Characterization of the Recurrent 8p11-12 Amplicon Identifies PPAPDC1B, a Phosphatase Protein, as a New Therapeutic Target in Breast Cancer

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Abstract

The 8p11-12 chromosome region is one of the regions most frequently amplified in breast carcinoma (10–15% of cases). Several genes within this region have been identified as candidate oncogenes, as they are both amplified and overexpressed. However, very few studies have explored the role of these genes in cell transformation, with the aim of identifying valuable therapeutic targets. An analysis of comparative genomic hybridization array and expression profiling data for a series of 152 ductal breast carcinomas and 21 cell lines identified five genes (LSM1, BAG4, DDHD2, PPAPDC1B, and WHSC1L1) within the amplified region as consistently overexpressed due to an increased gene copy number. The use of small interfering RNA to knock down the expression of each of these genes showed the major role played by two genes, PPAPDC1B and WHSC1L1, in regulating the survival and transformation of two different cell lines harboring the 8p amplicon. The role of these two genes in cell survival and cell transformation was also confirmed by long-term knockdown expression studies using short hairpin RNAs. The potential of PPAPDC1B, which encodes a transmembrane phosphatase, as a therapeutic target was further shown by the strong inhibition of growth of breast tumor xenografts displaying 8p11-12 amplification induced by the silencing of PPAPDC1B. The oncogenic properties of PPAPDC1B were further shown by its ability to transform NIH-3T3 fibroblasts, inducing their anchorage-independent growth. Finally, microarray experiments on PPAPDC1B knockdown indicated that this gene interfered with multiple cell signaling pathways, including the Janus-activated kinase–signal transducer and activator of transcription, mitogen-activated protein kinase, and protein kinase C pathways. PPAPDC1B may also potentiate the estrogen receptor pathway by downregulating DUSP22. [Cancer Res 2008;68(17):7165–75]

Introduction

Recurrent chromosomal alterations are a hallmark of cancer cells and represent critical events in tumor development. In particular, oncogene activation through increased gene copy number resulting in overexpression contributes to the malignant transformation of various human solid cancers, including breast cancer (1–3). In breast cancer, major recurrent amplicons include 17q12 (ERBB2/HER-2), 8q24 (MYC), 11q13 (CCND1), 20q13, and 8p11-12 (4). The 17q12 amplicon, which has been extensively characterized, provides a clear illustration of the potential of tumor genomics for identifying drug targets. Indeed, this amplification consistently leads to overexpression of the ERBB2 receptor, which can be efficiently targeted by specific inhibitors, such as Herceptin, now used to treat ERBB2-positive breast carcinoma (5). Two other genes within the 17q12 amplicon, GRB7 and STARD3, have also recently been shown to play a causal role in tumor development (6). Major efforts are now underway to characterize potential drug targets in other breast cancer amplicons (6–8).

The 8p11-12 amplicon is observed in approximately 10% to 15% of breast carcinomas and is correlated with histologic grade, Ki-67 proliferation index, and poor prognosis (9–15). Recent studies have improved the definition of this amplicon and have identified several candidate oncogenes based on significant correlations between gene amplification and gene overexpression (13–17). However, no clear consensus about the minimal region of amplification or about the driver genes of the amplicon has emerged from these expression/amplification correlation studies. This lack of consensus highlights the need for functional experiments to validate the oncogenic properties of the various candidate oncogenes. The functional role of several putative oncogenes has already been explored. The inhibition of FGFR1, a candidate driver gene, does not slow the proliferation of all breast cancer cell lines with 8p11-12 amplification (16, 18), whereas LSM1 has been shown to display transforming activity in all human breast cancer cell lines tested harboring the 8p amplicon (19). The first systematic functional study of the genes overexpressed as a consequence of the 8p11-12 amplification in breast tumors was recently published (15). Based on their ability to transform the immortalized human mammary epithelial cell line MCF10A, rendering it independent of growth factors in vitro, three genes were identified as breast cancer oncogenes: BAG4, LSM1, and C8orf4. Using a very different functional approach, based on the small interfering RNA (siRNA)-mediated knockdown of gene expression, we also aimed to identify putative oncogenes and therapeutic targets among genes amplified and overexpressed in breast tumors...
with 8p amplification. We report the analysis, by array comparative genomic hybridization (CGH) and gene expression profiling, of a series of 152 ductal breast carcinomas and 21 cell lines. This analysis identified five genes (LSM1, BAG4, DDHD2, PPAPDC1B, and WHSC1L1) within the amplified region as consistently overexpressed due to an increase in gene copy number. Knocking down the expression of each gene, using siRNA, showed the major oncogenic role of two genes, PPAPDC1B and WHSC1L1, both regulating the cell survival and transformation of two different cell lines harboring the 8p amplicon. These results were also confirmed by long-term knockdown expression analysis with short hairpin RNA (shRNA). PPAPDC1B encodes a transmembrane protein phosphatase, a potential therapeutic target. We therefore explored its potential role in tumor growth in vivo using a siRNA approach in a xenograft model. We also investigated the mechanisms and pathways deregulated by PPAPDC1B in tumor progression by carrying out microarray experiments following PPAPDC1B knockdown with shRNAs.

Materials and Methods

Breast tumor samples and cell lines. We used frozen samples of 152 primary infiltrating ductal breast carcinomas (1 T0, 80 T1, 63 T2, 7 T3, and 1 T4b) and 11 normal breast tissues. Normal breast samples were obtained during reduction mammoplasty. Samples of tumor or normal breast tissues were flash frozen and stored at −80°C. All tumor samples contained >50% cancer cells and the normal samples contained >50% breast epithelial cells. This study was approved by the Institutional Review Boards of Institut Curie. In total, 19 breast cancer–derived cell lines (184B5, BT474, BT549, CAMA-1, HCC1957, HeLa, MCF7, MDA-MB-134V1, MDA-MB-157V1, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MDA-MB-468, SKBR3, T47D, ZR-75-1, and ZR-75-30), one chemically transformed breast cell line (184B5), and one normal breast cell line (MCF 10A) were obtained from the American Type Culture Collection or from the German Resource Centre for Biological Material. The mouse fibroblastic cell line NIH-3T3 was kindly provided by Dr. S. Belluscí (Saban Research Institute, Los Angeles, CA).

DNA and RNA extraction from breast tumor samples and cell lines. DNA and RNA were extracted from frozen samples of breast carcinomas and normal breast tissue by cesium chloride protocol (20, 21). RNA was extracted from cell lines by the conventional phenol-chloroform procedure. For quantitative PCR experiments, whereas DNA was extracted with a bacterial artificial chromosome array and 1 T4b) and 11 normal breast tissues. Normal breast samples were obtained during reduction mammoplasty. Samples of tumor or normal breast tissues were flash frozen and stored at −80°C. All tumor samples contained >50% cancer cells and the normal samples contained >50% breast epithelial cells. This study was approved by the Institutional Review Boards of Institut Curie. In total, 19 breast cancer–derived cell lines (184B5, BT474, BT549, CAMA-1, HCC1957, HeLa, MCF7, MDA-MB-134V1, MDA-MB-157V1, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MDA-MB-468, SKBR3, T47D, ZR-75-1, and ZR-75-30), one chemically transformed breast cell line (184B5), and one normal breast cell line (MCF 10A) were obtained from the American Type Culture Collection or from the German Resource Centre for Biological Material. The mouse fibroblastic cell line NIH-3T3 was kindly provided by Dr. S. Belluscí (Saban Research Institute, Los Angeles, CA).

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CGH array. The 3:4K CIT (Carte d’Identité des Tumeurs program of the Ligue Nationale Contre le Cancer) bacterial artificial chromosome array and the experimental procedures for spotting, hybridization, and washing were as previously described (22).

DNA microarrays and probe set filter criteria. Microarray experiments were performed as previously described (23). For tumors, we used the Affymetrix Human Genome U133 set (HG-U133 A and B), consisting of two GeneChip arrays and containing 44,692 probe sets, corresponding to >16,000 unique genes for DNA microarray analysis. For cell lines, we used the Affymetrix Human Genome U133 Plus 2.0 DNA microarray containing 54,319 probe sets corresponding to ~19,000 unique genes. The CGH and expression data sets used in this study are available online.

Cell culture. NIH-3T3, CAMA-1, and MCF7 cells were cultured in DMEM supplemented with 10% FCS and 2 mmol/L L-glutamine; ZR-75-1 cells were grown in RPMI 1640 supplemented with 10% FCS, 1 mmol/l sodium pyruvate, 4.5 g/l glucose, and 10 mmol/l HEPES. Media were supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. All reagents were purchased from Life Technologies.

RNA interference. Transient transfections were performed using Oligofectamine according to the manufacturer’s instructions (Invitrogen), with 100 nmol/l siRNA. A negative control siRNA (UCUCCGAAACGGU-
Figure 1. Genetic aberrations on chromosome 8p11-12 in breast cancers. A, CGH array of the 8p11-12 chromosome in breast cancer samples (top) and cell lines (bottom). Each column represents a clone on the array. Clones are ordered according to genome position based on the NCBI human genome reference sequence 36 (May 2006) from telomere (left) to centromere (right). Three clones were localized to the same position (CTD-2139K9, and CTD-2092C4). We therefore considered, for this position, the mean signal for the three clones: mean ratio. Log2 values for the Cy5 (tumor DNA)/Cy3 (control DNA) ratio are depicted on a color scale in which blue indicates loss, red indicates gain, and gray indicates an absence of data. B, genomic profiles of 8p11-12 amplification in selected cell lines or tumor samples, defining a common region of amplification between clone RP11-962G15 (chr8:36653064-36836067) and clone RP11-470M17 (chr8:40010617-40185770). C, known genes present in the common region of amplification based on build 36 from NCBI.
Table 1. Pearson’s correlation between DNA copy number and gene expression for the 28 genes of the minimal 8p region of amplification

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NOTE: R values of >0.4 (P < 0.05) are highlighted in gray. If R > 0.4 in both cell lines and tumors, the gene name is highlighted in gray. If several probe sets were available for one gene, we used only the probe set yielding the highest correlation coefficient to ensure that no candidate gene was spuriously excluded. Abbreviation: NA, probe set not available.

facility of the Curie Institute in specified pathogen-free conditions. Animals were housed and cared for in accordance with the institutional guidelines of the French National Ethics Committee (Ministère de l’Agriculture et de la Pêche, Direction Départementale des Services Vétérinaires, Paris, France) under the supervision of investigators with the required accreditation. Slow-release estradiol pellets (1.7 mg, 90-d release; Innovative Research of the French National Ethics Committee (Ministère de l’Agriculture et de la Pêche, Direction Départementale des Services Vétérinaires, Paris, France) were used to estimate tumor volume according to the formula a/2 × b/2, where a is the largest diameter and b is the smallest diameter.

Tumor formation was monitored for up to 48 d and tumor size was determined twice weekly with Vernier calipers; two perpendicular diameters were used to estimate tumor volume according to the formula a/2 × b/2, where a is the largest diameter and b is the smallest diameter.
Production of PPAPDC1B-expressing NIH-3T3 clones. The PPAPDC1B cDNA (cDNA clone IMAGE:5267610) in the pBluescriptR vector was purchased from Geneservice and the sequence was verified by sequencing. This cDNA was excised from the vector and inserted into pcDNA3.1. NIH-3T3 cells were transfected with empty pcDNA3.1 or with PPAPDC1B-pcDNA3.1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. G418 (400 μg/mL) was added to culture medium 48 h after transfection, and drug selection was continued for 2 wk. Single colonies were ring cloned and expanded. Two control clones were obtained (Neo1 and Neo2) and three PPAPDC1B-expressing clones presenting different levels of PPAPDC1B mRNA were selected.

Statistics. For each gene in the common amplification region, the RNA levels measured by Affymetrix arrays of the 9 8p-amplified samples were compared with those of 114 tumor samples with an unchanged 8p DNA copy number and with those of normal samples using the Wilcoxon rank sum test. The Affymetrix signal and CGH log₂ ratio were compared using Pearson's correlation test. For each gene with an R value of >0.4 (P < 0.05), the level of RNA in each amplified tumor sample was compared with the distribution of RNA levels for the same gene in normal samples. The difference was considered significant if it exceeded two SDs (z score > 2; P < 0.0214). We evaluated the results of the functional experiments using χ² tests, Student's t test, or the Wilcoxon rank sum test depending on the experiment considered. Differences were considered significant if P < 0.05. Affymetrix expression data for PPAPDC1B knockdown experiments in cell lines were analyzed using Student's t test and the same significance threshold as above. The genes identified as differentially expressed by this method for each shRNA were listed in order of P values and log₂ fold change sign. The lists were then compared using hypergeometric tests and Bayesian methodology (25) to evaluate their concordance for the two shRNAs.

Results

We used CGH on DNA microarrays (array CGH) to map regions of amplification (identified based on a normalized array CGH log₂ ratio higher than 1.0) in 152 ductal breast carcinomas and 21 cell lines. Consistent with previous studies, the most frequently amplified regions were 17q13 (13%), 8p11-12 (9%), and 11q13 (10%), and the most frequently lost regions were 16q (50%), 8p (40%), and 11q (38%). We focused our study on the chromosome 8 amplicon and delineated a 3.2-Mb region of DNA amplification on chromosome 8p11-12 common to 11 of the 152 infiltrating ductal breast carcinomas (7.2%) and to 3 of the 21 breast cancer cell lines (14%) studied (Fig. 1A and B). This region encompassed the DNA fragments from clone RP11-962G15 to clone RP11-470M17 (Fig. 1A and B) and contained 28 known genes according to the Build 36 Human Reference Sequence from National Center for Biotechnology Information (NCBI; May 2006; Fig. 1C).

Affymetrix DNA microarrays were used to analyze RNA levels in 128 of the 152 breast carcinomas, in 18 of the 21 cell lines studied on CGH arrays, and in 11 normal breast samples. Affymetrix data were thus obtained for 9 of the 11 tumors and two of the three cell lines.
lines for which CGH array-based evidence of 8p11-12 amplification was obtained. Eleven of the 28 genes encompassed by the common region of amplification presented a direct relationship between DNA copy number and expression level in both tumors and cell lines, with a correlation coefficient $R > 0.4$ ($P < 0.05$), indicating that DNA copy number had a significant effect on gene expression level (Fig. 2A; Table 1; Supplementary Fig. S1). Accordingly, these 11 genes were more strongly expressed in tumors with 8p amplification than in tumors without amplification or in normal breast tissue, and in breast cancer cell lines with 8p amplification than in nonamplified cell lines (Supplementary Table S1). Consistent results were obtained for all Affymetrix probe sets, when available, for a given gene (data not shown). Given that candidate driver genes should be systematically overexpressed owing to gene amplification, we were able to exclude 6 of these 11 genes based on data from two particularly informative cases (S15 and S80; Fig. 2B). Indeed, the SPPH2, PROSC, BRF2, and ASH2L genes were not always significantly overexpressed (Fig. 2B) despite the high level of amplification of the 8p11-12 region observed in S15 (Fig. 1B). Similarly, the EIF4EBP1 and ADAM9 genes could be excluded based on the data for sample S80 (Fig. 2B). ADAM9 was also excluded based on the data for ZR-75-1 and CAMA-1, as it was not more strongly expressed in these cell lines with 8p amplification than in MCF10A, a normal breast epithelium–derived cell line (Fig. 2C). The 14 other genes of the region, which presented no significant correlation between DNA and RNA levels in cell lines and tumors and for which we had Affymetrix data for the tumors, were not systematically overexpressed in amplified tumors (Supplementary Fig. S2). Five genes, LSM1, BAG4, DHH2D2, PPAPDC1B, and WHSC1L1, were therefore identified as candidate drivers by this genomic and transcriptomic analysis of breast tumors.

RNA interference technology (26) was then used to investigate the role of these five genes in the cell growth and tumorigenic properties of two cell lines with 8p11-12 amplification (CAMA-1 and ZR-75-1) and in one control cell line without this amplicon (MCF7). The transfection of CAMA-1 and ZR-75-1 cells with specific siRNAs markedly decreased mRNA levels, resulting in levels similar to those observed in untransfected MCF7 cells (70–90% inhibition; Fig. 3A). The silencing of each gene individually had no significant effect on the levels of expression of the other four genes (data not shown). The specific knockdown of LSM1, BAG4, PPAPDC1B, or WHSC1L1 yielded fewer viable ZR-75-1 and CAMA-1 cells than transfection with the control siRNA (Fig. 3B). None of these siRNAs had any effect on MCF7 cells, suggesting that the effect observed following transfection with a specific siRNA was due to the silencing of a specific gene rather than to an off-target effect (Fig. 3B). This decrease in the number of viable cells could be attributed to an increase in the rate of apoptosis, with no significant change in cell cycle progression (Fig. 3C; data not shown). Thus, each of these four genes seems to regulate cell survival in breast carcinomas with 8p amplification. However, they differ in terms of their role in cloning efficiency. Indeed, PPAPDC1B silencing significantly decreased the colony-forming ability of CAMA-1 and ZR-75-1 cells in both anchorage-dependent (focus formation on plates) and anchorage-independent (soft agar) conditions, whereas it had no effect on MCF7 cells (Fig. 3D and E). WHSC1L1 knockdown also inhibited focus formation by CAMA-1 cells (Fig. 3D) and, to a lesser extent, the growth of both CAMA-1 and ZR-75-1 cells in soft agar (Fig. 3E). In contrast, the silencing of LSM1, BAG4, and DHH2D2 had no effect on the cloning efficiency of cells with and without 8p11-12 amplification.

Our results, obtained with a siRNA approach, suggest that PPAPDC1B and WHSC1L1 are two major driver genes of the 8p11-12 amplicon. Both are strongly overexpressed in breast carcinomas with 8p amplifications and regulate cell survival and transformation. The ability of these two major genes to regulate 8p-amplified breast cancer cell proliferation/survival and cell transformation was confirmed by long-term knockdown of the expression of these genes following the stable expression of shRNAs in ZR-75-1 and CAMA-1 cell lines (Supplementary Fig. S3), fully confirming the effects observed with siRNAs. Indeed, both WHSC1L1 and PPAPDC1B shRNAs efficiently knocked down WHSC1L1 and PPAPDC1B transcript levels by 80% to 90% (Supplementary Fig. S3A). Cells expressing WHSC1L1 or PPAPDC1B shRNAs grew much slower than control cells, resulting in a 4-fold difference in the number of shRNA-expressing cells 2 weeks after seeding (Supplementary Fig. S3B). Moreover, cells expressing WHSC1L1 or PPAPDC1B shRNAs formed fewer colonies in focus formation assay than control cells (Supplementary Fig. S3C).

However, as PPAPDC1B knockdown had a greater effect on cloning efficiency than WHSC1L1 knockdown, and as the phosphatase domain of PPAPDC1B may be an appropriate
pharmacologic target, we decided to focus our study on the role of this gene in breast cancer cell aggressiveness in vivo. ZR-75-1 cells were implanted s.c. in athymic nude mice. Once tumors were established, mice were randomly selected for treatment with control or PPAPDC1B-specific siRNAs, both complexed with atelocollagen, a reagent shown to increase mRNA knockdown efficiency in vivo. We used a combination of two different siRNAs targeting two different exons of PPAPDC1B (exons 2 and 3), as this approach is more efficient for gene silencing than the use of a single siRNA (27). The second anti-PPAPDC1B siRNA (siRNA2) knocked down mRNA levels and decreased the ability of CAMA-1 and ZR-75-1 cells to form colonies in anchorage-independent conditions as efficiently as the first (data not shown). After 48 days of treatment, tumor volume was 80% lower in mice treated with PPAPDC1B siRNAs than in mice treated with control siRNAs (P < 0.001, Wilcoxon rank sum test; Fig. 4A and B). No histologic difference was observed between treated and control tumors (data not shown). This inhibition of tumor growth was associated with a significant decrease in PPAPDC1B mRNA levels (P = 0.0035, Student’s t test; Fig. 4C).

Our results clearly identify PPAPDC1B as a major gene within the 8p11-12 amplicon in breast carcinoma regulating cell survival and cell transformation both in vitro and in vivo, suggesting that PPAPDC1B may play an oncogenic role. The potentially oncogenic properties of this protein were further shown by the induction of NIH-3T3 cell transformation by stable PPAPDC1B expression (Supplementary Fig. S4A and B). Indeed, three independent PPAPDC1B-expressing clones formed colonies in anchorage-independent conditions,
whereas control clones formed far fewer, smaller colonies (Supplementary Fig. S4B).

Our results, based on both the knockdown and expression of PPAPDC1B in different cell lines, clearly show that the product of this gene is involved in cell survival and transformation. We investigated the mechanism by which PPAPDC1B regulates these processes in a 8p11-12 amplified cell line (ZR-75-1) by carrying out expression profiling experiments with Affymetrix U133 Plus 2.0 DNA microarrays following PPAPDC1B knockdown with two different shRNAs (sh242 and sh558, targeting exon 3 and 6, respectively; Fig. 5A). The two ordered lists of genes displaying a significant change in expression following PPAPDC1B knockdown (P < 0.05, t test) overlapped considerably and significantly, as shown by hypergeometric test (P < 10e−22) and Bayesian methodology (data not shown; ref. 25). We found that 150 of the 614 probe sets displaying significant modulation by both PPAPDC1B shRNAs presented a more than 1.5-fold change in expression. These 150 probe sets formed five clusters: two small nonspecific clusters containing genes in which expression levels changed in opposite directions with the two shRNAs (28 probe sets/19 genes), one cluster of up-regulated genes (48 probe sets/37 genes), and two clusters of down-regulated genes (74 probe sets/59 genes; Fig. 5B). The last three of these clusters, corresponding to 122 probe sets displaying alterations (96 genes; Supplementary Table S2), were thus considered to be significantly and specifically altered (by both shRNAs, in the same direction in each case) by PPAPDC1B knockdown. They were analyzed further with OnTo-Express (Fig. 5C, top) and Pathway-Express (Fig. 5C, middle) software (28–35) to identify enrichment in Gene Ontology (GO) biological processes and in functional pathways, respectively. This approach suggested the probable involvement of calcium-mediated signaling, mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and Janus-activated kinase (JAK)-signal transducer and activator of transcription (STAT) pathways in PPAPDC1B-mediated tumorigenesis. Based on GO annotations, a more general significant enrichment (P < 0.01) in probe sets involved in signal transduction (Fig. 5C, bottom) was observed. We also investigated the genes that were both altered following PPAPDC1B knockdown and differentially expressed in 8p11-12-amplified tumors and either normal tissue (26 genes) or non–8p11-amplified samples (4 genes; Fig. 5D). Like PPAPDC1B, the other two genes down-regulated by PPAPDC1B knockdown and overexpressed in 8p11-12-amplified tumors belonged to the 8p11-12 amplicon, suggesting that PPAPDC1B regulation acts together with gene amplification to increase the expression levels of ZNF703 and AP3M2. A finding of even greater importance was the demonstration that DUSP22, which encodes a dual-specificity phosphatase that negatively regulates the estrogen receptor (ER) signaling pathway (38), is both up-regulated by PPAPDC1B knockdown and underexpressed in 8p-amplified tumors (Fig. 5D).

Discussion

Oncogene activation through increased gene copy number contributes to malignant transformation in human cancers. The 8p11-12 chromosome region is one of the most frequently amplified genomic fragments in breast carcinoma. It therefore probably contains one or possibly several oncogenes.

By combining analyses of RNA levels and copy number changes for 152 ductal breast cancer samples and 21 breast cancer cell lines, we were able to identify five candidate oncogenes in the 8p11-12 region: LSM1, BAG4, DDHD2, PPAPDC1B, and WHSC1L1. These genes were identified based on significant correlations between expression levels and copy number and systematic and significant overexpression in all tumor samples and cell lines presenting 8p11-12 amplification with respect to their 8p11-12 normal counterparts. Several groups have reported analyses of gene expression as a function of copy number changes in the 8p11-12 region in breast carcinomas and have also identified candidate oncogenes based on overexpression as a consequence of amplification. Two of the genes we identified, LSM1 and PPAPDC1B, were also identified as candidate oncogenes by several other groups (13–17, 36). DDHD2 and WHSC1L1 were identified by two groups (14, 15) and BAG4 was identified by Garcia and colleagues (13), Yang and colleagues (15), and Still and colleagues (36). A systematic functional study by siRNA inactivation of the five candidate oncogenes identified in this study indicated that the silencing of PPAPDC1B, WHSC1L1, and, to a lesser extent, LSM1 and BAG4 impaired the clonogenicity and/or survival of breast cancer cell lines harboring 8p11-12 amplifications. The absence of a clear phenotype following the invalidation of these genes in the MCF7 cell line (with no 8p11-12 amplification) provides strong evidence for the gene-specific effects of these siRNAs and against significant off-target effects. Moreover, the targeting of WHSC1L1 and PPAPDC1B by transfection with siRNAs and by infection with lentiviruses encoding two different siRNAs for each gene had very similar phenotypic consequences. The silencing of any one of the four genes identified promoted the apoptosis of 8p-amplified cell lines, but only WHSC1L1 or PPAPDC1B silencing impaired colony formation. This suggests that the cell population is not homogeneous and that these last two genes regulate clonogenic cell survival, whereas LSM1 and BAG4 regulate only the survival of nonclonogenic cells.

WHSC1L1 has already been identified as an oncogene; it is amplified and overexpressed in lung carcinoma and its transforming abilities have been described in a lung carcinoma cell line (37). We confirm previous reports that PPAPDC1B is consistently overexpressed when amplified (13–17, 36) and provide the first functional evidence of its involvement in breast cancer cell transformation. Knockdown experiments with two different shRNAs in a PPAPDC1B-amplified cell line indicated that this gene interfered with critical signaling pathways, including calcium-mediated signaling, PKC, JAK-STAT, and MAPK pathways. The expression of DUSP22, which encodes a dual-specificity phosphatase that negatively regulates the estrogen receptor (ER) signaling pathway (38), is both up-regulated by PPAPDC1B knockdown and down-regulated by 8p11-12 amplification, as shown by comparisons with other breast tumors and normal tissues. This suggests that PPAPDC1B amplification may increase ER activity in ER-positive tumors. It is interesting to note that in our series of tumors, the 8p amplicon was always associated with ER-positive status (all 11 cases). Together, these observations strongly suggest that PPAPDC1B amplification contributes to oncogenesis through multiple cell signaling pathways. The potential of PPAPDC1B as a therapeutic target was further shown by the dramatic inhibition of growth in vivo following the silencing of PPAPDC1B in breast tumor xenografts displaying 8p11-12 amplification. In this model, PPAPDC1B mRNA level at the end of the experiment was about 35% lower after the injection of PPAPDC1B siRNAs than after control siRNA injection. This difference, which is significant (P = 0.0035), may, however, seem too modest to account for the observed phenotype, but siRNA injections were administered on a weekly basis and inhibition levels were measured 6 days after the last siRNA injection when the effects of the siRNA would be expected to be the weakest. Moreover, the results presented are an...
average for different tumor cells and the extinction of PPAPDC1B is probably heterogeneous due to differences in the accessibility of different parts of the tumors to siRNA.

A systematic functional study of the genes overexpressed as a consequence of the 8p11-12 amplification in breast tumors was recently published (15). It identified 21 genes for which copy number and expression were strongly correlated. With the exception of ADAM9, all the genes identified in this study were also identified by Yang and colleagues. Based on unclearly defined criteria, Yang and colleagues investigated the ability of a subset of 8 of these 21 genes to promote the insulin-independent or epidermal growth factor (EGF)-independent growth of the human mammary MCF10A cell line in vitro. PPAPDC1B, BAG4, and LSM1 were included in this panel, but neither WHSC1L1 nor DDHD2 was included despite displaying strong correlations between copy number and expression level. Yang and colleagues finally identified BAG4, LSM1, and C8orf7 as breast cancer oncogenes. Thus, two genes, LSM1 and BAG4, were identified in both our study and in that by Yang and colleagues. Unlike us, Yang and colleagues did not identify PPAPDC1B as an oncogene. This difference in results may be linked to differences in the functional tests used in the two studies. Indeed, Yang and colleagues assessed the growth factor–independent proliferation of MCF10A cells (on insulin and EGF), whereas we assessed the loss of transforming properties after gene knockdown in 8p-amplified breast cancer cell lines and carried out NIH-3T3 cell transformation assays. The difference in the results obtained in these two studies suggests that PPAPDC1B promotes cell transformation without influencing growth factor–dependent cell growth. The role of the various isoforms described for PPAPDC1B should also be investigated in this context. PPAPDC1B seems hence to have effects on both cell survival and cell transformation, which could be accounted for by the activation of different signaling pathways, such as the MAPK and JAK-STAT signaling pathways. Such dual effects on cell survival and cell transformation are not unique to PPAPDC1B and have already been reported for another gene of the 8p11-12 amplicon, LSM1. Indeed, the down-regulation of LSM1 expression, using siRNA, in the 8p-amplified breast cell line SUM44 results in the inhibition of growth in soft agar (19), and antisense LSM1 has been shown to decrease cell viability and to increase apoptosis in pancreatic cancer (39).

The only published report on PPAPDC1B suggests, based on the down-regulation of this gene in metastatic human hepatocellular carcinoma (HCC) and on experimental criteria, that it may act as a metastasis suppressor in HCC (40). In contrast, its overexpression in a subset of breast cancers due to gene amplification, together with the experimental data reported here, indicates that it may have an opposite, oncogenic role in a different cell background. Alternatively, there may be functional differences between the different PPAPDC1B isoforms. Such isoform-specific properties have been described for BCL2L1, which encodes both apoptotic, Bcl-X(S), and antiapoptotic, Bcl-X(L), proteins (41). Yang and colleagues also identified C8orf7 as a putative oncogene. This gene is located outside the minimal region reported here and was therefore not studied further. However, nonoverlapping minimal regions of amplification have been described in the 8p11-12 region, and it is therefore likely that genes other than LSM1, BAG4, PPAPDC1B, and WHSC1L1, such as C8orf7, also contribute to breast cancer development. The presence of several driver genes is reminiscent of the situation observed for the 17q12 amplicon, in which the overexpression of STARD3 and GRB7, in addition to ERBB2, contributes to cell proliferation (6). Moreover, several other regions of amplification within 17q have been described. They may occur in association with or independently of the ERBB2 amplicon. This report, together with previous publications focusing on the 8p11-12 amplicon, suggests that 8p may display similar variability. Indeed, the 8p amplicon is reported to be large in most cases, encompassing the entire region, and rare amplicons limited to regions proximal (42) or distal (13, 17) to PPAPDC1B have been described. One group has also suggested that the 8p11-12 amplicon could be subdivided into different amplicons (14).

We nevertheless believe that, like ERBB2 for the 17q12 amplicon, PPAPDC1B constitutes a major potential therapeutic target in the 8p12 amplicon. Indeed, in addition to the effects of this gene on apoptosis and cloning efficiency in vitro and on tumor growth in vivo, PPAPDC1B encodes a putative transmembrane phosphatase that may constitute an interesting target for small molecules or antibodies directed against its catalytic and extracellular domains, respectively.

Little is currently known about PPAPDC1B. Its identification as a potential therapeutic target in breast cancer highlights the need for further studies investigating its role in cancer, normal development, and tissue homeostasis. It will also be of great interest to assess its potential role in the various cancers harboring an amplification of the 8p11-12 region (43, 44).

Finally, there is growing interest in the potential of phosphatases as drug targets in various diseases, including diabetes, obesity, and cancer (45–47). In cancer, two recent reports have highlighted the role of the PTP1B phosphatase, encoded by a gene in the 20q13 region, which is also amplified in breast cancer, in ERBB2-induced mammary tumorigenesis (7, 8). This rising interest in this class of molecules should facilitate the development of specific drugs against PPAPDC1B.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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