An Exportin-1–dependent microRNA biogenesis pathway during human cell quiescence

Ivan Martinez[a,1,2], Karen E. Hayes[a,1], Jamie A. Barr[a], Abby D. Harold[a], Mingyi Xie[b], Syed I. A. Bukhari[c], Shobha Vasudevan[c], Joan A. Steitz[d,e,f,2], and Daniel DiMaio[d,f,g,h]

[a]Department of Microbiology, West Virginia University Cancer Institute, West Virginia University, Morgantown, WV 26506; [b]Department of Biochemistry and Molecular Biology, University of Florida Health Cancer Center, University of Florida, Gainesville, FL 32610; [c]Cancer Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114; [d]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06536; [e]Howard Hughes Medical Institute, Yale University, New Haven, CT 06536; [f]Yale Cancer Center, New Haven, CT 06520; [g]Department of Genetics, Yale School of Medicine, New Haven, CT 06510; and [h]Department of Therapeutic Radiology, Yale School of Medicine, New Haven, CT 06510

Contributed by Joan A. Steitz, May 11, 2017 (sent for review November 14, 2016; reviewed by Judith Campisi, Richard I. Gregory, and Narry Kim)

The reversible state of proliferative arrest known as "cellular quiescence" plays an important role in tissue homeostasis and stem cell biology. By analyzing the expression of miRNAs and miRNA-processing factors during quiescence in primary human fibroblasts, we identified a group of miRNAs that are induced during quiescence despite markedly reduced expression of Exportin-5, a protein required for canonical miRNA biogenesis. The biogenesis of these quiescence-induced miRNAs is independent of Exportin-5 and depends instead on Exportin-1. Moreover, these quiescence-induced primary miRNAs (pri-miRNAs) are modified with a 2,2,7-trimethylguanosine (TMG)-cap, which is known to bind Exportin-1, and knockdown of Exportin-1 or trimethylguanosine synthase 1, responsible for (TMG)-capping, inhibits their biogenesis. Surprisingly, in quiescent cells Exportin-1–dependent pri-miR-34a is present in the cytoplasm together with a small isoform of Drosha, implying the existence of a different miRNA processing pathway in these cells. Our findings suggest that during quiescence the canonical miRNA biogenesis pathway is down-regulated and specific miRNAs are generated by an alternative pathway to regulate genes involved in cellular growth arrest.

XPO5 | XPO1 | (TMG)-cap | pri-miRNA | quiescence

Most metazoan cells enter a reversible cell-cycle arrest known as "cellular quiescence" when they are exposed to antimitogenic signals or an environment unfavorable for proliferation (1, 2). In mammalian cells, quiescence (also known as "G_0 arrest") is characterized by reduced DNA replication, altered metabolism, increased autophagy, and increased expression of cyclin-dependent kinase inhibitors such as $p27^{Kip1}$ (3, 4). In vitro, quiescence can be induced in primary cells by serum starvation, contact inhibition, and loss of adhesion to a substrate (5). Quiescence is involved in important cellular processes, including the balance between differentiation and self-renewal in different types of stem cells, and dysregulation of quiescence could favor carcinogenesis. However, the molecular mechanisms that regulate quiescence are poorly understood (6).

miRNAs are small, noncoding RNAs ∼22-nt long that regulate the expression of protein-coding genes by base-pairing with the 3′ UTR of mRNAs, repressing the translation and/or inducing the degradation of the target mRNA (7, 8). In canonical miRNA biogenesis in mammalian cells, miRNAs are transcribed by RNA polymerase II to produce 7-methylguanosine (m^7G)-capped primary miRNAs (pri-miRNAs) containing one or more bulged hairpin structures that are recognized by the nuclear microprocessor, which consists of the RNase III enzyme Drosha and the dsRNA-binding protein DGCR8 (DiGeorge syndrome critical region gene 8) (9–12). Specific cleavage of the pri-miRNA by the nuclear microprocessor generates an ∼70-nt stem–loop structure known as the "precursor miRNA" (pre-miRNA), which is recognized and transported to the cytoplasm by Exportin-5 (3, 10, 11, 13). Subsequently, pre-miRNA is cleaved by the cytoplasmic RNase III enzyme Dicer (14–17), generating a double-stranded mature miRNA. Finally, the guide miRNA strand is loaded into the RNA-induced silencing complex (RISC) containing one of four Argonaute proteins and GW182 protein at its core (18–20).

Recent studies have identified alternative pathways of miRNA biogenesis in different cell and animal models: pre-miRNA/intron miRNAs (miRtrons), which are Drosha and DGCR8 independent (21); Drosha- and DGCR8-independent viral miRNAs that use the Integrator complex to produce viral pre-miRNAs (22); miRNAs independent of DGCR8, Exportin-5, Dicer and Argonaute 2 (AGO2), known as "Simtrons" (23), which are exported from the nucleus by an unknown mechanism; Dicer-independent miRNAs, such as miR-451, which are generated by the catalytic activity of AGO2 (24, 25); and cytoplasmic relocalization of Drosha by RNA viruses, with no changes in endogenous miRNA expression but robust synthesis of viral miRNAs (26). Recently, we reported a novel class of (m^7G)-capped pre-miRNAs in human cells whose 5′ end corresponds to the transcription initiation site (27); these pre-miRNAs are exported from the nucleus by Exportin-1 rather than by Exportin-5.

Exportin-1 recognizes certain RNAs, including viral mRNAs, cellular mRNAs, and small nuclear RNAs (snRNAs), and exports

Significance

Quiescence is a growth-arrested cellular state; genes involved in this process are finely regulated by several factors, including miRNAs. During miRNA biogenesis, Exportin-5 transports miRNA precursors from the nucleus to the cytoplasm. In this study, we demonstrated the existence of an alternative miRNA biogenesis pathway in quiescent primary human cells. This pathway involves the repression of Exportin-5 expression by autophagy and miRNAs and the 2,2,7-trimethylguanosine-cap modification of specific primary miRNAs (pri-miRNAs), which signal their export to the cytoplasm by Exportin-1. We further showed that these pri-miRNAs are processed rapidly in the cytoplasm by a small isoform of Drosha. Collectively, these results reveal an alternative mechanism of miRNA biogenesis that will expand our understanding of miRNA regulation in normal or disease-related cells.

Author contributions: I.M., K.E.H., and J.A.B. designed research; I.M., K.E.H., J.A.B., A.D.H., M.X., S.I.A.B., and S.V. performed research; I.M., K.E.H., J.A.B., A.D.H., M.X., S.I.A.B., S.V., J.A.S., and D.D. analyzed data; and I.M., J.A.S., and D.D. wrote the paper.

Reviewers: J.C., Buck Institute for Research on Aging/Lawrence Berkeley National Laboratory; R.I.G., Boston Children's Hospital, Harvard Medical School; and N.K., Seoul National University.

The authors declare no conflict of interest.

Data deposition: Array data have been deposited into ArrayExpress (accession no. E-MTAB-2760).

[1]I.M. and K.E.H. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: ivmartinez@hsc.wvu.edu or joan.steitz@yale.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1618732114/-/DCSupplemental.

them from the nucleus to the cytoplasm (28). For example, precursors of snRNAs involved in mRNA processing such as U1, U2, U4, and U5 have a (m^7G)-cap, which is recognized by cap-binding complex and the phosphorylated adaptor for RNA export (PHAX) in the nucleus to enable their export to the cytoplasm (29) by Exportin-1. These snRNAs then are recognized by Sm proteins in the cytoplasm, and trimethylguanosine synthase 1 (TGS1) is recruited to hypermethylate the (m^7G)-cap into an [$m^{2,2,7}$G, 2,2,7-trimethylguanosine (TMG)]-cap. This modification is recognized by Snuportin-1 in association with Importin-β and other factors to import the snRNAs back into the nucleus (30, 31). Interestingly, Exportin-1 also has high affinity for the (TMG)-capped small nucleolar RNA (snoRNA) U3 in the nucleus and transports it from Cajal bodies to the nucleoli (32). Another study showed that TGS1 enhances Rev-dependent HIV-1 RNA expression by (TMG)-capping viral mRNAs in the nucleus, thereby increasing recognition by Exportin-1 for transport to the cytoplasm (33).

miRNAs play an important role in regulating cellular quiescence. For example, it has been shown that overexpression of miR-221 and miR-222, which target the 3′ UTRs of p27 and p57 mRNA, respectively, induce S-phase entry in quiescent cells (34). Another study reported up-regulation of let-7 and miR-125 and down-regulation of the miR-29 family in quiescent fibroblasts (35). Furthermore, overexpression of miR-29 increased cell-cycle reentry in quiescent cells, whereas overexpression of let-7 and miR-125 delayed cell-cycle reentry (35). Modulation of miR-29 can also influence senescence, an irreversible growth-arrested state, by affecting B-myb expression (36). We also showed that during quiescence some miRNAs up-regulate the translation of their target mRNAs (37). Finally, Exportin-5 protein expression is reduced during quiescence and is induced promptly during cell-cycle entry, causing a global increase in miRNA expression (38).

Here, we report the existence of an alternative miRNA biogenesis pathway in primary human foreskin fibroblasts (HFFs) during quiescence. We confirmed that the level of Exportin-5 is substantially reduced during quiescence and that certain miRNAs are induced during quiescence despite this paucity of Exportin-5. We also demonstrate that the biogenesis of pri-miRNAs corresponding to these miRNAs is dependent not on Exportin-5 but rather on Exportin-1. Furthermore, the Exportin-1–dependent pri-miRNAs have a trimethylguanosine ($m^{2,2,7}$G, TMG)-cap and are found in the cytoplasm during quiescence, as is a smaller isoform of Drosha. Together, our findings suggest that in quiescent cells an alternative miRNA biogenesis pathway selectively processes and transports a specific set of miRNAs, which could be essential for reversible G_0 arrest.

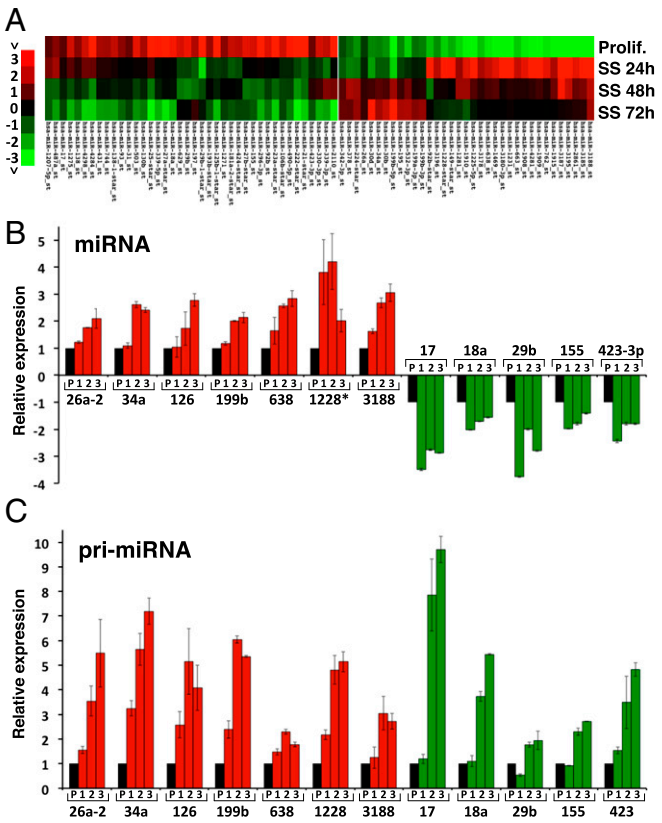

Fig. 1. Differential expression of mature miRNAs and their corresponding pri-miRNAs during quiescence. (*A*) Heat map of mature miRNA microarray data comparing proliferating HFFs to HFFs serum-starved (SS) for 24, 48, or 72 h. Only miRNAs differentially expressed during quiescence are shown. (*B*) qRT-PCR validation of representative differentially expressed mature miRNAs during quiescence. (*C*) qRT-PCR quantitation of pri-miRNAs corresponding to differentially expressed mature miRNAs (using primers that bind upstream and downstream of the miRNA stem loop). Red bars represent the miRNAs up-regulated during quiescence, and the green bars represent the miRNAs down-regulated during quiescence, as determined by miRNA microarray analysis. Numbers refer to days of serum starvation. Black bars represent miRNAs in proliferating (P) cells. RNU43 and GAPDH mRNA were used to normalize the qRT-PCR analyses, which are shown relative to expression in proliferating cells. Similar results were obtained in three independent experiments. Prolif, proliferating.

Results

Differential Expression of Specific pri-miRNAs and their Corresponding Mature miRNAs During Quiescence. To examine global miRNA expression during quiescence, miRNA microarrays that interrogated 1,105 mature human miRNAs were used to compare proliferating HFFs and quiescent HFFs after 12, 48, and 72 h of serum starvation. We identified 88 up-regulated and 94 down-regulated miRNAs that changed 1.5-fold or more for at least two time points of serum starvation (Fig. 1*A* and *SI Appendix*, Tables S1 and S2). As assessed by qRT-PCR, mature miR-26a-2, miR-34a, miR-126, miR-199b, miR-638, miR-1228*, and miR-3188 were induced two- to fourfold by serum starvation, and miR-17, miR-18a, miR-29b, miR-155, and miR-423-3p were repressed two- to threefold, confirming the microarray data (Fig. 1*B*). There was a similar pattern of miRNA expression (with the exception of miR-26a-2 and miR-126) when quiescence was induced by confluency (*SI Appendix*, Fig. S1*A*). Northern blot analysis confirmed the induction of representative miRNAs in quiescence induced by serum starvation or confluency (*SI Appendix*, Fig. S2). By using specific primers to amplify the pri-miRNAs of these differentially expressed miRNAs (*SI Appendix*, Table S3), we found that the mature miRNAs induced during quiescence are also induced at the pri-miRNA level (Fig. 1*C* and *SI Appendix*, Fig. S1*B*). In contrast, rather than being repressed, the levels of the pri-miRNAs corresponding to the repressed mature miRNAs, miR-17, miR-18a, miR-29b, miR-155, and miR-423-3p were induced during quiescence (Fig. 1*C* and *SI Appendix*, Fig. S1*B*), suggesting that repression of these miRNAs is caused by posttranscriptional regulation, presumably during their processing through the miRNA biogenesis pathway.

Exportin-5 Expression Is Down-Regulated by Autophagy and miR-34a During Quiescence. To explore the mechanism of miRNA synthesis in quiescent cells, the abundance of proteins involved in miRNA biogenesis was examined in quiescent HFFs. Confirming the previous findings of Iwasaki and collaborators (38), Exportin-5 protein levels were dramatically reduced (~30-fold) during quiescence induced by serum starvation (Fig. 2*A* and *SI Appendix*, Fig. S3*A*) or contact inhibition (*SI Appendix*, Fig. S3*B*), as assessed by immunoblotting and immunofluorescence. Exportin-5 protein also was reduced in serum-starved HeLa (human cervical carcinoma) and C127 (murine mammary tumor) cell lines (*SI Appendix*, Fig. S3*C*).

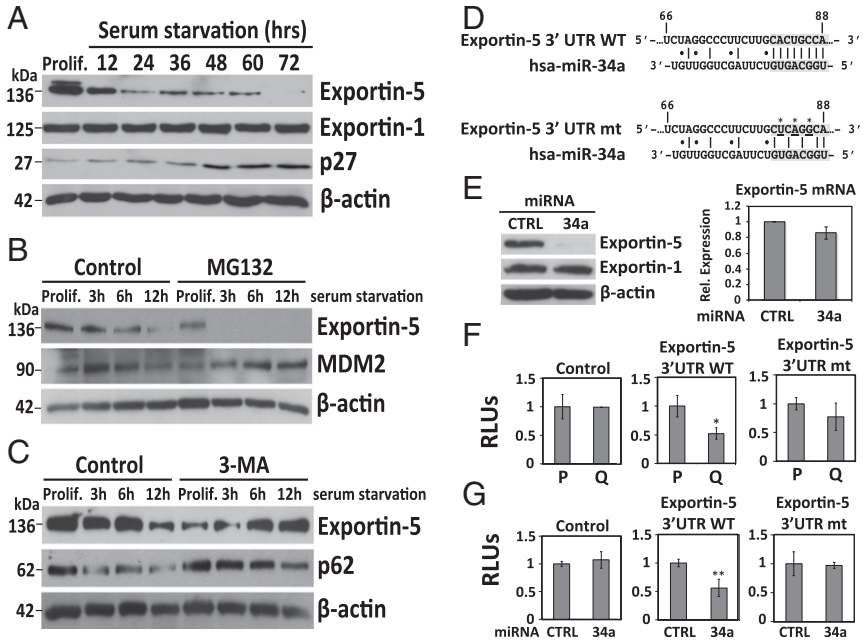

Fig. 2. Regulation of Exportin-5 during quiescence by autophagy and miR-34a. (*A*) Western blot showing the reduction of Exportin-5, but not Exportin-1, levels during the induction of quiescence by serum starvation. p27 was used as a marker of quiescence induction, and β-actin was used as a loading control. (*B*) Western blot showing Exportin-5 and MDM2 in proliferating (Prolif) and quiescent HFFs treated with 10 μM of the proteasome inhibitor MG132. Cells were serum-starved for 3, 6, or 12 h as indicated to induce quiescence. (*C*) Western blot of Exportin-5 and p62 in proliferating and quiescent HFFs treated with 10 μM of the autophagy inhibitor 3-MA. Similar results were obtained in three independent experiments. (*D*) The sequence of hsa-miR-34a and its putative wild-type (WT) or mutant (mt) binding site in the Exportin-5 3′ UTR are shown. Vertical lines indicate predicted base pairing between hsa-miR-34a and the Exportin-5 3′ UTR; dots indicate potential G-U base pairs. Point mutations in the Exportin-5 3′ UTR are indicated by asterisks. Numbers represent the nucleotide sequence of the Exportin-5 3′ UTR numbered from the beginning of the untranslated region. (*E*) miR-34a regulates the expression of Exportin-5. (*Left*) Western blot of Exportin-5 and Exportin-1 48 h after transfection of exogenous miR-34a. (*Right*) qRT-PCR analysis of Exportin-5 mRNA 48 h after transfection of miR-34a. (*F*) The activity of the Exportin-5 3′ UTR is regulated by the cell growth state. Luciferase activity from the empty reporter vector (Control), the vector containing the wild-type Exportin-5 3′ UTR (WT), or the Exportin-5 3′ UTR containing a mutant miR-34a binding site (mt) is shown. Assays were conducted in proliferating (P) or quiescent (Q) HFFs as described in *SI Appendix, Materials and Methods*. (*G*) miR-34a regulates the Exportin-5 3′ UTR. Luciferase activity from the reporter vectors described in *F* was measured in proliferating HFFs transfected with a scrambled miRNA control (miR-CTRL) or miR-34a. In *F* and *G* firefly luciferase activity was measured and normalized to Renilla luciferase activity expressed from the same plasmid (relative luciferase units, RLUs). Data were obtained in triplicate, and the graph shows the average of two individual experiments. Two-tailed *t* test results are indicated as $*P < 0.05$ and $**P < 0.01$ in *F* and *G*. CTRL, control; Prolif, proliferating.

The level of Exportin-5 mRNA did not change during quiescence (*SI Appendix*, Fig. S3 *D* and *E*), indicating that Exportin-5 is regulated at the posttranscriptional level. The level of Exportin-1, a protein involved in protein and RNA export (28), did not change during quiescence (Fig. 2*A* and *SI Appendix*, Fig. S3*B*). The quiescence marker p27 was induced, as expected (39).

To determine the basis for the reduction in Exportin-5 expression during quiescence, we blocked two major pathways of protein degradation, the proteasome and the autophagy pathways. Treatment of quiescent cells with the proteasome inhibitor MG132 did not restore Exportin-5 expression, whereas the protein MDM2, which is known to be degraded by the proteasome, is maintained upon MG132 treatment, as expected (Fig. 2*B* and *SI Appendix*, Fig. S4*A*). In fact, the level of Exportin-5 was further reduced in response to MG132 treatment, suggesting that a down-regulation pathway is induced by this drug (Fig. 2*B*). Treatment with 3-methyladenine (3-MA), an autophagy inhibitor, reduced the level of Exportin-5 in proliferating cells (Fig. 2*C*). Strikingly, treatment of quiescent HFFs with 3-MA restored the levels of Exportin-5 and of p62, a protein specifically degraded by autophagy (Fig. 2*C* and *SI Appendix*, Fig. S4*B*). These data suggest that the autophagy pathway is responsible for accelerated degradation of Exportin-5 during quiescence.

We also tested whether Exportin-5 levels are modulated by miRNAs. The 3′ UTR of the Exportin-5 mRNA contains a conserved predicted binding site for miR-34a (Fig. 2*D* and *SI Appendix*, Fig. S5), which, as shown above, is induced during quiescence (Fig. 1*B* and *SI Appendix*, Fig. S1*A*). To determine if miR-34a affects the expression of Exportin-5, we transfected synthetic miR-34a into proliferating HFFs and examined the expression of Exportin-5 after 48 h. As shown in Fig. 2*E*, transfected miR-34a reduced the amount of endogenous Exportin-5 protein but not Exportin-5 mRNA, suggesting translational repression of Exportin-5 by miR-34a. In contrast, there was no change in the expression of Exportin-1, which does not contain a miR-34a–binding site in its 3′ UTR. To determine if there is a direct functional interaction between miR-34a and Exportin-5 mRNA, we cloned the complete 3′ UTR of the Exportin-5 gene (1,537 nt) into a luciferase reporter vector and transfected quiescent HFFs with the empty reporter vector, the reporter vector containing the Exportin-5 3′ UTR, or a reporter vector containing the Exportin-5 3′ UTR with three point mutations in the miR-34a seed sequence binding site (Fig. 2*D*). As shown in Fig. 2*F*, luciferase activity from the vector containing the wild-type Exportin-5 3′ UTR was reduced about twofold during quiescence compared with the vector lacking the 3′ UTR, and there was partial recovery of luciferase activity in response to the mutant 3′ UTR. These results suggest that regulation of Exportin-5 expression in quiescent cells is mediated in part by miR-34a. We also cotransfected proliferating HFFs with the Exportin-5 3′ UTR luciferase reporter vectors and synthetic miR-34a or scrambled control miRNA. miR-34a, but not the control miRNA, caused a reduction in luciferase activity from the wild-type Exportin-5 3′ UTR reporter but not from the miR-34a-binding site mutant or empty vector (Fig. 2*G*). Together these

results demonstrate a functional interaction between the Exportin-5 3′ UTR and miR-34a. We conclude that Exportin-5 expression is regulated at two different levels during quiescence, at the protein level by autophagy and at the mRNA level by miR-34a.

Expression of Quiescence-Induced miRNAs Is Not Dependent on Exportin-5. The increase in the levels of specific mature miRNAs in quiescent cells expressing little Exportin-5 raised the possibility that these quiescence-induced miRNAs are generated by an Exportin-5–independent pathway. To investigate this possibility, we transiently transfected proliferating HFFs with a siRNA against Exportin-5 and 48 h later measured miRNA levels. Exportin-5 knockdown was confirmed by Western blot (Fig. 3*A*). As expected, Exportin-5 knockdown in proliferating cells lowered levels of mature miR-17, miR-18a, miR-29b, miR-155, and miR-423-3p, which are repressed during quiescence (Fig. 3*A*, dark gray bars). In contrast, levels of their corresponding primary miRNAs did not decline (Fig. 3*A*, light gray bars), as is consistent with Exportin-5 being required for maturation of these miRNAs, as it is for all canonically processed miRNAs. Strikingly, however, Exportin-5 knockdown did not repress the levels of several mature miRNAs and pri-miRNAs that are induced during quiescence, including miR-26a, miR-34a, miR-126, miR-199b, miR-638, and miR-3188 (Fig. 3*A*). Similar results were obtained with a second Exportin-5 siRNA in an independent experiment (*SI Appendix*, Fig. S6*A*). Although, we cannot exclude other possibilities, such as a higher affinity for Exportin-5 by some quiescence-induced miRNAs

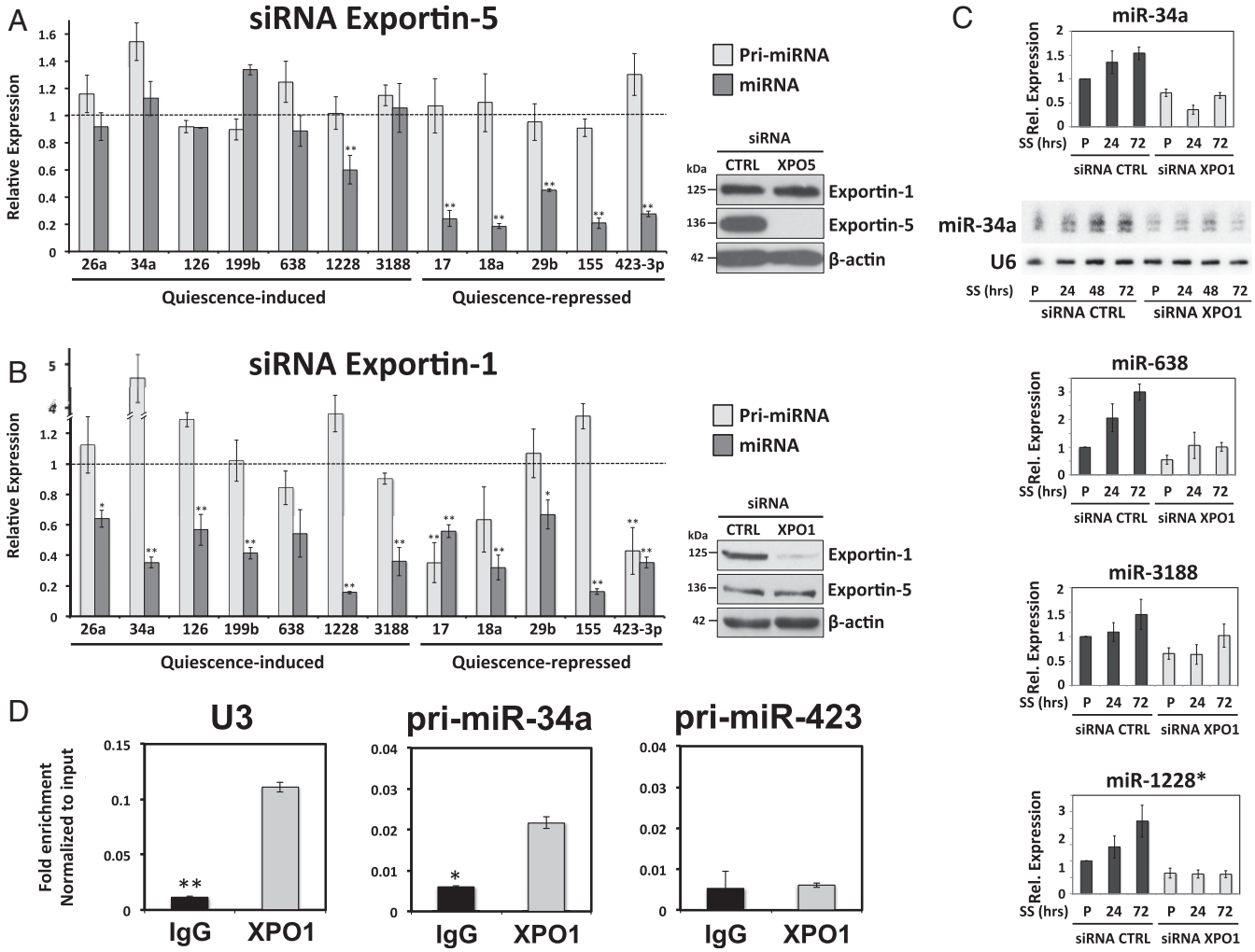

Fig. 3. Biogenesis of quiescence-induced miRNAs requires Exportin-1 but not Exportin-5. (*A*, *Left*) qRT-PCR analysis showing relative expression of primary miRNAs (light gray bars) and their corresponding mature miRNAs (dark gray bars) in proliferating HFFs 48 h after transfection with an siRNA scrambled control (dashed line) or an siRNA targeting Exportin-5 (Dharmacon) (dark and light gray bars). (*Right*) Western blot analysis of Exportin-1 and Exportin-5 after siRNA transfection. (*B*, *Left*) qRT-PCR analysis showing relative levels of primary miRNAs (light gray bars) and their corresponding mature miRNAs (dark gray bars) in proliferating HFFs 48 h after transfection with an siRNA scrambled control (dashed line) or an siRNA targeting Exportin-1 (s14937; Ambion) (dark and light gray bars). (*Right*) Western blot analysis of Exportin-1 and Exportin-5 is also shown after siRNA transfection. (*C*, *Upper*) qRT-PCR analysis of quiescence-induced miRNAs and miR-34a Northern blot 48 h after transfection of an siRNA scrambled control (black bars) or an siRNA targeting Exportin-1 (s14937; Ambion) (gray bars) into proliferating HFFs in the presence of serum (P) or into HFFs serum-starved (SS) for 24 or 72 h or for 24, 48, or 72 h for the miR-34a Northern blot (*Lower*). Transfection of quiescent cells was done 24 h before serum starvation. (*D*) Exportin-1 physically associates with pri-miR-34a in quiescent cells. qRT-PCR was used to detect U3 snoRNA, pri-miR-34a, or pri-miR-423 in control Flag-Vector (IgG, black bars) or Flag-Exportin-1 (XPO1, gray bars) immunoprecipitates from quiescent HFFs (expression data are shown in *SI Appendix*, Fig. S7*E*). An exogenous spike of *C. elegans* RNA was used for qRT-PCR normalization by amplifying Ama-1 mRNA. Similar results were obtained in two independent experiments. In *A*, *B*, and *D*, two-tailed *t* test results are indicated as $*P < 0.05$ and $**P < 0.01$. CTRL, control.

(which can thus tolerate lower Exportin-5 levels) or differences in the stability of the mature miRNA (40), our findings suggest that the biogenesis of these miRNAs can proceed via an Exportin-5–independent mechanism, even in proliferating cells.

Exportin-1 Is Required for Processing of Quiescence-Induced miRNAs. The results in the preceding section imply that an export factor other than Exportin-5 is required for the synthesis of mature miRNAs during quiescence. Previous studies showed that Exportin-1, an essential transport factor for certain proteins and RNAs, supports pri-miRNA processing in *Caenorhabditis elegans* and *Drosophila*, as well as shuttling of mature miRNA from the cytoplasm to the nucleus (28, 41, 42). Recently, we showed that Exportin-1 is involved in the nuclear export of a group of mammalian (m^7G)-capped pre-miRNAs in proliferating cells (27).

To test whether Exportin-1 is involved in the biogenesis of the miRNAs induced in quiescent cells, we transiently transfected proliferating HFFs with a siRNA targeting Exportin-1 and confirmed siRNA-mediated reduction of Exportin-1 but not Exportin-5 expression (Fig. 3*B*). Strikingly, Exportin-1 knockdown in proliferating cells inhibited the expression of several of the mature miRNAs that are induced during quiescence, namely miR-26a, miR-34a, miR-126, miR-199b, miR-638, miR-1228*, and miR-3188 (Fig. 3*B*, dark gray bars). Notably, the levels of the pri-miRNAs corresponding to these miRNAs did not decline (Fig. 3*B*, light gray bars); in fact, pri-miR-34a increased five- to ninefold in the absence of Exportin-1. The levels of the Exportin-5–dependent miRNAs miR-17, miR-18a, miR-29b, miR-155, and miR-423-3p were also reduced in proliferating cells by Exportin-1 knockdown (Fig. 3*B*, dark gray bars), but the corresponding primary miRNAs of these mature miRNAs were decreased also, suggesting a reduction of transcription or changes in pri-miRNA turnover in the absence of Exportin-1. Mature miR-155 and the Simtron miR-1228*, but not their corresponding pri-miRNAs, were reduced by both Exportin-5 and Exportin-1 knockdown (Fig. 3 *A* and *B*, dark gray bars), suggesting that both exportins are involved in the biogenesis of these miRNAs. Similar results were obtained with a second Exportin-1 siRNA experiment (*SI Appendix*, Fig. S6*B*).

To test the contribution of Exportin-1 to miRNA levels during quiescence, we knocked down Exportin-1 in serum-starved cells and measured the levels of mature miRNAs. Exportin-1 knockdown in quiescent cells did not affect the level of Exportin-5 or p27 mRNA or other proteins involved in miRNA biogenesis such as DGCR8, Drosha, Exportin-5, Dicer, and AGO2 (*SI Appendix*, Fig. S7 *A–D*). Nevertheless, Exportin-1 knockdown prevented or attenuated the increase of the quiescence-induced miRNAs: miR-34a, miR-638, miR-3188, and miR-1288* (Fig. 3*C*). The Exportin-1 dependence of miR-34a expression in quiescent cells was confirmed by Northern blot (Fig. 3*C*). Because the expression of the other quiescence-induced miRNAs was difficult to detect by Northern blotting, we focused on miR-34a in subsequent experiments. Interestingly, Exportin-1 knockdown did not change the percentage of HFFs entering quiescence after 24 h of serum starvation but caused a twofold decrease in the percentage of cells in S phase 24 h after the addition of serum, suggesting that Exportin-1–mediated miRNA biogenesis may be required for normal exit from quiescence (*SI Appendix*, Fig. S7*E*).

We used protein–RNA crosslinking followed by Exportin-1 immunoprecipitation to determine if there is a physical interaction between Exportin-1 and pri-miR-34. For this experiment we stably expressed FLAG-tagged Exportin-1 and immunoprecipitated cell extracts with an antibody recognizing the FLAG epitope (*SI Appendix*, Fig. S7*F*). The control snoRNA U3, which is normally transported by Exportin-1 (32), was coimmunoprecipitated, as expected (Fig. 3*D* and *SI Appendix*, Fig. S7*G*). Strikingly, the Exportin-1–dependent pri-miR-34a but not the Exportin-5–dependent pri-miR-423-3 was coimmunoprecipitated from quiescent HFFs by anti–Flag-Exportin-1 (Fig. 3*D* and *SI Appendix*, Fig. S7*G*). Together, these results suggest that Exportin-1 is required for the export and/or processing of miRNAs induced during quiescence and that this activity involves a physical association between these pri-miRNAs and Exportin-1.

Exportin-1–Dependent pri-miRNAs Have a (TMG)-Cap During Quiescence. Previous reports showed that Exportin-1 can bind to (TMG)-capped RNAs such as snoRNA U3 and viral Rev-dependent HIV-1 RNAs (32, 33). Therefore, we investigated whether pri-miRNAs of the Exportin-1–dependent miRNAs contain a (TMG)-cap. We used an antibody against (TMG)-caps [which does not cross-react with the (m^7G)-cap (43)] and nonimmune rabbit serum to perform immunoprecipitation from extracts of proliferating or quiescent HFFs. Specific immunoprecipitated pri-miRNAs were detected by RT-PCR amplification followed by gel electrophoresis. As shown in the top panel of Fig. 4*A*, the control (TMG)-capped U3 snoRNA was amplified following anti–(TMG)-cap pulldown from both proliferating and quiescent HFFs, as expected. Strikingly, pri-miR-34a and pri-miR-3188, two quiescence-induced miRNAs, were also detected in the anti–(TMG)-cap immunoprecipitate from quiescent and, to a lesser extent, from proliferating HFFs. qRT-PCR data from a replicate experiment confirmed that pri-miR-34a and pri-miR-3188 were immunoprecipitated by the anti–(TMG)-cap antibody during proliferation and quiescence (*SI Appendix*, Fig. S8 and Table S4). In contrast, pri-miR-29b and pri-miR-423, which are repressed during quiescence, were not detectable in the anti–(TMG)-cap pull-downs. These results indicate that some quiescence-induced pri-miRNAs are specifically modified with a (TMG)-cap.

TGS1, the enzyme that catalyzes 5′-cap hypermethylation, has two active isoforms, a short isoform in the nucleus and a full-length isoform in the cytoplasm (44). We found that the short form of TGS1 is induced during quiescence, suggesting it may be responsible for cap hypermethylation of Exportin-1–dependent pri-miRNAs (*SI Appendix*, Fig. S9*A*). Indeed, the ability of the anti-(TMG) antibody to immunoprecipitate pri-miR-34a and pri-miR-3188 (as well as the control U3 RNA) was dramatically diminished by siRNA-mediated knockdown of TGS1 in proliferating and quiescent HFFs [Fig. 4*A*, compare siRNA CTRL anti-(TMG) immunoprecipitation lanes with siRNA TGS1 anti-(TMG) immunoprecipitation lanes, and *SI Appendix*, Figs. S8 and S9 *B* and *C*], arguing that this enzyme is required for cap hypermethylation of these miRNAs.

To test further the model that (TMG)-capping is required for miRNA biogenesis in quiescent cells, we examined levels of mature miRNAs after knockdown of TGS1 in quiescent HFFs. As shown in Fig. 4*B*, TGS1 knockdown inhibited the induction of the quiescence-induced miR-34a and miR-3188 but did not affect the abundance of the quiescence-repressed miR-423-3p and miR-29b. The knockdown of TGS1 did not affect the expression of DGCR8, Drosha, or Exportin-5 and actually increased the expression of Dicer and AGO2 (*SI Appendix*, Fig. S9*D*). To investigate whether the (TMG)-cap modification in quiescence-induced pri-miRNAs is important for the interaction with Exportin-1, we used protein–RNA crosslinking followed by immunoprecipitation of FLAG-Exportin-1 in quiescent HFFs that had been treated with control scrambled siRNA or siRNA against TGS1. snoRNA U3, which contains a (TMG)-cap, showed a reduced level of coimmunoprecipitation from cells treated with siRNA against TGS1, as expected (Fig. 4*C*). Notably, Exportin-1–dependent pri-miR-34a was also coimmunoprecipitated to a lesser extent in cells after TGS1 knockdown (Fig. 4*C*), whereas the Exportin-5–dependent pri-miR-423 was not coimmunoprecipitated in the presence or absence of TGS-1, reaffirming the hypothesis that this pri-miRNA does not contain a modified cap.

Taken together, these findings indicate that the pri-miRNAs of quiescence-induced miRNAs are modified with a specific hypermethylation in their 5′-cap by TGS1, a modification important for Exportin-1 recognition (32).

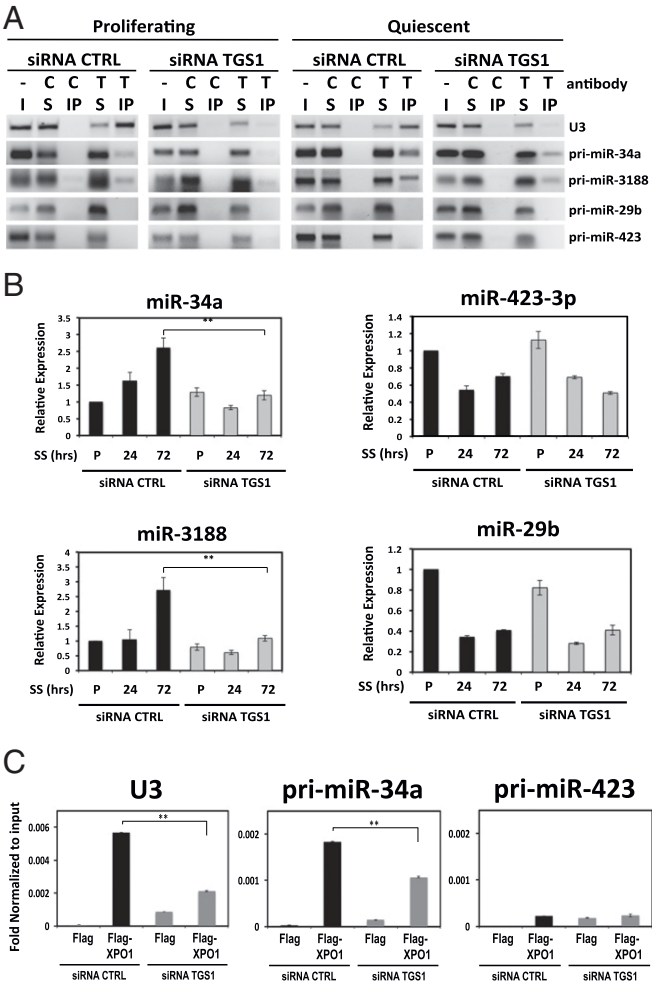

Fig. 4. Quiescence-induced primary miRNAs are (TMG)-capped by TGS1. (*A*) Quiescence-induced pri-miRNAs contain a (TMG)-cap. Extracts were prepared from proliferating or serum-starved quiescent HFFs transfected with control siRNA or with siRNA targeting TGS1 (s41313; Ambion). siRNAs were transfected 24 h before serum starvation. RNA was harvested 48 h after transfection of siRNAs in proliferating HFFs or 72 h after the removal of serum. Extracts then were immunoprecipitated with control antibody or with antibody recognizing the (TMG)-cap, and immunoprecipitated U3 snoRNA and pri-miRNAs were amplified by RT-PCR and visualized after gel electrophoresis. C, control anti-rabbit serum; I, input; IP, immunoprecipitate; S, supernatant; T, anti-TMG antibody. (*B*) TGS1 knockdown inhibits the expression of quiescence-induced miRNAs. HFFs were transfected with a siRNA scrambled control (black bars) or a siRNA targeting TGS1 (sc-45875; Santa Cruz) (gray bars). Twenty-four hours later, cells were serum starved (SS) for 24 or 72 h or were maintained in serum (P). RNA then was prepared and quantified by qRT-PCR. (*C*) TGS1 knockdown affects the binding of pri-miR-34a to Exportin-1 in quiescent cells. qRT-PCR was used to detect U3 snoRNA, pri-miR-34a, or pri-miR-423 in immunoprecipitate samples of control Flag-Vector (Flag) or Flag-Exportin-1 (Flag-XPO1) in quiescent HFFs. Cells were transfected with siRNA control (black bars) or siRNA against TGS1 (gray bars). In *B* and *C*, two-tailed *t* test results are indicated as $^{**}P < 0.01$. Similar results were obtained in two independent experiments. CTRL, control.

Pri-miR-34a Is Present in the Cytoplasm of Quiescent Cells Together with a Small Isoform of Drosha. To examine whether quiescence-induced pri-miRNAs are exported to the cytoplasm as a consequence of (TMG)-capping and interaction with Exportin-1, we prepared nuclear and cytoplasmic RNA from proliferating and quiescent HFFs pretreated with control siRNA or a pool of siRNAs against Drosha to minimize possible rapid processing of these pri-miRNAs (*SI Appendix*, Fig. S10). As expected, Exportin-1–dependent pri-miR-34a and Exportin-5–dependent pri-miR-29b and pri-miR-423 were detected in the nuclear fraction of both proliferating and quiescent HFFs (Fig. 5*A*). These nuclear pri-mRNAs accumulated in response to Drosha knockdown, supporting previous studies that showed their Drosha dependency (45, 46). Strikingly, we also detected pri-miR-34a in the cytoplasmic fraction of proliferating and quiescent HFFs following Drosha knockdown, whereas pri-miR-29b and pri-miR-423 were not detectable in this cellular fraction (Fig. 5 *A* and *B*). These data suggest that pri-miR-34a and possibly other quiescence-induced pri-miRNAs are transported into the cytoplasm where they are processed by an uncharacterized cytoplasmic form of Drosha.

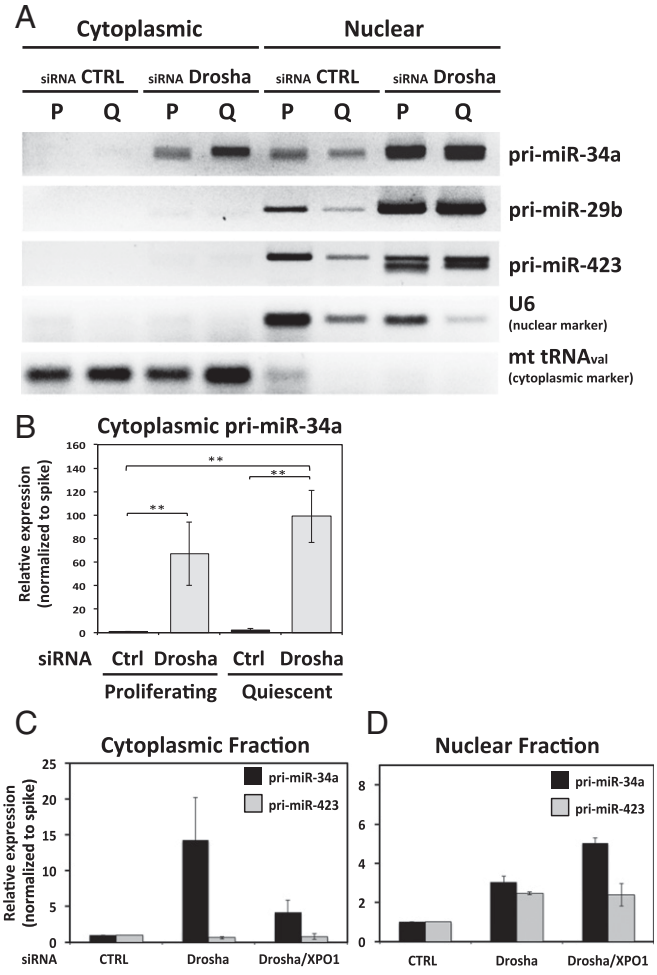

Fig. 5. Detection of cytoplasmic pri-miR-34a is dependent on Exportin-1 expression. (*A*) Detection of pri-miR-34a in the cytoplasmic fraction of Drosha knock-down HFFs. Proliferating (P) and quiescent (Q) HFFs were transfected with control siRNA or siRNA targeting Drosha (sc-44080; Santa Cruz) and after 48 h were subjected to cytoplasmic and nuclear RNA fractionation. RT-PCR was used to detect pri-miR-34a, pri-miR-29b, and pri-miR-423. U6 snRNA is a nuclear control, and mitochondrial (mt) tRNA-val is a cytoplasmic marker. (*B*) qRT-PCR of cytoplasmic fractions as in *A* was used to quantify the levels of pri-miR-34a. Two-tailed *t* test results are indicated as $^{**}P < 0.01$. (*C* and *D*) Exportin-1 knockdown affects the distribution of pri-miR-34a in quiescent HFFs. Cells were transfected with an siRNA scrambled control, an siRNA targeting Drosha (sc-44080; Santa Cruz), or siRNAs targeting Drosha (sc-44080; Santa Cruz) and Exportin-1 (s14937; Ambion) (Drosha/XPO1). Twenty-four hours later, cells were serum starved for 72 h. RNA then was prepared from the cytoplasmic (*C*) or nuclear (*D*) fraction for the quantification of pri-miR-34a and pri-miR-423 by qRT-PCR. Normalization was done using *C. elegans* total RNA as an exogenous spike for the amplification of the worm-specific *ama-1* gene. Similar results were obtained in two independent experiments. CTRL, control.

We also observed a reduction of all the pri-miRNAs in the nuclear fraction of the quiescent cells (Fig. 5*A* and *SI Appendix*, Fig. S11).

To determine if the presence of cytoplasmic pri-miR-34a required Exportin-1, we simultaneously knocked down Exportin-1 and Drosha in quiescent HFFs and determined the level of cytoplasmic pri-miRNAs compared with cells treated with control siRNA or Drosha siRNA alone (*SI Appendix*, Fig. S12). As shown in Fig. 5 *C* and *D*, knockdown of Exportin-1 expression in quiescent HFFs greatly attenuated the increased amount of cytoplasmic pri-miR-34a in the Drosha-knockdown cells and caused the accumulation of this pri-miRNA in the nuclear fraction, suggesting that Exportin-1 is involved in the transport of pri-miR34a from the nucleus to the cytoplasm.

To explore the processing of pri-miRNAs in quiescent HFFs further, we analyzed Drosha protein during quiescence. Western blotting showed the presence of full-length Drosha (~160 kDa) in proliferating and quiescent cells (Fig. 6*A*). In addition, quiescent cells displayed induction of a smaller Drosha band (~130 kDa) (Fig. 6*A*). A pool of three siRNAs that target Drosha mRNA in different regions caused a reduction in both bands, suggesting that the smaller band is a distinct splicing isoform or cleavage product of Drosha (*SI Appendix*, Fig. S13 *A* and *B*). An earlier publication

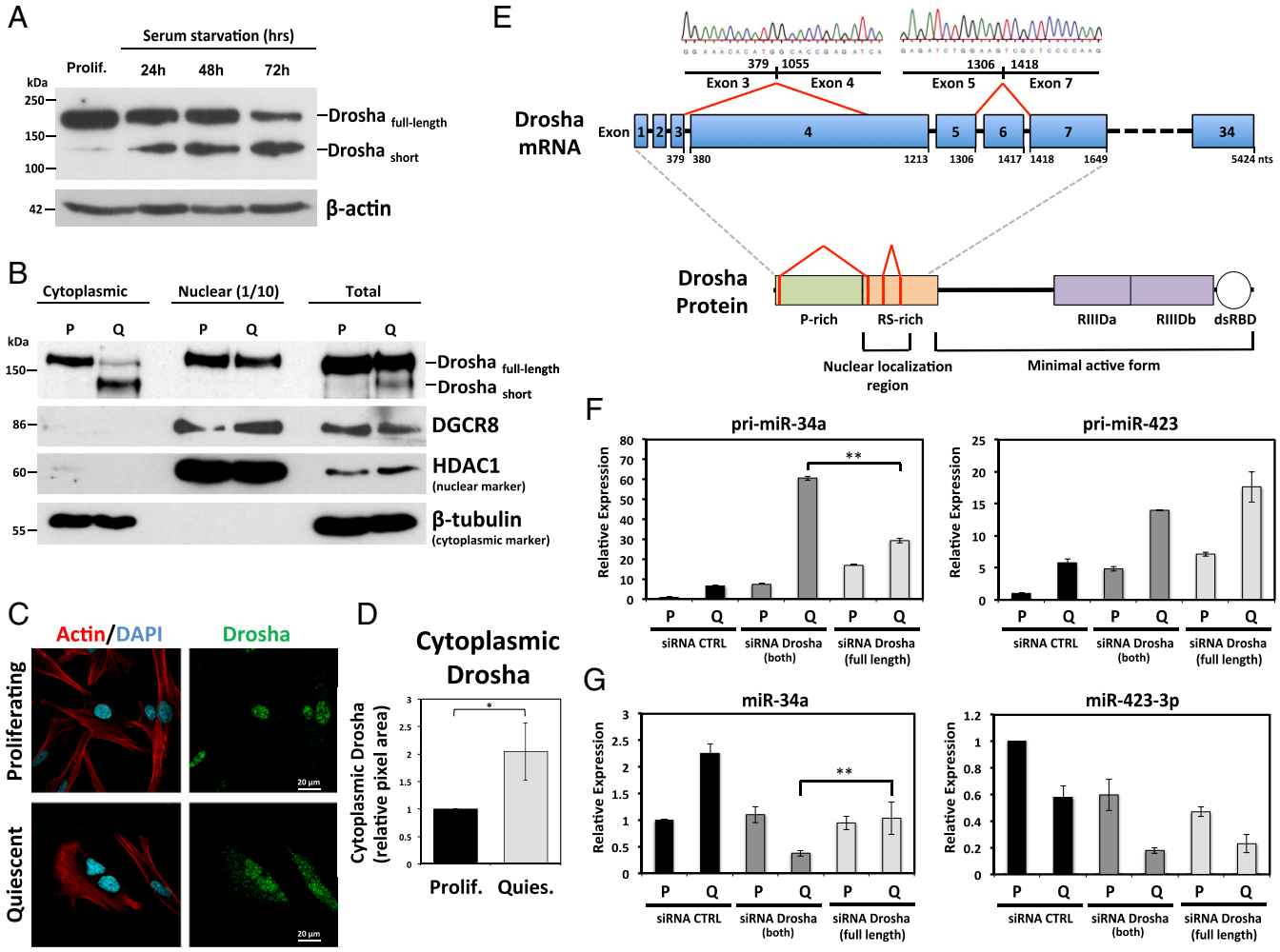

Fig. 6. Detection of a small cytoplasmic isoform of Drosha during quiescence. (*A*) Western blot showing the reduction of full-length Drosha and the appearance of a more rapidly migrating form of Drosha ($Drosha_{short}$) during the induction of quiescence by serum starvation. (*B*) A small form of Drosha ($Drosha_{short}$) is found in the cytoplasmic fraction of quiescent cells. Western blot analysis of Drosha and DGCR8 in total, nuclear, and cytoplasmic fractions in proliferating (P) and quiescent (Q) HFFs. Ten percent of the total loading was used for the nuclear fraction in the Drosha Western blot. Histone deacetylase 1 (HDAC1) was used as the nuclear marker, and β-tubulin was used as the cytoplasmic marker. (*C*) Cytoplasmic localization of Drosha in quiescent HFFs. Immunofluorescence of Drosha (green) in proliferating and quiescent HFFs. Nuclear fluorescence (DAPI, blue) and cytoskeleton immunofluorescence (actin, red) were used to visualize the localization of Drosha. (Scale bars, 20 μm.) (*D*) Quantification of cytoplasmic Drosha immunofluorescence. Proliferating and quiescent HFFs were incubated with digitonin (to permeabilize selectively only the plasma membrane) to diminish masking of cytoplasmic Drosha staining with nuclear Drosha signal. Confocal microscopy was used to visualize the cells, and fluorescent images were used to obtain relative pixel area quantification using ImageJ software. Two-tailed *t* test results are indicated as $*P < 0.05$. Similar results were obtained in three independent experiments. (*E*) Drosha mRNA exon skipping during quiescence. Illustration and sequencing data show two alternative splicing events (red lines) between exons 3 and 4 and between exons 5 and 7 in Drosha mRNA and the affected regions in Drosha protein. Numbers represent the nucleotide sequence of Drosha mRNA. dsRBD, dsRNA-binding domain; P-rich, the proline-rich region; RIIIDa and RIIIDb, RNase III domains a and b, respectively; RS-rich, the arginine- and serine-rich region. (*F*) The short isoform of Drosha is responsible for the processing of pri-miR-34a. qRT-PCR of pri-miR-34a and pri-miR-423 was conducted in quiescent HFFs transfected with control siRNA or siRNA against the full-length Drosha (s26491; Ambion) or both Drosha isoforms (both) (sc-44080; Santa Cruz). Reduced accumulation of pri-miR-34a in cells treated with siRNA targeting full-length Drosha compared with siRNA against both Drosha isoforms (both) suggests the short Drosha isoform can process pri-miR-34a. (*G*) qRT-PCR of mature miR-34a and miR-423-3p was conducted from the experiments shown in *F*. Two-tailed *t* test results are indicated as $*P < 0.01$ in *F* and *G*. Similar results were obtained in two independent experiments. CTRL, control; Prolif, proliferating; Quies, Quiescent.

also detected a smaller form of Drosha (~145 kDa) in HEK-293 cells, which was observed in a different protein complex than full-length Drosha, suggesting that it associates with different binding partners (11).

To investigate the localization of these two different forms of Drosha, we examined nuclear and cytoplasmic fractions from proliferating and quiescent HFFs. The short form of Drosha was found specifically in the cytoplasmic fraction of quiescent HFFs (Fig. 6*B*). In addition, immunofluorescent staining of Drosha in proliferating and quiescent HFFs revealed a cytoplasmic signal during quiescence (Fig. 6 *C* and *D*), which was reduced by siRNA against Drosha (*SI Appendix*, Fig. S13*E*). Because DGCR8 is essential for Drosha activity, we analyzed the cytoplasmic fraction of proliferating and quiescent HFFs for DGCR8. We were unable to detect DGCR8 in the cytoplasmic fractions by direct Western blotting (Fig. 6*B*) or by blotting after immunoprecipitation (*SI Appendix*, Fig. S14*A*), suggesting that Drosha may interact with a different dsRNA-binding protein in the cytoplasm, as was previously suggested (26). It is noteworthy that two recent publications demonstrated that alternative splicing of Drosha could affect its subcellular localization; these reports are consistent with our finding that a specific Drosha isoform exists in the cytoplasm of quiescent cells (47, 48). Furthermore, siRNA against DGCR8 in quiescent HFFs did not affect the biogenesis of the quiescence-induced miR-34a but altered the processing of the quiescence-repressed miR-423-3p (*SI Appendix*, Fig. S14*B*), supporting the existence of functional cytoplasmic Drosha without DGCR8.

To characterize the short form of Drosha in quiescent cells, we amplified the Drosha ORF mRNA by tiling RT-PCR (*SI Appendix*, Fig. S15). Cloning and sequencing identified two exon-skipping events in the same transcript, occurring in the 5′ half of the Drosha mRNA, during quiescence. These events disrupt the proline-rich (P-rich) region as well as the nuclear localization signal in the arginine- and serine-rich (RS-rich) portion of Drosha (Fig. 6*E*) but leave intact the previously described minimal active fragment of the protein (17). To measure the activity of the short isoform of Drosha indirectly, we measured the levels of pri-miR-34a and pri-miR-423 after treating cells with siRNAs against both Drosha isoforms or with an siRNA that specifically targets the exon 6 region that is present only in the full-length Drosha but not in the short isoform (*SI Appendix*, Fig. S13 *C* and *D*). siRNA against both forms of Drosha caused a marked increase in the level of pri-miR-34a and pri-miR423 in quiescent cells, confirming that Drosha contributes to the processing of these miRNAs (Fig. 6*F*). Notably, siRNA specifically targeting full-length Drosha was much less effective in inducing pri-miR-34a or miR-34a in quiescent cells than was siRNA targeting both Drosha isoforms, implying that the short cytoplasmic form of Drosha was competent to process pri-miR-34a to mature miR-34a. In contrast, the short isoform of Drosha does not appear to be involved in processing pri-miR-423, because specific knockdown of the full-length form was sufficient to cause pri-miR-423 accumulation. Similarly, siRNA against full-length Drosha allows partial induction of mature miR-34a but not miR-423 in quiescent cells (Fig. 6*G*), supporting the hypothesis that the short isoform of Drosha is able to process pri-miR-34a but not pri-miR-423 during quiescence.

Discussion

When cells reversibly exit the cell cycle and enter quiescence, they remain in a nonproliferative state until they receive appropriate mitogenic signals but must avoid irreversible states such as apoptosis, differentiation, and senescence. Because miRNAs regulate many cellular pathways, changes in miRNA biogenesis likely play an important role in controlling these important cellular processes. This study revealed a number of unique features of miRNA biogenesis in proliferating and quiescent cells.

Exportin-5 Levels Are Regulated During Cellular Quiescence by Autophagy and miR-34a. We confirmed the findings of Iwasaki and collaborators (38) that the amount of Exportin-5 protein decreases during quiescence (Fig. 2*A* and *SI Appendix*, Fig. S3). We showed that at least two posttranscriptional mechanisms contribute to the reduction of Exportin-5 expression during quiescence. Because a specific inhibitor of autophagy prevented the loss of Exportin-5, a major regulator of Exportin-5 levels during quiescence appears to be protein degradation by autophagy. In fact, autophagy is induced during quiescence to protect cells from apoptosis mediated by the proteasome (49), and serum starvation or treatment with an mTOR inhibitor activates autophagy, resulting in the degradation of Dicer and AGO2 (50). Together with our findings, these results indicate that several proteins involved in miRNA biogenesis are regulated by autophagy during quiescence to avoid the induction of apoptosis. In addition, our studies suggest that miR-34a binds to an evolutionarily conserved binding site in the Exportin-5 mRNA 3′ UTR and represses Exportin-5 expression (Fig. 2 *D*–*G*). miR-34a–mediated repression of Exportin-5 appears to act in conjunction with autophagy-mediated degradation to ensure low levels of Exportin-5 throughout quiescence. Consistent with this idea, miR-34a is induced relatively late after the onset of quiescence, when Exportin-5 levels are already low (Fig. 2*A* and *SI Appendix*, Fig. S3). Taken together, our data suggest that Exportin-1 contributes to Exportin-5 down-regulation during quiescence by supporting the synthesis of miR-34a, which binds to Exportin-5 mRNA and reinforces the block of Exportin-5 expression. An interesting topic for future studies will be determining if the regulation of Exportin-5 is related exclusively to the induction of quiescence or instead to the absence of specific growth factors such as IGF1, which have been shown to be important in the regulation of autophagy in HFFs (51).

Exportin-1–Dependent miRNA Biogenesis Occurs During Cellular Quiescence. Our major finding is the discovery of an alternative miRNA biogenesis pathway involving Exportin-1 (also known as "CRM1") during quiescence. Exportin-1, the major factor responsible for the export of proteins from the nucleus, is also involved in the export of certain RNAs such as ribosomal RNAs, snRNAs, and mRNAs encoding some proteins involved in cell-cycle regulation (28, 52). Previous studies in *C. elegans* and *Drosophila* showed that Exportin-1 is involved in pri-miRNA processing and biogenesis (41). Our findings demonstrate that Exportin-1 is also involved in the biogenesis of specific miRNAs in proliferating mammalian cells and that this activity is enhanced during quiescence. First, there is no change in Exportin-1 levels during quiescence (Fig. 2*A* and *SI Appendix*, Fig. S3*B*). Second, Exportin-1 knockdown reduced the levels of specific quiescence-induced mature miRNAs in proliferating and quiescent HFFs (Fig. 3 *B* and *C*), although the effect was not as dramatic as the reduction of quiescence-repressed miRNAs by Exportin-5 knockdown (Fig. 3*A*), Third, the Exportin-1–dependent pri-miR-34a, but not the Exportin-5–dependent pri-miR-423, is found in a stable complex with Exportin-1 (Fig. 3*D*). Our data further demonstrate that some miRNAs, including miR-26a, miR-34a, miR-126, miR-199, miR-638, and miR-3188, can use an Exportin-5–independent biogenesis pathway in proliferating cells. We also identified miRNAs subject to complex regulation during quiescence. For example, siRNA-mediated knockdown of either Exportin-5 or Exportin-1 in proliferating HFFs reduced the expression of mature miR-1228* but did not affect levels of pri-miR-1228 (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S6 *A* and *B*). Thus, both Exportin-5 and Exportin-1 are involved in the biogenesis of miR-1228*, which is considered a Simtron, a class of miRNAs that are produced in the absence of splicing and are independent of DGCR8, Dicer, Exportin-5, and Argonaute (23). Kim and collaborators (47) demonstrated that Exportin-5 knockout in the colorectal cancer cell line HCT116 only modestly affects the processing of several miRNAs, as is consistent with the existence of Exportin-5–independent miRNA maturation in these cells.

Quiescence-Induced pri-miRNAs Contain a (TMG)-Cap and Bind Exportin-1. We discovered that the pri-miRNAs of Exportin-1–dependent quiescence-induced miRNAs contain a (TMG)-cap, which may be a signal for recognition by Exportin-1, whereas this cap structure is absent from the Exportin-5–dependent pri-miRNAs (Fig. 4*A* and *SI Appendix*, Fig. S8 and Table S4). We also found that a short nuclear isoform of TGS1 is up-regulated during quiescence (*SI Appendix*, Fig. S9*A*). Importantly, knocking down the expression of TGS1 in HFFs reduced (TMG)-capping of quiescence-induced pri-miRNAs, diminished induction of the corresponding mature miRNAs during quiescence, and reduced the interaction of pri-miR-34a with Exportin-1 (Fig. 4). Because most RNAs need to be exported to the cytoplasm to be modified with a (TMG)-cap, we have not ruled out the possibility that some pri-miRNAs could be exported by Exportin-1 with a (m^7G)-cap and then undergo cap hypermethylation in the cytoplasm. We recently described microprocessor-independent human and mouse pre-miRNAs that are specifically (m^7G)-capped and transported by the PHAX–Exportin-1 pathway and cleaved by Dicer before loading into the RISC complex (27). The miRNAs described here are processed differently, because TMG caps are added at the pri-miRNA level, indicating that human cells contain at least two alternative Exportin-1–dependent pathways of miRNA biogenesis.

Discovery of pri-miRNAs and a Smaller Drosha Isoform in the Cytoplasm of Quiescent Cells. Another important discovery is finding pri-miR-34a in the cytoplasm. We detected cytoplasmic pri-miR-34a in proliferating and quiescent HFFs only after knocking down Drosha, suggesting that this pri-miRNA is processed rapidly once it leaves the nucleus (Fig. 5 *A* and *B*). We were unable to detect other quiescence-induced pri-miRNAs in the cytoplasm consistently, presumably because they are less abundant than pri-miR-34a. Drosha-mediated rapid cleavage of these cytoplasmic (TMG)-capped pri-miRNAs may prevent their recognition by Snuportin-1, which potentially could return the pri-miRNAs to the nucleus (30, 31). Moreover, we observed a reduction in the amount of pri-miR-34a in the cytoplasmic fraction of cells knocked down for both Exportin-1 and Drosha (Fig. 5*C*). This observation, together with the discovery of an interaction between pri-miR-34a and Exportin-1 (Fig. 3*D*), suggests that Exportin-1 plays a role in transporting pri-miR-34a from the nucleus to the cytoplasm. Further studies are necessary to elucidate the mechanism involved in the transport of quiescence-induced pri-miRNAs to the cytoplasm, their (TMG)-capping, and their cytoplasmic processing.

We also discovered the preferential expression of a smaller, alternatively spliced cytoplasmic isoform of Drosha during quiescence (Fig. 6 *A*–*E*). Recent studies demonstrated the existence of splicing isoforms of Drosha found in the cytoplasm in several cell lines (47, 48). These Drosha isoforms contain deletions in the nuclear localization (RS-rich) region similar to the small isoform of Drosha found in quiescent HFFs. These data support the hypothesis that splicing of specific genes, such as Drosha, by the spliceosome machinery is important in the regulation of cell-cycle progression (53). Importantly, our data expand the concept that a specific Drosha isoform can be found in the cytoplasm of normal human cells in a particular stress condition such as serum starvation. Another study showed that certain RNA viruses trigger Exportin-1–dependent translocation of Drosha to the cytoplasm, providing a potential antiviral mechanism that acts by cleaving viral genomic RNA (54). Consistent with this model, Exportin-1 knockdown reduced the amount of cytoplasmic pri-miR-34a (Fig. 5*C*), and a short cytoplasmic isoform of Drosha appeared to be involved in pri-miR-34a processing during quiescence (Fig. 6 *F* and *G*). Our discovery of pri-miRNAs and a small isoform of Drosha in the cytoplasm suggest the existence of a previously unrecognized miRNA biogenesis pathway during quiescence.

The Contribution of Quiescence-Induced miRNAs to Cellular Growth Arrest. Do the quiescence-induced miRNAs play a role in growth arrest? These miRNAs may be essential for the induction of quiescence, thereby necessitating the use of an alternative miRNA biogenesis pathway when the canonical pathway is repressed. For example, the p53-regulated miR-34a regulates cellular pathways such as cell cycle, differentiation, and apoptosis (55, 56); it is a master regulator of tumor suppression in different human cancers and is being evaluated as a miRNA therapeutic (57). Similarly, miR-638 up-regulation in vascular smooth muscle cells was recently reported to inhibit cell proliferation and migration by targeting the orphan nuclear receptor 1 (NOR1) and consequently affecting the expression of cyclin D1 mRNA (58). In addition, miR-1228* overexpression suppresses xenograft tumor formation by negatively regulating NF-KB activity (59). Finally, miR-3188 was found to reduce cell-cycle transition and proliferation in nasopharyngeal carcinoma by targeting mTOR mRNA and regulating the PI3K/AKT signaling pathway through FOXO1 (60). In addition, it is possible that impaired processing of specific miRNAs in certain growth conditions may contribute to the embryonic lethality of TGS1-knockout mice (61).

The results of the cell-cycle analysis in *SI Appendix*, Fig. S7*E* show that Exportin-1–regulated miRNAs are not required for entry into quiescence. However, the reduced number of cells in S phase 24 h after serum addition suggests that Exportin-1 is required for proper exit from quiescence. We speculate that the Exportin-1–dependent, quiescence-induced miRNAs are important not to maintain growth arrest but rather to poise cells so that they can reenter the cell cycle efficiently when favorable growth conditions are restored.

Not only do miRNAs affect cell growth; the cell growth state also can affect miRNAs. We reported previously that growth arrest can convert some miRNAs from repressors into activators of translation (37), and we show here that quiescence inhibits canonical miRNA biogenesis but stimulates an alternative miRNA biogenesis pathway. Thus, the cellular growth state may globally affect miRNA synthesis and function, which in turn may profoundly influence cellular gene expression and phenotype.

In summary, we have shown that a set of miRNAs is processed independently of the canonical miRNA pathway in quiescent cells via (TMG)-cap modification of their pri-miRNA, interaction with Exportin-1, and cytoplasmic processing by a small isoform of Drosha (*SI Appendix*, Fig. S16). This pathway is constitutively active in proliferating cells, but its activity is enhanced during quiescence, presumably to regulate key processes involved in cellular growth arrest. Conversely, in quiescent cells the canonical miRNA pathway is turned off, likely contributing to the quiescent state. Future investigation will be necessary to identify additional components of this miRNA Exportin-1–dependent pathway and its impact on cell physiology.

Materials and Methods

Detailed experimental protocols are described in the *SI Appendix*. All experiments were performed in compliance with the Institutional Biosafety Committee at West Virginia University, number 15-03-03.

Cell Culture and Reagents. Normal primary HFFs (obtained from the Yale Skin Disease Research Center, New Haven, CT) and HeLa/E6-5K, 293T, and C127 cells (obtained from the D.D. laboratory, Yale University, New Haven, CT) were cultured in DMEM-10 at 37 °C in the presence of 5% CO_2. Further details are in the *SI Appendix*.

Quiescence Induced by Serum Starvation and Confluency. HFFs and HeLa and C127 cells were rinsed with Dulbecco's phosphate buffered saline, detached with 0.25% trypsin-EDTA, and plated in serum-free DMEM at low density. For quiescence induced by confluency, we plated HFFs (2×10^6 cells per 100-mm plate) in DMEM-10. Further details are in the *SI Appendix*.

Immunoprecipitation of Trimethylated-Capped RNA. HFFs were incubated for 72 h in serum-free DMEM to induce quiescence. RNA was extracted using TRIzol Reagent, and 10 μg of RNA was diluted in NET-2 buffer, precleared and incubated with Protein G Sepharose 4 Fast Flow beads loaded with 15 μL normal rabbit serum or 15 μL rabbit m3G-cap antibody. Beads were rinsed five times with NET-2 buffer and were resuspended in G-50 buffer. RNA was extracted from the beads by phenol-chloroform-isoamyl alcohol extraction. Further details are in the *SI Appendix*.

1. Pardee AB (1974) A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci USA* 71:1286–1290.
2. O'Farrell PH (2011) Quiescence: Early evolutionary origins and universality do not imply uniformity. *Philos Trans R Soc Lond B Biol Sci* 366:3498–3507.
3. Coller HA (2011) Cell biology. The essence of quiescence. *Science* 334:1074–1075.
4. Valcourt JR, et al. (2012) Staying alive: Metabolic adaptations to quiescence. *Cell Cycle* 11:1680–1696.
5. Coller HA, Sang L, Roberts JM (2006) A new description of cellular quiescence. *PLoS Biol* 4:e83.
6. Cheung TH, Rando TA (2013) Molecular regulation of stem cell quiescence. *Nat Rev Mol Cell Biol* 14:329–340.
7. Bohnsack MT, Czaplinski K, Gorlich D (2004) Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* 10:185–191.
8. Krol J, Loedige I, Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* 11:597–610.
9. Lee Y, Han J, Yeom KH, Jin H, Kim VN (2006) Drosha in primary microRNA processing. *Cold Spring Harb Symp Quant Biol* 71:51–57.
10. Lee Y, et al. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425:415–419.
11. Gregory RI, et al. (2004) The Microprocessor complex mediates the genesis of microRNAs. *Nature* 432:235–240.
12. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature* 432:231–235.
13. Yi R, Qin Y, Macara IG, Cullen BR (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* 17:3011–3016.
14. Hutvágner G, et al. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 293:834–838.
15. Grishok A, et al. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell* 106:23–34.
16. Knight SW, Bass BL (2001) A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. *Science* 293:2269–2271.
17. Han J, et al. (2004) The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* 18:3016–3027.
18. Lian SL, et al. (2009) The C-terminal half of human Ago2 binds to multiple GW-rich regions of GW182 and requires GW182 to mediate silencing. *RNA* 15:804–813.
19. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293:1146–1150.
20. Mourelatos Z, et al. (2002) miRNPs: A novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev* 16:720–728.
21. Ruby JG, Jan CH, Bartel DP (2007) Intronic microRNA precursors that bypass Drosha processing. *Nature* 448:83–86.
22. Cazalla D, Xie M, Steitz JA (2011) A primate herpesvirus uses the integrator complex to generate viral microRNAs. *Mol Cell* 43:982–992.
23. Havens MA, Reich AA, Duelli DM, Hastings ML (2012) Biogenesis of mammalian microRNAs by a non-canonical processing pathway. *Nucleic Acids Res* 40:4626–4640.
24. Cifuentes D, et al. (2010) A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science* 328:1694–1698.
25. Cheloufi S, Dos Santos CO, Chong MM, Hannon GJ (2010) A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature* 465:584–589.
26. Shapiro JS, Langlois RA, Pham AM, Tenoever BR (2012) Evidence for a cytoplasmic microprocessor of pri-miRNAs. *RNA* 18:1338–1346.
27. Xie M, et al. (2013) Mammalian 5′-capped microRNA precursors that generate a single microRNA. *Cell* 155:1568–1580.
28. Hutten S, Kehlenbach RH (2007) CRM1-mediated nuclear export: To the pore and beyond. *Trends Cell Biol* 17:193–201.
29. Ohno M, Segref A, Bachi A, Wilm M, Mattaj IW (2000) PHAX, a mediator of U snRNA nuclear export whose activity is regulated by phosphorylation. *Cell* 101:187–198.
30. Palacios I, Hetzer M, Adam SA, Mattaj IW (1997) Nuclear import of U snRNPs requires importin beta. *EMBO J* 16:6783–6792.
31. Kiss T (2004) Biogenesis of small nuclear RNPs. *J Cell Sci* 117:5949–5951.
32. Boulon S, et al. (2004) PHAX and CRM1 are required sequentially to transport U3 snoRNA to nucleoli. *Mol Cell* 16:777–787.
33. Yedavalli VS, Jeang KT (2010) Trimethylguanosine capping selectively promotes expression of Rev-dependent HIV-1 RNAs. *Proc Natl Acad Sci USA* 107:14787–14792.
34. Medina R, et al. (2008) MicroRNAs 221 and 222 bypass quiescence and compromise cell survival. *Cancer Res* 68:2773–2780.
35. Suh EJ, et al. (2012) A microRNA network regulates proliferative timing and extracellular matrix synthesis during cellular quiescence in fibroblasts. *Genome Biol* 13:R121.
36. Martinez I, Cazalla D, Almstead LL, Steitz JA, DiMaio D (2011) miR-29 and miR-30 regulate B-Myb expression during cellular senescence. *Proc Natl Acad Sci USA* 108: 522–527.
37. Vasudevan S, Tong Y, Steitz JA (2007) Switching from repression to activation: MicroRNAs can up-regulate translation. *Science* 318:1931–1934.
38. Iwasaki YW, et al. (2013) Global microRNA elevation by inducible Exportin 5 regulates cell cycle entry. *RNA* 19:490–497.
39. Link S, Grund SE, Diederichs S (2016) Alternative splicing affects the subcellular localization of Drosha. *Nucleic Acids Res* 44:5330–5343.
40. Rissland OS, Hong SJ, Bartel DP (2011) MicroRNA destabilization enables dynamic regulation of the miR-16 family in response to cell-cycle changes. *Mol Cell* 43: 993–1004.
41. Büssing I, Yang JS, Lai EC, Grosshans H (2010) The nuclear export receptor XPO-1 supports primary miRNA processing in C. elegans and Drosophila. *EMBO J* 29: 1830–1839.
42. Castanotto D, Lingeman R, Riggs AD, Rossi JJ (2009) CRM1 mediates nuclear-cytoplasmic shuttling of mature microRNAs. *Proc Natl Acad Sci USA* 106:21655–21659.
43. Luhrmann R, et al. (1982) Isolation and characterization of rabbit anti-m3 2,2,7G antibodies. *Nucleic Acids Res* 10:7103–7113.
44. Girard C, et al. (2008) Characterization of a short isoform of human Tgs1 hypermethylase associating with small nucleolar ribonucleoprotein core proteins and produced by limited proteolytic processing. *J Biol Chem* 283:2060–2069.
45. Chong MM, et al. (2010) Canonical and alternate functions of the microRNA biogenesis machinery. *Genes Dev* 24:1951–1960.
46. Kawai S, Amano A (2012) BRCA1 regulates microRNA biogenesis via the DROSHA microprocessor complex. *J Cell Biol* 197:201–208.
47. Kim YK, Kim B, Kim VN (2016) Re-evaluation of the roles of DROSHA, Export in 5, and DICER in microRNA biogenesis. *Proc Natl Acad Sci USA* 113:E1881–E1889.
48. Ha M, Kim VN (2014) Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol* 15: 509–524.
49. Legesse-Miller A, et al. (2012) Quiescent fibroblasts are protected from proteasome inhibition-mediated toxicity. *Mol Biol Cell* 23:3566–3581.
50. Gibbings D, et al. (2012) Selective autophagy degrades DICER and AGO2 and regulates miRNA activity. *Nat Cell Biol* 14:1314–1321.
51. Bitto A, et al. (2010) Long-term IGF-I exposure decreases autophagy and cell viability. *PLoS One* 5:e12592.
52. Verheggen C, Bertrand E (2012) CRM1 plays a nuclear role in transporting snoRNPs to nucleoli in higher eukaryotes. *Nucleus* 3:132–137.
53. Karamysheva Z, Díaz-Martínez LA, Warrington R, Yu H (2015) Graded requirement for the spliceosome in cell cycle progression. *Cell Cycle* 14:1873–1883.
54. Shapiro JS, et al. (2014) Drosha as an interferon-independent antiviral factor. *Proc Natl Acad Sci USA* 111:7108–7113.
55. He L, et al. (2007) A microRNA component of the p53 tumour suppressor network. *Nature* 447:1130–1134.
56. Chen F, Hu SJ (2012) Effect of microRNA-34a in cell cycle, differentiation, and apoptosis: A review. *J Biochem Mol Toxicol* 26:79–86.
57. Bader AG (2012) miR-34 - a microRNA replacement therapy is headed to the clinic. *Front Genet* 3:120.
58. Li P, et al. (2013) MicroRNA-638 is highly expressed in human vascular smooth muscle cells and inhibits PDGF-BB-induced cell proliferation and migration through targeting orphan nuclear receptor NOR1. *Cardiovasc Res* 99:185–193.
59. Jia L, et al. (2013) Restoration of miR-1228* expression suppresses epithelial-mesenchymal transition in gastric cancer. *PLoS One* 8:e58637.
60. Lund E, Güttinger S, Calado A, Dahlberg JE, Kutay U (2004) Nuclear export of microRNA precursors. *Science* 303:95–98.
61. Jia Y, et al. (2012) Early embryonic lethality of mice with disrupted transcription cofactor PIMT/NCOA6IP/Tgs1 gene. *Mech Dev* 129:193–207.