**SUPPLEMENTARY METHODS**

***Patients***

In addition, patient molecular data including SNP microarray, mutation and RNA-Seq results produced by The Cancer Genome Atlas Network were retrieved from the Genomic Data Commons Data Portal (National Cancer Institute) for the following cohorts: Adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), diffuse large B-cell lymphoma (DLBC), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), acute myeloid leukemia (LAML), low grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), thyroid carcinoma (THCA), thymoma (THYM), uterine corpus endometrial carcinoma (UCEC), uterine carcinosarcoma (UCS) and uveal melanoma (UVM).

***Generation of isogenic HCT116 MACROD2 knock-out cells***

HCT116-Cas9 stable cells were generated using the FUCas9Cherry lentivirus system (Dr Marco Herold, Addgene #70182) by expansion of a single-cell derived RFP-positive clone isolated using fluorescence-activated cell sorting (FACS) (BD FACSAria III, Becton Dickinson). To generate *MACROD2* knock-out cells, guide RNA was designed against *MACROD2* exon 2: gRNA-F 5’-TCCCGAACAGCATTCTATCATGGA-3’, gRNA-R 5’-AAACTCCATGATAGAATGCTGTTC-3’. Guide RNA was cloned into the FgH1tUTG vector (Dr Marco Herold, Addgene #70183) and the insert confirmed by DNA sequencing. HCT116-Cas9 cells were infected with guide RNA lentivirus and induced with doxycycline. On day 6 post-induction, single RFP/GFP double-positive cells were FACS sorted in to 96 well plates. Single-cell derived clones with heterozygous or homozygous *MACROD2* knock-out were identified using DNA sequencing for *MACROD2* exon 2: forward primer 5’-AATGGAGATTCTGCTTTTATTTTGC-3’; reverse primer 5’-ACTTACCATCATTTTGGCCCT-3’. Details of the selected isogenic HCT116 clones are provided in **Supplementary** **Fig. S4**.

***Cell line transfections and plasmids***

CRC cell line transfections with siRNAs and plasmids were conducted using Lipofectamine 2000 (Invitrogen) as per manufacturer’s instructions for the following gene expression vectors, shRNA constructs and siRNAs: pEGFP (ClonTech), pMACROD2-EGFP (kindly provided by Dr Ivan Ahel), pMDC1-EGFP (Dr Eric Campeau, Addgene # 26427), DR-GFP (Dr Maria Jasin, Addgene # 26475), pCBASce (Dr Maria Jasin, Addgene # 26477), pMACROD2Δex11-17-EGFP (provided by Dr Tero Ahola), siRNA-MACROD2 (target sequence 5’-AAGAGAATGATTCAACGAAGA-3’, Life Technologies Australia) and siRNA-Control (target sequence 5’-AATTCTCCGAACGTGTCACGT-3’, Life Technologies Australia). The plasmid constructs, pRETROSUPER-NEO-GFP-shMACROD2 (target sequence 5’-AAGAGAATGATTCAACGAAGA-3’), pRETROSUPER-NEO-GFP-shNEG (target sequence 5’-AATTCTCCGAACGTGTCACGT-3’) were generated by cloning of the target sequences into pRETROSUPER-NEO-GFP vector (Oligoengine). pMACROD2Δex4-EGFP, pMACROD2Δex5-EGFP, pMACROD2Δex4-5-EGFP and pMACROD2Δex1-8-EGFP were generated by PCR amplification of the respective *MACROD2* wild-type and deletion transcripts from CRC cell lines cDNA and cloning into pEGFP vector (ClonTech). All vectors were verified by DNA sequencing. For gene expression and shRNA constructs, transfected cells were maintained in selective media to achieve stable integration, and single-cell derived clones isolated using FACS. Control (D-001206-14-05) and MACROD2 (MQ-006656-01-0002) siRNAs were obtained from Dharmacon.

***Mutation detection and mRNA transcript analysis***

*MACROD2* mutation detection (all exons) for tumor samples was performed using high resolution melting (HRM) analysis with SsoFast EvaGreen Supermix (Bio-Rad) on a 7500 Fast Real-Time PCR system (Applied Biosystems). Primer sequences are provided in **Supplementary** **Table S9**. For amplification quality control, PCR products were subsequently run on an agarose gel. Tumor samples with melting curve shifts and matched normal samples were sequenced in forward and reverse orientations from new PCR products and mutations identified. DNA sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and samples were analyzed on a 3130xl Genetic Analyzer (Applied Biosystems). *APC* and *TP53* mutation detection (all exons) was performed by direct DNA sequencing on a 3730xl Genetic Analyzers (Applied Biosystems) as described previously ([1](#_ENREF_1)). For CRC cell lines *MACROD2* mutations were identified from previously generated whole-exome sequencing data ([2](#_ENREF_2)). Sequencing of MACROD2 transcripts was performed on cDNA produced using the SuperScript® III First Strand Synthesis Kit (Thermo Fisher Scientific).

***Macrod2tm1.1(KOMP)Vlcg and ApcMin/+ mice***

All animal procedures were approved and conducted in accordance with the Animal Ethics Committee of the Walter and Eliza Hall Institute of Medical Research (Parkville, VIC, Australia). *Macrod2* knock-out mice (Macrod2tm1.1(KOMP)Vlcg) were obtained from the Knockout Mouse Program Repository (KOMP) of the Jackson Laboratory. In this strain, a *lacZ* reporter (ZEN-UB1 Velocigene cassette) has been inserted into the *Macrod2* locus, creating a deletion of size 19,224 base pairs between positions 140226712-140245935 of chromosome 2 (Genome Build37) resulting in premature truncation of the Macrod2 protein at amino acid 53. Mice heterozygous for *Apc* (*ApcMin/+*) have been described previously ([3](#_ENREF_3)). Both *ApcMin/+* and Macrod2tm1.1(KOMP)Vlcg mice are on an inbred C57BL/6 background. Animals were genotyped for *Apc* and *Macrod2* with three-primer, allele-specific PCR assays. Primer details are provided in **Supplementary** **Table S10**.

Male *ApcMin/+* mice were bred with female *Macrod2-/+*mice. Of the F1 generation, male *ApcMin/+*/*Macrod2-/+* mice were intercrossed with *Apc+/+*/*Macrod2-/+* females. Our three study groups were *ApcMin/+/Macrod2+/+*, *ApcMin/+/Macrod2-/+* and *ApcMin/+/Macrod2-/-* mice; our three control groups were *Apc+/+/Macrod2+/+*, *Apc+/+/Macrod2-/+* and *Apc+/+/Macrod2-/-* mice. Animals of the six groups arose at the expected Mendelian frequencies (*p*=1).

*Macrod2+/+*, *Macrod2-/+* and *Macrod2-/-* MEFs were generated by the collection of day 13.5 embryos. Embryos were washed with phosphate-buffered saline (PBS) and head and visceral tissues removed from isolated embryos. The head from each embryo was used for genotyping. The remaining bodies were washed in fresh PBS, minced using a pair of scissors, transferred into a 0.1 mM trypsin/1 mM EDTA solution (3 ml per embryo), and incubated at 37°C for 20 min. After trypsinization, an equal amount of medium (6 ml per embryo DMEM containing 10% FBS) was added, tissues disassociated by pipetting and supernatant transferred into a new tube. Cells were collected by centrifugation (200 × g for 5 min at 4°C) and resuspended in fresh medium. 1 × 106 cells (passage 1) were cultured on 100 mm dishes at 37°C with 10% CO2.

***Immunohistochemistry***

For immunohistochemical analysis, mouse tissue sections were prepared and de-paraffinized. Heat-induced antigen retrieval was performed in 10 mM citrate buffer (pH 6, Thermo Scientific). Endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide (v/v; BioLab). Immunohistochemical stains were performed with antibodies against β-catenin (Becton Dickinson, #610153, 1:500) and γ-H2AX (Cell Signaling Technologies, #9718, 1:500). Immunoperoxidase staining was detected with the 3-3’-Diaminobenzidine (DAB) Liquid Substrate System (Dako), and sections were counterstained with hematoxylin. Staining was quantified using MetaMorph software (Molecular Devices) blinded to animal genotype.

***Quantitative real-time RT-PCR***

QRT-PCR for gene expression was conducted on cDNA (SuperScript® III First Strand Synthesis Kit, Thermo Fisher Scientific) using SYBR Green Reagent (BioRad) on a 7500 Fast Real-time PCR System (Applied Biosystems). Details of the human and mouse primers used for the WNT target genes *CD44*, *AXIN2* and *DVL1*, and for the housekeeping gene *HMBS* (human) or *Hprt* (mouse) are provided in **Supplementary Table S11**. For WNT3A stimulation assays, CRC cell lines were incubated with 50% WNT3A condition medium or 50% control medium for 24 hours, cell pellets collected and RNA extracted with the QIAGEN RNeasy Minikit (Qiagen) following manufacturer instructions.

***TCF reporter assay***

HCT116, LOVO and HEK293T TCF/LEF reporter cells were generated using a Cignal T cell factor/lymphoid enhancer factor (LEF/TCF) luciferase reporter consisting of tandem TCF/LEF binding sites (containing the sequence AGATCAAAGGGGGTA) with a cytomegalovirus (CMV) minimal promoter and a separate human phosphoglycerate kinase (PGK) promoter that constitutively drives the expression of a puromycin resistance gene packaged into a vesicular stomatitis virus G protein (VSV-G) pseudotyped lentivirus (Qiagen). Stable TCF/LEF lines were maintained under selection for three weeks, single cell sorted to generated clones which were then cryogenically preserved. Frozen stocks were subsequently used for assays. Impact of MACROD2 on WNT3A responsiveness was assessed by transfection of reporter cell lines with siRNA-MACROD2 or siRNA-Control, serum starvation for 24hrs prior to addition of WNT3A conditioned medium (50%) for 48hrs. Cells were then subjected to flow cytometry analysis (FACScan; Becton Dickinson) using CellQuest 3.2 software (Becton Dickinson), 20000 events were collected and analyzed using FloJo X software (FloJo LLC).

***RNA-Seq analysis***

RNA samples from intestinal tumors of *ApcMin/+/Macrod2-/-*,*ApcMin/+/Macrod2-/+* and *ApcMin/+/Macrod2+/+* mice (6 tumors per group) were prepared for sequencing using the TruSeq Stranded Total RNA Library Preparation Kit. Libraries were pooled and clustered using the cBot system (Illumina) with TruSeq SR Cluster Kit v3 reagents (Illumina). Sequencing was performed with HiSeq Kit v4 reagents (Illumina) on the Illumina HiSeq 2500 system at the AGRF. Each sample was sequenced to a depth of at least 26 million single end reads of 100bp. Sequencing reads were quality assessed using FASTQC ([4](#_ENREF_4)) and trimmed for sequencing adaptors using Trimmomatic (v0.22) ([5](#_ENREF_5)); reads shorter than 50 bp were removed. Reads were aligned to mouse genome build mm10 using Tophat v2.0.8 ([6](#_ENREF_6)) with parameters -g 1, --library-type fr-firststrand. Gene level expression was quantified using UCSC mm10 annotation and featureCounts with a parameter for stranded counting (-s 2) ([7](#_ENREF_7)). Data have been deposited in the Gene Expression Omnibus (GSE93514).

***Western blot analysis***

Total cellular proteins were extracted by solubilizing cells in protein lysis buffer (1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM MgCl2, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na3VO4, 10% glycerol) in the presence of protease inhibitors (Sigma, #04693116001 and #04906845001). Extracts were collected by centrifugation, and quantified with the BCA Protein Assay Reagent kit (Thermo Fisher Scientific). For PARP1 immunoprecipitation,cell lysates (100ug) were pre-cleared with Protein A beads (Sigma) for 1hr at 4ºC, prior to formation of immune complex with mouse anti-PARP1 antibody (2.5ug, Abcam, ab110915) overnight at 4ºC. Lysates were boiled in 5x SDS sample buffer and ran on 3-8% Tris-Acetate SDS PAGE gel. The following primary antibodies were used: rabbit anti-MACROD2 (in house, see below), rabbit anti-(mono)ADP ribose (Merck Milipore, MABE1076), mouse anti-poly(ADP)ribose (Enzo, ALX-804-220-R100), mouse anti-vinculin (Sigma, V9264), mouse anti-GFP (OriGene, TA150052) and rabbit anti-PARP1 (Abcam, ab6079). Western blot detection was performed with corresponding mouse or rabbit secondary antibodies conjugated to IRDye infrared fluorescent dyes (Licor).

***Generation of immunopurified polyclonal anti-MACROD2 antibody***

Polyclonal anti-MACROD2 antibody was produced from rabbit serum immunized with MACROD2 peptide (45-60: EEMKGKGQNDEENTQE) (Mimotopes) conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce). Immunoglobulins were first purified from serum using Protein-A Sepharose 4 Fast Flow antibody purification resin (GE Healthcare). Anti-MACROD2 antibodies were then immunopurified using MACROD2-peptide conjugated to NHS-activated Sepharose (GE Healthcare). Antibody immunopurification was monitored using biosensor analysis with immobilized MACROD2 peptide conjugated to a carboxymethylated dextran sensor surface (CM5, GE Healthcare) using NHS/EDC chemistry. Anti-MACROD2 IgG was then purified by size-exclusion chromatography using a Superose 12 HR 3.2/30 column equilibrated in PBS and connected to an Agilent 1100 HPLC (Agilent Technologies). Immunopurified anti-MACROD2 antibody was validated by Western blot using HeLa cells transfected with FLAG-MACROD2 fusion protein (**Supplementary Fig. S23**).

***Clonogenic assay***

Cells were seeded at a density of 200 cells/well in a 24-well plate and incubated overnight. Cells were treated with either doxorubicin (6 point, 4-fold dilution series starting at 1.25uM) or γ-irradiation (0, 2, 4, 8 and 10Gy) and cultured for 10 days to allow colony formation. Colonies were fixed with 10% buffered formalin and stained with crystal violet (0.5% w/v). Plates were scanned on an Odyssey Imaging System (Licor) and the number of colonies counted using Image J software (FiJi).

***PARP1 activity assay***

CRC cells (104 cells/well) were seeded into 96 well plates and either γ-irradiated with 10Gy using a Cobalt 60 source or treated with 0.5 µM doxorubicin. PARP1 activity was measured at 2h and 4h using the Universal Chemiluminescent PARP Assay Kit (Trevigen).

**Caspase-3/7 activity assay**

CRC cells (3x104 cells/well) and MEF cells (5x104 cells/well) were seeded into 96 well plates and γ-irradiated with 10Gy using a Cobalt 60 source or treated with 0.5µM doxorubicin. At 72hr after treatment, Caspase-3/7 activity was measured using Caspase-Glo® 3/7 Assay Kit (Promega, Madison, WI) as per the manufacturer’s instructions.

***Annexin V apoptosis assay***

CRC cells (1x105 cells/well) and MEF cells (1x105 cells/well) were seeded into 24 well plates and γ-irradiated with 10Gy using a Cobalt 60 source or treated with 0.5 µM doxorubicin. At 24 hr after treatment, apoptosis was assessed by staining with annexin V-fluorescein isothiocyanate (FITC) and the nucleic acid dye 7-aminoactinomycin D (7-AAD; Apoptosis Detection Kit; BD PharMingen). Stained cells were analyzed by flow cytometry on a FACScalibur using CellQuest software (Becton Dickinson). A total of 20,000 events were collected.

***Immunofluorescence microscopy***

Cells were grown overnight on glass cover-slips, stimulated with WNT3A conditioned medium for 16 hours or subjected to γ-irradiation with 2Gy or treatment with 0.5µM Doxorubicin (0, 1 h, 2 h and 4 h). Cells were fixed in 4% paraformaldehyde and permeabilized with 100% ice-cold methanol for 30 min. Samples were blocked in 4% BSA/TBS/0.05% Tween-20 for 30 min, and then labelled with mouse anti-β-catenin (BD Transduction Laboratories, #610153, 1:500 dilution), mouse anti-γH2A.X (phospho S140) [3F2] (Abcam, ab22551, 1:1000 dilution), rabbit anti-phospho-histone H2A.X (Ser139) (20E3) (Cell Signaling Technology, #9718, 1:200 dilution), mouse anti-BRCA1 (Sigma, SAB2702136, 1:1000 dilution), rabbit anti-RAD51 (Abcam, ab133534, 1:1000 dilution) or rabbit anti-pATM antibody (EP1890Y) (Abcam, ab81292, 1:1000 dilution). After incubation with Alexa Fluor-conjugated secondary antibodies (Life Technologies, Alexa 488 anti-rabbit A11008, 1:2000 dilution; Alexa 546 anti-mouse A11003, 1:2000; Alexa 647 anti-rabbit A21245, 1:1000; Alexa 647 anti-mouse A21235, 1:1000) and DAPI (Roche, 1:5000) the slides were mounted with DPX (Sigma-Aldrich). Fluorescence analyses for γ-H2AX, pATM and BRCA1 foci were performed with a Deltavision Elite microscope (GE Lifesciences) equipped with a 60x/1.2NA magnification objective. Foci per cell were counted with Image J software (FiJi). Fluorescence analyses for β-catenin were performed on an Olympus FV1000 inverted confocal microscope equipped with a 63x/1.4NA oil immersion objective. β-catenin localization and intensity were analyzed with an Image J script to measure red channel fluorescence for the nucleus versus cytoplasm with manual thresholding.

***Flow cytometry analysis for cellular DNA content***

Analysis of cellular DNA content was performed by flow cytometry using a BD FACS Calibur instrument (BD Biosciences). CRC cell lines and MEFs were disaggregated into a cellular suspension by dispase treatment (1mg/10ml DMEM), fixed in 80% ethanol, washed in PBS and stained with 50 μg/ml propidium iodide (PI) in the presence of 10 μg/ml of DNase-free RNase. Cells were excited with an argon laser emitting at 488 nm, and PI fluorescence was detected by using a 670-nm long pass filter. Forward and right-angle light scatter were used to set a gate including all cells but excluding debris. A second gate set on area and width of PI fluorescence, to further define the single cell population. PI fluorescence was collected in linear mode, and acquisition was stopped after 20,000 gated events had been acquired. Data were analyzed for aneuploidy [DNA index (DI)] by using MODFIT LT v3.0 (Verity Software House, Topsham, ME).

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