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TITLE: CtIP (RBBP8), a Critical Player in the Development of Tamoxifen Resistance in Human Breast Cancer

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14. ABSTRACT
Tamoxifen resistance constitutes a major clinical challenge in breast cancer therapy. However, the mechanisms involved are still poorly understood. Using serial analysis of gene expression (SAGE) based profiling of our recently developed tamoxifen resistant human breast cancer cell line, we identified CtIP, a BRCA1- and CtBP-interacting protein, as one of the most significantly down-regulated transcripts in tamoxifen resistant cells. This result was independently confirmed by quantitative real-time RT-PCR and Western blot analyses. Therefore, we hypothesized that down-regulation of CtIP constitutes a critical event for the development of tamoxifen resistance in breast cancer cells. Our results demonstrated that silencing CtIP by means of siRNA in tamoxifen sensitive cells confers tamoxifen resistance and estrogen independence in vitro, and reintroduction of CtIP into tamoxifen resistant cells restores sensitivity to the inhibitory growth effects of tamoxifen. Importantly, immunohistochemical studies of CtIP expression in primary breast carcinomas show that CtIP status strongly correlates with clinical response to endocrine therapy, and patients with progressive diseases have significantly lower CtIP levels than those who completely respond. These findings indicate that CtIP silencing may be a novel mechanism of the development of tamoxifen resistance in breast cancer cells and suggest that CtIP maybe a potential biomarker to predict clinical response to endocrine therapy. In addition, we also found that silenced expression of CtIP in tamoxifen resistant cells is not due to promoter CpG islands methylation, suggesting that other mechanisms, such as transcriptional repression, may be involved.

15. SUBJECT TERMS
CtIP, tamoxifen resistance, breast cancer

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Introduction

The role of estrogen (E_2) as a fundamental factor in the etiology and progression of human breast cancer has been well documented. The extent of exposure to ovulary cycles is one of the most important endogenous causes associated with a higher risk for development of sporadic breast cancer [1]. Therefore, for a long time, treatment of breast cancer has been directed towards inhibiting the tumor promoting effects of estrogen. Tamoxifen, an antiestrogen, has been the gold standard for endocrine treatment of all stages of estrogen receptor (ER) positive breast cancer for more than 25 years and was the first approved drug by the FDA as a cancer chemopreventive for reducing breast cancer incidence in both pre- and post-menopausal women at high risk [2]. As adjuvant therapy, tamoxifen reduces the risk of recurrence and improves overall survival in early breast cancer. It is also effective for patients with untreated metastatic breast cancer [3]. However, most of the tumors that initially respond to tamoxifen therapy will develop resistance. This constitutes a major clinical challenge in breast cancer therapy. Unfortunately, our understanding of the mechanisms involved in tamoxifen resistance is very limited. In order to better understand this complex process, we recently generated the MCF-7 breast cancer cell line variants that are resistant to the inhibitory growth effects of tamoxifen. This new isogenic breast cancer cell lines represents a unique model that closely resembles the *in vivo* scenario. We performed extensive Serial Analysis of Gene Expression (SAGE) on these tamoxifen resistant variants comparing with their parental MCF-7 cell line. Among the various genes that change expression, we first focus in genes related to cell cycle and cell proliferation. Among these genes we observed that a commonly dysregulated gene is CtIP (RBBP8), a BRCA1- and CtBP-interacting protein, which is down-regulated 15-fold in tamoxifen resistant cells when compared to their parental MCF-7 cells. This result was independently confirmed using quantitative real-time PCR and Western blot analyses. Based on these findings, we hypothesized that down-regulation of CtIP constitutes a critical event for the development of tamoxifen resistance in breast cancer cells.

Body

To test our hypothesis, we first examined whether silencing the expression of CtIP in tamoxifen sensitive cells can induce a tamoxifen resistant phenotype. Knockdown of CtIP protein levels in tamoxifen sensitive parental MCF-7 cells was achieved using stable RNA interference techniques. Mammalian expression pSEC-puro vectors containing siRNA Expression Cassettes (SECs) that express either siRNA against CtIP or a negative control siRNA were transfected into parental MCF-7 cells. After selection, the stable clone (MCF-7/CtIP siRNA) in which CtIP protein levels were significantly knocked down was picked up (Fig. 1A). The negative control siRNA stable clone (MCF-7/(-) control siRNA) had unchanged CtIP protein levels when compared to parental MCF-7 cells (Fig. 1A). To test whether silenced CtIP expression in parental MCF-7 cells leads to the tamoxifen sensitive-to-tamoxifen resistant transition, we next directly compared cell

proliferation between MCF-7/CtIP siRNA and parental MCF-7 cells under different stimuli. Hormone-starved MCF-7/CtIP siRNA and parental MCF-7 cells were exposed to 4-OH-TAM (10 nM) for 2 weeks and cells were counted on days as indicated. CtIP silenced MCF-7/CtIP siRNA cells grew significantly faster than parental MCF-7 cells in the presence of tamoxifen ($P<0.05$). In fact, growth of parental MCF-7 cells was inhibited by tamoxifen, whereas MCF-7/CtIP siRNA cells showed significantly less growth inhibition by tamoxifen, indicating acquired resistance to tamoxifen (Fig. 1B). Interestingly, when cultured in conditions completely devoid of estrogen, inhibition of the growth of parental MCF-7 cells was observed as expected, indicating absolute estrogen dependence. In contrast, CtIP silenced MCF-7/CtIP siRNA cells grew continuously and much faster than parental cells in the absence of estrogen, suggesting gained capability of estrogen independent growth ($P<0.01$) (Fig. 1C). In addition, estrogen-stimulated cell proliferation was observed in MCF-7/CtIP siRNA cells (Fig. 1D), indicating that ER is still functional and regulating cell growth. Similar results were obtained with MCF-7/(-) control siRNA cells (data not shown). Thus, these data indicated that CtIP silencing leads to resistance to the inhibitory growth effects of tamoxifen and estrogen independent growth *in vitro*.

To further support our hypothesis, we also tested, in the opposite way, the effect of restoration of CtIP expression in tamoxifen resistant cells. We employed a Tetracycline-Off inducible expression system to introduce CtIP back to the tamoxifen resistant cells. We cloned the N-terminal Flag-tagged full length CtIP cDNA into the Tet-Off response plasmid, which was then co-transfected with the Tet-Off regulatory plasmid into the tamoxifen resistant cells. By using the anti-Flag M2 antibody, we confirmed that the expression of the Flag-tagged CtIP was tightly controlled under the presence or absence of doxycycline, a water-soluble analog of tetracycline, in the resistant cells (Fig. 2A). In a transient cell proliferation assay, we found that TAMR1 cells transiently restored with CtIP showed more growth inhibition by tamoxifen than control cells having no CtIP restoration (Fig. 2B). Without tamoxifen treatment (vehicle control), transient CtIP restoration had no significant effect on TAMR1 cell proliferation (Fig. 2C). Thus, tamoxifen appeared to inhibit the growth of the tamoxifen resistant cells that had the restoration of CtIP under the control of doxycycline. To further confirm our observation from the above transient experiment, we developed double-stable TAMR1 Tet-off FLAG-CtIP cells. Among stable clones identified, clone 32 was selected for further study. This clone showed no CtIP expression in the presence of doxycycline but similar CtIP protein level to that produced in the parental MCF-7 cells upon withdrawal of doxycycline, as determined by Western blot analysis using anti-CtIP antibody (14-1) (Fig. 2D). Next, we measured the growth of the double-stable TAMR1 Tet-off FLAG-CtIP cells under conditions in which CtIP restoration was either induced or repressed. Cells from clone 32 were first divided into two parts. Half of the cells were grown in hormone-free medium containing doxycycline while the other half were cultured in the same medium without doxycycline. After induction for 3 days, cells were treated with E2, 4-OH-TAM or vehicle. As shown in Fig. 2E, the growth of TAMR1 Tet-off FLAG-CtIP cells was significantly inhibited by tamoxifen when doxycycline was removed from the medium as compared to cells from the same clone but cultured in the presence of doxycycline ($P<0.05$). Cells still responded well to estrogen regardless of doxycycline

status (P>0.05) (Fig. 2F). Taken together, these results demonstrate that sensitivity to the inhibitory growth effects of tamoxifen in previously tamoxifen-resistant cells is restored, at least in part, by CtIP restoration.

It is well known that methylation of cytosine residues at cytosine-guanine sites (CpG islands), usually located in the promoter region of genes, can be a specific mechanism of transcriptional repression [4]. To investigate whether CpG methylation could be the mechanism causing CtIP down-regulation in tamoxifen resistant TAMR1 cells, we performed bisulfite genomic DNA sequencing and methylation specific PCR in CtIP gene promoter region containing CpG islands. As a result, we didn't observe any evidence of CpG islands methylation in such region.

We also analyzed CtIP expression by immunohistochemistry (IHC) in 59 hormone receptor-positive primary breast carcinomas from patients who subsequently developed metastatic breast cancer and received endocrine therapy (tamoxifen LY353381 or letrozole). Patients were categorized into 4 groups based on their clinical response to the therapy (complete response, partial response, stable disease, and progressive disease). Immunoreactive scores (IRS) was used to semiquantify the IHC staining for CtIP [5, 6]. IRS of 0 to 12 represents CtIP staining from undetectable to the highest expression level (Fig. 3A). Oneway ANOVA analysis on the raw CtIP IRS first revealed a significantly different CtIP expression in the above 4 response groups (P=0.01). In a Tukey's post test following ANOVA, we found that patients who had the worst response to endocrine therapy (defined as progressive disease) had significantly lower CtIP expression than those who completely responded (defined as complete response) (P=0.006) (Fig. 3B). Moreover, a Pearson's correlation analysis showed a significant correlation between CtIP status and clinical response to endocrine therapy (p=0.004). These data indicate that poor response to endocrine therapy is associated with CtIP deficiency and suggest that CtIP may be a potential marker of endocrine resistant phenotype in breast cancer.

Key Research Accomplishments

- Demonstrated that silencing CtIP in tamoxifen sensitive MCF-7 breast cancer cells confers tamoxifen resistance and estrogen independence in vitro.
- Demonstrated that reintroduction of CtIP into tamoxifen resistant cells restores sensitivity to the inhibitory growth effects of tamoxifen.
- Silenced expression of CtIP in tamoxifen resistant cells is not due to promoter CpG islands methylation.
- Demonstrated that poor clinical response to endocrine therapy is associated with CtIP deficiency in breast cancer patients.

Reportable Outcomes

- **Name of Meeting:**
  Era of Hope 2005 DoD breast cancer meeting, Philadelphia, PA, June 8-11, 2005

  Abstract/Presentation title:
  Silencing the expression of CtIP confers tamoxifen resistance and estrogen independence in human breast cancer cells.

- Manuscript in preparation entitled: **'CtIP Silencing as a Novel Mechanism of Tamoxifen Resistance in Breast Cancer'.** Minhao Wu, David R. Soler, Martin C. Abba, Maria I. Nunez, Richard Baer, Christos Hatzis, John H. Ludes-Meyers, Antonio Llombart-Bosch, Antonio Llombart-Cussac and C. Marcelo Aldaz

Conclusion

Our data from this research demonstrate that CtIP silencing is critical for the development of tamoxifen resistance in breast cancer cells. These findings indicate that CtIP silencing may be a novel mechanism of the development of tamoxifen resistance in breast cancer cells and suggest that CtIP may be a potential biomarker to predict clinical response to endocrine therapy. Our results also show that promoter CpG islands methylation is not the main mechanism for CtIP silencing in tamoxifen resistant cells, suggesting that other mechanisms, such as transcriptional repression, may be involved.

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Appendices

None.

Supporting Data

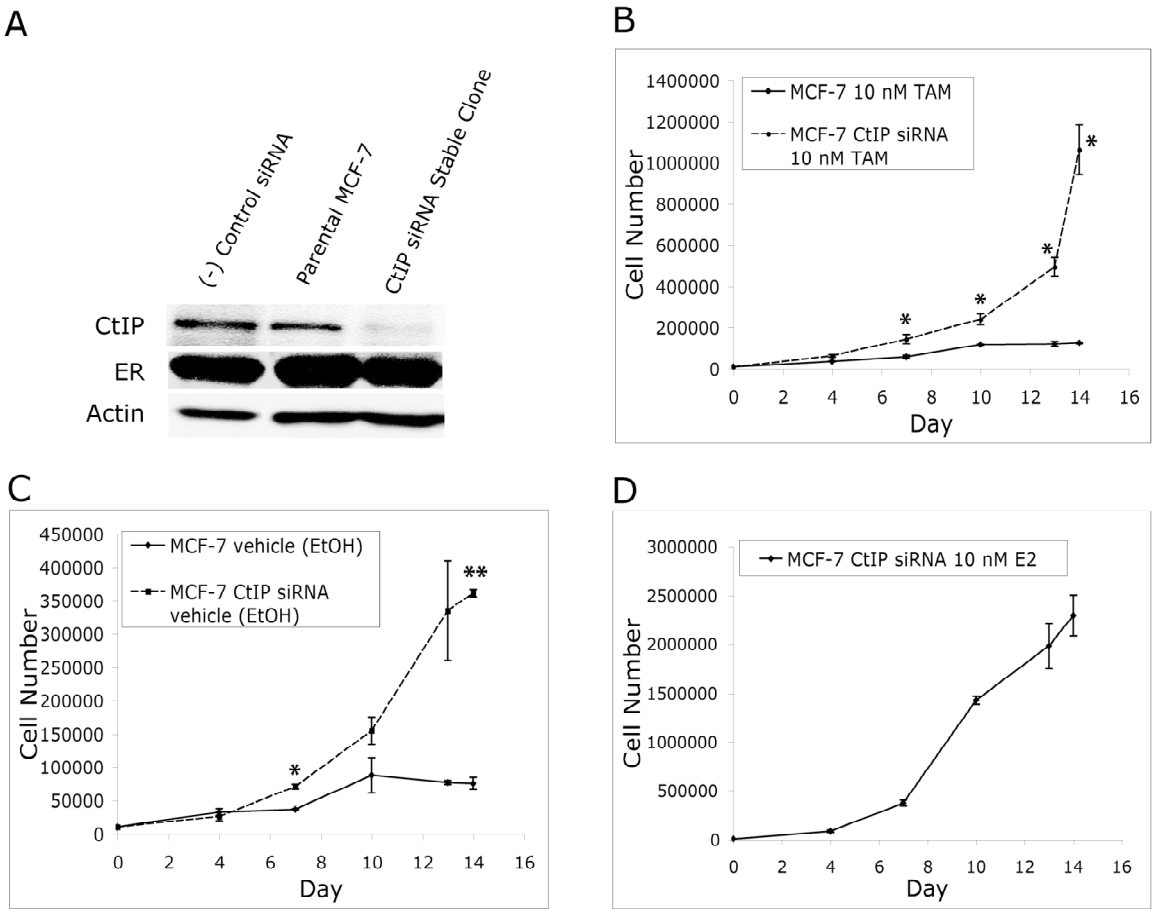

Figure 1

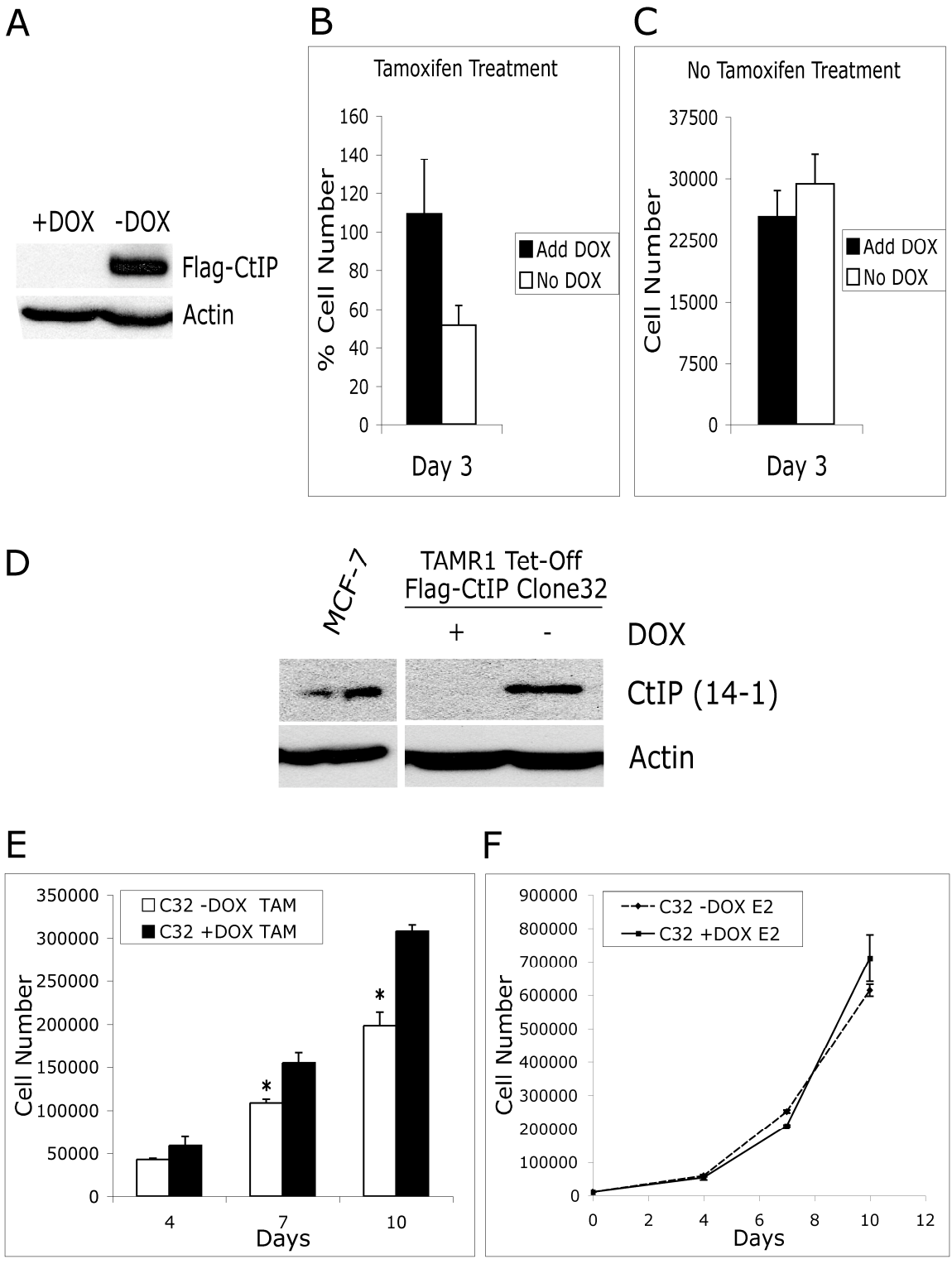
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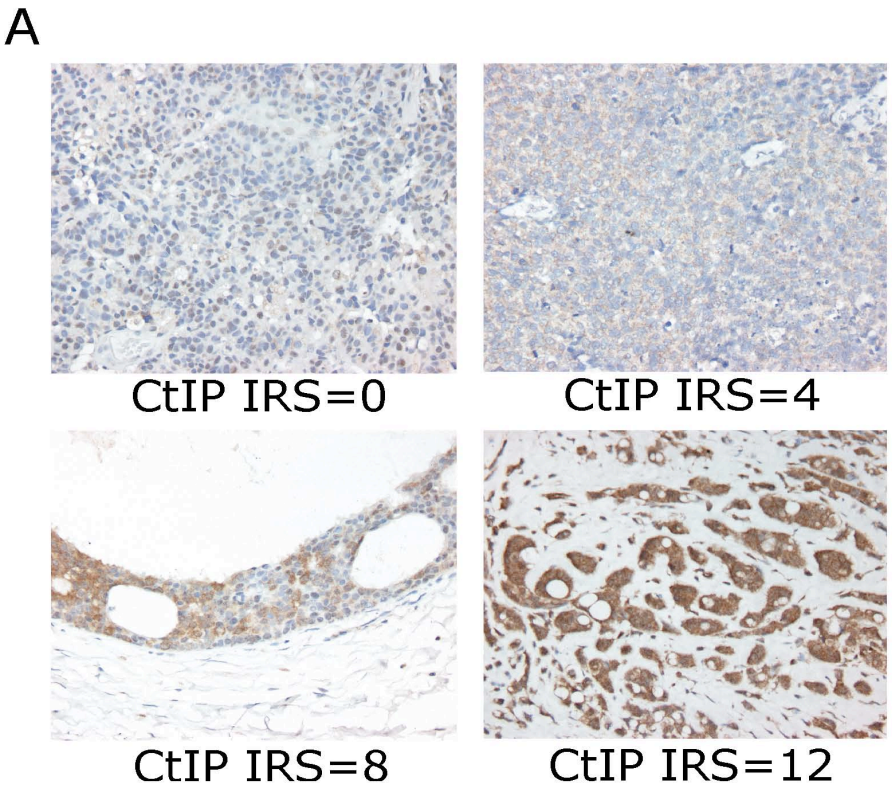
Figure 2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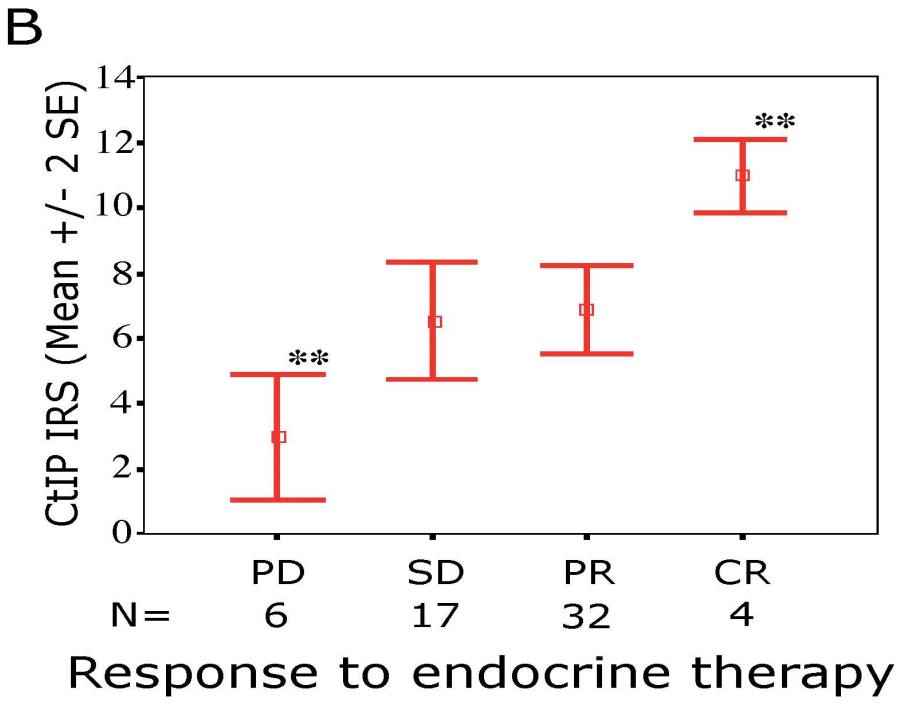
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Response to endocrine therapy

Figure 3

Figure Legend:

Figure 1. Silencing CtIP in tamoxifen sensitive MCF-7 cells confers tamoxifen resistance and estrogen independence in vitro. (A) Western blot analysis of CtIP and ER proteins in parental MCF-7 cells, MCF-7/(-) control siRNA and MCF-7/CtIP siRNA stable clones. β-Actin was used as a loading control. (B), (C) Steroid-starved MCF-7/CtIP siRNA and parental MCF-7 cells were plated and treated with 4-OH-TAM (10 nM) (B) or ethanol (C) for 2 weeks. Cell proliferation was determined by counting cells on days as indicated with a hemacytometer. All experiments were done in triplicate with each data point and error bar representing the mean ± s.e.m. * and ** indicate $p<0.05$ and $p<0.01$ by t-test, respectively. (D) Steroid-starved MCF-7/CtIP siRNA cells were exposed to E_2 (10 nM) for 2 weeks. Cell numbers were counted on days as indicated. Each data point and error bar show the mean ± s.e.m.

Figure 2. CtIP restoration in tamoxifen resistant cells regains sensitivity to the inhibitory growth effects of tamoxifen. (A) TAMR1 cells were transiently co-transfected with pTet-Off and pTRE2hyg-FLAG-CtIP vectors by electroporation and cultured in the presence or absence of doxycycline (DOX) for 3 days. Expression of FLAG-CtIP was analyzed by immunoblotting using anti-FLAG M2 antibody. β-Actin was used as a loading control. (B) Transient CtIP restoration appeared to abrogate resistance to tamoxifen in TAMR1 cells. TAMR1 cells transiently co-transfected with pTet-Off and pTRE2hyg-FLAG-CtIP vectors were treated with tamoxifen (1 μM) in the presence or absence of doxycycline for 3 days. Cell growth was determined by counting cells on days as indicated with a hemacytometer. Percent cell number represents % cell counts relative to vehicle control treated cells. The bar chart was plotted based on the mean values + 2 s.e.m. (C) Cell growth of TAMR1 cells that were transiently co-transfected with pTet-Off and pTRE2hyg-FLAG-CtIP vectors and treated with vehicle control in the presence or absence of doxycycline for 3 days. (D) Total CtIP protein expression in the double-stable TAMR1 Tet-off FLAG-CtIP clone 32 cells was analyzed using anti-CtIP antibody (14-1) in the presence or absence of doxycycline. CtIP expression in parental MCF-7 cells was used as a control. (E) CtIP reintroduction upon doxycycline withdrawal restores sensitivity to the inhibitory growth effects of tamoxifen in TAMR1 Tet-off FLAG-CtIP clone 32 cells. The proliferation of steroid-starved cells under the treatment of tamoxifen was determined in the presence (black bars) or absence (white bars) of doxycycline. Each point represents the mean + s.e.m of triplicate determinations. *, $p<0.05$ by t-test. (F) Effect of E_2 (10 nM) on cell growth of the TAMR1 Tet-off FLAG-CtIP clone 32 cultured with or without doxycycline.

Figure 3. Poor clinical response to endocrine therapy is significantly associated with CtIP deficiency in breast cancer patients. (A) CtIP expression patterns in human breast carcinomas. CtIP expression was evaluated by immunohistochemistry and semi-

quantified using immunoreactive scores (IRS) in 59 hormone receptor-positive primary breast carcinomas from patients who subsequently developed metastatic breast cancer and received endocrine therapy (tamoxifen LY353381 or letrozole). Representative tumor CtIP stainings (IRS 0, 4, 8 and 12) are shown. (B) CtIP status strongly correlates with clinical response to endocrine therapy (Pearson Correlation, p=0.004), and patients with progressive disease (PD) have significantly lower CtIP IRS than those who completely respond (CR). **, p=0.006 by ANOVA and Tukey's post tests. Complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD).