AWARD NUMBER: W81XWH-18-1-0034

TITLE: Mismatch Repair Loss Renders ER+/HER2- Breast Cancer Susceptible to HER2/3 Inhibition

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CONTRACTING ORGANIZATION: Sanford Burnham Prebys Medical Discovery Institute

REPORT DATE: NOVEMBER 2021

TYPE OF REPORT: Final Technical Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
NOVEMBER 2021	Final	1MAR2018 - 14JUL2021

4. TITLE AND SUBTITLE

Mismatch Repair Loss Renders ER+/HER2- Breast Cancer Susceptible to HER2/3 Inhibition

5a. CONTRACT NUMBER
W81XWH-18-1-0034

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The objective of this proposed study is to investigate a role for HER2/3 activation in MutLdefective ER+ breast cancer progression and resistance to endocrine therapy. By targeting HER2/3 signaling and key nodes of adaptive kinome response, we aim to significantly improve patient disease-specific survival.

15. SUBJECT TERMS
HER2 inhibitors, endocrine treatment resistance, growth factor signaling, ER+ breast cancer, DNA damage repair, mismatch repair

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	19	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION

More than 70% of breast cancer is estrogen receptor positive (ER+) and is treated with endocrine therapy, which targets the ER-pathway. While the majority of patients respond to treatment, ~30% of patients are resistant. This resistant subset is a significant contributor to the >40,000 breast cancer-related deaths that occur every year in the US. Activation of HER signaling has been previously suggested to induce endocrine therapy resistance. The HER family of tyrosine kinase receptors consists of EGFR, HER2, HER3 and HER4, and they are all known oncogenes and growth promoters. However, clinical trials incorporating drugs targeting EGFR/HER2 and/or downstream signaling pathways (PI3K/AKT/mTOR) into endocrine treatment regimens have obtained mixed results. This failure is potentially explained by a lack of predictive biomarkers to demarcate patients most likely to benefit from such targeted therapies.

Recently, we identified that loss of mismatch repair (MMR), specifically of the MutL complex consisting of MLH1, PMS1 and PMS2 genes, causes endocrine therapy resistance in ER+ breast cancer cells. To identify more efficacious, preferably cytotoxic therapeutic targets in MutL-defective ER+ breast tumors, we performed a proteomics screen on MCF7 cells stably engineered to downregulate MLH1, PMS1 or PMS2 (shMLH1, shPMS1, shPMS2) collectively termed shMutL cells. The proteomic response of these cells to the endocrine therapy, fulvestrant (an ER degrader), differed from that of control (shLuc) MCF7 cells in one important, druggable way: shMutL cells upregulated HER2/3 signaling. This finding is completely novel and presents a unique opportunity to exploit existing HER inhibitors to successfully treat endocrine therapy resistant ER+ breast cancer patients using rational drug combinations.

While HER2/3 inhibitors have been recommended for endocrine therapy resistant ER+ breast tumors in the past, clinical trials suggest that only an undefined subset of patients respond to this treatment, indicating a critical need for stratification based on predictive biomarkers. In the proposed study, we will investigate a role for MutL in predicting response to HER2/3 inhibitors in up to 30% of endocrine therapy resistant ER+/HER2- breast cancer.

2. KEYWORDS

HER2 inhibitors, endocrine treatment resistance, growth factor signaling, ER+ breast cancer, DNA damage repair, mismatch repair

3. ACCOMPLISHMENTS

What were the major goals of the project?

Major goals of the project were to (a) Validate activation of HER2/3 signaling in MutL-defective ER+/HER2- breast cancer cells (b) Investigate HER2/3 activation and signaling mechanisms in MutL-deficient ER+ breast cancer (c) Test efficacy of HER inhibition in decreasing MutL-defective ER+ breast cancer growth on endocrine treatment.

What was accomplished under these goals?

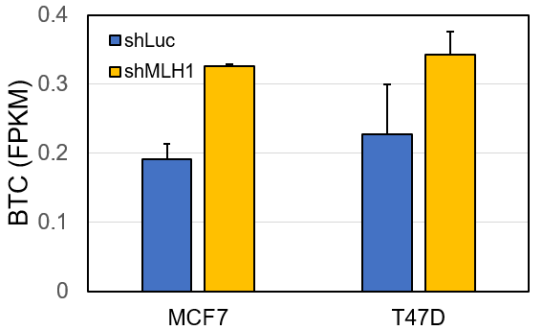
Year 1: Major Task 1 as outlined in the SOW was completed. Informatics analysis of patient tumors outlined in task 1.1 was conducted by Dr. Haricharan, surprisingly revealing that HER4, rather than HER3 is upregulated along with HER2 in ER+ patient tumors that are defective in mismatch repair (Fig 1+Fig S1B+E, Punturi et al, 2021). This finding was also confirmed in TCGA dataset (Fig 1A+FigS1A, C-D, F, Punturi et al, 2021). Accordingly, we pursued investigations into HER2 and HER4 co-upregulation in experimental model systems thereafter, rather than HER3 as initially outlined, with a main focus on HER2. As specified in task 1.2, IF (Fig 2D, Punturi et al, 2021) experiments were conducted in tumor sections from previously generated WHIM20 PDX line after fulvestrant treatment to optimize antibodies against HER2. HER3 was not assessed since there was no clinical correlations identified. Next as per task 1.3, IF was conducted after fulvestrant treatment in vitro

(Fig 3C, Fig S3D-E, Punturi et al, 2021). These data indicated significant co-upregulation of active pHer2 after fulvestrant treatment in MutL-defective ER+ breast cancer cells. The final task completed, as per the SOW was 2.1. Western blotting was conducted and confirmed upregulation of pHER2 specifically after fulvestrant treatment (Fig 2A, Fig S3A&F, Punturi et al, 2021).

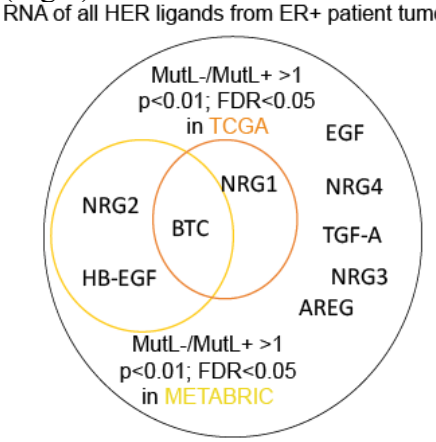
Year 2: Milestone#1 was in the process of being achieved in that a co-corresponding manuscript describing activation of HER2 in MutL- ER+ breast cancer cells was submitted and in review at *Science Translational Medicine.* In terms of experiments, two more MutL- PDX lines were identified and IF of these lines for HER2 and MLH1 were conducted (Fig 5D, Fig S7A-B, Punturi et al, 2021) to further support our hypothesis. Major task 3 subtask 1 (Fig 1, 3-4) was also completed and we identified BTC as one ligand that is upregulated in response to fulvestrant in shMLH1 ER+ breast cancer cell lines and xenograft tumors. These data were validated by analysis of patient tumors as well (Fig 2). Finally, major task 3 subtask 2 was completed and upregulation of HER2 in response to BTC was confirmed by immunofluorescence (Fig 5) and Western blotting (Fig 6).

Major Task 3 Subtask 1

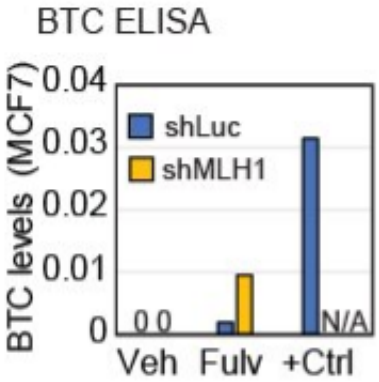
(Fig 1) RNAseq data for BTC in MCF7 (p=0.04) and T47D (p=0.03) shLuc and shMLH1 ER+ breast cancer cell lines after treatment with fulvestrant.



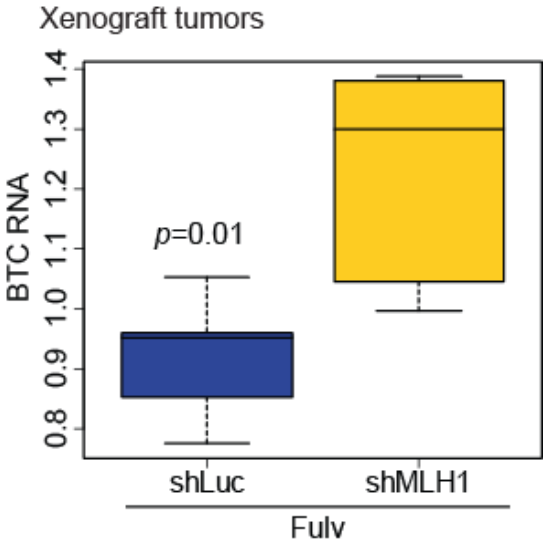
(Fig 2) Validation of BTC RNA upregulation in MutL- ER+ patient tumors from two independent datasets.



(Fig 3) ELISA for BTC in MCF7 shLuc and shMLH1 ER+ breast cancer cell lines.

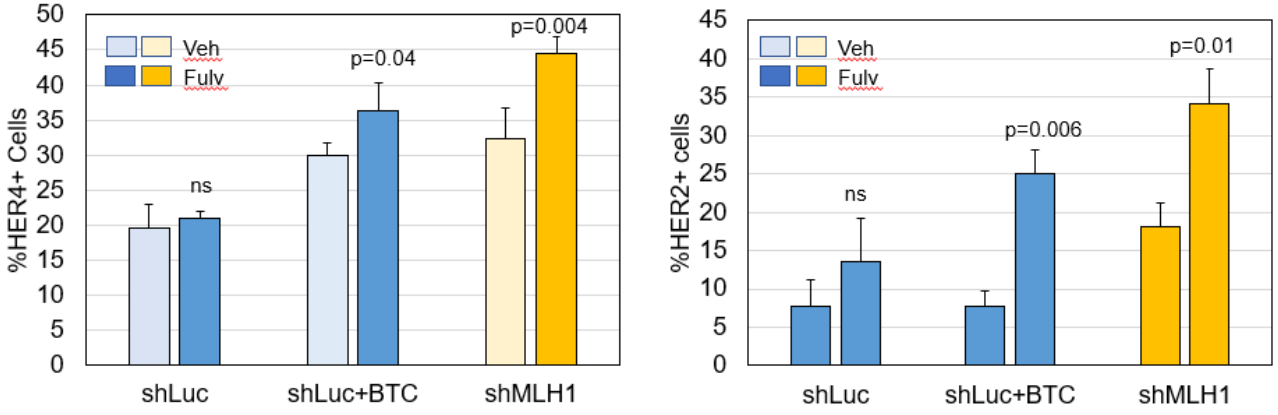


(Fig 4) qRT-PCR data validating upregulation of BTC RNA levels in MCF7 shMLH1 xenograft tumors after fulvestrant treatment.

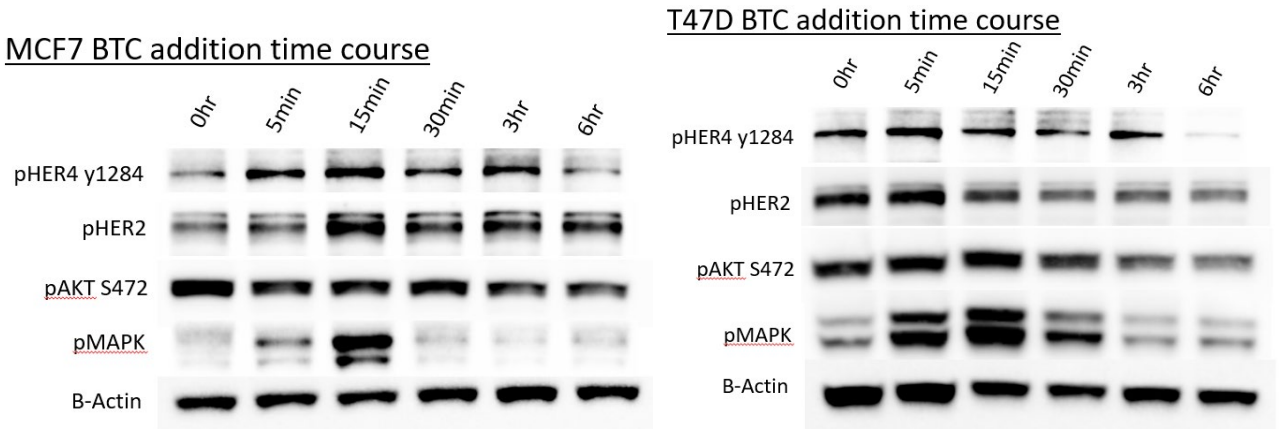


Major Task 3 Subtask 2

(Fig 5) Upregulation of HER2 in response to BTC by immunofluorescence in MCF7 cells.



(Fig 6) Upregulation of HER2 in response to BTC by Western blotting.



Year 3: Milestone#5 was achieved in that a co-corresponding manuscript describing efficacy of HER2 inhibitors in MutL- ER+ breast cancer cells was submitted and accepted for publication in *Nature Communications.* In response to reviewer comments, we conducted additional experiments demonstrating a role for autophagy and protein degradation in the upregulation of HER2 in the shMLH1 cells (see Fig 3 and Fig S4B-C, Punturi et al, 2021).

In terms of proposed experiments, all Major Tasks under Aims 1 and 3 have been completed and the results included in the manuscript mentioned above (Figs 4E&5A, Fig S5C-F&S6A). Aim 1 Major Task 2 is in the final phase of completion with our collaborator Dr. Fusco completing the IHC optimization on patient tumor samples currently. For Aim 2 Major Task 3, we have validated the upregulation of BTC, NRG-1, NRG-2 and HB-EGF in both MCF7 and T47D shMLH1 cells. We also downregulated HER2 in shMLH1 cells using RNAi to demonstrate the necessity of this heterodimerization for endocrine treatment resistance and sustained HER2 activation in shMLH1 cells (Fig 4A-D, Fig S5A-B, Punturi et al, 2021). For Aim 2 Major Task 4, Dr. Kavuri is in process of completing IP/Mass spec experiments to guide future experiments that build on the work proposed here.

Overall, in years 1-3, we have accomplished all the major goals and completed significant amounts of work towards the outstanding major tasks as demonstrated in the SOW reproduced below. We have generated a peer-reviewed publication (Punturi et al, 2021) and have co-authored several review articles (Mazumder et al, 2021; Sajjadi et al, 2021, and Venetis et al, 2020). Once our collaborator, Dr. Fusco completes IHC for MLH1 on patient tumor samples these results will be written up as a manuscript for peer-reviewed publication.

STATEMENT OF WORK	**Timeline** (Months)	**Partnering PI**	**Completion**
Specific Aim 1: Validate the ability of MutL-defective $ER^+/HER2^-$ breast cancer cells to activate HER2/3 signaling.			
Major Task 1: Implement IHC for HER proteins on patient tumors.			
Subtask 1: Bioinformatics analysis of patient tumor datasets Analysis of RNA and protein level data from Z1031, TCGA and METABRIC clinical trial data sets Groups: ER+/HER2 non-amplified tumors Proteins assayed: EGFR, HER2, HER3, HER4	7-10	Dr. Haricharan	Completed

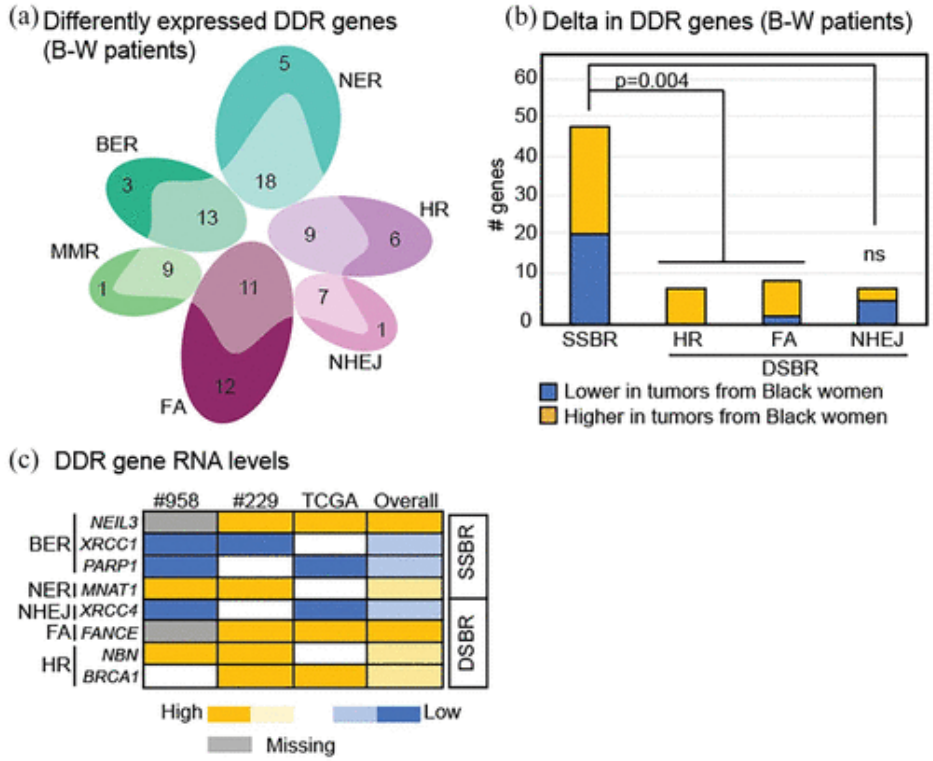
Subtask 2: Optimization of antibodies against HER2, HER3 and HER4 for IHC using xenograft tumor sections Groups: WHIM8 (HER2+), WHIM18 (HER2-) and WHIM20 (MutL-) tumors +/-E2 HCI-003 (HER2+) and HCI-005 (MutL-) tumors +/- E2 Proteins assayed: HER2, HER3, HER4	7-9	Dr. Haricharan	Completed
Subtask 3: Conduct Immunofluorescence on MCF7 and T47D shLuc and shMLH1 treated with or without endocrine interventions in vitro and in xenograft tumors. In vitro treatment groups: MCF7 and T47D shLuc cells+/-fulvestrant (1uM) in 10%FBS, shMLH1 cells+/-fulv (1uM) in 10% FBS In vivo treatment groups for MCF7 shLuc and shMLH1 xenograft tumors and WHIM20 xenograft tumors (already generated): Xenograft tumors +/-E2 and -E2+Fulv Proteins assayed: HER2, HER3, HER4	9-12	Dr. Haricharan	Completed
Subtask 4: IHC on patient tumor sections Participating teams: • Team A (Dr. Kavuri's lab): Oversee optimization and IHC • Team B (Pathology Core): Perform optimization, troubleshooting and IHC Tumor sections used from Z1031 and P024 clinical trial data Groups: Baseline and post-neoadjuvant aromatase inhibitor treatment Proteins assayed: HER3, HER4	5-12	Dr. Kavuri	Ongoing (Optimization was completed, our collaborator Dr. Fusco is completing the patient tumor studies currently)
Milestone #1: Corresponding author brief report on frequency of HER2/3/4 activation in MutL-deficient ER$^+$ breast cancer.	14	Dr. Haricharan / Dr. Kavuri	Ongoing
Major Task 2: Validate differences in HER activation *in vitro*			
Subtask 1: Perform Western blotting on MCF7 and T47D shLuc and shMLH1 cells treated with or without endocrine interventions in vitro Treatment groups: MCF7 and T47D shLuc cells+/-fulvestrant (1uM) in 10%FBS, shMLH1 cells+/-fulv (1uM) in 10% FBS Proteins assayed: EGFR, HER2, HER3, HER4, MAPK, AKT (phosphor- and total antibodies), ER, GAPDH	10-12	Dr. Haricharan	Completed
Subtask 2: Generate MutL-defective xenograft tumors and perform Western blotting for HER family members Treatment groups for WHIM20: +/-E2 and -E2+Fulv Proteins assayed: EGFR, HER2, HER3, HER4, MAPK, AKT (phosphor- and total antibodies), ER, GAPDH	12-15	Dr. Haricharan	Completed
Specific Aim 2: Investigate HER2/3 activation and signaling mechanisms in MutL-deficient ER$^+$ breast cancer.			
Major Task 3: Conduct "secretome" analysis			
Subtask 1: Document ligand secretion by qRT-PCR and ELISA for all HER family ligands in MCF7 and T47D shLuc and shMLH1 cells with or without endocrine intervention Cell lines (MCF7 and T47D shLuc and shMLH1)	15-20	Dr. Haricharan	Completed

Treatment groups: Veh, 1uM fulvestrant in 10% FBS Assays used: qRT-PCR for 10 HER ligands ELISA for 10 HER ligands			
Subtask 2: Functionally validate role of ligands in changing growth factor receptor composition by IF. Cell lines (MCF7 and T47D shLuc and shMLH1) PDX tumors (WHIM20, WHIM18, WHIM8 from Dr. Ellis) Treatment groups: In vitro → veh, fulv (1uM) in 10% FBS In vivo → +E2, -E2, -E2+Fulvestrant	20-24	Dr. Haricharan	Completed
Subtask 3: Functionally validate role of ligands in conditioned media experiments. Cell lines (MCF7 and T47D shLuc and shMLH1) Treatment groups: Vehicle, Fulvestrant (1uM) in 10% FBS	24-26	Dr. Haricharan	Completed
Subtask 3: Validate necessity and sufficiency of ligand secretion in endocrine response of MutL- cells. Cell lines (MCF7 and T47D shLuc and shMLH1) Growth assays: 2D, 3D soft gar and Matrigel growth in MCF7 and T47D shLuc and shMLH1 cells +/-fulvestrant (1uM), CSS+/-E2 Sufficiency assays: +/- BTC (three concentration dose curve) in MCF7 and T47D shLuc cells+/-fulvestrant (1uM) in 10% FBS Necessity assays: +/- BTC neutralizing antibody in MCF7 and T47D shMLH1 cells +/-fulvestrant (1uM) in 10% FBS	26-28	Dr. Haricharan	2D assays completed, 3D assays ongoing
Major Task 4: Conduct "interactome" analysis			
Subtask 1: Uncover HER-interactome in MutL-deficient ER^+ breast cancer cells. Cell lines (MCF7 shLuc and shMLH1) PDX tumors (WHIM20, WHIM18, WHIM8) Proteomics assays: immunoprecipitation Western blots using HER2, HER3 and HER4 antibodies In vitro treatment groups: MCF7 shLuc and shMLH1 cells +/-fulvestrant (1uM) in 10% FBS In vivo treatment groups: WHIMs 8, 18, and 20 +/-E2	13-16	Dr. Kavuri	Partially completed (in vitro experiments were completed but had no conclusive outcome so in vivo experiments were not pursued)
Subtask 2: Uncover HER-interactome in MutL-deficient ER^+ breast cancer cells. Cell lines (MCF7 shLuc and shMLH1) PDX tumors (WHIM20, WHIM18, WHIM8) Proteomics assays: Immunoprecipitation/mass spectrometry In vitro treatment groups: MCF7 shLuc and shMLH1 cells +/-fulvestrant (1uM) in 10% FBS Validation in vivo treatment groups: WHIMs 8, 18, and 20 +/-E2	16-22	Dr. Kavuri	Ongoing
Subtask 3: Functionally validate HER-interactome in MutL-deficient ER^+ breast cancer cells. Assays: Growth under 2D, soft agar and 3D conditions Treatment groups: Vehicle, 1uM fulvestrant in 10% FBS, CSS+/- 1nM estradiol Cell lines (MCF7 and T47D shLuc and shMLH1cells)	22-24	Dr. Kavuri	Not completed (will be completed based on results of subtask 2)

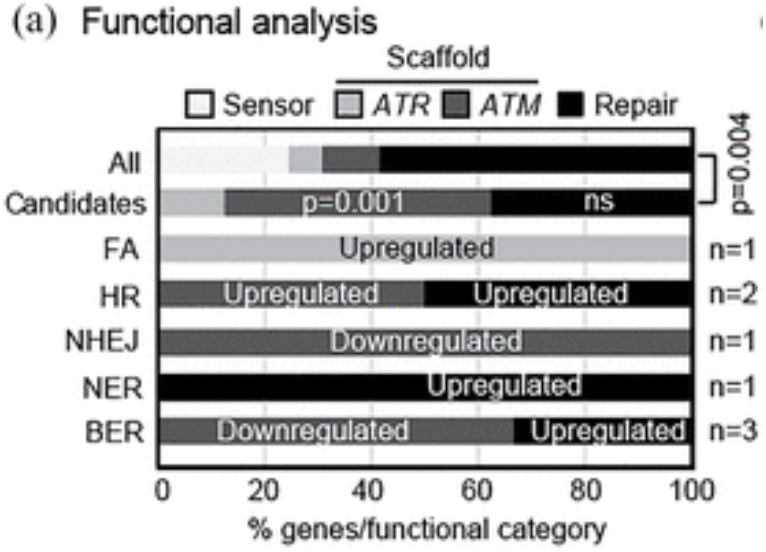
Milestone #2: Corresponding author manuscript on HER ligand secretome of MutL-defective ER$^+$ breast cancer cells [Dr. Haricharan] *Milestone #3: Corresponding author manuscript on HER interactome in MutL-defective ER$^+$ breast cancer cells [Dr. Kavuri]*	28-30	Dr. Haricharan / Dr. Kavuri	Not completed
Specific Aim 3: Test efficacy of HER inhibition in MutL-defective ER$^+$ breast cancer.			
Major Task 5: Establish sensitivity of MutL-defective cells to HER inhibitors			
Subtask 1: Test necessity of HER2/3/4 for growth and endocrine therapy resistance of MutL-defective ER$^+$ breast cancer cells Assays: 2D, soft agar and 3D growth after transient transfection with siRNA against HER2/3/4, and after stable infection with CRISPR Treatment groups: Vehicle, 1uM fulvestrant in 10% FBS, CSS+/- 1nM estradiol Cell lines (MCF7 and T47D shLuc and shMLH1 cells)	28-32	Dr. Haricharan	Completed
Subtask 2: Conduct dose curves with HER family pharmacological inhibitors and monoclonal antibodies Assays: 2D, soft agar and 3D growth Treatment groups: Vehicle, 1uM fulvestrant in 10% FBS, CSS+/- 1nM estradiol Cell lines (MCF7 and T47D shLuc and shMLH1 cells)	25-30	Dr. Kavuri	Completed
Subtask 3: Test necessity for HER2/3/4 for growth and endocrine therapy resistance of MutL-defective ER+ breast cancer cells Assays: Tumor growth after stable infection with CRISPR against candidate HER proteins (4 groups with 8 groups/mice=32 mice) Treatment groups: +E2, -E2 Cell lines (MCF7 shMLH1 cells+/- CRISPR-HER)	32-36	Dr. Haricharan	Ongoing (cell lines have been made and in vitro assays have been completed, in vivo expts are ongoing)
Subtask 4: Conduct PDX experiments with HER family pharmacological inhibitors Assays: Tumor growth rate (30 mice/expt with 10 mice/group in 3 treatment groups) Treatment groups: +E2, -E2, -E2+HER inhibitor Xenograft lines (WHIM20 and HCI-005)	30-36	Dr. Kavuri	Completed
Milestone #4: Joint R01 grant proposal to study MMR-HER connection in colorectal and bladder cancer. *Milestone#5: Joint co-corresponding author manuscript on efficacy of HER therapy in MutL-deficient ER+ breast cancer.*	38	Dr. Haricharan / Dr. Kavuri	Completed

The milestone tasks proposed by Initiating PI, Dr. Haricharan, were completed or ongoing to be completed shortly. Partnering PI, Dr. Kavuri at Baylor College of Medicine is still working on completing the major tasks associated with Aim 2. These delays did not impact Dr. Haricharan's ability to complete the proposed major tasks at SBP.

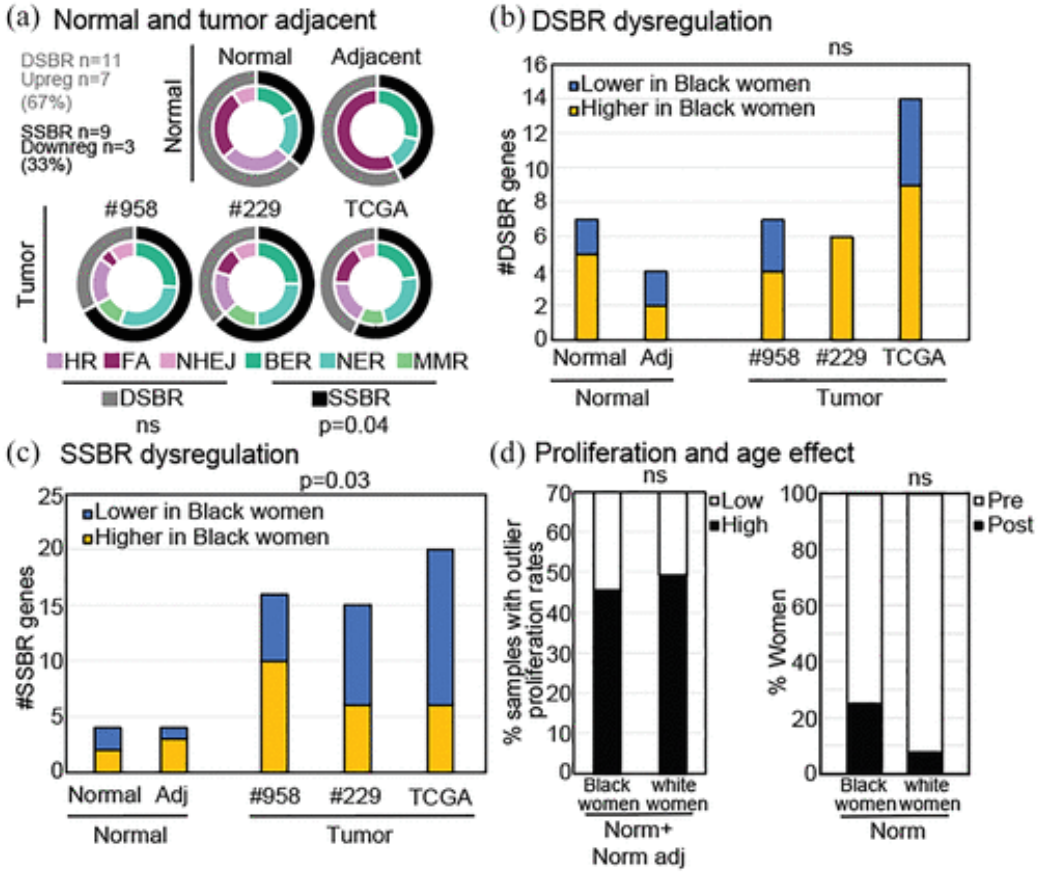
In addition, during the COVID lockdown, Dr. Mazumder, the postdoctoral trainee on this grant, worked on informatics analysis of the DNA damage repair landscape in breast cancer cells from black women. This analysis also resulted in a publication (Mazumder et al, 2022). Dr. Mazumder contributed to the following figures in the manuscript:



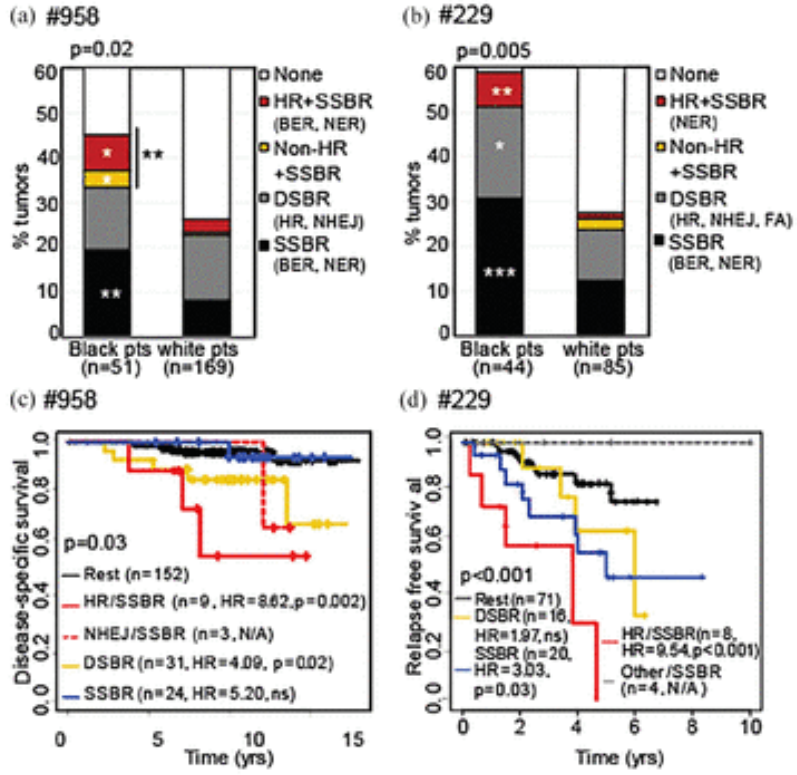
(Fig 7) Panels from Fig 2 of the published manuscript demonstrating difference in DNA repair gene expression in ER+ tumors from black women compared to those from white women.



(Fig 8) Panel from Fig 3 of the published manuscript demonstrating that those DDR genes that are differently expressed in ER+ tumors from black women compared to those from white women are enriched for functions as cell cycle checkpoint kinase scaffolds.



(Fig 9) Panels from Fig 4 of the published manuscript demonstrating that DDR genes are also differently expressed in the normal breast tissue of black vs white women, and these differences are not fully explained by proliferation rates of normal cells or by the age of the women studied.



(Fig 10) Panels from Fig 5 of the published manuscript demonstrating that dysregulation of those DDR genes that are differently expressed in the ER+ breast tumors of black vs white women associates with significantly worse

outcomes in multiple independent patient datasets.

What opportunities for training and professional development has the project provided?

The work we've done on this project was presented at the SABCS at a Spotlights session in 2020 and at the GRASP poster highlight session in 2021. The paper we published in Nature Communications on this project was also highlighted by the Nature Publishing Group on their website and was reported on by several news channels. The publication of the paper also resulted in several invited talks including the Scholars in Cancer lecture series (U Conn, 2022) and the UT Southwestern Komen Breast Cancer Forum (2022). Finally, the work allowed us to establish several collaborations with Drs. Nicola Fusco and Konstantinos Venetis at the IRCCS European Institute of Oncology, and with Dr. Stephen Shiao at Cedar Sinai in LA. Together, they plan to conduct a preliminary Phase II clinical trial to test efficacy of HER2 inhibition in combination with endocrine therapy in MutL- ER+/HER2- breast cancer patients. They are applying for an internal grant to provide preliminary supportive data for this project, as well as Department of Defense Breakthrough Level 3 grant to support further studies and the proposed clinical trial. The collaboration with Dr. Fusco has also resulted in Dr. Haricharan being invited to serve as a review editor for Frontiers in Oncology, a peer-reviewed journal. In addition, the informatics paper published in 2022 garnered a lot of media attention, being highlighted in major news outlets including Metro UK, USA Today and BBC World. These publications have therefore allowed Dr. Haricharan to establish expertise in the fields of DNA repair and breast cancer signaling.

The Sanford Burnham Prebys Medical Discovery Institute (SBP) Office of Education, Training & International Services (OETIS) oversees and coordinates an annual individual development planning (IDP) process for all postdocs at the Institute. The focus of the IDP process at SBP is the career goal of the postdoc; identification of what skills, knowledge, and accomplishments will be necessary for the postdoc to obtain a desired independent position following training; and identification of training and professional development opportunities that are available for the postdoc to obtain the necessary skills and knowledge. The SBP Office of Education, Training & International Services provides guidance and advising to both postdocs and PIs throughout the postdoc's training with respect to developing IDPs and preparing for a successful transition to independence post-training. The SBP Office of Education, Training & International Services also maintains webpages containing comprehensive resources on career path identification, career planning, and creating an IDP that can be utilized in conjunction with the formal annual IDP process.

The SBP IDP process includes two components:

I) <u>First-Year IDP (effective in 2014)</u>. Within the first 3 months of beginning postdoctoral training at SBP, all postdocs receive and fill out an initial "planning and expectations" document to discuss with their PI. This document serves as the foundation for their postdoctoral IDP and is designed to facilitate discussion between the PI and new postdoc regarding goals and expectations for the first year of training, as well as stimulate initial discussions about long-term career goals and training plans.

II) <u>Postdoctoral IDP (effective January 2013)</u>. At the end of the first year of training SBP postdocs receive notification that it is time to update their IDP, and they receive the information they included in their first-year planning and expectations document in the form of a full IDP that they can update with their accomplishments over the past year and their goals for the coming year, mid-term future, and long-term future. Each subsequent year of their postdoctoral training, postdocs will receive notification and the previous year's IDP form to update and expand. The IDP forms are designed to build upon each previous year as well as provide a solid foundation from which a postdoc can easily build his or her CV/resume.

During the three years of funding I participated in Parts 1&2) of the IDP process with Aloran Mazumder.

How were the results disseminated to communities of interest?

In the past year, Dr. Haricharan has presented some of this work in two SBP Spotlight events. In 2020, Dr. Haricharan was an invited speaker at the Fleet Science Lecture series at the San Diego Museum of Science, and

the Komen Race for the Cure Inauguration ceremony where she presented some of her work to the general public. Further, Dr. Haricharan and her lab participated in an interview for ABC describing recent breakthroughs in breast cancer research (https://abc30.com/5347545/) and in the Komen Race for the Cure Public Service Announcement aired on CBS (https://www.youtube.com/watch?v=p2AzReqsmB8&feature=youtu.be).
She has also been featured along with her patient advocate, Ms. Karen McDonald for Breast Cancer Awareness month (https://www.sbpdiscovery.org/news/beaker-blog/how-a-breast-cancer-advocate-shapes-research-at-sanford-burnham-prebys) and had her work featured on the Nature Communications website for Breast Cancer Awareness month (https://www.nature.com/collections/ehagdbgjgi). Additionally, the informatics analysis on DNA repair in breast tumors from black women was highlighted by several media outlets including a featured segment on BBC World Radio (https://www.bbc.co.uk/programmes/w3ct1nwl) and several pieces highlighting the work in New Scientist, USA Today, Metro UK, Huffington Post etc. It also led to Dr. Haricharan participating in a live stream event held by the patient advocacy group for black breast cancer patients, Touch BBCA (https://www.facebook.com/BlackDoctor.org/videos/464668028471717/?extid=NS-UNK-UNK-UNK-IOS_GK0T-GK1C&ref=sharing).

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

We have demonstrated a novel mechanism by which HER2 is activated in DNA repair defective ER+ breast cancer cells with potential to change therapy in the clinic. We have also identified differences in DNA repair gene expression in the tumors from black and white women. This study is a seminal proof of concept of differences in the molecular biology of breast cancer in black women that could result in poor outcome on standard therapies.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Breast cancer patients with MMR-defective ER+ disease might now be candidates for HER2 inhibitor therapy. An ongoing collaboration aims to conduct a proof of concept clinical trial to test this therapeutic hypothesis. The second paper also highlights the importance of streamlining diagnostics and therapeutics for different demographics of breast cancer patients.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change.

None.

Actual or anticipated problems or delays and actions or plans to resolve them.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

6. PRODUCTS

Publications, conference papers, and presentations.

Publications:

1. Punturi, N., Seker, S., Devarakonda, V., Mazumder, A., Kalra, R., Chen, C.H., Li, S., Primeau, T., Ellis, M.J., Kavuri, S.M., Haricharan, S. "Mismatch repair deficiency predicts response to HER2 blockade in HER2- negative breast cancer." *Nature Communications* (2021).
2. Mazumder A, Jimenez A, Ellsworth RE, Freedland SJ, George S, Bainbridge MN, Haricharan S. "The DNA damage repair landscape in Black women with breast cancer." *Therapeutic Advances in Medical Oncology* (2022).
3. Mazumder A, Shiao SL, Haricharan S. "HER2 Activation and Endocrine Treatment Resistance in HER2-negative Breast Cancer". *Endocrinology* (2021).
4. Sajjadi E, Venetis K, Piciotti R, Invernizzi M, Guerini-Rocco E, Haricharan S, Fusco N. "Mismatch repair-deficient hormone receptor-positive breast cancers: Biology and pathological characterization". *Cancer Cell International* (2021).

Presentations: Abstracts accepted for a short poster talk at Gordon Research Conference (2018), poster presentation at AACR (2020), poster presentation at SABCS 2020 (Spotlight session) and 2021 (GRASP poster highlights session), and for invited talks at Komen Metastatic Breast Cancer Conference panels in 2019, 2021 and 2022. Research presented in invited talks at many research institutions including the University of Milan (Italy), Scholars in Cancer lecture series (U Conn), and Komen Breast Cancer Forum (UT Southwestern).

Website(s) or other Internet site(s).

Nothing to Report.

Technologies or techniques.

Nothing to Report.

Inventions, patent applications, and/or licenses.

1. Invention Disclosure 21-007, IDENTIFICATION OF PROGNOSTIC BIOMARKERS OF POOR OUTCOME SPECIFIC TO AFRICAN AMERICAN BREAST CANCER PATIENTS

2. Provisional Patent Application No. 63/106,777, filed 10/28/20202 - PROGNOSTIC BIOMARKERS FOR BREAST CANCER

Other products.

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Haricharan, Svasti, Principal Investigator – 0.91 person months
Punturi, Nindo, Research Assistant – 12 person months
Mazumder, Aloran, Postdoctoral Associate – 1.80 person months

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Baylor College of Medicine, Dr. Kavuri, Partnering PI

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

9. APPENDICES

- Award Chart
- Award Expiration Transition Plan

BC170276: Mismatch Repair Loss Renders ER+/HER2- Breast Cancer Susceptible to HER2/3 Inhibition

PI: Svasti Haricharan, Sanford Burnham Prebys Medical Discovery Institute, CA **Budget:** $474,593

Topic Area: Breast Cancer Research Program **Mechanism:** Breakthrough Award Levels 1 & 2

Research Area(s): 0103; 0502 **Award Status:** 01 March 2018 – 14 July 2021

Study Goals: The goals of the project were to (a) Validate activation of HER2/3 signaling in MutL-defective ER+/HER2- breast cancer cells; (b) Investigate HER2/3 activation and signaling mechanisms in MutL-deficient ER+ breast cancer; (c) Test efficacy of HER inhibition in decreasing MutL-defective ER+ breast cancer growth on endocrine treatment.

Specific Aims: The aims of the project were (1) Validate the ability of MutL-defective ER+/HER2- breast cancer cells to activate HER2/3 signaling; (2) Investigate HER2/3 activation and signaling mechanisms in MutL-deficient ER+ breast cancer; (3) Test efficacy of HER inhibition in MutL-defective ER+ breast cancer.

Key Accomplishments and Outcomes:

Publications:

1. Punturi, N., Seker, S., Devarakonda, V., Mazumder, A., Kalra, R., Chen, C.H., Li, S., Primeau, T., Ellis, M.J., Kavuri, S.M., Haricharan, S. "Mismatch repair deficiency predicts response to HER2 blockade in HER2- negative breast cancer." Nature Communications (2021).
2. Mazumder A, Jimenez A, Ellsworth RE, Freedland SJ, George S, Bainbridge MN, Haricharan S. "The DNA damage repair landscape in Black women with breast cancer." Therapeutic Advances in Medical Oncology (2022).
3. Mazumder A, Shiao SL, Haricharan S. "HER2 Activation and Endocrine Treatment Resistance in HER2-negative Breast Cancer". Endocrinology (2021).
4. Sajjadi E, Venetis K, Piciotti R, Invernizzi M, Guerini-Rocco E, Haricharan S, Fusco N. "Mismatch repair-deficient hormone receptor-positive breast cancers: Biology and pathological characterization". Cancer Cell International (2021).
5. Mazumder, A., Jimenez, A., Ellsworth, R.E., Freedland, S., George, S., Bainbridge, M., Haricharan, S. "Race specific differences in DNA damage repair dysregulation in breast cancer and association with outcome." bioRxiv (2020).

Patents: US Provisional 63/106777 ("IDENTIFICATION OF PROGNOSTIC BIOMARKERS OF POOR OUTCOME SPECIFIC TO AFRICAN AMERICAN BREAST CANCER PATIENTS")

Funding Obtained: Award In Process – Department of Defense Log #BC210783 (Breast Cancer Research Program; Expansion Award)

Transition Plan Questionnaire

Directions: Please answer all questions that apply for each product under development. Please fill out one document per product.
This is not an application for funding; however, answers will help us understand the outcomes and products from your award.

1. After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? **Yes** ☑ or **No** ☐
These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.

2. What **conclusion(s)** does your final data support?

Our data support the conclusion that loss of MLH1 in ER+/HER2- breast cancer cells reactivates HER2 signaling to serve as an alternate pro-growth stimulus that enables these cells to evade endocrine therapy, which is standard of care for these patients. However, this finding also implies that loss of MLH1 in ER+/HER2- breast cancer cells makes them dependent on HER2 when exposed to endocrine therapy. Therefore, MLH1 loss can serve as a predictive biomarker for the combinatorial use of endocrine therapy and HER2 inhibition for ER+/HER2- breast cancer patients.

3. Will you/have you applied for/obtained follow-on-funding for this project? **If yes**, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. *This information will be recorded as an outcome to this award*.

Yes, we have obtained follow-on funding through a Department of Defense Expansion award requested for $974,997 (total costs). The funded proposal is titled "Mismatch repair loss, the immune environment and the breast cancer secretome".

4. What will be **the next step(s)** for this project?

We are in process of applying for NCI R01 funding to further understand the mechanistic implications of this funding, and DoD Level 3 funding to support a clinical trial to test our therapeutic hypothesis.

5. How would you classify your **lead candidate product**? b
(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy): Please choose, if applicable
(b) Diagnostic
(c) Device
(d) Research Tool to Address a Research Bottleneck
(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)
(f) Other - Please Specify:

6. How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

Our candidate product allows the use of MLH1 loss as a diagnostic to predict a better precision medicine alternative for a subset of ER +/HER2- breast cancer patients who are most likely to be resistant to standard of care. ER+/HER2- breast cancer is one of the most common malignancies diagnosed in warfighter, veteran, beneficiary and general populations, and is a leading cause of cancer-related death. By identifying better therapeutics to treat these women, thousands of lives could be saved.

7. Therapy / Product Development, Transition Strategies, and Intellectual Property

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.

We require funding to conduct a proof of concept clinical trial to demonstrate that MLH1 loss can, indeed, predict response to the combination of endocrine therapy and HER2 inhibition. If the results of this clinical trial are positive, then we will proceed towards a multi-institutional large-scale clinical trial to validate these findings. Since both the therapeutics and the clinical diagnostic are FDA-approved for cancer patients, once the clinical trials are completed, the results could directly impact the treatment options for ER +/HER2- breast cancer patients, with no further approvals required or regulatory constraints.

nature COMMUNICATIONS

ARTICLE

https://doi.org/10.1038/s41467-021-23271-0 OPEN

Mismatch repair deficiency predicts response to HER2 blockade in HER2-negative breast cancer

Nindo B. Punturi[1], Sinem Seker[1], Vaishnavi Devarakonda[2,3], Aloran Mazumder[1], Rashi Kalra[2,3], Ching Hui Chen[2,3], Shunqiang Li[4], Tina Primeau[4], Matthew J. Ellis[2,3], Shyam M. Kavuri[2,3]✉ & Svasti Haricharan[1]✉

Resistance to endocrine treatment occurs in ~30% of ER$^+$ breast cancer patients resulting in ~40,000 deaths/year in the USA. Preclinical studies strongly implicate activation of growth factor receptor, HER2 in endocrine treatment resistance. However, clinical trials of pan-HER inhibitors in ER$^+$/HER2$^-$ patients have disappointed, likely due to a lack of predictive biomarkers. Here we demonstrate that loss of mismatch repair activates HER2 after endocrine treatment in ER$^+$/HER2$^-$ breast cancer cells by protecting HER2 from protein trafficking. Additionally, HER2 activation is indispensable for endocrine treatment resistance in MutL$^-$ cells. Consequently, inhibiting HER2 restores sensitivity to endocrine treatment. Patient data from multiple clinical datasets supports an association between MutL loss, HER2 upregulation, and sensitivity to HER inhibitors in ER$^+$/HER2$^-$ patients. These results provide strong rationale for MutL loss as a first-in-class predictive marker of sensitivity to combinatorial treatment with endocrine intervention and HER inhibitors in endocrine treatment-resistant ER$^+$/HER2$^-$ breast cancer patients.

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Estrogen receptor positive (ER$^+$) breast cancer is one of the most common cancers in women worldwide[1]. ER$^+$ breast cancer patients are treated with endocrine therapy, which interrupts ER signaling[2]. A subset of ER$^+$ breast tumors also amplify the tyrosine kinase receptor and oncogene, HER2[3,4]. These ER$^+$/HER$^+$ breast cancer patients are less responsive to endocrine therapy but respond extremely well to combinatorial treatment with HER inhibitors, a seminal discovery[5]. However, the majority of ER$^+$ breast cancer is HER2$^-$ at diagnosis, and while ~70% of ER$^+$/HER2$^-$ breast cancer patients respond well to endocrine therapy, ~30% of patients become resistant to endocrine treatment resulting in relapse, metastasis, and death[2,6].

The discovery that HER2 amplification induces endocrine therapy resistance in ER$^+$ breast cancer spurred research into other means of HER2 activation. These studies identified mutation and phosphorylation as mechanisms by which ER$^+$ HER2 non-amplified (henceforth referred to as ER$^+$ HER2$^-$) breast cancer cells could activate HER2 signaling to resist endocrine treatment[4,7]. However, translation of these findings proved challenging with results from clinical trials failing to live up to preclinical promise[8,9]. There is recognition now that this is likely because only a subset of ER$^+$ breast cancers activate HER2 to resist endocrine therapy. Finding this subset is complicated by the fact that ER$^+$/HER2$^-$ breast cancer cells likely activate HER2 only in response to endocrine therapy, making identification of these patient cohorts from diagnostic biopsies challenging. Without identifying this patient subset, it is difficult to design a clinical trial with sufficient resolution to uncover real improvement in patient outcome.

Continuing efforts to identify alternate therapies for endocrine-therapy-resistant ER$^+$/HER2$^-$ breast cancer patients have largely failed to show real improvement in the clinic. The only targeted therapy to prove effective to date is CDK4/6 inhibitors[10]. However, these inhibitors have to be administered constantly to be effective, and are, therefore, a financially and physically costly treatment modality that postpones resistance, metastasis, and death but does not remove this threat[11]. Moreover, some endocrine-therapy-resistant patients do not respond to CDK4/6 inhibitors at all[12]. Hope of curing endocrine-therapy-resistant patients with HER2 inhibitors, therefore, remains a tantalizing challenge with clinical impact.

Defects in the MutL complex of mismatch repair, comprised of *MLH1* and *PMS2*, were recently identified as drivers of endocrine treatment resistance in 15–17% of ER$^+$/HER2$^-$ breast cancer patients[13,14]. Mismatch repair is a fundamental DNA repair pathway conserved between pro- and eukaryotes, and essential for guarding the genome during cellular replication[15]. Here, we demonstrate a non-genomic role for MutL loss in activating HER2 in ER$^+$ HER2$^-$ cells exposed to endocrine therapies. Moreover, using multiple experimental model systems, we provide strong evidence for MutL loss as a stratifier of response to HER inhibitors in endocrine-therapy-resistant, nominally HER2$^-$ ER$^+$ breast cancer patients.

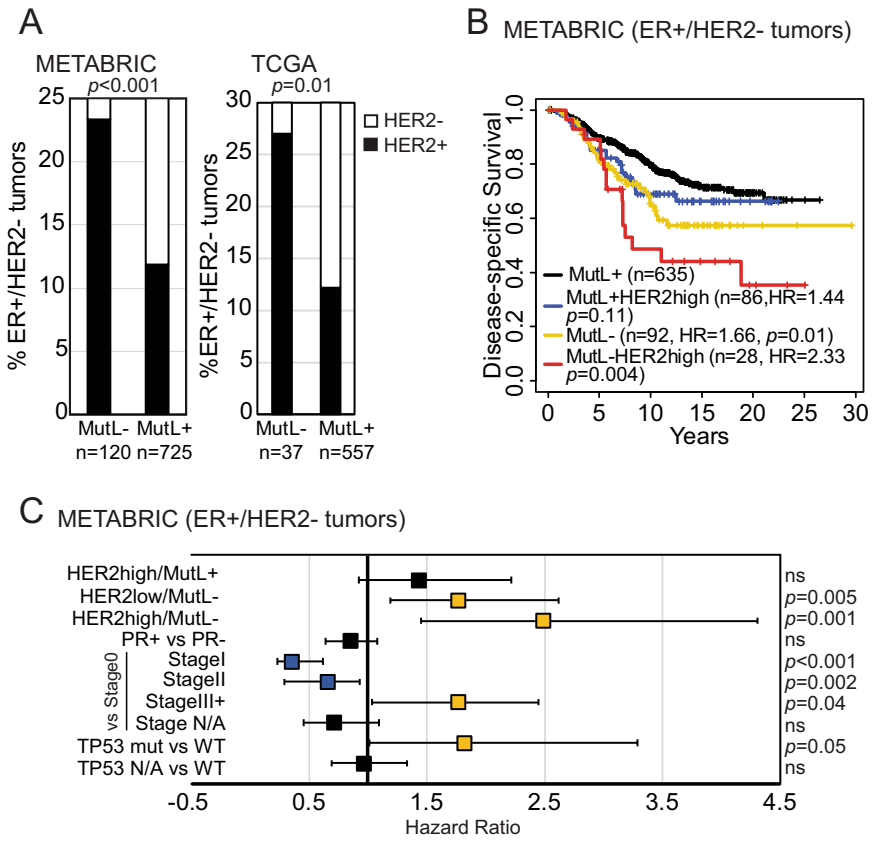

Fig. 1 ER$^+$, HER2$^-$ (non-amplified) breast cancer patients whose tumors are MutL$^-$ have elevated RNA levels of *HER2* and associate with significantly worse disease-specific survival. A Incidence of tumors with elevated *HER2* RNA levels within MutL$^-$ and MutL$^+$ ER$^+$/HER2$^-$ breast tumors from METABRIC ($p = 0.0006$) and TCGA. Pearson Chi-Square test identified p values. Corresponding RPPA data in Fig. S2A and contextualization with HER2$^+$ subset in Fig. S2B, C. Kaplan–Meier survival curves (**B**) and proportional hazard assessment (**C**) demonstrating differences in disease-specific survival between specified groups within the ER$^+$/HER2$^-$ breast tumor cohort from METABRIC. Boxes in (**C**) indicate the hazard ratio calculated using the Cox Proportional Hazards Regression analysis and error bars indicate the 95% confidence interval. Stage I p value = 0.0003. Supporting data from TCGA presented in Fig. S2D and proliferation controls in Fig. S2E, F. All statistical tests were two-sided. Source data for this figure are available with paper.

Results

Loss of mismatch repair associates with HER2 activation in HER2$^-$ breast cancer cells. To understand mechanisms underlying MutL loss-induced endocrine treatment resistance, we analyzed previously generated reverse phase protein array (RPPA) data to compare ER$^+$/HER2$^-$ MCF7 breast cancer cells engineered to carry shRNA against *MLH1* or *PMS2* against control isogenic cells with shRNA against Luciferase[13]. This model system has been extensively validated using orthogonal approaches, with pooled RNAi and with rescue using wild-type *MLH1* and is continually revalidated[13,14]. Analysis of the RPPA data identified significant upregulation of phosphorylated HER2 (pHER2) in response to endocrine treatment (fulvestrant) in sh*MLH1* and sh*PMS2* MCF7 cells but not in sh*Luc* cells (Fig. S1). To test whether an association between MutL loss and HER2 activation is also detectable in patient tumors, we analyzed HER2 protein levels from RPPA data in ER$^+$ breast tumors that were nominally HER2$^-$ (non-amplified) from TCGA. We observed that ~70% of MutL$^-$ patient tumors have positive HER2 levels compared to ~50% of MutL$^+$ patient tumors (Fig. S2A). These tumor samples are largely treatment-naïve, and therefore correspond more closely to the RPPA data generated from vehicle-treated controls in our model system, where we observe modest upregulation of HER2 protein levels, than to the more robust HER2 upregulation observed in fulvestrant-treated samples (Fig. S1).

Encouraged by this observation, we compared RNA levels using gene expression microarray data from two independent patient tumor datasets: METABRIC and TCGA. We chose to compare RNA levels as these data are more abundant in multiple datasets and permit correlations with patient outcomes. In both cases, we observed that ~25% of MutL$^-$ ER$^+$/HER2$^-$ patient tumors have relatively high RNA levels of HER2 compared to ~10% of MutL$^+$ patient tumors (Fig. 1A). While neither RNA nor protein levels in this heterogeneous collection of treatment-naïve and pre-treated patient tumors are as high as that seen in HER2$^+$ breast cancer (contextualized in Fig. S2B, C), nonetheless they consistently show modest increase in total HER2 RNA and protein levels in MutL$^-$ ER$^+$/HER2$^-$ patient tumors.

MutL$^-$ patient tumors with relatively high *HER2* RNA also associate with significantly worse disease-specific survival in METABRIC (Fig. 1B) and in TCGA (Fig. S2D). Upregulation of *HER2* in MutL$^-$ patient tumors also independently prognosticates worse disease-specific survival in Cox Proportional Hazards analyses when considering PR status, tumor stage, and *TP53* mutational status as confounding variables (Fig. 1C). MutL loss as assayed by low gene expression levels is not an artifact of low basal proliferation since RNA levels of *MKI67* (a proliferation marker) are either higher in MutL$^-$ patient tumors, or comparable between MutL$^-$ and MutL$^+$ patient tumors (Fig. S2E, F). Together, these data suggest that the association between MutL loss and HER2 upregulation is of clinical relevance.

Inhibition of mismatch repair activates HER2 in response to endocrine treatment in ER$^+$/HER2$^-$ breast cancer cells. We next tested the causality of this relationship in two independent cell line models of ER$^+$/HER2$^-$ breast cancer: MCF7 and T47D. Data from these experimental model systems mirror that observed in patient datasets. In both cell lines, Western blotting identified higher baseline levels of pHER2 in cells with stable knockdown of *MLH1* (sh*MLH1*), the principal component of the MutL complex, relative to isogenic MLH1-proficient (sh*Luc*) cells, with further increase upon treatment with ER degrader, fulvestrant (Figs. 2A and S3A). Downstream signaling to pAkt and pS6k is also upregulated in sh*MLH1* cells after fulvestrant treatment (Fig. 2A). In addition, we confirmed increased HER2 protein at the membrane of sh*MLH1* cells after fulvestrant treatment using both immunofluorescence (Fig. 2B) and flow cytometry (Fig. S3B, C). Increase in membrane HER2 in sh*MLH1* cells after exposure to endocrine treatment was consistent in xenograft tumors from MCF7 sh*Luc* and sh*MLH1* cells (Fig. 2C). This increase in membrane-bound HER2 remained consistent with use of antibodies against either total HER2 (Fig. 2B) or against pHER2 (Figs. 2C and S3E). Also, the same increase in membrane HER2 levels after fulvestrant treatment was seen in tumors from an ER$^+$/HER2$^-$ patient-derived xenograft (PDX) model of MutL loss (WHIM20[13,16]) (Fig. 2D). We did not observe changes in levels of MLH1 protein in response to HER2 inhibition, validating the directionality of the observed relationship (Fig. S3F). These data indicate that MutL loss directly activates HER2 signaling in ER$^+$/HER2$^-$ breast cancer cells upon endocrine treatment.

MutL$^-$ cells engage HER2 signaling by protecting HER2 from lysosomal protein trafficking. Since MutL$^-$ ER$^+$/HER2$^-$ tumors have higher mutation load than MutL$^+$ tumors[13,17], we tested whether *HER2* activation in these tumors occurs via activating mutations in *HER2*, a previously established mechanism of HER2 activation in HER2 non-amplified cancer cells[7]. We found no enrichment for *HER2* mutations in ER$^+$/HER2$^-$ MutL$^-$ primary patient tumors relative to MutL$^+$ ones (TCGA: 0 vs. 1.8%, METABRIC: 2.2% vs. 2.8% in MutL$^-$ vs. MutL$^+$). Further, HER2 activation induced by loss of MLH1 in our experimental model systems is reversible when MLH1 is re-expressed in sh*MLH1* cells (Fig. S3D), arguing against an irreversible mutational change as the underlying mechanism. In addition, acute loss of MLH1 by transient transfection of parental MCF7 cells with sgRNA against *MLH1* immediately upregulates pHER2 to similar levels as those seen in cells with stable knockdown of *MLH1* (Fig. S3E). These data both confirm the specificity of the link between MLH1 loss and HER2 activation and argue against an underlying mechanism of mutagenesis. This suggests that MutL loss activates HER2 through non-mutational mechanisms. To identify alternate mechanisms by which MutL loss activates HER2 signaling in conjunction with endocrine treatment, we conducted RNAseq analysis of sh*MLH1* MCF7 cells relative to isogenic sh*Luc* controls at baseline and after fulvestrant treatment (Supplementary Data 1). RNAseq analysis of signatures identified significant enrichment of protein trafficking pathways in MutL$^-$ relative to MutL$^+$ cells after fulvestrant treatment (Fig. 3A). We found similar enrichment for autophagy and protein trafficking pathways in Reactome analysis of RPPA data comparing MutL$^-$ and MutL$^+$ cells after fulvestrant treatment (Fig. S4A). Therefore, we next tested whether loss of the MutL complex prevents the targeting of HER2 for lysosomal degradation after endocrine therapy in ER$^+$/HER2$^-$ breast cancer cells.

First, we conducted a time course immunofluorescence experiment testing colocalization of HER2 with the lysosomal marker, LAMP1 in MCF7 and T47D sh*Luc* and sh*MLH1* cells at baseline and at 18, 36, and 54 h post treatment with fulvestrant. At baseline and at 18 h post treatment, both sh*Luc* and sh*MLH1* cells exhibit low levels of HER2, however by 36 h post treatment, HER2 positivity increases in both cell types. However, 60–80% of sh*Luc* cells with HER2 expression demonstrate colocalization of HER2 with LAMP1 (Figs. 3B and S4B). By 54 h post treatment, HER2 continues to colocalize with LAMP1 in sh*Luc* cells, whereas sh*MLH1* counterparts have HER2 at the membrane, distinct from the perinuclear LAMP1 immunostain (Fig. 3B). Next, we used chloroquine, a known autophagy inhibitor[18,19], to test whether inhibition of lysosomal degradation pathways in

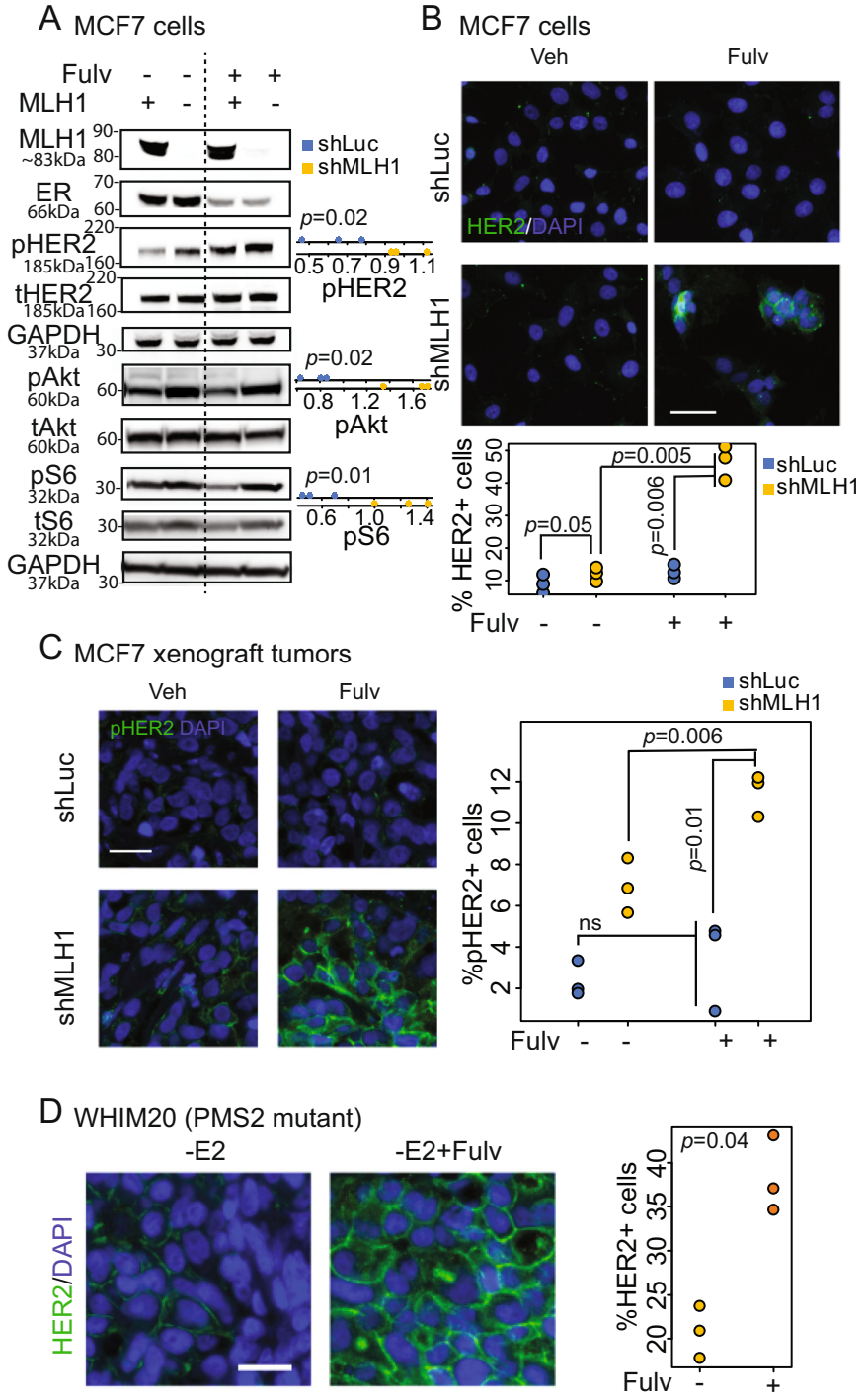

Fig. 2 MLH1 loss in ER$^+$, nominally HER2$^-$ breast cancer cells upregulates membrane-bound HER2. A Western blots demonstrating increase in pHER2 and downstream signaling in sh*MLH1* MCF7 cells treated with fulvestrant relative to sh*Luc* cells. Quantification of four independent replicates conducted through ImageJ in accompanying dot plots. Validation in T47D cells in Fig. S3A. Immunofluorescent staining for HER2 in MCF7 sh*Luc* and sh*MLH1* cells in vitro (**B**), in MCF7 sh*Luc* and sh*MLH1* xenograft tumors (**C**), and in WHIM20, *PMS2* mutant, ER$^+$/HER2$^-$ PDX tumors (**D**), grown with or without fulvestrant. Accompanying quantification presented as strip charts. Three independent experiments or tumors from each group were quantified. Two-sided Student's *t* test determined *p* values. Supporting data from FACS analysis are presented in Fig. S3B, C. Scale bars represent 50 μ. Source data for all figures available with paper.

sh*Luc* cells can rescue HER2 positivity after endocrine therapy. In both MCF7 and T47D cells, sh*Luc* cells treated with a combination of fulvestrant and chloroquine demonstrate significant increase in membrane HER2 positivity relative to those treated with fulvestrant alone (Figs. 3C and S4C). Indeed, membrane HER2 positivity is at levels comparable to that of sh*MLH1* counterparts in both cell lines tested, with the addition of chloroquine.

Finally, we directly tested whether MutL loss prevents targeting of HER2 to autophagosomes by assessing colocalization of transiently transfected *HER2-GFP* and *LC3-RFP* for up to 36 h after administration of fulvestrant in sh*Luc* and sh*MLH1* MCF7

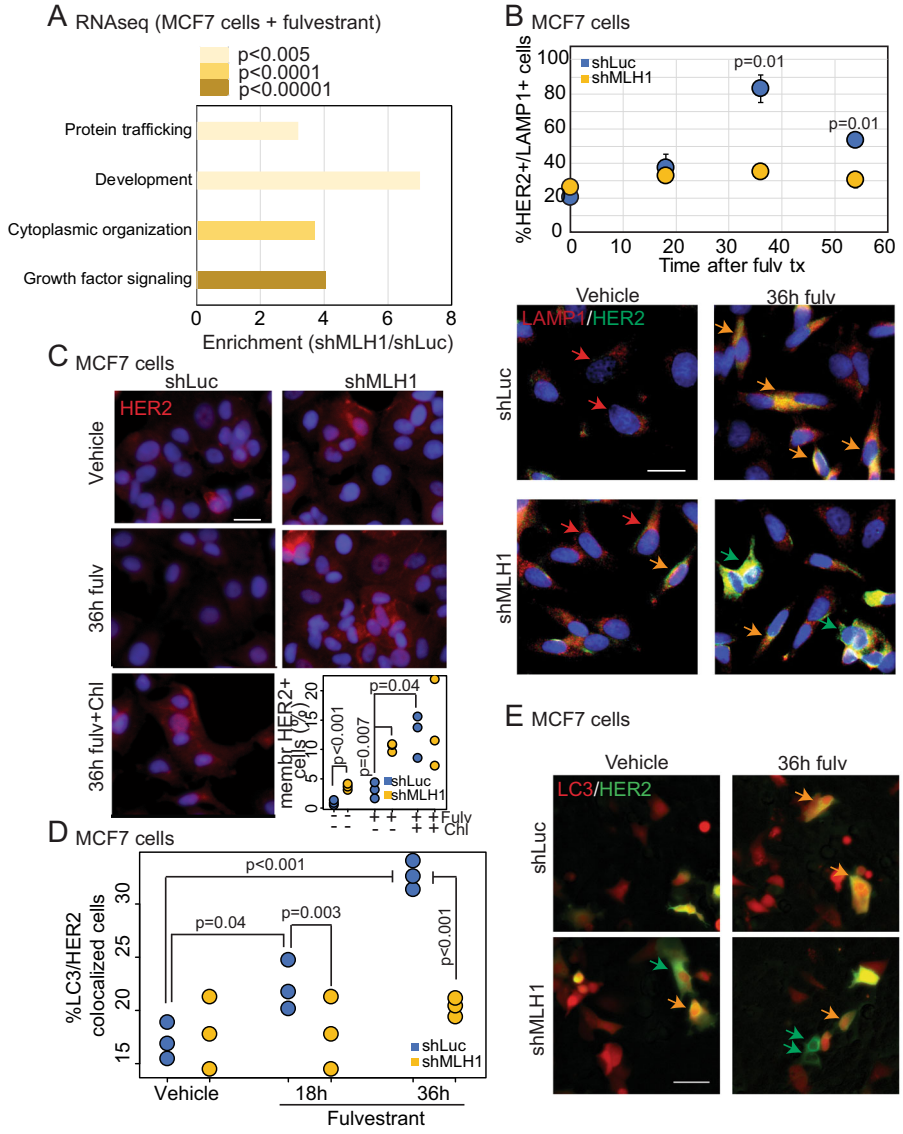

Fig. 3 MLH1 regulates protein trafficking of HER2. A Gene set enrichment analysis of RNAseq data comparing isogenic sh*Luc* and sh*MLH1* MCF7 cells after treatment with fulvestrant for 4 days. *P* values were generated using DESeq2 R package and adjusted for multiple comparison using Benjamini–Hochberg. Comparable RPPA data analysis in Fig. S4A. Raw read counts available as supplementary data. **B** Co-immunofluorescence for HER2 and lysosomal marker, LAMP1 (orange arrows) at baseline and after 18, 36, and 54 h of fulvestrant treatment. Green arrows indicate HER2 that is not colocalized with LAMP1, and red arrows indicate LAMP1 positivity alone. Validation in T47D cells in Fig. S4B. **C** Immunofluorescence staining for HER2 in MCF7 sh*Luc* and sh*MLH1* cells treated with vehicle and 36 h of fulvestrant alone or a combination of fulvestrant and chloroquine, an autophagy inhibitor. For sh*Luc* vs. sh*MLH1* vehicle, $p = 0.0006$. Validation in T47D cells in Fig. S4C. Quantification (**D**) and representative photomicrographs (**E**) from 36 h of live cell tracking of colocalization of HER2 and LC3 (orange arrows), a marker of autophagosomes, in MCF7 sh*Luc* and sh*MLH1* cells treated with vehicle or fulvestrant. Green arrows indicate $HER2^+$ $LC3^-$ cells. Cells were tracked after administration of fulvestrant. All quantification is of three independent biological replicates conducted through ImageJ and is represented as strip charts. Two-sided Student's *t* test determined all *p* values. For sh*Luc* vehicle vs. 36-h fulvestrant treatment, $p = 0.0003$ and for sh*Luc* vs. sh*MLH1* at 36-h fulvestrant treatment, $p = 0.0005$. Scale bars represent 50 μ. Source data for all figures available with paper.

cells. While sh*Luc* cells demonstrate increasing colocalization of HER2 and LC3 with time after treatment with fulvestrant, sh*MLH1* cells do not (Fig. 3D, E). In fact, by 36 h after fulvestrant treatment, sh*MLH1* cells with defined membrane HER2 staining are detectable with no LC3 colocalization, whereas this is undetectable in sh*Luc* counterparts (green arrows, Fig. 3E). Together, these data indicate that both sh*Luc* and sh*MLH1* ER^+/$HER2^-$ breast cancer cells upregulate HER2 upon ER degradation through endocrine therapy. However, while sh*Luc* cells rapidly target HER2 to lysosomal protein trafficking, sh*MLH1* cells maintain HER2 at the membrane, thereby upregulating HER2-mediated signaling and inducing endocrine therapy resistance.

HER2 is required for endocrine treatment resistance of MutL$^-$ ER$^+$/HER2$^-$ breast cancer cells. To test whether HER2 activation in MutL$^-$ cells is required for endocrine-therapy-resistant growth, we used siRNA to decrease endogenous *HER2* in MCF7 sh*Luc* and sh*MLH1* cells, and then assayed growth in presence of fulvestrant. We observed complete rescue of endocrine treatment sensitivity in sh*MLH1* cells transfected with si*HER2*, with no observable change in endocrine therapy response

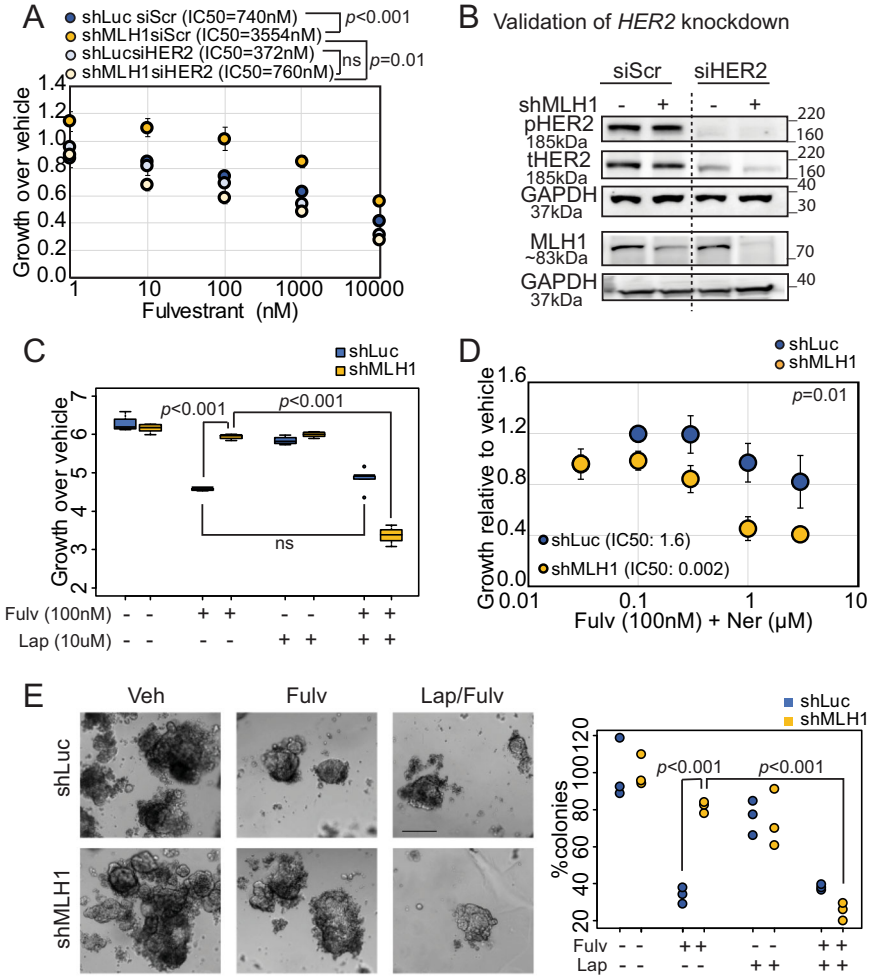

Fig. 4 HER2 is required for endocrine-therapy-resistant growth of ER$^+$ MLH1$^-$ breast cancer cells. Knockdown of endogenous *HER2* using siRNA against *HER2* or a scrambled control in MCF7 sh*Luc* and sh*MLH1* cells validated by Western blotting (**B**) and followed by 2D growth assays for dose response to fulvestrant treatment (**A**). For sh*Luc* vs. sh*MLH1* with siScr, $p = 0.0001$. Supporting data demonstrating similar response to tamoxifen and estrogen deprivation in Fig. S5A, B. **C** Growth of MCF7 sh*Luc* and sh*MLH1* cells in response to specified therapeutic combinations represented as a bar graph. For sh*Luc* vs. sh*MLH1* with fulvestrant treatment, $p = 0.0009$, and for sh*MLH1* fulvestrant vs. lapatinib + fulvestrant treatment, $p = 3.15e-05$. Supporting data from T47D in Fig. S5C. **D** Dose curve demonstrating response to neratinib and fulvestrant in MCF7 sh*Luc* and sh*MLH1* cells. Supporting data demonstrating similar results in T47D cells and in response to tamoxifen in Fig. S5D, E. **E** 3D growth in Matrigel of MCF7 sh*Luc* and sh*MLH1* cells in response to specified treatments. Representative images for each treatment group (except lapatinib, which showed no visible difference from vehicle-treated) shown alongside quantification. For both sh*Luc* vs. sh*MLH1* fulvestrant-treated, and sh*MLH1* fulvestrant vs. fulvestrant + lapatinib-treated, $p = 0.0002$. Supporting data in T47D cells in Fig. S6A. For dose curve experiments **A, C, D** three independent biological replicates were quantified for each group and each dose point. IC50 values were determined over three independent experiments and compared for statistical differences. Circles (**A**, **D**) represent mean growth relative to vehicle-treated cells over 7 days of treatment and error bars the standard deviation. Box plots show median, quartiles, minima and maxima, and outliers at 1.5× IQR. All statistical comparisons used the two-sided Student's *t* test. All experiments were conducted >2 times. Source data for this figure available with paper.

in sh*Luc* cells under the same conditions (Fig. 4A, B). This rescue of sensitivity to endocrine therapy extends to tamoxifen (Fig. S5A) and estrogen deprivation, a surrogate for aromatase inhibitors (Fig. S5B). In keeping with this observation, both MCF7 (Fig. 4C) and T47D (Fig. S5C) sh*MLH1* cells grown in 2D are sensitive to combinatorial administration of fulvestrant and lapatinib, a HER inhibitor used in clinic. In addition, MCF7 (Fig. 4D) and T47D (Fig. S5D) sh*MLH1* cells demonstrate increased sensitivity to fulvestrant when treated with neratinib, another HER inhibitor. Similar results were obtained when neratinib was combined with tamoxifen treatment (Fig. S5E).

To test specificity of MLH1 loss in inducing therapeutic vulnerability to HER2 inhibitors, we also tested growth response to lapatinib in two previously established endocrine therapy resistance models: MCF7 cells harboring either an ESR1-YAP1 or an ESR1-PCDH11X fusion[20]. Both these model systems with no known defects in mismatch repair are resistant to endocrine therapy, fulvestrant, as expected, but remain resistant to lapatinib compared to MCF7 sh*MLH1* cells (Fig. S5F). Finally, both MCF7 (Fig. 4E) and T47D (Fig. S6A) sh*MLH1* cells demonstrated persistent 3D growth relative to sh*Luc* cells in response to fulvestrant, but this growth was significantly suppressed by adding lapatinib.

These data suggest that loss of MutL predisposes ER$^+$/HER2$^-$ breast cancer cells to respond to HER inhibitors in concert with endocrine therapies. To test this proposition in vivo, we randomized mice with MCF7 sh*Luc* and sh*MLH1* xenograft tumors into five treatment arms: control (with estrogen supplementation), estrogen deprivation, fulvestrant (and estrogen deprivation), lapatinib (and estrogen deprivation), and a

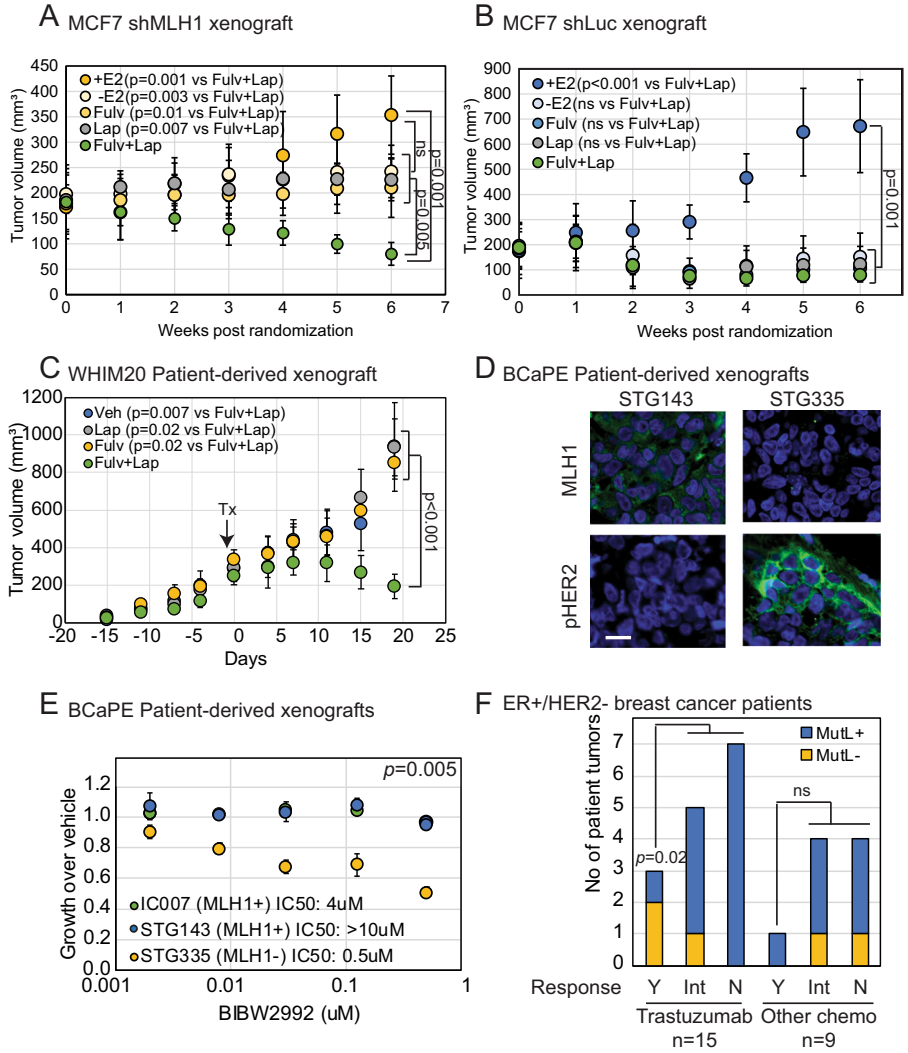

Fig. 5 MLH1 loss predicts sensitivity to HER inhibitors in endocrine-therapy-resistant ER$^+$, HER2$^-$ breast cancer cells in vivo and in patient tumors. In vivo xenograft experiments of MCF7 sh*MLH1* (**A**), sh*Luc* (**B**) cells, and WHIM20 PDX line (**C**) demonstrating response in tumor growth to specified treatments. Control group: $n = 5$ mice; estrogen-deprived group: $n = 4$ (**A**), $n = 5$ (**B**), and $n = 7$ (**C**); lapatinib group: $n = 6$ (**A**), $n = 5$ (**B**), $n = 4$ (**C**); fulvestrant group: $n = 5$ (**A**), $n = 6$ (**B**), $n = 3$ (**C**); fulvestrant + lapatinib group: $n = 8$ (**A**), $n = 6$ (**B**), $n = 5$ (**C**). Circles represent the mean and error bars the standard deviation. Student's *t* test determined *p* values by comparing slopes. For (**B**), $+E_2$ vs. fulvestrant + lapatinib-treated tumors, $p = 0.0005$ and for (**C**), fulvestrant + lapatinib-treated tumors vs. rest, $p = 4.7e-05$. **D** Immunofluorescence depicting protein levels of MLH1 in two ER$^+$/HER2$^-$ PDX lines associating with HER2 activation after estrogen deprivation. Scale bar = 50 μ. **E** Growth curves demonstrating sensitivity of these PDX lines to HER inhibition ex vivo. IC50 calculated using regression analysis over three independent experiments and compared using two-sided Student's *t* test. Circles represent the mean and error bars the standard deviation. Supporting data in Fig. S7A, B. **F** Categorical analysis supporting increased sensitivity of ER$^+$/MutL$^-$ patient tumors to trastuzumab in combination with other chemotherapy. Supporting data in Fig. S7E, F. Y yes, Int intermediate, N no. Two-sided Fisher's Exact test determined *p* values. For all regression analyses, individual *p* values and multiple adjusted R^2 values were derived using a linear model in R. Source data available with paper.

combination of fulvestrant and lapatinib (and estrogen deprivation). As expected from previous experiments[13], we observed estrogen independent and fulvestrant-resistant growth in MCF7 sh*MLH1* tumors, and little response to lapatinib alone (Fig. 5A). However, there was striking response with tumor shrinkage to the combination of fulvestrant and lapatinib (Fig. 5A). In contrast, MCF7 sh*Luc* tumors demonstrated tumor shrinkage in response to either estrogen deprivation or fulvestrant treatment alone and no further response to the addition of lapatinib (Fig. 5B), in keeping with previous literature[21–23].

Loss of mismatch repair increases sensitivity to HER inhibitors in vivo and in patient tumors. We next tested whether MutL defects had similar associations with sensitivity to HER inhibitors in PDX tumors. In vivo growth of WHIM20, *PMS2* mutant, ER$^+$/HER2$^-$ PDX tumors xenografted into mouse mammary fat pads demonstrated a similar pattern of tumor regression in response to combination of lapatinib and fulvestrant but not in response to either treatment alone (Fig. 5C). To test whether loss of PMS2 causally activates HER2 and induces response to HER2 inhibitors, similarly to MLH1, we tested our previously established and validated MCF7 cells with stable knockdown of *PMS2*[13] (Fig. S6B). We observed high baseline levels of pHER2 in sh*PMS2* cells and further induction after fulvestrant treatment (Fig. S6C). This upregulation of HER2 levels was reflected in increased sensitivity to HER inhibitor, lapatinib, in sh*PMS2* MCF7 cells relative to isogenic sh*Luc* cells (Fig. S6D).

An additional ER$^+$/HER2$^-$ PDX line[24] with low MLH1 protein (Fig. 5D) and low *MLH1* RNA levels (Fig. S7A) also has increased membrane-bound HER2 (Fig. 5D). This increase in HER2 protein at the membrane also associates with increased sensitivity to BIBW2992 (or afatinib, a second generation pan-HER inhibitor), as assayed by ex vivo 3D growth (Fig. 5E). We also observed significant correlation between sensitivity to three HER inhibitors, including lapatinib, and low RNA levels of *MLH1*/*PMS2* across seven PDX models of luminal breast cancer[24] grown in estrogen-deprived conditions (Fig. S7A). Of note, there was no such correlation across 11 PDX models of basal-like breast cancer (Fig. S7B). Together, these data demonstrate that MutL loss predisposes ER$^+$/HER2$^-$ PDX tumors to respond to a combination of HER inhibitors and endocrine treatment.

We also validated our findings in transcriptomics data from ER$^+$/HER2$^-$ patient tumors biopsied at diagnosis and after 4–6 weeks of neoadjuvant aromatase inhibitor treatment (Z1031[25]). We first confirmed inverse association between RNA levels of *HER2* and *MLH1* in these tumors at diagnosis (Fig. S7C), indicating that tumors with low *MLH1* have relatively higher *HER2* at baseline (as observed in our experimental model systems and in TCGA and METABRIC patient tumor datasets). Next, we identified direct association between RNA levels of *HER2* and proliferation as measured by immunohistochemistry for Ki67 after endocrine treatment (Fig. S7C). Importantly, this association is restricted to MutL$^-$ tumors and not seen in tumors that are MutL$^+$ (Fig. S7C).

These data suggest that loss of MutL induces HER2-associated proliferation in ER$^+$/HER2$^-$ breast cancer cells treated with endocrine intervention. As an additional control, we found no significant associations between levels of *HER2* RNA and those of another mismatch repair gene, *MSH2*, which is not part of the MutL complex (Fig. S7D). This specificity increases confidence in the association between HER2 activation and MutL loss. Second, association between *HER2* RNA levels and Ki67 in MutL$^-$ ER$^+$/HER2$^-$ breast tumors is only significant after exposure to endocrine treatment and not in pre-treatment biopsies (Fig. S7D). We confirmed that loss of MutL in patient tumors is not merely a consequence of low proliferation (Fig. S7E). This attests to the role of endocrine therapy in catalyzing reliance on HER2 for proliferation in MutL$^-$ ER$^+$/HER2$^-$ tumors.

We also analyzed a second dataset[26] where metastatic, treatment-resistant breast cancer patients, irrespective of HER2 status, were randomized to two arms of treatment: anthracyclines and taxanes or anthracyclines, taxanes and trastuzumab, a HER2 inhibitor. From this dataset, we parsed the subset of patients whose cancer was ER$^+$/HER2$^-$ for further analysis. Strikingly, all patients with MutL$^-$ ER$^+$/HER2$^-$ breast cancer demonstrate at least partial response to trastuzumab, compared to less than half of patients with MutL$^+$ ER$^+$/HER2$^-$ disease (Fig. 5F). In addition, 2/3rd of MutL$^-$ patients has complete response to the trastuzumab combination compared to less than a tenth of MutL$^+$ patients (Fig. 5F). This disparate response was only observed in the treatment group where trastuzumab was added to the chemotherapy administered to patients. Concomitant downregulation of *HER2* RNA in response to the trastuzumab combination, but not in response to anthracyclines/taxanes alone was confirmed in the MutL$^-$ ER$^+$/HER2$^-$ tumors (Fig. S7F). These data, while of small sample size, provide support for a role for MutL loss in sensitizing endocrine-therapy-resistant ER$^+$/HER2$^-$ breast cancer to a combination of HER inhibitors and endocrine therapy.

Discussion

Taken together, results presented here suggest that *MLH1*/*PMS2* downregulation could constitute a first-in-class predictive marker for response to HER2 inhibition in endocrine-therapy-resistant ER$^+$/HER2$^-$ breast cancer. The only other biomarkers proposed to predict response to HER2 inhibitors in the endocrine-therapy-resistant ER$^+$/HER2$^-$ setting are low ER/PR but these markers are not specific to HER2 activation and have mixed associations across clinical trials decreasing their feasibility for clinical use[8,27]. The impact of the discovery presented here could be substantial, given that loss of nuclear MLH1 and PMS2 occurs in 15–17% of ER$^+$/HER2$^-$ breast cancer[28]. Importantly, it is clinically feasible to assess these markers at diagnosis to tailor therapy since diagnostic assays for MLH1 and PMS2 loss are routinely implemented in clinic for colorectal and endometrial cancer patients[29,30]. Coopting these diagnostic tests for ER$^+$/HER2$^-$ breast cancer patients is, therefore, relatively straightforward and could benefit a significant subset of patients.

The mechanism underlying HER2 activation in response to endocrine therapy in MutL$^-$ ER$^+$/HER2$^-$ breast cancer cells is through lysosomal protein trafficking. Data from Western blots and immunofluorescence of cell lines and PDX tumors suggest that total HER2 levels increase with MutL loss even before endocrine therapy. Concordantly, baseline levels of HER2 appear higher in ER$^+$/HER2$^-$ MutL$^-$ breast cancer cells in patient tumor gene expression data, although many orders lower than levels in HER2-amplified patient tumors. These data suggest that even at baseline, MutL$^-$ cells are less likely to target HER2 for protein degradation. However, with endocrine therapy, HER2 is upregulated in both MutL$^+$ and MutL$^-$ ER$^+$/HER2$^-$ breast cancer cells as predicted by the literature[3]. In the context of this HER2 upregulation, the protection of HER2 from protein trafficking in MutL$^-$ cells allows HER2-mediated growth signaling to compensate as a cell-cycle cue for the loss of ER driven by standard endocrine therapies. These data provide an explanation for the lack of positive clinical trial data from using HER inhibitors in the endocrine-therapy-resistant ER$^+$/HER2$^-$ breast cancer setting, in spite of strong preclinical links between ER loss and upregulation of HER2[3,4]. The link between loss of a DNA damage repair pathway and targeting of growth factor proteins for protein trafficking requires further investigation.

A significant limitation of this study is the lack of specific clinical trial data with which to test the hypothesis raised by the molecular biology described above. Very few clinical trials have been performed to test efficacy of HER inhibitors in ER$^+$/HER2$^-$ breast cancer patients[8,31]. None of these trials include transcriptomic or genomic data accrual from tumor biopsies and since MutL status is not routinely tested in clinic for breast cancer patients, this data is missing from all existing trials. The strength of preclinical data presented here and the strong associations observed in the limited clinical trial data available make a compelling case for revisiting HER inhibitors in the ER$^+$/HER2$^-$ breast cancer setting but this time in context of MutL status.

These results also have significance beyond ER$^+$ breast cancer. Our data provide support for a recent report on Lynch syndrome colorectal cancer suggesting a link between loss of mismatch repair and response to HER inhibitors[32]. Lynch syndrome is one of the most common causes of inherited cancers at many sites and is caused by hereditary defects in mismatch repair genes[33]. In addition, mismatch repair loss drives a significant proportion of sporadic colorectal, ovarian, and endometrial cancer[34]. If *MLH1*/*PMS2* loss serves as a predictive marker for sensitivity to HER inhibitors across cancer types, the already routine identification of these markers in these other cancer types can be married to a clinically feasible targeted therapy.

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[35]. *Mycoplasma* tests were performed on parent cell lines and stable cell lines every 6 months (latest test: 02/19) with the Lonza Mycoalert Plus Kit (cat# LT07-710) as per the manufacturer's instructions. Cell lines were discarded at <25 passages, and fresh vials were thawed out. Key experiments were repeated with each fresh thaw. Transient transfection with siRNA against HER2 was conducted using JetPrime PolyPlus transfection reagent[35], and siRNA pools were purchased from Sigma-Aldrich. Stable cell lines were maintained in presence of specified antibiotics at recommended concentrations. Knockdown was validated using qRT-PCR (list of primers used in Supplementary Table 1) and/or Western blotting. Growth assays were conducted in triplicate and repeated independently using Alamar blue to identify cell viability[13]. Growth assay results were plotted as fold change in growth from day 1 to day 7 and normalized as specified. Three-dimensional growth assays were conducted over 4–6 weeks with weekly drug treatments using standard protocols[7]. Images were captured when colonies had established (at 2 weeks), and then treatment was administered, with images taken again at 1 and 3 weeks post treatment. Fold change in area of colonies was calculated over time and represented as %growth. Tumor growth assays in vivo were carried out by injecting $2–5 \times 10^6$ 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 or SBP animal facility). For WHIM20 PDX experiments, 6- to 8-week female SCID/Bg mice were purchased from Jackson laboratory. Tumor volume was measured twice or thrice weekly using calipers to make 2 diametric measurements. Tumors were randomized for treatment at 50–150-mm^3 volume for MCF7 xenografts and 100–300-mm^3 volume for WHIM20 PDX experiments. Tumors were harvested at <2-cm diameter and were embedded in paraffin blocks, OCT, and snap-frozen[36]. 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. STG335, STG143, and VHIO244 PDX experiment results were kindly provided by the BCaPE consortium, but tumor sections were stained in house. All mouse experiments were performed in compliance with all relevant ethical regulations for animal testing and research, and all experiments conducted in the study received approval from the respective Institutional Animal Care and Use Committee boards (protocols# AN-6934 for Baylor College of Medicine and 18-065 for Sanford Burnham Prebys).

Inhibitors and agonists. All drugs were maintained as stock solutions in DMSO, and stock solutions were stored at −80 and working stocks at −20 unless otherwise mentioned. 4-OHT (Sigma-Aldrich, cat# H7904) and fulvestrant (SelleckChem, cat# I4409) were purchased, and stocks were diluted to 10-mmol/L working stocks for all experiments other than dose curves, where specified concentrations were used. For all experiments, cells were treated 24 h after plating, and thereafter every 48 h until completion of experiment. For mouse xenograft experiments, fulvestrant concentrations of 250-mg/kg body weight were prepared in corn oil, freshly on day of injection and administered subcutaneously. Beta-estradiol was purchased from Sigma-Aldrich (cat# E8875), maintained in sterile, nuclease-free water, and diluted to obtain 10-mmol/L stocks for in vitro experiments. For mouse xenograft experiments, 17 β-estradiol was maintained in 200-proof ethanol at 2.7-mg/ml stock solution and added to drinking water twice a week at a final concentration of 8 μg/mL (cat# E2758; Sigma). For experiments involving Chloroquine (Selleckchem, cat#S4157), cells were treated at 50 μM for 16 h before end of assay. Lapatinib (SelleckChem, cat#S2111) and Neratinib were used at specified concentrations. Lapatinib tablets were used at 100 mg/kg in chow from Research Diets, Inc for tumor growth assays.

Flow cytometry, immunostaining, and microscopy. Flow cytometry for membrane-bound HER2 was performed based on manufacturer's instructions. After fulvestrant treatment, cells were detached from plates using StemPro™ Accutase™ Cell Dissociation Reagent (cat#A1110501). Cells were washed with chilled PBS and suspended in antibody solution, as per the manufacturer's instructions, in 5-mL flow cytometry tubes and incubated on ice for 20 min. Live cells were then run through BD Accuri C6 cytometer to assess only membrane-bound HER2 protein levels. IF was performed based on the manufacturer's instructions. Cells were washed in PBS; fixed for 20 min at room temperature in 4% PFA; blocked for 1 h at room temperature in 5% goat serum and 1% Triton X-100 in 1x PBS; incubated with primary antibody overnight at 4° in 1% goat serum and 1% Triton X-100 in 1x PBS antibody diluent; incubated with secondary antibody in diluent for 1 h at RT; and then mounted with DAPI-containing mounting media (cat# P36935). Tumor section staining was done using a standard protocol. Briefly, slides were incubated at 65° for 4 h and deparaffinized. Antigen retrieval was done with 10-mM Sodium Citrate (pH 6) for 25 min in pressure cooker. Hereafter, the cells were treated the same as the 2D IF. Primary antibodies used include pHER2 (EMD millipore; cat# 06-229; 1:200) and Ki67 (Novus Biologics, cat# NB500-170SS, 1:250). Cells were treated with fulvestrant for 24 h before evaluation. Fluorescent images were captured with a Nikon microscope and quantified with ImageJ. Representative images were translated into figures using Adobe Photoshop and Adobe Illustrator.

RNAseq analyses. RNAseq data were generated from two replicates each of MCF7 shLuc and shMLH1 cells treated with either vehicle or 100-nM fulvestrant for 4 days on the Illumina NovaSeq platforms with paired-end 150-bp sequencing. Downstream analysis was performed using a combination of programs including STAR, HTseq, Cufflink, and Novogene's wrapped scripts. Alignments were parsed using STAR program and differential expressions were determined through DESeq2/edgeR. FPKM of each gene was calculated based on the length of the gene and read counts mapped to this gene. GO and KEGG enrichment were implemented by the ClusterProfiler. Source data available in supplementary files.

Lysosomal analyses. Immunofluorescence of LAMP1/HER2 was conducted by plating 20k cells per well/per condition in a 96-well plate and treated with 100-nM fulvestrant (SelleckChem, cat# I4409) for 36 h. Cells were then probed ON at 4 °C with LAMP1 (proteintech, cat# 21997-1-AP) and HER2 (Invitrogen, cat# MA5-13105) antibodies used at a 1:750 and 1:250 dilution, respectively, diluted in 1x TBST with 5% Goat Serum. For HER2 and LC3 immunofluorescent images, cells were transiently transfected with mCherry-hLC3B-pcDNA3.1, a gift from David Rubinsztein (Addgene plasmid # 40827; http://n2t.net/addgene:40827; RRID: Addgene_40827) and pCMV3-C-GFPSpark-HindIII-XbaI (SinoBiological, cat# HG10004-ACG) using jetPRIME transfection reagent (Polyplus, cat#114-07) as per manufacturers' instructions. Thirty-six hours 100-nM Fulvestrant treatment started 16 h after transfection. Both assays were imaged using BioTek Cytation 5 Imaging Reader.

Protein analyses. Western blotting was conducted as described[35]. Cells were exposed to 18–24 h of fulvestrant treatment administered 40 h after plating. For pHER2 Western blots, primary antibody was incubated for 48 h at 4°. For all other antibodies, primary incubation was 2 h at room temperature. All antibodies diluted in 1x TBST and incubated overnight at 4 °C. Antibodies used were pHER2 Y1196 (D66B7) (Cell Signaling; cat# 6942S), total HER2 (Thermo Scientific; NeoMarkers; cat# MS-730-P1ABX), pAkt S473 (D9E) XP (Cell Signaling; cat#4060S), total Akt (Cell Signaling; cat#9272S), pS6 (S235/236) (Cell Signaling; cat# 2211S), total S6 (5G10) (Cell Signaling; cat# 2217S), MLH1 (1:2,000, Sigma-Aldrich; cat# WH0004292M2), ER clone 60C (EMD Millipore; cat# 04-820), and GAPDH (0411) (Santa Cruz; cat# sc-47724). Unless otherwise specified, primary antibodies were diluted 1:1000 for Western blotting. RPPA assays were carried out as described previously with minor modifications[37].

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 publically available resources: Oncomine, cBio[38], and COSMIC. Z1031 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 from each of the institutions involved in this previously published study. $MutL^-$ tumor from METABRIC, TCGA, and Z1031 datasets was determined in a case list containing all ER^+ sample IDs based on gene expression less than mean—1.5 × standard deviation and/or the presence of nonsilent mutations in *MLH1* and *PMS2*. For the multivariate analysis, we analyzed ER^+ tumor samples, extracting mutation data from the cBio portal, and corresponding clinical data through Oncomine. Only samples with survival metadata were included in the analysis. Gene expression, and survival data for TCGA samples were downloaded from cBio portal. 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 and edited in Adobe Photoshop or Illustrator. Z1031ClinicalTrials.gov Identifier: NCT00265759. Data for Z1031 samples available in dbGaP (phs000472.v2.p1).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The patient datasets analyzed during the current study are all publicly available from cBio data portal at cbioportal.org (TCGA and METABRIC), or from Gene Expression Omnibus (GEO) (https://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-28826/). Z1031 ClinicalTrials.gov Identifier: NCT00265759. Data for Z1031 samples available in dbGaP (phs000472.v2.p1). Raw read count data from RNAseq that support the findings of this study are available in Supplementary data. Source data are provided with this paper.

Received: 15 September 2020; Accepted: 22 April 2021;
Published online: 19 May 2021

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Acknowledgements

We would like to acknowledge the Patient-derived Xenograft and Advanced In Vivo Models core (funded by P30 Cancer Center Support Grant NCI-CA125123, CPRIT Core Facilities Support Grant RP170691) and Dr Michael T. Lewis, Ph.D., Academic Director, Lacey E. Dobrolecki, MS, Core Director at Baylor College of Medicine for helping us in engrafting WHIM20 PDX explants. We also thank Dr Alejandra Bruna (CRUK, UK) and Dr Violeta Serra (VHIO, Barcelona) for providing PDX drug response data and tumor sections for the STG and VHIO PDX lines. Work in this study was funded by Department of Defense Breast Cancer Research Program Breakthrough awards (W81XWH-18-1-0034 to S.H., W81XWH-18-1-0035 to S.M.K.), NCI K22 Career Development award (CA229613 to S.H.), Susan G. Komen Promise Grant (PG12220321 to M.J.E.), SPORE grant (P50CA186784-06), and Cancer Prevention and Research Institute of Texas (CPRIT) Recruitment of Established Investigators award (RR140033 to M.J.E.), National Cancer Institute of the National Institutes of Health under Award Numbers U10CA180821 and U10CA180882 (to the Alliance for Clinical Trials in Oncology), U24CA196171.

Author contributions

N.B.P. designed and performed experiments, analyzed data, and helped write the manuscript. S.S. helped design, conduct, and analyze data from Western blots and xenograft experiments. V.D. and A.M. helped design and conduct 3D Matrigel assays and immunofluorescence experiments. S.L., T.P., R.K. and C.-H.C. conducted WHIM20 patient-derived xenograft experiment. M.J.E. and S.M.K. helped design experiments and interpret results, and edit the manuscript. S.H. designed and performed experiments, analyzed and interpreted data, and wrote and edited the manuscript.

Competing interests

M.J.E. has intellectual property ownership and received royalties for the PAM50-based breast cancer test "Prosigna." In the last 5 years he has received ad hoc consulting fees and meals (<$5000 per year) from Abbvie, Novartis, AstraZenica, Pfizer, Sermonix, and Puma. S.M.K. is a stakeholder in NeoZenome Therapeutics Inc. S.L. has received license fee from Envigo. He received research funding from Pfizer, Takeda Oncology, Zenopharm, NIH, and DOD, outside of this project. The other authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-23271-0.

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Peer review information *Nature Communications* thanks Sarah Martin and the other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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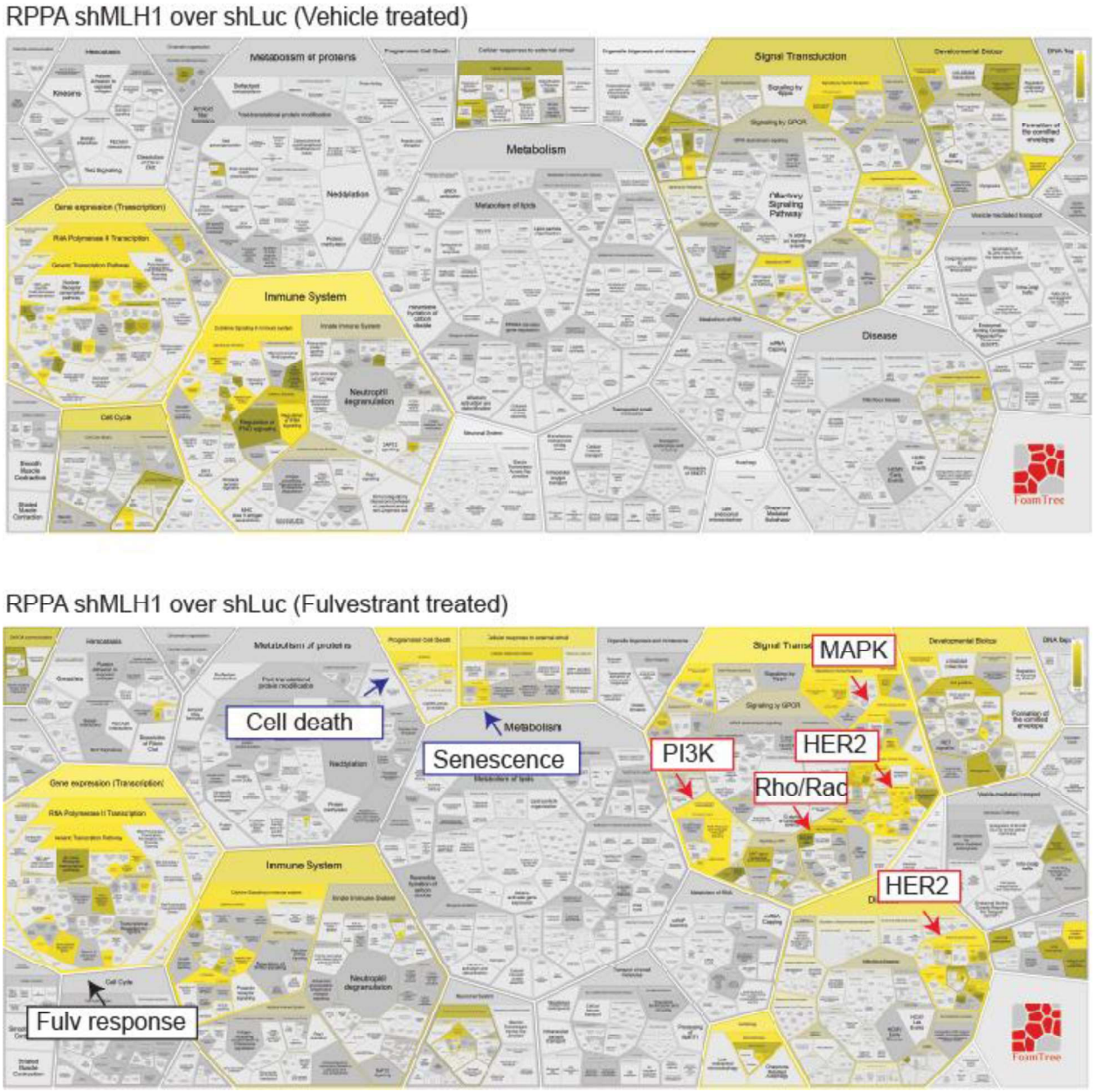
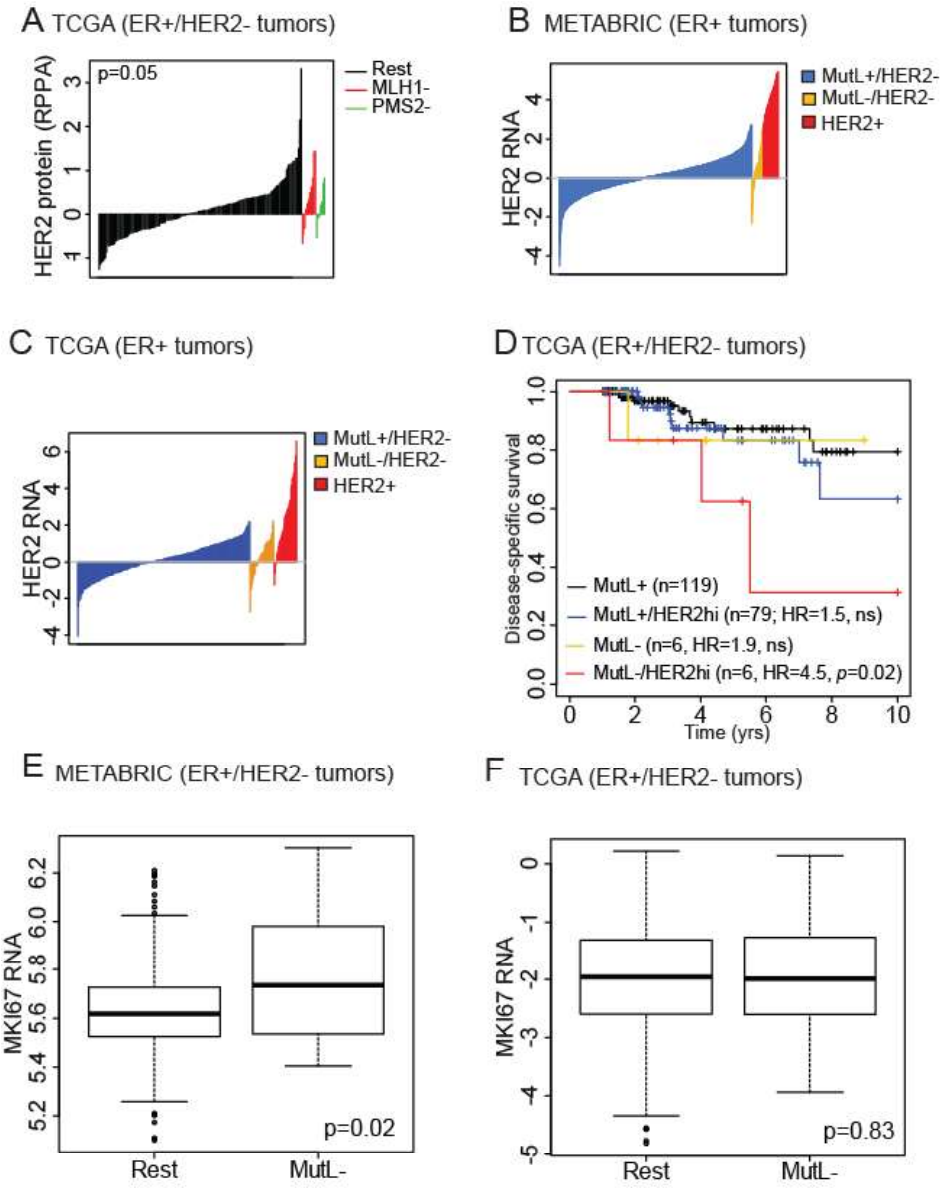
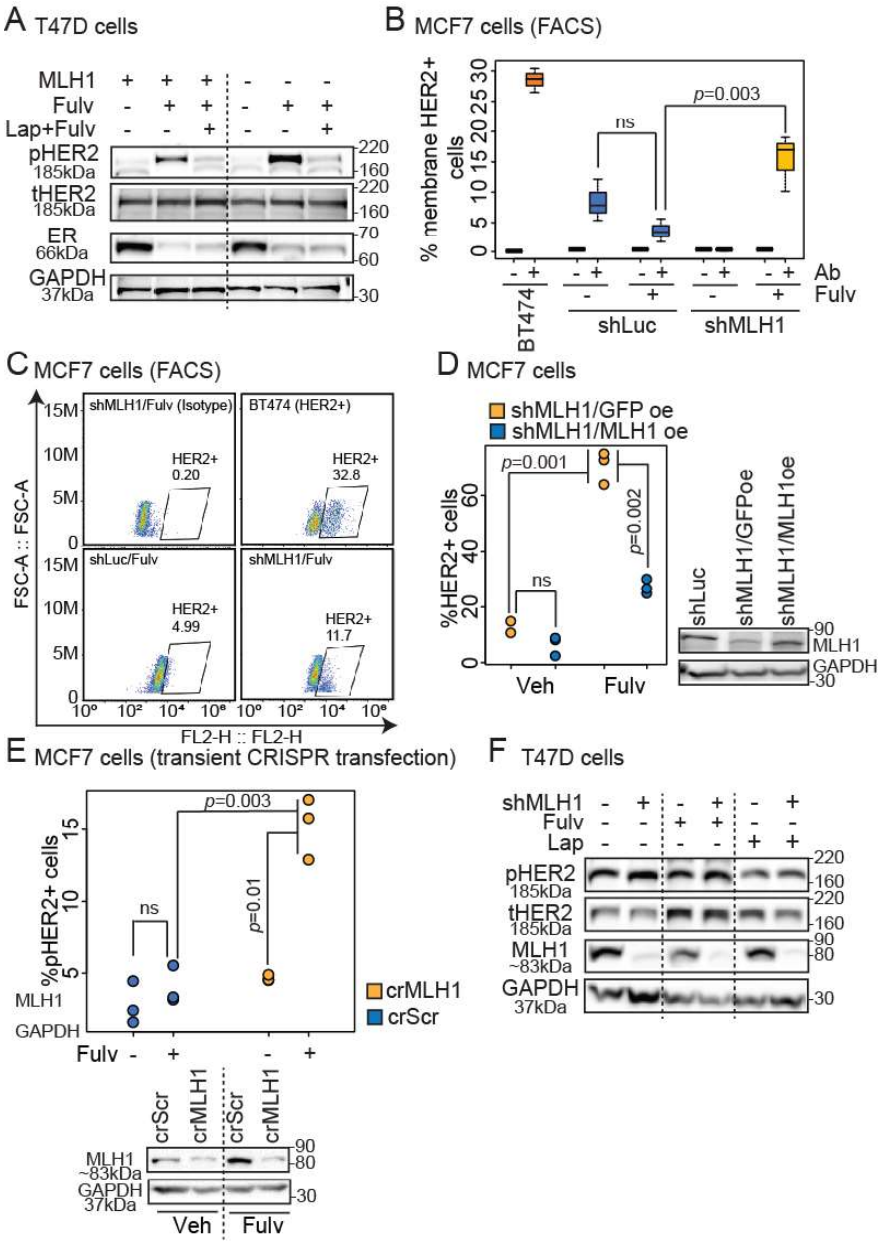
Supplementary Materials:



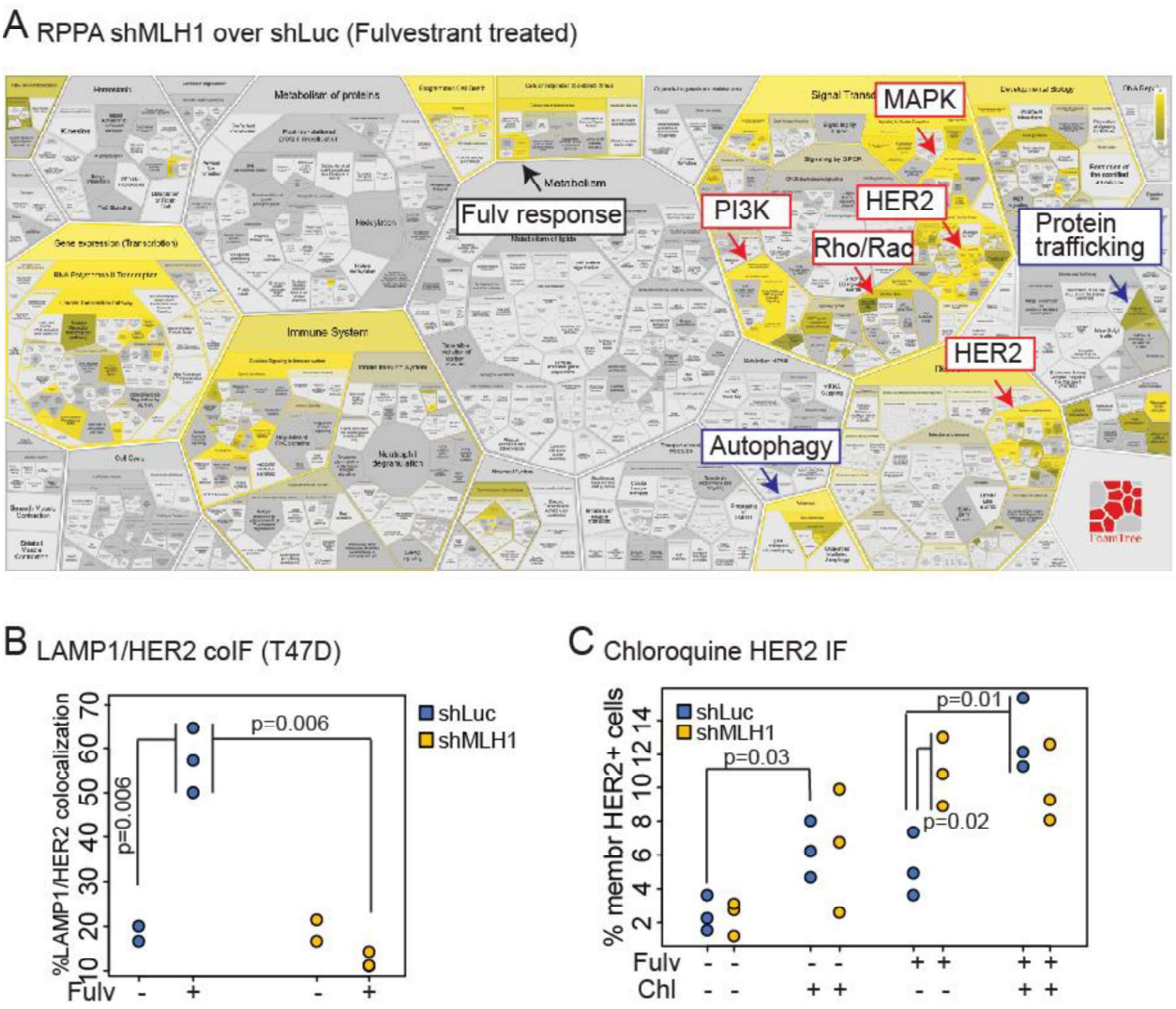
Supplementary Figure 1: ER^{+}, nominally HER2^{-} breast cancer cells engineered to harbor stable RNAi against the MutL complex upregulate HER2 signaling pathways upon treatment with endocrine intervention, fulvestrant. Reactome analysis of RPPA data from MCF7 sh*Luc*, sh*MLH1* and sh*PMS2* cells grown +/- 100 nM fulvestrant and analyzed for proteins whose levels increase specifically in response to fulvestrant in sh*MutL* cells relative to sh*Luc*. P-value generated using two-tailed Student's t-test and corrected for multiple comparison using Bonferroni. All proteins with $p<0.05$ after adjustment for multiple comparison were used in Reactome analysis. Four replicates assayed per cell line per treatment. Source data provided with paper.



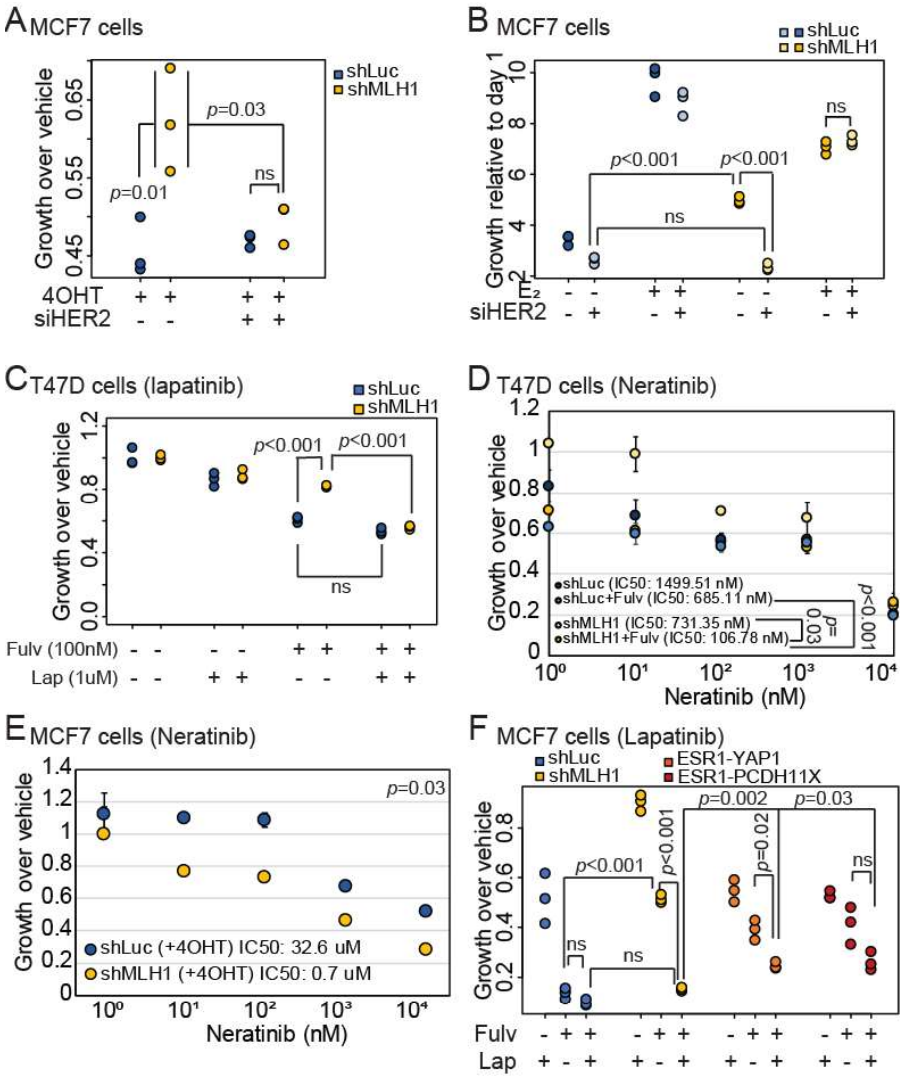
Supplementary Figure 2: ER$^+$, nominally HER2$^-$ breast cancer patients whose tumors are MutL$^-$ have relatively high levels of HER2 and significantly worse disease-specific outcomes. (A) Index plot depicting protein (RPPA) levels of HER2 in MLH1$^-$ (red) and PMS2$^-$ (green) ER$^+$/nominally HER2$^-$ patient tumors from TCGA. MutL$^+$ tumors shown for comparison in black. P-value generated using two-sided Pearson's chi-square test comparing proportion of tumors with positive values for HER2 vs those with negative values in each subgroup. (B-C) Index plots depicting RNA (microarray) levels of *HER2* in MutL$^-$ (gold) and MutL$^+$ (blue) ER$^+$/nominally HER2$^-$ patient tumors from METABRIC (B) and TCGA (C). HER2$^+$ (or amplified) patient tumors (red) are included to provide context. Supports data in Fig 1A. (D) Kaplan-Meier survival curves of indicated groups of patients from TCGA demonstrating differences in disease-specific survival. Cox Regression analysis determined p-values and hazard ratios. Supports data in Fig 1B. (E-F) Boxplots demonstrating comparable levels of gene expression of the proliferation marker, Ki67 in two independent datasets (METABRIC, E and TCGA, F) between MutL$^-$ (n=23 for METABRIC and n=31 for TCGA) and MutL$^+$ (n=594 for METABRIC and n=287 for TCGA) ER$^+$/HER2$^-$ patient tumors. Two-sided Wilcoxon Rank sum test determined p-values. Box plots show median, quartiles, minima and maxima, and outliers at 1.5xIQR. Source data provided with paper.



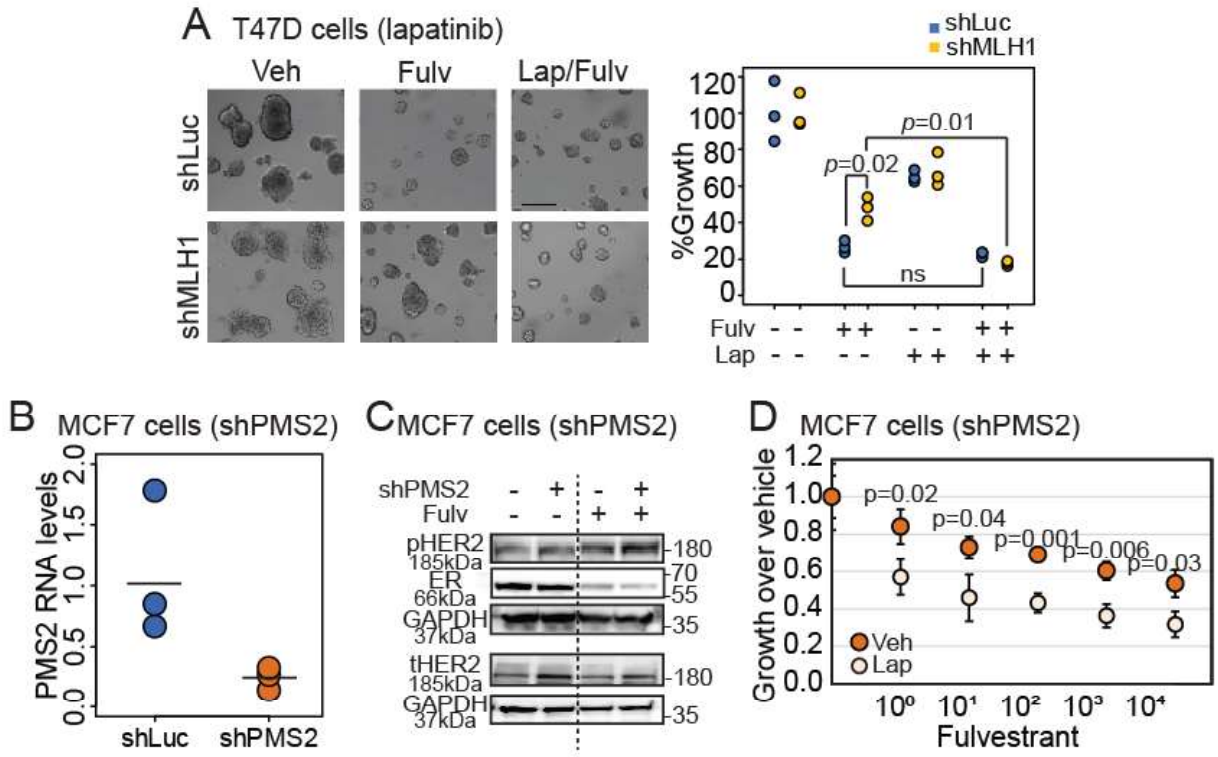
Supplementary Figure 3: MLH1 loss in ER$^+$, nominally HER2$^-$ breast cancer cells upregulates membrane-bound HER2 upon endocrine treatment. (A) Western blot demonstrating increased pHER2 in sh*Luc* and sh*MLH1* T47D cells treated with vehicle, fulvestrant (Fulv) or a combination of lapatinib, a HER inhibitor, and fulvestrant (Lap+Fulv). Supports data in Fig 2A. (B-C) Boxplots representing percent cells with membrane bound HER2 detected by FACS in sh*Luc* and sh*MLH1* MCF7 cells treated with or without fulvestrant (Fulv). Each group has an isotype control (Ab) and BT474, HER2-amplied cells are included as positive control. Accompanying plots demonstrate gating strategy (C). Box plots show median, quartiles, minima and maxima. Supports data in Fig 2B. (D-E) Quantification of HER2 positivity using immunofluorescence with a total HER2 antibody (D) and a pHER2 antibody (E) in MCF7 cells with re-expression of sh-resistant wildtype *MLH1* cDNA (D) or transient transfection with a CRISPR plasmid with sgRNA against *MLH1* (E), represented as strip charts. Accompanying Western blots validating MLH1 re-expression and knockdown respectively. (F) Western blot demonstrating MLH1 levels in T47D cells treated with lapatinib (Lap) to inhibit HER2 signaling. ns, not significant. For panels B-E, three biological replicates were assayed in each group for each cell line. For panel A, two independent replicates were assayed and for panel F, four independent replicates. Two-sided Student's t-test determined p-values.



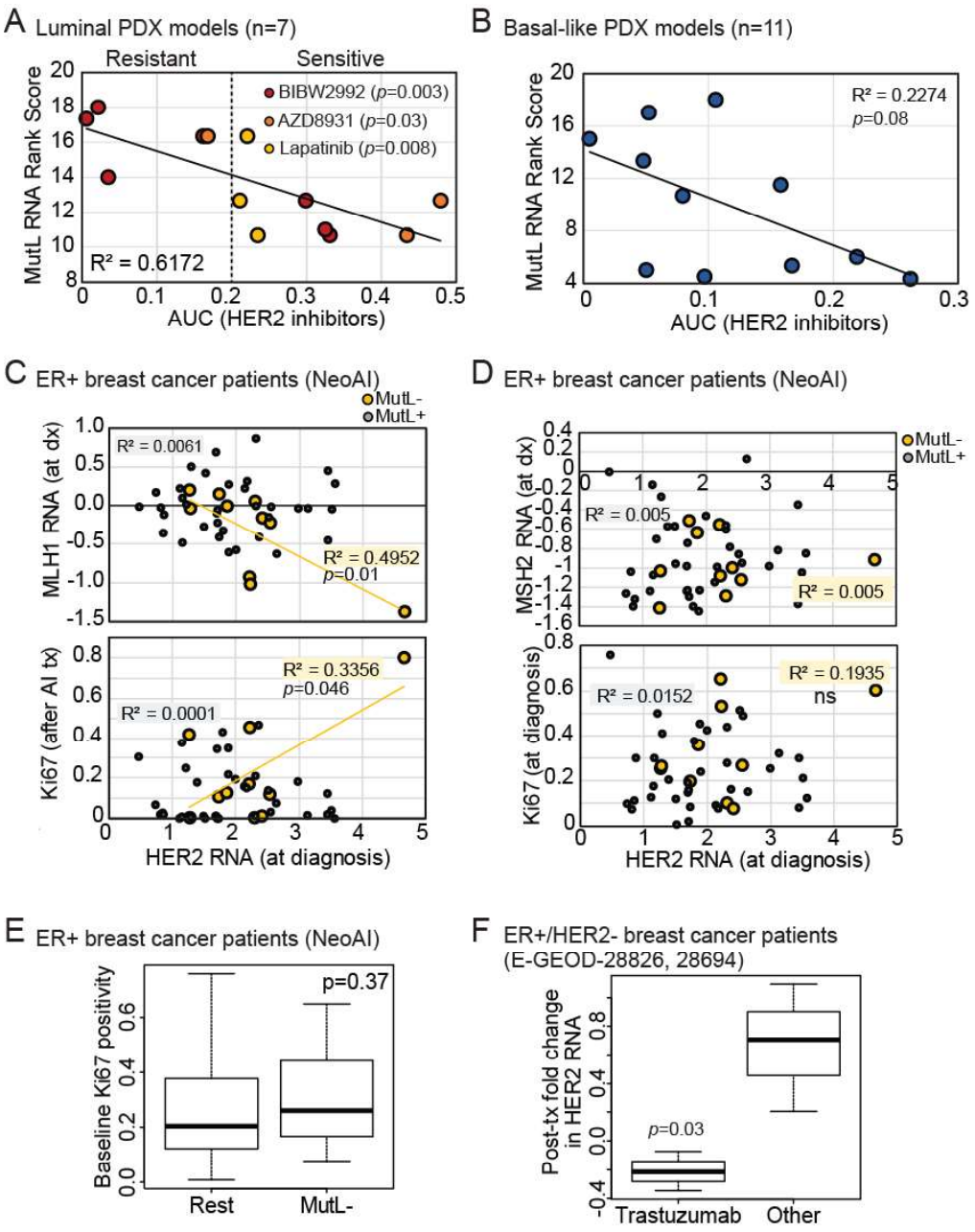
Supplementary Figure 4: HER2 is protected from lysosomal trafficking in ER$^+$ MLH1$^-$ breast cancer cells. (A-B) Reactome analysis of RPPA data demonstrating enrichment for protein trafficking and autophagy pathways in MutL$^-$ MCF7 cells. Corresponding RNAseq data in Fig 3A. (B-C) Strip charts representing quantification of colocalization of LAMP1 and HER2 (B) and membrane HER2 (C) immunofluorescent signaling in shLuc and shMLH1 T47D cells before and after endocrine therapy in the form of 100 nM fulvestrant (Fulv), and with or without treatment with chloroquine, an autophagy inhibitor (C). For panels B and C, three biological replicates were included in each group for each cell line. Corresponding data in MCF7 cells in Figs 3B-C. Two-sided Student's t-test determined all p-values. Source data available with paper.



Supplementary Figure 5: HER2 is required for endocrine therapy resistant growth of ER^+ $MLH1^-$ breast cancer cells. (A-B) Bar graphs representing relative growth of MCF7 sh*Luc* and sh*MLH1* cells transiently transfected with either scrambled siRNA or siRNA against *HER2*, and then treated with tamoxifen (4-OHT, A) or grown in charcoal stripped serum and deprived of estrogen (E_2, B). For panel B, shLuc vs shMLH1 estrogen deprived cells, p=0.0008, and for shMLH1 siScr vs siHER2 estrogen deprived cells, p=2.4e-05. Western blotting validating knockdown and response to fulvestrant presented in Fig 4A-B. (C) Strip chart demonstrating increased sensitivity of T47D sh*MLH1* cells to a combination of fulvestrant (Fulv) and lapatinib (Lap). For shLuc vs shMLH1 fulvestrant treated, p=0.0009 and for shMLH1 fulvestrant vs fulvestrant+lapatinib treated, p=3.15e-05. Supports data presented in Fig 4C. (D-E) Dose response curves of sh*Luc* and sh*MLH1* T47D (D) and MCF7 (E) cells treated with neratinib in combination with fulvestrant (Fulv, D) or tamoxifen (Tam, E). IC50 values were calculated and differences in IC50 from three independent experiments was statistically compared. For shLuc vs shMLH1 fulvestrant treated cells, p=0.0001. Circles represent mean relative growth and error bars the standard deviation. Supports data presented in Fig 4D. (F) Strip chart demonstrating specificity of lapatinib response in $MutL^-$ cells when compared to non-mismatch repair related mechanisms of endocrine therapy resistance (*ESR1* fusions). For shLuc vs shMLH1 fulvestrant treated cells, p=4.46e-05, and for shMLH1 fulvestrant vs fulvestrant+lapatinib treated cells, p=7.37e-05. Three biological replicates included in each group for each cell line. Two-sided Student's t-test determined all p-values. Source data available with paper.



Supplementary Figure 6: HER2 is required for endocrine therapy resistant growth of ER^{+} $MLH1^{-}$ breast cancer cells. (A) 3D growth of sh*Luc* and sh*MLH1* T47D cells in Matrigel shown with representative photomicrographs and accompanying quantification. Scale bars represent 50µ. Supports data presented in Fig 4E. (B-D) Knockdown of *PMS2* in MCF7 cells validated by qRT-PCR (B) with Western blot showing increased HER2 activation in sh*PMS2* cells (C) and increased sensitivity to the combination of HER inhibition (lapatinib, Lap) and endocrine therapy (fulvestrant, Fulv) (D). Two-sided Student's t-test determined all p-values. For panels A, B and D, three biological replicates were included in each group for each cell line. Source data available with paper.



Supplementary Figure 7: MLH1 loss predicts sensitivity to HER2 inhibitors in endocrine therapy resistant ER$^+$, nominally HER2$^-$ breast cancer cells *in vivo* and in patient tumors. (A-B) Regression analysis depicting lack of significant correlation between *MLH1* and *PMS2* RNA levels, and sensitivity to HER2 inhibitors in luminal (A) and basal-like (B) PDX tumors grown *in vivo*. Supports data in Fig 5D-E. (C-D) Regression analysis of correlation between *MLH1* RNA levels at diagnosis (top) and Ki67 levels after treatment with endocrine interventions (bottom) (C) and *MSH2* RNA levels (top) and proliferation marker, Ki67 before endocrine treatment (bottom) (D) with *HER2* RNA levels. For all graphs, linear regression model analysis in R was used to determine R^2 and p-values. (E-F) Boxplots demonstrating comparable levels of Ki67 positivity between MutL$^-$ (n=11) and MutL$^+$ (n=37) ER$^+$/HER2$^-$ breast tumors from the NeoAI database (E) and downregulation of *HER2* RNA levels in response to trastuzumab (n=10), but not in response to anthracyclines or taxanes (Other, n=8, F). Supports data in Fig 5F. Box plots show median, quartiles, minima and maxima, and outliers at 1.5xIQR. Two-sided Wilcoxon Rank Sum test determined p-value. P-values were not adjusted for multiple comparisons. Source data for all panels except those dealing with Z1031 data available with paper. Access to source data from Z1031 trial available upon request with permission from Alliance Clinical Trials.

Supplementary Table 1

Name	Sequence	Catalogue Number
CRISPR MLH1 Target Sequence	Target 3: GATGGAGCGAATATTGTCCA	301901110595
	Target 2: CAGATCCAAGACAATGGCAC	
	Target 1: AGTGGTGAACCGCATCGCGG	
PMS2 Oligo Reverse	TTATCAGTTCTGAGAAATGACACCCAGGTTGG	
PMS2 Oligo Forward	CACCATGGAGCGAGCTGAGAGCTCGAGTA	
esiRNA toward HER2 Target sequence	CCATCTGCACCATTGATGTCTACATGATCATGGTCAAATGTTGGATGATTGACTCTGAATGTCG GCCAAGATTCCGGGAGTTGGTGTCTGAATTCTCCCGCATGGCCAGGGACCCCCAGCGCTTTGT GGTCATCCAGAATGAGGACTTGGGCCCAGCCAGTCCCTTGGACAGCACCTTCTACCGCTCACTG CTGGAGGACGATGACATGGGGGACCTGGTGGATGCTGAGGAGTATCTGGTACCCCAGCAGGG CTTCTTCTGTCCAGACCCTGCCCCGGGCGCTGGGGGCATGGTCCACCACAGGCACCGCAGCTC ATCTACCAGGAGTGGCGGTGGGGACCTGACACTAGGGCTGGAGCCCTCTGAAGAGGAGGCCC CCAGGTCTCCACTGGCACCCTCCGAAGGGGCTGGCTCCGATGTATTTGATGGTGACCTGGGAA T	EHU078751-50UG
PMS2 shRNA	CCGGGGACTATGGAGTGGATCTTATCTCGAGATAAGATCCACTCCATAGTCCTTTTTTG	TRCN0000425805
MLH1 shRNA	CCGGGTGTTCTTCTTTCTCTGTATTCTCGAGAATACAGAGAAAGAAGAACACTTTTTG	SHC007
Luciferase shRNA	CCGGCGCTGAGTACTTCGAAATGTCCTCGAGGACATTTCGAAGTACTCAGCGTTTTT	TRCN0000288641