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ZIP7-Mediated Intracellular Zinc Transport Contributes to Aberrant Growth Factor Signaling in Antihormone-Resistant Breast Cancer Cells

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Antiestrogens such as tamoxifen are the mainstay of treatment for estrogen receptor-positive breast cancer. However, their effectiveness is limited by the development of endocrine resistance, allowing tumor regrowth and progression. Importantly, *in vitro* MCF7 cell models of acquired tamoxifen resistance (TamR cells) display an aggressive, invasive phenotype in which activation of epithelial growth factor receptor/IGF-I receptor/Src signaling plays a critical role. In this study, we report that TamR cells have increased levels of zinc and zinc transporter, ZIP7 [solute carrier family 39 (zinc transporter) member 7, also known as SLC39A7 and HKE4], resulting in an enhanced response to exogenous zinc, which is manifested as a greatly increased growth factor receptor activation, leading to increased growth and invasion. Removal of ZIP7, using small interfering RNA, destroys this activation of epithelial growth factor receptor/IGF-I receptor/Src signaling by reducing intracellular zinc levels. Similarly, it also blocks the activation of HER2, -3, and -4. These data suggest that intracellular zinc levels may be a critical factor in determining growth factor responses and that the targeting of zinc transporters may have novel therapeutic implications. We show that ZIP7 is a critical component in the redistribution of zinc from intracellular stores to the cytoplasm and, as such, is essential for the zinc-induced inhibition of phosphatases, which leads to activation of growth factor receptors. Removal of ZIP7 therefore offers a means through which zinc-induced activation of growth factor receptors may be effectively suppressed and provides a mechanism of targeting multiple growth factor pathways, increasing tumor kill, and preventing further development of resistance in breast cancer. (*Endocrinology* 149: 4912–4920, 2008)

ESTROGEN RECEPTOR-POSITIVE breast cancers are routinely treated clinically with antihormones such as tamoxifen. However, with time, tumors develop resistance to these agents, leading to their regrowth and progression (1). To better understand the mechanisms underlying the development of resistance, we generated a tamoxifen-resistant breast cancer model (TamR) derived from the estrogen receptor-positive human breast cancer cell line MCF-7, which we previously described (2). TamR cells grow in the presence of tamoxifen with an altered and more aggressive phenotype, which shows reliance on epithelial growth factor receptor (EGFR) (2), IGF-I receptor (IGF-1R) (3), and c-Src (4) signaling. Such cells also exhibit an increased motility and invasiveness (5) and a morphological appearance consistent with that of cells undergoing an epithelial-to-mesenchymal transition (4). Significantly, in these tamoxifen-resistant variants, we demonstrated that IGFs can improve the efficiency of the EGFR signaling by a Src-dependent phosphorylation of tyrosine 845 on the EGFR, with interactions between these signaling elements impinging on both their proliferative and invasive properties. This IGF-1R/EGFR cross talk mechanism is unidirectional whereby IGF-II regulates basal and ligand-activated EGFR signaling and cell proliferation in a Src-dependent manner in TamR cells (6). Consequently, TamR cells are inhibited by drugs that target the EGFR (2), IGF-1R (3), and Src (4) signaling pathways.

Zinc is essential for cell growth and is a cofactor for more than 300 enzymes, representing more than 50 different enzyme classes (7). Zinc is involved in protein, nucleic acid, and carbohydrate and lipid metabolism as well as the control of gene transcription, growth, development, and differentiation (8). As such, zinc deficiency can be detrimental to cells, causing stunted growth and serious metabolic disorders (9). Zinc is unable to passively diffuse across cell membranes; therefore, two families of mammalian zinc transporters are required to traffic zinc across cellular membranes; the ZnT (SLC30A) family of zinc efflux transporters (10) and the zinc influx transporter (ZIP; SLC39A) family (11). There are nine human ZIP family members, most of which have been localized to the plasma membrane (11) and transport zinc into cells (12–15). However, one ZIP family member, ZIP7 [solute carrier family 39 (zinc transporter) member 7, also known as SLC39A7 and HKE4], has been demonstrated by us to reside on the endoplasmic reticulum (16) and is thus expected to transport zinc from intracellular compartments to the cytoplasm.

These zinc transporters play a crucial role in maintaining the cellular balance between cell growth and cell death, and

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* K.T. and P.V. contributed equally to this paper.

Abbreviations: DAPI, 4′,6′-Diamino-2-phenylindole; EGFR, epithelial growth factor receptor; FACS, fluorescence-activated cell sorter; IGF-1R, IGF-I receptor; IRS1, insulin receptor substrate 1; 4-OH tamoxifen, 4-hydroxytamoxifen; PTP1B, protein tyrosine phosphatase 1B; siRNA, small interfering RNA; TamR, tamoxifen-resistant; TPEN, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl) ethylenediamine; wRPMI, RPMI 1640 with fetal calf serum, L-glutamine, penicillin, streptomycin, and Fungizone; ZIP7, solute carrier family 39 (zinc transporter) member 7, also known as SLC39A7 and HKE4.

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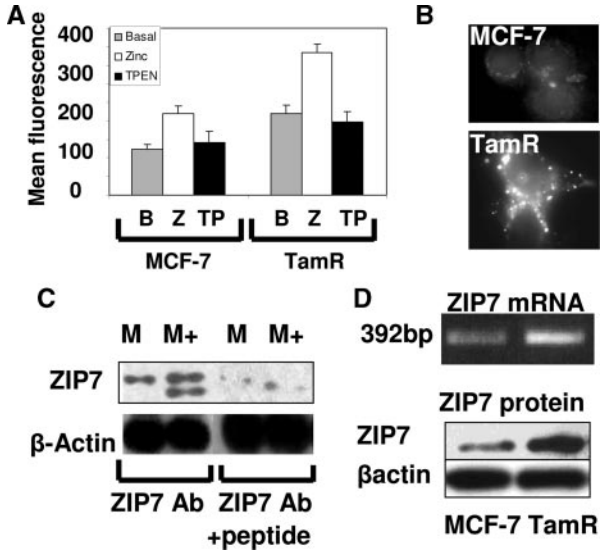

FIG. 1. TamR cells have increased zinc and ZIP7 expression. A, Basal (B, *gray bars*) TamR cells loaded with Newport green have increased intracellular zinc, compared with basal MCF-7 cells, as measured by mean fluorescence using FACS analysis. Both cell lines have increased fluorescence when treated with 100 μM zinc with ionophore (Z, *white bars*), which was reduced by addition of TPEN (TP, *black bars*). B, TamR cells loaded with Newport green exhibit increased green fluorescence by fluorescent microscopy, compared with MCF-7 cells. C, Our ZIP7 antibody recognizes ZIP7 in MCF-7 (M) cells with an additional band for the recombinant ZIP7 in transfected MCF-7 cells (M+), both of which are absent when the antibody was preabsorbed with the peptide (*right-hand lanes*). D, Confirmation of increased expression of ZIP7 in TamR compared with MCF-7 cells at both mRNA (*top panel*) and protein (*bottom panel*) level.

aberrations in zinc transport have been linked to diseases such as cancer. In this context, it is noteworthy that zinc appears to be elevated in human breast tumors when compared with benign breast tissue (17–19), and an *N*-methyl-*N*-nitrosourea-induced model of rodent mammary tumorigenesis, which has been widely used for investigating breast cancer development (20), shows up to 19-fold zinc accumulation in mammary tumors, compared with their normal counterparts, irrespective of zinc dietary intake (21). Interestingly, zinc is known to inhibit multiple phosphatases (22) and thus prevents the dephosphorylation of tyrosine kinase receptors, which are often aberrantly expressed and activated in cancer. Recently a calcium-dependent zinc wave has been described in mast cells (23), which defines zinc as a second messenger involved in intracellular signaling events primarily by its ability to inhibit phosphatases. This zinc wave was shown to originate in the endoplasmic reticulum, although the molecule responsible for its initiation was not determined. Previous studies from our group identified ZIP7, a member of the LIV-1 subfamily of ZIP zinc transporters, as residing in the endoplasmic reticulum (16), and we postulate that it may play a role in promoting altered zinc levels in antihormone-resistant cells. Moreover, because zinc inhibits phosphatases and thus activates tyrosine kinase receptors, which are raised in our antihormone-resistant cells, we sought to investigate the controlling role of ZIP7 in driving IGF-1R/EGFR/Src signaling and thus in promoting the aggressive behavior of TamR cells. Additionally, a new model has recently been proposed that demonstrates how zinc is buffered and stored within cells (24). This proposed muffler model suggests that zinc entering the cell is trafficked via a muffler with high affinity for zinc to intracellular storage sites (24) from which it is subsequently released. However, the molecule responsible for zinc release from these intracellular stores was not determined.

Given these data plus the previous observations that zinc is able to activate aspects of growth factor signaling such as EGFR (25), IGF-1R (26), and Src (27) and that these pathways are responsible for the growth and aggressive behavior of tamoxifen-resistant breast cancer cells (2–4), we postulate that altered zinc levels in antihormone-resistant cells may contribute to these unfavorable properties.

Materials and Methods

Cell lines and materials

Wild-type MCF-7 breast cancer cells, a gift from AstraZeneca (Macclesfield, UK), were cultured in phenol-red-free RPMI 1640 with 5% fetal calf serum plus 200 mM [scap]l[r]-glutamine, 10 IU/ml penicillin, 10 μg/ml streptomycin, and 2.5 μg/ml Fungizone (wRPMI) at 37 C in a humidified 5% CO_2 atmosphere. The development of tamoxifen-resistant cells (2), faslodex-resistant cells (27), and estrogen-deprived cells (28), MCF-7X cells has been described. Tissue culture media and constituents were obtained from Life Technologies Europe Ltd. (Paisley, UK) and plasticware from Nunc (Roskilde, Denmark); gefitinib (Iressa, ZD1839) was a gift from AstraZeneca Pharmaceuticals (Cheshire, UK); Su6656 was obtained from Calbiochem (Merck Chemicals Ltd., Nottingham, UK) and zinc chloride, zinc ionophore (sodium pyrithione), and zinc chelator, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) from Sigma-Aldrich (Poole, UK). Total RNA was extracted and reverse transcribed to cDNA using random hexamers as previously described (29) before performing PCR for 30 cycles using oligonucleotides; forward, 5′-ATC GCT CTC TAC TTC AGA TC-3′ and reverse, 5′-CTC TTC TGA ACC CCT CTT G-3′ producing a band of 392 bp.

Experimental cell culture, treatments, and cell lysis for Western blotting

TamR cells were seeded at 2×10^5 /cm^2, grown in wRPMI until 60–70% confluent, washed with PBS, and transferred to phenol-red-free and serum-free DCCM media (Biological Industries Ltd., Kibbutz Beit Haemek, Israel) supplemented with 200 mM [scap]l[r]-glutamine, 10 IU/ml penicillin, 10 μg/ml streptomycin, 2.5 μg/ml Fungizone (DCCM), and 4-hydroxytamoxifen (4-OH tamoxifen) for 24 h. This medium contains 1.5 μM free zinc and is depleted of growth factors. EGFR-specific tyrosine kinase inhibitor gefitinib (1 μM) was added for 1 h and c-Src kinase inhibitor Su6656 (5 μM) for 24 h before treatments. Twenty-minute treatments were 20 μM zinc with ionophore (sodium pyrithione), 50 μM TPEN, or 10 ng/ml EGF in serum-free wRPMI + 4-OH tamoxifen. Cells were harvested, washed with PBS, lysed for 1 h at 4 C with 5.5 mM EDTA/0.6% Nonidet P-40/10% mammalian protease inhibitor cocktail (Sigma-Aldrich) in Krebs-Ringer HEPES buffer (30), and centrifuged at 13,000 rpm for 15 min at 4 C. Protein was measured using the DC assay kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

Western blotting

Samples were separated by 10% SDS-PAGE, as described previously (13). Primary antibodies used were diluted 1:10,000 for β-actin or 1:1000 for EGFR, c-Src, and ERK1/2 MAPK. Primary antibodies were as follows: EGFR^{Y1068}, p-ERK1/2$^{T202/Y204}$, and AKT^{S473} were from Cell Signaling, New England Biolabs Ltd., Hitchin, Hertfordshire, UK; EGFR^{Y845} and c-Src^{Y418} were from BioSource Europe S.A. (Fleures, Belgium), ErbB2^{Y1248} was from Upstate Biotechnology (Lake Placid, NY); and β-actin was from Sigma-Aldrich. The IGF-1R^{Y1316} antibody specific to IGF-1R was a gift from AstraZeneca (6). Samples for ErbB3 and ErbB4 analysis were immunoprecipitated with either ErbB3 or ErbB4 antibody,

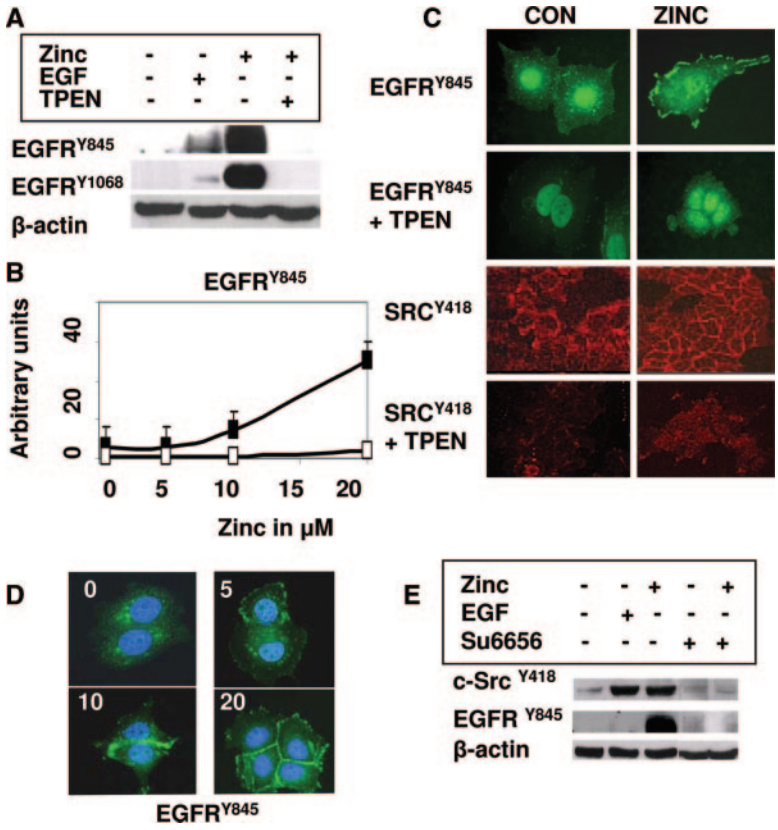

FIG. 2. TamR cells have increased zinc and activated EGFR and Src. A, Western blot results showing activation of $EGFR^{Y845}$ and $EGFR^{Y1068}$ after incubation with either EGF or 20 μM zinc and ionophore for 20 min, which was blocked by TPEN. B, Densitometric values from Western blot results normalized to β-actin demonstrates zinc-dependent activation of $EGFR^{Y845}$ in TamR cells (■), compared with MCF-7 cells (□). Results are expressed as mean ± SD. C, Fluorescent microscopy showing 20 μM zinc-induced activation of $EGFR^{Y845}$ and Src^{Y418} in TamR cells in 20 min, which is reduced by TPEN treatment. D, Time course (0–20 min) of increasing activation of $EGFR^{Y845}$ in TamR cells treated with 20 μM zinc. E, zinc-induced activation (20 μM) of $EGFR^{Y845}$ and Src^{Y418} in TamR cells is eradicated by pretreatment with the Src inhibitor SU6656.

respectively, and probed with phospho tyrosine antibody (Insight Biotechnology Ltd., Wembly, Middlesex, UK). We confirmed that our purified polyclonal antibody (1:100) to the peptide GRQERSTKEKQSSE present on the large cytosolic loop between TM III and IV of ZIP7 recognized recombinant ZIP7 with a C-terminal V5 tag by probing CHO cells transfected with ZIP7 for 24 h as described previously (16) with this ZIP7 antibody (1:100) and the mouse V5 antibody (1:1000; Invitrogen, Paisley, UK). Quantification of Western blot results was performed by normalization to β-actin values.

Fluorescent microscopy and fluorescence-activated cell sorter (FACS) analysis

Cells were cultured on 0.17-mm-thick glass coverslips until 50–60% confluent and treated as described previously (13). For imaging zinc, cells were loaded with cell-permeable zinc-specific dye Newport Green diacetate DCF (5 μM; Invitrogen), Fluozin-3 (5 μM; Invitrogen), or Zinquin (25 μM; Cambridge Bioscience, Cambridge, UK) for 30 min at 37 C before fixing and assembling on slides with Vectorshield ± 4′,6′-diamino-2-phenylindole (DAPI; Vector Laboratories, Peterborough, UK). For zinc assay by FACS analysis cells were loaded with Newport Green diacetate and values of zinc calculated as described previously (12) and expressed as percent control. For imaging EGFR or Src cells were treated ± 20 μM zinc and ionophore for 20 min before fixing as described previously (13), and primary antibodies were conjugated to Alexa-fluor 488 or 594 (1:2000; Invitrogen), respectively, before assembling on slides with Vectorshield ± DAPI.

Growth and invasion studies

Cells, grown for 24 h in wDCCM medium with 4-OH tamoxifen as required before agents were added to cells, were grown for 6 d before trypsin dispersion of cell monolayers and cell counting using a Beckham Coulter counter (Luton, UK). All experiments were performed in triplicate. The *in vitro* invasiveness of cells through Matrigel was determined over 72 h as previously described (4) preceded by 72 h of treatment with small interfering RNA (siRNA).

Statistical analysis

Statistical analysis was performed using ANOVA with *post hoc* Dunnett and Tamhane tests. Significance was assumed with $P < 0.05$. Error bars are SD with at least n = 3 different experiments.

Results

TamR cells have increased intracellular zinc and increased ZIP7 expression

To examine any variation in intracellular zinc levels between wild-type MCF-7 and TamR cells, cells were loaded with the membrane-permeable zinc-specific indicator Newport green diacetate, which increases in green fluorescence in proportion to levels of zinc. The observed mean fluorescence of TamR cell populations when analyzed by FACS was double that of wild-type cells (Fig. 1A), indicating a 2-fold increase in intracellular zinc. The Newport green indicator was specific for zinc as the green fluorescence from TamR cells increased when additionally incubated with 100 μM zinc and ionophore and decreased when incubated with zinc chelator TPEN (Fig. 1A), a recognized method for increasing and decreasing intracellular zinc, respectively (13). This result was confirmed by imaging the green fluorescence of Newport green loaded cells using fluorescent microscopy (Fig. 1B). To define a mechanism for this increase in intracellular zinc in TamR cells, we compared the expression of the LIV-1 family of ZIP zinc influx transporters between MCF-7 wild-type and TamR cells (31) and observed a significant increase in expression in TamR, compared with MCF-7 cells of ZIP7 (SLC39A7/HKE4) RNA. To verify this at both RNA and protein level, we first characterized our ZIP7

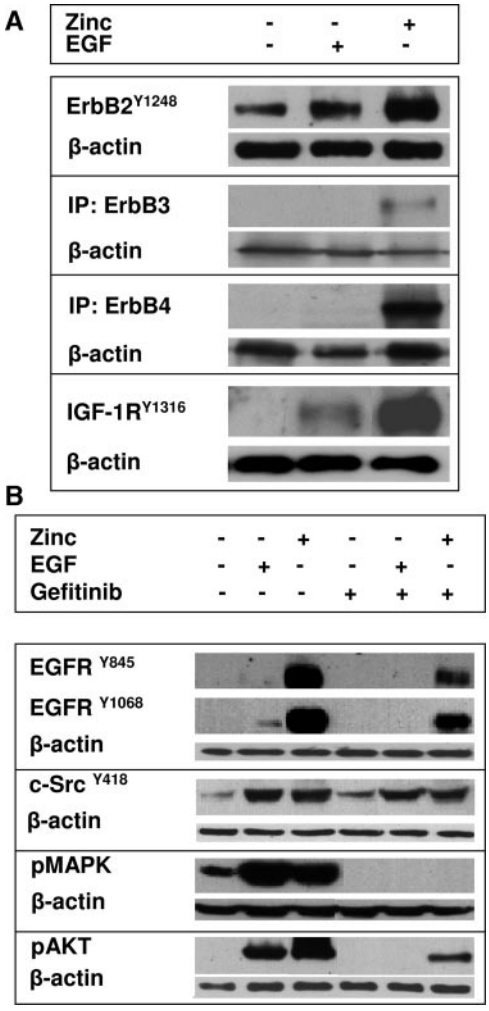

FIG. 3. Zinc activation of tyrosine kinases is reduced by gefitinib. A, Zinc (20 μM) induces activation of other tyrosine kinase receptors ErbB2, ErbB3, ErbB4, and IGF-1R in TamR cells. B, Zinc induces activation of downstream signaling molecules MAPK (pERK1/2) and AKT^{S743}. Zinc-induced activation of EGFR^{Y845}, EGFR^{Y1068}, Src^{Y418}, MAPK (pERK1/2), and AKT^{S743} is reduced by gefitinib.

polyclonal antibody generated against a peptide mapping to the cytoplasmic loop of ZIP7 (see *Materials and Methods*) to show that it recognized both endogenous and recombinant ZIP7, which was absent after peptide preabsorption (Fig. 1C). We then demonstrated increased expression of ZIP7 in TamR, compared with MCF-7, at both the RNA and protein level (Fig. 1D).

EGFR is activated by zinc

We previously demonstrated the increased activation of EGFR in TamR cells and a dependence of these cells on this pathway for cell growth (2). To test the hypothesis that this may be due in part to the observed increased intracellular zinc levels, we treated TamR cells with EGF, 20 μM zinc, or zinc with the zinc-specific chelator, TPEN, for 20 min before examining EGFR activation at Y845 and Y1068. To enable extracellular zinc to penetrate inside the cell in the short 20-min treatments, the zinc ionophore, sodium pyrithione, was included. Control experiments with sodium pyrithione alone produced no alteration in EGFR activation (results not shown). Zinc was able to activate EGFR^{Y845} and EGFR^{Y1068} to a level considerably greater than that observed with EGF alone (Fig. 2A). However, the activation of EGFR by zinc was not observed in the presence of the zinc chelator TPEN, confirming its zinc dependency. The activation of EGFR observed with EGF treatment was small, compared with that stimulated with zinc. This could have been due to the removal of serum and hence zinc from the medium for these short 20 min incubations.

A graph depicting densitometric evaluation of Western blot results, normalized to β-actin, of analysis of EGFR activation on Y845 across a range of zinc treatments (0–20 μM) is shown in Fig. 2B. Incubation of TamR cells with zinc activated EGFR^{Y845} in a zinc concentration-dependent manner. This zinc-dependent activation of EGFR^{Y845} was also confirmed by fluorescent microscopy (Fig. 2C). TamR cells on coverslips were imaged either untreated or treated with zinc in the presence or absence of the zinc chelator TPEN for 20 min. We observed considerable activation of plasma-membrane EGFR^{Y845} (Fig. 2C, *green*) by zinc (Fig. 2C, *top panel*), which was abolished in the presence of TPEN (Fig. 2C, *second panel*), confirming that this was a zinc-dependent phenomenon.

To investigate a more detailed time course of this activation, we treated TamR cells on coverslips with zinc and ionophore for 0–20 min after which cells were fixed, permeabilized, and probed for EGFR^{Y845}. The nucleus was counterstained blue with DAPI. We observed an increase of EGFR^{Y845} phosphorylation, localized predominantly to the plasma membrane, after 5 min, which became more prominent with time up to 20 min (Fig. 2D).

Zinc-induced activation of EGFR is c-Src dependent

Because phosphorylation of EGFR^{Y845} has previously been shown to be attributed to c-Src transactivation, we next investigated whether Src^{Y418} was also activated under these experimental conditions. Fluorescent confocal microscopy of TamR cells stained red for Src^{Y418} showed clear plasma membrane-associated staining when stimulated with zinc and ionophore (Fig. 2C). This activation, similar to the profile observed for EGFR^{Y845} in the same figure, was also abolished in the presence of the zinc chelator TPEN (Fig. 2C, *bottom panel*), suggesting a zinc-dependent mechanism. We next confirmed this zinc-induced activation of Src^{Y418} by Western blot (Fig. 2E) and inhibition by 24 h pretreatment with the Src kinase inhibitor SU6656. Additionally, we demonstrated that the zinc-dependent activation of EGFR^{Y845} was also inhibited by pretreatment with this Src kinase inhibitor, suggesting that zinc activates the EGFR^{Y845} in a Src-dependent manner.

Zinc-induced activation of growth factor receptors and downstream signaling events

Zinc has been shown to inhibit protein tyrosine phosphatases (22), which have a role in regulating multiple receptor tyrosine kinases. We therefore investigated whether zinc treatment could change the activity of other receptor tyrosine kinases, such as ErbB2, -3, and -4 and IGF-1R. ErbB2 was probed with an antibody to ErbB2^{Y1248}, whereas ErbB3 and ErbB4 were immunoprecipitated with respective antibodies before blotting with a phosphotyrosine antibody. We observed a substantial zinc-induced

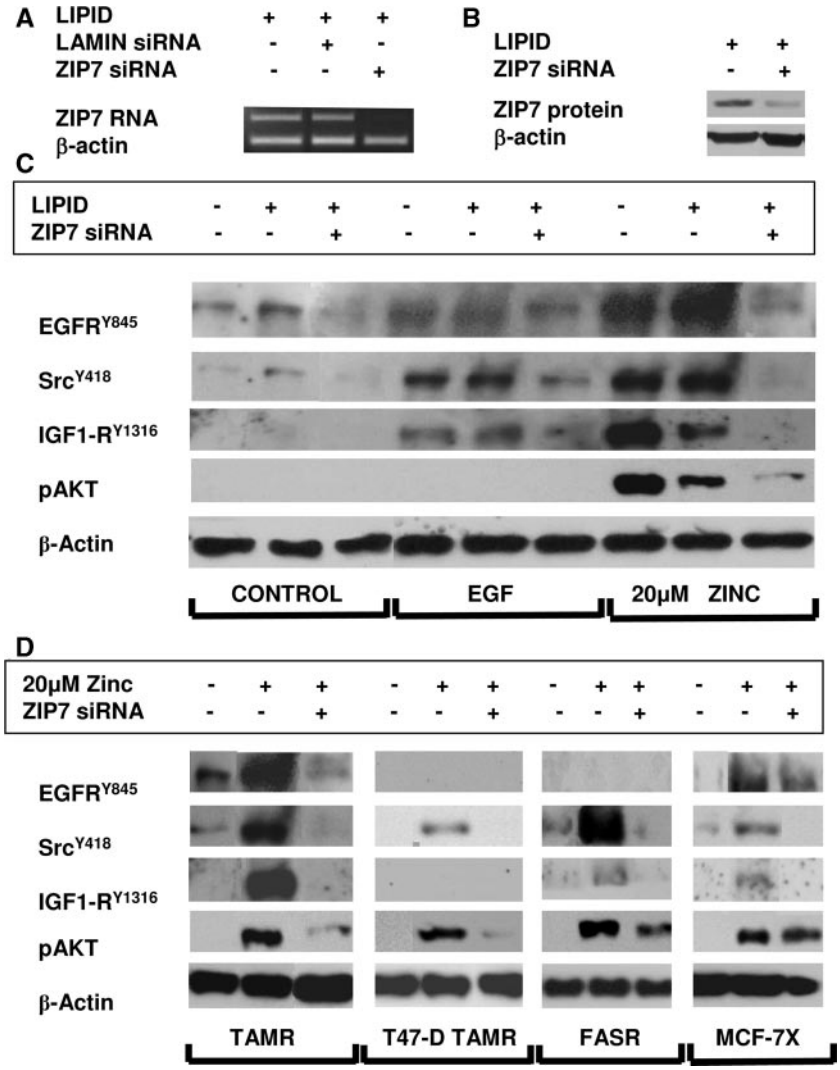

FIG. 4. ZIP7 is essential for zinc-dependent increase in signaling. A, PCR for ZIP7 48 h after TamR cells were transfected with siRNA for ZIP7, siRNA for lamin or lipid alone shows complete knockdown of ZIP7. B, Western blot for ZIP7 protein with ZIP7 antibody demonstrates a 75% reduction of ZIP7 protein after 72 h transfection with ZIP7 siRNA. C, Comparison of Western blot probed for activated $EGFR^{Y845}$, Src^{Y418}, $IGF\text{-}1R^{Y1316}$, and pAKT after transfection with siRNA for ZIP7 or compared with controls. Cells were either untreated or treated with EGF or 20 μM zinc and ionophore for 20 min before harvest. The presence of siRNA for ZIP7 prevents the zinc-induced activation of signaling molecules. D, ZIP7 siRNA reduces activation of signaling molecules in TamR, tamoxifen-resistant cells derived from T47-D, faslodex-resistant MCF-7 cells (FasR), and estrogen-deprived MCF-7 cells (X cells).

activation of ErbB2 and ErbB4 and some increase in ErbB3 (Fig. 3A). Additionally, zinc also promoted the activation of $IGF\text{-}1R^{Y1316}$. In all cases the zinc-induced activation was greater than that of EGF stimulated. Importantly, zinc also promoted the activation of MAPK (ERK1/2 phosphorylated at T202 and Y204) and AKT on S473 (Fig. 3B), as growth factor-induced downstream signaling events, although these molecules were also activated substantially by EGF. We further investigated whether the EGFR-specific tyrosine kinase inhibitor gefitinib would have any effect on the zinc-induced activation. Gefitinib reduced the zinc-induced activation of EGFR and AKT and eradicated MAPK activation but did not reduce the activation of Src (Fig. 3B).

ZIP7 is essential for zinc-induced activation of signaling pathways

To investigate whether the increased levels of ZIP7 observed in TamR cells had any effect on the activation of the signaling pathways demonstrated above, we reduced levels of ZIP7 using siRNA before harvesting the cell lysate and probing for signaling pathway activation. A considerable reduction in ZIP7 mRNA was observed after 48 h transfection (Fig. 4A) whereas presence of transfection lipid or lamin siRNA had no effect. Because the effects of siRNA on protein expression occur subsequent to gene knockdown, we investigated ZIP7 protein expression after 72 h transfection. Western blotting with the ZIP7Ab and subsequent densitometry revealed that ZIP7 protein was reduced by about 75% over this time, compared with control treatments (Fig. 4B). Having established the effectiveness of siRNA as a means of suppressing ZIP7 expression, cells were treated with ZIP7 siRNA for 72 h before treatment with EGF or zinc (20 μM) and ionophore *vs.* no treatment for 20 min to investigate the effect of ZIP7 loss on activation of intracellular signaling events. Cells untreated or treated with EGF exhibited some activation of signaling molecules with an apparent small reduction in the presence of siRNA for ZIP7 (Fig. 4C). However, the presence of siRNA for ZIP7 prevented zinc-induced activation of EGFR, Src, IGF-1R, and AKT. This same trend was observed in other cell lines such as TamR cells derived from T47-D, faslodex-resistant cells (FasR), and our in-house estrogen-deprived cells (MCF-7X cells, Fig. 4D).

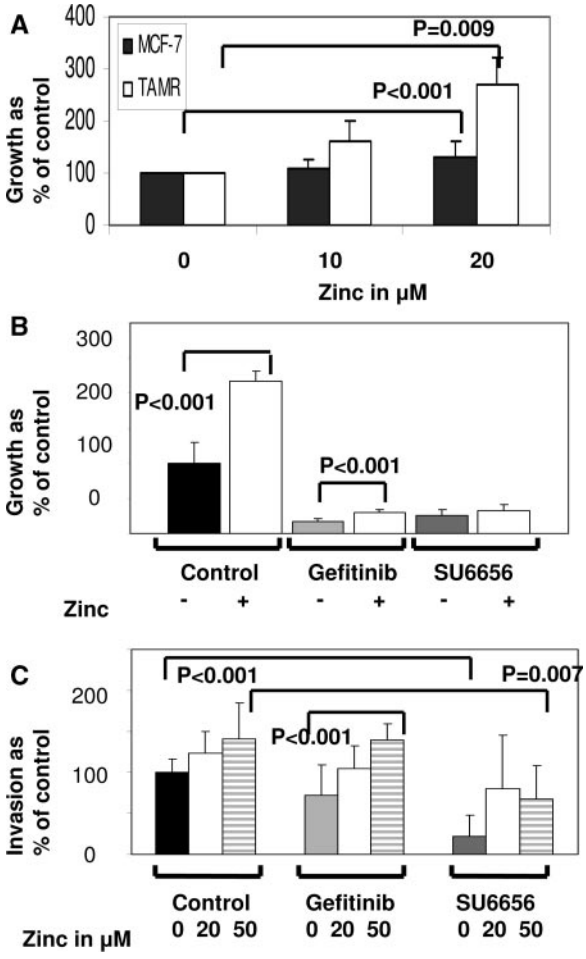

FIG. 5. Zinc induces growth and invasion of TamR cells. A, Effect of zinc concentration on growth of MCF-7 (■) and TamR (□) cells for 6 d. Graph depicts cell number as a percentage of untreated cells. Data are represented as mean ± SD. Zinc (20 μM) significantly increases the growth of both MCF-7 and TamR cells. B, Addition of 20 μM zinc (□) to TamR cells (■) does not prevent growth inhibition by either gefitinib or the Src inhibitor SU6656 as measured by cell count as a percentage of control over 6 d. C, Addition of 20 μM zinc (□) and 50 μM zinc (*striped bars*) increased the number of cells able to migrate through polycarbonate inserts coated with Matrigel after 72 h treatment, compared with controls (■). Addition of 20 μM (□) or 50 μM zinc (*striped bars*) prevented the gefitinib-induced reduction in invasive capability and lessened the SU6656-induced reduction in invasive capability.

Zinc increases growth and invasion of TamR cells

Having clearly demonstrated stimulatory effects of zinc on signaling molecules linked to cellular growth, we next investigated whether zinc would also have an effect on the actual rate of growth of either MCF-7 or TamR cells. MCF-7 and TamR cells were exposed to 0–20 μM zinc for 6 d (Fig. 5A). We observed a significant elevation in growth rate in zinc-treated TamR cells in comparison with untreated TamR and wild-type MCF-7 cells treated with 20 μM zinc. The elevated growth rate in TamR cells was considerably reduced by both gefitinib and the Src inhibitor, SU6656 (Fig. 5B). This result agrees with the observed gefitinib-induced reduction in activation of EGFR, AKT, and MAPK (Fig. 3B) and the SU6656-induced reduction in activation of EGFR and Src (Fig. 2E).

Given that zinc activates EGFR and Src and that both EGFR and Src can promote an aggressive invasive phenotype (4), we investigated the possible effects of zinc on tamoxifen-resistant cell invasiveness. In these assays, zinc treatment produced a small dose-dependent increase in invasive behavior (Fig. 5C), an effect that was significantly reduced by inclusion of the Src inhibitor, SU6656 (5 μM), but not gefitinib. We previously observed the more important role of Src than EGFR in driving invasion of TamR cells (4), which explains why gefitinib has less effect on reducing zinc-induced invasion than SU6656 (Fig. 5C).

ZIP7 controls intracellular zinc levels

To investigate whether the genetic manipulation of ZIP7 could alter the levels of intracellular zinc, cells were loaded with zinc-specific dyes and either imaged by fluorescent microscopy or analyzed by FACS. We observed that transfecting MCF-7 cells with ZIP7 for 24 h produced an increase in basal activation of EGFR, IGF-1R, and Src (Fig. 6A). This effect of ZIP7 was matched by a 50% basal increase in intracellular zinc in MCF-7 cells, comparable with that seen in the TamR cells, and a considerably enhanced ability to increase intracellular zinc levels when ZIP7 transfected MCF-7 cells were treated with zinc (Fig. 6B) and compared with controls. Furthermore, the presence of siRNA for ZIP7 was able to decrease zinc-induced increases in intracellular zinc as observed by fluozin-3 or zinquin fluorescence (Fig. 7A). The presence of siRNA for ZIP7 additionally reduced the zinc-induced increase of intracellular zinc when measured by FACS analysis (Fig. 7B) using cells loaded with Newport green. Furthermore, the presence of siRNA for ZIP7 was able to reduce by 40% the number of cells able to migrate across fibronectin (Fig. 7C).

Discussion

Here we demonstrate that our model of tamoxifen-resistant (TamR) breast cancer cells have increased intracellular zinc, compared with their hormone-responsive (wild type) counterparts; accompanying this is an increased expression of ZIP7 (SLC39A7/HKE4), a member of the LIV-1 family of ZIP zinc transporters, previously demonstrated capable of increasing intracellular zinc concentration (16). Consequently, treatment of TamR cells with physiological levels of zinc promotes the Src-dependent stimulation of tyrosine kinase receptor signaling pathways, which may contribute to the aggressive phenotype of these cells. Furthermore, reduction of ZIP7 levels in TamR cells by siRNA not only prevents the observed zinc-stimulated activation of receptor and nonreceptor tyrosine kinases associated with the aggressive phenotype of these cells (2–4) but also prevents the necessary increase in intracellular zinc needed to achieve this. These results raise the intriguing possibility that altered zinc levels, as a direct result of the aberrant expression of a member of the LIV-1 family of zinc transporters, may promote resistance to agents such as tamoxifen and provides ZIP7 as a new clinical target for diseases such as cancer, in which it would

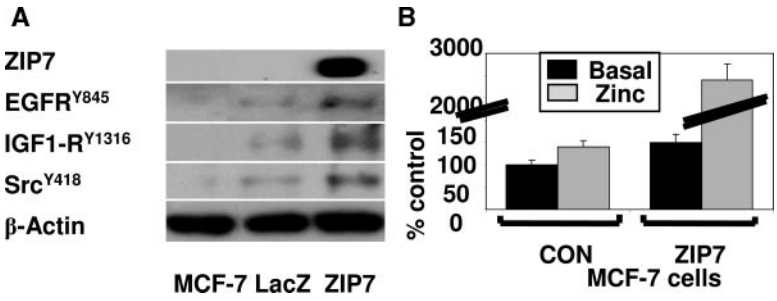

FIG. 6. ZIP7 transfection into MCF-7 cells increases intracellular zinc levels. A, Increased expression of recombinant ZIP7 in MCF-7 cells transfected with ZIP7 for 24 h that was not evident in the controls or those transfected with LacZ (*top row*). MCF-7 cells transfected with ZIP7 showed increased basal activation of EGFR, IGF-1R, and Src. B, MCF-7 cells transfected with ZIP7 for 24 h, loaded with the zinc-specific dye Newport green, were treated with 20 μM zinc and ionophore for 20 min and mean fluorescence measured by FACS analysis. Results are expressed in percent control. *Lines across scale* were necessitated by the large increase in fluorescence in transfected cells. Results are means of three separate experiments.

be advantageous to prevent zinc-induced inhibition of multiple phosphatases.

Interestingly, although ZIP7 is a member of a family of ZIP transporters present in breast cancer cells, its cellular location is unique. Thus, whereas most of the other ZIP transporters are present on the plasma membrane, we suggest that the location of ZIP7 on the endoplasmic reticulum membrane enables it to play a critical role in mobilizing intracellular zinc and thereby regulating its potential role in growth factor signaling. In this context two pieces of data have recently come to light. First, Yamasaki *et al.* (23) demonstrated the presence of a calcium-dependent zinc wave in mast cells, accounting for the widespread inhibition of phosphatases that has been previously attributed to zinc (22). This zinc wave was proposed to originate in the endoplasmic reticulum, yet the exact mechanism of its release was unknown. Second, a model for zinc handling within cells has been proposed by Colvin *et al.* (24), which predicts that intracellular zinc is associated with a muffler in the cytoplasm such as metallothionen that allows it to be strongly buffered and subsequently distributed into a deep store, such as the endoplasmic reticulum compartment before release to the cytoplasm. Our proposed role for ZIP7 in the control of intracellular zinc homeostasis (Fig. 8) is consistent with both these models and relies on the ability of ZIP7 to release zinc from intracellular stores, such as the endoplasmic reticulum. This fits the data presented here providing the zinc entering the cell is distributed directly to the endoplasmic reticulum via some unknown mechanism, such as the proposed muffler (24) and then released in a wave by a ZIP7-dependent mechanism, causing the zinc-induced inhibition of phosphatases, consistent with other reports (23).

The lack of an apparent rise in intracellular zinc levels in the presence of siRNA for ZIP7 may be considered confusing in light of the fact that there are other zinc transporters present on the plasma membrane capable of transporting

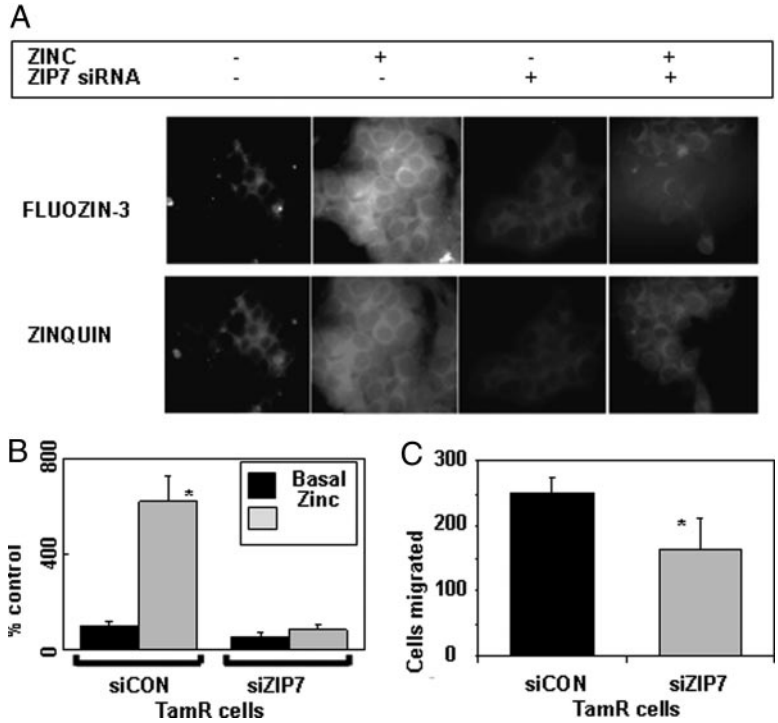

FIG. 7. ZIP7 siRNA reduces intracellular zinc levels and migration of TamR cells. A, TamR cells on coverslips ± ZIP7 siRNA, loaded with the zinc-specific dyes fluozin-3 or zinquin, were treated with 20 μM zinc and ionophore for 20 min and imaged for fluorescence by microscopy. The presence of siRNA for ZIP7 reduced intracellular zinc. B, TamR cells ± ZIP7 siRNA, loaded with the zinc-specific dye Newport green, were treated with 20 μM zinc and ionophore for 20 min and mean fluorescence measured by FACS analysis. Results are means of three experiments expressed as percent control ± SD. *, $P < 0.001$. ZIP7 siRNA prevents intracellular zinc increase. C, The presence of siRNA for ZIP7 significantly reduces the number of TamR cells able to migrate through matrigel in 72 h. *, $P = 0.013$.

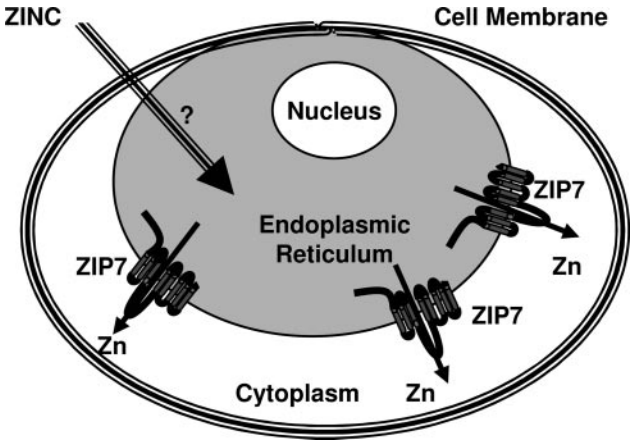

FIG. 8. Proposed mechanism of action of ZIP7 in distributing intracellular zinc. In these experimental conditions, the only possible explanation for the results reported here is depicted in this schematic. The zinc entering the cell must be distributed directly to the endoplasmic reticulum in which it is released into the cytoplasm by ZIP7. Removal of ZIP7 by siRNA reduces a detectable pool of intracellular zinc, whereas transfection of ZIP7 into cells increases this pool. *Zn* represents labile Zn^{2+}.

zinc into the cells. We believe that our proposed model (Fig. 8) is consistent with this for the following reason. Our data collectively agrees with the work of Hirano's group (23) and Colvin *et al.* (24) that all zinc that gets transported into cells is muffled and transported to the endoplasmic reticulum store, by a currently unknown mechanism, before release into the cytoplasm by ZIP7 assuming that the muffled zinc and that within the store are not accessible to the zinc dyes used here. In the time frame of these experiments, the zinc indicators are therefore measuring zinc released from the endoplasmic reticulum rather than the amount of zinc transported into the cell. It is probable that other zinc transporter family members on the plasma membrane are also transporting zinc into these cells; however, using this model, any such transported zinc would not be accessible to the zinc dyes if it was muffled and consolidated within the endoplasmic reticulum, and thus in agreement with the data presented here. The emergence of zinc dyes that are capable of measuring zinc in stores such as the ER will aid confirmation of this mechanism. This is an important finding with implications for treatment of diseases such as cancer and provides ZIP7 as a potential new and novel target for inhibiting intracellular zinc-dependent mechanisms, such as activation of tyrosine kinases.

We recently studied the expression of all nine human members of the LIV-1 family of ZIP zinc transporters in endocrine-resistant breast cancer (31), and ZIP7 is the only family member increased in all resistant models, suggesting an important mechanism with clinical relevance to different types of antihormone resistance. This was confirmed here by observing that removal of ZIP7 was able to prevent signaling activation in other cell models (Fig. 4D).

There is a paucity of information available in the literature concerning zinc-induced effects on signaling molecules. However, our observed ligand-independent activation of EGFR by zinc in a Src-dependent manner in tamoxifen-resistant breast cancer cells is in agreement with that observed by others in different cell lines. Zinc ions have been shown to induce gene expression as a downstream effect of activating growth factor signaling pathways (32), including activation of EGFR at Y845 by transactivation via c-Src in human epidermoid carcinoma A431 cells (25, 33) and human bronchial epithelial cells, BEAS (34). Downstream activation of protein tyrosine phosphorylation and MAPK signaling by zinc in Swiss 3T3 fibroblasts (35) has also been demonstrated. Although the mechanism of action of zinc impact on cell signaling is only now beginning to be elucidated, to our knowledge, zinc has not previously been linked to the response of breast cancer cells to antihormonal therapy or disease progression in such cells. Significantly, in previous studies using levels of zinc as high as 200 μM, the activation of receptor tyrosine kinases was shown to be due to the zinc-induced inhibition of the protein tyrosine phosphatase (PTP)-1B (22, 36). This mechanism of action would agree with the results reported here and explain the apparent prolonged activation of EGFR (Fig. 2D) observed because we previously demonstrated that the optimum time for EGFR activation by EGF is 5 min or less in our TamR cells (2). This is consistent with zinc inhibiting phosphatases and delaying the removal of activated signaling molecules such as EGFR from the plasma membrane. Because the known substrates for the phosphatase PTP1B include EGFR, insulin receptor substrate (IRS)-1/2, IGF-1R, and Src (36), inhibition of PTP1B in our cells might affect EGFR directly or indirectly via Src. Additionally, however, we have recently shown that TamR cells harness IGF-1R/IRS-1 signaling to support EGFR activation (6), and a prolongation of such responses would thus also aid aggressive cell behavior. A role of zinc in insulin and IGF receptor-1 signaling has already been documented. Zinc has an insulin-like effect and is essential for the induction of cell proliferation by IGF-I (37, 38), whereas dietary zinc deficiency causes growth retardation associated with reduced circulating IGF-I concentrations (39). Increase of cellular zinc caused by incubation with zinc and ionophore, sodium pyrithione, augments protein tyrosine phosphorylation, in particular the phosphorylation of three activating tyrosine residues of the IGF-1R and IRS-1, whereas zinc chelation attenuates insulin/IGF-I-induced phosphorylation signals (26).

The zinc-induced activation of the nonreceptor tyrosine kinase, Src, will have additional consequences because Src is increasingly implicated in the activation of proliferation, angiogenesis, survival, and increases in motility and invasive capability (40). As a result, Src is already a target in clinical disease. However, our results (Fig. 5C) demonstrate that even zinc levels as low as 20 μM were able to partially reverse the inhibition of invasion by use of a Src inhibitor, suggesting that targeting the zinc levels in cells may be extremely fruitful.

In summary, this study provides evidence that the level of intracellular zinc may be pivotal to characterizing the response of antihormone-resistant cancers to treatment with agents that target growth factor pathways. We suggest a mechanism for the observed increase in intracellular zinc in our TamR cells due to the increased expression of the zinc transporter ZIP7 with the consequence of activation of a variety of signaling pathways that in turn increase growth and invasion, leading to a more aggressive phenotype, which can be associated with antihormone resistance in the clinic.

Given its ubiquitous and physiological role in cells, it would likely prove difficult to selectively target zinc in cancer cells in which elevated levels may be a problem. However, our data demonstrate that effective suppression of zinc-induced signaling pathway activation can be achieved by targeting ZIP7 with siRNA. This strategy would have the additional bonus, due to the proposed zinc-induced inhibition of phosphatases, of an ability to target multiple growth factor pathways, increasing tumor kill and preventing the subsequent development of resistance.

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