

ORIGINAL ARTICLE

Targeting of the adaptor protein Tab2 as a novel approach to revert tamoxifen resistance in breast cancer cells

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Pharmacological resistance is a serious threat to the clinical success of hormone therapy for breast cancer. The antiproliferative response to antagonistic drugs such as tamoxifen (Tam) critically depends on the recruitment of NCoR/SMRT corepressors to estrogen receptor alpha (ER α) bound to estrogen target genes. Under certain circumstances, as demonstrated in the case of interleukin-1 β (IL-1 β) treatment, the protein Tab2 interacts with ER α /NCoR and causes dismissal of NCoR from these genes, leading to loss of the antiproliferative response. In Tam-resistant (TamR) ER-positive breast cancer cells, we observed that Tab2 presents a shift in mobility on sodium dodecyl sulfate–PAGE (SDS-PAGE) similar to that seen in MCF7 wt upon stimulation with IL-1 β , suggesting constitutive activation. Accordingly, TamR treatment with Tab2-specific short interfering RNA, restored the antiproliferative response to Tam in these cells. As Tab2 is known to directly interact with the N-terminal domain of ER α , we synthesized a peptide composed of a 14-aa motif of this domain, which effectively competes with ER α /Tab2 interaction in pull-down and co-immunoprecipitation experiments, fused to the carrier TAT peptide to allow internalization. Treatment of TamR cells with this peptide resulted in partial recovery of the antiproliferative response to Tam, suggesting a strategy to revert pharmacological resistance in breast cancer. Silencing of Tab2 in TamR cells by siRNA caused modulation of a gene set related to the control of cell cycle and extensively connected to BRCA1 in a functional network. These genes were able to discern two groups of patients, from a published data set of Tam-treated breast cancer profiles, with significantly different disease-free survival. Altogether, our data implicate Tab2 as a mediator of resistance to endocrine therapy and as a potential new target to reverse pharmacological resistance and potentiate antiestrogen action.

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Introduction

Tamoxifen (Tam) is by far the most widely used drug for hormone-dependent breast cancer and the use of this or similar drugs since 1980 has led to reduction in the annual breast cancer death rate by nearly a third (ECBCT, 2005) and contributed significantly to improvement in long-term survival. However, primary or acquired resistance to Tam severely limits its clinical effectiveness (Hughes-Davies *et al.*, 2009). Recently, another class of antiestrogenic drugs was introduced, namely the so-called third-generation aromatase inhibitors, showing very good efficacy (ECBCT, 2005; Dowsett *et al.*, 2010). Significant advantage is obtained by switching from initial Tam to AI, demonstrating that late failure of Tam could be attributed, at least in part, to acquired pharmacological resistance.

When estrogen receptor alpha (ER α) binds to Tam or other selective ER modulators it undergoes activation, dimerization and binding to cognate elements in DNA. Tam-bound ER α displays increased affinity for corepressor proteins and decreased affinity for coactivators (Shiau *et al.*, 1998; Perissi and Rosenfeld, 2005), as opposed to estrogen-bound ER α . As a result, estrogen-dependent genes are actively repressed and cell growth stops. In the breast, Tam-repressive effect is due, in part, to interaction with NCoR and SMRT corepressors, possibly showing a remarkable gene specificity (Keeton and Brown, 2005). NCoR and SMRT make part of large multiprotein complexes, where various components are sensors for other perceptive or proprioceptive signals coming to the genome (Perissi *et al.*, 2010). These components may alter the phosphorylation state, the stability and the distribution of corepressors, changing their activity to various degrees, up to the complete reversal of gene repression. One of these proteins, Tab2, was characterized as a mediator of both TGF- β and inflammatory cytokines (Takaesu *et al.*, 2000; Broglie

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et al., 2010), but was also found as a facultative component of the NCoR complex (Baek *et al.* 2002). Studying antiandrogen-resistant prostate cancer cells, it was found that interleukin-1 β (IL-1 β) induces phosphorylation of Tab2 engaged in the nucleus with NCoR complexes, unmasking Tab2 ability to translocate to the cytoplasm together with NCoR, thus dismissing repression from androgen-responsive genes and functionally converting antiandrogenic compounds to androgenic (Zhu *et al.*, 2006). Interestingly, deletion of Tab2 resulted in reversal of IL-1 β effect, demonstrating that Tab2 is not essential to transcriptional repression by the androgen receptor (AR). A similar mechanism was observed in MCF7 breast cancer cells in response to Tam, and indeed a conserved short motif was found in the N-terminal domain of steroid receptor proteins (AR, PgR, ER α) responsible for direct interaction between the receptor and Tab2. In fact, a peptide mimic of this region reverts IL-1 β -induced NCoR dismissal when microinjected in prostate cancer cell nuclei (Zhu *et al.*, 2006). A second interaction was demonstrated between Tab2 and the first RID of NCoR (Baek *et al.*, 2002).

The balance of coactivator to corepressor available at the gene level defines a threshold from activation to repression and may represent a general feature of hormone resistance. Among the mechanisms proposed to explain primary and acquired Tam resistance in breast cancer cells that maintain expression of ER α , there are constitutive activation of components of signaling pathways stemming from membrane growth factor receptors, resulting in re-distribution of coactivators or corepressors (Privalsky, 2004; Frogne *et al.*, 2005; Schiff *et al.*, 2005; Frankel *et al.*, 2007; Yue *et al.*, 2007; Pancholi *et al.*, 2008; Musgrove and Sutherland, 2009). In addition, it was shown that the coactivator SRC3/AIB1, which is often amplified and overexpressed

in breast cancer, induced Tam resistance *in vitro* (Osborne *et al.*, 2003), in a similar way as it did a decreased level of NCoR corepressor (Lavinsky *et al.*, 1998).

In order to understand the possible role of Tab2 in the control of Tam response in breast cancer cells, we set out to interfere with Tab2 action in MCF7 derivatives that were rendered resistant to Tam by continuous drug exposure. We present evidence that Tab2 has a general role in Tam resistance that bypasses its role in inflammatory response and describe a lead compound to reverse Tam resistance in breast cancer cells.

Results

Tab2 is involved in Tam resistance

In order to study the role of Tab2 in Tam resistance, we used cell lines obtained from MCF7 breast carcinoma cells by continuous passage in the presence of sub-lethal doses of Tam (Lykkesfeldt and Briand, 1986; Madsen *et al.*, 1997). These cells, indicated here as TAMR-4.1, TAMR-4.2 and TAMR-8, are slightly different in terms of cell morphology, gene expression and growth rate (for details, see Materials and methods). They all maintain expression levels of ER α and NCoR similar to wild-type MCF7 cells (MCF7 wt), while showing lower expression of SMRT corepressor protein (not shown), suggesting a more strict dependence on NCoR. We have studied Tab2 expression in these TamR sublines in the nuclear and cytoplasmic fractions, as compared with MCF7 wt, by immunoblotting (Figure 1). IL-1 β -treated MCF7 wt cells, where Tab2 is fully phosphorylated through MEKK1 (Zhu *et al.*, 2006), were used for comparison. As shown in Figure 1, whereas in untreated MCF7 wt cells the prevalent Tab2 form is the

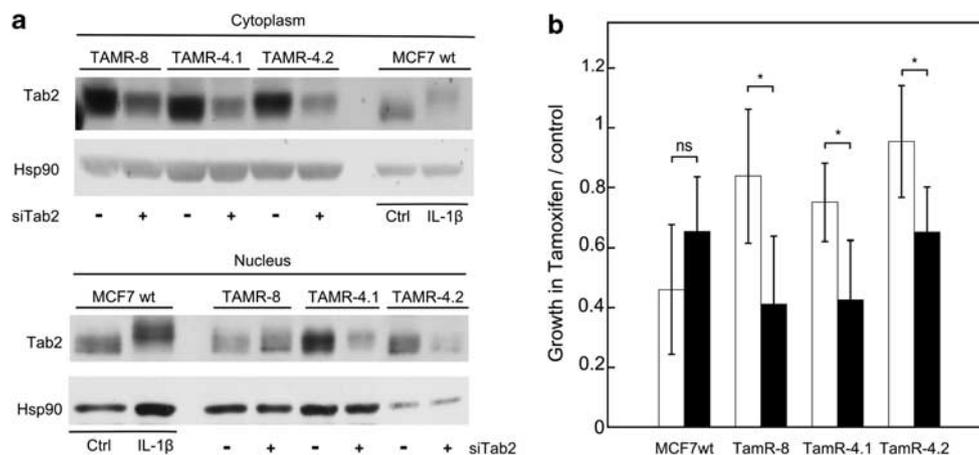


Figure 1 (a) Immunoblotting analysis of Tab2 in TamR cells as compared with MCF7 wt responding to IL-1 β treatment. TamR cells were transfected with control or Tab2 siRNA and kept in 1% DC-FBS for 48 h. MCF7 wt cells were kept in 1% DC-FBS for 3 days then treated with 10 ng/ml IL-1 β for 10 min. MCF7 wt and TamR cells were subjected to cellular fractionation and the Tab2 protein analyzed by immunoblotting in the cytoplasmic and nuclear fractions. (b) Tab2 downregulation restores tamoxifen response in TamR cells. MCF7 wt and TamR cells were transfected with control or Tab2 siRNA and 48 h later treated with 10⁻⁸ M E2 (white bars) or 10⁻⁸ M E2 plus 10⁻⁸ M 4OHT (black bars). After 24 h, the proliferation was measured by 2 h bromo-deoxyuridine incorporation. Data are means \pm s.d. of replicate experiments (MCF7wt and TamR-4.2 $N=4$; TamR-4.1 $N=3$; TamR-8 $N=5$). *Denotes $P \leq 0.05$ (Mann-Whitney U test). More details are given as Supplementary Figure S2.

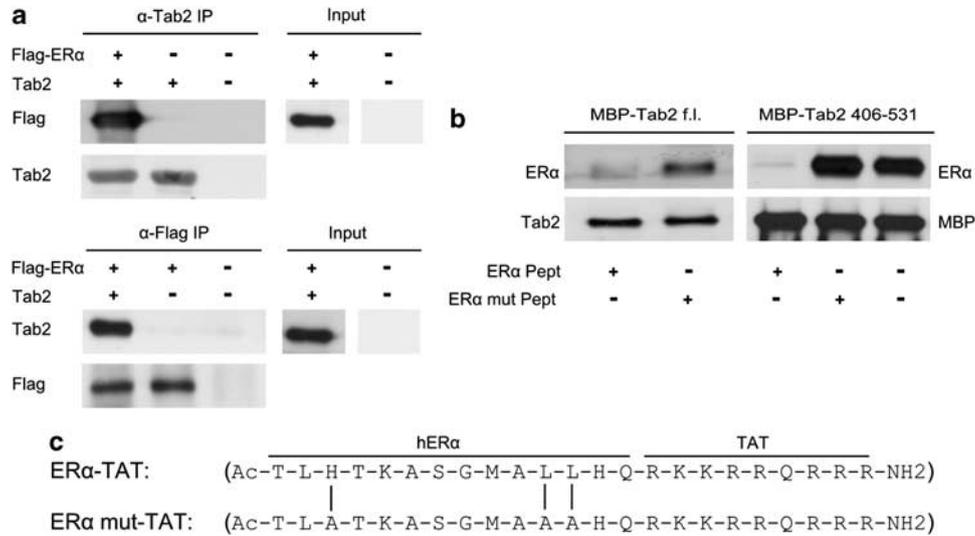


Figure 2 *In vivo* and *in vitro* interaction between Tab2 and ERα. (a) Coimmunoprecipitation of Tab2 and Flag-tagged ERα. Full length Tab2 and Flag-tagged ERα were transiently overexpressed in HEK293T cells. Anti-Flag and anti-Tab2 immunoprecipitates were carried out from nuclear extracts and were analyzed by immunoblotting with anti-Flag and anti-Tab2 antibodies. (b) Competition of ERα peptide for Tab2/ERα interaction. MBP pull-down assays, using full length Tab2 or the Tab2 central domain (aa 406-531) fused to MBP, and ERα overexpressed in HEK293T cells, in the presence of ERα-peptide or ERα mut-peptide. (c) Amino-acid sequences of ERα-TAT peptide and mutated ERα-TAT peptide. The vertical bars indicate amino-acid changes.

lower band (unphosphorylated), in untreated TamR cells Tab2 migrates as a doublet band in both subcellular fractions, and the upper band is often prevalent. Tab2 can be phosphorylated through different pathways involving MEKK1 and p38 at different sites (Baek *et al.*, 2002; Rush *et al.*, 2005; Mendoza *et al.*, 2008; Shin *et al.*, 2009) so that our result may indicate that Tab2 is constitutively activated in TamR cells. To further verify this possibility, we have performed experiments using the MEKK1 inhibitor U0126 and the p38 inhibitor SB203580 in TamR cells, obtaining visible reduction of the upper band in all cases (Supplementary Figure S1).

Based on these results, we next examined how Tab2 may influence the response to Tam. TamR cells were then transfected with Tab2-specific double helix interfering RNA (siRNA) and after 48 h, when Tab2 protein levels were 60–80% reduced, as evaluated by immunoblotting (Figure 1a), cells were treated with 4-OH-tamoxifen (4OHT) and the rate of proliferation measured. Downregulation of Tab2 led to a significant recovery of the antiproliferative response to Tam, which was maximal in TAMR-8 and less accentuated in TAMR-4.1 and TAMR-4.2 sublines (Figure 1b and Supplementary Figure S2). It should be noted that in these experiments, we compared 4OHT plus estradiol with estradiol alone and, in such conditions, 4OHT shows a little antiproliferative response (5–25%). However, in all cases downregulation of Tab2 significantly increased the inhibitory effect of Tam. Similar results were obtained in the presence of 4OHT only, although in general somewhat less evident (not shown). These results demonstrate a role of Tab2 in the resistance mechanisms in these cells.

An ERα mimic peptide restores Tam response

As seen above, Tab2 is not required to obtain antiproliferative response to Tam, suggesting that functional NCoR corepressor complexes exist devoid of Tab2, as also observed in different contexts (Baek *et al.*, 2002). Therefore, we reasoned that ERα/Tab2 could be the interaction responsible of recruiting Tab2 to corepressor complexes, causing Tab2 binding to NCoR through interaction with the RID1 domain (Zhu *et al.*, 2006) and, if phosphorylated, eliciting NCoR dismissal and export. Consequently, we hypothesized that interfering with ERα/Tab2 interaction in TamR cells would restore Tam response.

ERα/Tab2 interaction was investigated in more detail. First, Flag-ERα efficiently coimmunoprecipitated Tab2, and vice-versa (Figure 2a) when overexpressed in HEK293T cells. Second, fragments of Tab2 of different length were expressed in bacteria as MBP fusion proteins. Both full length Tab2 and its central domain (aa 406-531) were found to pull-down efficiently ERα from lysates of overexpressing HEK293T cells (Figure 2b). Taking advantage of the conservation among sex steroid receptors, we selected a 14-aa region of the hERα, corresponding to the androgen receptor motif shown to be essential for AR/Tab2 interaction (Zhu *et al.*, 2006). A synthetic peptide corresponding to this conserved sequence of human ERα, thereafter called ERα-peptide, but not a mutated version (first 14 aa in Figure 2c), was able to compete out efficiently the *in vitro* biochemical Tab2/ERα interaction (Figure 2b).

Next, we addressed the question whether such an interfering peptide could relieve Tab2 inhibition of Tam response in cultured cells. In order to get internalization, the 14-aa sequence used in *in vitro* experiments was

fused N-terminally to the minimized carrier sequence of the viral TAT protein (Patel *et al.*, 2007), to give the 23-aa sequence shown in Figure 2c, called ER α -TAT peptide, and the corresponding mutated version. Cells were then treated with 1 and 100 μ M concentrations of these peptides for 1 h in the absence of serum, then serum was added back and the effect on cell proliferation was measured 24 h later. As shown in Figure 3a, the ER α -TAT peptide, but not the mutated version, was able to reduce the growth of TamR cells in the presence of Tam. Cell treatment with 100 μ M peptide produced a 21–84% growth inhibition when compared with cells treated with the ER α mut-TAT control peptide. To obtain proof of the action of ER α -peptide *in vivo*, we overexpressed Flag-ER α and full length Tab2 in HEK293T cells and treated them with 100 μ M ER α -TAT or ER α mut-TAT peptides for 3 or 6 h. Co-immunoprecipitation analysis of nuclear extracts showed that the ER α -peptide, but not the mutated version, appreciably competed out the interaction (Figure 3b). Altogether, these results suggest that ER α /Tab2 interaction may take place in TamR cells, and that interfering with this interaction can recover Tam response in these cells. This observation may have some importance for the development of pharmacologically useful compounds. To extend the generalization of our observations, we examined the established breast cancer cell line BT474, which shows amplification of the *ERBB2* gene, while maintaining ER expression, and whose growth is not affected by Tam treatment. Also in this case, the ER α -TAT peptide was able to recover the antiproliferative response to Tam (23–44 vs 0–11% with the mutated peptide) (Figure 3c), and similar results were obtained by Tab2 downregulation using siRNA (40–43 vs 12–15% with control siRNA) (Figure 3d), suggesting a general role of Tab2 in the pharmacological response to Tam in breast cancer cells.

Gene expression programs controlled by Tab2

In prostatic cancer cells treated with IL-1 β , downregulation of Tab2 restored NCoR-mediated repression of several genes in response to antiandrogenic compounds (Zhu *et al.*, 2006). Therefore, we examined gene expression in TamR cells, under continuous Tam treatment, after transfection of control or Tab2 siRNA. Gene expression in TAMR-4.1, TAMR-4.2 and TAMR-8 was analyzed using a 44 K feature microarray platform, to ensure complete genomic coverage, by direct comparison of control siRNA with Tab2 siRNA using the double-color method and dye-swapping technical replicate. We obtained a list of 282 probes concordantly regulated in at least 2 out of 3 lines: of these, 51 were significantly regulated in all 3 lines, and 239 showed concordant regulation. The complete list of probes, with the corresponding gene name, is given in Supplementary Table S1. In order to get functional information from this gene list, we carried out Gene Ontology and pathway analysis using the Ingenuity platform. Table 1 shows top enriched molecular and cellular functional categories that clearly point out to the main biological

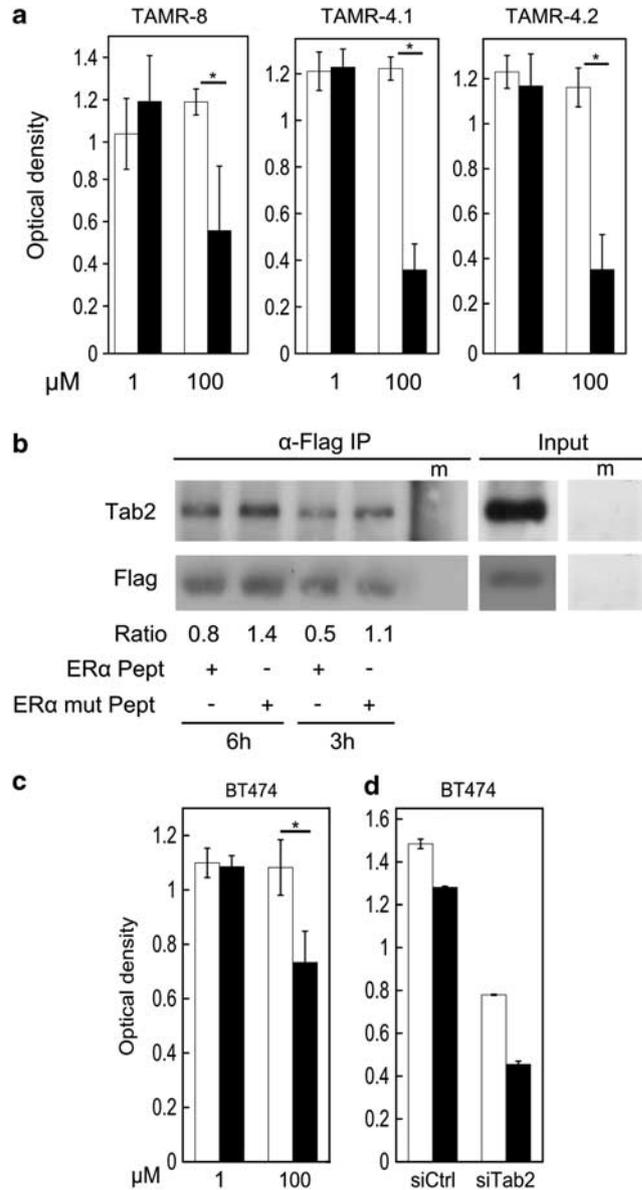


Figure 3 An ER α mimic peptide restores tamoxifen response in TamR cells. **(a)** TamR cells were treated with 1 μ M or 100 μ M ER α -TAT peptide (black bars) or ER α mut-TAT peptide (white bars) in absence of serum. After 1 h, 1% DC-FBS and 10⁻⁶ M 4OHT were added for 24 h, then cell growth was measured as described in Figure 1. Data are means \pm s.d. of triplicate experiments. **(b)** ER α -TAT peptide competition on Tab2/ER α interaction *in vivo*. Full length Tab2 and Flag-tagged ER α were transiently overexpressed in HEK293T cells. The cells were treated with 100 μ M of ER α -TAT or ER α mut-TAT peptides for 3 or 6 h in the presence of 4OHT. Anti-Flag immunoprecipitates from nuclear extracts were analyzed by immunoblotting with anti-Flag and anti-Tab2 antibodies. 'm' indicates untransfected HEK293T lysate. The ratio between anti-Tab2 and anti-Flag for each treatment is indicated. **(c)** BT474 cells were treated with ER α -TAT peptide (black bars) or ER α mut-TAT peptide (white bars) and their growth was analyzed as above. Data are from triplicate experiments. **(d)** BT474 cells were transfected with control or Tab2 siRNA and then treated with 10⁻⁸ M E2 (white bars) or 10⁻⁸ M E2 plus 10⁻⁶ M 4OHT (black bars). Data are means \pm s.d. of duplicate experiments. *Denotes $P \leq 0.05$ as in Figure 1.

effect of Tab2 silencing in TamR cells, that is, antiproliferative. Top network was ‘DNA Replication, Recombination, and Repair, Cellular compromise, Cell cycle’; top disease was ‘Cancer’; and top physiological system was ‘Hair and skin development and function’ (Supplementary Table S2). Pathway analysis showed that the described interaction network containing the highest number of Tab2-regulated genes is centered around the important breast cancer susceptibility gene *BRCA1* (Figure 4) that has important roles in transcription, DNA repair of double-stranded breaks and recombination. It is worth of note that, of the 35 proteins interconnected in this graph, 28 show some degree of regulation by Tab2 siRNA treatment and, most importantly, 21 of these are concordantly down-regulated. The genes involved in the canonical BRCA1

pathway are shown in Supplementary Figure S3. We also asked if the list of 282 Tab2-regulated genes contain some known estrogen-regulated genes by comparing it with the GEMS database (Ochsner *et al.*, 2009). Using a q-value < 0.05 for the choice, 49 common genes (Supplementary Table S3) were found, comprising—among the most known—*CCNG2*, *DICER1* and *IL1R1*.

Finally, we reasoned that, if Tab2 has a general role in Tam response in breast cancer, thus the expression profile of the genes showing dependency on Tab2 in the experimental setting should correlate with the relapse rate in patients treated with adjuvant Tam. Hence, we explored the data set published by Sotiriou *et al.* (2006) that contains data on 64 ER+ patients, treated with Tam in the adjuvant setting. A group of 212 probes, among the 282 probes regulated by Tab2 siRNA in TamR cells, selected on the basis of the ‘P’ value (see Materials and methods) were able to discriminate very significantly relapse-free survival of these patients (Figure 5a), suggesting that their expression profile in tumor cells may unravel a condition of relative resistance to the treatment. As a very high number of Tab2 siRNA-regulated genes belong to the BRCA1 network shown in Figure 4, we investigated whether this group of genes was able to predict response. As shown in Figure 5b, this subset (26 genes) was extremely efficient in identifying relapsing patients; one branch was completely free of events up to 7 years, whereas the other felt down quickly, suggesting that this expression profile marks hormone-resistant tumors.

Table 1 Molecular and cellular functions associated with Tab2 siRNA-regulated gene set (IPA, Ingenuity Systems)

Name	P-value	# Molecules
Cell Cycle	4.18E-06–4.63E-02	36
Cellular Assembly and Organization	1.51E-04–3.93E-02	20
DNA Replication, Recombination, and Repair	1.51E-04–3.93E-02	23
Cell Death	1.56E-04–3.41E-02	39
Cellular Development	5.22E-04–4.69E-02	29

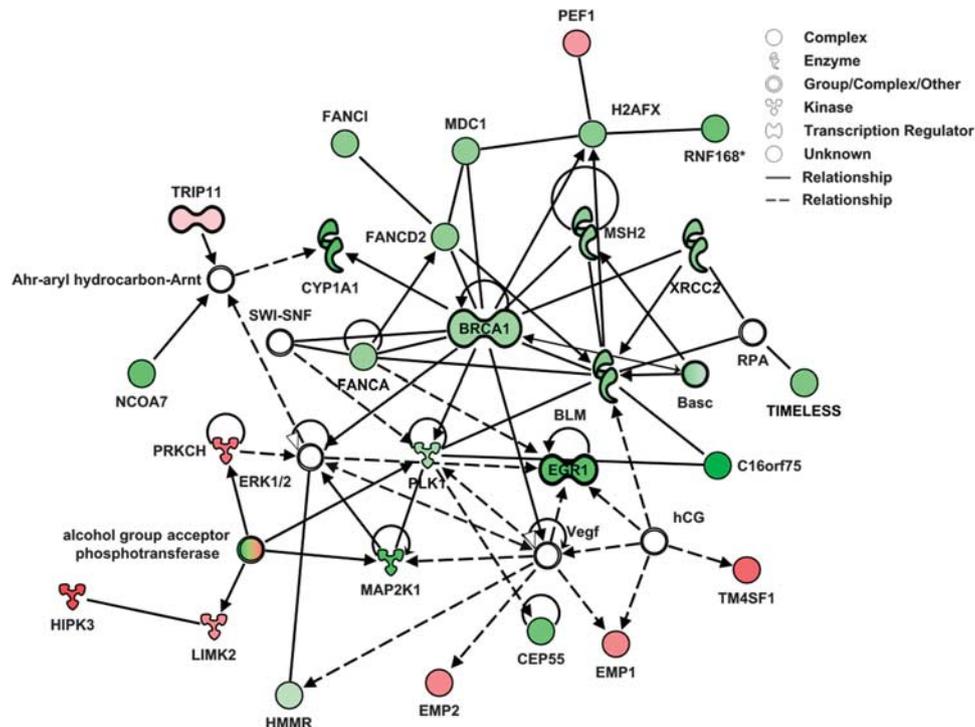


Figure 4 Top network of Tab2 siRNA-regulated genes (IPA, Ingenuity Systems). Down (green) and up (red) regulated genes are connected with continuous or dashed lines, indicating direct interactions experimentally proven and indirect connections, respectively. Basc indicates the BRCA1 associated complex.

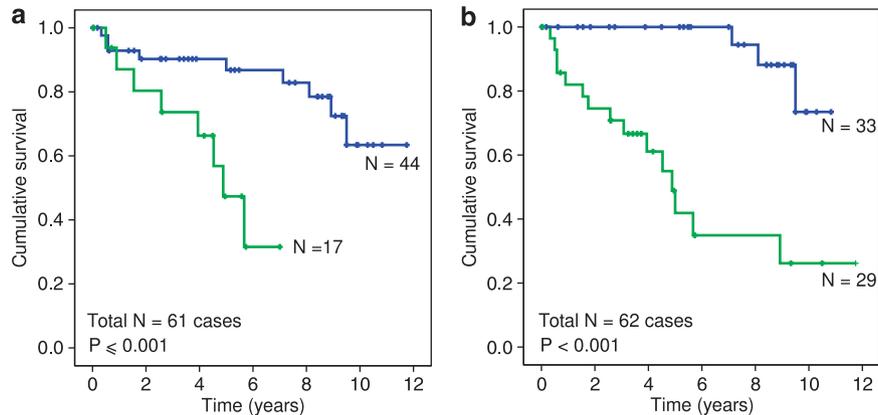


Figure 5 Association of Tab2 siRNA-regulated genes with survival in breast cancer patients treated with adjuvant tamoxifen (Sotiriou *et al.*, 2006). Regulation curves were calculated using the Kaplan–Meier statistics. (a) Sample clusters were obtained using 264 probes, corresponding to a subset of 212 Tab2 siRNA-regulated genes, selected on the combined *P* value (<0.05). (b) Sample clusters were obtained using 43 probes, corresponding to 22 genes belonging to the network in Figure 4.

Discussion

The data presented here demonstrate a role for Tab2 in the pharmacological resistance to Tam, as its silencing by siRNA leads to recovered inhibition of cell growth and altered expression of cell cycle-related genes in Tam-resistant breast cancer cells. In addition, we present evidence that a peptide mimicking the Tab2-interacting domain of ER α can represent a lead compound to reverse the pharmacological resistance to Tam in breast cancer cells.

Tab2 is known as a player in the inflammatory signal transduction pathway and was implicated in the reversal of NCoR-dependent and NF κ B- or APP-mediated gene repression (Baek *et al.*, 2002) and androgen antagonist response in prostate cancer (Zhu *et al.*, 2006). In addition, it was identified as a cofactor for ERBB4 to assemble NCoR corepressor complex in response to neuregulin (Sardi *et al.*, 2006). Although Tab2 was previously shown to mediate IL-1 β abrogation of Tam response in breast cancer cells (Zhu *et al.*, 2006), to our knowledge this is the first report demonstrating that Tab2 is directly involved in the acquisition of Tam resistance. In prostate cancer, a very clear relationship between macrophage infiltration and endocrine resistance was described (Zhu *et al.*, 2006), whereas in breast cancer this is less understood. However, it may be of interest the fact that, looking for signatures predictive of Tam response, Sotiriou and co-workers identified a cluster of genes related to cellular inflammation, further suggesting a link between these pathways (Loi *et al.*, 2008).

We did not directly evaluate differentially expressed genes in our TamR cells in comparison to MCF7 wt, so that we have no hint on which pathway(s) are activated in these cells. Other TamR sublines derived in our laboratories were shown to have constitutive activation of Akt (Frogne *et al.*, 2005) and increased level of PKC δ (Nabha *et al.*, 2005) and PKC α (Frankel *et al.*, 2007). Other groups have reported that activation of several growth factor receptor pathways, including ERBB2, EGFR and PKA can lead to Tam resistance (Shou *et al.*,

2004; Yue *et al.*, 2007; Pancholi *et al.*, 2008). Interestingly, we could demonstrate Tab2-dependency also in BT474 cells, which carry amplified HER2. Together with the observation that Tab2 migrated in SDS-PAGE gel electrophoresis as a phosphorylated form, these data suggest that Tab2 may be constitutively activated by unknown pathway(s) in TamR cells. Intriguingly, it has been shown that EGFR may lead to Tab2 phosphorylation through p38/MAPK (Shin *et al.*, 2009). In BT474 cells, downregulating Tab2 reduced the growth even in the absence of Tam. It is conceivable that Tab2-driven NCoR export is in part needed for the proliferative response to the ERBB2-dependent pathway in these cells. This effect was much less pronounced, yet present, in our TamR sublines (Supplementary Figure S2).

Phosphorylation of Tab2 by MEKK1 following IL-1 β stimulation in prostate cancer cells led to delocalization of NCoR to the cytoplasm (Baek *et al.*, 2002; Zhu *et al.*, 2006). The same observation was reported by others in MCF7 cells in response to insulin (Carroll *et al.*, 2003). We sought to find evidence of NCoR delocalization in our TamR cells, as well as reversal after Tab2 silencing by both immunofluorescence and immunoblotting using cell fractionation. Although some results could confirm this hypothesis, data were not completely consistent, possibly due in part to the fact that TamR and MCF7 wt grow optimally in very different conditions, and do not allow robust conclusions, so that we prefer not to present these data here.

Tab2 downregulation elicited some degree of growth inhibition in all experimental conditions we tested, in both TamR and MCF7 wt cells, suggesting that Tab2 may have a role in cell growth that is not strictly dependent on its function on Tam-regulated genes. However, the fact that an ER α mimic peptide that competes with ER α /Tab2 interaction *in vitro* and *in vivo* equally inhibits growth in the presence of Tam strongly suggest that NCoR dismissal from estrogen-regulated genes is, at least in part, the mechanism operating in these cells.

The effect of Tab2 downregulation in TamR cells is fully supported by microarray analysis results, showing

that the mostly over-represented molecular and cellular functions within the genes regulated by Tab2 siRNA were related to cell cycle. Interestingly, the canonical network with the highest enrichment score was centered around *BRCA1* that is the most important breast cancer susceptibility gene and that is centrally involved in DNA recombination and repair. The majority of genes in this network were downregulated in response to Tab2 silencing, that is, when response to Tam is partially restored. A negative effect of *BRCA1* downregulation on cell cycle is not straightforward, as it has been shown that *BRCA1* acts as a coactivator of p21(WAF1/CIP1) (Lee *et al.*, 2001). Nevertheless, this is consistent with the fact that *BRCA1* and most of the connected genes are upregulated in response to estrogen, as we determined exploring the GEMS database as well as our own data (Cicatiello *et al.*, 2004, 2010; Ochsner *et al.*, 2009). Furthermore, the association we have calculated between the expression of genes in *BRCA1* network and survival in breast cancer patients treated with Tam in the adjuvant setting is impressive (Figure 5b). To our knowledge, this is the first report linking these genes to prediction of response to endocrine therapy. In general, the subset of genes that are regulated following Tab2 silencing in TamR cells shows the same property, further confirming that Tab2 may be critically linked to Tam resistance in breast tumors.

In conclusion, we present here evidence that Tab2 is a new player in pharmacological resistance to Tam in breast cancer patients. Today, Tam is still first line endocrine treatment for premenopausal women and, even though selective ER modulators are more and more replaced with third generation aromatase inhibitors, there are suggestions that they still represent a valid alternative (Hackshaw *et al.*, 2011), especially if predictive markers are identified to sort out patients to the most appropriate endocrine treatment. Gene signatures as those presented here may be further refined and validated. Other groups have also presented interesting results on Tam response prediction (Jansen *et al.*, 2005; Loi *et al.*, 2008; Henriksen *et al.*, 2009). Finally, we have demonstrated that a cell permeable peptide, ER α -TAT, that effectively competes out ER α /Tab2 interaction *in vivo*, and restores in part Tam sensitivity in Tam-resistant cells, is a promising peptide for further pharmacological developments.

Materials and methods

Chemicals and antibodies

4-OHT and 17 β -estradiol were purchased from Sigma-Aldrich (St Louis, MO, USA). Human recombinant interleukin-1 β was from Calbiochem (San Diego, CA, USA). Polyclonal anti-ER α antibody (H-184), anti-Tab2 (K-20), anti-Tab2 (H-300) and anti-Tab2 (E-20) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). We used also a polyclonal anti-Tab2 antibody from Bethyl Laboratories (Montgomery, TX, USA) and a monoclonal anti-Hsp90 from Stressgene Bioreagents Corp. (Victoria, BC, Canada). Monoclonal antibody anti-Flag was from Sigma-Aldrich. The ER α peptides were synthesized and purchased from PolyPeptide Group SAS

(Strasbourg, France). The Tab2 (pool of three different Tab2 siRNAs) and untargeting control siRNAs were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA).

Plasmids

The pCMV-T7 containing the full length hTab2 cDNA, the hER α expression vector pHEGO, the p3XFLAG-CMV containing the hER α cDNA were generous gifts from Professor MG Rosenfeld (UCSD, La Jolla, CA, USA), Professor P Chambon and Professor A Weisz (University of Naples, Italy), respectively. The cDNA encoding human Tab2 full-length and the middle domain 406-531 Tab2 were amplified by PCR and cloned into pMALC2 vector. All constructs were verified by automated DNA sequencing.

Cell lines and treatments

Tam-resistant cells were obtained by continuous passage of MCF7 in the presence of sub-lethal doses of Tam (Lykkesfeldt and Briand, 1986; Madsen *et al.*, 1997). For the experiments, we used two independent subcultures from MCF7/TAMR-4 (independently passaged >15 times), here indicated as TAMR-4.1 and TAMR-4.2, and the MCF7/TAMR-8 cell line, indicated as TAMR-8. Resistant cells, collectively called TamR, were continuously propagated in phenol red-free Dulbecco's modified Eagle's medium (DMEM) /F12 1:1, supplemented with 1% fetal bovine serum (FBS) and 10⁻⁶M 4OHT. BT474 cell line was obtained from ATCC (HBT-20). MCF7 wt and HEK293T cells were grown in high glucose DMEM supplemented with 10% FBS. For Tab2 phosphorylation, MCF7 wt cells were starved in 1% dextran/charcoal-treated (DC) FBS for 3 days and then treated with 10 ng/ml IL-1 β in phenol red-free DMEM, 1% DC-FBS. For proliferation assays, MCF7 wt and TamR cells were maintained in phenol red-free DMEM supplemented with 1% DC-FBS for 48 h, then 10⁻⁸M E2 \pm 10⁻⁶M 4OHT were added. Proliferation was measured in quintuplicate by 2 h bromo-deoxyuridine incorporation (Cell Proliferation Biotrak ELISA System kit, GE-Healthcare, Bio-Sciences AB, Uppsala, Sweden) followed by chemi-luminescence detection on a TECAN Infinite200.

Sub-cellular fractionation

Cells were lysed with 20 mM Tris-HCl pH 8.0, 20 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, 2 mM Na₃VO₄, 50 mM NaF, 1X PIC for 10 min and centrifuged at 12000 \times g for 10 s. The cytoplasmatic fraction was further cleared at 12000 \times g for 5 min. The nuclear pellet was resuspended in 250 mM Sucrose, 10 mM MgCl₂, 1X PIC, layered on a cushion of 350 mM Sucrose, 0.5 mM MgCl₂, 1X PIC and centrifuged at 8000 \times g for 10 min. Nuclei were then resuspended in 20 mM Hepes pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.1% NP-40, 1 mM PMSF, 2 mM Na₃VO₄, 50 mM NaF, 1X PIC, put on ice for 30 min and centrifuged at 12000 \times g for 2 min. The supernatant nuclear extracts were diluted 1:3 in 20 mM Hepes pH 8.

siRNA and gene Transfection

MCF7 wt, TamR and BT474 cells were plated at 6 \times 10³ cells/well in 96-well plates, and after 18 h transfected with 20 nM siRNAs. After 7 h, the medium was changed to phenol red-free DMEM 1% DC-FBS, and 48 h after transfection cells were treated for 24 h with 10⁻⁸M E2 \pm 10⁻⁶M 4OHT. HEK293T cells were plated at 15 \times 10⁵ cells/plate in 100 \times 20 mm² plates and after 18 h transfected with 6 μ g pCMV-T7-Tab2, 6 μ g p3XFLAG-CMV-ER α or 6 μ g pHEGO using LipofectA-MINE2000 (Invitrogen) according to the manufacturer's

instructions. After 4 h, the medium was changed to phenol red-free DMEM 1% DC-FBS + 10^{-6} M 4OHT for 48 h.

Treatment with peptides

1 μ M and 100 μ M of ER α -TAT or ER α mut-TAT peptides were added to TamR, BT474 and HEK293T cells in phenol red-free DMEM + 10^{-6} M 4OHT in the absence of FBS, due to the low stability of the peptide in serum. After 1 h, 1% DC-FBS was added.

Coimmunoprecipitation, pull-down and immunoblotting

Coimmunoprecipitations of ER α and Tab2 were carried out from nuclear extracts of HEK293T cells, using 1 μ g of anti-Flag antibody or a pool of 3 μ g of anti-Tab2 K-20, 3 μ g of anti-Tab2 E-20 and 3 μ g of anti-Tab2 H-300 antibodies. *In vitro* pull-down assays were done using MBP-Tab2 proteins, ER α overexpressed in HEK293T cells, and synthetic ER α and ER α mut peptides at a final concentration of 10 μ M. Coimmunoprecipitation and pull-down assays were performed using standard techniques as described in more details in the Supplementary Methods. Specific bands were quantified by densitometry, using the Quantity One 1-D Analysis Software 4.6.9 (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Gene expression profiling and bioinformatic analysis

For microarray analysis, RNA quality, concentration and labeling were checked by means of RNA 6000 nano chip assays (Agilent Technologies, Palo Alto, CA, USA) and NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples were checked for Tab2 mRNA expression using real-time RT-PCR: reactions were carried out according to the LightCycler Real-Time PCR System (Roche Diagnostic, Indianapolis, IN, USA), using the SYBR Green method and 18S RNA as normalizing target. Primers for Tab2 and 18S were purchased from Qiagen, Hilden, Germany. Each RNA sample pair (siTab2/siCtrl) was labeled with Cy3 and Cy5 using the indirect method (Amino Allyl MessageAmp II aRNA Kit, Ambion Inc., Grand Island, NY, USA) and cohybridized to oligonucleotide glass arrays representing 41 K human unique genes and transcripts (Human Whole Genome Oligo Microarray Array 4x44 K, Agilent Technologies). Two replicates, with dye swap, were performed for each sample. Slides were scanned with the dual-laser microarray scanner Agilent G2505B (Agilent Technologies) and data normalized and statistically evaluated using the statistical computing software 'R'. The Limma package (Smyth *et al.*, 2005) was used for preprocessing and differential expression analysis. Further details on microarray analysis and statistical calculations are available as Supplementary Methods. In order to find a group of genes regulated in the three TamR cells examined, the following procedure was adopted. Modulated gene lists for each subline were obtained using the modified *t*-test supplied by the Limma package. Afterwards, a final gene list was created by selecting genes with *P*-value less

than 0.1 in at least two of three TamR lines, to avoid bias due to differences in sublines (Supplementary Table S1).

Gene ontology and molecular pathways analysis was performed using Ingenuity Pathway Analysis software (version 9.0) (<http://www.ingenuity.com>). The analysis of publicly available breast cancer data set was done by extracting normalized values from (GSE2990) (Sotiriou *et al.*, 2006), containing data from 64 patients with primary breast tumors treated with Tam in the adjuvant setting. Probes were extracted from Tab2 downregulation experiments based on the combined '*P*' value in the three cell sublines examined. Probes were referred to single entries in the NCBI genomic database and mapped in the tumor microarray data set with no assay to reduce multiple probes. Clustering was done using TMEV (<http://www.tm4.org/mev/>) by Pearson or Spearman correlation analysis and average linkage. The two major branches of samples obtained were directly compared with relapse-free survival or distant metastasis free-survival data, by using the Kaplan–Meier statistics (PASW Statistics 18, SPSS, Inc., 2009, Chicago, IL, USA, <http://www.spss.com>).

Abbreviations

4OHT, 4-OH-tamoxifen; AI, aromatase inhibitors; AR, androgen receptor; E2, 17 β -estradiol; ER α , estrogen receptor α ; IL-1 β , interleukin-1 β ; PgR, progesterone receptor; SERMs, selective estrogen receptor modulators; Tam, tamoxifen; TamR, tamoxifen-resistant cells.

Conflict of interest

Drs Caizzi, Agati and Ricci received compensation for this work by the Bioindustry Park Silvano Fumero S.p.A, owner of the Patent IT TO20080989. Drs Cutrupi, Reineri, Panetto and De Bortoli are inventors of the Patent IT TO20080989. Drs Grosso, Friard, Scatolini, Chiorino and Lykkesfeldt declare no potential conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)