LINC00355 regulates p27 KIP expression by binding to MENIN to induce proliferation in late-stage relapse breast cancer

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INTRODUCTION
Breast cancer is the most common cancer diagnosed among US women with ~276,480 estimated new cancer cases in 2020 and is the second leading cause of cancer deaths among women.1 The 5-year relative survival for localized disease is above 98%. However, this decreases significantly to 28% for distant metastasis2. Breast cancer relapse occurs within the first 3–5 years after initial treatment; however, those that relapse after five years are termed late-stage relapse (LSR) breast cancer.3 Trials of patients treated with adjuvant endocrine therapy for five years have shown a 50% reduction in the risk of relapsing, but the risk of relapse is 10% and as high as 41% depending on initial tumor node status and tumor grade.4–6 Additionally, there are limited treatment options for patients with LSR breast cancer and few of these patients cannot undergo chemotherapy owing to organ dysfunction or lower performance status as a result of widespread metastasis.7 Overall, more studies are needed to understand the benefits of long-term hormone therapy and the underlying molecular and genetic mechanisms promoting LSR.

RESULTS
Identification of long non-coding RNAs in late-stage relapse breast cancer

In order to identify which lncRNAs are associated with LSR, we analyzed transcriptome sequencing data from 72 early-stage patient samples from two preoperative neoadjuvant aromatase inhibitor treatment trials (ACOSOG-Z1031/NCT00265759 and NCT00084396532,33, termed “early-stage” and 24 LSR patient samples sequenced at Washington University34, termed “late-stage” (Supp. Table 1). Our analysis revealed 1,127 differentially expressed (DE) lncRNAs (FDR < 0.001, absolute log2FC > 2, Supp. Table 2) between the early-stage and late-stage relapse breast cancer patient samples (Fig. 1a, Supp. Fig. 1).
Additionally, due to the complex subtypes in breast cancer, we also evaluated the expression of the DE lncRNAs associated with LSR breast cancer in a panel of publicly available RNA sequence data from 31 nonmalignant and malignant ER+ breast cancer cell lines. We were able to identify 68 deregulated lncRNAs associated with LSR and highly expressed in cell lines with Fragments Per Kilobase of transcript per Million mapped reads (FPKM) > 5 in at least two breast cancer cell lines (Fig. 1a, b, and Supp. Fig. 2). We identified several known lncRNAs previously identified to promote breast cancer including LINCO2582, CASC9, PDXDC2P, NNT-AS1, AP000439.3, and lncRNAs found to be expressed in other cancer types (Fig. 1b). Next, we...

Fig. 1 Identification of long non-coding RNAs driving late-stage relapse breast cancer. a Schematic of the pipeline used to identify lncRNAs driving late-stage relapse breast cancer. b Heatmap showing the mean expression of patient samples and cell lines for the lncRNAs in late-stage relapse breast cancer. Side bar represent the log fold-change. c Top upregulated gene sets found to be correlated with late-stage relapse as determined by GSEA. d Top GSEA enrichment up-regulated gene sets.
performed a gene set enrichment analysis (GSEA) to evaluate the association of the identified lncRNA genes with cancer gene signatures (Fig. 1c). Our analysis found enrichment of multiple gene sets that were associated with breast cancer and estrogen receptor including MORF_ESR1 (nominal \( p = 0.001 \)) and GSEA sets indicating roles in FIRESTEIN_PROLIFERATION (nominal \( p = 0.001 \)), and TAVAZOIE_METASTASIS (nominal \( p = 0.001 \), Fig. 1c and d). Using the unbiased approach of transcriptome sequencing and unique patient samples from the two clinical trials of preoperative neoadjuvant aromatase inhibitor treated and late-stage relapse patients, we identified 68 lncRNAs that we believe are associated with late-stage relapse breast cancer signaling and phenotypes.

**LINC00355 is the most up-regulated lncRNA in LSR breast cancer**

We focused on characterizing the top upregulated lncRNA LINC00355 (NR_145420.1, Fold change \( = 7.21, p = 2.7e-13 \)), in LSR breast cancer when comparing early-stage patient samples (Figs. 1b and 2a). LINC00355 is a previously annotated 1878 nucleotide long lncRNA first identified as an oncogene in bladder cancer\(^4\). Similar to our early-stage patient cohort, LINC00355 was downregulated in 480 early-stage breast cancer samples from The Cancer Genome Atlas (TCGA) from multiple breast cancer subtypes (Fig. 2a): triple negative (\( n = 72 \)), mean FPKM = 0.151, HER2\(^+\) (\( n = 29 \)), mean FPKM = 0.215, luminal A (\( n = 298 \)), mean FPKM = 0.237, and luminal B (\( n = 81 \)), mean FPKM = 0.299, and normal tissue (\( n = 77 \)), mean FPKM = 0.174. LINC00355 was only highly expressed in the late-stage relapse breast cancer patient samples (mean FPKM = 28.092, Fig. 2a). Additionally, we detected increased LINC00355 expression in publicly available RNA-Seq data\(^{35} \) of malignant breast cell lines compared to non-malignant cell lines (\( p = 0.047 \), Fig. 2b, Supp. Fig. 2). To better assess LINC00355 cell type-specific expression and show expression levels are not due to contamination of using bulk patient tissue from RNA-Seq, we also assessed its expression in single-cell RNA-Seq data from breast tissues downloaded from Wu et al.\(^{44} \). We detected very low, 0.90% or less, of cells expressing LINC00355, which was restricted to cancer epithelial cells (Supp. Fig. 3a–c).
contrast, XIST lncRNA is expressed in 35% of cancer epithelial cells and is over 50% expressed in myeloid, cancer-associated fibroblast, and endothelial cells (Supp. Fig. 3a, d, and e). We further assessed expression of LINC00355 in normal tissue using RNA-Seq data from Genotype-Tissue Expression (GTEx) and see slightly higher expression in whole blood, omentum tissue, subcutaneous adipose tissue, and higher expression in EBV-transformed lymphocytes and testis compared to breast mammary tissue (Supp. Fig. 4). Overall, LINC00355 is more highly expressed in LSR compared to early-stage breast cancer patient samples, and in malignant compared to non-malignant cell lines and normal tissues.

Next, we assessed LINC00355 expression in a panel of breast cancer cell lines by quantitative PCR (qPCR) that included both luminal A and luminal B subtype cell lines. LINC00355 is expressed greater than tenfold in breast cancer cell lines compared to the non-tumorigenic cell line MCF10-A (Fig. 2c). Since subcellular localization may provide insight into lncRNA putative functions, we fractionated estrogen receptor positive (ER $\alpha$) T47D cells, which had highest endogenous levels of LINC00355, and found that LINC00355 is pre-dominantly expressed in the nucleus (Fig. 2d). In summary, we show that LINC00355 previously found to have oncogenic potential is also highly expressed in breast cancer patient samples and cell lines. Specifically, we were able to determine LINC00355 is selectively highly expressed in LSR breast cancer.

**LINC00355 expression promotes proliferation and invasion in malignant breast cancer cell lines**

In order to gain a better understanding of the role LINC00355 plays in LSR breast cancer, we transiently silenced its expression with two siRNAs (siRNA1 and siRNA2, Supp. Table 3) in malignant breast cancer cell lines (T47D and CAMA-1) which have high endogenous LINC00355 expression (Fig. 3a). As LINC00355 was previously found to be associated with cellular proliferation45–47, we conducted EdU (5-ethynyl-2$'$-deoxyuridine) proliferation assays using flow cytometry in T47D and CAMA-1 cells with at least 50% silenced LINC00355 expression. We observed a significant decrease of proliferation in T47D cells lines with silenced LINC00355 compared to negative control scrambled siRNAs (siRNA1 $p = 4.99e-05$, siRNA2 $p = 0.006$, two-tailed paired t-test; Fig. 3b). Next, we detected a significant decrease in cell viability for 3 days post 72-h LINC00355 knockdown compared to control siRNAs by Alamar Blue Assay (Day 5, siRNA1 $p = 0.0007$, siRNA2 $p = 0.008$; Day 6, siRNA1 $p = 0.02$, siRNA2 $p = 0.02$, two-tailed paired t test; Supp. Fig. 5a). In addition, we assessed DNA content in T47D cells with siRNAs targeting LINC00355 and detected a decrease in the S phase of cell cycle (siRNA1 $p = 0.002$, siRNA2 $p = 0.0003$, two-
tailed paired t test, Fig. 3c). A significant decrease in proliferation (siRNA1 p = 0.0002, siRNA2 p = 0.001, two-tailed paired t test, Fig. 3b) and S phase of cell cycle (siRNA1 p = 0.007, siRNA2 p = 0.03, two-tailed paired t test, Fig. 3c) was similarly seen in the second breast cancer cell line CAMA-1.

Due to the importance of the cyclin-dependent kinase inhibitor p27Kip as one of the key regulators of progression from G1 to S phase in cell cycle and its frequent decreased concentration in human malignancies, we confirmed protein expression of p27Kip in T47D and CAMA-1 cell lines. Silencing LINC00355 increased protein levels of p27Kip in both cell lines (T47D, siRNA1 fold = 1.45, siRNA2 fold 1.79; CAMA-1 siRNA1 fold = 1.97, siRNA2 fold = 2.49, Fig. 3d). There was also more than 1.5-fold increase of CDKN1B mRNA, which encodes transcription for p27Kip in LINC00355 silenced cell lines (Fig. 3e).

Next, we evaluated whether LINC00355 promotes cellular invasion by seeding Matrigel-coated transwells in a modified Boyden chamber assay. We found decreased cellular invasion when LINC00355 is silenced in T47D cells (siRNA1 p = 2.98e-06, siRNA2 p = 4.56e-05, two-tailed paired t test, Fig. 3f and g), and CAMA-1 cells (siRNA1 p = 0.01, siRNA2 p = 0.006, two-tailed paired t test, Fig. 3f, h) compared to negative controls.

Further, we developed a MCF10A stable cell line with more than 11,500-fold LINC00355 overexpression compared to empty vector cells that had negligible LINC00355 expression (Fig. 3i). We similarly detect an increase in proliferation in overexpression cell lines compared to empty vector by EdU assay (p = 0.002, two-tailed paired t-test, Fig. 3j), Alamar Blue assay (Day 5, p = 0.0006; Day 6, p = 0.00007, two-tailed paired t test; Supp. Fig. 5b), and S phase in cell cycle by assessing DNA content (p = 2.33 e-05, two-tailed paired t test, Fig. 3k). There was also more than 1.5-fold decrease of p27Kip protein levels (Fig. 3l) and CDKN1B mRNA (p = 0.0019, two-tailed paired t test, Fig. 3m) in LINC00355 overexpressed cell lines. Finally, we found increased cellular invasion when LINC00355 is overexpressed when compared to empty vector (p = 0.003, two-tailed paired t test, Fig. 3n and o) by modified Boyden chamber assay. These data indicate that LINC00355 induces proliferation and invasion in cell lines possibly through the regulation of p27Kip.

**LINC00355 expression promotes proliferation and invasion in long-term estrogen deprived cell lines**

To further assess the metastatic behavior associated LSR breast cancer, we determined if LINC00355 also induces aggressive phenotypes in a late-stage relapse setting by using two ER+ cell lines T47D and MCF7 that were deprived of estrogen for longer than 3 years, termed long-term estrogen deprived (LTED) cells. The LTED model has been developed to recapitulate the acquired resistance to aromatase inhibitors. However, the two LTED models have independent mechanisms of resistance. The T47D LTED model lacks ESR1 locus amplification whereas the MCF7 LTED model has ESR1 amplification leading to increased ERα protein expression. Wild type T47D cell lines when deprived of estrogen, termed T47D LTED, show a loss of ERα protein (Fold = 0.21) compared to its wild type counterpart (Fig. 4a). In contrast, MCF7 cell lines when deprived of estrogen, termed MCF7 LTED, show an amplified ERα protein level (Fold = 1.88) compared to its wild-type counterpart (Fig. 4a). LINC00355 expression was decreased by 65-fold in T47D LTED when compared to wild type T47D cells as measured by qPCR, and conversely there was a 60-fold increase of LINC00355 expression in and MCF7 LTED cell lines compared to wild type MCF7 (Fig. 4b). These results demonstrate an association of LINC00355 with ERα.

Due to the increased levels of LINC00355 expression in MCF7 LTED cells, we assessed whether LINC00355 promoted aggressiveness of MCF7 LTED cells. Greater than 70% silencing of LINC00355 in MCF7 LTED cell lines (Fig. 4c) resulted in a significant decrease of cellular proliferation (siRNA1 p = 0.0003, siRNA2 p = 0.02, two-tailed paired t test, Fig. 4d), Alamar Blue assay (Day 5, siRNA1 p = 0.015, siRNA2 p = 0.038; Day 6, siRNA1 p = 0.03, siRNA2 p = 0.0008, two-tailed paired t test; Supp. Fig. 5c), and S phase of cell cycle (siRNA1 p = 0.0006, siRNA2 p = 0.013, two-tailed paired t test, Fig. 4e) in MCF7 LTED cells compared to the negative control scrambled siRNAs. Additionally, we saw more than twofold increase in p27Kip protein levels (Fig. 4f) and more than 1.5-fold increase in CDKN1B mRNA expression (Fig. 4g) in the MCF7 LTED cell line with silenced LINC00355. Further, we evaluated cellular invasion in MCF7 LTED cells lacking expression of LINC00355. We observed a significant decrease of invasion (siRNA1 p = 0.0002, siRNA2 p = 0.004, two-tailed paired t test) in MCF7 LTED cells with siRNAs targeting LINC00355 as compared to cells treated with negative control scrambled siRNA (Fig. 4h and i). Last, silenced LINC00355 expression in MCF7 LTED cells then re-introduction of LINC00355 expression, restores invasiveness of MCF7 LTED cells compared to silenced cells (siRNA p = 0.0118, siRNA plus overexpression p = 0.012, Supp. Fig. 6).

We overexpressed LINC00355 in the T47D LTED cell lines more than 15.5-fold compared to empty vector cells that had negligible LINC00355 expression (Fig. 4j). We detect an increase in proliferation in T47D LTED overexpressed cells compared to empty vector by EdU assay (p = 0.019, two-tailed paired t test, Fig. 4k) and S phase in cell cycle by assessing DNA content (p = 0.007, two-tailed paired t test, Fig. 4l). There was also more than 60% decrease of CDKN1B mRNA (Fig. 4m) in LINC00355 T47D LTED overexpressed cell lines. We lastly found an increase in cellular invasion in T47D LTED overexpressed cells when compared to empty vector (p = 0.0003, two-tailed paired t test, Fig. 4n and o).

Taken together, LINC00355 induces cellular proliferation and invasion in both malignant breast cancer cell lines and long-term estrogen-deprived cell lines that mimic late-stage relapse.

**LINC00355 binds to MENIN to regulate CDKN1B expression**

Since LINC00355 is localized in the nucleus, increases cellular proliferation, and decreases p27Kip protein levels, we hypothesize that LINC00355 may transcriptionally regulate CDKN1B, the gene that encodes for p27Kip protein. Previously it was shown that MENIN, encoded by MEN1 (multiple endocrine neoplasia 1), is required for its transcriptional activation of p27Kip by increasing histone H3 lysine 4 methylation (H3K4me3) at the promoter of CDKN1B. Thus, we assessed if LINC00355 may directly bind to MENIN. We first assessed MENIN and ERα protein levels to show that protein levels did not change upon silencing of LINC00355 in both the highly expressed primary cell line T47D and the LSR model MCF7 LTED cell line (Fig. 5a and b). We next conducted an RNA immunoprecipitation coupled with qPCR (RIP-qPCR) with qPCR in MENIN in the T47D cell line. Indeed, we detected a more than 21-fold enrichment of LINC00355 by RIP-qPCR compared to IgG control and do not see enrichment of XIST RNA, as a negative control of MENIN binding (Fig. 5c). We also show LINC00355 increased MENIN binding in MCF7 LTED cells (Fold change = 10.4 in MENIN compared to IgG; Fig. 5d). To orthogonally validate these findings, we conducted a RNA pull-down assay utilizing a S′ Bromo-UTP full-length LINC00355 sense labeled probe and a negative control antisense probe to pull-down proteins that may be bound to LINC00355. We found that the LINC00355 sense probe was bound to MENIN protein compared with the control probe (Fig. 5e) by Western blot of nuclear lysates. To identify the regions of LINC00355 that bind to MENIN, we conducted cross-linking immunoprecipitation and qPCR in MCF10A cells transfected with an empty vector and LINC00355 full length (overexpression) using eight primers tiling LINC00355 (Supp. Fig. 7a). We detected Primer 4 and Primer 5 tiling 627-1023 nucleotides had higher fold enrichment (Fold >3 and Fold >5, respectively) of binding to MENIN (Supp. Fig. 7b). We re-validated our previous findings that full-length LINC00355 binds to MENIN around 789 nucleotides.
These orthogonal methods support LINC00355 binding to MENIN protein. To determine the effects of LINC00355-MENIN binding at the promoter of CDKN1B to regulate the expression of p27Kip protein, we silenced LINC00355 to show a significant increase in MENIN occupancy at the CDKN1B promoter in T47D cells (siRNA1 $p = 7.33 \times 10^{-07}$, siRNA2 $p = 0.0006$, two-tailed paired $t$-test, Fig. 5f) as compared to IgG control. We also observed an increase in H3K4me3 at the promoter of CDKN1B with silenced LINC00355 (siRNA1 $p = 0.004$, siRNA2 $p = 0.01$, two-tailed paired $t$-test, Fig. 5g). The increase in MENIN and H3K4me3 occupancy was further detected in MCF7 LTED cells with silenced LINC00355 in chromatin immunoprecipitation (ChIP) with MENIN (siRNA1 $p = 0.03$, siRNA2 $p = 0.001$) and ChIP with H3K4me3 (siRNA1 $p = 0.0001$, siRNA2 $p = 0.002$, two-tailed paired $t$-test, Fig. 5h and i). Decreased MENIN and H3K4me3 occupancy was detected in MCF10A cells with overexpressed LINC00355 (MENIN; $p = 0.001$, and H3K4me3; $p = 0.002$, two-tailed paired $t$-test, Fig. 5j and k).
H3K4me3; p = 0.01, two-tailed paired t test, Fig. 5j and k). We additionally identified two other genes regulated by MENIN including AGR3 and FOXA1, that are also shown to be regulated by LINC00355. We show a significant decrease of expression of CDKN1B (p = 0.04), AGR3 (p = 0.005) and FOXA1 (p = 0.02) using a siRNA targeting MEN1 (Supp. Fig. 8). We again validate a significant increase of CDKN1B expression (p = 0.02) with silenced LINC00355 expression and additionally detected an increase of AGR3 (p = 0.02) and FOXA1 (p = 0.004) genes (Supp. Fig. 8). Taken together, we provide evidence that LINC00355 functions by binding to the MENIN protein, which decreases its occupancy at the promoter of CDKN1B, decreasing protein levels of p27Kip, increasing proliferation, and cellular invasion in late-stage relapse breast cancer models (Fig. 6).

DISCUSSION

Although most relapses occur during the first 5 years after breast cancer diagnosis about 30% of ER+ positive breast cancer patients relapse more than 5 years and up to 10 years after their 5-year endocrine therapy treatment. Since distant metastases are responsible for the majority of breast cancer deaths, finding ways to reduce the risk of distant metastases associated with late relapse is critical in improving survival rates from the disease. Our
study used ER + early-stage (II and III) tumor tissues isolated from two neoadjuvant aromatase inhibitor (AI) therapy trials. This unique set of tumor samples allowed us to assess IncRNA expression in a preoperative AI treatment setting that is currently being assessed as a new treatment option. Additionally, we incorporated a unique very limited cohort of LSR patients. Thus, our study provides a systematic transcriptome analysis of IncRNAs expression in early-stage and late-stage relapse breast cancer patients to detect deregulated IncRNAs significantly altered in LSR breast cancer.

Through our analysis of IncRNAs in LSR breast cancer, we prioritized the most up-regulated IncRNA, LINC00355. Notably, LINC00355 was previously reported to promote proliferation in multiple cancer types including bladder cancer, colorectal cancer, prostate cancer, lung adenocarcinoma, and head and neck squamous cell carcinoma,43,45,47,57–59 and induce epithelial to mesenchymal transformation, and serve as a diagnostic biomarker in colon cancer45,47,57–60; however, it has not yet been identified in breast cancer and identified here specifically in ER + late-stage relapse breast cancer. High nuclear enrichment of LINC00355 was also previously detected in bladder cancer43, which we confirmed in breast cancer. This led us to hypothesize that LINC00355 may also play a role in inducing proliferation in LSR breast cancer through transcriptional regulation of key genes. Interestingly, we found that LINC00355 induced proliferation and cellular invasion in malignant breast cancer cell lines. We also utilized MCF7 and T47D long-term estrogen receptor deprived cells lines that mimic late-stage relapse patient samples as they have been deprived of estrogen for longer than three years and have previously been shown to provide an in vitro parallel of patients treated with aromatase inhibitor having post-treatment loss or amplification of estrogen receptor61. When using the LTED cell lines, we showed LINC00355 induced proliferation and cellular invasion. Because the MCF7 LTED cell line has amplified estrogen receptor protein, we have also provided evidence that LINC00355 may have a role in an ER + amplified setting, a hypothesis that needs further investigation.

As LINC00355 was able to induce proliferation, we decided to investigate a key cell signaling regulator, p27Kip, p27Kip signaling is highly complex and has multiple modes of both transcriptional and post-transcriptional regulation62–64. Indeed, we found that our breast cancer cell lines with high levels of LINC00355 also had decreased expression p27Kip. Moreover, we determined that LINC00355 expression is also inversely correlated with CDKN1B gene expression. This indicated that LINC00355 might transcriptionally regulate CDKN1B to alter cellular proliferation. We hypothesized that LINC00355 may interact with a negative regulator of cell cycle separating the protein from its native target, thus causing an uncontrolled increase in cellular proliferation. There are numerous well-known examples of IncRNAs that function as negative regulators or decoys including: CISAL63, ROR64, PANDA65, and MEG366. Previous research determined that the CDKN1B gene is transcriptionally negatively regulated by MENIN in ER + breast cancer67 through epigenetic modifications and maintenance of transcription at multiple loci for cell cycle regulators65,67,68. Given this, we focused our study on MENIN to determine if LINC00355 interacts with MENIN sequestering it from the promoter of CDKN1B gene leading to decreased p27Kip protooncogene levels and disruption of the cell cycle control and increasing proliferation. This study indicates a IncRNA to bind to MENIN which provides evidence of the important functional regulation of IncRNAs. An important unanswered question is the stoichiometry of LINC00355 in regulating the association of MENIN with the CDKN1B locus. Further, we highlight the importance of IncRNA expression in LSR breast cancer that may be used as novel therapies in the future. In conclusion, our study provides a landscape of IncRNAs in LSR and provides key evidence of their contribution to LSR breast cancer.

METHODS
Clinical samples and cell lines
The primary breast cancer samples for this study were accrued from two neoadjuvant endocrine therapy trials32,33. RNA-Seq data accrued from two neoadjuvant aromatase inhibitor therapy trials (ACOSOG-Z1031, NCT00084396, and NCT00265759)32–34 are available via the dbGAP database (with accession phs000472). The studies were supported by the Clinical Trials Support Unit and approved by the institutional review boards of all participating institutions; all patients provided signed informed consent. Twenty-four late-stage relapse patient samples were enrolled under a banking protocol approved by the Washington University School of Medicine Institutional Review Board (approval number 201102244, Supp. Table 1), their tumors were resected for RNA sequencing. PolyA RNA was isolated and created into Illumina TruSeq libraries run on Illumina HiSeq 2000 platform (accession GSE189389). The RNA-Seq gene expression data (FPKM) and the clinical data of TCGA BRCA samples were downloaded from the TCGA data portal. RNA-Seq data of the 32 breast cancer cell lines were obtained from the NCBII Gene Expression Omnibus (accession GSE482163). All cell lines with “unknown” subtype were removed and a representative number of cell lines from each subtype were retained.

Sequence alignment and transcript quantification
All sequencing reads from both patient and cell lines data were aligned to the human reference genome hg19 Ensembl release 75 using TopHat version v2.0.869. TopHat was run according to an in-house pipeline at The McDonnell Genome Institute (bowtie-version = 2.1.0, library-type fr-unstranded, mate-inner-dist 254, and mate-std-dev 50). The remaining TopHat parameters were left to their default values. For accurate alignment, transcriptome index file (transcript sequences) was provided to guide the alignment. Raw read counts for transcripts were generated using featureCounts version v1.4.6-p369 and were used to compute transcript expression levels as normalized in FPKM (Fragments Per Kilobase of transcript per Million mapped reads) format.

RNA-sequencing data analysis
Differential expression analysis was performed between early-stage and late-stage relapse samples using the negative binomial generalized log-linear model and likelihood ratio tests capabilities of edgeR version v3.8.670 using the raw read counts. To correct for batch effect, RUVSeq R package version v1.0.071 was used with a list of negative control genes72. To enhance our confidence in the differentially expressed genes we discovered, locally expressed transcripts were removed and only transcripts with expression > 1 FPKM in at least 50% of samples in either group were retained for downstream analysis. All transcripts with FDR < 0.001 and absolute log fold change ≥ 2 were considered differentially expressed transcripts. TCGA subtypes and cell lines expression difference significance was calculated specifically for LINC00355 using the nonparametric Wilcoxon rank-sum test.

Gene enrichment analysis
To identify gene signatures that are associated with the identified differentially expressed genes, a gene set enrichment analysis (GSEA)73 was performed on the list of up and down regulated IncRNAs with 1000 gene set permutations using Sigma2Noise metric. Significantly enriched gene sets were determined by false discovery rate (FDR) adjusted P ≤ 0.1 and normalized enrichment score (NES) ≥ 1.5.

Cell Culture
All breast cancer cell lines were a kind gift from Dr. Matthew Ellis and Jieya Shao at Washington University in St. Louis. T47D, MCF7, HCC1428, BT483, ZR75B, and HCC1500 cell lines were grown in RPMI 1640 media (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (Sigma, St. Louis, MO), 1% HEPES (Sigma), 0.5% glucose (Sigma), 1% sodium pyruvate (Sigma), 1% L-glutamine (Sigma), and 1% penicillin/streptomycin (Sigma, Carlsbad, CA). T47D LTED and MCF7 LTED cells were grown in phenol red free RPMI 1640 media (Invitrogen), 10% Charcoal stripped bovine serum (Sigma), 1% HEPES, 0.5% glucose, 1% sodium pyruvate, 1% L-glutamine, and 1% penicillin/streptomycin. CAMA-1 and BT-474 cells were grown in DMEM (Invitrogen), 10% fetal bovine serum, and 1% penicillin/streptomycin. MDA175 were grown in DMEM F12 (Invitrogen), 10% fetal bovine serum, and normalized for downstream analysis. All transcripts with FDR < 0.001 and absolute log fold change ≥ 2 were considered differentially expressed transcripts. TCGA subtypes and cell lines expression difference significance was calculated specifically for LINC00355 using the nonparametric Wilcoxon rank-sum test.

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and 1% penicillin/streptomycin. MCF10A cells were grown in DMEM F12 (Invitrogen), 10% horse serum serum, 0.5 µg/mL hydrocortisone, 10 µg/mL insulin, 20 ng/mL EGF and 1% penicillin/streptomycin. We silenced expression of LINC00355 using custom silencer select RNAs (siRNAs) targeting LINC00355 or Silencer Select Negative Control No.1 siRNA (Thermofisher, Austin, TX). siRNA sequences are listed in Supplementary Table 3.

Full-length LINC00355 transcript was PCR amplified from T47D cells and cloned into the pCFG5-IEG vector (a kind gift from Dr. Ron Bose, Washington University). Full-length LINC00355 inserts were confirmed with Sanger sequencing at GeneWiz. Retroviral infection of cells was performed according to Kauri et al. Briefly, the amphotropic phoenix cell line was transfected with 10 µg of pCFGS-LINC00355 or empty vector control by calcium phosphate precipitation and incubated for 24-h. Viral supernatants were harvested after an additional 24-h incubation. Virus was added to cells seeded in six-well dishes in the presence of 8 µg/mL polybrene (Sigma), centrifuged at 300 Å~ for 90 min, and fresh media was added to the plate. After 14 days of Zeocin (Invitrogen) selection cells were used for assays. MCF10A cells or T47D LTED cells that had low endogenous expression of LINC00355 were infected with virus expressing LINC00355 or empty vector for 48 h and selected with 100 µg/mL Zeocin.

Nuclear cytoplasmic isolations
Nuclear and cytoplasmatic isolations were conducted using the PARIS Kit (Thermo Fisher, Waltham, MA) following the manufacturer’s protocol. Total RNA was collected as described below. Nuclear and cytoplasmatic isolations were calculated by normalizing respective gene to total RNA expression.

Quantitative real-time PCR
Total RNA was isolated for each breast cancer cell line using NucleoSpin RNA plus with DNA removal column (Macherey-Nagel, Duren). Total RNA was then transcribed to cDNA with SuperScript III First-strand cDNA system (Invitrogen) to verify expression of genes and verify knockdown efficiencies using Fast SyberGreen Master Mix (Invitrogen) as per the manufacturer’s protocol. Primer sequences are available in Supplementary Table 3.

Modified Boyden chamber assay
Cell lines were seeded at 350,000 cells in a six-well dish. The next day cells were transfected at 50 nM with two independent custom designed siRNAs or a negative scramble control (Supplementary Table 3) with Lipofectamine RNAiMax (Invitrogen) for 72-h or 2 µg pCFGS-LINC00355 or empty vector control with Lipofectamine 3000 (Invitrogen) for 72-h. Cells were then harvested and re-seeded in complete media at 200,000 cells on an 8.0 µm permeable membrane support transwell (Corning, Corning, NY) pre-coated with 200 µg/mL Matrigel (Corning) in 24-well plates creating a modified Boyden chamber assay. A serum gradient was established with cells plated in serum-free media added to the bottom of the well. Cells were allowed to invade overnight and then fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Next, nuclei were stained with DAPI (Sigma) (1 µg/µL). A cotton swab was used to remove non-invading cells from the top of the membrane. Invaded DAPI-stained cells were then imaged with Q-Capture Pro software on an Olympus IX70 microscope, quantified using ImageJ software (http://imagej.nih.gov/ij/), and statistical significance was determined by a student’s t test. Five or more images were taken per transwell membrane at 20× magnification. Assays were repeated two to three times.

Proliferation assay
Cell lines were seeded at 350,000 cells in a six-well dish. The next day cells were transfected at 50 nM with siRNAs targeting LINC00355 or negative control pCFGS-LINC00355 empty vector control as described above in Modified Boyden chamber assay section. Seventy-two hours later cells were pulsed with Edu (5-ethyl-2′-deoxyuridine) (Thermo Fisher, Carsbad, CA, cat# C10420) for 3 h and harvested by trypsinization. Cells were then fixed, permeabilized, and washed following manufacturer’s instructions. Cells were stained for DNA content with FxCycle Violet (Thermo). Analysis of Edu and cell cycle was assessed by measuring DNA content on a flow cytometry machine (FACSscan, Becton Dickinson) at the Siteman Cancer Center Flow Cytometry Core. We collected a minimum of 25,000 cells per sample in triplicate. FlowJo Version10 (Becton Dickinson) was used to analyze data.

Western blot
Protein was collected by plating 300,00–350,000 representative cancer cells in a six-well dish. Cells were transfected as described above. Cells were then lysed with Tris Lysis buffer (50 mM Tris-HCl, 1% Triton X-100, 131 mM NaCl, 1 mM sodium orthovanadate, 10 mM Na2P04, 10 mM NaF, 1 mM EDTA, and proteasome inhibitor), run on agarose gel and transferred to nitrocellulose membranes. Blots were then probed overnight at four degrees with respective antibodies. All antibodies and concentrations are listed in Supplementary Table 4. Blots were then washed with TBST buffer and then applied with secondary goat anti-rabbit HRP linked or goat anti-mouse HRP-linked antibodies (Thermo Fisher, Waltham, MA). Lastly, blots were washed, visualized with Clarity Western ECL Substrate (BioRad, Hercules, CA) and imaged using the ChemiDoc XRS + System (BioRad). Blots were derived from the same experiment. Raw western blots are shown in Supplementary Fig. 9 were processed in parallel and derived from the same experiment.

RNA immunoprecipitation (RIP)
RIP coupled to qPCR assays were conducted by isolating nuclear lysates from ten million T47D or MCF7 LTED cells following the NER-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher). Nuclear lysates were then incubated overnight rotating with 5 µg of Anti-Menin antibody or IgG antibody isotype control in RIPA wash buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM MgCl2, 1% NP40, 0.5% Na-Deoxycholate, 0.05% SDS, 1 mM EDTA) and SUPERase-in RNase inhibitor (Invitrogen). The next day 50 µL of Invitrogen Dynabeads Protein G were added to the antibody mixture/lysatse and rotated for 1–2 h at 4 °C. Next, beads were washed six times with RIPA wash buffer using a magnetic bead separator. Protein was then digested with Proteinase K buffer (RIPA buffer, 10% SDS, 10 mg/ml Proteinase K), at 55 °C for 30 min shaking. RNA was phenolchloroform: isooval alcohol extracted following the general protocol (Thermo Fisher). Last, gDNA was removed from RNA using Articzymes Heat and Run qDNA removal kit following the manufacturer’s protocol (Tromso, Norway). cDNA was made using SuperScript III First strand cDNA system as indicated above and qPCR was run with Fast SyberGreen MasterMix and indicated primers (Supplementary Table 3). Fold enrichment of qPCR results were calculated following Sigma-Aldrich Data Analysis Calculation Shell by comparing non-specific control IgG antibody raw CTS to MENIN or H3K4me3 RNA binding protein CT normalized against 1% input.

BrU-labeled RNA pull-down
Full-length LINC00355 RNA probes were made using the Promega Riboprobe in vitro transcription kit from 2.5 µg of linearized DNA in the pCDS vector (Madison, WI). Control antisense probes were made by in vitro transcription from the SP6 promoter. LINC00355 RNA pull-down experiments were performed in MCF7 LTED nuclear lysates following the RiboTrap Kit manufacturer’s protocol (MBL, Woburn, MA).

Chromatin immunoprecipitation (ChIP)
ChIP coupled to qPCR assays were conducted by first sonication five million cells in SDS lysis buffer (1% SDS, 500 mM EDTA, 50 mM Tris-HCl pH4). Next, immunoprecipitation with 5 µg of IgG, MENIN, or H3K4me3 antibodies was done by incubating sonicated lystate with indicated antibody in ChIP Dilution Buffer (0.01% SDS, 1.10% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH8, 167 mM NaCl), and 1X Halt Protease and Phosphatase inhibitors overnight with rotation at 4°. The next day 50ul of Dynabeads Protein G (Invitrogen) were added to the antibody lystate mixture and rotated for 1 h. Bead/lystate mixture was then washed once with Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8, 167 mM NaCl), and 1X Halt Protease and Phosphatase inhibitors overnight with rotation at 4°. The next day 50ul of Dynabeads Protein G (Invitrogen) were added to the antibody lystate mixture and rotated for 1 h. Bead/lystate mixture was then washed once with Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8, 167 mM NaCl), then High Salt Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8, 500 mM NaCl), Lithium Chloride (150 mM Lithium chloride, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH8), and finally two washes with Tris-HCl EDTA Buffer (1% SDS, 1% Triton X-100, 1 mM EDTA). DNA was eluted by incubating beads for 30 min at room temperature with SDS Elution Buffer (1% SDS, 0.1 M sodium bicarbonate) followed by 1.25 M NaCl and 2.5 mg/ml RNase A at 95° for 15 min shaking followed by addition of Proteinase K Buffer (1 µL 10mg/ml Proteinase K, 5 µL 50ul EDTA, 10 µL 1 M Tris pH7.5) shaking at 60° for 15 min. DNA was then isolated using phenol:chloroform:isoval alcohol extraction following the general protocol as mentioned above. DNA was diluted by five and used for qPCR. The % input calculation was determined by comparing CT values from input DNA

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npj Breast Cancer (2022) 49
and ChiP DNA for the CDKN1B target promoter region using the following equation:

\[ \%\text{Input} = \% \text{of starting input fraction} \times 2^{(\text{CT(input)} - \text{CT(ChiP)})} \]

Prior sequences are available in Supplementary Table 3.

**Single-cell RNA sequencing analysis**

Breast single-cell data from 26 patients using the 10x chromium platform was downloaded from Gene Expression Omnibus (GSE176078) from Wu et al.\(^7\) Seurat v4.1.0 (Butler et al.;\(^7\)\) Hafemeister and Satija\(^7\)\) was used for all subsequent analyses. We applied a series of quality filters to the data to remove barcodes which fell into any one of the following categories recommended by Seurat: transcript counts below 300; total genes expressed below 200 and above 10,000; UMI count below 1000 or above 10,000; mitochondrial gene expression larger than 10%. The Seurat object was filtered to data to remove barcodes which fell into any one of the following categories.

**References**


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AUTHOR CONTRIBUTIONS

COMPETING INTERESTS
J.S.R-F. reports receiving personal/consultancy fees from Goldman Sachs, REPARE Therapeutics and Paige.AI, membership of the scientific advisory boards of VolitionRx, REPARE Therapeutics, Paige.AI and Personalis, membership of the Board of Directors of Grupo Oncoclinicas, and ad hoc membership of the scientific advisory boards of Roche Tissue Diagnostics, Ventana Medical Systems, Novartis, Genentech and Invicro, outside the scope of this study.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41523-022-00412-2.