Published in final edited form as:

Nat Struct Mol Biol. 2016 June ; 23(6): 522–530. doi:10.1038/nsmb.3211.

Long noncoding RNA LINP1 regulates double strand DNA break repair in triple negative breast cancer

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Abstract

Long noncoding RNAs (lncRNAs), which are transcripts that are larger than 200 nucleotides but do not appear to have protein-coding potential, play critical roles during tumorigenesis by functioning as scaffolds to regulate protein-protein, protein-DNA or protein-RNA interactions. Using a clinically guided genetic screening approach, we identified (lncRNA in Non-homologous end joining [NHEJ] pathway 1) as a lncRNA that is overexpressed in human triple-negative breast cancer. We found that LINP1 enhances double-strand DNA break repair by serving as a scaffold that links Ku80 and DNA-PKcs, thereby coordinating the NHEJ pathway. Importantly, blocking LINP1, which is regulated by the p53 and epidermal growth factor receptor (EGFR) signaling, increases sensitivity of tumor cell response to radiotherapy in breast cancer.

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AUTHOR CONTRIBUTIONS

Y.Z., C.V.D and L.Z conceptualized and designed the experiments. Y.Z., Q.H., L.F., S.W. and C.L. performed the molecular and cellular biology experiments. Z.H., Y.F., Z.T. and J.Y performed bioinformatics analysis. Y.Z., Z.H., J.Y., X.H., J.L.T., Y.F., Q.H., and K.M. analyzed and interpreted data. Y.Z., Y.F., C.V.D. and L.Z. wrote the manuscript.

Keywords

noncoding RNA; triple-negative breast cancer; DNA damage

INTRODUCTION

Triple-negative breast cancer (TNBC), an aggressive subtype with poor clinical outcome, represents approximately 10–20% of breast cancer cases^{1–3}. A large proportion of TNBC (50–75%) matches the molecular subtype known as basal-like breast cancers, which are characterized by the high expression of genes that are normally expressed in the basal epithelial layer. The absence of estrogen receptor (ER) and progesterone receptor (PR) expression, and Her2 amplification limits therapeutic options for this disease to surgery with adjuvant chemotherapy and radiotherapy^{1–3}. Currently, there are no effective targeted therapies, although *EGFR* amplification, *TP53* mutation, *BRCA* loss and PI3-kinase pathway activation have been exploited for TNBC treatment. Given the lack of recurrent targetable genomic alterations, functional characterization of the TNBC genome to identify driver genomic events is critically important^{1–3}. The human genome contains ~20,000 protein-coding genes (PCGs), representing less than 2% of the total genome, whereas up to 70% of the human genome is transcribed into RNA, yielding thousands of noncoding RNAs⁴. However, genomic studies on TNBC have mainly focused on PCGs and the function of noncoding genes is still largely unknown.

Long noncoding RNAs (lncRNAs) are defined as RNA transcripts larger than 200 nucleotides (nt) and that do not appear to have protein-coding potential^{5–13}. More than 15,900 lncRNA genes have been recently identified in the human genome based on GENCODE annotations⁴. Notably, the expression of lncRNAs is strikingly cell type- and tissue-restricted, and in many cases, even primate-specific. Investigations on lncRNAs have demonstrated that these noncoding transcripts can serve as scaffolds or guides to regulate protein-protein or protein-DNA, interactions; as decoys to bind proteins or miRNAs; or as enhancers to influence gene transcription, when transcribed from enhancer regions or their neighboring loci. Due to the highly dysregulated expression of lncRNAs in cancer^{14,15}, it is surmised that lncRNAs contribute to tumorigenesis. In fact, certain lncRNAs have been shown to function as oncogenes or tumor suppressors^{5–13}. For example, HOTAIR can induce breast cancer metastasis¹⁶ by operating as a tether that links EZH2 (PRC2) and LSD1, thereby coordinating their epigenetic regulatory functions¹⁷. *LINK-A* promotes glycolysis reprogramming and tumorigenesis, and its expression was increased in TNBC¹⁸.

DNA repair, a collection of processes by which the damaged DNA is identified and corrected in cells, is vital to genomic integrity and involved in tumorigenesis. Many proteins that were initially shown to influence life span have turned out to be involved in DNA damage repair. However, beyond protein whether RNA molecule is directly involved in DNA repair machinery is still largely unknown. The non-homologous end joining (NHEJ) is one of the major mechanisms for repairing damaged DNA in cancer cells^{19–26}. In response to double strand DNA breaks (DSBs), Ku80-Ku70 associate with the broken ends, forming a clamp-like complex, and then recruit DNA-PKcs to the damaged site. Other processing

proteins, including Artemis, DNA ligase IV, XRCC4 and XLF, assemble with the Ku80, Ku70 and DNA-PK complex, permitting repair the DNA^{19–26}. To identify the lncRNAs that are functionally involved in tumorigenesis of TNBC, we analyzed the expressional profile of lncRNAs in TCGA breast cancer dataset and performed a clinically guided genetic screening in TNBC cell line.

RESULTS

Identification of the TNBC-associated lncRNA *LINP1*

To identify the lncRNAs associated with TNBC, we analyzed lncRNA expression differences among the distinct pathological and molecular subtypes of breast cancers in the Cancer Genome Atlas (TCGA) dataset³ (Fig. 1a). We found 330 (fold change ≥ 2) and 45 (fold change ≤ 5) lncRNAs whose expression was significantly higher in TNBC compared to non-TNBC tumors. When the non-basal tumors (Luminal_A, Luminal_B and Her2_enriched) were treated as a whole, a total of 402 (fold change ≥ 2) and 69 (fold change ≤ 5) lncRNAs were found to express at a significantly higher level in basal tumors. When comparing four molecular subtypes individually, we identified 164 (fold change ≥ 2) and 75 (fold change ≤ 5) lncRNAs that were specifically enriched in basal subtypes. By cross-comparing the three gene lists, we found an overlap of 154 (fold change ≥ 2) and 35 (fold change ≤ 5) lncRNAs whose expressions were enriched in TNBC tumors (Fig. 1b, Table S1), including the most recently identified TNBC-enriched lncRNA *LINK-A*¹⁸. To identify the lncRNAs that are functionally involved in TNBC, we performed siRNA screening in the MDA-MB-231 cells. Of the 35 highly enriched lncRNA candidates, the expression of 20 was detected in MDA-MB-231 cells. Forty siRNAs targeting 20 lncRNA candidates were designed (Table S2) and individually transfected into MDA-MB-231 cells, which were treated with doxorubicin (a first line chemotherapy drug for TNBC^{1–3}) 48 hours after siRNA transfection (Supplementary Fig. 1a). Apoptosis via caspase3 activity was measured 24 hours post doxorubicin treatment, and the lncRNA *ENSG00000223784* (lncRNA in NHEJ pathway 1, *LINP1*), was identified as a strong candidate.

To further corroborate the above findings, we analyzed the RNA-seq data of breast cancer cell lines (n=46) from the Cancer Cell Line Encyclopedia (CCLE) dataset²⁷. Consistent with TCGA data, we found that *LINP1* was expressed at a significantly higher level in basal lines than in non-basal lines (Fig. 1c and Supplementary Fig. 1b). We choose two TNBC lines (MDA-MB-231 and MDA-MB-468), a triple-negative immortalized breast line (MCF10A), and one ER-positive line (MCF7) as the models for functional experiments. By Northern analysis, we confirmed the RNA-seq results and found that *LINP1* expression was highly expressed in TNBC lines as well as MCF10A cells and was undetectable in MCF7 cells (Supplementary Fig. 1b,c). As anticipated, transduction of *LINP1* siRNAs in the three triple-negative lines significantly enhanced doxorubicin induced apoptosis but had no effect on MCF7 cells by both the caspase3 assay and Western (Fig. 1d). Importantly, overexpressing *LINP1* RNA in MCF7 cells by lentiviral infection protected the cells from doxorubicin induced apoptosis compared to both empty vector and antisense controls (Fig. 1e). Together, through a combination of lncRNA profiling and functional screening, we identified *LINP1* as a lncRNA that may be functional involved in TNBC.

Expression and genomic alteration of *LINP1* in breast cancer

To define the molecular and pathological value of *LINP1* in TNBC, the expression and copy number alteration of *LINP1* and key known breast cancer-associated genomic alterations, along with the clinical annotations, were extracted from TCGA (Fig. 2a to 2d). Consistent with the findings in Fig. 1a, a significantly higher *LINP1* expression was observed in basal breast cancer (Fig. 2e). Notably, the somatic copy number of *LINP1* was significantly amplified in basal breast cancer (Fig. 2f), and there is a significant positive correlation between the gene copy number and RNA expression of *LINP1* in the breast cancer samples ($R=0.26$). These observations suggest that gains in the somatic copy number of the *LINP1* gene is a mechanism by which the RNA expression level of *LINP1* was increased in basal breast tumors. We further analyzed the correlation between the *LINP1* expression and expression of key molecular markers for breast cancer. We found that while *LINP1* expression was positively correlated with the RNA expression of *EGFR* and *CDKN2A*, it was negatively correlated with *RB1* (Fig. 2g). Intriguingly, we observed that the expression of *LINP1* was significantly higher in those with *TP53* mutations than in that with wild type (WT) *TP53* (Fig. 2h). Finally, we found that the *LINP1* RNA was detectable in normal breast tissues (Supplementary Fig. 2a) and distributed in both nuclear and cytoplasmic fractions of cells (Supplementary Fig. 2b).

LINP1 associates with proteins in the NHEJ pathway

To explore the molecular mechanisms underlying the biological activity of *LINP1*, we used an RNA pulldown assay followed by mass spectrometry (MS) to identify proteins associated with *LINP1* (Fig. 3a and Table S3). Interestingly, two proteins involved in the NHEJ pathway^{19–26}, Ku80 and DNA-PKcs, were identified as proteins that were present only in *LINP1*-associated samples (Supplementary Fig. 3a). To verify the result, we analyzed the lncRNA-pulldown protein samples by Western analysis with Ku80 and DNA-PKcs antibodies. Strong signals for Ku80 and DNA-PKcs were observed in proteins pulled down with *LINP1* RNA, but not in proteins associated with either antisense *LINP1* or beads alone (Fig. 3b), confirming that Ku80 and DNA-PKcs are indeed specifically present in the *LINP1*-associated protein complex. To confirm that the association between *LINP1* and Ku80 DNA-PKcs is not an artifact due to the use of *in-vitro* synthesized RNA, we tested the interaction between endogenous *LINP1* and these two proteins using the capture hybridization analysis of RNA targets (CHART)²⁸ (Fig. 3c,d). As shown in Fig. 3e, *LINP1* was enriched from the cross-linked chromatin extracts with the C-oligos 1.2 and 1.4, but not with C-oligo 1.2S and 1.4S. Furthermore, Western analysis detected the Ku80 and DNA-PKcs only in the complexes that were enriched by C-oligo 1.2 and 1.4 (Fig. 3f). Together, these results strongly suggest that endogenous *LINP1* can interact with Ku80-Ku70 heterodimer and DNA-PKcs. To further confirm the interaction between *LINP1* and Ku80 DNA-PKcs, we performed an RNA-immunoprecipitation assay (RNA-IP) in which the RNA-protein complex was immunoprecipitated with Ku80, Ku70 or DNA-PKcs-specific antibodies (Fig. 3g,h). Compared to the IgG-bound sample, the Ku80, Ku70 and DNA-PKcs antibody-bound complex had a significantly higher level of *LINP1* RNA. As a negative control, GAPDH RNA was also quantified in the complexes co-precipitated by IgG or the Ku80, Ku70 and DNA-PKcs antibodies; no significant enrichment of GAPDH was observed in the complex immunoprecipitated by either Ku80, Ku70 or DNA-PKcs specific antibodies

(Fig. 3i). In addition, UV-crosslinked RNA-IP experiment indicates that LINP1 is associated with Ku80-Ku70 heterodimer, and LINP1 may directly bind to Ku80 but not Ku70 (Fig. 3i). Finally, we mapped the regions of LINP1 that interact with Ku80 and DNA-PKcs by RNA pulldown assays. It revealed that a 300-nt region in the 5' of the LINP1 transcript (nt 1–300) was essential to the interaction with Ku80 and a 317-nt region in the 3' of the LINP1 transcript (nt 600–917) was essential to the interaction with DNA-PKcs (Supplementary Fig. 3b). In aggregate, our findings indicate that Ku80 and DNA-PKcs are LINP1-associated proteins and that LINP1 uses different regions to interact with these two proteins.

LINP1 serves as a modular scaffold in the NHEJ pathway

Given that LINP1 RNA binds to Ku80 and DNA-PKcs, two proteins well known for their roles in the NHEJ pathway^{19–26}, we hypothesized that LINP1 may play a role in double-strand DNA break (DSB) repair. Using a comet assay, we examined the impact of LINP1 knockdown on DNA repair activity in ionizing radiation (IR) induced DNA damage. While the level of DNA damage gradually returned to the baseline in the control cells 24 hours after IR treatment, it remained high in the LINP1 knockdown cells, suggesting there were delays in DNA repair in the cells with LINP1 inhibition (Fig. 4a,b). This was further confirmed by differences in the levels of γ H2AX at different time points after IR treatment; the LINP1 knockdown cells had higher levels of γ H2AX for prolonged time periods compared to control cells (Supplementary Fig. 4a). While the level of γ H2AX in the control cells at 24 hours after IR treatment was comparable to that at 0 hour, γ H2AX levels remained high in the LINP1 knockdown cells. Furthermore, we counted the number of γ H2AX-positive foci formed in the control and LINP1 knockdown cells in response to the IR treatment. Consistently, the number of γ H2AX-positive foci quickly diminished in the control cells but was sustained in the LINP1 knockdown cells (Fig. 4c,d). Together, all these observations strongly suggest that DSB repair activity is impaired by LINP1 knockdown. To test whether NHEJ is the pathway affected by LINP1, a NHEJ reporter assay²⁹ demonstrated that, in MDA-MB-231 cells, where LINP1 is highly expressed, we observed a decrease of NHEJ activity in cells with LINP1 knockdown (Fig. 4e); in MCF7 cells, where LINP1 expression is undetectable, the level of NHEJ activity increased dramatically when the cells were transduced with LINP1, but not with control or antisense LINP1 (Fig. 4e). These results support the idea that LINP1 enhances DSB repair via the NHEJ pathway.

Next, we analyzed the dynamic levels of chromatin-associated LINP1, Ku80, DNA-PKcs, and γ H2AX in response to IR treatment. Chromatin-associated complexes were isolated 0 to 60 minutes after IR treatment at 10-minute intervals. The levels of Ku80, DNA-PKcs, and γ H2AX in chromatin-associated complex were measured by Western and the level of LINP1 was measured by qRT-PCR. In response to IR treatment, the levels of Ku80, DNA-PKcs, γ H2AX and LINP1 all increased in the chromatin-associated complex (Fig. 4f). Since the level of LINP1 remained unchanged in the whole cell lysates (Fig. 4f), this observation suggests that LINP1 was recruited to the chromatin upon IR treatment. Furthermore, we found that IR treatment induced the association between LINP1 and Ku80 or DNA-PKcs as measured by RNA-IP analysis (Fig. 4g).

To further define the mechanism responsible, we analyzed the impact of LINP1 knockdown on the level of chromatin-associated Ku80 and DNA-PKcs after IR treatment. While LINP1 knockdown significantly decreased the level of chromatin-associated DNA-PKcs, it had no effect on Ku80 (Fig. 4h). Then we knocked down Ku80 or DNA-PKcs, respectively, to test whether Ku80 or DNA-PKcs affects the chromatin recruitment of LINP1 induced by IR treatment. Interestingly, we found that Ku80 knockdown resulted in a significant decrease in chromatin-associated LINP1 (Fig. 4i), but DNA-PKcs knockdown resulted in no significant change of LINP1 on chromatin (Fig. 4j). Finally, we examined the effect of LINP1 on the interaction of Ku80 and DNA-PKcs by IP followed by Western. In cells with LINP1 knockdown, there is less association between Ku80 and DNA-PKcs after IR treatment (Supplementary Fig. 4b). Collectively, our results suggest that LINP1 may serve as an RNA scaffold to enhance the molecular interaction between Ku80 and DNA-PKcs in the NHEJ pathway.

LINP1 expression is activated by the EGF signaling pathway

The observation of a correlation between LINP1 and EGFR expression (Fig. 2g) is intriguing as overexpressed or amplified *EGFR* has been reported in TNBC³. To further confirm, we analyzed LINP1 and EGFR RNA expression in the CCLE dataset. Consistent with primary specimens, the expression of LINP1 positively correlated with the expression of EGFR in cancer cell lines (Fig. 5a), indicating that EGFR-LINP1 correlation is cell autonomous and with no contribution from tumor stromal RNA. We treated three breast lines with EGF and measured the level of LINP1 RNA in response to EGF treatment. While EGF was able to significantly induce the expression of LINP1 in triple-negative lines MDA-MB-468 and MCF10A, it had no effect in MCF7 (Fig. 5b). The downstream effector signaling pathways of EGFR have been well characterized and multiple small molecules have been developed to inhibit the activities (Fig. 5c). We looked for correlations between the expression of LINP1 and expression of transcription factors downstream of the EGF pathway in the CCLE dataset. Significant and positive correlations were found between LINP1 and c-Jun, c-Fos (both $p < 0.01$, $R > 0.25$) but not with other transcription factors (Fig. 5c), suggesting that EGF may regulate LINP1 expression via the RAS-MEK-JNK pathway. We treated MDA-MB-468 and MCF10A cells with inhibitors to the RAS-MEK-JNK pathway as well as the PI3K-AKT pathway as a control. As anticipated, the EGFR inhibitor (Gefitinib), the MEK inhibitor (AZD6244) and the JNK inhibitor (SP600125) all significantly reduced the expression of LINP1 in triple-negative lines, while the PI3K inhibitor (LY294002) had little effect on the expression of LINP1 (Fig. 5d). By analyzing the bindings of c-Jun or c-Fos from the ChIP-seq data of ENCODE, we observed a strong enrichment of c-Jun or c-Fos in MCF10A cells but not in MCF-7 cells (Supplementary Fig. 5). Then we validated the above ChIP-seq data using ChIP-qPCR, and we found strong binding of both c-Jun and c-Fos to the promoter region of *LINP1* in MDA-MB-231, MDA-MB-468 and MCF10A cells but not in MCF7 cells (Fig. 5e). Importantly, we found that EGF treatment further increased the binding between c-Jun or c-Fos and the *LINP1* promoter in MDA-MB-468 cells but had no effect in MCF7 cells (Fig. 5e). We found that a consensus AP1 binding site at -102 bp from the transcription start site (TSS) of *LINP1* (Fig. 5f). EGF treatment significantly increased the WT, but not AP1 mutated *LINP1* promoter-luciferase activity (Fig. 5f). Consistent with the influence of EGF treatment, the EGFR

inhibitor (Gefitinib), the MEK inhibitor (AZD6244) and the JNK inhibitor (SP600125) all significantly reduced the activities of the reporter, while the PI3K inhibitor (LY294002) had little effect on the activity (Fig. 5f). Together, these findings demonstrate that EGF signaling, specifically its activation of the RAS-MEK-JNK pathway, is involved in regulating LINP1 expression in TNBC.

LINP1 expression is repressed by the p53 signaling pathway

TNBC shows a high frequency of *TP53* mutations³. The differential expression of LINP1 in breast cancer specimens of different *TP53* mutation status (Fig. 2h) suggests that the p53 pathway might play a role in regulating the expression of LINP1. To delineate the possible link to p53, we looked for a correlation between LINP1 expression and *TP53* mutation status in breast cancer cell lines from CCLE. Consistent with what we found in primary tumors, the breast cancer cells with mutant *TP53* have a significantly higher expression of LINP1 (Fig. 6a). Next, we found that nutlin-3a treatment significantly decreased the expression of LINP1 in MCF10A and HCT116 cells, which express WT p53, but not in MDA-MB-231 cells with mutated *TP53* and HCT116 cells with homozygous *TP53* deletion (Fig. 6b), suggesting that LINP1 expression is negatively regulated by the WT p53 pathway. We used the *LINP1* promoter-luciferase reporter in cells transduced with WT *TP53* cDNA. A luciferase construct containing a known p53 binding site was used as the positive control. While the activity of the construct containing a known p53 binding site dramatically increased upon *TP53* expression, the luciferase activity of the *LINP1* construct remained unchanged (Fig. 6c), suggesting that p53 may not directly regulate the transcription of *LINP1*. Consistent with this observation, ChIP-seq data from HCT116 cells also revealed an obvious p53-binding signal in the *CDKN1A* (p21) promoter in *TP53* WT cells, but no such signal in the *LINP1* promoter in any of the cell lines studied. Taken together, these results suggested that p53 may regulate LINP1 expression via an indirect pathway.

Next, we observed two regions in LINP1 exon 2 (Fig. 6d) that are completely complementary to the seed sequence of miR-29, which is positively and directly regulated by p53³⁰. To test whether p53 regulates LINP1 expression via miR-29, we treated the cells with nutlin-3a and confirmed an increase of miR-29 expression in *TP53* WT but not mutant or null cells (Supplementary Fig. 6). Then, we transduced miR-29 mimic into MCF10A and HCT116 cells, as well as in HCT116 cells with homozygous *TP53* deletion, and measured the level of LINP1. In all three lines, the expression of miR-29 mimic was able to decrease the expression of LINP1 in cells (Fig. 6d). Finally, we generated reporter constructs in which the WT LINP1 or LINP1 with miR-29 seed sequence mutation was inserted into the 3' UTR of a luciferase reporter gene. After we co-transfected the reporter construct with miR-29 mimics into cells, we found that the expression of the wide type construct was significantly reduced by co-transfection with miR-29 mimic. Importantly, unlike the WT LINP1 control construct, the mutant LINP1 construct retained a high level of expression despite the expression of miR-29 mimic (Fig. 6d). Together, this confirms a role for the p53 pathway in repressing LINP1 expression and indicates that miR-29 is a mediator of p53-regulated LINP1 expression.

Alteration of LINP1 modulates radiation sensitivity

Radiation treatment is currently one of the standard therapies for patients with TNBC^{1–3}. Previous studies have demonstrated that NHEJ is a key determinant of IR resistance in cancer cells^{19–26}. We hypothesized that LINP1 may regulate IR response via increasing NHEJ activity. First, we assessed the impact of LINP1 knockdown on the IR sensitivity of three cancer cell lines: MDA-MB-231 and MDA-MB-468 cells, which express high levels of LINP1; and MCF7, which has undetectable levels of LINP1 expression. LINP1 specific shRNAs were introduced into these cells, and different doses of IR were then used to treat the cells. Cell survival was assessed by survival assay a week after the IR treatments. As shown in Fig. 7a, LINP1 shRNA expression significantly decreased cell survival after IR treatments in MDA-MB-231 and MDA-MB-468 but not in MCF7 cells. Expressing LINP1 RNA in MCF7 cells, on the other hand, rendered the cells more resistant to IR (Fig. 7b). Next, we established control or LINP1 shRNAs-expressing MDA-MB-231 cell derived xenografts in nude mice. A single dose of 8Gy IR was administered to each tumor when the tumor reached 50mm³, and the mice were monitored till the tumor reached 900mm³ in size. In the untreated groups, a slight delay of growth was observed in the LINP1 knockdown tumors compared to the control (Fig. 7c). In the IR treated groups, however, the re-growth of LINP1 knockdown tumors were significantly attenuated. At the time when all IR-treated control growths reached 900mm³, the LINP1 knockdown tumors just started to regrow. In fact, 2 of the 7 LINP1 knockdown tumors became undetectable after IR treatment and never reemerged during the 64 day post-IR observation period (Fig. 7c). qRT-PCR analysis demonstrated that the tumors expressing LINP1 shRNAs had lower endogenous LINP1 expression than controls (Fig. 7d). Next, we implanted MDA-MB-231 derived tumors as described above and administered a single dose of 8Gy IR when the tumors reached 100 mm³. The tumor tissues were harvested either 0.5 hour or 24 hours after the IR treatment. Phospho-H2AX staining was used to assess the level of DSB in the tumor tissues at different time points. At 0.5 hours post IR treatment, a significant amount of γ -H2AX was observed in both control and LINP1 knockdown tumors. At 24 hours after IR treatment, the level of γ -H2AX in the control tumors was significantly decreased but the level in LINP1 knockdown cells remained high (Fig. 7e,f). These observations indicated that compared to the control tumors, LINP1 knockdown tumors impaired ability to repair DSBs present in the tumor tissues. Collectively, our results suggest that suppressing LINP1 expression can impair DNA repair activity *in vivo*, which in turn sensitizes tumors to IR treatment.

DISCUSSION

Triple negative breast cancer is a clinically challenging disease that involves multi-step changes of the genome^{1–3}. To date, genomic changes in protein-coding genes (PCGs) in TNBC genomes have been the major focus³. By sifting through genomic alterations and distinguishing ‘driver’ from ‘passenger’ alterations, a number of key PCG hubs have been uncovered including gain of *EGFR* signaling or loss of *TP53*. However, despite these pivotal findings, resistance of TNBC to standard therapy, particularly radiation and chemotherapy, remained poorly understood at the mechanistic level. Because PCGs constitute only 2% of the human genome, it is likely that noncoding RNAs play as yet undefined roles in TNBC ‘phenome’ of therapeutic resistance. In this regard, we report here a strategy to use a

clinically guided genetic screening approach to identify functional lncRNAs in TNBC. Using the lncRNA expression profile as initial clinical filter, we were able to generate a relatively short list of lncRNA candidates for more extensive testing in siRNA-based functional genetic screening. Based on a chemotherapy drug (Doxorubicin)-induced apoptosis screening, we successfully identified LINP1 as a potential lncRNA candidate that may be involved in cell death and DNA damage response in TNBC. Importantly, LINP1 can enhance NHEJ activity by providing a scaffold for Ku80 and DNA-PKcs (Fig. 8). Once DSB occurs, Ku80-Ku70 heterodimer recruits LINP1 to the damaged DNA; LINP1 then stabilizes the Ku80 and DNA-PKcs complex, increasing the NHEJ-mediated DNA repair activity (Fig. 8). Because cells without LINP1 expression (e.g. MCF7) can still repair DNA via the NHEJ pathway, LINP1 does not appear to be prerequisite for the NHEJ process. However, enforced expression of LINP1 in non-LINP1 expressing cells can enhance NHEJ mediated DNA repair activity. Interestingly, Ting *et al.* also showed that the Ku80 and Ku70 complex interacts with *hTR* (*TERC*), a lncRNA component of telomerase, in human cells³¹.

We also uncover functional links between the noncoding (LINP1 lncRNA) and protein coding (*EGFR* and *TP53*) genomic hubs. *EGFR* has been reported to be highly amplified in TNBC, serving as a potential target for treatment¹⁻³. Notably, the EGFR pathway is known to enhance NHEJ-mediated DNA repair, and high EGFR activity is associated with radiation resistance^{32,33}. Here, we uncover an additional mechanism for EGFR-induced radiation resistance, whereby EGFR activation upregulates *LINP1* transcription via the activation of the RAS-MEK-ERK pathway and AP1 transcription factors. Thus, in cells with EGFR activation, increased levels of LINP1 stabilizes the Ku80 and DNA-PKcs interaction and enhances the NHEJ-mediated DNA repair activity. Our study also revealed that p53 activation can down-regulate LINP1 expression via miR-29 via induction miR-29 that targets LINP1 RNA. Since LINP1's enhancement on NHEJ activity takes place immediately after DNA damage, while the mir29-mediated LINP1 downregulation happens at a much later time point, we speculate that the p53-mir29-mediated LINP1 regulation may serve as a negative feedback mechanism to restrict the level of NHEJ-mediated DNA repair activity in cells long after damage. High frequency of *EGFR* amplification and *TP53* mutations in TNBC may increase LINP1 expression at the transcriptional and post-transcriptional levels, respectively. Meanwhile, copy number amplification of the *LINP1* gene itself may further enhance the response to increased *EGFR* activity and loss of *TP53* repression in TNBC.

Because of limited therapeutic targets, TNBC is typically treated with surgery and a combination of radiation and chemotherapy which induce various types of DNA damage¹⁻³. The NHEJ pathway, which repairs double-strand breaks in DNA, is one of the major pathways in tumor cells which respond to radiation treatment and chemotherapeutic agents¹⁹⁻²⁶. Inhibition of the NHEJ pathway has been proposed to synergize with DNA-damaging therapies for TNBC¹⁻³. In addition, the NHEJ pathway may also be implicated as a key source of genomic rearrangement and instability¹⁹⁻²⁶, the fundamental feature of TNBC¹⁻³. We believe that a better understanding of the role of lncRNA in the NHEJ pathway will not only provide a deeper understanding TNBC development but will also help to refine the classification as well as the treatment of this disease.

ONLINE METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

RNA-seq data processing

The poly(A)⁺ RNA-seq (Illumine) data in BAM format of human breast tumor specimens was generated and processed by University of North Carolina (UNC) as part of the TCGA project. The poly(A)⁺ RNA-seq (Illumina HiSeq) data in BAM format of 935 human cancer cell lines across 21 cancer types was generated and processed by the Cancer Cell Line Encyclopedia (CCLE) project, a collaboration between the Broad Institute, the Novartis Institutes for Biomedical Research, and the Genomics Institute of the Novartis Research Foundation. RNA-seq files were downloaded from the Cancer Genomics Hub (<http://cghub.ucsc.edu>). We imported the aligned reads of each BAM file to the Partek Genomic Suite (<http://www.partek.com/>) to obtain the expression levels for genes by summarizing the reads per kb per million mapped reads (RPKM) values. GENCODE annotations (version 18; <http://www.gencodegenes.org/releases/18.html>) were used to define lncRNAs and PCGs. The log-transformed RPKM values of genes were further analyzed using Partek Genomic Suite and BRB-ArryTools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>).

SNP array data processing and copy number analysis

The TCGA SNP array (Affymetrix Genome-Wide Human SNP Array 6.0) data in CEL format of patients' paired breast tumor and germline-derived DNA specimens was downloaded from the TCGA Data Portal (<https://tcga-data.nci.nih.gov/tcga/>). The CEL files were imported to the Partek Genomic Suite to perform segmentation and calculate the predicted copy number for each given gene. A predicted copy number larger than 2.3 or smaller than 1.7 was considered as a copy number gain or loss for each gene, respectively. Amplified segments and GISTIC scores were visualized with IGV (<http://www.broadinstitute.org/igv/>).

Cell culture

Cancer cell lines were purchased from the ATCC without further authentication. HCT116 WT and HCT116 *TP53* cell lines were from Dr. Bert Vogelstein without further authentication. MDA-MB-231, MDA-MB-468, MCF7, HCT116 WT and HCT116 *TP53* were cultured in RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). MCF10A was cultured in DMEM/F12 medium (Invitrogen) containing 5% horse serum, 20 ng/ml EGF, 0.5 mg/ml Hydrocortisone, 100 ng/ml Cholera Toxin, 10 µg/ml Insulin. Cells were routinely tested for mycoplasma contamination using Mycoplasma Plus PCR Primer Set (Agilent, Santa Clara, CA) and were found to be negative.

siRNA screening in MDA-MB-231 cell line

A total of 20 lncRNAs were included in our initial screening in MDA-MB-231 cells (Table S2). To reduce the off-target effect of the siRNAs, we designed two independent siRNA

sequences targeting for each lncRNA gene candidate. qRT-PCR was used to monitor the siRNAs' knockdown efficiency. We found that 11 of 20 (55%) lncRNAs can be efficiently knocked down by siRNAs. *LINP1* and *ENSG00000227036* were initially identified from the screening. The non-targeting siRNA controls (which do not target any human or mouse genes) were used as negative controls in the screening. *LINP1* was the only positive lncRNA identified from the initial screening. Cells were seeded in 6-well plates in antibiotic-free media overnight, and then transfections were performed using the Lipofectamine RNAiMAX transfection reagent (Invitrogen). 24 hours after transfection with the indicated siRNAs, cells were trypsinized and plated in 96-well plates in triplicates. 24 hours later, 1 μ M doxorubicin was added. After growing for another 24 hours, caspase3 activity was assessed by Caspase-Glo3 Assay Kit (Promega). MTT was conducted in parallel for normalization with the Cell Proliferation Kit (I) (Roche) according to the manufacturer's instructions. Caspase3 activity was measured by Fluoroskan Ascent FL (Thermo), and MTT was quantified using an ELx800 Absorbance Microplate Reader (BioTek) at 570 nm.

Plasmid Construction

For pull-down assay, full length (1~917 bp), 5' (1~300 bp), 5' (1~600 bp), 5' (300~600 bp), 5' (600~917bp) and full length antisense LINP1 were cloned with 5' BamHI and 3' XhoI sites of pBluescript II SK (+) vector. For LINP1 overexpression, full-length sense and antisense LINP1 were cloned into CD513B vector (System Biosciences). For TP53 activation luciferase reporter, -4000 ~ +300 bp of *LINP1* promoter was cloned into pGL3-basic (Promega); -200 ~ +300bp was cloned into pGL3-basic for the study of EGF activation, drug inhibition and AP1 trans-activation. To generate AP1 binding site mutation reporter vector, two point mutations were introduced into the putative AP1-binding site of *LINP1*-promoter-WT (-200 ~ +300bp) vector by QuikChange Lightning Site-Directed mutagenesis kit (Agilent Technologies). The following primers were used for *LINP1*-P-MUT vector: *LINP1*-P-MUT F GAGCCAATGGGTAACATCACTGCTCTGTTCTTAGCCTCT; *LINP1*-P-MUT R AGAGGCTAAGA ACAGAGCAGTGATGTTACCCATTGGCTC. For miRNA Luciferase reporter assay of LINP1, double strand DNA sequence from LINP1 containing two wide type (psiCheck-LINP1 WT: TCGAGTGTGCTT TCCAGGATGGTGCTGAGATCTTAGCCGGGTTTACGGTGCTGGC) or two mutant target sequences (psiCheck-LINP1 mutant: TCGAGTGTGCTTTCCAGGATATGAAGTAGATCTTAGCCG GG TTTTACATGAAGTGC) of miR-29 were synthesized by IDT and cloned into psiCheck 2 plasmid.

shRNA lentiviral transduction

The Lentiviral vector (pLKO.1) and packaging vectors were transfected into 293T cells. The medium was changed 8 hours after transfection, and the medium containing lentivirus was collected 48 hours later. Cancer cells were infected with lentivirus in the presence of 8 mg/ml polybrene. The following shRNA oligonucleotides were used:

LINP1 sh1 F	CCGGTCTTGATTGAGCTGCATAAATCTCGAGATTATGCAGCTGAATCAA GATTTTGG
LINP1 sh1 R	AATTCAAAAATCTTGATTGAGCTGCATAAATCTCGAGATTATGCAGCTG AATCAAGA

LINP1 sh2 F	CCGGATGTAAGTATCCTTAGATATCTCGAGATATCTAAGGATCAGTTAC ATTTTTTG
LINP1 sh2 R	AATTCAAAAAATGTAAGTATCCTTAGATATCTCGAGATATCTAAGGATC AGTTACAT
Ku80 sh1 F	CCGGCGTGGGCTTTACCATGAGTAACTCGAGTTACTCATGGTAAAGCCCA CGTTTTTG
Ku80 sh1 R	AATTCAAAAACGTGGGCTTTACCATGAGTAACTCGAGTTACTCATGGTAA AGCCACAG
Ku80 sh2 F	CCGGAGAAGAGGCATATTGAAATATCTCGAGATATTTCAATATGCCTCTT CTTTTTG
Ku80 sh2 R	AATTCAAAAAGAAGAGGCATATTGAAATATCTCGAGATATTTCAATAT GCCTCTTCT
DNA-PKcs sh1 F	CCGGCCATCCCTTATAGGTTAATATCTCGAGATATTAACCTATAAGGGAT GGTTTTTG
DNA-PKcs sh1 R	AATTCAAAAACCATCCCTTATAGGTTAATATCTCGAGATATTAACCTATA AGGGATGG
DNA-PKcs sh2 F	CCGGGAAACAGCTGTCTCCGTAAATCTCGAGATTTACGGAGACAGCTGTT TCTTTTT
DNA-PKcs sh2 R	AATTCAAAAAGAAACAGCTGTCTCCGTAAATCTCGAGATTTACGGAGAC AGCTGTTTC

RNA isolation and qRT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen) and reverse transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). cDNA was quantified by an ABI ViiA 7 System (Applied Biosystems).

Protein isolation and Western blot

Western blotting was performed using the following primary antibodies: anti-Ku80 (Cat No:MA5-12933, Clone No:111, Thermo); anti-Ku70 (Cat No:MA5-13110, Clone No:N3H10, Thermo); anti-DNA-PKcs (Cat No: MA5-13404, Thermo); anti-phospho-H2AX(S139) (Cat No:05-636, Clone No: JBW301, Millipore); anti-PARP (Cat No:9542, CST); anti- β -Tubulin (Cat No:2128, Clone No:9F3, CST); anti-Lamin B (Cat No:ab8982, Clone No:119D5-F1, abcam) were used, followed by incubation with secondary antibodies conjugated with horseradish peroxidase (HRP, GE Healthcare Life Sciences). Immunoreactive proteins were visualized using the LumiGLO chemiluminescent substrate (Cell Signaling). The antibody information was provided in the Supplementary Note 1.

Northern blot

561-bp LINP1 cDNA fragment (266bp ~ 826bp) was cloned into pBluescript II SK(+). DIG-labeled RNA probe was transcribed in vitro and purified. 20 μ g of the total RNA was fractionated on a 2% agarose gel containing 1 x Denaturing Gel Buffer (Invitrogen). After visualizing 28S and 18S rRNAs by Sybr gold staining to check the integrity of RNA samples and equal loading, the RNA was blotted onto nylon membrane (Whatman, Sanford, ME). After UV cross-linking, membranes were placed into a hybridization bag containing pre-warmed DIG Easy Hyb buffer (Roche) and incubated for 30 min at 68°C, followed by incubation with DIG-labeled LINP1 RNA probe (final concentration 50ng/ml) for 14 hours at 68°C. The membranes were washed in 2 $\times$ SSC–0.1% SDS for 10 min twice at room temperature and in 0.1 $\times$ SSC–0.1% SDS for 10 min twice at 68°C and detection was performed using the CDP-Star, ready-to-use buffer (Roche).

RNA pull-down assay

The cDNA sequence of LINP1 was cloned into pBluescript II SK (+). Biotin-labeled RNAs were transcribed in vitro and purified. 3 μ g of biotinylated RNA was mixed with precleared human MDA-MB-231 whole cell lysate (containing 1 mg proteins) in 500 μ l RIP buffer and

then mixed with 50 µl washed Streptavidin agarose beads at RT for 1 hour. Beads were washed briefly with RIP buffer for five times and boiled in SDS buffer. Then the retrieved proteins were detected by Western blot or by mass spectrometry (MS) identification.

RNA-immunoprecipitation (RIP)

For native RIP, MDA-MB-231 extract was incubated with 10 µg of anti-Ku70 (Cat No:MA5-13110, Clone No:N3H10, Thermo), anti-Ku80 (Cat No:MA5-12933, Clone No:111, Thermo), anti-DNA-PKcs (Cat No: MA5-13404, Thermo) antibody or control IgG (Cat No:5415S, CST) and then with Protein A Sepharose beads. After a total of three washes in RIP buffer, beads were boiled in SDS buffer for Western blot, or resuspended in TRIzol reagent for real-time RT-PCR. UV-Crosslink RIP (CLIP) was performed as described^{34–36}. Briefly, UV-irradiated MDA-MB-231 cells were lysed in RSB-Triton buffer, incubated with anti-Ku70 (Cat No:MA5-13110, Clone No:N3H10, Thermo), anti-Ku80 (Cat No:MA5-12933, Clone No:111, Thermo), anti-DNA-PKcs (Cat No: MA5-13404, Thermo) antibody or control IgG (Cat No:5415S, CST), and then precipitated with Protein A Sepharose beads. Beads were then extracted for Western blot or real-time RT-PCR.

Capture hybridization analysis of RNA targets (CHART)

Experiment was performed as previously described³⁷. Briefly, cells were washed with PBS and nuclei were enriched by disrupting cells with a Dounce homogenizer in sucrose buffer, diluted with an equal volume of glycerol buffer, and layered on top of glycerol buffer (4 mL). The crosslinked nuclei were collected by centrifugation and further cross-linked in 3% formaldehyde diluted in PBST for 30 minutes. Cross-linked nuclei were washed in PBST and resuspended in sonication buffer and then sheared using a Misonix sonicator 3000. CHART nuclear extracts were diluted 1:4 in NRB buffer. RNase H mapping reactions were performed and analyzed as previously described³⁷. RNase H mapping oligonucleotides and sequences of qPCR primers are listed below:

LINP1 qPCR F1	AGCCGGTCCAGTACACCTTT
LINP1 qPCR R1	GGAAAGCACCGTCTGTGTGTT
LINP1 H1.1	TGTTCAATCAATTGCATCCG
LINP1 H1.2	CCGGCACGTAGAGGACATTA
LINP1 H1.3	ATCCGGGCTACTTCCACAC
LINP1 H1.4	AAAGGTGTACTGGACCGGCT
LINP1 qPCR F2	CCCAGAAATCAAGCCACACA
LINP1 qPCR R2	TCCCCATACCCTCTCCTACC
LINP1 H2.1	TAGCAGGGAGTCCAAGCCTA
LINP1 H2.2	AGGGCTGTTTGTGCTGACAGT
LINP1 H2.3	TCCTCCTTCTTTGTGTGGC
LINP1 H2.4	GCAGCTGAATCAAGATGCTG
LINP1 qPCR F3	GCTCGCATATCTCCACTTGC
LINP1 qPCR R3	GCTCTGTTCTGGGTGACACT
LINP1 H3.1	GAGCTGGCTGAGCAAGTTCT
LINP1 H3.2	TTCTTCTAATGGCAGTGGCA
LINP1 H3.3	ACACTTGTCTCTTTCTCTGGG

Capture oligonucleotides were synthesized (Integrated DNA Technologies) to incorporate an internal hexa-ethyleneglycol spacer (iSp18) and a 3' biotin label with an extended spacer arm (3Bio-TEG). LINP1 CO1.2 and CO1.4 and the sense sequences were listed below:

LINP1 CO 1.2	CCACACCGGCACGTAGAGGACATTA/iSp18//3BioTEG
LINP1 CO 1.4	AAAAGGTGTACTGGACCGGCTGGGC/iSp18//3BioTEG
LINP1 sense CO 1.2	TAATGTCCTCTACGTGCCGGTGTGG/iSp18//3BioTEG
LINP1 sense CO 1.4	GCCCAGCCGGTCCAGTACACCTTTT/iSp18//3BioTEG

For each CHART reaction, 100 pmol of capture oligonucleotides were added to the extract from 10^7 cells and hybridized overnight at room temperature with gentle shaking. Hybridized material was captured with 60 μ l streptavidin resin (Invitrogen) 8 hours at room temperature. Bound material was washed five times with WB250 buffer. Streptavidin resin was boiled in SDS buffer for western blot, or resuspended in TRIzol reagent for real-time RT-PCR.

Chromatin fractionation

MDA-MB-231 cells were fractionated as previously described³⁸ with modification. Briefly, cells were resuspended in Cytoplasmic Extract (CE) buffer and incubated on ice for 5 min. Cell lysates were centrifuged at 300 g for 2 min, and the supernatant (cytoplasm fraction) was removed. The remaining pellet (enriched with nuclei) was washed with CE buffer once and then lysed in buffer B on ice for 5 min. The nuclei lysate was then centrifuged at 1700 g for 4 min, and the supernatant (soluble nuclear fraction) was removed. The final pellet is the chromatin fraction.

Comet assays

MDA-MB-231 cells were transfected with anti-LINP1 siRNAs or control siRNA 48 hours before irradiation. Cells were treated with 10 Gy of IR, and harvested at 0 hour (before radiation), 0.5 hour, 4 hour, or 24 hour after IR. Neutral comet assays with Sybr gold staining (Invitrogen) were performed. The quantitation of tail DNA was done using CASP software.

Immunofluorescence

siRNA treated cells were seeded on coverslips, treated with 10 Gy of IR the next day, and then harvested at 0 h (before radiation), 0.5 h, 4 h, or 24 h for immunofluorescence. Cells were fixed in solution containing 3% paraformaldehyde, 2% sucrose for 10 min at room temperature. Cells were subsequently permeabilized with 0.5% Triton solution for 5 min at 4°C and then incubated with anti- γ H2AX antibody ((Cat No:ab81299, abcam); (Cat No: 05-636, Clone No: JBW301, Millipore)) with the dilution of 1:1000 by PBST buffer (PBS plus 0.1% Tween-20, 0.02% NaN₃) overnight at 4°C. Cells were then washed three times with PBST and next incubated with secondary antibody for 1 hour at room temperature. After four washes with PBST, coverslips were mounted onto glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories) and visualized using an Axiovert 200M inverted microscope (Zeiss).

Co-Immunoprecipitation (Co-IP)

MDA-MB-231 cells with stable expression of LINP1 shRNA1, LINP1 shRNA2 or control shRNA were treated with 10 Gy of IR. Cells were recovered in 37°C incubator for 0.5 hour after IR, then lysed in Co-IP buffer (50 mM Tris HCl (pH7.5), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% NP40, 1 x PIC) with disruptive sonication. After pre-clear, 10 µg of anti-Ku80 (Cat No:MA5-12933, Clone No:111, Thermo), anti-DNA-PKcs (Cat No: MA5-13404, Thermo) antibody or control IgG (Cat No:5415S, CST) was added to 5 mg supernatant and incubated overnight at 4 °C with gentle rotation. 50 µg supernatant from each samples was saved as input for the following Western blot. Protein A Sepharose beads were added to each sample and incubated at 4°C for 1 hour. After three times of wash, proteins were extracted for Western blot.

Non-homologous end joining Assay and FACS Analysis

The experimental strategy for the NHEJ assay was performed as described³⁹. LINP1-shRNA treated MDA-MB-231 or LINP1 overexpressed MCF7 cells were transfected with HindIII digested plasmid along with 0.1 µg of control pDsRed2-N1. Expression of GFP and DsRed was monitored by fluorescence microscopy (Nikon, Eclipse TE2000-U). 48–72 hours after transfection, cells were harvested, resuspended in 0.5 ml of PBS, pH 7.4 (GIBCO, Invitrogen), and analyzed by FACS (BD FACS Canto).

TaqMan miRNA assays

Briefly, single-stranded cDNA was synthesized from 5 ng of total RNA in a 15 µl reaction volume using a TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed with TaqMan microRNA assay mix.

miRNA mimic transfection

The hsa-miR-29a mimic and control mimic were purchased from Sigma. For transient transfections, cells were plated 24 hours before transfection at 50% confluence. miRNA mimic transfections were performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA).

Luciferase Assays

For TP53 activation assay of *LINP1* promoter, 500 ng pGL3.0-basic or pGL3.0-*LINP1* vector plus 5 ng of the Renilla luciferase plasmid with or without 1µg or 5µg pcdna3.1-*TP53* vector was transfected to 293 cells using FuGENE 6 (Roche). For measurement of promoter activity in cells treated with EGF or inhibitors, MDA-MB-468 cells were first transfected with *LINP1*-P-WT or *LINP1*-P-MUT for 24 hours prior to adding EGF or inhibitors for another 24 hours incubation. For miRNA Luciferase reporter assay, 293 cells were plated on a 24-well plate 24 h before transfection at 50% confluence. 30 nM miR-29a mimics or control mimics (Sigma) were transfected using Lipofectamine RNAiMAX (Invitrogen). 24 hours post-transfection, 0.125 µg of psiCheck-LINP1 WT or psiCheck-LINP1 MUT reporter vector was transfected using FuGENE6 transfection reagent (Roche). 48 hours after reporter vector transfection, cells were harvested, and reporter assays were performed using a dual

luciferase reporter assay system (Promega) by Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific, Rockford, IL, USA).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described⁴⁰ with the following modifications. 3×10^7 of MDA-MB-231, MDA-MB-468, MCF10A or MCF7 cells treated with or without 200 ng/ml of EGF was harvested for ChIP experiment. Cells were cross-linked with 1% formaldehyde at room temperature for 10 min, and then neutralized with 125 mM glycine for 5 min. Cells were rinsed with ice-cold PBS twice and scraped into 1 ml of ice-cold PBS. Cells were resuspended in 0.3 ml of lysis buffer and sonicated. After centrifugation, supernatants were collected and diluted in IP dilution buffer followed by immunoclearing with protein A-sepharose for 2 hour at 4°C. 5 µg anti-c-Jun (Cat No:9165S, Clone No:60A8, CST) or anti-c-Fos (Cat No:2250S, CST) antibody (Cell Signaling Technology) or control IgG (Cat No: 2729S, CST) was used for immunoprecipitation. After immunoprecipitation, 45 µl protein A-Sepharose was added and incubated for another 1 hour. Precipitates were washed, and DNA was purified after de-crosslinking for real-time PCR. Primers were listed are listed below:

<i>LINP1</i> AP1 ChIP qPCR F	AGCTCCCAGAAATGTCAGCT
<i>LINP1</i> AP1 ChIP qPCR R	GGAAGTCAAAGTCACTGGG
<i>GAPDH</i> ChIP F	GCTTGCCCTGTCCAGTTAAT
<i>GAPDH</i> ChIP R	TAGCTCAGCTGCACCCCTTA

In vivo tumor experiments

MDA-MB-231 cells were transduced with lentiviruses expressing anti-LINP1 or control shRNA, and selected in puromycin for 7 days. Three million tumor cells were injected subcutaneously into 6-weeks-old athymic female nu/nu mice (Stock No: 002019, Jackson Labs). An 8Gy single dose will be precisely delivered to the tumors of anaesthetized mice using a Small Animal Radiation Research Platform (SARRP) when tumors grow to about 50 mm³. Tumor growth will be monitored every other days with a digital vernier caliper, and tumor volumes were calculated according to the formula: Tumor volume [mm³] = $(1/6) \times \pi \times (\text{tumor length}) \times (\text{tumor width})^2$. For xenograft immunofluorescence, an 8Gy single dose will be delivered when tumors grow to 100mm³. 0.5 hour or 24 hours after irradiation, mice were sacrificed and tumors were harvested for immunofluorescence of γH2AX. The intensity of fluorescence will be quantified with ImageJ. Statistical significance of the differences was evaluated using two-tailed Students *t*-test. For all the xenograft studies, the sample size of each group is indicated in the figures. We performed pilot experiments using a few mice per group followed by larger studies if needed to reach statistical significance and repeated experiments to ensure reproducibility. Due to the nature of the performed experiments, no randomization and no blinding was used as it was deemed unfeasible. However, the resulting tumors were analyzed in a blinded manner. We treated a p-value of less than 0.05 as a significant difference. All experiments were performed at least twice. All animal procedures were in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Statistical analysis

Statistical analysis was performed using SPSS and SAS software. All results were expressed as mean $\pm$ SD, and $p < 0.05$ indicated significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the TCGA and CCLE project teams. This work was supported, in whole or in part, by the Bassett Center for BRCA (LZ), the US National Institutes of Health (R01CA142776 to LZ, R01CA190415 to LZ, P50CA083638 to LZ, P50CA174523 to LZ, R01CA148759 to QH, R01NS094533 to YF), the Breast Cancer Alliance (LZ and CVD), the Ovarian Cancer Research Fund (XH), the Foundation for Women's Cancer (XH), and the Marsha Rivkin Center for Ovarian Cancer Research (LZ). SW was supported by the China Scholarship Council.

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Figure 1. Identification of the TNBC-associated lncRNA *LINP1*

(a) Heat map of lncRNAs whose expression was significantly (fold change ≥ 5 ; p-value < 0.05) different between groups stratified by histological or PAM50 molecular subtypes. The expression differences among the distinct pathological and molecular subtypes of breast cancers were analyzed by BRB-ArrayTools. Upper panel: TNBC vs non-TNBC samples; Middle panel: basal vs non-basal samples; Bottom panel: comparison across all four subtypes. Yellow indicates high expression; blue, low expression. (b) Venn diagram of the three groups of genes identified through the comparisons in panel (a) as those with significantly (fold change ≥ 5 ; p-value < 0.05) enriched expression in TNBC tumors. (c) Violin plot of LINP1 expression in basal (n=26) vs non-basal (n=20) breast cancer cell lines from CCLE. Two-tailed Student's *t*-test; p-value=0.046. (d) Upper panel: Caspase3 activity assay in cells expressing control or LINP1 siRNAs, treated with doxorubicin or DMSO. Error bars indicate SD; two-tailed Student's *t*-test; * indicates p-value < 0.05 ; n=3 independent cell cultures; Lower panel: PARP and cleaved-PARP expression by Western blots in cells expressing control or LINP1 siRNAs. (e) Upper panel: Caspase3 activity assay in MCF7 cells expressing control vector, sense LINP1, or antisense LINP1. Error bars indicate SD; two-tailed Student's *t*-test; * indicates p-value < 0.05 ; n=3 independent cell cultures; Lower panel: PARP and cleaved-PARP expression by Western blots in MCF7 cells expressing control vector, sense LINP1, or antisense LINP1. Uncropped images of gels are shown in Supplementary Data Set 1.

Figure 2. Expression and genomic alteration of LINP1 in breast cancer
 (a to d) Heat maps of the RNA expression (a) somatic gene copy number alterations (b) and mutation status (c) of LINP1 and key molecular alterations of breast cancer and the clinical annotation (d) in breast cancer samples. The genomic information was retrieved from the TCGA and was visualized by MeV: Multi-Experiment Viewer. The samples were grouped by their molecular subtypes in rows and ranked by LINP1 RNA expression level in each subtype. The genomic alteration of other genes and clinical profile were mapped in columns. (e) Violin plot showing LINP1 expression in different breast cancer subtypes. Two-tailed Student's *t*-test; *p*-value<0.01. (f) Violin plot showing *LINP1* gene copy number alterations in different breast cancer subtypes. Two-tailed Student's *t*-test; *p*-value<0.0001. (g) Correlation between LINP1 expression and expression of *EGFR*, *CDKN2A*, *MYC*, *RB1*, *TP53*, *PIK3CA*, *PTEN* and *HER2* in the breast cancer samples. (h) Violin plot showing LINP1 expression in breast cancer with wild-type or mutant *TP53*. Two-tailed Student's *t*-test; *p*-value<0.0001.

Figure 3. LINP1 RNA associates with Ku80 and DNA-PKcs

(a) A schematic representation of the RNA pull-down assay. (b) Western analysis of Ku80 and DNA-PKcs levels in the protein complexes pulled down by either LINP1, antisense LINP1, or empty beads from whole cell extracts of MDA-MB-231 cells. "Input" refers to 5% input of whole cell lysates. (c) A schematic representation of CHART assay. (d) A schematic diagram of LINP1 RNA, the eleven C-oligos (red bars) designed for CHART assay, and the three primer sets (convergent arrows) used in the RNase-H sensitivity assay. RNase-H sensitivity was indicated with blocks with different shades of red, with darker shade indicating higher sensitivity. (e) Enrichment of LINP1 with C-oligo 1.2 and 1.4, as measured by qRT-PCR. GAPDH was used as negative control. (f) Presence of Ku80 and DNA-PKcs in C-oligo 1.2 and 1.4 enriched complexes, detected by Western analysis. C-oligo 1.2S and 1.4S were used as non-specific controls. (g) A schematic representation of an RNA-immunoprecipitation (RNA-IP) assay. (h) Results from native RNA-IP (upper panel) and CLIP (lower panel) with Ku80, Ku70, and DNA-PKcs specific antibodies. Western blots showing the immunoprecipitation efficiency of Ku80, Ku70 and DNA-PKcs. (i) Results from native RNA-IP (upper panel) and CLIP (lower panel) with Ku80, Ku70, and DNA-PKcs specific antibodies. qRT-PCR assays showing the level of LINP1 and GAPDH (non-specific control) in the co-precipitates. Ku80 (left), Ku70 (middle) and DNA-PKcs (right). Error bars indicate SD; two-tailed Student's *t*-test; * indicates *p*-value<0.05; *n*=3 independent technical replicates. Uncropped images of gels are shown in Supplementary Data Set 1.

Figure 4. LINP1 serves as a modular scaffold in the NHEJ pathway

(a) IR-induced DNA damage in control and LINP1 knockdown MDA-MB-231 cells, measured by the comet assay. Scale bar indicates 10 μ m. (b) Levels of IR-induced DNA damage, quantified by the tail moment in the comet assay. Two-tailed Student's *t*-test; * indicates *p*-value<0.05; *n*=3 independent cell cultures. (c) Quantification of the number of γ H2AX-positive foci in the control and LINP1 knockdown cells. Error bars indicate SD; two-tailed Student's *t*-test; * indicates *p*-value<0.05; *n*=3 independent cell cultures. (d) Representative pictures of γ H2AX-positive foci in the control and LINP1 knockdown cells. Scale bar indicates 10 μ m. (e) NHEJ-mediated DNA repair activity, measured by NHEJ reporter, in MDA-MB-231 cells expressing control or LINP1 specific shRNAs (left), and in MCF7 cells expressing control vector, sense LINP1 and antisense LINP1 (right). Error bars indicate SD; two-tailed Student's *t*-test; * indicates *p*-value<0.05; *n*=4 independent cell cultures. (f) Levels of LINP1, Ku80, DNA-PKcs, and γ H2AX in the chromatin-associated complex at different time points after IR treatment. (g) Levels of LINP1 associated with Ku80 and DNA-PKcs in control and IR-treated cells. Error bars indicate SD; two-tailed Student's *t*-test; * indicates *p*-value<0.05; *n*=3 independent technical replicates. (h) Levels of chromatin-associated Ku80 and DNA-PKcs in control and LINP1 knockdown cells after

IR-treatment. **(I and j)** Levels of Ku80, DNA-PKcs, and LINP1 in control and Ku80 or DNA-PKcs knockdown cells. Left panel: the protein levels of Ku80 (i) or DNA-PKcs (j) in the control and Ku80 (i) or DNA-PKcs (j) knockdown cells. Right panel: the levels of LINP1 and GAPDH (as control) in the control and Ku80 knockdown cells (i) or in the control and DNA-PKcs knockdown cells (j). Error bars indicate SD; two-tailed Student's *t*-test; * indicates p -value<0.05; $n=3$ independent technical replicates. 10 Gy of irradiation was used for all experiments. Uncropped images of gels are shown in Supplementary Data Set 1.

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Figure 5. LINP1 is activated by the EGF signaling pathway

(a) Correlation between LINP1 and EGFR expression in CCLE. Expression levels were determined by the RPKM from RNA-seq. R-value was calculated by Pearson test. (b) Expression of LINP1 in MDA-MB-468, MCF10A, and MCF7 cells treated with EGF or control. Error bars indicate SD; two-tailed Student's *t*-test; * indicates p -value <0.05 ; $n=3$ independent technical replicates. (c) A schematic diagram of EGFR signaling. Small molecule inhibitors of EGFR signaling and their specific targets are indicated. (d) Expression of LINP1 in MDA-MB-468 and MCF10A cells treated with different small molecules that inhibit different parts of the EGFR pathway. RNA expression was analyzed by qRT-PCR. Error bars indicate SD; two-tailed Student's *t*-test; * indicates p -value <0.05 ; $n=3$ independent technical replicates. (e) Left panel: Quantification of the amount of LINP1 promoter bound to c-Jun or c-Fos in MDA-MB-468, MCF10A, and MCF7 cells. The promoters were pulled down by c-Jun or c-Fos antibodies and measured by qPCR analysis. GAPDH was used as a negative control; Middle and Right panels: Quantification of the amount of LINP1 promoter bound to c-Jun or c-Fos in MDA-MB-468 (middle) and MCF7 (right) cells which were treated with EGF. Error bars indicate SD; two-tailed Student's *t*-test; * indicates p -value <0.05 ; $n=3$ independent technical replicates. (f) Left panel: An illustration

of an AP1 binding site at –102 (red indicates the consensus motif) and the sequences of the AP1 mutations (blue indicates the mutant nucleotides); Middle panel: Luciferase reporter assay of the promoter activities of the *LINP1* core promoter construct and its AP1 mutant counterpart in MDA-MB-468 cells treated with EGF; Right panel: Luciferase reporter assay assessing the promoter activities of the *LINP1* core promoter construct in MDA-MB-468 cells with different small molecules that inhibit different parts of the EGFR pathway. Error bars indicate SD; two-tailed Student's *t*-test; * indicates p -value<0.05; $n=3$ independent technical replicates.

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Figure 6. LINP1 is repressed by the p53 signaling pathway

(a) LINP1 expression in the CLE breast cancer cell lines in which the *TP53* mutation status is known. Two-tailed Student's *t*-test; p-value<0.01. (b) LINP1 expression in cells of different *TP53* status, in response to nutlin-3a treatment. MCF10A and HCT116, *TP53* WT cells; MDA-MB-231, *TP53* mutant; HCT116 with *TP53* deletion, *TP53* null. Error bars indicate SD; two-tailed Student's *t*-test; * indicates p-value<0.05; n=3 independent technical replicates. (c). Luciferase assay measuring the transcription activity of *LINP1* and construct containing a known p53 binding site in cells expressing control vector or WT *TP53*. Error bars indicate SD; two-tailed Student's *t*-test; * indicates p-value<0.05; n=3 independent cell cultures. (d) Left panel: Sequence alignment showing the complementarity between LINP1 exon 2 and miR-29. Red: seed sequence of miR-29; Middle panel, LINP1 expression in cells of different *TP53* status after treated with vehicle or miR-29; Right panel: Luciferase assay measuring the activity of WT or mutant LINP1-luciferase fusion reporter constructs in response to treatment with miR-29 mimic. Error bars indicate SD; two-tailed Student's *t*-test; * indicates p-value<0.05; n=3 independent cell cultures.

Figure 7. Alteration of LINP1 modulates IR sensitivity

(a) Survival of control or LINP1 knockdown MDA-MB-231, MDA-MB-468, and MCF-7 cells in response to IR treatments. Cells expressing control or LINP1 specific shRNAs were treated with different doses of IR, and the post-treatment survival was measured by survival assay. Error bars indicate SD; n=3 independent cell cultures. (b) Survival of control or LINP1 expressing MCF-7 cells in response to IR treatments. Cells expressing control vector, sense LINP1 or antisense LINP1 were treated with different doses of IR and the post-treatment survival was measured by colony formation assay. Error bars indicate SD; n=3 independent cell cultures. (c) Growth of control and LINP1 expressing MDA-MB-231 tumors with and without IR treatment. IR treatment (single dose 8Gy) was administered when the tumor reached 50mm³. Tumor growth was monitored for over 2 months. Animals bearing tumors larger than 900mm³ were euthanized. Animal number for each group was shown in figure. (d) LINP1 expression in control and LINP1 knockdown tumors was detected by qRT-PCR. Error bars indicate SD; two-tailed Student's *t*-test; p-value<0.001; n=5 independent tumors. (e) Phospho-H2AX staining in the control and LINP1 expressing MDA-MB-231 tumors at different points after IR treatment. IR treatment was administered when the tumor reached 100mm³, and tissues were harvested 0.5 hour and 24 hours after the IR treatment (single dose 8Gy). Scale bar indicates 40 μm. Blue, DAPI; red, phospho-H2AX staining. (f) Quantitation of phospho-H2AX staining in control and LINP1 expressing MDA-MB-231 tumors at 0.5 and 24 hours post IR treatment. Error bars indicate SD; two-tailed Student's *t*-test; p-value<0.001; n=6 independent tumors.

Figure 8. Role of LINP1 in the NHEJ DNA repair pathway

In response to DSBs, Ku80 and Ku70 associate with the broken ends, forming a clamp-like complex, and then recruit DNA-PKcs to the damaged site. Other processing proteins, including Artemis, DNA ligase IV, XRCC4 and XLF, assemble with the Ku80-70 and DNAPK complex, permitting repair the DNA. The lncRNA LINP1 can enhance NHEJ activity by providing a scaffold for Ku80 and DNA-PKcs. Once DSB occurs, Ku80 but not Ku70 recruits LINP1 to the damaged DNA; LINP1 then stabilizes the Ku80 and DNA-PKcs complex, increasing the NHEJ-mediated DNA repair activity.