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ORIGINAL ARTICLE

The branched-chain amino acid transaminase 1 sustains growth of antiestrogen-resistant and ERα-negative breast cancer

V Thewes[1], R Simon[2], M Hlevnjak[1], M Schlotter[1], P Schroeter[1], K Schmidt[3], Y Wu[1], T Anzeneder[4], W Wang[1], P Windisch[1], M Kirchgäßner[1], N Melling[2], N Kneisel[1], R Büttner[5], U Deuschle[6], HP Sinn[7], A Schneeweiss[8], S Heck[1], S Kaulfuss[9], H Hess-Stumpp[9], JG Okun[3], G Sauter[2], AE Lykkesfeldt[10], M Zapatka[1], B Radlwimmer[1], P Lichter[1] and M Tönjes[1]

Antiestrogen-resistant and triple-negative breast tumors pose a serious clinical challenge because of limited treatment options. We assessed global gene expression changes in antiestrogen-sensitive compared with antiestrogen-resistant (two tamoxifen resistant and two fulvestrant resistant) MCF-7 breast cancer cell lines. The *branched-chain amino acid transaminase 1* (*BCAT1*), which catalyzes the first step in the breakdown of branched-chain amino acids, was among the most upregulated transcripts in antiestrogen-resistant cells. Elevated *BCAT1* expression was confirmed in relapsed tamoxifen-resistant breast tumor specimens. High intratumoral BCAT1 levels were associated with a reduced relapse-free survival in adjuvant tamoxifen-treated patients and overall survival in unselected patients. On a tissue microarray ($n = 1421$), BCAT1 expression was detectable in 58% of unselected primary breast carcinomas and linked to a higher Ki-67 proliferation index, as well as histological grade. Interestingly, BCAT1 was predominantly expressed in estrogen receptor-α-negative/human epidermal growth factor receptor-2-positive (ERα-negative/HER-2-positive) and triple-negative breast cancers in independent patient cohorts. The inverse relationship between BCAT1 and ERα was corroborated in various breast cancer cell lines and pharmacological long-term depletion of ERα induced BCAT1 expression *in vitro*. Mechanistically, BCAT1 indirectly controlled expression of the cell cycle inhibitor $p27^{Kip1}$ thereby affecting pRB. Correspondingly, phenotypic analyses using a lentiviral-mediated BCAT1 short hairpin RNA knockdown revealed that BCAT1 sustains proliferation in addition to migration and invasion and that its overexpression enhanced the capacity of antiestrogen-sensitive cells to grow in the presence of antiestrogens. Importantly, silencing of BCAT1 in an orthotopic triple-negative xenograft model resulted in a massive reduction of tumor volume *in vivo*, supporting our findings that BCAT1 is necessary for the growth of hormone-independent breast tumors.

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INTRODUCTION

Breast cancer is the leading cause of cancer deaths in women in Western societies.[1] In the past years, transcriptional-profiling revealed that breast cancer comprises a heterogeneous group of tumors. Based on the differential expression of the ligand-activated transcription factors estrogen receptor-α (ERα) and progesterone receptor (PR), as well as the receptor tyrosine kinase human epidermal growth factor receptor-2 (HER-2), breast cancers are classified into distinct molecular subgroups, which are linked to distinct gene expression profiles, clinical outcomes and response to therapies.[2,3] These are luminal A/B (ERα and/or PR positive), HER-2-enriched and basal-like-breast carcinomas, which often present as triple-negative breast cancer (TNBC: ERα-negative, PR-negative, HER-2-negative).

ERα exerts its driving function as key transcriptional regulator of the luminal subtypes in about 70% of breast tumors. This dependency on estrogen signaling has been therapeutically exploited for many decades and the administration of endocrine therapies resulted in a 25–30% decrease in mortality.[4] The antiestrogen tamoxifen serves as a prototype of a selective estrogen receptor modulator that competitively blocks binding of estrogens. Moreover, it induces an ERα conformation that favors binding of corepressors instead of coactivators, thereby inhibiting the transcription of genes that promote breast cancer progression.[5]

The 'pure antiestrogen' fulvestrant (Fulv; ICI182,780) represents another effective therapy that results in destabilization and degradation of ERα (selective estrogen receptor downregulator), abrogating ERα-mediated target gene regulation completely.[6] Despite the significant improvements in patient survival because of endocrine agents,[4] a substantial number of patients (about 50%) with advanced disease is *de novo* resistant and one-third of initially responsive tamoxifen-treated patients relapse within 15 years.[4,7]

Moreover, ERα-negative/-HER-2-positive and TNBC represent a serious clinical problem because of limited molecular treatment options. Whereas patients with HER-2-positive tumors are

[1]Division of Molecular Genetics, German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany; [2]Institute of Pathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [3]Division of Inherited Metabolic Diseases, University Children's Hospital, Heidelberg, Germany; [4]PATH Foundation Biobank—Patients' Tumor Bank of Hope, Munich, Germany; [5]Institute of Pathology, University Hospital Cologne, Cologne, Germany; [6]Phenex Pharmaceuticals AG, Heidelberg, Germany; [7]Institute of Pathology, University of Heidelberg, Heidelberg, Germany; [8]Gynecologic Oncology, National Center for Tumor Diseases, University of Heidelberg, Heidelberg, Germany; [9]Bayer Pharma AG, Berlin, Germany and [10]Breast Cancer Group, Danish Cancer Society Research Center, Copenhagen, Denmark. Correspondence: Dr P Lichter, Division of Molecular Genetics, German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg 69120, Germany.
E-mail: peter.lichter@dkfz-heidelberg.de
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conventionally treated with chemotherapy in combination with the monoclonal antibody trastuzumab, for patients with TNBC cytotoxic chemotherapy is the only approved treatment option.[8] Both subtypes are often poorly differentiated and highly aggressive and consequently characterized by high mortality rates.[3,8,9] However, the underlying molecular features that govern these ERα-negative or endocrine-resistant breast tumors are poorly understood.

The antiestrogen-resistant phenotype can be modeled *in vitro* using ERα-positive breast cancer cells that have undergone a selection process because of long-term treatment with the respective antiestrogens. In order to identify molecular events that fuel endocrine resistance, we determined transcriptome-wide gene expression changes in antiestrogen-responsive and -resistant MCF-7 cells. Within the screening approach, the metabolic enzyme branched-chain amino acid transaminase 1 (BCAT1) appeared to be strongly expressed in resistant cells. BCAT1 has been recently identified by us as a crucial factor for aggressive IDH1-Wt glioma,[10] and has been further implicated in the progression of nasopharyngeal carcinomas.[11] However, its contribution to the pathogenesis of breast cancer has been so far largely unexplored.

This prompted us to perform a systematic analysis of BCAT1 in breast tumor specimens *in vitro* and in *vivo* to assess its pathobiological relevance in breast carcinomas. Altogether, our analyses point at a critical role of BCAT1 in the progression of antiestrogen-resistant and estrogen-independent breast carcinomas.

RESULTS

Transcriptional profiling of antiestrogen-resistant MCF-7 breast cancer cells uncovers upregulation of BCAT1 in resistant breast cancer cell lines and tumors

In order to identify gene expression differences and novel markers associated with antiestrogen resistance, we analyzed gene expression patterns of antiestrogen-resistant MCF-7 breast cancer cells using the Agilent (Santa Clara, CA, USA) 4x44k platform, which encompasses about 41 000 coding transcripts. The screening approach included one corresponding parental antiestrogen-sensitive (#Wt), two tamoxifen-resistant (#TamR1 and #TamR8), as well as two fulvestrant-resistant (#FulvR6 and #FulvR7) MCF-7 cell lines. These had undergone a selection process because of long-term treatment with the respective antiestrogens and were previously characterized in detail.[12,13] To exclude confounding effects of the culture conditions, expression analyses were performed of cells maintained in steroid-rich fetal calf serum (FCS) as well as in steroid-depleted charcoal stripped fetal calf serum (CCS) medium for the screening. In the latter condition, cells were further subjected to short-term treatment with 4-hydroxytamoxifen (4OHT) or fulvestrant.

Hierarchical clustering of the 100 most deregulated transcripts revealed separate branches for tamoxifen- and fulvestrant-resistant MCF-7 cells, suggesting resistance mechanisms specific for each treatment modality (Figure 1a). Irrespective of the culture and treatment conditions, *BCAT1* emerged as one of the most strongly upregulated transcripts in resistant cells in comparison with sensitive cells (Figure 1b, Supplementary Table S1).

Compared with #Wt cells, *BCAT1* was moderately upregulated in #TamR1 cells, and strongly in #TamR8, as well as in #FulvR6 and #FulvR7 cells (Figure 1c). These pronounced upregulations of BCAT1 were validated on mRNA level using quantitative real-time PCR (qRT–PCR; Supplementary Figure S1a) and also reflected on protein level (Figure 1d). Analysis of a published data set of an *in vitro* model of tamoxifen resistance (GSE26459),[14] confirmed our findings (Supplementary Figure S1b).

To further validate our findings in clinical specimens, we investigated BCAT1 expression in endocrine-resistant breast tumors. These tumors had undergone tamoxifen therapy and patients experienced a relapse during or after the course of treatment. In comparison with a treatment-naive primary breast carcinoma cohort (tumor set I), resistant carcinomas (tumor set II) possessed elevated *BCAT1* mRNA levels (Figure 1e). The respective protein product catalyzes the breakdown of branched-chain amino acids (Figure 1f).

BCAT1 association with clinicopathological parameters in primary breast cancer specimens

To analyze BCAT1 in all breast cancer subtypes — irrespective of treatment — we immunostained a tissue microarray (TMA) comprising 1421 evaluable primary breast tumors for BCAT1 (tumor set III, Table 1). When stratifying the BCAT1 immunohistochemistry (IHC) staining intensity into four categories (Figure 2a): 0 = negative (42.22%), 1 = weak (36.31%), 2 = moderate (15.55%) and 3 = strong (5.91%), BCAT1 is expressed in 57.8% of unselected primary breast carcinomas.

In order to determine whether BCAT1 is specifically enriched in cancerous tissue, we compared its expression in normal breast tissue specimens ($n = 37$) versus breast tumors of all histological and molecular subtypes on the same TMA. The analysis revealed considerably higher protein abundance in tumor tissue, indicative for a tumor-specific expression (Figure 2b).

Moreover, we observed an association between high BCAT1 expression and increased levels of the proliferation marker Ki-67, suggestive for a pro-proliferative role of BCAT1 in breast carcinomas (**$P < 0.001$; Figure 2c). This finding is in agreement with the worse histological differentiation (increased grade) of tumors with enhanced BCAT1 expression (**$P < 0.001$; Figure 2d). Therefore, BCAT1 expression is linked to aggressive disease features (see also Supplementary Table S2).

To assess whether BCAT1 protein expression has a prognostic impact on overall survival (OS), we stratified patients into high and low BCAT1 expression. We univariately observed a tendency for a diminished OS for patients with high BCAT1 protein-expressing tumors (Figure 2e, **$P = 0.0008$) with the restriction that the hazards were not proportional. In a multivariate Cox hazard analysis, including pT, pN, grade, age, ERα, HER-2, p53 and BCAT1, the underlying assumptions were fulfilled and we observed a significant independent influence of BCAT1 on OS (Table 2a; **$P = 0.0030$). In addition, we assessed the influence of BCAT1 on OS in different subtypes in the large TCGA patient cohort, which also uncovered a tendency for a shortened OS within ERα-positive and TNBC subtypes as well (Supplementary Figure S2a and Supplementary Table S3).

Furthermore, we conducted survival analyses in patients with a secured tamoxifen treatment history. For this purpose, we used a publicly available patient cohort ($n = 136$; GSE12093)[15] of primary breast tumors, which received adjuvant tamoxifen treatment (Figure 2f). Stratification of individuals into high and low *BCAT1* mRNA expression showed that patients with high *BCAT1* expression had a shortened relapse-free survival (*$P = 0.034$). Multivariate analyses confirmed that BCAT1 was independent of the available clinical variables T-stage and age (Table 2b; *$P = 0.0393$). Furthermore, we analyzed the impact of BCAT1 on relapse-free survival in unselected primary breast cancer patients in the TCGA cohort and again observed that high *BCAT1* mRNA levels associate with a shortened relapse-free survival (Supplementary Figure S2b and Supplementary Table S3).

Elevated expression of BCAT1 in ERα-negative primary breast tumors and cell lines

As ERα is a pivotal determinant in primary breast carcinomas, we set out to analyze the interplay between ERα and BCAT1 in breast

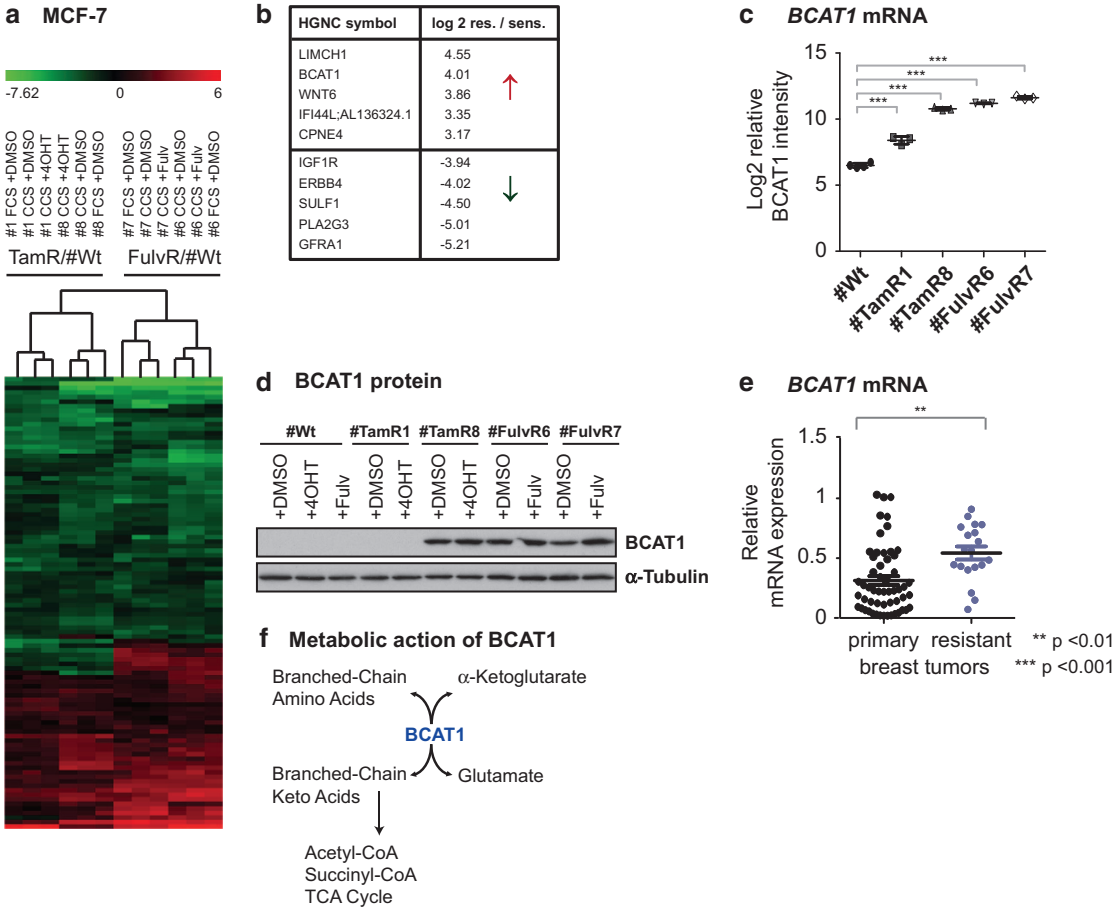


Figure 1. Gene expression profiling of parental and antiestrogen-resistant MCF-7 cell lines identifies pronounced BCAT1 upregulation. One antiestrogen-sensitive (#Wt), two tamoxifen-resistant (#TamR1/8) and two fulvestrant-resistant (#FulvR6/7) MCF-7 cells were cultured in FCS and CCS conditions and exposed to DMSO, 4OHT (1 μM) or Fulv (100 nM) for 1 h. (**a**) For hierarchical clustering, resistant cells were normalized to sensitive cells and 100 transcripts with the highest variance included. (**b**) Mean expression of the top five upregulated and downregulated transcripts in #TamR1/8 and #FulvR6/7 cells after normalization to #Wt cells. (**c**) *BCAT1* probe (A_24_P935986) intensity in individual cell lines. Replicates include the following conditions: FCS+DMSO, CCS+DMSO, CCS+4OHT or CCS+Fulv, respectively. (**d**) BCAT1 western blotting in MCF-7 sublines under the same conditions as for microarray analyses. (**e**) qRT–PCR measurement of *BCAT1* mRNA expression in primary untreated (tumor set I, $n = 55$) versus relapsed tamoxifen-treated (tumor set II, $n = 19$) breast carcinomas. *BCAT1* expression was normalized to *HPRT1, LMNB1* and *PGK1* housekeepers. (**f**) Schematic representation of the mechanistic action of BCAT1. BCAT1 catabolizes the reversible transamination of the branched-chain amino acids leucine, isoleucine and valine into the downstream branched-chain keto acids, which might serve as substrates to maintain homeostasis in the TCA cycle. This step is coupled to the concomitant conversion of α-ketoglutarate to the versatile bioenergetic substrate glutamate.[34] CCS, charcoal stripped fetal calf serum; FCS, fetal calf serum.

Table 1. Prevalence of BCAT1 and ERα in primary breast tumors (TMA, tumor set III)

TMA	*Evaluable*	*BCAT1-positive*	*%*	*BCAT1-negative*	*%*
Primary breast tumors	1421	821	57.78	600	42.22
ERα-positive[a]	1024	528	51.56	496	48.44
HER-2-positive	103	83	80.58	20	19.42
TNBC	194	147	75.77	47	24.23

Abbreviations: BCAT1, branched-chain amino acid transaminase 1; ERα, estrogen receptor-α; HER-2, human epidermal growth factor receptor 2; TMA, tissue microarray; TNBC, triple-negative breast cancer. [a]Breast tumors were separated into subtypes according to immunohistochemistry. One hundred samples lacked sufficient clinical annotations for the stratification into subtypes and were accordingly removed. Overall, 1096 tissues on the TMA were removed from the initial analysis due to insufficient staining quality, low tumor content or missing clinical variables.

tumors and cell culture models. For this purpose, we interrogated the BCAT1 expression in breast cancer subtypes on the TMA (Table 1). Overall, BCAT1 was detectable in 51.6% of ERα-positive and 75.4% of ERα-negative breast tumors. The classification into ERα-positive, HER-2-enriched (ERα-negative, PR-negative, HER-2-positive) as well as TNBCs (ERα-negative, PR-negative, HER-2-negative) confirmed elevated BCAT1 expression in both ERα-negative subtypes (***$P < 0.001$; Figure 3a).

Probing this question further, we explored *BCAT1* mRNA expression in breast cancer subtypes using publicly available

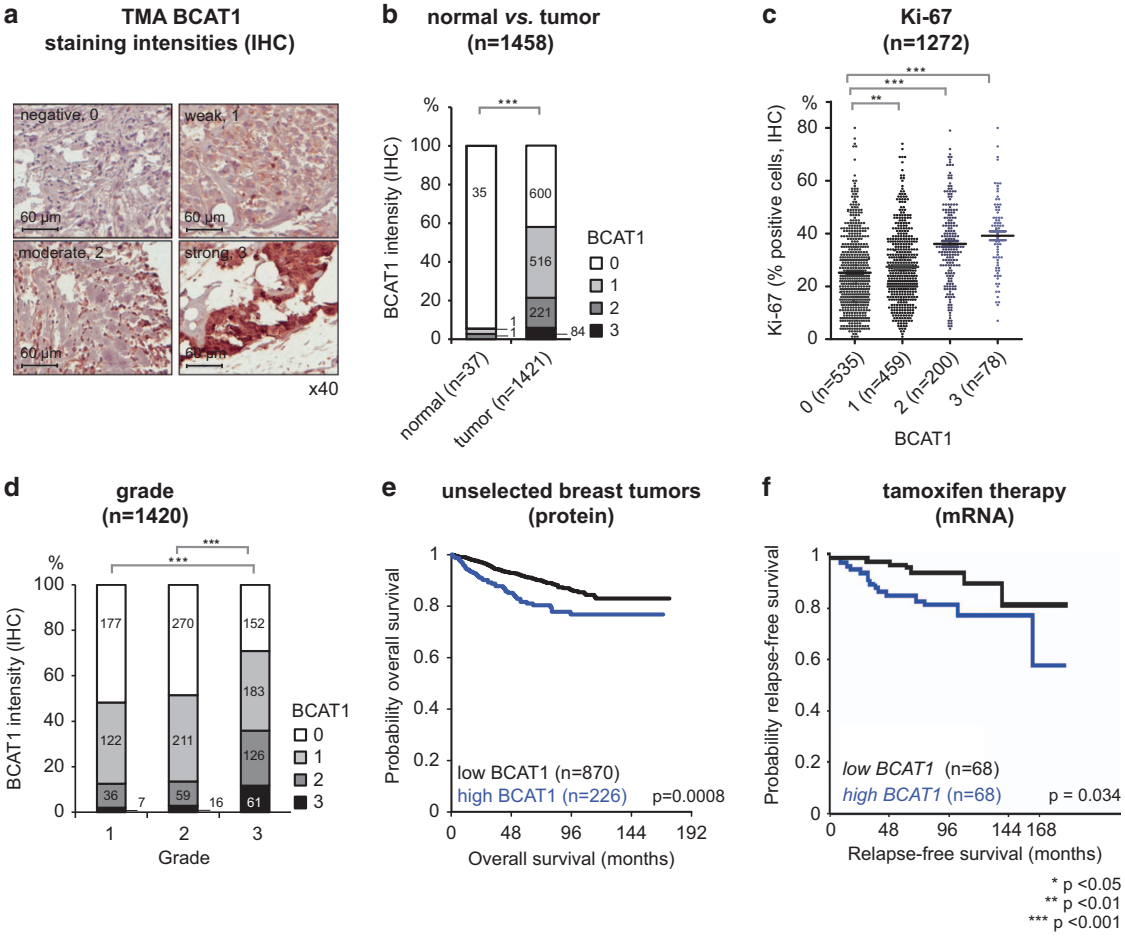


Figure 2. BCAT1 in clinical patient cohorts associates with aggressive disease parameters. (**a**) Representative IHC illustrations of BCAT1 scoring intensities on the TMA. Original magnification is × 40. (**b**) Bar chart of BCAT1 protein expression in normal breast tissue ($n = 37$) compared with breast tumor tissue ($n = 1421$) on the TMA. In normal breast spots, only stroma expression was included because of the lack of tumor tissue, whereas for tumors, the combination of tumor and stroma expression was scored. Percentages are shown on the *y* axis and absolute numbers within the bar diagram. (**c**, **d**) Association of BCAT1 protein expression on the TMA with the proliferation marker Ki-67 (**c**) and histological grade (**d**). Patient numbers do not always add up to the total sum of normal and tumor ($n = 37+1421$) because of missing clinical values. (**e**) Kaplan–Meier OS from TMA BCAT1 protein expression of unselected breast cancer patients ($n = 1096$). Tumors with staining intensity 0+1 were considered as low, whereas 2+3 was counted as high. (**f**) Relapse-free survival of *BCAT1* mRNA expression of individuals who received tamoxifen-monotherapy from a publicly available microarray data set ($n = 136$; GSE12093).[15] Patients were stratified according to median *BCAT1* mRNA expression.

data[16] and observed increased BCAT1 expression in HER-2-positive and TNBC tumors compared with ERα-positive tumors (***$P < 0.001$; Figure 3b). Furthermore, we retrieved the relative *BCAT1* mRNA expression from an open-source microarray data set of primary breast tumors ($n = 123$; GSE5460)[17] and detected a higher *BCAT1* mRNA expression in ERα-negative tumors compared with ERα-positive ones (***$P < 0.001$; Figure 3c). Western blotting in a small cohort of primary breast tumors again verified an enrichment of BCAT1 expression in ERα-negative tumors (tumor set IV; Figure 3d).

As ERα classically functions as a ligand-activated transcription factor, we determined the possibility of BCAT1 being a direct target gene of ERα. However, short-term (1 or 24 h) induction of ERα with an agonist (estradiol) or antagonist (4OHT) in MCF-7 cells did not result in marked changes in BCAT1 expression (Figure 1d and Supplementary Figure S2c), arguing against a direct regulation by ERα. This finding is supported by the notion that ERα binding has not been reported in the *BCAT1* regulatory regions in the ENCODE database.

Nevertheless, BCAT1 expression could be induced in antiestrogen-responsive MCF-7 #Wt cells after long-term depletion of ERα with the selective estrogen receptor downregulator fulvestrant (Figure 3e). Effects of treatment on ERα expression were visible after 6 days, but high levels of BCAT1 did not appear before 35 days of drug administration. From these long-term treated cells, we further generated eight single-cell-derived MCF-7 cell lines, which were continuously exposed to fulvestrant for several months. The majority also expressed high levels of BCAT1 (Supplementary Figure S3a). In order to complement the pharmacological approach by RNA interference data, we analyzed a publicly available data set generated upon stable silencing of ERα in MCF-7 cells (GSE27473)[18]. Consistent with our data, the knockdown of ERα over longer period-of-time resulted in significantly elevated *BCAT1* mRNA expression (Supplementary Figure S3b), confirming the inverse nature of BCAT1 and ERα.

For further analyses, we elucidated the BCAT1 expression pattern in a panel of common breast cancer cell lines (Figure 3f). Importantly, BCAT1 was not expressed in MCF-10A cells that serve as model for non-transformed, non-malignant normal breast epithelial cells, confirming the tumor-specific expression seen in patient specimens (Figure 2b). In line with the tumor findings, ERα-positive MCF-7 and T47D cells did not show strong BCAT1 expression, whereas ERα-negative JIMT, HDQ-P1 and MDA-MB231

cells displayed high BCAT1 protein abundance. BT474 and SK-BR3 cells that express high HER-2 levels were deficient in BCAT1.

In addition, we analyzed antiestrogen-sensitive versus -resistant MCF-7 cells (Figure 3g) and again observed an inverse relationship between ERα and BCAT1. One tamoxifen-resistant cell line (#TamR8) constituted an exception because it co-expresses BCAT1 and ERα. However, we have observed that classical ERα target gene regulation is not functional in this cell line as assessed by the surrogate target gene *PgR* (Supplementary Figure S4a and Lykkesfeldt *et al*.[19,20]).

BCAT1 expression can be also regulated by DNA methylation of its promoter and three different *BCAT1* transcript isoforms (v1, v4 and v6) are expressed in glioma.[10] We quantified these isoforms using qRT–PCR and identified *BCAT1* isoform v1 (ENST00000261192) as the most prevalent (Supplementary Figures S4b–d).

Correspondingly, we explored the DNA methylation patterns of the respective promoter region (−990 bp to +612 bp) in breast cancer cell lines with known BCAT1 expression (see Figures 3f–g) using MassARRAY. Overall, hypomethylation appeared to be linked with BCAT1 expression in ERα-negative cells, whereas we observed hypermethylation in BCAT1-negative ERα-positive cell lines (Figure 3h). Surprisingly, BCAT1 expression in the antiestrogen-sensitive and -resistant MCF-7 cells did not correspond with the methylation status, indicating that mechanisms other than epigenetic regulation govern BCAT1 expression in these cells.

To obtain an insight into the underlying metabolic processes that are controlled by BCAT1, we suppressed BCAT1 by lentiviral-induced silencing in an antiestrogen-resistant cell line (#TamR8) and triple-negative (MDA-MB231) cells and analyzed the effects on the intracellular amino acid composition by tandem mass spectrometry (Supplementary Figures S5a and b). The respective metabolic profiles after BCAT1 silencing show increased levels of alanine, glutamate and proline, which are markers for a potential mitochondrial dysfunction.[21]

To further analyze potential downstream effectors of BCAT1, we analyzed gene expression profiling data, which pointed at the cell cycle inhibitor $p27^{Kip1}$ as being indirectly regulated by BCAT1 (independent manuscript in preparation). Consequently, we conducted a lentiviral-mediated BCAT1 knockdown in antiestrogen-resistant (#TamR8, #FulvR6) and MDA-MB231 cells that show high BCAT1 expression. Loss of BCAT1 induced upregulation of $p27^{Kip1}$ (Figure 3i). Moreover, we observed a corresponding downregulation of phosphorylated retinoblastoma protein (pRB Ser807/811) in MDA-MB231 cells (Supplementary Figure S5c) indicating that BCAT1 controls passage through the cell cycle in breast cancer cells, which is consistent with the clinical data that point at a pro-proliferative function of BCAT1 (Figure 2c).

BCAT1 sustains proliferation, migration and invasion *in vitro*

Correspondingly, we analyzed the phenotypic effects of modulation of BCAT1 expression in breast cancer cell lines. Therefore, we quantified the fraction of cells in S-phase after BCAT1 knockdown with two independent short hairpin RNAs (shRNAs; shBCAT1#1 and shBCAT1#2) compared with a non-target control (shNTC) in antiestrogen-responsive and antiestrogen-resistant MCF-7 sublines. Effective silencing of BCAT1 was confirmed by western blot analysis (Figure 4a) and resulted in a significant decrease in proliferation in all cell lines as monitored by 'Click-iT-Edu' assays (Figure 4b). Surprisingly, antiestrogen-sensitive #Wt cells were affected to the same extent as resistant cells from loss of BCAT1. However, we observed that residual amounts of BCAT1 were also present in sensitive #Wt cells after longer exposure of western blots with a high sensitivity substrate and that BCAT1 silencing also resulted in upregulation of $p27^{Kip1}$ (Supplementary Figures S6a and b). This finding indicates that also sensitive cells are dependent on BCAT1, but that resistant cells have an increased requirement for the metabolic products of BCAT1. Moreover, the knockdown efficiency in resistant cells with high BCAT1 expression was not that strong as in sensitive cells, which might further explain the observed phenotype.

Table 2. Multivariate analyses (MVA): impact of co-variables on patient outcome in primary breast tumors

	Coefficient	*Confidence interval (95%)*	*Hazard ratio*	P-*value*
(A) Protein (TMA)				
Age	−0.0016	0.0084	0.9984	0.8465
pT-stage	0.306	0.105	1.3580	0.0036
pN-stage	1.3835	0.1711	3.9888	< 0.0001
Grade	0.4694	0.1664	1.5990	0.0048
ERα	−0.4803	0.2507	0.6186	0.0553
p53	0.0974	0.2325	1.1023	0.6752
HER-2	0.1465	0.2626	1.1578	0.5770
BCAT1	0.6894	0.232	1.9925	0.0030
(B) mRNA BCAT1[a]				
pT-stage	−0.0745	0.3975	0.9282	0.8513
Age	−0.0015	0.0268	0.9985	0.9545
BCAT1	1.0224	0.4960	2.7798	0.0393

Abbreviations: BCAT1, branched-chain amino acid transaminase 1; HER-2, human epidermal growth factor receptor 2; MVA, multivariate analyses; TMA, tissue microarray. [a]*BCAT1* mRNA expression values were retrieved from public data of primary breast tumors treated adjuvant with tamoxifen (GSE12093). Only limited clinical values for MVA were available.

To clarify whether BCAT1 indeed sustains growth under antiestrogen treatment, we stably overexpressed BCAT1 (pVLX-BCAT1) or the control vector (pVLX-empty) in antiestrogen-sensitive MCF-7 #Wt cells and treated them over 6 weeks (d42) with 4OHT (Figure 4c). Remarkably, whereas the amount of BCAT1 overexpression constructs was similar between DMSO and 4OHT-treated cells at earlier time points (d6), as well as in DMSO-treated cells at d42, 4OHT-treated cells showed increased BCAT1 expression, suggestive for a selection toward BCAT1-expressing cells under 4OHT treatment. In concordance with these findings, the proliferation rate between BCAT1-overexpressing and control cells was similar at d6. However, after 42 days of treatment, BCAT1-overexpressing cells (compared with empty vector control cells) gained an increased capacity to grow in the presence of 4OHT, which underlines the contribution of BCAT1 to the acquisition of resistance.

As an additional *in vitro* model for triple-negative breast tumors, we employed MDA-MB231 cells. Again, silencing of BCAT1 with both shRNAs (Figure 4d) significantly slowed-down proliferation (Figure 4e).

As MCF-7 cells display a poor migratory and invasive capacity *in vitro*,[22,23] we analyzed these phenotypes in MDA-MB231 cells (Figure 4e). The lentiviral BCAT1 knockdown strongly abrogated migration in Boyden chambers (middle) as well as invasion through matrigel (right).

Suppression of BCAT1 inhibits the growth of orthotopic human tumor cell xenografts *in vivo*

Finally, to confirm the significance of our findings *in vivo*, we engineered MDA-MB231 cells to express a doxycycline-inducible *BCAT1* shRNA (shBCAT1#1) or the corresponding control vector (shNTC). These stable cell lines were injected orthotopically into the mammary fat-pads of female nude Balb-c mice. Tumor-bearing mice were killed 28 days after transplantation (11 animals per group). At this endpoint, tumors were collected and sections hematoxylin and eosin-stained to ascertain the tumor content (Figure 4f). Further, we verified the maintenance of a robust BCAT1

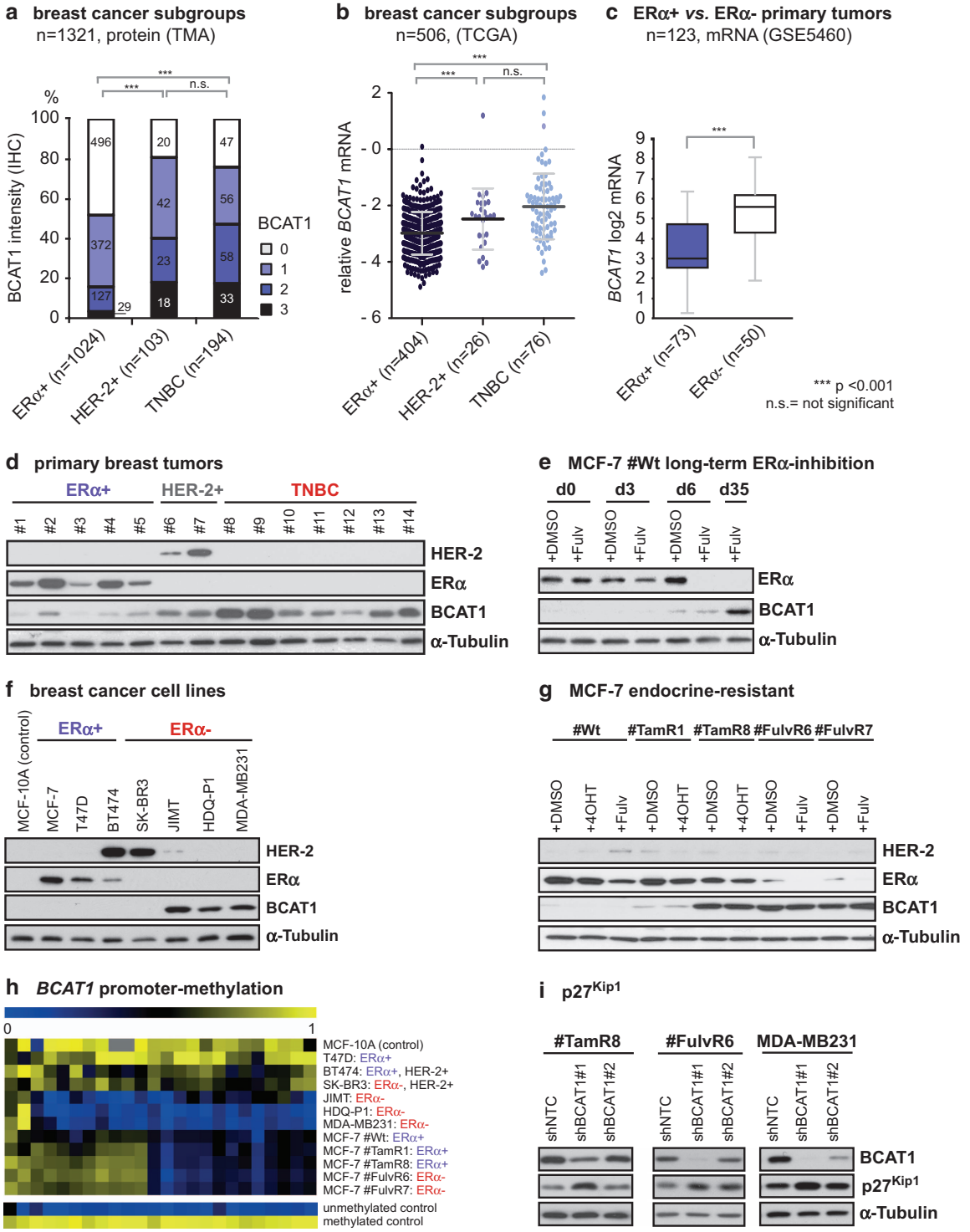
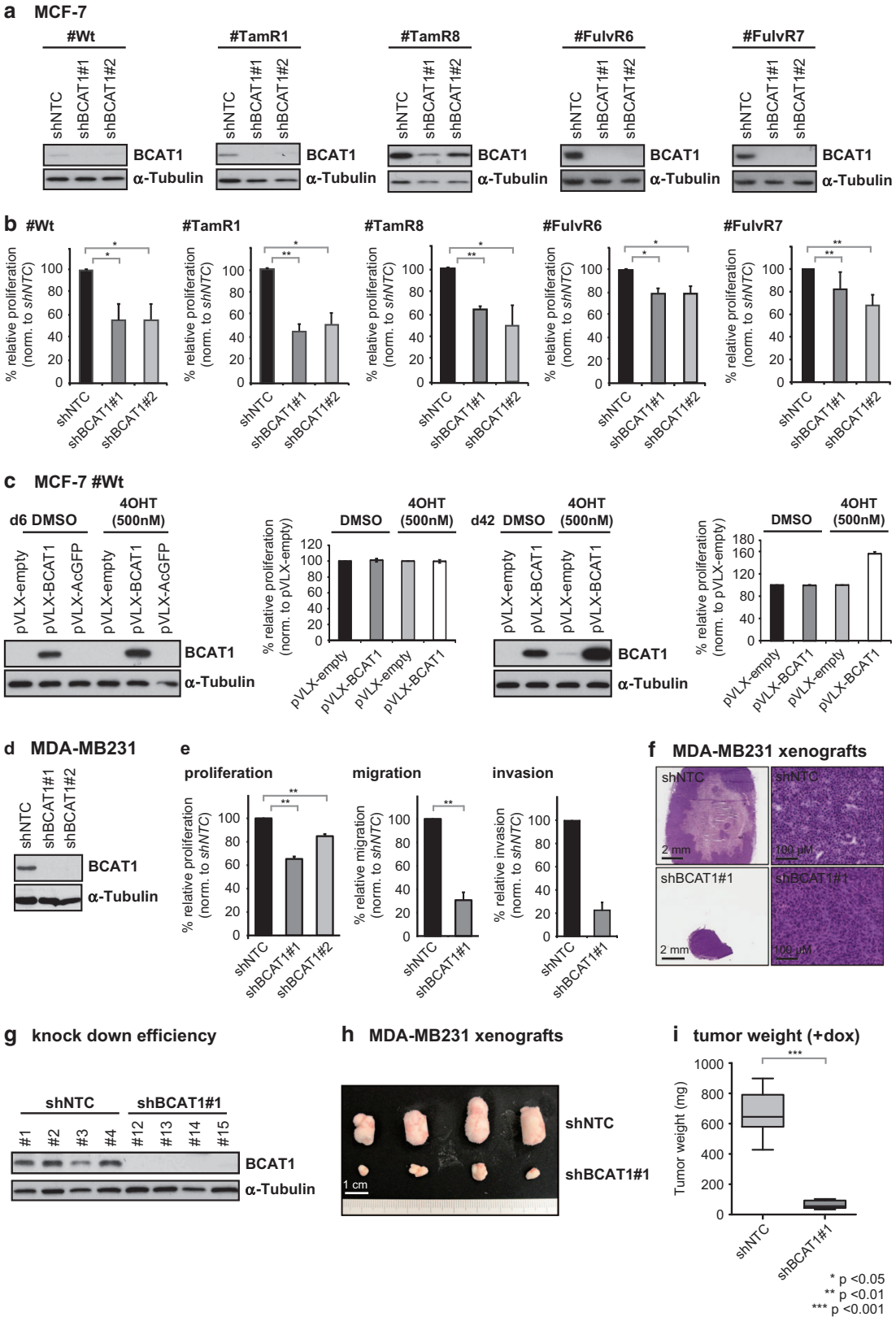


Figure 3. BCAT1 in IHC-defined breast cancer subtypes, impact of ERα modulation on BCAT1 expression and regulation of $p27^{Kip1}$ by BCAT1. (**a**) Bar chart showing BCAT1 protein expression on the TMA. (**b**) Relative *BCAT1* mRNA expression in the TCGA data set. (**c**) *BCAT1* mRNA expression in public primary untreated breast tumors ($n = 123$; GSE5460). Raw data were retrieved from the R2-microarray analysis and visualization platform. Breast cancer subgroups in (**a**–**c**) were classified according to IHC. (**d**) Western blotting to detect BCAT1, ERα and HER-2 expression in primary breast tumors. (**e**) Western blot analyses of ERα and BCAT1 in MCF-7 #Wt after treatment with DMSO and Fulv (100 nM) for 0, 3, 6 and 35 days. Protein lysates from d0, 3 and 6 have been also used for the Western blot in Supplementary Figure S3a. (**f**, **g**) Immunoblotting in a panel of (**f**) breast cancer cell lines and (**g**) sensitive and resistant MCF-7 cells. Blue marks ERα-positive, red marks ERα-negative cells. (**h**) MassARRAY to determine methylation status of the *BCAT1* promoter in breast cancer cell lines. Blue indicates hypomethylation, yellow indicates hypermethylation. (**i**) Western blotting upon lentiviral BCAT1 knockdown in MCF-7 #TamR8, #FulvR6 and MDA-MB231 cells to detect BCAT1 and $p27^{Kip1}$ protein expression.

protein knockdown in mouse tumor homogenates harboring the shBCAT1 construct after the indicated time frame (Figure 4g). Expression of the shNTC control resulted in the emergence of large tumors (mean weight: 658 mg), compared with a substantial reduction in the mean tumor size (mean weight: 64 mg) upon BCAT1 knockdown (Figures 4h and i). This 10-fold reduction in tumor volume is highly indicative that BCAT1 also promotes tumor progression *in vivo*.



Overall, our findings underline the hypothesis that BCAT1 supports the growth of highly aggressive breast cancer cells such as antiestrogen-resistant and hormone-independent breast tumors.

DISCUSSION

ERα-positive breast cancer that escaped hormone dependency, as well as ERα-negative tumors such as the HER-2-positive and TNBC subtypes represent a major clinical burden.[7,8,24] In this study, we broadly surveyed BCAT1 in breast carcinomas. Although anti-estrogen resistance is presumably driven by multiple factors, our findings argue for a necessary role of BCAT1 in these disease entities. To fuel cell proliferation and survival, cancer cells frequently adapt their metabolic demands and recent studies highlighted the importance of alterations in amino acid metabolism in breast cancer.[25,26]

We generated global gene expression profiles in different tamoxifen- and fulvestrant-resistant MCF-7 sublines to account for the diversity of resistant cell populations. In concordance with the situation in recurrent tamoxifen-resistant breast tumors, most resistant breast cancer cells are characterized by elevated BCAT1 expression, underlining the branched evolution of resistance.

In accordance with an earlier report in rodents,[27] BCAT1 was hardly expressed in normal mammary tissue, confirming the cancer-specific expression.

We observed predominant BCAT1 expression in hormone-independent ERα-negative breast carcinomas and tumor cell lines. Despite the opposite pattern of BCAT1 and ERα expression, our findings argue against a direct transcriptional repression of BCAT1 by ERα since short-term modulation of ERα activity did not induce notable changes in BCAT1 expression.

Nevertheless, pharmacological long-term ERα depletion by fulvestrant resulted in a marked increase in BCAT1 in conjunction with ERα-loss. Two scenarios can be envisaged that could explain this phenotype: first, ERα might indirectly act on BCAT1 expression. Second, ERα-negative, BCAT1-positive subclones might emerge as an outgrowth of a pre-existing inhomogeneous cell population under the selection of antiestrogen treatment in line with the widely documented polyclonal heterogeneity in breast tumor specimens and MCF-7 cells.[28,29] Hence, future investigations will have to delineate whether the resistant phenotype is a primary *de novo* or rather a newly acquired feature.

Furthermore, amplifications of the genomic region harboring *BCAT1* (12p12.1) have been correlated with BCAT1 expression in a variety of cancers such as testicular germ-cell and nasopharyngeal tumors.[11,30] Interestingly, a recent integrative genomic analysis identified copy number gains in 12p in TNBC,[16] which might also affect BCAT1.

Our functional data together with the Ki-67 status observed in a large patient cohort demonstrate that BCAT1 is necessary to fuel ERα-independent cell proliferation. The pro-proliferative function of BCAT1 presumably results from alterations in the amino acid metabolism. Endocrine-resistant and TNBC tumors have a higher dependency on the glutamine/glutamate-axis,[31,32] which serves as a building block for the synthesis of other amino acids and for replenishing anaplerosis in the TCA cycle.[26] BCAT1 functions as a major nitrogen donor for glutamate synthesis,[33] which is concomitantly produced by BCAT1 through the amino group transfer from valine, leucine or isoleucine to α-ketoglutarate.[34] Hence, BCAT1 upregulation might compensate the increased demand for the versatile metabolic substrate glutamate.

Moreover, the metabolic data indicate that BCAT1 sustains mitochondrial homeostasis. The importance of numerous mitochondrial functions such as regulation of energy production and biosynthesis of building blocks for tumor growth is well established.[35] Hence, a potential mitochondrial dysfunction, induced by BCAT1 silencing, might result in the disruption of vital parameters and proliferation. In accordance with this data, we provide a link that BCAT1 indirectly suppresses $p27^{Kip1}$ and maintains RB phosphorylation, thereby enabling S-phase transition.[36]

The $p27^{Kip1}$ induction upon interference with BCAT1 might also arise from the perturbations in mitochondrial homeostasis, since the latter was shown to induce the AMP-activated protein kinase, which in turn can upregulate $p27^{Kip1}$ expression and thereby promote cell cycle arrest.[37,38]

Apart from proliferation, we observed that BCAT1 promotes the migratory and invasive capacity in MDA-MB231 cells, indicating that BCAT1 might fuel metastasis in breast cancer as well, which is in good agreement with published data that correlates BCAT1 with metastasis in colon cancer.[39] Importantly, BCAT1 was indispensable for the growth of aggressive triple-negative MDA-MB231 breast cancer cells in mouse xenografts underlining its biological relevance *in vivo*.

Recently, our laboratory identified a central contribution of BCAT1 to the growth of glioblastoma, which is characterized by a very poor prognosis.[10] Moreover, increased BCAT1 expression emerges an indicator for an aggressive disease course in bladder and ovarian cancer.[40,41] The latter is known to share molecular and clinicopathological features with basal-like breast cancers,[16] suggesting that common driver events such as BCAT1 might underlie their pathogenesis. In accordance with our finding that BCAT1 affects response to antiestrogens, elevated BCAT1 expression has been listed together with other genes in endocrine neoadjuvant-treated patients that did not respond to therapy.[42] Similarly, BCAT1 has been implicated in chemoresistant ovarian carcinomas.[41]

Figure 4. Phenotypic analyses upon BCAT1 silencing in breast cancer cells *in vitro* and *in vivo*. (**a**, **b**) Lentiviral-induced BCAT1 knockdown in MCF-7. (**a**) Western blotting to ascertain protein knockdown. (**b**) Cell proliferation was monitored 6 days upon transduction in MCF-7 sublines by 'Click-iT-Edu' cell proliferation assays. Data were normalized to shNTC. (**c**) Lentiviral overexpression of BCAT1 (pVLX-BCAT1) and the respective control vector (pVLX-empty) in MCF-7 #Wt. Protein lysates were harvested and the proliferation rate measured using 'Click-iT-Edu' at d6 (left) and d42 (right) after beginning of treatment. Error bars indicate technical replicates of representative experiments. pVLX-AcGFP served as reporter to monitor transduction efficiency at the beginning of the experiment. Proliferation assays were normalized to the respective pVLX-empty vector controls. Raw values, which also show the effect of 4OHT are shown in Supplementary Figure S6c. (**d**) Effect of lentiviral-induced BCAT1 knockdown in MDA-MB231 cells on (**e**) cell proliferation measured by 'Click-iT-Edu', migration and invasion in transwell chambers. Invasion experiments were conducted twice with multiple replicates. (**f**–**i**) Dox-inducible BCAT1 knockdown in human MDA-MB231 xenograft mice. (**f**) Representative hematoxylin and eosin (HE)-stained cryosections of xenograft tumors without magnification in the original image (left) and with ×20.8 magnification (right). Mice were killed 28 days after transplantation and simultaneous doxycycline administration (2 mg/ml). (**g**) Confirmation of BCAT1 knockdown efficiency in representative tumor homogenates at the endpoint using western blotting. (**h**) Representative pictures of whole shNTC (upper panel) and shBCAT1#1 (lower panel) tumors. Scale represents 1 cm. (**i**) Box-plot showing the mean tumor weight (mg) in shNTC and shBCAT1#1 MDA-MB231 xenografts at d28. shNTC- and shBCAT1#1-bearing mice included 11 animals for each group.

To date, drugs suited to target BCAT1 efficiently are not available. However, the BCAT1 knockout mouse is viable and shows a tolerable phenotype,[43] suggesting that future efforts should be devoted to exploit a potential therapeutic-window for targeting of BCAT1.

In addition, monitoring of hyperpolarized BCAT1 substrates (for example, [1-^{13}C] α-KG) in glioma, which are converted into hyperpolarized [1-^{13}C] glutamate improved detection of residual tumors *in vivo*.[44] Accordingly, BCAT1 activity might also serve as a surrogate metabolic imaging biomarker in breast cancer.

In conclusion, this study provides evidence that BCAT1 is inversely expressed to ERα and that it supports proliferation in tumors that escaped antiestrogen therapy, as well as ERα-negative breast cancer. The data further pave the way to investigate the biological function of BCAT1 and possible paths for therapeutic interference.

MATERIALS AND METHODS

Cell lines

Antiestrogen-responsive MCF-7/S0.5 (#Wt), tamoxifen-resistant MCF-7/TAM^R-1 and MCF-7/TAM^R-8 (#TamR1 and #TamR8), and fulvestrant-resistant MCF-7/182^R-6 and MCF-7/182^R-7 (#FulvR6 and #FulvR7) cells were generated in the laboratory of Anne Lykkesfeldt.[19,20,45] Routine culture conditions were: Dulbecco's modified Eagle's medium/F12 (phenolred-free) with 1% FCS, glutamine (2 mM), penicillin/streptomycin (1%) and insulin (6ng/ml), supplemented with 1 μM tamoxifen or 100 nM fulvestrant for tamoxifen- and fulvestrant-resistant cell lines, respectively.

For microarray experiments, cells were cultured in parallel under steroid-depleted conditions: Dulbecco's modified Eagle's medium/F12 (1% charcoal-stripped, steroid-depleted FCS (CCS) with glutamine (2 mM), penicillin/streptomycin (1%) and insulin (6 ng/ml)) for 3 days and tamoxifen was replaced by 4OHT (1 μM). Authentication of cell lines was done using short-tandem-repeat or MCA profiling[46] within 1 year of usage of the respective strains (last time 2016). Cell lines were obtained between 2004 and 2013 either from American Type Culture Collection (ATCC, Manassas, VA, USA), German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK).

Purity of cell lines was assessed using the multiplex cell contamination test by Multiplexion (Heidelberg, Germany). No mycoplasma, squirrel monkey retrovirus or interspecies contamination was detected. Further details are given in the Supplementary Materials and Methods.

Microarray analyses and qRT–PCR

RNA isolation, microarray (Agilent 4x44k) and qRT–PCR analyses were conducted as previously described[47,48] and outlined in the Supplementary Information with primers given in Supplementary Table S4. Variance stabilizing normalization was conducted on microarray data and sensitive cells were normalized to resistant cells under the respective treatment conditions. Hierarchical clustering was performed on the basis of the 100 transcripts with the highest variability within the data set. Detailed information on bioinformatic processing is described in the supplements. Microarray data are deposited under Array-Express accession number E-MTAB-4426.

Western blotting

Immunoblotting was performed as described before[10] with antibodies specified in Supplementary Table S5.

Patient characteristics

Cryopreserved primary untreated ($n = 55$: tumor set I; $n = 14$: tumor set IV) and relapsed locoregional tamoxifen-resistant ($n = 19$: tumor set II) breast carcinomas used for qRT–PCR or western blotting were retrospectively gathered at the University Women's Hospital Heidelberg (Heidelberg, Germany) between 2001 and 2012 or obtained from the PATH Biobank.[49] Tumor set I+II have been described previously.[48] Collection of samples was approved by the local ethics committees in Heidelberg (S-541/2011) and Bonn (255/06) and all patients provided written informed consent. Primary breast tumors incorporated in the TMA ($n = 2197$, tumor set III) are detailed below.[50] Clinical annotations are provided in Supplementary Table S6.

IHC on a TMA

BCAT1 IHC was conducted on a TMA (0.6 mm cores), encompassing >2000 primary untreated breast tumors (tumor set III)[50] as outlined in the supplements at a 1:3000 antibody dilution (BCAT1 #51; BD Biosciences, San Jose, CA, USA). On the TMA, 1421 tumors and 37 normal breast tissues were evaluable. Accordingly, if patient numbers do not add up to the total number, respective clinical variables were missing or the staining or tumor content of the spots was insufficient. Molecular subgroups were defined according to immunohistochemical surrogate markers.[51]

Survival analyses

Kaplan–Meier survival plots and long-rank *P*-values were generated using R (Vienna, Austria) with rms and survival packages. Impact of covariates in multivariate analyses was computed based on Cox proportional hazards. *BCAT1* mRNA expression (probe: 214390) was retrieved from public microarray data (GSE12093)[15] of lymphnode-negative patients who received adjuvant tamoxifen monotherapy.

DNA methylation analyses

Genomic DNA was isolated using the 'AllPrep-DNA/RNA/Protein-Mini-Kit' (Qiagen, Hilden, Germany) according to the guidelines of the manufacturer. Methylation analyses on the MassARRAY-platform (EpiTYPER-Sequenom, San Diego, CA, USA) was performed as described before[10] and in the supplements.

Lentiviral shRNA knockdown and 'Click-iT-Edu' cell proliferation assays

Lentiviral particles containing shRNAs directed against *BCAT1* (TRCN0000005907 and TRCN0000005909, Sigma-Aldrich, St Louis, MO, USA) and a control non-target shRNA (SHC002) were produced as described previously[10,48] and in the supplements. S-phase index was determined with the 'Click-iT-EdU' cell proliferation kit (Life Technologies, Carlsbad, CA, USA) in a Canto II flow cytometer (BD Biosciences).

Lentiviral BCAT1 overexpression

Cells were transduced with plasmids either containing the empty vector (pVLX-empty), *BCAT1* isoform v1 (pVLX-BCAT1) or GFP (pVLX-AcGFP) to monitor transduction efficiency. After puromycin selection, cells were treated from d10 onward after initial transduction with 4OHT (500 nM) or DMSO every 2–3 days.

Migration and invasion assays

Boyden chamber migration assays were performed using transwells with 8 μm polycarbonate-membrane inserts (Corning, New York, NY, USA). Invasion was determined using BD BioCoat-Matrigel invasion-chambers (BD Biosciences). Cells were serum-starved (12 h), harvested and resuspended in serum-free medium. In all, 1×10^5 cells were seeded to the upper chambers and FCS medium was added to the lower reservoir. After 40 h, migrated cells were fixed with methanol, stained with haematoxylin and mounted on glass slides for cell counting.

MDA-MB231 tumor xenografts

In all, 5×10^6 MDA-MB231 cells, stably transduced with dox-inducible pLKO.1-Tet-On-shNTC or -shBCAT1#1 constructs (detailed in Tonjes *et al.*[10]), were diluted in matrigel and orthotopically injected into the mammary fat pads of female nude BALB-c mice (Charles-River, Sulzfeld, Germany; 11 animals per group) at 8 weeks of age. Doxycycline (2 mg/ml) was administered in the drinking water from the time-point of tumor cell injection. After tumors developed, caliper measurement of tumors was conducted twice a week. Mice were either killed at day 28 after transplantation or if tumors reached 200 mm^2. Animal experiments were carried out under the approval of the 'State-Department of Health and Social-Welfare' (Berlin; A 0293/12).

Statistical analyses

T-test, Mann–Whitney or Wilcoxon Rank Sum tests were used to determine significance ($P < 0.05$). Error bars indicate s.d. If not otherwise specified, three independent experiments were conducted encompassing at least two technical replicates each.

CONFLICT OF INTEREST

Stefan Kaulfuss and Holger Hess-Stumpp are employees of Bayer Pharma AG. However, no respective chemical compounds were used in the manuscript and the collaboration was for scientific purposes. The remaining authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

VT, MH, UD, AS, SH, AEL, MZ, BR, PL and MT conceived the project and designed the experiments. VT, MS, PS and MT performed *in vitro* experiments. YW and WW conducted MassARRAY analyses. KS and JGO conducted mass spectrometry analyses. TA, NM, RB, HPS, AS and GS provided and evaluated tumor specimens and clinical data. VT, RS, NM, HPS, AS, RB and MZ generated and analyzed clinical data. MH, MZ and RS performed computational analyses. MS prepared lentiviral particles with help of PW, SK and HH-S conducted animal experiments. PS and MK prepared xenograft sections with the help of NK. VT, UD, SH, MZ, BR, PL and MT analyzed data and wrote the manuscript with the help of other authors.

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