Hyperactive mTOR and MNK1 phosphorylation of eIF4E confer tamoxifen resistance and estrogen independence through selective mRNA translation reprogramming

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The majority of breast cancers expresses the estrogen receptor (ER⁺) and is treated with anti-estrogen therapies, particularly tamoxifen in premenopausal women. However, tamoxifen resistance is responsible for a large proportion of breast cancer deaths. Using small molecule inhibitors, phospho-mimetic proteins, tamoxifen-sensitive and tamoxifen-resistant breast cancer cells, a tamoxifen-resistant patient-derived xenograft model, patient tumor tissues, and genome-wide transcription and translation studies, we show that tamoxifen resistance involves selective mRNA translational reprogramming to an anti-estrogen state by *Runx2* and other mRNAs. Tamoxifen-resistant translational reprogramming is shown to be mediated by increased expression of eIF4E and its increased availability by hyperactive mTOR and to require phosphorylation of eIF4E at Ser209 by increased MNK activity. Resensitization to tamoxifen is restored only by reducing eIF4E expression or mTOR activity and also blocking MNK1 phosphorylation of eIF4E. mRNAs specifically translationally up-regulated with tamoxifen resistance include *Runx2*, which inhibits ER signaling and estrogen responses and promotes breast cancer metastasis. Silencing *Runx2* significantly restores tamoxifen sensitivity. Tamoxifen-resistant but not tamoxifen-sensitive patient ER⁺ breast cancer specimens also demonstrate strongly increased MNK phosphorylation of eIF4E. eIF4E levels, availability, and phosphorylation therefore promote tamoxifen resistance in ER⁺ breast cancer through selective mRNA translational reprogramming

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Estrogen receptor-positive (ER⁺) breast cancers comprise the majority (70%–80%) of breast cancers and the majority of breast cancer deaths resulting from metastatic disease (Fisher et al. 2004; Early Breast Cancer Trialists' Collaborative Group 2005). Anti-estrogen therapy with tamoxifen remains a cornerstone of therapy for ER⁺ premenopausal breast cancer, but resistance occurs in a third of patients and often progresses to metastasis and death (Musgrove and Sutherland 2009; Droog et al. 2013). ER drives survival and proliferation pathways in breast cancer (Fullwood et al. 2009), functions as a nuclear hormone receptor responsible for integrating signals relayed by estrogen, and plays a critical role in breast cell transformation and carcinogenesis (Sommer and Fuqua 2001). Of the two main isoforms, ERα is implicated primarily in the onset of breast cancer (Sommer and Fuqua 2001). ERα binds transcriptional coactivators and regulators (e.g., NCOA1, NCOA2, and NCOA3) that specify differential transcriptional activity (Sommer and Fuqua 2001; Oxelmark et al. 2006; Simpson et al. 2010). Tamoxifen is an ER-

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antagonizing small molecule that blocks ER transcriptional activity (Osborne et al. 2000) and inhibits ER^+ breast cancer cell proliferation and survival (Osborne et al. 2000).

Most tamoxifen resistance in breast cancer does not involve loss of ERα receptor expression, although mutations are common (Garcia-Quiroz et al. 2014). Tamoxifen resistance often involves hyperactivation of epidermal growth factor receptors (EGFRs) through a variety of mechanisms, including PI3K mutation and activation and MAPK–ERK activation (Campbell et al. 2001; Clark et al. 2002; Miller et al. 2010), resulting in uncoupled signaling from tamoxifen blockade (deGraffenried et al. 2004; Miller et al. 2011; Osborne and Schiff 2011). Both EGFR and ERα signaling can hyperactivate the MAPK–ERK and mTOR pathways, which are known effectors of tamoxifen resistance (Schiff et al. 2004; Massarweh et al. 2008; Miller et al. 2010, 2011; Sabnis and Brodie 2011; Baselga et al. 2012; Bostner et al. 2013; Beelen et al. 2014a,b; Karthik et al. 2015). mTOR consist of two complexes: mTORC1 and mTORC2. mTORC1 regulates protein synthesis, lipid synthesis, and ribosome biogenesis (Dancey 2010; Laplante and Sabatini 2012) and includes the proteins mTOR, Raptor, and GβL, among others (Sabatini 2006). mTORC1 phosphorylates (inactivates) the negative regulator of cap-dependent mRNA translation known as the eIF4E-binding protein (4E-BP1). mTORC2 includes the proteins mTOR, Rictor, and GβL, among others; regulates cytoskeleton organization in response to growth signals; and promotes cell survival and proliferation through activation of AKT (Zoncu et al. 2011). Increased signaling through these pathways is often caused by up-regulated expression of EGFR and IGF-IR proteins, which is also seen in the majority of tamoxifen-resistant ER^+ breast cancers and tamoxifen-resistant cell lines (Treeck et al. 2006; Cottu et al. 2014).

Investigation of mTOR-directed endocrine resistance mechanisms have focused primarily on pathway cross-talk and activating upstream mutations. Phosphorylation by S6 kinase 1 (S6K1), a target of mTORC1, establishes endocrine-independent activation of ERα (Yamnik and Holz 2010). Similarly, EGFR activation by dimerization through activating mutations or ligands stimulates both mTORC1/2 and MAPK–ERK pathways and is also associated with tamoxifen and endocrine therapy resistance (Nicholson et al. 2004). Moreover, increased AKT signaling is associated with resistance to anti-hormonal therapy, and, accordingly, inhibition of mTOR partially restores sensitivity (deGraffenried et al. 2004; Beeram et al. 2007). Furthermore, inhibiting both mTORC1/2 complexes blocks upstream AKT activation and increases resensitization to anti-endocrine agents (Leung et al. 2010; Jordan et al. 2014). However, little is known regarding the molecular basis for tamoxifen resistance downstream from mTOR, apart from pathway cross-talk. Downstream effectors of mTOR activity in tamoxifen and endocrine resistance remain unknown. mTOR and ERK signaling pathways converge on the control of mRNA translation (Silvera et al. 2010). Translation of mRNA begins with recognition of the 5′ inverted methyl^7-GTP "cap" structure by the translation initiation complex consisting of cap-binding protein eIF4E, RNA helicase eIF4A, and scaffolding protein eIF4G, which recruits the 40S ribosomal subunit and other initiation factors. Many of the translation initiation factors are regulated by mTORC1 activity. In particular, mTOR hyperphosphorylates the 4E-BPs (4E-BP1 is the major form in epithelial cells), preventing them from sequestering eIF4E by competing with the scaffolding protein eIF4G, thereby promoting translation. mRNA translation is also regulated by the MAPK/ERK pathway in response to growth factors, cytokines, and oncogenic signaling (Topisirovic and Sonenberg 2011b). ERK acts on mRNA translation by activating the eIF4G-associated kinase MNK1. MNK1 phosphorylates eIF4E at S209, which is associated with increased transformation potential, although a mechanism is lacking (Wendel et al. 2007; Silvera et al. 2010; Wheater et al. 2010; Konicek et al. 2011; Topisirovic and Sonenberg 2011b; Wolfe et al. 2014). The nature of the increased requirement for eIF4E with malignancy is thought to involve selective mRNA translation, but the mechanism is complex and remains only partially understood (Waskiewicz et al. 1999; Topisirovic and Sonenberg 2011a; Bhat et al. 2015). Up-regulation of the abundance and/or activity of eIF4E, eIF4A, and/or eIF4G occurs widely in breast and other cancers and selectively up-regulates translation of certain mRNAs involved in survival, proliferation, and metastasis (Avdulov et al. 2004; Braunstein et al. 2007; Kim et al. 2009; Silvera et al. 2010; Badura et al. 2012; Decarlo et al. 2015). In fact, increased abundance of eIF4E has been shown to be important in resistance to a variety of PI3K–AKT–mTOR inhibitors (Avdulov et al. 2004; Kim et al. 2009; Silvera et al. 2009a, 2010; Bitterman and Polunovsky 2010; Hsieh et al. 2010; Ilic et al. 2010; Burris 2013; Bhat et al. 2015; Fagan et al. 2017).

Certain mRNAs possess long or structured 5′ untranslated regions (UTRs) that serve to more highly regulate their translation and often encode transforming and survival proteins important for cancer development and progression (Koromilas et al. 1992; Svitkin et al. 2005; Badura et al. 2012). These mRNAs typically display a greater requirement for eIF4E, often a result of increased secondary structure close to the cap (Koromilas et al. 1992; Badura et al. 2012; Hsieh et al. 2012). Certain sequence motifs are also thought to increase the requirement for eIF4E interaction, although direct binding by eIF4E has not been shown (Hsieh et al. 2012). Here we show that an essential mechanism of tamoxifen resistance involves genome-wide translational reprogramming to select for the translation of mRNAs that specifically provide anti-estrogen and ER activities and requires increased expression and availability of eIF4E and its increased phosphorylation by MNK1. Only blockade of both mTORC1 and MNK1 re-establishes tamoxifen sensitivity and blocks selective translation of the small group of mRNAs that provide tamoxifen resistance. We propose a mechanism by which increased levels and phosphorylation of eIF4E promote selective translation of certain mRNAs.

Results

Increased eIF4E abundance, eIF4E S209 phosphorylation, and mTORC1 and MNK activity in tamoxifen-resistant breast cancers and cell lines

We characterized established MCF7 tamoxifen-responsive LCC1 cells (referred to here as TamS cells) and isotype-matched tamoxifen-resistant LCC9 cells (referred to here as TamR cells) used widely as a clinically relevant model of tamoxifen therapy resistance (e.g., Brunner et al. 1997; Clarke et al. 2003; Howell et al. 2004). Tamoxifen-sensitive cells demonstrate impaired growth and fail to transition through G_1 to S phase during treatment (Brunner et al. 1997), consistent with the primary inhibitory effect of tamoxifen. TamR cells are resistant to inhibition and maintain normal proliferation and survival to clinically relevant doses of tamoxifen (Supplemental Fig. S1A–C). An ER+ tamoxifen-resistant patient-derived xenograft (PDX) known as BR7 was also insensitive to tamoxifen, as shown by normal cell cycle distribution despite tamoxifen treatment (Supplemental Fig. S1D). TamR and BR7 cells were insensitive to tamoxifen-induced repression of canonical ER signaling as well, in contrast to TamS cells (Supplemental Fig. S1E,F). mTORC1 activity, measured by phosphorylation of 4E-BP1 (S65) and ribosomal protein S6, was elevated in TamR compared with TamS cells (Fig. 1A), as were eIF4E levels (Fig. 1B) and eIF4E phosphorylation (Fig. 1C, normalized for eIF4E levels). eIF4E levels and S209 phosphorylation were also increased in BR7 (PDX) cells compared with TamS cells (Fig. 1D). Therefore, increased mTORC1 and MNK pathway activity is associated with endocrine resistance in cell lines and in a PDX model of endocrine-resistant disease. Increased levels of eIF4E do not typically increase overall protein synthesis very strongly, which was also observed here (Fig. 1E). However, increased levels of eIF4E can selectively increase the translation of specific mRNAs in different physiological conditions (Avdulov et al. 2004; Holcik and Pestova 2007; Pelletier et al. 2015; Truitt et al. 2015). Importantly, there were no significant differences in the levels of other key translation factors in TamR compared with TamS cells, apart from the increased expression of eIF4E (Supplemental Fig. S2A).

We investigated biopsy specimens from ER+ invasive intraductal breast cancer patients who progressed on treatment (de novo resistance) or recurred within 5 yr of tamoxifen treatment, a standard for resistance, compared with nonrecurrent treated tumors at 10 yr. Despite the small sample size (due to difficulty in obtaining well-validated resistant and sensitive tumor specimens), tamoxifen-resistant tumors showed significantly increased eIF4E S209 phosphorylation (Mnk1 activation) compared with tamoxifen-sensitive tumors (Supplemental Table S1, $P = 0.05$), as did tamoxifen- or aromatase-resistant tumors ($P = 0.016$) (Supplemental Table S2). Given the fact that mTORC1 is already highly active and that eIF4E is already overexpressed as a driver of breast cancer, it is not surprising that there was only a trend toward increased mTORC1 activity (P-4E-BP1) and slightly increased eIF4E levels with tamoxifen or aromatase resistance that

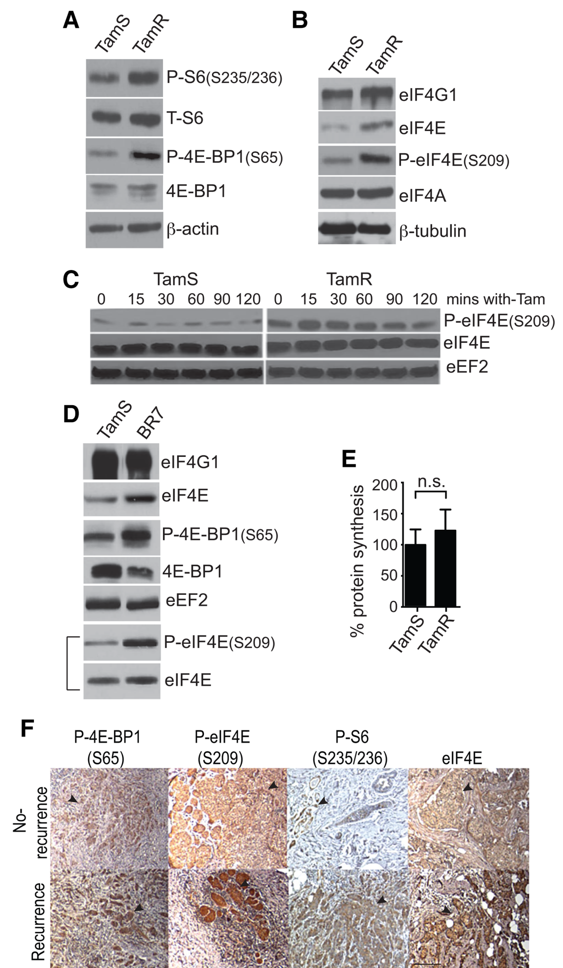

Figure 1. mTORC1 and MAPK pathway hyperactivation in tamoxfien resistance in ER+ breast cancer. (*A*) Immunoblot of TamS and TamR cells lysed in NP-40 buffer during exponential growth and probed for mTORC1 target proteins. β-Actin was used as a loading control. (*B*) Immunoblot of representative TamS and TamR cells lysed in NP-40 buffer and probed for the eIF4F complex proteins. eIF4A was also used as a loading control. (*C*) Immunoblot analysis of protein lysates from TamS and TamR cells following 4-hydroxytamoxifen (4-OHT) treatment in serum-free medium. Cells were treated using serum-free medium, with the 0 time point indicating untreated control samples using ImageJ software. eEF2 was used as a loading control. The bracketed eIF4E-P and eIF4E blots were normalized to loading equal levels of eIF4E. All other blots used equal protein amounts. The image is representative of three independent experiments. (*D*) NP40 cytoplasmic protein extracts were subjected to immunoblot as shown. Representative results comparing TamS with BR7 PDX cells are shown. eIF4E-P and eIF4E blots were normalized to loading equal levels of eIF4E. All other blots used equal protein amounts. (*E*) The overall protein synthesis activity of TamS and TamR cells was measured by [^{35}S]-methionine metabolic labeling normalized to TamR cells. A representative of three independent experiments is shown. (n.s.) Not significant. (*F*) Immunohistochemical staining of representative recurrent and nonrecurrent tumor specimens for P-4E-BP1 (S65), P-S6 (S235/236), P-eIF4E (S209), and total eIF4E. Bar, 20 µm. The immunoblots shown are representative of three independent experiments.

did not reach statistical significance. The lower saturation level of immunohistochemistry compared with immunoblot may also contribute to the smaller detectable increase in eIF4E levels, although it was apparent in many of the specimens (Fig. 1F).

Reduced overexpression of eIF4E and its S209 phosphorylation are required to restore tamoxifen sensitivity to resistant cells

The role of eIF4E-selective mRNA translation in endocrine therapy resistance was tested by stably transducing TamS and TamR cells with doxycycline (Dox)-inducible shRNAs targeting the 3′ UTR of eIF4E. Quantitative RT–PCR (qRT–PCR) and immunoblot analysis showed an average fourfold reduction of eIF4E mRNA and protein levels (Fig. 2A,B). Interestingly, whereas levels of eIF4E silencing were similar in both cell lines, it resulted in a larger (50% greater) reduction in overall protein synthesis only in TamR cells, indicating a moderate addiction to elevated levels of eIF4E with the acquisition of tamoxifen resistance (Fig. 2C).

We therefore asked whether the increased expression and/or phosphorylation of eIF4E is essential for selective mRNA translation and tamoxifen resistance. Silencing eIF4E in the presence of 4-hydroxytamoxifen (4-OHT), the active metabolite of tamoxifen, reduced clonogenic cell survival and cell growth of TamR cells by fourfold compared with the nonsilencing control and threefold to fourfold compared with silenced but untreated TamR cells (Fig. 2D; Supplemental Fig. S2B,C). Similarly inducible overexpression of 4E-BP1 in TamR-resistant cells (Fig. 2E) reduced eIF4E cap-binding complexes (Supplemental Fig. S2D) and resulted in a fourfold reduction in cell proliferation and survival of tamoxifen-treated TamR cells compared with controls (Fig. 2F,G). Moreover, silencing eIF4E in the BR7 PDX model restored tamoxifen sensitivity, as shown by delayed cell cycling in response to treatment (Supplemental Fig. S2E). Everolimus (RAD001) is an inhibitor of the mTORC1 signaling pathway and is currently approved for the treatment of hormone receptor-positive endocrine therapy-resistant breast cancers. To confirm the involvement of mTORC1 in endocrine resistance, TamR cells were treated with RAD001, which partially resensitized them to tamoxifen, as shown by the restoration of inhibition of cell proliferation and decreased survival (Supplemental Fig. S2F,G). Both increased mTORC1 activity and eIF4E availability are therefore required for tamoxifen resistance. Collectively, these data suggest a critical role for the eIF4E/4E-BP1 balance in regulating tamoxifen resistance and responsiveness by mTORC1 activity.

A requirement for eIF4E S209 phosphorylation (MNK1-mediated) has been implicated in tumorigenesis and metastasis (Bianchini et al. 2008; Wheater et al. 2010; Robichaud et al. 2015), and we observed increased eIF4E S209 phosphorylation in tamoxifen-resistant breast tumor tissues and cell lines. We therefore examined the role of eIF4E phosphorylation in tamoxifen resistance. A serine-to-alanine HA-tagged eIF4E protein (S209A) or a serine-to-aspartic acid protein (S209D) was expressed in

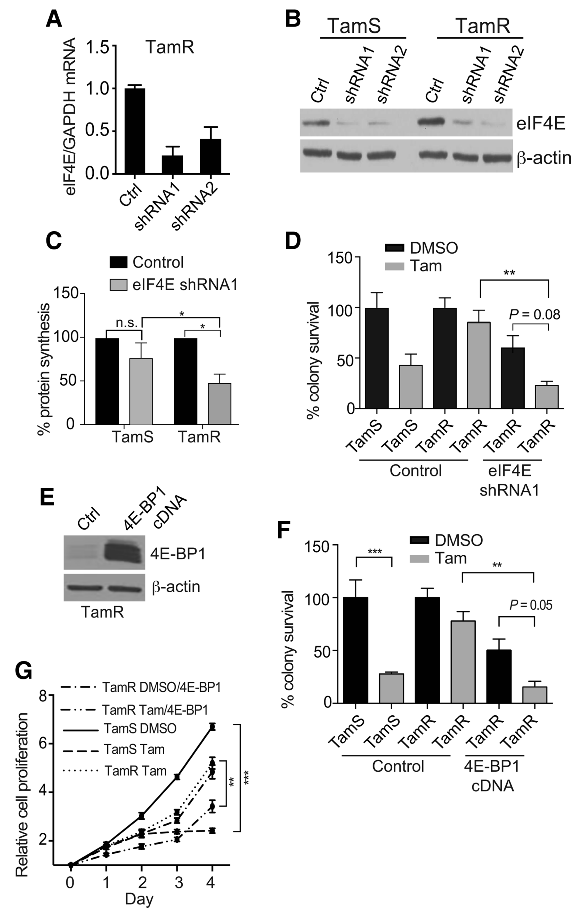

Figure 2. Blocking eIF4F complex formation by targeting eIF4E partially restores tamoxifen sensitivity. (*A*) mRNA expression of eIF4E in TamR cells following 72 h of 1 µg/mL Dox induction of eIF4E shRNAs. Equal amounts of RNA were quantified by quantitative real-time PCR (RT-qPCR) and normalized to GAPDH using the −ΔΔCt method. (*B*) Immunoblot of equal amounts of protein from NP-40-extracted TamS and TamR sh-control or sh-eIF4E cells 72 h after Dox addition. β-Actin was used as a loading control. Representative immunoblots are shown. (*C*) Overall protein synthesis activity of TamS and TamR cells with or without eIF4E silencing by [^{35}S]-methionine metabolic labeling. Three independent studies were averaged. (*) $P < 0.05$ by two-way ANOVA; (n.s.) not significant. (*D*) Colony survival growth assay was performed by low-density seeding (1000 cells) of stably transduced TamS and TamR cells treated with vehicle (DMSO) or 1 µM 4-OHT 24 h after plating. Dox (1 µg/mL) was administered 24 h after plating and removed at 72 h. Colonies were scored after 10 d, counting only ≥50 cells per colony. Results from three independent experiments were normalized to DMSO control. (**) $P < 0.01$. Comparisons were by two-way ANOVA. (*E*) Representative immunoblot of equal amounts of protein lysate from 4E-BP1-overexpressing cells. Dox (1 µg/mL) was added 72 h prior to lysis in NP-40 buffer. β-Actin was used as a loading control. (*F*) Colony survival assays were performed as in *D* after plating with Dox-induced 4E-BP1 expression. (**) $P < 0.01$; (***) $P < 0.001$ by two-way ANOVA. (*G*) Cell proliferation was assayed with Dox-induced overexpression of 4E-BP1, as shown. Cell proliferation was assayed by MTT and treated with vehicle (DMSO) or 1 µM 4-OHT. Dox was added at day 0 to induce 4E-BP1 expression. Results from three independent experiments were normalized to day 0. (**) $P < 0.01$; (***) $P < 0.001$ by *t*-test.

TamS and TamR cells (Supplemental Fig. S3A). Endogenous eIF4E was silenced to eliminate its contribution, and cells were assayed for proliferation in the presence or absence of 4-OHT (Fig. 3A,B). Notably, TamR cells were blocked in proliferation by expression of the nonphosphorylated S209A eIF4E mutant only in the presence of tamoxifen (implicating an essential role for MNK1-mediated eIF4E phosphorylation at S209) and overexpression of eIF4E in tamoxifen resistance. However, expression of the S209D phosphomimetic eIF4E protein in TamS cells did not confer tamoxifen-resistant proliferation. These data suggest two possibilities: that acquisition of tamoxifen resistance is multigenic and not solely the result of eIF4E overexpression and phosphorylation (whereas resistance can be reversed by impairing either because both are important) and/or that the phospho-mimetic eIF4E variant protein cannot fully recapitulate the effects of eIF4E phosphorylation, consistent with a previous report (Topisirovic et al. 2004).

We next investigated the effect of inhibition of MNK1 on tamoxifen sensitivity by the small molecule MNK1 inhibitor CGP57380. Dose escalation studies on both TamS and TamR cells established a concentration of 10 µM for complete inhibition of eIF4E phosphorylation by CGP57380 (identical in dose to previously reported) (Fig. 3C), where it has no inhibitory activity on other families of related kinases (p38, JNK1, and RSKs) (Knauf et al. 2001; Rowlett et al. 2008). This was confirmed in TamR cells by examining ATF2 phosphorylation at Thr69/71 (a target of RSK, JNK1, and p38 MAPK) and eIF4B S422 (a target of RSKs)—the next most sensitive kinases of CGP57380 inhibition. There was no change in phosphorylation of either protein with treatment (Supplemental Fig. S3B). Combined treatment with tamoxifen and CGP57380 significantly resensitized TamR cells to tamoxifen, as shown by a >60% reduction in proliferation and clonogenic survival compared with untreated controls (Fig. 3D,E). As described previously, cotreatment with mTORC1 inhibitor RAD001 produced a further additive reduction in cell survival (Fig. 3F; Supplemental Fig. S3C), with only a 10% reduction in global protein synthesis despite complete ablation of eIF4E phosphorylation (Supplemental Fig. S3D). Polysome profiling of tamoxifen-resistant cells following CGP57380 treatment showed no significant differences (Supplemental Fig. S3E), suggesting a role for eIF4E phosphorylation in selectively reprogramming the translation of a small subset of mRNAs involved in tamoxifen resistance.

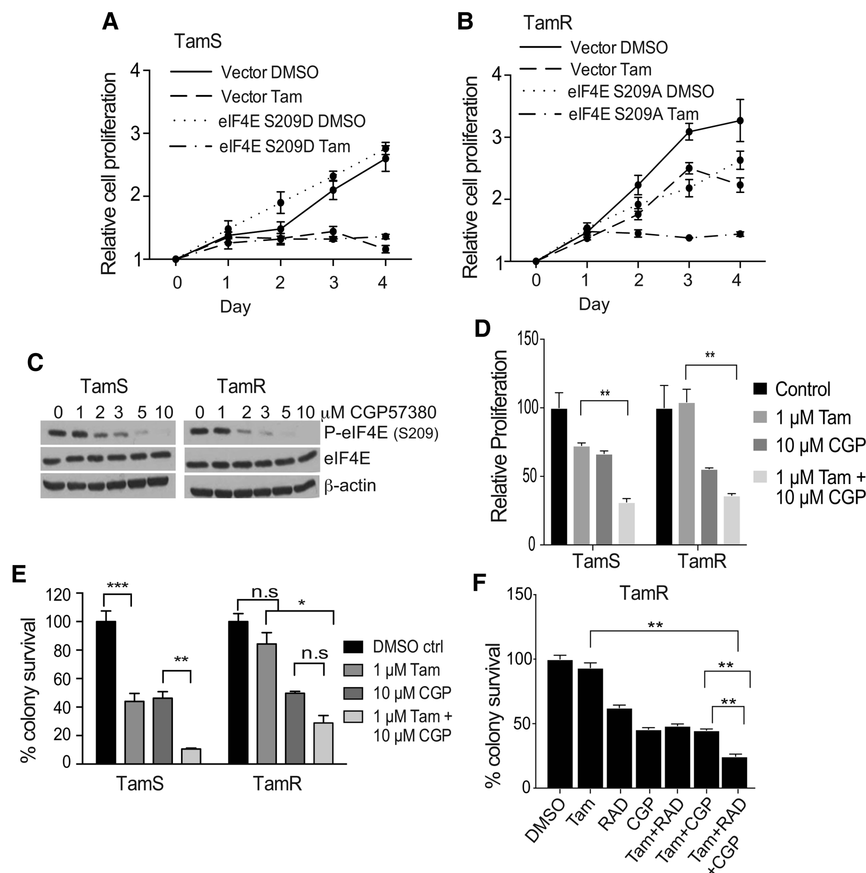

Figure 3. eIF4E S209 phosphorylation promotes tamoxifen resistance. (*A*) TamS cells were transfected with either empty vector or an eIF4E S209D-expressing construct 48 h prior to proliferation assay. Endogenous eIF4E was silenced by shRNA. Cell proliferation was assayed by MTT assay with cells treated with DMSO vehicle or 1 µM 4-OHT. Results are from three independent experiments. (*B*) TamR cells were transfected with empty vector or eIF4E S209A-expressing vector 48 h prior to proliferation assay. Endogenous eIF4E was silenced by shRNA. Cell proliferation was assayed by MTT assay using conditions described in the legend for Figure 2. Results from three independent experiments are shown. (*C*) Immunoblot of TamS and TamR cells treated with escalating doses of CGP57380 (GCP) for 2 h and lysed in NP-40 buffer; equal protein amounts were probed for P-eIF4E, total eIF4E, and β-actin (loading control). (*D*) Cell proliferation was assayed as above. Cells were treated with DMSO, 1 µM 4-OHT, or 4-OHT and MNK1 inhibitor CGP. Results of three independent experiments are shown. (**) $P < 0.01$ by *t*-test. (*E*) Colony survival assays were performed as described in the legend for Figure 2. Cells were treated with DMSO, 1 µM 4-OHT, 10 µM CGP, or combination therapy 24 h after plating. Drugs were restored every 72 h. Data from three independent experiments were normalized to DMSO control. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ by two-way ANOVA; (n.s.) not significant. (*F*) TamR colony survival assays were performed as described in the legend for Figure 2 and treated as in *E* plus 20 mM RAD001. Data from three independent experiments were normalized to DMSO control. (**) $P < 0.01$ by *t*-test.

Both overexpression of eIF4E and its phosphorylation are required to promote tamoxifen resistance in normally sensitive ER⁺ breast cancer cells

Since merely expressing a phospho-mimetic eIF4E is insufficient to confer tamoxifen resistance to normally sensitive cells, we determined whether both overexpression of eIF4E and increased eIF4E S209 phosphorylation were required. TamS cells were stably transfected with a Dox-inducible HA-tagged eIF4E cDNA that tripled eIF4E levels (Fig. 4A). Tamoxifen-sensitive TamS cells were unable to proliferate in the presence of tamoxifen regardless of eIF4E overexpression (Fig. 4B). However, since eIF4E availability and phosphorylation are limited by 4E-BP1, we hyperactivated mTORC1 by disrupting the repressing TSC1/TSC2 complex through shRNA silencing of *Tsc2* (Fig. 4C). Silencing *Tsc2* strongly increases mTORC1 signaling (Sato et al. 2012), demonstrated here by increased phosphorylation of 4E-BP1 and ribosomal protein S6. Importantly, *Tsc2* silencing conferred tamoxifen resistance to normally sensitive ER⁺ breast cancer cells (Fig. 4D). Cosilencing *Tsc2* and overexpressing eIF4E slightly reduced tamoxifen resistance for unknown reasons but might be related to homeostatic regulation of eIF4E levels. We noted somewhat lower levels of eIF4E and 4E-BP1 phosphorylation in *Tsc2* silenced eIF4E-overexpressing cells, consistent with this possibility. The importance of eIF4E S209 phosphorylation using a phospho-dead protein could not be tested due to the inability to sufficiently silence endogenous eIF4E in cells that were already drug-selected twice. Nevertheless, eIF4E and its phosphorylation, increased mTORC1 activity, and increased levels of available eIF4E and its phosphorylation can confer tamoxifen resistance. We note somewhat less eIF4E and 4E-BP1 phosphorylation in *Tsc2* silenced cells with eIF4E overexpression, supportive of this possibility (Fig. 4C). There was no change in basal ER signaling under these conditions, as shown by induction of ER biomarker mRNAs (Fig. 4E).

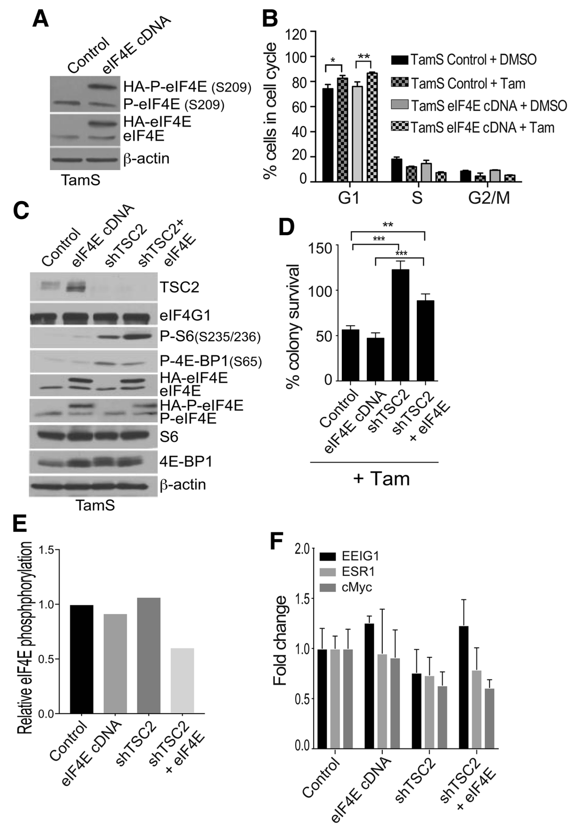

Figure 4. Hyperactivation of mTORC1 and eIF4E overexpression reprogram the cancer genome to mimic tamoxifen resistance. (*A*) Representative immunoblot analysis of equal amounts of protein lysate from eIF4E-overexpressing cells. Dox (2 µg/mL) was added 72 h prior to lysis in NP-40 buffer. Equal protein amounts were immunoblotted as shown. β-Actin was used as a loading control. (*B*) Cell cycle analysis of TamS control and TamS cells overexpressing eIF4E and treated with DMSO (vehicle) or 1µM 4-OHT for 72 h in 1% CS-FBS. Dox (2 µg/mL) was added for 72 h. Cells were subjected to exhaustive RNase A and stained with propidium iodide (PI). Flow cytometry data were collected using a FACScalibur and analyzed with FloJo software. The average of three studies is shown. (*C*) Immunoblot analysis of mTORC1 pathway proteins in TamS control, eIF4E-overexpressing, TSC2 silenced, or TSC2 silenced and eIF4E-overexpressing cells. Cells were treated with 2 µg/mL Dox for 72 h and lysed in NP-40 buffer. Equal protein amounts were immunoblotted. β-Actin was used as a loading control. Representative results are shown. (*D*) Colony survival assays from three studies were performed as described in the legend for Figure 2. TamS sh-control, eIF4E-overexpressing, shTSC2, and shTSC2 and eIF4E-overexpressing cells were treated with either DMSO or 1 µM 4-OHT. (**) $P < 0.01$; (***) $P < 0.0001$ by two-way ANOVA. (*E*) Quantitation of eIF4E S209 phosphorylation in the cells treated in *C*. (*F*) Markers of ER signaling in TamS cells were quantified by RT-qPCR of mRNAs with cDNA overexpression of eIF4E, shRNA silencing of TSC2, or both. Results are the average of three independent studies.

mRNAs are altered in abundance and translation in tamoxifen-resistant compared with tamoxifen-sensitive breast cancer cells

Research on tamoxifen-resistant disease has not yet been focused on differential mRNA translation. Here we sought to identify mRNAs that are selectively altered in translation in tamoxifen-resistant cells. We conducted a genome-wide translatome and transcriptome analysis using RNA sequencing (RNA-seq) of TamR and TamS cells. Three sets of conditions were analyzed to fully represent the genome-wide changes in mRNA abundance and translation: (1) expression levels for total mRNA (transcription), (2) changes in translation (heavy polysome fraction) regardless of mRNA abundance or translational regulation, and (3) translation-specific changes (ratio of heavy polysome mRNA/total mRNA) (Fig. 5A,B; Supplemental Table S3). Analyses used a cutoff of $\log_2$ 1.0 (two-fold) for total mRNA and $\log_2$ 0.6 (1.5-fold) for heavy polysome association; the latter was set lower because smaller changes in protein expression can have significant physiological effects. Significance was set at $P < 0.05$ for both mRNA and polysome analysis. Gene ontology (GO) analyses of significantly altered genes in both transcription and translation revealed an enrichment of

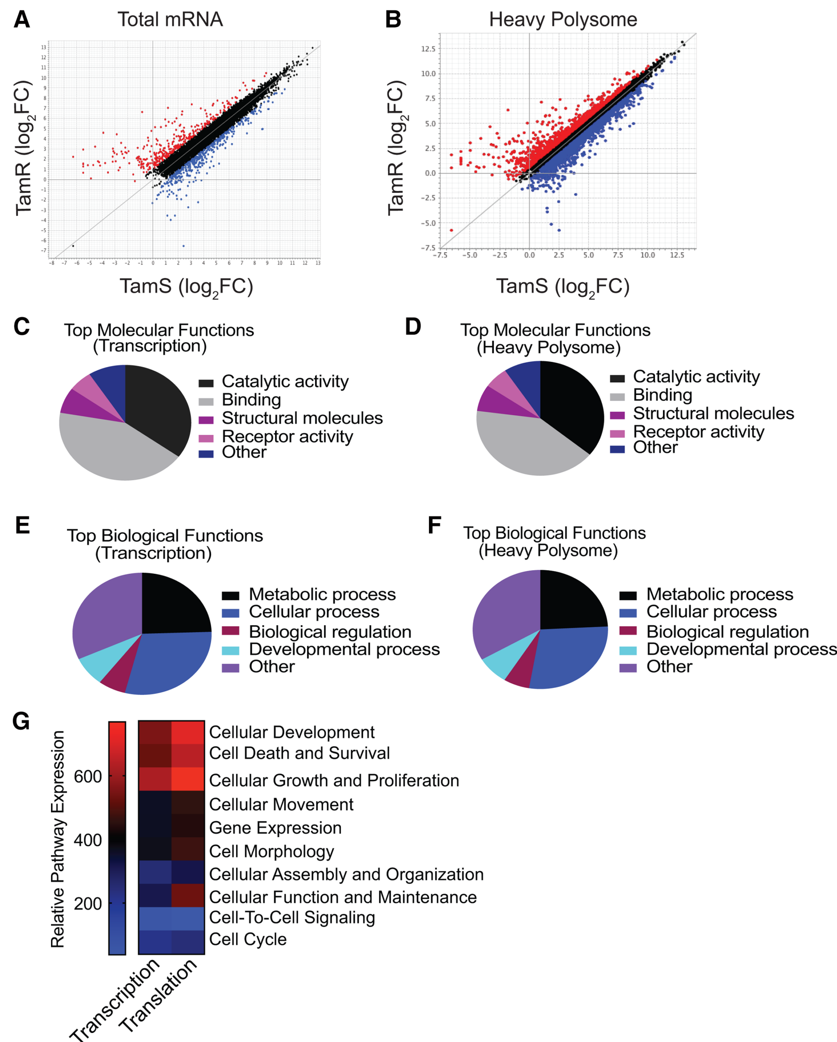

Figure 5. Selective translation of mRNAs important in cell proliferation, survival, and genomic reprogramming in tamoxifen-resistant compared with tamoxifen-sensitive breast cancer cells. (*A*,*B*) Genome-wide transcription and translation mRNA profiling of TamS compared with TamR cells with 1 µM 4-OHT for 48 h. Results are from two independent studies. Total mRNA and purified fractions containing four or more bound ribosomes (heavy) were sequenced using Illumina HiSeq 2500 single read. Volcano plots represent differences in transcription and translation. Blue dots identify mRNAs significantly changed in abundance. Transcription parameters were $P \leq 0.05$ and $-1.0 \leq \log_2 \geq 1.0$, translation parameters were $P \leq 0.05$ and $-0.6 \leq \log_2 \geq 0.6$. Red dots identify mRNAs not significantly changed in abundance. Statistical analysis was performed using the limma R package. (*C*,*D*) The top molecular functions of mRNAs significantly altered in total abundance and translation from heavy (well-translated) fractions (four or more ribosomes), respectively. (*E*,*F*) The top biological functions of mRNAs significantly altered in total abundance and translation from heavy (well-translated) fractions (four or more ribosomes), respectively. (*G*) Relative pathway summation of transcriptional and translational changes in mRNAs in TamR cells relative to TamS cells.

developmental, cell survival, and differentiation pathways in endocrine therapy-resistant cells (Fig. 5C–G). We note specific enrichment in up-regulated *Hox* and DNA recombination genes, with a concomitant repression of estrogen and *Tgfβ* genes (Supplemental Table S4). Moreover, *Hox* genes encode transcription factors that specify stem cell fate determination and are also important in oncogenesis (Shah and Sukumar 2010). Both the ER and TGF-β pathways play a pivotal role in tumor suppression (Bachman and Park 2005; Berger et al. 2013).

Identification of mRNAs highly dependent on overexpression of P-eIF4E for translation in tamoxifen-resistant breast cancer cells

We next identified mRNAs associated with tamoxifen resistance that were selectively altered in translation resulting from increased expression, availability, and phosphorylation of eIF4E. We used genome-wide transcriptomic and translatomic analyses in TamR cells with and without modest eIF4E reduction to identify this data set by silencing eIF4E to levels similar to TamS cells (Fig. 6A). Total mRNA and polysomal mRNA profiling showed only a very slight overall reduction in mRNA and polysome content in tamoxifen-resistant cells after eIF4E silencing (Fig. 6A–C; Supplemental Fig. S4A–C). Surprisingly, the number of mRNAs that changed significantly only at the translation-specific level was small (with most of them down-regulated) but included a small number that were translationally up-regulated as well (Fig. 6C; Supplemental Fig. S4A; Supplemental Table S6). Select genes from RNA-seq analysis were validated by qPCR of total and heavy polysome fractions (Supplemental Fig. S4B,C). GO analyses and Ingenuity Pathway Analyses (IPAs) revealed similar biological and molecular functions for transcriptionally and translationally increased mRNAs associated with tamoxifen resistance (Fig. 6D–F; Supplemental Fig. S4E–G).

We assessed the biological functions of mRNAs identified as highly eIF4E-dependent in tamoxifen-resistant cells. Supplemental Table S5 lists mRNAs that meet these stringent criteria. Of these, *Runx2* was particularly notable because it encodes a protein with a number of activities that could play a role in tamoxifen resistance, as it is an

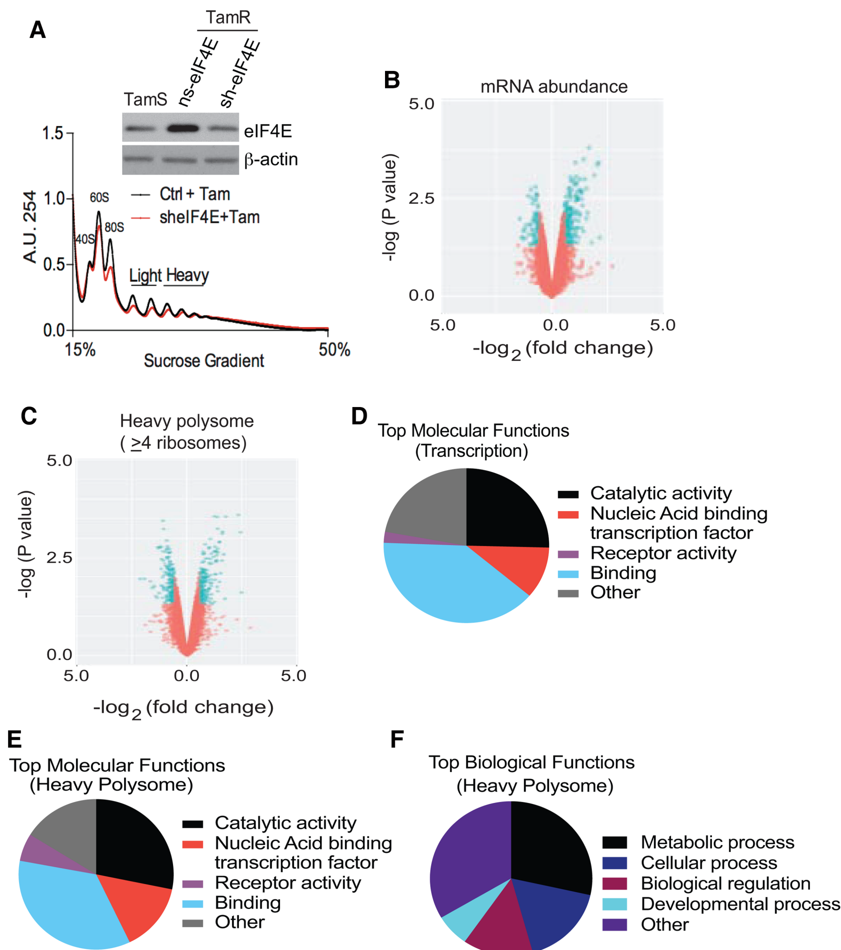

Figure 6. Tamoxifen resistance is associated with eIF4E overexpression and selective mRNA translation. (*A*) Polysome profiles of TamR cells without and with eIF4E silencing. Representative results are shown. (*Inset*) Immunoblot showing eIF4E levels and control β-actin. (*B*,*C*) Genome-wide transcription and translation mRNA profiling of TamR cells with or without eF4E silencing plus 1 µM 4-OHT for 48 h. Results are from two independent studies. Total mRNA and purified fractions containing four or more bound ribosomes (heavy) were sequenced and analyzed using the same parameters as described in the legend for Figure 5. (*D*,*E*) The top molecular functions of mRNAs significantly altered in total abundance (*D*) and in translation (*E*) from heavy (well-translated) fractions (four or more ribosomes), respectively. (*F*) The top biological functions of mRNAs significantly altered in translation from heavy polyribosomes.

important inhibitor of estrogen signaling and stimulates oncogenic pathways. RUNX2 is a transcription factor involved in regulating cell determination (Young et al. 2007; Blyth et al. 2010) and the TGF-β and Wnt/β-catenin pathways (which are also involved in cancer development, progression, and metastasis) (Young et al. 2007; Yang et al. 2015) and opposes ER signaling, leading to more aggressive ER+ breast cancer (Tandon et al. 2014). Interestingly, recent studies have also shown that RUNX2 plays a crucial role in regulating mammary stem cell regeneration (Ferrari et al. 2015). Surprisingly, we found *Runx2* to be the only *Runx* gene within the family to be transcriptionally or translationally up-regulated in both TamR and PDX tamoxifen-resistant cell lines (Fig. 7A,B; Supplemental Table S4). Total *Runx2* mRNA levels were unchanged with eIF4E reduction in TamR cells, but heavy polysome association was reduced threefold, which corresponds to a fourfold to fivefold reduction in RUNX2 protein levels (Fig. 7C, D). We also determined whether *Runx2* mRNA requires eIF4E S209 phosphorylation by Mnk1. Cells were untreated or treated with CGP57380, and *Runx2* mRNA and protein levels were determined. There was no statistically significant difference in *Runx2* mRNA levels with drug treatment, whereas RUNX2 protein levels were reduced more than threefold (Fig. 7E). Therefore, increased levels or availability of eIF4E and increased eIF4E S209 phosphorylation by Mnk1 promote selectively increased translation of *Runx2* mRNA.

Computational analysis of the *Runx2* 5′ UTR is consistent with a greater eIF4E dependency for translation, showing significant secondary structure and a high GC content within 30 nucleotides of the cap as well as a ΔG of approximately −40 kcal/mol (Supplemental Fig. S4H). To this end, we asked whether mRNAs that are translationally down-regulated upon eIF4E reduction have features in their 5′ UTRs that dictate a strong dependence on eIF4E levels. We conducted a genome-wide analysis of the 5′ UTR of mRNAs translationally down-regulated with eIF4E silencing to the level of tamoxifen-sensitive cells; however, we did not observe any statistical differences in GC content or length when compared with all cellular mRNAs (Supplemental Fig. S5A,B). This is consistent with previously published results regarding 5′ UTR analysis of translationally altered mRNAs upon eIF4E down-regulation (Truitt et al. 2015). To investigate the importance of RUNX2 in tamoxifen resistance in ER+ cells, we used shRNA to reduce *Runx2* mRNA levels approximately threefold, to levels found in tamoxifen-sensitive TamS cells and TamR cells silenced for eIF4E (Fig. 7F). The threefold reduction in RUNX2 in TamR cells resulted

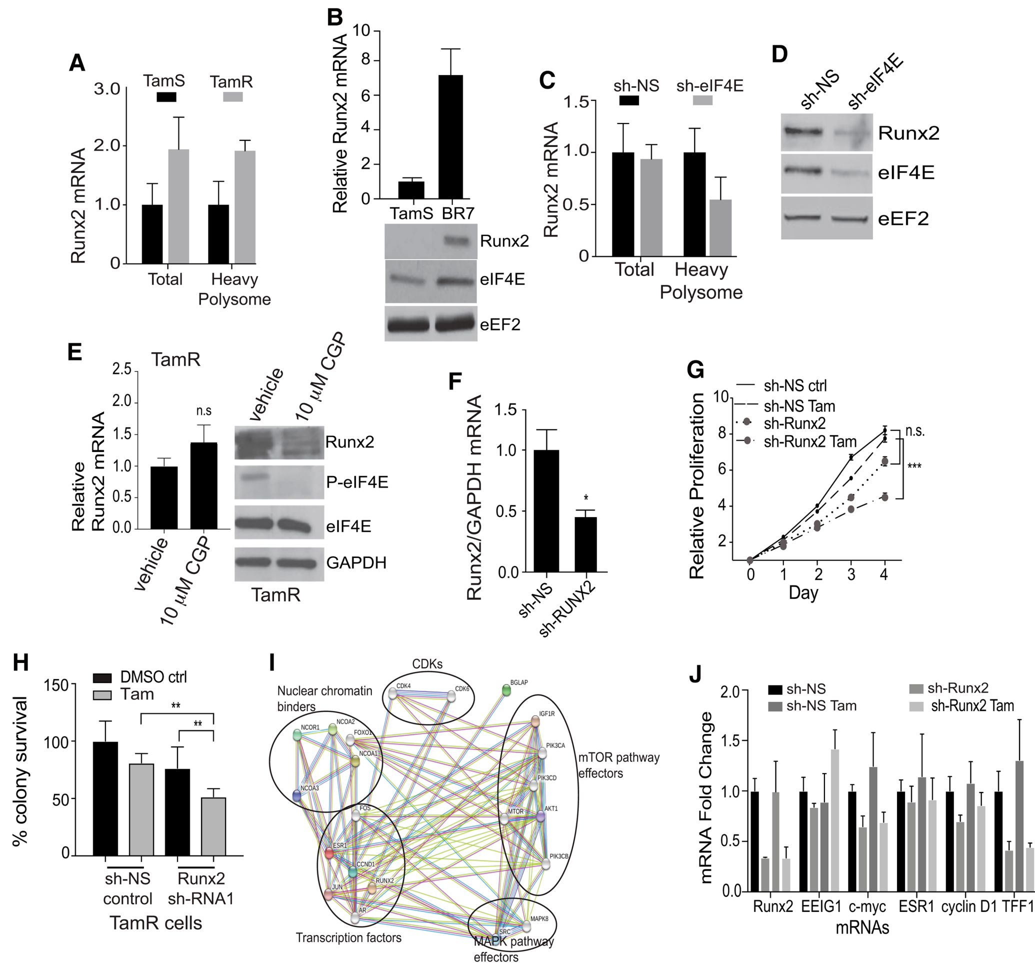

Figure 7. Silencing Runx2 mRNA partially restores tamoxifen sensitivity to resistant cells. (*A*) Relative levels of Runx2 mRNA and levels in heavy polysomes in TamR compared with TamS cells. (*B*) Levels of Runx2 mRNA and protein in BR7 compared with TamS cells. mRNA levels were determined by RT-qPCR, as above. The average of three studies is shown. The immunoblot is representative of three independent studies. (*C*) Relative levels of Runx2 mRNA in total and heavy polysomes in TamR cells in Dox-inducible sh-control nonsilencing (NS) and sh-eIF4E silencing for 72 h. Equal amounts of RNA were quantified by RT-qPCR and normalized to GAPDH using the −ΔΔCt method. An average of three studies is shown. (*D*) Representative immunoblot analysis of equal amounts of protein lysate from TamR cells silenced with nonsilencing (NS) control or sh-eIF4E for 72 h. Equal protein amounts were immunoblotted as shown. eEF2 was used as a loading control. (*E*) TamS and TamR cells were treated with CGP57380 for 6 h, and equal protein amounts of lysates were examined by immunoblot as shown. (*F*) Relative levels of Runx2 mRNA in TamR cells silenced with nonsilencing (NS) control or sh-Runx2 were analyzed as above. (*G*) Cell proliferation was assayed as described in the legend for Figure 2. Cells were treated with DMSO and 1 µM 4-OHT and silenced for Runx2 or nonsilencing (NS). The results of three independent experiments are shown. (*H*) Colony survival assays from three studies were performed as described in the legend for Figure 2 using TamR cells with sh-nonsilencing (sh-NS) control or sh-Runx2 in the presence of 1 µM 4-OHT. (*I*) STRING analysis of the top 20 protein interactors of the Runx2–ERα complex. Light-blue and purple lines represent validated interactions, and green, red, and dark-blue lines represent predicted interactions based on past literature. (*J*) Levels of identified mRNAs in TamR cells treated with DMSO, 1 µM 4-OHT, or 4-OHT and silenced for Runx2 or nonsilencing (NS) after 72 h of treatment. (**)$P < 0.01$; (***)$P < 0.001$ by *t*-test.

in a strong impairment in proliferation in the presence of tamoxifen (Fig. 7H) as well as a significant reduction in clonogenic cell survival of normally drug-resistant cells (Fig. 7I).

RUNX2 establishes a molecular program that opposes ERα signaling in both the normal and transformed settings (Chimge and Frenkel 2013; McDonald et al. 2014). In support of these studies, using computational STRING analysis, we identified interactions between RUNX2 and ERα, including established tamoxifen resistance genes (Fig. 7I). Furthermore, ERα–RUNX2 interaction analysis is consistent with RUNX2 stimulation of both the mTORC1 and MAPK translational control pathways (Fig. 7I) to promote drug resistance. In fact, it has been reported that breast tumors expressing high levels of RUNX2 generally express low levels of ERα and vice versa. To this end, we performed an extensive bioinformatics search of the TCGA (The Cancer Genome Atlas) breast cancer database as well as analysis of breast cancer cell lines and found an almost perfect inverse correlation

between ERα (ESR1) and *Runx2* mRNA expression in 594 patients diagnosed with ER⁺ breast cancer (Supplemental Fig. S5C,D). To understand the significance of the RUNX2–ERα axis in relation to tamoxifen responsiveness, we investigated whether silencing *Runx2* in TamR cells reverses the RUNX2 blockade of canonical ERα signaling as a mechanism to re-establish drug sensitivity. qRT–PCR analysis of ER target genes indicated that reduction of RUNX2 did not restore ERα signaling in resistant cells (Fig. 7J), indicating that RUNX2 expression may permanently overwrite classical ERα signaling, leading to genomic changes that establish permanent anti-estrogen resistance.

Discussion

The majority of ER⁺ breast cancer patients treated with tamoxifen will relapse with resistant disease even decades after curative care (Ali and Coombes 2002). Evidence has shown that a cross-talk exists between the ER and the PI3K/Akt/mTOR and MAPK signaling pathways in promoting tamoxifen resistance (Sommer and Fuqua 2001; Fan et al. 2014). Our findings indicate that tamoxifen resistance also involves the mRNA translational regulation of these pathways and is manifested by increased eIF4E levels, availability, and phosphorylation, resulting in selective mRNA translational reprogramming that establishes an anti-ER and anti-estrogen signaling state. Furthermore, these results have broader implications in understanding resistance to other endocrine therapies; notably, resistance to aromatase inhibitors. While aromatase inhibitors are also clinically used for the treatment of metastatic ER⁺ breast cancers, resistance occurs and is thought to arise through mechanisms similar to tamoxifen; namely, mTORC1 inhibition in combination with aromatase inhibitors leads to an overall increase in patient survival similar to results obtained in this and other studies regarding anti-estrogen resistance.

Alterations in eIF4E-dependent translation can promote selective translation of mRNAs that reprogram cancer cells for survival, invasion, metastasis, and possibly drug resistance (Silvera et al. 2009b; Hsieh et al. 2012; Boussemart et al. 2014). We previously showed a causal role for selective mRNA translation in therapy resistance in breast and other cancers (Braunstein et al. 2009; Ramírez-Valle et al. 2010; Badura et al. 2012; Korets et al. 2014). Here we disclose a mechanism by which increased expression, availability, and phosphorylation of eIF4E form a regulatory nexus important in anti-estrogen resistance in ER⁺ breast cancer. During therapeutic treatment, when cell surface EGF and IGF receptors are activated, they transactivate downstream MAPK/ERK/MNK and PI3K/Akt/mTOR pathways and ultimately converge on eIF4E, increasing its activity, phosphorylation, and availability. This leads to eIF4E-mediated selective translation of key mRNAs, such as *Runx2*. Our genome-wide transcription/translation analysis of tamoxifen-resistant cells revealed that several key pathways are down-regulated upon eIF4E silencing, with a majority of the down-regulated pathways with eIF4E silencing involved in cellular organization and motility, genetic recombination, and developmental processes (Ramírez-Valle et al. 2008; Cao et al. 2016). Most of these mRNAs are involved in DNA–protein interactions and the regulation of transcription factor binding. From these findings, we identified RUNX2 at the intersection of these molecular functions and demonstrated a strong translational down-regulation with eIF4E silencing in tamoxifen-resistant cells.

RUNX2 belongs to the family of RUNX transcription factors (RUNX1,2,3), which are involved in lineage-specific cell fate determination that is recapitulated in cellular transformation and tumorigenesis. RUNX proteins regulate gene expression by functioning as molecular scaffolds to recruit chromatin remodeling enzymes (e.g., SWI/SNF and CTCF) and modulate promoter accessibility (Young et al. 2007; Wu et al. 2014). Studies involving the role of RUNX2 in breast cancer have demonstrated the importance of overexpression of RUNX2 in regulating tumor growth, epithelial–mesenchymal transition, and metastasis (Pratap et al. 2009; Chimge et al. 2011; Karlin et al. 2014). Furthermore, RUNX2 has been shown to regulate the expression of genes involved in WNT/β-catenin and TGF-β signaling—two key pathways known to be dysregulated in many cancers, particularly breast cancer (Chimge and Frenkel 2013; Ferrari et al. 2015). Importantly, WNT and TGF-β signaling has been shown to promote cancer progression to a more poorly differentiated state (Barcellos-Hoff and Akhurst 2009; Ferrari et al. 2015). Notably, other studies describing RUNX2 transcriptional activity have shown that it regulates ER signaling by direct and indirect interactions (McDonald et al. 2014; Jeselsohn et al. 2017). Thus, our studies demonstrate that established genes and signaling pathways that confer tamoxifen resistance (and possibly other forms of endocrine therapy resistance) do so by acting on eIF4E abundance and phosphorylation to selectively translationally reprogram the breast cancer cell for estrogen- and ER-opposing activities.

Materials and methods

Chemicals and inhibitors

Final concentrations of chemicals and inhibitors used were 0.02% DMSO, 1 µM 4-OHT (Millipore), 20 nM RAD001 (Selleck Chemicals), and 10 µM CGP57380 (Sigma).

Cell lines and cell culture

MCF7 and BR7 cells were maintained in improved MEM (IMEM) with L-glutamine without phenol red (Cellgro), 5% fetal bovine serum (FBS) (Gibco), 0.4% gentamicin sulfate (Lonza), 0.5 µg/mL fungizone (Gibco), and 5 µg/mL plasmocin at 37°C in a 5% CO_2 tissue culture incubator. 4-OHT (1 µM) was added to TamR cells every 72 h. HEK293FT cells were maintained in DMEM with L-glutamine (Corning), 10% FBS, 1% penicillin–streptomycin (Life Technologies), 1 mM sodium pyruvate (Thermo Scientific), and 1% MEM nonessential amino acids (Thermo Scientific). Cells were routinely checked for mycoplasma contamination.

Patient cohorts and tissues

Archival tumor tissue specimens were obtained with prior Institutional Review Board (IRB) approval for patients ≥18 yr of age with ER^+ (≥5% ER^+ staining) invasive ductal breast cancer (IDC) stage II/III treated with adjuvant tamoxifen and/or aromatase inhibitor (Supplemental Tables S1, S2). Patients who recurred within 5 yr were considered resistant. A pathology database of all available treated tumor specimens was queried to identify cases between 2002 and 2011 that had a clinical description of <5-yr recurrence or no recurrence at 10 yr.

Anchorage-dependent colony formation assays

Cells were trypsinized, filtered, and counted using an automated cell counter (Bio-Rad). Cells (1×10^3) were seeded in triplicate in six-well culture dishes using IMEM supplemented with 5% charcoal-stripped FBS and 0.4% gentamicin sulfate and allowed to adhere overnight. The medium was changed, and the indicated treatments were carried out. The medium and treatments were changed every 72 h for 10–12 d. Colonies were washed, fixed, and stained with 0.5% Crystal Violet in 6% glutaraldehyde. Colonies containing ≥50 cells were scored.

Cell cycle analysis

Cells were trypsinzed, filtered, and counted using an automated cell counter (Bio-Rad). Cells (7×10^5) were seeded on 10-cm culture plates in IMEM (Corning) with 5% FBS (Gibco) and 0.4% gentamicin sulfate (Lonza) and allowed to adhere overnight. The medium was changed to IMEM (Corning) with 1% charcoal-stripped FBS (HyClone) and 0.4% gentamicin sulfate for 48 h. Cells were treated with the appropriate drug for 72 h and with fresh drug after 48 h. Cells were trypsinized and fixed in 70% ethanol overnight at 4°C. Cells were washed with PBS and treated with 0.5 mg/mL RNase A for 30 min at 37°C. Cells were washed again with PBS and stained with 50 µg/mL propidium iodide (PI) or Hoechst 33342 for 45 min at room temperature and protected from light. Data were collected using a FACScalibur or LSRII UV and analyzed with FlowJo 10.0.

Polysome-associated mRNA isolation

Isolation of ribosome-bound mRNA by polysome separation was performed as described previously with minor modifications (Silvera et al. 2017). Briefly, MCF7 cells were seeded 48 h prior to treatment, and cells were treated with 100 µg/mL cycloheximide for 10 min at 37°C, trypsinized, and collected in ice-cold PBS containing protease inhibitor cocktail and EDTA-free (Roche Diagnostics). All subsequent steps contained 100 µg/mL cycloheximide. Cells were resuspended in low-salt buffer (LSB; 20 mM Tris at pH 7.4–7.5, 10 mM NaCl, 3 mM $MgCl_2$, ribonuclease inhibitor [Thermo Scientific]) and incubated for 3–5 min on ice. Detergent buffer (LSB with 1.2% Triton X-100, 0.2 M sucrose) was then added, and cells were lysed with 15–20 strokes in a sterilized Dounce homogenizer at 4°C. Lysates were cleared by microfuge centrifugation at maximum speed for 5 min, and supernatant was combined with 100 µL of heparin buffer (LSB with 10 mg/mL heparin, 1.5 M NaCl) and then layered on a 15%–50% sucrose gradient in LSB using equal OD_{260} units of samples. Gradients were centrifuged at 36,000 rpm for 2 h in a SW40Ti rotor (Beckman Coulter), and polysome profiles were done at UV absorbance 254 nm by continuous flow cell monitoring and collected using an Isco UA-6 absorbance detector (Teledyne ISCO) and with a Foxy R1 fraction collector (Teledyne ISCO) at 1.5 mL/min.

RNA-seq and analysis

RNA was extracted and purified from pooled polysome fractions using the RNeasy minikit (Qiagen) as per the manufacturer's instructions. RNA quality was measured by a Bioanalyzer (Agilent Technologies). Fractions containing two to three bound ribosomes were considered poorly translated (light fractions), and those containing four or more bound ribosomes were considered well translated (heavy fraction). RNA-seq was carried out by the New York University School of Medicine Genome Technology Core using the Illumina HiSeq 2500 single read. To quantify translational efficiency, the difference in $\log_2$ intensity between matched polysomal mRNA and total mRNA was determined. To examine differences in transcription and translation, total mRNA and polysome mRNA were quantile-normalized separately. Statistical analysis was performed using the limma R package (Ritchie et al. 2015). Gene enrichment analysis was performed using IPA software, and GO analysis was performed using the DAVID online tool.

Quantitative real-time PCR (RT-qPCR) and analysis

RNA was extracted using Trizol as per the manufacturer's instructions. One microgram of RNA was used for reverse transcription reaction using Promega GoScript, as per the manufacturer's instructions. qRT–PCR was completed using iTaq Universal 2× SYBR Green qPCR master mix (Bio-Rad) and an Applied Biosystems 7500 Fast RT–PCR machine as per the manufacturers' instructions. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. A list of the primers used is available on request.

Cap chromatography

In brief, cells were lysed in NP-40 buffer (50 mM HEPES at pH7.0, 150 mM NaCl, 2 mM EDTA, 25 mM NaF, 25 mM β-glycerophosphate, 2 mM Na_3VO_4, 1% IGEPAL, Complete miniprotease inhibitor cocktail tablet ± EDTA [Roche]), and lysates were cleared by microcentrifugation at 13,000 rpm for 10 min at 4°C. Lysate protein concentration was determined by BCA assay, equal amounts were incubated with m^7GTP Sepharose beads for 1 h at 4°C, and beads were collected by centrifugation, washed three times with lysis buffer, resolved by 10% or 12% SDS-PAGE, and transferred to a PVDF transfer membrane (Millipore). The membrane was blocked in 5% BSA in TBS-T at 4°C. Primary antibodies were incubated overnight at 4°C. Secondary ECL antibodies (GE Healthcare) were incubated for 1 h at room temperature in 5% reconstituted dried milk in TBS-T. Protein was imaged using the chemiluminescence method and Genemate autoradiography film.

Cell proliferation assay

Cell proliferation was measured using the CellTiter 96 nonradioactive cell proliferation assay kit (Promega) according to the manufacturer's instructions. MCF7 cells were plated at 1500 cells per well in triplicate in 96-well culture plates. Cells were allowed to attach overnight. On day 0, 15 µL of dye solution containing tetrazolium was added to each well and incubated for 4 h at 37°C. One-hundred microliters of Stop Six was added to each well to solubilize the formazan products using the overnight method in a humidified chamber. Absorbance was measured at 570 nm. Four more time points were collected on days 1–4. Time points were normalized to day 0.

Statistical analysis

Unpaired *t*-test and two-way or one-way ANOVA were used for biological studies when applicable to determine statistical significance. Biomarkers were of ordinal measurements and used Fisher's exact test. Data were analyzed using GraphPad Prism 6.0e. Significant values were considered $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

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