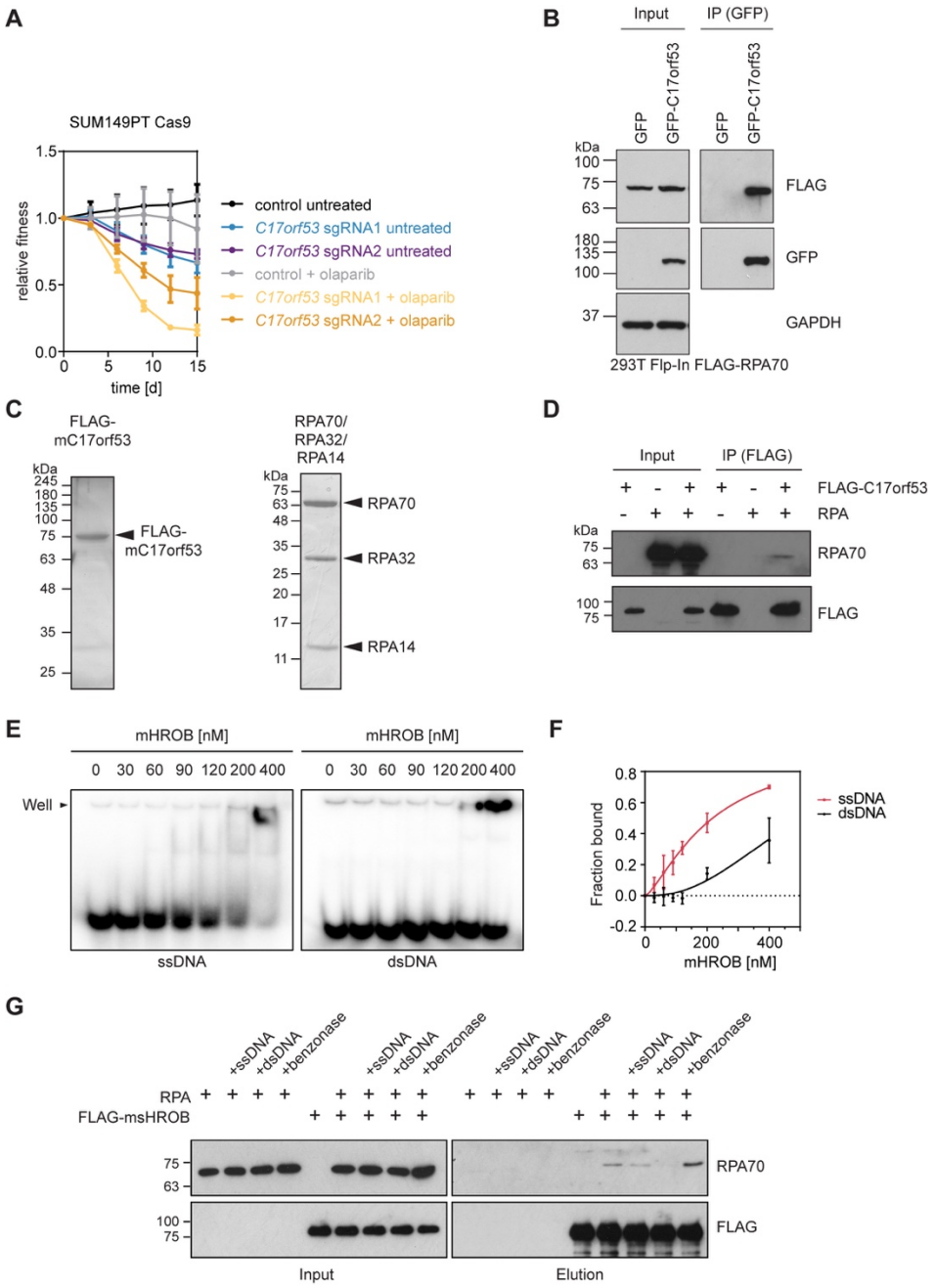
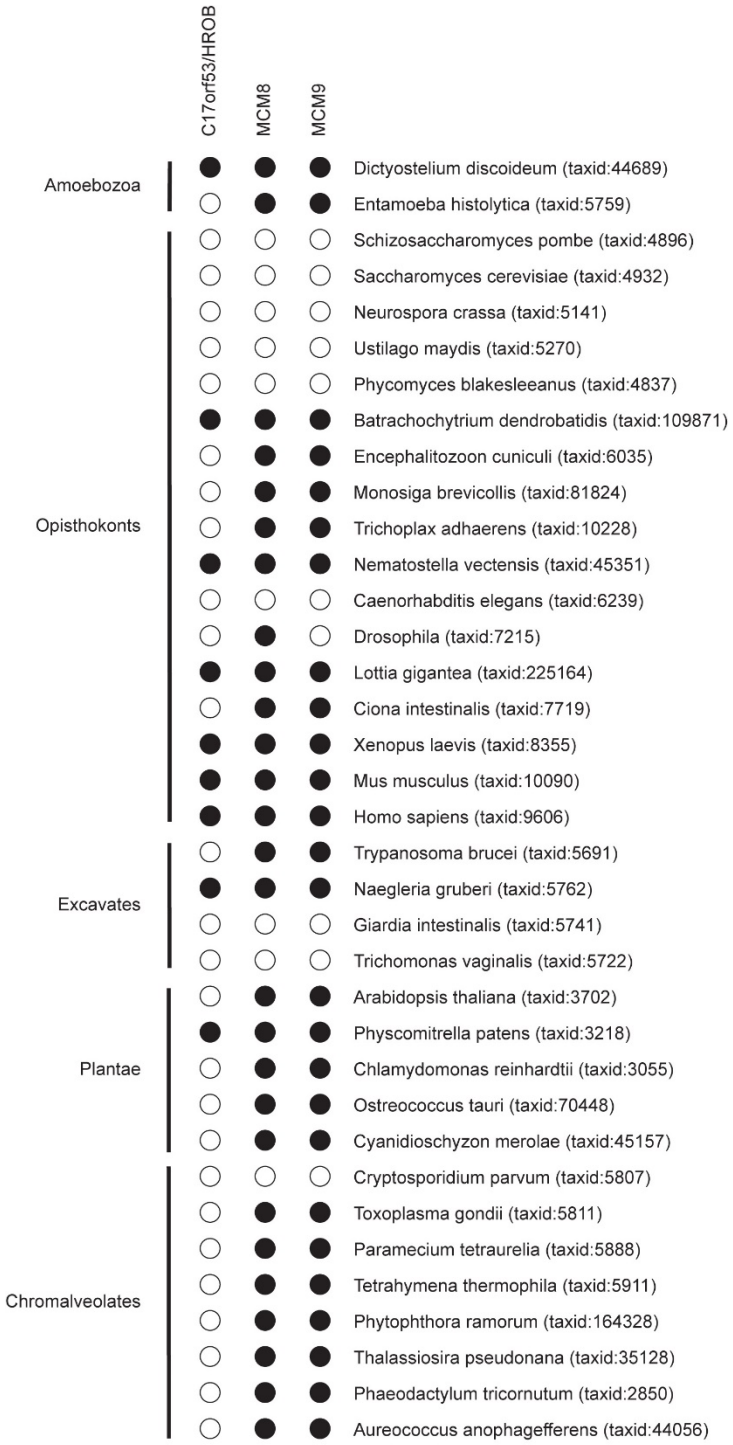
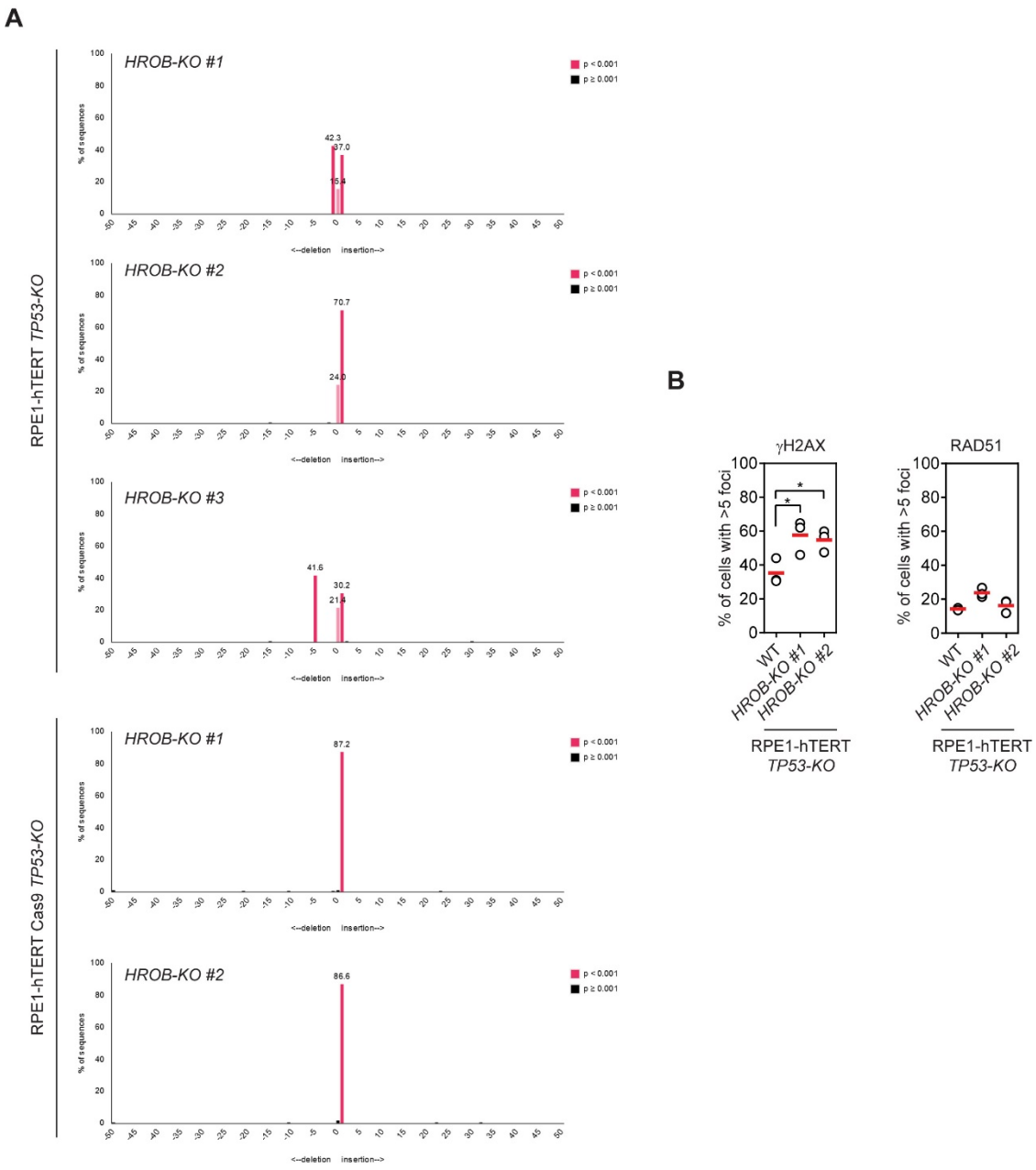


Supplemental Figure S1. HROB mutation confers sensitivity to olaparib in SUM149PT cells and HROB interacts with RPA and DNA. This figure is related to Figures 1A, B and 2A. **A**, Competitive growth assay determining the capacity of the indicated sgRNAs to cause sensitivity to 200 nM olaparib in SUM149PT Cas9 cells. Data is presented as the fraction of GFP-positive cells normalized to day 0. Data are presented as the mean of three independent experiments. Error bars indicate standard deviation. **B**, Whole cell extracts of 293T Flp-In FLAG-RPA70 cells transfected with either GFP or GFP-HROB plasmids were subjected to anti-GFP immunoprecipitation (IP). Immunoblots of input (left) or IP (right) samples were probed with the indicated antibodies. GAPDH served as a loading control. **C**, SDS-PAGE analysis of purified, FLAG-tagged mouse C17orf53 (mC17orf53) (left), and RPA70/RPA32/RPA14 complex (right). Arrowheads mark bands corresponding to either FLAG-mC17orf53 or the respective RPA complex subunits. **D**, Purified FLAG-mC17orf53 and RPA complex were mixed as indicated and subjected to anti-FLAG immunoprecipitation and immunoblotting with the indicated antibodies. **E**, Representative images of electrophoretic mobility shift assays with the ^{32}P-labelled single-stranded DNA (ssDNA, left) and double-stranded (dsDNA, right) incubated with the indicated amounts of purified FLAG-tagged mouse HROB (mHROB). **F**, Quantification of FLAG-mHROB bound DNA fraction. Data are presented as the mean of three independent experiments. Error bars indicate standard deviation. **G,** The HROB-RPA interaction occurs independently of DNA. In vitro interaction assay done as in (D) but with addition of 100 nM ss or dsDNA (59nt/bp), or 10 units of benzonase. The HROB IP buffer was supplemented with 5mM MgCl2 to facilitate benzonase activity.



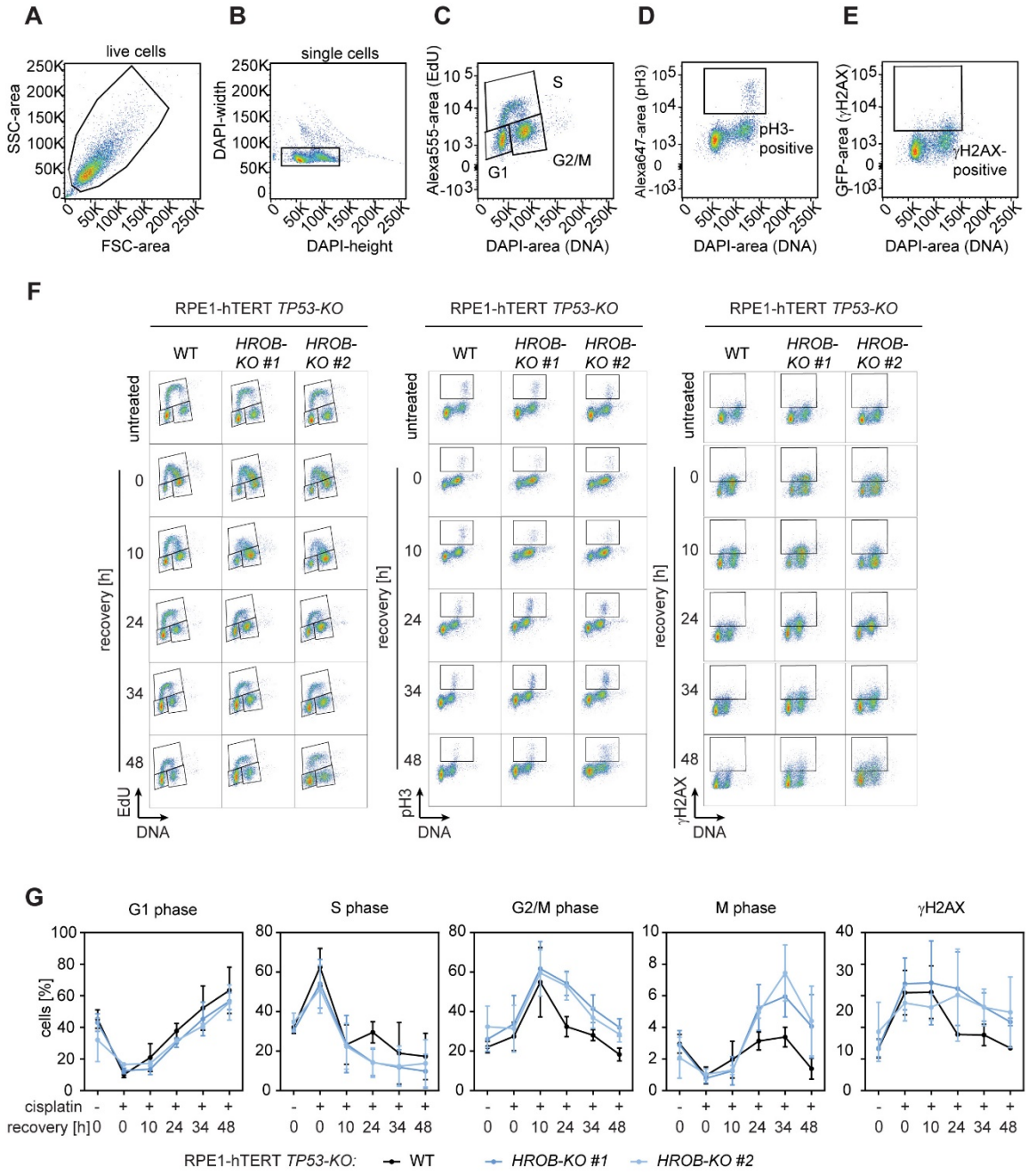
Supplemental Figure S2: HROB homologs were found in many metazoan organisms.
This figure is related to Figure 2A.
The human HROB amino acid sequence was used as input for a BLASTP search of the non-redundant protein sequence (nr) databases of the indicated organisms. Black circles: a protein with at least 25% sequence identity to human HROB and an E-value ≤ 0.001 was found; white circles, no protein with the above characteristics was found. Data for MCM8 and MCM9 are from ref(Liu et al. 2009).



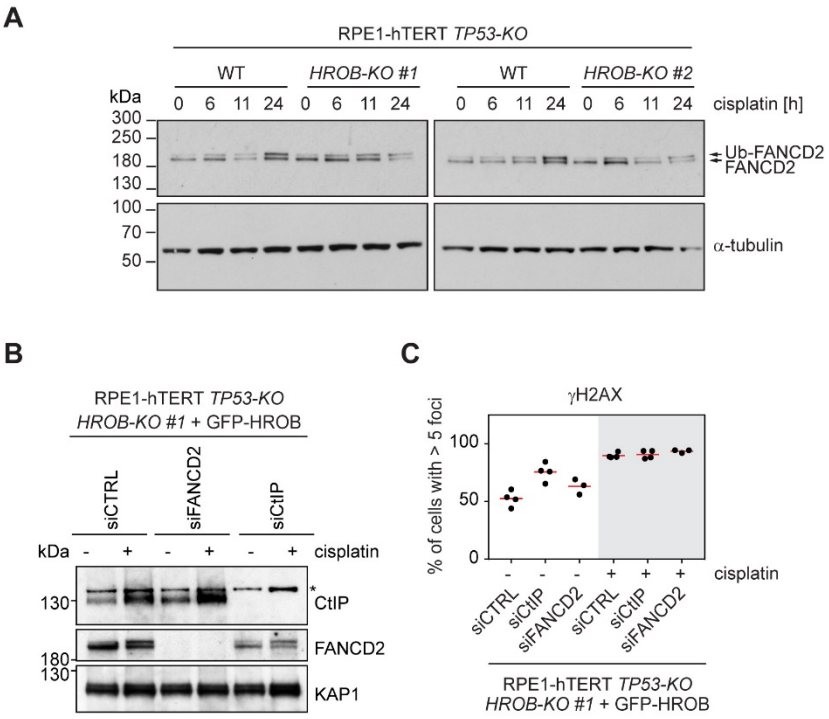
Supplemental Figure S3: Validation of clonal *HROB-KO* cell lines by TIDE analysis and elevated levels of spontaneous γH2AX in *HROB-KO* cells

This Figure is related to Figure 2B.

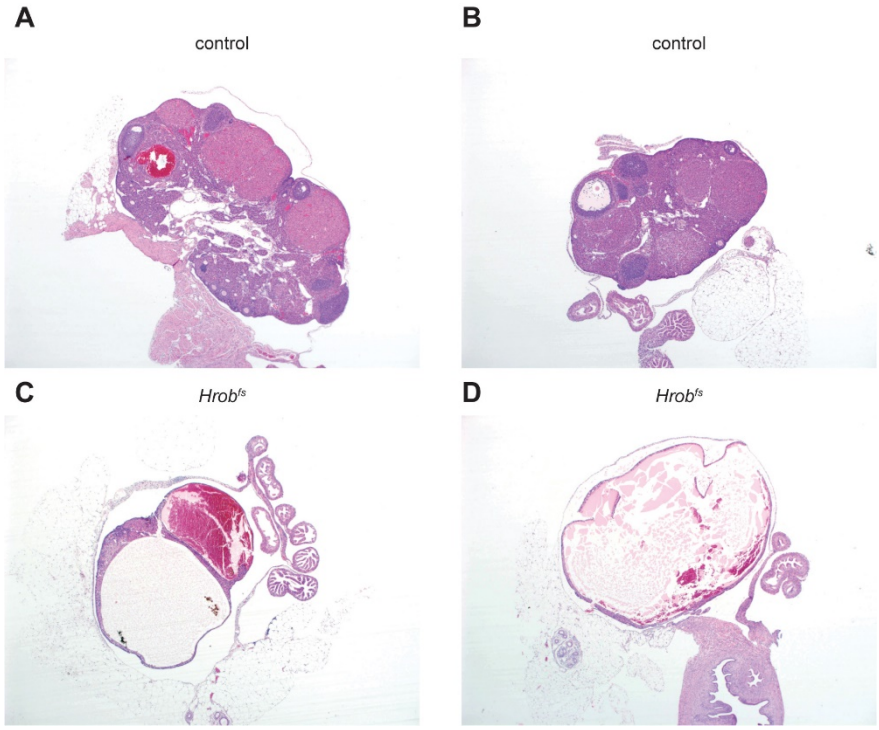
A, PCR was used to amplify the region surrounding the *HROB* sgRNA targeting site. TIDE analysis of sequences from the indicated clones that was used to assess indel formation in *HROB* gene knockouts. **B**, Quantification of spontaneous γH2AX (left) or RAD51/γH2AX colocalizing (right) foci in the indicated RPE1 cell lines. Red line indicates mean (n=3 biologically independent experiments). *$P \leq 0.05$. *P*-values are from paired two-tailed t-tests.



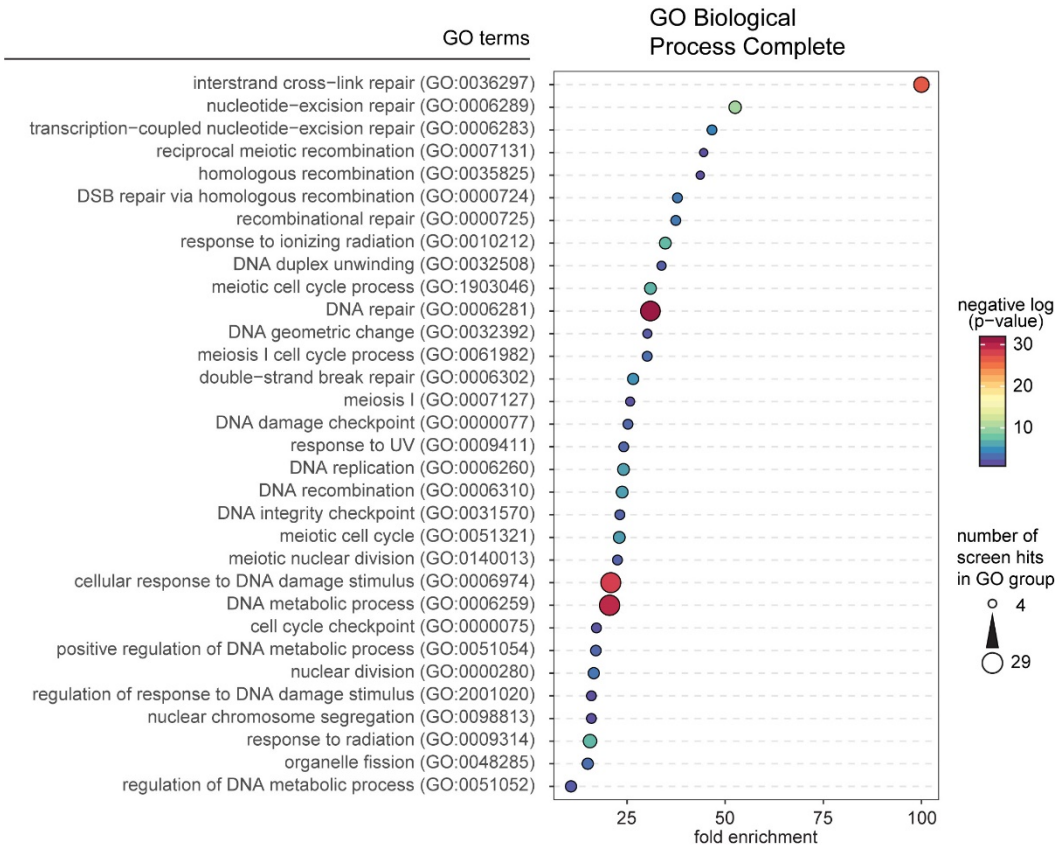
Supplemental Figure S4: *HROB-KO* cells accumulate in G2/M phase after cisplatin treatment.
This figure is related to Figure 3B.
A-E, Gating strategy for fluorescence activated cell sorting (FACS) analysis of different cell cycle stages and DNA damage signalling. **A**, Live cells were gated from total cell populations as indicated. **B**, Single cells were gated from live cells as indicated. **C-E**, G1 phase, S phase, G2/M phase (from EdU incorporation), phospho-histone H3 (pH3)-positive (M phase) and γH2AX-positive cells were gated from single cells as indicated. **F**, Representative graphs of FACS analysis for cell cycle stages and γH2AX formation in RPE1 wild type (WT) and the indicated *HROB-KO* cell lines after the indicated times of recovery from cisplatin treatment (for details of experimental setup see Figure 3A). Left, EdU analysis. Middle, pH3 analysis. Right, γH2AX analysis. Shown are representative data from one experiment. **G**, Quantification of the experiment shown in **F**. Lines connect means. Error bars indicate standard deviation (n=3 biologically independent experiments).



Supplemental Figure 5: HROB acts downstream of FANCD2 in the response to DNA inter-strand crosslinks.
This figure is related to Figure 3J.
A, Lysates were prepared from RPE1 wild type (WT) or *HROB-KO* cells treated with 4 μM cisplatin for the indicated periods of time and analyzed by immunoblotting with antibodies to FANCD2 or alpha-tubulin (loading control). **B, C**, RPE1 *HROB-KO #1* + GFP-HROB cells were transfected with the indicated siRNAs and were left untreated or were treated with 8 μM cisplatin for 4 h before recovery in medium without cisplatin for 20 h. **B**, Lysates from cells transfected with the indicated siRNAs were analyzed by immunoblotting with antibodies to CtIP, FANCD2 or KAP1 (loading control). **C**, Quantification of γH2AX foci in RPE1 *HROB-KO #1* + GFP-HROB cells transfected with the indicated siRNAs, with and without cisplatin treatment. Red line indicates mean (n ≥3 biologically independent experiments). siCTRL, non-targeting control siRNA.



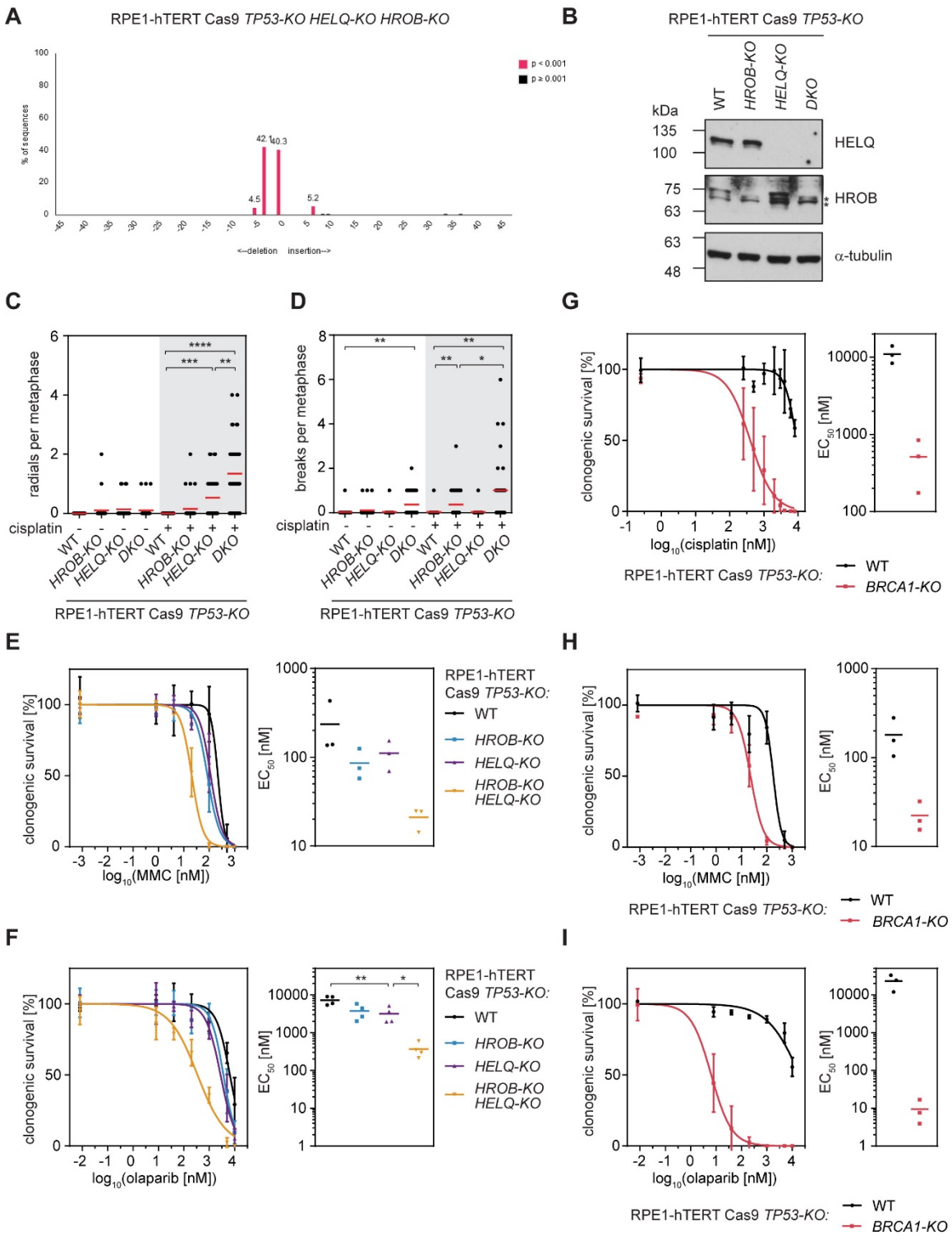
Supplemental Figure S6: Cystic ovaries in female *HROB*^{fs} mutant mice.
This figure is related to Figure 4.
A-D, Ovary sections stained with hematoxylin/eosin. **A, B**, Ovary sections from age-matched C57BL6 control animals. **C, D,** Ovary sections from HROB^{fs} mice. **C**, Ovary with two large cysts, one filled with red blood cells. Both cysts contain cells with dark pigment, possibly macrophages. **D**, Ovary with one very large cyst filled with eosinophilic material and some red blood cells. Original images were acquired using a 4x objective.



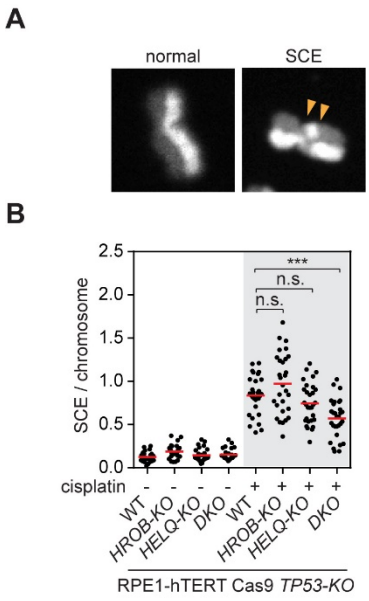
Supplemental Figure S7: Gene mutations causing cisplatin sensitivity are enriched for ICL repair, NER and HR factors.

This figure is related to Figure 5A.

Gene Ontology (GO) term enrichment analysis of Biological Process Complete terms of 37 genes that were hits (normZ ≤ -7.5) in both RPE1-hTERT Cas9 *TP53-KO* (WT) and RPE1-hTERT Cas9 *TP53-KO HROB-KO #1* (*HROB-KO*) cisplatin sensitivity screens using default settings. Shown are GO terms that are significantly enriched (>10-fold enrichment and $P < 0.05$; binomial test with Bonferroni correction). Circle size indicates number of genes from 37-gene hit list included in each GO term, color indicates negative log *P*-value and x-axis position indicates the fold enrichment compared to the whole genome reference set.


A
RPE1-hTERT Cas9 TP53-KO HELQ-KO HROB-KO
% of sequences
p < 0.001
p ≥ 0.001
42.1
40.3
4.5
5.2
<--deletion insertion-->
B
RPE1-hTERT Cas9 TP53-KO
WT
HROB-KO
HELQ-KO
DKO
kDa
135
100
HELQ
75
63
HROB
63
48
α-tubulin
C
radials per metaphase
cisplatin
WT
HROB-KO
HELQ-KO
DKO
RPE1-hTERT Cas9 TP53-KO
D
breaks per metaphase
RPE1-hTERT Cas9 TP53-KO
G
clonogenic survival [%]
log10(cisplatin [nM])
EC50 [nM]
RPE1-hTERT Cas9 TP53-KO:
WT
BRCA1-KO
E
clonogenic survival [%]
log10(MMC [nM])
EC50 [nM]
RPE1-hTERT Cas9 TP53-KO:
WT
HROB-KO
HELQ-KO
HROB-KO HELQ-KO
H
log10(MMC [nM])
RPE1-hTERT Cas9 TP53-KO:
WT
BRCA1-KO
F
log10(olaparib [nM])
RPE1-hTERT Cas9 TP53-KO:
WT
HROB-KO
HELQ-KO
HROB-KO HELQ-KO
I
log10(olaparib [nM])
RPE1-hTERT Cas9 TP53-KO:
WT
BRCA1-KO

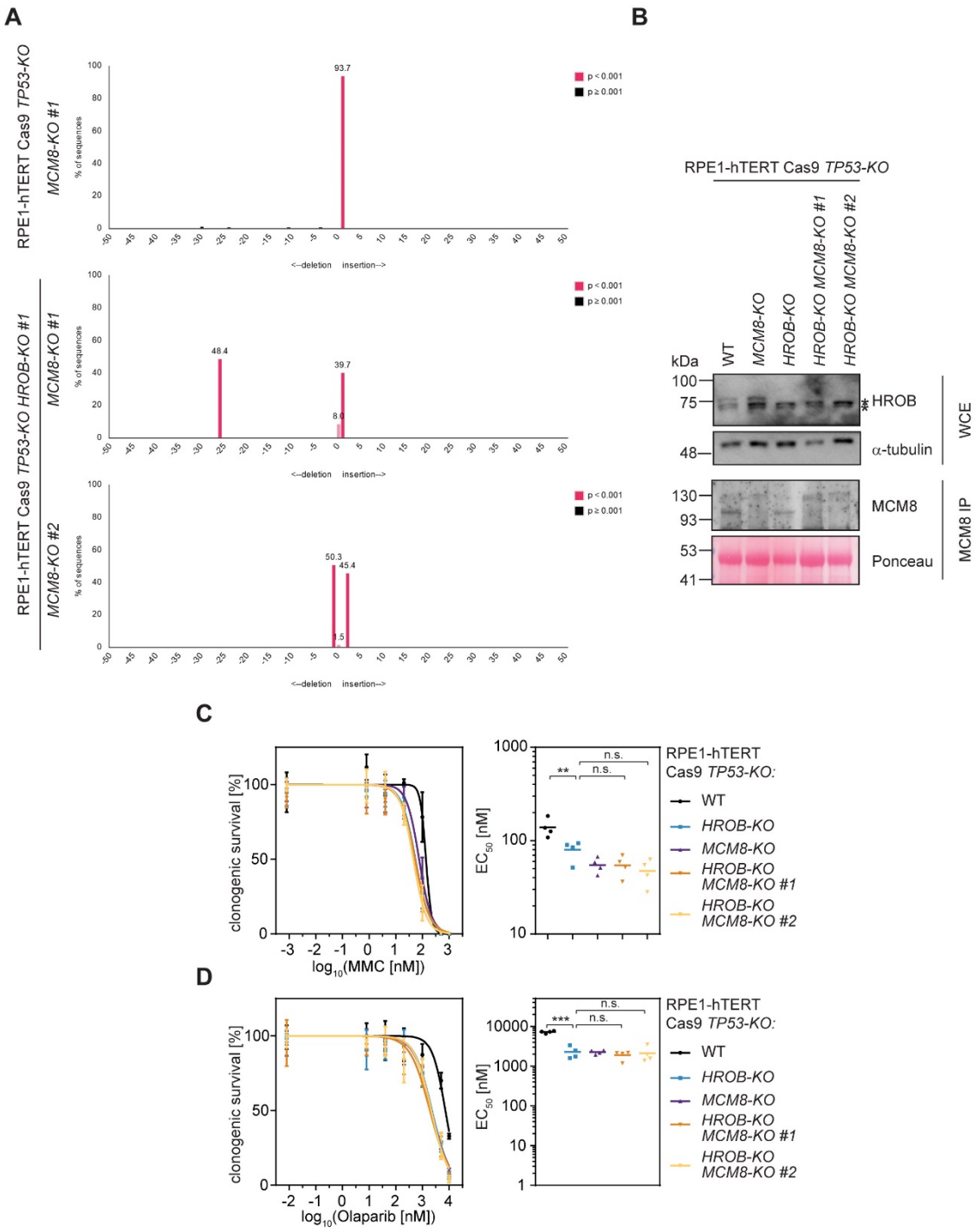
Supplemental Figure S8: *HROB-KO* is synergistic with *HELQ-KO*.
This figure is related to Figure 5B, C, D, E.
A, PCR was used to amplify the region surrounding the *HROB* sgRNA targeting site in HELQ-KO cells. Data shown is TIDE analysis of sequences that was used to assess indel formation in the *HROB* gene to create the *HELQ-KO HROB-KO* double knockout cell line. **B**, Lysates of RPE1 cells of the indicated genotypes were analyzed by immunoblotting with antibodies to HELQ, HROB or alpha-tubulin (loading control). DKO, *HELQ-KO HROB-KO* double knockout. Asterisks indicate non-specific bands. **C, D**, Quantification of radial chromosomes (**C**) and chromosome breaks (**D**) in metaphase spreads of RPE1 cells of the indicated genotypes, with and without cisplatin treatment. Data are pooled from three biologically independent experiments (≥ 30 metaphases scored per experiment). **E, F**, Mitomycin C (MMC) (**E**) and olaparib (**F**) sensitivity of RPE1 cells of the indicated genotypes. Left panels, quantification of colony formation. Lines show a nonlinear least-squares fit to a normalized dose–response model with a variable slope. Right panels, EC_{50} values calculated from fitted curves. Lines indicate mean. Data are from three (**E**) or four (**F**) biologically independent experiments. **G, H, I,** Cisplatin (**G**), MMC (**H**) and olaparib (**I**) sensitivity of RPE1wild type (WT) and RPE1 *BRCA1-KO* (*BRCA1-KO*) cells. Left, quantification of colony formation. Lines show a nonlinear least-squares fit to a normalized dose–response model with a variable slope. Right, EC_{50} values calculated from fitted curves. Lines indicate mean. Error bars indicate standard deviation (n=3 biologically independent experiments). *$P \leq 0.05$, **$P \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.0001$. *P* values in **B** and **C** are from unpaired two-tailed t-tests. *P* values in **F** are from paired two-tailed t-tests.



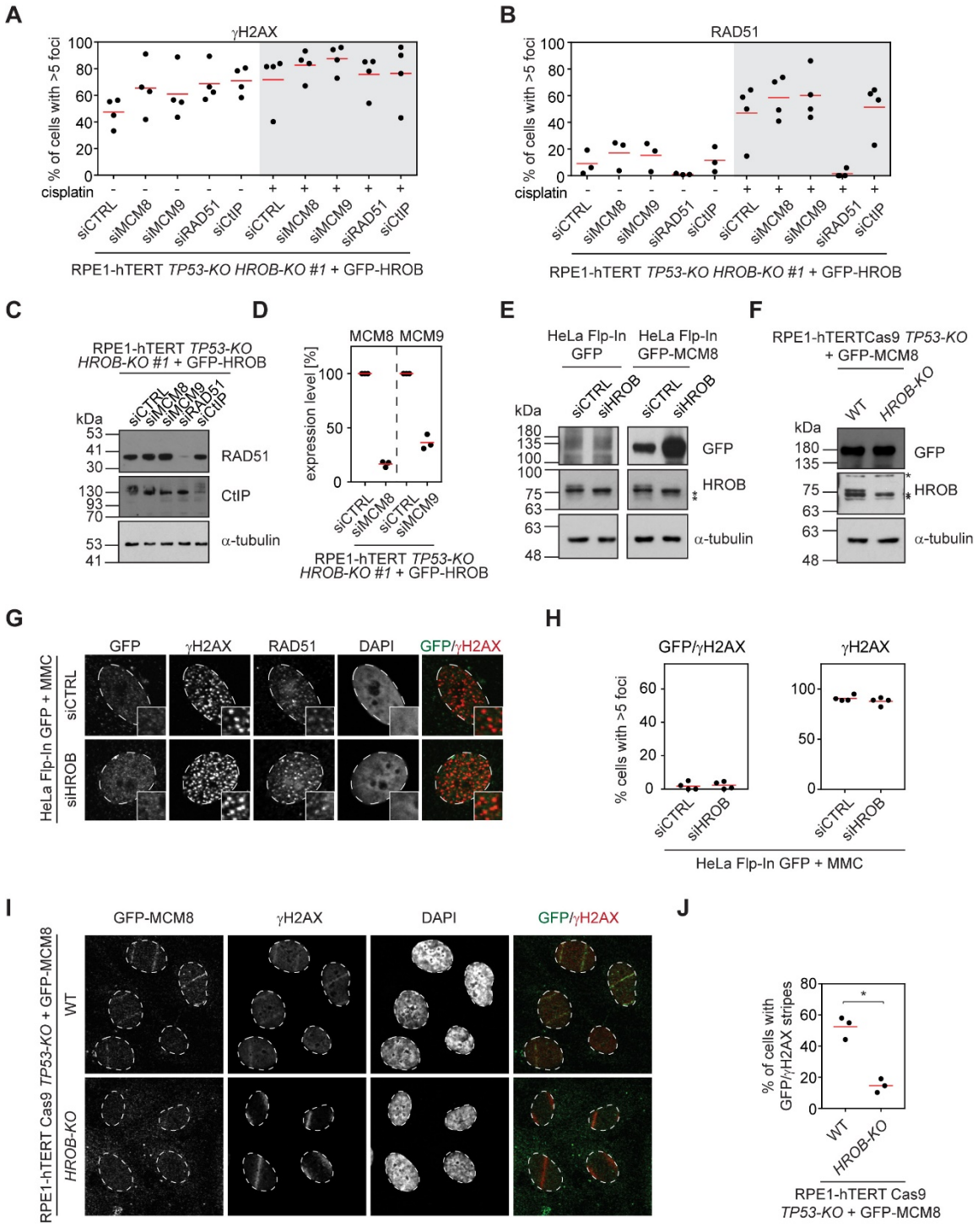
Supplemental Figure S9: *HROB-KO HELQ-KO* double knockout cells show a reduced number of sister chromatid exchanges after cisplatin treatment.

This figure is related to Figure 5 D, E.

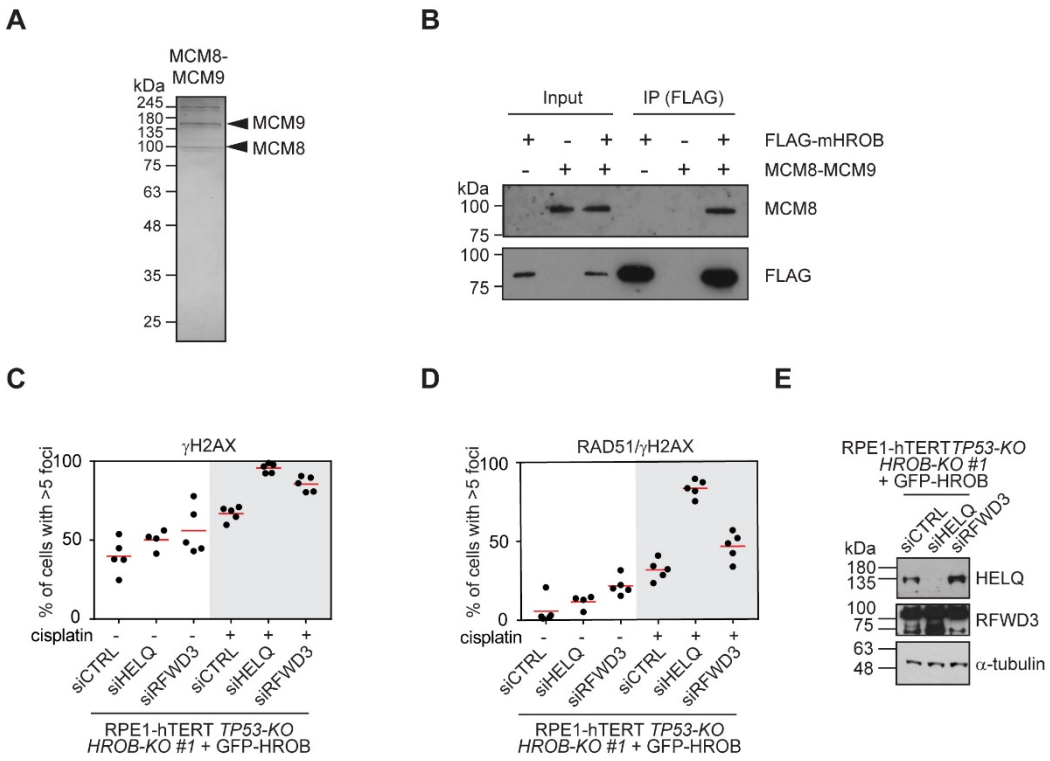
A, B, RPE1 cells of the indicated genotypes were treated with 8 μM cisplatin for 1 h and recovered in medium without cisplatin for 24 h before metaphase spread preparation. **A,** Representative images of a normal chromosome and a chromosome with sister chromatid exchanges (SCE). **B,** Quantification of sister chromatid exchanges on metaphase spreads of untreated or cicplatin-treated cells. DKO, *HELQ-KO HROB-KO* double knockout. Red lines indicate means (n=3 biologically independent experiments). *** $P \leq 0.001$, n.s. $P > 0.05$. *P*-values are from non-paired two-tailed t-tests.



Supplemental Figure S10: *HROB-KO* is epistatic with *MCM8-KO*.
This figure is related to Figure 5F, G.
A, PCR was used to amplify the region surrounding the *MCM8* sgRNA targeting site in the indicated clones. Data shown is TIDE analysis of sequences that was used to assess indel formation in the *MCM8-KO* single knockout and the *HROB-KO MCM8-KO* double knockout cell lines. **B**, whole cell extracts (WCE, upper two panels) from RPE1cells of the indicated genotypes were analyzed by immunoblotting with antibodies to HROB and alpha-tubulin (loading control). MCM8 was only detected by immunoblotting after prior enrichment by immunoprecipitation (IP, lower two panels). Anti-MCM8 immunoprecipitates from cell lysates were probed with anti-MCM8 antibodies. Ponceau S staining served as a loading control. Asterisks indicate nonspecific bands. **C, D**, Mitomycin C (MMC) (**C**) and olaparib (**D**) sensitivity of RPE1 cells with indicated genotypes. Left panels, quantification of colony formation. Lines show a nonlinear least-squares fit to a normalized dose–response model with a variable slope. Right panels, EC_{50} values calculated from fitted curves. Lines indicate mean. Error bars indicate standard deviation (n=4 biologically independent experiments). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, n.s. $P > 0.05$. *P* values in c and d are from paired two-tailed t-tests.


A
γH2AX
% of cells with >5 foci
cisplatin
siCTRL
siMCM8
siMCM9
siRAD51
siCtIP
RPE1-hTERT TP53-KO HROB-KO #1 + GFP-HROB
B
RAD51
C
RPE1-hTERT TP53-KO HROB-KO #1 + GFP-HROB
kDa
RAD51
CtIP
α-tubulin
D
MCM8 MCM9
expression level [%]
E
HeLa Flp-In GFP
HeLa Flp-In GFP-MCM8
GFP
HROB
F
RPE1-hTERTCas9 TP53-KO + GFP-MCM8
WT
HROB-KO
G
GFP
γH2AX
RAD51
DAPI
GFP/γH2AX
HeLa Flp-In GFP + MMC
H
% cells with >5 foci
I
GFP-MCM8
RPE1-hTERT Cas9 TP53-KO + GFP-MCM8
J
% of cells with GFP/γH2AX stripes
RPE1-hTERT Cas9 TP53-KO + GFP-MCM8

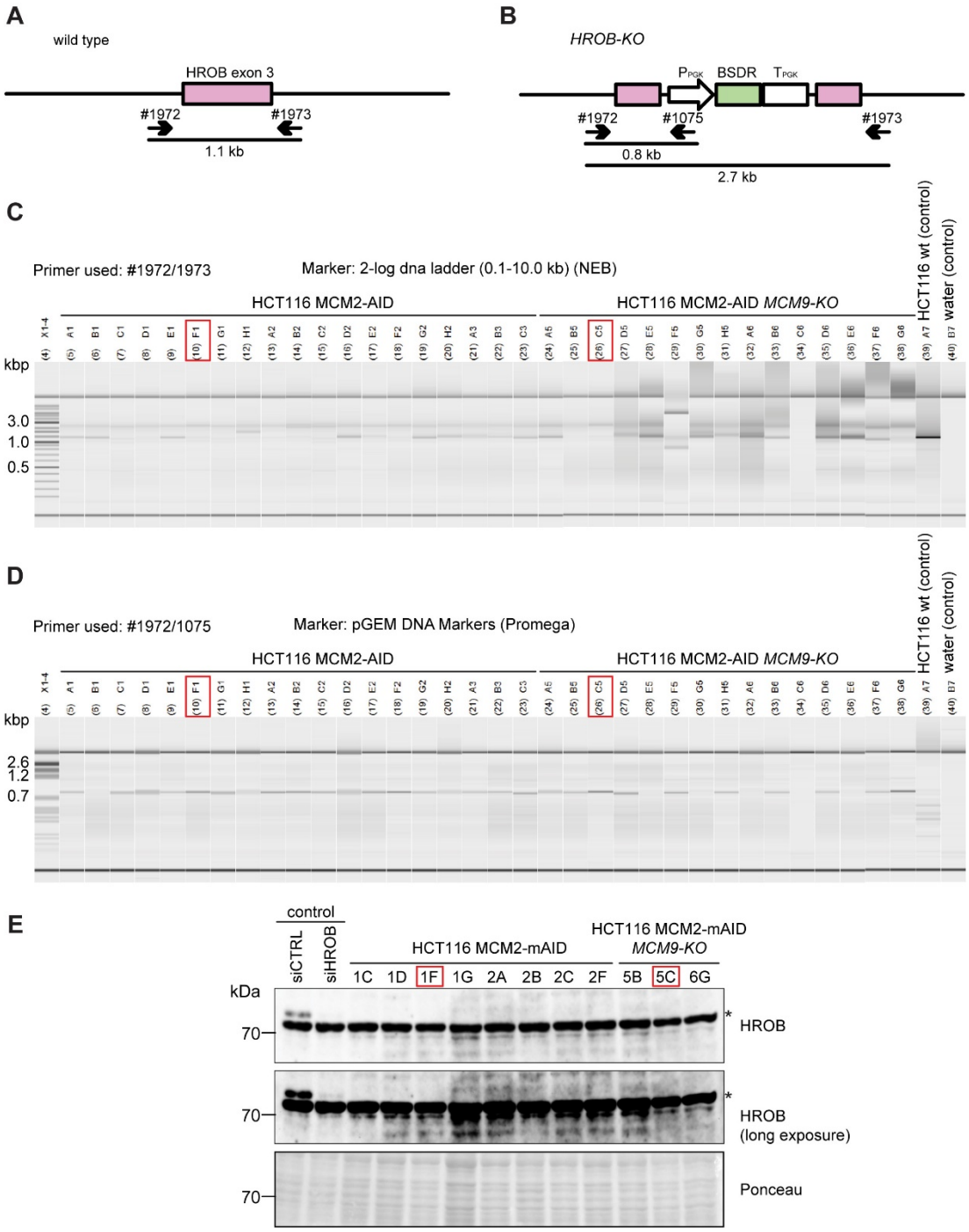
Supplemental Figure S11: Controls for GFP-HROB and GFP-MCM8 localization.
This figure is related to Figure 6A, B, C.
A, B, RPE1 *HROB-KO #1* + GFP-HROB cells were transfected with the indicated siRNAs and were left untreated or were treated with 8 μM cisplatin treatment for 4 h prior to recovery in medium without cisplatin for 20 h. Quantification of γH2AX (**A**) or RAD51/γH2AX colocalizing (**B**) foci in cells with and without cisplatin treatment. Red line indicates mean (n=4 biologically independent experiments. **C**, Lysates of RPE1 *HROB-KO #1* + GFP-HROB cells transfected with the indicated siRNAs were analyzed by immunoblotting with antibodies to RAD51, CtIP and alpha-tubulin (loading control). **D,** Quantification of relative MCM8 and MCM9 mRNA levels in RPE1-hTERT Cas9 *TP53-KO* cells transfected with indicated siRNAs. GAPDH mRNA served as internal normalization standard for each sample. Data are represented as percentage of mRNA levels in cells transfected with non-targeting siRNA (siCTRL). Red line indicates mean (n=3 biologically independent experiments). **E**, Lysates of HeLa Flp-In GFP and HeLa Flp-In GFP-MCM8 cells transfected with indicated siRNAs were analyzed by immunoblotting with antibodies to GFP, HROB or alpha-tubulin (loading control). Asterisks indicate non-specific bands. **F,** Lysates of RPE1 cells with the indicated genotype were analyzed by immunoblotting as in **E**. **G**, **H**, HeLa Flp-In GFP cells were transfected with the indicated siRNAs and treated with 60 nM mitomycin C (MMC) for 24 h. **G**, Representative images of cells stained with the indicated antibodies. **H**, Quantification of GFP/γH2AX colocalizing foci (left) or γH2AX foci (right). Red line indicate means. Data are from four biologically independent experiments. **I**, Representative images of cells stained with indicated antibodies after laser microirradiation. **J**, Quantification of cells with GFP-MCM8/γH2AX colocalizing stripes. Red line indicates mean (n=3 biologically independent experiments). $*P \leq 0.05$. *P*-value in **D** is from paired, two-tailed t-tests. WT, wild type.



Supplemental Figure S12: HROB and the MCM8-MCM9 complex interact direcly *in vitro* and controls for GFP-HROB localization experiments in Figure 6.

This figure is related to Figure 6D, E, F.

A, SDS-PAGE analysis of the purified MCM8-MCM9 complex. **B**, Purified FLAG-mHROB and MCM8-MCM9 were mixed as indicated, subjected to FLAG-immunoprecipitation (IP), and analyzed by immunoblotting with antibodies to MCM8 and FLAG. **C, D,** RPE1 *HROB-KO #1* + GFP-HROB cells were transfected with indicated siRNAs and were left untreated or treated with 8 μM cisplatin for 4 h prior to recovery in medium without cisplatin for 20 h. Quantification of γH2AX (**c**) and RAD51/γH2AX colocalizing (**D**) foci. Red line indicates mean (n=5 biologically independent experiments). **E**, Lysates from RPE1 *HROB-KO #1* + GFP-HROB cells transfected with the indicated siRNAs were analyzed by immunoblotting with antibodies to HELQ, RFWD3 and alpha -tubulin (loading control).



Supplemental Figure S13: Validation of HCT116 *MCM2-mAID HROB-KO* clones.
This figure is related to Figure 6G, H.
A, Schematic representation of wild type genomic HROB exon 3. **B**, Schematic representation of the *HROB* exon 3 genomic locus after integration of the blasticidin selection cassette at the CRISPR guide RNA targeting sequence to create *HROB-KO* mutants in HCT116 *MCM2-mAID* cell lines. P_{PGK}, PGK promoter; BSDR, blasticidin resistance gene; T_{PGK}, PGK terminator. Arrows indicate primer annealing sites. Horizontal black lines indicate resulting PCR products. **C, D**, PCR primers surrounding the targeting site were used to amplify the region of interest from isolated clones and were analyzed by MultiNA. **E**, Immunoblotting of whole cell extracts from isolated clones using the HROB antibody. Red boxes indicated cell clones used for the EdU incorporation assay in Fig. 6G, H. HCT116 *MCM2-mAID* cells transfected with negative control (siCTRL) or HROB siRNA were used as controls for the presence of HROB. Asterisks indicated non-specific bands. Loading was controlled by staining the membrane with Ponceau S.