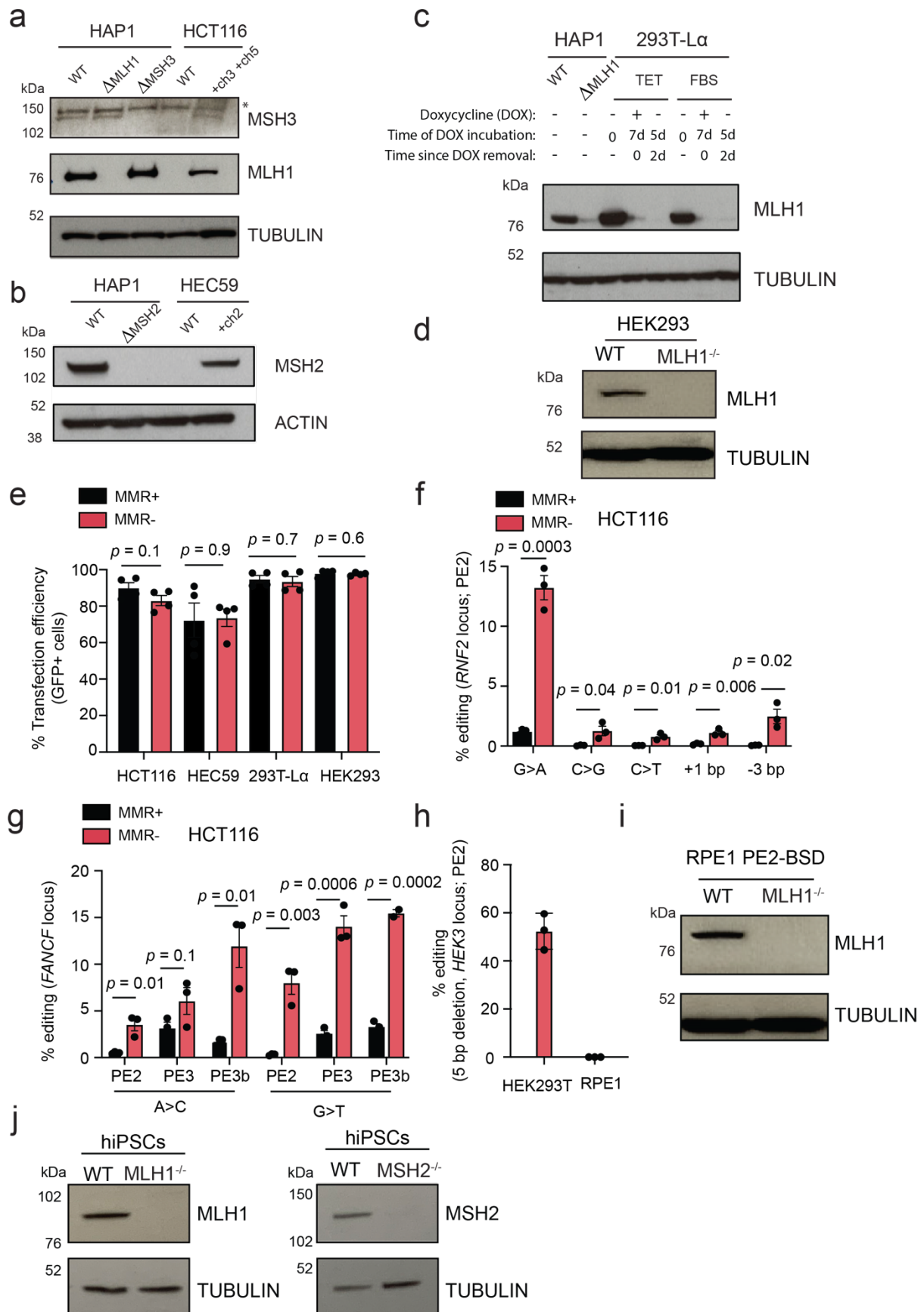
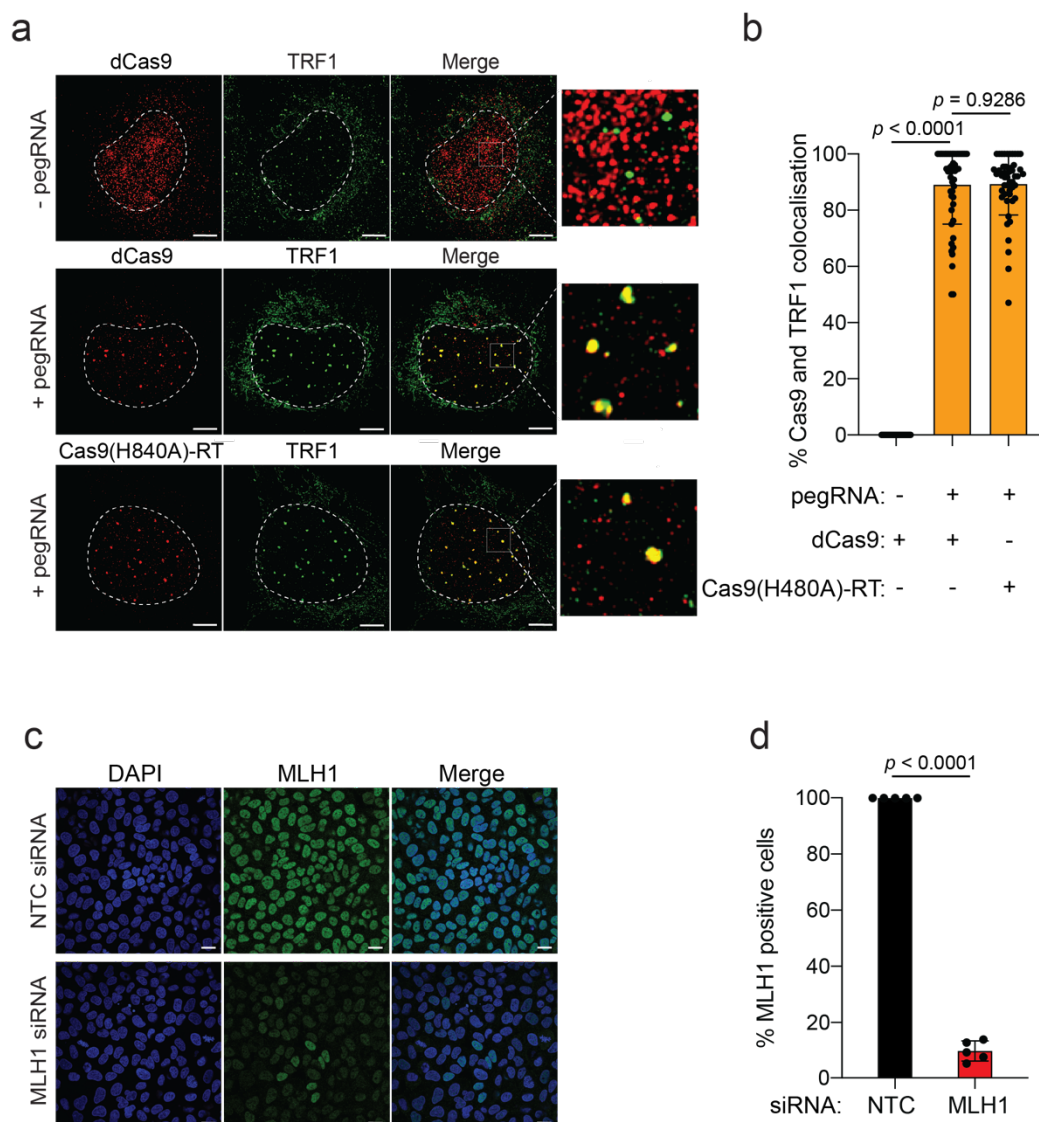


Supplementary Information



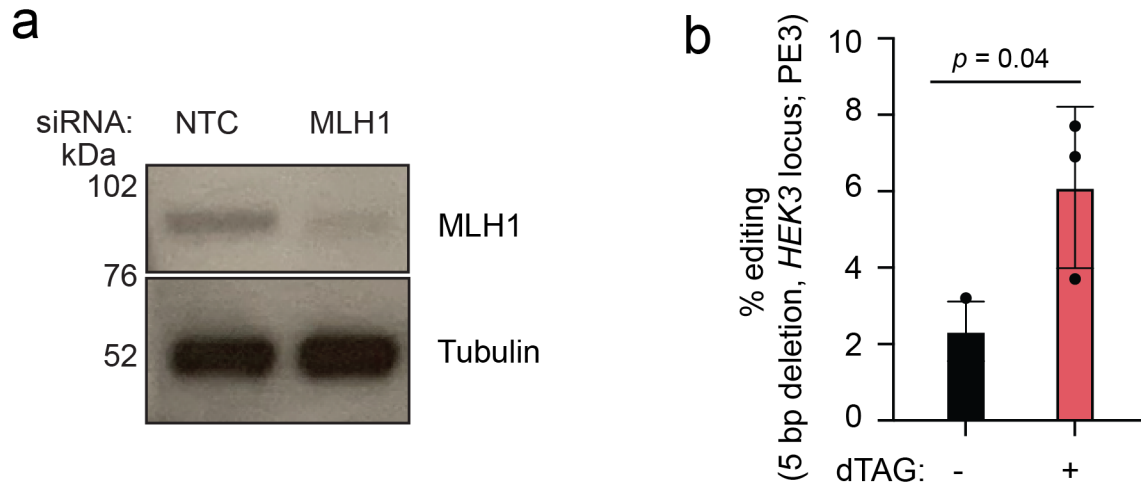
Supplementary Figure 1: Characterisation of mismatch repair-proficient cell lines.

A) Immunoblot for MSH3, MLH1 and Tubulin in HAP1 (WT, Δ MLH1, Δ MSH3) and HCT116 (WT and complemented with chromosome 3 and 5) cell extracts. * denotes a non-specific band. n=1 biologically independent experiment. **B)** Immunoblot for MSH2 and Actin in HAP1 (WT, Δ MSH2) and HEC59 (WT and complemented with chromosome 2) cell extracts. n=2 biologically independent experiments. **C)** Immunoblot for MLH1 and Tubulin in HAP1 (WT, Δ MLH1) and 293T-L α cell extracts. 293T-L α were cultured with or without doxycycline (DOX) in 10% of Tet-approved FBS (TET) or regular FBS. n=2 biologically independent experiments. **D)** Immunoblot for MLH1 and tubulin in HEK293 WT cells, as well as an MLH1-isogenic knockout (MLH1^{-/-}). n=1 biologically independent experiment. **E)** Transfection efficiency of indicated cell lines, measured by flow-cytometry, after transfection of a GFP-encoding plasmid, for n=4 biologically independent experiments. **F)** Efficiency of PE2 for different mutations in the *RNF2* locus, measured in HCT116 cells complemented with chromosomes 3 and 5 (MMR+), as well as HCT116 WT (MMR-). Editing efficiency measured for n=3 biologically independent experiments. **G)** Efficiency of PE2, PE3 and PE3b after installation of an A>C or a G>T mutation in the *FANCF* locus, in HCT116 cells complemented with chromosomes 3 and 5 (MMR+), or HCT116 WT (MMR-). Editing efficiency measured for n=3 biologically independent experiments. **H)** PE2 efficiency after installation of a 5 bp deletion in the *HEK3* locus, in HEK293T cells as well as RPE1 cells. These cell lines express Cas9(H840A)-RT constitutively (PE2-BSD). Editing efficiency measured by Sanger sequencing and analysed by TIDE, for n=3 biologically independent experiments. **I)** Immunoblot for MLH1 and Tubulin in RPE1 WT cells, as well as an MLH1 isogenic knockout (MLH1^{-/-}). These cell lines express Cas9(H480)-RT constitutively (RPE1 PE2-BSD). n=1 biologically independent experiment. **J)** Immunoblot for MLH1 (left) and MSH2 (right) in WT human induced pluripotent stem cells (hiPSCs) as well as isogenic knockouts for MLH1 (MLH1^{-/-}) and MSH2 (MSH2^{-/-}). n=2 biologically independent experiments. Statistical analysis using unpaired two-tailed Student's *t*-test across biological replicates only. Error bars reflect mean $\pm$ s.e.m. Source data are provided as a Source Data file.

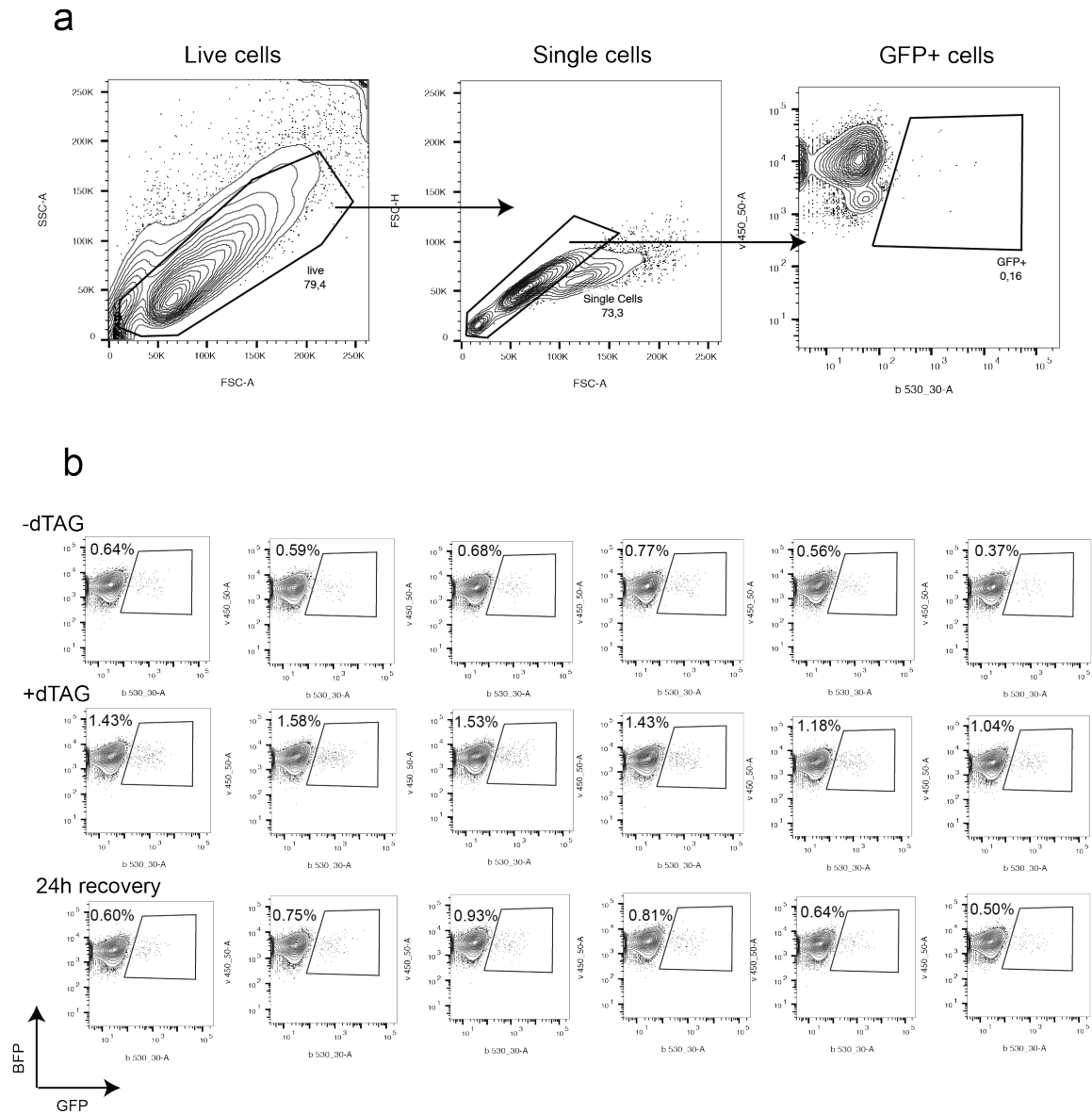


Supplementary Figure 2: Localisation of proteins to sites of active prime editing. A)

Representative super-resolution images of dCas9, or Cas9(H840A)-RT, and TRF1 in U2OS cells, 24 hours following reverse transfection in the presence and absence of a pegRNA targeting telomeric repeats. n=minimum 50 cells examined over 3 biologically independent experiments. Scale bars=5 μ m. **B)** Quantification of A, indicating the percentage of dCas9 and Cas9(H840A)-RT foci that co-localise with TRF1, in the presence or absence of a pegRNA targeting telomeric regions. n=minimum 50 cells examined over 3 biologically independent experiments. **C)** Representative images of MLH1 staining in U2OS cells transfected with either a non-targeting control siRNA (NTC) or an siRNA targeting MLH1. Scale bars=20 μ m. n=minimum 50 cells examined over 3 biologically independent experiments. **D)** Quantification of C indicating percentage of MLH1-positive cells. n=minimum 50 cells examined over 3 biologically independent experiments. Statistical analysis using unpaired two-tailed Student's *t*-test across biological replicates only. Error bars reflect mean $\pm$ s.e.m. Source data are provided as a Source Data file.

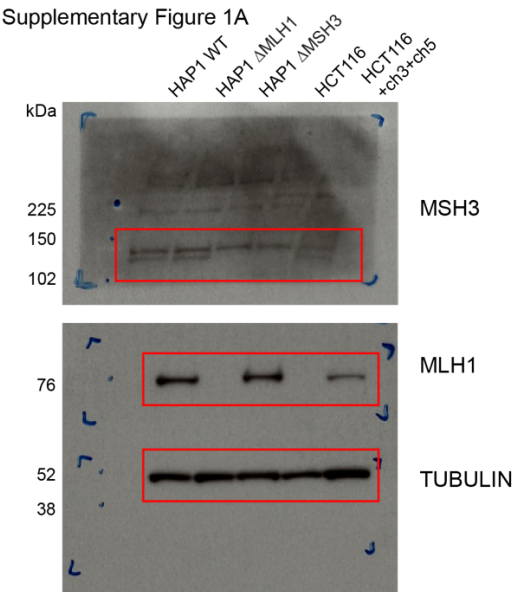


Supplementary Figure 3: Strategies to improve prime editing efficiency by transient ablation of MLH1. A) Immunoblot for MLH1 and Tubulin in HEK293 cell extracts, three days post transfection with non-targeting control (NTC) or MLH1 siRNA pools (of four siRNAs). $n=3$ biologically independent experiments. **B)** PE efficiency (PE3) of a 5 bp deletion in the *HEK3* locus in HAP1 dTAG-MLH1 cells in the presence and absence of 500 nM dTAG-7. Values correspond to editing efficiency, measured by Sanger sequencing and analysed by TIDE (Brinkman et al., 2014), for $n=3$ biologically independent experiments. Statistical analysis using unpaired two-tailed Student's *t*-test across biological replicates only. Error bars reflect mean $\pm$ s.e.m. Source data are provided as a Source Data file.

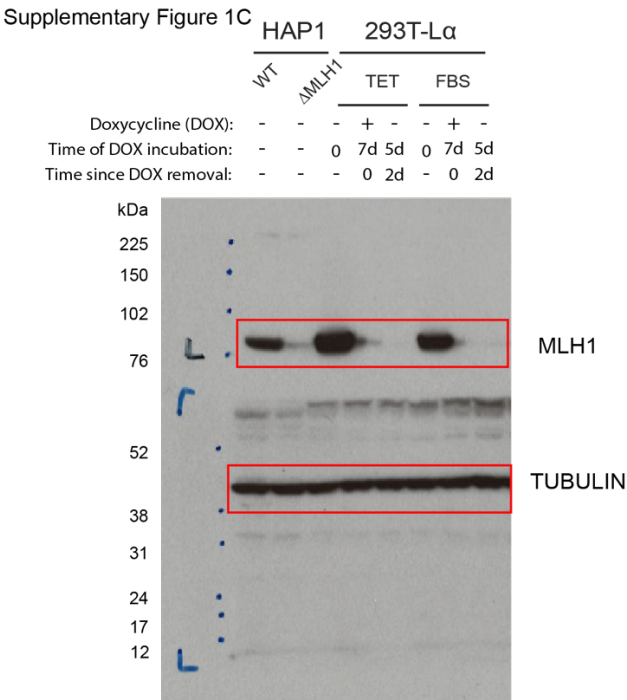


Supplementary Figure 4: Gating strategy and flow cytometry plots. A) Gating strategy to assess BFP>GFP conversion by PE2. Live cells were gated on FSC-A and SSC-A profiles. Within this gate, doublets were excluded based on the FSC-A and FSC-H profile. BFP/GFP-double positive cells were gated within single cells, using a GFP-negative sample as a control. The population within the BFP/GFP-double positive gate was used to determine the percentage of BFP>GFP conversion depicted in Figure 3C. **B)** FACS plots showing the percentage of BFP>GFP conversion by PE in dTAG-MLH1 HAP1 cells, treated with the dTAG ligand ('+dTAG'), untreated ('-dTAG'), or after 24 hours in ligand-free media ('24h recovery'). N=3 biologically independent experiments, with two technical replicates each.

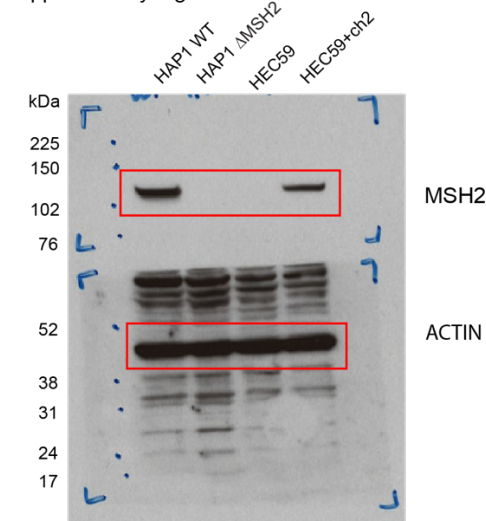
Supplementary Figure 1A



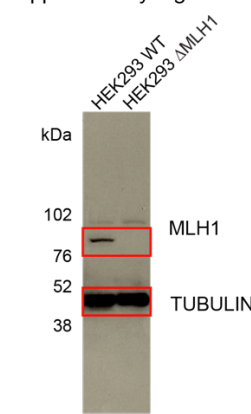
Supplementary Figure 1C



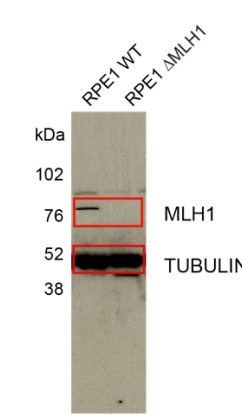
Supplementary Figure 1B



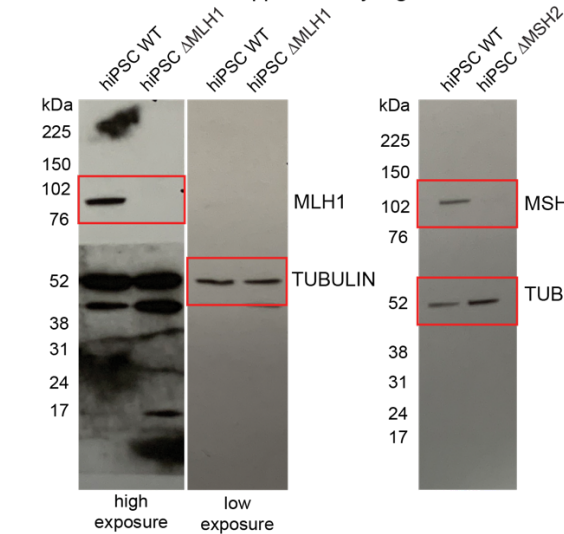
Supplementary Figure 1D



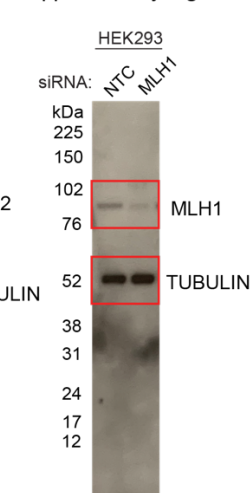
Supplementary Figure 1I



Supplementary Figure 1J



Supplementary Figure 3A



Supplementary Figure 5: Uncropped immunoblots displayed in the supplementary figures.