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Ars2 links the nuclear cap binding complex to RNA interference and cell proliferation

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SUMMARY

A partial cDNA for Arsenic resistance protein 2 (Ars2) was originally identified in a screen for genes that conferred arsenic resistance. Here we show that Ars2 is a component of the nuclear RNA cap binding complex (CBC) and is critical for proliferation. Unlike other components of the CBC, Ars2 expression is linked to the proliferative state of the cell. Deletion of Ars2 causes developmental lethality. In adult mice, deletion of Ars2 led to bone marrow failure, while parenchymal organs composed of non-proliferating cells were unaffected. Depletion of Ars2 or CBP80 from proliferating cells impairs miRNA-mediated repression. Ars2 functions in miRNA biogenesis at the level of nuclear miRNA processing. Depletion of Ars2 protein led to alterations in primary miRNA processing and reduced levels of several miRNAs implicated in cellular transformation, including miR-21, let-7, and miR-155. These findings provide evidence for a role for Ars2 in RNA interference regulation during cell proliferation.

INTRODUCTION

Arsenic resistance protein 2 (Ars2/Asr2) was originally cloned in a screen for cDNAs that could confer sodium arsenite resistance in a hamster cell line (Rossman and Wang, 1999), but the protein has remained poorly characterized. It is conserved throughout metazoa and has homologs in plants and fission yeast, but not budding yeast. Ars2 deletion in metazoans is associated with developmental lethality in *Drosophila* (Oh et al., 2003), zebrafish (Golling et

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Supplemental Data
Supplemental data include Supplemental Experimental Procedures and 5 Supplemental Figures.

al., 2002) and mouse (Wilson et al., 2008). Mutations of *SERRATE*, a plant gene with homology to Ars2, result in broad developmental defects and hypersensitivity to the plant stress hormone abscisic acid, whereas enforced expression of *SERRATE* enhanced plant growth (Bezerra et al., 2004; Grigg et al., 2005; Prigge and Wagner, 2001).

Several lines of evidence support a role for Ars2 in RNA metabolism. First, Ars2 contains a number of domains common in RNA binding proteins including an amino-terminal arginine-rich domain, a central RNA-binding domain and a zinc finger. Second, *SERRATE* was recently found to be required for the biogenesis of miRNAs in Arabidopsis (Grigg et al., 2005; Lobbes et al., 2006; Yang et al., 2006). Third, reports have identified Ars2 as a component of RNA-protein complexes enriched for spliceosomes (Rappsilber et al., 2002; Zhou et al., 2002).

The current experiments began by investigating the role of Ars2 in mediating cell survival in response to arsenic treatment. Unlike the reported partial clone, a full-length Ars2 cDNA did not confer arsenic resistance. To examine the possibility that the partial clone had functioned as a dominant-negative in previous studies, we suppressed Ars2 using RNAi and observed increased survival of cells treated with arsenic. However, Ars2 suppression leads to a profound defect in cell proliferation. To investigate the molecular basis of the essential role for Ars2 in proliferation we studied the expression, localization and biochemical properties of Ars2. These studies reveal that Ars2 interacts with the nuclear CBC and components of the nuclear pri-miRNA processing complex. Unlike the core components of the CBC, CBP80 and CBP20, Ars2 is not constitutively expressed. Ars2 is selectively expressed in proliferating cells and its expression is required for the maintenance of cell proliferation. Ars2 expression contributes to the stability and delivery of capped primary miRNA transcripts to the pri-miRNA processing complex containing Drosha and DGCR8 (Pasha). Furthermore, depletion of Ars2 is sufficient to reduce the pri-miRNA processing and miRNA levels of a number of miRNAs implicated in transformation including miR-21, let-7 and miR-155. Together, these data suggest that Ars2 is a proliferation-inducible component of the CBC that contributes to regulation of RNA interference.

RESULTS

Ars2 modulates arsenic sensitivity and colony formation

When we isolated Ars2 cDNAs from a murine hematopoietic cell line the clones obtained had an open reading frame coding for a predicted protein of 875 amino acids, in contrast to the 225 amino acids coded for by the open reading frame of the cDNA originally isolated (Rossman and Wang, 1999). The predicted protein contained an amino-terminal arginine-rich domain and an RNA recognition motif in addition to the carboxy-terminal zinc finger domain originally identified. When expressed in either murine fibroblasts or the human cell line K562 this clone of Ars2 resulted in no increase in resistance to arsenic trioxide compared to control transfected cells at a range of doses (data not shown).

Since the original reported Ars2 clone appeared to be a truncation of the full-length gene product, we reasoned that it may have functioned as a dominant negative. 3T3 MEFs infected with shRNA to Ars2 (shArs2-1) showed effective depletion of the protein after four days in culture as determined by Western blot analysis (Figure 1A) compared to cells treated with empty vector control retrovirus (shVec). When Ars2 depleted cells were treated with arsenic trioxide for 48 hours the cells exhibited a slower rate of cell death when compared to control infected cells treated with arsenic (Figure 1B). However, few of the cells infected with Ars2 shRNA were able to proliferate when subsequently passaged in arsenic-free medium (data not shown).

To extend these findings, 3T3 clonal cell lines were generated (hp1–11 and hp2–9) expressing shRNAs targeted to two independent sequences within the Ars2 mRNA. These stable cell line clones showed reduced levels of Ars2 in comparison to two different vector control clonal cell lines (vec-7, vec-12) (Figure 1C). The sensitivity of these cell lines to arsenic trioxide was assessed by colony forming assay. Ars2 knockdown cell lines consistently formed one-half the number of colonies as control cell lines (Figure 1D).

Ars2 is required for cell proliferation

The inability of viable Ars2-deleted cells to recover following treatment with arsenic trioxide suggested the possibility that these cells had a proliferative defect. 3T3 MEFs were treated with Ars2 shRNA and population doublings measured over time in culture. shArs2-1 caused a near complete loss of detectable Ars2 protein (Figure 1E) and suppressed proliferation for four days in culture, while shArs2-2 caused a lesser amount of Ars2 depletion and had a more modest effect on cell proliferation (Figs. 1E and 1F). The proliferative defect of Ars2-deficient cells was associated with a reduced ability of Ars2 knockdown cells to incorporate BrdU following serum stimulation (Figure 1G). Ars2 deficient cells did not arrest at a particular stage in the cell cycle or exhibit a significant amount of cell death until after they had ceased progressing through the cell cycle.

To independently confirm the effects of Ars2 on proliferation, a floxed allele of Ars2 was generated in murine embryonic stem cells by homologous recombination (Supplemental Figure 1). Injection of these cells into blastocysts resulted in chimeric animals and further breeding established mice homozygous for the targeted allele ($Ars2^{flox/flox}$) from which MEFs were generated and immortalized by transfection with SV40 large T antigen.

Genetic deletion of Ars2 in the $Ars2^{flox/flox}$ (fl) MEFs was accomplished by infecting the cells with a retrovirus expressing Cre recombinase. By 72 hours post-infection Ars2 protein levels were significantly diminished compared to wild-type (wt) MEFs infected with Cre (Figure 2A). Prior to this timepoint, there was no difference in the number of cells that had accumulated in culture. However, between 72 and 120 hours post-infection, the number of $Ars2^{flox/flox}$ cells that had accumulated was significantly diminished compared to wt cells (Figure 2B). Over this timecourse, there was an inhibition of cell proliferation comparable to that observed using Ars2 shRNA.

Analysis of Ars2 expression in vivo revealed high expression in hematopoietic tissues and reduced or absent expression in parenchymal organs like liver and kidney (Figure 2C and data not shown). To examine the effects of Ars2 deletion in proliferating tissues in vivo $Ars2^{flox/-}$ mice were crossed to mice harboring an MxCre transgene to obtain mice of the compound genotype $Ars2^{fl/-}$; MxCre (fl/−; MxCre). The MxCre transgene has been used to mediate inducible recombination in a wide variety of tissues and with high efficiency in the liver and hematopoietic compartments (Kuhn et al., 1995). Injection of these mice with the nucleotide analog polyinosinic-polycytidylic acid (pIpC) led to induction of Cre recombinase and deletion at the Ars2 locus as evidenced by Western analysis from hematopoietic tissues (Figure 2C, lane 6). Mice harboring one wt allele and one floxed allele of Ars2 (fl/wt) served as controls and did not have depletion of the Ars2 protein, even in the presence of the MxCre transgene (Figure 2C, lanes 4 and 5). Histological examination of $Ars2^{flox/-}$;MxCre mice sacrificed 9 days following pIpC treatment revealed decreased bone marrow cellularity compared to control (fl/−) injected mice (Figure 2D). Other hematopoietic organs including the thymus and spleen showed histological evidence of increased apoptosis (not shown). In contrast, the histology of other organs including the liver, heart, brain, kidney and lungs was unaffected in both $Ars2^{flox/-}$;MxCre and control mice following pIpC injections (Figure 2D and data not shown). Despite the ability of MxCre to induce recombination in liver following pIpC (Kuhn et al., 1995), liver function tests including serum alanine aminotransferase and

alkaline phosphatase levels did not show significant differences between fl/−;MxCre and control mice following pIpC injection.

Ars2 interacts with the nuclear cap binding complex

To gain further insight into Ars2 function a biochemical screen for interacting proteins was performed. Flag-Ars2 was transiently transfected into 293T cells and immunopurified with anti-Flag antibody. Co-precipitated proteins were identified by liquid chromatography coupled to mass spectrometry. Two proteins that specifically enriched with Flag-Ars2 immunoprecipitation but not with control immunoprecipitation were identified: the 80 kDa subunit of the nuclear cap binding complex (CBC) and importin α.

Immunoprecipitation of Flag-Ars2 followed by Western blot analysis with specific antibodies confirmed that Ars2 co-precipitates both the 80 kDa subunit of the nuclear cap binding complex (CBP80), as well as the 20 kDa CBC subunit (CBP20) and importin α/β heterodimeric nuclear import receptor (Figure 3A). Importin α has been previously shown to be a constitutive component of the nuclear CBC (Gorlich et al., 1996). Under the conditions tested Flag-Ars2 did not co-immunoprecipitate other molecules that have been reported to bind the nuclear cap binding complex, including the mRNA export adaptor ALY/REF (Cheng et al., 2006) or the nonsense-mediated decay factor Upf1 (Hosoda et al., 2005) (data not shown). Furthermore, Ars2 did not co-immunoprecipitate the cytoplasmic 7-methyl guanosine (7mG)-associated complexes eIF4A or eIF4E (data not shown), nor the highly abundant RNA binding protein hnRNP A1 (Figure 3A).

To verify that Ars2 interacts with the nuclear CBC, Flag-tagged CBP80 was immunopurified from transfected 293T cells using anti-Flag antibody. Ars2 was enriched along with other CBC complex components in this immunoprecipitate (Figure 3B). Furthermore, the sedimentation profile of endogenous Ars2 and CBP80 proteins was compared using sucrose gradient analysis. Ars2 was concentrated in the light fractions, whereas CBP80 was detected throughout the gradient with a significant amount unresolved in the pellet fraction (Figure 3C). Sucrose gradient analysis carried out in the presence of RNase A revealed that both Ars2 and CBP80 sedimentation profiles collapsed to fractions 3 to 5 at the top of the gradient (Figure 3C). Despite the observation that Ars2 sedimentation profiles were sensitive to RNase A treatment, the ability of Ars2 to co-immunoprecipitate with CBP80 was not dependent on RNA. Immunoprecipitation of Flag-Ars2 in the presence of RNase A did not abolish the Ars2-CBC interaction (Figure 3D). In contrast, a small amount of eIF4G immunoprecipitated by Ars2 was found to be sensitive to RNase treatment and served as a positive control for RNase A activity (Figure 3D).

Ars2 interacts with 7-methyl guanosine capped RNAs

To confirm that endogenous Ars2 interacts with the nuclear CBC, the ability of Ars2 antibodies to induce a gel shift in CBC-RNA assembly reactions was examined. Using previously published conditions (Izaurralde et al., 1992), a 7mG-dependent gel shift of capped and radiolabeled RNA probe was resolved by native gel electrophoresis (Figure 4A, complex, lane 3). This shift was 7mG-dependent as a significantly diminished shift was observed using 2,2,7-trimethylguanosine (TMG)-capped RNAs (Figure 4A, lane 4), for which the nuclear CBC has a low affinity. The 7mG-dependent shift has been reported to contain the nuclear CBC complex proteins CBP80 and CBP20 (Izaurralde et al., 1995). This was confirmed by incubating assembly reactions with a rabbit antibody raised against full-length CBP20 which resulted in an inhibition of the 7mG-dependent shift (data not shown) as has been previously reported for similar antibodies (Izaurralde et al., 1995). An Ars2-specific antibody reproducibly supershifted a fraction of the RNA binding complex (Figure 4A, supershift, lane 11). A

supershift was not observed with control rabbit antiserum (lanes 5–8), TMG-capped RNA (lanes 2, 4, 6, 8, 10, 12) or in the absence of nuclear extract (lanes 1, 2, 5, 6, 9, and 10).

The protein components of the cap binding complex shuttle between the nucleus and the cytoplasm. If Ars2 is associated with the CBC during nuclear export it should also undergo shuttling. Expression of V5-tagged Ars2 in 3T3 MEFs showed Ars2 to be a predominately nucleoplasmic protein, with faint staining of the cytoplasm (data not shown). In heterokaryon shuttling experiments, MEFs stably expressing V5-tagged Ars2 were fused to HeLa cells in the presence of cycloheximide to inhibit new protein synthesis. After two hours, immunofluorescence showed that Ars2 had accumulated in HeLa nuclei of heterokaryons (Figure 4B, upper panels). Addition of leptomycin B to inhibit Crm1-dependent nuclear export strongly decreased the shuttling of Ars2 (Figure 4B, lower panels).

Ars2 and the nuclear CBC are required for miRNA-mediated RNA interference

The data suggest Ars2 binds the nuclear CBC (Figs. 3 and 4A). In Arabidopsis, mutations in genes encoding the plant homologues of CBP80 (called ABH1) and Ars2 (SERRATE) have similar phenotypes (Bezerra et al., 2004;Hugouvieux et al., 2001;Papp et al., 2004). Mutation of *SERRATE* is proposed to result in developmental defects because of its required role in the processing of miRNAs (Grigg et al., 2005).

Based on these observations the effect of Ars2 on miRNA-mediated gene silencing in mammalian cells was tested. Two luciferase reporters that are sensitive to let-7-mediated miRNA repression were used. The first (“lin28 site”) contains Renilla luciferase with three let-7 target sites in the 3′UTR derived from the lin28 transcript. The second (“perfect match”) is a similar construct with the lin28 site mutated to be perfectly complementary to let-7 to trigger Argonaute 2 (Ago2)-dependent slicer activity and reporter mRNA cleavage. Transfection of siRNAs was first performed to deplete Ars2 or CBP80 from HeLa cells (Supplemental Figure 2) and then the cells were transfected with the reporter constructs. siRNAs to Ago2 were used as a control and caused a 1.5-2 fold loss of repression compared to control siRNAs for both reporters (Figure 5A). Two siRNAs targeted to Ars2 (Ars2-1, Ars2-2) or siRNA to CBP80 inhibited miRNA-mediated repression to an equal or greater extent compared to Ago2 siRNA for the lin28 site reporter and had a significant, but lesser, effect on the perfect match reporter (Figure 5A). A mutant reporter with a single base pair insertion in the lin28 site seed region to disrupt let-7 binding led to a loss of repression under all conditions tested and verified that our assay was responsive to let-7 (data not shown). Addition of excess let-7/let-7* duplex to the transfection mix rescued the loss of repression afforded by Ars2, but not Ago2, depletion (Figure 5B).

Depletion of either Ars2 or DGCR8 with two independent siRNAs led to a decrease in let-7 miRNA levels compared to control treated cells (Figure 5C, D). miR-21, another miRNA whose expression has also been implicated in the regulation of cell proliferation, was also decreased by depletion of Ars2 from cells. Although pre-let-7 was not detectable by Northern blotting, depletion of Ars2 protein also led to a clear reduction of pre-miR-21 (Supplemental Figure 3). However, not all miRNAs were similarly affected; the levels of several housekeeping miRNAs were not found to be affected by depletion of Ars2 (Supplemental Figure 3).

In the nucleus, primary miRNA transcripts are processed by the Microprocessor complex that contains Drosha and DGCR8, whereas Dicer functions to process pre-miRNAs in the cytoplasm. To determine whether Ars2 could interact with either the Dicer or Drosha processing complexes, immunoprecipitation of Flag-Drosha and Flag-Dicer was performed from 293T cells. Western blotting on these immunoprecipitates revealed that endogenous Ars2 and CBP80 co-precipitated with Drosha, but not Dicer (Figure 6A). RNase A treatment failed to disrupt co-precipitation of endogenous Ars2 and CBP80 with Drosha. Similarly when

extracts from 293T cells transfected with V5-tagged Ars2 (V5-Ars2) were used for immunoprecipitation, Drosha was co-precipitated (Figure 6B).

To test whether miRNA maturation downstream of Drosha was preserved in the absence of Ars2 or CBP80, the let-7-responsive luciferase reporters were transfected into cells in the presence or absence of excess pre-let-7 that is not dependent on Drosha for activity. When Ars2, CBP80 or Ago2 were depleted from cells a loss of let-7-mediated repression was observed for both lin28 site and perfect match reporters (Figure 6C). Addition of excess pre-let-7 to the transfection mix rescued the loss of repression in cells that had been depleted of Ars2 or CBP80 for both reporters (Figure 6C). When the levels of endogenous pri-miRNA transcripts were analyzed, depletion of either Ars2 or CBP80 led to a decrease in pri-miR-21 RNA (Figure 6D). In contrast, as previously reported (Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004), depletion of DGCR8 led to increased levels of pri-miR-21 compared to control treated cells as detected by RT-PCR (Figure 6D).

The above data indicate that the Ars2/CBC complex may be involved in the stability of pri-miRNAs and/or their processing. Recently, the plant Ars2 homolog SERRATE has been implicated in regulating the fidelity of pri-miRNA processing by the DCL1 complex (Dong et al., 2008). To assess whether Ars2 plays a similar role in mammalian cells, primary miRNA processing assays were performed. As shown in Figure 6E, when pri-miR-155 (pri) was incubated with a 293T extract under processing conditions, a cleavage product of approximately 65 nucleotides (pre) was obtained that was indistinguishable from that generated by incubation with immunopurified Drosha complexes, whereas control immunoprecipitations contained no activity. Extract prepared from MEF cells gave rise to an identical cleavage product as that generated by immunopurified Drosha complexes (Figure 6E).

To determine if Ars2 contributed to the primary miRNA processing activity in MEFs, extracts were generated from $Ars2^{flox/flox}$ cells infected with Cre recombinase (KO). These KO extracts contained diminished processing activity compared to control extracts prepared from wt cells infected with Cre recombinase which generated the correct 65 nucleotide cleavage product (Figure 6F). KO extracts generated a substantial fraction of cleavage products of smaller size than that generated by wt extracts. No processing was observed in the absence of extract. Quantitation of the indicated pri and pre bands revealed that KO extracts contained approximately 3 fold less correct processing activity compared to wt extracts (Figure 6G). Comparable results were obtained with pri-miR-21.

Ars2 and CBC are differentially regulated during cell quiescence and proliferation

To examine whether Ars2 is a constitutive component of the nuclear CBC or a more specialized component, the expression of Ars2 and other components of the CBC were examined as cells underwent transitions between quiescence and proliferation. MEFs undergoing exponential proliferation in 10% serum contain readily detectable protein levels of CBP80 and CBP20, as well as Ars2. However, upon serum starvation in 0.1% serum for 48 hours, a treatment that causes exit from the cell cycle, Ars2 protein levels decreased substantially (Figure 7A). Under the same conditions, levels of nuclear CBC were only modestly affected. As a control, the levels of actin were slightly increased by serum starvation whereas the levels of the RNA binding protein hnRNP A1 were unchanged (Figure 7A).

Regulation of Ars2 protein levels were also examined in an independent cell system. Hematopoietic cells derived from $bax^{-/-}bak^{-/-}$ mice have been shown to reliably exit the cell cycle upon IL-3 withdrawal and then re-enter cell proliferation in a synchronized fashion following restimulation with IL-3 (Lum et al., 2005). Cells maintained in the presence of IL-3 (day 0) had a mean resting cell size of approximately 700 femtoliters (fL) that rapidly declined upon IL-3 withdrawal as the cells exited the cell cycle. In these cultures proliferation

reproducibly ceased 3 days later (Figure 7B). Cell size then slowly declined as the cells engaged in macroautophagy to survive over the next 11 days in culture. At day 14 IL-3 was added back to the medium and the cells began to reaccumulate cell mass without proliferating. By day 18 the cells had achieved their original cell size and proliferation resumed one day later (Figure 7B). Ars2 was highly expressed at day 0 in the presence of IL-3 and was depleted by day 3 when proliferation ceased (Figure 7C). Furthermore, Ars2 protein levels were not initially induced during cell growth (days 15–18), but became highly expressed upon resumption of proliferation at day 19 (Figure 7C). In contrast, the levels of CBP20 did not correlate with cell proliferation, cell growth or quiescence and were maintained invariantly throughout the course of the experiment. CBP80 levels were maintained for a longer period following growth factor withdrawal than Ars2 levels, but quickly recovered upon IL-3 readdition during the cell growth phase (days 15–18).

Whether the downregulation of Ars2 protein levels associated with withdrawal from the cell cycle affected the ability to process proliferation-associated miRNAs was investigated using primary miRNA processing assays. Extracts prepared from MEFs undergoing proliferation in 10% serum efficiently processed a miR-155 pri-miRNA substrate to the correct pre-miRNA product (Figure 7D). In contrast, extracts from cells cultured in 0.1% serum for 48 hours gave rise to a decreased proportion of correctly processed pre-miRNA products and increased heterogeneity of the products of primary miRNA processing (Figure 7D). To determine whether the alteration in primary miRNA processing observed during cell cycle arrest could be rescued by addition of excess Ars2 protein, extracts prepared from MEFs cultured in 0.1% serum was mixed with extracts prepared from wt or Ars2-depleted (KO) MEFs grown in 10% serum and primary miRNA processing assays were performed. Mixture of extract from wt cells grown in 0.1% serum with extract from wt cells grown in 10% serum gave rise to correctly processed pre-miRNAs when incubated with pri-miR-155 substrate (Supplemental Figure 4). In contrast, mixture of extract from wt cells grown in 0.1% serum with extract from KO cells grown in 10% serum failed to generate properly processed pre-miRNAs.

DISCUSSION

The above studies demonstrate that the Ars2 protein plays a critical role in the ability of mammalian cells to proliferate. In the absence of Ars2, mammalian cells are incapable of maintaining proliferative expansion in vitro. Consistent with a critical role in proliferation, Ars2 is selectively expressed in proliferating cells. In the absence of Ars2 cells undergo cell cycle slowing at all stages of the cell cycle as evidenced by impairment of proliferation without discernable changes in the cell cycle profile (Figure 2 and data not shown). A biochemical screen for Ars2-interacting proteins yielded CBP80, a protein previously shown to assemble on 7mG-capped transcripts. Further immunoprecipitation experiments revealed that Ars2 efficiently immunoprecipitates both core components of the CBC, CBP80 and CBP20.

An independent line of evidence supporting Ars2-CBC interactions comes from studies of Arabidopsis. Plants deficient in SERRATE, an Ars2 homolog, partially phenocopy plants with CBP80 (ABH1; ABA HYPERSENSITIVE 1) mutations. Both plants have pleiotropic developmental defects including increased cauline leaf number and serrated leaf morphology (Bezerra et al., 2004). SERRATE has been genetically implicated in a nuclear step of miRNA biogenesis in plants and SERRATE-deficient plants have reduced mature miRNA levels (Grigg et al., 2005; Lobbes et al., 2006; Yang et al., 2006). To examine whether Ars2, as a constituent of the nuclear CBC, might have a similar effect on miRNA-mediated gene silencing in proliferating cells we examined the ability of Ars2 to affect let-7-mediated repression in proliferating cells. Suppression of either CBP80 or Ars2 was sufficient to disrupt let-7-mediated repression of a reporter transcript (Figure 5). These effects are likely due to a defect in miRNA biogenesis as Ars2-depleted cells had decreased levels of a subset of miRNAs

including let-7 and miR-21. In contrast, miR-30a, which is implicated in the regulation of hepatobiliary development (Hand et al., 2008) and miR-16, which has been implicated in the regulation of apoptosis and proliferation (Cimmino et al., 2005; Linsley et al., 2007) were not affected by depletion of Ars2 (Supplemental Figure 3).

Several lines of evidence indicate that Ars2 functions at the level of the primary miRNA transcript to promote miRNA maturation. The decreased levels of primary miRNA transcripts in the absence of Ars2 is different from the effect of Drosha or DGCR8 depletion, which causes stabilization of primary transcripts (Figure 6D; (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004; Lee et al., 2003)). This suggests that Ars2 and CBC contribute to the stability of pri-miRNAs in proliferating cells. As the CBC binds and protects the 5′ cap structure of PolII transcripts, depletion of Ars2 or CBC may lead to increased decapping and transcript decay.

As demonstrated in the accompanying manuscript (Sabin et al.), Ars2 and the CBC are also required for miRNA function in *Drosophila* cells and interact with *Drosophila* homologs of the evolutionarily conserved Microprocessor complex. In addition, Sabin et al. demonstrated in *Drosophila* that Ars2 and CBP20 are required for miRNA processing at the level of the pri-miRNA and that pri-miRNA levels decline in the absence of Ars2. Because the nuclear CBC is co-transcriptionally recruited to nascent transcripts (Visa et al., 1996; Wong et al., 2007) it is ideally positioned to promote recruitment of the Microprocessor complex to pri-miRNA transcripts. However, the observed alteration in pri-miRNA processing in Ars2-depleted extracts argues for a more direct role for Ars2 in modulating the activity of the Microprocessor complex. Consistent with our findings, recent work in plants has shown that the Ars2 homolog SERRATE increases the processing efficiency and accuracy of DCL1 *in vitro* (Dong et al., 2008). Sabin et al. also report decreased processing activity of Dcr-2 in extracts depleted of *Drosophila* Ars2. Taken together, these observations suggest that Ars2 homologs may contribute to the regulation of RNase III enzyme complexes in several species.

The data from Sabin et al. in the accompanying paper suggest that Ars2 also is a required component of small RNA-mediated viral suppression. While depletion of Ars2 from adult flies had no effect on organismal viability under baseline conditions, a requirement for Ars2 emerged during viral infection. Adult flies do not depend on cell proliferation for organismal viability or viral immunity. Thus, Ars2 may play an inducible role in small RNA regulation in non-proliferating cells when infected. Based on the data, Ars2 would not appear to be essential for miRNA processing during cell quiescence, but is required to maintain the efficiency of miRNA-mediated silencing as cells commit to engage in cell division or are subjected to viral infection. Recent studies have shown that translational repression of target mRNAs by miRNAs is relieved upon exit from the cell cycle (Vasudevan et al., 2007). Induction of Ars2 expression upon entry to cell proliferation may regulate the repression of genes specific to states of cellular quiescence. Alternatively, as cells engage in increased and more complex gene expression associated with cell division, compensatory upregulation of the efficiency or specificity of miRNA production may be required to meet the complex demands of exponential growth.

EXPERIMENTAL PROCEDURES

Cell lines, transfection and retrovirus

HeLa, immortalized MEFs and HEK-293T were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 10 U/ml penicillin/streptomycin and 10 mM HEPES. IL-3-dependent cell lines derived from $Bax^{-/-}Bak^{-/-}$ mice were maintained as previously described (Lum et al., 2005). Plasmid transfections were performed with Lipofectamine 2000 and reverse transfection of siRNA were performed with Lipofectamine RNAiMAX. Retrovirus for mammalian infection was generated

by co-transfection of HEK-293T cells with pCL-Eco helper virus and retroviral constructs and infections were performed as described (Zong et al., 2003). Cell proliferation, cell size, and viability were determined as previously described (Lum et al., 2005).

Colony formation assay and cell cycle analysis

Cells were plated at a density of 100 cells per well of a 12-well plate and maintained for 10 days without changing the media. Cells were fixed with ice-cold methanol and stained with 0.5% crystal violet. Cell cycle analysis was performed as previously described (Fox et al., 2003).

Immunoprecipitation, Western blotting, sucrose gradients, and gel shifts

Cells were lysed in RIPA and Western blots were prepared as previously described (Fox et al., 2003). Antibodies used were anti-Ars2 (custom made by Quality Controlled Biochemicals using a peptide corresponding to amino acids 76–93 of mouse Ars2), actin (Santa Cruz), CBP80 and CBP20 (generously provided by Iain Mattaj), importin-α (5C9), anti-importin-β (BD Biosciences), hnRNP A1 (4B10). For immunoprecipitation cells were lysed in RSB-100 buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$) containing 0.02% Triton X-100). Immunoprecipitation was carried out with FLAG M2 agarose or anti-V5 antibody. For gradients, extracts were prepared in BC100 buffer (100 mM KCl, 0.2% NP-40, 10% glycerol, 20 mM Tris, pH 7.5, 0.5 mM DTT) and centrifuged at 33,000 rpm for 15 hours and 20 minutes at 4°C using a SW41 Ti rotor. Equal volumes of each fraction were analyzed. For gel shifts, in vitro transcription of U1 Sm DNA template was performed in the presence of 7mG or TMG cap analog and ^{32}P-UTP. Conditions for CBC gel shift reactions were based on previously published works (Izaurralde et al., 1992; Ohno et al., 1990).

Northern blotting and RT-PCR

Total RNA was fractionated on 15% Urea-PAGE gels and transferred to Hybond+ membrane and crosslinked by UV irradiation. Hybridization of ^{32}P-end labeled DNA oligonucleotide probes antisense to let-7 or miR-21 was performed using Ultrahyb-Oligo buffer (Ambion) at 37°C overnight. For RT-PCR, total RNA was subject to reverse transcription using Superscript II Reverse Transcriptase. A range of PCR cycle numbers were examined to determine the range of linear amplification for each primer pair. For specific primer sequences see Supplemental Experimental Procedures.

Heterokaryon Assay

3T3 cells expressing Ars2-V5 were seeded together with HeLa cells on coverslips. Cycloheximide (50 μg/mL) was applied 30 minutes prior to fusion and throughout the experiment. Cells were fused with 50% polyethylene glycol 8000 in DMEM for 2 minutes, then washed extensively and incubated for a further 2 hours at 37°C in complete media. Immunofluorescence was performed using anti-V5 antibody as previously described (Zong et al., 2003).

Generation of Ars2^{flox} mice

The locus was targeted for Cre-recombinase-mediated deletion by flanking the region between Exons 2 through 19 with LoxP sites (Supplementary Figure 1). Two correctly targeted and karyotypically normal clones were injected into C57Bl/6 blastocysts to obtain chimeric mice, which were bred to C57Bl/6 females to obtain germline transmission. The resulting heterozygous offspring harboring the targeted allele were interbred to mice heterozygous for the Ars2 constitutive null allele and subsequently to mice carrying the MxCre transgene. Ars2$^{flox/flox}$, Ars2$^{flox/-}$ and Ars2$^{flox/wt}$ mice developed normally and appeared healthy. In vivo deletion was induced by intraperitoneal administration of 100 mg pIpC every other day for a

total of 3 times per week for two consecutive weeks to 6–8 week-old mice. Animals were sacrificed 9 days following the last injection.

Luciferase assays

Twenty-four hours after siRNA transfection HeLa cells were split and 24 hours later transfected with luciferase reporters with or without 20 pmol of let-7/let-7* duplex or 10 pmol pre-let-7. Let-7 reporters were previously described as lin-28-M1 (“mutant”), lin-28-wt (“lin28 site”) and let7-TA (“perfect match”) (Kiriakidou et al., 2005). pGL3 was co-transfected to normalize for transfection efficiency and cell number. Let-7/let-7* duplex was prepared by annealing let-7a and let-7a* synthetic RNAs (Dharmacon); these RNAs, and pre-let-7a were described previously (Maniataki and Mourelatos, 2005). Cells were harvested 16 hours after transfection and processed with Promega Dual Luciferase Assay reagents.

Primary miRNA processing assay

To generate pri-miR-155 substrate PCR was performed using primers flanking the pre-miR-155 hairpin of the human locus. The T7 promoter was incorporated into the forward primer. PCR was performed using a plasmid containing the full-length human pri-miR-155 sequence as a template and the product was used for *in vitro* transcription in the presence of ^{32}P-UTP. Cell extracts were prepared by lysing cells in Microprocessor buffer (100 mM KCl, 20 mM Tris pH 7.5, 0.2 mM EDTA, 0.01% Triton X-100) followed by sonication and centrifugation. A 30 μL processing reaction containing 15 μL of cell extract or IP material, 10.25 μL of water, 6.4 mM $MgCl_2$, and 10,000 C.P.M. of radiolabeled substrate was incubated at 37°C for 90 minutes. RNA was extracted with Trizol and resolved by 12% urea-PAGE.

Statistical methods

Error bars of experiments performed in triplicate or duplicate indicate standard deviation. P values were calculated using paired, two-tailed Student’s t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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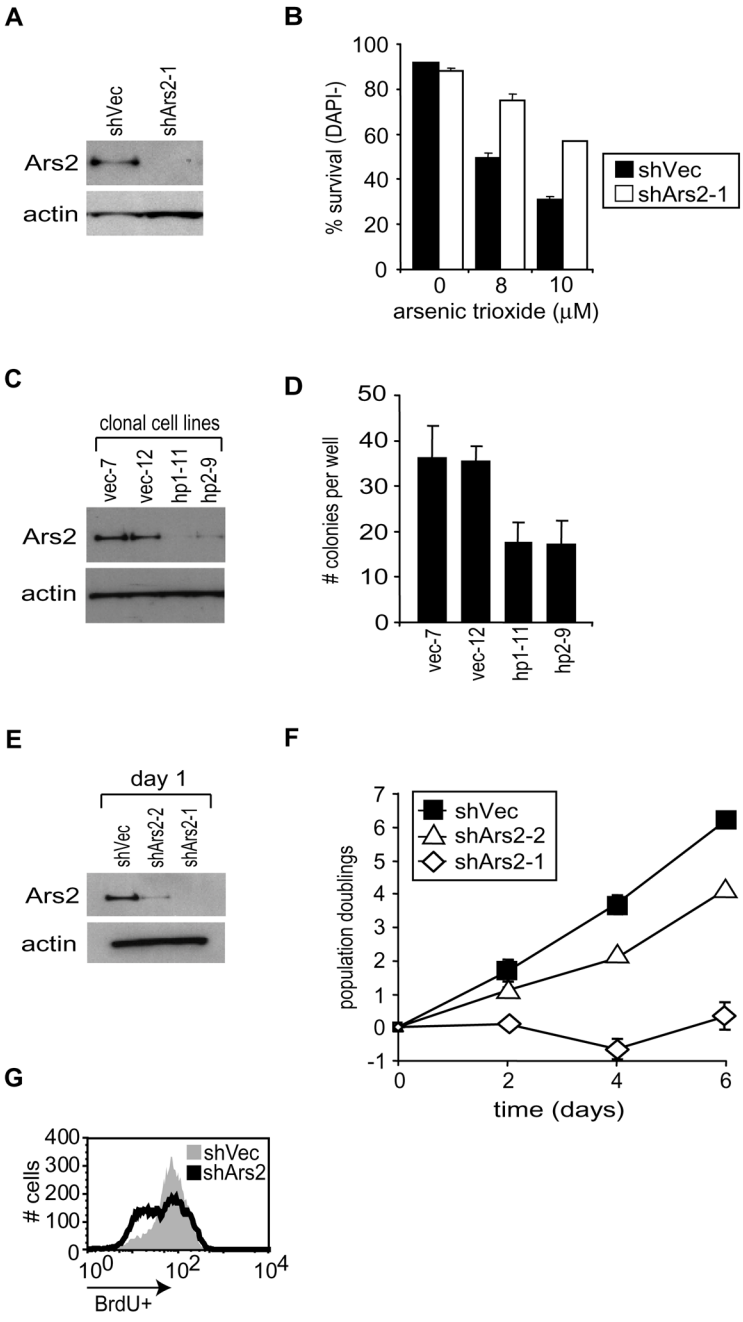

Figure 1. Ars2 is required for cell proliferation

A) 3T3 MEFs were infected with retroviral shRNA targeted to Ars2 (shArs2-1) or control empty vector (shVec) and expanded for four days. Western blot analysis was performed to determine Ars2 protein levels and actin as a loading control.

B) Cells were infected with retroviral shRNAs (shVec and shArs2-1) and treated with arsenic trioxide at the doses indicated for 48 hours and viability was determined by DAPI exclusion and FACS analysis (mean ± SD).

C) MEF stable clones were generated expressing vector control shRNA (two representative clones shown; vec-7, vec-12) or shRNAs targeting two independent sequences of Ars2 (hp1–11, hp2–9). Ars2 protein levels were determined.

D) The proliferative potential of the MEF stable shRNA clones (vec-7, vec-12, hp1–11, hp2–9) was measured by colony forming assay in 12-well plates and the number of colonies per well is plotted. The experiment was performed in triplicate and the data is expressed as mean ± SD.

E) 3T3 MEFs were infected with retroviral shRNAs to Ars2 (shArs2-1, shArs2-2) or vector control (shVec) and expanded for 4 days to allow depletion of Ars2 protein to occur and then plated for the proliferation assay. Western blotting was performed from cell extracts collected on day 1.

F) 3T3 cells were infected with retroviral shRNAs as in (E) and plated for proliferation assay. Population doublings were determined as described in experimental procedures. The data represent mean ± SD of triplicate samples.

G) Cells were infected with shVec or shArs2-1 as in (E), then subjected to serum starvation overnight in 0.1% serum followed by serum stimulation (10% FBS) in the presence of BrdU for 12 hours. BrdU incorporation was determined by FACS.

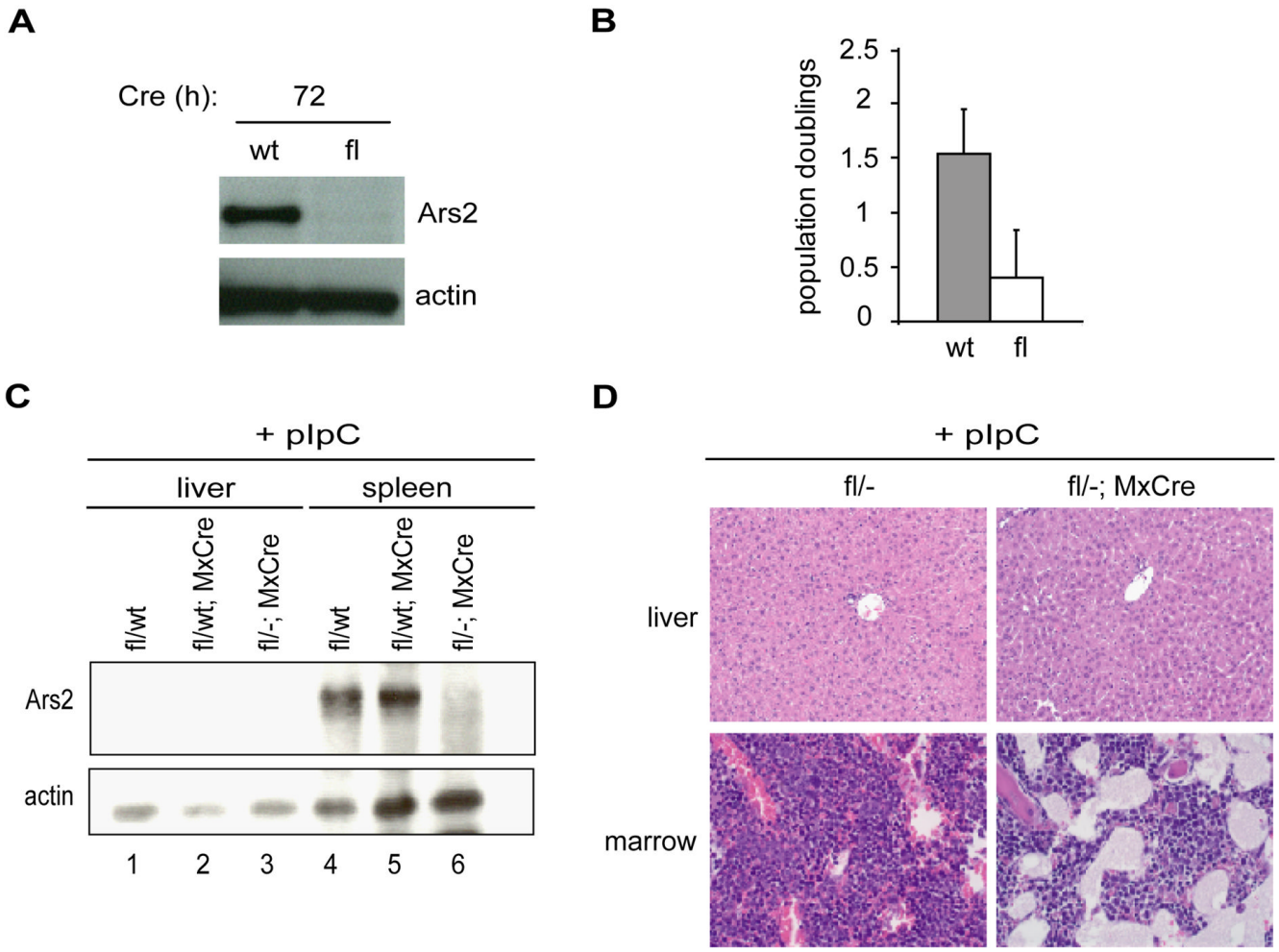

Figure 2. Ars2 genetic deletion causes proliferative arrest and bone marrow hypoplasia

A) Ars2$^{flox/flox}$ (fl) and wild-type (wt) control MEFs were infected with retrovirus expressing Cre recombinase and expanded for 72 hours and then protein extracts were analyzed by Western blot.

B) Wt and fl MEFs were infected with retroviral Cre as in (A). Between 72 and 120 hours after infection population doublings were measured by modified 3T3 assay. The data presented are the mean ± SD of triplicate samples.

C) In vivo deletion of Ars2 was achieved by injection of 6–8 week-old mice with polyinosinic-polycytidylic acid (pIpC) every other day over a two week period. Mice bearing one floxed allele of Ars2 (fl) and one null allele (−) (fl/−) were crossed to mice harboring an MxCre transgene to create mice with the compound genotype fl/−; MxCre. Controls included mice of the genotypes fl/wt and fl/wt; MxCre. The mice were sacrificed 9 days after the last injection and tissues (spleen, liver) were harvested for protein extraction. Ars2 levels were determined by Western blot.

D) pIpC injection was performed as in (C) and mice were sacrificed 9 days following the last pIpC injection. Liver and bone marrow were harvested and histological staining was performed with hematoxylin and eosin.

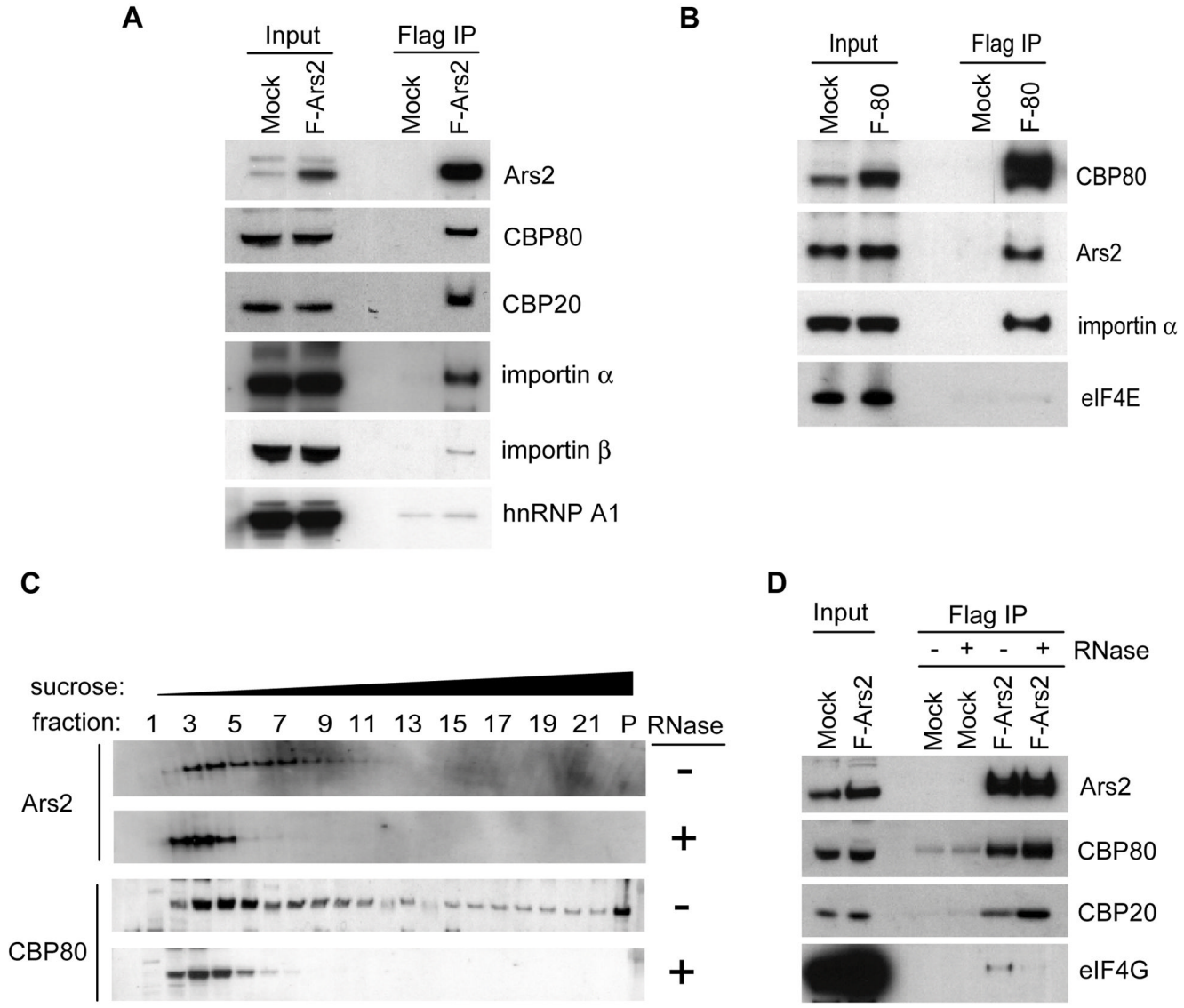

Figure 3. Ars2 interacts with the nuclear cap binding complex

A) 293T cells were transfected with Flag-Ars2 (F-Ars2) and immunoprecipitations were performed with Anti-Flag beads. Following elution with Flag peptide proteins were fractionated by SDS-PAGE and detected by Western blot using the antibodies indicated. Untransfected cells (Mock) were used as a control. Approximately 3% of the protein loaded into the IP was run as input.

B) Anti-Flag immunoprecipitation from 293T cells transiently transfected with Flag-CBP80 (F-80) followed by Flag peptide elution, SDS-PAGE and Western blotting using the antibodies indicated. One-half of the input material was loaded for Ars2 Western blot compared to the other Western blots.

C) Sucrose gradient (5–20%) analysis of 3T3 MEF whole cell extracts were carried out in the presence or absence of RNase A. Each fraction was separated by SDS-PAGE followed by Western blotting for Ars2 and CBP80. The percent of sucrose in the fractions increases from left to right as indicated. P indicates pellet.

D) Anti-Flag immunoprecipitations were carried out in the presence or absence of RNase A and analyzed by SDS-PAGE and Western blotting. The data are representative of three independent experiments.

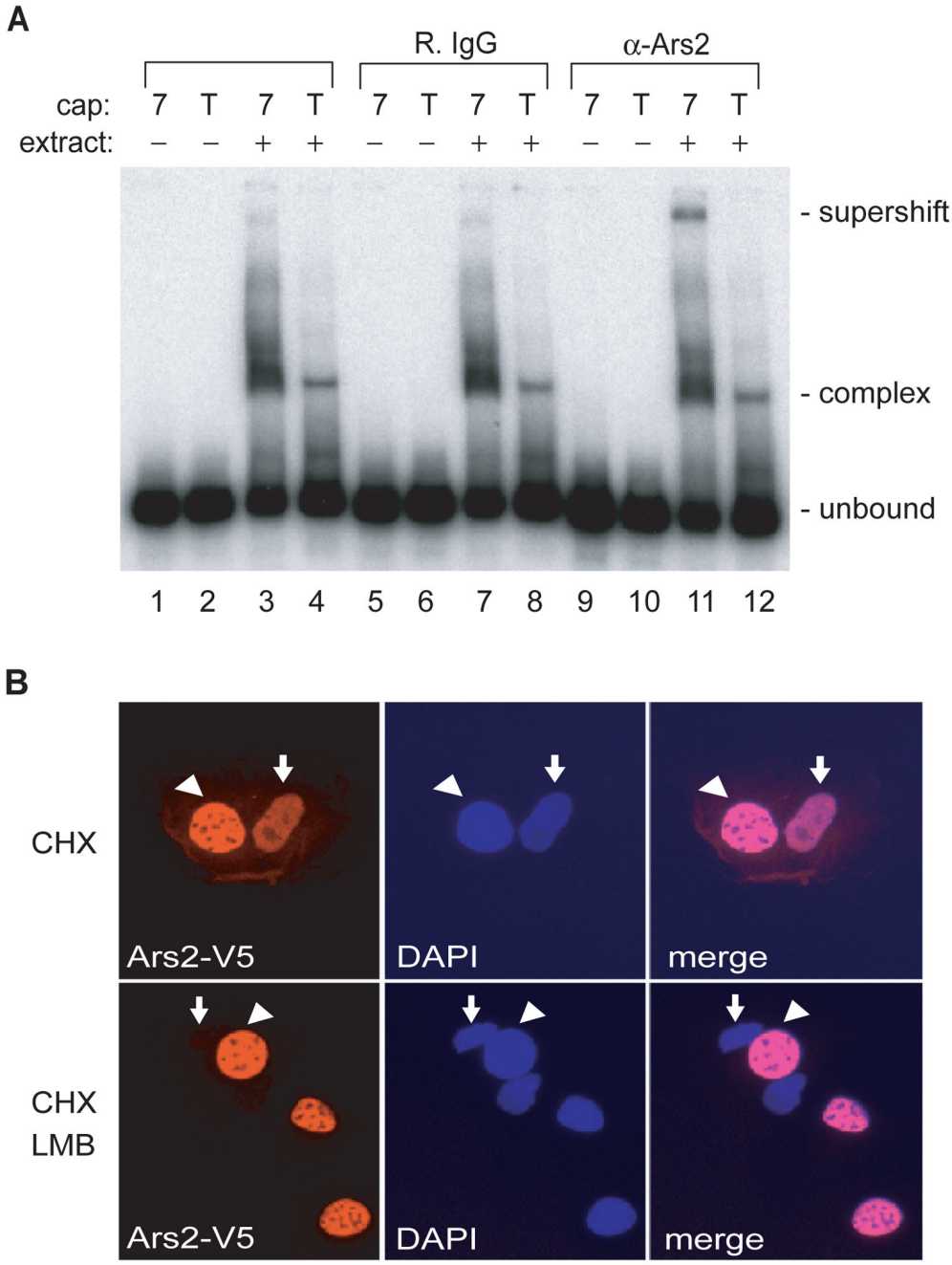

Figure 4. Like other CBC components, Ars2 interacts with 7mG-capped RNAs and shuttles in a Crm1-dependent fashion

A) Ars2 assembles on RNAs in a cap-dependent manner. Gel shift assay was performed with in vitro transcribed, radiolabeled probe RNA containing either 7-methylguanosine (7) or 2,2,7-trimethylguanosine (T) caps incubated with HeLa nuclear extract as indicated. RNA bound complexes were resolved by native PAGE. Rabbit IgG or Ars2 antibodies were added to the assembly reactions as indicated. Unbound probe (unbound), the cap binding complex shift (complex) and the supershift detected with Ars2 antibody (supershift) are indicated. The data are representative of four independent experiments.

B) Ars2 shuttling between the nucleus and cytoplasm is leptomycin B sensitive. 3T3 MEFs with stable expression of Ars2-V5 were fused to HeLa cells in the presence of cycloheximide (CHX), or cycloheximide plus leptomycin B (LMB) as indicated. DAPI counterstaining allowed HeLa and 3T3 nuclei to be distinguished. Arrowheads indicate 3T3 nuclei and arrows indicate HeLa nuclei.

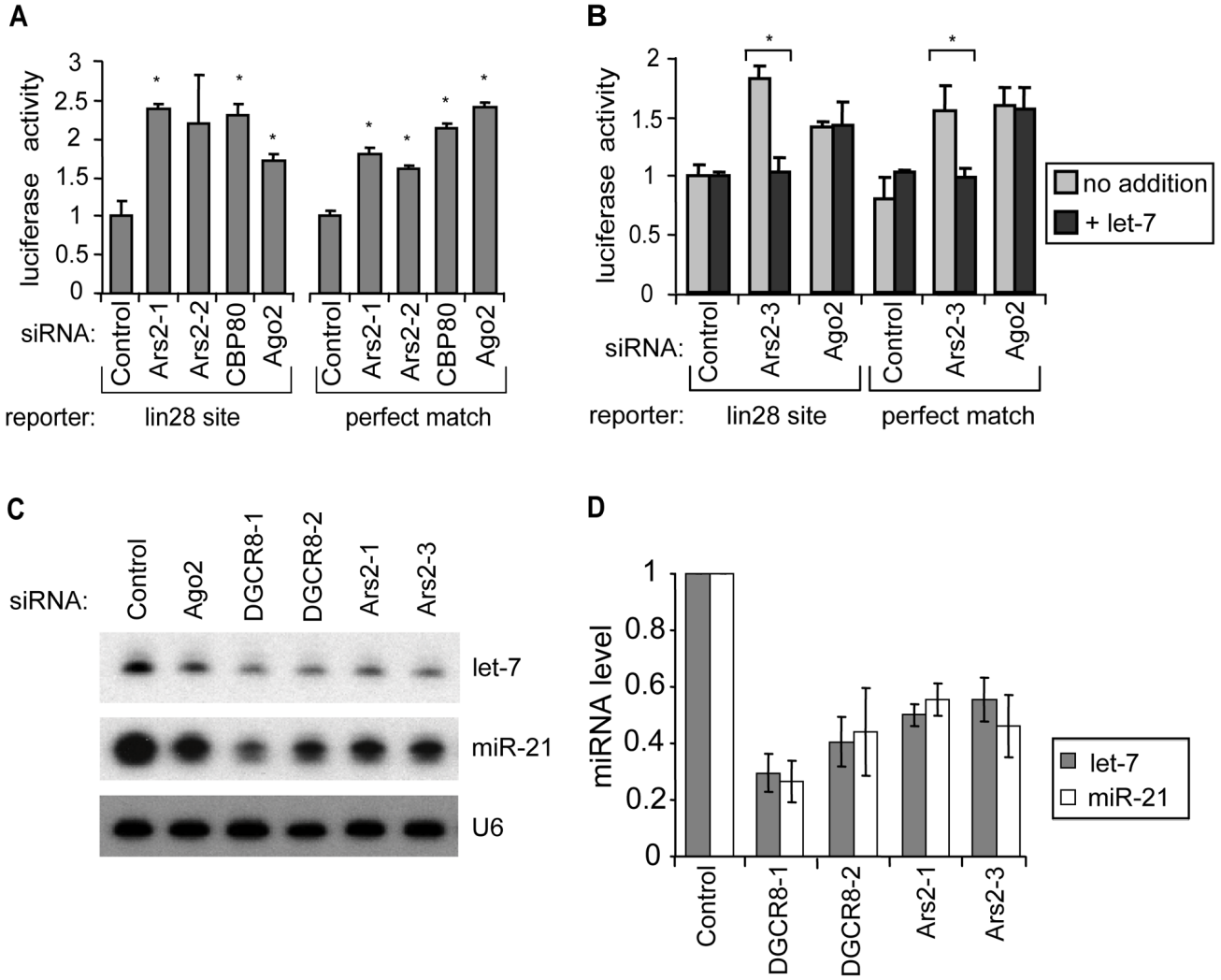

Figure 5. Ars2 and CBC contribute to miRNA-mediated gene silencing

A) HeLa cells were transfected with the siRNAs indicated and 48 hours later transfected with lin28 site or perfect match renilla luciferase reporters along with firefly luciferase as a normalization control. Luciferase activity was analyzed 16 hours after transfection and is presented as a ratio of renilla/firefly. The experiment was performed in triplicate and error bars represent standard deviation. * indicates $p < 0.03$ in comparison to control.

B) HeLa cells were transfected with siRNAs and 48 hours later luciferase reporters were transfected as in A (no addition) or with excess let-7/let-7* duplex included in the transfection mix (+ let-7). * indicates $p < 0.01$.

C) HeLa cells were transfected with siRNAs and harvested after 72 hours. Total RNA was fractionated by 15% urea-PAGE and blotted using specific probes to the RNAs indicated.

D) Quantitation of (C) was performed by phosphorimager or densitometry with equivalent results. Data represent mean ± SD of 2 independent experiments.

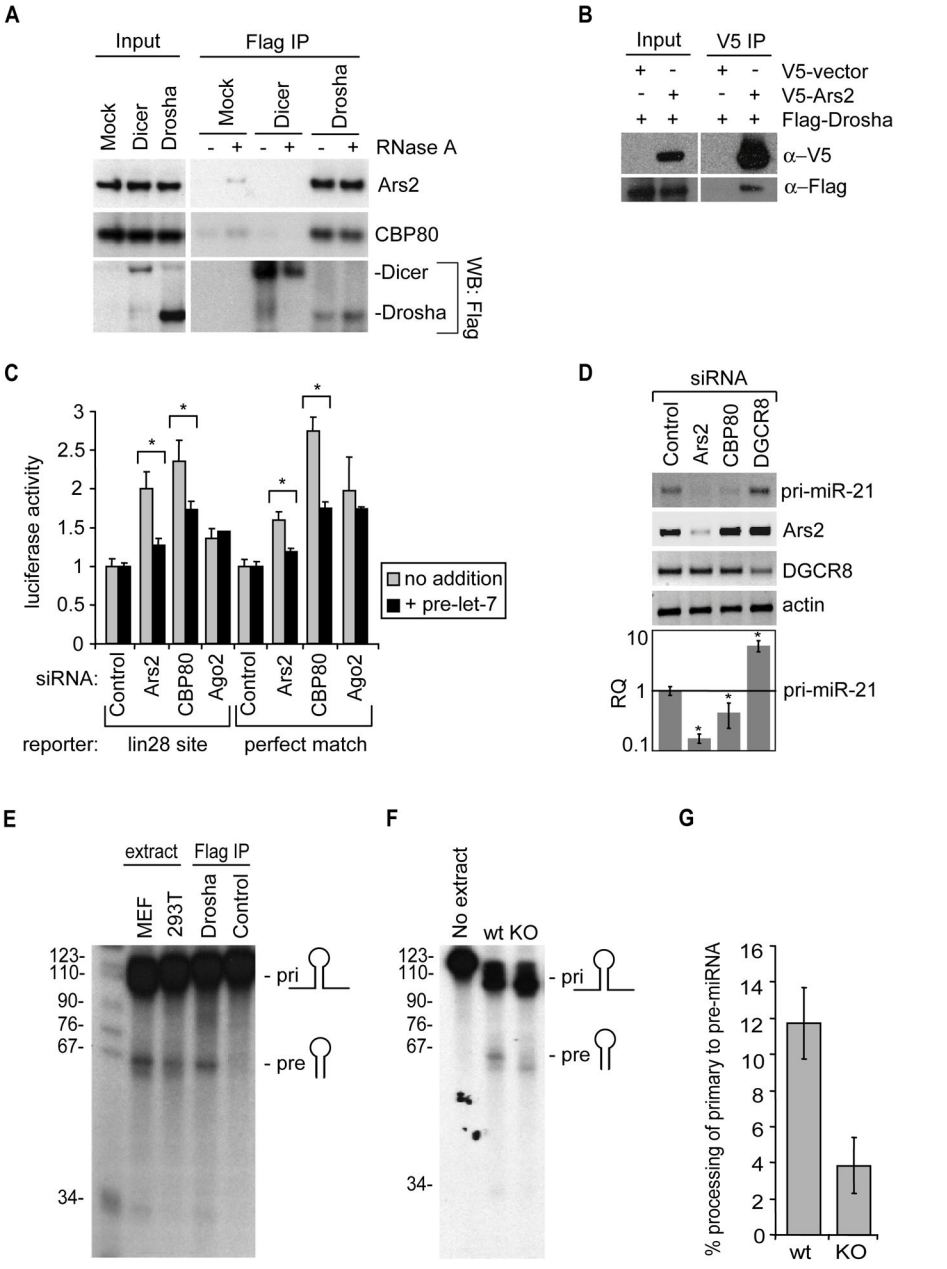

Figure 6. Ars2 functions at the level of the primary miRNA transcript

A) Anti-Flag immunoprecipitations were performed from 293T cells transfected with plasmids encoding Flag-Dicer or Flag-Drosha or non-transfected cells (Mock). Bound protein complexes were eluted with Flag peptide, fractionated by SDS-PAGE and Western blotting was performed with the indicated antibodies. RNase A was added as indicated. Input represents 1% of the material added to the IP. Data is representative of 3 independent experiments.

B) Anti-V5 immunoprecipitations were performed from 293T cells transfected with the indicated plasmids. Proteins were eluted in loading buffer, fractionated by SDS-PAGE and probed with the antibodies indicated.

C) Luciferase assays were performed as in (Figure 5B) except excess pre-let-7 was included in the transfection mix (+ pre-let-7). The experiment was performed in triplicate and error bars represent standard deviation. * indicates $p < 0.01$.

D) HeLa cells were transfected with siRNAs and harvested after 72 hours. RT-PCR and qRT-PCR was performed for the transcripts indicated. Data is representative of 3 independent experiments. RQ indicates relative quantitation in comparison to actin. Error bars represent SD of triplicate samples. * indicates $p < 0.01$ in comparison to control.

E) Primary miRNA processing assay. Radiolabeled pri-miR-155 was incubated with the cell extracts or immunoprecipitates indicated and RNAs were resolved by 12% urea-PAGE followed by autoradiography. Primary miRNA substrate (pri) and product (pre) are indicated. Flag Drosha was immunoprecipitated from transfected 293T cells. Flag Control indicates IP performed on mock transfected 293T cells using anti-Flag antibody.

F) miRNA processing assay was performed on extracts prepared from wild-type (wt) or $Ars2^{flox/flox}$ MEFs (KO) MEFs infected with Cre retrovirus for 72 hours and results determined by autoradiography as above. (A lighter exposure to visualize the pri-miRNAs is included in Supplemental Figure 5).

G) Quantitation of (F). Error bars indicate the average deviation of 3 independent experiments. Pri and pre bands were quantitated by exposure of dried gels to phosphorimager in two cases and exposure to film and densitometry in one case. The ratio pre/(pri+pre) is plotted.

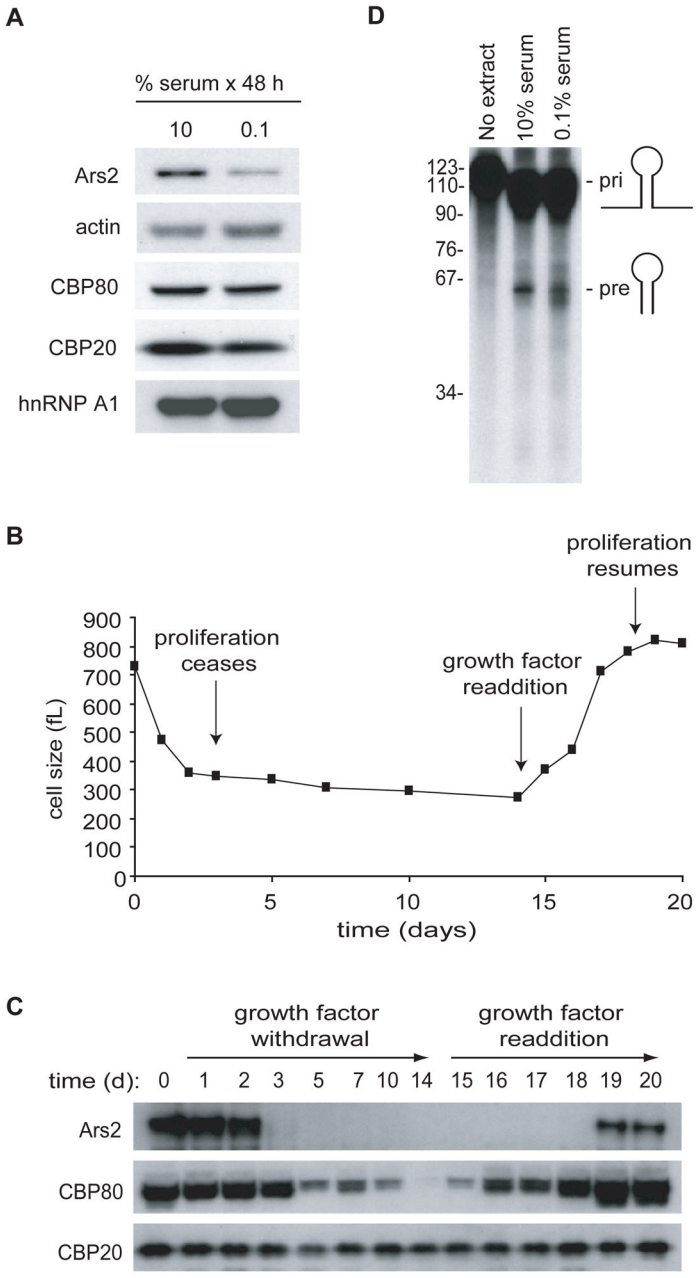

Figure 7. Non-proliferating cells down-regulate Ars2 and display altered miRNA processing

A) Exponentially growing MEFs were switched to 0.1% serum for 48 hours or maintained in 10% serum and protein levels of Ars2, CBP80 and CBP20 were analyzed by SDS-PAGE and Western blot. Actin and hnRNP A1 were probed as controls.

B) IL-3-dependent bax$^{-/-}$bak$^{-/-}$ hematopoietic cells were grown in IL-3 at day 0, then subjected to IL-3 withdrawal for a period of two weeks followed by restimulation with IL-3 at day 14.5. At the timepoints indicated cell size and cell number was determined. Points at which cell proliferation ceased and resumed are noted above the graph, as is the point of growth factor readdition. fL = femtoliters.

C) At the times indicated, aliquots from (B) were removed from the culture for protein extraction and Western analysis using antibodies to Ars2, CBP80 and CBP20. The periods of

growth factor (IL-3) withdrawal and readdition are noted. The data is representative of four independent experiments.

D) Primary miRNA processing assay. Pri-miR-155 was incubated with or without extracts of MEFs cultured in 10% or 0.1% serum for 48 hours. RNA was resolved by 12% urea-PAGE followed by autoradiography. Primary miRNA substrate (pri) and product (pre) are indicated.