

# E2F7 regulates transcription and maturation of multiple microRNAs to restrain cell proliferation

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## ABSTRACT

**E2F transcription factors (E2F1-8) are known to coordinately regulate the expression of a plethora of target genes, including those coding for microRNAs (miRNAs), to control cell cycle progression. Recent work has described the atypical E2F factor E2F7 as a transcriptional repressor of cell cycle-related protein-coding genes. However, the contribution of E2F7 to miRNA gene expression during the cell cycle has not been defined. We have performed a genome-wide RNA sequencing analysis to identify E2F7-regulated miRNAs and show that E2F7 plays as a major role in the negative regulation of a set of miRNAs that promote cellular proliferation. We provide mechanistic evidence for an interplay between E2F7 and the canonical E2F factors E2F1-3 in the regulation of multiple miRNAs. We show that miR-25, -26a, -27b, -92a and -7 expression is controlled at the transcriptional level by the antagonistic activity of E2F7 and E2F1-3. By contrast, let-7 miRNA expression is controlled indirectly through a novel E2F/c-MYC/LIN28B axis, whereby E2F7 and E2F1-3 modulate c-MYC and LIN28B levels to impact let-7 miRNA processing and maturation. Taken together, our data uncover a new regulatory network involving transcriptional and post-transcriptional mechanisms controlled by E2F7 to restrain cell cycle progression through repression of proliferation-promoting miRNAs.**

## INTRODUCTION

Since the initial identification of E2F as the cellular factor required for activation of the E2 adenoviral promoter, the E2F family of transcription factors has expanded through

the addition of new members in mammals and through the discovery of homologs in other eukaryotes. Eight mammalian E2F family members (E2F1-8) have been identified, which orchestrate a complex gene regulatory network to ensure proper cell cycle progression, cellular differentiation and development (1,2). However, it is still unclear what the precise roles of each individual E2F member are, and how the activity of the whole E2F family is coordinated to achieve an integrated regulation of gene expression.

Canonical E2F proteins (E2F1-6) bear one DNA-binding domain (DBD) immediately followed by a dimerization domain, which mediates interaction with the dimerization partner protein (DP). This dimerization enables E2Fs to bind DNA with high affinity, and to function as transcriptional regulators (3). According to the prevailing model, transcriptional regulation by canonical E2Fs is controlled through association with the retinoblastoma (RB) family of tumor suppressor proteins (pRB, p107 and p130) in the case of E2F1-5, or with polycomb group (PcG) proteins, in the case of E2F6 (4). These associations facilitate recruitment of histone deacetylases and methyltransferases to target promoters and subsequent transcriptional repression. Disruption of repressor complexes unleashes E2F activity, thereby triggering target gene transcription (3).

By contrast to canonical E2Fs, the atypical members E2F7 and E2F8, display two tandem DBDs and lack sequences that mediate RB and DP binding (5). The mechanisms by which atypical E2Fs regulate gene expression as well as their biological roles are still unclear. Gain-of-function experiments have revealed that E2F7 and E2F8 are recruited to promoters of several E2F target genes involved in DNA replication and DNA repair, and repress E2F site-dependent transcription in a RB-independent manner (6–11). Furthermore, overexpression of either E2F7 or E2F8 disrupts cell cycle progression, suggesting that they might promote negative cell cycle control through transcriptional repression of cell cycle genes (6–11). However, knockout

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(KO) of E2F7 or E2F8 in mice has no significant effect on cell cycle progression, and a concomitant inactivation of E2F7 and E2F8 is needed to impact on cell cycle progression *in vivo* (12). This is probably due to compensatory mechanisms between both E2Fs, a common outcome in constitutive KO mouse models. Thus, the specific contribution of E2F7 and E2F8 to cell cycle control remains to be elucidated.

Significant progress in the understanding of E2F-mediated regulation of gene expression has been achieved by the finding that many microRNA-coding genes are *bona fide* E2F target genes (13–20). In line with the complex nature of the E2F pathway, many reports have uncovered an essential role for E2F-regulated microRNAs in modulating distinct cellular processes, most notably pathways involved in neoplastic transformation (21,22). Some of these E2F-regulated miRNAs, including miR-17-92, miR-106b-25, miR-15b-16-2 and miR-15a-16-1, appear to function as tumor suppressors that modulate and restrict progression through the cell cycle by limiting the expression of E2Fs themselves as well as other pathway components, thereby creating negative feedback loops (14,16,18). By contrast, there is also evidence for an oncogenic potential for some E2F-dependent miRNAs. For instance, miR-17-92 and miR-106b-25 clusters have been found to suppress the expression of anti-proliferative and pro-apoptotic genes, such as p21<sup>CIP1</sup>, pRB, p130, p57<sup>KIP2</sup>, PTEN and BIM (13,17,23–25). Given that each miRNA can regulate the expression of numerous genes, the list of genes regulated by miRNAs under E2F control is likely to include other, yet to be identified, targets.

The contribution of atypical E2F factors to miRNA expression regulation, and the effect that target miRNAs have on the biological roles mediated by E2F7 and E2F8, are still unknown. In this work, we have investigated the role of E2F7 in the regulation of miRNA-coding gene expression. We show that E2F7 is required for the timely repression of a set of miRNAs that function to promote cell proliferation. Importantly, our data uncover both transcriptional and post-transcriptional mechanisms for E2F7-mediated regulation of these miRNAs, and provide new insights to the understanding of E2F-regulated gene network.

## MATERIALS AND METHODS

### Cell culture conditions and flow cytometry

Human U2OS osteosarcoma cell line and human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). For cell synchronization in G1/S, exponentially growing cells were incubated with 4 mM hydroxyurea (HU) for 24 h and subsequently washed and cultured in complete medium. For cell synchronization at mitosis, cell cultures were incubated with thymidine (2 mM) for 18 h. Subsequently, cells were washed and cultured for an additional 20 h in fresh medium. Nocodazole (50 ng/ml) was added to the cultures for the last 16 h. Mitotic cells were collected by shaking off the plates and seeded in complete medium for subsequent analyses. To assess the cell cycle distribution, cells were fixed with chilled 70% ethanol, stained with 50 µg/ml propidium iodide (PI) and analyzed by flow

cytometry (FACSCalibur, BD). To analyze the percentage of cells in mitosis, ethanol-fixed cells were stained with an antibody recognizing Histone H3 phosphorylated on Serine 10 (p-H3) conjugated with FITC (06-570, Millipore), subsequently incubated with PI and analyzed by flow cytometry. Cell cycle distribution and mitotic index analysis was performed with Summit 4.3 software. For cell proliferation assays, cells were stained with 0.5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (MolecularProbes) in phosphate buffered saline for 15 min at 37°C, washed with complete medium for 20 min and then treated and cultured as indicated. Cells were fixed for 10 min in a solution of buffered formaldehyde (3.7%) and fluorescence was detected and analyzed by flow cytometry. Proliferation Wizard software was used to identify cells in different cellular generations and determine proliferation index, which was calculated as the sum of the cells in all generations including the parental divided by the computed number of original parent cells present at the start of the experiment.

### Transfections

Plasmid transfection was performed using XtremeGENE HD (Roche) transfection reagent following manufacturer's recommendations. Mammalian expression plasmids pRc-CMV-HA-E2F1, pRc-CMV-HA-E2F2, pRc-CMV-HA-E2F3, pCEFL-MYC and pFRT/FLAG/HA-DEST-LIN28B have been previously described (26–28). For exogenous expression of miRNAs, miRNA genes were expressed in the pMirVec vector (29). To silence endogenous expression of E2F1, E2F2, E2F3, E2F7, c-MYC and LIN28B, and to inhibit endogenous microRNA activity, cells were transfected with commercial siRNAs and with miRvana microRNA Inhibitors, respectively (Life Technologies), at a final concentration of 10 nM using Lipofectamine RNAiMAX (Life Technologies) following manufacturer's recommendations.

### RNA expression analyses

Total RNA extraction was performed with TRIzol Reagent (Life Technologies) and purified using the miRNeasy kit (Qiagen) following the manufacturer's recommendations. For small RNA-Sequencing (RNA-Seq), 2 µg of total RNA containing the small RNA fraction including miRNAs was processed using the TruSeq Small RNA Sample Preparation kit from Illumina. The resulting libraries were sequenced on the Genome Analyzer IIX with SBS TruSeq v5 reagents following manufacturer's protocols. To test for differential miRNA expression between different samples the Bioconductor DESeq package was used (30). The list of differentially expressed miRNAs produced by DESeq was further filtered to remove miRNAs with fewer than 10 reads in the different samples under comparison (31). Clustering analysis of differentially expressed miRNAs was performed with Perseus software (<http://www.perseus-framework.org/>).

Predicted targets of microRNAs were identified using the DIANA-microT-CDS miRNA target prediction server (32) and then analyzed for pathway enrichment using terms from the Reactome database (33). We used low *P*-values (*P*

< 0.001) to avoid inconsistent results due to the use of different databases or algorithms, as suggested previously (34). Gene ontology analysis was performed using the FatiScan algorithm (35).

Mature microRNA and Primary microRNA (Pri-miRNA) RT-Q-PCR analyses were performed using specific TaqMan microRNA and Pri-miRNA assays, respectively (Life Technologies) (Supplementary Table S1). For mRNA expression analysis, RNA was reverse-transcribed into cDNA with the High-Capacity cDNA RT Kit (Life Technologies) and Q-PCR was performed as described previously (36). Sequences of Q-PCR primers are listed in Supplementary Table S2.

### Protein expression analyses

For western blot analyses, cells were lysed in buffer containing 10 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.2; 1 mM EDTA; 1 mM EGTA; 150 mM NaCl; 1% NP-40 and a cocktail of protease and phosphatase inhibitors (Roche). Protein concentrations in supernatants were determined using a commercially available kit (DC Protein Assay from Bio-Rad). A total of 20 µg of protein were loaded per lane, fractionated in 8–10% sodium dodecyl sulphate-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). Antibodies against the following proteins were used: E2F7 (sc-32574, Santa Cruz), Cyclin E1 (4129, Cell Signaling), c-MYC (sc-42, Santa Cruz), LIN28B (4192, Cell Signaling), HA (MMS-101R, Covance), p-H3 (06-570, Millipore), α-Tubulin (T-9026, Sigma), β-Actin (A5441, Sigma). Immunocomplexes were visualized with horseradish peroxidase-conjugated anti-mouse, anti-goat or anti-rabbit IgG antibodies (Santa Cruz), followed by chemiluminescence detection (ECL, Amersham) with a ChemiDoc camera (Bio-Rad).

### Chromatin immunoprecipitation

Chromatin immunoprecipitations (ChIPs) and the quantification of immunoprecipitated DNA sequences by Q-PCR were performed as described previously (36). The localization of E2F motifs in E2F7-regulated miRNAs was carried out with the MotifLocator tool of the TOUCAN program (37). The search was restricted to the proximal promoter region (–1000 and +500 bp relative to the transcription start site) (38). Sequences of Q-PCR primers are listed in Supplementary Table S3. Antibodies used for ChIP analysis were: E2F1 (sc-193, Santa Cruz), E2F2 (sc-633, Santa Cruz), E2F3 (sc-878, Santa Cruz), E2F4 (sc-1082, Santa Cruz), E2F7 (sc-66870, Santa Cruz), RB (sc-50 Santa Cruz), p107 (sc-318 Santa Cruz), p130 (sc-317 Santa Cruz), MYC (sc-764 Santa Cruz), RNA polymerase II (sc-899, Santa Cruz) and SV40LT (sc-147, Santa Cruz).

### Statistical analysis

Data are presented as mean ± SD. The significance of the difference between two groups was assessed using the Student two-tailed *t*-test. A *P* < 0.05 was considered statistically significant.

## RESULTS

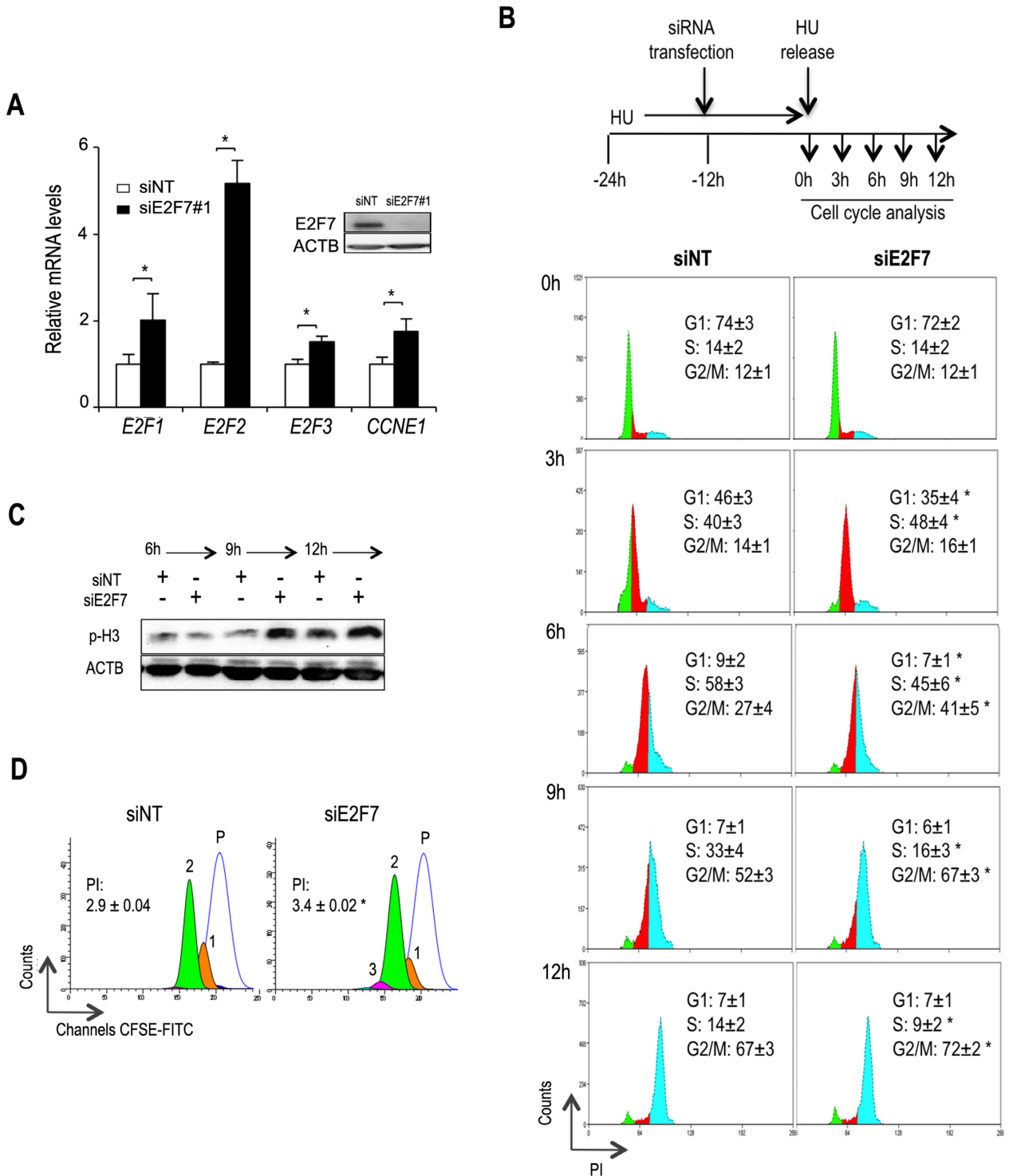
### Acute loss of E2F7 accelerates cell cycle progression

E2F7 gene expression is regulated in a cell cycle-dependent manner in U2OS cells, with reduced levels at M and G1 phases and a peak expression in G1/S transition and S phase (Supplementary Figure S1 A and B), consistent with previous reports (11). We assessed whether E2F7 is required for timely cell cycle progression by acutely depleting E2F7 and examining cell cycle distribution over time. Endogenous E2F7 was depleted very efficiently in U2OS cells individually transfected with three independent RNAi molecules specific for E2F7 (siE2F7), but not in cells transfected with an oligonucleotide whose sequence has no specificity to any human protein (siNT) used as a control (Figure 1A and Supplementary Figure S2). Importantly, E2F7 depletion resulted in substantially increased mRNA levels of known E2F7-downregulated genes (*E2F1*, *E2F2*, *E2F3* and *Cyclin E1*), confirming loss of E2F7-mediated repression in siE2F7-transfected cells (Figure 1A and Supplementary Figure S2).

To monitor cell cycle progression upon acute silencing of E2F7, U2OS cells were HU-synchronized at G1/S boundary and subsequently transfected with E2F7-specific siRNAs. Upon removal of the drug, cells were harvested every 3 h for FACS analyses (Figure 1B). DNA content analyses revealed a comparable block in G1/S in non-target control and E2F7 siRNA transfected cells. Remarkably, upon HU release, entry into S-phase in E2F7-depleted cells was significantly accelerated compared to control cells. This effect was visible as early as 3 h after HU release (48% in siE2F7 versus 40% siNT). Likewise, E2F7 depleted cells showed an earlier entry into G2 phase 6 h after exiting from HU-induced block (41 versus 27%) and it was also evident at the 9 h time point (67 versus 52%). Similarly, E2F7 depleted cells synchronized in M-phase with nocodazole showed accelerated entry and progression into S phase as well as into G2 (50% in siE2F7 versus 39% siNT at the 15 h-time-point following exit from mitotic arrest) in comparison with control siRNA transfected cells (Supplementary Figure S3). Consistent with these results, E2F7 siRNA transfected cells exhibited earlier and increased levels of the mitotic marker p-H3 after cell cycle re-entry from a HU-induced block (Figure 1C). By contrast, E2F8 depletion in U2OS cells did not result in a significant impact on cell cycle progression, even though E2F7 and E2F8 showed similar expression levels in U2OS cells (Supplementary Figure S4).

In parallel, we examined the rate of cell proliferation by labeling the cells with the vital fluorescent dye CFSE, which is diluted ~2-fold with each cell division. Consistent with cell cycle analyses, E2F7-depleted cells displayed a higher proliferation rate compared to siNT cells (Figure 1D). Thus, by inducing acute loss of E2F7, our data reveal that E2F7 is indispensable for correct progression through the cell cycle and for cellular proliferation, and that this role is not satisfied by E2F8 or by other E2F family members upon E2F7 knockdown.





**Figure 1.** Accelerated cell cycle progression and increased proliferation in cells lacking E2F7. (A) RT-Q-PCR analyses of *E2F1*, *E2F2*, *E2F3* and Cyclin E1 (*CCNE1*) in cells treated with siE2F7 #1 or a non-target siRNA (siNT) for 12 h. Western blot analysis shows efficient depletion of E2F7 by transfection of specific siRNA molecules. mRNA expression values are normalized to the expression of *EIF2C2*, used as a standard control. Results are expressed as fold over siNT values (mean ± SD) from three independent experiments. (B) U2OS cells were treated with 4 mM HU and 12 h later were transfected with NT siRNA and E2F7 siRNA (#1). Cells were washed after 24 h of HU treatment, harvested every 3 h and processed for FACS analysis. Shown is the experimental design followed. The percentage of cells in G1 (green), S (red) and G2/M (blue) ± SEM (standard error of the mean) is indicated and correspond to the average of three independent replicates. (C) Lysates from cells treated as in panel B, harvested at indicated times after HU release were used for western blot analyses of p-H3 (Ser 10). (D) Proliferation of representative siNT and siE2F7 transfected cultures. U2OS cells were incubated with CFSE, transfected with indicated siRNAs and cultured for 24 h. Proliferation Index (PI) corresponds to the average of three experiments. Shown are representative images of the parental population (P) and the proliferative cellular generations in each condition (indicated with numbers). Asterisks (\*) indicate significant differences ( $P < 0.05$ ), and were derived from a two-tailed *t*-test between siE2F7- and siNT-transfected cells.

## Identification of E2F7-regulated miRNAs

We set out to identify microRNAs that could contribute to E2F7-mediated cell cycle and proliferation control. To this end, unbiased RNA-Seq experiments were conducted using RNA derived from E2F7-competent and E2F7-depleted cells at G1/S transition (0 h), S phase (3 h) and G2/M boundary (12 h) of cell cycle following exit from HU-induced block. The expression level of endogenous E2F7 was appreciable in all three time-points (Supplementary Figure S1B). Three independent RNA-Seq experiments were performed for each condition (siE2F7 versus siNT) and time-point. Close to 1100 miRNAs were identified in the three time-points analyzed. A list of differentially expressed miRNAs between control and E2F7-depleted cells was produced by *Bioconductor DESeq* package (30) and only those miRNAs with fold-changes higher than 1.5 (siE2F7 versus siNT) in at least two of the three experiments were considered. Using these criteria a total of 18 miRNAs were found to be consistently deregulated upon E2F7 knockdown (Figure 2A and Supplementary Table S4), 15 of which were upregulated in at least two time-points of the cell cycle. These data suggest a major role for E2F7 as a negative regulator of miRNA expression throughout the cell cycle.

Within the set of E2F7-regulated miRNAs, miR-25, let-7f and miR-92a have been previously identified as E2F1 and E2F3 targets (14,17–19,39). Of note, whereas E2F1 and E2F3 are known to induce the expression of these miRNAs, our data indicate that E2F7 represses their expression. In addition, our deep-sequencing analyses produced many other miRNAs that represent potentially novel E2F-regulated miRNAs (Figure 2A and Supplementary Table S4). E2F7 has been reported to repress E2F site-dependent transcription (7,8,11). To identify potential E2F motifs within E2F7-repressed microRNAs, we made use of the MotifLocator tool provided by TOUCAN program (37). Using a threshold level of 0.8 for similarity with the canonical E2F motif recorded in the TRANSFAC database, we found that 67% of E2F7-repressed genes harbored at least one canonical E2F motif within the –1000/+500 bp regulatory region (Supplementary Table S5).

From the collection of miRNAs that were differentially expressed in E2F7-depleted cells, we selected those that have been previously related with E2F (miR-25, let-7f and miR-92a) as well as a set of miRNAs bearing E2F motifs in their promoter regions (let-7b, miR-26a, miR-27b and miR-7) (Supplementary Table S5) for further analyses. Conventional RT-Q-PCR assays of the selected miRNAs showed significantly increased expression levels upon E2F7 knockdown in the three cell cycle phases analyzed (Figure 2B), thus validating the small RNA-Seq experiment results. We subsequently examined potential pathways regulated by these miRNAs by performing a bioinformatics analysis of their predicted targets. Interestingly, Gene Ontology analysis of the combined predicted targets revealed that E2F7-repressed miRNAs preferentially modulate genes involved in cell cycle and mitotic regulation (Figure 2C). Other biological processes including hemostasis, signaling by Nerve Growth Factor (NGF) or transmembrane transport also appeared enriched in this analysis, suggesting that E2F7

regulates a diversity of functions through control of microRNA expression.

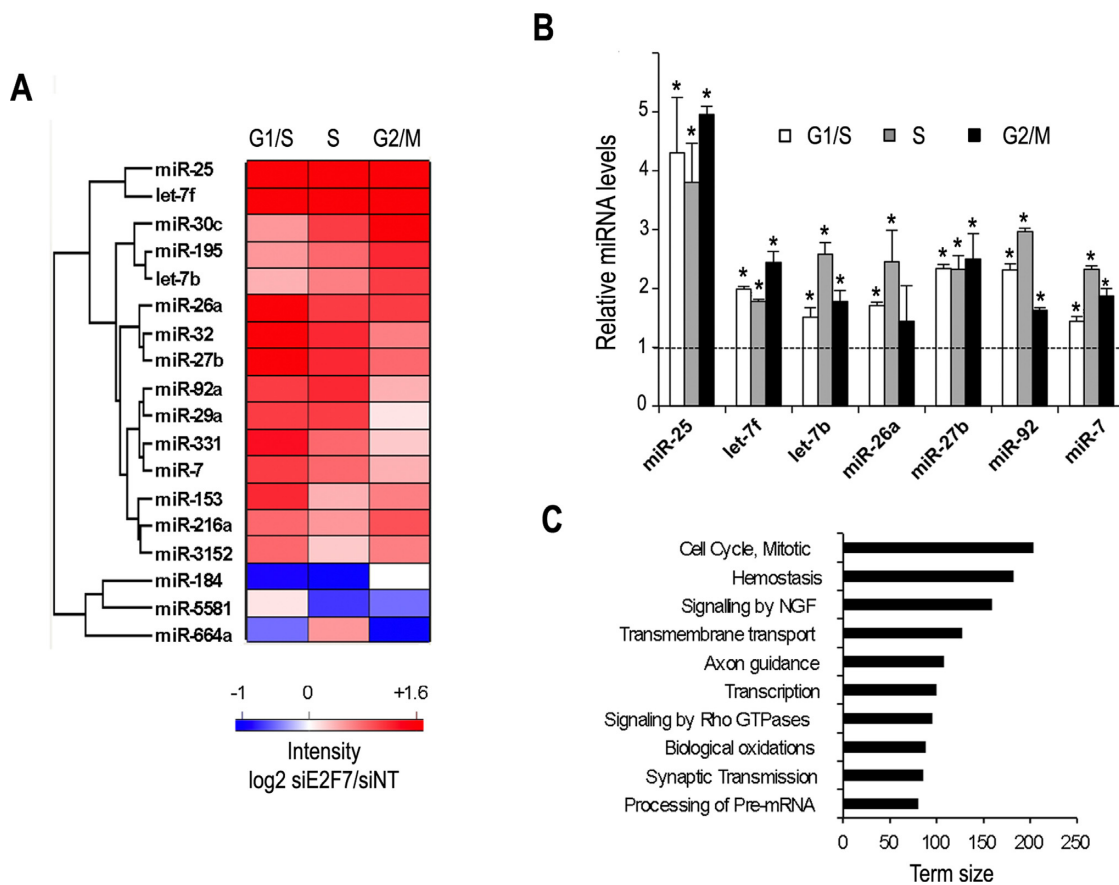
## E2F7-repressed miRNAs modulate cell proliferation

We tested whether miRNAs repressed by E2F7 (miR-25, let-7f, let-7b, miR-26a, miR-27b, miR-92a and miR-7) could contribute to E2F7-dependent control of the cell cycle. U2OS cells were transfected with expression vectors coding for these miRNAs, and cell cycle distribution profiles were analyzed. Ectopic expression of individual miRNAs gave rise to a slight acceleration of the first cell division cycle relative to *scramble* control-transfected cells (Supplementary Figures S5, S6 and data not shown). Importantly, this effect was amplified after several cell division cycles, and we observed significantly increased proliferation rates when fluorescence of CFSE-stained cells was quantified after overexpression of individual miRNAs (Figure 3A and Supplementary Table S6). Furthermore, blocking endogenous miRNA activity with a pool of anti-miRNA oligonucleotides reversed the accelerated cell cycle progression induced by E2F7 knockdown (Supplementary Figure S7).

We next assessed whether E2F7-regulated miRNAs could promote cell proliferation by limiting the expression of miRNA target genes involved in cell growth inhibition. Several critical cell cycle inhibitors reported to be regulated by these miRNAs, such as p21<sup>Cip1</sup>, p57<sup>Kip2</sup>, PTEN and p130, were indeed downregulated in U2OS cells overexpressing individual E2F7-repressed miRNAs (Figure 3B). p18<sup>INK4C</sup>, which has not been reported to be regulated by microRNAs, showed no differences in this assay, ruling out possible general effects due to an overall proliferation increase. Collectively, these results point to a role for these microRNAs in E2F7-mediated negative regulation of cell proliferation and cell cycle control by modulating the levels of critical cell cycle inhibitors.

## E2F factors are bound to the promoter region of miR-25, miR-26a, miR-27b, miR-92a and miR-7

To begin to dissect the mechanism by which E2F7 represses the expression of miRNAs during the cell cycle, we examined binding of E2F7 to the regulatory regions of the validated miRNAs. Binding of E2F7 was examined by ChIP analyses followed by Q-PCR with specific oligonucleotides for each miRNA regulatory region bearing E2F consensus sites (Figure 4A). The regulatory region of let-7f lacks putative E2F binding sites, and was therefore discarded for binding studies. The  $\beta$ -actin gene (ACTB), whose promoter lacks active E2F sites (36), was used as a negative promoter control. We made use of chromatin derived from cells collected at 3 h following HU release (corresponding to S-phase cells). Additionally, as a control for non-specific ChIP, parallel ChIP assays were carried out with an irrelevant antibody (SV40LT). As shown in Figure 4B, ChIP analyses revealed robust E2F7 binding to the regulatory region of miR-25, miR-92a and miR-7 (compare binding to ACTB), suggesting that E2F7 represses miR-25, miR-92a and miR-7 expression by directly binding to their regulatory region. By contrast, we did not detect binding of E2F7 to the regulatory regions of miR-26a, miR-27b and let-7b, which harbor consensus E2F sites (Figure 4A and B).



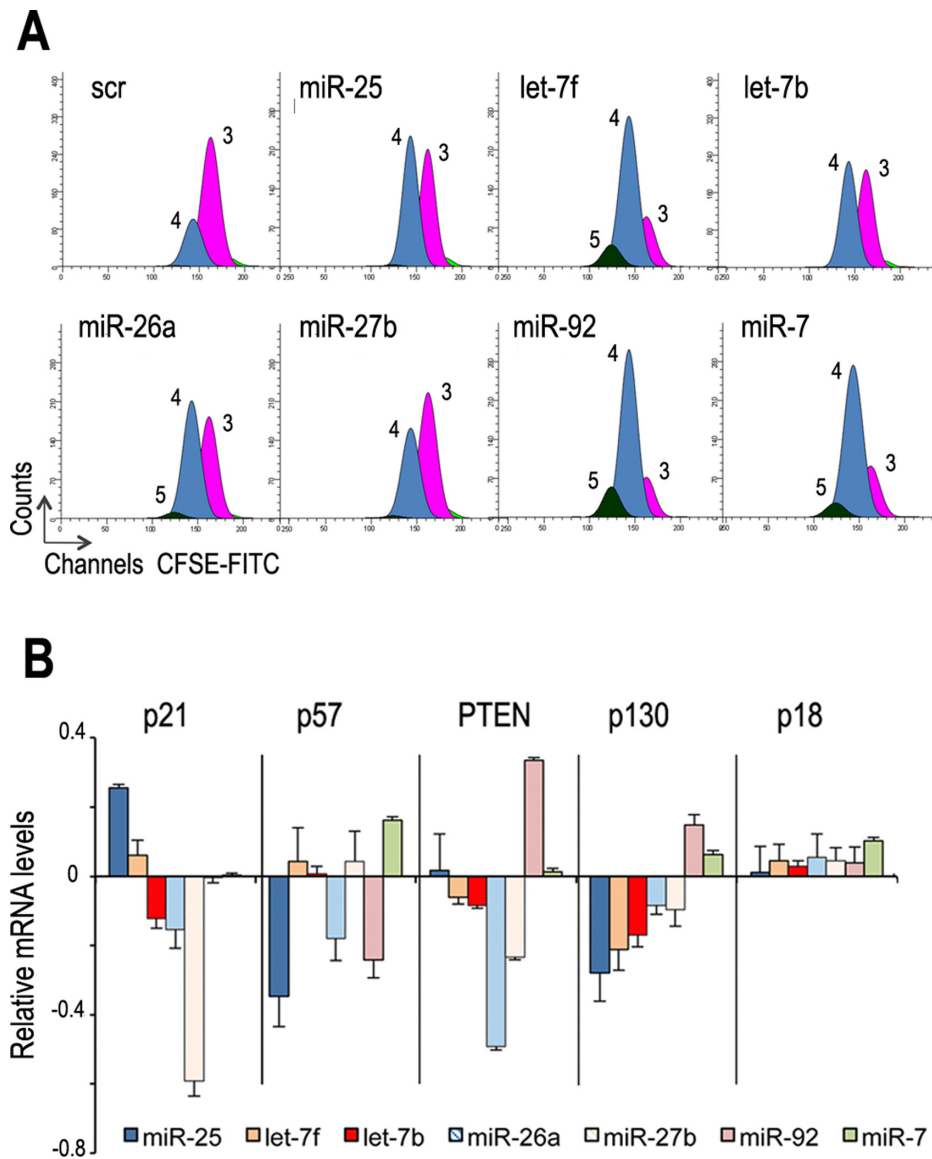
**Figure 2.** Identification of E2F7-regulated miRNAs by small RNA-Seq analysis. (A) MicroRNA expression profiles in siE2F7#1 transfected cells at various time-points after HU release. Data are normalized to the levels of siNT-treated cells. Red indicates upregulation and blue indicates downregulation. (B) Confirmatory RT-Q-PCR expression analyses in E2F7-depleted cells at various time-points after HU release. Mature miR-25, let-7f, let-7b, miR-26a, miR-27b, miR-92a and miR-7 expression levels were analyzed and normalized to RNU6B and RNU19 levels. Data are represented as fold-change relative to siNT-transfected samples (\* $P < 0.05$ ). (C) Gene ontology (GO) analysis of predicted targets of E2F7-repressed miRNAs using Fatigo tool. Only terms with adjusted  $P$ -value of  $> 0.001$  were considered.

It has been shown that individual E2F target promoters are bound by multiple E2Fs *in vivo* (36,40). Therefore, we tested whether other E2F family members could occupy the regulatory regions of these miRNAs. We focused on E2F1, E2F2 and E2F3 because they are regulated by E2F7 (Figure 1A), and thus, could potentially be involved in E2F7-dependent miRNA regulation. As shown in Figure 4C, we found that E2F1, E2F2 and E2F3 were efficiently recruited to the promoter region of miR-25 and miR-92a ( $>2$ -fold over  $\beta$ -actin promoter amplification), supporting previous data (14,18,19). Interestingly, miR-26a regulatory region was bound by E2F3, and both, E2F1 and E2F3, occupied miR-27b and miR-7 promoters. None of them was significantly recruited to let-7b. Remarkably, E2F7 depletion led to a dramatic increase in recruitment of E2F1, E2F2 and E2F3 to miR-25, miR-26a, miR-27b, miR-92a and miR-7 promoters (Figure 4C, note scale difference), consistent with the increased expression of these E2Fs in E2F7-knockdown cells (Figure 1A). Moreover, ectopic expression of E2F1-3 factors led to an induction of E2F7-regulated miRNAs (Supplementary Figure S8). These results point to a direct role for E2F1, E2F2 and E2F3 in the transcriptional activation of E2F7-repressed miRNAs.

### E2F7 regulates let-7 microRNA maturation

The absence of consensus E2F motifs in let-7f regulatory region and the lack of binding of E2F factors to let-7b, points to an indirect mechanism for E2F7 in the regulation of these miRNAs. We examined the abundance of let-7f and let-7b immature primary transcripts in U2OS cells transfected with non-target or E2F7-specific siRNAs. We included miR-25 in our assay as a control of a miRNA whose promoter is bound by E2F7 (Figure 4B). Unprocessed pri-miR-25 levels were increased in E2F7-depleted cells (Figure 5A), demonstrating that E2F7 regulates miR-25 expression at the transcriptional level. By contrast, pri-let-7f and pri-let-7b levels remained unaffected upon E2F7 knockdown. These findings rule out a transcriptional regulation of let-7f and let-7b by E2F7 and point to a role of this E2F factor in the maturation pathway of let-7 miRNAs.

The RNA binding proteins LIN28A and LIN28B have been reported to directly bind to let-7 precursor miRNA molecules and inhibit their processing into mature and functional miRNAs (41–45). We tested whether E2F7 depletion had an effect on LIN28 expression. Interestingly, RT-Q-PCR and western blot analyses showed a substantial reduction of LIN28B mRNA and protein levels (both al-



**Figure 3.** E2F7-regulated miRNAs target critical cell cycle regulators and enhance cell proliferation. (A) U2OS cells were transfected with various miRNA-coding plasmids and incubated with CFSE. A vector coding a scramble sequence (scr) was used as a negative control. Cells were harvested 24, 48 and 72 h after transfection and CFSE fluorescence was determined by flow cytometry. Shown are representative images of the distribution of cellular generations 48 h after transfection (indicated with numbers). (B) p21<sup>CIP1</sup>, p57<sup>KIP2</sup>, PTEN, p130 and p18<sup>INK4C</sup> mRNA levels were assessed by RT-Q-PCR in RNA samples extracted from cells treated as in panel A. Data are represented as normalized log<sub>2</sub>-ratios over control scr transfection.

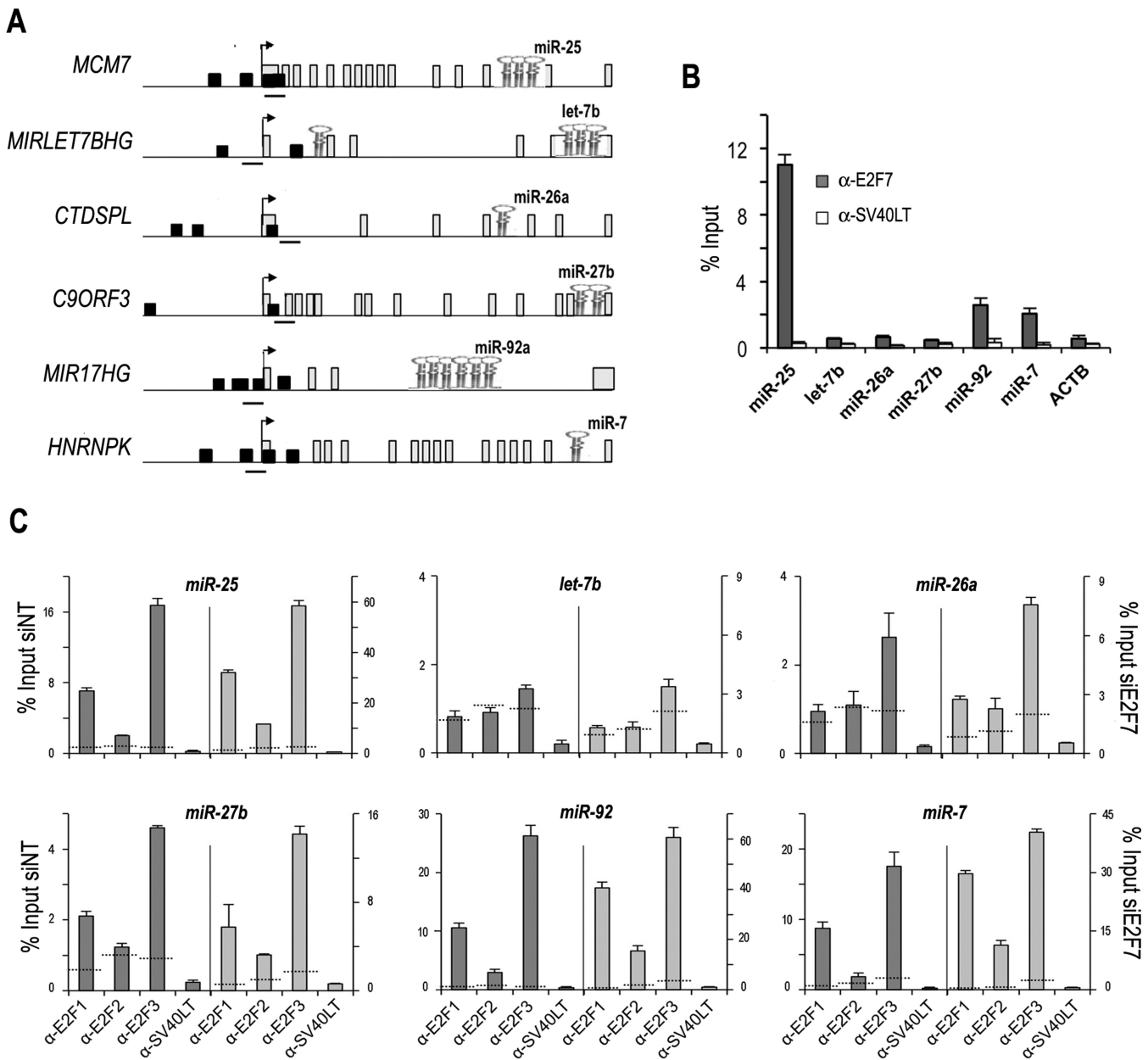
ternatively spliced forms) upon E2F7 knockdown (Figure 5B). LIN28A levels were not detected in U2OS cells (data not shown).

We next assessed whether LIN28B was required for downregulation of let-7b and let-7f in cell cycle synchronized U2OS cells. Knockdown of LIN28B by RNAi led to an increased expression of endogenous let-7b and let-7f (Figure 5C). Conversely, ectopic LIN28B expression abolished the increased expression levels exhibited by let-7f and let-7b in cells lacking E2F7, but not the levels of miR-25 (Figure 5D). Collectively, these results imply a post-transcriptional pathway regulated by E2F7 and LIN28B in dictating the levels of let-7 miRNAs.

### E2F7 regulation of let-7 involves the LIN28/c-MYC axis

LIN28B expression has not been previously linked to E2F. Instead, LIN28B expression is known to be induced by c-MYC (46). In addition to the c-MYC binding site previously reported (46), inspection of LIN28B promoter region revealed three putative E2F-recognition sites near the transcription start site (Figure 6A). However, ChIP analyses did not detect endogenous E2F7 bound to LIN28B promoter. Likewise, E2F1, E2F2 and E2F3 were absent from LIN28B promoter both in control- and E2F7-depleted cells (Supplementary Figure S9 and data not shown). In contrast, c-MYC was efficiently recruited to the promoter region of LIN28B (Figure 6A), and subsequent functional analyses showed that knockdown of c-MYC led to decreased





**Figure 4.** E2F factors are recruited to the promoter region of multiple microRNAs. (A) Schematic representation of human miR-25, let-7b, miR-26a, miR-27b, miR-92a and miR-7 loci within the human *MCM7*, *MIRLET7BHG*, *CTDSPL*, *C9ORF3*, *MIR17HG* and *HRNP7K* transcription units, respectively. The predicted E2F recognition sites are indicated by small filled boxes. Horizontal lines depict the chromatin sequences amplified by Q-PCR. (B) ChIP-Q-PCR analyses of E2F7-regulated miRNAs. Cell lysates were harvested 3 h after HU release and used for ChIP assays with an antibody against E2F7. Promoter regions near E2F consensus sites were amplified by Q-PCR. The promoter of  $\beta$ -Actin (*ACTB*) was used as a negative control. An unrelated antibody against the SV40 large T antigen (SV40LT) was used as a control for background immunoprecipitation. Data are presented as percentage of input chromatin (representative experiment of three independent experiments where the values are the mean  $\pm$  SD of triplicate determinations). (C) Cell lysates from siNT and siE2F7-transfected cells were harvested 3 h after HU release and used for ChIP assays with antibodies against E2F1, E2F2 and E2F3. Note the Y-axis scale difference in the siE2F7-treated samples. *ACTB* amplification values are represented as dotted horizontal lines.

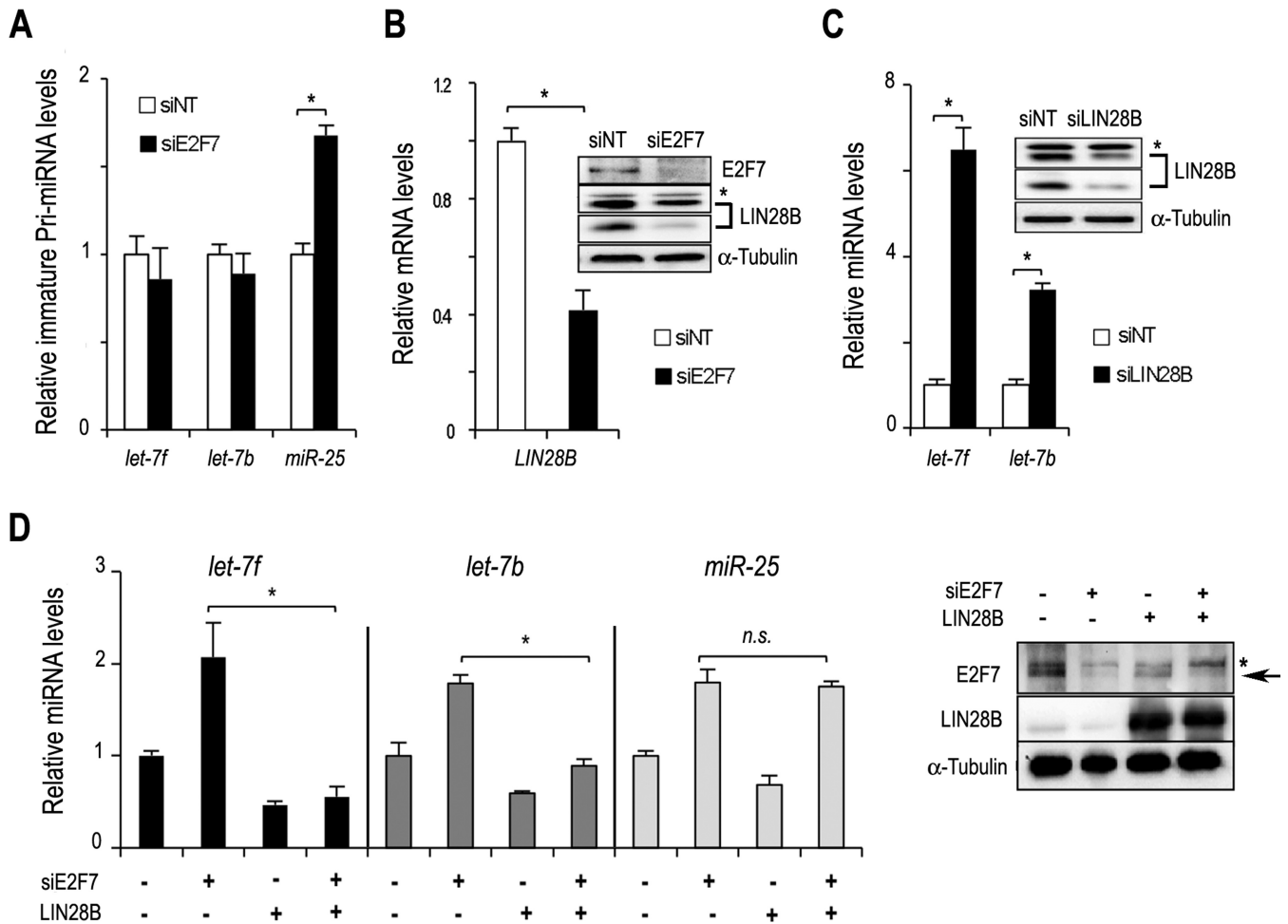
LIN28B mRNA and protein levels (Figure 6B), suggesting that c-MYC directly transactivates LIN28B expression in U2OS cells.

Importantly, ectopic expression of c-MYC impaired the reduction of LIN28B expression in E2F7-depleted cells, and led to a recovery of LIN28B levels close to those in siNT-treated cells (Figure 6C), suggesting that E2F7 could control LIN28B expression indirectly through the modula-

tion of c-MYC levels. Furthermore, let-7b upregulation in siE2F7 treated cells was partially reversed upon c-MYC expression, whereas miR-25 expression was not negatively affected by c-MYC (Figure 6D).

The above results raised the possibility that E2F7 may control let-7 and LIN28B expression through c-MYC. Indeed, we found that c-MYC expression was significantly decreased upon knockdown of endogenous E2F7 by two inde-



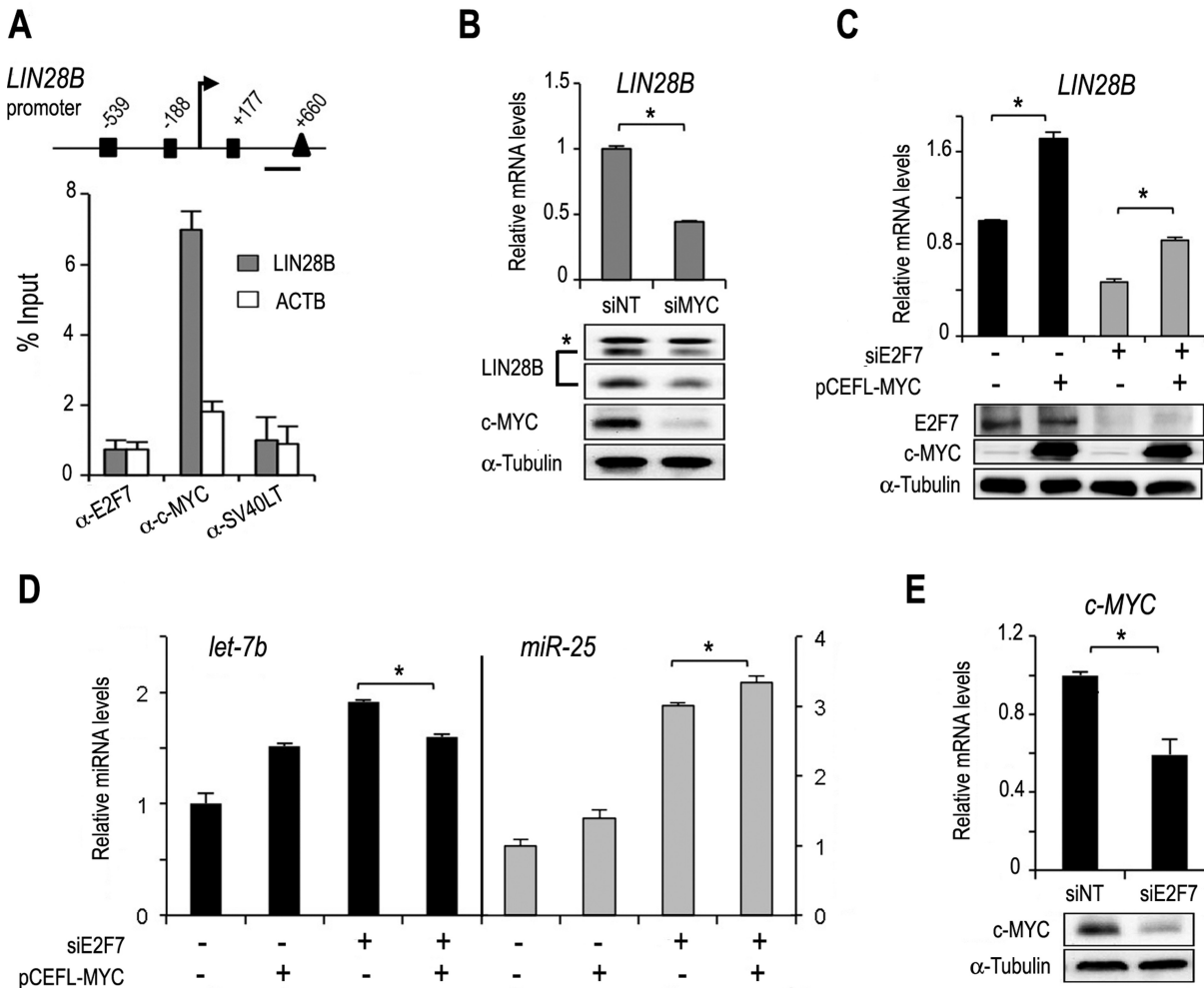


**Figure 5.** E2F7 controls *let-7f* and *let-7b* maturation through LIN28B (A) E2F7 does not regulate *let-7f* and *let-7b* transcription. HU-synchronized cells were transfected with siNT and siE2F7 and RNA was purified 3 h after cell cycle re-entry. Expression analyses of the indicated pri-miRNAs were performed using specific Taqman assays. (B) LIN28B expression was analyzed by RT-Q-PCR in cells transfected with siNT or siE2F7 RNAs. mRNA expression values are normalized to the expression of *EIF2C2*, used as a standard control. Western blot analysis shows significant downregulation of LIN28B expression after E2F7 depletion. Specific bands corresponding to two isoforms of LIN28B are indicated with arrows. A non-specific band is indicated with an asterisk. (C) LIN28B controls *let-7f* and *let-7b* expression in U2OS cells. U2OS cells were synchronized in mitosis by nocodazole treatment and transfected with LIN28B siRNA molecules. RT-Q-PCR analyses of *let-7f* and *let-7b* were carried out with RNA samples after 6 h of mitotic block release. *let-7f* and *let-7b* expression was normalized to *RNU6B* and *RNU19* small RNAs expression, used as standard controls. Data are represented as fold change relative to siNT. Western blot shows efficient siRNA-mediated knockdown of LIN28B. (D) E2F7 and LIN28B cooperate to regulate *let-7f* and *let-7b* expression. *let-7f*, *let-7b* and miR-25 levels were determined in U2OS cells synchronized in mitosis and transfected with E2F7 siRNA together with an expression plasmid encoding LIN28B (pFRT-LIN28B). Cells were harvested 6 h after block release. miRNA expression levels are shown over the empty transfection. (\* $P < 0.05$  in all graphs). Western blot shows expression of E2F7 and LIN28B in the samples used for miRNA expression analysis. A non-specific band in E2F7 blot is indicated with an asterisk.

pendent siRNA molecules (Figure 6E and Supplementary Figure S10). This surprising result prompted us to examine c-MYC regulation in more detail. c-MYC promoter region has been reported to contain several E2F elements (47), and our bioinformatic analysis confirmed this point (Figure 7A). However, whether E2Fs are involved in c-MYC regulation has not been clarified. We assessed c-MYC promoter occupancy by E2F7 as well as by its targets E2F1, E2F2 and E2F3. ChIP analyses revealed robust binding by all four E2Fs to c-MYC promoter. In addition, E2F7-depleted cells exhibited dramatically increased promoter binding by E2F1, E2F2 and E2F3 factors (Figure 7A, note scale difference), suggesting that E2F7-dependent c-MYC regulation might be mediated, at least in part, by E2F1-3. Ac-

cordingly, c-MYC mRNA and protein levels were increased upon E2F1, E2F2 and E2F3 depletion (Figure 7B and Supplementary Figure S11A). By contrast, another E2F target (Cyclin E1) showed decreased expression upon the combined knockdown of E2F1-3 (Figure 7B), as previously reported (48). Moreover, c-MYC expression was negatively affected by the ectopic expression of E2F1-3, both at the mRNA and protein levels (Figure 7C and Supplementary Figure S11B).

Modulation of c-MYC levels by E2F1-3 was not the result of altered cell-cycle profiles in these cells (Supplementary Figure S11C) and it did not involve increased promoter binding by classical E2F repressors, such as E2F4, or by pocket proteins (Supplementary Figure S12 and data



**Figure 6.** E2F7 controls LIN28B expression through c-MYC. (A) Schematic representation of LIN28B promoter region, indicating the localization of consensus E2F (boxes; -539: TTTCGGGC; -188: GCACGAAA; +177: TTTGGAGC) and c-MYC (triangle; TCCTCGTGCCC) binding motifs. ChIP analyses were performed with the indicated antibodies and Q-PCR was performed using primers spanning genomic regions around or close to E2F and c-MYC sites. The horizontal line depicts the chromatin sequence amplified by Q-PCR. Data correspond to a representative experiment of three independent replicates. (B) RT-Q-PCR and western blot analyses of LIN28B levels in c-MYC depleted cells. Cells blocked in mitosis were transfected with siNT or siMYC molecules and RNA and protein extracts were harvested 9 h after block release. Western blot shows efficient knockdown of c-MYC in U2OS cells. mRNA data are shown as fold-change over siNT. (C) RT-Q-PCR analysis of LIN28B mRNA levels in U2OS cells synchronized in mitosis and transfected with E2F7 siRNA along with an expression plasmid encoding c-MYC (pCEFL-MYC). Cells were harvested 9 h after block release. Data are shown as fold over the empty vector transfection. Western blot shows c-MYC and E2F7 expression levels in samples used for LIN28B expression analysis. (D) miRNA levels were assessed in cells treated as in panel C. (E) c-MYC mRNA and protein levels were analyzed in cells synchronized in the cell cycle by HU treatment and transfected with siNT or siE2F7. mRNA expression values are normalized to the expression of *EIF2C2*, used as a standard control. (\* $P < 0.05$ ).

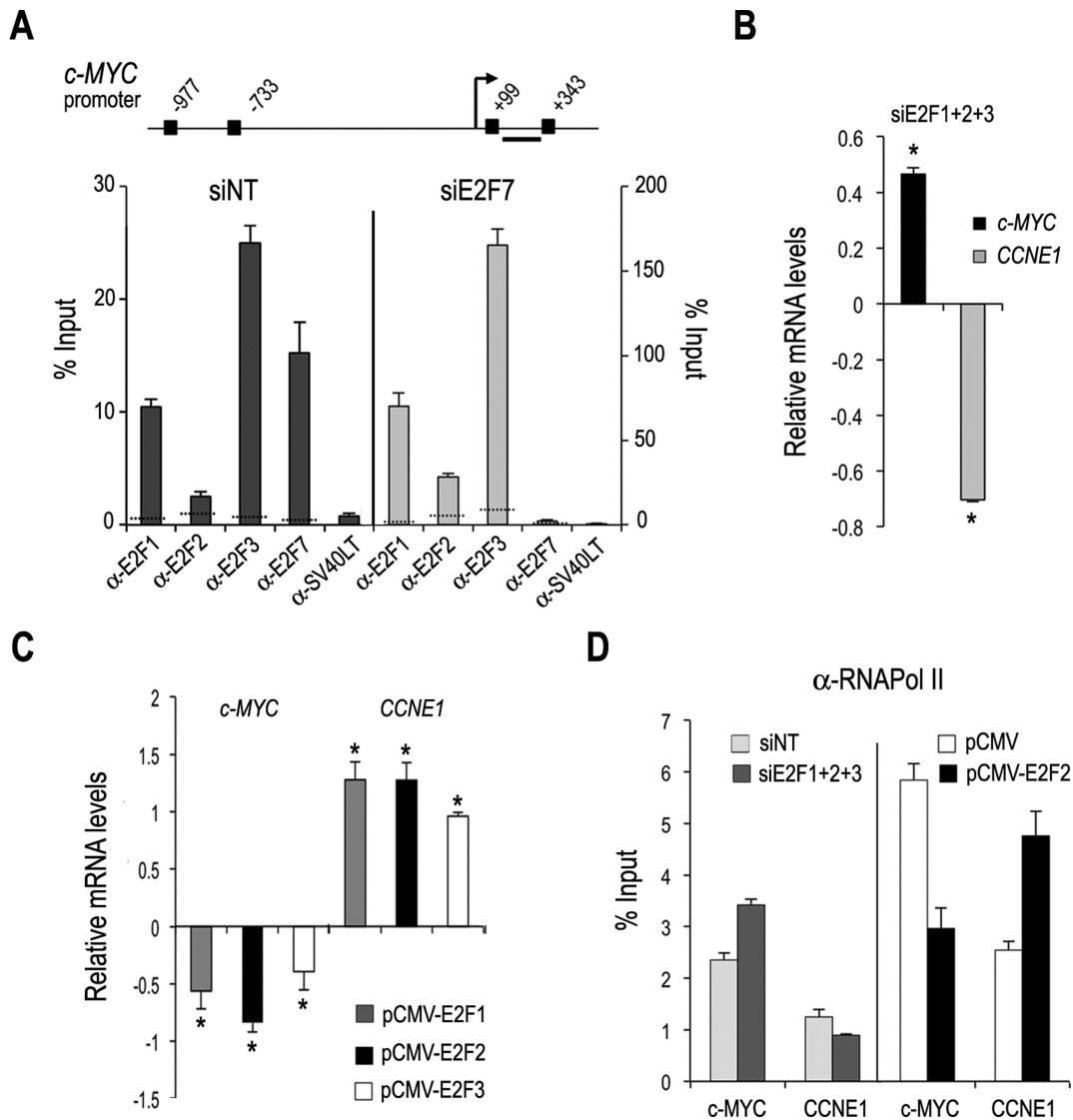
not shown), suggesting that repression of c-MYC by E2F1-3 could involve RB-independent mechanisms. Consistent with this, a similar level of c-MYC repression by E2F1-3 was detected in HEK293T cells (Supplementary Figure S13), which harbor inactive RB (49).

Importantly, modulation of E2F1-3 levels affected RNA Pol II occupancy downstream of c-MYC transcription start site (Figure 7D), which is a measure of c-MYC transcription rate (50). ChIP data revealed increased association of RNA Pol II with c-MYC gene in E2F1-3 depleted cells, whereas RNA Pol II occupancy in Cyclin E1 gene was decreased in the same experiment. Conversely, ectopic E2F2 expression negatively impacted on RNA Pol II association to c-MYC promoter, while Cyclin E1 gene showed an increased occu-

pancy by RNA Pol II in these samples. These results further demonstrate that E2F1-3 repress c-MYC at the transcriptional level. Thus, we conclude that E2F7 regulates let-7 miRNA expression through a miRNA maturation pathway involving several intermediate steps controlled by the transcriptional activity of E2F and c-MYC factors.

## DISCUSSION

In this work, we have analyzed the contribution of E2F7 transcription factor to the regulation of a subset of novel target miRNAs during the cell division cycle. We have discovered transcriptional and post-transcriptional mechanisms by which E2F7 modulates target miRNA expression. Our data support a model whereby E2F7 ensures repression



**Figure 7.** E2F1, E2F2, E2F3 and E2F7 bind to *c-MYC* promoter and regulate its expression. (A) Schematic representation of *c-MYC* promoter region, indicating the localization of consensus E2F motifs (filled boxes; -977: GCGCCACA; -733: GCAGCAAA; +99: GCGGGAAA; +343: CTTGCCGC). The horizontal line depicts the ChIP-Q-PCR amplicon. Binding of E2F1, E2F2, E2F3 and E2F7 was assessed by ChIP-Q-PCR in HU-synchronized cells transfected with E2F7 siRNA or control NT siRNAs. Cell lysates of siNT and siE2F7 treated cells were harvested 3 h after HU release. Note scale difference between siNT and siE2F7. Dotted horizontal lines represent *ACTB* amplification values. Data correspond to a representative experiment of three independent replicates. (B) *c-MYC* and *CCNE1* expression was analyzed by RT-Q-PCR in cells transfected with siNT or a pool of E2F1, E2F2 and E2F3 specific siRNAs. mRNA data are presented as normalized log<sub>2</sub>-ratios over siNT transfection. (C) U2OS cells were synchronized in mitosis and transfected with plasmids expressing HA-tagged E2F1, E2F2 and E2F3. *c-MYC* and *CCNE1* levels were analyzed by RT-Q-PCR in samples harvested 6 h after block release. mRNA data are shown as log<sub>2</sub>-ratios over the empty pCMV transfection. (D) ChIP analyses of RNA-Pol II binding at downstream regions (>1 kb) of *c-MYC* and *CCNE1* genes. U2OS cells transfected with E2F1, E2F2 and E2F3 specific siRNAs or pCMV-E2F2-HA were used for ChIP assays with an antibody against RNA-Pol II. Immunoprecipitated DNA was analyzed by Q-PCR using primers in *c-MYC* and *CCNE1* +1 kb region. Data correspond to a representative experiment of three independent replicates.

of a set of miRNA genes throughout the cell cycle, which in turn may finely tune pathways controlling cell proliferation.

The role of E2F7 in cell cycle progression has not been clearly established. Early overexpression experiments suggested that E2F7 could be a negative regulator of the cell cycle (7,8). However, chronic ablation of E2F7 did not impact cellular proliferation (12). By inducing acute depletion of E2F7, and thus largely avoiding compensatory mechanisms that are common after chronic ablation, our data clearly establish a unique requirement for E2F7 in dictating proper

cell cycle kinetics, a role that is not shared with E2F8 in U2OS cells. Our observation that E2F7 restrains cell cycle progression raises the possibility that E2F7 could function as a tumor suppressor gene, and is consistent with recent data showing that E2F7 loss together with RB inactivation promotes oncogenic transformation of murine cells (51).

E2F7 has been shown to repress a set of protein-coding genes involved in DNA replication and metabolism (11). The miRNA expression profiling analysis performed in the present work significantly expands our understanding of

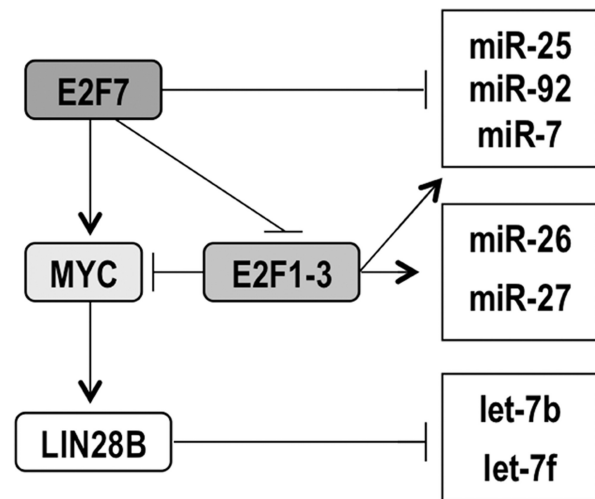


E2F7 function, by providing evidence that this factor has a major role as a negative regulator of miRNA expression. miR-25 and let-7f exhibited the highest levels of overexpression upon E2F7 loss. Interestingly, these miRNAs have previously been identified as induced by E2F1 and E2F3 in S phase entry (14), indicating that E2F7 might repress the expression of miRNAs activated by canonical E2Fs. Other miRNAs previously related to E2F, such as miR-449a/b and miR-15 (15,16,20), were not detected in our work, perhaps because of the restrictive criteria that we used in the different steps of RNA-Seq data analysis. On the other hand, our small RNA-Seq experiment has revealed many other differentially expressed miRNAs that have not been previously linked to E2F activity, and thus represent potentially novel E2F-regulated miRNAs.

Several of the miRNAs that we found to be repressed by E2F7 have previously been described as regulators of proliferation pathways (13,14,17,23,24,52–59). However, their potential roles in cancer have not been clearly established, as these miRNAs appear to have both oncogenic and anti-oncogenic functions in different cellular contexts. Our data evidence that miR-25, let-7f, miR-27b, let-7b, miR-92a, miR-7 and to a lesser extent miR-26a, promote cell proliferation in U2OS cells, at least in part by silencing the expression of anti-proliferative cell cycle regulators. Thus, by downregulating miRNA expression, E2F7 would indirectly upregulate the levels of cell cycle inhibitors to restrain cell cycle progression. These findings suggest that E2F7 controls cell cycle progression and cellular proliferation through a coordinated performance of both protein-coding and non-coding genes.

According to our promoter occupancy analyses, the chromatin binding properties of E2F7 and canonical E2F factors in the regulation of target genes appear to be different. The finding that E2F7 is only bound to E2F sites present in miR-25, miR-92a, miR-7 and c-MYC, suggests that the affinity of E2F7 for its binding site could be more restricted than that of canonical E2F1-3 factors. The basis for this selectivity remains unknown, and could involve unique interactions between E2F7 and other transcription factors at a particular promoter, as it has been proposed for other members of the E2F family (60).

miRNA biogenesis is thought to be regulated at multiple levels through mechanisms that are still not well understood. Our work reveals that regulation of let-7f and let-7b maturation by E2F7 involves both transcriptional and post-transcriptional mechanisms mediated by c-MYC and LIN28B, adding a new level of complexity to E2F-mediated miRNA regulation. The mechanism by which E2F7 modulates c-MYC expression is presently unknown, although our findings suggest that it involves negative regulation of E2F1-3 and RNA Pol II activities. Interestingly, an interplay between E2F7 and c-MYC activity has recently been suggested (61). Interfering with E2F7 expression resulted in inhibition of c-MYC functional activity in acute myeloid leukemia (AML) cells by an unknown mechanism. However, the authors did not report changes in c-MYC or LIN28B expression. It would be interesting to examine if E2F7 regulates c-MYC gene expression in AML cells, similarly to what we have observed in U2OS cells, and inquire



**Figure 8.** Model summarizing the mechanism of action of E2F factors, c-MYC and LIN28B in miRNA expression regulation described in this study.

into the biological relevance of this novel E2F7-c-MYC-LIN28B axis in AML cells.

Overall, our study identifies E2F7 as a critical regulator of miRNA biogenesis throughout the cell cycle (Figure 8). Interestingly, we have uncovered a novel interplay between E2F7 and E2F1-3 in the regulation of miRNAs to ensure induction and repression of miRNA genes during the cell division cycle, which in turn could contribute to cell growth control. In this regard, E2F7 might repress miRNA gene expression through multiple mechanisms: by binding to its target genes and directly repressing their transcription (miR-25, miR-92a and miR-7); by repressing the expression of E2F1-3, and indirectly suppressing miRNA expression at the level of transcription (miR-25, miR-26a, miR-27b) or maturation (let-7b, let-7f); or probably by a combination of both mechanisms. These findings support a model in which the transcriptional activity of E2F-target miRNAs may be dictated by an ‘E2F-network’ in which E2F1-3 and E2F7 play antagonistic roles. A similar mechanism may also be operating in E2F7-mediated regulation of protein-coding genes. Future studies may help to further identify the components of this novel molecular network as well as its biological relevance.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Conflict of interest statement.* None declared.

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