Molecular Biology of the Cell
Vol. 21, 2138–2149, July 1, 2010

MiR-322/424 and -503 Are Induced during Muscle Differentiation and Promote Cell Cycle Quiescence and Differentiation by Down-Regulation of Cdc25A

Sukumar Sarkar, Bijan K. Dey, and Anindya Dutta

Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, VA 22908

Submitted January 27, 2010; Revised April 5, 2010; Accepted May 3, 2010
Monitoring Editor: Mark J. Solomon

Induction of a G1 phase cell cycle arrest, caused primarily by the inhibition of cyclin-dependent-kinase 2 (cdk2), is a critical step in the differentiation of myoblasts into myotubes. Here, we report that two microRNAs, miR-322/424 and miR-503, are induced and promote cdk2 inhibition during myogenesis. These microRNAs down-regulate Cdc25A, the phosphatase responsible for removing inhibitory phosphorylation of cdk2, both in myoblasts differentiating into myotubes and in nonmuscle cells. Cdc25A is down-regulated during muscle differentiation by multiple pathways: action of these two microRNAs, proteasomal degradation of Cdc25A protein and transcriptional repression. Overexpression of Cdc25A or of cdk2 with mutations on T14 and Y15 (cdk2-AF), so that it cannot be inhibited by phosphorylation, decreases differentiation and differentiation-induced cell cycle quiescence. Introduction of miR-322/424 and miR-503 in heterologous cancer cells induces G1 arrest, which is also attenuated by overexpression of the cdk2-AF mutant. Until now Cdc25A and the inhibitory phosphorylation on T14 and Y15 of cdk2 have only been implicated in the intra-S phase checkpoint pathway after DNA damage. Our results reveal an unexpected role of Cdc25A down-regulation and the inhibitory phosphorylation of cdk2 T14 and Y15 in cell cycle quiescence during muscle differentiation and implicate two muscle differentiation-induced microRNAs in the process.

INTRODUCTION

A complex interplay of cell proliferation and cell differentiation is essential to make an organism from a single fertilized egg. Proliferation increases the number of cells available for making up different tissues and organs. Yet, differentiation of proliferating cells into specific tissue types is always accompanied by an arrest of the cell cycle in the G0/G1 stage.

C2C12 myoblasts can be induced to differentiate into myotubes by serum depletion. This differentiation model has been very useful for discovering both the transcription factors and microRNAs important for differentiation, and the mechanism by which the cells are arrested in G1 as a prelude to differentiation. It is in this system that hypophosphorylation of the retinoblastoma protein Rb was shown to be important of cell cycle quiescence during differentiation (De Falco *et al*., 2006; Khidr and Chen, 2006). Hypophosphorylation of Rb is a direct result of inhibition of cyclin-dependent kinase (cdk), especially the cdk2 kinase that is active during the G1–S transition in undifferentiated cells. Cyclin-dependent kinases can be inhibited by interaction with inhibitory proteins CDKN1A (p21) or CDKN1B (p27) and CDKN2 proteins (p15/16), and by inhibitory phosphorylation on T14 and Y15 of the kinase (Nilsson and Hoffmann, 2000; Vidal and Koff, 2000). The cdk inhibitors p21, p27, and p15/16 have been found to be important for cell cycle quiescence during myoblast to myotube transition (Hawke *et al*., 2003; Myers *et al*., 2004), but the role of the inhibitory phosphorylation on T14 and Y15 of cdk2 at this stage of differentiation has not yet been explored. Inhibitory phosphorylation on T14 and Y15 of cdk2 has been reported only after DNA damage as part of the intra-S phase checkpoint response, and this phosphorylation is brought about by the degradation of Cdc25A, the phosphatase responsible for removing the phosphates from T14 and Y15 (Mailand *et al*., 2000).

The role of microRNAs in the regulation of gene expression has been uncovered in the last decade. MicroRNAs are 20- to 24-base-long single-stranded RNAs generated by the concerted action of the RNAses Drosha and Dicer on RNA transcripts. The microRNAs are incorporated into an RNA-induced silencing complex and usually exploit their 5′-most seven to eight nucleotides (the seed sequence) to pair with target mRNAs, which are then silenced both at the translational level and by RNA degradation (reviewed in Lee and Dutta, 2009). MicroRNAs have been implicated in varied processes: differentiation, proliferation, apoptosis, carcinogenesis, and metastasis, to name a few. Indeed, microRNAs have been implicated in the cell cycle quiescence and the differentiation pathways that underlie the myoblast to myotube transition. For example, miR-1, -206, and -133 are induced during this transition and are shown to promote the transition to myotubes in skeletal muscle. MiR-1/206 (these two microRNAs share a common seed sequence) promotes cell cycle quiescence, both in myoblasts and in heterologous cancer cell lines. Although many targets of these microRNAs have been predicted by computational programs, targets proved by experiment include the p180 catalytic subunit of DNA polymerase α, connexin-43, butyrate-induced tran-

This article was published online ahead of print in *MBoC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10–01–0062) on May 12, 2010.

Address correspondence to: Anindya Dutta (ad8q@virginia.edu).

script-1, monocyte-macrophage differentiation factor, and others (Kim *et al*., 2006). miR-1 also targets the histone deacetylase HDAC4, a transcriptional repressor of muscle differentiation, whereas miR-133 paradoxically promotes myoblast proliferation by repressing serum-response-factor (Chen *et al*., 2006). miR-133 targets an alternative splicing factor, n-polypyrimidine tract-binding protein, thus contributing to changes in splicing patterns that are observed during muscle differentiation (Boutz *et al*., 2007) and inhibits a factor that slows down muscle differentiation, uncoupling protein 2 (Chen *et al*., 2009). miR-26a and miR-181 are also induced during muscle differentiation. miR-26a represses a histone methyltransferase, enhancer of zeste homologue 2, a known repressor of muscle differentiation (Wong and Tellam, 2008). miR-181 represses another repressor of muscle differentiation, HoxA11 (Naguibneva *et al*., 2006). Furthermore, miR-424 regulates human monocyte/macrophage differentiation (Rosa *et al*., 2007). Finally, miR-27b, induced during muscle differentiation, represses Pax3, a proproliferative transcription factor that inhibits differentiation (Crist *et al*., 2009). Perhaps the most stringent evidence of the role of microRNAs in skeletal muscle development comes from the targeted deletion of dicer in the myogenic compartment during embryogenesis (O'Rourke *et al*., 2007). The mice died perinatally with reduced skeletal muscle mass and abnormalities in the morphology of the muscle fibers.

MicroRNAs are also important for muscle differentiation in nonmammalian organisms, such as *Drosophila* (Kwon *et al*., 2005; Nguyen and Frasch, 2006) and *Caenorhabditis elegans* (Ketting *et al*., 2001). Furthermore, an important role of microRNA has become evident in cardiac myogenesis, with cardiac problems in mice lacking only one copy of two miR-1 genes (Zhao *et al*., 2007). Knockout of a subset of miR-133 genes also results in mice with ventricular septal defects, dilated cardiomyopathy and aberrant cardiomyocyte proliferation (Liu *et al*., 2008). A more extensive discussion of microRNAs in the differentiation of skeletal and cardiac muscle can be found in several reviews (Callis *et al*., 2008; Chen *et al*., 2009; Williams *et al*., 2009).

The role of muscle differentiation-induced microRNAs in suppressing cell proliferation is particularly evident when one considers changes in microRNA expression during the dedifferentiation process seen in malignancies of the skeletal muscle. Rhabdomyosarcomas epigenetically silence the miR-29 microRNA, which has a positive role in muscle differentiation (Wang *et al*., 2008). Similarly, miR-1/206 down-regulates the c-MET oncogene and acts as a tumor suppressor for rhabdomyosarcomas (Taulli *et al*., 2009; Yan *et al*., 2009).

In our search for anti-cell cycle effects of microRNAs, we examined various muscle differentiation-induced microRNAs for their role is stopping cell cycle progression. In this article, we report that two such microRNAs target the cell cycle in an unexpected manner through the inhibition of Cdc25A, the phosphatase that removes the inhibitory phosphates on T14 and Y15 of cdk2. Following up on this, we discovered that Cdc25A is repressed by multiple mechanisms during muscle differentiation, and the importance of this repression is highlighted by the negative effect on cell cycle quiescence and muscle differentiation that is seen after either the overexpression of Cdc25A or of mutant cdk2 (that cannot be inhibited by phosphorylation on T14 and Y15).

MATERIALS AND METHODS

Cell Culture

Human embryonic kidney (HEK)-293T and U2OS (osteosarcoma) cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% of fetal bovine serum and 1% penicillin/streptomycin. HCT116 and HCT116 $^{p21-/-}$ (colon cancer) cells were maintained in McCoy's 5A media (Invitrogen) supplemented with 10% of fetal bovine serum and 1% penicillin/streptomycin. C2C12 mouse skeletal myoblasts (American Type Culture Collection; Yaffe and Saxel, 1977) were maintained in DMEM supplemented with 20% fetal calf serum (GM) and 1% penicillin/streptomycin. Myogenic differentiation (Andres and Walsh, 1996) into myotubes was induced by changing subconfluent cells to DMEM containing 2% heat-inactivated horse serum (DM).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cells were collected at different days of differentiation and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR for microRNA was performed using Ncode miRNA first-strand cDNA synthesis and qRT-PCR kit (Invitrogen). cDNA synthesis for mRNA detection was carried out using SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). mRNA was detected by quantitative (q)PCR using SYBR Green PCR master mix in an ABI cycler and quantified with ABI 7300 software (Applied Biosystems, Foster City, CA).

Luciferase Reporter Assays

Human and mouse Cdc25A 3′ untranslated regions (UTRs) (41-1000 bp and 51-1369 bp, respectively) were amplified from cDNA and cloned into modified pRL-CMV (MCS) described previously (Kim *et al*., 2006) by using EcoRI and XhoI restriction sites. U2OS cells were transfected with microRNAs using RNAiMAX transfection reagent (Invitrogen). After 48 h, luciferase plasmids were transfected using Lipofectamine 2000 (Invitrogen), pGL3 control vector (Promega, Madison, WI) was transfected as internal control. At 24 h after plasmid transfection, luciferase assays were performed with Dual-Luciferase Reporter Assay system (Promega) per the manufacturer's instructions. Luminescent signal was quantified by luminometer (Monolight 3020; BD Biosciences, San Jose, CA). Each value from *Renilla* luciferase construct (Rr) was first normalized to the firefly (*Photinus pyralis*) luciferase assay value (Pp) from the cotransfected pGL3-control vector (Promega). Each Rr/Pp value in the microRNA transfections was again normalized to the Rr/Pp value obtained in control small interfering RNA (siRNA)-transfected cells.

Plasmid Construction

Cdk2-wild type (WT) and cdk2-AF were subcloned to pMSCV retroviral vector using EcoRI/NotI site; Cdc25A and mutants were subcloned to either pLPCX or pMSCV retroviral vectors using BamHI/NotI site. Retrovirus was made in HEK-293T cells cotransfected with virus packaging plasmids.

Plasmid, siRNA, and microRNA Mimic and Antisense Transfection

Plasmids were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) siRNA, and microRNA mimics were transfected into U2OS or HCT116 $^{p21-/-}$ using RNAiMAX (Invitrogen) as per manufacturer's protocol. The antisense oligonucleotides (locked nucleic acid or 2′*O*-methyl) were transfected into C2C12 at 0 and 48 h in serum-containing medium. At 72 h cells were differentiated by serum depletion (day 0 in Figures 1C or 5E).

Western Blotting and Antibodies

For Western blotting, cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, and 10% glycerol) supplemented with protease inhibitor mix (Sigma-Aldrich, St. Louis, MO). Unless otherwise mentioned, 30 μg of protein was resolved by SDS-polyacrylamide gel electrophoresis, transferred, and immunoblotted with various antibodies. The antibodies used were anti-p21 (C-19), anti-p27 (C-19), anti-Cdk2 (M2), anti-cyclin E (HE111), anti-cyclin A (H-432), anti-Cdc25A (F-6), anti-Wee1 (H-300) (all from Santa Cruz Biotechnology, Santa Cruz, CA); and anti-Cdc2-phospho-Tyr15 (Cell Signaling Technology, Danvers, MA). Mouse monoclonal antibody anti-myosin heavy chain (MHC), anti-myogenin, and anti-β-actin antibody were from Sigma-Aldrich.

Fluorescence-activated Cell Sorting (FACS) Analysis

Cells were harvested by trypsinization and then fixed with 70% ethanol for 24 h at 4°C. Fixed cells were stained in 1 ml of propidium iodide solution (0.05% NP-40, 50 μg/ml propidium iodide, and 10 μg/ml RNase A) at least for 2 h at 4°C. Stained cells were analyzed with a flow cytometer using CellQuest software (both from BD Biosciences).

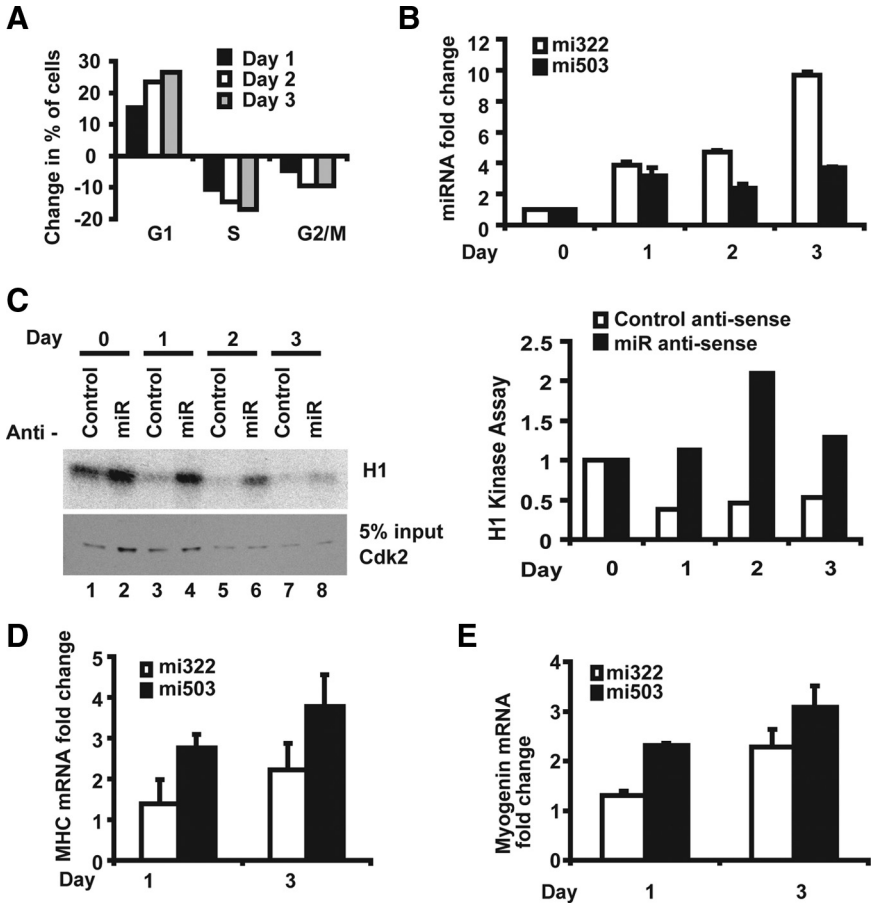

Figure 1. miR-322 and -503 are induced during the differentiation of C2C12 myoblasts, required for optimal inhibition of cdk2 kinase and accelerate expression of myogemic markers. (A) Increase in G1 population cells during differentiation. Days of serum deprivation are indicated. The results are expressed as percentage of change of cells in a given phase of the cell cycle in differentiating cells relative to undifferentiated C2C12. The primary FACS results are in Supplemental Figure S1. (B) Quantitative RT-PCR of the miR-322 and -503 during differentiation of C2C12 myoblasts. Days of serum deprivation are indicated. Fold changes of these microRNAs, normalized to U6 sRNA, are expressed relative to day 0 of serum depletion. (C) Left, total cdk2 was immunoprecipitated by anti-cdk2 antibody from C2C12 cells transfected with locked nucleic acid antisense to miR-322 and -503, and cdk2 kinase activity measured in vitro on histone H1. Autoradiogram of phospho-H1 is shown. Western for cdk2 shows amounts of the kinase in the input lysates. Day 0: 72 h after first anti-sense transfection and harvested before adding differentiation medium. Days of differentiation are indicated. Right, quantification of the H1 kinase activity (normalized to cdk2 quantity) is shown. The results are expressed relative to normalized kinase activity on day 0. (D and E) Transfection of miR-322 and -503 accelerate expression of myogenic markers MHC and myogenin. Fold change in the expression of MHC (D) and myogenin (E) mRNA compared with control siRNA-treated cells are shown. Days of differentiation are indicated.

Immunoprecipitation and cdk2 Kinase Assays

For total cdk2, cyclin E- or cyclin A-associated kinase assays 100 μg of protein was immunoprecipitated and pulled down on G-Sepharose beads. The beads were washed three times with lysis buffer and twice with kinase buffer (50 mM HEPES-NaOH, pH 7.4, and 25 mM $MgCl_2$). The beads were then incubated with 25 μl of kinase reaction mixture (50 mM HEPES-NaOH, pH 7.4, 25 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 2 μg of histone H1) at 30°C for 30 min. The reaction was stopped by the addition of 12.5 μl of 3× SDS sample buffer. Samples were resolved by SDS-PAGE, and the gel was dried before autoradiography.

RESULTS

MiR-322 and -503 Induced during Muscle Differentiation Are Required for cdk2 Inhibition

On depletion of serum, proliferating mouse C2C12 myoblasts stop proliferation and differentiate in 1–3 d into myotubes that are positive for MHC and myogenin (Kim *et al*., 2006). Propidium iodide staining of nuclei for DNA content and flow cytometry for the distribution of cells in different phases of the cell cycle revealed that the G1 population increased and the S and G2/M population decreased concurrently (Figure 1A and Supplemental Figure S1A). Quantitative RT-PCR was used to screen twenty microRNAs known to have cell cycle targets in these differentiating myoblasts. The screen revealed that two microRNAs, miR-322 and miR-503, were increased during this differentiation process (Figure 1B).

The cdk2 kinase activity of C2C12 cells undergoing differentiation was followed by immunoprecipitating cdk2 and assaying kinase activity on histone H1 (Figure 1C, left). In control antisense-transfected cells (same as untransfected cells), the H1 kinase activity declined rapidly by 1–2 d of differentiation. To ascertain whether miR-322 and -503 had any role in the cdk2 repression, in parallel experiments we transfected C2C12 cells with locked nucleic acid (LNA) antisense oligonucleotides to miR-322 and -503. The anti-microRNA oligonucleotides (LNA) caused the kinase activity of cdk2 to persist up to day 3 of the differentiation (compare lanes 3 and 4, 5 and 6, and 7 and 8). Quantification of this Western blot (Figure 1C, left) revealed that H1 kinase activity (normalized to input) was higher in cells with anti-microRNA oligonucleotides in differentiation medium (Figure 1C, right). We thus hypothesized that the induction of miR-322 and -503 contributed to the cdk2 inhibition during myotube differentiation.

We next tested whether these microRNAs accelerated muscle differentiation. Transfection of synthetic RNA duplexes mimicking the miR-322 and -503 in C2C12 cells indeed accelerated myogenic differentiation as determined by the induction of MHC and myogenin mRNA (Figure 1, D and E; and Supplemental Figure S2). Reciprocally inhibition of miR-322 and -503 using 2-*O*′-methyl antisense oligonucleotides decreased expression of myogenin and MHC mRNA (Supplemental Figure S3).

MiR-322/424 and -503 Promote G1 Arrest in Osteosarcoma Cells

To determine whether these microRNAs could induce a G1 block in another cell lineage, we transfected synthetic RNA duplexes mimicking the microRNAs in rapidly proliferating cell cycle-asynchronous human U2OS osteosarcoma cells.

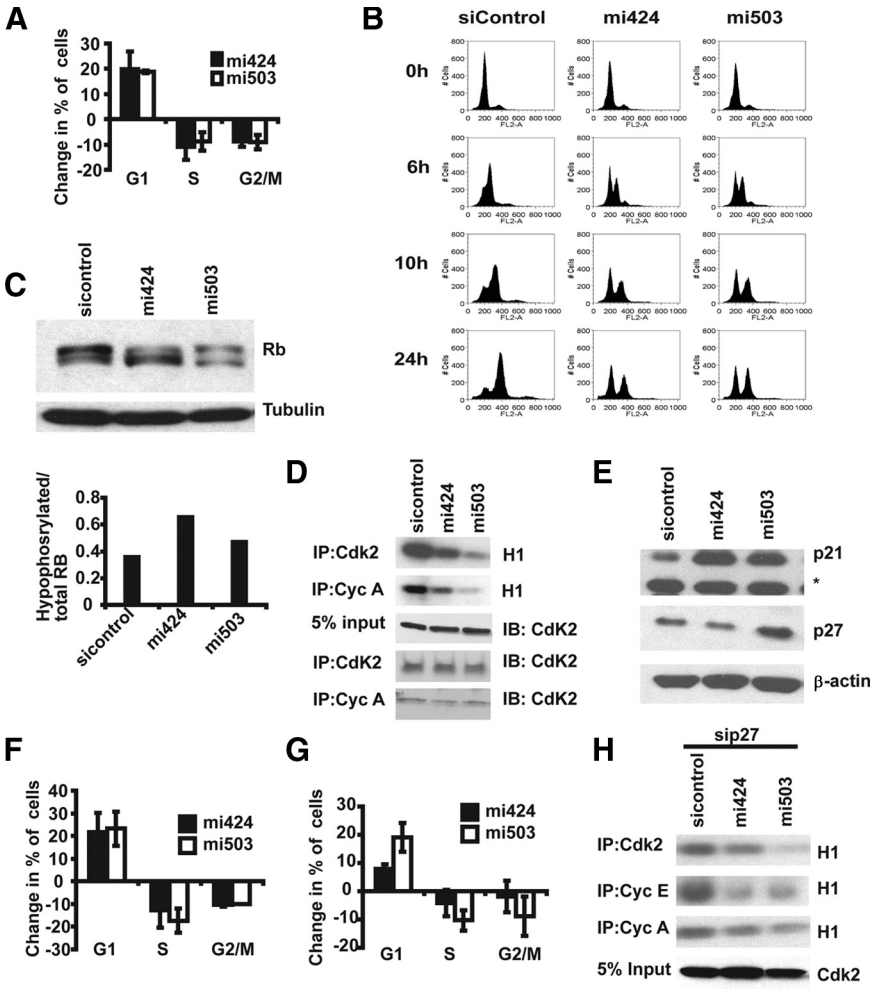

Figure 2. The G1 accumulation and cdk2 inhibition upon miR-424 or miR-503 transfection in U2OS and HCT116 cells are independent of p21 and p27. (A) Change in cell cycle stage after miR-424 and -503 were transfected in U2OS cells. DNA contents were measured after 72 h, and the percentage of change in microRNA-treated cells relative to control siRNA treated cells was plotted. Mean ± SD of at least three independent experiments. Primary FACS results are in Supplemental Figure S1. (B) U2OS cells collected in mitosis by nocodazole and shake-off were put into fresh medium, transfected with control or microRNA mimic and aphidicolin added to the medium to hold cells in G1/S. Twenty four hours later, cells were released from aphidicolin block and placed in nocodazole to trap cells moving from S phase to mitosis. FACS analysis for DNA content at indicated time points after release from aphidicolin. (C) Top, protein lysate were extracted from U2OS cells 72 h after transfection of sicontrol (GL2), miR-424, and -503. Western blot analysis for anti-Rb shows hypophosphorylated (faster migrating) Rb protein in cells transfected with the microRNAs. Bottom, quantification of the hypophosphorylated Rb normalized to total Rb. (D) Total and cyclin A-associated cdk2 were immunoprecipitated (IP), and kinase assays performed as mentioned in *Materials and Methods* (H1: autoradiogram of phospho-H1). Five percent input and corresponding IP for cdk2 or cyclin A were immunoblotted for cdk2 as loading controls. (E) Immunoblot with indicated antibodies of lysates extracted from U2OS cells transfected with indicated RNA duplexes. Loading control, nonspecific band (*) in the anti-p21 blot and β-actin in the anti-p27 blot. (F) Change in percentage of HCT116 $^{p21-/-}$ cells in different phases of cell cycle upon overexpression of miR-424 or -503 relative to control RNA duplex. Mean ± SD of at least three independent experiments. Primary FACS results are in Supplemental Figure S1. (G) Same as in F except that p27 was initially depleted by si-p27 (see Supplemental Figure S1D) before transfecting miR-424 and -503. Primary FACS results are in Supplemental Figure S1. (H) Cdk2 kinase activity of HCT116 $^{p21-/-}$ cells depleted of p27 and overexpressing miR-424 or -503.

Transfection of miR-424 and miR-503 independently increased the G1 and decreased the S and G2/M population of cells (Figure 2A and Supplemental Figure S1B).

Unless mentioned specifically, we transfected 75 nM of miR-322/424 or 25 nM of miR-503. Quantitative RT-PCR showed similar levels of miR-322 and -503 in C2C12 cells on day 3 of differentiation and in microRNA transfected U2OS cells (Supplemental Figure S4).

To further establish whether these microRNAs caused a G1 accumulation, U2OS cells were synchronized in mitosis by nocodazole block and shake off and then transfected with either negative control GL2 duplex or the microRNA mimics as the cells transited through G1 and accumulated at the G1/S transition because of exposure to aphidicolin, an inhibitor of the replicative DNA polymerase. After giving the microRNAs ~18 h to exert their effect, the cells were released from the aphidicolin block (0 h) into a new nocodazole block, and their progression through S phase and G2 was followed by propidium iodide FACS (Figure 2B). Ninety percent of the cells transfected with the negative control duplex moved through S and G2 and accumulated in mitosis with a G2 content of DNA at 10–24 h after release from aphidicolin. In contrast, nearly 50% of the cells transfected with miR-424 or miR-503 remained stuck at the G1-S boundary. Thus, the two microRNAs block the progression of cells into S phase of the cell cycle.

We then focused on the cell cycle proteins that are important for progression from G1 to S. Rb is hyperphosphorylated and migrates in SDS-PAGE as a slower moving band upon phosphorylation by cdk2 at the G1–S transition. Consistent with miR-424 and -503 inhibiting this transition, a faster moving, hypophosphorylated Rb band was visible in the U2OS cells transfected with these microRNAs (Figure 2C, top). This hypophosphorylation was more pronounced in miR-424– and -503–transfected lanes compared with sicontrol lane as determined by quantification of this Western blot (Figure 2C, bottom). When cell lysates were immunoprecipitated by antibodies to cdk2 or cyclin A, although equal amounts of cdk2 protein were precipitated from all three lysates, the kinase activity in vitro on histone H1 was significantly attenuated in miR-424– or -503–transfected cells (Figure 2D). Cyclin A level went down in these samples (Supplemental Figure S5). Therefore, to immunoprecipitate equal amount of Cyclin A we used more cell lysate in miR-424– and -503–treated samples compared with sicontrol-treated sample. Thus, transfection of either microRNA

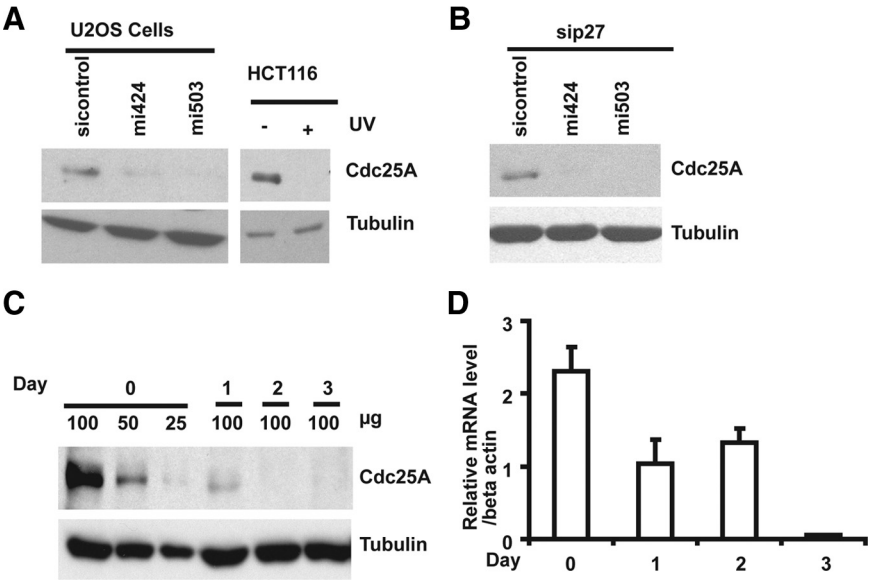

Figure 3. miR-424 and miR-503 down-regulate Cdc25A. (A) Western blot for Cdc25A and tubulin (loading control) on lysate from U2OS cells transfected with indicated RNA duplexes. Right, HCT116 cells were treated with UV, and the degradation of Cdc25A used to indicate specificity of the immunoblot signal. (B) Same as in A except HCT116 $^{p21-/-}$ cells were depleted of p27 protein by siRNA against p27 and indicated RNA duplexes transfected. (C) Western blot analysis of Cdc25A protein in C2C12 cells at indicated times after serum deprivation. Anti-tubulin is shown as loading control. Dilutions of the day 0 extract are loaded in the first three lanes to show the signal expected when Cdc25A protein is 50% the level in day 0. (D) Real-time qRT-PCR of mRNA at indicated times upon differentiation. Cdc25A mRNA level was plotted after normalizing to that of β-actin.

inhibits cdk2 kinase activity, perhaps suggesting how they might interfere with G1–S transition.

Cdk2 Inhibitors Are Induced but Dispensable for the G1 Arrest

Transfection of miR-424 or -503 into U2OS cells induced the cdk inhibitors p21 and p27 (Figure 2E). However, because degradation of both the inhibitors is dependent on active cdk2 kinase, their induction could be secondary to cdk2 inhibition from a different proximate cause. Indeed, even after double knockdown of p21 and p27 in U2OS cells, transfection of miR-424- or of -503 still caused an accumulation of the cells in the G1 phase of the cell cycle and inhibition of cdk2 kinase activity (Supplemental Figures S1K and S6, A–C).

To independently examine whether p21 or p27 were necessary for the G1 arrest by miR-424 or -503, we turned to HCT116 colon cancer cells that had been engineered by homologous recombination to be null for the p21 locus (HCT116 $^{p21-/-}$) (Waldman *et al*., 1995). Mir-424 or -503 transfection increased the G1 population and decreased the post-G1 population of cell (Figure 2F and Supplemental Figure S1C). We repeated the experiment after knocking down p27 by siRNA (Supplemental Figure S6D). Despite the fact that p21 was absent and p27 had been virtually eliminated, transfection of miR-424 or -503 still increased the G1 population and decreased the percentage of cells in the later stages of the cell cycle (Figure 2G and Supplemental Figure S1D). In vitro kinase assays with immunoprecipitates of cdk2 or of cyclin E or A revealed that the microRNAs continued to decrease cdk2 kinase activity despite the absence of p21 and decrease of p27 (Figure 2H). We concluded that cdk2 kinase was being inhibited by the microRNAs by a mechanism independent of the cdk2 inhibitors.

Cdc25A Is Repressed by miR-424 and -503 in Human Cancer Cell Lines

The microRNA target prediction algorithms suggested that cyclins D and E and Cdc25A are potential targets of these two microRNAs. Levels of cyclin D or E were unchanged in the cells transfected with the microRNAs (data not shown). In contrast, immunoblots of extracts of U2OS cells show that transfection of miR-424 or -503 represses the Cdc25A protein and mRNA levels (Figure 3A and Supplemental Figure S7). Identical results were obtained in HCT116 $^{p21-/-}$ cells with a knockdown of p27 (Figure 3B). These results are consistent with the notion that the microRNAs target Cdc25A.

Cdc25A Protein Is Decreased Posttranscriptionally during Differentiation of C2C12 Myoblasts

When C2C12 cells are induced to differentiate by serum depletion, Cdc25A protein decreased to levels below detection within 2 d (Figure 3C). Interestingly, qRT-PCR of mRNA reveals that Cdc25A mRNA declines in a more gradual manner, with a 50% reduction on day 1 that is then repressed even further on day 3 of serum depletion (Figure 3D). The faster kinetics of repression of Cdc25A protein relative to that of the RNA suggests that posttranscriptional mechanisms are at play in inhibiting Cdc25A during muscle differentiation. This, too, is consistent with the idea that muscle differentiation-induced microRNAs may target Cdc25A during differentiation.

Cdc25A Is a Direct Target of the Two MicroRNAs

The 3′UTR of both human and mouse Cdc25A had sequences that could anneal to the seed sequences of miR-424/322 or -503 (Figure 4A). A luciferase reporter plasmid with the 3′UTR of human Cdc25A was repressed by cotransfection of miR-503 (Figure 4B) or miR-424 (Figure 4C). Mutation of both the putative target sequences for miR-503 attenuated the repression by the microRNA (Figure 4B), demonstrating that we have identified the relevant target sites for this microRNA. In contrast, mutation of the putative target site for miR-424 did not reprieve the repression (Figure 4C), suggesting that miR-424 may act through a nonseed matching sequence in the 3′UTR that we have not yet identified. Such nonseed match mediated repression of targets by microRNAs has been described previously (Lal *et al*., 2009).

The results were reinforced by analogous effects of miR-503 and miR-322 on a luciferase reporter with the 3′UTR of mouse Cdc25A (Figure 4, D and E). In both these cases, mutation of the putative target sites attenuated the repression by the microRNA. Despite the extensive variation in the sequences of the 3′UTRs of human and mouse Cdc25A, the

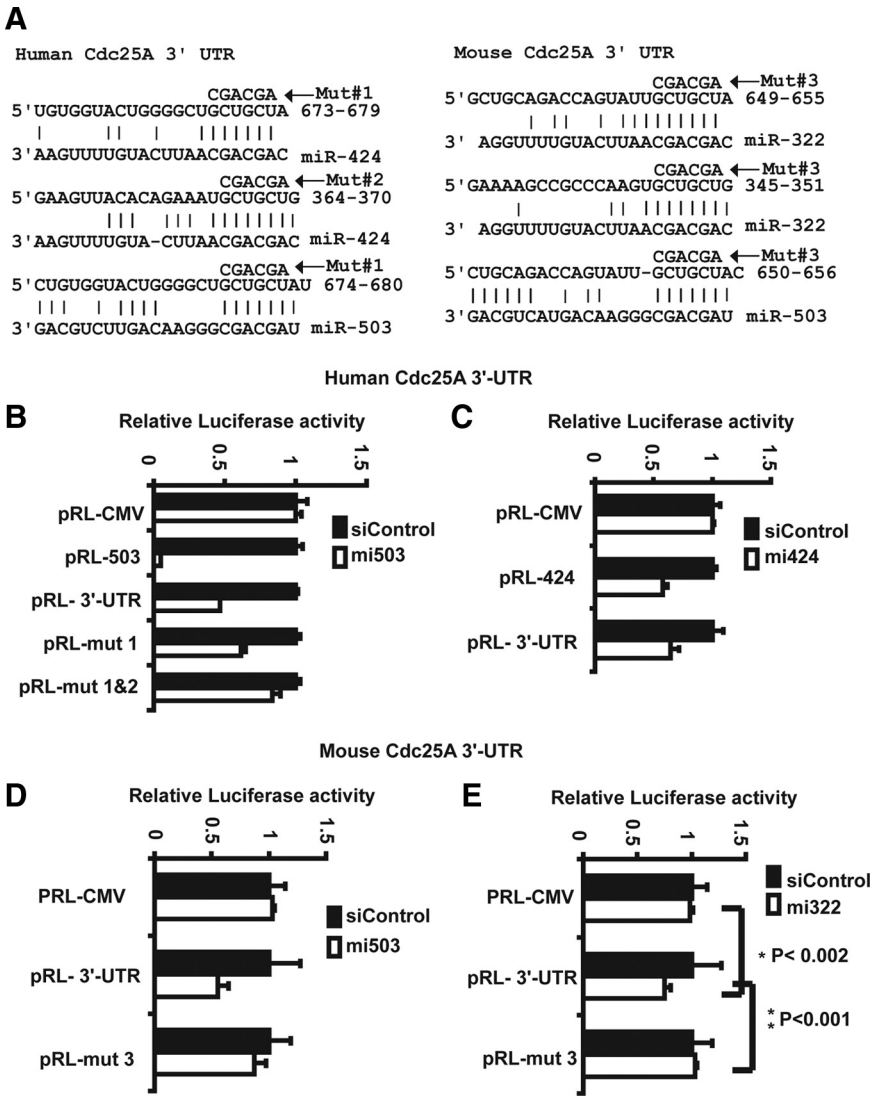

Figure 4. miR-424 and -503 directly target Cdc25A. (A) Two predicted target sites of miR-424/322 and -503 in the 3′UTR of human and mouse Cdc25A. Mutant 1, mutant 2 (in human Cdc25A) and mutant 3 (in mouse Cdc25A) are indicated. (B–E) Luciferase assays were performed to measure the effect of miR-424/322 and -503 on a *Renilla* luciferase reporter fused to a perfect match to the microRNA (pRL-503 or pRL-424) or to human or mouse Cdc25A 3′UTR (see *Materials and Methods*). The results are expressed as *Renilla* luciferase activity relative to the firefly luciferase expressed from a cotransfected plasmid and serving as a transfection control. The ratio seen in control duplex transfected cells is 1. (B) Luciferase with human Cdc25A 3′UTR repressed by miR-503 mimic. (C) Same reporter as in B repressed by miR-424 mimic. (D) Luciferase with mouse Cdc25A 3′UTR repressed by miR-503 mimic. (E) Same as in D, except with miR-322 mimic. Because the differences were small in E, the p values in a Fisher's test are shown to indicate statistical significance.

evolutionary conservation of repression by both the microRNAs suggested that repression of Cdc25A by these microRNAs could be developmentally important.

Overexpression of Cdc25A Delays Differentiation but Also Reveals That the Protein Is Degraded by Proteasomes during Differentiation

To test whether the decrease in Cdc25A is important for the normal kinetics of differentiation of C2C12 myoblasts to myotubes, we established C2C12 derivatives infected with either an empty retroviral vector (vector) or a retrovirus overexpressing Cdc25A from its open reading frame alone. The latter construct is missing the 3′UTR of Cdc25A. When these cells were induced to differentiate by serum depletion, the appearance of myogenin, a differentiation marker, was significantly delayed in the C2C12 cells overexpressing Cdc25A (Figure 5A). This result is consistent with the hypothesis that repression of Cdc25A is an important element of the differentiation program.

Immunoblots of the C2C12 cells indicated that, consistent with Figure 3C, the endogenous Cdc25A protein decreased by day 1 of serum depletion (Figure 5B). The overexpressed exogenous Cdc25A persisted until day 3 of differentiation. Surprisingly, however, although the exogenous Cdc25A was expressed from a heterologous promoter and did not have a 3′UTR that could be targeted by muscle-differentiation-induced microRNAs, the protein still decreased during differentiation. Cdc25A is ubiquitinylated by multiple pathways and is targeted for proteasome-mediated proteolysis following DNA damage. This made us wonder whether proteasome-mediated degradation was also important for Cdc25A decrease during differentiation. Indeed, the addition of MG132, an inhibitor of proteasomes, increased both endogenous and exogenous Cdc25A in C2C12 myoblasts (Figure 5C). When the myoblasts were induced to differentiate by serum depletion, it was interesting to note that the endogenous Cdc25A protein was undetectable even after MG132 treatment after day 2 of differentiation, suggesting that nonproteasome mediated pathways (such as microRNAs and transcriptional repression) come into effect to keep Cdc25A repressed during differentiation. The proteasome-mediated degradation, however, continues to be active, because the exogenous Cdc25A protein is readily induced even after 3 d of differentiation when the proteasomes are inhibited by MG132.

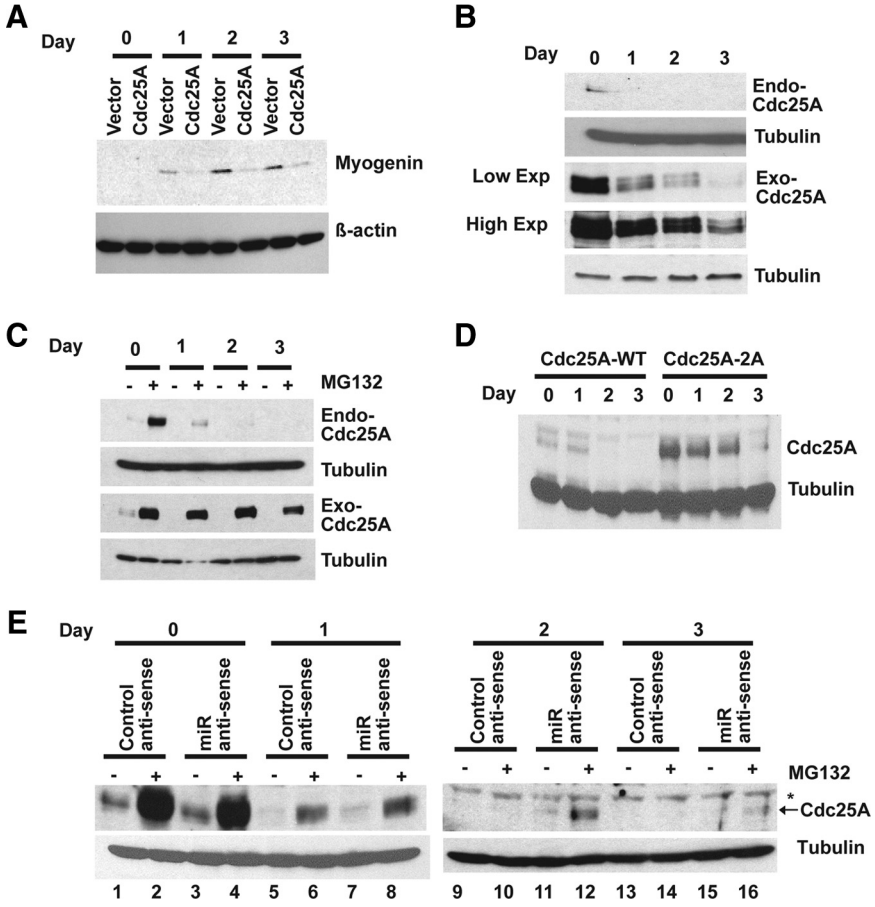

Figure 5. Ectopic expression of Cdc25A delays differentiation, although Cdc25A protein is still degraded in a proteasome-dependent manner. (A) C2C12 cells stably infected with either empty retroviral vector or retrovirus expressing Cdc25A were differentiated and assayed for induction of myogenin, a differentiation marker on indicated days after serum deprivation. Anti-actin is shown as loading control. (B) Lysates from the experiment in A were immunoblotted for anti-Cdc25A. 100 μg of total protein from vector-infected cells (endogenous Cdc25A) and 30 μg from Cdc25A-overexpressing cells (exogenous Cdc25A) were resolved. (C) Cdc25A overexpressing C2C12 cells were differentiated by serum deprivation. Cells were treated with or without proteasome inhibitor MG-132 (5 μg/ml) for 4 h before harvest at indicated times. Cells were lysed and immunoblotted for anti-Cdc25A. The rest are as described in B. (D) Cdc25A-WT and Cdc25A-2A were stably expressed in C2C12 cells, which were then differentiated, harvested at indicated times, lysed, and immunoblotted from Cdc25A. (B–D) Anti-tubulin blots serve as loading control. (E) C2C12 myoblasts were preloaded with 2′-*O*-methyl oligonucleotide antisense to either control RNA (luciferase) or a mixture of antisense to miR-322 and -503 and then induced to differentiate. Day 0, first day of serum deprivation, which is 72 h after first antisense transfection. Cells were treated with MG-132 as described in C, and 100 μg of cell lysate was immunoblotted for Cdc25A and tubulin (loading control).

S82 in the DSG motif of Cdc25A is phosphorylated by Chk1 to promote its degradation by proteasomes after DNA damage (Kanemori *et al*., 2005). This site and a related DDG motif are recognized by the βTRCP subunit of CRL1$^{\beta TRCP}$ for polyubiquitination. To test whether these two sites were relevant for Cdc25A degradation during muscle differentiation, we used C2C12 cells overexpressing wild-type Cdc25A or Cdc25A-2A mutated at the two sites (S82A and D220A) (Figure 5D, which is a much lighter exposure than Figure 5B). Compared with the wild-type exogenous Cdc25A, the mutant exogenous protein was more stable during the earlier stages of differentiation, but still disappeared on day 3 of the differentiation regime. MG132 treatment revealed that the disappearance of Cdc25A-2A on day 3 was still dependent on proteasomes (data not shown), suggesting that another ubiquitylation pathway becomes active after three days of serum depletion to target Cdc25A protein for degradation.

Antisense to miR-322 and -503 Sustains Cdc25A Levels in Differentiating C2C12 Myoblasts

We next tested whether antisense to the two microRNAs will slow down the rate of disappearance of Cdc25A during differentiation. A control antisense 2′*O*-methyl oligonucleotide or a mixture of antisense to miR-322 and -503 was transfected into C2C12 myoblasts, which were then induced to differentiate by serum depletion (Figure 5E). Because Cdc25A is also targeted by proteasomes during differentiation, we neutralized the proteasome activity by treating the cells for 4 h with MG132 before harvest. On days 0 and 1, the anti-miR-322 and -503 did not seem to increase Cdc25A protein level relative to control anti-sense RNA even in the presence of MG132. On days 2 and 3 of differentiation (compare lane 10–12 and 14–16), however, transfection of the anti-miR oligonucleotides caused a detectable persistence of Cdc25A protein relative to the control oligonucleotide when the proteasomes were inhibited by MG132. Note that transfection of antisense to 2′-*O*-methyl oligonucleotides to microRNAs is an imperfect tool for knocking down microRNA activity.

Thus, despite the fact that Cdc25A (and most likely other cdk2 regulators) are regulated by multiple microRNA- and nonmicroRNA-dependent pathways, neutralization of miR-322 and -503 by imperfect tools showed lesser repression of Cdc25A (Figure 5E) and greater persistence of cdk2 kinase activity (Figure 1C) in the differentiating myoblasts.

Inhibitory Phosphorylation on cdk2 T14 and Y15 Is Important for the Differentiation of C2C12 Myoblasts

We next investigated whether Y15 on cdk2 is phosphorylated in the myoblasts. After immunoprecipitating with anti-cdk-phosphotyrosine15 antibody, immunoblot with anti-cdk2 revealed a weak but detectable phosphotyrosine signal on endogenous cdk2 in lysates of C2C12 on days 0, 1, and 2 of serum depletion (Figure 6A, top). Reasoning that the signal was weak because of rate-limiting quantities of cdk2, we overexpressed exogenous FLAG-tagged wild-type cdk2 in C2C12 and then measured phosphoY15 levels in these cells as they differentiated. A clear phosphotyrosine signal was detected on cdk2 at days 0, 1, and 2 of differentiation. Cdk2 paired with a cyclin is the preferential substrate of the

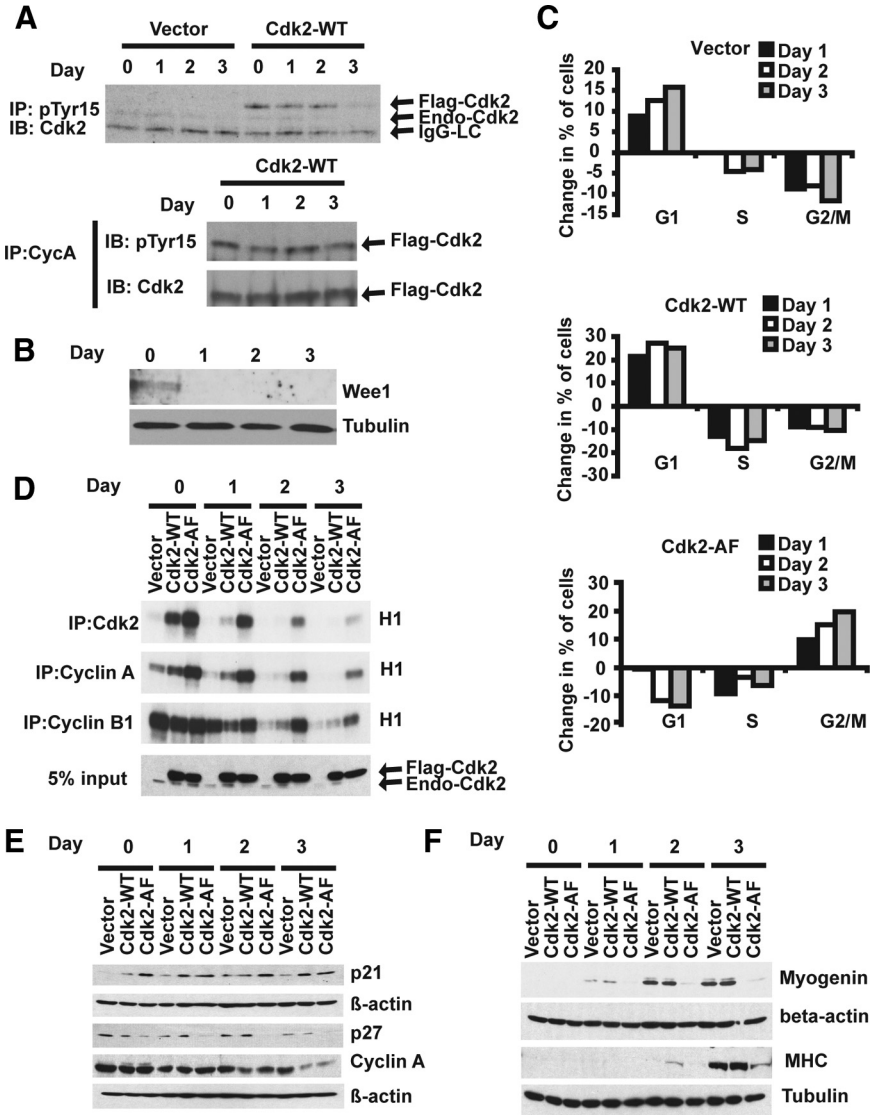

Figure 6. Cdk2-AF mutation delays differentiation of C2C12 cells. (A) Cdk2 is phosphorylated on tyrosine in differentiating C2C12 cells. C2C12 cells infected with empty retroviral vector or retrovirus expressing FLAG-tagged cdk2-WT were differentiated. Top, cell lysates at indicated times were immunoprecipitated with anti-cdc2-phosphotyrosine15 antibody and immunoblotted for cdk2. Bottom, cell lysates were immunoprecipitated with anti-cyclin A antibody and the tyr15 phosphorylation of cyclin A-associated cdk2 assessed as in the top panel. (B) Western blot analysis of Wee1 protein in C2C12 cells at indicated times after serum deprivation. Anti-tubulin serves as loading control. (C) Overexpression of cdk2-AF mutant represses G1 block in differentiating C2C12 cells. C2C12 cells with empty vector (top) or overexpressing cdk2-WT (middle) or cdk2-AF (bottom) were induced to differentiate. The rest are as described in Figure 1A. Primary FACS results are in Supplemental Figure S1. (D) Cell lysates from C were immunoprecipitated with indicated antibodies, and in vitro histone H1-kinase activities were measured. The phospho-H1 autoradiogram is shown in the top three panels, whereas 5% of input lysate immunoblotted with anti-cdk2 antibody is shown in the bottom panels. (E) Western blot analyses were performed for p21, p27, and cyclin A on the same cell lysates described in C and D. (F) Cell lysates from C were immunoblotted for differentiation markers myogenin and MHC. β-Actin and tubulin serve as loading controls.

Wee1 kinase that phosphorylates cdk2 on Y15. The disappearance of the phosphoY15 signal at day 3 made us wonder whether this was due to a decrease in partner cyclins that are transcriptionally down-regulated during differentiation. Indeed, when we immunoprecipitated cyclin A from the C2C12 cells and exclusively examined the cdk2 that was associated with cyclin A, the phosphorylation on tyrosine was discernible on all the days examined (Figure 6A, bottom). The failure to see an increase in phospho-tyrosine during the differentiation process despite the loss of Cdc25A made us wonder whether the kinase that phosphorylates the tyrosine, Wee1, was decreasing concurrently. Indeed the Wee1 kinase decreased upon serum depletion (Figure 6B). The persistence of the inhibitory phosphorylation during the differentiation program despite the loss of Wee1, could only be explained by the decrease in Cdc25A.

Thus, the decrease in Wee1 kinase masked the increase of Y15 phosphorylation that would have been expected from Cdc25A decrease. The question then is whether the persistence of Y15 phosphorylation generated by Cdc25A loss is important for differentiation. To answer this, we created C2C12 cells stably overexpressing wild-type cdk2 (cdk2-WT) or cdk2 mutated as T14A+Y15F (cdk2-AF). Cdk2-AF will not show the persistent phosphorylation onT14/Y15 and so will not be inhibited by Cdc25A loss. For comparison, we used C2C12 cells infected with the empty retroviral vector (vector). On serum depletion, the vector-infected or the cdk2-WT–infected C2C12 cells showed an increase in G1 content and decrease in the other phases of the cell cycle (Figure 6C and Supplemental Figure S1, E–G). Overexpression of cdk2-AF, however, significantly impaired the G1 accumulation, strongly suggesting that phosphorylation of cdk2 on T14 and Y15 is essential for the cell cycle quiescence during muscle differentiation. As expected, the kinase activity of cdk2, cyclin A-associated cdks or cyclin B-associated cdks decreases rapidly upon serum depletion of vector or cdk2-WT infected C2C12 (Figure 6D). In contrast, the overexpression of cdk2-AF resulted in significant persistence of histone H1 kinase activity in all three immunoprecipitates all the way to day 3 of serum depletion. Moreover, Rb, a substrate for cdk2, remained phosphorylated in cells expressing Cdk2-AF compared to vector alone or Cdk2-WT–expressing cells upon myoblast to myotube differentiation (Supplemental Figure S8). The kinase activity persisted despite the induction of the cdk inhibitor p21 and the decrease in cyclin A (Figure 6E), showing that although other mech-

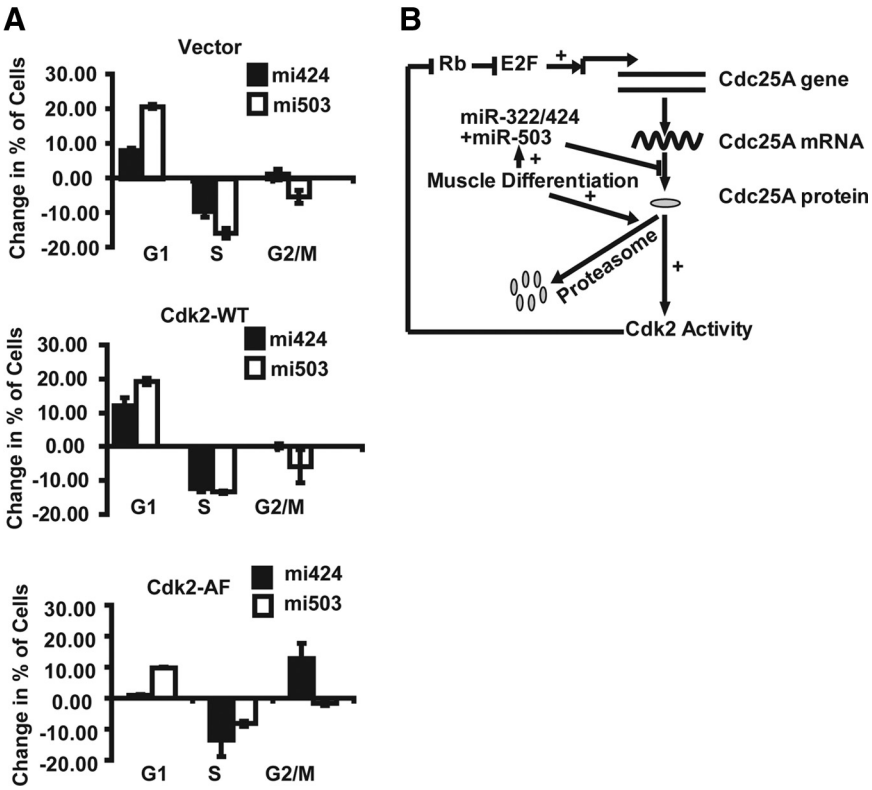

Figure 7. Accumulation of cancer cells in G1 upon transfection of miR-424 or -503 also requires phosphorylation of cdk2 on T14 and Y15; schematic of Cdc25A down-regulation during differentiation. (A) HCT116 cells expressing cdk2-AF were resistant to G1 arrest induced by miR-424 or -503. Cells stably infected with empty vector or viruses containing cdk2-WT or cdk2-AF were transfected either with control siRNA or miR-424 or si-503. The percentage of difference in miR-424 or -503–transfected cells in the indicated part of the cell cycle relative to control siRNA treated cells at 72 h is shown. Data are mean ± SD of least three independent experiments. Primary FACS results are in Supplemental Figure S1. (B) Cdc25A is down-regulated in differentiating myoblasts at the transcriptional, posttranscriptional and protein levels. The induced microRNAs miR-424 and -503 down-regulated Cdc25A protein synthesis, whereas existing protein is targeted to proteasomes by polyubiquitination-dependent pathways. The decrease in cdk2 kinase activity (which is facilitated by the concurrent induction of cdk2 inhibitors that is not shown) leads to E2F repression and repression of transcription from the Cdc25A promoter.

anisms for inhibiting the kinase were active in the differentiating C2C12 cells, phosphorylation of cdk2 on T14 and Y15 is a critical event for kinase inhibition. Thus, cdk2 has to be phosphorylated on T14 and Y15 for optimal G1 block in differentiating myoblasts.

The overexpression of cdk2-AF, but not cdk2-WT, also delayed the appearance of muscle-specific differentiation markers myogenin and MHC (Figure 6F). Consistent with the expectation that inhibition of Cdk2 will promote differentiation, acute inhibition of Cdk2 by roscovitine promotes C2C12 differentiation (Supplemental Figure S9). Thus persistent phosphorylation of cdk2 on T14 and Y15 due to the down-regulation of Cdc25A and resulting Cdk2 inhibition is important for optimal differentiation of myoblasts to myotubes.

Accumulation of Cancer Cells in G1 upon Transfection of miR-424 or -503 Also Requires Phosphorylation of cdk2 on T14 and Y15

Finally, we went back to the heterologous HCT116 colon cancer cells to test whether here, too, the G1 block created by these two microRNAs was dependent on the inhibitory phosphorylation of cdk2 on T14 and Y15. HCT116 cells infected with empty vector, or viruses overexpressing cdk2-WT or cdk2-AF were transfected with the two microRNAs, and the change in cell cycle distribution was measured relative to the transfection of a negative control siGL2 duplex (Figure 7A and Supplemental Figure S1, H–J). Both microRNAs increased the G1 population and decreased the S and G2 population of cells in the vector-infected or cdk2-WT–overexpressing C2C12. In contrast, overexpression of cdk2-AF diminished the G1 accumulation after transfection of miR-424 or -503. Thus, in the heterologous system, too, phosphorylation of cdk2 on T14 and Y15 is important for the G1 accumulation induced by the two microRNAs, consistent with our suggestion that Cdc25A is an important target for these microRNAs for their effect on the cell cycle.

DISCUSSION

We report that repression of Cdc25A, a key cell cycle regulator important for the activation of the cell cycle-dependent kinases (Cdks), is important for myogenic differentiation. It has been known for some time that Cdc25A is important for the intra-S phase checkpoint response after genotoxic stress, which results in the phosphorylation of and degradation of Cdc25A and subsequent inactivation of cdk2 kinase (Mailand *et al*., 2000). In mammals, there are three genes coding for the Cdc25A, B, and C proteins. Cdc25A acts during G1 phase and G1/S transition by activating cyclin E/cdk2 and cyclin A/cdk2, a function not shared by Cdc25B or C (Fernandez-Vidal *et al*., 2008). Cdk2 kinase is inhibited by phosphorylation of thr14/tyr15 of cdk2 by Wee1/Myt1 kinase (Krek and Nigg, 1991; Gu *et al*., 1992; Morgan, 1995), which is reversed by Cdc25A phosphatase. Cdk2 kinase is also inhibited by binding to Cip/Kip family of small inhibitor proteins $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (Mainprize *et al*., 2001). The role of cdk2 inhibitory proteins in induction of muscle differentiation has been well studied (Hawke *et al*., 2003; Myers *et al*., 2004). Our results suggest that inhibitory phosphorylation of T14 and Y15 on cdk2 plays an additional role during the differentiation of C2C12 myoblasts to myotubes. The delayed differentiation seen after overexpression of cdk2 AF mutant or of Cdc25A highlights the importance of Cdc25A in this process.

The mechanism of Cdc25A repression during the differentiation process is, however, complicated and multilayered (Figure 7B). Cdc25A is repressed posttranscriptionally by microRNAs and the protein targeted for degradation by proteasomes. Cdk2 kinase activity decreases (aided by the

induction of cdk inhibitors), resulting in the hypophosphorylation of Rb and repression of E2F. Because Cdc25A is an E2F target gene (Wu *et al*., 2000), this is expected to decrease Cdc25A expression further, but now at the transcriptional level. Our data suggest that all these layers of repression of Cdc25A are active in differentiation myoblasts.

Of these mechanisms, the microRNA-mediated repression is the most novel. Many other microRNAs, such as miR-135b, -338 (Wienholds *et al*., 2005) -181 (Naguibneva *et al*., 2006) -1, -206, and -133 (Kim *et al*., 2006), are reported to be involved in the regulation of myogenesis. Our findings add miR-424 and -503 to this list. Because microRNAs play a role in diverse cellular processes such as proliferation and differentiation, it is expected that many more microRNAs will emerge to be important for myogenesis (Lee and Dutta, 2009). However, it is striking that 1) miR-424 and -503 directly repress a reporter linked to the Cdc25A 3′ UTR, and 2) antisense to the microRNAs delay the disappearance of Cdc25A during myogenesis despite the persistence of other mechanisms to repress Cdc25A.

Recent reports show that many microRNAs regulate cell cycle regulatory proteins. For example, miR-24 regulates expression of E2F2, MYC, AURKB, CCNA2, CDC2, CDK4, and FEN1 by recognizing seedless but highly complementary sequences (Lal *et al*., 2009), and miR-92b directly down-regulates levels of the G(1)/S checkpoint protein p57 (Sengupta *et al*., 2009). miR-21 negatively regulates Cdc25A and cell cycle progression in colon cancer cells (Wang *et al*., 2009). Furthermore overexpression of let-7 in A549 lung carcinoma and HepG2 cells arrest cells in G0/G1 phase of cells cycle. Microarray analysis of A549 and HepG2 cells overexpressing let-7 revealed that the cell cycle arrest was due to the repression of several cell cycle regulatory proteins, including Cdc25A (Johnson *et al*., 2007), although the authors did not explore whether Cdc25A is a direct target of let-7 or the consequence of Cdc25A repression. We show here that Cdc25A is a target of miR-424 and -503 by luciferase reporter assays (Figure 4). The conservation of the repression in mouse and human Cdc25A adds to significance of this finding.

Transfection of miR-424 and -503 to human U2OS osteosarcoma cells and HCT116 colon cancer cells promoted a G1 block, lending credence to the hypothesis that these microRNAs are important for inducing G1 block during normal muscle differentiation. Not only was the target for the microRNAs still Cdc25A in these heterologous cells, but bypassing inhibitory phosphorylation on cdk2 by the A14, F15 mutant decreased the G1 block induced by these microRNAs. We suspect that many other differentiation-induced microRNAs are likely to have an anti-cell cycle effect and could be harnessed as antiproliferative agents on cancer cells.

Although the microRNAs were important for Cdc25A repression during C2C12 differentiation, they were clearly not the only agents promoting the repression. Most notably, even when Cdc25A was expressed from a heterologous promoter Cdc25A protein levels still declined during muscle differentiation in a proteasome-dependent manner.

Although Cdc25A undergoes degradation by proteasome pathways upon DNA damage (Busino *et al*., 2003; Jin *et al*., 2003; Kanemori *et al*., 2005), such degradation has not been implicated in a differentiation pathway. Besides Chk1 kinase, a recent report suggests that never in mitosis gene A-related kinase 11 regulates CDC25A degradation by β-transducin repeat-containing protein (TRCP) during the ionizing radiation-induced G2/M checkpoint (Melixetian *et al*., 2009). The extracellular signal-regulated kinase is also reported to be involved in Cdc25A degradation (Isoda *et al*., 2009). Future work will address which of these kinases are involved in Cdc25A degradation in differentiating myoblasts. There are two sites on Cdc25A protein reported to be important for its degradation after genotoxic stress by targeting the protein to β-TRCP-containing Skp1-Cullin 1-F-box protein complex (Kanemori *et al*., 2005). Mutation of these two sites stabilizes the protein after DNA damage. Interestingly, the same mutations stabilized Cdc25A during the first 2 d of differentiation (Figure 5D, Cdc25A-2A), and yet the protein was degraded on day 3. We know that the degradation of Cdc25A-2A on day 3 of differentiation was also inhibited by MG132 (data not shown), suggesting that an alternate pathway of Cdc25A polyubiquitination becomes active on this day and promotes Cdc25A degradation even in the 2A mutant. Cdc25A is a target of the anaphase-promoting complex (APC) ubiquitin ligase in G1 phase (Donzelli *et al*., 2002) so that APC-Cdh1 may be responsible for this alternative pathway of degradation. Future work will pursue this additional pathway of Cdc25A degradation on day 3 of differentiation.

Cdc25A knockout mice exhibited early embryonic lethality (Ray *et al*., 2007). Cdc25A$^{+/-}$ mice develop normally, although the mouse embryonic fibroblasts demonstrated delayed cell cycle progression that corresponded to the decrease in Cdc25A protein. Cdk2$^{-/-}$ mice develop normally when cdk2 has been knocked out (Berthet *et al*., 2003; Ortega *et al*., 2003). Here, however, the loss of cdk2 is believed to be compensated for by other cdk genes (Sherr and Roberts, 2004). Thus, the engineered mice have not yet tested our proposal that Cdc25A repression is critical for muscle differentiation.

Another interesting finding from this article is that Wee1 kinase is decreased during differentiation, so that the importance of T14-Y15 phosphorylation has been missed because of the absence of any increase in this phosphorylation during differentiation. The approaches we took, Cdk2-AF expression or Cdc25A overexpression, are essential in such circumstances to uncover a role of persistent T14-Y15 phosphorylation in cell cycle quiescence. It will be interesting to generate transgenic mice overexpressing degradation resistant Cdc25A or Cdk2-AF to test the importance of Cdc25A down-regulation in animals.

In summary, we implicate Cdc25A repression as an important step in the G1 quiescence and myogenic differentiation of C2C12 cells. Differentiation was delayed by overexpression of constitutively active mutant of cdk2 (cdk2-AF) and Cdc25A itself. We also identify multiple mechanisms by which Cdc25A protein is repressed during the differentiation including direct targeting by microRNA-322/424 and -503. This result adds to the list of differentiation-induced microRNAs that can block cell cycle progression both in normally differentiating cells and in heterologous cancer cells. The redundancy of pathways by which Cdc25A is down-regulated during differentiation may be another factor in why the repression of this cell cycle regulator has not been recognized as being important for differentiation.

ACKNOWLEDGMENTS

Cdc25A-2A mutant constructs were kindly provided by Dr. Noriyuki Sagata (Kyushu University, Fukuoka, Japan). We thank the members of Dutta laboratory for helpful discussion and comments. This work was supported by National Institutes of Health National Institute of Arthritis and Musculoskel-

etal and Skin Diseases grant R01AR-053948 (to A. D.) and a Heart and Stroke Foundation of Canada grant (to B.K.D.).

REFERENCES

Andres, V., and Walsh, K. (1996). Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J. Cell Biol. *132*, 657–666.

Berthet, C., Aleem, E., Coppola, V., Tessarollo, L., and Kaldis, P. (2003). Cdk2 knockout mice are viable. Curr. Biol. *13*, 1775–1785.

Boutz, P. L., Chawla, G., Stoilov, P., and Black, D. L. (2007). MicroRNAs regulate the expression of the alternative splicing factor nPTB during muscle development. Genes Dev. *21*, 71–84.

Busino, L., Donzelli, M., Chiesa, M., Guardavaccaro, D., Ganoth, D., Dorrello, N. V., Hershko, A., Pagano, M., and Draetta, G. F. (2003). Degradation of Cdc25A by beta-TrCP during S phase and in response to DNA damage. Nature *426*, 87–91.

Callis, T. E., Deng, Z., Chen, J. F., and Wang, D. Z. (2008). Muscling through the microRNA world. Exp. Biol. Med. *233*, 131–138.

Chen, J. F., Callis, T. E., and Wang, D. Z. (2009). microRNAs and muscle disorders. J. Cell Sci. *122*, 13–20.

Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L., and Wang, D. Z. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat. Genet. *38*, 228–233.

Crist, C. G., Montarras, D., Pallafacchina, G., Rocancourt, D., Cumano, A., Conway, S. J., and Buckingham, M. (2009). Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. Proc. Natl. Acad. Sci. USA *106*, 13383–13387.

De Falco, G., Comes, F., and Simone, C. (2006). pRb: master of differentiation. Coupling irreversible cell cycle withdrawal with induction of muscle-specific transcription. Oncogene *25*, 5244–5249.

Donzelli, M., Squatrito, M., Ganoth, D., Hershko, A., Pagano, M., and Draetta, G. F. (2002). Dual mode of degradation of Cdc25 A phosphatase. EMBO J. *21*, 4875–4884.

Fernandez-Vidal, A., Mazars, A., and Manenti, S. (2008). CDC25A: a rebel within the CDC25 phosphatases family? Anticancer Agents Med. Chem. *8*, 825–831.

Gu, Y., Rosenblatt, J., and Morgan, D. O. (1992). Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. EMBO J. *11*, 3995–4005.

Hawke, T. J., Meeson, A. P., Jiang, N., Graham, S., Hutcheson, K., DiMaio, J. M., and Garry, D. J. (2003). p21 is essential for normal myogenic progenitor cell function in regenerating skeletal muscle. Am. J. Physiol. Cell Physiol. *285*, C1019–C1027.

Isoda, M., Kanemori, Y., Nakajo, N., Uchida, S., Yamashita, K., Ueno, H., and Sagata, N. (2009). The extracellular signal-regulated kinase-mitogen-activated protein kinase pathway phosphorylates and targets Cdc25A for SCF beta-TrCP-dependent degradation for cell cycle arrest. Mol. Biol. Cell *20*, 2186–2195.

Jin, J., Shirogane, T., Xu, L., Nalepa, G., Qin, J., Elledge, S. J., and Harper, J. W. (2003). SCFbeta-TRCP links Chk1 signaling to degradation of the Cdc25A protein phosphatase. Genes Dev. *17*, 3062–3074.

Johnson, C. D., Esquela-Kerscher, A., Stefani, G., Byrom, M., Kelnar, K., Ovcharenko, D., Wilson, M., Wang, X., Shelton, J., and Shingara, J. (2007). The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res. *67*, 7713–7722.

Kanemori, Y., Uto, K., and Sagata, N. (2005). Beta-TrCP recognizes a previously undescribed nonphosphorylated destruction motif in Cdc25A and Cdc25B phosphatases. Proc. Natl. Acad. Sci. USA *102*, 6279–6284.

Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J., and Plasterk, R. H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. Genes Dev. *15*, 2654–2659.

Khidr, L., and Chen, P. L. (2006). RB, the conductor that orchestrates life, death and differentiation. Oncogene *25*, 5210–5219.

Kim, H. K., Lee, Y. S., Sivaprasad, U., Malhotra, A., and Dutta, A. (2006). Muscle-specific microRNA miR-206 promotes muscle differentiation. J. Cell Biol. *174*, 677–687.

Krek, W., and Nigg, E. A. (1991). Differential phosphorylation of vertebrate p34cdc2 kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites. EMBO J. *10*, 305–316.

Kwon, C., Han, Z., Olson, E. N., and Srivastava, D. (2005). MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. Proc. Natl. Acad. Sci. USA *102*, 18986–18991.

Lal, A., *et al*. (2009). miR-24 Inhibits cell proliferation by targeting E2F2, MYC, and other cell-cycle genes via binding to "seedless" 3′UTR microRNA recognition elements. Mol. Cell *35*, 610–625.

Lee, Y. S., and Dutta, A. (2009). MicroRNAs in cancer. Annu. Rev. Pathol. *4*, 199–227.

Liu, N., Bezprozvannaya, S., Williams, A. H., Qi, X., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2008). microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. Genes Dev. *22*, 3242–3254.

Mailand, N., Falck, J., Lukas, C., Syljuasen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000). Rapid destruction of human Cdc25A in response to DNA damage. Science *288*, 1425–1429.

Mainprize, T. G., Taylor, M. D., Rutka, J. T., and Dirks, P. B. (2001). Cip/Kip cell-cycle inhibitors: a neuro-oncological perspective. J. Neurooncol. *51*, 205–218.

Melixetian, M., Klein, D. K., Sorensen, C. S., and Helin, K. (2009). NEK11 regulates CDC25A degradation and the IR-induced G2/M checkpoint. Nat. Cell Biol. *11*, 1247–1253.

Morgan, D. O. (1995). Principles of CDK regulation. Nature *374*, 131–134.

Myers, T. K., Andreuzza, S. E., and Franklin, D. S. (2004). p18INK4c and p27KIP1 are required for cell cycle arrest of differentiated myotubes. Exp. Cell Res. *300*, 365–378.

Naguibneva, I., Ameyar-Zazoua, M., Polesskaya, A., Ait-Si-Ali, S., Groisman, R., Souidi, M., Cuvellier, S., and Harel-Bellan, A. (2006). The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. Nat. Cell Biol. *8*, 278–284.

Nguyen, H. T., and Frasch, M. (2006). MicroRNAs in muscle differentiation: lessons from *Drosophila* and beyond. Curr. Opin. Genet. Dev. *16*, 533–539.

Nilsson, I., and Hoffmann, I. (2000). Cell cycle regulation by the Cdc25 phosphatase family. Prog. Cell Cycle Res. *4*, 107–114.

O'Rourke, J. R., Georges, S. A., Seay, H. R., Tapscott, S. J., McManus, M. T., Goldhamer, D. J., Swanson, M. S., and Harfe, B. D. (2007). Essential role for Dicer during skeletal muscle development. Dev. Biol. *311*, 359–368.

Ortega, S., Prieto, I., Odajima, J., Martin, A., Dubus, P., Sotillo, R., Barbero, J. L., Malumbres, M., and Barbacid, M. (2003). Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. Nat. Genet. *35*, 25–31.

Ray, D., Terao, Y., Nimbalkar, D., Hirai, H., Osmundson, E. C., Zou, X., Franks, R., Christov, K., and Kiyokawa, H. (2007). Hemizygous disruption of Cdc25A inhibits cellular transformation and mammary tumorigenesis in mice. Cancer Res. *67*, 6605–6611.

Rosa, A., *et al*. (2007). The interplay between the master transcription factor PU. 1 and miR-424 regulates human monocyte/macrophage differentiation. Proc. Natl. Acad. Sci. USA *104*, 19849–19854.

Sengupta, S., Nie, J., Wagner, R. J., Yang, C., Stewart, R., and Thomson, J. A. (2009). MicroRNA 92b controls the G1/S checkpoint gene p57 in human embryonic stem cells. Stem Cells *27*, 1524–1528.

Sherr, C. J., and Roberts, J. M. (2004). Living with or without cyclins and cyclin-dependent kinases. Genes Dev. *18*, 2699–2711.

Taulli, R., Bersani, F., Foglizzo, V., Linari, A., Vigna, E., Ladanyi, M., Tuschl, T., and Ponzetto, C. (2009). The muscle-specific microRNA miR-206 blocks human rhabdomyosarcoma growth in xenotransplanted mice by promoting myogenic differentiation. J. Clin. Invest. *119*, 2366–2378.

Vidal, A., and Koff, A. (2000). Cell-cycle inhibitors: three families united by a common cause. Gene *247*, 1–15.

Waldman, T., Kinzler, K. W., and Vogelstein, B. (1995). p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res. *55*, 5187–5190.

Wang, H., *et al*. (2008). NF-kappaB-YY1-miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma. Cancer Cell *14*, 369–381.

Wang, P., Zou, F., Zhang, X., Li, H., Dulak, A., Tomko, R. J., Jr., Lazo, J. S., Wang, Z., Zhang, L., and Yu, J. (2009). microRNA-21 negatively regulates Cdc25A and cell cycle progression in colon cancer cells. Cancer Res. *69*, 8157–8165.

Wienholds, E., Kloosterman, W. P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H. R., Kauppinen, S., and Plasterk, R. H. (2005). MicroRNA expression in zebrafish embryonic development. Science *309*, 310–311.

Williams, A. H., Liu, N., van Rooij, E., and Olson, E. N. (2009). MicroRNA control of muscle development and disease. Curr. Opin. Cell Biol. *21*, 461–469.

Wong, C. F., and Tellam, R. L. (2008). MicroRNA-26a targets the histone methyltransferase Enhancer of Zeste homolog 2 during myogenesis. J. Biol. Chem. *283*, 9836–9843.

Wu, L., Goodwin, E. C., Naeger, L. K., Vigo, E., Galaktionov, K., Helin, K., and DiMaio, D. (2000). E2F-Rb complexes assemble and inhibit cdc25A transcription in cervical carcinoma cells following repression of human papillomavirus oncogene expression. Mol. Cell. Biol. *20*, 7059–7067.

Yaffe, D., and Saxel, O. (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature *270*, 725–727.

Yan, D., Dong, X. D., Chen, X., Wang, L., Lu, C., Wang, J., Qu, J., and Tu, L. (2009). MicroRNA-1/206 targets c-Met and inhibits rhabdomyosarcoma development. J. Biol. Chem. *284*, 29596–29604.

Zhao, Y., Ransom, J. F., Li, A., Vedantham, V., von Drehle, M., Muth, A. N., Tsuchihashi, T., McManus, M. T., Schwartz, R. J., and Srivastava, D. (2007). Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell *129*, 303–317.