Cell

Phosphorylation of the Human MicroRNA-Generating Complex Mediates MAPK/Erk Signaling

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DOI 10.1016/j.cell.2009.06.044

SUMMARY

MicroRNAs (miRNAs) govern an expanding number of biological and disease processes. Understanding the mechanisms by which the miRNA pathway is regulated, therefore, represents an important area of investigation. We determined that the human miRNA-generating complex is comprised of Dicer and phospho-TRBP isoforms. Phosphorylation of TRBP is mediated by the mitogen-activated protein kinase (MAPK) Erk. Expression of phospho-mimic TRBP and TRBP phosphorylation enhanced miRNA production by increasing stability of the miRNA-generating complex. Mitogenic signaling in response to serum and the tumor promoter PMA was dependent on TRBP phosphorylation. These effects were accompanied by a coordinated increase in levels of growth-promoting miRNA and reduced expression of let-7 tumor suppressor miRNA. Conversely, pharmacological inhibition of MAPK/Erk resulted in an anti-growth miRNA profile. Taken together, these studies indicate that the MAPK/Erk pathway regulates the miRNA machinery and suggest a general principle, wherein signaling systems target the miRNA pathway to achieve biological responses.

INTRODUCTION

miRNAs are ~22 nucleotide cellular RNAs that target mRNAs to regulate protein expression. miRNAs are derived from genome-encoded primary transcripts that are processed to ~65 nucleotide stem-loop precursor (pre-) miRNAs by a processing complex comprised of the ribonuclease III Drosha and double-strand RNA-binding protein (dsRBP) DGCR8/Pasha (Lee et al., 2003; Denli et al., 2004; Gregory et al., 2004; Han et al., 2004). Exportin-5/Ran-GTP translocates pre-miRNA from nucleus to cytoplasm where they serve as substrates for the miRNA-generating machinery (Yi et al., 2003; Lund et al., 2004). The miRNA-generating complex consists of the ribonuclease III Dicer (Bernstein et al., 2001; Hutvagner et al., 2001; Lee et al., 2004; Zhang et al., 2004) and a partner dsRBP (Liu et al., 2003; Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005). In humans, Dicer is accompanied by the HIV TAR RNA-binding protein (TRBP; Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). Mature miRNAs direct sequence-specific silencing of target transcripts through Argonaute-containing effector complexes by means that are dependent and independent of mRNA stability (Filipowicz et al., 2008).

The importance of the miRNA pathway in mammalian biology was first highlighted through genetic studies. Chromosomal ablation of the RNaseIII catalytic domain of murine Dicer resulted in stem cell depletion and embryonic lethality (Bernstein et al., 2003). Independent targeting of Dicer and DGCR8/Pasha alleles in murine embryonic stem cells resulted in deficiencies in centromeric silencing and differentiation (Kanellopoulou et al., 2005; Wang et al., 2007). Related approaches further established the importance of Dicer and partner dsRBPs in murine and *Drosophila* germline stem cell division and maintenance (Hatfield et al., 2005; Murchison et al., 2007; Park et al., 2007) and zygotic development (Tang et al., 2007).

Identification of the first miRNA and its biological target preceded the discovery of the miRNA machinery. The *C. elegans lin-4* locus, and encoding miRNA, was found to direct developmental timing through recognition of homologous repeat elements within the lin-14 3′-untranslated region (UTR; Lee et al., 1993; Wightman et al., 1993). Subsequent work revealed that lin-4 and the highly evolutionarily conserved let-7 miRNA govern a network of regulatory factors in directing temporal development (Reinhart et al., 2000). Recent studies have demonstrated the necessity of numerous other miRNAs in organismal development.

Aberrant expression of developmental genes is often related to disease etiology including cancer. Determination of let-7-mediated regulation of let-60/Ras in *C. elegans* development led to an observed inverse correlation between let-7 and Ras expression in normal and human lung tumor tissue (Johnson et al., 2005). 3′UTR engineering to mimic chromosomal translocations found in some human tumors revealed let-7-mediated silencing of high-mobility group A2-mediated oncogenic transformation (Mayr et al., 2007). Oligonucleotide-mediated inhibition of let-7 enhanced lung cancer cell proliferation, and let-7 overexpression inhibited cell division (Johnson et al., 2007; Yu et al., 2007). These and other studies (He et al., 2007; Tavazoie et al., 2008) provided clear demonstration of miRNAs functioning as tumor suppressors.

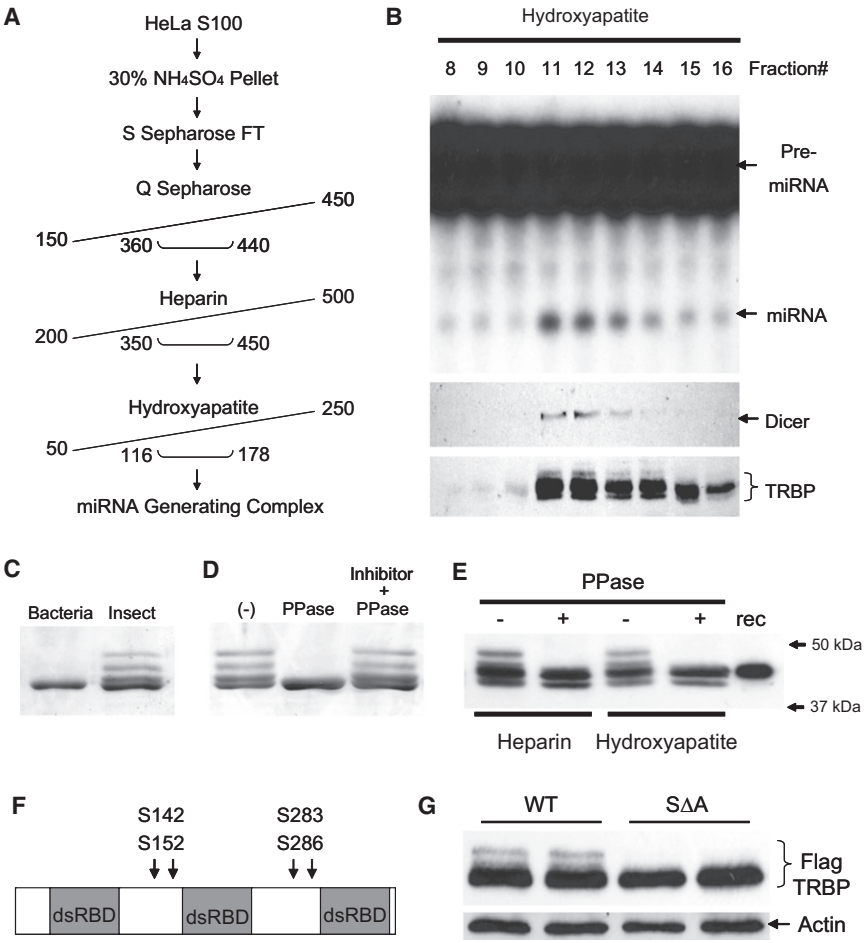

Figure 1. The Human miRNA-Generating Complex Contains Dicer and Phospho-TRBP Isoforms

(A) Purification scheme of the miRNA-generating complex from HeLa cytoplasmic (S100) extract. Numbers indicate salt (NaCl) concentration (mM).

(B) Correlation of the miRNA-generating activity peak with Dicer and TRBP immunoblots following hydroxyapatite column fractionation.

(C) Coomassie-stained polyacrylamide gel illustrating the difference in banding pattern between recombinant TRBP produced in bacteria and insect cells.

(D) Insect-cell-produced recombinant TRBP was subjected to phosphatase (PPase) treatment alone or in combination with phosphatase inhibitors.

(E) Heparin and hydroxyapatite column fractions of HeLa extract were subjected to phosphatase treatment and immunoblotting performed with anti-TRBP antibody. Nonphosphorylated recombinant (rec) TRBP was run as a marker.

(F) Schematic domain structure of TRBP with arrows indicating the positions of four phosphoserine residues identified by mass spectrometry. Shaded boxes represent annotated double-strand RNA-binding domains (dsRBD).

(G) HeLa cells were transfected with constructs encoding Flag-tagged wild-type (WT) or phospho-mutant TRBP bearing serine-to-alanine (SΔA) mutations at the four phospho-residues identified. Immunoblotting was performed with anti-Flag antibody.

miRNAs have also been found to promote oncogenesis. Elevated expression of the *miR-17-92* "oncomir" cluster was found in hematopoietic, colorectal, and lung cancers and transgenic expression of this polycistron promoted cancer cell growth (Ota et al., 2004; He et al., 2005; Hayashita et al., 2005). Through miRNA microarray profiling and a forward genetic screen miR-10b, miR-373, and miR-520c were found to initiate tumor cell invasion and metastasis (Ma et al., 2007; Huang et al., 2008). Thus, miRNAs appear to share a paradigm similar to that of protein (proto-) oncogenes and tumor suppressors in regulating developmental and cancer biology (Hanahan and Weinberg, 2000; Pardal et al., 2005).

In addition to those outlined above, there is growing understanding of the importance of miRNAs in regulating a spectrum of biological and pathophysiological processes. Among these are cardiopathology (van Rooij et al., 2006), neurodegeneration (Bilen et al., 2006), skeletal muscle hypertrophy (Clop et al., 2006), viral pathogenicity (Triboulet et al., 2007), and innate (Pedersen et al., 2007) and adaptive immunity (Koralov et al., 2008). These and numerous other studies outlining the role of miRNAs as key biological regulators raise important questions as to how the miRNA pathway is regulated. Moreover, there is an enormous void in our understanding of the relationship between the miRNA pathway and other cell signaling pathways. In the present study, we identify MAPK/Erk-mediated phosphorylation of the human miRNA-generating complex and demonstrate the importance of this regulation in effecting mitogenic signaling. This concept may be a general principle, wherein a myriad of signaling pathways target the miRNA machinery to achieve biological responses.

RESULTS

The Human miRNA-Generating Complex Consists of Dicer and Phospho-TRBP Isoforms

We used sequential chromatography to isolate the human miRNA-generating complex from HeLa cytoplasmic extract (Figure 1A). Throughout the purification process, peak miRNA-generating activity cofractionated with Dicer and TRBP (Figure 1B). Further, reconstitution studies demonstrated that both Dicer and TRBP are required for efficient interaction with pre-miRNA substrate and miRNA production (Figure S1 available online).

Purified miRNA-generating fractions immunoblotted for TRBP revealed a multiple banding pattern (Figure 1B) similar to that observed by others (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). Recombinant TRBP produced in bacteria yielded a single product of the expected size (Figure 1C). In

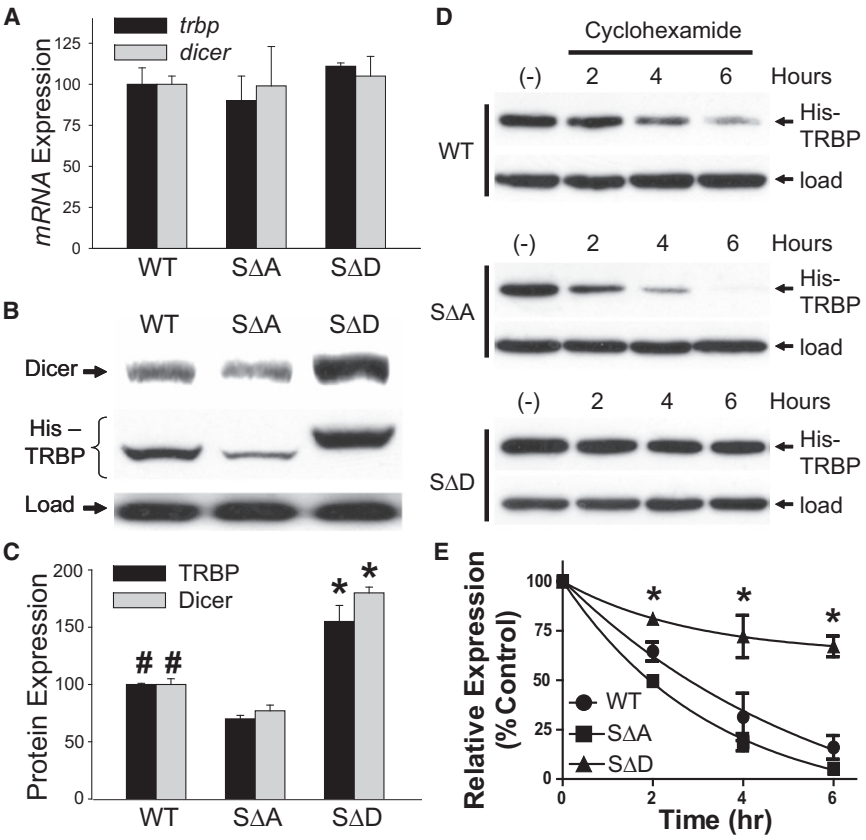

Figure 2. Expression of Phospho-Mimic TRBP Enhances Stability of the miRNA-Generating Complex

(A) Quantitative RT-PCR analysis of *trbp* (solid bars) and *dicer* (light bars) mRNA relative to *β-actin* from isogenic Flp-In 293 cells expressing wild-type (WT), phospho-mutant (SΔA) and phospho-mimic (SΔD) TRBP. Values represent means ± standard deviation (SD), n = 3.

(B) Immunoblots of Dicer and TRBP from isogenic Flp-In 293 cells expressing WT, SΔA, and SΔD TRBP. Multiple bands for WT TRBP in 293 cells are visualized with longer exposure.

(C) Quantitative analysis of TRBP (solid bars) and Dicer (light bars) expression. * indicates greater than WT and SΔA. # indicates greater than SΔA ($p < 0.05$). Values represent means ± SD, n = 3.

(D) Cells were treated with cyclohexamide for 2, 4, or 6 hr to determine stability of WT, SΔA, and SΔD TRBP.

(E) Quantitative analysis of protein decay (t1/2: SΔA (squares) 1.7 ± 0.2 hr, WT (circles) 3.0 ± 0.1 hr, and SΔD (triangles) >6 hr]. * indicates SΔD greater than WT and SΔA ($p < 0.02$). Values represent means ± standard deviation (SD), n = 3.

contrast, recombinant TRBP generated in an insect cell (eukaryotic) expression system exhibited a multiple banding pattern, suggesting the possibility of posttranslational modifications. Treatment of insect-cell-produced TRBP with phosphatase resulted in a collapse of this multiple banding pattern, whereas cotreatment with phosphatase and phosphatase inhibitors abrogated this effect (Figure 1D). Similarly, phosphatase treatment of purified HeLa fractions also resulted in a collapsed TRBP banding pattern (Figure 1E). Mass spectrometry analysis identified four phospho-serine residues (Figure 1F and Table S1). Authenticity of the identified sites was validated by the different banding profiles between wild-type (WT) and serine-to-alanine mutant TRBP expressed in human cells (Figure 1G). The number of sites mutated inversely correlated with the number of isoforms detected (not shown), indicating that all four identified sites were important in producing phospho-isoforms. Collectively, these results indicate that the human miRNA-generating complex consists of Dicer and phospho-TRBP isoforms.

TRBP Phosphorylation Stabilizes the miRNA-Generating Complex

To investigate functionality of TRBP phosphorylation, we generated isogenic cell lines expressing WT, phospho-mutant (serine-to-alanine (SΔA)), and phospho-mimic (serine-to-aspartate (SΔD)) TRBP. We employed a Flipase (Flp)/Flp recognition target (FRT) site-directed recombination system to achieve single copy integration at the same genomic locus driven by the same promoter. As expected, these isogenic cell lines expressed similar levels of *dicer* and *trbp* mRNA (Figure 2A). However, immunoblot analysis revealed higher expression of the phospho-mimic relative to WT and phospho-mutant proteins (Figures 2B and 2C). Expression of phospho-mimic TRBP also increased Dicer expression relative to that for WT and phospho-mutant TRBP (Figures 2B and 2C). These findings are consistent with previous reports of interdependent expression between RNaseIII enzymes and partner dsRBPs (Liu et al., 2003; Chendrimada et al., 2005; Haase et al., 2005; Jiang et al., 2005; Lee et al., 2006; Park et al., 2007; Han et al., 2009).

These differences in protein levels suggested that phospho-mimic TRBP may be more stable than WT and phospho-mutant TRBP. To test this hypothesis, cells were treated with the translation inhibitor cyclohexamide and protein decay was monitored. As shown in Figures 2D and 2E, phospho-mutant TRBP had the shortest half-life (1.7 ± 0.2 hr), followed by WT (3.0 ± 0.1 hr) and phospho-mimic (>6 hr; $p < 0.015$). Taken together, these findings indicate that expression of phospho-mimic TRBP enhances stability of the miRNA-generating complex.

Consistent with elevated levels of Dicer and TRBP, phospho-mimic TRBP-expressing cells demonstrated higher in vitro miRNA-generating activity compared to WT and phospho-mutant (Figure 3A). miRNA-generating complex formation assays indicated a higher capacity to interact with pre-miRNA substrate in phospho-mimic compared with WT and phospho-mutant TRBP-expressing cells (Figure S2). Recombinant WT and phospho-mimic TRBP yielded similar effects in reconstituting miRNA-generating activity in vitro (Figure S3). Thus, although phosphorylation state may alter some intrinsic property of the miRNA-generating enzyme, stabilization and elevated expression of Dicer-TRBP is a principal cause for increased miRNA

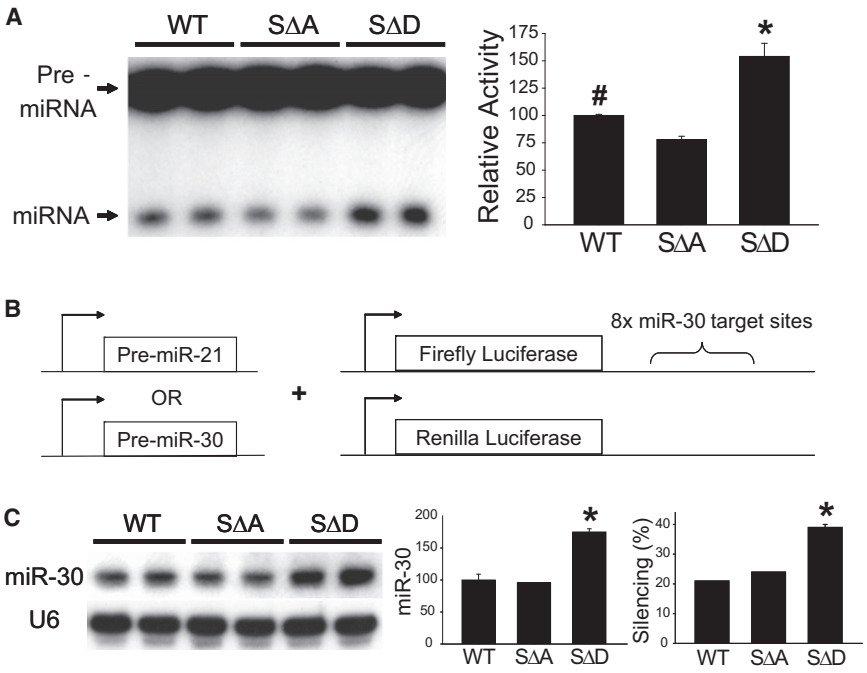

Figure 3. Expression of Phospho-Mimic TRBP Enhances miRNA Production and miRNA-Mediated Target Silencing

(A) In vitro miRNA-generating assays were performed with 15 μg of extracts prepared from isogenic Flp-In 293 cells expressing WT, SΔA, and SΔD TRBP. * indicates greater than WT and SΔA. # indicates greater than SΔA ($p < 0.04$). Values represent means ± SD, n = 3.

(B) Cells were cotransfected with plasmids expressing pre-miR-21 or pre-miR-30, *Renilla* luciferase to normalize and firefly luciferase encoding eight miR-30 target sites in the 3′UTR.

(C) miR-30 production was assessed by northern blot (left) and quantified (middle). U6 RNA was used as a loading control. miR-30-mediated silencing was measured by the ratio of firefly to *Renilla* luciferase activity and expressed as percent silencing relative to pre-miR-21 control (right). * indicates greater than WT and SΔA ($p < 0.01$). Values represent means ± SD, n = 4.

production in phospho-mimic TRBP-expressing cells. Expression of phospho-mimic TRBP also resulted in greater cellular production of miRNA relative to that for WT and phospho-mutant (Figure 3C). To determine if the observed differences in miRNA production impacted downstream miRNA function, miRNA-mediated silencing assays were conducted using a reporter construct under the control of miR-30 (Yi et al., 2003). In line with a higher level of miR-30 production, phospho-mimic TRBP-expressing cells exhibited greater miR-30-mediated silencing relative to WT and phospho-mutant (Figures 3B and 3C). These results were consistent for all miRNAs tested including miR-21, miR-133, and miR-206 (Figure S4). Collectively, these findings suggest that phosphorylation of TRBP enhances stability of the miRNA-generating complex, resulting in enhanced miRNA production and miRNA-mediated target silencing.

The miRNA-Generating Complex Is a Target of the MAPK/Erk Pathway

To identify kinases that phosphorylate TRBP, we performed computational analysis (Huang et al., 2005) of phospho-TRBP peptides (Table S1). This analysis indicated potential MAPK substrate sequences. Coimmunoprecipitation studies using cells stably expressing Flag-TRBP demonstrated cellular interaction between TRBP and phospho(p)-Erk1/2 (Figure 4A). Next, we developed an in vitro TRBP phosphorylation assay using recombinant TRBP generated in *E. coli* as substrate and HeLa cell extract as source material for kinase activity. Peak TRBP phosphorylation activity corresponded with pErk2 following Superdex 200 column fractionation (Figure 4B).

Activation of MAPKs such as Erk requires phosphorylation by an upstream kinase known as a MAPK kinase (MKK). Constitutively active mutant MKK1 (MKK1*; ΔN3 [deleted residues 32–51], S218D, S222D] is commonly used to specifically activate Erk1/2 (Mansour et al., 1994; Khokhlatchev et al., 1997). Recombinant MKK1* and Erk2 were both required to reconstitute in vitro TRBP phosphorylation (Figure 4C).

To determine the role of MKK1/Erk in modifying cellular TRBP, we treated cells stably expressing Flag-TRBP with the mitogen and tumor promoter phorbol 12-myristate 13-acetate (PMA). PMA-induced activation of pErk1/2 resulted in phosphorylation and accumulation of TRBP (Figure 4D). Pretreatment of cells with the MKK1 inhibitor U0126 attenuated PMA-induced phosphorylation of TRBP, whereas pretreatment with the MKK3/p38 inhibitor SB203580 did not. Moreover, cellular expression of MKK1* resulted in phosphorylation and accumulation of TRBP (Figure 4E). Collectively, these studies indicate that TRBP is a target of the MKK1/Erk pathway.

Concomitant with MKK1*-induced phosphorylation of TRBP was an accumulation of TRBP and Dicer proteins (Figures 5A and 5C, top). Such changes occurred without any increase in *trbp* or *dicer* transcript levels (not shown). These findings parallel those of the aforementioned studies involving expression of phospho-mimic TRBP (Figure 2) and indicate that phosphorylation of TRBP stabilizes the miRNA-generating complex.

Functionally, MKK1*-induced phosphorylation of TRBP resulted in elevated production of miRNA and enhanced miRNA-mediated silencing (Figures 5B and 5C). To determine if the MKK1*-mediated enhancement of miRNA pathway activity was dependent on phosphorylation of TRBP, parallel studies were performed using cells stably expressing phospho-mutant TRBP. Despite activation of pErk1/2, expression of MKK1* in these cells did not result in changes in TRBP or Dicer expression, miRNA production, or miRNA-mediated silencing (Figures 5A–5C). The basis for dominance of transgenic TRBP is depicted in Figure S5. Column fractionation of cell extracts derived from cells stably expressing transgenic TRBP revealed that

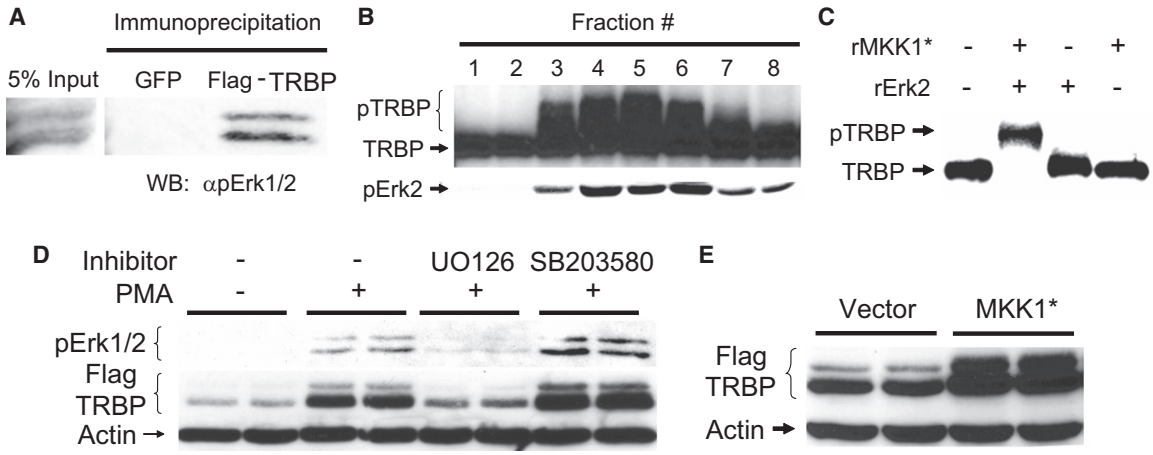

Figure 4. TRBP Is Phosphorylated by MAPK/Erk

(A) HeLa cell extracts from cells stably expressing Flag-TRBP were immunoprecipitated using anti-GFP or anti-Flag antibodies. Western blots (WB) were performed using anti-phospho(p)-Erk1/2 antibody.

(B) Correlation of in vitro TRBP phosphorylation activity and pErk2 following Superdex 200 fractionation of HeLa cell extract.

(C) In vitro reconstitution of TRBP phosphorylation activity using constitutively active recombinant MKK1 (rMKK1*) and recombinant Erk2 (rErk2).

(D) HeLa cells stably expressing Flag-TRBP were serum-starved for 24 hr, treated with DMSO, U0126, or SB203580 for 1 hr, followed by PMA treatment for 24 hr.

(E) HeLa cells stably expressing Flag-TRBP were transfected with an empty vector or one encoding MKK1*.

miRNA-generating activity exhibited perfect correlation with Dicer and transgenic TRBP. Thus, in these cells, the miRNA-generating enzyme is comprised mostly of Dicer and transgenic TRBP. In this way, phosphorylation of background WT TRBP in phospho-mutant TRBP-expressing cells would not be expected to influence miRNA production. Taken together, these findings demonstrate that the MKK1/Erk pathway enhances the capacity of the miRNA pathway via phosphorylation of TRBP.

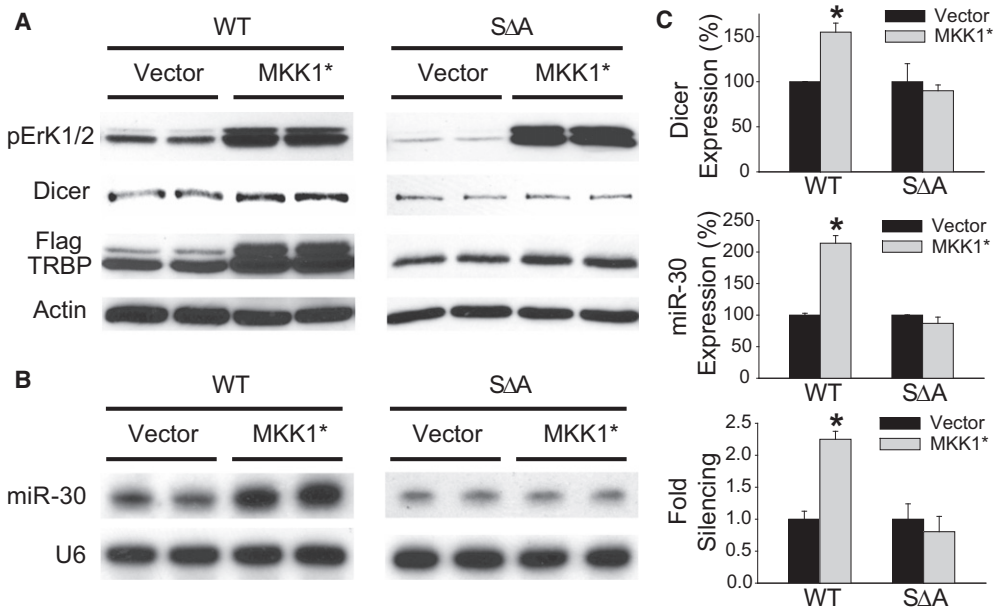

Figure 5. Activation of MKK1/Erk Enhances miRNA Pathway Activity in a Phospho-TRBP-Dependent Manner

(A) HeLa cells stably expressing WT or SΔA TRBP were transfected with an empty vector or one encoding MKK1*. Immunoblots were performed for pErk1/2, Dicer, TRBP, and actin.

(B) miR-30 northern blots were performed to compare the level of miR-30 production following cotransfection of either empty vector or MKK1* and pre-miR-30 plasmid. U6 RNA was used as a loading control.

(C) Quantitative representation of Dicer expression (top), miR-30 expression (middle), and miR-30-mediated silencing (bottom) as described in Figure 3B. * indicates MKK1* (light bars) greater than empty vector (black bars; $p < 0.02$). Values represent means ± SD, n = 4.

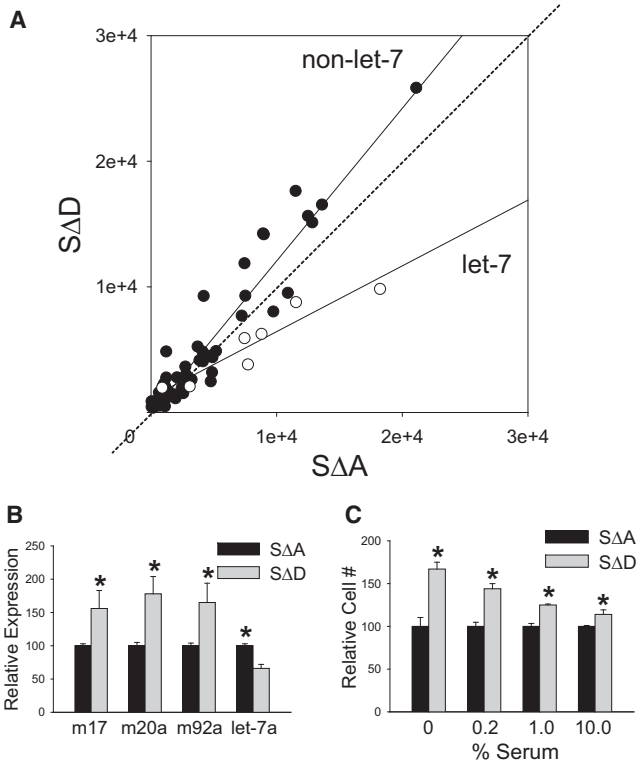

Figure 6. Cells Expressing Phospho-Mimic TRBP Exhibit a Pro-Growth miRNA Expression Profile and Higher Levels of Cellular Growth and Serum-Restricted Survival

(A) Regression analysis of miRNA microarray studies from Flp-In 293 cells expressing SΔA and SΔD TRBP. The dotted line represents equivalent miRNA levels between cell lines. Data points above the dotted line (64.6% of data points) indicate higher miRNA expression in SΔD versus SΔA TRBP-expressing cells. Data points below the dotted line (35.4%) indicate lower expression in SΔD compared with SΔA TRBP-expressing cells. The equation of the non-let-7 (closed circles) regression line is $y = 1.22x - 93.5$ with a correlation coefficient of 0.96 ($p < 0.0001$). The let-7 family of miRNA (let-7a, b, c, d, e, f, g; open circles) demonstrated the opposite pattern of expression. The equation of the let-7 regression line is $y = 0.52x + 1202.2$ with a correlation coefficient of 0.95 ($p < 0.002$). Data are means of three independent analyses for each cell line. For simplicity, error bars are not shown (but see Table S2).

(B) miRNA quantitative PCR was performed to assess expression of miR-17, miR-20a, miR-92a, and let-7a. * indicates differences in expression between HeLa cells stably expressing SΔA (black bars) versus SΔD TRBP (light bars; $p < 0.03$; $n = 3$). Values represent means ± SD.

(C) HeLa cells were incubated in serum-free media overnight and cultured in media containing 0%, 0.2%, 1.0%, and 10% serum for 48 hr. Cell counts at 48 hr were normalized to those at 0 hr. * indicates cells stably expressing SΔD TRBP (light bars) that demonstrated greater cell populations relative to those expressing SΔA TRBP (black bars; $p < 0.02$; $n = 3$). Values represent means ± SD.

The miRNA-Generating Complex Is an Effector of the MAPK/Erk Pathway

Having established regulation of miRNA expression via MAPK/Erk-mediated phosphorylation of TRBP, we wanted to determine the effect of TRBP modification on global miRNA expression. We conducted miRNA microarray studies of phospho-mutant and phospho-mimic TRBP-expressing cells. Consistent with the findings from the above-mentioned experiments employing expressed miRNA, phospho-mimic TRBP-expressing cells exhibited higher miRNA levels relative to phospho-mutant (Figure 6A). Given the importance of the MAPK/Erk pathway in mediating cell growth and proliferation, we were particularly interested in miRNAs that have been demonstrated to function in these processes. Among the miRNAs that were upregulated in phospho-mimic TRBP-expressing cells were growth-promoting miR-17, miR-20a, and miR-92a. Interestingly, there was one very notable exception to this pattern. Levels of the let-7 tumor suppressor miRNA family were lower in phospho-mimic compared to phospho-mutant TRBP-expressing cells (Figure 6A). These results suggested a mitogenic miRNA profile including a coordinated upregulation of pro-growth miRNAs and downregulation of anti-growth miRNAs in response to phosphorylation of TRBP.

To validate these microarray data and to track miRNA changes in subsequent experiments, we used miRNA northern blot hybridization and miRNA quantitative RT-PCR to examine expression of growth-promoting miR-17, miR-20a, and miR-92a and growth-suppressing let-7a. These studies confirmed that expression of phospho-mimic TRBP resulted in higher levels of pro-growth miR-17, miR-20a, and miR-92a and lower levels of let-7a tumor suppressor miRNA relative to phospho-mutant (Figures 6B and S6A). Quantitative RT-PCR for pri-let-7a (Figure S7) and miRNA northern blot analyses (Figure S6A) indicated that these changes were mediated posttranscriptionally. The let-7 miRNA family continues to demonstrate unique characteristics. For example, the stem cell marker Lin28 specifically regulates let-7 biogenesis (Heo et al., 2008; Newman et al., 2008; Viswanathan et al., 2008) and distinct let-7 ribonucleoprotein complexes have been identified in *C. elegans* (Chan et al., 2008). Thus, TRBP phosphorylation may influence Lin28 activity or let-7 RISC turnover or may alter the interface between pre-let-7 and the miRNA-generating enzyme. Although the underlying mechanisms for differential regulation of let-7 in response to expression of phospho-mimic TRBP are not known, there is clear logic in coordinating levels of pro- and anti-growth miRNA.

Given the growth promoting miRNA profile of phospho-mimic TRBP expressing cells, we wanted to determine whether these cells exhibited any differences in growth characteristics. Indeed, phospho-mimic TRBP expressing cells exhibited higher levels of expansion relative to phospho-mutant (Figure 6C, 1% and 10% serum). Under serum-restricted conditions, phospho-mimic TRBP-expressing cells also demonstrated enhanced cell viability relative to phospho-mutant (Figure 6C, 0% and 0.2% serum). These growth and survival differences were observed for all media conditions tested. However, the magnitude of this disparity was inversely related to serum concentration. We reasoned that in the presence of high levels of serum, miRNA expression represents one of a myriad of mitogenic signals. As serum levels are progressively restricted, these other inputs are attenuated and the role of the miRNA machinery in regulating cell signaling becomes more pronounced. These findings suggest that phosphorylation of TRBP is important in mediating the hallmarks of MAPK/Erk signaling including proliferation and cell survival (Anjum and Blenis, 2008).

As a key stimulus for MAPK/Erk, we wanted to determine if phosphorylation state of TRBP could be perturbed through

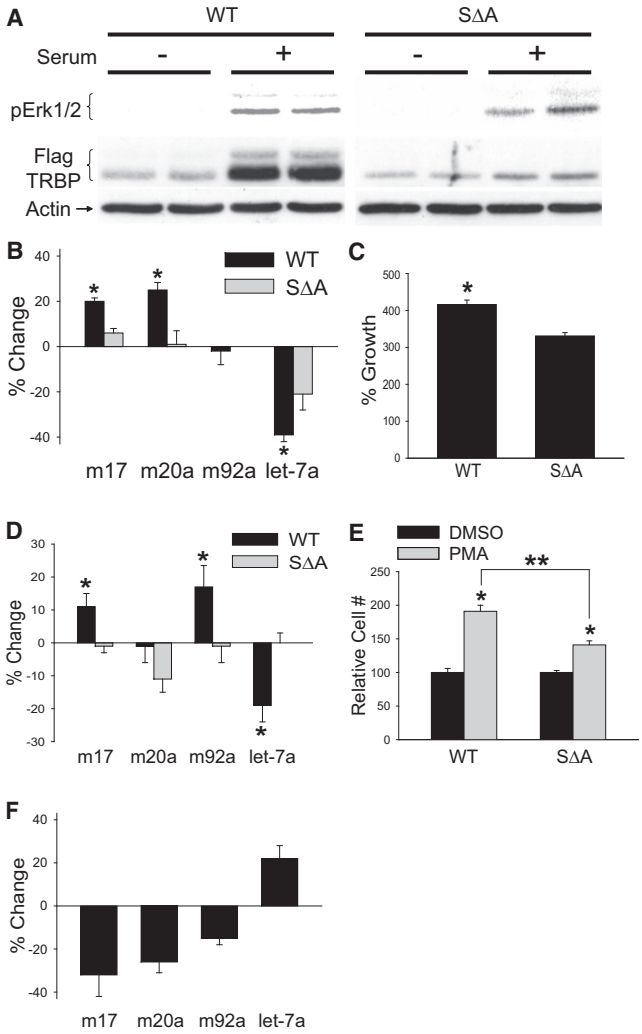

Figure 7. Phosphorylation of TRBP Mediates MAPK/Erk Signaling

(A) Immunoblots from cells stably expressing WT and SΔA TRBP incubated in media containing 0% or 10% serum.

(B) Serum-induced changes in miRNA expression in WT (black bars) and SΔA (light bars) TRBP-expressing cells. Cells were washed with PBS twice and incubated in 0% or 10% serum for 48 hr. Data are presented as percent change relative to serum-free levels where * indicates WT changes greater than those for SΔA ($p < 0.01$; $n = 4$). Values represent means ± SD.

(C) Cells expressing WT and SΔA TRBP were washed with PBS twice, incubated in serum-free media overnight, and plated in media containing 10% serum for 48 hr. Cell growth is expressed relative to cell count at 0 hr. * indicates $p < 0.001$ ($n = 3$). Values represent means ± SD.

(D) PMA-induced changes in miRNA expression in cells stably expressing WT (black bars) and SΔA (light bars) TRBP. Data are percent change relative to DMSO treatment where * indicates $p < 0.05$ ($n = 4$). Values represent means ± SD.

(E) Cells stably expressing WT or SΔA TRBP were cultured in serum-free media overnight and seeded in serum-free media containing DMSO or PMA, and cell populations were counted after 24 hr. * indicates that PMA treatment (light bars) resulted in greater cell viability relative to DMSO (black bars; $p < 0.001$). ** indicates that PMA treatment yielded greater cell counts for WT versus SΔA TRBP-expressing cells ($p < 0.01$; $n = 3$). Values represent means ± SD.

(F) HeLa cells were treated with DMSO or U0126 for 48 hr. Data are expressed as percent change relative to DMSO ($n = 4$). All data shown are at the level of $p < 0.05$. Values represent means ± SD.

serum. Consistent with the effects of MKK1* expression, activation of pErk1/2 with serum stimulation resulted in phosphorylation and accumulation of TRBP (Figure 7A). In line with the effects of phospho-mimic TRBP expression, serum-induced TRBP phosphorylation increased expression of pro-growth miRNA and decreased expression of let-7a growth suppressor miRNA (Figure 7B). These serum-induced changes were largely attenuated in phospho-mutant TRBP-expressing cells. To determine if serum-induced mitogenic signaling was dependent on phosphorylation of TRBP, WT and phospho-mutant TRBP-expressing cells were cultured in serum-free media overnight followed by serum exposure for 48 hr. Cells expressing phospho-mutant TRBP demonstrated a ~25% growth disadvantage relative to WT (Figure 7C). These findings indicate that serum-induced mitogenic signaling is, at least in part, phospho-TRBP dependent.

We performed similar studies using another mitogen. PMA-induced phosphorylation of TRBP (Figure 4D) resulted in a pro-growth miRNA profile including an upregulation of growth-promoting miRNA and a decrease in let-7a growth-suppressor miRNA in WT but not phospho-mutant TRBP-expressing cells (Figure 7D). PMA treatment enhanced cell survival during serum starvation in both WT and phospho-mutant TRBP-expressing cells (Figure 7E). However, the magnitude of this effect was greater for WT than for phospho-mutant TRBP-expressing cells. Similar to findings with serum stimulation, these findings indicate that the mitogenic effects of PMA are partially mediated through phosphorylation of TRBP. Taken together, these results indicate that phosphorylation of TRBP is important in effecting cellular proliferation and serum-restricted survival.

As expression of phospho-mimic TRBP, serum stimulation, and PMA treatment resulted in a coordinated pro-growth miRNA profile, we sought to determine if pharmacological inhibition of the MAPK/Erk pathway would yield a reciprocal anti-growth miRNA response. Indeed, treatment of cells with the MKK1/2 inhibitor U0126 resulted in downregulation of pro-growth miR-17, miR-20a, and miR-92a and an increase in let-7a miRNA tumor suppressor expression (Figures 7F and S6B). As MKK1 inhibitors are currently being assessed in clinical oncology trials, we tested six cancer cell lines to determine the generality of this pharmacological response. Human cervical (HeLa), gastric (KatoIII), and lung carcinoma (A549) cells exhibited this coordinated anti-growth miRNA expression pattern, and mammary adenocarcinoma (MDA MB231) and glioblastoma (T97G) cells produced a decrease in growth-promoting miRNA following U0126 treatment (Figure S8). Of the six cancer cell lines evaluated, only one osteosarcoma cell line (U2OS) did not demonstrate changes in the miRNAs examined. These findings indicate a concerted miRNA regulatory program capable of responding positively in response to mitogenic signals and negatively following inhibition of these signals. Collectively, these data indicate that the MAPK/Erk pathway targets the human miRNA-generating complex to effect cell signaling.

DISCUSSION

Our understanding of the importance of miRNA in regulating development, homeostasis, and pathophysiology continues to

expand. Therefore, elucidating the mechanisms by which the miRNA pathway is governed represents a critical area of investigation. A key component of these endeavors is understanding the inter-relationship between the miRNA machinery and other cellular systems. The present study indicates that the miRNA-generating complex is regulated by MAPK/Erk and that this regulation is important in effecting mitogenic signaling. To our knowledge, the current work provides the first demonstration of a direct connection between a cell signaling pathway and the core miRNA machinery and suggests that other cellular networks also target the miRNA pathway to carry out functional cellular responses.

The principle of miRNA pathway regulation is in its infancy. Clearly, transcriptional control is important. Two protein factors have been shown to modulate expression of specific miRNAs through posttranscriptional mechanisms. Lin28 has been shown to modulate pri-let-7 and pre-let-7 processing (Heo et al., 2008; Newman et al., 2008; Viswanathan et al., 2008). Smad transcriptional transducers have been shown to facilitate pri-miR-21 processing in mediating transforming growth factor β stimulation (Davis et al., 2008). Prolyl hydroxylation has been shown to govern stability of Argonaute2 and siRNA-mediated silencing (Qi et al., 2008). An autoregulatory feedback loop regulating expression of the microprocessing complex has been outlined (Han et al., 2009). Dead End 1 has been demonstrated to modulate interactions between the miRNA silencing machinery and target mRNA (Kedde et al., 2007). The present work introduces new principles in understanding miRNA regulatory mechanisms. Genetic studies of the miRNA machinery and, later, of specific miRNAs have been instrumental in elucidating the importance of miRNA in biology. Here, we utilize the study of posttranslational modification to identify upstream signaling-mediated regulation of the miRNA machinery and demonstrate that this governance is important in effecting downstream cellular signaling. It is likely that other cellular systems similarly target the miRNA pathway in order to achieve biological responses.

The major function of the MAPK/Erk pathway is to appropriately respond to cellular signals with respect to cellular growth, survival, proliferation, and differentiation (Roux and Blenis, 2004). Although these processes are tightly connected, specific events for each have been identified (Bonni et al., 1999; Chen et al., 2008). Effectors of the MAPK/Erk pathway include mediators of the transcriptional and translational apparatus (Anjum and Blenis, 2008). That serum stimulation resulted in reduced cellular expansion in phospho-mutant TRBP-expressing cells relative to WT indicates that cellular proliferation is mediated, in part, through phosphorylation of the miRNA-generating complex. Further, that PMA preferentially enhanced serum-restricted cell viability in WT relative to phospho-mutant TRBP-expressing cells demonstrates that phosphorylation of the miRNA-generating complex is also important for cell survival signaling. Thus, in addition to regulating transcription and translation, MAPK/Erk also acts on the miRNA-generating complex to effect mitogenic signaling. Interestingly, a recent report identified a transcriptional connection between the Raf/MAPK/Erk pathway and miRNA expression (Dangi-Garimella et al., 2009). The *lin28* locus was identified as a transcriptional target of c-Myc providing a means through which Raf signaling can modulate expression of let-7. This study and the current work suggest that the Raf/MAPK/Erk cascade may regulate miRNA expression through parallel mechanisms.

The loss of balance between activities of oncogenes and tumor suppressors is a key occurrence in neoplastic progression (Hanahan and Weinberg, 2000; Pardal et al., 2005). Expression of phospho-mimic TRBP and mitogenic stimuli produced a concerted upregulation of miRNAs that have previously been demonstrated to promote tumor progression and downregulation of growth-retarding miRNA. A reciprocal anti-growth miRNA profile was observed in response to pharmacological inhibition of MAPK/Erk. These findings suggest a bidirectional miRNA-generating logic that corresponds with and/or directs cellular behavior. Therefore, a potential mechanism of action of MAPK inhibitors, including those in clinical development, may be through targeting of the miRNA machinery. Moreover, there is widespread interest in the development of miRNA-based therapeutics. Numerous groups have reported the potential of miRNA mimics and anti-miRNA oligonucleotides to drive desired outcomes in preclinical studies. Though advances in oligonucleotide drug development have been made, significant challenges remain. That a small molecule compound produced targeted and coordinated reprogramming of miRNA expression indicates that miRNA-based therapeutic interventions need not rely solely on oligonucleotide drug development. Purposeful miRNA changes may be achievable through traditional pharmacological approaches.

Elucidating the signaling systems, including identification of modifications, modified targets, and modifiers, represents an important direction in understanding regulation of the miRNA pathway. Moreover, determining the requirement of these post-translational controls in producing functional cellular responses will advance understanding of these signaling systems. Just as other genomic regulatory mechanisms such as transcription, splicing, pre-mRNA processing, and export can be modulated through signaling-induced modifications, the micro-RNA apparatus appears to demonstrate similar tunability. The current work establishes a direct connection between a cell signaling pathway and the core miRNA machinery. These studies indicate that the MAPK/Erk pathway regulates the miRNA-generating complex and that this regulation is important in effecting mitogenic signaling.

EXPERIMENTAL PROCEDURES

General Procedures

Flag, His, and pErk antibodies were purchased from Sigma. Actin and Dicer antibodies were obtained from Abcam. TRBP antiserum was raised against purified full-length recombinant TRBP. Phosphatase was obtained from NEB. Phosphatase inhibitor treatment included 10 mM sodium fluoride (Sigma), 4 mM sodium orthovanadate (Sigma), and 4 mM β-glycerophosphate (Calbiochem). Serine-to-alanine (SΔA) phospho-mutant and serine-to-aspartate (SΔD) phospho-mimic TRBP were constructed by the "QuikChange" system (Stratagene). Immunoprecipitation was performed using Protein A agarose from Santa Cruz Biotechnology.

Cell Culture Procedures

Transfections were performed using Lipofectamine 2000 (Invitrogen). Cyclohexamide (Sigma) treatment was performed using 100 μg/ml. U0126 (Promega) was used at a concentration of 20 μM and PMA (Sigma) was used at

100 ng/ml. Unless otherwise stated, cell treatments were administered for a duration of 48 hr with a media change, including fresh dosage of compounds, after 24 hr. Cell populations were assayed using a Cell Titer Glo luminescent cell viability assay (Promega). Typically, cells were cultured in serum-free media overnight and plated the following day at a density of 1000 cells/96-well in experimental media. Cell counts were taken at 48 hr and normalized to those obtained at 0 hr.

Two sets of stable cell lines were employed in the current work. Isogenic cell lines expressing WT, phospho-mutant, and phospho-mimic His-TRBP were produced using a Flipase (Flp)/Flp recognition target site-directed recombination system (Invitrogen). Briefly, Flp-In 293 cells (Invitrogen) were cotransfected with pcDNA5/FRT containing TRBP cDNA and Flp recombinase. Stable clones were selected using 200 μg/ml hygromycin. HeLa cells stably expressing $(Flag)_3$-TRBP were generated from a modified pCI-neo vector encoding WT, phospho-mutant, or phospho-mimic TRBP. Clones were selected using 500 μg/ml G418.

mRNA and miRNA Analysis

Northern blots were performed as previously reported (Park et al., 2007). Probe sequences were as follows: miR-30—gcugcaaacauccgacugaaag; U6—gcaggggccatgctaatcttctctgtatcg. mRNA and miRNA RT-quantitative PCR studies were carried out using Taqman assay systems (Applied Biosystems) where mRNA expression was normalized to *β-actin* and miRNA expression was normalized to RNU44. miRNA microarray analysis was conducted by LC Sciences. The platform database used was Sanger miR Base 10.0. Least-squares linear regression analysis was performed with Sigma Plot 10.0. p values were obtained with the corresponding F statistic derived from the analysis of variance for each regression.

miRNA-Mediated Silencing

Cells were transfected with constructs encoding pre-miR-21 (control) or pre-miR-30, firefly luciferase under the control of eight miR-30 target sites encoded in the 3′UTR and *Renilla* luciferase to normalize results (Yi et al., 2003). Reporter activity was assayed using a Dual Luciferase Reporter System (Promega). Pre-miR-30-mediated silencing was determined by the ratio of firefly to *Renilla* luciferase activity and expressed as percent silencing relative to pre-miR-21 control.

Statistical Analysis

Experiments were run in duplicate or triplicate and repeated in a minimum of three independent trials. Image quantitation was performed using Scion Image analysis software (NIH). Data are represented as means ± standard deviation (SD). Two-tailed t tests were employed where the minimum level of significance was $p < 0.05$.

ACCESSION NUMBERS

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE16442.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two tables, and eight figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00791-0.

ACKNOWLEDGMENTS

We thank Drs. Dangsheng Li, Yi Liu, Eric Olson, Michael White, and Hongtao Yu for critical reading of the manuscript. We thank Fenghe Du and Drs. Yue Chen, Melanie Cobb, Bryan Cullen, Witold Filipowicz, Eric Olson, Patrick Provost, Ramin Shiekhattar, Bing Su, and Yingming Zhao for providing reagents and assistance in the development of this work. We thank Phi Luong, Andrew Williams, and Drs. Gaya Amarasinghe, Alex Pertsemlidis, Joan Reisch, and Michael White for insightful discussion. This work was conducted in a facility renovated with support from the National Center for Research Resources, National Institutes of Health (C06-RR15437-01) and was supported by the Welch Foundation (I-1608) and National Institutes of Health grants (GM078163 and GM084010) awarded to Q.L.

Received: February 11, 2009
Revised: April 8, 2009
Accepted: June 22, 2009
Published: October 1, 2009

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