Isakoff et al, Platinum for Metastatic Triple Negative Breast Cancer

Inventory of Supplemental Data:

Supplementary Tables 1-5

Supplementary Figures 1-5

Supplementary Figure Legends

Supplementary Methods

Supplementary Table 1:
All grade toxicity in >10% of population.

Toxicity	All patients (n=86)		Cisplatin (n=43)		Carboplatin (n=43)	
	No. of patients	%	No. of patients	%	No. of patients	%
Hemoglobin	63	73%	35	81%	28	65%
Fatigue	57	66%	29	67%	28	65%
Nausea	57	66%	30	70%	27	63%
Hyperglycemia	40	47%	21	49%	19	44%
Neutrophils	36	42%	21	49%	15	35%
Leukocytes	33	38%	21	49%	12	28%
Neuropathy	31	36%	16	37%	15	35%
Thrombocytopenia	28	33%	8	19%	20	47%
Hypomagnesemia	28	33%	18	42%	10	23%
Constipation	26	30%	15	35%	11	26%
Dyspnea	25	29%	13	30%	12	28%
AST	25	29%	12	28%	13	30%
Lymphopenia	20	23%	11	26%	9	21%
Alk Phos	19	22%	8	19%	11	26%
Tinnitus	16	19%	16	37%	0	0%
ALT	16	19%	7	16%	9	21%
Vomiting	16	19%	6	14%	10	23%
Anorexia	15	17%	11	26%	4	9%
Hyponatremia	15	17%	9	21%	6	14%
Cough	14	16%	6	14%	8	19%
Headache	12	14%	5	12%	7	16%
Hypocalcemia	12	14%	7	16%	5	12%
Constitutional	12	14%	8	19%	4	9%
Back- pain	11	13%	7	16%	4	9%
Febrile neutropenia	11	13%	7	16%	4	9%
Hypoalbuminemia	10	12%	5	12%	5	12%
Edema limb	10	12%	5	12%	5	12%
Chest wall- pain	10	12%	6	14%	4	9%
Insomnia	10	12%	5	12%	5	12%
Rash	9	10%	4	9%	5	12%
Dizziness	9	10%	7	16%	2	5%
Hypokalemia	9	10%	4	9%	5	12%
Diarrhea	9	10%	6	14%	3	7%
Abdomen- pain	9	10%	6	14%	3	7%

Supplementary Table 2:
Grade 3/4 adverse events in >5% of patients

Toxicity	All patients (n=86)		Cisplatin (n=43)		Carboplatin (n=43)	
	No. of Patients	%	No. of patients	%	No. of patients	%
Fatigue	7	8%	5	12%	2	5%
Neutrophils	6	7%	2	5%	4	9%
Dyspnea	5	6%	3	7%	2	5%
Hemoglobin	5	6%	1	2%	4	9%
Hyperglycemia	5	6%	2	5%	3	7%
Hyponatremia	4	5%	3	7%	1	2%

Supplementary Table 3:
Association of genetic and tumor molecular features with RR

Molecular Parameter	N evaluated	Absent			Present			*P*
		Resp.	total	%	Resp.	total	%	
BRCA1/2 germline mutation	77	13	66	20%	6	11	55%	0.02
p53 mutation	52	3	16	19%	10	36	28%	0.73
PIK3CA mutation	53	12	44	27%	1	9	11%	0.42
PAM50 Basal	55	2	21	10%	8	34	24%	0.29
p63/p73 > 2	61	9	33	27%	5	28	18%	0.54

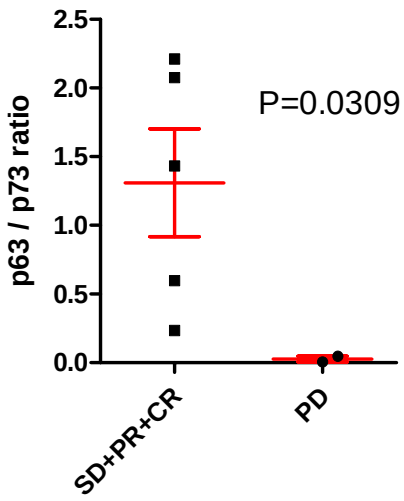
Supplementary Table 4:
Summary of Lehmann expression signatures' association with RR

Signatures	*P* value	RR associated with high subclass score
Basal_like1	0.89	sensitive
Basal_like2	0.55	resistant
Immunomodulatory	0.6	resistant
Mesenchymal	0.22	sensitive
Mesench_Stem_Like	0.37	sensitive
Luminal_AR	0.0078	resistant

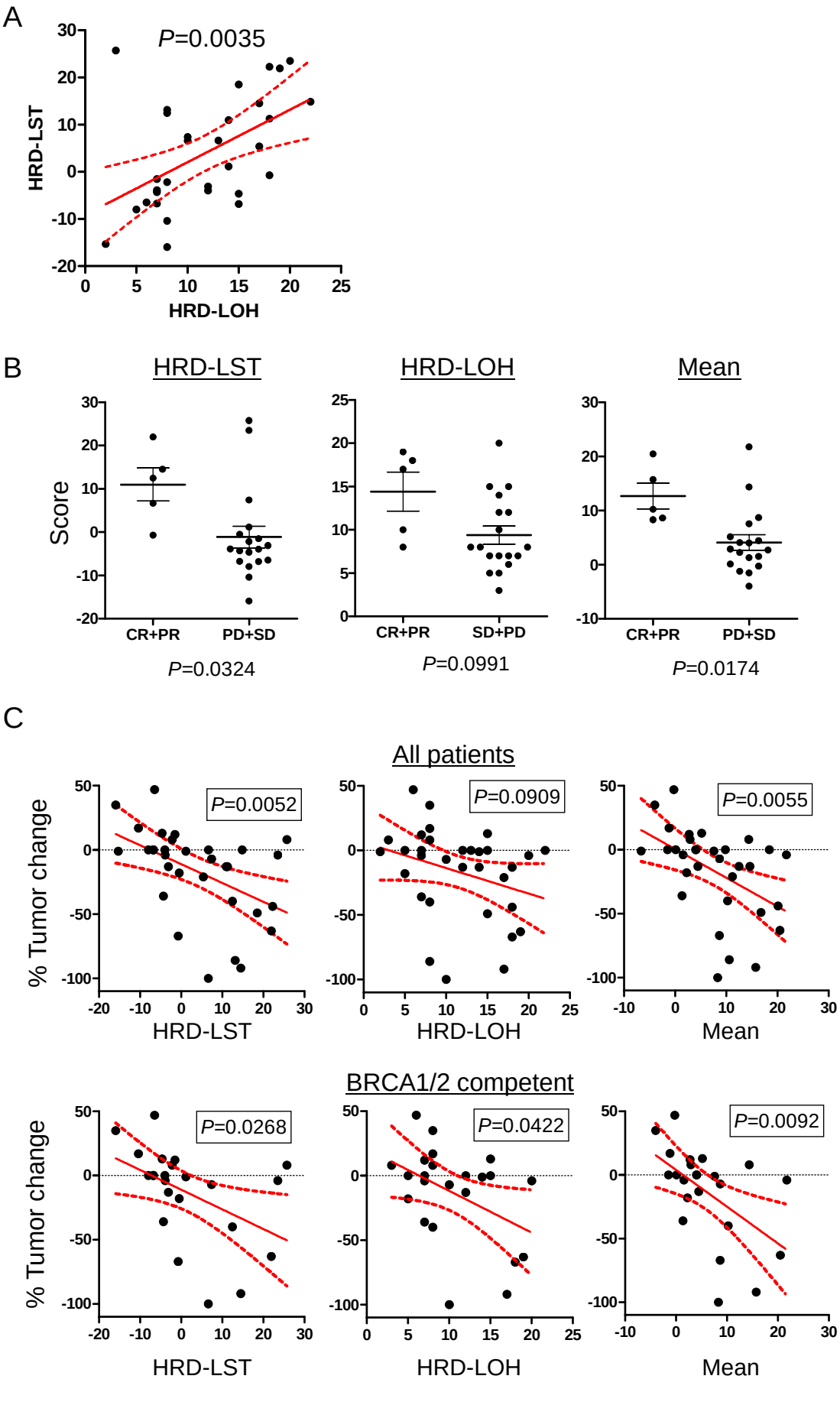
Supplementary Table 5:
Baseline characteristics by BRCA status

Characterstic	BRCA WT/UNK (N=75)		BRCA MUT (N=11)		
	No.	%	No.	%	p value
First Line Therapy	61	81%	8	73%	0.45
Prior Adjuvant Therapy	64	85%	10	91%	1
Sites of disease					
Lung	39	52%	5	45%	0.75
Liver	21	28%	4	36%	0.72
Bones	21	28%	4	36%	0.72
Lymph nodes	44	59%	9	82%	0.19
Mean # Sites of disease	2.5		2.8		0.64
Median Relapse Free Interval (days)	644		781		0.54

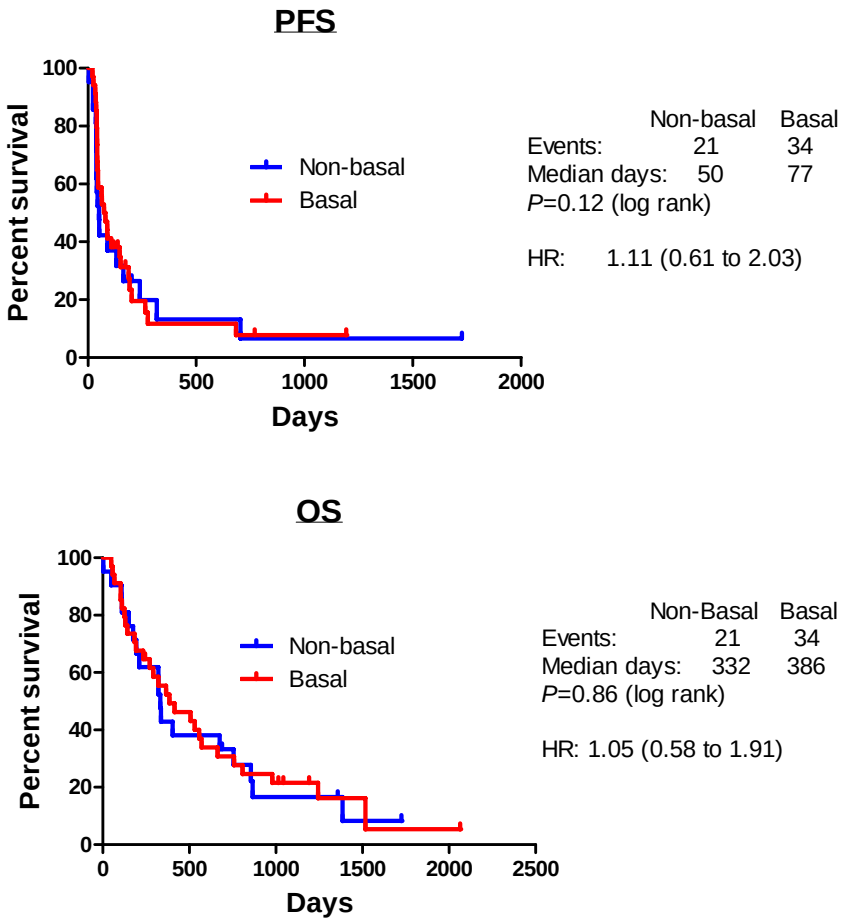
Supplementary Figure 1



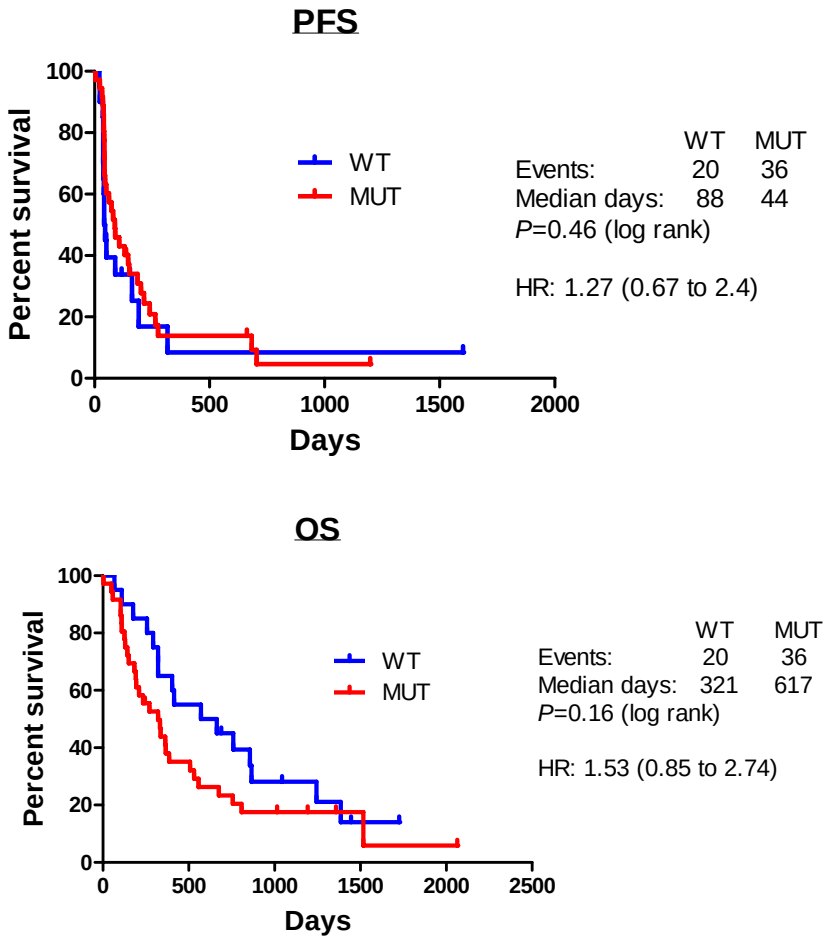
Supplementary Figure 2



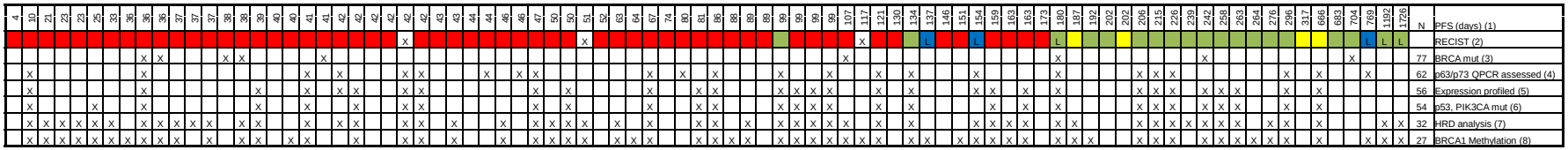
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure Legends

Supplementary Table 3:

Fisher's exact t test was used to calculate P value.

Supplementary Table 5

Fisher's exact t test and Wilcoxon test were used to calculate P values.

Supplementary Fig 1:

Association of p63/p73 ratio with clinical benefit in previously untreated patients. Tumor ΔNp63/TAp73 mRNA expression ratio measured by quantitative RT-PCR. Unpaired test (Welch's correction) was used to calculate p value.

Supplementary Fig 2:

Summary of HRD-LST and HRD-LOH analysis. Tumor DNA was analyzed for HRD-LST and HRD-LOH genomic aberration patterns as described in text and methods. A. Correlation between HRD-LST and HRD-LOH scores among all tested tumors. B. High HRD-LST/HRD-LOH scores are associated with platinum sensitivity in BRCA1/2 competent tumors (tumors without BRCA1/2 mutation or BRCA1 promoter methylation). C. High HRD-LST/HRD-LOH scores are associated with regression of pre-specified RECIST-measured lesions among all patients (top) and those with BRCA1/2 competent tumors. D. Correlation of LST score with BRCA1 mRNA level among BRCA1/2 wild-

type tumors. Unpaired t test (Welch's correction) was used to calculate P value for B. A linear regression model was used for 2A and 2C.

Supplementary Fig 3:

PAM50 basal signature does not predict PFS or OS. Kaplan-Meier estimates of PFS and OS of basal versus non-basal tumors (see methods).

Supplementary Fig 4:

Association of tumor p53 mutation with PFS and OS (N=56). Kaplan-Meier esitmates of PFS and OS of p53 mutant versus wild-type tumors.

Supplementary Fig 5:

Summary of correlative studies performed on available samples. Each column represents one patient: all 86 cases are presented in ascending order of PFS from left to right (row 1). RECIST category of each sample (row 2). PD ( ), SD ( ), PR ( ), CR ( ). "L" denotes long term responders. "X" indicates "not available". For rows 3 through 8, "X" denotes sample was not available, blank denotes sample was tested. N = number of specimens successfully assayed for the correlative studies denoted for that row. Germline BRCA testing from blood (row 3); p63/p73 QPCR (row 4); Gene expression profiled (row 5); p53 and PIK3CA mutation testing (row 6); HRD assay tested (row 7); BRCA1 methylation assayed (row 8). Rows 4 through 8 utilized tumor tissue samples and are listed in order of priority of testing based on sample availability.

Supplementary Methods

Data Analysis and Interpretation

The co-primary objectives of this study were to determine the objective response rate of single agent platinum and to determine the correlation of the p63/p73 expression ratio to the RR. For the primary endpoint analyses, all (cohort 1 and 2) patients were planned to be considered together. A Simon optimal 2-stage design with a maximal sample size of 82 patients and a target overall RR of 22% allowed for early closure for futility if <4 patients among the first 27 achieved a response. If at least 13 of the total 82 patients achieved a response, there is <5% probability (alpha 0.05) of accepting a true response rate of <10%, and 84% probability (beta 0.16) of rejecting a true response rate of 22%. In addition, with a target of 40% of patients testing positive for the p63/73 biomarker, the study had at least 91% power to detect a response rate of ≥40% in biomarker-positive patients and ≤10% in biomarker negative patients using a Fisher Exact test with a one-sided significance level of .05. Accrual overrun was allowed to ensure at least 27 and 55 eligible and evaluable patients enrolled in each stage, and to allow enrollment of any patients who signed consent prior to the 82nd eligible patient initiating treatment. Response was assess according to RECIST 1.0 as complete response (CR), partial response (PR), Progressive Disease (PD) or Stable Disease for >24 weeks (SD). Patients who developed early clinical or objective disease progression, regardless of duration of study treatment, prior to response evaluation were considered to have PD. Preplanned subgroup analysis for RR included cisplatin, carboplatin, all first line patients, and all second line patients. Progression free survival (PFS) was defined as the time from

enrollment until disease progression, death, or date of last patient contact. Patients were censored if they remained alive and free from disease progression, or at the time of initiation of subsequent therapy (surgery, radiation, or chemotherapy) if they did not experience a progression event. Overall survival (OS) was estimated from the time of enrollment until death from any cause. PFS and OS were estimated according to the method of Kaplan and Meier by means of the log-rank test. All enrolled patients were evaluable for safety. Adverse events were recorded at each visit and graded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. The study was monitored by the Dana Farber/Harvard Cancer Center Data Safety Monitoring Committee.

Determination of Triple Negative status

Determination of ER, PR and HER were determined locally and were not centrally reviewed. However, estrogen and progesterone receptors were <1% in all cases in accordance with current guidelines (except for 1 patient with ER 1-4%, PR 0%).

Somatic mutation detection of PIK3CA and p53

Sections (5μm) were obtained from formalin-fixed, paraffin-embedded tissue. Following pathological review, total nucleic acid was extracted from macrodissected tumor-enriched portions. Nucleic acid was further fragmented using mechanical shearing method. Library for targeted DNA next-generation sequencing was prepared using two rounds of nested multiplexing PCR reactions. Sequencing of the libraries was then performed on a MiSeq

desktop sequencer. Data was processed and analyzed using an in-house bioinformatics pipeline.

Gene Expression Array Analysis

Nucleic acid samples were further treated with DNase I (QIAGEN 79254) and RNA was purified according to manufacturer's suggestion. RNA samples were amplified NuGEN Ovation® FFPE WTA System followed by biotinylation with the NuGEN Encore Biotin Module. Gene expression profiling was then performed on Affymetrix GeneChip® Human Gene 1.0 ST Array at the Dana-Farber/Harvard Cancer Center array core facility. The complete gene expression array dataset will be available on the NCBI GEO database (accession submission in progress).

Taqman PCR for BRCA1, ΔNp63 and TAp73 analysis

cDNA was synthesized from total RNA using random hexamer primers and the SuperScript II system for RT-PCR (Invitrogen). Q-PCR analysis was carried out using iQTM SYBR Green mix (Bio-Rad). The b-actin transcript was used as an internal control for normalization of relative expression levels. Primers used for qPCR were designed across exons to avoid amplification of genomic DNA. Primer sequences for ΔNp63 were: GGAAAACAATGCCCAGACTCA and TGTTCAGGAGCCCCAGGTT), probe sequence is TTAGTGAGCCACAGTACAC. Primer sequences for TAp73: GATTCCAGCATGGACGTCTTC and GAACTGGGCCATGACAGATGT , probe sequence is ACCTGGAGGGCATGAC. Primer sequence for β-actin is CTTCCTGGGCATGGAGTCC and ACGTCACACTTCATGATGGAGTT, the probe

sequence is ATCCACGAAACTAC. Primer and probe for BRCA1 were purchased from Taqman, Hs01556193_m1.

Tumor subtype determination

Raw expression values in the form of CEL files were processed and normalized using RMA in the R Bioconductor package (data used in the analysis is available in GEO under accession GSEXXX). PAM50 basal signature was obtained from Parker et al., 2009. The meta-gene values basal subtype was calculated by the mean of the log2 expression of the up genes minus the mean of the log2 expression of the down genes. A sample was called PAM50 Basal-like when the meta-gene score for the Basal-like subtype was greater than any other subtype's meta-gene scores. Meta-gene values for the 7 TNBC subtypes defined in Lehmann et al. 2013 were created from the lists of up and down genes of Lehmann et al. 2013 Table S3B, which listed the top 20% of genes expressed in 50% of the samples for each cluster (subtype), and finding the mean of the up genes minus the mean of the down genes. Meta-gene values for the claudin-low subtype defined in Prat et al., 2010 were created by taking the mean of the positive genes minus the mean of the negative genes for each sample where the lists of negative and positive genes from their supplemental data file 2 that listed genes from their unpaired SAM analysis for claudin-low vs. the rest. When multiple probe sets were available for a particular gene in a subtype, the probe set with the maximum variance in the data set was chosen. The meta-gene scores for both the Lehmann et al., 2013 and the Prat et al., 2010 signatures were globally scaled to a zero-to-one range to make a uniform color scale for all meta-genes in the heatmap image of Fig 2.

HRD-LST and HRD-LOH assay analysis

Nucleic acid samples were treated with RNaseA (Promega, Wisconsin) and DNA was purified using Ampure beads (Beckman Coulter, Brea, CA). Genomic libraries were made using a Kapa HTP kit (Kapa Biosystems,Wilmington, NA) with 50 – 200 ng DNA input. The libraries were pooled and hybridized to a custom SureSelect XT2 library (Agilent Technologies, Santa Clara, CA). Libraries were sequenced on an Illumina HiSeq 2500 (Illumina, San Diego CA).

Sequence reads were trimmed for quality and aligned to BRCA1/2 or SNP target regions. BRCA1/2 variants were classified as deleterious or suspected deleterious based on previously described criteria (Beauet and Tsui, 1993). Sequence reads overlapping a SNP position were used to count the SNP alleles. The resulting read counts were used to reconstruct allele specific copy number at each SNP location using an algorithm described in Abkevich et al., 2012.

HRD score was defined as the number of LOH regions >15Mb, but shorter than the length of a whole chromosome (Abkevich et al., 2012). LST score is the number of break points between regions longer than 10 Mb after filtering out regions shorter than 3 Mb (Popova et al., 2012).

BRCA1 promoter methylation assay

50 – 300 ng DNA was subjected to bisulfite conversion using an EpiTect Bisulfite kit (Qiagen, Venlo, Netherlands). PCR primers specific for bisulfite converted DNA were designed to the promoter region in exon 1A of BRCA1 (5'-

TGAGAGGTTGTTGTTTAG-3' and 5'-CTAAAAAACCCCACAACCTATC-3'). Converted DNA was amplified, and then secondary PCR performed using Fludigm access array index primers (Fluidigm, South San Francisco, CA). The product was size fractionated (Pippin Prep, Sage Science, Beverly, MA) to isolate product between 250 – 300 bp, and sequenced using a MiSeq sequencer (Illumina, San Diego, CA).

Sequence reads were aligned to the BRCA1 promoter amplicon. Reads with 0 or 1 methylated CpG sites were considered unmethylated. Reads with 9 or 10 methylated CpG sites were considered methylated. All other reads were discarded. A methylation score was calculated as the proportion of methylated reads relative to the total number of reads that were either methylated or unmethylated.