Condensin Complex and Molecular Motor KIF4A New players in CRC under Wnt signalling regulation

Papel del Complejo Condensina y el Motor Molecular KIF4A en el Cáncer Colorrectal como nuevas dianas de la vía de señalización Wnt

Lucía Suárez López

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Abbreviations

ACF	Aberrant crypt foci
ALP	Alkaline phosphatase
ATTC	American Type Tissue collection
AJCC	American Joint Committee on Cancer
bp	Base pair
BSA	Bovine serum albumin
ChIP	Chromatin immunoprecipitation
CIMP	CpG Methylator phenotype
CPT	Camptothecin
CPT-11	Irinotecan
CRC	Colorectal cancer
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbbeco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DSB	Double strand break
DTT	Dithiothreitol
EMT	Epithelium-mesenchymal transition
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
IF	Immunofluorescence
IHC	Immunohistochemistry
Kb	Kilobase
KDa	Kilodalton
LOH	Loss of heterozygosis
MMR	Missmatch repair
MSI	Microsatellite instable
MSS	Microsatellite stable
NaB	Sodium butyrate
NEBD	Nuclear envelope breakdown
PFA	Paraformaldehyde
PI	Propidium iodide
pKIF4A	KIF4A promoter cloned into PGL3 vector
PMSF	Phenylmethylsulfonyl fluoride
pSMC2	SMC2 promoter cloned into pGL3 vector
Q-PCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation lysis buffer
RLU	Relative luciferase units
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SMC	Structural maintenance of chromosomes
SRB	Sulforhodamine B
TA	Transit amplifying
TBE	TCF-4 binding element
TMA	Tissue microarray
TSS	Transcription start site
WB	Western blot
WHO	World health organization

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Introduction

1. Colorectal cancer

According to World Health Organization (WHO), colorectal cancer was the third most commonly diagnosed cancer in men (663,000 cases, 10 % of the total) and the second in women (571,000 cases, 9.4 % of the total) worldwide in 2008.

The highest incidence rate is found in Australia and New Zealand, Europe, and North America, whereas the lowest rates are found in Africa and South-Central Asia. About 608,000 deaths from colorectal cancer are estimated worldwide, accounting for 8 % of all cancer deaths, making it the fourth most common cause of death from cancer (1).

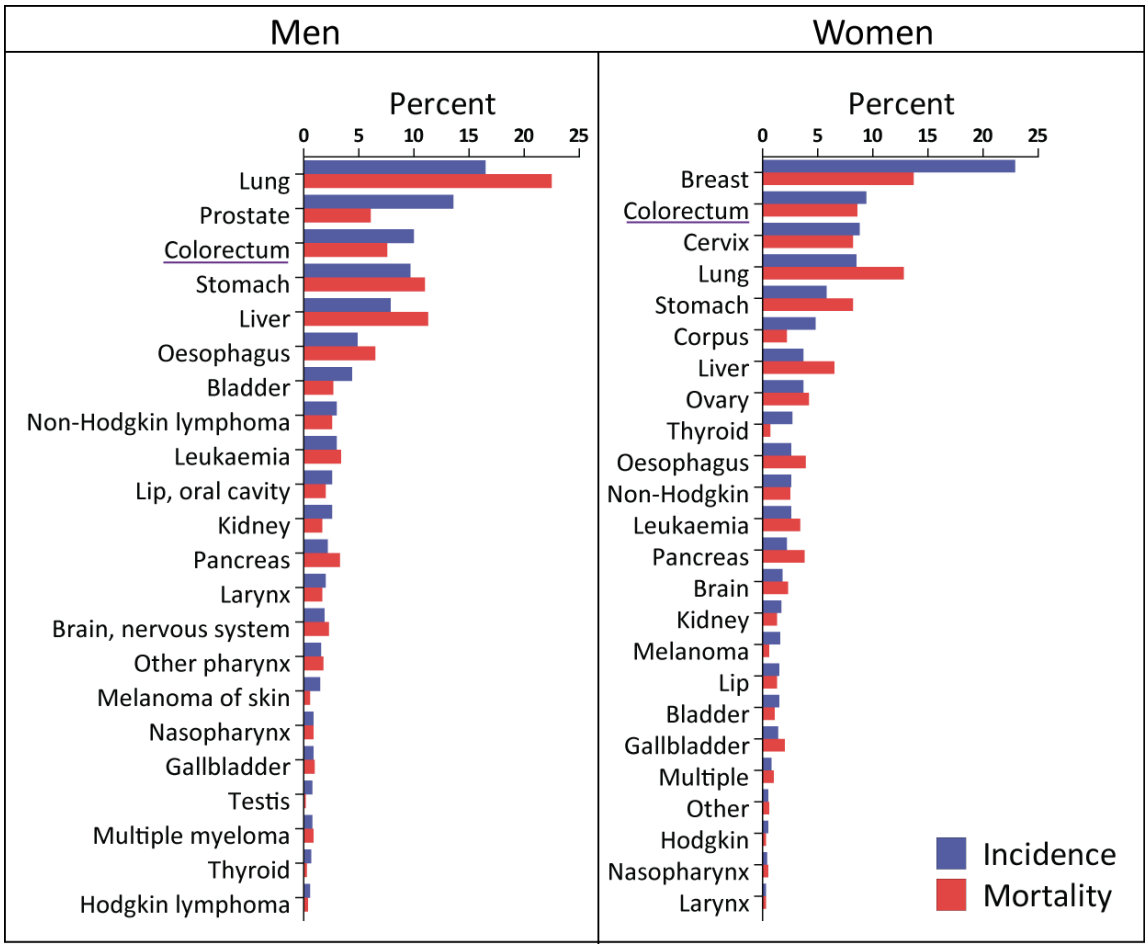

Fig. 1. Cancer incidence and mortality in men and women worldwide according to Globocan 2008 project (1).

Colorectal cancer incidence rates are rapidly increasing in several areas historically at low risk, including Spain, and a number of countries within Eastern Asia and Eastern Europe; such unfavourable trends are thought to reflect a combination of factors including changes in dietary patterns (red meat and alcohol consumption, low dietary fibre), obesity, and an increased prevalence of smoking among others (2).

Mortality rates are decreasing in Western developed countries due to improved treatments and early detection. Colorectal cancer survival is highly dependent upon stage of disease at diagnosis, and typically ranges from a 90% 5-year survival rate for cancers detected at a localized stage, to 70% for regional, and to 10% for people diagnosed for distant metastatic cancer. In general, the earlier the stage at diagnosis, the higher the probability of survival (3). As a consequence, mortality rates are increasing in developing countries with aged population and increasing westernized lifestyle, but limited in economic resources and health infrastructures, to improve the treatment/diagnosis (4).

1.1. Colon cancer staging

Staging of colorectal cancer refers to how far a cancer has spread on a scale from 0 to IV, with 0 meaning a cancer that has not invade the colon wall and IV describing cancer that has spread beyond the original site to other far parts of the body (frequently to lungs and liver). Two staging systems are used in the clinics to define the extent of invasion of colorectal cancer: Dukes' classification and TNM staging (Table 1). The TNM system was developed by the American Joint Committee on Cancer (AJCC); it is the most widely used and considered the most precise and descriptive. T stands for tumour invasion through bowel wall layers, from mucosa to outer serosa; N stands for lymph node involvement and M for metastases.

Table 1. TNM and Dukes' classification system for colorectal cancer staging. (Tis: carcinoma in situ).

	TNM classification			Dukes' classification
Stages	T	N	M	Stages
Stage 0	Tis	N0	M0	
Stage I	T1	N0	M0	A
	T2	N0	M0	B1
Stage II	T3	N0	M0	B2
	T4	N0	M0	B2
Stage III	T1,T2	N1 or N2	M0	C1
	T3,T4	N1 or N2	M0	C2
Stage IV	Any T	Any N	M1	D

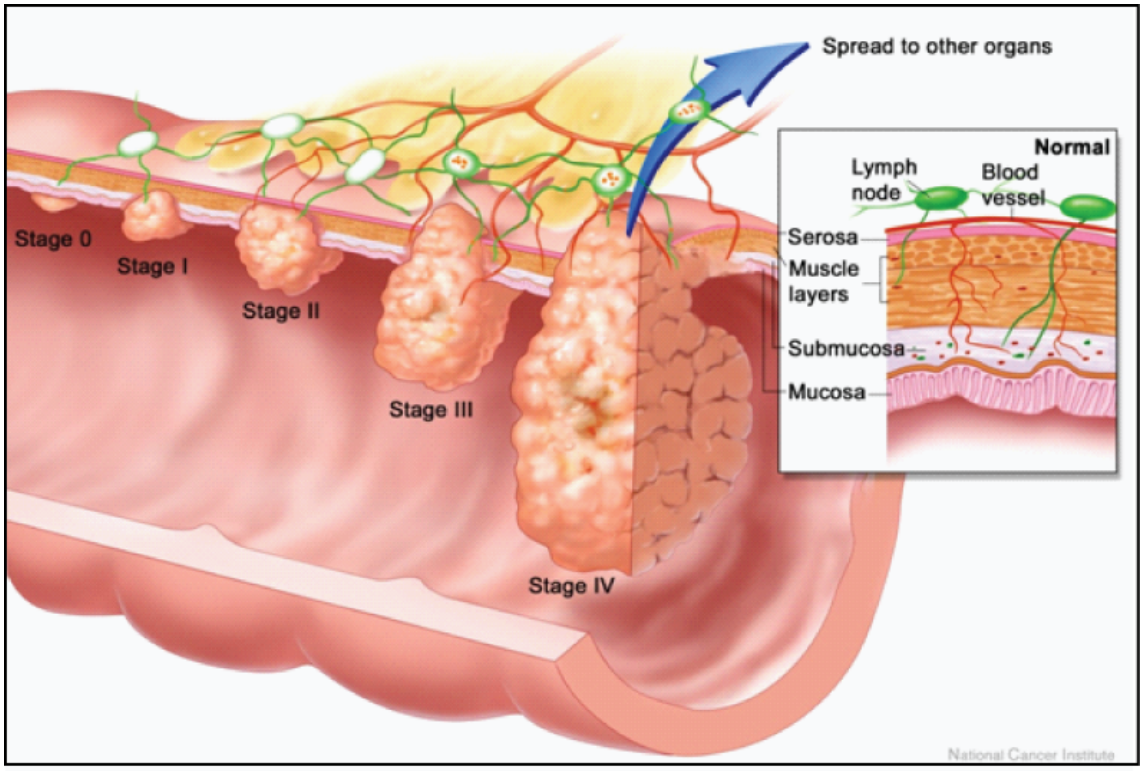

Fig. 2. The growth from polyp to metastatic tumour. In stage 0, abnormal cells are found in the mucosa of the colon wall. These abnormal cells may become cancer and spread. Stage 0 is also called carcinoma in situ. In stage I, cancer has formed in the mucosa of the colon wall and has spread to the submucosa. Cancer may have spread to the muscle layer of the colon wall. Stage II tumours have spread through the muscle layer of the colon wall to the serosa. In stage III, cancer has spread through the mucosa to the submucosa and to nearby lymph nodes. In stage IV the cancer has spread through the blood and lymph nodes to other parts of the body, such as the lung, liver, abdominal wall, or ovary. Image from Terese Winslow, US Govt.

1.2. Colorectal cancer treatment

Treatment for patients with cancers of the colon and rectum varies by tumour location and stage at diagnosis. Surgery to remove the cancer and nearby lymph nodes is the most common treatment for early stage (stage I and II) colon (94%) and rectal (74%) cancer (5).

Chemotherapy alone, or in combination with radiation therapy, is often given to patients with late-stage disease (50%-70%) before or after surgery. In Europe, the first line of treatment is mainly based in FOLFOX (5-FU, leucovorin and oxaliplatin) or FOLFIRI (5-FU, leucovorin and irinotecan) backbones (6).

The administration of capecitabine is recommended as adjuvant chemotherapy in stage III patients. For these patients, capecitabine provides equivalent outcome to intravenous 5-FU and leucovorin, with significantly less side effects (7).

Monoclonal antibodies-based therapies have been recently included in clinics in combination with chemotherapy. The European Society of Medical Oncology recommends the use of anti-VEGF antibodies (Bevacizumab) and anti-EGFR antibodies (cetuximab and panitumumab) in combination with cytotoxic treatments in selected patients with metastatic disease, as these regimens have been associated with improved outcomes compared with chemotherapy alone (8).

1.3. Colon tumorigenesis

Early in the nineties, Bert Vogelstein and Eric R. Fearon postulated a model for colon cancer progression, based on the adenoma-carcinoma sequence (9). Upon this model, colon cancer progression could be explained as a sequence of genetic changes or mutations on particular genes or crucial pathway's alterations. Albeit the model has been revised and modified in the latter years (Fig. 3), assuming that the scenario is much more complex, its basis is still accepted (10). Colorectal tumours are characterized by their high genomic instability; such an environment is presumed to favour the appearance of those mutations/molecular alterations responsible for cancer progression. In the last decades, different molecular pathways have been postulated to underlie this phenomenon: chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP). It is important to note that these three phenotypes are not mutually exclusive and may coexist in the same tumour to some extend.

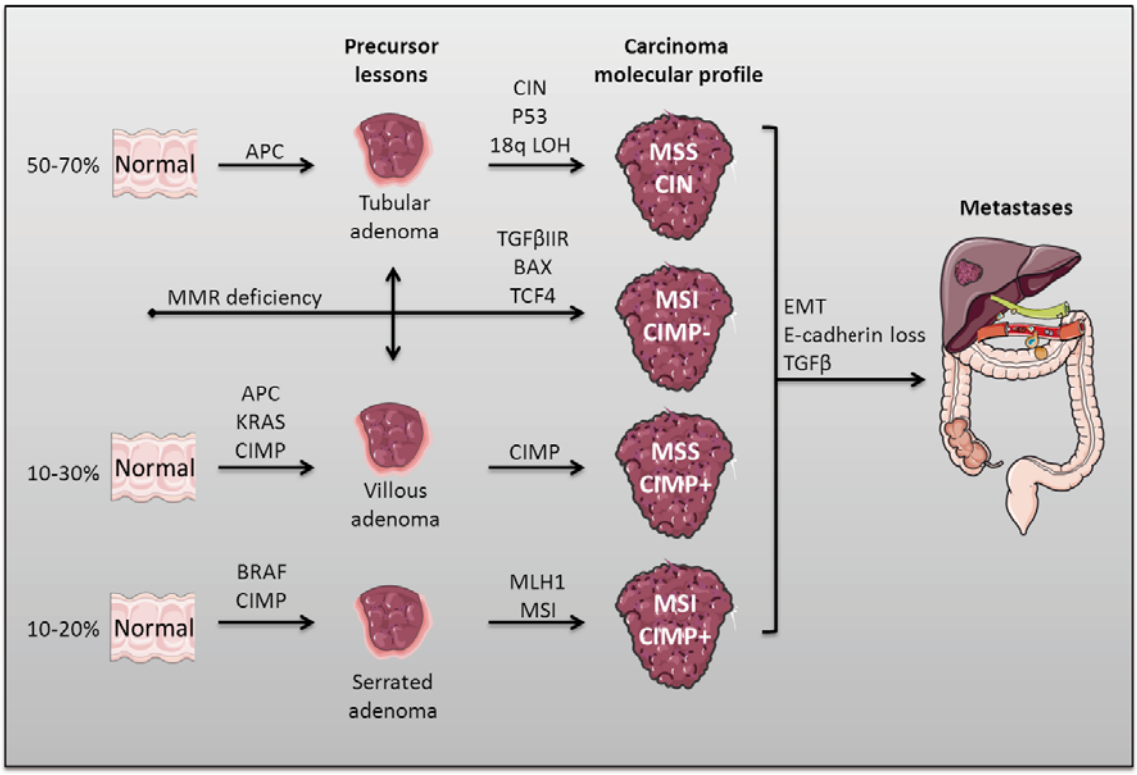

Fig. 3. Adenoma-carcinoma sequence reviewed. Vogelstein's model has become more complex the last decades. Instead of a linear, single progression model, sporadic colorectal cancer seems to arise from (at least) three distinct parallel modes. The top and bottom pathways are the most homogeneous, with clear distinctions in precursor lesions (tubular vs. serrated adenomas), genetics (APC and p53 vs. BRAF mutations, MSI vs. CIN) and epigenetics (CIMP negative vs. positive). The middle pathway is more heterogeneous than depicted and not fully understood yet. It may arise mostly from villous adenomas, but perhaps also from serrated adenomas. It has a different form of CIMP, predominant KRAS but occasional BRAF mutations and usually lacks CIN. MSI tumours lacking CIMP phenotype arise from either villous or tubular adenomas by MMR system deficiency. This type of tumour follows a particular progression pathway and rarely derives to metastases. Figure adapted from (11), (10),(12) and (13). EMT, epithelium-mesenchymal transition.

CIN is the more frequent cause for genomic instability in colorectal tumours, being present in most sporadic CRC cases (nearly 85%), and also in familiar adenomatous polyposis (FAP) cases with germline *APC* mutations. It is defined by the presence of numerous chromosomal aberrations, including gain and losses of chromosomes, translocations and aneuploidy. Allelic losses are also frequent (LOH, loss of heterozygosity) predominantly in chromosomal arms 5q, 8p, 17p and 18q. These tumours are thought to arise from truncating mutations in the *APC* gene, which results in benign tubular adenomas. The malignant lesions carry additional mutations most frequently in the tumour suppressor gene *TP53*, oncogenes like *KRAS*, kinase *PIK3CA*, E3 ubiquitin ligase complex member *FBXW7*, TGF-β signal transducer *SMAD4*, and transcription factor *TCF7L2* (14). This subtype of tumours is the one that fits the best with Vogelstein's model for CRC progression.

Microsatellite instability is present in 15% of CRC cases and in the Lynch syndrome, as an hereditary non-polyposic form of CRC. It is characterized by mostly stable karyotypes, even though they show widespread insertion/deletion mutations in short, repeated nucleotide sequences (microsatellites) in tumour DNA as opposed to normal DNA. In these tumours, the DNA mismatch repair system (MMR) is impaired, so fails to correct matching failures occurring during normal DNA replication. The MSI phenotype is strongly associated with mutations in specific oncogenes and tumour suppressor genes, especially *BRAF* (V600E), TGFB receptor II and IGF receptor II, the pro-apoptotic factor *BAX*, the mismatch repair genes *MSH3* and *MSH6* and the histone modifier *HD2* (12,15).

The methylator phenotype, also known as CIMP (CpG island methylator phenotype), is present in around 35% of CRC tumours. It was first described in 1999 (16) and is characterized by aberrantly increased gene silencing due to hypermethylation of CpG islands. In these tumours the activating mutation in *BRAF* is considered as the initiating event, which inhibits apoptosis in the normal colonic epithelium and results in sessile-serrated polyps. As these lesions are highly prone to CpG island methylation in promoter regions for multiple genes, *MLH1* gene promoter is frequently silenced, conferring additionally the MSI phenotype because *MLH1* is a mismatch repair gene (13,17). These tumours have also been considered by some as "epigenomic instable"(10). Gene silencing by promoter CpG hypermethylation is presumed to occur randomly. Target genes like cell-cycle regulator p16 (*CDKN2A*), the glycoprotein *THBS1*, growth factor *IGF2* and transcriptional regulators *NEUROG1* and *RUNX3*, are found silenced very frequently in this subtype of tumours (16,18). *APC* tumour suppressor gene is also found silenced in CRC by promoter hypermethylation, but rarely (19).

It is important to note that despite the origin of the malignant lesion or the molecular origin of the inherent genomic instability, the alteration in *APC* or other members of the Wnt pathway (i.e., β-catenin) is found in 93% of CRC cases (14). As detailed below, Wnt pathway is the major regulator of the intestinal homeostasis.

2. Intestinal epithelium homeostasis

The mammalian intestine, which consists of the small intestine (duodenum, jejunum and ileum) and the large intestine or colon (ascending, transverse and descending colon, sigmoid flexure and rectum), is lined by a monolayer of epithelial cells (or mucosa). The absorptive epithelium of the small intestine is ordered into flask-shaped submucosal invaginations known as crypts of Lieberkühn, and finger-like luminal protrusions termed villi. Spatially, the villi surround the entrance of each crypt. In the colon, the crypts are larger than in the small intestine, and there is a flat surface epithelium facing the lumen instead of villi (20). The crypt is mainly a proliferative compartment, whereas the villus represents the differentiated compartment. These two morphologically distinct compartments ensure different functions in the intestinal epithelium.

Almost all epithelial cells in the intestinal lining are replaced on a weekly basis (21), which puts great demands on the cellular organization of this tissue; homeostasis of the intestinal epithelium is maintained by an intestinal stem cell (ISC) compartment that resides at the bottom of the crypts, safely tucked away from the shear stresses and potentially toxic agents that pass through the intestinal tract. These ISCs are at the top of a cellular hierarchy and are crucial for the renewal of the differentiated progeny within the intestinal layer.

ISCs cycle infrequently and produce rapidly proliferating daughter cells, referred to as Transit Amplifying (TA) cells, which fill the crypts with committed precursor cells gradually differentiating into the two main epithelial lineages upon reaching the crypt–villus junction (22). First, the absorptive lineage that entails all enterocytes, and second, the secretory lineage which is composed of Goblet cells (secreting protective mucins), and enteroendocrine cells [they represent less than 1% of all epithelial cells and secrete hormones like serotonin or secretin (23)]. Additionally, only in the small intestine reside the Paneth cells, epithelial cells that secrete antimicrobial agents such as cryptidins, defensins and lysozyme (24), and can be found immediately below the stem cell compartment

The differentiation process is then completed by a bidirectional migration: Paneth cells migrate to the bottom of the crypt where they reside for about 20 days, while the three other differentiated cell types migrate upward in coherent bands from the upper third of the crypt to the apex of the villus. At the villus tip, this continuous influx of new cells is compensated by cell loss: the differentiated epithelial cells are removed by apoptosis and lost into the gut lumen (25).

ISCs were first defined by label-retention techniques as those cells at position +4 in the crypt (considering position +1 the Paneth cells located at the bottom of the crypt) (26). However, the identification of the intestinal stem cell marker Lgr5, revealed that also the crypt base columnar cells (CBCCs) located in between the Paneth cells behave also as intestinal stem cells (27) and give rise to all the cell types in the intestinal mucosa. In the colon, Lgr5+ cells are found in the bottom of colonic crypts, where Paneth cells are absent; thus colon ISC compartment is defined as the bottom of the crypt (28).

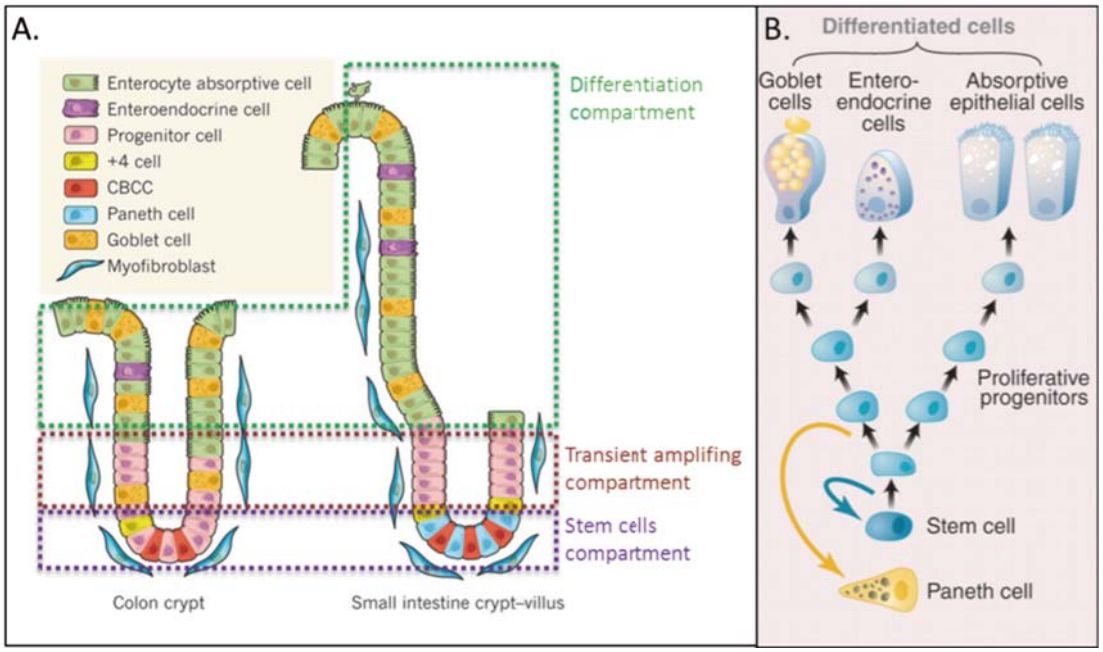

Fig. 4. Distribution of cellular subtypes in intestinal mucosa. A, The bottom of the intestinal crypts constitute the stem cells compartment, where reside the crypt base columnar cells (CBCC) and the +4 cells. Small intestine paneth cells are also found in this compartment. The proliferative progenitor cells, located in the transient amplifying compartment, have limited self-renewal capacity. When cells reach to the crypt-villus axis in the small intestine or to the upper third of the colonic crypt, they commit to different cell linages. B, Cell lineage scheme depicts the stem cell, the transit-amplifying cells, and the two differentiated branches. The right branch constitutes the enterocyte lineage; the left branch is the secretory lineage. Figure adapted from (29) and (30).

As explained bellow, Wnt/β-catenin signalling pathway is the dominant force in controlling cell fate along the crypt–villus axis, and by extension, the major regulator of intestinal epithelium homeostasis.

2.1. Wnt/β-catenin signalling pathway

Wnt proteins constitute a highly conserved family of secreted glycoproteins that regulate cell fate decisions during development of vertebrates and invertebrates. The Wnt signalling network regulates diverse processes during development such as cell fate determination, structural remodelling, cell polarity and morphology, cell adhesion, and growth. Moreover, they are responsible for stem cell number regulation and differentiation in adult tissues [reviewed in (31)].

The central player in Wnt signalling pathway is β–catenin, and its stabilization and nuclear accumulation is a hallmark of activated canonical Wnt signalling (Fig. 5). In the absence of Wnt ligand, β-catenin is sequestered in a multiprotein degradation complex containing the scaffold protein AXIN, the tumour suppressor genes Adenomatous Polyposis Coli (*APC*) and WTX, as well as the kinases casein kinase I (CKI) and glycogen synthase kinase 3β (GSK3β). Upon sequential phosphorylation at a set of conserved amino-terminal Serine and Threonine residues, β-catenin is ubiquitinated by the β-TrCP containing E3 ubiquitin ligase and subsequently degraded by the proteasome machinery (32).

When secreted Wnt ligands bind to an heterodimeric receptor in the target cell surface (formed by the frizzled (Fz) seven-span transmembrane receptors and LPR5/6, members of the single-span transmembrane LDL receptor family), Axin is recruited to the plasma membrane by its binding to the

cytoplasmic tail of LRP6 (33). This results in the inhibition of β–catenin ubiquitination, saturation of the multiprotein degradation complex by the phosphorilated form of β-catenin and accumulation of the newly synthesized form in the cytoplasm (34).

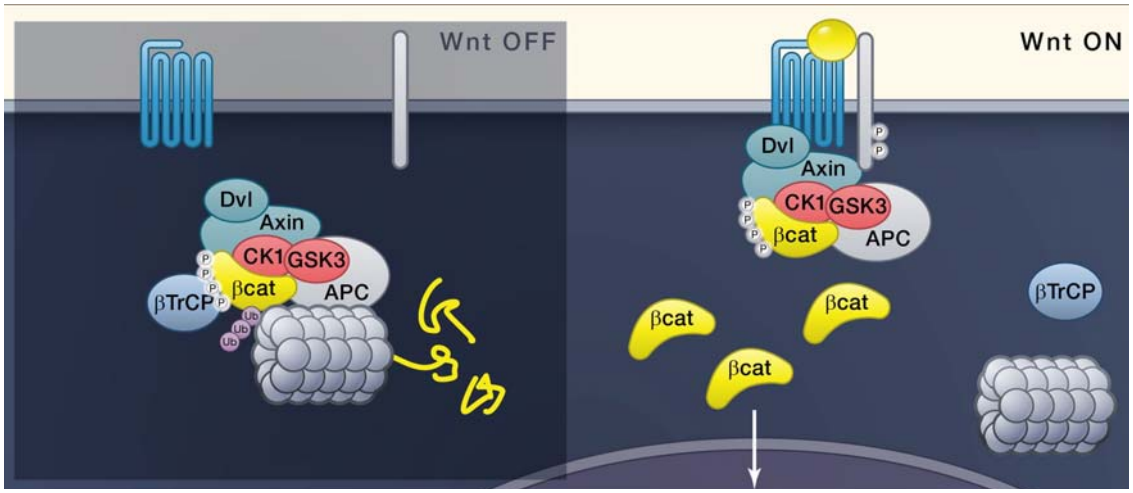

Fig. 5. Wnt signalling in the cytoplasm. In the absence of Wnt, the destruction complex resides in the cytoplasm, where it binds, phosphorylates, and ubiquitinates β-catenin by β-TrCP. The proteasome recycles the complex by degrading β-catenin. Wnt induces the association of the intact complex with phosphorylated LRP. After binding to LRP, the destruction complex still captures and phosphorylates β-catenin, but ubiquitination by β-TrCP is blocked. Newly synthesized β-catenin accumulates. Figure taken from (35).

β-catenin then enters the nucleus, where it engages DNA-bound TCF/LEF transcription factors (36,37), which interact with Groucho transcriptional repressors (38,39); in the intestine, the predominant TCF factor is TCF-4 (40); TCF-4 is bound to DNA canonical binding site (T/A)(T/A)CAAAG or the evolutionary conserved elements A(C/G)(T/A)TCAAAG (41). The association with β-catenin transiently converts TCF into a transcriptional activator, replacing Groucho from TCF and recruiting transcriptional coactivators and histone modifiers such as Brg1, CBP, Cdc47, Bcl9, and Pygopus to drive target gene expression (Fig. 6) [reviewed in (42)].

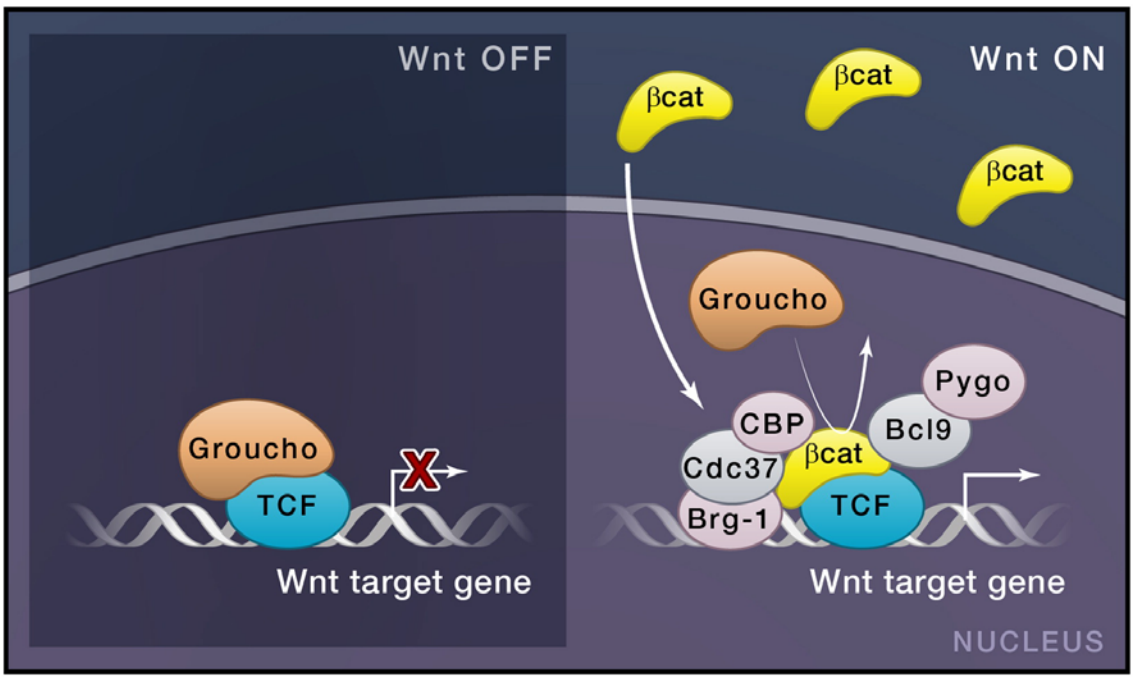

Fig. 6. Wnt signalling in the nucleus. In the absence of Wnt signals, TCF occupies and represses its target genes, helped by transcriptional corepressors such as Groucho. Upon Wnt signalling, β-catenin replaces Groucho from TCF and recruits transcriptional coactivators and histone modifiers such as Brg1, CBP, Cdc47, Bcl9, and Pygopus to drive target gene expression. Figure taken from (35).

The β-catenin/TCF complexes activate transcription of many different target genes; indeed, an increasing number of Wnt target genes are discovered lately thanks to new high-throughput techniques and bioinformatics tools (summarized at Wnt homepage: http://www.stanford.edu/~rnusse/pathways/targets.html.) Products of Wnt target genes unfold a large variety of biochemical functions including cell cycle regulation, cell adhesion, hormone signalling and transcription regulation (Table 2). The plurality and diversity of the biochemical functions reflect the variety of different biological effects of the Wnt pathway, including activation of cell cycle progression and proliferation (C-MYC, CYCLIN-D1, C-JUN), inhibition of apoptosis (SURVIVIN), regulation of embryonic development (SOX-2, SOX-9), cell differentiation (EPHB/EPHRIN-B), cell growth, and cell migration (MMP-7, MMP-26), [reviewed in (43)].

Table 2. List of selected target genes (from Wnt homepage) with corresponding biochemical functions and regulation trend, adapted from (43). (Trend: purple= up-regulated; green= down-regulated).

Cell cycle kinase regulators	cyclin D1 p21
Cell adhesion proteins	Claudin-1, connexin-30, connexin-43, L1CAM, Nr-CAM E-cadherin, periostin
Receptors	CD44, Dfz3, EGF, Fz7, receptor, Met, Ret, retinoic acid receptor gamma, Stra6 Arrow/LRP, Dfz2, Fz
Factor synthases	COX2, NOS2
Hormones, growth factors	Gastrin, BMP4, CCN1/Cyr61, Dickkopf-1, Dll1, Eda, endothelin-1, EphB/ephrin-B, FGF18, FGF20, FGF4, FGF9, follistatin, IGF-I, IGF-II, IL-6, IL-8, jagged 1, nanog, proglucagon, proliferin-2, proliferin-3, s-FRP, Stra6, TNF family 4-1BB ligand, VEGF, wingful/notum, wingless, WISP-1, WISP-2, Xnr3 BMP4, osteocalcin, RANK, wingless
Transcription regulators	c-Myc, brachyury, Cdx1, Cdx4, c-jun, dharma/bozozok, engrailed-2, FoxN1, fra-1, Id2, Irx3, ITF-2, LEF-1, mBTEB2, MITF/nacre, movo, myogenic bHLH, neurogenin 1, Pitx2, PTTG, Runx2, SALL4, Sox2, SOX9, TCF-1, twin, Twist, Ubx Hath1, nanog, Ubx, Six3, SOX9
Proteases, protease inhibitors, protease receptors	CD44, MMP-7, MMP-26, stromelysin-1, survivin, uPAR
Matrix proteins	Fibronectin, keratin, versican
GTPase, GTPase regulator	Tiam, Wrch-1
Others	Axin-2, MDR1, nemo, siamois, β-TRcP, twin

2.2. Wnt signalling in CRC

Several findings have positioned Wnt/β-catenin pathway as a key player in colon cancer onset and development. First, it was discovered that germline mutations in the *APC* gene cause a hereditary cancer syndrome termed familiar adenomatous polyposis (FAP) (44,45). FAP patients carry heterozygous *APC* mutations. The second allele is frequently lost in individual cells, which grow into colon adenomas, polyps, in early adulthood. Additional mutations in genes like *KRAS*, *TP53*, and *SMAD4* induce some of these polyps to progress toward malignancy.

Moreover, Wnt signalling appears aberrantly activated in 93 % of sporadic colon cancer cases (14), being the inactivating mutation of *APC* or the activating mutation of *CNNTB1* gene (β-catenin) present in a 80 % of cases.

On the other hand, recent findings postulate Wnt pathway as the central regulator of intestinal stem cells proliferation and maintenance (46–50). Indeed, proliferative cells at the bottom of the small intestine (51) and the colon crypts (52) accumulate nuclear β-catenin and mutation of TCF-4 leads to the depletion of intestinal proliferative compartments in fetal mice (53).

Interestingly, TCF-4/β-catenin target genes in CRC cells are also expressed in normal proliferating cells of the crypt, while repressed genes are expressed in normal villus-associated differentiated cells (Fig. 7). Thus, an activated Wnt cascade drives a very similar genetic program in CRC cells as in crypt stem cells/progenitors (52).

Taken together, it clearly appears that any mutational event stabilizing nuclear β-catenin in the intestinal epithelium, which leads to constitutively activated canonical Wnt signalling, represents the initiating event of intestinal tumorigenesis, conferring the cells a crypt stem cell/progenitor phenotype highly proliferative that give rise to aberrant crypt foci lesions (ACF) and later on, adenomas.

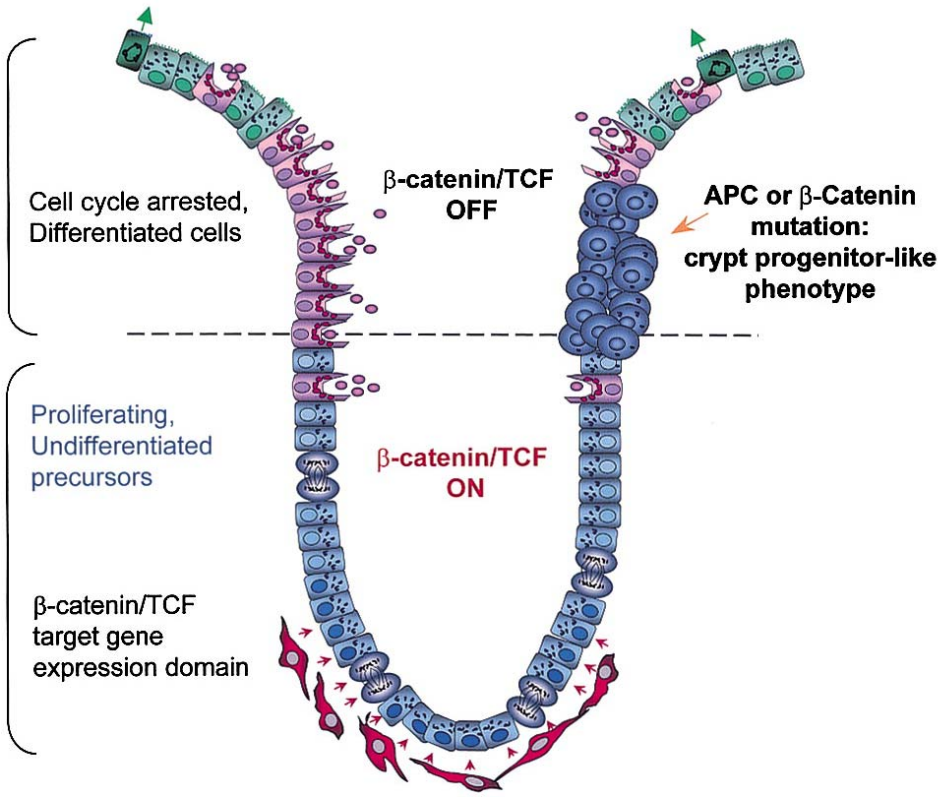

Fig. 7. Schematic representation of a colon crypt and proposed model for polyp formation. At the bottom third of the crypt, the progenitor proliferating cells accumulate nuclear β-catenin. Consequently, they express β-catenin/TCF target genes. Mesenchymal cells surrounding the bottom of the crypt (depicted in red) secret Wnt ligands. As the cells reach the midcrypt region, β-catenin/TCF activity is downregulated and this results in cell cycle arrest and differentiation. Cells undergoing mutation in APC or β-catenin become independent of the physiological signals controlling β-catenin/TCF activity. As a consequence, they continue to behave as crypt progenitor cells in the surface epithelium, giving rise to ACFs. Figure taken from (52).

3. The family of SMC proteins: Global organizers of the Genome.

Structural Maintenance of Chromosomes (SMC) proteins are highly conserved proteins crucial for chromosome structure and dynamics, gene regulation and DNA damage repair. Members of this family can be found along the entire phylogenetic tree, from bacteria and archaea to human (54).

SMC proteins share similar domain structures: they consist of N-terminal and C-terminal domains that fold back onto each other to create an ATPase 'head' connected to a central 'hinge' via extended coiled-coils (Fig. 8). The hinge domain mediates the heterodimerization of eukaryotic SMC proteins, allowing the two ATPase heads from two SMC proteins to transiently interact with each other to bind and hydrolyze ATP. As revealed by electron microscopy, the SMC heterodimers can adopt different conformations, including V-shaped dimers and ring-like structures, possibly depending on the nucleotide-binding states of their ATPase heads (55–57).

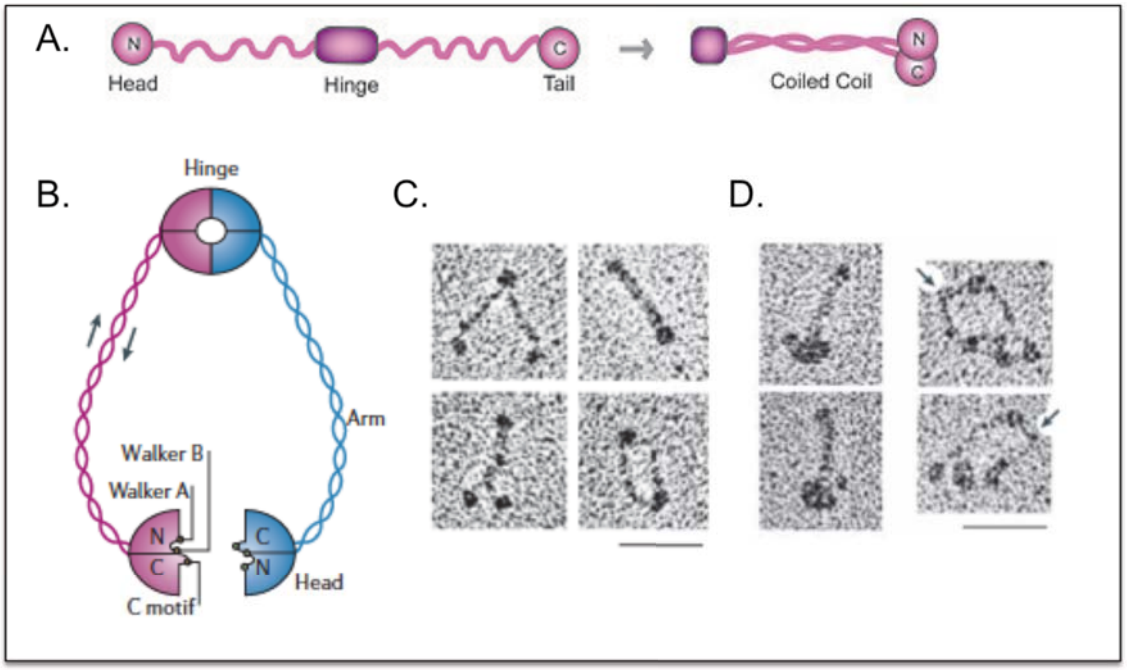

Fig. 8. Structure of a SMC heterodimer. A, Each SMC protein folds back on itself through antiparallel coiled-coil arm interactions. This forms an SMC dimerization hinge domain from the central part at one end, and an ATPase head domain from association of the terminal globular parts at the other. B, SMC proteins heterodimerize to form the core of the complex. Walker A and B domains (in N terminus) and the C motif (in C terminus) are responsible for ATP molecules binding. C, Electron micrographs of the *Bacillus subtilis* SMC (BsSMC) homodimers show a wide variety of conformations. Bar, 50 nm. D, Examples of rotary-shadowed images of condensin I (left) and cohesin (right) purified from human tissue culture cells. Condensin usually adopt a "lollipop" structure whereas cohesin forms a ring-like structure. A sharp kink in one of the coiled-coil arms of SMC3 is indicated by the arrow. Bar, 50 nm. Figure adapted from (57) and (58).

Each SMC heterodimer associates with non-SMC subunits to form functional SMC complexes (Fig. 9): the cohesin complex, the condensin complex, and the SMC5-6 complex (59); despite different roles were assigned to each complex when they were first described, recent evidences demonstrate that the three complexes are involved in DNA repair mechanisms, gene regulation in interphase, spatial organization of interphase chromatin and chromosome segregation and dynamics (60–62). It is not surprising that SMC complexes are increasingly pointed as "global organizers of the genome" (63).

Cohesin complex is formed by the SMC1-SMC3 heterodimer and non-SMC proteins named Rad21 and SA1/2 in vertebrates (64). The main function of cohesin complex is to generate sister chromatid cohesion, which holds sister chromatids together from S phase until mitosis, when cohesion is removed to allow chromosome segregation (65).

SMC5-SMC6 complex is formed by the SMC5-SMC6 heterodimer and several non-SMC elements (Nse), including Nse1-6. It is involved in DNA recombination and DNA repair mechanisms (60).

Condensin complex is formed by the SMC2-SMC4 heterodimer and three non-SMC regulatory subunits. In vertebrates, there are two types of condensin complexes: condensin I and condensin II (66). They share two core subunits, SMC2 and SMC4, but differ in the other three non-SMC subunits. Condensin I contains two HEAT subunits (CAP-D2 and CAP-G) and the kleisin CAP-H, while condensin II contains HEAT subunits CAP-D3 and CAP-G2 and the kleisin CAP-H2. The main function of Condensin complex is to regulate chromosome organization and condensation during mitosis and meiosis in eukaryotic cells. They are responsible for folding chromatin fibre into highly compact chromosomes to ensure their faithful segregation and they are necessary also for resolution of sister chromatids during anaphase [reviewed in (56,63)].

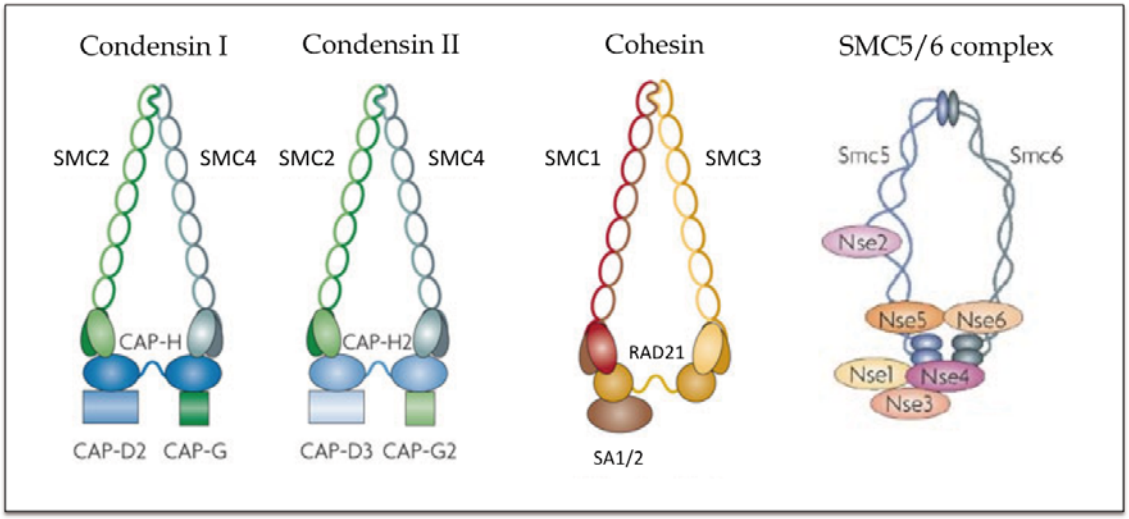

Fig. 9. Arquitecture of the SMC complexes. Two paralogous condensin complexes (condensin I and condensin II) have been identified in many metazoans. Both condensins contain an heterodimer of SMC2 and SMC4 but associate with a distinct set of non-SMC subunits. The cohesin complex consists of an heterodimer of SMC1 and SMC3, the Rad21 kleisin protein, and stromalin (SA 1/2). SMC5 and SMC6 core subunits and Non-SMCs Elements NSE1-4 form SMC5/6 complex in humans. Well-characterized yeast SMC5/6 complex is represented. Figure adapted from (61) and (67).

3.1. The condensin complex

To ensure the faithful inheritance of genetic information to daughters cells in mitosis, disorganized interphase chromatin must be packaged into discrete units named chromosomes. This highly organized packaging provides mitotic chromosomes with mechanical strength and reduces their length so that they can be transported effectively to opposite poles of the dividing cell by the mitotic spindles.

As mentioned above, condensin complex is the major actor in chromosome condensation, and additionally maintains their condensed state until the end of mitosis.

In vertebrates, the two types of condensin complexes (I and II) exhibit distinct spatial staining patterns on chromosome axes, as well as differing temporal localisation patterns throughout the cell cycle. Condensin I is sequestered in the cytoplasm during interphase and gains access to chromosomes only after the nuclear envelope breaks down in prometaphase. In contrast, condensin II localizes to the nucleus from interphase through prophase and participates in an early stage of chromosome condensation within the prophase nucleus (Fig. 10, A). After nuclear envelope breakdown (NEBD), condensins I and II collaborate to support proper assembly of chromosomes in which sister chromatids are well resolved by metaphase and to promote faithful segregation in anaphase, but they localize differentially on the chromosomes, in an alternate pattern (66, 68, 69). Indeed, depletion of condensin I- or condensin II-specific subunits produces a highly characteristic chromosome morphology (i.e., swollen or curly chromosomes, respectively). Recent studies support the fact that the two complexes play distinct roles in mitotic chromosome structure; it has been proposed that condensin I mediates more-frequent short-range lateral interactions among chromatin loops, whereas condensin II mediates axial stacking of the laterally assembled configurations (70) (Fig. 10, B).

The protein levels of condensin complex subunits remain almost constant throughout the cell cycle in mammalian cells (71). Several authors have shown how phosphorylation of the different subunits, mainly the non-SMC subunits, regulates condensin localization and function in mitosis and interphase (71–73).

The exact mechanism by which condensin complex is able to compact DNA fibres is still under debate. Nevertheless, it has been well characterised the condensin ability to introduce positive superhelical tension into dsDNA *in vitro*, using naked circular DNA as template (74,75). This positive supercoiling activity demands the five-subunit holocomplex and ATP hydrolysis by the SMC subunits. It has been proposed that the positive supercoiling activity could promote ordered folding of chromatin fibres to initiate the formation of chiral (positively supercoiled) loops in eukaryotes' chromosomes.

A three-step model for post-translational regulation of condensin function has been recently proposed (76). First, during interphase condensin complex is inhibited by casein kinase II (CKII) phosphorylation in order to prevent premature condensation before mitosis onset. This is followed by a stage of condensin activation during early prophase, which requires the phosphorylation by the cyclin-dependent kinase CDK1, together with dephosphorylation of CKII sites by an unknown phosphatase. When CDK1 levels drop in the metaphase-to-anaphase transition, Polo/Cdc5 then phosphorylates condensin complex; it is thought that this regulation by Polo/Cdc5 allows condensin to maintain its activity from anaphase until the end of mitosis (Fig. 11).

Different regulators other than CDK1 and Polo/Cdc5 are also implicated in mitotic condensin loading into DNA, but they were not included in this model: Aurora B kinase for condensin I (72,77) and Protein Phosphatase 2A (PP2A) for condensin II (78) loading. Additionally, Retinoblastoma protein (RB) has been implicated in interphase loading of condensin II complexes to DNA (79) .

It is important to note that transcriptional regulators of condensin complex were unknown before our data was published in Journal of Biological Chemistry (80), (see appendix for details).

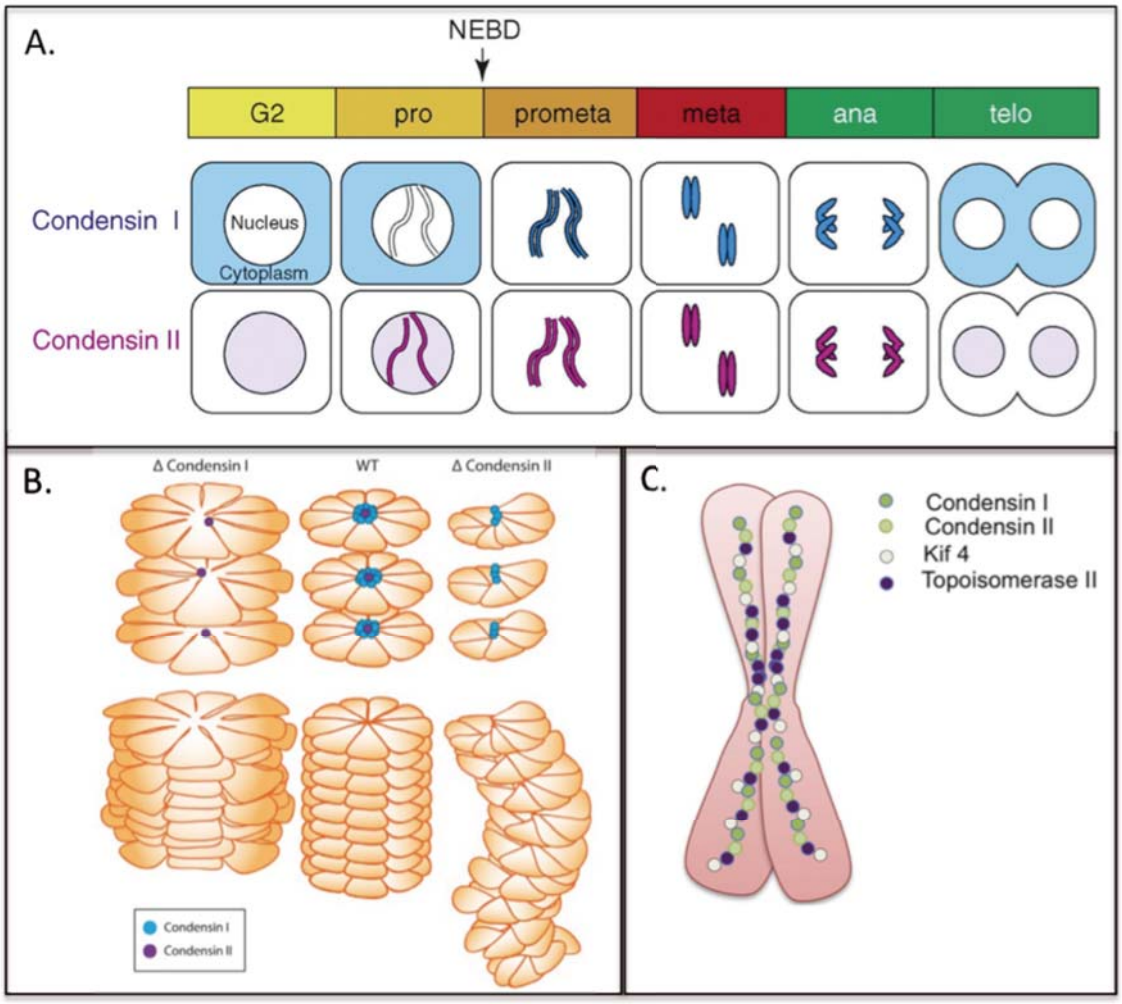

Fig. 10. Different contributions of condensin I and II in chromosome structure. A, In vertebrate cells, condensin II is predominantly nuclear, whereas condensin I is sequestered in the cytoplasm during interphase. Condensin I gains access to the chromosomes only after the nuclear envelope breaks down (NEBD) in prometaphase, and the two complexes alternate along the chromatid axis by metaphase. B, Model for contrasting roles of both complexes: in wild-type mitotic chromosomes, condensin I stabilises and nucleates short-range loops, promoting compaction of chromosome rosettes. Condensin II provides the long-range linkage and alignment between the rosettes, thus facilitating chromosome longitudinal compaction. Chromosomes deficient of condensin I (Δ condensin I) are unable to link and nucleate short-range loops, resulting in a fatter and disorganized chromosome scaffold. Chromosomes deficient of condensin II (Δ condensin II) are unable to provide regular structural linkage between rosettes. Discrete rosettes are unable to form, resulting in a thinner chromosome lacking structural integrity. C, Differential distributions of the "chromosome scaffold" along the metaphase chromosome. Figure adapted from (70,81,82).

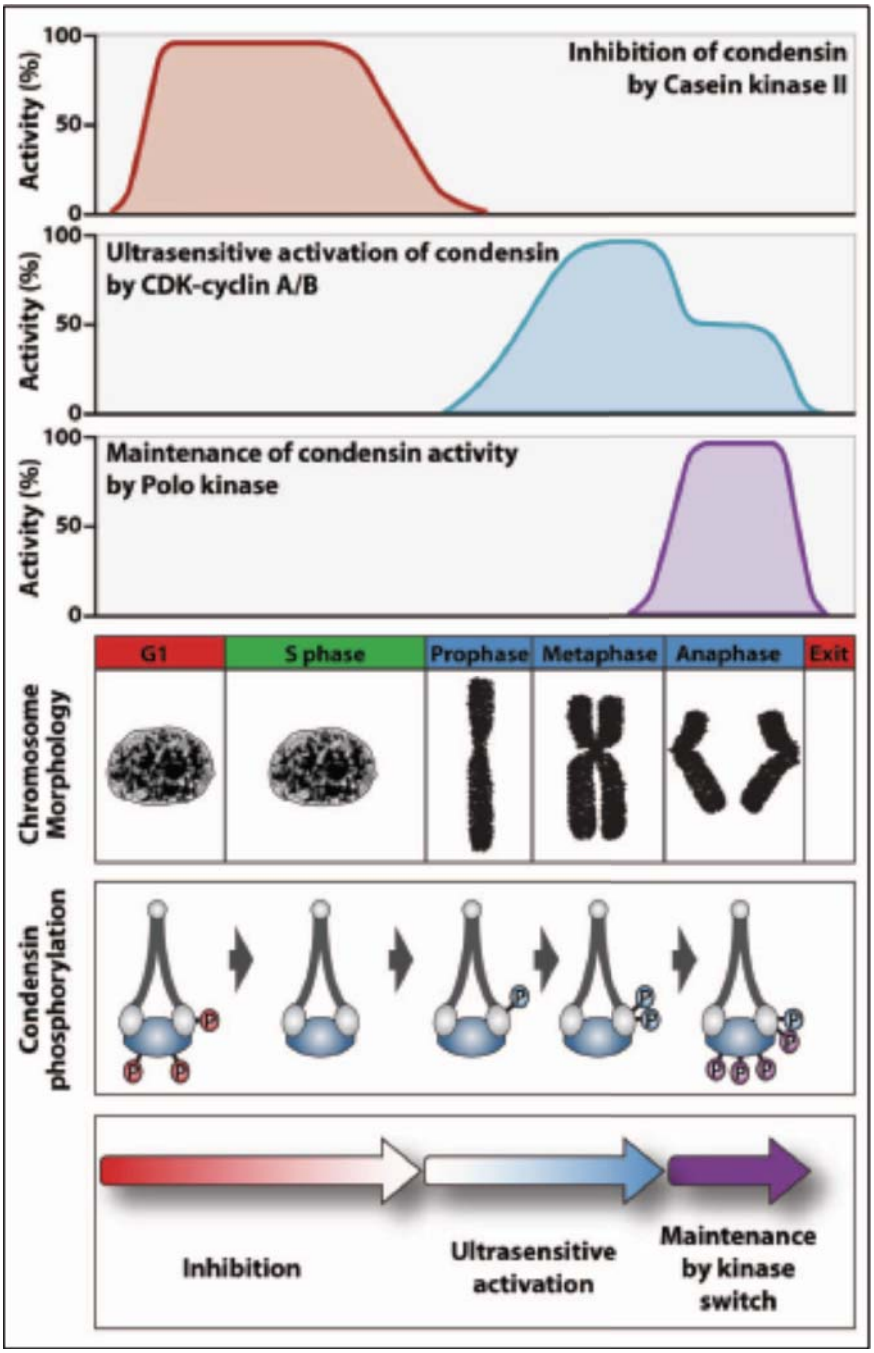

Fig. 11. The ultrasensitive/kinase switch model for chromosome condensation. Cell cycle-dependent regulation of condensin by CKII (top part), CDK1-cyclin A/B (second part) and Polo/Cdc5 (third part). These graphs represent the levels of phosphorylation of condensin subunits by various kinases during interphase and mitosis. The fourth part depicts the morphology of chromosomes in relation with the levels of modification of condensin by cell cycle kinases. The fifth part is a graphical representation of the phosphorylation state of condensin subunits throughout the cell cycle. The last part is a schematic representation of the inhibition, activation and maintenance stages of chromosome condensation according to the ultrasensitive/kinase switch model. The intensity of the color in the inhibition and activation arrows represents decrease and increase, respectively, in the intensity of the stimulus provided by CKII and CDK1. Figure taken from (76).

4. The Molecular Motor KIF4A in chromosome condensation

The molecular motor protein KIF4A was identified by Misteli's group as a novel interactor of condensin complexes I and II in 2004 (83). Indeed, KIF4A belongs to the so-called "chromosome scaffold", that is biochemically defined as the insoluble protein fraction of a mitotic chromosome after the extraction of histones and DNA digestion (84). The most abundant scaffold proteins were first identified as the condensin core subunit SMC2, topoisomerase IIα (TOPO IIα) (85,86) and KIF4 (83,87). These proteins were also shown to be the major components of *in vitro* assembled mitotic chromosomes.

Misteli's study describes how KIF4A contributes to proper chromosome condensation, stability and segregation in mitosis. KIF4A-depleted cells delocalize condensin subunits from the chromosome axis, chromosomes appeared shorter and hipercondensed. Moreover, KIF4A-depleted cells showed mitotic spindle defects, anaphase bridges and aneuploidy (83). The authors postulated that KIF4A might function as a molecular linker and/or spacer between chromosome condensation machinery and DNA to contribute to higher order organization of metaphase chromosomes. A study raised in 2012, confirmed Misteli's group results and also described how KIF4A is needed for correct condensin distribution along chromosomes and how it cooperates with condensin on the lateral compaction of chromosomes; KIF4A works in opposition to topoisomerase IIα action, which shortens chromosomes arms (88). As condensin II, KIF4A loading on mitotic chromosomes is regulated by PP2A (78).

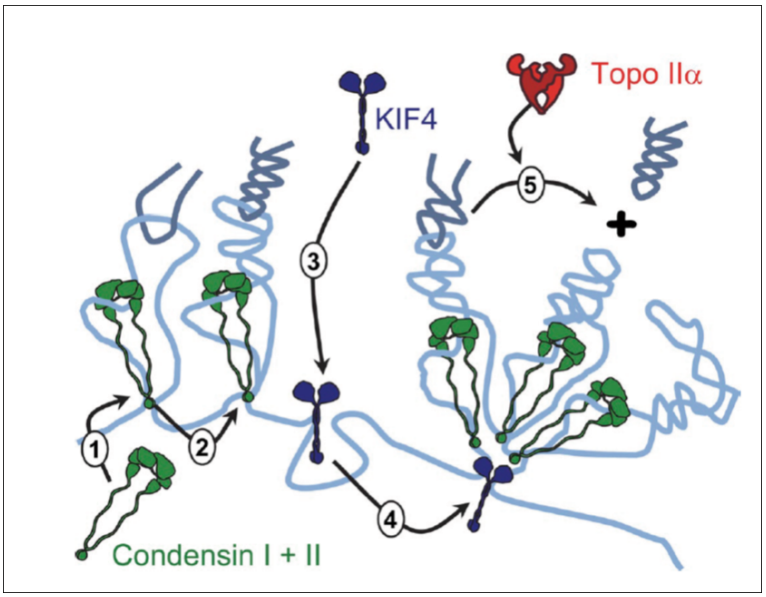

Fig. 12. Interplay between KIF4A, condensin and Topo IIα in shaping mitotic chromosomes. Condensin binds to chromatin, forming loops that it then compacts by supercoiling (1 and 2). KIF4 also independently binds to DNA, possibly forming higher-order loops and promoting their supercoiling by interactions with condensin (3 and 4). Thus, condensin and KIF4 independently and additively contribute to lateral chromatid compaction. Lastly, Topo IIα decatenates the loops in a step required for axial shortening. Figure taken from (88).

KIF4A belongs to the Kinesin (KIF) superfamily. Kinesins are a conserved class of microtubule-dependent molecular motor proteins that have adenosine triphosphatase (ATPase) activity and motion characteristics. KIFs transport cargos along the microtubules transforming ATPase hydrolysis energy into mechanical force. They support several cellular functions, such as mitosis, meiosis, and the transport of macromolecules and vesicles (89).

KIF4A has the typical kinesin structure: an N-terminal motor domain, a stalk domain containing predicted coiled-coil regions and a C-terminal tail domain (90). The motor domain, containing the ATPase activity and the microtubule binding capacity, is highly conserved along kinesin family members whereas tail domain is divergent and confers cargo-specificity. The stalk domain serves for dimerization, as KIF4A functions as homodimers, and also contains a nuclear localization signal (87) for nuclear transport (Fig. 13, A).

KIF4A was first described as a chromokinesin, as it binds to chromosomes during mitosis. As other kinesin proteins, it is involved in several mitotic processes: spindle assembly, proper chromosomes alignment in metaphase and pulling forces generation to separate sister chromatids to opposite spindle poles. Nevertheless, the main function of KIF4A takes place in cytokinesis, when accumulates in the midzone to form the cytokinetic cleavage furrow (Fig. 13, C). Unlike other spindle kinesins, KIF4A is localized into the nucleus during interphase and participates actively in chromosome condensation, as explained before.

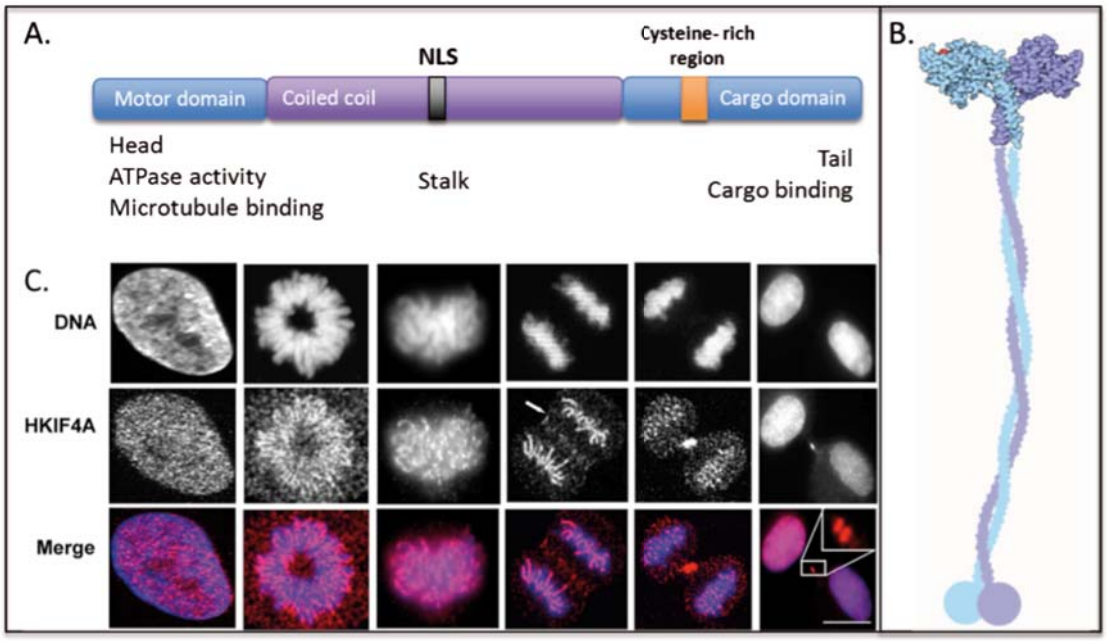

Fig. 13. KIF4A structure and cell cycle distribution. A, Schematic representation of KIF4A protein. NLS: Nuclear localization signal. B, KIF4A forms homodimers by the stalk domain to be functionally active. Red dot represents an ATP molecule. (Illustration by David S. Goodsell of The Scripps Research Institute). C, Cell cycle distribution. During interphase the protein is prominently nuclear but from prophase to telophase KIF4A is present on chromosome arms. In addition, the protein accumulates in the mid-zone (arrow) and forms the cytokinetic ring until cytokinesis. The inset shows an amplified image of the midbody that appears as two rings. Bar, 5 μm. Figure adapted from (83).

The nuclear localization of KIF4A during interphase has been recently related to other cellular processes distinct from mitosis, like DNA damage repair, neuronal survival and gene expression regulation:

a) KIF4A interacts with BRCA2 and it is involved in homologous recombination repair of DNA double-strand breaks, modulating the Rad51/BRCA2 pathway (91).
b) In murine juvenile neurons, KIF4A binds to and inhibits PARP-1, which regulates cell survival. In this tissue, those cells where PARP-1 is inactive are prone to apoptosis. The membrane polarization activates PARP-1 and dissociates it from KIF4A. Active PARP-1 triggers cell survival in active neurons, thus regulating brain homeostasis (92).
c) KIF4A has additionally been implicated in gene expression regulation, acting as a modulator of chromatin structure and accessibility. KIF4A interacts the DNA methyl-transferase DNMT3B, the chromatin remodellers SIN3A and hSNF2H and the histone deacetylase HDAC1 (93). KIF4A is also bound to the apolipoprotein D promoter under growth conditions (94), possibly regulating Apo-D gene expression.

5. Might Chromosome Condensation proteins be involved in Cancer?

5.1. Condensin complex in cancer

No experimental evidences had related condensin complex to cancer development or progression until our group described how colon cancer cells require the expression of the core member of condensin complex, SMC2, to progress. When injected into a xenograft tumour model, SMC2-depleted cells generated significantly smaller tumours compared to the control cell population (Fig. 14). We found that siRNA mediated depletion of SMC2 in colon cancer cell lines impaired cell proliferation (Fig. 15), as SMC2 depleted cells entered into apoptosis, G2/M stop and aneuploidy. These all features presumably indicated mitotic catastrophe (80), (see appendix section for details).

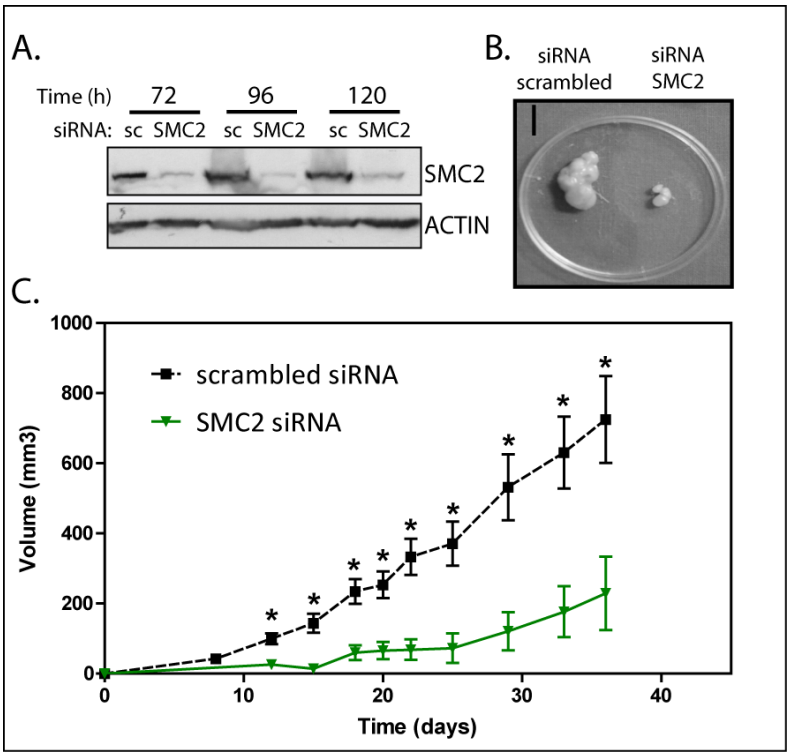

Fig. 14. siRNA mediated knockdown of SMC2 impairs tumour growth in xenograft mice models. A, SMC2 knockdown was assessed by western blot. C, Representative resected tumours from the same animal at day 40 post-injection. D, Tumour growth curves. Figure adapted from (80).

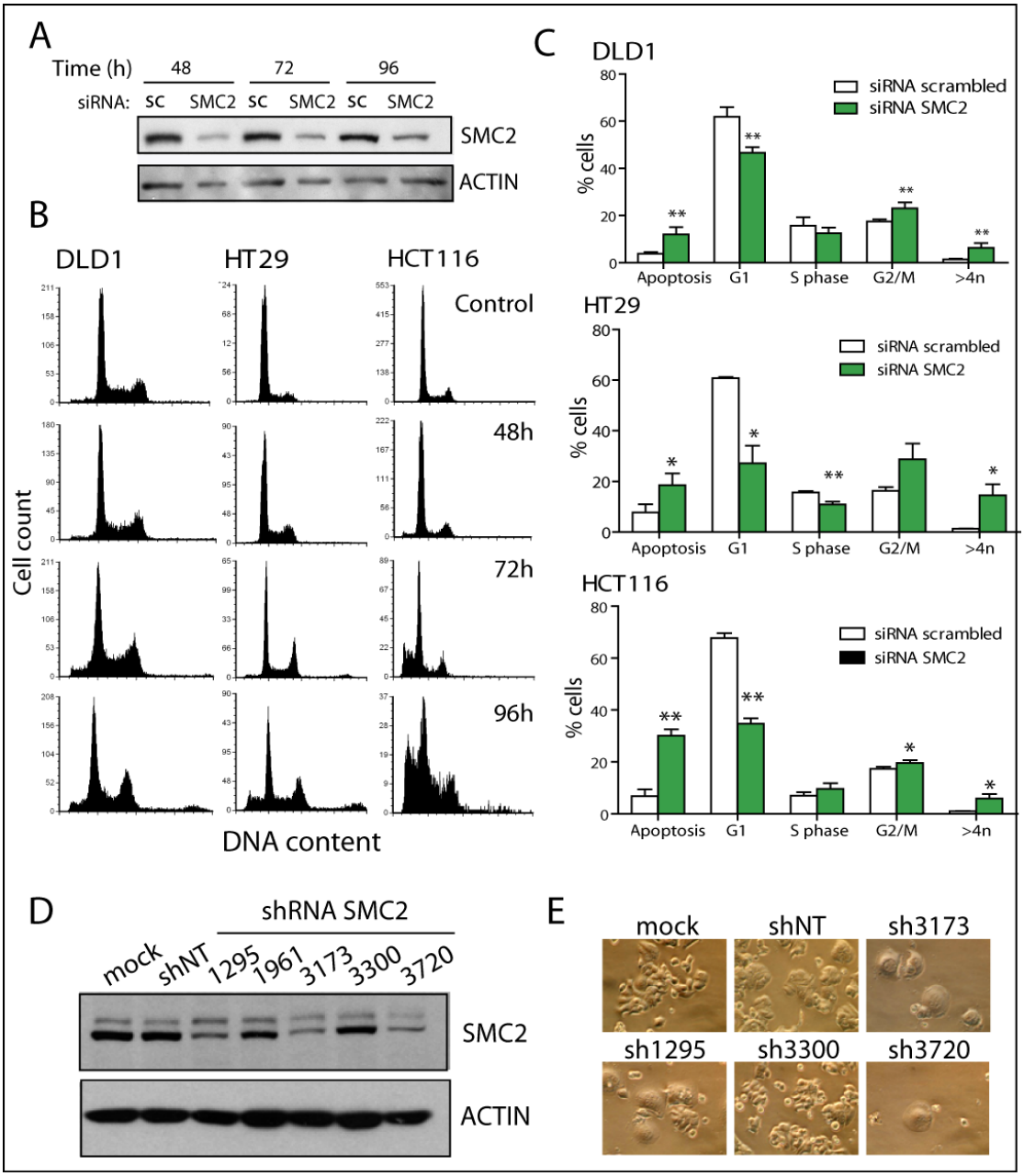

Fig. 15. SMC2 knockdown effect on cell cycle profile. A, SMC2 knockdown analysis by western blot after transfection of an siRNA targeted to SMC2 at the indicated time points in DLD1 cells (sc=siRNA scrambled, used as control). B, Analysis of cell cycle distribution of DLD1, HT29 and HCT116 cell lines 48, 72 or 96 hours post-siRNA SMC2 transfection. C, Cell population distribution 96 hours post-siRNA transfection in DLD1, HT29 or HCT116 cell lines. D, Stable knockdown of SMC2 in HT29 cells. E, The stable knockdown of SMC2 impairs cell viability. Morphological changes in SMC2-downregulated cells could be appreciated after one week in culture; enlarged-multinucleated, non-viable cells resulted from stable knockdown of SMC2. Figure taken from (80).

Furthermore, previous data from our group revealed that SMC2 and SMC4 seemed to be under Wnt signalling regulation (Fig. 16). Both proteins were down-regulated in cellular models for Wnt pathway inhibition, and TCF-4 transcription factor is bound to SMC2 promoter (Fig. 17). It was uncertain, however, the exact regulation of SMC2 promoter under Wnt stimulation.

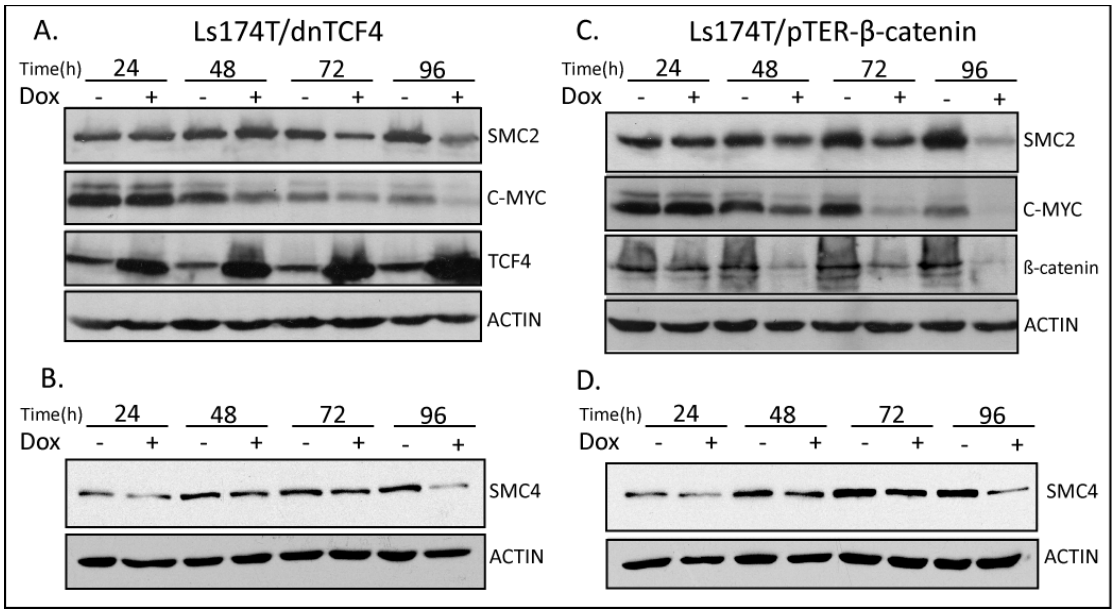

Fig. 16. SMC2 and SMC4 as putative target genes for Wnt signalling. SMC2 and SMC4 proteins are downregulated in cellular models for Wnt signalling inhibition. Tet-on system Ls174T dnTCF4 (A, B) and Ls174T-pTER-β-catenin (C, D) cell lines were cultured in absence or presence of 5 μg/μl doxycycline (Dox) during the indicated times followed by western blot analysis of whole-cell lysates with antibodies against SMC2, C-MYC, as positive control of Wnt signalling blockade, TCF-4 or β-catenin as Tet-On system functionality controls, and ACTIN, as loading control. Figure taken from (80).

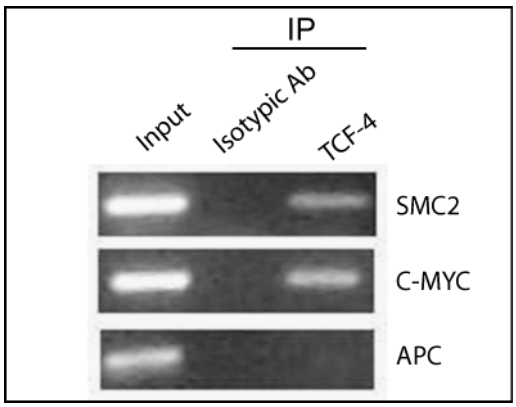

Fig. 17. TCF-4 transcription factor is bound to SMC2 promoter in colon cancer cells. ChIP experiments on DLD-1 cells demonstrated that TCF-4 is bound to SMC2 promoter region. IgG antibody was used as negative control in the immunoprecitation. C-MYC promoter sequence containing TBE1 and region 1B of APC promoter were used as PCR positive and negative controls, respectively. Figure taken from (80).

5.2. KIF4A in cancer

Recent publications have linked kinesin KIF4A to cancer. Narayan et al. (95) reported that the expression of KIF4A mRNA in cervical cancer was much higher than in normal tissues. Taniwaki et al. (96) demonstrated that KIF4A gene was activated in non-small cell lung cancer (NSCLC) cells and the treatment of NSCLC cells with specific siRNA to knockdown KIF4A expression resulted in the suppression of cancer cell growth. Moreover, patients with NSCLC who had KIF4A-positive tumours had a shorter cancer-free survival than patients who had KIF4A-negative tumours. In addition, the same authors classified KIF4A as one of the typical cancer testis antigens. The selective inhibition of KIF4A activity by molecular-targeted agents was proposed as promising therapeutic strategy that was expected to have powerful biologic antitumor activity with minimal adverse events. However, some conflicting results have been reported also. For instance, Mazumdar et al. (97) performed *in vivo* and *in vitro* experiments to demonstrate that loss of KIF4A leads to multiple mitotic defects, including chromosome misalignments, spindle defects, and aberrant cytokinesis, which may cause tumorigenesis. Furthermore, Gao and collaborators (98) showed in 2011 that KIF4A is downregulated in gastric cancer and its overexpression inhibits proliferation of human gastric carcinoma cells both *in vitro* and *in vivo*.

Due to this controversial data, further studies are required to gain a better understanding of the role of KIF4A in cancer development and progression.

Thesis Purpose

Condensin complex and kinesin KIF4A cooperate in chromosome condensation during mitosis, but this interaction is not restricted to mitosis. Nuclear localization of both KIF4A and at least one type of condensin complex during interphase gives information about additional cooperative roles of these proteins out from mitosis. A recent paper points out a role of KIF4 in determining higher order chromatin structure during interphase by dynamic interaction with condensin. Moreover, the authors postulate that this interaction is also responsible for chromatin accessibility regulation, as condensin and KIF4A are found in larger complexes containing PARP-1, ATP-dependent chromatin remodelling factors, histone modifiers (HDAC1), DNMT3B and S phase replication machinery members (99).

On the other hand, chromosomal instability is the main hallmark in colorectal tumours harbouring APC mutations or Wnt signalling deregulation [reviewed in (100)]. Many studies propose APC interaction with microtubules and its mitotic localization to centromeres, kinetochores and mitotic spindle to be under CIN phenotypes present in intestinal polyps and APC-depleted ES cells (101–103). However, many colorectal tumours with MMR-deficiency have APC gene mutations, but remain diploid and do not manifest CIN (104). Thus, other mechanisms probably underlie this kind of genomic instability.

Condensin dysfunction has also been related to genomic instability. Depletion of condensin in higher eukaryotes cells leads to delayed anaphase with prominent centromere defects and uncoordinated chromosome movement. This results in chromosomal bridges arising from missegregated centromeres [reviewed in (105)]. The authors postulate condensin missfunction as potential generator of wide genome instability signatures seen in many cancers (105,106). Indeed, mutations in condensins have been found in 5% (8 of 159) of cancer genomes sequences in COSMIC database (106). It remains to be elucidated, however, whether they are passengers or true drivers of genome instability. Also, chromokinesins, like KIF4A, are crucial components of the mitotic machinery and are required for accurate genome segregation, and by extension, genomic stability. Not surprisingly, loss of KIF4A function leads to deleterious genome defects, particularly an increased number of anaphase bridges, micronuclei and aneuploidy (83).

Another chromosome passenger, SMC3 -member of cohesin complex- has been recently involved in colon tumorigenesis (107). Additionally cohesin missfunction leads to chromosomal instability seen in this kind of tumours (108). Furthermore, SMC3 is a direct transcriptional target of Wnt/β-catenin pathway (109).

The crucial role of condensin complex and KIF4A in chromosome organization and euploidy maintenance, their cooperative function during interphase and mitotic functions, their putative regulation by the Wnt signalling pathway, that could underlie CIN phenotypes seen in CRC tumours, make these proteins good candidates to study their role in colorectal cancer progression and exactly decipher their Wnt-dependent regulation.

Aims of the study

1. Assessment of expression levels of condensin complex and kinesin KIF4A in colon cancer samples.
2. Study of transcriptional regulation of condensin complex and kinesin KIF4A.
3. Determine the role of KIF4A in colorectal tumorigenesis.

Material and Methods

1. Materials

1.1. Colorectal tissue samples

Tumour and normal counterpart samples for Q-PCR, Western blot and IHC analysis were provided by the Surgery and Pathology Departments of the Vall d'Hebron Universitary Hospital (Barcelona, Spain) and Trias i Pujol Hospital (Badalona, Spain). Patients gave written consent before their inclusion in the analysis and the Hospital Ethics Committee approved the study.

Tissue microarrays (TMA) were constructed as described previously (110). A total of 92 colorectal cancer patients with metastatic disease receiving Irinotecan-based chemotherapy at Vall d'hebrón University Hospital (Barcelona, Spain) were included in the TMA. Response to the chemotherapeutic treatment was evaluated by computed tomography using response evaluation criteria in solid tumours (RECIST) criteria (111). The median follow-up time of the patients in this study was 4.6 years. The study was carried out according to Human Investigations and Ethical Committee–approved research protocols.

1.2. Human cancer cell lines and cell culture

HeLa, HEK293T and colorectal cancer cell lines were purchased from the American Type Culture Collection (ATCC). Ls174T/dnTCF4, Ls174T/pTER-β-catenin and Ls174T-W4 cells were kindly provided by Prof H. Clevers (Hubrecht Institute, The Netherlands). HEK293FT variant used for lentiviral particles production was purchased from Invitrogen.

Cell lines were cultured in DMEM or RPMI-1640 (Ls174T variants) medium supplemented with 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin at 37 ºC under 5 % CO_2. In order to induce dnTCF4, siRNA-β-catenin or STRAD/LKB1 expression, Ls174T cells were treated with 5 µg/ml doxycycline.

Cell cycle synchronization of HeLa cells was based on the thymidine double-blocking method: 800.000 cells were plated in 60 mm Petri dishes, and thymidine was added to a final concentration of 2 mM after cell adherence (about 6-8 h). The cells were cultured for 19 h. After removal of the thymidine and incubation for 8 h in fresh DMEM solution, thymidine was again added to a final concentration of 2 mM for an additional 16 h. After second removal of thymidine, synchronized cells were cultured in fresh DMEM and collected at different times for analysis.

Differentiation studies were performed as follows: Caco-2 BBe and HCT8 cells were grown to confluence in DMEM medium to induce dome formation, being the medium changed every day. Butyrate induced-differentiation was performed in SW620 and Ls174T plating one million of cells on 6-well plates and treating them with 1 mM or 5 mM of Sodium Butyrate [Na (C3H7COO)] for 24 h.

1.3. Antibodies

Table 3. Primary antibodies used in the study

Primary Antibody	Source	Reference code	Host	Application (dilution)
KIF4A	Protein Atlas	HPA035517	Rabbit	WB (1:2000) IHC (1:300) IF (1:200)
SMC2	Abcam	ab10412	Rabbit	WB (1:2000) IHC (1:200)
SMC4	Abcam	ab17958	Rabbit	WB (1:2000)
NCAPH	Protein Atlas	HPA003008	Rabbit	IHC (1:50)
Ki67	Santa Cruz Biotechnology	ab833	Rabbit	IHC (1:50)
PCNA	Chemicon International	MAB424R	Mouse	WB (1:1000)
β-catenin	BD-Transduction Laboratories	610154	Mouse	WB (1: 2000)
β-catenin	Santa Cruz Biotechnology	sc-7963	Mouse	IHC (1:100)
TCF-4	Usptate-Millipore	05-511	Mouse	WB (1:500)
PARP	Cell signaling	9542	Rabbit	WB (1:2000)
Cleaved PARP	Cell signaling	9541	Rabbit	WB (1:2000)
C-MYC	Hybridome	Clon 9E10	Mouse	WB (1:10)
GAPDH	Santa Cruz Biotechnology	sc-32233	Mouse	WB (1:2000)
ACTIN	Sigma	A5060	Rabbit	WB (1:5000)

1.4. Primers

Table 4. Primers used in the study. Restriction enzymes sites are highlighted in purple; TBEs mutagenesis is underlined.

Primer Name	Application	Sequence 5´-3'
SMC2p-KpnI- FW	SMC2 promoter cloning	GGGGTACCGACGTGGAAACTTCAG
SMC2p-BglII-RV	SMC2 promoter cloning	GAAGATCTCATTTTCGATACTGTCTTGGG
Δ1-KpnI-SMC2p	SMC2 promoter deletion	GGGGTACCCTTTGAGGAGAGAAAAGTAAG
Δ2-KpnI-SMC2p	SMC2 promoter deletion	GGGGTACCAGGAGCTTTTGGGGTGCGTC
Δ3-BglII-SMC2p	SMC2 promoter deletion	GAAGATCTACGCACCCCAAAAGCTCCT
SMC2prom-TCF4BOX1mut (FW)	SMC2 promoter mutagenesis	TCCACTTCCTAACTGTCGCGCTGAGGAGAGAAAAGTAAGC
SMC2prom-TCF4BOX1mut (RV)	SMC2 promoter mutagenesis	GCTTACTTTTCTCTCCTCAGCGCGACAGTTAGGAAGTGGA
SMC2prom-TCF4BOX2mut (FW)	SMC2 promoter mutagenesis	TGGAGGTGGGGTCCTCTACTCGCGCCGAAATTC
SMC2prom-TCF4BOX2mut (RV)	SMC2 promoter mutagenesis	GAATTTCGGCGCGAGTAGAGGACCCCACCTCCA
SMC2prom-TCF4BOX3mut (FW)	SMC2 promoter mutagenesis	GTCCTTTGCTCGCGCCGAAATTCATTGGAATAAATAGTTCC
SMC2prom-TCF4BOX3mut (RV)	SMC2 promoter mutagenesis	GGAACTATTTATTCCAATGAATTTCGGCGCGAGCAAAGGAC
SMC2prom-TCF4BOX4mut (FW)	SMC2 promoter mutagenesis	TGGTGAAGTTCGCTGCGTAGCGGCCCCGGC
SMC2prom-TCF4BOX4mut (RV)	SMC2 promoter mutagenesis	GCCGGGGCCGCTACGCAGCGAACTTCACCA
SMC2prom-TCF4BOX5mut (FW)	SMC2 promoter mutagenesis	TTCTGTTCCCTGCCTATGTGACCCGGAGG
SMC2prom-TCF4BOX5mut (RV)	SMC2 promoter mutagenesis	CCTCCGGGTCACATAGGCAGGGAACAGAA
KIF4Ap_KpnI_FW	KIF4A promoter cloning	CGGGGTACCGCTAGCTGGTTCGGG
KIF4Ap_KpnI_RV	KIF4A promoter cloning	CGGGGTACCCTCGAGGATCCTATC
KIF4A_001_FW	KIF4A cDNA cloning	CACCATGAAGGAAGAGGTGAAGGG
KIF4A_001_RV	KIF4A cDNA cloning	ACTCCAACTTCAGTGGGC

Primer Name	Application	Sequence 5´-3'
KIF4A_Seq_1	KIF4A cDNA sequencing	GCGCCACTCATAAAAGGTGT
KIF4A_Seq_2	KIF4A cDNA sequencing	TATGAACTCCCAGTCGTCCC
KIF4A_Seq_3	KIF4A cDNA sequencing	GCAAGAAAAATCAAGAACAAACCTA
KIF4A_Seq_4	KIF4A cDNA sequencing	TGCAGCAATTGATTACCCAG
KIF4A_Seq_5	KIF4A cDNA sequencing	AGCTGGAGGGTCAAATTGCT
KIF4A_Seq_6	KIF4A cDNA sequencing	GTGGAATGGAAGGCACTGCAGC
KIF4A_Seq_7	KIF4A cDNA sequencing	ATTTGATTGGAGAGCTGGTC
KIF4A_Seq_8	KIF4A cDNA sequencing	GCAGACAGAAACATCTTCCTAAG
Snail-RT-FW	Q-PCR	CACTATGCCGCGCTCTTTC
Snail-RT-RV	Q-PCR	GCTGGAAGGTAAACTCTGGATTAGA
Slug-RT-FW	Q-PCR	GGACACATTAGAACTCACACGGG
Slug-RT-RV	Q-PCR	GCAGTGAGGGCAAGAAAAAGG
GAPDH-RT-FW	Q-PCR	ACCCACTCCTCCACCTTTGAC
GAPDH-RT-RV	Q-PCR	CATACCAGGAAATGAGCTTGACAA

Table 5. TaqMan Gene Expression Assays used in the study

Taqman Probe (Applied Biosystems)	Code
KIF4A	HS01020169_m1
SMC2	Hs00374522_m1
NCAPH	Hs00379340_m1
NCAPG	Hs00254617_m1
NCAPG2	Hs00214861_m1
18S	4333760F

2. Methods

2.1. RNA extraction and Quantitative PCR (Q-PCR)

Total RNA was extracted with Trizol® (Invitrogen), and further treated with DNase I Amplification Grade (Invitrogen) and retrotranscribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real time PCR reactions were performed in triplicate on an ABI PRISM 7500 Real-Time System (Applied Biosystems), using TaqMan Gene Expression Assays listed in Table 5.

according to the manufacture's protocol. Data were normalized to 18S rRNA expression. For SNAIL (*SNAI1*) and SLUG (*SNAI2*) quantification, SyBrGreen method was used, using GAPDH amplification as endogenous control. The relative mRNA levels were calculated using the comparative Ct method (2e-ΔΔCt) as described previously (112).

2.2. Protein extraction and Western blotting (WB)

Cell pellets and tissue homogenates were lysed in RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM DTT, 1 mM sodium orthovanadate, 0.5 % deoxycholate, 1 % Triton X-100, 0.1 % SDS) containing protease inhibitors (2 μg/ml Aprotinin, 1 μg/ml Pepstatin, 1 μg/ml Leupeptin, 1 mM PMSF, 1 mM EDTA and 1 mM EGTA). Proteins in the crude lysates were quantified using the BCA Protein Assay (Pierce Biotechnology) and 50 μg of whole-cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blots were probed using primary antibodies listed in Table 3 3. Proteins were detected using corresponding HRP-conjugated secondary antibodies, anti-mouse (P0447, Dako) or anti-rabbit (P0217, Dako). The intensity of the bands on the blots was quantified using the GeneTools Program (SynGene).

2.3. Alkaline phosphatase assay

Cell pellets were lysed in cold Mannitol buffer (50 mM D-Mannitol, 2 mM Tris, 0.1 % Triton X-100, pH 7.4) supplemented with protease inhibitors (0.3 mM pepstatin, 1 μg/ml aprotinin and 100 μM sodium orthovanadate). For enzimatic activity assesment 50μg of protein were mixed with 200 μl of p-Nitrophenyl Phosphate Liquid Substrate System (Sigma N7653) and incubated at 37°C for 1 h. Absorbance was measured at 405 nm. Each lysate was run in triplicate.

2.4. Immunohistochemistry (IHC)

Paraffin-embebed tissue samples were incubated at 55ºC overnight prior to xilene mediated de-waxing and serial diluted ethanol hydration. Epitope retrieval was heat induced in citrate buffer pH 6.0. Immunohistochemistry was performed using the Novolink polymer detection system (Novocastra laboratories). Samples were additionally counterstained with hematoxilin.

2.5. Cell cycle analysis by FACS (Fluorescence-activated cell sorting)

One million of cells were fixed in 70% ethanol prior to RNAse treatment (0.1 mg/ml) and Propidium Iodide (PI) staining (40 μg/ml) for 30 min at 37ºC. Distribution of cell cycle phases with different DNA contents upon PI intensity was determined using a flow cytometer FACScalibur (Becton-Dickinson). Analysis of cell cycle distribution and the percentage of cells in the G1, S, and G2/M phases of the cell cycle were determined using the FCS Express cell cycle platform and ModifLT software. Apoptosis percentage was calculated measuring the haplo-diploid population using ModifLT software as well.

2.6. Immunofluorescence (IF)

Cells were grown on gelatin-coated coverslips before 4 % PFA fixation for 10 min. Then, cells were permeabilized with 0.05 % Triton X-100 and blocked for antibody unspecific binding with 0.2 % BSA at room temperature for 30 min. An additional step of blocking was performed by 10 % FBS incubation for 30 min. Then, the coverslips were incubated with anti-KIF4A antibody for 1 h at room temperature. Primary antibody was detected with Alexa Fluor 594–labeled secondary antibodies (Molecular Probes).

To assess Ls174T-W4 polarization scores, F-actin was detected using rhodamine–phalloidin (Cytoskeleton). Polarized cells were defined by the characteristic accumulation of actin in one pole of the cell as previously described (113).

DNA was visualized using DAPI (4',6-diamidino-2-phenylindole, Sigma) at a concentration of 1 μg/ml. Images were taken with a DP70 camera coupled to a Olympus BX61 fluorescence microscope.

2.7. Promoters cloning and Luciferase reporter assays

SMC2 promoter was obtained from DLD-1 cell line by nested PCR amplification of genomic DNA followed by cloning into pGL3-Basic Firefly luciferase reporter vector (Promega) using the primers listed in Table 4. Deletion mutants of SMC2 promoter were obtained by PCR amplification and subcloned into pGL3-Basic vector. Substitution mutants affecting the TCF4-binding sites on SMC2 promoter regions were generated with mutagenic oligonucleotides in Table 4, using QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene).

2000 bp upstream transcription start site of human KIF4A gene was synthesized by GenScript Corporation and subcloned into pGL3-Basic vector as well using the primers listed in Table 4. Cloned promoter sequences were verified by sequencing.

Promoter's activity was evaluated by Luciferase reporter assay as follows: 50,000 cells were seeded into 96-well microtiter plates. The day after, cells were transfected using Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. After 24 h, cells were lysed and Luciferase activity was measured with Dual-Luciferase Reporter Assay (Promega) in a FB12 luminometer (Berthold Detection System), using Renilla (pRL-TK) as an internal control.

In order to evaluate response to TCF-4 or β-catenin, co-transfection with expression vectors VP16-TCF4 or pBCAT was performed. These vectors were kindly provided by Prof. Antonio García de Herreros (IMIM-Hospital del Mar, Barcelona, Spain). VP16-TCF4 vector was constructed by inserting the VP-16 activating domain with a Kozak sequence just upstream of the initiation codon of TCF-4 cloned into pcDNA3. As a result, a constitutively active TCF-4 protein is produced. pBCAT vector consists of the β-catenin cDNA cloned into pcDNA3.

To assess KIF4A promoter activity after lithium chloride-mediated Wnt pathway stimulation, 50 mM LiCL was added to cell culture six hours after transfection. Luciferase activity was measured 24h later.

Luciferase reporter protocol in Tet-On system cells Ls174T-dnTCF4 and Ls174T/pTER-β-catenin was slightly modified. Cells were doxycycline (5 μg/ml) treated 72 h prior to transfection in order to induce dnTCF4 protein or siRNA targeting β-catenin, respectively. Doxycycline was removed for transfection but restored six hours later. Luciferase activity was measured 24 h post-transfection.

pTOP-Flash and pFOP-Flash were used as positive and negative luciferase reporter controls, respectively. pTOP-Flash contains 3 TCF-4 responding elements upstream the luciferase reporter gene. pFOP-Flash contains 3 mutated binding sites for TCF-4 upstream the reporter gene. These vectors were kindly provided by Prof. Hans Clevers (Hubrecht Institute, The Netherlands).

2.8. Stable KIF4A down-regulation

Short hairpin targeting KIF4A mRNA was purchased from Sigma (Track number TRCN0000074163, clon ID: NM_012310.2-4179s1c1; Sequence: CCGGCCTCAGGAATGAGGTTGTGATCTCGAGA TCACAACCTCATTCCTGAGGTTTTTG) cloned into PLKO.1-puro vector, which allows for transient transfection or stable selection via puromycin resistance. In addition, the plasmid may be used to generate lentiviral transduction particles in packaging cell lines. The last strategy was chosen following manufacturer instructions. Briefly, packaging cell line HEK293FT was lipotransfected (Lipofectamine 2000, Invitrogen) with pLKO.1-shKIF4A and proper packaging vectors (pVSV-G and pCMV-dR8.91). 48 h later, culture supernatant containing lentiviral particles was recovered and filtered to proceed to CRC cell lines transduction in presence of Polybrene (6 μg/ml). After 5 days of puromycin selection (DLD1: 2 μg/ml, HCT116: 0.5 μg/ml, SW620: 1.5 μg/ml, Ls174T-W4: 3 μg/ml), polyclonal cell populations were evaluated for KIF4A knockdown. Simultaneously, lentiviral particles containing non-targeting shRNA (pLKO.1-shNT) were used to obtain control cell lines.

2.9. KIF4A cloning and overexpression

KIF4A cDNA was obtained from HEK293T cell line by retrotranscription form total RNA extracts using Transcriptor First Strand cDNA Synthesis Kit (Roche ®). Specific primers were used to amplify KIF4A cDNA and clone it into a pcDNA3.1/V5-His TOPO vector (Invitrogen ®) including a Kozak sequence (Table 4).

After checking KIF4A cDNA sequence by sequencing, HEK293T cells were transiently transfected using Lipofectamine (Invitrogen ®), following manufacturer instructions. pcDNA3.1/V5-His TOPO/LacZ was used as control vector.

2.10. Doubling time calculation-SRB method

The doubling time is the period of time required for a cell population to double. Cell line's doubling times were calculated using SRB (sulforhodamine B) method as described previously (114). SRB stains protein content and the absorbance measurement at 590 nm can be used for cell density calculation.

2,000 cells were seeded on 96 well microtiter plates. One plate was TCA (10 %) fixed every 24 h for 6 days. Once all plates were fixed, they were SRB stained and washed in 1 % acetic acid. SRB precipitates were dissolved in 10 mM Tris pH10 and absorbance was measured at 590 nm.

Absorbance measurements were plotted versus time and non-linear regression was applied to calculate doubling time value for each cell line.

2.11. Anchorage-independent Growth Assay

One of the hallmarks of cell transformation is the capacity of cells to grow on a semi-solid substrate or the anchorage-independent growth. The assay was done in 6-well plates with a base layer containing 0.6 % agar in complete DMEM or RPMI (Ls174T variants). This layer was overlaid with a second layer of 1.5 ml of 0.3 % agar containing a suspension of 30,000 cells. The plates were incubated at 37°C for 10–14 days and tumour colonies were overnight stained with 1 mg/ml nitroblue tetrazolium chloride monohydrate (Sigma). Plates were scanned and colonies were automatically scored using Clono-Counter software (115).

2.12. Matrigel Invasion assay

The Matrigel invasion Assay provides an *in vitro* system to study cell invasion because it allows the assessment of the metastatic potential of tumour cells. The ability of cells to invade through Matrigel-coated filters was determined using 24-well Boyden chamber (Beckton Dickinson; 8 µm pore size) covered by 1 mg/ml Matrigel. CRC cells were seeded at different densities (HCT116: 100 cells/μl, SW620: 100 cells/μl, Ls174T-W4: 500 cells/μl) in 100 µl DMEM containing 1 % FBS in the upper compartment of transwell. pcDNA-TOPO-KIF4A transfected HEK293T cells were seeded 24h post-transfection at 50 cells/μl. The lower compartment was filled with DMEM 10 % FBS, acting as attractant. After incubation for 48 h at 37°C in 5 % CO2, the cells that not penetrated the filter were wiped out with a cotton swab, whereas the cells that had migrated to the lower surface of the filter were methanol fixed and Giemsa stained. Filters were mounted on microscope slides to enable cell counting under a contrast phase microscope (10X). Four fields covering filter's central area were counted on each case.

2.13. MTT assay

The MTT assay is a colorimetric assay that measures cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and reflects the number of viable cells. It was used to measure cytotoxicity or cytostatic activity of camptothecin-derived drugs.

Cells were seeded in 96-well plates at a density of 3000 cells/well and treated with increasing amounts of CPT or CPT-11 in six replicates. 1 % SDS was used as mortality control. After 72 h, 3-(4, 5)-dimethylthiahiazo(-z-y1)-3,5-diphenyl-tetrazoliumromide (MTT) at 5 mg/ml was added into each well and incubated for 4 h at 37ºC. After adding 180 μl dimethyl sulfoxide (DMSO) to each well, the absorbance was measured at 590 nm. Survival percentages were calculated upon 0% (1 % SDS) and 100% (no drug) survival controls. Non-linear regression adjustment was used to compare the different curves obtained and to calculate IC50 values.

2.14. Statistical analyses

All statistical analyses were performed using GraphPad Prism software 5.0; statistical test applied in each experiment is depicted in figure legends. Statistical significance was set up to $p<0.05$. Multivariate Cox regression analyses were run in SPSS software package. Unless stated differently, results are expressed in terms of mean +/- standard deviation.

Results

1. Condensin complex and KIF4A are up-regulated in human CRC

Condensin complex and kinesin KIF4A cooperate in chromosome condensation and dinamycs during mitosis. KIF4A has been previously related to cancer progression, but data is conflicting, as explained before. Our focus is colorectal cancer, so we decided to study condensin complex and *KIF4A* expression in colorectal tumours.

Expression levels of condensin complex subunits were initially evaluated on 15 Normal-Tumour paired samples from patients that had undergone surgery for colon carcinoma in Vall d'Hebrón hospital. Q-PCR measurements showed that different members of condensin complex were clearly up-regulated in the tumour counterparts comparing to the matched normal tissues (Fig. 18): core member *SMC2* was up-regulated in 12 out of 15 cases (80 %), HEAT subunits *NCAPG* and *NCAPG2* were up-regulated in 12 (80 %), whereas kleisin subunit *NCAPH* was up-regulated in 8 (53 %) cases.

Kinesin *KIF4A* expression levels were also evaluated in 24 patient samples (Fig. 19), in which *KIF4A* was significantly over-expressed in 15 tumoral counterparts (62.5 %). Raw data from Q-PCR studies are depicted in Table 6.

These observations were further confirmed on an independent study from Denmark and Finland (Fig. 20). This study consisted in the transcriptome evaluation by expression microarrays (Human Genome U133A GeneChip array, Affymetrix) of 122 non-matched CRC samples (17 normal, 105 tumours) previously described (116). Clinico-pathological features are summarized in Table 7. Briefly, the readings from the quantitative scanning were analysed by the Affymetrix Software MAS 5.0 and normalized using the quantile normalization procedure implemented in robust multiarray analysis (RMA). The RMA scores of the whole condensin complex and KIF4A were extracted from the total array data for our study.

Arrays scores showed that the expression of these genes was significantly higher in the tumour than in the normal tissues studied. Moreover, significant positive correlations were found between the expression levels of condensin subunits and also between those and *KIF4A*. Spearman correlation coefficients are depicted in Table 8. These results pointed towards a significant associated expression of this group of genes.

Table 6. Raw data in Q-PCR studies. Average fold change in the tumoral counterparts for each gene is shown, being the normal counterpart normalized to 1 for each case. Significant overexpression is highlighted in purple ($p<0.05$, Student's t test). N/A, not available.

Patient ID	*SMC2*	*NCAPG*	*NCAPG2*	*NCAPH*	*KIF4A*
17	**2,82**	**5,26**	**6,17**	1,96	4,09
26	**6,29**	N/A	N/A	N/A	N/A
31	N/A	**10,98**	**11,27**	**5,26**	**7,86**
35	**2,72**	**8,92**	**11,00**	**4,71**	**7,86**
36	3,38	**3,14**	**3,02**	**2,03**	N/A
60	N/A	**3,17**	**6,13**	**3,81**	1,97
66	**10,67**	**8,94**	**12,33**	**6,00**	**7,92**
67	N/A	N/A	N/A	N/A	**27,76**
79	**39,92**	N/A	N/A	N/A	N/A
85	N/A	**45,10**	**26,66**	**19,45**	**30,79**
86	N/A	**36,33**	**12,50**	**4,45**	**11,09**
91	1,09	0,79	1,22	0,21	0,83
94	**6,48**	N/A	N/A	N/A	N/A
95	**3,04**	N/A	N/A	N/A	**4,66**
149	N/A	N/A	N/A	N/A	**3,16**
162	**7,15**	**3,50**	**5,41**	1,94	**7,71**
213	0,64	1,18	0,59	0,69	0,75
227	**2,17**	**3,10**	**5,21**	1,26	**3,66**
233	**2,72**	**3,74**	**2,41**	2,02	**4,51**
234	N/A	N/A	N/A	N/A	**4,90**
236	**2,55**	1,38	1,59	**3,17**	1,85
237	N/A	N/A	N/A	N/A	0,80
241	N/A	N/A	N/A	N/A	1,12
252	N/A	N/A	N/A	N/A	0,43
253	N/A	N/A	N/A	N/A	**12,14**
255	**2,36**	**3,29**	**4,09**	0,20	0,60
270	N/A	N/A	N/A	N/A	**1,76**
279	N/A	N/A	N/A	N/A	1,08

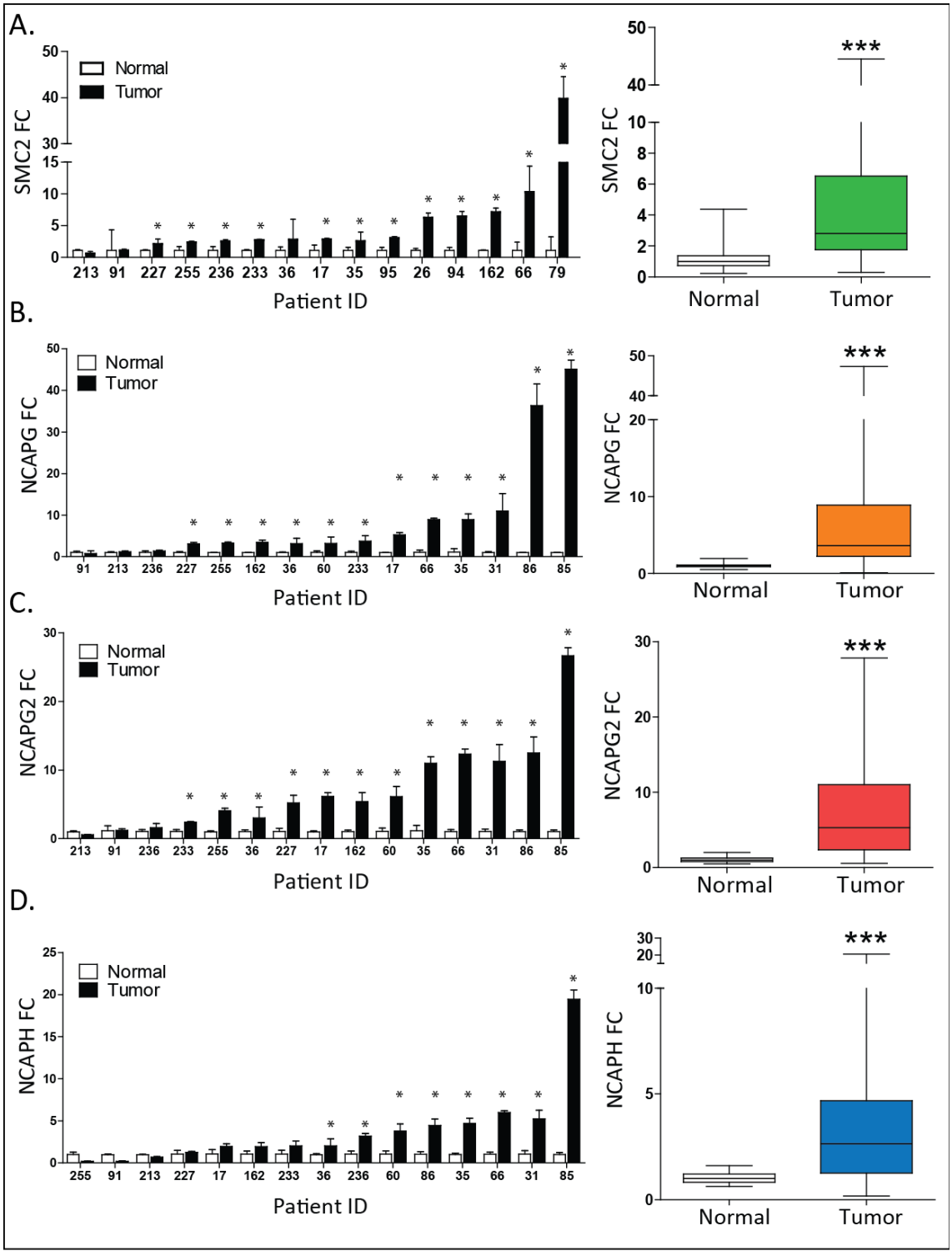

Fig. 18**. Condensin complex is up-regulated in CRC**. Core subunit *SMC2* (A) and three non-SMC subunits *NCAPG* (B), *NCAPG2* (C) and *NCAPH* (D) were evaluated on 15 paired CRC samples by QPCR. Fold increase relative to the normal counterpart is represented (FC, Fold change) (*, $p<0.05$). Total mean values of 15 pairs were compared using a Student's t test (right panels) Boxplots represent minimum and maximum values (***, $p<0.001$).

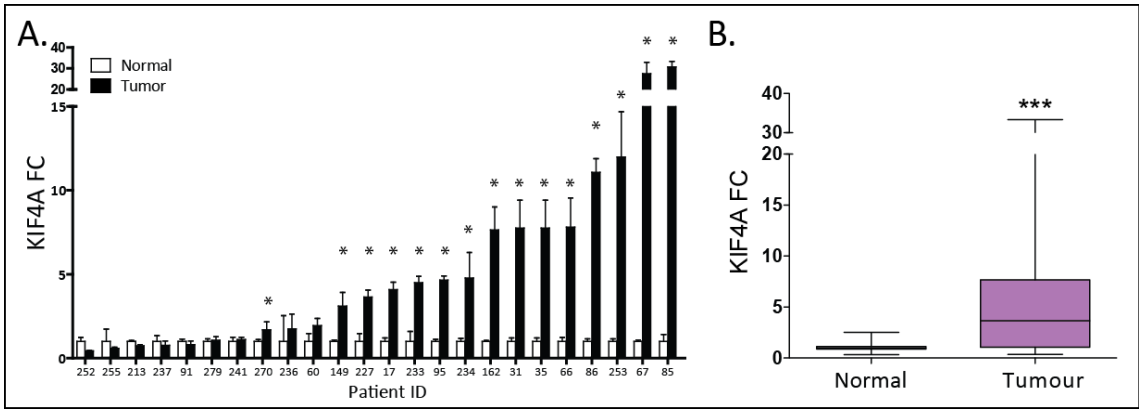

Fig. 19**. Kinesin *KIF4A* is up-regulated in CRC**. *KIF4A* expression was studied in 24 paired CRC samples by Q-PCR. Fold increase relative to the normal counterpart is represented (FC, Fold change). Total mean values of 24 pairs were compared using a Student's t test (right panels). Boxplots represent minimum and maximum values. (***, p-value<0.001).

Table 7. Clinical features of 105 CRC patients dataset from Denmark and Finland.

Age (Average, Min-Max)		59.32 (32-87)	
Stage	I	2	2%
(n , %)	II	36	34%
	III	65	62%
	N/A	2	2%
Grade (n, %)	Good	6	6%
	Moderate	78	74%
	Poor	20	19%
	N/A	1	1%
Location (n, %)	Right	29	28%
	Left	31	30%
	N/A	45	43%
MSS status	Sporadic MSI	20	19%
(n, %)	Hereditary MSI	17	16%
	MSS	61	58%
	N/A	7	7%

Table 8. Spearman correlation coefficients between expression levels of *KIF4A* and condensin complex members in the danish/finish study. Statistically significant coefficients (p<0.05) are highlighted in bold

	SMC4	***NCAPD2***	***NCAPD3***	***NCAPG***	***NCAPG2***	***NCAPH***	***KIF4A***
SMC2	**0.43**	**0.40**	**0.29**	**0.47**	**0.40**	0.13	**0.53**
SMC4		**0.51**	**0.62**	**0.62**	**0.43**	**0.42**	**0.52**
NCAPD2			**0.55**	**0.50**	**0.45**	**0.47**	**0.71**
NCAPD3				**0.60**	**0.68**	**0.46**	**0.63**
NCAPG					**0.67**	**0.56**	**0.71**
NCAPG2						**0.45**	**0.67**
NCAPH							**0.53**

Microarray expression data was interrogated using Student's t test to explore any association with the different clinico-pathological characteristics in this set of patients. No significant association was found between condensin complex or *KIF4A* expression levels and any clinical feature (Table 9).

Multivariate Cox regression analyses were run to explore the influence of expression levels of the different condensin complex members and *KIF4A* on patient overall survival or disease free survival. As expected, location (p= 0.03), age (p= 0.02) and stage (p= 0.015) were significantly associated with patient overall survival, whereas only age was significantly associated to disease free survival (p= 0.022). Regarding condensin complex and *KIF4A*, no independent significant association was found between patient overall- or disease free-survival and the expression levels of these genes.

Table 9. p-values from Student's t test analyses of Condensin complex/KIF4A association to patient's clinical features. Age threshold was set based on average age (59.32 years).

Gene	**Stage** (II vs. III)	**Grade** (Well and Moderate vs. Poor differentiated)	**Age** (Threshold 59 years)	**Location** (Right vs. Left)	**MSI status**
SMC2	0.436	0.830	0.282	0.261	0.801
SMC4	0.595	0.590	0.201	0.615	0.616
NCAPD2	0.633	0.817	0.365	0.179	0.130
NCAPD3	0.497	0.132	0.637	0.285	0.519
NCAPG	0.464	0.800	0.831	0.849	0.177
NCAPG2	0.724	0.681	0.498	0.231	0.994
NCAPH	0.390	0.872	0.701	0.926	0.443
KIF4A	0.583	0.057	0.758	0.355	0.507

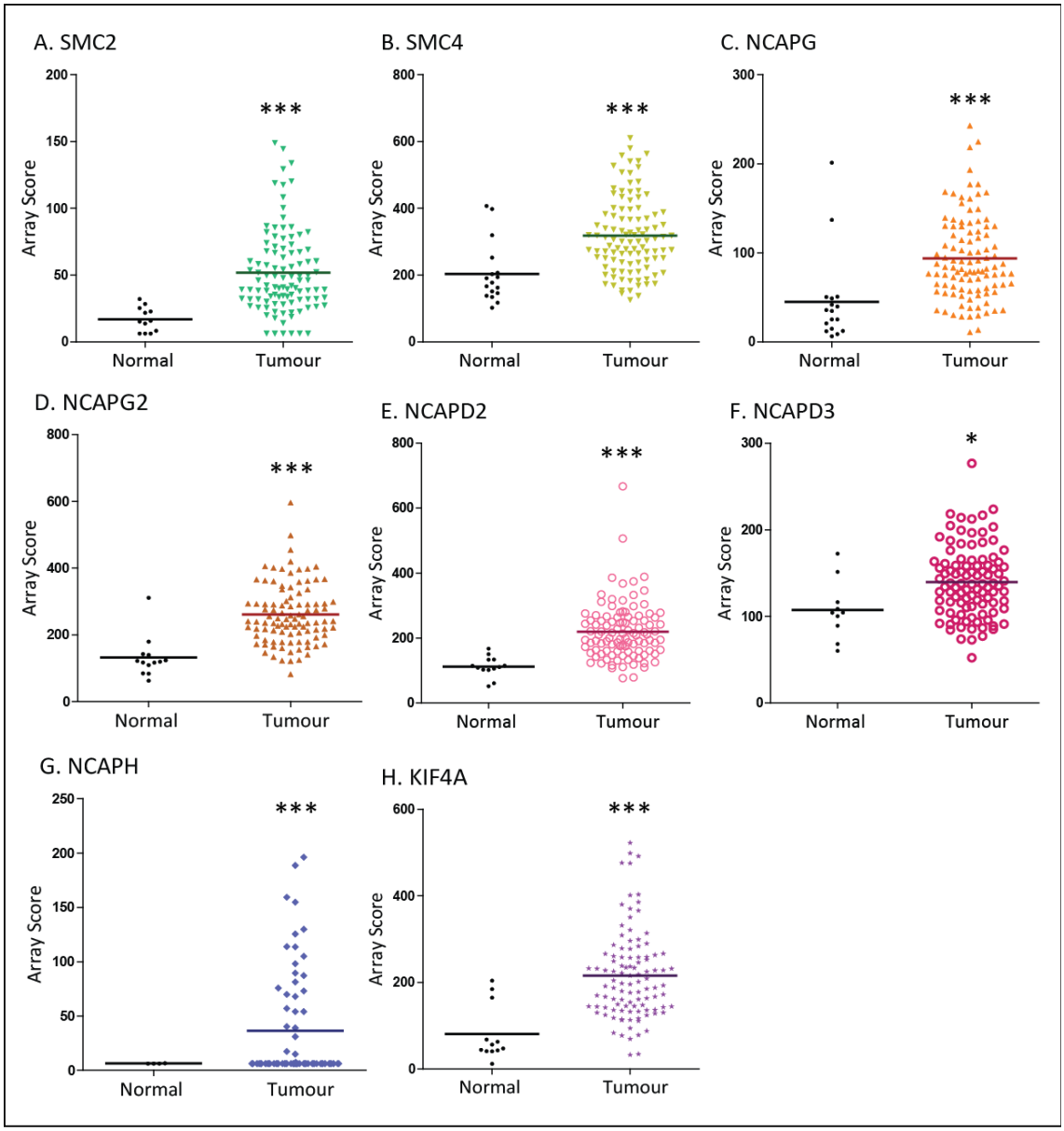

Fig. 20**. Condensin complex and *KIF4A* expression in the Finish/Danish microarray**. Expression data of condensin complex and *KIF4A* was extracted from an independent expression microarray-based study. Relative expression levels (array scores) are represented for 17 normal and 105 tumour samples. Average scores are represented for those genes that had more than one probe in the array. Student's t test was used to compare normal versus tumour expression scores (*, $p < 0.05$; ***, $p < 0.001$).

In order to validate gene expression data at protein level, SMC2, NCAPH and KIF4A proteins were measured by IHC on formalin-fixed, paraffin embedded CRC tissues (Fig. 21). Tumour cells showed strong SMC2 staining both in the nuclei and cytoplasm compartments (Fig. 21 B, C). NCAPH was concentrated also in tumoral cells, but mainly at cytoplasm level (Fig. 21 E, F). KIF4A was also over-expressed in the tumoral counterparts but its expression was limited to nuclei compartment (Fig. 21 H, I). SMC2, NCAPH and KIF4A levels could be also evaluated in normal intestinal mucosa, and we found that these three proteins were also up-regulated in the lower part of the colon crypts (Fig. 21 A, D, G), where Wnt signalling is active and cells actively proliferate in order to maintain the normal epithelial homeostasis (117).

This particular staining at the bottom of normal crypts together with a progressive decreased expression towards the top of the crypt, which is similar to the expression profile described in target genes of the Wnt signalling pathway, suggested that condensin complex and KIF4A could be under Wnt signalling regulation. Hence, we explored Wnt signalling influence on condensin complex and kinesin KIF4A expression.

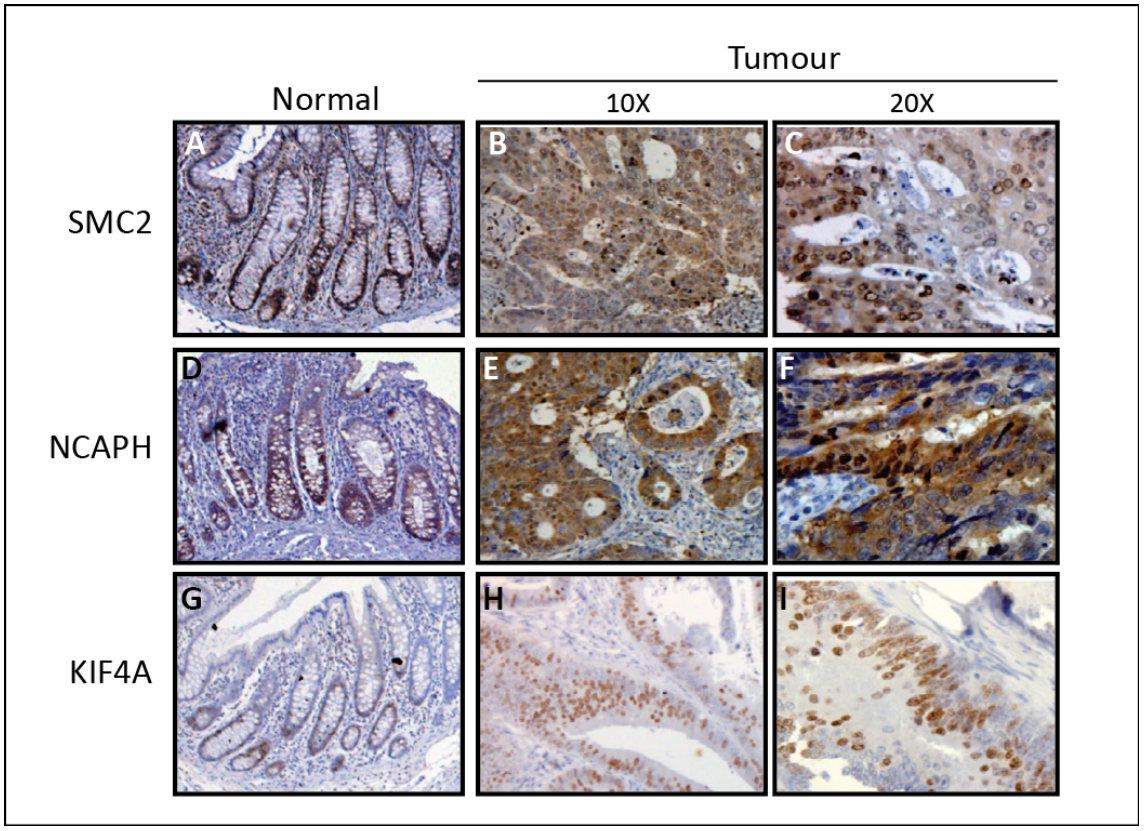

Fig. 21**. IHC on normal and tumoral tissue from CRC biopsies**. Normal (A, D, G) and tumoral tissues (B-C, E-F, H-I) were subjected to IHC staining for SMC2 (A-C), NCAPH (D-F) or KIF4A (G-I). Images from normal samples were taken under bright field microscope at 10X. For tumour tissues two different magnifications, 10X and 20X, are shown. SMC2 is concentrated on nuclei in the bottom part of the crypts (A), and both in normal and cytoplasmic compartment in tumour cells (B-C). NCAPH is mainly cytoplasmic both in normal (D) and tumour specimens (E-F). KIF4a is predominantly nuclear in the lower part of the normal crypts (G) and tumour cells (H-I).

2. Wnt regulation of Condensin complex

2.1. Expression of SMC2 and SMC4 proteins correlates with β-catenin.

Condensin complex regulation along the cell cycle has already been described (71),(118). However, little is known about the particular effectors that regulate the expression of this protein complex at transcriptional level.

Spatial distribution of condensin complex along the intestinal crypts resembled the expression pattern of genes under Wnt signalling regulation. To test if SMC2/SMC4 expression was in concordance to Wnt signalling activity, their protein levels were measured by western blot, along with the central regulator of the Wnt pathway, β-catenin, on 14 pairs of normal-tumour samples (Fig. 22 A) and 14 different colon cancer cell lines (Fig. 22, B). As expected, western blots showed that SMC2 and SMC4 were up-regulated in the tumoral counterparts (in 69% and 48.1% of cases, respectively). Furthermore, a strong positive correlation was found between SMC2/SMC4 and β-catenin levels, both in tissue samples and in colon cancer cell lines (Fig. 22, C).

2.2. Overexpressed SMC2 and SMC4 proteins co-localize with nuclear β-catenin staining

Although β-catenin levels could serve as Wnt signalling activity indicator, its transcriptional activity depends on its nuclear accumulation as Wnt target genes expression depends on nuclear β-catenin transcriptional activity. So, the main hallmark of Wnt activation is the cytoplasmic and nuclear accumulation of β-catenin. To test if condensin complex could be under β-catenin regulation, we investigated SMC2 and NCAPH protein expression in tumours exhibiting different β-catenin localizations. After blinded IHCs evaluation of 43 tumour samples arrayed on a TMA (Fig. 23), we found a significant association between nuclear β-catenin localization and high expression of SMC2 and NCAPH, whereas membrane localized β-catenin was found in conjunction with low levels of SMC2 and NCAPH ($p= 0.0464$ and $p= 0.0014$, respectively; Fisher exact test).

Summing up, condensin core members SMC2/SMC4 expression significantly correlated with β-catenin expression in primary colon tissues and colon cancer cells lines; moreover, the nuclear accumulation of β-catenin correlated to SMC2 and NCAPH overexpression, indicating that this complex could be under direct regulation of the Wnt pathway; hence, we next explored these genes promoters in order to identify functional TCF-4 responding elements.

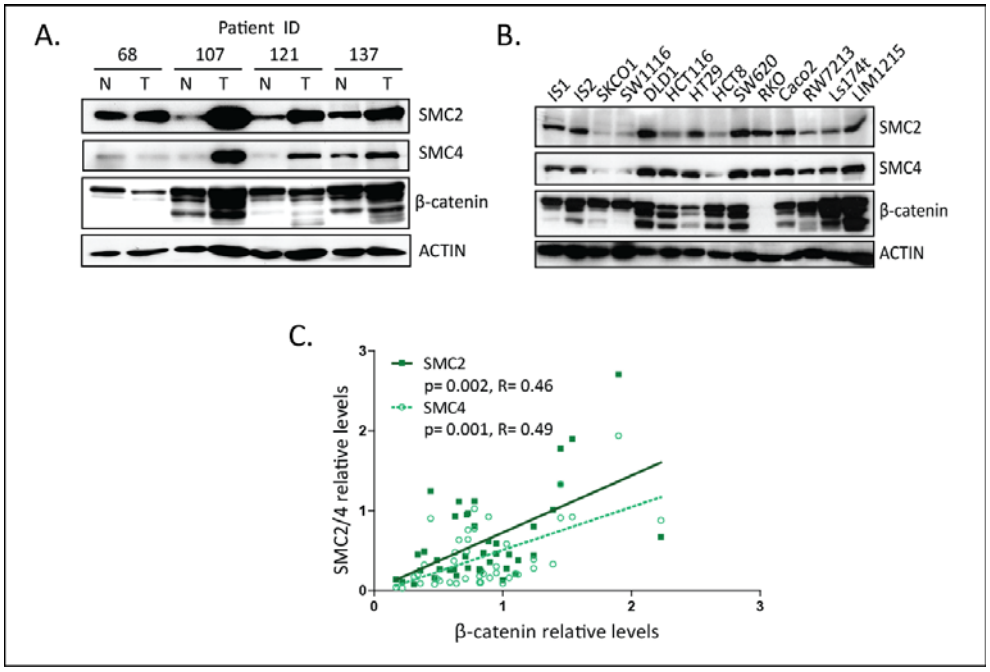

Fig. 22**. Correlated expression of SMC2, SMC4 and β-catenin proteins**. SMC2, SMC4 and β–catenin levels were evaluated by WB in samples from CRC patients (n=27, a representative subset is shown, N= Normal, T= Tumour) (A) and colorectal cancer cell lines (n=14) (B). Actin was used as loading control. C, SMC2, SMC4 and β–catenin protein levels on WB were determined by gel band quantification and normalized to the corresponding actin levels. Values were used to perform correlation studies following Spearman test.

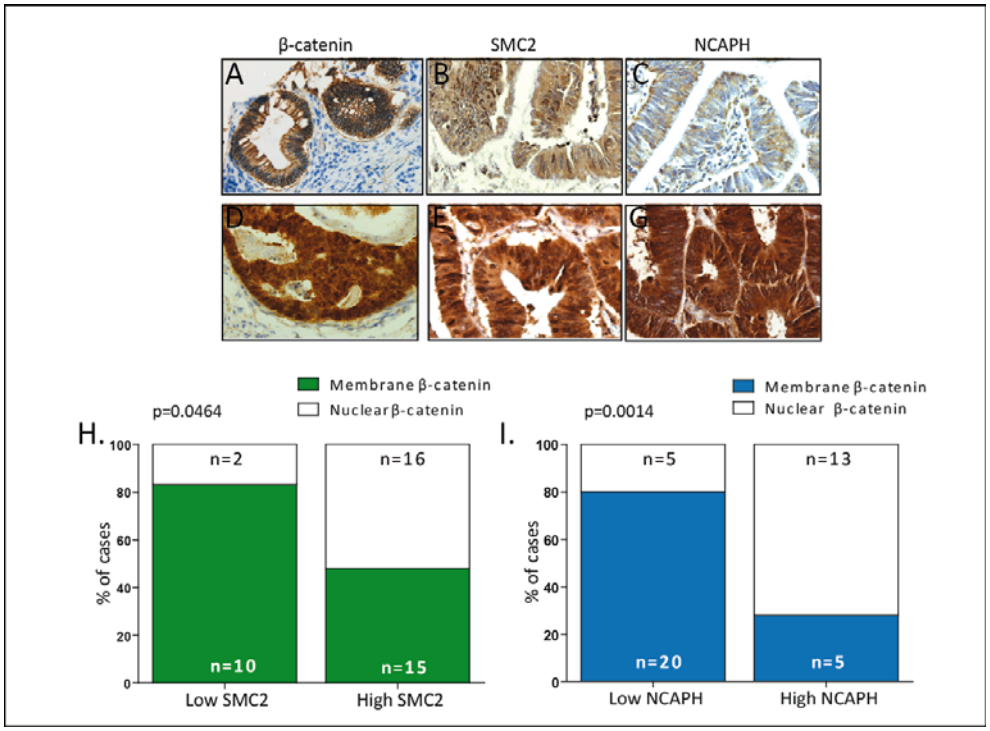

Fig. 23**. Increased levels of condensin subunits correlate with β-catenin subcellular location**. Immunohistochemistry using antibodies against β-catenin (A, D), SMC2 (B, F) and NCAPH (C, G) are shown. Cell-junctional localization of β-catenin in human CRC tumour samples was associated with low levels of SMC2 and NCAPH proteins (A-C). However, nuclear β-catenin localization was associated with high levels of SMC2 and NCAPH (D-G). H, I, Quantification of the correlation between SMC2 (p = 0.0464) and NCAPH (p = 0.0014) protein expression and β-catenin localization, as observed in (B-G). Data analysed using Fisher Exact Test. Images taken under a bright field microscope at 20X.

2.3. *In silico* analysis of SMC2 and SMC4 promoters.

As explained before, previous studies of our laboratory demonstrated that SMC2 and SMC4 levels were down-regulated in cellular models for Wnt pathway inhibition. Moreover, ChIP studies demonstrated that the transcription factor TCF-4 binds to SMC2 promoter. In order to determine whether SMC2/4 could be direct targets of the β-catenin/TCF-4 transcription factor complex, transcription start site (TSS) upstream sequences of the human *SMC2* and *SMC4* genes were obtained from Ensembl database (119). Three different software packages were used for *in silico* prediction of the *SMC2* promoter: Gene2Promoter recognized a very highly promoter-like region between the -308bp and +420bp region (considering 0bp the transcription start site); Promoter 2.0 predicted a promoter region starting in the -476bp position; lastly, PromoterScan located two putative regulatory regions, from the -597bp to -348bp position, and from -313bp to -64bp, respectively. For subsequent studies, we compiled a SMC2 promoter based on the different predictions, which was determined to be from position -597bp to the translation start site (+1059 bp) (Fig. 24, A). In this region, two putative TATA boxes were identified at positions -591bp and -12bp and three recognition sites for the Sp1 transcription factor were situated at -561bp, -301bp and +219bp positions. The predicted *SMC2* promoter was subjected to a screen *in silico* for putative TCF binding elements (TBE). rVista 2.0 (NCBI DCODE, http://rvista.dcode.org/), TESS (Transcription Element Search System, http://www.cbil.upenn.edu/cgi-bin/tess/tess) and Matinspector software (Genomatix, http://www.genomatix.de) were used and predicted four different elements: TBE1 (-389bp), TBE3 (-20bp), TBE4 (+57bp) and TBE6 (+724bp). Additionally, MatInspector located two further TBEs: TBE2 (-37bp) and TBE5 (+98bp). Interspecies conservation analysis showed that the TBEs located closer to transcription start site, TBE2 and TBE3, were highly conserved in orthologous *SMC2* promoters of mouse, rat, macaque and chimpanzee (Fig. 24, B).

Promoter 2.0 software predicted that the region from the -1500bp position to the transcription start site (0bp) of *SMC4* was highly likely to be a promoter region, in which one TATA box (-837bp) and three Sp1 sites (-1066bp, -21bp and -5bp) could be identified. Two putative TBE were predicted in this region (-1270bp and -1294bp), but none of them were phylogenetically conserved in mammals (data not shown), so we continued our study by focusing on Wnt pathway regulation of SMC2 expression.

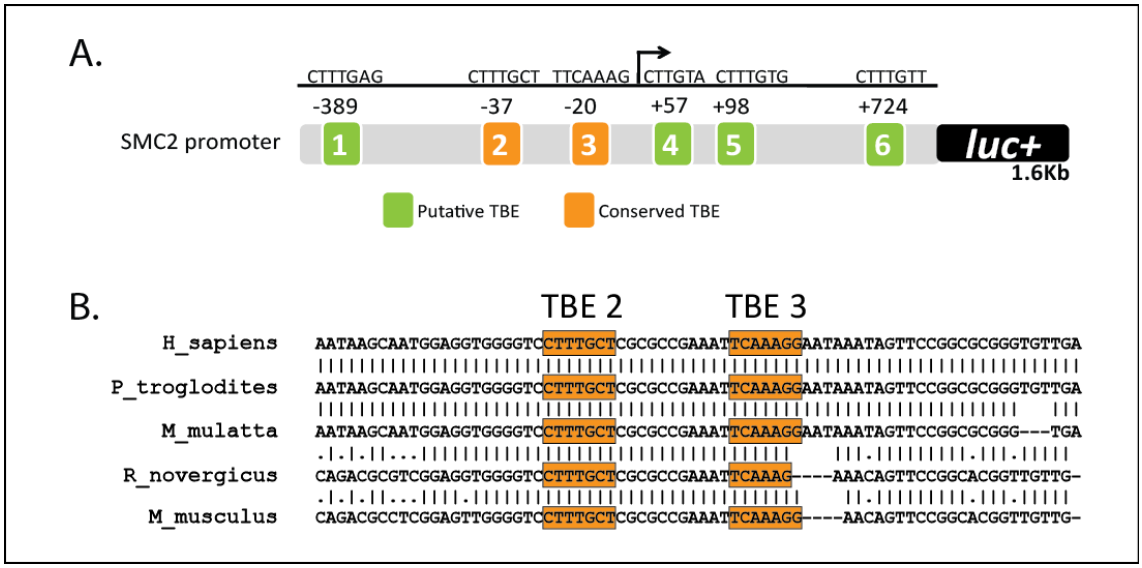

Fig. 24**. SMC2 promoter structure**. A, Schematic representation of predicted SMC2 human promoter. Sequence and position of putative TCF-4 responding elements are indicated. Arrow indicates the transcription start site. B, Sequence alignment of SMC2 promoter orthologous from human (*H_sapiens*), chimpanzee (*P_troglodites*), macaque (*M_mulatta*), rat (*R_novergicus*) and mouse (*M_musculus*). Conserved TBE sites are highlighted in orange.

2.4. SMC2 promoter activity assessment

The full-length promoter of *SMC2* (from -597 bp- to +1509 bp) was cloned into a pGL3 Firefly luciferase reporter vector (pSMC2), and its activity was assayed in cells following transient transfection alone, or in combination with β–catenin expression vector (pBCAT) or alternatively, a constitutively active form of TCF-4 (VP16-TCF4).

First, pSMC2 activity was assessed in human embryonic kidney cells (HEK293T), where Wnt signalling is not aberrantly activated by mutation. As shown in Fig. 25, increasing concentrations of β-catenin or VP16-TCF4 resulted in a significant increase in pSMC2 activity in a dose-dependent manner. Vectors pTOP-flash and pFOP-flash were run in parallel as positive and negative controls, respectively.

Next, pSMC2 activity was evaluated in two colon carcinoma cell lines, DLD1 and HCT116, carrying respectively an activating mutation in β-catenin or an inactivating mutation in APC (120), (Fig. 25, B-C). Again, TOP-flash vector was run in parallel as positive control of Wnt pathway stimulation.

In both cell lines, SMC2 promoter showed a significant transactivation increase after the co-transfection with the β-catenin expression vector or the constitutively active form of TCF-4; this supports the hypothesis that the activated Wnt pathway can drive transcription from the SMC2 promoter via the β-catenin/TCF4 transcription complex, additionally in a colorectal cancer cell context.

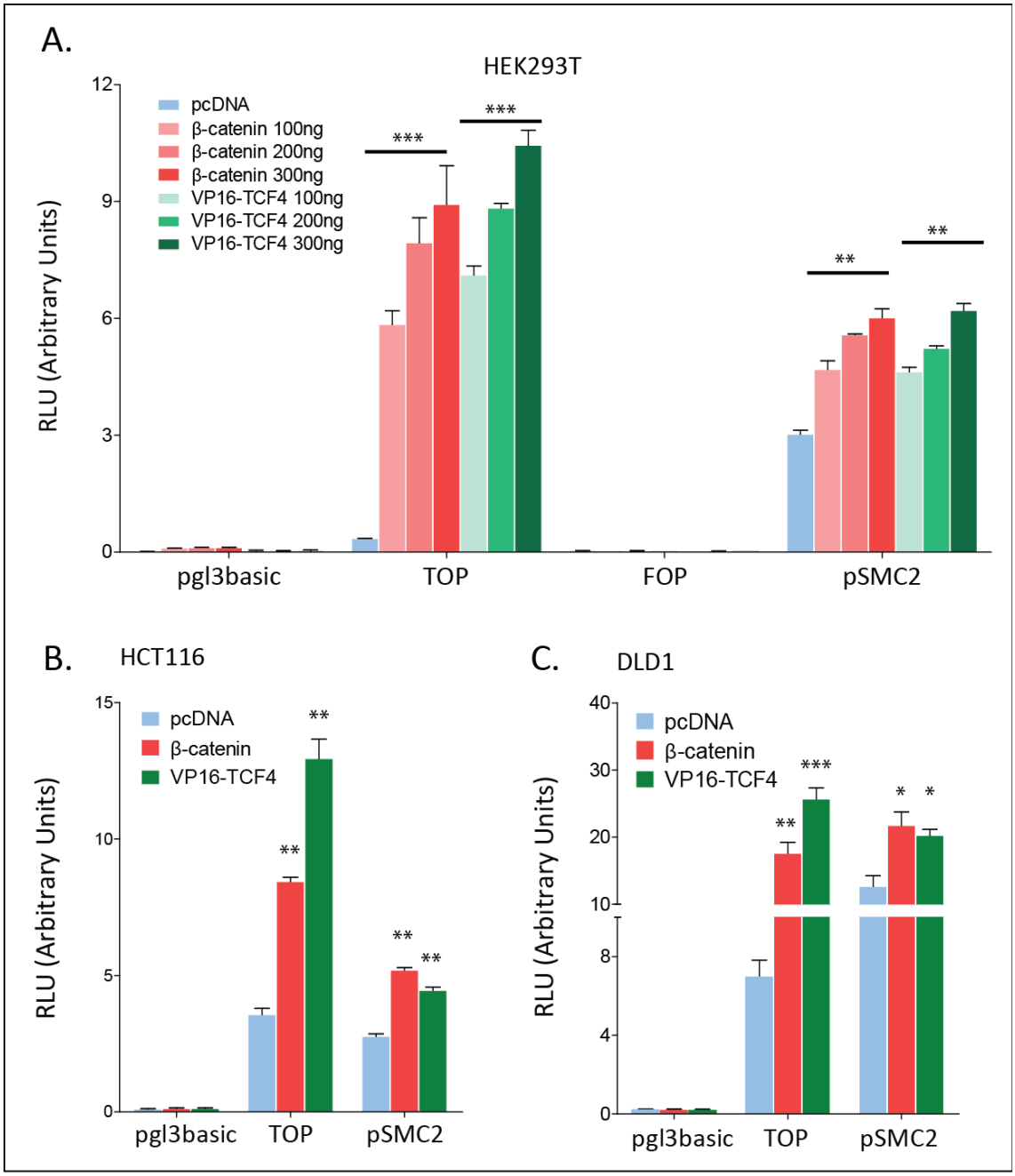

Fig. 25**. Functional study of *SMC2* promoter activity**. HEK293T (A), HCT116 (B) and DLD1 (C) cell lines were transfected with SMC2 promoter-luciferase reporter construct together with control Renilla luciferase reporter pRL-TK for normalization (RLU: relative luciferase units). pSMC2 was tested alone or in combination with increasing or fixed amounts of expression plasmids for β-catenin, VP16-TCF4 or the empty vector pcDNA3 (pcDNA), were indicated. Reporter vectors pTOP-flash (TOP) and pFOP-flash (FOP) were used as positive and negative control, respectively (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

2.5. Identification of active regulatory TCF4 responding element in pSMC2

In order to define the minimal transcriptional regulatory region in the *SMC2* promoter, we generated a series of terminal deletions of the full-length sequence based on the position of the predicted TCF response elements (Fig. 26, A). DLD1 and HCT116 cells were transfected with the three different deletion mutants, and luciferase activity was measured. Deletion of the first 100 base pairs in the *SMC2* promoter resulted in decreased luciferase activity, and the promoter activity was almost lost when the deletion removed all of the putative TBEs (except TBE6). It was also confirmed that the 0.5 Kb (Δ3 sequence), that contains TBEs 1, 2, 3, 4 and 5, maintained the maximal activity in both cell lines. Fragment Δ3 showed a luciferase activity similar to the full-length sequence. Thus, we defined Δ3 as the minimal regulatory region and used it for further mutational studies (Fig. 26, B-C).

As mentioned before, interspecies conservation analysis showed that 2 out of the six TBEs predicted, TBE2 and TBE3, were highly conserved in orthologous *SMC2* promoters (Fig. 24, A), and both were present in the minimal regulatory region, Δ3. Additionally, we had previously detected the promoter region where TCF-4 is bound to *SMC2* promoter (Fig. 17), which coincides within that minimal region Δ3.

To investigate whether those conserved TBEs were functionally relevant, we performed site-directed mutagenesis in order to disrupt TCF-4 binding ability (Fig. 27, A). We detected a significant decrease in luciferase activity when TBE3, located at -20bp, was mutated. However, mutations in all other TBEs did not affect luciferase activity driven by the *SMC2* promoter (Fig. 27, B-C).

To confirm TBE3 susceptibility to Wnt signalling transactivation, we measured luciferase activity after co-transfection of β-catenin or VP16-TCF4 expression plasmids and different mutational combinations in Δ3 fragment. The enhancement of luciferase activity in response to Wnt/β-catenin stimulation, observed in Δ3, was lost when TBE3 was disrupted (Fig. 27, D-E). As expected, mutations in TBE 1, 2, 4 and 5 did not affect promoter response to β-catenin or VP16-TCF4 stimulation. Thus, we identified the TCF response element located at -20bp (TBE3) as responsible for β-catenin/TCF4 transactivation of the *SMC2* promoter.

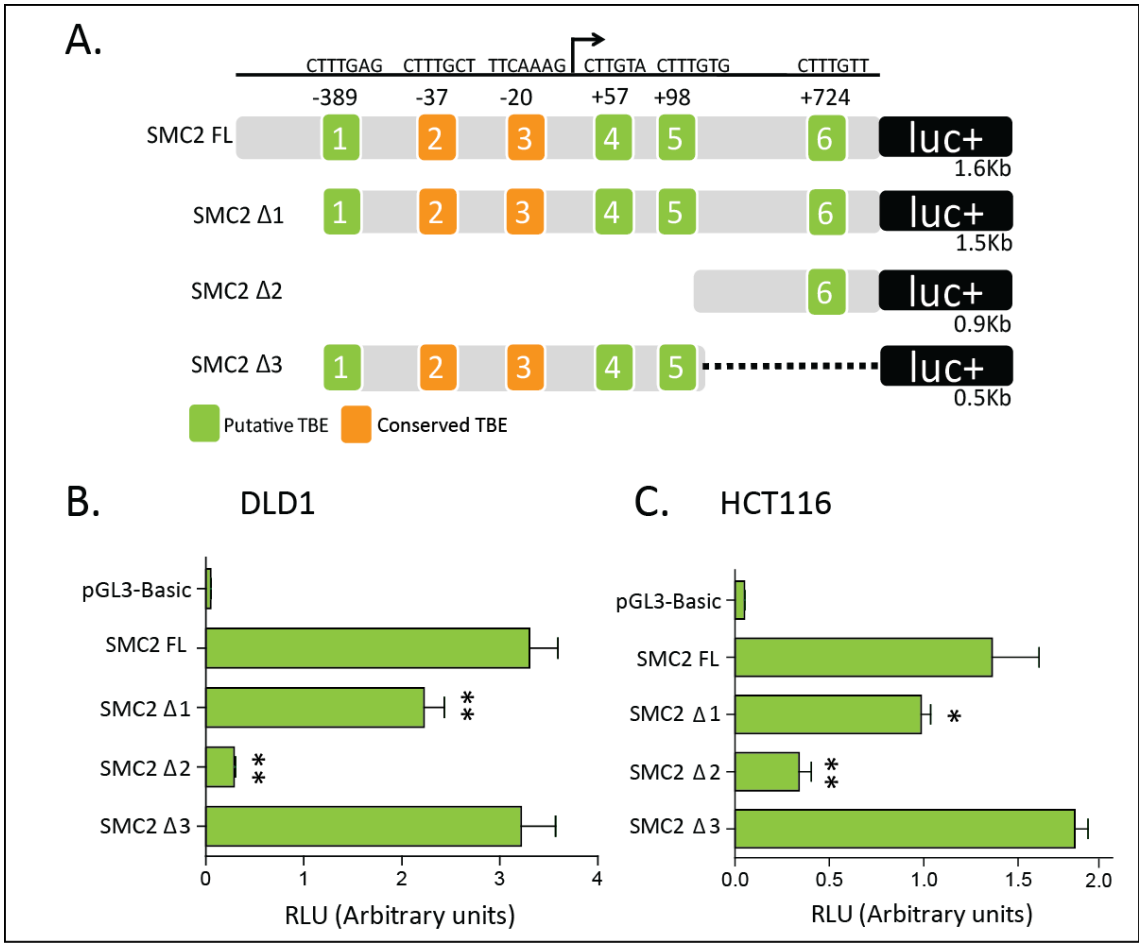

Fig. 26**. Determination of the minimal regulatory region of *SMC2* promoter**. A, Relative position and sequences of the putative TBEs predicted *in silico* in the SMC2 promoter and deletion mutants for luciferase reporters performed. B, C, Determination of fragment 3 as the minimal regulatory region of SMC2 promoter. Luciferase activity of each deletion mutant was normalized to Renilla luciferase internal control (RLU: relative luciferase units) in DLD1 (B) or HCT116 (C) cell lines; a representative result is shown out of at least 3 independent experiments. *, $p<0.05$; **, $p<0.01$; t Student test, (promoter activity versus full length SMC2 promoter (SMC2 FL).

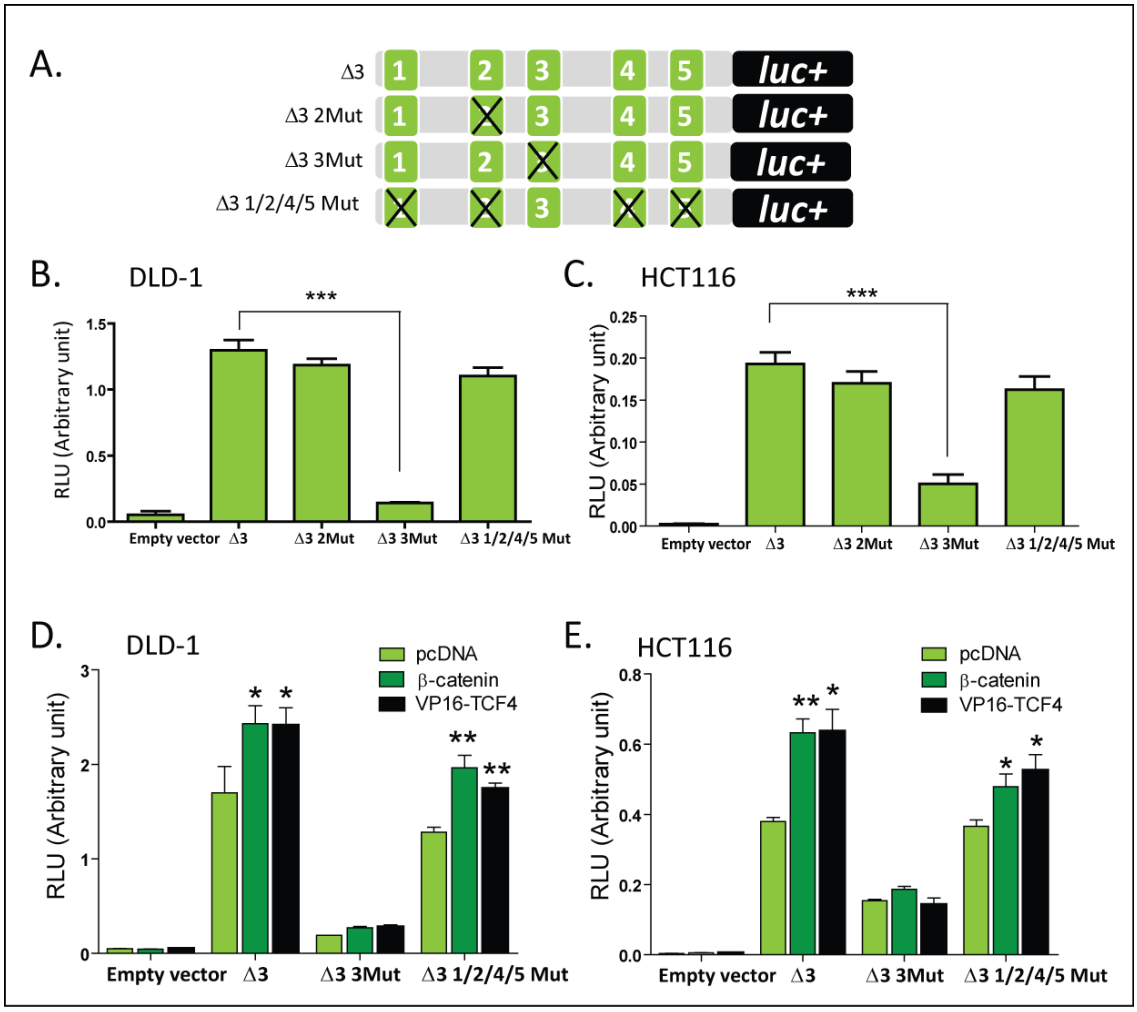

Fig. 27**. Elucidation of the TBE responsible for β-catenin/TCF4 transactivation in the human *SMC2* promoter**. A, Schematic representation of SMC2 promoter mutant variants. DLD1 (B) or HCT116 (C) cell lines were transfected with the constructs shown in A. Luciferase activity was normalized to Renilla activity (RLU: relative luciferase units); a representative result is shown out of at least 3 independent experiments. DLD1 (D) or HCT116 (E) cell lines were co-transfected with Δ3 fragment mutational combinations and expression vectors for β-catenin, VP16-TCF4 (constitutively active form of TCF4) or the empty vector pcDNA3 (pcDNA); a representative result is shown out of at least 3 independent experiments. (*, p<0.05; **, p<0.01,***, p<0.001).

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Discussion

1. Expression of the chromosome condensation machinery in CRC

Condensin complex members -SMC2, SMC4, NCAPG, NACPG2, NCAPD2, NCAPD3, NCAPH- and kinesin KIF4A are significantly up-regulated at mRNA level in colorectal cancer in a high percentage of cases. Two independent sets of patient samples have been evaluated, showing the same results (Figs. 18, 19 & 20) Moreover, we find a significant positive correlation in the expression levels of these genes, possibly as a consequence of their coordinated function in mitosis and chromosome dynamics. In addition, we demonstrate that the over-expression of these genes in colon tumours is also present at protein level, at least for SMC2, SMC4, NCAPH and KIF4A (Fig. 21 & 22).

To date, the condensin complex has not been linked to tumorigenesis. There is only one study that identifies point mutations in SMC2 and SMC4 in two cell lines derived from phytorax-associated lymphoma (133). The authors describe how these mutations conduct to reduced expression levels of both SMC2 and SMC4, causing aberrant chromosomal morphology and inaccurate chromosome segregation in mitosis. The authors postulate that this phenotype could contribute to the chromosomal instability present in this type of tumours. Certainly, several *in vitro* studies had previously described how impaired function of condensin results in chromosomal abnormalities and genomic instability (134,135). Condensin depletion in metazoan's cells results in stretched centromeres and massive merotelic attachments (105). Unlike yeast condensin mutants, metazoans' spindle checkpoint does not correct the miss-attachments and cells enter in a delayed anaphase. As a consequence, anaphase bridges and lagging chromosomes are frequently observed after condensin depletion, which later give rise to chromosome breaks and non-disjuntion, ultimately resulting in aneuploidy (80,106,136).

It is feasible to hypothesize that condensin loss of function could be under the genomic instability observed in colon tumours. However, contrary to condensin depletion, we have observed an up-regulation of these genes in colorectal cancer. Despite various mutations have been found in condensin complex members in various cancer genomes (106), it has not been clarified if these mutations confer actually a loss or a gain of function, except those found in phytorax-associated lymphoma mentioned above.

In order to assess the expression levels of condensin and KIF4A not only in CRC, but also in other tumour types, we consulted the Oncomine database (137). Oncomine is a cancer microarray database and web-based data-mining platform. Oncomine allows researchers to explore gene expression across the increasing number of publications based on cancer expression microarray studies. It integrates data from genome-wide expression microarrays, including those from the Cancer Genome Atlas (TCGA) project (website: www.oncomine.org).

Oncomine data confirmed that the over-expression of the condensin complex is more common that its under-expression, not only in CRC but also in other types of cancer.

Table 13. Oncomine results. Number of studies where the specified gene was found over-expressed (in red) or under-expressed (in blue) comparing tumour versus normal tissues. Selected thresholds: Fold change = 2, p-value < 1e-4.

Cancer Type	SMC2		SMC4		NCAPG		NCAPG2		NCAPD2		NCAPD3		NCAPH		NCAPH2		KIF4A	
Bladder	2		2		4		1		1		2		2				2	
Brain & CNS	2		8		5		3	1	1		1		1	1		1	5	1
Breast	1	1	7	1	4	1	1	1	1	1		1	6	1			6	1
Cervix	3		4		4		3		1		1		3		1		4	
Colorectal	4		9		6		10		5		7		10				7	
Esophaegal	1			2	1		1		1				1				2	
Gastric	1		2		1		1		1		1		2		1		2	
Head and Neck	2		5		4		1						1				1	
Kidney			4				1									2		
Leukaemia		2		2		3		2		2	1			3	1		3	2
Liver			3		3		1		1				1				6	
Lung	2		5		4		6		3		1		7				3	
Lymphoma	3		2	2	3		3		3				2					
Melanoma					1				1				1					
Myeloma	1		1															
Ovarian			3		1		1		2		1		1					
Pancreatic			1		1												1	
Prostate					1								1				1	
Sarcoma	9		9		9		8		4				2				9	
Other	2	2	4	3	2	1	3	1	2		1		2	4	1		5	

Further, supporting the oncogenic role of other SMC proteins, overexpression of SMC3 has been reported in 70% of colon cancer specimens (107). The study describes how SMC3 is aberrantly up-regulated in colon cancer cell lines and in intestinal tumours derived from APC*min* mice. Moreover, SMC3 overexpression induces oncogenic transformation in murine fibroblasts, even though the exact mechanism by which SMC3 up-regulation leads to cell transformation is still unknown.

SMC3 forms the core of the cohesin complex with SMC1; this complex is essential for sister chromatid cohesion after DNA replication until anaphase. The loss of function of cohesin leads to chromosome miss-segregation and aneuploidy (138,139), similarly as condensin depletion does.

It is believed that cohesin down-regulation contributes to cancer by leading to chromosome instability. For cancers with loss of cohesin function, this idea seems plausible (108). However, overexpression of cohesin in cancer appears to be more significant for prognosis than its loss (140–142). Increased levels of cohesin subunits correlate with poor prognosis and resistance to drug, hormone, and radiation therapies

(143). Multiple lines of evidence show that cohesin function does not limit to chromatid cohesion; it has been recently involved in DNA damage repair (60,144), hormone-dependent gene expression (145,146) and tissue-specific gene regulation (147). It is hypothesized that interphase functions of cohesin are under its implication in tumorigenesis.

Clearly, SMC complexes are essential for genome integrity maintenance, and their miss-function can lead to cell transformation. siRNA-mediated depletion of SMC2 impairs the viability of CRC cells, as huge chromosomal aberrations lead cells to G2/M abrogation and apoptosis (Fig. 15), (77); but this effect does not inform us about a putative role of SMC2 in cell transformation.

No experimental data is available to explain the role of condensin overexpression in tumorigenesis. Aberrant high amount of condensin complexes could lead to premature condensation, centromeres dysfunction or sister chromatid resolution defects, ultimately leading to CIN. Additionally, as cohesin complex, altered interphase functions recently assigned to condensin complexes could be also implicated in cancer development, like DNA damage repair (60), rDNA stability (148), gene repression (149–151) or chromatin 3D-structure organization (61,99).

The case of KIF4A is a different one, as there is more evidence about its implication in tumorigenesis. Nevertheless, its exact role in tumour progression is still under debate. On the one hand, KIF4A depletion leads murine stem cells to tumorogenic transformation *in vitro* (97) and its overexpression in gastric cancer cells impairs cell proliferation (98), postulating KIF4A as a tumour suppressor. On the other hand, studies on patient samples show that KIF4A is overexpressed in non-small cell lung cancer (96) and cervix cancer (95). Also, if we consider the genome-wide expression studies included in the Oncomine database (Table 13), it is clear that KIF4A up-regulation is a common feature in cancer.

Similarly, our data demonstrates that KIF4A is overexpressed in colon cancer. The putative role of KIF4A on colon tumorigenesis will be discussed below.

2. Wnt regulation of the chromosome condensation machinery

Wnt signalling is the major regulator of intestinal homeostasis. Wnt ligands promote cell proliferation in the base of the colonic crypt. As long as cells move upwards to the intestinal lumen, Wnt signalling activity decreases and cells enter into differentiation. The gradient of Wnt ligands along the crypts explains the differential spatial expression of Wnt target genes, highly expressed in the bottom of the crypt and in the transient amplifying cells compartment.

The hallmark for Wnt activation is the cytoplasmic and nuclear accumulation of β–catenin. When a Wnt ligand binds to the heterodimeric membrane receptor LRP5/6-Frizzled, the β–catenin degradation complex function is disrupted, β–catenin protein accumulates in the cytoplasm and traslocates to the nucleus to trigger proliferative transcriptional programs.

We observe that SMC2, NCAPH and KIF4A protein are highly expressed in those crypt compartments, with their expression decreasing towards the intestinal lumen (Fig. 21). SMC2, SMC4 and KIF4A protein expression correlate significantly with β–catenin protein levels in normal and tumoral tissue samples and also in CRC cell lines (Fig. 22 & 31); moreover, we find a strong association between SMC2, NCAPH and KIF4A protein levels and nuclear distribution of β–catenin in human colon tumours (Fig. 23 & 31).

These results prompt us to investigate if Wnt signalling might regulate the expression of the condensin complex and kinesin KIF4A.

2.1. SMC2 is a novel transcriptional target of canonical Wnt signalling

In vitro studies performed previously in our group demonstrated that SMC2 and SMC4 protein expression were down-regulated upon Wnt signalling blockade. Both dnTCF4 induction and siRNA-mediated depletion of β-catenin reduced significantly SMC2 and SMC4 protein levels (Fig. 16).

We further focused in SMC2 promoter activity, as SMC2 promoter sequence contain putative TCF-4 responding elements that are evolutionary conserved. Luciferase reporter assays demonstrated that β-catenin and TCF-4 drive SMC2 promoter activity, and the blockade of TCF-4 binding to SMC2 promoter disrupts its transcriptional activity. We had previously observed that TCF-4 factor is bound to SMC2 promoter *in vivo*. In this study we have additionally been able to identify the TCF-4 binding element responsible for SMC2 promoter response to Wnt signalling activation (Fig. 27).

Parallel experiments in which SMC2 expression is depleted in DLD1 cells show that there is a corresponding reduction in the levels of SMC4 and the non-SMC regulatory subunit NCAPH (80). However, even though the expression of other condensin subunits appears to be very tightly linked to the expression of SMC2 (Table 8), no conserved TBE sites were located within SMC4 promoter.

Therefore, additional studies should be performed to investigate the transcriptional regulation of the whole condensin complex and its putative crosstalk with the canonical Wnt pathway.

In summary, this study has identified SMC2, one of the core members of condensin complex, as a novel, bone fide target of β–catenin/TCF4 transcription, which could explain its frequent overexpression in colonic tumours.

It is known that Wnt signalling promotes cell proliferation because stimulates cell division. G1 progression is triggered by active transcription of cell cycle effectors like cyclin D and C-MYC, and inhibition of cell cycle repressors, like p21 and p27. Thus, it is logical that canonical Wnt target genes oscillate during the cell cycle, peaking at G1/S boundary (C-MYC) or G2/M (LGR-5, AXIN2) to prepare cells for division. Contrary to expected, Takemoto et al. observed that in HeLa cells, condensin protein levels remain stable throughout the cell cycle (71). However, a recent study on enterocitic differentiation showed that all condensin members' expression decrease when Caco-2 cells differentiate (152). In agreement to this, parallel studies in our group demonstrated that condensin complex expression decreases under replicative senescence (Kandhaya–Pillai, R. Doctoral thesis, 2011). These data, together with spatial distribution of condensin along the intestinal crypt argues in favour a cell-cycle dependent regulation of the condensin complex.

Similarly, another SMC protein from the cohesin complex, SMC3, has also been identified as a β–catenin/TCF-4 target gene (109). Despite their differential roles in chromosome conformation and dynamics, both condensin and cohesin complexes are essential to ensure faithfully segregation of DNA into the two daughter cells. Under this scenario, Wnt-dependent transcription of members from both complexes might ensure adequate levels of chromosome scaffold proteins to drive appropriate cell division.

2.2. KIF4A expression is β–catenin regulated, independently from TCF-4

Data presented in this study demonstrates that KIF4A expression is tightly related to cell proliferation. First, KIF4A is highly expressed in the transit-amplifying compartment of colonic crypts, correlative to the proliferative marker Ki67. Moreover, KIF4A expression significantly correlates to PCNA in colon cancer cell lines (Fig. 29). Not surprisingly, KIF4A is highly expressed in proliferative tissues, like hematopoietic tissues, fetal liver, spleen, thymus and bone marrow, whereas lower levels are found in heart, testis, kidney, colon and lung (90). Furthermore, KIF4A expression is reduced under replicative senescence (153).

Secondly, *in vitro* models for enterocytic differentiation reveal that KIF4A expression is lost when cell cycle stops and cells differentiate, either when induced by cell-to-cell contact or in response to butyrate treatment (Fig. 30)

Accordingly, KIF4A is cell cycle regulated. KIF4A protein increases in S-phase and remains stable until early G2/M; but latter in this phase, protein levels back to the interphase ones. It is important to note that

a significant amount of KIF4A persists in interphase, localized in the nucleus. KIF4A gene transcription follows an alternate dynamic, as it peaks at G2/M phase (Fig. 28). This would be a consequence of the complex post-translational regulation of KIF4A protein. KIF4A is phosphorylated specifically in mitosis, probably to regulate its function (154,155). In late mitosis, KIF4A could be actively degraded by the proteasome after accomplishing its function in cytokinesis. Hence, the peak of transcription observed in G2/M might ensure KIF4A G1 levels present in the nucleus in interphase.

KIF4A association to β-catenin expression and nuclear localization in tumours (Fig. 31) encouraged us to explore the putative Wnt-dependent regulation of KIF4A.

Not surprisingly, KIF4A protein decreases upon Wnt activity blockade in Ls174T-derived cell lines (Fig. 32), as these cells enter in cell cycle arrest under these conditions. Remarkably, KIF4A protein inhibition is more prominent under β-catenin depletion than under TCF-4 blockade. We then explored if this effect was directly or indirectly mediated by TCF-4 exploring the promoter sequence of KIF4A.

We found only one evolutionary conserved TBE in KIF4A promoter sequence at -906bp position, considering 0bp the transcription start site (Fig. 33). Reporter assays demonstrated that KIF4A responds to β-catenin and LiCl stimulation, whereas TCF-4 co-transfection does not alter KIF4A promoter activity. Moreover, TCF-4 blockade does not supress KIF4A promoter transactivation as β-catenin inhibition does (Fig. 34). Therefore, we conclude that KIF4A transcription is regulated by β-catenin but independently from TCF-4 transcription factor; hence, KIF4A protein inhibition observed in Ls174T/dnTCF4 cells might be an indirect consequence of the cell cycle arrest.

Despite most known β-catenin target genes require TCF/LEF factors for their activation, recent findings show that β-catenin is able to induce gene transcription independently of TCF-4. This is the case for $p16^{INK4A}$, WISP-1, LEF-1 and PML genes, although the particular transcription factors that mediates their β–catenin dependent transcription is not clear yet (156–159).

There is growing evidence that the relationship between β-catenin and TCF is not monogamous and several nuclear receptors and transcriptional factors apart from TCF/LEF family interact with β-catenin to transactivate gene expression; for instance: retinoic acid receptor RARα (160), the vitamin D receptor VDR (161), the androgen receptor (162), the liver receptor homologue LRH1 (163), the hypoxia induced factor HIF1α (164) or the transcription factor FOXO (165). Interestingly, some of these interactions are really significant for colon cancer progression, as the case of FOXO3, which in cooperation with β–catenin, triggers a potent metastatic transcriptional program (166).

We checked if any of these factors could bind to KIF4A promoter by additional *in silico* analyses of the KIF4A promoter but no evolutionary-conserved binding sites were identified within KIF4A promoter for any of them.

Therefore, despite β–catenin regulates KIF4A promoter activity, it is still unclear if this action is mediated by direct binding of β–catenin to KIF4A promoter or which alternative transcription factors might act as mediators of KIF4A transcription. Further investigation is needed to unveil this question.

In summary, KIF4A overexpression in colon tumours might be a consequence of the aberrantly activated Wnt signalling pathway, present in the majority of CRC cases. Under normal conditions, Wnt signalling could be actively promoting KIF4A expression to ensure an efficient chromosome condensation and faithful cytokinesis.

Given that SMC2, member of the condensin complex, is also under direct regulation of β–catenin/TCF-4, reinforces the mitogenic role of the canonical Wnt signalling in the intestinal homeostasis and colon tumours.

3. KIF4A role in intestinal tumorigenesis

Data presented in this study clearly show that KIF4A overexpression is a common feature in CRC, but we wanted to know if KIF4A could be an active driver or a passenger in the colorectal tumorigenic process.

KIF4A depletion impairs cell proliferation in three different CRC cell lines. DLD-1 cells are not affected, even though KIF4A levels are efficiently decreased after shRNA lentiviral transduction (Fig. 35, A). This could be due to particular cellular compensatory mechanisms, only present in this cell line. However, the other three cell lines tested show the same phenotype after KIF4A inhibition: slower cell cycle -with less cells in S-phase and higher proportion of cells in G1- that results in a higher cell population doubling time for shKIF4A cells (Figs. 37 & 39). Anchorage-independent growth and invasive capacity are also affected after KIF4A depletion, probably as a consequence of the lower cell proliferation rate in absence of KIF4A (Fig. 40 & 41).

Our results are in concordance with other studies where KIF4A is depleted. KIF4A depletion in mice ES cells results in a S-phase reduction, probably due to a delay in S-phase entry (99). The authors postulate that KIF4A is necessary for recruitment of replication machinery factors to DNA, as KIF4A binds to histone chaperone Asf-1 and NURD chromatin-remodelling complexes during replication. In lung cancer cell lines, RNAi depletion of KIF4A results in a dramatic loss of cell viability (96). HCT116 KO-KIF4A cells also proliferate slower, with lacked organized spindle midzone but displaying only a mild increase in bi-nucleated cells (167). We do not observe an increase in the >4n population, namely aneuploid cells, despite RNAi-mediated KIF4A depletion in HeLa cells results in multinucleated cells, due to cytokinesis failure (168). We tried to measure the multinucleation rates in CRC lines by IF, but we were not able to detect any significant increase in multinucleated cells after KIF4A down-regulation (data not shown).

Preliminary experiments of transient KIF4A overexpression in non-tumoral cells show that ectopic expression of KIF4A enhances their invasive capacity. This cannot be explained by an increased proliferation rate, as cell cycle is not affected after KIF4A overexpression (Fig. 38). Moreover, cells overexpressing KIF4A might activate the epithelium-mesenchymal transition program, as SNAIL and SLUG transcriptional repressors are significantly induced (Fig. 42).

These transcriptional repressors act at the core of several signalling pathways proposed to mediate epithelial to mesenchymal transition or EMT, which is implicated in tumour metastasis. EMT

involves an alteration from an organized, epithelial cell structure to a mesenchymal, invasive and migratory phenotype.

Different stimuli have been implicated on EMT induction: inflammation (via TGF-β and NFκB), hypoxia (via HIF1 and Notch), oncogene or tumour suppressors mutations (p53, Ras, ErbB2) and growth factors signalling activation (FGF, EGF, IGF, HGF, PDGF) [reviewed in (169)]. The vast majority of those signalling pathways that trigger EMT converge at the induction of the E-cadherin repressors, in particular, the Snail genes (*SNAI1*/SNAIL and *SNAI2*/SLUG) mentioned above (170). Loss of E-cadherin at the adherent junctions marks the onset of a series of dramatic changes that include: loss of cell-cell adhesion structures (including adherent junctions and desmosomes), polarity modulation and rearrangement of the cytoskeleton. Cells become isolated, motile, and resistant to apoptosis (171). The acquired mesenchymal phenotype confers tumour cells the ability to invade, migrate and generate distant metastasis.

How exactly ectopic expression of KIF4A induces Snail family expression is completely uncertain. These results, still preliminary, should be confirmed on CRC cell lines, by stable overexpression of KIF4A.

KIF4A is not associated to tumour staging (Table 9 & 10), but we and others have observed that its expression is elevated in invasive CRC cell lines [Fig. 29 and (172)]. It would be certainly interesting to decipher the exact role of KIF4A on EMT and invasion.

Since KIF4A is linked to proliferative phenotypes and its expression is reduced upon cell differentiation, we also explored the effect of KIF4A depletion on enterocytic differentiation. Surprisingly, KIF4A depletion impairs Butyrate-mediated differentiation and LKB1-induced polarization (Fig. 43). Actually, KIF4A protein levels increase at the first phases of cell differentiation (Fig. 30). These data argue in favour of an active role of KIF4A during differentiation.

Sodium Butyrate (NaB) is a short-chain fatty acid produced in the colonic lumen by fermentation of dietary fibre. It was also discovered as a potent histone deacetylase (HDAC) inhibitor (173), and, despite the exact mechanism by which modulates gene expression is complex and remains elusive, it is accepted that NaB triggers global gene transcriptional programs that lead to enterocytic differentiation (174). In the absence of KIF4A, colon cells are less prone to differentiate in response to NaB; KIF4A interacts with gene expression related proteins like HDAC1, SIN3A, hSNF2H and DNMT3B (93), so it is possible that KIF4A could be actively participating in the gene transcriptional program induced by NaB that lead to enterocytic differentiation.

Ls174T-W4 cells polarize in response to STRAD/LKB1 expression induction. LKB1 is a serine/threonine kinase that is normally sequestered in the nucleus. When the pseudokinase STRAD is over-expressed, it traslocates LKB1 to the cytoplasm and activates its kinase activity (175). Once in the cytoplasm, LKB1 triggers the cell-polarity pathway, which consists in actine cytoskeleton rearrangement, brush border constitution and membrane localization of apical and baso-lateral protein markers (113,176). The role of KIF4A in this process is uncertain. Another kinesin family member, KIF3 has been related to rat neuronal polarity, as it interacts with PAR family proteins (the orthologue of *LKB1* is *par-4*) (177). However, KIF3 carries out its function in the cytoplasm and KIF4A is mainly nuclear, so a similar function would not be achieved by KIF4A. It is important to note that KIF4A function is not restricted to mitosis; during

interphase, KIF4A participates in chromatin structure maintenance, DNA replication (99), DNA damage repair (91) and gene regulation (93,94). Anyhow, the exact role of KIF4A in the differentiation process is still enigmatic.

Because KIF4A is also implicated in DNA damage repair, we also investigated the effect of KIF4A on the cellular response to DNA damage. Wu and collaborators described how KIF4A localizes to DNA damage sites upon laser micro-irradiation and participates in the homologous recombination repair, presumably modulating the BRCA2/Rad51 pathway (91).

Camptothecin (CPT) derivatives are highly selective topoisomerase I (TOP1) inhibitors. Topoisomerase I is an enzyme that alleviates the superhelical tensions of DNA by producing transient single-strand breaks. As soon as the DNA is relaxed, TOP1 re-ligates the single-stranded DNA ends, reverses its covalent binding and dissociates from the DNA. CPT analogues selectively bind to TOP1 and fix the enzyme on DNA during the enzymatic cleavage intermediate step, which leads to the formation of reversible TOP1 cleavable complexes. The cytoxicity of these drugs resides in the formation of DNA double strand breaks (DBS) when replication and/or transcription machineries collide with these complexes (178).

Expression of DBS repair proteins in cancer cells, both participating in homologous recombination (HR) and non-homologous end-joining (NHEJ) seems to be crucial for resistance to CPTs, since deficiency of those proteins leads to CPT sensitivity (179).

KIF4A depletion in HCT116 and DLD1 cells confers more resistance to TOP1 inhibitors (Fig. 44). If we consider KIF4A as a modulator of HR mediated DNA repair, we would expect the contrary effect from KIF4A depletion. This led us to speculate that KIF4A may have a more complex role in the cellular response to TOP1 inhibitors.

As mentioned above, KIF4A inhibition decreases the number of cells in S-phase. This might explain why HCT116 KIF4A-depleted cells are more resistant to CPT/CPT-11, as these drugs perform their toxicity during replication. But the same cannot be applied for DLD1 cells, whose cell cycle is not affected after KIF4A depletion.

Apoptotic levels in DLD1 cells after CPT-11 treatment (Fig. 45) are reduced in those cells KIF4A-depleted, and the corresponding PARP-1 cleavage is partially inhibited. We measured PARP-1 cleavage just as a marker of apoptosis, but Midorikawa and collaborators had already demonstrated that KIF4A binds to and inhibits PARP-1 activity, and neurons lacking KIF4A are resistant to physiological apoptosis (92). PARP-1 activity is essential to promote juvenile neurons survival after membrane depolarization; this mechanism ensures that active neurons survive along brain development. In a steady state, KIF4A binds to PARP-1 and suppresses PARP-1 activity. Cells are then prone to apoptotic death; but membrane depolarization disrupts this binding and PARP-1 promotes cell survival. Thus, KIF4A is favouring apoptosis in this cellular context.

Our scenario is different, as CPT/CPT-11 induce DNA damage. Under CPT exposition, PARP-1 is involved in the resolution of TOP-1-DNA-CPT complexes, avoiding the production of DNA double strand breaks (180) and, by extension, reducing the cytotoxic effects of these drugs. Indeed, PARP inhibition enhance CPT/CPT-11 cytotoxicity (181).

As mentioned above, KIF4A binds to and inhibits PARP-1. The absence of KIF4A could be favouring PARP-1 repairing activity, preventing DSB formation and conferring more drug resistance to shKIF4A cells. Alternatively, KIF4A could be actively involved in the apoptotic program. Anyhow, further experiments are needed to unveil if KIF4A could directly modulate PARP-1 activity under CPT/CPT-11 exposure.

CPT derivatives, like Irinotecan (CPT-11), have been FDA approved for metastatic CRC treatment about 15 years ago. These agents have increased significantly the percentage of patients with an objective response and better overall survival (182,183) compared with patients treated with 5-fluorouracil alone. Unfortunately, only 20% to 30% of patients show an objective response (110), and there is still a lack of knowledge of markers capable of predicting response to CPTs-based treatment. Since KIF4A seemed to be involved in cellular response to CPTs, we examined the value of KIF4A expression as a predictive marker for CPT-11 response in advanced CRC patients. We found no association between KIF4A expression levels in tumours and patient overall survival or time to progression after CPT-11-based therapy administration (Fig. 47); hence, we should discard KIF4A as a molecular marker to predict patient response to Irinotecan (CPT-11).

Contrary to CPT-11 prediction outcome, high expression of KIF4A predicts poor overall survival in advanced CRC patients (Fig. 46). Data obtained in this study also suggests an active role of KIF4A in the metastatic process, as it is found more expressed in metastatic cell lines (Fig. 29) an its ectopic expression favours cell invasion *in vitro* (Fig. 42).

Others had previously linked KIF4A expression to poor overall survival in non-small cells lung cancer patients (96). The exact mechanism underlying this effect of KIF4A expression in advanced tumours opens an exciting line of investigation and possibly new treatment strategies for these patients.

Conclusions

1. The condensin complex and the molecular motor KIF4A are significantly up-regulated in CRC.
2. SMC2 is a novel transcriptional target of Wnt/β-catenin.
3. TCF-4 binding element TBE3 in SMC2 promoter is responsible for Wnt/β-catenin transcriptional activity.
4. KIF4A expression is cell cycle regulated and tightly linked to proliferative phenotypes.
5. KIF4A is under Wnt/β-catenin regulation, independently from the transcriptional factor TCF-4.
6. KIF4A depletion impairs cell proliferation, cell anchorage-independent growth and cell invasion of colon cancer cell lines.
7. KIF4A depletion impairs cell differentiation *in vitro*.
8. KIF4A ectopic expression promotes cell invasion *in vitro*.
9. KIF4A expression is associated to poor overall survival in advanced CRC patients.
10. KIF4A modulates the cellular response to Topoisomerase I inhibitors, but it is not a useful molecular marker for patient response prediction to Irinotecan-based treatments.

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Summary in Spanish

Antecedentes

Los tumores colorectales se caracterizan por su alta inestabilidad genómica, principalmente de dos tipos: la inestabilidad de microsatélites y la inestabilidad cromosómica. Los microsatélites son elementos presentes en el ADN caracterizados por la repetición en tándem de secuencias cortas de nucleótidos. El defecto en los mecanismos de reparación de errores por deslizamiento (*mismatch repair* o MMR) da lugar a la aparición de multitud de alteraciones en estas secuencias repetidas. Esto ocurre aproximadamente en un 15 % de tumores. En el 85 % restante, tiene lugar la inestabilidad cromosómica, que se caracteriza por la pérdida o ganancia de grandes elementos cromosómicos, resultando en importantes aneuploidias de las células tumorales. Estos tumores se caracterizan, a su vez, por presentar alteraciones de la vía de señalización Wnt, fundamental en el mantenimiento de la homeóstasis intestinal.

El epitelio intestinal humano se renueva completamente aproximadamente cada semana. Esta alta demanda requiere la eficiente actividad de las células madre intestinales, que residen en el fondo de las criptas colónicas. Esta zona de la cripta se halla bajo la influencia de los ligandos Wnt, que estimulan la proliferación de las células madre intestinales. A medida que se asciende hacia el lumen intestinal, los ligandos Wnt disminuyen en concentración y las células entran en diferenciación para dar lugar a los distintos tipos celulares del epitelio intestinal: células absortivas o enterocitos, células de Paneth, células globet (secretoras de mucus) y células enteroendocrinas, repoblando así la mucosa intestinal.

Cuando un ligando Wnt se une a los receptores de membrana heterodiméricos (formados por una proteína LPR5/6 y un receptor de la familia Frizzled) en la célula diana se produce la inhibición del complejo de degradación de β-catenina (formado por APC, GSK3-β, CKIα y Axin), ésta se acumula en el citoplasma y se trasloca al núcleo, donde se une a factores de transcripción TCF/LEF y activa la transcripción de genes relacionados con la proliferación celular, como C-MYC o ciclina-D.

La activación aberrante de esta vía de señalización, comúnmente por mutación en las proteínas APC o β-catenina, da lugar a células hiperproliferativas, resultando en la generación de focos de criptas aberrantes y posteriomente adenomas. La mutación adicional en otros genes, como por ejemplo, KRAS, p53 o TFG-β, da como resultado la progresión de adenoma a carcinoma in situ, carcinoma invasivo y metástasis, sucesivamente.

Datos previos de nuestro laboratorio indicaban que el complejo condensina podría estar bajo la influencia de la vía Wnt. El complejo condensina es esencial para el buen reparto de la información genética en las dos células hijas en la división celular, ya que es el principal encargado de dar estructura y estabilidad a los cromosomas. Este complejo está formado por dos proteínas que forman el núcleo y pertenecen a la familia SMC, SMC2 y SMC4, y junto a ellas, se encuentran proteínas reguladoras no-SMC (NAPG, NACPD2 y NCAPH, en el complejo condensina tipo I; y NCAPG2, NCAPD3 y NCAPH2, en el tipo II).

La alteración en la función de este complejo da lugar a graves aberraciones cromosómicas y aneuploidas, similares a las observadas en los tumores colorrectales.

Junto con el complejo condensina, la kinesina KIF4A es también esencial para el mantenimiento estructural de la cromatina en interfase, así como la condensación de los cromosomas y la citokinesis durante la división celular.

La desregulación de estas proteínas podría estar bajo la inestabilidad cromosómica observada en aquellos tumores en los que la vía Wnt se encuentra desregulada, es decir, en el aproximadamente en el 85% de los tumores colorrectales.

Objetivos

-Estudiar los niveles de expresión en el cáncer colorrectal de las proteínas implicadas en el mantenimiento cromosómico, tanto el complejo condensina como la kinesina KIF4A.

-Estudiar la posible regulación transcripcional de estos complejos dependiente de la vía de señalización Wnt.

-Estudiar el papel que podría estar ejerciendo la kinesina KIF4A en el proceso tumorogénico intestinal.

Resultados

El complejo condensina y la kinesina KIF4A están sobreexpresados en el cáncer colorectal

En una primera aproximación, la expresión distintas subunidades del complejo condensina y la kinesina *KIF4A* fue evaluada en muestras de pacientes con cáncer colorectal mediante PCR a tiempo real en parejas de muestras normal-tumor. Se observó que tanto el complejo condensina como *KIF4A* se encuentran significativamente sobreexpresados en las muestras tumorales un alto número de casos. Esta sobreexpresión se confirmó además en un estudio paralelo consistente en la medición global del transcriptoma en 122 pacientes de Finlandia y Dinamarca. Asímismo, se comprobó que estos genes también están activados en muestras tumorales a nivel proteico, al menos en el caso de SMC2, SMC4, NCAPH y KIF4A.

Se encontró una fuerte correlación positiva entre los niveles de expresión de todos los genes estudiados. Sin embargo, no se encontró asociación con ninguna característica clínico-patológica de los pacientes evaluados.

Regulación transcripcional del complejo condensina: Influencia de la vía de señalización Wnt.

En las tinciones inmunohistoquímicas de SMC2, KIF4A y NCAPH, se observó un patrón de tinción en las criptas intestinales característico de los genes diana de la vía Wnt; es decir, genes que están altamente expresados en la base de las criptas colónicas, y que muestran una disminución en gradiente a medida que nos aproximamos al lumen intestinal.

Debido a este patrón de tinción, se investigó si el complejo condensina y KIF4A podrían estar bajo la influencia de la vía Wnt, responsable del mantenimiento de la homeóstasis intestinal y frecuentemente hiperactivada en los tumores colorrectales.

En primer lugar, se observó que la expresión proteica de SMC2 y SMC4 , componentes del núcleo del complejo condensina y β-catenina, regulador central de la vía Wnt, correlacionan positivamente, tanto en muestras procedentes de pacientes como en líneas celulares. Además, la expresión en tumores de SMC2 y NCAPH está fuertemente asociada a la localización nuclear de β-catenina.

Datos previos del laboratorio mostraban que la expresión de SMC2 y SMC4 disminuye en modelos celulares de inhibición de la vía Wnt; estos modelos consisten en células colorectales en las que la adición de doxyciclina induce la expresión de un siRNA dirigido contra β-catenina o bien la inducción de una variante dominante-negativa del factor de transcripción TCF-4. Adicionalmente, habíamos observado mediante inmunoprecipitación de cromatina (ChIP) que TCF-4 se une a la secuencia promotora de SMC2 *in vivo*.

Para elucidar si la vía Wnt podría activar la transcripción génica de *SMC2*/*SMC4* de forma directa, se llevaron a cabo estudios *in silico* para localizar posibles sitios de unión a TCF-4 (TBE) en el promotor de SMC2 y SMC4. En el promotor de SMC2 se encontraron 6 posibles sitios de unión, dos de los cuáles se encuentran muy próximos al inicio de transcripción del gen y están altamente conservados evolutivamente. En el promotor de SMC4 no se encontraron TBEs evolutivamente conservadas, por ello se centró el estudio en SMC2.

Estudios reporteros con luciferasa nos permitieron establecer que tanto β–catenina como TCF-4 son capaces de transactivar el promotor de *SMC2*. Mediante delecciones seriadas del promotor pudimos establecer que la secuencia mínima que mantiene la actividad promotora contiene 5 de los 6 TBEs identificados previamente. Mediante mutagénesis dirigida en esa secuencia mínima promotora fuimos capaces de identificar exactamente el TBE responsable de la actividad basal del promotor de *SMC2* y de la transactivación dependiente de β-catenina y TCF-4. Este TBE se encuentra a -20 pb del inicio de transcripción, y uno de los evolutivamente mejor conservados en el promotor de SMC2.

Regulación transcripcional de KIF4A: papel de la vía Wnt.

Diversos datos apuntan que la expresión de KIF4A está íntimamente relacionada con la proliferación celular. Tanto a nivel de ARNm como proteína, su expresión varía según la fase del ciclo celular: su máximo de expresión tiene lugar al principio de la fase S y presenta un pico de transcripción en la fase G2/M. Mediante técnicas inmunohistoquímicas pudimos comprobar que se encuentra altamente expresado en el compartimento proliferativo de las criptas intestinales, correlacionando su expresión con el marcador proliferativo Ki67. En líneas celulares colorrectales, su expresión correlaciona positivamente con el marcador proliferativo PCNA. Asimismo, KIF4A disminuye drásticamente en los procesos de diferenciación enterocítica, tanto en aquellos inducidos por butirato de sodio como en aquellos inducidos por confluencia en las líneas Caco 2-Bbe y HCT8.

La expresión de KIF4A en tumores correlaciona además con los niveles de expresión de β–catenina. Asimismo, los mayores niveles de KIF4A se encuentran en aquellos tumores que presentan localización nuclear de β–catenina. En modelos celulares de inhibición de a vía Wnt, KIF4A disminuye tras la expresión de la forma dominante negativa de TCF-4, si bien la inhibición es más significativa cuando se bloquea mediante siRNA la expresión de β–catenina.

Para elucidar si la regulación de *KIF4A* dependiente de Wnt es directa o indirecta, se aisló el promotor de *KIF4A*, que contenía tan sólo una TBE evolutivamente conservada. Ensayos reporteros de luciferasa demostraron que la actividad del promotor de KIF4A aumenta cuando la vía Wnt es estimulada mediante cloruro de litio o mediante la expresión ectópica de β-catenina. Sin embargo, la sobreexpresión de TCF-4 no afecta a la actividad del promotor. Incluso cuando se inhibe la vía Wnt, el promotor de KIF4A se ve afectado tras la depleción de β-catenina, pero no ante el bloqueo de la acción de TCF-4. Estos resultados nos llevaron a concluir que *KIF4A* está regulado por β-catenina a nivel transcripcional, pero independientemente del factor TCF-4.

Papel de KIF4A en la tumorogénesis colorrectal

Se generaron líneas celulares colorrectales isogénicas en las que la expresión de KIF4A está inhibida. Pudimos comprobar que en ausencia de KIF4A el ciclo celular se ve afectado (más proporción de células en interfase y menos en fase S), las células proliferan menos, tienen menor capacidad de crecimiento en sustrato semi-sólido y menor capacidad invasiva. Por el contrario, la expresión ectópica de KIF4A en células HEK293T no altera el ciclo celular, pero aumenta la capacidad invasiva de las células, probablemente induciendo cambios del tipo “transición epitelio-mesénquima”, ya que la expresión de los represores transcripcionales de la familia Snail se ve aumentada.

Por otro lado, hay datos que apuntan a que KIF4A podría estar directamente implicado en los procesos de diferenciación, ya que las células donde se ha inhibido la expresión de KIF4A muestran menores niveles de diferenciación, ya sea inducida por butirato de sodio o por la expresión forzada de los factores inductores de polarización STRAD/LKB1.

Se estudió además cómo influían los niveles de KIF4A en la respuesta a inhibidores de topoisomerasa I (CPT y CPT-11), ya que KIF4A participa en la respuesta a daño a ADN. Estos agentes son regularmente utilizados en el tratamiento del cáncer colorrectal y su mecanismo de acción se basa en la inducción de roturas de doble cadena en el ADN.

Comprobamos que las células en las que la expresión de KIF4A está inhibida son más resistentes a CPT y CPT-11. En el caso de la línea DLD-1, se comprobó además que esta mayor resistencia puede deberse a que las células entran menos en apoptosis, de acuerdo con los menores niveles de células haplo-diploides observados y una menor expresión del marcador apoptótico *cleaved*-PARP-1 tras el tratamiento con el análogo de CPT utilizado en clínica, Irinotecan.

KIF4A como marcador de pronóstico en pacientes de cáncer colorrectal

Dado que los resultados obtenidos *in vitro* indicaban que KIF4A podría ejercer un papel en el proceso tumorogénico, especialmente en la invasión celular y la respuesta a inhibidores de topoisomerasa I, se

evaluó si KIF4A podría ser un nuevo marcador molecular de pronóstico en cáncer colorrectal. Para ello, se midió la expresión de KIF4A mediante tinción inmunohistoquímica en un *array* de tejidos de 91 tumores de pacientes con enfermedad avanzada. En 82 de los pacientes se conocía además su evolución clínica tras la administración de quimioterapia basada en Irinotecan (CPT-11).

Se descubrió que aquellos pacientes con altos niveles de KIF4A tienen peor pronóstico, en términos de supervivencia total desde el momento del diagnóstico, particularmente en los diagnosticados en estadío IV. Este resultado, ligado a la mayor expresión de KIF4A en líneas celulares metastásicas y a la inducción de los factores Snail tras su expresión ectópica en células HEK293T, nos llevan a hipotetizar que KIF4A puede estar ejerciendo un papel activo en los procesos metastásicos.

Sin embargo, no observamos asociación alguna entre la expresión de KIF4A y la respuesta a Irinotecan, ni en términos de supervivencia total ni en el tiempo de progresión del tumor desde el inicio del tratamiento con Irinotecan.

Discusión

La vía de señalización Wnt es vital para el mantenimiento de la homeostasis intestinal, promoviendo la división de las células madre intestinales, residentes en el fondo de las criptas de Lieberkühn, para sostener la continua regeneración del epitelio intestinal.

Hemos demostrado que el complejo condensina y KIF4A se hallan bajo la influencia de esta vía de señalización, bien directamente mediando la transcripción génica de SMC2 por el tándem β–catenin/TCF-4, o bien indirectamente como en el caso de KIF4A y presumiblemente el resto de las subunidades del complejo condensina, ya que la expresión de todos los miembros del complejo está íntimamente relacionada. Por tanto, la activación aberrante de la vía Wnt podría contribuir activamente a la sobreexpresión de estas proteínas presente en los tumores colorrectales.

El claro papel mitogénico de esta vía de señalización explica su influencia sobre proteínas que participan activamente en la división celular. Sin embargo, queda por resolver si estas proteínas son "pasajeros" del proceso tumoral o contribuyentes activos de la transformación celular. La inhibición de estos genes conlleva la pérdida de viabilidad celular, hecho que nosotros hemos observado y también otros autores. En el caso particular de KIF4A, su inhibición provoca un enlentecimiento de la progresión del ciclo celular, que se traduce en tasas de proliferación más lentas. Además, la depleción de KIF4A provoca una menor eficiencia de crecimiento celular en sustratos semi-sólidos y menos capacidad invasiva. No sabemos con certeza qué efecto puede tener la sobreexpresión del complejo condensina en la progresión tumoral; sin embargo, en el caso de KIF4A, su sobreexpresión podría estar favoreciendo la invasión celular y, por tanto, la aparición de metástasis distantes, a juzgar por los estudios preliminares de expresión ectópica de KIF4A *in vitro*. Apoyando esta hipótesis, hemos observado que aquellos pacientes en estadío IV con altos niveles de KIF4A muestran menor tasa de supervivencia.

Hemos observado también que KIF4A podría estar implicada activamente en mecanismos de diferenciación celular; su expresión disminuye durante el proceso de diferenciación pero su presencia parece ser importante para el proceso, al menos en su inicio.

Con respecto a la modulación de la respuesta a daño a ADN, la depleción de KIF4A confiere mayor resistencia celular a los tratamientos con inhibidores de TOP1. La depleción de proteínas implicadas en la reparación de ADN mediante recombinación homóloga, como es el caso de KIF4A, suelen conferir mayor sensibilidad a este tipo de drogas; sin embargo nosotros observamos lo contario. Probablemente la función de KIF4A en este proceso es más compleja de lo esperado inicialmente, y los datos indican que también podría estar implicada en la señalización a apoptosis, ya que en ausencia de KIF4A los niveles de apoptosis son menores tras el tratamiento con Irinotecan.

En resumen, la sobre-activación del complejo condensina y KIF4A en el tumor podría provocar una división celular aberrante, en forma de inestabilidad genómica y generación de aneuploidias. Sin embargo, no se descartan otras funciones interfásicas como responsables de su contribución en la tumorogénesis. Entre estas funciones se encuentran: el mantenimiento de la estructura tridimensional de la cromatina en interfase, la regulación de la expresión génica, la diferenciación celular y la participación en la reparación de daño en el ADN.

Este estudio abre nuevas e interesantes líneas de investigación, incluyendo posibles alternativas terapéuticas, basadas en la inhibición de estos complejos indispensables en la división celular.

Conclusiones

1. Tanto el complejo condensina como la kinesina KIF4A se encuentran sobreexpresadas en el cáncer colorrectal.
2. SMC2, componente del núcleo del complejo condensina, es una diana transcripcional directa de la vía de señalización Wnt/β-catenina.
3. El elemento de unión a TCF-4, TBE3, del promotor de SMC2 es la única entidad responsable de la regulación dependiente de Wnt//β-catenina.
4. KIF4A está regulada a nivel transcripcional por β-catenina, regulador central de la vía Wnt, aunque independientemente del factor de transcripción TCF-4.
5. La inhibición de la expresión de KIF4A *in vitro* afecta la tasa proliferativa celular, su capacidad invasiva y su potencial de diferenciación.
6. La expresión ectópica de KIF4A promueve la capacidad celular invasiva.
7. KIF4A modula la respuesta celular a los inhibidores de Topoisomerasa I, que inducen daño al ADN.
8. KIF4A podría ser empleado como marcador molecular de mal pronóstico en estadíos avanzados de cáncer colorrectal. Sin embargo, sus niveles de expresión en el tumor no se asocian con la respuesta a Irinotecan.

Appendix

Publication:

Human SMC2 Protein, a Core Subunit of Human Condensin Complex, Is a Novel Transcriptional Target of the WNT Signalling Pathway and a New Therapeutic Target.

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Abstract:

Human SMC2 is part of the condensin complex, which is responsible for tightly packaging replicated genomic DNA prior to segregation into daughter cells. Engagement of the WNT signalling pathway is known to have a mitogenic effect on cells, but relatively little is known about WNT interaction with mitotic structural organizer proteins. In this work, we described the novel transcriptional regulation of SMC2 protein by direct binding of the β–catenin/TCF4 transcription factor to the SMC2 promoter. Furthermore, we identified the precise region in the SMC2 promoter that is required for β-catenin-mediated promoter activation. Finally, we explored the functional significance of down-regulating SMC2 protein *in vivo*. Treatment of WNT-activated intestinal tumor cells with SMC2 siRNA significantly reduced cell proliferation in nude mice, compared with untreated controls ($p=0.02$). Therefore, we propose that WNT signalling can directly activate SMC2 transcription as a key player in the mitotic cell division machinery. Furthermore, SMC2 represents a new target for oncological therapeutic intervention.

Summary in Spanish:

SMC2 forma parte del complejo condensina, encargado del empaquetamiento del ADN replicado en forma de cromosomas, facilitando el reparto de la información genética en las dos células hijas durante la división celular. La activación aberrante de la vía WNT tiene un claro papel mitogénico en las células tumorales; sin embargo, no se conoce con claridad el papel de esta vía en la regulación de los organizadores estructurales mitóticos, como es el caso del complejo condensina. En este estudio, describimos como el miembro SMC2 del complejo condensina está regulado directamente a nivel transcripcional por el complejo β–catenina/TCF-4. Además, se identifica el elemento responsable de esta regulación en el promotor de *SMC2*. Asimismo, se investiga el significado funcional de la inhibición de *SMC2* en células tumorales *in vivo*. En células tumorales donde la vía WNT está hiperactivada, la inhibición de SMC2 reduce de forma significativa su proliferación en ratones inmunodeprimidos en comparación con los correspondientes controles (p= 0.02). En resumen, se propone que la vía WNT regula directamente la transcripción de *SMC2* como componente de la maquinaria mitótica y que la inhibición de éste podría utilizarse en un futuro como estrategia terapéutica en el cáncer colorrectal.

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Human SMC2 Protein, a Core Subunit of Human Condensin Complex, Is a Novel Transcriptional Target of the WNT Signaling Pathway and a New Therapeutic Target*[S]

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Background: Condensin SMC proteins are frequently overexpressed in WNT-activated hyperplastic cells.
Results: The SMC2 promoter is a novel target on the β-catenin·TCF4 transcription complex.
Conclusion: β-Catenin·TCF4 may drive production of condensin in hyperplastic cells. SMC2 is required to ensure cellular mitosis and fast proliferation.
Significance: Down-regulation of SMC2 expression can repress cell proliferation in WNT-activated cells and represents a new therapeutic target in cancer treatment.

Human SMC2 is part of the condensin complex, which is responsible for tightly packaging replicated genomic DNA prior to segregation into daughter cells. Engagement of the WNT signaling pathway is known to have a mitogenic effect on cells, but relatively little is known about WNT interaction with mitotic structural organizer proteins. In this work, we described the novel transcriptional regulation of SMC2 protein by direct binding of the β-catenin·TCF4 transcription factor to the *SMC2* promoter. Furthermore, we identified the precise region in the *SMC2* promoter that is required for β-catenin-mediated promoter activation. Finally, we explored the functional significance of down-regulating SMC2 protein *in vivo*. Treatment of WNT-activated intestinal tumor cells with *SMC2* siRNA significantly reduced cell proliferation in nude mice, compared with untreated controls ($p = 0.02$). Therefore, we propose that WNT signaling can directly activate *SMC2* transcription as a key player in the mitotic cell division machinery. Furthermore, SMC2 represents a new target for oncological therapeutic intervention.

SMC (structural maintenance of chromosomes) proteins are a family of DNA-binding ATPases that are essential for maintenance of chromosomal integrity during cell division (1). Eukaryotes express at least six SMC proteins (SMC1–6), which form three heterodimers (SMC1/3, SMC2/4, and SMC5/6 (2)). SMC5/6 is part of a complex involved in DNA repair and checkpoint responses. The SMC1/3 heterodimer associates with two regulatory non-SMC proteins, SCC1 and SCC3, and collectively, this complex is known as cohesin. Cohesin holds sister chromatids together until they are physically segregated during anaphase (3). The SMC2/4 heterodimer associates with three non-SMC proteins to form a five-member complex known as condensin. Lower eukaryotes have a single condensin complex, but metazoans have two. In humans, both condensin I and condensin II contain the core SMC2/4 subunits, but have different regulatory non-SMC subunits. As the name suggests, condensin has DNA supercoiling activity, which is essential for packaging of chromatin prior to cell division. Condensin has also been shown to be necessary for resolution of sister chromatids during anaphase (4, 5). Condensin supercoiling activity is spatially and temporally regulated by mitotic kinases (6–10), which ensure DNA condensation only occurs at appropriate stages of the cell cycle.

Mutations in condensin subunits are likely to drive chromosomal destabilization and are found in some cancer genomes (11, 12). Furthermore, activated WNT signaling in colorectal tumors are considered to cause chromosomal instability. Upon investigation of normal human intestine and colorectal tumor samples, we noted that high SMC2 protein expression appeared to coincide with nuclear β-catenin localization in dividing cells. Therefore, we decided to investigate whether WNT signaling and β-catenin might transcriptionally regulate condensin.

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[S] This article contains supplemental Tables 1 and 2 and Figs. S1–S6.
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Secreted WNT ligands are essential morphogens that control patterning and cell division during embryogenesis (13). WNT signals are principally transduced by two classes of cell surface receptors; Frizzled (Fz) proteins and low density lipoprotein receptor-related proteins 5 and 6 (LRP5/6). In canonical, β-catenin-dependent signaling, phosphorylation of LRP6 leads to release of cyctoplasmic β-catenin from the prodegradatory Axin complex (which includes glycogen synthase 3 and adenomatous polyposis coli (APC)[3] protein). Free β-catenin translocates to the nucleus, where it acts as a transcriptional coactivator of target genes in combination with TCF/LEF transcription factors (14, 15).

WNT signaling is well known to promote cell cycle progression by up-regulating proliferation-stimulating target genes *e.g.* cyclin D and c-*myc*. However, it has become apparent that the cell cycle and WNT signaling are intrinsically linked (16). In a seminal study, WNT-β-catenin signaling (and β-catenin protein levels) were noted to oscillate during the cell cycle, peaking at the G_2/M transition (17). Since that initial observation, many of the components of the WNT pathway have been shown to play an integral role during cell division. In addition to their function as activators of WNT target gene transcription, APC protein and β-catenin are important constituents of the centrosome complex (18–20). β-Catenin is also essential for centrosomal separation at the onset of spindle formation (21). Moreover, glycogen synthase 3 binds to and regulates microtubules, thereby contributing to mitotic spindle alignment (22).

For WNT-stimulated cells to undergo mitotic division, the genome must be faithfully replicated and packaged up prior to cytokinesis. By definition, this is a complex and highly regulated process, and failure to control each stage can lead to aneuploidy, chromosomal instability, and/or cell death. Chromosomal architecture during cell division is maintained in part by SMC proteins, and in this study, we provide evidence that canonical WNT signaling is directly driving SMC2 expression and that depleting tumor cells of SMC2 effectively drives a tumor xenograft model into mitotic catastrophe. Therefore, modulating cellular levels of condensin subunits may provide a novel chemotherapeutic tool for controlling the rate of cell division and/or critically destabilizing chromosomal organization.

EXPERIMENTAL PROCEDURES

Human Cancer Cell Lines and Cell Culture—Colorectal cancer (CRC) cell lines were purchased from the American Type Culture Collection (ATCC). Ls174T/dnTCF4 and Ls174T/pTER-β-catenin cells were kindly provided by Prof H. Clevers (Hubrecht Institute, Utrecht, The Netherlands). Cell lines were cultured in DMEM or RPMI 1640 (Ls174T variants) medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C under 5% CO_2. To induce dnTCF4 or siRNA-*BCAT*, Ls174T cells were treated with 5 μg/ml doxycycline. Doubling time calculations were performed as described by Bex *et al.* (23).

Colorectal Tissue Samples—Tumor and counterpart normal samples were provided and analyzed by the Surgery and Pathology Departments of the Vall d'Hebron Universitary Hospital (Barcelona, Spain) respectively. Patients gave written consent before their inclusion in the analysis, and the study was approved by the Hospital Ethics Committee.

DNA Reagents—pTOPFLASH and pFOPFLASH plasmids were generously provided by Prof H. Clevers (24). *VP16-TFC4* and p*BCAT* expression vectors were kindly supplied by Antonio García de Herreros (IMIM-Hospital del Mar, Barcelona, Spain). *SMC2* promoter regions were amplified by PCR using the pairs of primers listed in supplemental Table 1. The products were directionally cloned in pGL3-basic vector (Promega) using KpnI and BglII restriction sites. Substitution mutants affecting the TCF4-binding sites on *SMC2* promoter regions were generated with mutagenic oligonucleotides in supplemental Table 1 using QuikChange II XL site-directed mutagenesis kit (Stratagene). All constructs were confirmed by DNA sequencing under Big Dye™ cycling conditions on an Applied Biosystems 3730xl DNA Analyzer (Macrogen, Inc.).

RNA Extraction and Real-time PCR—Total RNA was extracted with Trizol® (Invitrogen) and further treated with DNase I amplification grade (Invitrogen) and retrotranscribed using a High Capacity cDNA reverse transcription kit (Applied Biosystems). Real time PCR reactions were performed in triplicate on an ABI PRISM 7500 real-time system (Applied Biosystems), using TaqMan gene expression assays (Applied Biosystems, catalog no. Hs00374522_m1, Hs00197593_m1, Hs00254617_m1, Hs00214861_m1, and Hs00379340_m1) according to the manufacturer's instructions. Data were normalized to 18 S rRNA (catalog no. 4333761F) expression but also confirmed with other endogenous controls: peptidylprolyl isomerase A (cyclophilin A) (catalog no. 4333763T) or β-actin (catalog no. 4333762T). The relative mRNA levels were calculated using the comparative C_t method ($2^{-\Delta\Delta Ct}$) as described by Arango *et al.* (25).

Protein Extraction and Western Blotting (WB)—Cell pellets and tissue homogenates were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM DTT, 1 mM sodium orthovanadate, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS) containing complete protease inhibitor mixture (Roche Diagnostics). Proteins in the crude lysates were quantified using the BCA protein assay (Pierce Biotechnology), and 50 μg of whole-cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose filters. Blots were probed using antibodies against SMC2 (ab10412, Abcam; and 07-710, Upstate-Millipore, dilution factor of 1:1000), SMC4 (ab17958, Abcam, dilution factor of 1:1000), TCF4 (05-511, Upstate-Millipore, dilution factor, 1:500), NCAPH (HPA003008, Sigma Aldrich, dilution factor, 1:2000), β-catenin (610154, BD Transduction Laboratories, dilution factor, 1:1000) or c-Myc (monoclonal 9E10, sc-40, Santa Cruz Biotechnology, 1:100). Proteins were detected using corresponding HRP-conjugated secondary antibodies, anti-mouse (P0447, Dako), or anti-rabbit (P0217, Dako). Actin was used as loading control (CP01, Calbiochem, 1:5000). The intensity of the bands on the blots was quantified using the GeneTools Program (SynGene).

[3] The abbreviations used are: APC, adenomatous polyposis coli; dnTCF4, dominant-negative transcription factor 4; CRC, colorectal cancer; WB, Western blot; TBE, TCF-binding element; NCAPH, non-SMC condensin I complex, subunit H.

β-Catenin·TCF4 Transcription Complex Drives SMC2 Expression

Immunohistochemistry—Paraffin-embedded tissues were provided by the archive tumor bank of the Department of Pathology of the Vall d'Hebron Universitary Hospital. Epitope retrival was heat induced in citrate buffer, pH 6.0. Immunohistochemistries were performed using EnVision + Dual Link System-HRP, DAB+ (Dako) according to the manufacturer's instructions, using the SMC2 antibody (ab10412, Abcam, 1:200), NCAPH antibody (HPA003008, Sigma Aldrich, dilution factor, 1:50), and β-catenin (610154, BD Transduction Laboratories, dilution factor, 1:*X*). Samples were additionally counterstained with hematoxilin. Anti-SMC2 antibody (ab10412) specificity was confirmed by immunocytochemistry of wt *versus* SMC2-depleted DLD-1 human colorectal cancer cells (supplemental Fig. 1).

Chromatin Immunoprecipitation (ChIP)—Cells were grown to 80% confluency in 15-cm dishes. Proteins and nucleic acids were cross-linked with formaldehyde (1%) for 10 min at 4 °C. Cross-linking was quenched by adding 125 mM glycine for 5 min. Following two washes with cold PBS containing protease inhibitors, cells were collected and resuspended in SDS lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS). Lysates were sonicated 12× for 10 s (60-s interval on ice between pulses) at 8 Å on a Soniprep 150 (MSE, Ltd., Kent, U.K.). Chromatin samples were diluted with chromatin immunoprecipitation buffer (20 mM Tris-HCl, pH 8, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) supplemented with protease inhibitors. Samples were precleared for 2 h at 4 °C with protein G-agarose/salmon sperm DNA beads (Upstate-Millipore) and incubated with 5 μg of the appropriate antibody overnight at 4 °C. Immunoprecipitation was carried out with protein G-agarose/salmon sperm DNA beads for 2 h at 4 °C. DNA·protein·antibody·bead complexes were washed out with low salt buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), high salt buffer (500 mM NaCl, 20 mM Tris-HCl, pH 8, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), LiCl buffer (250 mM LiCl, 10 mM Tris-HCl, pH 8, 1 mM EDTA, 1% Igepal, 1% sodium deoxycholate), and TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). Proteins were eluted with elution buffer (100 mM $NaHCO_3$, 1% SDS). Cross-linking was reversed incubating samples with 200 mM NaCl overnight at 65 °C. Before DNA purification (phenol-chloroform-isoamilic alcohol), proteins were digested with 20 μg of proteinase K (Roche Diagnostics) for 2 h at 45 °C. Immunoprecipitated DNA was used as template in the PCR reactions. The primers are listed in supplemental Table 1.

Luciferase Reporter Assays—Cells were transiently co-transfected with pGL3-basic-*SMC2* promoter (1 μg/10^6 cells) alone or in combination with *VP16-TCF4* (3 μg/10^6 cells) or *pBCAT* (3 μg/10^6 cells) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. *pRL-TK Renilla* (0.2 μg/10^6 cells) was introduced in all samples to allow data normalization. pTOPFLASH and pFOPFLASH were used as positive and negative luciferase reporter controls, respectively. 24 h post-transfection, cells were lysed, and luciferase activity was measured according to the Dual-Luciferase reporter assay using a Clarity Luminescence Microplate Reader (BioTek Instruments).

SMC2 Knockdown—Cells were transiently transfected with 20 μM siRNA using HiPerfect Transfection Reagent (Qiagen®) according to the manufacturer's instructions. *SMC2* and scrambled siRNA were purchased from Qiagen® (catalog no. SI02654260 and 1027281, respectively). Cells used in the xenograft assays were cultured for 48 h and subjected to a second round of transfection. For stable knockdown, cells were transduced with lentiviral particles containing five different shRNAs targeting *SMC2* (MISSION shRNA, Sigma-Aldrich, clone IDs NM_006444.1-3720s1c1, -1295s1c1, -1961s1c1, -3173s1c1, and -3300s1c1) prior to puromycin selection.

Assessment of Cell Cycle Profile—Cells transiently silenced for 24, 48, 72, or 96 h were trypsinized, washed with cold PBS, fixed with 70% ethanol, and stained with propidium iodide (40 μg/ml). DNA content was assessed using a FACSCalibur instrument and CellQuest software (BD Biosciences).

Xenograft Study—Female athymic nude mice (Hsd:athymic nude-Foxn1 nu/nu; Harlan Interfauna Iberica) were maintained in pathogen-free conditions and used at 5–6 weeks of age. Animal care was handled in accordance with the Guide for the Care and Use of Laboratory Animals of the Vall d'Hebron Hospital Animal Experimentation Ethical Committee. 1.5×10^6 silenced DLD1 cells were injected subcutaneously in the rear flanks of mice. Tumor growth was monitored three times per week for 5 weeks by conventional caliper measurements (tumor volume = $D \times d^2/2$, where D is the major diameter and d is the minor diameter).

Statistical Analysis—Unless stated differently, descriptive data were expressed as mean ± S.D. The GraphPad Prism statistical package was used to investigate group differences by unpaired Student's *t* test. *p* values are indicated for statistically different means.

RESULTS

The Core Subunit of Human Condensin Complex, SMC2, Is Overexpressed in CRC—SMC2 protein expression was evaluated in clinical samples from 29 patients that had undergone surgery for colon carcinoma. Protein detection by WB showed that SMC2 was up-regulated in 20 of the 29 tumor samples (69%) compared with the matched normal controls (subset shown in Fig. 1*A*). SMC2 overexpression in CRC was further confirmed by quantitative PCR of 16 clinical samples, showing also a clear up-regulation of SMC2 in the tumor counterpart samples in 11 cases (68.5%) (Fig. 1*B*). As SMC4 is the natural partner in the core of the condensin complex, its levels were also studied in 27 clinical samples and found to be overexpressed in 13 tumor counterparts (48.1%) (Fig. 1*C*). Further analysis of non-SMC subunits in patient samples confirmed the trend for increased expression of all condensin complex members in tumor samples *versus* normal tissue (supplemental Fig. 2). A strong positive correlation between the protein levels of SMC2 and SMC4 in clinical samples and also in CRC cell lines was identified (Fig. 1*E*). Interestingly, levels of SMC2/SMC4 protein negatively correlated with population doubling times in CRC cell lines (supplemental Fig. 3). Nevertheless, neither SMC2 nor SMC4 overexpression could be correlated to any clinicopathological variables (age, sex, tumor stage, or tumor location; supplemental Table 2) in the clinical samples studied.

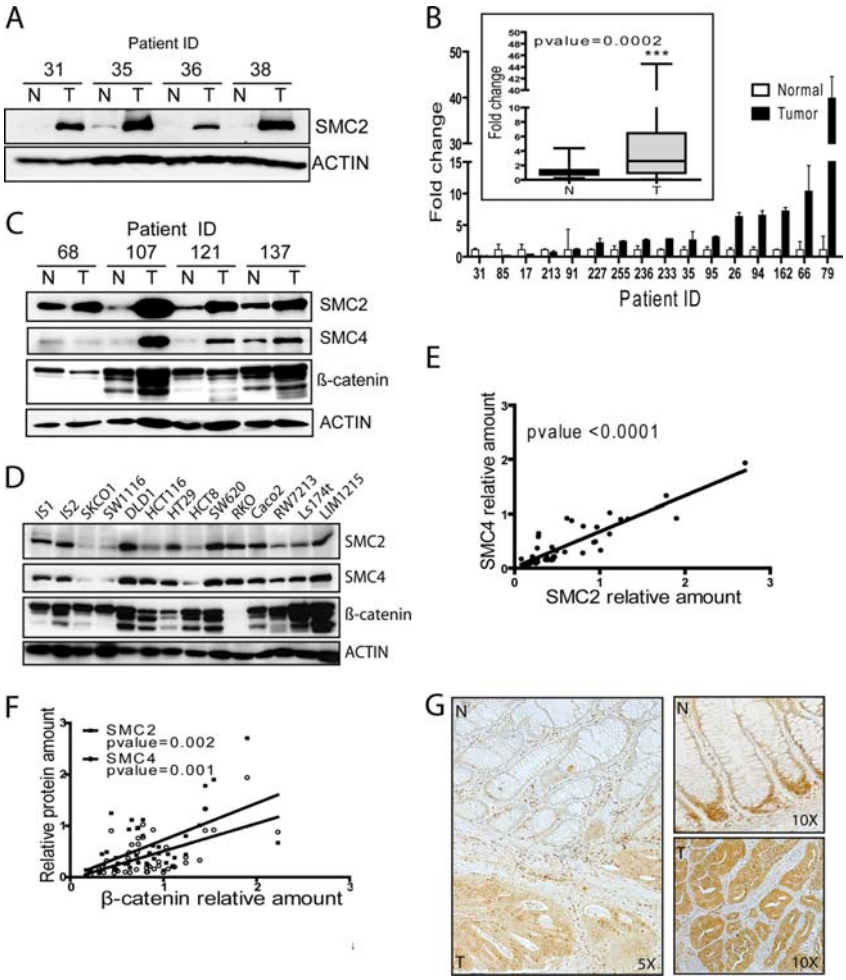

FIGURE 1. **SMC2 is up-regulated in human CRC.** *A*, WB analysis of SMC2 in human CRC. A representative subset of 29 cases studied is shown. Actin was used as loading control. *B*, quantitative real-time PCR for *SMC2* in 16 pairs of colon adenocarcinoma tumors and matched adjacent normal colonic tissues. Data are representative of three independent experiments. The mean values of *SMC2* levels were compared using Student's *t* test (*upper boxplot*). *C* and *D*, SMC2, SMC4, and β-catenin levels were evaluated by WB in both colorectal cancer cell lines ($n = 14$) and samples from CRC patients ($n = 27$, a representative subset is shown). Actin was used as loading control. *E* and *F*, SMC2, SMC4, and β-catenin protein levels on WB were determined by gel band quantification and normalized to the corresponding actin levels. Values were used to perform correlation studies following Spearman test. *G*, immunohistochemistry of SMC2 in paraffin-embedded tissue. A representative specimen is shown. Magnified regions of the normal and tumor mucosa are shown on the *right*. *N*, normal tissue; *T*: tumor tissue (adenocarcinoma).

Furthermore, additional immunohistochemistry studies were performed in paraffin embedded sections of normal colon mucosa and tumor tissue. SMC2 protein was up-regulated in tumor cells (Fig. 1*G*), both in the cytoplasmic compartment and nuclei. Normal tissue staining confirmed β-catenin, SMC2, and NCAPH (a non-SMC subunit of the condensin complex) accumulation in cells located in the lower part of the intestinal crypts, where WNT signaling is active and cells proliferate actively to maintain the normal epithelial homeostasis (supplemental Fig. 4*A*) (26). Our observation that SMC2 is naturally expressed in cells where WNT pathway is engaged, prompted us to examine whether there was a correlation between the levels of SMC2/SMC4 and β-catenin in a panel of 14 CRC cell lines, and also in a subset of 14 pairs (normal/tumor) of clinical samples by WB (Fig. 1, *C* and *D*). It was confirmed that there was a strong positive correlation between the protein levels of β-catenin and SMC2 and SMC4 (Fig. 1*F*). Furthermore, immunohistochemical analysis of tumor samples confirmed that membrane-localized β-catenin corresponded to low levels of SMC2 and NCAPH, predominantly localized in the cytoplasm, whereas nuclear β-catenin staining was found in conjunction with increased levels of SMC2 and NCAPH expression, predominantly in the nucleus (supplemental Fig. 4, *B–I*). Because SMC2 was up-regulated in cells actively proliferating in response to WNT signaling and correlated with β-catenin levels in CRC cell lines and clinical samples, we were interested to determine whether SMC2 expression could be directly regulated by the WNT/β-catenin pathway.

SMC2 Is Down-regulated in Cellular Models for WNT Pathway Inhibition—First, we wanted to determine whether disruption of WNT/β-catenin signaling could affect *SMC2*/*SMC4* transcription. For this purpose, we used two *in vitro* systems,

β-Catenin·TCF4 Transcription Complex Drives SMC2 Expression

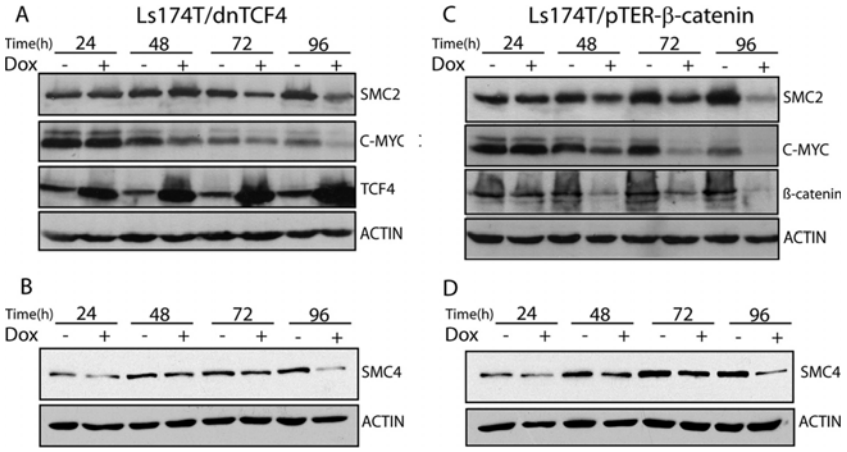

FIGURE 2. **SMC2 protein is down-regulated upon WNT signaling inhibition.** Ls174T/dnTCF4 (*A* and *B*) and Ls174T/pTER-β-catenin (*C* and *D*) cell lines were cultured in absence or presence of 5 μg/μl doxycycline (*Dox*) during the indicated time points. Cells were lysed and analyzed by WB using the indicated antibodies. Representative data from three replicates/independent experiments are shown.

Ls174T/dnTCF4 and Ls174T/pTER-β-catenin cell lines, for WNT pathway inhibition. Ls174T/dnTCF4 cells carry a doxycycline-inducible expression plasmid encoding N-terminally truncated version of TCF4, which acts as a dominant negative form of TCF4 (dnTCF4). Even though dnTCF4 protein binds to DNA it does not bind B-catenin acting as a potent inhibitor of endogenous β-catenin·TCF4 complexes (26). Induction of dnTCF4 after 96 h of doxycycline treatment resulted in a decrease in SMC2 protein levels in a dnTCF4 protein dose-dependent manner (Fig. 2*A*). Longer inductions were not tested as these cells rapidly undergo G_1 arrest (26, 27). To confirm inhibition of β-catenin·TCF4 activity, the levels of c-Myc, a well characterized WNT target gene, were evaluated (28). The same effect could be observed in the SMC4 protein levels under the same conditions (Fig. 2*B*).

To substantiate the dnTCF4 result, we examined the levels of SMC2/4 in Ls174T/pTER-β-catenin, a cellular model that expresses a doxycycline-inducible form of the RNA polymerase III H1 promoter to drive expression of an siRNA, directed to β-catenin. Addition of doxycycline to the growth medium induced rapid down-regulation of β-catenin messenger RNA (27) and protein (Fig. 2*C*) in these cells. In this context, following 96 h of doxycycline treatment, we observed a down-regulation in SMC2 (Fig. 2*C*) and SMC4 protein levels (Fig. 2*D*) that correlated to decreased β-catenin protein levels and implied a strong association between SMC subunit expression and β-catenin·TCF4 transcription factor.

SMC2 Promoter Responds to WNT Pathway Activation/Inhibition—To determine whether *SMC2/4* could be targets of the β-catenin·TCF4 transcription factor complex, upstream sequences of the human *SMC2* and *SMC4* genes were obtained from the Ensembl database (30). Three different software packages were used for *in silico* prediction of the *SMC2* promoter: Gene2Promoter recognized a very highly promoter-like region between the −308 bp and +420 bp region (considering 0 bp the transcription start site); promoter 2.0 predicted a promoter region starting in the −476 bp position; finally, promoterScan located two putative regulatory regions, from the −597 bp to

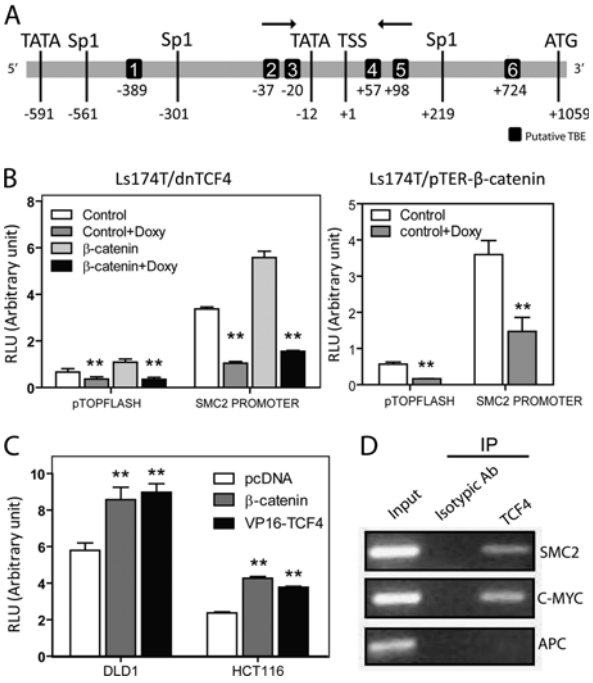

FIGURE 3. **Functional study of *SMC2* promoter activity.** *A*, schematic representation of human *SMC2* promoter. Predicted TCF response elements are also indicated; *arrows* indicate target sequence for ChIP PCR amplification. *B*, Ls174T/dnTCF4 (*left*) and Ls174T/pTER-β-catenin (*right*) cell lines were transfected with *SMC2* promoter-luciferase reporter construct together with control *Renilla* luciferase reporter pRL-TK for normalization (*RLU*, relative luciferase units). Where indicated, cells were doxycline (*Doxy*)-treated to induce the TCF4 dominant-negative form (*left*) or a siRNA targeting β-catenin (*right*). TOP-flash vector was used as positive control for WNT signaling activity/repression. A representative result out of at least three different experiments run in triplicates is shown. *C*, DLD-1 or HCT116 cell lines were co-transfected with *SMC2* promoter luciferase construct and pcDNA (empty vector), β-catenin, or *VP16-TCF4* expression vectors. *D*, PCR analyses of DNA pulled down by isotypic antibody (negative control) or anti-TCF-4 monoclonal antibody in ChIP assay. c-*myc* promoter sequence containing TBE1 element and APC promoter region 1B sequences were amplified as positive and negative controls, respectively. *Error bars* indicate S.D. (Student's *t* test; **, $p < 0.01$).

−348 bp position, and from −313 bp to −64 bp, respectively. For subsequent studies, we compiled an *SMC2* promoter based on the different predictions, which was determined to be from position −597 bp to the translation start site (+1059 bp) (Fig. 3*A*). In this region, two TATA boxes were identified at positions −591 and −12 bp, and three recognition sites for the Sp1 transcriptional factor were situated at −561 bp, −301 bp, and +219 bp positions. The predicted *SMC2* promoter was subjected to an *in silico* screen for TCF binding elements (TBE). rVista (version 2.0, NCBI DCODE), TESS (Transcription Element Search System), and Matinspector software (Genomatix), which predicted four different elements: TBE1 (−389 bp), TBE3 (−20 bp), TBE4 (+57 bp), and TBE6 (+724 bp). Additionally, Matinspector located two further TBEs: TBE2 (−37 bp) and TBE5 (+98 bp) (Fig. 3*A*).

Promoter software (version 2.0) predicted that the region from the −1500 bp position to the transcription start site (0 bp) of *SMC4* was highly likely to be a promoter region, in which one TATA box (−837 bp) and three Sp1 sites (−1066 bp, −21 bp, and −5 bp) could be identified. Two putative TBE were predicted in this region (−1270 bp and −1294 bp), but none of these were phylogenetically conserved in mammals (data not shown), so we continued our study by focusing on WNT pathway regulation of *SMC2* expression.

The full-length promoter of *SMC2* was cloned into a pGL3 firefly luciferase reporter vector, and its activity was assayed in Ls174T/dnTCF4 cells following transient transfection alone, or in combination with β-catenin expression vector (*pBCAT*). This promoter was active under normal conditions, even more than the positive control TOPFLASH (run in parallel). After doxycycline induction of the dnTCF4 form, luciferase activity was significantly reduced. Moreover, the promoter was able to respond to β-catenin transduction, but this capacity was lost when the dnTCF4 form was induced by doxycycline (Fig. 3*B*, *left*). *SMC2* promoter was also tested in Ls174T/pTER-β-catenin cells. Doxycycline-induced down-regulation of β-catenin also diminished the luciferase activity of the full-length promoter of *SMC2* (Fig. 3*B*, *right*).

To confirm that the *SMC2* promoter was a target of activated WNT signaling, we tested the luciferase activity of the promoter in two colon carcinoma cell lines, DLD1 and HCT116, carrying an activating mutation in β-catenin or a deactivating mutation in APC (31), respectively. Cells were co-transfected with pGL3-*SMC2* promoter and expression vectors for β-catenin (*pBCAT*) or *VP16-TCF4* (a constitutively active form of TCF4). In both cell lines, a significant gene transactivation increase could be observed after the co-transfection (Fig. 3*C*), supporting the hypothesis that the activated WNT pathway can drive transcription from the *SMC2* promoter via the β-catenin·TCF4 transcription complex.

TCF4 Transcription Factor Is Bound to the SMC2 Promoter in Vivo—We aimed to determine whether TCF4 interaction with the *SMC2* promoter was direct or indirect. Therefore, ChIP experiments were used to test whether TCF4 could occupy the *SMC2* promoter. Chromatin from DLD1 cells was cross-linked prior to anti-TCF4 antibody immunoprecipitation of DNA·protein complexes. The *SMC2* promoter sequence that contains TBE 2, 3, 4, and 5 was present in the TCF4 eluate (Fig. 3*D*), confirming that this transcription factor can bind to the *SMC2* promoter *in vivo*. Primers for c-*myc* and APC promoter amplification were used as positive and negative controls, respectively (28).

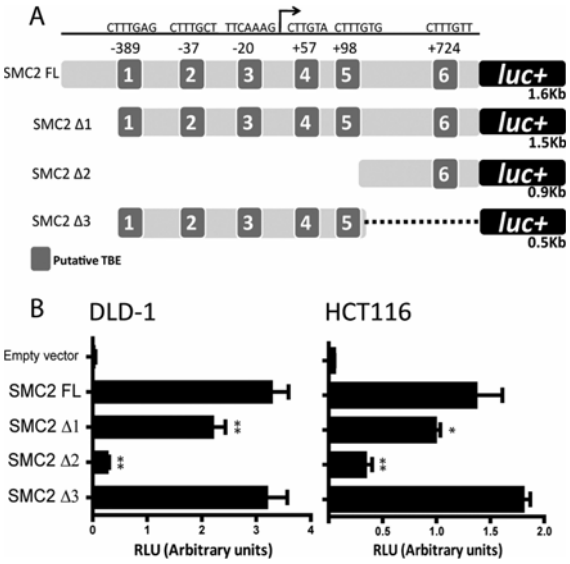

FIGURE 4. **Determination of the minimal regulatory region of *SMC2* promoter.** *A*, relative position and sequences of the putative TBEs predicted *in silico* in the *SMC2* promoter and deletion mutants for luciferase (*luc*) reporters performed. *B*, determination of fragment 3 as the minimal regulatory region of the *SMC2* promoter. Luciferase activity of each deletion mutant was normalized to *Renilla* luciferase internal control (*RLU*, relative luciferase units) in DLD1 (*left*) or HCT116 (*right*) cell lines; a representative result is shown of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$; Student's *t* test (promoter activity *versus* full-length *SMC2* promoter (*SMC2 FL*).

The Region Located between −389 bp and +98 bp in SMC2 Promoter Is Defined as the Minimal Regulatory Fragment of the SMC2 Gene—To define the minimal transcriptional regulatory region in the *SMC2* promoter, we cloned a series of terminal deletions of the full-length sequence based on the position of the predicted TCF response elements (Fig. 4*A*). DLD1 and HCT116 cells were transfected with three different deletion mutants, and luciferase activity was measured.

Deletion of the first 100 base pairs in the *SMC2* promoter resulted in decreased luciferase activity, and the promoter activity was almost lost when the deletion removed all of the putative TBEs (except TBE6). It was also confirmed that the 0.5 kb (Δ3 sequence), which contains TBEs 1, 2, 3, 4, and 5, maintained the maximal activity in both cell lines. Fragment Δ3 showed a luciferase activity similar to the full length sequence. Thus, we defined Δ3 as the minimal regulatory region and used it for further mutational studies (Fig. 4*B*).

The TCF Response Element Located at −20 bp (TBE3) Is Susceptible to β-Catenin·TCF4 Transactivation—Interspecies conservation analysis showed that two of the six TBEs predicted, TBE2 and TBE3, were highly conserved in ortholog *SMC2* promoters of mouse, rat, macaque, and chimpanzee (Fig. 5*A*), and both were present in the minimal regulatory region, Δ3. Interestingly, these two TBEs are the closest ones to the transcription start site.

β-Catenin·TCF4 Transcription Complex Drives SMC2 Expression

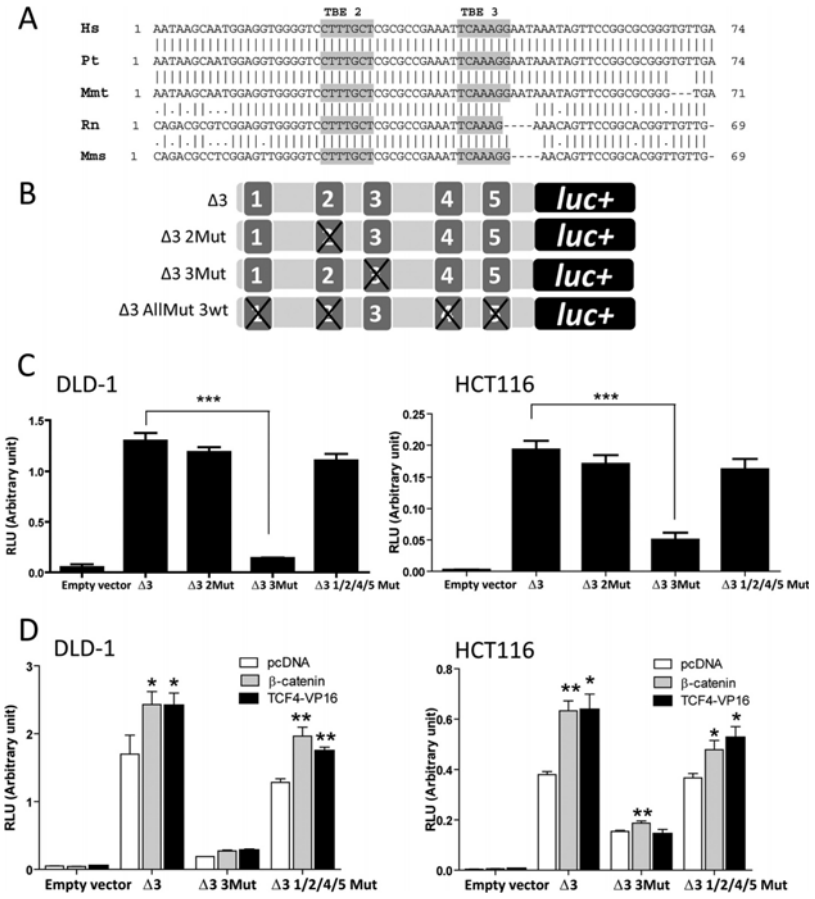

FIGURE 5. **Elucidation of the TBE responsible for β-catenin·TCF4 transactivation in the *SMC2* promoter.** *A*, sequence alignment of *SMC2* promoter in different species; *Hs*, *Homo sapiens*; *Pt*, *Pan troglodytes*; *Mmt*, *Macaca mulatta*; *Rn*, *Rattus novergicus*; *Mms*, *Mus musculus*. Conserved TBEs are highlighted in *gray background*. *B*, schematic representation of *SMC2* promoter mutant variants. *C*, DLD1 (*left*) or HCT116 (*right*) cell lines were transfected with constructs above. Luciferase activity was normalized to *Renilla* activity (*RLU*, relative luciferase units); a representative result is shown out of at least three independent experiments. *D*, DLD-1 (*left*) or HCT116 (*right*) cell lines were co-transfected with Δ3 fragment mutational combinations and expression vectors for β-catenin, *TCF4-VP16* (constitutively active form of TCF4), or the empty vector pcDNA3 (pcDNA); a representative result is shown out of at least three independent experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

To study the functionality of those conserved TBEs, we performed site-directed mutagenesis to disrupt TCF4 binding ability (Fig. 5*B*). We detected a significant decrease in luciferase activity when TBE3, located at −20 bp, was mutated. However, mutations in all other TBEs did not affect luciferase activity driven by the *SMC2* promoter (Fig. 5*C*).

To confirm TBE3 susceptibility to WNT signaling transactivation, we measured luciferase activity after co-transfection of β-catenin or *VP16-TCF4* expression plasmids and different mutational combinations in Δ3 fragment. Enhancement of luciferase activity in response to WNT/β-catenin stimulation was lost when TBE3 was disrupted (Fig. 5*D*). As expected, mutations in TBE 1, 2, 4, and 5 did not affect promoter response to β-catenin or VP16-TCF4 stimulation. Thus, we identified the TCF response element located at −20 bp (TBE3) as the sole entity responsible for β-catenin·TCF4 transactivation of the *SMC2* promoter.

SMC2 Knockdown Results in Decreased Tumor Growth in Vivo—Because we had established that the *SMC2* promoter could be driven by WNT signaling, and SMC2 has a clear role in mitosis, we hypothesized that perturbing SMC2 expression may reduce WNT-induced cell proliferation. Therefore, we investigated the effect of SMC2 down-regulation in WNT-activated CRC cell lines. DLD1, HT29, and HCT116 cells were transiently transfected with an siRNA targeting *SMC2* for 48, 72, and 96 h. SMC2 knockdown efficiency was assessed by WB. Furthermore, a decrease in SMC4 and NCAPH protein expression was also detected, implying a reduction in the condensin complex as a whole (supplemental Fig. 5). Cell cycle profile was studied by FACS determination of propidium iodide stained DNA. A significant increase in haplo-diploid (apoptosis), 4n (G_2/M), and aneuploid (>4n) DNA content populations could be observed along treatments, whereas the 2n DNA content population (G_1) decreased drastically (supplemental Fig. 6,

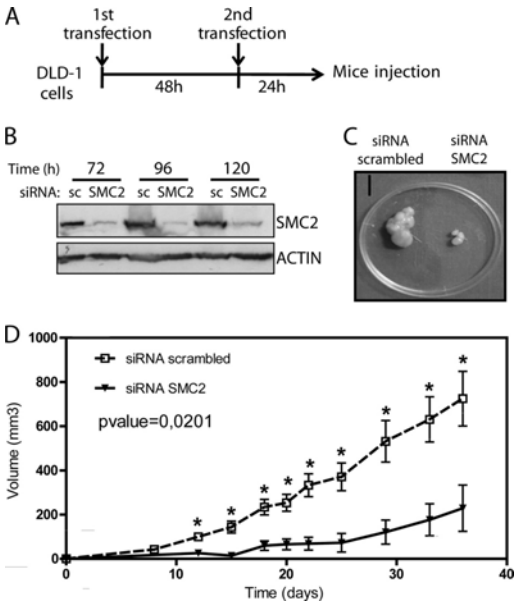

FIGURE 6. **siRNA knockdown of *SMC2* impairs tumor growth in a xenograft mouse model.** *A*, schematic representation of the experimental design. DLD1 cells were transiently transfected with an siRNA targeting *SMC2* or a scrambled sequence. After 48 h, a second round of transfection was performed. 24 h later, 1.5×10^6 cells were injected subcutaneously in the dorsal flanks of athymic nude mice. *B*, SMC2 knockdown was assessed by WB using whole cell extracts from *in vitro* culture until 120 h post-tranfection (*sc*, scrambled siRNA). *C*, representative resected tumors from the same animal at day 40 post-injection. *Scale bar*, 1 cm. *D*, tumor growth curves. Tumor volume was measured every 2–3 days for 36 days. The graph is representative of two independent experiments. *Error bars* represent S.E. ($n = 11$). Differences were evaluated with paired Student's *t* test ($p = 0.0201$); (*, $p < 0.05$, *t* test in each time point).

A–C). For stable SMC2 knockdown, lentiviral particles containing an shRNA targeted to *SMC2* were used to transduce HT29 cells. Five different sequences targeting different regions of *SMC2* mRNA were tested, but only three regions were able to down-regulate SMC2 efficiently. As expected, stable knockdown of SMC2 impaired HT29 cell viability. Morphological changes in SMC2-down-regulated cells could be appreciated after 1 week in culture in terms of enlarged multinucleated and non-viable cells, a phenotype clearly associated to shRNA-*SMC2* knockdown efficiency (supplemental Fig. 6, *D* and *E*). These results implied that decreasing SMC2 protein levels could attenuate cell division even in cells that are receiving strong proproliferation signals, such as the WNT/β-catenin pathway.

To test this concept *in vivo*, we investigated whether CRC tumor cells require SMC2 expression to proliferate in a xenograpft model of tumor progression. To prolong the knockdown effect, two rounds of transfection were performed in DLD1 cells before injection into athymic nude mice (Fig. 6*A*). To asses SMC2 knockdown durability, SMC2 protein was evaluated by WB of whole cell extracts 72, 96, and 120 h post-transfection, confirming that SMC2 levels remained down-regulated for at least 120 h under these experimental conditions (Fig. 6*B*). siRNA-*SMC2* or scrambled siRNA transfected DLD1 cells were injected into the flanks of 11 nude mice and tumor growth was measured over 5 weeks. Transient knockdown of SMC2 was enough to significantly reduce tumor size compared with controls even at 12 days post-injection, and this difference became more pronounced after 35 days, the point at which the animals were sacrificed (Fig. 1, *C* and *D*). Although further investigation is required, the significantly tumor growth-retarding effect of SMC2 knockdown *in vivo* could make SMC2 an interesting novel chemotherapeutic target.

DISCUSSION

It is becoming apparent that the WNT signaling pathway appears to be intimately linked with the mitotic machinery. In this study, we have demonstrated that the TCF4 transcription factor can bind to and drive the *SMC2* promoter *in vitro* and that preventing β-catenin binding to TCF4 markedly reduces SMC2 protein levels. Our *in vivo* study suggests that depletion of SMC2 levels in human CRC cells expressing constitutively active β-catenin significantly affected tumor growth in an immunodeficient mouse model.

In this study, we observed SMC2 protein levels to correlate directly with SMC4 protein levels in a panel of colorectal cell lines and tumor lysates, in accordance with the heterodimeric structure of the condensin SMC2/4 core. We were unable to locate a conserved TCF4 transcription element within the *SMC4* promoter; however, expression of either condensin SMC subunit appears to be very tightly linked to expression of its partner. Indeed, preliminary experiments in which SMC2 expression is depleted in DLD1 cells using siRNA to *SMC2* show that there is a corresponding reduction in the levels of SMC4 and the non-SMC regulatory subunits (supplemental Fig. 5).

Chromatin is generally thought to be transcriptionally silent around the G_2/M transition. Furthermore, Takemoto *et al.* (32) demonstrated that in unstimulated cells, SMC protein levels remained stable throughout the cell cycle. However, in WNT-activated cells, the situation may be different. WNT signaling is enhanced by cyclin γ and peaks around G_2/M (16). Therefore, it is important to consider whether WNT target genes, such as SMC2, could be actively transcribed during this phase of the cell cycle. A recent study using conditional gene knock-out (KO) mice highlighted the link between cell cycle regulators and WNT signaling, and goes some way to answering the question. Deletion of all three members of the CDC25 protein phosphatase family led to a lethal reduction in enterocyte proliferation due to arrest at G_2/M. Notably, in the same animals, WNT target gene expression was up-regulated in putative epithelial crypt progenitor cells, and there was a 50% increase in the total number of crypt cells staining positive for nuclear β-catenin (34). This result confirms the possibility that β-catenin·TCF4 could be actively driving transcription of *SMC2* and other target genes during G_2/M *in vivo*. The physiological significance of *SMC2* transcription at this point in the cell cycle is unclear; however, it could be a method of ensuring that sufficient levels of DNA-condensing proteins are available at the juncture where they are required most.

Our initial immunohistological observations of normal human intestine confirmed that SMC2 protein expression was

up-regulated in crypt cells staining positive for nuclear β-catenin. As WNT signaling drives cell proliferation, it is not particularly surprising that higher levels of condensins are required by tissues with elevated cell turnover such as the gut. However, it is exciting to note that SMC2 expression can be directly driven by TCF4 transcription factor. It is possible that WNT signaling can drive a positive feedback loop, whereby rapidly dividing cells are induced to produce elevated levels of proteins involved in the cell division machinery.

Previously, Ghiselli and co-workers (29) found that one of the cohesin SMC subunits, SMC3, was up-regulated in human colorectal adenocarcinomas and APC^{Min} mouse adenomas. The *SMC3* promoter also contains two conserved transcriptional binding sites for β-catenin·TCF4 in the human and mouse promoters, which could be driven by elevated β-catenin (29, 33). Our data confirms that the promoter of a condensin subunit, *SMC2*, can also be a target of β-catenin·TCF4 activation, and our *in vivo* knock-down experiment suggests that reducing SMC2 levels could be an effective way of retarding or ablating tumor growth.

Our analysis of a bank of human CRC cell lines showed that SMC2 and SMC4 proteins are highly expressed in many transformed cells. Interestingly, there appeared to be a correlation between the level of SMC protein expressed, and the rate of cell division (*i.e.* cells with higher levels of SMC2 tended to be the fastest growing; supplemental Fig. 3). Furthermore, SMC2 levels are significantly reduced in non-dividing senescent cells (data not shown), supporting the positive feedback hypothesis suggested above. Moreover, our analysis of human colorectal tissue samples implies that up-regulation of SMC2 and SMC4 is a common occurrence in human intestinal cancer, corroborating the idea that up-regulation of condensin can be linked to β-catenin-induced hyperplasia. Analysis of non-SMC condensin subunits at the mRNA and protein levels confirmed up-regulation of the condensin complex as a whole in tumor *versus* normal tissue samples (supplemental Figs. 2 and 4). Our observation that SMC2 expression is up-regulated in cells with nuclear β-catenin suggests that β-catenin·TCF4 may drive production of condensin, which might be required to allow rapid cell division.

Interestingly, knockdown of the SMC2 subunit alone was sufficient to cause a significant reduction in proliferation of an APC mutant colorectal cell line *in vivo* (confirmed by FACS, supplemental Fig. 6*B*). Upon further analysis, we found that two additional CRC tumor cell lines treated with *SMC2* siRNA appeared to be undergoing aneuploid division and apoptosis, most likely as result of mitotic catastrophe (supplemental Fig. 6, *B* and *C*). Therefore, given that reducing SMC2 expression alone is enough to induce growth arrest and apoptosis of tumor cells, the SMC2 condensin subunit could be an attractive novel target for therapeutic intervention in cancer treatment. Of particular significance is the fact that SMC2 is highly expressed alongside nuclear β-catenin in a few cells located at the base of intestinal crypts, which are putative stem cells. This suggests that high expression of SMC2 may be a characteristic of stem cells in normal colon tissues.

In summary, this study has identified SMC2, one of the condensin ATPase subunits, as a novel, *bone fide* target of β-catenin·TCF4 transcription. Furthermore, overexpression of condensin appears to be a frequent feature of human CRC. Our data suggests that elevated levels of condensin may be required to allow WNT-driven cell proliferation and that reducing SMC2 expression can lead to tumor cell apoptosis. Therefore, modulation of condensin SMC protein expression may offer exciting novel therapeutic potential in the treatment of human neoplasia.

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Supplementary Material

Supplementary Tabl e 1 :Oligonucleotides used in the study. Restriction enzymes sites are indicated underlined; TBEs mutagenesis are highlighted in grey background.

Use	Primer ID		Sequence
ChIP	*SMC2* prom-ChIP TCF4	Sense	GGCACCAGCACAGGAAATAAG
		Antisense	GCTTGACGCACCCCAAAAG
	c-myc prom	Sense	TTTCACAAGGGTCTCTGCT
		Antisense	TGCTCTCTGCCAGTCTGTA
	APC prom	Sense	GCCAGTAAGTGGTGCAACTG
		Antisense	TGTGGGAGGTGGGAAGACTA
SMC2 promoter cloning and deletions	Full length	Sense (KpnI)	GGGGTACCGACGTGGAAACTTCAG
		Antisense (BglII)	GAAGATCTCATTTTCGATACTGTCTTGGG
	Δ1-KpnI-SMC2p	Sense (KpnI)	GGGGTACCCTTTGAGGAGAGAAAAGTAAG
	Δ2-KpnI-SMC2p	Sense (KpnI)	GGGGTACCAGGAGCTTTTGGGGTGCGTC
	Δ3-BglII-SMC2p	Antisense (BglII)	GAAGATCTACGCACCCCAAAAGCTCCT
SMC2 promoter site-directed mutagenesis	*SMC2*prom-TCF4BOX1mut	Sense	TCCACTTCCTAACTGTCGCGCTGAGGAGAGAAAAGTAAGC
		Antisense	GCTTACTTTTCTCTCCTCAGCGCGACAGTTAGGAAGTGGA
	*SMC2*prom-TCF4BOX2mut	Sense	TGGAGGTGGGGTCCTCTACTCGCGCCGAAATTC
		Antisense	GAATTTCGGCGCGAGTAGAGGACCCCACCTCCA
	*SMC2*prom-TCF4BOX3mut	Sense	GTCCTTTGCTCGCGCCGAAATTCATTGGAATAAATAGTTCC
		Antisense	GGAACTATTTATTCCAATGAATTTCGGCGCGAGCAAAGGAC
	*SMC2*prom-TCF4BOX4mut	Sense	TGGTGAAGTTCGCTGCGTAGCGGCCCCGGC
		Antisense	GCCGGGGCCGCTACGCAGCGAACTTCACCA
	*SMC2*prom-TCF4BOX5mut	Sense	TTCTGTTCCCTGCCTATGTGACCCGGAGG
		Antisense	CCTCCGGGTCACATAGGCAGGGAACAGAA

Supplementary Table 1: Clinical features of the patients used in this study.

		Low SMC2	High SMC2	Total	p	Low SMC4	High SMC4	Total	p
Sex (nº)	Female	4	10	14	1$	5	5	10	1$
	Male	10	18	28		9	8	17	
Age (average)		59.4	67.35	65.26	0.17&	63.7	58.23	61.11	0.34&
Location	Right	3	7	10	0.7$	4	3	7	0.67$
	Left+Rectum	7	20	27		8	10	18	
Stage	I+II	5	9	14	0.45$	6	5	11	1$
	III+IV	5	18	23		7	8	15	
Grade	Good	5	0	5	0.089+	1	3	4	0.11+
	Poor	16	9	25		10	7	17	
	Moderate	5	0	5		0	3	3	

$: Fisher exact test

&: Mann-Whitney test

+: Chi-square test for trend

SUPPLEMENTARY FIGURE LEGENDS

SUPPLEMENTARY FIGURE 1. Confirmation of SMC2 antibody specificity: DLD-1 cells were treated with SMC2 siRNA or control scrambled siRNA (sc) and subject to immunocytochemistry 96h after transfection. Anti-SMC2 antibody staining (ab10412; 1:200 dilution) revealed nuclear SMC2 staining in cells treated with control siRNA (A and C). However, cells that were depleted of SMC2 using SMC2 siRNA showed greatly reduced peroxidase staining, indicating that the anti-SMC2 primary antibody specifically recognized SMC2 (B and D). Images were taken under bright field microscope at 100X.

SUPPLEMENTARY FIGURE 2. Expression of non-SMC condensin complex subunits was generally higher in colorectal tumor samples in comparison to their normal counterparts. qPCR of NCAPG (A), NCAPG2 (B) and NCAPH (C) showed a trend (≥12/15 patient samples) for increased mRNA levels of all non-SMC condensin subunits in human tumor samples, mirroring the data for SMC2 and SMC4. Inter-patient disparity in the fold change of each condensin subunit is likely to reflect technical differences in the affinity of each qPCR assay, and would best be addressed using multiple primer-probe sets for all the condensin subunits.

SUPPLEMENTARY FIGURE 3. Negative correlation between SMC2/SMC4 protein levels and population doubling time in 14 colorectal cancer cell lines. SMC2/4 protein levels were analyzed by WB, quantified and normalized to actin levels by gel band quantification. p-values were calculated using the Spearman Correlation Test.

SUPPLEMENTARY FIGURE 4. Nuclear localization of β-catenin correlates with increased levels of condensin subunits and redistribution of their subcellular location. (A) Immunohistochemistry using antibodies against β-catenin, SMC2 and NCAPH confirmed that condensin subunit expression mirrored β-catenin expression in normal human crypts; ie a gradient of expression along the crypt axis, strongest at the base. (B-G) In human colorectal tumor samples, cell-junctional localization of β-catenin was associated with low levels of SMC2 and NCAPH proteins (B-D). However, nuclear β-catenin localization was associated with high levels of SMC2 and NCAPH (E-G). (H and I) Quantification of the correlation between SMC2 ($p = 0.0464$) and NCAPH ($p = 0.0014$) protein expression and β-catenin localization, as observed in (B-G). Data interrogated using the Fisher Exact Test. Images taken under a bright field microscope at 10X (A) and 100X (B-G).

SUPPLEMENTARY FIGURE 5. Expression of condensin complex members appears to be coupled at the protein level. Transient SMC2 siRNA treatment of DLD-1 cells resulted in a matching decrease of SMC2, SMC4 and NCAPH protein levels from 48-72h post-transfection, compared to cells treated with control scrambled siRNA (sc).

SUPPLEMENTARY FIGURE 6. SMC2 knockdown effect on cell cycle profile. (A) SMC2 knockdown analysis by WB after transfection of an siRNA targeted to SMC2 at the indicated time points in DLD1 cells (sc=siRNA scrambled, used as siRNA control). Similar results were obtained in HT29 and HCT116 cell lines. Actin served as loading control. (B) Analysis of cell cycle distribution of DLD1, HT29 and HCT116 cell lines 48, 72 or 96 h post-siRNA SMC2 transfection. One representative experiment is shown out of three independent experiments. (C) Cell population distribution 96 hours post-siRNA transfection in DLD1, HT29 or HCT116 cell lines. (*,$p<0.05$; **,$p<0.01$). (D) Stable knockdown of SMC2 in HT29 cells. Lentiviral particles containing five different shRNA sequences targeting SMC2 were used to transduce HT29 cells. shRNA non-targeted was lentivirally delivered as control (shNT). Protein expression was measured by WB using extracts of the polyclonal populations obtained after puromycin selection. Actin served as loading control. (E) Morphological changes in stably SMC2-downregulated cells. Images were taken under a phase-contrast microscope at 20X.

Figure S1

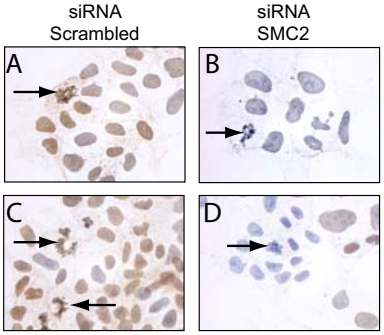


Figure S2

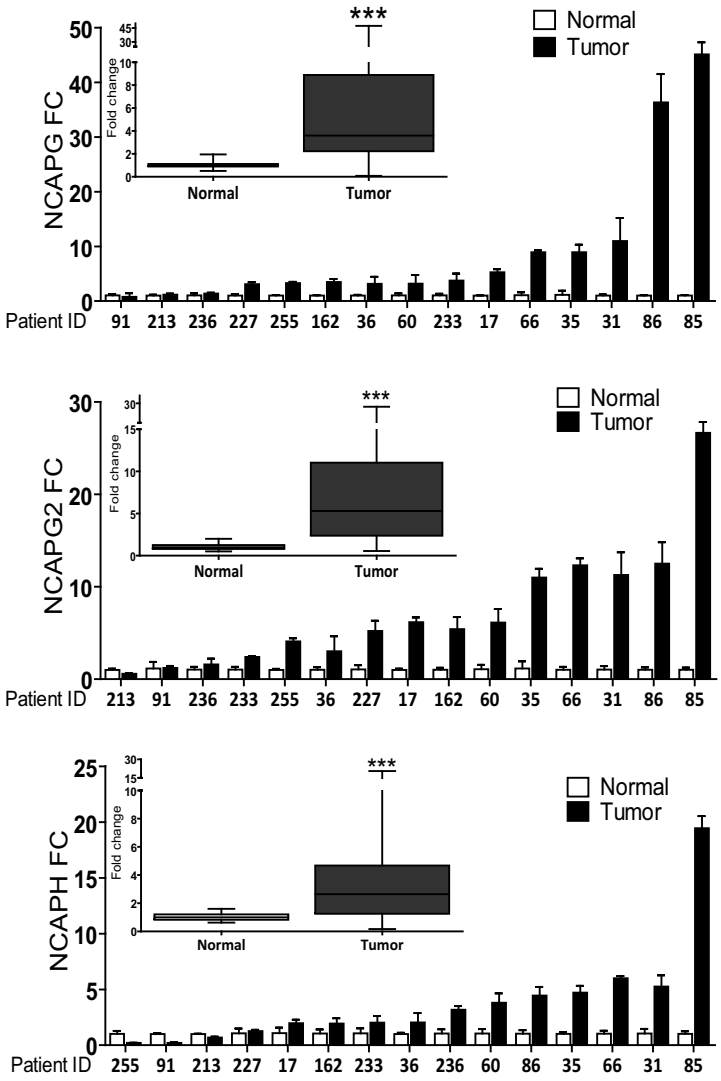


Figure S3

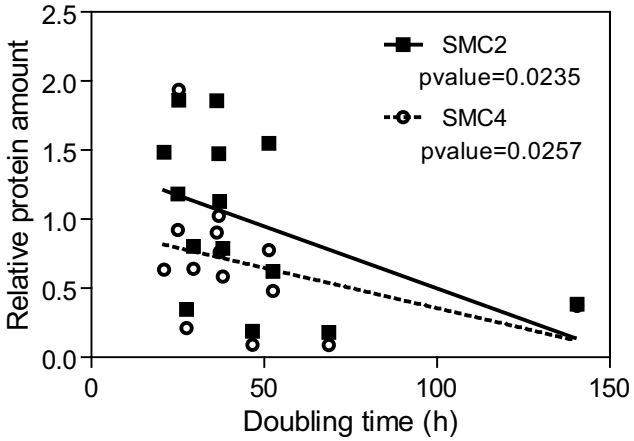

2.5
2.0
1.5
1.0
0.5
0.0
Relative protein amount
0
50
100
150
Doubling time (h)
SMC2
pvalue=0.0235
SMC4
pvalue=0.0257

Figure S4

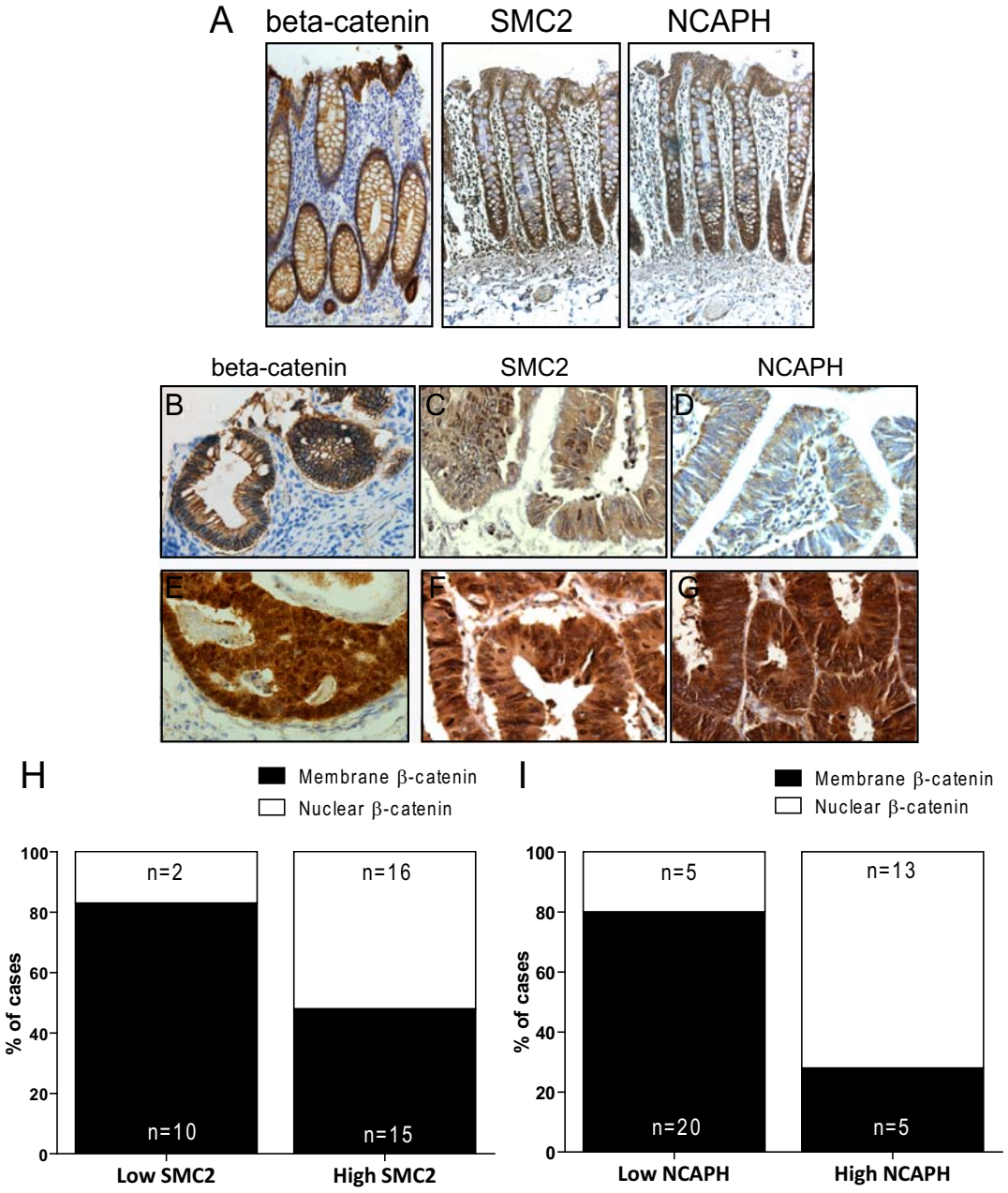

A
beta-catenin
SMC2
NCAPH
B
C
D
E
F
G
H
Membrane β-catenin
Nuclear β-catenin
% of cases
n=2
n=16
n=10
n=15
Low SMC2
High SMC2
I
Membrane β-catenin
Nuclear β-catenin
% of cases
n=5
n=13
n=20
n=5
Low NCAPH
High NCAPH

Figure S5

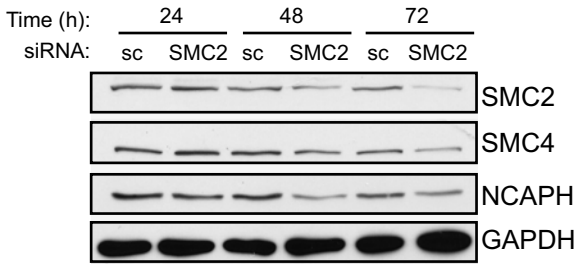
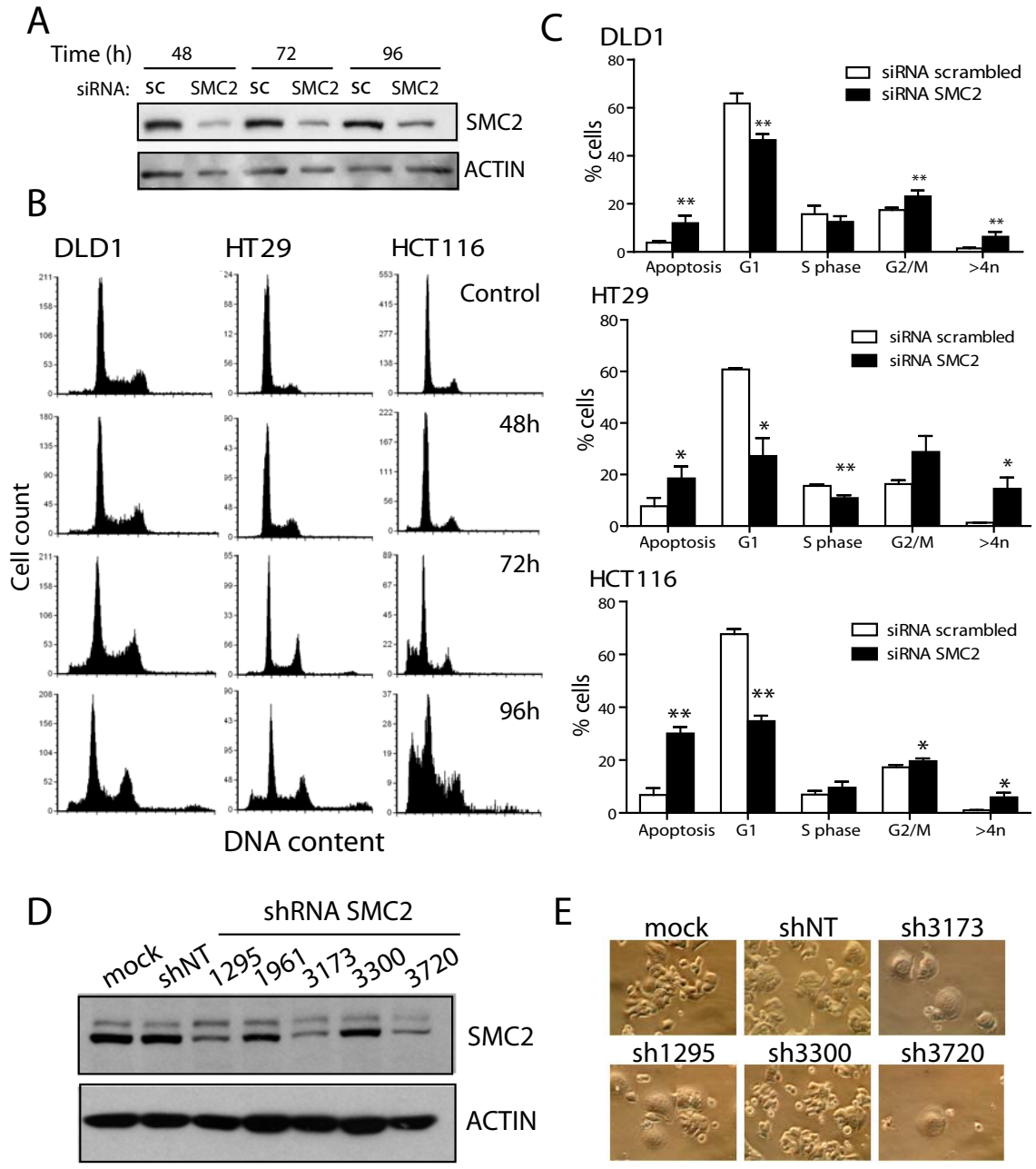

Time (h):
24
48
72
siRNA:
sc
SMC2
sc
SMC2
sc
SMC2
SMC2
SMC4
NCAPH
GAPDH

Figure S6


A
Time (h)
48
72
96
siRNA:
sc
SMC2
SMC2
ACTIN
B
DLD1
HT29
HCT116
Control
48h
72h
96h
Cell count
DNA content
C
DLD1
HT29
HCT116
siRNA scrambled
siRNA SMC2
% cells
Apoptosis
G1
S phase
G2/M
>4n
D
shRNA SMC2
mock
shNT
1295
1961
3173
3300
3720
SMC2
ACTIN
E
mock
shNT
sh3173
sh1295
sh3300
sh3720

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