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Regulation of microRNA activity by translation initiation factors in melanoma

A dissertation presented

by

Adrienne Gail Yanez

to

The Division of Medical Sciences

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in the subject of

Cell and Developmental Biology

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Regulation of microRNA activity by translation initiation factors in melanoma

Abstract

microRNAs (miRNAs) are small, noncoding RNAs that may regulate more than half of human genes, yet the molecular mechanism of miRNA-mediated repression remains obscure. Using a cell-free assay of miRNA activity, we show that miRNA-targeted mRNAs are enriched for components of the 40S, but not 60S ribosomal subunit. Additionally, a molecular toeprint of 18 nucleotides 3′ relative to the start codon, consistent with nucleotide protection by 40S ribosomal subunits, is enriched on miRNA-targeted mRNAs. Our results suggest that miRNAs repress translation initiation in a cell-free system by preventing 60S ribosomal subunit joining to 40S subunits positioned at the start codon.

The effect of miRNAs on translation initiation led us to investigate the influence of initiation factors on miRNA function in cells. Misregulation of protein translation is a common feature of many cancers. eIF4A and eIF4E-driven oncogenesis is thought to occur through differential regulation of mRNA 5′ untranslated regions (UTRs) based on sequence or secondary structural determinants. Here we show that modulation of eIF4A1 and eIF4E expression positively regulates melanoma cell proliferation, invasion, and miRNA activity. Transcriptome-wide measurements of mRNA abundance and

translation efficiency identified over 1400 common mRNAs with significantly increased translation efficiency in both eIF4A1 and eIF4E knockdown. Endogenous miRNA target sites are enriched within this mRNA subset, including putative melanoma tumor suppressors. Moreover, there is substantial overlap in transcripts with significantly increased translation efficiency in eIF4A1, eIF4E and Ago2 knockdown. Our data establish eIF4A1 and eIF4E as positive regulators of endogenous miRNA-activity.

These findings reveal that miRNAs repress translation initiation in a cell-free system by inhibiting 60S ribosome subunit joining. Conversely, translation initiation factors regulate miRNA activity in multiple cancer cell lines. Our results show that eIF4A1 and eIF4E regulate mRNA translation efficiency through both the 5′ and 3′ UTRs, and that misregulation of miRNA targets is a novel mechanism of eIF4A1 and eIF4E-dependent melanoma phenotypes.

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List of Figures

1. Introduction

1.1. Introduction to microRNAs

In 1993, the Ruvkun and Ambros labs described a *Caenorhabditis elegans* small-temporal RNA (stRNA), lin-4, and its novel mode of action; post-transcriptional repression of target gene lin-14 through partial sequence complementarity to elements in the lin-14 mRNA 3' UTR (Lee et al., 1993; Wightman et al., 1993). Five years later, Fire and colleagues refined the model of antisense gene regulation with a report that double-stranded RNA causes greater 'interference' with endogenous gene function than single stranded RNA (Fire et al., 1998). The subsequent identification of let-7, another small, noncoding RNA, in a wide range of animal species revealed the ubiquity and high degree of evolutionary conservation of this large class of regulatory RNAs, now called microRNAs (miRNAs) (Pasquinelli et al., 2000). It is now well-documented that miRNA-mediated regulation of gene expression is essential to the normal development and physiology of all plants and metazoans.

miRNAs are 19-24 nucleotide regulatory RNAs that bind to complementary sites within a target mRNA to reduce transcript stability and translation. Collectively, miRNAs fine tune gene expression and contribute to the robustness of gene expression programs essential for cellular function (Ebert and Sharp, 2012). The average decrease in target protein output per miRNA binding site is <2-fold, but miRNA networks display

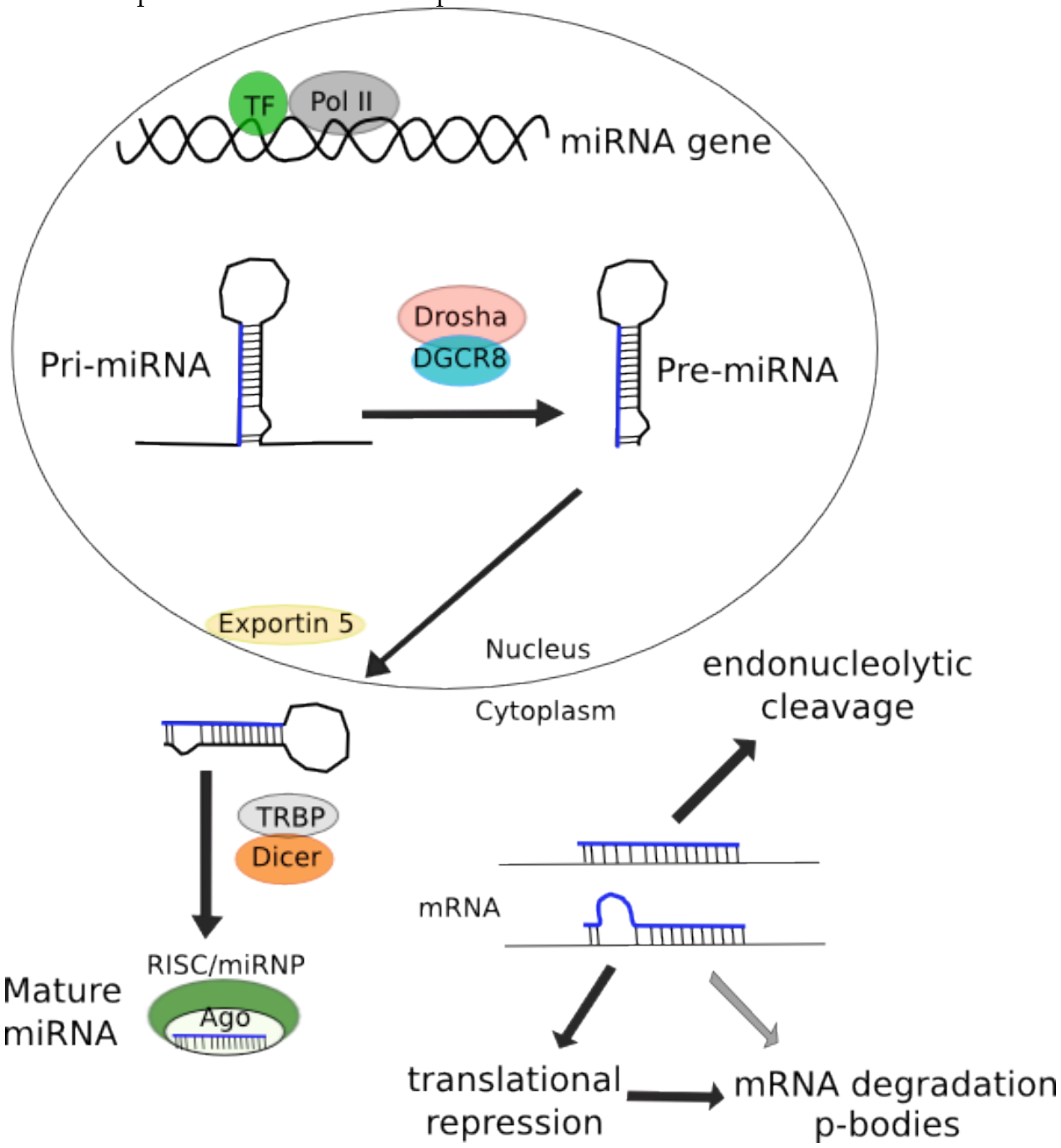
combinatorial regulation, meaning repression of mRNAs that are targeted by multiple miRNAs is enhanced (Baek et al., 2008; Ebert and Sharp, 2012). There are currently ~1900 annotated human miRNAs, which are predicted to target up to 60% of human genes (miRbase v20, (Friedman et al., 2008; Griffiths-Jones and Grocock, 2006; Griffiths-Jones et al., 2008; Jones, 2004; Kozomara and Griffiths-Jones, 2011; 2014; Lewis et al., 2005)

Most miRNAs are derived from stem loops within long, non-coding RNA polymerase II transcripts, called primary miRNA (pri-miRNA) transcripts, while a subset of miRNAs are processed from the introns of host protein coding genes (Fig. 1.1) (Borchert et al., 2006). In the cell nucleus, the canonical biogenesis pathway is initiated by an RNase III enzyme, Drosha, with binding partner, DGCR8 (Gregory et al., 2004; Lee et al., 2003). Drosha rapidly cleaves the stem loop base from pri-miRNA transcripts, creating ~70 nucleotide precursor miRNAs (pre-miRNAs). A small subset of miRNAs, called miRtrons, bypass Drosha processing and are instead derived from very small (~70 nucleotide) introns and are generated solely by spliceosome activity (Ruby et al., 2007). Pre-miRNAs are exported to the cytoplasm by Exportin 5, where they are cleaved by an RNase III enzyme, Dicer, into a mature miRNA duplex.

Mature miRNAs are loaded into Argonaute (Ago) proteins (Yi, 2003). Ago proteins are the evolutionarily conserved core components of the miRNA-induced

silencing complex (miRISC), or miRNP. There are four ubiquitously-expressed mammalian Argonaute proteins, Ago1-4, whose distinct functions remain unclear. Ago3 and Ago4 are consistently expressed at lower levels than Ago1 and Ago2 across various tissues (Valdmanis et al., 2012). Ago1-4 seem to be capable of miRNA-mediated translational repression, while only Ago2 has a functional RNase H domain that is capable of endonucleolytic cleavage of a target RNA with perfect sequence complimentarity to a loaded siRNA or miRNA (Fig. 1.1) (Pfaff and Meister, 2013). miRNAs that bind with imperfect complementarity to target mRNAs lead cause translational repression and mRNA degradation in P-bodies (Fig. 1.1).

Figure 1.1. miRNA biogenesis and function.
Adapted from Gurtan & Sharp 2013. See text for details.



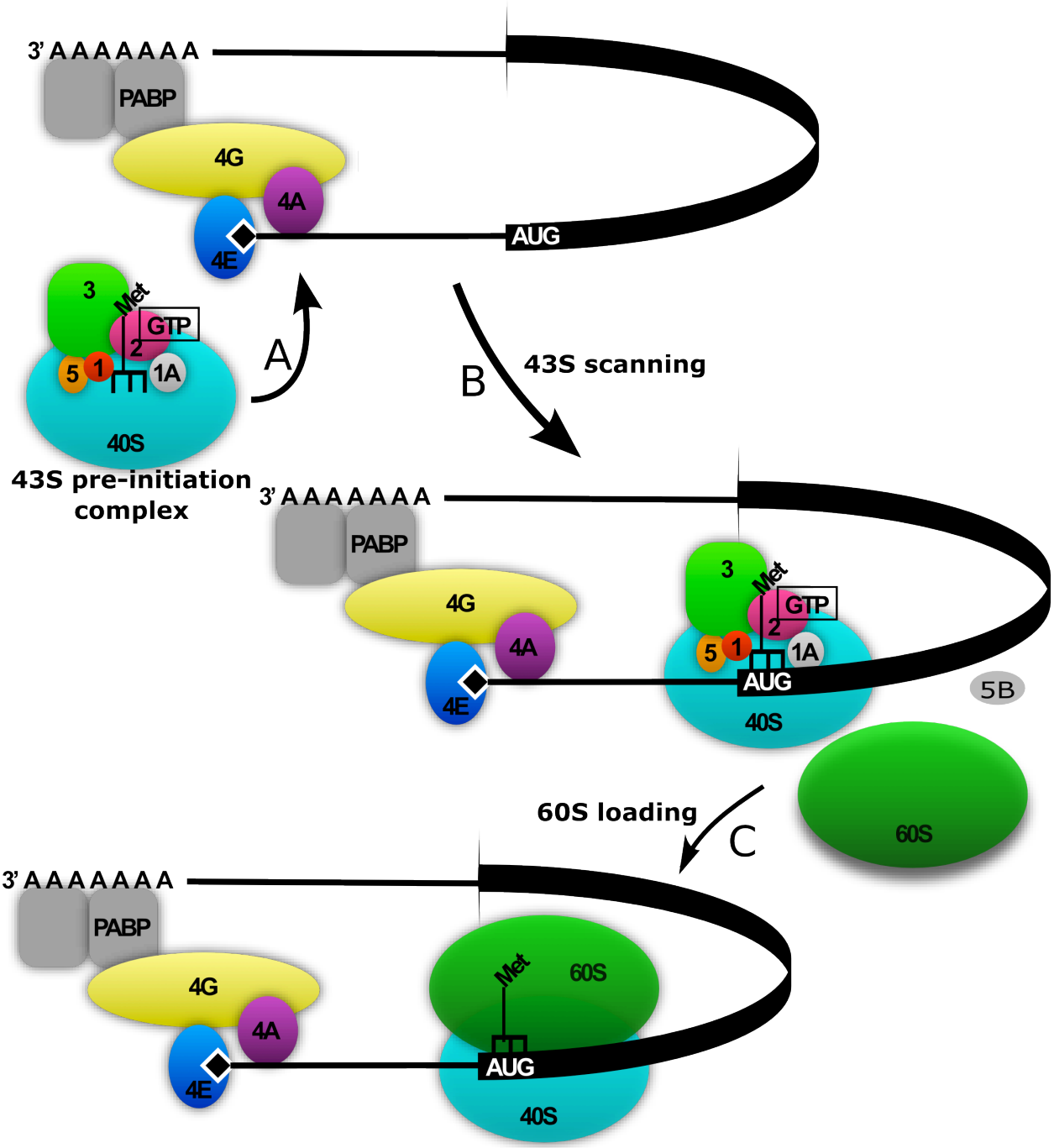
1.2. An overview of translation initiation

Three ribonucleoprotein (RNP) complexes must converge for the complicated macromolecular ballet that is translation initiation (Fig. 1.2, reviewed in (Hershey et al., 2012; Hinnebusch and Lorsch, 2012; Pestova et al., 2007)). First, the messenger RNP (mRNP); a 5′ 7-methyl guanosine capped (m^7GpppN) and polyadenylated mRNA in a closed, circular conformation due to connections between cap-bound eIF4F (comprised of eIF4E, eIF4G and eIF4A and associated with eIF4B) and poly-A tail-bound PABPC1. Second, the 43S preinitiation complex; composed of the ternary complex (initiator methionyl tRNA and eIF2 bound to GTP) bound to the 40S ribosomal subunit and eIF1, eIF1A, eIF5, and eIF3. Third, the 60S ribosomal subunit and unassociated eIF5B bound to GTP.

The 43S preinitiation complex is loaded onto the mRNP through an eIF3-eIF4G interaction. It scans the 5′ UTR with assistance from eIF4A, an ATP-dependent RNA helicase, and pauses when the methionyl-tRNA anticodon pairs with the mRNA start codon, AUG. eIF2-bound GTP is hydrolyzed with eIF5 (a GTP-ase activating protein), triggering the release of pre-initiation complex-associated factors. eIF5B-driven GTP hydrolysis facilitates 60S subunit joining and the initiation complex is formed and ready for elongation.

Figure 1.2. An overview of eukaryotic translation initiation.
(A) The 43S pre-initiation complex (comprised of the 40S ribosomal subunit, eIF3, eIF5, eIF1, eIF1A, [eIF2+GTP+tRNAi-Met]) is recruited to a 5′ 7-methyl guanosine-capped and polyadenylated messenger mRNA, creating a 48S complex.
(B) The 43S complex scans the 5′ UTR and pauses at the AUG codon.
(C) eIFs dissociate from the 40S ribosomal subunit as the 60S subunit joins, creating an 80S monosome.

Figure 1.2 (continued)



1.3. Inhibition of translation by microRNAs

Since 1999, much effort has focused on identifying the stage of translation that is inhibited by miRNAs. There is a current consensus that miRNAs interfere with the process of translation initiation, but other mechanisms for post-initiation repression may exist. Early studies focused on a few miRNA-targeted genes or reporters and may have over interpreted data to draw sweeping conclusions about the molecular mechanism of miRNA inhibition. More novel techniques to measure genome-wide changes in mRNA levels and translation efficiency have provided great insight into the effects of miRNA-mediated repression (Bazzini et al., 2012; Guo et al., 2010; Hendrickson et al., 2009; Ingolia et al., 2009).

Data addressing the molecular mechanism(s) of miRNA function from cells or *in vitro* reactions generally fall under the following two categories; biophysical or functional. Biophysical experiments measure the distribution of miRNAs, target mRNAs and miRNP factors across polyribosomal gradients. For target mRNAs, this has also been analyzed on a transcriptome-wide level by measurement of ribosome density across the length of individual transcripts (Bazzini et al., 2012; Guo et al., 2010; Hendrickson et al., 2009). Functional experiments measure changes in RNA or protein levels of miRNA-targeted reporter mRNAs that have varied determinants of translation efficiency, such as viral internal ribosome entry sites (IRESes), 5′ cap structures, and

differing polyadenylic (poly[A]) tail status or length. Reporter mRNAs with IRESes or modified cap structures undergo cap-independent, or non-canonical, translation, which is independent of eIF4E recruitment for 43S preinitiation complex recruitment to mRNAs (Figure 1.2A). Unlike the mRNA depicted in Figure 1.2 reporter mRNAs without poly(A) tails are not circularized and have lower translational efficiency than those with poly(A) tails. Increased poly(A) tail length generally leads to increased translational efficiency.

Biophysical assays. Biophysical assays from multiple groups show that miRNAs, Ago2, and target mRNAs co-sediment with polysomes from *C. elegans* larvae and mammalian cells (Kim et al., 2004; Maroney et al., 2006; Nelson et al., 2004; Nottrott et al., 2006; Olsen and Ambros, 1999; Petersen et al., 2006). This led some groups to conclude that miRNAs repress translation after initiation. Upon treatments that induce ribosome runoff, miRNA-targeted mRNAs, miRNAs and Ago proteins shift to lighter fractions in polysome gradients, indicating these factors are associated with actively-translating ribosomes(Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006). We have reinterpreted some of the original data described above in light of more recent studies and present our working model in Figure 1.3. First, the presence of miRNAs, Ago2 and target mRNAs in actively translating polysomes does not necessarily indicate that miRNA-mediated translational repression occurs post-initiation. These data are

consistent with a model in which miRNA-mediated repression of translation initiation causes reduced ribosome density on mRNAs. These mRNAs (Figure 1.3A & B) have reduced protein output and ribosome density compared to untargeted mRNAs (Figure 1.3C) , but could still sediment into polysomal fractions. This interpretation is further supported by genome-wide studies showing that there is, in fact, uniform reduction of ribosome density across the length of miRNA-targeted transcripts (Bazzini et al., 2012; Guo et al., 2010; Hendrickson et al., 2009). Other groups have reported that miRNA-targeted reporter and endogenous mRNAs do shift toward lighter fractions in a polysomal gradient compared to untargeted mRNA (Janas et al., 2012a; Pillai, 2005). This could indicate more efficient repression of translation initiation (Figure 1.3A vs B). Furthermore, our model accounts for the co-sedimentation of Ago proteins with polysomes, as miRNA-targeted mRNAs and associated miRNP complexes sediment to the polysome fractions (Nottrott et al., 2006) (Figure 1.3A & B). It is important to note that while the majority of biophysical and functional evidence supports miRNA-mediated repression of translation initiation, we do not rule out the existence of additional post-initiation mechanisms of repression.

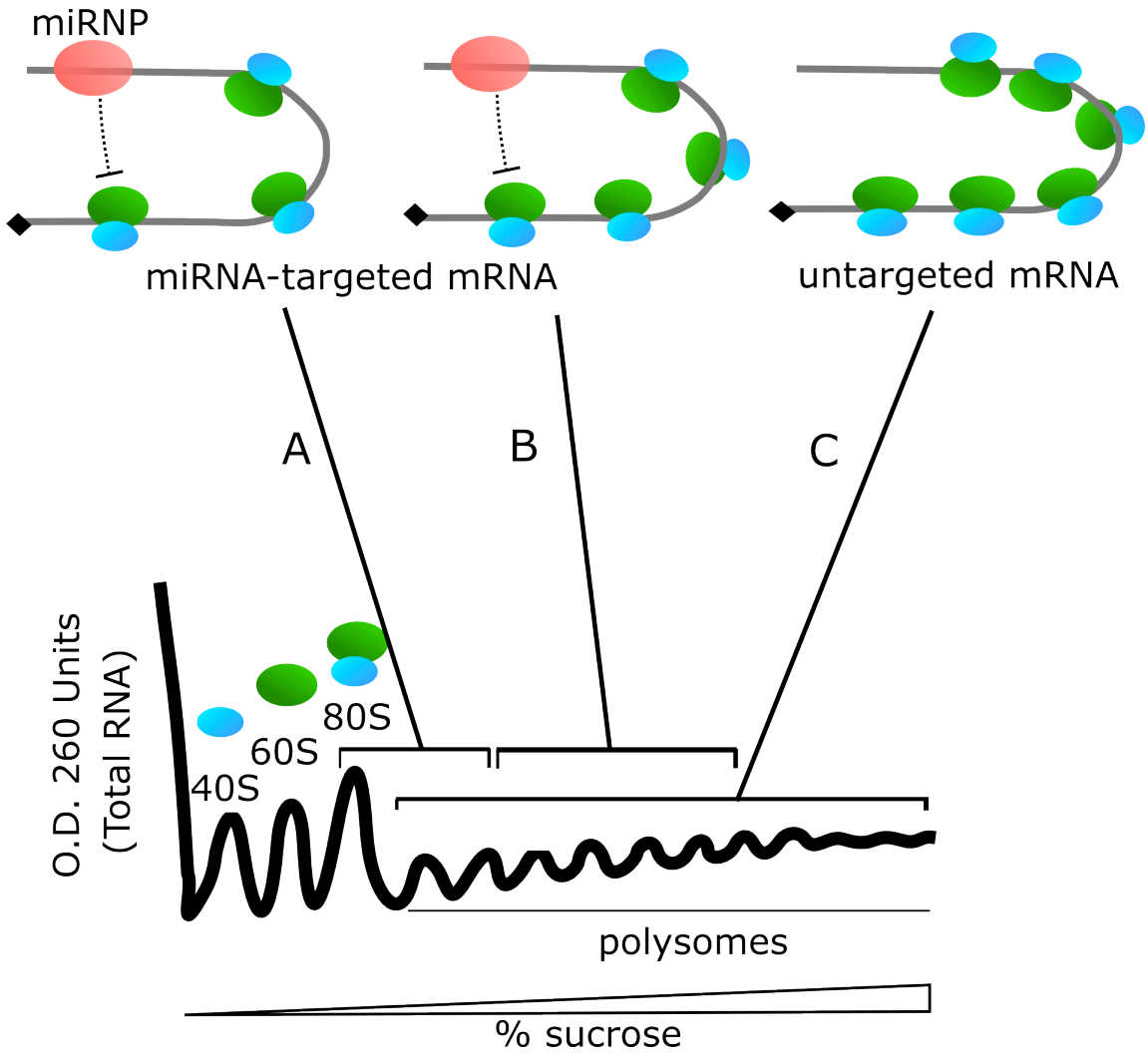
Figure 1.3. A model for miRNA-mediated translational repression
miRNA targeting leads to reduced ribosomal density on a target mRNA, resulting in a shift toward lighter polysomes or monosome fractions. Shown is the artistic rendition of a polysome profile typically obtained by resolving post-nuclear cell lysate on a sucrose gradient, then measuring total RNA across the gradient. The number of ribosomes per mRNA corresponds to the relative translation rate, as measured by protein output.

(A) Translation of a miRNA-targeted mRNA is efficiently repressed and the mRNA associates with fewer ribosomes than untargeted mRNA.

(B) Translation of a miRNA-targeted mRNA is repressed (to a lesser degree than in [A]) and the mRNA associates with fewer ribosomes than an untargeted mRNA.

(C) An untargeted mRNA associates with relatively more ribosomes than miRNA-targeted mRNA.

Figure 1.3 (continued)



Functional assays. Functional assays interrogate mRNA target determinants of miRNA-mediated repression. Viral IRES-driven translation is cap-independent, but the processes of elongation and termination proceed in the same manner as cap-dependent translation. If miRNAs affect translation after initiation, they should also repress translation of IRES-driven reporters. Multiple groups have shown that miRNAs do not repress IRES-driven translation in cells or *in vitro*, consistent with an initiation block (Humphreys et al., 2005; Kamenska et al., 2013; Mathonnet et al., 2007; Pillai, 2005; Wang et al., 2006). However, two groups have reported that miRNA-targeted-IRES driven reporters are repressed, suggesting a post-initiation block (Meijer et al., 2013; Petersen et al., 2006). The reasons for these discrepancies remain unclear, but may be due to differences in experimental design or systems between the studies.

More compelling evidence for miRNA-mediated repression of translation initiation comes from experiments interrogating the mRNA requirements for miRNA-mediated repression. Cellular mRNAs have a 5′ 7-methyl-guanosine cap (m^7GpppN) which is recognized by eIF4E during translation initiation to stimulate ribosome recruitment. Synthetic cap analogues have been extensively used to compete out this interaction and therefore guage the requirement for eIF4E in biological processes. Multiple groups, including our own, have shown that a 5′ m^7GpppN cap and 3′ poly(A) tail is required for miRNA-mediated repression in cells and *in vitro* (Humphreys et al.,

2005; Wang et al., 2006). Interestingly, one report suggests that the mechanism of translational repression is determined by the nuclear history, specifically the promoter, of a target gene (Kong et al., 2008). This finding has not been validated by other groups. Taken together, the results from various experimental systems indicate that an mRNA 5' m^7GpppN and 3' poly(A) tail are required for miRNA-mediated repression, implying that miRNAs target the process of translation initiation.

How do miRNAs repress translation? After 15 years of vigorous study, there is not a clear, detailed answer. There is, however, ample biophysical and functional evidence for repression of translation initiation by miRNAs, while far fewer data support a post-initiation mechanism of repression. The discrepancies cannot be easily explained by differences in experimental systems and it is possible that multiple mechanisms of translational repression co-exist. Within the process of translation initiation, miRNAs may repress eIF4E-mRNA cap recognition, 43S preinitiation complex loading (Figure 1.2A) or scanning (Figure 1.2B), or 60S ribosomal subunit joining (Figure 1.2C). Further study of the molecular mechanism(s) of miRNA-mediated repression *in vitro* and in cells is necessary to understand the basis of this widespread and important level of gene regulation.

1.4. Factors involved in miRNA-mediated translational repression

A consensus regarding factors required for miRNA function has not been reached, testifying to the complex and possibly dynamic nature of the miRNP. Ago proteins are necessary components of the miRNP, as they bind to mature miRNAs and thus facilitate target-miRNA interaction (Peters and Meister, 2007). Mammalian Ago1 and Ago2 are generally more abundant than Ago3 or Ago4, although all can associate with endogenous miRNAs and repress target genes (Meister, 2013). It was reported that human Ago2 may be capable of binding cap analogue *in vitro*, suggesting that Ago2 could competitively inhibit eIF4E-cap binding (Kiriakidou et al., 2007). However, this conclusion was strongly challenged by contradictory results in a sequence-structure analysis and a subsequent report showing that Ago2 cap-binding activity was non-specific and conformationally implausible when an siRNA is loaded (Frank et al., 2011; Kinch and Grishin, 2009). There is no convincing data to support direct binding of Ago proteins to mRNA 5′ m^7GpppN cap structures.

In cells, Ago proteins localize to the rough endoplasmic reticulum and RNA processing bodies (P-bodies) and exist in distinct protein complexes, which are not well-characterized. Immunoprecipitation of Ago proteins from total lysate therefore captures and pools distinct complexes, while resolution of cell lysate on sucrose gradients prior to immunoprecipitation partially purifies distinct Ago complexes. One report found that

in HeLa cells, Ago1 and Ago2 associate with three distinct complexes ranging in size from 250-900 kDa (Höck et al., 2007). The authors immunopurified these complexes to identify associated factors. The smallest complex contains miRNA biogenesis factors Dicer and TRBP, and thus likely represents a RISC/miRISC loading complex (Peters and Meister, 2007). Chaperone protein Hsp90 associates with Ago2 independently of miRNAs and may enhance RISC loading (Frohn et al., 2012; Meister, 2013). Numerous RNA granule components (EDC4 and GW proteins TNRC6A, TNRC6B, TNRC6C), DEAD/DEAH box proteins (Rck/p54 [DDX6]), and RNA binding proteins (PABPC1 and RBM) have also been shown to interact with Ago2 (Frohn et al., 2012; Meister, 2013).

Additionally, factors that are functionally, but not necessarily physically, involved in miRNA activity have been identified in RNAi screens as enhancers of RNAi (Eri genes) or miRNA activity in *C. elegans*. Multiple translation initiation factors and ribosomal protein genes are positive modulators of miRNA-mediated repression and enhance let-7 function in *C. elegans* (Chan and Slack, 2009; Ding et al., 2008). In mammalian cells, knockdown of small or large subunit ribosomal protein genes, eIF4GI, eIF4A2, and poly(A) tail binding proteins PABPC1 and hnRNP-Q causes the derepression of miRNA-targeted reporters and/or endogenous mRNAs (Janas et al., 2012a; Jannot et al., 2011b; Meijer et al., 2013; Ryu et al., 2013; Svitkin et al., 2013). Additionally, knockdown of eIF6, a ribosome dissociation factor which associates with

TRBP (which associates with Dicer) relieves miRNA-mediated repression (Chendrimada et al., 2007). The effect of overexpression of these factors on miRNA activity has not been studied. Differences in methods and conditions used in each study hinder comparisons between findings, and to date there is no consensus regarding the factors required for miRNA function.

1.5. miRNA-mediated translational repression and mRNA decay

In addition to miRNA-mediated translational repression, there is mRNA decay through deadenylation, decapping, and localization to P-bodies in multiple experimental systems (Anderson and Kedersha, 2009; Pillai, 2005; Wu and Belasco, 2008). Genome-wide studies have revealed that introduction or deletion of a miRNA causes reduced or increased, respectively, protein and mRNA levels for most targets in mammalian cells (Baek et al., 2008; Hendrickson et al., 2009; Selbach et al., 2008). Recent work has utilized non-steady state systems to investigate the kinetics of miRNA-mediated repression. Translational repression precedes mRNA deadenylation and decay of miRNA-targeted reporters in human and *D. melanogaster* cells, and endogenous miRNA targets in zebrafish (Bazzini et al., 2012; Bethune et al., 2012; Djuranovic et al., 2012). Furthermore, blocking deadenylation of target mRNAs does not cause full derepression of miRNA-targeted mRNA, indicating that deadenylation partially accounts for the total magnitued of miRNA-mediated repression (Bazzini et al., 2012; Beilharz et al., 2009). The most widely-accepted model is that miRNA-mediated translational repression precedes RNA decay and the relative contributions of these activities to the overall reduction of the target gene varies for different transcripts.

1.6.Translation initiation factors and cancer

In healthy cells, the complex process of mRNA translation is extensively regulated by cell growth and stress signaling pathways. In cancer cells, this regulation is perturbed (reviewed in (Silvera et al., 2010; Stumpf and Ruggero, 2011)). Misregulation of translation by altered activity or expression of translation and ribosomal factors is a common feature of many cancers (reviewed in (Stumpf and Ruggero, 2011)). Eukaryotic translation is regulated primarily at the rate-limiting step of initiation- the recruitment of the 43S preinitiation complex to mRNAs (Figure 1.2). eIF4F is a heterotrimeric complex comprised of eIF4E, the mRNA 5' cap binding protein; eIF4G, a molecular bridge between eIF4E and the 43S preinitiation complex; and eIF4A (paralogues 1 and 2), an ATP-dependent RNA helicase. Among all the initiation factors, eIF4E is the least abundant and therefore eIF4F formation is limited by eIF4E availability.

The overexpression of two components of eIF4F, eIF4G or eIF4E can promote malignant transformation (Fukuchi-Shimogori et al., 1997; Lazaris-Karatzas et al., 1990). Although it was initially thought that increased eIF4A1 and eIF4E expression was a consequence, rather than a cause of malignant transformation, several reports suggest oncogenic roles for both factors (Avdulov et al., 2004; Larsson et al., 2007; 2006). eIF4E expression is increased in up to 30% of human cancers and many melanoma samples (Carroll and Borden, 2013; Yang et al., 2007). Increased eIF4E activity via 4E-BP1

phosphorylation is associated with poor survival of melanoma patients (O'Reilly et al., 2009). eIF4A1 expression is increased in melanoma cell lines compared to melanocytes, with intermediate expression in benign melanocytic nevi (Eberle et al., 1997). Suppression of eIF4E activity or expression inhibits proliferation, invasion, and migration of cancer cells *in vitro* as well as tumor initiation and growth of human xenografts *in vivo* (Chen et al., 2012b). Likewise, decreased eIF4A1 expression or activity decreases proliferative and invasive phenotypes of cancer cell lines, including melanoma (Eberle et al., 2002; Nasr et al., 2012a).

eIF4E is a downstream effector of both the phosphoinositide 3-kinase-mammalian target of rapamycin (PI3K-mTOR) and Ras-mitogen activated protein kinase (MAPK) pathways, while eIF4A is a downstream effector of PI3K-mTOR signaling. The MAPK pathway was found to be aberrantly activated by BRAF or NRAS mutations in 50% and 15%, respectively, of melanoma cases (Tsao et al., 2012). Signaling flux through the mTORC1 pathway increases the availability of eIF4E and eIF4A through phosphorylation and release of two repressor proteins; eIF4E binding proteins (4E-BPs), and PDCD4, a tumor suppressor gene product and inhibitor of eIF4A, respectively (Dennis et al., 2012). Activation of the mTORC1 pathway is associated with poorer survival of melanoma patients (O'Reilly et al., 2009). Treatment with mTORC1 ATP-site inhibitors decreases the invasive phenotype in a human prostate cancer cell

line, primarily through the eIF4E-4EBP axis (Hsieh et al., 2013). Additionally, activation of both the PI3K-mTORC1 and Ras-MAPK pathways increases activity of eIF4B, an enhancer of eIF4A helicase activity (Dennis et al., 2012).

As major effectors of the PI3K-mTOR and/or Ras-MAPK pathways, eIF4E and eIF4A1 are promising therapeutic targets. Suppression of eIF4A1 or eIF4E reduces proliferation and sensitizes cancer cell lines to chemotherapy (Cencic et al., 2013a; Hayman et al., 2012; Yang et al., 2012). One promising therapy, an anti-sense oligo targeting eIF4E, reduced eIF4E expression in human tumor xenografts, leading to reduced tumor growth (Graff et al., 2007). It also induced apoptosis and chemosensitivity while reducing proliferation in mesothelioma cells (Jacobson et al., 2013). In a phase I clinical trial in patients with advanced cancer, this anti-sense oligo was well tolerated and reduced eIF4E expression in tumor biopsies, but did not significantly affect tumor response (Hong et al., 2011). This eIF4E inhibitor is currently in two phase II clinical trials in combination with other chemotherapeutic agents (ISISPharmaceuticals).

Despite their roles in malignant transformation and potential as therapeutic targets, the precise roles of eIF4A1 and eIF4E in tumor initiation and/or maintenance remain unclear. Upon eIF4E modulation, the translation efficiency of a subset of mRNAs is differentially regulated. Initial studies showed that mRNAs with highly

structured 5′ UTRs, including proto-oncogenes and growth factors are among this subset (Koromilas et al., 1992). In contradiction to the long-standing model that 5′UTR length or secondary structure was the major mRNA determinant of translational efficiency, genome-wide studies have identified mRNAs with 5′ terminal oligopyrimidine (TOP) tracts or pyrimidine-rich translational elements (PRTE) as differentially sensitive to eIF4E activity (Hsieh et al., 2013; Huo et al., 2012b; Mamane et al., 2007; Santhanam et al., 2009; Thoreen et al., 2012). Although little is known about the mechanism of eIF4A1 proto-oncogenic activity, it is thought to increase translation efficiency of transcripts with highly structured 5′ UTRs (Svitkin et al., 2001). A comprehensive understanding of the quantitative proteomic changes caused by eIF4A1 or eIF4E modulation has not been reached.

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2. microRNA-repressed mRNAs contain 40S but not 60S components.

2.1. Abstract

microRNAs (miRNAs) are small, noncoding RNAs that may regulate more than half of human genes, yet the molecular mechanism of miRNA-mediated repression remains obscure. Using a cell-free assay of miRNA activity, we show that miRNA-targeted mRNAs are enriched for components of the 40S, but not 60S ribosomal subunit. Additionally, a molecular toeprint of 18 protected nucleotides 3' relative to the start codon, consistent with nucleotide protection by 40S ribosomal subunits, is enriched on miRNA-targeted mRNAs. Our results suggest that miRNAs repress translation initiation in a cell-free system by preventing 60S ribosomal subunit joining to 40S subunits positioned at the start codon.

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2.2.Introduction

miRNAs bind to target mRNAs with imperfect sequence complementarity and repress translation through mechanisms that are incompletely understood. Intensive efforts have focused on determining the precise stage of translation that is repressed by miRNAs. We have developed a cell-free translation repression reaction (Wang et al., 2006) in mammalian extracts which faithfully recapitulates important properties of miRNA function in cells, including dependence on miRNA 5' phosphates and perfect seed region complementarity between miRNAs and target mRNAs. Importantly, translation is repressed without reduction in target mRNA levels. However, addition of siRNAs that are perfectly complementary to the reporter mRNA leads to significant reduction in target mRNA levels. Additionally, translational repression is dependent on a 7-methyl guanosine cap (m^7GpppN) and a polyA tail on target mRNAs for translational repression as observed in cells. Other cell-free translation repression reactions in mammalian and *D. melanogaster* cell extracts also demonstrate a requirement for m^7GpppN capped target mRNAs for translational repression, further supporting a model of miRNA repression of translation initiation (Mathonnet et al., 2007; Thermann and Hentze, 2007). Still, the precise mechanisms of miRNA function are unknown. We define a mechanism of miRNA-directed repression of translation initiation by decreased 60S ribosome recruitment to target mRNAs in our cell-free system using ribosome binding assays, precipitation of miRNA-targeted mRNAs

followed by Northern blotting, Western blotting, and toeprinting analyses of miRNA-targeted mRNAs.

2.3.Results

2.3.1. miRNA-targeted mRNAs contain reduced amounts of 60S ribosome components.

To investigate the mechanism of repressed translation initiation, we measured association of the 40S and 60S ribosomal subunits with miRNA-targeted mRNAs in the cell-free translation repression reactions (Wang et al., 2006). To enable recovery of miRNA-targeted mRNAs, firefly luciferase reporter mRNAs were polyadenylated with an average of two biotinylated adenosines incorporated into the approximately 200 nucleotide polyA tail. These reporter mRNAs (FL6X) contained six imperfectly complementary binding sites to the CXCR4 miRNA (miR-CXCR4). Consistent with previous observations, the FL6X reporter mRNA was repressed upon addition of CXCR4 miRNAs when normalized to an untargeted *Renilla* luciferase reporter (RL0X, Fig. 2.1B)(Wang et al., 2006).

Figure 2.1. miRNA-targeted mRNAs have reduced association with 60S ribosome components.

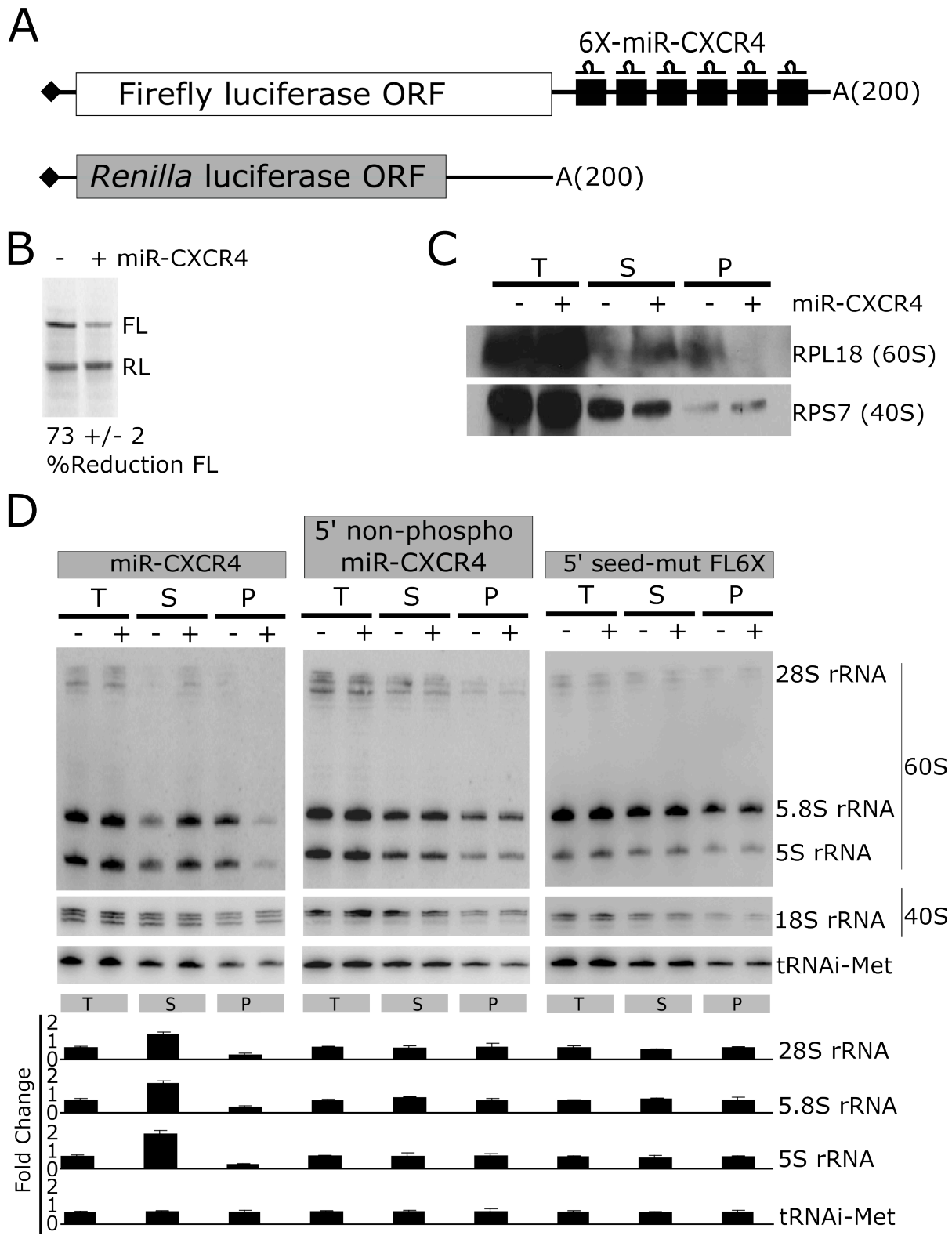
(A) Schematic representation of firefly luciferase mRNA reporter construct with six miR-CXCR4 binding sites in the 3′ UTR (FL6X, top) and *Renilla* luciferase construct with no miRNA binding sites (RL0X, bottom). Both mRNAs had 7-methyl-guanosine caps and polyA tails. The FL6X mRNA contained biotinyl-adenosines (99:1 adenosines:biotinyl-adenosines).

(B) Translation repression reactions using FL6X and RL0X mRNAs without (-) and with (+) CXCR4 miRNA (miRCXCR4). Translation repression of firefly luciferase (FL, % Reduction FL) is normalized by *Renilla* luciferase (RL) and is calculated by the equation [1-FL/RL(+miRCXCR4):FL/RL(-miRNA)] x 100.

(C) Northern blotting for 40S and 60S rRNAs in total (T) translation repression reactions, supernatants (S), and precipitates (P) following streptavidin precipitation of biotinylated mRNA reporters from total translation repression reactions. Streptavidin-precipitated FL6X mRNAs from reactions with (+) phosphorylated miR-CXCR4 (left panels) or unphosphorylated CXCR4 miRNA (5′ nonphospho. miR-CXCR4, middle panel) or without miRNA (-) . Streptavidin precipitated FL6X mRNAs with point mutations in the 5′ seed region (5′ seed mut. FL6X) from reactions with (+) and without (-) CXCR4 miRNA (right panel). Signal intensities were normalized by calculating 60S (5S, 5.8S, and 28S):40S (18S) rRNAs and initiator methionine tRNA (tRNAi-Met):40S rRNA. The fold changes in signal intensities between reactions containing and lacking siRNAs were calculated by the equation [(60S/40S+miR-CXCR4):(60S/40S-miR-CXCR4)] or [(tRNAi-Met/40S+miRCXCR4):(tRNAi-Met/40S-miRCXCR4)]. Results are presented as bar graphs below each panel as an average of n=3 trials (left panel), n=2 trials (middle panel), and n=2 trials (right panel).

(D) Western blotting for 40S-associated and 60S-associated proteins in T, S, and P following streptavidin precipitation of biotinylated mRNA reporters from total translation repression reactions. In corresponding lanes between reactions without (-) or with (+) CXCR4 miRNAs in T, S, and P, the same amount of total protein was loaded into each lane.

Figure 2.1 (continued)



Upon completion of the translation repression reactions, reporter mRNAs were precipitated with streptavidin beads and precipitates were analyzed by Northern blotting to detect ribosomal RNAs (Fig. 2.1D). A fixed amount of RNA was loaded in each lane and 60S rRNA and $tRNA_i$-Met band intensities was normalized to 40S rRNA. 18S rRNA, a component of the 40S ribosome subunit, was not significantly altered in reactions containing or without siRNAs. However, 60S ribosomal rRNAs (28S, 5.8S, and 5S) were reduced relative to the 18S rRNA in reactions with miRNA-targeted mRNAs (Fig. 2.1D). Additionally, the reduction in 28S, 5.8S, and 5S rRNAs associated with FL6X was approximately the same as the degree of translational repression observed in Figure 2.1B (~60%). These results suggest that miRNAs reduce 60S ribosome subunit loading on target mRNAs. Conversely tRNAi-Met was not changed relative to the 18S rRNA in reactions containing miRNA-targeted mRNAs compared to untargeted mRNAs. This suggests that miRNAs permit 43S ribosome subunit loading on target mRNAs.

Several control reactions confirm the specificity of reduced 60S ribosome recruitment to miRNA-targeted mRNAs. (1) There was no significant change in the 40S or 60S ribosomes rRNA content in Northern blots of total lysates from translation repression reactions, suggesting that the ribosome subunits are not being lost due to degradation. Relative to reactions with siRNAs, there was no change in the amounts of 60S ribosome subunits between reactions using (2) FL6X mRNAs with

unphosphorylated CXCR4 miRNAs (Fig. 2.1D middle panel), (3) FL6X mRNAs containing point mutations in the 5′ seed region of the miRNA binding sites in the 3′ UTR (Fig. 1D right panel), (4) FL6X mRNAs with non-specific control miRNAs (Fig. S2.1C), and (5) FL0X mRNAs with CXCR4 miRNAs (Fig. S2.1C). The absence of significant changes in 40S and 60S rRNA content in these control reactions is consistent with the absence of translational repression in these control reactions (Fig. 2.1B and Fig. S2.1B) and indicates that the reduction in 60S ribosome RNAs is specific to miRNA-repressed mRNAs.

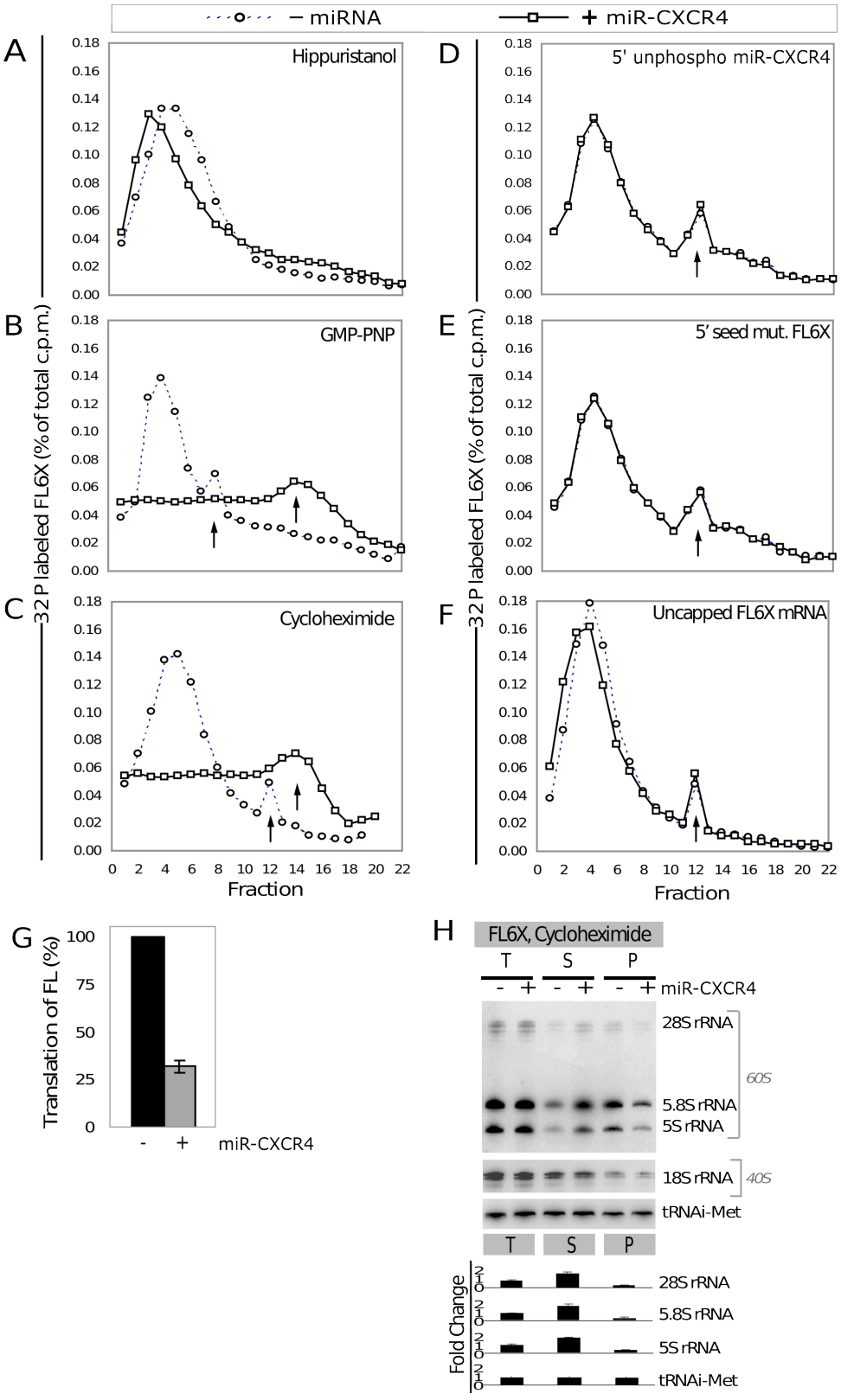
To independently confirm these observations, precipitated reporter mRNAs were subjected to Western blotting for 40S-associated and 60S-associated proteins (Fig. 2.1C). Whereas slightly increased amounts of RPS7 were associated with miRNA-repressed mRNAs, strongly decreased amounts of RPL18 were detected on these same mRNAs. Together, these observations demonstrate that miRNA-targeted mRNAs have steady-state levels of 40S ribosome components but reduced levels of 60S ribosome components relative to untargeted mRNAs.

2.3.2.Chemical inhibitors identify high molecular mass complex formation on miRNA-targeted mRNAs that depends upon 40S but not 60S ribosomes.

To more precisely define the stage of translation initiation affected by miRNAs, we used ribosome binding assays to analyze the sedimentation profiles of radiolabeled miRNA-targeted mRNAs from translation repression reactions (Fig. 2.2). As in all reports of polysome profiling of miRNA targeted mRNAs (Chendrimada et al., 2007; Olsen and Ambros, 1999; Petersen et al., 2006; Pillai, 2005; Seggerson et al., 2002; Thermann and Hentze, 2007), ribosome binding assays require chemical inhibitors to stabilize intermediates of monosome (80S) assembly. In ribosome binding assays, addition of CXCR4 miRNAs to translation repression reactions without chemical inhibitors is not sufficient to capture complexes at specific stages of ribosome assembly during translational repression (data not shown). Complexes stabilized at specific stages of ribosome assembly on radiolabeled mRNAs are resolved on a glycerol gradient and detected by Cerenkov scintillation counting of individual glycerol gradient fractions.

Figure 2.2. A high molecular mass complex containing 40S but lacking 60S ribosome subunits forms on miRNA-repressed mRNAs.
(A-F) Horizontal axis indicates fraction number. Vertical axis indicates the counts per minute (c.p.m) of each fraction as a percent of total counts recovered (% of total c.p.m.). Reactions were performed without (-, dotted line) or with (+, solid line) the indicated miRNA.
(B-F) The upward pointing arrows indicate peak fractions of mRNA sedimentation in glycerol gradients.
(A) Ribosome binding analysis of translation repression reactions containing hippuristanol identifies a peak (fraction 4) corresponding to unbound mRNAs in reactions without and with miR-CXCR4.
(B) Ribosome binding analysis of translation repression reactions containing GMP-PNP identifies a 48S complex (fraction 8) in reactions without, and a high molecular mass complex formed on FL6X mRNAs in reactions with miR-CXCR4.
(C) Ribosome binding analysis of translation repression reactions containing cycloheximide identifies an 80S complex (fraction 12) in reactions without, and a high molecular mass complex formed on FL6X mRNAs in reactions with miR-CXCR4.
(D) An 80S complex is formed on FL6X mRNAs in reactions containing cycloheximide and and unphosphorylated miR-CXCR4.
(E) An 80S complex is formed on FL6X mRNAs with three point mutations in the 5′ seed region (5′seed mut. FL6X) in reactions containing cycloheximide and miR-CXCR4.
(F) An 80S complex is formed on uncapped FL6X mRNAs in reactions containing cycloheximide and miR-CXCR4.
(G) Dual luciferase assay using FL6X mRNAs with ^{32}P-labeled polyA tails in the presence of miR-CXCR4. Firefly luciferase (FL) measurements were normalized to *Renilla* luciferase expressed from RL0X mRNAs with an unlabeled cap and a polyA. Bars indicate percent translation of FL (%, Y-axis).
(H) Northern blotting analysis of translation repression reactions in the presence of cycloheximide using FL6X mRNAs with 7-methyl guanosine caps and biotinylated polyA tails from reactions without (-) or with (+) miRCXCR4, as in Figure 2D.

Figure 2.2 (continued)



First, translation repression reactions were performed in the presence of the eIF4A inhibitor hippuristanol (Bordeleau et al., 2006). Hippuristanol blocks 43S recruitment to mRNAs resulting in a free mRNP peak in fraction four of the glycerol gradient. Addition of CXCR4 miRNA to translation repression reactions did not alter the sedimentation of mRNAs in the presence of hippuristanol (Fig. 2.2A), suggesting that any complexes that form on repressed mRNAs require 43S recruitment. Next, translation repression reactions were performed in the presence of the non-hydrolyzable GTP analog GMP-PNP, which blocks 60S ribosome recruitment, resulting in the capture of 48S initiation complexes in a peak in fraction eight. When CXCR4 miRNA was added to translation repression reactions containing GMP-PNP, the mRNA sedimentation peak was shifted from fraction 8 to fraction 14, indicating the formation of a high molecular mass complex (Fig. 2.2B). These results suggest that this high molecular mass complex is formed on miRNA-targeted mRNA after 43S recruitment but before 60S recruitment.

Finally, translation repression reactions were performed in the presence of the translation elongation inhibitor cycloheximide, which traps fully-assembled 80S monosomes at the initiation codon of mRNAs in gradient fraction twelve. Reactions without added miRNA led to the expected mRNA sedimentation, consistent with captured 80S complexes (Fig. 2.2C-F). Addition of CXCR4 miRNAs to translation repression reactions containing cycloheximide, however, generated an mRNA

sedimentation profile identical to the profile observed with GMP-PNP (Fig. 2.2C). This observation indicates that 80S monosomes do not form in these reactions and that the repression occurs at an earlier step of translation initiation.

The high molecular mass complex was not formed in a variety of control reactions. Reactions with unphosphorylated CXCR4 miRNAs (Fig. 2.2D), a triple point mutant in the 5′ seed region of the miRNA binding site in the FL6X mRNA 3′ UTR (Fig. 2.2E), a non-specific control miRNA (Fig. S2.1C), or fully phosphorylated CXCR4 miRNAs with FL0X mRNA (Fig. S2.2D) all resulted in the formation of an 80S complex in the presence of cycloheximide and had no translation repression measured by luciferase activity (Figs. 2.2G, S2.1B). Consistent with the lack of translational repression of uncapped mRNAs (Wang et al., 2006), an 80S complex was formed in reactions with uncapped FL6X (Fig. 2.2F). These results demonstrate that the high molecular mass, or repressive complex forms only on translationally-repressed mRNAs, contains the 40S ribosome subunit, and lacks the 60S ribosome subunit. This further supports a model that miRNAs repress translation by preventing 60S ribosome subunit recruitment to target mRNAs. Additionally, because the high molecular complex is not formed in reactions with the eIF4A inhibitor, hippuristanol, it seems that miRNA-directed repression of translation occurs after cap-facilitated, 40S ribosome subunit recruitment.

To rule out any effect of cycloheximide in the reactions in Figure 2.2, we performed Northern blotting analysis on precipitated complexes from translation repression reactions containing cycloheximide (Fig. 2.2H). Consistent with data presented in Figure 2.1D, Northern blotting of precipitated FL6X demonstrated similar levels of 18S rRNA but reduced 28S, 5.8S and 5S rRNAs (with no change in tRNAi-met) associated with target mRNAs in reactions with CXCR4 miRNAs compared to reactions without CXCR4 miRNAs. Also consistent with data presented in Figure 2.1D, 60S ribosome association was reduced to a similar degree (~70%) as translational repression observed in Figures 2.1B and 2.2G.

2.3.3. 48S complexes are positioned at AUG on miRNA repressed miRNAs

To identify the position of 40S ribosomes assembled on miRNA-repressed mRNAs, we performed primer extension analysis (Fig. 2.3A). In this assay, a radiolabeled primer hybridizing to sequences 3′ relative to the AUG codon of reporter mRNAs was used to initiate reverse transcription without extraction from associated proteins. A molecular 'toeprint,' of bound protein complexes is generated when steric hindrance prevents reverse transcriptase from transcribing cDNA from regions of the mRNA. Translation repression reactions with CXCR4 siRNAs generated bands at 18 nucleotides 3′ relative to the AUG codon (lanes 5 versus 6). This toeprint was identical to translation repression reactions containing GMP-PNP (lanes 10-12), which marks 40S ribosomes positioned at the start codon after completion of scanning. In reactions containing hippuristanol, 40S ribosomal subunit loading is blocked and the toeprint was not present (lanes 7 & 8), as expected. Addition of miR-CXCR4 did not induce a toeprint (lane 9). This is consistent with ribosome binding assay results that show hippuristanol blocks formation of the high molecular mass complex on miRNA-targeted mRNA (Fig. 2.2A).

The protected nucleotide 'toeprint' was quantified using the ratio of the 3′ (specific) protected band relative to the 5′ (non-specific) band. RNA secondary structure causes MMLV reverse transcriptase to drop-off of its template, generating a non-specific 5′ band that can be used to normalize a specific ribosome toeprint. By this

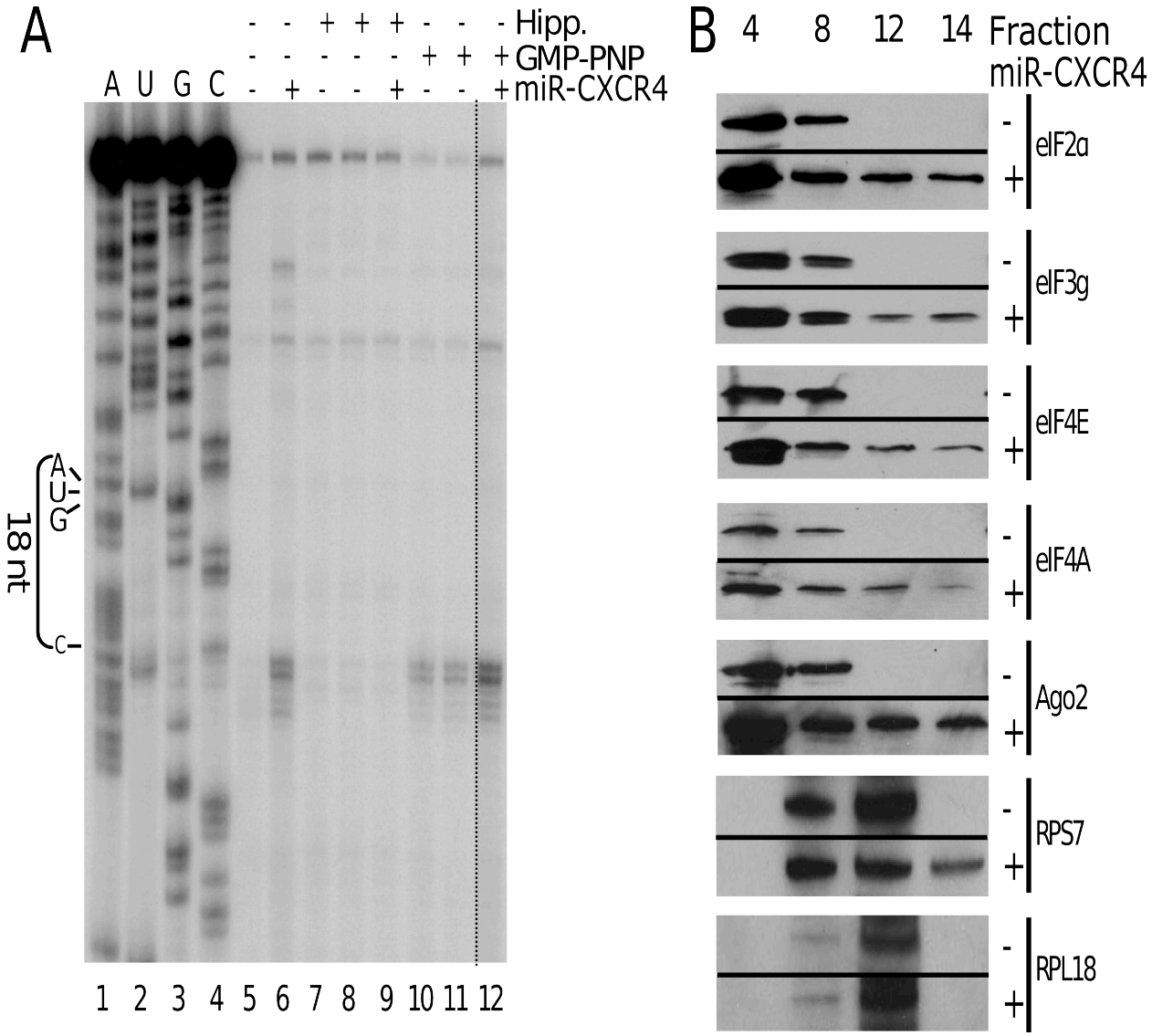
measure, the CXCR4 siRNA-induced toeprint ratio was 2.3 (lane 6), the GMP-PNP-induced toeprint ratio was 6.9 (lanes 10 & 11), and the combined ratio was 7.1 (lane 12), three-fold more than the toeprint ratio induced by CXCR4 siRNA alone. These data indicate that GMP-PNP more strongly stabilizes 40S complexes positioned at AUG compared to miRNAs alone and may help explain why the addition of miRNAs alone is not sufficient to capture complexes in ribosome binding assays.

Figure 2.3. miRNAs repress translation after 48S scanning but before 60S subunit joining.

(A) Molecular toeprinting assay of FL6X mRNAs with a 7-methyl guanosine cap and polyA tail. Compared to reactions without (-) CXCR4 miRNA (miR-CXCR4, lane 5), reactions with (+) CXCR4 miRNA (lane 6) demonstrate a strong ribosomal toeprint encompassing the initiating AUG codon. Addition of hippuristanol (hipp.) to translation repression reactions does not lead to a toeprint (lanes 7 and 8) and blocks the CXCR4 miRNA-induced toeprint (lane 9). The 40S toeprint is present in translation repression reactions containing GMP-PNP (lanes 10 and 11) and augmented in reactions containing CXCR4 miRNA (lane 12). A labeled ladder (lanes 1-4) indicates nucleotide positions relative to AUG. Lane 12 was run on the same gel, but is cropped into the figure (indicated by dotted line).

(B) Western blotting analysis using antibodies to eIF2α, eIF3g, eIF4E, eIF4A, Ago2, RPS7 (40S-associated protein), and RPL18 (60S-associated protein) detects proteins that co-precipitate with FL6X mRNAs from glycerol gradient fractions 4 (free mRNA), 8 (48S peak), 12 (80S peak) and 14 (high molecular mass complex peak) in ribosome binding assays. The same amount of total protein was loaded into each lane from reactions without (-) or with (+) CXCR4 miRNAs. Although panels are presented one above the other, for each antibody, data was obtained from the same blot.

Figure 2.3 (continued)



2.3.4. eIF2 and eIF3 are associated with miRNA-targeted mRNAs.

To investigate the complement of translation initiation factors associated with mRNAs in fraction 4 (free mRNA), fraction 8 (48S complexes), fraction 12 (80S complexes), and fraction 14 (high molecular mass complex), mRNA precipitates from glycerol gradient fractions in ribosome binding assays were analyzed by Western blotting. Because many translation initiation and miRNP factors are highly conserved through evolution, antibodies against these human and mouse proteins cross-react with their rabbit homologs. The translation initiation factors eIF2 and eIF3 are recruited to the 43S preinitiation complexes prior to joining mRNAs and dissociate from 48S ribosome complexes just prior to (or concomitant with) 60S ribosome subunit joining mRNAs (reviewed in (Pestova et al., 2007)). The eIF2 subunit eIF2α, and the eIF3 subunit, eIF3g, were enriched in fractions 12 and 14 from reactions with CXCR4 miRNA compared to those without miRNA (Fig. 2.3B). Together with toeprinting analysis, this supports a model in which miRNAs block translation after 43S subunit joining and scanning, but prior to eIF2 and eIF3 release and 60S ribosome subunit joining.

The cap-binding protein eIF4E and RNA helicase eIF4A were enriched in gradient fractions 12 and 14 from reactions with CXCR4 siRNA compared to those without miRNA (Fig. 2.3B). This suggests that eIF4E and eIF4A are still bound to translationally-repressed mRNAs after 40S subunit joining and raises the possibility

that interaction of these proteins with the cap are important for translational repression by miRNAs.

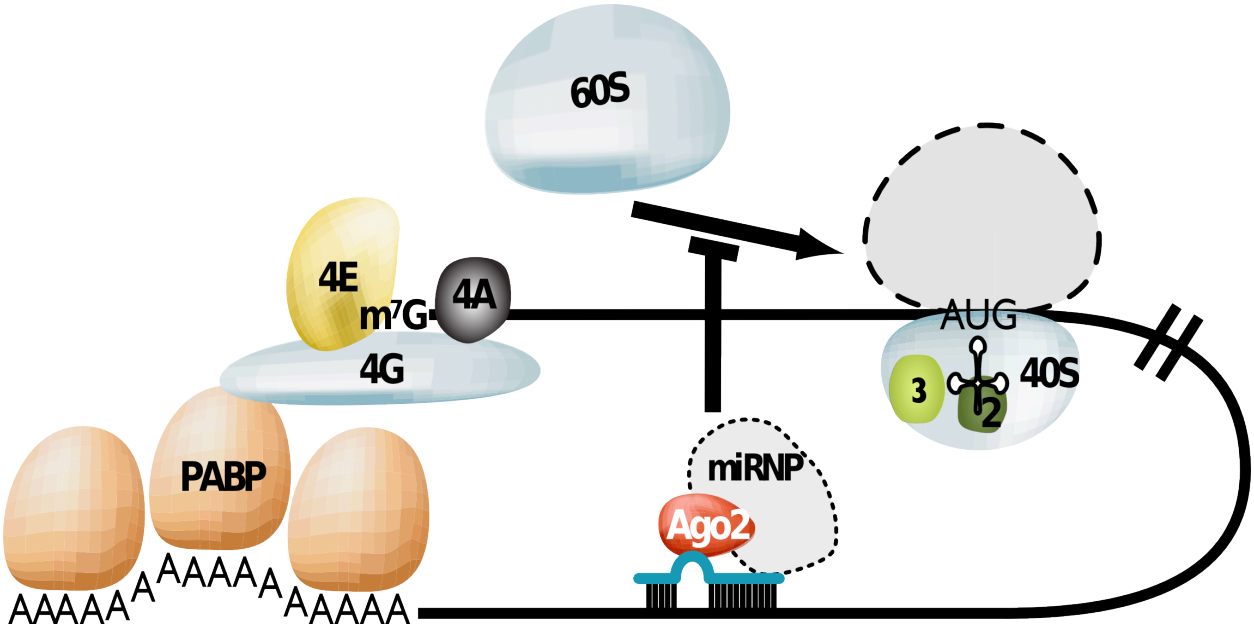
In all species capable of small RNA-directed gene silencing, microribonucleoprotein (miRNP) complexes contain a member of the Ago family of proteins. Ago2 was enriched in gradient fractions 12 and 14 from reactions with CXCR4 miRNA compared to those without miRNA (Fig. 2.3B). This indicates that the high molecular mass complex formed on miRNA-repressed mRNAs is a bona fide miRNP. To demonstrate that Ago proteins recruited to miRNAs pre-annealed to mRNAs are functional, Ago2-dependent RNA cleavage assays were performed. Our data indicate that Ago2-mediated cleavage of target RNAs *in vitro* maps to the exact position as reported for Ago2-dependent cleavage in cells (Fig. S2.4). Our group has subsequently shown that Ago2 is capable of binding pre-annealed miRNA:mRNA duplexes *in vitro* and in cells (Janas et al., 2012a). These data indicate that these reaction conditions permit formation of functional miRNP/RISC on miRNA-repressed mRNAs.

2.4.Conclusions

The process of translation initiation is typically regulated at one of two steps: either 43S pre-initiation complex formation or recruitment to mRNA(Pestova et al., 2007). However, more specialized mechanisms of translational control have been reported. The mechanism for miRNA-directed translation repression proposed here is analogous to a previously reported mechanism by a 3'UTR regulatory ribonucleoprotein complex that represses translation by inhibiting 60S subunit joining with the 40S subunit positioned at the AUG codon of lipooxygenase mRNA (Ostareck et al., 2001). Because miRNAs may regulate large networks of genes, the mechanism of blocked 60S recruitment may be far more prevalent than previously thought.

A model integrating the observations reported here is presented in Figure 2.4.

Figure 2.4. A model of miRNA-directed repression of translation initiation.
Several translation initiation factors may interact with a recruited Ago protein to repress translation including the cap binding factor, eIF4E; the protein associated with the polyA tail, PABP; the bridging protein between cap structures and the polyA tails, eIF4G; the RNA helicase that unwinds local mRNA secondary structure, eIF4A; and the multi-component proteins associated with the 40S ribosome, eIF3 and eIF2.



Two other groups have proposed mechanisms of miRNA repression consistent with reduced 60S ribosome recruitment to translationally-repressed mRNAs in *C. elegans*, human cells and *D. melanogaster* embryonic lysate (Chendrimada et al., 2007; Thermann and Hentze, 2007). One study found that 60S anti-association factor eIF6 associates with RNA-induced silencing complexes, but not necessarily with miRNA-targeted mRNAs. In agreement with the data presented in this chapter, another group reported that large (>80S, or 'pseudopolysome') complexes are formed on miRNA-targeted mRNAs, even when 60S ribosomal subunit joining or translation elongation is inhibited (Thermann and Hentze, 2007). However, contrary to our data showing that high molecular mass complex formation is cap-dependent, pseudopolysomes form on mRNAs lacking a 7-methyl guanosine cap. These observations imply important similarities between miRNA-mediated translation repression across species, but also suggest distinguishing details in the mechanisms of miRNA-mediated repression in these organisms. We believe that the high molecular mass complex that forms on miRNA-targeted mRNAs in mammalian lysate contains the miRNP, and that a similar complex forms on miRNA-targeted mRNA in cells. All evidence to date supports the model that miRNAs repress, rather than completely abolish the translation of most target mRNAs. In our model, repressing the efficiency of 60S subunit joining causes reduced translation initiation, and therefore reduced ribosome density on miRNA-targeted mRNAs. We speculate that this is why miRNA-targeted mRNAs co-sediment

with polysomes on polysome gradients from *C. elegans* and mammalian cells (Olsen and Ambros, 1999; Petersen et al., 2006; Seggerson et al., 2002). Further analyses in cell-based and cell-free systems will more precisely define the mechanism(s) of miRNA function in mammals and their similarities and differences across species.

Ago2 (co-eIF2A) was originally defined as a ribosome-associated protein that eluted in high salt (Chakravarty et al., 1985)and stabilized 40S-containing complexes in the presence of mRNAs (Roy et al., 1988). We have shown that the high molecular mass complex formed on translationally-repressed mRNAs contains 40S ribosome subunits but lacks 60S subunits. Consistent with a role in stabilizing 40S ribosomes associated with mRNAs, Ago2 is recruited to unrepressed mRNAs (fraction 8, Ago2 upper panel (-), Figure 2.3B). Ago2 is also recruited to translationally-repressed mRNA (fraction 14, Figure 2.3B) possibly because these mRNAs contain 40S subunits without joined 60S subunits. It has been shown that Ago2 interacts with the anti-association factor eIF6 through TRBP and prevents 60S subunit joining to translationally-repressed mRNAs (Chendrimada et al., 2007). Our data supports a possibly related or complimentary mechanism that Ago2 interacts with translationally-repressed mRNAs and prevents 60S subunit joining. Together, these data suggest that Ago2 may function in more than one way to repress translation.

2.5. Author attributions

Data presented in Chapter 2 was produced in collaboration with Drs. Bingbing Wang and Carl Novina and is reprinted from the following published work: Wang B, Yanez A, Novina CD. Proc Natl Acad Sci U S A. 2008 Apr 8;105(14):5343-8. Epub 2008 Apr 4. BW, and CN designed the experiments. BW and AY performed the experiments and data analysis. BW, AY and CN wrote the manuscript. The data presented in each figure was generated by the authors as follows:

Figure 2.1B-C: BW
Figure 2.1D: BW and AY
Figure 2.2A-F: BW and AY
Figure 2.2G: BW
Figure 2.2 H: BW and AY
Figure 2.3A: BW
Figure 2.3B: BW and AY
Figure S2.1A-B, D: BW
Figure S2.1C: BW and AY
Figure S2.2A-B: BW
Figure S2.2C-D: AY
Figure S2.3B: BW
Figure S2.4: BW

2.6.Experimental procedures

Translation repression reactions. All mRNA reporters used in these studies were prepared and used as described previously (Wang et al., 2006). Plasmids expressing all of these mRNA reporters are available from (www.addgene.org). The sequences of the CXCR4 miRNA were 5'P-GUUUUCACUCCAGCUAACACA-3 (sense strand) and 5'P-UGUUAGCUGGAGUGAAAACUU-3' (antisense strand). The sequences of the GFP siRNA were 5'P-GGCUACGUCCAGGAGCGCACC-3' (sense strand) and 5'P-UGCGCUCCUGGACGUAGCCUU-3' (antisense strand). The control mRNA reporter used in Supporting Online Fig. 3 was human CD3 (kind gift of Dr. Chenqi Xu).

Translation repression reactions were performed as described previously (Wang et al., 2006). Briefly, pre-annealed mRNA reporter (0.025 pmole) and CXCR4 miRNA (0.15 pmole) were incubated with a master mix containing 7 μL nuclease-treated rabbit reticulocyte lysate (RRL, Promega), 4-8 U RNase Out (Invitrogen), 20 μM amino acid mixture (complete or minus methionine and cysteine, Promega), and 0.4 μL (5.7 μCi) Promix L-[^{35}S] *in vitro* cell labeling mix (Amersham Biosciences) at 30^{o}C for 10 min. Reaction products were separated on 12% SDS-PAGE and transferred onto PVDF (BioRad) or subjected to dual luciferase assay.

Dual luciferase assay. Dual luciferase assays were performed according to manufacturer's protocols (Promega). Firefly luciferase activity was measured by adding

2 μL of each reaction with LAR I (20 μL) into one well of a 96-well plate and read in Victor^3 V (PerkinElmer) for 5 sec. *Renilla* luciferase activity was measured by adding Stop & Glo (20 μL) to each well and re-read for 5 sec.

Ribosome binding assay. Ribosome binding assays were performed as described previously (18). *In vitro* translation repression reactions supplemented with Cycloheximide (600 μM), GMP-PNP (1 mM), or Hippuristanol (50 μM) were loaded onto 10-30% glycerol gradient containing 1X HSB (500 mM NaCl, 20 mM Hepes-KOH, pH 7.5, 30 mM MgOAc, and 2 mM DTT). Glycerol gradients were ultracentrifuged using an SW41 rotor (Beckman) at 39,000 rpm for 3.5 hrs, sequentially fractionated (500 μL) from the top, and subjected to Cerenkov scintillation counting.

Precipitation of biotinylated mRNAs. Streptavidin agarose (SAA) beads (Invitrogen) were used to precipitate biotinylated mRNA reporters. SAA beads (100 μL) were washed in 1X HSB buffer three times and incubated with glycerol gradient fractions (200 μL) or whole lysate reactions at 4^{o}C for 60 min. Reactions were centrifuged and supernatants were removed. Precipitates were washed twice in 1X HSB and subjected to RNA extraction and precipitation or to Western blot analysis.

Northern blotting analysis. The Northern blotting analysis was performed by PAGE as described previously (Wang et al., 2006). RNAs extracted from SAA precipitates were separated on 8% PAGE containing urea (7M) and transferred to Hybond N+ membranes

(Amersham Biosciences) for 2.5 hr at 350 mA. After UV cross-linking, membranes were hybridized with 5′ end labeled primers for 5S rRNA, 5′-TTAGCTTCCGAGATCA-3′; 5.8S rRNA, 5′-GCTAGCGCTGCGTTCTTCATCGACGC-3′; 28S rRNA5′-AACGATCAGAGTAGTGGTATTTCACC-3′; 18S rRNA, 5′-CGGAACTACGACGGTATCTG-3′; and tRNAi-Met, 5′-GGTAGCAGAGGATGGTTTCGATCC-3′. Membranes were washed, visualized, and analyzed by PhosphorImager (Molecular Dynamics).

Western blot analysis. The SAA precipitates were resuspended in 1X SDS loading buffer, boiled at 95^{o}C for 5 min., and centrifuged. Supernatants were resolved on SDS-10% PAGE and transferred onto PVDF membranes (BioRad). Membranes were blocked in 5% nonfat milk powder in PBST (10 mM phosphate buffer, pH 7.2, 150 mM NaCl, and 0.1% Tween 20) for 60 min., washed twice with PBST, and incubated with antibodies in 1% nonfat milk powder-PBST at 4^{o}C overnight. The anti-Ago2 antibody (Upstate) recognizes residues 7-48 of human Ago2, which are conserved amino acids between human, mouse, cattle, dog, and frog. Anti-eIF2α, anti-eIF4A, and anti-eIF4E were kind gifts from Dr. Jerry Pelletier. The anti-eIF3g antibody was a kind gift from Dr. Hiroaki Imataka. Anti-RPS7 and anti-RPL18 antibodies were used according to manufacturer's protocol (Abnova). Membranes were washed three times with PBST, incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson

ImmunoResearch) at 1:5,000 in 1% nonfat milk powder-PBST, and were developed by ECL (Pierce).

To strip Western blots of antibody complexes, membranes were incubated in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) at 50°C for 30 min. These membranes were washed with PBST for 2 X 10 min., blocked in 5% milk-PBST, and re-probed with appropriate antibodies.

Toeprinting assay. Translation repression reactions containing mRNA (0.1 pmole) and CXCR4 siRNA (0.6 pmole), RRL (7 μL) and MgOAc (2 mM) with or without GMP-PNP (1 mM) or Hisppuristanol (50 μM) proceeded for 5 min. at 30°C. Then, reverse transcription (RT) mix containing dNTPs (5 mM), 1X reconstitution buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl and 1 mM DTT), 5′ end labeled primer (0.2 pmole, 5′-TTATGCAGTTGCTCTCCAGCG-3′), and M-MLV RT (1 μL, Invitrogen) was added to translation repression reactions. These mixtures were incubated for 15 min. at 30°C and were subjected to deproteinization and ethanol-precipitation. RNAs were resolved on a 10% sequencing gel (National Diagnotics) and visualized by Phosphorimager analysis (Molecular Dynamics).

Argonaute2-Mediated Cleavage Assays of siRNA Targeted RNA. The miR21 target RNA was based upon the substrate described by Meister et al. (Meister et al., 2004). An oligodeoxyribonucleotide corresponding to the miR-21 target RNA with the sequence 5′

GA ACA AT TGCT T T TACAGATGCACATATCGAGGTGA-

ACATCACGTACGTCAACATCAGTCTGATAAGCTATC-

GGTTGGCAGAAGCTAT-3′ (miR-21 complementary site underlined), was synthesized (Integrated DNA Technologies) and gel-purified. This DNA template was amplified by PCR using T7 promoter-containing primers 5′-TAATACGACTCACTATAG-

GAACAATTGCTTTTACAG-3′ (forward, T7 promoter se- quence underlined) and 5′-

ATAGCT TCTGCCA ACCGA-3′ (reverse).

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3. eIF4A and eIF4E modulate miRNA mediated repression and transformation phenotypes in melanoma cells

3.1. Abstract

The effect of miRNAs on translation initiation led us to investigate the influence of initiation factors on miRNA function in cells. Misregulation of protein translation is a common feature of many cancers. eIF4A1 and eIF4E-driven oncogenesis is thought to occur through differential regulation of mRNA 5′ untranslated regions (UTRs) based on sequence or secondary structural determinants. Here we show that modulation of eIF4A1 and eIF4E expression positively regulates melanoma cell proliferation, invasion, and miRNA activity. Transcriptome-wide measurements of mRNA abundance and translation efficiency identified over 1400 common mRNAs with significantly increased translation efficiency in both eIF4A1 and eIF4E knockdown. Endogenous miRNA target sites are enriched within this mRNA subset, including putative melanoma tumor suppressors. Moreover, there is substantial overlap in transcripts with significantly increased translation efficiency in eIF4A1, eIF4E and Ago2 knockdown. Our data establish eIF4A1 and eIF4E as positive regulators of endogenous miRNA-activity.

3.2. Introduction

In healthy cells, the complex process of protein translation is extensively regulated by cell growth and stress signaling pathways. In cancer cells, this regulation is perturbed (reviewed in (Silvera et al., 2010; Stumpf and Ruggero, 2011)). Misregulation of translation by altered activity or expression of translation and ribosomal factors is a common feature of many cancers (reviewed in (Stumpf and Ruggero, 2011)). Eukaryotic translation is regulated primarily at the rate-limiting step of initiation- recruitment of the 43S preinitiation complex, which is facilitated by eIF4F, the heterotrimeric mRNA cap-binding complex. eIF4F is comprised of eIF4E, the mRNA 5′ cap binding protein; eIF4G, a molecular bridge between eIF4E and the 43S preinitiation complex; and eIF4A (paralogues 1 and 2), an ATP-dependent helicase. eIF4F formation is limited by the availability of eIF4E.

The expression and activity of eIF4E and eIF4G promote tumorigenesis and metastasis (Fukuchi-Shimogori et al., 1997; Lazaris-Karatzas et al., 1990). Although it was initially thought that increased eIF4A1 and eIF4E expression was a consequence, rather than a cause of malignant transformation, several reports suggest oncogenic roles for both factors (Avdulov et al., 2004; Larsson et al., 2006; 2007). eIF4E expression is increased in up to 30% of human cancers and many melanoma samples (Carroll and Borden, 2013; Yang et al., 2007). Increased eIF4E activity via 4E-BP1 phosphorylation is

associated with poor survival of melanoma patients (O'Reilly et al., 2009). eIF4AI expression is increased in melanoma cell lines compared to melanocytes, with intermediate expression in benign melanocytic nevi (Eberle et al., 1997). Suppression of eIF4E activity or expression inhibits proliferation, invasion, and migration of cancer cells *in vitro* as well as tumor initiation and growth of human xenografts *in vivo* (Chen et al., 2012a). Likewise, decreased eIF4A1 expression or activity decreases proliferative and invasive phenotypes of cancer cell lines, including melanoma (Eberle et al., 2002; Nasr et al., 2012b).

eIF4A1 and eIF4E are downstream effectors of the phosphoinositide 3-kinase-mammalian target of rapamycin (PI3K-mTOR) pathway, and indirectly regulated by the Ras-mitogen activated protein kinase (MAPK) pathway. The MAPK pathway was found to be aberrantly activated by BRAF or NRAS mutations in 50% and 15%, respectively, of melanoma cases (Tsao et al., 2012). Signaling flux through the mTORC1 pathway increases levels of active eIF4E and eIF4A through phosphorylation and release of two repressor proteins; eIF4E binding proteins (4E-BPs), and PDCD4, an inhibitor of eIF4A, respectively. (Dennis et al., 2012). Activation of the mTORC1 pathway is associated with poor survival of melanoma patients (O'Reilly et al., 2009). Treatment with mTORC1 ATP-site inhibitors decreased the invasive phenotype in a human prostate cancer cell line, primarily through the eIF4E-4EBP axis. (Hsieh et al., 2013). Additionally, activation

of both the PI3K-mTORC1 and Ras-MAPK pathways increases activity of eIF4B, an enhancer of eIF4A (Dennis et al., 2012).

Despite their fundamental roles in malignant transformation and potential as therapeutic targets, the detailed mechanisms of eIF4A1 and eIF4E oncogenic activity remain unclear. Upon eIF4E modulation, the translation of a subset of mRNAs is differentially regulated. Initial studies showed that mRNAs with highly structured 5′ UTRs, including proto-oncogenes and growth factors are among this subset (Koromilas et al., 1992). However, in contradiction to the long-standing model that 5′UTR length or secondary structure was the major mRNA determinant of translational efficiency, genome-wide studies have identified mRNAs with 5′ terminal oligopyrimidine (TOP) tracts or pyrimidine-rich translational elements (PRTE) as sensitive to eIF4E activity (Hsieh et al., 2013; Huo et al., 2012a; Mamane et al., 2007; Santhanam et al., 2009; Thoreen et al., 2012). Although little is known about the mechanism of eIF4A1 proto-oncogenic activity, it is thought to increase translation efficiency of transcripts with highly structured 5′ UTRs (Svitkin et al., 2001).

3.3.Results

3.3.1.eIF4A1 and eIF4E are positive regulators of melanoma cell proliferation and invasion

To interrogate the effect of eIF4A1 and eIF4E modulation on melanoma cell proliferation, migration, and invasion, we utilized A375, a melanoma cell line; WM46, a short term culture with low eIF4E expression; and WM1575, a short term culture with high eIF4E expression. Melanoma short term cultures are grown directly from patient samples without further manipulation and have undergone a limited number of passages in culture. Knockdown of eIF4A1 or eIF4E (Fig. 3.1A & B) leads to decreased proliferation relative to the nonspecific siRNA control in both A375 and WM1575 cells (Fig. 3.2A).

Figure 3.1. Knockdown or stable overexpression of eIF4A1 and eIF4E in melanoma cell lines.

(A) Immunoblot analysis of transient knockdown of eIF4A1 (si4A1), eIF4E (si4E), Ago1 (siAgo1), Ago2 (siAgo2) or 'scrambled' nonspecific siRNA control (siSCR) in A375 cells. Primary antibodies are labeled to the left of the blots, transfected siRNAs are listed on the top.

(B) Immunoblot analysis of transient knockdown of factors as in (A) in WM1575 melanoma short term culture.

(C) Immunoblot analysis of stable overexpression of eIF4A1, eIF4E or empty vector in A375 cells.

(D) Immunoblot analysis of stable overexpression of eIF4E in WM46 melanoma short term culture.

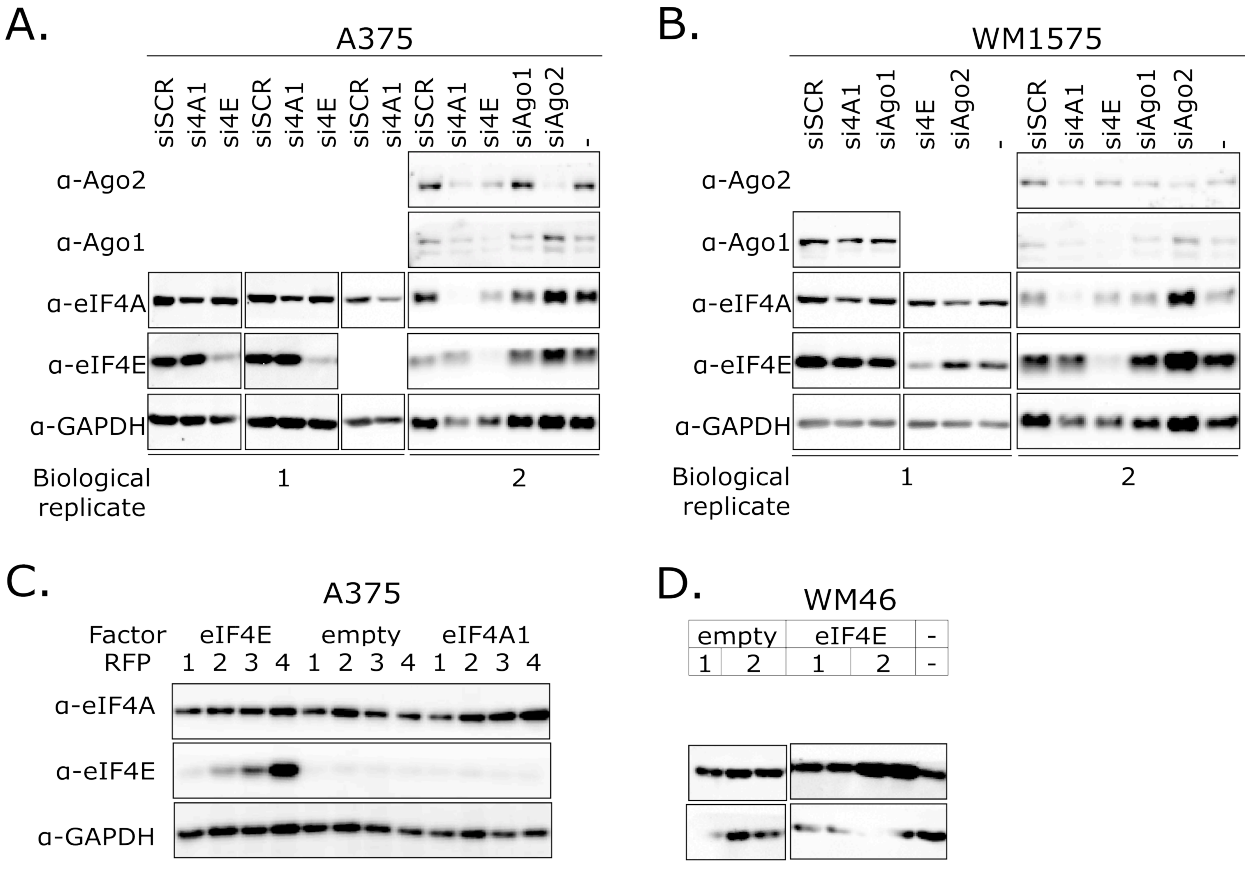


Figure 3.2. eIF4AI and eIF4E are positive regulators of melanoma cell proliferation and invasion.

(A) Melanoma cell line (A375, left panel) or short term culture (WM1575, right panel) proliferation following siRNA knockdown of eIF4A1 or eIF4E. Absorbance values were measured after incubation with Wst-1 colorimetric reagent, normalized to day one and are shown relative to Control (si-SCR) transfection. Points represent mean, error bars are S.E.M. from three independent experiments.

(B) Melanoma cell line (A375, left panel) or short term culture (WM46, right panel) proliferation with stable eIF4A1 or eIF4E overexpression. Values derived as in A and shown relative to empty vector control.

(C-F) Line represents mean number of cells per field from 6-8 image fields, error bars are S.E.M.

(C) Melanoma cell line (A375, left panel) or short term culture (WM1575, right panel) migration through a control insert membrane following siRNA knockdown of eIF4A1 or eIF4E.

(D) Melanoma cell line (A375, left panel) or short term culture (WM46, right panel) migration with stable eIF4A1 or eIF4E overexpression.

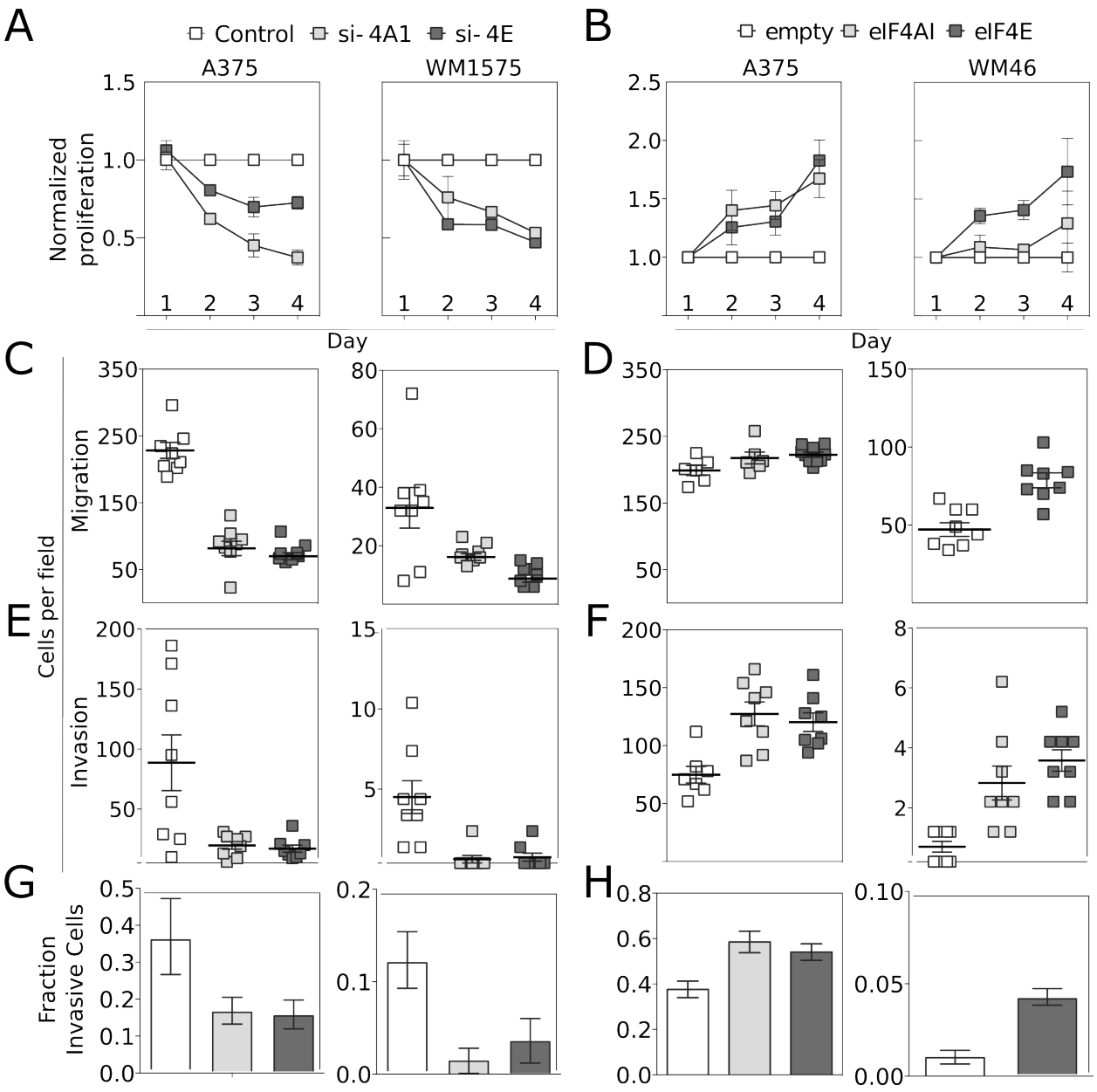
(E) Melanoma cell line (A375, left panel) or short term culture (WM1575, right panel) invasion through a Matrigel-coated membrane following siRNA knockdown of eIF4A1 or eIF4E.

(F) Melanoma cell line (A375, left panel) or short term culture (WM46, right panel) invasion with stable eIF4A1 or eIF4E overexpression.

(G) The fraction of invasive per total migrated cells for melanoma cell line A375 (left panel) or short term culture WM1575 (right panel) following siRNA knockdown of eIF4A1 or eIF4E. Bar represents mean, error bars are S.E.M.

(H) The fraction of invasive to total migrated cells for melanoma cell line A375 (left panel) or short term culture WM46 (right panel) with stable overexpression of eIF4A1 or eIF4E. Values represented as in G.

Figure 3.2 (continued)



We created A375 and WM46 cell lines that stably overexpress eIF4A1, eIF4E, or the empty expression vector (Fig. 3.1C & D). Overexpression of eIF4A1 or eIF4E leads to increased cell proliferation relative to empty vector in A375 cell lines (Fig. 3.2B, left panel). In WM46, overexpression of eIF4E, but not eIF4A1, increases cell proliferation (Fig. 3.2B, right panel). Knockdown of eIF4A1 or eIF4E leads to decreased migration (Fig. 3.2C) and Matrigel invasion (Fig. 3.2E) of A375 and WM1575 cells. In both cell lines, the fraction of invasive cells is reduced by more than two-fold (Fig. 3.2G). In A375 cells, overexpression of eIF4A1 or eIF4E leads to increased invasion (Fig. 3.2F, left panel) and percent invasive cells (Fig. 3.2H, left panel), but not migration (Fig. 3.2D, left panel). Overexpression of eIF4E leads to increased migration and invasion of WM46 cells (Fig. 3.2D, F, right panels) and a four-fold increase in percent invasive cells (Fig. 3.2 H, right panel). In A375 cells, eIF4A1 and eIF4E knockdown causes greater phenotypic changes than overexpression, possibly due to relatively high endogenous eIF4A and eIF4E expression. Together, these data show that eIF4A1 and eIF4E positively regulate melanoma cell proliferation and invasion.

3.3.2. eIF4A1 and eIF4E modulate miRNA activity

To determine the effect of eIF4A1 and eIF4E modulation on miRNA activity, we transfected a miRNA-targeted reporter construct with control or miR-CXCR4 siRNA (Fig. 3.3A) into cells following knockdown or overexpression of the initiation factor.

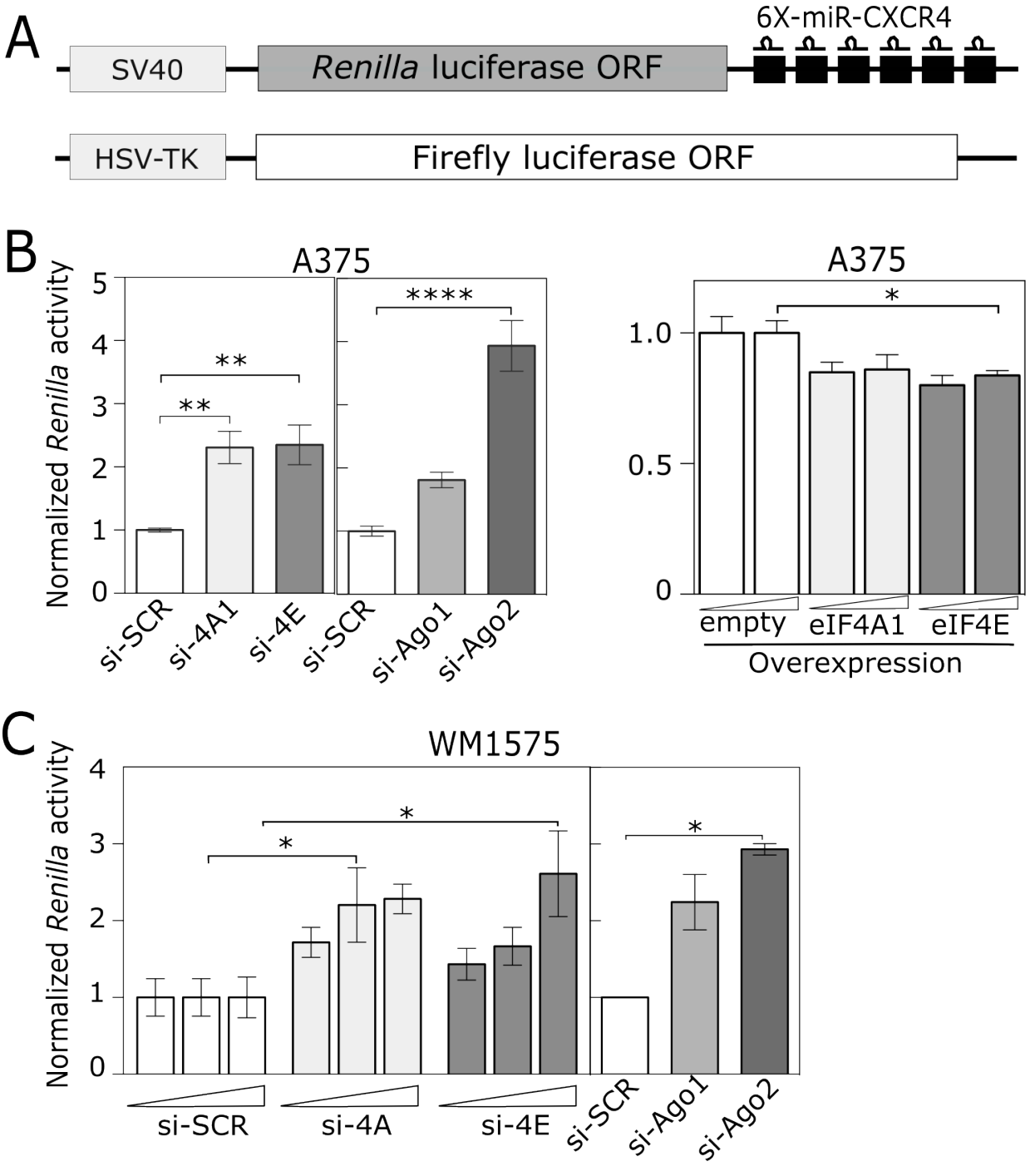
Figure 3.3. Modulation of eIF4A1 and eIF4E affects miRNA-mediated repression.

(A) Schematic representation of SV40-driven *Renilla* luciferase reporter construct with six miR-CXCR4 target sites in the 3′ UTR and HSV-TK-driven firefly luciferase construct with no miRNA binding sites.

(B) Luciferase activity for miRNA-targeted reporter normalized to untargeted reporter in A375 cell line following transient knockdown (left panel) of eIF4A1 (si-4A1), eIF4E (si-4E), Ago1 (si-Ago1), Ago2 (si-Ago2) or increasing levels of overexpression (right panel) of empty vector, eIF4A1 or eIF4E. Values are shown relative to indicated control (si-SCR or empty vector) and represent the mean of 2-3 independent experiments, n=6-9, error bars show standard error of the mean. *p<0.05, **p<0.01, ****p<0.0001 as determined by one-way ANOVA.

(C) Reporter assay as in (B) in melanoma short term cell culture WM1575 following transient knockdown of eIF4AI, eIF4E, Ago1 or Ago2. Increasing amounts of si-4A1 and si-4E were used (1 nM, 7.5 nM, 15 nM). Values are shown relative to control (si-SCR) and represent the mean of 2-3 independent experiments, n=5-9, error bars show standard error of the mean. *p<0.05 as determined by one-way ANOVA.

Figure 3.3 (continued)



Knockdown of eIF4A1 and eIF4E causes decreased miRNA activity, with derepression of the reporter compared to control siRNA transfection in A375 (Fig. 3.3B, left panel) and WM1575 cells (Fig. 3.3C). We verified the specificity of this effect by using a second siRNA to knockdown eIF4A1 or eIF4E in A375 cells and found that the miRNA-targeted reporter is derepressed to a similar degree (Fig. S3.2, left panel). Similarly, knockdown of Ago2 individually or in combination with Ago1 leads to derepression of the miRNA-targeted reporter (Fig. S3.2, left panel). Overexpression of eIF4A1 and eIF4E has the opposite effect and increases miRNA activity, with enhanced repression of the reporter compared to empty expression vector (Fig. 3.3B, right panel). As observed with the changes in melanoma phenotypes, knockdown of eIF4A1 and eIF4E causes greater fold changes in miRNA activity than overexpression. Together, these data show that eIF4A1 and eIF4E regulate miRNA-mediated repression of a reporter gene in melanoma cells.

3.3.3. Knockdown of eIF4A1 or eIF4E in melanoma leads to bi-directional changes in mRNA translation

To measure the effect of eIF4A1 and eIF4E knockdown on mRNA translation efficiency, we resolved total A375 cell lysate on 10-50% sucrose gradients (Fig 3.4A), then extracted RNA for cDNA library preparation from total lysate and pooled monosomal or polysomal gradient fractions (Fig. 3.5A).

Figure 3.4 Global translation is not significantly affected in response to eIF4A or eIF4E knockdown or overexpression.

(A) Polysome profiles from knockdown (si, top panels) and overexpression (bottom panels) of eIF4A1, eIF4E or control treatments in A375 cells.

(B) Quantification of peak height.

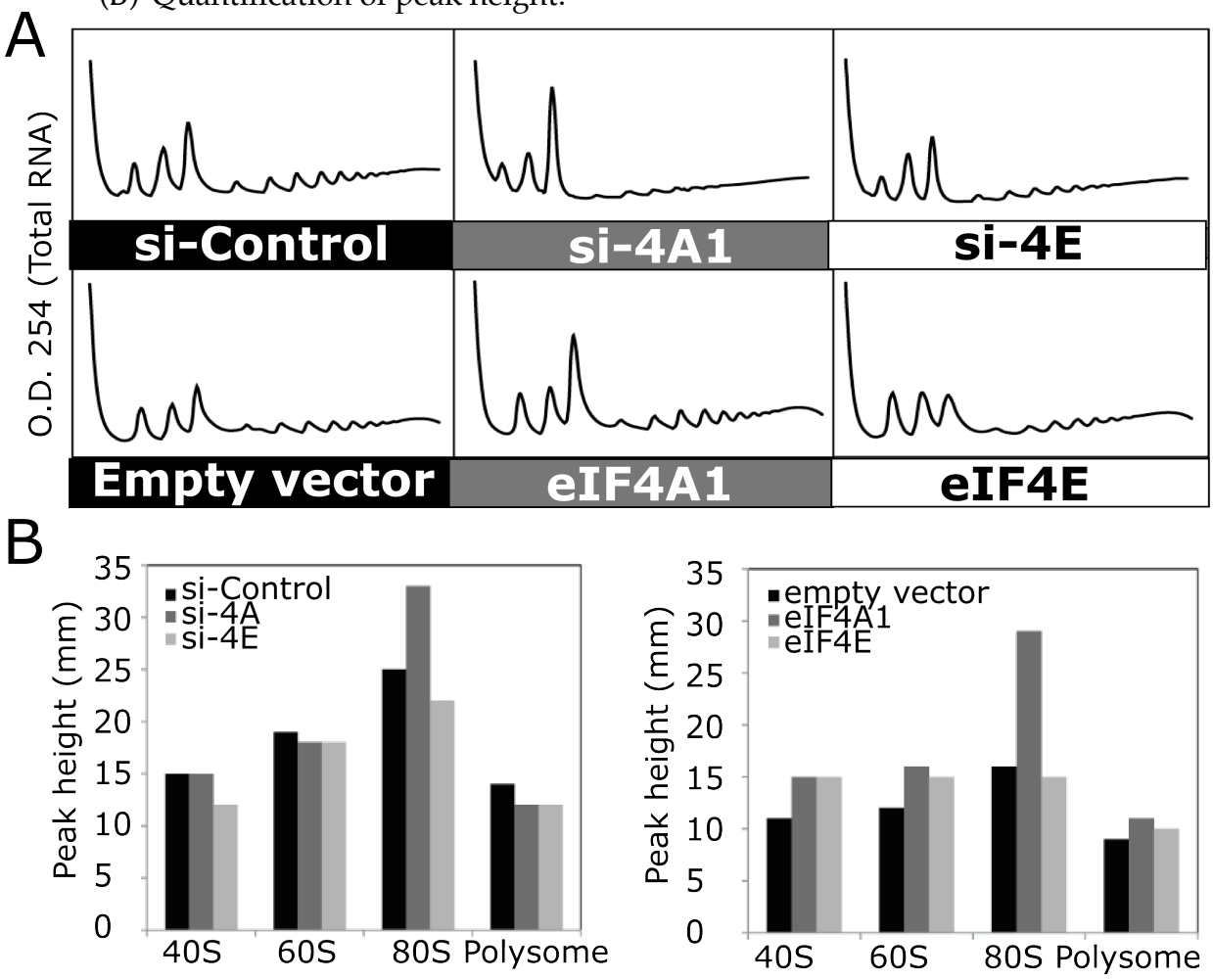


Figure 3.5. Knockdown of eIF4A1 or eIF4E in melanoma leads to bi-directional changes in mRNA translation.

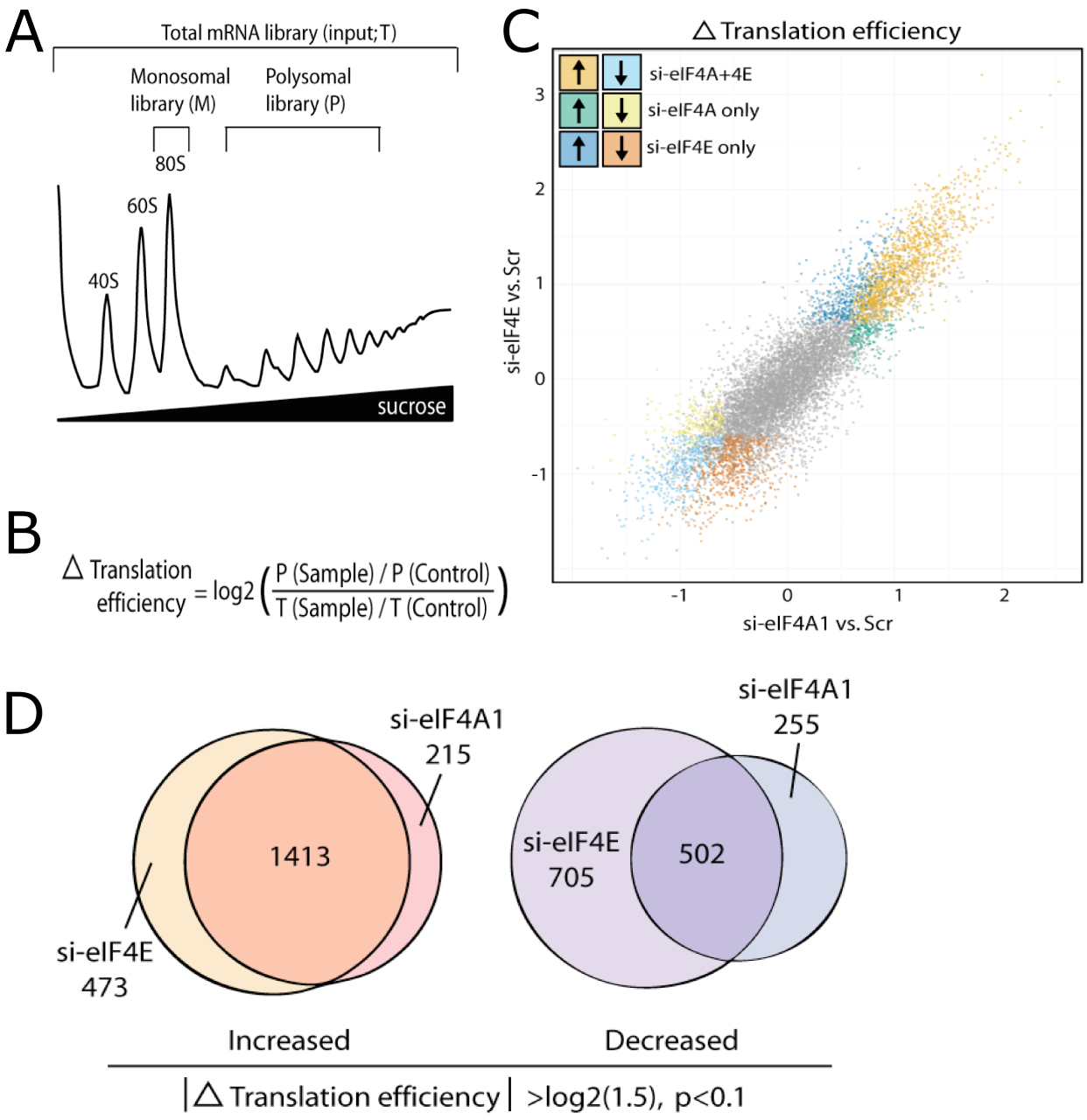
(A) Schematic representation of polysome profiling. Total cell lysate was resolved on 10-50% sucrose gradients and monosomal and polysomal fractions were pooled prior to RNA extraction and cDNA library preparation.

(B) Equation for calculating the translation efficiency for mRNA transcripts.

(C) Definition of eIF4A1- and eIF4E-responsive mRNA subsets based on translation efficiency. Transcripts were assigned to six different subsets based on fold-change and p-value cut-offs ($|\text{Fold change}| > 1.5$, $p < 0.1$).

(D) Overlap in mRNAs with significantly increased (left) or decreased (right) translation efficiency in eIF4AI or eIF4E knockdown treatments.

Figure 3.5 (continued)



There were no obvious differences in 40S, 60S and 80S peak heights of the polysome profiles from each sample (Fig. 3.4B). In both knockdown and overexpression of eIF4A1, there are increased 80S peaks. We did not observe corresponding increased mRNA content in gradient fractions containing 80S ribosomes, suggesting that these ribosomes are not loaded onto mRNA. The increased 80S peak seems to be eIF4A1 specific, as we did not see similar increases in eIF4E knockdown or overexpression. Generally, the large 80S peak corresponds to 80S ribosomes formed by aberrant and mRNA-independent joining of the 40S and 60S ribosomal subunits during lysate preparation. RNA sequencing was performed on each library and the change in translation efficiency for each transcript was calculated as the ratio of the fragment per kilobase of million mapped reads (FPKM) value from the polysome fraction of test to control sample divided by the same value for the total fraction (Fig 3.5B). This metric isolates the change translation efficiency from transcriptional changes. An overview of eIF4A1 and eIF4E-responsive transcripts reveals bidirectional changes in translation efficiency, with six mRNA subsets defined by increased or decreased translation efficiency in eIF4A1, eIF4E or both knockdowns (Fig. 3.5C). The translation efficiency of 1,462 transcripts was significantly decreased in either or both knockdowns (Fig. 3.5D, right panel), with 502 transcripts common to this subset in both eIF4A1 and eIF4E knockdown. The translation efficiency of 2,101 transcripts was significantly increased in either or both knockdown (Fig. 2D, left panel), with 1,413 transcripts common to this

subset in both eIF4A1 and eIF4E knockdown. Additionally, we calculated the change in monosome loading efficiency analogously to that of translation efficiency by using the FPKM values from the monosome fraction (Fig. S3.3A). The changes in translation versus loading efficiency are shown for mRNA transcripts that were changed (| Fold change | >1.5, $p<0.1$) in both eIF4A1 and eIF4E knockdowns.

3.3.4. eIF4A1 and eIF4E are positive regulators of endogenous microRNA-mediated translational repression

Because knockdown of eIF4A1 and eIF4E caused derepression of miRNA-targeted reporter genes, we wanted to determine if derepressed targets of endogenous miRNAs were among the mRNA subset with increased translation efficiency in both eIF4A1 and eIF4E knockdowns. Indeed, among this subset, there is an enrichment ($p=2.6 \times 10^{-16}$) of transcripts with one or more A375-expressed miRNA binding sites ('observed') than predicted from control sets of transcripts with matched 3' UTR length ('expected,' Fig. 3.6A, upper panel). Among the mRNA subset with decreased translation efficiency in eIF4A1 and eIF4E knockdown, there is no significant difference ($p=0.24$) in the fraction of transcripts with at least one A375 miRNA binding site (observed) compared to the control sets of transcripts (expected, Fig. 3.6A, lower panel). There are 590 transcripts that are commonly increased in eIF4A1, eIF4E and Ago2 knockdown, further supporting a role for eIF4A1 and eIF4E in endogenous miRNA-mediated repression (Fig. 3.6B). It is important to note that the expression of the majority of miRNAs did not change upon eIF4A1 or eIF4E knockdown (Fig. S3.3B), therefore changes in translational efficiency of transcripts with miRNA binding sites are more likely reflect changes in miRNA activity level rather than altered miRNA expression levels.

Changes in translational efficiency were of a lesser degree in eIF4A1 or eIF4E overexpression compared to knockdown in A375 cells (Fig. S3.5A & B). This is

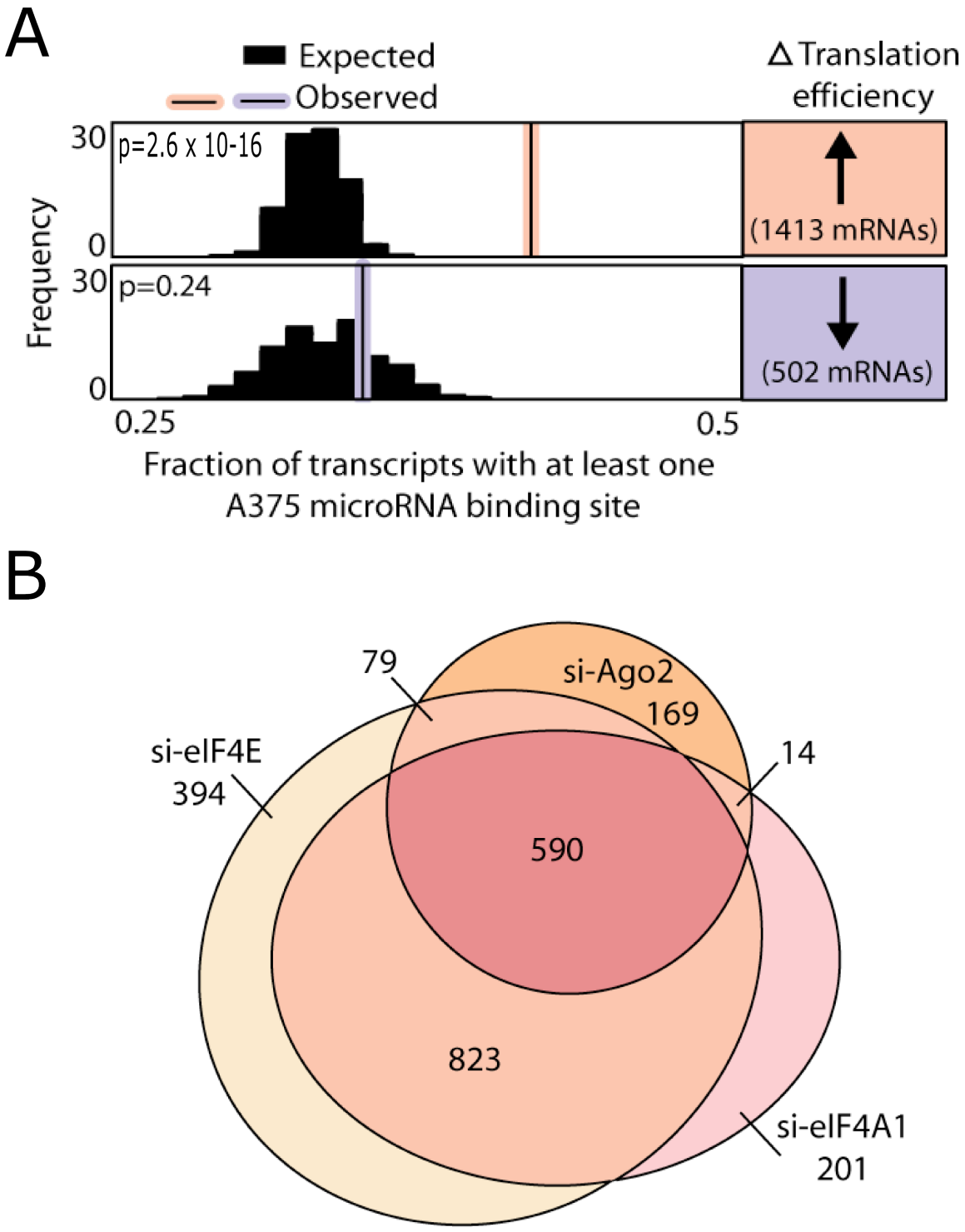
consistent with our data from reporter assays (Fig. 3.3B), where the fold change of derepression is greater than that of enhanced repression in eIF4A1 and eIF4E knockdown and overexpression, respectively. Similarly, there is a greater change in A375 cellular proliferation and invasion when eIF4A1 and eIF4E are knocked down versus overexpressed (Fig 3.1) It is important to note that knockdown and overexpression have generally opposite effects on the translation efficiency of the mRNA subset that is discordantly regulated by eIF4A1 and eIF4E modulation. That is, this mRNA subset has *increased* translation efficiency in eIF4A1 and eIF4E knockdown, while it has *decreased* translation efficiency in eIF4A1 and eIF4E overexpression (Fig. S3.5C).

Figure 3.6. eIF4AI and eIF4E are positive regulators of endogenous miRNA-mediated translational repression

(A) Proportion of binding sites for A375-expressed microRNAs in discordant transcripts commonly regulated by eIF4AI and eIF4E. The same proportion was determined for 1,000 sets of control genes that matched the original increased or decreased sets in 3'UTR length (black histogram).

(B) Overlap between discordant transcripts commonly regulated by eIF4AI and eIF4E and transcripts with increased translation efficiency following Ago2 knockdown (Fold change>1.5; si-eIF4AI and si-eIF4E, $p<0.1$; si-Ago2, no p-value cut-off).

Figure 3.6 (continued)



3.4.Conclusions

We have shown that the translation efficiency of an mRNA subset *increases* significantly upon knockdown of eIF4A1 or eIF4E, and generally *decreases* upon eIF4A1 or eIF4E overexpression. Importantly, A375-expressed miRNA binding sites are enriched in the subset with increased translation efficiency. This is consistent with our results from reporter assays, where miRNA activity is decreased upon eIF4A1 or eIF4E knockdown and enhanced in cells that overexpress eIF4A1 or eIF4E. Taken together, these data reveal that eIF4A1 and eIF4E are enhancers of miRNA activity. The only previously previous study on this subject reported that transcripts with increased translation efficiency upon eIF4E overexpression or activation showed negative selection for miRNA binding sites (Santhanam et al., 2009). This is analogous to our findings that transcripts with increased translation efficiency in eIF4E knockdown are enriched for miRNA binding sites. Taken together, these data support the role of eIF4E as a positive regulator of miRNA activity.

This is the first transcriptome-wide study of changes in mRNA translation efficiency caused by eIF4A1 modulation. Because eIF4A1 is an RNA helicase, it was expected that mRNAs with long, highly-structured 5′ UTRs would shift concordantly with eIF4A1 modulation. We found this to be partially correct; GC content is greater in transcripts with reduced translation efficiency compared to those with increased

translation efficiency in eIF4A1 and eIF4E knockdown (Fig. S3.4). That is, 5′ UTR, coding sequence and 3′ UTR GC content are correlated with transcript sensitivity to eIF4A1 and eIF4E levels. This sensitivity is defined by concordant changes in mRNA translational efficiency upon eIF4A1 and eIF4E modulation. This is consistent with a previous report that increased mRNA translation efficiency upon eIF4E overexpression or activation is positively correlated with higher mRNA GC content (Santhanam et al., 2009).

It was recently reported that knockdown of eIF4A2, but not eIF4A1, in Hela cells causes derepression of reporter and endogenous miRNA targets (Meijer et al., 2013). In agreement with this, we found that in A375 and WM1575 cell lines, knockdown of eIF4A2 leads to derepression of a miRNA-targeted reporter (Fig. S3.2). The reason for the discrepancy in our reporter assay results following knockdown of eIF4A1 is unclear, but our findings strongly suggest that eIF4A1 does modulate both reporter and endogenous miRNA activity. It is important to reiterate that in mammalian cells, knockdown of many factors involved in translation, including small or large subunit ribosomal protein genes, eIF4GI, and poly(A) tail binding proteins PABPC1 or hnRNP-Q, has been reported to cause derepression of miRNA-targeted reporter gene and/or endogenous mRNAs (Janas et al., 2012b; Jannot et al., 2011a; Ryu et al., 2013; Svitkin et

al., 2013). Taken together, these findings suggest that interference with the translation initiation pathway leads to derepression of miRNA targets.

We have shown that eIF4A1 and eIF4E are positive regulators of miRNA activity. This finding functionally connects two determinants of mRNA translation; expression levels of eIFs and global miRNA activity. Individual miRNAs, like protein-coding genes, can act as tumor suppressor genes or oncogenes. Altered global or individual miRNA expression levels have been shown to affect cellular transformation, tumor formation and metastasis in many cancers (Kumar et al., 2007; Loreni et al., 2014). Altered expression or activity of factors involved in miRNA biogenesis and function, including Drosha, DGCR8, Dicer, Agos 1 and 2, TNRC6, PABPC1 also affect tumorigenesis (Naoghare et al., 2011). It follows that changes in global miRNA activity may contribute to tumorigenesis and metastasis. We have established the role of eIF4A1 and eIF4E modulation in miRNA activity. It will be important to characterize the downstream effects of misregulated miRNA targets and implications on the mechanism of eIF4A1 and eIF4E-driven oncogenesis.

To investigate the role of Ago1 and Ago2 in melanoma proliferation, we performed knockdown of each protein individually or together, then measured cell proliferation relative to control siRNA transfection (Fig. S3.1). Knockdown of Ago1, Ago2, or both does not significantly affect proliferation. There are conflicting reports on

the role of Ago proteins in various types of cancer. Some studies suggest that Ago1 or Ago2 may have tumor suppressive functions, while others suggest they may have oncogenic roles. Overexpression of Ago2 has been reported to have a tumor suppressive role in melanoma (Voller et al., 2013). Further study is necessary to understand the role of Ago1 and Ago2 modulation in miRNA activity and transformation phenotypes.

3.5. Author attributions

Data presented in Chapter 3 was produced in collaboration with Drs. Cailin Joyce and Carl Novina. Computational analyses of RNA-Seq data were performed by Drs. Cailin Joyce and Yogesh Saletore in collaboration with Dr. Christopher Mason. AY, CJ, and CN designed and performed the experiments. AY, CJ, YS and CM analyzed the data. AY wrote the introduction, results and conclusions sections. CJ wrote the figure legends for figures 3.4, 3.5, 3.6, S3.3, S3.4, and S3.5. The data presented in each figure was generated by the authors as follows:

Figure 3.1 A-D: AY
Figure 3.2 A-H: AY
Figure 3.3 B-C: AY
Figure 3.4 A-B: CJ
Figure 3.5 C,D: CJ & YS
Figure 3.6 A-B: CJ & YS
Figure S3.1 A-B: AY
Figure S3.2 A-B: AY
Figure S3.3 A-B: CJ & YS
Figure S3.4: CJ & YS
Figure S3.5: CJ & YS
Figure 4.4 B: AY

WM1575 and WM46 melanoma short term cultures (Figures 3.1, 3.2, 3.3) were obtained from the lab of Dr. Levi Garraway.

3.6.Experimental Procedures

Reagents and cell culture

Cell culture. Cells were cultured in Dulbecco's modified Eagle's medium high glucose (Cellgro) supplemented with 10% fetal bovine serum (Invitrogen) and 1% L-glutamine. A375 and WM46 cell lines were transduced with lentivirus containing empty SparQ IRES RFP vector (System Biosciences, catalog # QM531A-2), or vector containing eIF4A1 (Locus BC009585,Open Biosystems catalog # 4096621) or eIF4E cDNA (Locus BC043226, Open Biosystems catalog # 5295521). Cells were sorted by flow cytometry for RFP expression.

Transient gene knockdown. A375 and WM1575 cells were transfected according to manufacturers instructions with scrambled (Allstars Negative Control siRNA, Qiagen) or test siRNA using Lipofectamine RNAiMax (Life Technologies). Final siRNA concentration was 7.5 nM unless otherwise indicated. All siRNAs listed in the following table are Qiagen FlexiTube siRNAs.

siRNA	Catalog Number
eIF4E #1	Hs_EIF4E_1
eIF4E #2	Hs_EIF4E_8
eIF4A1 #1	unavailable for order
eIF4A1 #2	unavailable for order
eIF4A2 #1	Hs_EIF4A2_2
eIF4A2 #2	Hs_EIF4A2_7
Ago1	Hs_EIF2C1_4
Ago2 #1	Hs_EIF2C2_6
Ago2 #2	Hs_EIF2C2_5
ITGA3	Hs_ITGA3_7
UBC #1	Hs_UBC_3
UBC #2	Hs_UBC_5
BRCA1 #1	Hs_BRCA1_13
BRCA1 #2	Hs_BRCA1_14

Reporter gene assays. Cells were transfected with 100 ng psiCheck2-6X-CXCR4 and 15 nM (final concentration) scrambled (Allstars negative control, Qiagen) or CXCR4 siRNA (5'P-GUUUUCACUCCAGCUAACACA-3 [sense strand], 5'P-UGUUAGCUGGAGUGAAAACUU-3' [antisense strand]) using Lipofectamine 2000 (Life Technologies) in 48-well plate format 24 hours after initial siRNA transfection in gene knockdown experiments. Dual luciferase assays (Promega) were performed 48 hours after reporter transfection according to manufacturers instructions.

Expression analysis. For immunoblotting, cells were collected in cold 1X PBS, pelleted by centrifugation, and resuspended in polysome lysis buffer (5 mM Tris pH 7.5, 2.5 mM

$MgCl_2$, 1.5 mM KCl, 2 uM DTT, 1X protease inhibitor). Samples were mixed by vortex for five seconds, Triton-X100 and sodium deoxycholate were added to final concentrations of 0.5%, and centrifuged at full speed at 4° C for 2 minutes. Cleared supernatant was collected after centrifugation. Protein concentrations were determined by Pierce microBCA assay. Total protein lysates (15-20 μg) were resolved by 8% or 10% SDS-PAGE, transferred to membranes, probed with the listed antibodies, and visualized using ECL detection.

Antibody	Company, product number
α -GAPDH	Cell Signaling, #2118
α -eIF4E	Cell Signaling, #2067
α -eIF4A	Cell Signaling, #2013
α -Ago1	Millipore, #07599
α -Ago2	Cell Signaling, #2897

Cell proliferation and invasion assays. For proliferation assays, 250, 500 or 1000 cells were plated in triplicate in 96-well plates on day 0. On days 1-4, cell culture medium was replaced with 10 μl Wst-1 reagent (Pierce) in 100 μl complete medium, samples were incubated in growth conditions for 30 minutes, and absorbance at 450 nm was measured for test and blank wells (Victor 3V plate reader, Perkin Elmer). A450 blank readings were subtracted from test readings. Fold change for each sample condition was calculated by normalizing to Day 1 value. Fold change relative to control condition was calculated by normalizing to matched day control condition value.

For invasion assays 4.0 x 10^4 (gene knockdown) or 2.0 x 10^4 (gene overexpression) cells were washed to remove serum, resuspended in 0.5 mL serum free medium, and added to rehydrated control (BD Biocoat) or Matrigel invasion (BD Biocoat) chambers in wells containing 0.9 mL complete medium. Following 20 hours of incubation at 37°C, 5% CO_2, non-invading cells were removed from inserts by scrubbing twice with a cotton swab, inserts were washed in cold PBS and fixed in cold 100% methanol. Inserts were mounted with Vectashield (Vector Labs) and DAPI staining was visualized. Eight or more visual fields were photographed for cell counting. Invasion assay was performed 24 hours after siRNA transfection for for gene knockdown experiments.

Polysome profiling. A375 cells were grown to 80% confluence in 10cm^2 plates. Cells were washed once with 5ml 100 μg/ml cyclohexamide/PBS and scraped into 1ml 100 μg/ml cyclohexamide/PBS. Cells were pelleted for 10min, 2000rpm, 4˚C. Cells were resuspended in 500 μl polysome lysis buffer (5mM Tris pH 7.4, 2.4 mM $MgCl_2$, 1.5mM KCl), freshly supplemented with 10 μg/ml cycloheximide, 2uM DTT, 0.5% Triton-X and 0.5% sodium deoxycholate. Lysates were cleared for 2min, 18,000 x g, 4˚C. 400 μl of cleared lysate was loaded onto 12ml 10-50% sucrose gradients (prepared in 15mM Tris pH 7.4, 15mM $MgCl_2$, 150mM NaCl) and ultracentrifuged 2h, 35000rpm, 4˚C. Immediately following centrifugation, 1ml fractions were collected.

Library construction. Cleared polysome lysate was adjusted to a volume of 250 μl and monosomal and polysomal RNA fractions were pooled in a total volume of 250 μl. RNA was extracted in 750 μl Trizol LS (Life Technologies), according to the manufacturer's instructions. 500 ng of total, monosomal or polysomal RNA was prepared for sequencing according to the manufacturer's instructions (TruSeq RNA Sample Preparation v2 LS Protocol, Illumina), except that RNA adapters were diluted 1:5 before use. Libraries were pooled and sequenced on a HiSeq 2000 instrument (Illumina).

miRNA profiling. 250 ng of total, monosomal or polysomal RNA and spike-in controls were labeled and hybridized to SurePrint G3 Human v16 miRNA microarrays, according to the manufacturer's protocol (Agilent Technologies).

miRNA analysis. Raw microarray profiling data was normalized and filtered using Bioconductor packages AgiMicroRna and Limma. A contrast matrix was used to compare the change in total RNA with the change in polysomal RNA between an effect and its corresponding control. Limma's generalized linear model was used with this contrast matrix and its Emprical Bayes method was used to compute p-values. The set of miRNAs present in A375 cells (A375 miRNAs) was determined by taking the top 75% of miRNAs when sorted descending by their normalized counts in the empty vector control cells.

RNA-Seq analysis. RNA-Seq reads were aligned to the hg19 reference genome (UCSC Genome Browser (Karolchik et al., 2013; Kent et al., 2002)), excluding the haplotype and random chromosomes, using the STAR aligner version 2.3.0e, with input RefSeq hg19 gene splicing annotations for only the protein coding genes and excluding multimapped reads. A custom script was used to compute read counts for these protein coding genes, considering a gene as the union of all of its transcript variants and excluding reads that mapped to more than a single gene. This raw count data was filtered at 1 count per million in all samples and normalized using Limma's voom function. Differentially translated protein-coding genes were found using the same contrast matrix used in the miRNA analysis with Limma's generalized linear model, computing p-values using Limma's Empircal Bayes method with Benjamini Hochberg p-value adjustment. Genes were considered as differentially translated in a given condition if they met a fold change of at least 1.5 (either direction) and had adjusted p-values less than or equal to 0.1.

miRNA target analysis. Targetscan was run on RefSeq protein coding gene 3' UTRs and the top 5% of all targets were used, when sorted by context score percentile descending. These targets were filtered using the A375 miRNAs to find the set of genes that have miRNA targets for miRNAs present in A375 cells.

miRNA binding site enrichment analysis. As in (Janas et al 2012), for each subset, the fraction of genes with miRNA targets was computed by dividing the number of genes with Targetscan-filtered target prediction sites for A375 miRNAs by the total number of genes present in that subset. Each random control was created by matching each gene in a given subset by choosing a gene from a set of control genes (not present in any differentially translated subset) by matching its RPKM expression level (binned at 10 bins) in both of the eIF4E and eIF4A knockdown total RNA-Seq data and the log 10 of the 3′ UTR length (binned at 10 bins). These random controls were generated 1,000 times for a 1,000 different control sets and the same fraction was computed as before. A p-value was computed by fitting a normal distribution to the fractions from the 1,000 controls and determining the probability of obtaining a fraction as extreme as found for the specific subset.

RNA Structural analysis. The longest transcript variant was used to determine RNA structural data for each protein coding gene. The length and GC content were computed for the entire gene, as well as the 5′ UTR, CDS, and 3′ UTR individually.

3.7.References

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4. Discussion, preliminary data and future directions

4.1. A model for eIF4A1 and eIF4E regulation of miRNA activity

43S preinitiation complex recruitment. Our data from cell-free and cell-based assays presented in this thesis support a model of miRNA-mediated repression of translation initiation specifically at the 60S ribosomal subunit joining step. We propose that eIF4A1 and eIF4E regulate miRNA activity in cells through 43S preinitiation complex recruitment to mRNA, or 48S complex formation. eIF4E enhances 43S complex recruitment to mRNAs, while eIF4A1 enhances 43S complex scanning of the 5' UTR. Counterintuitively, it seems that 43S complex recruitment is *necessary* for miRNP formation *in vitro*. That is, recruitment of a translation initiation complex to an mRNA is actually necessary for formation of a repressive complex on that mRNA. This is supported by the finding that miRNA activity is enhanced in cells which overexpress eIF4A1 or eIF4E, where, presumably, 43S complex loading is more efficient. One possible mechanism for this observation, that miRNAs hinder 60S subunit joining by Ago protein interaction with components of the 43S complex, is discussed in the following subsection.

When eIF4A1 or eIF4E expression is reduced, miRNA activity is reduced, as indicated by increased expression of miRNA targets. A combination of direct and secondary effects of eIF4A1 and eIF4E modulation may contribute to the positive regulation of miRNA activity in cells. Untargeted mRNAs are efficiently translated, with

eIF4E and eIF4A1 enhancing 43S preinitiation complex recruitment and scanning, respectively (Fig. 4.1A). miRNA-targeted mRNAs have reduced ribosome density and a lower translation efficiency than untargeted mRNAs (Fig. 4.1B). A direct effect of eIF4A1 and eIF4E modulation is altered rate of 43S preinitiation complex joining or scanning, respectively (Fig. 4.1, "43S recruitment"). When eIF4A1 and eIF4E expression is decreased, miRNA activity is reduced, as indicated by increased expression of miRNA targets (Fig. 4.1C). We propose that knockdown of eIF4E or eIF4A1 leads to reduced 43S preinitiation complex recruitment or scanning, respectively, on all mRNAs, including miRNA-targeted mRNAs (Fig. 4.1D), and reduction of miRNA activity (Fig. 4.1F). Conversely, when eIF4A1 or eIF4E expression is increased, miRNA activity is enhanced, as indicated by decreased expression of miRNA targets (Fig. 4.1G). We posit that overexpression of eIF4E or eIF4A1 leads to enhanced 43S complex recruitment or scanning, respectively, on all mRNAs including miRNA targeted mRNAs (Fig. 4.1H), and enhanced miRNA activity (Fig. 4.1J).

Figure 4.1. A model for eIF4A1 and eIF4E regulation of miRNA activity. eIF4A1 and eIF4E expression levels regulate miRNA activity through recruitment of the 43S preinitiation complex to mRNA and/or indirect regulation from secondary effects on cellular signaling pathways. The number of ribosomes per mRNA represents relative translation rates. eIF4E and eIF4A1 enhance 43S preinitiation complex recruitment and scanning, respectively (labeled '43S recruitment').

(A) An untargeted mRNA is actively translated in normal cellular levels of eIF4A1 and eIF4E.

(B) In normal cellular levels of eIF4A1 and eIF4E, a miRNA-targeted mRNA has lower relative translation efficiency compared to an untargeted mRNA.

(C) Decreased eIF4A1 or eIF4E expression, relative to normal expression, leads to reduced miRNA activity, as indicated by increased expression of miRNA targets.

(D) Decreased eIF4A1 or eIF4E expression causes reduced recruitment of 43S complexes to mRNA.

(E) Secondary, cellular effects of eIF4A1 or eIF4E knockdown may also lead to reduced miRNA activity.

(F) Decreased eIF4A1 or eIF4E expression causes decreased miRNA activity.

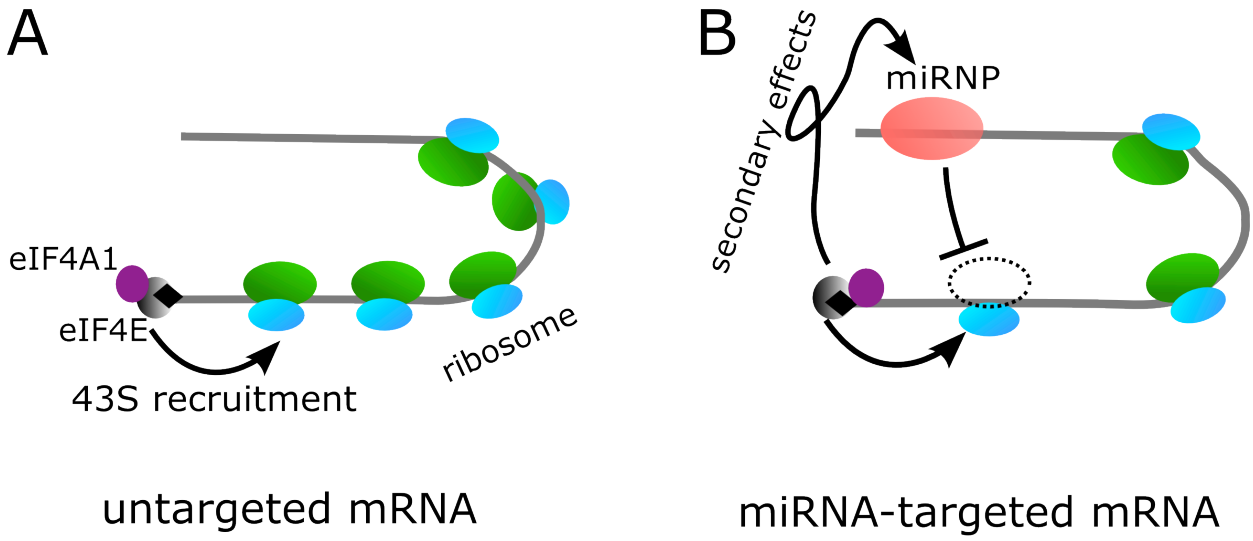
(G) Increased eIF4A1 or eIF4E expression, relative to normal expression, leads to increased miRNA activity, as indicated by decreased expression of miRNA targets.

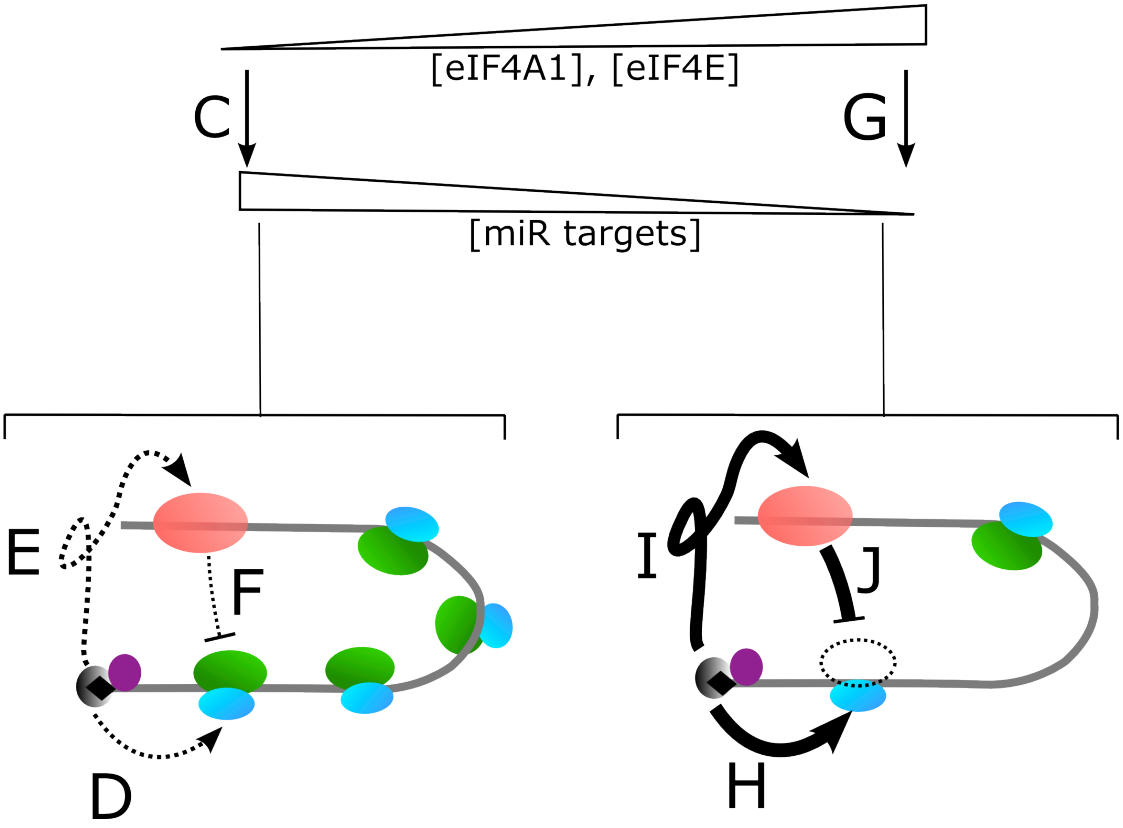
(H) Increased eIF4A1 or eIF4E expression causes increased recruitment of 43S complexes to mRNA.

(I) Secondary, cellular effects of eIF4A1 or eIF4E overexpression may also lead to enhanced miRNA activity.

(J) Increased eIF4A1 or eIF4E expression causes increased miRNA activity.

Figure 4.1 (continued)





Secondary (cellular) effects. It is important to restate that a combination of direct and secondary effects of eIF4A1 and eIF4E modulation may contribute to the positive regulation of miRNA activity in cells. Our model does not imply direct physical binding between Ago proteins and eIF4A1 or eIF4E, but allows for secondary, cellular-level effects of eIF4A1 or eIF4E modulation to also regulate miRNA activity. Modulation of eIF4A1 or eIF4E positively regulates cellular proliferation, and the state of cellular proliferation and stress seems to affect miRNA activity. miRNA activity is higher in proliferative compared to quiescent cells (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). Our lab has shown that knockdown of ribosomal protein genes leads to reduced miRNA activity through induction of nucleolar stress (Janas et al., 2012b). This is an example of secondary, or cellular effects mediating changes in miRNA activity. Therefore, changes in cellular state caused by eIF4A1 or eIF4E modulation may lead to changes in miRNA activity. These could be mediated via changes in expression or post-translational modification of Ago proteins, or other miRNP or initiation factors which affect miRNA activity. Furthermore, it has been shown that knockdown of many factors involved in translation, including small or large subunit ribosomal protein genes, eIF4A2, eIF4G1, and poly(A) tail binding proteins PABPC1 or hnRNP-Q, causes derepression of miRNA-targeted reporter genes and/or endogenous genes (Janas et al., 2012b; Jannot et al., 2011a; Ryu et al., 2013; Svitkin et al., 2013). This suggests that general perturbation of the translation initiation pathway leads to derepression of

miRNA targets. Prior to the studies described in this thesis, the effect of overexpression of these factors on miRNA activity has not been addressed. In conclusion, a combination of direct and secondary effects of eIF4A1 and eIF4E modulation may contribute to the positive regulation of miRNA activity in cells.

4.2. miRNA-mediated repression of 60S ribosomal subunit joining

Since our report was published in 2008, genome-wide studies have shown that there is uniform reduction of ribosome density across the length of miRNA-targeted transcripts (Bazzini et al., 2012; Guo et al., 2010; Hendrickson et al., 2009). This suggests that miRNAs partially repress translation initiation rather than post initiation, which is consistent with our model that miRNAs repress 60S subunit joining. If miRNAs repress translation post initiation by ribosome stalling, the expected change in ribosome density on a targeted compared to an untargeted transcript would be an accumulation of ribosomes along the open reading frame, resulting in unevenly increased ribosome density. If miRNAs repress translation post initiation by ribosome drop-off, the expected change in ribosome density would be decreased ribosome density toward the 3′ end of the open reading frame relative to the 5′ end. In agreement with our model presented here, the uniform reduction of ribosome density across the length of miRNA-targeted compared to untargeted transcripts suggests that miRNAs partially repress translation initiation, but do not interfere with translation post initiation. Because partial repression of translation initiation would reduce the rate of ribosome joining, rather than completely abolish the process, we speculate that this is why miRNAs and targeted mRNAs cosediment with polysomes on polysome gradients from *C. elegans* (Olsen and Ambros, 1999; Seggerson et al., 2002) and mammalian cells (Petersen et al., 2006). Further analyses in cell-based and cell-free systems will more precisely define the

mechanism(s) of miRNA function in mammals and their similarities and differences across species.

The potential role of Ago2 in miRNA-mediated repression. Ago2 (co-eIF2A) was originally described as a ribosome-associated protein that eluted in high salt (Chakravarty et al., 1985) and stabilized 40S-containing complexes in the presence of mRNAs (Roy et al., 1988). We have shown that the repressive complex formed on miRNA-targeted mRNAs contains 40S ribosome subunits but lacks 60S subunits. Consistent with a role in stabilizing 40S ribosomes associated with mRNAs, Ago2 is recruited to unrepressed mRNAs that cosediment with 48S initiation complexes (Fig. 2.3B). Ago2 is also recruited to translationally-repressed mRNAs by miRNAs and may serve to stabilize 40S subunits and hinder joining of 60S subunits (Fig. 2.3B). It has been shown that Ago2 interacts with the anti-association factor eIF6 through TRBP, which may prevent 60S subunit joining to translationally-repressed mRNAs through an unknown mechanism (Chendrimada et al., 2007). Our data support a complimentary mechanism where Ago2 interacts with translationally-repressed mRNAs and prevents 60S subunit joining. Together, these data suggest that Ago2 may function in more than one way to repress translation.

Evidence for miRNA-mediated repression of 60S subunit joining. The results presented in chapter 2 showing that miRNAs repress 60S subunit joining are consistent

with reports of reduced 60S ribosome recruitment to translationally-repressed mRNAs in *C. elegans*, human cells (Chendrimada et al., 2007), and *D. melanogaster* embryonic lysate (Thermann and Hentze, 2007). 60S ribosomal anti-association factor eIF6 associates with RNA-induced silencing complexes, but not necessarily with miRNA-targeted mRNAs (Chendrimada et al., 2007). eIF6 may be involved in an alternative or supplemental mechanism of repression to that proposed in our model, where Ago interaction with 43S initiation complexes hinders 60S subunit joining. Similar to our observations in mammalian lysate, in *D. melanogaster* embryonic lysate, large (>80S, or 'pseudopolysome'), putative miRISC/ miRNPs complexes are formed on miRNA-targeted mRNAs, even when 60S ribosomal subunit joining or translation elongation is inhibited (Thermann and Hentze, 2007). However, in contrast to mammalian lysate, miRNPs form on mRNAs without a 7-methyl guanosine cap in *D. melanogaster* lysate. The source of this discrepancy may be a species-specific mechanism of miRNA-mediated repression in *D. melanogaster* versus mammalian lysate or a difference in lysate incubation and reaction times. Our *in vitro* repression reactions measure the initial mechanism of miRNA-mediated repression after ten minute incubation, while the Hentze lab reaction has an initial three hour pre-incubation step and a one hour incubation (Thermann and Hentze, 2007). Taken together, our findings in a mammalian lysate system are consistent with data from *C. elegans*, human cells and *D. melanogaster*

embryonic lysate and support a model of miRNA-mediated repression of 60S ribosomal subunit joining.

Interestingly, our results are also consistent with a recent study utilizing an *in vitro* repression reaction in non-nuclease-treated rabbit reticulocyte lysate in which a reporter mRNA is targeted by an endogenous miRNA (Ricci et al., 2012). In this study, the effect of various translation initiation inhibitors on miRNA function was measured. Endogenous miRNA-targeted reporter is derepressed only in the presence of AMP-PNP, an inhibitor of 43S complex scanning, but not MDMP, an inhibitor of 60S subunit joining or cycloheximide, an inhibitor of translation elongation (Ricci et al., 2012). These functional data are relevant to our measurements of translation initiation complex formation in the presence of hippuristanol, an inhibitor of 43S complex joining/scanning, GMP-PNP, an inhibitor of 60S subunit joining, and cycloheximide. It is crucial to restate that we found that formation of a putative miRNP complex on miRNA-targeted mRNAs was *dependent* on 43S complex joining. This is consistent with functional data that miRNA activity is reduced in reactions containing AMP-PNP, an inhibitor of 43S complex scanning. If 43S loading and scanning are essential to miRNA function, as our data suggests, then it is expected that AMP-PNP would inhibit miRNA function. Furthermore, Ricci, Ohlmann, and colleagues showed that miRNA activity is not reduced in reactions containing inhibitors of 60S subunit joining (MDMP) or

translation elongation (cycloheximide) (Ricci et al., 2012). This is in agreement with our findings that repressive complexes form on miRNA-targeted mRNAs even when these processes are inhibited. Additionally, miRNA-targeted reporter constructs containing a viral IRES and scanning region within the 5′ UTR are repressed by miRNAs, while those lacking a scanning region are not (Ricci et al., 2012). Ricci, Ohlmann and colleagues conclude that miRNAs inhibit 43S ribosomal scanning, although our reinterpretation reveals that their more compelling data measuring miRNA activity in the presence of translation inhibitors are consistent with our model of miRNA-mediated repression of 60S subunit joining.

In conclusion, our results show that large, repressive, putative miRNP complexes form on miRNA-targeted mRNAs. These complexes contain Ago2 and components of the 43S initiation complex, including 18S rRNA, RPS7, eIF2α, and eIF3g. The complexes have reduced components of the 60S ribosomal subunit, including 28S, 5.8S, and 5S rRNAs, and RPL18, compared to untargeted mRNAs. Loading of the 40S ribosome, but not 60S ribosome is required for complex formation, and the 40S molecular toeprint is enhanced on miRNA-targeted mRNAs. Taken together, these results support the conclusion that miRNAs repress 60S ribosomal subunit joining on target mRNAs. We propose that Ago2 is recruited to the 3′ UTR of target mRNAs by miRNAs, and

associates with components of the 43S pre-initiation complex, thereby hindering 60S ribosomal subunit joining.

4.3. Comparison of cap-dependent translation and miRNA function *in vitro* and in cells

The relative concentrations of translation initiation factors determine translation efficiency in cell-free and cell-based systems. Our *in vitro* system utilizes nuclease-treated rabbit reticulocyte lysate, which is optimized for high translation activity with high stoichiometric excess of eIF4E:mRNA molecules. In contrast, cellular translation occurs in the setting of a large mRNA pool (the transcriptome) and therefore, lower stoichiometry of eIF4E:mRNA. It is also important to note that our ribosome binding assay data showing that repressive complexes form on miRNA-targeted mRNAs *in vitro* was performed in the presence of translation inhibitors to allow the study of initiation complex intermediates. The ability to study initiation complex intermediates is a key advantage of cell-free systems. In these assays, we see that a large, repressive complex forms on miRNA-targeted mRNAs. We speculate that a similar complex forms on miRNA-targeted mRNA in cells, and mediates partial translational repression, rather than complete inhibition. This is in agreement with findings by numerous groups that miRNAs partially repress, rather than completely abolish translation of target transcripts, as measured by the protein output and ribosome density of target transcripts. Furthermore, this accounts for the multiple reports that miRNA-targeted mRNAs and Ago proteins cosediment with polysomes (Kim et al., 2004; Maroney et al.,

2006; Nelson et al., 2004; Nottrott et al., 2006; Olsen and Ambros, 1999; Petersen et al., 2006).

Another key difference between our experimental systems is the typical assay duration and system complexity. Our *in vitro* repression reactions are incubated for ten minutes to measure the initial effects of miRNA-mediated translational repression. In contrast, in cell-based assays, miRNA activity is measured 48 hours after reporter transfection into cells or, for endogenous genes, at steady-state levels of miRNA-targeting. Finally, cell-free assays are less complex than cell-based assays as they are not regulated by cellular signaling pathways or spatial organization of translation. Secondary effects of eIF4A1 and eIF4E modulation, possibly including post-translational modification of Ago proteins, induction of nucleolar stress, or altered activity of other translation initiation factors, may affect miRNA activity in our cell-based assays. These key differences between cell-free and cell-based assays highlight the utility and respective advantages of the two experimental systems.

4.4.Regulation of miRNA activity by eIF4A1 and eIF4E

In normal cells, protein translation is extensively regulated by cellular microenvironmental conditions which control signaling pathways for cell proliferation. In cancer cells, this regulation is perturbed. Translation initiation factors eIF4A1 and eIF4E are extensively regulated by cell signaling pathways and are components of the rate-limiting translation factor; eIF4F. Unsurprisingly, the expression and activity of eIF4F components promote tumorigenesis and metastasis, yet the mechanisms for eIF4A1 and eIF4E-induced oncogenesis are not well understood.

Upon eIF4E overexpression, the translation of a subset of mRNAs is differentially reduced. In various cell types, transcripts with high 5′ UTR secondary structure or signal sequences are positively regulated by eIF4E expression (Koromilas et al., 1992; Santhanam et al., 2009)(Fig. 4.2B). In agreement with this model, our results indicate that transcripts which are positively regulated by eIF4E expression have higher 5′ UTR GC content than those which are negatively regulated. Although fewer studies exist for the changes in translation efficiency caused by eIF4A1 modulation, it is thought that because it is a helicase, eIF4A1 modulation also differentially affects transcripts with highly structured 5′ UTRs (Svitkin et al., 2001). We found that transcripts which are positively regulated by eIF4A1 expression have higher 5′ UTR GC content than those

which are negatively regulated (Fig. 4.2A). Ours is the first study to measure transcriptome-wide changes in translation efficiency upon eIF4A1 modulation.

Figure 4.2. Mechanisms of eIF4A1 and eIF4E-driven oncogenesis.
The effects of eIF4A1 and eIF4E expression and activity on mRNA translation efficiency are mediated through 5′ and 3′ determinants. Black arrows indicate the previously known relationships between these factors and activities. Red arrows indicate those relationships that have been reported for the first time in this thesis.

(A) It was hypothesized that, because it is a helicase, eIF4A1 activity or expression levels differentially affect the translation efficiency of mRNAs with highly structured 5′ UTRs. Our data support this hypothesis.

(B) There is ample evidence that eIF4E activity or expression levels differentially affect the translation efficiency of mRNAs with high secondary structure or signal sequences in their 5′ UTRs.

(C) We have shown that eIF4A1 and eIF4E expression levels regulate miRNA activity.

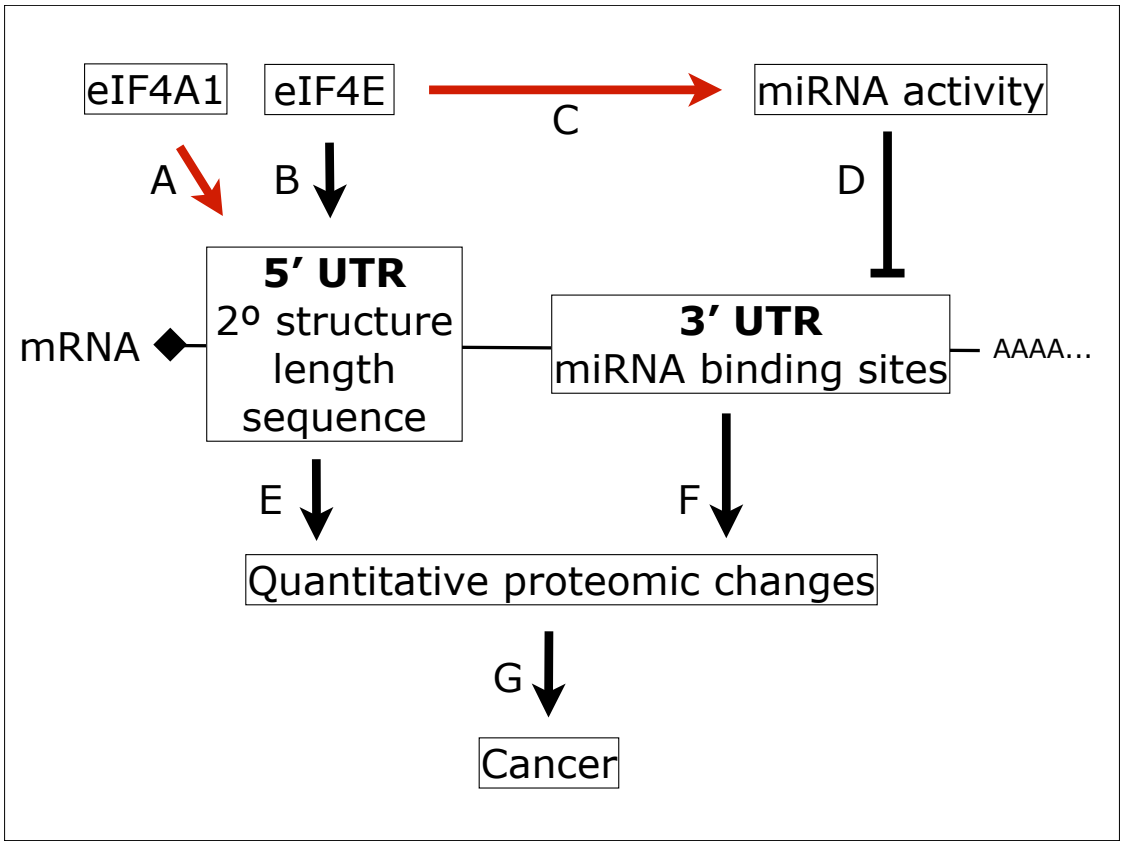
(D) miRNA activity decreases translation efficiency of mRNAs with bindings sites in the 3′ UTR.

(E) Regulation of mRNAs through 5′ UTR determinants is generally positively correlated with changes in translation efficiency.

(F) Regulation of mRNAs through 3′ UTR determinants is generally inversely correlated with changes in translation efficiency.

(G) Quantitative proteomic changes affect tumorigenesis and metastasis.

Figure 4.2 (continued)



miRNAs affect tumorigenesis at individual, network and global levels. Individual miRNAs act as oncogenes if they target tumor suppressor genes or tumor suppressor genes, if they target oncogenes. On a network level, miRNAs may be concordantly regulated or converge on the same mRNA target, resulting in combinatorial regulation of cellular pathways. On a global level, miRNA activity is regulated by the expression or activity of Ago proteins or factors that enhance miRNA activity. Changes in global miRNA activity may occur independently of the expression levels of individual miRNAs.

Our results indicate that eIF4A1 and eIF4E *positively* regulate translation efficiency through mRNA 5′ UTR determinants (Fig. 4.2A & B) and *negatively* regulate translation efficiency through 3′ UTR determinants (Fig. 4.2C & D). Increased mRNA translational efficiency through 5‘ UTR regulation (Fig. 4.2E), and decreased mRNA translation efficiency through 3′ UTR regulation (Fig. 4.2F) lead to quantitative proteomic changes that affect tumorigenesis and metastasis (Fig. 4.2G). It is important to emphasize that in our model, we discern between regulation of mRNA 5′ UTR sequence or structure from that due to miRNA-targeting because they cause *opposite* changes in translational efficiency (Fig. 4.2A & B compared to D). Our findings establish the interplay between mRNA 5′ and 3′ UTR regulation of translation efficiency (Fig. 4.2C). The previously unknown link between eIFA1 and eIF4E expression levels and

miRNA activity reveals crosstalk between two ubiquitous determinants of post-transcriptional gene regulation; initiation factor expression levels and miRNA activity.

We did not specifically search for the presence of 5′ UTR features that affect translation such as upstream open reading frames (uORFs) or cellular IRESes in our analysis of mRNA subsets. uORFs typically inhibit downstream expression of the primary open reading frame, so transcripts with uORFs may be differentially sensitive to eIF4A1 or eIF4E expression levels. Therefore, it is possible that transcripts with uORFs are among the subset of mRNAs with concordant, or decreased translational efficiency in eIF4A1 and eIF4E knockdown. The degree of translational regulation by cellular IRESes remains under debate. Cellular IRESes may facilitate 5′ cap-independent translation initiation. This translation is relatively inefficient compared to cap-driven translation (Gilbert, 2010). It is possible, though not likely, that the increased relative expression of a transcript upon eIF4A1 and eIF4E knockdown may be due to the presence of a cellular IRES. To address these possibility, we will analyze the presence of uORFs and cellular IRESes in mRNA subsets with increased and decreased translation efficiency.

A recent report shows that knockdown of eIF4A2, but not eIF4A1, in Hela cells causes derepression of reporter and endogenous miRNA targets (Meijer et al., 2013). We found that knockdown of eIFA1 *or* eIF4A2 causes derepression of a miRNA-targeted

reporter gene. Although we didn't measure the effect of eIF4A2 modulation on endogenous miRNA targets, we have shown on a transcriptome-wide level that knockdown of eIF4A1 causes derepression of endogenous miRNA targets. eIF4A1 (mRNA variant 1) and eIF4A2 are protein paralogues which are collectively referred to as eIF4A. They share 91% amino acid identity and are functionally interchangeable *in vitro*. However, eIF4A1 and eIF4A2 display tissue-specific distinct transcriptional expression patterns and increased expression of eIF4A2 does not restore translation levels that are reduced in knockdown of eIF4A1 (Galicia-Vazquez et al., 2012). The reason for the discrepant findings for eIF4A1 regulation of miRNA activity remain unclear. Unfortunately, Meijer, Bushell, and colleagues did not test the effect of eIF4E knockdown on miRNA-mediated repression, so their conclusion that, "eIF4A2 is the only component of eIF4F required for miRNA-mediated repression" is overstated (Meijer et al., 2013). It is important to reiterate that in mammalian cells, knockdown of many factors involved in translation, including small or large subunit ribosomal protein genes, eIF4GI, and poly(A) tail binding proteins PABPC1 or hnRNP-Q, has been reported to cause derepression of miRNA-targeted reporter gene and/or endogenous mRNAs (Janas et al., 2012b; Jannot et al., 2011a; Ryu et al., 2013; Svitkin et al., 2013). Our model presented in figure 4.1 accounts for these findings in that knockdown of various translation initiation or ribosomal factors may lead to decreased efficiency of 43S preinitiation complex loading (as in Figure 4.1D) and/or may lead to changes in cell

stress or proliferative state which affect miRNA activity (as in Figure 4.1E). Taken together, these findings suggest that interference with the translation initiation pathway leads to derepression of miRNA targets.

4.5.Preliminary data for future analysis

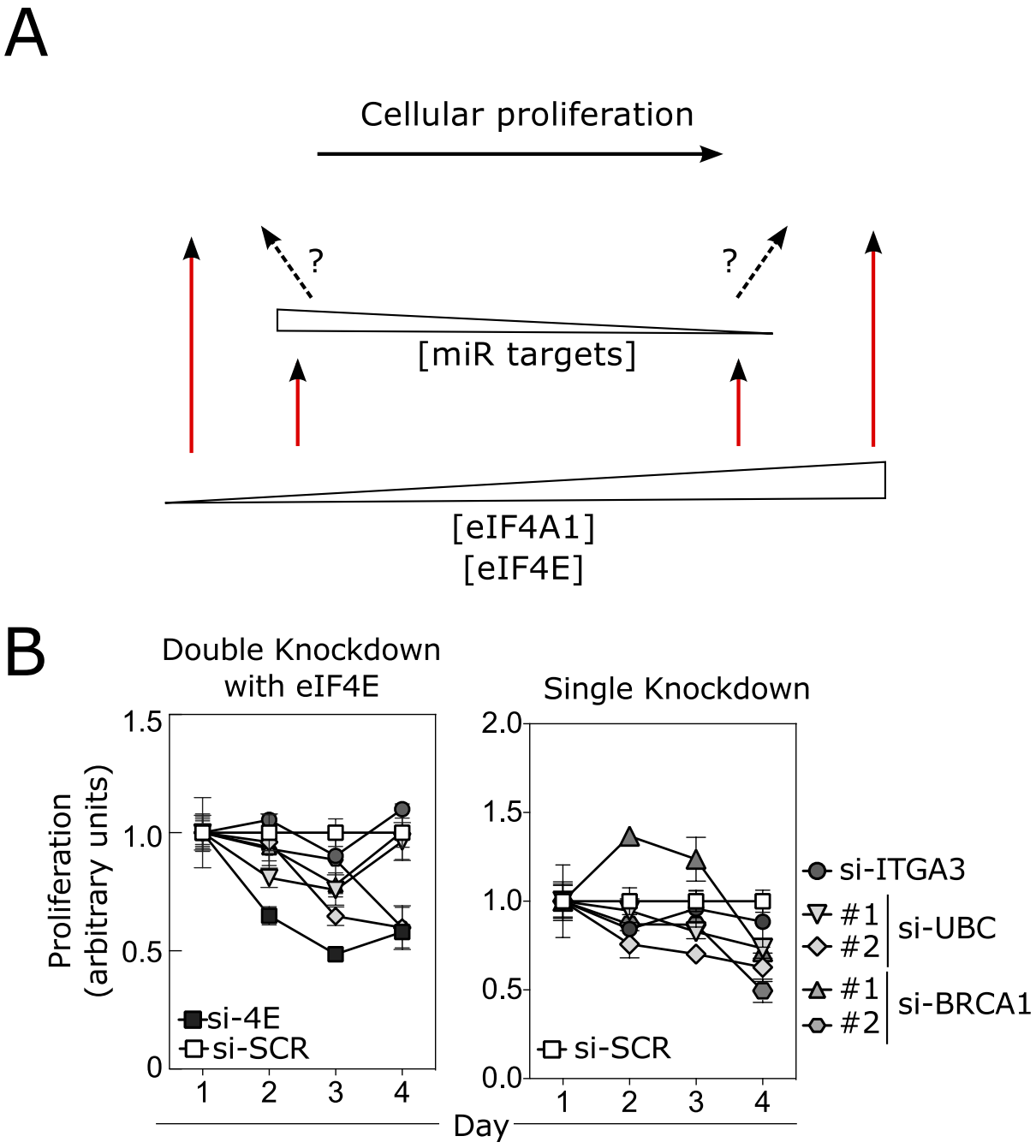
In chapter 3, we show that eIF4A1 and eIF4E knockdown cause reduced cellular proliferation, invasion and miRNA activity. We are in the process of characterizing the potential role of miRNA target misregulation in the eIF4E-dependent decrease in cell proliferation. We have identified genes with increased translation efficiency in eIF4A1 and eIF4E knockdown which are putative miRNA targets and tumor suppressors. We reasoned that if derepression of a miRNA target contributes to the decreased proliferation phenotype in eIF4E knockdown, then double knockdown of this gene

Figure 4.3. eIF4A1 and eIF4E knockdown leads to derepression of miRNA targets, including putative tumor suppressors.

(A) Schematic of the experimental rationale. Our results show that modulation of eIF4A1 or eIF4E expression ([eIF4A1], [eIF4E]) affects cellular proliferation and miRNA activity (red arrows). miRNA activity is measured as the inverse expression of miRNA target expression ([miR targets]). Does the derepression of miRNA targets in eIF4E knockdown contribute to the effect on cellular proliferation (left dotted arrow)?

(B) Melanoma cell line A375 proliferation following double knockdown of eIF4E with each factor of interest (left panel) or single knockdowns of each factor (right panel). Absorbance values were measured after incubation with Wst-1 colorimetric reagent, normalized to day one and are shown relative to control (si-SCR) transfection. siRNAs against ITGA3 (si-ITGA3), UBC (si-UBC), and BRCA1 (si-BRCA1) genes are indicated in the legend. Points represent mean, error bars are S.E.M., n=6 from two independent experiments.

Figure 4.3 (continued)



with eIF4E would diminish the eIF4E-dependent phenotype and restore cellular proliferation (Fig. 4.3A). The genes we identified include breast cancer 1, early onset (BRCA1), integrin alpha-3 (ITGA3), and ubiquitin C (UBC). To assess their possible roles in the decreased proliferative phenotype induced by eIF4E knockdown, we performed double knockdowns of each gene with eIF4E (Fig. 4.3B, left panel, see chapter three for experimental methods). Knockdown of each gene should mimic normal miRNA-mediated repression that is lost in eIF4E knockdown. Our preliminary data show that knockdown of each gene partially reverses the effect of eIF4E knockdown on cellular proliferation (Fig. 4.3B, left panel). The effect of the single knockdown of each gene on cellular proliferation is shown for comparison (Fig. 4.3B, right panel). It does not seem that any of these genes have a tumor suppressive role in the physiology of untreated A375 cells, as the individual knockdown does not increase proliferation. We are in the process of validating BRCA1 and UBC as miRNA targets through reporter assays with their cloned 3' UTRs. ITGA3 is a validated target of miR-214 in melanoma (Penna et al., 2011). We will measure changes in expression of each gene between the different conditions of eIF4A1 or eIF4E knockdown or overexpression by immunoblot to validate our initial measurements of translation efficiency through RNA sequencing. We will further investigate the effects of double knockdown of each factor with eIF4E on melanoma invasion. In summary, our preliminary data suggests that misregulation of miRNA targets is a novel mechanism of eIF4E-dependent melanoma phenotypes.

4.6. Future directions

Cell free assays. We have identified a repressive, Ago2-containing, putative miRNP complex that assembles on miRNA-targeted mRNAs *in vitro*. To further characterize the structural and functional determinants of this complex, we will first perform immunoblots for known miRNP factors on glycerol gradient fractions containing the semi-purified repressive complex (as in Figure 2.3B). We will also perform mass-spectroscopy analysis on these samples to identify novel factors that are associated with miRNA-targeted mRNAs. After optimizing stabilization and purification of the complex, we would like to further characterize it by imaging the gross structure by electron microscopy.

Cell-based assays. We have created and characterized experimental systems in which eIF4A1 and eIF4E modulation directs cellular transformation phenotypes and miRNA activity (Figure 3.1). These systems will be used to interrogate qualitative and quantitative differences between mRNA localization and miRNP composition in various cellular levels of miRNA activity. Using the reporter system described in figure 3.3 and/or validated endogenous miRNA target genes, we will interrogate miRNA-targeted mRNA localization upon eIF4A1 or eIF4E knockdown (low miRNA activity) or overexpression (high miRNA activity) in A375 cells. We will use both fluorescently-labeled miR-CXCR4 and fluorescently-labeled probes to detect the miRNA and reporter

mRNA, respectively, and determine if there are changes in mRNA localization between baseline (control knockdown or empty vector overexpression), low (eIF4A1 and eIF4E knockdown) and high (eIF4A1 and eIF4E overexpression) cellular miRNA activity. Additionally, we will measure colocalization of miR-CXCR4 and targeted mRNA with known P-body and stress granule factors in baseline, low and high miRNA activity. It will be interesting to determine whether miRNA-targeted mRNA recruitment to P-bodies or stress granules corresponds to cellular levels of miRNA activity.

We found that knockdown of eIF4A1 or eIF4E reduced melanoma proliferation (Fig. 3.2A), while knockdown of Ago1 and Ago2 individually or together did not affect cellular proliferation (Fig. S3.1). Knockdown of eIF4A1, eIF4E, Ago2 (and Ago1 to a lesser extent) causes derepression of miRNA-targeted reporter and/or endogenous mRNAs. There are conflicting reports on the oncogenic or tumor suppressive roles of Ago1 and Ago2. Overexpression of Ago2 has been reported to have a tumor suppressive role in melanoma (Voller et al., 2013). This does not agree with our data showing that knockdown of Ago2 does not affect cellular proliferation. Further study is necessary to understand the role of Ago1 and Ago2 modulation in miRNA activity and transformation phenotypes, therefore, we will interrogate the effects of Ago1 and Ago2 overexpression on miRNA activity in our cell-based experimental systems. Moreover, we will perform a detailed analysis of the transcripts that are commonly and

differentially regulated by eIF4A1, eIF4E and Ago2 knockdown in melanoma (Fig. 3.6B). We will perform gene ontology analysis on the 590 transcripts with increased translation efficiency in the three knockdowns and the 823 transcripts with increased translation efficiency only in eIF4A1 and eIF4E knockdown. Because eIF4A1 and eIF4E knockdown cause decreased proliferation, while Ago2 knockdown does not, this analysis may reveal pathways that contribute to these differences. We will also perform gene ontology analysis on the mRNA subsets with decreased translation efficiency in all three or eIF4A1 and eIF4E knockdowns to further understand the proteomic changes that lead to differential proliferation phenotypes in eIF4A1, eIF4E and Ago2 knockdowns.

Relevance to cancer therapy. As major effectors of the PI3K-mTOR pathway, eIF4E and eIF4A1 are promising therapeutic targets. Suppression of eIF4A1 or eIF4E reduces proliferation and sensitizes cancer cell lines to chemotherapy (Cencic et al., 2013b; Hayman et al., 2012; Yang et al., 2012). One promising therapy, an anti-sense oligo targeting eIF4E, reduced eIF4E expression in human tumor xenografts, leading to reduced tumor growth (Graff et al., 2007). It also induced apoptosis and chemosensitivity while reducing proliferation in mesothelioma cells (Jacobson et al., 2013). In a phase I clinical trial in patients with advanced cancer, this anti-sense oligo was well-tolerated and reduced eIF4E expression in tumor biopsies, but did not

significantly affect tumor response (Hong et al., 2011). It is currently in two phase II clinical trials in combination with other chemotherapeutic agents (ISISPharmaceuticals). Our study of transcriptome-wide changes in translation efficiency provides specific insight into the effects of treatment with the eIF4E antisense oligo. Our finding that miRNA activity is reduced in the setting of eIF4E knockdown may prove essential to understanding the tumor suppressive mechanisms of eIF4E inhibition. The gene ontology analyses proposed in the preceding subsection will provide insight into the cellular changes caused by eIF4E knockdown.

4.7.References

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5. Appendix A. Supplemental figures relating to chapter 2.

Figure S2.1. miRNA-targeted mRNAs have reduced 60S ribosome components.

(A) Ethidium bromide-stained agarose gel of 7-methyl-guanosine-capped FL6X and FL0X mRNAs without (-) and with (+) a ~200 nucleotide polyadenylated tail ((A99-bi1)2; 99:1 adenosine:biotinyl-adenosine) used for the experiments in B–D.

(B) Autoradiography of [^{35}S]-labeled proteins synthesized in translation repression assays and resolved by SDS-PAGE. Translation repression reactions contained FL6X or FL0X mRNA as in (A) and *Renilla* luciferase (RL) mRNA with and without test (miR-CXCR4) or control (siGFP) mi/siRNAs. Firefly and *Renilla* luciferase protein levels were measured by [^{35}S]-methionine incorporation and quantitation of translational repression is indicated (% reduction FL, left). Quantitation of synthesized firefly and *Renilla* luciferase proteins (translation of FL %, Right). Error bars indicate standard deviation of three independent experiments.

(C) Northern blot analysis of components of the 40S and 60S ribosomal subunits in total (T) translation repression assays, and in supernatant (S) or mRNA-containing precipitate (P) following streptavidin precipitation of biotinylated mRNA reporters from total translation repression assays. Reactions contained FL6X mRNA with CXCR4 miRNA (left panel) or non-specific GFP siRNA (middle panel) and FL0X mRNA with CXCR4 miRNA (right panel) The dotted lines facilitate visualization of lane pairs. Signal intensities were normalized by calculating 60S (5S, 5.8S, and 28S):40S (18S) rRNA and initiator methionine tRNA (tRNAi-Met):40S rRNA. The fold changes in signal intensities between reactions containing and lacking siRNAs were calculated by the equation [(60S/40S+miR-CXCR4):(60S/40S-miR-CXCR4)] or [(tRNAi-Met/40S+miR-CXCR4):(tRNAi-Met/40S-miR-CXCR4)]. Results are presented as bar graphs below each image.

(D) Northern blot analysis of FL mRNA (upper panel) and CXCR4 miRNA (lower panel) in total (T) supernatant (S) or precipitate (P) fractions from a translational repression reaction shows a ~1:6 ratio of mRNA:miRNA. The numbers below each image indicate the amount of mRNAs or miRNAs calculated by in vitro transcribed mRNA or miRNA, average from two independent experiements.

Figure S2.1 (continued)

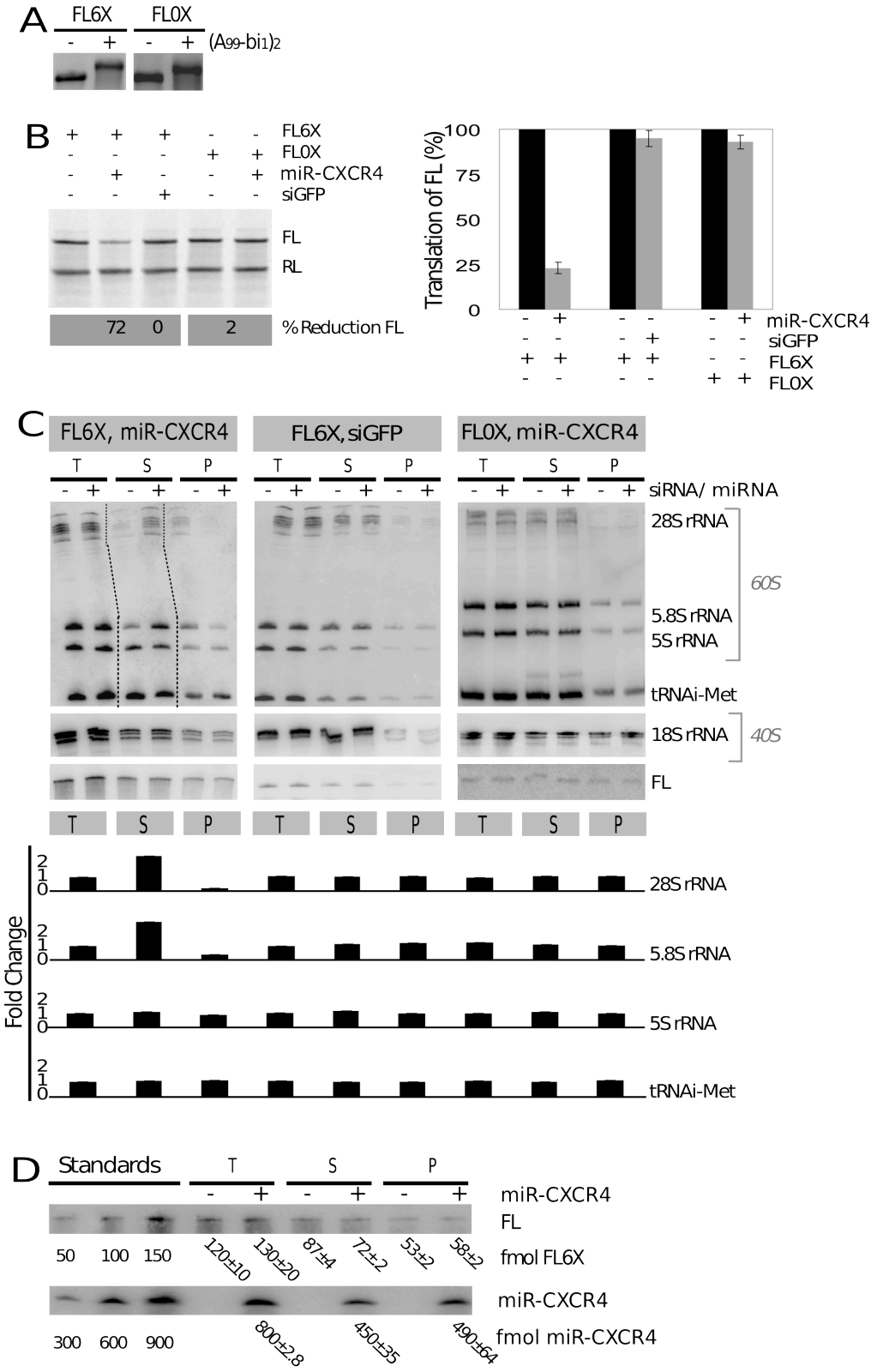


Figure S2.2. A high molecular mass complex containing 40S ribosomal components but lacking 60S components forms on miRNA-repressed mRNAs.

Sedimentation velocity analysis of 7-methyl-guanosine-capped firefly luciferase (FL) mRNAs containing ^{32}P-labeled polyA tails. Error bars indicate standard deviation of three independent experiments.

(A) Ribosome-binding profiles of FL6X mRNA from translation repression reaction assays containing GMP-PNP with (+, solid line) or without (-, dotted line) CXCR4 siRNA.

(B) Ribosome-binding profiles of FL6X mRNA translation repression reactions containing cycloheximide with (+, solid line) or without (-, dotted line) CXCR4 miRNA.

(C) Ribosome-binding profiles of FL6X mRNA from translational repression reactions containing cycloheximide and with (+, solid line) or without (-, dotted line) control GFP siRNA.

(D) Ribosome-binding profiles of FL0X mRNA from translation repression reactions containing cycloheximide and with (+, solid line) or without (-, dotted line) CXCR4 miRNA. In (C) and (D), arrow marks peak containing the 80S monosome, which sediments to fraction 12.

Figure S2.2 (continued)

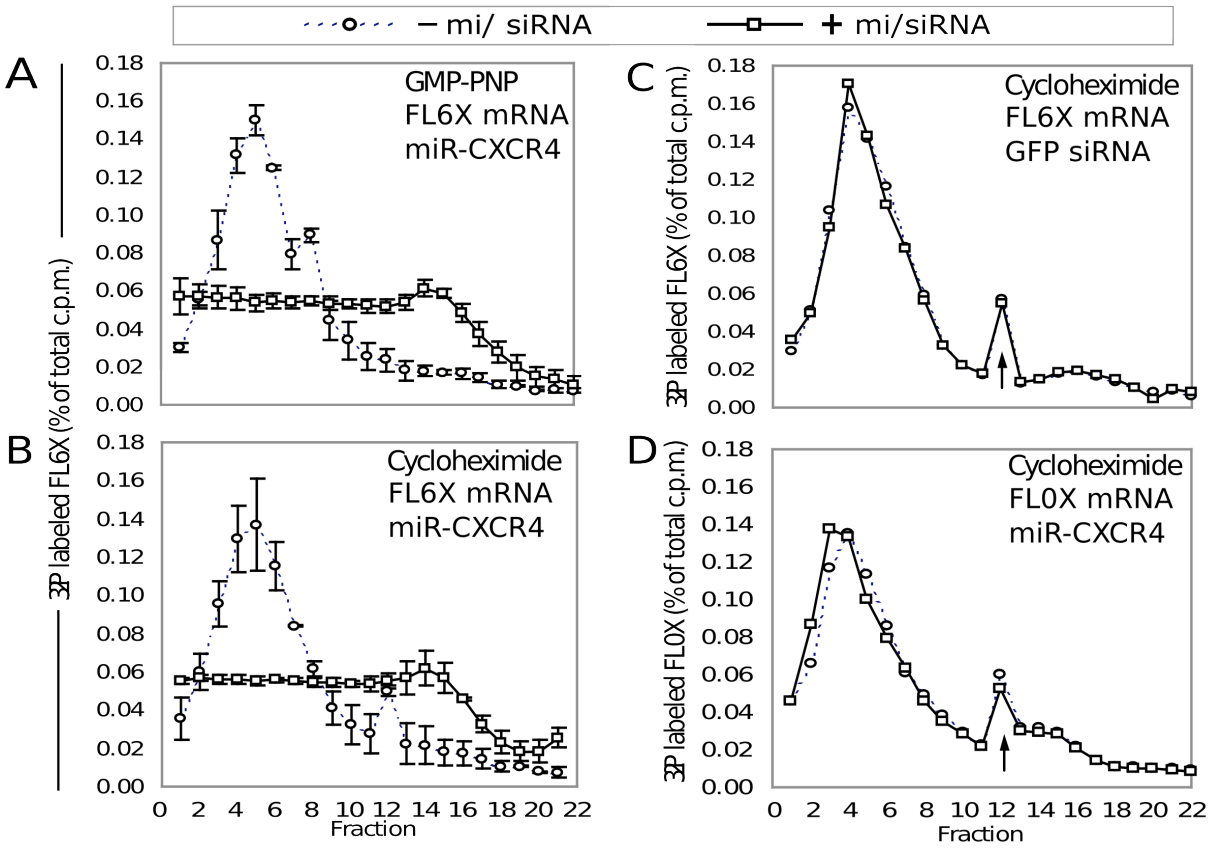


Figure S2.3. miRNAs repress 7-methyl-guanosine cap-mediated, but not IRES-mediated, translation.

(A) Schematic presentation of bicistronic mRNA reporter construct with cap-driven firefly luciferase (FL, black rectangle), IRES-driven (gray oval) *Renilla* luciferase (RL, white rectangle), and six miRNA binding sites (miRCXCR4, black squares) in the 3′ UTR.

(B) Autoradiograph of [^{35}S]-labeled proteins synthesized in translation repression assays and resolved by SDS-PAGE. Translation repression reactions contained capped (+cap) and polyadenylated [200 adenosines (A200), upper panel] or uncapped (- cap) and polyadenylated [800 adenosines (A800), lower panel] bicistronic reporters driven by cricket paralysis virus (CrPV), hepatitis C virus (HCV), or encephalo- myocarditis virus (EMCV) IRESes. Firefly and *Renilla* luciferase protein levels were measured by [^{35}S]-methionine incorporation and quantitation of translational repression is indicated (% reduction FL, RL). FL and RL protein levels were normalized to a control (CTRL) protein (human CD3) expressed from an mRNA lacking miRNA-binding sites.

Figure S2.3 (continued)

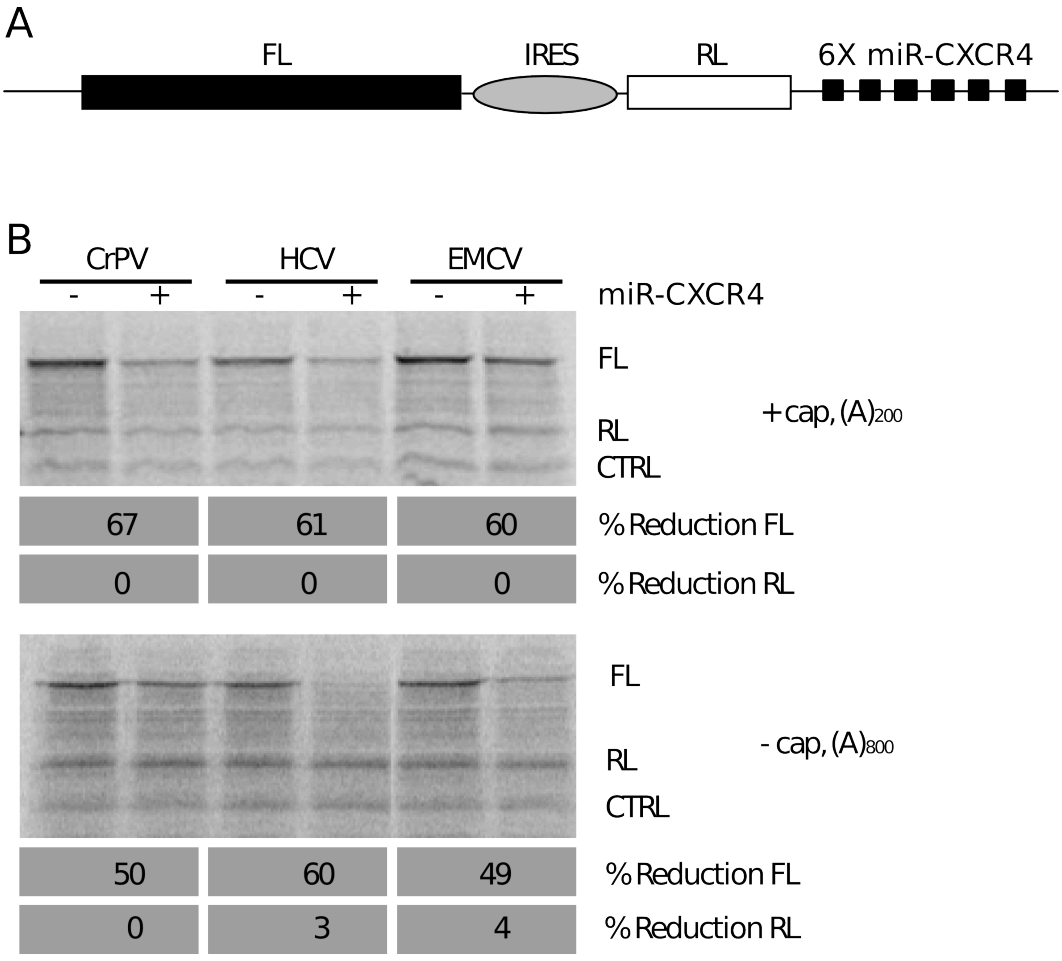


Figure S2.4. Ago2 mediates siRNA-guided cleavage activity with or without guide strand preannealing to target mRNAs

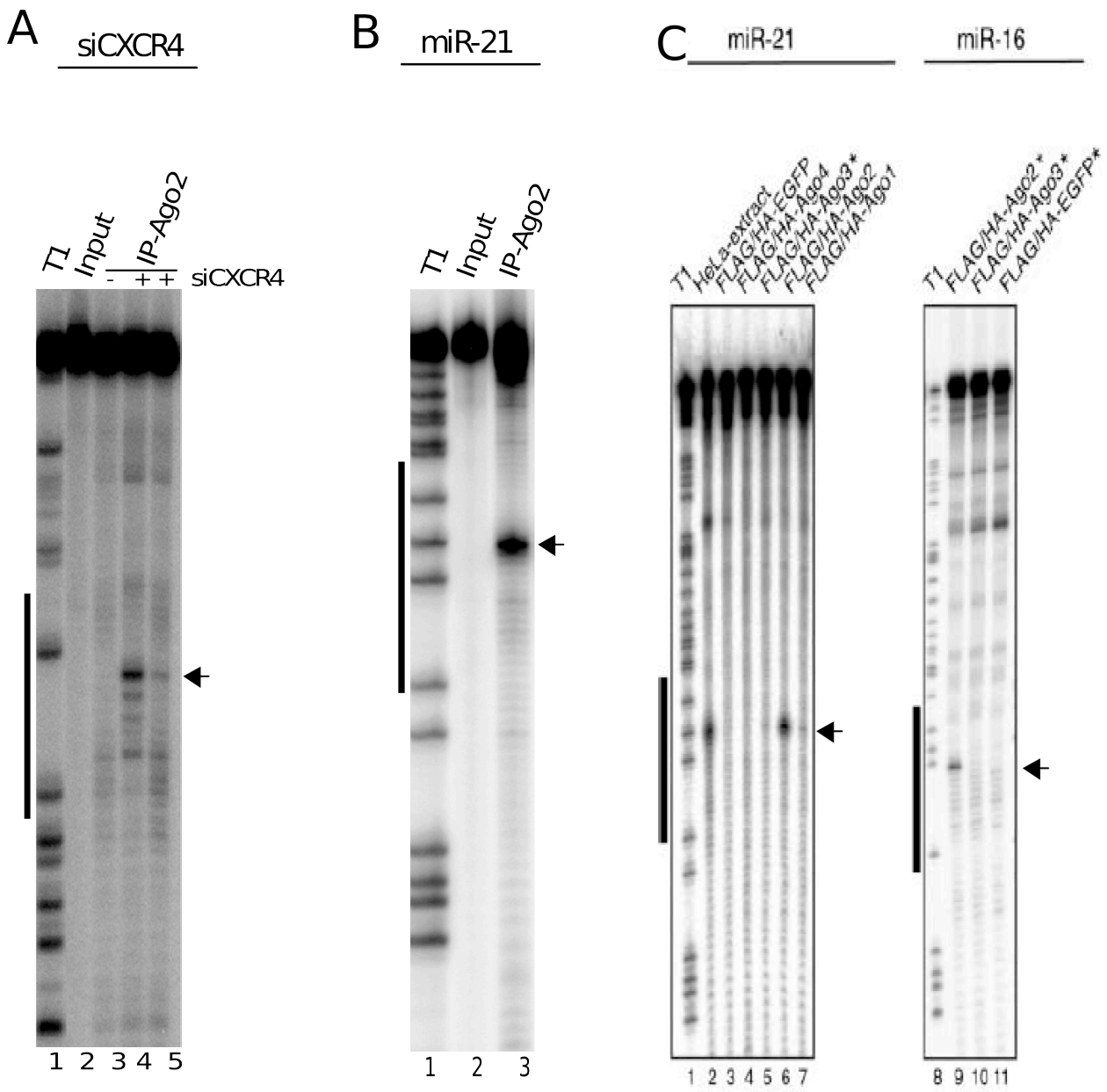
^{32}P-cap-labeled target RNAs containing perfectly complementary binding sites for siCXCR4 (A) or endogenous miR-21 (B) were subjected to RNase T1 hydrolysis of the target RNAs to show the G ladder (lanes 1), or conditions without RNase T1 hydrolysis (lanes 2) to show the integrity of the labeled target RNAs. siRNA-targeted mRNA cleavage products were separated on 8% PAGE containing 7 M urea.

(A) Ago2 immunoprecipitates from RRL were added to ^{32}P-Cap-labeled target RNAs containing perfectly complementary binding sites for siCXCR4 either without siCXCR4 (lane 3), with preannealing of siCXCR4 to target mRNAs (lane 4), or without preannealing of siCXCR4 to target mRNAs (lane 5). The black bar represents the target RNA binding site complementary to siCXCR4, and the arrow indicates the predicted cleavage product of siCXCR4.

(B) Ago2 immunoprecipitates from RRL were incubated with ^{32}P-cap-labeled target RNAs containing perfectly complementary binding sites for endogenous miR-21 (no preannealing). The black bar represents the target RNA binding site complementary to miR-21 and the arrow indicates the predicted cleavage product of miR-21.

(C) Adapted with permission from Meister et al. (Meister et al., 2004). miRNA- and siRNA-guided cleavage activity is mediated by the Ago2 protein complex in vitro. ^{32}P-cap-labeled target RNAs containing miR-21 or miR-16 complementary sites were incubated with the FLAG-tag-purified Ago complexes. The cleavage products were resolved on 8% sequencing gels. The Ago complexes labeled with asterisk were purified from transiently transfected HEK 293 cells rather than stably transfected HeLa cells. miR-21 is abundant in HeLa cells but of low abundance in HEK 293 cells, miR-16 is abundant in both cell lines. Lanes 1 and 8 show the T1 hydrolysis ladder of the cleavage substrates. The black bars to the left side of the images represent the region of the target RNA complementary to miR-21 or miR-16.

Figure S2.4 (continued)



Adapted from Meister et al (2004). Mol Cell 15:185 (Figure 5)

6. Appendix B. Supplemental figures relating to chapter 3.

Figure S3.1. eIF4A1 and eIF4E, but not Ago1 or Ago2 are positive regulators of melanoma cell proliferation

(A) Melanoma cell line (A375) proliferation following siRNA knockdown of eIF4A1 or eIF4E with secondary siRNAs (#2). Absorbance values were measured after incubation with Wst-1 colorimetric reagent, normalized to day one and are shown relative to control (si-Scr) transfection. Points represent mean, error bars are S.E.M.

(B) Melanoma cell line (A375) proliferation following siRNA knockdown of Ago1 or Ago2 individually or combined. Values are derived as in A.

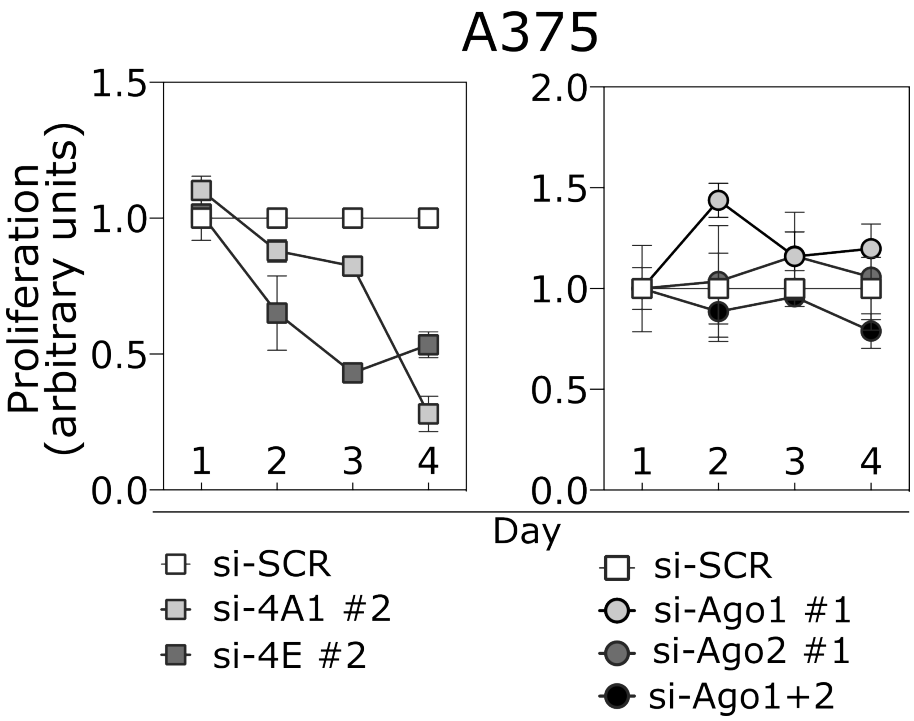


Figure S3.2. eIF4A2 is a positive regulator of miRNA-mediated repression

(A) *Renilla* luciferase activity for a miRNA-targeted reporter relative to untargeted firefly luciferase reporter in A375 cell line following transient knockdown of eIF4A1 (si-4A1), eIF4E (si-4E), eIF4A2 (si-4A2), Ago1 (siAgo1), Ago2 (siAgo2). Results for primary (#1) or secondary (#2) siRNAs are shown.

(B) Data as in (A) for WM1575 melanoma short term culture.

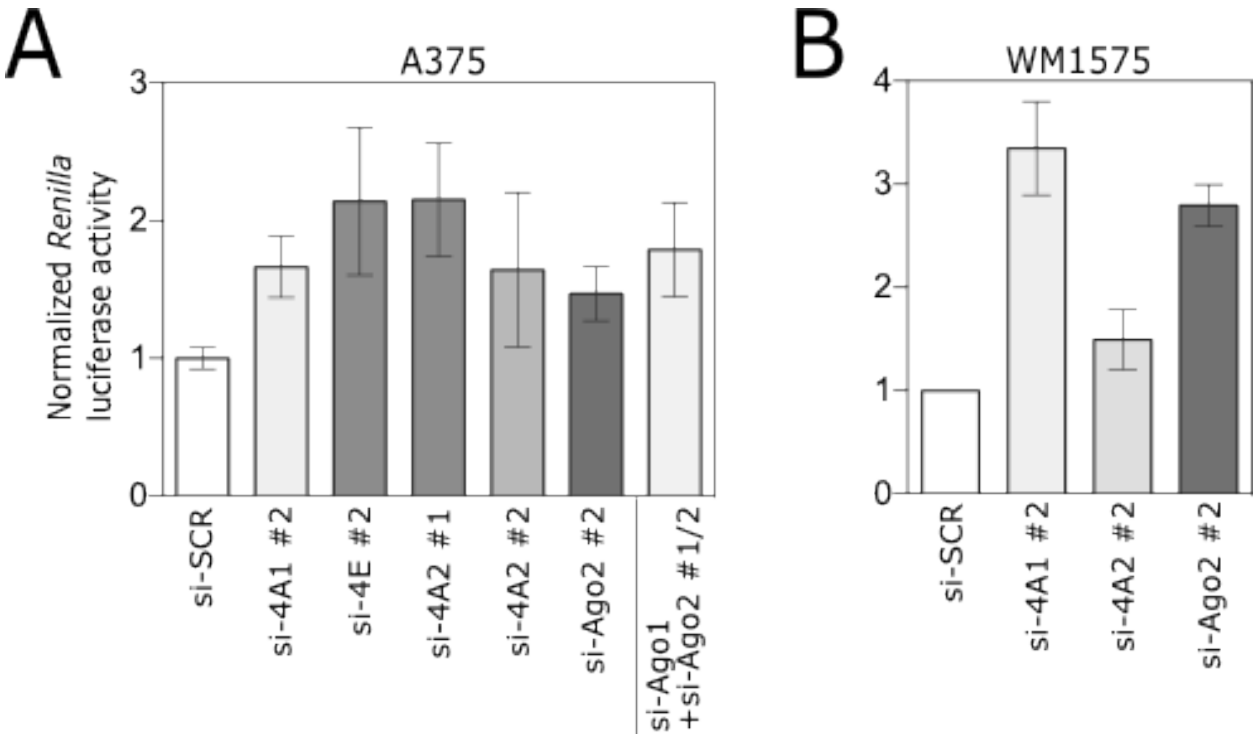


Figure S3.3. eIF4AI- and eIF4E-dependent changes in mRNA translation efficiency and mature miRNA levels.

(A) Summary of significant changes in either translation or loading efficiency in eIF4AI and eIF4E knockdown treatments. Shown are mRNAs that were significantly changed in both treatments (| Fold change | >1.5, $p<0.1$). Equations for calculating efficiency of translation or monosome loading for mRNA transcripts are shown.

(B) eIF4A1 and eIF4E-dependent changes in mature miRNA levels. Six miRNAs with significantly altered expression following eIF4AI and eIF4E knockdown (|Fold change| >1.5, $p<0.1$) are highlighted in black (si-4A1) or red (si-4E).

Figure S3.3. (continued)

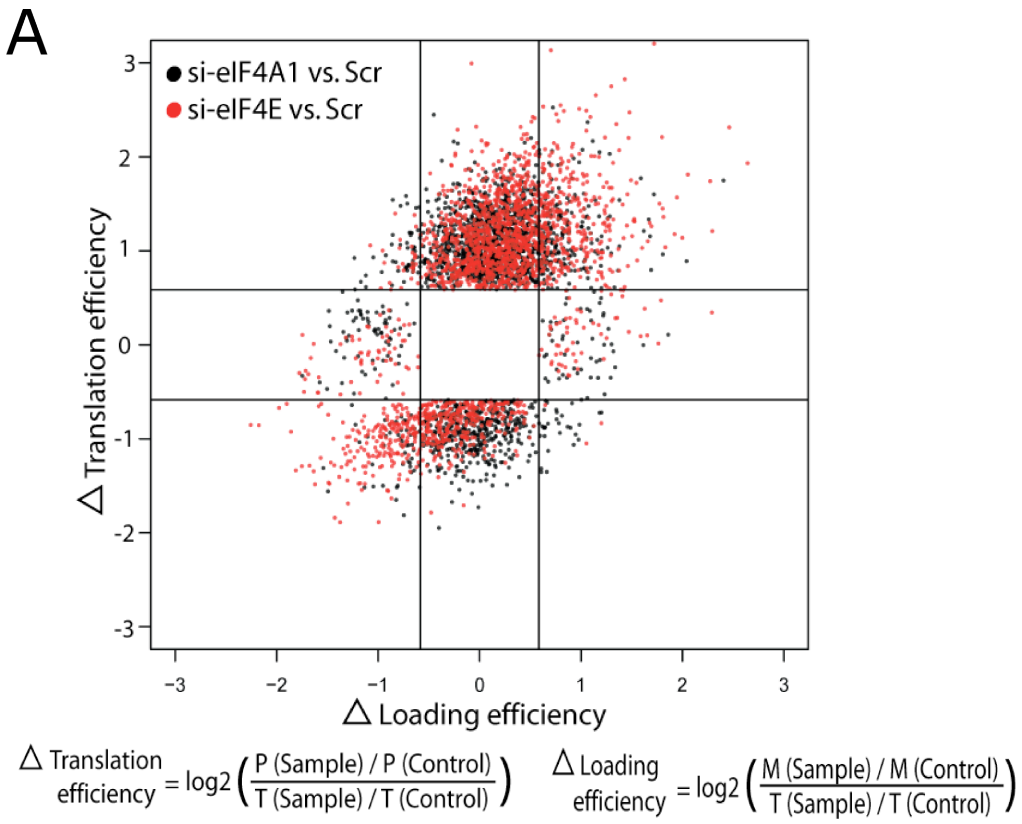


Figure S3.4. Discordant transcripts that are commonly regulated by eIF4A1 and eIF4E have lower GC content and longer CDS.
Fraction of GC content (top) and log(10) transcript length (bottom) are shown for 5′UTR, CDS and 3′UTR of discordant (orange) and concordant (purple) transcripts.

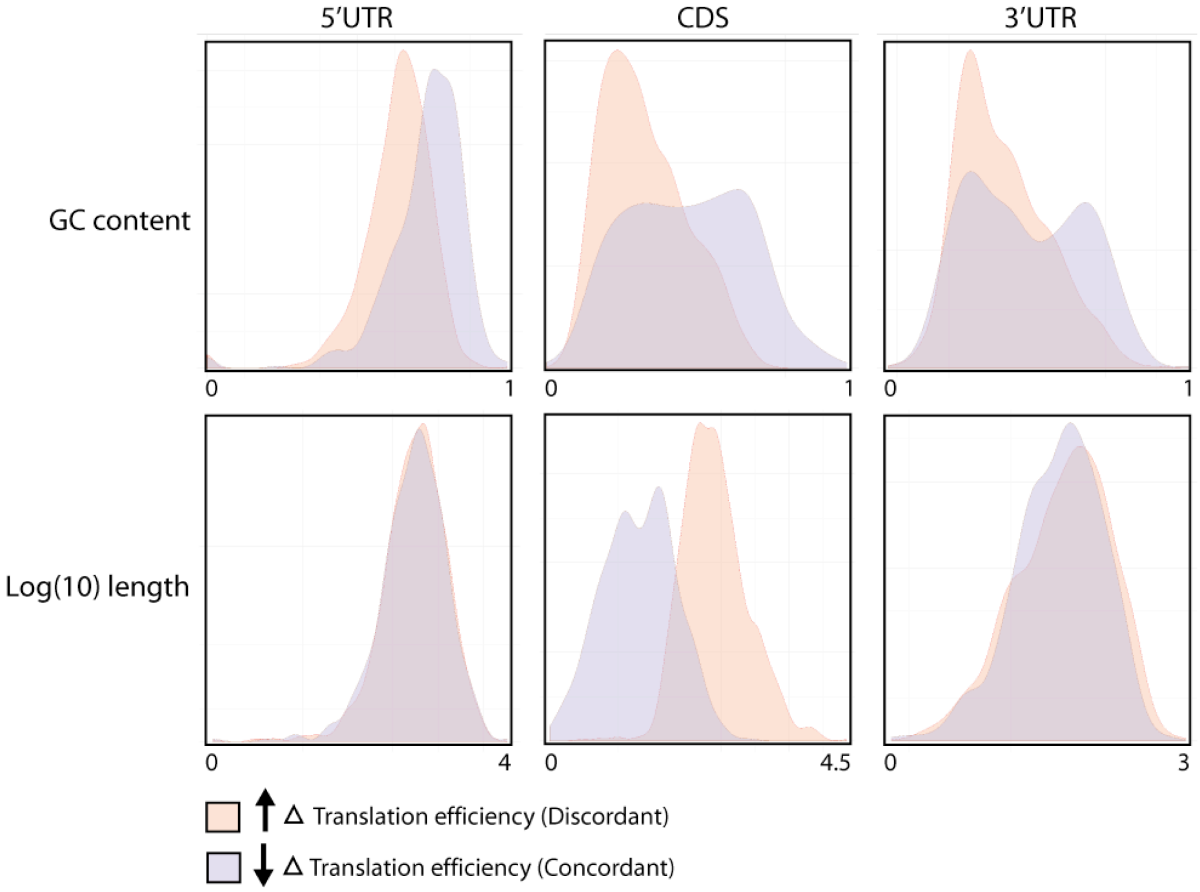
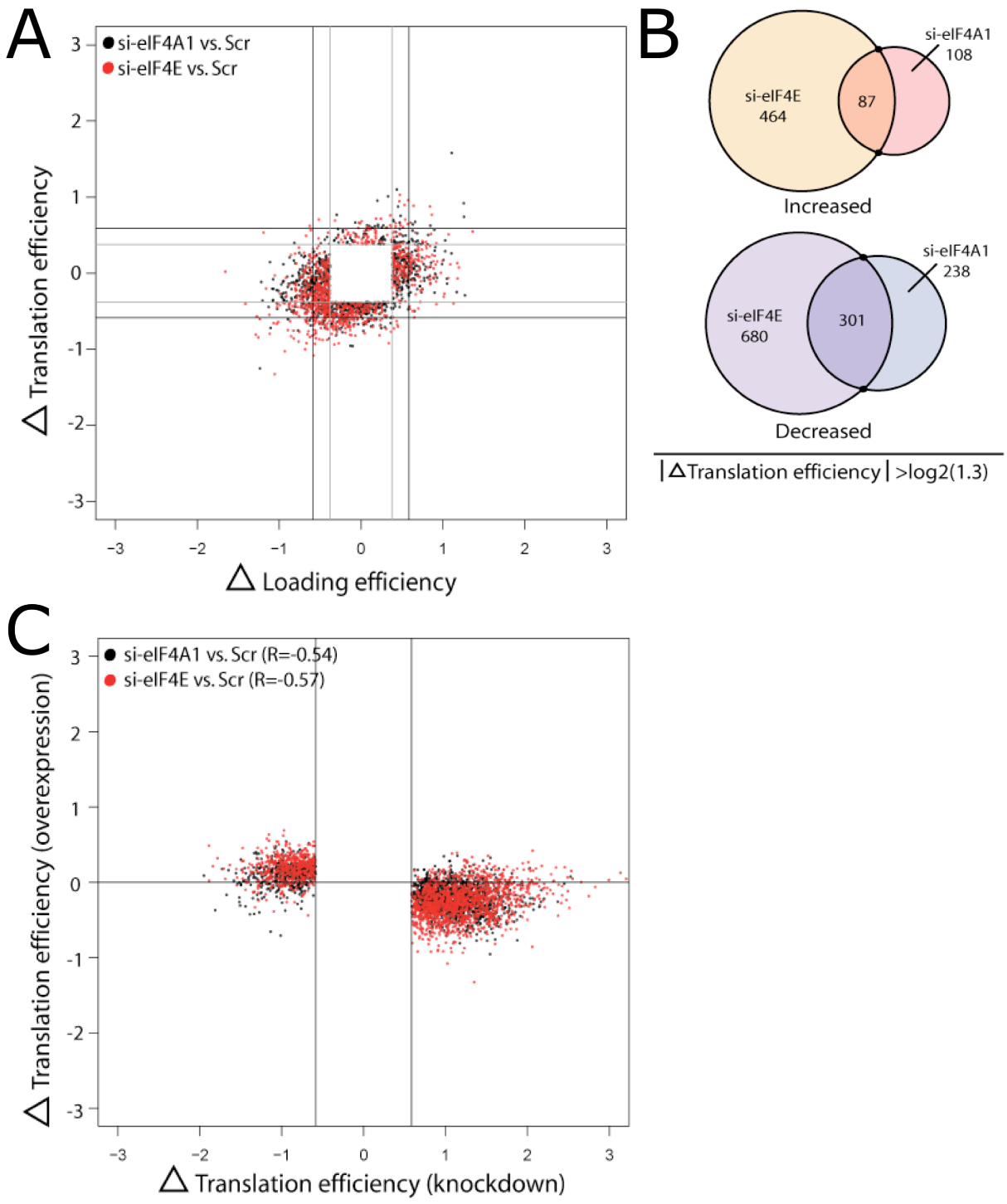


Figure S3.5. Translational changes in stable eIF4A and eIF4E overexpression lines are dampened compared to knockdown treatments.
(A) Summary of changes in translation and/or loading efficiency in A375 cells stably overexpressing eIF4AI or eIF4E. Shown are mRNAs with altered translation and/or loading efficiency in both knockdown treatments (|Fold change| >1.3, no p-value cut-off). Black lines denote |Fold change| >1.5 fold cut-off applied to knockdown treatments in Fig. 2C.
(B) Overlap in mRNAs with increased (left) or decreased (right) translation efficiency in eIF4AI or eIF4E overexpression lines.
(C) Inverse correlation between translation efficiency in transient knockdowns versus overexpression lines. Shown are mRNAs with significantly altered altered translation efficiency in both knockdown treatments (|Fold change| >1.5, $p<0.1$).

Figure S3.5 (continued)



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