

**POLIPOSI ADENOMATOSA FAMILIAR I CÀNCER
COLORECTAL**
Estudi genòmic i anàlisi d'alteracions de la via de Wnt

Memòria presentada per

Antònia Obrador Hevia

Per optar al Grau de

Doctor

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Dr. Gabriel Capellà i Munar
a l'Institut Català d'Oncologia

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Tutora: Dra. Marta Pascual

Gabriel Capellà

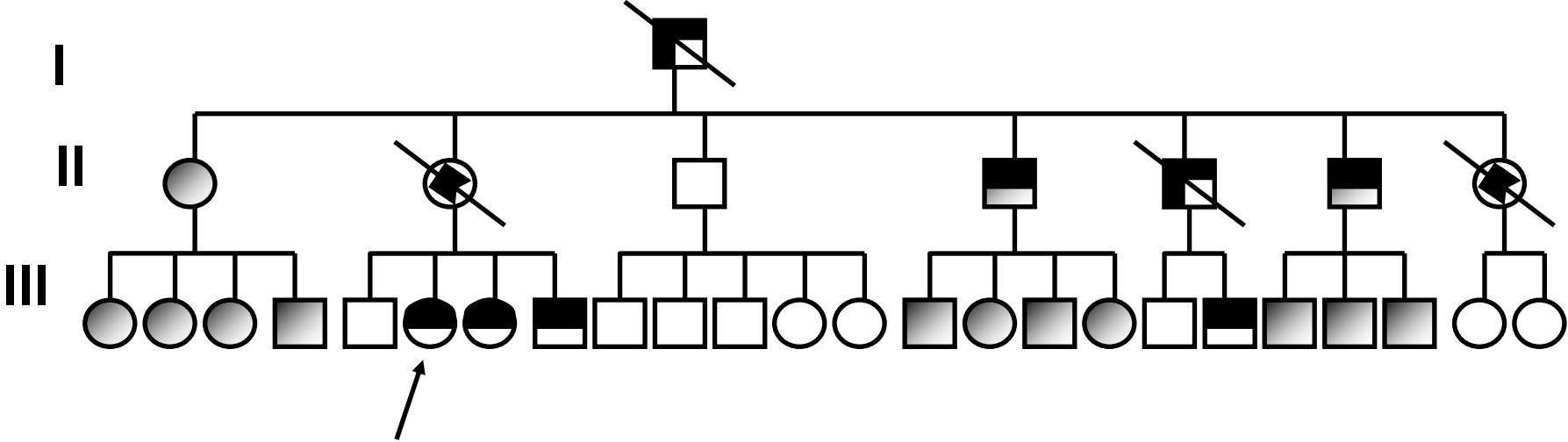
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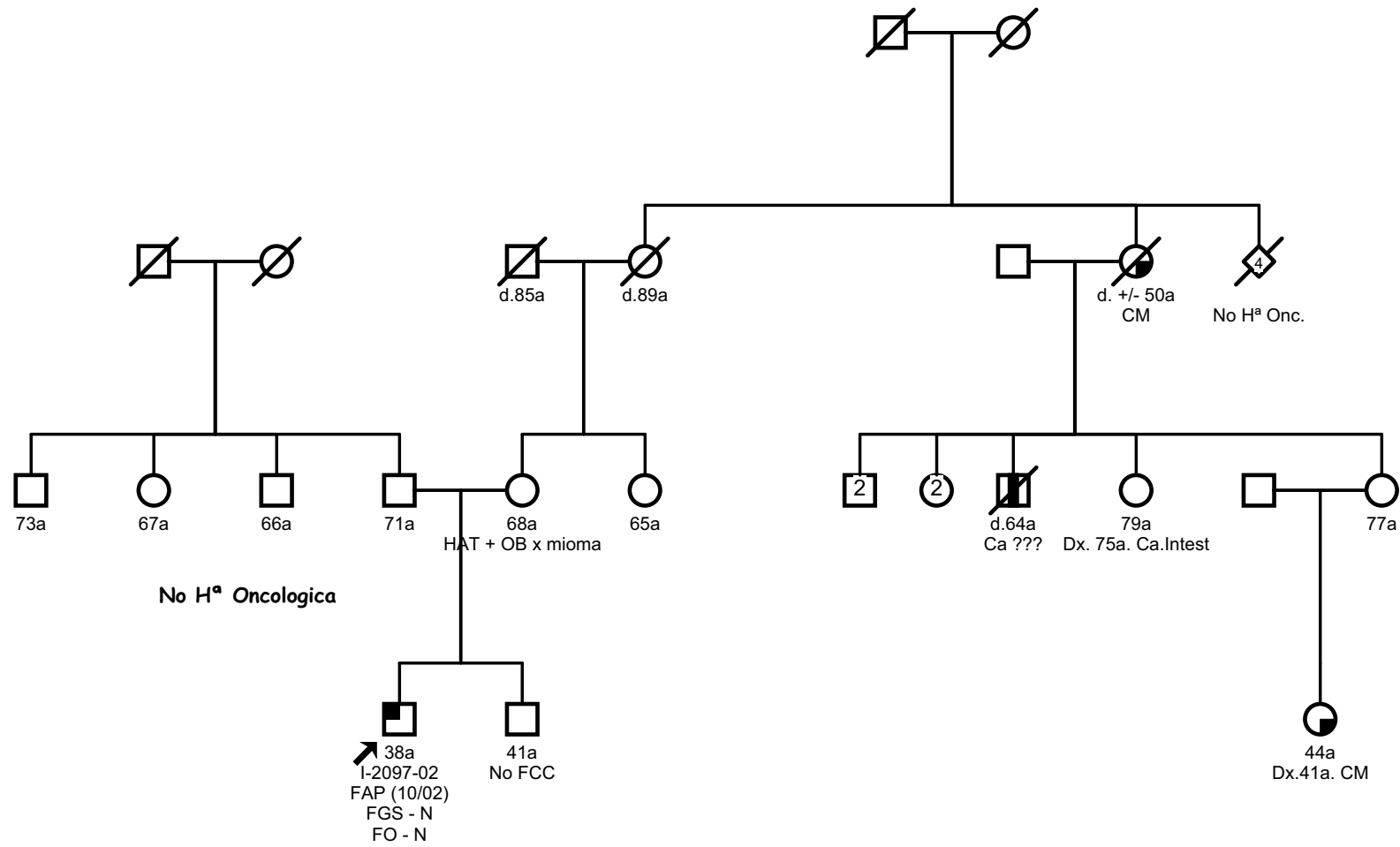
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Annex I

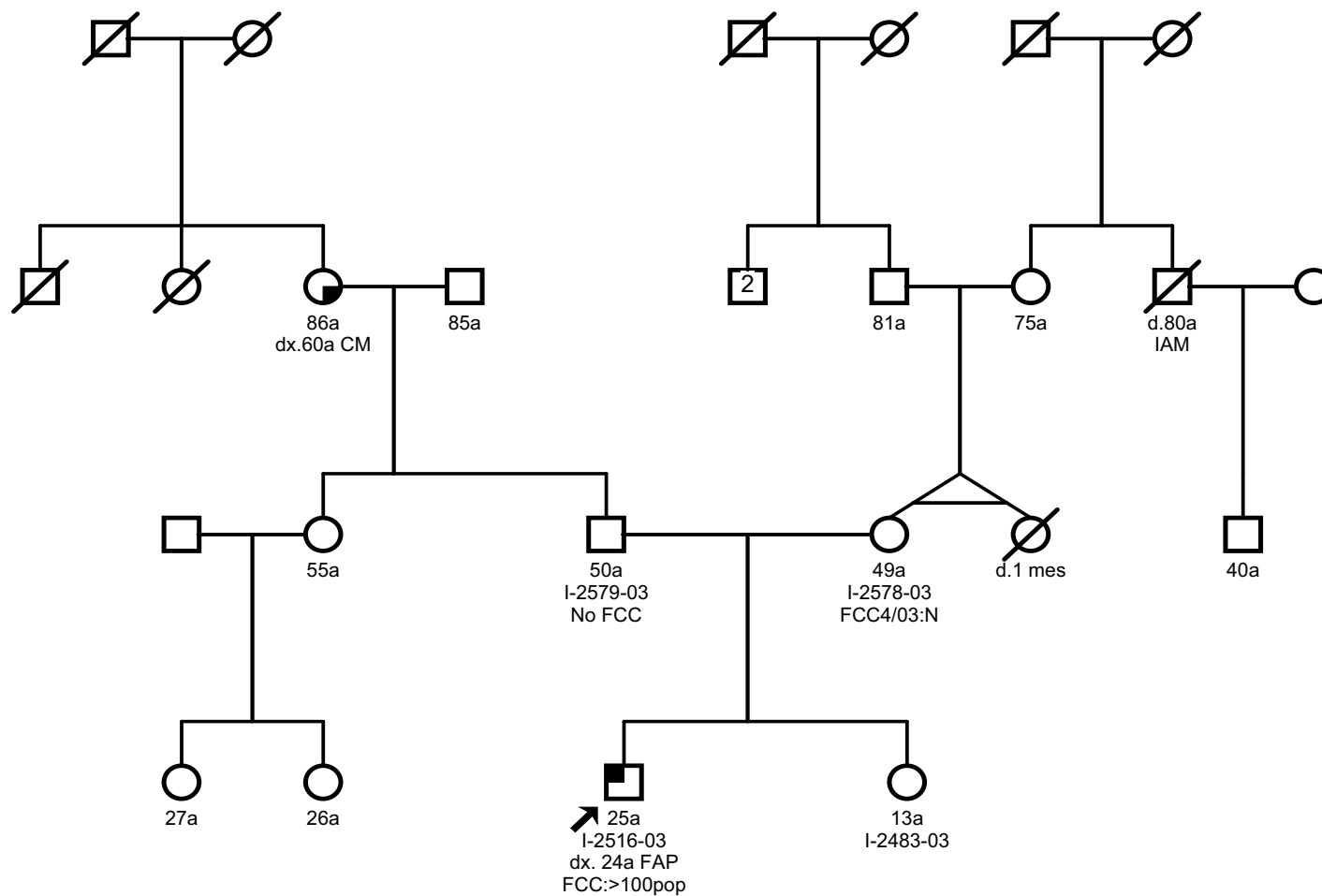
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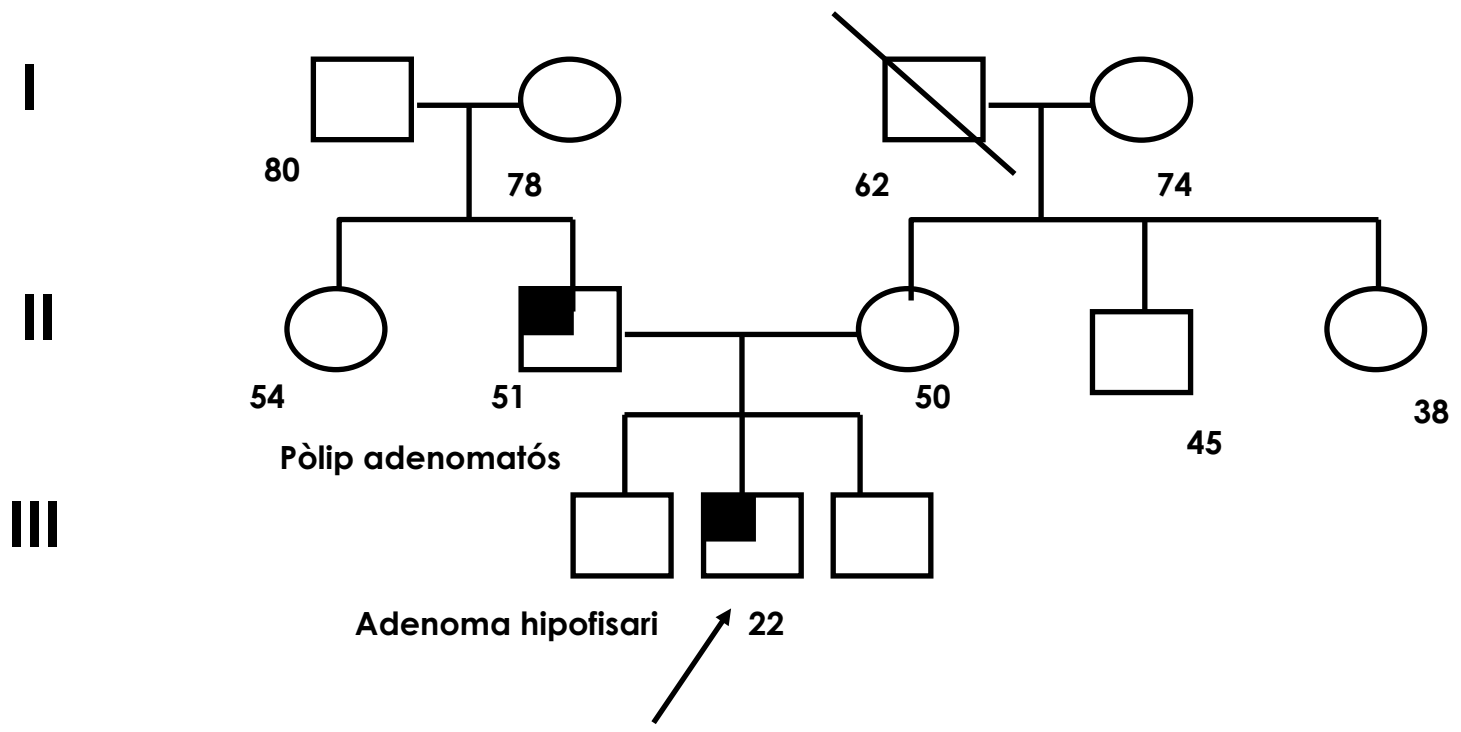
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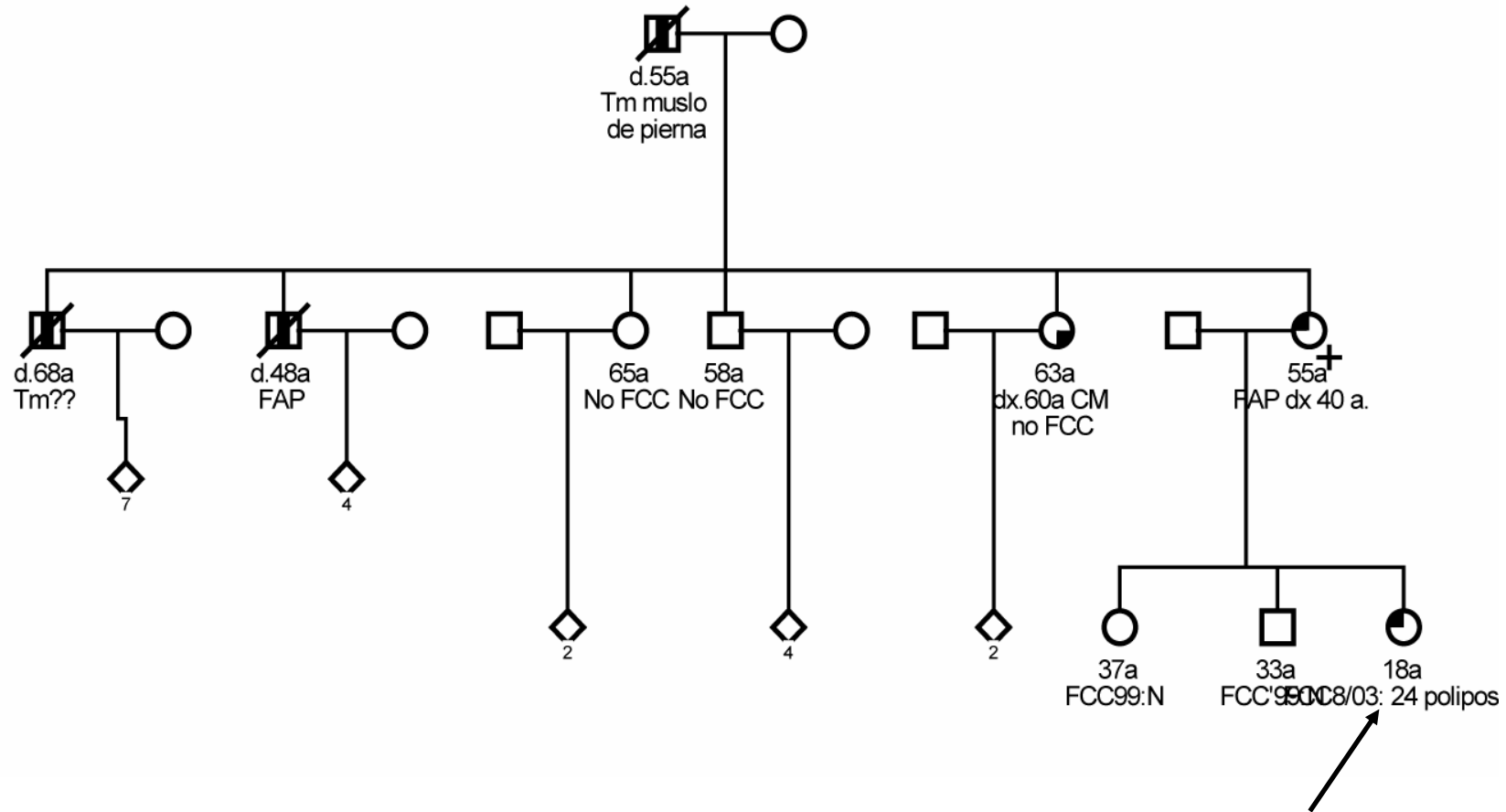
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Annex 2

APC Inactivation Associates With Abnormal Mitosis Completion and Concomitant BUB1B/MAD2L1 Up-Regulation

MIGUEL ABAL,^{*,**} ANTONIA OBRADOR-HEVIA,[‡] KLAUS-PETER JANSSEN,^{*,§} LAURA CASADOME,[‡] MIREIA MENENDEZ,[‡] SABRINA CARPENTIER,^{||} EMMANUEL BARILLOT,^{||} MECHTHILD WAGNER,[¶] WILHELM ANSORGE,[¶] GABRIELA MOESLEIN,[#] HAFIDA FSIHI,^{*} VLADIMIR BEZROOKOVE,^{**} JAUME REVENTOS,^{**} DANIEL LOUVARD,^{*} GABRIEL CAPELLA,[‡] and SYLVIE ROBINE^{*}

^{*}Morphogenesis and Intracellular signalling, UMR 144, Institut Curie-CNRS, Paris, France; [‡]Translational Research Laboratory, IDIBELL-ICO, Barcelona, Spain; [§]Department of Surgery, Klinikum rechts der Isar, TUM, Munich, Germany; ^{||}Equipe Bioinformatique, Research Institute, Institut Curie; [¶]European Molecular Biology Laboratory, Heidelberg, Germany; [#]Department of General Surgery and Traumatology, Heinrich Heine University, Duesseldorf, Germany; ^{**}Department of Dermatology, UCSF Comprehensive Cancer Center, University of California, San Francisco, California; and ^{**}Unitat de Recerca Biomedica, Research Institute Vall d'Hebron University Hospital, Barcelona, Spain

Background & Aims: Chromosomal instability, a hallmark of most colorectal cancers, has been related to altered chromosome segregation and the consequent deficit in genetic integrity. A role for the tumor suppressor gene *APC* has been proposed in colorectal cancer that leads to compromised chromosome segregation even though the molecular mechanism is not yet understood. Here, we tackled the genetic basis for the contribution of *APC* to chromosomal instability in familial adenomatous polyposis and sporadic colorectal cancer. **Methods:** We have used video-microscopy of primary cultures and molecular genetic methods to address these issues in human samples and in genetically defined mouse models that either recapitulate the familial adenomatous polyposis syndrome (*Apc*^{1638N}), or develop tumors in the absence of *APC* mutations (*p*villin-KRASV12G). **Results:** Mutations in *APC* were associated with an increased incidence in cell cycle defects during the completion of cytokinesis. Transcriptome analysis performed on mouse models indicated a significant up-regulation of genes that regulate accurate mitosis. Notably, we identified up-regulated expression of *BUB1B* and *MAD2L1*, 2 genes that are involved in the mitotic checkpoint, but have so far not been implicated in chromosomal instability induced by *APC* loss of function. *In vitro* modulation of *APC* expression suggested a causal association for this upregulation, which was consistently found in sporadic and familial adenomatous polyposis lesions, as an early event in colorectal tumorigenesis. **Conclusions:** In addition to the known function of *APC* during correct spindle assembly and positioning, we propose a concomitant involvement of *APC* in the surveillance mechanism of accurate mitosis.

genetic integrity. CIN is the hallmark of most colorectal cancers. Both qualitative and quantitative variations in chromosome numbers are observed in early colonic adenomas. This is consistent with CIN playing a potential role in tumor progression.¹ Moreover, the CIN phenotype seems to be dominant, as it can be conferred on a chromosomally stable, diploid cell when it is fused with a CIN cell.²

A role for the tumor suppressor gene *adenomatous polyposis coli* (*APC*) has been proposed in colorectal CIN. This role was suggested after observing mouse embryonic stem cells carrying *APC* mutated alleles, which lead to truncated *APC* proteins lacking carboxyl-terminal sequences. Extensive chromosome and spindle aberrations provided genetic evidence for a possible role played by *APC* in chromosome segregation through kinetochore-microtubule attachment.^{3,4} In addition to its prominence in the Wnt signaling pathway as a regulator of β -catenin transcriptional activity,⁵ *APC* has been described to bind to and stabilize microtubules, to localize to clusters at the ends of microtubules, and to be an important regulator of the cytoskeletal function. By modulating microtubule plus-end attachments during mitosis, mutant *APC* dominantly compromises astral and mid-zone microtubules in mitotic tumor cells with CIN.⁶ More precisely, *APC* mutants interfere with the function of EB1, a plus-end microtubule-binding protein that interacts with *APC* and is required for normal microtubule dynamics.⁷

Despite these insights regarding the contribution of *APC* to genomic instability in colorectal cancer, CIN is still not completely understood at the molecular level. A recent report presented evidence for the genetic basis of

A large percentage of cancers are characterized by chromosomal instability (CIN) and aneuploidy. This can be linked to a laxity in high-fidelity chromosome segregation and the consequent inability to maintain

Abbreviations used in this paper: *APC*, adenomatous polyposis coli; CIN, chromosomal instability; FAP, familial adenomatous polyposis; PCR, polymerase chain reaction.

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chromosomal instability, categorizing 3 classes of genes mutated in colorectal cancers with chromosomal instability. This study, based on the instability phenotypes identified in the *Saccharomyces cerevisiae* and *Drosophila melanogaster* model systems, included genes involved in double-strand break repair, genes that control chromosome segregation, and genes essential for proper chromosome disjunction.⁸ In the present investigation, we tackled the genetic basis for the contribution of APC to CIN in both genetically defined mouse models and in human colorectal samples. We were able to relate mitotic defaults associated with nonfunctional APC to an altered expression of the genes involved in the pathways that regulate accurate mitosis completion. In addition to the genes that participated in proper spindle function and chromosome segregation, we also identified altered genes that were related to the mitotic checkpoint, among them, BUB1B and MAD2L1.

Materials and Methods

Familial Adenomatous Polyposis (FAP) and Tumor Samples From Patients

FAP patient specimens for video-microscopy analysis were obtained from the Department of Surgery at the Heinrich Heine University in Germany. In addition, a panel of 60 fresh-frozen FAP adenomas from 6 different FAP patients (10 adenomas each) and their corresponding normal mucosa were obtained from the IDIBELL-Institut Catala d'Oncologia in Barcelona, Spain. All patients harbored 100 or more adenomas and carried a pathogenic germ-line mutation in the APC gene. Finally, a panel of 10 fresh-frozen paired normal-mucosa/adenoma/carcinoma series from colorectal cancer patients was also analyzed. The protocol was previously approved by the institutional review boards, and informed consent was obtained from all of the patients involved in the study.

Animal Models

All experiments on mice were performed in accordance with institutional and national guidelines and regulations. The *Apc*^{1638N} mouse lineage of the inbred C57Bl/6J background⁹ was bred with the transgenic model pVillin-KRAS^{V12G} of the genetic background B6D2 (C57Bl/6J × DBA/2).¹⁰ To control for genetic background effects, littermates were always employed as controls. Mice were maintained under a 12-hour light-dark cycle and fed with a standard diet and water ad libitum.

Primary Cultures

Polyps were isolated after the colectomy of a FAP patient and histology-matched adenomas from *Apc*^{1638N}, pVillin-KRAS^{V12G}, and compound *Apc*^{+ /1638N}/pVillin-KRAS^{V12G} mice were used. Human and animal samples

were collected in DMEM medium supplemented with 20% fetal bovine serum and penicillin and streptomycin. Polyps were cut into pieces and processed for enzymatic digestion in culture medium with 0.5 fetal calf serum supplemented with insulin 0.25 μg/mL and collagenase 0.2 mg/mL, for 1 hour at 37°C in rotation. Tissue masses were washed twice, resuspended in complete medium with 20% fetal calf serum supplemented with insulin 0.25 μg/mL, seeded onto collagen-treated coverslips, and incubated for 48 hours at 37°C and in 5% CO₂.

Video-Microscopy, Immunofluorescence, and Immunohistochemistry

Analysis of cell cycle progression was accomplished by collecting phase-contrast images every 10 minutes on a Leica (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) DMIRBE microscope controlled by Metamorph software (West Chester, PA). The microscope was equipped with an open chamber equilibrated in 5% CO₂ and maintained at 37°C, and images were taken with a 40×/0.70 PL Fluotar (Leica Microsystems Wetzlar GmbH) objective and a MicroMax (Princeton Instruments, Trenton, NJ) CCD camera.

Primary culture cells were fixed with 3% paraformaldehyde at room temperature for 20 minutes, treated with 50 mmol/L NH₄Cl in PBS for a further 20 minutes, and permeabilized with 0.1% Triton X-100 for 5 minutes. Cells were incubated with primary antibodies for β-catenin (1:200 Clone 14, BD Transduction Laboratories, Franklin Lakes, NJ), Villin (1:500 monoclonal antibody ID2C3¹¹), cytokeratin (1:200 Clone LP34; Dako Corp, Carpinteria, CA), and γ-tubulin (1:200 ab11319, Abcam, Cambridge, UK), and with Cy3- or Alexa488-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA), diluted in PBS containing 3% bovine serum albumin for 60 minutes at room temperature. The whole-cell volume was scanned using a piezo device mounted at the base of a Leica DMRXA microscope; 63×/1.32 or 100×/1.4–0.7 PL-APO objectives and a MicroMax CCD camera (Princeton Instruments) were used for acquisition; images were analyzed with Metamorph software (Universal Imaging, West Chester, PA) and processed using Adobe Photoshop (San Jose, CA).

Immunohistochemistry was performed on 5-μm OCT sections of human adenomas and carcinomas from FAP patients and from sporadic colorectal cancer patients. Samples treated with 3% formaldehyde and 3% hydrogen peroxide were evaluated for BUB1B and MAD2L1 expression with the anti-BUB1B rabbit polyclonal antibody (1:50; Abgent, San Diego, CA) and the anti-MAD2L1 rabbit polyclonal antibody (1:400; kindly provided by E.D. Salmon¹²). After washing in 0.1% PBS Tween 20, the samples were incubated for 30 minutes with the antirabbit secondary antibody EnVision HRP system of Dako. Omission of the antibody served as a negative control.

Transcriptome Analysis

We obtained a 15,247 mouse cDNA clone set from the National Institute of Aging (<http://lgsun.grc.nia.nih.gov/index.html>), and we used it to generate custom cDNA microarrays. The establishment, testing, and analysis of these custom microarrays are described in detail elsewhere (manuscript in preparation). Briefly, polymerase chain reaction (PCR) fragments were spotted onto homemade amino-silanized glass or epoxy glass slides (Quantifoil, Jena, Germany). Spotting was performed on an OmniGrid Spotter (GeneMachines, San Carlos, CA).

For direct labeling without amplification, we reverse transcribed the total RNA from normal/tumor samples into cDNA with oligo (dT)₁₂₋₁₈ primers using Superscript II (Invitrogen, Carlsbad, CA). For most of the labeling, total RNA was amplified first. Therefore, RNA was converted into cDNA with a T7-oligo (dT)₂₄ primer using Superscript II, followed by second-strand synthesis using DNA polymerase I and T4 DNA ligase, as well as RNase H. cDNA was purified by phenol/chloroform extraction and amplified using the Ambion MEGAScript Kit. Cleaning of the antisense RNA was accomplished using the Qiagen RNeasy Kit. Quantity was checked by spectrophotometrical measurement, and quality was analyzed by microelectrophoresis (Bioanalyzer 2100, Agilent Technologies, Waldbronn, Germany). Five micrograms of aRNA were directly labeled using random primers (Invitrogen). Purified Cy3- and Cy5-labeled probes were combined, mixed with 10 μ g poly (dA) (Amersham, Buckinghamshire, United Kingdom) and 3 μ g mouse Cot-1 DNA (Invitrogen) and evaporated in a vacuum concentrator (5301, Eppendorf, Hamburg, Germany).

Microarrays were scanned using a GenePix 4000B Scanner (Axon Instruments, Union City, CA). All arrays were scanned with a resolution of 10 μ m. For image analysis we used the ChipSkipper microarray analysis software (<http://www.ansorge-group.embl.de/services/chipskip.htm>).

To identify genes that were differentially expressed, we used the detection procedure called significance analysis of microarrays,¹³ as implemented in the R package *siggenes*.¹⁴ We used the following parameters for significance analysis of microarrays: the vector of values for the threshold $\Delta_i/5$ for $i = 1 \dots 10$ and 100 permutations. We retained the list of significant differentially expressed genes for a false discovery rate equal to 0.001.

APC Reconstitution Assay and Blocking of APC Function by siRNA

SW480 cells were transiently transfected with 50 ng of phRL-TK as an internal control for transfection efficiency and 3 μ g of the expression plasmid. Three different plasmids were transfected using the LipofectAMINE PLUS method (Invitrogen), according to the manufacturer's instructions: pCMV-APC, containing full-length APC; pCMV-APC1309 Δ , which encodes a C-terminal truncated APC

mutant as negative control; and pCDNA 3.1 vector (Invitrogen), used as a transfection control. Experiments were performed on 4 independent days, and 11 replicas were obtained.

Alternatively, 293 human embryonic kidney cells were transfected overnight with the following siRNAs (50–75 nM final concentration) using LipofectamineTM 2000 protocol (Invitrogen): 2 validated siRNA against human APC (siAPC1 ID 42812, and siAPC2, ID 122391, both from Ambion, Austin, TX) and a negative control siRNA (ID 4626, Ambion). Forty-eight hours after transfection, RNA was extracted and target mRNA levels were measured by quantitative real-time PCR. Experiments were performed on 3 independent days, and 3 replicas were obtained.

Real-Time Quantitative PCR

Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed into cDNA using pdN6 primers and the MMLV reverse transcriptase (Invitrogen). Subsequent real-time PCR reactions were performed in the LightCycler 2.0 System (Roche, Indianapolis, IN) using the SYBR Green detection methodology. Primer sets were designed to specifically amplify cDNA by using oligos complementary to different exons of the gene: MAD2L1 forward, 5' GGTCCCTGGAAAGATGGCAG 3' and reverse, 5' ATCACTGAACGGATTTTCATCC 3'; BUB1B forward 5' AGGATCTGCCCGCTTCCC 3' and reverse, 5' GTCGTCTGATAGGTTACTGG 3', Stathmin forward, 5' GAACGTTTGGCAGAGAAGGATAA 3' and reverse, 5' GTCAGCTTCAGTCTCGTCAGCA 3'. β -2-Microglobulin and c-myc were used as controls.

Threshold cycle data were analyzed using the following formula: ratio = $((E_{target})^{\Delta C_{P_{target}}(control-sample)}) / ((E_{ref})^{\Delta C_{P_{ref}}(control-sample)})$ ¹⁵ to quantify the level of gene expression changes in adenomas and carcinomas relative to their corresponding normal mucosa. The gene expression levels were log₂ ratios. The log₂ ratios were applied to a *t*-test and a Wilcoxon test to evaluate their significance.

To evaluate the differences between APC1309 Δ and APC WT, with respect to pCDNA 3.1, we fitted a linear regression model adjusted by the effect of day. Significance was assessed by using the estimated parameters and standard error from the model. Box plots were used to visualize and interpret the observed effects with respect to the basal category represented by pCDNA 3.1. A significance level of 0.05 was set for the *P* values. Alternatively, expression levels after siRNA transfection for APC are expressed as log ratio with respect to the negative control siRNA.

Results

APC Loss of Function Is Associated With Failed Mitosis Completion

First, we sought to characterize whether eventual abnormalities during mitosis progression were associated

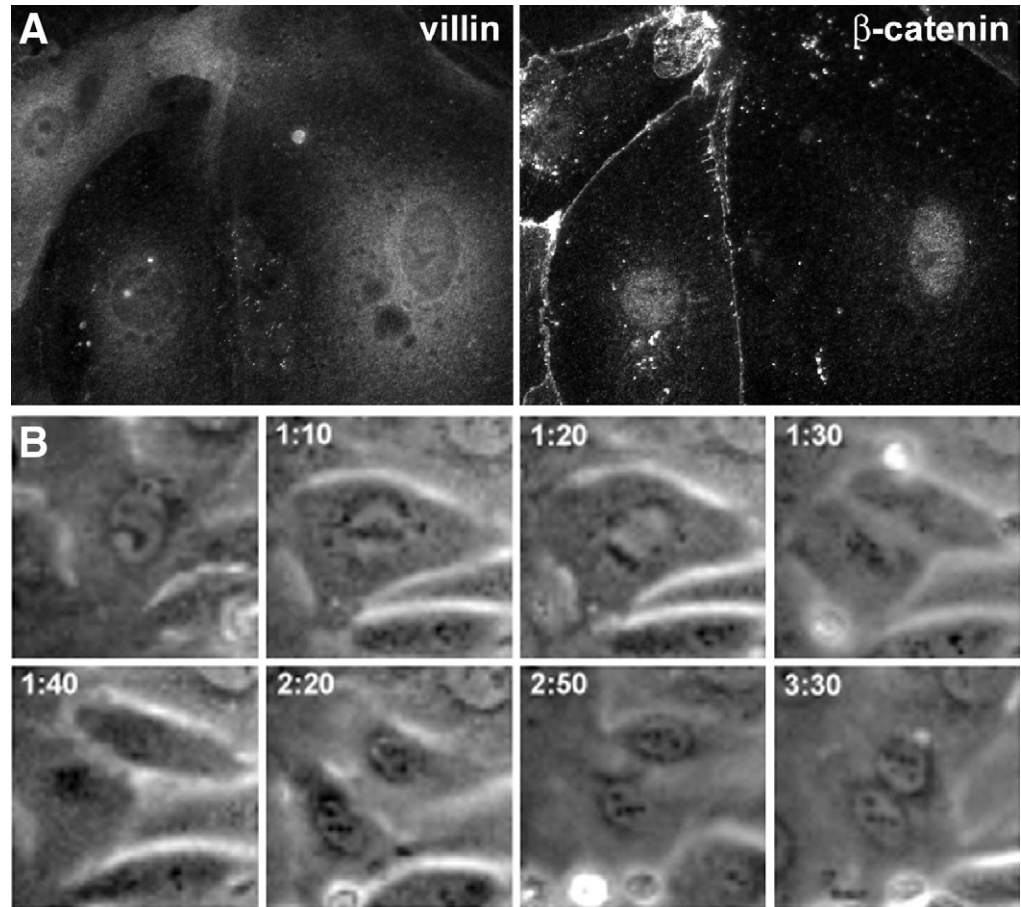


Figure 1. Mitotic defects in FAP primary cultures. (A) Primary epithelial cells from polyps isolated after the colectomy of an FAP patient, stained for the epithelial specific marker villin (*left*) and β -catenin (*right*). Note that β -catenin nuclear staining is present as a surrogate marker of mutated nonfunctional APC. (B) A phase-contrast video-microscopy sequence of a representative example of FAP primary epithelial cells showing failure during mitotic completion. Cells were able to enter mitosis, to congress chromosomes in a metaphase plate, and to separate sister chromatids, but they failed to finish the segregation of the presumptive daughter cells and ended up as polynucleated cells.

with inactivated APC in human and mouse digestive tumors. For this, we prepared primary short-term cultures from polyps isolated after the colectomy of an FAP patient and analyzed them by video-microscopy. FAP is 1 of the hereditary factors accounting for about 1% of all colorectal cancers, and the genetic basis for FAP lies in the germ-line (inherited) mutation of APC. The epithelial origin of the primary cultured cells was confirmed by labeling with the intestinal epithelial-specific marker villin (Figure 1A, left panel). The mutated status of APC was elicited by the inability to induce β -catenin degradation, as detected by prominent nuclear accumulation of β -catenin (Figure 1A, right panel). Two days after seeding, the cell cycle progression was analyzed by phase-contrast video-microscopy for up to 24 hours. Evidence of cell cycle defaults in FAP-epithelial primary cultures was observed during the completion of cytokinesis (Figure 1B). Approximately 20% of the cells that progressed through mitosis during the video recording were able to enter mitosis and to separate sister chromatids, but they failed to finally complete cell segregation and ended up as polynucleated cells (Figure 1B). Correspondingly, a quantification of nuclei per cell in fixed cells yielded essentially the same number of binucleated cells.

We have tried a variety of different culture conditions but we have not succeeded to obtain primary cultures of

normal human colonocytes, so to further confirm whether mitosis defaults were associated with APC loss of function, we turned to a mouse model that carried a targeted loss-of-function mutation at the endogenous *Apc* gene. The mouse line *Apc*^{1638N} has an autosomal dominant predisposition toward development of spontaneous colonic and intestinal tumors.⁹ These animals progressively develop intestinal tumors in a manner that is similar to that observed in patients with FAP. Likewise, abnormalities during the mitosis completion of primary epithelial cells derived from these lesions were observed. Epithelial cells typically presented abnormal multiple nuclei and supernumerary centrosomes (Figure 2A), a phenotype that could have been the result of an eventual incomplete cytokinesis and the fusion of presumptive daughter cells, as previously shown in the FAP films (see Figure 1B). Consistent with the mitotic defects observed in the FAP primary cultures, around 20% of the mitoses in the *APC*^{1638N} cells recapitulated the errors in cell segregation completion and the refusion of presumptive daughter cells observed in the human cells (Figure 2B). Correspondingly, a quantification of nuclei per cell in fixed cells yielded essentially the same number of binucleated cells (Figure 2B).

To exclude the possibility that the observed mitotic defaults in the digestive cancer cells could be due to the

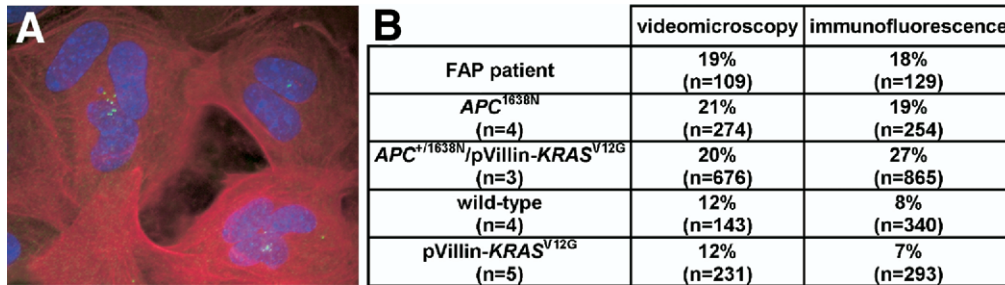


Figure 2. Transgenic mice carrying a mutated APC allele accurately recapitulate the human mitotic defaults associated with nonfunctional APC. (A) Polynucleated cells from *Apc*^{+1638N} primary tumor cells, most likely resulting from incomplete mitosis (as shown in Figure 1B for FAP), labeled with cyokeratin for epithelial origin (red) and presenting abnormal nuclei (DAPI, blue) and supernumerary centrosomes (anti- γ -tubulin staining, green). (B) Percentages of abnormal mitosis assessed by both video-microscopy and immunofluorescence in primary cultures from wild-type littermates (C57Bl/6J \times DBA/2) mice, histology-matched lesions from single transgenic *Apc*^{1638N} and pVillin-KRAS^{V12G} models, from the compound *Apc*^{+1638N}/pVillin-KRAS^{V12G} model, and from the FAP polyps. Note that similar percentages of mitotic abnormalities were found among human tumors and in mouse models presenting APC deactivation, but not in a mouse model with functional APC (pVillin-Kras^{V12G} line).

preparation of the primary cultures, we set up primary cultures from normal intestinal epithelial cells generated from wild-type littermates. The percentage of abnormal mitoses in normal epithelial primary culture cells was indeed only half as high as observed in the APC^{1638N} cells, as judged from both video-microscopy and immunofluorescence (Figure 2B) analysis.

Furthermore, primary tumor cultures were derived from transgenic pVillin-KRAS^{V12G} mice, which have been reported to be diploid and do not display genetic instability.¹⁰ The pVillin-KRAS^{V12G} mice express oncogenic human KRAS in the intestinal epithelium and develop spontaneous digestive adenocarcinomas in the presence of functional *Apc*.¹⁰ The tumors from KRAS-induced tumors did not show elevated numbers of aberrant mitoses or binucleated cells, when compared to the wild-type littermates (Figure 2B). Thus, in primary cultures derived from digestive murine tumors with nonmutated, functional *Apc*, we could not observe mitotic aberrations above the levels found in wild-type primary cells.

We finally evaluated the contribution of nonfunctional *Apc* in a compound animal model, the *Apc*^{+1638N}/pVillin-KRAS^{V12G} mice, that carried both the loss-of-function mutation at the endogenous *Apc* gene and the transgene encoding for the activated form of the human KRAS oncogene, and develop adenomas and adenocarcinomas in the gastrointestinal tract.¹⁶ Video-microscopy, performed on primary cultures from the compound *Apc*^{+1638N}/pVillin-KRAS^{V12G} mice, showed similar mitotic defaults and similar percentages of incomplete mitoses to those found in the FAP patient and the APC^{1638N} animals (20%; Figure 2B). Similar results were also found when we compared the percentages of polynucleated cells (27%; Figure 2B).

No difference in the time of mitosis completion between wild-type and APC-deficient cultures was observed. All of these data indicated a specific involvement for the inactivation of APC, but not oncogenic KRAS, in the increased incidence of compromised mi-

tos completion. When using a test for given proportions based on the chi-squared distribution, we analyzed the incidence of abnormalities during mitosis for those samples harboring mutant APC (FAP patient, *Apc*^{1638N}, *Apc*^{+1638N}/pVillin-KRAS^{V12G}) and compared them to those with functional APC (wild-type, pVillin-KRAS^{V12G}), significant statistical differences were found (*P* value <.0006, video-microscopy; *P* value <.00001, immunofluorescence).

Mitotic Gene Expression Profiling Associated With APC Loss of Function

To further explore the molecular basis of the observed abnormalities during mitosis in primary cells with mutated APC, we conducted a series of gene expression profiling experiments. Lesions from single transgenic pVillin-KRAS^{V12G} animals, which are diploid, did not present signs of genetic instability,¹⁰ and were found to have no obvious defects in mitosis. They were compared to age-, sex-, and histology-matched tumors dissected from the compound mutant *Apc*^{+1638N}/pVillin-KRAS^{V12G} littermates. The analysis was carried out on custom-generated arrays containing a 15k mouse gene set (described in detail: Janssen & Wagner et al, in preparation). Briefly, to extract the biologic information and remove experimental biases, microarrays were normalized; the differential expression of genes was assessed as described in the Materials and Methods section; and finally, we carried out our analysis using gene ontology¹⁷ in the functional annotation of the differentially expressed genes found by significance analysis of microarrays. We thus applied the method Gostat¹⁸ to obtain the gene ontology annotations that were significantly over-represented among the differentially expressed genes. We analyzed 8 tumors from pVillin-KRAS^{V12G} animals and 6 tumors from compound *Apc*^{+1638N}/pVillin-KRAS^{V12G} mice against the normal mucosa from wild-type littermates. This transcriptome analysis of tumor samples

Table 1. A List of Mitosis-Related Genes Found Altered in Association With APC-Dependent CIN

Gene code	Gene description	Increase
STMN1	stathmin 1/oncoprotein 18	4.7702
CCNB2	cyclin B2	3.6262
RANBP1	RAN binding protein 1	3.4847
BUB1B	budding uninhibited by benzimidazoles 1 homolog beta	3.4311
CCNB1	cyclin B1	3.2234
NUSAP1	nucleolar- and spindle-associated protein 1	2.9636
WEE1	WEE1 homolog	2.704
SMC4L1	SMC4 structural maintenance of chromosomes 4-like 1	2.6086
RAN	RAN, member RAS oncogene family	2.5616
CNAP1	chromosome condensation-related SMC-associated protein 1	2.554
MAD2L1	MAD2 mitotic arrest deficient-like 1	2.487
CETN3	centrin, EF-hand protein, 3	2.1549
ANAPC4	anaphase-promoting complex subunit 4	2.0603

Transcriptome analysis revealed that the genes related to mitosis were significantly up-regulated in the tumor samples from the compound *Apc*^{+/-1638N}/*pVillin-KRAS*^{V12G} mice, compared to the genetically stable tumors of the *pVillin-KRAS*^{V12G} mice.

dissected from transgenic mouse models revealed altered expression in a group of genes related to mitosis (Table 1).

The catalog of mitotic genes altered in a nonfunctional APC scenario included some related to spindle assembly, such as RAN, RANBP1, NUSAP1, and STMN1, genes associated with spindle formation and maintenance, such as CETN3, SMC4L1, and CNAP1, and genes of the regulatory machinery, such as B-type cyclins, WEE1, and ANAPC4. The alteration of the genes involved in spindle formation and positioning is consistent with the observations that a truncation in APC dominantly interferes with the mitotic spindle.

More interestingly, and closely related to the described stepwise signaling that directs final mitosis segregation and the completion of cytokinesis, 2 genes related to the control of the mitotic checkpoint, BUB1B and MAD2L1, resulted in up-regulation in the APC inactivated scenario. BUB1B encodes a kinase localized to the kinetochore, which plays a role in the inhibition of the anaphase-promoting complex/cyclosome and delays the onset of anaphase, ensuring proper chromosome segregation. MAD2L1 is also a component of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate.

Modulation of APC Expression Levels Induces Changes in the Transcription of BUB1B, MAD2L1, and STMN1

To investigate the eventual causative relationship between the inactivation of APC and the up-regulation of these mitotic related proteins, we assessed whether their

expression levels changed by transfecting wild-type APC in the APC mutant SW480 cells. Transfection of functional APC is known to restore the cellular capacity to down-regulate β -catenin, and subsequently reducing β -catenin/Tcf-4 signaling. A decrease in the levels of *c-myc* expression, a known β -catenin/Tcf-4 target, was used as a control of the efficiency of wild-type APC introduction. This resulted in a reduced expression of BUB1B, MAD2L1, and STMN1, with statistical significance for BUB1B and STMN1 (Supplementary Figure 1; see supplemental material online at www.gastrojournal.org).

Furthermore, silencing of APC by siRNA transfection in 293 cells, which express wild-type APC, resulted in a 50% to 65% reduction of endogenous expression. This down-regulation of APC induced a 1.5-fold increase of *c-myc* expression levels, demonstrating a reduction in β -catenin/Tcf-4 signaling. Interestingly, a modest but consistent increase for MAD2L1 (1.22-fold) and STMN1 (1.17-fold) expression levels was detected. However, heterogeneous results were obtained for BUB1B expression (Supplementary Figure 2; see supplemental material online at www.gastrojournal.org).

Altogether, slight but consistent changes were found in the expression levels of mitosis-related genes when APC expression was experimentally modified. These results suggest a causal role of APC in the transcriptional regulation of BUB1B, MAD2L1, and STMN1.

MAD2L1, BUB1B, and STMN1 Expression in Human Tumors

To confirm and validate findings observed in the animal model, we analyzed a panel of human adenomas from 6 different FAP patients (10 adenomas each) and their corresponding normal mucosa (see the Materials and Methods section). Within these samples, using real-time quantitative PCR, we evaluated the expression of the most up-regulated gene, involved in spindle formation and positioning, STMN1, and investigated whether BUB1B and MAD2L1 up-regulation also correlated to the loss of function of APC during human colorectal tumorigenesis.

Information regarding the relative expression values of the above-mentioned genes in the 60 FAP adenomas analyzed is summarized in Figures 3 and 4B. The median values of BUB1B, MAD2L1, and STMN1 expression were significantly higher in the majority of adenomas when compared to corresponding mucosa, although some intracase and intercase variability was observed. Noteworthy was the fact that the expression profiles of the 3 analyzed genes were very similar for each subject, suggesting possible coexpression (Figure 4B). Interestingly, when we compared macroscopically normal colonic mucosa from each FAP patient to a pool of different normal mucosa from sporadic colorectal cancer patients, we detected higher expression values for the 3 genes in the nor-

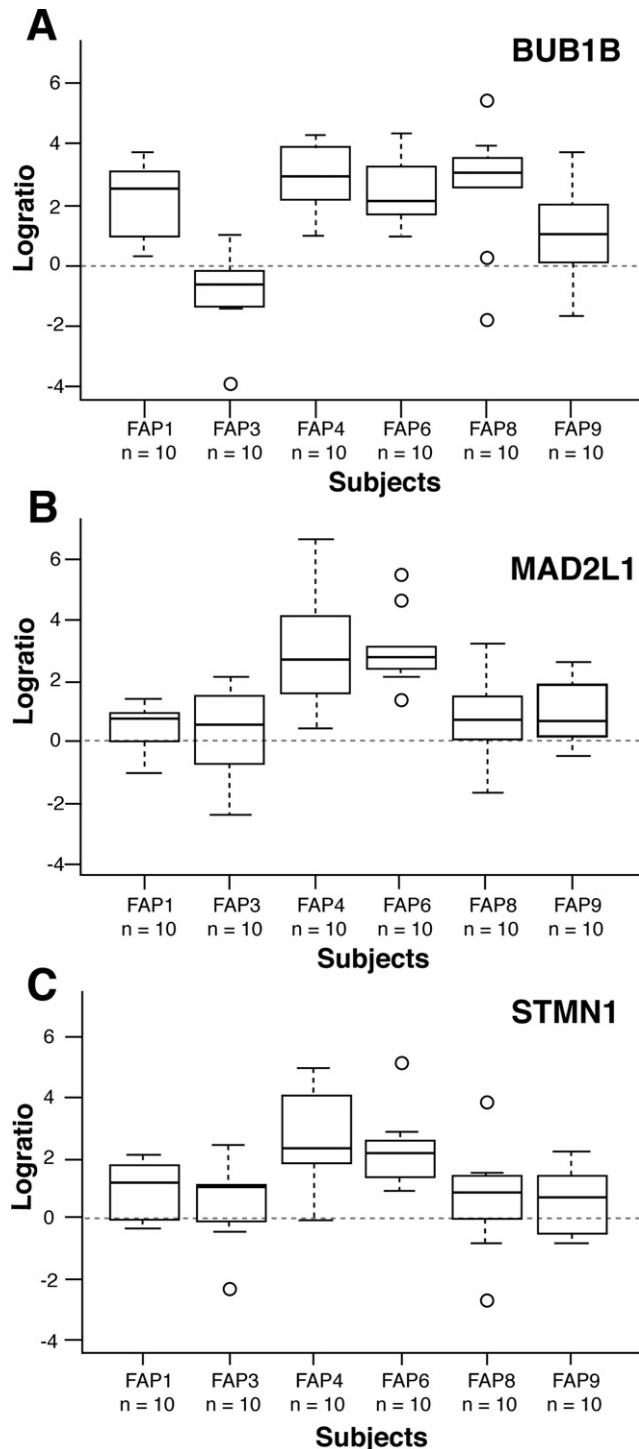


Figure 3. The expression levels of BUB1B (A), MAD2L1 (B), and STMN1 (C) in FAP adenomas. Ten adenomas per FAP case were analyzed. Box plots indicate the median value (center line), and the first and third quartiles are the edges of the box area (interquartile range). Points at a greater distance from the median than 1.5 times the interquartile range have been plotted individually as dots and are considered to be potential outliers. The level of expression is expressed in a log₂ ratio (adenoma/normal mucosa). Most of the values are over 0, thus, indicating an overexpression of these 3 genes in almost all of the subjects (BUB1B $P = .03$; MAD2L1 $P = .03$; STMN1 $P = .02$).

mal FAP mucosa (Figure 4A; t -test BUB1B P value = .002; MAD2L1 P value = .008; STMN1 P value = .008). Because each analyzed FAP patient harbored a known pathogenic germ-line APC mutation (Supplementary Table 1; see supplemental material online at www.gastrojournal.org), these results suggested that the inactivation of 1 allele could be sufficient to promote higher levels of transcription.

To study the behavior of BUB1B, MAD2L1, and STMN1 expression levels in colorectal cancer progression, we expanded our analysis to a panel of 10 sporadic colorectal adenomas and carcinomas (Figure 4C and D, respectively). Both sporadic adenomas and carcinomas showed increased levels for the 3 genes when compared to their corresponding normal tissue in the majority (8 out of 10) of the analyzed cases. All differences were statistically significant with the exception of MAD2L1 and STMN1 in carcinomas (adenomas: t -test; BUB1B P value = .01 MAD2L1 P value = .02; STMN1 P value = .04) (carcinomas: t -test BUB1B P value = .02; MAD2L1 P value = .06; STMN1 P value = NS). It is of note that in 2 cases (cases 220 and 731), the down-regulation of these genes was observed. Similar to what occurred in the FAP adenomas (Figure 4B), the 3 genes followed a similar pattern of expression in all of the sporadic adenoma-carcinoma paired samples, suggesting putative coexpression.

To further explore whether this up-regulation was present at the protein level, BUB1B and MAD2L1 immunohistochemical staining was performed on a set of 4 paired adenomas and mucosa FAP samples and on 10 paired sporadic colorectal adenomas, carcinomas, and normal mucosa (Figure 5). BUB1B and MAD2L1 protein overexpression was clearly detectable in all of the adenomas and carcinomas tested. Taken together, we were able to identify specific changes in gene expression in the APC-mutated intestinal cancer that occurred in both the human patients, as well as in the preclinical mouse models.

Discussion

Our results indicate a specific association between mutations in the tumor suppressor gene APC and an increased incidence in mitotic defects, as demonstrated both in clinical samples of colorectal cancer as well as in genetically defined mouse models. The occurrence of cell cycle defects was independent of the oncogene KRAS. It is conceivable that mutated APC could play a dual role for the observed phenotype: APC is not only a master regulator of the Wnt signaling pathway, but it is directly involved in the mitotic spindle apparatus. However, the direct contribution of the Wnt pathway to the mitotic alterations seems to be unlikely, as β -catenin is absent from the kinetochores.⁴

To get a broader overview of the molecular pathways involved in the observed mitotic aberrations, we carried out a gene expression profiling approach. Interestingly, we detected the differential expression of several key

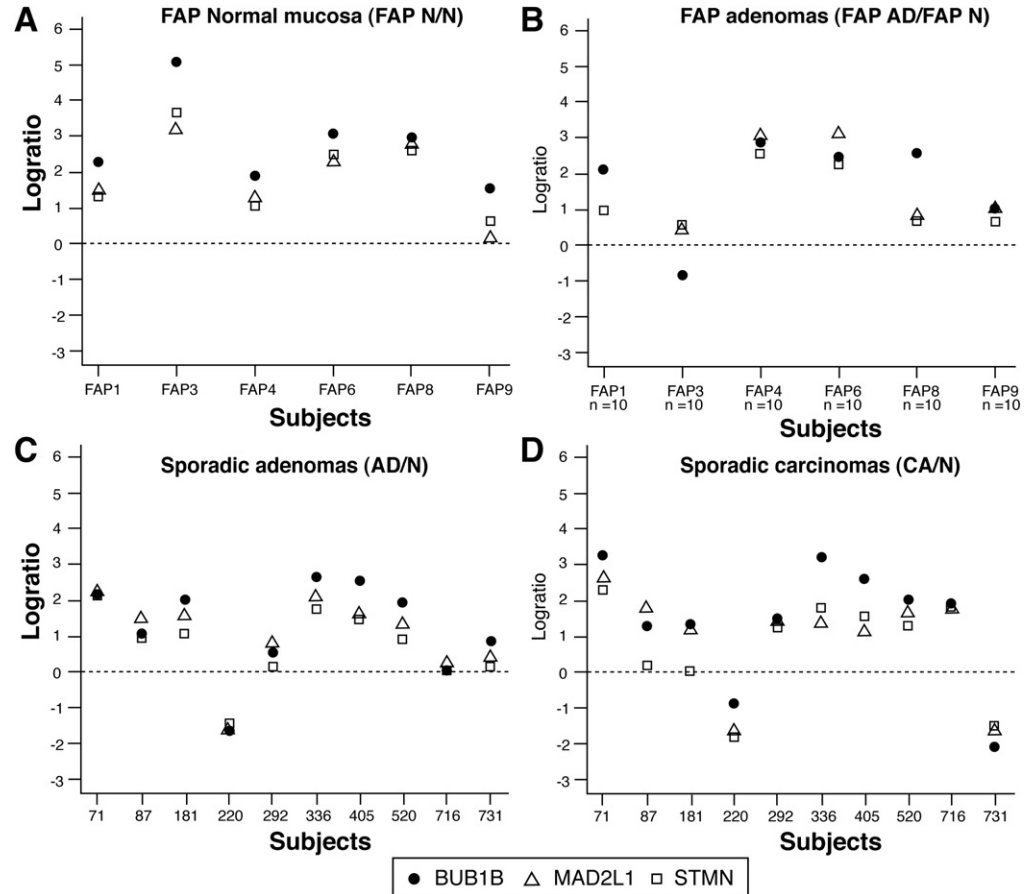


Figure 4. The relative expression levels of BUB1B, MAD2L1, and STMN1 in normal mucosa (FAP and sporadic), FAP adenomas, sporadic adenomas, and sporadic carcinomas. (A) Log2 ratio for each normal FAP mucosa (n = 6)/pooled normal colorectal mucosa. (B) Median log2 ratio values for 10 FAP adenomas of a given case (n = 6)/corresponding normal mucosa. (C) Log2 ratio values for each (n = 10) sporadic adenomas/corresponding normal mucosa. (D) Log2 ratio values for each (n = 10) sporadic colorectal carcinoma/corresponding normal mucosa.

genes involved in cell division and mitosis. To the best of our knowledge, so far most of the genes from this group have not been described as target genes of the canonical Wnt pathway (<http://www.stanford.edu/~rnusse/pathways/targets.html>). The up-regulation of the mitotic checkpoint proteins, BUB1B and MAD2L1, suggests that they are related with the increased incidence of mitotic abnormalities associated with the APC loss of function. Accordingly, Kaplan and coworkers described that during mitosis, APC localized to the ends of microtubules embedded in kinetochores, forming a complex with the checkpoint proteins, Bub1 and Bub3. Moreover, in vitro, APC was a high-affinity substrate for Bub kinases.⁴ It is conceivable that APC sustains or amplifies the BUB1/BUBR1 checkpoint signal, thus contributing to the fidelity of chromosome segregation. These authors also described the retention of proteins like BUB1B and MAD2L1 in CIN-positive cells, although not due to a general failure in the down-regulation of checkpoint proteins during anaphase onset.⁶ In fact, a normal checkpoint response was described for APC mutant ES cells displaying extensive chromosome and spindle aberrations,³ as well as in *Min/Min* embryonic cells.⁴ More recently, Tighe and coworkers associated shorter mitotic spindles in cells expressing APC fragments with reduced tension across the metaphase kinetochores and increased

levels of kinetochore-bound BUB1 and BUB1B. Because the dissociation of BUB1 and BUB1B from the kinetochores of aligned chromosomes appeared to be dependent on microtubule occupancy and tension, respectively,¹⁹ the expression of the APC mutants may weaken the kinetochore-microtubule interactions. This would reduce interkinetochore tension, resulting in an increased level of checkpoint proteins bound to the kinetochores.²⁰

Transcriptional analysis obtained in animal models for the FAP syndrome (*Apc*^{1638N}) corresponded indeed to the transcriptional changes observed in FAP patients. In accordance, in vitro experiments to either reconstitute or block APC function also suggested that APC is involved in transcriptional control of mitotic checkpoint genes.

Taken together, inactivated APC may be a key element contributing to an aberrant up-regulation of genes involved in the correct completion of mitosis. Importantly, STMN1, BUB1B, and MAD2L1 up-regulation represented an early event in colorectal tumorigenesis, because it was already present in the microscopically normal mucosa of FAP patients (Figure 4A). Very recently, Mad2 overexpression in mice led to tumor initiation, most likely through the acquisition of a CIN phenotype, which may take place prior to overt transformation.²¹ A single hit on the APC gene may be sufficient to promote this up-regulation, which is maintained during tumorigenesis

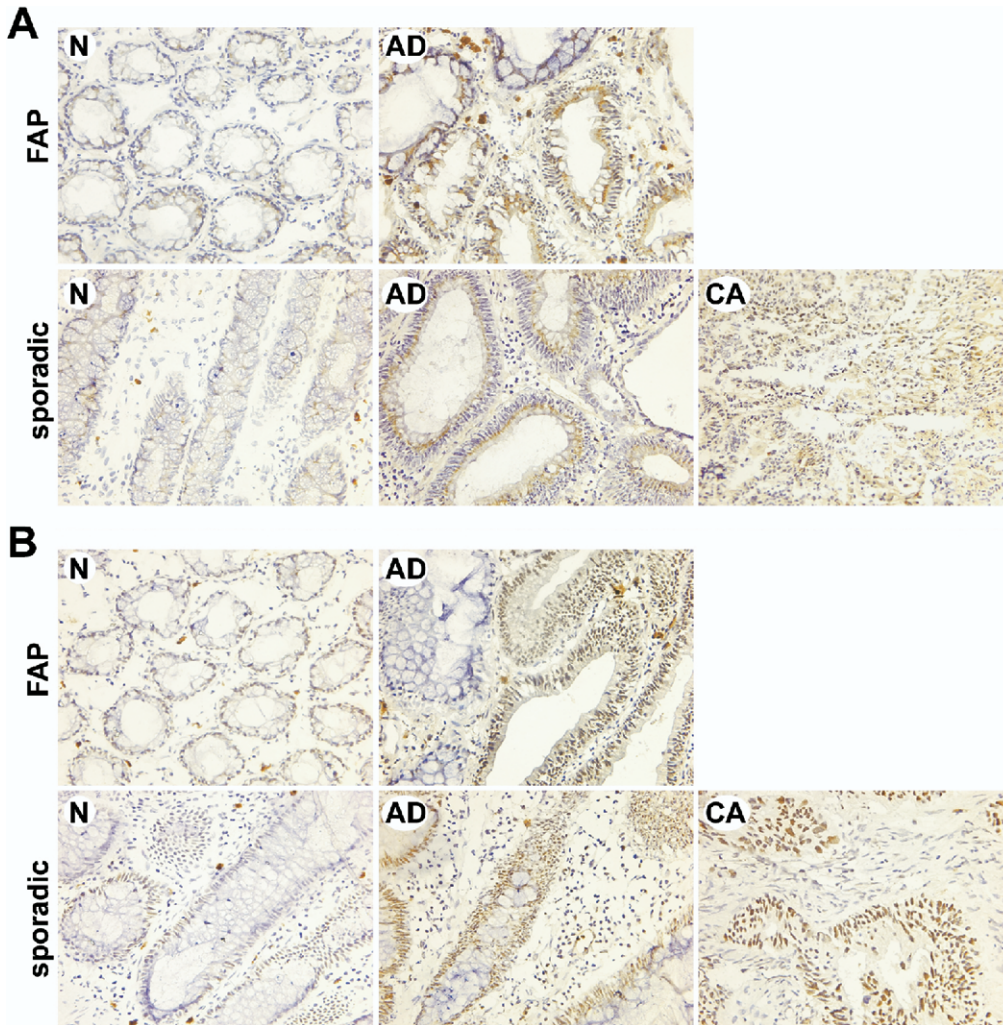


Figure 5. The immunostaining pattern of BUB1B (A) and MAD2L1 (B) in representative examples of FAP and sporadic adenomas and sporadic carcinomas from the analyzed series. (A) In all adenomas (AD) and carcinomas (CA), the cytoplasmic overexpression of BUB1B was evident, while weak immunostaining was observed in the nonneoplastic cases corresponding to colonic epithelium (N) (magnification 40 \times). (B) MAD2L1 showed a nuclear localization in adenomas and in the carcinomas. There was an increase in immunostaining in tumor tissues in comparison with the corresponding normal tissues. Stromal regions of the tumors did not show immunostaining. Non-specific macrophage staining was present in both nonneoplastic epithelium and adenomas.

in adenomas and in more advanced stages of tumor progression. This is in accordance with a possible role for APC in chromosomal instability as an early step related to mitotic abnormalities.^{22,23} The deletion of 1 MAD2 allele has been shown to result in an elevated rate of chromosome missegregation and the implication of mitotic checkpoint impairment in tumorigenesis.²⁴ The overexpression of MAD2 in colorectal cancer has been reported to correlate to p53 expression,²⁵ and aberrant expression of the protein has been postulated to compromise mitotic events, predisposing cells to genomic instability.²⁶ Overexpression of the homologous gene MAD2L2 has been shown to coincide with the occurrence of anaphase bridges and bad prognosis in human colorectal cancer.²⁷ Interestingly, the inactivation of both the MAD2- and p53-dependent checkpoint pathways exhibits an extraordinarily high level of CIN, due to the lack of a functional spindle checkpoint.²⁸ Along the same line of evidence, results obtained from *BUB1B*^{+/-} *APC*^{Min/+} compound mice suggested a functional interaction between these 2 genes in regulating the metaphase to the anaphase transition. The impairment of this in-

teraction could play a role in genomic instability and the development and progression of colorectal cancer.²⁹ The analysis of the signaling pathways involved in the abortion of cytokinesis will clarify the association of incomplete cytokinesis and the up-regulation of the mitotic proteins. Defects in the mitotic spindle triggering the abortion signals, lagging chromosome leading to furrow regression, chromosome nondisjunction, or the presence of a hyperactive checkpoint,²¹ are possible mechanisms that deserve further investigation.

As suggested by the group of K.B. Kaplan, the simultaneous failure to properly align chromosomes and the inability of the spindle checkpoint to sense these failures represent a potent combination that could contribute to chromosome instability in tumor cells.⁷ Even though further work must be done to completely understand how APC may regulate the metaphase to anaphase transition, we speculate that APC-related abnormalities during proper chromosome segregation must be accompanied by an adjustment of the machinery that surveys the genetic integrity. This could balance the increasing genomic instability and ensure a real growth advantage for tumor cells (Figure 6).

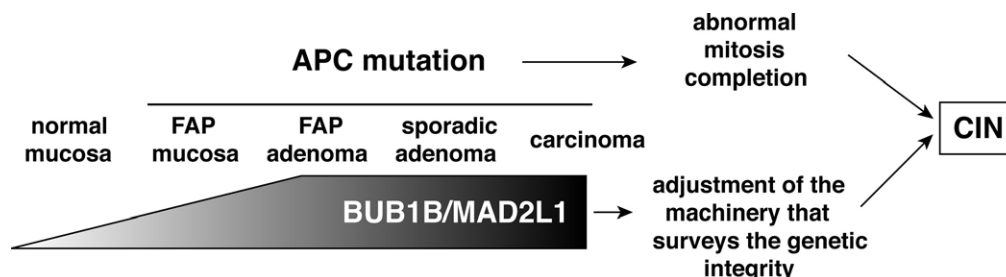


Figure 6. A model for the contribution of mutated APC to CIN. Video-microscopy on both human colorectal samples and on genetically defined mouse models showed that mutations in APC were associated with an increased incidence in cell cycle defects during cytokinesis completion. These defects were not observable in tumors with functional APC alleles. Transcriptome analysis performed on animal models that accurately mimicked the human clinics, demonstrated an increase in BUB1B and MAD2L1 expression on both FAP and sporadic colorectal samples. This was confirmed by real-time quantitative PCR and immunohistochemistry. Therefore, we propose the following model: APC has a concomitant role in correct spindle assembly and positioning, and in a consequent readjustment of the surveillance mechanism that integrates the spindle checkpoint. This dual role is the proposed basis of the contribution of mutated APC to CIN in colorectal cancer.

The triggering of apoptosis and the clearance of highly unstable cells were reportedly linked to the presence of extensive numerical and structural chromosomal rearrangements in APC mutant ES cell lines. Accordingly, an increase in ploidy was observed when these cells were rescued from apoptosis by overexpressing Bcl-2.^{3,22} The analysis of chromosomal instability in human colorectal adenomas with 2 mutational hits at APC suggests a subtle effect in vivo for APC mutations leading to CIN.³⁰ Subtle changes that affect chromosome segregation could balance cell viability with the potential selective advantages derived from changes in ploidy. Genetic instability thus accompanies tumor progression, as it continuously ensures sufficient genetic variability to overcome additional selection barriers.³¹ This hypothesis also includes the possibility of a negative feedback mechanism resulting from CIN.

In conclusion, we found that the APC-mutant animal models accurately mimicked both the mitotic defects found in the FAP human primary cultures, as well as the changes in mitosis-related gene expression. These changes, validated in a large set of human tumor samples, provide additional evidence on the involvement of APC during correct spindle assembly and positioning, and further support a key role of APC in the generation of chromosomal instability.

Appendix

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1053/j.gastro.2007.03.027](https://doi.org/10.1053/j.gastro.2007.03.027).

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Address requests for reprints to: Sylvie Robine, PhD, UMR 144 CNRS/Institut Curie, 26 rue d'Ulm, 75248 Paris Cedex-05, France. e-mail: Sylvie.Robine@curie.fr; fax: (33) 1 42346377.

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