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Inactivating *NF1* mutations are enriched in advanced breast cancer and contribute to endocrine therapy resistance

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Competing interests:

Nicholas Turner declares receiving advisory board honoraria from Pfizer, Novartis and Lilly, and research funding from Pfizer.

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Statement of translational relevance:

We show that the molecular profile of advanced breast cancer is enriched for multiple potentially targetable genetic events, which are associated with poor prognosis and resistance to adjuvant therapy, with increased frequency of *HER2, AKT1 and NF1* mutations. Among these, truncating mutations in *NF1* can be selected in advanced breast cancer, not present in original matched primaries, and are associated with poor prognosis and endocrine resistance that may be overcome through inhibition of CDK4/6.

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ABSTRACT

Purpose: Advanced breast cancer (ABC) has not been subjected to the same degree of molecular scrutiny as early primary cancer. Breast cancer evolves with time and under the selective pressure of treatment, with the potential to acquire mutations with resistance to treatment and disease progression. To identify potentially targetable mutations in advanced breast cancer, we performed prospective molecular characterisation of a cohort of patients with ABC.

Experimental Design: Biopsies from patients with advanced breast cancer were sequenced with a 50 gene targeted panel in the Advanced Breast Cancer Biopsy (ABC-Bio) study. Blood samples were collected at disease progression for circulating tumour DNA (ctDNA) analysis, along with matched primary tumour to assess for acquisition in ABC in a subset of patients.

Results: We sequenced 210 ABC samples, demonstrating enrichment compared to primary disease for potentially targetable mutations in *HER2* (in 6.19% of samples), *AKT1* (7.14%) and *NF1* (8.10%). Of these enriched mutations, we show that *NF1* mutations were frequently acquired in ABC, not present in the original primary disease. In ER positive cancer cell-line models, loss of *NF1* resulted in endocrine therapy resistance, through both ER dependent and independent mechanisms. NF1 loss promoted ER-independent cyclin D1 expression, which could be therapeutically targeted with CDK4/6 inhibitors *in vitro*. Patients with *NF1* mutations detected in baseline circulating tumour DNA had a good outcome on the CDK4/6 inhibitor palbociclib and fulvestrant.

Conclusions: Our research identifies multiple therapeutic opportunities for advanced breast cancer and identifies the previously underappreciated acquisition of *NF1* mutations.

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INTRODUCTION

As breast cancer evolves from primary to metastatic breast cancer, and through the selective pressure of treatment, the genetic drivers may change (1,2). The genomics of primary breast cancer has been well established through multiple large studies including TCGA (3) and METABRIC (4), and yet the acquired genetic events of advanced breast cancer have been investigated less thoroughly (5). Mutations in the oestrogen receptor are acquired in advanced ER positive breast cancer, especially during treatment with aromatase inhibitors (6,7). Mutation in the oestrogen receptor influence sensitivity to subsequent endocrine therapies, suggesting that acquired genetic events may be critical to predicting outcome on subsequent therapy.

Breast cancer is characterised by a large number of relatively rare genetic events that may both predict for adverse outcome and be potentially targetable with novel therapies. Yet few studies have examined how these genetic events may change in metastatic breast cancer, whether such genetic events may be enriched through inherent poor prognosis, and therefore relative enrichment, or through acquisition by tumour evolution. Here in a clinical sequencing program, we identify acquired mutations in the *NF1* tumour suppression gene in advanced breast cancer, demonstrating that such mutations are enriched in the metastatic setting.

NF1 is a tumour suppressor gene that encodes for neurofibromin protein which acts as a repressor of RAS-GTP activation, with loss of NF1 resulting in RAS activation and downstream to the MAPK pathway activation (8). *NF1* germline mutations are associated with neurofibromatosis type 1 (NF1), a dominant autosomal disorder clinically characterized by pigmentary changes in the skin and typically the apparition of multiple peripheral nerve sheath tumours (neurofibromas) and other benign nervous system tumours like optic gliomas. Germline *NF1* mutation increases the risk of breast cancer especially in women under 50 years old that could lead to an increased risk of cancer related death (9-11). Somatic mutations in *NF1* are rare in primary cancer, but are associated with poor prognosis and an increased risk of recurrence (12). Loss of *NF1* expression results in tamoxifen resistance in pre-clinical models (13). Here we, elucidate the functional consequences of *NF1* loss in ER positive breast cancer, and identify therapeutic approaches to treat *NF1* mutations.

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MATERIALS AND METHODS.

Study design and Patients

Patients with advanced breast cancer were recruited into a clinical sequencing study, the Advanced Breast Cancer Biopsy (ABC-Bio) trial (CCR3991, REC ID: 14/LO/0292), a prospective tissue collection study at The Royal Marsden Hospital, London, UK. The study protocol was approved by the NHS Health Research Authority, Research Ethics Committee London-Chelsea. Written informed consent was obtained from each patient in accordance with regulatory requirements, good clinical practice and the Declaration of Helsinki. Patients consented to either a biopsy of metastatic disease or access to an archival biopsy of recurrent disease. Blood was collected in EDTA blood tubes at disease progression for circulating tumour DNA (ctDNA) analysis. Immunohistochemical analysis and assessment of tumour samples was performed by the Histopathology Department, Royal Marsden Hospital. ER and PR scoring were assessed following the Allred/Quick Score, which gives a scoring range of 0-8. Scores 3-8 were considered positive. In cases with ER-, only a strong score in PR (defined as >5) allocated the sample as HR+. IHC analyses of HER2 were reported as a score ranging from 0–3. Scores 0 and 1+ were considered negative, 3+ positive and borderline 2+ results were retested with in situ hybridization methods to confirm HER2 positivity. Cases included using external analysis had been performed under standard local practise and according to general recommendations.

Additional paired samples before and after resistance to aromatase inhibitors (AI) were collected in a retrospective tissue collection study, the AI pairs study. These paired tumour biopsy samples were obtained from patients pre- and post- progression (either locally advanced or metastatic disease) whilst receiving treatment with an AI (14,15). A total of 48 paired samples were subjected to molecular characterisation by next generation sequencing and gene expression analysis (15).

Baseline plasma samples from the PALOMA-3 trial were analysed. PALOMA-3 was a multicenter, randomized phase III trial assessing palbociclib and fulvestrant in premenopausal and postmenopausal women (n=331) with advanced, hormone receptor–positive breast cancer who had progressed during prior endocrine therapy, as previously reported (16). Patients were assigned 2:1 to palbociclib (125 mg orally for 3 weeks followed by 1 week off) and fulvestrant (500 mg intramuscularly every 14 days for the first three injections, then 500 mg every 28 days), or matching placebo plus fulvestrant. Written informed consent was obtained from all participants.

Next generation sequencing (NGS)

Formalin-fixed paraffin embedded (FFPE) tissue blocks were reviewed for tumour content by a pathologist and tumour rich areas marked. Tumour sections were macrodissected to enrich for tumour content.

DNA was extracted from 10 micron sections of FFPE tumour samples using QIAamp DNA FFPE tissue kit (56404 QIAGEN) and quantified using the Qubit dsDNA High Sensitivity Assay Kit with the Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA). Samples were sequenced using a targeted capture panel (The Breast NGS v1.0 panel) consisting of 41 breast cancer driver genes

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(Supplementary Table 1) selected based on either being frequently mutated in breast cancer or rare but potentially targetable (3,17,18). NGS libraries were prepared from 50-400ng DNA using the KAPA HyperPlus Kit (Kapa Biosystems, Wilmington, MA, USA) and SeqCap EZ adapters (Roche, NimbleGen, Madison WI, USA), following the manufacturer's protocol, including dual-SPRI size selection of the libraries (250-450 bp). To optimise enrichment and reduce off-target capture, pooled, multiplexed, amplified pre-capture libraries (up to 13 samples per hybridization) were hybridized overnight using 1 µg of total DNA to a custom design of DNA baits complementary to the genomic regions of interest (NimbleGen SeqCap EZ library, Roche, Madison, WI, USA). Hybridised DNA was PCR amplified and products purified using AMPure XP beads (Beckman Coulter, Danvers, MA, USA) and quantified using the KAPA Quantification Q-PCR Kit (KAPA Biosystems, Wilmington, MA, USA).

Sequencing was performed on a MiSeq (Illumina, San Diego, CA, USA) with 75 bp paired-end reads and v3 chemistry, or NextSeq (Illumina, San Diego, CA, USA) with 75 bp paired-end reads and v2 chemistry, according to the manufacturer's instructions. For samples where germline matched control was available, pools from tumour and control DNA libraries were multiplexed separately for hybridization and combined prior sequencing at a ratio of 4:1, increasing the relative number of reads derived from tumour DNA.

Miseq runs were analysed using MiSeq Reporter Software (v2.5.1; Illumina), to generate nucleotide sequences and base quality scores in Fastq format. Resulting sequences were aligned against the human reference genome build GRCh37/Hg19 to generate binary alignment (BAM) and variant call files (VCF). Secondary analysis was carried out using Molecular Diagnostics Information Management System to generate QC, variant annotation, data visualisation and a clinical report. Reads were deduplicated using Picard (http://broadinstitute.github.io/picard/), and metrics generated for each panel region. Oncotator (v1.5.3.0) (https://portals.broadinstitute.org/oncotator) was used to annotate point mutations and indels using a minimum variant allele frequency (VAF) of 5% and a minimum number of 10 variant reads as a cut-off (19). Manta (https://github.com/Illumina/manta) was used for the detection of structural variants (20). Variants were annotated for gene names, functional consequence (e.g. Missense), PolyPhen-2 predictions, and cancer-specific annotations from the variant databases including COSMIC (https://cancer.sanger.ac.uk/cosmic), Tumorscape (21), and published MutSig results (22). Copy number variation (CNV) was assessed by measuring the coverage ratio between each tumour probe target and the average coverage of all probe targets in the normal (when a normal sample was available). If a normal sample was not available the ratio between each tumour probe target and the average of all probe targets in the tumour was used instead. Ratios below 0.5 fold were defined as a potential deletion whereas a ratio above 2.4 was flagged as a potential amplification if 80% of the target regions had exceeded the thresholds. Borderline genes with less than but almost 80% of the targets showing amplification/deletion were not automatically flagged but assessed individually. All potential mutations, structural variants and CNVs were visualised using Integrative Genomics Viewer (IGV; (23,24)) and two individuals were required to review the mutation report

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independently. VCF files from unpaired samples were annotated using Illumina VariantStudio v3.0, and checked manually on IGV.

NextSeq runs were analysed using an in-house pipeline. For the demultiplexing bcl2fastq (v2.19) was used to isolate reads for each sample. The reads were aligned to the reference genome build GRCh37/Hg19 using Burrows-Wheeler Aligner (BWA-MEM), followed by the marking of PCR duplicates and calculation of various quality control (QC) metrics using Picard. Copy number was estimated as described above for the analysis of Miseq runs. Manta (v.0.29.6) was used for the detection of structural variants. Genom Analysis ToolKit (GATK) was used for re-aligning around indels to improve indel calling and base quality score recalibration for adjusting systematic errors made by the sequencer when estimating quality scores of each base call (25). Finally, GATK was also used for variant calling using HaplotypeCaller for tumour only analysis (limit of detection ~10%) and MuTect2 for tumour paired analysis. VCF files from unpaired samples were annotated using Illumina VariantStudio v3.0, and checked manually on IGV.

The Breast NGS v1.0 panel could detect single nucleotide variants at >5% allele frequency with >99% sensitivity (95% CI) and >98% specificity (95% CI). Small indels could be detected with sensitivity >95% and specificity >81% at >5% variant allele frequency. High-level gene amplifications (>8 copies) could be detected in samples with >30% neoplastic nuclei. For each patient, germline DNA was sequenced to allow subtraction of single nucleotide polymorphisms, thus only somatic variants were reported.

The sequencing strategies used in the molecular characterisation of ctDNA in the PALOMA3 study are described in detail by O'Leary *et al* (26).

Mutation detection using digital droplet PCR

ctDNA was extracted from plasma using either the QIAamp circulating nucleic acid kit (Qiagen) or the QIASymphony SP Instrument using QIAsymphony DSP Circulating DNA Kit (Qiagen) according to manufacturer's guidelines. Concentrations of extracted ctDNA were estimated using either a TaqMan™ Copy Number Reference Assay (4403326, Life technologies) for *RPPH1*(27-29) or the Qubit hsDNA quantification kit and Qubit instrument (Life Technologies). Mutations in *PIK3CA* (p.E542K, *c.1624G > A*; p.E545K, *c.1633G > A*; p.H1047R, *c.3140 A > G*; p.H1047L, *c.3140A>T*)(26) and *ESR1* (p.E380Q, *c.1138G>C*; p.L536R, *c.1607T>G*; p.Y537C, *c.1610A>G*; p/D538G, *c.1613A>G*. p.S463P, *c.1387T>C*; p.Y537N, *c.1609T>A*; p.Y537S, *c.1610A>C*) were interrogated by digital PCR (dPCR) using custom assays as previously described (6,26,27,30). *AKT1* hotspot mutation (p.E17K, *c.49G>A*; E17K) were interrogated using a commercial dPCR (dHsaCP2000031 and WT: dHsaCP2000032, BIORAD) as per manufacturer instructions dPCR was conducted as previously described (14)

RNA extraction and Nanostring gene expression on tumours

RNA was extracted from tumour samples using RNeasy Mini Kit (74104, Qiagen) and quantified using the Qubit RNA High Sensitivity Assay Kit with the Qubit 3.0 fluorometer (Life technologies). RNA from tumours with *NF1* mutations was run on a NanoString nCounter™ with a custom codeset

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comprised of 70 genes (Supplementary Table 2; (15)), according to manufacturer's guidelines. Expression data from *NF1* mutant samples was combined and normalised with an existing expression data set (AI pairs study cohort, n=30), generated using the same codeset (15). The AI pairs cohort, contained 3 *NF1* mutant tumours, expression data from which were added to the ABC-bio NF1 mutant dataset.

Cell Lines

MCF7 and T47D cell lines were obtained from ATCC and cultured in phenol free RPMI media (32404-014, Life technologies) supplemented with 10% dextran/charcoal stripped FBS (12676029, Life Technologies), 1nM oestradiol (Sigma), glutamine (25030149, Life technologies), penicillin and streptomycin (15140-122, Life technologies). Cell lines were banked in multiple aliquots on receipt to reduce risk of phenotypic drift and identity confirmed by STR profiling with the PowerPlex 1.2 System (Promega).

Antibodies, RNAi and Drugs

Antibodies used were phosphorylated (p) AKT S473 (4058), pAKT T308 (2965), AKT (4691), CCND1 (2978), CCNE1 (4129), CCNE2 (4132), pCDK2 T160 (2561), CDK2 (2546), pERa S118 (2511), pERa S167 (64508), ERa (13258), pERK1/2-Thr202/Tyr204 (4370), ERK1, 2 (9102), NF1 (14623), pRB S780 (3590), pRB S807 (8516), Rb (9313), PGR (8757), p-mTOR S2481 (2974), mTOR (2983), phospho-ribosomal protein S6 (5364), ribosomal protein S6 (2217; all Cell Signaling Technology). Fulvestrant (S1191), tamoxifen (S1238) and palbociclib (S1116) were obtained from Selleck Chemicals. siRNAs were from Dharmacon: siGENOME non-targeting siRNA Pool#2 (D-001210-02), siGENOME NF1 set of 4 (MQ-003916-03). NF1 shRNA constructs, shLuc-72243, shNF1-39714 and shNF1-39717 (31,32) were a kind gift from Dr Steven Whittaker, Institute of Cancer Research. The vectors were packaged into lentivirus in 293-T cells and MCF7 cells were infected with shLuc-72243 MCF7-LucB2.2, shNF1-39714 (MCF7-shNF1_14B2.2) and shNF1-39717 (MCF7-shNF1_17B2.2). At 96h after infection, 2μg/mL puromycin was added, and a polyclonal stable pool was established under continuous selection.

Gene Expression using digital PCR

cDNA was prepared using the SuperScript III First Strand Kit (Life Technologies; 18080-051) according to the manufacturer's guidelines, using 50 to 200 ng total RNA primed with random hexamers. dPCR gene expression reactions were typically set up with 1 to 5 ng RNA equivalent of cDNA. Taqman gene expression assays for NF1 (Hs01035108_m1), NCOR1 (Hs01094541_m1) and NCOR2 (Hs00196955_m1) were run a duplex reaction and normalized using GUSB reference assay (Hs99999908_m1) were obtained from Life Technologies Ltd. dPCR was conducted as previously described(14).

Human Estrogen Receptor RT^2 Profiler PCR Array

RNA was extracted from cells using RNeasy Mini kit (74104, Qiagen), and genomic DNA eliminated and cDNA prepared with 500ng template RNA using RT^2 First strand Kit (330401, Qiagen), according to manufacturer's guidelines. cDNA samples were prepared for qPCR using RT^2 SYBR Green qPCR Mastermix (330523, Qiagen) and run on the Human Estrogen Receptor RT^2 Profiler

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PCR Array (330231, PAHS-005ZA-24, Qiagen) comprising 84 target genes and 5 housekeeping genes (Supplementary Table 3). For each sample, gene expression data was adjusted using the geometric mean of the housekeeping genes, the delta C_t calculated and data presented as the Log2 fold change.

Western Blotting

Cells were lysed in NP40 lysis buffer (1% v/v NP40, 10 mmol/L Tris–Cl pH8, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT) supplemented with protease/phosphatase inhibitor cocktail (5872, Cell Signaling Technologies). Western blots were carried out with precast TA or Bis-Tris gels (Life Technologies). Cells were reverse transfected with siRNA 72 hours prior to lysis.

Colony formation assays

Colony formation assays were conducted in 6-well plates, seeded with 1000–2500 cells prior to exposure to the indicated experimental conditions. Plates were fixed with tricyclic acid (10%), stained with sulforhodamine B (SRB) and colonies counted using a GelCOUNT instrument (Oxford Technologies.

Bromo deoxyuridine incorporation assays

Cells were seeded into 96 well plates and S-phase fraction assayed after 24 hours exposure to compounds, with the addition of 10 μmol/L bromodeoxyuridine (BrdU) for 2 hours prior to fixing. BrdUrd incorporation was assessed with Cell Proliferation chemiluminescent ELISA-BrdUrd assay (Roche 11 669 915 001) according to the manufacturer's instructions and adjusted for viable cells in parallel wells assessed with CellTiter-Glo (33,34).

Statistics, Databases and analysis tools

Mutation and expression data from TCGA (Provisional, 1105 samples) was extracted from cBIOportal (http://www.cbioportal.org/) (35,36). ER positive samples only were extracted and the remaining samples were divided into NF1 truncated and nontruncating with samples with missense NF1 mutations removed from the analysis. Data was normalised and differential expression was investigated between NF1 mutated and non-mutated samples using the voom function from the LIMMA R package. Further pathways analysis on the differentially expressed genes was carried out using Ingenuity pathway analysis (IPA, QIAGEN, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/). Graphical presentation of mutations in context with protein domains was performed using ProteinPaint (https://pecan.stjude.cloud/pp). Other statistical analysis was performed as indicated using Graphpad Prism v7.05 and custom scripts in R version 3.4.3. Correction for multiple comparisons was performed using either Sidak test for multiple comparisons or the method of Benjamin-Hochberg for false discovery as indicated.

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RESULTS

Genetic profile of advanced breast cancer

A total of 246 patients with metastatic breast cancer gave consent and were recruited into a clinical sequencing study (ABC-Bio study, Figure 1A), with sequencing data obtained for 210 patients. The clinical demographics of the 210 patients are shown in Table 1. Sequencing revealed mutations in 33 genes, including *TP53* (44.8%, 98 mutations in 94 patients), *PIK3CA* (37.1%, 93 mutations in 78 patients), *ESR1* (10.0%, 22 mutations in 21 patients), *NF1* (8.1%, 17 mutations in 17 patients), *HER2* (6.2%, 13 mutations in 13 patients) and *AKT1* (7.1%, 16 mutations in 15 patients; Figure 1B). Comparison with the mutation incidence in primary cancers in the TCGA dataset, revealed higher mutations rates in advanced breast cancer in *TP53* (q=0.0011), *ESR1* ($q=5.26x10^{-11}$), *NF1* (q=0.0078), *AKT1* ($q=4.76x10^{-9}$), *HER2* (q=0.0207), PTEN (q=0.0195) and *SF3B1* (q=0.041; all Fisher's exact test with FDR correction using Benjamini Hochberg method; Figure 1B).

Of the mutations found at higher frequency in advanced breast cancer, *NF1* was characterised by frequent inactivating, truncating or nonsense mutations (Figure 1C). *AKT1* and *HER2* were dominated by known hot-spot activating mutations, while in *PTEN* frameshift, nonsense and deletions accounted for the majority of identified mutations (Figure 1B and Supplementary Figure 1A). *ESR1* mutations were found at a high prevalence only in HR positive/HER2 negative tumours (20/22 mutations HR+/HER2-, p=0.0278, Fisher's exact test Supplementary Figure 1A). HR+/HER2- tumours had significantly lower incidence of *TP53* mutations (40/143, 27.97%) than both HER2+ tumours (16/19, 84.21%, p<0.0001, Fisher's exact test) and triple negative breast cancer (TNBC) (37/45, 82.22%, p<0.0001, Fisher's exact test), with subtype determined in metastatic sample. HR+/HER2- tumours had a similar rate of *PIK3CA* mutations (57/143, 39.86%) to HER2+ tumours (7/19, 36.84%), and non-significantly higher rate than TNBC (12/45, 26.67%, p=0.1555, Fisher's exact test), in part as comparison made to metastatic TNBC which in turn had a higher rate of *PIK3CA* mutations than primary TNBC in TCGA. Incidence of *NF1* mutations was similar in HR+/HER2-, HER2+ tumours and TNBC (Supplementary Figure 1A). Comparison of mutation frequency between ABC-Bio and TCGA by tumour subtype showed comparable mutation frequencies with significant increase identified in *ESR1* and *AKT1* in HR+/HER2- tumours after adjusting for multiple comparisons. The rate of *NF1* mutations increased from 2.5% in TCGA to 7.0% in ABC-Bio (p= 0.021, q= 0.127; Supplementary Figure 1B). Similarly, ABC-Bio sequencing was highly comparable to the MSKCC dataset (37), with increased frequency of mutations noted in *ESR1*, *AKT1* and *BRCA1* compared to primary breast cancers (Supplementary Figure 1C). *HER2* amplification status had very high agreement with clinical *HER2* amplification status determined by IHC or FISH (sensitivity=1, specificity=0.9746, p<0.0001: Supplementary Figure 1D).

We next looked at factors that influenced the genomic profile. *ESR1* mutations were only rarely identified in patients with newly relapsed disease, and were frequent in patients with more heavily pre-treated cancer (Supplementary Table 4). Similarly, *ESR1* mutations were rare in *TP53* mutant advanced HR+/HER2-breast cancer (1/40) and common in *TP53* wildtype HR+/HER2- breast cancer (18/142, 12.6%, p=0.0455 Fisher's exact test, Figure 2A). This suggested that *ESR1* mutations are acquired through prior endocrine therapy in the metastatic setting, principally in *TP53*

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wild-type cancers. In contrast, *NF1* mutations rates did not differ across line of therapy, nor by *TP53* mutation status. *NF1* mutations were frequently associated with mutations in genes in the PI3K pathway (11/17 patients, 64.7%), including *PIK3CA* (6/17), *AKT1* (3/17) and *PTEN* (4/17), but rarely associated with *ESR1* mutations (1/17, 5.9%).

In the cohort, 10/132 primary HR+/HER2- tumours switched phenotype to be classified as TNBC in the metastatic setting (Figure 2A). These "acquired TNBC" reflected 21.7% (10/46) of advanced TNBC as a whole. The mutational profile of these "acquired TNBC" more closely resembled that of stable HR+/HER2- tumours (both primary and recurrent HR+/HER2-) rather than stable TNBC tumours (both primary and recurrent) TNBC (Supplementary Figure 2A), suggesting the elevated rate of *PIK3CA* mutation observed in advanced TNBC may in part reflect subtype switching.

Prognostic implications of genomic profiles

We investigated the influence of mutational profile on outcome, both from time of diagnosis of the original primary to relapse (disease free survival, DFS), and the time from relapse to death (advanced overall survival, advanced OS). We note that all patients in this series relapsed, and analysis of DFS assessed risk of early *versus* later relapse. DFS and advanced OS data for all mutations found with a frequency of ≥5% are presented in Supplementary Tables 5 and 6 respectively. In patients with HR+/HER2- tumours, truncating *NF1* mutations were associated with shorter DFS compared to wild type *NF1* (HR 4.46, 95% CI 1.65-12.08, Log rank p=0.0031; Figure 2B), whilst *MAP3K1* mutations were associated with longer DFS (HR 0.53, 95% CI 0.3012 - 0.9411, Log rank p=0.030). These data reflect similar poor prognosis in the adjuvant setting associated with *NF1* mutations in other data sets (12,38). *NF1* mutant patients had frequently received adjuvant chemotherapy (88.2%, 15/17) and adjuvant endocrine therapy (100%, 17/17). In the advanced setting, these patterns were maintained although without statistical significance (Supplementary Table 6). In patients with HER2+ tumours, the 3 cancers with *HER2* mutations (both *HER2* amplified and mutant cancers) were associated with dramatically shorter DFS (HR 7.548 (95%CI 0.3983-143, Log rank p=0.0001; Figure 2C). Though limited in number, these findings suggest a rare but important subset of breast cancers that may do poorly on current treatment. Interestingly *HER2* mutant HR+/HER2- breast cancers also had significant worse DFS and advanced OS. Analysis of outcome for TNBC was limited by small numbers (Supplementary Tables 5 and 6).

Acquisition of NF1 mutations in advanced breast cancer

We investigated whether genes mutated at higher incidence in advanced breast cancer were mutated at higher incidence due to acquisition of the mutation in the metastatic setting, or whether the mutation was present in the original primary tumour but enriched in the metastatic setting due to a higher propensity to relapse. We focused our analysis on tumour samples with mutations in *NF1*, *AKT1* and *HER2* – rare, but potentially targetable mutations. We did not further investigate *ESR1* mutations, as it is well documented these are acquired in the advanced setting following endocrine therapy (6,7,15,30). Primary tumour samples for 34 patients were retrieved and sequenced, including samples for 13/17 *NF1*, 12/15 *AKT1* and 6/12 *HER2* mutant cases identified in the sequencing of metastatic tumours. Of the 13 patients with *NF1* mutations in their metastatic samples, 8/13 (61.5%) patients had *NF1* mutation in the primary tumour sample (Figure 2D),

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indicating acquisition of *NF1* mutations continues in the advanced setting (5/13, 38.5%). In addition, one primary tissue sample was found to have an *NF1* mutation that was lost in the paired metastatic sample. In contrast, *AKT1* (10/12, 83.3%) and *HER2* (5/6, 83.3%) mutations were largely shared with the primary sample. Consistent with being truncal driver mutations *TP53* mutations (12/13, 92.3%) were largely shared in both primary and metastatic tumour sample. *PIK3CA* mutations (5/13, 38.5%) are also acquired in the metastatic setting (26).

Gene expression Analysis

Our genomic analysis suggested that *NF1* mutations may be acquired in the metastatic setting, are frequently truncating mutations predicted to inactivate *NF1* function, and are associated with marked shorter DFS in HR+/HER2- breast cancers with relapse during adjuvant endocrine therapy. We next investigated the functional impact of *NF1* mutations on oestrogen receptor positive breast cancer.

RNA from 8 tumour samples with truncating *NF1* mutations, were analysed with a custom Nanostring ER signalling gene expression codeset, along with 30 *NF1* wildtype metastatic breast cancers that had relapsed after AI therapy (15) (Figure 3A). Tumours with truncating *NF1* mutations had lower NF1 expression ($p=2.74x10^{-6}$, Wilcoxon signed rank test Figure 3B). In the series of *NF1* wild-type cancers 7/30 cancers had acquired very low ER signalling in advanced cancer (Figure 3A left hand branch), effectively becoming genomically ER negative. All *NF1* mutations had some maintained ER signalling (Figure 3A). *ESR1* mutations have been shown to significantly increase expression of oestrogen regulated genes (ERGs) and proliferation genes (15). The presence of a truncating *NF1* mutation resulted in substantially less ER signalling than *ESR1* mutations, with *NF1* mutant cancers having broadly similar expression of ERGs and proliferation genes compared to wild-type for both *ESR1* and *NF1* ($p=0.1572$ and $p=0.1123$ respectively, Wilcoxon test, Figure 3D). Tumours with *NF1* mutations had significantly lower expression of the nuclear co-repressor proteins *NCOR1* ($p=0.021$, Wilcoxon test) and *NCOR2* ($p=0.011$, Wilcoxon test) than *ESR1* mutant tumours or wild type tumours (Figure 3E). These data suggested that *NF1* mutant tumors had down regulated ER signalling in metastases, but without the acquisition of ER negative phenotypes prevalent in tumors wildtype for *NF1* and *ESR1* mutations.

To corroborate our findings, we analysed gene expression and mutation data from primary tumours in TCGA. Similar to our analysis of metastatic tumours, primary tumours with truncating *NF1* mutations had decreased expression of *NF1* (Wilcoxon test, $p=0.000159$; Supplementary Figure 3A). Cancers with truncating *NF1* mutations had enrichment of differentially regulated genes associated with canonical oestrogen receptor signalling (Figure 3F), and decreased *NCOR1* compared to wildtype tumours (Supplementary Figure 3B and C).

***NF1* silencing results in resistance to endocrine therapy**

Prior research has identified that *NF1* silencing results in resistance to tamoxifen therapy (13). Our findings on short DFS in *NF1* mutant cancer included 14/17 (82.4%) patients treated with adjuvant endocrine therapy, with early relapse during endocrine therapy, suggested a potential for more general endocrine therapy resistance in the clinic. To investigate the consequence of *NF1* loss on

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endocrine therapy resistance, we silenced *NF1* with NF1 siRNA SMARTpool in ER positive cell lines MCF7 and T47D, or siCON non-targeting control, and performed clonogenic assays. The individual siRNAs that comprised the SMARTpool all decreased NF1 expression (Supplementary Figure 4A). Silencing *NF1* resulted in resistance to tamoxifen and withdrawal of oestrogen from medium to mimic aromatase inhibition, with partial resistance to fulvestrant (Figure 4A and B). Assessment using the Bliss independence model indicated that NF1 knockdown was antagonistic of endocrine therapies (Supplementary Figure 4B)

We generated MCF7 cells with stable knock down of *NF1* using two different shRNA constructs (shNF1-14B and shNF1-17B) and a non-targeting control (LucB) (31,32). Stable silencing of *NF1* similarly resulted in stable, long term resistance to oestrogen deprivation, fulvestrant and tamoxifen (Figure 4C), despite NF1 shRNA stable cell lines having only partial *NF1* silencing (Supplementary Figure 4C and D).

We next investigated the signalling consequences of *NF1* loss, and the impact on ER signalling. Silencing *NF1* using siRNA in MCF7 decreased expression of NF1 and increased levels of phospho-ERK1,2 and phospho-AKT, which was sustained when cells were treated with fulvestrant, tamoxifen or oestradiol-depleted media for 24 hours (Figure 4D). However, AKT phosphorylation was also induced by NF1 loss, likely reflecting the well described role of RAS signalling in controlling PI3 kinase activity, and suggesting that NF1 loss may possibly broadly activate both MAPK and AKT signal transduction. We performed a time course experiment treating MCF7 cells with the MEK inhibitor, trametinib. Trametinib treatment resulted in sustained inhibited phosphorylation of ERK1,2 up to 72h, with strong induction of NCOR2 (Supplementary Figure 4E). Knock down of NF1 decreased NCOR1 and NCOR2 expression which was increased by treatment with trametinib (Supplementary Figure 4F). ER signalling after *NF1* silencing was investigated with RT2 profiler array (methods). *NF1* silencing down regulated *ESR1* expression (Figure 4E), and ER signalling (Supplementary figure 4E), whilst upregulating CCND1 and MYC gene expression (Figure 4E and Supplementary figure 4F). Inhibition of MEK with trametinib largely reversed the gene expression changes of *NF1* silencing (Figure 4E and Supplementary Figure 4G), implicating increased MEK-ERK signalling as the major driver of endocrine resistance.

We further investigated signalling effects of NF1 loss. *NF1* silencing resulted in increased cyclin D1 expression, which was not suppressed after 72 hours of treatment in both MCF7 and T47D cells with fulvestrant, tamoxifen or oestradiol-depletion (Figure 5A). *NF1* silencing did not appreciably alter expression of cyclin E1 or E2 (Figure 5B). In stable knockdown *NF1* shRNA MCF7 cells, long term *NF1* silencing resulted in higher cyclin D1 protein expression, which suppressed incompletely on endocrine therapies (Figure 5B). In keeping with elevated cyclin D1 expression, Rb phosphorylation was increased at both S780 and 807 (Figure 5B), with modestly elevated phosphorylation of CDK2 T180. Cells with stable *NF1* knockdown had decreased ER expression, but increased phospho-ER, which was exaggerated compared to control when treated with tamoxifen or oestradiol-depletion (Figure 5B). Expression of *NCOR1* and *NCOR2* were decreased in cells with stable knock down of *NF1* (Supplementary Figure 4D), as predicted by our tumour analysis, which was reversed by treatment with trametinib (Supplementary Figure 4F).

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In summary, *NF1* loss resulted in increased MAPK pathway signalling, that downregulated ER expression and signalling, but with residual ER hyperphosphorylation. NF1 silencing resulted in ER independent activation of cyclin D1 expression, with increased Rb phosphorylation, suggesting that *NF1* loss promoted endocrine resistance through both ER dependent and independent mechanisms.

Combating NF1 loss in breast cancer therapy

We next investigated therapeutic approaches that may overcome endocrine resistance in *NF1* mutant cancers. We noted that *NF1* silencing resulted in marked overexpression of cyclin D1 and increased RB1 phosphorylation, and we therefore investigated whether CDK4/6 inhibition may overcome the adverse effects on endocrine therapy resistance after *NF1* silencing. In short-term BrdU incorporation assays, NF1 siRNA blocked the anti-proliferative effects of tamoxifen; BrdU positive cells were reduced in control siCON cells with tamoxifen, whereas there was no reduction in siNF1 cells. Palbociclib, and the combination of palbociclib and tamoxifen substantially reduced proliferation in siNF1 cells (Figure 5C). Similarly, in long-term clonogenic assays palbociclib reduced colony formation of MCF7 cells after *NF1* silencing and further mitigated resistance to fulvestrant, tamoxifen and oestrogen depletion (Figure 5D; Supplementary Figure 5A). Using the Bliss independence model, palbociclib was found to combine with the endocrine targeted treatments in an additive manner (Supplementary Figure 5B). In contrast, in cells with NF1 knockdown the effect of combining palbociclib with the endocrine treatments was synergistic.

We then investigated the effect of *NF1* mutations on the survival in patients enrolled in the PALOMA-3 randomised phase III trial, of fulvestrant plus placebo versus fulvestrant plus palbociclib. We have previously reported ctDNA sequencing in the PALOMA-3 trial, and we analysed the effects of *NF1* mutation detection in baseline ctDNA (39). Overall *NF1* mutations were detected in 6.34% (21/331) baseline plasma samples. In patients with available end of treatment samples, the baseline *NF1* mutations (11/11) were detected at end of treatment, suggesting stability through treatment (26,40). Two mutations in *NF1* were selected through treatment, present at end of treatment but not in baseline ctDNA. There were too few patients with *NF1* mutations to make meaningful assessment in the placebo and fulvestrant control arm (Supplementary Figure 5B). Patients with baseline *NF1* mutations detected had a similar outcome on palbociclib plus fulvestrant, compared to patients without *NF1* mutations detected (Log rank, p=0.71, 5/16 stopgain, 11/16 nonsynonymous; Figure 5E), supporting our pre-clinical experiments that CDK4/6 inhibition in part overcame the effects of NF1 loss on endocrine resistance.

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DISCUSSION

Here we present the molecular characterisation of 210 metastatic breast cancers, and demonstrate that multiple targetable mutations are detected at increased frequency in metastatic disease as compared to archival primary cancers. *NF1* mutations may be acquired in the metastatic setting and loss of *NF1* function results in resistance to all commonly used endocrine therapies, although combination of fulvestrant and CDK4/6 inhibition presents a therapeutic strategy to overcome resistance.

Our findings on acquired *NF1* mutations adds to increasing evidence that mutations in the MAPK pathway are enriched in advanced ER positive breast cancer. We previously demonstrated that *KRAS* mutations, highly likely sub-clonal, may be detected at relatively high frequency after progression on AI therapy for advanced breast cancer (30). Mutations in the fibroblast growth factors receptor genes *FGFR2* and *FGFR3* may be found in ctDNA of endocrine resistant cancers (41), with FGFR signalling canonically activating MAPK pathway signalling (42). Similarly, a large recent series of metastatic biopsy sequencing, without paired primary sequencing, demonstrated frequent mutational activation of the pathway in advanced ER positive breast cancer (37). These data demonstrate opportunities to develop targeted therapeutic approaches. The majority of *NF1* mutations are truncating mutations, and therefore highly likely inactivating. Although likely that loss of heterozygosity is required to inactive NF1 function, our data on resistance to endocrine therapy despite only partial knock down of NF1 with shRNA (Figure 4C) suggests the possibility of heterozygous effects of NF1 loss. Missense mutations in *NF1* are relatively frequent, and although the majority of these may be non-pathogenic, further research will be required to establish if some *NF1* missense mutations are functional. Finally, whether the clonality of these mutations is important for outcome and treatment will need to be addressed.

HR+/HER2 breast cancer is the most frequent phenotype of breast cancer, accounting for approximately 70% of cases. *NF1* mutation confers poor prognosis in terms of shorter time to relapse in HR+/HER2- patients, with relapse occurring frequently on endocrine therapy reflecting endocrine resistance (Figure 1). Loss of NF1 results in endocrine resistance likely both through ER-dependent mechanisms and ER independent mechanisms, likely with MAPK pathway driven expression of cyclin D1 and ER independent S phase entry. Of all endocrine therapies fulvestrant is the least resistant pre-clinically (Figure 4). Although ER expression and signalling was partially down-regulated with *NF1* silencing, residual ER was hyper-phosphorylated likely reflecting ligand independent activation of residual ER by enhanced signal transduction, which would be most effectively inhibited by fulvestrant. Combination with CDK4/6 inhibitors, which target ER independent cyclin D1 transcription (Figure 4), results in substantial enhanced efficacy of endocrine therapy *in vitro* (Figure 5). Consistent with these observations, the prognosis of patients with baseline or pre-treatment detection of *NF1* mutation in the PALOMA-3 phase III trial (16) suggested that combined fulvestrant and palbociclib may mitigate the adverse prognostic effects of *NF1* mutations. This suggests the possibility that fulvestrant and palbociclib could be investigated in the adjuvant setting in *NF1* mutant cancers, in an attempt to overcome the risk of early relapse (37).

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Our data has limitations; we focused our analysis of primary-metastasis pairs on those potentially targetable genetic events present at increased frequency in advanced breast cancer, and have therefore not performed an exhaustive investigation of discordance of genetic events. Our sequencing strategy was a targeted approach, again to investigate potential targetable genetic events, and has not interrogated genetic events outside the gene panel which would be addressed by either larger panel or whole exome sequencing. Our analysis of the clinical impact of *NF1* mutations on fulvestrant and palbociclib is limited by small numbers, and these findings would need validation in additional studies of fulvestrant and CDK4/6 inhibitors. However, these studies also indicate that addition of a MEK inhibitor to CDK4/6 inhibition may offer further benefit, which could be explored in the clinic.

Breast cancers evolve through treatment, with endocrine therapy for hormone receptor positive breast cancer driving diversification and acquisition of resistant mutations. This selection of resistance mutations presents substantial challenges treatment, but also opportunities to develop new therapeutic strategies. Mutations in *NF1* mutations, both those detectable in primary cancer and acquired in the metastatic setting, induce resistance to endocrine therapy, and may be targetable to reverse resistance in progressing cancers.

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TABLES

Table 1. The clinical demographics of the 210 patients with sequencing data from the ABC-Bio study presented by *NF1* mutation: *NF1* wild type; *NF1* mutant predicted truncating (N=9; 6 Non-sense, 2 Frameshift and 1 Stop-gain); *NF1* mutant not truncating (N=8; 5 Missense + 2 Splice Site + 1 In-frame deletion). Comparisons using Chi-square test.

	NF1 wild-type N=193	**NF1 mutant predicted truncating N=9**	**NF1 mutant not truncating N=8**	**q value**
Age at inclusion (years), median				
	56	55	51	
Hormone receptor status on primary, n (%)				
HR+/HER2-	121 (63)	7 (78)	4 (50)	0.44*
HER2+	23 (12)	1 (11)	1 (12)	0.98*
HR-/HER2-	33 (17)	1 (11)	3 (38)	0.34*
UK	16 (8)	0 (0)	0 (0)	NA
Total	193 (100)	9 (100)	8 (100)	
Hormone receptor status on metastatic, n (%)				
HR+/HER2-	133 (69)	6 (67)	4 (50)	0.48*
HER2+	16 (8)	1 (11)	2 (25)	0.27*
HR-/HER2-	41 (21)	2 (22)	2 (25)	0.97*
UK	3 (2)	0 (0)	0 (0)	
Total	193 (100)	9 (100)	8 (100)	
Presentation at diagnosis, n (%)				
Early	167 (87)	8 (89)	8 (100)	0.53
Metastatic	26 (13)	1 (11)	0 (0)	0.53
Total	193 (100)	9 (100)	8 (100)	
Nodal status if early presentation, n (%)				
Positive	100 (60)	5 (63)	6 (75)	0.72*
Negative	64 (38)	3 (37)	2 (25)	0.72*
Missing/Unknown	3 (2)	0 (0)	0 (0)	0.86
Total	167 (100)	8 (100)	8 (100)	
Germline BRCA1/2 status, n (%)				
Positive	12 (6)	0 (0)	1 (12)	0.49*
Negative	59 (31)	4 (44)	2 (25)	0.49*
Unknown	122 (63)	5 (56)	5 (63)	0.89
Total	193 (100)	9 (100)	8 (100)	

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Adjuvant treatment if early presentation, n (%)				
Yes	164 (98)	8 (100)	7 (88)	0.11
No	3 (2)	0 (0)	1 (12)	0.11
Total	167 (100)	8 (100)	8 (100)	
Adjuvant ET if early presentation, n (%)				
Yes	123 (74)	8 (100) - 1 TNBC on primary received adjuvant ET	6 (75) - 1 TNBC on primary received adjuvant ET	0.24
No	44 (26)	0 (0)	2 (25)	0.24
Total	167 (100)	8 (100)	8 (100)	
Type of adjuvant ET if adjuvant ET, n (%)				
Tamoxifen only	71 (58)	6 (75)	3 (50)	0.57
AI only	19 (15)	2 (25)	1 (17)	0.77
Tamoxifen + AI	33 (27)	0 (0)	2 (33)	0.31
Total	123 (100)	8 (100)	6 (100)	
Resistance to adjuvant ET, n (%)				
Yes	74 (60)	6 (75)	6 (100)	0.10
No	49 (40)	2 (25)	0 (0)	0.10
Total	123 (100)	8 (100)	6 (100)	
Type of endocrine resistance to adjuvant ET, n (%)**				
Primary resistance	23 (31)	1 (17)	1 (17)	0.59
Secondary resistance to adjuvant ET, n (%)	51 (69)	5 (83)	5 (83)	0.59
Total	74 (100)	6 (100)	6 (100)	
Prior neoadjuvant/adjuvant CT if early presentation, n (%)				
Yes	132 (79)	8 (100)	7 (88)	0.30
No	35 (21)	0 (0)	1 (12)	0.30
Total	167 (100)	8 (100)	8 (100)	
Prior metastatic +/- adjuvant CT before sequencing, n (%)				
Yes	158 (82)	8 (89)	8 (100)	0.36
No	35 (18)	1 (11)	0 (0)	0.36
Total	193 (100)	9 (100)	8 (100)	
Metastatic CT after sequencing, n (%)				

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Yes	91 (47)	3 (33)	7 (88)	0.05
No	102 (53)	6 (67)	1 (12)	0.05
Total	193 (100)	9 (100)	8 (100)	
Lines of ET therapy for metastatic disease before sequencing, n (%)				
0	132 (69)	9 (100)	5 (63)	0.11
1	39 (20)	0 (0)	2 (25)	0.30
2	16 (8)	0 (0)	1 (12)	NA
3+	6 (3)	0 (0)	0 (0)	NA
Total	193 (100)	9 (100)	8 (100)	
Lines of CT for metastatic disease before sequencing, n (%)				
0	122 (63)	8 (89)	2 (25)	**0.02**
1	36 (19)	1 (11)	3 (38)	0.34
2	21 (11)	0 (0)	1 (12)	0.57
3+	14 (7)	0 (0)	2 (25)	0.12
Total	193 (100)	9 (100)	8 (100)	

NA, does not meet requirements for chi-square test* Unknown excluded from analysis. **Only patients with endocrine resistance considered.

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FIGURES

Figure 1. Genetic profile of advanced breast cancer

A) CONSORT diagram showing the structure and the patient numbers of the ABC-Bio clinical sequencing study. B) *Left hand panel*, number and type of mutations identified in advanced breast cancer within ABC-Bio; *Right hand panel*, comparison of the incidence of mutations identified in ABC-Bio (green bars) with the TCGA primary breast cancer (grey bars), p value Fisher's exact test with Benjamini Hochberg false discovery correction. C) *NF1* mutations detected in the ABC-Bio study, with mutation type, functional domain and reference to amino acid residue.

Figure 2. Mutational profile impact on outcome and agreement with targetable mutations between paired primary and metastatic samples.

A) Co-occurrence of mutations in metastatic setting and tumour subtype of both primary and metastatic samples, presented by subtype of primary tumour. B) *NF1* mutation status and Overall survival (top) and disease free survival - time to recurrence – (bottom) in HR+HER2- tumours (Log rank test, p=0.436 and p=0.0031 respectively). C) *ERBB2* mutation status and overall survival (top) and disease free survival - time to recurrence - (bottom) in HER2+ tumours (Log rank test, p=0.6857 and p=0.0001 respectively). D) Mutation concordance between primary and advanced tumour samples for 34 patients with targetable mutations in NF1, AKT1, and ERBB2 in advanced breast cancer. The type of NF1 mutation and subtype of the tumour samples are indicated.

Figure 3. Gene expression profiling of *NF1* mutant breast cancers

A). Effect of truncating *NF1* mutations on *NF1* expression (Log2 ratio) compared to wild type *NF1* tumours; p value as indicated, Wilcoxon test. B) Differential gene expression in *NF1* wild type (n=30) versus patients with truncating *NF1* mutations (n=8). Indicated genes (p<0.1 Wilcoxon signed rank test) with increased (•) and decreased (•) expression in truncating *NF1* mutations. C) Effect of *NF1* truncating mutations on averaged ER gene expression (ERG) and proliferation genes; p value as indicated, Wilcoxon test. D) Expression of the nuclear receptor corepressors in *NF1* truncating mutations, *Left hand panel NCOR1* and *Right hand panel NCOR2*; p value as indicated, Wilcoxon test. E) Gene expression analysis of TCGA data, signalling pathways enriched for genes with differential expression in *NF1* mutated samples (Fisher exact test, p value as indicated).

Figure 4. Loss of NF1 causes resistance of endocrine therapy mediated by both ER dependent and independent mechanisms.

A) Colony formation assay of MCF7 transfected with siCON or siNF1 and treated with either fulvestrant, tamoxifen, oestradiol depletion, or control. Box 25-75th percentiles, bar median and

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whiskers Min-Max, n=8, ANOVA with Sidak multiple comparisons, p values as indicated. B) Colony formation assay of T47D transfected with siCON or siNF1 and treated with either fulvestrant, tamoxifen, oestradiol depletion or vehicle. Box 25-75th percentiles, bar median and whiskers Min-Max n=4, ANOVA with Sidak multiple comparisons, p values as indicated. C) Long term treatment of MCF7 with stable NF1 knock down (shNF1-14B and shNF1-17B) and control cells (LucB2.2) with fulvestrant (500nM), tamoxifen (100nM), oestradiol depletion and vehicle. Colonies highlighted in yellow. D) Western blot of whole cell lysates from MCF7 transfected with siCON or siNF1 and treated for 24hr with either fulvestrant, tamoxifen, oestradiol depletion or control, and probed for the indicated proteins. E) Gene expression analysis of ER pathway genes in MCF7 cells transfected 96 hours earlier with indicated siRNA, treated with trametinib (100nM) or vehicle for 72 hours. q values, t Test with Benjamini Hochberg false discovery correction.

Figure 5. CDK4/6 inhibition overcomes the adverse impact of NF1 loss in ER positive breast cancer

A) Western blot of whole cell lysates from MCF7 (left panel) and T47D (right panel), transfected with siCON or siNF1 and treated for 72hr as indicated and probed for the indicated proteins. B) Western blot of whole cell lysates from MCF7-LucB2.2, MCF7-shNF1 14B2.2 and MCF7 17B2.2, treated for 72hr as indicated and probed for the indicated proteins. C) MCF7 transfected with siCON2 or siNF1, treated with tamoxifen, palbociclib, combination tam+palbo, or vehicle for 24hr and assessed for BrdU incorporation. D) Colony formation assay of MCF7 transfected with siCON or siNF1 and treated with either fulvestrant, tamoxifen, oestradiol depletion, or control on their own or in combination with palbociclib. n=4; 2 way ANOVA with Sidak comparisons, p values as indicated. E) *NF1* mutation status and progression free survival in patients enrolled in the PALOMA-3 trial treated with palbociclib and fulvestrant (Log rank test, p=0.71).

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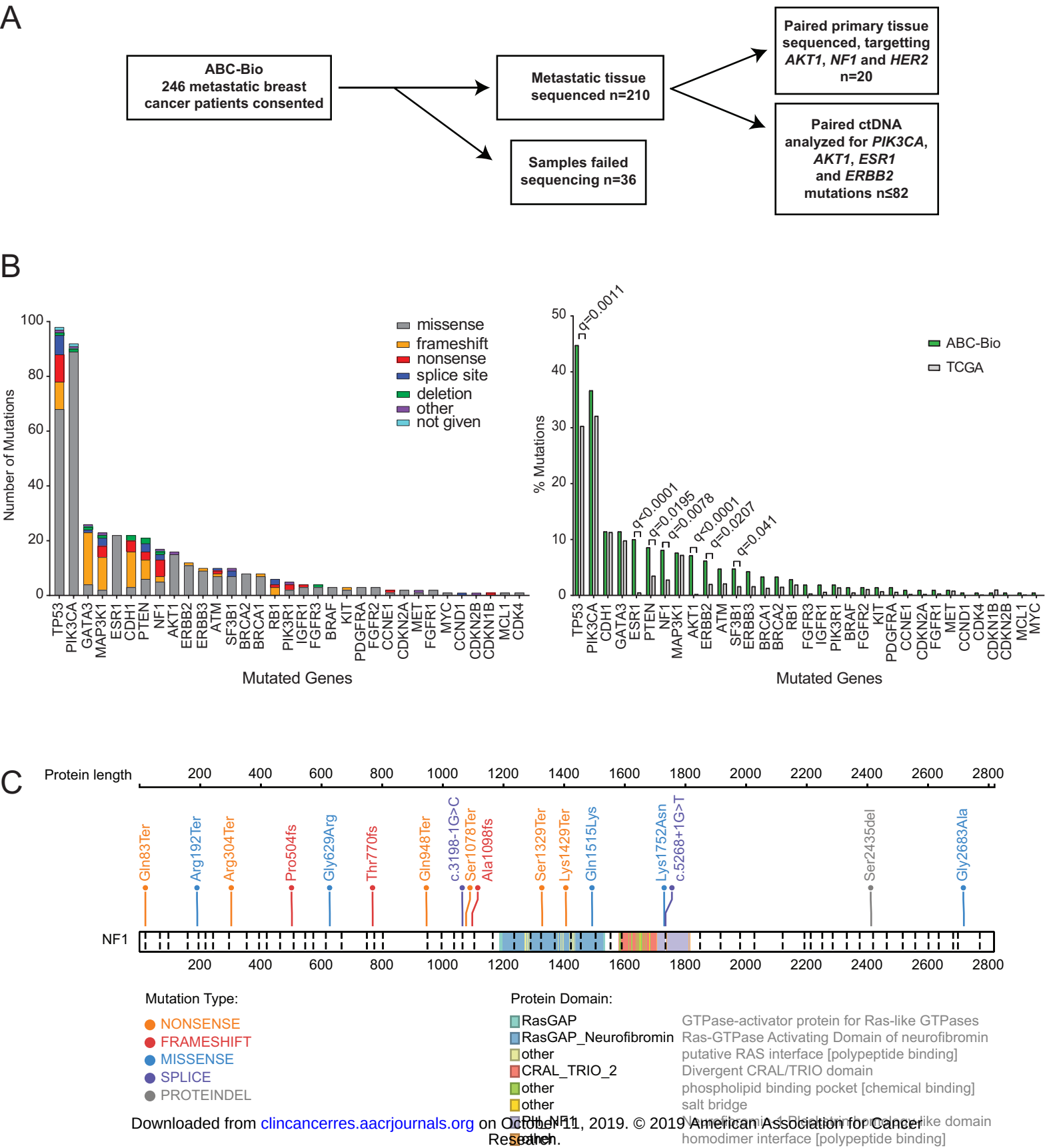
Figure 1



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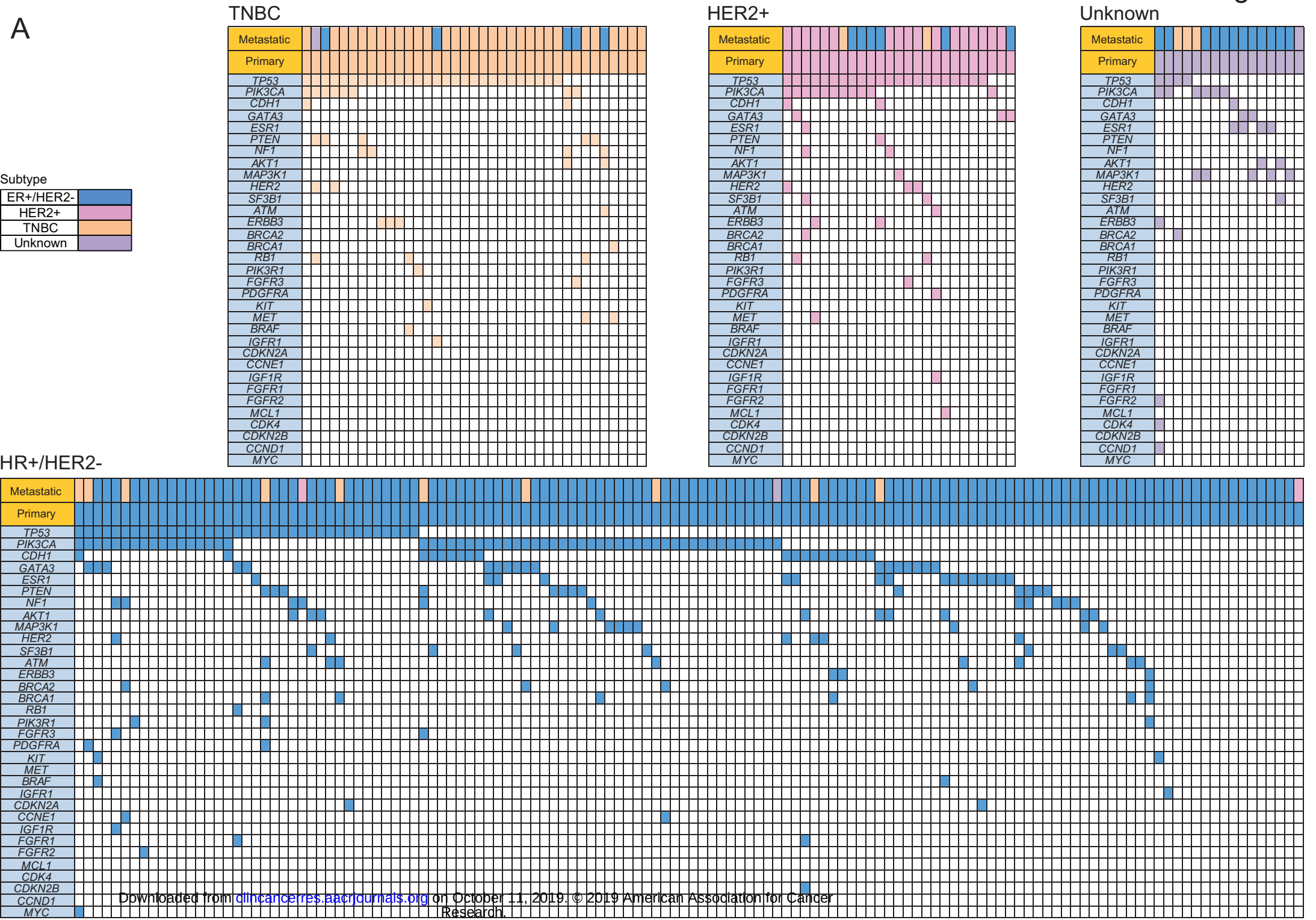

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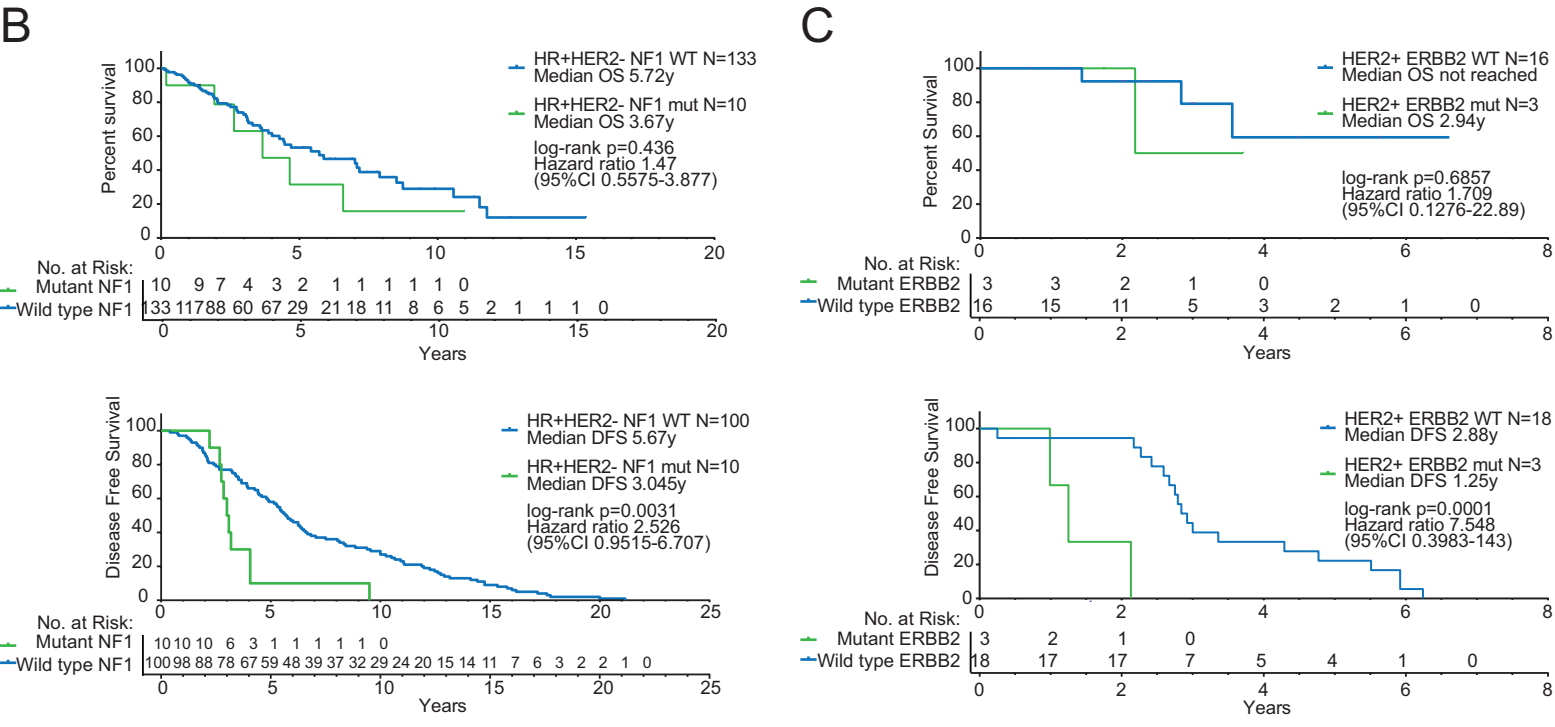

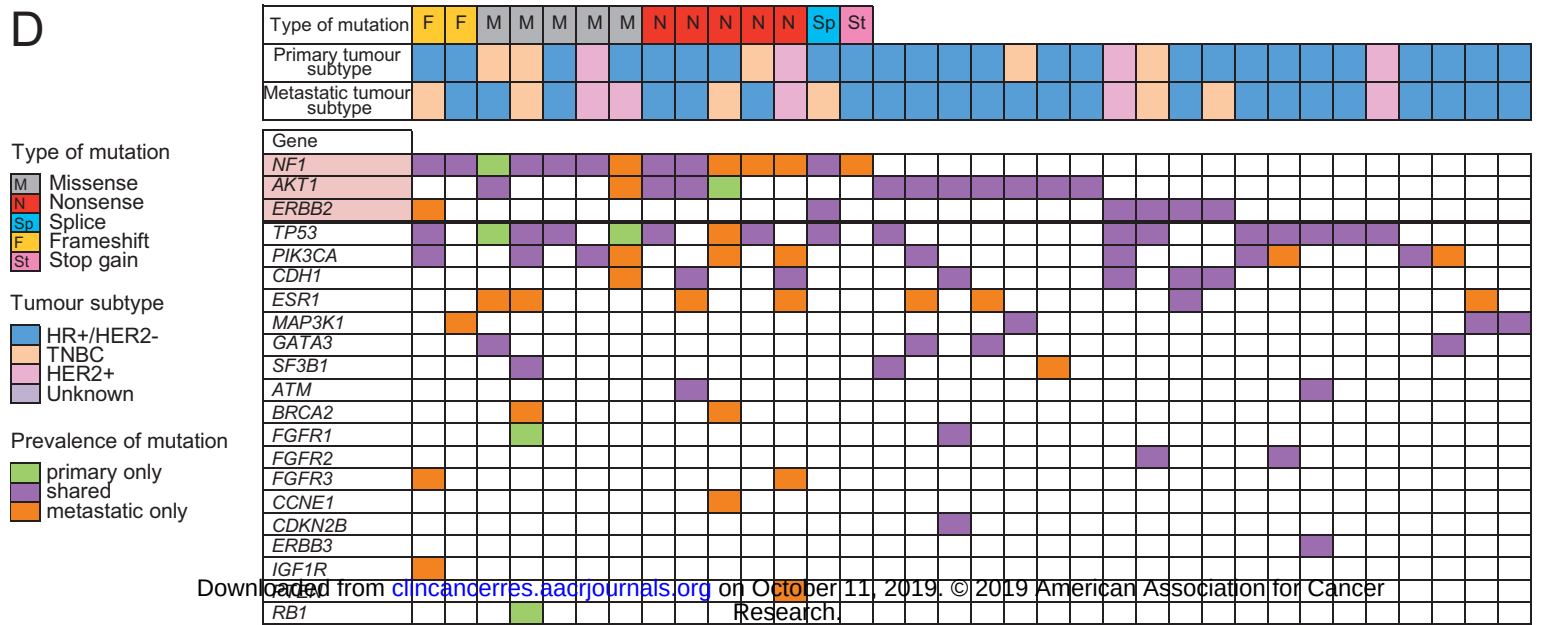

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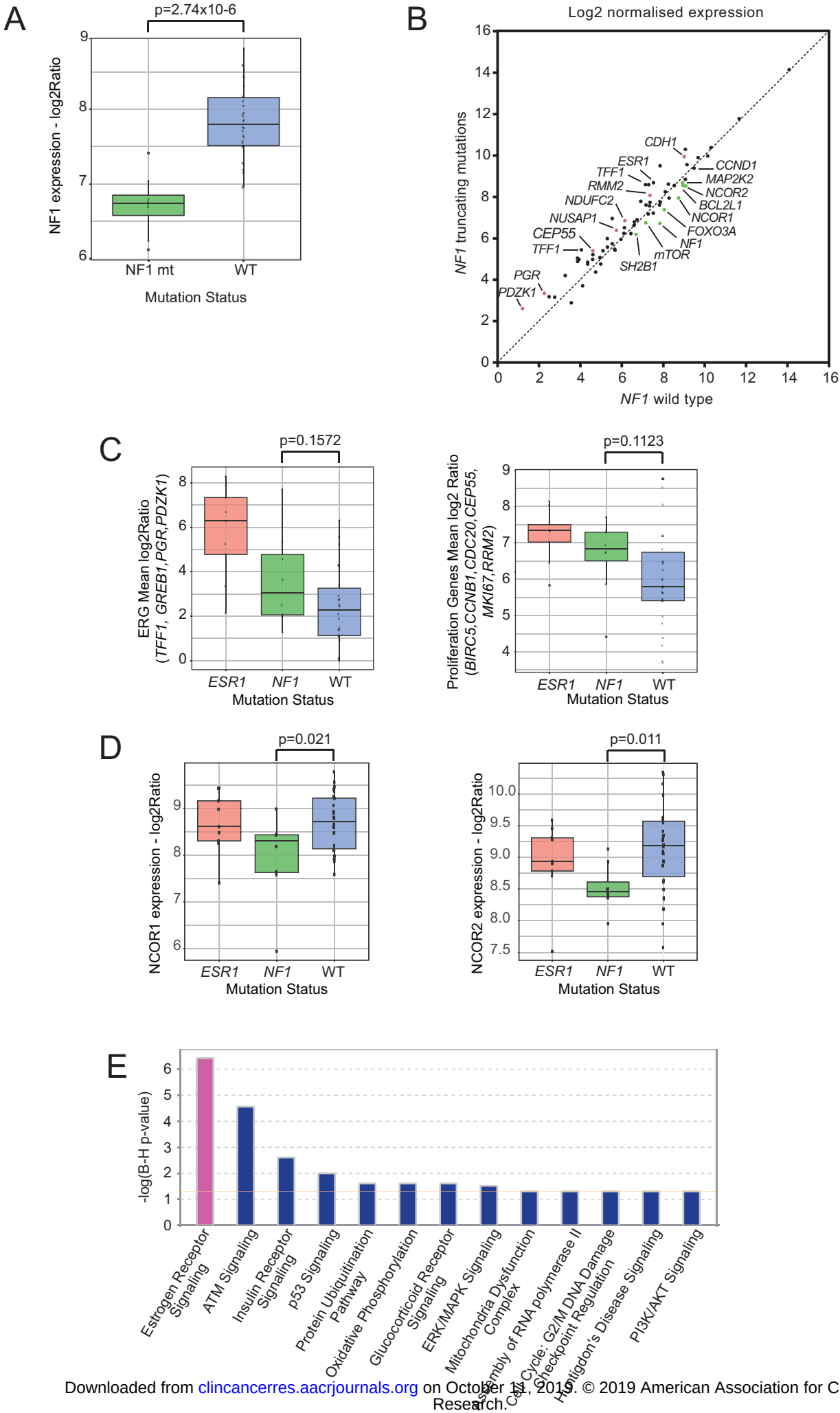
NF1_Figure 3



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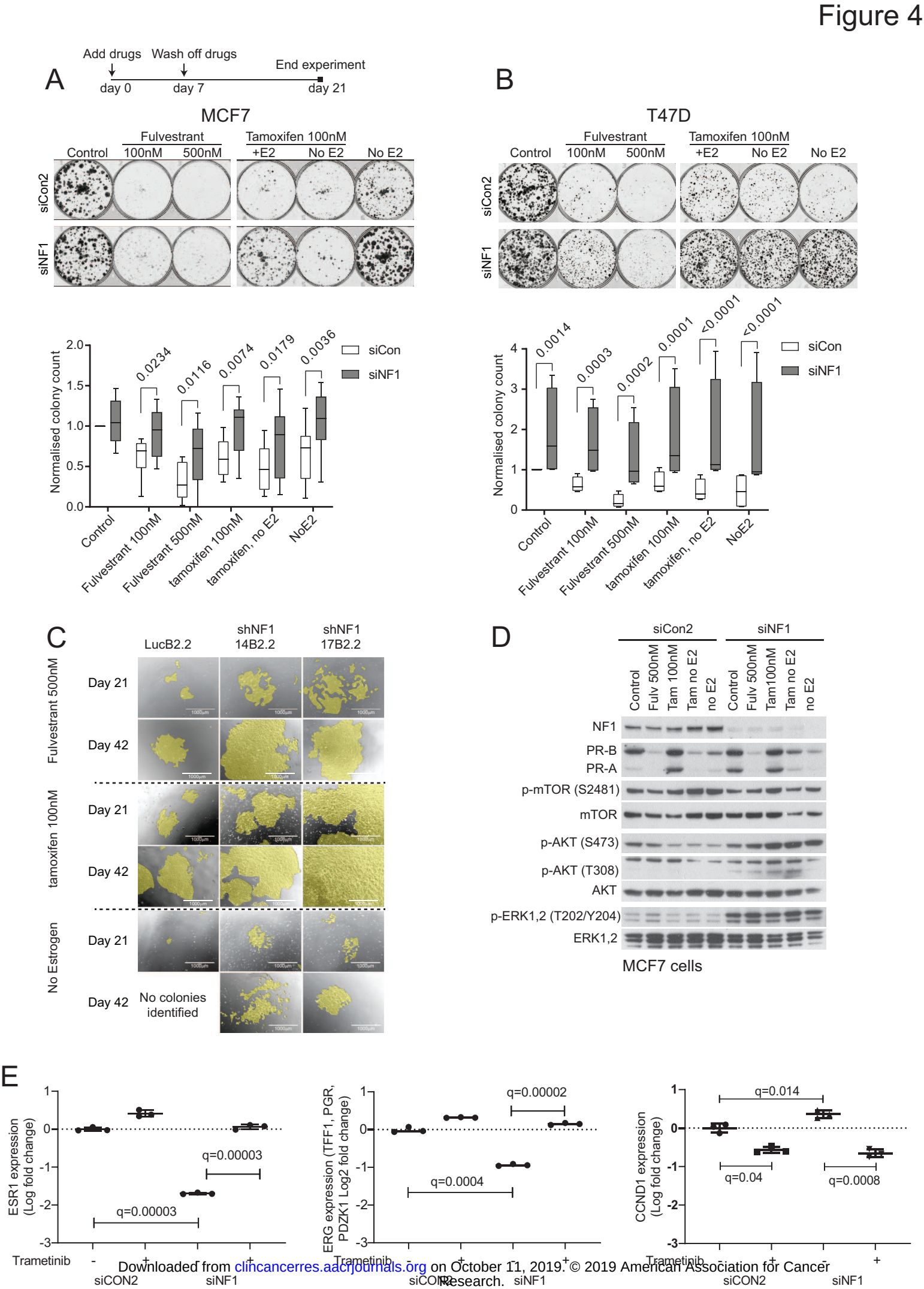

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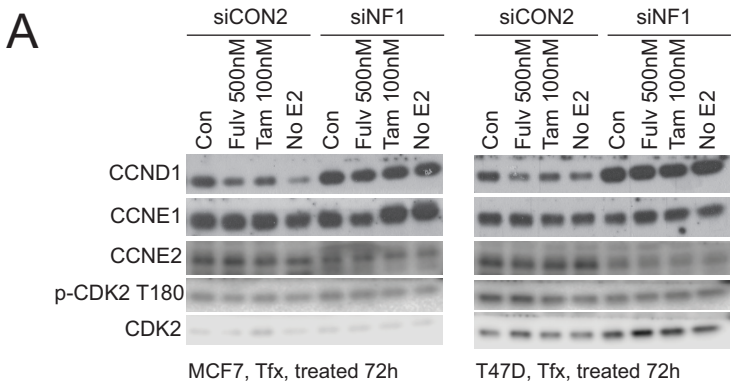

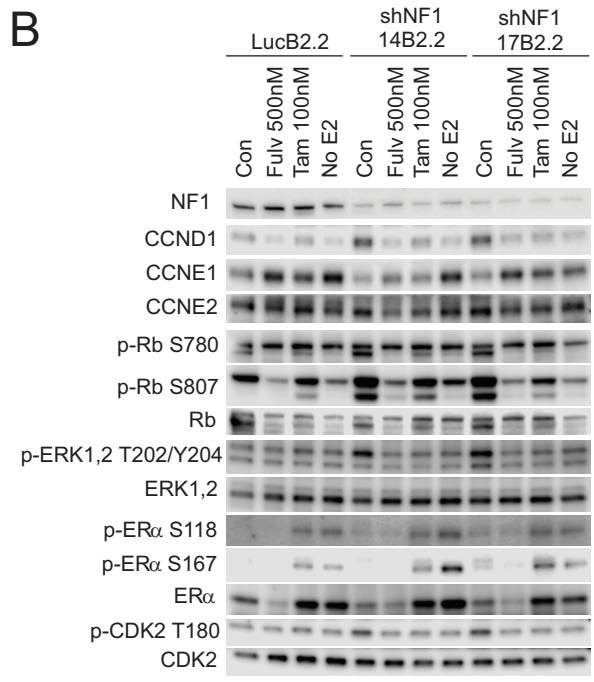

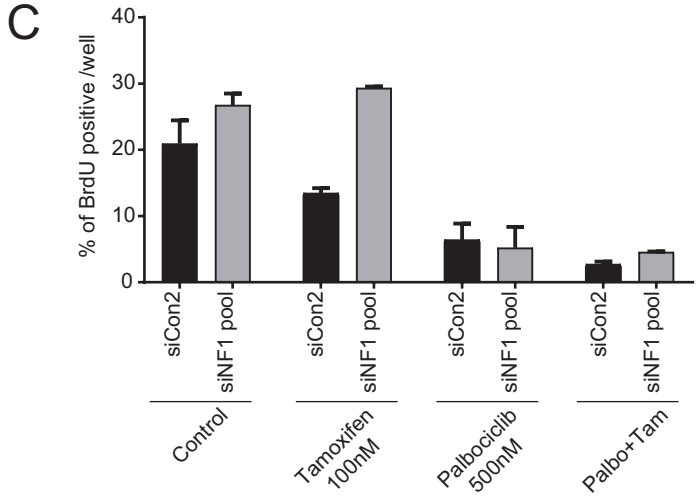

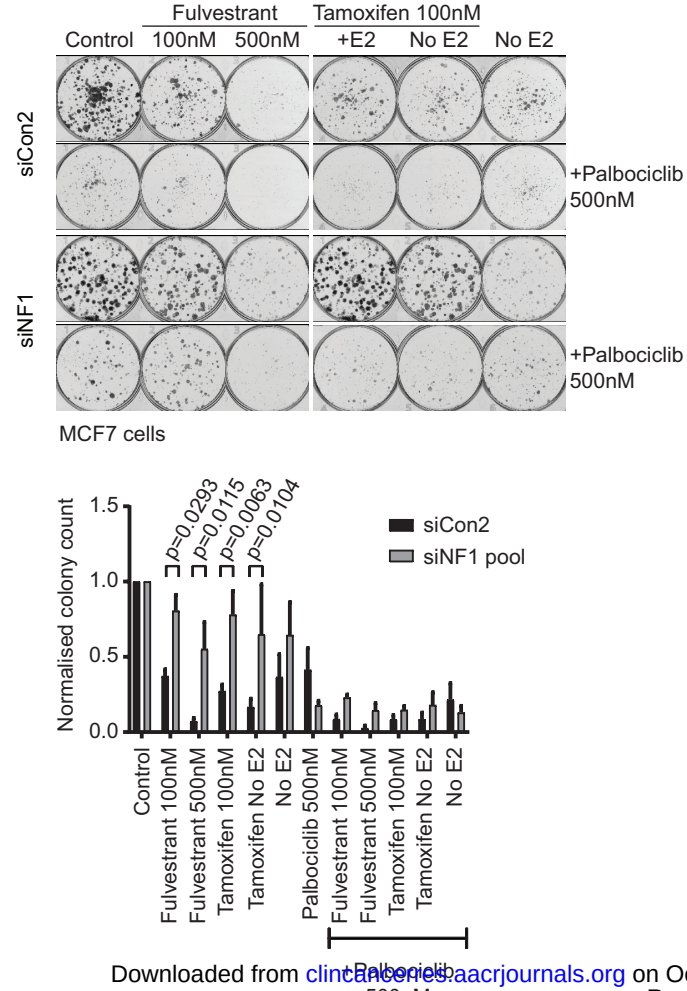

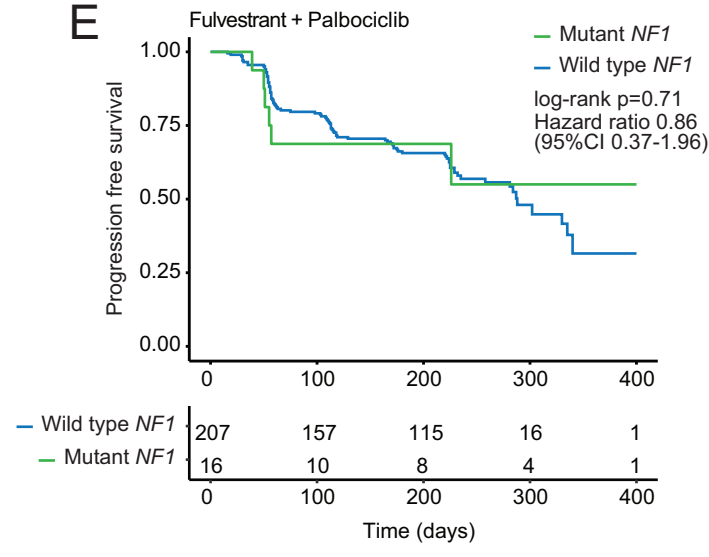

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