Supplemental Information

Supplemental Figure Legends

Figure S1. Loss of MMR genes increase ATRi sensitivity.

(A) mRNA levels of *ATR, ATRIP*, and regulators of the ATR pathway in MSS or MSI tumor samples from 91 patients with colorectal or endometrial cancers. RPKM (Reads per kilobase per million mapped reads).

(B-C) Western blots confirming the loss of MLH1 protein in CT26 (B) and B16 (C) *Mlh1 KO* cells derived from single-cell clones. GAPDH was used as a loading control.

(D-E) Relative viability of CT26 and CT26 *Mlh1 KO* cells (D), or B16 and B16 Mlh1 KO cells (E) 5 days after ATRi (VE-821) treatment at the indicated concentrations. Cell viability was measured using CellTiter-Glo.

(F) Relative viability of MC38 and MC38 Mlh1 KO cells 5 days after ATRi (AZD6738) treatment at the indicated concentrations.

(G) Western blots confirming the knockdown of MSH2 and MLH1 by siRNAs in U2OS cells.

(H). Relative viability of U2OS cells treated with control, MSH2, or MLH1 siRNA 5 days after ATRi (AZD6738) treatment at the indicated concentrations.

(I) Western blot confirming the knockdown of MLH1 by siRNA in human MSS cell lines SW620, MKN45, and LS513.

(J) Relative viability of SW620, MKN45, and LS513 cells treated with control or MLH1 siRNA 5 days after ATRi (AZD6738) treatment at the indicated concentrations.

(K) Western blots confirming the knockdown of MLH1, Rad17, and Tipin by siRNAs in U2OS cells.

(L) Relative viability of U2OS cells treated with various combinations of control, MLH1, Rad17, and Tipin siRNAs 5 days after ATRi (AZD6738) treatment at the indicated concentrations.

Figure S2. ATRi preferentially induces DNA damage in MMR-d cells.

(A) Relative viability of CT26 and CT26 *Mlh1 KO* cells after ATRi (AZD6738) treatment at the indicated concentrations and timepoints. Cell viability was measured with CellTiter-Glo.

(B) Western blot confirming the knockdown of MSH2, MLH1, and MUS81 by siRNAs in U2OS cells. GAPDH was used as a loading control.

(C) Images of γ-H2AX-MSH2 PLA foci in cells analyzed in Fig. 2E. Scale bar = 20μm.

(D) Images of γ-H2AX-PCA PLA foci in cells analyzed in Fig. 2F. Scale bar = 20μm.

Figure S3. ATRi reduces growth of MMR-d tumors in vivo.

(A-B) B16 *Mlh1 KO* (clone #6 in A and #2 in B) xenograft growth with or without treatment of 50 mg/Kg ATR inhibitor (AZD6738) by oral gavage every 2 days after tumor size reached 70-100 mm^3.

(C-D) Curves of overall survival for mice bearing CT26 *Mhl1 KO* [clone #6 in (C) and #2 in (D)] tumors with or without ATRi (AZD6738) treatment as in (A).

(E) Images for CT26 *Mlh1 KO* tumors in the absence or presence of CD8+ T cells, and with or without ATRi (AZD6738) treatment as in (A).

Figure S4. ATRi increases cytosolic DNA and triggers interferon responses in MMR-d cells.

(A-B) CT26 or CT26 *Mhl1 KO* cells were labeled with EdU for 2h and then treated with ATRi for 3h. Cells were analyzed by PLA assay with antibodies to biotin (EdU) and actin. Images of cells with or without PLA foci are shown in (A). The number of PLA foci per cell was quantified in (B) as in Fig. 4B. Red bars: mean number of PLA foci per cells in cell populations.

(C) CT26 cells were treated as in (A) and analyzed by PLA using anti-biotin or anti-actin antibody (not combination). Images of random cells are shown.

(D-E) MC38 cells were treated ana analyzed as in (A). Images of cells with or without PLA foci are shown in (D). The number of PLA foci per cell was quantified in (E). Red bars: mean number of PLA foci per cells in cell populations.

Figure S5. ATRi stimulates cGAS-mediated interferon response and augments immunotherapy of MMR-d tumors.

(A) Images of CT26 and CT26 *Mlh1 KO* tumors after treatments with ATRi, anti-PD-1, or the combination as in Fig. 5D.

(B) Curves of overall survival of mice bearing CT26 or CT26 *Mlh1 KO* tumors after treatments with ATRi, anti-PD-1, or the combination.