Oncogenic IncRNA downregulates cancer cell antigen presentation and intrinsic tumor suppression

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How tumor cells genetically lose antigenicity and evade immune checkpoints remains largely elusive. We report that tissue-specific expression of the human long noncoding RNA LINK-A in mouse mammary glands initiates metastatic mammary gland tumors, which phenotypically resemble human triple-negative breast cancer (TNBC). LINK-A expression facilitated crosstalk between phosphatidylinositol-(3,4,5)-trisphosphate and inhibitory G-protein-coupled receptor (GPCR) pathways, attenuating protein kinase A-mediated phosphorylation of the E3 ubiquitin ligase TRIM71. Consequently, LINK-A expression enhanced K48-polyubiquitination-mediated degradation of the antigen peptide-loading complex (PLC) and intrinsic tumor suppressors Rb and p53. Treatment with LINK-A locked nucleic acids or GPCR antagonists stabilized the PLC components, Rb and p53, and sensitized mammary gland tumors to immune checkpoint blockers. Patients with programmed cell death protein-1 (PD-1) blockade-resistant TNBC exhibited elevated LINK-A levels and downregulated PLC components. Hence we demonstrate IncRNA-dependent downregulation of antigenicity and intrinsic tumor suppression, which provides the basis for developing combinatorial immunotherapy treatment regimens and early TNBC prevention.

The poor prognosis of triple-negative breast cancer (TNBC), hallmarkmed by the absence of estrogen receptor (ER), progesterone receptor (PR) and HER2 expression, and its resistance to standard chemotherapies have considerably hindered overall survival rates for this disease1,2. Immunotherapy, including programmed cell death protein-1 and programmed death ligand-1 (PD-1–PD-L1) blockade, has been demonstrated to inhibit cancer progression1. However, less than 20% of TNBC tissues are PD-L1+, and the overall response rate of patients with PD-L1+ TNBC to blockade strategies ranges from 10% to 18.5% (ref. 1). These setbacks demand definition and genetic evidence of the molecular mechanisms of immunosuppression during tumor initiation. One of the central roles of the immune system is the surveillance and elimination of malignant transformations1. To escape immunosurveillance, nascent malignant cells may develop diverse mechanisms, including reducing antigenicity so that antitumor lymphocytes fail to detect transformed cells, eliminating immunogenicity by upregulating immunoinhibitory molecules, and recruiting immunosuppressive cells to establish an immunosuppressive microenvironment5,7. Mutation-derived tumor antigens, also
known as neo-antigens, are produced through proteasome-mediated degradation, then transported into the endoplasmic reticulum, where the antigenic peptides are loaded onto the synthesized major histocompatibility complex class I (MHC class I) molecules and migrate to the cell surface to be recognized by cytotoxic T cells. The presentation of neo-antigens derived from mutated proteins leads to tumor suppression, indicating that mutation burden functions as a predictor of neo-antigens and sensitivity to immunotherapy. However, how tumor cells lose antigenicity is unknown, and therapeutic strategies that restore the antigen presentation pathway and sensitize cancers to immunotherapy are missing.

It has become increasingly apparent that many long non-coding RNAs (lncRNAs) are aberrantly expressed in a broad spectrum of cancers and play key roles in promoting and maintaining cancer characteristics. An increased understanding of lncRNAs should stimulate new directions for future research and therapeutic options that focus on lncRNAs as prognostic markers and therapeutic targets for human cancer. Although our previous data have indicated that a lncRNA, LINK-A (long intergenic noncoding RNA for kinase activation), is involved in breast cancer drug resistance and hypoxia, genetic mouse models of lncRNAs with spontaneous tumor development remain elusive and are crucial for developing a proof of concept that lncRNAs function as oncogenes that drive tumor initiation.

Here we investigated the role of LINK-A using a transgenic (Tg) mouse model that represents human TNBC. LINK-A facilitated the association between phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns(3,4,5)P3) and inhibitory GPCRs, leading to reduced cyclic AMP (cAMP) concentrations and protein kinase A (PKA)-mediated phosphorylation of a E3 ligase, TRIM71. As a consequence, TRIM71 catalyzed the K48-linked polyubiquitination (poly-Ub) and proteasome-mediated degradation of Rb, p53 and peptide-loading complex (PLC) components, thereby contributing to decreased immunosurveillance.

**Results**

**LINK-A correlates with immunosuppression.** We previously demonstrated that LINK-A is upregulated in TNBC compared with non-TNBC breast cancer tissues and is correlated with poor outcomes for patients with breast cancer. To investigate potential relationships between LINK-A and the immune microenvironment, we performed a TCGA (The Cancer Genome Atlas) pan-cancer analysis, finding that LINK-A is upregulated in multiple cancer types (Supplementary Fig. 1a). The expression of LINK-A was substantially correlated with relative immune cell abundance (see Methods) and GZMB/CD8A messenger RNA expression ratio across multiple cancer types, and specifically anticorrelated with antigen-presenting cell (APC) and CD8+ T cell abundance in breast-like breast cancer (Fig. 1a and Supplementary Fig. 1b). The top 25% of breast tumors with higher infiltration of activated CD8+ T cells and APCs exhibited substantially reduced LINK-A expression compared with the bottom 25% of breast tumors (Supplementary Fig. 1c). Fluorescent multiplex (anti-PD-L1, CD3, CD8) immunohistochemistry staining and RNAscope indicated that human breast cancer tissues with high LINK-A expression exhibited low CD8+CD3+ lymphocyte infiltration (Fig. 1b,c). Thus, the expression of LINK-A is correlated with an immunosuppressive microenvironment.

To demonstrate the potential prognostic value of LINK-A in patients with TNBC who need immunotherapy, we determined the infiltration of CD8+ T cells in patients with TNBC who are receiving pembrolizumab (anti-PD-1) treatment. The patients with TNBC who responded to pembrolizumab exhibited relatively lower expression of LINK-A and higher CD8+ T cell infiltration compared with non-responders (Fig. 1d,e and Supplementary Fig. 1d). CD8+ T cell infiltration in this cohort of patients with TNBC negatively correlated with LINK-A expression (Fig. 1f).

The decreased APC infiltration in LINK-A-high TNBC suggested potentially impaired antigen presentation machinery, prompting an investigation into the status of the PLC components within the tumors. The protein levels of the PLC components, including TPSN, TAP1, TAP2 and CALR, were all downregulated in TNBC non-responders compared with responders upon pembrolizumab treatment (Fig. 1g). Using modification-specific antibodies, we demonstrated that the K48-linked poly-Ub of TPSN (Lys537), TAP1 (Lys245), TAP2 (Lys213) and CALR (Lys48) was upregulated in non-responders, although the level of total polyubiquitinated proteins remained unaltered (Fig. 1g), suggesting proteasome-regulated degradation of these components. Furthermore, the expression of LINK-A negatively correlated with the protein expression of TPSN, TAP1, TAP2 and CALR, as well as β2-microglobulin (β2M) and MHC class I in this cohort (Fig. 1h and Supplementary Fig. 1e–i). These observations suggested the importance of LINK-A in modulating immune balance in favor of immunosuppression, and that the expression of LINK-A potentially modulates the protein levels of the PLC.

**LINK-A drives basal-like breast cancer.** Previous studies using mouse mammary tumor virus (MMTV) long terminal repeat-driven Neu (Erbb2), Ras and Myc Tg mice demonstrated the development of tumors in mouse mammary glands by 6–12 months. Tg mice harboring a LINK-A-containing ‘flox-stop-flox’ cassette...
were bred with MMTV-Cre mice to induce expression of LINK-A in mammary tissues (referred to as MMTV-Tg(LINK-A) mice)\(^{(20)}\) (Fig. 2a). Two male founder Tg animals were generated (Fig. 2a and Supplementary Fig. 1j), and both founder animals passed the transgene to their offspring in accordance with Mendelian inheritance. All of the data reported are based on observations of the progeny of the two founder animals.

Moderate expression of LINK-A in mouse mammary glands following crossing with MMTV-cre was confirmed by RNA blot (Fig. 2b). Virgin female mice with Tg expression of LINK-A developed mammary gland adenocarcinomas, which involve the entire epithelium of the gland (Fig. 2c). Histologic evaluation indicated that at 8 weeks of age, the majority of mammary gland ducts appear normal (referred to as normal-like) (Fig. 2d). Mice that were 3, 4...
and 5 months old exhibited a reduced percentage of normal-like mammary gland ducts with a concurrently increased percentage of hyperplasia, ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) over the time, respectively (Fig. 2d,e). The median time until development of hyperplasia, DCIS and IDC in virgin female MMTV-Tg(LINK-A) animals was 85, 136 and 161 days, respectively (Fig. 2f–h). The primary mammary gland tumors metastasized to the lungs, which exhibited adenocarcinoma (Fig. 2i). Of tumor-bearing MMTV-Tg(LINK-A) tumors, 22%, compared with no lung metastasis in the control MMTV-cre and Tg(LINK-A) animals (Fig. 2j–l). The copy number of LINK-A in MMTV-Tg(LINK-A) mouse tumors was comparable with that in human TNBC tissues (Supplementary Table 1 and Supplementary Fig. 1k–m). Immunohistochemistry indicated that MMTV-Tg(LINK-A) tumors exhibited similar expression of estrogen receptor-α, PR-A/B and HER2 as normal mammary glands (Supplementary Fig. 2a,b), suggesting that the MMTV-Tg(LINK-A) malignancies show no estrogen receptor, PR and HER2 amplification or upregulation.

We observed that MMTV-Tg(LINK-A) tumors harbored a similar number of missense somatic mutation burdens compared with human TNBCs (Supplementary Fig. 2c). Furthermore, MMTV-Tg(LINK-A) tumors harbored non-silencing somatic mutations on Trp53 and Pik3ca genes that are frequently mutated in human TNBCs (Supplementary Fig. 2d). These observations suggested that the MMTV-Tg(LINK-A) tumors exhibit genetic similarity to human TNBCs, mimicking the bona fide tumor initiation and somatic mutation generation processes.

RNA-sequencing (RNA-seq) analyses of MMTV-Tg(LINK-A) tumors exhibited distinct transcriptional profiling compared with non-LINK-A-expressing Tg(LINK-A) mouse mammary glands (Supplementary Fig. 2e). The transcriptome of MMTV-Tg(LINK-A) tumors was co-clustered with human basal-like breast cancers compared with other subtypes (Supplementary Fig. 2e). Notably, upregulated genes in tumors versus normal tissues were enriched in cell-cycle and redox homeostasis, whereas downregulated genes were enriched in lipid metabolism, T cell activation and immunoresponse (Supplementary Fig. 2f).

Human TNBCs also exhibit a metabolic signature that is hallmarked by glycolysis and accumulation of redox-1. Although the relative abundance of overall metabolites was comparable, MMTV-Tg(LINK-A) tumors were enriched with metabolites of glycolysis and redox homeostasis compared with normal tissues (Supplementary Fig. 2g–i). Hence our findings suggested that the MMTV-Tg(LINK-A) tumors model human basal-like breast cancer metabolically.

**LINK-A mediates PtdIns(3,4,5)P_3–G-protein–coupled receptor crossstalk.** LINK-A associates with PtdIns(3,4,5)P_3 and regulates the activation of the AKT pathway. To dissect the signaling pathway mediated by PtdIns(3,4,5)P_3–bound LINK-A in MMTV-Tg(LINK-A) tumors, we surveyed the PtdIns(3,4,5)P_3–binding proteins in Tg(LINK-A) mouse mammary glands and MMTV-Tg(LINK-A) tumors using liquid chromatography–mass spectrometry (LC–MS) (Table 1 and Supplementary Table 2). Without Cre recombination, PtdIns(3,4,5)P_3 associated with a cohort of lipid-interacting proteins, which is consistent with previous findings (Table 1). Upon expression of LINK-A, PtdIns(3,4,5)P_3 associated with G-protein–coupled receptors (GPCRs), which included cannabinoid receptor 2 (CNR2), γ-amino butyric acid type B receptor subunit 1 (GABRB1), α2A-adrenergic receptor (ADAA2), muscarinic acetylcholine receptor M4 (ACM4) and μ-type opioid receptor (OPRM) (Table 1). Upon ligand binding, GPCRs activated the associated G protein, in which the G-protein alpha subunit (Gα) can be classified as Gαq, Gαi, Gαq or G12/13 (ref. 2). Interestingly, CNR2, GABRB1, ADAA2, ACM4 and OPRM all associate with Gαi, leading to inhibition of adenyl cyclase and reduced cAMP production upon ligand binding-3,4. Consistent with this notion, PtdIns(3,4,5)P_3 and Gαi exhibited robust interactions with GPCRs in MMTV-Tg(LINK-A) tumors compared with the mammary glands of Friend leukemia virus B (FVB) mice (background control mice) or MMTV-PyVT tumors (Fig. 3a), despite similar PtdIns(3,4,5)P_3 enrichment (Supplementary Fig. 3a). The presence of exogenous ligands may further facilitate these interactions.

To validate that the Gαi–GPCRs interaction is dependent on PtdIns(3,4,5)P_3–bound LINK-A, we observed that full-length LINK-A facilitated the interactions between glutathione S-transferase (GST)-tagged bacterially expressed human GPCRs and Gαi (Fig. 3b). Conversely, the presence of a LINK-A PtdIns(3,4,5)P_3–binding motif deletion mutant (nucleotides 1081–1140) (referred to as ΔPIP_3) failed to do so (Fig. 3b). In LNK-A low mouse/human mammary gland epithelial NMuMG/MCF10A cells, exogenous expression of the full-length IncRNA, but not the ΔPIP_3 mutant, enhanced the recruitment of GPCRs to Gαi and PtdIns(3,4,5)P_3 (Fig. 3c and Supplementary Fig. 3b). In LNK-A high MDA-MB-231 cells with a LINK-A PtdIns(3,4,5)P_3–binding motif depletion (referred to as PIP_3–BM–<>) , reintroduction of full-length LINK-A, but not the mutant, rescued these interactions (Supplementary Fig. 3c).

Activation of Gαi results in reduced cellular CAMP concentrations (ref. 6), MCF10A–NMuMG cells harboring ~100 copies of exogenous full-length LINK-A, but not the ΔPIP_3 mutant, showed considerably reduced CAMP concentrations and PKA phosphorylation at Thr197 (ref. 6) with minimally altered cellular PtdIns(3,4,5)P_3, (Fig. 3d,e and Supplementary Fig. 3d–i).

To determine the binding affinity of PtdIns(3,4,5)P_3–GPCRs interactions, we applied an alpha assay. This assay uses 'donor' and 'acceptor' beads to capture interacting biomolecules in proximity that leads to an energy transfer from one bead to the other.
ultimately producing a luminescent/fluorescent signal. For these experiments, we used GST-tagged bacterially expressed GPCRs and biotinylated-PtdIns(3,4,5)P₃ as donor–acceptor pairs (Fig. 3f–h and Supplementary Fig. 3j,k). Gαi exhibited non-detectable interactions with PtdIns(3,4,5)P₃; however, all five GPCRs showed moderate interactions with PtdIns(3,4,5)P₃ (Kᵩ 3.96–8.75 µM) and strong interactions with PtdIns(4,5)P₂ (Kᵩ 0.08–0.42 µM) (Fig. 3f,g and Supplementary Fig. 3j). Next, we determined the Kᵩ value of PtdIns(3,4,5)P₃–GPCRs interactions in the presence of LINK-A or a cardiolipin-binding lncRNA, RP11-383G10.5 (ref. 15), as a control (Fig. 3h and Supplementary Fig. 3k). In the presence of full-length LINK-A, but not the ΔPIP₃ mutant, the Kᵩ values of

![Image](93x510 to 238x585)

![Image](388x510 to 509x632)

![Image](95x428 to 507x488)

![Image](344x287 to 507x409)

![Image](95x65 to 307x138)

![Image](273x517 to 342x588)
Mutants with deletions in the C-terminal lipid raft binding domain (Supplementary Fig. 3l–q), followed by expression of wild-type or ΔCNR2, were required for the crosstalk with LINK-A, affecting upon ΔCNR2. We used single-guide RNAs (sgRNAs) to deplete ΔCNR2, i–GPCRs interactions, resulting in reduced intracellular concentrations of cAMP. The intracellular C termini of GPCRs harbor hydrophobic acyl binding proteins, A dash indicates that no proteins in the categories shown were pulled down with control or PIP3 beads.

| Experimental setting | MMTV-cre mammary gland | MMTV-Tg(LINK-A) mammary gland |
|----------------------|-------------------------|-------------------------------|
|                      | Control beads           | PtdIns(3,4,5)P3 Control beads | PtdIns(3,4,5)P3 P3 beads |
| PtdIns(3,4,5)P3       | -                       | AKT1                          | AKT1                      |
| -                    | -                       | -                             | -                          |
| -                    | -                       | -                             | -                          |
| -                    | -                       | -                             | -                          |
| -                    | -                       | -                             | -                          |
| -                    | -                       | -                             | -                          |
| -                    | -                       | -                             | -                          |
| Inhibitory GPCRs     | -                       | CNR2                          | -                          |
| -                    | -                       | -                             | -                          |
| -                    | -                       | -                             | -                          |
| -                    | -                       | -                             | -                          |
| -                    | -                       | -                             | -                          |
| -                    | -                       | -                             | -                          |
| -                    | -                       | -                             | -                          |
| Table 1 | Summary of protein identification of PIP3 pulldown using Tg(LINK-A) normal mammary gland or MMTV-Tg(LINK-A) tumor | | |

PKA stabilizes Rb and p53. We demonstrated that LINK-A activates GPCRs, leading to downregulation of cAMP and inactivation of PKA. Although PKA depletion leads to carcinogenesis, the underlying molecular mechanism of how PKA prevents tumor initiation is unknown. Using LC–MS as an open-ended technology, we identified PKA-binding proteins and the post-translational modifications of these proteins in MMTV-Cre mammary glands, MMTV-PyVT tumors and MMTV-Tg(LINK-A) tumors (Table 2 and Supplementary Table 3). Whereas the catalytic and regulatory subunits of PKA were detected in all three types of tissues (Table 2), PKA catalytic subunits (PKA-Cα, PKA-Cβ) exhibited phosphorylation at the Thr197 residues of both subunits in normal mammary glands and MMTV–PyVT tumors, but not in MMTV-Tg(LINK-A) tumors (Table 2). Consistently, cAMP levels were reduced in MMTV-Tg(LINK-A) tumors compared with MMTV-Cre mammary glands and MMTV–PyVT tumors (Fig. 5a), validating the conclusion that expression of LINK-A inactivates the cAMP–PKA pathway.

PKA associated with TRIM71, an E3 ligase, in all three types of tissues; however, in MMTV-Tg(LINK-A) tumors, PKA associated with the ubiquitin pathway components, RL40 (ubiquitin precursor), UBA1 (E1), UB3D2 (E2) and TRIM71 (E3) (Table 2), suggesting that the ubiquitination pathway was activated in MMTV–Tg(LINK-A) tumors. Furthermore, TRIM71 was phosphorylated at Ser3 in normal tissues and MMTV–PyVT tumors, but not in MMTV-Tg(LINK-A) tumors (Table 2 and Fig. 5b,c). These data suggested that TRIM71 was phosphorylated by 50%; however, a combined treatment consisting of rauwolscine with abemaciclib or erlotinib substantially repressed cell proliferation (Supplementary Fig. 4c). These findings suggested that inhibiting GPCR signaling may improve the sensitivity of TNBCs to anti-CDK4/CDK6 and anti-EGFR targeted treatments.

GPCR antagonist represses tumor cell proliferation. Our findings suggested that LINK-A-positive basal-like breast cancer may be addicted to GPCR signaling, which could be repressed with GPCR antagonists or inhibitors. We screened and determined the effect of a cohort of GPCR antagonists/inhibitors using isolated tumor cells derived from individual MMTV-Tg(LINK-A) tumors (referred to as MMTV–LINK-A cells) and MDAMB-231 (Fig. 4a). Compared with the vehicle, the ADA2A antagonist rauwolscine exhibited potent effects on restoring cAMP concentrations in both MMTV-LINK-A and MDA-MB-231 cells (Fig. 4a). Rauwolscine is a natural alkaloid that acts as a selective and reversible ADA2A antagonist (K_i = 12 nM). The effect of rauwolscine on restoring the phosphorylation of PKA (Thr197) was validated in MMTV-LINK-A cells derived from individual MMTV-Tg(LINK-A) tumors, in which the protein expression of ADA2A or the PKA catalytic subunit was unaltered (Fig. 4b).

Rauwolscine exhibited minor effects on the cell viability of MCF10A–NMuMG cells but robust cell cytotoxicity against MMTV–LINK-A and MDA-MB-231 tumor cells with half-maximal inhibitory concentration (IC50) values of 30 and 40 nM, respectively (Fig. 4c,d). We then determined the efficacy of rauwolscine in modulating cell proliferation using a panel of TNBC and non-TNBC breast cancer cell lines, finding that treatment with rauwolscine specifically repressed the cell proliferation of TNBC cells, but not non-TNBC cells or normal cells (Fig. 4e,f). Treatment with LINK-A locked nucleic acids (LNAs) showed similar repression of cell proliferation in TNBC cells, but not non-TNBC cells (Fig. 4g). We then tested combinatorial strategies against the growth of MDA-MB-468 cells, which do not respond to current anti-epidermal growth factor receptor (EGFR) targeted therapies. Individually, rauwolscine and the CDK4/CDK6 inhibitor abemaciclib, but not erlotinib, inhibited cell proliferation by 50%; however, a combined treatment consisting of rauwolscine with abemaciclib or erlotinib substantially repressed cell proliferation (Supplementary Fig. 4c). These findings suggested that inhibiting GPCR signaling may improve the sensitivity of TNBCs to anti-CDK4/CDK6 and anti-EGFR targeted treatments.
Fig. 3 | LINK-A mediates crosstalk between PttdIns(3,4,5)P₃ and GPCRs. a, Immunoblotting (IB) detection using indicated antibodies of immunoprecipitates with indicated beads or antibodies using MMTV-cre or Tg(LINK-A) normal mammary gland, MMTV-PyVT tumor or MMTV-
Δ(GABR1) PtdIns(3,4,5)P₃ mutants. Three independent experiments were performed and yielded similar results. b, GST-pulldown using His-tagged Gαi, GST-tagged GPCRs and PttdIns(3,4,5)P₃, in the presence of biotinylated LINK-A full length (fl) or ΔPtdIns(3,4,5)P₃ mutant. Three independent experiments were performed and yielded similar results. c, Immunoblotting detection using indicated antibodies of immunoprecipitates with indicated beads or antibodies of MCF10A cells delivered with vehicle, LINK-A fl or ΔPtdIns(3,4,5)P₃ mutant. Three independent experiments were performed and yielded similar results.

d–e, Measurement of LINK-A copy number (d) or cellular cAMP level (e) of MCF10A cells delivered with vehicle, LINK-A fl or ΔPtdIns(3,4,5)P₃ mutant (NS, P=0.399, ***P<0.001). Results are mean±s.e.m. of n=3 independent experiments. P values were determined by one-way ANOVA. f–g, Competition binding assay to determine Kᵦ for interaction between GST-tagged Gαi (left top), or GST-tagged GABR1 (f) or GST-tagged ADA2A (g) and biotinylated PIPs as indicated. The Kᵦ values (μM) are shown. Results are mean±s.d. of n=3 independent experiments.

h–i, Competition binding assay to determine Kᵦ for interaction between GST-tagged GPCRs and biotinylated-PttdIns(3,4,5)P₃ in the presence of RP11-383G10.5, LINK-A fl or LINK-A ΔPtdIns(3,4,5)P₃ mutant. Results are mean±s.d. of n=3 independent experiments. j–l, Immunoblotting detection using indicated antibodies in MDA-MB-231 cells harboring sgRNAs knocking out GABR1 (j) and ADA2A (l), respectively, followed by expression of GPCRs wild-type or ΔPtdIns(3,4,5)P₃ mutants. The lysates were subjected to immunoblotting detection using indicated antibodies (k) or measurement of cAMP concentration (l) (NS, P>0.05, ***P<0.001). Ctl: control. Results are mean±s.d. of n=3 independent experiments; P values were determined by one-way ANOVA.
Fig. 4 | Identification and characterization of GPCR antagonists. a, Fold change of cellular cAMP concentration of MMTV-LINK-A and MDA-MB-231 cells treated with indicated GPCR antagonists (NS, P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001). Results are mean ± s.d. of n = 3 independent experiments. P values were determined by unpaired two-tailed Student’s t-test. b, Immunoblotting detection using indicated antibodies using MMTV-LINK-A tumor cells subjected to vehicle (Veh.) or rauwolscine (Rau) treatment. Tumor cells isolated from 5 individual MMTV-Tg(+-+) tumor-bearing mice were used. Three independent experiments were performed and yielded similar results. c, Measurement of viability index of three-dimensional spheroid formation assay of NMuMG and MMTV-LINK-A cells (left), or MCF10A or MDA-MB-231 cells (right) in the presence of a serial twofold dilution of rauwolscine; n.d., not determined. Results are mean ± s.d. of n = 3 independent experiments. d, Representative images at days 0–5 (left) and measurement of proliferation index (right) of three-dimensional spheroid formation assay of NMuMG and MMTV-LINK-A cells (top), or MCF10A or MDA-MB-231 cells (bottom) in the presence of vehicle or rauwolscine 30 nm (top) or 40 nM (bottom) (***P < 0.001). Results are mean ± s.d. of n = 3 independent experiments. P values were determined by unpaired two-tailed Student’s t-test. e, Representative images of day 8 (left) and measurement of proliferation index (right) of indicated normal or breast cancer cells in the presence of vehicle or rauwolscine (Rau, 40 nM) (NS, P > 0.05, **P < 0.01, ***P < 0.001). Results are mean ± s.d. of n = 8 spheroids per experimental condition; P values were determined by one-way ANOVA. g, Measurement of proliferation index of indicated normal or breast cancer cells in the presence of Scr or LINK-A LNAs (5 nM) (NS, P > 0.05, **P < 0.01). Results are mean ± s.d. of n = 8 spheroids per experimental condition; P values were determined by one-way ANOVA.
identify the proteins with peptide adducts derived from ubiquitin. The C-terminus of the mature ubiquitin has the amino acid sequence KESTHLVLRLLGG, in which the last Gly can be conjugated to lysine residues on target proteins. When the conjugated ubiquitin is cleaved with trypsin, it leaves Gly–Gly (GG) residues on the modified lysine residues of the target proteins. Rb and p53 exhibited GG modifications at Lys803 and Lys126, respectively, which suggested ubiquitination modification of these proteins (Table 2 and Fig. 5f). Ubiquitin contains seven lysine residues, K6, K11, K27, K29, K33, K48 and K63, through which a specific polyubiquitin chain can be formed upon a target protein. To determine the types of polyUb chains on the target proteins in MMTV-Tg(LINK-A) tumors, we analyzed which lysine residues of ubiquitin are predominantly conjugated with the GG dipeptide, finding that lysine 48 of ubiquitin is largely modified by GG (Supplementary Fig. 4f). These data indicated that Ub-Rb and Ub-p53 are modified with K48-linked poly-Ub and are potentially subjected to proteasomal degradation. Consistently, the protein status of Rb and p53, which have been shown to be downregulated in human breast tumors compared with mouse mammary glands (Fig. 5g–i). Taken together, our data suggested that expression of LINK-A downregulates intrinsic tumor suppressor barriers via the GPCR–PKA–TRIM71 signaling axis during tumorigenesis.

**LINK-A-dependent degradation of PLC components.** To address the hypothesis that LINK-A-mediated hypophosphorylation of TRIM71 in MMTV-Tg(LINK-A) tumors may catalyze poly-Ub chain formation in a panel of substrates, we identified TRIM71-binding proteins with post-translational modifications using MMTV-Tg(LINK-A) tumors pretreated with scramble (Scr) or LINK-A LNAs (Table 3 and Supplementary Table 4), which have been shown to efficiently knockdown LINK-A in vivo. Although the regulatory and catalytic subunits of PKA were detected under both conditions, the catalytic subunits exhibited phosphorylated-Thr197 following LINK-A LNAs treatment (Table 3). Furthermore, the LINK-A LNAs treatment restored the phosphorylation of TRIM71 (Ser3), with concurrent disassociation of ubiquitin, UBA1 and UB2D3 (Table 3). These observations suggested that the LINK-A LNAs treatment effectively reversed the inactivation of the cAMP–PKA pathway in MMTV-Tg(LINK-A) tumors.

In addition to Rb and p53, TRIM71 associated with all six components of the PLC, namely, TPSN, TAP1, TAP2, CALR, ERAP1 and PDIA3 in MMTV-Tg(LINK-A) tumors (Table 3). The PLC components facilitate the folding and loading of antigenic peptides and the transportation of the MHC class I complex to the cellular surface. TPSN, TAP1, TAP2 and CALR were all subjected to GG modification at the Lys213 (TPSN), Lys537 (TAP1), Lys245 (TAP2) and Lys48 (CALR) residues (Table 3), and all ubiquitin that associated with TRIM71 or TRIM71-binding proteins was K48-linked (Supplementary Fig. 4g). Upon LINK-A LNAs treatment, the GG modifications at Lys213 (TPSN), Lys537 (TAP1), Lys245 (TAP2) and Lys48 (CALR) were all abolished (Table 3 and Supplementary Fig. 4h). These observations indicated that the TRIM71-associated PLC components were modified with K48-linked poly-Ub chains, which were diminished upon LINK-A knockdown. We developed modification-specific antibodies targeting Ub-TPSN (Lys213), Ub-TAP1 (Lys537), Ub-TAP2 (Lys245) and Ub-CALR (Lys48) (Supplementary Fig. 4i).

To validate these observations, we observed that the protein levels of TPSN, TAP1, TAP2 and CALR were downregulated in MMTV-Tg(LINK-A) tumors, but not in MMTV-PyVT tumors or normal mammary gland tissues (Fig. 6a). We further confirmed that in human breast cancer tissues (Duke cohort; Supplementary Table 1), the expression of LINK-A negatively correlated with the protein levels of TPSN and CALR (Supplementary Fig. 5a). In TNBC resistant to anti-PD-1 blockage, Ub-TPSN, Ub-TAP1, Ub-TAP2 and Ub-CALR were all substantially increased compared with patients with anti-PD-1-sensitive TNBC (Supplementary Fig. 5b).

To demonstrate that expression of LINK-A downregulates PLC components during tumor initiation, we took advantage of the mammary ductal transformation process of the normal-like, hyperplastic, DCIS and IDC morphologies of MMTV-Tg(LINK-A) mice. Compared with the normal ducts of Tg(LINK-A) mice (Fig. 6b, first column), although the ducts of MMTV-Tg(LINK-A) mouse mammary glands at 8 weeks of age were morphologically similar to normal ducts, the protein status of TPSN and CALR was considerably downregulated in the epithelial cells of these normal-like ducts (Fig. 6b, second column, and 6c), suggesting that the PLC complex is likely to be downregulated in mammary gland epithelial cells without substantial lymphocyte infiltration or accelerated cell division. In hyperplastic ducts, the protein status of TPSN and CALR was...
Fig. 5 | LINK-A represses PKA-dependent TRIM71 phosphorylation. a. Measurement of cAMP concentration in MMTV-cre normal mammary gland, MMTV-PyVT or MMTV-Tg(LINK-A) tumor lysates (NS, \( P > 0.05 \)). ***\( P < 0.001 \)). Results are mean ± s.e.m. of \( n = 4 \) animals per experimental condition; \( P \) values were determined by one-way ANOVA. b. Percentage of modified versus total number of peptides harboring indicated residues of PKA catalytic unit α (left) or TRIM71 (right) in MMTV-cre mammary gland, MMTV-PyVT or MMTV-Tg(LINK-A) tumors. c. Annotated MS/MS spectrum assigned to the TRIM71 peptide MApSFETDFQICLLCK acquired from analysis of tryptic digest by high-sensitivity LC–MS/MS on an Orbitrap Elite high-resolution mass spectrometer. d. Pearson correlation between immunoblotting staining of p-PKA-C (Thr197) (d) or p-TRIM71 (Ser3) (e) and LINK-A copy number of human TNBC (left) or p53 staining intensity (right) per cell (×200). e. Statistical analysis (f,i) of immunohistochemistry staining using indicated antibodies of Tg(LINK-A) normal mammary gland and MMTV-Tg(LINK-A) tumors. Scale bars: 100 µm (g). ***\( P < 0.001 \)). Results are mean ± s.d. of \( n = 6 \) animals per experimental condition; \( P \) values were determined by unpaired two-tailed Student’s t-test.

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Further downregulated compared with normal-like ducts, whereas the atypical hyperplastic ducts, DCIS and IDCs all exhibited similar low-PLC status (Fig. 6bc and Supplementary Fig. 5c,d). Hence it is highly possible that one of the mechanisms by which malignant cells lose antigenicity during tumor initiation is through LINK-A-dependent downregulation of antigen-presenting machinery.

Proteasome-mediated protein degradation occurs in the cytosol;$^{13}$ it is highly likely that the translated MHC class I complex and PLC components are subject to proteasome-mediated protein degradation before the assembly on the endoplasmic reticulum membrane. We addressed the hypothesis that the expression of LINK-A facilitates the interactions between TRIM71 and PLC components, which could be reduced upon LINK-A knockdown using Duolink proximity ligation assay (PLA) signals (Supplementary Fig. 5e). Robust PLA signals were detected in MDA-MB-231 cells harboring Scr LNAs, suggesting protein proximity between TRIM71:TAP1, TRIM71:CALR, TRIM71:TAP2 and TRIM71:TPSN, respectively, which were substantially reduced (Supplementary Fig. 5f). The majority of PLA signals did not overlap with endoplasmic reticulum markers, suggesting that a portion of PLC components could be subjected to TRIM71-mediated poly-Ub and protein degradation on the exterior of the endoplasmic reticulum, which is consistent with previous literature suggesting that a portion of the MHC class I complex overlaps with the endoplasmic reticulum marker$^{16}$.

Tumor-bearing MMTV-Tg(LINK-A) mice treated with LINK-A LNAs showed restored phosphorylation of PKA (Thr197) and p-TRIM71 (Ser3), and elevated protein levels of TPSN, TAP1, TAP2 and CALR without affecting the total protein levels of PKA and TRIM71 (Fig. 6d and Supplementary Fig. 6a,b). To determine the poly-Ub types of the Ub-TPSN, Ub-TAP1, Ub-TAP2 and Ub-CALR, we performed denaturing immunoprecipitation to immunoprecipitate similar amounts of TPSN, TAP1, TAP2 and CALR proteins in MMTV-Tg(LINK-A) tumors. Upon Scr LNAs treatment, the immunoprecipitated proteins exhibited K48-linked

### Table 3 | Summary of protein identification and post-translational modification of TRIM71 pulldown of MMTV-Tg(LINK-A) tumor upon Scr or LINK-A LNA treatment

| Experimental setting | MMTV-Tg(LINK-A) tumor Scr LNA | MMTV-Tg(LINK-A) tumor LINK-A LNA |
|----------------------|-----------------------------|---------------------------------|
| PKA complex          | IgG                         | Anti-TRIM71                     | IgG                           | Anti-TRIM71                     |
|                      | KAP3                        | –                               | KAP3                          | –                              |
|                      | KAP2                        | –                               | KAP2                          | –                              |
|                      | KAP0                        | –                               | KAP0                          | –                              |
|                      | PKA-Cα                      | –                               | p-PKA-Cα (Thr197)             | –                              |
|                      | PKA-Cβ                      | –                               | p-PKA-Cβ (Thr197)             | –                              |
|                      | KAP1                        |                                  | KAP1                          | –                              |
| Ubiquitin machinery  | TRIM71                      | –                               | p-TRIM71 (Ser3)               | –                              |
|                      | Ubiquitin                   | –                               | –                             | –                              |
|                      | UBA1                        | –                               | –                             | –                              |
|                      | UB2D3                       | –                               | –                             | –                              |
| Ubiquitin substrates | Ub-Rb (Lys803)              | –                               | Rb                            | –                              |
|                      | Ub-p53                      | –                               | PS3                           | –                              |
|                      | Ub-TPSN (Lys213)            | –                               | TPSN                          | –                              |
|                      | Ub-TAP2 (Lys245)            | –                               | TAP2                          | –                              |
|                      | Ub-CALR (Lys48)             | –                               | TAPI                          | –                              |
|                      | Ub-TAP1 (Lys537)            | –                               | CALR                          | –                              |

A dash indicates that no proteins in the categories shown were pulled down with IgG or anti-TRIM71.
poly-Ub chains, which were diminished upon LINK-A LNAs treatment (Supplementary Fig. 6b). Treatment with rauwolscine efficiently restored the protein levels of the PLC components, as well as inhibited the K48-linked poly-Ub of these proteins without affecting LINK-A expression in these tumors (Fig. 6e and Supplementary Fig. 6c,d).

The PLC complex is vital for the stabilization and cellular presentation of the MHC class I complex. To determine the functional role of LINK-A and the TRIM71-dependent molecular mechanism in antigen presentation, we determined the cellular surface MHC class I complex of the B16F10 cells with Tet-on-induced expression of LINK-A, finding that expression of LINK-A suppressed the cellular surface expression of H-2Kb with or without interferon-γ (IFN-γ) stimulation (Fig. 6f and Supplementary Fig. 7a). Similarly, expression of exogenous TRIM71 led to reduced cell surface expression of H-2Kb (Supplementary Fig. 7b,c). Furthermore, Trim71 knockdown blocked the LINK-A-dependent cellular surface suppression of H-2Kb in B16F10 cells (Supplementary Fig. 7d).
Exogenous expression of LINK-A in low-LINK-A-expressing MCF7 cells led to reduced cell surface expression of β2M and HLA-ABC (Supplementary Fig. 7e–g); on the contrary, knockdown of LINK-A in high-LINK-A-expressing MDA-MB-231 cells resulted in increased cell surface expression of β2M and HLA-ABC (Supplementary Fig. 7h–j). Treatment with rauwolscine considerably improved the cellular surface expression of MHC class I and β2M (Fig. 6g and Supplementary Fig. 7k). Using B16-ovalbumin (OVA) cells, we observed that knockdown of Trim71 increases cellular surface expression of the chicken OVA peptide (Fig. 6h). Hence, LINK-A downregulates PLC components and impairs antigen presentation, leading to tumor immunosurveillance evasion.

**LINK-A LNAs suppress tumor initiation and progression.** Administration of LINK-A or rauwolscine exhibited minimal effects on the body weight, liver functions and renal functions of the mice (Supplementary Fig. 8a–f). As a prevention trial, 12-week-old female MMTV-Tg(LINK-A) mice without palpable mammary gland tumors were treated with LINK-A LNAs or rauwolscine for a 16-week period. Mice given the vehicle treatment exhibited hyperplasia and atypical hyperplasia throughout their mammary glands with the presence of DCISs and IDCs (Fig. 7a,b). The ducts of mice subjected to LINK-A LNAs or rauwolscine treatment exhibited a low degree of atypical hyperplasia and DCIS with more than 80% normal-like ducts (Fig. 7a,b). The LINK-A LNAs- or rauwolscine-treated mice also exhibited reduced tumor incidence (Fig. 7c,d). LINK-A LNA- or rauwolscine-treated mammary glands exhibited restored protein statuses of CALR and TPSN in ductal epithelial cells and increased CD8+ T cell infiltration compared with the vehicle-treated group (Fig. 7a,c,f).

In our regression model, the tumor-bearing MMTV-Tg(LINK-A) mice treated with LINK-A LNAs exhibited inhibited tumor growth and reduced lung metastasis compared with those treated with Scr LNAs (Fig. 7g–i and Supplementary Fig. 8g). The LINK-A LNA-treated tumors showed increased protein levels of CALR and TPSN (Fig. 7j–l).

We then developed a syngeneic breast tumor model derived from MMTV-Tg(LINK-A) genetic mice using orthotopic transplantation of the mouse tumors to female FVB mammary glands. Treatment with LINK-A LNAs, rauwolscine or immune checkpoint blockers (ICBs) inhibited tumor growth in the syngeneic MMTV-Tg(LINK-A) tumors, which was synergistically suppressed by a combinational treatment of LINK-A LNAs/rauwolscine and ICBs (Fig. 8a,b). The combined treatment substantially extended the survival time of the tumor-bearing mice (Fig. 8b). We then determined the antitumor effect of small molecular inhibitors against CNR2, GABR1, ADA2A, ACM4 and OPRM using JTE907 (CNR2 inhibitor), CPG54626 (GABR1 inhibitor), rauwolscine (ADA2A inhibitor), tropicamide (ACM4 inhibitor) and cypropramide (OPRM inhibitor), respectively, finding that individual administration of these inhibitors substantially inhibited the growth of MMTV-Tg(LINK-A) syngeneic tumors (Supplementary Fig. 8h).

We then determined the status of tumor-resident CD8+ T cells in MMTV-Tg(LINK-A) tumors, finding that the LNAs treatment substantially improved CD8+CD3+ T cell infiltration and cytotoxicity but exhibited minimal effects on the expression of PD-L1 in these tumors (Fig. 8c–f and Supplementary Fig. 8i). The LINK-A LNAs treatment showed minimal effects on the infiltration of CD8+ T cells, macrophages and myeloid-derived suppressor cells (MDSCs) in non-tumor-bearing mammary glands (Supplementary Fig. 8j–o). Taken together, we demonstrated a lncRNA-mediated molecular mechanism that modulates intrinsic tumor suppressors and antigen presentation in breast tumor initiation and progression (Supplementary Fig. 8p). Therefore, LINK-A may serve as a valuable biomarker for predicting the outcome of patients with TNBC who require immunotherapy, and targeting LINK-A further sensitizes breast tumors to immune checkpoint inhibitors.

**Discussion**

The battle between the immune system and malignant cells is constantly evolving, and cancer cells develop a variety of mechanisms to escape immunoediting. Although genetic evidence has indicated that malignant cells with restored antigenicity are subject to anti-tumor immunity, whether cancer cells passively or actively downregulate antigenicity remains elusive. We demonstrated that during breast cancer initiation, the transformed mammary gland epithelial cells downregulate antigen presentation machinery upon expression of LINK-A, illustrating one of the initial and important mechanisms through which cancer cells escape from immune checkpoints. We reasoned that mammary gland epithelial cells expressing LINK-A may exhibit antigenicity loss, which contributes to the survival and expansion of malignant cells. The PLC plays vital roles in antigen presentation and transportation of MHC class I. Posttranslational modifications of PLC components in cancer cells may serve as an advantageous mechanism for downregulating antigenicity without losing achieved genomic mutations and mutation-derived growth advantages. Treatment with LINK-A LNAs or GPCR antagonists like rauwolscine in vivo substantially improved the protein stability of the PLC components and MHC class I complex, leading to sensitization of mammary gland tumors to immunotherapy. Most importantly, the enhanced CD8+ T cell infiltration was specific to tumor tissues; the LINK-A LNAs treatment did not affect the distribution of CD8+ T cells, macrophages, or MDSCs in normal mammary glands. Hence our results suggested promising therapeutic strategies for improving antigen presentation and the efficacy of immunotherapy, which could be synergistic with immune checkpoint inhibitors.

![Fig. 7](https://example.com/fig7.png) **Targeting LINK-A prevents mammary gland tumor initiation and progression.** a. H&E staining, immunohistochemistry staining of CALR and TPSN, and fluorescent multiplex immunohistochemistry labeling of CD3 and CD8 in MMTV-Tg(LINK-A) mammary gland treated with indicated treatments. Six random fields per animal and 20 or 24 animals per experimental condition were analyzed. Scale bars: 50 μm. b. Percentage of normal-like, hyperplastic, atypical hyperplasia and DCIS of MMTV-Tg(LINK-A) mammary gland tumors were treated with LINK-A LNAs or rauwolscine for a 16-week period. Mice given the vehicle treatment exhibited hyperplasia and atypical hyperplasia throughout their mammary glands with the presence of DCISs and IDCs (Fig. 7a,b). The ducts of mice subjected to LINK-A LNAs or rauwolscine treatment exhibited a low degree of atypical hyperplasia and DCIS with more than 80% normal-like ducts (Fig. 7a,b). The LINK-A LNA- or rauwolscine-treated mice also exhibited reduced tumor incidence (Fig. 7c,d). LINK-A LNA- or rauwolscine-treated mammary glands exhibited restored protein statuses of CALR and TPSN in ductal epithelial cells and increased CD8+ T cell infiltration compared with the vehicle-treated group (Fig. 7a,c,f).

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Expression of \textit{LINK-A} mediated the crosstalk between inhibitory GPCRs and PtdIns(3,4,5)P\(_3\), leading to inactivation of the cAMP/PKA pathway. The \textit{LINK-A}-dependent suppression of PKA-dependent phosphorylation of TRIM71 resulted in the K48-linked poly-Ub and protein degradation of the intrinsic tumor suppressors Rb and p53 and the components of PLC. TRIM71 has been shown to modulate p53 degradation\(^4\). It is likely that \textit{LINK-A}/TRIM71-dependent p53 ubiquitination is independent of MDM2 (ref. \(^5\)), which is consistent with the notion that the major ubiquitination residues of p53 mediated by MDM2 are located within the tetramerization domain of p53 (Lys320, Lys321, Lys351, Lys357, Lys370, Lys372, Lys373)\(^6\), whereas \textit{LINK-A}-dependent, TRIM71-mediated p53 poly-Ub occurs at Lys126, which is within the DNA-binding domain of p53.
Fig. 8 | Targeting LINK-A sensitizes mammary gland tumor to immunotherapy. a, Tumor volumes of syngeneic MMTV-Tg(LINK-A) mice treated with LINK-A LNA, rauwolscine (Rau), ICB alone or in combination (n = 8, 8, 8, 8, 12 and 12 animals). b, Kaplan–Meier survival analysis of syngeneic MMTV-Tg(LINK-A) mice treated with LINK-A LNA, rauwolscine (Rau), ICBs alone or in combination (n = 8, 8, 8, 8, 12 and 12 animals), log rank test, ***P < 0.001. c, Flow cytometry detection of CD3+ /CD8+ cells (top panel) or CD45− /PD-L1+ cells (bottom panels) isolated from MMTV-Tg(LINK-A) tumors with Scr or LINK-A LNA treatment (5 mg kg−1, subcutaneously, every other day, total of seven doses) (n = 10 and 10 animals (top); n = 11 and 11 animals (bottom); ****P < 0.0001). Results are mean ± s.d. P values were determined by unpaired two-tailed Student’s t-test. Numbers in quadrants indicate percentage of cells. d–f, Representative images (d) and statistical analysis of CD8 (e) and granzyme B (GB) (f) of syngeneic MMTV-Tg(LINK-A) mice treated with LINK-A LNA, ICB alone or in combination (n = 7, 7, 7 and 7 animals). Scale bars: 100 μm (d). e, f, **P < 0.01, ***P < 0.001. Results are mean ± s.d. P values were determined by one-way ANOVA.
Despite the genome-wide identification of noncoding RNAs in human diseases, limited genetic evidence has demonstrated the biological importance of IncRNAs in cancer initiation and progression. Tissue-specific expression of LINK-A in mouse mammary glands led to mammary gland carcinogenesis, implicating it as an oncogene. Genome-wide analysis indicated that MMTV-Tg(LINK-A) tumors represent human TNBC genetically, transcriptionally and metabolically. Hence the MMTV-Tg(LINK-A) mouse model provides a valuable tool for studying the underlying molecular mechanisms of TNBC. The molecular mechanisms of LINK-A-driven breast tumors are mediated through multiple signaling pathways: we previously demonstrated that LINK-A expression mediates non-canonical HIP1rl signaling and hyperactivation of the AKT pathway. With genetic evidence, our results suggested that LINK-A inactivates tumor suppressor pathways and downregulates antigen presentation through inactivation of PKA pathways, which is consistent with the previous notion that PKA knockout leads to carcinogenesis. Although the total protein level of TRIM71 exhibited minimal changes under our experimental conditions, phosphorylated TRIM71 faithfully correlated with the outcome of patients with TNBC treated with immunotherapy, suggesting that the LINK–PKA–TRIM71 signaling axis likewise correlates with patient outcomes. These results implicated the potential for these molecules to serve as biomarkers for predicting the outcome of patients with cancer treated with immune checkpoint inhibitors.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41590-019-0400-7.

Received: 12 September 2018; Accepted: 12 April 2019; Published online: 3 June 2019

**References**

1. Dent, R. et al. Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin. Cancer Res.* 13, 4429–4434 (2007).
2. Foulkes, W. D., Smith, I. E. & Reis-Filho, J. S. Triple-negative breast cancer. *N. Engl. J. Med.* 363, 1938–1948 (2010).
3. Chen, L. & Han, X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. *J. Clin. Invest.* 125, 3384–3391 (2015).
4. Jia, H. et al. Immunotherapy for triple-negative breast cancer: existing challenges and exciting prospects. *Drug Resist. Update* 32, 1–15 (2017).
5. Burnet, F. M. The concept of immunological surveillance. *Prog. Exp. Tumor Res.* 13, 1–27 (1970).
6. Leach, D. R., Krummel, M. F. & Allison, J. P. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271, 1734–1736 (1996).
7. Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P. & Yamaguchi, T. Regulatory T cells: how do they suppress immune responses? *Int. Immunol.* 21, 1105–1111 (2009).
8. Johnsen, A. K., Templeton, D. L., Sy, M. & Harding, C. V. Deficiency of transporter for antigen presentation (TAP) in tumor cells allows evasion of immune surveillance and increases tumorigenesis. *J. Immunol.* 163, 4224–4231 (1999).
9. Tran, E. et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science* 344, 641–645 (2014).
10. Snyder, A. et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N. Engl. J. Med.* 371, 2189–2199 (2014).
11. Premsner, I. R. & Chinnaiyan, A. M. The emergence of IncRNAs in cancer biology. *Cancer Discov.* 1, 391–407 (2011).
12. Lin, C. & Yang, L. Long noncoding RNA in cancer: wiring signaling circuitry. *Trends Cell Biol.* 28, 287–301 (2018).
13. Wapinski, O. & Chang, H. Y. Long noncoding RNAs and human disease. *Trends Cell Biol.* 21, 354–361 (2011).
14. Lin, A. et al. The LINK-A IncRNA activates normoxic normoxic HIF1alpha signalling in triple-negative breast cancer. *Nat. Cell Biol.* 18, 213–224 (2016).
15. Lin, A. et al. The LINK-A IncRNA interacts with PtdIns(3,4,5)P3 to hyperactivate AKT and confer resistance to AKT inhibitors. *Nat. Cell Biol.* 19, 238–251 (2017).
45. Nguyen, D. T. T. et al. The ubiquitin ligase LIN41/TRIM71 targets p53 to antagonize cell death and differentiation pathways during stem cell differentiation. *Cell Death Differ.* **24**, 1063–1078 (2017).

46. Honda, R., Tanaka, H. & Yasuda, H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.* **420**, 25–27 (1997).

47. Brooks, C. L. & Gu, W. p53 regulation by ubiquitin. *FEBS Lett.* **585**, 2803–2809 (2011).

**Acknowledgements**

The Proteomics and Metabolomics Facility was supported in part by a Cancer Prevention Research Institute of Texas (CPRIT) grant (no. RP130397) and a National Institutes of Health (NIH) grant (no. 1S10OD012304-01 to D.H.H.). This project is partially supported by University of Houston (UH) Seq-N-Edit Core with funding from UH Division of Research; UH College of NSM and Department of Biology & Biochemistry; NRUF MINOR CORE-17 Grant (to P.H.G.) and UH Small Core Equipment Program Grant (to P.H.G.). We thank the core facilities at BCM: Metabolomics Core (NIH grant no. P30CA125123), CPRIT Proteomics and Metabolomics Core Facility (grant no. RP170005 to N.P.) and Dan L. Duncan Cancer Center. This work was supported by grant nos. R01CA216426, R01CA220297 and U01CA214263 from NIH and L27430-RSG-15-105-01-CNE from the American Cancer Society (to N.P.). This project was partially supported by the NIH T32 Training Grant in Cancer Biology (grant no. ST32CA186892 to L.-C.C.). This project was also supported by Cancer Prevention & Research Institute of Texas grant (no. RR150085 to L.H.). This work was supported by NIH R01 awards (nos. 1R01CA218025-01 and 1R01CA231011-01), CPRIT individual investigator research award (nos. RP150094 and RP180259) and Department of Defense Breakthrough Award (no. BC180196 to L.H.). This work was also supported by NIH R01 awards (nos. 1R01CA218036-01), CPRIT First-time Faculty Recruitment Award (no. R1218), Department of Defense Breakthrough award (no. BC151465), The American Association for Cancer Research-Bayer Innovation and Discovery Grant (no. 18-80-44) and Andrew Sabin Family Foundation Fellows Award (to L.Y.).

**Author contributions**

L.Y. and C.L. conceived the project and designed the experiments. Q.H. and L.-C.C. executed the primary studies. Q.H., T.K.N. and A.L. developed genetic mouse models and related experiments with assistance of Y.L. and Y.Z. Y.Y. and L.H. performed bioinformatics analysis with assistance of J.G., J.L. and J.Y. The histological staining and corresponding analysis were performed by W.X., K.L. and Q.H. D.H.H. executed MS analysis. RNA-seq was performed by Y.P., S.S.C. and P.H.G. Metabolic profiling was performed and analyzed by C.C., S.R.D., C.R.A., V.P., A.S. and N.P. K.W.E. and E.M. assisted with the syngeneic mouse model. Clinical specimens were ascertained and processed by A.S.C. A.A.S. J.R.M. M.-C.H., D.Y., G.N.H., L.C. and M.A.C. contributed to experimental design and data interpretation. P.K.P. and S.D.E. assisted with manuscript drafting and figure presentation. L.Y. and C.L. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41590-019-0400-7.

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**Methods**

**In vivo murine models and treatment procedures.** All animal-based research was conducted according to the guidelines and requirements set forth by the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 as amended by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center. Applied STEMCELL Inc’s proprietary TARGATT Tg mouse technology was used to generate the LINK-A Tg mouse model. In brief, the generated pBET7R-PGK-LaserLINK-A vector contains the transgene for qC31 integrase-mediated recombination. A mixture of the construct and qC31 integrase mRNA was microinjected into the pronucleus of each of 80 zygotes in the FVB genetic background using TARGATT Technology, and the injected zygotes were implanted into four CD1 foster mice. Twenty-three mice were born from the microinjection. Successful integration from the founder mice was identified by PCR analysis of genomic DNA using primers spanning LINK-A. After breeding, two male positive founders (Tg-LINK-A mice) were identified. All mice had an FVB genetic background. Tg-LINK-A mice were crossed with MMTV-cre mice (Tg(MMTV-cre)1Mam; The Jackson Laboratory) to produce mice with LINK-A transgene expression in the mammary glands. MMTV-Tg(LINK-A) and Tg(LINK-A) female mice were used as experimental and control mice, respectively. All of the mice genotyping primer sequences can be found in Supplementary Table 5.

For the prevention treatment, MMTV-Tg(LINK-A) mice started the LINK-A LNAs (5 mg/kg (mg per kg body weight), subcutaneously (SubQ), every other day), or rauwolscine (5 mg/kg intraperitoneally (i.p.), daily) treatment from 12 weeks of age, and the treatment was terminated at 28 weeks of age. After treatment, mammary gland tissues were collected for morphology and immunohistochemistry analysis. For the regression treatment, MMTV-Tg(LINK-A) mice bearing mammary tumors up to 150 mm³ were randomly assigned to treatment groups and injected with the following drugs: Scr LNAs or LINK-A LNAs (5 mg/kg SubQ, every other day). Tumors were measured three times per week, and mice were euthanized once the ethical endpoint was reached (tumor volume of 1,500 mm³, as determined by measuring the minimum and maximum tumor diameters and using the following formula: \( V = \frac{4}{3} \pi r^3 \)). Mice mammary glands and lungs were collected for morphology and immunohistochemistry analysis.

For establishment of the syngeneic MMTV-Tg(LINK-A) model, MMTV-Tg(LINK-A) mice bearing mammary tumors up to 600 mm³ were euthanized, and the tumors were excised. Tumors were dissociated as a single cell using the gentle MACS Dissociator (Miltenyi Biotec) with a mouse Tumor Dissociation kit (Miltenyi Biotec). A single-cell suspension was generated after filtration through a 70 μm cell strainer (BD Falcon). Single-cell suspensions were used for primary culture or transplant injection. Cells (40,000 per gland) were injected into the right inguinal fat pad of 4-week-old female FVB/N recipients. Three weeks after transplantation, when mammary tumors had reached a size of about 150 mm³, mice were randomly assigned to treatment groups and injected with combinations of the following drugs: Scr or LINK-A LNAs (5 mg/kg SubQ, every other day; QIAGEN), rauwolscine hydrochloride (5 mg/kg i.p., daily; Tocris), JTE 907 (5 mg/kg i.p., daily; Tocris), CGP 54626 hydrochloride (5 mg/kg i.p., daily; Tocris), tropicamide (5 mg/kg i.p., daily; Tocris), cyprophenyl hydrochloride (5 mg/kg i.p., daily; Tocris), and anti-PD-1 (clone RMP1-14; Bio X Cell) and anti-CCL2 (clone 2F10-11; Bio X Cell) antibodies in 100 μg/mouse ip. For the syngeneic model, an isotype control antibody (clone LTF-2; Bio X Cell) 200 μg every 72 h. Tumors were measured three times per week, and mice were euthanized once the ethical endpoint was reached. After treatment, mice tissues were collected for morphology, flow cytometry and immunohistochemistry analysis.

**Tissue samples.** TNBC tissues from patients who responded or did not respond to pembrolizumab (10 mg/kg, every 2 weeks, total of 3–4 months) were purchased from Boston Biosource Inc. Fresh frozen breast carcinomas and their adjacent normal tissues were obtained from Duke University. The study protocol was approved by the Institutional Review Board of Duke University Health System. All tissue use procedures were in compliance with informed consent policy. Clinical information is summarized in Supplementary Table 1.

**Immunohistochemistry, immunofluorescence and Duolink PLA.** For multiplex immunohistochemistry staining, formalin-fixed, paraffin-embedded (FFPE) human tissues cut at a thickness of 5μm were prepared, and the staining was conducted using a PD-L1, CD3e and CD8 Multiplex IHC Antibody Panel (65713; Cell Signaling Technology) according to vendor’s instructions and imaged with a confocal microscope (Zeiss). Duolink PLAs were performed following the manufacturer’s instructions (Sigma), using antibodies targeting TRIM71, TAP1, TAP2, CALR and TPSN. In brief, cells on round-cover glass slips were fixed in 4% PFA at 25°C for 1 h after PBS washing followed by 0.5% Triton X-100 for 10 min and then treated in accordance with the Duolink assay kit instructions. Antibody information is summarized in Supplementary Table 6. The confocal microscope (LSM700; Carl Zeiss) was used for image analysis. The number of PLA signals per cell was calculated. Immunofluorescence and immunohistochemistry were performed as previously described. Antibody information is summarized in Supplementary Table 6.

**Cell lines, transfection, treatment and cellular assays.** Human TNBC cell lines MDA-MB-231, MDA-MB-468, BT549 and HCC-1187; human HER2ʰ breast cancer cell lines BT474 and SK BR-3; human estrogen receptor (ER)ʰ breast cancer cell lines MCF7, T47D and ZR-75-1; human normal mammary gland epithelial cell line MCF10A; and mouse mammary gland epithelial cell line NMuMG were purchased from American Type Culture Collection. SJ/CL1 immortalized normal mouse TNBC cell line, B16-OVA (mouse melanoma cell line, a gift from H. Patrick) and B16F10 (mouse melanoma cell line) were maintained using standard media and conditions, and the Characterized Cell Line Core Facility (MD Anderson Cancer Center). Lincode SMARTpool short interfering RNA targeting Trim71 (636931) was used in this study, and the LNAs targeting LINK-A and the scrambled sequence were purchased and synthesized by Exiqon. Short interfering RNA, LNA and plasmid transfections were performed using DharmaFECT4 (Dharmacon) and Lipofectamine 3000 (Life Technologies), respectively. B16F10 was used to stably express LINK-A by selection with G418 (1,500 μg/mL). To induce LINK-A overexpression, we added 50 mM 5'-doxycycline (Sigma) to the culture medium at the indicated time. For the IFN-γ treatment, cells were treated with mouse IFN-γ (10 ng/mL).

**Plasmid construction, protein recombinantion and purification.** Mammalian expression vectors for full-length LINK-A and the deletion mutants were constructed by subcloning the gene sequences into a pcDNA3.1(+) backbone and pludn20 inducible lentiviral expression vector (Life Technologies). Mammalian expression of full-length TRIM71, PKA-Gα, CNR2, GABR1, ADBA2, ACMA, OPRM and mutant vectors was constructed by subcloning the corresponding gene sequences into the His-tagged expression vector (pcDNA-DEST40) using the Gateway system (Life Technologies). Bacteria expression of full-length CNR2, GABR1, ADBA2, ACMA, OPRM and mutant vectors was constructed by subcloning the corresponding gene sequences into the GST-tagged expression vector pGEX-5X-1. Single-point and deletion mutations were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Recombinant proteins were expressed in the E. coli strain BL21 CodonPlus (DE3) -RIPL (Agilent Technologies) and purified using the Protein Purification Kit (Chontec).

**Immunoprecipitation and immunoblotting.** Cells, human tumor samples, mouse normal mammary gland tissues and mouse breast tumor samples were homogenized in 1x RIPA buffer (EMD Millipore) supplemented with Protease/Phosphatase Inhibitor Cocktail (Pierce, Thermo Scientific), panobinostat (Selleck Chemicals) and methylstat (Sigma-Aldrich). Lysates were cleared by centrifugation at 13,000g for 15 min at 4°C. Supernatants were analyzed for immunoblotting or immunoprecipitation with the indicated antibodies, and the immunoprecipitated protein was subjected to either SDS-PAGE or protein identification by MS. For the denaturing immunoprecipitation, tissues were lysed using TSD buffer (50 mM Tris-HCL, pH 7.5, 1% SDS, 5 mM DTT) boiled for 10 min. The cleared lysates were used for immunoprecipitation. The elutions were loaded on NuPAGE 4–12% Bis-Tris Gel (GeneScript) and then analyzed for immunoblotting with the indicated antibodies summarized in Supplementary Table 6.

**RNA biology assays.** Total RNA isolation and quantitative PCR with reverse transcription were performed as previously described. Total RNAs in mouse normal mammary glands and mouse breast cancer samples were analyzed for LINK-A and Actb expression using biotin-labeled LNA probes (Exiqon) according to the manufacturer’s instructions for the NormazMax Kit (Ambion). Mammalian expression using RNAscope probe (designed by Advanced Cell Diagnostics) and image quantification were performed as previously described using a RNAscope 2.5 High Definition Assay kit according to the manufacturer’s instructions (Advanced Cell Diagnostics). For DNA fluorescence in situ hybridization (FISH), single-cell suspensions from freshly harvested Tg(LINK-A) mammary
gland epithelial cells or MMTV-Tg(LINK-A) mammary tumor cells were used to
hybridize probes targeting full-length LINK-A using a FISH Tag DNA Kit
according to the manufacturer’s instructions (F32947; Thermo Scientific). The
RNA counts were measured as previously described.19 All of the primer
and probe sequences are listed in Supplementary Table 5.

Alpha assay. Alpha assays were performed in accordance with the manufacturer’s
instructions (PerkinElmer).20 The Kd of the interaction between biotin-labeled
phosphatidylinositol phosphates and GST-tagged GPCR recombinant proteins
was determined in the Alpha indirect format using a competition experiment
in which untagged GPCR recombinant proteins were titrated from 0.4 mM to
0.05 nM. More specifically, triplicate samples containing the indicated GPCRs
and phosphatidylinositol phosphates at the indicated concentrations diluted in
protein-liquid binding buffer (25 mM Tris-C1, 150 mM NaCl, 0.1% Tween 20, 1%
non-fat milk, 2 mM calcium chloride, 1 mM magnesium chloride, 100 µM
sulfate, a volume of 10 µl to each well of a 96-well assay plate and then incubated at
25°C for 1 h. A total of 10 µl streptavidin AlphaLISA acceptor beads (100 µg/ml)−1 was
added to each well. The plate was placed on an orbital shaker for 10 min and then
incubated at 25°C for 1 h. Following incubation, 10 µl Alpha glutathione donor
beads (100 µg/ml)−1 were added for 30 min at 25°C. The plate was read on the EnSpire Multimode Plate Reader (PerkinElmer) (wavelength: 615 nm). The competitive inhibition curves were generated based on Alpha
signal readings by fitting to a non-linear regression saturation binding model and a log
(inhibitor) versus response-variable slope (four parameters) model, respectively
(GraphPad Prism 7 software).

Flow cytometry. Cell lines of 1 × 10⁶ cells per condition were stained with the
appropriate antibodies diluted in DPBS (Corning) plus 2% FBS (Gibco) for
30 min at 25°C. Matched fluorescence minus one staining for each condition was
performed as a control. Mouse tissues and tumors were dissociated as a single
cell using the gentleMACS Dissociator (Miltenyi Biotec) with the mouse Tumor
Dissociation kit (Miltenyi Biotec). After lysis of red blood cells (RBC Lysis Buffer;
BioLegend), single-cell suspensions were blocked with anti-CD16/32 (BioLegend)
for 20 min on ice and then incubated with the appropriate antibodies for 30 min
at 25°C. Mouse antibodies were purchased from BioLegend unless otherwise
indicated: CD45, CD3, CD8 and H-2K/K. H-2K/K bound to SIFPEKL, β-M, PD-L1,
F4/80, CD11b and Ly6G/Ly6C. Human antibodies were β-M, HLA-A, HLA-B and
HLA-C. To distinguish live/dead cells, we used Zombie Violet (BioLegend) fixable
viability dyes. Flow cytometry was performed on an LSRII (BD Biosciences),
30 min at 25°C. Matched fluorescence minus one staining for each condition was
performed as a control. Mouse tissues and tumors were dissociated as a single
sample, which was referred to as relative immune cell abundance. Then, we a-fat
factor (M2 macrophages) were transplanted into each sample and inoculated
macrophages; monocytes; mast cells; eosinophils; neutrophils; activated,
plasmacytoid and immature dendritic cells; natural killer cells; natural killer T
cells and myeloid-derived suppressor cell). We used the gene set variance analysis
(GSVA) program to calculate the absolute enrichment score of gene signatures
for immune cells in each sample, which was referred to as relative immune
cell abundance. We used the gene set variance analysis (GSA) to identify LINK-A
expression level and GSVA score for each type of immune cell across cancer
samples and or different subtypes of breast cancer, considering FDR < 0.05 as
statistical significance.

Mutation calling. Whole exome sequencing reads were aligned to the mouse
reference (GRCm38/38) using Burrows-Wheeler Aligner (BWA 0.7.17).21 BAM
files were processed using the Genome Analysis Toolkit22 to improve alignment
accuracy. We identified somatic point mutations through four popular callers,
including VarScan2 (ref. 23), MuTect2 (ref. 24), MuSE (ref. 25) and SomaticSniper
(ref. 26), and only reported mutations called by at least two callers. To further
reduce false positives and missed germline events, we removed any mutations
called by MuTect2 in at least two normal samples24. We obtained TCGA TNBC
mutations from http://gdac.broadinstitute.org/ as previously described.27 Whole
exome sequencing data were deposited to the NCBI Sequence Read Archive
(PRJNA453620).

Statistics and reproducibility. The experiment was set up to use three to eight
samples per repeats per experiment/group/condition to detect a twofold difference
with a power of 0.8 and a significance level of 0.05 using a two-sided test for
significance studies. Each of these experiments was independently repeated three
times. Analyses of relative gene expression were determined using the 2−ΔΔCt
method with GAPDH as the internal reference gene. Results are reported as
mean ± s.e.m. or s.d. of at least three independent experiments, as indicated by the
figure legends. Each exact n value is indicated in the corresponding figure legend.
Statistical analysis was performed using GraphPad Prism 7 software. Comparisons
were analysed using unpaired Student’s t-test or one-way analysis of variance
(ANOVA) test (NS, P > 0.05, *P < 0.05, **P < 0.01 and ***P < 0.001), as indicated in the
individual figures. Fisher’s exact test was implemented for statistical analyses of
the correlation between markers and clinical parameters. Kaplan–Meier survival
curves were compared using the log rank test.

Reporting Summary. Further information on research design is available in the
Nature Research Reporting Summary linked to this article.

Data availability
The breast cancer RNA-seq data used to analyze LINK-A expression were derived from
the TCGA Research Network (http://cancergenome.nih.gov/), and the breast cancer RNA-seq BAM files were downloaded from the UCSC Cancer
Genome Browser (CGHub; CGHub.ucsc.edu) database. RNA-Seq data for all human
tissue experiments have been provided as Supplementary Table 1. Supplementary
Tables 5 and 6 provide information about the oligonucleotides and antibodies
used in this study, respectively. The raw RNA-seq data for this manuscript
are available at GEO under the accession number GSE113143. Whole exome
sequencing data were deposited to the NCBI Sequence Read Archive, with the ID
PRJNA453620. All other data are available from the corresponding author upon
reasonable request.

References
48. Yasgar, A., JadHAV, A., Simeonov, A. & Coussens, N. P. Alphascreen-based
assays: ultra-high-throughput screening for small-molecule inhibitors of
challenging enzymes and protein-protein interactions. Methods Mol. Biol. 439,
77–98 (2016).
49. Xing, Z. et al. IncRNA directs cooperative epigenetic regulation downstream
of chemokine signals. Cell 159, 1110–1125 (2014).
50. Hu, Q. et al. LncRNAs-directed PTEN enzymatic switch governs epithelial-
mesenchymal transition. Cell Res. 29, 266–304 (2019).
51. Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from
RNA-Seq data with or without a reference genome. BMC Bioinform. 12,
323 (2011).
52. Li, J. et al. TANRIC: an interactive open platform to explore the function of
lncRNAs in cancer. Cancer Res. 75, 3728–3737 (2015).
53. Kim, D., Langmead, B. & Salzberg, S. L. HISAT2: a fast spliced aligner with
low memory requirements. Nat. Methods 12, 357–360 (2015).
54. Pertea, M. et al. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* **33**, 290–295 (2015).
55. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754–1760 (2009).
56. DePristo, M. A. et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat. Genet.* **43**, 491–498 (2011).
57. Koboldt, D. C. et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res.* **22**, 568–576 (2012).
58. Cibulskis, K. et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat. Biotechnol.* **31**, 213–219 (2013).
59. Fan, Y. et al. MuSE: accounting for tumor heterogeneity using a sample-specific error model improves sensitivity and specificity in mutation calling from sequencing data. *Genome Biol.* **17**, 178 (2016).
60. Deng, J. et al. Comparative genomic analysis of esophageal squamous cell carcinoma between Asian and Caucasian patient populations. *Nat. Commun.* **8**, 1533 (2017).
61. Ye, Y. et al. The genomic landscape and pharmacogenomic interactions of clock genes in cancer chronotherapy. *Cell Syst.* **6**, 314–328.e312 (2018).
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a  Confirmed

☐  The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐  A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐  The statistical test(s) used AND whether they are one- or two-sided

☐  Only common tests should be described solely by name; describe more complex techniques in the Methods section.

☐  A description of all covariates tested

☐  A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐  A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐  For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.

☐  For Bayesian analyses, information on the choice of priors and Markov chain Monte Carlo settings

☐  For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐  Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  Proteome Discoverer v1.4, EnSpire Multimode Plate Reader Software, Bio-Rad CFX Manager Software, BD FACSDiva software

Data analysis  Microsoft Excel, GraphPad Prism v7.0, MasterPlex ReaderHit v2.0, ImageJ v1.52c, MaxQuant v1.5.8.3, metaX, Inform Analysis Software, Flowio

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The breast cancer RNA-seq data used to analyze LINK-A expression were derived from the TCGA Research Network: http://cancergenome.nih.gov/, and the breast cancer RNA-seq BAM files were downloaded from the UCSC Cancer Genomics Hub (CGHub, https://cgub.ucsc.edu/). Source data for all human tissue experiments have been provided as Supplementary Table 1. Supplementary Tables 5,6 provide information of oligonucleotides and antibodies used in this study, respectively. The raw RNA-seq data for this manuscript are available at GEO under the accession number GSE113143. Whole exome sequencing data was deposited to NCBI Sequence Read Archive, with ID as [PRJNA453620]. All other data are available from the corresponding author on reasonable request.
Field-specific reporting

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- Ecological, evolutionary & environmental sciences

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

No statistical method was used to predetermine sample size. Sample sizes were determined based on previous studies in the field to enable statistical analyses and ensure reproducibility.

Data exclusions

No data were excluded from this study.

Replication

Each of these experiments was independently repeated for 3-5 times. H & E staining, immunohistochemistry staining or immunofluorescence staining were representative of 3-7 animals.

Randomization

Experiments described here were not randomized.

Blinding

The investigators were not blinded to allocation during experiments and outcome assessment, because all data were acquired from cell or tissue samples of specific genotypes or with designated genetic manipulations.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a                              | n/a     |
| ☐ Antibodies                     | ☐ ChiP-seq |
| ☐ Eukaryotic cell lines          | ☐ Flow cytometry |
| ☐ Palaeontology                  | ☐ MRI-based neuroimaging |
| ☐ Animals and other organisms    |         |
| ☐ Human research participants    |         |
| ☐ Clinical data                  |         |

Antibodies

Antibodies Company Catalogue Number Clone Dilution Application

- Elk1 Alpha Invitrogen MAS-13191 1DS 1:100 (IHC), 1:1000 (IB) IHC, IB
- PR Cell Signaling Technology 3153 C39F7 1:100 (IHC), 1:1000 (IB) IHC, IB
- HER2 Invitrogen MAS-13675 385 1:100 (IHC), 1:1000 (IB) IHC, IB
- TAP1 LSBio LS-B14012 Polyclonal 1:200 IHC
- TAP2 LSBio LS-B14864 Polyclonal 1:200 IHC
- TAPBP/Tapasin LSBio LS-C331792 Polyclonal 1:250 IHC
- CALR / Calreticulin LSBio LS-B9387 Polyclonal 1:100 IHC
- CD8α Cell Signaling Technology 70306 CE-144B 1:250 IHC
- CD8α Cell Signaling Technology 98941 D4W2Z 1:200 IHC, IF
- CD3ε Cell Signaling Technology 85661 D7A6E 1:250 IHC, IF
- PD-1 Cell Signaling Technology 13684 11L3N 1:250 (IHC, IF), 1:1000 (IB) IHC, IF, IB
- EpCAM Cell Signaling Technology 93790 E6V8Y 1:250 IHC
- PD-1 Cell Signaling Technology 64988 D5V3B 1:200 IHC
- CD3 Novus Biologicals N0600 1A41 1P7 1:200 IHC
- CTLA-4 Santa Cruz Biotechnology sc-376016 F-8 1:100 IHC
- Granzyme B Abcam ab4059 Polyclonal 1:200 IF
- p53 Invitrogen MAS-12453 PAb 122 1:50 IHC
- Rb Invitrogen MAS-11387 F18 (Rb1) 1:50 IHC
- PCNA Cell Signaling Technology 13110 D3H8P 1:200 IHC
- Cleaved Caspase-3 Cell Signaling Technology 9529 D3E9 1:250 IHC
- Phospho-PKA C (Thr197) Cell Signaling Technology 5661 D45D3 1:1000 IB
- Phospho-TRIM71 (Ser53) Yenzy Antibodies Custom generated Polyclonal 1:1000 IB
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

Human TNBC cell lines: MDA-MB-231, MDA-MB-468, BT549, HCC-1187; human HER2-positive breast cancer cell lines: BT474, SK-BR-3; human ER-positive breast cancer cell lines: MCF7, T47D, ZR-75-1; human normal mammary gland epithelial cell line: MCF10A and mouse mammary gland epithelial cell line: NuMuMG were purchased from American Type Culture Collection (ATCC); SUM-149 (human TNBC cell line), B16-OVA (mouse melanoma cell line, a gift from H. Patrick), B16F10 (mouse melanoma cell line) were maintained using standard media and conditions.

B16F10 were constructed to stably express LINK A by selection with G418 (1500 µg/mL).

Human target PKAalpha cat, PKAbeta cat, CNR2, ADA2A, GABR1, ACM4 and OPRM specific sgRNA sequences are listed in Supplementary Table 5. MDA-MB-231 and MCF10A cells were constructed to stably express Cas9 and sgRNAs by selection with puromycin (1µg/mL). Single clones were obtained by serial dilution. LINK-A Poltns[3,4,5]P3 binding motif deficient cell lines were generated using the CRISPR/Cas9 genome editing system by the Gene Editing/Cellular Model Core Facility (MD Anderson Cancer Center).

Authentication

The cell lines were authenticated by short tandem repeats (STR) profiling performed by MDACC Characterized Cell Line Core Facility.

Mycoplasma contamination

All of the cell lines were free of mycoplasma contamination tested by MDACC Characterized Cell Line Core Facility using MycoAlert kit.
Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

For experiments involved LINK-A knock-in model 12-weeks old female FVB mice were used. All mice were on a FVB genetic background. We crossed Tg-LINK-A mice with MMTV-cre mice [Tg(MMTV-cre);Mam, The Jackson Laboratory] to produce mice with LINK-A transgene expression in the mammary glands.

For establishment of syngeneic MMTV-Tg[LINK-A] model, MMTV-Tg[LINK-A] mice bearing mammary tumors up to 600 mm3 were euthanized, and the tumor was excised. Tumors were dissociated as a single cell using the gentle MACS Dissociator (Miltenyi Biotec Inc) with the mouse Tumor Dissociation kit (Miltenyi Biotec). A single-cell suspension was generated after filtration through a 70-mm cell strainer (BD Falcon). Single-cell suspensions from freshly harvested MMTV-Tg[LINK-A] mammary tumors were counted and resuspended in transplantation buffer containing 50% growth factor-reduced Matrigel (BD Pharmingen). Cells (40,000 per gland) were injected into the right inguinal fat pad of 4-week-old female FVB/N recipients.

Wild animals

This study does not include wild animals.

Field-collected samples

This study does not include field-collected samples.

Ethics oversight

Institutional Animal Care and Use Committee (IACUC) of the University of Texas M.D. Anderson Cancer Center (MDACC)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cell lines: 1 x 10^6 cells per condition were stained with the appropriate antibodies diluted in DPBS (Corning) plus 2% FBS (Gibco) for 30 min at 25°C. Matched fluorescence minus one (FMO) staining for each condition was performed as a control. Mouse tissues and tumors: mouse tissues and tumors were dissociated as a single cell using the gentleMACS Dissociator (Miltenyi Biotec Inc) with the mouse Tumor Dissociation kit (Miltenyi Biotec), after lysis of red blood cells (RBC Lysis Buffer, Biolegend), single-cell suspensions were blocked with anti-CD16/32 (BioLegend) for 20 min on ice and then incubated with appropriate antibodies for 30 min on 25°C. Mouse antibodies: antibodies were purchased from BioLegend unless otherwise indicated: CD45, CD3, CD8, H-2Kb, H-2Kb bound to SINFEKL, beta2-microglobulin, PD-L1, F4/80, CD11b, Ly6G/Ly6C. Human antibodies: beta2-microglobulin and HLA-A, -B, -C. To distinguish live/dead cells, Zombie Violet (BioLegend) fixable viability dyes were used.

Instrument

BD LSR II flow cytometer

Software

BD FACSDiva software and FlowJo

Cell population abundance

The single cell suspension from mouse tumor tissues were staining with APC-conjugated anti-CD3, APC-Cy7-conjugated anti-CD8 and PE-conjugated anti-CD45, then CD3+CD8+CD45.1+ cells were sorted in BD LSR II with purity of priority.

Gating strategy

A gate was set on the live cell population based on the forward and side scatter properties, and the gating strategy has been provided in Supplementary Fig.8i

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.