

The Molecular and Functional Landscape of Resistance to FOLFIRI Chemotherapy in Metastatic Colorectal Cancer



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ABSTRACT

The combination of 5-fluorouracil and irinotecan (FOLFIRI) remains a standard-of-care treatment for metastatic colorectal cancer (mCRC) yet benefits only about half of patients. Using patient-derived xenografts, we investigated the biological underpinnings of this heterogeneous response. FOLFIRI-resistant models showed transcriptional upregulation of innate immunity and mitochondrial metabolism genes, together with reduced expression of the DNA polymerase POLD1. Sensitive counterparts exhibited a BRCAness-like phenotype with genomic scars of homologous recombination (HR) deficiency, not caused by genetic or epigenetic loss of HR genes but by low abundance of the RAD51 recombinase. In tumoroids, forced RAD51 overexpression attenuated HR deficiency-related scars and chemotherapy-induced damage, whereas HR inhibition through ATM blockade enhanced drug sensitivity. The predictive relevance of key response determinants was validated in clinical samples. This work illuminates functional, nongenetic facets of BRCAness in mCRC and introduces actionable biomarkers and targets, offering prospects to improve clinical decision-making and broaden therapeutic options for chemorefractory patients.

SIGNIFICANCE: FOLFIRI response biomarkers in mCRC are lacking. Evidence in patient-derived xenografts, tumoroids, and patients shows that chemosensitivity arises from functional relaxation, rather than (epi)genetic inactivation, of the HR DNA repair pathway. Integrative analyses yield a chemopredictive algorithm centered on the expression of the RAD51 recombinase, with potential to refine patient stratification.

INTRODUCTION

Colorectal cancer ranks as the third most prevalent cancer globally, accounting for approximately 10% of all cancer cases and standing as the second leading cause of cancer-related deaths worldwide (1). Around 20% of colorectal cancers present as metastatic at the time of initial diagnosis, and nearly half of patients with localized colorectal cancer will eventually develop metastases (2, 3). Systemic chemotherapy is the primary treatment modality for patients with microsatellite stable (MSS) metastatic disease who are not eligible for surgery, with sequential or combined administration of fluoropyrimidines, oxaliplatin, and irinotecan resulting in median overall survival (OS) ranging from 18 to 26 months (4). However, the current paradigm of cytotoxic treatments follows a “one-size-fits-all” approach, with only a subset of patients achieving a clinical benefit, whereas many endure unnecessary exposure to the toxic effects of treatment.

For example, response rates to first-line FOLFOX [the combination of the fluoropyrimidine 5-fluorouracil (5-FU) and oxaliplatin] or FOLFIRI (the combination of 5-FU and irinotecan) hover around 55% and drop to less than 10% in the second-line setting (5).

The biological mechanisms underlying sensitivity and resistance to chemotherapy in metastatic colorectal cancer (mCRC) remain elusive, and clinically useful response biomarkers are remarkably absent. Early studies in small patient cohorts proposed *ERCC1*, encoding an endonuclease involved in nucleotide excision repair of DNA damage, as a potential determinant of resistance to oxaliplatin (6, 7). However, the association between excision repair cross-complementation group 1 expression and the efficacy of oxaliplatin was not confirmed in a larger randomized trial (8). For irinotecan, poor responses have been associated with high expression of the drug efflux transporter ABCG2 and low levels of

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topoisomerase I (TOP-I), the primary molecular target of the compound (9). Furthermore, gene expression-based classifier models have proposed an association between FOLFIRI response and transcriptional signatures enriched for genes implicated in cell adhesion and WNT signaling (10, 11). Despite these advances, such predictive models remain largely exploratory and have yet to be translated into clinical practice. Therefore, the search for predictive biomarkers capable of informing treatment decisions and aiding in the triage of patients who would experience therapy failure remains an unmet medical need.

Large collections of patient-derived xenografts (PDX) faithfully mirror the molecular diversity of human tumors on a scale compatible with that achievable in clinical trials and, at the same time, allow for systematic drug response annotation. Consequently, they have proven valuable as experimentally testable pharmacogenomic platforms to pinpoint molecular features that are preferentially represented in defined response categories (12, 13). In the case of mCRC, PDX resources have contributed to the nomination and clinical development of biomarkers of poor responsiveness to the anti-EGFR antibody cetuximab, a targeted agent that is often used in the metastatic setting (14–19). PDX-based research has also documented a more pronounced expression of transcriptional signatures reminiscent of those exhibited by nonmalignant enterocytes and goblet cells in colorectal tumors sensitive to 5-FU (17) or patterns similar to those of a quiescent subpopulation of normal intestinal secretory precursors in mCRC residual cells that survive cetuximab treatment (20).

In this study, we aimed to comprehensively portray the spectrum of responses to chemotherapy in a vast series of mCRC PDX models. This undertaking, coupled with multidimensional profiling and biological experiments in matched tumoroids, established a methodologic foundation for exploring mechanisms and discovering biomarkers of response and resistance. For practical and ethical reasons related to the observed mild toxicity in mouse hosts, we elected FOLFIRI as the reference treatment for the PDX population trial and analyzed molecular enrichments differentially stratified by treatment outcome.

RESULTS

Distribution of FOLFIRI Response in a PDX Population Trial

We assessed the response to FOLFIRI in a set of 127 mCRC PDX models, mostly from MSS lesions, each originating from a distinct patient and capturing the spectrum of interpatient and intertumor heterogeneity observed in mCRC (Fig. 1A; Supplementary Tables S1 and S2). Each sample was utilized to establish subcutaneous xenografts in NOD/SCID mice and propagated until an experimental group of seven animals was formed. Within this group, two animals received a placebo, whereas five animals were administered FOLFIRI once the tumors reached an approximate average volume of 300 mm³.

Dosages and schedules for 5-FU and irinotecan were adopted from prior studies (21, 22) and were intended to reflect plasma concentrations in humans (23, 24). The treatment

regimen extended for a duration of 6 weeks, with an initial evaluation scheduled 3 weeks following the first dose. Alternatively, the treatment was terminated upon reaching predefined humane endpoints (see “Methods” section; Supplementary Fig. S1A). To classify response patterns, we employed categories inspired by clinical criteria, consistently used in our previous work to annotate mCRC PDX response to cetuximab (14–16, 20): (i) Regression indicated a reduction of at least 50% in the average tumor volume for the treated cohort of each model, measured against the baseline (pretreatment) tumor volume; (ii) progression denoted a volume increase of at least 35%; and (iii) stabilization encompassed intermediate responses that did not fulfill the criteria for regression or progression.

Figure 1B illustrates the distribution of early (3-week) response rates to FOLFIRI across the entire set of PDXs, with models ranked by changes in relative tumor volume from baseline on the first day of treatment. Within this population, 54 cases (42.5%) showed progression, 57 cases (45%) displayed disease stabilization, and 16 cases (12.5%) demonstrated regression (Supplementary Table S1). Mice xenografted with 35 models (27.5%) reached the humane endpoint before the conclusion of the 6-week evaluation period, either due to chemotherapy-resistant tumors exceeding the maximum volume threshold ($n = 12$, 9.4%) or clinical signs of health deterioration ($n = 23$, 18%). Consequently, long-term (6 weeks) efficacy data were available for 92 cases. In this subset, the depth of response heightened, with 33 models (36%) showing more than 50% tumor shrinkage (Fig. 1C). Specifically, during the second observation period, 19 cases previously categorized as stabilized displayed regression, and 14 cases that had initially progressed became stabilized (Supplementary Fig. S1B; Supplementary Table S1). Progressors totaled 26 (28%; Fig. 1C), but it is important to acknowledge that this category is spuriously reduced as mice with large tumors were prematurely sacrificed before the final analysis, as previously explained. Overall, these response rates align with the heterogeneous chemosensitivity observed in patients with mCRC who are treated with FOLFIRI (25–27).

For most of the models ($n = 86$), we had accompanying mutational information obtained by whole-exome or targeted next-generation sequencing (Supplementary Table S2). We therefore sought to explore whether some high-frequency or biologically relevant gene mutations were differentially represented across the distinct response categories. The only significant enrichment observed was for *KRAS* mutations in resistant cases (Fig. 1B and C). Interestingly, *KRAS*^{G12} mutations have recently emerged as biomarkers of resistance to the 5-FU–related combination therapy with trifluridine, a nucleoside analog, and tipiracil, a thymidine phosphorylase inhibitor (28).

Gene Expression Signatures and Response to FOLFIRI

We performed bulk RNA sequencing profiling on 84 untreated PDX models to identify differentially expressed genes (DEG) between chemoresistant tumors ($n = 33$, with a tumor volume increase ranging from 52.62% to +297.09% after 3 weeks of treatment) and chemosensitive tumors ($n = 33$, with

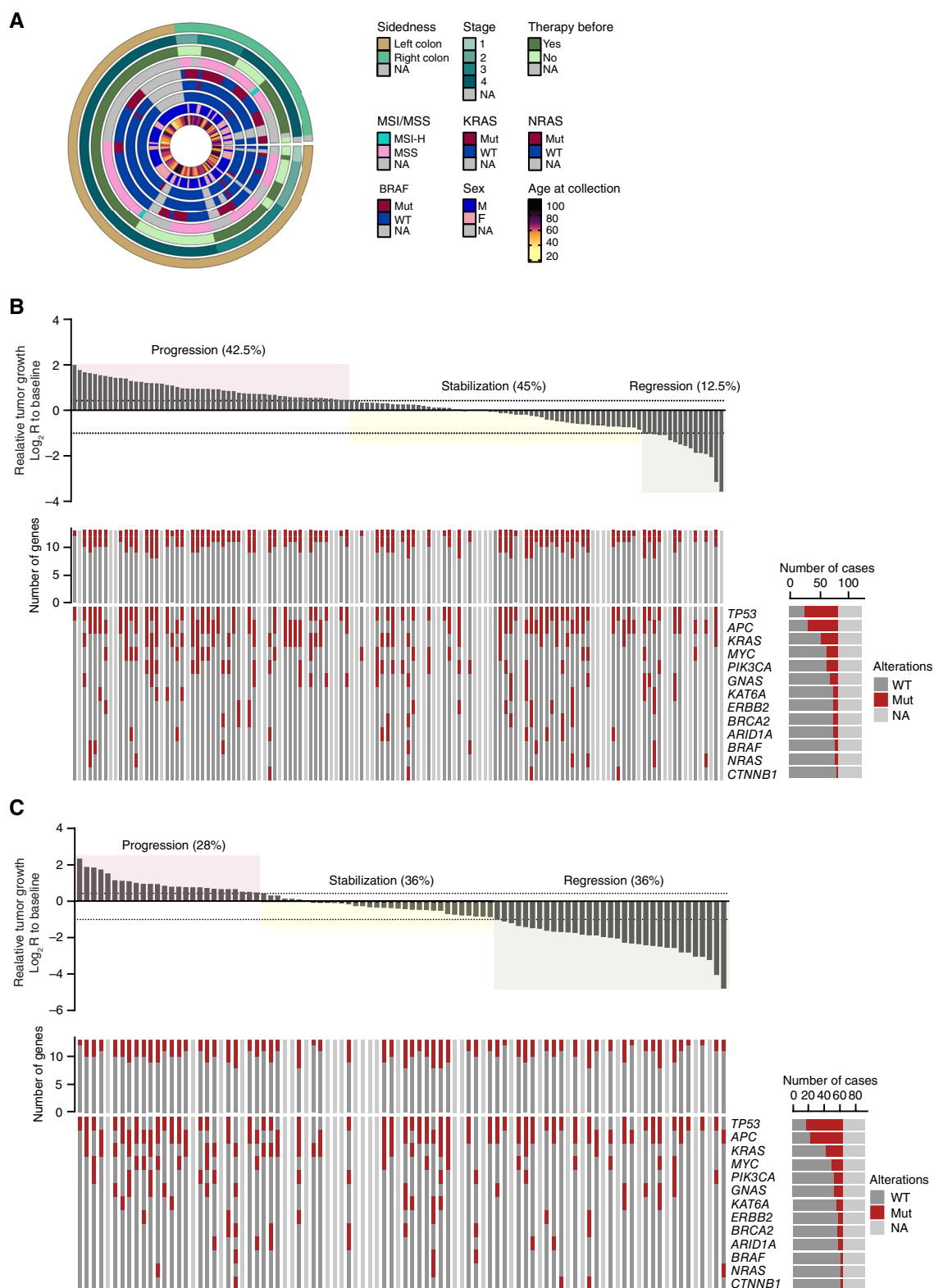


Figure 1. Model characteristics and distribution of FOLFIRI response in a PDX population trial. **A**, Key clinical and molecular features of the mCRC tumors from which PDX models were derived. F, female; M, male; MSI-H, microsatellite instability high; Mut, mutant; NA, not available; WT, wild-type. **B** and **C**, (Top) Tumor response to FOLFIRI after 3 weeks (**B**) or 6 weeks (**C**) of treatment, calculated as the $\log_2$ ratio ($\log_2 R$) of posttreatment to basal tumor volumes, in a population of 127 PDX models (**B**) or 92 models (**C**) ($n = 3$ –5 mice for each bar in both **B** and **C**). Dotted lines indicate the cutoff values for arbitrarily defined categories of therapy response. (Bottom) Distribution of high-frequency or biologically relevant gene mutations according to FOLFIRI response. KRAS mutation enrichment in resistant PDXs: $P = 0.048$ at 3 weeks of treatment and $P = 0.0016$ at 6 weeks. Statistical analysis was done using Fisher's exact test.

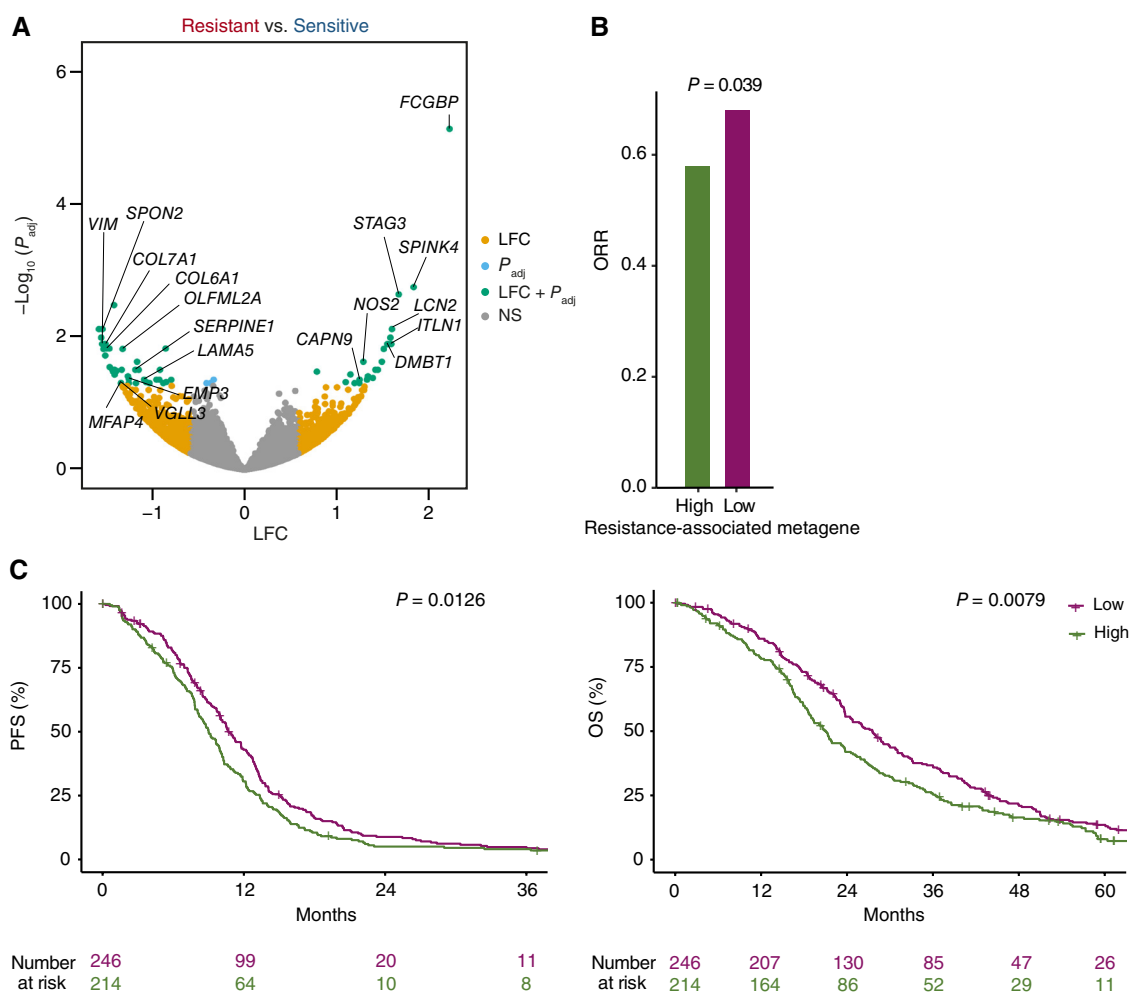


Figure 2. Gene expression differences between FOLFIRI-resistant and FOLFIRI-sensitive tumors in PDXs and patients. **A**, Genes with baseline differential expression between resistant ($n = 33$) and sensitive ($n = 33$) PDX models. Response annotation refers to tumors exposed to 3 weeks of FOLFIRI. Colored dots denote genes with an absolute $\log_2$ fold change ($|LFC| \geq 0.585$) (orange), genes with an adjusted P value (P_{adj}) < 0.05 (Wald test, implemented by DESeq2; pale blue), and genes with both an $|LFC|$ higher than 0.585 and an adjusted P value < 0.05 (green). Grey dots indicate genes without significant differential expression. NS, nonsignificant. **B**, Objective response rates (ORR) among patients from the FIRE-3 trial stratified by expression of the PDX-derived resistance-associated metagene. Statistical analysis was done using Fisher's exact test. **C**, PFS and OS in FIRE-3 trial patients according to the expression of the same metagene. Statistical analysis was done using the Wald test on the univariate Cox model coefficient of the expression of the resistance-associated metagene. In **B** and **C**, High and low refer to GSVA enrichment scores above or below the defined threshold (0 natural cutoff), respectively.

tumor volume shrinkage ranging from -7.81% to -88.62%). This analysis identified 22 genes with significant upregulation in resistant models and 40 genes with significant upregulation in responsive models (19 and 36 protein-coding genes, respectively; Fig. 2A; Supplementary Table S3). To determine whether the degree of gene expression modulation was associated with the extent of FOLFIRI response, we calculated Pearson's correlation coefficients between the percentage change in tumor volume after treatment and the expression levels of differential genes in resistant versus sensitive PDX models. Genes upregulated in resistant models tended to positively correlate with tumor volume change (i.e., greater expression associated with tumor progression), whereas genes enriched in sensitive models showed negative correlations (i.e., greater expression associated with tumor regression). Specifically, 11 of the 22 genes upregulated in resistant models and 23 of

the 40 genes upregulated in sensitive models demonstrated nominally significant associations with the magnitude and direction of treatment response across the entire cohort (Supplementary Fig. S2A).

Among the genes significantly upregulated in resistant tumors, we found commonalities related to intestinal inflammation, innate immunity, and type 1 IFN genes. Specific examples include the bacteriostatic iron transporter lipocalin-2 (*LCN2*; ref. 29), the antimicrobial agents CRP-ductin (*DMBT1*; ref. 30) and inducible nitric oxide synthase (*NOS2*; ref. 31), the proinflammatory mediators calpain-9 (*CAPN9*; ref. 32) and SPINK4 (33), and goblet cell-derived molecules that help maintain intestinal mucosal integrity, such as the glycan-binding lectin intelectin-1 (*ITLN1*; ref. 34) and the mucin-like glycoprotein IgG Fc-binding protein (*FCGBP*; Fig. 2A; ref. 35). Another gene significantly upregulated in

resistant PDXs was *STAG3*, which encodes a protein involved in sister chromatid cohesion (Fig. 2A; ref. 36). In contrast, many of the genes significantly upregulated in responsive tumors encode cytoskeletal and extracellular matrix proteins involved in epithelial regeneration, cell motility, and wound healing. These include plasminogen activator inhibitor-1 (*SERPINE1*), vimentin (*VIM*), microfibril-associated protein 4 (*MFAP4*), vestigial-like family member 3 (*VGLL3*), spondin-2 (*SPON2*), olfactomedin-like 2A (*OLFML2A*), epithelial membrane protein 3 (*EMP3*), collagen alpha-1(VI) chain (*COL6A1*), collagen alpha-1(VII) chain (*COL7A1*), and laminin subunit alpha-5 (*LAMA5*; Fig. 2A; refs. 37–42).

Gene set enrichment analysis (GSEA) confirmed that host defense pathways—including IFN α and IFN γ responses and JAK-STAT signaling—were upregulated in resistant tumors, whereas signatures indicative of epithelial cell motility and invasion [epithelial–mesenchymal transition (EMT)] were enriched in responsive tumors. Other signatures associated with poorly responsive tumors included hallmarks of mitochondrial fatty acid metabolism, whereas pathways related to high proliferative activity in colorectal cancer (MYC and WNT/LEF1 targets; β -catenin signaling) were enriched in responders (Supplementary Fig. S2B; Supplementary Table S3). It is noteworthy that similar resistance-associated pathways of mitochondrial metabolism have been documented in patients with triple-negative breast cancer (TNBC) who did not achieve a pathologic complete response after platinum-based neoadjuvant chemotherapy, whereas sensitivity-associated cell-cycle pathways were also prominent in patients with TNBC who had a pathologic complete response (43).

To assess the relationship between the observed gene set enrichment patterns and FOLFIRI response across the PDX population, we calculated pathway activity at the individual PDX level using single-sample GSEA (ssGSEA). A general trend of concordance emerged between the distribution of ssGSEA scores and response categories; in most cases, the largest differences occurred between the extreme groups (resistant and sensitive tertiles), rather than between the extremes and the intermediate group (mid tertile). These differences were statistically significant for some cell proliferation-related GSEA signatures (Supplementary Fig. S2C). Overall, although correlations between GSEA-enriched signatures and the spectrum of FOLFIRI response were evident, they were less robust than those observed for individual genes, likely reflecting the broader biological processes captured by the GSEA-defined pathways examined.

Our results in PDX models align with preclinical and clinical observations in colorectal cancer. In cell-based experiments, lipocalin-2, *STAG3*, IFN-stimulated genes, and JAK-STAT signaling have been linked to 5-FU and irinotecan resistance (44–48), whereas EMT induction has been shown to increase chemosensitivity (49, 50). Clinically, plasminogen activator inhibitor-1 is part of a four-protein signature that identifies patients with high-risk early-onset colorectal cancer with a favorable response to adjuvant chemotherapy (51), whereas high expression of fatty acid oxidation genes correlates with poor response (52). Furthermore, in line with our finding of elevated WNT target gene expression in responsive PDX models, mCRC tumors belonging to the C5

transcriptional subtype by Marisa's classification—characterized by high WNT signaling—are more prevalent among FOLFIRI-responsive patients (11, 53).

The clinical utility of our transcriptomic profiles for predicting FOLFIRI resistance is further supported by the analysis of patient datasets. We mined gene expression data from 460 patients with *KRAS* exon 2 wild-type mCRC enrolled in the FIRE-3 trial, who were randomized to receive either cetuximab or the anti-VEGFA antibody bevacizumab in combination with FOLFIRI as first-line therapy (54). Using gene set variation analysis (GSVA), we computed single-sample enrichment scores for two metagenes: one comprising all significantly upregulated genes in FOLFIRI-resistant PDXs and another encompassing all upregulated genes in FOLFIRI-sensitive PDXs. By applying a natural cutoff of 0, we found that patients whose tumors displayed an above-threshold score for the resistance-associated metagene were significantly enriched for lower objective response rates and shorter progression-free survival (PFS) and OS (Fig. 2B and C). This enrichment was not biased by treatment with cetuximab or bevacizumab (Supplementary Fig. S3A), indicating that the observed predictive value is specific to FOLFIRI response. Although our PDX-based transcriptomic analyses proved useful for predicting resistant patients, they did not reliably identify those who derived clinical benefit from FOLFIRI (Supplementary Fig. S3B and S3C), implying that the biology underlying sensitivity genes may be less generalizable.

Validation of a Reference Subset of PDX Models for Biomarker Discovery Studies

We designed a series of systematic studies for biomarker discovery and validation using a reference subset of PDX models that exhibited either overt progression ($n = 14$) or massive regression ($n = 15$) after 6 weeks of exposure to FOLFIRI (Supplementary Fig. S4A). To confirm that this subset was representative of the larger cohort, we first verified that the transcriptional patterns distinguishing sensitive from resistant tumors were preserved in these selected models. Indeed, the gene expression differences observed across the entire collection were maintained in the reference subset (Supplementary Fig. S4B). Of the 55 protein-coding genes significantly modulated between sensitive and resistant tumors in the full cohort, 26 (47.3%) also showed nominally significant, directionally consistent changes in the reference subset (Supplementary Table S3), and all GSEA pathways differentiating responders from nonresponders in the full cohort were similarly enriched in the reference subset as well (Supplementary Fig. S4C).

Among the genes with baseline differential expression, a minority (10/26, 38.5%) were significantly upregulated by treatment, either exclusively in sensitive models (four genes: *CHRD*, *COL7A1*, *GALNT8*, and *STAG3*) or in both sensitive and resistant models (five genes: *ENO3*, *KIAAIP49*, *QKI*, *VGLL3*, and *VIM*), with overall stronger induction observed in responders (Supplementary Fig. S4D; Supplementary Table S3). Only one gene, *ITLN1*, was selectively upregulated in resistant cases. Of the 10 treatment-modulated genes, seven were part of the sensitivity-associated signature and were predominantly involved in cell motility and invasion, whereas three were linked to resistance. Notably, none of

the baseline DEGs was significantly downregulated by treatment (Supplementary Fig. S4D; Supplementary Table S3). Therefore, FOLFIRI exposure induced a modest yet discernible modulation of the analyzed genes, primarily involving the adaptive overexpression of response-associated subsets, with a more pronounced effect in sensitive tumors.

IHC evaluation informed by transcriptomic observations confirmed that sensitive tumors displayed stronger positivity for canonical markers of active cell-cycle phases (cyclin A2, cyclin B1, and phospho-H3, indicative of the S-, G₂-, and M-phase, respectively), unlike resistant counterparts (Supplementary Fig. S5A). As expected, the levels of S, G₂, and M cell-cycle markers were diminished in posttreatment (6 weeks) samples from sensitive tumors, whereas they remained substantially unaltered in resistant tumors (Supplementary Fig. S5A). Flow cytometry–based cell-cycle analysis on tumoroids derived from four responsive PDXs and four resistant models revealed that FOLFIRI sensitivity was associated with a higher proportion of cells in the G₂ and M-phases (Supplementary Fig. S5B), supporting the link between accelerated mitotic activity and favorable treatment outcomes.

Functional and Molecular Analysis of DNA Damage Response according to FOLFIRI Sensitivity

Irinotecan stabilizes TOP-I–DNA complexes, resulting in the formation of one-ended DNA double-strand breaks (DSB) during the S-phase of the cell cycle (55). Similarly, 5-FU promotes DSB accumulation through inhibition of thymidylate synthase and misincorporation of fluorodeoxyuridine triphosphate into genomic DNA, driven by nucleotide imbalance (56). These replication-associated DSBs are primarily repaired via the homologous recombination (HR) pathway (57, 58); if left unrepaired, however, they can become lethal lesions that compromise genomic integrity. Although DNA repair pathways are increasingly recognized as modulators of chemosensitivity in various cancers, their contribution to chemotherapy response in colorectal cancer remains poorly understood (59–61). To investigate whether tumors sensitive to FOLFIRI were more vulnerable to its genotoxic effects due to a limited capacity to repair DSBs, underpinned by defects in the HR machinery, we undertook several orthogonal approaches revolving around functional readouts and molecular interrogation of HR deficiency (HRD).

Biological Assessment of DNA Damage Response

We first analyzed baseline DSB abundance and postchemotherapy DSB accrual in samples from sensitive and resistant PDXs of the reference subset using γ -H2AX staining, which provides an indirect measure of DSB generation and repair. The levels of γ -H2AX expression before treatment were comparable between the two cohorts (Fig. 3A). However, consistent with the hypothesis that FOLFIRI susceptibility depends on genomic damage, we observed considerably higher γ -H2AX positivity in treated tumors that shrank following treatment, as compared with those that progressed (Fig. 3A). Notably, the magnitude of γ -H2AX induction after treatment was significantly correlated with the extent of tumor chemosensitivity (Fig. 3B), supporting an association between the cell's ability to accumulate DNA DSBs and the depth of

FOLFIRI response. These variations in DSB formation after chemotherapy between responders and nonresponders were further confirmed using the neutral comet assay, an independent method for detecting DSBs, in a collection of 20 PDX-derived tumoroids (Fig. 3C).

We next examined the response to PARP blockade using tumoroids derived from 8 FOLFIRI-sensitive and 8 FOLFIRI-resistant PDX models, capitalizing on the knowledge that PARP is a synthetic lethal target in HR-deficient tumors (62). Although responses to the PARP inhibitor olaparib were variable across individual models, the FOLFIRI-sensitive group exhibited overall greater susceptibility, as reflected by significantly lower IC₅₀ values (Fig. 3D). These findings indicate that despite model-to-model heterogeneity, FOLFIRI-sensitive tumors were generally more vulnerable to PARP inhibition than their FOLFIRI-resistant counterparts.

Multidimensional Molecular Evaluation of HR Gene Status

Collectively, the above observations indicate that FOLFIRI-sensitive mCRC tumors are less proficient in DSB repair than FOLFIRI-resistant tumors, possibly due to HR gene defects. We therefore scrutinized a manually curated panel of 44 relevant DNA damage response genes with direct or indirect implications in the HR pathway for the presence of nonsynonymous somatic mutations in a subset of 84 PDX models with varying responses to FOLFIRI. Among these, 40 models progressed by 35% to 297% after 3 weeks of treatment, whereas 23 exhibited tumor shrinkage ranging from 18.4% to 88.6% (Fig. 4A). Forty-three models (51.2%) harbored a total of 132 HR gene mutations, with most displaying alterations in more than one gene (Fig. 4A; Supplementary Table S2). The vast majority of these mutations (89%) were heterozygous, and only 29 of them (22%) were consistently predicted to be deleterious by multiple independent algorithms (Fig. 4A; Supplementary Table S2). When all mutations were considered, no enrichment of HR gene variants was observed in relation to treatment outcome (Fig. 4A). Predicted loss-of-function variants in homozygosity were infrequent—affecting only 12 genes—and occurred as isolated events in 12 individual models, with no clear link to sensitivity or resistance (Supplementary Fig. S6A). We note, however, that one model harboring a biallelic deleterious mutation in *TOP3A* and another with an analogous mutation in *TOP3B* were both refractory to FOLFIRI (Supplementary Fig. S6A).

To complement mutational profiling, we employed low-pass whole-genome sequencing to explore recurrent copy-number changes in the same HR gene panel. This analysis revealed a homozygous deletion (GISTIC threshold –2) of the *RAD51* paralog *RAD51B* in two FOLFIRI-sensitive models and a high-level gene amplification (GISTIC threshold +2) of the *BRCA2* locus in one FOLFIRI-resistant model (Fig. 4A). Additional high-level gene amplifications were detected for the *RAD54B* helicase in seven models, the nuclear foci-associated cofactor *NBN* in three models, and the *RAD51* interactor *RAD52* in one model, but these alterations showed no consistent association with FOLFIRI response (Fig. 4A). Hemizygous deletions (GISTIC threshold –1) were identified for 35 genes in 72 models, again without a significant correlation with treatment outcome (Supplementary Fig. S6B).

Overall, the predominance of mutations categorized as variants of unknown significance, the rarity of widespread homozygous inactivation of the analyzed genes, and the lack of strong associations between mutational or copy-number alterations and treatment response suggest that FOLFIRI sensitivity is not primarily driven by HR gene inactivation. Nonetheless, anecdotal evidence points to alterations in specific genes, such as those affecting *RAD51B*, *BRCA2*, and *TOP3A/TOP3B*, as potential determinants of response in selected cases.

As an additional layer of analysis, we investigated whether the panel of HR genes subject to mutational and copy-number annotation exhibited differential methylation and mRNA expression between chemorefractory and chemosensitive tumors. There was a large concordance in baseline methylation patterns between the two categories ($n = 27$ nonresponders and $n = 27$ responders), with only two genes displaying nominally significant differences: the DNA damage sensor *ATR* (more methylated in responders) and the DNA topoisomerase *TOP3A* (more methylated in nonresponders; Supplementary Table S4). At the gene expression level ($n = 33$ nonresponders and $n = 33$ responders), we observed nominally significant up-regulation of *POLD3*, a component of the DNA polymerase delta complex, and *ABRAXAS1*, a partner of the BRCA1 complex, in resistant models. Conversely, genes with nominally significant lower expression in resistant tumors included *TOP3A* (consistent with its higher methylation) and *POLD1*, the catalytic subunit of the DNA polymerase delta complex (Fig. 4B; Supplementary Table S4).

Although *POLD1* was not the top gene showing differential expression between sensitive and resistant PDX models (Supplementary Table S4), we chose to explore its association with FOLFIRI response further, prompted by prior evidence linking low *POLD1* expression to chemotherapy resistance in breast cancer. In particular, a recent study (43) reported reduced expression of gene products located in the 19q13.31-33 cytoband, encompassing *POLD1*, in tumors from patients with TNBC who experienced residual disease after neoadjuvant carboplatin chemotherapy. Additionally, clones featuring single-copy loss and reduced expression of *POLD1* expanded in PDXs as tumors advanced toward a carboplatin-resistant state (43). In line with these observations, we found that *POLD1* protein abundance was markedly lower in baseline tumor samples from FOLFIRI-refractory mCRC PDXs compared with responsive models in the reference subset (Fig. 4C), with a significant correlation between the intensity of *POLD1* positivity and the extent of tumor response (Fig. 4D). In posttreatment (6-week) samples, *POLD1* protein levels were generally reduced, with a more pronounced and consistent decrease in sensitive tumors (Fig. 4E). More than 50% (8/15) of mCRC PDXs displaying *POLD1* single-copy loss (GISTIC threshold -1) were categorized as FOLFIRI-resistant; however, nearly half (32/69) of the models with normal *POLD1* copy number also exhibited poor response (Fig. 4F). This suggests that hemizygous *POLD1* loss alone does not fully account for its reduced expression in FOLFIRI-resistant mCRC tumors, whereas transcriptional and posttranscriptional regulation likely play a more prominent role. Finally, a survey of drug screen results from the Cancer Dependency Map project (63, 64) revealed a significant correlation between

low *POLD1* gene and protein expression and reduced sensitivity to irinotecan in a pan-cancer cell line collection (Fig. 4G and H), further confirming the robustness of this association and potentially extending its reach to other tumor types. These findings underscore a commonality between FOLFIRI-resistant mCRC and carboplatin-resistant TNBC, consistent with our analysis of transcriptional signatures linked to therapeutic response.

Genomic Scars of HR Deficiency

Growing knowledge indicates that BRCAness is more accurately captured by evaluating the genomic scars caused by HRD rather than focusing solely on individual mutations in HR genes, which, with the exception of *BRCA1* and *BRCA2*, are rare and often entail uncertain functional effects (65). This evidence prompted us to explore whether FOLFIRI-sensitive tumors displayed genomic characteristics indicative of BRCAness, even in the absence of clear pathogenic alterations in HR genes. In particular, we determined the levels of genomic instability arising from loss of heterozygosity, telomeric-allelic imbalance, and large-scale state transitions (the most relevant genomic scars in tumors with BRCAness) in PDX models of the reference subset. Results from two NGS-based research assays in clinical development (AmoyDX HRD Focus and Illumina TruSight Oncology 500 HRD) revealed markedly higher HRD scores in responders compared with nonresponders (Fig. 5A; Supplementary Table S5), with very high analytic concordance despite the two assays employing different wet methodologies and computational algorithms (Fig. 5B).

Importantly, in line with our findings, the downregulation of *POLD1* observed in TNBC samples was not only associated with carboplatin resistance but was also mutually exclusive with the presence of mutational signature 3 (43), a genomic scar typically associated with an HR-deficient BRCAness phenotype (66).

RAD51 Expression as a Biomarker of FOLFIRI Response

The previous results indicate that FOLFIRI-sensitive PDX models exhibited a BRCAness phenotype, which was not, however, driven by loss-of-function mutations, deletions, or promoter hypermethylation of HR genes. The DNA recombinase RAD51 is the central catalyst of the HR pathway. In particular, RAD51 loads onto single-stranded DNA tracts at DSB sites resected by nucleolytic degradation to form a nucleoprotein filament, which mediates strand invasion to initiate HR repair (67). Recent work has shown that low nuclear abundance or nuclear foci counts of RAD51 predict clinical benefit from platinum-based chemotherapy or PARP inhibitors in patients with breast and ovarian cancers exhibiting *BRCA1/BRCA2* wild-type status or a subthreshold genomic scar HRD score (68–74). This suggests that low RAD51 expression may serve as a marker for HR-defective tumors, regardless of the underlying mechanisms compromising HR function. In light of this, we assessed the protein amount of RAD51 in PDX models of the reference subset by IHC. This analysis revealed considerable differences in RAD51 positivity between responders and nonresponders. Under basal (untreated) conditions, nuclear RAD51 intensity was significantly higher in resistant tumors than in sensitive tumors (Fig. 5C). Similarly, in

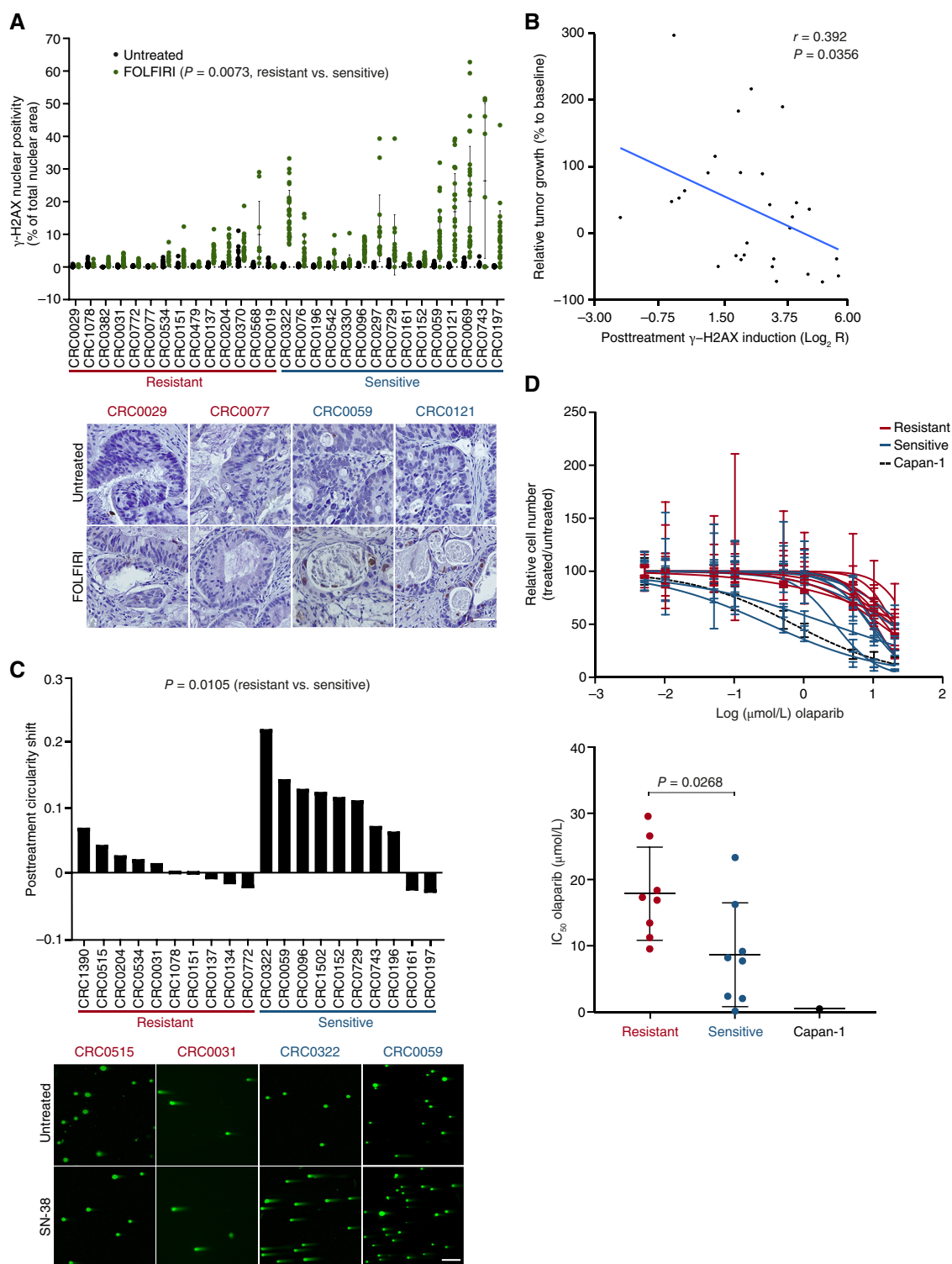


Figure 3. Functional assessment of DNA damage response after FOLFIRI treatment in PDXs and tumoroids. **A**, Morphometric quantification (top) and representative images (bottom) of γ -H2AX nuclear positivity in FOLFIRI-resistant ($n = 14$) and FOLFIRI-sensitive ($n = 15$) PDXs of the reference subset under basal (untreated) conditions and after treatment with FOLFIRI for 6 weeks. For each PDX model, individual dots represent the average γ -H2AX nuclear positivity in one optical field (40 $\times$). Ten fields were quantified per section, with analysis conducted on 1 section from 1 to 3 randomly chosen tumors ($n = 10$ –30). Statistical analysis was done using a two-tailed unpaired Welch's t test. Scale bar, 50 μm . **B**, Correlation between FOLFIRI sensitivity and posttreatment γ -H2AX induction, calculated as the $\log_2$ ratio ($\log_2 R$) of posttreatment to basal γ -H2AX nuclear positivity. Statistical analysis was done using a Pearson's correlation test. **C**, Morphometric quantification (top) and representative images (bottom) of comet circularity shifts in tumoroids from FOLFIRI-resistant ($n = 10$) or FOLFIRI-sensitive ($n = 10$) PDXs after treatment for 4 hours with 10 nmol/L SN-38, the active metabolite of irinotecan. (continued on following page)

posttreatment samples, the formation of RAD51 nucleoprotein filaments (microscopically visualized as nuclear foci) was more pronounced in tumors that progressed on FOLFIRI compared with those that regressed (Fig. 5D). Both basal and posttreatment RAD51 nuclear positivity were significantly anticorrelated with the extent of tumor chemosensitivity (Supplementary Fig. S7A), indicating that higher RAD51 expression was generally associated with greater resistance. Although some individual models deviated from this trend, the overall pattern supports RAD51 as a key predictor of FOLFIRI resistance across our PDX cohort. Consistently, despite individual variability, RAD51 was, on average, more abundant in nuclear extracts of tumoroids derived from FOLFIRI-resistant PDXs than in those from FOLFIRI-sensitive PDXs (Supplementary Fig. S7B).

This evidence suggests that tumors with marked RAD51 abundance are “primed” to withstand the genotoxic impact of FOLFIRI by effectively engaging the cellular DSB repair machinery. In line with this hypothesis, exogenous stable overexpression of *RAD51* in tumoroid models from FOLFIRI-sensitive MSS PDXs led to reduced genomic scar HRD scores and either prevented or attenuated DNA DSB formation following treatment with SN-38, the active metabolite of irinotecan (Fig. 5E and F; Supplementary Fig. S7C and S7D). This enhancement of DSB repair capability after ectopic introduction of *RAD51* resulted in decreased sensitivity to SN-38 (Fig. 5G; Supplementary Fig. S7E). Collectively, these findings indicate that high RAD51 nuclear positivity is correlated with—and causally sustains—FOLFIRI resistance by fostering a state of DSB repair proficiency that counteracts the DNA-damaging effects of chemotherapy.

The differential expression of RAD51 between responders and nonresponders was evident at the protein level but not at the transcript level, both in baseline and posttreatment tumors (Fig. 5C and D; Supplementary Fig. S8A and S8B). To explore potential posttranslational regulation of RAD51 in the two response categories, we analyzed the mRNA expression of a set of ubiquitin ligases, deubiquitinases, and helicases known to directly or indirectly affect RAD51 protein stability (75). Among these regulators, the ubiquitin ligase genes *RING1* and *FBXO18* stood out for being significantly more expressed in FOLFIRI-sensitive PDX models, both across the entire collection (Supplementary Fig. S8C) and within the reference subset (Supplementary Fig. S8D), suggesting their potential involvement in reducing RAD51 protein abundance in responsive tumors. This differential expression pattern was further validated by IHC analysis in PDXs from the reference subset (Supplementary Fig. S8E).

To assess RING1-dependent regulation of RAD51 protein levels, we examined the ubiquitination status of RAD51 in 293T cells transiently transfected with a RING1 lentiviral vector

and treated with MG132 to inhibit proteasome-dependent degradation. This analysis revealed an accumulation of higher-order ubiquitinated RAD51 species in RING1-overexpressing cells (Supplementary Fig. S8F), supporting a role for RING1 in targeting RAD51 for ubiquitin-mediated proteasomal degradation. Consistently, in tumoroid models, proteasome inhibition increased RAD51 protein stability (Supplementary Fig. S8G). Moreover, although enforced RING1 overexpression raised RAD51 steady-state levels—possibly due to acute compensatory mechanisms following lentiviral transduction—it shortened RAD51 half-life after cycloheximide-induced inhibition of protein synthesis (Supplementary Fig. S8H). We were unable to determine whether this regulation also applies to FBXO18, as tumoroids proved refractory to its lentiviral transduction. Overall, these findings suggest that proteostatic regulation of RAD51 may contribute to the differential protein expression observed in colorectal tumors with distinct FOLFIRI responses.

Clinical Validation of FOLFIRI Response Biomarkers

To validate RAD51 and POLD1 as predictive biomarkers for FOLFIRI response in a clinical setting, we conducted IRInotecan Sensitivity (IRIS), a real-world, retrospective, multicenter study involving 82 patients with mCRC with annotated response to first- or later-line FOLFIRI-based therapy (Supplementary Table S6). In this cohort, RAD51 and POLD1 expression was evaluated through blinded, centralized IHC assessment of baseline marker positivity in formalin-fixed, paraffin-embedded (FFPE) diagnostic specimens from the patients' primary tumors. As both markers displayed uniform staining intensity within individual samples and a continuous distribution across the cohort (Supplementary Fig. S9A), expression levels were dichotomized at the median cutoff and treated as categorical variables.

Consistent with our findings in PDXs, RAD51 expression significantly correlated with FOLFIRI response. Among RAD51-low tumors ($n = 41$), the majority of patients achieved an objective response (26 responders, 63.4%; 15 nonresponders, 36.6%). Conversely, RAD51-high tumors ($n = 41$) were predominantly observed in patients with disease stabilization or progression as their best response (26 nonresponders, 63.4%; 15 responders, 36.6%; Fig. 6A; Supplementary Table S6). POLD1 expression showed an opposite pattern, with POLD1-low tumors being more common in nonresponders (28/41 cases, 68.3%) and POLD1-high tumors being more prevalent in responders (28/41 cases, 68.3%; Fig. 6B; Supplementary Table S6). Importantly, uni- and multivariate regression analyses adjusted for clinical and pathologic variables that could influence therapeutic outcomes—including tumor sidedness,

Figure 3. (Continued) The circularity shift was calculated by subtracting posttreatment values from pretreatment values in a range of increasing circularity from 0 to 1, where 1 is a perfect circle. For each tumoroid, bars represent the average circularity shift values of at least 56 objects visualized in at least 10 optical fields. Statistical analysis was done using a two-tailed unpaired Welch's *t* test. Scale bar, 10 μ m. **D, (Top)** Quantification of cell viability [measured by adenosine triphosphate (ATP) content] in tumoroids derived from FOLFIRI-resistant ($n = 8$) or FOLFIRI-sensitive ($n = 8$) PDXs. Tumoroids were seeded at a density of 5,000 cells/well in 96-well plates, allowed to recover for 2 days, and then treated with olaparib at the indicated concentrations for 7 days. Capan-1, a *BRCA2*-deficient pancreatic cell line, was used as a positive control. Results represent the means $\pm$ SD of at least two independent experiments, each performed in at least three biological replicates. (Bottom) IC_{50} values of tumoroid sensitivity to olaparib. Each dot represents the average IC_{50} value, calculated from the data shown in the top, for an individual tumoroid. The plots show means $\pm$ SD. Statistical analysis was done using a two-tailed unpaired Welch's *t* test.

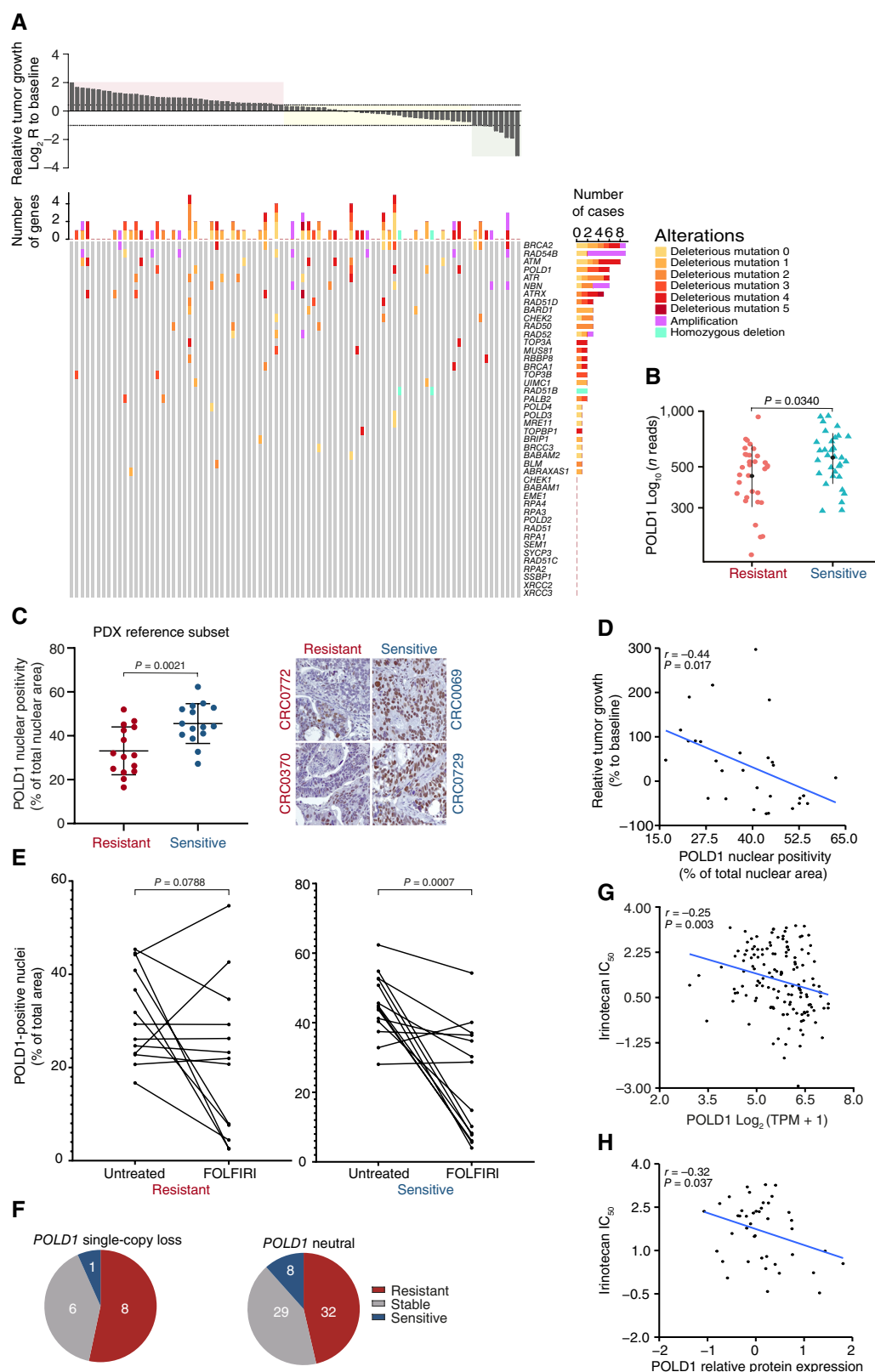


Figure 4. Multidimensional evaluation of HR gene status in PDX models. **A**, Distribution of HR gene mutations and copy-number alterations according to FOLFIRI response in a cohort of 84 PDXs. The waterfall plot depicts tumor response after 3 weeks of treatment, calculated as the log₂ ratio (Log₂ R) of posttreatment to basal tumor volumes. The potential pathogenicity of mutations was assessed using five predictive tools; shades of red to orange indicate the number of tools that classified each mutation as deleterious. (continued on following page)

KRAS/NRAS and *BRAF* mutation status, histology, disease stage, site of metastasis, line of treatment, prior therapies, and FOLFIRI combinations—confirmed that RAD51 and POLD1 independently predict response to FOLFIRI (Fig. 6C). Their predictive value remained significant even after accounting for differences across recruiting centers (Fig. 6C), indicating that the observed biomarker–response correlations were not biased by subcohort-specific factors.

Although no significant anticorrelation between RAD51 and POLD1 was observed across the entire IRIS cohort (Supplementary Fig. S9B), likely due to signal dilution in patients with intermediate responses, a combined analysis identified a subset of patients with inversely polarized marker profiles. Specifically, 22 patients exhibited RAD51-high/POLD1-low tumors, whereas 22 patients had RAD51-low/POLD1-high tumors. This composite biomarker demonstrated enhanced predictive accuracy: 77.3% of patients in the RAD51-high/POLD1-low subgroup ($n = 17$) experienced disease progression or stabilization on FOLFIRI, whereas 81.8% of those in the RAD51-low/POLD1-high subgroup ($n = 18$) achieved an objective response (Fig. 6D; Supplementary Table S6).

Kaplan–Meier analyses demonstrated that RAD51-high tumors were significantly associated with earlier disease progression and shorter OS compared with RAD51-low tumors (Fig. 6E; Supplementary Table S6). In contrast, although POLD1 positivity predicted an objective response, it did not correlate with either PFS or OS (Supplementary Fig. S9C; Supplementary Table S6). This divergence suggests that high POLD1 expression primarily influences early treatment response rather than long-term outcomes, potentially due to the observed decline in POLD1 expression during therapy (Fig. 4E), which may reflect a transient chemosensitizing effect. Notably, in the reference PDX subset, RAD51 expression was correlated with genomic scar HRD scores, whereas no such association was observed for POLD1 (Supplementary Fig. S10A and S10B). This distinction underscores the relevance of RAD51 as a biologically meaningful chemoresistance biomarker and supports the interpretation of POLD1 as a surrogate determinant of initial treatment response. However, the interpretation of PFS and OS data remains limited, as time-related variables in real-world retrospective studies like IRIS are inevitably affected by subsequent treatments and other confounding factors. With this caveat, the correlation between RAD51/POLD1 expression and objective response should be considered more reliable and regarded as the primary endpoint of the IRIS study.

Exploratory Analysis of Response Biomarkers in RAD51 “Lowish” and RAD51/POLD1 “Neutral” Tumors

RAD51 and POLD1 emerged as strong predictors of FOLFIRI response, with an accuracy that was further enhanced by combining the two biomarkers. However, in both PDX models and patients, some resistant cases could not be clearly explained by high RAD51 expression or by opposing expression patterns of RAD51 and POLD1. To investigate potential additional response determinants in these borderline cases, we focused on the 29 PDX models of the reference subset for which RAD51/POLD1 protein expression data, transcriptomic profiles, and mutational information were available. From this subset, we first excluded four resistant outliers with markedly elevated basal RAD51 protein overexpression (CRC0029, CRC0151, CRC0204, and CRC0479; mean positivity >2% of total nuclear area per tissue section; Fig. 5C), which could have disproportionately influenced the observed association between RAD51 and resistance in the entire subset. Notably, even after removing these outliers, RAD51 IHC levels in the remaining resistant models—though relatively “lowish” (mean positivity = 0.80%)—were still significantly higher than those in sensitive models (mean positivity = 0.21%; Supplementary Fig. S11A). Transcriptomic differences between the RAD51-lowish resistant models and sensitive models were highly concordant with those of the full reference subset (Supplementary Fig. S11B), and no differential enrichment in HR gene mutations or gene copy-number alterations was detected (Supplementary Fig. S11C and S11D). Together, these findings suggest that the FOLFIRI-resistant subgroup was molecularly homogeneous, irrespective of variability in basal RAD51 positivity. This interpretation is further supported by the observation that all resistant models efficiently formed RAD51 foci after FOLFIRI treatment (Fig. 5D), indicating a shared capacity for RAD51 activation despite a certain degree of heterogeneity in basal expression levels.

As a second approach, we examined models lacking obvious RAD51/POLD1 antiregulation. Post-FOLFIRI RAD51 focus positivity had a broader dynamic range than basal RAD51 intensity, thus providing a more suitable metric for defining threshold-based grouping. Accordingly, we used posttreatment RAD51 positivity and basal POLD1 expression for model selection and excluded models with combined RAD51 positivity exceeding 15% and POLD1 positivity below 35%, as well as models with combined RAD51 positivity below 10%

Figure 4. (Continued) For copy-number representation, only homozygous deletions and high-level gene amplifications were included, as identified by -2 and $+2$ thresholds in the GISTIC output. **B**, Expression levels of the *POLD1* gene in FOLFIRI-resistant ($n = 33$) and FOLFIRI-sensitive ($n = 33$) PDX models. Each dot represents the number of reads aligned to the *POLD1* gene for each sample, normalized by library depth and presented on a logarithmic scale. The plots show means $\pm$ SD. Statistical analysis was done using the Wald test, implemented by DESeq2. **C**, Morphometric quantification (left) and representative images (right) of POLD1 nuclear positivity in FOLFIRI-resistant ($n = 14$) and FOLFIRI-sensitive ($n = 15$) PDXs of the reference subset under basal (untreated) conditions. Individual dots represent the average POLD1 nuclear positivity for each PDX, quantified in 10–30 optical fields (40 $\times$) in 1 section from 1 to 3 randomly chosen tumors. The plots show means $\pm$ SD. Statistical analysis was done using a two-tailed unpaired Welch's *t* test. Scale bar, 50 μ m. **D**, Correlation between FOLFIRI sensitivity and basal POLD1 nuclear positivity. Statistical analysis was done using Pearson's correlation test. **E**, Changes in POLD1 nuclear positivity in pre- vs. posttreatment PDXs ($n = 13$ FOLFIRI-resistant and $n = 14$ FOLFIRI-sensitive PDXs). Individual dots represent the average POLD1 nuclear positivity for each PDX, quantified in 10–30 optical fields (40 $\times$) in 1 section from 1 to 3 randomly chosen tumors. The values of untreated PDXs are the same as those shown in **C**. Statistical analysis was done using a two-tailed paired Welch's *t* test. **F**, Distribution of *POLD1* gene copy number (assessed by GISTIC) according to FOLFIRI response in the subset of 84 PDXs shown in **A**. **G** and **H**, Correlation between irinotecan sensitivity and *POLD1* mRNA (**G**) and protein (**H**) expression in a pan-cancer cell line collection ($n = 141$ cell lines for mRNA and 42 cell lines for protein). Data were extracted from the Cancer Dependency Map dataset (63). Statistical analysis was done using Pearson's correlation test. TPM, transcript per million.

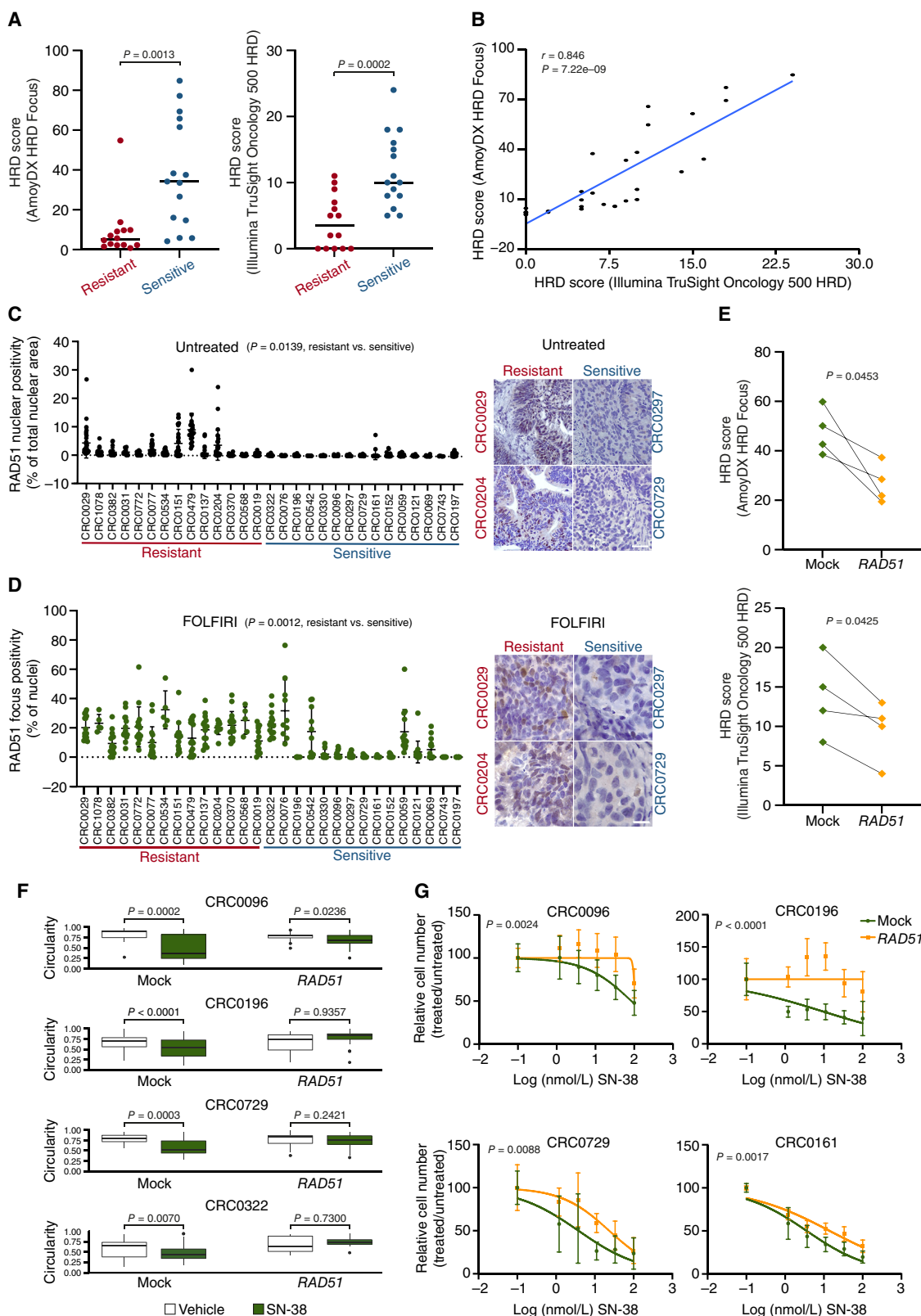


Figure 5. Low HRD scores and high RAD51 as biomarkers of FOLFIRI resistance in PDXs and tumoroids. **A**, Genomic scar HRD scores in FOLFIRI-resistant ($n = 14$) and FOLFIRI-sensitive ($n = 15$) PDXs of the reference subset under basal (untreated) conditions using two different assays. Statistical analysis was done using a two-tailed unpaired Welch's t test. **B**, Correlation between the HRD scores obtained using the two assays. Statistical analysis was done using Pearson's correlation test. (continued on following page)

and POLD1 positivity above 40%. These cutoffs were chosen to approximate the central distribution of each biomarker, yielding a “gray zone” of 12 remaining models (4 responders and 8 nonresponders) in which RAD51 and POLD1 expression levels were not antiregulated (Supplementary Fig. S12A).

Differential gene expression analysis in this biomarker-neutral subgroup confirmed the global gene expression differences between responders and nonresponders observed in the PDX reference subset (Supplementary Fig. S12B). However, it also unveiled a distinct block of DEGs that were not identified in either the full PDX cohort or the reference subset (Supplementary Fig. S12C; Supplementary Table S3). Consistent with findings from our initial differential expression analysis across the full PDX cohort, several genes significantly upregulated in resistant tumors were linked to inflammation and innate immunity, such as *LYZ* (lysozyme), members of the Schlafen gene family (*SLFN5*, *SLFN11*, *SLFN13*; ref. 76), the IFN γ -inducible gene *SOCS2* (77), and the NF- κ B activator *BIRC3* (78). Notably, *BIRC3* has been implicated in 5-FU resistance in *Fusobacterium nucleatum*-infected colorectal cancer cells (79). Conversely—and again in accordance with transcriptional patterns observed in the entire PDX dataset—some of the genes significantly overexpressed in sensitive tumors were associated with heightened mitotic activity and tumor aggressiveness, including the ferroptosis-related molecule *TTC7B* (80); the NAD⁺ synthase *NMNAT2* (81, 82); the transcription factors *NFIX* (83), *ZNF114* (84), and *PDX1* (85, 86); and the autophagy protein *ATG9A* (87). High levels of *NFIX* and *PDX1* have previously been linked to colorectal cancer sensitivity to cytotoxic drugs in cell line-based experiments (86, 88).

At the gene pathway level, most GSEA signatures that differentiated responders from nonresponders in the larger datasets remained similarly enriched in the biomarker-neutral subgroup. Interestingly, however, EMT-related traits—associated with response in the broader populations—were instead more enriched in nonresponders within this subgroup (Supplementary Fig. S12D), suggesting a context-dependent shift in their association with treatment outcome.

When HR gene mutations were assessed within the biomarker-neutral subgroup, a predicted deleterious *BRCA1* mutation was identified in one resistant model, and a similar *BRCA2* mutation was identified in one sensitive model; however, both variants were heterozygous (Supplementary Fig. S12E). No homozygous mutations with putative pathogenic

significance or homozygous deletions were detected. Hemizygous deletions affecting 27 genes were observed in 7 models, with no clear association with treatment response (Supplementary Fig. S12E). Notably, of the seven high-level *RAD54B* amplifications found in the entire PDX cohort—which were evenly distributed between sensitive and resistant models (Fig. 4A)—two were selectively enriched in the biomarker-neutral subgroup, and both were associated with FOLFIRI sensitivity (Supplementary Fig. S12E). In this context, it is noteworthy that *RAD54B* amplification has been linked to enhanced WNT/ β -catenin signaling (89), a pathway also enriched in biomarker-neutral responders (Supplementary Fig. S12D). Although anecdotal, this finding reinforces the notion that distinct molecular features may underlie FOLFIRI response in tumors lacking the canonical RAD51/POLD1 anticorrelation.

Taken together, results from both general and stratified analyses support the existence of core transcriptional programs distinguishing FOLFIRI-resistant tumors (marked by innate immunity activation) from FOLFIRI-sensitive tumors (characterized by increased cell proliferation). This discriminatory capacity persists even in cases where the response was not predicted by RAD51/POLD1 anticorrelation although the specific genes involved may differ. Moreover, the opposite enrichment pattern of EMT traits in biomarker-neutral tumors, along with the selective association of *RAD54B* gene amplification with FOLFIRI sensitivity in this subgroup, underscores the molecular heterogeneity of response mechanisms beyond RAD51/POLD1-defined contexts.

Homologous Recombination Inhibition Efficacy in FOLFIRI-Resistant Tumors

The observation that FOLFIRI resistance is sustained by DSB repair through RAD51 suggests that chemorefractory patients with RAD51-high tumors might be sensitized to FOLFIRI by targeting key effectors of HR-mediated DNA repair. In agreement with this hypothesis, the RAD51-specific inhibitor B02 showed minimal efficacy as a single agent but potentiated the cytotoxic activity of SN-38 in four independent tumoroids derived from FOLFIRI-resistant PDXs (Supplementary Fig. S13A and S13B). Although this chemosensitizing effect was reproducible, the drug interaction was additive and modest in magnitude. We attribute this outcome

Figure 5. (Continued) C, Morphometric quantification (left) and representative images (right) of RAD51 nuclear positivity in FOLFIRI-resistant ($n = 14$) and FOLFIRI-sensitive ($n = 15$) PDXs of the reference subset under basal (untreated) conditions. For each PDX model, individual dots represent the average RAD51 nuclear positivity in one optical field (40 $\times$). Ten fields were quantified per section, with analysis conducted on one section from one to three randomly chosen tumors ($n = 10$ –30). Statistical analysis was done using a two-tailed unpaired Welch's t test. Scale bar, 50 μ m. **D**, Morphometric quantification (left) and representative images (right) of RAD51 nuclear foci positivity in FOLFIRI-resistant ($n = 14$) and FOLFIRI-sensitive ($n = 15$) PDXs of the reference subset after treatment with FOLFIRI for 6 weeks. For each PDX model, individual dots represent the average RAD51 nuclear foci positivity in one optical field (100 $\times$). Five fields were quantified per section, with analysis conducted on one section from one to three randomly chosen tumors ($n = 5$ –15). RAD51 positivity in nuclear foci was calculated as the percentage of cells showing at least one RAD51 nuclear focus, divided by the total cell number. Statistical analysis was done using a two-tailed unpaired Welch's t test. Scale bar, 20 μ m. **E**, Genomic scar HRD scores in four tumoroids from FOLFIRI-sensitive PDXs transduced with a control vector (mock) or RAD51 using the same assays as in **A** and **B**. Statistical analysis was done using a two-tailed paired Welch's t test. **F**, Morphometric quantification of comet circularity in four tumoroids from FOLFIRI-sensitive PDXs transduced with a control vector (mock) or RAD51 and treated for 4 hours with vehicle or 10 nmol/L SN-38. For each tumoroid, circularity values of at least 11 objects visualized in at least 10 different optical fields are represented via boxplots with default thresholds for whiskers. Statistical analysis was done using a one-tailed Mann-Whitney U test. Scale bar, 10 μ m. Representative images are shown in Supplementary Fig. S7D. **G**, Quantification of cell viability (measured by ATP content) in four tumoroids from FOLFIRI-sensitive PDXs transduced with a control vector (mock) or RAD51. Tumoroids were seeded at a density of 5,000 cells/well in 96-well plates, allowed to recover for 2 days, and then treated for 7 days with SN-38 at the indicated concentrations. Results represent the means $\pm$ SD of three independent experiments, each performed in at least two biological replicates. Statistical analysis was done using two-way ANOVA. Results from three additional tumoroids are shown in Supplementary Fig. S7E.

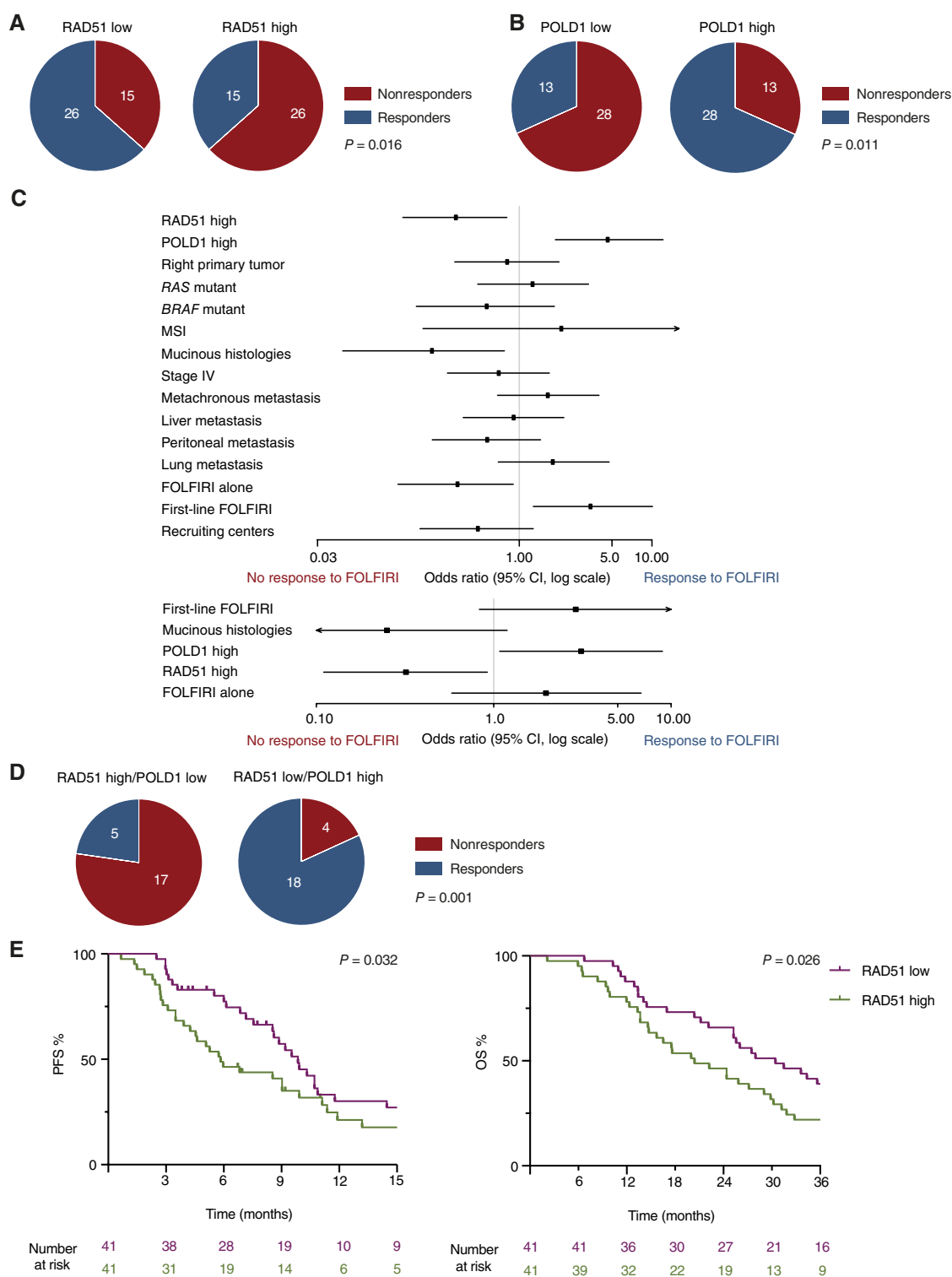


Figure 6. Clinical validation of RAD51 and POLD1 as predictive biomarkers of FOLFIRI response. **A** and **B**, Distribution of tumors with high or low expression of RAD51 (**A**) and POLD1 (**B**) in 82 patients who did or did not respond to FOLFIRI in the IRIS study. Expression levels were dichotomized at the cohort median. Statistical analysis was done using Mantel-Haenszel χ^2 test. **C**, Uni- and multivariate regression analyses of the association between FOLFIRI response and clinical, pathologic, and biomarker variables in IRIS tumors. Only variables with a univariate $P < 0.05$ were included in the multivariate model. The variable "Recruiting centers" compares patients enrolled in Italian sites ($n = 24$) vs. Spanish sites ($n = 58$). The variable "Mucinous histologies" compares not otherwise specified adenocarcinomas with tumors exhibiting mucinous or signet-ring cell features. CI, confidence interval. Statistical analysis was done using the Wald test. **D**, Distribution of tumors with high RAD51/low POLD1 and low RAD51/high POLD1 expression in IRIS patients ($n = 44$). Statistical analysis was done using the Mantel-Haenszel χ^2 test. **E**, PFS and OS of IRIS patients ($n = 82$), stratified by RAD51 expression. Statistical analysis was done using the log-rank test.

to the limitations of current RAD51-targeting drugs like B02, which interfere with RAD51 strand exchange and gene conversion functions but do not disrupt its critical role in protecting nascent DNA strands from degradation at stalled forks and facilitating replication restart (90–92). To achieve more complete RAD51 inactivation, we employed RNAi-based silencing (Supplementary Fig. S13C). Consistent with the notion that homozygous RAD51 knockout is embryonic lethal in mice (93), this approach led to near-complete inhibition of cell viability in two tumoroids, with more than a 75% reduction in proliferation compared with controls, thereby precluding further experiments with SN-38 (Supplementary Fig. S13D). However, in the remaining two models in which RAD51 knockdown was better tolerated (proliferation reduced by less than 50%), sensitization to SN-38 was more pronounced than that induced by B02 (Supplementary Fig. S13E), supporting the idea that pharmacologic RAD51 blockade remains functionally incomplete. These constraints, together with the challenges posed by incomplete target specificity and toxicity concerns in the clinical development of RAD51 inhibitors (94), prompted us to explore alternative strategies to intercept the HR pathway more comprehensively.

The ATM kinase serves as a sensor of DSB damage and acts as the apical enzyme triggering the HR cascade. Beyond its involvement in initiating DNA end resection and facilitating RAD51 nucleofilament formation (95), ATM also participates in the later HR phases, possibly by favoring RAD51 displacement from DNA break ends (96). Moreover, ATM acts in the early steps of the DNA damage response by promoting the recovery of stalled replication forks prior to fork collapse (97). Several ATM inhibitors are currently in pre-clinical development, with some undergoing clinical trials (98). Motivated by the dual role of ATM as both an upstream and downstream regulator of RAD51, which extends to aiding stalled fork reversal, and recognizing the clinical potential of ATM inhibitors, we opted to indirectly interfere with RAD51 activity by blocking ATM. The ATM-specific inhibitor AZD0156—recently evaluated in a first-in-human clinical trial (NCT02588105)—demonstrated limited efficacy when tested individually at near-micromolar concentrations in eight tumoroid models derived from FOLFIRI-resistant MSS PDXs (Fig. 7A; Supplementary Fig. S14A). However, its coadministration substantially enhanced the growth inhibitory effect of SN-38 (Fig. 7B; Supplementary Fig. S14B). A similar sensitizing effect was observed in four of five tumoroids from FOLFIRI-sensitive PDXs (Supplementary Fig. S14C and S14D), indicating that exacerbating HR defects with ATM inhibition can further amplify SN-38 cytotoxic activity, even in tumors showing some degree of HR impairment. Interestingly, the only FOLFIRI-sensitive model in which AZD0156 failed to enhance the response to SN-38 (CRC0196) also exhibited complete resistance to SN-38 following ectopic RAD51 expression (Fig. 5G) and had one of the highest HRD scores (Supplementary Table S5). Moreover, this model harbored a homozygous deleterious *ATM* mutation (Supplementary Fig. S6A). This anecdotally suggests that this specific tumor may possess a distinct “all-or-none” HRD state, rendering it fully susceptible to the genotoxic effect of SN-38 alone, whereas the introduction of RAD51 provides sufficient repair capacity to revert this vulnerability.

The sensitizing effect of ATM blockade was further validated *in vivo* in three chemorefractory PDX models, in which AZD0156 invariably enhanced the therapeutic efficacy of FOLFIRI (Fig. 7C; Supplementary Fig. S15A and S15B). Analysis of end-of-treatment samples revealed that γ -H2AX positivity was more pronounced in tumors exposed to the combination of FOLFIRI + AZD0156 compared with tumors exposed to FOLFIRI alone (Fig. 7D), consistent with the assumption that HR inactivation potentiates the genotoxic effect of chemotherapy. Therefore, disruption of the HR pathway renders chemorefractory mCRC tumors more vulnerable to the effects of FOLFIRI, highlighting possible therapeutic opportunities for FOLFIRI-resistant patients.

DISCUSSION

The lack of reliable predictors of response to irinotecan-based chemotherapy remains a major barrier to advancing precision therapy in mCRC. To address this challenge, we conducted multidimensional molecular analyses in a large cohort of PDXs representing a diverse spectrum of responses to FOLFIRI, complemented by functional and pharmacologic investigations in matched tumoroids.

Our first observation was the enrichment of genes associated with innate defense pathways and fatty acid metabolism in FOLFIRI-resistant PDX models. Notably, these genes were also upregulated in tumors from patients with lower objective response rates and shorter PFS and OS on FOLFIRI, highlighting the clinical relevance of this association. The link between chemoresistance and heightened innate immune activation aligns with evidence that chronic inflammation dampens cancer cell responses to genotoxic agents (99, 100). Likewise, chemoresistant cancer cell lines commonly exhibit lipid droplet accumulation and increased fatty acid oxidation (101).

Our findings also unveiled defects in the HR machinery as hallmarks of FOLFIRI responsiveness in mCRC tumors. This BRCAness-like state manifested as discernible genomic scars indicative of HRD-driven genomic instability, yet it did not seem to arise from obvious genetic or epigenetic alterations in the HR pathway. In particular, we found no clear segregation of HR gene loss-of-function pathogenic variants or homozygous copy-number losses with FOLFIRI response, nor did we observe systematic variations in HR gene promoter methylation or transcript expression between responders and nonresponders.

Unlike cancers in which HRD is a clearly defined heritable trait—such as those harboring deleterious *BRCA1/BRCA2* mutations—FOLFIRI-sensitive colorectal cancers seem to exhibit a more variable and incomplete form of HR dysfunction. For example, although tumoroids derived from FOLFIRI-responsive PDX models generally displayed greater sensitivity to the PARP inhibitor olaparib than those from resistant models, only a subset of them showed IC₅₀ values comparable with the *BRCA2*-null pancreatic ductal adenocarcinoma cell line used as a positive control (Fig. 3D). Similarly, the HRD scores in FOLFIRI-sensitive colorectal tumors were lower on average than those typically predictive of PARP inhibitor responsiveness in *BRCA1/BRCA2*-mutant breast and ovarian cancers (Fig. 5A; ref. 102). These considerations suggest

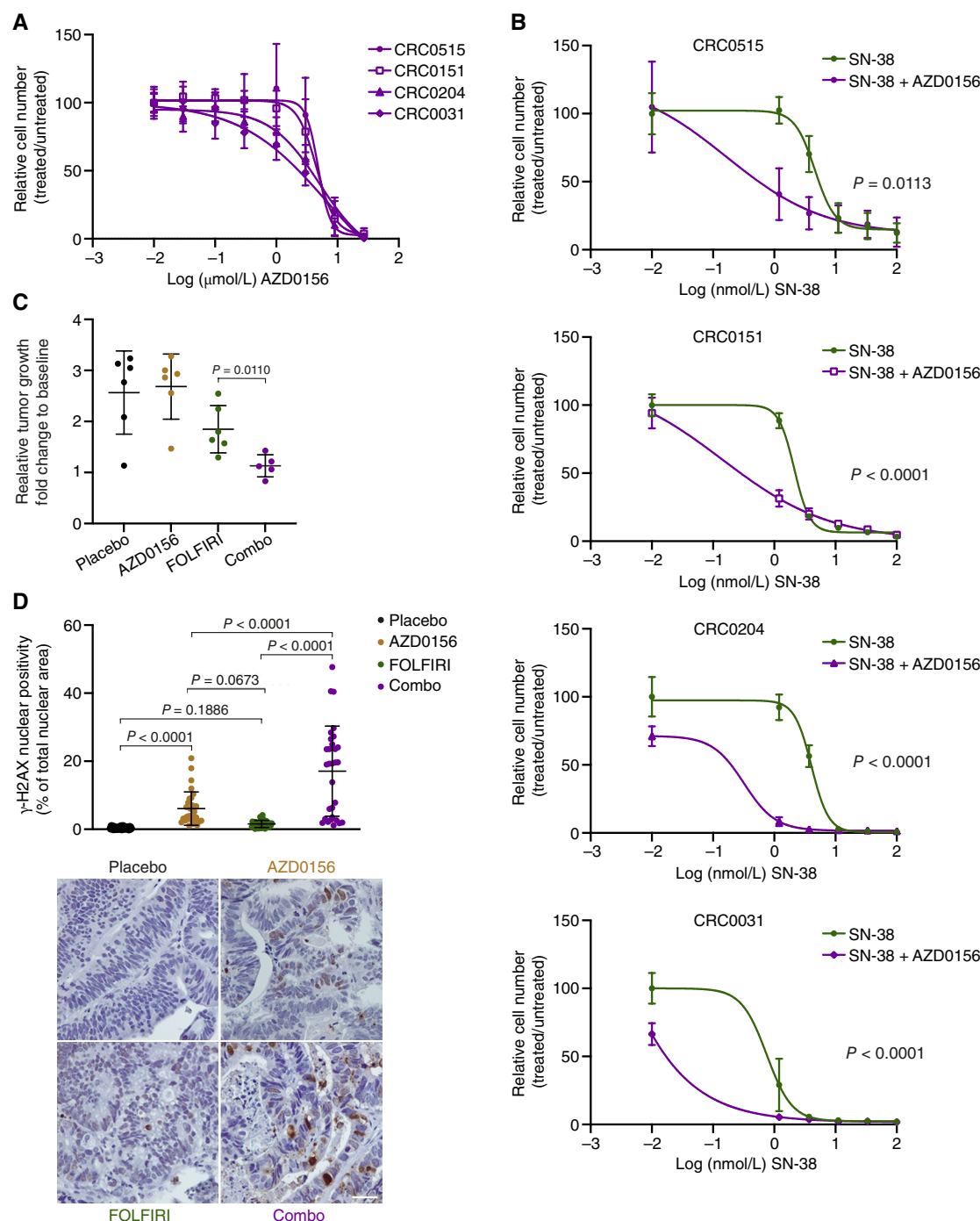


Figure 7. ATM inhibition efficacy in FOLFIRI-resistant PDXs and tumoroids. **A**, Quantification of cell viability (measured by ATP content) in four tumoroids from FOLFIRI-resistant PDXs treated with the ATM inhibitor AZD0156 at the indicated concentrations. Results represent the means $\pm$ SD of at least three independent experiments, each performed in at least two biological replicates. **B**, Quantification of cell viability in the same tumoroids shown in **A** treated with the indicated concentrations of SN-38 in the presence of 1 μ mol/L AZD0156. Results represent the means $\pm$ SD of at least three independent experiments, each performed in at least two biological replicates. Statistical analysis was done using two-way ANOVA. In **A** and **B**, tumoroids were seeded at a density of 5,000 cells/well in 96-well plates, allowed to recover for 2 days, and then treated with the indicated drugs for 7 days. **C**, Endpoint tumor volume changes compared with baseline (start of treatment) measurements in the PDX model CRC0031 from mice treated with the indicated modalities for 3 weeks ($n = 5-6$ animals per treatment arm). Dots represent volume changes of PDXs from individual mice, and plots show means $\pm$ SD for each treatment arm. Statistical analysis was done using a two-tailed unpaired Welch's *t* test. Detailed tumor growth curves for each treatment arm are shown in Supplementary Fig. S15A. **D**, Morphometric quantification (top) and representative images (bottom) of γ -H2AX nuclear positivity in PDX tumors from model CRC0031 treated with the indicated modalities for 3 weeks. At the end of treatment, three tumors from three different mice were explanted and subjected to IHC analysis. Each dot represents the value measured in one optical field (40 $\times$), with 10 optical fields per tumor ($n = 30$). The plots show means $\pm$ SD. Statistical analysis was done using one-way ANOVA with Dunnett's multiple comparison test. Results from drug efficacy studies in additional tumoroids and PDX models are shown in Supplementary Figs. S14 and S15B, respectively.

that FOLFIRI-sensitive colorectal cancers have developed a “hypomorphic” BRCAness phenotype with milder characteristics than those exhibited by HR-deficient cancer subtypes associated with *BRCA1/2* mutations.

Basal (pretreatment) nuclear positivity for RAD51, a key enzyme in the HR pathway, emerged as a standalone determinant of FOLFIRI resistance, validated across both PDX models and patient samples, and holds promise as a robust, scalable biomarker for clinical deployment at the point of care. RAD51 forms discrete nuclear foci upon HR activation, a process commonly utilized as a functional measure of recombination proficiency following genotoxic insults, both *in vitro* and in on-treatment patient biopsies (103). The RAD51 foci counting assay has also been integrated into pretreatment diagnostic protocols and proven to be a dependable predictor of response to PARP inhibitors and platinum-based chemotherapy in breast and ovarian cancer, showing superior performance over the assessment of HR gene mutations in predicting therapeutic outcomes (68, 69, 71–74). Specifically, patients with *BRCA1/2* wild-type tumors and low RAD51 foci scores tend to exhibit favorable responses to therapy, whereas those with *BRCA1/2* mutant tumors and high RAD51 foci scores often demonstrate resistance. Recent research has also demonstrated that quantifying basal RAD51 nuclear intensity, akin to our approach, serves as an alternative to foci counts. Accordingly, strong basal RAD51 nuclear intensity has been significantly associated with platinum resistance in patients with ovarian cancer, particularly in tumors with subthreshold genomic scar HRD scores (70). These findings collectively indicate that a subset of HR-deficient tumors, sensitive to PARP inhibitors or platinum compounds, lack underlying HR gene alterations yet exhibit sensitivity markers such as low RAD51 IHC expression. Our observations suggest that colorectal tumors may fall into this category. The concept that FOLFIRI-sensitive colorectal cancers exhibit HR functional deficiencies due to reduced RAD51 activity is reinforced by two key findings: the mitigation of chemotherapy-induced DNA damage and cytotoxicity upon forced overexpression of RAD51 and the chemosensitizing effect of blocking ATM, a critical HR effector. The ability of ATM inhibition to enhance the FOLFIRI response is consistent with previous studies in colorectal cancer cell line spheroids and PDXs (104, 105).

Although RAD51 alone shows promise as an individual biomarker for distinguishing FOLFIRI-responsive patients from nonresponders, its predictive power could be further enhanced by incorporating POLD1 nuclear expression into a composite algorithm. Findings from the IRIS study suggest that approximately 80% of colorectal tumors exhibiting high RAD51 and low POLD1 nuclear positivity are poorly responsive to FOLFIRI, whereas 80% of tumors with low RAD51 and high POLD1 abundance are sensitive. Although additional work is needed to establish cutoffs of biomarker positivity that ensure adequate diagnostic sensitivity and specificity in patients with mCRC, the composite RAD51/POLD1 biomarker is detectable in approximately half of real-world cases and is expected to facilitate effective patient stratification into response-enriched subgroups.

Biologically, the potential functional relationship between elevated RAD51 and diminished POLD1 expression remains unclear. POLD1 is a versatile DNA polymerase with a pivotal

role in various aspects of the DNA damage response (106). Consequently, tumors with low POLD1 expression may be predisposed to genotoxic cell death unless they develop dependence on alternative DNA repair pathways to sustain survival. We hypothesize that in some cases, RAD51 overexpression confers an evolutionary advantage to colorectal cancer by promoting the positive selection of cells with reduced POLD1 activity.

In conclusion, our findings underscore the value of PDX-based association studies, complemented by functional investigation in *ex vivo* models and clinical validation in patients, as a powerful strategy for the discovery and experimental interrogation of chemotherapy response biomarkers in colorectal cancer. Moreover, our delineation of the BRCAness phenotype in FOLFIRI-sensitive tumors is poised to spur further investigations into the impact of DNA damage response pathways on susceptibility to genotoxic agents, with implications for the elucidation of new disease mechanisms and the design of additional therapeutic opportunities.

METHODS

Specimen Collection and Annotation for PDX Studies

Tumor samples were obtained from patients undergoing liver metastasectomy at the Candiolo Cancer Institute, Candiolo, Torino, Italy; Ospedale Mauriziano Umberto I, Torino; and Città della Salute e della Scienza di Torino–Presidio Molinette, Torino. Written informed consent was obtained from all patients prior to sample collection. Samples were procured, and the study was conducted within the observational trial “Prospective study for the determination of the molecular profile of resistance to antineoplastic treatments”—PROFILING protocol No. 001-IRCC-00IIS-10—approved by the Ethics Committee of the Candiolo Cancer Institute FPO IRCCS (authorization v. 11.0, dated July 13, 2022), in accordance with the Declaration of Helsinki guidelines. Clinical and pathologic data were recorded and maintained in our prospective database. The deidentified clinical information presented in this study adheres to the ethics guidelines outlined in the PROFILING protocol.

PDX Models and In Vivo Treatments

In the FOLFIRI PDX study, tumor implantation and expansion were performed in 6-week-old male and female NOD/SCID mice (Charles River Laboratories, RRID: IMSR_CRL:394), following previously established protocols (14). Once tumors reached an average volume of approximately 300 mm³, mice were randomized into two treatment arms (*n* = 2 for placebo; *n* = 5 for FOLFIRI) and subjected to treatment with either vehicle (physiologic saline, intraperitoneally, twice a week) or the combination of 5-FU (100 mg/kg in 5% dextrose, intraperitoneally, once a week) and irinotecan (25 mg/kg in 5% glucose, intraperitoneally, twice a week). For the drug efficacy study involving FOLFIRI and the ATM inhibitor AZD-0156, 6-week-old male *nu/nu* mice were employed. When the average tumor volume approached 200 mm³, mice were randomized into treatment arms (*n* = at least 5 per arm) and administered the following treatments: (i) vehicle (physiologic saline, intraperitoneally, once a week), (ii) AZD-0156 (10 mg/kg in 10% DMSO and 30% hydroxypropyl- β -cyclodextrin, intraperitoneally, 5 days on/2 days off), (iii) the combination of 5-FU (100 mg/kg in 5% dextrose, intraperitoneally, once a week) and irinotecan (25 mg/kg in 5% glucose, intraperitoneally, once a week), and (iv) AZD-0156 + 5-FU + irinotecan. All procedures involving live animals were reviewed and approved by the Candiolo Cancer Institute Institutional Animal Care and Use Committee and the Italian Ministry of Health (authorization 432/2020-PR).

and complied with relevant ethical regulations. Tumor volumes were measured weekly using a caliper. Volume calculations were based on the following formula: $4/3\pi(d/2)^2 \cdot D/2$, where d and D represent the minor and major tumor axes, respectively. All experiments complied fully with the maximum tumor size limits established by the Institutional Animal Care and Use Committee and the Italian Ministry of Health. For the PDX population trial, results were deemed interpretable when at least three mice per treatment arm reached the prespecified endpoints: a minimum of 3 weeks on therapy or the development of tumors with the largest diameter ≥ 15 mm. For AZD0156 efficacy studies, at least five animals in each treatment arm were required to reach the specified endpoints for results to be considered evaluable. No statistical methods were used to predetermine sample sizes; sample sizes were determined according to previous publications (15, 20) and conformed to PDX minimal information standards (107). Operators were not blinded during measurements. *In vivo* procedures, including animal randomization and related biobanking data, were managed using the Laboratory Assistant Suite (108).

Patient-Derived Tumoroid Cultures

mCRC tumoroids were established from PDX explants. Tumor specimens (0.5 × 0.5 cm) were chopped with a scalpel and then washed with PBS. Following centrifugation, the resulting cell preparation was embedded in Growth Factor Reduced Matrigel (Corning) or Cultrex Basement Membrane Extract (BME Type II or Ultimatix RGF BME, R&D Systems) and dispensed onto 24-well plates (Corning). Tumoroids were allowed to settle for 10 to 20 minutes at 37°C before the addition of DMEM/F12 medium (Sigma-Aldrich) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine, 1 mmol/L n-acetyl cysteine, B27 (Thermo Fisher Scientific), N2 (Thermo Fisher Scientific), and 20 ng/mL EGF (Sigma-Aldrich). Tumoroids were routinely tested for *Mycoplasma* contamination and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Periodic verification of sample identity with the original human specimen (liver metastasis and, if available, normal liver) was performed using a 24-SNP custom genotyping panel (Diatech Pharmacogenetics), and results were analyzed using the MassARRAY Analyzer 4 (Sequenom Inc.). Culture expansions and biobanking were managed using the Laboratory Assistant Suite (108).

Vectors and Tumoroid Genetic Manipulation

The cDNA of human RAD51 (NM_002875) was cloned into the pLenti-C-Myc-DDK-P2A-Puro lentiviral vector (PS100092, OriGene). The cDNA of human RING1 (NM_002931) was cloned into the pLenti-C-Myc-DDK-P2A-BSD lentiviral vector (PS100103, OriGene). The pLVX-IRES-puro lentiviral vector (PT4063-5, Clontech) was used as a control (mock) vector. The RAD51 lentiviral pLKO.1-puro short hairpin RNA (shRNA) vectors were purchased from Sigma-Aldrich (target sequences: shRNA_RAD51_0, GCTAAGACT AACTCAAGATAA; shRNA_RAD51_1, CGGTCAGAGATCATACA GATT; shRNA_RAD51_2, CGGTCAGAGATCATACAGATT). Lentiviral particles were produced by Lipofectamine 2000 (Thermo Fisher Scientific)-mediated transfection of 293T cells (ATCC, #CRL-3216, RRID: CVCL_0063). Tumoroid lentiviral transduction was carried out for 6 hours in the presence of 8 µg/mL polybrene (Millipore). Transduced tumoroids were then selected with 8 µg/mL puromycin (Sigma-Aldrich) or 10 µg/mL blasticidin (Thermo Fisher Scientific) to establish stably expressing lines.

Viability and Cell-cycle Analysis Assays

Viability assays were performed in 96-well plates coated with a thin layer of Matrigel. Tumoroids were washed with PBS, incubated with trypsin-EDTA solution for 5 minutes at 37°C, and then

vigorously pipetted to obtain a single-cell suspension. Cells were seeded in 2% Matrigel with complete culture medium and treated with the modalities indicated in the figures or figure legends. In fixed-dose experiments, SN-38 was used at a concentration of 10 nmol/L to maximize the window of effect, ensuring sufficient variance while avoiding concentrations that could obscure differential responses among tumoroids by being either too low or generically cytotoxic. Previous studies have demonstrated that a single concentration of 3.2 nmol/L SN-38 correctly classifies colorectal tumoroids in predicting irinotecan response in approximately 80% of donor patients (109). However, given that the maximum plasma concentration of irinotecan in patients can reach 26 nmol/L (109), we empirically increased the SN-38 concentration to 10 nmol/L. This adjustment was intended to better reflect clinical dosing conditions while remaining below universally lethal thresholds. Cell viability was assessed by ATP content (CellTiter-Glo Assay, Promega), and luminescence was measured using a GloMax Discover microplate reader (Promega). SN-38 and olaparib were purchased from SelleckChem; AZD0156 was purchased from Cayman Chemical Company. Cell-cycle analyses were conducted using the Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific) and a rabbit polyclonal anti-phospho-histone H3 antibody (Ser10, Cell Signaling Technology, #9701).

Neutral Comet Assays

Neutral comet assays were performed using a CometAssay Kit (Trevigen) following the manufacturer's instructions. Briefly, after 4 hours of treatment, cells were enzymatically dissociated with trypsin-EDTA solution, combined with low-melting agarose, and spread onto comet slides. Slides were then placed in an ice-cold lysing solution overnight at 4°C. Following lysis, slides were immersed in freshly prepared neutral electrophoresis buffer (pH 9.0, 500 mmol/L Tris base, 150 mmol/L sodium acetate) for 30 minutes at 4°C in the dark. Subsequently, slides were transferred to an electrophoresis chamber filled with cold neutral buffer and run for 45 minutes at 21 V at 4°C. After the electrophoresis run, slides were fixed with 70% ethanol, dried, and stained with SYBR Green I (Thermo Fisher Scientific). Images were acquired using a Leica DMI4000 B microscope equipped with a DFC350 FX camera (Leica).

Western Blot Analysis

Proteins were extracted with boiling Laemmli buffer [1% SDS, 50 mmol/L Tris HCl (pH 7.5), 150 mmol/L NaCl]. Lysates were boiled for 10 minutes and briefly sonicated, and the protein amounts were normalized using the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific). Total proteins were separated on precast SDS-polyacrylamide gels (Invitrogen) and transferred onto nitrocellulose membranes (Bio-Rad) using a Trans-Blot Turbo Blotting System (Bio-Rad). Membrane-bound antibodies were detected using the enhanced chemiluminescence system (Promega). Primary antibodies were the following: mouse anti-cMYC (clone 9E10, Santa Cruz Biotechnology, #sc-40, RRID: AB_627268), rabbit anti-RING1 (clone EPRI3047, Abcam, #ab180170, RRID: AB_2716574), rabbit anti-RAD51 (clone D4B10, Cell Signaling Technology, #8875, RRID: AB_2721109), and mouse anti-vinculin (clone hVIN-1, Sigma-Aldrich, #V9131, RRID: AB_477629). To detect ubiquitinated proteins, 293T cells were lysed with a buffer containing 50 mmol/L Tris HCl (pH 7.6), 150 mmol/L NaCl, 1% NP40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₃VO₄, and protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were then incubated on reconstituted UBA01B ubiquitin affinity beads or CUB02B control beads (Cytoskeleton), and ubiquitinated RAD51 was detected with the RAD51 antibody.

IHC and Morphometric Analyses

Tumor specimens were formalin-fixed, paraffin-embedded, and subjected to immunoperoxidase staining with the following antibodies: rabbit monoclonal anti-POLD1 (clone EPR15118, Abcam, #ab186407, RRID: AB_2921290), rabbit monoclonal anti-RING1 (clone EPR13047, Abcam, #ab180170, RRID: AB_2716574), mouse monoclonal anti-RAD51 (clone 14B4, Abcam, #ab213, RRID: AB_302856), rabbit monoclonal anti-cyclin B1 (clone Y106, Abcam, #ab32053, RRID: AB_731779), rabbit monoclonal anti-cyclin A2 (clone Y193, Abcam, #ab32386, RRID: AB_2244193), rabbit monoclonal anti-cyclin D1 (clone E3P5S, Cell Signaling Technology, #55506, RRID: AB_2827374), rabbit polyclonal anti-phospho-histone H3 (Ser10, Cell Signaling Technology, #9701, RRID: AB_331535), rabbit polyclonal anti-FBXO18 (Novus Biologicals, #NBPI-83921, RRID: AB_11056055), and rabbit monoclonal anti-phospho-histone H2A.X (Ser139, clone 20E3, Cell Signaling Technology, #9718, RRID: AB_2118009). Following secondary antibody incubation, immunoreactivity was visualized with DAB chromogen (Dako), and images were captured with the Leica LAS EZ software using a Leica DM LB microscope. Morphometric analysis was performed using ImageJ software (RRID: SCR_003070), with nuclear staining assessed by spectral separation of DAB-colored (marker positive) and hematoxylin-colored (marker negative) nuclei. The percentage of immunoreactive cells was calculated as the DAB-positive nuclear area divided by the total nuclear area. RAD51 immunoreactivity in clinical diagnostic blocks from the IRIS study was assessed exclusively based on nuclear staining, without quantification of cytoplasmic staining. Posttreatment RAD51 positivity in nuclear foci was calculated as the percentage of cells showing at least one RAD51 nuclear focus divided by the total cell number (103).

Molecular Analyses

For qRT-PCR experiments, total RNA was extracted using the Maxwell Instrument (Promega) and reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Gene expression was measured using specific primers by SYBR technology in custom-made PrimePCR plates (Bio-Rad). Results were normalized to the average of two housekeeping genes.

The mutational, transcriptional, and methylation profiles illustrated in this article represent a subset of a larger collection of PDX and tumoroid models characterized in our laboratory (110, 111). Specifically, the mutational annotations presented in Fig. 1 were obtained from the targeted next-generation sequencing data available in ref. 110; methylation profiles were originally reported in ref. 111; the transcriptional dataset was described in both refs. 110 and 111. Bioinformatic analyses were performed on the whole collection, with specific details available in refs. 110 and 111. Briefly, state-of-the-art analytic pipelines, such as GATK (RRID: SCR_001876), Mutect2 (RRID: SCR_026692), STAR (RRID: SCR_004463), DESeq2 (RRID: SCR_015687), and minfi (RRID: SCR_012830), were applied. Adaptations were made to these pipelines to exclude data originating from mouse tissues in PDX samples.

Differential Gene Expression Analyses. Differential gene expression analyses were conducted using the R package DESeq2 (version 1.26.0; ref. 112) with the formula “~batch + type.” This formula incorporated “batch” to correct for sequencing batches and “type” to specify sample resistance or sensitivity to chemotherapy. Genes with more than five reads in only one sample were removed before testing for differential expression. DEGs were identified based on an absolute $\log_2$ fold change ≥ 0.585 and adjusted P values < 0.05 . The resulting DEGs were used for GSEA (RRID: SCR_003199) with R libraries ClusterProfiler (version 3.14.3, RRID: SCR_016884;

refs. 113, 114), DOSE (version 3.12.0; ref. 115), msigdb (version 7.4.1, RRID: SCR_022870; ref. 116), and enrichplot (version 1.6.1, RRID: SCR_026996). Volcano plots were generated by manually highlighting a subset of relevant terms with ggplot (RRID: SCR_014601). The list of samples subjected to differential gene expression analyses is presented in Supplementary Table S7. To analyze the relationship between gene set enrichment patterns and FOLFIRI response across the PDX population, pathway activity at the individual PDX level was calculated using ssGSEA. To assess signatures in individual patient samples, GSVA scores were generated using the “gsva” method from the GSVA R package (version 1.44.1, RRID: SCR_021058; ref. 117) on the robust fragments per kilobase of transcripts per million fragments mapped values.

Differential Methylation Analyses. Differential methylation analyses were conducted in 27 untreated PDX samples categorized as chemoresistant and 27 untreated PDX samples categorized as chemosensitive after 3 weeks of treatment. Models were selected based on available methylation profiles from tumors that progressed or regressed on FOLFIRI treatment. Methylation levels, expressed as M values, were analyzed with the R package limma (version 3.42.2, RRID: SCR_010943; ref. 118), using the formula “~ response,” where response identifies whether the sample was resistant or sensitive to chemotherapy. Probes with absolute $\log_2$ fold change ≥ 1 and P value < 0.05 were considered differential. One-to-one gene-probe associations were defined by selecting the probe with the largest SD and falling outside the gene body (defined as the region between the most 5' transcription start site and the most 3' transcription end site annotated for each gene transcript) among all the probes with reported associations to a specific gene based on the Illumina Human Methylation EPIC annotation. In cases where no probes fell outside the gene body, the probe with the largest SD within the gene body was retained.

Mutational Analysis of HR Genes. Mutational analysis of HR genes was conducted on 69 PDX models using a manually defined panel covering exons of 44 HR-relevant genes (Twist Bioscience). Targeted sequencing was performed on Illumina HiSeq 4000/NovaSeq 6000 instruments by IntegraGen SA. Paired-end 2×100 bp reads were obtained, aiming for $\sim 1,000\times$ depth for each sample in the targeted regions (113 kbp). Initial quality control was performed with FastQC (version 0.11.9, RRID: SCR_014583) by IntegraGen. Murine reads were filtered using Xenome (version 1.0.0, RRID: SCR_026266) with default parameters after building k-mer indexes for the human genome (GRCh38, downloaded from <https://gdc.cancer.gov/about-data/gdc-data-processing/gdc-reference-files>) and the murine genome (mm10, obtained from iGenomes). The GATK Best Practices Workflow for somatic mutation calling was followed to perform mutation calling with Mutect2 (bwa version 0.7.17-r1188; parameters -K 100000000 -Y, GATK 4.1.4.0), with alignment performed versus GRCh38. The Single Nucleotide Polymorphism Database (for quality recalibration, downloaded from NCBI, https://ftp.ncbi.nih.gov/snp/organisms/human_9606/VCF/All_20180418.vcf.gz) and Gnomad (release 2.1.1, RRID: SCR_014964) were used as external references for common human polymorphisms. The resulting mutations were annotated using ANNOVAR (version 2018-04-16, RRID: SCR_0001821), and their potential deleteriousness was assessed using five different prediction algorithms: “SIFT_pred,” “fathmm.MKL_coding_pred,” “LRT_pred,” “FATHMM_pred,” and “PROVEAN_pred.” The mutational status of the 44 HR genes was analyzed in an additional 15 PDX models (for a total number of 84 models) through manually curated integration of the mutation calls from a previous whole-exome sequencing dataset (15). The variant allele frequencies of examined mutations and predicted effects on protein function are available in Supplementary Table S2.

Low-Pass Whole-Genome Sequencing. Copy-number information was initially accessible for 57 out of the 84 PDX models with known HR gene mutational status (110). To comprehensively annotate HR gene copy numbers across the entire dataset of 84 PDX samples, low-pass whole-genome sequencing was conducted on an additional 27 samples. Sequencing was performed on Illumina NovaSeq S4 instruments by Biodiversa srl. Paired-end 2 × 150 bp reads were obtained, aiming for 0.65× depth for each sample. Initial quality control was performed with FastQC (version 0.11.8). Murine reads were filtered using Xenome (version 1.0.0) with the same procedure described above for targeted sequencing of HR genes. Total or human-classified reads were aligned to GRCh38 with bwa (v0.7.17-r1188, RRID: SCR_010910 - parameters -K 100000000 -Y) following GATK best practices. Then, duplicates were marked using Picard (2.18.25, RRID: SCR_001876). Segmented fold changes were obtained with QDNAseq (version 1.22.0, RRID: SCR_003174) using default parameters and pairedEnds = TRUE, with annotations obtained from QDNAseq.hg38 and a bin size of 15 kb. Log₂ values with a pseudocount of 1 in .seg format were input into the GISTIC2.0 module (version 2.0.23, RRID: SCR_000151) in GenePattern (<https://cloud.genepattern.org>, RRID: SCR_003201) with default parameters and TRHuman_Hg38.UCSC.add_miR.160920.refgene.mat. The GISTIC2.0 module was run on the entire cohort of 84 xenografts.

Homologous Recombination Deficiency Scores. HRD status was determined using the TruSight Oncology 500 HRD panel (Illumina) and the E-IVD AmoyDx HRD Focus Panel (Amoy Diagnostics), according to the manufacturer's instructions. Genomic DNA libraries were sequenced on the Novaseq 6000 instrument (Illumina), aiming for a minimum of 500× read depth. TruSight raw data were processed on a local DRAGEN server version 3 by the DRAGEN TruSight Oncology 500 version 2 analysis software, which incorporates a proprietary genomic instability score algorithm powered by Myriad Genetics for HRD assessment. AmoyDX raw data were analyzed using the AmoyDx NGS Data Analysis System software with a proprietary algorithm.

IRIS Translational Study in Patients

The IRIS study is a translational research initiative aimed at validating candidate response biomarkers in diagnostic specimens from real-world patients with clinically annotated mCRC. As a subproject of the master observational trials AlfaOmega (NCT04120935) and AlfaOmega-Retro (NCT05101382), IRIS benefits from their established procedures for patient enrollment and biological specimen retrieval. We retrospectively identified 169 patients with mCRC who were treated with first- or subsequent-line FOLFIRI, either alone or in combination with molecular agents, across four cancer centers in Italy and Spain: Grande Ospedale Metropolitano Niguarda, Milano, Italy; Vall'Hebron Institute of Oncology and Hospital del Mar, Barcelona, Spain; and INCLIVA Biomedical Research Institute, Valencia, Spain. Patient eligibility was based on their best response to FOLFIRI and the availability of pretreatment archival tumor FFPE specimens from primary tumor resections or metastasectomies. Fifty-three patients were excluded due to FFPE specimens solely from tumor biopsies, which exhibited heterogeneous RAD51 positivity patterns compared with primary tumor diagnostic blocks, thus hindering side-by-side comparative assessment. Twelve additional patients were excluded due to the unavailability of any tumor FFPE block. The remaining 104 patients were enrolled in the AlfaOmega and AlfaOmega-Retro protocols, which were already approved by the local ethical committees and active at each institution. Following the retrieval of FFPE blocks and quality assessment for IHC analysis, 22 patients were further excluded due to poor quality of the archival specimens

and technical issues. The study adhered to the principles of the Declaration of Helsinki and the International Conference on Harmonization and Good Clinical Practice guidelines.

Statistical and Bioinformatics Analyses

Error bars indicate SDs unless otherwise indicated. The number of biological (nontechnical) replicates for each experiment is reported in the figure legends. For experiments with two groups, statistical analysis was performed using a two-tailed Student *t* test, Welch's *t* test, one-sided Mann-Whitney *U* test, or Wilcoxon matched pairs signed-rank test, unless otherwise indicated. For experiments with more than two groups, one-way ANOVA was used. Two-way ANOVA was applied for experiments in which the determinations were considered interdependent. In the case of multiple testing, we adopted Dunnett's multiple comparison test or the Benjamini-Hochberg FDR test. Correlations were calculated using Pearson's coefficients. The enrichments of specific mutational alterations, gene sets, or IHC scores in FOLFIRI-resistant versus FOLFIRI-sensitive PDX models and patients were evaluated using the Mann-Whitney *U* test, Fisher's exact test, or Mantel-Haenszel χ^2 test. The correlation between the main clinicopathologic variables and response to FOLFIRI within the IRIS study was assessed using both uni- and multivariate logistic regression, and *P* values were calculated using the Wald test. Patient survival in the FIRE-3 trial was assessed using the Wald test on the univariate Cox model coefficient of the expression of the resistance-associated or the sensitivity-associated metagene. Patient survival in the IRIS study was assessed using the log-rank (Mantel-Cox) test. The level of statistical significance was set at *P* < 0.05. For multiple comparisons, the results were considered significant when Dunnett's multiple comparison test was <0.05 and when the Benjamini-Hochberg FDR was <0.1. Graphs were generated, and statistical analyses were performed using GraphPad Prism (version 9.0, RRID: SCR_002798), R (version 3.6.3), R base packages, and the following libraries: precrec (0.12.9, RRID: SCR_018659), ggplot2 (version 3.3.0, RRID: SCR_014601), pheatmap (v1.0.12, RRID: SCR_016418), ComplexHeatmap (version 2.2.0, RRID: SCR_017270), sjPlot (2.8.10, RRID: SCR_024307), and circlize (version 0.4.15, RRID: SCR_002141).

Data Availability

All sequencing data reported here have been deposited in the European Genome-phenome Archive (RRID: SCR_004944) under the following accession numbers: EGAD50000000107 (targeted DNA sequencing of HR genes), EGAD00001009653 (targeted DNA sequencing of genes recurrently mutated in colorectal cancer), EGAD50000000277, and EGAD00001009661 (low-pass whole-genome sequencing). Access to these datasets will be granted upon registration to the European Genome-phenome Archive and submission of a request to access the specified studies. Processed expression levels and raw read counts are publicly accessible in the Gene Expression Omnibus database (RRID: SCR_005012) under accession number GSE204805. Methylation data are publicly available in the Gene Expression Omnibus under accession number GSE208713. All analysis pipelines were developed and run with Snakemake (version 5.4.0, RRID: SCR_003475; ref. 119), with codes available at the following repositories: (i) https://github.com/vodkatad/biodiversa_DE (RNA sequencing differential analysis) and (ii) <https://github.com/vodkatad/strata>.

Authors' Disclosures

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