An Integrated Genomic Screen Identifies LDHB as an Essential Gene for Triple-Negative Breast Cancer

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Abstract
Breast cancer has been redefined into three clinically relevant subclasses: (i) estrogen/progesterone receptor positive (ER+/PR+), (ii) HER2/ERBB2 positive, and (iii) those lacking expression of all three markers (triple negative or basal-like). While targeted therapies for ER+/PR+ and HER2+ tumors have revolutionized patient treatment and increased lifespan, an urgent need exists for identifying novel targets for triple-negative breast cancers. Here, we used integrative genomic analysis to identify candidate oncogenes in triple-negative breast tumors and assess their function through loss of function screening. Using this approach, we identify lactate dehydrogenase B (LDHB), a component of glycolytic metabolism, as an essential gene in triple-negative breast cancer. Loss of LDHB abrogated cell proliferation in vitro and arrested tumor growth in fully formed tumors in vivo. We find that LDHB and other related glycolysis genes are specifically upregulated in basal-like/triple-negative breast cancers as compared with other subtypes, suggesting that these tumors are distinctly glycolytic. Consistent with this, triple-negative breast cancer cell lines were more dependent on glycolysis for growth than luminal cell lines. Finally, we find that patients with breast cancer and high LDHB expression in their tumors had a poor clinical outcome. While previous studies have focused on the ubiquitous role of LDHA in tumor metabolism and growth, our data reveal that LDHB is upregulated and required only in certain cancer genotypes. These findings suggest that targeting LDHB or other components of lactate metabolism would be of clinical benefit in triple-negative breast cancer. Cancer Res; 72(22); 5812–23. ©2012 AACR.

Introduction
In the past decade, molecular profiling has redefined breast cancer as a heterogeneous group of diseases. Gene expression microarray analyses have identified at least 5 different breast cancer subtypes: luminal A, luminal B, HER2+ (or ERBB2+), basal-like (or triple negative), and claudin-low (1–4). The molecular distinction between these subtypes is defined by a tissue-specific gene signature and by the status of estrogen receptor (ER), progesterone receptor (PR), and ERBB2 (or HER2). In the clinic, these molecular subgroups translate into 3 major categories of breast cancer; (i) those that express ER or PR and are termed hormone receptor positive–dependent (HR+), (ii) those with ERBB2/HER2 amplification (HER2+), and (iii) those that lack expression of all 3 markers and are referred to as triple negative. Each of these breast cancer subgroups has distinct clinicopathologic features and prognostic implications (5).

The molecular classification of these breast tumors is of profound predictive value in determining patient response to targeted therapies. For example, patients with HER2+ breast tumors are frequently treated with Herceptin (trastuzumab), a monoclonal antibody that blocks the growth promoting effects of the amplified HER2 kinase (6). Likewise, HR+ tumors respond to antiestrogen therapy (7). As neither hormone receptors nor the HER2 oncogene is expressed in triple-negative breast cancer, a rational therapy for these tumors remains elusive. This is underscored by the poor patient outcomes seen in the triple-negative breast cancer population (8).

The goal of this study was to identify new therapeutic targets for triple-negative breast cancers. Starting with an RNA interference (RNAi) screen conducted on both triple-negative and luminal breast cancer cell lines, we identified the lactate dehydrogenase B isoform (LDHB) as an essential gene of triple-negative breast cancer. LDHB is overexpressed in triple-negative breast tumors as compared with normal and luminal breast tumors, and importantly, this overexpression correlates with a poor clinical outcome. Knockdown of LDHB selectively reduced proliferation of triple-negative breast cancers both in vitro and in established tumors in vivo. Interestingly, we find that triple-negative breast cancers have a strong predilection for the glycolytic pathway as an energy source. Our work identifies LDHB as an important mediator of triple-
Materials and Methods

Cell lines  
All breast cancer cells were grown in RPMI, 10% FBS, 2 mmol/L L-glutamine (Invitrogen), and 1% penicillin–streptomycin (Invitrogen). 293T human embryonic kidney packaging cells were grown in Dulbecco’s Modified Eagle’s Medium (high glucose), 10% FBS, 100 μmol/L nonessential amino acids (Invitrogen), and 1% penicillin–streptomycin.

Antibodies  
The following antibodies were used for immunoblot analysis: actin (MP Biomedicals; 69100), LDHA (Santa Cruz Biotechnology; sc-133123), LDHB (Epitomics; 2090-1), MCT1 (Sigma; HPA003324), MCT4 (Santa Cruz Biotechnology; sc-50329), tubulin (Sigma; T6074), and horseradish peroxidase–conjugated anti-mouse and anti-rabbit secondary antibodies (Jackson ImmunoResearch).

Compilation of genes for RNAi screen  
To identify genes that are specifically required in triple-negative versus luminal breast cancers, a targeted siRNA library was assembled on the basis of 3 main criteria (mRNA overexpression, genes residing in genomic amplicons, and literature supported). Public microarray datasets were retrieved from Oncomine (9–11) and were used to identify genes overexpressed in triple-negative breast tumors as compared with luminal tumors. Genes were filtered and selected if the Q value was less than 0.1, and at least more than 2-fold change was observed over luminal tumors. Genes located in copy number gain regions (defined by GISTIC analysis with Q value less than 0.1) were identified using in-house array comparative genomic hybridization (CGH) datasets from triple-negative breast tumors and from 2 published array CGH datasets (12, 13). In addition, correlation analysis between expression and copy number gain was conducted in a panel of 3 triple-negative and 4 luminal breast cancer cell lines. After cross-referencing the tumor gene set with the cell line analysis, a total of 39 genes were selected on the basis of overexpression, and 30 genes were selected on the basis of gene copy number gain in triple-negative versus luminal breast cancer. Finally, 10 genes were selected on the basis of published data, which implicate Notch and Wnt-β–catenin signaling pathways in triple-negative breast cancer (14).

siRNA screen and data analysis  
Appropriate cell number and transfection conditions were optimized for each cell line (Supplementary Table S1). Briefly, cell number for plating was determined by quantifying cell growth on an IncuCyte (Essen BioScience) so that the cell density was approximately 80% 4 days after plating (duration of screen). Transfection conditions were optimized using 5 different lipid transfection reagents (Dharmafect 1–4 and Lipofectamine RNAiMAX) and appropriate positive and negative siRNAs (siNTC, Tox, siPLK1, and siCDK1). For the screen, cells were reverse transfected in 96-well plates in triplicate using the Dharmacon siGENOME.SMARTpool library (4 siRNAs/gene) at a final concentration of 100 nmol/L and the appropriate lipid. Cells were incubated for 4 days before 5-ethyl-2′-deoxyuridine (EdU) labeling. As internal positive controls for the screen, Tox (to ensure transfection efficiency) and siCDK1 were included. CDK1 is a key cell-cycle regulator and upon knockdown significantly reduces the S-phase index (15).

EdU labeling was carried out according to the manufacturer’s instructions with minor modification (Invitrogen). Briefly, cells were incubated for 30 minutes with EdU before fixation and permeabilization. Nuclei were labeled with Hoechst 33342. Cell number and EdU-positive cells were quantified using an ImageXpress system (Molecular Devices). One image per well was collected for a total of 3 images per gene per cell line. For each gene, an EdU-positive percentage was calculated by dividing the EdU-positive nuclei by the total number of nuclei.

Data were normalized on each plate by dividing the percentage EdU-positive nuclei by the average of 2 nontargeting controls. Normalized values from each plate were then averaged and Z-scores were calculated using the following formula: (gene value – plate average)/plate SD (Supplementary Table S2). Z-scores from the basal and luminal lines were independently averaged. To reduce the Z-score to a single, comparable value, the basal Z-score was subtracted from the basal Z-score. Conducting a t test between the 2 groups identified statistically significant hits. Genes with a Z-score less than −0.9 and a P value more than 0.05 were considered hits.

Cell proliferation assays  
Validation of LDHB knockdown with individual siRNA oligos was conducted in 96-well format. Briefly, cells were reverse transfected with a single siGENOME oligo at a final concentration of 25 nmol/L using 0.11 μL of Dharmafect 4 (Dharmacon) per well. For LDHB, 2 independent siRNAs from the siGenome pool were used for subsequent analysis (Dharmacon D-009779-01 and D-009779-02). Cells were plated at 2,750 cells per well and incubated for 6 days. Cell proliferation was measured at the end of study using CellTiter-Glo (Promega) according to manufacturer instructions.

For 2-deoxyglucose (2DG) studies, cells were seeded in 45 μL at 3.4 × 104 cells per mL (in a 384-well plate) in RPMI containing 5% FBS, 6 mmol/L glucose, 2 mmol/L glutamine, and 1× antibiotic. 2DG was added 12 hours after plating at a final concentration of either 5 mmol/L or 20 mmol/L. (2DG was prepared as a 10× stock). Cell growth was quantified 5 days later with Cyquant Direct (Invitrogen). Relative cell growth was calculated by averaging normalized values from the 2 drug concentrations.

Construction of stable cell lines with inducible knockdown  
For the inducible knockdown experiments, the lentivirus pHush–shRNA system was used (16). Two independent short hairpin RNA (shRNA) sequences were constructed for LDHB (shLDHB-1 target sequence: GGATATACCAACTGGGCTA and shLDHB-2 target sequence: GTACAGTCCTGATTGCATC).

LDHB Is a Regulator of Triple-Negative Breast Cancer
MCT1 shRNA target sequence was GCAGTATCTGGTGAA-TAA and the MCT4 shRNA target sequence was CCGCAAGT-TACAAGGCAT. Sequences were first cloned into the pSHUTTLE-H1 vector and then the pSHUTTLE-H1–shRNA was Gateway (Invitrogen) recombined into a puromycin-selectable pHush vector (16). The pHush–shNTC control was obtained from David Davis (Genentech).

To make lentiviral particles, the pHUSH–shRNA construct was transfected into 293T cells along with pCMV–VSVG and pCMV–dR8.9 plasmids. Viral particles were added to cells with 8 μg/mL polybrene and spin infected at room temperature (1,800 rpm, 30–45 minutes). Stable integration of shRNAs was selected with 2 μg/mL puromycin 48 hours after infection.

Xenograft tumor models

For each cell line, 5 × 10^5 cells were injected subcutaneously into the flank of 26 female NCr nude mice (Taconic) to initiate tumor growth. Tumor size was monitored using a calliper. Once tumors reached 200 mm² in size, the animals from each cell line were split into 2 groups. One group was given 5% sucrose in their water (control group) and the other group was given 5% sucrose in their water to induce hairpin expression. Eleven days after mice were started on doxycycline, 3 mice from each group were euthanized and the tumors were harvested to examine LDHB knockdown. The remaining 10 mice in each group were monitored for tumor growth for the remainder of the study (39 days). Mouse weight was measured at each time point to monitor weight changes throughout the study.

Microarray data analysis

The mRNA expression dataset to examine LDHB and related lactate pathway genes in different breast tumor subtypes was downloaded from Gene Expression Omnibus, accession GSE18229 (3). Mean-centered log2 expression values of LDHB were sorted from high to low for each tumor subtype. Pearson correlation was calculated between LDHB and MCT1, MCT2, MCT3, and MCT4. This is essentially a score for the expression level of lactate-pathway genes in each tumor as compared with LDHB expression, in which a high correlation value indicates that a gene is coregulated when LDHB is overexpressed. Pearson values were calculated specifically in basal-like tumors, in which LDHB is significantly upregulated, and across all tumor subtypes. Survival curves were calculated by the Kaplan–Meier method. High and low LDHB and LDHA expression was defined by values above or below the mean expression of all tumors, respectively (the log2 mean was set to 0).

Immunohistochemistry

Immunohistochemistry (IHC) was conducted on 4 μm thick formalin-fixed paraffin embedded tissue sections mounted on glass slides. All IHC steps were carried out on the Ventana Discovery XT autostainer (Ventana Medical Systems). Pretreatment was done with Cell Conditioner 1 with the standard time. Primary antibodies were used at the following concentrations: MCT1 (Santa Cruz Biotechnology; sc-365501) at 1 μg/mL, MCT4 (Santa Cruz Biotechnology; sc-50329) at 0.1 μg/mL, LDHA (Cell Signaling Technologies; 3882) at 0.081 μg/mL, and LDHB (Epitomics; 2090-1) at 0.04 μg/mL. Slides were incubated with primary antibody for 60 minutes at 37°C. Ventana Mouse or Rabbit OmniMap was used as the detection system. Ventana 3,3′-diaminobenzidine (DAB) and Hematoxylin II were used for chromogenic detection and counterstain.

Results

LDHB is overexpressed in triple-negative breast cancer and essential for tumor growth

We conducted a loss of function RNAi screen to identify genes that are selectively required for cell proliferation in triple-negative breast cancer. Target genes for the screen were selected on the basis of their genomic amplification status or noncopy number dependent overexpression in triple-negative as compared with luminal tumors. An additional set of genes, representing key cancer-signaling pathways implicated in triple-negative breast cancer growth, were also included (Fig. 1A). On the basis of this analysis, 79 genes in total were screened across 8 triple-negative and 3 luminal cell lines and cell proliferation was quantified using an EdU incorporation assay 4 days posttransfection. By comparing the average Z-score of triple-negative and luminal cell lines, genes with a differential cell proliferation effect between triple-negative and luminal breast cancer cell lines were identified (Fig. 1B and Supplementary Table S2). Of the 4 genes that met our significance criteria of scoring at a ΔZ-score (Z-score triple-negative–Z-score luminal) of −0.9 or below, we found that only 1, LDHB, was specifically required for proliferation of triple-negative breast cancers (Fig. 1C–E and Supplementary Fig. S1). LDHB is a member of the LDH family of enzymes and functions as a metabolic regulator (Fig. 1F) to interconvert pyruvate and lactate (17, 18).

To validate LDHB as a regulator of triple-negative breast cancer, a panel of triple-negative breast cell lines were transfected with 2 independent LDHB siRNAs. Both LDHB siRNAs exhibited robust knockdown of LDHB protein and significantly reduced cell proliferation on average 3- to 4-fold in a 6-day assay (Fig. 2A). To determine the genotype–phenotype relationship between LDHB and its knockdown effect, LDHB expression was analyzed in basal-like/triple-negative and luminal cell lines and tumors. Gene expression and immunoblot analysis of both LDH isoforms showed that while LDHA expression is ubiquitous, LDHB is specifically upregulated in basal-like/triple-negative breast cancer cell lines and tumors as compared with luminal cancers (Fig. 2B–D). Even within the triple-negative breast cancer subset, the variability of LDHB expression correlated well with its knockdown phenotype, as 5 of the highest expressing triple-negative cell lines (BT549, CAL51, CAL120, MDA-MB-231, and MDA-MB-436) showed the strongest LDHB knockdown effect on proliferation (Fig. 1E). These effects are specific to LDHB isoform inhibition, as LDHA levels did not change upon LDHB knockdown (Supplementary Fig. S3). These results show that triple-negative breast cancer cell lines express elevated LDHB levels and are highly dependent on its expression for proliferation.

To extend our in vitro observations, the effect of acute loss of LDHB on xenograft tumor growth and maintenance was
A doxycycline-inducible shRNA lentiviral system was used to introduce 2 independent shRNAs to LDHB (shLDHB) or a nontargeting control (shNTC) into a human triple-negative breast cancer cell line (MDA-MB-231). Resulting cell lines were injected into the flanks of mice. Once tumors were established (measuring 200 mm³), a subset of mice in each group was treated with doxycycline to induce shRNA expression. Gene expression changes were assessed using RNA sequencing and qPCR. The results showed significant downregulation of LDHB expression in the shLDHB group compared to the shNTC group.

Figure 1. RNAi screening strategy to identify regulators of basal-like breast cancer. A, schematic highlighting the criteria for gene selection and experimental setup of the RNAi screen. B, distribution curve showing the average Z-score of triple negative minus the average Z-score of luminal cell lines for all the genes included in the RNAi screen. The dashed line indicates the Z-score cutoff for genes that preferentially affected triple-negative cell lines. As an additional threshold, a gene also had to pass a t test (P < 0.05) comparing triple negative and luminal Z-scores. C, representative images from the primary RNAi screen of a triple-negative (CAL120) and luminal (MCF-7) cell line. Nuclear staining with Hoechst 33342 and EdU incorporation after knockdown with siNTC (nontargeting control), siCDK1 (positive control), and siLDHB are shown. D, average triple-negative and luminal Z-scores for all cell lines for LDHB. P value indicates a statistically significant difference in EdU incorporation between triple-negative and luminal lines. Error bars represent SD. E, normalized Z-scores for LDHB siRNA across cell lines in the RNAi screen. Corresponding immunoblot of LDHB levels are shown for each cell line: BT549 (1), CAL51 (2), CAL120 (3), HCC1395 (4), HCC38 (5), MDA-MB-231.x1 (6), MDA-MB-436 (7), SW982 (8), KPL1 (9), MCF7 (10), and T47D (11). F, schematic of LDH function in glycolysis.
cohort was administered doxycycline to initiate LDHB knockdown. LDHB knockdown in fully formed tumors led to profound tumor growth inhibition (tumor growth inhibition for shLDHB-1 = 75% and shLDHB-2 = 66%), when compared with the shNTC control (\(P < 10^{-10}\) for both hairpins) or the nondoxycycline-induced shLDHB tumors (\(P < 10^{-9}\) for both hairpins; Fig. 3A and Supplementary Fig. S2). Acute and sustained LDHB knockdown in the tumors was confirmed by immunoblot at day 11 and day 39 after doxycycline treatment, respectively (Fig. 3B). No significant weight changes were observed in the mice throughout the study for any of the treatment groups (Supplementary Fig. S2). These observations indicate that LDHB is required for triple-negative breast tumor growth in vivo.

Basal-like/triple-negative tumors and cell lines show glycolytic dependence

It has been known for over half a century that tumor cells display an altered cellular metabolism (19, 20). Instead of using pyruvate oxidation in the mitochondria as their primary means of generating energy, many tumor cells rely exclusively on a high rate of glycolysis for ATP production (18). We hypothesized that upregulation of LDHB in triple-negative breast cancer may reflect a more global shift in cellular metabolism. To examine whether the breast cancer subtypes differ in their glycolytic dependence, we analyzed and compared the expression of defined metabolic gene signatures across breast cancer subtypes using a breast cancer microarray dataset (gene signatures adapted from the Broad Institute Molecular Signatures Database (MSigDB) gene sets for Glucose_Metabolic_Process, (21), and shown in Supplementary Table S3). Strikingly, basal-like, claudin-low, which are representative of triple-negative breast cancer (3, 22), and HER2+ breast tumors exhibit an elevated glycolytic gene signature and concomitant lower oxidative phosphorylation signature compared with luminal tumors (Fig. 4A and B). These results imply that triple-negative breast cancers have altered their transcriptional profiles to accommodate a dependence on glycolysis.

To functionally test these changes in gene expression, we profiled the glycolytic dependence of a panel of 23 triple negative and 6 luminal cell lines by examining their sensitivity...
Cancers have an altered metabolic gene profile (3). Together, these data reveal that triple-negative breast cancer is specifically reliant on glycolysis for their metabolic demand. To examine whether specifically other glycolysis genes in triple-negative breast cancer, we investigated whether LDHB expression correlates to clinical outcome. In the luminal tumor category (Fig. 6C and D). In contrast, the other MCT proteins (Fig. 4D and Supplementary Fig. S5). MCT1 expression significantly correlated to LDHB expression in both basal-like and claudin-low tumors. In contrast, the other MCT proteins showed either no correlation (MCT2 and MCT3) or a negative correlation (MCT4) to LDHB in basal-like tumors. Interestingly, while MCT4 and LDHA are upregulated in response to hypoxia (18, 25), both MCT1 and LDHB are expressed in normoxic regions of breast cancer (data not shown), suggesting that different isoforms of LDH and MCTs function together in distinct tumor microenvironments.

To extend these coexpression findings at the protein level, we conducted immunohistochemical analysis of LDHA, LDHB, MCT1, and MCT4 in ER/PR, and HER2-annotated breast tumors. The specificity of immunohistochemical staining for each antibody was validated by the use of cell line controls (Supplementary Fig. S6). Consistent with our data in breast cancer cell lines, LDHB was expressed at moderate or high levels (having an IHC score of 2 or 3; \( P = 10^{-5} \)) in 64% of triple-negative breast cancers (\( n = 31 \) tumors), with weak or no expression detected in normal breast, HER2+ tumors, or HR+ tumors (Fig. 5 and Table 1). Furthermore, MCT1, but not MCT4, was significantly upregulated in triple-negative breast tumors, as 26% of triple-negative breast cancers (\( P = 0.02; n = 31 \) tumors) exhibited an IHC score of 2 or 3. Moreover, MCT1 correlated to LDHB expression (\( P = 0.001 \)) within the triple-negative tumor subset (Fig. 5C–E). These data indicate that LDHB and MCT1 are coexpressed in triple-negative breast tumors and suggest a codependent function for these 2 genes in triple-negative breast cancer.

**LDHB expression in breast cancer correlates to a poor clinical outcome**

Cancers with a high glycolytic rate have been noted to have worse clinical outcome (26, 27). To determine whether LDHB expression correlates with prognosis in patients with breast cancer, we analyzed a cohort of 227 patients with outcome data (3). We segregated patients into 2 categories: those with LDHB expression above the mean were considered high expressors and those below the mean were considered low expressors. We find that high expression of LDHB is associated with both poor overall survival (\( P = 0.0001 \)) and progression-free survival (\( P = 0.0004 \); Fig. 6A and B). Because LDHB is primarily upregulated in basal-like/triple-negative breast cancer and these cancers are typically more aggressive than other subtypes, we also examined if LDHB expression correlated to clinical outcome independent of the basal-like breast cancer subtype. High expression of LDHB was associated with a poor overall survival (\( P = 0.00003 \)) and progression-free survival (\( P = 0.00006 \)), even in the luminal tumor category (Fig. 6C and D). In contrast, LDHA expression did not correlate with clinical outcome in patients with breast cancer (Fig. 6E–H). Taken together, these data indicate that LDHB expression correlates to poor patient outcome in breast cancer.
Patients with triple-negative breast cancers experience poor clinical outcome and an aggressive tumor pathology, highlighting the need for an effective treatment (5). In this study, we have identified LDHB as an essential gene in triple-negative breast cancer. LDHB was selectively required for the growth of triple-negative breast cancers both in vitro and in vivo and specifically was overexpressed in triple-negative breast cancers.

Figure 4. Basal-like breast cancers are dependent on glycolysis. A and B, bar graph depicts glycolysis (A) and oxidative phosphorylation (B) gene signatures in 5 breast cancer subtypes. Average log2 expression of the signature genes is shown for basal-like (n = 68), claudin-low (n = 27), HER2+ (n = 39), luminal A (n = 95), and luminal B (n = 57) breast tumors (3). Error bars represent SEM. C, sensitivity of 21 triple-negative cell lines and 5 luminal cell lines to 2DG. Graph represents the average cell proliferation of all cell lines in each cancer subtype after 5 days of growth in 2DG. Data are the average of 3 independent experiments (each experiment carried out in triplicate) and was normalized to cells treated with dimethyl sulfoxide. Each dot or square denotes an individual cell line and error bars represent SEM. P value indicates a statistically significant difference. D, distribution plot showing LDHB mRNA expression in 5 breast cancer subtypes. LDHB expression was normalized to its average expression across all breast tumors and sorted from high to low for each breast cancer subtype (n = 286 tumors). Below, MCT1-4 expression is shown for each corresponding tumor. Pearson correlation between LDHB and MCT expression in the basal-like cohort is indicated. P values for each correlation are as follows: MCT1: Pearson = 0.48, P = 0.00002; MCT2: Pearson = −0.22, P = 0.04; MCT3: Pearson = −0.15, P = 0.1; or MCT4: Pearson = −0.42, P = 0.0002.

Discussion

Patients with triple-negative breast cancers experience poor clinical outcome and an aggressive tumor pathology, highlighting the need for an effective treatment (5). In this study, we have identified LDHB as an essential gene in triple-negative breast cancer. LDHB was selectively required for the growth of triple-negative breast cancers both in vitro and in vivo and specifically was overexpressed in triple-negative breast cancers.
tumors. Gene expression analysis indicates that triple-negative breast tumors upregulate a number of genes important for glycolysis. In support of this expression profile, we show that triple-negative breast cell lines are more sensitive to the glycolysis inhibitor 2-deoxyglucose than luminal cell lines, suggesting that triple-negative tumors exploit glycolysis for their energy demands. Finally, we show that LDHB overexpression affords patients with breast cancer an overall poor clinical outcome, regardless of tumor subtype.

Over the last decade, there has been a tremendous transformation in the way we research and treat breast cancer. We now recognize that it is not a homogenous disease, but rather a diverse set of diseases that arise from the same tissue. Most clinical advancements have benefited patients that present with intact ER/PR or overexpress HER2. However, patients that lack these growth factor receptors currently have few therapeutic options. Notably, many triple-negative breast cancers display aberrant p53, BRCA1 mutations, and express intact EGFR/HER1 (28). Recent reports have found that basal-like cancers contain activated KRAS and MYC gene signatures and are sensitive to mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibition, despite the lack of KRAS mutation (29–31). While targeting these signaling pathways is an active area of investigation, we took an unbiased approach to discovering novel therapeutic targets by identifying genes that exhibit differential expression or genomic amplification between luminal and triple-negative/basal-like cancers. Our hypothesis was that as tumor cells become dependent on cellular activities for cell growth or survival, they find ways to upregulate components in that pathway. Using this integrated genomic approach, we identified LDHB and other metabolic pathway genes that are upregulated and essential for triple-negative cell proliferation.

In adult somatic tissue, LDHA and LDHB are the predominant LDH isoforms that are expressed. While many tissues express both isoforms, LDHB is exclusively expressed in heart and LDHA is exclusively found in skeletal muscle (32). Interestingly, patients have presented in the clinic that lack functional LDHA or LDHB, suggesting that both are individually dispensable for normal adult physiology (33, 34). Importantly,
this also suggests that small molecules that target LDH activity should afford patients few off-target effects. While our data using RNAi to deplete LDHB are compelling, the development of such molecules will be an important tool to validate the phenotype associated with RNAi mediated loss of LDHB.

LDHA has garnered the most attention in tumor biology, as it is broadly upregulated in many tumors, is a downstream target of hypoxia-inducible factor-1 (HIF-1), and plays an essential role in tumor initiation and growth (18, 35). The significance of LDHB in tumor biology is not well defined. Our IHC data show that LDHB is expressed at low levels in normal breast tissue. This would argue that luminal tumors are more like normal tissue and that triple-negative/basal-like tumors actively upregulated LDHB expression. Recent reports indicate that LDHB expression and function in tumors may be context or tissue specific. In mouse immortalized cell lines, it was found that LDHB expression was controlled by the mTOR pathway and that mTOR-mediated tumor formation depended on LDHB (36). Our in vitro and in vivo LDHB knockdown data extend on this initial observations and implicate LDHB as a metabolic regulator in triple-negative breast cancer. It is worth noting that LDHB downregulation has been reported in the context of other tumor types. For example, LDHB is expressed in normal prostate tissue, but silenced by promoter methylation in prostate carcinomas (37). In the future, it will be of interest to determine the upstream pathways that regulate LDHB expression in both luminal and triple-negative breast cancer.

A key question remains how LDHB inhibition causes reduced tumor growth. Because LDHA is present in all breast cancers and cell lines we have examined, we speculate that LDHA has a nonredundant function. LDHA and LDHB can form homo or heterotetramers with each other to generate a functional enzymatic complex. One possible mechanism to account for the essential effects of LDH in triple-negative breast cancer is that in these tumors, LDH homotetramers or LDHB–LDHA heterotetramers are preferred or have increased catalytic activity in comparison with LDHA only homotetramers.

A recent set of publications has provided new perspectives on tumor metabolism, which offers an alternative hypothesis for how loss of LDHB may impact tumor metabolism in triple-negative breast cancer (18, 38, 39). In what has been termed the "reverse Warburg effect," tumor cells that are hypoxic and glucose-deprived can use lactate that was derived from aerobic glycolysis from surrounding stromal or tumor cells. This mechanism requires the concerted effort of the monocarboxylate transporters MCT4, which favors lactate export, and MCT1, which favors lactate import (24, 25, 40, 41). Of particular interest, in triple-negative tumors it was observed that MCT1 is expressed on tumor cells and MCT4 is expressed on the surrounding stromal cells (39). Our findings that MCT1 is coexpressed in LDHB-high tumors are consistent with the hypothesis that these tumors use pyruvate or lactate as an energy source (18, 42). It has been postulated that LDHB in tumor cells functions to convert lactate to pyruvate (18) and is supported by biochemical studies (43). LDHB exhibits a lower affinity for pyruvate and is more sensitive to substrate inhibition by pyruvate, compared with LDHA. Thus, an alternative hypothesis is that LDH might be overexpressed in glycolytic tumors to counteract the activity of LDHA and provide cells' metabolic flexibility. Further work is required to test these hypotheses and is beyond the scope of this present work. Taken together, we postulate that the concerted upregulation of LDHB and MCT1 may provide tumors with alternative means of energy generation and the ability to adapt to the tumor microenvironment.

Our data show that on a molecular level, LDHB expression represents a more global shift in tumor metabolism in triple-negative tumors. We find that triple-negative breast tumors and

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<td></td>
<td>2+</td>
<td>37 (16)</td>
<td>45 (31)</td>
<td>23 (31)</td>
<td>13 (31)</td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>25 (16)</td>
<td>19 (31)</td>
<td>3 (31)</td>
<td>0 (31)</td>
</tr>
</tbody>
</table>

NOTE: LDHB, LDHA, MCT1, and MCT4 protein expression scores are shown for the different breast cancer subtypes. Expression was ranked from 0 (no expression) to 3 (highest expression). Tumor subtypes were defined by HR+ (expressing ER or PR), HER2 positive (HER2+), or triple negative (TN; lacking ER, PR, and HER2). For each gene, the percentage of tumors with the indicated expression is shown. Percentages were calculated within each subtype and n designates the number of tumors for each subtype.
cell lines upregulate glycolytic genes and become more dependent on glycolysis for growth. In addition to our findings, 2 recent studies reported that a subset of triple-negative breast cancers is marked by amplification and overexpression of phosphoglycerate dehydrogenase (PHGDH; refs. 44–46). PHGDH is a key enzyme in serine biosynthesis and shunts glucose toward glycine production. Similar to our findings with LDHB, triple-negative tumors with high PHGDH levels were dependent on PHGDH expression and showed metabolic addiction to serine. These converging data indicate that triple-negative breast cancers are distinctly wired for dependence on key metabolic components of glucose metabolism, and interference with these pathways presents a plausible therapeutic approach.

In conclusion, our work identifies LDHB as an important mediator of triple-negative tumor growth and supports the idea that triple-negative breast cancers have unique metabolic profiles that may be exploited for therapeutic intervention.

Disclosure of Potential Conflicts of Interest
All authors are employed by Genentech, Inc.

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Figure 6. LDHB expression correlates to a poor clinical outcome in patients with breast cancer. A–H, Kaplan–Meier curves depicting the correlation between LDHB (A–D) or LDHA (E–H) expression and overall patient survival (A, C, E, and G) or progression-free survival (B, D, F, and H) in primary breast tumors. LDHB and LDHA expression were normalized to their average expression across all breast tumors. For A, B, E and F, all tumors with survival data were analyzed (n = 227). For C, D, G and H, only luminal (A) and tumors (B) with survival data were analyzed (n = 125). P values indicate differences between the 2 patient populations.
Acknowledgments

The authors thank James Lee for help and advice with the RNAi screen, David Davis for reagents, William Forrest for biostatistical advice, and Pete Haverty for his assistance in creating metabolic gene signatures.

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Received March 22, 2012; revised July 16, 2012; accepted September 1, 2012; published OnlineFirst November 8, 2012.

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