Estrogen Mediated Coordination of Post-Transcriptional Regulation

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Introduction

Breast Cancer

Breast cancer is a leading cause of morbidity and mortality for women in the United States. It is the second leading cause of cancer-related deaths in women in the US and the most common cancer in women younger than 55 years old [1]. Women in the US have roughly a 1 in 8-lifetime risk of being diagnosed with breast cancer, with 254,650 women being diagnosed in 2009 alone [1]. The relative five year survival rate after diagnosis for women is 89% [1]. In 2009, an estimated 40,170 women died from breast cancer. Due to earlier detection and the development of new efficacious treatments, mortality rates have been dropping steadily since 1990 [1, 2].

While relatively stable since 2003, there was a 6.7% decrease in age-adjusted breast cancer incidence rates between 2002 and 2003. This decrease was most evident in women aged 50-69 years whose tumors were estrogen receptor positive (ER+). The sharp decline has been attributed to the diminished use of hormone replacement therapy (HRT) in menopausal women after the publication of the Women's Health Initiative randomized clinical trial in 2002. The trial showed that the use of estrogen and progestin significantly increased the risks of breast cancer and other ailments [1, 3].

Discovery of Endocrine Therapy

Hormones were first implicated as carcinogenic agents in breast cancer in 1896 when Dr. George Beatson preformed bilateral oophorectomies on premenopausal women with advanced metastatic breast cancer. He observed tumor regression and improved patient prognosis in a subset of these women [4]. By 1911, Beatson and others had found that oophorectomy only benefited one-quarter to one-third of patients. Despite a high rate of recurrence within a few years, these observations clearly linked an unknown ovarian factor to the growth of a subset of breast tumors [5].

Discovering Estrogen and the Estrogen Receptor

Hormones act as specific messenger molecules and their signaling is important in development, growth, metabolism, and reproduction. They mediate their biological activity through specific cell surface, and intracellular, receptors [6]. Edward Doisy first isolated 17-beta-estradiol (E2), a steroid hormone, in 1936 from 8,000 kg of sow ovary [7]. While not appreciated at the time, E2 was Beatson's unknown ovarian factor. Estrogen is an important regulator of breast tissue homeostasis and other reproductive organs, as well as non-reproductive tissues such as bone and brain tissue, and the cardiovascular system [8, 9]. It is well established that E2 stimulates the growth and proliferation of the reproductive organs including the uterus, vagina, and breast [8, 9]. In addition, E2 has been shown to drive the growth and progression of many breast cancers [8, 9].

In 1962, Elwood V. Jensen discovered the estrogen receptor (ER) by measuring the specific uptake and retention of radiolabelled estradiol [10]. His discovery in 1971 that in breast tumor tissue the presence of ER was a prognostic marker of E2 response, transformed the treatment of breast cancer by allowing for predictive and specific individual treatment regimens [11]. The development of a prognostic test had been a major goal in breast cancer research since Beatson established the benefit of endocrine therapy 75 years earlier.

Using ER antibodies developed by Jensen, Pierre Chambon isolated the cDNA of estrogen receptor alpha (ERα) from an MCF-7 cDNA library in 1985 [12]. Once the ERα cDNA was sequenced [12-14], it quickly became apparent that ERα, shared significant regional homology with the steroid glucocorticoid receptor [15], thyroid hormone receptor [16, 17], and with the retinoic acid receptor [18, 19]. These receptors shared a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD), and were grouped together as a family of genes termed the nuclear receptor superfamily [20]. A second ER, estrogen receptor beta (ERβ), was cloned in 1996 [21]. In addition, three estrogen-related receptors have been identified, which share a high degree of amino acid homology to the ERs but do not bind natural estrogens or any other endogenous ligand [22, 23] . The cloning and characterization of the ERs has allowed the study of E2 action at the molecular level.

Defining the Estrogen Receptor

The two ER subtypes, ERα and ERβ, mediate E2 action. The two proteins share similar functional organization; however, the degree of homology between domains varies. Both receptors contain six distinct functional regions denoted A-F. The A/B region contains a ligand-independent transactivation region, AF-1. Region C contains the DNA binding domain. Region E contains the hormone-binding region, a ligand-dependent transcription activation function, AF-2, and a corepressor binding domain [24]. Coactivator binding sites are located in regions A/B, D, and E [24]. When comparing amino acid sequences, ERα and ERβ are 97% homologous in the DNA-binding domain, 56% in the ligand-binding domain, and 24% in the N-terminal domain [25, 26], [25, 26]. Despite having only 56% amino acid homology in the ligand-binding domain, the residues in the hydrophobic binding pocket are highly conserved and both ERα and ERβ bind E2 with similar affinities [25, 26].

The uses of ERα and/or ERβ knockout animals and tissue expression studies have shown that the two receptors have distinct biological activities and organ distributions [25-27]. ERα is often highly expressed in breast tissue and typically promotes cell cycle progression and proliferation [25-27]. In contrast, ERβ protein is not commonly found in breast tissue, and is pro-apoptotic and anti-proliferative [28, 29]. The functional differences between the two receptors might result from the sequence differences within the corepressor and coactivator binding sites; facilitating differential interactions with a unique set of transcription factors and regulatory proteins.

Classic Model of Estrogen Receptor Signaling

The ERs are predominately nuclear-localized proteins, however, both nuclear and membrane/cytoplasmic forms of ERα and ERβ have been reported [30]. Until recently, most efforts have focused on understanding their function as nuclear transcription factors. The classic genomic mechanism of E2 activation involves the formation of a tripartite complex. Upon ligand binding, ERα undergoes a conformational change that induces the formation of an ERα homo- or heterodimer with ERβ. The activated complex can bind either directly or indirectly, with the aid of co-factors, to regulatory DNA sequences, known as ligand responsive elements. The sequence of the minimal consensus estrogen responsive element (ERE) is a palindromic inverted repeat of: 5'-GGTCAnnnTGACC-'9, where *n* is any nucleotide. While in association with regulatory DNA, the active complex interacts with the basal transcription factor machinery through complex combinatorial interactions with co-repressors and co-activators, which can enhance or inhibit target gene transcription. The transcriptional activity of the ER complex can also be further regulated by specific ligand binding, the presence of anti-estrogens, or receptor modifications. The ER can also modulate transcriptional responses in a ligand independent manor, through interactions with other classes of DNA-bound transcription factors [24, 26, 31].

Estrogen Receptor Ligands: Modulating ER Activity

Antiestrogen pharmacologic therapy is most efficacious in tumors that are ER+ (containing ERα). About 50-80% of breast cancers are ER+ +, depending on the cohort studied, and of these roughly two-thirds respond to endocrine therapy [3, 32]. These compounds function primarily by competing with E2 in binding the ER and thereby inhibiting E2 mediated transcriptional response. Binding of antiestrogens to the ER induces a unique conformational change in the ER. These compounds are more appropriately termed selective estrogen receptor modulators (SERMs) as they can have agonist activity, antagonist activity, or mixed agonist/antagonist activity depending on factors such as the cellular context, which ER is occupied, and the structure of the ligand [33].

The first SERM, tamoxifen (TAM), was developed in the 1960's by a team of researchers at ICI in Macclesfield England (now AstraZeneca PLC) [34]. The FDA first approved TAM as a treatment for ER+ breast cancer in 1977. It is currently the most prescribed SERM and, as of 2002, there has been over 12 million patient years of therapy [35]. Treatment with TAM in women with ER+ breast tumors results in a 41% reduction in the annual recurrence rate and a 33% reduction in the death rate [1]. Unfortunately, TAM acts as an ER agonist in the uterus and elevates the risk of endometrial cancer. Additionally, many patients' tumors exhibit de novo resistance to TAM. The majority of tumors that are initially sensitive to TAM develop resistance to therapy causing tumor

relapse that often requires treatment with a different class of drugs [36]. While various factors to tamoxifen resistance, the precise mechanisms remain unknown [26, 36, 37].

In vitro, TAM causes G0/G1 phase cell cycle arrest in hormone-dependent cancer cell lines [38-40]. *In vivo*, TAM is a pro-drug that is converted to its active metabolite 4-hydroxytamoxifen (4-OHT) in the liver [41]. The active metabolite causes tumor regression through cytotoxic effects and by inhibiting E2-mediated proliferation [26, 42]. The active metabolite acts as an ER antagonist in the breast by competitively inhibiting E2 binding to the ERs, and by recruiting co-repessors, rather than co-activators, to target promoters [26]. While TAM can reduce or abrogate the transcriptional activity of the ER-E2 complex, it has been shown that TAM can also induce ERα-mediated transcriptional regulation of a unique set of target genes that are minimally affected by E2 treatment. There is a correlation between elevated levels of some of these genes and disease recurrence. These genes could potentially serve as predictors of both initial TAM responsiveness and risk of development of TAM resistance in breast cancer [33].

ER Mediated Non-genomic Actions of NRs Ligands

In addition to the classic genomic pathway of ER mediated regulation of gene expression, E2 and other SERMs, including TAM, can also modulate cell function through non-genomic pathways. Estrogen receptor dependent non-genomic responses have

traditionally been defined as a collection of rapid transcription independent hormonal responses occurring within seconds to minutes [43].

Szego and Davis demonstrated evidence of non-genomic signaling in 1967. They showed that within 15 seconds after intravenous administration of E2, levels of 3'-5'-cyclic adenosine monophosphate (cAMP) approximately double in the uterus of oophorectomized rats. Due to the rapid response and the observed tissue and ligand specificity, they hypothesized that E2 must have been interacting with a cell membrane receptor [44]. Initially, this observation was thought to be an indirect action of E2. However, nearly 30-years later, Benita Katzenellenbogen's group showed that E2 and antiestrogens have stimulatory effects on cAMP levels in ER+ breast and uterine cancer cells. They also showed that this effect was likely modulated by ER, as the phenomenon was only observed ER+ cell lines. The response was also independent of RNA and protein synthesis [45]. It is now accepted that although ERs are predominately-nuclear proteins, membrane/cytoplasmic forms of ERs participate in signal transduction pathways that contribute to cellular response [43]. The precise mechanisms and relative contributions of NR ligand modulated non-genomic actions remains poorly understood [46].

Non-classical Non-genomic Signaling

The classic definition of non-genomic E2 mediated action fails to encompass the broad range of biological responses observed after E2 stimulation. Recent studies have revealed many complex and diverse mechanisms through which NR ligands induce both genomic and non-genomic responses. These pathways, triggered in response to stimulation, may function in complementary or overlapping ways. For example, non-genomic kinase signaling could rapidly activate transcription, which can then be sustained by nuclear receptor transcriptional activity [46]. While there are many mechanisms by which genomic and non-genomic actions ultimately facilitate cell response, it is likely that many more await discovery. Adding to the complexity, the cell-type dependent nature of E2 and TAM response increases the diversity of outcomes.

In both ER+ and ER- cell lines, E2 and TAM have been shown to bind to and initiate cell membrane signaling pathways, through the G protein-coupled receptor (GPCR) named GPR30 [31, 46]. This receptor antagonizes the growth of the ERα+, ERβ+, and GPR30+ breast cancer cell line, MCF-7, by inducing sustained increases in cytosolic $Ca2^{+}$ concentrations, while in the ERα-, ERβ-, and GPR30+ cell line, SKBr3, it enhances proliferation [47]. In addition to triggering a broad range of rapid non-genomic activity originating at the plasma membrane, in SKBr3, E2 and TAM stimulation of GPR30 leads to the modulation of unique gene transcription profiles [48]. Likewise, it has been shown that the extranuclear pathways of ER activation induce gene expression profiles distinct from the patterns of the classic NR mediated expression profile [49].

Determination of Transcriptional Activity

First developed in 1995, DNA microarrays provide a means of simultaneously investigating global gene response [50]. In an effort to understand the global gene responses evoked by E2 and TAM stimulation, numerous genome wide DNA microarray experiments have been preformed. These studies have proven valuable in the elucidation of discrete patterns of hormone dependent gene activation and inhibition by monitoring total RNA. Several distinct programs have been identified and include, but are not limited to, those involved in proliferation, survival, and SERM resistance [33, 51-54]. While informative, these studies have neglected the contributions of important aspects of gene expression regulation in defining cellular phenotype. Regulation of gene expression is dependent not only on transcriptional activity, but also post-transcriptional processing, which require additional complementary experiments beyond just measuring total RNA, such as regulation of translation.

Post-transcriptional Gene Regulation

Cellular phenotype depends not only on the genes that are transcribed, but also on the subset of genes that are actually translated into proteins. Post-transcriptional gene regulation is an umbrella term used to describe all the layers of cellular control that mediate the differential fates of transcribed messages. The processes are generally grouped into the following categories: mRNA processing, mRNA export and localization, mRNA surveillance, mRNA silencing, mRNA turnover, and mRNA translation [55].

Once translated, each protein is then also subject to post-translational regulation including protein modification, activation, localization, and degradation [56].

The importance of the contribution of translation and translational regulation in gene expression is evident by the observation that all of these processes ultimately determine if, where, and how efficiently a given mRNA is translated into protein [55]. Of the studies that have attempted to correlate the ratio of protein concentration to mRNA concentration, two have significant correlations (Spearman rank correlation coefficient of >0.24, $P>0.001$ and 0.46%, $P<0.001$) [57]. While these studies did not separate the contributions of translational regulation and protein degradation, it is obvious that steady state mRNA measurements do not accurately represent the proteome, suggesting the importance of these processes in the regulation of protein expression levels.

Translational regulation allows the cell to alter the efficiency at which mRNAs are translated into protein. The regulation of translation is advantageous for a number of reasons. For example, it allows for a rapid response to external stimuli without invoking nuclear pathways of mRNA synthesis, processing, and transport [58]. Translation regulation is usually reversible and, as such, affords the cell flexibility in precisely tuning responses, as well as the ability to conserve energy. The targets and mechanism of translational control include altering the availability and activity of both ribosomes and translation factors, as well as intrinsic properties of the mRNA itself [58].

While translational initiation is the major rate-determining step in many causes regulation can also occur during the elongation and termination phases of translation [58]. In addition to altering the rate of global protein synthesis, translational regulation can modulate mRNA specific responses. Global control of translation efficiency often results from changes in the phosphorylation state of initiation factors, and affects the translation of most cellular mRNAs in a similar way. The translational efficiency of specific transcripts is determined by a variety of *cis*-regulatory elements within an mRNA and any associated *trans*-acting elements. These elements allow for a differential utilization of mRNA in various cellular contexts [58]. In metazoans, for example, the translational efficiencies of mRNAs vary over a 100-fold range [59]. The common elements of an mRNA that determine its association with ribosomes, and its translational efficiency, are the 5' cap, 3'-poly (A) tail, internal ribosome entry sites (IRESs), upstream open reading frames (uORFs), secondary or tertiary RNA structures, and sequences that bind RNA binding proteins (RBPs) or microRNAs. These elements are found within the 5' and especially within the 3' untranslated regions (UTRs) of mRNAs [58]. Interestingly, across all organisms studied to date, overall gene length is the sequence characteristic that best correlates with protein-per-mRNA ratios [60].

Similar to translational efficiency, *cis*-regulatory elements within an mRNA, and any associated *trans*-acting elements, can alter the stability of a transcript independent of transcription, and therefore can alter the rate at which new protein levels are achieved [61]. This is a common form of regulation in many genes involved in metabolism, the

cell cycle, inflammation, and differentiation. In fact, protein abundance can fluctuate several fold due to a change in mRNA half-life, without any change in transcription [58]. These regulatory elements can also alter a critical, though often overlooked, step in post-transcriptional gene regulation: the transport of specific mRNAs from the nucleus to the cytoplasm [62]. While most cellular mRNAs appear to be exported at constant rates, in certain genes, export rates have been shown to regulate expression patterns [58, 63]. The enrichment of specific regulatory motifs that contribute to translation efficiency, mRNA stability, or mRNA transport might define global or specific programs of regulation [60].

Parallel Transcription and Translation Analysis

The ultimate goal of translational control research is to determine which fraction of the genome is actually made into proteins and to determine the efficiency at which this occurs [64]. This requires measures of both mRNA and protein concentrations. The determination of the absolute number of mRNA transcripts is complicated both by inherent biological variation and by experimental variation stemming from technical noise occurring during hybridization. While models that estimate absolute mRNA levels require additional refinement, DNA microarrays are nevertheless a powerful tool for studying relative transcript concentrations between samples [65]. Indeed, as mentioned earlier, transcriptional profiling has provided many important insights into E2/TAM regulation of global gene response.

Unfortunately, no currently available high-throughput method can provide large-scale quantitative measurements of expressed cellular proteins. The absolute protein expression measurement (APEX) method has provided the largest data sets of this kind, measuring just over 1000 proteins [57]. Polysome profile analysis using sucrose density gradient centrifugation is a validated approach diagnosing and investigating translational control [58]. This method separates the population of mRNAs by their association with ribosomes and has been shown to be a reliable indicator of protein synthesis. Untranslated and poorly translated mRNA remains towards the top of the gradient (0-80S), while transcripts undergoing active translation are associated with multiple ribosomes (polysomes) and are located towards the bottom of the gradient [58]. In the vast majority of cases, the rate of initiation limits translation, and therefore a redistribution of an mRNA within the gradient is indicative of altered translation efficiency [64]. DNA microarrays can be used to globally quantify the translation states of mRNAs by measuring the expression levels of transcripts isolated from monosome and polysome regions within a sucrose gradient. This method has been shown to have increased sensitivity, compared to transcriptional profiling, in identifying pathways and genes responding to drug treatment [66]. This method has also allowed for the detection of polysome-associated transcripts that were not identified when measuring the transcriptome alone [67].

Combining transcriptional and translational analysis leads to insights into how these two levels of gene expression regulation are coordinated [64]. The simplest expected

mechanism is that some specific pathways and genes would show correlated regulation in both the transcriptome and translational responses. Such homodirectional changes have been termed potentiation [68]. Alternatively, translation regulation may be independent of apparent transcriptome level changes as observed in a number of systems [66, 69-73].

E2 and TAM Mediate Post-transcriptional Gene Regulation

In general, E2's role in regulating post-transcriptional gene processing remains under-explored. There are, however, a few examples of important post-transcriptional processing that have been identified. Interestingly, in the 1960s, there was an active debate regarding whether or not E2 acted directly as a translation factor [74]. While E2 does not directly act as a translation factor, E2 does have the ability to induce rapid responses that mediate translational and other post-transcriptional gene processing activity. This activity could be independent of ERα, as both E2 and TAM have been shown to bind many intracellular proteins in addition to ER [37]. For example, independent of transcription, TAM greatly enhances protein expression of transforming growth factor ß1 (TGF-ß1) by enhancing its translation efficiency in ER- human fetal stromal cell lines [75].

E2 can also act through non-genomic signaling proteins. Within 10 minutes of E2 treatment, there is a rapid increase in the phosphorylation of translation machinery, including some of the downstream targets of mTOR, ribosomal protein S6 kinase, and

eukaroyotic translation initiation factor 4E binding protein in MCF-7 [76]. The phosphorylation status of these factors modulates global and specific mRNA translation efficiency [77]. These observations suggest a direct mechanism linking E2 with the regulation of protein synthesis. In fact, E2 has been shown to translationally upregulate the synthesis of PSD-95 in neurons, by facilitating Akt phosphorylation dependent protein translation [78]. E2 stimulation induces positive potentiation by upregulating the progesterone receptor both transcriptionally and translationaly [79]. Fibulin-1 mRNA is upregulated during transcription, but one of its two major splice variants is negatively regulated post-transcriptionally by decreasing its stability [80]. E2 regulates ERα mRNA post-transcriptionally by decreasing its stability [81]. Only one other group has performed parallel analysis of transcription and translation profiles in MCF-7 [72]. While they were not investigating E2 or SERM response, but rather TNF stimulation, they found that the cell line has the ability to regulate genes at both the transcriptional and translational levels.

Research Aims

The aim of the following research is to gain a more complete understanding of how E2 drives the proliferation of breast cancer. This work achieves that goal by examining the extent and significance of E2 mediated post-transcriptional gene regulation, and by investigating how E2 coordinates transcriptional and post-transcriptional regulation.

We have investigated post-transcriptional regulation of E2 response in the breast cancer cell line MCF-7 by performing genome-wide microarray analysis comparing total mRNA levels with that of monosomal and polysomal mRNA levels after E2 treatment. Our data have identified two transcription factors mediating E2 stimulation, SOX2 is translationally repressed, while expression of NRSF target genes expression is repressed by E2. We have demonstrated that these two transcription factors mediate E2 stimulation of the cell cycle. These observations highlight a novel level of control mediating E2 stimulation of breast cancer cells.

To improve our understanding of the role of translation in endocrine therapy, we have extended our analysis to explore TAM controlled translation regulation in order to identify directly regulated genes and any potential sequences involved in antiestrogen induced growth arrest.

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Chapter 2

Estrogen Coordinates Translation and Transcription Revealing a Role for NRSF in Human Breast Cancer Cells

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Abstract

Posttranscriptional regulation may enhance or inhibit estrogen transcriptional control to promote proliferation of breast cancer cells. To understand how transcriptome and translational responses coordinate to drive proliferation, we determined estrogen's global and specific effects on translation regulation by comparing the genome-wide profiles of total mRNA, polysome-associated mRNA, and monosome-associated mRNAs in MCF-7 cells after stimulation by 1 h of 10 nM 17β-estradiol (E2). We observe three significant, novel findings. 1) E2 regulates several transcripts and pathways at the translation level. 2) We find that polysome analysis has higher sensitivity than total RNA in detecting E2-regulated transcripts as exemplified by observing stronger E2-induced enrichment of E2 expression signatures in polysomes more than in total RNA. This increased sensitivity allowed the identification of the repression of neural restrictive silencing factor (NRSF) targets in polysome-associated RNA but not total RNA. NRSF activity was required for E2 stimulation of the cell cycle. 3) We observe that the initial translation state is already high for E2 up-regulated transcripts before E2 treatment and vice versa for E2 down-regulated transcripts. This suggests that the translation state anticipates potential E2-induced transcriptome levels. Together, these data suggest that E2 stimulates breast cancer cells by regulating translation using multiple mechanisms. In sum, we show that polysome profiling of E2 regulation of breast cancer cells provides novel insights into hormone action and can identify novel factors critical for breast cancer cell growth.

Introduction

Estrogen influences many cell types and is a critical driver of many breast cancers. Estrogen stimulates 70% of breast cancers through the transcription factor estrogen receptor-α (ER). Expression profiling has provided many important insights into genes and networks controlling breast cancer proliferation [1, 2, 3, 4]. It has become clear that a complex network of genes and pathways is controlled by 17β-estradiol (E2) to promote breast cancer cell growth. Although transcriptional regulation is clearly central to E2 stimulation of proliferation, little is understood about the coordination with the myriad downstream posttranscriptional processes necessary to transduce these signals and synthesize proteins.

Estrogen stimulation of protein synthesis was first observed in the 1960s, leading to active discussion about whether estrogen directly stimulates transcription or translation [5, 6, 7]. The discovery of ER solidified the idea that estrogen can directly regulate RNA synthesis [8, 9]. Since then, most efforts have focused on understanding transcriptional regulation and how this leads to proliferation of breast cancer cells [1, 2, 3, 4]. Complementing transcription, protein synthesis stimulation is vital for rapid cancer cell growth. The regulation of translation has emerged as a vital player in driving tumors [10], as exemplified by the role of mammalian target of rapamycin (mTOR) [11], the overexpression of initiation factors such as eukaryotic initiation factor 4E (eIF4E) [12, 13, 14], and the role of micro-RNAs in numerous cancers including ER+ breast tumors

[15, 16]. Recently, miR-21 has been shown to be directly regulated by ER [17, 18], providing a mechanism linking ER transcription control and translation regulation.

Most regulation of translation occurs during initiation as extensively reviewed [19]. Cell cycle-dependent activation of translation initiation has been described [14, 20]. High expression levels of translation initiation factors such as eIF4E have transforming and strong proliferative properties, suggesting that these proteins may be important oncogenes [13, 21]. From these observations and the targeting of mTOR in breast cancer [22], translational control is emerging as a potential therapeutic target to regulate cancer cell growth. Homodirectional coordination between transcription and translation has been described in yeast [23] and makes intuitive sense to drive gene expression. The term potentiation was suggested by Preiss and co-workers [23] to describe this phenomenon.

Estrogen control of posttranscriptional regulation, including translation, has been reported for a number of transcripts [24, 25, 26]. In Xenopus, polysomal ribonuclease 1 is a nuclease, activated by E2, that degrades polysome-associated transcripts [27]. A second example is the E2-inducible protein vigilin, which binds 3'-untranslated regions to control pre-mRNA processing [28]. These examples highlight how E2 action involves specific posttranscriptional events as well as transcriptional events. In cancer, E2 may reduce protein expression of ABC transporters posttranscriptionally to mediate drug sensitivity [29, 30].

More recently, a new appreciation has emerged for the role of nongenomic, transcription-independent control of cell behavior by estrogens [31, 32]. Both genomic and nongenomic pathways are thought to contribute to breast cancer growth [33, 34]. Within 10 min of E2 treatment, rapid phosphorylation of translation machinery including some of the downstream targets of mTOR, ribosomal protein S6 kinase, and eukaryotic translation initiation factor 4E binding protein in MCF-7 cells has been reported, suggesting a direct mechanism linking E2 with regulation of protein synthesis [35].

Translational profiling is one of the best ways to determine which fraction of the genome is actually made into proteins [36]. This method determines the population of actively translated mRNAs by microarray analysis after sucrose gradient fractionation. Translational profiling has been applied to many model systems, from yeast [37, 38] to cell lines [39, 40], to uncover novel pathways and genes involved in stem cell differentiation [41] and apoptosis [40]. Often, translational profiling is applied to systems where global translation is decreased, such as during apoptosis or other stress conditions [40, 42, 43, 44]. Other studies have examined specific translation regulation upon significant increase in protein synthesis such as during growth stimulation [23, 41, 45]. All of these studies have found that transcripts loaded onto polysomes are likely translated [46]. Specific putative internal ribosome entry sites such as polypyrimidine-rich elements [40] and 5' terminal oligopyrmidine tract sequences [45] have been identified in mammalian transcripts as drivers of cap-independent translation. Specific

regulation of translation by general translation factors, such as eIF4E, has been reported [45, 47].

As a first step toward understanding how estrogens may control translation of specific transcripts and pathways in breast cancer cells, we probed RNA isolated from polysomes, monosomes, and total RNA on Affymetrix microarrays from MCF-7 breast cancer cells E2 stimulated for 1 h. We hypothesized that many key factors and pathways are translationally regulated by E2, complementing classic transcription control through estrogen receptors. The simplest expected mechanism is that some specific pathways and genes would show correlated regulation in both the transcriptome and translational responses. Such homodirectional changes have been termed potentiation, [23] and have been observed in a number of systems [48, 49]. Alternatively, transcripts may be stimulated translationally but not in the transcriptome. Consistent with this possibility, we observe pathways and genes likely critical for MCF-7 growth including ribosomal proteins, energy metabolism pathways, and the ERK pathway stimulated by E2 at the translation but not transcriptome level. Examination of E2-induced changes in polysome and total RNA revealed a second mechanism of apparent increased sensitivity of E2-regulated transcripts in polysomes, including known E2 expression signatures. We took advantage of this apparent amplification and observed that targets of neuron-restrictive silencer factor (NRSF)/RE-1 silencing transcription factor (REST) are down-regulated by E2 in polysomes more so than in total RNA. NRSF is a transcriptional repressor that suppresses neuronal-specific genes in nonneuron cells [50] and can regulate cancer cell

proliferation [51]. To test whether transcription factor networks detected in polysomes, but not total RNA, are important for E2 action, we knocked down NRSF and found that NRSF activity is important for E2 stimulation. Interestingly, these observations correlate with higher NRSF expression in relapsed ER+ breast tumors. Surprisingly, we observe a third mechanism coordinating the transcriptome and translational responses. The translation state of E2 up-regulated transcripts that were already high before E2 treatment, anticipating the eventual expression changes. Together, these data highlight how transcription and translation coordinate at multiple levels and demonstrate how polysome analysis can be used to identify key regulators of steroid hormone action.

Results

Hours of E2 stimulation significantly affects the expression of thousands of genes, whereas far fewer are observed to change significantly at 1 h. This suggests that many of the early responders are more likely to be directly regulated by ER. Here, we aimed to understand what the contribution of translational regulation may be to E2 stimulation of breast cancer cell proliferation. To probe E2 translational regulation, we compared transcript levels in polysomes and monosomes with total RNA between E2-treated and vehicle control MCF-7 cells (Fig. 1). MCF-7 cells were hormone deprived in charcoal-dextran-treated (CDT) serum for 3 d before addition of 10 nM E2. We chose these conditions to compare these data with the vast array of transcription and gene expression data [3, 4, 52, 53, 54]. Polysomes and lighter subpolysome fractions containing monosomes were isolated as described in Materials and Methods by linear 10–45%

(wt/vol) sucrose gradient fractionation (Fig. 1). We observe that 1 h of E2 stimulation induces no obvious, significant increase in polysome/monosome ratios in MCF-7 breast cancer cells (Fig. 1). Addition of the vehicle, 0.1% ethanol, does not significantly affect the polysome profile compared with untreated hormone-deprived MCF-7 cells (data not shown). Consistent with these observations, we typically isolate similar amounts of polysome RNA from 1 h E2-treated and vehicle-treated cells. The observed peaks are likely active polysomes, because their formation is puromycin dependent. Puromycin significantly reduces the appearance of polysomes by greater than 100-fold, suggesting that most of the mRNA cosedimenting with polysomes is likely associated with the polysomes (Supplemental Fig. 1). [35S]Methionine and cysteine labeling of total protein after 5 h of high 100 nM concentration of E2 suggests a modest 10% increase in protein levels, whereas slightly lower stimulation with 10 nM E2 is observed (data not shown). This is consistent with small increases in polysome/monosome ratios by 3 h (data not shown). Together, these observations suggest that as E2 stimulates hormone-deprived MCF-7 cells, resulting in only modest increases in global translation, indicating that any changes in the first hour of E2 stimulation are likely specific.

RNA was isolated from pooled polysome fractions by guanidine hydrochloride ethanol precipitation followed by purification through a QIAGEN RNeasy column. Biological triplicates were prepared and hybridized to Affymetrix Human Gene ST 1.0 arrays. Arrays were all of high quality and reproducibility, as determined by Affymetrix metrics and replication plots (Supplemental Fig. 2). As internal controls, we observe stimulation

of well-known E2-regulated genes, including XBP1 (2-fold) and MYC (3-fold), in both total and polysome RNA (Supplemental Table 1).

To analyze the polysome data, we compare three values for each transcript: the signal in total RNA (T), the signal in the polysomes (P), and the signal in the monosomes (M). Total RNA measures the transcriptome and is indicative of the balance of transcription and degradation in all cellular compartments. Polysome RNA is indicative of transcripts engaged with ribosomes during the elongation phase. The light subpolysome fractions, monosomes, are dominated by interactions with preinitiation ribosomal complexes. The distribution between the polysome and monosome fraction (P/M) indicates the translation state in that condition [23]. The polysome and total RNA (P/T) also can indicate the fraction of the RNA in the cell that is associating with polysomes and represents a second measure of translation state [45]. In sum, there are two major approaches that we use to characterize how E2 may coordinate the transcriptome and translational responses. 1) Changes in the polysome fractions (PE2/PVeh) are sensitive to both translation and overall mRNA level changes. Comparing this ratio to changes in total RNA (TE2/TVeh) can identify candidate translationally regulated genes. 2) The translation state of each transcript defined as P/T or P/M indicates the fraction of the transcript cosedimenting with polysomes. Changes in these ratios would indicate translation regulation. Each analysis is sensitive to different dynamic range and noise, and thus, each analysis complements the other in understanding translation regulation and identifying gene expression regulators important for E2 action.

Because the polysome traces did not show significant differences when treated with E2, we did not expect to see changes in the global distribution of transcripts between the E2 and vehicle conditions. Figure 2 shows that the distribution of transcripts in both total vs. total and polysome vs. polysome comparisons of E2 to vehicle is not globally shifted, suggesting that the observed differences are relatively specific in this background (Fig. 2, A and B). This also allows us not to use a scale factor to correct for significant mass differences in the E2 and vehicle conditions to identify specific translation state regulation.

To test the quality of the microarray data, we sampled transcripts by quantitative PCR (qPCR). To perform qPCR, luciferase mRNA was added to each fraction to normalize for purification yields. We sampled 13 genes in each pooled fraction and observe high correlation ($R2 > 0.8$; R2, coefficient of determination which is square to the correlation coefficient) between the microarray and qPCR data, suggesting that the array data are accurate (Supplemental Table 1). To determine whether the microarray data accurately portray the translation state of transcripts, we compared the polysome to monosome ratio from the microarray to qPCR measurements for selected transcripts (Fig. 2D). This requires comparing RNA isolated from polysomes and monosomes, which originate from different yields of mass and volume. Figure 2D shows that although the absolute ratios differ between fractions for eight genes selected for significantly high and low translation states ($P < 0.001$, fold change (Fc) > 2) in the microarray data, the two measurements accurately determine the relatively high and low translation states for each transcript. It is

not clear why the absolute ratios differ between the two measurements. Most reports compare microarray data to Northern blots without spike-in transcripts for normalization control and thus may not be accounting for variable purification yields from each fraction. These data indicate that relatively high and low translation states are accurately measured by two independent assays.

E2 regulates specific genes and pathways at the translation level

One mechanism for E2 regulation of translation is to control initiation, the major rate-limiting step in protein synthesis. Polysome cosedimentation indicates transcripts that have entered the elongation phase of the translation cycle. Exclusive changes in polysome RNA or total RNA would indicate translation or transcriptome regulation, respectively. We examined the correlation between E2 regulation in total and polysome RNA for the most significantly changing genes (Fig. 3A). We observed only a small number of significantly changing genes using thresholds of $P < 0.05$ and fold changes greater than 1.5, with a strong bias toward up-regulated genes in polysomes and total RNA (Supplemental Tables 2 and 3). Figure 3A shows that most transcripts are stimulated in both the total RNA and polysome RNA fractions, consistent with potentiation, where transcriptome and polysome changes are significantly correlated.

We identified the genes with the highest and lowest translation states using a t test ($P < 0.05$) and fold change (>50% change) thresholds. The distribution of the translation states

in E2- and vehicle-treated cells shows that genes with high translation states in the vehicle-treated cells are not stimulated to even higher translation states by E2 treatment (Supplemental Fig. 3A). On the other hand, transcripts with low translation states in the vehicle condition are modestly driven toward higher translation states at this early time point (Supplemental Fig. 3B). At longer times, we see a larger global shift into polysomes consistent with increased polysome/monosome ratios seen on the sucrose gradients (data not shown).

Because few genes are significantly changing, we sought to examine the overall trends in the data. To gain insight into the pathways that may be regulated at the transcriptome or translation levels, we compared E2 polysome vs. vehicle polysome and E2 total vs. vehicle total changes using gene set enrichment analysis (GSEA) [55]. GSEA uses all the data to determine whether specific gene sets, derived from pathways or transcription factor networks, may be biased in one condition compared with another. GSEA can therefore identify regulated networks without arbitrary thresholds but, rather, by determining trends using all the microarray data. This approach has proven invaluable to identify and experimentally confirm the role of critical pathways in numerous systems [56, 57]. Table 1 summarizes some of the top pathways translationally affected by E2, i.e. in polysomes only, such as oxidative phosphorylation, or in total RNA only, such as the cAMP response element-binding protein (CREB) pathway, or in both, such as the MAPK pathway. E2 appears to stimulate energy metabolism and ribosomal proteins in

polysomes but not total RNA. In sum, these observations suggest that E2 uses different mechanisms to stimulate genes required for growth and survival.

A second approach to examine translation regulation is to compare the translation state of pathways in the E2- and vehicle-treated cells. Pathways that show changes in both translation state and polysome levels, but not total RNA, are the most likely to be truly translationally regulated. Table 2 lists pathways significantly enriched in P/T or P/M measurements of the translation state. Pathways highlighted in bold or italics show different enrichments in the vehicle and E2 conditions. These include some of the same pathways observed to change in polysomes but not total RNA in Table 1, including the ERK pathway, whereas others change in total RNA but not polysomes such as the p38MAPK pathway. Similar to past studies [23], some enrichment is seen in one approach but not the other. This is likely due to experimental noise in each analysis. Thus, the most likely candidates to be translationally regulated are those showing significant responses in both approaches, such as the ERK pathway, which shows changes in polysome vs. polysome [normalized enrichment score (NES) = 1.80], but not total vs. total (NES = 1.25), and changes in translation state (E2 NES = 1.47 vs. vehicle NES = 1.28).

E2-regulated genes are more enriched in polysomes than in total RNA

Correlated changes in both the transcriptome and translation state is a phenomenon termed potentiation [23]. We anticipated that many pathways and expression signatures stimulated by E2 would show both increased transcriptome expression and translation states, consistent with potentiation. This is the case for some pathways, such as the MAPK pathway, where upon E2 treatment, the translation state increases (E2 NES = 1.82 and vehicle NES = 1.62), transcriptome levels increase (NES = 1.43), and polysome levels increase (NES = 1.43). The data in Tables 1 and 2 also suggest that some pathways are more enriched in polysome comparisons than in total RNA. Most striking is the observed stronger enrichment of known E2 expression signatures [4, 53] in polysomes compared with transcriptome level changes. This is shown in Fig. 3B, where more of the genes in the Frasor E2 expression signature are biased toward E2 in polysomes than in total RNA. To test whether ER-regulated genes are also enriched in polysomes, we created gene sets for all genes with transcription start sites within 10 kb of chromatin immunoprecipitation observed binding regions [3, 58]. Stronger biases are observed in E2 polysome vs. vehicle polysome compared with E2 total vs. vehicle total (Table 1). These observations suggest that increased expression of transcripts previously associated with E2 action have large fold changes in polysomes from two possible mechanisms. 1) Even before total mRNA levels of ER-bound genes are observed to increase, these transcripts are translationally stimulated in the polysomes. This model implies that the cells may be specifically stimulating ER genes translationally before responding to E2 genomic action. 2) A more likely explanation may be that detection of mRNA level changes is more sensitive in polysome RNA than total RNA, perhaps because small

changes in total RNA are amplified at the translational level, enhancing E2-induced transcriptome changes. Polysome mRNA may also represent a higher level of purification of mature mRNA, without confounding effects of nuclear fractions as recently suggested by examining cytoplasmic vs. nuclear RNA [59]. In sum, E2-stimulated genes are observed with higher sensitivity in polysomes compared with total RNA after 1 h of E2 treatment.

Enhanced sensitivity of gene expression regulation in polysomes reveals NRSF

We hypothesized that the higher apparent sensitivity observed for E2 expression signatures and ER-bound genes in polysomes could be useful to identify novel networks that may mediate E2 action. Therefore, we investigated whether enrichment of gene sets with common transcription factor-binding promoter motifs is observed in total and/or polysome RNA fractions. Transcription factors are often key regulators controlling cell fate, and significant precedent exists for identifying them using GSEA [56]. GSEA reveals a number of gene sets with common transcription factor motifs in promoters enriched in polysome RNA but not total RNA (Table 3). E2F1-regulated genes are enriched in polysomes, but not total RNA, at 1 h. E2F1 has been observed to be enriched in E2-stimulated MCF-7 cells from a long time-course experiment [60]. Genes regulated by the aryl hydrocarbon receptor are also enriched in E2 polysome compared with vehicle polysome RNA. Aryl hydrocarbon receptor has previously been associated with E2 signaling [61]. These data suggest that polysome profiling has increased sensitivity in detecting significant changes in specific gene expression programs known to be

important for E2 action. Together, these findings suggest that even at 1 h, E2 is rapidly stimulating many of the specific gene expression programs critical for E2 action and that these are more readily detected in polysome RNA than in total RNA.

To determine whether transcription factor networks enriched in translational responses are important for E2 stimulation, we tested the function of NRSF. NRSF-regulated genes are repressed in E2 polysomes, compared with vehicle polysomes, whereas no significant change is observed in total RNA (Fig. 4). Moreover, NRSF-regulated genes have lower (NES = –2.32) P/M translation states in E2 compared with vehicle (NES = –2.20). To our knowledge, NRSF, a transcription repressor of neuron-specific genes in nonneuronal tissue [50], has not previously been associated with E2 action, although it has been suggested to be a tumor suppressor in breast cancer [62, 63]. However, NRSF has also been shown to be oncogenic in some contexts (as reviewed in Ref. 64]. NRSF can mediate glucocorticoid receptor action [65], providing precedent for direct connections to nuclear receptors.

NRSF is required for E2 stimulation of ER+ breast cancer cells

The repression of transcripts with NRSF promoter motifs in polysomes predicts that NRSF activity may be crucial for E2 stimulation of the cell cycle in MCF-7 cells. To determine whether NRSF expression is required for E2 action, we knocked down NRSF with small interfering RNAs (siRNAs) in hormone-deprived MCF-7 cells and tested for

effects on E2 stimulation of the cell cycle by DNA content fluorescence-activated cell sorting (FACS). Figure 4A shows that significant knockdown of NRSF is observed at the protein level in typical experiments. The DNA content cell cycle data suggest that the cells were synchronized upon hormone removal with approximately 90% of the cells in G1 similar to previous reports [66]. Reduced NRSF expression reproducibly inhibited E2 stimulation of the cell cycle, compared with control scrambled siRNA sequences (Fig. 4, B and C). NRSF knockdown did not significantly affect the growth arrest induced by hormone deprivation but inhibited the E2 response. NRSF also affects E2 action in a second ER+ cell line, T47D, suggesting that NRSF's importance in mediating the cell cycle is not MCF-7 specific (Supplemental Fig. 4). We tested whether NRSF affects MCF-7 growth in rich medium and observed that knockdown of NRSF causes a modest 30% decrease in the number of viable cells after 3 d (data not shown). Because NRSF is typically thought to be a transcriptional repressor, these observations are consistent with a model of decreased expression of target genes being important for ER+ breast cancer cell growth.

NRSF may mediate both E2 and tamoxifen (TAM) action. We tested whether NRSF affects TAM activity using a 4',6-diamidino-2-phenylindole (DAPI) assay to detect apoptotic cells (Supplemental Fig. 5). Only a modest number of apoptotic cells are induced by 2 μM TAM as previously reported [67]. When NRSF is knocked down, an increase in the number of apoptotic nuclei is observed compared with negative siRNA controls with and without TAM (Supplemental Fig. 5). These data indicate that NRSF

induces MCF-7 cell death with no significant synergistic, but perhaps additive, effects on TAM sensitivity in hormone-deprived conditions. Because TAM has mild agonist activities, this could be complicating the balance of effects observed under these conditions. Additional experiments testing for TAM and NRSF effects on caspase-3, caspase-7, and caspase-9 activity did not reveal any significant effects (data not shown). These data suggest that NRSF is important for E2 stimulation of ER+ breast cancer cells but may not be critical for TAM-induced cell death.

These in vitro data suggest a possible role for NRSF in controlling growth of ER+ breast cancer cells. To test whether NRSF may be important for breast tumor progression and patient prognosis, we examined NRSF expression in breast tumors using Oncomine [68]. For all breast carcinomas, NRSF expression is significantly higher for patients who did not survive 5 yr compared with those who did (Supplemental Fig. 6A). In particular, NRSF is more highly expressed in primary ER+ tumors in relapsed patients compared with those with no recurrent disease with modest significance (Supplemental Fig. 6B). Interestingly, NRSF is more highly expressed in ER– breast carcinomas compared with ER+ breast carcinomas (Supplemental Fig. 6C). Because ER– carcinomas are typically more aggressive and lethal than ER+ tumors, higher NRSF expression may be contributing to faster growth. NRSF expression is also significantly higher in breast carcinomas compared with normal breast tissue (Supplemental Fig. 6D). In sum, higher NRSF expression in poor-prognosis breast carcinomas is consistent with our findings that NRSF is necessary for maximal E2 stimulation of ER+ breast cancer cells. These data

suggest that additional investigation of NRSF is warranted to understand the role of the NRSF network in breast cancer.

E2 coordination of transcription and translation

The translation state may be reflecting mass action driving transcripts into polysomes such that higher translation state and expression are correlated. To determine whether the translation state of these clusters is correlated with the expression level, we compared the translation state to the total RNA expression levels as defined by the hybridization score (Supplemental Figs. 7 and 8). Up-regulated genes are slightly biased toward lower initial expression levels compared with down-regulated genes. This may reflect that stimulated transcripts are selected to go up upon E2 stimulation and thus may start from a lower position than down-regulated transcripts. In any case, transcripts with a higher translation state are not biased toward higher expression levels as may have been expected. In fact, E2-stimulated transcripts have lower expression levels at the zero time point compared with repressed transcripts, further suggesting that the initial translation state is independent of the expression level. These findings suggest that the translation state of each transcript is independent of expression levels in these conditions.

After 1 h of E2 stimulation, there is not a significant increase in global translation as indicated by comparing E2 polysome vs. vehicle polysome transcripts (Fig. 2) and by the distribution of translation states in each condition (Supplemental Fig. 3). When

examining the translation state of E2-stimulated transcripts, we noticed that the translation state was higher in the vehicle control for E2-stimulated transcripts than for E2-repressed transcripts. We considered RefSeq genes measured in Affymetrix U133 plus 2.0 microarray time-course data from Carroll et al. [3] and compared the time patterns to the initial translation state observed in the vehicle control. A sampling of transcripts by qPCR suggested that 0.1% ethanol and untreated MCF-7 cells are very similar (data not shown). We thus equate vehicle-treated cells with untreated cells as equivalent to the cell state before the addition of E2. Upon E2 stimulation of MCF-7 cells, hundreds of genes are stimulated, whereas others are down-regulated, as shown in Fig. 5A. Significantly changing transcripts were selected by ANOVA with P values <0.1 and fold changes more than 1.5-fold and clustered into six distinct groups (Fig. 5A). We compared the initial translation state at the zero time point to the time-course behavior of E2-regulated genes. Figure 5B shows that stimulated transcripts (clusters 1 and 2) have high initial translation states in the absence of E2. On the other hand, transcripts that will be down-regulated by E2 in the transcriptome (clusters 5 and 6) have lower initial translation states. These observations suggest that the inherent translation state of transcripts predicts the direction that E2 will drive expression in total RNA. Comparing the polysome to total (P/T) ratio indicates the fraction of the cellular RNA engaged with active ribosomes. Recent data suggest that total RNA is significantly different from cytoplasmic RNA [59]. An alternative indicator of the translation state is the P/M ratio. Similar trends are observed, albeit with slightly more moderate biases as indicated by the higher, but still significant P values, as shown in Fig. 5C. The P/M ratio reflects how each transcript fractionates in the sucrose gradient and is a refined metric of translation state

and indicates the ratio of transcripts that are before or after initiation of translation. The difference between the P/M and P/T distributions may indicate that P/T is sensitive to all processes that can affect polysome and total mRNA levels including presence in the cytoplasm, whereas P/M is more specific for translation state. Therefore, the reduced bias seen in P/M compared with P/T may indicate the importance of other steps such as nuclear export in controlling mRNA levels. In sum, these data suggest that under these cell conditions, E2 increases the overall expression of some genes by increasing their transcriptome levels, leading to higher levels in both polysomes and monosomes. On the other hand, transcripts down-regulated in the transcriptome have low initial translation states yet shift toward higher translation states, suggesting that transcriptome and translational responses may be anti-correlated.

We expected genes stimulated by E2 in total RNA would show higher translation states after E2 stimulation, reflecting potentiation. To determine how E2 may affect the global translation state, we compared the translation state before and after E2 treatment. Stimulated transcripts in clusters 1 and 2 show a very modest decrease in translation state, whereas significantly down-regulated transcripts in clusters 5 and 6 show a modest increase in translation state (Supplemental Figs. 9 and 10). Similar patterns are observed for both P/T and P/M definitions of translation state. The up-regulated transcripts are already shifted to a much higher translation state before E2 treatment and therefore may not be able to have a further significant increase (Supplemental Fig. 9, clusters 1 and 2). On the other hand, the down-regulated transcripts start at a lower translation state and

then have the potential to be driven into a higher translation state (Supplemental Fig. 9, clusters 5 and 6). Similar trends are seen for all transcripts that are in high and low translation states (Supplemental Fig. 11). Therefore, although total RNA levels may be decreasing for the transcripts in clusters 5 and 6, the overall expression may not be down-regulated because the translation state is increasing, counteracting the transcription effects. These observations suggest that the translation state is not correlated with expression level but is an independent indicator of gene expression, consistent with the well-known low correlation between total RNA and protein expression levels [69].

To understand why MCF-7 cells appear to be trying to translate E2-stimulated transcripts before the addition of E2, we investigated whether there is functional organization to these observed patterns. It is possible that mRNAs primed for E2 stimulation with higher translation states are transcripts that MCF-7 cells likely need to survive when hormone deprived and growth arrested. We examined gene ontology (GO) enrichment using GOStat [70] to compare the genes in each cluster to all 3273 E2-regulated genes measured on both array experiments. Up-regulated genes in clusters 1 and 2, with higher translation states before the addition of E2, are enriched for genes involved in cell growth and survival including the cell cycle, ribosome biogenesis, and DNA replication (Supplemental Table 4). Only modest enrichment of vacuole, lysosome, and membrane-associated transcripts in clusters 5 and 6 is observed. Clusters 3 and 4 do not show any significant enrichment. In sum, these observations suggest that the translation state predicts or anticipates the subsequent direction of expression upon E2 treatment.

Discussion

Our findings suggest that E2 uses multiple mechanisms to coordinate transcription and translation to stimulate ER+ breast cancer cell growth. They also illustrate the value of genomic analysis of polysome profiling to identify novel factors and networks important in the regulation of breast cancer cell proliferation. Together, these findings highlight the importance of understanding translation regulation as a key contributor to hormonal regulation of proliferation in breast cancer.

Our approach focuses on changes occurring within 1 h of E2 stimulation, allowing detection of several E2-regulated genes and programs at the expression and translation levels. This early time point increases the probability that these observations are more directly controlled by E2. The mechanisms leading to translation control by E2 are not yet clear. This could be regulated by either rapid transcriptional control of key regulators such as MYC, which promotes ribosome biogenesis and translation [71]. It could also be through rapid induction of signaling pathways including mTOR, whose targets are reported to be rapidly phosphorylated within 10 min in MCF-7 cells, likely leading to increased polysome association and stimulation of some transcripts [35]. Global initiation factors appear to regulate specific transcripts as recently suggested for eIF4E, which stimulates ribosomal proteins and other transcripts critical for cell growth more quickly than others, suggesting an element of specificity [45]. E2 increases polysome association for transcripts involved in energy metabolism and the ERK pathway without significantly

affecting transcriptome levels, suggesting that these pathways are regulated translationally.

We used multiple approaches to examine translation regulation including two measures of the translation state. The P/T and P/M ratios indicate the fraction of each transcript in the cell associating with polysomes. The P/T ratio can be influenced by transcription, stability, and subcellular location. This metric indicates how the expression level in the transcriptome compares to transcript levels associating with polysomes. The P/M ratio is a more direct measure of translation state because it indicates how each transcript migrates in the sucrose gradient in pre-initiation (monosomes) and post-initiation (polysome) complexes. Similar global trends are seen for both analyses as shown in Fig. 4. Stronger biases are seen in the P/T comparison, perhaps because the total RNA is starting at lower or higher expression values (Supplemental Fig. 7), which affects the P/T ratio. This likely reflects how transcriptome levels are uncoupled from translational responses because the polysome levels do not necessarily follow the transcriptome levels. The genes that appear to be E2 up-regulated in total RNA appear to have a combination of biases including low starting expression compared with down-regulated genes (Supplemental Fig. 7) and high translation states compared with down-regulated genes (Fig. 4). Thus, translational profiling may be a general approach to identify E2-regulated transcripts, even those that may be directly transcriptionally controlled by ER. Proving ER's role in driving the observed amplification of predicted ER gene sets (Table 1 and

Fig. 3B) would require additional experiments such as translational profiling to test the role of ER specifically.

A second general approach to determine how translational responses and transcriptome levels are being regulated by E2 is to compare changes in polysomes to changes in the total RNA as highlighted in Table 1. The polysome fold change vs. total RNA fold change and the translation state approach each are subject to different experimental noise and caveats. We therefore present both, similar to past studies [23]. Candidate translationally regulated pathways are those showing significant regulation in both approaches such as the ERK pathway. These data strongly suggest that specific E2 translational regulation is a key component of E2's ability to drive breast cancer cell growth.

Polysome analysis reveals apparent increased sensitivity to detect E2-regulated gene sets compared with total RNA. Transcriptional mechanisms have been shown to influence posttranscriptional processes through control of poly(A) tail lengths, alternative 3' processing, and formation of specific messenger ribonucleoprotein that can all influence and enhance translational regulation [72, 73]. Some E2-stimulated genes, including many potentially directly regulated by ER, are likely transcriptionally stimulated yet are detectable more significantly in polysomes than in total RNA at an early 1-h time point. This type of enhanced expression in polysomes compared with total RNA has been

reported in yeast, where coordinated transcriptome and translation was found to be part of a transcription factor's regulation of gene expression in the stress response [48]. Thus, not only do these transcripts show correlated up-regulation at the transcriptome and translation levels, but they also appear to amplify the degree of change in polysomes more significantly compared with other genes in the transcriptome. Although the mechanism of polysome but not total RNA enrichment is not clear, previously known key regulators of E2 action are more significantly enriched in polysome RNA compared with total RNA (Tables 1 and 3). This increased sensitivity may be indicative of an amplification of small transcriptome changes. Additional time points may provide further insight into the coordination between transcriptome and translational responses. At later times, more global increases in translation state may be observed. This would suggest that translational responses and transcriptome changes respond to E2 with different kinetics. Based on the observations at the 1-h time point, it seems likely that a variety of translation state responses would also be observed at later time points. Another possibility is an amplification model where small changes in total RNA levels lead to much larger changes in polysomes for some transcripts. Although true for some pathways such as glycolysis, this is not always the case, as seen for transcripts coding for the CREB pathway. This amplification model highlights how transcription and translation can be sensitive to E2-stimulated gene expression to different degrees at each time point.

We can take advantage of this increased sensitivity in polysomes to detect novel networks regulated by E2. We tested the role of NRSF in E2 action and found it to be

necessary for maximal E2 stimulation of the cell cycle. We suspect that NRSF was not previously seen in gene expression studies because it fell below the sensitivity of total RNA comparisons but was detectable in polysomes. NRSF has previously been implicated as both a tumor suppressor and oncogene in tumors [51]. Here, we find NRSF to be promoting E2-driven growth. NRSF has been found to be a tumor suppressor in rich-media conditions in ER– and ER+ cell lines [63] as well as in a RNA interference screen [62]. Our results differ for ER+ breast cancer cells, perhaps because we focus on the response to E2 under hormone-reduced conditions where the effects of E2 are isolated. Moreover, specific genetic backgrounds are known to lead to NRSF having oncogenic or a tumor suppressor activity [50, 51]. These in vitro findings correlate with NRSF mRNA expression levels in breast tumors. As a putative transcriptional repressor, higher NRSF would lead to suppression of the expression of many neuron-specific genes. Further investigation of the NRSF gene expression program in breast cancer would address these apparent discrepancies linking neuroendocrine systems, cancer biology, and estrogen action. NRSF may be developed as a breast cancer biomarker that could improve breast cancer treatment and diagnosis. Investigating the NRSF network may also identify key novel genes important for breast cancer growth and E2 action.

One of the most striking observations is that E2-regulated genes are biased toward higher translation states primed before the addition of E2. Transcripts that will be stimulated by E2 are biased toward polysomes, whereas those that will be repressed are biased away from polysomes. These data suggest that the translation state of many transcripts

anticipates the eventual expression changes induced by E2. This coordination may be mediated by sequence elements in either the 5'- or 3'-untranslated region; however, we have yet to identify a specific sequence enriched in the data. Gene ontology enrichment analysis suggests that many of the E2-stimulated, high-translation-state transcripts include cell cycle regulators and DNA replication machinery likely required for MCF-7 cells to reenter the cell cycle. Preliminary data suggest that at longer E2 treatment times where many genes are up-regulated by E2, no significant changes in translation state are observed, consistent with the translation state already being very high for most of these mRNAs (data not shown). Even under these cell conditions, the common expectation would have been that E2 would stimulate both the expression and translation state. Instead, the translation state of most E2-stimulated transcripts is already very high before the addition of E2. This could be because the cells are primed for the addition of a mitogen like E2 in an effort to survive during hormone deprivation. Alternatively, this observed priming of the translation state may be due to incomplete E2 deprivation during the 3 d of CDT treatment. The translation state could be very sensitive to low-level E2 signaling leading to the observed high translation states before the addition of E2. It is known that longer estrogen deprivation can further reduce E2 signaling. We used these short-term deprivation conditions to be able to compare with the vast array of previous genomic studies that have focused on transcription control including expression and chromatin immunoprecipitation experiments [3, 4, 52, 53, 54]. These short-term deprivation experiments suffer from incomplete removal of estrogen signaling, which requires longer, 2-wk hormone withdrawal. However, long-term deprivation may lead to complications from selecting cell populations that may be resistant to E2 [74]. We do not

know whether longer hormone deprivation may reduce the translation state, which would allow E2 to then stimulate the translation state of E2 transcriptionally regulated genes. These same E2-stimulated genes appear to have lower translation states in log-phase MCF-7 cells grown in 10% fetal bovine serum (FBS) with phenol red medium compared with 3-d CDT-treated, hormone-deprived cells (data not shown). This suggests that the whole balance of transcription and translation for E2-regulated genes is significantly different in hormone-deprived, growth-arrested cells compared with log-phase growing MCF-7 cells. It may also imply that the translation state is sensitive in unknown ways to a combination of E2 and other growth factor signaling driving MCF-7 growth in rich media. These findings are currently being further investigated to understand the relationship between E2 signaling and translational responses.

The classic model of transcription and translation coordination, potentiation, predicts that E2 would stimulate both the transcriptome and translation state. Our findings suggest that under these experimental conditions, used for the majority of expression profiling and transcription analysis of E2 action, E2-stimulated transcripts already have a high translation state before the addition of E2. Thus, increased expression for transcriptionally up-regulated transcriptions appears to be driven by mass action where increased transcriptome levels lead to higher levels in polysomes and not a change in translation initiation rates for these mRNAs.

In sum, we demonstrate the power of translation profiling to gain insight into the coordination of transcription and translation as a new approach to identify factors important for steroid hormone action. Together, these findings also suggest that translation control may be particularly sensitive to cell conditions and E2 signaling. Translational profiling can reveal novel E2-regulated genes and pathways. We believe that higher sensitivity in the polysomes and amplification of gene expression changes in the polysomes can help identify novel factors important for E2 signaling as exemplified by NRSF. Our data reveal novel mechanisms of E2 control of breast cancer cell growth, including specific translation regulation and surprising mechanisms to coordinate transcription and translation regulation of gene expression. Further investigation to understand how different estrogens and antiestrogens may coordinate transcription and translation may uncover novel mechanisms important to understand the full gene expression picture and regulators of cell state that may be relevant to breast cancer *in vivo*.

Materials and Methods

Antibodies, Western blots, and cell culture

MCF-7 cells were cultured in DMEM (Mediatech, Manassas, VA) with 10% FBS (HyClone, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin (GIBCO, Carlsbad, CA). Cells were hormone deprived in phenol red-free DMEM with 5% CDT FBS (Omega Scientfic, Tarzana, CA), 100 U/ml penicillin, and 100 mg/ml streptomycin.

Cells were treated with E2 (Sigma-Aldrich, St. Louis, MO) or 4-hydroxytamoxifen (Sigma-Aldrich), anti-NRSF antibody [Millipore Corp., Billerica, MA (07-579)], anti-β-tubulin antibody [Abcam Inc., Cambridge, MA (ab6046)], and antirabbit IgG and horseradish peroxidase (Cell Signaling Technology, Inc., Danvers, MA).

Determination of protein synthesis rates

A total 100 μCi EasyTag EXPRESS35S protein labeling mix (PerkinElmer, Waltham, MA) was added to hormone-deprived MCF-7 cells. Protein lysate was trichloroacetic acid/acetone precipitated, resuspended in guanidinium-HCl and added to scintillation fluid for counting. Counts were normalized to microliters of extract, and percentage of trichloroacetic acid incorporation was determined by dividing the trichloroacetic acid precipitate counts per minute per milliliter extract by total counts per minute per milliliter extract.

Sucrose gradient density centrifugation and RNA analysis

To prepare polysomes, MCF-7 cells were hormone deprived for 3 d before addition of 10 nM E2. Cycloheximide (CHX) (100 μg/ml) was added for 3 min, medium was removed, and the cells were washed with PBS with 100 μg/ml CHX. Lysis buffer [15 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 300 mM KCl, 100 μg/ml CHX, and 1% Triton X-100] was added. Equivalent OD260 units of total lysate was separated on a 10–45% continuous,

linear sucrose gradient prepared with lysis buffer without Triton X-100, prepared with a Gradient Master (BioComp Instruments, Fredericton, New Brunswick, Canada), by ultracentrifugation in a SW40 Ti rotor (Beckman Coulter, Brea, CA) for 3 h at 36,000 rpm at 4 C. Polysome profiles were collected with a Brandel gradient fractionator (Gaithersburg, MD) and determined by monitoring RNA absorbance at 254 nm. Fractions 3–6 were pooled to represent the subpolysomes, and fractions 7–13 were pooled to represent the polysomes. For puromycin experiments, 1 mM puromycin (Sigma-Aldrich) was added to the cells for 5 min and to the lysis buffer for 15 min at 37 C while mixing before ultracentrifugation.

Microarrays and analysis

RNA was purified by precipitation with guanidinium-HCl and ethanol followed by an RNeasy column (QIAGEN, Valencia, CA) according to manufacturer's instructions including treatment with deoxyribonuclease I. RNA quality was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). All RNA processing, array hybridizations, and scanning were performed by the Brown University Center for Genomics and Proteomics core facility. RNA was prepared for hybridization using Affymetrix standard protocols and applied to Human Gene 1.0 ST microarrays (Affymetrix, Santa Clara, CA). We quantile normalized all 18 arrays and then used the Affymetrix Probe Logarithmic Intensity Error (PLIER) algorithm to generate signal estimates for all RefSeq genes. To select actual signal, we discarded those transcripts belonging to the lower quartile of their respective datasets. Analysis of significantly

changing genes was determined using R (http://www.r-project.org/). Gene attributes were downloaded from UCSC Genome Browser [75]. Complete microarray data have been deposited at the National Center for Biotechnology Information's Gene Expression Omnibus (accession no. GSE18592).

To identify gene expression patterns in the U133 time-course data, we applied a correlation analysis to separate transcripts into groups with similar expression profiles as previously reported [76]. Briefly, each probe set expression profile was normalized by subtracting its mean and dividing by its SD. All normalized expression values of all probe sets in the class in question were then pooled together, and the mean and SD was computed at each time point.

Quantitative real-time PCR

Equal amounts of RNA from polysomes or total RNA were reverse transcribed using Superscript III and random hexamers (Invitrogen, Carlsbad, CA). Resulting cDNA was renormalized using Quant-iT PicoGreen (Invitrogen) before mixing with primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Reactions were performed in an Applied Biosystems 7900HT Fast Real-Time PCR system. The fold change for total vs. total RNA was calculated, using spiked-in luciferase mRNA for normalization. To control for differential masses and purification yields from polysomes,

luciferase mRNA was spiked in as a normalization control. Primer sequences are list in Supplemental Table 5.

siRNA knockdown, cell cycle, and apoptosis assays

Cells were plated in phenol red-free medium containing 5% CDT FBS at 450,000 and 10,000 cells per well in six- or 96-well plates, respectively. Silencer siRNAs targeting NRSF and control were purchased from Ambion/ABI (Austin, TX). Three siRNAs (siRNA ID 115696, 115695, and 107778) were pooled, and 75 nM total concentration was transfected using DharmaFECT 1 (Dharmacon, Inc., Lafayette, CO) according to the manufacturer's instructions. After 48 h, cells were treated with E2 or vehicle (0.1% ethanol). Cell cycle was determined by propidium iodine staining, using a BD FACSCalibur flow cytometer (BD, Franklin Lakes, NJ). FACS data were modeled using ModFIT LT software (Verity Software House, Topsham, ME). For the DAPI assay, cells were serum starved in phenol red medium with no FBS for 24 h before treatment with tamoxifen for 24 h. Cells were fixed in paraformaldehyde, stained with DAPI, and imaged with a Zeiss Axiovert 200M fluorescence microscope.

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Table 1. GSEA reveals stronger stimulation of known E2-regulated genes in polysomes

	E2 polysome *vs.* vehicle polysome		E2 total *vs.* vehicle total	
Pathway	NES	*P* value	NES	*P* value
ER binding (genes within 10 kb)	2.06	0.000	1.61	0.000
Stossi E2 UP signature	2.35	0.000	1.74	0.000
Frasor E2 UP signature	3.04	0.000	2.00	0.000
GenMapp				
Ribosomal proteins	**2.87**	**0.000**	**0.68**	**0.975**
Proteasome	**2.07**	**0.000**	**–0.80**	**0.754**
Oxidative phosphorylation	**1.75**	**0.000**	**0.69**	**0.952**
Glycolysis	**1.65**	**0.023**	**0.51**	**1.000**
DNA replication reactome	*–0.83*	*0.738*	*1.42*	*0.034*
G1 to S cell cycle reactome	0.87	0.723	1.20	0.168
ATP synthesis	1.17	0.261	0.51	0.985
RNA transcription reactome	1.44	0.060	1.59	0.016
Apoptosis	1.30	0.109	1.34	0.078
Biocarta				
ERK pathway	**1.80**	**0.003**	**1.25**	**0.163**
WNT pathway	1.55	0.044	1.51	0.037
MAPK pathway	1.43	0.022	1.43	0.021
P38MAPK pathway	*1.17*	*0.209*	*1.82*	*0.004*
CREB pathway	*0.99*	*0.462*	*1.58*	*0.025*

NES higher than 1.4 suggests significant bias toward E2. Positive NES indicates bias toward E2, whereas negative values indicate vehicle bias. *P* values <0.1 are significant. *Bold* indicates possible translational regulation with significant E2 stimulation in the polysome level only. *Italics* indicates transcriptome-level E2 stimulation but not a translational response in the polysome comparisons.

Table 2. Transcriptome or translation level analysis of specific pathways by GSEA

	E2 polysome *vs.* E2 total		Vehicle polysome *vs.* vehicle total		E2 polysome *vs.* E2 monosome		Vehicle polysome *vs.* vehicle monosome	
Pathway	NES	*P* value	NES	*P* value	NES	*P* value	NES	*P* value
Genes within 10 kb ER binding site	1.03	0.421	1.00	0.503	1.33	0.003	1.39	0.001
Stossi E2 UP signature	1.05	0.375	1.32	0.068	1.06	0.359	1.02	0.441
Frasor E2 UP signature	**1.49**	**0.041**	**1.50**	**0.041**	**1.98**	**0.000**	**1.81**	**0.000**
GenMapp								
Ribosomal proteins	2.48	0.000	2.96	0.000	3.69	0.000	3.77	0.000
Proteasome	1.73	0.011	1.11	0.331	2.02	0.000	2.08	0.000
Oxidative phosphorylation	1.15	0.219	1.15	0.211	1.92	0.000	1.92	0.000
Glycolysis	0.99	0.441	0.96	0.525	1.16	0.239	1.17	0.228
DNA replication reactome	1.18	0.183	1.45	0.045	1.67	0.002	1.45	0.044
G1 to S cell cycle reactome	**1.29**	**0.080**	**1.27**	**0.130**	**1.56**	**0.002**	**1.33**	**0.068**
ATP synthesis	**0.80**	**0.715**	**0.95**	**0.514**	**2.04**	**0.004**	**1.83**	**0.002**
RNA transcription reactome	**1.95**	**0.000**	**1.43**	**0.043**	**1.93**	**0.000**	**1.66**	**0.008**
Apoptosis	*1.41*	*0.044*	*1.41*	*0.048*	*1.25*	*0.108*	*1.49*	*0.020*

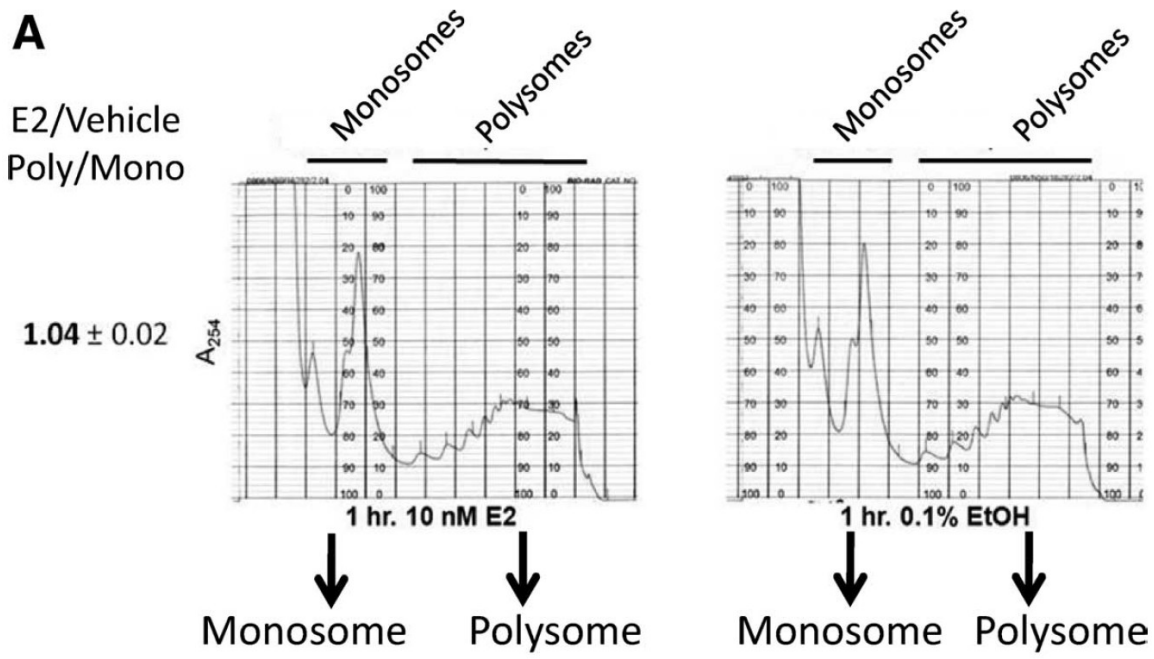
Biocarta								
ERK pathway	**0.57**	**0.971**	**0.77**	**0.8**	**1.47**	**0.046**	**1.28**	**0.143**
WNT pathway	0.87	0.644	1.10	0.346	1.29	0.146	1.24	0.175
MAPK pathway	**1.88**	**0.000**	**1.44**	**0.011**	**1.82**	**0.000**	**1.62**	**0.004**
P38MAPK pathway	1.64	0.005	1.56	0.091	1.79	0.002	1.69	0.004
CREB pathway	1.49	0.044	1.53	0.015	1.90	0.000	1.87	0.002

NES higher than 1.4 suggests significant bias toward E2. Positive NES indicates bias toward E2, whereas negative values indicate vehicle bias. *P* values <0.1 are significant. Changes in translation state in between conditions indicate possible translation regulation of the indicated pathway. *Bold* indicates translationally up-regulated by E2, and *italics* indicates translational down-regulation by E2.

Table 3. GSEA reveals enrichment of transcription factor programs

	E2 Polysome *vs.* vehicle polysome		E2 Total *vs.* vehicle total		E2 polysome *vs.* E2 total		Vehicle polysome *vs.* vehicle total		E2 polysome *vs.* E2 monosome		Vehicle polysome *vs.* vehicle monosome	
Transcription factor	NES	*P* value	NES	*P* value	NES	*P* value	NES	*P* value	NES	*P* value	NES	*P* value
NRSF	1.68	0.000	1.18	0.170	2.32	0.000	2.02	0.000	2.32	0.000	2.20	0.000
E2F1	1.53	0.000	1.10	0.211	1.65	0.000	1.64	0.001	1.76	0.000	1.86	0.000
AHR	1.69	0.000	0.89	0.699	1.36	0.023	1.48	0.008	1.31	0.031	1.28	0.051

Gene sets are defined by common sequence motifs in promoter regions. NES higher than 1.4 suggests significant bias toward E2. Positive NES indicates bias toward E2, whereas negative values indicate bias toward vehicle. *P* values <0.1 are significant.



B

RNA	Analysis
E2 Total vs. Veh Total	Transcriptome levels
Polysome vs. Total	Translation State/transcriptome level
Polysome vs. Monosome	Translation State
E2 Polysome vs. Veh Polysome	Translation State/Transcriptome levels

Figure 1. E2 effect on polysomes and microarray analysis.
A, Representative fractionation profile from 10–45% linear sucrose gradients from MCF-7 cells treated with 10 nM E2 or 0.1% ethanol (vehicle) for 1 h. The average ratio with the SD of triplicates of the P/M ratio in E2 *vs.* 0.1% ethanol (vehicle) is shown. Sucrose density gradients separated the mRNAs into the subpolysomal/monosome and polysome fractions. Sucrose fractionation was monitored at 254 nM. **B**, Scheme of the procedure of translational profiling. Unfractionated total RNA was isolated in each condition and hybridized on Affymetrix microarrays. Veh, Vehicle condition.

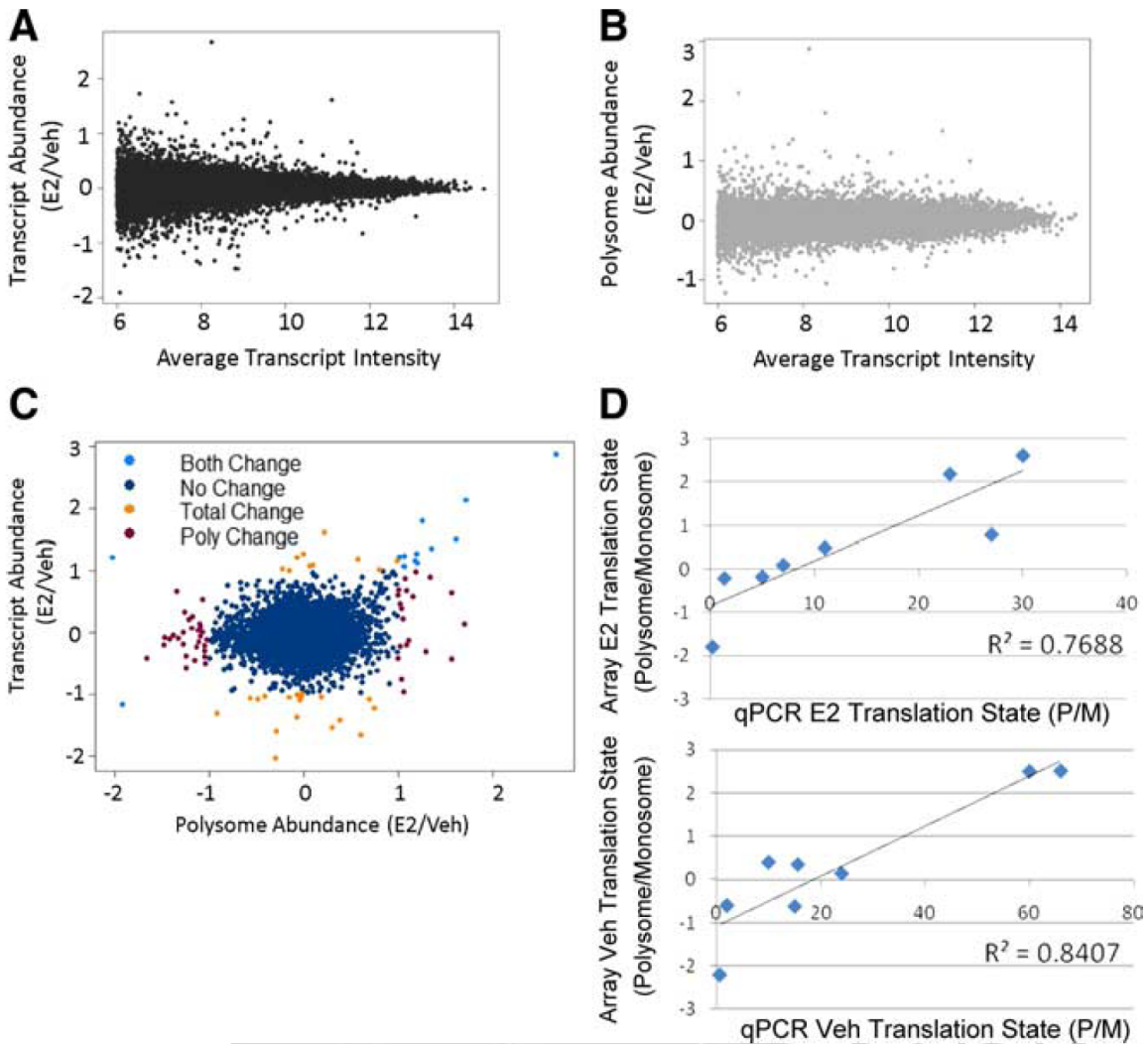


Figure 2. No significant global upregulation is observed suggesting specific changes. Graphical representations of the relationship between transcriptome and translational changes reveal no dramatic shift in the signal in polysome or total RNA after 1 h of 10 nM E2 treatment. **A**, Total RNA fold change compared with total RNA transcript signal shows no significant global shift in transcript expression levels. **B**, Polysome fold change compared with total RNA transcript signal indicates no significant global shift in polysome association. **C**, Color coding indicates whether the changes are in total RNA (orange), polysome RNA (red), both (light blue), or unchanged (dark blue) in E2 vs. vehicle. Only signals from RefSeq transcripts with average log intensity higher than 6 are included. Each point represents an individual transcript. D, qPCR analysis of the translation state correlates with microarray signals. Luciferase RNA was spiked in before purification for normalization. The y-axis indicates the log of the P/M ratios in the designated condition. The x-axis indicates the qPCR-determined P/M ratio. A linear relationship between the qPCR and array observations is indicated by least squares fitting of a line with high R2 to the data. Veh, Vehicle.

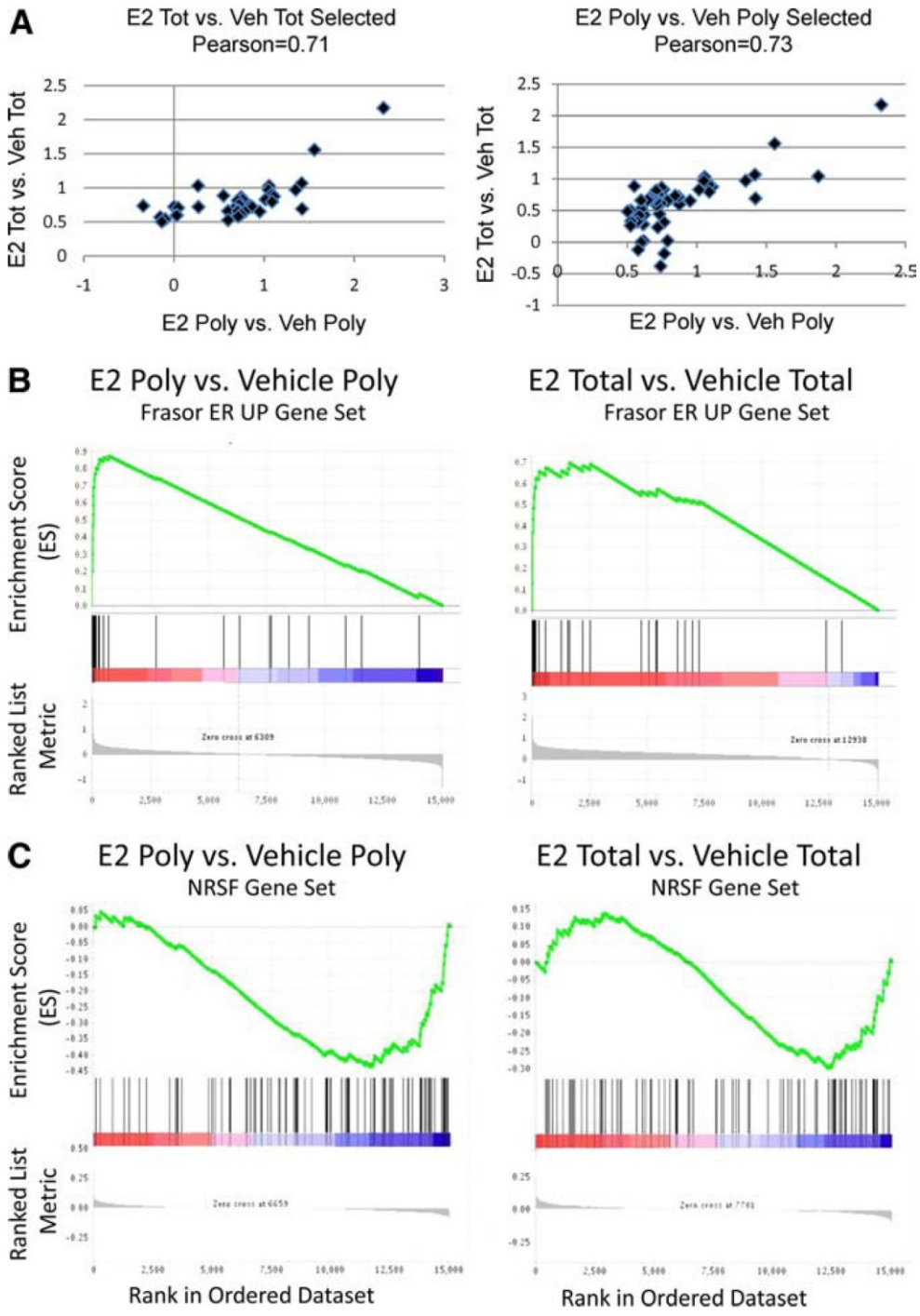


Figure 3. E2 induces specific translational responses and transcriptome level changes. A, Significantly changing genes in the transcriptome and translational responses are plotted against each other to show that they are correlated. The y-axis indicates the log fold change in total RNA, transcriptome, and the x-axis is the log fold change in polysomes in the E2 and vehicle (Veh) conditions. The left graph compares transcripts with significant (P value <0.05; fold change >0.5) changes in the transcriptome. The right

graph compares transcripts with significant changes (P value <0.05; fold change >0.5) in the polysomes. The Pearson correlation coefficient is indicated above each graph. **B**, GSEA expression analysis of a representative E2 expression signature [53] in the transcriptome and translational responses. The black lines demonstrate where each gene in the gene set falls within the 15,000 probe sets probing RefSeq transcripts ordered from left to right based on the E2 polysome/vehicle polysome ratio with gene 1 the most highly expressed in E2 polysomes. The green line represents the running enrichment score (ES) that becomes more negative as probe sets are identified toward the bottom of the list. Significant up-regulation is seen in both the transcriptome and polysomes. More significant bias of the Frasor ER expression signature is observed in the polysomes compared with the bias observed in the E2 Total vs. vehicle total, transcriptome comparison. More black lines are observed near the top of the rankings in E2 (left side) than in the vehicle (right side). **C**, Significant bias of the NRSF gene set is observed in the polysomes but not the total RNA because more black lines are near the bottom of the list in the E2 polysome vs. vehicle polysome comparison. The NRSF gene set is a group of genes with NRSF-binding motifs in their promoter regions. The NRSF gene set is fairly evenly distributed in the E2 total vs. vehicle total comparison.

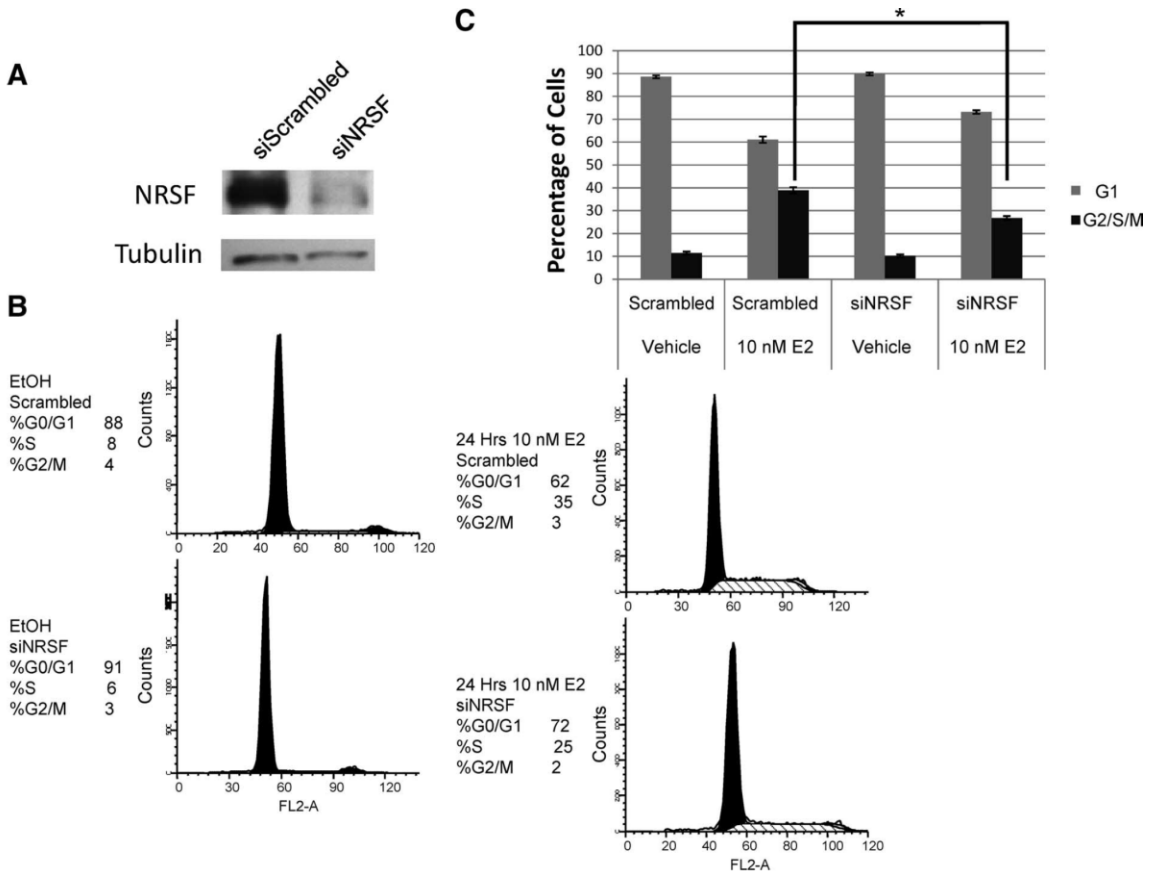


Figure 4. NRSF enhances E2 stimulation of the cell cycle over 24 hours. A, Representative Western blot with anti-NRSF antibody shows significant knockdown. NRSF runs at approximately 180 kDa. qPCR RNA level knockdown is typically about 8-fold. B, Representative propidium iodine staining DNA content FACS shows that NRSF knockdown inhibits E2 stimulation of the cell cycle. MCF-7 cells were hormone deprived in phenol red-free CDT serum for 3 d before 10 nM E2 treatment. Almost 90% of the cells became G1 growth arrested. The percentage of S-phase cells observed after E2 treatment is reduced when NRSF is knocked down before E2 stimulation. The acquired FACS data were modeled with ModFit LT software. EtOH, Ethanol. C, Significantly fewer S-phase cells are observed when NRSF is knocked down, as indicated by the black lines comparing the two conditions. *, $P < 0.05$. Error bars are SD from triplicate experiments indicating that the NRSF effect is reproducible.

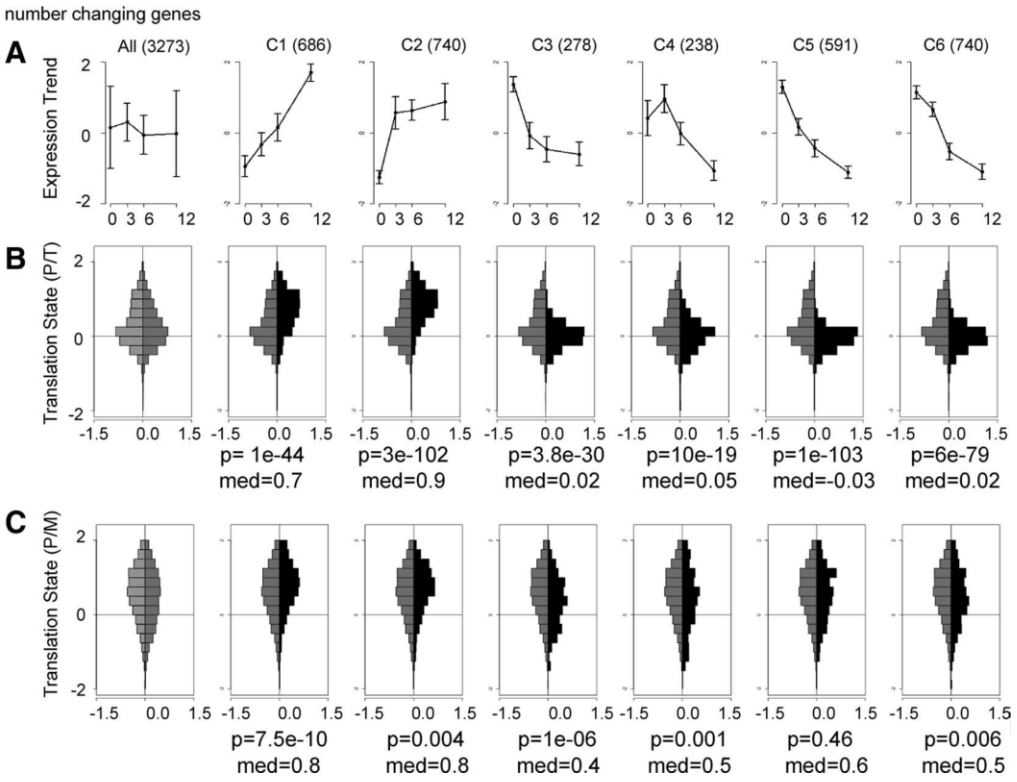


Figure 5. Translation state anticipates the direction of transcriptome changes upon E2 stimulation. A, Clustering of significantly changing genes from Affymetrix U133 microarray expression data [3] classifies genes into six major patterns of gene expression over a 12-h time course. The number of transcripts in each cluster is indicated in parentheses. Shown in each plot is the averaged expression behavior of all transcripts in the indicated cluster, with error bars indicating the deviation from the mean for the entire cluster. Expression of each transcript was obtained by subtracting the mean of all points at all times from each expression value in the original time series and dividing by the SD. **B**, The distribution of fraction of transcript associating with polysomes (P/T), translation state, for each cluster is shown as histograms. The y-axis indicates the translation state, whereas the x-axis is the probability density ranging from 0–1.5. The left side of each panel is the translation state distribution for all the genes in gray, whereas the right side of each panel, in black, is the translation state distribution for the genes in the cluster. The P value testing the difference between the distributions of translation states for the cluster compared with all E2-regulated transcripts is shown below each panel along with the median (med) translation state for the transcripts in each cluster. E2 up-regulated genes, clusters 1 and 2, are strongly biased toward higher translation states, whereas E2-down-regulated genes, clusters 5 and 6, are biased toward lower translation states. **C**, The distribution of the P/M translation state of the transcripts in each cluster is shown. The P value, testing the difference between the distributions of translation states for the cluster compared with all E2-regulated transcripts, is shown below the density distribution, along

with the median translation state for the transcripts in each cluster. Similar patterns are seen for both the P/M and P/T ratios in each cluster with stronger biases seen for the P/T distributions.

Supplemental Data

Supplemental Table 1. Log fold changes for selected transcripts by array and qPCR. The log fold change (Fc) on the array and by qPCR are indicated. Often the fold changes are larger by qPCR but the microarray data correctly indicate the direction of significant fold changes. N.S. indicates not significantly changing on the microarray.

Gene	Array Total Fc	qPCR Total Fc	Array Poly Fc	qPCR Poly Fc
Tff1	0.38	1.58	0.68	0.68
XBP1	0.85	1.68	1.14	1.93
TNFRSF11B	-0.74	-0.74	-0.74	-2.18
GADD45b	0.68	1.26	0.70	2.07
MYC	0.68	1.85	0.68	1.38
RBM24	0.69	2.68	1.42	2.10
TIPARP	1.00	1.54	1.07	1.07
STC2	0.97	1.14	1.05	0.77
GPR132	N.S.		0.77	0.77
SKIV2L	-1.87	-1.00	N.S.	
EGR3	0.70	0.81	0.70	2.20
SOCS3	0.80	0.77	1.10	1.20
SGK	1.00	2.81	1.40	2.58

Supplemental Table 2. Genes Significantly Changing in Total RNA

RefSeq ID	Gene Name	Log(E2 Total/Veh Total)	P-value E2 Total/Veh Total
Up			
NM_003740	KCNK5	2.17304	0.003586
NM_002467	MYC	1.558743	0.001266
NM_005252	FOS	1.068011	0.00019
AK090412	LOC375010	1.031428	0.04938
NM_015508	TIPARP	1.028771	0.000465
NM_005627	SGK	0.971892	0.001497
NM_003714	STC2	0.971064	0.013021
NM_005220	DLX3	0.906264	0.000967
NM_004694	SLC16A6	0.885244	0.019875
NM_001079539	XBP1	0.875844	0.000124
NM_005375	MYB	0.863766	0.023626
NM_001039	SCNN1G	0.832873	0.005916
AK096336	C8orf46	0.83009	0.009985
NM_005655	KLF10	0.815531	0.015935
NM_003955	SOCS3	0.797472	0.000357
NM_002163	IRF8	0.753726	0.005466
M16768	TRGV9	0.73655	0.049665
NM_001165	BIRC3	0.73419	0.025881
BC127725	hCG_2042718	0.720688	0.040014
NM_012259	HEY2	0.718133	0.030169
uc003wrd.1	LOC349196	0.708013	0.00247
NM_153020	RBM24	0.691651	0.007324
NM_001218	CA12	0.689948	0.0214
NM_005931	MICB	0.686362	0.006818
NM_001006641	SLC25A25	0.685065	0.024017
NM_004430	EGR3	0.675911	0.015785
NM_005749	TOB1	0.65997	0.003013
NM_015675	GADD45B	0.656421	0.00273
NM_138440	VASN	0.653516	0.016657
NM_018724	IL20	0.653338	0.047543
NM_004235	KLF4	0.614677	0.009494
NM_198486	RPL7L1	0.603802	0.049272
NM_177980	CDH26	0.595788	0.004641
NM_004566	PFKFB3	0.580922	0.002234
XM_942240	LOC650557	0.571107	0.049739
NM_014996	PLCH1	0.54067	0.010087

NM_000964	RARA	0.526897	0.00582
NM_002030	FPRL2	0.505292	0.007884
Down			
NM_006929	SKIV2L	-1.87442	0.033876
NM_001632	ALPP	-1.12746	0.020896
NM_001012423	GOLGA8E	-0.99388	0.039139
AK091124	EFCAB4B	-0.94462	0.021617
NM_002546	TNFRSF11B	-0.7535	0.03836
XR_019031	LOC649115	-0.74956	0.032784
NM_000039	APOA1	-0.72789	0.035946

Supplemental Table 3. Significantly Changing Genes in Polysomes

RefSeq ID	Gene Name	Log(E2 Poly/Veh Poly)	P-value E2 Poly/Veh Poly
Up			
NM_003740	KCNK5	2.325537	0.003241
NM_002467	MYC	1.559021	0.007755
NM_153020	RBM24	1.421299	0.003777
NM_005252	FOS	1.417247	0.00238
NM_005627	SGK	1.350881	0.009003
NM_001079539	XBP1	1.109847	0.005345
NM_005220	DLX3	1.088154	0.002813
NM_003955	SOCS3	1.088132	0.002374
NM_015508	TIPARP	1.055746	0.000433
NM_003714	STC2	1.050277	0.000568
AK096336	C8orf46	1.015917	0.024248
NM_138440	VASN	0.950863	0.013775
NM_015516	TSKU	0.873806	0.046159
NM_012259	HEY2	0.868557	0.018528
NM_001165	BIRC3	0.843752	0.029356
NM_173078	SLITRK4	0.789138	0.008164
NM_018724	IL20	0.788567	0.022757
NR_002755	RNU5D	0.766	0.035277
NM_013345	GPR132	0.762962	0.025004
NM_001039	SCNN1G	0.759688	0.021193
NM_005375	MYB	0.746762	0.046591
NM_004235	KLF4	0.74398	3.32E-05
XM_001129363	FAM90A17	0.738815	0.027924
NM_024762	ZNF552	0.730357	0.002082
NM_002163	IRF8	0.721375	0.026975
NM_052864	TIFA	0.718764	0.03352
NM_004566	PFKFB3	0.713085	0.012659
NM_004430	EGR3	0.711851	0.047303
NM_005655	KLF10	0.707475	0.020514
NM_015675	GADD45B	0.706783	0.006684
NM_001218	CA12	0.680689	0.000586
NM_001006641	SLC25A25	0.663159	0.000887
NM_005931	MICB	0.657838	0.001567
NM_198461	LONRF2	0.645497	0.024976
NM_014566	OR1D5	0.620933	0.033133
NM_001455	FOXO3	0.616927	0.008696

XM_001126897	LOC728220	0.615383	0.011515
NM_000964	RARA	0.600328	0.010648
NM_001085489	hCG_2028557	0.599176	0.033629
NM_005749	TOB1	0.599118	0.014534
NM_001453	FOXC1	0.58702	0.038202
NM_025069	ZNF703	0.586888	0.036466
NM_021220	OVOL2	0.578431	0.015042
NM_005904	SMAD7	0.571006	0.022821
NM_004694	SLC16A6	0.549974	0.004748
NM_005524	HES1	0.531998	0.009979
NM_005194	CEBPB	0.526918	0.028505
NM_021205	RHOU	0.525604	0.021262
XM_926027	LOC642538	0.523185	0.037592
NM_145283	NXNL2	0.502115	0.047718
Down			
NM_138960	TGIF2LX	-1.43147	0.048502
NM_033468	ZNF257	-1.34894	0.019714
NM_007028	TRIM31	-0.7635	0.012305
NM_003106	SOX2	-0.75124	0.021651
NM_030965	ST6GALNAC5	-0.75106	0.002319
NM_002546	TNFRSF11B	-0.73774	0.022147

Supplemental Table 4. Enrichment of Gene Ontologies in time course clusters. P-values are generated by GOStat and are FDR corrected. No GO categories are significantly enriched ($p<0.01$) in clusters 3, 4 and 6.

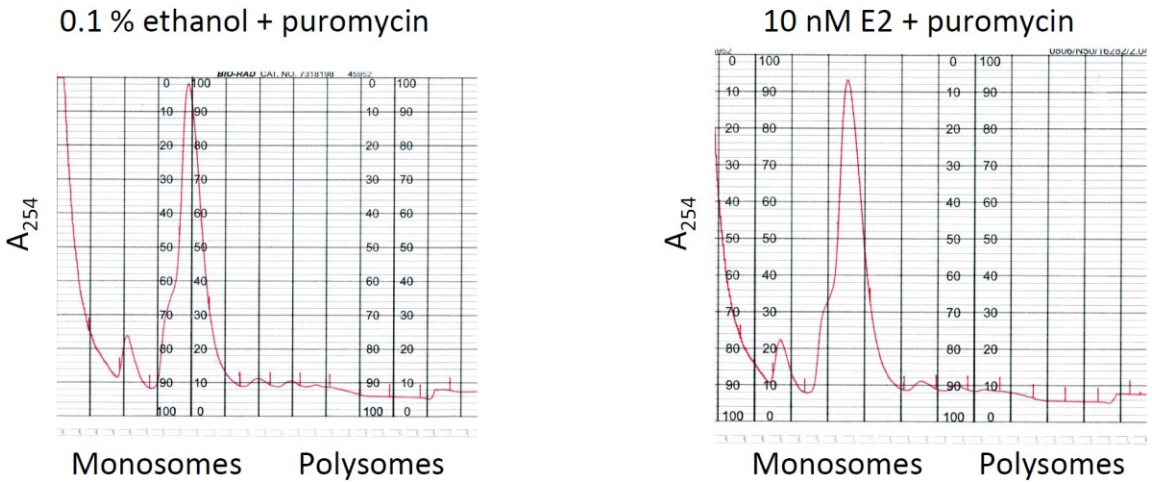
Cluster 1	**P-value**
Cell cycle	4.2e-24
M Phase	1.1e-22
Cell division	4.8e-20
DNA replication	4.2e-19
Cell cycle checkpoint	2e-8
Cluster 2	
Ribosome biogenesis and assembly	1.7e-11
Nucleolus	1.6e-11
RNA splicing	1.9e-9
Translation initiation factor activity	2.2e-4
Cluster 5	
Membrane	3e-7
Vacuole	0.008
Lysosome	0.01

Supplemental Table 5. List of all primer sequences used for qPCR in this study. F and R indicate forward and reverse primers. CANX is used a normalization control in total RNA.

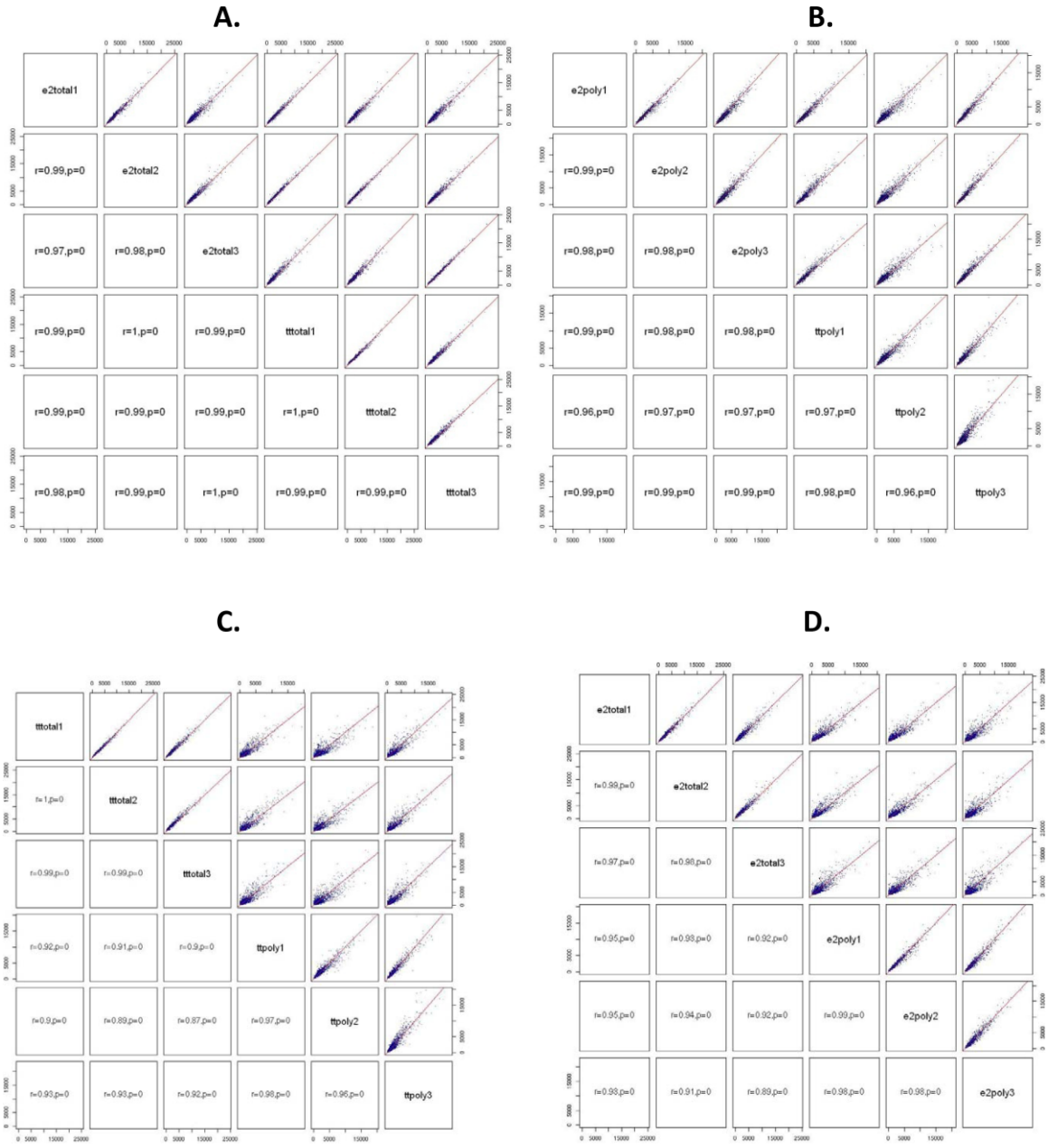
Refseq ID	Gene Name	Primer Name	Sequence
NM_002467	MYC	MB1F	TTCGGGTAGTGGAAAACCAG
		MB1R	CAGCAGCTCGAATTTCTTCC
NM_001079539	XBP1	MB2F	GCGCCTCACGCACCTG
		MB2R	GCTGCTACTCTGTTTTTCAGTTTCC
NM_002546	TNFRSF11B	MB4F	GGCAACACAGCTCACAAGAA
		MB4R	CGCTGTTTTCACAGAGGTCA
NM_003225	TFF1	MB5F	GTGTCACGCCCTCCCAGT
		MB5R	GGACCCCACGAACGGTG
NM_001453	FOXC1	MB6F	CGGGTTGGAAAGGGATATTT
		MB6R	CTCCTCTCGCCTTCCTTCTT
NM_001024649	CANX	MB7F	GCCTGAAGAAAGCTGCTGAT
		MB7R	GGAACACAGGAAGGGCTACA
NM_005612	NRSF	MB8F	CACCACTGCCAAAGGAAAAT
		MB8R	CCAGGTAGGCTCTCATCTGC
NM_015508	TIPARP	MB9F	CCACACCACCCTCTAGCAAT
		MB9R	CGGGATACTCTCTCCATCCA
NM_003714	STC2	MB10F	ATTCTCCTGCGTTGCTGAGT
		MB10R	TGGAAATGTGATGCCTTTCA
NM_015675	GADD45B	MB11F	GTGTACGAGTCGGCCAAGTT
		MB11R	TGTCACAGCAGAAGGACTGG
NM_013345	GPR132	MB12F	CGCACAGAGACAAAGTGGAA
		MB12R	TCATCTTCCCAAACGGAGAC
NM_004430	EGR3	MB13F	CTCTTCCCCATGATTCCTGA
		MB13R	AATGCCTTGATGGTCTCCAG
NM_003955	SOCS3	MB14F	ATGGTCACCCACAGCAAGTT
		MB14R	GGTACTCGCTCTTGGAGCTG
NM_153020	RBM24	MB15F	AGGGAATACAGAGCGAGCAA
		MB15R	GCTTTCGCCATTTTGTTTGT
NM_006929	SKIV2L	MB16F	CCAACCACAGGCCAGATACT
		MB16R	GCGAAGAGACAAGGAGGTTG
NM_005627	SGK	MB17F	ACCCTTCTCCTCCACCAAGT
		MB17R	CCCTTTCCGATCACTTTCAA
	luciferase	MB18F	CGCTTCCGGATTGTTTACAT
		MB18R	GCTGGGCGTTAATCAGAGAG
NM_001873	CPE	MB19F	CTCCTGAGACCAAGGCTGTC
		MB19R	TAATTGGCCACAAGGTCTCC
NM_003878	GGH	MB20F	CTGCAGGTGCGAGAGTTGTA
		MB20R	ACTTCCTCCAGGGAAAAGGA
NM_001006	RPS3A	MB21F	CGAGAGGTGCAGACAAATGA

		MB21R	CAAACTTGGGCTTCTTCAGC
NM_005257	GATA6	MB22F	GGGCTCTACATAGGCGTCAG
		MB22R	CAAAAGCAGACACGAGTGGA
NM_002872	RAC2	MB23F	AATGTGATGGTGGACAGCAA
		MB23R	GAAGACGTCCGTCTGTGGAT
NM_032118	WDR54	MB24F	AGGTCCACTGGTGTGTCCTC
		MB24R	GGTGTAGCCATTGGACTCGT
NM_025202	EFHD1	MB25F	TCAACCCCTACACGGAGTTC
		MB25R	CATCAGCTTCAGCTCCATCA
NM_175854	PAN3	MB26F	AGGGCTCAGCAAATGCTTTA
		MB26R	CAAAGTGCATGGTGTTGGTC

Supplemental Figures



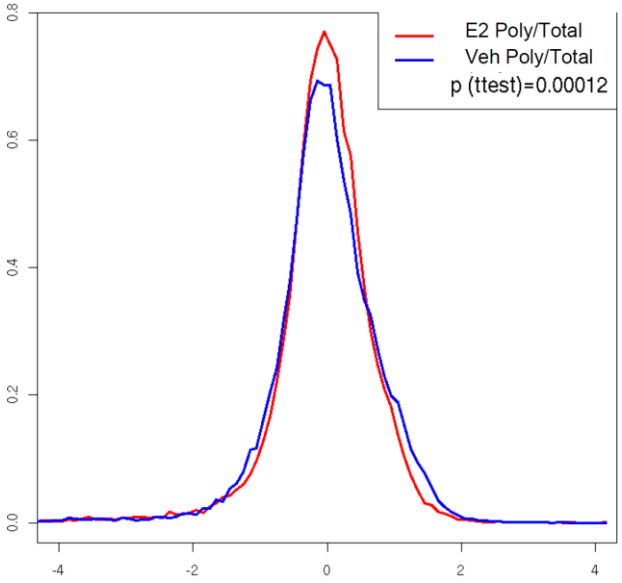
Supplemental Figure 1. Polysome traces are puromycin dependent indicating that they are active ribosomes translating mRNAs. 1 mM puromycin was added to MCF-7 cells for 5 minutes before lysis. Polysome signal is significantly reduced indicating that a large proportion of the observed A254 signal originates from actively translating ribosomes.



Supplemental Figure 2. Replication plots. Upper panels show pairwise scatter plots between the plier scores for each array in the set. The lower panels show the Pearson correlation coefficents and corresponding P-values. All of the arrays were processed together and are split here only for easy visualization. **A**. All of the arrays measuring total RNA. **B**. All of the arrays measuring polysome-associated RNA. **C**. All of the ethanol-treated RNA samples. **D**. All of the estrogen treated samples.

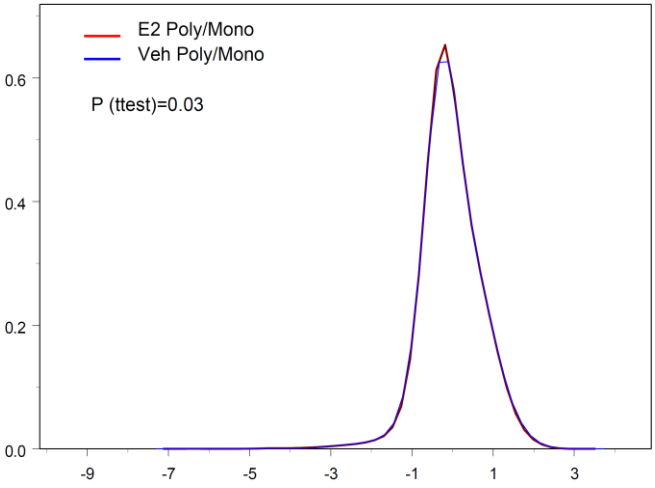
A.

	E2	Vehicle
Median	0.23	0.20
Top Quartile	2.57	2.41
Bottom Quartile	-0.05	-0.10

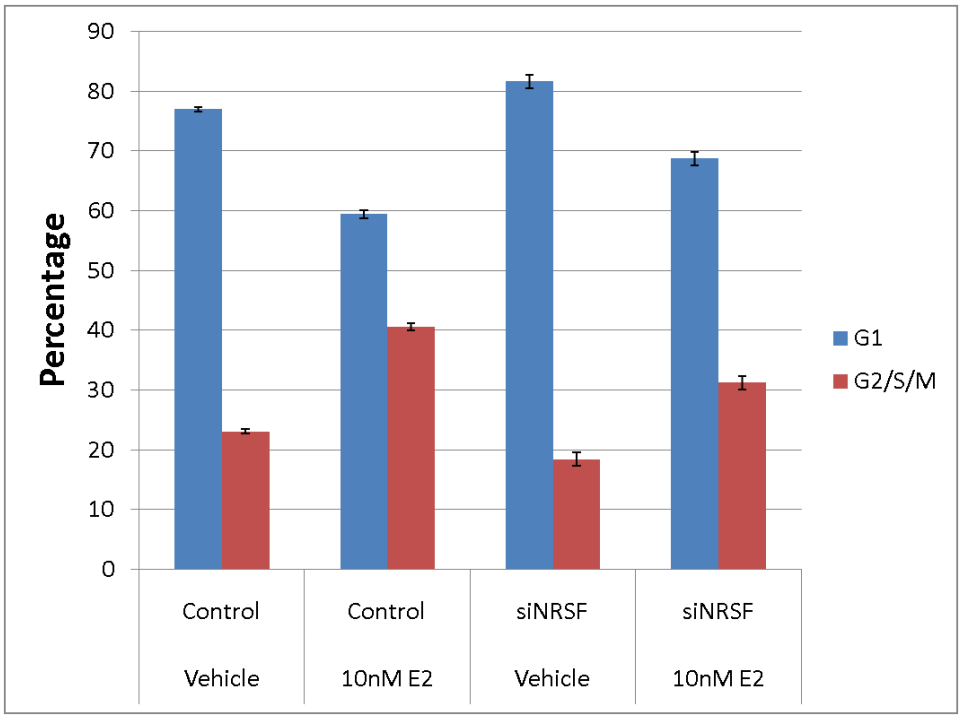


B.

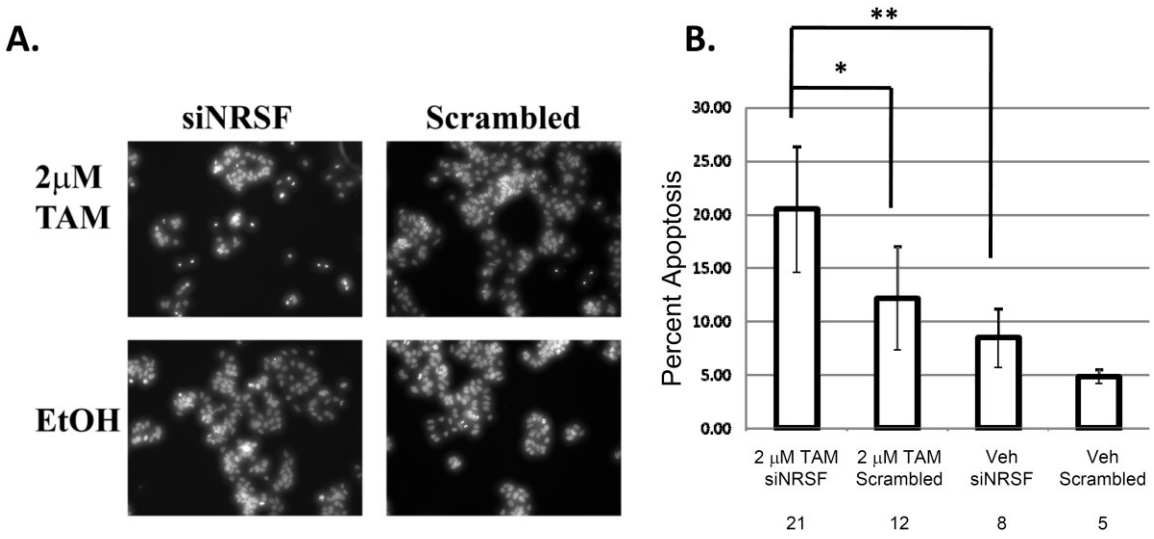
	E2	Vehicle
Median	-0.12	-0.12
Top Quartile	2.63	2.82
Bottom Quartile	-0.42	-0.42



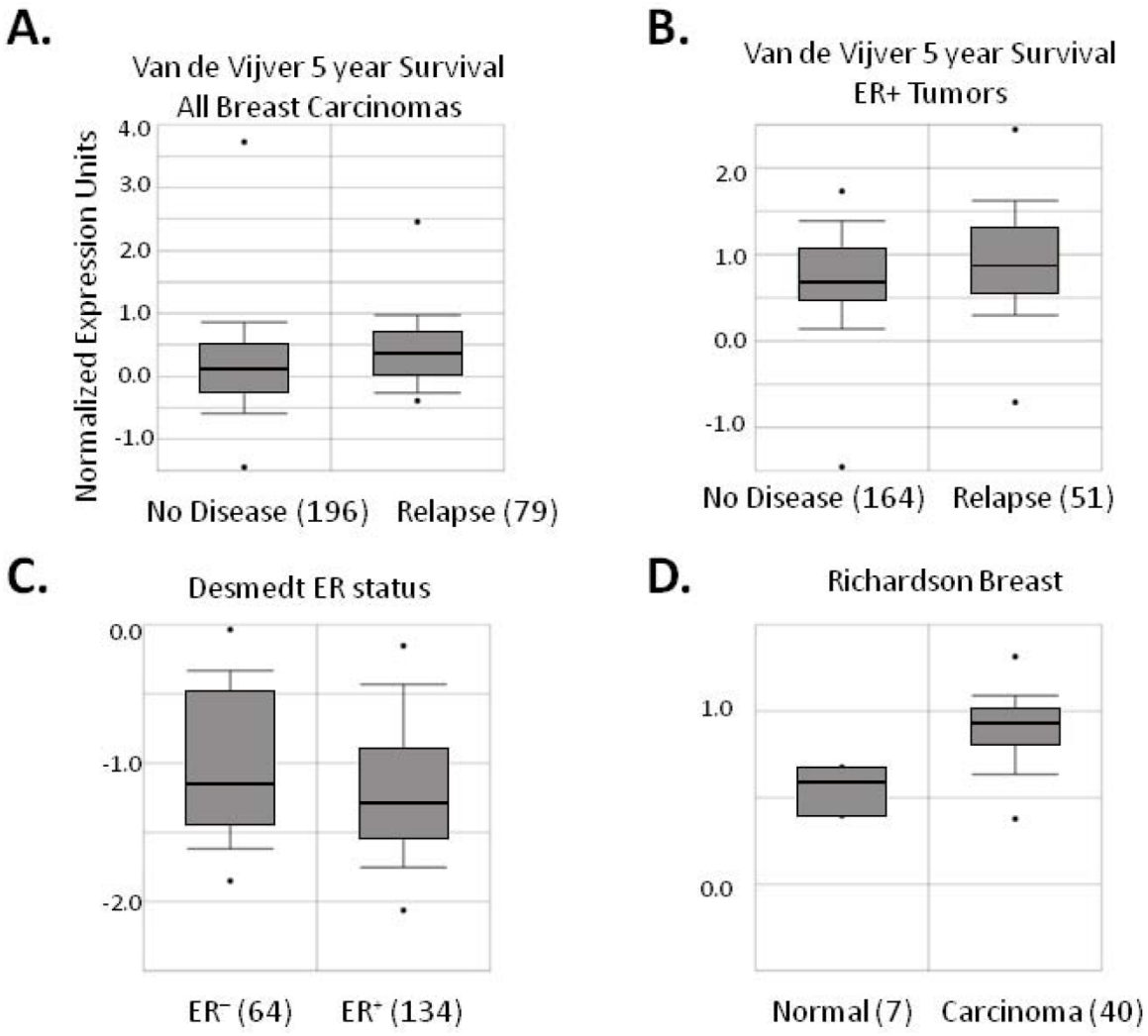
Supplemental Figure 3. E2 modestly affects global translation states. A. The distribution of the translation state (Polysome/total) for the E2 (red) and vehicle (blue) conditions reveals a modest, but significant, overall increase in polysome association relative to total cellular mRNA levels. This graph includes all measured RefSeq transcripts. **B**. The distribution of the Polysome/Monosome translation state for the E2 (red) and vehicle (blue) conditions indicate no significant change in translation state for all measured RefSeq transcripts.



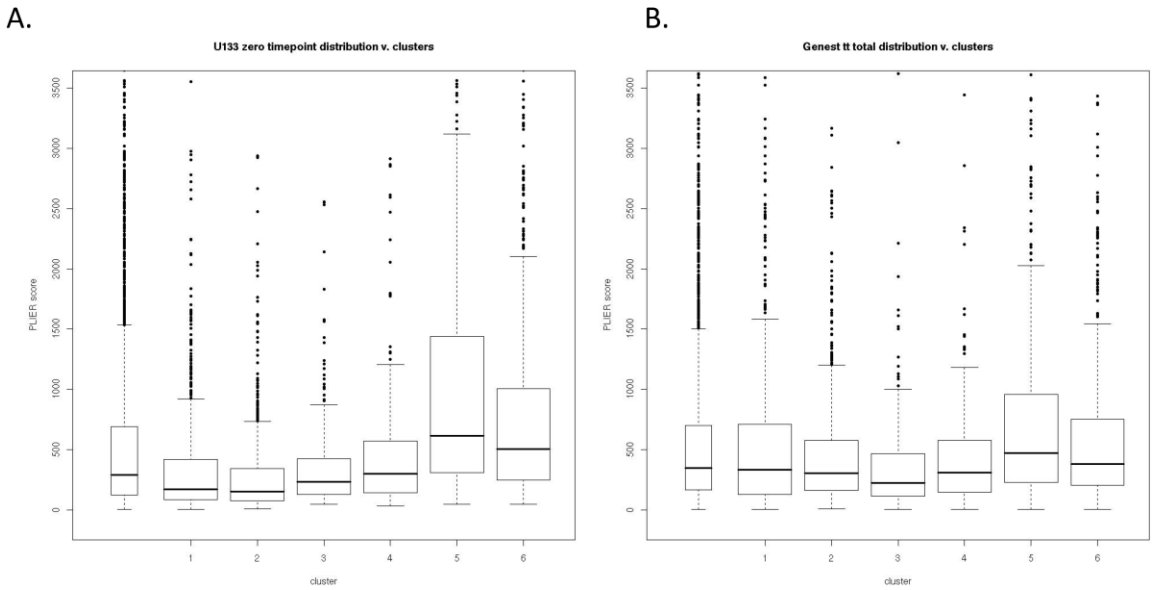
Supplemental Figure 4. Knockdown of NRSF in T47-D cells inhibits E2 stimulation of the cell cycle. T47D cells are not as E2 responsive as MCF-7. Control indicates transfection with scrambled non-targeting siRNA. As T47-D cells do not growth arrest as significantly as MCF-7 in hormone deprived conditions, a small decrease in S phase cells may be induced by NRSF knockdown in the absence of E2. Duplicate experiments are averaged with error bars indicating standard error.



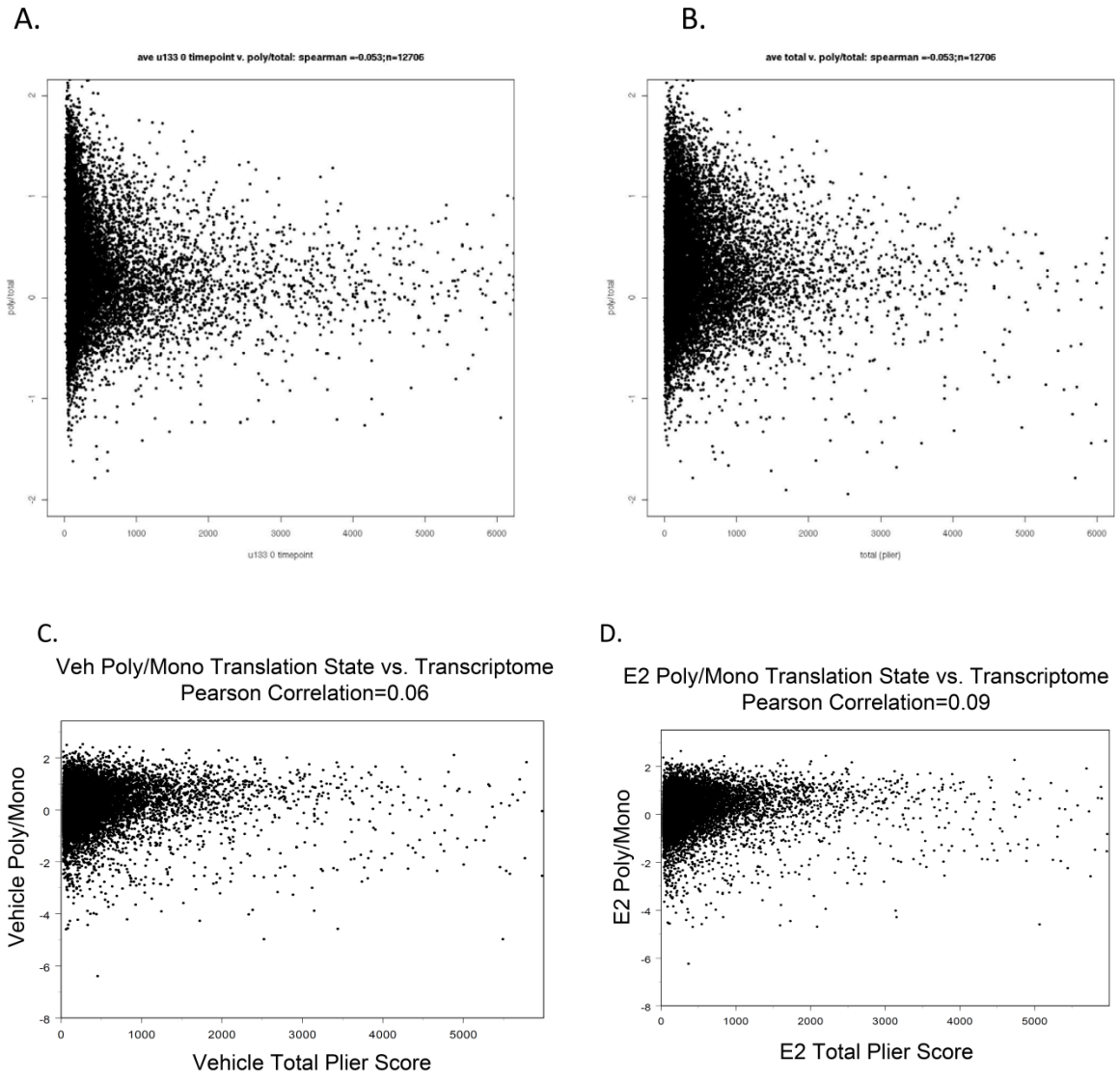
Supplemental Figure 5. NRSF and TAM induce cell death. Knockdown of NRSF increases MCF-7 cell death in the presence of 4-hydroxytamoxifen (Sigma-Aldrich) as determined by DAPI assay. We also notice less cells remaining on the plate when NRSF is knocked down compared to a scrambled, non-targeting siRNA sequence. **A**. Representative views of DAPI stained nuclei. **B**. Percent of cells showing clear apoptotic nuclei. At least 100 cells were counted in each condition from three randomly selected fields. * indicates p-value<0.05. ** indicates p-value ≤ 0.01.



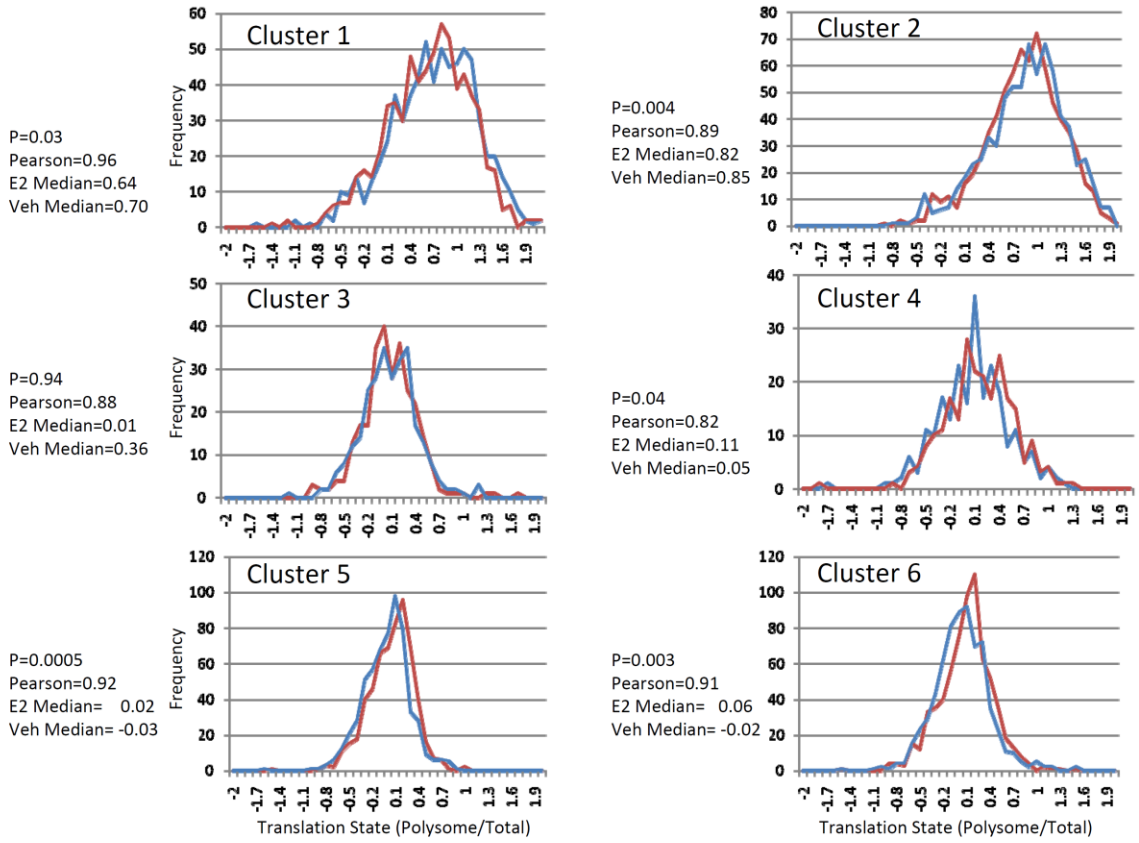
Supplemental Figure 6. Higher NRSF expression in breast tumors correlates with poorer outcomes. Data and statistics were obtained from www.oncomine.org. **A**. Box plot of NRSF mRNA levels show higher expression in relapsed patients, compared to patients in remission for all human breast carcinomas [1] P=0.005. **B**. NRSF expression is higher in poor surviving ER+ breast tumors [1] P=0.05. **C**. NRSF is higher in typically more aggressive ER- tumors compared to ER+ tumors [2]. P=0.007 **D**. NRSF is expressed higher in carcinomas compared to normal breast tissue [3] P<1e-6.



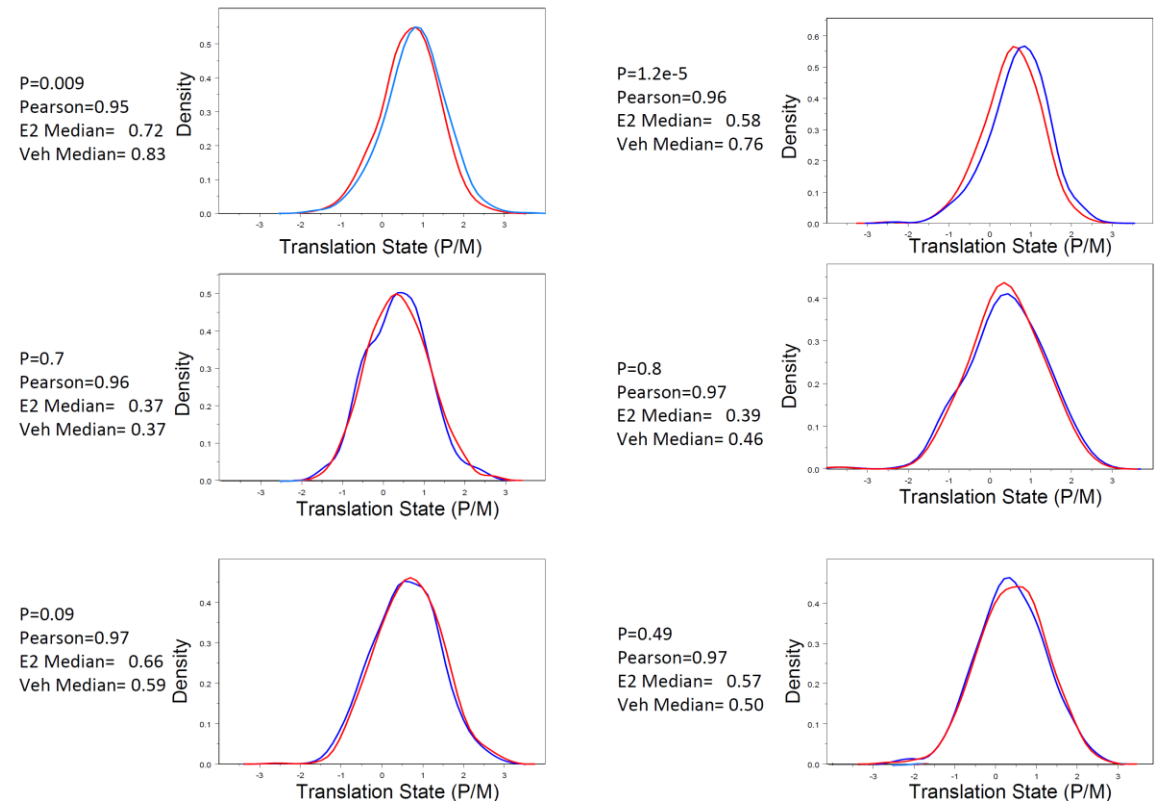
Supplemental Figure 7. Boxplot of average expression in the absence of E2 suggest that stimulated transcripts with higher translation states are not biased towards higher expression before E2 treatment. A. All 3273 E2 regulated transcripts that were clustered from the U133 time course microarray data as shown in Figure 6 along and the total RNA expression score (PLIER) from the U133 microarray data for each individual cluster is plotted as a boxplot. The upregulated C1 and C2 clusters are biased towards lower expression at the zero time point while the downregulated C5 and C6 clusters are biased towards higher initial expression. This starting values may be expected to be able to detect the subsequent up or down trend. **B**. Boxplot comparing the average vehicle control total RNA PLIER scores from the Affymetrix Human Gene 1.0 ST arrays. The upregulated clusters are not biased lower very significantly but the downregulated clusters (5 and 6) are biased towards higher hybridization scores. Similar trends are seen in both array types suggesting that the cross-platform analysis for these genes is accurate.



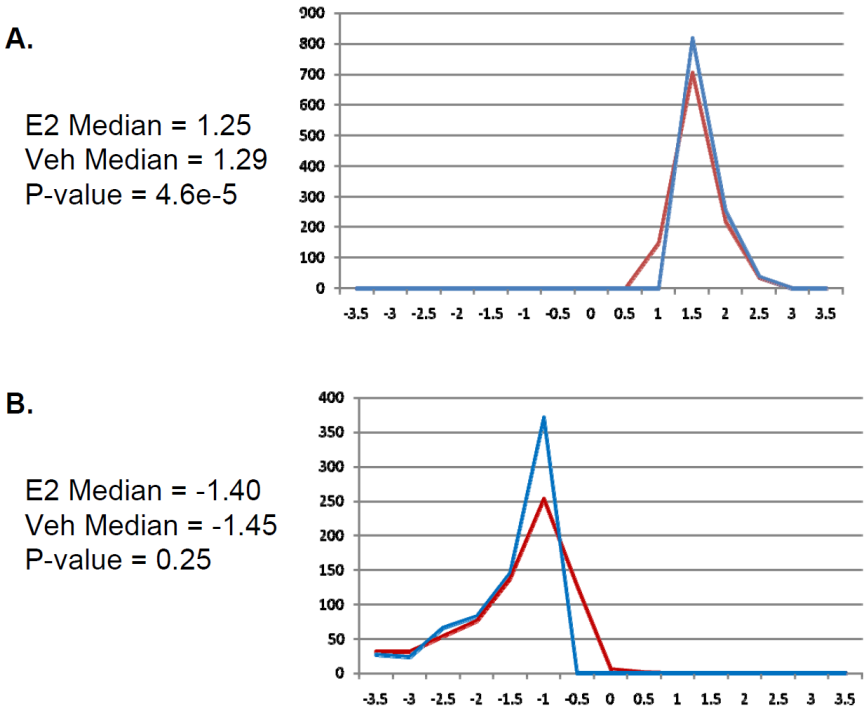
Supplemental Figure 8. Translation state vs. hybridization scores shows no significant correlation. A. Translation state compared to the zero time point U133 microarray data. **B**. Translation state compared to the vehicle control Gene 1.0 ST microarray data. **C**. The Vehicle Polysome/Monosome translation state is compared to the total RNA, transcriptome levels. The Pearson Correlation indicates that the translation state and transcriptome expression are independent. **D**. The E2 Polysome/Monosome translation state is compared to the total RNA, transcriptome levels. No significant correlation is induced by E2 as similar overall trends are observed as in the vehicle condition.



Supplemental Figure 9. The translation state (Polysome/Total) distribution for each E2 expression cluster, as defined in Figure 3, reveals that the degree of E2 stimulated changes in translation state vary for each cluster. E2 (red) and vehicle (blue) histograms show increased polysome association for transcripts in the downregulated clusters 5 and 6 while E2 stimulated transcripts in clusters 1 and 2 show a modest shift towards less association with polysomes relative to the amount of transcript. The transcripts in clusters 1 and 2 have significantly higher translation states and are thus likely being translated more than transcripts in the other clusters. These data indicate that increased association with polysomes relative to the total RNA occurs for E2 downregulated genes but not upregulated genes.



Supplemental Figure 10. The translation state (Polysome/Monosome) distribution for each E2 expression cluster. Similar, though more modest, trends as seen in the Polysome/Total translation state are observed for the Polysome/Monosome translation state compared to the Polysome/Total translation state. E2 (red) and vehicle (blue) density plots show decreased translation state for E2 upregulated transcripts in clusters 1 and 2. E2 downregulated transcripts, clusters 5 and 6, however, show no significant change in Polysome/Monosome translation state, unlike the Polysome/Total translation state. The y-axis indicates the probability density. The x-axis is the Polysome/Monosome (P/M) translation state. The median for each distribution is indicated along with a P-value from a t-test comparing the E2 and Vehicle distribution of translation states.



Supplemental Figure 11. The most significant high and low Polysome/Monosome translation states are compared and show small changes in opposite directions. A. Transcripts with high translation states are selected (Log fold change > 1, P-value<0.001) indicate that transcripts already in a high translation state actually shift lower upon E2 treatment. E2 is in red and vehicle in blue. **B**. Transcripts with low translation states are selected (Log fold change < -1, P-value < 0.001) and are shifted slightly higher, but statistically insignificant, by E2 indicating a translational response. E2 is in red and vehicle in blue.

Supplemental References

1. van de Vijver, M.J., et al., *A gene-expression signature as a predictor of survival in breast cancer.* N Engl J Med, 2002. **347**(25): p. 1999-2009.
2. Desmedt, C., et al., *Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series.* Clin Cancer Res, 2007. **13**(11): p. 3207-14.
3. Richardson, A.L., et al., *X chromosomal abnormalities in basal-like human breast cancer.* Cancer Cell, 2006. **9**(2): p. 121-32.

Chapter 3

The Stem Cell Transcription Factor SOX2 Mediates Estrogen Function

Abstract

The SOX2 transcription factor plays a key role in maintaining pluripotency in human embryonic stem cells. We observe that SOX2 is translationally repressed after E2 stimulation. We demonstrate that SOX2 inbibits E2 stimulation of the cell cycle. This finding is consistent with ER+ Breast cancers not expressing SOX2. Finally, we observe that E2 may differentially regulate alternatively processed 3'-UTR variants of SOX2.

Introduction

The sex-determining region Y-box 2 (SOX2) gene plays a key role in maintaining pluripotency in human embryonic stem cells (hESCs) [1]. SOX2 is a transcription factor that can directly interact and collaborate with other transcription factors, including Oct4, Nanog, Klf4, and others, to regulate stem cell pluripotency and differentiation [2]. Additionally, downstream targets of SOX2 include many regulators of the cell cycle [3]. Downregulation of SOX2 correlates with the loss of pluripotency and self-renewal, inducing subsequent differentiation [4]. SOX2 contains a high mobility group (HMG) box motif that allows for sequence specific DNA binding; however, its transcriptional activity requires the recruitment of cell-specific transcription factors. These combinatorial interactions facilitate the modulation of distinct tissue specific gene expression profiles [5].

Recent evidence suggests that SOX2 expression may also be important in cancer. For example, SOX2 has been shown to be overexpressed in many different types of solid tumors and its overexpression can be oncogenic in the lung [6]. In contrast, SOX2 has also been shown to be down regulated in other cancers [4]. It is frequently downregulated in gastric cancers, and its' overexpression induces cell-cycle arrest and apoptosis [7]. Additionally, tumors that develop from the same organ tissue can exhibit large variations in SOX2 expression [4, 8]. Clearly, SOX2 can act as either an oncogene or a tumor suppressor: a difference that is likely driven by the co-expression of cell specific transcription factors.

Breast cancer is a heterogeneous disease, and can be divided into at least five subgroups based on gene expression patterns associated with different clinical outcomes. The groups are luminal A, luminal B, triple negative/basal-like, HER2 overexpressing, and unclassified. Many basal tumors (ER-, progesterone receptor-negative (PR-) and HER2/neu-negative (HER2-)) exhibit HESCS–like gene expression signatures [3]. These tumors are poorly differentiated, tend to have a higher incidence of visceral metastasis, are highly proliferative, and often present as late stage, with high-grade tumors [9, 10]. In contrast, luminal A tumors (ER+ and/or PR+, HER2-) have gene expression patterns that are anti-correlated with HESCS-like gene expression signatures [3]. These tumors tend to be fairly well differentiated, less aggressive, and have the best prognosis [11].

The human MCF-7 breast carcinoma cell line (ER+, PR+, HER2-) is among the most frequently used model systems studied in breast cancer. This cell line has a luminal-A like phenotype as it is a fairly well differentiated, epithelial-like, non-invasive, and has a high expression of the ERα and related genes [12]. In MCF-7, E2 treatment has been shown to have no effect on SOX2 transcription levels, while in the luminal-A like T47D (ER+, PR+, HER2-nonoverexpssing [13]) breast cancer cell line, SOX2 is somewhat down regulated [14]. Downstream targets of SOX2 are lower in ER+ and luminal-A breast cancers versus ER- and basal types, respectively [3]. In agreement with these observations, SOX2 protein is preferentially expressed in tumors with basal-like phenotype [4]. The increased expression of SOX2 and its downstream targets likely plays

a role in defining the less differentiated/stem cell-like phenotype that is typical in basal breast cancers [3, 4].

In a recent publication, we [15] performed parallel analysis of transcription and translation profiles. Among our findings, we observed that E2 regulates several transcripts and pathways at the translation level. Polysome analysis also had higher sensitivity than total RNA in detecting E2-regulated transcripts. The increased sensitivity allowed us to identify the repression of NRSF targets in polysome-associated RNA, but not in total RNA. We then were able to show that NRSF activity was required for E2 stimulation of the cell cycle. In this paper, we used the transcription and translation profile data sets we collected previously to identity and characterize E2 mediated translational repression of SOX2.

Results

Briefly, in Bronson et al. (2010), we probed rapid E2 mediated translational regulation by comparing transcript levels in polysomes and monosomes with total RNA between one hour E2-treated and vehicle control MCF-7 cells (Fig. 1A). MCF-7 cells were hormone deprived in 5% CDT serum for three days before addition of 10 nM E2. Any changes after one hour of E2 stimulation were likely specific, as we did not observe significant upregulation of global protein synthesis. Sucrose density gradients separated the mRNAs into subpolysomal/monosomal and polysomal fractions as indicated by horizontal bars

above the profiles. We characterized how E2 may coordinate the transcriptome and translational responses by comparing changes in the polysome (P) fractions (P_{E2}/P_{Veh}), which are sensitive to both translation and overall mRNA level changes.

We then compared this ratio to changes in total (T) RNA (T_{E2}/T_{Veh}) in order to identify candidate translationally regulated genes. Comparing this ratio to changes in total RNA (T_{E2}/T_{Veh}) can identify candidate translationally regulated genes. The translation state of each transcript defined as P/M indicates the fraction of the transcript cosedimenting with polysomes. Changes in these ratios would indicate translation regulation. Each analysis is sensitive to different dynamic range and noise, and thus, each analysis complements the other in understanding translation regulation and identifying gene expression regulators important for E2 action. We focused our analysis on genes that were translationally, but not transcriptionally, regulated as genes that likely represent hitherto unidentified E2 responsive genes. We further focused on transcription factors, as they are often key regulators controlling cell fate, and play important roles in mediating E2 driven proliferation [15, 16].

SOX2 is Translationally Repressed upon Stimulation with E2

Our microarray analysis revealed that the SOX2 gene was highly repressed during translation, while being relatively unchanged in overall mRNA level, after E2 stimulation (Fig. 1B). The array data was consistent with measurements using pooled polysome

fractions compared to unfractionated total RNA (Fig. 1B). Next, we analyzed the translation state ratios, in higher-resolution, by measuring the distribution of SOX2 within the profiles using individual sucrose gradient fractions (Fig. 1C). The redistribution of SOX2 from the heavy polysomal region to the lighter subpolysomal region after E2 stimulation suggests reduced translational efficiency of SOX2 mRNA after one hour of E2 stimulation.

Next, we sought to quantify the degree of SOX2 translational repression by performing anti-SOX2 Western blots after time course treatment with 10 nM E2 (Fig. 2). SOX2 protein levels are highly variable in different biological preparations of the MCF-7 cell line. The exact reason why different preparations of the same cell line have various levels of SOX2 protein is unclear. This could be caused by inherent differences in the rate and degree to which MCF-7 preparations undergo G0/G1 growth arrest in response to hormone deprivation. Preparations that are weakly E2 responsive undergo growth arrest that is slower and less complete (data not shown). These preparations might represent a subpopulation of MCF-7 that have lost some of their ER expression, and represent more basal-like forms of breast cancer, which have been shown to have higher SOX2 protein levels [3]. Independent of the basal level of SOX2 in these preparations, its protein level is at least slightly reduced at 12 hours after treatment. It might be surprising that such strong translation repression does not cause a greater degree of protein level reduction, however, the steady state level of a protein is also dependent upon the its rate of decay. Even if decay rates are not affected by E2, subtle changes in transcription factor mRNA

concentration [17] and protein levels [18] are known to have large effects on the cellular phenotype. This may be particularly relevant in the case of SOX2, as it interacts with a large number of other transcription factors through a hierarchy of transcription regulatory networks [5, 19]. Differences in translation efficiencies are more readily detected as they result from a zero sum change in the polysome/monosome ratio. Despite a large change in efficiency of translation, there is still considerable SOX2 signal in the polysomal region allowing for active translation.

Since *cis*-acting elements, which can regulate the translational efficiency of an mRNA, often reside within the 3'-UTR of target genes, we tested whether the 3'-UTR after SOX2 was sufficient to convey translational repression to a luciferase reporter vector (Fig. 3.) *A* plasmid containing either luciferase fused SOX2 3'-UTR or β-Actin control 3'-UTR was transfected into MCF-7 cells 72 hours before treatment and subsequent collection of translation profiles. Luciferase signal was used to normalize for global transfection efficacy between the one hour 10 nM E2 and vehicle treatment conditions. There was no translational repression observed in the SOX2 3'-UTR of E2 stimulation. Several possibilities could account for this. The control element regulating translational repression is either not in the 3'-UTR or it requires additional SOX2 sequences to regulate translational repression. Alternatively, it is possible that our transfection saturated the SOX2 translational repressive capacity of the cell. SOX2 is in low abundance and therefore we chose to transfect 50 ng of reporter plasmid per 150,000 cells, which has been shown to be the upper limit before saturation of AU-rich element

(ARE) mediated decay machinery [20], however, the relevance of this is unclear, as SOX2 does not have any AREs in its 3'-UTR. Since we are transfecting arresting cells, we have only ~35-50% transfection efficiency (data not shown). Unfortunately, it is difficult to use less material and still obtain reliable detection by quantitative real-time PCR.

SOX2 Inhibits E2 Stimulation of ER+ Breast Cancer Cells

To determine whether SOX2 expression modulates E2 stimulation of the cell cycle, we knocked down SOX2 with siRNAs or overexpressed it with a CMV-promoter plasmid in hormone-deprived MCF-7 cells, and tested for effects on E2 stimulation of the cell cycle by DNA content FACS. Figure 4A shows modest knockdown and overexpression of SOX2 at the protein level. The DNA content cell cycle data suggests that the cells were synchronized upon hormone removal, with approximately 90% of the cells in G1. Overexpression of SOX2 significantly reduces the number of S phase cells (Fig. 4D). SOX2 knock-down shows a small, but significant and reproducible, increase in S phase cells. SOX2 also affects E2 action in a second ER+ cell line, T47D, suggesting that the importance of SOX2 in mediating the cell cycle is not MCF-7 specific (data not shown). Because SOX2 has been shown to regulate NRSF levels [3] and because we have shown NRSF to be important for regulating E2 stimulation of the cell cycle, we measured NRSF levels after both SOX2 knockdown and overexpression; NRSF levels were relatively unchanged (data not shown).

SOX2 May Have Alternative 3' Ends That Are Differently Regulated By E2

One of the ultimate goals of translational control research is to determine which fraction of the genome is actually made into proteins. A first step in this process is active gene transcription. The addition of the polyadenine tract [poly(A)] to the 3'end of a newly transcribed gene is tightly associated with the termination of transcription [21]. In addition to having a canonical polyadenylation site, nearly all human genes also have several alternative polyadenylation sites in their 3′UTRs [22]. The use of shortened alternative 3'end proximal poly (A) sites has been shown to be a mechanism of oncogene activation, through escape from regulatory potential in the longer 3'-UTR [22]. The lost sequences can influence microRNA interactions, mRNA nuclear export and cytoplasmic localization, and alter mRNA stability and translational efficiency [22, 23].

SOX2 has three validated alternative poly(A) sites [24] (Fig. 5). To gain additional insight as to how E2 might coordinate transcription and post-transcriptional processing, we sought to determine if these alternative SOX2 products were similarly translationally repressed after E2 stimulation. Our microarray signal showed that SOX2 was strongly repressed, but microarrays have limited capacity to reveal alternative processing. The array measurement represents a weighted sum of multiple probes for a given gene, and thus the contribution of a particular sequence variant can be overlooked. The primer set, MB5F and MB6R, was designed to amplify a region 3' to the product of primers MB3F and MB4R, and the site of an alternative poly(A) site (Fig. 5). This primer set reports similar distribution of SOX2 between treatment conditions (Fig. 6). This suggests

alterative SOX2 3'UTRs have different translational efficiencies. This could explain the lack of translational repression observed in the full length *SOX2* 3'-UTR reporter (Fig. 3). While not yet performed, the exact sequences of all SOX2 3'-UTRs should be determined. The use of 3'-rapid amplification of cDNA ends (RACE) is a useful tool that can provide information as to the 3' sequences of SOX2. The significance of this shorter 3'-UTR is not yet clear, nor is the mechanism by which it displays a greater degree of translational repression.

Discussion

Translational profiling has proven to be a powerful tool in uncovering hitherto unidentified E2 responsive genes. In these studies, we have used translational profiling to identify the stem cell transcription factor, SOX2, as being an important mediator of E2 function. Transcription factors are often key regulators controlling cell fate and mediators of proliferation. The deregulation of transcription factors in human cancers is, therefore, neither surprising, nor uncommon.

Translational regulation is emerging as an important contributor to E2 driven proliferation in breast cancer [15]. Translational profiling is a highly sensitive method of detecting changes in the translational efficiencies of an mRNA. After the addition of 10 nM E2 for one hour, in hormone deprived G0/G1 growth arrested MCF-7 cells, a population of SOX2 mRNA is translationally repressed (Fig. 1B and C). This repression

leads to a reduction in SOX2 protein levels at 12 hour (Fig 2). Although the absolute level of SOX2 varies between biological replicates of MCF-7 cells, the ability of E2 to reduce the translational efficiency of SOX2 is consistently observed. Unfortunately, we have not yet identified the mechanism by which E2 reduces the translational efficiency of SOX2.

The SOX2 transcription factor plays a key role in maintaining pluripotency in human embryonic stem cells [1]. In breast cancer, higher SOX2 levels [4], or increased levels of its downstream targets [3], have been associated with a more basal-like phenotype. The higher level of SOX2 in basal-like tumors likely contributes to the HESCS-like gene expression patterns [3]. The absence of E2 driven proliferation in basal-like breast cancer can, at least partially, be conveyed to the luminal A breast cancer cell lines MCF7 and T47D through the manipulation of SOX2 levels (Fig. 4). We show that SOX2 inhibits E2 mediated stimulation of the cell cycle. This finding is consistent with the reduced ER signaling found in SOX2 overexpressing basal-like breast cancers. This suggests that SOX2 contributes to defining the ER non-responsive state found in these less differentiated/stem cell-like basal breast tumors [3, 4].

In the hormone-deprived condition in which our cell cycle experiments were conducted, E2 stimulation induces ERα mediated transcriptional activation of cyclin D1, which is a critical promoter of G0/G1-to-S transition of the cell cycle [25]. Cyclin D1 is amplified in 15–20% of breast cancers [26]. In hESCs, SOX2 was shown to be required for, and enhance, the transcriptional expression of the miR-302 cluster of microRNAs. This

cluster functioned to post-transcriptionally repress cyclin D1, abrogating its ability to promote G0/G1-to-S transition [27]. In our system, SOX2 overexpression could act by enhancing the expression of miR-302 cluster and thus interfering with the ability of E2 to increase cyclin D1, levels resulting in the reduction of S-phase that we observe. Although this cluster of microRNAs is not normally expressed in MCF-7 cells [28], endogenous SOX2 levels are low (Fig. 2). The overexpression of SOX2 in other breast tumors is associated with a hESCs-like expression profile [3, 4], and therefore SOX2 overexpression in MCF7 might facilitate the transcription of this cluster of microRNAs.

Materials and Methods

Antibodies, Western blots, and cell culture

MCF-7 cells were cultured in DMEM (Mediatech, Manassas, VA) with 10% FBS (HyClone, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin (GIBCO, Carlsbad, CA). Cells were hormone deprived in phenol red-free DMEM with 5% CDT FBS (Omega Scientfic, Tarzana, CA), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were treated with E2 (Sigma-Aldrich, St. Louis, MO), anti-SOX2 antibody [Millipore Corp., Billerica, MA (07-579)], anti-β-tubulin antibody [Zymed (Invitrogen) San Francisco, CA (48-1400)] and antirabbit IgG and horseradish peroxidase (Cell Signaling Technology, Inc., Danvers, MA).

Sucrose gradient density centrifugation and RNA analysis

To prepare polysomes, MCF-7 cells were hormone deprived for 3 d before addition of 10 nM E2. Cycloheximide (CHX) (100 µg/ml) was added for 3 min, medium was removed, and the cells were washed with PBS with 100 µg/ml CHX. Lysis buffer [15 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 300 mM KCl, 100 µg/ml CHX, and 1% Triton X-100] was added. Equivalent OD260 units of total lysate was separated on a 10–45% continuous, linear sucrose gradient prepared with lysis buffer without Triton X-100, prepared with a Gradient Master (BioComp Instruments, Fredericton, New Brunswick, Canada), by ultracentrifugation in a SW40 Ti rotor (Beckman Coulter, Brea, CA) for 3 h at 36,000 rpm at 4 C. Polysome profiles were collected with a Brandel gradient fractionator (Gaithersburg, MD) and determined by monitoring RNA absorbance at 254 nm. Fractions 3–6 were pooled to represent the subpolysomes, and fractions 7–13 were pooled to represent the polysomes. For puromycin experiments, 1 mM puromycin (Sigma-Aldrich) was added to the cells for 5 min and to the lysis buffer for 15 min at 37 C while mixing before ultracentrifugation.

Microarrays and analysis

RNA was purified by precipitation with guanidinium-HCl and ethanol followed by an RNeasy column (QIAGEN, Valencia, CA) according to manufacturer's instructions including treatment with deoxyribonuclease I. RNA quality was determined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). All RNA

processing, array hybridizations, and scanning were performed by the Brown University Center for Genomics and Proteomics core facility. RNA was prepared for hybridization using Affymetrix standard protocols and applied to Human Gene 1.0 ST microarrays (Affymetrix, Santa Clara, CA). We quantile normalized all 18 arrays and then used the Affymetrix Probe Logarithmic Intensity Error (PLIER) algorithm to generate signal estimates for all RefSeq genes. To select actual signal, we discarded those transcripts belonging to the lower quartile of their respective datasets. Analysis of significantly changing genes was determined using R (http://www.r-project.org/). Gene attributes were downloaded from UCSC Genome Browser. Complete microarray data have been deposited at the National Center for Biotechnology Information's Gene Expression Omnibus (accession no. GSE18592).

Quantitative real-time PCR

Equal amounts of RNA from polysomes or total RNA were reverse transcribed using Superscript III and random hexamers (Invitrogen, Carlsbad, CA). Resulting cDNA was renormalized using Quant-iT PicoGreen (Invitrogen) before mixing with primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Reactions were performed in an Applied Biosystems 7900HT Fast Real-Time PCR system. The fold change for total vs. total RNA was calculated, using spiked-in luciferase mRNA for normalization. To control for differential masses and purification yields from polysomes, luciferase mRNA was spiked in as a normalization control.

siRNA knockdown and plasmid overexpression, cell cycle, and apoptosis assays

Cells were plated in phenol red-free medium containing 5% CDT FBS at 450,000 and 10,000 cells per well in six- or 96-well plates, respectively. Silencer siRNAs targeting SOX2 and control were purchased from Ambion/ABI (Austin, TX). Three siRNAs (siRNA ID s13295, s13296, and s13294) were pooled, and 45 nM total concentration was transfected using DharmaFECT 1 (Dharmacon, Inc., Lafayette, CO) according to the manufacturer's instructions. After 48 h, cells were treated with E2 or vehicle (0.1% ethanol). Cell cycle was determined by propidium iodine staining, using a BD FACSCalibur flow cytometer (BD, Franklin Lakes, NJ). FACS data were modeled using ModFIT LT software (Verity Software House, Topsham, ME). SOX2 ORF was cloned into pCMV6-XL4's multiple cloning site using NotI. This plasmid or empty control were transfected using Fugene HD (Roche Diagnostics. Indianapolis, IN) according to the manufacturer's instructions. Cells were processed that same as siRNA treated samples.

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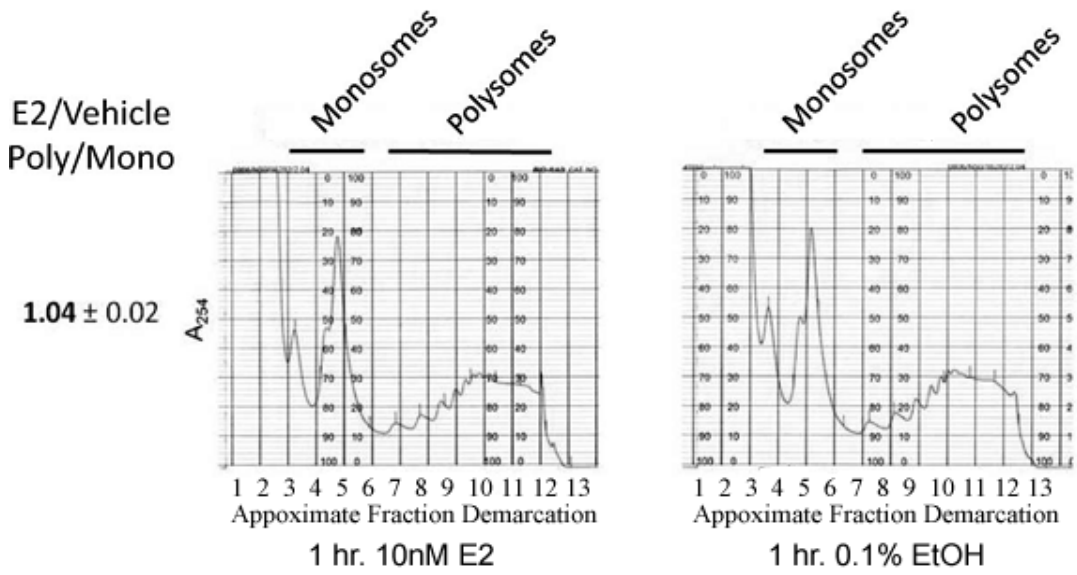
Tables

Table 1. List of all primer sequences used for qPCR in this study. F and R indicate forward and reverse primers.

Refseq ID	Gene Name	Primer Name	Sequence
	luciferase	MB1F	CGCTTCCGGATTGTTTACAT
		MB2R	GCTGGGCGTTAATCAGAGAG
NM_003106	SOX2 3'-UTR	MB3F	GGAGCTTTGCAGGAAGTTTG
		MB4R	TTTTTCGTCGCTTGGAGACT
NM_003106	SOX2 3'-UTR	MB5F	GCGAACCATCTCTGTGGTCT
		MB6R	GGAAAGTTGGGATCGAACAA
NM_003106	SOX2 ORF	MB7F	GCCGAGTGGAAACTTTTGTCG
		MB8R	GCAGCGTGTACTTATCCTTCTT
	SwitchGear pSGG_3UTR plasmid Promega Luc2P reporter	MB9F	TGCAAAAGATCCTCAACGTG
		MB10R	AATGGGAAGTCACGAAGGTG

Figure 1

A



B

	E2 Total vs Veh total	E2 poly vs Veh Poly
Array	0.9	0.57
qPCR	0.7	0.3

C

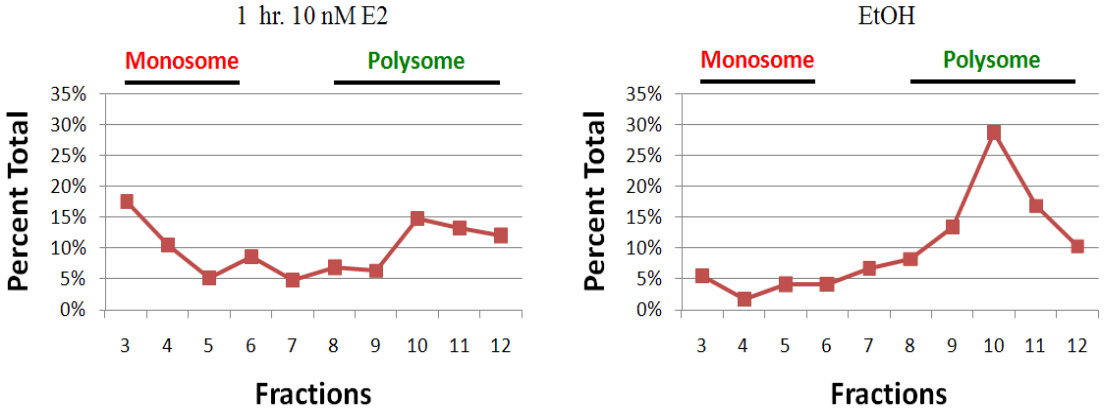


Figure 1. **E2 effect on translation profiles and SOX2 levels mRNA distribution**. Three independent measurements suggest that E2 represses SOX2 protein synthesis. **A**, Representative fractionation profile from 10–45% linear sucrose gradients from MCF-7 cells treated with 10 nM E2 or 0.1% ethanol (vehicle) for 1 h. The average ratio with the SD of triplicates of the P/M ratio in E2 vs. 0.1% ethanol (vehicle) is shown. Sucrose density gradients separated the mRNAs into subpolysomal/monosome and polysome fractions or were collected as individual fractions as indicated. Sucrose fractionation was monitored at 254 nM. **B**, Pooled polysome fraction compared to unfractionated total RNA suggest that SOX2 polysome levels are reduced after treatment with 10 nM E2 as measured by microarray and quantitative real-time PCR. **C**, The relative reduction in polysome signal and increased monosome signal, as measured by quantitative real-time PCR using primers MB3F and MB4R, suggests SOX2 is translationally repressed. Note: Polysome Association is Puromycin dependent (data not shown). SOX2 was measured using MB3F and MB4R primers.

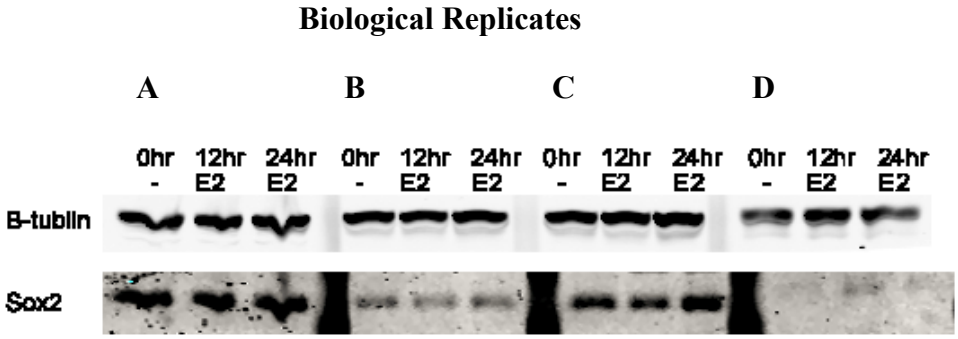


Figure 2. Detection of SOX2 protein levels after treatment with 10nM E2. MCF-7 cells were hormone deprived in phenol red-free CDT serum for at least 4 d before 10 nM E2 treatment. Representative Western blots with anti-SOX2 antibody shows SOX2 levels fluctuate relative to b-tublin in different biological replicates (A-D) of MCF-7 cell lines. SOX2 protein levels are transiently reduced at 12 hours after treatment.

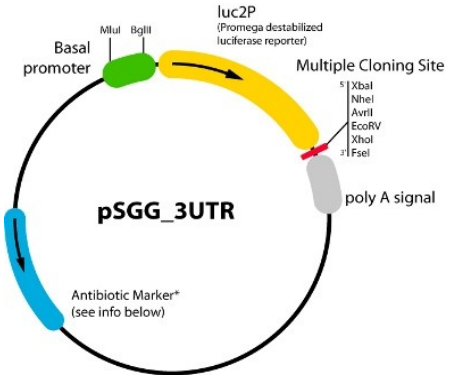


Figure 3. SwitchGear pSGG_3UTR luciferase reporter vector. SOX2 or B-Actin control 3'-UTRs were cloned in to the multiple cloning site creating a fused luciferase-3'-UTR product. Each plasmid was transfected using Fugene HD about 72 h before treatment with 10 nM E2 or vehicle for 1 h in hormone deprived MCF-7 cells. Individual fractions from a sucrose gradient translation profile was collected and LU2P and gene specific 3'-UTR were measured using quantitative real-time PCR. Transfection did not alter translation profiles (data not shown).

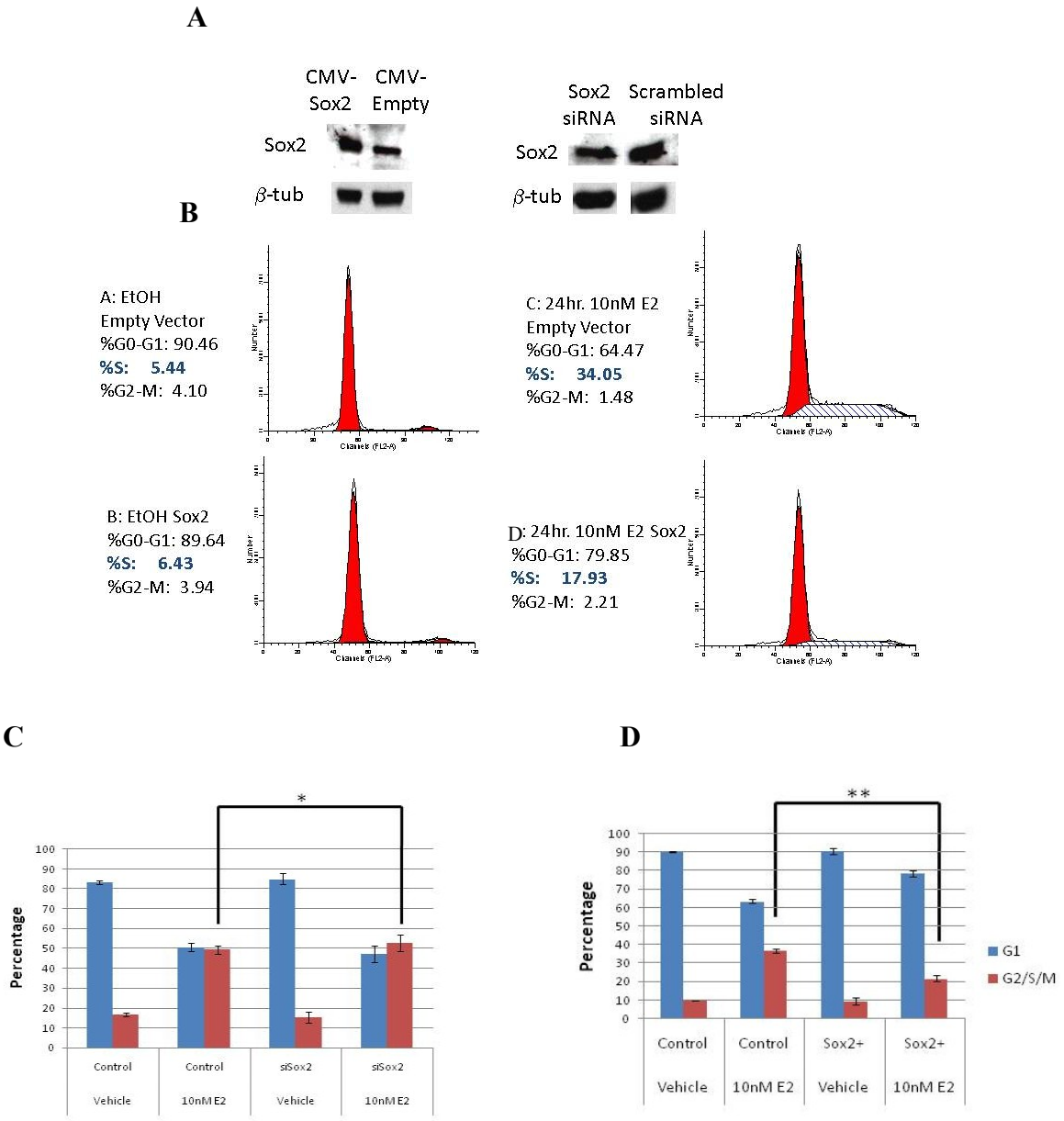


Figure 4. **SOX2 inhibits E2 stimulation of the cell cycle over 24 hours**. MCF-7 cells were hormone deprived in phenol red-free CDT serum for at least 3 d before 10 nM E2 treatment. Almost 90% of the cells became G1 growth arrested. **A**, Representative Western blot with Zymed anti-SOX2 antibody shows overexpression (right) and knockdown (left) SOX2 runs at approximately 37 kDa. qPCR RNA level overexpression is at least 8-fold while knockdown is typically about 4-fold. **B** and **D**, Representative propidium iodine staining DNA content FACS shows that overexpression of SOX2 significantly reduces the number of S phase cells. **C**, SOX2 knock-down shows a small but significant and reproducible increase in S phase cells. ** indicate t-test p-values < 0.01 and * indicate $p<0.05$. Error bars are standard deviation.

GACTTCACATGTCCCAGCACTACCAGAGCGGCCCGGTGCCCGGCACGGCCA
TTAACGGCACACTGCCCCTCTCACACATGTGAgggccggacagcgaactggaggggggag
aaattttcaaagaaaaacgagggaaatgggaggggtgcaaaagaggagagtaagaaacagcatggagaaaacccggtac
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agtctttcatttat→→ttggataacactgtgattatacataacttttcggggaattataatactgtgctcagccaagaaagc

Figure 5. **Sequence of SwitchGear pSGG_ SOX2_3UTR Sequence**. SOX2 has three validated alternative polyadenylation sites as indicated "→→" beaks in the sequence. The SOX2 open reading frame "UPPERCASE", SOX2 3'-UTR "lowercase", intergenic sequence 3' promixal to SOX2 3'-UTR "lowercase italics", MB3F and reverse compliant of MB4F in "red", and MB5F and reverse compliant of MB6R in "blue". Sequence information and location of poly(A) sites was collected from, The AceView genes [http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly] [24].

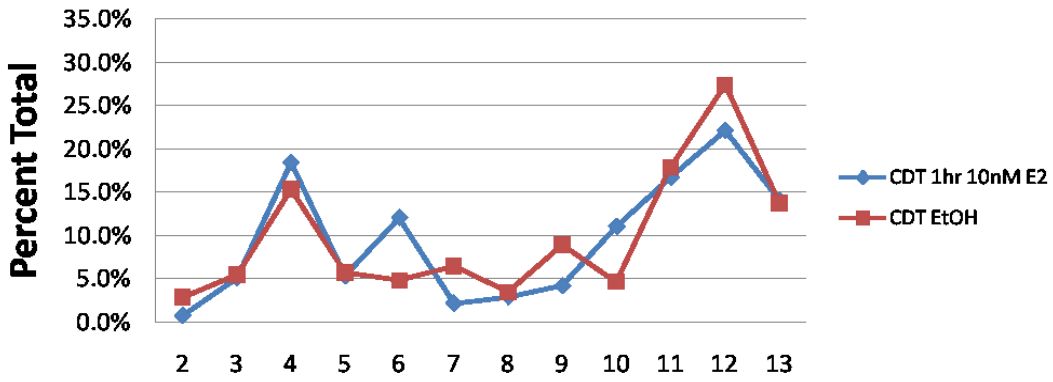


Figure 6. **E2 effect on SOX2 mRNA distribution as measured by primers MB5F and MBR6**. Similar distribution of SOX2 signal throughout translation profile as measured by quantitative real-time PCR. While SOX2 appears to be translationally repressed after stimulation with E2 in Fig. 1C, this SOX2 3'-UTR primer set reports similar distribution of SOX2 between treatment conditions. The intervening sequence between the amplicons of the two PCR primer sets contains a validated alternative poly(A) site. Together, these observations suggest that SOX2 may undergo alternative 3'end processing. The products of which display different translational efficiencies. See all primer and their sequence used in the study in Table 1.

Chapter 4

Discussion and Future Directions

Discussion

Even before the isolation of 17-β-estradiol in 1936 [1], its action has been shown to be intimately involved in a plethora of complex biological responses. The diversity of responses continues to grow, and has proven to be far more complex than was previously thought. The aim of the research presented within this thesis was to gain a more complete understanding of how E2 drives the proliferation of breast cancer. This work achieves that goal by examining the extent and significance of E2 mediated post-transcriptional gene regulation, and by investigating how E2 coordinates transcriptional and post-transcriptional regulation. Prior to this study, there has been no other comprehensive investigation of the role of E2 in these processes.

Estrogen (E2) stimulates 70% of breast cancers through the transcription factor, Estrogen Receptor-alpha (ERα) [2]. Most efforts to examine global effects of E2 have focused on total mRNA levels and transcription mediated by ERα [2-5]. These and other studies have identified numerous factors critical for E2 and tamoxifen (TAM) action, including E2F1 [6] and PAX2 [7].

Our hypothesis, that E2 plays an important role in the regulation of the transcriptome by modulating post-transcriptional processing, prompted our studies. We have investigated post-transcriptional regulation of E2 response in the breast cancer cell line MCF-7 by performing microarray analysis comparing total mRNA levels with monosomal and

polysomal mRNA levels after E2 treatment. We observed several significant, novel findings. We find that polysome analysis has higher sensitivity than total RNA analysis in detecting E2-regulated transcripts, and it allowed us to discover several transcripts and pathways regulated by E2 at the translation level. This increased experimental sensitivity is exemplified by observations of stronger E2-induced enrichment of E2 expression signatures in polysomes than in total RNA. We observed that the initial translation state is already high for E2 upregulated transcripts before E2 treatment and vice versa for E2 down-regulated transcripts. This suggests that the translation state anticipates potential E2-induced transcriptome levels. Our data have identified two transcription factors mediating E2 stimulation, SOX2, which is translationally repressed, and NRSF, whose target genes' expression are repressed by E2. We have demonstrated that these two transcription factors mediate E2 stimulation of the cell cycle. The effects of SOX2 and NRSF on E2 mediated stimulation of the cell cycle is shown in Fig. 1. Together, these data suggest that E2 stimulates breast cancer cells by regulating translation through multiple mechanisms. In sum, we show that polysome profiling of E2 regulation of breast cancer cells provides novel insights into hormone action, and can identify novel factors critical for breast cancer cell growth.

Future Directions

The observations presented within this thesis highlight a novel level of control mediating E2 stimulation of breast cancer cells. To improve our understanding of the role of translation in endocrine therapy, we have extended our analysis to explore TAM controlled translation regulation in order to identify directly regulated genes and any potential sequences involved in antiestrogen induced growth arrest.

We have also collected an additional three-hour E2 time point. Additional time points may provide further insight into the coordination between transcriptome and translational responses. At later time points, more global increases in translation state may be observed. This would suggest that translational responses and transcriptome changes respond to E2 with different kinetics. Based on the observations at the one-hour time point, it seems likely that a variety of translation state responses would also be observed at later time points. Another possibility is an amplification model, where small changes in total RNA levels lead to much larger changes in polysomal RNA levels for some transcripts. Although true for some pathways, such as glycolysis, this is not always the case, as seen for transcripts coding for the CREB pathway. This amplification model highlights how transcription and translation can be sensitive to E2-stimulated gene expression, to different degrees, at each time point.

Results

To probe E2 and TAM translational regulation, we compared polysomal and monosomal transcript levels with total RNA levels between E2-treated and TAM-treated MCF-7 cells (Fig. 2). MCF-7 cells were hormone deprived in charcoal-dextran-treated (CDT) serum for three days before addition of either 10 nM E2 or 1 uM 4-hydroxy-tamoxifen (TAM) for three hours. One mechanism for E2/TAM regulation of translation is to control initiation, the major rate-limiting step in protein synthesis [8]. We observe that, after three hours of E2 stimulation, there is an increase in polysome/monosome ratios in MCF-7 breast cancer cells compared to TAM treated cells (Figs. 2 and 6). After three hours of 10 nM E2 treatment, increased polysome/monosome ratios suggest increased protein synthesis compared to one hour of E2 stimulation (Fig. 6).

GSEA expression analysis of defined expression signatures in the transcriptome and translational responses shows that TAM and E2 induce specific translational responses and transcriptome level changes (Fig. 3). Consistent with our observation, at one hour after 10 nM E2 treatment, ribosomal proteins are translationally upregulated, but are not transcriptionally upregulated when compared to TAM. Post-transcriptional regulation seems to be different, and more sensitive, than transcriptional regulation. Also consistent with E2 driving proliferation, there is significant upregulation of cell cycle genes in both the transcriptome and polysomes.

Figures 4 and 5 show Ingenuity Pathway Analysis (IPA), which is a database and analysis system for understanding how proteins work together to effect cellular changes. We observe that TAM downregulates MYC and progesterone receptor (PGR) networks in total RNA. It also translationally upregulates genes, including E2F7, which has been shown to repress transcription and delay cell-cycle progression [9]. These two observations are consistent with TAM inhibition of ER+ tumor growth. These preliminary data suggests that TAM influences translation regulation of specific networks to complement transcriptional (total RNA) regulation to control the cell cycle. These exciting observations will require validation and are subject to further refinement, including validation with quantitative real time PCR and western blots to measure protein levels. The functional significance of translationally regulated genes will also be tested for affects on E2 or TAM sensitivity.

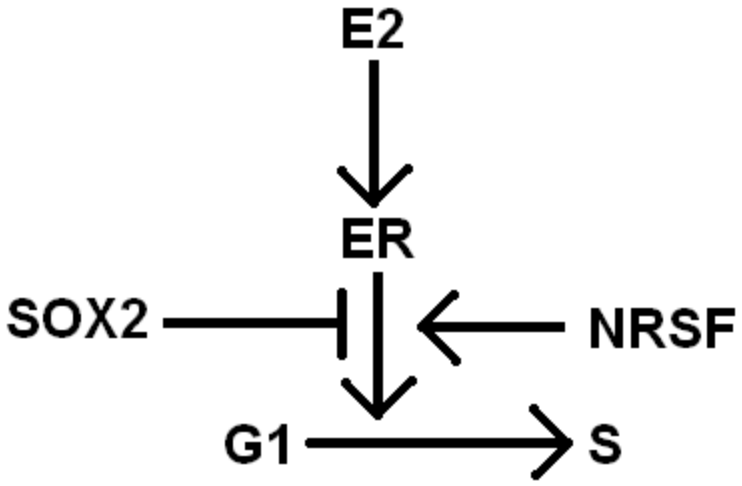
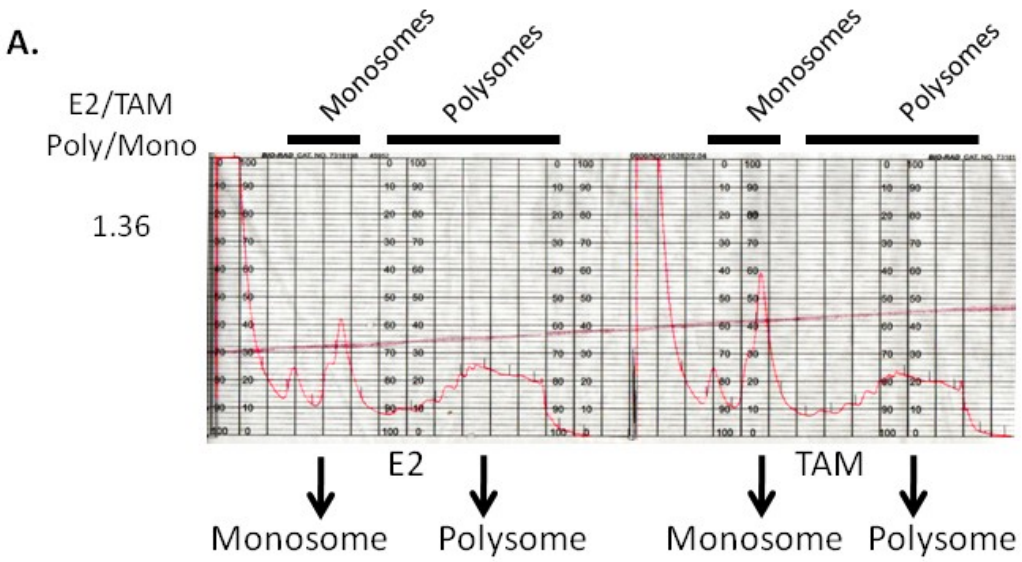


Figure 1. Working model of the effects of SOX2 and NRSF on E2 mediated stimulation of the G1/S cell cycle transition.



B.

RNA	Analysis
E2 Total vs. Veh Total	Transcriptome levels
Polysome vs. Total	Translation State/Transcriptome levels
Polysome vs. Monosome	Translation State
E2 Polysome vs. Veh Polysome	Translation State/Transcriptome levels

Figure 2. E2 and TAM effect on polysomes and microarray analysis. A, Representative fractionation profile from 10–45% linear sucrose gradients from MCF-7 cells treated with 10 nM E2 or 1 μM for 3 hours.. The average ratio of the P/M ratio in E2 *vs.* TAM is shown. Sucrose density gradients separated the mRNAs into the subpolysomal/monosome and polysome fractions. Sucrose fractionation was monitored at 254 nM. B, Scheme of the procedure of translational profiling. Comparisons to be made are (EtOH, one hour, E2 three hour), (EtOH, TAM), and (TAM, 3hr E3) These was removed from the figure to simplify its layout. Unfractionated total RNA was isolated in each condition and hybridized on Affymetrix microarrays.

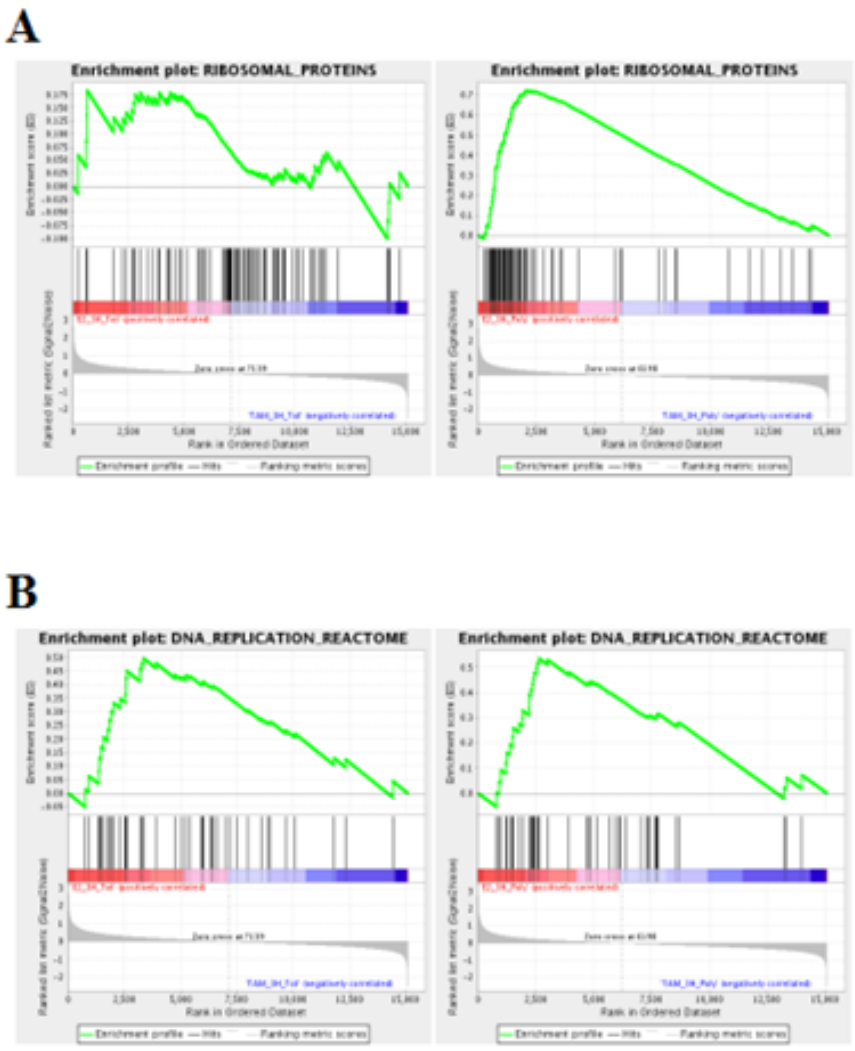


Figure 3. TAM and E2 induce specific translational responses and transcriptome level changes. GSEA expression analysis of indicated protein expression signatures in the transcriptome and translational responses. The *black lines* demonstrate where each gene in the gene set falls within the probe sets probing RefSeq transcripts ordered from *left to right* based on the E2 polysome/TAM polysome ratio with gene 1 the most highly expressed in E2 polysomes. The *green line* represents the running enrichment score (ES) that becomes more negative as probe sets are identified toward the bottom of the list. Significant up-regulation is seen in both the transcriptome and polysomes. A, Ribosomal proteins translationally stimulated by E2, but not in total when compared to TAM or vehicle. Translational stimulation by E2 and not the transcroptome at 1 hour of E2 treatment. B, E2 drives proliferation. There is significant up-regulation in cell cycle genes in both the transcriptome and polysomes.

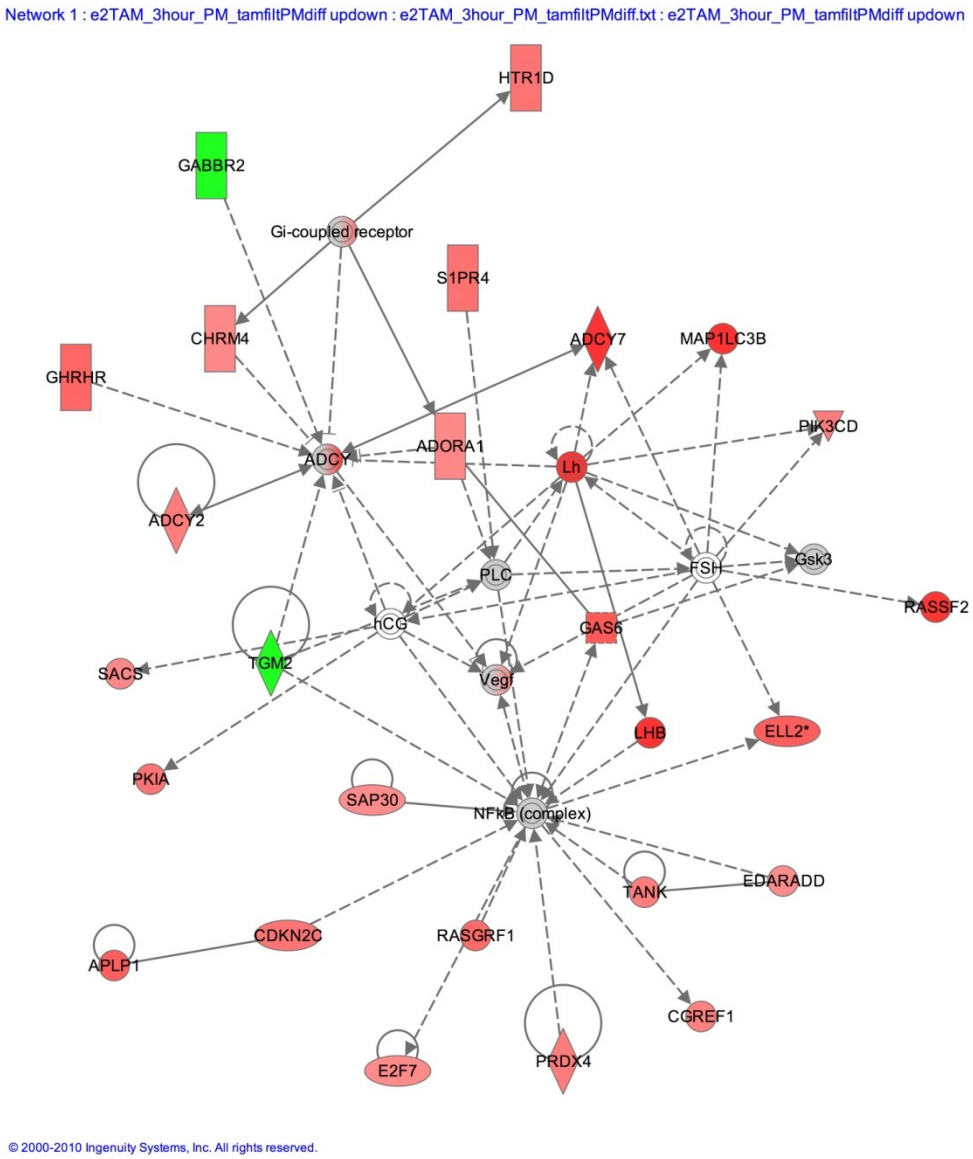


Figure 4. TAM translationally up-regulates genes including E2F7, which has been shown to repress transcription and delay cell-cycle progression. Genes were selected by significant differences in Polysome/Monosome ratio. Relative translation state gene fold expression data from MCF-7 treated with either 10 nM E2 or 1 uM TAM for 3 hours were analyzed through the use of Ingenuity Pathway Analysis (Ingenuity Systems). Differentially expressed (E2 vs. TAM) genes from hormone deprived MCF-7 cells. Solid line, direct interaction; *dotted line,* indirect interaction. *Green nodes,* down-regulated P/M ratio after TAM treatment. *Red nodes,* up-regulated P/M ratio after TAM treatment (for more information, see also http://www.ingenuity.com/). $q<0.05$, Diff in log2 > 0.5, 310 genes included

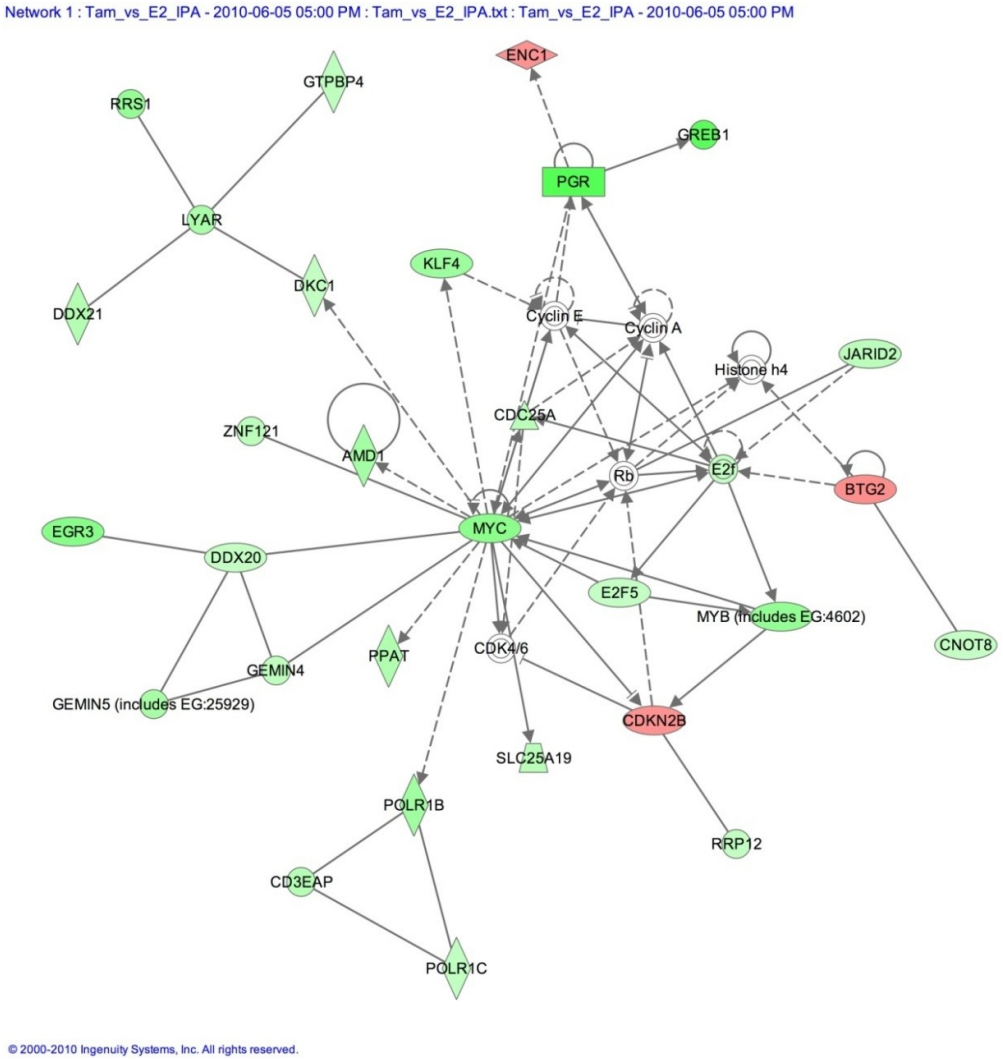


Figure 5. TAM down-regulates the translation state of MYC and PGR networks in total RNA, consistent with inhibition of cell growth. Signaling pathways affected by TAM and E2. Genes were selected by significant differences in Polysome/Monosome ratio. Relative translation state gene fold expression data from MCF-7 treated with either 10 nM E2 or 1 uM TAM for 3 hours were analyzed through the use of Ingenuity Pathway Analysis (Ingenuity Systems). Differentially expressed (E2 vs. TAM) genes from hormone deprived MCF-7 cells. Solid line, direct interaction; *dotted line,* indirect interaction. *Green nodes,* down-regulation in P/M ratio after TAM treatment. *Red nodes,* up-regulation in P/M ratio after TAM treatment (for more information, see also http://www.ingenuity.com/). 351 genes, fold change >1.5, $p<0.05$ (t-test)

Translation State	Samples (Used in Comparisons)
1.04	1 hr. 10 nM E2 vs. 1 hr. EtOH
1.17	3 hr. 10 nM E2 vs. 1 hr. 10 nM E2
1.36	3 hr. 10 nM E2 vs. 3 hr. 1 uM TAM
4.88	Difference between hormone enriched and puromycin treatment

Figure 6. Translation states (Mono/Poly ratio) compared between different treatment conditions reveals a sequential gradual increase in global translation rate after the addition of E2.

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Appendix 1

Detailed Protocols

Polysome Protocol

Cell Culture

1. Split cells to be in log growth with 75-90% confluence for use, uniformly distributed
    a. For CDT: Plate directly into CDT media for 3-6 days and change media daily
        i. Dependent on growth arrest rate
    b. For Red: Plate cells at least 48 hours before use, change media 24 hr before use

Preparing Gradients (10-45%)

1. Make gradients the day you harvest cells, using cold previously prepared solutions
2. Use 10% and 45% stock sucrose solutions previously prepared (include 10 mL extra 10%) ensure the solution is mixed before use
3. For two gradients, make 25 ml and 15 ml of 10% and 45% respectively.
    a. For cycloheximide, remove 1% volume of gradient and add cycloheximide
    b. Other conditions: remove 1% volume of gradient and add diH20 (BTV)
4. Mark the half volume line on tube with thin marker (highest level of tube holder (Beckman, Polyallomer, # 331374 14 ml))
5. Place tubes into six tube magnetic holder and pipet ~6.7 ml of 10% sucrose into tube

6. Using a 10 ml syringe and a cannula carefully underlay the 10% sucrose with 45% sucrose to the marked half volume line, only insert cannula 1 mm into 45% stock.
7. Put on cap (do not trap any air bubbles), aspirate excess sucrose out of cap
8. Use the GRADIENTMASTER to form continuous gradients
    a. Turn on and level the platform
    b. Place six tube magnetic holder with gradients onto platform
    c. Select: “GRAD”, “RCNT”, “SW40”, “Short Sucr 10-45% wv 1st”, “RUN”
9. Cool gradients at 4°C

Lysis/Sample Preparation

1. If adding a translation inhibitor, add inhibitor
2. Aspirate media from plate and wash with original plate volume of ice cold PBS
3. Place each plate onto ice, all remaining steps to be done at 4°C
4. Aspirate PBS, and again wash the plate with PBS (thoroughly aspirate PBS)
5. Add concentrated lysis buffer to plate, scrape down cells
    a. 200 ul for a 10 cm plate
    b. 250 ul for a 15 cm plate
6. Collect into microcentrifuge tube
    a. ~340 ul for a 10 cm plate
    b. ~460 ul for a 15 cm plate
7. Place the microcentrifuge tube onto tube rotator for 10 min (no longer than 15 min)
8. Lyse nuclei using 1 ml syringe with a 26&1/2 G needle
9. Pellet nuclei at 10K rpm for 11+ min. and transfer supernatant into new tube (take care not to collect all of the aqueous which will contaminate your sample with DNA)

Loading and Running Gradients

1. Load the SW-40Ti rotor into the Beckman ultra-centrifuge and set to vacuum at 4°C
2. Determine the OD260 and ABS 260/280 ratio of the extract (2 ul lysate in 198 ul diH20) using BioRAD trUView Cuvette (170-2511)
3. OD per gradient is calculated by: Desired OD/(Abs 260 Reading) = ul of sample to load
4. Check rotor tube/gradient weights, the final weight must be within 0.1 g after the addition of sample, adjust weight with lysis buffer)
5. Dropwise add lysate onto top sucrose gradient, do not load more than 290ish ul, but adjust paired samples for OD and weight
6. Load rotor tubes onto SW-40Ti rotor in Beckman ultra-centrifuge set instrument to: 36K, 3 hours, 4°C. with max acceleration and deceleration

Fractionation of Gradients

1. Assemble piercer system, and load polyallomer tube
2. Wash the system with diH2O for 15+ min and remove air bubbles
3. Reverse pump diH20 into polyallomer tube
4. Run your 10 mL excess of 10% sucrose into UV detector
5. Set UV monitor sensitively level
    a. Puromycin 15 cm (Auf 0.5), 100-200 OD (Auf 0.2), 50-100 OD (Auf 0.1)
6. Autozero the UV detector with 10% sucrose, then remove all 10% sucrose
7. Displace the washing diH2O with 55% sucrose until past needle
8. Load sample tube into tube piercer, and pierce tube
9. Upwardly display your gradient with 55% sucrose
10. Pump speed 4.5 rpm, collector speed at 0.89, make sure the pen recorder is down and recording and that the fraction collector tip is dried before your sample elutes
    a. Advance fraction collector when sample is at yellow cap of collector
    b. Start fraction timer with the first drop of material
11. Collect 14 fractions (~950 ul), run two more fractions as waste
12. If you wish spike-in luciferase mRNA as a normalization control (0.001 ng)
13. Reverse pump the 55% to one inch above needle
14. Wash the top portion of the flow cell with diH20
15. Repeat from steps 3-13 with any remaining gradients
16. Wash system when done with diH2O

Stock Solutions (use acid/base washed glass, do not put anything into solutions (stir bars, pH meters, etcetera) into solutions. Tap out compound when making solutions:

10X Polysome Salts Stock (500 ml):

150 mM Tris-Cl [pH 7.4]	75 ml 1M
100 mM MgCl	50 ml 1M
2.0 M KCl	333.333 ml 3M KCl
diH20 (BTV)	
Filtersterilize	

55% Sucrose Stock (1000 ml):

diH20	needs heat (90C), 450 ml
Sucrose	550 g
Polysome salts stock,	100 ml
diH20 (BTV) (let cool to room temp. first)	
Filtersterilize	

45% Sucrose Stock (297 ml):

55% Sucrose	245.5 ml
10X Polysome Salt Sucrose	5.45 ml
3 M KCl	10 ml
diH20	36.05 ml

Filtersterilize

10% Sucrose Stock (495 ml):

55% Sucrose	90.91 ml
10X Polysome Salt Sucrose	40.91 ml
3 M KCl	16.667 ml
diH20	363.18 ml

Filter sterilize

Lysis Buffer: **Gradient Buffer:**

15 mM Tris [pH 7.4]	15 mM Tris [pH 7.4]
350 mM KCl	10 mM MgCl2
15 mM MgCl2	300 mM KCl
1% Triton X-100	
40 U/ml Optizyme Ribonuclease Inhibitor	

Translation Inhibitors:

Cycloheximide:

1. 10 mg/ml cycloheximide in PBS day of use, add to:
    a. Media for three min. at 37°C, final concentration of 100 ug/ml
    b. To lysis buffer, final concentration of 100 ug/ml
    c. To PBS wash buffer, final concentration of 100 ug/ml
    d. To 10% and 45% sucrose, final concentration of 100 ug/ml

Puromycin:

1. Make puromycin in 50 mg/ml in diH2O, add to:
    a. Media for five min. at 37°C, final concentration of 1 mM
    b. To lysis buffer, final concentration of 1 mM
2. After needle lysis, place samples into vortexing heat block at 37°C for 10 minutes

Polysome RNA purification: (non microRNA, can be for microRNAs if EtOH is increased to at least 60%)

1. Collect 1 ml fractions into tubes containing 2 ml 8M guanidinium-HCl

2. Precipitate by adding equal volume of 100% Ethanol and incubation overnight at-20°

3. Centrifuge samples at 4,000 rpm for 1 hr

4. Pour off supernatant carefully. Remove the residual solution by pipette

5. Invert falcon tube and let the pellet dry for 15 min

6. Prepare extraction buffer (EXB) by adding β-mercaptoethanol to RLT (QIAGEN) (10 ul/1 mL RLT).

7. Add 450 uL EXB to the sample and vortex vigorously

8. Add 275 uL 100% EtOH to the sample. Mix well by pipetting (do not vortex)

9. Apply sample (750 uL) to an RNeasy mini spin column (pink). Let it sit for 5 min

10. Spin the column for 15 sec at 14K rpm

11. Take the flow through and reload the column with sample, let sit for 5 min.

12. Spin the column for 15 sec at 14K rpm.

13. Add 700 uL RW1 let sit 3 min & spin for 30 sec at 14 K rpm. Discard the flow through.

14. Add 80 ul DNaseI mix (70 ul RDD+10 DNaseI) let sit for 15 min

15. Add 350 ul RW1 spin for 30 sec at 14K rpm. Discard the flow through.

16. Transfer the column into a new 2 mL microtube. Add 500 uL RPE (QIAGEN) onto the column and spin for 15 sec at 14K rpm.

17. Add 500 RPE to the column and spin for 2 min at 14K rpm, you can do extra RPE washes

19. Place the column in a new 2 mL microtube and spin for 1 min at 14K rpm.

20. Transfer the column in a new 1.7 mL microtube and add 30 uL RNase free water Let it sit for 5 min.

19. Elute RNA by centrifuge for 1 min at 14K rpm.

20. Reload column with the same 30 ul elution from step 19, let sit 5 min, elute RNA

21. Nanodrop and Agilent 2100 Bioanalyzer

Polysome RNA purification: (Trizol LS for microRNA)

1. Collect 14 fractions with volume of ~1 ml. (Collect fractions into tubes containing 3 ml (fractions 2-7), and 6ml (fractions 1, 8-14 and add 1ml diH2O) of Trizol LS))

2. Add chloroform and mix (0.75 ml per ml Trizol LS)

3. Centrifuge samples at 4,000 rpm for 1 hr.

4. Collect aqueous solution by pipette.

5. Add 1.5 times 100% EtOH to collected aqueous and mix

6. Place QIAGEN RNeasy mini spin column onto vacuum manifold and add sample to column

7. Apply vacuum (I like to do rest of prep by spin prep, but you can do it by vacuum manifold)

8. Add 350 uL RWT let sit 3 min & spin for 30 sec at 14K rpm.

9. Add 80 ul DNaseI mix (70 ul RDD+10 DNaseI) let sit for 15 min

10. Add 350 ul RWT spin for 30 sec at 14 K rpm. Discard the flow through.

11. Transfer the column into a new 2 mL microtube (without a cap). Add 500 uL RPE

12. Add 500 RPE to the column and spin for 2 min at 14K rpm.

13. You can do extra RPE washes

14. Place the column in a new 2 mL microtube and spin for 1 min at 14K rpm.

15. Transfer the column to a new microtube and add 30 uL RNase free water, let it sit for 5 min.

16. Elute RNA by centrifuge for 1 min at 14 K rpm (repeat 15-16)

17. Reload column with the same 30 ul elution let sit 5 min, elute RNA

18. Nanodrop and Agilent 2100 Bioanalyzer

Trichloroacetic Acid (TCA) Polysome Fraction Protein Purification

1. Collect fractions into 15 mL tubes

2. Dilute 950 ul protein sample by adding 950 ul diH2O

3. Add 475 ul of 100% TCA stock to sample.

2. Incubate 30 min at 4°C or overnight at -20°C, you might need BSA or tRNA as carrier

3. Spin tube in microcentrifuge at 14K rpm, 5 min.

4. Remove supernatant, leaving protein pellet intact.

5. Wash pellet with 900 ul cold acetone.

6. Spin tube in microfuge at 14K rpm, 5min.

7. Repeat steps 4-6 for at least four acetone washes.

8. Dry pellet by placing tube in 95°C heat block for 30 sec to drive off acetone.

9. For SDS-PAGE, 4X sample buffer (with BME) and boil sample for 10 min at 85°C

10. You may need to sonicate sample to get them into solution

Determination of Protein Synthesis Rates

1. Label cells by adding 100 uCi/mL EasyTag EXPRESS^{35}S protein labeling mix (PerkinElmer, Waltham, MA) to culture media in a 12-well plate.

2. After labeling, add cycloheximide to 100 ug/ml to the culture media and incubate for five min.

3. Cells were then washed twice with PBS with 100 ug/ml of cycloheximide

4. Cells were lysed in 1 mL of RIPA buffer plus protease inhibitors and 100 ug/ml cycloheximide

5. The lysate was transferred to a microcentrifuge tube, and spun at 14K rpm for 10 min

6. Transfer supernatant, to a new microcentrifuge tube

7. TCA precipitation: 300 uL of extract were added to 300 uL of ice-cold 20% trichloroacetic acid and incubated on ice for 30 min.

8. Spin tube in microcentrifuge at 14K rpm, 5 min.

9. Remove supernatant, leaving protein pellet intact.

10. Wash pellet with 900µl cold acetone.

11. Spin tube at 14K rpm, 5 min.

12. Repeat steps 9-11 for two acetone washes.

13. Dry pellet by placing tube in 95°C heat block for 5 seconds to drive off acetone.

14. Resuspend the pellet in 600 ul 4M gHCL

15. Once resuspended, add 600 ul of 10 mM Tris-HCl [pH 8.0]

16. For total counts per minute, 20 uL of total extract was added to scintillation vials with 5 mL of scintillation fluid and mixed before reading on a scintillation counter.

17. For incorporation counts per minute, 80 uL of total extract was added to scintillation vials with 5 mL of scintillation fluid and mixed before reading on a scintillation counter.

18. Counts were normalized to microliters of extract, and percentage of trichloroacetic acid incorporation was determined by dividing the trichloroacetic acid precipitate counts per minute per milliliter extract by total counts per minute per milliliter extract.

Measurement of DNA Content Using Propidium Iodide (PI) Staining of Fixed Whole Cells

1. From a six well plate, typsinise cells thoroughly to obtain a single cell suspension

2. Transfer cells to a 15 ml conical tube with 3X culture media

3. Centrifuge at 1000g for five minutes

4. Wash cells two times in PBS

5. Resuspend the cell pellet in ~500 ul of PBS

6. Add 5 mL of 100% ethanol slowly while vortexing to prevent clumping

7. Fix for at least one day 4°C

8. Centrifuge at 1000g for five minutes and remove ethanol

9. Wash the cells two times in 5 ml of PBS + 1% BSA

10. Resuspend the pelleted cells in 300 µl of PBS containing 1% BSA

11. Add 70 µl of 10X PI solution.

12. Add 5 µl of boiled RNase A and incubate at 37°C for 30 minutes.

13. Analyze the fixed samples by flow cytometry

14. Model the flow cytometry data using ModFIT LT software

Immunofluorescence of Apoptosis using DAPI

1. Spin down plate at 200g for 5 min
2. Aspirate medium, wash with PBS, remove solutions carefully
3. Fix cells with 4% paraformaldehyde for 30 min. at room temperature
4. Wash with PBS
5. Permeabilize cells with 0.5% Triton X-100 for 5 min
6. Wash twice with PBS
7. Block with 3% BSA in PBS for 30 min. at room temperature (if sealed with parafilm@ 4^{o}C refresh BSA PBS every 1-2 weeks)
8. Wash with PBS
9. Incubate with DAPI (1:3000 of 1 mg/ml stock) for 60 min. at room temperature, keep protected from light
10. Wash 2x with PBS
11. Add PBS to well
12. Image using Zeiss Axiovert 200M fluorescence microscope
13. Record images in MetaMorph and convert to ImageJ files and count nuclei

4% Paraformaldehyde (100 ml PBS)

1. Dilute 10x PBS with dH_20, but not to final volume
2. Add 2 g sucrose

3. Add 4 g paraformaldehyde while warming the solution, do not heat above 70˚C
4. Stir for 20 min. until cool
5. Adjust pH to 7.4 and BTV with diH2O
6. Aliquot and store in -20˚C

cDNA Normalization using Quant-iT PicoGreen (Invitrogen) for qPCR

1. Make 1X TE pH 8.0 (10 mM TRIS 1 mM EDTA),
2. Dilute PicoGreen 1:200 in 1X TE
3. Add 25 ul of diluted PicoGreen to sample wells of costar 3694 plate
4. Use 100 ng/ml DNA stock to create standard curve by serial 1:2 dilutions in TE
5. Add 25 ul standards to PicoGreen in sample wells
6. Take 3 ul of 1:30 dilution (10 mM Tris-HCl pH 7.4) from a 500 ng reverse transcription reaction, add 22 ul of 1X TE and add to PicoGreen sample well
7. Spin plate down at 1000 rpm for 10 seconds
8. Using plate reader, read fluorescence and set the instrument:

    a. Read from top of plate (Purple Adaptor In)
    b. Mix 1 min before reading
    c. Excitation @ 488 nm
    d. Emission @ 534 nm
    e. Cut @ 515 nm

9. Calculate the concentration of cDNA and dilute for qPCR at 1 ng/15 ul reaction

FuGENE® HD Transfection Reagent / DNA Complex and Transfection Protocol

MCF7 and T47D:

1. Plate cells to be at least 30% confluent on day of transfection (30-80%)

2. Allow FuGENE® HD, DNA, and diluent to adjust to room temperature.

2. Vortex FuGENE® HD for one second

2. Dilute expression plasmid in diH2O (160 ul final volume), to a final concentration of 2 µg / well (tested down to 25 ng/150K cells)

3. Add 6 µl FuGENE® HD Transfection Reagent

4. Do not allow the FuGENE® HD to contact the plastic walls of the tube

5. Mix carefully by pipetting (15 times) or vortex 3 seconds

6. Quick spin the tube for 2 second and incubate for 5 minutes at room temperature

8. Pipet transfection complex to cells drop-wise below the surface of the medium. Swirl the wells to ensure distribution over the entire plate surface.

7a. 96 Well → Add 8 µl complex per well to the cells and mix thoroughly

7b. 6 well →Add 160 µl complex per well to the cells and mix thoroughly

9. Incubate the cells in a CO_2 incubator for 48 hours; you can change media after 24 hrs.

10. Monitor

DharmaFECT 1 Transfection Reagent / siRNA complex and transfection protocol

1. Prepare working stock of siRNA solution in RNase-free diH2O

2. In separate tubes, dilute siRNA and DharmaFECT 1 using serum and antibiotic free medium.

a. siRNA (5-100 nM final siRNA concentration)
    a. 96 well (10 ul final), 24 well (50 ul final), 6 well (200 ul final)
b. DharmaFECT 1 transfection reagent
    a. 96 well (0.2 ul in 10 ul final,), 24 well (1.5 ul in 50 ul final), 6 well (4.0 ul in 200 ul final)

3. Gently mix the contents of each tube by pipetting carefully up and down. Incubate for 5 minutes at room temperature.

4. Add the content of two tubes, mix by pipetting carefully up and down and incubate for 20 minutes at room temperature.

5. Add antibiotic-free complete medium to the mix in step 4

a. 96 well (80 ul), 24 well (400 ul), 6 well (1600 ul)

6. Remove culture medium from the wells of the plate and add transfection medium to each well.

7. Incubate cells at 37°C in 5 % CO2 for 24–48 hours (for mRNA analysis) or 48–96 hours (for protein analysis).

8. If necessary, the transfection medium may be replaced with complete medium after 24 hours to reduce cytotoxicity.