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An RNAi screen identifies metabolic regulators *NR1D1* and *PBP* as novel survival factors for breast cancer cells with the *ERBB2* signature

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Abstract

Overexpression of the adverse prognostic marker *ERBB2* occurs in 30% of breast cancers, however, therapies targeting this gene have not proven to be as effective as was initially hoped. Transcriptional profiling meta-analyses have shown that there are approximately 150 genes co-overexpressed with *ERBB2*, suggesting that these genes may represent alternative factors influencing *ERBB2*-positive tumors. Here we describe an RNA interference-based analysis of these genes that identifies transcriptional regulators of fat synthesis and storage as being critical for the survival of these cells. These transcription factors, *NR1D1* and *PBP*, both reside on *ERBB2*-containing 17q12-21 amplicons and are part of the *ERBB2* expression signature. We show that *NR1D1* and *PBP* act through a common pathway in upregulating several genes in the de novo fatty acid synthesis network that is highly active in *ERBB2*-positive breast cancer cells. MDH1 and ME1, enzymes that link glycolysis and fatty acid synthesis, are also regulated by *NR1D1*. The resulting high-level fat production from increased expression of these genes likely contributes to an abnormal cellular energy metabolism based on aerobic glycolysis. Together, these results demonstrate that the cells of this aggressive form of breast cancer are genetically preprogrammed to depend on *NR1D1* and *PBP* for the energy production necessary for survival.

Keywords

functional genomics; breast cancer; metabolism

Introduction

Overexpression of the *ERBB2* oncogene is one of the most clinically relevant abnormalities in breast cancer. It occurs in 20–30% of breast cancer cases and is an established adverse predictor of relapse time and survival (1). A meta-analysis of the relationship between *ERBB2* status and breast cancer prognosis revealed that in 90% of the studies either *ERBB2* amplification or protein overexpression was correlated with poor clinical outcome of the patients (2). This association with aggressive disease has made *ERBB2* an attractive therapeutic target for breast

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cancer. Herceptin, a monoclonal antibody targeting the extracellular domain of *ERBB2*, was widely hailed as the first “next generation” cancer therapy. However, its success has been tempered by response rates of only 11–26% when used as a single agent therapy in patients with *ERBB2*-positive breast cancer (3), leading other groups to propose that factors beyond *ERBB2* and its downstream signaling pathway influence therapy escape of these tumors (4).

Clues to other causes that might contribute to the aggressiveness of *ERBB2*-positive breast cancer have come from transcriptional profiling meta-analyses that have demonstrated the existence of more than a hundred genes consistently co-overexpressed with *ERBB2* in this tumor type (5–7). Overexpression of a number of these genes is the result of amplification of the 17q12-q21 region that contains *ERBB2* (8). Most, however, do not map to this region. We have performed a functional RNAi screen (9–11) to determine whether any of the genes overexpressed with *ERBB2* are causal to the aggressiveness of this type of breast cancer. In the study, each overexpressed gene was systematically targeted one at a time in cells of this tumor type so that new therapeutic targets could be identified for *ERBB2*-positive breast cancer whether or not they are functionally related to the *ERBB2* protein itself (12). The screen reveals genes that induce an abnormal cellular physiology as critical for the survival of this type of cells.

Materials and Methods

Cell culture and chemicals

Breast cancer cell lines were obtained from ATCC. HMECs were obtained from Cambrex. HEK 293FT cells were obtained from Invitrogen. MCF10A cells were a gift of the Julio Aguirre-Ghisso lab. All cell lines were cultured in DMEM (Hyclone) supplemented with 10% FBS (Hyclone) and 100U/μl of penicillin-streptomycin (Cellgro), except MDA-MB-361 that were cultured in RPMI-1640 (Hyclone) supplemented with 20% FBS, HMEC that were cultured in MEGM medium (Cambrex) and MCF10A cells that were cultured as indicated in (13). The PPAR γ antagonists GW9662 and T0070907 were obtained from Sigma-Aldrich.

Constructs, RNAi screen and transfections

The flag-RevErb α (flag-NR1D1) construct was a gift of M. Lazar. The LSXN-neu* (*ERBB2*) construct was a gift of L. Petti. To overexpress NR1D1 in MCF10A cells, the NR1D1 cDNA was obtained from the Dana-Farber/Harvard Cancer Center (DF/HCC) DNA Resource Core (clone ID: HsCD00005059) and subcloned into the pMARXIV-Puro retroviral expression vector using standard cloning techniques. ShRNA constructs were expressed from the pSHAG-MAGIC 2 (pSM2) vector and derived from a genome-wide shRNA library (14) (Open Biosystems). We targeted all the genes overexpressed in *ERBB2*-positive breast cancer with constructs from the initial release of this library available at the time of the screen, irrespectively of their level of overexpression and without posing any other criteria. For mature sequences of shRNAs see Supplementary Table S1. ShRNAs targeting the firefly luciferase gene were used as negative controls. Transfection efficiency was monitored by co-transfection with a MSCV-Puro vector expressing green fluorescent protein (GFP). Transfections were performed using FuGENE 6 and HD (Roche) according to the manufacturer’s protocol in an EpMotion 5070 fluidics station (Eppendorf). For quantification of alamarBlue we used a BioTek HT Synergy plate reader. The alamarBlue (Biosource) assay was performed 96h post-transfection, since BT474 cells have a population doubling time of ~100 hours. The 10% of the shRNAs that resulted in the highest decrease in alamarBlue signal (alamarBlue<55%, $P<0.05$) were subjected to a second round of transfections to confirm the initial results. The hits that consistently produced a close to or more than 50% decrease on cell proliferation are shown in Figure 1.

Cell viability - apoptosis assays - immunoblotting

For live cell counts, cells grown on 96-well plates were fixed with 2.5% formaldehyde and stained with Hoechst 33342 (Molecular Probes-Invitrogen). Pictures of cells were acquired using an In Cell Analyzer 1000 (GE Healthcare) high-content imaging system, with a 20x objective. At least 30 fields were imaged per single experiment. Statistics were performed using the In Cell Investigator 3.4 image analysis software (GE Healthcare). Cleaved Caspase-3 and activated Bax immunofluorescence was performed at 48h of treatments, using standard protocols. Cells were imaged using the In Cell Analyzer 1000. At least 500 cells were counted for each experiment. For western blots, cell extracts were obtained using RIPA buffer supplemented with cOmplete cocktail of proteinase inhibitors (Roche). Protein extracts were separated by SDS-PAGE, transferred to Immobilon-P (Millipore) membranes, blotted according to standard protocols and imaged using a FluorChem HD (Alpha Innotech) imaging system. Antibodies used: cleaved Caspase-3 (Asp175, #9661; Cell Signaling Technology), Bax (6A7; BD, San Jose, CA), anti-flag (M2; Stratagene), PBP (TRAP220, C-19; Santa Cruz Biotechnology), GAPDH (V-18; Santa Cruz), Alexa Fluor 568 goat anti-rabbit IgG (#A-11011; Invitrogen), Alexa Fluor 568 goat anti-mouse IgG (#A-11004; Invitrogen), anti-rabbit IgG-HRP (sc-2204, Santa Cruz), anti-goat IgG-HRP (sc-2768, Santa Cruz), anti-mouse IgG-HRP (#31430; Pierce Biotechnology).

qRT-PCR

For all reactions, cells were harvested 48h post-transfection. Due to low shRNA transfection efficiency of BT474 cells, GFP-positive cells were sorted using a BD FACSaria sorting system. Final population enrichment of transfected cells was 65–70%. Total RNA was extracted using TRIzol (Invitrogen) and cDNA was synthesized by reverse transcription of 2 µg of RNA in a 20 µl reaction using MMLV reverse transcriptase (Promega) at 42°C for 1h. QRT-PCR was performed on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), using SYBR Green PCR Master Mix (Applied Biosystems). The primer pairs used were designed using ABI's Primer Express software (Supplementary Table S2). After initial incubation at 95°C for 2½ min, the amplification protocol consisted of 40 cycles of a 95°C - 15 sec step and a 60°C - 60 sec step. Product levels were calculated after normalization with β-actin control.

Metabolic assays

For detection of neutral fat stores, cells were either stained with 60% Oil Red O (Fisher Chemicals) or with 10µg/ml 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503; Molecular Probes). For Oil Red O staining, cells were counter-stained for nuclei with Mayer's Hematoxylin (Sigma-Aldrich) and visualized using the 60x objective of an Arcturus Veritas (Molecular Devices Corporation) microdissection system. For BODIPY assays, cells were grown on 96-well plates, fixed with 2.5% formaldehyde, stained with 10µg/ml BODIPY 493/503 and counter-stained for nuclei with Hoechst 33342. Transfected cells were monitored by co-transfecting with the pDsRed-Monomer-N1 vector (Clontech). Cells were imaged and analyzed using the In Cell Analyzer 1000 - In Cell Investigator 3.4 system. For total fatty acid detection and quantification, total cellular lipids were extracted according to a standard procedure previously described (15).

Statistics

In all experiments, standard deviations (s.d.) from 3 individual experiments were calculated and presented as error bars. The Student's two-tailed *t*-test was employed for *P* value calculations. Comparisons in each case refer to the respected controls, unless otherwise indicated.

Results

A function-based screen of genes overexpressed in *ERBB2*-positive breast cancer

A total of 309 available short-hairpin RNAs (shRNAs) (9) were used to target 141 of the 154 genes identified in a total of 22 studies (5–7); Supplementary References) as being upregulated in *ERBB2*-positive breast cancer cell lines and tumors (Supplementary Table S3). These constructs were individually used to silence each gene in the well-characterized, *ERBB2*-positive, BT474 breast cancer cell line. The effects on cells were monitored using alamarBlue, a fluorimetric indicator of cell proliferation and viability (11). While most of the shRNAs corresponding to the overexpressed genes were without significant effect, shRNAs targeting ten genes result in more than a 50% decrease in proliferation (Fig. 1A; Table 1). This group of shRNAs includes an *ERBB2* shRNA, as would be predicted. Other shRNAs with significant effects on proliferation silence genes with previously reported roles in cancer, such as *KI67*, *TPD52* and *CA9* (16–18) (Table 1). Knockdown of *ERBB3*, another member of the EGFR family that heterodimerizes with *ERBB2* (19) has a modest impact on cell viability (Table 1). ShRNAs targeting *GRB7* and *STARD3*, two genes that are closely linked to *ERBB2* on the 17q12 amplicon (20), have a moderate effect on cell proliferation in our screen (Table 1), which agrees with previous studies (20).

Surprisingly, three of the shRNAs that have the most dramatic effect on cell proliferation target genes are related to adipogenesis, including the nuclear receptor subfamily 1, group D, member 1 (*NR1D1*) (21), the PPAR γ binding protein (*PBP*) (22) and the MAP kinase, *MAP2K6* (23) (Table 1). Three others, fatty acid desaturase 2 (*FADS2*), StAR-related lipid transfer domain containing 3 (*STARD3*) and fatty acid synthase (*FASN*), which, when silenced, have a moderate effect on cell proliferation, are also involved in lipid metabolism (Table 1).

NR1D1 and *PBP* are required specifically for *ERBB2*-positive cell survival

To assess the specificity of effect of each shRNA, those causing the greatest decrease in BT474 cell proliferation were transfected in *ERBB2* overexpressing and non-overexpressing breast cancer cell lines (24), in normal human mammary epithelial cells and a non-breast cell line (Fig. 1B). Proliferation rates of shRNA-transfected cells were measured for each cell line (Supplementary Table S4) and the results were subjected to cluster analysis and displayed as a heat map and dendrogram (Fig. 1B). The *NR1D1* and *PBP* shRNAs cluster with *ERBB2*, since each specifically decreases the viability of *ERBB2*-positive cells, yet is without effect on other cell lines or HMECs (Fig. 1B). Notably, their effect on cell survival is independent of the estrogen receptor (ER) status of the cell lines used (Fig. 1B).

NR1D1 and *PBP* are tightly linked to *ERBB2* and frequently reside on the 17q12-21 amplicons found in *ERBB2*-positive tumors (4,8). Several studies have shown that irrespective of the amplicon size they are consistently co-overexpressed with *ERBB2* (25,26) and are among the six genes that comprise the “*ERBB2* gene expression signature” seen in breast cancers (7). These genes are also functionally linked. *PBP* is a co-activator of peroxisome proliferator activated receptor gamma (PPAR γ) (22) which upregulates 30 genes related to adipogenesis (27) including *NR1D1* (28). *NR1D1* is a member of the steroid receptor supergene family that promotes adipocyte differentiation and has been also identified as a component of the circadian clock (29). However, its downstream effectors are poorly characterized (21).

NR1D1 and *PBP* promote survival of *ERBB2*-positive cells through the same pathway

Decreased alamarBlue staining can result from slowed growth, quiescence or increased cell death. Cells transfected with *NR1D1* and *PBP* shRNAs were monitored for 72hs after transfection using microscopic imaging (Supplementary Fig. S1A). Cell counts show a time dependent decrease in the number of cells transfected with the *NR1D1* and *PBP* shRNAs (Fig.

2A), indicating that cell death is the cause of the decrease in alamarBlue staining. Increased cleaved Caspase-3 (Fig. 2B) and activated Bax signals (Supplementary Fig. S1B) indicate that the cell death is apoptotic. A 2.5 to 3.5-fold increase in apoptosis, which is initially observed within 48h of shRNA transfection, results in extensive cell death after 72h (Fig. 2A). Three other shRNAs targeting *NR1D1* and two targeting *PBP* also result in apoptosis specifically of BT474 cells confirming the result (Supplementary Fig. S2 A and B). These results indicate that *NR1D1* and *PBP* are survival factors for these cells.

Transfection with *NR1D1* or *PBP* shRNAs results in more than 60% decreased mRNA levels of their targets in BT474 cells (Fig. 2C), as well as in decreased protein levels (Supplementary Fig. S2C). Interestingly, the *PBP* shRNA also causes a significant decrease in the message levels of *NR1D1*, while the *NR1D1* shRNA does not have a significant effect on *PBP* mRNA levels (Fig. 2C). Since *PBP* is a coactivator of PPAR γ , which is known to regulate *NR1D1* expression (28), it is likely that *PBP* indirectly regulates expression of *NR1D1*. Simultaneous transfection of the *NR1D1* and *PBP* shRNAs does not have an additive effect on either the cell loss from the population or the apoptosis (Fig. 2D). This indicates that the two genes promote survival of these cells via the same pathway. Since PPAR γ is also part of this pathway in other cell types, we examined its potential role in breast cell survival. Inhibition of PPAR γ activity using an antagonist (GW9662) (30) dramatically decreases *NR1D1* message levels (Supplementary Fig. S3A). Similar to the effects seen with *NR1D1* and *PBP* shRNAs, *ERBB2*-positive cells are specifically sensitive to GW9662 treatment (30) (Supplementary Fig. S3B). A second PPAR γ antagonist produces the same effects (Supplementary Fig. S3C). For either drug, PPAR γ inhibition results in apoptosis of BT474 cells (Supplementary Fig. S3D).

Genetic interaction experiments indicate that *ERBB2* is not a downstream mediator of the survival advantage that *NR1D1* and *PBP* confer in BT474 cells. Although *PBP* knockdown decreases *ERBB2* message levels, *NR1D1* knockdown does not have any effect on *ERBB2* expression (Supplementary Fig. S4A). Since the effect of *PBP* and *NR1D1* appears to occur through a common pathway (Fig. 2 C and D), it is unlikely that the effect on cell survival is caused by *ERBB2*. The different phenotypic effects of *NR1D1* and *PBP* inhibition compared to *ERBB2* inhibition support this notion, since *ERBB2* inhibition does not result in significant increase in apoptosis ($P=0.08$; Supplementary Fig. S4 B and C), but only in attenuation of proliferation (Table 1). Furthermore, the effect on growth rate observed in the proliferation of the *ERBB2*-negative MCF-7 cells, when *ERBB2* and *NR1D1* are simultaneously overexpressed, is additive (Supplementary Fig. S4D). In the same context, simultaneous knockdown of *ERBB2* and *NR1D1* had an additive effect on BT474 cell viability (Supplementary Fig. S4E), further supporting the notion that the two genes regulate different pathways. It is also unlikely that *NR1D1* and *PBP* are effectors of *ERBB2*, since an *ERBB2* shRNA does not alter message levels of *PBP* or *NR1D1* in BT474 or HEK 293 cells (Supplementary Fig. S4F) and since overexpression of either wild-type or constitutively active forms of *ERBB2* in MCF10A cells does not increase levels of *NR1D1* and *PBP* (data not shown). However, we still cannot formally exclude the possibility that the different phenotypes observed are due to bifurcation of a single pathway.

***NR1D1* and *PBP* induce excessive fat accumulation in *ERBB2*-positive cells**

The emergence of genes related to lipid metabolism in the RNAi screen suggests that fat production is critically important to *ERBB2*-positive cell survival. Stains of neutral fat reveal that *ERBB2*-positive cells exhibit an about 20-fold higher level of accumulated fat in lipid stores, when compared to HMEC cells and a 10-fold increase when compared to other breast cancer cell lines (Fig. 3 A and B and Supplementary Fig. S5A). When quantified by mass spectrometry, differences are also observed in total cellular fats, since BT474 cells are found to contain more than twice the amount of fatty acids of MCF-7 cells (Supplementary Fig.

S5B). Targeting *NR1D1* and *PBP* with shRNAs causes a modest though significant decrease in fat stores in BT474 cells of 47% and 30%, respectively, in the first 48h after transfection (Fig. 4 A and B and Supplementary Fig. S5C). The decrease in cellular fats begins at 24h (Fig. 4 A) and maximizes at 48h after *NR1D1* and *PBP* shRNA transfection, whereas apoptosis under the same conditions is not significant at 24h, but maximizes after 48h (Supplementary Fig. S1B). This indicates that apoptosis caused by *NR1D1* and *PBP* knockdown is a result of the observed changes in the metabolic state of the cells. Knockdown of *ERBB2* results only in 1% and 17% decreases in fat stores of BT474 cells, after 24h and 48h respectively Supplementary Fig. S5D). In addition, when *NR1D1* is overexpressed in the immortalized, non-tumorigenic, MCF10A breast cells (Fig. 4C), a 4-fold increase in fat accumulation is observed in these cells, compared to the control cells (Fig. 4D). In contrast, overexpression a constitutively active *ERBB2* causes only a 1.6x increase in fats in MCF10A cells. Wild-type *ERBB2* is without effect (Supplementary Fig. S5E).

The fat synthetic process is required for survival of *ERBB2*-positive breast cancer cells

The importance of fatty acid synthesis to the survival of *ERBB2*-positive cancer cells is not due to the level of stored fats but rather the synthetic process itself. Targeting *NR1D1* and *PBP* with shRNAs significantly decreases viability (Fig. 2 A and B) and fat stores in BT474 cells (Fig. 4 A and B). However, similar decreases in fat stores of BT474 cells grown in media containing the alternative fuel sources galactose or fructose (Supplementary Fig. S6 A–C) do not lead to cell death (Supplementary Fig. S6D). These results indicate that the survival function provided by *NR1D1* and *PBP* is due to increased activity of the fatty acid synthesis pathway and not to the increased levels of the end products of this pathway.

To identify potential downstream targets of *NR1D1* and *PBP* that are related to fat metabolism and pertinent to breast cancer cell survival we looked at mRNA levels of the three major enzymes of *de novo* fatty acid synthesis, ATP citrate lyase (*ACLY*), acetyl-coenzyme A carboxylase alpha (*ACACA*) and fatty acid synthase (*FASN*). The transcript level of each enzyme is significantly reduced after transfection with *NR1D1* and *PBP* shRNAs (Fig. 5A) or PPAR γ antagonist treatment (Supplementary Fig. S7A). Transcript levels of *FADS2*, a fat metabolism-related gene that was identified in the screen (Table 1), are also downregulated under the same conditions (Fig. 5A and Supplementary Fig. S7A). ShRNAs targeting *ACLY*, *ACACA* and *FASN* significantly decrease BT474 cell viability (Fig. 5A and Supplementary Fig. S7B), demonstrating that these genes are mediators of the survival advantage that *NR1D1* and *PBP* confer to *ERBB2*-positive cells. It has already been established that *FASN* (31,32) and *ACACA* (4) are upregulated in *ERBB2*-positive breast tumors. A search in the Oncomine database (Edition 4) also revealed strong correlations of *PBP* and *NR1D1* to *ACLY* and *ACACA* expression in *ERBB2*-positive breast tumors (see Supplementary Table S5).

Since both synthesis and elongation of fatty acids use NADPH as a cofactor, it is necessary that these reactions be continuously supplied with NADPH. This also predicts that enzymes that generate cytoplasmic pools of NADPH using NADH might be required for fat synthesis and consequently for *ERBB2*-positive breast cancer cell survival. Two genes involved in these processes are malate dehydrogenase 1 (*MDH1*), the key enzyme in the production of cytoplasmic malate from oxaloacetate using NADH (33), and malic enzyme 1 (*ME1*), which converts malate to pyruvate and is the primary source of NADPH required by *FASN* for palmitate synthesis (34). Message levels of the *MDH1* and *ME1* genes after *NR1D1* shRNA treatment are significantly decreased (Fig. 5B). Although *PBP* acts upstream of *NR1D1*, we did not observe the same effect on *MDH1* and *ME1* message levels, after *PBP* knockdown. These results may suggest that *MDH1* and *ME1* are more acutely affected by loss of *NR1D1* than *PBP*. Such a situation might be caused by the kinetics of the pathway. Importantly, targeting *MDH1* and *ME1* with shRNAs, results in decreased viability of BT474 cells but not

MCF-7 cells (Fig. 5B and Supplementary Fig. S7C). Simultaneous knockdown of *MDH1* or *ME1* with either *NR1D1* or *PBP* did not result in an additive effect on cell viability (Supplementary Fig. S7D), consistent with the notion that these two genes are downstream targets of *NR1D1*. Together, these results suggest that *NR1D1* and *PBP* maximize fatty acid synthesis in favor of an altered physiology that provides a survival advantage to the *ERBB2*-positive breast cancer cells (Fig. 5C).

Discussion

Our functional genomics approach revealed that a small fraction of the genes that are overexpressed in *ERBB2*-positive breast cancer plays a role in the aggressiveness of this type of cancer. Down-regulation of 10 out of the 141 genes targeted in our RNAi screen resulted in 50% or more decrease in proliferation of the *ERBB2*-positive, BT474 breast cancer cells. Most interestingly, the screen revealed a number of genes associated to fatty acid metabolism as being necessary for the survival of this type of cells. Three of them, *NR1D1*, *PBP* and *MAP2K6*, were among the most significant hits of the screen. Our interrogation of the system identified not just individual genes that are the most important for *ERBB2*-positive breast cancer, but also a whole pathway and a particular metabolic state that is required for the survival of the cells of this type of cancer, demonstrating the power of this approach.

Knockdown of *NR1D1* and *PBP* induces apoptosis specifically in *ERBB2*-positive cells, indicating that these genes are required for the survival of these cancer cells and fundamentally contribute to their aggressiveness. Although the effects on viability of inhibiting *NR1D1* and *PBP* are seen only in *ERBB2*-positive cells, they are not mediated by *ERBB2* itself. Our results indicate that *NR1D1* and *PBP* are factors that contribute to the aggressiveness of what are clinically designated "*ERBB2*-positive breast cancer". *NR1D1* and *PBP* are part of the *ERBB2*-positive gene expression signature (7), which has been established to better describe *ERBB2*-positive tumors. One reason of *NR1D1* and *PBP* being highly active in this type of cancer cells is their tight genetic linkage to *ERBB2* in the 17q12 amplicon. This tight genetic linkage between *NR1D1*, *PBP* and *ERBB2* causes co-overexpression of these gene products such that, as indicated by the present study, *ERBB2*-positive breast cancer cells are genetically preprogrammed to depend on *NR1D1* and *PBP* for survival. It is unlikely, however, that these genes drive cells to become tumorigenic by themselves. For example, overexpression of *NR1D1* in MCF10A cells does not confer the ability to form colonies in soft agar (data not shown).

We have shown that overexpression of *NR1D1* and *PBP* are responsible for the up-regulation of all three major enzymes of the *de novo* fatty acid synthesis pathway. The phenotypic outcome of *NR1D1* and *PBP* overexpression is that *ERBB2*-positive breast cancer cells possess extraordinarily high levels of fats. Downregulation of *NR1D1* or *PBP* directly results in rapid decrease of fatty acid stores within 48h. These results support the notion that the major effect of inhibition of *NR1D1* and *PBP* is an acute inhibition of the *de novo* fatty acid synthesis pathway that is coordinated by these transcription factors. This has a more profound effect on *ERBB2*-positive breast cancer cells causing apoptosis, which likely reflects the overall higher activity of the fatty acid synthesis pathway in these cells and their dependence upon this pathway for survival. Although the mechanism of *NR1D1* and *PBP* regulating fat metabolism is at present unclear, our results are in accord with a recent study which finds that *NR1D1* overexpression in the livers of transgenic mice causes the upregulation of several genes related to fat synthesis through a poorly understood effect on the sterol response element binding protein SREBP1c (35).

Our approach also identified an extended metabolic network required for the high level fat production that is found in *ERBB2*-positive breast cancer cells. The role of fatty acid synthesis

in the overall metabolic balance of these cells provides a rationale for its essential function. Our finding that it is not the decrease in the amount of stored fats followed by *NR1D1-PBP* inhibition that is responsible for the apoptosis observed in the *ERBB2*-positive breast cancer cells, but the process of synthesizing these fats, supports this notion. Recently published results showing that exogenous supplementation of fatty acids is toxic to *ERBB2*-positive cells is also indicative of this physiology (30). A rationale for fat production as a survival mechanism has been suggested by studies showing that increased fatty acid synthesis in some cancer cells is a feature of aerobic glycolysis (36), the altered tumor cell energy metabolism first proposed by Warburg (37). Oxygen does not serve as the terminal electron acceptor in cells with this physiology. To avoid low NAD⁺/NADH ratios that would eventually feed back to inhibit glycolysis, electrons are incorporated into other molecules such as lactate with the concomitant regeneration of NAD⁺. Since both synthesis and elongation of fatty acids use NADPH as a cofactor, it is thought to play a role in indirectly regenerating NAD⁺ under these conditions. This implies that enzymes that might couple the transfer of electrons from glycolysis to fat synthesis would also be required for *ERBB2*-positive breast cancer cell survival. MDH1 and ME1 are two such enzymes that mediate inter-conversion of NAD-NADH used in glycolysis to NADP-NADPH used in fatty acid synthesis. Our results demonstrate that both these genes are regulated by *NR1D1* and are critical for the survival specifically of the *ERBB2*-positive breast cancer cells. Overall, the transcriptional regulation by *NR1D1* and *PBP* of enzymes involved in fatty acid synthesis and storage and whose concerted action is required for clearing the electrons that are produced during glycolysis, provides an explanation for the essential nature of these genes. These results suggest that there is a greater metabolic requirement served by maximized fatty acid synthesis in the *ERBB2*-positive breast cancer cells, and that this requirement is satisfied by *NR1D1* and *PBP* overexpression.

The central role that the *NR1D1-PBP* pathway plays in the physiology of these cells may have several implications for this type of cancer. The action of *NR1D1* and *PBP* establishes a physiological program that causes products of glycolysis to be stored as triglycerides, rather than to be exported as lactate. This may bestow *ERBB2*-positive breast cancer cells with increased metabolic autonomy *in vivo*, which could directly improve clonal dissemination and survival during metastasis and tumor relapse. Supporting this notion, recent expression-profiling studies identified *PBP* in the group of genes that are differentially expressed in invasive ductal carcinoma (38) and in brain metastases of breast cancer (39). This altered physiological program may also implicate *NR1D1*, a proposed link between the circadian clock and cellular metabolism (29) as a potential mediator of the tumorigenic influences of diet and the “night shift” that have been recently established for breast cancer (40). Further study of *NR1D1* and *PBP* promises the elucidation of key functions associated to breast cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
(A) The RNAi screen targeting genes overexpressed in *ERBB2*-positive breast cancer. Three transfection mixes were produced for each shRNA and each was transfected into triplicate wells of BT474 cells. AlamarBlue was used to monitor cell proliferation and viability. The averages of the nine parallel cultures were calculated for each shRNA, normalized to transfection efficiency, expressed as % of the control shRNA (luciferase) and sorted on the basis of effect. (B) *NR1D1* and *PBP* are specifically necessary for viability of *ERBB2*-positive cells. Several breast cancer (BC) cell lines as well as HMEC and HEK 293 cells were transfected and assayed as in Figure 1A. Effects of each shRNA on each cell line were subjected

to hierarchical cluster analysis using Cluster 3.0 (M. Eisen) and displayed using Treeview. The ERBB2 and estrogen receptor (ER) status of each cell line is indicated at the bottom.

Figure 2.

NR1D1 and *PBP* influence survival of *ERBB2*-positive cells. (A) BT474 cells were transfected with luciferase (control), *PBP* and *NR1D1* shRNAs and co-transfected with GFP. Green cells were counted at intervals and expressed as percentage of control (* $P < 0.03$). (B) BT474 cells were transfected with luciferase (control), *PBP* or *NR1D1* shRNAs for 48h.

Immunofluorescence was performed for cleaved Caspase-3 signal; cell nuclei were stained with Hoechst 33243. Cells were counted for cleaved Caspase-3 signal (* $P < 0.0003$). (C) *PBP* and *NR1D1* mRNA levels of BT474 cells transfected with luciferase (control), *PBP* or *NR1D1* shRNAs for 48h, analyzed by qRT-PCRs. (* $P < 0.003$). (D) BT474 cells were transfected with luciferase (control) *PBP*, *NR1D1* or *PBP* and *NR1D1* shRNAs together and

co-transfected with GFP. Transfected cells were counted at 24h and 72h and the 72h-to-24h ratio for each shRNA was calculated and expressed as % of the control shRNA (top panel; $*P<0.01$). Transfected cells were also assayed by immunofluorescence and counted for cleaved Caspase-3 signal at 48h of transfection (bottom panel; $*P<0.0001$).

Figure 3.
ERBB2-positive breast cancer cells possess high levels of fats (A) Several breast cancer cell lines and normal HMEC cells were stained for fat stores with BODIPY 493/503 lipid stain. Hoechst 33243 was used for nuclei staining. The *ERBB2* and ER status of each cell line is indicated on the left. (B) Quantification of BODIPY signal from (A).

Figure 4.

NR1D1 and *PBP* overexpression induces fat accumulation in *ERBB2*-positive cells. (A) BODIPY images and (B) quantification of BT474 cells transfected with luciferase (control), *PBP* or *NR1D1* shRNAs and co-transfected with DsRed (**P*<0.03). Arrows indicate transfected cells. (C) *NR1D1* qRT-PCR of MCF10A cells transduced with either empty vector (control) or a construct expressing *NR1D1* (**P*=0.03). (D) BODIPY images (top panel) and quantification (bottom panel) of MCF10A cells overexpressing *NR1D1* compared to control. Hoechst 33243 was used for nuclei staining (**P*=0.001).

Figure 5.

NR1D1 and *PBP* upregulate the *de novo* fatty acid synthesis pathway. (A) qRT-PCRs of BT474 cells treated with luciferase (control), *PBP* or *NR1D1* shRNAs for detection of *ACLY*, *ACACA*, *FASN* and *FADS2* mRNAs (left panel; * $P < 0.005$). BT474 cells were also transfected with *ACLY*, *ACACA* and *FASN* shRNAs, co-transfected with GFP and counted as in Fig. 2B (right panel; * $P < 0.01$). (B) *MDH1* and *ME1* qRT-PCRs of BT474 cells treated with luciferase (control), *PBP* or *NR1D1* shRNAs (left panel; * $P < 0.002$). BT474 cells were also transfected with luciferase (control), *MDH1* and *ME1* shRNAs and assayed as in Fig. 2B (right panel; * $P = 0.0003$). (C) The model of *NR1D1* and *PBP* - mediated regulation of fatty acid metabolism and energy balance in *ERBB2*-positive cells.

Table 1

The shRNAs that consistently decreased BT474 cell proliferation in the RNAi screen

Symbol	Name	Function	% proliferation of control*	Z-score
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2	Receptor Tyrosine Kinase	27.04	-2.86
MKI67	Antigen identified by monoclonal antibody Ki-67	Proliferation	32.99	-2.59
NR1D1	Nuclear receptor subfamily 1, group D, member 1	Adipogenesis - Fat metabolism	35.83	-2.46
PBP	PPAR binding protein PPARBP	Adipogenesis - Fat metabolism	37.08	-2.40
MAP2K6	Mitogen-activated protein kinase 6	Adipogenesis - Signal Transduction	37.23	-2.39
SLPI	Secretory leukocyte protease inhibitor	Proteolysis	38.65	-2.33
PFN2	Profilin 2	Actin polymerization	41.06	-2.22
SPINT1	Serine protease inhibitor, Kunitz type 1	Proteolysis	42.87	-2.14
LASP1	LIM and SH3 protein 1	Actin cytoskeleton organization	43.27	-2.12
TPD52	Tumor protein D52	Secretion	46.50	-1.97
H2AFY	H2A histone family, member Y	Nucleosome assembly	50.46	-1.79
SIAT4C	Sialyltransferase 4C	Transferase activity	53.30	-1.66
SFRS7	Splicing factor, arginine/serine-rich 7, 35kDa	Splicing	53.87	-1.64
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	Apoptosis	54.62	-1.61
CA9	Carbonic anhydrase IX	Secretion	54.01	-1.61
RPL19	Ribosomal protein 19	Ribosome structure	55.23	-1.60
FADS2	Fatty acid desaturase 2	Fat metabolism	63.54	-1.20
STARD3	StAR-related lipid transfer (START) domain containing 3	Fat metabolism	66.88	-1.00
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3	Receptor Tyrosine Kinase	69.33	-0.94
GRB7	Growth factor receptor bound protein 7	Signal Transduction	71.41	-0.84
FASN	Fatty acid synthase	Fat metabolism	75.40	-0.66

* Results shown are statistically significant ($P < 0.05$). Dotted line indicates genes that produced close to or more than 50% decrease in proliferation