MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression through partial complementary base-pairing to the 3′ untranslated region (UTR) of target mRNAs. Inhibition of translation initiation has been identified as an early event of miRNA-mediated gene repression, but the underlying mechanistic details of this process are not well understood. Recently, eukaryotic initiation factor (eIF) 4AII was identified as a critical modulator of miRNA activity with depletion of this factor alleviating miRNA-mediated gene repression. Using the CRISPR/Cas9-editing system, we generated a novel cell line in which expression of eIF4AII was eliminated. The absence of eIF4AII does not affect cell viability, proliferation, or global mRNA translation. Importantly, we show that eIF4AII is dispensable for miRNA-mediated gene silencing.

Keywords: eIF4AII; RISC; miRNA; CRISPR; Cas9; genome editing

Among the mechanisms implicated in translational repression by miRNAs, inhibition of cap-dependent initiation has been invoked from in vitro and in vivo studies (Pillai et al. 2005; Wang et al. 2006; Mathonnet et al. 2007). Of note is the finding that type II internal ribosome entry sites (IRESes), which direct translation initiation in the absence of the cap-binding protein, eukaryotic initiation factor (eIF) 4E, are refractory to miRNA-mediated repression (Humphreys et al. 2005; Mathonnet et al. 2007) thus implicating a role for eIF4E or the 5′ m7G cap structure in this process. Consistent with this is the requirement of a cap structure for efficient miRNA-mediated silencing and the finding that the eIF4F complex (consisting of eIF4E, the DEAD-box eIF4A helicase, and the eIF4G scaffolding protein) can alleviate miRNA activity repression in vitro (Pillai et al. 2005; Wang et al. 2006; Mathonnet et al. 2007). Taken together, these results suggest that miRNAs may target the translation initiation apparatus by promoting disassembly of, or impairing cap recognition by, the eIF4F complex (Mathonnet et al. 2007). Recent experiments have also implicated a critical role for the eIF4A subunit of the eIF4F complex in this process (Meijer et al. 2013; Fukao et al. 2014).

In mammals, two eIF4A paralogs (eIF4AI and eIF4AII) sharing >90% identity, assemble into the eIF4F complex (Yoder-Hill et al. 1993; Rogers et al. 2002). The translation of most cellular mRNAs is dependent on eIF4A, although the degree to which they require eIF4A is related, in part, to the presence of a secondary structure within the mRNA 5′ untranslated region (UTR) (Bhat et al. 2015; Pelletier et al. 2015). The levels of eIF4AI and eIF4AII have been assessed in various tissues and cell lines, and generally eIF4AI is the more abundant paralog (Nielsen et al. 1985; Nielsen and Trachsel 1988; Galicia-Vázquez et al. 2012). Although both proteins are thought to participate in translation initiation, there is evidence suggesting that eIF4AI and eIF4AII are
not redundant proteins and may possess unique biological properties (Li et al. 2001; Galicia-Vázquez et al. 2012). The dissociation of eIF4AI and eIF4AII from target mRNAs is thought to be an early step of miRISC-mediated translational repression (Fukao et al. 2014; Fukaya et al. 2014). Consistent with this, the stabilization of eIF4A onto mRNAs using the pharmacological inhibitor, silvestrol, has been shown to dampen miRNA repression (Fukao et al. 2014). However, another study invoked a critical role for eIF4AII, but not eIF4AI, in miRNA-mediated repression (Meijer et al. 2013). Here, it was proposed that eIF4AII is the only eIF4F component required for miRNA-mediated repression and that it interacts with CNOT1 to induce deadenylation (Meijer et al. 2013). To test the relevance of eIF4AII in miRNA silencing, we used CRISPR/Cas9-mediated genome editing to generate an eIF4AII null cell line. We show that the absence of eIF4AII does not affect cell viability or global protein synthesis, and is not required for efficient siRNA- or miRNA-mediated gene regulation.

RESULTS

We utilized CRISPR/Cas9-editing technology to eliminate eIF4AII in NIH/3T3 cells to dissect potential biological differences between eIF4AI and eIF4AII. Our approach was to design a sgRNA that targeted exon 5, which contains the conserved RNA-binding TPGT motif 1b to disrupt expression of full-length wild-type protein (Fig. 1A). Following introduction of the CRISPR/Cas9-editing system into NIH/3T3 cells, 168 GFP+ single-cell clones were isolated by limiting dilution and screened by PCR using Primers 4AII (Table 1; Fig. 1A). Genomic DNA from one of the isolated clones failed to yield a PCR product with this primer pair, despite being of sufficient quality to produce a PCR product from the eIF4AI locus (Fig. 1B, cf. lane 3 to lanes 1 and 2). This clone, designated eIF4AIIem1JP (for the first endonuclease-induced mutation [em1] of the eIF4AII gene produced in the JP laboratory) (http://www.informatics.jax.org/mgihome/nomen/gene.shtml#endim) was chosen for further characterization. Using a more recessed, second primer pair (4AIIext), two shorter than expected PCR products were obtained (Fig. 1B). Sequencing of the larger PCR product revealed a duplication of the target region that likely led to microhomology-mediated NHEJ to generate Δ1 (see text for details). The TPGT motif 1b is shown in green. (B) PCR analysis of eIF4AIIem1JP. DNA was isolated from parental NIH/3T3 cells (lane 1), NIH/3T3 cells expressing Cas9 and an sgRNA targeting the neutral Rosa26 locus (Malina et al. 2013) (lane 2), or eIF4AIIem1JP cells (lane 3) and used in PCRs with the indicated primer pairs (Table 1). PCR products were analyzed on 1% agarose gels. Products were excised from the gel and analyzed by direct sequencing. (C) Schematic diagram representing configuration of the Δ1 eIF4AII allele in eIF4AIIem1JP. (D) Schematic diagram representing configuration of the Δ2 eIF4AII allele in eIF4AIIem1JP. (E) RT-PCR analysis of mRNA isolated from the indicated cell lines. Complementary DNA was generated as indicated in Materials and Methods using an eIF4AII-specific primer. PCRs were performed using the indicated primer pairs and products analyzed on a 1.2% agarose gel. Products were excised from the gel and analyzed by direct sequencing. (F) RT-qPCR analysis of eIF4AII mRNA in NIH/3T3 and eIF4AIIem1JP cells. Expression of eIF4AII was calculated relative to GAPDH levels and is based on ΔΔCT values. (G) Western blot probing of extracts prepared from NIH/3T3 (lane 1) or eIF4AIIem1JP (lane 2) using antibodies directed to the proteins shown to the right. (H) Western blot probing of extracts prepared from NIH/3T3 (lane 1) or eIF4AIIem1JP (lane 2) using an N-terminal directed antibody (ab31218, Abcam). Arrow indicates position of migration of eIF4AII.
product revealed a 673 bp product with a 109 bp deletion within exon 5, which we refer to as Δ1 (Fig. 1C). In this case, repair of the double-stranded break generated by Cas9 may have been mediated by microhomology-mediated NHEJ (see highlighted sequences in Fig. 1A,C). Sequencing of the shorter 4AIIext product corresponded to a 588 bp fragment that arose as a result of mispriming of Primer 4AIIext-Rev (Table 1) within eIF4AII intron 8. This fortuitous event uncovered a second larger deletion, Δ2, extending from exon 5 to exon 8 with an extra adenine inserted at the junction point (Fig. 1D). Deletions Δ1 and Δ2 were also detected using a different primer pair spanning from intron 4 to intron 8 (4AIIext-b) (Fig. 1B), which upon sequencing, yielded products harboring the expected mutations.

We then analyzed the structure of the expected eIF4AII mRNA using primers spanning from exons 4 to 9 and obtained cDNA products and confirmed the presence of Δ1 and Δ2 mRNA transcripts in eIF4AIIem1JP cells via sequencing (Fig. 1E). An additional product (554 bp) was obtained following RT-PCR, which upon sequencing, corresponded to an mRNA lacking exon 5 (ΔExon 5: exon 4 was fused to exon 6) (see Discussion). We saw no evidence for a full-length eIF4AII transcript spanning exons 4–9 in eIF4AIIem1JP cells and all mutations are predicted to cause frameshifts resulting in early termination of translation (Δ1, 14.2 kDa; ΔExon 5, 14.6 kDa; Δ2, 20.2 kDa).

Quantitative RT-PCR analysis of the eIF4AII mRNA in eIF4AIIem1JP cells, with primers targeting exon 1 or spanning exons 2–4, revealed an approximately fourfold decrease in mRNA levels (Fig. 1F). Although we have not investigated the mechanism responsible for this decrease, it may be attributable to nonsense-mediated decay, effects on transcriptional output, and/or mRNA stability. Importantly, Western blot analysis of total protein extracts from NIH/3T3 and eIF4AIIem1JP cells revealed the absence of eIF4AII in the latter, with no significant changes in eIF4A1, eIF4E, and eIF4GI levels (Fig. 1G, P-values: 0.90 for eIF4A1, 0.74 for eIF4E, and 0.93 for eIF4GI). Using an antibody targeting the eIF4AII N-terminal domain, we failed to note the presence of truncated products arising from Δ1, ΔExon 5, or Δ2 mRNA transcripts (~14–20 kDa), indicating that, if produced, these are below the limits of detection of our probed (Fig. 1H).

We previously documented that knockdown of eIF4AI, but not eIF4AII, dramatically inhibits protein synthesis and is lethal in Myc-driven tumors (Galicia-Vázquez et al. 2012; Cencic et al. 2013). We took advantage of the complete absence of eIF4AII in eIF4AIIem1JP to assess the consequences on global protein synthesis and cellular proliferation. We found that relative to the parental NIH/3T3 cell line or to a cell line in which Cas9 had been targeted to the neutral Rosa26 locus (referred to hereafter as NIH/3T3[sgROSA]), eIF4AIIem1JP showed no detectable differences in 35S-methionine incorporation or cellular proliferation (Fig. 2). We conclude that eIF4AI is not essential and that its loss in NIH/3T3 cells has no noticeable impact on global protein synthesis.

These results were surprising since eIF4AII has been reported to be essential for microRNA-mediated gene regulation (Meijer et al. 2013) and loss of miRNA function is associated with perturbed proliferative and survival responses (e.g., as documented in Dicer−/− cells) (Ravi et al. 2012). We thus took advantage of eIF4AIIem1JP cells to characterize siRNA and miRNA activity in the absence of eIF4AII. To this end, we assessed gene expression from two reporter systems in NIH/3T3[sgROSA] or eIF4AIIem1JP cells transduced with shRNAs targeting GFP or Ago2 (Fig. 3A). First, we probed for effects of eIF4AII loss on siRNA-mediated gene

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**TABLE 1. PCR Primers used in the current study**

| Primer          | Sequence (5’–3’) | Expected product size (bp) | Product size obtaineda (bp) |
|-----------------|-----------------|----------------------------|---------------------------|
| 4AII-For        | CTGTGATCGTCTGCATTGGAG | 131                        | 131                       |
| 4AII-Rev        | GAAGGACCATATAGTGCATC | 782                        | 588 and 673               |
| 4AIIext-For     | GCCTGAACTTTATCGGCGT | 1738                       | 623 and 1629              |
| 4AIIext-Rev     | GCCTGAACTTTATCGGCGT | 184                        | 184                       |
| 4AIIext-b-Rev   | GGCAGATGCGAGCAAGATAT | 184                        | 184                       |
| eIF4AII-For     | GTATGTTTCCAGTTTCTCCTGGGC | 723                        | 614, 554, and 326         |
| eIF4AIIext-4/9 For | TTGCTCAAGCTCAGTCAGGT | 118                        | N/A                       |
| eIF4AIIext-4/9 Rev | TGCATCTCCCTTCCTGGTCCC | 163                        | N/A                       |
| eIF4AIIexon-1 For | GCTGAAATATTCTCCTGGCCG | 163                        | N/A                       |
| eIF4AIIexon-1 Rev | TTACCGGAGGTAGAACCTTA | 106                        | N/A                       |
| eIF4AIIext-2/4 For | TGGTGTACCTAGAGCAACTGGAA | 106                        | N/A                       |
| eIF4AIIext-2/4 Rev | TTGGCCTACCTCAGCTGAGCTTG | 225                        | N/A                       |
| HMG2A-For       | GTGGCCGGTTTTTCTCCTCGAGTC | 225                        | N/A                       |
| HMG2A-Rev       | GTGGCCGGTTTTTCTCCTCGAGTC | 225                        | N/A                       |

aInformation obtained after sequencing of PCR product.
repression. We introduced a *Renilla* luciferase (RLuc)-based reporter carrying six artificial CXCR4 siRNA-binding sites, along with a control scrambled (siScr) or CXCR4 siRNA, into each of the cell lines, and assessed subsequent repression by measuring luciferase activity (Fig. 3B; Wang et al. 2009). A plasmid coding for HCV-Firefly Luciferase (FLuc) was used as a transfection control. The RLuc expression data were normalized to FLuc activity and indicated significant loss of siRNA-mediated repression upon Ago2 suppression in both NIH/3T3[sgROSA] and elf4AIIem1JP cells (Fig. 3B). There was no significant difference in response between NIH/3T3[sgROSA] and elf4AIIem1JP cells transduced with shGFP. Second, we utilized a set of reporters whose translation is regulated by endogenous let-7 (Fig. 3C; Mathonnet et al. 2007). In both shGFP infected cell lines, expression from the pRL 6xLet7 reporter was significantly reduced compared to pRL mm6xLet7, indicating regulation by endogenous mouse Let-7 (Fig. 3D). Of note, derepression of the pRL 6xLet7 reporter was observed upon knockdown of Ago2 in both cell lines, whereas expression from reporters lacking Let-7 binding sites (pRL) or harboring mutated seed regions (pRL mm6xLet7) were unchanged (Fig. 3D). No significant difference in response was noticed between the shGFP infected NIH/3T3[sgROSA] and elf4AIIem1JP cells (Fig. 3D). Taken together, these results indicate that elf4AII is not necessary for siRNA- or miRNA-mediated regulation of gene expression.

Although none of the alleles disrupted in elf4AIIem1JP cells can produce functional elf4AII protein, it is a formal possibility that a truncated form could be generated (albeit at significantly reduced levels [Fig. 1F]) that could participate in RNA-mediated silencing—perhaps as a scaffold for complex assembly. We therefore suppressed elf4AII mRNA levels in NIH/3T3[sgROSA] and elf4AIIem1JP cells using two previously characterized independent shRNAs (Fig. 4A; Galicia-Vázquez et al. 2012) and monitored the
consequences on siRNA- and miRNA-mediated silencing (Fig. 4B,C). We saw no evidence for changes to suppression of our reporter constructs when eIF4AII mRNA levels were further suppressed in eIF4AIIem1JP cells, indicating that any truncated products, if synthesized, are unlikely to be participating in RNA-mediated regulation of gene expression. These results were not cell type specific as potent knockdown of eIF4AII in HeLa cells showed no effects on siRNA- or miRNA-mediated gene expression when compared to control cells (Fig. 4D,E).

Finally, to assess whether the loss of eIF4AII altered miRNA-mediated repression of endogenous transcripts, we transfected NIH/3T3[sgROSA] and eIF4AIIem1JP cells with an antagonim to let-7c (referred to hereafter as Anti-let-7c) and measured the consequences on the expression of HMG A2, a well-characterized target of let-7 miRNAs (Lee and Dutta 2007; Mayr et al. 2007). RT-qPCR shows that introduction of Anti-let-7c led to a sixfold to sevenfold increase in HMG A2 mRNA levels relative to the mock-transfected cells, irrespective to the presence of eIF4AII (Fig. 5A). Accordingly, we also observed a 1.5- to 1.6-fold increase in HMG A2 protein expression in NIH/3T3[sgROSA] and eIF4AIIem1JP cells transfected with Anti-let-7c. Taken together, our data demonstrate that miRNA-mediated repression is not impaired in eIF4AIIem1JP cells, suggesting that eIF4AII is not an essential factor for miRNA silencing.

DISCUSSION

Here, we used CRISPR/Cas9 to generate a novel NIH/3T3 cell line in which expression of eIF4AII is ablated (Fig. 1). Genomic PCR analysis of this line indicated the presence of at least two mutated eIF4AII alleles harboring intragenic deletions, whereas RT-PCR analysis indicated the presence of three truncated mRNA transcripts. Deletion Δ1 is a 109 bp deletion that is within the size range usually obtained using the CRISPR/Cas9-editing system (Mali et al. 2013; Malina et al. 2013). The serendipitous mispriming event with primers 4AIIext, however, uncovered a larger intragenic deletion, Δ2 (Fig. 1). We do not have insight into the mechanism that led to the generation of Δ2, but it could have been the result of unpredicted off-target cutting by Cas9 near the 3’ border of the deletion. However, we failed to detect any sequence target site of sufficient homology with the eIF4AII sgRNA used in our approach (data not shown). RT-PCR analysis revealed the presence of a third transcript lacking exon 5 (Fig. 1E, ΔExon5). Whether this is the result of inefficient recognition
of the splice acceptor site in exon 5 of Δ1 leading to exon skipping or the presence of a third mutated eIF4AII allele in eIF4AIIem1JP cells is currently unknown (since there is no a priori reason to assume that NIH/3T3 are diploid for eIF4AII). These results highlight that CRISPR/Cas9-editing can lead to unpredicted and complex gene rearrangements. Nonetheless, Western blot analysis indicated complete loss of all wild-type eIF4AII alleles (Fig. 1G,H).

We did not detect any significant consequence of eIF4AII loss on global protein synthesis or cellular proliferation (Fig. 2). These results reinforce our previous findings in which we used siRNAs and shRNAs to suppress eIF4AII and saw little effect on cell proliferation and viability (Galicia-Vázquez et al. 2012; Cencic et al. 2013). However, the differential regulation of eIF4AII and eIF4AI during differentiation (Galicia-Vázquez et al. 2014), growth status (Williams-Hill et al. 1997), and in response to MYC (Lin et al. 2008) are suggestive of specialized functions for eIF4AII which have not been uncovered by our assays. MicroRNAs mediate translational repression and deadenylation/mRNA decay with the sum of these processes measured by the assays utilized in our study. eIF4AII has been reported to be critical to miRNA-mediated gene regulation and Meijer et al. (2013) reported derepression of RNA silencing upon knockdown of eIF4AII. In our hands, we were unable to observe this phenomenon in HeLa cells (Fig. 4D,E). The underlying basis for this discrepancy is unclear, although our results in HeLa cells are consistent with the bulk of the data presented herein using NIH/3T3 cells lacking eIF4AII, and indicate that eIF4AII is dispensable for RNA-mediated silencing. Our data do not allow us to rule out a specialized role for eIF4AII, but it does not appear to be essential to the process. The current study also exemplified how the ability to rapidly generate isogenic cell lines using CRISPR/Cas9 is a powerful addition to the somatic cell genetics toolkit to help elucidate gene function.

CONCLUSIONS

Our results demonstrate that eIF4AII activity is neither essential nor required for miRNA-mediated gene silencing or reporter mRNAs in mammalian cells.

MATERIALS AND METHODS

Generation of eIF4AIIem1JP cells

NIH/3T3 cell lines were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, and 4 mM L-glutamine at 37°C and 5% CO2. A previously described pQCX-based All-In-One vector system (Malina et al. 2013) expressing an sgRNA targeting eIF4AII exon 5 (5′-GCATTGTTGTTGGTACTCCA-3′) (Fig. 1A), human codon-optimized Cas9, and GFP was introduced into NIH/3T3 cells by nucleofection of 2.5 µg of plasmid (Amaza Cell Line Nucleofector Kit R for NIH/3T3, VCA-1001, Lonza) using program U-030 and an Amaza Nucleofector device. After 48 h, GFP+ cells were isolated by cell sorting with a FACSAria-Cell Sorter (BD Biosciences). One hundred and sixty-eight single-cell clones were isolated by limiting dilution. Upon expansion, DNA was isolated and screening performed by PCR using Phusion High-Fidelity DNA polymerase (New England BioLabs) (Table 1).

Retroviral transfections

Twenty-five micrograms of MLS-empty, MLS-sh4AII.485 or MLS-sh4AII.592 was transfected into ectropic Phoenix cells using calcium phosphate in the presence of 25 μM chloroquine. The media was changed the following day and 48 h post-transfection, the viral supernatant was collected, filtered, and added to NIH/3T3[sgROSA] or eIF4AIIem1JP cells in the presence of 4 μg/mL polybrene. Cells were infected six times every 8 h by spinoculation at 1000 g for 1 h at 30°C.

Cell doubling assays

NIH/3T3 (10,000 cells) were seeded in 12-well plates at the beginning of each passage. After 2 d, cells were trypsinized, counted by flow cytometry (GUAVA EasyCyte Plus; Millipore) and the data analyzed using Cytosoft 5.3 Software. Cells were then reseeded for the next passage analysis. Population doubling level for each passage was calculated with the following equation: Cumulative PDL = [Log
Monitoring siRNA and miRNA activity

To assess siRNA activity, a previously reported Renilla luciferase expression vector (RL6X) harboring six CXCR4 siRNA-binding sites was utilized (Wang et al. 2006). Twelve hours prior to transfection, 50,000 cells were seeded per well in a 12-well plate. In each well, 1 μg RL6X, 1 μg pcDNA3-HCV-Fluc, and 12 pmol of siRNA were co-transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Life Technologies). In experiments involving transient suppression of elf4AII using siRNAs, HeLa cells were transfected with siRNAs targeting human elf4AII (M-013758-01) using Lipofectamine 2000 24 h prior to transfection of the luciferase reporters.

To assess miRNA activity, we utilized previously described Renilla luciferase reporters harboring either no (pRL) or 6 (pRL 6xLet7) let-7 target sites in addition to a luciferase reporter with 6 let-7 target sites containing mutations within its seed sequence (pRL mm6xLet7). The reporter plasmids were co-transfected using Lipofectamine 2000 2000 according to the manufacturer’s instructions (Life Technologies). In experiments involving transient suppression of elf4AII using siRNAs, HeLa cells were transfected with siRNAs targeting human elf4AII (M-013758-01) using Lipofectamine 2000 24 h prior to transfection of the luciferase reporters.

Immunoblotting analysis

Protein samples were fractionated on SDS-polyacrylamide gels, transferred to PVDF membrane (Millipore), and probed with the indicated antibodies. Antibodies used in this study were directed against: eIF4AI (ab31217, Abcam), eIF4AII (ab31218, Abcam), elf4E (Cell Signaling), elf4G1 (A300-502A, Bethyl Labs), PDCD4 (sc9535, Cell Signaling), Tubulin (TS168, Sigma), Actin (A5441, Sigma), GAPDH (ab8245, Abcam), Ago2 (C34C6, Cell Signaling), and HMGA2 (ab97276, Abcam). For statistical analysis, Student t-tests were performed using GraphPad InStat version 3.10.

[^35S]-Methionine/cysteine metabolic labeling

Cells were preincubated in methionine/cysteine-free DMEM with 10% dialyzed FBS and 4 mM l-glutamine for 40 min prior to the addition of [^35S]-methionine/cysteine (150–200 μCi/mL, 1175 Ci/mmol) (PerkinElmer). Cells were labeled for 20 min and levels of [^35S]-methionine/cysteine incorporation were determined from cell extracts by TCA precipitation followed by scintillation counting. CPMs obtained were normalized to protein levels in the extracts and are plotted relative to the values obtained with the parental NIH/3T3 cells.

RT-PCR

Cellular RNA was isolated using TRIzol (Invitrogen). Complementary DNA was generated by reverse transcription using SuperScript III (Invitrogen) and 4AII-Exon9 Rev primer (5′-AGTGATCAGAA CACGGCTTGA-3′) according to the manufacturer’s instructions. Amplifications were performed by Touchdown PCR using Phusion High-Fidelity DNA Polymerase (New England BioLabs) and 4Alleonx-4/9 primers, with annealing temperatures ranging from 67°C to 57°C (Table 1). To quantify elf4AII mRNA levels, reverse transcription was performed using Superscript III (Invitrogen) and Oligo dT (Invitrogen). Quantitative PCRs were performed using SsoFast EvaGreen Supermix (Bio-Rad). Reactions were performed using a CFX96 PCR System (Bio-Rad), and data analyzed using Bio-Rad CFX Manager 2.1 software. The threshold cycles (Ct) were determined by single threshold, and the expression of elf4AII was determined by the ΔΔCT method using GAPDH as the reference target. Primer efficiency was determined and taken into account in the CT expression determinations.

Probing endogenous miRNA repression

Approximately, 200,000 cells were seeded per well in a 6-well plate and transfected with 250 pmol of Anti-miR hsa-let-7c miRNA Inhibitor (Ambion) using Lipofectamine 2000 according to the manufacturer’s instructions. Media was changed 24 h later and a second transfection performed 48 h later. RNA and protein were extracted 24 h after the final transfection. For mRNA expression analysis, total RNA was extracted using TRIzol and cDNA was produced using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative PCRs were performed with SsoFast EvaGreen Supermix (Bio-Rad) using primers specific to HMGA2 and GAPDH. Relative expression of HMGA2 mRNA was determined using the 2^−ΔΔCT method (Livak and Schmittgen 2001), with GAPDH as the reference transcript. To assess HMGA2 protein levels, cells were lysed in RIPA buffer (20 mM HEPEs, 150 mM NaCl, 1% Triton-X100, 10% glycerol, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate, 1 mM PMSF, 4 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL pepstatin) and processed for immunoblotting. Blots were scanned and quantitated using the LI-COR Odyssey Image system (v3.0).

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