus and stabilization of AXIN1 in the cytoplasm of DLD1F and HT29F cells upon indicated treatments. The amounts of β -TUBULIN and LAMIN A/C were evaluated to confirm the purity of nuclear and cytoplasmic extracts and as loading control. (C) Left panel: Representative pictures of DLD1F cells expressing fluorescent green protein (eGFP) under the control of seven upstream TCF/ β -catenin binding sites (7xTOP-eGFP) upon 5 days of NVP-TNKS656 treatment. Scale bar, 200 mm. Right panel: 7xTOP-eGFP activity after treating DLD1F cells with vehicle or NVP-TNKS656 was assessed by flow cytometry. Bars indicate S.D. of experimental triplicates. (D) Apoptosis was measured in DLD1F and HT29F cells upon indicated treatments. Data are represented as fold change of apoptotic cells induced by the treatment compared to cells treated with vehicle. Bars show $\pm$ S.D. of five replicates in six independent experiments. *P* values correspond to unpaired t tests.

Furthermore, NVP-TNKS656 promoted apoptosis in patient-derived sphere cell cultures proportionally to the amount of nuclear FOXO3A detected in their correspondent patients' tumors (Fig. 5A and B). Apoptosis was also proportional to FOXO3A content when sphere cultures were treated with a different tankyrase inhibitor, NVP-XAV939 (Supplementary Fig. S1). Similarly to β -catenin (10), patient-derived xenografts models and primary sphere cultures faithfully preserved the levels of nuclear FOXO3A detected in their correspondent original patient samples (Supplementary Fig. S6). We also observed that nuclear FOXO3A content positively correlated with the expression of *SLC2A3* mRNA, a FOXO3A/ β -catenin target gene (10) that was repressed upon NVP-TNKS656 treatment *in vivo* (Fig. 3).

Interestingly, we observed that paired primary tumors and liver metastases accumulated similar nuclear FOXO3A amounts, showing that its activation could be durable and occur prior progression to metastatic stages (Supplementary Fig. S7).

Lastly, by profiling 130 CRC cases, we identified a distinctive population of patients with tumors presenting high expression of FOXO3A/ β -catenin target genes (Fig. 5C). The expression of TCF/ β -catenin target genes also identified such a population but showed lower signal. Interestingly, FOXO3A/ β -catenin and TCF/ β -catenin target genes clustered separately among all cases profiled. Samples were also evaluated for their microsatellite instability and mutational status of *KRAS*, *BRAF* and *PIK3CA*, all molecular features relevant for CRC tumors. Any obvious correlation was observed between them and gene expression signatures distinctive of active FOXO3A/ β -catenin transcription (Fig. 5C and Supplementary Table S12).

These data suggest that patients with tumors that are active for FOXO3A/ β -catenin transcription can be identified by transcriptional profiling, whereby reduction of nuclear β -catenin by Wnt inhibitors could promote FOXO3A-dependent apoptosis.

Comparative analysis of β -catenin as potential biomarker of resistance to PI3K and AKT inhibitory drugs

We decided to compare the potency of nuclear β -catenin in predicting resistance to PI3K or AKT inhibitors with the most frequent mutations or histological traits observed in CRC tumors. Limited availability of samples from clinical trials only permitted the study of tumors at baseline from a cohort of 40 CRC patients treated with PI3K/AKT/mTOR pathway inhibitors in several phase I clinical trials (Fig. 6A, upper panel and

FIGURE 5

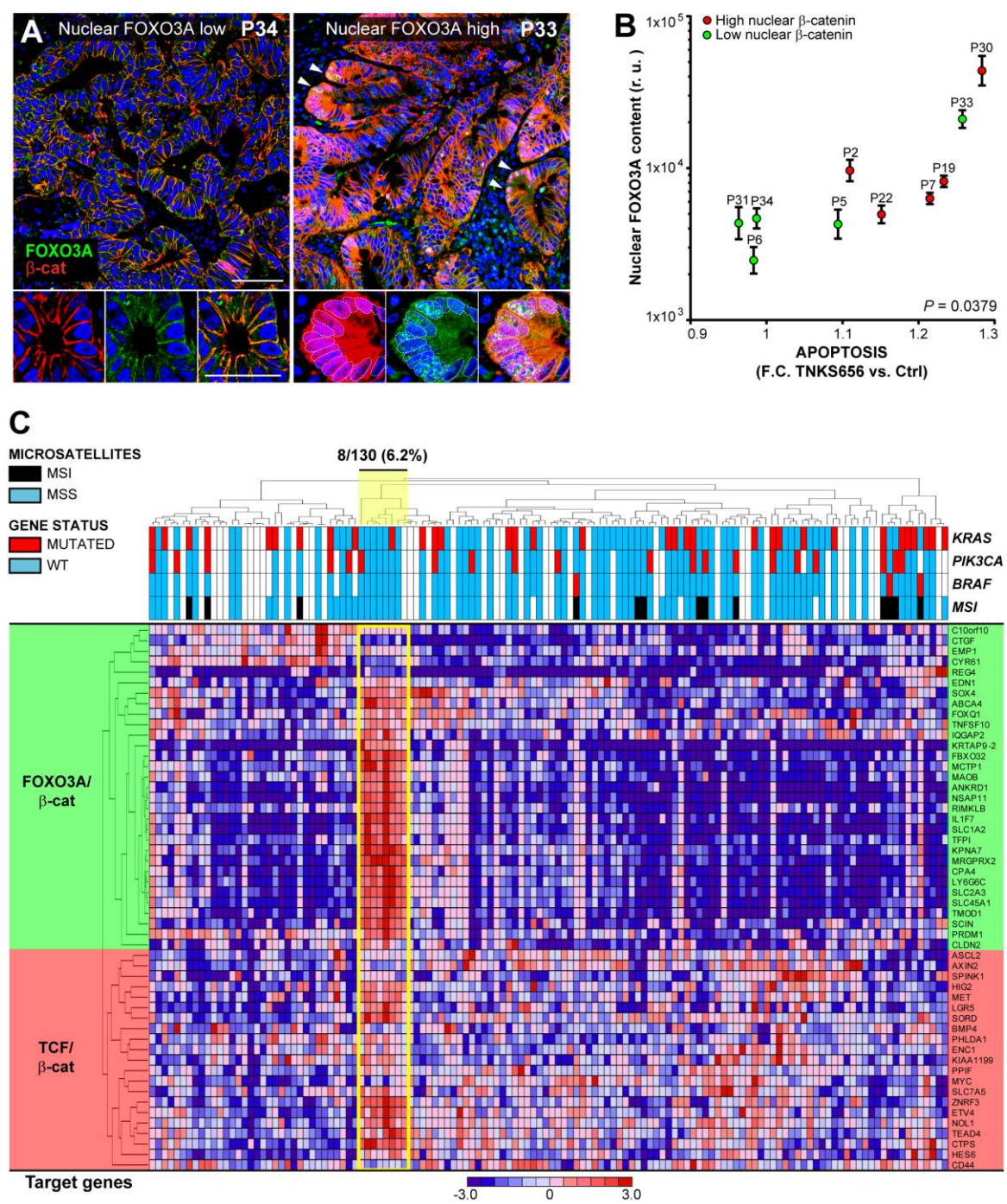


Figure. 5. High FOXO3A activity in CRC patient-derived cells determines the apoptosis induced by NVP-TNKS656. (A) Representative pictures of double immunofluorescence and confocal microscopy to detect β-catenin (red) and FOXO3A (green) in models PDX-P33 and PDX-P34. Arrowheads point cancer cells in PDX-P33 presenting high nuclear FOXO3A and β-catenin accumulation. PDX-P33 presented high and PDX-P34 low nuclear FOXO3A and β-catenin content. Lower panels show magnifications to better visualize β-catenin and FOXO3A subcellular localization. White dotted lines delineate nuclei stained with Hoechst 33342 (blue). Scale bars, 100 mm; magnifications, 50 mm. **(B)** Scatter plot comparing apoptosis induced by NVP-TNKS656 in xenograft-derived sphere cell cultures versus the histological amount of nuclear FOXO3A in the corresponding PDX models. FOXO3A relative units (r. u.) were calculated as described in methods. Apoptosis is represented as fold change of apoptotic cells induced by NVP-TNKS656 compared to the cells treated with vehicle. *P* values correspond to Pearson's correlation test. Nuclear β-catenin content for each model is also indicated. **(C)** Nanostring platform was used to analyze the expression of 31 FOXO3A/β-catenin and 21 TCF/β-catenin target genes in FFPE tumor samples from 130 colorectal cancer patients. Samples and genes were ordered by hierarchical clustering using uncentered Pearson correlation distance and complete linkage. Microsatellite stability and mutational status of *KRAS*, *PIK3CA* and *BRAF* were evaluated as indicated in methods. Patients showing highest expression of FOXO3A/β-catenin and TCF/β-catenin target genes are highlighted with a yellow box. Gene expression scaling is shown from low (blue) to high (red).

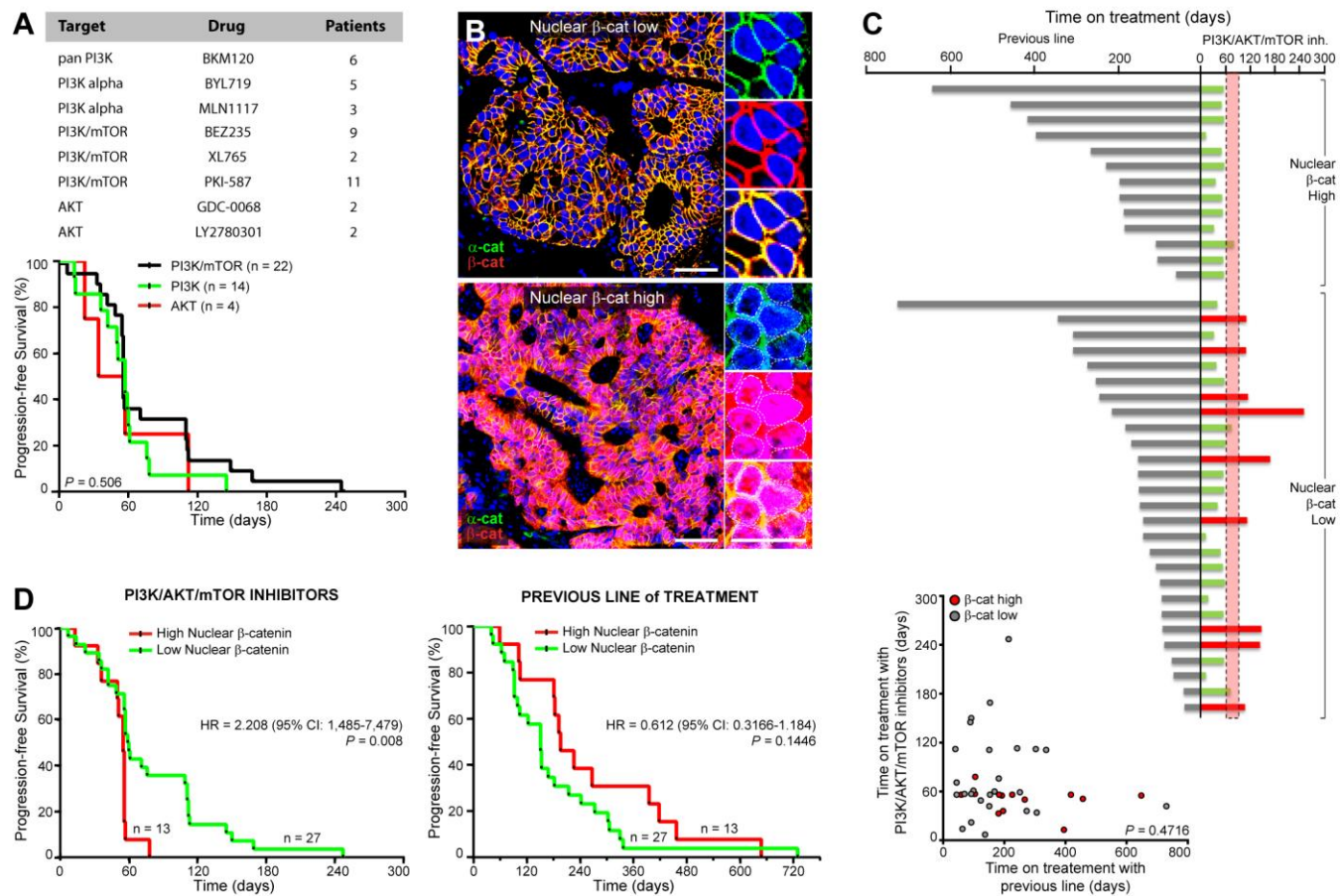
Supplementary Table S13). We selected tumors from patients treated with half the maximum tolerated dose aiming to homogenize the study cohort. There was no significant difference in progression free survival (PFS) between patients treated with different PI3K, AKT or dual PI3K/mTOR drug subtypes (Fig. 6A, lower panel). We performed a double-blinded evaluation of nuclear β -catenin content by two independent pathologists using immunohistochemistry and immunofluorescence on all baseline tumor samples (Fig. 6B and Supplementary Fig. S8). We classified tumors into two histological categories: high or low, depending on the number of cells positive for nuclear β -catenin accumulation. Out of 40 cases, 13 were high, and 27 were low in content.

After the first clinical diagnosis of disease progression by Computerized Axial Tomography (CT) scan, all 13 patients presenting tumors with high nuclear β -catenin content had progressed despite treatment with PI3K/AKT/mTOR pathway inhibitors. Contrarily, nine out of 27 patients with tumors presenting low β -catenin continued treatment after CT scan evaluation, showing some degree of stabilized disease (Fig. 6C, upper panel). Such longer-lasting response to PI3K/AKT/mTOR pathway inhibitors did not correlate with better response to the previous line of treatment, ruling out the possibility that those nine patients were either more sensitive to anti-tumoral drugs in general or had slower tumor growth independently of treatment (Fig. 6C, lower panel and D). Contrary to nuclear β -catenin content, relevant oncogenic mutations in *PIK3CA*, *KRAS*, *APC* or *TP53* genes, the site of tumor samples (primary tumor or metastasis) or TNM stage (T3 or T4) at the time of diagnosis did not correlate with any significant difference in response to PI3K/AKT/mTOR pathway inhibitors (Fig. 6C and D, Supplementary Fig. S9A-F, Supplementary Tables S13 and S14). Only two out of 28 samples analyzed presented MSI, preventing the possibility to evaluate its impact on drug response (Supplementary Table S13).

Equivalent analyses were performed separately in patients treated with PKI-587 (n = 11), BEZ235 (n = 9) or NVP-BKM120 (n = 6). We observed that cases with high nuclear β -catenin presented a shorter PFS upon PKI-587 or BEZ235 treatment (Supplementary Fig. S9G-I). However, results were not statistically significant as expected from the small number of cases available for the analyses.

To confirm the higher risk of progression in the β -catenin high group, we performed a Cox Proportional Hazards analysis on a subset of our cohort (n = 27) with complete annotation for variables potentially linked to outcome: age, gender, therapy (AKT, PI3K alpha, pan-PI3K, PI3K-mTOR inhibitor), number of prior treatment lines, presence of liver metastasis, and molecular profile (*PIK3CA*, *KRAS*, *TP53*, *APC* mutations, PTEN loss). Even after

FIGURE 6



multivariate adjustment, patients whose tumors had high nuclear β -catenin content still displayed a significantly worse outcome (PFS HR 3.96, CI95% 1.03-15.27; log rank p value 0.0158) (Supplementary Table S15).

Concerning gene expression patterns, we could analyze 31 out of the initial 40 baseline samples and observed that tumors responding to PI3K/AKT/mTOR pathway inhibitors clustered together (Supplementary Fig. S10A). The oncogenic mutations detected did not correlate with any of the gene expression clusters observed. We finally observed that the expression profile of a reduced set of genes could also help identifying tumors presenting high or low nuclear β -catenin content, cross-validating the initial cut-off selected to catalogue tumors histologically (Fig. 6B, Supplementary Figs. S8 and S10B).

DISCUSSION

The efficacy of several PI3K and AKT inhibitors is being tested in multiple clinical trials worldwide. Although initial results were promising in some tumor types, clinical responses are null or limited in most CRC patients (4-8). A reasonable hypothesis for such limited responses is the absence of a universal biomarker to select drug-sensitive patients or discard resistant cases. Since activating mutations in *PIK3CA* gene confer sensitivity to PI3K/AKT pathway inhibitors in preclinical assays, they have been commonly used as inclusion criteria in clinical trials. However, *PIK3CA* mutations showed conflicting results in predicting response to single agent PI3K or AKT inhibitors in early phase clinical trials (7, 35-37). Similarly, we show here that mutations in genes frequently altered in CRC including *KRAS*, *TP53* or *PIK3CA* do not predict response to PI3K/AKT pathway inhibitors. Interestingly, multivariate analysis indicates that *APC* mutations are a risk factor for patients treated with these inhibitors (Supplementary Table S15). This would suggest that oncogenic activation of the Wnt/ β -catenin pathway could be a mechanism of resistance to PI3K and AKT inhibitors.

Indeed, we previously described that nuclear accumulation of β -catenin conferred resistance to PI3K and AKT inhibitors in colon cancer cells (10). In the present study we observed that CRC patients with high nuclear β -catenin present a shorter progression free survival when treated with PI3K/AKT/mTOR pathway inhibitory drugs in the context of clinical trials. Such differential response contrasts with the fact that all CRC patients in our cohort progressed to previous lines of treatment irrespective of their nuclear β -catenin content. These results on clinical samples indicate that nuclear β -catenin accumulation

could be an independent predictive biomarker of resistance to PI3K and AKT inhibitors beyond other molecular alterations frequent in CRC.

In accordance, we explored here the therapeutic potential of overcoming such resistance by reducing nuclear β -catenin content with a new Wnt/tankyrase inhibitor, NVP-TNKS656. We show that it sensitizes patient-derived cells to PI3K or AKT inhibitors *in vitro* and *in vivo*, especially those with high accumulation of nuclear β -catenin and, thus, high oncogenic Wnt/ β -catenin pathway activity.

Our results on Wnt/tankyrase inhibitors reveal a new therapeutic opportunity for the treatment of advanced CRC patients beyond their combination with PI3K or AKT inhibitors. Indeed, we previously showed that cancer cells with high pro-apoptotic FOXO3A transcriptional activity require high levels of nuclear β -catenin to preserve a viable balance between survival and apoptosis (10). Here we observed that NVP-TNKS656 treatment was particularly effective in cells and tumors with high endogenous nuclear β -catenin and FOXO3A content, where the pharmacological reduction of nuclear β -catenin promoted FOXO3A-dependent apoptosis. Therefore, it is expected that cancer cell survival in such endogenous FOXO3A-active scenario, more frequent in advanced metastatic CRC tumors (10), would rely on Wnt/ β -catenin pathway activity. This could represent an exceptional opportunity to compromise cancer cell viability by treating patients with tankyrase inhibitors. Indeed, we identify by gene expression profiling a population of patients presenting tumors with high FOXO3A/ β -catenin activity who could potentially respond best to the treatment with these Wnt/ β -catenin pathway inhibitors.

However, very little is known about the determinants of nuclear FOXO3A activation in CRC. It is well described that oxidative stress promotes FOXO3A translocation from the cytoplasm to the nucleus and the consequent induction of genes involved in cells cycle arrest, survival, apoptosis and metastasis (9). Future investigations should reveal the precise contribution of oxidative stress and other stimuli originated in the surrounding tumor stroma on determining the final levels of activated FOXO3A. Our results suggest that describing FOXO3A activating factors would be of particular interest to understand the response to Wnt/ β -catenin inhibitors. The use of PDX models could be pivotal in these investigations since they preserve equivalent levels of FOXO3A than the original patients' tumors. In fact, the efficacy of Wnt/tankyrase inhibitors observed in cancer cell lines or genetic mouse models has been modest (18-20), since they may not recapitulate tumoral FOXO3A activity as consistently as PDX. Indeed, PDX are generally considered the best pre-clinical models to evaluate drug-response since they faithfully recapitulate patient's disease preserving their molecular alterations and histopathological traits (24, 38, 39).

Other alterations frequent in CRC could also be relevant determinants of response to Wnt/ β -catenin inhibitors. For instance, truncating mutations in *APC* gene are present in more than 80% of CRC patients and constitutively activate the oncogenic Wnt/ β -catenin pathway (3). Here we show that NVP-TNKS656 can reduce nuclear β -catenin content and repress tumor growth even in *APC* mutant PDX models. This data suggests that the therapeutic potential of tankyrase inhibitors could be extended to the majority of CRC tumors. This wide spectrum would contrast with the lack of activity in *APC*-mutated tumors expected from the treatment with NVP-LGK974. This is the first Wnt/ β -catenin pathway inhibitor tested in clinical trials (NCT01351103), which blocks porcupine activity and the maturation of Wnt ligands upstream in the oncogenic signaling. As tankyrases polysylate and commit other proteins to degradation in addition to AXIN1 and 2 (40, 41), it could be of interest to evaluate to what extent the anti-tumoral capacity of tankyrase inhibitors rely on affecting any other cell processes beyond Wnt/ β -catenin signaling.

Aiming to define clinically useful biomarkers to predict the response to PI3K/AKT and Wnt/ β -catenin pathway inhibitors, we investigated the potential of gene expression signatures. Our initial results suggest that specific gene expression profiles could identify CRC tumors with high FOXO3A and β -catenin content and transcriptional activity. In particular, we observed that a particular set of genes was overexpressed in CRC tumors with high nuclear β -catenin content. Our drug treatment experiments indicate that such Wnt/ β -catenin activated tumors could be resistant to PI3K and AKT inhibitors. Furthermore, we observed a significant correlation between nuclear FOXO3A accumulation and mRNA expression of one of its target genes, *SLC2A3* (10). These results suggest that nuclear accumulation of FOXO3A and β -catenin observed by histology reflects their activation as transcription factors inducing the expression of their corresponding target genes. Finally, we could identify, using Nanostring nCounter platform, a population of CRC patients with tumors presenting high expression of FOXO3A/ β -catenin and TCF/ β -catenin target genes. Our preclinical data indicate that these patients could benefit from the treatment with Wnt/tankyrase inhibitors.

Together, these results suggest that gene expression profiling could help to build complex predictive biomarkers of response to PI3K/AKT and Wnt/ β -catenin pathway inhibitors. However, selecting a precise set of genes to build robust signatures would require evaluating whole gene expression patterns by microarrays or RNAseq in prospective studies with fresh-frozen tumor samples. These expression profiles should be cross-compared with histological evaluation of nuclear FOXO3A and β -catenin content and the response to treatment, to finally define those gene sets associated to such histological

and clinical traits. These biomarker exploratory studies should ideally focus on analyzing tumor biopsies taken from progressive lesions at the time of inclusion in clinical trials and not from archival samples. In the particular case of nuclear FOXO3A, we have observed that advanced metastatic tumors present the highest proportion of cases activated for this transcription factor (10). Therefore, validating the use of nuclear FOXO3A accumulation and its associated gene expression signatures as potential biomarker of sensitivity to Wnt/ β -catenin pathway inhibitors would require taking biopsies preferentially from metastatic lesions that will actually be progressing at the time of patients' inclusion in clinical trials.

Hence, we suggest combining histological evaluation of FOXO3A and β -catenin with functional gene expression signatures to build complex predictive biomarkers of response to PI3K/AKT and Wnt/ β -catenin pathway inhibitors in CRC, which may help the design of future clinical trials with this family of drugs. Similarly, current studies are analyzing the expression of 50 selected genes by Nanostring nCounter platform to facilitate a more precise definition of breast cancer subtypes (42, 43), whose differential response to treatment is currently under validation in several clinical trials. In the same line as our proposal, such gene expression signatures are being combined with clinical evaluation of hormone receptors and HER2 protein levels by histopathological techniques.

In summary, we propose combining gene expression profiling and histology to define nuclear β -catenin and FOXO3A content and activity as predictive biomarkers of drug-response (Supplementary Fig. S11). We hypothesize that this molecular prescreening could establish three groups of patients presenting tumors with: 1) low nuclear β -catenin and FOXO3A activity, more suitable for the treatment with PI3K or AKT inhibitors alone, 2) high β -catenin and FOXO3A activity who could benefit from Wnt/ β -catenin inhibitors alone and 3) high nuclear β -catenin but low FOXO3A activity who may benefit from combined treatments. Such molecular stratification of patients could represent a significant improvement in response to therapy and an important step forward towards reverting the long-stalled scenario of CRC therapy.

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plasmid. We acknowledge Javier Hernández Losa for evaluation of MSI status and Amanda Wren for her valuable assistance in the preparation of the English manuscript.

AUTHOR CONTRIBUTIONS

O.A. performed most of the experiments and helped to interpret the results and write the manuscript. I.C., I.P. and S.P.T. helped to perform some experiments. G.A., R.D. and J.R. provided clinical follow up of all patients included in the study. S.L. selected the tissue specimens from which to derive cancer cells. A.P. Helped analyzing gene expression profiles by Nanostring. E.E. and R.C. performed surgery on colon cancer patients from whom PDX models were derived. P.N. performed histopathological evaluation and immunohistochemistry of β -catenin on tissue sections from CRC patients included in clinical trials with PI3K/AKT/mTOR inhibitors. A.V. performed mutational analysis of human colon carcinomas. W.S. provided the tankyrase inhibitor NVP-TNKS656 developed by Novartis and supervised the project. J.T. supervised the project. H.G.P designed all the experiments, interpreted all resulting data, supervised the whole project and wrote the manuscript.

ABBREVIATIONS

CRC, colorectal cancer; CT, computerized axial tomography; FFPE, formalin-fixed paraffin-embedded; NOD-SCID, nonobese diabetic/severe combined immunodeficiency; PDX, patient-derived xenograft; TNKS, tankyrase; TNM, tumor, Nodes and Metastasis.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011;61:69-90.
2. Janne PA, Mayer RJ. Chemoprevention of colorectal cancer. *N Engl J Med.* 2000;342:1960-8.
3. TCGA. Comprehensive molecular characterization of human colon and rectal cancer. *Nature.* 2012;487:330-7.
4. Courtney KD, Corcoran RB, Engelman JA. The PI3K Pathway As Drug Target in Human Cancer. *Journal of Clinical Oncology.* 2010;28:1075-83.
5. Markman B, Atzori F, Perez-Garcia J, Tabernero J, Baselga J. Status of PI3K inhibition and biomarker development in cancer therapeutics. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO.* 2010;21:683-91.

6. Bendell JC, Rodon J, Burris HA, de Jonge M, Verweij J, Birlle D, et al. Phase I, Dose-Escalation Study of BKM120, an Oral Pan-Class I PI3K Inhibitor, in Patients With Advanced Solid Tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2012;30:282-90.
7. Juric D. BYL719, a next generation PI3K alpha specific inhibitor: preliminary safety, PK, and efficacy results from the first-in-human study. . *Cancer research*. 2012;72, Abstr. CT-01
8. H. Burris JR, S. Sharma, R. S. Herbst, J. Tabernero, J. R. Infante, A. Silva, D. Demanase, W. Hackl, J. Baselga. First-in-human phase I study of the oral PI3K inhibitor BEZ235 in patients (pts) with advanced solid tumors. *J Clin Oncol* 2010;28:15s (suppl; abstr 3005).
9. Eijkelenboom A, Burgering BM. FOXOs: signalling integrators for homeostasis maintenance. *Nature reviews Molecular cell biology*. 2013;14:83-97.
10. Tenbaum SP, Ordonez-Moran P, Puig I, Chicote I, Arques O, Landolfi S, et al. beta-catenin confers resistance to PI3K and AKT inhibitors and subverts FOXO3a to promote metastasis in colon cancer. *Nature medicine*. 2012;18:892-901.
11. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell*. 2012;149:1192-205.
12. Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature*. 2005;434:843-50.
13. Palmer HG, Gonzalez-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, et al. Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J Cell Biol*. 2001;154:369-87.
14. Essers MA, de Vries-Smits LM, Barker N, Polderman PE, Burgering BM, Korswagen HC. Functional interaction between beta-catenin and FOXO in oxidative stress signaling. *Science*. 2005;308:1181-4.
15. Voronkov A, Krauss S. Wnt/beta-catenin signaling and small molecule inhibitors. *Curr Pharm Des*. 2013;19:634-64.
16. Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. *Nature reviews Cancer*. 2013;13:11-26.
17. Huang SM, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature*. 2009;461:614-20.
18. Lau T, Chan E, Callow M, Waaler J, Boggs J, Blake RA, et al. A novel tankyrase small-molecule inhibitor suppresses APC mutation-driven colorectal tumor growth. *Cancer research*. 2013;73:3132-44.
19. Waaler J, Machon O, Tumova L, Dinh H, Korinek V, Wilson SR, et al. A novel tankyrase inhibitor decreases canonical Wnt signaling in colon carcinoma cells and reduces tumor growth in conditional APC mutant mice. *Cancer research*. 2012;72:2822-32.
20. Morrissey E, James RG, Davidson KC, Bosch KA, Biechele TL, Robin NC, et al. WIKI4, a Novel Inhibitor of Tankyrase and Wnt/ β -Catenin Signaling. *PLoS ONE*. 2012;7:e50457.

21. Shultz MD, Cheung AK, Kirby CA, Firestone B, Fan J, Chen CH-T, et al. Identification of NVP-TNKS656: The Use of Structure–Efficiency Relationships To Generate a Highly Potent, Selective, and Orally Active Tankyrase Inhibitor. *Journal of Medicinal Chemistry*. 2013;56:6495-511.
22. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *Journal of the National Cancer Institute*. 2000;92:205-16.
23. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *European journal of cancer*. 2009;45:228-47.
24. Isabel Puig, Irene Chicote, Stephan P. Tenbaum, Oriol Arqués, José Raúl Herance, Juan D. Gispert, et al. A Personalized Pre-clinical Model to Evaluate the Metastatic Potential of Patient-derived Colon Cancer Cells. *Clinical Cancer Research*. 2013;2013:6787-801.
25. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008;3:1101-8.
26. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome research*. 2012;22:568-76.
27. Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, Handsaker RE, et al. An integrated map of genetic variation from 1,092 human genomes. *Nature*. 2012;491:56-65.
28. Arqués O, Chicote I, Tenbaum S, Puig I, G. Palmer H. Quantitative Procedure to Analyze Nuclear β -Catenin Using Immunofluorescence Tissue Staining. 2014.
29. Arqués O, Chicote I, Tenbaum S, Puig I, G. Palmer H. Standardized Relative Quantification of Immunofluorescence Tissue Staining. 2012.
30. Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffey PJ. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Current biology : CB*. 2000;10:1201-4.
31. Fuerer C, Nusse R. Lentiviral vectors to probe and manipulate the Wnt signaling pathway. *PLoS One*. 2010;5:e9370.
32. Bartucci M, Dattilo R, Moriconi C, Pagliuca A, Mottolese M, Federici G, et al. TAZ is required for metastatic activity and chemoresistance of breast cancer stem cells. *Oncogene*. 2014.
33. Wang L, Shi S, Guo Z, Zhang X, Han S, Yang A, et al. Overexpression of YAP and TAZ is an independent predictor of prognosis in colorectal cancer and related to the proliferation and metastasis of colon cancer cells. *PloS one*. 2013;8:e65539.

34. Mishra SK, Siddique HR, Saleem M. S100A4 calcium-binding protein is key player in tumor progression and metastasis: preclinical and clinical evidence. *Cancer metastasis reviews*. 2012;31:163-72.
35. Dienstmann R, Serpico D, Rodon J, Saura C, Macarulla T, Elez E, et al. Molecular profiling of patients with colorectal cancer and matched targeted therapy in phase I clinical trials. *Molecular cancer therapeutics*. 2012;11:2062-71.
36. Elkabets M, Vora S, Juric D, Morse N, Mino-Kenudson M, Muranen T, et al. mTORC1 inhibition is required for sensitivity to PI3K p110alpha inhibitors in PIK3CA-mutant breast cancer. *Science translational medicine*. 2013;5:196ra99.
37. Janku F, Wheler JJ, Westin SN, Moulder SL, Naing A, Tsimberidou AM, et al. PI3K/AKT/mTOR inhibitors in patients with breast and gynecologic malignancies harboring PIK3CA mutations. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2012;30:777-82.
38. Bertotti A, Migliardi G, Galimi F, Sassi F, Torti D, Isella C, et al. A molecularly annotated platform of patient-derived xenografts ("xenopatients") identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. *Cancer Discovery*. 2011;1:508-23.
39. Julien S, Merino-Trigo A, Lacroix L, Pocard M, Goere D, Mariani P, et al. Characterization of a large panel of patient-derived tumor xenografts representing the clinical heterogeneity of human colorectal cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2012;18:5314-28.
40. Lehtio L, Chi NW, Krauss S. Tankyrases as drug targets. *The FEBS journal*. 2013;280:3576-93.
41. Riffell JL, Lord CJ, Ashworth A. Tankyrase-targeted therapeutics: expanding opportunities in the PARP family. *Nature reviews Drug discovery*. 2012;11:923-36.
42. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2009;27:1160-7.
43. Prat A, Adamo B, Fan C, Peg V, Vidal M, Galvan P, et al. Genomic analyses across six cancer types identify basal-like breast cancer as a unique molecular entity. *Scientific reports*. 2013;3:3544.
44. Van der Flier LG, Sabates-Bellver J, Oving I, Haegebarth A, De Palo M, Anti M, et al. The Intestinal Wnt/TCF Signature. *Gastroenterology*. 2007;132:628-32.

SUPPORTING INFORMATION

SUPPLEMENTARY METHODS

Gene expression

Gene expression profiles were acquired in baseline tumors from patients treated with PI3K/ATK/mTOR inhibitors. To be able to visualize, select and isolate areas enriched in tumor tissue from tumor stroma, sections of each formalin-fixed paraffin-embedded (FFPE) tumor sample were stained with Hematoxylin and Eosin (H/E). Two tumor tissue cores ($\varnothing = 1$ mm) were collected from each FFPE block and total RNA was purified using the HighPure FFPE Micro Kit (Roche). 100 ng of total RNA was used to measure the expression levels of 292 selected genes using the nCounter platform from Nanostring Technologies. In short, fluorescently labeled probes are designed for specific genes and allowed to hybridize and capture target RNAs. Individual RNA molecules are counted using unique color-coded probe pairs (1). Raw data was log₂-transformed and normalized using five house-keeping transcripts using nSolver2 Software. Cluster 3.0 (2) and Genepattern software (3) were used to generate hierarchical cluster heatmaps and dendrograms.

We used a genome wide Human Gene 1.0 ST Array (Affimetrix) to determine the genomewide transcriptome of PDX-P2, PDX-P5 and PDX-P30 xenograft tumors growing in mice treated with vehicle or NVP-TNKS656. Total RNA from endpoint tumor xenografts was isolated using TRIZOL reagent (Life Technologies). Microarray data was acquired using the Affimetrix GeneChip/GeneTitan platforms. Hierarchical clustering was performed with the expression values of 3110 probes in PDX-P2, 2290 probes in PDX-P30 and 1996 probes in PDX-P5 in vehicle and NVP-TNKS656 treated samples (ANOVA significance level of $P < 0.05$). Rows and columns were clustered using Euclidian distance algorithm with the average linkage method using Partek Genomics Suite Software.

To perform quantitative RT-PCR, RNA from endpoint tumor xenografts was used to synthesize cDNA using Superscript-III reverse transcriptase with oligo-dT and random hexamer primers (Life Technologies). We applied geNorm algorithms (4) to select succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*) and peptidylprolyl isomerase A (Cyclophilin A, *PPIA*) as the most stable reference transcripts. We used the geometric mean of their relative expression values for normalization and for calculating the respective normalized standard deviation from triplicates of three biological replicates. Primer pairs for qPCR detection of human transcripts were as following: *AXIN2*-fw: 5'-AGTCAGCAGAGGGACAGGAA-3'; *AXIN2*-rev: 5'-GTGGACACCTGCCAGTTTCT-3'; *CAV2*-fw: 5'-AAGAGCGATACACAAAGC-3'; *CAV2*-rev: 5'-GATCACAGCCAGAAAAATGTC-3'; *PPIA*-fw: 5'-CAGAGGCAGGAAAAGCAA-3'; *PPIA*-rev: 5'-AACCGCAACAGATGTCTC-3'; *CLIC3*-fw: 5'-TGCAGATCGAGGACTTTC-3'; *CLIC3*-rev: 5'-GGAGAACTTGTGGAAAACG-3'; *CYR61*-fw: 5'-ATTGTAGAAAGGAAGCCTTGCTCAT-3'; *CYR61*-rev: 5'-TCCAATCGTGGCTGCATTAG-3';

EPHB3-fw: 5'-GCACCTGCCACAATAACT-3'; *EPHB3*-rev: 5'-CATTCACATTGGAGATCACACC-3';
ID3-fw: 5'-CCTGAAGAGCCAGAGCTA-3'; *ID3*-rev: 5'-ATAGTTTGGAGAGCAGCC-3'; *S100A4*-fw:
5'-GCAAAGAGGGTGACAAGT-3'; *S100A4*-rev: 5'-TCCAAGTTGCTCATCAGC-3'; *SDHA*-fw: 5'-
TGGGAACAAGAGGGCATCTG-3'; *SDHA*-rev: 5'-CCACCACTGCATCAAA TTCATG-3'; *SLC2A3*-fw:
5'-CTCTTCGTCAACCGCTTT-3'; *SLC2A3*-rev: 5'-AGTCCCATAAAGCAGCCA-3'.

Genotyping

Amplicon sequencing: DNA extraction was performed from five 10 µm sliced sections of FFPE material using the Maxwell FFPE Tissue LEV DNA Purification Kit. Tumor area content was evaluated by a pathologist. Minimum tumor content was set to 30%, in order to better detect somatic mutations. An initial multiplex-PCR using a proof-reading polymerase was performed on samples.

Indexed libraries were pooled and loaded onto a MiSeq instrument (Illumina). Initial alignment was performed with BWA after primer sequence clipping and variant calling was done with the GATK Unified Genotyper and VarScan2 followed by ANNOVAR annotation. Mutations were called at a minimum 3% allele frequency. SNPs were filtered out with dbSNP and 1000 genome datasets. All detected variants were manually revised and confirmed.

Exome sequencing: Patients provided written informed consent for somatic and germline DNA analysis. Fresh frozen tumor samples, along with a blood sample from patients P5 and P30, were subjected to whole Exome sequencing. Samples were initially assessed for tumor content based on a Hematoxylin and Eosin staining. Genomic DNA was extracted from frozen samples with the QIAamp DNA mini kit (Qiagen) according to manufacturer's instructions. Germline DNA was extracted from whole blood using the DNA QIAampDNA blood midi kit (Qiagen). Library preparation was performed following the standard Illumina protocol (Genomic Sample Prep). One µg of DNA was fragmented, end-repaired and an adenine was ligated to each of the 3' ends, where sample-specific adaptors were linked. Libraries were amplified using eight cycles of PCR, and Exome enrichment was performed using specific biotinylated probes (SureSelect XT Human All Exon 50Mb, Agilent). After enrichment, the Exome libraries were PCR-amplified (eight cycles), quantified and loaded in a HiSeq2000 sequencer (Illumina). Paired-end 100 base sequences were obtained and aligned to the reference genome using BWA. This yielded a median depth of coverage in targeted regions of 60-120x, with more than 80% of the Exome having at least 10 reads. Predefined filtering (minimum 8x coverage, five reads supporting the variant allele, strand-bias filtering) was applied, previous to final manual review to check for local indel misalignment and homopolymer false positive calls.

β-catenin immunohistochemistry evaluation

β-catenin staining was evaluated by a pathologist. Tumors were scored for active β-catenin according to the percentage of cells positive for nuclear staining and the pattern of localization in cell membrane versus cytoplasm. This scoring was based on the observation that in tumors with less than 50% of cells presenting nuclear β-catenin, the protein was predominantly localized in the cell membrane. Those samples were classified as “low”. In contrast, when nuclear β-catenin staining was observed in more than 50% of the cells they were considered “high” and membrane localization was almost lost.

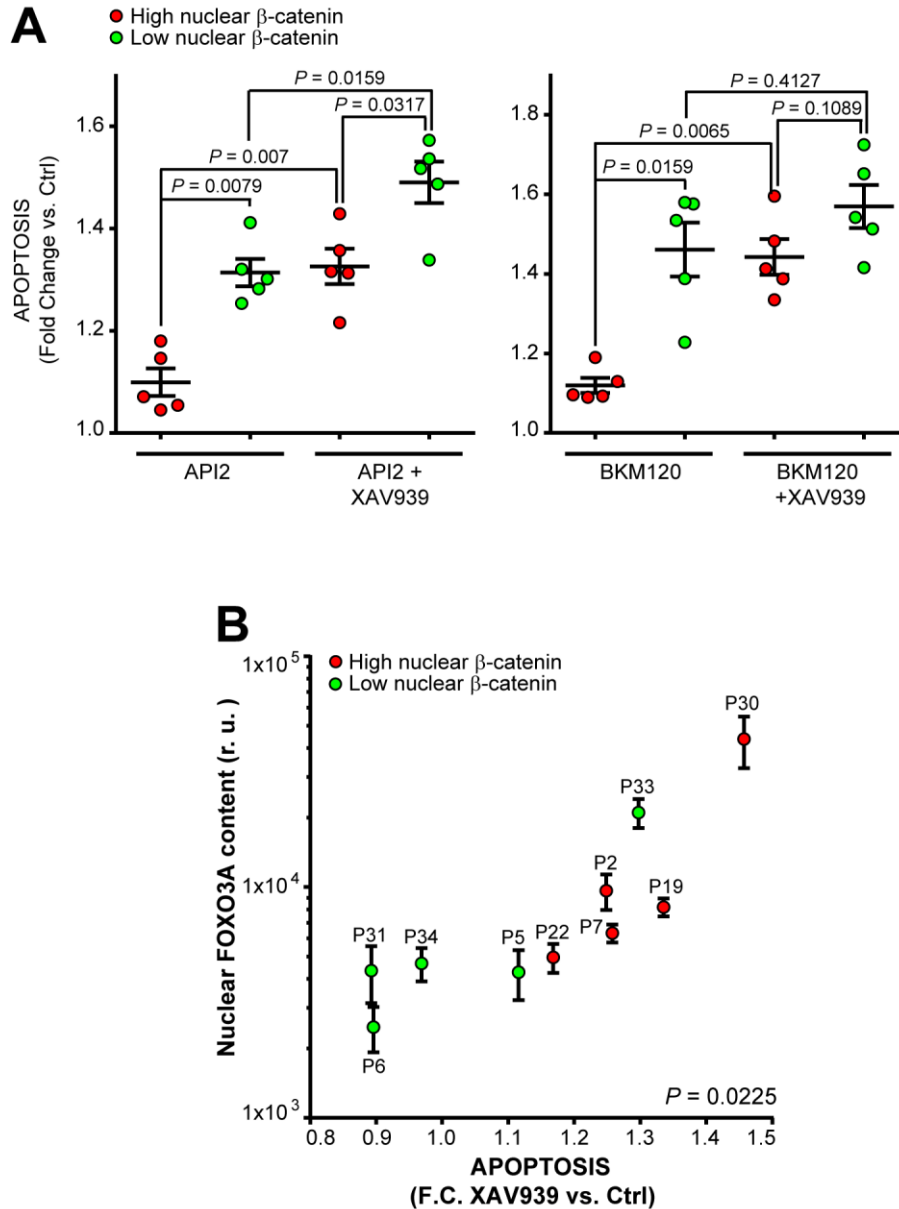
Protein extraction for western blot

Protein extracts from tumor samples were obtained by homogenizing tumors in a lysis buffer containing 50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 1% SDS. After homogenization samples were boiled 5 min at 95°C and centrifuged at 13,500 rpm for 20 min at 4°C. To obtain cytosolic and nuclear fractions, cells were scraped in PBS, centrifuged at 1,500 rpm for 10 min and incubated for 15 min at 4°C in a lysis buffer containing 10 mM Hepes pH 7.5, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA. After adding 10% NP40, samples were centrifuged at 15,000 rpm for 10 min at 4°C. Nuclear pellets were washed twice with the lysis buffer and incubated twice at 4°C for 30 min with a buffer containing 20 mM Hepes pH 7.5, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA. Nuclear extracts were obtained by centrifuging the samples 15,000 rpm for 10 min 4°C. For all the lysis buffers used, 1 mM DTT and protease inhibitors (Complete, Mini, EDTA-free protease inhibitor cocktail, Roche) were added.

SUPPLEMENTARY METHODS REFERENCES

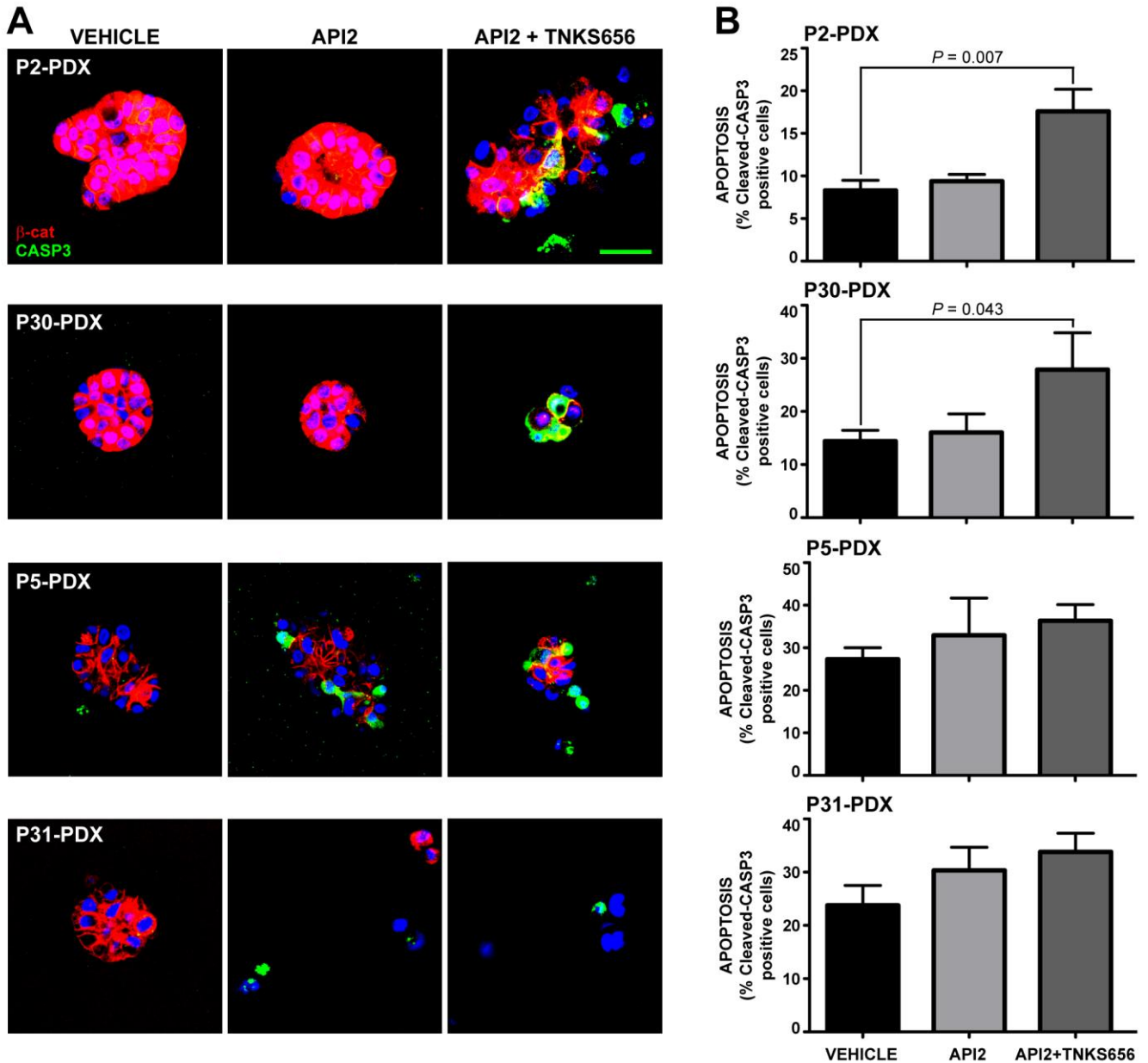
1. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nature Biotechnology*. 2008;26:317-25.
2. de Hoon MJ, Imoto S, Nolan J, Miyano S. Open source clustering software. *Bioinformatics*. 2004;20:1453-4.
3. Reich M LT, Gould J, Lerner J, Tamayo P, Mesirov JP. GenePattern 2.0. *Nature Genetics*. 2006;38:500-1.
4. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3:RESEARCH0034.

SUPPLEMENTARY FIGURE 1



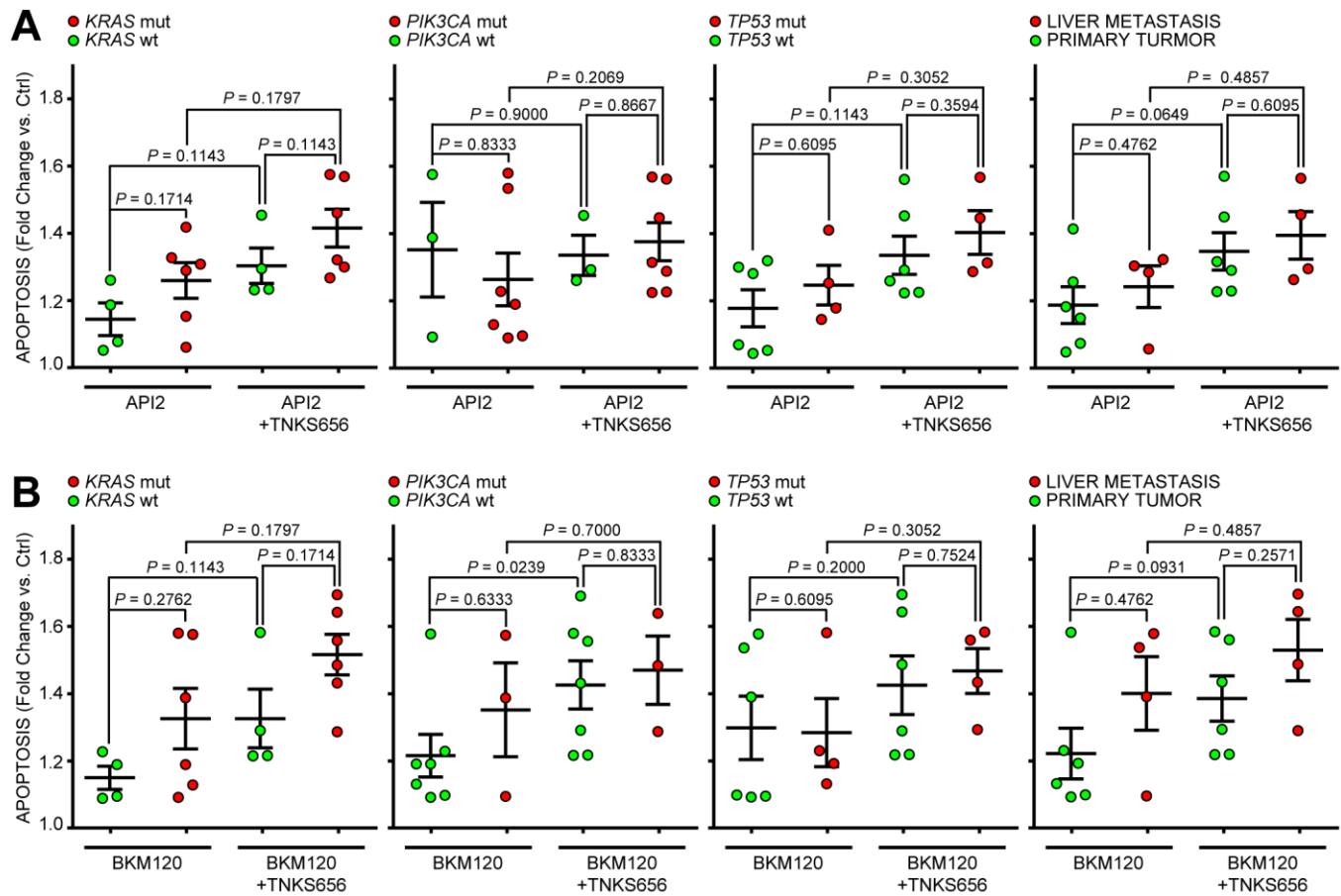
Supplementary Figure S1. Apoptosis of CRC patient-derived cells upon treatment with API-2 or NVP-BKM120 in combination with XAV939. Upper panel: Column scatter plot showing the apoptosis induced in sphere cell cultures of the 10 patient-derived models treated as indicated. Data are represented as fold change of apoptotic cells induced by the treatment compared to cells treated with vehicle. Horizontal lines indicate arithmetic mean values, and error bars SEM. P values correspond to unpaired t tests. Lower panel: Scatter plot comparing apoptosis induced by XAV939 in xenograft-derived sphere cell cultures versus the histological amount of nuclear FOXO3A in the corresponding PDX models. FOXO3A relative units (r. u.) were calculated as described in methods. Apoptosis is represented as fold change of apoptotic cells induced by XAV939 compared to the cells treated with vehicle. P values correspond to Pearson's correlation test. Nuclear β -catenin content for each model is also indicated.

SUPPLEMENTARY FIGURE 2



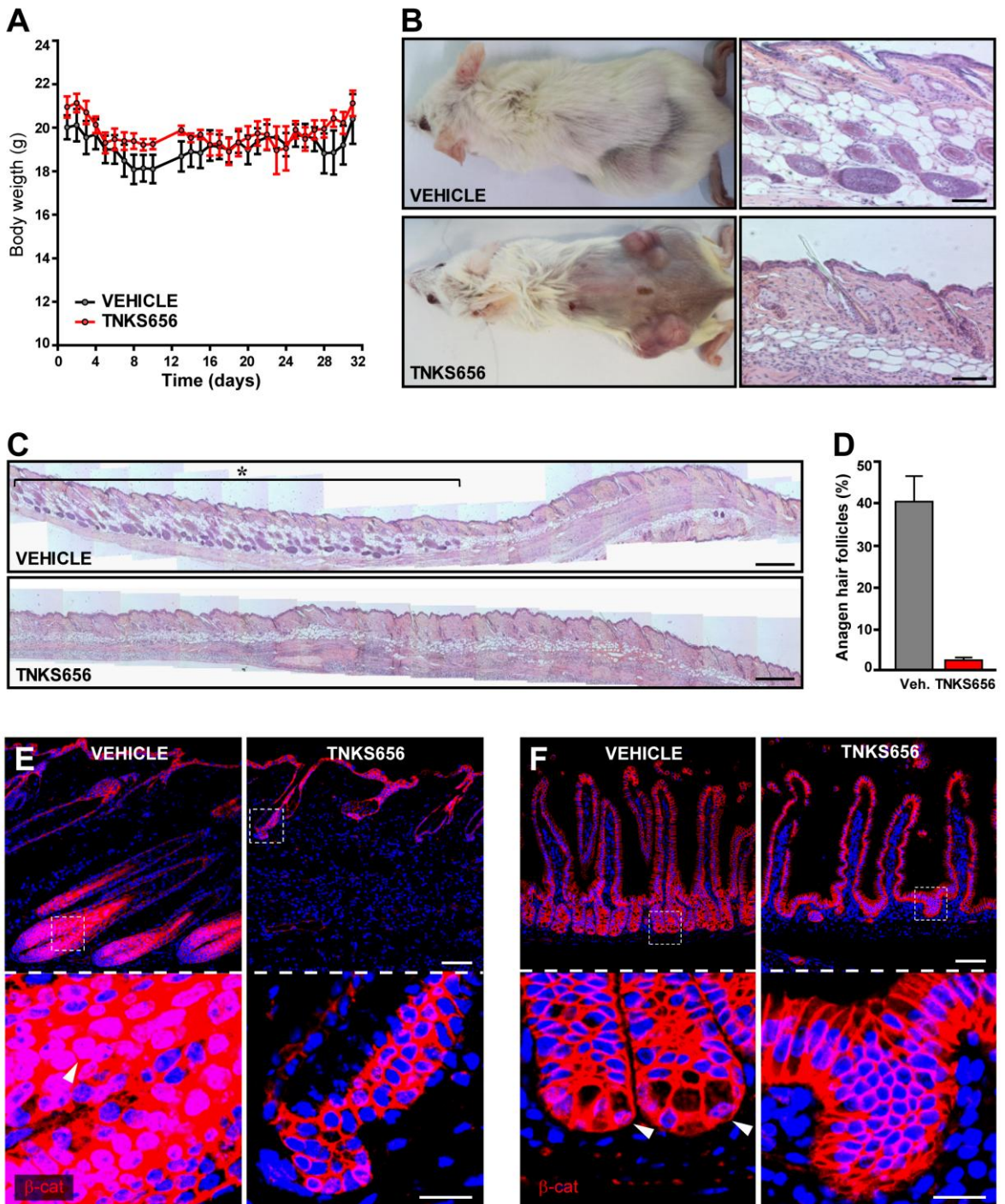
Supplementary Figure S2. Evaluation of apoptosis in primary sphere cell cultures by immunofluorescent quantification of cleaved-CASPASE3. Cells from four patient-derived xenografts models (P2, P5, P30, P31) were cultured as sphere cultures. They were treated with API-2 and NVP-TNKS656 and by the end of the experiment spheroids were included in matrigel and stained as described in methods. **(A)** Representative pictures of immunofluorescence and confocal microscopy of spheroids from all four models stained for β -catenin (red) and cleaved-CASPASE3 (green) in each treatment condition. Nuclei were stained with Hoechst 33342 (blue). Scale bars, 50 mm; **(B)** Graphs representing the percentage of cleaved-CASPASE3 positive cells in sphere cell cultures treated as indicated. Error bars show $\pm$ S.D. in five images. P values correspond to unpaired t tests.

SUPPLEMENTARY FIGURE 3



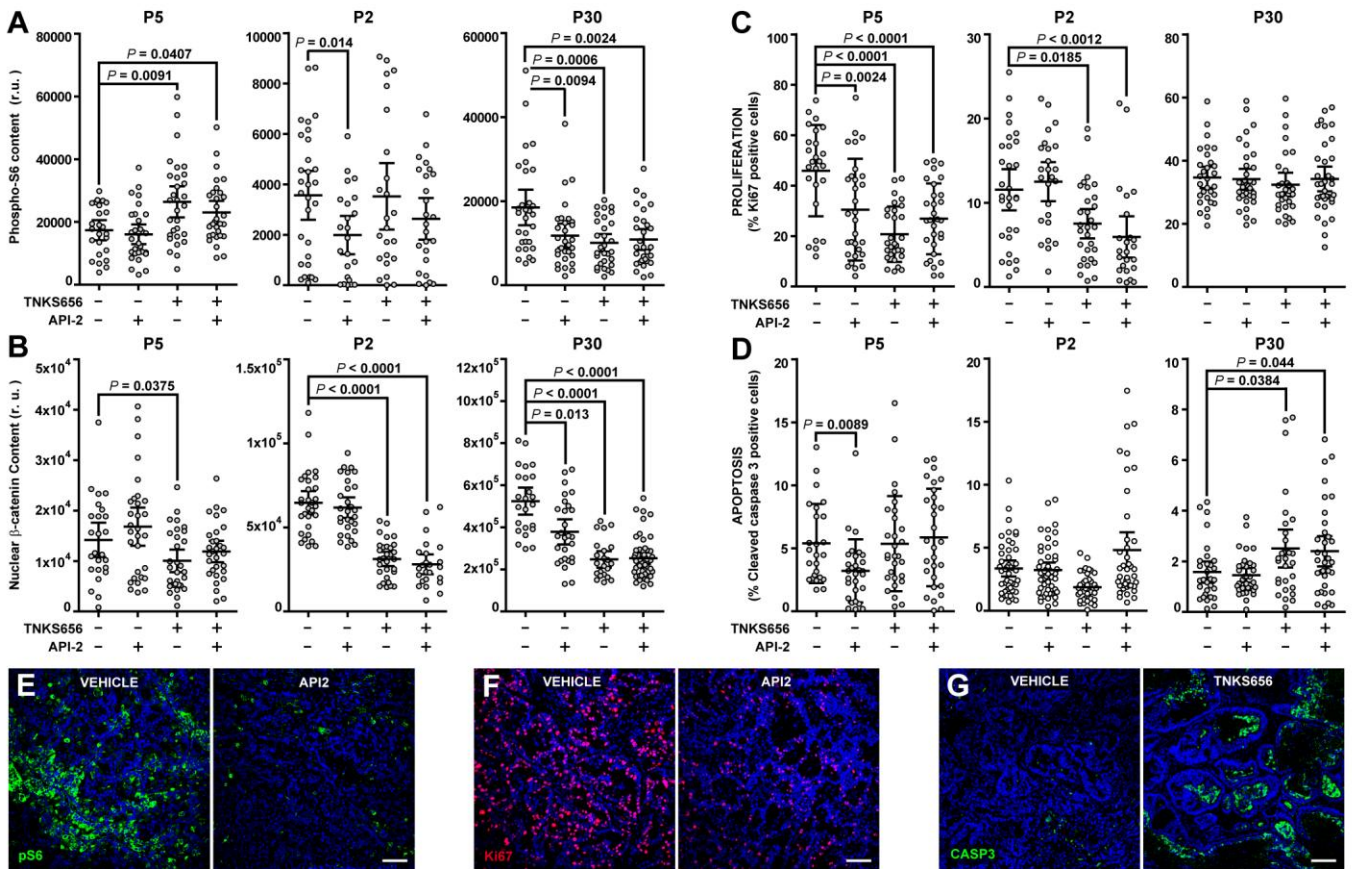
Supplementary Figure S3. Frequent mutations in CRC do not determine the response to API2, NVP-BKM120 or NVP-TNKS656. (A, B) Column scatter plots showing the apoptosis induced in sphere cell cultures of 10 patient-derived models treated as indicated. Cells are divided in groups depending on their mutational status for *KRAS*, *PIK3CA*, *TP53* or the site of the tumor from which cells were derived (primary tumor or metastasis) as indicated. Data are represented as fold change of apoptotic cells induced by the treatment compared to cells treated with vehicle. Horizontal lines indicate arithmetic mean values, and bars show standard error. *P* values correspond to unpaired *t* tests.

SUPPLEMENTARY FIGURE 4

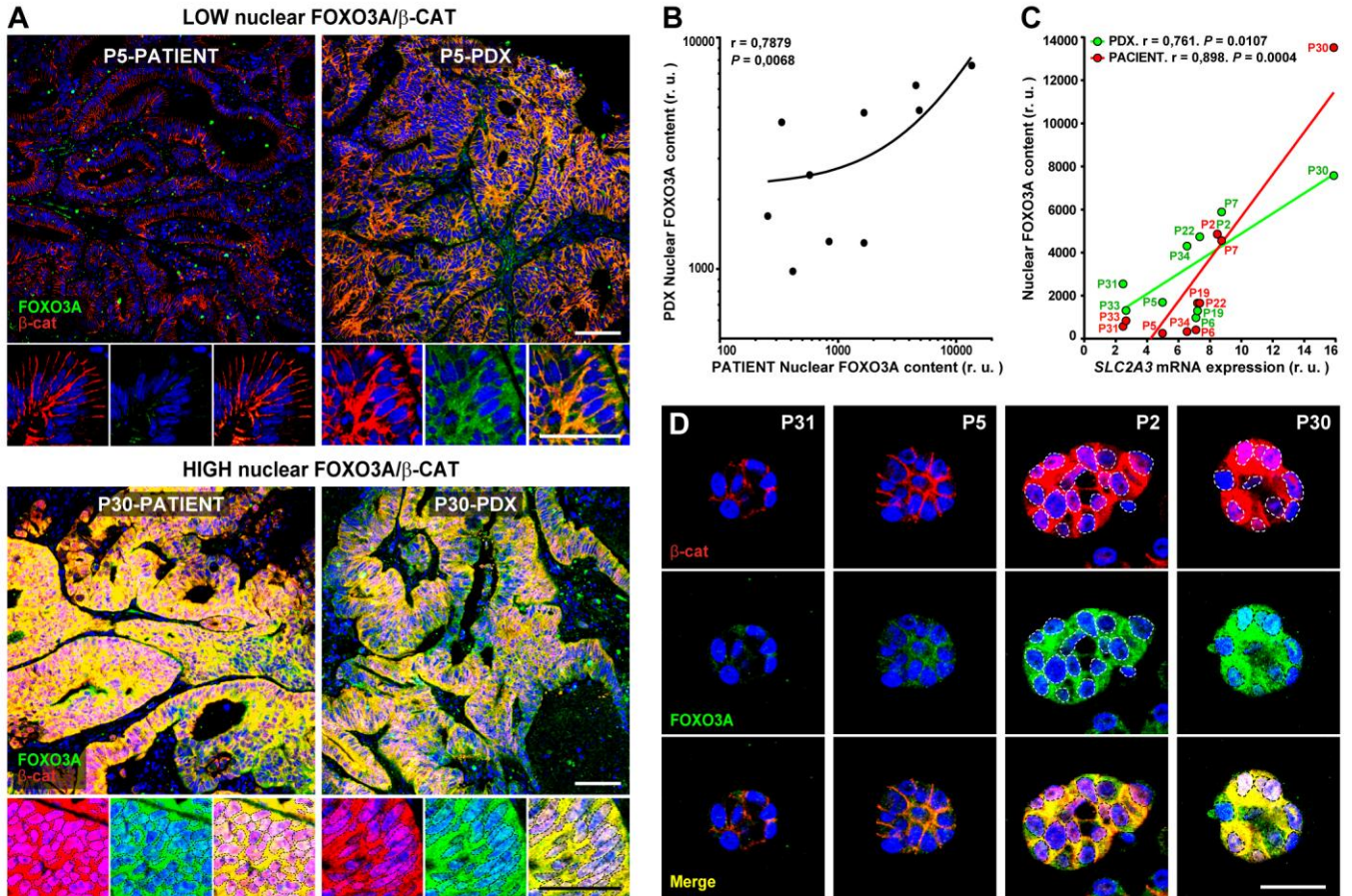


Supplementary Figure S4. NVP-TNKS656 is not toxic and reduces nuclear β -catenin in the skin and small intestine in NOD-SCID mice. (A) Graph representing body weight of NOD-SCID mice treated with vehicle (black line) or NVP-TNKS656 (red line) from one representative experiment. Error bars and $\pm$ S.D. are shown at each given time point. (B) Mice were shaved to facilitate cell injections before initiating treatments. Left pictures show two representative NOD-SCID mice treated with vehicle or NVP-TNKS656 at experiment endpoint. Right images show a representative Hematoxylin & Eosin staining of the correspondent mouse back skin. Scale bar, 200 mm. (C) Pictures showing Hematoxylin & Eosin staining of a long piece of back skin from mice treated as indicated. Black bar with an asterisk indicates an area full with growing anagen hair follicles. Scale bars, 1mm. (D) Quantification of hair follicles in anagen phase in the back skin of NOD-SCID mice treated as indicated. A minimum of 1 cm of back skin was analyzed in three mice per group of treatment. Bars indicate S.D. (E, F) Representative pictures of immunofluorescent staining and confocal microscopy to detect β -catenin (red) in histological sections of skin (E) or small intestine (F) from animals treated with vehicle or NVP-TNKS656. Lower panels are magnifications of the area highlighted in upper images with a dash-lined square and show hair follicles (E) or intestinal crypts (F). Scale bar, 100 mm; magnifications, 50 mm. Nuclei were stained with Hoechst 33342 (blue). Arrowheads indicate cells accumulating nuclear β -catenin.

SUPPLEMENTARY FIGURE 5

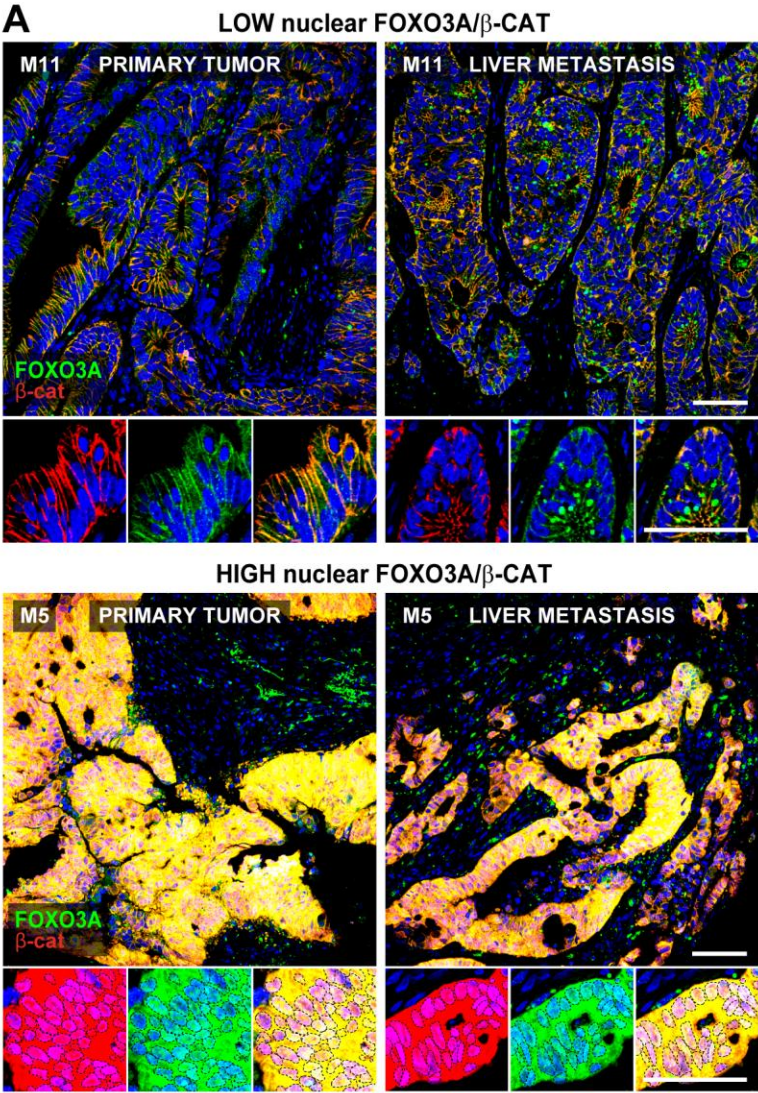


SUPPLEMENTARY FIGURE 6

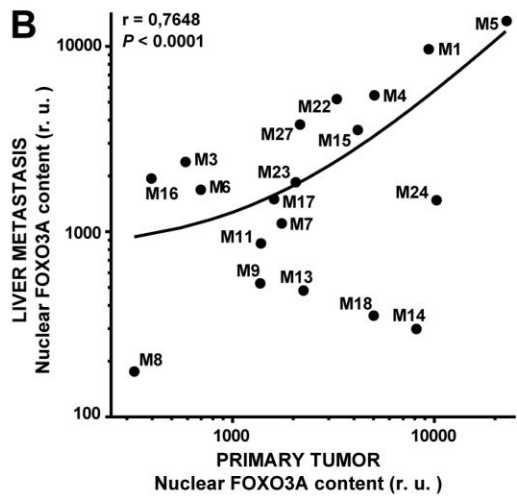


Supplementary Figure S6. Nuclear FOXO3A content is preserved in PDX and primary sphere cultures. Nuclear FOXO3A and β -catenin content was detected by immunofluorescence and confocal microscopy in ten original patients' samples and their correspondent PDX tumors (FFPE). **(A)** Representative pictures showing one case with low (P5) and one with high (P30) nuclear accumulation of both proteins. Lower panels show magnifications to better visualize β -catenin and FOXO3A subcellular localization. Dotted lines delineate nuclei stained with Hoechst 33342 (blue). Scale bars, 200 μ m; magnifications, 100 μ m. **(B)** Nuclear FOXO3A content was quantified in five pictures from the same 20 paired samples (A) and values are represented as a scatter plot comparing patients' and PDX tumors. Relative units (r. u.) were calculated as described in methods. P value was calculated by Pearson's correlation test. **(C)** Scatter plot comparing the histological amount of nuclear FOXO3A in original patient samples (red) or in the corresponding PDXs (green) with the expression of *SLC2A3* mRNA. *SLC2A3* expression was measured in PDX tumor samples by Nanostring nCounter platform. Relative units (r. u.) were calculated using nSolver software. P values correspond to a Pearson's correlation test. **(D)** Representative pictures of immunofluorescent staining and confocal microscopy for β -catenin (red) and FOXO3A (green) in primary cultured spheroids derived from the indicated patients. Dotted lines delineate nuclei stained with Hoechst 33342 (blue). Scale bars, 100 μ m.

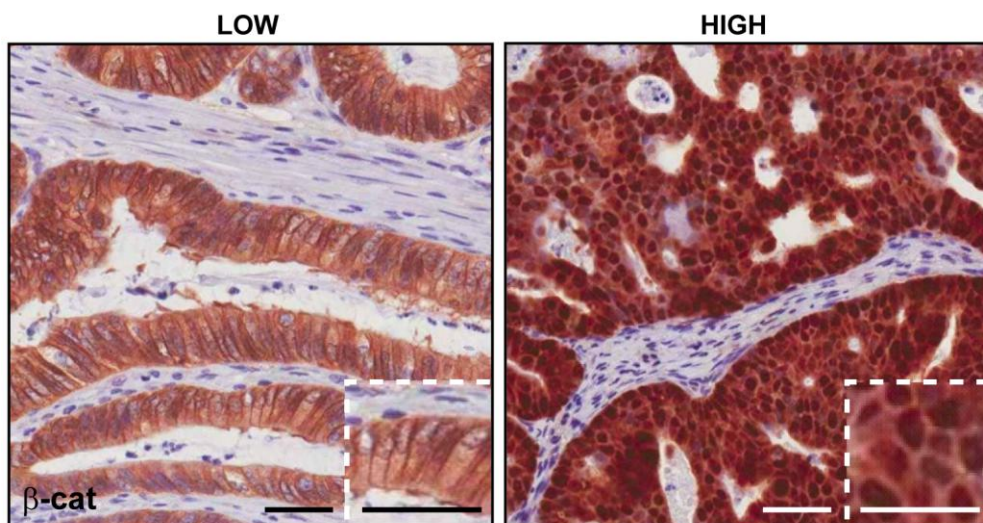
SUPPLEMENTARY FIGURE 7



Supplementary Figure S7. Nuclear FOXO3A in paired primary tumors and liver metastases. (A) Representative pictures of double immunofluorescent staining and confocal microscopy of FFPE samples from primary tumors or a liver metastases from one patient with low (M11) and one with high (M5) nuclear FOXO3A (green) and β -catenin (red) content. Lower panels show magnifications to better visualize subcellular localization. Black dotted lines delineate nuclei stained with Hoechst 33342 (blue). Scale bars, 200 mm; magnifications, 100 mm. (B) Nuclear FOXO3A content was quantified from four pictures of paired primary tumors and liver metastases from 19 patients. Average values are represented as a scatter plot comparing primary tumors and their corresponding metastases. Relative units (r. u.) were calculated as described in methods. *P* value was calculated by Pearson's correlation test.

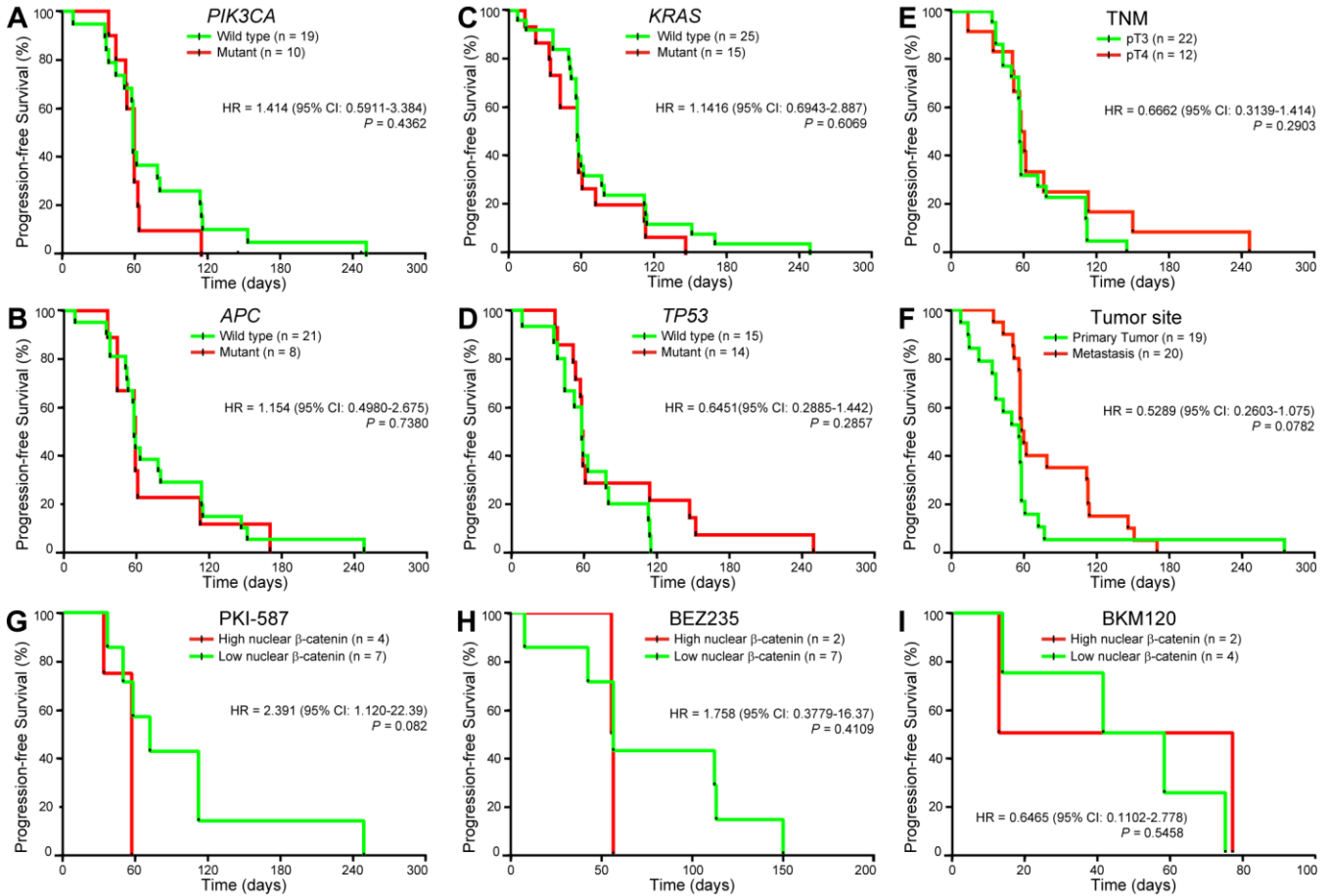


SUPPLEMENTARY FIGURE 8



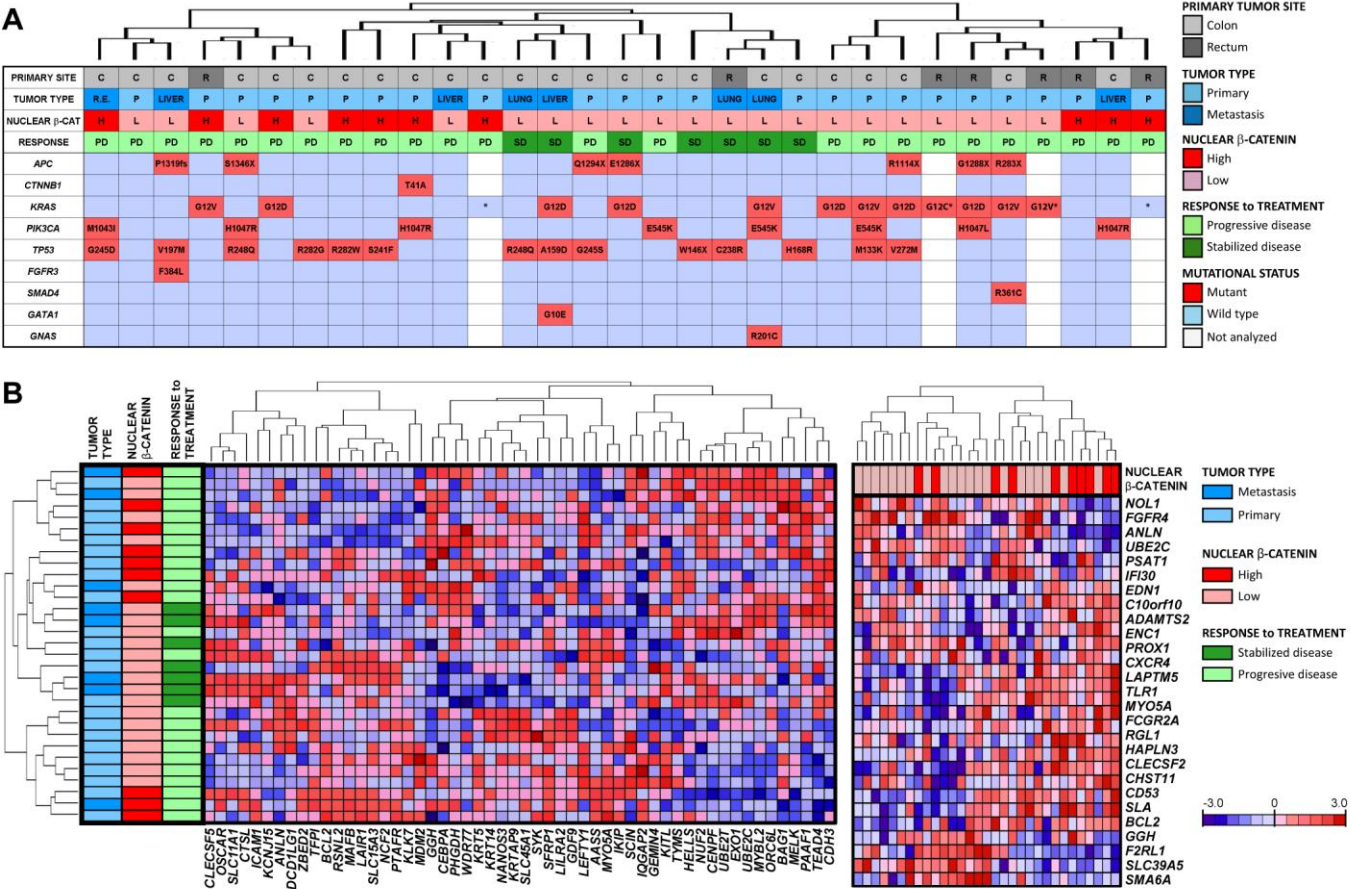
Supplementary Figure S8. Immunohistochemical staining of β -catenin in CRC tumors. (A) Representative pictures of β -catenin immunohistochemical staining of patients' tumors with low (left) or high (right) amounts of nuclear β -catenin. Inserts show magnifications to better visualize β -catenin subcellular localization. Scale bars, 100 μ m; magnifications, 50 μ m.

SUPPLEMENTARY FIGURE 9



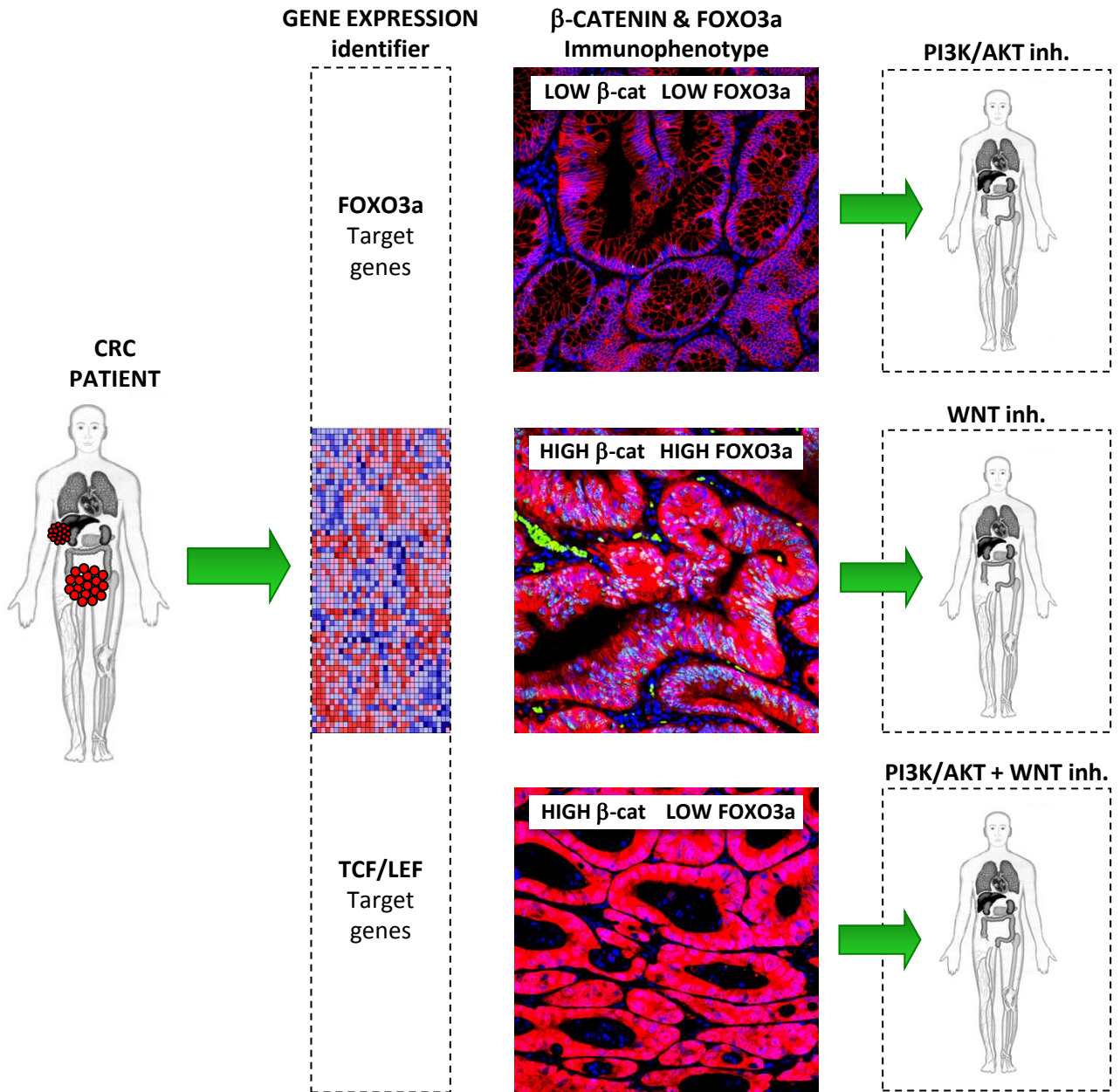
Supplementary Figure S9. β -catenin in single-agent treatments, relevant mutations, TNM stage or tumor site do not associate with a differential response to PI3K/AKT/mTOR inhibitors. Kaplan-Meier analysis representing progression-free survival (PFS) of patients separated by their mutational status in *PIK3CA* (A), *APC* (B), *KRAS* (C), *TP53* (D) genes, TNM stage (T3 or T4) (E) or the site of tumor samples (primary tumor or metastasis) (F). Kaplan-Meier analysis representing PFS of patients separated by their nuclear β -catenin content and treated in monotherapy with PKI-587 (G), BEZ235 (I) or BKM120 (H). Number of patients (n) is shown for each cohort. P value, Hazard Ratio (HR) and 95% confidence interval (CI) are shown and calculated by Log-rank (Mantel-Cox) test.

SUPPLEMENTARY FIGURE 10



Supplementary Figure S10. Gene expression clustering and mutational status of baseline tumor samples from patients treated with PI3K/AKT/mTOR inhibitors. 31 out of the initial 40 tumor samples at baseline had enough material to be analyzed for the expression of 293 genes using the Nanostring nCounter platform. (A) 54 genes were selected to order samples by hierarchical clustering using uncentered Pearson's correlation distance and complete linkage. The cluster tree was represented by the relative distance-similarities of samples. Twenty-seven out of the 31 samples were genotyped by amplicon sequencing (Supplementary Table 13) and mutations in oncogenes and tumor suppressor genes are indicated. Asterisks indicate *KRAS* genotype evaluated by Sequenom. The site of primary tumor (C, colon; R, rectum), location of metastases (R.E. retroexternal), nuclear β-catenin content (H, high; L, low) and response to treatment (PD, progressive disease; SD, stabilized disease) are indicated. (B) Left panel: Equivalent hierarchical clustering and corresponding heatmap showing the set of 54 genes selected to profile the same 31 samples (A). Right panel: A subset of 27 genes permit to cluster tumors with low and high nuclear β-catenin content. Relative gene expression scaling was indicated from low (blue) to high (red).

SUPPLEMENTARY FIGURE 11



Supplementary Figure S11. Molecular prescreening scheme to select CRC patients eligible for the treatment with PI3K/AKT and/or Wnt/ β -catenin inhibitory drugs. We propose a histological quantification of nuclear β -catenin and FOXO3A content and profiling the expression of a short set of FOXO3A/ β -catenin and TCF/ β -catenin target genes in baseline tumor samples prior treatment selection. Such molecular characterization would establish three groups of patients presenting tumors with: 1) low nuclear β -catenin and FOXO3A activity, more suitable for the treatment with PI3K or AKT inhibitors alone, 2) high β -catenin and FOXO3A activity who could benefit from Wnt/ β -catenin inhibitors alone and 3) high nuclear β -catenin but low FOXO3A activity who may benefit from combined treatments.

SUPPLEMENTARY TABLE S1

Gene Symbol	RefSeq	Residues interrogated by amplicons							
<i>ABL1</i>	NM_005157.4	M237-E258	E275-K285	G303-L324	S348-H361	M388-F401			
<i>AKT1</i>	NM_005163.2	G16-P24	L166-Y175						
<i>AKT2</i>	NM_001626.4	G16-K20	I167-Y177	T292-G304	F369-A382				
<i>AKT3</i>	NM_005465.4	G16-F27	K161-Y174						
<i>APC</i>	NM_00038.5	Q203-Q215	S280-L293	S295-H307	P865-L878	E1111-N1124	A1283-S1355	S1362-T1380	
		R1386-S1398	S1403-M141	S1421-P143	P1443-A147	E1530-S1539	E1554-K1561		
<i>BRAF</i>	NM_004333.4	K439-D445	Q461-H477	I582-F610	Q612-M620				
<i>CDH1</i>	NM_004360.3	S337-P339	Y380-Q383						
<i>CDKN2A</i>	NM_000077.4	A13-R22	L32-A73	P75-L91	H98-R112				
<i>CSF1R</i>	NM_005211.3	S298-Q307	L964-C972						
<i>CTNNB1</i>	NM_001904.3	D6-S45							
<i>EGFR</i>	NM_005228.3	I106-N115	P281-V292	P589-G601	V674-R686	A702-V726	G729-D761	E762-K823	
		R831-V843	Q849-L861						
<i>ERBB2</i>	NM_004448.2	N302-P316	G462-P481	G668-Q680	K753-S779	V839-L852			
<i>ERBB3</i>	NM_001982.3	E82-S95	N101-D112	F294-P306	C331-T342	F351-T366	W373-L384	I454-H466	
		G526-L536	T662-R675	L775-L796	K926-I938	T1169-L1177	D1259-P1272		
<i>ESR1</i>	NM_000125.3	R300-D313							
<i>FBXW7</i>	NM_033632.3	Q275-I281	T463-K472	H495-V507	H580-I593				
<i>FGFR1</i>	NM_023110.2	A100-S115	P123-D132	E126-N143	R250-A263	P469-S477			
<i>FGFR2</i>	NM_000141.4	R251-L262	P303-K313	P363-F387	K543-T555	L576-P594	N653-N662		
<i>FGFR3</i>	NM_000142.4	R248-A261	E362-V393	V390-S408	Y648-N653	L690-E702	H791-S801		
<i>FGFR4</i>	NM_002011	S123-D135							
<i>FLT3</i>	NM_004119.2	D829-S840							
<i>GATA1</i>	NM_002049.3	G7-V19	A68-P73						
<i>GNA11</i>	NM_002067.3	G207-H218							
<i>GNAQ</i>	NM_002072.4	G207-H218							
<i>GNAS</i>	NM_001077488	D197-L204	F209-F220						
<i>GSK3B</i>	NM_002093.3	D260-K271							
<i>HRAS</i>	NM_001130442	V7-L19	A59-S65						
<i>IDH1</i>	NM_005896.3	G123-Y135							
<i>IDH2</i>	NM_002168.2	P162-Q178							
<i>JAK1</i>	NM_002227.2	Q134-T147	V427-G439						
<i>JAK3</i>	NM_000215.3	L129-Q140	S720-A728	S568-S574					
<i>KIT</i>	NM_000222.2	V50-D60	T500-K509	M557-Y570	T574-F584	N649-T661	L663-Y668	A814-N828	
		Y835-Y846							
<i>KRAS</i>	NM_004985.3	Y4-G15	S17-F28	I55-S65	Y137-T148				
<i>MAG</i>	NM_080600.2	Q198-C217							
<i>MAP2K1</i>	NM_002755.3	Q116-V127							
<i>MET</i>	NM_001127500	K368-N381	V941-I953	S984-H994	S1006-E1017	H1106-K1128	R1245-N1257	V1265-K1277	
<i>MLH1</i>	NM_000249.3	S374-T386							
<i>MPL</i>	NM_005373.2	A506-Q516							
<i>MSH6</i>	NM_000179.2	P1077-L1089							
<i>MYC</i>	NM_002467.4	Q48-S61	P72-G83	G99-A111					
<i>NF2</i>	NM_000268.3	W191-A199	E206-V219	I254-I264	M334-E348	E393-R411	L458-Q470		
<i>NOTCH1</i>	NM_017617.3	S1689-V1676							
<i>NOTCH4</i>	NM_004557.3	P1970-A1989							
<i>NRAS</i>	NM_002524.4	V8-I21	D57-D69						
<i>PDGFRA</i>	NM_006206.4	R560-P577	L839-K852						
<i>PIK3CA</i>	NM_006218.2	L58-F70	E80-C90	N107-N114	F119-A120	Y343-K353	A399-G411	E418-L422	
		V448-I459	T536-W552	T898-L911	S1015-L1028	E1037-W1051			
<i>PIK3R1</i>	NM_181523.2	L573-M582							
<i>PIK3R5</i>	NM_014308.3	R19-T31							
<i>PTEN</i>	NM_000314.4	M1-K13	E40-D51	K66-L70	L98-L108	H118-F154	I168-Y180	N212-V216	
		V222-R234	K260-K267	D268-F279	S287-S302	E314-A328			
<i>RB1</i>	NM_000321.2	H129-N146	V193-K202	L317-K327	F351-S360	L452-S459	E545-M558	L569-E580	
		L657-L670	L743-S758						
<i>RET</i>	NM_020975.4	P628-V642	A764-S774	R873-G885	V915-I927				
<i>RUNX1</i>	NM_001754.4	S100-P113	R157-S167	A187-R204					
<i>SMAD4</i>	NM_005359.5	I240-N251	S343-S368	I383-G395					
<i>SMARCB1</i>	NM_003073.3	G41-R52	R155-P165						
<i>SRC</i>	NM_198291.1	S525-G533							
<i>STK11</i>	NM_000455.4	S31-K44	G47-G58	K83-N94	V133-P144	G163-H174	G187-E199	Y253-E265	
		G270-S283	P323-S334						
<i>TP53</i>	NM_000546.5	M1-D393							
<i>VHL</i>	NM_000551.3	R58-Q73	S80-R108	H115-D121	P146-P154	V155-R167			

Supplementary Table S1. Genes and positions analyzed by amplicon sequencing. A panel over 600 PCR primer pairs targeting frequent mutations in oncogenes and tumor suppressors was used to genotype genes in FFPE colorectal cancer samples. Gene symbol, transcript identifier (NM) and aminoacid sequence analyzed are indicated.

SUPPLEMENTARY TABLE S2

Genes							
ABL1	CDC27	EPHA7	GOLPH3	LRP1B	NOTCH4	RARA	SYNE1
ABL2	CDC42EP2	EPHA8	GPC3	LRP5	NPM1	RASA1	TBK1
ABCA7	CDC73	EPHB1	GPC6	MAGI2	NRAS	RASA2	TCERG1
ABCA12	CDH1	EPHB4	GRIK3	MAPK12	NSD1	RASA3	TCF7L2
ACVR1B	CDH10	EPHB6	GRIN2A	MAP2K1	NTRK1	RASA4	TEK
ACVR2A	CDK12	ERBB2	GSK3B	MAP2K2	NTRK2	RB1	TERT
ADAMTS20	CDK4	ERBB3	H3F3A	MAP2K4	NTRK3	RECQL4	TET1
AFF2	CDK5	ERBB4	HDAC2	MAP3K5	OR10R2	REL	TET2
AKT1	CDK6	ERCC2	HIF1A	MAP3K8	PAK7	RET	TET3
AKT2	CDK8	ERCC3	HMGAA2	MAP3K9	PALB2	RICTOR	TGFBR1
AKT3	CDKN2A	ERCC4	HNF1A	MAP7	PARK2	RNF43	TGFBR2
ALK	CDKN2B	ERCC5	HRAS	MCL1	PARP1	RPTOR	TMPPRSS2
ALOX12B	CDKN2C	ERG	HSP90AA1	MDM2	PAX5	RUNX1	TNFAIP3
APC	CEBPA	ESR1	IDH1	MDM4	PBRM1	RYR2	TNFRSF14
AR	CHEK1	ETV1	IDH2	MEN1	PCDH15	RYR3	TOP1
ARAF	CHEK2	ETV6	IGF1R	MET	PDGFRA	SBDS	TP53
ARHGAP26	CIC	EXT1	IGFBP7	MIER3	PDGFRB	SCN5A	TP63
ARID1A	CREBBP	EXT2	IKBKE	MITF	PDZRN3	SDHB	TSC1
ARID1B	CRKL	EZH2	IKZF1	MLH1	PHF6	SDHC	TSC2
ARID2	CRLF2	FAM123B	IL7R	MLH3	PHOX2B	SDHD	TSHR
ASXL1	CSF1R	FAM46C	INSR	MLL	PIK3C2G	SERPINA9	TTN
ATM	CSMD1	FANCA	IRS1	MLL2	PIK3CA	SERPINB1	U2AF1
ATP6V0D2	CTNNA2	FANCC	IRS2	MLL3	PIK3CB	SERPINB2	VHL
ATR	CTNNB1	FANCD2	JAK1	MLST8	PIK3CD	SERPINB3	WAS
ATRX	CYLD	FANCE	JAK2	MPL	PIK3CG	SERPINB4	WBSCR17
AURKA	DAXX	FANCF	JAK3	MSH2	PIK3R1	SERPINB5	WHSC1
AXIN1	DDR2	FANCG	JUN	MSH3	PIK3R2	SERPINE1	WHSC1L1
AXIN2	DICER1	FAS	KAT6A	MSH6	PIK3R3	SERPINI1	WRN
BAI3	DIS3	FBN1	KAT6B	MTOR	PIM1	SERPINI2	WT1
BAP1	DKK1	FBN2	KDM1A	MUC16	PKM2	SETD2	XIRP2
BARD1	DKK2	FBXO11	KDM2A	MUTYH	PLK2	SF3B1	XPA
BCL2L1	DKK3	FBXW7	KDM2B	MYB	PLK3	SHQ1	XPC
BCL6	DKK4	FGFR1	KDM3B	MYC	PMS1	SKI	XPO1
BCOR	DMD	FGFR2	KDM4A	MYCL1	PMS2	SKIL	YAP1
BIRC2	DNAH5	FGFR3	KDM4B	MYCN	PNRC1	SLC16A4	YES1
BLM	DNM2	FGFR4	KDM4C	MYD88	POLE	SLC9A9	ZIM2
BMPR1A	DNMT1	FH	KDM5A	MYO1B	PPP2R1A	SMAD2	ZNRF3
BRAF	DNMT3A	FLCN	KDM5B	NALCN	PRDM1	SMAD3	ZRSR2
BRCA1	DNMT3B	FLT1	KDM5C	NBN	PREX2	SMAD4	
BRCA2	DOCK2	FLT3	KDM6A	NCOA2	PRKAR1A	SMAD7	
BRIP1	DPP6	FMN2	KDM6B	NEB	PRKCI	SMARCA4	
BUB1B	ECT2L	FOXL2	KDM8	NF1	PTCH1	SMARCB1	
CARD11	EDNRB	FUBP1	KDR	NF2	PTEN	SMO	
CASP8	EGFR	FZD3	KEAP1	NFE2L2	PTPN11	SOCS1	
CBL	EIF4EBP1	GATA1	KIT	NFKB1	PTPN12	SOX2	
CBLB	EP300	GATA2	KLF6	NFKB2	PTPRD	SPOP	
CBLC	EPC1	GATA3	KLHDC4	NKX2-1	PTPRS	SRC	
CCND1	EPHA3	GNA11	KRAS	NOTCH1	PXDN	SRSF2	
CCNE1	EPHA5	GNAQ	LDHA	NOTCH2	RAD51	STK11	
CD79B	EPHA6	GNAS	LGR6	NOTCH3	RAF1	SUFU	

Supplementary Table S2. Genes sequenced by Haloplex Target Enrichment System. The complete coding sequence of 388 genes was sequenced by a MiSeq instrument in patient-derived colorectal cancer cells.

SUPPLEMENTARY TABLE S3

Primary antibodies

Protein	Clone	Provider	Host	Dilution	Technique
β-Catenin	14	BD Transduction Laboratories	Mouse	1:100 / 1:2000	Immunostaining
α-Catenin	EP1783Y	Abcam	Rabbit	1:100	Immunostaining
FOXO3a	GTX100277	GeneTex	Rabbit	1:100	Immunostaining
P-S6 Ribosomal Protein (Ser240/244)	#2215	Cell Signaling Technology	Rabbit	1:25	Immunostaining
Cleaved Caspase-3	9661	Cell Signaling Technology	Rabbit	1:100	Immunostaining
Ki67	MIB-1	DAKO	Mouse	1:100	Immunostaining
AXIN1	AF3287	R&D Systems	Goat	1:2000	Western blot
β-Tubulin	TUB2.1	Sigma Aldrich	Mouse	1:10000	Western blot
LAMIN A/C	sc-6215	Santa Cruz Biotechnology	Goat	1:2000	Western blot
HA-probe	sc-805	Santa Cruz Biotechnology	Rabbit	1:200	Western blot

Secondary antibodies

Antibody	Provider	Dilution	Technique
Alexa Fluor 488 goat anti-rabbit IgG	Invitrogen	1:250	Immunostaining
Alexa Fluor 555 goat anti-mouse IgG	Invitrogen	1:250	Immunostaining
Donkey anti-goat IgG HRP conjugated	Santa Cruz Biotechnology	1:2500	Western blot
Goat anti-mouse IgG HRP conjugated	Life Technologies	1:5000	Western blot
Goat anti-rabbit IgG HRP conjugated	Jackson ImmunoResearch	1:5000	Western blot

Supplementary Table S3. Antibodies. All primary and secondary antibodies against the indicated proteins used in the present study are listed next to the corresponding clone number, provider, host species, working dilution and technique in which they were used.

SUPPLEMENTARY TABLE S4

		P5	P31	P33	P6	P34	P2	P7	P19	P30	P22
% APOPTOSIS	VEHICLE	21.50	19.80	16.40	12.30	15.70	22.60	18.40	21.70	18.90	6.20
	API2	26.96	25.39	21.66	17.36	20.43	25.90	19.71	22.68	19.93	7.32
	BKM120	26.40	27.48	25.84	19.43	24.09	25.52	20.05	23.78	20.65	7.38
	TNKS656	23.52	19.08	20.63	12.09	15.49	25.08	22.35	26.76	24.26	7.14
	API2+TNKS	27.69	28.78	21.20	19.29	24.53	29.70	22.57	26.58	23.82	8.97
	BKM+TNKS	27.76	29.40	26.92	19.16	26.59	32.36	22.39	26.38	24.32	9.81
APOPTOSIS FOLD CHANGE	VEHICLE	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	API2	1.25	1.28	1.32	1.41	1.30	1.15	1.07	1.05	1.05	1.18
	BKM120	1.23	1.39	1.58	1.58	1.53	1.13	1.09	1.10	1.09	1.19
	TNKS656	1.09	0.96	1.26	0.98	0.99	1.11	1.21	1.23	1.28	1.15
	API2+TNKS	1.29	1.45	1.29	1.57	1.56	1.31	1.23	1.22	1.26	1.45
	BKM+TNKS	1.29	1.48	1.64	1.56	1.69	1.43	1.22	1.22	1.29	1.58

Supplementary Table S4. Treatment-induced apoptosis in 10 primary patient-derived sphere cell cultures. Apoptosis is indicated as percentage of Annexin V positive cells (upper table) or as fold change comparing the number of Annexin V positive cells present upon each treatment with those treated with vehicle (lower table).

SUPPLEMENTARY TABLE S5

	Frequency (%)			P2	P5	P6	P7	P19	P22	P30	P31	P33	P34
	Total	Non-hypermuted	Hypermuted										
<i>APC</i>	75	79	51	Q532*	P2831H	R1450*	R232*	E1408*	R232*	Q236*		Y935*, P1439fs*	M1413fs
<i>TP53</i>	54	60	20	P278A	R282Q	V216M			P250L				
<i>KRAS</i>	42	44	31	G13D		G12D				G13D	G12V	G12D	G12S
<i>TTN</i>	38	30	80										
<i>PIK3CA</i>	20	17	34							112_113del	E545K	H1047R	
<i>FBXW7</i>	17	11	46			K524N							
<i>SMAD4</i>	12	10	20										
<i>TCF7L2</i>	12	9	29										
<i>ACVR2A</i>	11	2	63		K435fs*					T175S			
<i>TGFBR2</i>	10	3	51		E125fs*							R528H	
<i>NRAS</i>	9	9	9										
<i>BRAF</i>	9	3	46					K601E					
<i>ARID1A</i>	9	5	34										
<i>SMAD2</i>	7	6	11										
<i>MSH3</i>	7	1	40										
<i>MSH6</i>	7	1	40		T1102fs*								
<i>ACVR1B</i>	6	4	20									G219E	
<i>ERBB3</i>	6	3	23										
<i>AXIN2</i>	6	3	23		N142S							P562L	A684V
<i>MYO1B</i>	6	1	31										
<i>MIER3</i>	5	1	29										
<i>TCERG1</i>	5	1	29	R155C									
<i>CDC27</i>	4	0	23										
<i>CASP8</i>	4	0	29	K180R									
<i>FZD3</i>	4	0	29										
<i>MAP7</i>	4	1	26										
<i>PTPN12</i>	4	1	26										
MSI Status	19	16	77	MSS	MSI	MSS	MSS	MSS	MSS	MSS	MSS	MSS	MSS

Supplementary Table S5. Genotype of patient-derived colorectal cancer models. Mutations in 27 genes considered significantly mutated in CRC, TCGA. *Nature*. 2012 (3) (http://cancergenome.broadinstitute.org/index.php?tttype=_CRC). Frequency of mutated tumors for each gene and microsatellite status are indicated (3). Percentages of mutations and microsatellite status are shown for all, hypermutated and non-hypermutated cases. All models were genotyped using Haloplex Target Enrichment System and a MiSeq instrument (Illumina) with the exception of PDX-P19 and PDX-P31 that were genotyped by Sequenom. Microsatellite status was analyzed as described in Methods section. MSS, Microsatellite Stable; MSI, Microsatellite Unstable. Asterisks indicate stopgain mutations.

SUPPLEMENTARY TABLE S6

Patient	Gene	Transcript	CDS Mutation	AA Mutation	Type	Polyphen	Position
P2	APC	NM_000038	c.C1594T	p.Q532*	stopgain SNV	0.735401	5:112163671-112163671
	KRAS	NM_033360	c.G38A	p.G13D	nonsynonymous SNV	0.998	12:25398281-25398281
	FOXP1	NM_032682	c.A1427C	p.Q476P	nonsynonymous SNV	0.999	3:71026795-71026795
	CASP8	NM_033356	c.A443G	p. K180R	nonsynonymous SNV	0.563	2:202137392-202137392
	JUN	NM_002228	c.C658G	p.P220A	nonsynonymous SNV	0.994	1:59248085-59248085
	NDRG1	NM_001135242	c.G574A	p.V192M	nonsynonymous SNV	0.15	8:134266802-134266802
	TCERG1	NM_001040006	c.C463T	p.R155C	nonsynonymous SNV	0.788948	5:145838471-145838471
	TP53	NM_00546	c.C436G	p.P278A	nonsynonymous SNV	1.0	17:7577106-7577106
P5	APC	NM_000038	c.C8492A	p.P2831H	nonsynonymous SNV	0.999	5:112179783-112179783
	ABL1	NM_0015157	c.G505T	p.G169W	nonsynonymous SNV	0.999	9:133730439-133730439
	ACVR2A	NM_001616	c.1303_1304del	p.K453fs	frameshift deletion	n. d.	2:148683686-148683687
	AKT2	NM_001626	c.G1036A	p.G346S	nonsynonymous SNV	0.994	19:40741936-40741936
	ARHGEF12	NM_015313	c.C1871T	p.A624V	nonsynonymous SNV	0.171153	11:120322248-120322248
	ARID2	NM_152641	c.C853T	p.R285W	nonsynonymous SNV	1.0	12:46230604-46230604
	ATM	NM_000051	c.C8762T	p.T2921M	nonsynonymous SNV	0.994	11:108224583-108224583
	AXIN2	NM_004655	c.A425G	p.N142S	nonsynonymous SNV	0.469	17:63554314-63554314
	C15orf55	NM_175741	c.G1872A	p.W624*	stopgain SNV	0.735505	15:34648165-34648165
	DDX5	NM_004396	c.C202T	p.R68C	nonsynonymous SNV	0.984	17:62500803-62500803
	DICER1	NM_177438	c.G3451A	p.V1151M	nonsynonymous SNV	0.395	14:95570282-95570282
	ETV6	NM_001987	c.T1173A	p.Y391*	stopgain SNV	0.609824	12:12038880-12038880
	EXT1	NM_000127	c.T677C	p.F226S	nonsynonymous SNV	0.984	8:119122609-119122609
	FIP1L1	NM_030917	c.G1550A	p.R517H	nonsynonymous SNV	0.555808	4:54324870-54324870
	IDH2	NM_002168	c.G179A	p.R60H	nonsynonymous SNV	0.415	15:90634813-90634813
	KIAA1549	NM_020910	c.1454delT	p.F485fs	frameshift deletion	n. d.	7:138602918-138602918
	MAFB	NM_005461	c.G846T	p.Q282H	nonsynonymous SNV	0.0	20:39316645-39316645
	MSH6	NM_000179.2	c.3306delT	T1102fs	frameshift deletion	n. d.	2:48030692-48030692
	NUP214	NM_005085	c.A2731G	p.S911G	nonsynonymous SNV	0.036	9:134039264-134039264
	PDGFRA	NM_006206	c.G1499A	p.R500Q	nonsynonymous SNV	0.999	4:55139838-55139838
	SMARCA4	NM_003072	c.C3236T	p.S1079L	nonsynonymous SNV	0.978	19:11138480-11138480
	TP53	NM_00546	c.G449A	p.R282Q	nonsynonymous SNV	1.0	17:7577093-7577093
	TCF3	NM_003200	c.G1515T	p.E505D	nonsynonymous SNV	0.0050	19:1615756-1615756
	TGFBR2	NM_003242	c.374_375del	p.E125fs	frameshift deletion	n. d.	3:30691872-30691873
	TFPT	NM_013342	c.C59T	p.A20V	nonsynonymous SNV	0.036	19:54618045-54618045
P30	AADACL3	NM_001103169	c.C667T	p.R223W	nonsynonymous SNV	1.0	1:12785786-12785787
	ABCA8	NM_007168	c.G1966A	p.A656T	nonsynonymous SNV	0.0010	17:66913553-66913554
	ABCC12	NM_033226	c.C3284T	p.S1095L	nonsynonymous SNV	0.0	16:48125031-48125032
	ACTR5	NM_024855	c.G434A	p.R145Q	nonsynonymous SNV	0.989	20:37378710-37378711
	ACVR2A	NM_001616	c.C524G	p.T175S	nonsynonymous SNV	0.0030	2:148657462-148657463
	ADAMTS12	NM_030955	c.G3559A	p.A1187T	nonsynonymous SNV	0.0060	5:33576571-33576572
	APC	NM_000038	c.C706T	p.Q236*	stopgain SNV	0.735307	5:112128202-112128203
	APC	NM_000039	c.3804_3805del	p.1268_1269del	frameshift deletion	n. d.	5:112175095-112175097
	BCAR3	NM_003567	c.C607T	p.R203W	nonsynonymous SNV	0.986	1:94054855-94054856
	C6orf136	NM_145029	c.G29T	p.R10L	nonsynonymous SNV	0.153	6:30615036-30615037
	CCDC147	NM_001008723	c.C1601G	p.S534C	nonsynonymous SNV	0.053	10:106153159-106153160
	CCR3	NM_178328	c.G931T	p.A311S	nonsynonymous SNV	0.422	3:46307516-46307517
	CCT8L2	NM_014406	c.C448A	p.L150M	nonsynonymous SNV	0.984	22:17072992-17072993
	COL22A1	NM_152888	c.G3214T	p.G1072C	nonsynonymous SNV	0.787343	8:139674298-139674299
	COL5A3	NM_015719	c.C4123T	p.P1375S	nonsynonymous SNV	0.463485	19:10080053-10080054
	CUBN	NM_001081	c.C9592A	p.H3198N	nonsynonymous SNV	0.0020	10:16893304-16893305
	DCAKD	NM_024819	c.C562G	p.Q188E	nonsynonymous SNV	0.997	17:43101934-43101935
	DOCK8	NM_001190458	c.C334T	p.R112C	nonsynonymous SNV	0.513	9:311962-311963
	DUSP9	NM_001395	c.G692A	p.R231H	nonsynonymous SNV	0.977	X:152915004-152915005
	FAM212B	NM_019099	c.G563A	p.R188H	nonsynonymous SNV	0.0060	1:112269920-112269921
	FILIP1L	NM_182909	c.C432A	p.D144E	nonsynonymous SNV	0.993	3:99643246-99643247
	FLVCR1	NM_014053	c.G1307T	p.G436V	nonsynonymous SNV	0.983	1:213061342-213061343
	GJA8	NM_005267	c.G1027A	p.E343K	nonsynonymous SNV	0.256	1:147381108-147381109
	HEATR5B	NM_019024	c.C4457T	p.A1486V	nonsynonymous SNV	0.303	2:37235818-37235819
	HELZ	NM_014877	c.C5501T	p.P1834L	nonsynonymous SNV	0.621287	17:65074695-65074696
	HLA-B	NM_005514	c.294_295insA	p.D98fs	frameshift insertion	n. d.	6:31324514-31324515
	IGSF3	NM_001542	c.C790T	p.R264*	stopgain SNV	0.725318	1:117156428-117156429
	KRAS	NM_004985	c.G38A	p.G13D	nonsynonymous SNV	0.994	12:25398280-25398281
	KRTAP5-10	NM_001012710	c.A92G	p.Y31C	nonsynonymous SNV	0.406626	11:71276724-71276725

SUPPLEMENTARY TABLE S6 (Continued)

Patient	Gene	Transcript	CDS Mutation	AA Mutation	Type	Polyphen	Position
P30	<i>LRP11</i>	NM_032832	c.G844A	p.D282N	nonsynonymous SNV	0.999	6:150164187-150164188
	<i>MMRN1</i>	NM_007351	c.C1957A	p.L653I	nonsynonymous SNV	0.042	4:90856787-90856788
	<i>NF1</i>	NM_000267	c.C2044T	p.Q682*	stopgain SNV	0.735393	17:29553494-29553495
	<i>NRG3</i>	NM_001010848	c.G292A	p.V98M	nonsynonymous SNV	0.728	10:83635387-83635388
	<i>PAMR1</i>	NM_015430	c.303delT	p.G101fs	frameshift deletion	n. d.	11:35513669-35513670
	<i>PCBP1</i>	NM_006196	c.T299A	p.L100Q	nonsynonymous SNV	0.993	2:70315173-70315174
	<i>PIK3CA</i>	NM_006218	c.334_336del	p.112_112del	nonframeshift deletion	n. d.	3:178916947-178916950
	<i>PKD2L2</i>	NM_014386	c.T1025G	p.F342C	nonsynonymous SNV	0.125	5:137243479-137243480
	<i>PLEC</i>	NM_201381	c.G12859T	p.G4287W	nonsynonymous SNV	1.0	8:144991033-144991034
	<i>PLEKHF1</i>	NM_024310	c.C94T	p.P32S	nonsynonymous SNV	0.133	19:30164839-30164840
	<i>RBM10</i>	NM_001204466	c.G17A	p.R6H	nonsynonymous SNV	0.727115	X:47006896-47006897
	<i>RIPK1</i>	NM_003804	c.G800T	p.C267F	nonsynonymous SNV	0.999	6:3085603-3085604
	<i>RNF182</i>	NM_001165032	c.C662T	p.S221L	nonsynonymous SNV	0.688	6:13978011-13978012
	<i>RP1L1</i>	NM_178857	c.C1477T	p.R493W	nonsynonymous SNV	0.0050	8:10470130-10470131
	<i>RRNAD1</i>	NM_015997	c.G775A	p.A259T	nonsynonymous SNV	0.014	1:156703938-156703939
	<i>SLC16A8</i>	NM_013356	c.C1400T	p.P467L	nonsynonymous SNV	0.088	22:38474509-38474510
	<i>SLC39A6</i>	NM_012319	c.G2248A	p.V750M	nonsynonymous SNV	0.937	18:33689575-33689576
	<i>SLITRK5</i>	NM_015567	c.A91G	p.T31A	nonsynonymous SNV	0.569	13:88327733-88327734
	<i>SPANXN2</i>	NM_001009615	c.G337A	p.E113K	nonsynonymous SNV	0.071464	X:142795340-142795341
	<i>SREK1</i>	NM_001077199	c.C449T	p.A150V	nonsynonymous SNV	0.074	5:65457973-65457974
	<i>TGIF1</i>	NM_003244	c.C494A	p.S165*	stopgain SNV	0.734866	18:3457612-3457613
	<i>TPTE</i>	NM_199261	c.C1130A	p.T377N	nonsynonymous SNV	0.17	21:10920123-10920124
	<i>TRAF3IP2</i>	NM_001164282	c.C374T	p.P125L	nonsynonymous SNV	0.916	6:111880707-111880708
	<i>TSHZ2</i>	NM_001193421	c.G2245A	p.V749M	nonsynonymous SNV	0.813	20:51872250-51872251
	<i>UBR5</i>	NM_015902	c.A3637G	p.R1213G	nonsynonymous SNV	0.99	8:103309148-103309149
	<i>ZC3H7B</i>	NM_017590	c.G1163A	p.R388H	nonsynonymous SNV	0.0020	22:41737115-41737116
	<i>ZNF256</i>	NM_005773	c.G1784A	p.R595Q	nonsynonymous SNV	0.952	19:58452391-58452392

Supplementary Table S6. Exome sequencing of colorectal cancer patient-derived models. The Exome of PDX-P2, P5 and P30 models was sequenced using a MiSeq instrument (Illumina). Gene symbol, transcript identifier (NM), mutations (CDS, Coding DNA Sequence; AA, Aminoacid), type of mutation, polyphen and genomic position of each alteration are shown. Polyphen is a score which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations (<http://genetics.bwh.harvard.edu/pph2/>).

SUPPLEMENTARY TABLE S7

Gene Symbol	Fold-change		Gene Symbol	Fold-change		Gene Symbol	Fold-change	
	TNKS656 vs. Vehicle	p-value		TNKS656 vs. Vehicle	p-value		TNKS656 vs. Vehicle	p-value
<i>IGFL4</i>	3.82	0.003	<i>PLEKHA2</i>	-1.50	0.031	<i>ID3</i>	-1.92	0.038
<i>GRB10</i>	2.94	0.029	<i>TFF3</i>	-1.51	0.027	<i>HIST1H2BM</i>	-1.92	0.016
<i>GDF15</i>	2.77	0.049	<i>HoxA11</i>	-1.52	0.032	<i>F10</i>	-1.95	0.027
<i>HBEGF</i>	2.50	0.026	<i>C9orf152</i>	-1.52	0.002	<i>GUCA2A</i>	-1.96	0.026
<i>HAS2</i>	2.34	0.017	<i>TPM2</i>	-1.53	0.004	<i>TAF1D</i>	-1.97	0.015
<i>RIPK2</i>	2.28	0.018	<i>ZDHHC2</i>	-1.53	0.006	<i>TLR3</i>	-1.97	0.006
<i>PMP22</i>	2.26	0.016	<i>KIAA0226L</i>	-1.53	0.027	<i>ARSE</i>	-2.03	0.009
<i>SLC7A11</i>	2.25	0.011	<i>PDK3</i>	-1.53	0.015	<i>GPER</i>	-2.06	0.011
<i>CLIP4</i>	2.09	0.014	<i>RNU2-6P</i>	-1.53	0.005	<i>LRP4</i>	-2.13	0.022
<i>VSNL1</i>	1.99	0.017	<i>ACVR1C</i>	-1.54	0.009	<i>LY6G6D</i>	-2.13	0.033
<i>PSAT1</i>	1.93	0.000	<i>MAB21L3</i>	-1.54	0.015	<i>CTTNBP2</i>	-2.16	0.005
<i>CHAC1</i>	1.92	0.010	<i>TLN2</i>	-1.54	0.015	<i>PHLDB2</i>	-2.28	0.034
<i>ASNS</i>	1.91	0.003	<i>FGGY</i>	-1.54	0.001	<i>SPTSSB</i>	-2.31	0.007
<i>CASP5</i>	1.86	0.020	<i>SNCAIP</i>	-1.54	0.013	<i>MAP1B</i>	-2.35	0.046
<i>MUC12</i>	1.85	0.015	<i>IL3RA</i>	-1.55	0.029	<i>ACOX2</i>	-2.42	0.027
<i>GCNT3</i>	1.83	0.038	<i>VAV3</i>	-1.56	0.039	<i>CYP2B6</i>	-2.49	0.029
<i>WEE1</i>	1.83	0.044	<i>AXIN2</i>	-1.56	0.009	<i>PDE5A</i>	-2.49	0.011
<i>F5</i>	1.82	0.034	<i>CA9</i>	-1.57	0.012	<i>C10orf99</i>	-2.60	0.030
<i>PLEKHG1</i>	1.81	0.008	<i>ATP10D</i>	-1.57	0.014	<i>TMEM211</i>	-2.66	0.011
<i>PYROXD1</i>	1.75	0.015	<i>FAIM2</i>	-1.59	0.007	<i>NPY4R</i>	-3.43	0.017
<i>SLC9A2</i>	1.69	0.010	<i>GMFG</i>	-1.59	0.037	<i>NOX1</i>	-4.08	0.015
<i>ZNF292</i>	1.68	0.042	<i>EPHB3</i>	-1.62	0.012	<i>NELL2</i>	-4.95	0.002
<i>MUC17</i>	1.65	0.006	<i>CLIC3</i>	-1.62	0.036	<i>HMGCS2</i>	-6.23	0.003
<i>LURAP1L</i>	1.64	0.014	<i>FABP3</i>	-1.62	0.035			
<i>GEM</i>	1.62	0.002	<i>HoxB3</i>	-1.64	0.002			
<i>FBXO32</i>	1.62	0.049	<i>QPRT</i>	-1.64	0.018			
<i>DSC3</i>	1.61	0.038	<i>MLEC</i>	-1.64	0.048			
<i>LACTB2</i>	1.61	0.040	<i>LOC10106062</i>	-1.64	0.047			
<i>LOC100996822</i>	1.59	0.038	<i>SNORA74A</i>	-1.65	0.001			
<i>TMC7</i>	1.59	0.049	<i>FOLR1</i>	-1.66	0.024			
<i>STC2</i>	1.58	0.008	<i>DLGAP1-AS1</i>	-1.68	0.010			
<i>NOS3</i>	1.58	0.002	<i>KIAA1147</i>	-1.69	0.024			
<i>SNX2</i>	1.58	0.001	<i>DDC</i>	-1.69	0.015			
<i>AREG</i>	1.58	0.049	<i>FRMPD3</i>	-1.70	0.007			
<i>FAM177A1</i>	1.57	0.009	<i>HoxA5</i>	-1.71	0.044			
<i>TRIB3</i>	1.57	0.032	<i>CYP2B7P1</i>	-1.71	0.005			
<i>GPCPD1</i>	1.56	0.001	<i>LOC280665</i>	-1.74	0.018			
<i>HERPUD1</i>	1.56	0.034	<i>HoxA7</i>	-1.74	0.034			
<i>IL6R</i>	1.56	0.007	<i>HSPA12A</i>	-1.74	0.011			
<i>IMPAD1</i>	1.55	0.048	<i>SLC39A8</i>	-1.74	0.001			
<i>IMPA1</i>	1.54	0.006	<i>SLC6A4</i>	-1.75	0.001			
<i>CA2</i>	1.54	0.024	<i>APOLD1</i>	-1.75	0.028			
<i>ZNF382</i>	1.54	0.009	<i>RNASE1</i>	-1.76	0.009			
<i>SLC31A1</i>	1.54	0.001	<i>HNF1A-AS1</i>	-1.77	0.010			
<i>DNM3</i>	1.53	0.006	<i>RASL11A</i>	-1.78	0.005			
<i>DOCK4</i>	1.53	0.005	<i>GPA33</i>	-1.80	0.025			
<i>SLC20A2</i>	1.52	0.020	<i>HUNK</i>	-1.80	0.003			
<i>TCP11L2</i>	1.52	0.024	<i>S100A4</i>	-1.80	0.028			
<i>NSMCE2</i>	1.52	0.012	<i>KRT31</i>	-1.82	0.022			
<i>MTHFD2</i>	1.52	0.003	<i>RNA5SP473</i>	-1.84	0.006			
<i>SLC16A4</i>	1.51	0.016	<i>AQP3</i>	-1.87	0.016			
<i>SESTD1</i>	1.50	0.034	<i>SNORD51</i>	-1.89	0.011			

Supplementary Table S7. Genes regulated by NVP-TNKS656 in PDX-P2. Microarray analyses revealed 127 genes regulated by NVP-TNKS656 in subcutaneous tumors generated with PDX-P2 model. Genes listed correspond to those differentially expressed at least 1.5 fold in tumors from NVP-TNKS656-treated versus vehicle-treated mice. *P* values are the result of one-way ANOVA. Official HUGO Gene Symbols are shown.

SUPPLEMENTARY TABLE S8

Fold change			<i>continued</i>		
Gene Symbol	TNKS656 vs. Vehicle	P value	Gene Symbol	TNKS656 vs. Vehicle	P value
PRUNE2	2.51	0.033	SLC2A14	-1.55	0.026
KIAA1324	2.46	0.040	ARSK	-1.55	0.017
DACH1	2.24	0.022	ARHGAP42	-1.55	0.041
DUOX2	2.17	0.005	MPP6	-1.56	0.016
SLC38A4	2.14	0.009	RNA5SP374	-1.56	0.013
LRRC19	2.12	0.006	KIRREL	-1.57	0.000
TOX	1.95	0.007	BAMBI	-1.58	0.010
ZDHHC2	1.90	0.018	CATSPERB	-1.58	0.021
GRM8	1.86	0.034	GABRA3	-1.58	0.000
CDHR1	1.85	0.010	GPR126	-1.59	0.004
SPINK1	1.83	0.003	WWTR1	-1.59	0.024
TMEM71	1.79	0.004	GCNT4	-1.60	0.031
METTL7A	1.77	0.003	SLC35D3	-1.61	0.021
IQGAP2	1.77	0.000	KIF3A	-1.61	0.007
LOC93432	1.77	0.049	MBOAT2	-1.62	0.026
PARM1	1.75	0.034	IL1R2	-1.62	0.022
NFATC2	1.73	0.003	AFAP1L2	-1.63	0.002
ENPP3	1.73	0.017	DSG3	-1.64	0.049
PHC3	1.73	0.038	SLPI	-1.64	0.050
PRLR	1.69	0.022	SEMA3A	-1.64	0.016
CEL	1.67	0.015	WWC2	-1.64	0.008
TM4SF20	1.67	0.037	PODXL	-1.68	0.002
NPY4R	1.64	0.030	NCF2	-1.70	0.046
TESC	1.61	0.039	ADAM19	-1.72	0.033
PGAP1	1.60	0.024	WNT11	-1.73	0.005
NXPE4	1.60	0.029	STS	-1.78	0.006
PAH	1.60	0.009	MSLN	-1.78	0.043
DCDC2	1.59	0.007	FLNA	-1.79	0.005
RPL17	1.58	0.002	B3GALT2	-1.79	0.021
AOAH	1.57	0.045	FRMD5	-1.80	0.048
ADH6	1.57	0.049	SYTL5	-1.85	0.035
CYP27A1	1.57	0.001	ASB4	-1.89	0.010
MIR95	1.56	0.023	S100A4	-1.91	0.011
MEP1A	1.56	0.012	EPHB6	-1.99	0.020
XDH	1.56	0.008	SLCO1B3	-2.00	0.049
MIR192	1.55	0.037	DCBLD2	-2.14	0.012
SNORA28	1.55	0.035	CD109	-2.15	0.032
PDK4	1.54	0.005	ARHGAP29	-2.17	0.004
DAPK2	1.54	0.009	PLK2	-2.25	0.006
CYP2B6	1.53	0.026	CSMD3	-2.28	0.005
SLC5A1	1.52	0.028	SLCO1B1	-2.36	0.039
COL28A1	1.51	0.005	RPTN	-2.40	0.032
MIRLET7G	1.50	0.005	CST7	-2.44	0.042
TMEM37	1.50	0.033	PPBP	-2.49	0.008
PPARGC1A	1.50	0.018	SLCO1B7	-2.69	0.041
DYNLT3	-1.50	0.008	CAV1	-3.17	0.005
MALT1	-1.52	0.012	CAV2	-3.46	0.016
PRKCDBP	-1.52	0.050	DKK1	-3.91	0.019
EREG	-1.53	0.033	SLC2A3	-4.34	0.023
CCDC68	-1.53	0.012			
PAQR3	-1.53	0.039			
KRT80	-1.53	0.049			
PLAU	-1.54	0.027			
ECI2	-1.54	0.009			
PCSK5	-1.55	0.000			

Supplementary Table S8. Genes regulated by NVP-TNKS656 in PDX-P30.

Microarray analyses revealed 104 genes regulated by NVP-TNKS656 in subcutaneous tumors generated with PDX-P30 model. Genes listed correspond to those differentially expressed at least 1.5 fold in tumors from NVP-TNKS656-treated versus vehicle-treated mice. *P* values are the result of one-way ANOVA. Official HUGO Gene Symbols are shown.

SUPPLEMENTARY TABLE S9

Fold change			<i>continued</i>		
Gene Symbol	TNKS656 vs. Vehicle	P value	Gene Symbol	TNKS656 vs. Vehicle	P value
<i>SLC26A3</i>	6.35	0.048	<i>SEPP1</i>	1.63	0.047
<i>ANXA10</i>	4.23	0.020	<i>HNFB4G</i>	1.62	0.035
<i>CEACAM7</i>	4.21	0.023	<i>ENTPD8</i>	1.61	0.048
<i>TM4SF4</i>	3.63	0.022	<i>C1orf106</i>	1.61	0.019
<i>FER1L6</i>	3.26	0.024	<i>P2RX4</i>	1.61	0.047
<i>PCK1</i>	3.25	0.027	<i>NAAA</i>	1.60	0.038
<i>ALDOB</i>	3.10	0.038	<i>CYP4F12</i>	1.60	0.049
<i>CDHR2</i>	3.02	0.035	<i>APOL6</i>	1.59	0.003
<i>TRIM31</i>	2.99	0.010	<i>BCMO1</i>	1.57	0.028
<i>UGT2B11</i>	2.84	0.028	<i>C12orf23</i>	1.56	0.009
<i>TFF2</i>	2.69	0.049	<i>TLR3</i>	1.56	0.032
<i>FABP1</i>	2.65	0.022	<i>GPR110</i>	1.56	0.011
<i>SMPDL3A</i>	2.64	0.030	<i>SPPL2A</i>	1.56	0.033
<i>PDK4</i>	2.56	0.000	<i>ABHD17B</i>	1.55	0.002
<i>RNF152</i>	2.50	0.038	<i>CREBRF</i>	1.55	0.034
<i>MEP1B</i>	2.48	0.028	<i>SAR1B</i>	1.55	0.046
<i>DDX60L</i>	2.47	0.046	<i>SNORD116-1</i>	1.55	0.046
<i>GCNT4</i>	2.42	0.009	<i>IL10RB</i>	1.54	0.040
<i>GPR128</i>	2.39	0.044	<i>UGCG</i>	1.54	0.050
<i>TRANK1</i>	2.36	0.021	<i>KLHL24</i>	1.54	0.013
<i>BIRC3</i>	2.20	0.011	<i>TMOD1</i>	1.54	0.015
<i>TNFSF15</i>	2.19	0.044	<i>FAM114A1</i>	1.54	0.043
<i>GK</i>	2.18	0.029	<i>C6orf222</i>	1.53	0.022
<i>TMEM45A</i>	2.13	0.000	<i>ZNF587</i>	1.53	0.008
<i>MEP1AP2</i>	1.99	0.005	<i>LYSMD3</i>	1.52	0.001
<i>CDHR5</i>	1.96	0.040	<i>STYK1</i>	1.52	0.031
<i>PDE5A</i>	1.96	0.042	<i>SESTD1</i>	1.51	0.009
<i>MGAT4A</i>	1.95	0.036	<i>RHOA</i>	1.51	0.002
<i>XKR9</i>	1.91	0.048	<i>LGALS9C</i>	1.51	0.027
<i>DDX60</i>	1.89	0.021	<i>SNRPN</i>	1.51	0.003
<i>SULT1B1</i>	1.89	0.042	<i>MPZL3</i>	1.50	0.025
<i>GRAMD1C</i>	1.89	0.001	<i>NCAPG2</i>	-1.50	0.046
<i>TCN2</i>	1.89	0.041	<i>KIAA0020</i>	-1.50	0.026
<i>SLC2A13</i>	1.88	0.047	<i>CMSS1</i>	-1.52	0.023
<i>SPINK1</i>	1.86	0.002	<i>VRK1</i>	-1.52	0.042
<i>SLC17A5</i>	1.85	0.038	<i>PLA2G2A</i>	-1.52	0.039
<i>MB21D2</i>	1.83	0.004	<i>ALDOC</i>	-1.52	0.008
<i>ACVR2A</i>	1.82	0.025	<i>CXCL1</i>	-1.54	0.018
<i>SECTM1</i>	1.81	0.004	<i>MT2A</i>	-1.55	0.049
<i>HSD17B11</i>	1.79	0.033	<i>ARHGAP11B</i>	-1.55	0.026
<i>LIPH</i>	1.79	0.038	<i>MLF1IP</i>	-1.56	0.013
<i>DNAJB4</i>	1.78	0.018	<i>CIRH1A</i>	-1.56	0.002
<i>LOC93432</i>	1.78	0.026	<i>SLC9B2</i>	-1.57	0.002
<i>YPEL2</i>	1.78	0.022	<i>TDO2</i>	-1.58	0.011
<i>C3orf79</i>	1.77	0.017	<i>RNA5SP63</i>	-1.58	0.011
<i>MIER3</i>	1.77	0.023	<i>KIF23</i>	-1.60	0.049
<i>CNKSR3</i>	1.74	0.015	<i>PYGL</i>	-1.61	0.002
<i>HCFC2</i>	1.73	0.009	<i>TRMT112</i>	-1.62	0.045
<i>IGFBP3</i>	1.73	0.015	<i>LY6G5B</i>	-1.63	0.041
<i>GCNT3</i>	1.73	0.005	<i>TYMS</i>	-1.66	0.042
<i>TCP11L2</i>	1.72	0.045	<i>CDKN3</i>	-1.69	0.031
<i>UGT2B15</i>	1.72	0.017	<i>ANXA3</i>	-1.69	0.011
<i>RASA4CP</i>	1.72	0.032	<i>SLC6A14</i>	-1.74	0.047
<i>ASTE1</i>	1.72	0.034	<i>WEE1</i>	-1.75	0.038
<i>RASSF6</i>	1.69	0.049	<i>RNA5SP172</i>	-1.84	0.029
<i>SMIM14</i>	1.68	0.008			
<i>PRAP1</i>	1.67	0.029			
<i>LRRRC19</i>	1.67	0.041			
<i>ARRDC4</i>	1.66	0.010			
<i>TXNIP</i>	1.66	0.022			
<i>TOR1AIP2</i>	1.65	0.046			
<i>HBD</i>	1.64	0.016			

Supplementary Table S9. Genes regulated by NVP-TNKS656 in PDX-P5.

Microarray analyses revealed 117 genes regulated by NVP-TNKS656 in subcutaneous tumors generated with PDX-P5 model. Genes listed correspond to those differentially expressed at least 1.5 fold in tumors from NVP-TNKS656-treated versus vehicle-treated mice. *P* values are the result of one-way ANOVA. Official HUGO Gene Symbols are shown.

SUPPLEMENTARY TABLE S10

Gene Symbol

<i>AIMP2</i>	<i>FAM89B</i>	<i>NOC3L</i>	<i>SRM</i>
<i>AMPD2</i>	<i>FARSA</i>	<i>NOP16</i>	<i>SRPRB</i>
<i>APEX1</i>	<i>FOXQ1</i>	<i>NOP2</i>	<i>SRSF7</i>
<i>ARID5B</i>	<i>FUT4</i>	<i>NOP56</i>	<i>STAMBPL1</i>
<i>ASCL2</i>	<i>GAR1</i>	<i>NPRL3</i>	<i>TBX3</i>
<i>AURKB</i>	<i>GEMIN4</i>	<i>NUDCD3</i>	<i>TDGF1</i>
<i>AXIN2</i>	<i>GEMIN5</i>	<i>NXT1</i>	<i>TEAD4</i>
<i>BAG2</i>	<i>GEMIN6</i>	<i>ODC1</i>	<i>TFB2M</i>
<i>BMP4</i>	<i>GIN51</i>	<i>OVOL2</i>	<i>TGIF1</i>
<i>BYSL</i>	<i>GLS2</i>	<i>PAAF1</i>	<i>TIMM10</i>
<i>C10ORF2</i>	<i>GNL3</i>	<i>PATZ1</i>	<i>TMEM97</i>
<i>C11ORF5</i>	<i>GNL3MF</i>	<i>PDCD11</i>	<i>TP53RK</i>
<i>CAD</i>	<i>GPX2</i>	<i>PHLDA1</i>	<i>TPST2</i>
<i>CBX2</i>	<i>HEATR1</i>	<i>PIGW</i>	<i>TREX2</i>
<i>CD320</i>	<i>HES6</i>	<i>PLK1S1</i>	<i>TRIM27</i>
<i>CD44</i>	<i>HILPDA</i>	<i>POLR1B</i>	<i>TRMT1</i>
<i>CDC25A</i>	<i>HIRA</i>	<i>POLR1C</i>	<i>TSPAN5</i>
<i>CDCA7</i>	<i>HNRNPA0</i>	<i>POLR1D</i>	<i>UBIAD1</i>
<i>CDK4</i>	<i>HNRNPDL</i>	<i>POLR3C</i>	<i>UCK2</i>
<i>CDKN3</i>	<i>HOXA11</i>	<i>POLRMT</i>	<i>UTP14A</i>
<i>CDT1</i>	<i>ID3</i>	<i>PPAN</i>	<i>WDR12</i>
<i>CEP76</i>	<i>IL20RA</i>	<i>PPIF</i>	<i>WDR3</i>
<i>CIRH1A</i>	<i>IMP4</i>	<i>PPIL1</i>	<i>WDR74</i>
<i>CLDN2</i>	<i>ISM1</i>	<i>PRMT5</i>	<i>WDR77</i>
<i>CMTM7</i>	<i>JAG1</i>	<i>PTMA</i>	<i>XPOT</i>
<i>COG8</i>	<i>KCNE3</i>	<i>RABEPK</i>	<i>YAP1</i>
<i>CRTAP</i>	<i>KIAA1199</i>	<i>RBM12</i>	<i>ZIC2</i>
<i>CTPS1</i>	<i>KITLG</i>	<i>RHEB</i>	<i>ZNF259</i>
<i>CXCL5</i>	<i>LEF1</i>	<i>RHOBTB3</i>	<i>ZNF275</i>
<i>DCUN1D5</i>	<i>LGR5</i>	<i>RRP12</i>	<i>ZNF503</i>
<i>DDX10</i>	<i>LIMS1</i>	<i>RRP15</i>	<i>ZNF511</i>
<i>DDX20</i>	<i>MAT2A</i>	<i>RRP1B</i>	<i>ZNF593</i>
<i>DDX21</i>	<i>MET</i>	<i>RRP7A</i>	<i>ZNF703</i>
<i>DDX56</i>	<i>METTL1</i>	<i>RRP9</i>	<i>ZNRD1</i>
<i>DHX33</i>	<i>METTL2A</i>	<i>SCD</i>	<i>ZNRF3</i>
<i>DIMT1</i>	<i>MRPL12</i>	<i>SCLY</i>	
<i>DKC1</i>	<i>MRPL15</i>	<i>SF3A2</i>	
<i>DOCK4</i>	<i>MRPL36</i>	<i>SLC19A1</i>	
<i>EIF5A2</i>	<i>MRPS12</i>	<i>SLC19A2</i>	
<i>EIF6</i>	<i>MRPS26</i>	<i>SLC1A5</i>	
<i>ENC1</i>	<i>MRPS30</i>	<i>SLC25A19</i>	
<i>EPDR1</i>	<i>MRT04</i>	<i>SLC29A2</i>	
<i>EPHB2</i>	<i>MTFP1</i>	<i>SLC39A10</i>	
<i>EPHB3</i>	<i>MYB</i>	<i>SLC3A2</i>	
<i>ETS2</i>	<i>MYBBP1A</i>	<i>SLC52A2</i>	
<i>ETV4</i>	<i>MYC</i>	<i>SLC5A1</i>	
<i>EXOSC4</i>	<i>NIFK</i>	<i>SLC7A5</i>	
<i>EXOSC9</i>	<i>NIP7</i>	<i>SORD</i>	
<i>FAM60A</i>	<i>NLE1</i>	<i>SOX4</i>	
<i>FAM84A</i>	<i>NOB1</i>	<i>SOX9</i>	

Supplementary Table S10. Intestinal β -catenin/TCF signature in human CRC. List of genes regulated by Wnt/TCF signaling as described in Van der Flier et al. *Gastroenterology*. 2007 (44). Gene names were actualized to Official HUGO Gene Symbols.

SUPPLEMENTARY TABLE S11

Gene Symbol

ABCA4	CYTL1	KRTAP9-2	RAB3B	TMCC3
ABHD3	DAAM1	KRTAP9-4	RAB43	TMEM140
ABHD4	DAB2	L3MBTL3	RAB4B	TMEM173
ABI1	DCAF6	LAMC2	RAB5A	TMEM59
ABLIM1	DDI2	CERS5	RAB9A	TMEM86A
ABTB1	DNAJB6	LDLR	RAET1L	TMEM92
ACPP	DOCK5	LGMN	RALGPS2	TMOD1
AFF1	DOCK9	LGR4	RARB	TMOD2
AFF4	DPYSL2	LGR5	RASA1	TMSB4X
AGR2	DUSP1	LIPH	RBMS2	TMSB4XP8
AKAP13	DUSP5	LMO7	RCAN3	TNFAIP1
ALDH3A1	DUSP6	LPHN3	REEP1	TNFRSF21
ALPP	EDN1	LRP10	REEP3	TNFSF10
ALS2CR12	EGLN3	LRRC8A	REG4	TNRC18
AMIGO2	EIF1AY	LY6G6C	REPS2	TNS1
AMOTL2	AGO4	LY6G6E	RERE	TNS4
ANGPT2	EMP1	MACF1	RGL1	TOR1AIP2
ANKRD1	EPHX4	MAGI1	RHOF	TP53INP2
ANXA1	EPS8L2	MALT1	RICTOR	TPRA1
APLF	ERBB3	MAML3	RIMKLB	TRAK2
ARHGAP17	ERRFI1	MAOB	RIOK3	TRAM1
ARHGAP21	ERV3-1	MAP1LC3B2	RND1	TRIM29
ARHGAP29	ETHE1	MAP2	RNF122	TSPAN1
ARHGEF12	FADS3	MAP4K4	RSPH3	TSPAN6
ARHGEF2	FANK1	MARCH3	RTN3	TSPAN8
ARID5B	FBXO30	MBNL2	RYBP	TUBA1A
ARRDC3	FBXO32	MCTP1	S100P	TUFT1
ATG2A	FCER1A	MEGF9	SASH1	UBE2B
ATP6V1E1	FCHO2	MFGE8	SAT1	UBE2D1
ATP8A1	FCHSD1	MIR222	SCARA3	UBE2H
ATP9A	FGD4	MMD	SCIN	UCA1
ATXN1	FLJ44874	MMP3	SDR16C5	ULBP2
AXL	FLNB	MRGPRX1	SEC14L1	UPK3B
B4GALNT3	FMNL2	MRGPRX2	SECISBP2L	VAMP5
BACH1	FOS	MS4A15	SELP	VEPH1
BACH2	FOSL2	MXI1	SEMA3C	VILL
BCL6	FOXO1	MXRA7	SEMA5A	VSIG1
BMPR2	FOXP1	NAPG	SEPP1	WDFY3
BTG1	FRYL	NAV2	SERINC2	WDR45
WBP1L	FSTL3	NAV3	SERPINB8	WDSUB1
RGCC	FYN	NBEAL1	SERPINE2	WDTC1
C1ORF116	GAB1	NDRG1	SESN3	WWC2
MAP3K7CL	GADD45B	NEBL	SESTD1	WWTR1
CREBRF	GALNT4	NECAP2	SHROOM3	XG
C6ORF15	GATA6	NEDD4L	SKIL	XPR1
CA5B	GATS	NPIPB3	SLC12A6	XRN1
CALCOCO1	GATSL1	NSAP11	SLC1A1	YIPF1
CALHM2	GBE1	NT5C2	SLC1A2	YOD1
CAV1	GCNT1	NT5E	SLC28A3	YPEL5
CAV2	GDA	NTAN1	SLC29A3	ZBED2
CBLB	GDF15	NTN4	SLC2A1	ZBTB38
CCDC50	GFPT2	NUDT4	SLC2A10	ZDHHC21
CCDC68	GLUL	NUP50	SLC2A12	ZFAND5

SUPPLEMENTARY TABLE S11 (continued)

Gene Symbol

CCNDBP1	GM2A	NABP1	SLC2A3	ZFAND6
CCNG2	GPR137B	OPTN	SLC31A2	ZFHX2
CD177	GPR161	OR2A20P	SLC35D2	ZFYVE1
CD55	GPRC5A	OR2A4	SLC43A2	ZNF185
CD59	GRB7	OR2A7	SLC45A1	ZSWIM4
CD99	GREB1	OSGIN2	SLC4A11	ZYX
CDC14A	GSN	OTUD1	SLC5A3	
CDC34	GTF2B	PARD6B	SLC7A11	
CDKN1A	GULP1	PAWR	SMURF2	
CDKN2B	HAPLN3	PCMTD1	SNAP23	
CEACAM1	HBEGF	PDIA6	SNX9	
CEP76	HECA	PDLIM5	SOD2	
CGA	HEG1	PDZK1IP1	SORL1	
CHIC2	HIST1H2BC	PHACTR2	SP100	
CHMP4C	HIVEP2	PHF21A	SPINK1	
CHMP5	HLA-E	PHLDB2	SPTAN1	
CHN2	HNMT	PIK3AP1	SPTB	
CHRN1	HSD17B11	PIK3C3	SSFA2	
CITED2	HSD17B2	PIK3CA	STARD13	
CLDN1	HSPG2	PIK3CB	STRADB	
CLDN10	ICK	PIK3IP1	STX11	
CLDN16	IGF1R	PLAT	STX7	
CLEC2D	IGFBP4	PLCG2	STXBP5	
CLIC3	IL10RA	PLEKHA1	SUN1	
CLIC5	IL18	PLK2	SWAP70	
CLTB	IL37	PLOD2	SYNE2	
CLU	IL2RG	PMAIP1	SYTL4	
COL10A1	INPP4B	PNRC1	TBC1D15	
COL17A1	IQGAP2	POC1B	TBC1D19	
CCP110	IRF6	POLB	TCF12	
CPA4	IRS2	PPAP2B	TCP11L2	
CRIM1	ITGA3	PPFIBP1	TDRD7	
CRIP1	ITGAX	PPP1R15A	TEAD1	
CRTAM	ITGB8	PPP2CB	TEK	
CSGALNACT2	ITLN2	PRDM1	TEP1	
CSRNP2	ITM2B	PRR15	TES	
CTGF	JAK1	PSG7	TFPI	
CTNNA1	JMJD1C	PSG9	TGFA	
CTSB	KCTD21	PTPN12	TGFBR2	
CTSL	KIF3C	PTPN14	TGFBR3	
CYBRD1	KLF5	PTPN21	THBS1	
CYP2S1	KLF6	PTPRG	TIMP2	
CYP39A1	KLF7	PTPRR	TIMP3	
CYR61	KLHL24	PTTG1IP	TINAGL1	
CYTH1	KLK10	PYGL	TIPARP	
CYTH2	KPNA7	RAB11FIP5	TJP1	
CYTH3	KRTAP2-4	RAB30	TM4SF1	

Supplementary Table S11. Genes regulated by β -catenin/FOXO3a signaling in human CRC. Genes up-regulated by FOXO3a/ β -Catenin expression in human DLD1F β colon cancer cells as described in Tenbaum et al. *Nature Medicine* . 2012 (10). Gene names were actualized to Official HUGO Gene Symbols.

SUPPLEMENTARY TABLE S12

CLUSTER ORDER	TUMOR TYPE	STAGE	KRAS	PIK3CA	BRAF	MSI STATUS
1	RECTUM	T3N0M0	WT	Q546R	WT	MSS
2	COLON	T3N0M0	WT	WT	WT	MSS
3	RECTUM	T3N0M0	WT	WT	WT	MSS
4	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
5	COLON	T3N2M0	WT	H1047L	WT	MSS
6	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
7	COLON	T3N0M0	G12D	WT	WT	MSI
8	RECTUM	T3N0M0	WT	WT	WT	MSS
9	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
10	COLON	T3N0M0	G12D	E545K	WT	MSI
11	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
12	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
13	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
14	COLON	T3N1M0	WT	WT	WT	MSS
15	COLON	T4N0M0	WT	WT	WT	MSS
16	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
17	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
18	COLON	T4N2M0	G12S	WT	WT	n. d.
19	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
20	RECTUM	TxNxM1	G12D	n. d.	n. d.	MSS
21	COLON	T3N2M0	G12D	WT	WT	MSS
22	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
23	COLON	T3N1M0	WT	WT	WT	MSS
24	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
25	COLON	T3N1M0	G12D	WT	WT	MSI
26	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
27	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
28	LIVER MET	T3N0M0	WT	WT	WT	MSS
29	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
30	LIVER MET	T4N1M1	G12V	E545K	WT	MSS
31	LIVER MET	n. d.	WT	WT	n. d.	MSS
32	COLON	T3N1M0	WT	WT	WT	MSS
33	LIVER MET	T4pN2M1	WT	H1047R	WT	MSS
34	COLON	T4bN2M1	G12V	n. d.	n. d.	MSS
35	COLON	T4N2M0	WT	WT	WT	MSS
36	COLON	T3N1M0	WT	WT	WT	MSS
37	COLON	T3N0M0	WT	WT	WT	MSS
38	COLON	T3N1M0	WT	WT	WT	MSS
39	COLON	T3N0M0	A146T	WT	WT	MSS
40	COLON	T3N0M0	G12D	WT	WT	MSS
41	COLON	T3N0M0	WT	WT	WT	MSS
42	COLON	T3N0M0	WT	WT	WT	n. d.
43	COLON	T3N0M0	G12V	WT	WT	n. d.
44	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
45	COLON	T3N2M0	G12D	WT	WT	MSS
46	COLON	T3N0M0	n. d.	n. d.	n. d.	n. d.
47	COLON	T3N0M0	G12S	E542K	WT	MSS
48	COLON	T3N0M0	Q61H	N345K	WT	MSS
49	COLON	T3N1M0	G12C	WT	WT	MSS
50	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
51	RECTUM	T3N2M0	WT	WT	WT	MSS
52	COLON	T3N0M0	WT	WT	WT	MSS
53	COLON	T3N0M0	WT	E545K	WT	MSS
54	COLON	T3N0M0	WT	WT	WT	MSS
55	n.d.	n.d.	n. d.	n. d.	n. d.	n. d.
56	RECTUM	T3N0M0	WT	WT	WT	MSS
57	n.d.	n.d.	n. d.	n. d.	n. d.	n. d.
58	n.d.	n.d.	n. d.	n. d.	n. d.	n. d.
59	COLON	T3N0M0	G13D	WT	WT	MSS
60	COLON	T3N0M0	WT	WT	WT	MSS
61	n.d.	n.d.	n. d.	n. d.	n. d.	n. d.
62	COLON	T3N0M0	WT	WT	WT	MSS
63	PERITONEAL MET	T4N2M1	G12D	WT	WT	n. d.
64	COLON	T4bN2M0	WT	WT	WT	MSS
65	n.d.	n.d.	n. d.	n. d.	n. d.	n. d.

SUPPLEMENTARY TABLE S12 (Continued)

CLUSTER ORDER	TUMOR TYPE	STAGE	KRAS	PIK3CA	BRAF	MSI STATUS
66	n.d.	n.d.	n. d.	n. d.	n. d.	n. d.
67	COLON	T2N1M0	WT	WT	WT	MSS
68	COLON	T2N1M0	WT	WT	WT	MSS
69	n.d.	n.d.	n. d.	n. d.	n. d.	n. d.
70	COLON	T3N1M0	WT	WT	WT	MSI
71	n.d.	T3N2M1	G12D	WT	WT	MSS
72	n.d.	n.d.	n. d.	n. d.	n. d.	n. d.
73	COLON	T3N1M0	WT	WT	WT	MSS
74	COLON	T3N0M0	WT	R88Q	WT	MSS
75	LIVER MET	T3N2M1	WT	WT	WT	MSS
76	LIVER MET	T3N0M0	WT	n. d.	n. d.	n. d.
77	COLON	T3N0M0	WT	WT	WT	MSS
78	LUNG MET	T3N0M0	WT	WT	WT	MSS
79	COLON	T3N0M0	WT	WT	WT	MSS
80	COLON	T3N0M0	WT	WT	WT	MSI
81	LIVER MET	T4N0M0	WT	WT	WT	MSI
82	LIVER MET	T4N2M1	WT	M1043I	WT	MSS
83	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
84	COLON	T3N1M0	WT	n. d.	n. d.	MSS
85	COLON	T3N2M0	G12V	WT	WT	MSS
86	COLON	T3N0M0	G12A	WT	WT	MSS
87	COLON	TxNxM1	WT	WT	WT	n. d.
88	LIVER MET	T3N2M1	G12D	WT	WT	MSS
89	COLON	T3N1M0	G12C	E545K	WT	MSS
90	COLON	T3N1M0	WT	R88Q	WT	MSI
91	COLON	T3N0M0	WT	H1047R	WT	MSI
92	COLON	T3N2M0	A146V	WT	WT	MSS
93	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
94	COLON	T3N0M0	WT	WT	WT	MSS
95	RECTUM	T3N0M0	WT	WT	WT	MSS
96	COLON	T3N1M0	WT	H1047R	WT	MSI
97	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
98	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
99	LIVER MET	T3N2M0	G12D	WT	WT	MSS
100	LIVER MET	n. d.	WT	WT	WT	MSS
101	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
102	COLON	T3N2M0	WT	WT	WT	MSS
103	COLON	T3N0M0	G12D	WT	WT	MSS
104	COLON	T3N2M0	WT	WT	WT	MSS
105	COLON	T4N1M1	WT	WT	WT	MSS
106	LIVER MET	T4bN2M0	WT	E545K	WT	MSS
107	LIVER MET	T3N1M0	G12D	n. d.	WT	MSS
108	COLON	T3N2M0	WT	WT	WT	MSS
109	RECTUM	T3N2bMx	WT	H1047R	WT	n. d.
110	COLON	T3N0M1	WT	WT	WT	MSS
111	COLON	TxNxM1	WT	n. d.	n. d.	n. d.
112	RECTUM	T3N1M0	G12V	WT	WT	MSS
113	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
114	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
115	LUNG MET	T4NxM0	WT	WT	WT	MSS
116	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
117	COLON	T4N1M1	WT	WT	WT	MSS
118	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
119	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
120	COLON	T3N0M0	G12V	WT	WT	MSI
121	COLON	T3N0M0	WT	WT	WT	MSI
122	COLON	T3N0M0	A146T	H1047R	WT	MSI
123	LIVER MET	T3N0M1	G12V	H1047L	WT	MSS
124	COLON	T3N0M0	WT	WT	V600V	MSS
125	COLON	T3N0M0	G13D	WT	WT	MSS
126	COLON	T3N2M0	WT	WT	V600E	MSI
127	COLON	T3N0M0	WT	R88Q	WT	MSS
128	COLON	T3N0M0	G12D	WT	WT	MSS
129	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
130	RECTUM	T3N2M1	G12C	n. d.	n. d.	MSS

Supplementary Table S12. Molecular profiling of 130 CRC tumors. Gene expression profiling of 130 FFPE tumor samples was performed using the nCounter platform (Nanostring). Samples were clustered by gene expression as shown in Figure 5C. This table shows such cluster order (1 to 130), tumor type (Primary colon or rectum and metastases), tumor stage (TNM), microsatellite status (MSI, microsatellite instable; MSS, microsatellite stable) and the mutational status of KRAS, BRAF and PIK3CA genes. Not determined (n. d.).

SUPPLEMENTARY TABLE S13

PATIENTS' CLINICAL DATA								SAMPLE'S HISTOPATHOLOGY					SAMPLE'S MUTATIONAL STATUS								PATIENTS' TREATMENTS				SAMPLE'S β-CATENIN		
Patient ID	Clinical trial	Drug	Molecular Mechanism	Sex	Age	Primary tumor site	Metastasis site at the time of study inclusion	Origin of biopsy analyzed	Histology	Grade	TNM at the time of diagnosis	PTEN (Hscore)	Microsatellites	PIK3CA	KRAS	TP53	APC	BRAF	OTHERS	Number of previous lines of treatment	Time on last previous treatment	Time on treatment	Response	% of nuclear β-catenin positive	Nuclear β-catenin Score	Nuclear β-catenin Category	
1	INK1117-001	MLN1117	PI3K ALPHA	M	46	Colon	Lymph nodes, peritoneal	Colon	ADC	G2	pT3N1M0	Normal (140)	MSI	H1047R	WT	WT	WT	WT	CTNNB1T41A	3	196	36	PD	80	4	HIGH	
2	CBYL719X2101	BYL719	PI3k ALPHA	F	59	Colon	Liver, peritoneal	Liver	ADC	G2	pT4pN2M1	Normal (90)	MSS	H1047R	WT	WT	WT	WT	WT		3	267	50	PD	50	4	HIGH
3	CBYL719X2101	BYL720	PI3k ALPHA	F	66	Colon	Liver	Liver	ADC	G3	pT4N2M1	Null (0)	MSS	M1043I	WT	G245D	WT	WT	WT		3	457	51	PD	50	4	HIGH
4	CBKM120X2101	BKM120	pan PI3K	M	65	Rectum	Lung, Liver, lymph nodes	Colon	ADC	G1	pT4bN2M1	Loss (10)	MSS	n.d.	G12V*	n.d.	n.d.	n.d.	n.d.		3	395	13	PD	90	4	HIGH
5	CBEZ235A2101	BEZ235	PI3K & mTOR	M	60	Colon	Lung, Liver, adrenal gland	Colon	ADC	G1	pT4N4M1	n.d.	n.d.	n.d.	WT*	n.d.	n.d.	n.d.	n.d.		3	418	56	PD	70	4	HIGH
6	I4H-MC-JWAA	LY2780301	AKT & S6	F	59	Colon	Liver, lymph nodes	Colon	ADC	G1	pT3N1M0	Normal (85)	MSS	n.d.	WT*	n.d.	n.d.	n.d.	n.d.		2	102	57	PD	60	4	HIGH
7	B2151001	PKI-587	PI3K & mTOR	M	61	Rectum	Lung, Liver	Liver	ADC	n.d.	pT3N1M0	Null (0)	n.d.	n.d.	WT*	n.d.	n.d.	WT*	n.d.		3	59	56	PD	50	4	HIGH
8	CBEZ235A2101	BEZ235	PI3K & mTOR	M	68	Colon	Lung, Liver, lymph nodes	Liver	ADC	G2	pT3N2M1	Null (0)	n.d.	n.d.	WT*	n.d.	n.d.	n.d.	n.d.		3	192	55	PD	50	4	HIGH
9	B2151001	PKI-587	PI3K & mTOR	M	63	Rectum	Lung, Liver	Colon	ADC	G2	pT3N2M0	Loss (20)	MSS	WT	G12V	WT	WT	WT	WT		4	181	33	PD	80	4	HIGH
10	XL765-001	XL765	PI3K & mTOR	M	57	Colon	Lung, Liver	Colon	ADC	G1	pT3N2M0	n.d.	MSS	WT	WT	S241L/S109F	WT	WT	WT		2	648	55	PD	50	4	HIGH
11	B2151001	PKI-587	PI3K & mTOR	M	60	Colon	Liver, Lymph nodes, peritoneal	Liver	ADC	G1	pT3N2M1	loss (40)	MSS	WT	WT	R282Y/R150W	WT	WT	WT		4	183	56	PD	70	4	HIGH
12	CBKM120X2101	BKM120	pan PI3K	M	71	Rectum	Lung, Liver	Liver	ADC	G2	pT3N0M0	loss (30)	MSS	WT	WT	WT	WT	WT	WT		3	105	78	PD	60	4	HIGH
13	B2151001	PKI-587	PI3K & mTOR	M	68	Colon	Lung, Liver, lymph nodes	Liver	ADC	G2	pT3N2M1	Null (0)	MSS	WT	G12D	WT	WT	WT	WT		3	226	56	PD	70	4	HIGH
14	I4H-MC-JWAA	LY2780301	AKT & S6	F	69	Colon	Lung	Lung	Mucinous ADC	G2	pT3N1M0	Normal (100)	n.d.	E545K	G12V	WT	WT	WT	GNAQ5 R201C	3	40	112	SD	5	1	LOW	
15	INK1117-001	MLN1117	PI3K ALPHA	M	69	Colon	Liver, lymph nodes	Liver	ADC	G2	pT4bN2M0	loss (40)	MSS	E545K	WT	WT	WT	WT	WT		2	99	61	PD	5	1	LOW
16	CBYL719X2101	BYL720	PI3K ALPHA	F	47	Colon	Lung, Liver	colon	ADC	G3	pT3N0M1	Normal (140)	n.d.	E545K	G12C	WT	R1450X	WT	WT		2	70	57	PD	10	3	LOW
17	CBYL719X2101	BYL720	PI3K ALPHA	F	77	Colon	Lung, Liver	Liver	ADC	G1	pT4N1M1	Loss (40)	MSS	E545K	G12V	M133K	WT	WT	WT		3	92	57	PD	5	1	LOW
18	INK1117-001	MLN1117	PI3K ALPHA	M	71	Colon	Liver, Lung, Pancreas, Peritoneal	colon	ADC	G1	pT4N1M1	Loss (10)	n.d.	E545K*	G12A*	n.d.	n.d.	n.d.	n.d.		2	168	60	PD	15	3	LOW
19	CBEZ235A2101	BEZ235	PI3K & mTOR	M	63	Colon	Lung, lymph nodes, bone	Liver	ADC	G1	pT3N0M1	Loss (10)	MSS	H1047L	G12V	WT	G1288X/Q1367X	WT	WT		5	730	42	PD	30	3	LOW
20	B2151001	PKI-587	PI3K & mTOR	M	51	Colon	Liver, Lung and pelvic	Rectum	ADC	G1	pT3N2bMx	Null (0)	n.d.	H1047R	WT	R248Q	S1346X	WT	WT		3	104	57	PD	1	1	LOW
21	CBYL719X2101	BYL720	PI3K ALPHA	M	67	Colon	Lung, Liver	Liver	ADC	G2	pT3N1M0	Loss (10)	MSS	n.d.	G12D	A27D/G159D	WT	WT	G474I G10E	6	89	145	SD	1	1	LOW	
22	B2151001	PKI-587	PI3K & mTOR	M	58	Colon	No met	Liver	ADC	G2	pT3N0M0	n.d.	n.d.	n.d.	WT*	n.d.	n.d.	n.d.	n.d.		2	151	111	SD	5	1	LOW
23	B2151001	PKI-587	PI3K & mTOR	M	27	Rectum	Lung, Liver	Rectum	ADC	G1	pT3N2M1	Loss (40)	MSS	n.d.	G12C*	n.d.	n.d.	n.d.	n.d.		3	44	71	PD	5	1	LOW
24	CBKM120X2101	BKM120	pan PI3K	F	48	Colon	Lung, bone	Colon	ADC	n.d.	pT4N4M1	Loss (5)	n.d.	n.d.	WT*	n.d.	n.d.	n.d.	n.d.		5	63	14	PD	0	0	LOW
25	XL765-001	XL765	PI3K & mTOR	M	68	Colon	Liver	Liver	ADC	G2	n.d.	n.d.	MSS	n.d.	WT*	n.d.	n.d.	n.d.	n.d.		3	153	169	SD	40	3	LOW
26	PAM4743g	GDC-0068	AKT	M	64	Rectum	Lung, Liver	Rectum	ADC	G1	pT4N4M1	Null (0)	MSS	n.d.	G12D*	n.d.	n.d.	n.d.	n.d.		7	92	22	PD	10	3	LOW
27	CBKM120X2101	BKM120	pan PI3K	M	69	Colon	Liver	Liver	ADC	n.d.	n.d.	Loss (5)	MSS	WT	WT	V197M	P1319fs	WT	FGFR3 F384L		3	44	59	PD	5	1	LOW
28	CBKM120X2101	BKM120	pan PI3K	M	63	Rectum	Lung, Liver, adrenal gland	Rectum	ADC	G1	pT3N1M0	Loss (10)	MSS	WT	G12V	WT	R283X	WT	SMAD4 R361C		1	151	42	PD	20	2	LOW
29	B2151001	PKI-587	PI3K & mTOR	F	57	Colon	No met	n.d.	ADC	G3	pT3N2M1	Null (0)	MSS	WT	G12D	WT	E1286X	WT	WT		2	337	111	SD	5	1	LOW
30	CBEZ235A2101	BEZ235	PI3K & mTOR	F	50	Rectum	Lung	Lung	ADC	G3	pT4N4M0	Loss (40)	MSS	WT	WT	C238R	WT	WT	WT		4	92	150	SD	5	1	LOW
31	CBEZ235A2101	BEZ235	PI3K & mTOR	F	57	Colon	Lung, Liver, lymph nodes	Colon	ADC	G1	pT4N1M1	Normal (140)	MSS	WT	WT	E245K	Q1294X	WT	WT		3	151	56	PD	40	3	LOW
32	CBEZ235A2101	BEZ235	PI3K & mTOR	M	54	Colon	Liver, bone	Liver	ADC	G2	pT4N0M0	Loss (19)	MSI	WT	WT	WT	WT	WT	WT		2	242	113	SD	15	2	LOW
33	CBEZ235A2101	BEZ235	PI3K & mTOR	F	75	Colon	Liver, lymph nodes	Colon	ADC	G2	pT4N4M1	Null (0)	n.d.	WT	WT	WT	WT	WT	WT		5	137	7	PD	5	1	LOW
34	CBKM120X2101	BEZ235	pan PI3K	M	54	Colon	Liver	colon	ADC	G1	pT4N1M1	Loss (10)	MSS	WT	WT	WT	WT	WT	WT		2	182	76	PD	0	0	LOW
35	CBEZ235A2101	BEZ235	PI3K & mTOR	F	60	Colon	Lung, Liver	Lung	ADC	G2	pT3N0M0	Loss (20)	MSS	WT	WT	R248Q	WT	WT	WT		4	303	112	SD	20	2	LOW
36	CBEZ235A2101	BEZ235	PI3K & mTOR	M	63	Colon	Lung, Liver, lymph nodes	Liver	ADC	G3	pT3N2M0	Loss (10)	MSS	WT	G12D	WT	WT	WT	WT		4	153	56	PD	5	1	LOW
37	PAM4743g	GDC-0068	AKT	M	63	Colon	Liver and peritoneal	peritoneal	ADC	G1	pT4N2M1	Null (0)	n.d.	WT	G12D	V272S/V140M	R1114X/R1450X	WT	WT		2	306	34	PD	0	0	LOW
38	B2151001	PKI-587	PI3K & mTOR	M	35	Colon	No met	colon	Mucinous ADC	G1	pT4bN2M0	Loss (40)	MSS	WT	WT	W146X	WT	WT	WT		4	214	247	SD	5	1	LOW
39	B2151001	PKI-587	PI3K & mTOR	M	41	Colon	Liver, lymph nodes, peritoneal	colon	ADC	G3	pT3N1M0	Null (0)	n.d.	WT	WT	E245K/G113S	WT	WT	WT		3	273	36	PD	1	1	LOW
40	B2151001	PKI-587	PI3K & mTOR	M	60	Colon	Liver	colon	ADC	G2	pT3N0M1	Null (0)	MSS	WT	WT	R282G	WT	WT	WT		3	123	49	PD	30	2	LOW

Supplementary Table S13. Clinicopathological data of 40 CRC patients treated with PI3K/AKT/mTOR inhibitors and their corresponding baseline tumor samples analyzed for mutations and nuclear β -catenin content. Clinical trial, drug and molecular target are indicated for each patient. Basic patient's information is included: age, sex, primary tumor site and metastases. Major histopathological information of baseline tumor samples is also shown: tissue origin; histology (ADC) adenocarcinoma, Grade (G1, 2 or 3), TNM classification and PTEN score (% positive cells). 28 out of 40 samples analyzed had enough material to evaluate microsatellite instability. Results are indicated as microsatellite instable (MSI) or stable (MSS). 29 out of the initial 40 samples analyzed had enough material to be genotyped by Amplicon sequencing. Mutations are specified and wild type samples indicated (WT). Mutations in tumor suppressor genes such as APC are underestimated due to the diversity of alterations occurring all along their coding sequence that are not covered by the amplicon sequencing panel (Methods). Asterisks indicate additional genetic analyses performed by Sequenom. Number of previous lines of treatment, days on previous line of treatment, time on treatment with PI3K/AKT/mTOR inhibitors and response (Stabilized Disease, SD; Progressive disease; PD) are indicated. The percentage of nuclear β -catenin positive cells, samples' β -catenin score and final classification (high or low) are shown. (n. d.) Not determined.

SUPPLEMENTARY TABLE S14

		RESPONSE		
		SD	PD	TOTAL
Nuclear β -cat	LOW	9	18	27
	HIGH	0	13	13
	TOTAL	9	31	40
	n. d.	0	0	0

$P = 0,0195$

		RESPONSE		
		SD	PD	TOTAL
TP53	WT	3	12	15
	MUT	4	10	14
	TOTAL	7	22	29
	n. d.	2	9	11

$P = 0,6817$

		RESPONSE		
		SD	PD	TOTAL
PIK3CA	WT	5	14	19
	MUT	1	9	10
	TOTAL	6	23	29
	n. d.	3	8	11

$P = 0,3880$

		RESPONSE		
		SD	PD	TOTAL
APC	WT	6	15	21
	MUT	1	7	8
	TOTAL	7	22	29
	n. d.	2	8	10

$P = 0,6349$

		RESPONSE		
		SD	PD	TOTAL
KRAS	WT	6	19	25
	MUT	3	12	15
	TOTAL	9	31	40
	n. d.	0	0	0

$P = 1$

		RESPONSE		
		SD	PD	TOTAL
TNM	T3	4	18	22
	T4	3	9	12
	TOTAL	7	27	34
	n. d.	1	5	6

$P = 0,6769$

Supplementary Table S14. Nuclear β -catenin content but not relevant mutations or TNM stage determine response of CRC patients to PI3K/AKT/mTOR inhibitors. Tables summarizing the number of cases presenting high or low nuclear β -catenin, mutations in *PIK3CA*, *KRAS*, *TP53* or *APC*, or different TNM stage among patients showing stabilized disease (SD) or progressive disease (PD). P values correspond to Fisher's exact test. (n. d.) Not determined.

SUPPLEMENTARY TABLE S15

Multivariate analysis (n = 27)

Overall *P* Value: 0.04484 - ANOVA (model with or without β -catenin)

		HR	95% CI		Log Rank <i>P</i> value
β -catenin	high versus low	3.9644	1.028	15.275	0.0158
Therapy	pan-PI3K versus AKT inh	0.102	0.005	2.023	0.134
	PI3K-mTOR versus AKT inh	0.431	0.019	9.448	0.593
	PI3K-alpha versus AKT inh	0.975	0.046	20.663	0.987
Gender	female vs. male	2.152	0.436	10.624	0.346
Age	continuous	1.067	0.971	1.173	0.177
Number of prior therapies	≥ 4 versus < 4	2.486	0.512	12.061	0.258
Liver metastasis	Yes versus No	3.556	0.661	19.126	0.139
KRAS mutations	Yes versus No	0.991	0.266	3.681	0.988
PIK3CA mutations	Yes versus No	0.744	0.117	4.712	0.753
PTEN loss	Yes versus No	0.608	0.033	11.125	0.737
APC mutations	Yes versus No	8.882	1.716	45.961	0.0092
TP53 mutation	Yes versus No	0.789	0.171	3.661	0.762

Supplementary Table S15. Multivariate analysis of baseline tumors from patients treated with PI3K/AKT/mTOR inhibitors.

We used R software version 3.1.1 and the "survival" package for Cox regression model analysis (Log-Rank test *P* values).

Features of interest in multivariable model were age (continuous), gender (female vs. male), type of therapy (AKT inhibitor vs. pan-PI3K inhibitor, PI3K-mTOR inhibitor or PI3K-alpha inhibitor), number of prior therapies (≥ 4 vs. < 4), presence of liver metastasis, KRAS mutations, PIK3CA mutations, PTEN loss, APC mutations, TP53 mutations and beta catenin class (high vs. low).

We compared a model with and without beta catenin class using ANOVA.

3. GLOBAL SUMMARY OF THE RESULTS

The data generated during this thesis led to the publication of two articles. In **article 1** we studied the crosstalk between β -catenin and FOXO3a and its implications in CRC.

β -catenin prevents FOXO3a-induced apoptosis and promotes metastasis

We showed that both factors, FOXO3a and β -catenin, were found in the nucleus of advanced colon adenomas, carcinomas and liver metastasis, with highest concentration at stages III and IV. Furthermore, patients with higher nuclear accumulation of both factors presented an average of eight months shorter overall survival.

We studied the effect of FOXO3a and β -catenin in colon cancer cells. We used an inducible expression system, where we could force nuclear translocation of exogenous FOXO3a-ER fusion protein, by treating cells with 4-hydroxy-tamoxifen (4OHT), and induce the expression of a stable mutant form of β -catenin upon doxycycline treatment. As expected, FOXO3a activation reduced cell proliferation, accumulating cells in G1 phase, and induced apoptosis. In contrast, β -catenin increased cell proliferation. Simultaneous accumulation of both factors in the nucleus revealed that FOXO3a was dominant on reducing cell proliferation. However, β -catenin conferred resistance to FOXO3a-induced apoptosis. Those cells lost their epithelial phenotype and scattered. Interestingly, E-cadherin was relocated from the membrane into cytoplasmic aggregates. We ruled out that those changes were due to E-cadherin proteolysis or canonical epithelial-to-mesenchymal transition.

Morphological changes induced by high nuclear concentrations of both factors also correlated with increased cell motility and adhesion *in vitro* and increased incidence of metastasis and tumor budding at the invasive front of tumors *in vivo*. At the transcriptional level, β -catenin modulated FOXO3a-dependent gene expression profile promoting regulation of genes controlling cell morphology, adhesion, motility and metastasis.

AKT inhibition induces metastasis in cooperation with β -catenin

It is known that AKT phosphorylates and inhibits FOXO3a, which leads to its translocation into the cytoplasm and blocking of FOXO3a-induced apoptosis. This rationale is the main reason for using AKT inhibitors in the clinic. To put our previous results into a clinically relevant context, we used the AKT inhibitor API-2 to block FOXO3a phosphorylation and to restore its function as a transcription factor. However, FOXO3a *in vivo* accumulation in the nucleus by API-2 treatment did not promote apoptosis but induced metastasis in tumors with high nuclear β -catenin. Additionally, resistance to apoptosis induced by API-2 correlated with nuclear β -catenin content *in vitro*. The use of a Wnt signaling inhibitor, the tankyrase inhibitor XAV-939, reduced nuclear β -catenin content and increased sensitivity to API-2. A similar effect was observed inhibiting the pathway upstream of AKT using the pan-PI3K inhibitor NVP-BKM120.

β -catenin confers resistance to PI3K and AKT inhibitors in CRC cancer PDX models.

Finally, we derived cells from CRC patient tumors and we injected them into immunodeficient NOD-SCID mice or seeded them in suspension. *In vivo* treatment of subcutaneous patient-derived xenografts (PDXs) showed that models derived from patients with high amounts of nuclear β -catenin were resistant to API-2 treatment whereas those with low amounts of nuclear β -catenin were sensitive. *In vitro*, we observed the same behavior when cells were treated either with AKT or PI3K inhibitors. However, by using the tankyrase inhibitor XAV-939 we could reduce β -catenin content and turn resistant cells sensitive to apoptosis induced by PI3K and AKT inhibition.

In **article 2** we sought to confirm the clinical relevance of the findings highlighted in article 1. We assessed the therapeutic potential of Wnt inhibition to overcome resistance to PI3K and AKT inhibition in CRC. We also evaluated β -catenin as a biomarker of resistance to those inhibitors in the clinic.

Pharmacological inhibition of Wnt/ β -catenin and PI3K/AKT pathways reduces tumor growth

For those purposes we used as a Wnt inhibitor NVP-TNKS656, a novel tankyrase inhibitor with high activity *in vivo*. Using this new drug we confirmed our previous results obtained *in vitro* with XAV-939. Using patient-derived sphere cell cultures, we observed that NVP-TNKS656 was not only capable of reducing β -catenin but it also sensitized cells with high amounts of nuclear β -catenin to apoptosis induced by PI3K and AKT inhibitors.

Our next step was to assess NVP-TNKS656 potential *in vivo*. Subcutaneous PDXs were generated in NOD-SCID mice with cells derived from three PDX models presenting known nuclear β -catenin and FOXO3a status and with poor response to AKT or PI3K inhibitors *in vitro*. We treated the animals with NVP-TNKS656 and API-2 and monitored tumor growth. We observed that NVP-TNKS656 in combination with API-2 reduced tumor growth in PDXs with high amounts of nuclear β -catenin. Surprisingly, it also had an effect as a single agent in the case of a PDX model with endogenous high nuclear β -catenin and high nuclear FOXO3a amounts.

FOXO3a/ β -catenin target genes are pharmacodynamic markers of response to tankyrase inhibition

Having seen those two different responses to the same drug, we wanted to address whether high FOXO3a content could determine first, the repression of a distinct set of Wnt/ β -catenin target genes by NVP-TNKS656 and second, an enhanced sensitivity to that treatment. We analyzed mRNA expression in tumors at the endpoint of the *in vivo* experiments using microarrays. Unexpectedly, tankyrase inhibition did not down-regulate many TCF/ β -catenin targets, considered as canonical Wnt target genes, but more FOXO3a/ β -catenin targets.

FOXO3a determines the response to tankyrase inhibition

To further characterize the role of FOXO3a in the context of tankyrase inhibition we used again engineered recombinant cell lines where we could force the translocation of exogenous FOXO3a-ER into the nucleus with 4OHT. We showed that tankyrase inhibition alone did not induce apoptosis but enhanced apoptosis induced by relocation of FOXO3a to the nucleus upon 4OHT treatment. We also observed in patient-derived sphere cell cultures a positive correlation between apoptosis induced by NVP-TNKS656 and nuclear FOXO3a content. Finally, we performed gene expression analysis of a cohort of 130 CRC patients using the nCounter platform (Nanostring). With that, we were able to identify a subset of patients that present high expression of FOXO3a/ β -catenin targets, highlighting the possibility of identifying patients potentially sensitive to tankyrase inhibitors by using gene expression profiling.

 β -catenin is a biomarker of resistance to PIK3 and AKT inhibitors

Having seen that high nuclear amounts of β -catenin could cause resistance to PI3K and AKT inhibitors we evaluated its potential as a biomarker of response to those therapies. We analyzed formalin fixed paraffin-embedded (FFPE) tumor samples at baseline from 40 CRC patients that underwent phase I clinical trials with drugs targeting PI3K, AKT and/or mTOR. We categorized those samples quantifying by IHC its nuclear β -catenin content, allowing us to separate patients in two groups with: high nuclear- β -catenin or low nuclear- β -catenin content. In terms of progression-free survival, patients with high amounts of nuclear- β -catenin responded worse to the treatment and progressed much faster than those patients with lower amounts. Among all factors evaluated in a multi-variant analysis including most commonly mutated genes in CRC (*PIK3CA*, *KRAS*, *APC* and *TP53*), tumor site or the TNM stage at the time of diagnosis, β -catenin was the only factor that could identify a subgroup of patients with worse prognosis when treated with PI3K, AKT or mTOR inhibitors.

DISCUSSION

FOXO transcription factors have classically been considered bona-fide tumor suppressor genes because they induce transcription of cell cycle inhibitors and pro-apoptotic genes (Paik et al., 2007). Additionally, they can induce resistance to oxidative stress and are also associated with longevity. The execution of such processes is tightly regulated by the interaction of FOXOs with several other proteins, including β -catenin (Eijkelenboom and Burgering, 2013). It was previously known that FOXO3a could interact with β -catenin under oxidative stress conditions; however, it was thought to be a competitive mechanism used by cells to inhibit Wnt signaling mediated by β -catenin-TCF/LEF (Essers et al., 2005; Hooigeboom et al., 2008). So far, the physiologic role of this interaction had only been studied in the context of osteoblast formation and differentiation (Almeida et al., 2007). In this thesis we studied the implications of the association between β -catenin and FOXO3a in the context of CRC. We showed that in CRC cells β -catenin modified FOXO3a pro-apoptotic activity, which is essential for its tumor suppressor effect. Actually, in the presence of both factors, the expression of pro-apoptotic genes is still promoted; however, the apoptosis program is not executed. This could be caused by the modulation of the FOXO3a transcriptional activity carried out by nuclear β -catenin, enhancing the transcription of different sets of genes than those induced by FOXO3a alone. Interestingly, many of these alternative gene sets are related to the acquisition of metastatic capacity. Among them we observed overexpression of genes involved in cytoskeleton reorganization or destabilization of cell-to-cell contacts which in turn increase cell motility and migration *in vitro* and *in vivo*. The fact that the execution of FOXO3a-induced apoptosis is blocked by nuclear β -catenin has implications in the clinic since high amounts of the latter can induce resistance to treatments with PI3K and AKT inhibitors. Part of the therapeutic effect shown by PI3K and AKT inhibitors relies on their capacity to relocate FOXO proteins in the nucleus potentiating their pro-apoptotic functions (Fruman and Rommel, 2014). However, we demonstrated that nuclear β -catenin confers resistance to FOXO-induced apoptosis promoted by the treatment with those inhibitors. In addition, we observed that such therapeutic re-localization of FOXO3a to the nucleus would have a pro-metastatic rather than a tumor suppressive effect in tumors with high nuclear β -catenin containing cells. In this sense, patients could face a treatment-induced metastasis phenomenon in tumors with high nuclear β -catenin content treated with PI3K or AKT inhibitors.

It is well accepted that alterations in the Wnt signaling pathway are essential for the initiation of CRC, but it is less clear their role in tumor progression (Fearon, 2011). Furthermore, it has been clearly demonstrated that β -catenin can also interact with other transcription factors in different cellular contexts driving different transcription programs (Valenta et al., 2012). However, the consequences of those interactions have not been deeply characterized. A recent study suggests that despite β -catenin can be recruited by several transcription factors, Wnt-induced transcription is exclusively mediated by TCF/LEF proteins (Schuijers et al., 2014). Another study indicates that Wnt pathway may not be required for late stages of the disease (Varnat et al., 2010). Contrarily, our work demonstrated that Wnt/ β -catenin signaling pathway is still important in the context of advanced mCRC but inducing a distinct transcriptional program through FOXO3a. This suggests that pharmacodynamic evaluation of response to Wnt

inhibitors should be focused not only on the repression of TCF/LEF target genes but also alternative genes controlled by other transcription factors such as FOXO3a.

It is possible that β -catenin/FOXO3a interactions are less abundant than β -catenin/TCF/LEFs, as seen in the study commented before (Schuijers et al., 2014). In any case it seems clear that modification of FOXO3a activity by β -catenin is enough to impact on the biology of CRC cells. Nevertheless, as we observed by gene expression profiling of a cohort of CRC patients, high expression of FOXO3a/ β -catenin target genes seems to be linked to increased levels of TCF/LEF/ β -catenin targets. This suggests that an increased Wnt signaling may lead to an over-expression of both programs controlled by β -catenin.

Our findings highlight the relevance of the Wnt signaling pathway and β -catenin beyond TCF/LEF transcription and are in line with other published studies. YAP1, a transcription factor regulated by the Hippo pathway, acts together with β -catenin to maintain survival and increase tumorigenesis in CRC cell lines (Rosenbluh et al., 2012). YAP1 homolog, WWTR1 (also known as TAZ) is also regulated by Wnt signaling (Azzolin et al., 2012). By analyzing the genes up-regulated by β -catenin and FOXO3a in our inducible cell lines we observed that WWTR1 is a target gene of both factors (**Supplementary table 2 in article 1**) and also that it is down-regulated by NVP-TNKS656 in PDX-P30 (**Figure 3B in article 2**), suggesting that WWTR1 may have a role in the effects driven by FOXO3a and β -catenin. It is important to mention that CRC tumors are still dependent on Wnt ligands even in the context of APC truncating mutations (Dow et al., 2015; Scholer-Dahirel et al., 2011; Voloshanenko et al., 2013). Indeed, most frequent mutations in APC preserve its β -catenin binding domains, retaining part of its capacity to promote β -catenin degradation. This means that although tumor cells present downstream alterations they are still sensitive to external signals that can modulated the pathway. Overall, our results highlight the necessity of evaluating Wnt signaling in the context of therapeutic use of PI3K and AKT inhibitors.

PI3K/AKT/mTOR signaling pathway inhibition is a promising therapeutic strategy since it is altered in most tumor types and is essential for survival of cancer cells. So far, results with PI3K pathway inhibitors as single agents have proved limited clinical efficacy (Bauer et al., 2015). This is especially evident in the context of CRC (Papadatos-pastos et al., 2015). Since mutations in components of the PI3K signaling pathway are very frequent in cancer, its inhibition was expected to have a strong anti-tumor effect comparable to that observed with BRAF inhibitors in melanoma patients harboring V600E mutation (Salama and Flaherty, 2013; Thiel and Ristimäki, 2013). In that context, patients have a very good response to BRAF inhibition, although acquired resistance frequently occurs. CRC patients present innate resistance to BRAF inhibitors due to a higher activity of receptors tyrosine kinase such as EGFR (Lo, 2012; Prahallad et al., 2012). A possible alternative to overcome these innate resistances could be the use of combinatory therapies. One of the main limitations in the use of PI3K pathway inhibitors is the lack of precise biomarkers to predict drug-response and select patients that could benefit the most from treatment. At the moment, there is not a well defined candidate. So far the predictive potential of the mutational status of *PIK3CA* or *PTEN* has been tested in several early-phase clinical trials with conflicting results (Dienstmann et al., 2014; Janku et al., 2012, 2013). Our data suggest that nuclear β -catenin is a biomarker of resistance to PI3K and AKT inhibitors in CRC. We observed, that patients containing high nuclear amounts of β -

catenin did not respond PI3K signaling inhibition in phase I clinical trials. Besides, other factors analyzed including the mutational status of *PIK3CA* and other frequent mutated genes in CRC (*KRAS* or *TP53*) did not predict drug-response. However, in the case of patients with tumors containing cells with low amounts of nuclear β -catenin we observed a heterogeneous response to PI3K signaling inhibition. While some patients responded to the therapy and presented a stabilized disease others progressed faster, suggesting that other elements apart from β -catenin can contribute to resistance to PI3K or AKT inhibition.

Data obtained in other studies using different types of treatments, such as EGFR inhibitors, suggest that PI3K pathway inhibitors could be combined with additional target-directed therapies in order to overcome innate or acquired resistances. Examples of therapeutic combinations include simultaneous blocking of EGFR and downstream elements in the same signaling pathway such as MEK, PI3K pathway or BRAF to prevent positive feedback loops. We propose to combine PI3K or AKT inhibitors with inhibitors of the Wnt signaling pathway, such as tankyrase inhibitors, as a promising new therapeutic option for mCRC treatment. Tankyrase inhibitors would lead to AXIN1/2 stabilization and an increased degradation of β -catenin. Reduction in β -catenin levels would deprive FOXO proteins from its transcriptional partner and prevent resistance mechanisms driven by the action of both factors. Indeed, our results using PDX models showed that resistant tumors, with high amounts of nuclear β -catenin, responded to the combination of PI3K or AKT inhibitors with a tankyrase inhibitor. In the case of PDX models with tumors containing low nuclear β -catenin concentrations we observed a heterogeneous response to PI3K signaling inhibition, similar to that seen in the retrospective study with patients in phase I clinical trials. One PDX model responded to AKT inhibition alone (model T61 in **article 1**) whereas another responded neither to AKT inhibition nor to its combination with a tankyrase inhibitor (model P5 in **article 2**). Resistance to the AKT inhibitor API-2 in the specific case of PDX-P5 could be explained by the fact that it presents an uncharacterized mutation in AKT2 protein. API-2 inhibits AKT by binding to its PH domain and blocking its recruitment to the plasma membrane, a step necessary for AKT activation. The mutation found in this PDX model consists in an amino acid change (glycine to serine) in the kinase domain, which could lead to API-2 resistance if it causes a constitutive activation of the protein. Further analyses should be done to validate this hypothesis. Altogether, these results indicate that it may be necessary to further investigate the determinants of resistance to PI3K pathway inhibitors in the case of tumors presenting low amounts of nuclear β -catenin since they may not rely as much on the effect of this protein to survive. However, it should be necessary to focus not only on the genetic alterations that tumors present but also analyze the molecular changes that PI3K signaling inhibition can induce in that cellular context. It has been described that acquired resistance to agents targeting EGFR, BRAF or mTOR can be caused by several mechanisms including the hyperactivation of compensatory pathways through the MET receptor (Sadanandam et al., 2013; Straussman et al., 2012) or through other compensatory feedback loops including MAPKs (Carracedo et al., 2008; Prahallad et al., 2012).

Targeting Wnt signaling has been a challenging process since this pathway was discovered more than 30 years ago. Together with Notch, Hedgehog and TGF β signaling pathways, Wnt signaling is a crucial developmental pathway highly conserved throughout evolution. This means that their inhibition could potentially have devastating effects in living organisms and

resulting in severe undesired effects in the clinic. Additionally, few components from the Wnt pathway have proved to be druggable. Many Wnt signaling inhibitors have been described to possess good *in vitro* activity, however, much less have proved to be effective *in vivo* and just few have reached clinical trials (Kahn, 2014). In this context, tankyrase inhibitors have arisen as very promising therapeutic agents for Wnt signaling blocking. We and others have proved that although their use can have side effects in organs that rely on Wnt signaling, such as the intestine and the skin, they are well tolerated in mice (**article 2** and (Lau et al., 2013; Waaler et al., 2012).

We showed that tankyrase inhibitors can be combined with PI3K and AKT inhibitors to sensitize tumors containing cells with high endogenous amounts of nuclear β -catenin. *In vitro*, combination of both classes of drugs sensitized cancer cells to apoptosis. *In vivo*, tankyrase inhibition additionally led to a reduction of tumor growth when combined with an AKT inhibitor. Surprisingly, we also demonstrated that, in a specific context *in vivo*, NVP-TNKS656 can efficiently reduce tumor growth acting as a single agent. Single agent activity was achieved in a PDX model with high endogenous nuclear amounts of both β -catenin and FOXO3a (model PDX-P30 in **article 2**). This effect could be consequence of restoring FOXO pro-apoptotic program upon reducing β -catenin by tankyrase inhibition. In this manner, the use of a PI3K signaling inhibitor to promote FOXOs accumulation in the nucleus would not be necessary. Additionally, in our inducible cellular models, we demonstrated that reduction in the activity of nuclear β -catenin by tankyrase inhibition increases apoptosis induced by FOXO3a. These results could position FOXO3a content as a biomarker of response to mono-therapy with Wnt/tankyrase inhibitors. Its validation would allow the identification of patients susceptible to this treatment and could speed up the introduction of this class of drugs in the clinic. However, at this stage we cannot rule out the possibility that the other members of the FOXO family could have the same predictive properties as FOXO3a. It has been well established the functional redundancy between FOXO3a and other FOXO proteins (Paik et al., 2007), however, complete expression patterns of all FOXO isoforms are not determined. A deeper evaluation of specific FOXO isoforms expression could help determining to which extent the effects observed in this thesis are restricted exclusively to FOXO3a.

It is important to highlight that the anti-tumor effect of tankyrase inhibition is effective even in cells containing APC truncating mutations. Around 80% of CRC cases present alterations in APC (TCGA, 2012). Therefore, tankyrase inhibitors would potentially be beneficial for the majority of CRC patients whereas Wnt inhibitors acting upstream in the signaling pathway would not. This would be the case of the PORCN inhibitor NVP-WNT974, that reduces the maturation and secretion of Wnt ligands (Chen et al., 2009; Liu et al., 2013), or antibodies blocking Wnt receptors (Fukukawa et al., 2008; Smith et al., 2013) that may only be effective in patients with APC wild type tumors. For this reason, the first clinical trial with NVP-WNT974 (CTID: NCT01351103) excluded CRC patients and focused on other APC wild type solid tumors showing activated Wnt pathway such as pancreatic tumors or melanomas (Jiang et al., 2013; Madan et al., 2015). Truncating mutations in *RNF43* and *ZNRF3* genes, coding for ubiquitin ligases that degrade FZD, or fusion in *RSPO* genes activate the oncogenic Wnt/ β -catenin signaling alternatively to APC mutations. Such less frequent upstream alterations confer sensitivity to Wnt/PORCN inhibitors (Madan et al., 2015; Wetering et al., 2015). It has also

been suggested that incomplete elimination of nuclear β -catenin by tankyrase inhibitors could confer resistance to these drugs (De La Roche et al., 2014). Indeed, we observed that NVP-TNKS656 is not able to completely eliminate β -catenin from the nucleus of tumor cells. This may be probably due to its retention by particular nuclear interacting proteins that prevent its degradation by the proteasome in the cytoplasm. However, we showed that in such scenario tumor growth is reduced after long-term treatments.

Overall, it seems clear that although tankyrase inhibitors are potent inhibitors of Wnt signaling they do not seem to induce massive cell death in tumor cells and tumor shrinkage (Lau et al., 2013). Tankyrases participate in many different cellular processes by controlling the stability or the function of several proteins. Mostly, these are processes that could favor tumor growth, such as promoting mitosis and telomere length or increase glucose intake. To which extend these Wnt-independent tankyrase functions are responsible for tumor growth reduction caused by tankyrase inhibitors is not well understood and has not been addressed in the present thesis. Further study of such effects would be necessary to fully characterize the potential of tankyrase inhibitors as anti-tumor therapeutic agents. So far, our data and that from other groups indicate that Wnt inhibition would be effective in rational combinations with other target-directed drugs inhibiting the PI3K, AKT, MEK or EGFR inhibitors (Casás-Selves et al., 2012; Schoumacher et al., 2014).

With the results of this thesis we propose to use a combination of gene expression profiling and histological methods to define nuclear β -catenin and FOXO3a content and activity as predictive biomarkers of response to PI3K, AKT and/or tankyrase inhibitors. We believe that this type of molecular prescreening could establish three groups of patients presenting tumors with:

1. **Low nuclear β -catenin and FOXO3a activity**, more suitable for the treatment with PI3K or AKT inhibitors as a monotherapy.
2. **High β -catenin and FOXO3a activity** who could benefit from Wnt/ β -catenin inhibitors alone.
3. **High nuclear β -catenin but low FOXO3a activity** who may benefit from combined treatments.

Such molecular stratification could help to precise the most appropriate therapy for each patient. Further validation of these biomarkers will require retrospective analyses of larger cohorts of patients treated with PI3K, AKT or Wnt inhibitors in the next future.

CONCLUSIONS

1. FOXO3a and β -catenin, are present and co-localize at high concentration in the nucleus of advanced colon carcinomas and liver metastases.
2. β -catenin modifies FOXO3a transcriptional activity rescuing cells from FOXO3a-induced apoptosis and increasing cell motility, adhesion and metastasis.
3. PDXs from CRC patients with high amounts of β -catenin are resistant to AKT inhibition while some models from patients with low amounts of β -catenin are sensitive.
4. Tankyrase inhibitors effectively overcome the resistance conferred by high nuclear β -catenin to FOXO3a-induced apoptosis promoted by PI3K or AKT inhibitors *in vitro* and *in vivo*.
5. Tankyrase inhibition is particularly effective in tumors with high endogenous nuclear β -catenin and FOXO3a content, promoting apoptosis and reducing tumor growth preferentially by repressing the expression of FOXO3a instead of TCF/LEF target genes.
6. Nuclear β -catenin is a biomarker of resistance to PI3K and AKT inhibition in CRC whereas FOXO3a is a biomarker of response to tankyrase inhibition.
7. By using gene expression profiling, it is possible to identify CRC patients with tumors active for FOXO3a/ β -catenin transcription. These patients are potentially susceptible to be treated with Wnt inhibitors.

REFERENCES

- Albuquerque, C., Bakker, E.R.M., van Veelen, W., and Smits, R. (2011). Colorectal cancers choosing sides. *Biochim. Biophys. Acta - Rev. Cancer* *1816*, 219–231.
- Almeida, M., Han, L., Martin-Millan, M., O'Brien, C.A., and Manolagas, S.C. (2007). Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting β -catenin from T cell factor- to forkhead box O-mediated transcription. *J. Biol. Chem.* *282*, 27298–27305.
- American Cancer Society (2013). Colorectal Cancer What is cancer ? Am. Cancer Soc.
- Anastas, J.N., and Moon, R.T. (2013). WNT signalling pathways as therapeutic targets in cancer. *Nat. Rev. Cancer* *13*, 11–26.
- Arena, S., Bellosillo, B., Siravegna, G., Martinez, A., Canadas, I., Lazzari, L., Ferruz, N., Russo, M., Misale, S., Gonzalez, I., et al. (2015). Emergence of multiple EGFR extracellular mutations during cetuximab treatment in colorectal cancer. *Clin. Cancer Res.* *21*, 2157–2167.
- Azzolin, L., Zanconato, F., Bresolin, S., Forcato, M., Basso, G., Bicciato, S., Cordenonsi, M., and Piccolo, S. (2012). Role of TAZ as mediator of wnt signaling. *Cell* *151*, 1443–1456.
- Barker, N., and Clevers, H. (2006). Mining the Wnt pathway for cancer therapeutics. *Nat. Rev. Drug Discov.* *5*, 997–1014.
- Bauer, T.M., Patel, M.R., and Infante, J.R. (2015). Targeting PI3 kinase in cancer. *Pharmacol. Ther.* *146*, 53–60.
- Behrens, J., von Kries, J.P., Kühl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of β -catenin with the transcription factor LEF-1. *Nature* *382*, 638–642.
- Berndt, N., Yang, H., Trinczek, B., Betzi, S., Zhang, Z., Wu, B., Lawrence, N.J., Pellecchia, M., Schönbrunn, E., Cheng, J.Q., et al. (2010). The Akt activation inhibitor TCN-P inhibits Akt phosphorylation by binding to the PH domain of Akt and blocking its recruitment to the plasma membrane. *Cell Death Differ.*
- Bertotti, A., Migliardi, G., Galimi, F., Sassi, F., Torti, D., Isella, C., Corà, D., di Nicolantonio, F., Buscarino, M., Petti, C., et al. (2011). A molecularly annotated platform of patient- derived xenografts (“xenopatients”) identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. *Cancer Discov.* *1*, 508–523.
- Bogaert, J., and Prenen, H. (2014). Molecular genetics of colorectal cancer. *Ann. Gastroenterol.* *27*, 9–14.

- Boland, C.R., and Goel, A. (2010). Microsatellite Instability in Colorectal Cancer. *Gastroenterology* 138, 2073–2087.
- Bregman, H., Gunaydin, H., Gu, Y., Schneider, S., Wilson, C., Dimauro, E.F., and Huang, X. (2013). Discovery of a class of novel tankyrase inhibitors that bind to both the nicotinamide pocket and the induced pocket. *J. Med. Chem.* 56, 1341–1345.
- Brenner, H., Kloor, M., and Pox, C.P. (2014). Colorectal cancer. *Lancet* 383, 1490–1502.
- Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96, 857–868.
- Callow, M.G., Tran, H., Phu, L., Lau, T., Lee, J., Sandoval, W.N., Liu, P.S., Bheddah, S., Tao, J., Lill, J.R., et al. (2011). Ubiquitin ligase RNF146 regulates tankyrase and Axin to promote Wnt signaling. *PLoS One*.
- Carracedo, A., Ma, L., Teruya-feldstein, J., Rojo, F., and Salmena, L. (2008). Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K- dependent feedback loop in human cancer. *118*, 3065–3074.
- Casagolda, D., Del Valle-Pérez, B., Valls, G., Lugilde, E., Vinyoles, M., Casado-Vela, J., Solanas, G., Batlle, E., Reynolds, A.B., Casal, J.I., et al. (2010). A p120-catenin-CK1epsilon complex regulates Wnt signaling. *J. Cell Sci.* 123, 2621–2631.
- Casás-Selves, M., Kim, J., Zhang, Z., Helfrich, B.A., Gao, D., Porter, C.C., Scarborough, H.A., Bunn, P.A., Chan, D.C., Tan, A.C., et al. (2012). Tankyrase and the canonical Wnt pathway protect lung cancer cells from EGFR inhibition. *Cancer Res.* 72, 4154–4164.
- Castells, A., Castellví-Bel, S., and Balaguer, F. (2009). Concepts in Familial Colorectal Cancer: Where Do We Stand and What Is the Future? *Gastroenterology* 137, 404–409.
- Chang, P., Coughlin, M., and Mitchison, T.J. (2009). Interaction between Poly(ADP-ribose) and NuMA Contributes to Mitotic Spindle Pole Assembly. *Mol. Biol. Cell* 20, 4575–4585.
- Chen, B., Dodge, M.E., Tang, W., Lu, J., Ma, Z., Fan, C.-W., Wei, S., Hao, W., Kilgore, J., Williams, N.S., et al. (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat. Chem. Biol.* 5, 100–107.
- Chiang, Y.J., Hsiao, S.J., Yver, D., Cushman, S.W., Tessarollo, L., Smith, S., and Hodes, R.J. (2008). Tankyrase 1 and tankyrase 2 are essential but redundant for mouse embryonic development. *PLoS One* 3, 1–10.
- Cho-Park, P.F., and Steller, H. (2013). Proteasome regulation by ADP-ribosylation. *Cell* 153, 614–627.

- Citri, A., and Yarden, Y. (2006). EGF-ERBB signalling: towards the systems level. *Nat. Rev. Mol. Cell Biol.* 7, 505–516.
- Clarke, S.J., Karapetis, C.S., Gibbs, P., Pavlakis, N., Desai, J., Michael, M., Tebbutt, N.C., Price, T.J., and Tabernero, J. (2013). Overview of biomarkers in metastatic colorectal cancer: Tumour, blood and patient-related factors. *Crit. Rev. Oncol. Hematol.* 85, 121–135.
- Clevers, H. (2006). Wnt/ β -Catenin Signaling in Development and Disease. *Cell* 127, 469–480.
- Clevers, H., and Nusse, R. (2012). Wnt/ β -catenin signaling and disease. *Cell* 149, 1192–1205.
- Cong, F., and Varmus, H. (2004). Nuclear-cytoplasmic shuttling of Axin regulates subcellular localization of beta-catenin. *Proc. Natl. Acad. Sci. U. S. A.* 101, 2882–2887.
- Curtin, J.C. (2013). Novel drug discovery opportunities for colorectal cancer. *Expert Opin. Drug Discov.* 8, 1–12.
- Van Cutsem, E., Borràs, J.M., Castells, A., Ciardiello, F., Ducreux, M., Haq, A., Schmoll, H.J., Tabernero, J., Cutsem, E. Van, Borra, J.M., et al. (2013). Improving outcomes in colorectal cancer: Where do we go from here? *Eur. J. Cancer* 49, 2476–2485.
- Van Cutsem, E., Cervantes, a., Nordlinger, B., and Arnold, D. (2014). Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* 25, iii1–iii9.
- Danielsen, S.A., Eide, P.W., Nesbakken, A., Guren, T., Leithe, E., and Lothe, R.A. (2015). Portrait of the PI3K/AKT pathway in colorectal cancer. *Biochim. Biophys. Acta - Rev. Cancer* 1855, 104–121.
- Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A., and Niehrs, C. (2005). Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* 438, 867–872.
- DeAlmeida, V.I., Miao, L., Ernst, J.A., Koeppen, H., Polakis, P., and Rubinfeld, B. (2007). The soluble Wnt receptor Frizzled8CRD-hFc inhibits the growth of teratocarcinomas in vivo. *Cancer Res.* 67, 5371–5379.
- Dienstmann, R., Rodon, J., Markman, B., and Tabernero, J. (2011). Recent Developments in Anti-Cancer Agents Targeting PI3K, Akt and mTORC1/2. *Recent Pat. Anticancer. Drug Discov.* 6, 210–236.
- Dienstmann, R., Rodon, J., Serra, V., and Tabernero, J. (2014). Picking the point of inhibition: a comparative review of PI3K/AKT/mTOR pathway inhibitors. *Mol. Cancer Ther.* 13, 1021–1031.

- Doble, B.W., and Woodgett, J.R. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. *J. Cell Sci.* *116*, 1175–1186.
- Doble, B.W., Patel, S., Wood, G.A., Kockeritz, L.K., and Woodgett, J.R. (2007). Functional Redundancy of GSK-3 α and GSK-3 β in Wnt/ β -Catenin Signaling Shown by Using an Allelic Series of Embryonic Stem Cell Lines. *Dev. Cell* *12*, 957–971.
- Dow, L.E., Rourke, K.P.O., Simon, J., Es, J.H. Van, Clevers, H., Lowe, S.W., Dow, L.E., Rourke, K.P.O., Simon, J., Tschaharganeh, D.F., et al. (2015). Apc Restoration Promotes Cellular Differentiation and Reestablishes Crypt Homeostasis in Colorectal Article Apc Restoration Promotes Cellular Differentiation and Reestablishes Crypt Homeostasis in Colorectal Cancer. *Cell* *161*, 1539–1552.
- Dregalla, R.C., Zhou, J., Idate, R.R., Battaglia, C.L.R., Liber, H.L., and Bailey, S.M. (2010). Regulatory roles of tankyrase 1 at telomeres and in DNA repair: Suppression of T-SCE and stabilization of DNA-pkcs. *Aging (Albany. NY)*. *2*, 691–708.
- Eijkelenboom, A., and Burgering, B.M.T. (2013). FOXOs: signalling integrators for homeostasis maintenance. *Nat. Rev. Mol. Cell Biol.*
- El-Khoueiry, A.B., Ning, Y., Yang, D., Cole, S., Kahn, M., Zoghbi, M., Berg, J., Fujimori, M., Inada, T., Kouji, H., et al. (2013). A phase I first-in-human study of PRI-724 in patients (pts) with advanced solid tumors. *J Clin Oncol abstract* 2501.
- Emami, K.H., Nguyen, C., Ma, H., Kim, D.H., Jeong, K.W., Eguchi, M., Moon, R.T., Teo, J., Oh, S.W., Kim, H.Y., et al. (2004). A small molecule inhibitor of β -catenin/CREB-binding protein transcription. *PNAS* *101*, 12682–12687.
- Essers, M.A.G., de Vries-Smits, L.M.M., Barker, N., Polderman, P.E., Burgering, B.M.T., and Korswagen, H.C. (2005). Functional interaction between beta-catenin and FOXO in oxidative stress signaling. *Science (80-.)*. *308*, 1181–1184.
- Fang, D., Hawke, D., Zheng, Y., Xia, Y., Meisenhelder, J., Nika, H., Mills, G.B., Kobayashi, R., Hunter, T., and Lu, Z. (2007). Phosphorylation of β -catenin by AKT promotes β -catenin transcriptional activity. *J. Biol. Chem.* *282*, 11221–11229.
- Fearon, E.R. (2011). Molecular genetics of colorectal cancer. *Annu. Rev. Pathol.*
- Fearon, E.R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell* *61*, 759–767.
- Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., and Bray, F. (2015). Cancer incidence and mortality worldwide : Sources , methods and major patterns in GLOBOCAN 2012. *Int. J. Biochem. Cell Biol.* *136*, E359–E386.
- Fodde, R., Smits, R., and Clevers, H. (2001). APC, signal transduction and genetic

instability in colorectal cancer. *Nat. Rev. Cancer* **1**, 55–67.

Fruman, D. a, and Rommel, C. (2014). PI3K and cancer: lessons, challenges and opportunities. *Nat. Rev. Drug Discov.* **13**, 140–156.

Fukukawa, C., Hanaoka, H., Nagayama, S., Tsunoda, T., Toguchida, J., Endo, K., Nakamura, Y., and Katagiri, T. (2008). Radioimmunotherapy of human synovial sarcoma using a monoclonal antibody against FZD10. *Cancer Sci.* **99**, 432–440.

Garcia-carbonero, R., and Supko, J.G. (2002). Current Perspectives on the Clinical Experience , Pharmacology , and Continued Development of the Camptothecins. *Clin. Cancer Res.* **8**, 641–661.

Goldstein, N.I., Prewett, M., Zuklys, K., Rockwell, P., and Mendelsohn, J. (1995). Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. *Clin. Cancer Res.* **1**, 1311–1318.

Guo, H.-L., Zhang, C., Liu, Q., Li, Q., Lian, G., Wu, D., Li, X., Zhang, W., Shen, Y., Ye, Z., et al. (2012). The Axin/TNKS complex interacts with KIF3A and is required for insulin-stimulated GLUT4 translocation. *Cell Res.* **22**, 1246–1257.

Haikarainen, T., Krauss, S., and Lehtiö, L. (2014). Tankyrases: Structure, Function and Therapeutic Implications in Cancer. *Curr. Pharm. Des.*

Hart, M., Concordet, J.P., Lassot, I., Albert, I., Del Los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R., et al. (1999). The F-box protein β -TrCP associates with phosphorylated β -catenin and regulates its activity in the cell. *Curr. Biol.* **9**, 207–210.

Henderson, B.R. (2000). Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nat. Cell Biol.* **2**, 653–660.

Herr, P., Hausmann, G., and Basler, K. (2012). WNT secretion and signalling in human disease. *Trends Mol. Med.* **18**, 483–493.

Hidalgo, M., Amant, F., Biankin, a. V., Budinska, E., Byrne, a. T., Caldas, C., Clarke, R.B., de Jong, S., Jonkers, J., Maelandsmo, G.M., et al. (2014). Patient-Derived Xenograft Models: An Emerging Platform for Translational Cancer Research. *Cancer Discov.* **4**, 988–1013.

Holash, J., Davis, S., Papadopoulos, N., Croll, S.D., Ho, L., Russell, M., Boland, P., Leidich, R., Hylton, D., Burova, E., et al. (2002). VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11393–11398.

Hoogeboom, D., Essers, M.A.G., Polderman, P.E., Voets, E., Smits, L.M.M., and Burgering, B.M.T. (2008). Interaction of FOXO with β -catenin inhibits β -catenin/T cell factor activity. *J. Biol. Chem.* **283**, 9224–9230.

- Hsiao, S.J., and Smith, S. (2009). Sister telomeres rendered dysfunctional by persistent cohesion are fused by NHEJ. *J. Cell Biol.* **184**, 515–526.
- Huang, S.-M.A., Mishina, Y.M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G.A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., et al. (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* **461**, 614–620.
- Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B.G., and Kemler, R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech. Dev.* **59**, 3–10.
- Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D., and Perucho, M. (1993). Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* **363**, 558–561.
- James, R.G., Davidson, K.C., Bosch, K.A., Biechele, T.L., Robin, N.C., Taylor, R.J., Major, M.B., Camp, N.D., Fowler, K., Martins, T.J., et al. (2012). WIKI4, a Novel Inhibitor of Tankyrase and Wnt/ β -Catenin Signaling. *PLoS One* **7**, 1–10.
- Janku, F., Wheler, J.J., Westin, S.N., Moulder, S.L., Naing, A., Tsimberidou, A.M., Fu, S., Falchook, G.S., Hong, D.S., Garrido-laguna, I., et al. (2012). PI3K/AKT/ mTOR Inhibitors in Patients With Breast and Gynecologic Malignancies Harboring PIK3CA Mutations. *J. Clin. Oncol.* **30**, 777–782.
- Janku, F., Wheler, J.J., Naing, A., Falchook, G.S., Hong, D.S., Stepanek, V.M., Fu, S., Piha-paul, S.A., Lee, J.J., Luthra, R., et al. (2013). PIK3CA Mutation H1047R Is Associated with Response to PI3K/AKT/ mTOR Signaling Pathway Inhibitors in Early-Phase Clinical Trials. *Cancer Res.* **73**, 276–285.
- Jass, J.R. (2007). Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* **50**, 113–130.
- Jiang, X., Hao, H.-X., Gowney, J.D., Woolfenden, S., Bottiglio, C., Ng, N., Lu, B., Hsieh, M.H., Bagdasarian, L., Meyer, R., et al. (2013). Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma. *Proc. Natl. Acad. Sci. U. S. A.*
- Johnson, J.I., Decker, S., Zaharevitz, D., Rubinstein, L. V, Venditti, J.M., Schepartz, S., Kalyandrug, S., Christian, M., Arbuck, S., Hollingshead, M., et al. (2001). Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. *Br. J. Cancer* **84**, 1424–1431.
- Julien, S., Merino-Trigo, A., Lacroix, L., Pocard, M., Goéré, D., Mariani, P., Landron, S., Bigot, L., Nemati, F., Dartigues, P., et al. (2012). Characterization of a large panel of patient-derived tumor xenografts representing the clinical heterogeneity of human colorectal cancer. *Clin. Cancer Res.* **18**, 5314–5328.

- Kahn, M. (2014). Can we safely target the WNT pathway? *Nat. Rev. Drug Discov.* **13**, 513–532.
- Kaidi, A., Williams, A.C., and Paraskeva, C. (2007). Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nat. Cell Biol.* **9**, 210–217.
- Kelly, K.F., Ng, D.Y., Jayakumaran, G., Wood, G.A., Koide, H., and Doble, B.W. (2010). β -Catenin Enhances Oct-4 Activity and Reinforces Pluripotency through a TCF-Independent Mechanism. *Cell Stem Cell* **8**, 214–227.
- Kikuchi, A., Yamamoto, H., and Sato, A. (2009). Selective activation mechanisms of Wnt signaling pathways. *Trends Cell Biol.* **19**, 119–129.
- Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., and Ferrara, N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* **362**, 841–844.
- Kim, M.K., Dudognon, C., and Smith, S. (2012). Tankyrase 1 regulates centrosome function by controlling CPAP stability. *EMBO Rep.* **13**, 724–732.
- Kinzler, K., and Vogelstein, B. (1996). Lessons from Hereditary Review Colorectal Cancer. *Cell* **87**, 159–170.
- Krasinskas, A.M. (2011). EGFR Signaling in Colorectal Carcinoma. *Patholog. Res. Int.* **2011**, 932932.
- De La Roche, M., Ibrahim, A.E.K., Mieszczanek, J., Bienz, M., Roche, M. De, Ibrahim, A.E.K., and Mieszczanek, J. (2014). LEF1 and B9L shield b-catenin from inactivation by axin, desensitizing colorectal cancer cells to tankyrase inhibitors. *Cancer Res.* **74**, 1495–1505.
- Labianca, R., Nordlinger, B., Beretta, G.D., Mosconi, S., Mandalà, M., Cervantes, A., and Arnold, D. (2013). Early colon cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **24**, vi64–vi72.
- De Lange, T. (2005). Shelterin: The protein complex that shapes and safeguards human telomeres. *Genes Dev.* **19**, 2100–2110.
- Lau, T., Chan, E., Callow, M., Waaler, J., Boggs, J., Blake, R.A., Magnuson, S., Sambrone, A., Schutten, M., Firestein, R., et al. (2013). A novel tankyrase small-molecule inhibitor suppresses APC mutation-driven colorectal tumor growth. *Cancer Res.* **73**, 3132–3144.
- Lawrence, M.S., Stojanov, P., Mermel, C.H., Robinson, J.T., Garraway, L. a, Golub, T.R., Meyerson, M., Gabriel, S.B., Lander, E.S., and Getz, G. (2014). Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* **505**, 495–501.
- Lee, E., Salic, A., Krüger, R., Heinrich, R., Kirschner, M.W., Lee, E., Salic, A., and Kru, R.

- (2003). The roles of APC and axin derived from experimental and theoretical analysis of the Wnt pathway. *PLoS Biol.* *1*, 116–132.
- Lehtiö, L., Chi, N.W., and Krauss, S. (2013). Tankyrases as drug targets. *FEBS J.* *280*, 3576–3593.
- Liu, J., Pan, S., Hsieh, M.H., Ng, N., Sun, F., Wang, T., Kasibhatla, S., Schuller, A.G., Li, A.G., Cheng, D., et al. (2013). Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. *Proc. Natl. Acad. Sci. U. S. A.* *110*, 20224–20229.
- Liu, P., Cheng, H., Roberts, T.M., and Zhao, J.J. (2009). Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat. Rev. Drug Discov.* *8*, 627–644.
- Liu, S., Knapp, S., and Ahmed, A.A. (2014). The structural basis of PI3K cancer mutations: From mechanism to therapy. *Cancer Res.* *74*, 641–646.
- Lo, R.S. (2012). Receptor tyrosine kinases in cancer escape from BRAF inhibitors. *Cell Res.* *22*, 945–947.
- Lupo, B., and Trusolino, L. (2014). Inhibition of poly(ADP-ribosyl)ation in cancer: Old and new paradigms revisited. *Biochim. Biophys. Acta - Rev. Cancer.*
- Lustig, B., Lustig, B., Jerchow, B., Jerchow, B., Sachs, M., Sachs, M., Weiler, S., Weiler, S., Pietsch, T., Pietsch, T., et al. (2002). Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol. Cell. Biol.* *22*, 1184–1193.
- MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/ β -Catenin Signaling: Components, Mechanisms, and Diseases. *Dev. Cell* *17*, 9–26.
- Madan, B., Ke, Z., Harmston, N., Ho, S.Y., Frois, A.O., Alam, J., Jeyaraj, D.A., Pendharkar, V., Ghosh, K., Virshup, I.H., et al. (2015). Wnt addiction of genetically defined cancers reversed by PORCN inhibition. *Oncogene* 1–11.
- Maira, S.-M., Pecchi, S., Huang, A., Burger, M., Knapp, M., Sterker, D., Schnell, C., Guthy, D., Nagel, T., Wiesmann, M., et al. (2012). Identification and Characterization of NVP-BKM120, an Orally Available Pan-Class I PI3-Kinase Inhibitor. *Mol. Cancer Ther.* *11*, 317–328.
- Markman, B., Atzori, F., Pérez-García, J., Tabernero, J., and Baselga, J. (2009). Status of PI3K inhibition and biomarker development in cancer therapeutics. *Ann. Oncol.* *21*, 683–691.
- McIntyre, R.E., Buczacki, S.J.A., Arends, M.J., and Adams, D.J. (2015). Mouse models of colorectal cancer as preclinical models. *BioEssays* *37*, 909–920.
- Meropol, N.J. (1998). Clinical Oncology Update Oral Fluoropyrimidines in the

Treatment of Colorectal Cancer. *Eur. J. Cancer* 34, 1509–1513.

Mojarad, E.N., Kuppen, P.J.K., Aghdaei, H.A., and Zali, M.R. (2013). The CpG island methylator phenotype (CIMP) in colorectal cancer. *Gastroenterol. Hepatol. from Bed to Bench*.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destrée, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. *Cell* 86, 391–399.

Montagut, C., Dalmases, A., Bellosillo, B., Crespo, M., Pairet, S., Iglesias, M., Salido, M., Gallen, M., Marsters, S., Tsai, S.P., et al. (2012). Identification of a mutation in the extracellular domain of the Epidermal Growth Factor Receptor conferring cetuximab resistance in colorectal cancer. *Nat. Med.* 18, 1445–1445.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997). Activation of β -catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* (80-.). 275, 1787–1790.

Mosimann, C., Hausmann, G., and Basler, K. (2009). β -catenin hits chromatin: regulation of Wnt target gene activation. *Nat. Rev. Mol. Cell Biol.* 10, 276–286.

Mouradov, D., Sloggett, C., Jorissen, R.N., Love, C.G., Li, S., Burgess, A.W., Arango, D., Strausberg, R.L., Buchanan, D., Wormald, S., et al. (2014). Colorectal cancer cell lines are representative models of the main molecular subtypes of primary cancer. *Cancer Res.* 74, 3238–3247.

Mulholland, D.J., Dedhar, S., Coetzee, G.A., and Nelson, C.C. (2005). Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? *Endocr. Rev.* 26, 898–915.

Myatt, S.S., and Lam, E.W.-F. (2007). The emerging roles of forkhead box (Fox) proteins in cancer. *Nat. Rev. Cancer* 7, 847–859.

Neufeld, K.L., Zhang, F., Cullen, B.R., and White, R.L. (2000). APC-mediated downregulation of beta-catenin activity involves nuclear sequestration and nuclear export. *EMBO Rep.*

Obsil, T., and Obsilova, V. (2008). Structure/function relationships underlying regulation of FOXO transcription factors. *Oncogene* 27, 2263–2275.

Ozaki, Y., Matsui, H., Asou, H., Nagamachi, A., Aki, D., Honda, H., Yasunaga, S., Takihara, Y., Yamamoto, T., Izumi, S., et al. (2012). Poly-ADP Ribosylation of Miki by tankyrase-1 Promotes Centrosome Maturation. *Mol. Cell* 47, 694–706.

Paik, J.H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., et al. (2007). FoxOs Are Lineage-Restricted Redundant Tumor

Suppressors and Regulate Endothelial Cell Homeostasis. *Cell* 128, 309–323.

Pálmer, H.G., González-Sancho, J.M., Espada, J., Berciano, M.T., Puig, I., Baulida, J., Quintanilla, M., Cano, A., de Herreros, A.G., Lafarga, M., et al. (2001). Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J. Cell Biol.* 154, 369–387.

Papadatos-pastos, D., Rabbie, R., Ross, P., and Sarker, D. (2015). The role of the PI3K pathway in colorectal cancer. *Crit. Rev. Oncol. / Hematol.* 94, 18–30.

Piedra, J., Martínez, D., Castaño, J., Miravet, S., Duñach, M., and García De Herreros, A. (2001). Regulation of β -Catenin Structure and Activity by Tyrosine Phosphorylation. *J. Biol. Chem.* 276, 20436–20443.

Pino, M.S., and Chung, D.C. (2010). The Chromosomal Instability Pathway in Colon Cancer. *Gastroenterology* 138, 2059–2072.

Pokutta, S., and Weis, W.I. (2000). Structure of the dimerization and beta-catenin-binding region of alpha-catenin. *Mol. Cell* 5, 533–543.

Prahallad, A., Sun, C., Huang, S., Di Nicolantonio, F., Salazar, R., Zecchin, D., Beijersbergen, R.L., Bardelli, A., and Bernards, R. (2012). Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature* 483, 100–103.

Price, M.A. (2006). CKI, there's more than one : casein kinase I family members in Wnt and Hedgehog signaling. *Genes Dev.* 15, 399–410.

Prior, I. a., Lewis, P.D., and Mattos, C. (2012). A comprehensive survey of ras mutations in cancer. *Cancer Res.* 72, 2457–2467.

Puig, I., Chicote, I., Tenbaum, S.P., Arqués, O., Herance, J.R., Gispert, J.D., Jimenez, J., Landolfi, S., Caci, K., Allende, H., et al. (2013). A personalized preclinical model to evaluate the metastatic potential of patient-derived colon cancer initiating cells. *Clin. Cancer Res.* 19, 6787–6801.

Rashid, a, Shen, L., Morris, J.S., Issa, J.P., and Hamilton, S.R. (2001). CpG island methylation in colorectal adenomas. *Am. J. Pathol.* 159, 1129–1135.

Raymond, E., Chaney, S.G., Taamma, A., and Cvitkovic, E. (1998). Oxaliplatin : A review of preclinical and clinical studies. *Ann. Oncol.* 9, 1053–1071.

Riffell, J.L., Lord, C.J., and Ashworth, A. (2012). Tankyrase-targeted therapeutics: expanding opportunities in the PARP family. *Nat Rev Drug Discov* 11, 923–936.

Roock, W. De, Vriendt, V. De, Normanno, N., Ciardiello, F., and Tejpar, S. (2011). KRAS, BRAF, PIK3CA, and PTEN mutations: Implications for targeted therapies in metastatic

colorectal cancer. *Lancet Oncol.* 12, 594–603.

Rosenbluh, J., Nijhawan, D., Cox, A.G., Li, X., Neal, J.T., Schafer, E.J., Zack, T.I., Wang, X., Tsherniak, A., Schinzel, A.C., et al. (2012). β -Catenin-driven cancers require a YAP1 transcriptional complex for survival and tumorigenesis. *Cell* 151, 1457–1473.

Rosin-Arbesfeld, R., Townsley, F., and Bienz, M. (2000). The APC tumour suppressor has a nuclear export function. *Nature* 406, 1009–1012.

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. (1996). Binding of GSK3 β to the APC- β -catenin complex and regulation of complex assembly. *Science* (80-.). 272, 1023–1026.

Rustgi, A.K. (2007). The genetics of hereditary colon cancer. *Genes Dev.* 21, 2525–2538.

Sadanandam, A., Lyssiotis, C. a, Homicsko, K., Collisson, E. a, Gibb, W.J., Wulschleger, S., Ostos, L.C., Lannon, W. a, Grotzinger, C., Del Rio, M., et al. (2013). A colorectal cancer classification system that associates cellular phenotype and responses to therapy. *Nat Med* 19, 619–625.

Salahshor, S., and Woodgett, J.R. (2005). The links between axin and carcinogenesis. *J. Clin. Pathol.*

Salama, A., and Flaherty, K. (2013). BRAF in melanoma: Current strategies and future directions. *Clin. Cancer Res.* 19, 4326–4334.

Samatar, A. a, and Poulikakos, P.I. (2014). Targeting RAS - – ERK signalling in cancer : promises and challenges. *Nat. Publ. Gr.* 13, 928–942.

Sato, T., Nakashima, A., Guo, L., Coffman, K., and Tamanoi, F. (2010). Single amino-acid changes that confer constitutive activation of mTOR are discovered in human cancer. *Oncogene* 29, 2746–2752.

Schmoll, H.J., Van cutsem, E., Stein, a., Valentini, V., Glimelius, B., Haustermans, K., Nordlinger, B., Van de Velde, C.J., Balmana, J., Regula, J., et al. (2012). Esmo consensus guidelines for management of patients with colon and rectal cancer. A personalized approach to clinical decision making. *Ann. Oncol.* 23, 2479–2516.

Scholer-Dahirel, A., Schlabach, M.R., Loo, A., Bagdasarian, L., Meyer, R., Guo, R., Woolfenden, S., Yu, K.K., Markovits, J., Killary, K., et al. (2011). Maintenance of adenomatous polyposis coli (APC)-mutant colorectal cancer is dependent on Wnt/ -catenin signaling. *Proc. Natl. Acad. Sci.* 108, 17135–17140.

Schoumacher, M., Hurov, K.E., Lehár, J., Yan-Neale, Y., Mishina, Y., Sonkin, D., Korn, J.M., Flemming, D., Jones, M.D., Antonakos, B., et al. (2014). Inhibiting tankyrases sensitizes KRAS-mutant cancer cells to MEK inhibitors via FGFR2 feedback signaling.

Cancer Res. 74, 3294–3305.

Schuijers, J., Mokry, M., Hatzis, P., Cuppen, E., and Clevers, H. (2014). Wnt-induced transcriptional activation is exclusively mediated by TCF/LEF. *EMBO J.*

Sharma, M., Jamieson, C., Johnson, M., Molloy, M.P., and Henderson, B.R. (2012). Specific Armadillo repeat sequences facilitate beta-catenin nuclear transport in live cells via direct binding to nucleoporins Nup62, Nup153, and RanBP2/Nup358. *J. Biol. Chem.* 287, 819–831.

Shultz, M.D., Cheung, A.K., Kirby, C.A., Firestone, B., Fan, J., Chen, C.H., Chen, Z., Chin, D.N., Dipietro, L., Fazal, A., et al. (2013). Identification of NVP-TNKS656: the use of structure-efficiency relationships to generate a highly potent, selective, and orally active tankyrase inhibitor. *J. Med. Chem.* 56, 6495–6511.

Sideris, M., and Papagrigoriadis, S. (2014). Molecular biomarkers and classification models in the evaluation of the prognosis of colorectal cancer. *Anticancer Res.* 34, 2061–2068.

Siegel, R., Desantis, C., Virgo, K., Stein, K., Mariotto, A., Smith, T., Cooper, D., Gansler, T., Lerro, C., and Fedewa, S. (2012). Cancer Treatment and Survivorship Statistics , 2012. *CA. Cancer J. Clin.* 62, 220–241.

Smith, S., and De Lange, T. (2000). Tankyrase promotes telomere elongation in human cells. *Curr. Biol.* 10, 1299–1302.

Smith, D.C., Rosen, L.S., Chugh, R., Goldman, J.W., Xu, L., Kapoun, A., Brachmann, R.K., Dupont, J., Stagg, R.J., Tolcher, A.W., et al. (2013). First-in-human evaluation of the human monoclonal antibody vantiactumab (OMP-18R5; anti-Frizzled) targeting the WNT pathway in a phase I study for patients with advanced solid tumors. *J Clin Oncol* 31, abstract 2540.

Smith, S., Gariat, I., Schmitt, A., and Lange, T. De (1998). Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science* (80-.). 282, 1484–1487.

De Sousa E Melo, F., Wang, X., Jansen, M., Fessler, E., Trinh, A., de Rooij, L.P.M.H., de Jong, J.H., de Boer, O.J., van Leersum, R., Bijlsma, M.F., et al. (2013). Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat. Med.* 19, 614–618.

Stamos, J.L., Weis, W.I., Zimmerman, Z.F., Moon, R.T., Chien, J., Whyte, J.L., Smith, A.A., Jill, A., and van Amerongen, R. (2012). Alternative Wnt pathways and receptors. *Cold Spring Harb. Perspect. Biol.* 4, a007914.

Straussman, R., Morikawa, T., Shee, K., Barzily-Rokni, M., Qian, Z.R., Du, J., Davis, A., Mongare, M.M., Gould, J., Frederick, D.T., et al. (2012). Tumour micro-environment

- elicits innate resistance to RAF inhibitors through HGF secretion. *Nature* **487**, 500–504.
- Tabernero, J. (2007). The role of VEGF and EGFR inhibition: implications for combining anti-VEGF and anti-EGFR agents. *Mol. Cancer Res.* **5**, 203–220.
- Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T., and Takada, S. (2006). Monounsaturated Fatty Acid Modification of Wnt Protein: Its Role in Wnt Secretion. *Dev. Cell* **11**, 791–801.
- TCGA (2012). Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **487**, 330–337.
- Thiel, A., and Ristimäki, A. (2013). Toward a Molecular Classification of Colorectal Cancer: The Role of BRAF. *Front. Oncol.*
- Valenta, T., Gay, M., Steiner, S., Draganova, K., Zemke, M., Hoffmans, R., Cinelli, P., Aguet, M., Sommer, L., and Basler, K. (2011). Probing transcription-specific outputs of β -catenin in vivo. *Genes Dev.* **25**, 2631–2643.
- Valenta, T., Hausmann, G., and Basler, K. (2012). The many faces and functions of β -catenin. *EMBO J.*
- Del Valle-Pérez, B., Arqués, O., Vinyoles, M., de Herreros, A.G., and Duñach, M. (2011). Coordinated action of CK1 isoforms in canonical Wnt signaling. *Mol. Cell. Biol.* **31**, 2877–2888.
- Varnat, F., Siegl-Cachedenier, I., Malerba, M., Gervaz, P., and Ruiz I. Altaba, A. (2010). Loss of WNT-TCF addiction and enhancement of HH-GLI1 signalling define the metastatic transition of human colon carcinomas. *EMBO Mol. Med.*
- Veeman, M.T., Axelrod, J.D., and Moon, R.T. (2003). A second canon: Functions and mechanisms of β -catenin-independent Wnt signaling. *Dev. Cell* **5**, 367–377.
- Verheyen, E.M., and Gottardi, C.J. (2010). Regulation of Wnt/ β -catenin signaling by protein kinases. *Dev. Dyn.* **239**, 34–44.
- Voloshanenko, O., Erdmann, G., Dubash, T.D., Augustin, I., Metzigg, M., Moffa, G., Hundsrucker, C., Kerr, G., Sandmann, T., Anchang, B., et al. (2013). Wnt secretion is required to maintain high levels of Wnt activity in colon cancer cells. *Nat. Commun.* **4**, 2610.
- Voronkov, A., and Krauss, S. (2013). Wnt/ β -catenin signaling and small molecule inhibitors. *Curr. Pharm. Des.* **19**, 634–664.
- van der Vos, K.E., and Coffey, P.J. (2008). FOXO-binding partners: it takes two to tango. *Oncogene* **27**, 2289–2299.
- van der Vos, K.E., and Coffey, P.J. (2011). The extending network of FOXO

transcriptional target genes. *Antioxid. Redox Signal.* **14**, 579–592.

Waalder, J., Machon, O., Von Kries, J.P., Wilson, S.R., Lundenes, E., Wedlich, D., Gradl, D., Paulsen, J.E., Machonova, O., Dembinski, J.L., et al. (2011). Novel synthetic antagonists of canonical Wnt signaling inhibit colorectal cancer cell growth. *Cancer Res.* **71**, 197–205.

Waalder, J., Machon, O., Tumova, L., Dinh, H., Korinek, V., Wilson, S.R., Paulsen, J.E., Pedersen, N.M., Eide, T.J., Machonova, O., et al. (2012). A novel tankyrase inhibitor decreases canonical Wnt signaling in colon carcinoma cells and reduces tumor growth in conditional APC mutant mice. *Cancer Res.* **72**, 2822–2832.

Wetering, M. Van De, Francies, H.E., Garnett, M.J., Wetering, M. Van De, Francies, H.E., Francis, J.M., Bounova, G., Iorio, F., Blokker, J., Jaksani, S., et al. (2015). Prospective Derivation of a Living Organoid Biobank of Colorectal Cancer Patients Resource Prospective Derivation of a Living Organoid Biobank of Colorectal Cancer Patients. *Cell* **161**, 933–945.

Van de Wetering, M., Cavallo, R., Dooijes, D., Van Beest, M., Van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., et al. (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. *Cell* **88**, 789–799.

Wilhelm, S.M., Dumas, J., Adnane, L., Lynch, M., Carter, C.A., Schütz, G., Thierauch, K.H., and Zopf, D. (2011). Regorafenib (BAY 73-4506): A new oral multikinase inhibitor of angiogenic, stromal and oncogenic receptor tyrosine kinases with potent preclinical antitumor activity. *Int. J. Cancer* **129**, 245–255.

Willert, K., Shibamoto, S., and Nusse, R. (1999). Wnt-induced dephosphorylation of Axin releases β -catenin from the Axin complex. *Genes Dev.* **13**, 1768–1773.

Wolpin, B.M., and Mayer, R.J. (2008). Systemic Treatment of Colorectal Cancer. *Gastroenterology* **134**, 1296–1310.

Wu, X., Tu, X., Joeng, K.S., Hilton, M.J., Williams, D.A., and Long, F. (2008). Rac1 Activation Controls Nuclear Localization of β -catenin during Canonical Wnt Signaling. *Cell* **133**, 340–353.

Xing, Y., Clements, W.K., Le Trong, I., Hinds, T.R., Stenkamp, R., Kimelman, D., and Xu, W. (2004). Crystal structure of a β -catenin/APC complex reveals a critical role for APC phosphorylation in APC function. *Mol. Cell* **15**, 523–533.

Xing, Y., Takemaru, K.I., Liu, J., Berndt, J.D., Zheng, J.J., Moon, R.T., and Xu, W. (2008). Crystal Structure of a Full-Length β -Catenin. *Structure* **16**, 478–487.

Xu, W., and Kimelman, D. (2007). Mechanistic insights from structural studies of β -

catenin and its binding partners. *J. Cell Sci.*

Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S., Kikuchi, A., Kishida, M., Ikeda, S., Takada, S., and Kikuchi, A. (1999). Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3 β regulates its stability. *J. Biol. Chem.* **274**, 10681–10684.

Yang, L., Dan, H.C., Sun, M., Liu, Q., Sun, X., Feldman, R.I., Hamilton, A.D., Polokoff, M., Nicosia, S. V, Herlyn, M., et al. (2004). Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. *Cancer Res.* **64**, 4394–4399.

Yang, X., Jia, X., Corvalan, J.R.F., Wang, P., Davis, C.G., and Jakobovits, A. (1999). Eradication of Established Tumors by a Fully Human Monoclonal Antibody to the Epidermal Growth Factor Receptor without Concomitant chemotherapy Eradication of Established Tumors by a Fully Human Monoclonal Antibody to the Epidermal Growth Factor Receptor w. 1236–1243.

Yang, X.D., Jia, X.C., Corvalan, J.R.F., Wang, P., and Davis, C.G. (2001). Development of ABX-EGF, a fully human anti-EGF receptor monoclonal antibody, for cancer therapy. *Crit. Rev. Oncol. Hematol.* **38**, 17–23.

Yeh, T.-Y.J., Sbodio, J.I., Tsun, Z.-Y., Luo, B., and Chi, N.-W. (2007). Insulin-stimulated exocytosis of GLUT4 is enhanced by IRAP and its partner tankyrase. *Biochem. J.* **402**, 279–290.

Zeineldin, M., and Neufeld, K.L. (2013). More than two decades of Apc modeling in rodents. *Biochim. Biophys. Acta - Rev. Cancer* **1836**, 80–89.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T.J., Perry, W.L., Lee, J.J., Tilghman, S.M., Gumbiner, B.M., and Costantini, F. (1997). The mouse Fused locus encodes axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell* **90**, 181–192.

Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J., and He, X. (2005). A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* **438**, 873–877.

Zhang, Y., Liu, S., Mickanin, C., Feng, Y., Charlat, O., Michaud, G.A., Schirle, M., Shi, X., Hild, M., Bauer, A., et al. (2011). RNF146 is a poly(ADP-ribose)-directed E3 ligase that regulates axin degradation and Wnt signalling. *Nat. Cell Biol.* **13**, 623–629.

Zhao, Y., Wang, Y., and Zhu, W.G. (2011). Applications of post-translational modifications of FoxO family proteins in biological functions. *J. Mol. Cell Biol.* **3**, 276–282.