



Published in final edited form as:

*Oncogene*. 2016 December 01; 35(48): 6189–6202. doi:10.1038/onc.2016.151.

## MiR-16 mediates trastuzumab and lapatinib response in ErbB-2-positive breast and gastric cancer via its novel targets CCNJ and FUBP1

L Venturutti<sup>1</sup>, RI Cordo Russo<sup>1</sup>, MA Rivas<sup>2</sup>, MF Mercogliano<sup>1</sup>, F Izzo<sup>1</sup>, RH Oakley<sup>3</sup>, MG Pereyra<sup>1,4</sup>, M De Martino<sup>1</sup>, CJ Proietti<sup>1</sup>, P Yankilevich<sup>5</sup>, JC Roa<sup>6,7,8</sup>, P Guzmán<sup>6</sup>, E Cortese<sup>9</sup>, DH Allemand<sup>10</sup>, TH Huang<sup>11</sup>, EH Charreau<sup>1</sup>, JA Cidlowski<sup>3</sup>, R Schillaci<sup>1</sup>, and PV Elizalde<sup>1</sup>

<sup>1</sup>Laboratory of Molecular Mechanisms of Carcinogenesis, Instituto de Biología y Medicina Experimental, CONICET, Buenos Aires, Argentina

<sup>2</sup>Department of Medicine, Weill Cornell Medicine, New York, NY, USA

<sup>3</sup>Department of Health and Human Services, National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC, USA

<sup>4</sup>Servicio de Anatomía Patológica, Hospital General de Agudos 'Juan A Fernández', Buenos Aires, Argentina

<sup>5</sup>Instituto de Investigación en Biomedicina de Buenos Aires, CONICET—Partner Institute of the Max Planck Society, Buenos Aires, Argentina

<sup>6</sup>Departamento de Anatomía Patológica (BIOREN), Universidad de La Frontera, Temuco, Chile

<sup>7</sup>Departamento de Anatomía Patológica, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago de Chile, Chile

<sup>8</sup>Advanced Center for Chronic Diseases (ACCDIS), Pontificia Universidad Católica de Chile, Santiago de Chile, Santiago, Chile

<sup>9</sup>Servicio de Ginecología, Hospital Aeronáutico Central, Buenos Aires, Argentina

<sup>10</sup>Unidad de Patología Mamaria, Hospital General de Agudos 'Juan A Fernández', Buenos Aires, Argentina

<sup>11</sup>Department of Molecular Medicine/Institute of Biotechnology, Cancer Therapy and Research Center, University of Texas, San Antonio, TX, USA

Correspondence: Dr PV Elizalde, Laboratory of Molecular Mechanisms of Carcinogenesis, Instituto de Biología y Medicina Experimental (IBYME), CONICET, Vuelta de Obligado 2490, Buenos Aires 1428, Argentina. [patriciaelizalde@ibyme.conicet.gov.ar](mailto:patriciaelizalde@ibyme.conicet.gov.ar)

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### AUTHOR CONTRIBUTIONS

LV, MAR, RS and PVE were responsible for the conception and design of the study. LV, RICR, MFM, RS and PVE developed methodology. LV, RICR, MAR, MFM, FI, RHO, MGP, MDM, CJP, JCR, PG, EC, DHA, TH-MH, EHC, JAC, RS and PVE acquired the data (and also provided animals, acquired and managed patients, provided facilities, and so on). LV, RICR, MAR, PY, RS and PVE analyzed and interpreted the data. LV and PVE wrote the manuscript. PVE supervised the study. All authors read and approved the final manuscript.

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

## Abstract

ErbB-2 amplification/overexpression accounts for an aggressive breast cancer (BC) subtype (ErbB-2-positive). Enhanced ErbB-2 expression was also found in gastric cancer (GC) and has been correlated with poor clinical outcome. The ErbB-2-targeted therapies trastuzumab (TZ), a monoclonal antibody, and lapatinib, a tyrosine kinase inhibitor, have proved highly beneficial. However, resistance to such therapies remains a major clinical challenge. We here revealed a novel mechanism underlying the antiproliferative effects of both agents in ErbB-2-positive BC and GC. TZ and lapatinib ability to block extracellular signal-regulated kinases 1/2 and phosphatidylinositol-3 kinase (PI3K)/AKT in sensitive cells inhibits c-Myc activation, which results in upregulation of miR-16. Forced expression of miR-16 inhibited *in vitro* proliferation in BC and GC cells, both sensitive and resistant to TZ and lapatinib, as well as in a preclinical BC model resistant to these agents. This reveals miR-16 role as tumor suppressor in ErbB-2-positive BC and GC. Using genome-wide expression studies and miRNA target prediction algorithms, we identified cyclin J and far upstream element-binding protein 1 (FUBP1) as novel miR-16 targets, which mediate miR-16 antiproliferative effects. Supporting the clinical relevance of our results, we found that high levels of miR-16 and low or null FUBP1 expression correlate with TZ response in ErbB-2-positive primary BCs. These findings highlight a potential role of miR-16 and FUBP1 as biomarkers of sensitivity to TZ therapy. Furthermore, we revealed miR-16 as an innovative therapeutic agent for TZ- and lapatinib-resistant ErbB-2-positive BC and GC.

## INTRODUCTION

Approximately 15–20% of breast cancers (BC) overexpress ErbB-2, a member of the ErbBs family of receptor tyrosine kinases, which also includes epidermal growth factor receptor/ ErbB-1, ErbB-3 and ErbB-4.<sup>1</sup> Until the development of ErbB-2-targeted therapies, ErbB-2-positive subtype was associated with increased metastatic potential and poor prognosis in BC patients with node-positive and -negative tumors.<sup>2</sup> Notably, the overall survival of metastatic patients and the cure rate in the adjuvant setting improved significantly after the development of such therapies.<sup>3</sup> Current therapeutic options for patients with ErbB-2-positive BC include monoclonal antibodies (trastuzumab (TZ) and pertuzumab), tyrosine kinase inhibitors (lapatinib) and TZ-DM1, an antibody–drug conjugate. Around 40–60% of patients with ErbB-2-positive metastatic BC do not respond to TZ, showing *de novo* or acquired resistance.<sup>3</sup> Although lapatinib, a dual epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, provides clinical benefit to a subset of patients progressing on TZ, <25% achieve an objective response, whereas most eventually develop lapatinib resistance.<sup>4</sup> Thus, resistance to available drugs is a mayor clinical issue in BC. ErbB-2 gene amplification and protein overexpression occur also in several tumor types, including gastric, bladder, cervix, colon, endometrium, germ cell, glioblastoma, head and neck, liver, lung, ovarian, pancreas, and salivary duct (reviewed in Yan *et al.*<sup>5</sup>). However, among all these cancer types, ErbB-2-targeted therapy has only been approved for gastric cancer (GC), currently targeted with TZ.<sup>6</sup>

MicroRNAs (miRNAs) are short non-coding endogenous RNAs with regulatory functions, whose abnormal expression in cancer is known to have a key role in growth, progression and resistance to classic therapies (reviewed in Berindan-Neagoe *et al.*<sup>7</sup>). Previous findings,

including our own, identified miR-16 as a potent tumor suppressor downregulated in various cancer types, such as BC and GC.<sup>8,9</sup> Our earlier discoveries demonstrated that progestins and ErbB's ligand heregulin downregulate miR-16, which inhibits BC growth induced by said mitogens.<sup>9</sup> MiR-16 reduced levels have also been implicated in resistance to radio-, chemo- and endocrine therapies in BC.<sup>10–12</sup> Interestingly, several miR-16 validated targets, such as cyclin D1, cyclin E1, B-cell lymphoma 2, survivin, vascular endothelial growth factor receptor 2, insulin-like growth factor 1 receptor and tumor necrosis factor alpha,<sup>9,13–16</sup> were found to participate in TZ resistance.<sup>17–20</sup>

The effects of ErbB-2 activation or of its blockade with TZ or lapatinib on the modulation of miRNAs remain poorly known. On the other hand, cancer progression in the metastatic setting raises a clinical challenge. In this framework, we explored the role of miR-16 in *in vitro* and *in vivo* ErbB-2-positive BC and GC growth. We revealed that miR-16 function as a tumor suppressor mediates TZ and lapatinib antiproliferative effects, and identified cyclin J (CCNJ) and far upstream element-binding protein 1 (FUBP1) as novel miR-16 targets. We disclosed miR-16 and FUBP1 clinical relevance in BC, and revealed miR-16 administration as a new therapy to overcome resistance to TZ and lapatinib in ErbB-2-positive BC and GC.

## RESULTS

### TZ induces miR-16 upregulation in ErbB-2-positive BC and GC sensitive to its antiproliferative effects

Our study of ErbB-2-positive and TZ-sensitive BT-474 and SKBR-3 human BC cells (Supplementary Figure S1a and O'Brien *et al.*<sup>21</sup>), revealed that TZ significantly increased miR-16 expression in both cell lines (Figure 1a). Knockdown of ErbB-2, which abolishes growth of said cells, led to a comparable increase in miR-16 levels (Supplementary Figure S1b shows results in BT-474 cells). Next, we established BT-474 tumor xenografts in nude mice and treated them with TZ or control IgG. Inhibition of tumor growth was associated with a ~ 3-fold increase of miR-16 levels in tumors from TZ-treated mice, compared with tumors from the IgG group (Figure 1b). We then assessed the effects of TZ in a panel of ErbB-2-positive BC cell lines with intrinsic TZ resistance<sup>21</sup> (JIMT-1, HCC-1569 and MDA-MB-453) and in a BT-474 HR clone we selected for its acquired TZ resistance (Supplementary Figure S1c). TZ failed to increase miR-16 expression in these cells (Figure 1c). In fact, TZ downregulated miR-16 in MDA-MB-453 cells (Figure 1c). Similar results were obtained using a preclinical model in which JIMT-1 cells were inoculated in nude mice and the developed tumors were treated with TZ. We found that TZ failure to inhibit tumor growth associated with its inability to upregulate miR-16 levels (Figure 1d).

TZ has also been approved for first-line treatment of ErbB-2-positive metastatic GC.<sup>6</sup> Thus, we assessed TZ effects on miR-16 levels in ErbB-2-positive GC cell lines NCI-N87 and SNU-I, sensitive and resistant to TZ, respectively (Supplementary Figure S1d and references Kim *et al.*<sup>22</sup> and Bae *et al.*<sup>23</sup>). In accordance with our results in BC, TZ upregulated miR-16 expression in NCI-N87 cells, but failed to modulate miR-16 levels in SNU-I cells (Figure 1e). Our findings *in vitro* and *in vivo* for the first time show that upregulation of miR-16 levels is a common mechanism of TZ action in ErbB-2-positive BC and GC sensitive to its antiproliferative effects.

We previously identified cyclin E1 as a relevant miR-16 target in progestin- and heregulin-mediated BC growth.<sup>9</sup> Cyclin E1 amplification/overexpression is associated with TZ resistance in BC.<sup>18</sup> Here, we found that *in vitro* and *in vivo* TZ treatment decreased cyclin E1 expression in sensitive BC cells (Supplementary Figure S1e), but not in the resistant cells (Supplementary Figure S1f).

### MiR-16 acts as a tumor suppressor in ErbB-2-positive BC and GC

We already demonstrated that miR-16 is a tumor suppressor in progestin-induced BC growth.<sup>9</sup> In the frame of our current findings, we here explored miR-16 role in ErbB-2-positive BC and GC growth. Overexpression of miR-16 by transfection with a precursor (pre-miR-16) inhibited proliferation of TZ- and of lapatinib-sensitive cells (Figure 2a and Supplementary Figure S2a). Moreover, miR-16 forced expression significantly decreased proliferation of cells with intrinsic or acquired TZ resistance (Figure 2b and Supplementary Figures S2a and b). Among these cells, HCC-1569, JIMT-1 and MDA-MB-453 are also lapatinib-resistant.<sup>21</sup> These data identify a completely novel role of miR-16 as a tumor suppressor in ErbB-2-positive BC, both sensitive and resistant to TZ and lapatinib. Furthermore, transfection with pre-miR-16 inhibited proliferation of NCI-N87 and SNU-I cells, extending our findings in BC to ErbB-2-positive GC (Figure 2c and Supplementary Figure S2c). Functional inhibition of miR-16 by transfection with an inhibitor (anti-miR-16), attenuated TZ antiproliferative effects in BT-474 and SKBR-3 cells, revealing the direct involvement of miR-16 in TZ action (Figure 2d).

In light of miR-16 inhibitory effects on the proliferation of cells unresponsive to anti-ErbB-2 therapies, we conducted a preclinical study where we established JIMT-1 tumor xenografts and, once tumors reached 100 mm<sup>3</sup>, animals were treated with TZ, received intratumoral pre-miR-16 injections or a combination of both. In order to define the protocol for miR-16 administration, we previously verified that a single injection resulted in miR-16 upregulation in tumor cells for at least 72 h post administration (Supplementary Figure S2d). Therefore, mice were treated with pre-miR-16 thrice a week. As described,<sup>24</sup> no significant differences were observed in either mean volumes or growth rates of tumors treated with TZ and those treated with IgG (Figures 2e and f). Contrastingly, volumes and growth rates of tumors injected with pre-miR-16 were significantly lower than those treated with TZ or IgG (Figures 2e and f). The combined administration of TZ and pre-miR-16 showed no further therapeutic benefit, compared with pre-miR-16 as a single agent (Figures 2e and f). MiR-16 levels in tumors injected with pre-miR-16 proved to be significantly upregulated at the end of the experiment, demonstrating the efficacy of our delivery strategy (Figure 2g). These results demonstrate that miR-16 inhibits tumor growth in TZ- and lapatinib-resistant BC. Our findings highlight miR-16 as a novel therapeutic agent in ErbB-2-positive BC and GC sensitive and resistant to ErbB-2-targeted therapies.

### C-Myc mediates TZ effects on miR-16 expression

The oncogenic transcription factor c-Myc has already been found to repress miR-16 expression.<sup>25</sup> Our own findings revealed that progestins induce BC growth via c-Myc downregulation of miR-16 levels.<sup>9</sup> Therefore, we explored whether c-Myc is involved in TZ-induced miR-16 upregulation. Knockdown of c-Myc significantly upregulated miR-16 and

inhibited proliferation in TZ-sensitive and -resistant cells (Figure 3a). On the other hand, TZ inhibited c-Myc expression in BT-474 cells, and restoration of c-Myc levels by transfection of a constitutively active c-Myc variant<sup>26</sup> abolished TZ ability to upregulate miR-16 levels (Figure 3b). This highlights c-Myc function as mediator of TZ-induced miR-16 upregulation. We then studied the mechanism of TZ regulation of c-Myc expression. ErbB-2 activation of extracellular signal-regulated kinases 1/2 (Erk1/2) and phosphatidylinositol-3 kinase (PI3K)/AKT cascades mediates its proliferative effects.<sup>1</sup> Both cascades have also been found to control the levels and transcriptional activity of c-Myc.<sup>27</sup> Moreover, 4D5, the parental murine version of TZ, was found to block said signaling pathways and to downregulate c-Myc expression in BT-474 cells.<sup>28</sup> Consistent with TZ inhibition of Erk1/2 and PI3K/AKT pathways,<sup>1</sup> we found that TZ significantly downregulated c-Myc, *in vitro* and *in vivo*, in TZ-sensitive cells (Figure 3c). Similar results were obtained upon ErbB-2 silencing in BT-474 cells (Figure 3d). On the contrary, we found that TZ inability to inhibit these pathways in BC cells with intrinsic or acquired resistance is associated with TZ failure to modulate c-Myc expression (Figure 3e). Our findings in GC cell lines were comparable to those obtained in BC cells (Figure 3f). In BT-474 cells, inhibition of PI3K activation with LY294002, or of Erk1/2 with the MEK1/2 inhibitor U0126, resulted in c-Myc downregulation, miR-16 upregulation and reduced cell proliferation (Figure 3g). These inhibitors also induced c-Myc downregulation, enhanced miR-16 expression and reduced cell proliferation in cell lines with intrinsic or acquired TZ resistance (Figure 3g). Our findings reveal that TZ failure to induce miR-16 upregulation in resistant BC lies on its inability to block PI3K/AKT and Erk1/2 pathways activation and, consequently, c-Myc expression.

Lapatinib reversibly inhibits ErbB-2 auto-phosphorylation and thereby inhibits the PI3K/AKT and Erk1/2 pathways, which underlies its growth inhibitory effects.<sup>1</sup> We found that in addition to inhibiting said pathways, lapatinib downregulated c-Myc and increased miR-16 levels in BT-474 and SKBR-3 cells, which are sensitive to its antiproliferative effects (Figure 3g and Supplementary Figures S3a and b). Inhibition of miR-16 function with anti-miR-16 reduced lapatinib effects in BT-474 cells, demonstrating miR-16 involvement in its therapeutic effects (Supplementary Figure S3c). As described for another BT-474 TZ-resistant clone,<sup>21</sup> lapatinib inhibited proliferation in our BT-474 HR clone, where it also abolished Erk1/2 and PI3K/AKT activation, reduced c-Myc and increased miR-16 levels (Figure 3g). On the contrary, and consistent with its inability to block Erk1/2 and PI3K/AKT signaling, lapatinib failed to alter c-Myc or miR-16 expression in lapatinib-resistant cells (Supplementary Figures S3d and S3e). JIMT-1 cells are resistant to lapatinib when used at a concentration of 1  $\mu$ M, but higher concentrations block their proliferation (Figure 3g and Koninki *et al.*<sup>29</sup>). We found that lapatinib growth inhibitory effects at 5  $\mu$ M are associated with the blockade of Erk1/2 and PI3K/AKT activation, downregulation of c-Myc and upregulation of miR-16 (Figure 3g). Our findings reveal that two different anti-ErbB-2 therapies share a common downstream growth inhibitory mechanism in ErbB-2-positive BC, where Erk1/2 and PI3K/AKT blockade results in c-Myc downregulation and the consequent enhancement of miR-16 expression.

### **TZ remodels the local chromatin architecture at the *DLEU2* promoter in BC cells sensitive to its growth inhibitory effects**

C-Myc represses miR-16 expression by binding to its response element (E-Box) at the proximal promoter of deleted in lymphocytic leukemia 2 (*DLEU2*), miR-16 host gene, and recruiting chromatin remodeling machinery to this site.<sup>30</sup> To explore TZ effects on the *DLEU2* promoter, we performed chromatin immunoprecipitation assays using primers spanning the E-Box located 510-bp upstream from exon 1A.<sup>9</sup> TZ treatment of BT-474 cells evicted c-Myc from the E-Box (Figure 4a). Moreover, analysis of the local chromatin architecture revealed a decrease in histone H3 trimethylation on lysine 9 (H3K9me3), a hallmark of transcriptional repression (Figure 4a). TZ simultaneously increased the levels of histone H4 acetylation (H4Ac) (Figure 4a), which, as reported by us and others, represents a mark of *DLEU2* promoter activation.<sup>9,30</sup> Contrastingly, TZ was unable to displace c-Myc from the *DLEU2* promoter in cells with intrinsic TZ resistance, and failed to alter H3K9me3 or H4Ac levels at this site (Figure 4b). C-Myc transcriptional activity is highly dependent on its phosphorylation at serine 62 (S62) by the Erk1/2 pathway.<sup>27</sup> We found that TZ rapid blockade of Erk1/2 activity significantly inhibits c-Myc S62 phosphorylation in BT-474 cells (Figure 4c). Consistent with its inability to suppress Erk1/2 activation, TZ had no effects on c-Myc phosphorylation in JIMT-1 cells (Figure 4c). These results provide a mechanistic explanation for TZ-induced eviction of c-Myc from the *DLEU2* promoter in sensitive cells. To further characterize TZ effects on chromatin architecture at the site, we assessed chromatin accessibility by performing DNase I sensitivity assays. In BT-474 cells, we found that TZ augmented DNase sensitivity at the E-Box, consistent with a more accessible chromatin environment (Figure 4d). TZ had no effects on DNase sensitivity at this site in resistant cells (Figure 4d). Our results indicate that in sensitive cells, TZ regulates miR-16 at the transcriptional level by evicting c-Myc from the *DLEU2* promoter and enhancing local chromatin accessibility.

### **CCNJ and FUBP1 constitute novel miR-16 targets in ErbB-2-positive BC**

To identify miR-16 targets responsible for its antiproliferative effects in ErbB-2-positive BC, we used ErbB-2-overexpressing C4HD murine BC cells, where we previously found that miR-16 acts as tumor suppressor.<sup>9</sup> We transfected primary cultures of C4HD tumors with pre-miR-16 or pre-miR-Control and studied their gene expression profile. Mir-16 overexpression resulted in 444 and 999 genes significantly downregulated and upregulated, (adjusted *P*-value<0.01 and absolute fold change >1.9), respectively (Supplementary Figures S4a and b and Supplementary Table S1). The gene expression profile was combined with target prediction algorithms for murine miR-16 (mmu-miR-16). To this end, we retrieved from miRecords<sup>31</sup> a list of mmi-miR-16 putative targets, predicted by at least four different algorithms (Supplementary Table S2). Among these, eight candidate genes were found to be significantly downregulated in the expression profile of pre-miR-16-transfected cells (Supplementary Table S3). The list included two previously validated miR-16 targets, growth hormone receptor<sup>32</sup> and caudal type homeobox 2,<sup>33</sup> whose involvement in BC has been described,<sup>34,35</sup> and two unreported targets, CCNJ and FUBP1, whose role in BC remains almost completely unexplored.<sup>36,37</sup> The latter two proved to be significantly downregulated upon validation of microarray data (Supplementary Figure S4c). Therefore, we focused our study on CCNJ and FUBP1 roles as miR-16 targets in ErbB-2-positive BC.



Since our expression profile studies were performed in a murine model, we verified sequence complementarity between human miR-16 (hsa-miR-16) and CCNJ or FUBP1 mRNAs. We found complementarity between hsa-miR-16 seed sequence and one site at the 3'UTR of each mRNA (positions 1834–1840 of CCNJ 3'UTR and 707–713 of FUBP1 3'UTR) (Figure 5a). MiR-16 overexpression reduced CCNJ and FUBP1 mRNA levels in human BC and GC cells (Figure 5b), and protein expression in *in vitro* and *in vivo* BC models (Figure 5c). Abrogation of miR-16 function augmented CCNJ and FUBP1 protein levels in TZ-sensitive and -resistant cells (Figure 5d). To assess whether CCNJ and FUBP1 are direct miR-16 targets, we performed reporter gene assays using constructs carrying the wild type 3'UTR sequence of CCNJ (luc-3'CCNJ-WT) or FUBP1 (luc-3'FUBP1-WT), cloned downstream of a luciferase gene. Transfection with pre-miR-16 reduced luciferase activity from both constructs, compared with pre-miR-control-transfected cells (Figure 5e). However, upon site directed mutagenesis of the putative miR-16 binding sites in these constructs (Figure 5a), miR-16 lost the capacity to downregulate luciferase activity (Figure 5e). Moreover, blockade of miR-16 function in cells transfected with luc-3'CCNJ-WT or luc-3'FUBP1-WT, resulted in enhanced luciferase activity, compared with anti-miR-Control-transfected cells (Figure 5f). Our findings identify CCNJ and FUBP1 as novel direct miR-16 targets in ErbB-2-positive BC.

Next, we explored whether TZ-induced miR-16 upregulation altered the expression of its targets. TZ decreased CCNJ and FUBP1 mRNA and protein levels, *in vitro* and *in vivo*, in sensitive cells (Figures 6a and b), but was unable to alter their expression in BC cells with intrinsic or acquired resistance (Figures 6c and d). Similar results were obtained in GC cells (Figure 6e). Similarly, lapatinib induced CCNJ and FUBP1 downregulation only in BC cells sensitive to its antiproliferative effects (Figures 6f and g). We next assessed whether TZ and lapatinib-induced downregulation of CCNJ and FUBP1 were mediated by miR-16. In BT-474 cells transfected with luc-3'CCNJ-WT or luc-3'FUBP1-WT, both TZ and lapatinib inhibited luciferase activity, compared with untreated cells (Figure 6h). Notwithstanding, these effects were completely lost when miR-16 binding sites in these constructs were mutated (Figure 6h), demonstrating that TZ and lapatinib inhibit CCNJ and FUBP1 expression in sensitive cells through miR-16 upregulation.

### CCNJ and FUBP1 are required for ErbB-2-positive BC growth

Next, we explored CCNJ and FUBP1 roles in ErbB-2-positive BC. CCNJ was previously implicated in the G2/M transition in MCF-7 BC cells,<sup>37</sup> whereas abrogation of FUBP1 was linked to cell proliferation inhibition in different cancer types (reviewed in Zhang *et al.*<sup>38</sup>). Here, we found that CCNJ or FUBP1 abrogation resulted in reduced cell proliferation in BC cells sensitive and resistant to ErbB-2-targeted therapies (Figure 7a). These results support CCNJ and FUBP1 roles as mediators of miR-16 anti-proliferative effects in ErbB-2-positive BC.

FUBP1 was originally described as a transcription factor promoting c-Myc expression through its binding to a far upstream element at the *MYC* promoter.<sup>38</sup> Our chromatin immunoprecipitation assays revealed that FUBP1 is recruited to this site in BT-474 cells (Figure 7b). Moreover, analysis of the local chromatin architecture showed high levels of

H4Ac, a mark of transcriptional activation (Figure 7b). In accordance with previous studies using FUBP1 dominant-negative variants,<sup>39</sup> FUBP1 silencing resulted in c-Myc downregulation (Figure 7c). Consistent with c-Myc role as a miR-16 repressor, c-Myc inhibition was accompanied by an increase in miR-16 levels (Figure 7d) and the consequent downregulation of CCNJ (Figure 7c). Our results unveil a positive feedback loop driving ErbB-2-positive BC growth, where c-Myc inhibition of miR-16 expression increases the levels of FUBP1, which in turn binds to a far upstream element at the *MYC* promoter and enhances c-Myc expression, resulting in further miR-16 downregulation (Figure 7e).

### MiR-16 and FUBP1 potential role as predictors of TZ response

MiRNAs are aberrantly expressed in BC and have been proposed as biomarkers in the clinic.<sup>7</sup> We found that miR-16 levels were higher in TZ-sensitive BT-474 and SKBR-3 cells than in a panel of cells with intrinsic or acquired resistance (Figure 8a). Then, we explored the clinical significance of miR-16 levels in the response to adjuvant therapy with TZ in a cohort of 19 primary ErbB-2-positive BC tumors (Supplementary Table S4). MiR-16 levels were assessed in extracts from formalin-fixed paraffin-embedded (FFPE) tumor samples. We found concordance when comparing miR-16 levels from different sections of the same tumors (data not shown), suggesting that tumor heterogeneity did not substantially influence our observations. MiR-16 levels were significantly higher in responsive patients than in those who relapsed after TZ treatment (Figure 8b). RNA integrity from FFPE samples can be affected by fixation and storage conditions.<sup>40</sup> Although long RNAs, such as mRNAs, are particularly vulnerable, we were able to measure CCNJ and FUBP1 mRNA levels in half of the tumor samples from our cohort and found an inverse correlation between miR-16 levels and those of its targets (Figure 8c). We validated these findings in data sets from two publicly available independent cohorts of ErbB-2-positive BC primary tumors (GEO accession numbers: GSE75685 and GSE19783)<sup>41</sup> (Figure 8d).

The incorporation of miRNA-based predictors may not yet be suitable for routine clinical practice. Thus, we studied FUBP1 nuclear expression by immunohistochemistry, as a surrogate of miR-16 levels, in a cohort of 42 ErbB-2-positive BCs treated with TZ in the adjuvant setting (Supplementary Table S5), and independent from our first cohort of 19 patients. FUBP1 immunohistochemistry was scored with a staining index, where an index of 4 was considered positive (Supplementary Figure S5a and Zhang *et al.*<sup>42</sup>). Kaplan–Meier analysis revealed that patients bearing FUBP1-positive tumors exhibited significantly shorter disease-free survival (log-rank test,  $P = 0.012$ ) than those with FUBP1-negative tumors (Figures 8e and f). FUBP1 expression showed no correlation with other clinical parameters, such as tumor grade or stage (Supplementary Table S6). Our results show that high expression levels of miR-16 and, accordingly, low levels of FUBP1, its downstream target, correlate with response to TZ in ErbB-2-positive BC, highlighting their potential role as biomarkers of therapeutic response.

## DISCUSSION

We revealed a novel mechanism driving ErbB-2-positive BC growth, where ErbB-2 stimulation of Erk1/2 and PI3K/AKT cascades induces c-Myc transcriptional activation via



its rapid phosphorylation at S62, as well as c-Myc expression. Activated c-Myc then binds to the *DLEU2* promoter and represses miR-16 expression. This results in increased levels of two newly identified miR-16 targets, FUBP1 and CCNJ, both of which promote proliferation. Notably, FUBP1 in turn binds to the *MYC* promoter and enhances c-Myc expression, which further represses miR-16, resulting in a positive feedback loop that stimulates growth. We found that TZ and lapatinib capacity to inhibit BC growth lies on their common ability to block Erk1/2 and PI3K/AKT. This inhibits c-Myc activation, which leads to upregulation of miR-16 and decrease of FUBP1 and CCNJ. We validated our findings in ErbB-2-positive GC. Our discoveries are depicted in Figure 8g.

Multiple mechanisms cause TZ resistance in BC, such as expression of drug-resistant ErbB-2 isoforms, presence of ligand-induced ErbB-2/ErbB-3 dimers and ErbB-2/insulin-like growth factor 1 receptor dimerization (reviewed in Spector *et al.*<sup>20</sup>). Hyperactivation of PI3K/AKT is the best-acknowledged alteration in downstream signaling associated to TZ resistance. It also has a role in the lack of response to lapatinib, through a mechanism involving FoxO3a-dependent ErbB-3 upregulation and the activation of the latter by residual ErbB-2 activity.<sup>43</sup> Our experimental models display many of the mentioned TZ resistance features, and several show lapatinib resistance. Notably, PI3K/AKT hyperactivation is present in all of them.<sup>21</sup> In particular, HCC-1419 cells express a constitutively active truncated ErbB-2, which correlates with lack of TZ effect, and also show reduced phosphatase and tensin homolog expression.<sup>21,44</sup> Increased epidermal growth factor receptor expression and phosphorylation, high levels of ErbB ligands, and cyclin E overexpression have been identified as mechanisms of acquired TZ resistance in BT-474 cells.<sup>18,45</sup> HCC-1569 cells show low phosphatase and tensin homolog expression and elevated levels of anti-apoptotic proteins, such as B-cell lymphoma 2.<sup>21,46</sup> MDA-MB-453 cells present ErbB-3-induced compensating signaling, an activating mutation in the PIK3CA gene, and low phosphatase and tensin homolog expression.<sup>21,47</sup> Finally, JIMT-1 cells present multiple molecular mechanisms underlying resistance to anti-ErbB-2 therapies, including an activating mutation of the PIK3CA gene, low expression of phosphatase and tensin homolog, and enhanced heregulin levels.<sup>29</sup> On the other hand, different reports have identified Erk1/2 aberrant activation as a major factor underlying lapatinib resistance.<sup>48–50</sup> Moreover, Erk1/2 activation as a compensatory mechanism, downstream of the tyrosine kinase EphA2 (EPH receptor A2), has been implicated in the development of acquired TZ resistance.<sup>51</sup> Here, we found that TZ and lapatinib failure to abrogate PI3K/AKT and Erk1/2 persistent activation in resistant cells, due precisely to the particular molecular traits present in these cells, results in sustained c-Myc activation and expression and, consequently, in the inability of both agents to upregulate miR-16. We also disclosed a novel mechanism that explains the capacity of high concentrations of tyrosine kinase inhibitors, which accomplish near total inhibition of ErbB-2/ErbB-3 activity, to abrogate lapatinib resistance in BC cells.<sup>52</sup> We found that a high dose of lapatinib, which abolishes Erk1/2 and PI3K/AKT activation, or specific inhibitors of said pathways, result in blockade of c-Myc activation with the consequent increase of miR-16, which in turn acts as a suppressor of lapatinib-resistant BC growth. In addition, our results with TZ-resistant cells in which lapatinib upregulated miR-16 and inhibited proliferation, provide a mechanistic insight into the benefit from lapatinib seen in patients with TZ-refractory disease.

Our findings revealed miR-16 as a novel miRNA regulated by TZ in BC and GC, and by lapatinib in BC. Ours is the first study to dissect the molecular mechanisms underlying anti-ErbB-2 therapy-induced regulation of any miRNA in BC, and the first report on miRNA regulation by TZ in GC. ErbB-2 drives the expression of miRNAs, which account for several of its effects (reviewed in Wang *et al.*<sup>53</sup>). Furthermore, differential sets of miRNAs have been found to be modulated in ErbB-2-positive BC and GC, compared with other subtypes.<sup>54</sup> The mechanisms underlying miRNAs modulation by ErbB-2 remain poorly explored. In GC, ErbB-2 was found to downregulate miR-139 via epigenetic silencing.<sup>55</sup> In BC, ErbB-2 upregulates miR-23b/27b expression through PI3K/AKT,<sup>56</sup> and miR-21 via Erk1/2.<sup>57</sup> Our own findings demonstrated that ErbB-2 nuclear function as a coactivator of Stat3 and as a transcription factor are mandatory for miR-21 upregulation.<sup>58</sup> Interestingly, several of the tumor types which, in addition to BC and GC, show ErbB-2 gene amplification or protein overexpression, display low levels of miR-16.<sup>59–65</sup> Given that miR-16 was found to act as a tumor suppressor in these cancers,<sup>59–65</sup> our findings raise the exciting possibility that ErbB-2 inhibition of miR-16 expression may be a common mechanism underlying ErbB-2-driven cancer growth. TZ has been found to regulate the expression of other miRNAs, which mediate its antiproliferative effects in BC.<sup>66,67</sup> Forced expression of miRNAs downregulated in TZ-resistant BC, restores sensitivity to TZ and suppresses growth and metastasis (reviewed in Wang *et al.*<sup>53</sup>). Similarly, miRNAs modulate TZ sensitivity in GC by downregulating genes such as ErbB-2 itself.<sup>68</sup>

c-Myc roles as effector of ErbB-2 and mediator of TZ response have been acknowledged (reviewed in Xu *et al.*<sup>69</sup>). Although preliminary reports from a BC clinical trial identified c-Myc/ErbB-2 co-amplification as a marker of response to adjuvant TZ, other studies did not find correlation between c-Myc amplification and additional TZ benefit (reviewed in Esteva *et al.*<sup>4</sup>). Here, we unraveled a new level of the ErbB-2/c-Myc interaction in BC. Our assessment of the mechanisms through which ErbB-2 controls miR-16 highlighted that c-Myc, via its role as transcriptional repressor, is a key factor mediating ErbB-2 growth effects and the response to TZ and lapatinib. Recent findings showed that TZ also inhibits the expression of c-Myc-induced genes in BC by inducing c-Myc association with a transcriptional corepressor.<sup>70</sup>

Our gene expression profile in ErbB-2-positive BC identified CCNJ and FUBP1 as novel miR-16 targets. We also discovered their downregulation contributes to miR-16 tumor-suppressor effects. Although CCNJ is a poorly characterized gene in mammals, it may have a role in BC.<sup>37</sup> FUBP1 is a well-acknowledged transcription factor and RNA-binding protein that regulates transcription and translation of various genes (reviewed in Zhang *et al.*<sup>38</sup>). Here, we revealed a positive feedback loop among miR-16, FUBP1 and c-Myc in BC. This class of regulatory architecture has been reported for other miRNAs in different cancer types,<sup>71–73</sup> suggesting that it may be a common trait enhancing/inhibiting miRNAs expression and function.

Our clinical findings revealed an inverse correlation between miR-16 and CCNJ or FUBP1 mRNA levels in ErbB-2-positive BC. In light of the major clinical issue of response to anti-ErbB-2 therapies in the adjuvant setting, and considering that ErbB-2 expression remains the only marker for patient selection in the neoadjuvant setting,<sup>74</sup> there is urgent need to identify

novel response markers. Our findings uncovered miR-16 and FUBP1 as potential novel predictive biomarkers, likely to succeed in the clinic since they are actual mediators of TZ and lapatinib effects. Finally, we demonstrated, using *in vitro* and preclinical models, that miR-16 is a strong tumor suppressor in ErbB-2-positive BC and GC. A phase I clinical trial assessing miR-16 therapeutic potential in malignant pleural mesothelioma is underway, with promising preliminary results.<sup>75</sup> MiR-16 ability to target multiple ErbB-2 downstream effectors, including those known to mediate TZ resistance, such as cyclin E and those identified here, turns it into a potential novel therapy for BC and GC sensitive and resistant to ErbB-2-targeted therapies.

## MATERIALS AND METHODS

### Reagents

Antibodies and reagents are listed under Supplementary Materials and methods.

### Cell lines

BT-474, SKBR-3, NCI-N87 and SNU-I cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and JIMT-1 cells from the German Resource Center for Biological Material (Braunschweig, Germany). MDA-MB-453, HCC-1419 and HCC-1519 cells were a gift from DJ Slamon (University of California, Los Angeles, CA, USA). Cells were maintained as described<sup>24</sup> and routinely tested for mycoplasma contamination. Cell lines were authenticated by short tandem repeat profiling. Experiments were performed in complete media.

### Transient transfections

Plasmids, small interfering RNAs and miRNA precursors or inhibitors are detailed in Supplementary Materials and methods. Transfections were performed as described.<sup>58</sup>

### RNA preparation and RT–qPCR

RNA was isolated from cells using the miRVANA PARIS Purification Kit (Ambion, Austin, TX, USA). RNA was extracted from FFPE samples using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion). Reverse transcriptase (RT)–quantitative PCR (qPCR) was performed as described.<sup>58</sup> Details are provided in Supplementary Materials and methods.

### Microarray samples

RNA was extracted from six independent primary cultures of epithelial cells from C4HD tumors,<sup>9</sup> transfected for 48 h with 30 nM pre-miR-16 or pre-miR-Control (Ambion). A detailed protocol is provided under Supplementary Materials and methods. Data were deposited into Gene Expression Omnibus<sup>76</sup> (GSE73900).

### Preclinical models

Experiments were approved by the IBYME Animal Research Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were

randomly assigned to treatment groups using a parallel groups design. Detailed protocols are provided in Supplementary Materials and methods.

## Patients

FFPE tissue samples from ErbB-2-positive invasive BC patients who received adjuvant TZ were selected from the files of Hospital de Temuco (Chile), Hospital 'Juan A. Fernández' and Hospital Aeronáutico Central (Argentina). Informed consents were obtained from all patients. Details are provided in Supplementary Materials and methods.

## Immunohistochemistry

Immunohistochemistry was performed as described.<sup>58</sup> The anti-FUBP1 antibody was validated by immunofluorescence and confocal microscopy (Supplementary Figure S5b). Details are provided in Supplementary Materials and methods.

## Statistics

When two groups were compared, the two-tailed Student's *t*-test was used. When three or more groups were compared, the one-way analysis of variance test was used. All tests were two-sided. Details are provided in Supplementary Materials and methods.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank AA Molinolo (UCSD, San Diego, CA, USA) for his constant help. This work was supported by KG090250 investigator-initiated research grant from Susan G Komen for the Cure, Financial Assistance for National Research Projects from the National Institute of Cancer (INC, Argentina), IDB/PICT 2012-668 and PID 2012-066 from National Agency of Scientific Promotion of Argentina (ANPCyT), awarded to PVE; IDB/PICT 2012-382 from ANPCyT, awarded to RS; Oncomed-Reno CONICET 1819/03, from Henry Moore Institute of Argentina, awarded to PVE and RS; IDB/PICT 2008-189 and 2012-1017 from ANPCyT, PIP 59 from CONICET, awarded to CJP.

## References

1. Arteaga CL, Engelman JA. ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics. *Cancer Cell*. 2014; 25:282–303. [PubMed: 24651011]
2. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987; 235:177–182. [PubMed: 3798106]
3. Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN. The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. *Oncologist*. 2009; 14:320–368. [PubMed: 19346299]
4. Esteva FJ, Yu D, Hung MC, Hortobagyi GN. Molecular predictors of response to trastuzumab and lapatinib in breast cancer. *Nat Rev Clin Oncol*. 2010; 7:98–107. [PubMed: 20027191]
5. Yan M, Parker BA, Schwab R, Kurzrock R. HER2 aberrations in cancer: implications for therapy. *Cancer Treat Rev*. 2014; 40:770–780. [PubMed: 24656976]
6. Gomez-Martin C, Lopez-Rios F, Aparicio J, Barriuso J, Garcia-Carbonero R, Pazo R, et al. A critical review of HER2-positive gastric cancer evaluation and treatment: from trastuzumab, and beyond. *Cancer Lett*. 2014; 351:30–40. [PubMed: 24943493]

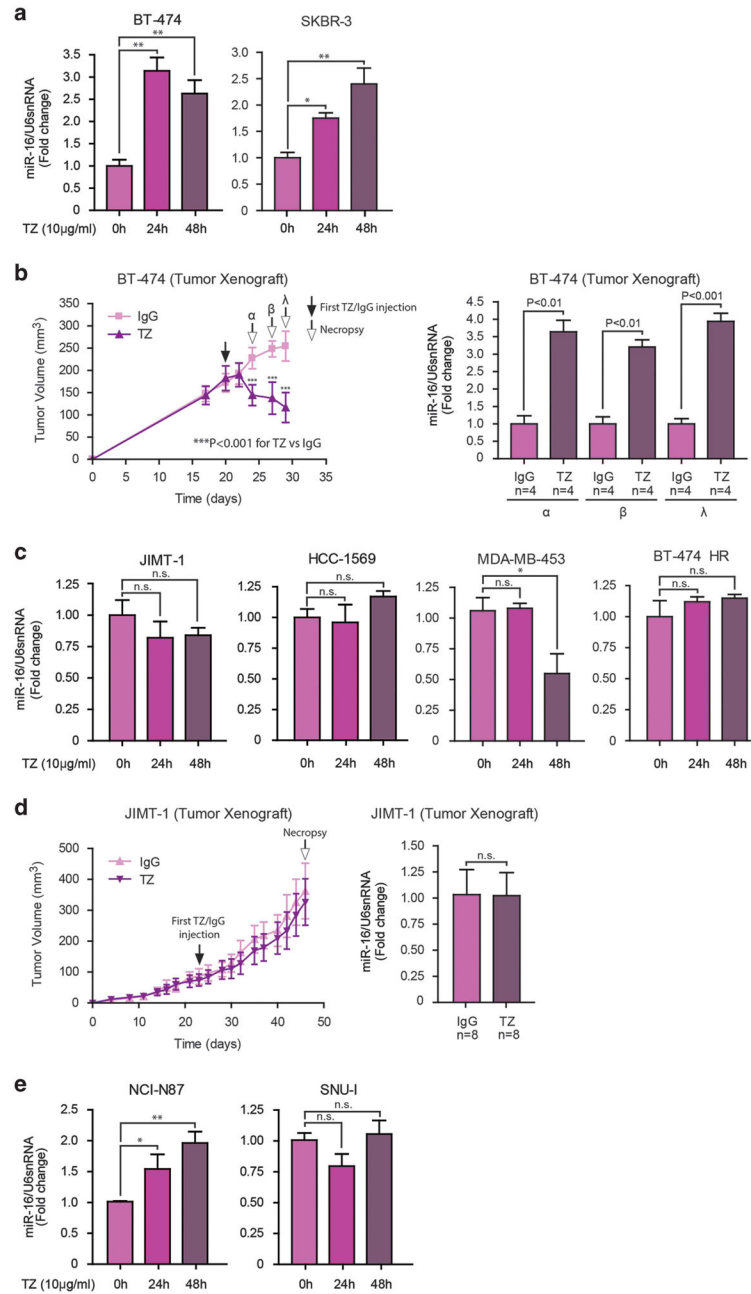
7. Berindan-Neagoe I, Monroig PC, Pasculli B, Calin GA. MicroRNAome genome: a treasure for cancer diagnosis and therapy. *CA Cancer J Clin.* 2014; 64:311–336. [PubMed: 25104502]
8. Kang W, Tong JH, Lung RW, Dong Y, Zhao J, Liang Q, et al. Targeting of YAP1 by microRNA-15a and microRNA-16-1 exerts tumor suppressor function in gastric adenocarcinoma. *Mol Cancer.* 2015; 14:52. [PubMed: 25743273]
9. Rivas MA, Venturutti L, Huang YW, Schillaci R, Huang TH, Elizalde PV. Downregulation of the tumor-suppressor miR-16 via progesterin-mediated oncogenic signaling contributes to breast cancer development. *Breast Cancer Res.* 2012; 14:R77. [PubMed: 22583478]
10. Cittelly DM, Das PM, Salvo VA, Fonseca JP, Burrow ME, Jones FE. Oncogenic HER2 {Delta}16 suppresses miR-15a/16 and deregulates BCL-2 to promote endocrine resistance of breast tumors. *Carcinogenesis.* 2010; 31:2049–2057. [PubMed: 20876285]
11. Kastl L, Brown I, Schofield AC. miRNA-34a is associated with docetaxel resistance in human breast cancer cells. *Breast Cancer Res Treat.* 2012; 131:445–454. [PubMed: 21399894]
12. Mei Z, Su T, Ye J, Yang C, Zhang S, Xie C. The miR-15 family enhances the radiosensitivity of breast cancer cells by targeting G2 checkpoints. *Radiat Res.* 2015; 183:196–207. [PubMed: 25594541]
13. Chamorro-Jorganes A, Araldi E, Penalva LO, Sandhu D, Fernandez-Hernando C, Suarez Y. MicroRNA-16 and microRNA-424 regulate cell-autonomous angiogenic functions in endothelial cells via targeting vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1. *Arterioscler Thromb Vasc Biol.* 2011; 31:2595–2606. [PubMed: 21885851]
14. Chen L, Wang Q, Wang GD, Wang HS, Huang Y, Liu XM, et al. miR-16 inhibits cell proliferation by targeting IGF1R and the Raf1-MEK1/2-ERK1/2 pathway in osteosarcoma. *FEBS Lett.* 2013; 587:1366–1372. [PubMed: 23507142]
15. Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, et al. Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell.* 2005; 120:623–634. [PubMed: 15766526]
16. Yu X, Zhang X, Dhakal IB, Beggs M, Kadlubar S, Luo D. Induction of cell proliferation and survival genes by estradiol-repressed microRNAs in breast cancer cells. *BMC Cancer.* 2012; 12:29. [PubMed: 22260523]
17. Rivas MA, Tkach M, Beguelin W, Proietti CJ, Rosemblyt C, Charreau EH, et al. Transactivation of ErbB-2 induced by tumor necrosis factor alpha promotes NF-kappaB activation and breast cancer cell proliferation. *Breast Cancer Res Treat.* 2010; 122:111–124. [PubMed: 19760502]
18. Scaltriti M, Eichhorn PJ, Cortes J, Prudkin L, Aura C, Jimenez J, et al. Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. *Proc Natl Acad Sci USA.* 2011; 108:3761–3766. [PubMed: 21321214]
19. Valabrega G, Capellero S, Cavalloni G, Zaccarello G, Petrelli A, Migliardi G, et al. HER2-positive breast cancer cells resistant to trastuzumab and lapatinib lose reliance upon HER2 and are sensitive to the multitargeted kinase inhibitor sorafenib. *Breast Cancer Res Treat.* 2011; 130:29–40. [PubMed: 21153051]
20. Spector NL, Blackwell KL. Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer. *J Clin Oncol.* 2009; 27:5838–5847. [PubMed: 19884552]
21. O'Brien NA, Browne BC, Chow L, Wang Y, Ginther C, Arboleda J, et al. Activated phosphoinositide 3-kinase/AKT signaling confers resistance to trastuzumab but not lapatinib. *Mol Cancer Ther.* 2010; 9:1489–1502. [PubMed: 20501798]
22. Kim SY, Kim HP, Kim YJ, Oh dY, Im SA, Lee D, et al. Trastuzumab inhibits the growth of human gastric cancer cell lines with HER2 amplification synergistically with cisplatin. *Int J Oncol.* 2008; 32:89–95. [PubMed: 18097546]
23. Bae CD, Juhnn YS, Park JB. Post-transcriptional control of c-erb B-2 overexpression in stomach cancer cells. *Exp Mol Med.* 2001; 33:15–19. [PubMed: 11322480]
24. Cordo Russo RI, Beguelin W, Diaz Flaque MC, Proietti C, Venturutti L, Galigniana NM, et al. Targeting ErbB-2 nuclear localization and function inhibits breast cancer growth and overcomes trastuzumab resistance. *Oncogene.* 2015; 34:3413–3428. [PubMed: 25174405]
25. Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet.* 2008; 40:43–50. [PubMed: 18066065]

26. Kapeli K, Hurlin PJ. Differential regulation of N-Myc and c-Myc synthesis, degradation, and transcriptional activity by the Ras/mitogen-activated protein kinase pathway. *J Biol Chem.* 2011; 286:38498–38508. [PubMed: 21908617]
27. Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes Dev.* 2000; 14:2501–2514. [PubMed: 11018017]
28. Motoyama AB, Hynes NE, Lane HA. The efficacy of ErbB receptor-targeted anticancer therapeutics is influenced by the availability of epidermal growth factor-related peptides. *Cancer Res.* 2002; 62:3151–3158. [PubMed: 12036928]
29. Koninki K, Barok M, Tanner M, Staff S, Pitkanen J, Hemmila P, et al. Multiple molecular mechanisms underlying trastuzumab and lapatinib resistance in JIMT-1 breast cancer cells. *Cancer Lett.* 2010; 294:211–219. [PubMed: 20193978]
30. Zhang X, Chen X, Lin J, Lwin T, Wright G, Moscinski LC, et al. Myc represses miR-15a/miR-16-1 expression through recruitment of HDAC3 in mantle cell and other non-Hodgkin B-cell lymphomas. *Oncogene.* 2012; 31:3002–3008. [PubMed: 22002311]
31. Xiao F, Zuo Z, Cai G, Kang S, Gao X, Li T. miRecords: an integrated resource for microRNA-target interactions. *Nucleic Acids Res.* 2009; 37:D105–D110. [PubMed: 18996891]
32. Elzein S, Goodyer CG. Regulation of human growth hormone receptor expression by microRNAs. *Mol Endocrinol.* 2014; 28:1448–1459. [PubMed: 25073105]
33. Tagawa T, Haraguchi T, Hiramatsu H, Kobayashi K, Sakurai K, Inada K, et al. Multiple microRNAs induced by Cdx1 suppress Cdx2 in human colorectal tumour cells. *Biochem J.* 2012; 447:449–455. [PubMed: 22849325]
34. Stajduhar E, Sedic M, Lenicek T, Radulovic P, Kerenji A, Kruslin B, et al. Expression of growth hormone receptor, plakoglobin and NEDD9 protein in association with tumour progression and metastasis in human breast cancer. *Tumour Biol.* 2014; 35:6425–6434. [PubMed: 24676793]
35. Bhat-Nakshatri P, Goswami CP, Badve S, Sledge GW Jr, Nakshatri H. Identification of FDA-approved drugs targeting breast cancer stem cells along with biomarkers of sensitivity. *Sci Rep.* 2013; 3:2530. [PubMed: 23982413]
36. Jang M, Park BC, Kang S, Chi SW, Cho S, Chung SJ, et al. Far upstream element-binding protein-1, a novel caspase substrate, acts as a cross-talker between apoptosis and the c-myc oncogene. *Oncogene.* 2009; 28:1529–1536. [PubMed: 19219071]
37. Feliciano A, Castellvi J, Artero-Castro A, Leal JA, Romagosa C, Hernandez-Losa J, et al. miR-125b acts as a tumor suppressor in breast tumorigenesis via its novel direct targets ENPEP, CK2-alpha, CCNJ, and MEGF9. *PLoS One.* 2013; 8:e76247. [PubMed: 24098452]
38. Zhang J, Chen QM. Far upstream element binding protein 1: a commander of transcription, translation and beyond. *Oncogene.* 2013; 32:2907–2916. [PubMed: 22926519]
39. He L, Liu J, Collins I, Sanford S, O'Connell B, Benham CJ, et al. Loss of FBP function arrests cellular proliferation and extinguishes c-myc expression. *EMBO J.* 2000; 19:1034–1044. [PubMed: 10698944]
40. von Ahlfen S, Missel A, Bendrat K, Schlumpberger M. Determinants of RNA quality from FFPE samples. *PLoS One.* 2007; 2:e1261. [PubMed: 18060057]
41. Enerly E, Steinfeld I, Kleivi K, Leivonen SK, Aure MR, Russnes HG, et al. miRNA-mRNA integrated analysis reveals roles for miRNAs in primary breast tumors. *PLoS One.* 2011; 6:e16915. [PubMed: 21364938]
42. Zhang F, Tian Q, Wang Y. Far upstream element-binding protein 1 (FUBP1) is overexpressed in human gastric cancer tissue compared to non-cancerous tissue. *Onkologie.* 2013; 36:650–655. [PubMed: 24192769]
43. Garrett JT, Olivares MG, Rinehart C, Granja-Ingram ND, Sanchez V, Chakrabarty A, et al. Transcriptional and posttranslational up-regulation of HER3 (ErbB3) compensates for inhibition of the HER2 tyrosine kinase. *Proc Natl Acad Sci USA.* 2011; 108:5021–5026. [PubMed: 21385943]
44. Scaltriti M, Rojo F, Ocana A, Anido J, Guzman M, Cortes J, et al. Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. *J Natl Cancer Inst.* 2007; 99:628–638. [PubMed: 17440164]



45. Ritter CA, Perez-Torres M, Rinehart C, Guix M, Dugger T, Engelman JA, et al. Human breast cancer cells selected for resistance to trastuzumab in vivo overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. *Clin Cancer Res.* 2007; 13:4909–4919. [PubMed: 17699871]
46. Belkhiri A, Dar AA, Peng DF, Razvi MH, Rinehart C, Arteaga CL, et al. Expression of t-DARPP mediates trastuzumab resistance in breast cancer cells. *Clin Cancer Res.* 2008; 14:4564–4571. [PubMed: 18579663]
47. Yao E, Zhou W, Lee-Hoeflich ST, Truong T, Haverty PM, Eastham-Anderson J, et al. Suppression of HER2/HER3-mediated growth of breast cancer cells with combinations of GDC-0941 PI3K inhibitor, trastuzumab, and pertuzumab. *Clin Cancer Res.* 2009; 15:4147–4156. [PubMed: 19509167]
48. Gayle SS, Castellino RC, Buss MC, Nahta R. MEK inhibition increases lapatinib sensitivity via modulation of FOXM1. *Curr Med Chem.* 2013; 20:2486–2499. [PubMed: 23531216]
49. Lee YY, Kim HP, Kang MJ, Cho BK, Han SW, Kim TY, et al. Phosphoproteomic analysis identifies activated MET-axis PI3K/AKT and MAPK/ERK in lapatinib-resistant cancer cell line. *Exp Mol Med.* 2013; 45:e64. [PubMed: 24263233]
50. Xiang B, Chatti K, Qiu H, Lakshmi B, Krasnitz A, Hicks J, et al. Brk is coamplified with ErbB2 to promote proliferation in breast cancer. *Proc Natl Acad Sci USA.* 2008; 105:12463–12468. [PubMed: 18719096]
51. Zhuang G, Brantley-Sieders DM, Vaught D, Yu J, Xie L, Wells S, et al. Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. *Cancer Res.* 2010; 70:299–308. [PubMed: 20028874]
52. Amin DN, Sergina N, Ahuja D, McMahon M, Blair JA, Wang D, et al. Resiliency and vulnerability in the HER2-HER3 tumorigenic driver. *Sci Transl Med.* 2010; 2:16ra7.
53. Wang SE, Lin RJ. MicroRNA and HER2-overexpressing cancer. *Microna.* 2013; 2:137–147. [PubMed: 25070783]
54. Kang HS, Kim J, Jang SG, Kwon SY, Park YS, Green JE, et al. MicroRNA signature for HER2-positive breast and gastric cancer. *Anticancer Res.* 2014; 34:3807–3810. [PubMed: 24982406]
55. Bao W, Fu HJ, Xie QS, Wang L, Zhang R, Guo ZY, et al. HER2 interacts with CD44 to up-regulate CXCR4 via epigenetic silencing of microRNA-139 in gastric cancer cells. *Gastroenterology.* 2011; 141:2076–2087. [PubMed: 21925125]
56. Jin L, Wessely O, Marcusson EG, Ivan C, Calin GA, Alahari SK. Prooncogenic factors miR-23b and miR-27b are regulated by Her2/Neu, EGF, and TNF-alpha in breast cancer. *Cancer Res.* 2013; 73:2884–2896. [PubMed: 23338610]
57. Huang TH, Wu F, Loeb GB, Hsu R, Heidersbach A, Brincat A, et al. Up-regulation of miR-21 by HER2/neu signaling promotes cell invasion. *J Biol Chem.* 2009; 284:18515–18524. [PubMed: 19419954]
58. Venturutti L, Romero LV, Urtreger AJ, Chervo MF, Cordo Russo RI, Mercogliano MF, et al. Stat3 regulates ErbB-2 expression and co-opts ErbB-2 nuclear function to induce miR-21 expression, PDCD4 downregulation and breast cancer metastasis. *Oncogene.* 2015
59. Dai L, Wang W, Zhang S, Jiang Q, Wang R, Dai L, et al. Vector-based miR-15a/16-1 plasmid inhibits colon cancer growth in vivo. *Cell Biol Int.* 2012; 36:765–770. [PubMed: 22574716]
60. Bhattacharya R, Nicoloso M, Arvizo R, Wang E, Cortez A, Rossi S, et al. MiR-15a and MiR-16 control Bmi-1 expression in ovarian cancer. *Cancer Res.* 2009; 69:9090–9095. [PubMed: 19903841]
61. Bandi N, Zbinden S, Gugger M, Arnold M, Kocher V, Hasan L, et al. miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer. *Cancer Res.* 2009; 69:5553–5559. [PubMed: 19549910]
62. Jiao LR, Frampton AE, Jacob J, Pellegrino L, Krell J, Giamas G, et al. MicroRNAs targeting oncogenes are down-regulated in pancreatic malignant transformation from benign tumors. *PLoS One.* 2012; 7:e32068. [PubMed: 22384141]
63. Yang TQ, Lu XJ, Wu TF, Ding DD, Zhao ZH, Chen GL, et al. MicroRNA-16 inhibits glioma cell growth and invasion through suppression of BCL2 and the nuclear factor-kappaB1/MMP9 signaling pathway. *Cancer Sci.* 2014; 105:265–271. [PubMed: 24418124]

64. Jiang QQ, Liu B, Yuan T. MicroRNA-16 inhibits bladder cancer proliferation by targeting cyclin D1. *Asian Pac J Cancer Prev.* 2013; 14:4127–4130. [PubMed: 23991964]
65. Agra AN, Motino O, Mayoral R, Llorente IC, Fernandez-Alvarez A, Bosca L, et al. Cyclooxygenase-2 is a target of microRNA-16 in human hepatoma cells. *PLoS One.* 2012; 7:e50935. [PubMed: 23226427]
66. Le XF, Almeida MI, Mao W, Spizzo R, Rossi S, Nicoloso MS, et al. Modulation of MicroRNA-194 and cell migration by HER2-targeting trastuzumab in breast cancer. *PLoS One.* 2012; 7:e41170. [PubMed: 22829924]
67. Ichikawa T, Sato F, Terasawa K, Tsuchiya S, Toi M, Tsujimoto G, et al. Trastuzumab produces therapeutic actions by upregulating miR-26a and miR-30b in breast cancer cells. *PLoS One.* 2012; 7:e31422. [PubMed: 22384020]
68. Nishida N, Mimori K, Fabbri M, Yokobori T, Sudo T, Tanaka F, et al. MicroRNA-125a-5p is an independent prognostic factor in gastric cancer and inhibits the proliferation of human gastric cancer cells in combination with trastuzumab. *Clin Cancer Res.* 2011; 17:2725–2733. [PubMed: 21220473]
69. Xu J, Chen Y, Olopade OI. MYC and breast cancer. *Genes Cancer.* 2010; 1:629–640. [PubMed: 21779462]
70. Collins DC, Cocchiglia S, Tibbitts P, Solon G, Bane FT, McBryan J, et al. Growth factor receptor/steroid receptor cross talk in trastuzumab-treated breast cancer. *Oncogene.* 2015; 34:525–530. [PubMed: 24469058]
71. Okada N, Lin CP, Ribeiro MC, Biton A, Lai G, He X, et al. A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression. *Genes Dev.* 2014; 28:438–450. [PubMed: 24532687]
72. Liu S, Sun X, Wang M, Hou Y, Zhan Y, Jiang Y, et al. A microRNA 221- and 222-mediated feedback loop maintains constitutive activation of NFkappaB and STAT3 in colorectal cancer cells. *Gastroenterology.* 2014; 147:847–859. [PubMed: 24931456]
73. Chen L, Min L, Wang X, Zhao J, Chen H, Qin J, et al. Loss of RACK1 promotes metastasis of gastric cancer by inducing a miR-302c/IL8 signaling loop. *Cancer Res.* 2015; 75:3832–3841. [PubMed: 26199092]
74. Schneeweiss A, Chia S, Hegg R, Tausch C, Deb R, Ratnayake J, et al. Evaluating the predictive value of biomarkers for efficacy outcomes in response to pertuzumab-and trastuzumab-based therapy: an exploratory analysis of the TRYPHAENA study. *Breast Cancer Res.* 2014; 16:R73. [PubMed: 25005255]
75. Kao SC, Fulham M, Wong K, Cooper W, Brahmabhatt H, MacDiarmid J, et al. A significant metabolic and radiological response after a novel targeted microRNA-based treatment approach in malignant pleural mesothelioma. *Am J Respir Crit Care Med.* 2015; 191:1467–1469. [PubMed: 26075427]
76. Edgar R, Domrachev M, Lash AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 2002; 30:207–210. [PubMed: 11752295]



**Figure 1.** TZ induces miR-16 expression in TZ-sensitive BC and GC. **(a)** Sensitive cells were treated with TZ. MiR-16 levels were determined by RT-qPCR. **(b)** Left panel: mice were inoculated subcutaneously (s.c.) with BT-474 cells and were then injected twice a week with TZ or control IgG. Each point represents mean tumor volume  $\pm$  s.e.m. from each group. Right panel: animals were killed at different time points ( $\alpha$ ,  $\beta$  and  $\gamma$ ) and miR-16 levels were measured in tumor extracts. **(c)** Resistant cells were treated with TZ. MiR-16 levels were determined as in **a**. **(d)** Left panel: mice were inoculated s.c. with JIMT-1 cells and were treated as in **b**. Each point represents mean tumor volume  $\pm$  s.e.m. ( $n=8$ ). Right panel:

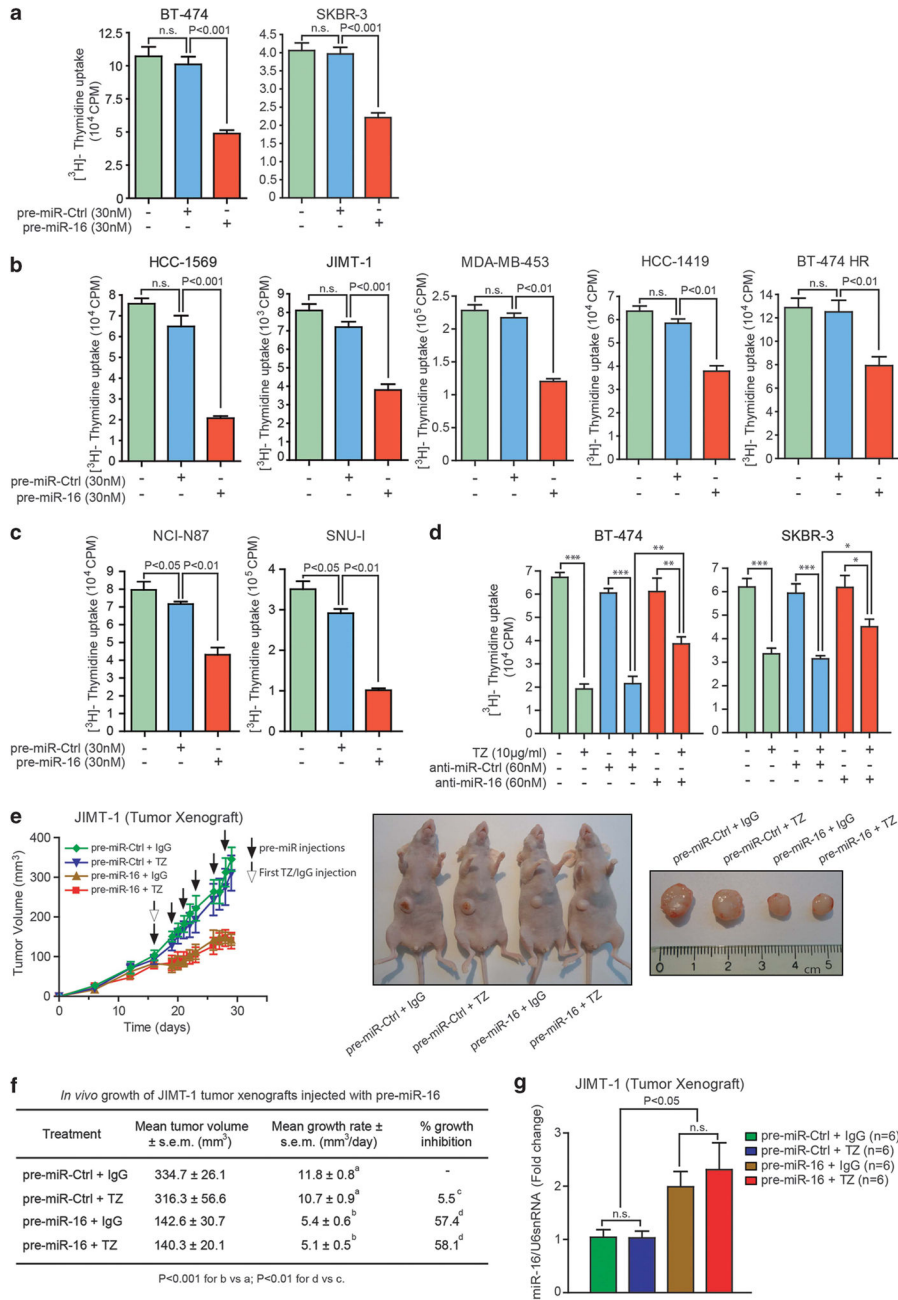
animals were killed at day 45 and miR-16 levels were measured as in **b**. **(e)** GC cells were treated with TZ. MiR-16 levels were determined as in **a**. Data in **a**, **c** and **e** represent mean  $\pm$ s.e.m. of three independent experiments. Experiments in **b** and **d** were repeated thrice with similar results. \* $P<0.05$ ; \*\* $P<0.01$ . See Supplementary Figure S1.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 2.** MiR-16 is a tumor suppressor in ErbB-2-positive BC and GC. (a–c) Cells were transfected as stated and proliferation was assessed by [<sup>3</sup>H]-Thymidine uptake. (d) Cells were transfected with a miR-16 inhibitor or control and treated with TZ for 48 h. Proliferation was assessed as in a. (e) Left panel: mice were inoculated s.c. with JIMT-1 cells and then received intratumoral pre-miR injections thrice a week, and intraperitoneal TZ or IgG injections twice a week. Each point represents mean tumor volume  $\pm$ s.e.m. ( $n=6$ ). Middle and right panels: images show representative animals and tumors at time of killing. (f) Animals in e were killed at day 29. Final tumor volumes, growth rates and percentage of

growth inhibition were calculated as stated in Materials and methods section. (g) MiR-16 levels were measured in tumors in e at day 29. Data in a–d represent mean  $\pm$  s.e.m. of three independent experiments. \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001. Experiments in e–g were repeated thrice with similar results. See Supplementary Figure S2.

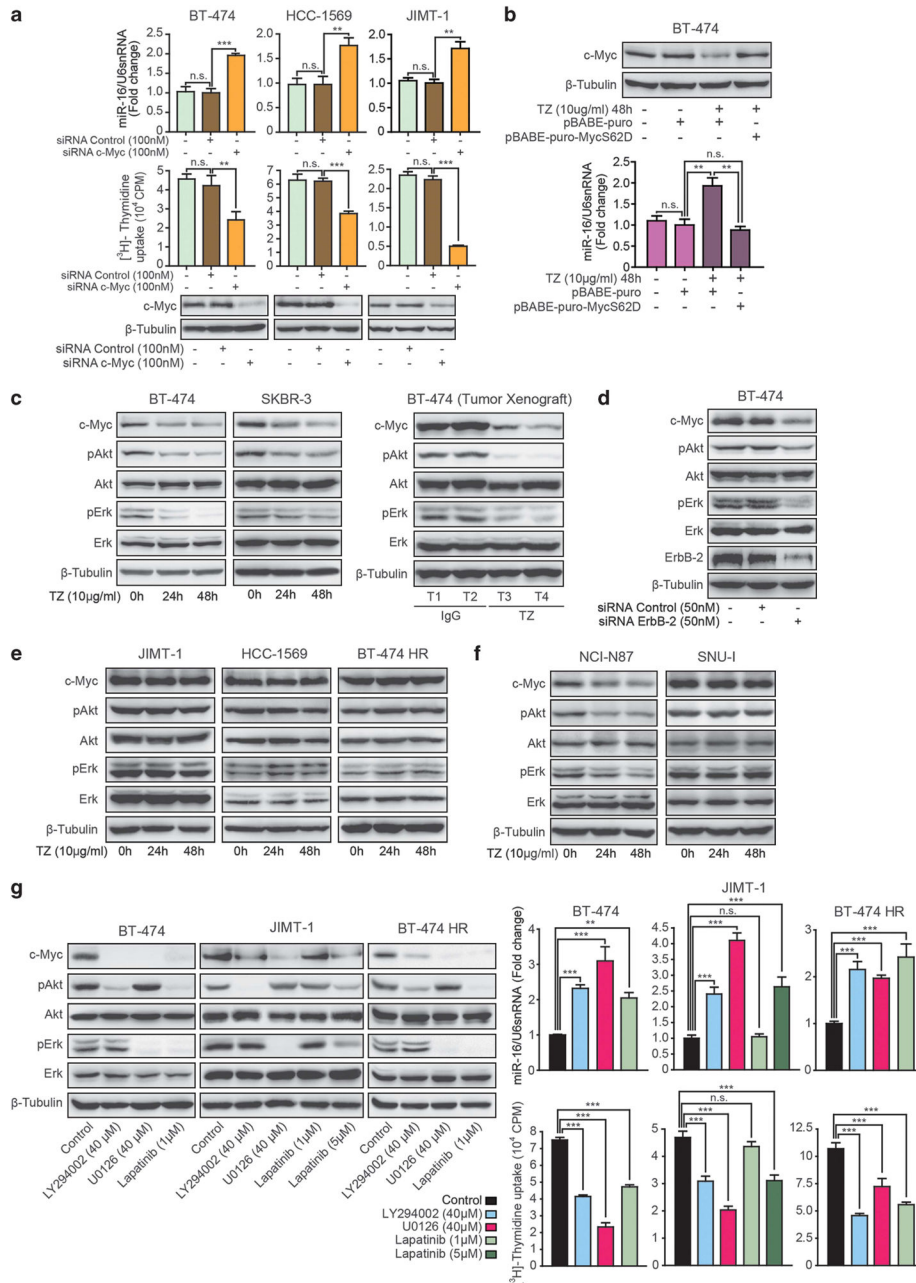
Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript





**Figure 3.** Lapatinib and TZ induce miR-16 upregulation in sensitive cells via inhibition of c-Myc and the PI3K/AKT and Erk1/2 pathways. **(a)** Upper panels: cells were transfected as stated and miR-16 levels were determined by RT-qPCR. Middle panels: proliferation was assessed as in Figure 2. Lower panels: efficiency of c-Myc inhibition. **(b)** c-Myc expression was reconstituted by transfection of TZ-treated cells with the pBABE-puro-MycS62D plasmid. Upper panel: efficiency of c-Myc reconstitution. Lower panel: miR-16 levels were determined as in **a**. **(c)** WB analyses in protein extracts from cells treated with TZ, or in tumor lysates from the experiment in Figures 1b. **(d)** Cells were transfected as stated and

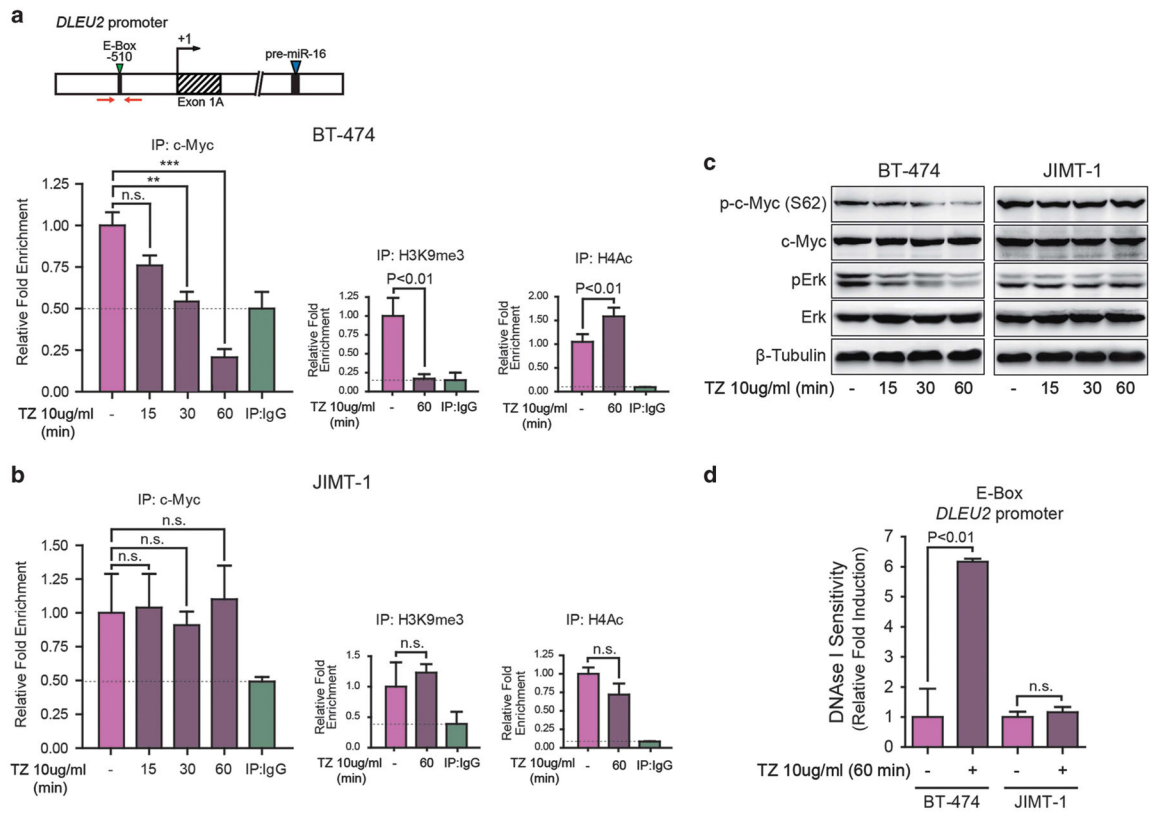
protein extracts were analyzed by WB. (e) TZ-resistant cells were treated with TZ. Protein extracts were analyzed by WB. (f) WB analysis in GC cells treated with TZ. (g) Cells were treated for 24 h with lapatinib or the stated inhibitors. Left panel: WB analysis was performed as in c. Top right panel: miR-16 levels were determined as in a. Bottom right panel: cell proliferation was assessed as in a. Images shown in a–g are representative of a total of three experiments, all with similar results. Data in a, b and g represent mean  $\pm$  s.e.m. of three independent experiments. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . See Supplementary Figure S3.

Author Manuscript

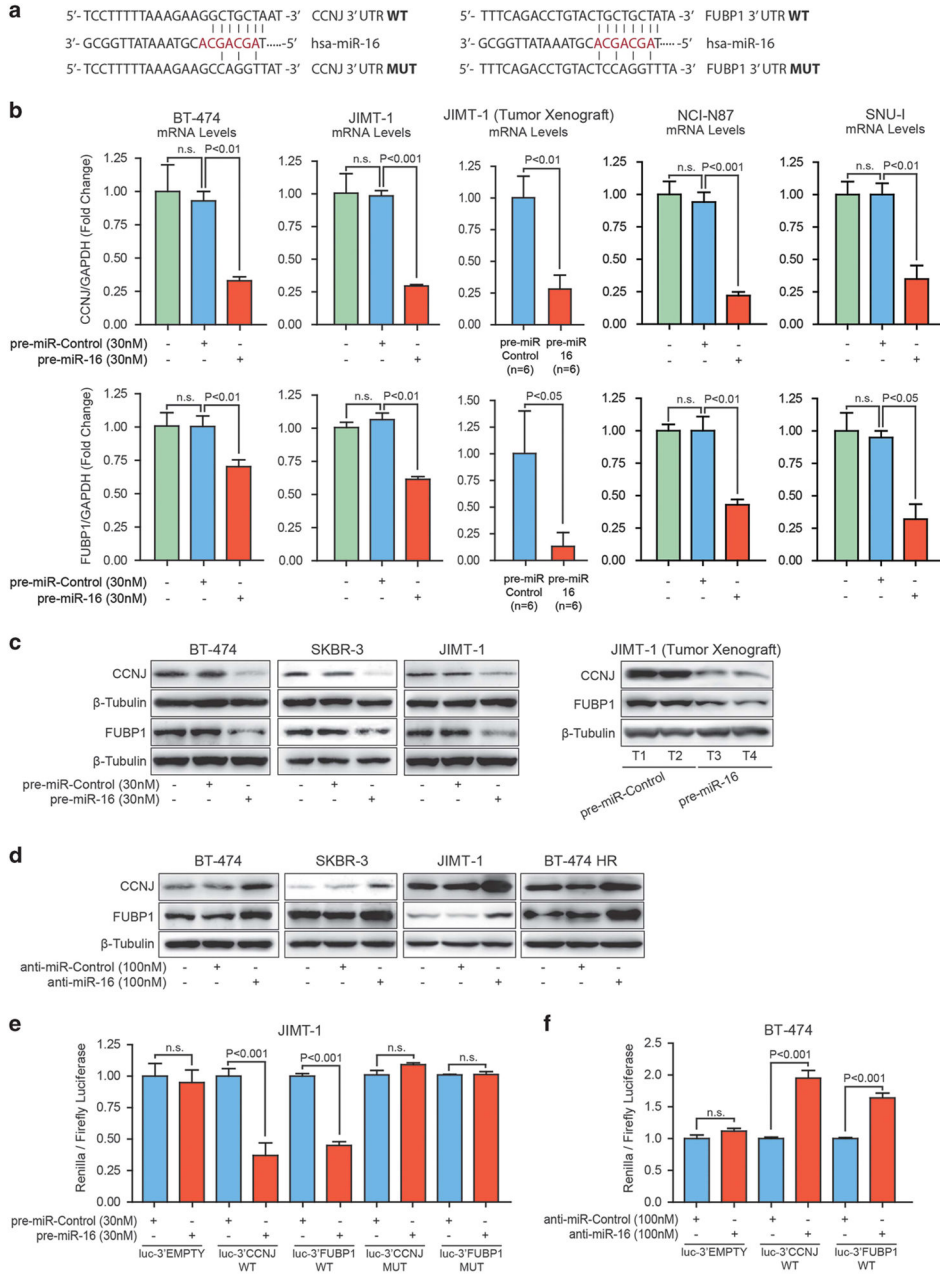
Author Manuscript

Author Manuscript

Author Manuscript



**Figure 4.** TZ controls c-Myc transcriptional activity and the local chromatin structure at the *DLEU2* promoter in TZ-sensitive cells. **(a, b)** Cells were treated with TZ. Recruitment of c-Myc or H3K9me3 and H4Ac levels at the *DLEU2* promoter were assessed by chromatin immunoprecipitation (ChIP). DNA was amplified using primers (red arrows) flanking the E-Box (green wedge) indicated in the diagram. **(c)** WB analysis of p-c-Myc (S62) and pErk levels in cells treated as in **a**. **(d)** DNase I sensitivity assays were performed using the same primers as in **a**. Images shown in **c** are representative of a total of three experiments, all with similar results. Data in **a, b** and **d** represent mean  $\pm$  s.e.m. of three independent experiments. \* $P < 0.05$ ; \*\*\* $P < 0.001$ .



**Figure 5.**

CCNJ and FUBP1 constitute novel miR-16 targets in ErbB-2-positive BC. **(a)** Sequence alignment between hsa-miR-16 and the wild type (WT) or mutated (MUT) sequences of human CCNJ and FUBP1 3'UTR. MiR-16 seed sequence is depicted in red. **(b)** CCNJ and FUBP1 mRNA levels were measured by RT-qPCR in cells transfected as stated, or in extracts from tumors in the experiment of Figure 2e. **(c)** CCNJ and FUBP1 protein levels in cells treated as in **b** or in lysates from tumors in the experiment of Figure 2e. **(d)** CCNJ and FUBP1 protein levels in cell lines transfected with a miR-16 inhibitor or control. **(e)** Luciferase assays in cells co-transfected with the indicated plasmids and pre-miR-16 or pre-miR-Control. **(f)** Luciferase assays in cells co-transfected with the indicated plasmids and a

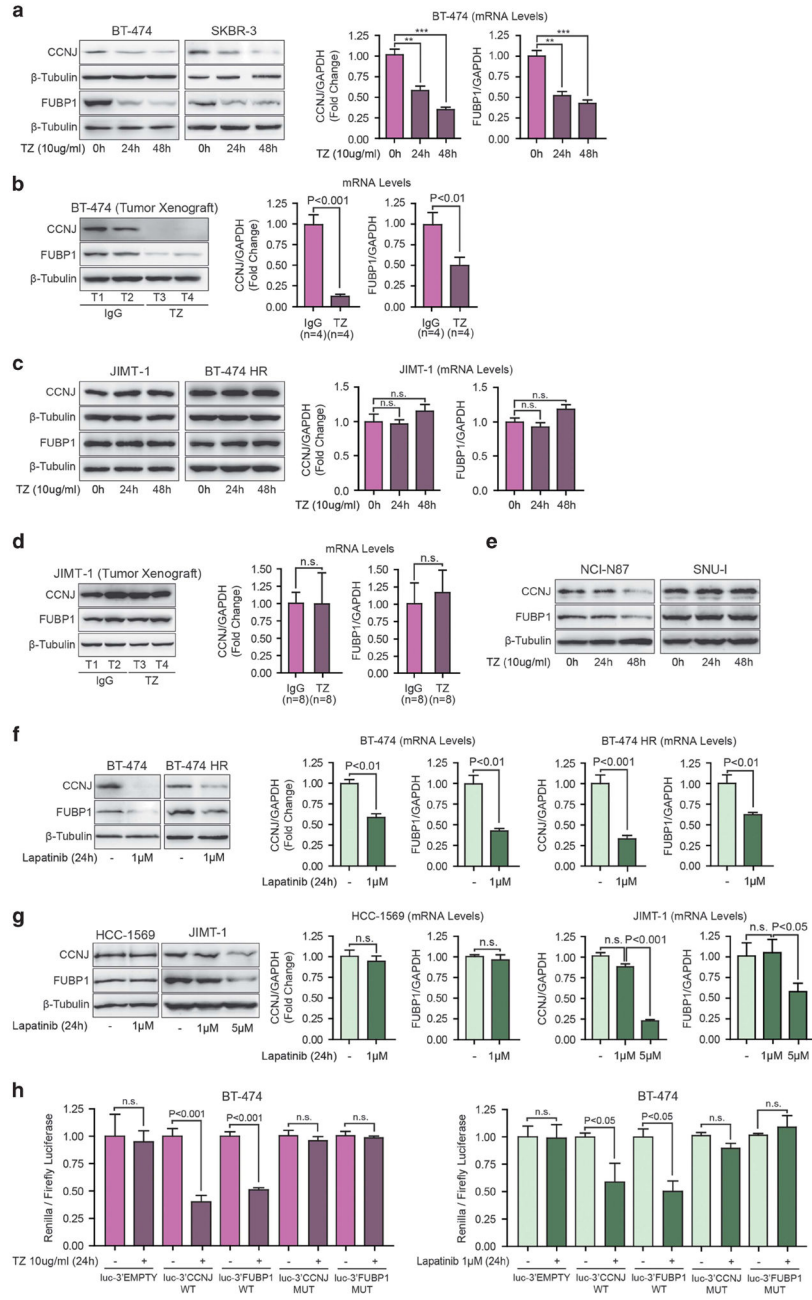
miR-16 inhibitor or control. Images shown in **c** and **d** are representative of a total of three experiments, all with similar results. Data in **b**, **e** and **f** represent mean  $\pm$ s.e.m. of three independent experiments. See Supplementary Figure S4 and Supplementary Tables S1–S3.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 6.** Lapatinib and TZ control CCNJ and FUBP1 expression through miR-16 upregulation in BC cells sensitive to these drugs. **(a, b)** Left panel: CCNJ and FUBP1 protein levels in cells treated with TZ, or in lysates from tumors in the experiment of Figure 1b. Right panel: CCNJ and FUBP1 mRNA levels were determined by RT-qPCR. **(c, d)** Left panel: CCNJ and FUBP1 protein levels in cells treated with TZ, or in protein extracts from tumors in the experiment of Figure 1d. Right panel: CCNJ and mRNA levels were determined as in **a**. **(e)** WB analysis of CCNJ and FUBP1 expression in GC cells treated with TZ. **(f, g)** Left panel: CCNJ and FUBP1 mRNA levels were measured in cells treated with lapatinib. Right panel:



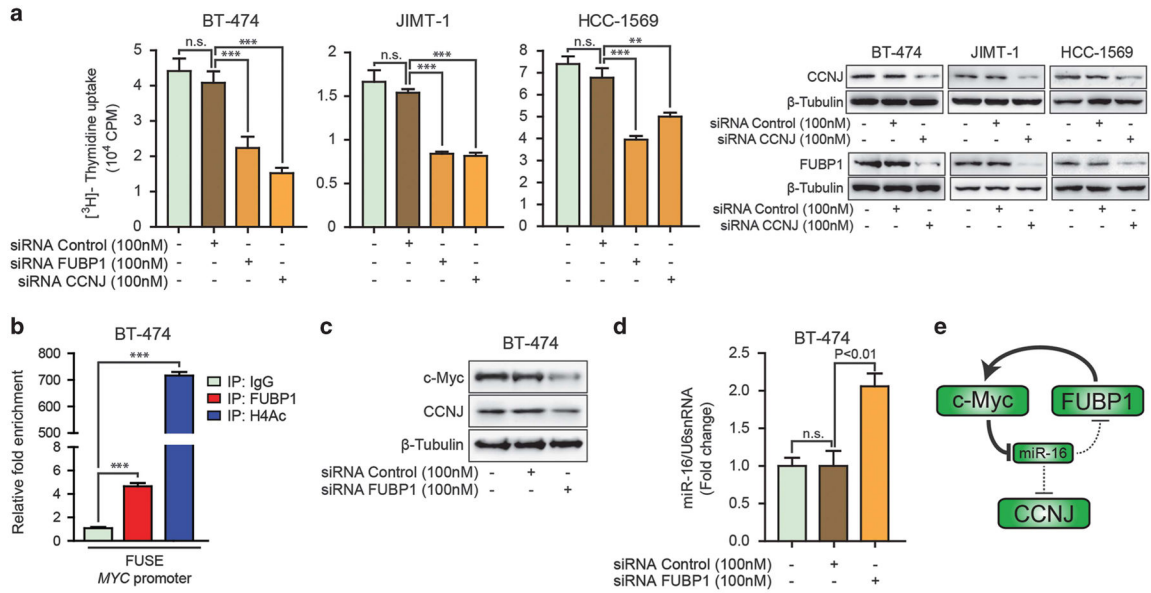
CCNJ and FUBP1 protein levels. **(h)** Luciferase assays in cells transfected with the indicated plasmids and then treated with TZ or lapatinib. Luciferase activity was measured as in Figure 5. Images shown in **a–g** are representative of a total of three experiments, all with similar results. Data in **a–d** and **f–h** represent mean  $\pm$ s.e.m. of three independent experiments. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

Author Manuscript

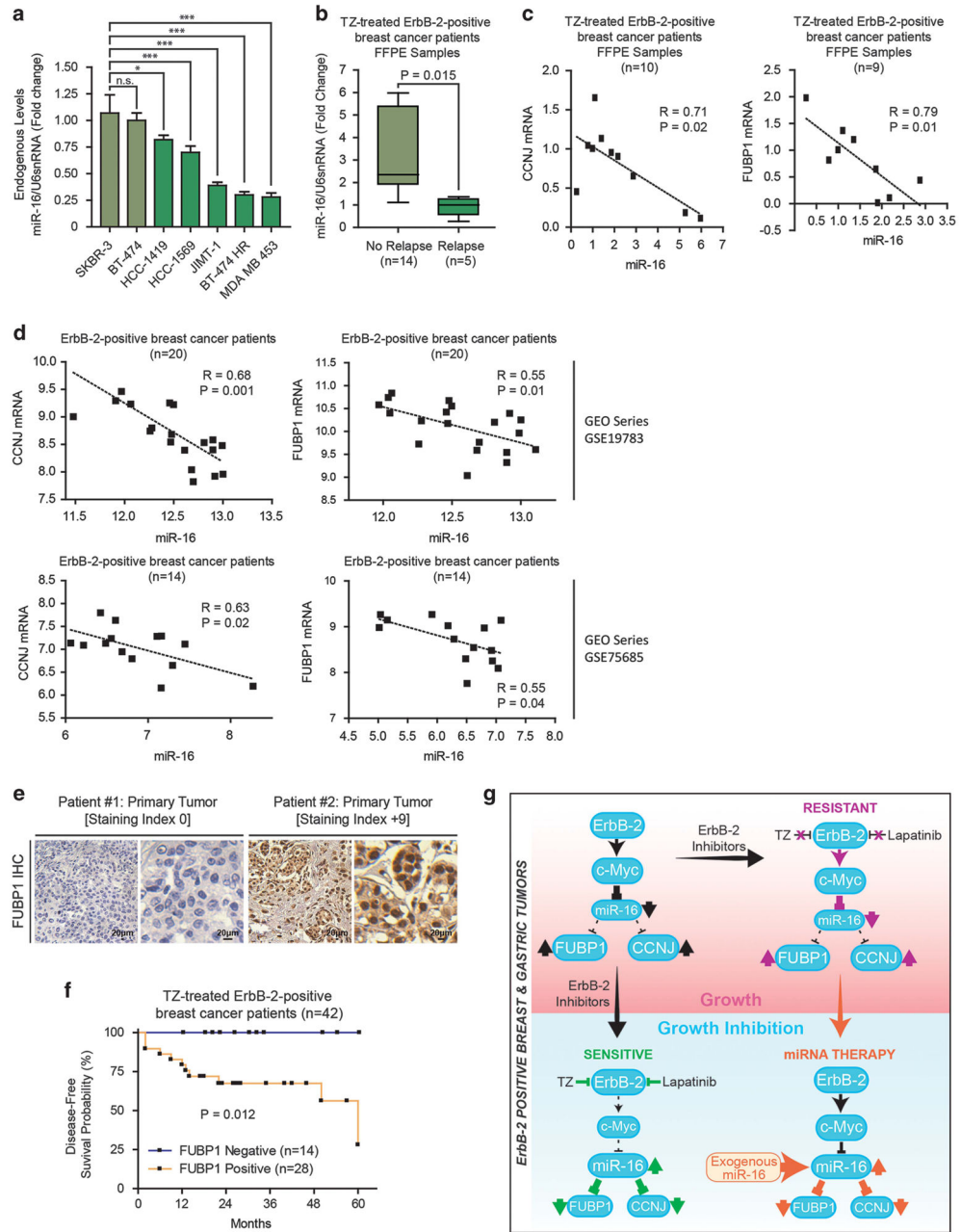
Author Manuscript

Author Manuscript

Author Manuscript



**Figure 7.** CCNJ and FUBP1 are required for ErbB-2-positive BC growth. **(a)** Cells were transfected as stated. Left panel: proliferation was measured as in Figure 2. Right panel: WB shows efficiency of CCNJ and FUBP1 inhibition. **(b)** Recruitment of FUBP1 and H4Ac levels at the *MYC* promoter were analyzed by ChIP. DNA was amplified using primers flanking the far upstream element (FUSE) site at the *MYC* promoter. **(c, d)** Cells were transfected for 48 h as stated. **(c)** WB shows c-Myc and CCNJ protein levels. **(d)** MiR-16 levels were assessed by RT-qPCR. **(e)** Scheme depicting the positive feedback loop involving FUBP1, c-Myc and miR-16. Images shown in **a** and **c** are representative of a total of three experiments, all with similar results. Data in **a, b** and **d** represent mean  $\pm$ s.e.m. of three independent experiments. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



**Figure 8.** miR-16 and FUBP1 levels predict adjuvant TZ efficacy in ErbB-2-positive BC. (a) MiR-16 levels were assessed by RT-qPCR 24 h after plating cells in complete growth media. (b) RNA was extracted from FFPE tumor samples from patients with ErbB-2-positive primary invasive BC who received adjuvant TZ. MiR-16 levels were measured by RT-qPCR. (c) Inverse correlation between endogenous CCNJ and FUBP1 mRNA levels and miR-16 levels, determined by RT-qPCR in the same cohort as in b. R, regression coefficient. (d) The expression levels of miR-16, and those of its target genes, were studied in two publicly available independent cohorts of ErbB-2-positive BC primary tumors. (e) FUBP1 expression

was assessed by immunohistochemistry (IHC) before TZ treatment in an independent cohort of 42 ErbB-2-positive BC patients who received adjuvant TZ. Shown are representative images of a tumor with null (left) or positive (right) FUBP1 staining. (f) Kaplan–Meier analysis was performed to correlate FUBP1 expression with disease-free survival in the same cohort as in e. (g) Schematic model of miR-16 role on the mechanisms of action and resistance to ErbB-2-targeted therapies. Data in a represent mean  $\pm$ s.e.m. of three independent experiments. \* $P$ <0.05; \*\*\* $P$ <0.001. See Supplementary Figure S5 and Supplementary Tables S4–S6.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript