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Supplemental Information

DNA Polymerases as Potential Therapeutic Targets for Cancers Deficient in the DNA Mismatch Repair Proteins MSH2 or MLH1

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Figure S1

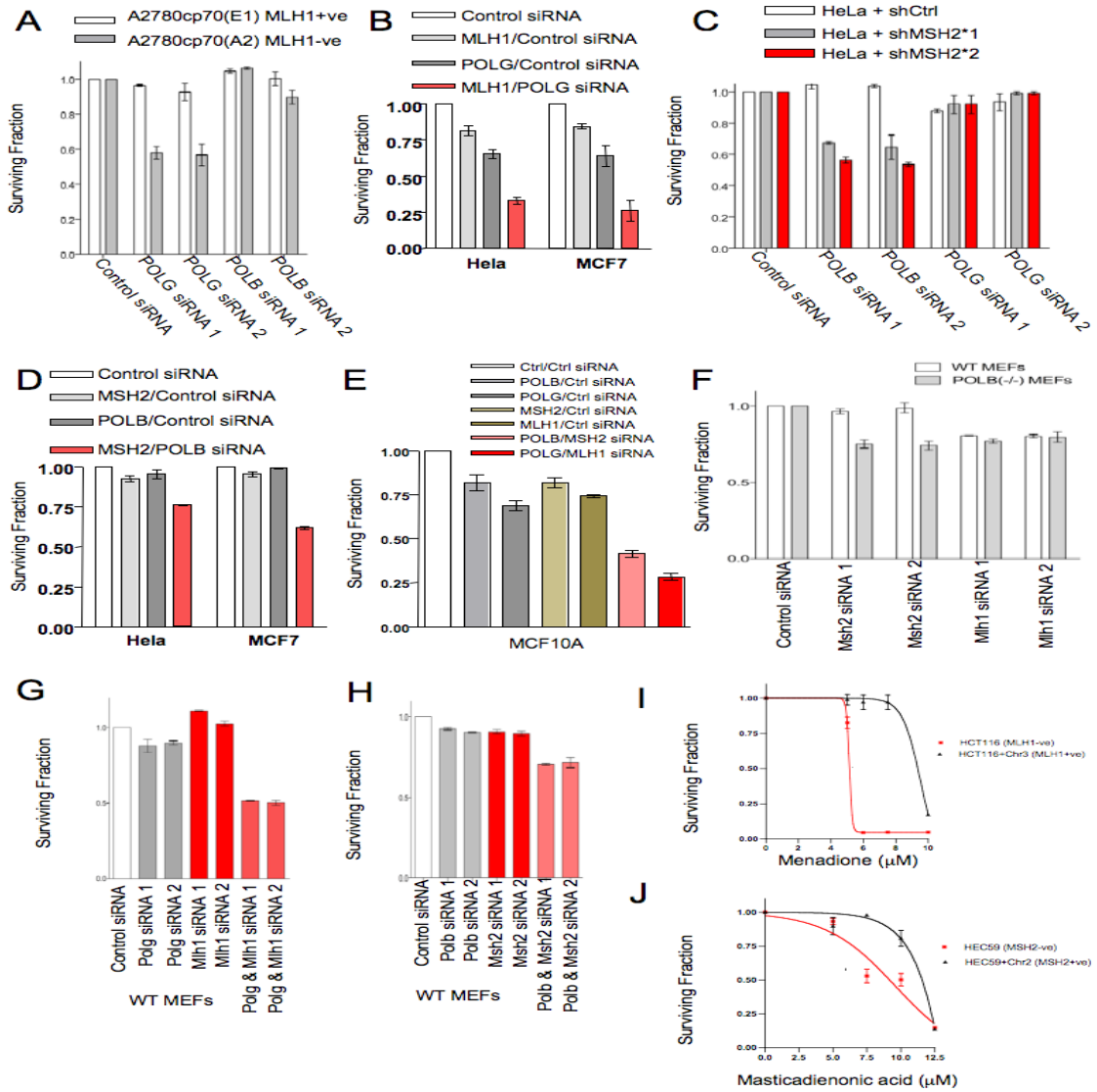


Figure S2

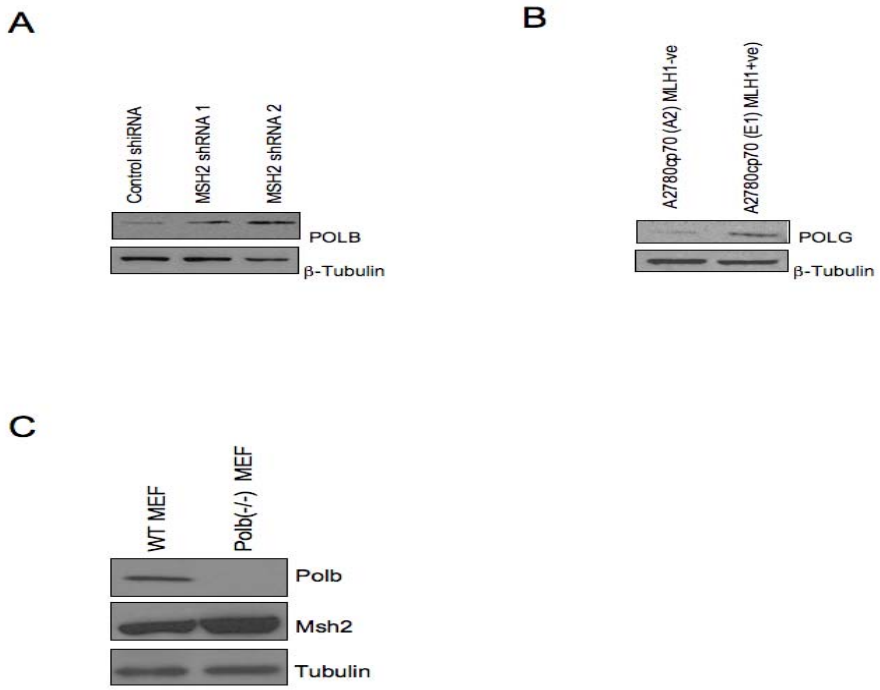

A
Control shiRNA
MSH2 shRNA 1
MSH2 shRNA 2
POLB
β-Tubulin
B
A2780cp70 (A2) MLH1-ve
A2780cp70 (E1) MLH1+ve)
POLG
β-Tubulin
C
WT MEF
Polb(-/-) MEF
Polb
Msh2
Tubulin

Figure S3

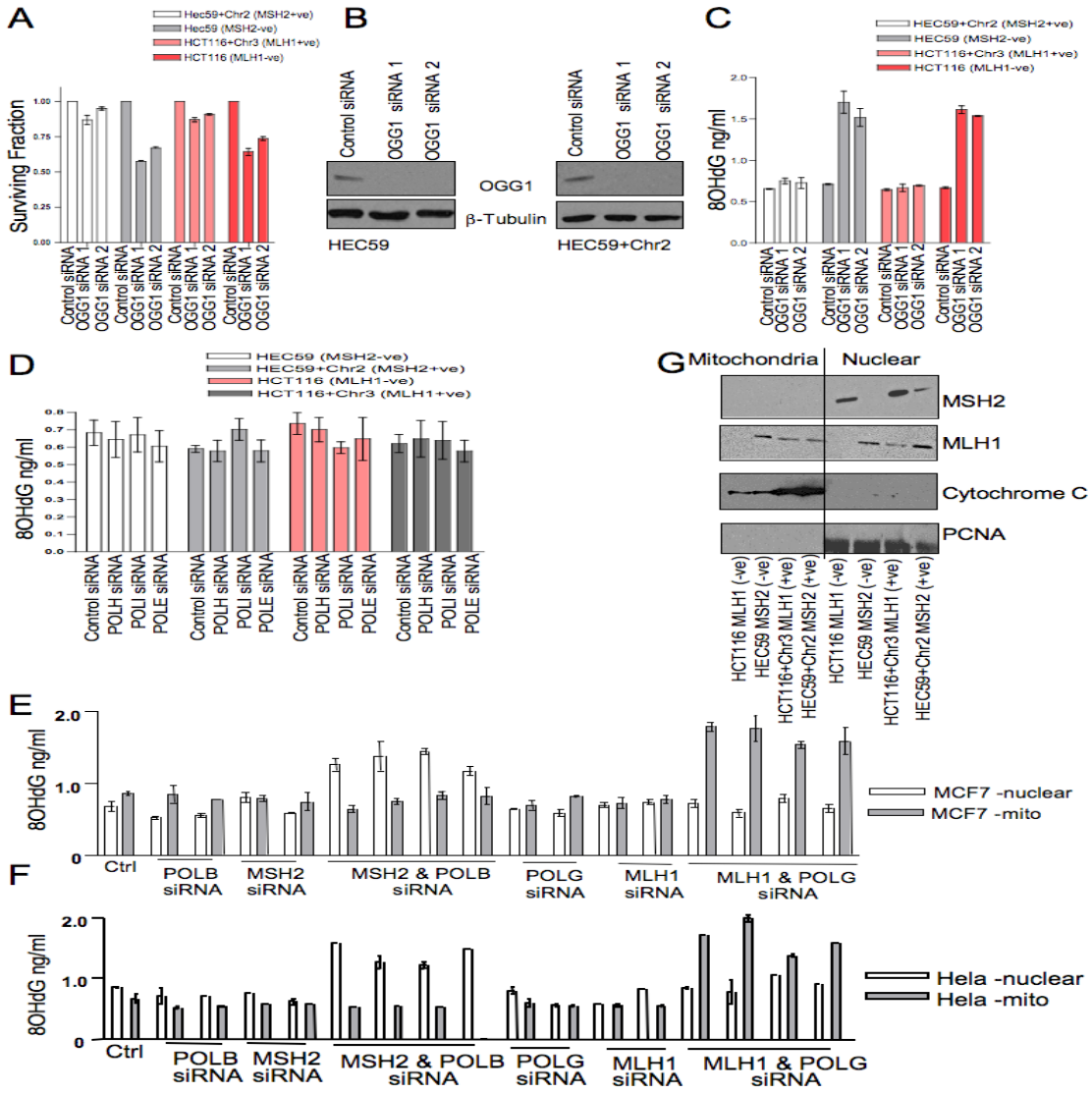

A
Hec59+Chr2 (MSH2+ve)
Hec59 (MSH2-ve)
HCT116+Chr3 (MLH1+ve)
HCT116 (MLH1-ve)
Surviving Fraction
Control siRNA
OGG1 siRNA 1
OGG1 siRNA 2
B
Control siRNA
OGG1 siRNA 1
OGG1 siRNA 2
OGG1
β-Tubulin
HEC59
HEC59+Chr2
C
HEC59+Chr2 (MSH2+ve)
HEC59 (MSH2-ve)
HCT116+Chr3 (MLH1+ve)
HCT116 (MLH1-ve)
8OHdG ng/ml
D
HEC59 (MSH2-ve)
HEC59+Chr2 (MSH2+ve)
HCT116 (MLH1-ve)
HCT116+Chr3 (MLH1+ve)
8OHdG ng/ml
Control siRNA
POLH siRNA
POLI siRNA
POLE siRNA
G
Mitochondria
Nuclear
MSH2
MLH1
Cytochrome C
PCNA
HCT116 MLH1 (-ve)
HEC59 MSH2 (-ve)
HCT116+Chr3 MLH1 (+ve)
HEC59+Chr2 MSH2 (+ve)
E
8OHdG ng/ml
MCF7 -nuclear
MCF7 -mito
Ctrl
POLB siRNA
MSH2 siRNA
MSH2 & POLB siRNA
POLG siRNA
MLH1 siRNA
MLH1 & POLG siRNA
F
8OHdG ng/ml
Hela -nuclear
Hela -mito
Ctrl
POLB siRNA
MSH2 siRNA
MSH2 & POLB siRNA
POLG siRNA
MLH1 siRNA
MLH1 & POLG siRNA

Figure S4

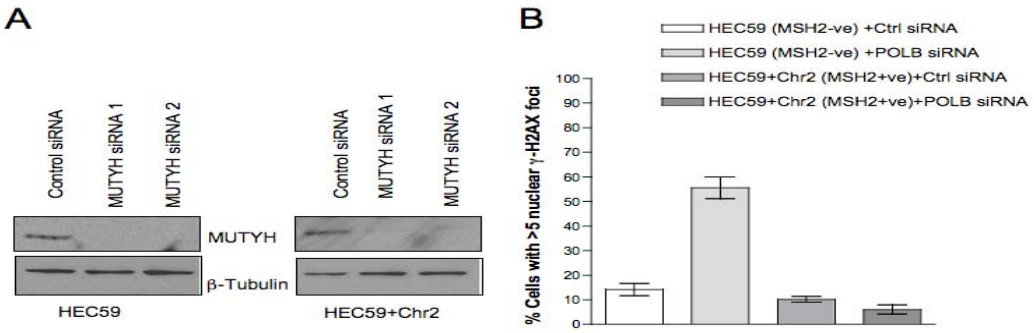
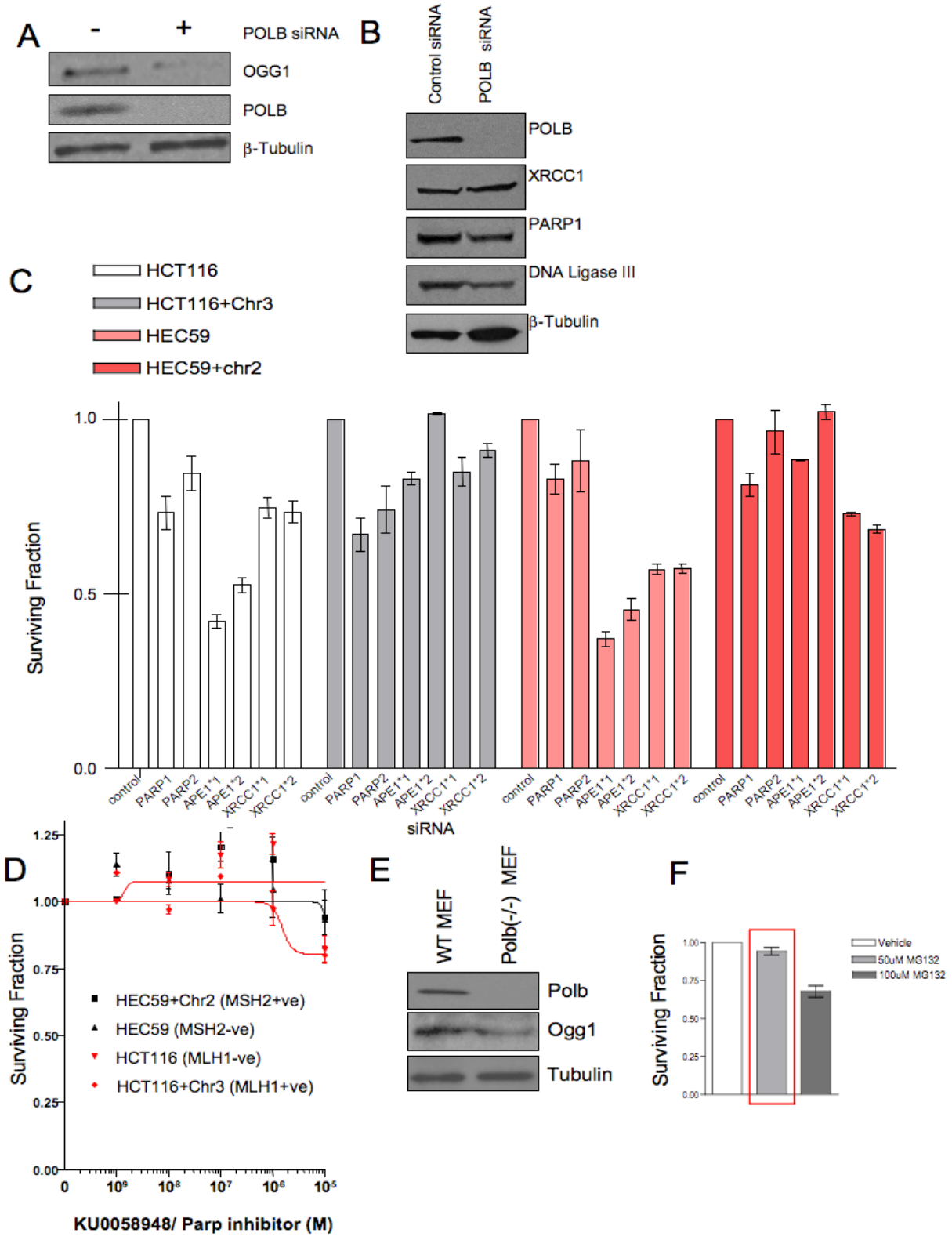

A
Control siRNA
MUTYH siRNA 1
MUTYH siRNA 2
Control siRNA
MUTYH siRNA 1
MUTYH siRNA 2
MUTYH
β-Tubulin
HEC59
HEC59+Chr2
B
HEC59 (MSH2-ve) +Ctrl siRNA
HEC59 (MSH2-ve) +POLB siRNA
HEC59+Chr2 (MSH2+ve)+Ctrl siRNA
HEC59+Chr2 (MSH2+ve)+POLB siRNA
% Cells with >5 nuclear γ-H2AX foci
100
90
80
70
60
50
40
30
20
10
0

Figure S5


A
-
+
POLB siRNA
OGG1
POLB
β-Tubulin
B
Control siRNA
POLB siRNA
POLB
XRCC1
PARP1
DNA Ligase III
β-Tubulin
C
HCT116
HCT116+Chr3
HEC59
HEC59+chr2
Surviving Fraction
1.0
0.5
0.0
control
PARP1
PARP2
APE1*1
APE1*2
XRCC1*1
XRCC1*2
siRNA
D
Surviving Fraction
1.25
1.00
0.75
0.50
0.25
0.00
HEC59+Chr2 (MSH2+ve)
HEC59 (MSH2-ve)
HCT116 (MLH1-ve)
HCT116+Chr3 (MLH1+ve)
KU0058948/ Parp inhibitor (M)
E
WT MEF
Polb(-/-) MEF
Polb
Ogg1
Tubulin
F
Surviving Fraction
Vehicle
50uM MG132
100uM MG132

SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1, related to Figure 1. MLH1/POLG and MSH2/POLB SSLs

A. MLH1 deficiency is synthetically lethal with POLG silencing in A2780 cells. A2780cp70 A2 (MLH1 –ve) and A2780cp70 E1 (MLH1 +ve) cells were transfected with either control, POLG or POLB siRNA and clonogenic assays performed.

B. MLH1 deficiency is synthetically lethal with POLG silencing in HeLa and MCF7 cells. Cells were transfected with either control siRNA, POLG siRNA, MLH1 siRNA or siRNA combinations as indicated and clonogenic assays performed.

C. MSH2 deficiency is synthetically lethal with POLB silencing. HeLa cells stably expressing either control shRNA or shRNA targeting MSH2 were transfected with either control, POLB or POLG siRNA and clonogenic assays performed.

D. HeLa and MCF7 cells were transfected with either control siRNA, POLB siRNA, MSH2 siRNA or in combination as indicated and clonogenic assays performed.

E. MCF10A cells were transfected with specific siRNA as indicated. After 14 days, colonies were stained and counted.

F. Polb deficiency is SSL with Msh2 silencing. Wild type (WT) and $Polb^{-/-}$ MEFs were transfected with either control, Msh2 or Mlh1 siRNA and cellular viability assays were performed.

G. Wild type (WT) MEFs were transfected with Polg and Mlh1 siRNA alone or in combination, as indicated and cellular viability assays were performed.

H. Wild type (WT) MEFs were transfected with Polb and Msh2 siRNA alone or in combination, as indicated and cellular viability assays were performed.

I. Deficiency in MLH1 is selectively lethal with the POLG inhibitor, menadione. HCT116 (MLH1 deficient) and HCT116+Chr3 (MLH1 proficient) cells were treated with increasing concentrations of menadione and clonogenic assays performed.

J. Deficiency in MSH2 is selectively lethal with the POLB inhibitor, masticadienonic acid. HEC59 (MSH2 deficient) and HEC59+Chr2 (MSH2 proficient) cells were treated

with increasing concentrations of masticadienonic acid and clonogenic assays performed.

Error bars for each individual experiment represent standard errors of the mean.

FIGURE S2, related to Figure 2. DNA polymerase expression in MSH2 and MLH1 deficient cells

A. Expression of POLB levels is elevated in MSH2 deficient cells. HeLa cells with stable MSH2 silencing were generated by infection with a lentiviral constructs expressing one of two MSH2-specific short-hairpin RNAs (shRNA). Cell lysates from MSH2-silenced cells were analysed by western blotting, using POLB and β-tubulin specific antibodies.

B. Expression of POLG levels are increased in MLH1 deficient cells. Cell lysates from MLH1 deficient A2780cp70(A2) and MLH1 proficient A2780cp70 (E1) cells were analysed by western blotting using POLG and β-tubulin specific antibodies.

C. Expression of Msh2 is increased in Polb deficient MEFs. Cell lysates from wild type (WT) and $Polb^{-/-}$ MEFs were analysed by western blotting using Polb, Msh2 and β-tubulin specific antibodies.

FIGURE S3, related to Figure 3. MLH1 or MSH2 deficiency is SSL with OGG1 silencing

A. HEC59, HEC59+Chr2, HCT116 and HCT116+Chr3 cells were transfected with either control siRNA or siRNA targeting *OGG1* and clonogenic assays performed.

B. HEC59 and HEC59+Chr2 cells were transfected with OGG1 siRNA and cell lysates were analysed 72 hours later by western blotting using OGG1 and β-tubulin specific antibodies.

C. OGG1 inhibition causes an accumulation of 8-oxoG lesions in MSH2 and MLH1 deficient cells. HEC59, HEC59+Chr2, HCT116 and HCT116+Chr3 cells were transfected with either control or *OGG1* siRNA. DNA from transfected cells was

analysed for 8-oxoG accumulation by ELISA, 72 hr after transfection. Oxidised lesions were quantified according to a standard curve generated using known amounts of 8-oxoG. Assays were performed in triplicate.

D. No increase in 8-oxoG accumulation upon MSH2 or MLH1 deficiency and silencing of POLH, POLI and POLE. HEC59, HEC59+Chr2, HCT116 and HCT116+Chr3 cells were transfected with either control, *POLH, POLI* or *POLE* siRNA. DNA from transfected cells were analysed for 8-oxoG accumulation by ELISA. Oxidised lesions were quantified according to a standard curve generated using known amounts of 8-oxoG. Assays were performed in triplicate.

E & F. Increased nuclear 8-oxoG accumulation upon MSH2 deficiency and silencing of POLB and increased mitochondrial 8-oxoG accumulation upon MLH1 deficiency and silencing of POLG in MCF7 (E) and HeLa (F) cells. MCF7 and HeLa cells were transfected with either control, *POLB*, *POLG, MSH2 or MLH1* siRNA or siRNAs in combination. Nuclear and mitochondrial DNA isolated from transfected cells were analysed for 8-oxoG accumulation by ELISA. Oxidised lesions were quantified according to an 8-oxoG standard curve. Assays were performed in triplicate.

G. MLH1 is found in both the nucleus and the mitochondria, whereas MSH2 expression is restricted to the nucleus. Nuclear and mitochondrial protein lysates isolated from HCT116, HEC59, HEC59+Chr2 and HCT116+Chr3 cells were analysed by western blotting. PCNA and cytochrome C specific antibodies were used to determine nuclear and mitochondrial fractionations, respectively.

Error bars for each individual experiment represent standard errors of the mean.

FIGURE S4, related to Figure 4.

A. Validation of MUTYH siRNA**.** HEC59 and HEC59+Chr2 cells were transfected with MUTYH siRNA and cell lysates were analysed 72 hours later by western blotting.

B**.** Quantification of γH2AX foci. HEC59 (MSH2 deficient) and HEC59+Chr2 (MSH2 proficient) cells were transfected with either control or *POLB* siRNA. Cells with γH2AX

foci were quantified as in Figure 4C with cells with >5 foci per nucleus being counted as positive. Bar chart showing frequencies is shown. Error bars represent standard errors of the mean from triplicate experiments.

FIGURE S5, related to Figure 6.

A. OGG1 expression is decreased upon silencing of *POLB*. Cell lysates from MCF7 cells were analysed 72 hours after transfection with siRNA oligonucleotides, by western blotting. Antibodies directed against OGG1, POLB and β-tubulin, were used to demonstrate reduction in expression of OGG1 after transfection with *POLB* siRNA.

B. BER protein expression. HeLa cells were transfected with siRNA and cell lysates were analysed 72 hours later. Antibodies directed against POLB, XRCC1, PARP1, DNA Ligase III and β-tubulin were used to demonstrate no change in expression of BER members after transfection with *POLB* siRNA.

C. HEC59, HEC59+Chr2, HCT116 and HCT116+Chr3 cells were transfected with either control, *PARP1, PARP2, APE1* or *XRCC1* siRNA. After 14 days, cells were analysed for cellular survival by staining with sulforhodamine-B.

D. HEC59, HEC59+Chr2, HCT116 and HCT116+Chr3 cells were treated with increasing concentrations of a specific PARP1/2 inhibitor (KU0058948) and clonogenic assays performed.

E. Expression of Ogg1 levels are decreased in Polb deficient MEFs. Cell lysates from wild type (WT) and $Polb^{-/-}$ MEFs were analysed by western blotting using Polb, Ogg1 and β-tubulin specific antibodies.

F. MG132 effect on cell viability (pertaining to Figure 6E). Cell lysates from HeLa cells were transfected with siRNA and after 48hr, cells were treated with MG132 (50μM). Figure shows cell viability 18 hours after MG132 treatment, the identical time point at which lysates analysed in Figure 6E were generated.

Error bars for each individual experiment represent standard errors of the mean.

TABLE S1, related to Figure 1.

Densitometric analysis of protein levels from western blots in Figure 1E & F.

Cell line	HEC59	HEC59 +Chr2	HEC59	HEC59 +Chr2	HCT116	HCT116+Chr3	HCT116	HCT1
Antibody	POLB	POLB	β-Tubulin	β-Tubulin	POLG	POLG	β-Tubulin	β-Tub
Ctrl siRNA	1	1	1	1	1	1	1	
POLB siRNA*1	0.2676	0.276	0.924688	1.0756	N/A	N/A	N/A	
POLB siRNA*2	0.2887	0.411	0.96461	1.0637	N/A	N/A	N/A	
POLG siRNA*1	N/A	N/A	N/A	N/A	0.14907	0.357	0.9921	
POLG siRNA*2	N/A	N/A	N/A	N/A	0.15777	0.313	0.95626	

"N/A: not applicable"

TABLE S2, related to Figure 1 & Figure S1.

Plating Efficiencies for each cell line.

Cell Line	MMR Status	Plating Efficiency	SEM
HEC59	MSH2 -ve	0.35	0.06
HEC59+Chr2	MMR+ ve	0.36	0.04
HCT116	MLH1 -ve	0.52	0.06
HCT116+Chr3	MMR+ ve	0.54	0.03
A2780cp70 (E1)	MMR+ ve	0.32	0.04
A2780cp70 (A2)	MLH1 -ve	0.31	0.02
HeLa shCtrl	MMR+ ve	0.71	0.05
HeLa shMSH2*1	MSH2 -ve	0.67	0.005
HeLa shMSH2*2	MSH2 -ve	0.65	0.03

TABLE S3, related to Figure 2.

Densitometric analysis of protein levels from western blots in Figure 2C & D.

	HEC59	HEC59+Chr2	HCT116	HCT116+Chr3
MSH2	0.00028	1	N/A	N/A
MLH1	N/A	N/A	0.00034	1
POLB	1.45	1	0.946	1
POLG	0.986	1	1.832	1
Beta-Tubulin	1.004	1	1.05	1

"N/A: not applicable"

Supplemental Experimental Procedures

Cell lines

The human endometrial cells HEC59+Chr2 and HEC59 cells were grown in DMEM F12 supplemented with FCS (10% v/v), glutamine and antibiotics. The human colon cancer cell line HCT116 and HCT116+Chr3 were grown in McCoys 5A supplemented with FCS (10% v/v), glutamine, and antibiotics. Cells containing human chromosome 2 and chromosome 3 were cultured under selective pressure of 400 µg/mL geneticin (G418 sulfate). The human ovarian tumor cell lines A2780cp70+chr3/A2 and A2780cp70+chr3/E1 were maintained in RPMI 1640 supplemented with FCS (10%, v/v), glutamine, and antibiotics. Cells were cultured under selective pressure of 200 µg/mL Hygromycin B (Invitrogen, UK). The Masticadienonic Acid was a kind gift of Dr. C Cazaux (Institut de Pharmacologie et de Biologie Structurale, France) and Dr. G Massiot (Institut de Recherche, Pierre Fabre). Menadione was purchased from Prestwick Chemicals (Saffron Walden, Essex, UK). Wild type and Polb(-/-) mouse embryonic fibroblasts were maintained in DMEM, supplemented with FCS (10% v/v), glutamine and antibiotics. MCF10A cells were grown in MEBM media, supplemented with BPE 13 mg/ml; hydrocortisone 0.5 mg/ml; hEGF 10 ug/ml and insulin 5 mg/ml (MEGM BulletKit, Clonetics). We also supplemented the media with the following, 100 ng/ml cholera toxin, horse serum (10%, v/v) glutamine and antibiotics. MCF7 and HeLa cells were grown in DMEM, supplemented with FCS (10% v/v), glutamine and antibiotics. shRNA expressing cells were established by infecting HeLa cells with shRNA expressing empty or hMSH2 vectors, which were generated by PCR amplification of 97mer DNA oligonucleotides as described (Paddison et al., 2004) and cloned into the LMP vector (Dickins et al., 2005) by EcoRI/XhoI subcloning. shRNA sequences were as follows:

shMsh2*1

TGCTGTTGACAGTGAGCGCCTCAGTGAATTAAGAGAAATATAGTGAAGCCACAGA

TGTATATTTCTCTTAATTCACTGAGATGCCTACTGCCTCGGA

shMsh2*2

TGCTGTTGACAGTGAGCGCAAAGTCCTTCGTAACAATAAATAGTGAAGCCACAGA

TGTATTTATTGTTACGAAGGACTTTTTGCCTACTGCCTCGGA

Protein Analysis

For western blotting, lysates were electrophoresed on Novex precast gels (Invitrogen) and immunoblotted using the following antibodies: anti-MSH2 (Ab-1, Calbiochem), anti-MmMsh2 (ab70270, Abcam) anti-POLB (ab3181, Abcam), anti-MLH1 (ab9144, Abcam), anti-POLG (Novus), anti-PCNA (SC7907, Santa-Cruz), anti-Cytochrome C (Pharmagen) anti-OGG1 (NB100-106, Novus biologicals), anti-MmOgg1 (ab204, Abcam), (anti-CHIP (ab39559, Abcam), and anti-β-tubulin, (T4026, Sigma). This was followed by incubation with anti-IgG-horseradish peroxidase and chemiluminescent detection (SuperSignal West Pico Chemiluminescent Substrate, Pierce). Immunoblotting for β-tubulin was used as a loading control.

Measurement of 8-oxoG

Genomic DNA was extracted using the QIamp DNA isolation kit (Qiagen) and digested with nuclease P1. Mitochondrial and nuclear DNA were extracted using the mitochondrial DNA isolation kit (ab65321, Abcam). An ELISA kit (Cell Biolabs) was used to determine levels of 8-oxoG in isolated DNA. The 8-oxoG standard (0.078–20 ng/ml) or 10 μg test DNA was incubated with an 8-OHdG monoclonal antibody in a microtiter plate precoated with 8-oxoG. Addition of 3,35,5-tetramethylbenzidine to replicate samples was followed by measurement of absorbance at 450 nm. Standard curves were calculated for all reactions with serial dilutions of 8-oxoG standard to calculate reaction efficiency. Samples were assayed in triplicate.

***In vitro* OGG1 assay**

OGG1 glycosylase activity was analysed using the OGG1 assay kit (Sigma Aldrich, UK). Briefly, protein was isolated from transfected cells as indicated in Figure 6B. The substrate is a 23 oligonucleotide containing 8-OHdG at its 11th base, labeled with ^{32}P at its 5' end, and annealed to its complementary strand (containing dC at the opposite base position to the 8-OHdG). Upon cleavage of the substrate by the OGG1 enzyme, the oligonucleotides were electrophoresed on a 15% polyacrylamide denaturing (7 M Urea) PAGE gel, followed by autoradiography.

Detection of γ-H2AX foci by Immunofluorescence

72hr post transfection, cells were fixed for 15 min with 4% paraformaldehyde in PBS. Slides were then permeabilized with TBS/Tween-20 and followed by washes with TBS/Tween-20, blocking for 1 h at 37°C and then incubated with **γ-**H2AX antibody (Milipore) for 24h at 4°C. Cover slips were stained with DAPI, mounted and viewed using a Leica TCS-SP2 confocal microscope.

Use of RNA interference to assess synthetic lethality

Cells were transfected with short interfering RNA (siRNA) (Qiagen or Dharmacon; CHIP siRNA) targeting the following genes (target sequences shown):

POLγ*1,5'-CACGAGCAAATCTTCGGGCAA-3';

POLγ*2,5'-CAGATGCGGGTCACACCTAAA-3';

POLβ*1,5'-CAAGATATTGTACTAAATGAA-3';

POLβ*2,5'-TACGAGTTCATCCATCAATTT-3';

POLι*1,5'-ACCGGGAACATCAGGCTTTAA-3';

POLι*2,5'-GCGGTTTATTAAGCTCTTCTA-3';

POLη*1,5'-ATCCATTTAGGTGCTGAGTTA-3';

POLη*2,5'-CTGGTTGTGAGCATTCGTGTA-3';

POLε*1,5'-CCGCATCATCCTCTGTACAAA-3';

POLε*2,5’-CCGCCTCTCCATTGACCTGAA-3’;

OGG1*1, 5’-CGGGACCTACACCTCAGGAAA-3’

OGG1*2, 5’-CACCGTGTGGGCGAGGCCTTA-3’

CHIP*1,5’-CCGCGGAGCGUAGAGAGGGA-3’

CHIP*2, 5’-GCAUUGAGGCCAAGCACGA-3’

MUTYH*1, 5’-CAGGAGGAATTTCACACCGCA-3’

MUTYH*2, 5’-CAAGCTGACATATCAAGTATA-3’

Control, 5’-CATGCCTGATCCGCTAGTC-3’

As a control for each experiment, cells were left un-transfected or transfected with a non-targeting Control siRNA and concurrently analysed. Twenty-four hours after transfection, cells were divided into three replica plates. Cell viability was measured five days after transfection using the 96-well plate CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's instructions. For clonogenic assays, exponentially growing cells were seeded at various densities in six-well plates. Cells were transfected with siRNA as before. Cell medium was replaced every four days. After ten to fourteen days, colonies were fixed in 10% trichloroacetic acid and stained with sulforhodamine-B. Surviving Fractions were calculated as the ratio of the plating efficiency (PE) of gene-specific siRNA transfected cells, divided by the PE of control siRNA transfected cells, where PE = colonies counted/cells plated.

cDNA Synthesis and Quantitative RT-PCR (qRT-PCR)

Total RNA from cell lines was extracted using Trizol (Invitrogen) according to manufacturers instructions. Total RNA from patient biopsies was purified from 10 µm sections using the High Pure RNA Paraffin Kit (Roche Diagnostic Ltd). cDNA was synthesized using Omniscript Reverse Transcriptase System for RT-PCR (Qiagen) with oligo dT as per manufacturer’s instructions. Assay-on-Demand primer/probe sets

were purchased from Applied Biosystems. Real-Time qPCR was performed on the 790DHT Fast Real-Time PCR System (Applied Biosystems), using *GAPDH* as an endogenous control. Standard curves were calculated for all reactions with serial dilutions of control cDNA to calculate reaction efficiency. Gene expression was calculated relative to expression of *GAPDH* endogenous control, and adjusted relative to expression in control cDNA. Samples were quantified in triplicate.

Immunohistochemical Staining

Immunohistochemistry was performed for anti-MSH2 antibody (Zymed; clone FE11, dilution 1/400; Antigen retrieval: ER1 20 minutes) and anti-MLH1 antibody (BD Transduction Laboratories; clone G168-15, dilution 1/150; Antigen retrieval: ER2 40 minutes) on an automated platform (BondMax™ system - Vision BioSystems™). Staining was performed according to the protocol as listed above with the antibody details. A polymer detection system was selected to avoid non-specific endogenous biotin staining. Cases with unequivocal nuclear staining were considered positive. Validation of staining was confirmed by expression in normal colonic mucosa cells, normal epithelial cells, stromal cells or lymphocytes (Mackay et al., 2000).

Supplemental References

Dickins, R. A. et al. (2005) Probing tumor phenotypes using stable and regulated synthetic microRNA precursors Nat. Genet. 37, 1289-1295.

Mackay, H. J., Cameron, D., Rahilly, M., Mackean, M. J., Paul, J., Kaye, S. B., and Brown, R. (2000). Reduced MLH1 expression in breast tumors after primary chemotherapy predicts disease-free survival. J Clin Oncol *18*, 87-93.

Paddison, P. J. et al. (2004) Cloning of short hairpin RNAs for gene knockdown in mammalian cells Nat. Methods 1, 163-167.