Dual Mechanisms of LYN Kinase Dysregulation Drive Aggressive Behavior in Breast Cancer Cells

Highlights

- LYN kinase is a downstream effector of the c-KIT receptor in normal breast cells
- Loss of BRCA1 function hyperactivates LYN via prolyl isomerase 1 upregulation
- The full-length LYN isoform promotes tumor cell invasion
- Time to breast cancer death is shorter in tumors with a high LYN-A::B isoform ratio

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In Brief

Tornillo et al. show that in aggressive breast cancers, LYN activity is deregulated by a change in patterns of splice isoform expression. In BRCA1-dysfunctional breast cancers, LYN activity is upregulated by a prolyl isomerase (PIN1) that is normally repressed by BRCA1.
Dual Mechanisms of LYN Kinase Dysregulation Drive Aggressive Behavior in Breast Cancer Cells

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SUMMARY

The SRC-family kinase LYN is highly expressed in triple-negative/basal-like breast cancer (TNBC) and in the cell of origin of these tumors, c-KIT-positive luminal progenitors. Here, we demonstrate LYN is a downstream effector of c-KIT in normal mammary cells and protective of apoptosis upon genotoxic stress. LYN activity is modulated by PIN1, a protein isomerase, and in BRCA1 mutant TNBC PIN1 upregulation activates LYN independently of c-KIT. Furthermore, the full-length LYN splice isoform (as opposed to the Δaa25–45 variant) drives migration and invasion of aggressive TNBC cells, while the ratio of splice variants is informative for breast cancer-specific survival across all breast cancers. Thus, dual mechanisms—uncoupling from upstream signals and splice isoform ratios—drive the activity of LYN in aggressive breast cancers.

INTRODUCTION

Breast cancers molecularly classified as basal-like breast cancer typically display the triple (ER/PR/HER2)-negative (TNBC) phenotype (Badve et al., 2011). The molecular etiology of sporadic TNBC is still poorly understood, although germline BRCA1 mutations predispose to TNBC, and BRCA1 silencing or dysfunction in the BRCA1 pathway can be found in sporadic TNBC (Badve et al., 2011). Limited therapeutic options are available for TNBC; chemotherapy is often initially beneficial, but TNBC has a high risk of relapse (Liedtke et al., 2008), emphasizing the need to elucidate its biology and identify targets for novel treatment options.

The mammary epithelium consists of luminal cells, including ER-negative (ER−) progenitor-like and ER-positive (ER+) differentiated cells, and basal cells. TNBC likely originates from luminal ER− progenitors, and the gene expression profile of both BRCA1 mutation-associated and sporadic TNBC reflects a luminal progenitor-like profile (Lim et al., 2009; Molyneux et al., 2010). Elucidating the molecular regulation of this cell subset is important to understand not only the normal mammary cell homeostasis but also the origins of TNBC.

Mammary ER− luminal progenitors are characterized by expression of the membrane tyrosine kinase receptor c-KIT (Regan et al., 2012; Smart et al., 2011), which is required for growth and survival of these cells (Regan et al., 2012; Tornillo et al., 2013) as well as the SRC family tyrosine kinase (SKF) LYN (Bach et al., 2017; Regan et al., 2012; Smart et al., 2011), a known effector of c-KIT signaling in hematopoietic cells (Shivakrupa and Linnekin, 2005). Other SFKs are expressed in the mammary epithelium, but other than LYN, only FYN has an expression pattern restricted to a specific population (basal epithelial cells) (Bach et al., 2017; Kendrick et al., 2008). Based on this co-expression, a c-KIT-LYN signaling axis in mammary epithelial progenitors is proposed.

Previous studies have largely focused on LYN function in hematopoietic cells and leukemia, and persistent activation and/or deregulation of LYN has been associated with imatinib resistance in BCR-ABL+ leukemia (Wu et al., 2008). In breast cancer, LYN has been reported as overexpressed and a potential drug target in TNBC by several studies (Choi et al., 2010; Hochgräfe et al., 2010; Molyneux et al., 2010; Regan et al., 2012; Smart et al., 2011). LYN point mutations in breast cancer are rare (0.6%) (https://cancer.sanger.ac.uk/cosmic), but have been associated with anti-estrogen resistance in a subset of ER+ tumors (Schwarz et al., 2014); only 6%–10% of breast cancers show LYN amplification (http://www.cbioportal.org/index.do; https://cancer.sanger.ac.uk/cosmic). Other mechanisms contributing to the underlying LYN dysregulation in TNBC remain to be defined, as does the potential wider role of LYN in breast cancer.

Here we demonstrate that LYN kinase is a transducer of c-KIT growth signals in the normal mammary epithelium. We show that LYN can also be activated by prolyl isomerase 1 (PIN1), normally transcriptionally repressed by BRCA1. In BRCA1-deficient TNBC, loss of this transcriptional repression results in increased PIN1 levels and thus in LYN activation independently of c-KIT. Furthermore, we address the role of the two LYN splice isoforms...
in breast cancer and find that only full-length LYN (LYNA), as opposed to LYN<sup>A25–45</sup> (LYNB), promotes cell migration and invasion. LYN is expressed more highly in TNBC than other breast cancer types; however, we find that a higher ratio of LYN over LYNB is present in breast cancers of patients with shorter survival times, irrespective of tumor subtype. Therefore, our findings demonstrate dual mechanisms, uncoupling from upstream signals and changing splice isoform ratios, driving the activity of LYN in aggressive breast cancers. These mechanisms have the potential to be targeted therapeutically, and the LYN:A:B ratio is a biomarker that could identify patients who would benefit from such interventions.

**RESULTS**

**LYN Kinase Is Regulated by c-KIT and Promotes Growth of Normal Mammary Epithelial Cells**

To define the major components of the c-KIT signaling network in the mammary epithelium, we examined expression of c-KIT and its ligand stem cell factor (SCF) in normal mouse mammary cell populations (Figure 1A). The two splice variants of c-KIT, GNNK<sup>+</sup> and GNNK<sup>−</sup>, were expressed primarily in luminal cells (particularly in the ER<sup>−</sup> luminal subpopulation) (Figure 1B). The two SCF isoforms, soluble SCF (sSCF) and membrane-bound SCF (mSCF), were present at low levels in luminal cells, whereas basal cells showed the highest levels of total SCF, with almost exclusive expression of the sSCF form (Figures 1B and 1C).

LYN is a key effector of c-KIT signaling in hematopoietic cells, and LYN expression and c-KIT expression in the mammary gland are correlated (Regan et al., 2012; Roskoski, 2005). LYN exists in two isoforms, LYNA (full-length LYN) and LYNB (LYN<sup>A25–45</sup>) (Figure 1D). When expression of these isoforms was analyzed by semiquantitative RT-PCR, both LynA and LynB were found in all mammary epithelial populations; however, there was an association between higher LynA and c-KIT expression in the luminal ER<sup>−</sup> compartment (Figure 1B). Therefore, the expression pattern of c-KIT, SCF, and full-length LYN in the mammary epithelium indicated the existence of a basal-to-luminal paracrine c-KIT signaling network, mediated by the soluble form of SCF (sSCF), along with an enrichment of a potential c-KIT effector, LYN, in the SCF-responsive luminal cells.

To determine the signaling cascade activated by c-KIT, we treated primary mouse mammary epithelial cells with SCF and assessed the phosphorylation status of a series of previously described c-KIT effectors (Roskoski, 2005). Addition of SCF caused a marked increase in c-KIT phosphorylation, as well as upregulation of phosphorylation levels of JAK2, STAT3, AKT, and ERK1/2 with distinctive kinetics (Figure 1E). Phosphorylation levels of LYN at its positive regulatory site Y397 were elevated approximately 6-fold within 60 min of stimulation with SCF (Figure 1F), and SCF treatment induced an increase in LYN kinase activity as measured by an immunoprecipitation (IP) kinase assay (Figure 1G). Conversely, c-KIT inhibition, by using short hairpin RNA (shRNA) against c-KIT or a specific anti-c-KIT blocking antibody (ACK2), led to a significant decrease in LYN phosphorylation (Figures 1H and 1I; Figure S1A).

Because c-KIT is required for growth of normal mammary cells in vitro (Regan et al., 2012) and positively regulated LYN activity, we tested whether LYN depletion also affected mammary cell growth. Following LYN knockdown with two distinct shRNAs (shLyn1 or shLyn2) (Figure 2A), primary mouse mammary epithelial cells exhibited defective growth (Figure 2B) and a significant reduction in the expression of the proliferation marker Ki67 (Figure 2C). This effect was observed both in unsorted primary mammary epithelial cells and in the purified luminal ER<sup>−</sup> progenitor population (Figures 2D and 2E; Figure S1B). Furthermore, knockdown of LYN in the human normal mammary epithelial cell line, MCF10A, with two distinct shRNAs caused a significant reduction in relative cell growth and in Ki67 expression compared to shScrambled (shScr) controls (Figures 2F and 2G), without obviously affecting acinar architecture (Figure S1C).

We next tested the ability of a constitutively active LYN (Y508F) mutant to rescue c-KIT knockdown. Whereas overexpression of wild-type LYN (LYNA WT) had no effect on the viability of c-KIT knockdown cells, constitutively active LYN (LYNA CA) rescued the growth defect (Figure S2A). In addition, when we examined the ability of LYN-depleted cells to activate c-KIT downstream effectors in response to SCF, we found that LYN knockdown specifically interfered with AKT phosphorylation upon c-KIT stimulation (Figure S2B). Overall, these findings support the model that c-KIT activates LYN kinase to transduce...
pro-growth and survival signals and activate the AKT pathway in mammary epithelial cells.

**LYN Is Required for Growth of BRCA1-Deficient Mammary Tumor Cells**

We have previously demonstrated that Brca1 mutation-associated breast cancers originate from luminal ER– progenitors (Molyneux et al., 2010) and that c-KIT and LYN are expressed in mouse Brca1 mammary tumor cells (Regan et al., 2012). To determine whether Brca1 mutant mammary cell growth depends on the activation of the c-KIT signaling pathway, primary mouse Brca1 mutant mammary tumor cells transduced with lentiviruses expressing either one of two shRNAs against c-Kit (shKit1 and shKit2) or a control shRNA (shScr) were analyzed. Despite reduced c-Kit expression, no change in cell growth was observed in shKit cells compared to shScr cells (Figure S2C). Furthermore, unlike normal cells, c-Kit-depleted tumor cells had phospho-LYN levels similar to those of control cells (Figure S2D) and treatment of Brca1 tumor cells with the ACK2 c-KIT blocking antibody did not alter LYN phosphorylation status (Figure S2E; contrast with Figure 1I). Likewise, c-KIT knockdown failed to affect phospho-LYN levels in three human c-KIT-positive breast cancer cell lines with low BRCA1 levels: HCC38 (BRCA1 silenced by methylation), HCC1187, and MDA-MB-157 (BRCA1 low due to downregulation by microRNA [miRNA]) (Garcia et al., 2011; Li et al., 2013) (Figure S3A). However, c-KIT knockdown in a c-KIT-positive BRCA1-wild-type cell line, HCC1187, suppressed LYN phosphorylation (Figure S3A). These results indicate that in Brca1/BRCA1 tumor cells, at least in vitro, c-KIT is dispensable for growth and does not regulate LYN activity.

Next, we evaluated the effects of LYN knockdown on Brca1 tumor cell growth. LYN knockdown markedly impaired growth of mouse Brca1 tumor-derived cells in monolayer culture (Figure 3A) and in three-dimensional (3D) culture conditions on Matrigel (Figure 3B). Staining of 3D-cultured tumor cells for the proliferation marker Ki67 showed that the number of proliferating cells was reduced by approximately 30% in shLyn-transduced cultures compared with control (Figure 3C). The kinase activity of LYN was required for its pro-survival functions, because expression of shRNA-resistant wild-type LYN (LYNA*WT) was able to rescue the effect of shLyn transduction, but expression of a kinase-dead LYN (T410K) mutant (LYNA*KD) was unable to do so (Figure 3D). The broad spectrum kinase inhibitor Dasatinib, which was able to block LYN Y397 phosphorylation in mammary epithelial cells in a dose-dependent manner (Figure S3B), inhibited growth of three mouse BRCA1 tumor-derived cell lines (half-maximal inhibitory concentration [IC50] 0.1–1 µM) and the human BRCA1 mutant HCC1937 line (IC50 0.1 µM) (Figures S3C and S3D).

Use of two short hairpins targeting human LYN (Figure 3E) demonstrated that LYN knockdown in human breast cancer cells also significantly impaired cell growth in the BRCA1-mutated HCC1937 human breast cancer cell line (Figure 3F) and in cells from a BRCA1 mutant breast cancer patient-derived xenograft (PDX) (Figure 3G). These effects, therefore, were consistent in both mouse and human cells.

Because LYN blockade effectively suppressed tumor cell growth in vitro, we next evaluated the effects of blocking LYN activity in vivo. Intraperitoneal (i.p.) injection of Dasatinib strongly reduced LYN phosphorylation in the normal mammary epithelium of wild-type mice (Figure 3E), and daily treatment with Dasatinib significantly inhibited growth of Brca1+/–, p53+/– tumors (Figures S3F and S3G). Immunohistochemical staining for phospho-histone H3 (phospho-H3) showed a lower number of mitotic cells in Dasatinib-treated compared to vehicle-treated tumors (Figure 3H).

Reduction of cell numbers following constitutive Lyn knockdown made testing the effects of specific Lyn depletion by shRNA on tumor cell growth in vivo difficult. Therefore, a conditional Lyn knockdown system in which mouse Brca1 tumor cells expressed shRNA against Lyn under the control of doxcycline was established. Analysis of Lyn transcript levels after exposure to doxcycline confirmed that Lyn expression was reduced in inducible shLyn-carrying cells in the presence of doxcycline.
(Figure 3H). Upon orthotopic cell injection in immunodeficient mice, administration of doxycycline resulted in a significant decrease in the growth of tumors derived from cells carrying inducible anti-Lyn shRNA (Figure 3I). Staining of tumor sections for the mitotic cell marker phospho-H3 revealed a reduction in the number of mitotic cells in samples from doxycycline-treated shLyn tumors compared with controls (Figure S3I). Therefore, LYN kinase depletion suppresses Brca1 mammary tumor cell growth both in vitro and in vivo.

**Brca1 Depletion Leads to Upregulation of LYN Kinase Activity in a PIN1-Dependent Manner**

Our data show that in normal mammary epithelial cells, LYN kinase activity is under the strict control of the c-KIT receptor, whereas in Brca1 mutant tumor cells, LYN functions independently of c-KIT. We hypothesized that inactivation of Brca1 might contribute to dysregulation of LYN kinase activity. First, we analyzed a panel of TNBC cell lines for LYN and phospho-LYN (Y397) levels. Three of the lines (MDA-MB-436, SUM-149, and HCC1937) carry inactivating BRCA1 mutations, one (HCC38) has BRCA1 promoter methylation, four (MDA-MB-157, HCC1806, MDA-MB-468, and HCC70) have been reported as having low BRCA1 expression (Buckley et al., 2016; Garcia et al., 2011; Gong et al., 2015; Li et al., 2013), and six (MDA-MB-231, MDA-MB-453, BT-20, BT-549, HCC1143, and HCC1187) are BRCA1 wild-type. Total LYN levels were variable across the lines; however, when phospho-LYN levels were normalized to total LYN, TNBC cells with defective BRCA1 had significantly higher levels of phospho-LYN than those of wild-type cells (Figure 4A). Furthermore, Brca1 knockdown in primary (normal) mouse mammary epithelial cells resulted in increased LYN phosphorylation but unchanged c-KIT phosphorylation (Figure 4B). Conversely, forced overexpression of hemagglutinin-tagged BRCA1 (HA-BRCA1) in primary mammary epithelial cells suppressed LYN phosphorylation (Figure 4C). The prolyl isomerase PIN1 recognizes specific serine-proline or threonine-proline sequences in proteins, changing the conformation of the prolines within these sequences and resulting in altered activity of the target protein (Zhou and Lu, 2016). LYN contains potential PIN1 consensus target sequences (Pro197 and Pro229), and PIN1 is transcriptionally repressed by BRCA1 (MacLachlan et al., 2000). Therefore, we hypothesized that increased LYN activity following BRCA1 inactivation or depletion results from increased PIN1 levels and that PIN1 was activating LYN. To test this, we first used phospho-protein arrays to demonstrate that both BRCA1 overexpression and PIN1 knockdown in MDA-MB-468 cells resulted in a significant reduction in phosphorylation of LYN, but not its close family member SRC (Figures S4A and S4B). Moreover, we confirmed that BRCA1 suppresses PIN1 expression by overexpressing BRCA1 in MDA-MB-468 cells and showing that PIN1 mRNA levels were reduced by approximately 50% (Figure S4C). We also compared PIN1 levels in mouse Brca1 tumor cells and normal mouse mammary epithelium and confirmed that PIN1 levels were significantly higher in the tumor cells (Figure S4D).

Next, we stained a tissue microarray consisting of 15 germline BRCA1 mutant and 15 sporadic TNBC cases. Cases from BRCA1 patients showed, overall, significantly more intense PIN1 staining than did sporadic tumors (Figures 4D and 4E). Given our findings that BRCA1 loss results in PIN1 upregulation, we hypothesized that even in sporadic breast cancers not linked to germline BRCA1 mutation but that have low BRCA1 levels through other mechanisms, levels of BRCA1 and PIN1 expression would be inversely correlated. We therefore normalized to total LYN, TNBC cells with defective BRCA1 had significantly higher levels of phospho-LYN than those of wild-type cells (Figure 4A). Furthermore, BRCA1 mutant PDX-derived cells (BCM 3887) were transduced with control (shScr) or Lyn knockdown (shLyn1 and shLyn2) lentiviruses, seeded at low density in adherent conditions (2D), and stained with crystal violet after 6 days. Viable cell density was determined after absorbance measurement following solubilization of the dye. Representative images of tumor cell colonies at day 6 of culture are shown.
investigated their expression patterns in sporadic TCGA breast cancer cases and, consistent with our hypothesis, observed an inverse correlation between BRCA1 and PIN1 expression levels (Figure S4E).

To demonstrate a direct functional link between PIN1 expression and LYN activity, we knocked down PIN1 in primary mouse Brca1 null cells (Figure 5A) and cells from a BRCA1 mutant human breast cancer cell line (HCC1937) (Figure 5B) and the BRCA1 mutant PDX (Figure 5C). In all cases, knockdown of PIN1 decreased active LYN phosphorylation and cell survival, mimicking the effect of LYN knockdown, but it did not change c-KIT phosphorylation.

To elucidate the relationship of LYN phosphorylation, PIN1, and BRCA1, we silenced PIN1 in a broader panel of TNBC cell lines. In HCC38, MDA-MB-436, HCC1395, and MDA-MB-468 cells (BRCA1 defective), PIN1 knockdown resulted in decreased LYN Y397 phosphorylation (Figures S4F–S4J). In HCC1187 cells (BRCA1 wild-type), PIN1 knockdown did not affect phosphorylation (Figure S4J); in this line, LYN was still regulated by c-KIT (Figure S3A).

Co-immunoprecipitation (coIP) demonstrated that in mouse Brca1 null tumor cells, PIN1 interacted with LYN (Figure 5D). Furthermore, generation of mutants in putative PIN1 consensus target sequences (Figure 5E) showed that proline-to-isoleucine mutation of either residue 197 or both 197 and 229 resulted in a significant increase in inhibitory LYN phosphorylation at the Y508 site (Figure 5F).

We next assessed whether the PIN1–LYN regulatory mechanism is likely to be more widely applicable than just to BRCA1 breast cancer. We therefore knocked down PIN1 in BRCA2 null mammary epithelial cells and in a panel of BRCA1 and BRCA2 null ovarian cancer cells. PIN1 knockdown significantly reduced LYN Y397 phosphorylation in a human BRCA2 mutant breast cancer cell line (Figure S5A) and in primary mouse Brca2 null tumor cells (Figure S5B). However, knockdown of Brca2 in primary normal mouse mammary cells did not alter PIN1 or phospho-LYN levels (Figure S5C). PIN1 knockdown suppressed LYN Y397 phosphorylation in COV 362 cells (BRCA1 mutant ovarian carcinoma) (Figure S5D) and PEO-1 and PEO-4 cells (BRCA2 mutant ovarian carcinoma) (Figures S5E and S5F), but not in KURAMOCHI cells (BRCA2 mutant ovarian carcinoma) (Figure S5G). Therefore, regulation of LYN by PIN1 is a general (but not universal) mechanism, but PIN1 is not regulated by BRCA2. These findings are consistent with transcriptional activity of BRCA1 being involved in PIN1 regulation, as previously shown (MacLachlan et al., 2000).

To further investigate the involvement of specific BRCA1 functional domains in the regulation of the PIN1–LYN axis, and the possibility that different clinically relevant BRCA1 mutants may have different effects on this axis, we re-expressed either the wild-type BRCA1 or clinically relevant BRCA1 missense mutants (C61G in the RING domain, L1407P in the CC motif, and A1708E in the BRCT domain) (Anantha et al., 2017) in the HCC1937 human BRCA1-deficient breast cancer cell line. We found that re-expression of both wild-type and C61G mutant BRCA1 resulted in both decreased PIN1 levels and decreased LYN phosphorylation, while expression of the L1407P and A1708E mutations showed no significant differences compared to control BRCA1 mutant cells (Figure 5G). Therefore, mutation of the N-terminal RING domain (which disrupts binding to BARD1) does not alter the ability of BRCA1 to suppress the PIN1–LYN activation pathway. In contrast, mutation of the coiled-coil domain, affecting PALB2 binding (suggested to be critical for the activation of the BRCA1 transcriptional program, as well as for DNA repair) (Anantha et al., 2017; Gardini et al., 2014), and of the C-terminal BRCT domains, important for interactions with Abraxas, BRIP1, and CtIP (Anantha et al., 2017) and known to be important for BRCA1 transcriptional activity (Hayes et al., 2000; Iofrida et al., 2012), result in elevated levels of PIN1 and LYN activation. These support a model in which the transcriptional activity of BRCA1 is critical in the control of PIN1–LYN pathway activation.

Having established the BRCA1–PIN1–LYN axis, and given the important role of BRCA1 in repair of double-stranded DNA breaks, we examined whether LYN activity could affect the normal mammary cell response to DNA damage. Primary normal mouse mammary epithelial cells expressing LYNA CA were treated with the DNA damaging agent methyl methane sulfonate (MMS), which causes double-stranded breaks. Expression of LYNA CA led to a marked transient increase in Akt phosphorylation, suggesting elevated levels of survival signaling, and a significant reduction in cleaved PARP levels (Figure S6A) and TUNEL staining (Figure S6B), both markers of apoptosis, after MMS treatment relative to control cells. Consistent with this, levels of cleaved caspase-3 were significantly reduced in normal mammary cells expressing LYNA CA, compared to control cells, following treatment with 10 μM cisplatin (Figure S6C) or exposure to 10 Gy of ionizing radiation (Figure S6D).

Figure 4. LYN Activity Is Regulated by BRCA1 via the Prolyl Isomerase PIN1

(A) Protein extracts from TNBC cells with either wild-type BRCA1 or impaired BRCA1 expression were analyzed for phospho-LYN (p-LYN) (Y397), total LYN, and GAPDH levels by western blot. Scatterplot shows quantification of p-LYN levels normalized to total LYN levels.

(B) Primary mouse mammary organoids were transduced with control (shScr) or Brca1 knockdown (shBrca1) lentiviruses. Knockdown was assessed by qRT-PCR relative to comparator shScr cells (left), shScr and shBrca1 cells were assessed for levels of phospho-c-KIT (Y719), phospho-LYN (Y397), LYN, and GAPDH by western blot after 4 days (middle).

(C) Western blot analysis and quantitation of LYN autophosphorylation levels in primary mouse mammary organoids transduced with control (Ctrl) or HA-tagged BRCA1 (HA BRCA1) expression lentiviruses.

(D) Examples of PIN1 immunohistochemistry scores in breast cancer TMAs: (i) 0, (ii) 1, (iii) 2, (iv) 3, and (v) 4. DAB staining of PIN1, and blue counterstaining of nuclei. Scale bar in main panels, 500 μm; scale bar in inset, 50 μm.

(E) Quantitation of PIN1 scoring in BRCA1 mutant and sporadic TNBC TMAs.

Blots in (B) and (C) are representative of three independent experiments. Quantitation is shown as mean and SD (n = 3; two-tailed unpaired t tests) except for gene expression analysis by quantitative real-time RT-PCR (mean ± 95% confidence intervals; significance of real-time RT-PCR data was determined from confidence intervals; n = 3 independent experiments for each of 3 technical replicates per sample) (Cumming et al., 2007). *p < 0.05; **p < 0.01. See also Figure S4.
LYNA Drives Breast Tumor Cell Aggressiveness

We next asked whether the two LYN isoforms, LYNA and LYNB (shown in detail in Figure S7A), play different roles in breast cancer biology, independent of the BRCA1-PIN1-LYN axis. First, we transiently expressed GFP-tagged variants of LYNA and LYNB in MDA-MB-231 cells. After 48 hr, cells were fixed, counterstained with DAPI, and analyzed by confocal microscopy. Both LYNA and LYNB were predominantly membrane localized, with additional foci of intracellular staining, under these conditions (Figure S7B).

Next, we used a LYNA-specific shRNA to knock down LYNA expression in MDA-MB-231 cells. shLynA cells displayed an approximately 60% reduction in LYN protein levels compared to control (shScr) cells (Figure 6A). LYNA knockdown resulted in an overall decrease in cell proliferation (Figure 6A) and a strong reduction in cell migration and invasion in vitro (Figure 6B). To exclude the possibility that the impaired growth, migration, and invasion of shLynA knockdown cells was due to a reduction in total LYN levels, rather than depletion of the LYNA form, and to determine the specific contribution of each LYN variant to the malignant behavior of the cells, we used a knockdown and reconstitution approach. Total LYN was knocked down in MDA-MB-231 cells, and then either a LYNA or a LYNB variant (LYNA* or LYNB*) not targetable by shLyn was re-expressed. We assessed cell growth and the ability of the cells to migrate and invade relative to control cells. Total LYN knockdown led to a decrease in cell growth, but this could be rescued by either LYNA* or LYNB* (Figure 6C), indicating that these two distinct LYN isoforms can compensate for each other in promoting tumor cell growth. LYN knockdown significantly reduced the ability of the cells to migrate and invade; this could be rescued by LYNA*, however, LYNB* was unable to do so (Figure 6D). Therefore, while both LYN isoforms promoted tumor cell growth, only LYNA drove aggressive behavior in these cells.

To determine whether LYNA and LYNB may associate with different protein partners, and whether this might explain their different effects on migration and invasion, we carried out a mass spectrometry analysis of proteins that interact with the two isoforms. LYNA was knocked down in MDA-MB-231 cells, and then either LYNA* or LYNB* was re-expressed. We also expressed a LYNA* variant, LYNA*-Y32F (Figure S7C), Y32 is located within the 21-amino acid segment present in LYNA and has been reported as being regulated by epidermal growth factor (EGF) signaling (Huang et al., 2013); if phosphorylation of this tyrosine was required for the differential behavior of LYNA compared to LYNB, then we would predict LYNA*-Y32F would behave like LYNB. Cultures were established in duplicate, and one set was treated with EGF before lysis (Huang et al., 2013). LYN was immunoprecipitated from these eight conditions (LYN KD, LYNA*, LYNB*, and LYNA*-Y32F; all ± EGF), and lysates were analyzed by tandem mass tagging. The full results and differentially enriched proteins are provided in Table S1. There was little difference between the proteins that co-immunoprecipitated with LYNA* and LYNA*-Y32F, arguing against the hypothesis that LYNA*-Y32F was like LYNB (Figure S7D). The outcome of the analysis of the LYNA*–EGF, LYNB*+EGF, LYNA*-Y32F–EGF, and LYNA*-Y32F+EGF pull-downs, four independent cell preparations, was similar. Furthermore, the list of co-immunoprecipitated proteins included eight previously characterized LYN-interacting proteins (ANKRD54, LIMA1, HNRNPK, MYH9, STAT3, PRKDC, EGF, and HSP90AB1) (Hein et al., 2015; Hornbeck et al., 2015; Huang et al., 2005; Kumar et al., 1998; Mertins et al., 2016; Petschnigg et al., 2014; Taipale et al., 2012; Tausz et al., 2008; Van Seuningen et al., 1995).

By comparing LYN knockdown samples with LYNA*‐ and LYNB*‐expressing samples, several proteins were identified that were differentially enriched in LYNA* samples. Using a cut-off for analysis of proteins that were enriched >1.2-fold both in the LYNA* pull-down compared to the LYN KD pull-down and in the LYNA* pull-down compared to the LYNB* pull-down, we identified 20 candidate LYNA-interacting proteins. We carried out a gene ontology analysis using DAVID (Huang et al., 2009) of the differentially interacting proteins to begin to understand their functional significance. The list of proteins and the results of this analysis are provided in Table S1. Six proteins (ACTC1, ACTG2, KRIT5, LIMA1, MYH3, and TUBA1A) are associated with the cytoskeleton and its regulation, and two proteins (LPXN and TNS1) are associated with integrins and cell adhesion. These findings suggest that LYNA and LYNB may interact differently with cell adhesions and the cytoskeleton, potentially explaining the effects of LYN on migration and invasion.

Figure 5. LYN Is Activated in BRCA1 Null Cells by the Prolyl Isomerase PIN1

(A–C) Primary cells from BlgCre Brca1fl/fl p53−/− mouse mammary tumors (A), human HCC1937 BRCA1-deficient breast cancer cells (B), and BRCA1 mutant PDX-derived cells (C) were transduced with control (shScr) or Pin1 knockdown (shPin1#1 and shPin1#2) lentiviruses and lysed after 72 hr. Protein extracts were assessed for levels of Pin1, LYN, phospho-LYN (Y397), and c-KIT (Y719) (PDX samples were not probed for phospho-KIT). Representative western blots and quantitation of phospho-LYN (Y397) levels are shown. GAPDH was used as loading control. shScr-, shPin1#1-, and shPin1#2-transduced BigCre Brca1fl/fl p53−/− tumor cells and HCC1937 cells were also seeded at low density in adherent conditions and stained with crystal violet after 6 days. Cell number was determined by absorbance measurement following solubilization of the dye. PDX-derived transduced cells were cultured for 10–12 days in 3D on Matrigel and then assayed for cell viability.

(D) Protein extracts from primary BigCre Brca1fl/fl p53−/− mouse tumor cells transduced with vectors carrying wild-type LYN were subjected to immunoprecipitation by anti-PIN1 or control (IgG) antibodies. Total proteins (input) and immunoprecipitates (IPs) were probed for PIN1 and LYN by western blot.

(E) Schematic of LYN showing the position of PIN1 consensus recognition sequences and the proline isoleucine mutants generated.

(F) Representative western blot analysis of LYN phosphorylation levels at the negative regulatory phosphorylation site (Y508) in primary BigCre Brca1fl/fl p53−/− transduced with vectors carrying wild-type LYN or LYNA proline mutants (LYN P229I, LYN P197I, or LYN P197I P229I).

(G) Western blot analysis of LYN autophosphorylation and PIN1 levels in human HCC1937 cells transduced with either control (Ctr) lentivirus or virus-carrying HA-tagged wild-type or mutant BRCA1 (C61G, A1708E, or L1407P). Blots are representative of three independent experiments. Quantitation is shown as mean and SD (n = 3; two-tailed unequal t tests). *p < 0.05; **p < 0.01; ***p < 0.001. See also Figures S5 and S6.
LYN Splicing Is Regulated by ESRP1
To determine what might regulate the balance between LYN A and LYN B expression, we first examined Affymetrix Human Exon 1.0ST array gene expression profiles of a breast cancer cohort from Guy’s Hospital, London, and the TNBC subset of these cancers. Cohorts were split into high-LYN A- and low-LYN A-expressing tumors (i.e., above and below median expression of Affymetrix probe 3098998, uniquely targeting the N-terminal region of LYN A), and the expression levels of 270 splicing regulators (the spliceosome) (Table S2) (Papasaikas et al., 2015) were interrogated. We found that in ‘all breast cancers’ (Figure S8A) and the TNBC subset (Figure S8B), high-LYN A tumors had significantly lower spliceosome levels than those of low-LYN A tumors, indicating that splicing in general might be compromised. Next, we examined the expression of a splicing regulatory protein (ESRP1/RBM35A) with putative consensus sequences in LYN intron 2 (Figure S8C). We found that ESRP1 levels were lower in high-LYN A breast cancers as a whole (Figure S8D) and in the high-LYN B TNBC subset (Figure S8E). When ESRP1 was knocked down in MCF7 cells (Figure S8F), which normally have a LYN A:B ratio of <2, the A:B ratio was significantly increased to a mean of 2.5±1 (Figure 6E). Furthermore, when ESRP1 was overexpressed in MDA-MB-231 cells, which normally have a LYN A:B ratio of >3, this ratio was significantly reduced (Figure 6F), Therefore, a decrease in the expression of the spliceosome in TNBC, and in particular ESRP1, could result in an increased LYN A:B ratio.

Patients with a High Tumor LYN A:B Ratio Have Shorter Survival
Because LYN A drives aggressive migratory and invasive properties in breast cancer, we asked whether total LYN A expression levels, the relative amounts of LYN A and LYN B, or the LYN A:B ratio might have prognostic potential. First, we analyzed the relative expression of the LYN A and LYN B isoforms in samples of human normal mammary tissue, as well as triple-negative (TN) and ER+PR+ primary breast cancers. The ratio of LYN A to LYN B transcripts was close to 1 in the normal samples, but LYN A was preferentially expressed in TNBC (Figure S9A). No significant difference in relative LYN A:B transcript ratio was observed in ER+:PR+ tumors compared to normal samples (Figure S9A). Similar results were observed in a small panel of human breast cancer cell lines (basal ER−MDA-MB-231, MDA-MB-468, and HCC1143 and luminal ER+ MCF7) (Figure S9B). However, while we could be confident that the tumor samples and cell lines in this analysis were predominantly composed of tumor cells, the normal tissue samples had not been purified and likely contained a mixture of normal epithelial cell populations and non-epithelial cells. Therefore, for a more accurate assessment of the LYN A:B ratio in normal human tissue, we used established flow cytometry protocols to purify the basal, luminal progenitor, luminal ER+ differentiated, and stromal cell populations from reduction mammoplasty samples from four individuals (Figures 7A–7C; Figure S10). Analysis of LYN A:B demonstrated that the luminal progenitor population had a significantly higher ratio compared with the other populations and that the LYN A:B ratio in normal cells was in a similar range to that of the tumor samples. To expand our analysis, we investigated a panel of breast cancer cell lines (Heiser et al., 2012) and the Guy’s Hospital TNBC-enriched breast cancer cohort (Gazinska et al., 2013) for the expression of the LYN A isoform using the Affymetrix probe 3098998. The LYN A sequence was significantly more highly expressed in basal and claudin-low cell lines than in luminal cell lines (Figure S9C), and in the Guy’s dataset, it was more highly expressed in tumors classified by PAM50 (Parker et al., 2009) as basal (Figure S9D) or by immunohistochemistry as TNBC (Figure S9E).

Next, we interrogated LYN A and LYN B expression in TCGA breast cancer RNA sequencing (RNA-seq) data. Consistent with the microarray-based results, LYN A was expressed more highly in TNBC than in non-TNBC (p = 6.528e−18, Wilcoxon rank-sum test). Moreover, LYN B was higher in TNBC than non-TNBC (p = 1.554e−20, Wilcoxon rank-sum test), although it showed overall lower expression levels than LYN A (p = 2.23e−3, Wilcoxon rank-sum test, for TNBC; p = 3.35e−22, Wilcoxon rank-sum tests, for non-TNBC) (Figure 7D). There was no difference in LYN A:B ratio for normal breast tissue, TNBC, and non-TNBC in the TCGA dataset (Figure 7E). When we investigated the distributions of LYN A:B ratios across all tumors, we noted that while most sample ratios were in the range seen in the purified normal breast cells (Figure 7C),
there was a distinct population of breast cancers with a log2 RSEM expression ratio of >7 (Figure 7F). When this population was compared for time to breast cancer-specific death with the remaining TCGA breast cancer cases, it had a shorter median time for survival (p = 0.032 for >7.3) (Figure 7G).

**DISCUSSION**

Although it has been previously reported that LYN is one of the most highly expressed SFKs in the normal mammary gland (Bach et al., 2017; Kendrick et al., 2008; Smart et al., 2011), its function in this tissue has not previously been investigated. LYN associates with c-KIT in hematopoietic cells and participates in numerous SCF-induced responses by promoting either positive or negative downstream signaling, depending on cell type and context (Shivakrupa and Linnekin, 2005). Our results demonstrate that LYN is activated by c-KIT and is critical for SCF:c-KIT-dependent phosphorylation of AKT in mammary progenitors. However, given that LYN has been implicated in other signaling pathways promoting cell survival and proliferation (Shivakrupa and Linnekin, 2005), it cannot be ruled out that additional pathways in mammary progenitors may be regulated by LYN.

c-KIT+ ER− mammary luminal progenitors are considered the cell of origin of BRCA1-mutated and sporadic TNBC (Lim et al., 2009; Molyneux et al., 2010). Although c-KIT is highly expressed in Brca1 mutant mammary tumors (Regan et al., 2012; Smart et al., 2011), as well as in a subset of breast cancers within the TNBC group (Jansson et al., 2014), targeting this receptor has not been an effective therapeutic approach (Yardley et al., 2006). Our findings may at least partly explain why these trials have failed. Although carriers of BRCA1 germline mutations have an 80% lifetime risk of breast cancer, such cases make a small contribution to breast cancer in the general population. However, BRCA1 was found to be silenced through promoter methylation in 14% of sporadic basal-like and 11% of non-basal-like breast cancers, while in two special subtypes of TNBC, medullary and metaplastic breast cancer, promoter methylation was found in >60% of cases (Badve et al., 2011; Turner et al., 2007). Furthermore, BRCA1 mRNA expression was two-fold lower in TNBC compared to matched controls, and this was suggested to depend on upregulation of ID4, a negative regulator of BRCA1 transcription (Turner et al., 2007).

BRCA1 levels can also be suppressed by other epigenetic mechanisms, such as activity of miRNAs (Garcia et al., 2011; Li et al., 2013). Therefore, activation of the PIN1-LYN axis by BRCA1 downregulation is more widely applicable than to BRCA1 germline mutation carriers alone.

PIN1 can be aberrantly activated in human cancers by various mechanisms, including changes in transcription, translation, and/or post-translational modifications (Zhou and Lu, 2016). In addition to being a target for BRCA1 transcriptional activity, PIN1 is a direct transcriptional target of E2F (Ryo et al., 2002). PIN1 mRNA stability is also inhibited by miRNAs, while the phosphorylation and/or sumoylation status of specific PIN1 residues has been reported to be critical for PIN1 substrate binding and/or catalytic activity (Zhou and Lu, 2016).

PIN1 specifically catalyzes cis-trans proline isomerization within phosphorylated Ser/Thr-Pro motifs with important effects on phosphorylation-dependent signaling. Numerous oncogenes and tumor suppressors are directly regulated by PIN1 (Zhou and Lu, 2016), and here we show that PIN1 is an important contributor to LYN hyperactivation in BRCA1 mutant tumor cells. Consistent with PIN1 substrates typically containing one or few target motifs, LYN has only two putative PIN1 consensus sites (Ser196-Pro197 and Ser228-Pro229). LYN phosphorylation at Ser196 is only predicted, but phosphorylation at Ser228 has been previously observed during cell-cycle progression (Daub et al., 2009), although the specific kinase or kinases involved are still unknown. These two sites are located in the SH2 domain and in the SH2-Kinase domain linker segment, respectively, which are involved in intra- and/or intermolecular interactions critical for the regulation of the open-closed LYN conformation, suggesting that local structural changes upon proline isomerization are likely to affect LYN activation status. Our findings suggest that regulation of LYN by PIN1 is a widely applicable mechanism of regulation of this SFK but that SRC is not a target of PIN1 (Figure S4B); whether other SFKs are PIN1 targets remains to be investigated.

The link between BRCA1 loss of function and LYN activation and the activation of LYN by signaling pathways that promote cell survival, growth, and invasion are important findings. In normal cells, the absence of functional BRCA1 results in genomic instability, which leads to p53 activation, followed by cell-cycle arrest and apoptosis (Roy et al., 2011), implying that additional molecular alterations are required for BRCA1 mutant...
cells to survive and undergo malignant transformation. Not surprisingly, TP53 mutations are frequently present in BRCA1-associated mammary tumors (Roy et al., 2011). As LYN hyperactivation suppressed cell death induced by DNA damage, aberrant LYN activation following BRCA1 loss could facilitate neoplastic progression, allowing BRCA1 loss-of-function cells to survive long enough to accumulate TP53 genetic alterations. Furthermore, activation of AKT downstream of LYN has been linked to ubiquitination and degradation of the p53 protein (Dos Santos et al., 2013; Iqbal et al., 2010), and this would enable functional suppression of the p53 pathway in BRCA1 mutant cells before genetic pathway suppression. There is some evidence that LYN is generally anti-apoptotic (Aira et al., 2018), and this warrants further investigation in breast cancer.

Alternative splicing is a critical post-transcriptional regulatory mechanism for many cancer-associated genes (Bonomi et al., 2013). LYN kinase exists as two isoforms, full-length LYN (LYNA) and LYN (LYNB), differing by a 21-amino acid insert found in the unique NH2-terminal domain (Alvarez-Errico et al., 2010). We have found that in breast epithelial cells, the balance between these transcripts is modulated by the splicing factor ESRP1. LYN has not been found among the ESRP1-regulated alternative spliced genes resulting from previous analyses (Shapiro et al., 2011; Warzecha et al., 2009, 2010), most likely due to the lack of representative probe sets in the array platforms used in those studies. Nevertheless, like LYN, known ESRP1 target genes play a role in cell motility, cell adhesion, and/or epithelial-mesenchymal transition (Shapiro et al., 2011; Warzecha et al., 2009, 2010), indicating that co-regulation by ESRP1 of splicing of transcripts for proteins that may function, together with LYN, in a pro-migratory and invasive pathway in TNBC cells.

We find that patients with breast cancer with a high LYN::LYN ratio have a shorter time to breast cancer death. Biologically, this clinical phenotype could be a result of LYN conferring migratory and invasive properties on breast cancer cells. How alteration of the LYN::LYNB ratio can generate signal outputs leading to cancer cell aggressiveness remains to be fully defined. Previous analysis of LYN and LYNB function in mast cells revealed the two isoforms associate differentially with phosphoproteins (Alvarez-Errico et al., 2010), indicating that the 21-amino acid sequence governs protein interactions. Moreover, LYNA was more potent than LYNB in activating Phospholipase C gamma (PLCγ) and downstream Ca2+ signaling (Alvarez-Errico et al., 2010). In addition, unlike LYNB, LYNA kinase activity can be enhanced through phosphorylation by EGFR at a specific tyrosine residue (Y32) within the 21-amino acid insert (Huang et al., 2013). However, in an analysis of proteins differentially interacting with the LYN isoforms, we saw little effect of either EGF stimulation or Y32F mutation in the 21-amino acid insert. We did find that LYN interacted more strongly with proteins associated with the cytoskeleton, integrins, and cell adhesion, pointing to differential effects of LYNA and LYNB on migration and invasion. This warrants further work.

Identification of patients who will respond to targeted, novel, or repurposed therapies remains a major goal of clinical research. Our findings demonstrate that patients with BRCA1 dysfunction or with a high LYN::B isoform ratio would be particularly likely to benefit from specific therapies targeting LYN kinase. Furthermore, our findings on the key dual mechanisms of LYN regulation, combined with knowledge of LYN interaction partners, will enable rational design of new compounds to specifically block the oncogenic signaling driven by LYN without the need to directly target the kinase domain, increasing treatment specificity and reducing the likelihood of off-target effects.

**STAR METHODS**

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  - In vitro and in vivo Dasatinib treatment
  - Immunofluorescence staining
  - Immunohistochemistry and analysis of BRCA1 tumor TMA
  - Isoform specific expression analysis by Affymetrix
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  - LYN pull-down for Tandem Mass Tag (TMT) labeling
  - TMT Labeling and High pH reversed-phase chromatography
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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes ten figures and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.11.103.

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES


programme is abrogated during the epithelial-mesenchymal transition. EMBO J. 29, 3286–3300.


### STAR★METHODS

#### KEY RESOURCES TABLE

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**Biological Samples**

| RNA samples from human breast tumor tissue and reduction mammoplasties | Breast Cancer Now Tissue Bank | Anonymized |
| Normal breast tissue from women (n = 4; aged 15, 24, 35, 39 years) undergoing reduction mammoplasty with no previous history of breast cancer | Cruz Roja, Clínica Indautxu | Anonymized |
| Human breast cancer patient-derived xenograft (PDX) BCM 3887 | Baylor College of Medicine; an MTA may be required for distribution of this material | Zhang et al., 2013 |
| Human BRCA1 breast cancer tissue microarray | Northern Ireland Biobank via Niamh Buckley, Queen’s University Belfast; an MTA may be required for distribution of this material | N/A |

**Chemicals, Peptides, and Recombinant Proteins**

| Dasatinib                  | Selleckchem, Strathech, Newmarket, Suffolk, UK | Cat# S1021 |
| Soluble murine SCF         | Peprotech, London, UK | Cat# 250-03 |

**Critical Commercial Assays**

| Proteome Profiler Human Phospho-Kinase Array Kit | R&D Systems, Abingdon, Oxford, UK | Cat# ARY003B |
| ApopTag® Red In Situ Apoptosis Detection Kit | Merck Millipore, Watford, Hertfordshire, UK | Cat# S7165 |
| Mouse Tumor Dissociation Kit for GentleMACS | Miltenyi Biotec, Bisley, Surrey, UK | Cat# 130-096-730 |

**Experimental Models: Cell Lines**

| COV362                     | European Collection of Authenticated Cell Cultures (ECACC) | Cat# 07071910 |
| PEO1                       | European Collection of Authenticated Cell Cultures (ECACC) | Cat# 10032308 |

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**Experimental Models: Organisms/Strains**

- 10 week old virgin female FVB mice
  - Charles River, Margate, Kent, UK
  - FVB/NCl
  - Stock# 012620

- *Trp53<sup>tm1Brd</sup> Brca<sup>1fl/fl</sup>Alav<sup>tg(LGB-cre)74Acl/J</sup> (BigCre Brca<sup>1fl/fl p53<sup>+/−</sup> </sup>) mice
  - The Jackson Laboratory, Bar Harbor, Maine, USA
  - Hay et al., 2009

- *BlgCre Brca2<sup>fl/fl</sup> p53<sup>+/−</sup> mice*
  - In house; an MTA may be required for distribution of this material

- *NOD SCID<sup>γ</sup> mice*
  - Charles River, Margate, Kent, UK
  - NSG

**Oligonucleotides**

- See Table S3 for details of oligos used for RT-PCR, site directed mutagenesis, PCR cloning, shRNA and siRNA knockdown, SYBR Green qRTPCR oligos and TAQman qrtPCR assays

**Non-Targeting siRNA Pool #1**
- Dharmacon, Cambridge, UK
  - Cat# D-001206-13-05

**ON-TARGETplus ESRP1 siRNA**
- Dharmacon, Cambridge, UK
  - Cat# L-020672-01-0005

**Recombinant DNA**

- **pEGFP-N3**
  - Prof Vladimir Buchman, Cardiff University
  - N/A

- **pENTR™/H1/TO**
  - Thermo Fisher Scientific, Life Technologies, Paisley, UK
  - Cat# K4920-00

- **ESRP1 cDNA**
  - Prof Klaus Holzmann, Institute of Cancer Research, Medical University of Vienna
  - Leontieva and Ionov, 2009

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Professor Matt Smalley (SmalleyMJ@cardiff.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All animal work was carried out under UK Home Office project and personal licenses following local ethical approval and in accordance with local and national guidelines, including ARRIVE guidelines. Normal primary mammary cells were prepared from fourth mammary fat pads of 10 week-old virgin female FVB mice. The BigCre Brca1^{fl/fl} p53^{+/−} and BigCre Brca2^{fl/fl} p53^{fl/fl} mice and the tumors they generate have been fully described previously (Hay et al., 2009; Molyneux et al., 2010).
Human Tissue
Normal breast tissue was obtained from women (n = 4; aged 15, 24, 35, 39 years) undergoing reduction mammoplasty with no previous history of breast cancer. Patients provided written informed consent and the procedures were approved by the local Hospital Research Ethics Committee and by the ‘Ethics Committee of Clinical Investigation of Euskadi’.

The human breast cancer patient-derived xenograft (PDX) BCM 3887 derived from a patient with a BRCA1 mutation (Zhang et al., 2013) was passaged in NOD scid gamma (NGS) mice.

RNA samples from human tumor tissue were obtained from Breast Cancer Now Tissue Bank. Normal tissue samples were from reduction mammoplasties, selected to contain > 50% epithelium. All tumor samples (10 ER+PR+HER2- and 10 Triple Negative) were from primary tumors of no specific type, grade III, from pre-menopausal patients.

Cell lines
Cells were maintained at 37°C in a 5% CO₂ atmosphere with the exception of MDA-MB-157, which were kept in L-15 medium with 10% FBS, streptomycin (100 µg/ml) and penicillin (100 U/ml) in a free gas exchange with atmospheric air.

MCF10A cells were maintained in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 10 µg/ml insulin, 1 ng/ml cholera toxin, 100 µg/ml hydrocortisone, 50 µM penicillin and 50 µg/ml streptomycin (growth medium). BT-549, KURAMOCHI, MCF-7, MDA-MB-231 and MDA-MB-436 cells were cultured in RPMI 1640 medium with 10% FBS, L-glutamine (4 mM), streptomycin (100 µg/ml) and penicillin (100 U/ml). HCC38, HCC-70, HCC1143, HCC1187, HCC1395, HCC1599, HCC1806 and HCC1937 cells were cultured in modified RPMI-1640 medium (ATCC 30-2001) supplemented with 10% FBS, streptomycin (100 µg/ml) and penicillin (100 U/ml). COV-362, MDA-MB-453 and MDA-MB-468 cells were grown in DMEM with 10% FBS, streptomycin (100 µg/ml) and penicillin (100 U/ml). COV3937 and MDA-MB-468 cells stably overexpressing BRCA1 were previously generated (Buckley et al., 2011) and were grown in the presence of puromycin (1 µg/ml). BT-20 cells were grown in MEM added with 10% FBS, non-essential amino acids (0.1 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), streptomycin (100 µg/ml) and penicillin (100 U/ml). PEO-1 and PEO-4 cells were cultured in RPMI 1640 medium with 10% FBS, L-glutamine (2 mM), sodium pyruvate (2 mM) streptomycin (100 µg/ml) and penicillin (100 U/ml). SUM-149 cells were grown in Ham’s F-12 medium containing 5% FBS, HEPES (10 mM), insulin (5 µg/ml), hydrocortisone (1 µg/ml), streptomycin (100 µg/ml) and penicillin (100 U/ml). See Key Resources Table for more details.

METHOD DETAILS
Isolation of and culture of normal mouse mammary epithelial cells
All animal work was carried out under UK Home Office project and personal licenses following local ethical approval and in accordance with local and national guidelines, including ARRIVE guidelines.

Single cells were prepared from fourth mammary fat pads of humanely killed 10 week-old virgin female FVB mice. Intramammary lymph nodes were removed prior to tissue collection. Fat pads were finely minced on a McIlwain Tissue Chopper and then digested for 1 hr at 37°C in 3 mg/ml collagenase A / 1.5 mg/ml trypsin (both from Sigma, Poole Dorset, UK) in serum-free L15 medium (ThermoFisher Scientific, Life Technologies, Paisley, UK) with gentle rotation. Tissue fragments (‘organoids’) released from the fat pad were washed and then incubated for 5 min in Red Blood Cell Lysis buffer (Sigma), washed and then plated for 1 hr at 37°C in DMEM/10%FBS (ThermoFisher) to partially purify fibroblasts by differential attachment. Organoids were then poured off, pelleted, washed twice with versene (ThermoFisher) and then incubated for 15 min in serum-free Joklik’s Low Calcium medium (Sigma) at 37°C. They were then pelleted and resuspended in 2mls of 0.25% trypsin / 0.02% EDTA in HBSS (Sigma) and incubated for 2 min 37°C to release single epithelial cells. 5 ml of 5 µg/ml DNase I (Sigma) in serum-free L15 was then added to digest DNA liberated from any lysed cells. Single epithelial cells were then pelleted and washed in L15/10% FBS (ThermoFisher Scientific, Life Technologies, Paisley, UK) and then resuspended at 10⁶ cells/ml in L15/10% FBS (Regan et al., 2012; Smalley, 2010; Smalley et al., 2012).

Cell suspensions were stained with combinations of anti-CD24-FITC (1.0 µg/ml; BD Biosciences, Oxford, UK), anti-CD45-PE-Cy7 (1.0 µg/ml; BD Biosciences), anti-SCA-1-APC (1.0 µg/ml; eBioscience, Hatfield, UK) or anti-SCA-1-PE (1.0 µg/ml; BD Biosciences) antibodies and DAPI. Cells were then sorted on a FACSAria flow cytometer (BD Biosciences) to partially purify fibroblasts by differential attachment. Organoids were then recovered, pelleted, washed twice with versene (ThermoFisher) and then incubated for 15 min in serum-free Joklik’s Low Calcium medium (Sigma) at 37°C. They were then pelleted and resuspended in 2mls of 0.25% trypsin / 0.02% EDTA in HBSS (Sigma) and incubated for 2 min 37°C to release single epithelial cells. 5 ml of 5 µg/ml DNase I (Sigma) in serum-free L15 was then added to digest DNA liberated from any lysed cells. Single epithelial cells were then pelleted and washed in L15/10% FBS (ThermoFisher Scientific, Life Technologies, Paisley, UK) and then resuspended at 10⁶ cells/ml in L15/10% FBS (Regan et al., 2012; Smalley, 2010; Smalley et al., 2012).

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For 3D cultures, cells were resuspended in complete growth medium (DMEM:F12 with 10% FBS (ThermoFisher Scientific), 5 µg/ml insulin (Sigma, Poole, UK), 10 ng/ml cholera toxin (Sigma) and 10 ng/ml epidermal growth factor) supplemented with 2.5% growth factor reduced Matrigel (BD Biosciences, Oxford, UK) and plated in 96- or 48- well plates onto Matrigel (40 ul or 100 ul per well, respectively). Cultures were maintained at 37 °C in a 5% CO₂/5%O₂ atmosphere in a Galaxy 170R Incubator (New Brunswick, Eppendorf, Stevenage, UK). Stimulation with soluble murine SCF (Peprotech, London, UK) (100 ng/ml) and treatment with anti-c-Kit (ACK2) or IgG isotype control antibodies (50 µg/ml) were carried out after starving cells for 12 hr.

Phase-contrast images were taken using a Leica MI6000B microscope (10X PH1 objective) and the LAS AF software.
**Preparation and flow cytometric separation of normal breast cells from reduction mammoplasty**

Normal breast tissue was obtained from pre-menopausal women undergoing reduction mammoplasty, with no previous history of breast cancer, who gave their informed consent. All samples were confirmed by histopathological examination to be free of malignancy. Immediately upon arrival at the laboratory, breast tissue was cut up manually into small pieces (approximately 0.5 cm cubed). Breast material was incubated in an equal volume of Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) supplemented with 5% fetal calf serum (FCS) and collagenase (Type I, Sigma) to a final concentration of 0.2 mg/ml, and digested (while shaking) overnight at 37°C. Following enzyme digestion, breast cells were washed and the organoids separated from any undigested material. The organoids were then isolated from blood cells, fibroblasts, and endothelial cells by sequential filtration and back flushing from 140 and 53 μm pore size polyester monofilament meshes. Organoids were then disaggregated with 0.05% trypsin-EDTA and finally filtered through a 40 μm sieve (BD) to yield a predominantly single cell suspension. Cells were immediately processed for flow cytometric cell sorting on the basis of CD49f, ESA and 7-AAD staining (see Figure 7 and Figure S10) (Iriondo et al., 2015).

For CD49f/ESA staining, FITC-conjugated anti-ESA antibody and APC-conjugated anti-CD49f antibody were used (see Key Resources Table). In all cases, control samples were stained with isotype-matched control antibodies; the viability dye 7-aminoactinomycin D (7AAD) (BD) was used for dead cell exclusion and fluorescence minus one (FMO) controls were used to define the gates (Iriondo et al., 2015). In all cases, cells were analyzed and sorted using a FACSAnia (Becton Dickinson) flow cytometer. Data were analyzed using FACSDiva software.

**Primary tumor cell isolation and culture**

Primary epithelial cells (from three distinct tumors (namely #1, #2, #3) from each mouse model or from three PDX implants) were obtained using the gentle MACSTM Dissociator and Mouse Tumor dissociation kit (Miltenyi Biotec, Bisley, Surrey, UK) following the manufacturer’s recommendations using the protocol for ‘Dissociation of Tough Tumors’ for mouse tumors and the protocol for ‘Dissociation of Soft and Medium Tumors’ for the PDX. To ensure efficient dissociation volumes of Enzyme D, Enzyme R and Enzyme A were scaled up according to the size of the tumor piece (100 μL, 50 μL and 12.5 μL respectively per each 0.5 cm³). The optional steps - the short spin for collection of the dissociated material at the bottom of the MACS tube and red blood cell lysis - were included in the procedure.

Mouse cells were cultured in complete growth medium in 2D adherent conditions for expansion or in 3D for functional studies. Cells up to passage 5 were used for all the experiments in this study. Freshly isolated human PDX cells were grown in HuMEC Ready Medium (Thermo Fisher Scientific) in Matrigel in 3D. Cultures were maintained at 37°C in a 5% CO2/5%O2 atmosphere in a Galaxy 170R incubator (New Brunswick, Eppendorf).

**Protein extraction and western blot analysis**

3D cultured primary mammary cells were released from Matrigel using the BD cell recovery solution and lysed in Laemmli buffer. Protein extracts were separated by SDS-PAGE, transferred to PVDF membranes (IPVH00010, Merck Millipore, Hertfordshire, UK) and immunoblotted with antibodies detailed in the Key Resources Table. GAPDH or alpha-tubulin were used as loading controls. Probing was performed using enhanced chemiluminescent (ECL) reagents (WBLUF0100, Merck Millipore). Protein extracts (400 μg) from Ctr, BRCA1-, siCtr- and siPin1-MDA-MB-468 cells were processed and analyzed for phosphorylation of LYN (Y397) and SRC (Y419) using the Human Phospho-Kinase Antibody Array (R&D Systems) following the manufacturer’s instructions.

**Immunoprecipitation (IP) LYN kinase assay**

Once recovered from Matrigel, 3D cultured cells were lysed in RIPA buffer (50mM Tris/HCl, pH 7.5, 150mM NaCl, 1% Triton X-100, 1% Na deoxycolate, 0.1% SDS) supplemented with 1mM Na orthovanadate and protease inhibitor-coccktail (Roche, Burgess Hill, West Sussex, UK). After centrifugation (14000 g for 10 min at 4°C), supernatants (150 μg of protein per sample) were pre-cleared with protein A-Sepharose beads (GE Healthcare) for 45 min at 4°C and washed twice with 20 mM HEPES, pH 7.4, 5 mM MgCl2, 3 mM MnCl2 1mM, 1mM Na orthovanadate (kinase buffer). Beads were then resuspended in 50 μL of kinase buffer with 2.75 μg of acid denatured enolase (Sigma), 5-10 μCi of γ32P ATP (PerkinElmer, Seer Green, Buckinghamshire, UK) and 1 μM cold ATP. After a 10 min-incubation at 30°C, the reaction was stopped by adding 13 μL of 10mM ATP, 50 mM EDTA and samples were subjected to SDS-PAGE on a 10% acrylamide gel. Gels were fixed in 10% methanol/ 10% acetic acid solution, then dried and developed by autoradiography. Intensities of bands corresponding to phosphorylated enolase were measured using the ImageJ software.

**LYN-PIN1 co-immunoprecipitation**

Primary BlgCre Brca1fl/fl p53−/− mouse tumor cells were collected in cold PBS pH 8.3 buffer with 10 mM EDTA, 0.1% Tween 20, 10 mM Sodium Fluoride, 1 mM Sodium Orthovanadate, 10 mM Sodium Pyrophosphate, 100 mM β-Glycerophosphate, 2 mM PMSF, complete Protease Inhibitors (Roche) and lysed by passing through a 26G needle. After centrifugation (14000 g for 15 min at 4°C), supernatants (3-4 mg of protein) were pre-cleared with protein A-Sepharose beads (GE Healthcare) for 45 min at 4°C prior to incubation with anti-Pin1 (rabbit polyclonal (H-123), sc-15340, Santa Cruz) or control (IgG) antibodies overnight at 4°C. After
incubation with protein A-Sepharose beads for 45 min at 4°C, immunoprecipitates were pulled down by centrifugation (900 g for 5 min at 4°C), washed five times with lysis buffer and eluted with Laemmli buffer. Samples were then resolved by SDS-PAGE on 10% polyacrylamide gels (15 × 15 cm). Western blot analysis was carried out as described above.

**Gene expression analysis**

With the exception of purified human primary cell populations (see below), RNA was extracted using the RNeasy Mini Kit (QIAGEN, Manchester, UK) from freshly isolated primary mouse mammary cells and 2D cultured cells. Alternatively, Trizol reagent (ThermoFisher Scientific, Paisley, UK) was used for RNA isolation from 3D cultured cells. cDNA synthesis was carried out using QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacturer’s instructions.

Semi-quantitative PCR reactions (28 cycles) were performed using GoTaq® PCR Core System reagents (Promega, Southampton, UK) and up to 120 ng of cDNA as template. Primers are listed in Table S3. PCR products were separated by electrophoresis on a 2% agarose gel with the exception of c-Kit PCR products, which were resolved on a 4% agarose gel.

Quantitative real-time PCR (qPCR) was carried out using TAQMAN (Applied Biosystems, Life Technologies, Paisley, UK) Assays-on-Demand probes or Fast SYBR green Master Mix (Table S3) on freshly isolated RNA. Results were analyzed using the Δ-ΔCt method normalized to β-actin or GAPDH and expressed as relative to a comparator sample.

For normal primary human breast cell populations purified by flow cytometry, RNA was isolated using the Machery-Nagel NucleoSpin RNA, according to instructions of the manufacturer. DNase-treated RNA was used to synthesize cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen, 11754050), following the manufacturer’s protocol. Semi-quantitative-PCR was performed using Phusion High-Fidelity DNA Polymerase (Thermofisher Scientific, F530S) and Deoxynucleotide (dNTP) Mix, PCR Reagents (Sigma, D7295) on a MyCycler thermal cycler (Bio-Rad). 10 ng of cDNA was used as template and amplified using the following conditions: 95°C for 15 min, 22 cycles of amplification (95°C for 30 s, 59°C for 30 s, 72°C for 1 min) and a final extension at 72°C for 5 min. Primer (Invitrogen) sequences can be found in the Table S3. Finally, PCR products were separated by 1.5% agarose gel and stained with GelRed Nucleic Acid Gel Stain (Biotium). GAPDH was used as an internal control.

**Cell viability and growth assays**

Cell density in 2D cultures of primary cells and HCC1937 was determined by absorbance measurement following fixation and staining with crystal violet. CellTiterGlo cell viability reagent (Promega, Southampton, UK) was used to assess relative cell number of 3D cultured primary cells and MDA-MB-231 cells. The GelCount platform and software (Oxford Optronix, Oxford, UK) were used to automatically determine the size of organoids grown in 3D.

**Cell migration and invasion assay**

Invasion and migration assays were performed using 24-well Transwell inserts (Corning, Amsterdam, the Netherlands) coated or not with Matrigel, respectively. After 24 hr-starvation cells (75,000) were resuspended in serum-free (250 μL) medium and seeded into the upper chamber. 750 μL of medium supplemented with 10% serum was added to the lower chamber. After 20 hr, cells on the lower side of the insert were fixed, stained with crystal violet and counted under a light microscope.

**siRNA Transfection**

MDA-MB-468 cells were transfected with Pin1 or control siRNA (Table S3) using Lipofectamine RNAiMax reagent (ThermoFisher Scientific) in Opti-MEM serum-free medium (ThermoFisher Scientific). MCF7 cells were transfected with control (Non-Targeting siRNA Pool #1, Dharmacon; see Key Resources Table) or ESRP1 siRNA (ON-TARGETplus, Dharmacon; see Key Resources Table) using DharmaFECT 4 Transfection Reagent (Dharmacon). All analyses were performed 72 hr after transfection.

**Lentiviral vectors and cell transduction**

pLKO.1 lentiviral vectors carrying shRNA directed to Brca1, c-Kit and Lyn were selected from the corresponding pLKO.1 target gene MISSION TRC shRNA sets (Sigma; see Key Resources Table). The c-Kit knockdown oligos target both c-Kit isoforms.

For Lyn, Pin1, c-Kit and Brca2, Pin1 knockdown experiments, DNA Oligonucleotide pairs for shRNA specifically targeting LynA, Pin1, Pin1 or shScr were ligated into the pENTR™/U6 Gateway system entry vector (ThermoFisher Scientific). Hairpin sequences were verified and then transferred, together with the U6 promoter, into a Gateway-modified pSEW lentiviral vector (Regan et al., 2012) by LR reaction (ThermoFisher Scientific). ORFs for Lyn mutants (LynCA and LynTK), mouse LynB, human LYNB, human LynA Y32F, Lyn variants resistant to shLyn and human BRCA1 (C61G, L1407P, A1708E) mutants were generated using the Quickchange Lightning site-directed mutagenesis kit (Agilent Technologies, Stockport, Cheshire, UK) according to the manufacturer’s instructions. Primers and templates used are listed in Table S3. Successful mutagenesis was verified by sequence analysis. WT or mutagenized ORFs were then inserted into a Gateway modified pWPI lentiviral vector (Regan et al., 2012) by LR reaction. WPI lentiviral vectors carrying HA-wt BRCA1, BRCA1 mutants (C61G, L1407P, A1708E) or ESRP1-FLAG ORFs were obtained following a similar strategy (further details in Table S3; the ESRP1 plasmid was kindly provided by Prof Klaus Holzmann) (Leontieva and Ionov, 2009).

Viral supernatants were generated by co-transfection of the expression vector and two packaging vectors (psPAX2 and pMD2.G) into HEK293T cells. Cells were refed with fresh medium (DMEM/10% FBS; ThermoFisher) after 24 hr. Supernatants were harvested.
48 and 72 hr after transfection, checked for absence of replication-competent virus and stored at −80°C until use. Lentiviruses derived from pWPI and pHIV-H2BmRFP plasmids were concentrated by ultracentrifugation (50,000g, 2 hr at 4°C). Relative lentiviral titer was determined by transducing NIH 3T3 cells using serial dilutions of the viral preparations. Freshly isolated primary cells were resuspended in viral supernatant (shRNA-carrying vectors) or concentrated viral particles in growth medium (overexpression vectors) and plated on to Matrigel or plastic as required for the specific assay. After 24 hr, medium was replaced with fresh medium (Regan et al., 2012). Puromycin (Sigma) (1.5 μg/ml) was added to culture medium of cells transduced PLKO.1 lentiviral vectors 36 hr after infection.

**Generation and expression of LYN-GFP fusion proteins**

ORFs for human LYN A and LYN B were cloned into pEGFP-N3 (EcoRI/BamHI). Primers and templates used are listed in Table S3. MDA-MB-231 cells were transiently transfected with pEGFP-N3-LYN A or pEGFP-N3-LYN B plasmids using Lipofectamine 3000 Reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. After 48 hr cells were fixed, counterstained with DAPI and analyzed by confocal microscopy.

**In vitro and in vivo Dasatinib treatment**

For in vitro experiments culture medium with a range of Dasatinib concentrations (Selleckchem, Stratech, Newmarket, Suffolk, UK) was added to cells 24 hr after plating and replaced every other day. Sigmoidal curves from dose-response data were generated using Prism software.

For in vivo treatment, Dasatinib monohydrate (Selleckchem) was dissolved in DMSO at 20 mg/mL and stored in aliquots at −20°C. Aliquots were thawed and diluted in 5.1% polyethylene glycol (PEG-400) and 5.1% Tween 80 (vehicle, VEH) before use. Mice were treated with a single intraperitoneal (IP) injection of Dasatinib (DAS) (15 mg/Kg) daily. Control mice were treated with an equivalent concentration of DMSO dissolved in vehicle. Caliper measurements of tumor width (W) and length (L) were recorded every other day and tumor volumes were calculated using the formula (L x W²)/2).

**In vivo conditional Lyn knockdown**

Pairs of complementary DNA oligonucleotides (Table S3), encoding shLyn#2 (shLyn) or shScr, were annealed and cloned into a pENTRTM/H1/TO vector (ThermoFisher Scientific). The H1/TO-shLyn or -shScr cassette was then transferred into a Gateway-modified pSEW lentiviral vector (Regan et al., 2012) via LR recombination. ORF of Tetracycline repressor (TetR) was amplified from e8 Cell Reports, C226/G0H3 diluted 1:100 in blocking buffer prior to incubation with Alexa Fluor 488 Goat Anti-Mouse or Donkey Anti-Rat secondary antibodies, respectively, for 1 hr. All incubation steps were carried out at room temperature. Counterstaining with DAPI was then followed by mounting using the ProLong Antifade agent (ThermoFisher Scientific).

Indirect TUNEL was performed using The ApopTag® Red In Situ Apoptosis Detection Kit (Merck Millipore) following the manufacturer’s protocol. Slides were analyzed on a Zeiss LSM 710 confocal microscope using a 20X objective.

**Immunofluorescence staining**

For immunofluorescence analysis cells were grown in 8-well chamber slides (BD Biosciences) in 3D culture conditions (BD Biosciences). Cells were fixed in 4% formalin for 20 min and washed with PBS-glycine (0.7%) before blocking with PBS-0.1% Bovine Serum Albumin (BSA)/0.2% Triton X-100/0.05% Tween-20/10% goat serum for 1.5 hr.

Cultured MCF10A 3D acini were incubated for 2 hr with antibodies to Ki-67 (clone MM1) diluted 1:50 or to integrin-alpha6 (clone GoH3) diluted 1:100 in blocking buffer prior to incubation with Alexa Fluor® 488 Goat Anti-Mouse or Donkey Anti-Rat secondary antibodies, respectively, for 1 hr. All incubation steps were carried out at room temperature. Counterstaining with DAPI was then followed by mounting using the ProLong Antifade agent (ThermoFisher Scientific).

**Phospho-Histone H3 immunohistochemical staining**

Immunohistochemistry was carried out following standard procedures. Fresh sections were cut from formalin-fixed and paraffin-embedded tumor tissue. Dewaxed and re-hydrated slices underwent antigen retrieval in citrate buffer, pH 6.0 (Sigma) in a pressure cooker for 5 min before incubation with 1% hydrogen peroxide solution for 20 min and then blocking in 1% BSA/0.1% Tween-20/ TBS for 1 hr. Incubation with anti-phospho-Histone H3 (S10) antibodies (rabbit polyclonal, #9701, Cell Signaling Technology; diluted 1:200 in blocking buffer) was performed overnight at 4°C. Detection was carried out using the EnVision-System-HRP kit for rabbit primary antibody (Dako, Ely, Cambridgeshire, UK). Sections were then counterstained with hematoxylin and mounted. Images were acquired using an Olympus BX43 microscope with a 20X/0.50 Ph1 objective.
PIN1 Immunohistochemistry and analysis of BRCA1 tumor TMA

PIN1 immunohistochemistry (IHC) was carried out by the Northern Ireland Biobank. Briefly, wax was removed from Formalin-Fixed Paraffin-Embedded (FFPE) tissue by three washes with Bond Dewax solution (Leica, Milton Keynes, UK) at 72 °C, three washes with alcohol, and three washes with Bond Wash solution (Leica). Proteins were prepared for antibody binding by incubating in Bond Epitope Retrieval 1 solution (Leica) at 100 °C for 20 min. Slides were then washed three times with Bond Wash solution. Incubation with primary antibody (anti-PIN1 Sc-46660) at 1:200 dilution was carried out for 15 min. The wash step was repeated before blocking in peroxide for 5 min, washing again, and incubating in Post Primary anti-mouse antibody for 8 min. Antibody detection with DAB was carried out using the Bond Polymer Refine Detection kit (Leica) according to the manufacturer’s instructions, counterstained in hematoxylin and mounted.

PIN1 scoring was based on a scale of 0–4 where 0 represented no visible staining of PIN1, 1 represents low, 2 represents medium, 3 represents high and 4 represents very high, as per the examples in Figure 4. Each of three cores per patient was scored independently; the highest score of the three was used as the overall score.

Isoform specific expression analysis by Affymetrix

The human LYN A isoform can be detected specifically by the microarray feature 3098998 on the Affymetrix Human Exon 1.0ST arrays. To establish LYN A’s levels in human breast cancers, we extracted its isoform-specific expression across 177 previously published breast carcinomas enriched for the triple negative phenotype (Brasó-Maristany et al., 2016; Gazinska et al., 2013) (ArrayExpress accession number E-MTAB-570) and across a panel of breast cancer cell lines (Heiser et al., 2012). For each breast cancer sample, immunohistochemistry-based and PAM50 derived breast cancer subtypes, as well as breast cancer cell line subtypes were retrieved from the original publications, respectively (Gazinska et al., 2013; Heiser et al., 2012).

Isoform specific expression analysis by RNaseq

Level-3 RNaseq data and overall survival was downloaded from TCGA breast cancer (https://cancergenome.nih.gov/). LYN A and LYNB isoforms were manually identified as uc003xsk.* and uc003xsl.* (see Figure S7A for details). Ratios were calculated using raw RSEM values and log transformed for brevity. PAM50 classification was performed as described (Perou et al., 2000). Statistical analyses and respective data plots were generated in R version 3.2.2.

LYN pull-down for Tandem Mass Tag (TMT) labeling

TMT enables robust quantitation and comparison by mass spectrometry of protein levels between samples. MDA-MB-231 (LYN KD, LYN-A*, LYN-B*, LYN-YF*) cells were plated in T175 flasks and after two days were either serum-starved or left untreated overnight. The following day starved cells were treated with 50 ng ul−1 EGF for two hr. Next, both treated and untreated cells were lysed in 1% IGEPAL CA-630, 150 mM NaCl, 1 mM MgCl2, 50 mM Tris pH 7.5, 5% glycerol, 10 mM Sodium Fluoride, 1 mM Sodium Orthovana- date, 10 mM Sodium Pyrophosphate, 100 mM (β-Glycerophosphate and Complete Protease inhibitor cocktail (Roche).

After centrifugation (14000 g for 15 min at 4 °C), cell lysates (3 mg of protein) were pre-cleared with protein A-Sepharose beads (GE Healthcare) for 45 min at 4 °C prior to incubation with anti-LYN antibodies (rabbit polyclonal (44), sc-15, Santa Cruz) overnight at 4 °C. After incubation with protein A-Sepharose beads for 45 min at 4 °C, immunoprecipitates were pulled down by centrifugation (900 g for 5 min at 4 °C), washed three times with lysis buffer, twice with lysis buffer devoid of IGEPAL CA-630 and after removal of the supernatants samples were stored at −80 °C until being processed for TMT labeling.

TMT Labeling and High pH reversed-phase chromatography

Pull-down samples were digested with trypsin while on the beads (2 μg trypsin; 37 °C, overnight), labeled with Tandem Mass Tag (TMT) ten plex reagents according to the manufacturer’s protocol (Thermo Fisher Scientific, Loughborough, LE11 5RG, UK) and the labeled samples pooled.

The pooled sample was evaporated to dryness, resuspended in 5% formic acid and then desalted using a SepPak cartridge according to the manufacturer’s instructions (Waters, Milford, Massachusetts, USA). Eluate from the SepPak cartridge was again evaporated to dryness and resuspended in buffer A (20 mM ammonium hydroxide, pH 10) prior to fractionation by high pH reversed-phase chromatography using an Ultimate 3000 liquid chromatography system (Thermo Scientific). The sample was loaded onto an XBridge BEH C18 Column (130A, 3.5 μm, 2.1 mm X 150 mm, Waters, UK) in buffer A and peptides eluted with an increasing gradient of buffer B (20 mM Ammonium Hydroxide in acetonitrile, pH 10) from 0%–95% over 60 min. The resulting fractions were evaporated to dryness and resuspended in 1% formic acid prior to analysis by nano-LC MSMS using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific).

Nano-LC Mass Spectrometry

High pH RP fractions were further fractionated using an Ultimate 3000 nano-LC system in line with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Peptides in 1% (vol/vol) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (vol/vol) acetonitrile 0.1% (vol/vol) formic acid peptides were resolved on a 250 mm × 75 μm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1%–6% solvent B over 1 min., 6%–15% B over 58 min., 15%–32%B over 58 min., 32%–40%B over 5 min., 40%–90%B
over 1 min., held at 90% B for 6 min and then reduced to 1% B over 1 min.) with a flow rate of 300 nL min$^{-1}$. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization at 2.0 kV using a stainless steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary temperature of 275 °C.

All spectra were acquired using an Orbitrap Fusion Tribrid mass spectrometer controlled by Xcalibur 2.0 software (Thermo Scientific) and operated in data-dependent acquisition mode using an SPS-MS3 workflow. FTMS1 spectra were collected at a resolution of 120 000, with an automatic gain control (AGC) target of 200 000 and a max injection time of 50 ms. Precursors were filtered with an intensity threshold of 5000, according to charge state (to include charge states 2-7) and with monoisotopic precursor selection. Previously interrogated precursors were excluded using a dynamic window (60 s ± 10 ppm). The MS2 precursors were isolated with a quadrupole mass filter set to a width of 1.2 m/z. ITMS2 spectra were collected with an AGC target of 10 000, max injection time of 70 ms and CID collision energy of 35%.

For FTMS3 analysis, the Orbitrap was operated at 50 000 resolution with an AGC target of 50 000 and a max injection time of 165 ms. Precursors were fragmented by high energy collision dissociation (HCD) at a normalized collision energy of 60% to ensure maximal TMT reporter ion yield. Synchronous Precursor Selection (SPS) was enabled to include up to 5 MS2 fragment ions in the FTMS3 scan.

**TMT Data Analysis**

The raw data files were processed and quantified using Proteome Discoverer software v2.1 (Thermo Scientific) and searched against the UniProt Human database (downloaded 14/09/17; 140 000 sequences) plus LYNA and LYNB and LYNA_YF sequences using the SEQUEST algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.6 Da. Search criteria included oxidation of methionine (+15.9949) as a variable modification and carbamidomethylation of cysteine (+57.0214) and the addition of the TMT mass tag (+229.163) to peptide N-termini and lysine as fixed modifications. Searches were performed with full tryptic digestion and a maximum of 2 missed cleavages were allowed. The reverse database search option was enabled and the data were filtered to satisfy false discovery rate (FDR) of 5%.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Unless otherwise stated, blots shown are representative of three independent experiments. Unless otherwise stated, all quantitation is shown as mean and SD from three independent experiments and statistical significance determined using two-tailed unpaired t tests. Gene expression analysis by quantitative real-time rtPCR is shown as mean ± 95% confidence intervals from three independent experiments, each of which was carried out using three technical replicates. Significance of real-time RT-PCR data was determined from confidence intervals (Cumming et al., 2007). *p < 0.05; **p < 0.01; ***p < 0.001.

Statistical analysis of tumor growth was conducted using the glmer function for generalized linear mixed models from the lme4 package (Bates et al., 2015) in the R software (version 3.2.2). The final model accounted for the change in tumor VOLUME with time (DAY) and a DAY-by-TREATMENT interaction as fixed effects using variable random intercepts and slopes for each tumor (TUMOUR_ID). This relationship was specified as glmer (VOLUME ~ DAY + DAY:TREATMENT + (DAY|TUMOUR_ID), family = Gaussian (link = “log)). All modelling assumptions were confirmed to be reasonable on diagnostic residual plots.

Number of phospho-H3-positive cells in FFPE sections of grafted tumors was determined by using ImageJ image analysis software (https://imagej.nih.gov/ij/). Automatic counting was performed on binary images (8-12 fields per tumor) after applying consecutive dilations to coalesce multiple dots within the same cell.

Band intensities on gels and western blots were also quantified using ImageJ.