MicroRNA-223 Regulates Cyclin E Activity by Modulating Expression of F-box and WD-40 Domain Protein 7*§

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F-box and WD-40 domain protein 7 (Fbw7) provides substrate specificity for the Skp1-Cullin1-F-box protein (SCF) ubiquitin ligase complex that targets multiple oncoproteins for degradation, including cyclin E, c-Myc, c-Jun, Notch, and mammalian target of rapamycin (mTOR). Fbw7 is a bona fide tumor suppressor, and loss-of-function mutations in FBXW7 have been identified in diverse human tumors. Although much is known about targets of the Fbw7 ubiquitin ligase pathway, relatively little is known about the regulation of Fbw7 expression. We identified a panel of candidate microRNA regulators of Fbw7 expression within a study of gene expression alterations in primary erythroblasts obtained from cyclin ET74A T393A knock-in mice, which have markedly dysregulated cyclin E expression. We found that overexpression of miR-223, in particular, significantly reduces FBXW7 mRNA levels, increases endogenous cyclin E protein and activity levels, and increases genomic instability. We next confirmed that miR-223 targets the 3′-untranslated region. We then found that reduced miR-223 expression in primary mouse embryonic fibroblasts leads to increased Fbw7 expression and decreased cyclin E activity. Finally, we found that miR-223 expression is responsive to acute alterations in cyclin E regulation by the Fbw7 pathway. Together, our data indicate that miR-223 regulates Fbw7 expression and provide the first evidence that activity of the SCFFbw7 ubiquitin ligase can be modulated directly by the microRNA pathway.

The SCFFbw7 ubiquitin ligase regulates the stability of multiple oncoprotein substrates, including cyclin E, c-Myc, Notch, c-Jun, and mammalian target of rapamycin (mTOR) (1–8). Recognition of substrates by SCFFbw7 is promoted by phosphorylation of these proteins at a conserved phosphoepitope, termed the Cdc4 phospho-degron (CPD),3 which directly interacts with the WD-40 domain of Fbw7 (also known as human Cdc4 or hCdc4) (9, 10). Loss-of-function mutations in Fbxw7 cause both hematopoietic and solid organ tumors in mouse models (11–13). Additionally, Fbw7 is frequently mutated in diverse human cancers, with most mutations introducing amino acid substitutions at the WD-40 domain (14). Thus, Fbw7 is a tumor suppressor, and the abundance of hotspot mutations in human cancers targeting the Fbw7 substrate-binding domain supports the notion that impaired substrate degradation mediates the oncogenicity associated with Fbw7 loss.

Three Fbw7 isoforms with distinct subcellular localizations result from alternative splicing of transcripts made from the FBXW7 gene (15). Currently, little is known about how Fbw7 expression is regulated. The cytoplasmic isoform of Fbw7 (Fbw7β) is induced by p53, whereas expression of the nuclear (α) isoform is repressed by UV radiation (16, 17). Additionally, impaired mitochondrial oxidative function induces the Drosophila FBXW7 homolog, ago, by a p53-dependent mechanism (18). Other than these reports, most current understanding of how Fbw7-mediated substrate degradation is regulated centers on mechanisms by which Fbw7-substrate interactions are driven (14).

In the case of cyclin E, its degradation via the SCFFbw7 isoform is directed by phosphorylation within two distinct CPDs based around threonines 62 and 380 (in human cyclin E, Thr-74 and Thr-39 in murine cyclin E (7, 8, 19, 20)). Fbw7 is necessary for the maintenance of cyclin E periodicity in normal cell cycles. In the absence of a functional Fbw7 pathway, caused either by mutating cyclin E at its CPDs or by deleting FBXW7, cyclin E activity is pathologically sustained throughout the cell cycle, leading to aberrant S-phase progression, delayed mitotic exit, and genome instability (21–24). In vivo, impaired Fbw7-mediated cyclin E degradation causes increased cell proliferation and impaired maturation in a cell type-specific manner as well as increased tumorigenesis (21, 25–27). The developmental abnormalities associated with impaired Fbw7-mediated regulation in a cyclin ET74A T393A knock-in mouse model are especially pronounced in erythroid cell precursors, which exhibit hyperproliferation, increased apoptosis, and dysplastic morphologies (25).

In this study, we profiled alterations in microRNA expression in erythroblasts obtained from cyclin ET74A T393A knock-in mice and identified down-regulated expression of a number of candidate regulators of Fbw7 gene expression. We found that two microRNAs, miR-25 and miR-223, when overexpressed, reduce FBXW7 mRNA levels and the activation of a reporter linked to the 3′-untranslated region (3′-UTR) of the FBXW7 gene. However, only miR-223 significantly increases endoge-
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nous cyclin E protein and activity levels and increases genomic instability. Conversely, reduced miR-223 expression in primary mouse embryonic fibroblasts results in increased Fbw7 protein expression and decreased cyclin E activity. Finally, we found that miR-223 expression responds to alterations in cyclin E protein levels as regulated by the Fbw7 pathway, suggesting that miR-223 regulates Fbw7 expression as part of a homeostatic response to acute fluctuations in cyclin E activity.

EXPERIMENTAL PROCEDURES

Mice—The cyclin ET74A T393A knock-in mouse has been previously described (25). Wild-type littermate controls (129Sv background) were used for comparison. Mice were housed in barrier facilities, and the Northwestern University Animal Care and Use Committee (ACUC) approved all animal studies. Euthanasia was performed by CO₂ asphyxiation according to ACUC guidelines. Murine bone marrow cells were harvested by grinding femurs and tibia using a mortar/pestle, suspending cells in 2% fetal bovine serum (FBS), and filtering suspensions through 70-micron nylon cell strainers.

Reagents—FITC-conjugated anti-CD71 and phycoerythrin-conjugated anti-Ter119 antibodies (BD Biosciences) were used for isolation of primary erythroblasts from bone marrow cell suspensions. Cell extracts for immunoblot and kinase assays were prepared using Nonidet P-40 lysis buffer (28). Antibodies used in immunoblots were: anti-cyclin E (human, HE-12, Santa Cruz Biotechnology), polyclonal anti-phospho-Thr-380 cyclin E (Santa Cruz Biotechnology), polyclonal anti-cyclin E (mouse, (29)), anti-FLAG (M2, Sigma), and anti-Grb2 monoclonal (BD Biosciences).

Cell Culture—Mouse embryo fibroblasts were obtained by standard methods at days 12–14 postcoitum. (25) and used within two passages. HCT116 and K562 cells were obtained from ATCC. FBXW7-null HCT116 cells were provided by Drs. Jon Grim and Bruce Clurman, Fred Hutchinson Cancer Research Center. All cells were grown in ATCC-recommended media supplemented with 10% fetal calf serum.

Vectors, Transfections, and Retroviral Transduction—The pBabe-cyclin E (T380A) retroviral construct was previously described (23). A retroviral construct encoding human FBXW7 was made by subcloning the FBXW7α reading frame into p RetroX-IRES-DsRedExpress (Clontech), allowing transduced cells to be sorted based on fluorescence signal. The retroviral construct expressing miR-223 and green fluorescent protein (GFP) was obtained from Dr. Chang-Zheng Chen (Stanford University) via Addgene (30). Viral supernatants were prepared using Nonidet P-40 lysis buffer (28). Antibodies used in immunoblots were: anti-cyclin E (human, HE-12, Santa Cruz Biotechnology), polyclonal anti-phospho-Thr-380 cyclin E (Santa Cruz Biotechnology), polyclonal anti-cyclin E (mouse, (29)), anti-FLAG (M2, Sigma), and anti-Grb2 monoclonal (BD Biosciences).

Isolation of RNA and Quantitative RT-PCR—For mRNA assays, RNA was isolated using RNeasy mini kit (Qiagen) and reverse transcribed using the AffinityScript quantitative PCR cDNA synthesis kit (Agilent Technologies). TaqMan probes for cyclin E1 and FBXW7 detection were purchased from Applied Biosystems. Expression levels of target transcripts were normalized to 18 S RNA. For miRNA analysis, total RNA was isolated using the mirVana miRNA isolation kit (Ambion) and reverse transcribed using the TaqMan microRNA reverse transcription kit (Applied Biosystems). Target rodent microRNA Megaplex assays were used according to the manufacturer’s instruction. Individual assays for microRNA detection were purchased from Applied Biosystems. Expression levels of miRNAs were normalized to U6 or sno234. All quantitative real-time PCRs were performed using the ABI 7900HT Fast real-time PCR system (Applied Biosystems).

Flow Cytometry and Cell Cycle Analysis—CD71high/Ter-119high erythroblasts were isolated by FACS on a MoFlo cell sorter (Dako), as were cells transfected or transduced with fluorescent markers. For cell cycle studies, asynchronous mouse embryonic fibroblasts (MEFs) or synchronized MEFs were trypsinized at the indicated times, fixed in 70% ethanol, washed, and incubated in PBS containing propidium iodide and RNase A. Analyses were performed on a BD Biosciences LS82 and using the Multicycle software. Statistical analyses of cell cycle alterations (one-way analysis of variance) or cell cycle phases-specific gene expression changes (Student’s t test) were performed using the Prism 5 software (GraphPad).

Pulse-Chase—HCT116s (in 6-cm dishes) were transduced with the indicated vectors, starved for 30 min in met-free Dulbecco’s modified Eagle’s medium (Invitrogen Gibco) containing 5% dialyzed FCS, and labeled with 1 ml of trans-35S label (400 mCi/ml, MP Biomedical) for 30 min. Dishes were washed, “chased,” and at the indicated time points, washed with cold PBS and then stored at −80 degrees prior to lysis and immunoprecipitation.

Kinase Assays—For kinase assays using purified histone subunit H1 (Sigma) as substrate, human cyclin E was immunoprecipitated from extracts normalized for total protein (Bradford protein assay, Bio-Rad) with the HE111 monoclonal antibody (Santa Cruz Biotechnology), and mouse cyclin E was immunoprecipitated using the affinity-purified polyclonal antibody. Buffer conditions for measuring cyclin E kinase activity in vitro are previously described (29). Quantitation of autoradiographs was performed using PhosphorImager and ImageJ software.

Micronucleation Assay—Cultured HCT116 cells were trypsinized and washed in PBS, and nuclei were fixed in 1:3 acetic acid:methanol and dropped onto slides at an approximate density of 5 × 10⁴ nuclei/slide. Nuclei were hybridized with a pancentromeric, FITC-labeled FISH probe (ID Labs) according to the manufacturer’s protocol and counterstained (VECTASHIELD with DAPI, Vector Laboratories). Using fluorescence microscopy, micronuclei were counted as described. Statistical significance was determined using one-way analysis of variance calculations (Prism 5).
Luciferase Assays—For the luciferase assays, 1 × 10^6 K562 or HCT116 cells were co-transfected in 6-well plates with 700 ng of Renilla reporter plasmid, 100 ng of pGL3c vector, expressing firefly luciferase (Promega), and 150 pmol of each pre-miRNA. Two days after transfection, cell luminescence was measured using a Dual-Luciminescence reporter assay kit (Promega) and a Moonlight 2010 luminometer (Analytic Luminescence Laboratory). Renilla reporter activities were normalized based on firefly luciferase expression (31).

RESULTS

A Screen for microRNAs Dysregulated in Cyclin E<sup>T74A T393A</sup> Erythroblasts Reveals Candidate Regulators of Fbw7 Expression—To identify alterations in gene expression associated with the defective maturation phenotype in cyclin E<sup>T74A T393A</sup> erythroid cells, we profiled global gene expression changes associated with dysregulated cyclin E, including for non-coding RNAs, for which we employed a multiplex real-time PCR (RT-PCR) assay. This multiplex assay allowed us to compare the expression of up to 518 microRNAs in primary erythroblasts of cyclin E<sup>T74A T393A</sup> mice with wild-type, littermate controls. We found 63 microRNAs whose expression was quantifiable in both the wild-type and the knock-in erythroblasts and determined to be at least 2-fold lower in the knock-in cells (Fig. 1A and supplemental Fig. 1). We used two target prediction tools, TargetScan (32) and PicTar (33), to help elucidate the functional significance of misregulating these microRNAs, by identifying their potential mRNA targets.

We noted that seven microRNAs down-regulated in the cyclin E knock-in erythroblasts are known to regulate inhibitors of cyclin/CDK activity: miR-222, which targets p27<sup>Kip1</sup> and p57<sup>Kip2</sup> (34–36), five members (miR-17, miR-19a, miR-19b, miR-20a, and miR-92a) of the miR-17–92a cluster, which target both p21<sup>Cip1</sup> and p130 (37, 38), and miR-26a, which targets retinoblastoma (39) (supplemental Fig. 1). We then hypothesized that among the microRNAs that are down-regulated in response to high cyclin E expression in primary erythroblasts would be one or more that regulate expression of Fbw7 itself. We identified four candidate microRNA regulators of Fbw7 from among those whose expression was significantly altered in the knock-in cells based on target sites predicted to lie within the Fbw7 3′-UTR: miR-25, miR-27a, miR-92a, and miR-223.

We first verified that these microRNAs were down-regulated in cyclin E<sup>T74A T393A</sup> erythroblasts, using separate quantitative RT-PCR assays (Fig. 1B). Next, we tested the ability of these microRNAs, when overexpressed individually in human proerythroblast K562 cells by transient transfection of synthesized, precursor microRNAs (pre-miRs), to repress the activity of a bioluminescent reporter linked to the 3′-UTR of Fbw7. We found that miR-25, miR-27a, and miR-223 effectively repressed reporter activity, whereas miR-92a did not (Fig. 1C).

Predicted MicroRNA Binding Sites Are Confirmed within the Fbw7 3′-UTR—We further characterized the functional consequences of microRNA-mediated down-regulation of the Fbw7 ubiquitin ligase pathway in HCT116 cells. We chose this cell line because it has been used extensively to study the Fbw7 pathway, with Fbw7-null HCT116 cells available (22, 40), and because we achieve uniformly high (>90%) transfection efficiencies with these cells. Using the luciferase reporter construct containing the Fbw7 3′-UTR, we confirmed that expression of miR-25, miR-27a, and miR-223 repressed reporter activity in HCT116 cells, comparable with our results in K562 cells (supplemental Fig. 2). We then introduced mutations into the predicted binding sites for miR-25 and miR-223. Because miR-27 overexpression elicited neither down-regulation of Fbw7 mRNA levels nor a significant effect on Fbw7 regulation of cyclin E (Figs. 2C and 3A), we chose not to further characterize its binding sites within the Fbw7 3′-UTR.

We confirmed both the predicted binding sites for miR-25 and miR-223 within the Fbw7 3′-UTR by showing that mutation of each of these sites relieved repression of reporter activity by the corresponding miRNA but not the other (Fig. 2, A and B). Thus, miR-25 and miR-223 are able to bind sites within the Fbw7 3′-UTR.

We found that overexpression of either miR-25 or miR-223 caused substantial down-regulation of Fbw7 mRNA levels, whereas miR-27a expression only modestly reduced Fbw7 message (Fig. 2C). We then measured Fbw7 protein abundance in cells transfected with miR-25, miR-27a, or miR-223. Because
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antibodies currently available for detecting endogenous Fbw7 protein require very large numbers of cells not feasible for these studies (40), we utilized a reporter construct, which encodes the FBXW7α cDNA with an N-terminal epitope tag and the full-length 3′-UTR of FBXW7. We found that miR-223 overexpression led to significant reduction in ectopic FBXW7α abundance. Despite repressing the activity of the FBXW7 3′-UTR reporter and mRNA levels, miR-25 did not significantly reduce Fbw7 protein abundance (Fig. 2D). This discrepancy either could be due to an indirect effect of miR-25 overexpression on FBXW7α translation and/or protein stability or it may reflect that miR-25 shows less robust targeting of FBXW7 mRNA than miR-223 in our assay.

MiR-223 Overexpression Causes Dysregulation of Cyclin E Activity and Abundance—The most readily identifiable abnormality in HCT116 cells with reduced Fbw7 expression is significantly increased total and phosphorylated cyclin E and cyclin E kinase activity (40). Because the Fbw7 pathway targets active, phosphorylated cyclin E for ubiquitin-mediated proteolysis, measuring both total and phosphorylated cyclin E protein and cyclin E kinase activity provides a readout of Fbw7 function (40). We found that overexpression of miR-223 in HCT116 cells produced the most significant increases in both total and Thr-380-phosphorylated cyclin E and cyclin E kinase activity, indicative of stabilization of the catalytically active, Cdk2-bound fraction of cyclin E (Fig. 3A, left panel). In contrast, overexpression of miR-25 or miR-27 did not result in statistically significant increases in cyclin E kinase activity (p values calculated from data obtained from multiple independent experiments). Cyclin E mRNA levels were not significantly altered by the overexpressed microRNAs (Fig. 3A, right panel), consistent with the idea that the observed increase in cyclin E abundance and activity with miR-223 overexpression is associated with stabilization of the catalytically active protein fraction.

We next measured cyclin E abundance and activity in Fbw7-null HCT116 cells transfected to overexpress miR-25 and miR-223 and found that, unlike the parental cells, there was no further increase in either cyclin E protein or kinase activity with expression of either microRNA (Fig. 3B). This finding further supports the hypothesis that overexpression of miR-223 increases cyclin E activity specifically by inhibiting the Fbw7 pathway. To demonstrate directly that cyclin E degradation is impaired by increased miR-223 expression, we measured endogenous cyclin E protein half-life by pulse-chase in HCT116 cells transduced with either control or miR-223-expressing retroviruses. We found a 2-fold increase in endogenous cyclin E half-life in miR-223-expressing HCT116 cells, consistent with down-regulation of Fbw7-mediated degradation (Fig. 3C).

Previous studies have identified genomic instability as a hallmark of dysregulated cyclin E kinase activity (23, 41). Moreover, Fbw7-null HCT116 cells show evidence of genomic instability, which is dependent on cyclin E (22). We employed a micro-nucleus assay to measure genome instability in cells expressing miR-25 and miR-223. Quantitative micronucleation assays consistently have been found to be concordant with results of chromosome enumerator and metaphase analyses for scoring cyclin E-associated genome instability (22, 23, 42). We found that miR-223 expression resulted in the greatest amount of micronuclei formation in HCT116 cells when compared with that seen with either miR-25 or the control microRNA, consistent with our finding that miR-223 produces the largest increase in cyclin E kinase activity (Fig. 3D).

Reducing miR-223 Levels Down-regulates Cyclin E Activity in Primary Fibroblasts—To further address the role microRNAs play in the physiologic regulation of Fbw7 expression and activity, we studied the consequences of reducing miR-25 and miR-223 levels in cells. Because HCT116 cells express very low levels of endogenous miR-223, we performed these experiments in primary MEFs, which we found expressed higher levels of miR-223 and readily detectable miR-25 as well (Fig. 4A).

Another advantage to using primary MEFs is that they can be synchronized in G0-phase to study miR-25 and miR-223 levels in relationship to Fbw7 expression during cell cycle progression. MEFs were made quiescent by serum starvation and then released into serum using a method that yields populations of cells in G0-phase (12 h after release), early S-phase (18 h), and late S/early G2-phase (24 h) (25). We identified a significant decrease in miR-223 levels from G0- to G1-phase, which is associated with an increase in Fbxw7 mRNA levels during the same transition. In contrast, miR-25 levels remained relatively constant during cell cycle progression (Fig. 4B). These data are consistent with the hypothesis that endogenous miR-223 modulates Fbw7 expression. To test this directly, we utilized complementary, single-stranded RNA analogs (“antagomirs”) to down-regulate the expression of endogenous microRNAs. Using transfected antagomirs, we were able to reduce endogenous levels of miR-25 and miR-223 by ~50% (Fig. 4C). We then utilized the reporter construct for measuring Fbw7 protein in antagomir-expressing MEFs. We found that expression of antagomirs targeting endogenous
miR-25 and miR-223 resulted in increased levels of Fbw7 protein (Fig. 4C).

Next, we studied the effect of miR-25 and miR-223 reduction in MEFs on endogenous cyclin E expression and activity. In MEFs, due to the high abundance of p21Cip1 bound to cyclin E-Cdk2, which renders a large pool of cyclin E insensitive to Fbw7, we measured changes in cyclin E kinase activity to discern changes in the catalytically active, Cdk2-bound cyclin E fraction that is susceptible to the Fbw7 pathway (20, 21). With expression of an antagonmir targeting miR-223 (anti-miR-223), we identified a moderate but statistically significant (40%) reduction in endogenous cyclin E kinase activity and less of an effect with down-regulation of miR-25, which did not achieve statistical significance (Fig. 4D, left panel). We also observed a modest increase in the number of anti-miR-223-expressing cells in G1-phase and significantly decreased frequencies of cells in S- and G2/M-phases when compared with control anti-mir- and anti-miR-25-expressing cells (Fig. 4D, right panel). These cell cycle distribution data are consistent with a decrease in cyclin E kinase activity because acute reduction in cyclin E is known to prolong G1-phase (43). Together, data from these experiments support the concept that endogenous miR-223 can regulate Fbw7 expression sufficiently to potentiate cyclin E activity in primary fibroblasts.

**MiR-223 Levels Are Responsive to Acute Changes in Cyclin E and Fbw7 Expression**—In considering the candidates from our screen of cyclin E(C393A)erythroblasts for Fbw7-regulating microRNAs, we hypothesized that expression of a physiologically relevant regulator of Fbw7 would itself be responsive to acute alterations in cyclin E levels as a means of maintaining homeostasis during normal cell cycle progression. Thus, we measured the levels of all four candidate microRNAs, which were originally nominated in our screen of cyclin E(C393A)erythroblasts, in K562 cells stably transduced either with an Fbw7-resistant cyclin E (T380A) mutant (23) or with a control vector. Only miR-223 levels decreased significantly in response to dysregulated cyclin E expression, in contrast to miR-25, miR-27a, and miR-92a. Thus, the down-regulated expression of these other microRNAs in cyclin E(C393A) erythroblasts is likely due to an indirect consequence of expression of the knock-in allele rather than a direct result of increased cyclin E activity.
Conversely, we overexpressed Fbw7α in K562 cells and found that miR-223 levels increased in response to enforced Fbw7-mediated cyclin E destruction, whereas expression of the other microRNAs in our panel of candidates did not change (Fig. 5B). Together, these data are consistent with a model in which miR-223 acts as a rheostat that regulates

![FIGURE 4. Endogenous miR-223 regulates Fbw7 expression in primary mouse embryonic fibroblasts. A, comparison of the indicated microRNA levels in HCT116 cells versus early passage MEFs using real-time PCR. B, levels of endogenous miR-25, miR-223, and Fbw7 mRNA (all isoforms) were measured in synchronized, passage 1 MEFs. Calculated p values are displayed for the expression differences between G0 and G1 for both miR-25 and miR-223 in triplicate assays from two experiments. n.s., not statistically significant. C, left, levels of endogenous miR-25 and miR-223 were measured following transfection of primary MEFs with antagonirs (a-m). Right, MEFs were transfected with the indicated antagonirs and the reporter construct expressing FLAG-Fbw7α. FLAG-Fbw7 signal was quantified and normalized using the Grb2 signal; expression of antagonirs targeting miR-25 and miR-223 resulted in 3.7- and 3.5-fold increases in FLAG-Fbw7 expression, respectively, when compared with control (ctrl) antagonirs-expressing cells. NS, nonspecific protein in MEF extract that cross-reacts with the FLAG antibody. D, left, primary MEFs were transfected with the indicated antagonirs and GFP plasmid. Transfected cells were sorted via flow cytometry, and lysates were prepared from collected cells, immunoblotted as shown, and immunoprecipitated for cyclin E kinase activity. Right, MEFs were co-transfected with the indicated antagonirs and CD20-expresssing plasmid, enabling gating of transfected cells. Cells were fixed following CD20 surface marker staining, and cell cycle distributions were measured. Data displayed represent mean values from triplicate experiments with p values for decrease in S/G2-M-phase populations indicated. Error bars indicating standard deviations are displayed in all panels. n.s., not statistically significant.](https://journal.aabb.org/content/285/45/34444)

![FIGURE 5. MiR-223 expression levels are responsive to acute alterations in cyclin E expression associated with manipulation of the Fbw7 pathway. A, K562 cells were transduced with control or cyclin E (T380A)-expressing retrovirus. Left panel, RT-PCR analyses were performed to measure the abundance of the indicated microRNAs in cyclin E (T380A)-expressing cells, relative to control vector-transduced cells. Right panel, immunoblot analysis of transduced K562 cells for cyclin E and Grb2 (loading control). Statistical significance for expression differences was determined as for Fig. 4B. B, K562 cells were transduced with control or FLAG-Fbw7α-expressing retrovirus followed by similar microRNA RT-PCR and immunoblot analyses as in A. Error bars indicating standard deviations and p values are displayed n.s., not statistically significant. C, schematic of proposed negative feedback loop connecting cyclin E levels to regulation of Fbw7 expression via miR-223.](https://journal.aabb.org/content/285/45/34444)
cyclin E activity through its ability to modulate Fbw7 expression. Thus, we propose that miR-223 is part of a feedback loop connecting cyclin E activity to the regulation of Fbw7 expression (Fig. 5C).

**DISCUSSION**

A growing body of evidence highlights the importance of microRNAs in regulating the expression of mediators of cell cycle progression (34–39, 44–47). A theme emerging from these studies is that microRNAs participate in feedback or feed-forward circuits to provide bistability for key transition points in the cell cycle (44–46). The expression of cyclin E is under partial control of a microRNA-regulated feed-forward loop, whereby miR-15a, which represses cyclin E expression, is itself negatively regulated by a protein kinase C α-dependent mechanism (46).

We observed that a number of microRNAs known to regulate inhibitors of cyclin/CDK activity, including p21 and p27, were down-regulated in cyclin E T74A T393A erythroblasts. When compared with wild-type erythroblasts, the knock-in cells express higher levels of these CDK inhibitors. These data are consistent with the hypothesis that dysregulated cyclin E activity provokes a physiologic response, directed at least in part by multiple microRNA-regulated mechanisms aimed at repressing inappropriately high cyclin E activity levels. We hypothesized that microRNAs regulating Fbw7, which regulates the destruction of catalytically active cyclin E, would similarly be down-regulated as part of the physiologic response to dysregulated cyclin E activity in cyclin E T74A T393A erythroblasts. In knock-in erythroblasts, however, the alanine substitutions at the cyclin E CPDs render the mutant cyclin E unresponsive to increased Fbw7 expression.

In this study, we found that miR-223 overexpression causes increased cyclin E abundance, stability, and activity and cyclin E-associated genome instability. Although overexpression of miR-25, another candidate Fbw7 regulator identified in our screen, elicited potent repression of the Fbw7 3′-UTR reporter activity and reduced Fbw7 mRNA levels, its effects in most of our assays of Fbw7 function did not achieve statistical significance. The regulation of cyclin E by miR-223 is Fbw7-dependent, as evidenced by the lack of impact of miR-223 overexpression on cyclin E abundance and activity in Fbw7-null cells. In contrast, acute depletion of miR-223 in primary cells leads to decreased cyclin E activity. Recently, miR-223 has been identified as an E2F transcriptional target (48); therefore, E2F activity likely links the effect of acute alterations in cyclin E activity to changes in miR-223 expression in the feedback circuit we propose (Fig. 5C). Indeed, this circuit should reinforce another negative feedback mechanism that ties cyclin E activation to its destruction via Cdk2 autophosphorylation within the cyclin E CPDs, which drives the association of cyclin E with the SCF<sup>FBW7</sup> ubiquitin ligase (29, 49).

Another possible utility for microRNA regulation of SCF<sup>FBW7</sup> activity would be to provide cells with a means of ‘fine-tuning’ individual Fbw7-mediated substrate degradation pathways in response to various stimuli. Importantly, Fbw7-dependent degradation of many of its proto-oncogenic substrates utilizes the same kinase, glycogen synthase kinase 3, to drive the state-SCF<sup>FBW7</sup> interaction by phosphorylating CPDs (4–6, 20). Thus, regulation of individual degradation pathways could be directed at the SCF<sup>FBW7</sup> rather than toward substrate phosphorylation. We speculate that miR-223-mediated down-regulation of Fbw7 synthesis could establish windows of opportunity for individual protein substrates of the SCF<sup>FBW7</sup>, which are synthesized themselves through distinct gene expression programs, to accumulate. Importantly, our experiments have focused solely upon cyclin E, downstream of Fbw7 modulation. Further work will be necessary to develop a more comprehensive understanding of the physiologic relevance of microRNA-mediated regulation of Fbw7 and its various substrates in different cell- and tissue-specific contexts.

Notably, miR-223, which has been implicated as part of the myeloid cell differentiation program (50–52), is overexpressed in solid tumors, including ovarian and bladder cancers (53, 54). Although FBXW7 is one of several identified targets of this microRNA (51, 55, 56), the potency of Fbw7 as a tumor suppressor and the frequency at which it is mutated in human cancers suggests that its down-regulation through miR-223 overexpression could be an important event in the development of some cancers. For example, a recent report has described increased expression of miR-223 in some T-cell acute lymphoblastic leukemia cases, a leukemia subtype in which FBXW7 loss-of-function mutations are frequently found (14, 57). Possibly, miR-223 dysregulation could provide a means for impairing the tumor suppressor activities of Fbw7 in cell transformation without direct mutation of FBXW7. Further study of miR-223 in animal models and of the relationship between miR-223 expression and that of Fbw7 and its substrates in primary tumors will clarify its relevance in regulating the tumor suppressor functions of the SCF<sup>FBW7</sup> ubiquitin ligase.

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