RESEARCH BRIEF

Werner Helicase Is a Synthetic-Lethal Vulnerability in Mismatch Repair–Deficient Colorectal Cancer Refractory to Targeted Therapies, Chemotherapy, and Immunotherapy

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ABSTRACT Targeted therapies, chemotherapy, and immunotherapy are used to treat patients with mismatch repair–deficient (dMMR)/microsatellite instability-high (MSI-H) colorectal cancer. The clinical effectiveness of targeted therapy and chemotherapy is limited by resistance and drug toxicities, and about half of patients receiving immunotherapy have disease that is refractory to immune checkpoint inhibitors. Loss of Werner syndrome ATP-dependent helicase (WRN) is a synthetic lethality in dMMR/MSI-H cells. To inform the development of WRN as a therapeutic target, we performed WRN knockout or knockdown in 60 heterogeneous dMMR colorectal cancer preclinical models, demonstrating that WRN dependency is an almost universal feature and a robust marker for patient selection. Furthermore, models of resistance to clinically relevant targeted therapy, chemotherapy, and immunotherapy retain WRN dependency. These data show the potential of therapeutically targeting WRN in patients with dMMR/MSI-H colorectal cancer and support WRN as a therapeutic option for patients with dMMR/MSI-H cancers refractory to current treatment strategies.

SIGNIFICANCE: We found that a large, diverse set of dMMR/MSI-H colorectal cancer preclinical models, including models of treatment-refractory disease, are WRN-dependent. Our results support WRN as a promising synthetic-lethal target in dMMR/MSI-H colorectal cancer tumors as a monotherapy or in combination with targeted agents, chemotherapy, or immunotherapy.

INTRODUCTION

DNA mismatch repair (MMR) is an evolutionarily conserved process that recognizes and repairs spontaneously misincorporated bases during DNA replication. Microsatellite instability (MSI) is caused by impaired MMR and is a ubiquitous feature in cancer, observed in more than 20 different tumor types and frequently present in colon, ovarian, endometrial, and gastric cancer, with hundreds of thousands of MSI cancer diagnoses worldwide each year.

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Lynch syndrome is caused by inherited MMR defects (1). Approximately 10% to 15% of sporadic colorectal cancer display mismatch repair–deficient (dMMR)/MSI, with important prognostic and therapeutic implications for patients (2).

Molecularly targeted therapies and chemotherapy agents are used to treat patients with dMMR colorectal cancer. Tumor evolution and resistance are major causes of treatment failure and mortality in patients with colorectal cancer (3, 4). For instance, activating KRAS mutations lead to primary and secondary resistance to EGFR-targeted therapies (5, 6). Combination therapies based on vertical suppression of the EGFR–MAPK pathway are effective in BRAF-mutated colorectal cancer tumors (7–10), but again resistance occurs in preclinical models and the clinical setting (11–13). Rearrangements in *ROS1, ALK*, or *NTRK* are also enriched in dMMR tumors (14, 15) and lead to hypersensitivity to matched kinase inhibitors (16). Resistance to these matched targeted agents can emerge due to *NTRK1* mutations or by genomic alterations that converge to activate the MAPK pathway (17–19). Immunotherapy with checkpoint inhibitors to PD-1 and PD-L1 are effective against dMMR colorectal cancer tumors due to their high mutational burden and increased numbers of neoantigens (20–22). While response rates to checkpoint inhibitors are high and durable for many patients with dMMR colorectal cancer, around half experience primary resistance and disease that is refractory to treatment (22–25), and secondary resistance is a problem (21, 26, 27). Thus, while advances in precision medicine have led to improved treatment options for patients with dMMR/MSI-high (MSI-H) colorectal cancer, a range of mechanisms can confer resistance and there remains an unmet clinical need for new therapeutic options for patients with disease that is refractory to currently available therapies.

We and others recently identified Werner helicase (WRN) as a synthetic-lethal target in dMMR/MSI-H cancers, with a large proportion of sensitivities in colorectal cancer cell lines (28–31). WRN is a member of the RecQ family of DNA helicases and has important but poorly understood roles in maintaining genome stability, DNA repair, replication, transcription, and telomere maintenance (32, 33). WRN is selectively essential for dMMR/MSI-H cell viability both *in vitro* and *in vivo*, and WRN knockout in dMMR/MSI-H cells induces double-stranded DNA breaks and widespread genome instability, promoting apoptosis (28–31). A previously unappreciated genetic feature of dMMR/MSI-H cancer cells, DNA $(TA)_n$-dinucleotide repeat expansions, has recently been reported to cause the selective vulnerability to WRN depletion (34). Given these promising results, translational efforts are needed to comprehensively evaluate the efficacy of WRN inactivation and the performance of dMMR/MSI status as a biomarker of response for patient stratification. In this context, targeting WRN potentially represents an effective option as first-line treatment in monotherapy or combinatorial regimens. Additionally, WRN dependency has not been evaluated in advanced or therapy-refractory tumors, such as in the context of primary and acquired resistance to targeted agents, chemotherapy, and/or immunotherapy.

In the present study, we determined the spectrum of WRN dependency in a broad collection of dMMR/MSI-H colorectal cancer models, including those derived from patients with disease refractory to targeted agents and chemotherapy or who displayed limited benefit from immune checkpoint inhibitors. We demonstrate that WRN dependency is widespread in a heterogeneous collection of dMMR models, supporting the use of MSI status for patient stratification. Additionally, we provide evidence that WRN synthetic lethality is retained in diverse models of primary and acquired resistance to targeted therapy, chemotherapy, and checkpoint inhibitor therapy, expanding the cohort of patients potentially benefiting from WRN-targeted therapies.

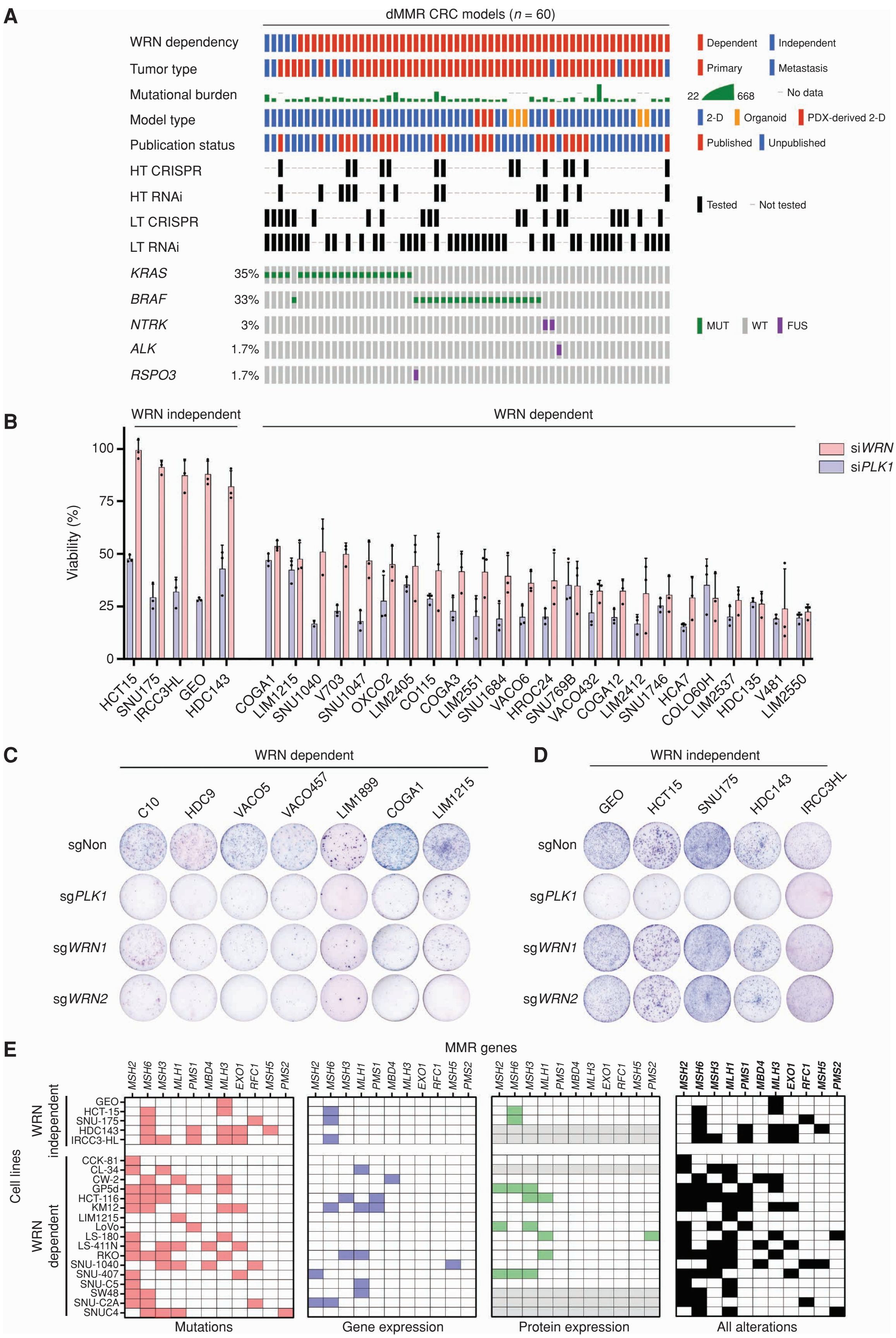
RESULTS

WRN Dependency in Heterogeneous dMMR Colorectal Cancer Preclinical Models

WRN helicase is a promising candidate drug target for dMMR cancers. A limited number of colorectal cancer cell lines have been used to evaluate WRN inhibition efficacy, and an in-depth evaluation of WRN dependency in a diverse set of preclinical models is missing. To assess the robustness of the WRN–dMMR association, we assembled the largest collection of dMMR colorectal cancer preclinical models to date, including 60 unique models (each from a different individual) derived from primary tumors and metastatic lesions and comprising both cancer cell lines and newly generated patient-derived 3-D organoid cultures (Fig. 1A; Supplementary Table S1). This collection reflects the genetic/molecular diversity observed in patients with dMMR/MSI-H colorectal cancer (Supplementary Fig. S1A). Pathogenic missense mutations in *KRAS* occurred in 35% (n = 21) of models, while $BRAF^{V600E}$ mutations were present in 33% (n = 20). Cell lines with oncogenic driver gene fusions in the *NTRK* gene (n = 2), as well as *ALK* and *RSPO3* genes (n = 1 of each), were represented (35, 36).

Of the 60 dMMR colorectal cancer models, we curated published WRN dependency data for 22 cell lines previously

Figure 1. Landscape of WRN dependency in dMMR colorectal cancer preclinical models. **A,** Oncoprint representation of WRN dependency and oncogenic driver mutations in dMMR colorectal cancer (CRC) models. For each model, WRN dependency status, type of model, tumor type, mutational burden, publication status of WRN dependency data, and assay types are annotated. Missense mutations in KRAS and BRAF and oncogenic rearrangements in NTRK1, ALK, and RSPO3 are indicated. **B,** WRN depletion assay in 29 dMMR colorectal cancer cell lines. Bars are normalized viability upon siRNA-mediated WRN depletion in WRN-dependent cell lines and WRN-independent cell lines, as indicated. Nontargeting siRNA or *PLK1* siRNAs (blue bars) were used as negative and positive controls, respectively. Dots represent mean and SD of three independent experiments with five technical replicates each. SNU1040 was tested twice. **C,** WRN dependency in hard to transfect cell lines and models displaying an intermediate response by RNA interference evaluated by CRISPR/Cas9-based clonogenic assays (14 days). **D,** Clonogenic assays of dMMR colorectal cancer models insensitive to WRN knockout. Clonogenic assays are representative of three independent experiments. **E,** Genomic and proteomic profile of MMR pathway gene alterations in dMMR colorectal cancer models. Colored (red, blue, green, and light black) boxes indicate the presence of the alteration. Light gray boxes represent data unavailable.


A
dMMR CRC models (n = 60)
WRN dependency
Tumor type
Mutational burden
Model type
Publication status
HT CRISPR
HT RNAi
LT CRISPR
LT RNAi
KRAS 35%
BRAF 33%
NTRK 3%
ALK 1.7%
RSPO3 1.7%
Dependent
Independent
Primary
Metastasis
22
668
No data
2-D
Organoid
PDX-derived 2-D
Published
Unpublished
Tested
Not tested
MUT
WT
FUS
B
WRN independent
WRN dependent
Viability (%)
100
75
50
25
0
siWRN
siPLK1
HCT15
SNU175
IRCC3HL
GEO
HDC143
COGA1
LIM1215
SNU1040
V703
SNU1047
OXCO2
LIM2405
CO115
COGA3
LIM2551
SNU1684
VACO6
HROC24
SNU769B
VACO432
COGA12
LIM2412
SNU1746
HCA7
COLO60H
LIM2537
HDC135
V481
LIM2550
C
WRN dependent
C10
HDC9
VACO5
VACO457
LIM1899
COGA1
LIM1215
sgNon
sgPLK1
sgWRN1
sgWRN2
D
WRN independent
GEO
HCT15
SNU175
HDC143
IRCC3HL
sgNon
sgPLK1
sgWRN1
sgWRN2
E
MMR genes
MSH2
MSH6
MSH3
MLH1
PMS1
MBD4
MLH3
EXO1
RFC1
MSH5
PMS2
Cell lines
WRN independent
GEO
HCT-15
SNU-175
HDC143
IRCC3-HL
WRN dependent
CCK-81
CL-34
CW-2
GP5d
HCT-116
KM12
LIM1215
LoVo
LS-180
LS-411N
RKO
SNU-1040
SNU-407
SNU-C5
SW48
SNU-C2A
SNUC4
Mutations
Gene expression
Protein expression
All alterations

measured by genome-wide CRISPR/Cas9 screens or siRNA-mediated *WRN* knockdown (28, 30, 37). Profiles of WRN dependency were generated by CRISPR/Cas9 and/or RNA interference for an additional 38 dMMR colorectal cancer preclinical models not included in previous studies, including models derived from metastatic lesions (Fig. 1A). Cell lines (n = 29) were tested by RNA interference (Fig. 1B), while patient-derived organoids (n = 5) were tested by either CRISPR/Cas9-based dropout screening or viability and co-competition assays (Supplementary Fig. S1B–S1D). Five additional difficult-to-transfect cell lines and models displaying an intermediate response by RNA interference were confirmed to be sensitive using CRISPR/Cas9-based clonogenic assays (Fig. 1C). Strikingly, altogether 92% (55 of 60) of dMMR/MSI colorectal cancer models were dependent on WRN for viability, irrespective of the presence of different cancer driver mutations or gene rearrangements (Fig. 1A). As expected, MMR-proficient models were not affected by WRN knockout (Supplementary Fig. S1B). Interestingly, 5 outlier dMMR models were not dependent on WRN, retaining more than 75% viability following depletion (Fig. 1B). We independently confirmed the lack of WRN dependency in these models by CRISPR/Cas9 clonogenic assays and efficient WRN downregulation and knockout by Western blot (Fig. 1D; Supplementary Fig. S1E and S1F). Moreover, in WRN-independent MSI-H cells, less than 10% of metaphases are affected by double-strand breaks (DSB) after WRN knockout, similar to what is detected in microsatellite-stable (MSS) cells (Supplementary Fig. S1G and S1H).

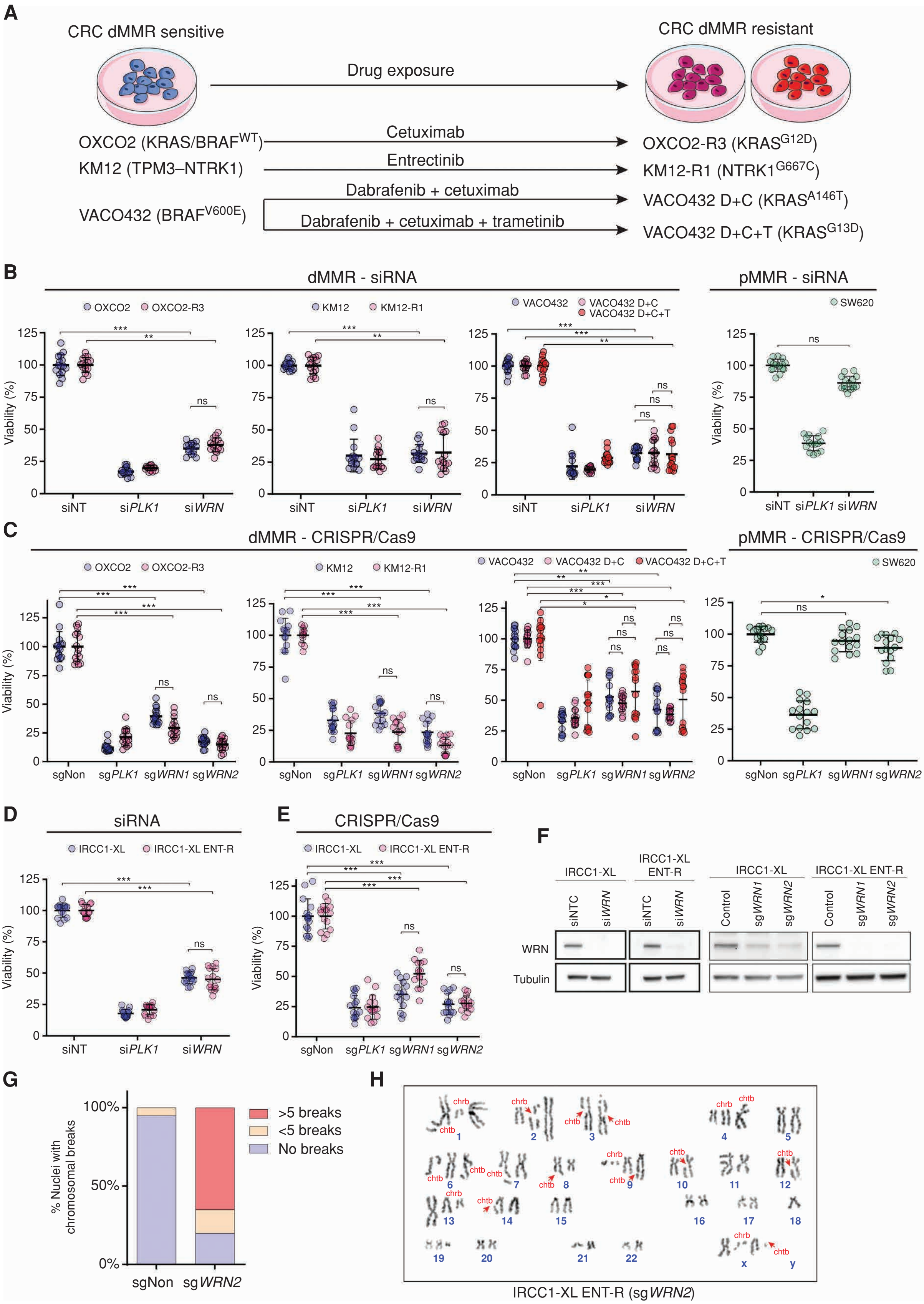
Integration of multiple mutation, gene, and protein expression data sets for the models confirmed that all had one or more alterations in a gene encoding a protein involved in MMR (Fig. 1E). WRN dependency was not associated with mutational burden ($P = 0.88$; Student t test). Interestingly, we observed a statistically significant enrichment for MSH2 ($P = 0.0048$ or 0.0357 excluding cell lines with missing data; Fisher exact test) and MLH1 ($P = 0.0096$ or 0.0625) alterations in WRN-dependent versus WRN-independent cell models. We reassessed MSI status by PCR and independently evaluated MLH1, MSH2, and MSH6 protein expression by Western blot for WRN-independent lines (Supplementary Fig. S2A). All the models were confirmed MSI-H except GEO, which was reclassified as MSI-low, explaining WRN independence and the absence of alterations in canonical MMR pathway genes in this model. An analogous analysis in an independent set of cancer models from non–colorectal cancer dMMR/MSI-H-predominant tissue lineages confirmed an enrichment for *MSH2* alterations (P value = 0.0391) in WRN dependent models, but not MLH1 (Supplementary Fig. S2B). We then performed PCR-based and whole-genome sequencing (WGS) coverage analysis to assess MSI cell lines for expanded TA repeats, a recently identified feature of MSI cells contributing to WRN synthetic lethality (34). WGS data were available for a subset of cell lines. We confirmed the presence of expanded TA repeats in MSI WRN-dependent cell lines compared with MSS cells, as evidenced by a failure to PCR amplify some broken repeat regions and reduced WGS sequencing coverage across broken repeats ($P < 0.001$; Supplementary Fig. S2C and S2D). Strikingly, MSI-H WRN–independent cells were most similar to MSS cells, with little or no evidence of expanded TA repeats with either analysis. The expanded TA-repeat phenotype was variable in cell lines within the MSI subgroups, but nonetheless our results suggest that repeat length is not altered, or at least not to the same extent, in WRN-independent MSI-H cell lines.

Overall, employing a heterogeneous collection of dMMR/MSI-H colorectal cancer models, including a large cohort of previously untested models, our results indicate that inhibiting WRN has a nearly universal synthetic-lethal effect, strongly supporting WRN as a target and dMMR as a therapeutic biomarker for patient selection. There exists, however, a rare subset of dMMR/MSI-H colorectal cancer, characterized by the absence of MLH1 and MSH2 alterations and expanded TA-repeat phenotype, which is not dependent on WRN and would presumably be refractory to WRN-targeted therapies.

WRN Inhibition Is Effective in dMMR Colorectal Cancer Models of Acquired Resistance to Targeted Therapies and Chemotherapy

New treatment options for patients with advanced and treatment-refractory disease represent an unmet clinical need. Given the diverse genetic background of tumors dependent on WRN, we hypothesized that dMMR tumors with acquired resistance to targeted therapies and chemotherapy may retain WRN dependency. To investigate this, we began by using isogenic dMMR colorectal cancer cell models of acquired resistance to clinically relevant single-agent or combination therapies (Fig. 2A; refs. 11, 18, 38). Specifically, cells were made resistant *in vitro* to the anti-EGFR mAb cetuximab, the combination of cetuximab and the BRAF inhibitor (BRAFi) dabrafenib (D+C), or the NTRK inhibitor entrectinib. We confirmed drug sensitivity of the parental cell lines and corresponding resistance of the derivative line (Supplementary Fig. S3A). Upon RNAi-mediated silencing of *WRN*, all models showed a marked reduction in fitness (Fig. 2B). To confirm these results, we independently

Figure 2. WRN dependence in models of acquired resistance to targeted agents. **A,** Representation of *in vitro* dMMR colorectal cancer (CRC) models of acquired resistance to EGFR, NTRK1, and BRAF target therapies. **B,** Cell viability in models of acquired resistance upon transfection of WRN-targeting siRNAs. PLK1 (si*PLK1*) siRNA were used as positive control. MMR-proficient (pMMR) cell line SW620 was included as a negative control. Data are the mean ± SD of three independent experiments with five technical replicates each and were analyzed with two-tailed Student t test comparing si*WRN* to nontargeting control. ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. **C,** Normalized viability data in models of acquired resistance upon WRN knockout. Nonessential (sgNon) and PLK1 (sg*PLK1*) sgRNAs were used as negative and positive controls, respectively. The pMMR SW620 cell line was a negative control. Data are mean and SD of three independent experiments with five technical replicates each. Statistical significance was evaluated comparing *WRN* sgRNAs versus nonessential gene sgRNA (sgNon) performing a two-tailed Student t test. ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. **D,** Viability of IRCC-1-XL-ENT-R cells upon transfection of *WRN*-targeting siRNAs. **E,** Normalized viability data of IRCC-1-XL-ENT-R cells upon WRN knockout. **F,** WRN reduction verified by Western blot analysis. siRNA nontargeting controls (siNTC), siRNA targeting *WRN* (si*WRN*). Tubulin is a loading control. Representative of two independent experiments. **G,** Quantification of metaphase chromatid breaks in IRCC1-XL-ENTR-R cells 96 hours after transduction with *WRN* sgRNA ($n \geq 20$ randomly selected metaphases analyzed). **H,** Representative metaphase karyotype of IRCC-1-XL-ENT-R cells after 96 hours of transduction with *WRN*-targeting sgRNA2. Red arrows indicate chromosome (chrb) and chromatid (chtb) breaks.


A
CRC dMMR sensitive
Drug exposure
CRC dMMR resistant
OXCO2 (KRAS/BRAF^WT)
Cetuximab
OXCO2-R3 (KRAS^G12D)
KM12 (TPM3–NTRK1)
Entrectinib
KM12-R1 (NTRK1^G667C)
Dabrafenib + cetuximab
VACO432 D+C (KRAS^A146T)
VACO432 (BRAF^V600E)
Dabrafenib + cetuximab + trametinib
VACO432 D+C+T (KRAS^G13D)
B
dMMR - siRNA
pMMR - siRNA
OXCO2 OXCO2-R3
KM12 KM12-R1
VACO432 VACO432 D+C VACO432 D+C+T
SW620
Viability (%)
siNT siPLK1 siWRN
C
dMMR - CRISPR/Cas9
pMMR - CRISPR/Cas9
sgNon sgPLK1 sgWRN1 sgWRN2
D
siRNA
IRCC1-XL IRCC1-XL ENT-R
E
CRISPR/Cas9
F
IRCC1-XL
IRCC1-XL ENT-R
siNTC siWRN
Control sgWRN1 sgWRN2
WRN
Tubulin
G
% Nuclei with chromosomal breaks
>5 breaks
<5 breaks
No breaks
sgNon sgWRN2
H
chrb
chtb
IRCC1-XL ENT-R (sgWRN2)

performed CRISPR/Cas9 knockout of WRN and observed a marked reduction in cell fitness in all drug-sensitive and drug-resistant lines (Fig. 2C). Downregulation or knockout of the WRN protein was confirmed by Western blot analysis (Supplementary Fig. S3B and S3C).

Triple therapy based on EGFR, BRAF, and MEK inhibitors recently demonstrated efficacy in patients with metastatic colorectal cancer with the $BRAF^{V600E}$ mutation (9). To validate WRN dependency in this setting, we selected drug-resistant BRAF-mutated VACO432 cells in the presence of D+C double therapy, and dabrafenib, trametinib, and cetuximab (D+C+T) triple therapy (Fig. 2A). The resulting resistant cells had a $KRAS^{G13D}$ mutation, which is a common mechanism of acquired resistance to this therapy regimen in patients with colorectal cancer (ref. 10; Supplementary Fig. S3D). Remarkably, cell lines resistant to double or triple therapy retained notable sensitivity to the loss of WRN (Fig. 2B and C). Finally, we used cell lines derived from a patient-derived xenograft (PDX) model generated from a patient with colorectal cancer positive for *LMNA–NTRK1* rearrangement, treated *in vivo* with entrectinib in a mouse–human coclinical trial (18). An $NTRK1^{G595R}$ mutation led to entrectinib resistance both in the patient and in the resistant cell line generated from the tumor that acquired resistance *in vivo* (Supplementary Fig. S3E and S3F). Again, both the entrectinib-sensitive and entrectinib-resistant cell lines showed a strong dependency on WRN (Fig. 2D–F). *WRN* knockout in LMNA–NTRK1 cells led to numerous chromosomal abnormalities, including chromatid and chromosome breaks and rearrangements (Fig. 2G and H; Supplementary Fig. S3G).

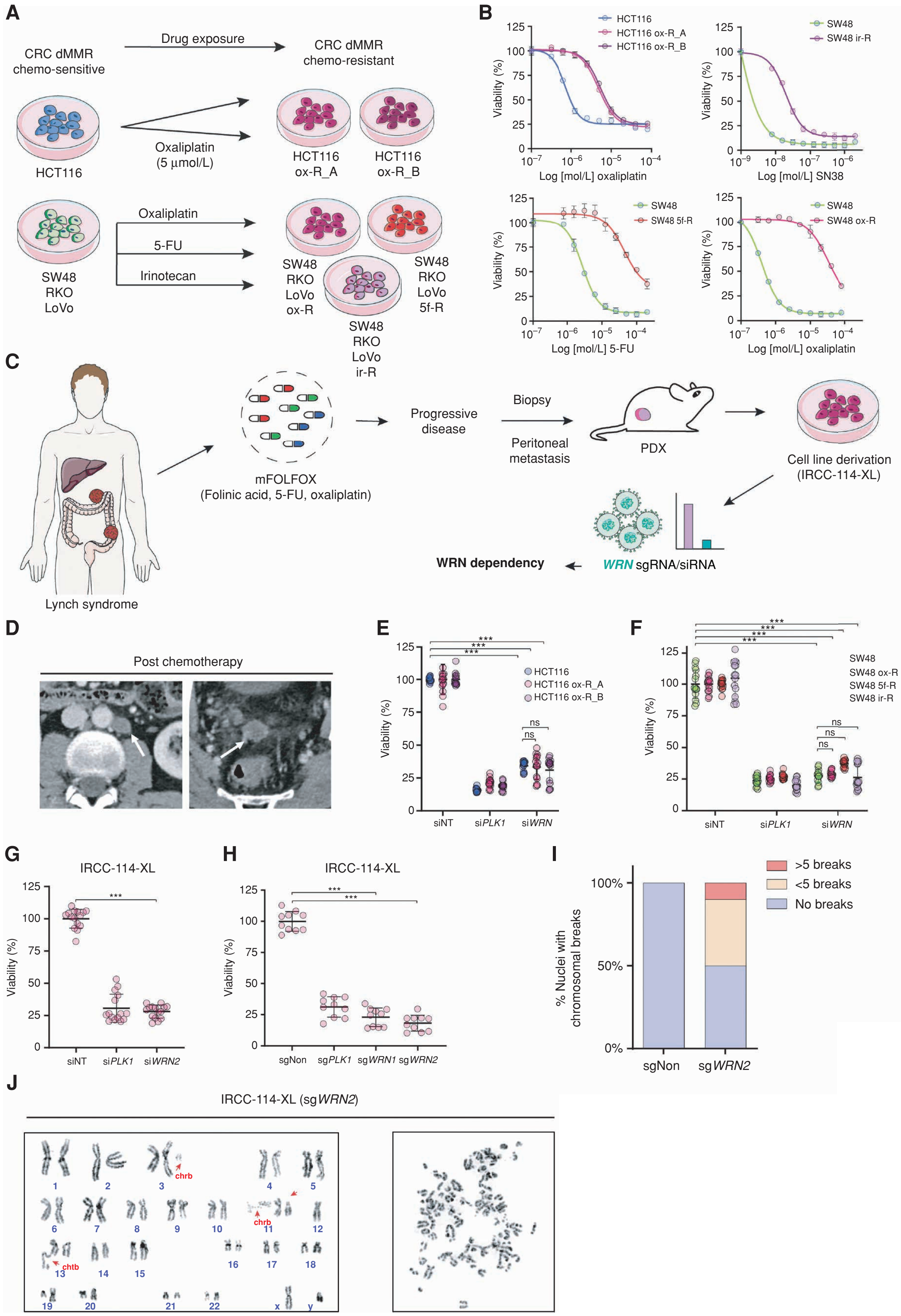
We next evaluated WRN dependency in the setting of acquired resistance to standard-of-care chemotherapeutic agents. We treated the MSI colorectal cancer cell line HCT116 with increasing doses of oxaliplatin (two independent selections) until resistant cells emerged. We also generated MSI colorectal cancer SW48, RKO, and LoVo cells resistant to irinotecan, oxaliplatin, or 5-fluorouracil (5-FU; Fig. 3A and B; Supplementary Fig. S4A). In addition, we established a cell line (IRCC-114-XL) from the PDX of a patient with a clinical history of Lynch syndrome, who experienced relapse after surgery and 6 months of treatment with mFOLFOX (folinic acid, 5-FU, and oxaliplatin), displaying no objective response and rapid progression of disease (Fig. 3C and D). Notably, WRN knockout or depletion markedly reduced the viability of all 12 chemotherapy-resistant dMMR/MSI-H colorectal cancer sublines and IRCC-114-XL cells (Fig. 3E–H; Supplementary Fig. S4B–S4D). *WRN* knockout in IRCC-114-XL cells promoted DSB formation and marked chromosomal defects (Fig. 3I and J; Supplementary Fig. S4E–S4G).

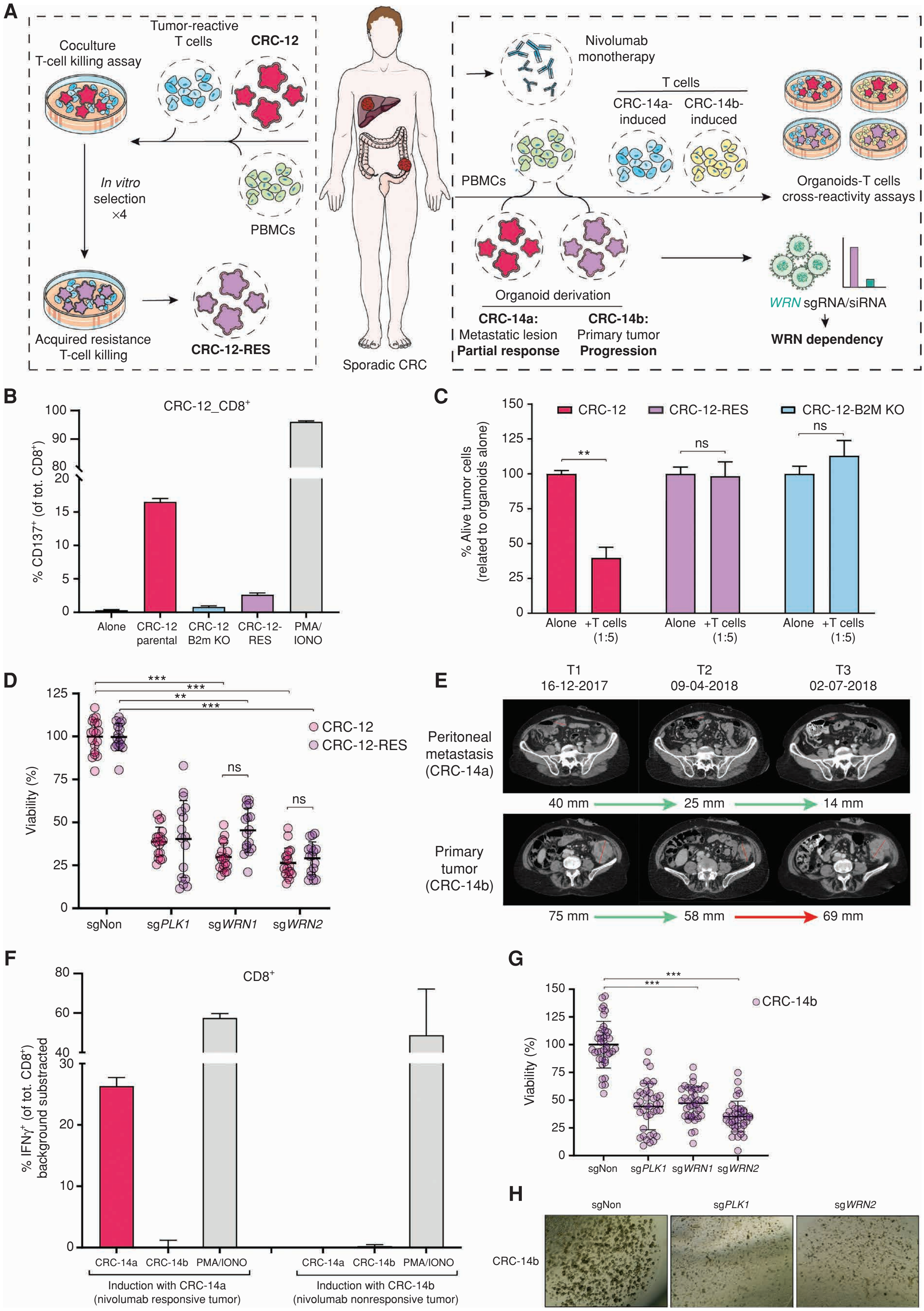
These results demonstrate that dMMR colorectal cancer cells resistant to clinically relevant targeted therapies or chemotherapy retain a synthetic-lethal dependency on WRN, irrespective of the mutational background of the tumor and the therapeutic regimen to which resistance was acquired.

Patient-Derived dMMR Colorectal Cancer Models Refractory to Immunotherapy Are WRN-Dependent

We next used multiple patient-derived organoid models to investigate whether dMMR colorectal cancer tumors responding poorly to immunotherapy are dependent on WRN. First, we evaluated WRN dependency in the setting of resistance to T cell–mediated tumor cell killing using an autologous tumor organoid and peripheral blood lymphocyte coculture system (39, 40). We made use of a previously established organoid model from a patient with dMMR colorectal cancer (CRC-12) together with matched tumor-reactive T cells generated by 2 weeks of coculturing peripheral blood mononuclear cells (PBMC) with tumor organoids (ref. 39; Fig. 4A, left). CRC-12 cells were killed by autologous tumor-reactive T cells in a dose-dependent manner. Killing was rescued by the addition of an MHC class I blocking antibody, confirming an antigen-specific $CD8^+$ T cell–mediated response (Supplementary Fig. S5A). To generate a model of resistance, we *in vitro* selected a subpopulation of CRC-12 organoids resistant to T-cell killing (CRC-12-RES). In addition, as a positive control for resistance, we knocked out the *B2M* gene to create an isogenic CRC-12 line (CRC-12-B2M) and confirmed loss of MHC-I expression (Supplementary Fig. S5B). CD137 surface expression was used as a marker for T-cell activation. Autologous $CD8^+$ T cells were reactive to CRC-12 tumor organoids, whereas no CD8-mediated reactivity was detected in the presence of CRC-12-RES or CRC-12-B2M organoids; $CD4^+$ T-cell reactivity remained unaffected (Fig. 4B; Supplementary Fig. S5C and S5D). Accordingly, while CRC-12 parental organoids were killed by autologous tumor-reactive T cells, CRC-12-RES and CRC-12-B2M KO organoids were unaffected by the presence of the reactive population (Fig. 4C). Resistance in CRC-12-RES organoids was not due to the loss of MHC-I or IFNγ receptor (Supplementary Fig. S5B and S5E), and *B2M* mutations were absent. Next, we used these advanced models to investigate WRN dependency. Strikingly, *WRN* knockout inhibited viability in the parental CRC-12 organoid, as well as CRC-12-RES, demonstrating that strong WRN dependency is retained in a model refractory to autologous T cell–mediated cytotoxicity (Fig. 4D).

Figure 3. WRN dependency in chemoresistant dMMR/MSI-H colorectal cancer sublines and patient-derived model. **A,** dMMR colorectal cancer (CRC) models of acquired resistance to chemotherapeutic agents. **B,** Proliferation assays of cell line models of acquired resistance to chemotherapies and parental counterparts. Data are average ± SD of three technical replicates and are representative of three independent experiments. **C,** The IRCC-114-XL cell line established from a PDX model of a patient with Lynch syndrome treated with mFOLFOX for 6 months after surgery. **D,** CT scan of the IRCC-114 patient displaying drug resistance and early tumor progression after chemotherapy. **E–G,** Normalized viability of upon siRNA-mediated *WRN* depletion in HCT116 and SW48 chemotherapy-resistant sublines and IRCC-114-XL cells. Nontargeting siRNA (siNT) and si*PLK1* were used as negative and positive controls, respectively. Data are mean and SD of three independent experiments with five technical replicates each. Statistical significance was evaluated using a Student *t* test. ns, not significant; *, $P \le 0.05$; **, $P \le 0.01$; ***, $P \le 0.001$. **H,** Normalized viability for IRCC-114-XL cells upon WRN knockout. Nonessential (sgNon) and PLK1 (sg*PLK1*) sgRNAs were negative and positive controls, respectively. Data are mean and SD of two independent experiments with 5 technical replicates each. Statistical significance was calculated using a two-tailed Student *t* test. ns, not significant; *, $P \le 0.05$; **, $P \le 0.01$; ***, $P \le 0.001$. **I,** Chromosome breaks in IRCC-114-XL cell line 96 hours after WRN depletion (≥20 metaphase spreads assayed). **J,** Representative images of IRCC-114-XL metaphases (left) and a pulverized metaphase (right) after 96 hours of transduction with an sg*WRN*. Red arrows indicate chromosome (chrb) and chromatid (chtb) breaks.


A
Drug exposure
CRC dMMR chemo-sensitive
CRC dMMR chemo-resistant
HCT116
Oxaliplatin (5 µmol/L)
HCT116 ox-R_A
HCT116 ox-R_B
Oxaliplatin
5-FU
Irinotecan
SW48 RKO LoVo
SW48 RKO LoVo ox-R
SW48 RKO LoVo 5f-R
SW48 RKO LoVo ir-R
B
HCT116
HCT116 ox-R_A
HCT116 ox-R_B
Viability (%)
Log [mol/L] oxaliplatin
SW48
SW48 ir-R
Log [mol/L] SN38
SW48 5f-R
Log [mol/L] 5-FU
SW48 ox-R
C
Lynch syndrome
mFOLFOX (Folinic acid, 5-FU, oxaliplatin)
Progressive disease
Biopsy
Peritoneal metastasis
PDX
Cell line derivation (IRCC-114-XL)
WRN sgRNA/siRNA
WRN dependency
D
Post chemotherapy
E
siNT
siPLK1
siWRN
F
SW48 ox-R
SW48 5f-R
SW48 ir-R
G
IRCC-114-XL
siWRN2
H
sgNon
sgPLK1
sgWRN1
sgWRN2
I
% Nuclei with chromosomal breaks
>5 breaks
<5 breaks
No breaks
J
IRCC-114-XL (sgWRN2)
chrb
chtb


A
Coculture
T-cell killing assay
Tumor-reactive
T cells
CRC-12
PBMCs
In vitro
selection
×4
Acquired resistance
T-cell killing
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Sporadic CRC
Nivolumab
monotherapy
T cells
CRC-14a-
induced
CRC-14b-
induced
PBMCs
Organoids-T cells
cross-reactivity assays
Organoid derivation
CRC-14a:
Metastatic lesion
Partial response
CRC-14b:
Primary tumor
Progression
WRN sgRNA/siRNA
WRN dependency
B
CRC-12_CD8+
% CD137+ (of tot. CD8+)
Alone
CRC-12 parental
CRC-12 B2m KO
CRC-12-RES
PMA/IONO
C
CRC-12
CRC-12-RES
CRC-12-B2M KO
% Alive tumor cells
(related to organoids alone)
Alone
+T cells (1:5)
ns
D
CRC-12
CRC-12-RES
Viability (%)
sgNon
sgPLK1
sgWRN1
sgWRN2
E
T1
16-12-2017
T2
09-04-2018
T3
02-07-2018
Peritoneal metastasis (CRC-14a)
40 mm
25 mm
14 mm
Primary tumor (CRC-14b)
75 mm
58 mm
69 mm
F
CD8+
% IFNγ+ (of tot. CD8+)
background substracted
CRC-14a
CRC-14b
PMA/IONO
Induction with CRC-14a
(nivolumab responsive tumor)
Induction with CRC-14b
(nivolumab nonresponsive tumor)
G
CRC-14b
Viability (%)
sgNon
sgPLK1
sgWRN1
sgWRN2
H
sgNon
sgPLK1
sgWRN2
CRC-14b

To corroborate our findings, we investigated WRN dependency in two organoids derived from a patient with sporadic dMMR colorectal cancer with variable clinical response to immunotherapy. CRC-14a and CRC-14b were derived from biopsies obtained from a peritoneal metastasis and primary tumor of a patient with a clinical treatment history of capecitabine, oxaliplatin, and bevacizumab, then treated with nivolumab monotherapy (Fig. 4A, right). The CRC-14a metastasis biopsy was taken before the start of the checkpoint blockade, and this lesion regressed on nivolumab, whereas the biopsy for CRC-14b was taken from the primary tumor upon progression on nivolumab (Fig. 4E). To induce (or enrich for) a tumor-reactive T-cell population, both organoids were individually cocultured with autologous PBMCs obtained before treatment with nivolumab (39, 40). After 2 weeks of coculture with CRC-14a (from the responsive metastatic lesion), we observed marked and selective $CD8^+$ T-cell reactivity against CRC-14a (but not CRC-14b) organoids (Fig. 4F). In contrast, when CRC-14b organoids (derived from the nonresponding primary tumor) were used in the coculture, no T-cell reactivity was detected against any of the organoid lines. Of note, $CD4^+$ T-cell reactivity remained unaltered (Supplementary Fig. S5F). Interestingly, loss of MHC-I expression was found in CRC-14b, potentially explaining the failure to generate tumor-reactive T cells from PBMCs and lack of clinical response to nivolumab treatment (Supplementary Fig. S5G). B2M protein expression in CRC-14b was confirmed by flow cytometry (Supplementary Fig. S5H), and no frameshift or nonsense mutations were detected, suggestive of a B2M-independent resistance mechanism, although a nonsynonymous variant of unknown significance (Y30C) was present. These results support CRC-14b as an *ex vivo* model to evaluate WRN dependency in an immune-refractory setting. Viability assays after CRISPR-based knockout of WRN in CRC-14b organoids revealed a strong dependency on the WRN helicase (Fig. 4G and H).

Altogether, these data provide multiple lines of evidence that WRN dependency is retained in patient-derived dMMR colorectal cancer preclinical models of resistance to immunotherapy.

DISCUSSION

We have investigated the potential of therapeutically targeting WRN in preclinical models of dMMR colorectal cancer, including in the setting of resistance to targeted therapies, chemotherapy, and immunotherapy. We used the largest collection of dMMR colorectal cancer preclinical models characterized to date, nearly tripling the number assessed for WRN dependency. More than 90% of models were WRN-dependent, including models with diverse genetic backgrounds, molecular contexts, and oncogenic alterations, suggesting that WRN dependency is an almost universal feature of dMMR/MSI colorectal cancer cells. This reinforces dMMR/MSI status as a robust biomarker for WRN synthetic lethality and to stratify patients for the clinical development of WRN-targeted therapies. Notably, for the approximately 7% of dMMR colorectal cancer models that were WRN-independent, functional expression of MSH2 and MLH1 was retained, suggesting that WRN dependency is influenced by the underlying MMR pathway genes altered. Moreover, TA repeats are differentially altered compared with MSI-H WRN-dependent lines, suggesting that loss of MSH2 or MLH1 might be of particular importance to generate TA-dinucleotide repeat expansions reported to confer WRN addiction (34). This observation warrants confirmation in larger cohorts but, if validated, could provide mechanistic insight into the WRN-MSI synthetic-lethal interaction and help refine patient selection strategies based on novel biomarkers of sensitivity.

Inhibition of WRN leads to genome instability in dMMR cells. This may be due to a catastrophic failure to process TA-dinucleotide expansions that accumulate in MSI cells (34). This is distinctive from targeted agents that inhibit specific oncogenic alterations in cancer cells and immunotherapies that suppress immune evasion and tolerance. Consistent with an orthogonal therapeutic activity, WRN is a synthetic lethality in preclinical models of resistance to molecular targeted therapies, including models addicted to a diverse set of oncogenic alterations and that acquire different genetic mutations to promote therapy escape. In addition, WRN is synthetic-lethal in patient-derived models from patients with dMMR colorectal cancer with limited clinical benefit from chemotherapy and PD-1 inhibitors or resistant to autologous T cell–mediated cancer cell killing. Resistance to targeted therapies can occur through a range of mechanisms, including through reactivation of the targeted pathway, whereas for immunotherapies, several mechanisms of resistance are emerging including loss of antigen processing and presentation (41). Our finding suggests that WRN inhibitors could be effective as a second- or third-line monotherapy for dMMR patients. Indeed, WRN sensitivity was not correlated with mutational load in dMMR tumors, whereas low mutability in dMMR tumors is negatively associated with response to immune checkpoint blockade (25, 42). Because of their independent modes of action, combining a checkpoint inhibitor, chemotherapy, or a targeted therapy with a WRN

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Figure 4. Patient-derived colorectal cancer dMMR organoid models refractory to immunotherapy are WRN-dependent. **A,** dMMR colorectal cancer (CRC) tumor organoid–T-cell cocultures from a sporadic dMMR primary tumor (left) or two lesions in a patient with a heterogeneous clinical response to nivolumab (right). **B,** CD137 expression of $CD8^+$ T cells upon stimulation with different CRC-12–derived organoid lines. Values are background corrected, and bars represent mean and SEM of two independent experiments. **C,** Organoid killing after 3 days of T-cell coculture. Error bars represent SEM of at least two biological replicates. **D,** Viability in CRC-12 and CRC-12-RES upon WRN knockout. Nonessential (sgNon) and *PLK1* (sg*PLK1*) sgRNAs were negative and positive controls, respectively. Data are the mean ± SD of three independent experiments with six technical replicates each. **E,** CT scans over an 8-month period of the peritoneal metastasis lesion and the primary tumor of CRC-14 patient treated with nivolumab. Red and green arrow indicates a size increase and reduction, respectively. **F,** IFNγ expression of $CD8^+$ T cells upon exposure to CRC-14a (responsive) or CRC-14b (nonresponsive) organoids. Stimulation with PMA/ionomycin is a positive control. Background IFNγ-positive cells (in unstimulated condition) were subtracted from the signal. Data are the mean and SEM of at least two independent experiments. **G,** Viability of CRC-14b organoids upon WRN knockout. sgNon and sg*PLK1* were used as negative and positive controls, respectively. Data are the mean ± SD of four independent experiments (10 technical replicates each). **H,** Representative images of CRC-14b (10× magnification) 10 days after transduction with indicated sgRNAs. **C**, **D**, and **G**, Significance was evaluated by two-tailed Student *t* test. ns, not significant; **, $P \leq 0.01$; ***, $P \leq 0.001$.

inhibitor may suppress cross-resistance and promote tumor eradication. Moreover, WRN inhibition may also be synergic with immunotherapy, as loss of DNA repair modulates the neoantigen landscape and increases mutational burden, leading to an enhanced immune response (43). DNA damage resulting from loss of WRN could likewise potentiate the effects of immunotherapy, similar to combining chemotherapeutics with immune-modulating agents (44). Investigations into the effects of WRN inhibition on immune recognition and surveillance to increase therapeutic efficacy for patients with dMMR colorectal cancer refractory to immunotherapy regimens are warranted. Collectively, our findings provide a rationale for the clinical development of WRN-targeted medicines in patients with advanced colorectal cancer and potentially in combination with existing therapies.

For our study, we exploited a tumor organoid T-cell coculture system as a preclinical tool to assess WRN dependence. We used, for the first time, an organoid coculture system to model *in vitro* acquired resistance to T-cell killing. Mechanisms driving resistance to immunotherapy and tumor-reactive T cells in these models are currently unverified, but loss of MHC-I expression in organoids derived from an anti–PD-1–resistant tumor points to a loss of antigenicity and immunogenicity due to immune selection pressure, favoring the growth of tumor cell clones with a nonimmunogenic phenotype, similar to what has been described clinically (45).

WRN has a role in maintenance of genome stability, and Werner syndrome is an autosomal-recessive disorder associated with premature aging caused by mutation in the WRN gene. Nonetheless, WRN mutations are compatible with human development well into the fourth decade of life, and disease-associated complications take decades to manifest, suggesting that a therapeutic window of activity could be achieved using WRN-targeted medicines in appropriately selected patients. WRN is the focus of ongoing drug discovery programs. Small-molecule WRN helicase inhibitors have been reported (46, 47), but their efficacy is impaired by lack of selectivity against dMMR cells, off-target effects, and cytotoxicity to normal cells (48). Our study provides new information to support the continued development of WRN-targeted medicines. Furthermore, as potent and selective WRN drugs are developed, our findings will inform patient selection strategies and provide a strong rationale for their clinical development in patients with dMMR tumors not benefiting from current therapeutics alone.

METHODS

Cell Models

A full description of cell models (cell lines and organoids) used in this study is provided in Supplementary Table S1. The majority of cell lines were curated from the Genomics of Drug Sensitivity 1000 cell line collection and are annotated in the Cell Model Passports database (https://cellmodelpassports.sanger.ac.uk/; ref. 49) or are from previously reported collections (35, 50). The LIM1215 parental cell line has been described previously (51) and was obtained together with LIM2405, LIM2412, and LIM2537 from Prof. Robert Whitehead, Vanderbilt University (Nashville, TN), with permission from the Ludwig Institute for Cancer Research (Zurich, Switzerland). LIM2550 and LIM2551 were obtained from CellBank Australia. Cell lines were maintained in their original culturing conditions according to supplier guidelines or as previously described (52). Cells were supplemented with 10% FBS, 2 mmol/L L-glutamine, and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) and grown at 37 °C and 5% CO_2 air incubator. Cells were routinely screened for the absence of *Mycoplasma* contamination using the VenorGeM Classic Kit (Minerva Biolabs). The identity of each cell line was checked before starting each experiment and after every genomic DNA extraction by PowerPlex 16 HS System (Promega), through short tandem repeats at 16 different loci (D5S818, D13S317, D7S820, D16S539, D21S11, vWA, TH01, TPOX, CSF1PO, D18S51, D3S1358, D8S1179, FGA, Penta D, Penta E, and amelogenin). Amplicons from multiplex PCRs were separated by capillary electrophoresis (3730 DNA Analyzer, Applied Biosystems) and analyzed using GeneMapper v 3.7 software (Life Technologies). The MSI status of the cell lines and organoids in Fig. 1 was previously reported (35, 39, 53) and/or is publicly available (Cell Model Passports database; https://cellmodelpassports.sanger.ac.uk/; ref. 49). The PDX-derived cell line IRCC-114-XL was generated following previously described procedures (37) approved by the Italian Ministry of the Health and the Local Ethics Committee (protocol no. 1014/2009 and 194/2010 of Grande Ospedale Metropolitano Niguarda, Milano, Italy) in accordance with generally accepted guidelines for the use of human material. Organoids were derived at the Sanger Institute by the Cell Model Network UK consortium as part of the Human Cancer Model Initiative, and genomic characteristics, such as microsatellite stability status, were downloaded from the Cell Model Passports website (49).

Patient-derived organoids for immuno-oncology studies were derived at the Netherlands Cancer Institute as previously reported (39, 40). Briefly, tumor tissue was mechanically dissociated and digested with 1.5 mg/mL of collagenase II (Sigma-Aldrich), 10 μg/mL of hyaluronidase type IV (Sigma-Aldrich), and 10 μmol/L Y-27632 (Sigma-Aldrich). Cells were embedded in Geltrex (Geltrex LDEV-free reduced growth factor basement membrane extract, Gibco) and placed in a 37 °C incubator for 20 minutes. Human colorectal cancer organoids medium is composed of Ad-DF+++ [Advanced DMEM/F12 (Gibco) supplemented with 2 mmol/L ultraglutamine I (Lonza), 10 mmol/L HEPES (Gibco), and 100/100 U/mL penicillin/streptomycin (Gibco), 10% Noggin-conditioned medium, 20% R-spondin1–conditioned medium, 1× B27 supplement without vitamin A (Gibco), 1.25 mmol/L N-acetylcysteine (Sigma-Aldrich), 10 mmol/L nicotinamide (Sigma-Aldrich), 50 ng/mL human recombinant EGF (PeproTech), 500 nmol/L A83–01 (Tocris), 3 μmol/L SB202190 (Cayman Chemicals), and 10 nmol/L prostaglandin E2 (Cayman Chemicals)]. Organoids were passaged every 1 to 2 weeks by incubating in TrypLE Express (Gibco) for 5–10 minutes followed by embedding in Geltrex. Organoids and cell lines were authenticated by SNP array and regularly tested for *Mycoplasma* using Mycoplasma PCR43 and the MycoAlert Mycoplasma Detection Kit (catalog no. LT07–318). In the first 2 weeks of organoid culture, 1× Primocin (Invivogen) was added to prevent microbial contamination. All the procedures performed with patient specimens were conducted under the approval of the institutions' local Ethical Committee, after the written informed consent of the patients. The study (NL48824.031.14) was approved by the Medical Ethical Committee of the Netherlands Cancer Institute–Antoni van Leeuwenhoek hospital, and written informed consent was obtained from all patients. Peripheral blood and tumor tissue were obtained from patients with a confirmed diagnosis of colorectal cancer.

Generation of Sublines Resistant to Chemotherapy

Colorectal cancer cell lines SW48, LoVo, RKO, and HCT116 were obtained from ATCC. SW48, LoVo, and RKO drug-resistant sublines were derived from the resistant cancer cell line collection (https://research.kent.ac.uk/industrial-biotechnology-centre/the-resistant-cancer-cell-line-rccl-collection/; ref. 54) and established by continuous exposure to stepwise increasing drug concentrations as previously described (55). SW48, LoVo, and RKO-resistant sublines were adapted to growth in the presence of 5-FU (8, 1.5, and 3 μmol/L; 5f-R), irinotecan (8, 0.34, and 1.7 μmol/L; ir-R), or oxaliplatin (5, 5, and 3.8 μmol/L; ox-R), respectively. SW48, LoVo, and RKO cells were propagated in DMEM/F-12 supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C. Similarly, HCT116-resistant sublines (HCT116 ox-R_A and B) were adapted to growth in the presence of 5 μmol/L oxaliplatin.

Molecular Characterization of dMMR Cancer Cell Lines

The MSI status of WRN-independent dMMR models (GEO, HCT15, HDC143, SNU175, and IRCC3HL) was reassessed and confirmed with the MSI Analysis System Kit (Promega). The analysis requires a multiplex amplification of seven markers, including five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) and two pentanucleotide repeat markers (Penta C and Penta D). The products were analyzed by capillary electrophoresis in a single injection (3730 DNA Analyzer, ABI capillary electrophoresis system; Applied Biosystems). Results were analyzed using GeneMapper V5.0 software. Mutations in MMR pathway genes were downloaded from The Cell Model Passport or Dependency Map (DepMap) websites. Mutations in IRCC3-HL and HDC143 cell lines were obtained by whole-exome sequencing data generated at the Candiolo Cancer Institute. Mutational burden of cancer cell lines was computed analyzing next-generation sequencing data previously published (56–58) and available at the European Nucleotide Archive (accession codes PRJEB33045 and PRJEB33640). Genetic analysis was performed as previously described (56–58). Mutations in VACO432 D+C+T cell model were detected through Sequenom analysis by using the Myriapod Colon status kit (Diatech Pharmacogenetics). SNP were excluded except if predicted as damaging. For gene expression, we used RNA-sequencing (RPKM) data previously generated (59). For GEO and HDC143, we used gene expression data obtained previously (35). Proteomics data were already available (60, 61). To identify which MMR pathway gene displayed altered gene or protein expression, we computed the *Z*-score by gene across all the cell lines in the respective data set and considered genes with Z-score or normalized values less than −2 to identify genes downregulated in a particular sample. WRN dependency was obtained mining essentiality data obtained from multiple sources: Project Score (https://score.depmap.sanger.ac.uk/) and Dependency Map (DepMap; https://depmap.org/portal/) websites or additionally available data sets (62). Cell lines were considered WRN-dependent if WRN essentiality reached threshold values of significance in at least one of the CRISPR (Sanger or DepMap Public 20Q2) or combined RNAi (Broad, Novartis, Marcotte) data sets. Statistical significance was computed by performing Fisher exact comparison for the presence of cumulative alterations (mutation, gene expression, and protein expression) detected in WRN-dependent versus WRN-independent cell lines. For TA-dinucleotide repeat expansion analysis, WGS data for cancer cell lines were downloaded from SRA study SRP186687 (https://trace.ncbi.nlm.nih.gov/Traces/study/?acc=SRP186687). WGS data for IRCC3_XL, HDC143, and SNU175 are available at the European Nucleotide Archive (ENA; accession code PRJEB43711). Fastq files were mapped to human genome reference GRCh38 using bwa-mem alignment algorithm (http://arxiv.org/abs/1303.3997) and then PCR duplicates were marked using the MarkDuplicates tool (http://broadinstitute.github.io/picard). The genomic coordinates of broken and unbroken regions were downloaded from Wietmarschen and colleagues (34) and then converted into the GRCh38 assembly version using the LiftOver tool (63). A total of 5,362 and 59,926 broken and unbroken regions were analyzed, respectively. For all WGS, the fragments per base per million were calculated in each interval as reported in Wietmarschen and colleagues (34), and, finally, the median values of broken and unbroken regions were estimated in each sample. PCR-based analysis of TA repeats were performed as previously reported (34), using the same PCR primer sequences. Samples were denatured at 95°C for 3 minutes and underwent 28 cycles of denaturation at 95°C for 30 seconds and annealing/extension at 60°C for 3 minutes, followed by an extension at 60°C for 10 minutes. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

IHC

IHC assessment of MMR status in patient-derived organoids derived at the Netherlands Cancer Institute was performed as follows. Formalin-fixed, paraffin-embedded sections were obtained from both pretreatment biopsies and resection specimens. Baseline tumor biopsies were used to assess MMR status using IHC for MLH1, PMS2, MSH2, and MSH6 according to standard protocols for the Ventana automated immunostainer (MLH1 Ready-to-Use, M1, 6472966001, lot no. G07286, Roche; MSH2, Ready-to-Use, G219–1129, 5269270001, lot no. 1616008C, Roche; MSH6, 1/50 dilution, EP49, AC-0047, lot no. EN020910, Abcam; PMS2, 1/40 dilution, EP51, M3647, lot no. 1012289, Agilent Technologies).

Generation of Cas9-Expressing Cell Lines

Cells (2–3 × 10^5) were transduced overnight with lentivirus containing Cas9 (Addgene, 68343) in a T25 flask, in the presence of polybrene (8 μg/mL). Lentivirus-containing medium was refreshed the following day with complete medium. Tumoral organoids were dissociated into single cells and incubated overnight in suspension and complete media. The following day, cells were seeded in Matrigel and grown as organoids. Positively transduced cells were selected for with blasticidin (20 μg/mL, Thermo Fisher Scientific, A1113903) starting 48 hours after transduction. Cas9 activity was determined as described previously (30). Briefly, cells or organoids were transduced with Cas9 reporter virus (pKLV2-U6gRNA(gGFP)-PGKBFP2AGFP-W), as described above. The number of BFP^+ and GFP-BFP double-positive cells were determined by flow cytometry on a BD LSR Fortessa instrument (BD Biosciences), and data were subsequently analyzed using FlowJo to determine the percentage of BFP^+ cells. All cell lines and organoid lines displayed Cas9 activity more than 75%.

Organoid Genome Editing and Genome-Wide CRISPR/Cas9 Screens

The genome-wide single-guide RNA (sgRNA) library transduction was adapted from a previous protocol recently reported to screen cancer cell lines (30). Briefly, tumor organoids were dissociated into single cells, and a total of 3.3 × 10^7 cells were transduced overnight, in suspension, with an appropriate volume of the lentiviral-packaged whole-genome sgRNA library to achieve 30% transduction efficiency (100× library coverage) and polybrene (8 μg/mL). The following day, cells were seeded in matrigel and grown as organoids. After 48 hours, organoids were selected with puromycin (2 μg/mL). After 14 days, approximately 2 × 10^7 cells were collected, pelleted, and stored at −80°C for DNA extraction. Genomic DNA was extracted using the Qiagen Blood & Cell Culture DNA Maxi Kit, 13362, as per the manufacturer's instructions. PCR amplification, Illumina sequencing (19-bp single-end sequencing with custom primers on the HiSeq2000 v.4 platform), and sgRNA counting were performed as described previously (30). To generate B2M knockout organoids lines, we used sgRNA targeting B2M (GGCCGAGATGTCTCGCTCCG), cloned into LentiCRISPR v2 plasmid, and the virus was produced by standard method. To express luciferase in the organoids, we used pLenti CMV Puro LUC (w168–1; Plasmid #17477; Addgene).

CRISPR/Cas9 Viability and Cocompetition Assay

Approximately 1.5–3 × 10^3 Cas9-expressing cells per well, of a 96-well plate, were transduced overnight in the presence of polybrene (8 μg/mL) with lentiviral constructs containing sgRNAs against a nonessential gene (CYP2A13, GTCACCGTGCGTGCCCCGG), an essential gene (PLK1, GCGGACGCGGACACCAAGG), and 2 sgRNAs against WRN (#1, GAGCATGAGTCTATCAGAT and #2, GTCCTGTGGAACATACCATG). Medium was refreshed for fresh complete medium the following day, and cells were treated with blasticidin (20 μg/mL) and puromycin (2 μg/mL, Thermo Fisher Scientific, A1113803) to select for Cas9-expressing cells carrying the sgRNAs. Cells were allowed to grow for approximately 7 to 10 days before cell viability was determined using the CellTiter-Glo 2.0 Assay (Promega, G9241). For the co-competition assay, organoids were transduced as above to achieve 50% of BFP^+ cells and seeded in 6-well plates the day after to form organoids. A co-competition score was determined as the ratio of the percentage of BFP^+ (sgRNA transduced) cells on day 14 compared with day 3, as measured by flow cytometry.

RNA Interference–Based Sensitivity Assay

Approximately 1.5–3.5 × 10^3 cells per well, of a 96-well plate, were reverse-transfected with ON-TARGETplus siRNA, to a final concentration of 20 nmol/L, using RNAiMAX (Invitrogen) as per manufacturer's instructions. Each experiment included transfection reagent only as mock control, a nontargeting pool as negative control (Dharmacon, D-001810–10–05), polo-like kinase 1 (PLK1) pool as positive control (Dharmacon, L-003290–00–0010), and the targeting pool against WRN (Dharmacon, L-010378–00–0005). siRNA sequences: nontargeting control pool (UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUUCCUA), PLK1 (GCACAUACCGCCUGAGUCU, CCACCAAGGUUUUCGAUUG, GCUCUUCAAUGACUCAACA, UCUCAA GGCCUCCUAAUAG), WRN (GAUCCAUUGUGUAUAGUUA, GCAC CAAAGAGCAUUGUUA, AUACGUAACUCCAGAAUAC, GAGGGUUU CUAUCUUACUA). Cells were grown for 5 to 7 days. Cell viability was assessed using the CellTiter-Glo 2.0 Assay (Promega, G9241) as described below.

Drug Sensitivity Assay

Drug sensitivity assays were performed to confirm the resistance of each cell line. For each pair of cell lines of interest, approximately 1.5–2.5 × 10^3 cells per well of a 96-well plate were seeded and grown for both the drug-sensitive and drug-resistant lines. The following day, a concentration range of the respective drug was added to the cells, in triplicate per concentration per line, and cells were allowed to grow for 7 to 10 days. Cell viability was assessed using the CellTiter-Glo 2.0 Assay (Promega, G9241).

Cell Viability Assay

Cell viability was determined using the CellTiter-Glo 2.0 Assay (Promega, G9241), as per manufacturer's instructions. Briefly, 25 μL of CellTiter-Glo 2.0 reagent was added to each well of a 96-well plate and incubated for at least 20 minutes at room temperature in the dark. After incubation, the luminescence signal was read out using an Envision Multiplate Reader.

Western Blotting

Western blotting was performed to confirm the absence of WRN in siRNA and CRISPR-treated cells. For siRNA-based knockdown, approximately 0.5–1 × 10^6 cells were seeded in a 6-well plate in Opti-Mem and treated as described above. This assay included siRNA pools targeting WRN and a nontargeting pool as negative control. For CRISPR-based knockdown, approximately 1 × 10^6 cells were seeded in a 10-cm cell culture dish and treated as described above. This assay included 2 sgRNAs against WRN and a negative control without virus. Protein was isolated 72 to 96 hours after seeding with 100 to 150 μL RIPA buffer supplemented with proteinase and phosphatase inhibitors. Lysate concentration was determined using the BCA Assay. Per sample, 20 to 30 μg of lysate was loaded onto a 4% to 12% Bis-Tris gel (Invitrogen) for SDS-PAGE followed by protein transfer from the gel onto a polyvinylidene difluoride membrane. Membranes were blocked in 5% milk (in TBST) and incubated overnight with the appropriate antibodies. Blots were washed in TBST and incubated with secondary antibody for 1 hour at room temperature. Blots were washed in TBST before the signal was enhanced with Super Signal Dura and visualized. The following primary antibodies were used for immunoblot analysis: anti-WRN antibody (Cell Signaling Technology, 4666, 1:2,000) and anti-β-tubulin (Sigma-Aldrich, T4026: 1:5,000) as loading control. Anti-mouse IgG HRP-linked secondary antibody (GE Healthcare, #NA931) was used as a secondary antibody. Precision Plus Protein Standards (Bio-Rad, 161–0373) were used as a molecular weight marker.

Karyotype Analysis with Human Multiplex FISH Probes

WRN was knocked out using CRISPR/Cas9 as described above. Puromycin selection (2 μg/mL) was initiated 48 hours after transduction, cells were harvested for metaphases 96 hours after transduction from control, and WRN knockout cell lines followed a standard protocol with modifications. Briefly, cells growing in T150 flasks were treated with colcemid (KaryoMax Colcemid Solution in PBS, 10 g/mL, Thermo Fisher Scientific), to a final concentration of 0.1 g/mL for 1.5 hours. TrypLE Express Enzyme (Thermo Fisher Scientific) was used to dissociate adherent cells to obtain a single-cell suspension, which was pelleted down and resuspended in a hypotonic solution (0.56% KCl in H2O) for 12 to 14 minutes and subsequently fixed with Carnoy fixative, 3:1 (v/v) methanol:acetic acid. FISH analysis was performed as previously reported (64). Metaphase slides were prepared and fixed in acetone (Sigma Aldrich) for 10 minutes followed by baking at 62°C for 30 minutes. Denaturation of metaphase spreads was carried out by immersing slides in an alkaline denaturation solution (0.5 mol/L NaOH, 1.0 mol/L NaCl) for 7.5 to 8 minutes followed by two subsequent washes in 1 mol/L Tris-HCl (pH 7.4) and 1× PBS, 4 minutes each. Slides were dehydrated in a 70%, 90%, and 100% ethanol series. The probe mix [24-color human multiplex FISH (M-FISH) paint] was denatured at 65°C for 10 minutes before applying onto the denatured slide. Hybridization was carried out at 37°C for 2 nights. Post-hybridization steps included a 30-minute (approximately) wash in 2× SSC at 37°C, to remove coverslips, followed by a 5-minute stringent wash in 0.5× SSC at 75°C, a 5-minute rinse in 2× SSC containing [0.05% Tween-20 (VWR)] and another 5-minute rinse in 1× PBS, both at room temperature. Slides were finally mounted in Vectashield Vibrance Antifade Mounting medium with 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Metaphases were imaged using AxioImager D1 microscope equipped with appropriate narrow-band pass filters for DAPI, Aqua, FITC, Cy3, Texas Red, and Cy5 fluorescence. Digital images were captured using the SmartCapture software (Digital Scientific) and 20 randomly selected metaphase cells were karyotyped and analyzed with particular interest in chromatid and chromosome breaks including complex rearrangements based on Multiplex FISH and DAPI banding pattern using the SmartType Karyotyper (Digital Scientific).

Organoid and T-cell Coculture

PBMCs and tumor organoids were generated and cocultured as previously described (39, 40). Briefly, PBMCs were isolated from peripheral blood using Ficoll–Paque and cryopreserved for later use. For patient CRC-14, blood was drawn before the first cycle of nivolumab. Culture media for PBMCs were composed of RPMI 1640 (Gibco), supplemented with 2 mmol/L ultraglutamine I, 1:100 penicillin/streptomycin, and 10% male human AB serum (Sigma-Aldrich; catalog no. H3667; "T-cell medium"). One day before coculture, PBMCs were thawed in prewarmed (37°C) T-cell medium (human serum was replaced with FCS during thawing) and incubated for 15 minutes with 25 U/mL benzonase (Merck; catalog no. 70746–3) at 37°C. After washing, cells were resuspended at 2–3 × 10^6 cells/mL in T-cell medium supplemented with 150 U/mL IL2 and cultured overnight at 37°C. Forty-eight hours prior to coculture, tumor organoids were isolated from Geltrex by incubation with 2 mg/mL dispase II and cultured in colorectal cancer medium. Prior to coculture, tumor organoids (isolated from Geltrex) were stimulated for 24 hours with 200 ng/mL human recombinant IFNγ (PeproTech; catalog no. 300–02). Then, 96-well U-bottom plates were coated with 5 μg/mL anti-CD28 (clone CD28.2, eBioscience; catalog no. 16–0289–81) and kept overnight at 4°C. The next day, tumor organoids were dissociated to single cells with TrypLE Express and resuspended in T-cell medium. Anti-CD28–coated plates were washed twice with PBS, and PBMCs were seeded at a density of 10^5 cells/well and stimulated with single cell–dissociated organoids at a 20:1 effector:target ratio. Cocultures were performed in the presence of 150 U/mL IL2 and 20 μg/mL anti–PD-1–blocking antibody (kindly donated by Merus; catalog no. 5C4). Half of the medium, including IL2 and anti–PD-1, was refreshed 2 to 3 times per week. Every week, PBMCs were collected, counted, and replated at 10^5 cells/well and restimulated with fresh tumor organoids, for a total of 2-week coculture.

Tumor Recognition Assay, Killing Assay, and Generation of Organoids Resistant to Autologous Reactive T Cells

For evaluation of tumor reactivity, 10^5 PBMCs were restimulated with tumor organoids (isolated from Geltrex and stimulated with IFNγ, as described before) at a 2:1 effector:target ratio and seeded in anti–CD28-coated plates in the presence of 20 μg/mL anti–PD-1 and cocultured for 5 hours for IFNγ evaluation. Golgi-Plug (1:1,000, BD Biosciences; catalog no. 555029) and Golgi-Stop (1:1,500, BD Biosciences, catalog no. 554724) was added after 1 hour and coculture continued for an additional 4 hours. Cells were washed twice in FACS buffer and stained with the following antibodies: anti-CD3-PerCP-Cy5.5 (BD Biosciences; catalog no. 332771), anti–CD4-FITC (BD Biosciences; catalog no. 555346), anti–CD8-BV421 (BD Biosciences; catalog no. 562429), and near-IR viability dye (Life Technologies) for 30 minutes at 4°C in the dark. Cells were washed twice in FACS buffer, fixed using the Cytofix/Cytoperm Kit (BD Biosciences, according to manufacturer's instructions), and stained for intracellular IFNγ (anti–IFNγ-APC, BD Biosciences; catalog no. 554702). PBMCs stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich; catalog no. 19–144) and 1 mg/mL ionomycin (Sigma-Aldrich; catalog no. I9657) served as positive controls and PBMCs cultured without tumor stimulation as negative controls. Cells were then washed twice with FACS buffer and recorded at a Becton Dickinson Fortessa or LSRII flow cytometer.

For CD137 expression evaluation, 10^5 PBMCs were restimulated with tumor organoids (isolated from Geltrex and stimulated with IFNγ, as described previously) at a 2:1 effector:target ratio and seeded in anti–CD28-coated plates in the presence of 20 μg/mL anti–PD-1 and cocultured for 24 hours. Cells were washed twice in FACS buffer and stained with the following antibodies: anti–CD3-PerCP-Cy5.5 (BD Biosciences), anti–CD4-FITC (BD Biosciences), anti–CD8-BV421 (BD Biosciences), anti–CD137-APC (BD Biosciences; catalog no. 550890), and near-IR viability dye (Life Technologies) for 30 minutes at 4°C in the dark. PBMCs stimulated with 50 ng/mL PMA (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) served as positive controls and PBMCs cultured without tumor stimulation as negative controls. Cells were then washed twice with FACS buffer and recorded with a Becton Dickinson Fortessa or LSRII flow cytometer. To determine the sensitivity of tumor organoids to T cell–mediated killing, flat-bottom non–tissue culture-treated plates were coated with 5 mg/mL anti-CD28 and kept at 4°C overnight prior to coculture. Tumor organoids were previously transduced with luciferase reporter gene. Organoids were isolated from Geltrex 48 hours prior to coculture and stimulated with 200 ng/mL IFNγ for 24 hours prior to coculture. The next day, part of the organoids were dissociated to single cells and counted using a hemocytometer. This was used to infer the number of tumor cells per tumor organoid to allow coculture of organoids and T cells at a 5:1 effector:target ratio. Next, tumor organoids were resuspended in the T-cell medium. T cells were collected after 2 weeks of coculture with tumor organoids and resuspended in the T-cell medium. Anti–CD28-coated plates were washed twice with PBS and 1 × 10^4 organoids were seeded for 72 hours in triplicate without T cells or with 5 × 10^4 autologous T cells obtained by 2 weeks of organoid coculture. To block MHC class I and II, organoids were preincubated for 30 minutes with 50 μg/mL pan–MHC-I blocking antibody W6/32, or pan–MHC-II blocking antibody T39 (blocking antibody remained present throughout the coculture; BD Biosciences; catalog no. 555556). At the end of the 72 hours, tumor cell viability in the different conditions was measured by luciferase reporter assay using 3 μg/mL luciferin (Promega; catalog no. E1605). Luminescence was measured with a Tecan reader (1,000 ms exposure).

Flow Cytometry

For evaluation of MHC-I, tumor organoids were dissociated to single cells using TrypLE Express, with or without overnight preincubation with 200 ng/mL IFNγ. Tumor cells were washed in FACS buffer (PBS, 5 mmol/L EDTA, 1% BSA) and stained with mouse anti-human HLA-A, B, C-PE (BD Biosciences; catalog no. 555553), or isotype controls (PE mouse IgG1, kappa; BD Biosciences; catalog no. 556650) for 30 minutes at 4°C. Cells were washed twice with FACS buffer and DAPI was added to exclude dead cells prior to recording at a Becton Dickinson Fortessa or LSRII flow cytometer.

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Authors' Contributions

G. Picco: Conceptualization, data curation, formal analysis, supervision, validation, investigation, visualization, writing–original draft, writing–review and editing. **C.M. Cattaneo:** Formal analysis, validation, investigation, visualization, methodology, writing–review and editing. **E.J. van Vliet:** Formal analysis, validation, investigation, visualization, writing–original draft. **G. Crisafulli:** Data curation, formal analysis, investigation, methodology, writing–review and editing. **G. Rospo:** Data curation, investigation, methodology, writing–review and editing. **S. Consonni:** Visualization, methodology. **S.F. Vieira:** Validation. **I. Sánchez Rodriguez:** Validation. **C. Cancelliere:** Resources, validation, project administration. **R. Banerjee:** Validation, methodology. **L.J. Schipper:** Validation, methodology. **D. Oddo:** Resources, validation. **K.K. Dijkstra:** Validation, methodology, writing–review and editing. **J. Cinatl:** Resources, validation. **M. Michaelis:** Resources, validation. **F. Yang:** Formal analysis, validation. **Cell Model Network UK:** Resources. **F. Di Nicolantonio:** Resources, validation, writing–review and editing. **A. Sartore-Bianchi:** Resources, methodology. **S. Siena:** Resources, visualization. **S. Arena:** Validation, investigation, methodology, writing–review and editing. **E.E. Voest:** Conceptualization, resources, supervision, funding acquisition, methodology, project administration, writing–review and editing. **A. Bardelli:** Conceptualization, resources, funding acquisition, methodology, project administration, writing–review and editing. **M.J. Garnett:** Conceptualization, supervision, funding acquisition, writing–original draft, project administration, writing–review and editing.

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