Loss of MutL Disrupts CHK2-Dependent Cell-Cycle Control through CDK4/6 to Promote Intrinsic Endocrine Therapy Resistance in Primary Breast Cancer

Svasti Haricharan¹,², Nindo Punturi¹,², Purba Singh¹,², Kimberly R. Holloway¹,², Meenakshi Anurag¹,², Jacob Schmelz¹,², Cheryl Schmidt¹,², Jonathan T. Lei¹,²,³, Vera Suman⁴, Kelly Hunt⁵, John A. Olson Jr⁶, Jeremy Hoog⁷,⁸, Shunqiang Li⁷,⁸, Shixia Huang⁹,¹⁰, Dean P. Edwards⁹,¹⁰,¹¹, Shyam M. Kavuri¹,², Matthew N. Bainbridge¹,²,¹²,¹³, Cynthia X. Ma⁷,⁸, and Matthew J. Ellis¹,²

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ABSTRACT

Significant endocrine therapy–resistant tumor proliferation is present in ≥20% of estrogen receptor–positive (ER+) primary breast cancers and is associated with disease recurrence and death. Here, we uncover a link between intrinsic endocrine therapy resistance and dysregulation of the MutL mismatch repair (MMR) complex (MLH1/3, PMS1/2), and demonstrate a direct role for MutL complex loss in resistance to all classes of endocrine therapy. We find that MutL deficiency in ER+ breast cancer abrogates CHK2-mediated inhibition of CDK4, a prerequisite for endocrine therapy responsiveness. Consequently, CDK4/6 inhibitors (CDK4/6i) remain effective in MutL-defective ER+ breast cancer cells. These observations are supported by data from a clinical trial where a CDK4/6i was found to strongly inhibit aromatase inhibitor–resistant proliferation of MutL-defective tumors. These data suggest that diagnostic markers of MutL deficiency could be used to direct adjuvant CDK4/6i to a population of patients with breast cancer who exhibit marked resistance to the current standard of care.

SIGNIFICANCE: MutL deficiency in a subset of ER+ primary tumors explains why CDK4/6 inhibition is effective against some de novo endocrine therapy–resistant tumors. Therefore, markers of MutL dysregulation could guide CDK4/6 inhibitor use in the adjuvant setting, where the risk benefit ratio for untargeted therapeutic intervention is narrow. Cancer Discov; 7(10); 1168–B3. © 2017 AACR.

INTRODUCTION

Resistance to endocrine therapy remains a significant cause of death for the approximately 175,000 women diagnosed each year with estrogen receptor–positive (ER+) breast cancer (1). Although some headway has been made in understanding underlying mechanisms, the majority of cases remain unexplained (1). Traditionally, growth factor receptor pathway activation has been implicated as one underlying mechanism of acquired resistance to endocrine therapy. Recently, studies of endocrine therapy resistance in metastatic breast cancer, where patients have been exposed to long periods of treatment, have identified the acquisition of mutations in the estrogen receptor gene (ESR1) causing ligand-independent activation and aromatase inhibitor (AI) resistance to constituting an alternative underlying mechanism for endocrine resistance in the advanced disease setting (2–4). However, ER+ primary breast cancer can also be endocrine therapy resistant at diagnosis (intrinsic resistance) where ESR1 mutations cannot be the sole explanation, as they are too rare. Intrinsic endocrine resistance is easily diagnosed, based on failure to fully suppress Ki67 (proliferation marker) in tumor biopsies after 2 to 4 weeks of neoadjuvant endocrine treatment (5), but it is relatively understudied. Intrinsic resistance, as assayed by Ki67, occurs in at least 20% of ER+HER2- tumors and is an established poor-prognosis marker. Unfortunately, these tumors also often fail to respond well when switched to neoadjuvant chemotherapy (6, 7). Consequently, patients diagnosed with intrinsically endocrine-resistant ER+HER2- primary breast cancer suffer high rates of relapse and death.

The recently noted correlation between high mutation load and poor prognosis in ER+ breast cancer suggests that defects in DNA damage repair (DDR) genes may constitute an underexplored driver of endocrine therapy resistance (8). Although the best-understood DDR defect in breast cancer concerns homologous recombination (HR) deficiency, due to BRCA1/2 loss (9), this mechanism is less pertinent to ER+ disease, which is largely HR-competent. A few preliminary epidemiologic studies have noted possible roles for base excision repair (BER) and nucleotide excision repair (NER) in ER+ disease pathogenesis (10, 11). However, our investigations, reported here, establish a new role for a subset of mismatch repair (MMR) pathway components in regulating intrinsic endocrine therapy resistance in ER+ disease. Using in vitro and in vivo models, we demonstrate that defects in MutL complex genes (MLH1, PMS1, and PMS2) directly induce endocrine therapy resistance because an intact MutL complex enables an endocrine therapy–activated ATM/CHK2-dependent cell-cycle checkpoint control to suppress CDK4 activity. We also use preclinical and neoadjuvant clinical trial data to demonstrate that deregulated CDK4 in MutL-deficient tumors remains targetable by CDK4/6 inhibition (CDK4/6i),
explaining the activity of these agents in a subset of endocrine therapy–resistant disease and thereby suggesting a new class of predictive markers for CDK4/6-targeted drugs.

RESULTS
Role of DDR Dysregulation in ER+ Breast Cancer

Because high mutation load is a marker of poor prognosis in ER+ breast cancer (8), we first assessed correlations between mutation load and incidence of nonsilent mutations in pathway-unique genes of the five major DDR pathways: MMR, BER, NER, nonhomologous end joining (NHEJ), and HR in ER+ human breast tumors. We chose two clinical datasets with whole-exome sequencing data for this analysis: (i) several neoadjuvant AI trials, collectively termed NeoAI [Z1031, a study from the American College of Surgeons Oncology Group, which is now part of the Alliance for Clinical Trials in Oncology (6), and the multi-institutional Preoperative Letrozole Phase 2 study (POL; ref. 12)] and (ii) The Cancer Genome Atlas (TCGA) dataset (13). Only mutations in MMR genes were significantly associated with increased tumor mutation load in both clinical datasets (Fig. 1A and B). Because MMR genes are known to be dysregulated at the gene expression level, the impact of low MMR gene expression (using TCGA definitions of mean-1.5x standard deviation to avoid threshold training) on mutation load was also assessed in both datasets, and a correlation between this expanded set of MMR-defective tumors and mutation load was confirmed (Supplementary Fig. S1A and S1B).

MMR Dysregulation and Endocrine Therapy Resistance in Patients with ER+ Breast Cancer

Next, the effect of low RNA levels of each unique MMR gene on ER+ breast cancer outcome of patients treated with hormone therapy (tamoxifen or AI) was assessed in the METABRIC dataset, chosen for its large sample size and long-term clinical follow-up (14). Analysis in this dataset using the same cut-point for low expression drawn from the TCGA analysis (Supplementary Fig. S1A and S1B) revealed significant association between low RNA and poor overall (Fig. 1C) and disease-free survival (Supplementary Fig. S1C and S1D) in 3 of 8 canonical MMR genes: MLH1, PMS1, and PMS2. The poor prognostic effects were independent for each gene and were significant after correction for multiple testing (Table 1).

MLH1, PMS1, and PMS2, along with MLH3, constitute the MutL complex of the MMR pathway. Therefore, the finding that only these three genes negatively affect overall and disease-free survival suggested a specific role for the MutL complex in poor-outcome ER+ disease. The MutL complex is recruited to DNA mismatches by the MutS complex (MSH genes), whereupon it performs two functions: first, to recruit repair proteins to the mismatched nucleotide, and second, to activate ATM/CHK2 signaling in the event of unsuccessful repair (15). The coordinating protein in the MutL complex is MLH1 which, when heterodimerized with PMS1/2, forms a stable complex that is translocated to the nucleus (12). In contrast, MLH3 is largely involved in postmeiotic recombination, although it can play a compensatory role in MMR in the absence of MLH1 (16), possibly explaining the more equivocal association of MLH3 with clinical outcome in the METABRIC database. Underexpression of any gene serving the MutL complex was used to define a MutL low signature (MutL−) versus a MutL normal group (MutL+). The adverse effects of MutL− status on prognosis were found to be independent of clinical variables [progesterone receptor (PR) positivity, HER2 positivity, tumor stage] as well as mutations known to affect breast cancer prognosis (MAP3K1, GATA3, and TP53; Fig. 1D). Interestingly, the HR for MutL− was comparable to the adverse effects of HER2 amplification (Fig. 1D; Supplementary Fig. S1E).

Next, MutL selectivity was assessed in the TCGA dataset where ER+ tumors (treated with tamoxifen or AI) with mutations and/or low mRNA levels for any one of MLH1, MLH3, PMS1, or PMS2 (MutL+ tumors) also associated with significantly worse survival than tumors without MutL dysregulation (MutL−; Supplementary Fig. S1F). Of note, the association of MutL downregulation with poor overall survival is specific to ER+ breast cancer, with no significant association observed in either METABRIC (P = 0.75) or TCGA (P = 0.5) datasets in ER+ breast cancer cohorts.

To examine whether MutL defects can predict intrinsic response to endocrine therapy (in the form of AI) in primary breast cancer, MutL status was analyzed in the NeoAI dataset (including data from the most recent clinical trial, NeoPaLana; ref. 17) focusing on cases where paired Ki67 data at diagnosis and after 2 to 4 weeks of AI, as well as exome sequencing and gene expression data, were available. Using the same mutation/expression-based definitions used in the TCGA and METABRIC analyses, MutL− tumors (n = 24) showed no significant fall in Ki67 levels despite AI treatment and less treatment-induced change in Ki67 compared with MutL+ tumors without MutL underexpression or mutation (P = 0.03, Wilcoxon Rank Sum test; Fig. 1E). Interestingly, 11 tumors assigned as MutS− by mutation or underexpression of MutS were largely endocrine therapy sensitive with a significant fall in Ki67 (P = 0.02) and no difference in Ki67 change compared with the MutS+ group (Fig. 1E).

To estimate the relative frequency of MutL− in endocrine therapy–resistant ER+ breast cancers, we examined the incidence of either nonsynonymous mutations or low mRNA levels in the subset of AI-resistant tumors from NeoAI (Ki67 > 10% on AI treatment). In this setting, MutL− status accounted for 27% of poor responders, and MutL+, but not MutS, dysregulation (4% of poor responders) was enriched in endocrine therapy–resistant tumors (Fig. 1F). Moreover, MutL dysregulation was enriched in endocrine therapy–resistant relative to endocrine therapy–sensitive tumors in the TCGA dataset as well (P < 0.001; MutL− occurring in 25% of tumors from patients who died within 5 years of diagnosis).

MutL Complex Inactivation Causes Intrinsic and Class-Independent Endocrine Therapy Resistance in ER+ Breast Cancer Cells

ER+ breast cancer cell lines were stably transfected with shRNA to suppress expression from each of the three Mutl genes most strongly linked to poor clinical outcome: MLH1, PMS1, and PMS2, and one MutS gene, MSH2, to serve as negative control (knockdown validation in Supplementary Fig. S2A and S2B). As previously published (16, 18, 19), PMS1 silencing also induced loss of stability of MLH1 protein (Supplementary
Figure 1. MMR dysregulation associates with high mutation load and poor clinical outcome in patients with ER+ breast cancer. A and B, ER+ tumors with nonsynonymous mutations in pathway-unique MMR genes from TCGA (A) and NeoAI (B) datasets have increased overall exomic mutation load compared with tumors without. Rest, all others. ANOVA with Tukey comparison determined P values.

C and D, Forest plots demonstrating that ER+ tumors with low MutL gene mRNA (<mean-1.5×StDev) in METABRIC associate selectively with poor overall survival (C) and that low MutL mRNA is an independent prognosticator of poor survival in ER+ breast cancer (D). Log-rank test was used to determine significance and Cox Regression Proportional Hazards for multivariate analysis. Benjamini–Hochberg correction was applied for multiple comparisons.

E, Box plots demonstrating that ER+ tumors from NeoAI (Z1031, POL, NeoPalAna) with mutations in and/or low expression of MutL genes are significantly more likely to be resistant to endocrine therapy as determined by change in Ki67 levels on AI treatment. Friedman Rank Sum test for repeated measures determined P values for pairwise comparisons, and Kruskal–Wallis test determined P value for between-group comparisons. BL, baseline; EoT, early on treatment.

F, Stacked column graph depicting frequency of incidence of MutL dysregulation in ER+ tumors from NeoAI. Fisher exact test determined P values. Accompanying information presented in Supplementary Fig. S1 and Table 1. Horizontal lines indicate mean, and error bars denote standard error for box plots.

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Cox regression was used to calculate outcomes measured by overall survival: the three MMR genes with significant effect on clinical Proportional hazard calculations for each of Table 1.

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**NOTE:** Cox regression was used to calculate P values.

*P < 0.05.

**P ≤ 0.1.**

Fig. S2B). Suppression of any one of the three MutL genes in either MCF7 or T47D cells induced resistance to all classes of endocrine interventions, i.e., estrogen deprivation, a surrogate for AI exposure (Fig. 2A, Supplementary Fig. S2C), fulvestrant, and tamoxifen (Fig. 2B; Supplementary Fig. S2D) within a week of administration. In contrast, suppression of MSH2 did not affect response to estrogen stimulation (Fig. 2A) or to fulvestrant-mediated ER degradation (Supplementary Fig. S2E).

All further experiments were conducted using pharmacologically relevant 100 nmol/L doses of fulvestrant and tamoxifen, and largely in shMLH1 (MLH1 WT) cells because of the central importance of MLH1 to MutL complex formation.

Pooled CRISPR-mediated disruption of each of MLH1, PMS1, and PMS2 genes in both MCF7 and T47D cell lines orthogonally supported a causal association between MutL− and response to fulvestrant (Supplementary Fig. S2F and S2G) and to estrogen stimulation (Supplementary Fig. S2H). Of note, CRISPR-mediated knockdown of MutL genes was less well tolerated in T47D than in MCF7 cells (Supplementary Fig. S2I), and PMS2 knockdown by shRNA or CRISPR was not well tolerated in either cell line (Supplementary Fig. S2B and S2I). These data might explain why PMS2 somatic mutations in human breast tumors are characteristically missense, as complete loss of function may reduce cell viability, but may also be a reflection of the low baseline levels of PMS2 in MCF7 and T47D cells (approximately 30-fold lower than other MutL genes), rendering the establishment of knockdown efficacy challenging.

MCF7 cells with MLH1 downregulation demonstrated fulvestrant-resistant growth in soft agar (Fig. 2C), and unpended xenograft tumor growth after estrogen deprivation, as well as fulvestrant resistance (Fig. 2D). Critically, introduction of shRNA-resistant MLH1 cDNA into MLH1− cells (validated in Supplementary Fig. S3A) restored sensitivity to fulvestrant under both 2-D (Supplementary Fig. S3A) and 3-D growth conditions (Fig. 2C).

**MutL Dysregulation in Patient-Derived Xenograft Models**

To confirm in vitro findings in a human-in-mouse breast cancer model, the occurrence of MutL gene mutations in patient-derived xenograft (PDX) tumors was examined. A missense mutation (ESK) in the MutL gene, PMS2, previously described in a hypermutator, MMR-deficient colorectal cancer cell line [HT-115; Catalogue of Somatic Mutations in Cancer (COSMIC)], was identified in WHIM20, an ERα PDX (4) with high mutation load (Supplementary Fig. S3D). Notably, WHIM20 exhibits resistance to both fulvestrant treatment (Supplementary Fig. S3B) and estrogen deprivation (4). A second MutL-mutant ERα PDX model, HCI-005 (20), was also identified, harboring a frameshift mutation (L160fs) in PMS1 and also associated with high mutation load (Supplementary Fig. S3C). HCI-005 tumors are less estrogen dependent than other ERα PDX, demonstrating 100% tumor outgrowth in ovariectomized mice (20). These data provide additional support for a role for MutL− in endocrine response as well as model systems to study MutL− deficient breast cancer.

**MutL Loss Decreases CHK2 Activation in Response to Endocrine Treatment**

Resistance to fulvestrant treatment in MutL− cells was not due to a failure to degrade ER given the comparable levels.
Figure 2. MutL deficiency induces endocrine therapy resistance in ER+ breast cancer cells by preventing proliferative block. 

A. Bar graph depicting growth of MCF7 cells stably expressing shRNA against luciferase control, MSH2, MLH1, PMS1, or PMS2, as indicated, at day 4 relative to day 1 after addition of 10 nmol/L beta-estradiol to cells grown in charcoal-stripped serum for 4 days. Accompanying knockdown validation in Supplementary Fig. S2A. B, Dose curves demonstrating increased growth of MCF7 cells stably expressing shRNA against MLH1 in response to increasing doses of 4-hydroxy tamoxifen (4-OHT) and fulvestrant (Fulv) relative to cells expressing shRNA against luciferase control. C, Bar graph depicting percentage of colonies in soft agar (3-D growth assay) generated from isotypic MCF7 cells with specified genotype after 4 to 6 weeks of endocrine treatment with accompanying representative images. D, Box plots depicting differences in tumor growth rate (calculated as slope from growth curve of each tumor) of MutL-proficient (shLuc) versus MutL-deficient (shMLH1) MCF7 cells, in the presence of estrogen (E2) and fulvestrant (Fulv) relative to cells expressing shRNA against MLH1 WT and MLH1 shLuc. E and F, Bar graphs depict fold change in percentage of pHistoneH3 (pH3, mitotic) and Ki67+ (proliferation marker) cells in MutL-proficient (shLuc) and MutL-deficient (shMLH1, shPMS1, shPMS2, as indicated) ER+ breast cancer cells and tumors with representative images. Scale bar, 20 μm. Associated data for T47D cells in Supplementary Figs. S2 and S3. For all graphs, error bars describe SD, and Student t-test determined P values.

of ER and ER downstream effectors (PGR and GREB1) at both RNA and protein level before and after fulvestrant treatment in MCF7 shLuc and shMLH1 cells and xenograft tumors (Supplementary Fig. S3E). In addition, comparable RNA levels for a panel of ER target genes including PGR and GREB1 were observed between MutL− and MutL+ human tumors from the TCGA dataset (Supplementary Fig. S3F), indicating that ER transcriptional activity is not significantly affected by defective MutL function. Because MLH1 re-expression rescued the endocrine therapy–sensitive phenotype of MLH1− cells, loss of MutL competence appears to have a direct and causal role in intrinsic endocrine therapy resistance rather than an indirect role through the induction of genome instability and evolution of secondary
mutations. This conclusion is supported by the rapidity with which resistance arises upon disruption of MutL function, because clonal selection of resistance mutations usually takes months or even years.

To understand the mechanism whereby MutL gene loss causes intrinsic endocrine therapy resistance, cell-cycle activity after fulvestrant treatment was determined by immunofluorescence (IF) for pHistoneH3 (mitotic marker) staining in vitro (Fig. 2E) and immunohistochemistry for Ki67 (proliferative marker) in xenograft tumors (Fig. 2F). Although basal cell-cycle profiles were comparable between shLuc and shMLH1 cells (Supplementary Fig. S3G), fulvestrant significantly inhibited proliferation of shLuc but not of shMLH1, shPMS1 or shPMS2 MCF7 and T47D cells (Fig. 2E; Supplementary Fig. S3H). Similarly, MCF7 shMLH1 xenograft tumors demonstrated no measurable inhibition of proliferation after fulvestrant treatment, unlike their shLuc counterparts (Fig. 2F). These data suggest that loss of any member of the MutL complex prevents the antiproliferative effects of ER blockade in ER+ breast cancer cells.

Two screens were performed to explore underlying mechanisms. The first approach used reverse phase protein array (RPPA) analysis, and the second compared gene expression of cell cycle–related genes in MCF7 MutL+ and MutL− cells. For both screens, cells were treated with either control or fulvestrant for 48 hours before harvest. Analysis of results identified that of 211 proteins screened in the RPPA array, the levels of 12 proteins were significantly different (depicted in Supplementary Fig. S4A), and of the 96 gene RNA levels assayed by qRT-PCR, 15 were significantly differentially regulated (shown in Supplementary Fig. S4B) by fulvestrant treatment in MutL− relative to MutL+ cells. Of these factors, CHK2 and p21 were the only two that were significantly downregulated in both screens in MutL− versus MutL+ cells after fulvestrant treatment (Fig. 3A).

MutL complex genes are known to activate CHK2 during MMR (21). We therefore proceeded to evaluate the role of CHK2 in mediating intrinsic endocrine therapy resistance. Downregulation of CHK2 in MutL+ relative to MutL− cells after fulvestrant treatment was observed in vitro by assessing levels of pCHK2 and its downstream effectors, p21 and p27, in MCF7/shLuc relative to either shMLH1 (Fig. 3B) or shPMS2 cells (Supplementary Fig. S4C). In addition to downregulation of pCHK2 and, more modestly, of p27/p21, upregulation of pRb was observed in MLH1+ versus MLH1− MCF7 xenograft tumors grown in the absence of estrogen (Fig. 3C).

In spite of the heterogeneity of PDX tumors (22), WHIM20 tumors (#20, PMS2 mutant) also exhibited an almost complete inhibition of both total CHK2 and pCHK2 protein levels after estrogen deprivation, and fulvestrant treatment, in contrast with a MutL−ER+ PDX tumor, WHIM16 (Fig. 3D). Both RPPA (Supplementary Fig. S4D) and mass spectrometry–based phosphoproteomics (ref. 22; Supplementary Fig. S4E) across PDX lines confirmed that WHIM20 tumors (−E) have downregulated pCHK2 and p-p27 at multiple phosphorylation sites. Finally, pCHK2 was significantly downregulated in primary MutL− ER+ breast tumors in TCGA RPPA data (Supplementary Fig. S5A), and mRNA from CDKN1A (p21) was upregulated after AI treatment in MutL+, but not in MutL−, tumors from the NeoAI dataset (Supplementary Fig. S5B).

**ATM/CHK2 Activation by the MutL Complex Is Required for Endocrine Therapy Response**

The data presented thus far are consistent with previously reported links between MMR signaling and CHK2 activation (23), and between ER signaling and CHK2 (24), presenting the hypothesis that MutL+ER+ breast tumors are less able to phosphorylate CHK2 in response to endocrine therapy thereby decreasing the strength of treatment-associated cell-cycle arrest (25). Previously published research suggests that MLH1 acts as a scaffold enabling ATM autophosphorylation and subsequent phosphorylation of CHK2 (21). To test this association in ER+ breast cancer cells, cellular localization of MLH1 and pCHK2 was analyzed after fulvestrant treatment in MCF7 MutL+ (shLuc and shMLH2) and MutL− (shMLH1 and shPMS2) cells. In response to fulvestrant treatment, both MutL− cell lines significantly increased MLH1 translocation to the nucleus where colocalization with pCHK2 was observed (Fig. 4A; Supplementary Fig. S5C). Unsurprisingly, shMLH1 cells did not upregulate MLH1 nuclear translocation (Supplementary Fig. S5C), but neither did shPMS2 cells (Fig. 4A; Supplementary Fig. S5C), supporting previously published evidence that the MutL complex requires heterodimerization to facilitate nuclear translocation (18). In corroboration, WHIM20 (PMS2 mutant) tumors had cytoplasmic but no detectable nuclear MLH1 on fulvestrant treatment, in contrast with MCF7 MutL+ xenograft tumors (Fig. 4A). Consistent with these data, nuclear colocalization of pATM and pCHK2 increased >4-fold (P < 0.001) in MutL+ cells after fulvestrant treatment but demonstrated no perceivable increase in MutL− (0.6-fold) MCF7 cells (Fig. 4B). The lack of pATM/pCHK2 foci in response to fulvestrant was also observed in MutL−, WHIM20 tumors where pATM was virtually undetectable, although attenuated pCHK2 nuclear foci were detected (Fig. 4B).

In support of these functional relationships, pooled siRNA used to transiently downregulate either ATM or CHK2 in MCF7 parental cells induced resistance to both fulvestrant and tamoxifen within 48 hours (Fig. 4C and D), although transient downregulation of ATM, but not CHK2, also inhibited baseline growth of MCF7 cells (Supplementary Fig. SSD). These results were confirmed in T47D cells (Supplementary Fig. S5E), where transient downregulation of either ATM or CHK2 significantly affected baseline growth (Supplementary Fig. S5G). Orthogonally, when ATM and CHK2 activation was inhibited pharmacologically (ref. 26; validated in Supplementary Fig. SSF), both MCF7 (Fig. 4E and F) and T47D (Supplementary Fig. S6A and S6B) cells lost the ability to respond to growth inhibition to fulvestrant treatment. This phenotype appeared specific to CHK2, as neither an ATR inhibitor (27) nor a CHK1 inhibitor (28) could induce endocrine treatment resistance (Fig. 4E and F; Supplementary Fig. S6A and S6B). Moreover, ATM/CHK2 activation using the small-molecule activator 3,3′-diindolyl methane (DIM; refs. 29, 30; validated in Supplementary Fig. SSE) rescued sensitivity of MCF7/MutL (Supplementary Fig. S6C and S6E) and T47D/MLH1 (Supplementary Fig. S6D and S6F) cells to endocrine treatment in a dose-dependent manner. These data suggest that CHK2 activation is both necessary and sufficient for MutL−-induced endocrine therapy resistance.
MutL, ATM, and CHK2 Regulate Endocrine Therapy Responsiveness

**RESEARCH ARTICLE**

MutL/CHK2-Dependent Inhibition of CDK4 Is Required for Response to Endocrine Therapy

Several lines of evidence support the hypothesis that the MutL/CHK2 axis regulates induction of a G1–S cell-cycle block when ER⁺ breast cancer cells are exposed to endocrine therapy. First, the two most significantly upregulated cell-cycle genes in MCF7 MutL− versus MutL⁺ cells after fulvestrant treatment were the cyclin-dependent kinase CDK4 and the cyclin CCND3 (Supplementary Figs. S5A and S7A), both integral for G1–S cell-cycle progression. Second, MutL/ER⁺ patient tumors from the NeoAI studies had increased levels of CDK4 and CCND3 mRNA relative to MutL⁺ tumors, and these levels did not significantly decrease upon AI treatment (Supplementary Fig. S7B). Third, and in support of previously published evidence (25, 29, 31, 32), a significant inverse correlation was observed between protein levels of pCHK2 and CDK4 in cells treated with CHK2 inhibitors and activators (Supplementary Fig. S7D). These lines of evidence are largely indirect, however, and could represent consequences of unchecked proliferation in MutL/ER⁺ breast tumors treated with endocrine therapy, rather than a causal occurrence. Therefore, we next tested whether the G1–S phase cyclin-dependent kinases CDK4 and CDK6 were critical...
for mediating endocrine therapy resistance in ER+ breast cancer cells.

When pooled siRNA was used to suppress CDK4 and CDK6 in MCF7 MutL+ and MutL− cells (Supplementary Fig. S7C), suppression of either gene singly resulted in partial rescue of endocrine response in the MutL+ cells (data not shown), but the combinatorial inhibition of both genes resulted in complete rescue of fulvestrant sensitivity in MCF7/MutL+ cells (Fig. 5B). The next step in the proposed genetic pathway, loss of CHK2, was also tested for its role in CDK4/6 regulation in ER+ breast cancer cells. Transient siRNA-induced suppression of CHK2 in MCF7 parental cells resulted in an immediate and significant increase in sensitivity to pharmacologic CDK4/6i (Fig. 5C), a result confirmed in T47D/MutL− cells (Supplementary Fig. S7E). These data suggested that loss of either MutL or CHK2 in ER+ breast cancer cells induces reliance on CDK4/6 upregulation for cell-cycle progression, resulting in resistance to endocrine therapy but sensitivity to CDK4/6i. Corroboratively, analysis of the response of 47 breast cancer cell lines to CDK4/6i with palbociclib (33)
indicated increased sensitivity to palbociclib in MutL−, but not MutS−, ER+ breast, and not in ER− breast cancer cell lines (Supplementary Fig. S7F).

MutL− ER+ Breast Tumors Are Sensitive to CDK4/6 Inhibition

To directly test whether MutL− ER+ breast cancer cell lines are sensitive to CDK4/6, both palbociclib and abemaciclib (ref. 34; validated in Supplementary Fig. S7G) were administered to MCF7 and T47D MutL− and MutL+ cells along with fulvestrant. Both palbociclib (Supplementary Fig. S7H) and abemaciclib (Fig. 5D; Supplementary Fig. S7I) profoundly and consistently inhibited 2-D growth of MutL− MCF7 and T47D cells. A striking difference in growth response in MutL− versus MutL+ cells was observed when comparing the effect of fulvestrant alone relative to the combinatorial use of CDK4/6i and fulvestrant.
along with fulvestrant treatment. In the case of MCF7 MutL\(^+\) cells, the combination of fulvestrant and abemaciclib induced approximately 50% growth inhibition relative to fulvestrant alone, whereas in MutL\(^-\) cells, the combination induced >80% growth inhibition over fulvestrant alone (Fig. 5D; \(P = 0.02\)). This difference was even more noticeable in T47D cells, where the combinatorial growth inhibition relative to fulvestrant alone went from approximately 25% in MutL\(^+\) to >50% in MutL\(^-\) cells (Supplementary Fig. S7I, \(P = 0.005\)). Abemaciclib, in combination with fulvestrant, also inhibited 3-D growth of MutL\(^+\) cells (Fig. 5E). Of note, because CDK2, another G1–S cyclin-dependent kinase, was also upregulated at RNA levels in the cell-cycle screen (Supplementary Fig. S7A), response to CDK2 inhibition in MutL\(^-\) cells was tested. In contrast to their response to CDK4/6 inhibitors, neither MCF7 nor T47d shMLH1 cells showed increased sensitivity to CDK2 inhibitors; rather, both MutL\(^+\) cell lines trended toward resistance to this inhibitor relative to MutL\(^+\) cells. IC\(_{50}\) for MCF7 shLuc cells was 429 nmol/L versus 3 μmol/L for shMLH1 cells (\(P = 0.05\)). Similarly, in T47D cells, IC\(_{50}\) for shLuc cells was 7 versus >50 μmol/L for shMLH1 cells (\(P = 0.04\)). These data suggested a specific role for CDK4/6 in inducing MutL\(^-\)-mediated intrinsic endocycle therapy resistance, and for CDK4/6 inhibitors in targeting MutL\(^-\) endocrine therapy-resistant tumors.

Three xenograft models were utilized for \textit{in vivo} validation of response to CDK4/6 inhibition. MCF7 MutL\(^+\) xenograft tumors regressed when treated with a combination of estrogen deprivation and palbociclib but not when treated with estrogen deprivation alone or with fulvestrant (Fig. 5F and G). Response of MutL\(^+\) xenograft tumors to palbociclib was significantly higher than MutL\(^+\) MCF7 xenograft tumors (Fig. 5F), although the effect size was moderate. CDK4/6i response was also validated in WHIM20 tumors, which demonstrated downregulation of pRb in response to a combination of fulvestrant and palbociclib treatment but not in response to fulvestrant alone (Fig. 5H). WHIM20 tumors also demonstrated significant inhibition of tumor growth in response to either palbociclib alone or the combination of palbociclib and fulvestrant (Fig. 5I). Finally, a second PDX model, HCI-005 with a frameshift mutation in \(\text{PMS1}\), also demonstrated endocycle therapy resistance and significant palbociclib sensitivity (Fig. 5I). These data together suggested that patients with MutL\(^+\) ER\(^+\) tumors can benefit from CDK4/6i despite intrinsic endocycle therapy resistance.

To obtain clinical data to support the postulate that MutL\(^-\) tumors are sensitive to CDK4/6i, data from the NeoPalAna trial were examined (17). In this study (Fig. 6A), 50 patients presenting with clinical stage 2/3 ER\(^+\)HER2\(^-\) breast cancer were treated with an AI, anastrozole, and biopsied after 1 month (C1D1). Palbociclib was then added to the treatment regimen with a further biopsy after approximately 2 weeks of combined treatment (C1D15). Patients then completed neoadjuvant treatment with the AI+CDK4/6i combination for approximately 16 weeks before surgery. Whole-exome sequencing and RNA expression analysis were conducted on tumors with sufficient material (Fig. 6B). The primary endpoint for this study was complete cell-cycle arrest (CCCA) defined as Ki67 < 2.7%. More tumors demonstrated CCCA after the combination of AI and CDK4/6i than after AI alone, confirming the activity of palbociclib in primary ER\(^+\) breast cancer.

First, the Ki67 response of tumors based on mutations in MutL and MutS genes alone was analyzed (Fig. 6C–E). To serve as rebinning points in the analysis, the MutL\(^+\) cases were divided into AI-sensitive and AI-resistant (AI-sensitive = Ki67 < 10% after 4 weeks of AI). When considering mutations alone, 6 MutS-mutant tumors were identified, 5 of which were AI-sensitive, including a tumor with a truncating mutation in \(\text{MSH5}\). The majority of the MutS-mutant tumors also demonstrated CCCA with AI alone, although the addition of CDK4/6i incrementally increased treatment efficacy (Fig. 6C and F). Four MutL-mutant tumors were identified (Fig. 6D). Consistent with our hypothesis, these tumors exhibited AI-resistant proliferation and none demonstrated CCCA with AI alone. However, in keeping with the experimental data presented above, all 4 MutL-mutant tumors demonstrated significant Ki67 inhibition when palbociclib was added, with all of them achieving CCCA (Fig. 6D). Importantly, tumors with inactivating (nonsense or frameshift) mutations in MutL genes demonstrated the strongest endocrine therapy–resistance phenotype and appeared most sensitive to CDK4/6i (Fig. 6D).

Next, dysregulation of MutL and MutS genes at both DNA and gene expression level (MutL\(^+\) and MutS\(^-\) groups) was determined as in previous analyses presented in Fig. 1 (described in Fig. 6F). Using the combined definition, 6 MutL\(^+\) and 6 MutS\(^-\) tumors were identified from 37 tumors examined (2 combined MutL and MutS deficient were excluded as not fitting a binary definition of deficiency). Again, 4 of the 6 MutS\(^-\) tumors were AI-sensitive, even exhibiting CCCA with AI alone (Fig. 6F), and only 1 of the tumors remained AI-resistant (Fig. 6E and F). In contrast, 3 of 6 MutL\(^+\) tumors were AI-resistant (Ki67 > 10%), and 0 of 6 MutL\(^-\) tumors demonstrated CCCA on AI alone (Fig. 6F). MutL\(^+\) tumors demonstrated mean Ki67 levels after AI treatment of 13%, comparable to MMR+/AI-resistant tumors (19%; \(P = 0.2\)) and significantly different from both MutS\(^-\) and MMR+/AI-sensitive tumors (4%; \(P = 0.003\)). However, upon addition of palbociclib, all 6 MutL\(^+\) tumors achieved CCCA, with mean Ki67 levels falling from 13% on AI treatment to 1.3% on the combination.

**DISCUSSION**

In this investigation, we delineate a pathway involving the MutL complex, along with ATM, CHK2, and CDK4/6 that is required for ER\(^+\)HER2\(^-\) tumors to respond to endocrine therapy (Fig. 6G). When components of this pathway are poorly expressed or lost through mutation, feedback control on CDK4/6 is defective. This allows the cell cycle to proceed toward resistance to this inhibitor relative to MutL\(^+\) cells. In contrast to their response to CDK4/6 inhibitors, neither MCF7 nor T47d shMLH1 cells showed increased sensitivity to CDK2 inhibitors; rather, both MutL\(^+\) cell lines trended toward resistance to this inhibitor relative to MutL\(^+\) cells. IC\(_{50}\) for MCF7 shLuc cells was 429 nmol/L versus 3 μmol/L for shMLH1 cells (\(P = 0.05\)). Similarly, in T47D cells, IC\(_{50}\) for shLuc cells was 7 versus >50 μmol/L for shMLH1 cells (\(P = 0.04\)). These data suggested a specific role for CDK4/6 in inducing MutL\(^-\)-mediated endocycle therapy resistance, and for CDK4/6 inhibitors in targeting MutL\(^-\) endocrine therapy-resistant tumors.

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In this investigation, we delineate a pathway involving the MutL complex, along with ATM, CHK2, and CDK4/6 that is required for ER\(^+\)HER2\(^-\) tumors to respond to endocrine therapy (Fig. 6G). When components of this pathway are poorly expressed or lost through mutation, feedback control on CDK4/6 is defective. This allows the cell cycle to proceed despite DNA mismatches, thereby promoting the growth of high mutation load ER\(^+\) breast cancers that are intrinsically resistant to endocrine treatment, but still sensitive to CDK4/6i.

The sequential triple biopsy design of the NeoPalAna trial was executed to determine the effects of CDK4/6i on ER\(^+\) HER2\(^-\) tumors where intrinsic endocrine therapy resistance had been demonstrated. Consistent with our model, CDK4/6i was uniformly effective in suppressing residual proliferation in MutL\(^+\) ER\(^+\) breast cancers, with almost undetectable Ki67.
MutL, ATM, and CHK2 Regulate Endocrine Therapy Responsiveness

Figure 6. MutL deficiency in ER+ breast cancer cells induces mismatches and activates the MMR pathway, which in turn activates CHK2 resulting in exit from the cell cycle. In the absence of either estrogen signaling or MutL complex activation, there is no/low CHK2 activation and cells do not respond to endocrine therapy.

levels after approximately 2 weeks of palbociclib exposure. In contrast, the degree to which each mutant MutL allele or expression level defect promoted AI-resistant proliferation was variable and likely dependent on the degree of dysfunction induced by the mutation or the degree of underexpression. A comprehensive predictive assay based on MutL gene status would have to combine expression analysis with mutation detection. A combination of somatic mutation detection and expression status is a reasonable proposition for a clinical assay, as both approaches have been adapted to routine...
pathologic material. Ultimately, large datasets from randomized trials of adjuvant CDK4/6i therapy that are currently under way will be required to dissect these relationships fully.

The efficacy of CDK4/6i in the advanced ER+ breast cancer setting has been validated in multiple clinical trials (35, 36), but as of yet there are no biomarkers to guide treatment. CDK4/6i are expensive, have more severe adverse events than endocrine therapy alone, and have to be administered chronically to control disease. Our findings open up new hypotheses for CDK4/6i predictive test discovery, which to date has not been successful (37). Although a weakness of our study concerns the sample size in the NeoPalAna study, the data are entirely consistent with our extensive preclinical investigation regarding the response of MutL+ tumors to CDK4/6i. Clearly, our work merits further correlative studies in the setting of both endocrine monotherapy studies and CDK4/6i combination studies with larger sample sizes and long-term outcomes to determine the prognostic and predictive impact of our findings. A further issue is that the MutL- mechanism clearly does not explain all cases of endocrine therapy-resistant yet palbociclib-sensitive ER+ breast cancer. Further exploration of the relationship between other DNA repair pathways and this phenotype may be fruitful in this regard.

The endocrine therapy sensitivity of MutS-ER+ breast cancers is an interesting and contrasting phenomenon compared with MutL- tumors. The molecular underpinnings of this gene selectivity is an important area for further research, and is perhaps linked to the canonical association of MLH1 with ATM activation and of MSH2 complex formation with ATR/CHK1 activation (21, 38). Consistent with this hypothesis, in this study, MutS- tumors appear to retain the ability to activate ATM and CHK2 in response to inhibition of ER. A further uncertainty is how ER is linked to ATM function. This has not been entirely delineated, but it has been proposed to be through ER-upregulated miR-18a and miR-106a (15). In this model, ER activation drives the miRNA-dependent loss of ATM function, allowing the cell cycle to proceed.

Despite the retention of endocrine therapy sensitivity in MutS- tumors, either MutL- or MutS- may promote secondary mutations though MMR defects that enhance the probability of acquired endocrine resistance. A potential incidence of this phenomenon is observed in WHIM20, where we have previously reported an ESRI ligand-binding domain mutation (Y537S) that causes endocrine therapy resistance (4). It is possible that the PMS2-ESR1 mutation in WHIM20 causes only a partial endocrine therapy resistance that is eventually made more profound due to the evolution of an ESRI gain-of-function mutation. It is clear from the relationships between Mutl status and Ki67 response that partial endocrine resistance is often seen, likely due to incomplete suppression of Mutl function. This possibility is illustrated by the HCl005 PDX model where, in the presence of wild-type ESRI but mutant PMS1, there is partial endocrine therapy resistance and significant palbociclib sensitivity. Even modest levels of persistent Ki67 activity despite AI therapy dramatically increase relapse risk (39).

The role of MMR in breast cancer has been underestimated for several reasons. First, there is only a weak relationship between germline defects in MMR genes and breast cancer susceptibility, although links to increased breast cancer risk have been observed particularly for families with defective MLH1 alleles (40). Second, our investigations on ER/HER2 breast cancer in the neoadjuvant setting illustrated here provide the only published datasets to date that can connect intrinsic endocrine therapy resistance and palbociclib to MMR gene mutation status. Third, an examination of classic markers of MMR dysregulation such as repeat expansion may be unhelpful in the breast cancer context, because MutL alleles are likely frequently hypomorphic, i.e., sufficient to be defective in CHK2 activation but having only relatively weak effects on promoting mutation load, unlike, for example, MMR-deficient in classic Lynch syndrome cancers such as endometrial and colorectal cancers (41). Simple immunohistochemistry that is typically used for the routine diagnosis of MMR in colorectal cancer will not identify Mutl, missense alleles with partially defective function.

Both CHK2 and ATM are well-established breast cancer susceptibility genes with low to medium penetrance (42). Interestingly, CHK2 germline variants specifically associate with increased incidence of ER+ tumors (43), suggesting that the role of CHK2 in connecting ER to the cell cycle highlighted in our investigation is a fundamental pathway that contributes to development of luminal tumors. Of concern is that endocrine drugs used for chemoprevention in patients with germline mutant MutL, CHK2, or ATM-related breast cancer might be less effective. However, we show that the CHK2 activator and dietary component DIM could provide an alternative agent in this setting, at least regarding the chemoprevention of MutL- and ATM-associated breast cancers.

METHODS

Cell Lines, Mice, CRISPR, si/shRNA Transfection, and Growth Assays

Cell lines were obtained from the ATCC (2015) and maintained and validated as previously reported (44). Mycoplasma tests were performed on parent cell lines and stable cell lines every 6 months (latest test: 05/17) with the Lonza Mycoalert Plus Kit (cat# LT07-710) as per the manufacturer’s instructions. Cell lines were discarded at c.5 passages, and fresh vials were thawed out. Key experiments were repeated with each fresh thaw. shRNA plasmids were purchased from Sigma-Aldrich. CRISPR plasmids were purchased from Santa Cruz Biotechnology. Transfection and validation of CRISPR and shRNA plasmids were conducted as previously (23). Transient transfection with siRNA against CHK2 was conducted as previously (44), and siRNA pools were purchased from Sigma-Aldrich. Stable cell lines were established in the presence of specified antibiotics at recommended concentrations. Growth assays were conducted in triplicate and repeated independently as previously using Alamar blue to identify cell viability (44). Growth assay results were plotted as fold change in growth from days 1 to 7, and normalized to vehicle control where specified. Three-dimensional growth assays were conducted over 4 to 6 weeks with weekly drug treatments as described previously (45). Tumor growth assays in vivo were carried out as described previously (46) by injecting 2 to 5 × 10^6 MCF7 cells into the L4 mammary fat pad/mouse. Mice for the MCF7 experiments were 4- to 6-week athymic nu/nu female mice (Envigo), and for the PDX experiments were 6- to 8-week female SCID/Bg mice, both from Charles River laboratory. Tumor volume was measured twice or thrice weekly using calipers to make 2 diametric measurements. Tumors were randomized for treatment at 50 to 150 mm³ volume. Tumors were harvested at <2 cm diameter and were embedded in paraffin blocks, OCT, and snap-frozen as described previously (47).
Mice that died within 3 weeks of tumor growth rate experiments were excluded from analysis. For all mouse experiments, investigator was blinded to groups and to outcomes. All mouse experiments were performed according to the Institutional Animal Care and Use Committee rules and regulations (protocol# AN-6934).

**Inhibitors and Agonists**

All drugs were maintained as stock solutions in DMSO, and stock solutions were stored at −80°C and working stocks at −20°C. Dose curves for cell line assays and at 70 mg/kg/day in chow (PD-0332991, cat# S1116; SelleckChem) were used at 100 nmol/L. Abemaciclib (LY2835219, cat# S7158; SelleckChem) and palbociclib hydrate (cat# C3742; Sigma-Aldrich), CHK1 inhibitor (PF-477736, stocks for all experiments. For mouse xenograft experiments, 17β-estradiol was purchased from Sigma-Aldrich (cat# E8875), main-tained in sterile, nuclease-free water, and diluted to obtain 10 mmol/L stocks for all experiments. For mouse xenograft experiments, 17β-estradiol was added to the drinking water twice a week at a final concentration of 0.8 μg/ml. (cat# E2758; Sigma). CHK2 Inhibitor II (cat# C3742; Sigma-Aldrich), CHK1 inhibitor (PF-477736, cat# S2904; SelleckChem), and CHK2 activator (3, 3-diiodotyrosine methane, cat# sc-204624; Santa Cruz Biotechnology) were used at 10 and 100 nmol/L concentrations, respectively. Abemaciclib (LY2835219, cat# S7158; SelleckChem) and palbociclib (PD-0332991, cat# S1116; SelleckChem) were used at 100 nmol/L; final concentrations for cell line assays and at 70 mg/kg/day in chow for tumor growth assays as described previously (46).

**Immunostaining, Comet Assay, and Microscopy**

IF was performed based on the manufacturer’s instructions. Cells were washed in PBS; fixed for 20 minutes at room temperature in 4% PFA; blocked for 1 hour at room temperature in 5% goat serum and 1% Triton X-100 in 1x PBS; incubated with primary antibody overnight at 4°C in goat serum and 1% Triton X-100 in 1x PBS antibody diluent; incubated with secondary antibody in diluent for 1 hour at RT; then placed on DAPI-containing mounting media (cat# P36935). Primary antibodies used include pHistoneH3 (Cell Signaling Technology; 1:500), pATM (cat# ab36810), pCHK2 (cat# 21997), and MLH1 (cat# WH0004292M2). Cells were treated with fluorverstatin for 48 hours before evaluation. Alkaline comet assay was performed as per the manufacturer’s instructions on a CometAssay Electrophoresis SystemII ( Trevigen; cat# 4250-050-IES). Calculation of nuclei with DNA damage was performed using CASPLab software (27) to calculate the ratio of DNA content in tail/head. Cutoffs for categorization were set at 0–0.5, 0.5–1, 1–3, and >3 for no, low, medium, and high DNA damage. Fluorescent images were captured with a Nikon microscope and quantified with ImageJ.

**Western Blotting, Gene Expression Array, RPPA, and Phosphoproteomics**

Western blotting was conducted as previously described (23). All antibodies were purchased from Cell Signaling Technology and used at 1:1,000 dilutions unless otherwise specified. Primary antibodies were incubated with the membrane overnight at 4°C and included pCHK2 (cat# 2197), total CHK2 (cat# 2662), p21 (cat# 2947), p27 (cat# 2552), MLH1 (1:2,000, Sigma-Aldrich; cat# WH0004292M2), PMS1 (1:2,000, Sigma-Aldrich; cat# WH0005378M1), PMS2 (1:2,000, Sigma-Aldrich; cat# SAB450223C), CDK2 (cat# 2546), CDK4 (cat# 12790), and CyclinD1 (cat# 2175, 1:5,000). The gene expression array (Qiagen) was used according to the manufacturer’s instructions. RPPA assays were carried out as described previously with minor modifications (48). Phosphoproteomics data and methodologies for analysis were performed also as described previously (22).

**Statistical Analysis**

ANOVA or Student t test was used for independent samples with normal distribution. Where distribution was not normal (assessed using Q-Q plots with the Wilk-Shapiro test of normality), either the Kruskal–Wallis or Wilcoxon Rank Sum test was used. All experiments were conducted in triplicate, and each experiment was duplicated independently ≥2 times. These criteria were formulated to ensure that results from each dataset were calculable within the range of sensitivity of the statistical test used. Databases used for human data mining are from publicly available resources: Oncomine, cbio (49), and COSMIC (50). Z1031/POL dataset was used with permission from the Alliance consortium. All patients provided informed consent, and studies were conducted according to ethical guidelines and with Institutional Review Board approval. Lists of DDR genes for initial analyses were obtained from the KEGG database, and the list of MMR genes was restricted to MLH1, PMS1, PMS2, MLH3, MSH2, MSH3, MSH4, MSH5, PMS2L3, and EXO1. MutL+ tumor from META-BRIC, TCGA, and Z1031/POL datasets was determined in a case list containing all ER+ sample IDs (6) based on gene expression less than mean ±1.5 x standard deviation and/or the presence of nonsilent mutations in MLH1, MLH3, PMS1, and PMS2. Mutations identified by next-generation sequencing in the NeoAI trials were validated previously (51), and mutations identified by TCGA whole-exome sequencing have been demonstrated to have approximately 95% validation efficacy (52). For the multivariate analysis, we analyzed 1,415 ER+ tumor samples, extracting mutation data from the cbio portal (53), and corresponding clinical data through Oncomine. Only samples with survival metadata were included in the analysis. RPPA gene expression, and survival data for TCGA samples were downloaded from cbio portal (49). Resistance in the Z1031/POL dataset was determined as previously (6). For combined NeoAI trial data analysis in Fig. 1E, a one-sided Wilcoxon Rank Sum test was used to confirm significance of change in Ki67 in MutL+ versus MutL+ tumors, and two-sided tests for all other analyses, with correction for multiple comparisons. For NeoPalAna trials, the combined RNA/mutation level analysis in Fig. 6F excluded all 4 tumors that had coincident mutations and/or downregulation of both MutL and MutS family members, including 2 tumors with MutS gene mutations (E284* and P295S in MSH5). All survival data were analyzed using Kaplan–Meier curves and log-rank tests. Proportional hazards were determined using Cox regression. Sample size for animal experiments was estimated using power calculations in R. P values were adjusted for multiple comparisons where appropriate using Benjamini–Hochberg. All graphs and statistical analyses were generated either in MS Excel or R (54) and edited in Adobe Photoshop or Illustrator.

**Disclosure of Potential Conflicts of Interest**

K. Hunt reports receiving a commercial research grant from Endomagnetics and is a consultant/advisory board member for Armada Health. C.-X. Ma is a consultant/advisory board member for AstraZeneca, Novartis, and Pfizer. M.J. Ellis is CEO at and has ownership interest (including patents) in Bioclassifier/PAM50 and is a consultant/advisory board member for AstraZeneca, Novartis, Pfizer, and Puma. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: S. Haricharan, K. Hunt, S.M. Kavurri, M.N. Bainbridge, C.X. Ma, M.J. Ellis


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Haricharan, N. Punturi, ...

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Haricharan, M. Anurag, J. Hoog, S. Huang, M.N. Bainbridge, C.X. Ma, M.J. Ellis

Writing, review, and/or revision of the manuscript: S. Haricharan, K.R. Holloway, J. Schmelz, C. Schmidt, J.T. Lei, V. Suman, K. Hunt, J.A. Olson Jr, S. Huang, S.M. Kavuri, M.N. Bainbridge, C.X. Ma, M.J. Ellis

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Punturi, K.R. Holloway, M. Anurag, J. Hoog, S. Huang, C.X. Ma, M.J. Ellis

Study supervision: S. Haricharan, C.X. Ma, M.J. Ellis

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References


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