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CONTRACTING ORGANIZATION: Baylor College of Medicine

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13. SUPPLEMENTARY NOTES					
14. ABSTRACT The key objective of this project was to define a resistance mechanism for treating ER+ breast cancer by endocrine therapy, such as tamoxifen. This project investigated the hypothesis that inactivating the tumor suppressor NF1 (neurofibromin), previously best known as a negative regulator for Ras by acting as a GAP (GTPase Activating Protein), can not only activate Ras, but also ER, by interacting with ER's co-regulators. This is a collaboration between two PIs with complementary expertise. Aim 1 is the main responsibility of Dr. Chang, a molecular biologist, to investigate NF1's interaction with known ER co-regulator. Dr. Ellis is a physician scientist who is responsible for Aim 2 to establish a treatment strategy to treat NF1-deficient ER+ breast cancer. We have made great stride in this study from both aims and the results have been published in a high impact journal <i>Cancer Cell</i> . Briefly, we discovered a surprising GAP-independent activity of NF1 that it is also a co-repressor for ER. As such, inactivating a single tumor suppressor NF1 can activate two powerful oncogenic pathways, which must be co-targeted for effective treatment. To this end, we demonstrate that using patient-derived xenograft model that this can be achieved by FDA-approved fulvestrant in combination with binimetinib, a new generation MEK inhibitor. This treatment strategy is the basis of a NCI ComboMATCH-supported phase-II trial, thus fulfilling the promise of translating laboratory study into clinical practice.					
15. SUBJECT TERMS NF1, Neurofibromatosis type 1, Ras GTPase, GAP, Neurofibromin, estrogen receptor, gene expression, targeted therapy, ER co-regulators, ER-co-repressors.					
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INTRODUCTION

Although great strides have been made in treating estrogen receptor- α positive (ER⁺) tumors, relapse and death is common and closely linked to resistance to ER-targeting endocrine therapies. The overarching **objectives** of this project are to (1) define mechanisms of endocrine therapy resistance, and (2) to design etiology-matched treatments to improve outcomes in estrogen receptor α positive (ER⁺) breast cancer. The focus of this project is the NF1/neurofibromin tumor suppressor, which was best known to repress Ras signaling as a GAP (GTPase Activating Protein). We have evidence supporting the **hypothesis** of this project that NF1 can also directly regulate ER transcriptional activity via interactions with co-activators. This project was funded as a multi-PI grant with Eric Chang as the initiating PI, who is responsible for the molecular study as proposed in Aim 1, and Matthew Ellis as the Patterning PI who is responsible for preclinical therapeutic study using animals as proposed in Aim 2.

These studies have led to a surprisingly discovery that NF1 is not only a Ras GAP but also

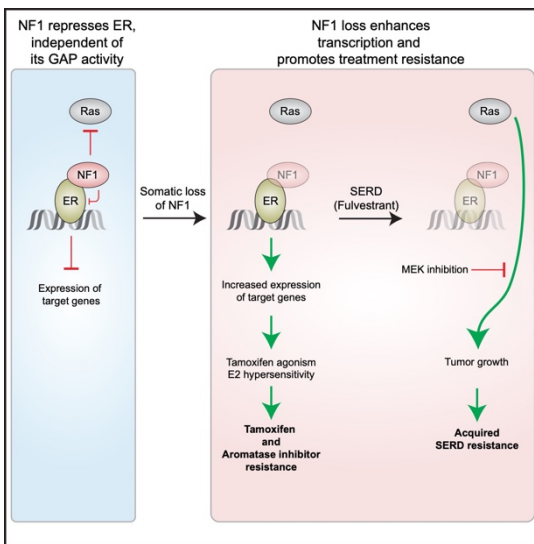


Figure 1. Model as appeared in Zheng et al 2020.

a co-repressor for ER. The paradigm-shifting concept was published in the prestigious journal *Cancer Cell* (Zheng et al, 2020, see “Products”, Appendix). Our public graphic abstract summarizing this work is shown in Figure 1. The loss of a single tumor suppressor NF1 stimulates both the Ras and ER signaling pathways to induce aggressive tumor activities such as resistance to endocrine therapy (tamoxifen and aromatase inhibitor). When these two pathways are properly inhibited by the combination of two FDA-approved drugs, fulvestrant (which induces ER turnover) and binimetinib (which inhibits MEK, a Ras effector kinase), full tumor regression was observed in a patient-derived mouse model.

Our task-specific progress is as follows:

KEYWORDS

NF1, Neurofibromatosis type 1, Ras GTPase, GAP, Neurofibromin, estrogen receptor, gene expression, targeted therapy, MEK inhibition, ER co-regulators, ER-co-repressors.

ACCOMPLISHMENTS

Major Task 1 (responsibility of the Chang lab): To define how NF1 regulates expression of ER target genes by investigating a direct interaction between NF1 and canonical ER transcriptional co-regulators.

We initially thought that NF1 can indirectly regulate ER by interacting with co-activators in the pathway. But we soon realized that NF1 interacts directly with ER as an authentic co-repressor, and our findings have recently been published in *Cancer Cell* (Zheng et al, 2020, PMID32142667, Appendix). Below we will outline key findings described in the paper:

(1) NF1 contains two Leu/IL-rich co-repressor motifs (M1 and M2) that bind ER directly. Unlike the GAP mutations which are infrequently in primary cancers, these co-repressor motifs (Figure 3A, Zheng et al) are subject to mutational inactivation in cancer. NF1 and ER coimmunoprecipitate in several breast cancer cell lines (Figure 3B, Zheng et al), and direct binding between them can be detected using purified ER and an NF1 fragment containing the M2 motif (Figure 3C, Zheng et al). These ER-NF1 interactions are stimulated by 4 hydroxyl-tamoxifen (4-OHT), but weakened by estradiol (E2), suggesting an antagonist-induced direct interaction, as expected of a co-repressor. Finally, 4-OHT, but not E2, stimulated NF1 recruitment to an ERE together with ER, as measured by both an ERE-pull down assay and by NF1-ChIP-qPCR. More NF1-ER interactions can be seen in Supplemental Figure S3 A-C in the same paper (Appendix).

(2) NF1 loss enhances ER-ERE recruitment to promote gene expression. To mimic the *NF1*^{low} state in ER⁺ cancer cells we used both DOX-inducible shRNA and CRISPR/CAS9-mediated knock out (KO) in several ER⁺ breast cancer cell lines and found that when *NF1* is depleted, there is a global increase in ER recruitment to an ERE, as detected by ER ChIP-seq (Figure. 2 C and D, Zheng et al) with validation by qPCR. This increase in DNA binding correlated with genome-wide enhanced expression of E2-responsive genes (Figure 2A, Zheng et al). Note that the great majority of genes upregulated after E2-stimulation in *NF1*⁺ cells were more strongly induced in *NF1* knock-down (*NF1*^{KD}) cells, and the great majority of E2-repressed genes were also more strongly repressed, consistent with bidirectional ER hyperactivity upon NF1-depletion¹⁰. These observations agree with clinical datasets: tumors carrying NS/FS *NF1* mutations in TCGA and METABRIC are enriched with an E2-responsive gene signature (Figure 2B, Zheng et al). More gene expression studies can be seen in Supplemental Figure S2.

(3) Ligand-dependent NF1 nuclear localization. In agreement as being transcription co-repressor, we performed cell fractionation and immunofluorescence microscopy to demonstrate that NF1 shuttles in and out of the nucleus (Figure 4, Zheng et al). More NF1 can be seen in the nucleus in the presence of 4-OHT, supporting the concept that 4-OHT promotes nuclear import of co-repressor. The immunofluorescence was conducted using a monoclonal antibody made by us with support of this grant. This antibody is now licensed and commercially available (Products)

(4) Ligand-dependent NF1 association with chromatin/ERE. We performed ERE-bead pull down and NF1 ChIP-qPCR, to show that in the presence of 4-OHT, more NF1 associates with EREs (Figure 5, Zheng et al). More NF1 ChIP-qPCR using a separate antibody can be found in Supplemental Figure S5.

(5) Repression of ER signaling by NF1 is independent of the GAP function. Site-directed mutagenesis was performed to show that key residues in M1 and M2 are necessary for efficient ER-binding without affecting GAP activity (Supplemental Figure S3 H and I). Conversely, two *NF1* GAP mutants were generated to show that functional GAP activity is not required for ER binding or ER antagonism. These mutants were also ectopically expressed in *NF1*-depleted cells to demonstrate that while the *NF1* GAP mutant, like wild type, can restore the E2-dependent phenotype, the ER-binding defective *NF1* mutant cannot.

One ER-binding (*NF1-I417M*) (mentioned above) and one GAP-inactivating mutant (*NF1-R1362Q*) were selected for detailed studies using CRISPR-mediated knock-in (KI) to replace both copies of endogenous *NF1* in MCF-7 cells (Supplemental Figure S3L). The resulting *NF1* ER-binding domain mutant had normal GAP activity, but defective ER binding; in contrast, the GAP domain mutant bound to ER as efficiently as wild type NF1 protein. While conducting the ER ChIP experiments (Figure 2D, Zheng et al), we observed that more ER was recruited to the chromatin when *NF1* was depleted, even when the cells were seeded in the absence of E2. We further examined these *NF1* mutant cells under E2-depleted conditions by RNA-seq and found that the majority of E2-induced genes were more highly expressed in the *NF1-I417M* mutant vs. wild type or *NF1-R1362Q* mutant (Figure 3 C-G, Zheng et al). Likewise, many E2-repressed

genes were more strongly repressed in the *NF1-I417M* mutant. Essentially, the *NF1-I417M* mutation mimics E2-stimulation.

Major Task 2 (Responsibility of the Ellis lab): To establish a strategy to treat NF1-deficient ER⁺ breast cancers by rationally combining anti-Ras and anti-ER approaches.

(1) Enhanced ER-dependent gene expression induces tamoxifen agonism and E2 hypersensitivity but retains SERD sensitivity. When *NF1* expression was inhibited by either shRNA or CRISPR-mediated knock-out, growth was *stimulated* by 4-OHT; furthermore, *NF1^{KD}* cells were capable of E2-stimulated growth at very low E2 levels (Figure 1 D-F, Zheng et al), which explains why *NF1* inactivation correlated with AI-resistance (Figure 1C, Zheng et al, Appendix) and tamoxifen resistance (Figure 3, Griffith et al, Appendix). Findings of tamoxifen agonism and E2 hypersensitivity *in vivo* were also observed in MCF-7 xenografts. Importantly, *NF1^{KD}* cells were still sensitive to fulvestrant (SERD) exposure, because this agent has no partial agonist activity and degrades ER (Supplemental Figure S6 A and G, Zheng et al).

(2) Co-targeting the ER and Ras pathways in *NF1*-depleted ER⁺ breast cancer cells with a SERD/MEK inhibitor combination. Our data suggest that *NF1^{low}* ER⁺ breast cancer will not be effectively treated with tamoxifen or AI, but fulvestrant could still be effective. However, acquired fulvestrant resistance was consequently observed in the *NF1^{KD}* MCF-7 xenograft model after an initial response (Supplemental Figure S6E, Appendix). The acquired fulvestrant resistance is driven by the Ras-Raf pathway because it is blocked by the combination of a MEK inhibitor (MEKi) trametinib in combination with a Raf inhibitor dabrafenib, a regiment approved by FDA to treat melanoma carrying oncogenic BRAF.

In our efforts to develop a clinical protocol, we found that the dabrafenib and trametinib combination (which is poorly tolerated in patients) can be replaced by a better tolerated single MEKi, such as selumetinib or binimetinib. These drugs are newer generation drugs that are less toxic than trametinib due to a shorter half-life. Importantly, selumetinib (S) is now approved for treating plexiform neurofibromas in pediatric patients with neurofibromatosis, while binimetinib (B) has been approved for melanoma treatment. Our data demonstrated that *in vitro* fulvestrant plus a MEKi strongly inhibited cell growth and induced apoptosis in *NF1*-silenced MCF-7, ZR-75B, and T47D cells whereas either single agent did not (Figure 6B, Appendix). In addition, we conducted a detailed study of WHIM16, an ER⁺ *NF1^{low}* patient-derived xenograft (PDX), which does not have any detectable NF1. WHIM16 was derived from a patient who died from progressive disease despite fulvestrant and AI treatment, so this model represents a late stage patient with acquired endocrine therapy resistance. *In vivo* while the WHIM16 model showed no response to fulvestrant (F) monotherapy, fulvestrant + MEKi (S or B) induced full and persistent tumor regression (even after the drugs were stopped at day 84, Figure 6 C-E, Zheng et al) with no appreciable mouse body weight loss, indicating acceptable toxicity for this combination.

IMPACTS

To effectively deliver targeted therapy, it is indispensable to define all the molecular activities in a tumor. Germline loss of the *NF1* gene is responsible for neurofibromatosis type 1, the world's most common genetic disorder, occurring in 1 out of 3,000 cases. NF1 is also somatically lost in a wide range of tumors. Thus, defining a new function of NF1 can have wide clinical impacts.

In the context of breast cancer, this project has demonstrated that loss-of-function mutations in the *NF1* gene promote tamoxifen agonism and estrogen- deprivation resistance

because NF1 is also a co-repressor of estrogen receptor (ER) therefore loss enhances ER transcriptional activity. While ER⁺ NF1-depleted cells are initially responsive to fulvestrant, a selective ER degrader, enhanced Ras activity promotes acquired resistance. When Ras pathway activation is inhibited with a well-tolerated MEK inhibitor, fulvestrant resistance is reversed and full tumor regression ensues. Thus, NF1-depletion represents a distinct breast cancer subset where the choice of endocrine treatment may be critical, and MEKi combinations require clinical investigation.

To this end, an NCI ComboMATCH-supported Phase-2 clinical trial will be launched to further investigate this strategy. A role as a dual repressor for both Ras and ER may also explain the sexually dimorphic characteristics of neurofibromatosis where tumorigenesis is promoted by female puberty.

CHANGES/PROBLEMS: NA.

KEY PRODUCTS:

Paper:

(1) Obi Griffith, Nick Spies, Meenakshi Anurag, Malachi Griffith, Jingqin Luo, Dongsheng Tu, Belinda Yeo, Jason Kunisaki, Christopher Miller, Kilannin, Krysiak, Jasreet Hundal, Benjamin Ainscough, Zachary Skidmore, Katie Campbell, Runjun Kumar, Catrina Fronick, Lisa Cook, Jacqueline Snider, Sherri Davies, Shyam Kavuri, Eric C. Chang, Vincent Magrini, David Larson, Robert Fulton, Shuzhen Liu, Samuel Leung, David Voduc, Ron Bose, Mitchell Dowsett, Richard Wilson, Torsten Nielsen, Elaine Mardis, Matthew J. Ellis. 2018. The prognostic effects of somatic mutations in ER-positive breast cancer, *Nature Communications*. PMID: 30181556. PMCID: PMC6123466.

(2) Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafaei, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis[†], Eric C. Chang[†]. 2020. Neurofibromin is an Estrogen Receptor α Transcriptional Corepressor in Breast Cancer. PMID: 32142667, PMCID:7286719. [†]Equal contribution. See Appendix for the main paper and supplemental data.

(3) Dr. Foulds is a key member of the team and was able to use the support of this grant to publish a paper focusing on profiling co-regulators associated with mutant ER:

Gates LA, Gu G, Chen Y, Rohira AD, Lei JT, Hamilton RA, Yu Y, Lonard DM, Wang J, Wang SP, Edwards DG, Lavere PF, Shao J, Yi P, Jain A, Jung SY, Malovannaya A, Li S, Shao J, Roeder RG, Ellis MJ, Qin J, Fuqua SAW, O'Malley BW, Foulds CE. 2018. Proteomic profiling identifies key coactivators utilized by mutant ER α proteins as potential new therapeutic targets. *Oncogene*, PMID: 29748621, PMCID: PMC6095836.

Meeting/seminar presentations:

Invited talk:

(1) Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafaei, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis[†], Eric C. Chang Direct regulation of estrogen receptor- α transcriptional activity by NF1. San

Antonio Breast Cancer Symposium 2017 as a platform speaker. San Antonio, TX.

(2) Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafae, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis^{1†}, Eric C. Chang. Neurofibromin is an Estrogen Receptor Alpha Transcriptional Corepressor in Breast Cancer. Hormone dependent cancers Gordon Research Conference, 2017.

(3) Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafae, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis^{1†}, Eric C. Chang. Neurofibromin is an Estrogen Receptor Alpha Transcriptional Corepressor in Breast Cancer. Metastatic Breast Cancer Conference, 2017.

(4) Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafae, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis, Eric C. Chang. Neurofibromin is an Estrogen Receptor Alpha Transcriptional Corepressor in Breast Cancer. GeneMed, 2018.

(5) Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafae, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis^{1†}, Eric C. Chang. Neurofibromin is an Estrogen Receptor Alpha Transcriptional Corepressor in Breast Cancer. “NF1/Neurofibromin is a co-repressor for ER α transcriptional activity” Drug Development joint Symposium, by MD Anderson Cancer Center and BCM, 2019.

(6) Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafae, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis^{1†}, Eric C. Chang. Neurofibromin is an Estrogen Receptor Alpha Transcriptional Corepressor in Breast Cancer “NF1/Neurofibromin is a transcription co-repressor for estrogen receptor- α in ER⁺ breast cancer,” NCI SPORE bi-monthly teleconference, 2019.

(7) Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafae, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis^{1†}, Eric C. Chang. Neurofibromin also has a GAP-independent activity, acting as a transcriptional co-repressor for estrogen receptor-alpha. NF International Conference. 2019.

(8) Eric Chang and Matthew Ellis. Seminar speaker for the SCC Excellence in Cancer Research Seminar. University of Oklahoma Heath Science Center. Dec. 2020. Title: “NF1/neurofibromin, more than just a Ras GAP in breast cancer and beyond — a lesson learned from patients.” 2020.

Additional meeting presentations:

(1) Zeyi Zheng, Philip Lavere, Burcu Cakar, Johnathan Lei, Purba Singh, Joseph Toonen, Svasti

(2) Haricharan, Meenakshi Anurag, Daniel Yan, Khushboo Shah, Doug Chen, Xi Chen, David Gutman, Charles E. Foulds, Matthew J. Ellis, and **Eric C. Chang**. Regulation of estrogen receptor- α by NF1. SABCS. 2016.

(3) Zheng, Zeyi^{*}; Lavere, Philip[‡]; Cakar, Burcu^{*}; Lei, Johnathan^{*}; Singh, Purba^{*}; Anurag, Meenakshi^{*}; Chan, Doug^{*}; Li, Jing^{*}; Chen, Xi[‡]; Banks, Kimberly[‡]; Lanman, Richard B.[‡]; Foulds, Charles E.^{‡§}; Ellis, Matthew J.^{*†}; **Chang, Eric C.**^{*‡†} Regulation of estrogen receptor- α activity by NF1. Dan Duncan Cancer Center Symposium. 2017

(4) Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar³, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly Banks³, Richard B. Lanman³, Maryam Nemati Shafae¹, Susan Hilsenbeck¹, Charles E. Foulds^{2, 4}, Matthew J. Ellis^{1†}, **Eric C. Chang**. Direct regulation of estrogen receptor- α transcriptional activity by NF1. AACR, 2018.

(5) Zheng ZY, Li J, Li F, Zhu Y, Cui K, Wong ST, **Chang EC** and Liao YH. Target N-Ras for degradation by flunarizine to treat basal-like breast cancer. AACR San Antonio Breast Cancer Symposium, San Antonio, TX. 2018

(6) Peng J, Zheng ZY, Cakar B, Li J, Singh P, Szafrain AT, Stossi F, Dubrulle J, Mancini MA, Mao R, Miles G, Ellis MJ and **Chang EC**. Developing an immunohistochemistry protocol to detect neurofibromin as an effective, simple, and rapid method to identify NF1-negative breast cancer patients. AACR San Antonio Breast Cancer Symposium, San Antonio, TX. 2018

(7) Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafae, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis^{1†}, Eric C. Chang. 2019. Neurofibromin is an Estrogen Receptor α Transcriptional Corepressor in Breast Cancer. Cell Hallmarks of Cancer Symposium.

(8) Ze-Yi Zheng^{1*}, Hanan Elsarraj^{2*}, Yan Hong², Yichao Shen¹, Flora Lo¹, Long Feng¹, Xian H. -F. Zhang¹, Fariba Behbod^{2‡}, Eric C. Chang. N-Ras as a marker for DCIS progression. San Antonio Breast Cancer Symposium 2020. San Antonio, TX.

Invention/commercial licensing: mAb against NF1. BCM Invention Disclosure #18-002 with this grant listed as one of the funding sources (Appendix). This antibody was later licensed to Millipore-Sigma, Cat #MAB2557.

Grants: totaling \$3.7 M in direct cost

With the support of this DOD grant, we have obtained the following grants, including a SPORC from NCI.

(1) Breast Cancer SPORC developmental project at BCM with a direct cost of \$50,000 for one year.

(2) A high risk/reward grant from Cancer Prevention and Research Institute of Texas (CPRIT) to investigate another aspect of NF1's biology. (RP180844- Total direct cost: \$190,000)

(3) This DoD has also helped to receive an R21 from NIH to investigate how Ras may promote DCIS progression in breast cancer. (R21CA226567- Total direct cost: \$281,405)

(4) DoD Expansion Award (PI: Chang) to investigate NF1's role in bone metastasis. (W81XWH-19-1-0527-Total direct cost: \$493,734)

(5) Project-1 in an NIH SPORE entitled "Translational research in breast cancer." (P50CA186784-Total direct cost (PJ1): \$1,169,861)

(6) An DoD Level-2 award entitled "Therapeutic Targeting of Nuclear Hormone Receptors in Neurofibromin/NF1-Depleted Breast Cancer" to investigate the possibility that NF1 can also regulate the androgen receptor, which is structurally related to ER. (W81XWH-21-1-0106-Total direct cost: \$1,020,000)

(7) An DoD Expansion Award (PI: Ellis) to establish proteomic technology to assist in identifying NF1-deficient tumors to support the clinical trial. (Total direct cost: \$500,000)

Clinical trial:

The results from this project have been translated into a Phase-II clinical trial to treat metastatic ER⁺ NF1-depleted patients by the fulvestrant and binimetinib combination with the support from the NCI ComboMATCH program (Protocol # EAY191-N2, Appendix). This trial is through CTEP, and the NRG Cooperative group is led by Baylor. The PI on the trial is Dr. Bora Lim, Associate Director of Clinical Science in our Breast Center. We are awaiting the final approval from CTEP.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Name	Project role	ORCID ID	Person Mon worked	Project contribution	Funding support
Eric Chang	PI	0000-0002-1375-5088	3.6	Design and execute all the studies in Aim 1, and write the paper.	This grant.
Matthew Ellis*	Partnering PI	0000-0002-8467-8534	0.24	Design and execute the studies in Aim 2, and write the paper.	This grant.
Purba Singh*	Tech	NA	3.0	Conduct animal Studies under the direction of Dr. Ellis in Aim 2.	This grant.

*Personnel supported by Ellis's portion of the grant.

SPECIAL REPORTING REQUIREMENTS

None.

APPENDIX:

- (1) Griffith et al as published in *Nature Communications*.
- (2) Zheng et al as published in *Cancer Cell*.
- (3) Gates et al as published in *Oncogene*.
- (4) Supplemental data in Zheng et al.
- (5) ComboMATCH approval letter.
- (6) Invention Disclosure from BCM regarding the creation of NF1 mAb.

ARTICLE

DOI: 10.1038/s41467-018-05914-x

OPEN

The prognostic effects of somatic mutations in ER-positive breast cancer

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Here we report targeted sequencing of 83 genes using DNA from primary breast cancer samples from 625 postmenopausal (UBC-TAM series) and 328 premenopausal (MA12 trial) hormone receptor-positive (HR+) patients to determine interactions between somatic mutation and prognosis. Independent validation of prognostic interactions was achieved using data from the METABRIC study. Previously established associations between MAP3K1 and PIK3CA mutations with luminal A status/favorable prognosis and TP53 mutations with Luminal B/non-luminal tumors/poor prognosis were observed, validating the methodological approach. In UBC-TAM, *NF1* frame-shift nonsense (*FS/NS*) mutations were also a poor outcome driver that was validated in METABRIC. For MA12, poor outcome associated with PIK3R1 mutation was also reproducible. DDR1 mutations were strongly associated with poor prognosis in UBC-TAM despite stringent false discovery correction ($q = 0.0003$). In conclusion, uncommon recurrent somatic mutations should be further explored to create a more complete explanation of the highly variable outcomes that typifies ER+ breast cancer.

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While recent genomic studies have provided a comprehensive catalog of genes that accumulate somatic point mutations and small insertions/deletions (indels) in estrogen receptor-positive (ER+) breast cancer, there remains considerable uncertainty as to how these newly discovered mutations relate to disease outcomes^{1–3}. Most genomic discovery cohorts were neither uniformly treated nor followed long enough. For ER+ disease in particular, prognostic studies require prolonged observation since relapses often occur after 5 years⁴. Uniform treatment was a feature of a whole-genome sequencing study of samples accrued from a neoadjuvant aromatase inhibitor (AI) clinical trial for ER+ clinical stage 2 or 3 disease, although only short-term anti-proliferative response to AI was reported. This investigation identified that mutations in *MAP3K1*, a tumor suppressor gene involved in stress kinase activation, were associated with indolent biological features and low proliferation rates⁵. The resulting hypothesis was that *MAP3K1* mutation would be associated with favorable outcomes. In contrast, *TP53* mutations associated with poor prognosis features and high proliferation rates.

To more comprehensively address the relationships between somatic mutations and outcomes in ER+ breast cancer, we report herein an approach to detect somatic mutations in DNA isolated from formalin fixed tumor blocks that were over 20 years old. After curating existing mutational data from breast cancer genomics discovery studies (Supplementary Data 1), 83 genes were chosen for analysis (Supplementary Table 1). We applied DNA hybrid capture, sequencing and somatic analysis to three ER+ breast cancer discovery cohorts with contrasting clinical characteristics: an older cohort treated with adjuvant tamoxifen and no chemotherapy (UBC-TAM series⁶), a premenopausal cohort uniformly treated with chemotherapy and randomized to tamoxifen versus observation (NCIC-MA12 clinical trial⁷); and a third mixed cohort that was used to expand the mutational landscape analysis (POLAR) (Supplementary Table 2). We report an analytical pipeline to identify somatic variants while compensating for the lack of matched normal DNA, which is generally unavailable in the setting of older formalin-fixed tumor material. Somatic mutations were analyzed for association with standard clinical variables, wherein mutated *TP53* and *MAP3K1* served as a priori hypotheses for poor and good outcome, respectively. Additional objectives were to identify new mutational hotspots, assess interactions with PAM50-based intrinsic subtypes and to determine mutation frequencies for therapeutic targets. Validation was possible by comparing our results to those in cBioPortal where the genes sequenced in the METABRIC cohort overlapped with the 83 genes investigated in the study described herein. We report consistent associations between NF1 frameshift/nonsense (FS/NS) and non-synonymous PIK3R1 mutations and poor outcomes that is independent of standard prognostic variables. DDR1 mutations were also associated with poor outcomes in the UBC-TAM series and significant despite stringent false discovery correction. We conclude that uncommon recurrent somatic mutations should be further explored to create a more complete explanation of the highly variable outcomes that typifies ER+ breast cancer.

Results

Sequencing and final study cohorts. University of British Columbia Tamoxifen Series (UBC-TAM): these cases were drawn from a well-annotated cohort of patients treated with adjuvant tamoxifen without chemotherapy⁶. A total of 625 of 632 (98.8%) patient samples that fully met study criteria passed a minimum sequencing quality cutoff of at least 80% of targeted exonic bases covered at greater than 20× with other quality metrics described

in the supplementary data (Supplementary Figure 1–5 and Supplementary Data 2). Mean depth was correlated with input DNA and negatively correlated with time since diagnosis (approximate age of sample) and duplication rates were negatively correlated with input DNA and positively correlated with sample age. However, despite these trends, overall metrics were excellent with an average of 135.8× coverage and 3.0% duplicate rate despite the generally low input amounts and old sample age. The final patient population had an average age of 67 at diagnosis (range: 40–89+). All were treated with five years of adjuvant tamoxifen, and were primarily postmenopausal, grade 2 or 3 cancers, of ductal histologic subtype (Supplementary Table 2). All were ER+ (>1% cells positive by IHC) and at least 88.6% were clinically HER2- (13/625 unknown). A subset of 463 of these patients had PAM50 subtyping data available from a previous study⁶. The median follow up in the cohort examined was 25 years and one month.

NCIC-MA12 Trial cohort: these cases were drawn from a clinical trial in premenopausal women treated with a standard adjuvant chemotherapy regimen and randomized to tamoxifen versus observation. A total of 459 patient samples passed the minimum sequencing quality threshold (mean coverage: 200×), of which 328 were hormone receptor positive (HR+; >1% cells positive for ER or PR by IHC), and only the HR+ cohort were included here for most analyses. The majority were premenopausal (mean age of 45). All patients received chemotherapy, and 48% were treated with 5 years of adjuvant tamoxifen. A subset of 255 of these patients had PAM50 subtyping data available. The median follow up in the cohort examined was 9.7 years.

POLAR cohort: this patient series was a case-control study of ER+ (>1% cells positive by IHC) breast tumors, 175 of 194 (90.2%) patient samples passed minimum sequencing quality thresholds (mean coverage: ×75). A case was defined as any patient who relapsed during follow-up, and controls were defined as lacking relapse through a similar follow-up duration. Based on these definitions, there were 91 cases and 84 controls. Of the cases, 43 were early relapses (<5 years since diagnosis) and 48 were late relapses (>5 years). Patients were only included if they received adjuvant endocrine therapy, but chemotherapy was not an exclusion criterion, nor was menopausal status. Because the POLAR study was a case-control design, outcome data could not be easily integrated into prognostic analysis. Therefore, these cases were used in the mutation landscape and hotspot analyses only.

Across the three cohorts, there were 1259 patient samples that passed minimum sequencing quality thresholds and 1128 of these were ER+ (UBC-TAM and POLAR) or ER and/or PgR+ (HR+) (MA12).

Variant calling and filtering. A total of over 62 million variants were identified in UBC-TAM. After extensive filtering against a set of nearly 70,000 unmatched normal samples and manual review to eliminate common polymorphisms and false positives (see methods), 1991 putative somatic variants were identified (0 to 26 variants per patient). A set of 1693 mutations was defined as the “non-silent” set for further analysis that excluded sequencing variants in splice regions (except proximal splice site), RNA genes (except *MALAT1*), UTRs, introns, and all silent mutations. Finally, a set of 408 frameshift or nonsense mutations was defined. The same filtering method was applied to both the POLAR and MA12 data sets. A total of 540 putative somatic mutations (436 non-silent, 145 FS/NS) were identified in POLAR, and 2104 (1753 non-silent, 610 FS/NS) in MA12. Full details on these variants are included in Supplementary Data 3 and summarized for key genes in Supplementary Figure 6.

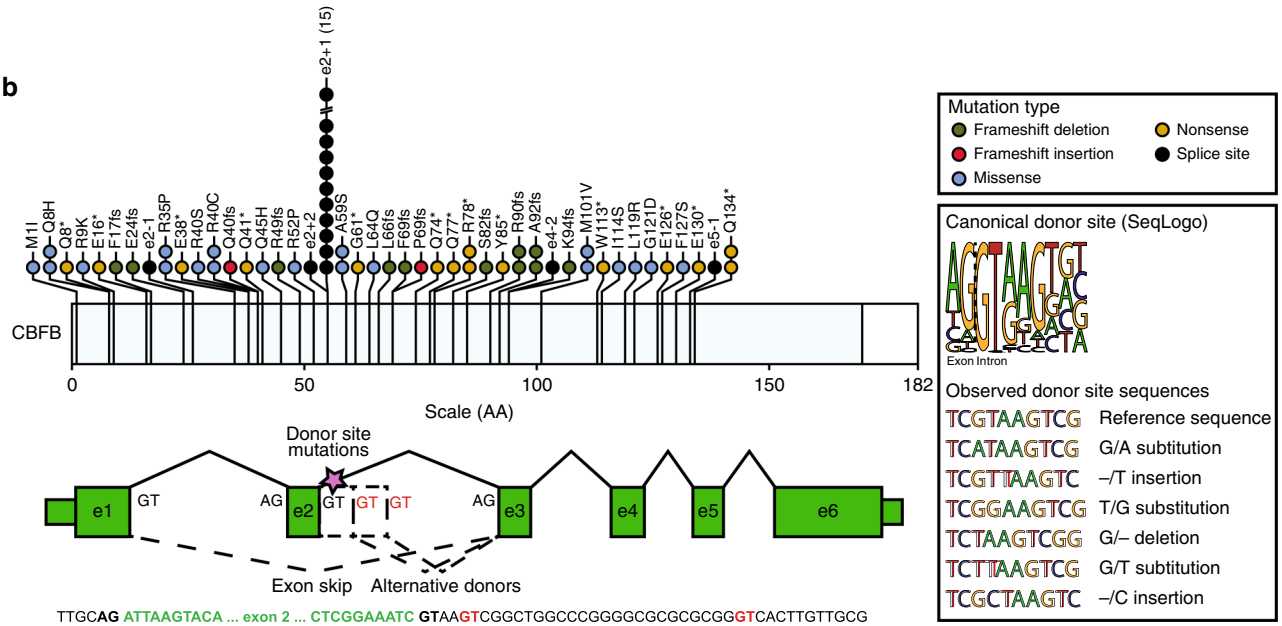
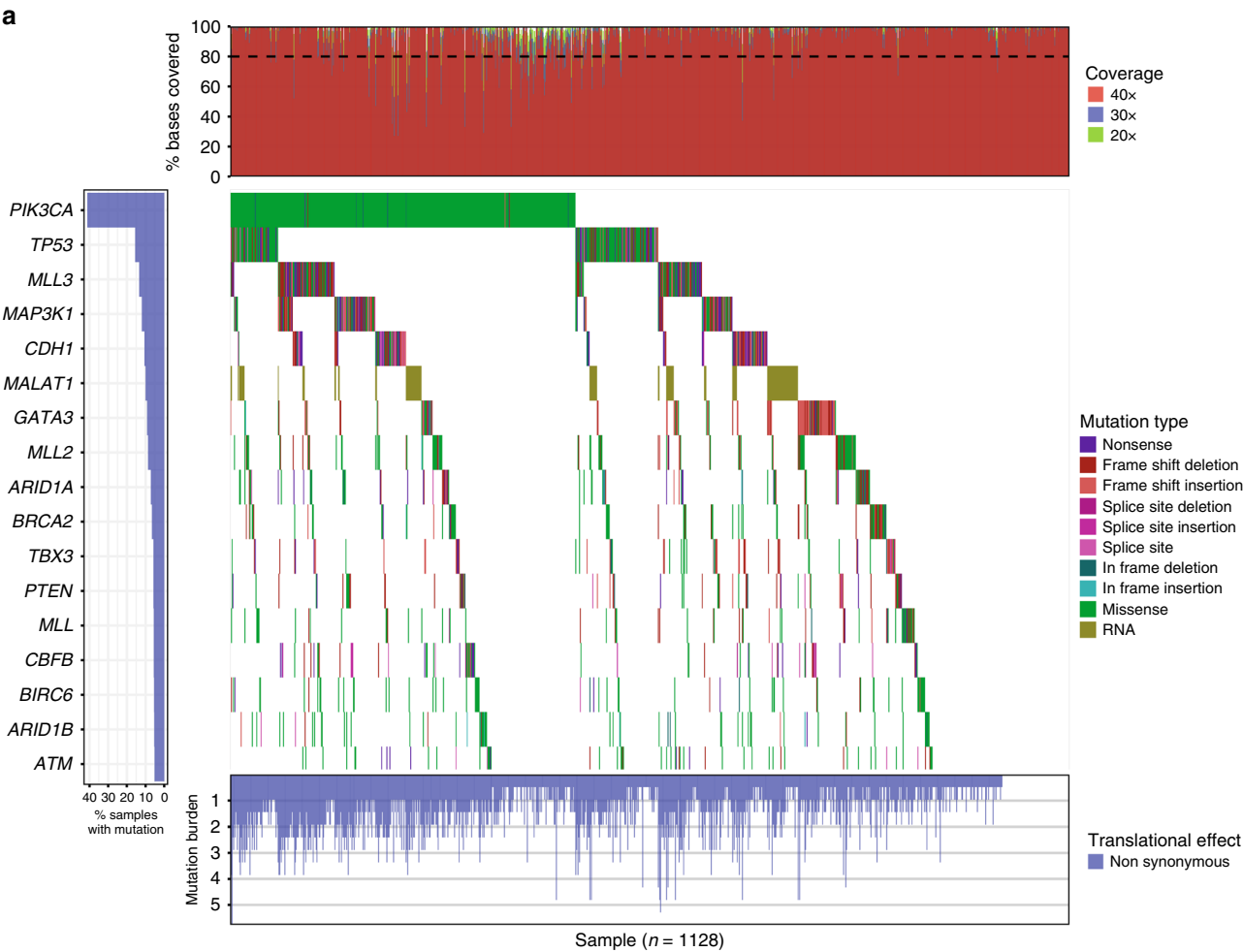
Mutation landscape analysis. In 1128 samples passing quality control standards, considering only non-silent mutations, 17 genes were mutated at a rate greater than 5%, and 6 at a rate greater than 10%; *PIK3CA* was the only gene mutated in greater than 20% of samples (Fig. 1a). The order from most recurrent to least for the 10 most frequently mutated genes was: *PIK3CA* (41.1%), *TP53* (15.5%), *MLL3* (13.4%), *MAP3K1* (12.0%), *CDH1* (10.5%), *MALAT1* (10.0%), *GATA3* (9.1%), *MLL2* (8.7%), *ARID1A* (7.2%), and *BRCA2* (6.6%). This list correlates well with previously reported recurrently mutated genes. For example, the top 4 most significantly mutated (non-silent) genes in the ER+ subset of TCGA breast project³ were *PIK3CA* (24.0%), *TP53* (14.6%), *GATA3* (8.6%), and *MAP3K1* (6.1%). Considering METABRIC ER+ patients, the most recurrently mutated genes were *PIK3CA* (~46%), *TP53* (~21%), *GATA3*, *MLL3*, *CDH1*, and *MAP3K1* (all ~12–14%) demonstrating slightly higher but very similar frequencies. The overall average non-silent mutation frequency was estimated as 1.6 per MB of coding sequence (range: 0.5–5.8 mutations per MB, excluding samples with no mutations called). In order to determine whether mutations in any gene pair were mutually exclusive or co-occurring in this data set, a pairwise Chi-squared or Fisher's exact test was performed. Mutations in *PIK3CA* and *MAP3K1* were significantly more likely to co-occur (after BH FDR correction) in the TAM dataset, and were near significance in MA12 although not after correction ($p = 0.08$). These results are summarized in Supplementary Data 4.

Hotspot analysis. As anticipated⁸, mutations in *PIK3CA* at E542K, E545K, and H1047R were highly recurrent in this study with 69/1259 (5.5%) E542K, 104 (8.3%) E545K, and 181 (14.4%) H1047R mutations (Supplementary Figure 6C). Mutations in the ligand-binding domain of *ESR1* (1.1%) were extremely rare^{3,9} (Supplementary Figure 6A). To uncover novel hotspots in these data, both Chi-squared and Fisher's exact tests were performed using mutation frequencies from previous sequencing studies as the expected values (see Methods for definition of multi-study MAF file) (Supplementary Table 3). The most notable novel finding was in *CBBF* (Fig. 1b). At least six different genomic alterations were observed in 15 patients (Supplementary Data 3) that affected the donor splice site of exon 2. Manual review of this splice site identified at least two additional patients with evidence for mutations at this location. The predicted effect of these mutations is skipping of exon 2 or alternate donor site usage, each likely resulting in loss-of-function of the *CBBF* protein. Additional splice site mutations were observed at the exon 2, exon 4, and exon 5 acceptor sites of *CBBF*. *ErbB2* exhibited the anticipated profile of activating mutations from earlier publications¹⁰.

Somatic mutation association with PAM50-based intrinsic subtype. PAM50 intrinsic subtype calls were obtained from previously published analyses to compare to their mutational profiles for UBC-TAM and MA12 (HR+ only) studies. In both studies about half the patients had luminal A tumors. However, the MA12 cohort had a higher proportion of non-luminal subtypes, with 19.8% HER2-E and 6.6% basal and fewer luminal B tumors (25.1% versus 42.4%) (Fig. 2a, b). As expected, patients with the HER2-E intrinsic subtype were enriched for HER2+ve status compared to other subtypes (Fisher's exact test $p < 0.0001$). Of interest, in the HER2-enriched group there were 51 tumors that were not HER2 amplified and of these 4 were HER2 mutant (~8%), indicating that HER2 mutation could be an occasional explanation for a HER2-E subtype assignment in the absence of HER2 amplification. For NF1 FS/NS mutations, there was also a statistically significant association with the HER2-E subtype ($p = 0.002$) (Supplementary Figure 7B and Supplementary Data 5).

Notably NF1 non-silent mutations were enriched in the HER2-E non-HER2 amplified subgroup, where they were present in 8/51 cases (16%). Compared to the frequency in all other subtypes 12/582 (2%), this enrichment was significant (Fishers exact test $p < 0.0001$) (Supplementary Figure 7A left panel). This association could be reproduced in the METABRIC data with an NF1 non-silent mutation incidence in the HER2-E non-HER2 amplified group of 8/80 (10%) versus 35/1283 (3%) in the rest of the subtypes ($p = 0.003$) (Supplementary Figure 7A right panel). Age-density plots by subtype serve to emphasize the large difference in the median age between the two sample cohorts (43 versus 65), and also the influence of age with respect to the intrinsic subtype incidence. Namely, in the older UBC-TAM cohort, an influence of age on intrinsic subtype was not observed (Fig. 2c). In contrast, in the younger MA12 cohort, there is a younger peak incidence with basal-like breast cancer than Luminal A disease (Fig. 2d). Relationships between intrinsic subtype and mutation patterns were also explored, classifying mutation positive status as “non-silent”, “missense”, nonsense/frame-shift (FS/NS), or FS/NS+ splice site (Supplementary Data 5). The FDR corrected p -value (q -value) took into account that 83 genes were examined. However, this level of false discovery detection could be viewed as overly conservative in an exploratory analysis. Therefore, any gene mutation with q -value association of < 0.2 was considered reportable for the purposes of subsequent validation efforts^{11–13}. For MA12, non-silent TP53 mutation was highly subtype-associated because of the very high incidence in non-luminal versus luminal subtypes. *PIK3CA* and *MAP3K1* mutations were associated with Luminal A disease in both cohorts (Supplementary Figure 7B). Finally, there was a strong association between Luminal B status and non-silent (Supplementary Figure 7C), as well as FS/NS mutations in *GATA3* (Supplementary Data 5, q -value = 0.006) for MA12 (but not UBC-TAM). *GATA3* mutations were present in 28–30% of Luminal B cases and less so in luminal A cases (5%). Considering q values of < 0.2 the associations between FS/NS and non-silent mutations in *ATM* and Luminal B tumors in MA12 (8–13%) suggests that *ATM* disruption is also a possible luminal B driver (Supplementary Figure 7C), at least in younger women (MA12). Relationships between age and mutation incidence were therefore also explored (Supplementary Figure 7D), with the finding that both *ATM* mutation and *GATA3* mutations were associated with an earlier age of onset within the luminal B category (Fig. 2e, f). Some *ATM* mutations are likely to be germline (see discussion below), which could partially explain the association with younger age.

Survival analysis according to somatic mutation. For the UBC-TAM Series (Fig. 3a) univariate analysis, favorable prognostic associations for breast-cancer-specific survival (BCSS) were detected for non-silent mutations in *MAP3K1*, *ERBB3*, *XBP1*, and *PIK3CA* (Fig. 3b, Supplementary Data 6). Adverse prognostic effects were observed for non-silent mutations in *DDR1* and *TP53*, as well as for frame-shift and nonsense (FS/NS) mutations in NF1. An analysis for recurrence-free survival (RFS) produced similar results, except for *ARID1B*, which was marginally associated with more favorable outcome. A multivariate Cox model was applied to put each gene in the context of clinical parameters (grade, tumor size, and node status). These analyses indicated that the prognostic effects of non-silent *DDR1*, *PIK3CA*, *GATA3* FS/NS, *TP53*, and *MAP3K1* mutations were independent of grade and pathological stage (Fig. 3c). Multiple correction testing, yielded *DDR1* as the only gene that remained significant with a q -value of 0.0003 (Supplementary Data 6). For the MA12 clinical trial cohort (Fig. 4a) we focused on overall survival associations, as this was the primary endpoint of the study and the most robust



endpoint. A number of rarely mutated genes were associated with poor outcome in univariate analysis as displayed in Fig. 4b. Multiple testing corrections indicated none of these findings could be considered significant^{11–13}. However, in multivariate analysis, based on the uncorrected *p*-value, the prognostic effects of mutations in ErbB2, ErbB4, LTK FS/NS, MAP3K4, PIK3R1,

RB1, RELN, and TGFB2 were independent of pathological stage and grade (Fig. 4b).

Verification of prognostic effects of mutations in METABRIC data. While few genes were significant in univariate analysis after

Fig. 1 Mutation recurrence and novel splice site mutation. **a** The overall mutation recurrence rate ranged from 41.1% of samples for *PIK3CA* to 0.0% for *PIN1*. The figure depicts non-silent mutations for all 1128 patients for the top 17 most recurrently mutated genes (>5% recurrence). If a patient had multiple mutations it is colored according to the “most damaging” mutation following the order presented in the Mutation Type legend (vertical color bar). Mutations per MB were calculated using the total number of mutations observed over the total exome space corresponding to the tiled space from “SeqCap EZ Human Exome Library v2.0”. A correction factor was applied to account for genes not assayed using the expected number of additional mutations based on ER+ TCGA data. The coverage histogram (top sidebar) shows the percent of targeted exonic bases with at least 20×, 30×, and 40× coverage. **b** Mutation recurrence frequencies (amino acid level) in this study were compared to previously reported mutation frequency from a multi-study MAF file of six reported breast cancer sequencing studies (Supplementary Data 1). An entirely novel mutation “hot spot” was discovered affecting the exon 2 splice (donor) site of *CBFB* in at least 15 patients. Six different single nucleotide substitutions, insertions, and deletions were observed, all affecting either the first or second base of the donor splice site. These mutations were most likely missed in previous studies because of a lack of sequencing coverage due to the GC-rich nature of exons 1 and 2 of *CBFB* (Supplementary Figures 9, 10). Such mutations are predicted to significantly alter the canonical donor site and result in either alternate donor usage or skipping of one or more exons of *CBFB*

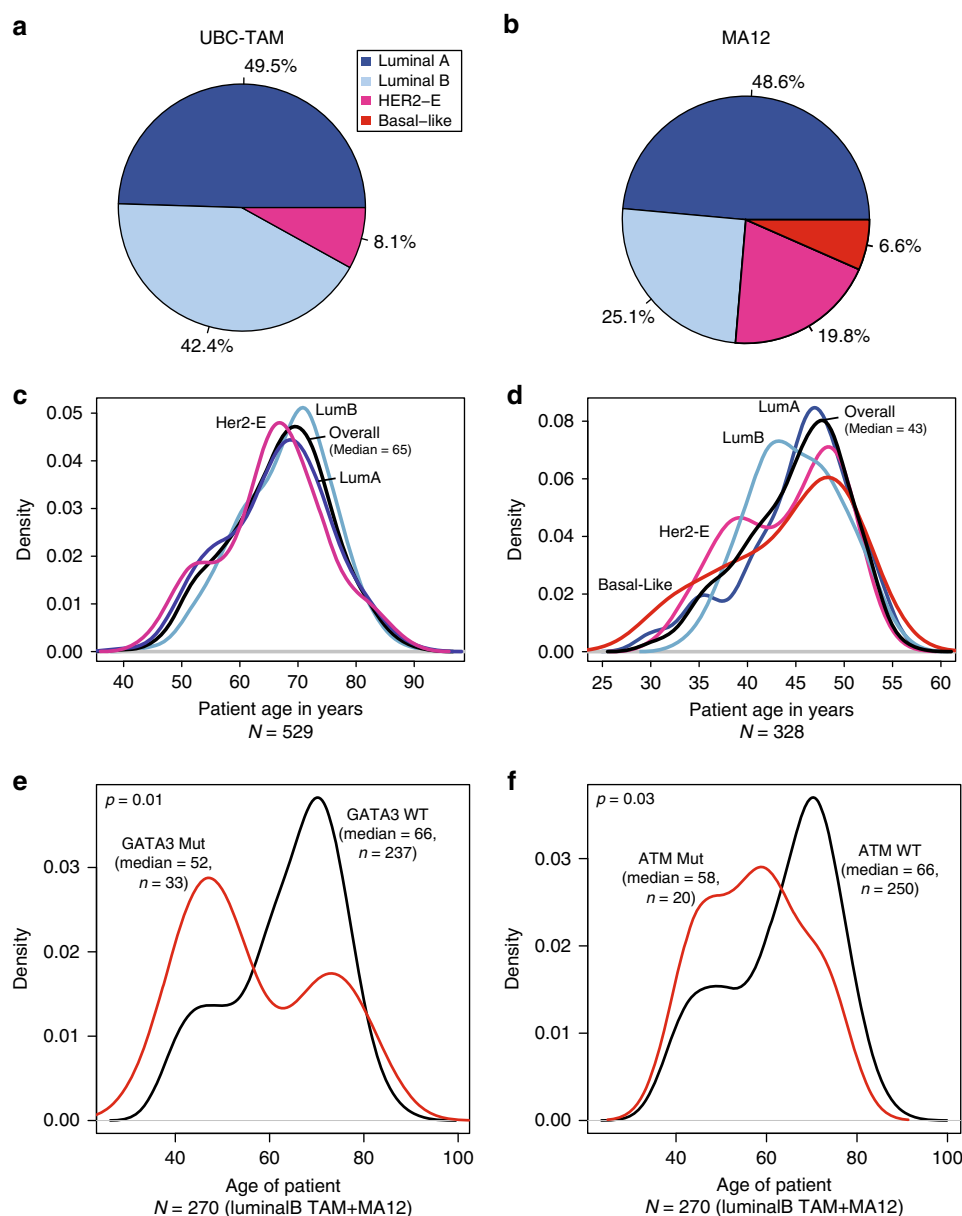
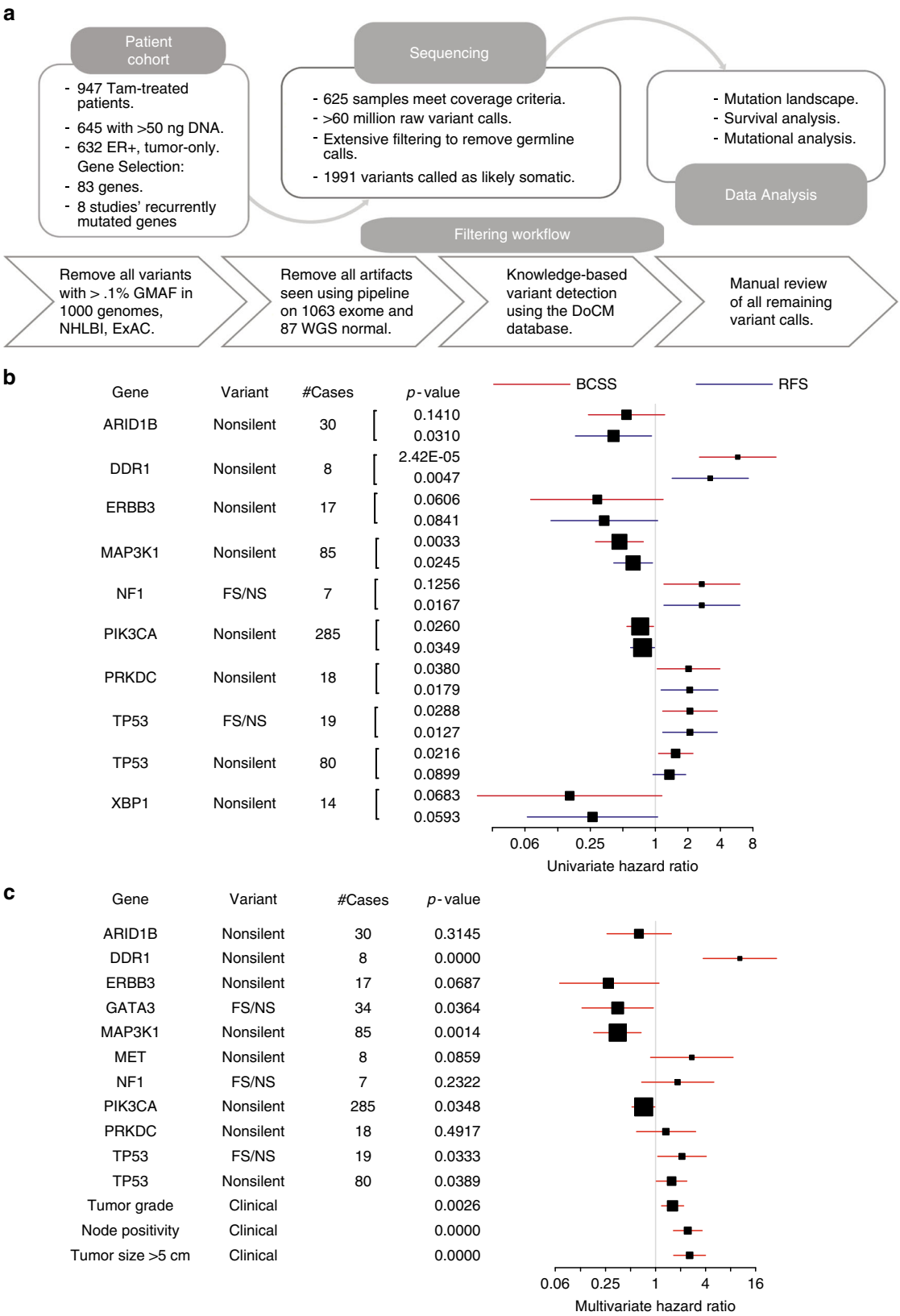


Fig. 2 Cross-cohort age and subtype analysis. **a, b** Percentage composition of samples by intrinsic subtype of the tumor in the two discovery cohorts for UBC-TAM (**a**) and MA12 (**b**) cohorts. **c, d** Age-density plots for patients categorized by intrinsic subtype in UBC-TAM (**c**) and MA12 (**d**) cohorts. The overall median age shows that UBC-TAM is constituted mostly of postmenopausal patients (median age = 65), in contrast to MA12, which has younger patients (median age = 43). **e, f** Younger luminal B subtype patients harbor GATA3 (**e**) and ATM (**f**) mutations in the combined set of UBC-TAM and MA12 Luminal B cases (median age = 52, $p = 0.01$; median age = 58, $p = 0.03$ for GATA3 and ATM, respectively). Significant p values were determined by Wilcoxon rank-sum test analysis



multiple testing correction, their identification provides valuable hypotheses for further testing and validation. We therefore sought additional data in the public domain to further assess the uncorrected *p*-value-based findings in our data set. The METABRIC consortium have reported somatic mutations in

cBioPortal¹⁴ with co-reported detailed hormone receptor status, age at diagnosis (median age = 64 years for ER+ patients), mean follow up of >8 years, and disease-specific (breast-cancer-specific) outcome^{15,16}. This data set provided the opportunity to conduct a validation exercise for overlapping genes in the two data sets. For

Fig. 3 Candidate discovery from UBC-TAM cohort and prognosis evaluation. **a** DNA was extracted from tumor specimens from 947 patients with ER+ breast cancer treated with tamoxifen monotherapy for 5 years. 632 samples with adequate yield were sequenced for 83 genes known to be recurrently mutated or breast cancer relevant. A total of 625 samples passed minimum quality checks and were sequenced to an average of 135.8× coverage. A total of ~62 million variants from the reference genome were identified. Extensive filtering and manual review reduced this list to 1991 putatively somatic variants. Survival analysis was applied to non-silent and truncating gene mutation status versus disease outcome (relapse or breast-cancer-specific death). In addition, mutations were analyzed for novel hotspots, patterns of mutual exclusivity or co-occurrence and association with clinical variables. **b** Forest plot of impact of mutations in candidate genes, identified using the UBC-TAM population, on breast-cancer-specific survival (red) and recurrence-free survival (blue). The variant types are characterized based on non-silent or nonsense/frameshift (FS/NS) mutations. The box size is relative to frequency of mutations in the analysis, with larger boxes representing higher incidence mutations. Log-rank test was used to determine significance and Cox regression proportional hazards generated univariate hazard ratios. **c** Multivariate forest plot of effect of mutations in UBC-TAM candidate genes on breast-cancer-specific survival when assessed together with clinical factors including Tumor Grade, Node positivity, and Tumor Size (>5 cm). Multivariate Cox proportional-hazard model was used in the multivariate analysis

the UBC-TAM series (Fig. 3), nine genes with a univariate p -value of <0.05 were brought forward for validation (Fig. 5). Of the six overlapping genes also examined in METABRIC, consistent prognostic effects independent of clinical variables were observed for non-silent mutations in three genes, *MAP3K1* (favorable), *TP53* (unfavorable), and *NF1* FS/NS mutations (unfavorable). In order to maintain coherence in discovery and validation patient cohorts, a similar analysis was carried out restricting the patient pool to postmenopausal patients only. No significant variation in hazard ratio for candidate genes was observed (Supplementary Table 4). For the MA12 series (Fig. 4), five shared genes were identified with univariate p values of <0.05, yet only *PIK3R1* mutations (non-silent or FS/NS) showed consistent adverse prognostic effects (Fig. 6). The Kaplan–Meier survival plots for the consistent adverse prognostic effects of *NF1* FS/NS (TAM vs METABRIC) and non-silent *PIK3R1* (MA12 vs METABRIC) mutations are illustrated in Fig. 7a–d. Copy number aberrations and chromosomal instability have been associated with prognosis across multiple cancer types, including ER-positive (ER+) breast cancer^{15,17,18}. To gauge the confounding nature of commonly amplified genes in breast cancer, we further performed multivariate analysis on the candidate genes with cases of amplification of *MYC*, *FGFR1*, *CCND1*, and *ERBB2* (Supplementary Table 5). We did not observe a significant change in the hazard ratios reported in Figs. 5b and 6b.

Prognostic interactions between *PIK3CA* and *MAP3K1*. Since *PIK3CA* and *MAP3K1* mutations co-associate, the combined effect of non-silent mutations in these genes was examined. Patients with tumors exhibiting both genes mutated have a more favorable clinical course than either singly mutant cases or cases without either gene mutated. While the prognostic effects were strongest in the UBC-TAM series, this result was also reproduced in the METABRIC data (Fig. 7e, f).

Mutation analyses for uncommon targetable kinases. Of the 83 genes analyzed, at least eight are directly targetable with small molecules or antibodies that are either FDA approved or in late-stage development (Fig. 8). Pre-existing data on these mutations is summarized (Supplementary Data 7). *PIK3CA* is not further discussed here, since the mutation spectrum is well described and large therapeutic studies are already underway. An examination of the 23 mutations in *ERBB2* revealed locations that were, as expected, clustered in two major domains, with 2 of 23 having extracellular domain mutations at residue 310 and 21 of 23 having kinase domain mutations between residues 755–842^{10,19}. To further investigate the preliminary finding of an adverse prognostic effect for *ERBB2* mutation in the MA12 series, an examination of the METABRIC data indicated that known

activating mutations in *ErbB2* were associated with a near significant adverse effect (HR = 1.71, $p = 0.075$) (Supplementary Figure 8).

For *ERBB3*, two known activating mutations were identified (V104L and E928A)²⁰. In total, *ErbB2* exhibited the anticipated profile of activating mutations from earlier publications¹⁰ with 22/1259 (1.7%) samples harboring known activating mutations and another six variants of unknown significance in the kinase domain or at the S310 residue (Fig. 8c). The *DDR1* kinase domain mutation, R776W, is possibly homologous to *EGFR* hotspot mutation L858R, but the remaining *DDR1* variants are of unknown significance. For the mutations in *JAK1*, 3 of 12 are loss-of-function mutations (frame shift or nonsense) and the S816* mutation has been reported in a lung adenocarcinoma sequencing data set²¹. The loss-of-function mutations in *JAK1* have been shown to associate with immunotherapy resistance^{22,23}. A few mutations identified in *ERBB4*, *MET*, and *PDGFRA* have been previously reported but those reported here have not been functionally tested.

Discussion

This investigation adds to the knowledge on the impact of somatic mutations in ER+ breast cancer by providing new data on almost a thousand samples with prolonged follow up and controlled adjuvant treatment. By combining these data with a comprehensive overview of published information we report herein the largest database studied to date. The size of our database is critical to our conclusions as the investigation of the prognostic effects of rarer mutations with an overall frequency of 5% or less requires very large sample sizes. Weaknesses include the lack of treatment prediction because endocrine treatment in UBC-Tam was uniform but not randomized. In MA12 the use of tamoxifen was randomized, but the numbers were too small to examine treatment interactions. The landscape of recurrently mutated genes in ER+ breast cancer observed in this study is consistent with reports where matched germline samples were available, indicating that our variant filters were effective for somatic mutation detection in a research setting. Overall, mutation frequencies were higher in our cohort (e.g., for *PIK3CA*, *MLL3*, *MAP3K1*) than the TCGA cohort, but were also lower for a few specific genes (e.g., *TP53* and *GATA3*). Due to higher sequencing data coverage of recurrently mutated target genes than TCGA and the use of a different hybrid capture reagent, we were likely able to detect mutations that were missed with lower-depth exome or whole-genome sequencing data. Differences in patient populations may also be a factor. Frequencies were much closer to reported values for METABRIC, which also used a targeted sequencing approach. It is also possible that in some instances we overestimated somatic mutation frequency, due to the lack of matched normal samples and imperfections in our

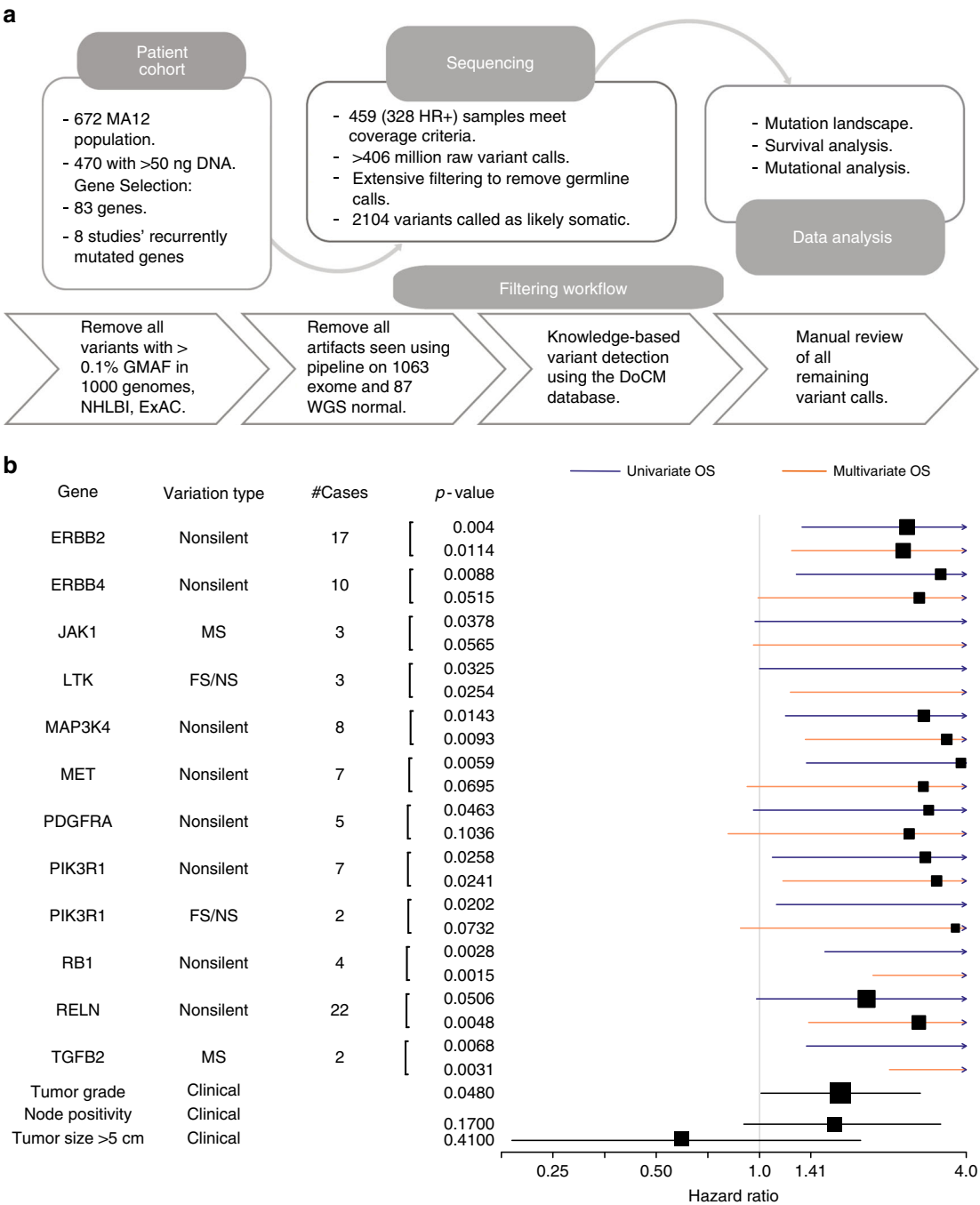


Fig. 4 Candidate discovery from MA12 cohort and prognosis evaluation. **a** DNA was extracted from tumor specimens and 470 samples with adequate yield were sequenced for 83 genes known to be recurrently mutated or breast cancer relevant. A total of 459 (328 HR+) samples passed minimum quality checks and were sequenced to an average of 272.6× coverage. A total of 406 million variants from the reference genome were identified. Extensive filtering and manual review reduced this list to 2104 putatively somatic variants. Survival analysis was applied to non-silent and truncating gene mutation status versus overall survival. **b** Forest plot showing effect of mutation in candidate genes on overall survival (univariate—blue, multivariate—orange), along with the clinical factors used in the multivariate analysis (black), tumor grade, node positivity, and tumor size (>5 cm). The box size is relative to frequency of mutations in the analysis, with larger boxes representing higher incidence mutations. Note: a few boxes are not shown if their hazard ratios were greater than 4.0. Log-rank test was used to determine significance and Cox regression proportional hazards generated univariate hazard ratios. Multivariate Cox proportional-hazard model was used in the multivariate analysis

germline polymorphism filtering. In particular, a significant number of *BRCA1*, *BRCA2*, and *ATM* mutations are likely de novo germline mutations that we would not be able to easily distinguish from somatic mutations. Of the 117 non-silent *BRCA1/2* mutations observed (from 110/1128 patients across all 3 cohorts; 7 patients had two hits) 74 were observed at a VAF greater than 40% and 31 were greater 60%. Additionally, of the 61 non-silent *ATM* mutations (from 58/1128 samples; 3 samples had 2 hits) 39 had VAF greater than 40% and 18 had VAF greater than 60 (Supplementary Data 9). Variants with VAFs this high are less likely to be somatic given the general expectation of impure tumor samples and heterozygous mutations. Indeed, the

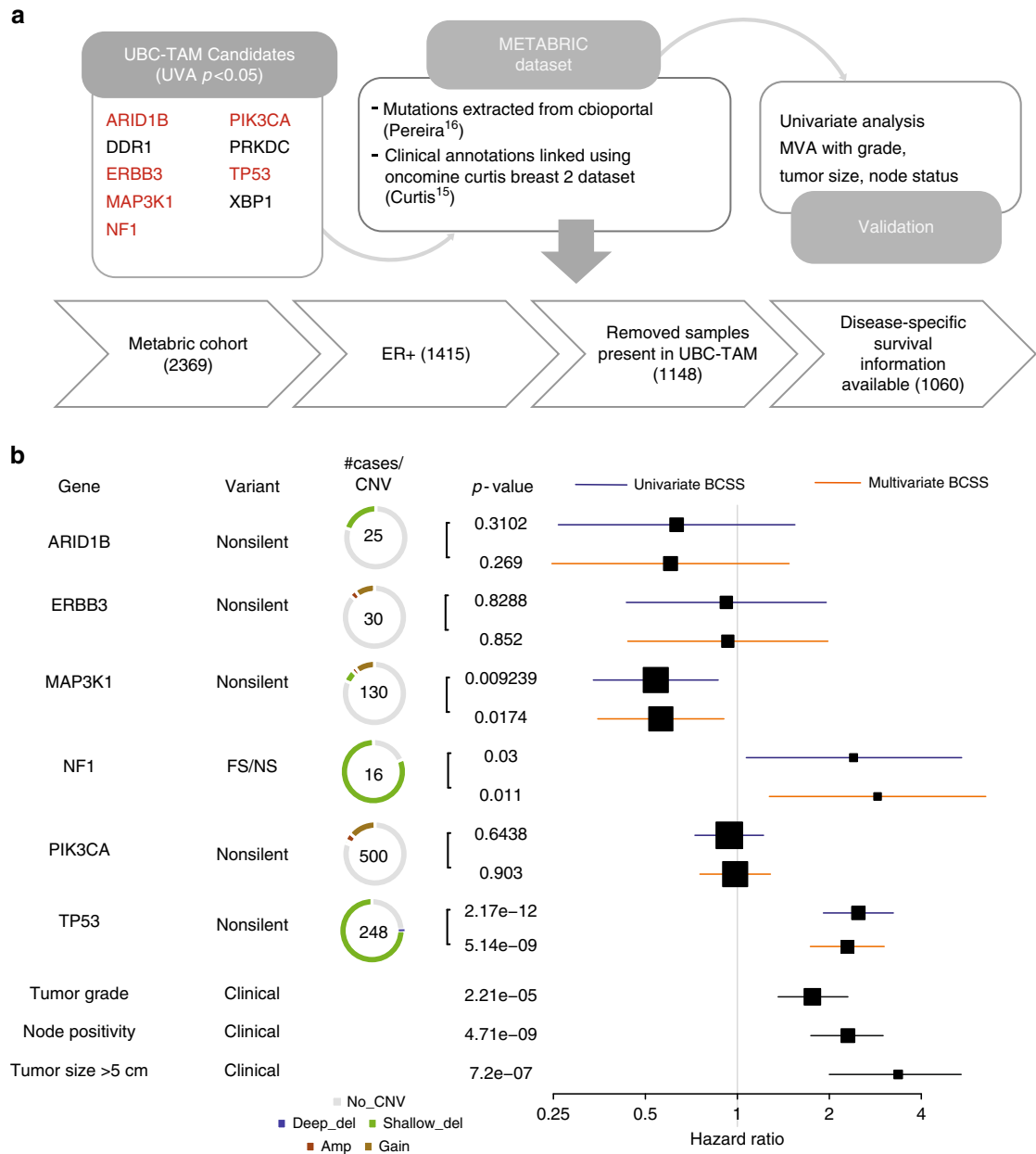


Fig. 5 Validation of UBC-TAM candidates in ER+ METABRIC. **a** Six out of nine candidate genes from UBC-TAM analysis had mutations reported in the METABRIC cohort. 1060 ER+ samples with breast-cancer-specific survival information were used to test the effect of mutations in the candidate genes on prognosis. **b** Forest plot shows effect of mutated candidate genes on breast-cancer-specific survival in METABRIC ER+ cohort with univariate cox proportional-hazard ratio in blue and multivariate in orange. The clinical factors used in the multivariate analysis, namely tumor grade, node positivity and tumor size (>5 cm), are shown in black. The box size is relative to frequency of mutations in the analysis, with larger boxes representing higher incidence mutations. The # cases/CNV column shows the total number of cases with the SNV/Indel variant surrounded by a ring chart indicating the proportion of total cases with CNV alterations. Log-rank test was used to determine significance and Cox regression proportional hazards generated univariate hazard ratios. Multivariate Cox proportional-hazard model was used in the multivariate analysis

VAFs for *BRCA1/2* and *ATM* non-silent mutations (mean = 46.0%) were significantly higher than for other genes (mean = 36.7%, $p = 5.92 \times 10^{-9}$). Even when considered separately, the VAFs for *BRCA1* (mean = 46.6%), *BRCA2* (mean = 43.8%), and *ATM* (mean = 48.2%) were significantly higher than the other genes ($p = 0.002$, $p = 0.0015$, and 5.27×10^{-5} , respectively). Among the *BRCA1/2* variants, there were eight known pathogenic (ENIGMA expert reviewed) mutations according to a search of the BRCA Exchange database (<http://brcaexchange.org>, 12 November 2017) and another 37 assumed pathogenic (FS/NS) mutations. Of the remaining, four were benign according to expert review

(ENIGMA), and eight benign, 15 likely benign and 45 variants of unknown significance according to all public sources. Out of the 61 *ATM* variants queried in ClinVar, four were designated as pathogenic, three were pathogenic/likely pathogenic, and two were likely pathogenic. Another seven were frameshift mutations and assumed pathogenic. Additionally, 23 variants had uncertain significance, eight variants had conflicting interpretations of pathogenicity (any combination of benign, likely benign, or uncertain significance), and the remaining 14 variants had no data. *ATM* variants were also queried in the Leiden Open Variation Database (LOVD)²⁴, which identified one variant that

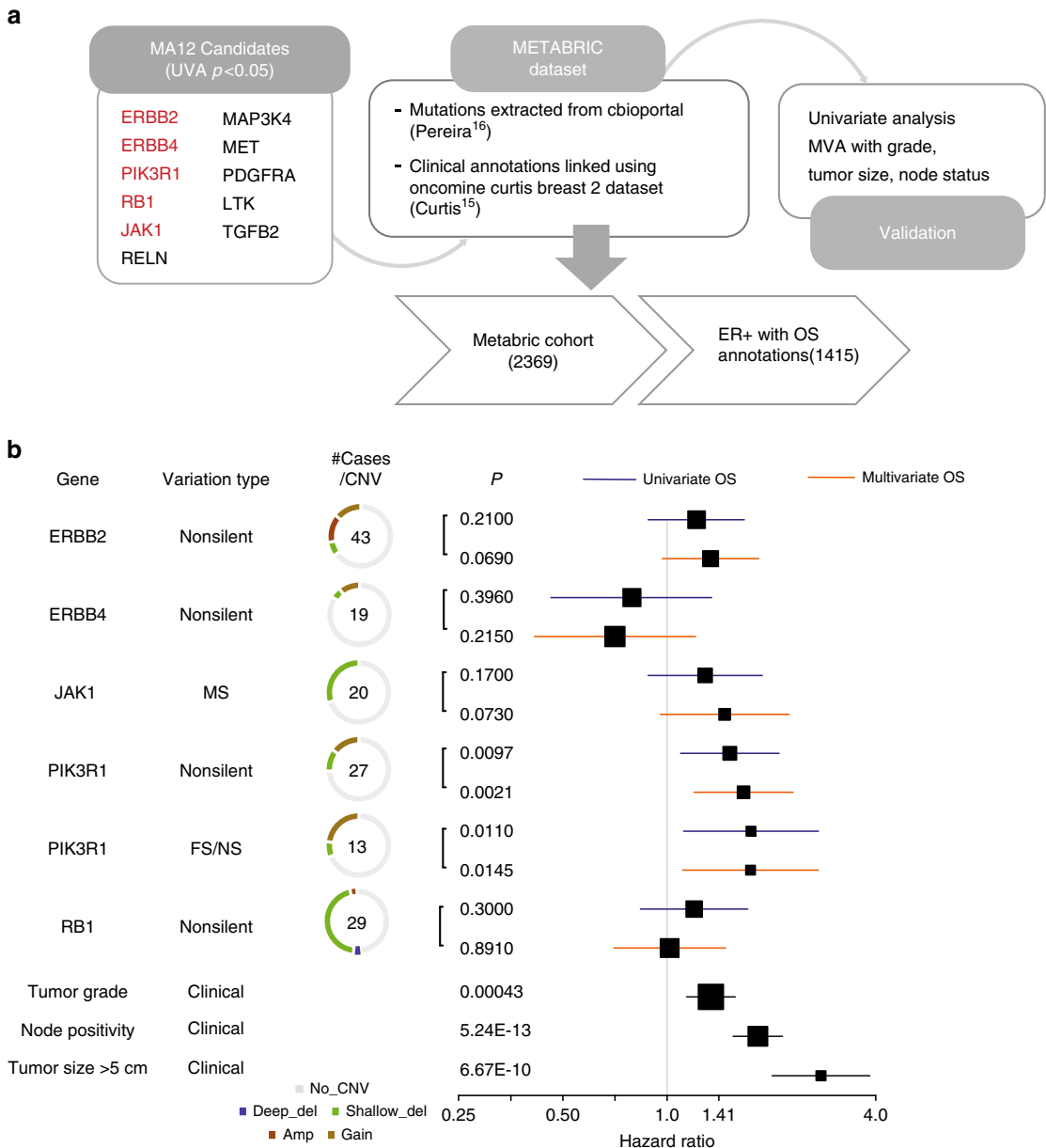


Fig. 6 Validation of MA12 candidates in ER+ METABRIC. **a** Five out of eleven candidates from MA12 analysis had mutations reported in the METABRIC cohort. 1415 ER+ samples with overall survival information were used to test the effect of mutations in the candidate genes on prognosis. **b** Forest plot shows effect of mutated candidate genes, shortlisted based on MA12 mutation analysis, on overall survival in METABRIC ER+ breast cancer patients. Univariate (blue) and multivariate (orange) cox proportional-hazard ratios depict the independent prediction of survival outcomes for the six candidate genes. The box size is relative to frequency of mutations in the analysis, with larger boxes representing higher incidence mutations. The # cases/CNV column shows the total number of cases with the SNV/Indel variant surrounded by a ring chart indicating the proportion of total cases with CNV alterations Log-rank test was used to determine significance and Cox regression proportional hazards generated univariate hazard ratios. Multivariate Cox proportional-hazard model was used in the multivariate analysis

affects function (designated as likely pathogenic by ClinVar), 10 variants with unknown effect, and one variant that probably does not affect function (uncertain significance in ClinVar). The remaining variants had no data in LOVD. Given these complexities the prognostic effects of somatic versus germline BRCA1/2 and ATM mutations remain unresolved, however attention should clearly be paid to therapeutic strategies for these patients. The ATM findings deserve a particular highlight because of the younger age/luminal B association and the current lack of studies devoted to this population.

The discovery of a novel recurrent *CBFB* (core binding factor subunit beta) splice site mutation in this cohort illustrates a

limitation of exome capture reagents. The affected bases in exon 2 of *CBFB* display reduced sequence coverage, possibly due to high GC content, in the breast TCGA exome dataset (Supplementary Figures 9–10). This site was mutated in at least 1.5% of ER+ breast cancers sequenced, bringing the overall rate of *CBFB* mutations to nearly 6%, which should drive further investigation of this gene in ER+ breast cancer pathogenesis. *CBFB* functions as a subunit in a heterodimeric core binding transcription factor that interacts with *RUNX1*²⁵. Consistent with this model, *CBFB* mutants were mutually exclusive from *RUNX1* mutants in this cohort with only a single sample harboring non-silent mutations in both *CBFB* and *RUNX1*.

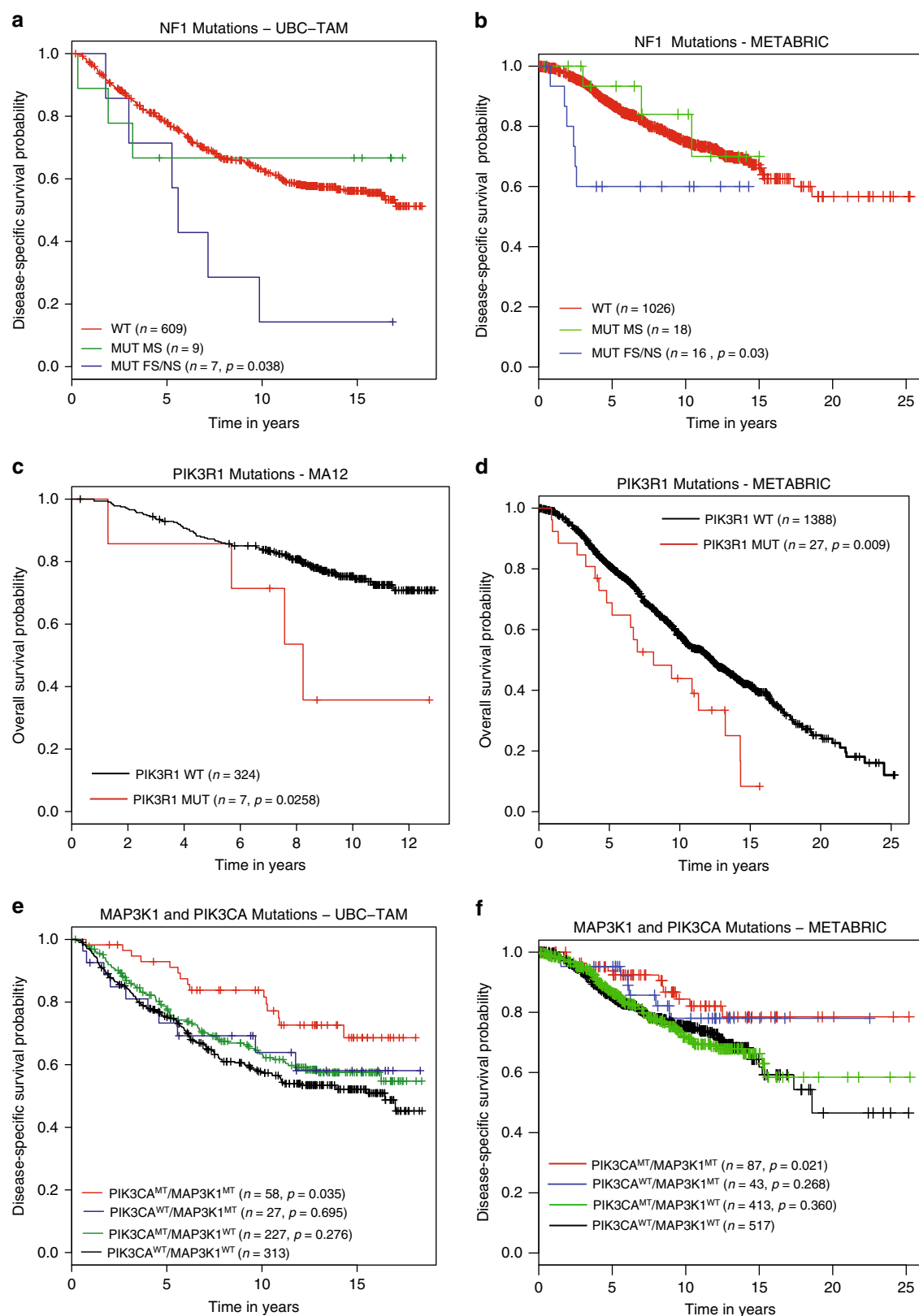


Fig. 7 Kaplan-Meier plots of candidate gene mutations in discovery and validation cohorts. **a, b** Kaplan-Meier graphs showing the prognostic role of NF1 mutations, separated by variant type—Missense (MUT MS, green), Frameshift/Nonsense (MUT FS/NS, blue) in ER+ breast cancer patients from **a** UBC-TAM and **b** METABRIC cohort establishing the association between FS/NS mutations in NF1 with poor prognosis. **c, d** Kaplan-Meier graph showing the prognostic role of PIK3R1 in **c** MA12 and **d** METABRIC ER+ breast cancer patients, categorized based on tumors with wild type (WT, black) or mutated PIK3R1 non-silent mutations (MUT, red). **e–f** Kaplan-Meier graph demonstrating co-occurrence of non-silent mutations in MAP3K1 and PIK3CA (red) in **e** UBC-TAM and **f** METABRIC associates with better survival when compared against tumors with mutations exclusively in MAP3K1 (blue) or PIK3CA (green) or wild type for both MAP3K1 and PIK3CA (black). p , log rank test p -value

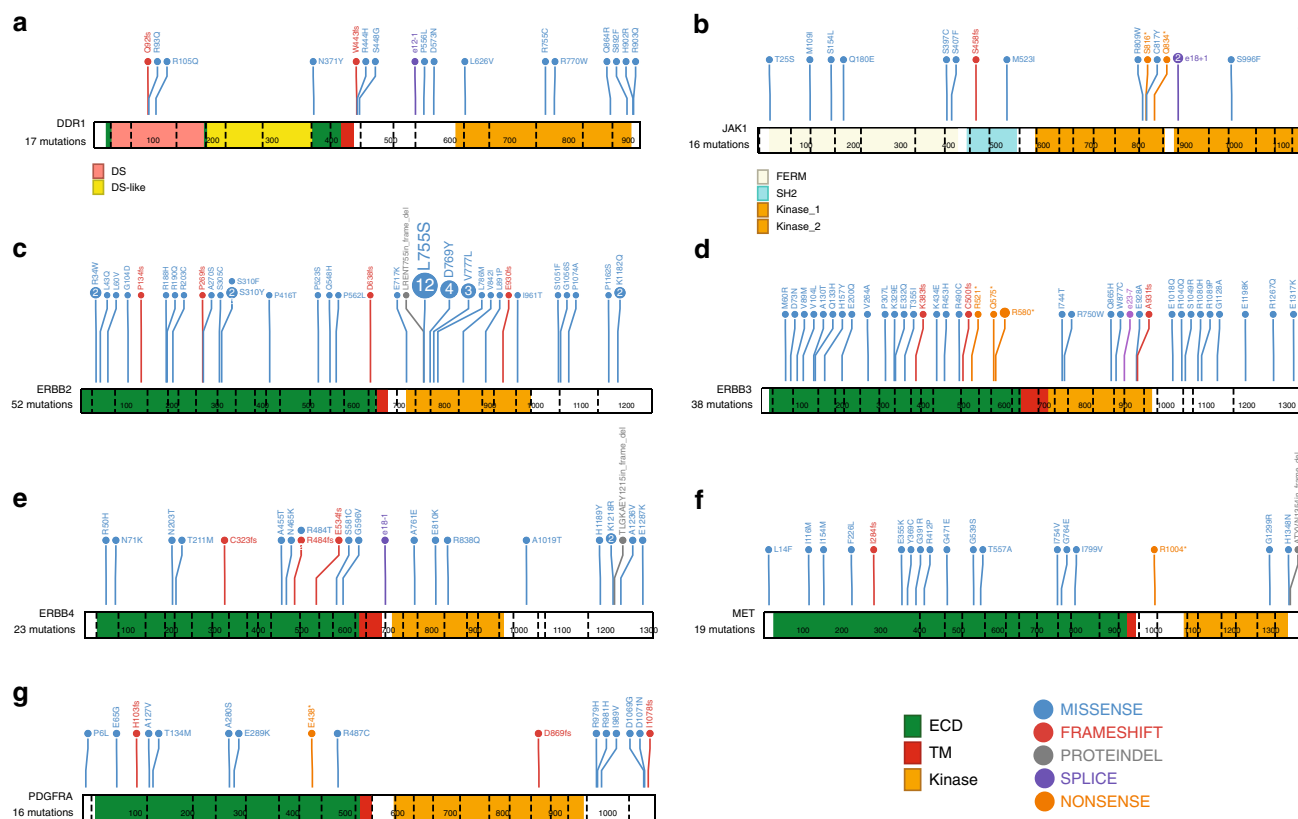


Fig. 8 Mutation profiles for selected genes. Mutation frequency plots illustrate all non-silent mutations (TAM, POLAR, and MA12; $n = 1259$) for representative transcripts for several kinase genes of interest. The domains belonging to **a** DDR1 (RefSeq ID: NM_013994) and **b** JAK1 (NM_002227) are indicated below the schematic diagram of each gene. The ECD (extracellular domain), TM (transmembrane domain), and kinase domain are depicted as green, red, and orange bars respectively for **c** ERBB2 (NM_004448), **d** ERBB3 (NM_001982), **e** ERBB4 (NM_005235), **f** MET (NM_000245), and **g** PDGFRA (NM_006206). The variant counts across the three data sets for each gene are provided below the gene's name. Note, in the mapping from Ensembl (Supplementary Data 3) to RefSeq annotations (required for use of ProteinPaint tool) a small number of variants annotations may have changed or been lost, despite selecting the most similar representative transcript possible

The UBC-TAM and MA12 studies revealed different lists of potentially prognostic mutations. Prognostic effects are likely to be strongly affected by the use of systemic therapy, as well as by patient age at diagnosis. The UBC-TAM series is the simplest study to interpret from a drug resistance perspective since the only systemic therapy was tamoxifen. Thus, the consistent adverse effect of NF1 FS/NS mutation on prognosis is intriguing as this result is consistent with results from an in vitro screen for tamoxifen resistance²⁶. Understanding why only FS/NS mutations predict poor outcome, rather than missense or other non-silent mutations, will require further investigation. The association of the HER2-E, non-HER2 amplified subset with non-synonymous NF1 mutations was observed in both the discovery and validation (METABRIC) data sets. It is a logical proposition that mutations that activate RAS, like NF1 mutation, could create a tumor with a similar transcriptional phenotype as some HER2 amplified breast cancers. PIK3R1 mutation also emerged as a consistent poor prognosis mutation from the MA12 analysis, with validation in METABRIC. The proposed favorable prognostic effects of PIK3CA mutation were observed in the UBC-TAM series, but were not found to be independent of stage and grade, and PTEN mutations were neutral.

According to our validation results, NF1, PIK3R1, PIK3CA, and TP53 are therefore likely to be prognostic drivers that are independent of clinical variables. In postmenopausal women treated with adjuvant endocrine therapy, DDR1, PRKDC, and XBP1 should be further studied and of these DDR1 is the

strongest candidate because it was significant despite strict false discovery correction. DDR1 is a collagen-binding receptor expressed in epithelial cells that stabilizes E-cadherin-mediated intracellular adhesion²⁷. DDR1 mutations also occur in endometrial cancer²⁸, acute leukemia²⁹, and lung cancer³⁰. Loss of DDR1 (DDR1-null mice) produces hyper-proliferation and abnormal branching of mammary ducts, suggesting DDR1 is a breast tumor suppressor³¹. Mutations in PRKDC will potentially produce a defective ATM response/low ATM levels³², which is interesting in the context of the finding herein that ATM mutations are a potential luminal B driver gene. The significance of a defective ATM pathway as a cause of endocrine resistance is highlighted by the recent finding that dysregulation of the MutL complex (MLH1, PMS1, and PMS2) causes failure of ATM/CHK2-based negative regulation of CDK4/6³³. Prognostic candidate mutations revealed by the MA12 analysis were different from the UBC-TAM series, likely reflecting the different patient profiles and adjuvant treatments illustrated in Fig. 2. The prognostic effects of mutations in ERBB2, ERBB4, JAK1, LTK, MAP3K4, MET, PDGFRA, RB1, RELN, and TGFβ2 all await further study with even larger sample sizes.

A limitation of this study is that the mutation data sets we generated for UBC-TAM and MA12 cohorts lack comprehensive assessment of copy number signatures that have been associated with prognosis in ER+ breast cancer^{15,17,18}. While multivariate analysis considering key CNVs did not appear to affect our prognostic associations, future studies may be needed to

completely understand the interplay between simple and large-scale variation for prognostic prediction. Another limitation to this study was the heterogeneity in the data sets in terms of age, treatment, and other factors that limited direct comparison and made validation with METABRIC somewhat challenging. The collection of sufficiently large, uniformly treated populations with long-term follow-up for discovery and validation remains a challenge that must be addressed to fully characterize the prognostic significance of somatic mutations, especially low frequency mutations.

In conclusion, we have successfully utilized clinically well-annotated, uniformly treated patient samples using DNA from archival material greater than 20 years old without a matched normal to explore the prognostic effects encoded by the mutational landscape of ER+ breast cancer. We were able to confirm our prospective hypothesis from our earlier studies⁵ that MAP3K1 is associated with indolent disease and TP53 with adverse outcomes. We also associated NF1 FS/NS mutations with strong adverse effects on prognosis. Similarly, PIK3R1 mutations were associated with an adverse prognosis, in contrast to PIK3CA mutation which were weakly favorable. This suggests somatic mutations in these two physically interacting gene products are not biologically equivalent with respect to PI3 kinase pathway activation and resistance effects. The possibility that the long tail of low frequency mutation events in luminal type breast cancer may harbor multiple molecular explanations for poor outcomes should spur new collaborative efforts to thoroughly screen thousands of properly annotated cases. Only after these iterative efforts of proposing and confirming candidates will a clinically useful and comprehensive somatic mutation-based classification of ER+ breast cancer emerge. In the meantime, functional studies should be pursued to understand the biological effects of low frequency somatic mutations, prioritizing these studies according to whether the mutations are driving an adverse prognostic effect and whether their disruption creates a therapeutic vulnerability.

Methods

Patient samples. The focus of this analysis was ER-positive breast cancer patients with long-term follow-up data available. As such, three main cohorts were available to us, the University of British Columbia Tamoxifen Cohort (UBC-TAM) cohort, the NCIC-MA12 trial (NCT00002542) cohort, and the POLAR cohort. Collection for each of these cohorts is described below. Ethics board approvals were obtained for all studies. For the UBC-TAM series, biomarker studies on the anonymized archival specimens and clinical data were approved by the Clinical Research Ethics Board of the British Columbia Cancer Agency. The MA12 trial was approved by the NCIC Clinical Trials Group (CTG). The details of the conduct of this study are published³⁴. Analysis on anonymized archival specimens and clinical data were approved by the Clinical Research Ethics Board of the British Columbia Cancer Agency. For the analysis on the POLAR study, biomarker studies on the anonymized archival specimens and clinical data were approved by the Clinical Research Committee, Royal Marsden Trust and the National Research Ethics Committee Research Ethics Committee, West Midlands, United Kingdom. The Human Research Protection Office of Washington University also approved these anonymized analyses.

UBC-TAM—In total, 947 samples were identified from the TAM series^{6,35} with sufficient materials available for DNA isolation. Archival formalin-fixed paraffin embedded (FFPE) blocks were subjected to histopathologic review and one to five cores (1.25 mm) taken from tumor rich areas (>50%) for DNA isolation. Briefly, the cores were de-paraffinized using Citri-Solv Clearing Agent (Fisherbrand # 22-143-975), the pellet washed with 100% ethanol and then dried at 55 °C. The residual tissue pellet was incubated overnight with proteinase K solution (40 µl) and the nucleic acid extracted per manufacturer's directions using the Qiagen Blood and Tissue Kit (catalog #69506). A total of 645 samples met the minimum DNA yield requirement of 50 ng. Of these, five samples were excluded as duplicates (higher yield sample was chosen per patient). An additional eight patients were excluded if they had a metastasis at diagnosis or were not treated with tamoxifen upon review of records. A minimum sequencing quality cutoff was applied, requiring at least 80% of targeted bases covered at greater than 20× with other quality metrics described in the supplementary data. The final population that met all study criteria and had sufficient coverage (See REMARKS summary in Fig. 3a for details) included 625 women diagnosed with estrogen receptor-positive invasive breast cancer in the province of British Columbia of Canada between 1986

and 1992, as previously described^{6,35–37}. These patients were treated with five years of tamoxifen as the adjuvant monotherapy. The mean age of the cohort at diagnosis of breast cancer was 66.9 years, and the median follow-up time was 11.7 years.

MA12—DNA isolations were performed as above. A total of 459 of 470 (97.7%) patient samples met study criteria and passed the minimum sequencing quality cutoff. The final patient population for most analyses only includes 328 HR positive patients (see REMARKS summary in Fig. 4a for details), who had an average age of 45 at randomization (range: 30–57). All received chemotherapy (27% CEF, 44% CMF, 29% AC) and 48% were treated with five years of adjuvant tamoxifen. Patients were primarily premenopausal, with grade 2 or 3 (63%), nodal positive (82%), and stage 2 (85%) breast cancers. 89% were clinically ER+ and 53% clinically PR+ (42% unknown). PAM50 molecular subtype by a non-commercial qRT-PCR assay was available from a previous study on 255 (78%) patients: 114 (44.7%) were luminal A, 61 (23.9%) luminal B, 48 (18.8%) Her2-enriched, 17 (6.7%) normal-like, and 15 (5.9%) basal-like subtype.

POLAR—DNA was isolated as above from 196 primary breast tumors from the POLAR study, a case-control study of ER+ primary breast tumors from patients diagnosed with breast cancer between 2000 and 2004 (to allow for long-term follow up). A case was defined as a patient who relapsed with any recurrence during follow-up. A control was defined as a patient who did not relapse during follow-up. Patients were included if they were treated with 5 years of adjuvant endocrine therapy. Pre and postmenopausal patients were eligible for the study and chemotherapy was also permitted. There are three main differences between the TAM series and the POLAR case-control cohort that warrant attention.

1. TAM had no patients treated with chemotherapy whereas in POLAR, 52% of patients received chemotherapy.
2. TAM is a cohort study whereas POLAR is a case-control design hence proportionally significantly more relapses are represented.
3. POLAR was initially designed to select cases and matched controls from a hospital cohort—matching on age at diagnosis, NPI category, type of endocrine therapy for 5 years, and whether chemotherapy was administered or not. Hence cases and controls (i.e., relapses and no relapses) have previously been matched.

There were 91 relapse events in POLAR and 84 controls (referred to as No Relapse). There were 43 early relapses (occurring up to five years after diagnosis) and 48 late relapses (occurring beyond 5 years). Two samples had insufficient material or failed at library construction. 175/194 samples passed sequence coverage thresholds. Baseline clinical and pathological characteristics of each study population are shown in Supplementary Table 2.

Gene panel and capture probe design. A meta-analysis of six published breast cancer sequencing studies^{1,2,5,38–40} was performed and a multi-study mutation annotation format (MAF) file compiled that included all somatic exome mutations from the six studies (Supplementary Data 1). Kan et al.³⁹ targeted ~1500 coding genes whereas the other five used “whole-exome” capture approaches. Data were gathered from supplementary materials of each study or the TCGA data portal for that study³. Gene names were harmonized to official gene symbols using the NCBI Human Gene Info table (available at http://ftp.ncbi.nih.gov/gene/DATA/GENE_INFO/Mammalia/Homo_sapiens.gene_info.gz as of 4/3/15). The columns for protein change, mutation type (e.g., SNP, INS, DEL, etc), and mutation class (e.g., Missense, Silent, Frameshift, etc) were edited to use TCGA nomenclature. Finally, clinical subtype data was gathered and appended to the dataset where available. Intrinsic subtype was available for the Banerji et al.¹, Ellis et al.⁵, and TCGA studies³, while immunohistochemistry (IHC) results were available for the Kan et al.³⁹, Shah et al.⁴⁰, Stephens et al.², and TCGA publications. Genes were selected from this multi-study MAF file that were (1) recurrently mutated in 2% or more of cases or (2) recurrently mutated in 1% or more and determined to be druggable by their membership in greater than two druggable gene categories or having greater than two interactions with known anti-cancer drugs according to DGIdb⁴¹. Genes or their obvious family members were excluded if known to be problematic for variant calling (e.g., highly exonically variable). Unnamed genes (ENSG, KIAA, LOC) were also excluded. Genes from two additional large-scale sequencing studies were also considered. We included 15 of 16 genes reported as somatically altered by Chanock et al. from their own targeted sequencing of a 21 gene panel⁴². Three genes that were reported as deleted by the Curtis et al. study of copy number data in ~2000 patients were also included¹⁵. Further literature review was performed to include genes of known relevance to breast cancer (e.g., *BRCA1*, *BRCA2*, *ERBB2*, *ESR1*, and *PRLR*⁴³), not meeting any of the criteria above. This resulted in a final list of 83 breast-cancer-related genes (Supplementary Table 1). Biotinylated 120 bp oligonucleotide probes were ordered from Integrated DNA Technologies (IDT, Coralville IA) and required to have no more than 50bp gaps between probes, and greater than 50% unique alignment to the reference genome and non-repetitive sequence content. These genes were targeted comprehensively with 337,144bp of unique coding exon sequences tiled by 3029 120 mer probes (a unique tiled space of 362,572 bp) (Supplementary Data 8).

Library construction and sequencing. TAM—Automated dual indexed libraries were constructed with 50–250 ng of genomic DNA utilizing the KAPA HTP Library Kit (KAPA Biosystems) on the SciClone NGS instrument (Perkin Elmer)

targeting 250 bp inserts. Libraries with a starting input between 50–149 ng were enriched for 10 PCR cycles and libraries with a starting input between 150–250 ng were enriched for eight PCR cycles. A total of 92 libraries were pooled pre-capture generating a 4.6 µg library pool. Library pools were hybridized with a custom set of probes (see above). The concentration of each captured library pool was determined through qPCR according to the manufacturer's protocol (KAPA Biosystems) to produce cluster counts appropriate for the Illumina HiSeq2000 platform. One lane of 2 × 100 sequence data generated an average of 375 Mb of data per sample. An average of 135.8× mean depth of coverage was achieved per sample.

MA12—Automated dual indexed libraries were constructed as above. A total of 90 libraries were pooled pre-capture generating a 5 µg library pool. Library pools were double hybridized with a custom set of probes (see above). The initial 4 h hybridization utilized the standard amount of probes followed by eight PCR cycles for enrichment. The second hybridization only required half of the standard probe concentration, followed by nine enrichment PCR cycles. The concentration of each captured library pool was determined as above to produce cluster counts appropriate for the Illumina HiSeq2500 platform. Two lanes of 2 × 125 sequence data generated an average of 668 Mb of data per sample. An average of 200× mean depth of coverage was achieved per sample.

POLAR—Automated dual indexed libraries were constructed with 25–250 ng of genomic DNA utilizing the KAPA HTP Library Kit (KAPA Biosystems) on the SciClone NGS instrument (Perkin Elmer) targeting 250 bp inserts. Libraries with a starting input between 25 and 149 ng were enriched for 11 PCR cycles and libraries with a starting input between 150 and 250 ng were enriched for eight PCR cycles. A total of 64 libraries were pooled pre-capture generating a 5 µg library pool. Library pools were hybridized with a custom set of probes (see above). The concentration of each captured library pool was determined as above to produce cluster counts appropriate for the Illumina HiSeq2500 platform. One lane of 2 × 125 sequence data generated an average of 750 Mb of data per sample. Approximately 75× mean depth of coverage was achieved.

Primary variant calling and pre-filtering. Variant calling was performed with the Genome Modeling System as previously described⁴⁴. Specifically, sequence data were aligned to reference sequence build GRCh37-lite-build37 using bwa version 0.5.9⁴⁵ (params: -t 4 -q 5:), merged using picard version 1.46 [<http://broadinstitute.github.io/picard/>], then deduplicated with picard version 1.46. SNVs were detected using the union of samtools version r963⁴⁶ and VarScan version 2.2.6⁴⁷ (params: -min-coverage 3 -min-var-freq 0.08 -p-value 0.10 -strand-filter 1 -map-quality 10) and processed through false-positive filter v1 (params: -bam-readcount-version 0.4 -bam-readcount-min-base-quality 15 -max-mm-qalsum-diff 100). Indels were detected using VarScan version 2.2.6 (params: same as for SNVs) and filtered by false-indel version v1 (params: -bam-readcount-version 0.4 -bam-readcount-min-base-quality 15 -max-mm-qalsum-diff 100). Variants were annotated using Ensembl version 70. The initial variant set was expected to be dominated by germline variants and false positives. Therefore, a series of filters were applied. Variants were restricted to the regions of targeted genes. Variants were further excluded if found to have a global minor allele frequency in 1000 genomes (1092 individuals)³⁸, NHLBI exomes (SP6500SI-V2, 6503 individuals)⁴⁸, or ExAC⁴⁹ data sets greater than 0.1%. The 1000 genomes data (phase 1, release v3) were obtained directly from <ftp://ftp.1000genomes.ebi.ac.uk> and via dbSNP (v137). NHLBI Exomes were obtained from the Exome Variant Server (NHLBI GO Exome Sequencing Project, Seattle, WA, <http://evs.gs.washington.edu/EVS/>), downloaded 23 July 2012). Finally, a set of exome data for 151 unmatched normal blood samples⁵⁰ were processed through the identical variant calling pipeline as above. Any variant called in this study that was also observed in 10 or more of the unmatched normal samples was eliminated. This last filter was an attempt to eliminate common pipeline-related artifacts and common polymorphisms not present (or called) in 1000 genomes or NHLBI exomes. Variants from samples that failed coverage QC (Supplementary Data 2) were excluded.

Variant post-filtering. Inspection of the most highly recurrent variants identified several unlikely events in *FRG1B*, *MAP3K4*, and *MLL* that were mutated in more than 95% of samples. It was hypothesized that additional technical (alignment related) artifacts were causing these likely false positives. Rather than rely on variant calls in unrelated normals, a read counting approach was used to look for evidence of pipeline-related artifacts that might explain these variants. First, all variants were assessed for readcount support in a set of exome sequence data for 912 blood normal samples from the TCGA Breast Cancer project³. Variants were excluded from our list if at least 1% of TCGA samples had evidence of the same variant with at least three supporting reads, 1% VAF and 20× total coverage. Further inspection of recurrent variants in this newly filtered set identified two indels in the genes *RELN* and *FOXCI* that remained at suspiciously high quantities. Upon review of the TCGA normal exome panel it was discovered that the positions for these indels had very poor sequence coverage preventing identification of artifacts in these regions. Therefore, we further filtered against a set of 87 whole-genome sequenced blood normals also from the TCGA breast project. The same criteria were used as above except for a sample threshold of 2 out of 87. As a final step of quality control, each of these variants were manually reviewed. The entire pipeline was used on a set 16 tumors, sequenced with the same panel, for which matched normal variant calls (filtered and reviewed) were also available⁵¹. This

identified a small number of remaining low-support false positives. Finally, a Bayesian classifier was applied as previously described⁵² to eliminate variants not predicted to be somatic with a binomial log likelihood ratio less than 10 for SNVs and less than six for indels.

Detection of low frequency variants of known or potential clinical relevance.

To ensure that variants of known clinical relevance were not missed by automated variant calling approaches, a knowledge-based variant calling strategy was performed focused on the mutations in the Database of Curated Mutations⁵³. This method scans directly for evidence of variants in raw alignment data using a database of mutations thought to be clinically or functionally relevant. At time of analysis the database included 413 total variant-disease entries for 35 genes from My Cancer Genome⁵⁴, Dienstmann et al.⁵⁵ and manual curation of the literature. These include well described variants of relevance in other cancers (e.g., *BRAF* V600E), as well as those specific to breast cancer such as *ESR1* ligand-binding domain^{10,56} and *ERBB2* kinase domain mutations⁵⁷. All samples were scanned for these known positions and potential variant calls made where at least five reads supported the variant with at least 1% VAF and 20× total coverage (Supplementary Data 3).

Hotspot analysis. The multi-study MAF file used for our original gene panel design (see above) was used as a reference to determine which variants in our study (if any) might represent novel sites of recurrent mutation, so called “hotspots”. A recurrent mutation was considered novel if it was significantly enriched in our cohort by Fisher's exact test. Variants in both data sets were aggregated by amino acid position and counted. Each position was considered separately for the purposes of testing, and a minimum of three mutations in either cohort was required for inclusion.

Univariate statistical analysis. Patients were divided by mutation status or truncating mutation status for each gene. Fisher's exact and chi-squared tests were used for hotspot analysis, mutual exclusivity or co-occurrence, and other categorical clinical statistics (e.g., mutation status vs intrinsic subtype) as appropriate. Mutual exclusivity or co-occurrence tests were only performed for the top seven genes with the highest non-silent mutation rate. Paired t-tests or Mann–Whitney U tests (depending on normality of distributions tested via Kolmogorov–Smirnov test) were used for comparing group means for age, number of positive nodes, and tumor size. Kaplan–Meier survival analysis was performed for breast-cancer-specific survival (BCSS), relapse free survival (RFS; any regional or distant relapse), or overall survival (OS) with non-silent or truncating mutation status as a factor. Significant survival differences between the groups were determined by log-rank (Mantel–Cox) test. Survival tests were only performed if at least 15 patients had a non-silent mutation or at least eight patients had a truncating mutation. The Benjamini–Hochberg method was performed for multiple testing corrections to report the false discovery rate adjusted *p*-value (*q*-value).

Multivariate statistical analysis. The multivariate Cox proportional-hazard model was fitted to BCSS and RFS separately on the mutation status of a gene, besides classical clinical variables including node status (0 vs. 1–3 vs. 4+ positive), grade (1 or 2 vs. 3), and tumor size (1 vs. 2 vs. 3 vs. 4). To avoid the common issue of non-proportional-hazard and to clarify whether a gene's mutation shows early or late prognostic effect, the survival endpoints were each analyzed using the Cox model splitting at year 5. To adjust for multiplicity, the permutation procedure was performed. At each permutation, the survival and event outcome, together with clinic data, was permuted while mutation data of all genes were untouched but their original association with survival adjusted for clinical data was changed due to the permutation. The same Cox model was fitted on the permuted data to have its accompanied Wald test *p*-value derived. The permuted *p*-value associated with a gene's mutation was finally calculated as the proportion of permutations where at least one gene showed better *p*-value than the gene's original *p*-value for a survival endpoint. All statistical analyses were performed in the R statistical programming language with core, “survival” and “multtest” libraries. Genomic visualizations were created with ProteinPaint⁵⁸ and GenVisR⁵⁹.

Data availability. All mutation calls are made available as a MAF file with this publication. The raw sequence data from UBC-TAM patients are available in the database of Genotypes and Phenotypes (dbGaP) under accession number [dbGaP: phs001234]. Raw sequence data from MA12 and POLAR could not be deposited in public repository due to patient consent issues and complexities of institutional certification. However, these data are available from the authors (contact Obi Griffith and Matthew Ellis). Primary clinical outcome data for UBC-TAM and MA12 can be made available to qualified researchers through application to the Canadian Cancer Trials Group. Primary clinical outcome data for POLAR can be made available to qualified researchers through application to Mitch Dowsett at the Ralph Lauren Centre for Breast Cancer Research.

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Author contributions

O.L.G., T.O.N., M.J.E., and E.R.M. designed the experiments; N.C.S., M.A., M.G., J. K., C. A.M., K.K., J.H., B.J.A., Z.L.S., K.C., R.K., C.F., L.C., J.E.S., S.D., V.M., D.E.L., R.S.F., S.L., and R.K.W. generated the sequencing data. T.O.N., B.Y., M.D., S.L., and D.V. orchestrated the sample pipeline, M.A., O.L.G., N.C.S., and J.K. prepared the figures and tables. O.L.G., N.C.S., M.A., J.L., J.K., and D.T. provided statistical analysis. S.M.K., R.B., and E. C.C. provided functional annotations. T.O.N. provided pathology analysis. M.J.E., N.C.S., M.A., and O.L.G. wrote the manuscript. E.R.M., T.O.N., and M.D., critically read and commented on the manuscript.

Additional information

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paper. The remaining authors declare no competing interests.

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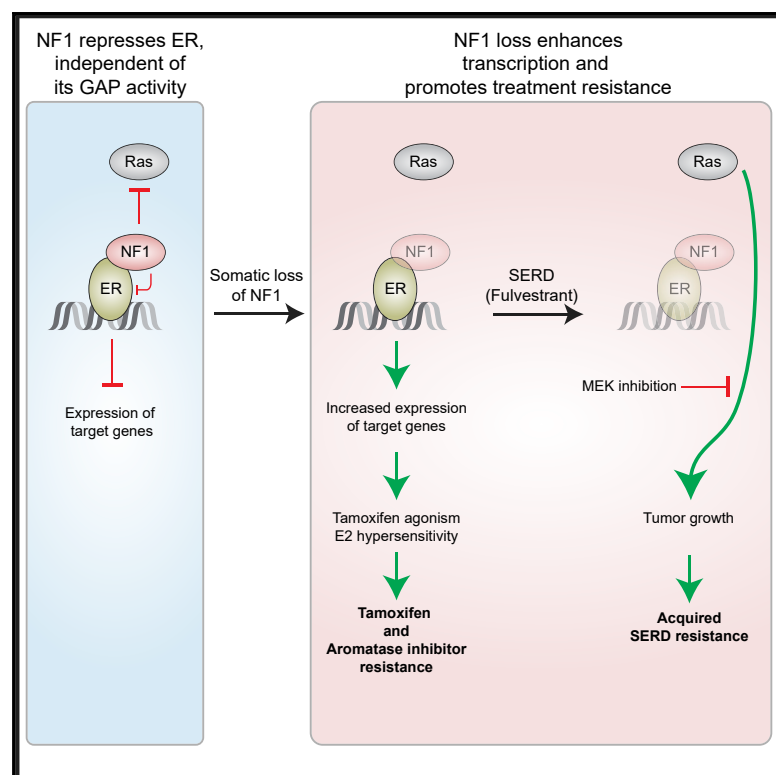


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Neurofibromin Is an Estrogen Receptor- α Transcriptional Co-repressor in Breast Cancer

Graphical Abstract



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In Brief

Zheng et al. find that the Ras-GAP NF1 is also a transcriptional co-repressor of estrogen receptor α (ER). NF1 loss leads to estradiol hypersensitivity and tamoxifen agonism. A selective ER degrader and MEK inhibitor combination induces tumor regression in mouse models of NF1-deficient ER⁺ breast cancer.

Highlights

- NF1 is a GAP-independent estrogen receptor transcription co-repressor
- Somatic NF1 loss causes tamoxifen/aromatase inhibitor resistance in ER⁺ breast cancer
- A MEK inhibitor plus a SERD is effective in NF1⁻ ER⁺ PDX and cell line models



Neurofibromin Is an Estrogen Receptor- α Transcriptional Co-repressor in Breast Cancer

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SUMMARY

We report that neurofibromin, a tumor suppressor and Ras-GAP (GTPase-activating protein), is also an estrogen receptor- α (ER) transcriptional co-repressor through leucine/isoleucine-rich motifs that are functionally independent of GAP activity. GAP activity, in turn, does not affect ER binding. Consequently, neurofibromin depletion causes estradiol hypersensitivity and tamoxifen agonism, explaining the poor prognosis associated with neurofibromin loss in endocrine therapy-treated ER⁺ breast cancer. Neurofibromin-deficient ER⁺ breast cancer cells initially retain sensitivity to selective ER degraders (SERDs). However, Ras activation does play a role in acquired SERD resistance, which can be reversed upon MEK inhibitor addition, and SERD/MEK inhibitor combinations induce tumor regression. Thus, neurofibromin is a dual repressor for both Ras and ER signaling, and co-targeting may treat neurofibromin-deficient ER⁺ breast tumors.

INTRODUCTION

Germline mutations in the *NF1* (neurofibromatosis type 1) gene are responsible for neurofibromatosis type 1, the most common inherited disorder that predisposes individuals to both benign

and malignant tumors of the nervous system (Ratner and Miller, 2015), as well as an increased risk for breast cancer (Madanik et al., 2012; Salemis et al., 2010; Sharif et al., 2007). Analysis of The Cancer Genome Atlas (TCGA) data has shown that *NF1* is mutated in a wide range of common cancers (e.g., melanoma,

Significance

Neurofibromin is a co-repressor of estrogen receptor (ER), therefore loss enhances ER transcriptional activity. Loss-of-function mutations in the *NF1* gene promote tamoxifen agonism and estrogen deprivation resistance. Although ER⁺ NF1-depleted cells are initially responsive to fulvestrant, a selective ER degrader, enhanced Ras activity promotes acquired resistance. When Ras pathway activation is inhibited with a well-tolerated MEK inhibitor, fulvestrant resistance is reversed and full tumor regression ensues. Thus, neurofibromin depletion represents a distinct breast cancer subset where the choice of endocrine treatment may be critical, and MEK inhibitor combinations require clinical investigation. A role as a dual repressor for both Ras and ER may also explain the sexually dimorphic characteristics of neurofibromatosis where tumorigenesis is promoted by female puberty.



lymphoma, and cancers of the lung, breast, and colon) (Yap et al., 2014). Thus, *NF1* deficiency underlies the formation and/or progression of a large number of cancers.

NF1 encodes neurofibromin, an established GTPase-activating protein (GAP), that attenuates Ras signaling (Maertens and Cichowski, 2014). The human genome contains up to 14 Ras GAPs that share no significant sequence homology beyond the GAP domain. However, across species there is extensive evolutionary sequence conservation outside of the relatively small GAP domain (e.g., 83% overall identity in amino acid sequence between human and salmon neurofibromins), suggesting that neurofibromin has other yet undisclosed functional domains.

Approximately 80% of breast cancers are positive for estrogen receptor- α (ER⁺), a ligand-dependent transcription factor that is activated by estradiol (E2) (Feng and O'Malley, 2014). The E2-liganded ER recruits co-activators (such as steroid receptor co-activators, SRC-1-3) to estrogen-responsive elements (EREs) in ER-regulated genes. Tamoxifen, a selective ER modulator (SERM), is predominantly antagonistic in breast cancer cells, hence its therapeutic effect. When tamoxifen binds to ER, co-activators are displaced by co-repressors in the ER-ERE complex (Shang et al., 2000). Established ER co-repressors bind ER via their leucine/isoleucine-rich motifs, and substitutions of L or I with an A can disrupt binding (Hu and Lazar, 1999). Interactions are also mediated by electrostatic interactions (Heldring et al., 2007; Shiau et al., 1998).

Besides tamoxifen, ER⁺ breast cancer can be treated with estrogen deprivation through ovarian suppression and aromatase inhibition (AI), and ER can be directly targeted by selective ER degraders (SERDs), such as fulvestrant, which induce ER protein turnover. However, relapse is common, and the majority of breast cancer deaths occur after a diagnosis of an ER⁺ primary cancer.

RESULTS

NF1 Loss Correlates with Poor Patient Outcome in ER⁺ Breast Cancer

While *NF1* mutation frequencies are low in primary ER⁺ breast cancer (2% in TCGA [TCGA, 2012] as shown in Figure 1A, and 4% in our patient cohort [Griffith et al., 2018]), *NF1* mutation frequency is higher in metastatic ER⁺ breast cancer patients ($n = 535$) (Lanman et al., 2015; Zill et al., 2018) when circulating cell-free tumor DNA (ctDNA) was sequenced (18%; Figure 1A; Table S1). Similar enrichment of *NF1* mutations in metastatic ER⁺ breast cancer has been reported by others (Bertucci et al., 2019; Meric-Bernstam et al., 2014; Pearson et al., 2019; Razavi et al., 2018; Sokol et al., 2019; Yates et al., 2017). These observations suggest that somatic *NF1* events are an important class of mutations driving breast cancer progression.

We have previously analyzed somatic mutations in primary tumors accrued from ER⁺ breast cancer patients treated with adjuvant tamoxifen monotherapy and found that *NF1* nonsense and frameshift mutations were associated with a markedly higher risk of breast cancer recurrence and death (Griffith et al., 2018). No GAP-inactivating missense mutations were found. When examining the COSMIC database, a C to T nonsense mutation at R2450 (R2450*) is the most frequent non-silent *NF1* mutation,

found 28 times (Figure S1A). Nonsense/frameshift mutations can trigger mRNA degradation through nonsense-mediated decay. Consistent with this mechanism, greatly reduced full-length *NF1* mRNA levels were observed in both TCGA (Figure 1B) and METABRIC (Curtis et al., 2012) (Figure S1B) samples when nonsense/frameshift mutations were present. Similar reduction of *NF1* mRNA in tumors carrying *NF1* mutations have also been reported in another patient cohort (Pearson et al., 2019). ER⁺ breast cancer is also treated with AI; therefore, we examined gene expression data from the ACOSOG Z1031 clinical trial, where patients were treated with an AI before surgery (Ellis et al., 2011). These data demonstrated that while a multigene proliferation score (MGPS) (Ellis et al., 2017) decreased after AI treatment in tumors with higher *NF1* mRNA levels, reduced suppression of proliferation (high MGPS) was observed in tumors with low *NF1* mRNA levels, indicating AI resistance (Figure 1C; Table S2). Finally, we found that *NF1* proteins missing a small portion of the C terminus caused by the *NF1*-R2450* mutation or by two other recurrent nonsense/frameshift mutations, *NF1*-R2258* and *NF1*-Y2285* (Figure S1A), could not be efficiently expressed unless a proteasome inhibitor was added (Figure S1C), supporting the possibility that some C-terminally truncated *NF1* proteins may be unstable.

Collectively, these findings raise the possibility that depletion of neurofibromin is a common consequence of nonsense/frameshift mutations and most associated with poor outcomes. Importantly, sequencing studies have found few recurrent inactivating mutations in the *NF1* GAP domain, leading us to speculate that *NF1* protein may harbor additional negative regulatory functions relevant to breast cancer pathogenesis.

NF1 Depletion Promotes Tamoxifen Agonism and E2 Hypersensitivity in ER⁺ Breast Cancer Cells

To investigate the consequences of *NF1* depletion on ER⁺ breast cancer, MCF-7 ER⁺ breast cancer cells were engineered to harbor lentiviruses expressing one of two doxycycline (DOX)-inducible small hairpin RNA (shRNA) clones (C5 and C6). Upon DOX addition (+DOX), *NF1* protein levels were reduced by ~70% as detected by a monoclonal antibody (mAb) we raised (Figures S1D and S1E). Increased ERK activating phosphorylation (pERK1/2) in DOX-treated cells versus vehicle-treated or scrambled shRNA controls indicated the anticipated reduction in GAP activity (Figure S1E). An *NF1* shRNA-resistant expression construct reversed these effects (Figure S1E).

Remarkably, when *NF1* expression was suppressed (+DOX) in MCF-7 cells as well as in two more ER⁺ breast cancer cell line, ZR-75B and T47D (Figure S1F), *in vitro* growth was consistently stimulated by 4-hydroxy-tamoxifen (4-OHT) under E2-deprived conditions (charcoal-stripped serum) (Figures 1D and S1G), in comparison with the non-silenced control (–DOX) or the scrambled shRNA +DOX control, indicating an *NF1* loss-mediated transition from 4-OHT antagonism to agonism. Furthermore, *NF1*-silenced cells proliferated at lower concentrations of E2 than controls, and higher E2 concentrations paradoxically inhibited cell growth indicating E2 hypersensitivity (Figures 1E and S1H). Two pools of *NF1* “knockout” (KO) MCF-7 cells were independently created by CRISPR-Cas9 (Figure S1D) with results similar to the shRNA data (Figures 1D and 1E). MDA-MB-231 cells are ER[–], and *NF1* expression is barely

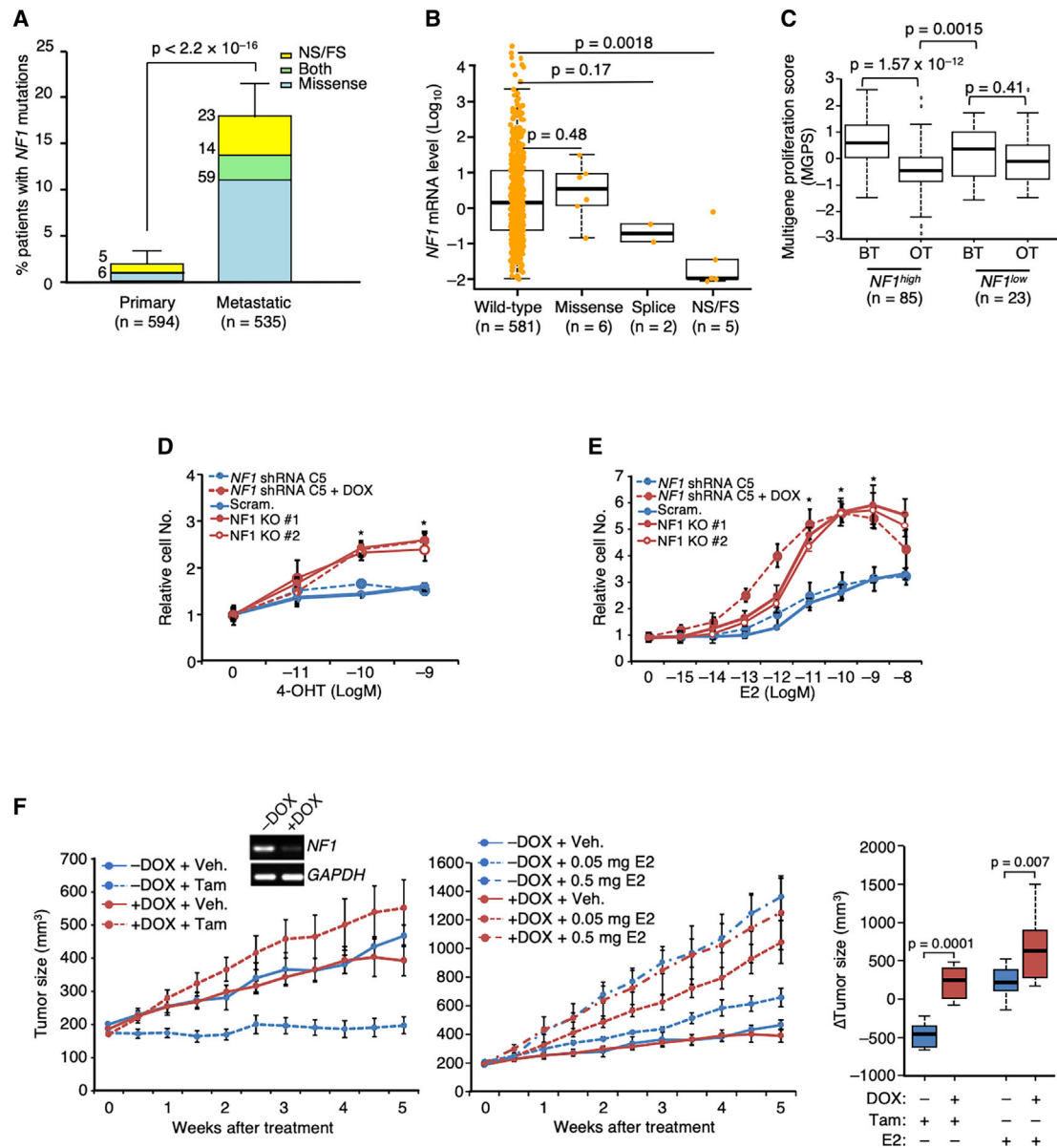


Figure 1. *NF1* Loss Promotes Tamoxifen Agonism and E2 Hypersensitivity Leading to Poor Patient Outcome in ER⁺ Breast Cancer

(A) The percentages of ER⁺ primary versus metastatic breast cancers carrying *NF1* mutations were analyzed by Fisher's exact test. NS and FS stand for nonsense and frameshift, respectively. The number of patients carrying a particular type of *NF1* mutation is shown at the upper left side of each column.

(B) Boxplot analysis of *NF1* mRNA levels in ER⁺ breast tumors carrying different *NF1* mutations in the RNA-seq database of TCGA. p value by Wilcoxon rank-sum test. The line in the middle of the box is the median. The box edges are the 25th and 75th percentiles and the whiskers denote 1.5 times the interquartile range.

(C) Patient samples were stratified by *NF1* mRNA levels according to TCGA definitions of high versus low expression (mean, $1.5 \times$ SD). The boxplot analysis was similarly performed as in (B) to compare multigene proliferation score (MGPS) in tumors before treatment (BT) and on treatment (OT) with AI. The differences in MGPS before and during treatment in each *NF1* group were analyzed by the Wilcoxon signed-rank test. The differences in MGPS as a result of treatment between the two *NF1* groups were further analyzed by Wilcoxon rank-sum test.

(D) DOX-inducible gene silencing using *NF1* shRNA clone C5 and CRISPR-mediated *NF1*-KO were performed in MCF-7 cells. These cells were seeded in E2-deprived medium, to which 4-OHT was added, and cultured for 6 days. Cell numbers relative to vehicle control are plotted. Experiments were conducted as biological triplicates (n = 3 experiments), except for MCF-7 cells carrying *NF1* shRNA C5 (n = 8 experiments).

(E) Cell growth in response to E2 was similarly analyzed as in (D). n = 3 experiments, except for MCF-7 cells carrying *NF1* shRNA C5 (n = 8 experiments).

(F) MCF-7 cells carrying DOX-inducible *NF1* shRNA were transplanted into the mammary fat pads of ovariectomized nude mice, supplemented by an E2 capsule. When tumors appeared, the original E2 capsule was removed, and the resulting mice were randomized, DOX or vehicle treated. Each set was then treated by either tamoxifen (5 mg/mouse, left), or E2 (at two doses, middle). For *NF1*⁺ (-DOX) tumors, n = 10, 12, 12, and 8 mice per group for treatment of vehicle, 0.05 mg E2, 0.5 mg E2, and tamoxifen; for *NF1*^{KO} (+DOX) tumors, n = 10, 13, 11, and 8 mice per group. The inset shows *NF1*-silencing validation by qPCR 2 weeks post-DOX addition. On the far right, " Δ tumor volumes" between vehicle and either tamoxifen or E2 treated were analyzed by boxplot as in (C).

Data are reported as mean $\pm$ SEM. *p < 0.05, **p < 0.01 by pairwise two-tailed Student's t test, unless otherwise indicated. See also Figure S1 and Tables S1 and S2.

detectable (Ogata et al., 2001) due to a frameshift mutation (*NF1*-T467^{fs}) (Neve et al., 2006). These ER⁺ “*NF1*^{low}” cells were unresponsive to 4-OHT or E2, indicating a requirement for ER for the endocrine effects of *NF1* perturbation (Figures S1G and S1H). E2 hypersensitivity and 4-OHT agonism were reproduced *in vivo* using an MCF-7-based xenograft mouse model—tamoxifen stimulated the growth of *NF1*-silenced tumors (Figure 1F, left), and these tumors grew better than control tumors at a lower dose of E2 (0.05 mg dose) (Figure 1F, right).

NF1 Depletion Globally Enhances ER Transcriptional Activity

Because the abnormal E2 and tamoxifen responses observed in *NF1*-depleted cells are ER dependent, we investigated whether *NF1* depletion affects ER-dependent transcription. First, the mRNA levels of established ER target genes were examined by qPCR in MCF-7 cells, and the expression levels of *GREB1* and *pS2/TFF1* in the presence of E2 were elevated when *NF1* was depleted by DOX-inducible shRNA (Figure S2A) or CRISPR-mediated KO (Figure S2B). This increase was not due to an increase in ER protein levels and could be reversed by the shRNA-refractory *NF1* construct (“*NF1*-rescue,” Figure S2A), indicating the specificity of the shRNA targeting. Gene expression was also stimulated by 4-OHT in MCF-7 cells depleted for *NF1* by shRNA (Figure S2C) or by CRISPR (Figure S2B), consistent with conversion to agonism. E2- and 4-OHT-stimulated gene expression were similarly observed in *NF1*-silenced ZR-75B cells (Figure S2C). ER-dependent transcription is promoted by co-activators SRC1-3, and these factors can be selectively degraded by bufalin (Wang et al., 2014). Our data showed that bufalin inhibited 4-OHT-induced enhanced gene expression in *NF1*^{KD} MCF-7 cells (Figure S2D), indicating that SRC1-3 are required for this activity. Finally, we examined tumor tissues from the MCF-7 xenograft model (Figure 1E) by qPCR and found that expression of two ER target genes *GREB1* and *TFF1* was greatly enhanced when *NF1* was silenced (Figure S2E).

To examine the effects of *NF1* on gene expression in a genome-wide fashion, RNA sequencing (RNA-seq) experiments were performed in control (–DOX) and *NF1* knockdown (+DOX, KD) MCF-7 cells with and without E2 stimulation. Overall, E2 altered expression from 540 genes in the control *NF1*⁺ cells and 955 genes in *NF1*^{KD} cells (Figure 2A; Table S3). There was an overlap of 388 genes between the two gene sets (Table S3), indicating that expression changes of 72% (388/540) of the observed E2-altered genes seen in *NF1*⁺ cells were also altered in *NF1*^{KD} cells. These overlapping genes, referred to as the “common E2-regulon,” were analyzed by gene set enrichment analysis (GSEA) Hallmark Pathway analysis and found to be highly enriched with well-established E2-responsive genes (Figure 2A; Table S3). Furthermore, nearly all genes upregulated after E2 stimulation in *NF1*⁺ cells were more strongly induced in *NF1*^{KD} cells, and the great majority of E2-repressed genes were also more strongly repressed, consistent with bidirectional ER hyperactivity upon *NF1* depletion (Zubairy and Oesterreich, 2005). The 415 (955–540) E2-altered genes that were selectively observed in the *NF1*-depleted state (referred to as the “*NF1*^{KD}-unique E2-regulon”) were also assessed by GSEA Hallmark Pathway analysis (Table S3). Aside from a predominance of additional E2-regulated genes, a K-Ras-dependent gene

expression signature was also observed (Figure 2A). Gene expression changes induced by 4-OHT were also examined by RNA-seq. Although 4-OHT overall displays weak agonist activity as expected (Figure S2F; Table S3), the overlap of 4-OHT-altered gene expression between *NF1*⁺ and *NF1*^{KD} cells was 65% and enriched with well-established E2-responsive genes (Figure S2G). Furthermore, genes that were induced by 4-OHT in *NF1*⁺ cells were more highly induced, while 4-OHT-repressed genes were more strongly repressed in *NF1*^{KD} cells (Figure S2G).

To determine whether the E2-induced gene expression patterns shown in Figure 2A could be replicated in patient samples, differentially expressed genes according to *NF1* status (with or without *NF1* nonsense/frameshift mutation) in the METABRIC and TCGA ER⁺ datasets were identified, and pathway enrichments were similarly assessed (Table S4). The results were compared with the two MCF-7 E2-regulons described above. Supporting the conclusion that *NF1* depletion dramatically affects the expression of E2-responsive genes in clinical ER⁺ specimens, E2-responsive pathway terms were most significantly modulated by *NF1* mutation status, followed by the K-Ras signature (Figure 2B). A similar conclusion has been obtained by others after examining ER⁺ breast tumors with *NF1* shallow deletion in METABRIC (Dischinger et al., 2018) and *NF1* “truncating” mutations in TCGA (Pearson et al., 2019).

NF1 Depletion Increases ER Recruitment to EREs

In the presence of E2, *NF1* depletion mostly affects expression of known E2-responsive genes. To assess whether this is due to direct ER binding to the EREs, we performed chromatin immunoprecipitation (ChIP)-qPCR to analyze ER recruitment to ten well-documented ERE sites in six genes, *TFF1*, *GREB1*, *PGR*, *MYC*, *XPB1*, and *CCDN1*. The data show that *NF1* depletion enhances ER recruitment to all of these sites (Figures 2C and 2D).

In the presence of 4-OHT, expression of 542 genes was mostly detected in *NF1*^{KD} cells (Figure S2G). We examined one 4-OHT-dependent ER ChIP sequencing (ChIP-seq) dataset (He et al., 2018) and found that 68% of these 542 genes (371/542) were also found in this ER ChIP-seq study (Table S3). All together, these data suggest that *NF1* depletion mostly *quantitatively* enhances ER-dependent transcription, whether it is stimulated by E2 or 4-OHT, but *NF1* loss does not substantially *qualitatively* alter the profile of genes that get transcribed under the experimental conditions tested.

Neurofibromin Contains Two Leucine/Isoleucine-Rich Co-repressor Motifs

Tamoxifen agonism and E2 hypersensitivity, together with the RNA-seq and ER ChIP-seq data, suggested that neurofibromin may function as a ligand-dependent co-repressor for ER transcriptional activity. Consistent with this postulate, there are two consensus co-repressor motifs in neurofibromin, designated here as M1 and M2 (Figure 3A). Notably the ER binding motifs in neurofibromin family members appear to have co-evolved with emergence of estrogen-dependent reproduction. M1/2 are highly conserved in salmon, the most human-distant species examined to have an ER homolog (Rogers et al., 2000), but not in fly or yeast (Figure 3A), which do not. Furthermore, mutations affecting key residues in M1 and M2 can be found in cancer (COSMIC and METABRIC) and some of these

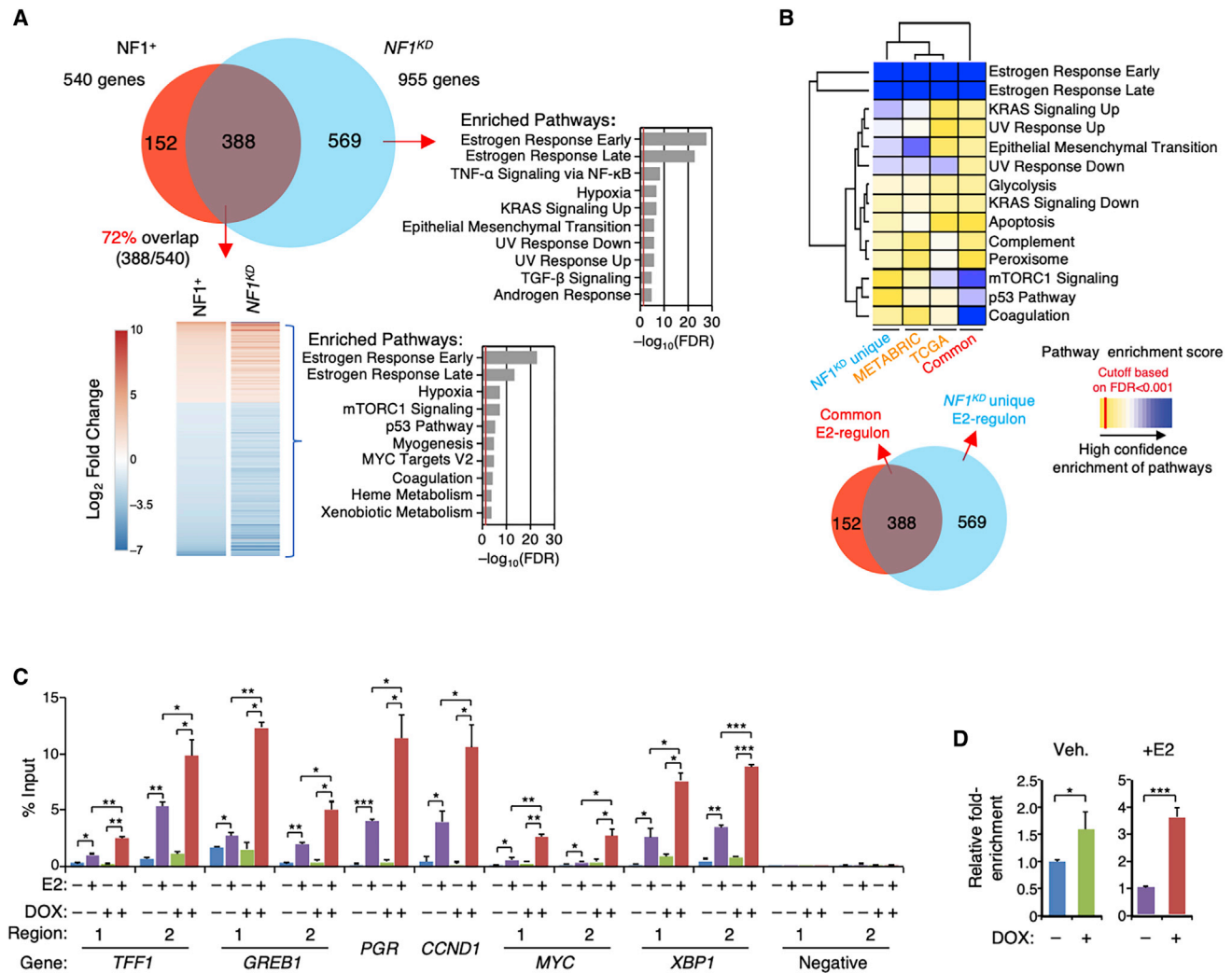


Figure 2. NF1 Depletion Globally Enhances ER Transcriptional Activity

(A) RNA-seq was performed on NF1⁺ or NF1^{KD} MCF-7 cells treated with E2 or vehicle. A Venn diagram depicts the number of E2-mediated differentially expressed genes in NF1⁺ (red), NF1^{KD} cells (blue), and those that overlap (common E2-regulon, purple). The common E2-regulon genes identified in NF1⁺ and NF1^{KD} cells were ranked by (Log₂) fold-change in gene expression, and enrichment for Hallmark Pathways by GSEA is shown to the right (red line marks a false discovery rate cutoff at 0.05). GSEA analysis was also performed to examine Hallmark Pathways selectively enriched in NF1^{KD} cells ("NF1^{KD} unique E2-regulon"). (B) Genes identified in (A) were examined in the TCGA and METABRIC ER⁺ breast cancer cases to identify those genes that are differentially expressed between tumors with wild-type NF1 and NF1 frameshift/nonsense mutations. The enriched Hallmark Pathways in the patient data are presented along with the results of the two E2-regulons identified in MCF-7 cells.

(C) ER was immunoprecipitated from crosslinked NF1⁺ or NF1^{KD} MCF-7 cells treated by E2 or vehicle, and ChIP-qPCR was performed to measure ER occupancy at ten ERE sites in six genes. Two previous known ERE⁻ regions (Carroll et al., 2005, 2006) were assessed as negative controls. n = 2 ChIP experiments.

(D) ER ChIP-qPCR fold-enrichment values from all ERE sites examined in (C) were averaged and compared in NF1⁺ versus NF1^{KD} (+DOX) cells seeded with or without added E2 (n = 10 sites).

Data are reported as mean $\pm$ SEM.

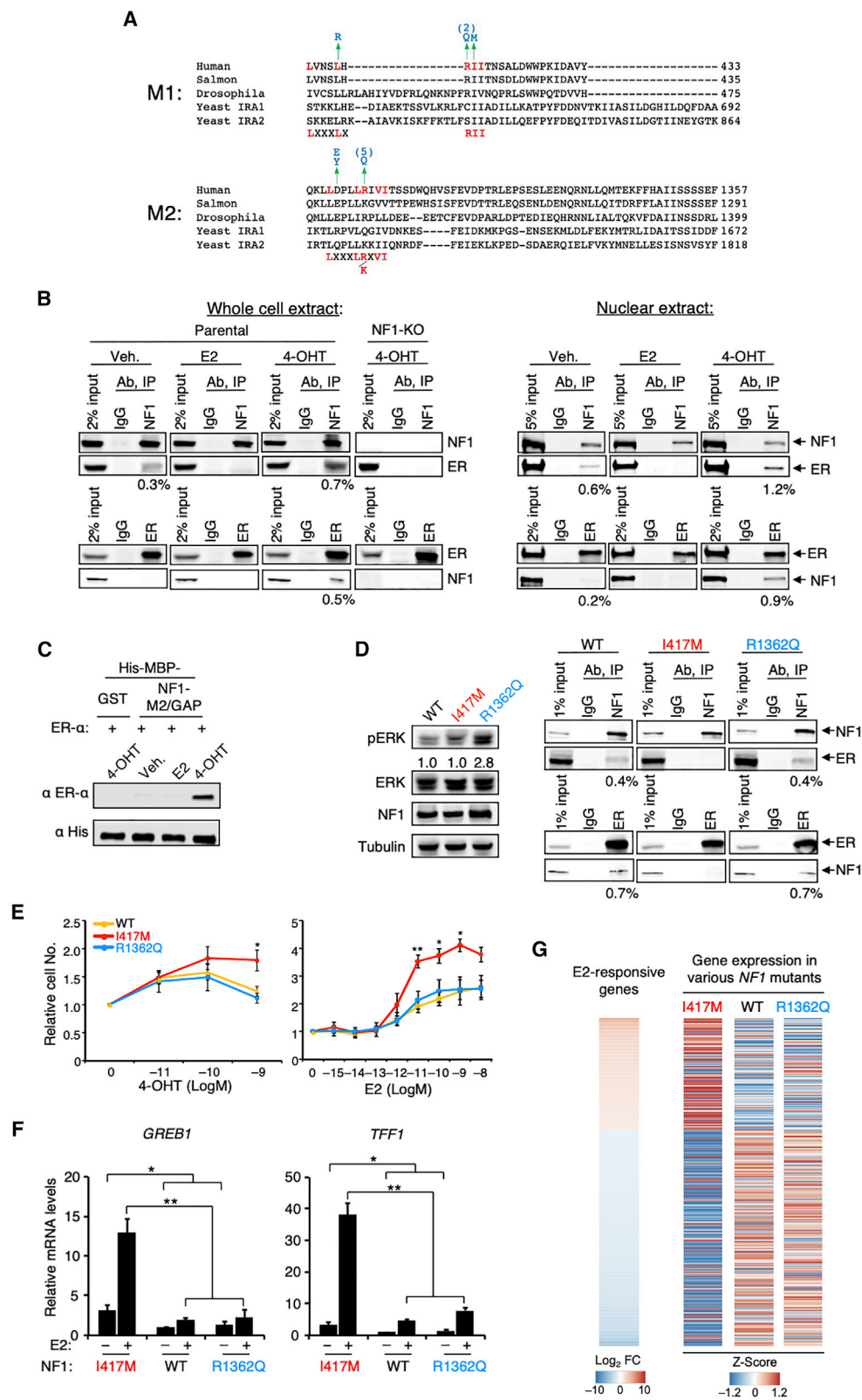
*p < 0.05, **p < 0.01, ***p < 0.001 by pairwise two-tailed Student's t test. See also Figure S2 and Tables S3 and S4.

mutations are recurrent. In particular, a somatic M1 I417M mutation was found in our patient cohort (Griffith et al., 2018), which coexisted with an NF1 nonsense mutation suggesting a biallelic event.

The Binding of ER to Neurofibromin Is Selective, Ligand Dependent, and Direct

Established ER co-repressors preferentially bind to the ER ligand-binding domain (LBD) in an interaction that is enhanced

by 4-OHT but not by E2 (while the reverse is the case for co-activators) (Huang et al., 2002; Shiao et al., 1998). Ligand-dependent binding of neurofibromin to the ER LBD was detected in cells using a mammalian two-hybrid assay (Chang et al., 1999), mimicking a known co-repressor NCoR1 and opposite to the behavior of the co-activator SRC-1 (Figures S3A and S3B). Reciprocal co-immunoprecipitation experiments in MCF-7 and ZR-75B cells were also performed to demonstrate that neurofibromin can co-immunoprecipitate ER using our mAb and vice



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versa (Figure S3C). NF1⁺ MCF-7 cells were used to illustrate the point that these interactions were enhanced by 4-OHT, but reduced by E2; in contrast, ER could not be pulled down in the NF1-KO line, indicating that our neurofibromin mAb is specific (Figure 3B). Finally, M2 is located in the GAP domain, which can be readily purified from *Escherichia coli* (Bollag et al., 1993). We thus determined that 4-OHT increased the efficiency of the interaction between purified NF1-M2/GAP domain and purified ER in a pull-down, confirming that the interaction between NF1 and ER is very likely direct (Figure 3C).

The human genome contains 14 potential Ras GAPs, but they do not share sequence homology beyond the GAP domain. For example, in p120GAP/RASA1 there is no identifiable M1; nor was there an M2 in a published GAP domain alignment study (Ballester et al., 1990) (Figure S3D). Although ER co-immunoprecipitated NF1, ER did not co-immunoprecipitate p120/RASA1 (Figure S3C). Protein co-expression is a strong predictor for co-functionality (Wang et al., 2017a). Therefore, we assessed gene ontology molecular function terms of proteins whose levels positively correlated with those of neurofibromin from proteomics analysis of >100 breast cancer patient-derived samples (Clinical Proteomic Tumor Analysis Consortium) (Mertins et al., 2016). Whereas NF1 protein levels highly correlated with a number of transcription factor functionalities, and the “ligand-dependent nuclear receptor binding” term in particular, p120GAP, mostly negatively correlated with these factors (Figure S3E).

ER Binding and GAP Activity Are Two Independent Functions of Neurofibromin

To determine whether neurofibromin can directly interact with ER via M1/2 without a requirement for GAP activity or Ras pathway activation, we first took a pharmacological approach using Raf and MEK inhibitors, dabrafenib and trametinib (Robert et al., 2015). Here we found that Raf/MEK inhibition did not affect E2-dependent transcription measured by qPCR in either NF1⁺ or NF1-silenced MCF-7 cells (Figure S3F). Immunoblotting detected substantial loss of pERK levels by dabrafenib and trametinib, suggesting that the Ras-Raf pathway was efficiently inhibited in these experiments (Figure S3F). Bufalin, which promotes degradation of SRC1-3 co-activators, was the positive

control for this study. One of the best-known examples of a Ras-ER interaction is phosphorylation at serine 118 (pS118) in ER by ERK after growth factor stimulation (Kato et al., 1995). However, this event seems to be context (e.g., cell type and/or culture conditions)-dependent and can be catalyzed by other kinases (Anbalagan and Rowan, 2015). We could not detect a significant increase in ER-pS118 in NF1 KO MCF-7 cells, despite strong increase in activating phosphorylation in ERK1/2 (Figure S3G). Finally, we note that the average half maximal inhibitory concentration (IC₅₀) of trametinib in commonly used ER⁺ breast cancer cell lines under normal culture condition is 300 versus 0.05 μ M in melanoma cells (<https://www.cancerrxgene.org>). Thus, under standard culture conditions, Ras-Raf signaling is not critical for either ER-dependent gene expression or growth of E2-sensitive ER⁺ breast cancer cells.

To directly ascertain whether M1 and M2 are authentic ER binding sites, residues I and V were substituted with A to create NF1-I417A-I418A and NF1-V1308A-V1309A. The I417M NF1 somatic M1 mutant found in an ER⁺ breast cancer was also generated. Conversely, two mutations found in metastatic breast cancer, NF1-R1362Q (Lanman et al., 2015) and NF1-K1444R (Li et al., 2013), were predicted (Wilkins et al., 2012) to inactivate GAP activity (Figure S3H). They were thus tested further to assess whether ER repression requires intact GAP activity. We confirmed that R1362Q and K1444R mutations greatly reduce the GAP activity of NF1, while the M1/2 mutations do not, by measuring pERK levels (Figure S3H).

Binding to ER (two-hybrid assay, Figure S3I) and repression of ER transcriptional activity (ERE-luciferase reporter assay [Hall and McDonnell, 1999]) (Figure S3J) were both greatly reduced by M1 and M2 mutations; in contrast, the two GAP mutants bound and suppressed ER activity as efficiently as wild-type NF1. One GAP and one M1 mutant were chosen for further study by expressing them in NF1-depleted cells to levels comparable with endogenous neurofibromin in control cells (Figure S3K). The enhanced *GREB1* and *TFF1* expression in NF1-silenced cells was completely repressed by wild-type NF1 and the GAP mutant, but only partially by the M1 mutant.

To ascertain whether ER repression and GAP activity are two independent functions of NF1 without using ectopic

Figure 3. Neurofibromin Binding to ER Is Selectively Mediated by Co-repressor Motifs

(A) Protein alignment was created by ClustalW (MUSCLE). NF1 has two potential co-repressor motifs, M1 and M2 (potential consensus sequences shown at the bottom). Mutations found in cancers (COSMIC) or neurofibromatosis are colored blue, and the numbers in parentheses are the number of times found in the COSMIC database.

(B) Parental MCF-7 or the NF1-KO (clone no. 1) MCF-7 cells were grown in E2-deprived medium to which E2, 4-OHT, or vehicle was added. Whole-cell or nuclear extracts were immunoprecipitated with NF1 or ER antibody, and the resulting samples were analyzed by immunoblotting. The number below the blot shows the percentage of co-immunoprecipitated protein.

(C) Recombinant purified ER- α preincubated with E2, 4-OHT, or vehicle was pulled down by amylose beads containing His-MBP-tagged NF1-M2/GAP domain, or His-MBP-GST control, and the results were analyzed by immunoblotting.

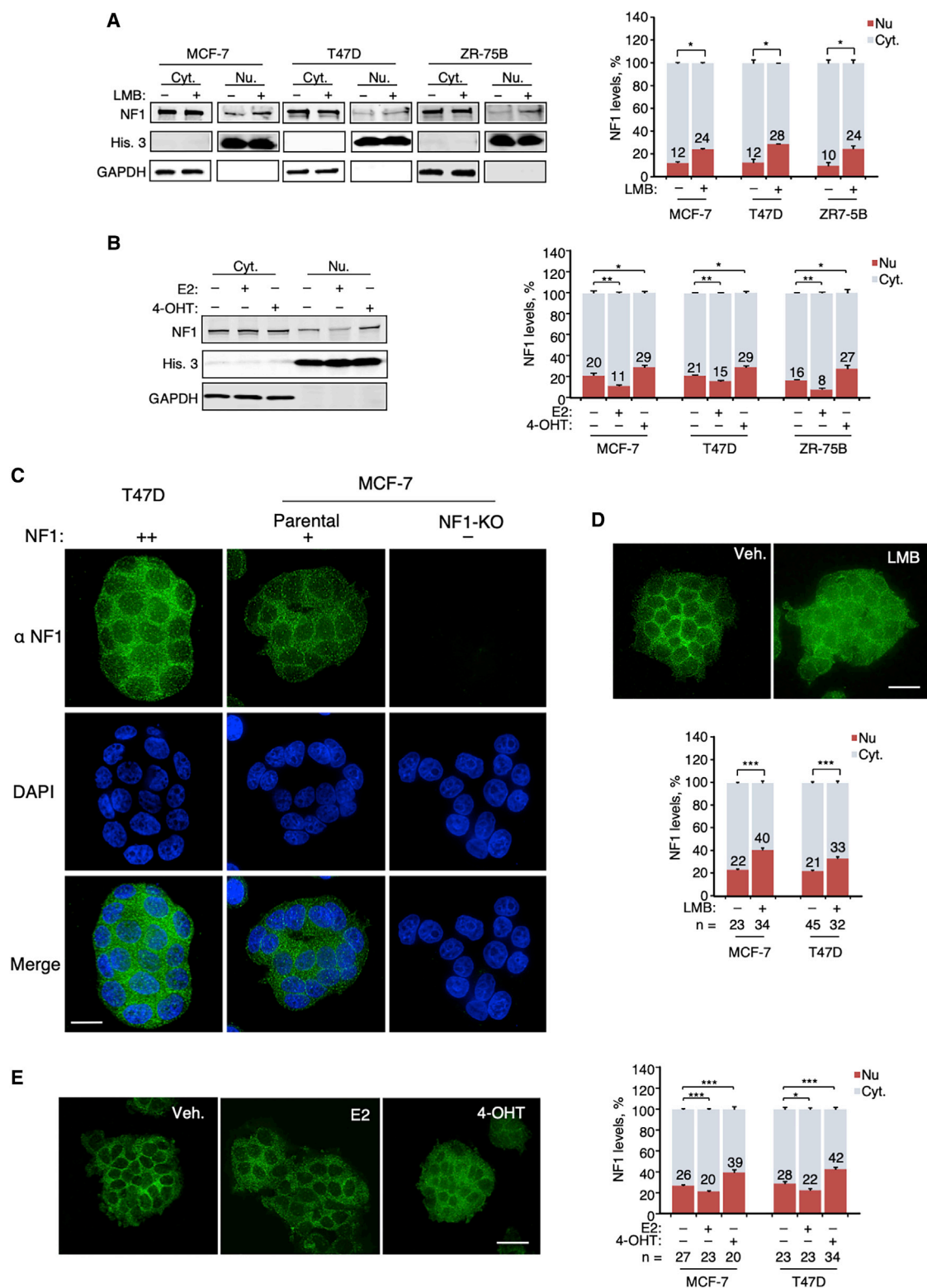
(D) Left: whole-cell lysates from parental MCF-7 cells (WT) or from a CRISPR “knockin” mutant carrying either an I417M (colored red) or an R1362Q (colored blue) mutation grown in full-serum medium were examined by immunoblotting to measure the pERK/total ERK ratios (below). Right: reciprocal co-immunoprecipitation experiments in the presence of 4-OHT similar to those in (B) assessed ER binding to various NF1 proteins.

(E) Parental or NF1 mutant cells were seeded in E2-deprived medium and were examined for cell growth in response to E2 or 4-OHT (6 days later) (n = 3 experiments).

(F) *GREB1* and *TFF1* mRNA levels from parental or NF1 mutant cells treated with vehicle or E2 were measured by qPCR (n = 3 independent experiments). Expression levels were normalized to those of vehicle-treated wild-type cells. Data are reported as mean $\pm$ SEM. *p < 0.05, **p < 0.01 by pairwise two-tailed Student's t test. NS, not significant.

(G) mRNAs from cells seeded in charcoal-stripped serum were analyzed by RNA-seq. “E2-responsive genes” as defined in Figure 2A are shown on the left to which expression levels of genes (row Z scores from “log₂ transcripts per million” values) in various strains were aligned.

See also Figure S3 and Table S5.



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overexpression, CRISPR-Cas9-mediated knockin was used to create MCF-7 cells carrying homozygous *NF1-I417M* (M1) mutation or *NF1-R1362Q* (GAP) mutation (Figure S3L). The former cells had normal GAP activity, but their NF1-I417M cannot co-immunoprecipitate ER (Figure 3D). Similar to cells in which NF1 is depleted by shRNA or CRISPR, the *NF1-I417M* (ER binding) mutant cells showed abnormal endocrine responses because cell growth was stimulated by 4-OHT and low levels of E2; in contrast, the *NF1-R1362Q* (GAP) mutant behaved like wild-type cells (Figure 3E). We examined expression of two well-established ERE genes (*TFF1* and *GREB1*) by qPCR (Figure 3F) and found that their expression after E2 stimulation was greatly enhanced by the I417M mutation, which again behaved like full NF1 depletion. In contrast, the *NF1-R1362Q* mutant behaved like wild-type cells showing only a modest increase in gene expression.

While conducting the ER ChIP experiments, we noted a trend that, when NF1 was depleted, more ER was recruited to the chromatin even when the cells were seeded in the presence of vehicle alone (Figure 2D). Consistent with this, expression of *GREB1* and *TFF1* was significantly stronger in vehicle-treated *NF1-I417M* mutant cells than in wild-type and the *NF1-R1362Q* mutant cells (Figure 3F). To further examine these findings in a transcriptome-wide manner, cells were seeded in charcoal-stripped serum-supplemented medium without adding ligand, and RNA-seq experiments were performed (Table S5; Figure 3G). When expression levels of genes (row Z scores from “Log2 transcripts per million” values) in various cell lines were aligned to the list of E2-responsive genes as identified previously (Figure 2A), it was evident that most of the E2-induced genes were more highly expressed and many E2-repressed genes were more strongly repressed in the *NF1-I417M* mutant (right panel, first column, Figure 3G). Essentially, the *NF1-I417M* mutation mimicked E2-stimulation. In contrast, the *NF1-R1362Q* mutant behaved like the wild-type, showing no detectable transcriptional phenotype (middle and right columns in the right panel, Figure 3G). These results clearly illustrate that ER repression and Ras repression are two independent activities of NF1 that are mediated by distinct structural motifs.

Ligand-Stimulated Nuclear Accumulation of Neurofibromin

For transcriptional regulation, neurofibromin must enter the nucleus. Although neurofibromin is mainly cytoplasmic, nuclear neurofibromin has been previously reported in a variety of cell types, including ER⁺ breast cancer cells (Beausoleil et al., 2004; Daston et al., 1992; Koliou et al., 2016; Kweh et al., 2009; Li et al., 2001; Nousiainen et al., 2006; Vandenbroucke et al., 2004). In one study, neurofibromin nuclear localization in neuronal cells was shown to be dependent on the Ran GTPase (Koliou et al., 2016). This mechanism may also operate in ER⁺ breast cancer, because neurofibromin co-immunoprecipitated

with Ran in MCF-7 cells (Figure S3C). Leptomycin-B (LMB) blocks CRM1-dependent nuclear export. MCF-7, ZR-75B, and T47D cells were treated with LMB, and cell fractionation was then used to demonstrate that nuclear neurofibromin is increased after nuclear export blockade (Figure 4A). We further investigated whether NF1 nuclear levels are also affected by E2. Nuclear neurofibromin was decreased by E2 but increased by 4-OHT, indicating that ligand-regulated modulation of nuclear levels is an aspect of the co-repressor function of neurofibromin (Figure 4B). 4-OHT-enhanced ER and neurofibromin co-immunoprecipitation can be readily detected in nuclear extracts, and the percentages of co-immunoprecipitated proteins were higher in the nuclear extracts than in whole-cell extracts (Figure 3B). To investigate neurofibromin subcellular localization by microscopy, an immunostaining protocol (Figure 4C) was developed using a set of cell lines with varying degrees of *NF1* expression as controls (Figure S4), which included NF1-KO cells. Two cell lines were then chosen to confirm by microscopy the impact of LMB and ER ligands on nuclear neurofibromin levels (Figures 4D and 4E). Overall these results suggest that, while neurofibromin is mostly cytoplasmic at steady state, it is shuttled in and out of the nucleus, possibly by Ran and CRM-1, in a manner controlled by ER ligand exposure.

Ligand-Dependent Association of Neurofibromin with the ER-ERE Complex

When E2 is present, E2-liganded ER recruits co-activators to the ERE; 4-OHT, in turn, displaces co-activators with co-repressors in the complex. These ligand-mediated actions can be recapitulated by a cell-free assay using HeLa cell nuclear extract (Foulds et al., 2013), which does not have endogenous ER. When purified recombinant ER, biotinylated ERE, and HeLa nuclear extract were combined, the E2-liganded ER-ERE complex could more efficiently pull down the co-activator SRC-1 by Streptavidin beads, while NF1, like the co-repressor HDAC1, was more strongly recruited to the ER-ERE complex in the presence of 4-OHT (Figure 5A). To assess whether neurofibromin is recruited to endogenous EREs in a ligand-dependent manner, we performed ChIP-qPCR using two separate polyclonal neurofibromin antibodies, one of which, Ab-2, was generated by us (Figure S5A), and found that neurofibromin was more efficiently recruited to the EREs of *GREB1* and *TFF1* when 4-OHT, but not E2, was added (Figure S5B). NF1-KO cells were examined as the control to show antibody specificity and neurofibromin dependency (Figure 5B). We then selected the same ten well-documented ERE sites as investigated in Figure 2C, and performed three separate neurofibromin ChIP-qPCR experiments to show that neurofibromin recruitment to these sites was enhanced by 4-OHT; in contrast, no neurofibromin recruitment was detected in other regions (Carroll et al., 2005, 2006) that do not contain an ERE (Figure 5C). Together, these results demonstrate that neurofibromin is an authentic ER co-repressor that localizes

(B) Cells after ligand stimulation were similarly analyzed as in (A), and an immunoblot of treated MCF-7 cells is shown as an example. *n* = 3 experiments.

(C) Immunofluorescence deconvolution microscopy was performed using our monoclonal NF1 antibody on cells with varying levels of NF1 protein. A single focal plane across the middle of the nucleus (marked by DAPI) is shown.

(D) Cells treated with LMB were examined by microscopy. NF1 nuclear fractions were quantified in the indicated number of cells.

(E) Cells after treatment with ER ligands were similarly examined by microscopy and quantified. Scale bars, 20 μ m.

Data are reported as mean $\pm$ SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 by pairwise two-tailed Student's *t* test. See also Figure S4.

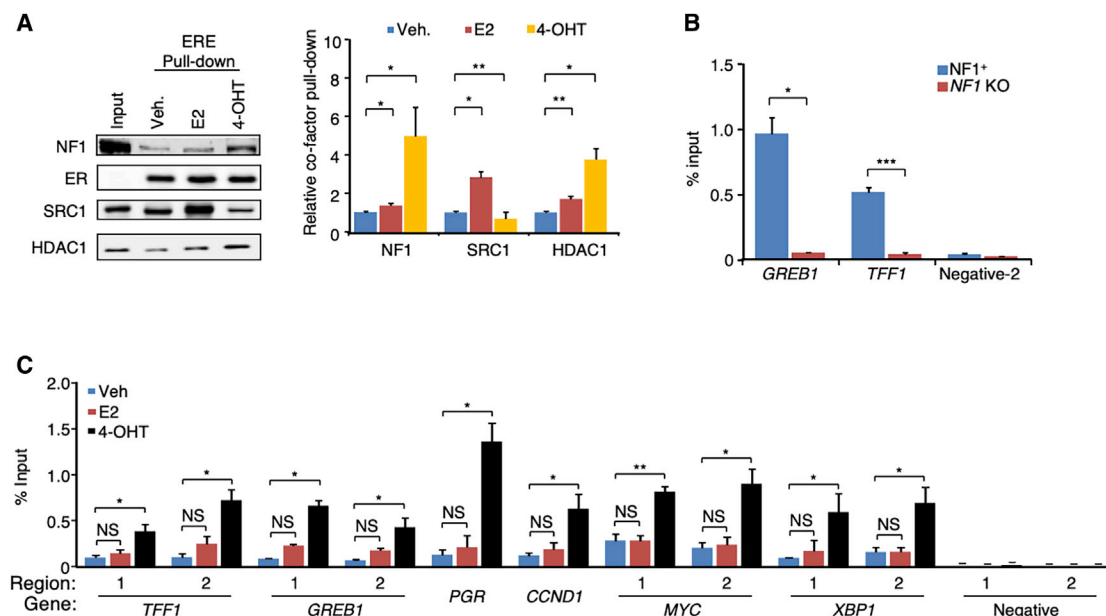


Figure 5. Ligand-Dependent Association of Neurofibromin with the ER-ERE Complex

(A) Ligands were added to the HeLa nuclear extract together with purified ER and biotinylated EREs immobilized onto streptavidin beads. After washing, the proteins bound to EREs with ER were analyzed by immunoblotting (left) and quantified (right). $n = 4$ experiments.

(B) ChIP-qPCR experiment was performed using NF1 antibody Ab-2 to assess NF1 occupancy at the EREs in *GREB1* (region 2, Figure 2C) or *TFF1* (region 1, Figure 2C) in parental or NF1-KO (clone 1) MCF-7 cells treated with 4-OHT. An ERE⁻ region (site 2) (Carroll et al., 2006) was also analyzed as negative control. $n = 2$ experiments.

(C) NF1 occupancy in MCF-7 cells at ten ERE sites in six genes and two negative control regions was assessed by qPCR ($n = 3$ separate ChIP experiments). Data are reported as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by pairwise two-tailed Student's t test. NS, not significant. See also Figure S5.

with ER on an ERE. These findings may explain the recent observation that *NF1* loss (e.g., by loss of heterozygosity) and *ESR1*-activating LBD mutations are mutually exclusive (Sokol et al., 2019), because they both lead to enhanced ER transcriptional activity (Gates et al., 2018; Jeselsohn et al., 2018).

Co-targeting Ras and ER to Treat NF1-Deficient ER⁺ Breast Tumors

The clinical and functional data presented thus far suggest that *NF1*-deficient ER⁺ breast tumors will not be effectively treated with tamoxifen or AI. However, when *NF1* was depleted by either shRNA or CRISPR, the resulting MCF-7 cells were still sensitive to the SERD fulvestrant (Figure S6A). Similar observations were made in *NF1*^{KD} ZR-75B and T47D cells (Figure S6B). However, in *NF1*-silenced MCF-7 cells fulvestrant produced an enhanced compensatory activation of the Ras-Raf-MEK-ERK pathway that may promote cell survival and/or drug resistance despite effective ER inhibition (Figure 6A). Thus, combinatorial targeting of both ER with fulvestrant and the Ras-Raf pathways was assessed to determine if *NF1*-deficient ER⁺ breast cancer can be effectively treated with this strategy. *In vitro*, the combination of Raf and MEK inhibitors, dabrafenib and trametinib, enhanced fulvestrant activity to inhibit cell growth by mostly increasing apoptosis in *NF1*^{KD} MCF-7 and ZR-75B cells (Figure S6C). Immunoblots showed that all drugs inhibited the intended targets (Figure 6A). To develop a clinical protocol, we next investigated whether the dabrafenib and trametinib combination could be replaced by a single MEK inhibitor (MEKi). Selumetinib is a new-

generation MEKi that is less toxic than trametinib due to a shorter half-life and is under consideration by the FDA for the treatment of plexiform neurofibromas in pediatric patients with neurofibromatosis (Dombi et al., 2016; Gross et al., 2018). Our data demonstrate that *in vitro* fulvestrant plus selumetinib also strongly inhibited cell growth and induced apoptosis in *NF1*-silenced MCF-7, ZR-75B, and T47D cells, whereas single-agent exposure did not (Figure 6B). Immunoblotting was performed to confirm that the drugs effectively target ER and ERK activation (Figures 6A and S6D).

In vivo, while tumors from the MCF-7 *NF1*^{KD} xenograft model (+DOX) initially responded to fulvestrant, at later time points these tumors acquired resistance (Figure S6E). Acquired fulvestrant resistance in *NF1*-depleted ER⁺ breast cancer cells has recently also been reported by others (Pearson et al., 2019). We further demonstrated that the fulvestrant-resistant tumor outgrowth in ER⁺ *NF1*^{low} cells *in vivo* can be blocked by dabrafenib and trametinib (Figure S6E). In addition, RNA-seq (Li et al., 2013) and mass spectrometry data (Huang et al., 2017) suggested that a patient-derived xenograft (PDX), WHIM16 (Li et al., 2013), is *NF1* deficient. Immunoblotting confirmed that *NF1* protein levels were barely detectable in WHIM16 (Figure S6F). This PDX line was derived from a patient who died after the development of resistance to multiple lines of endocrine therapies, including fulvestrant and AI.

An initial *in vivo* assessment of dabrafenib-trametinib in WHIM16 suggested activity for Ras-dependent kinase inhibition with fulvestrant over that observed for fulvestrant alone

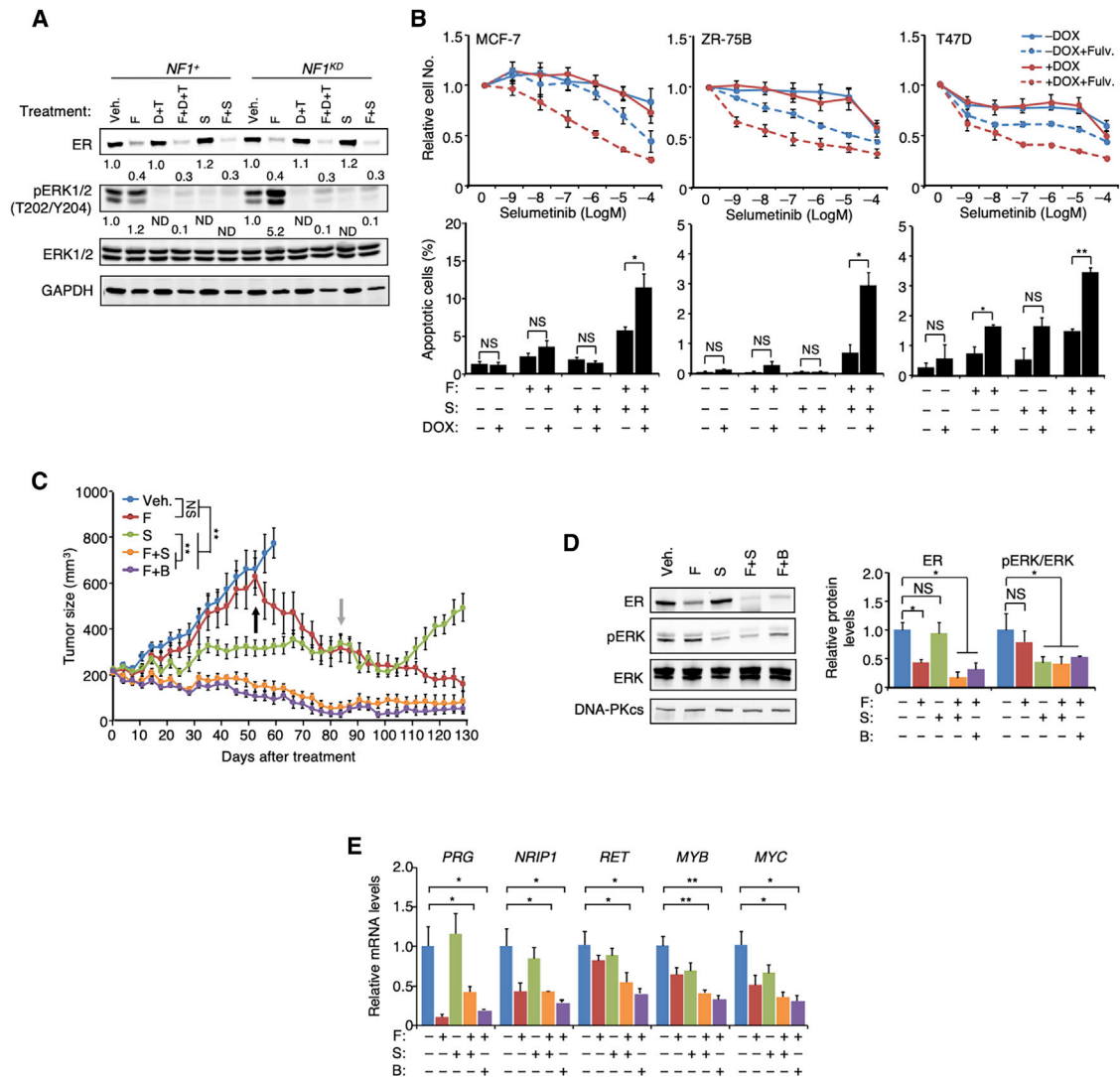


Figure 6. Co-targeting Ras and ER to Treat NF1-Deficient ER⁺ Breast Cancer

(A) MCF-7 cells were seeded in 10^{-11} M E2 to which fulvestrant (F), dabrafenib (D), trametinib (T), or selumetinib (S) were subsequently added at 10^{-9} , 10^{-6} , 10^{-7} , or 10^{-6} M, respectively. After 6 days, proteins were measured by immunoblotting, and phosphorylation levels (as defined by the levels of the phosphorylated form over total protein) relative to the vehicle-treated cells were set to 1. ND, not detectable.

(B) Top: cells were grown for 6 days in the presence of 10^{-11} M E2 and 10^{-9} M fulvestrant, to which increasing concentration of selumetinib was added. Cell numbers relative to the vehicle control are plotted. $n = 3$ experiments. Bottom: the cells were treated similarly except 10^{-6} M selumetinib was used, and apoptosis was measured 6 days post-treatment. $n = 2$ experiments.

(C) WHIM16 tumors were transplanted into cleared mammary fat pads of mice and later randomized to receive treatment ($n = 15$ per treatment arm) when tumor volumes reached 200 mm^3 . Tumor volume comparison was performed at 52 days post-treatment by t test between indicated treatment groups, and at the same time, binimetinib (B) was added to the fulvestrant-only arm (marked by the black arrow). After 84 days (gray arrow), treatments were withdrawn from all treatment groups except the group receiving late binimetinib after initial fulvestrant monotherapy.

(D) WHIM16 tumors from each treatment arm at week-4 post-treatment in (C) were analyzed by immunoblot (one representative tumor shown on the left), and the results relative to those treated by the vehicle control were quantified on the right ($n = 3$ tumors).

(E) qPCR was performed to analyze expression levels of indicated genes from the same tumor samples as in (D). mRNA levels in vehicle-treated samples were set to 1.

Data are reported as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ by t test. NS, not significant.

(Figure S6F), but longer-term exposure was limited by weight loss and diarrhea. We therefore replaced the dabrafenib-trametinib combination with selumetinib-laced chow and conducted a four-arm study to compare single-agent efficacy versus the combination. These data demonstrate that when selumetinib was combined with fulvestrant, efficient and long-term inhibi-

tion/regression of tumor was achieved (Figure 6C) without weight loss (Figure S6G) despite only 50% inhibition of ERK activity. In contrast, selumetinib alone slowed growth but did not induce tumor regression. Western blot confirmed ER degradation by fulvestrant and pERK inhibition by selumetinib (Figure 6D). Selumetinib did not reduce ER phosphorylation at

S118 (Figure S6H), suggesting the MEKi efficacy cannot be easily explained on this basis. Binimetinib, another shorter half-life and better-tolerated MEKi (Trojaniello et al., 2019), also efficiently inhibited tumor growth when combined with fulvestrant (Figures 6C and 6D). Expression of several ERE-containing genes in treated tumors was examined by qPCR. Overall, the lowest level of expression correlated with combination fulvestrant and MEKi exposure (Figure 6E). Although fulvestrant was ineffective as a single-agent, fulvestrant-treated tumors regressed with later addition of binimetinib (Figure 6C, black arrow), mimicking the treatment of ER⁺ NF1^{low} tumors progressing on fulvestrant monotherapy. Furthermore, while selumetinib alone can modestly inhibit tumor growth, growth soon resumed after the treatment was withdrawn; in contrast, no relapse was detected in the fulvestrant + MEKi groups after both agents were withdrawn (Figure 6C, gray arrow).

DISCUSSION

This study presents comprehensive evidence that neurofibromin is a transcriptional co-repressor of ER in ER⁺ breast cancer, independent of its GAP activity. Consequently, when NF1 is depleted through somatic mutation, ER function is enhanced, leading to tamoxifen agonism, estradiol hypersensitivity, AI resistance, and poor outcome. These data suggest that the NF1-depleted ER⁺ breast cancers represent a distinct molecular subset of the disease that will require the development of a new standard of care since tamoxifen is likely contraindicated and AI ineffective.

Our data suggest that, in most cases, complete loss of the NF1 protein is responsible for enhanced ER transcriptional activity and endocrine therapy resistance. Such an NF1-null or NF1^{low} state can be caused by nonsense and frameshift mutations due to nonsense mRNA decay and/or protein instability. Although nonsense and frameshift mutations are detectable by ctDNA sequencing on metastatic patients, NF1 depletion can also be caused by mechanisms not detectable by targeted sequencing. In a study of breast cancer by whole-genome sequencing Nik-Zainal et al. (2016) have described a wide range of structural variations affecting NF1 (tandem duplications, deletions, translocations, and inversions). Furthermore, in METABRIC database NF1 nonsense and frameshift mutations are also usually associated with shallow deletion (Griffith et al., 2018), suggesting biallelic loss-of-function events.

Human and salmon NF1 protein sequences share over 80% identity, which extends well beyond the GAP domain. However, defining a GAP-independent activity in neurofibromin has been difficult because of the broad range of cellular functions influenced by Ras. In hindsight, the commonly used ER⁺ breast cancer cells are ideal for investigating GAP-independent activities of neurofibromin because under standard culture conditions (e.g., in the absence of fulvestrant) growth and ER-dependent transcription operate independently of Ras. This is consistent with the observation that oncogenic mutations in RAS, RAF, MEK, and ERK almost never occur in primary ER⁺ breast cancer. However, Ras-dependent cell survival becomes evident when the ER-dependent growth is inhibited by fulvestrant. Activation of Ras is made more efficient by NF1 loss, and NF1^{low} cells appear to most addicted to this pathway for survival when ER activity is efficiently in-

hibited. *In-vivo*-acquired fulvestrant resistance modeled in the NF1-depleted MCF-7 tumors was efficiently inhibited by dabrafenib + trametinib. WHIM16 represents metastatic ER⁺ breast cancer that already had acquired fulvestrant resistance when the tumor was accrued from the patient. Fulvestrant has little effect on tumor growth unless the MEK is co-inhibited.

As ER⁺ NF1^{low} breast cancer acquires fulvestrant resistance, ER and MEK/ERK crosstalk likely occurs, but the finer details of this mechanism are beyond the scope of this paper. We investigated ER phosphorylation at S118 because this can be directly catalyzed by ERK. However, we could not detect a clear increase in pS118 levels in NF1-KO MCF-7 cells; conversely, in the presence of an MEKi, no decrease in pS118 can be seen in the WHIM16 tumors. Interpretation of WHIM16 experiments is complicated by the presence of fulvestrant, which greatly lowers total ER levels. There is a potential for selective stabilization of the S118-phosphorylated form of ER by other protein kinases, such as GSK3 (Grisouard et al., 2007).

In our experiments, estrogen deprivation therapy is mimicked by incubating cells in charcoal-stripped serum-containing medium, as is customary in the field (Martin et al., 2017). We demonstrate that growth-simulation can be observed in NF1-depleted cells *in vitro* with as little as 10⁻¹² M E2, which parallels E2 levels seen in AI-treated patients. We also present evidence from a neoadjuvant trial that AI resistance can be seen in NF1^{low} tumors defined at the mRNA level.

Our data suggest that most of the 569 NF1^{KD}-unique genes are well-established E2-responsive genes. We speculate that the reason the expression of these genes was not readily detected unless NF1 is depleted is due to the low E2 concentration (10⁻¹¹ M, see the STAR Methods) used to stimulate gene expression in this experiment. This E2 concentration was chosen because it best differentiates the growth of NF1⁺ from NF1^{KD} cells, making it possible to more easily detect the differences in gene expression pattern we display. However, 10⁻¹¹ M E2 is typical for the postmenopausal state and therefore may be too low to affect expression of some E2-responsive genes, until a co-repressor (NF1) is depleted. Of interest, the 569 genes also include genes responsible for EMT, a phenomenon that has also been seen in other resistance settings, i.e., the presence of ER mutants (Jeselsohn et al., 2018) and ER fusions (Lei et al., 2018).

The expression of the rest of the “NF1 knockdown only” genes that do not harbor an ERE is harder to decipher, and many of these genes appear in more than one functional category. Ras does not directly regulate gene expression, so gene expression influenced by Ras activity is likely to be context dependent. Of the 12 “K-RAS signaling up genes,” three (*ID2*, *SERPINA3*, and *PCP4*) are also defined as “estrogen response late,” so only the remaining nine genes may be controlled by Ras activation.

In experimental systems, fulvestrant resistance can be efficiently inhibited by further targeting MEK in both the MCF-7 and WHIM16 mouse models. These results strongly suggest that activation of the Ras-Raf pathway is the major driver for fulvestrant resistance in NF1-deficient ER⁺ breast cancer. On this basis, there is adequate clinical rationale to activate a clinical trial of an MEKi and an SERD specifically in patients with evidence for a loss of NF1 due to somatic mutation. Of note, WHIM16 regression was achieved with a well-tolerated dose of short half-life

MEKi that inhibited ERK signaling by only 50%. This suggests that long-term exposures to lower-dose MEKi necessary for management of advanced breast cancer will be feasible, and the pediatric experience of neurofibromatosis-related plexiform neurofibroma treatment is relevant here (Gross et al., 2018). We note that a fulvestrant and selumetinib combination failed in a randomized phase 2 trial (23 patients per arm) in unselected postmenopausal patients (Zaman et al., 2015). This study does not exclude a benefit in ER⁺ NF1^{low} tumors, however, as it is likely that less than five patients harbored NF1^{low} tumors in this study. Since the prevalence of ER⁺ advanced breast cancer in the USA exceeds 100,000 patients, the ctDNA-based evidence suggests at least 7,000 to 10,000 of these individuals will have an NF1^{low} tumor in which an SERD/MEKi combination could be beneficial.

Further research may connect the ER hyperactivity induced by NF1 depletion to other paradoxical or unexplained observations in ER⁺ breast cancer, for example, regression of endocrine therapy-resistant ER⁺ breast cancer with estradiol treatment (Ellis et al., 2009). In addition, the role of neurofibromin as an ER co-repressor may underlie the sexually dimorphic features of neurofibromatosis, including tumor growth and the preponderance of optic chiasm gliomas during female puberty (Diggs-Andrews et al., 2014).

SUPPORTING CITATIONS

Berto et al., 2018; Borromeo et al., 2016; de Cremoux et al., 2004; Ding et al., 2019; Glont et al., 2019; Harrod et al., 2017; Karmakar et al., 2013; Kininis et al., 2009; Lin et al., 2004; Paschou and Doxakis, 2012; Wang et al., 2018; Won Jeong et al., 2012; Zheng et al., 2005.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.ccell.2020.02.003>.

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AUTHOR CONTRIBUTIONS

Z.Y.Z., M.J.E., and E.C.C. are responsible for the design, analysis, and overall execution of the experiments. Z.Y.Z., M.A., J.C., P.S., J.P., J.T.L., N.-C.N., P.L., Y.C., J.L., X.-H.D., B.C., H.K., W.S., B.-J.K., J.S., S.S., D.W.C., C.-Q.Z., X.C., K.C.B., R.B.L., M.N.S., X.H.-F.Z., S.V., W.L., B.Z., C.E.F., and S.G.H. conducted the experiments and/or data analysis. The paper was mostly written by Z.Y.Z., M.J.E., and E.C.C. and reviewed by all authors.

DECLARATION OF INTERESTS

K.C.B. and R.B.L. are stockholders and employees of Guardant Health, Inc., C.E.F. discloses an equity position in Coactigon, Inc., and M.J.E. received consulting fees from Abbvie, Sermonix, Pfizer, AstraZeneca, Celgene, NanoString, Puma, and Novartis, and is an equity stockholder, consultant, and Board Director member of BioClassifier, and inventor on a patent for the Breast PAM50 assay.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal NF1 (against aa2471-2839, 1:200)	This study	N/A
Rabbit polyclonal NF1 (against aa2471-2839)	This study	N/A
NF1 (against aa2760-2839)	Bethyl Laboratories	Cat#A300-140A; RRID:AB_2149790
NF1 (against N-terminus, 1:500)	Cell Signaling Technology	Cat#14623; RRID:AB_2798543
ER- α (1:500)	Cell Signaling Technology	Cat#8644; RRID:AB_2617128
ER- α (HC-20)	Santa Cruz Biotechnology	Cat#sc-543; RRID: AB_631471
ER- α (F-10, ChIP grade, 1:5000 for Western blots)	Santa Cruz Biotechnology	Cat# sc-8002X; RRID:AB_627558
phospho-ER- α (Ser118, 1:500)	Millipore-Sigma	Cat#05-793; RRID:AB_310004
ERK1/2 (1:500)	Cell Signaling Technology	Cat#9102; RRID:AB_330744
Phospho-ERK1/2 (Thr202/Tyr204, 1:500)	Cell Signaling Technology	Cat#9101; RRID:AB_331646
AKT (1:500)	Cell Signaling Technology	Cat#4691; RRID:AB_915783
Phospho-AKT (Ser473, 1:500)	Cell Signaling Technology	Cat#4060; RRID:AB_2315049
His-tag (1:1000)	Cell Signaling Technology	Cat#2365; RRID:AB_2115720
Ran (1:1000)	Cell Signaling Technology	Cat#4462; RRID:AB_2284873
Histone-3 (1:1000)	Cell Signaling Technology	Cat#4499; RRID:AB_10544537
DNA-PKcs (1:1000)	Cell Signaling Technology	Cat#4602; RRID:AB_10692482
K48-linkage specific polyubiquitin (1:1000)	Cell Signaling Technology	Cat#8081; RRID:AB_10859893
α / β -tubulin (1:2000)	Cell Signaling Technology	Cat#2148; RRID:AB_2288042
α -tubulin (1:10,000)	Santa Cruz Biotechnology	Cat#sc-5286; RRID:AB_628411
p120/RasGAP (1:1000)	Santa Cruz Biotechnology	Cat#sc-63; RRID:AB_628206
SRC-1 (1:1,000)	Santa Cruz Biotechnology	Cat#sc-6096; RRID:AB_661355
HDAC1 (1:1,000)	Santa Cruz Biotechnology	Cat#sc-81598; RRID:AB_2118083
GAPDH (1:10,000)	Santa Cruz Biotechnology	Cat#sc-32233; RRID:AB_627679
Normal Mouse IgG	Santa Cruz Biotechnology	Cat# sc-2025; RRID:AB_628411
Normal Rabbit IgG	Millipore-Sigma	Cat# 12-370; RRID:AB_145841
Bacterial and Virus Strains		
BL21 (DE3)	Agilent	Cat#200131
Biological Samples		
Patient-derived xenografts (PDX) WHIM16	(Li et al., 2013)	N/A
Chemicals, Peptides, and Recombinant Proteins		
17 β -estradiol (E2)	Sigma-Aldrich	Cat#E8875
Cyclodextran-encapsulated “water-soluble” E2	Sigma-Aldrich	Cat#E4389
(Z)-4-hydroxytamoxifen (4-OHT)	Sigma-Aldrich	Cat#H7904
Doxycycline (DOX)	Sigma-Aldrich	Cat#D9891
Human epidermal growth factor (EGF)	Sigma-Aldrich	Cat#E4127
Leptomycin B (LMB)	Sigma-Aldrich	Cat#L2913
Bufalin	Sigma-Aldrich	Cat#B0261
Phenylmethanesulfonylfluoride (PMSF)	Sigma-Aldrich	Cat#78830
DMSO	Sigma-Aldrich	Cat#D8418
Ethanol	Sigma-Aldrich	Cat#E7023
MG132	Millipore-Sigma	Cat#474790
Polybrene	Millipore-Sigma	Cat#TR-1003-G
Trametinib	Selleck Chemicals, MedChem Express, or Novartis	Cat#S2673 or Cat#HY-10999A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dabrafenib	Selleck Chemicals or MedChem Express	Cat#S2807 or Cat#HY-14660A
Fulvestrant	MedChem Express	Cat#HY-13636
Binimetinib	MedChem Express	Cat#HY-15202
Selumetinib	Selleck Chemicals or MedChem Express	Cat#S1008 or Cat#HY-50706
Recombinant ER α protein	ThermoFisher Scientific	Cat#A15674
Critical Commercial Assays		
TruSeq RNA Library Prep Kit	Illumina	Cat# RS-122-2001
KAPA Library Quantification Kit	Kapa Biosystems	Cat# KR0405
Deposited Data		
RNA-Seq	GEO	GSE142479
Experimental Models: Cell Lines		
MCF-7	ATCC	Cat#HTB-22; RRID:CVCL_0031
ZR-75B	A gift from Marc E. Lippman (Georgetown Univ., Med. Cntr.)	RRID:CVCL_5614
T47D	ATCC	Cat#HTB-133; RRID:CVCL_0553
Experimental Models: Organisms/Strains		
Athymic nude mice	Envigo	N/A
SCID/bg	Envigo	N/A
Oligonucleotides		
See Table S6 .	This study	N/A
Recombinant DNA		
pMD2.G	Addgene	Cat#12259
pSPAX2	Addgene	Cat#12260
pINDUCER11-scrambled shRNA	This study	N/A
pINDUCER11-NF1 shRNA clone 5	This study	N/A
pINDUCER11-NF1 shRNA clone 6	This study	N/A
pLentiCRISPR v2-scramble	This study	N/A
pLentiCRISPR v2-NF1 clone 1	GenScript	N/A
pLentiCRISPR v2-NF1 clone 2	GenScript	N/A
pDONR225-NF1	A gift from the RAS Initiative at the Frederick National Laboratory for Cancer Research at NCI	N/A
pCL-FLAG	Zheng et al., 2012	N/A
pCL-FLAG-NF1	This study	N/A
pCL-FLAG-NF1-R2258*	This study	N/A
pCL-FLAG-NF1-Y2285*	This study	N/A
pCL-FLAG-NF1-R2450*	This study	N/A
pCL-FLAG-NF1-R1362Q	This study	N/A
pCL-FLAG-NF1-K1444R	This study	N/A
pCL-FLAG-NF1-I417M	This study	N/A
pCL-FLAG-NF1-V1308A/I1309A	This study	N/A
pM-NF1	This study	N/A
pM-NF1-R1362Q	This study	N/A
pM-NF1-K1444R	This study	N/A
pM-NF1-I417M	This study	N/A
pM-NF1-I417A/I418A	This study	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
pM-NF1-V1308A/I1309A	This study	N/A
pDEST566-GST	This study	N/A
pDEST566-NF1-M2/GAP	A gift from the RAS Initiative at the Frederick National Laboratory for Cancer Research at NCI	N/A
SpCas9(BB)-2A-GFP(PX458)	Addgene	Cat# 48138; RRID: Addgene_48138
pGL4.70-RLuc	Promega	Cat#E688A
pGL2-ERE-Luc	A gift from Donald P. McDonnell (Duke Univ.)	Hall and McDonnell, 1999
5xGal4-Luc	Promega	Cat#E2440
pVP16-ER α	A gift from Donald P. McDonnell	Chang et al., 1999
pVP16-SRC1	A gift from Donald P. McDonnell	Chang et al., 1999
pVP16-NCOR1	A gift from Donald P. McDonnell	Chang et al., 1999
Software and Algorithms		
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/
SoftWorx v7.0	GE Healthcare	N/A
FlowJo	FlowJo	N/A
Bowtie2	Langmead and Salzberg, 2012	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
RSEM v1.2.31	Li and Dewey, 2011	https://deweylab.github.io/RSEM/
R 3.3.4	R Core Team	N/A

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources or reagents should be directed to and will be fulfilled by the lead contact Dr. Eric C. Chang at echang1@bcm.edu. All materials generated by this study are available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Cell Lines**

The human cell lines, growth media, and general methods of cell culture have been previously reported ([Zheng et al., 2015](#)), unless otherwise indicated. All lines were routinely tested for mycoplasma contamination. Estrogen deprivation was achieved by seeding cells in phenol red-free media (DMEM for ZR-75B, MDA-MB-231, and RPMI1640 for MCF-7 and T47D cells) containing 10% charcoal-stripped fetal bovine serum (Sigma-Aldrich) for two days prior to the experiment. Note that MDA-MB-175VII is an NF1-null cell line, apparently carrying biallelic frameshift mutation *p.Y2285FS*5* ([Neve et al., 2006](#)).

The procedures for producing lentivirus, infecting breast cancer cells, and selecting for stable integrants were also conducted as previously described ([Zheng et al., 2015](#)). The cells stably expressing inducible shRNA were collected using FACS (Aria II, Becton Dickinson) two days after DOX treatment (2 μ g/ml) by examining the presence of turboRFP, which is expressed together with the shRNA from the same bicistronic transcript. Typically, >85% of transduced cells were RFP⁺. For *NF1* overexpression and CRISPR-mediated knock-out by two gRNA sequences, cells were transduced and selected in puromycin (1 μ g/ml) and pooled.

Animals

All animal work was approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. For MCF-7 xenografts, prior to transplantation, 5–6 week old female nude mice (Envigo International) were ovariectomized and left to recover for two weeks before an estrogen “capsule” ([Robinson and Jordan, 1989](#)) (0.7 cm silicone tubing, Dow Corning) was inserted. These capsules contain 0.5 mg E2 mixed with silicone gel (Factor II) to keep the final weight of the filler at 2 mg. Three days later, one million human cancer cells were suspended in Growth Factor-Reduced Matrigel (BD Biosciences) and PBS and injected into the No. 9 mammary glands. When average tumor volume reached ≈ 200 mm³, the mice were randomized into different treatment groups, and the E2 capsule in the tumor-bearing mice was replaced with a fresh one, or with a tamoxifen pellet (5 mg/pellet, Innovative Research of America). DOX was added in the drinking water at 0.2 mg/ml to silence *NF1* expression. For PDX studies, WHIM16 tumors were engrafted into cleared mammary fat pads of SCID/bg female mice (Envigo International) and allowed to grow without exogenous E2 supplementation until tumors reached ≈ 200 mm³. Mice were then randomized into different treatment groups. Fulvestrant

was injected subcutaneously at 250 mg/kg weekly. Trametinib (1 mg/kg), dabrafenib (30 mg/kg), binimetinib (20 mg/kg), and selumetinib (20 mg/kg) were given daily as chow (Research Diets Inc.). Mouse body weight was measured twice weekly to monitor treatment toxicity.

METHOD DETAILS

Chemicals

Chemicals used are listed in [Key Resources Table](#). Note that with the exception of the animal experiments, in all other experiments involving E2, the water-soluble version was used. Ethanol was used to dissolve 4-OHT, while DMSO was used to dissolve trametinib, dabrafenib, fulvestrant, and selumetinib. We note that drug concentrations were selected such that fulvestrant plus the selumetinib combination can more easily produce an effect on the cells that is greater than either group alone.

Plasmids

Scrambled and *NF1*-targeting shRNAs (clone C5 and C6) in pGIPZ were obtained from Dharmacon. All nucleotide sequences used in this study are described in [Key Resources Table](#) and [Table S6](#). The shRNA clones were subcloned into pINDUCER11, with which cell lines stably expressing DOX-inducible scrambled or *NF1* shRNA were later created as described ([Zheng et al., 2015](#)). Unless otherwise mentioned, data obtained from cell lines expressing *NF1* shRNA C5 are shown in the figures, while the cells expressing C6 were also tested in most experiments. *NF1* CRISPR-Cas9 knock-out (KO) constructs (clone #1 and #2 in pLentiCRISPR v2) were purchased from GenScript, and scrambled gRNA sequences were synthesized and then cloned into pLentiCRISPR v2. pDONR225-*NF1* was a kind gift from the RAS Initiative at the Frederick National Laboratory for Cancer Research at NCI. The full-length *NF1* coding sequence in this vector has been extensively modified to facilitate expression studies without changing the amino acid sequence. To analyze its expression, an “*NF1* (NCI)” primer set was used for RT-PCR. This *NF1* coding sequence is also refractory to both the C5 and C6 shRNA clones. *NF1* mutants were created by site-directed mutagenesis (QuickChange II XL Site-Directed Mutagenesis Kit, Agilent Technologies) using pDONR225-*NF1* as the template. These cDNAs were finally transferred into destination vectors pCL-FLAG and pM ([Chang et al., 1999](#)) to create pCL-FLAG-*NF1*, pM-*NF1*, pCL-FLAG-*NF1*-R2258*, pCL-FLAG-*NF1*-Y2285*, pCL-FLAG-*NF1*-R2450*, pCL-FLAG-*NF1*-R1362Q, pM-*NF1*-R1362Q, pCL-FLAG-*NF1*-K1444R, pM-*NF1*-K1444R, pCL-FLAG-*NF1*-I417M, pM-*NF1*-I417M, pM-*NF1*-I417A/I418A, pCL-FLAG-*NF1*-V1308A/I1309A, and pM-*NF1*-V1308A/I1309A. pDEST566-*NF1*-M2/GAP ([Bollag et al., 1993](#)), which expresses recombinant 6×His-MBP tag at the N-terminus of *NF1*-M2/GAP in *E. coli*, was also a gift from the NCI RAS Initiative. pDEST566-GST was built by transferring GST cDNA from pENTR-GST to pDEST566.

Generation of *NF1* Point Mutation Knock-in (KI) Cells Using CRISPR-Cas9

MCF-7 cells carrying homozygous mutations were generated by the Cell-Based Assay Screening Service core at Baylor College of Medicine (BCM). The general procedure is as described previously ([Ran et al., 2013](#)) and depicted in the right figure. Briefly, gRNA sequences were cloned into SpCas9(BB)-2A-GFP (PX458, a gift from Feng Zhang, Addgene plasmid #48138; <http://n2t.net/addgene:48138>; RRID: Addgene_48138). In addition to silent mutations that prevent Cas9 cleavage, each single-stranded oligodeoxynucleotide (ssODN) repair template also contains an engineered restriction site to facilitate identification of the KI allele. Cells were co-transfected with the CRISPR/gRNA plasmid and its corresponding ssODN template using the Neon transfection system (Thermo Fisher Scientific). The resulting GFP⁺ cells were plated by limiting dilution for single clone isolation. The genomic region surrounding each target site was PCR-amplified for validation by sequencing.

Assays for Cell Growth and Apoptosis

To compare cell growth between *NF1*⁺ and *NF1*^{KD} cells, cells stably expressing *NF1* shRNA were first seeded in estrogen-deprived medium, typically in triplicate, with DOX added at the same time. Two days later (Day-0), the medium was replaced with a fresh medium containing either vehicle control or compound(s) under investigation, with medium change every three days thereafter. The number of viable cells was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega) six days later (Day-6). Cell growth between parental and *NF1* knock-out cells was similarly examined except no DOX was added. Apoptosis was measured by FITC-Annexin (Becton Dickinson) staining followed by FACS.

Generation of *NF1* Antibodies

A cDNA encoding amino acid residues 2471-2839 in *NF1* was subcloned into pRP259 ([Chang et al., 1994](#)) to express this C-terminal fragment of *NF1* (*NF1*C) as a GST-tagged protein in *E. coli* (BL21 DE3). Purified GST-*NF1*C was injected into rabbits (Thermo Fisher Scientific) or mice (Protein and Monoclonal Antibody Production Shared Resource at BCM) to generate polyclonal and monoclonal antibodies, respectively. The polyclonal antibody from one rabbit (*NF1* Ab-2, [Figure S5A](#)) was later affinity-purified against the same immunizing antigen. One hybridoma clone (mAb-376) was expanded *in vitro* and IgG was purified from culture medium by protein-G Sepharose column chromatography.

Immunoprecipitation (IP)

Cells grown in E2-deprived medium were treated with E2 (10⁻¹¹ M), 4-OHT (10⁻⁷ M), or the vehicle (ethanol) for 4 hrs before being isolated for whole cell or nuclear extracts. To conduct IP using whole cell extracts, cells were lysed in RIPA buffer supplemented with 1 mM

PMSF and protease and phosphatase inhibitors (Roche Applied Science). The cell lysates were pre-cleared with mouse IgG beads (Sigma, 3 hours at 4°C) before NF1 (mAb-376) or ER (F-10 monoclonal, Santa Cruz) antibody or the mouse IgG control (Santa Cruz) was added. Samples were incubated at 4°C overnight, followed by incubation with protein A/G agarose beads (Santa Cruz) for another 4 hours. All beads were finally washed three times with TBS (20 mM Tris-HCl (pH 7.5) and 150 mM NaCl). The bound proteins were eluted with 2× SDS sample buffer by boiling for 5 minutes and then examined by immunoblotting. To conduct IP using nuclear extracts, the same antibodies were pre-incubated with the protein-G Dynabeads™ (Thermo Fisher Scientific) overnight at 4°C before mixing with nuclear extracts which were prepared as described previously (Lanz et al., 2010). All beads were washed twice with NETN buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 0.5% NP-40), and once with PBS before elution and immunoblotting.

Immunoblotting

Cell lysates were generally prepared in RIPA buffer supplemented with protease and phosphatase inhibitors, and proteins were separated by SDS-PAGE before being transferred to nitrocellulose membranes. The primary antibodies used are listed in [Key Resources Table](#). The fluorescein-conjugated secondary antibodies were from Li-COR Biosciences, and the protein levels were quantified by an Odyssey infrared imaging system (Li-COR Biosciences).

RNA-seq

Estrogen-deprived MCF-7 cells were treated with E2 (10^{-11} M), 4-OHT (10^{-7} M), or ethanol (vehicle) before RNA was extracted after 24 hrs using RNeasy Mini Kit (Qiagen) according to manufacturer's directions. The E2 concentration used in this and the ChIP experiment was chosen because it can efficiently differentiate the growth between NF1⁺ and NF1-depleted cells (Figure 1E). Because the E2 concentration used was low, pilot qPCR experiments were conducted to determine that it takes 24 hrs after E2 stimulation to observe substantial difference in gene expression between these cells. MCF-7 CRISPR KI cells were simply grown in estrogen deprivation medium for 72 hrs before RNA extraction. The Genomic and RNA Profiling Core (GARP) at BCM constructed libraries with 250 ng of total RNA using the TruSeq RNA Library Prep Kit (Illumina). cDNA was generated from poly(A)-selected RNA. Libraries were quantified with the KAPA Library Quantification Kit (Kapa Biosystems) and were sequenced on the NextSeq 500 (Illumina) with paired-end 75bp reads and aligned to the hg19 (GRCh37) reference genome using RSEM v1.2.31 (Li and Dewey, 2011) and Bowtie 2 (Langmead and Salzberg, 2012).

Differential gene expression and fold change analysis was performed using EBseq (Leng et al., 2013) with FDR < 0.2 as a cutoff. Differentially expressed genes were subsequently used in Gene Set Enrichment Analysis (GSEA) for “Hallmark Pathways” in the Molecular Signatures Database (MSigDB) (Subramanian et al., 2005) and the top 10 enriched pathways were reported. For validation in clinical datasets, mRNA levels of the 1,107 genes identified from the MCF-7 cell line study were analyzed in TCGA and METABRIC ER⁺ breast cancer cohorts using cBioportal (Cerami et al., 2012; Gao et al., 2013). Genes in these cohorts that are differentially expressed between NF1-FS/NS and NF1-WT tumors were identified by t-test with p values cutoff at < 0.05. The differentially expressed genes in these clinical validation datasets were each similarly analyzed for “Hallmark” pathways enrichment as above (FDR<0.05). Clustering was performed using Hierarchical Clustering in R.

ChIP

The cells were seeded in E2-deprived medium. After 48 hrs, the cells were treated with E2 (10^{-11} M), 4-OHT (10^{-7} M), or the vehicle control (ethanol) for 45 min before ChIP, which was performed as described (Chen et al., 2008). Specifically, crosslinking was performed using 1% formaldehyde for 10 min at room temperature and quenched by glycine (final concentration of 125 mM). Cells were then scraped off the plate with ice-cold TBSE buffer (20 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 150 mM NaCl) and pelleted by centrifugation. Next, pelleted cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 0.25% Triton X-100, 10 mM EDTA, 100 mM NaCl) to release nuclei. The nuclei were further lysed in 1% SDS buffer (50 mM HEPES-KOH, pH 7.5, 1% Triton X-100, 0.1% sodium deoxycholate, 1% SDS, 2 mM EDTA, 150 mM NaCl) and sonicated using a Branson Sonicator to shear genomic DNA to an average fragment size of 200 to 500 bp. ChIP was performed with a commercially available antibody against ER- α (HC-20 and F10 for ChIP-qPCR, and F10 for ChIP-seq, Santa Cruz), a rabbit polyclonal NF1 antibody (A300-140A from Bethyl Laboratories; Ab-1), a polyclonal NF1 antibody made by us (above; Ab-2), a rabbit IgG control (Millipore) or a mouse IgG control (Santa Cruz) added to the same amount of chromatin after SDS was diluted to 0.1%. The precipitated DNA was isolated by phenol-chloroform extraction. To assess ERE occupancy by qPCR, fold-enrichment of amplified ERE levels in the ChIPed samples vs. those in inputs were calculated.

qPCR

RNA was isolated using the RNeasy kit (Qiagen), and cDNAs were synthesized from 5 μ g of total RNA using the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific). Real-time PCR was performed with Fast SYBR Green Master Mix on a Light Cycle 96 (Roche Life Science) or CFX (Bio-Rad) Real-Time PCR system. The PCR primers used are described in [Table S6](#). The relative amounts of PCR products generated from each primer set were determined on the basis of threshold cycle (Ct) using GAPDH as the loading control.

ERE-Luciferase Reporter Assay

MCF-7 cells were seeded in E2-deprived medium for one day before being transfected with pGL2-ERE-Luc (Hall and McDonnell, 1999), which contains 3× vitellogenin EREs, for another day. The cells were then treated with 10^{-11} M E2 for 24 hrs. Cells were

also co-transfected with pGL4.70-RLuc (Promega, Madison, WI, USA) to express *Renilla* luciferase as an internal transfection efficiency control. The firefly and *Renilla* luciferase levels were measured using the Dual-Luciferase Reporter Assay kit (Promega).

The Mammalian Two-Hybrid Assay

The mammalian two-hybrid assays measuring the binding between ER and its coregulators have been described (Chang et al., 1999). Briefly, HEK293 cells were co-transfected by pM vectors expressing various versions of NF1 fused to the Gal4 DNA binding domain (DBD), as well as pVP16 vectors expressing various ER fragments fused to the VP16 transcriptional activation domain. These cells were also co-transfected to carry the firefly luciferase reporter 5×Gal4-Luc3 and the pGL4.70-RLuc, whose *Renilla* luciferase activity was measured to control transfection efficiency. Fusion protein expression levels were also assessed by Western blot. Luciferase activities were measured two days later. To determine whether the NF1-ER interaction can be modulated by E2 and 4-OHT, cells were estrogen-starved before transfection. E2 (10^{-11} M), 4-OHT (10^{-7} M), or the vehicle control was added one day post-transfection. The luciferase activity was measured after another day.

ER and NF1-M2/GAP In Vitro Binding Assay

E. coli BL21 (DE3) cells carrying either pDEST566-GST or pDEST566-NF1-M2/GAP were induced by 1 mM IPTG at 30°C for 4 hrs. The cell pellets were then resuspended in ice-cold MBP binding buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitors) and sonicated. The cell lysates were incubated with amylose magnetic beads (New England Biolabs) for 4 hrs at 4°C and washed by the MBP binding buffer. After elution (2× SDS sample buffer), the concentrations of bound proteins on the beads were quantified using BSA as the standard. Purified recombinant ER- α (10 ng, Thermo Fisher Scientific) was pre-incubated with 100 nM E2, 5 μ M 4-OHT, or ethanol for 1 hr at 4°C, and then mixed with 200 ng bead-bound NF1-M2/GAP or the GST control, which was pre-incubated with 1 mg/ml BSA in MBP binding buffer for 1 hr at 4°C to reduce non-specific binding. After 1 hr at 4°C, the bound proteins were eluted with 2× SDS sample buffer and examined by immunoblotting.

Cell Fractionation

The experiments were conducted as previously described (Suzuki et al., 2010). Leptomycin B (LMB) at 40 nM or ethanol (vehicle) was given to the cells grown in regular growth medium for 6 hours. To assess the influence of ER ligands, cells were first starved in E2-deprived medium and then treated with E2 (10^{-11} M), 4-OHT (10^{-7} M), or the vehicle control for 16 hours.

Immunofluorescence Deconvolution Microscopy

Cells were seeded and treated by LMB or ER-ligands (above) in 12-well plates with an 18-mm No. 1.5 coverslip. The fixation (4% formaldehyde), permeabilization (200 mM glycine/1× PBS, 0.1% Triton X-100), and blocking (5% goat serum) were performed as previously described (Cheng et al., 2011). The sample was incubated with NF1 antibody mAb-376 at room temperature for 2.5 hrs. We found that it is important to optimize the dilution factor for the secondary antibody to reduce background. Alexa-Fluor™ 488 conjugated goat anti-mouse IgG secondary Ab (Thermo Fisher Scientific) was added after a 1:1,000 dilution in the blocking solution and incubation was performed in the dark for 1 hr. The samples were then mounted to slides with a DAPI-containing mounting medium (Vector Laboratories). Imaging was performed at the Integrated Microscopy Core at BCM on a DVLIVE epifluorescence image restoration microscope (GE Healthcare). The system is equipped with an Olympus PlanApo 60×/1.42 N.A. objective and a pco.EDGE sCMOS_5.5 camera with a resolution up to 2,560 × 2,160 pixels. The filter sets used were: DAPI and FITC. Z stacks (0.2 μ m) covering the whole cell (~12 μ m) were acquired before applying a conservative restorative algorithm for quantitative image deconvolution using SoftWorx v7.0. Image J was used to quantify NF1 levels. For each cell a single focal plane dissecting the middle of the nucleus was chosen, from which two ROIs were created: ROI-1 encircles the whole cell while ROI-2 encircles just the nucleus. The reported relative NF1 nuclear levels were defined as: ROI-2/ROI-1.

ERE Pull-down Using HeLa Cell Nuclear Extract

These assays were conducted similarly to those previously described (Foulds et al., 2013). Briefly, 1 mg of HeLa S3 nuclear extract and 0.5 μ g of purified recombinant ER were added to 4 μ g of a 921 bp biotinylated 4× ERE DNA fragment immobilized onto 60 μ l Dynabeads™ M280 (Thermo Fisher Scientific). Different ligands were included in these recruitment assays. After washing twice in NETN and once in DPBS, the retained proteins were detected by immunoblotting.

QUANTIFICATION AND STATISTICAL ANALYSIS

All reported data were typically presented as mean $\pm$ s.e.m. and pair-wise comparisons were analyzed by two-sided Student's t-test unless otherwise mentioned. The n values are either separate biological replicates or numbers of cells or mice, as indicated. Human tumor databases were analyzed using the R statistical packages. We note that throughout this paper *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, Not Significant ($p \geq 0.05$).

NF1 Mutation Frequency in Patients

The extraction and general sequencing pipeline of cell-free DNA has been described previously (Lanman et al., 2015). Specifically, for NF1, most of its exons (except exons 1, 7, 15, 19, 22, 23, 29, 31, 51, and 56-61) were completely sequenced, and single nucleotide

variants as well as small indel events were analyzed. M1 and M2 are encoded by exons 11 (25 amino acids) and 29 (35 amino acids). cBio (Cerami et al., 2012) was used for somatic mutation and clinical data mining. Mutations were visualized using the GeneVisR (Skidmore et al., 2016) Bioconductor package. The cohort of primary breast cancers was from TCGA (TCGA, 2012), while the metastatic cohort was described (Lanman et al., 2015).

Patient Proliferation Status after Aromatase Inhibitor (AI) Treatments

Patients in the ACOSOG Z1031 trial (Ellis et al., 2011) were treated by AIs in a neoadjuvant setting, and their mRNAs before and after treatment were analyzed by Agilent microarray. A multigene proliferation score (MGPS) derived from mRNA expression of 772 genes was used to analyze proliferation status in the patient to assess treatment response (Ellis et al., 2017).

NF1 Expression Level Analysis in Patients

NF1 mutation types in TCGA (Ciriello et al., 2015) and METABRIC (Pereira et al., 2016) were mined from cBio (Cerami et al., 2012; Gao et al., 2013). NF1 mRNA levels in ER⁺ breast tumors from the METABRIC cohort (Curtis et al., 2012) together with survival information were obtained from Oncomine (Rhodes et al., 2007). High and low NF1 mRNA levels were as defined above. Univariate Cox regression was analyzed using the Survival package for R.

Protein Levels Correlation Analysis

We have previously shown that protein co-expression is a strong predictor for co-functionality (Wang et al., 2017a). To identify proteins co-expressed with NF1, we used the LinkedOmics tool (<http://www.linkedomics.org>) (Vasaikar et al., 2018) to calculate the Pearson's correlation coefficient between NF1 and all other proteins in the CPTAC (Clinical Proteomic Tumor Analysis Consortium) breast cancer mass spectrometry dataset, which contains 105 breast tumor samples (Mertins et al., 2016). We ranked the proteins based on correlation coefficient scores and then analyzed them using Gene Set Enrichment Analysis (GSEA) in WebGestalt (Wang et al., 2017b) to identify enriched Gene Ontology (GO) molecular functions. As a control, we similarly analyzed p120GAP/RASA1.

DATA AND CODE AVAILABILITY

RNA-seq data are deposited in Gene Expression Omnibus (GEO) database, accession number GSE142479.



Proteomic profiling identifies key coactivators utilized by mutant ER α proteins as potential new therapeutic targets

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Abstract

Approximately 75% of breast cancers are estrogen receptor alpha (ER α)-positive and are treatable with endocrine therapies, but often patients develop lethal resistant disease. Frequent mutations (10–40%) in the ligand-binding domain (LBD) codons in the gene encoding ER α (*ESR1*) have been identified, resulting in ligand-independent, constitutively active receptors. In addition, *ESR1* chromosomal translocations can occur, resulting in fusion proteins that lack the LBD and are entirely unresponsive to all endocrine treatments. Thus, identifying coactivators that bind to these mutant ER α proteins may offer new therapeutic targets for endocrine-resistant cancer. To define coactivator candidate targets, a proteomics approach was performed profiling proteins recruited to the two most common ER α LBD mutants, Y537S and D538G, and an *ESR1*-YAP1 fusion protein. These mutants displayed enhanced coactivator interactions as compared to unliganded wild-type ER α . Inhibition of these coactivators decreased the ability of *ESR1* mutants to activate transcription and promote breast cancer growth in vitro and in vivo. Thus, we have identified specific coactivators that may be useful as targets for endocrine-resistant breast cancers.

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Introduction

Approximately 75% of breast cancers express estrogen receptor alpha (ER α) and new therapies are needed for the ~50% of ER-positive tumors that acquire endocrine resistance [1]. Current endocrine therapies include selective ER α

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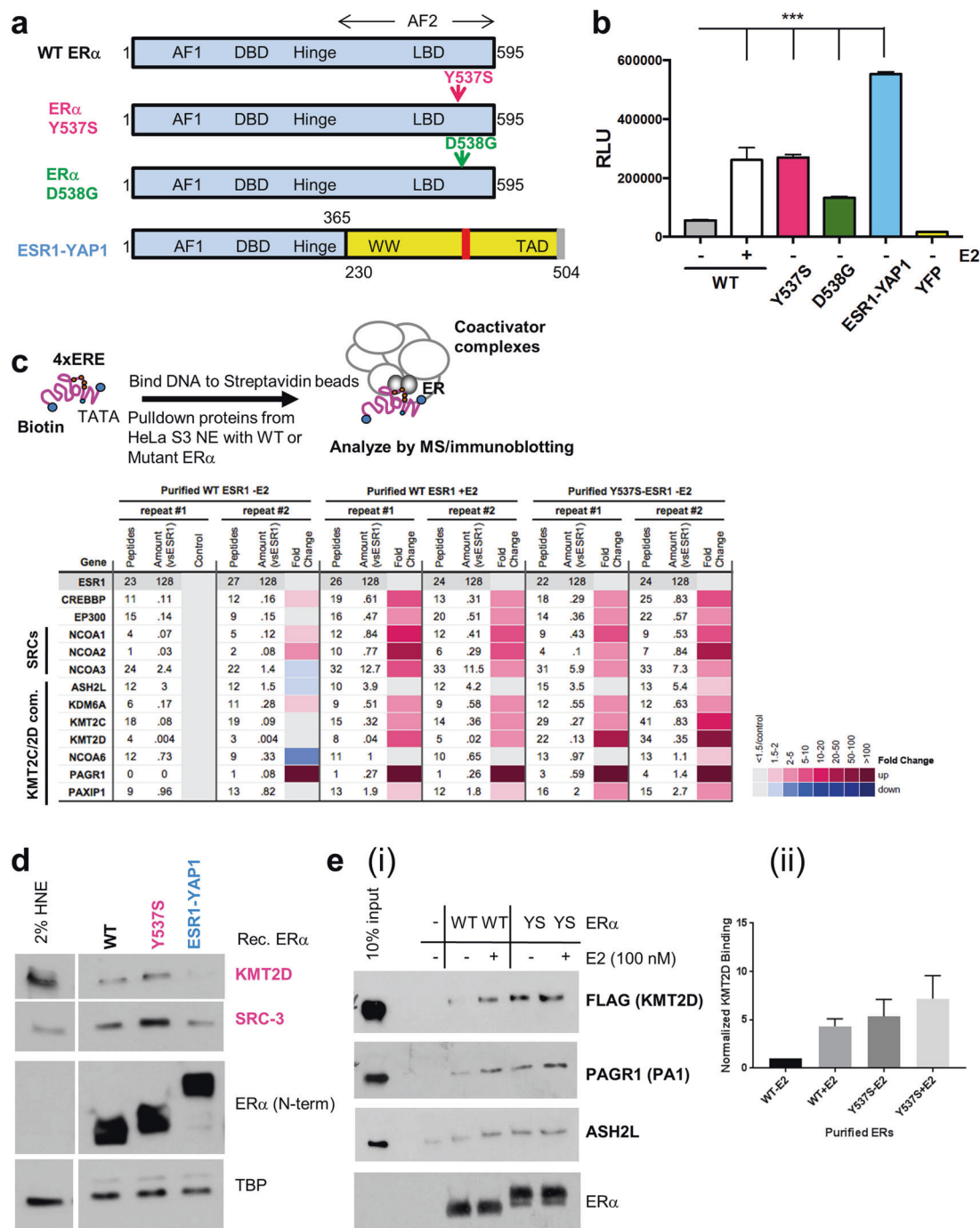
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modulators [2], aromatase inhibitors (AIs) [3], and selective ERα downregulators (SERDs) [4]. ERα plays a major role in the development of therapy-resistant tumors, and its activity is mediated by binding of 17-β estradiol (E2) to its ligand-binding domain (LBD) for the recruitment of steroid receptor coactivators (SRCs) and other coactivators for transcriptional activation of estrogen response element (ERE)-containing genes [5].

One mechanism for endocrine resistance is thought to be through acquired mutations in *ESR1* (gene encoding ERα).

The first *ESR1* mutation (Y537N) was identified in a metastatic breast cancer patient and conferred ligand-independent transcriptional activity [6]. Since then, additional LBD point mutations have been identified that are expressed in a subset (10–40%) of metastatic tumors [7–14]. *ESR1* mutations appear to be acquired in response to AI treatment during metastatic progression [15] and are associated with poor survival [15–17]. The Y537S and D538G mutant receptors possess constitutive ERE-driven transcriptional activity [7–11]. These mutations lock helix 12 of

Fig. 1 Proteomics of co-regulators recruited to the Y537S ER α LBD point mutant. **a** Schematic diagram of WT, Y537S, and D538G ER α point mutants (mutations indicated by arrows), and the ESR1-YAP1 fusion protein. Numbers refer to amino-acid residues in ER α and YAP1. *AF1* activation function 1, *DBD* DNA-binding domain. *Hinge*, region between DBD and ligand-binding domain (LBD); *AF2* Activation function 2, *WW* WW domain, *TAD* transcription activation domain. In ESR1-YAP1, blue represents ER α residues (1–365); yellow indicates YAP1 residues (230–504). **b** Mutant ER α proteins display E2-independent transcriptional activity. Vectors expressing YFP or YFP-tagged WT, Y537S, D538G, or ESR1-YAP1 proteins were co-expressed with an ERE-dependent luciferase reporter (pERE-E1b-luc) in HeLa cells grown in charcoal-stripped fetal bovine serum (FBS). Cells were then treated with/without 10 nM E2 for overnight. Luciferase activity (*RLU* relative light units) was assayed from whole-cell extracts of the cells transfected in triplicate. Data are represented as mean $\pm$ SEM ($n = 3$); *** $p < 0.001$. **c** MS data depicted as a heatmap for WT or Y537S ER α -dependent coactivators recruited to EREs. (Top) Schematic of ERE DNA pulldown assay using HeLa S3 NE as the source of co-regulators (modified from ref. [21]) and purified ER α proteins. (Bottom) MS data were analyzed from duplicate reactions using a label-free method and depicted as in ref. [21]. *Peptides* number of peptides detected; *amount (vsESR1)* amount normalized by sum of area under the curve for six N-terminal ESR1 peptides (see Supplementary Table 2). Fold change represents the ratio of amount detected normalized to unliganded WT ER α . Fold change cutoff used was ≥ 1.5 . SRC-1 to -3 (gene symbols: NCOA1, NCOA2, NCOA3), p300 (gene symbol: EP300), CBP (gene symbol: CREBBP). **d** Immunoblotting validation of KMT2D and SRC-3 enrichment with purified Y537S ER α bound to EREs using independent DNA pulldown samples in the absence of E2. ER α protein binding was assayed by an N-terminal (N-term) antibody. TBP serves as a loading control. 2% input represents 2% of the starting HNE employed in the ERE DNA pulldown. **e** Y537S ER α and the KMT2D complex interact directly in an enhanced manner relative to unliganded WT ER α . ERE DNA pulldown assays were performed with purified ER α proteins and a purified KMT2D “fusion” complex [22], and then analyzed by immunoblotting. (i) Representative immunoblot probing for select KMT2D complex members and ER α . (ii) Quantification of KMT2D signal across three independent pulldown assays. Binding was quantified using Image J and normalized to ER α signal

the ER α LBD into an agonist-type conformation [7, 9, 18] and have higher affinities for the SRC-3 coactivator compared to unliganded wild-type (WT) ER α [14, 19].

ESR1 chromosomal translocation events also occur in endocrine-resistant, metastatic breast cancer patients resulting in fusion proteins possessing the N terminus and DNA-binding domain (DBD) of ER α , but containing partners from various genes that replace the LBD [11, 20]. One such fusion, ESR1-YAP1, induced expression of ERE-containing target genes in a ligand-independent manner, and cannot be targeted with standard endocrine therapies since it lacks a LBD [11].

Understanding how ER α mutant proteins function is essential for the development of new therapeutics to treat endocrine-resistant tumors. To define co-regulators binding mutant ER α proteins as potential new targets, we profiled their recruitment to Y537S ER α , D538G ER α , and ESR1-YAP1 proteins bound to EREs using mass spectrometry (MS). We show that inhibition of the most-enhanced

binding coactivators reduced ERE-driven transcription and *ESR1* mutant expressing breast cancer cell growth.

RESULTS

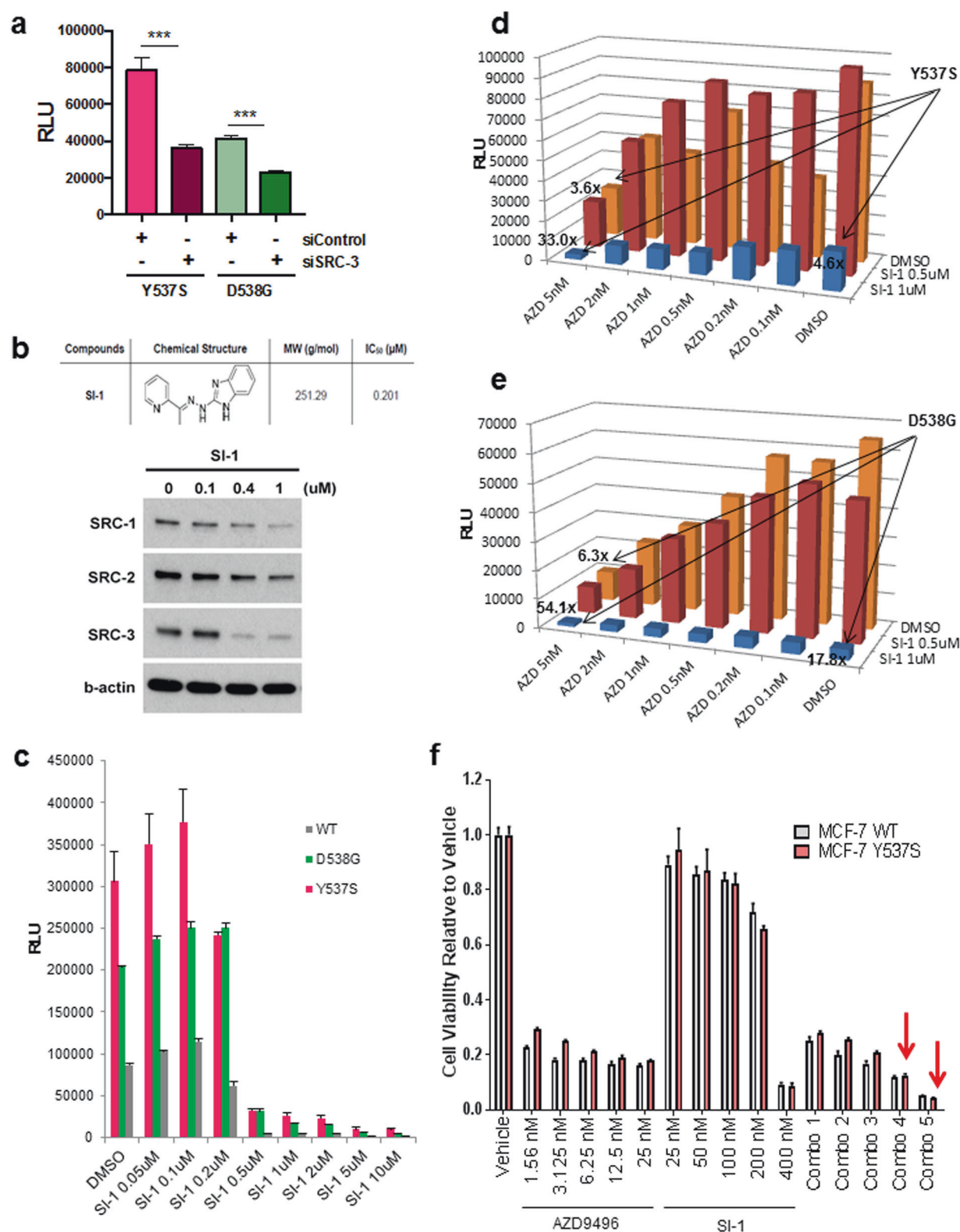
Identification of co-regulators recruited to ER α mutants

Since Y537S, D538G, and ESR1-YAP1 ER α (Fig. 1a) promote E2-independent transcriptional activation of an ERE-dependent reporter (Fig. 1b), we tested whether this activation is through recruiting or repelling distinct co-regulators (coactivators and co-repressors). Using our 4xERE DNA pulldown assay to identify co-regulators recruited to ER α [21], we first utilized recombinant WT and Y537S ER α , along with HeLa S3 nuclear extract (HNE) to form complexes. Washed complexes were then subjected to label-free, quantitative MS and bound proteins were normalized by the amount of ESR1 N-terminal peptides bound (Fig. 1c, top; Supplementary Table 2).

Compared with unliganded WT ER α , a subset of coactivators were recruited in an enhanced manner to Y537S ER α (Fig. 1c, bottom). Namely, the histone H3 lysine 4 (H3K4) methyltransferase KMT2D complex displayed the greatest enrichment with Y537S ER α , along with SRC-1, -2, and -3, p300, CBP, and KMT2D's paralog, KMT2C. Immunoblotting validated the enhanced KMT2D and SRC-3 recruitment (Fig. 1d).

As SRC-3 directly binds Y537S ER α [14, 19], we tested whether a KMT2D complex [22] would directly interact with Y537S ER α . Indeed, the binding of the KMT2D complex was enhanced with Y537S, along with E2-bound WT, compared to unliganded WT ER α (Fig. 1e). We found other potential coactivators with enhanced binding to Y537S ER α (Supplementary Figure 1a). We further validated PELP1 recruitment by immunoblotting, as an inhibitor disrupting this interaction is described [23] (Supplementary Figure 1b). We found very few co-repressors had reduced recruitment to Y537S ER α (Supplementary Figure 1c).

As purified D538G ER α failed to recruit SRC-3 despite binding EREs (data not shown), we resorted to using extracts from transfected 293T cells as sources of WT, Y537S, and D538G ER α proteins. We again observed enhanced recruitment of KMT2D and SRCs to Y537S compared to unliganded WT ER α (Supplementary Figure 2). The D538G mutant also recruited these coactivators, but three to four times less than that of Y537S. Additional potential co-regulators displayed enhanced binding to both ER α mutants. However, for subsequent functional characterization, we chose to focus on SRCs and the KMT2D complex.



◀ **Fig. 2** SRCs are key co-regulators of WT, Y537S, and D538G ER α transcriptional activity and are essential for breast cancer cell growth. **a** Knockdown of SRC-3 by co-transfection of HeLa cells with an siRNA targeting pool reduced transcriptional activity of LBD point mutant ER α proteins, as assayed by an ERE-luciferase reporter, in hormone-depleted media. Data are represented as mean $\pm$ SEM ($n = 3$); *** $p < 0.001$. *RLU* relative light units. **b** A “pan-SRC” inhibitor, SI-1, reduced all three SRC protein levels in MCF-7 breast cancer cells treated overnight, as assayed by immunoblotting. β -actin serves as a loading control. **c** SRC inhibitor SI-1 reduced WT, Y537S, and D538G ER α transcriptional activity at concentrations higher than the established IC₅₀. Vectors expressing YFP-tagged WT, Y537S, and D538G ER α proteins were co-expressed with pERE-E1b-luc in HeLa cells, and then cells were treated with dimethyl sulphoxide (DMSO, vehicle control) or SI-1 overnight. Luciferase activity was measured as in Fig. 1b. Data are represented as mean $\pm$ SEM ($n = 3$). **d,e** Combination of an SRC inhibitor (SI-1) and an oral SERD (AZD9496) synergistically reduce Y537S (**d**) and D538G (**e**) mutant ER α transcriptional activity. Experiment was done as in (**c**), except that AZD9496 was added with or without SI-1 to co-transfected HeLa cells. Data are represented as mean $\pm$ SEM ($n = 3$). **f** The oral SERD AZD9496 is more effective than the SRC inhibitor SI-1 in reducing cell viability of MCF-7 lines expressing WT or Y537S ER α . The lentiviral transduced MCF-7 stably expressing cell lines were treated with vehicle (DMSO) or different concentrations of AZD9496 or SI-1 as indicated. After 6 days of treatment, viability was assayed by a MTT assay. Data are represented as mean $\pm$ SEM ($n = 3$). Synergism was observed with combination (Combo) treatments 4 and 5 (12.5/200 nM AZD9496/SI-1; 25/400 nM AZD9496/SI-1) in Y537S ER α -

SRCs are critical for ER α LBD mutant activity and cell growth

We next tested the functional consequence of enhanced SRC-ER α mutant interactions on transcription. Knockdown of SRC-3 using published siRNAs [24] significantly reduced both Y537S and D538G ER α -mediated transcriptional activity on the ERE-Luc reporter (Fig. 2a). Treatment with a small molecule inhibitor (SMI), SI-1, which inhibits the activities of all three SRCs (IC₅₀ = 0.2 μ M) [25] and reduces SRC protein levels (Fig. 2b), severely reduced the transcriptional activities of WT and mutant ER α (Fig. 2c). Cell viability was minimally affected (Supplementary Figure 3a). We also tested whether the combination of an oral SERD and SI-1 would further reduce LBD mutant ER α transcriptional activity. We focused on AZD9496 [26] (AZD) as it: (1) reduced endogenous ER α protein, (2) was significantly more potent than ICI182,780 (ICI, fulvestrant) in reducing mutant ER α transcriptional activities (unlike another SERD GDC-0810 [27] (GDC; Supplementary Figure 3b-d), and (3) AZD was reported as more effective than ICI at inhibiting tumor growth promoted by Y537S ER α [14]. The combination of SI-1 and AZD synergistically reduced both Y537S and D538G activities on the ERE-luciferase (Luc) reporter (Fig. 2d, e, Supplementary Table 4).

We tested whether SRC inhibition would affect growth of stably expressing WT or Y537S ER α MCF-7 cell lines [28]. SI-1 at 400 nM reduced viability in both WT and Y537S ER α -expressing cells by 91% (Fig. 2f). When combinations of SI-1 with AZD or ICI were tested, a synergistic reduction in viability of Y537S ER α -expressing cells was found at the two highest combined doses or highest combined dose, respectively (Fig. 2f and Supplementary Figure 3e; Supplementary Tables 5 and 6). Thus, SI-1 combined with AZD was most effective in reducing both transcription and cell growth mediated by mutant ER α proteins.

Inhibiting SRCs and mutant ER α most effectively reduces patient-derived xenograft tumor growth

We next tested the efficacy of an improved pan-SRC inhibitor (SI-2) [25], AZD, or the combination in a patient-derived xenograft (PDX) expressing Y537S ER α (WHIM 20 [11]) for tumor reduction (Fig. 3a). SI-2 was chosen instead of SI-1, given its reduced IC₅₀ (3.4 nM) and ability to reduce ER α tumor growth [25]. After tumors grew to 350 mm³, mice were randomized, E2 was withdrawn to mimic AI treatment, and mice were then treated with control vehicle, SI-2, AZD, or the combination. After 4.5 weeks, SI-2 alone significantly reduced tumor volume, AZD gave a larger reduction, but the combination gave the most significant reduction in tumor growth.

We next confirmed that the drugs indeed affected their intended targets and tested for effects on apoptosis and proliferation. First, as expected, AZD treatment significantly reduced ER α expression in tumor lysates. Unexpectedly, AZD treatment upregulated SRC expression (Fig. 3b, Supplementary Figure 4a), which may have relevance for patients receiving AZD monotherapy in clinical trials (NCT02248090/NCT03236974). Second, we tested the expression of a classical ER target gene, PR (Fig. 3c, Supplementary Figure 4a). While SI-2 did not affect PR expression, AZD clearly did. Third, we found that SI-2 increased an apoptosis marker (cleaved PARP protein), while AZD decreased proliferation as measured by BrdU incorporation (Fig. 3d, e, Supplementary Figure 4b). Finally, we did not observe any significant toxicity with any drug treatment after examining recipient mouse livers by histochemistry and measuring body weights (Supplementary Figures 4c-d). Thus, our PDX data support a potential new treatment regime for breast cancers bearing ESR1 LBD mutations, which is to combine a SRC inhibitor with an oral SERD.

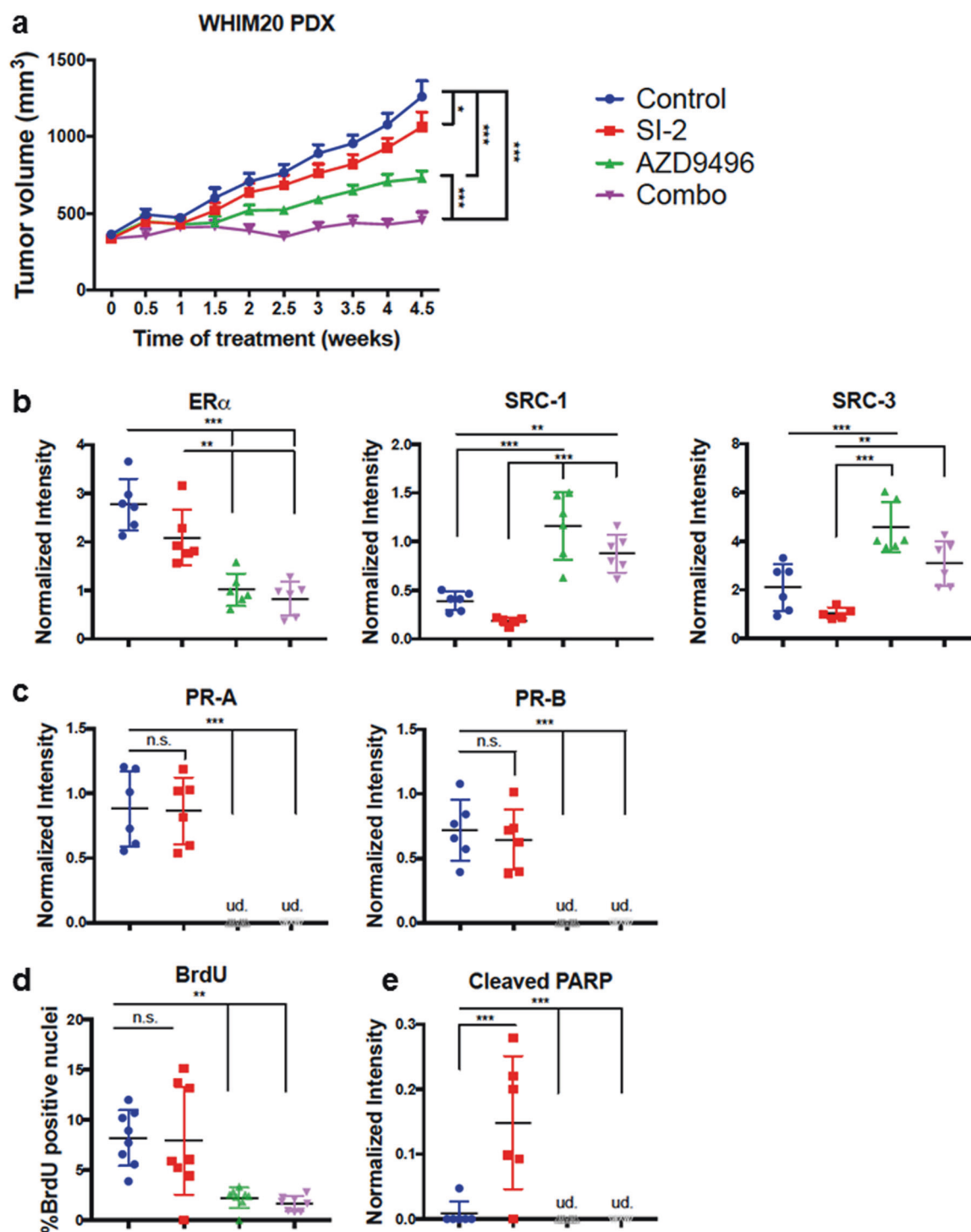


Fig. 3 Inhibition of SRCs and ER α reduces tumor burden in a PDX model of Y537S ER α breast cancer. Significance was determined using one-way ANOVA and Tukey's test to correct for multiple comparisons. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **a** Tumor volume ($n = 8$) is reduced with treatment of either SI-2 or AZD9496, and further reduced upon treatment with a combination of both inhibitors. Data are represented as mean $\pm$ SEM. **b–e** Quantification of tumor immunoblotting ($n = 6$ /treatment) performed with Image J analysis relative to GAPDH expression or imaging data ($n = 8$) unless otherwise indicated. Data are represented as mean $\pm$ SD. ud. undetected. Color legend

is the same as in (a). **b** Quantification of ER α , SRC-1, and SRC-3 protein expression across tumors. By Dixon's Q test, the tumor samples in the last lane probed for SRC-1 and SRC-3 (Supplementary Figure 4a) were outliers at 99% confidence and thus excluded from the Image J analysis. **c** The ER α target gene PR displays reduced expression with AZD9496 and combination treatment. **d** BrdU is decreased in the AZD9496 and combination therapy groups ($n = 8$ mice per group, with mean $\pm$ SD of three slides for each mouse counted). **e** Cleaved PARP is increased with SI-1 treatment and decreased with AZD9496 and combination therapies

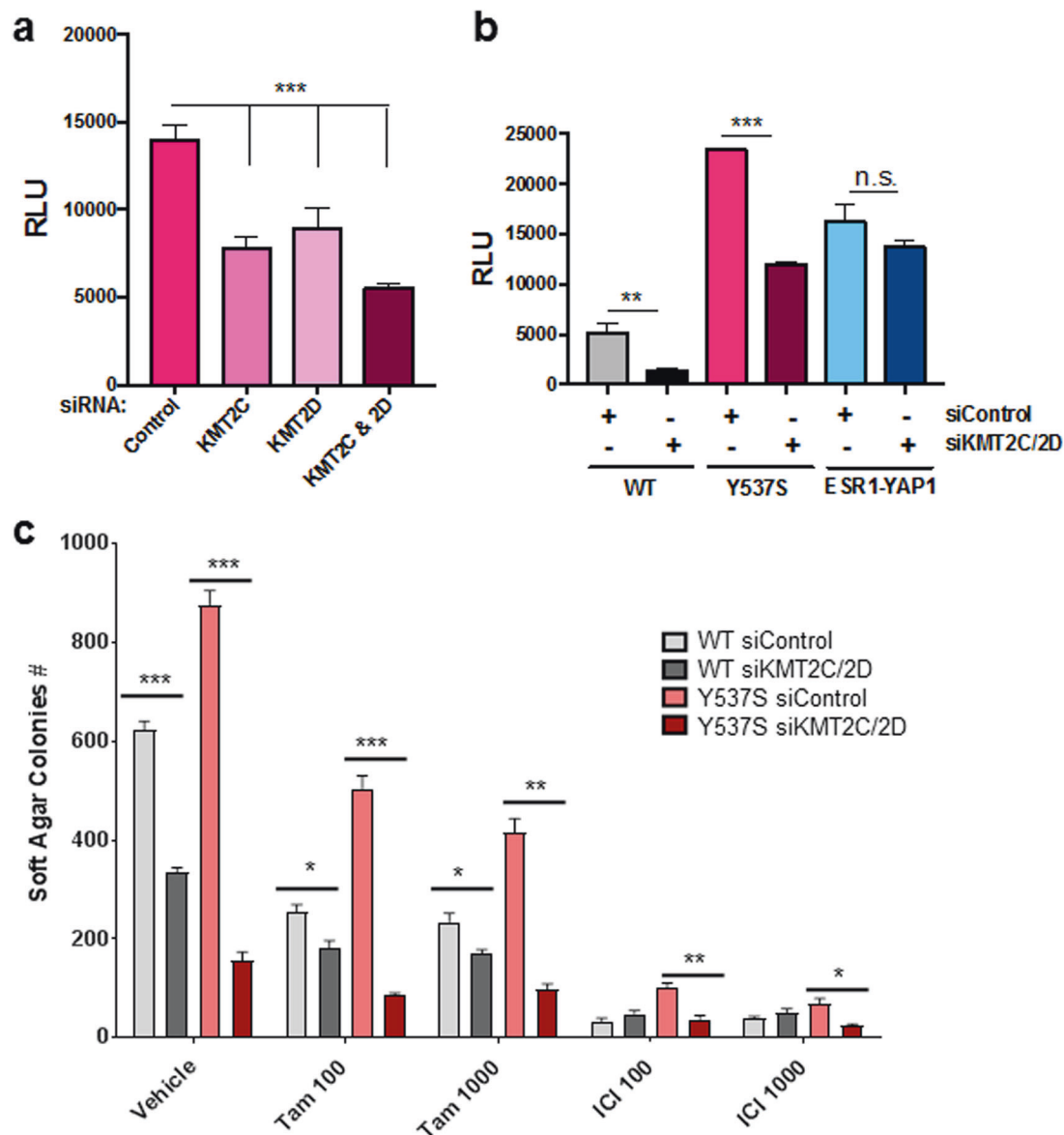


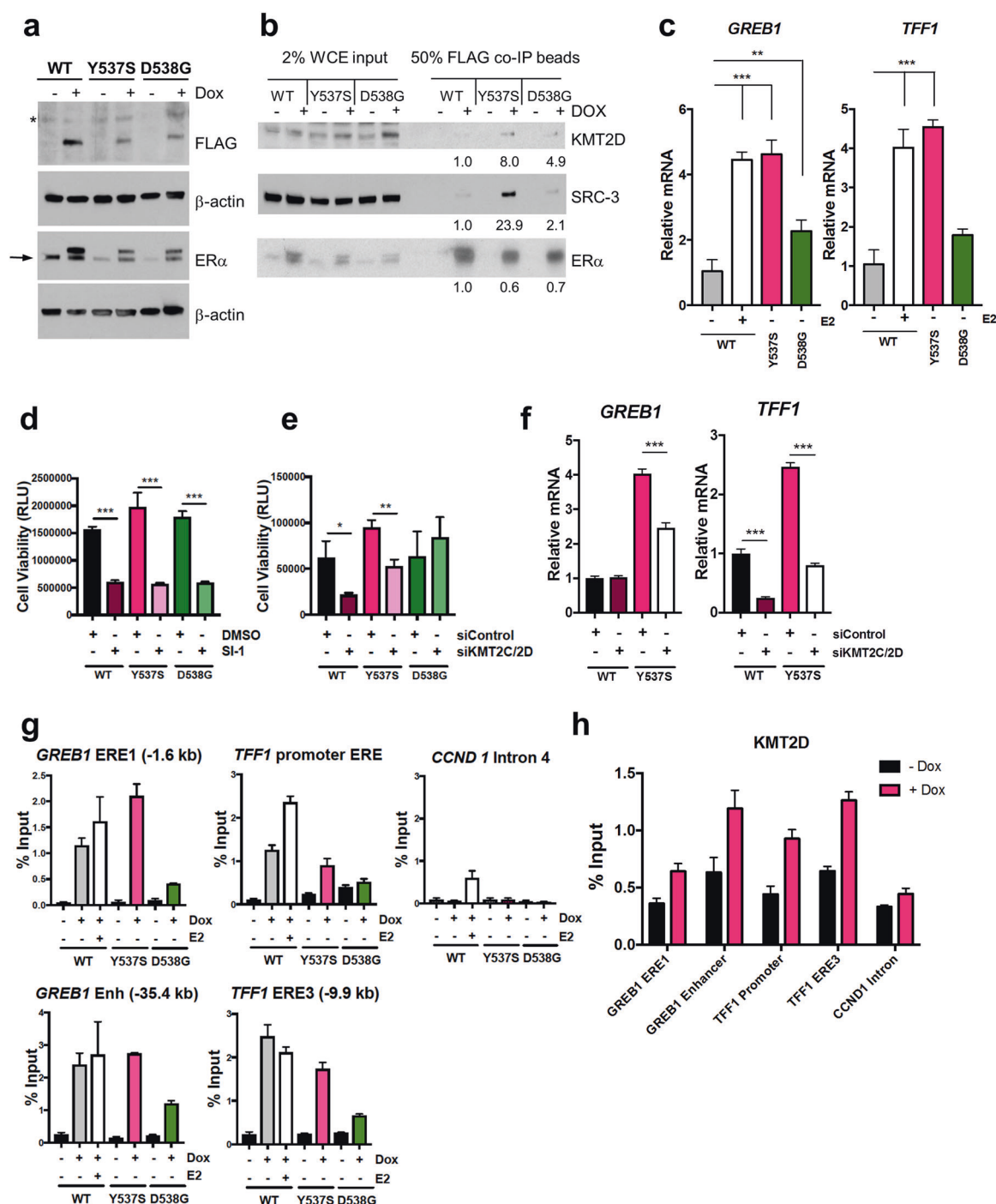
Fig. 4 Knockdown of KMT2C/2D reduces WT and Y537S ER α transcriptional activity and breast cancer cell growth. **a** HeLa cells grown in phenol red-free, charcoal-stripped media were co-transfected with pERE-E1b-luc, YFP-tagged Y537S ER α , and different siRNAs (25 nM each for 50 nM total). Cell lysates were assayed for luciferase activity (RLU). Data are represented as mean $\pm$ SEM ($n = 3$); *** $p < 0.001$. NC#1 siRNA served as the negative control for KMT2D targeting siRNA, while Control-A siRNA pool served as the negative control for KMT2C targeting siRNA pool. **b** Transfection of siRNAs targeting KMT2C and KMT2D reduced expression of the pERE-E1b-luc reporter, as compared to non-targeting siRNAs (siControl), in HeLa cells co-transfected with YFP-tagged WT or Y537S ER α

vectors, but not an YFP-tagged ESR1-YAP1 fusion. Luciferase activity was measured as in Fig. 1b. Data are represented as mean $\pm$ SEM ($n = 3$); ** $p < 0.01$; *** $p < 0.001$. **c** Knockdown of KMT2C and KMT2D in lentiviral transduced stably expressing WT or Y537S MCF-7 cells results in reduced anchorage-independent growth in soft agar and confers sensitivity to anti-estrogens. siRNAs (same as above) were transfected into the two cell lines at a final concentration of 100 nM (50 nM each), and then re-plated in 24 well plates 24 h later. After 1 week with either vehicle or anti-estrogen (100 or 1000 nM 4-hydroxytamoxifen (Tam) or ICI) treatment, colonies formed in soft agar were counted and quantified. Data are represented as mean $\pm$ SEM ($n = 4$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

KMT2C/2D are novel coactivators for Y537S ER α

From above, we found that the KMT2C/2D complexes were preferentially enriched with Y537S ER α (Fig. 1c, Supplementary Figure 2a). To determine the functional role of KMT2C/2D, we tested whether their depletion would affect

Y537S ER α -mediated reporter expression. Knocking down KMT2C, KMT2D, or both together reduced Y537S ER α transcriptional activity (Fig. 4a). Upon double KMT2C/2D knockdown, WT ER α transcriptional activity was also reduced (Fig. 4b). However, ESR1-YAP1 transcriptional



activity was not affected, ruling out a general transcriptional effect.

We additionally found that KMT2C/2D knockdown reduced anchorage-independent growth of WT, Y537S, and D538G ERα-expressing cells (Supplementary Figure 5), with significant differences observed between WT and mutant ERα data. More importantly, knockdown of KMT2C/2D significantly sensitized the partially resistant Y537S ERα cells to anti-estrogens currently given in the

clinic (Fig. 4c). Overall, our data reveal an important functional role for KMT2C/2D with Y537S ERα in transcription and cell growth.

SRCs and KMT2D are crucial for growth of inducible LBD mutant ERα cells

As our above cell lines stably overexpressed LBD ERα mutants [28], we created MCF-7 cell lines supporting

Fig. 5 SRCs and KMT2C/2D promote cell growth and transcription of ERE-containing target genes in conditionally expressed (Dox-inducible) WT and mutant ER α MCF-7 cell lines. Prior to all assays, cell lines were grown in charcoal-stripped, phenol red-free media with/without Dox for at least 2 days. **a** Confirmation of FLAG-tagged WT, Y537S, and D538G ER α Dox-inducible expression in MCF-7 cells transduced with specific lentiviruses. Immunoblotting was performed on whole-cell extracts with FLAG, ER α (HC-20 antibody), or β -actin (loading control) antibodies. *, Nonspecific band. $\rightarrow$, Position of endogenous ER α . **b** KMT2D and SRC-3 co-immunoprecipitate with mutant ER α . Whole-cell extracts from Dox-inducible MCF-7 cells were subject to immunoprecipitation of FLAG, followed by immunoblotting. For analysis, intensities were normalized for different precipitated ER α levels by Image J. **c** Dox-inducible expression of Y537S and D538G ER α in MCF-7 cells results in activation of two canonical ER α target genes independent of E2. Cell lines were treated with Dox and the WT ER α line was treated ($\pm$) 10 nM E2 overnight, followed by RNA isolation. Relative levels of *GREB1* or *TFF1* mRNAs were determined by RT-qPCR with *ESR1* mRNA as the normalizer. Data are represented as mean $\pm$ SEM ($n = 3$); ** $p < 0.01$; *** $p < 0.001$. **d, e** Cell viability (measured by Cell Titer Glo as RLU) of Dox-inducible WT, Y537S, and D538G ER α expressing MCF-7 lines (induced as above). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **d** Cell viability is reduced in all three cell lines upon treatment with SRC inhibitor SI-1 (1 μ M) after 3 days' exposure. DMSO served as the vehicle control. Data are represented as mean $\pm$ SEM ($n = 3$). **e** Cell viability of Dox-inducible WT and Y537S ER α -expressing MCF-7 lines, but not the D538G-expressing ER α line, is reduced upon knockdown of KMT2C and KMT2D after 3 days exposure to 100 nM total siRNA (50 nM each). Data are represented as mean $\pm$ SEM ($n = 3$). **f** Knockdown of KMT2C and KMT2D reduces Y537S ER α -enhanced expression of *GREB1* and *TFF1*. Cells were transfected with siRNAs (100 nM total, 50 nM each). Relative levels of *GREB1* or *TFF1* mRNAs were determined by RT-qPCR using *ACTB* mRNA as the normalizer. Data are represented as mean $\pm$ SEM ($n = 3$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **g** Dox-induced FLAG-tagged WT, Y537S, and D538G ER α proteins occupy EREs of *GREB1* and *TFF1* genes in MCF-7 cells, implying direct transcriptional regulation. In contrast, ER α proteins minimally occupy a negative control region from intron 4 of the *CCND1* gene, which lacks endogenous ER α binding [36]. Where indicated, WT ER α cells were treated with 100 nM E2 for 45 min. ChIP assays employed an antibody against FLAG to IP the FLAG-tagged ER α proteins and associated DNA. Isolated DNA was assayed by ChIP-qPCR. Representative data were plotted relative to percentage of starting input chromatin and are represented as mean of triplicate qPCR reactions $\pm$ SEM. Supplementary Figure 7d shows a repeated ChIP assay. **h** KMT2D occupies EREs of *GREB1* and *TFF1* genes in a Dox-dependent manner correlating with increased Y537S ER α occupancy. ChIP-qPCR was performed using an antibody to KMT2D. Representative data were plotted as above and the *CCND1* gene intron 4 served as a negative control region. Supplementary Figure 7e shows a repeated ChIP assay

conditional (doxycycline (Dox)-inducible) expression of FLAG-tagged WT, Y537S, or D538G ER α . The FLAG tag did not impair the transcriptional activities of ER α proteins (Supplementary Figure 6). Dox addition to cells grown in charcoal-stripped media induced expression of these ER α proteins, with Y537S and D538G ER α accumulating to a similar extent but less than WT (Fig. 5a). We next performed co-immunoprecipitations to test whether SRC-3 and KMT2D displayed enhanced association with inducible

mutant ER α proteins under hormone-depleted conditions. We found greatest KMT2D and SRC-3 association with the Y537S mutant, with less recruited to D538G ER α (Fig. 5b). Importantly, the Dox-inducible mutant ER α proteins activated endogenous ER α target genes (*GREB1* and *TFF1*) in a hormone-independent manner and D538G ER α had a weaker effect than Y537S, in accordance with other models [7, 9, 29–31] (Fig. 5c and Supplementary Figure 7a). We next asked whether ablation of these key co-regulators would inhibit the viability of these cells. Consistently, SI-1 significantly reduced the viability of all ER α -expressing cells (Fig. 5d), while knockdown of KMT2C/2D selectively affected WT and Y537S ER α -expressing cells (Fig. 5e).

Knockdown of KMT2C/2D modulates Y537S ER α direct target gene expression

We next tested the effect of KMT2C/2D depletion on *GREB1* and *TFF1* gene expression in our Dox-inducible MCF-7 cells. KMT2C/2D knockdown reduced Y537S ER α -mediated transcription of both genes (Fig. 5f and Supplementary Figure 7b). In WT ER α cells, the loss of KMT2C/2D also reduced *TFF1* mRNA, but *GREB1* or *ESR1* were unaffected. We extended our analysis to 10 total ER α target genes by depleting only KMT2D using a validated siRNA [32], as KMT2D had greater recruitment than KMT2C to Y537S ER α (Fig. 1c). We observed that Y537S mutant expression regulates select ER α targets, a subset of which is reduced by KMT2D depletion (Supplementary Figure 7c).

Chromatin immunoprecipitation (ChIP) was used to examine whether the ER α mutants bound EREs [33–35] located upstream of the *GREB1* and *TFF1* gene transcription start sites (TSS) in a Dox-dependent manner (Fig. 5g and Supplementary Figure 7d). All ER α proteins displayed Dox-dependent enrichment on EREs upstream of the *GREB1* and *TFF1* TSS, but not on a negative control region [36]. Thus, the binding of the LBD mutant ER α proteins to multiple EREs suggests direct regulatory roles in regulation of these genes.

Chromatin occupancy of Y537S ER α and KMT2D are positively correlated

As SRC-3 and p300 are co-localized with Y537S ER α on chromatin [29], we next tested whether KMT2D chromatin occupancy correlated with Y537S ER α . We performed ChIP using a validated KMT2D antibody [37, 38] (Fig. 5h and Supplementary Figure 7e). KMT2D occupancy increased in Y537S ER α cells in a Dox-dependent manner for all EREs assayed, but not for the control region, without increased KMT2D protein expression (Supplementary Figure 7f). Thus, Dox-induced occupancy of EREs by Y537S

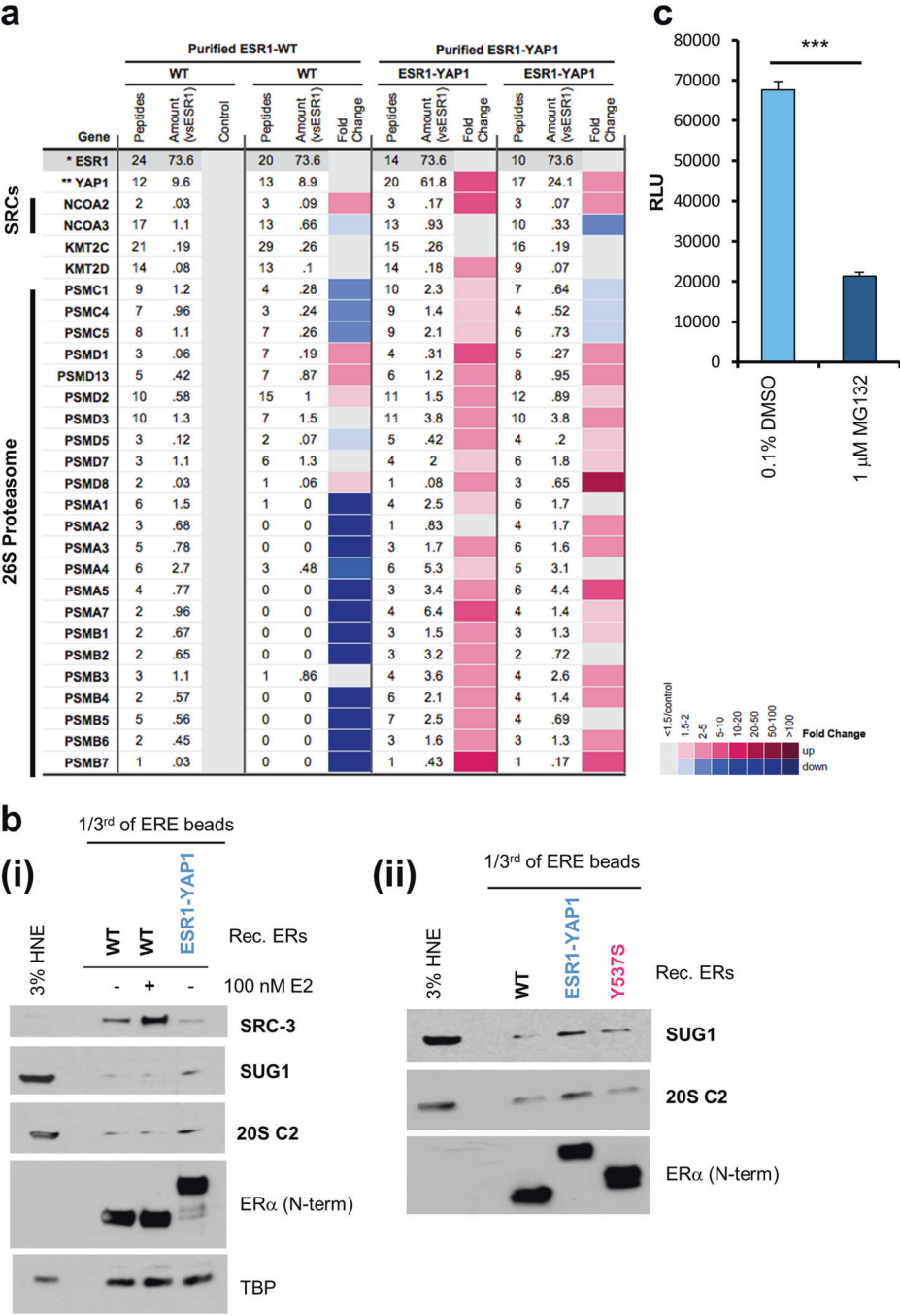


Fig. 6 The ESR1-YAP1 fusion protein recruits the 26S proteasome for activated transcription of an ERE-driven luciferase reporter. **a** MS data depicted as a heatmap (displayed as in Fig. 1c) for ESR1-YAP1-dependent coactivators recruited to EREs from HNE. Recombinant purified WT or ESR1-YAP1 proteins were added to duplicate ERE DNA pulldown reactions. Fold change cutoff was ≥ 1.5 for enrichment over unliganded WT ER α . **ESR1* normalized ESR1 (see Supplementary Table 2). ***YAP1* YAP1 corrected for ESR1. **b** Immunoblotting validation of the 26S proteasome being recruited to ESR1-YAP1. Independent ERE DNA pulldown samples were used to detect proteins recruited to ESR1-YAP1 compared to WT with/without E2 (i) or compared to WT or Y537S ER α (ii). Levels of ER α bound to the

ERE were determined with an ER α antibody recognizing an N-terminal (N-term) epitope. TBP served as a loading control. 3% HNE, 3% of the starting HeLa S3 NE employed in the ERE DNA pulldown. **c** A 26S proteasome inhibitor, MG132, reduces ESR1-YAP1 transcriptional activity on an ERE-driven luciferase reporter. HeLa cells grown in phenol red-free, charcoal-stripped media were co-transfected with a vector expressing YFP-tagged ESR1-YAP1 protein and pERE-E1b-luc. Cells were then treated with vehicle (0.1% DMSO) or 1 μ M MG132 for overnight. Luciferase activity (RLU relative light units) was assayed from whole-cell lysates. Data are represented as mean $\pm$ SEM ($n = 3$); *** $p < 0.001$

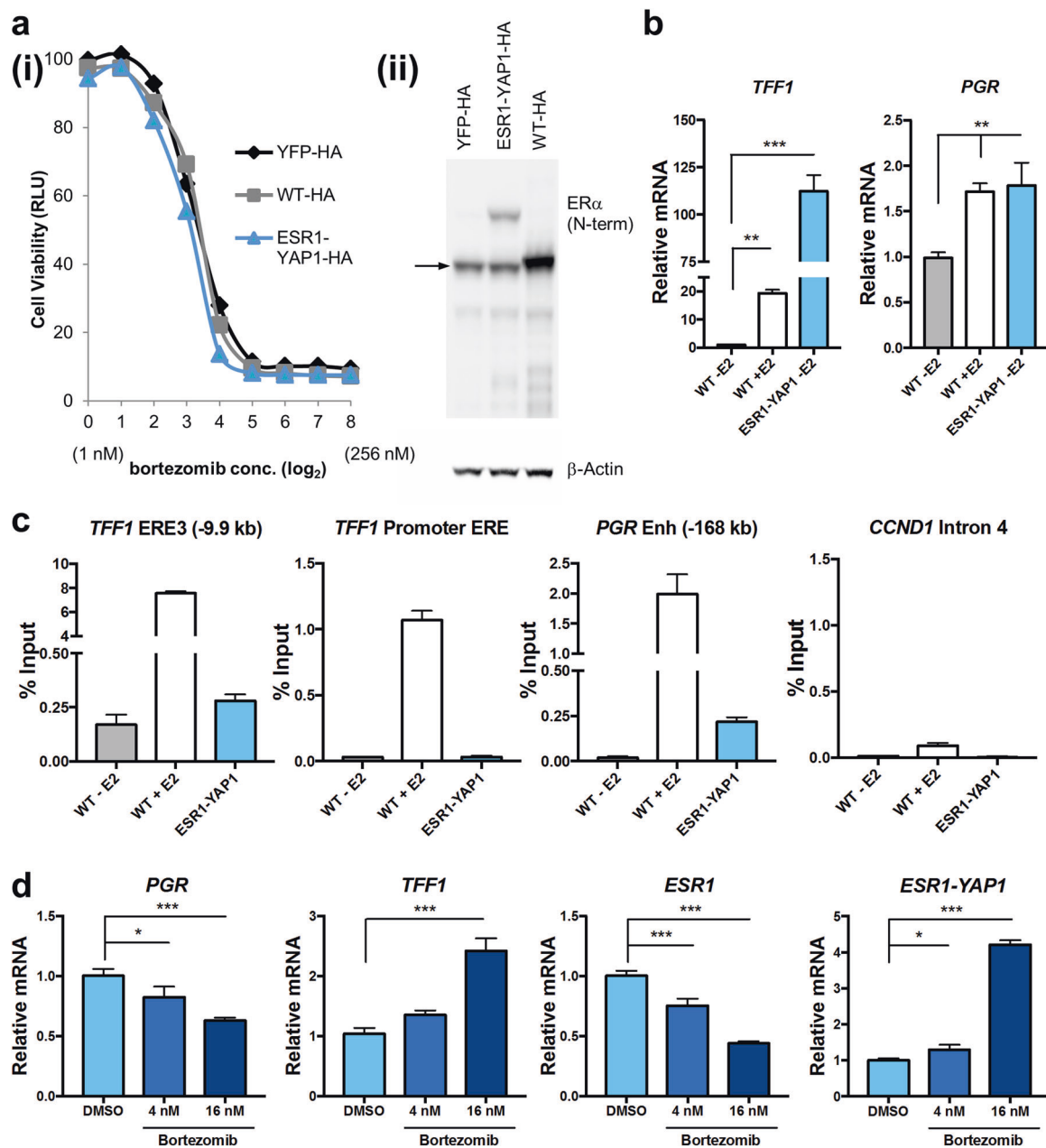


Fig. 7 Proteasome inhibition reduces viability of breast cancer cells expressing the ESR1-YAP1 fusion and modulates ESR1-YAP1 target gene expression. T47D cell lines expressing these constructs were grown in phenol red-free, charcoal-stripped media for at least 2 days. **a** Increasing concentrations of bortezomib reduce cell viability of HA epitope-tagged YFP, WT ER α , and ESR1-YAP1 stably expressing T47D cell lines. **(i)** Cell viability was measured (via Cell Titer Glo as RLU) after 3 days of bortezomib or vehicle (DMSO) treatments. Data are represented as mean $\pm$ SEM ($n = 3$). **(ii)** Expression levels of ER α proteins were assayed in whole-cell extracts made from HA-tagged YFP, WT ER α , and ESR1-YAP1 expressing T47D cell lines. The N-terminal ER α antibody was used to detect endogenous ER α (denoted by $\rightarrow$) as well as the ESR1-YAP1 fusion. β -actin served as a loading control. **b** Expression of HA-tagged ESR1-YAP1 activates expression of two classical ERE-containing target genes as compared to E2-deprived HA-tagged WT ER α . WT ER α -expressing cells were treated with/without 10 nM E2 for 24 h. Relative levels of *TFF1* or *PGR*

mRNAs were determined by RT-qPCR using *ACTB* mRNA as the normalizer. Data are represented as mean $\pm$ SEM ($n = 3$); ** $p < 0.01$; *** $p < 0.001$. **c** ESR1-YAP1 directly occupies certain EREs of the *TFF1* and *PGR* genes. Where indicated, HA-tagged WT ER α cells were treated with 100 nM E2 for 45 min before ChIP assays. ChIP-qPCR assays employed an antibody against HA to IP the HA-tagged ER α proteins and associated DNA. Representative data were plotted relative to percentage of starting input chromatin, which was represented as the mean of triplicate qPCR reactions $\pm$ SEM. *CCND1* gene intron 4 served as a negative control region. Supplementary Figure 8d shows a repeated ChIP assay. **d** Proteasome inhibitor treatment of T47D cells expressing HA-tagged ESR1-YAP1 modulates ERE-containing target gene expression. Cells were treated with vehicle (0.1% DMSO) or 4 or 16 nM bortezomib for 17 h. Relative levels of *PGR*, *TFF1*, *ESR1*, or *ESR1-YAP1* mRNAs were determined by RT-qPCR using *GAPDH* mRNA as the normalizer. Data are represented as mean $\pm$ SEM ($n = 3$); * $p < 0.05$; *** $p < 0.001$

ER α is correlated with increased recruitment of KMT2D and enhanced transcription of *GREB1* and *TFF1*.

Co-regulators recruited to the ESR1-YAP1 fusion

We next investigated co-regulator recruitment to ESR1-YAP1, which contains the N terminus and DBD, but lacks the LBD, of ER α fused in-frame to the C terminus of the Yes-associated protein 1¹¹ (Fig. 1a). ESR1-YAP1 promotes high levels of expression of an ERE-dependent reporter without E2 (Fig. 1b and Supplementary Figure 6a). We compared co-regulator recruitment to either purified WT ER α or ESR1-YAP1 bound to ERE DNA in the absence of E2 (Fig. 6a). After normalization for ER α -binding differences, the ESR1-YAP1 protein did not recruit more SRC-3 and KMT2D as compared to WT ER α , unlike the LBD ER α mutants (validated in Fig. 1d). Instead, the ESR1-YAP1 protein recruited many subunits of the 26S proteasome (Fig. 6a). Immunoblotting validated enhanced proteasome recruitment (SUG1/PSMC5 and 20S C2/PSMA1) of ESR1-YAP1 vs. WT ER α , even with E2 (Fig. 6b). We further validated that the 26S proteasome was recruited to ESR1-YAP1 in E2-deprived T47D stable cell line [11] extracts (Supplementary Figure 8a).

The 26S proteasome plays a role in WT ER α degradation that is linked with the receptor's ability to activate ERE-driven reporter genes [39–41]. We thus tested whether a proteasome inhibitor, MG132, would similarly inhibit ESR1-YAP1 transcriptional activity. Indeed, MG132 treatment of cells transfected with an ERE-driven reporter and an ESR1-YAP1 expression plasmid reduced Luc activity, as compared to the vehicle control (Fig. 6c). Interestingly, MG132 or the FDA-approved proteasome inhibitor, bortezomib, could inhibit the transcriptional activity of a GAL4 DBD-YAP1 fusion (Supplementary Figure 8b).

Proteasome activity is important for ESR1-YAP1-mediated cell growth and gene expression

We next tested the functional significance of the ESR1-YAP1: 26S proteasome interaction in T47D stable lines expressing HA-tagged YFP, WT ER α , or ESR1-YAP1 proteins (Fig. 7a_{ii} and Supplementary Figure 8c). Increasing concentrations of bortezomib treatment for 3 days efficiently reduced the growth of all three T47D lines compared to the vehicle control (Fig. 7a_i).

We next wanted to define the effect of ESR1-YAP1 expression in T47D cells grown in an E2-deprived state on ER α target gene expression. The ESR1-YAP1 fusion promoted the expression of two target genes, *TFF1* and *PGR*, significantly above the level of unliganded WT ER α -expressing cells (Fig. 7b), even though much less ESR1-YAP1 fusion was expressed (Fig. 7a, ii and Supplementary

Figure 8c). The regulation of these two genes is likely direct, as ChIP assays revealed ESR1-YAP1 occupancy at two defined ERE enhancer-like sequences [35, 42] (Fig. 7c and Supplementary Figure 8d).

The effect of proteasome inhibition on *TFF1* and *PGR* gene expression in E2-deprived ESR1-YAP1-expressing cells was tested by treating cells with bortezomib (Fig. 7d). Proteasome inhibition had both a dose-dependent and gene-specific effect on the ESR1-YAP1 targets, decreasing *PGR* and increasing *TFF1*, which resembles the effect of proteasome inhibitors on these genes after E2 treatment of MCF-7 cells [43–46]. Bortezomib decreased endogenous *ESR1* mRNA expression, consistent with prior reports [43, 47]. Finally, we observed that bortezomib stimulated *ESR1-YAP1* mRNA expression (driven by the CMV promoter), which may explain why bortezomib did not severely reduce ESR1-YAP1 cell viability as compared to overexpressed WT ER α (Fig. 7a). Thus, the proteasome modulates ESR1-YAP1 target genes and cell growth, suggesting a new approach for treating tumors bearing this class of resistance mutation.

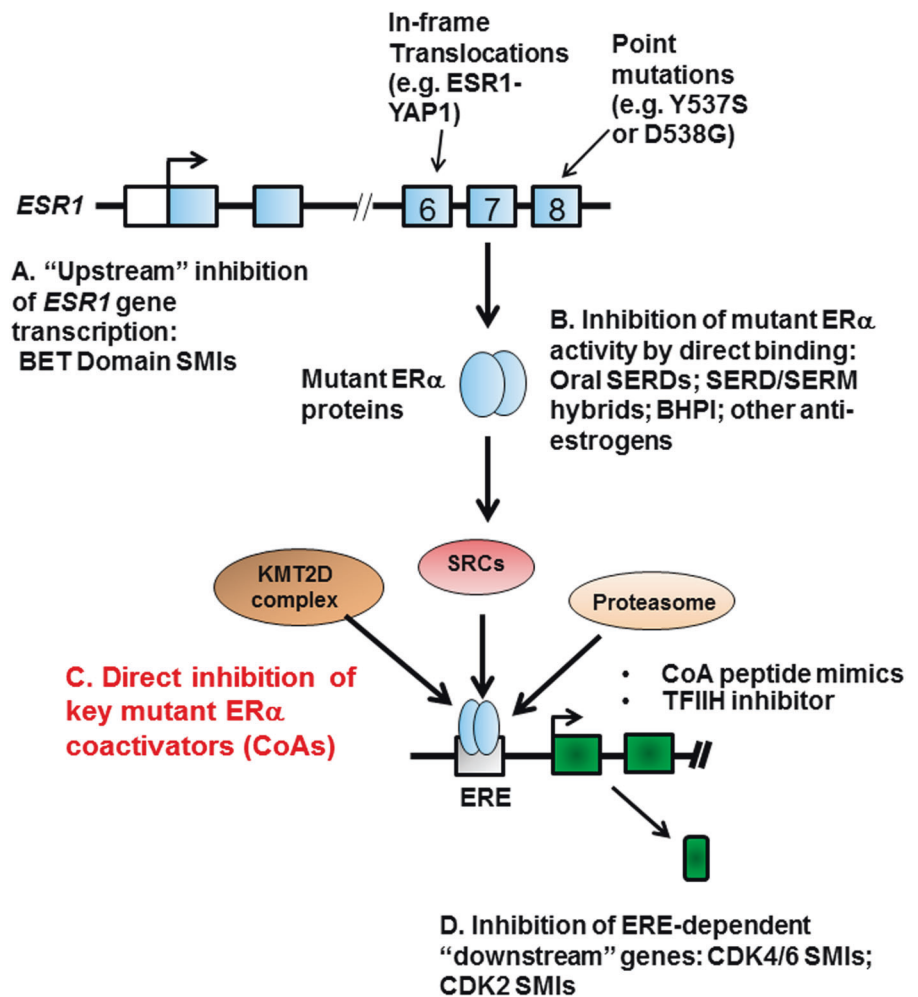
Potential clinical relevance of mutant ER α -binding coactivators

To investigate whether the expression levels of the coactivators identified in this study correlate with patient outcomes, we queried two existing expression data sets—the Symmans Breast 2 ER-positive tamoxifen-treated patients [48] or ER-positive breast cancer patients treated with endocrine therapy (in KM plotter [49]; Supplementary Figure 9). In the Symmans data set, we found significantly higher KMT2D mRNA levels in patients who had a metastatic occurrence after 3 years of tamoxifen treatment vs. those that had not (panel a). We also found that higher SRC-3 and proteasomal subunit mRNA levels correlated with reduced survival from distant metastasis (panels b, c). In the KM plotter analysis, we found that higher KMT2D, but not KMT2C, mRNA significantly correlated with reduced recurrence-free survival (panel d). Finally, we assayed a metastatic breast cancer mutation/amplification database [50] and found that the ESR1 LBD mutations were not present in patients with mutations in either KMT2C or KMT2D genes (panel e).

DISCUSSION

Different therapeutic approaches have been proposed to inhibit LBD mutant ER α proteins in breast cancers (Fig. 8) [23, 26, 27, 29–31, 51–54]. However, resistance to these therapies will occur. We envisioned that by defining the co-

Fig. 8 Potential ways to inhibit mutant ER α -driven breast cancer cell growth. In metastatic breast cancer, the *ESR1* gene encoding ER α is mutated in exon 6 (by translocations creating in-frame fusion proteins such as ESR1-YAP1) or in exon 8 (by point mutation in the LBD creating Y537S or D538G ER α mutants). While other laboratories have published pharmacological inhibitor strategies for reducing *ESR1* mutant gene transcription (a), for inhibiting the LBD mutant ER α proteins directly (b), and for inhibiting downstream gene products whose expression is driven by LBD mutant ER α proteins (d), we propose that inhibition of key mutant ER α coactivators, such as KMT2C/2D, SRC coactivators, and the proteasome, is a new therapeutic approach for inhibiting mutant ER α -driven breast cancer cell growth (c). See also Discussion



regulator "complexome" of each *ESR1* mutant protein we could identify new potential therapeutic targets (Fig. 8c).

We identified coactivators that exhibit enhanced binding to ER α mutant proteins. Consistent with prior literature [7, 9, 14, 18, 19, 55, 56], SRCs display enhanced recruitment to EREs bound by the two LBD ER α mutants as compared with unliganded WT ER α . Since all three SRCs are recruited to the two LBD ER α mutants, therapeutic intervention likely must be directed toward all three proteins. Accordingly, we show that a pan-SRC SMI can effectively inhibit LBD ER α mutant activity and breast cancer cell growth (Fig. 2). Furthermore, combining pan-SRC SMI and oral SERD treatments have a synergistic effect on both LBD mutant transcriptional activity and cell growth (Fig. 2), and, more importantly, Y537S ER α -expressing PDX tumor growth (Fig. 3).

Our results further reveal coactivator complexes preferentially interacting with ER α mutants. First, we show that KMT2C/2D H3K4 methyltransferases are preferentially recruited to Y537S (Fig. 1c, Supplementary Figure 2a). These complexes were previously shown to coactivate E2-

bound WT ER α in MCF-7 cells [57–60]. Although Y537S and D538G are one amino-acid residue apart in helix 12, it is striking that the KMT2C/2D complex is preferentially recruited to Y537S, and that it promotes the growth of Y537S ER α -expressing cells (Fig. 5e, Supplementary Figure 5). Detailed structural analysis is needed to examine how such coactivator-binding selectivity is achieved. As expression of Y537S ER α confers poor prognosis in metastatic breast cancer [16], our data suggest that developing targeted inhibitors of KMT2C/2D would be worthwhile for inhibition of Y537S ER α mutant activity. Furthermore, KMT2D is oncogenic in ER-positive breast cancers resistant to PI3K α inhibitors [61].

Second, we have profiled coactivators bound to ESR1-YAP1, as it represents a "paradigmatic" gene fusion that activates ERE-driven transcription (Figs. 1b, 7b, Supplementary Figure 6). The ESR1-YAP1 fusion biochemically behaved in a distinct manner from the two LBD ER α mutants, as it displayed enhanced recruitment of the 26S proteasome (Fig. 6, Supplementary Figure 8a). Furthermore, proteasome activity is important for both ESR1-

YAP1 transcriptional activity and growth of breast cancer cells (Figs. 6c, 7a, b). As the proteasome inhibitor bortezomib is in clinical trial for ER α -positive, metastatic breast cancer (NCT01142401), we propose that it may reduce ESR1-YAP1-mediated tumor growth. Recently, other *ESR1* gene fusions [20] have been identified in ER-positive metastatic disease and whether they also utilize the proteasome for their activity should be further investigated.

In summary, our study utilized an MS approach profiling three different mutant ER α proteins, which identified coactivators preferentially recruited to LBD mutants vs. an ESR1 gene fusion. We further showed that inhibition of these coactivators decreased ERE-driven transcription and reduced growth of breast cancer cells expressing these ER α mutants. Importantly, the combination of a pan-SRC inhibitor and oral SERD reduced the tumor growth of a human PDX expressing the Y537S ER α mutant, suggesting this as a potential new therapeutic strategy. We further identify additional potential therapeutic targets for Y537S ER α -expressing breast cancers (KMT2C/2D complexes) and for the ESR1-YAP1 fusion (26S proteasome) for which no prior targets were known. Together, our data support the idea that differential coactivator recruitment may be partly responsible for the ability of ER α mutant proteins to potentially drive metastatic breast cancer.

Materials and methods

Cell lines

Lines were obtained from BCM tissue culture core (originally from ATCC) unless otherwise indicated. STR profiling validated MCF-7 cell line authenticity. All lines tested negative for mycoplasma. MCF-7 and T47D cell lines stably expressing WT, Y537S, and D538G ER α were described [28]. C-terminal HA-tagged YFP, ESR1-WT, and ESR1-YAP1 lentiviral T47D cell lines were generated as in [11] using the pCD516B-2 vector (System Biosciences). Dox-inducible N-terminal FLAG-tagged WT, Y537S, and D538G ER α stable MCF-7 cell lines were constructed with pCW-FLAG-ER α lentiviruses. All lines were maintained in media containing 10% fetal bovine serum and switched to phenol red-free, hormone-depleted media before treatments.

Plasmids

pCW-FLAG-WT, Y537S, or D538G were created from pCW-Cas9 [62] (Addgene). pFLAG-CMV constructs were made from pFLAG-CMV2 (Sigma). YFP-tagged ESR1-

YAP1 was constructed by PCR of the ESR1-YAP1 open reading frame (ORF) [11], followed by ligation into digested pECFP-C1 (Clontech). pBIND-YAP1 (GAL4 DBD-YAP1 amino acid 230–504 fusion expression plasmid) was constructed by ligation into pBIND (Promega). All constructs were sequenced.

ERE DNA pulldown assays

The ERE DNA pulldown was described [21]. One microgram of purified receptor was added to 2–2.5 mg HNE and 15 pmol 4xERE-E4 DNA immobilized on Dynabeads M-280 streptavidin (Invitrogen). For Supplementary Figure 8a, NE was made as described [63]. For direct interaction assays, 10 μ l of the purified recombinant KMT2D “fusion” complex [22] was incubated with 0.25 μ g of purified ER α for 1.5 h at 4 °C followed by washes and elution [21].

Mass spectrometry

MS was performed and analyzed as described [21]. All data sets are summarized in Supplementary Tables 1, 2, and 7. The raw MS proteomics data were deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>), data set identifier: PXD005887.

Recombinant ER α proteins

Flag-tagged ER α ORFs were expressed using the Baculo-Direct N-terminal Expression kit (Invitrogen). Infected *Sf9* cells were lysed in lysis buffer (50 mM Tris-Cl, pH 7.5, 500 mM NaCl, 15% glycerol, 0.01% NP40, 10 mM β -glycerophosphate and protease inhibitor cocktail (Roche)), followed by purification using anti-FLAG M2 antibody-conjugated beads and 3 $\times$ FLAG peptide (Sigma).

Co-immunoprecipitations

Dox-treated MCF-7 cells expressing WT or LBD mutant ER α proteins were lysed in NETN buffer [21]. Three milligrams whole-cell extract was incubated for 4 h at 4 °C with 5 μ g mouse anti-FLAG-pre-bound Protein G Dynabeads (Invitrogen) and washed with NETN and PBS.

Reagents

Dimethyl sulfoxide, 17 β -estradiol (E2), 4-hydroxytamoxifen, ICI182,780 (ICI), MG132, and Dox were purchased from Sigma. Bortezomib (Selleckchem), AZD9496 (MedChemExpress), and GDC0180 (Active Biochem) were from listed vendors. SI-1 and SI-2 have been described [25]. Antibodies are listed in Supplementary Table 3.

Luc reporter assays and siRNA transfections

For ERE-Luc assays, HeLa cells were transfected with pERE-E1b-Luc [39] and ER α expression vector using Lipofectamine LTX (Invitrogen), and lysed in Glo Lysis Buffer (Promega). For GAL4-Luc assays, cells were transfected with pBIND-YAP1 and pG5luc (Promega). Luc activity was measured on a Berthold luminometer as described [64]. Cells were co-transfected with pERE-E1b-luc, ER α expression plasmids, and siRNAs using Trans-IT-TKO (Mirus Corp.) or RNAimax (Invitrogen). siRNAs are listed in Supplementary Table 3.

Cell viability and soft agar assays

Cell viability or soft agar anchorage-independent cell growth was measured after 3 days by either a CellTiter-Glo[®] Luminescent assay (Promega) or by a MTT assay [65].

Chromatin immunoprecipitation

Cells in hormone-depleted media were subject to ChIP assays performed using the EZ-ChIP kit (Millipore) as described [21]. See Supplementary Table 3.

Calculations of synergism and IC₅₀

Synergism between two drugs was defined as a combination index < 1 using CompuSyn (<http://www.combosyn.com/>) or Calcsyn (<http://www.biosoft.com/w/calcsyn.htm>) software [66]. IC₅₀ values were determined by using Very Simple IC50 Tool Kit (<http://www.ic50.tk/>).

Statistical analysis

P values were calculated using Student's *t*-test (two-sample, two-tailed) to compare two means or ANOVA (ordinary, one-way) followed by adjusting for multiple comparisons using the Dunnett or Tukey method. *P* values < 0.05 were considered significant. For statistics, experiments were performed with three to eight biological replicates (see figure legends).

Real-time reverse transcription

Total RNA was isolated with Direct-zol RNA MiniPrep (Zymo Research) or RNeasy (Qiagen) kits. cDNA synthesis and real-time reverse transcription (RT-qPCR) data analysis were carried out as described [64]. See Supplementary Table 3 for primers.

PDX experiments

Experiments were carried out in accordance with protocol AN-1875 approved by the BCM Institutional Animal Care and Use Committee. WHIM 20 PDX was transplanted as described [11] into 4–5-week-old female SCID/Beige mice (Envigo). Mice were palpated semi-weekly, and tumor growth measured using calipers. When tumors reached ~350 mm³ (volume = $L \times ((W \times W)/2)$), the mice were randomized into four treatment groups (*n* = 10 each): (1) AZD9496 5 days/week by oral gavage once daily, 5 mg/kg body weight (b.w.) [14, 26]; (2) SI-2 twice daily 5 days/week by intraperitoneal (i.p.) injection at 2 mg/kg b.w. [25]; (3) combination of both inhibitors as described; and (4) control PBS vehicle (oral and i.p.). Mice were sacrificed and tissues harvested at 4 months post transplantation. Tumor volume of eight mice per group (which completed at least 4.5 weeks of treatment) was analyzed. Representative tumors (*n* = 6/treatment) were lysed in RIPA buffer with phosphatase and protease inhibitors (Calbiochem). Hematoxylin and eosin staining and immunohistochemistry were performed by the Lester and Sue Smith Breast Center Pathology Core at BCM. After staining, images were captured on an OLYMPUS DP73 microscope. For BrdU analysis, 7 mg/ml BrdU (Sigma) was prepared in PBS and i.p. injected at 10 μ l/g b.w. After 2 h, the mice were sacrificed (*n* = 8). Slides were stained by BD Pharmingen in Situ-Detection KIT II and were visualized with diaminobenzidine tetrahydrochloride (Dako), and counterstained with Harris hematoxylin. Images were analyzed in Matlab by the Integrated Microscopy Core at BCM. The investigators were not blinded to allocation during experiments and outcome assessment. No statistical methods were used to predetermine sample size estimate.

Patient data analysis

Oncomine was used to query the Symmans Breast 2 data set. KM plotter (kmplot.com) was used to query the ER-positive patients treated with endocrine therapy. See Supplementary Figure 9 for more details.

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Compliance with ethical standards

Conflict of interest B.W.O., D.M.L., J.W., A.D.R., Y.Y., and C.E.F. disclose an equity position in Coactigon, Inc.; M.J.E. received a consulting fee from AstraZeneca.

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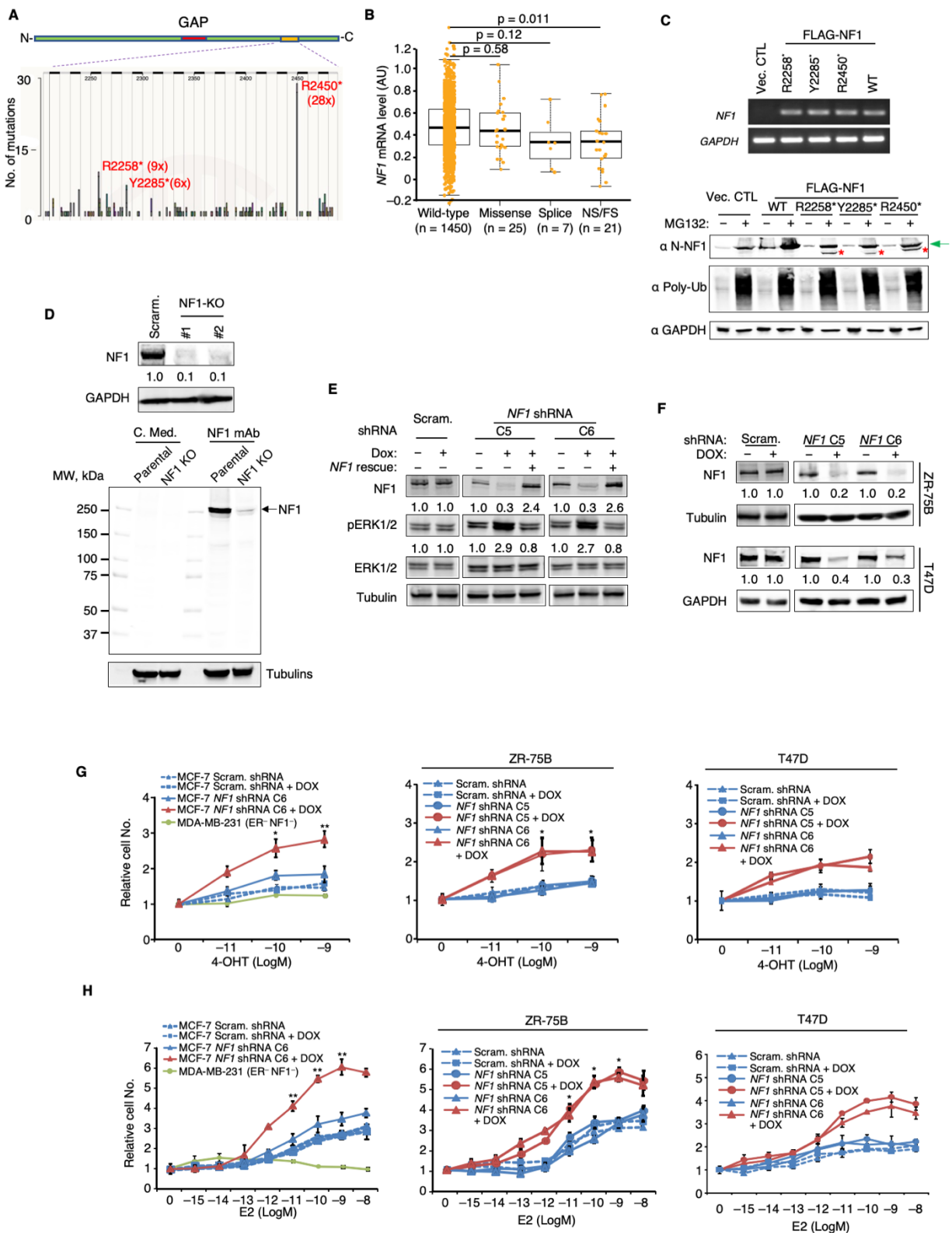


Figure. S1, related to Figure 1. A. The marked C-terminal region in the NF1 protein contains several recurring nonsense (NS) and frameshift (FS) mutations, including R2450*. The numbers

in parentheses are the number of times mutations were found in the COSMIC database. **B.** Analysis of *NF1* expression in patient tumors with wild type or mutant *NF1*. Data analysis was similar to Figure 1B except that microarray data from the METABRIC dataset were employed. **C.** MCF-7 cells were transduced to express FLAG-tagged *NF1* cDNA carrying a stop codon in place of the normal codon affected by the three recurrent nonsense mutations affecting NF1's C-terminus, as seen in (A). These cells were treated with or without MG132 (1 μ M, 16 hrs) and the cellular mRNA samples were later examined by RT-PCR (top) using primer set "NF1-NCI", or by immunoblotting (bottom) using an antibody targeting its N-terminus. The green arrow marks wild-type NF1, while red asterisks mark C-terminally truncated NF1 proteins. An antibody against the polyubiquitin chain was used to assess proteasome inhibition, and GAPDH was measured as the loading control. **D.** Top, MCF-7 cells were stably transfected with scrambled gRNA or *NF1*-targeting gRNA KO constructs (#1 and #2), and neurofibromin-depletion was validated by immunoblotting. Bottom, KO #1 was used to validate the specificity of mouse monoclonal NF1 antibody (mAb-376) by immunoblotting. Since the mAb-376 was purified from the culture medium of the hybridoma cells, the culture medium was used here as the antibody control. Tubulins were examined as the loading control. **E.** MCF-7 cells stably transfected with DOX-inducible scrambled or *NF1*-targeting shRNA clones were treated with DOX or vehicle for 2 days and analyzed by immunoblotting. *NF1*^{KD} (+DOX) cells were also transfected with an *NF1* cDNA refractory to the shRNA ("*NF1 rescue*") and similarly analyzed. The numbers below show protein levels normalized to tubulin relative to the vehicle control. The ERK phosphorylation levels were also normalized to total ERK levels. **F.** *NF1* silencing by the same shRNA clones in ZR-75B and T47D cells were analyzed by immunoblotting. **G.** Cells were analyzed as those in Figure 1D. n = 3 experiments. **H.** Cells were analyzed as in Figure 1E. n = 3 experiments. Data are generally reported as mean $\pm$ SEM. *p<0.05, **p<0.01 by pair-wise two-tailed Student's t-test, unless otherwise indicated.

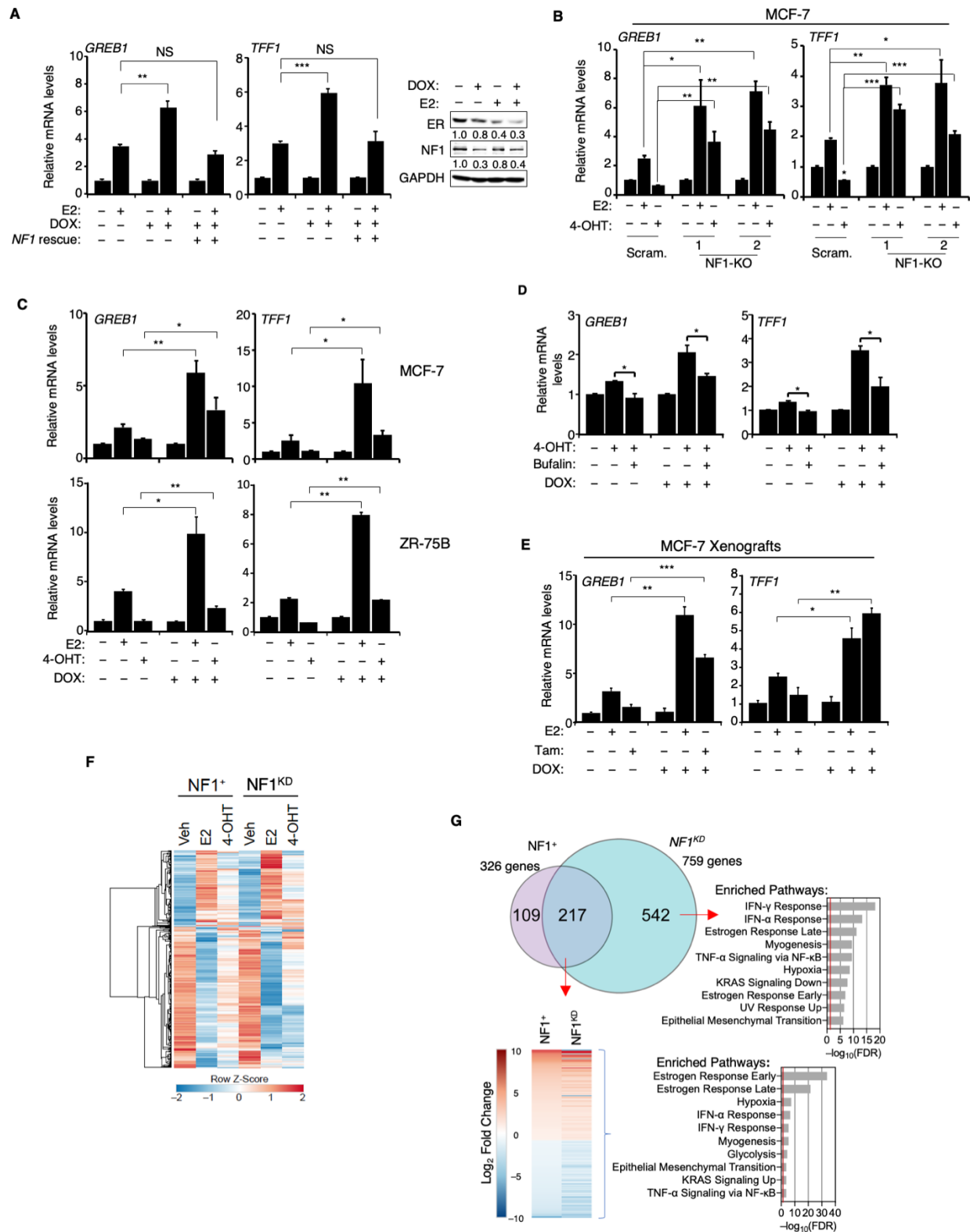


Figure S2, related to Figure 2. A. E2-induced *GREB1* and *TFF1* mRNA level changes in normal or *NF1*-silenced (+DOX) MCF-7 cells were measured by qPCR. The altered gene expression can be “rescued” by an *NF1* cDNA refractory to the shRNA. n = 6 experiments. Immunoblotting on

the right assessed ER and NF1 levels (relative to GAPDH). **B.** *GREB1* and *TFF1* expression relative to vehicle control was measured as in (A) except NF1-KO cells were examined. n = 2 experiments. **C.** *GREB1* and *TFF1* expression in response to E2 or 4-OHT in indicated cell lines was measured as in (A). n = 3 experiments. **D.** Gene expression was similarly measured as in (C) except bufalin was added to inhibit SRCs. **E.** *GREB1* and *TFF1* mRNA levels in MCF-7 xenograft tumors at week-2 from Figure 1F were examined by qPCR in 3 mice (n = 3). **F.** Hierarchical clustering conducted on RNA-seq expression of E2-responsive genes detected in NF1⁺ cells (Table S3, last tab). Row Z-scores were generated from Log₂TPM. **G.** 4-OHT-induced gene expression changes analyzed as in Figure 2A. Data are reported as mean±SEM. *p<0.05, **p<0.01, ***p<0.001 by pair-wise two-tailed Student's t-test.

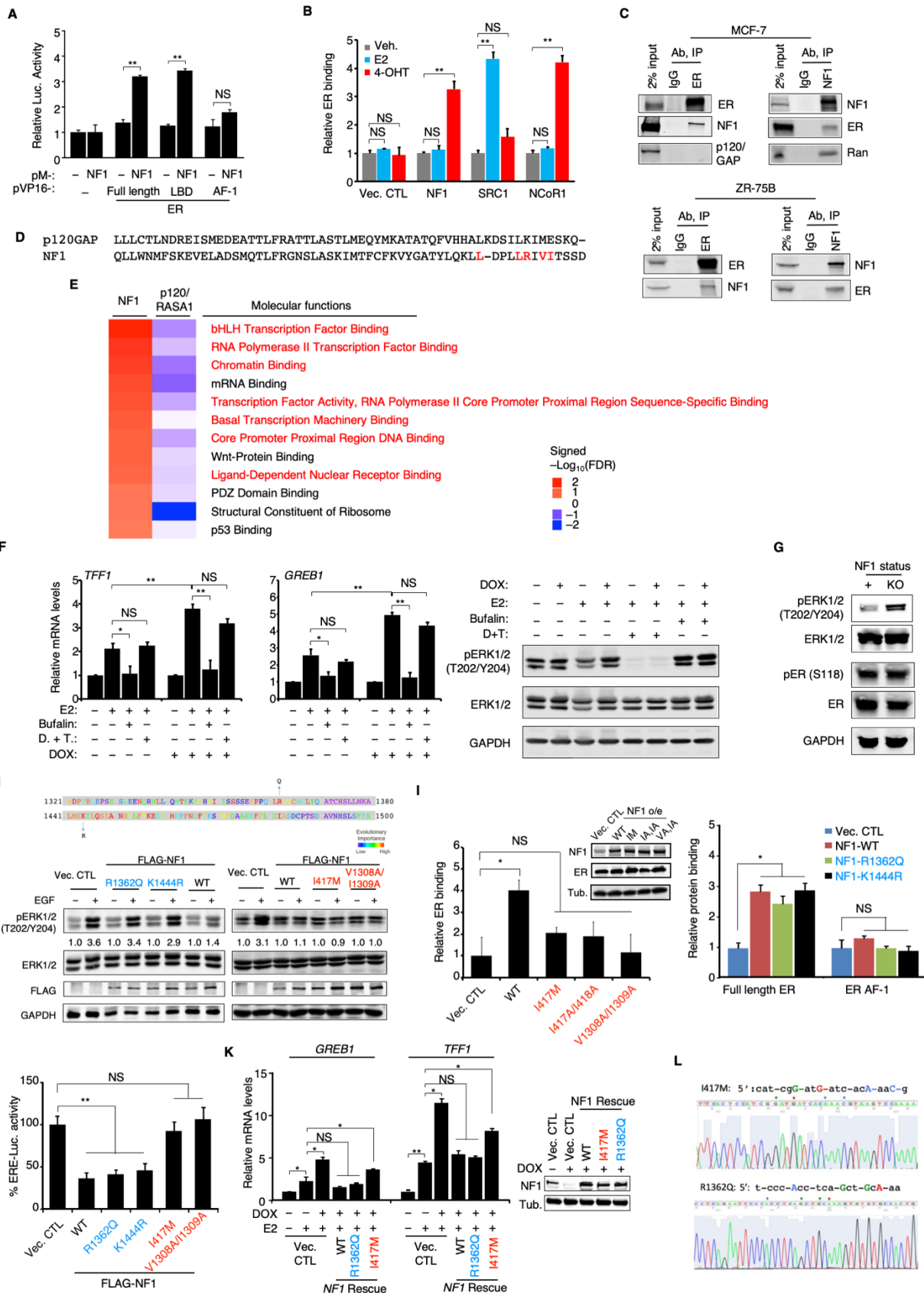


Figure S3, related to Figure 3. **A.** Various fragments of DNA encoding full length or indicated ER domains were expressed by the pVP16 vector in 293 cells as the transcription activation domain fusion proteins. NF1 was expressed from pM as a fusion to the GAL4 DNA binding domain. Note that the LBD and AF-1 constructs contain ER amino acid residues 312-595 and 1-184. Protein binding leads to expression of the luciferase reporter gene. $n = 4$ experiments. NS, not significant. **B.** The same two-hybrid assay was used to measure ligand-dependent binding of ER to NF1 and to known co-regulators (co-activator SRC-1 and co-repressor NCoR1) as controls. Cells were first seeded in E2-deprived medium before E2 or 4-OHT was added. $n = 3$ experiments. NS, not significant. **C.** Cells grown in full serum medium were harvested, immunoprecipitated with antibody against NF1 (mAb-376) or ER, and examined by immunoblotting. **D.** Protein sequence alignment between human NF1 and p120GAP was derived from a published study (see main text), and key residues in NF1's M2 co-repressor motif (Figure 3A) are colored red. **E.** The top 12 Gene Ontology molecular function terms were enriched with proteins in breast tumors whose levels are highly positively correlated with those of NF1 (GSEA, $FDR < 0.05$), of which seven are evidently related to transcription (colored red). The results are ranked color-coded based on the signed and Log_{10} transformed FDR from the GSEA analysis. RASA1/p120GAP enrichment results for the same molecular function terms were co-visualized, which showed a very different enrichment pattern (e.g., no significant enrichment for transcriptionally related factors). **F.** Gene expression relative to vehicle control was measured by qPCR in the presence of indicated drugs (D and T are dabrafenib and trametinib, respectively). Western blot on the right assessed pERK levels to validate D+T activity. NS, not significant. **G.** Lysates from parental and NF1-KO MCF-7 cells were analyzed by Western blot using indicated antibodies to assess ERK and ER phosphorylation at S118. **H.** Top: The relative conservation of a given amino acid residue in NF1 family members was computed by Evolutionary Trace analysis and color-coded (see heatmap below). The two substitutions found in metastatic tumors (arrows) affect residues that are colored red, indicating they are essentially invariant among NF1 family members. Bottom: FLAG-tagged wild type and mutant NF1 proteins (GAP mutants are colored blue and ER binding mutants are colored red) were expressed in serum-starved MCF-7 cells, and ERK phosphorylation after EGF stimulation (50 nM, 15 min) was measured by immunoblotting. ERK phosphorylation levels of vehicle-treated cells were set to 1 after normalization to GAPDH and total ERK. EGF stimulation was needed to assess GAP activity because these cells contain endogenous wild type NF1. **I.** Left, two-hybrid interaction was performed as in (A), and NF1 protein levels are assessed by immunoblotting (inset). o/e, over-expressed. $n = 2$ experiments. Right, the two-hybrid interaction was similarly measured; $n = 3$ experiments. **J.** MCF-7 cells stably expressing FLAG-tagged wild type or mutant NF1 were also transfected to carry an ERE-luciferase reporter. E2-stimulated luciferase activity in cells carrying the vector control was set as 100%. $n = 2$ experiments. **K.** FLAG-tagged shRNA-refractory wild type or mutant NF1 was expressed in NF1^{KD} MCF-7 cells to comparable levels (right). qPCR was performed to measure *GREB1* and *TFF1* expression (left). $n = 2$ experiments. Data are reported as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ by pair-wise two-tailed

Student's t-test, unless otherwise indicated. NS, not significant. **L.** Sequencing validation of identified *NFI* mutant KI clones in which the wild-type *NFI* allele was undetectable.

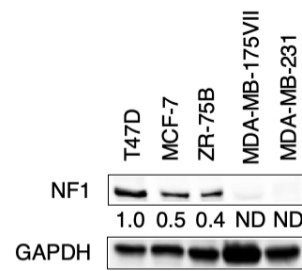


Figure S4, related to Figure 4. NF1 levels were measured in several commonly used breast cancer cell lines. MDA-MB-231 and MDA-MB-175VII cell lines are both *NF1*-null and included here as negative controls. ND, not detectable.

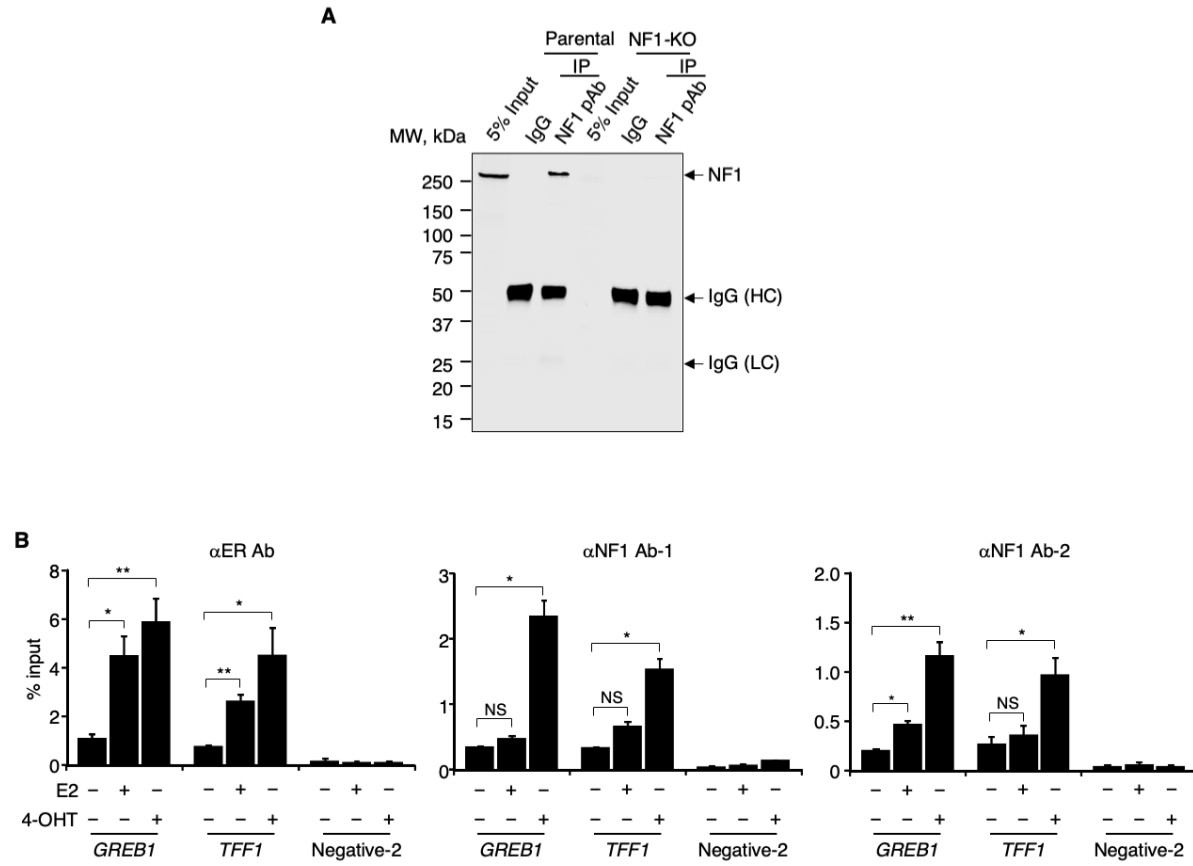


Figure S5, related to Figure 5. A. Test of the specificity of a rabbit polyclonal antibody against NF1 (Ab-2). Lysates from parental and NF1-KO (clone #1) MCF-7 cells grown under conditions similar to the ChIP experiment were immunoprecipitated by Ab-2 and the results were analyzed by immunoblot. **B.** NF1 or ER was chromatin-immunoprecipitated from MCF-7 cells stimulated by E2 or 4-OHT, and the associated chromatin was examined by qPCR using primers targeting established EREs in *GREB1* (Region 2) or *TFF1* (Region 1) as well as a previously known ERE-negative region. NF1 Ab-1 is from a commercial stock (see Methods) while Ab-2 was made by us for this project, see (A). $n = 3$ experiments. Data are reported as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ by pair-wise two-tailed Student's t-test.

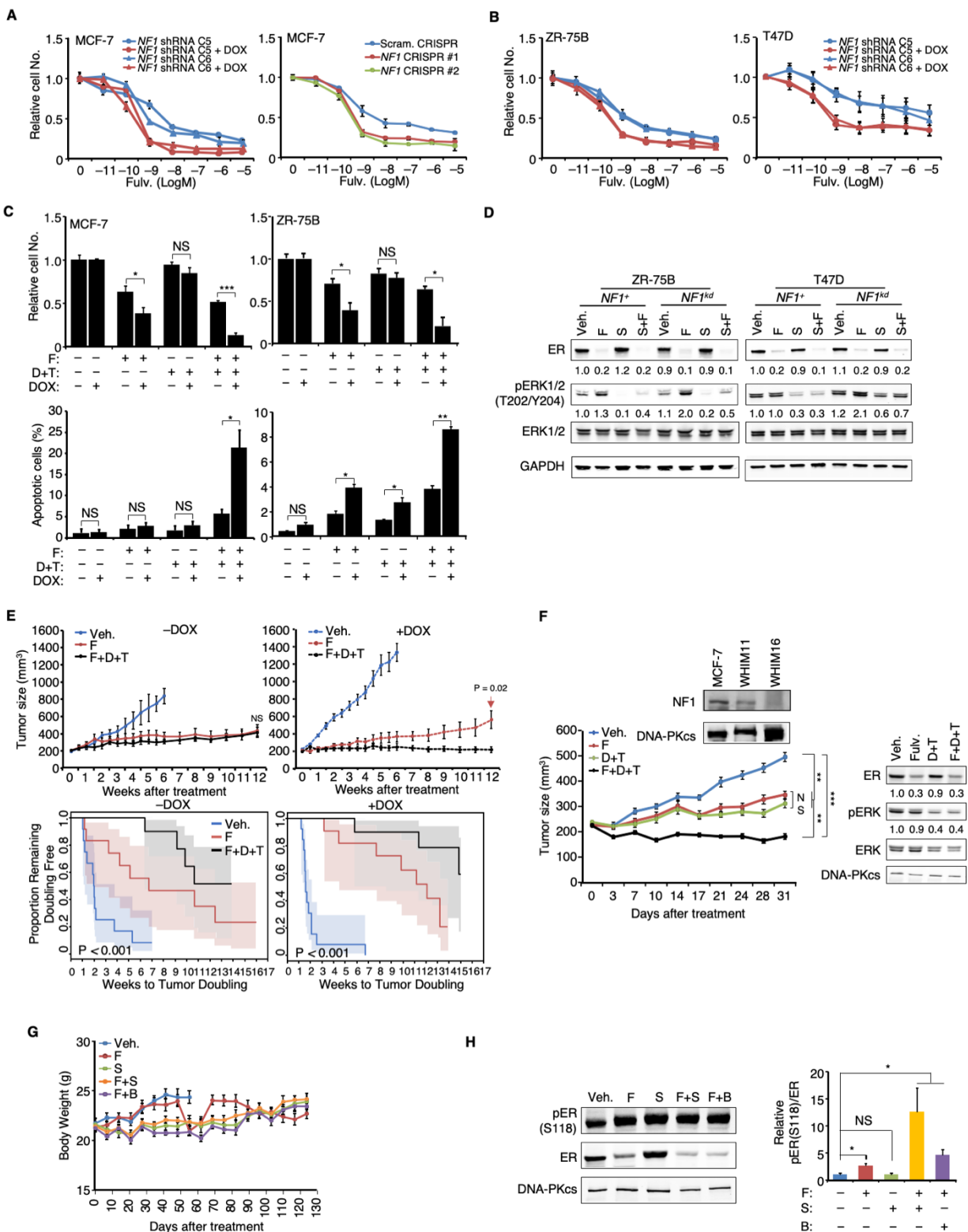


Figure S6, related to Figure 6. A. MCF-7 cells carrying inducible *NF1* shRNAs or CRISPR KO constructs were seeded in the presence of 10^{-11} M E2, to which fulvestrant at the indicated concentrations was added. Cell numbers were measured as in Figure 6B. $n = 3$ experiments for

all, except *NF1* shRNA C5-transfected cells (n = 8 experiments). **B.** ZR-75B and T47D cells were examined as in (A). n = 3 experiments. **C.** MCF-7 and ZR-75B cells carrying *NF1* shRNA (C5) were seeded with E2 (10^{-11} M). Fulvestrant (10^{-9} M), dabrafenib (10^{-6} M), and trametinib (10^{-7} M) were added. Cell numbers and apoptosis were measured 6 days later. n = 3 experiments. NS, not significant. **D.** ER and pERK levels were measured as Figure 6A. **E.** MCF-7 xenograft experiment was performed similarly as in Figure 1F, except that when tumors became palpable, the original E2 capsule was replaced by one that contained 0.05 mg E2, which can fully support the growth of *NF1*^{KD} tumors (Figure 1F). The resulting mice were then randomized ($\pm$ DOX). All *NF1*⁺ tumors were transplanted into 13 mice per group. The vehicle-treated *NF1*^{KD} tumors also represented 13 mice per group, while drug-treated groups had only 12 mice per group. Tumor sizes at week-12 of mice treated by fulvestrant alone and the 3-drug combination were compared to show that only toward the end of this experiment the former were becoming substantially larger than the latter. The tumor growth curves are shown on the top. Bottom: time from initiation of treatment to doubling in size for the tumors in Figure 5C (estimated by linear interpolation when doubling occurred between measurements) was summarized by Kaplan-Meier survival curves with shaded 95% confidence intervals (log-log method) and compared by Generalized Wilcoxon test with Holm's adjustment for multiple comparisons. **F.** Inset: Lack of *NF1* protein in WHIM16 was confirmed by immunoblotting using an antibody against human DNA-PKcs as a human-specific protein loading control. Tumor growth and treatment were similarly measured and plotted as in Figure 6C (n = 10 mice). Tumor samples were analyzed by immunoblotting for treatment validation (right). Tumor volumes at the last time point were analyzed by t-test. **G.** Body weight of mice carrying the WHIM16 tumor as in Figure 6C was measured weekly. **H.** WHIM16 tumors as in Figure 6C were analyzed by immunoblots to measure the ratio of ER-pS118 vs. total ER relative to vehicle control. A representative immunoblot was shown on the left and the data from three separate tumors were quantified on the right. NS, not significant. Data are reported as mean $\pm$ SEM. *p<0.05, **p<0.01 by pair-wise two-tailed Student's t-test, unless otherwise indicated.

NCI-ComboMATCH Steering Committee Protocol Proposal Review Decision

LEAD NETWORK: NRG

TITLE: Binimetinib and Fulvestrant for ER+ HER2-, NF1 deleted metastatic breast cancer

STUDY PI(S): Matthew Ellis & Bora Lim

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REVIEW DECISION: Approval
ASSIGNED SUB-PROTOCOL NUMBER: EAY191-N2

The NCI-ComboMATCH Steering Committee (SC) has granted approval of NRG's ComboMATCH protocol proposal from Matthew Ellis and Bora Lim to proceed with study development.

REQUIRED SC COMMITTEE APPROVALS:

1. Initiation of study development: **Obtained** ✓
2. Activation: **Pending**

TEMPLATES:

To facilitate standardization amongst the ComboMATCH protocols, templates were created for use by all participating Networks. Use of ComboMATCH templates is mandatory. Templates are available at the following URL and can be distributed as necessary amongst Network team members:

<https://nc.ecog-acrin.org/index.php/s/EzXNHCGqRNNinmA>

Password: oat-2QJhn

COMMITTEE COLLABORATIONS:

While designing a ComboMATCH clinical trial, the Lead Network must work in partnership with the:

- ComboMATCH Molecular Biomarker and Specimen Management (MBSM) Committee
- ComboMATCH Precision Medicine Analysis Coordination Committee (PMACC)
- ComboMATCH Protocol Logistics Working Group (PLWG)
- Assigned CTEP IDB Drug Monitor and CTEP CIB Disease Monitor

CTEP IDB Drug Monitor	Helen Chen
CTEP CIB Disease Monitor	Larissa Korde

PROTOCOL DEVELOPMENT:

- Protocol Development Guidance document available on ECOG-ACRIN website listed above
- Questions: ComboMATCHPLWG@ecog-acrin.org

CC: Lead Network Group Chair; Study Chairs: James Ford and Funda Meric-Bernstam; ComboMATCH Leadership: Jeffrey Moscow, Peter O'Dwyer, Lyndsay Harris, and Donna Marinucci; NCI: Sherry Ansher, Lisa McShane, and Jian Zhang; MBSM Committee Co-Chairpersons; PMACC Co-Chairpersons; Assigned CTEP IDB Drug Monitor and CTEP CIB Disease Monitor; and ComboMATCHPLWG@ecog-acrin.org

BAYLOR LICENSING GROUP



View Disclosure Detail (18-002)

New Disclosure

Title: Antibodies targeting NF1 (neurofibromin)

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% contribution:	10%		
Funding Sources			
Federal Funds:			

Funding Agency: DOD
Grant Number: BC151851 % of overall funding for invention: 50%
BCM Funds:
Other Sources – i.e. non-profit Foundations
Source: CPRIT % of overall funding for invention: 50%
Contact person at funding source:
Funding Agreement: No funding agreement
Summary of the Invention
Attachments (manuscript, abstract, disclosure, etc.):
Summary (a short paragraph describing the unique/proprietary aspects of the invention): Germline NF1 mutations cause neurofibromatosis type-1, the world most common genetic disorder, leading to neuronal tissue outgrowth that sometimes can be malignant. More recent studies have shown that NF1 is also mutated in 20% of all cancers, suggesting that NF1 inactivation is also a powerful driver for the formation of many common cancers. We have come upon NF1 when studying breast cancer, which is diagnosed annually in 250,000 women in the US alone. We have evidence that NF1 inactivation can be seen in over 20% metastatic breast cancer alone because its loss promotes aggressive tumor activities. In order to facilitate the study of NF1, we have generated monoclonal and polyclonal antibodies that can be used for Western blot, immunoprecipitation, and ChIP. We believe that the quality of our antibody is outstanding that will benefit a wide range of scientific and commercial applications.

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