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Jumonji/ARID1 B (JARID1B) Protein Promotes Breast Tumor Cell Cycle Progression through Epigenetic Repression of MicroRNA let-7e*[S]

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Background: The transcriptional repressor and histone demethylase JARID1B promotes G_1 progression and breast tumor cell proliferation.
Results: JARID1B regulates cyclin D1 expression and cell cycle progression through epigenetic suppression of the let-7e tumor suppressor microRNA.
Conclusion: Epigenetic suppression of let-7e by JARID1B allows G_1 progression of breast tumor cells.
Significance: The mechanism of JARID1B oncogenic activity involves suppression of a tumor suppressor microRNA.

MicroRNAs (miRs) function as tumor suppressors or oncogenes in multiple tumor types. Although miR expression is tightly regulated, the molecular basis of miR regulation is poorly understood. Here, we investigated the influence of the histone demethylase Jumonji/ARID1 B (JARID1B) on miR regulation in breast tumor cells. In MCF-7 cells with stable RNAi-mediated suppression of JARID1B expression we identified altered regulation of multiple miRs including let-7e, a member of the let-7 family of tumor suppressor miRs. Chromatin immunoprecipitation analysis demonstrated JARID1B binding to the let-7e promoter region as well as removal of the of H3K4me3 histone mark associated with active gene expression. These results suggest that JARID1B epigenetically represses let-7e expression. JARID1B stimulates tumor cell proliferation by promoting the G_1 to S transition. As predicted, suppression of JARID1B resulted in an accumulation of MCF-7 cells in G_1. We confirmed that cyclin D1, which also promotes G_1 progression, is a direct target of let-7e, and we show that cyclin D1 expression is suppressed in JARID1B knockdown cells. Cyclin D1 expression and cell cycle progression were restored following inhibition of let-7e, suggesting that JARID1B repression of let-7e contributes to cyclin D1 expression and JARID1B-mediated cell cycle progression. Our results indicate that the JARID1B demethylase contributes to tumor cell proliferation through the epigenetic repression of a tumor suppressor miR.

MicroRNAs (miRs)[2] are short, ~22 nucleotides long, noncoding RNAs that suppress target gene expression by binding to the 3′-UTR of mRNAs and inhibiting translation and/or promoting mRNA degradation (1). MiRs regulate multiple diverse cellular physiological processes including proliferation, stem cell self-renewal, differentiation, apoptosis, and metabolism (1).

MiRs also influence several human diseases including cancer, where they may function as tumor suppressors or oncogenes (2). In breast cancer miRs appear to regulate tumor cell proliferation, evasion of apoptosis, metastasis, and therapeutic evasion (3–7). The let-7 family of miRs has emerged as a family of tumor suppressor miRs (8), and in breast cancer let-7 suppresses breast stem cell self-renewal, tumorigenesis, and metastasis (9). Accordingly, down-regulation of let-7 family members is observed in multiple carcinomas, including carcinoma of the breast (8); however, the molecular mechanisms regulating let-7 expression remain poorly defined. One possibility is that similar to miR-127 (10) and miR-124a (11), let-7 expression is epigenetically regulated during tumorigenesis.

JARID1B (PLU-1) is a transcriptional repressor that harbors intrinsic histone demethylase activity. Trimethylation at H3K4 is an important histone mark associated with actively transcribed genes (12), and JARID1B specifically demethylates H3K4me3 to a transcriptionally inactive state (13–15). JARID1B is up-regulated in multiple breast tumor cell lines (16) as well as primary breast carcinomas (17). Furthermore, JARID1B promotes proliferation of MCF-7 breast cancer cells (15, 18), and knockdown of JARID1B causes a significant delay in the G_1/S transition in these cells (15).

JARID1B appears to promote breast tumorigenesis through the specific repression of antiproliferative genes including the *BRCA1* breast tumor suppressor gene (13, 15). Here, we have expanded the repertoire of tumor suppressors transcriptionally repressed by JARID1B in breast tumor cells to include members of the let-7 family. Our results suggest that JARID1B promotes cell cycle progression in part through epigenetic suppression of let-7e, thereby derepressing expression of cyclin D1.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–4 and Figs. 1 and 2.
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[2] The abbreviations used are: miR, microRNA; JARID1B; Jumonji/ARID1 B; qRT-PCR, quantitative RT-PCR.

EXPERIMENTAL PROCEDURES

Cell Lines—MCF-7 and T47D cells were obtained from ATCC and maintained according to the manufacturer's instructions. The IMG-800-JARID1B knockdown construct was generated exactly as described elsewhere (13). The IMG-800 negative control construct contains a sequence that has no significant homology with any known human gene sequence (Imgenex Corp., San Diego).

MCF-7 and T47D cells were transfected with IMG-800-JARID1B (Imgenex), the JARID1B-targeting MISSION shRNA clone TRCN0000014761 in pLKO.1-puro vector (Sigma), or IMG-800 (Imgenex) with FuGENE6 (Roche Applied Science) according to the manufacturer's instructions, and an MCF-7 cell clone containing IMG-800-JARID1B (JKD1), or IMG-800 (NC1), and pooled T47D cells containing IMG-800 (NCP) were isolated by Geneticin selection. The MCF-7 cell clone containing TRCN0000014760 (JKD2) and the pooled T47D cells containing TRCN0000014760 (JKDP3) or TRCN0000014760 and the let7e targeting miRZip-let-7e (System Biosciences) (JKDP3/anti-let-7e) were isolated by puromycin selection.

Quantitative Reverse Transcription PCR (qRT-PCR)—Analysis of gene expression by qRT-PCR was performed exactly as described elsewhere (3) using the gene specific oligonucleotides listed in supplemental Table 1.

Western Blot Analysis of Cell Lysates—Total cell lysates were prepared and analyzed by Western blotting exactly as described elsewhere (19). Primary antibodies used for Western blot analysis included cyclin D1 (sc-718; Santa Cruz Biotechnology), α-tubulin (05-829; Millipore), and JARID1B (H00010765-M02; Abnova). Secondary antibodies were Alexa Fluor 680-conjugated affinity-purified anti-rabbit or anti-mouse IgG (Invitrogen) detected using an Odyssey infrared imaging system (LiCor Biosciences, Lincoln, NE).

Cell Cycle Analysis—Cells were synchronized in media without FBS for 24 h and then cultured in MEM with 10% FBS for 48 h. Cells were harvested, stained with 20 μg/ml propidium iodide for 1 h, and analyzed by FACS at the University of Colorado Cancer Center Flow Cytometry Core using a Beckman FC500. Cell cycle data were analyzed using ModFitLT version 3.2 (Verity Software House, Topsham, ME). Alternatively, synchronized cells were stained with Guava Cell Cycle Reagent (Millipore), assayed in a Guava Easycyte Mini Flow Cytometer (Millipore), and analyzed using Guava Cytosoft™ version 4.2 software (Millipore), all according to the manufacturer's instructions.

MiR Expression Profiling—Biological triplicates of total RNA isolated from JKD1 or NC1 cells were profiled for miR expression using a service provider (LC Sciences) on LC-Science miR arrays miRHuman_11.0, which detect 856 unique human miR transcripts listed in Sanger miRBase Release 11.0 exactly as described elsewhere (4). We observed altered expression of 1.2-fold or greater in 12 miRs (supplemental Table 2).

Chromatin Immunoprecipitation (ChIP)—ChIP was performed as described elsewhere (20, 21) using the immunoprecipitation antibodies JARID1B (H00010765-M02; Abnova) and H3K4me3 (ab12209; Abcam). Eluted chromatin was analyzed by qPCR using the oligonucleotide primers listed in supplemental Table 3.

Inhibition of hsa-let-7e—Cells were transfected with 50 or 100 nM miRIDIAN miRNA inhibitor nonspecific control 1 or miRIDIAN miRNA inhibitor hsa-let-7e (Dharmacon) using Hyperfect Reagent (Qiagen) according to the manufacturer's instructions.

Expression of Pre-let-7e—Cells were transfected with 3 or 30 nM pre-miR nonspecific control 1 or pre-miR hsa-let-7e (Qiagen) using Hyperfect Reagent as described by the manufacturer.

Cyclin D1 3′-UTR Reporter Assay—Independent firefly luciferase reporter constructs harboring two regions of the cyclin D1 3′-UTR, each with a putative let-7e target sequence as well as mutated sites (supplemental Table 4), was inserted into the multiple cloning site of pMIR-REPORT (Applied Biosystems). NC1 cells were transfected with precursors of pre-let-7e (Ambion) or negative control precursor 1 (Ambion) using HiPerfect Reagent and then transfected the next day with a pMIR-REPORT plasmid and the *Renilla* luciferase expression plasmid pRL-SV40 (Promega) using FuGENE6. At 48 h after transfection, the cells were assayed for *Renilla* and firefly luciferase activity using the Dual Luciferase assay kit (Promega) according to the manufacturer's protocol.

RESULTS

JARID1B Targets H3K4 Demethylation of miR-let-7e—To investigate the influence of JARID1B on the regulation of breast tumor cell miR expression, we stably suppressed JARID1B expression in two independent MCF-7 and one T47D breast tumor cell lines (supplemental Fig. 1*A*).

To confirm JARID1B loss of function in MCF-7/JKD1 (JKD1) cells, we examined expression of the JARID1B-repressed genes metallothionein-1F (*MT1F*) and caveolin 1 (*CAV1*), and we performed a cell cycle analysis of all three independent JARID1B knockdown cell lines. As predicted from previously published results (13, 15), expression of both *MT1F* and *CAV1* was derepressed in the JKD1 cells (S1B), and the JKD1, MCF-7/JKD2–5 (JKD2), and T47D/JKDP3 (JKDP3) cells accumulated in G_1 (S1C).

To identify miRs deregulated by JARID1B knockdown, we performed a miR expression profile comparing miR expression in MCF-7/NC1 (NC1) to JKD1 cells. Interestingly, we observed altered regulation of several members of the let-7 family of tumor suppressor miRs in the JKD1 cells (supplemental Table 2). let-7e was the let-7 family member most dramatically up-regulated in the JKD1 cells. We identified five potential JARID1B binding sites (13) in an 816-bp region upstream of let-7e (Fig. 1*A*). We confirmed let-7e up-regulation in the absence of JARID1B by qRT-PCR where we observed a 3.1-fold increase in let-7e expression in the JKD1 cells compared with the NC1 cells (Fig. 1*B*). Similar results were obtained in the JKD2 and JKDP3 cell lines (supplemental Fig. 2*A*), indicating that JARID1B regulates let-7e expression in multiple breast tumor cell lines. The genomic region of let-7e also harbors miR-99b and miR-125a (Fig. 1*A*). Similar to let-7e, we observed a 1.9-fold increase in miR-99b expression by miR array profiling,

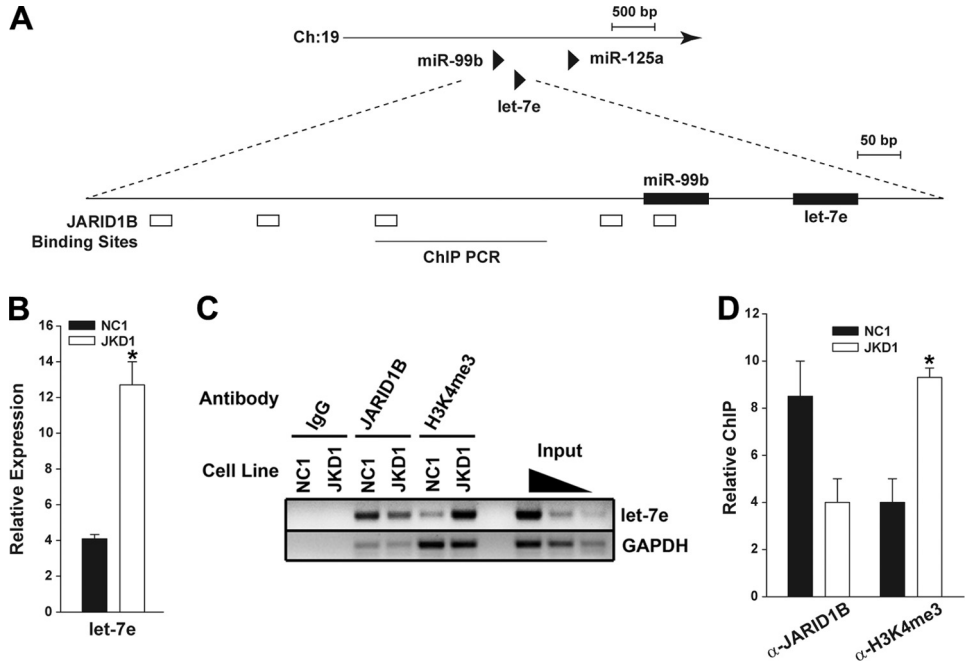

FIGURE 1. **JARID1B epigenetically regulates let-7e expression.** *A*, schematic of the intergenic let-7e miR cluster on chromosome 19 shows expanded details of potential JARID1B binding sites and position of the ChIP PCR product. *B*, qRT-PCR shows expression of let-7e in NC1 and JKD1 cells. Data are represented as expression relative to RNU24. *Asterisk* indicates significant difference by paired Student's *t* test ($p = 0.003$). *C*, PCR of ChIP analysis shows let-7e upstream region with antibodies directed against nonspecific IgG, JARID1B, or H3K4me3. Input was diluted 1-, 10-, and 100-fold. *D*, qPCR was performed on ChIP, and data represent α-JARID1B or α-H3K4me3 ChIP relative to ChIP using nonspecific control antibody. *Asterisk* indicates significant difference by paired Student's *t* test ($p = 0.008$). *B–D*, each entire experiment was repeated at least three times. Mean ± S.E. (*error bars*) are shown.

and by qRT-PCR we observed a 2.0-fold increase in miR-125a expression in the JKD1 cells (data not shown).

To explore the possibility that let-7e expression is directly suppressed through JARID1B demethylase activity, we first performed ChIP analysis of the let-7e promoter using antibodies directed against JARID1B. We observed an 8-fold enrichment of JARID1B ChIP of the let-7e upstream region compared with control nonspecific antibody in the NC1 cells which was reduced by 2-fold in the JKD1 cells (Fig. 1, *C* and *D*). We next determined whether JARID1B binding upstream of let-7e altered H3K4 methylation. Indeed, we observed a dramatic enrichment of H3K4me3 in the let-7e upstream region in ChIPs from JKD1 cells (Fig. 1, *C* and *D*), suggesting that JARID1B binding to the region upstream of let-7e in the NC1 cells results in loss of H3K4me3. Taken together, our results suggest that JARID1B directly represses expression of let-7e in breast tumor cells by promoting H3K4me3 demethylation.

Suppression of let-7e Restores Cell Cycle Progression—In concordance with published data (13, 15) we have shown that JARID1B suppression in multiple breast tumor cell lines results in a G_1/S delay (supplemental Fig. 1*C*). To determine whether G_1 accumulation of JKD1 cells was due to enhanced expression of let-7e in the absence of JARID1B, we suppressed let-7e expression in JKD1 cells and performed a cell cycle analysis. Suppression of let-7e with 50 or 100 nm of inhibitor restored cell cycle progression in the JKD1 (Fig. 2). Cell cycle progression was also restored in JKDP3 cells with stable suppression of let-7e (supplemental Fig. 2*B*). Taken together, our results suggest that JARID1B promotes breast tumor cell cycle progression through the epigenetic suppression of let-7e, an inhibitor of the G_1/S transition.

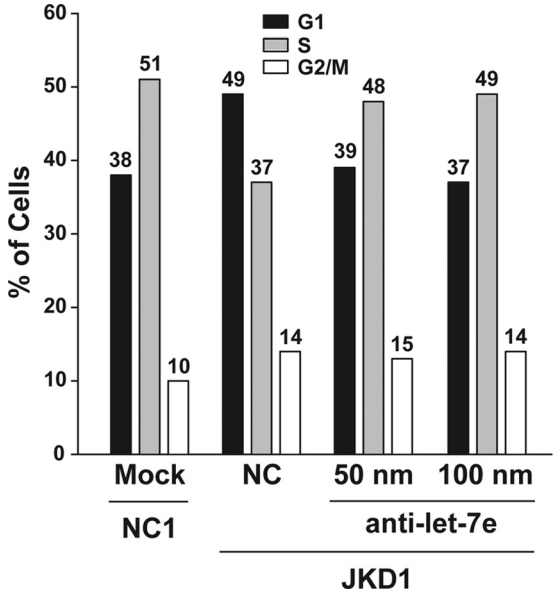

FIGURE 2. **Inhibition of let-7e restores cell cycle progression in JARID1B-deficient MCF-7 cells.** JKD1 cells were transfected with nonspecific control (*NC*) or let-7e miR inhibitor (anti-let-7e) in serum-free medium. The next day cells were released into medium with 10% FBS, transfected again with nonspecific control or anti-let-7e, and incubated for 48 h. Fixed cells were stained with propidium iodide, and cell cycle distribution was determined by flow cytometry. Mock-treated NC1 cells were included as a control.

Cyclin D1 Is a Direct Target of let-7e—To clarify the mechanistic basis of let-7e-induced cell cycle delay, we used bioinformatics to identify potential let-7e target genes with roles in cell cycle regulation. Target prediction software identified between 602 and 1012 potential targets for let-7e in the human genome with 17 cell cycle genes. Interestingly, cyclin D1 (*CCND1*) was

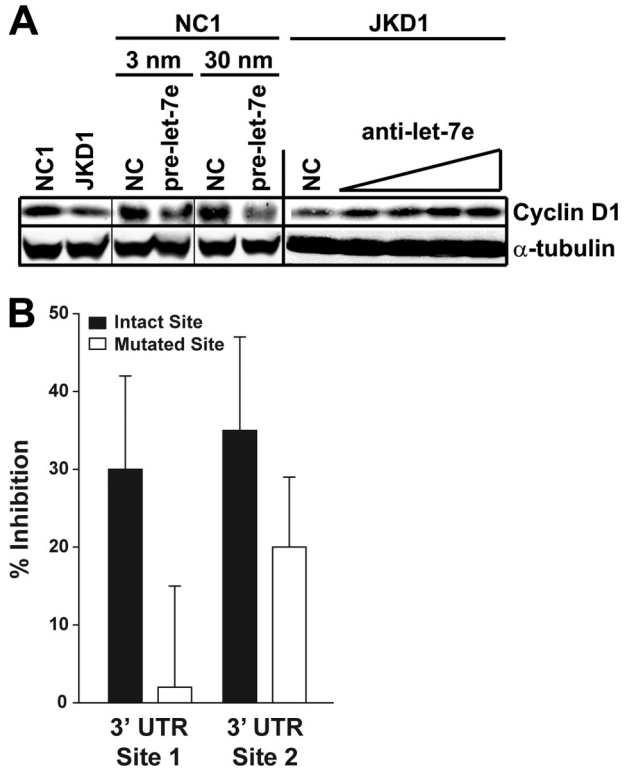

FIGURE 3. **Cyclin D1 is a let-7e target gene.** *A*, altered let-7e expression regulates cyclin D1 protein levels. Cyclin D1 Western blot analysis was performed of the indicated cell lines treated with nonspecific controls (*NC*), pre-let-7e miR, or increasing amounts (15–100 nm) of let-7e inhibitor (*anti-let-7e*). Analysis of α-tubulin was included as a loading control. *Vertical lines* indicate the positions of omitted gel lanes. *B*, two regions of the cyclin D1 3′-UTR predicted to bind let-7e were independently cloned into the pMIR-REPORT firefly luciferase reporter construct (*Intact Site*). Bases complementary to the let-7e seed sequence were altered in each construct (*Mutated Site*). NC1 cells were cotransfected with 1 μg of a cyclin D1 3′-UTR reporter construct, 50 nm of nonspecific control or pre-let-7e, and a *Renilla* luciferase control construct. At 48 h after transfection the cells were lysed and assayed for *Renilla* and firefly luciferase activity using the Dual Luciferase assay kit. Each entire experiment was repeated at least three times. Mean ± S.E. (*error bars*) of percentage inhibition of pre-let-7e luciferase activity relative to the nonspecific control are shown. Differences were not significantly different by paired Student's *t* test.

among the potential cell cycle target genes. Cyclin D1 functions by promoting the G_1 to S phase transition, and suppression of cyclin D1 in breast tumor cells results in delayed G_1/S transition (22), a phenotype reminiscent of cell cycle defects associated with let-7e gain of function. We therefore determined whether cyclin D1 was a target of let-7e by first examining the impact of altered let-7e expression on cyclin D1 protein levels. Consistent with a role for let-7e in cyclin D1 regulation, cyclin D1 protein levels were reduced in JKD1 (Fig. 3*A*), JKD2, and JKDP3 cells (supplemental Fig. 2*C*) when compared with the negative control cell lines. We next introduced pre-let-7e into the NC1 cells and observed reduced cyclin D1 protein levels (Fig. 3*A*). In the converse experiment, we inhibited let-7e in the JKD1 cells, and cyclin D1 protein levels were restored in a dose-dependent manner (Fig. 3*A*). Taken together, these results indicate that let-7e regulates cyclin D1 protein levels in breast tumor cells.

To assess whether the cyclin D1 transcript is a direct target of let-7e, we independently cloned two putative let-7e target sites and flanking sequences, located within the cyclin D1 3′-UTR (TargetScan version 5.1) as well as versions with mutated sites complementary to the let-7e seed sequence into the pMIR-REPORT luciferase reporter vector. Expression of the cyclin D1 3′-UTR construct containing let-7e site 1 in NC1 cells was inhibited by 30% when cotransfected with pre-let-7e, and this inhibition was abolished when the let-7e seed sequence was mutated (Fig. 3*B*). Although expression of the cyclin D1 3′-UTR construct containing let-7e site 2 was inhibited by 35% when cotransfected with pre-let-7e, mutation of the let-7e seed sequence only partially restored expression (Fig. 3*B*). Taken together, these results provide evidence that cyclin D1 is a direct target of let-7e, and binding of let-7e to at least one site within the cyclin D1 3′-UTR may be sufficient to reduce cyclin D1 protein levels.

DISCUSSION

Accumulating evidence implicates the JARID1B transcriptional repressor as a novel breast oncogene. JARID1B has been shown to promote breast tumor proliferation, in part, through the repression of breast tumor suppressor genes including *BRCA1* (15). Here, we provide evidence that the repertoire of JARID1B targets can be expanded to include tumor suppressor miRs. Accordingly, we demonstrate that JARID1B represses transcription of multiple members of the let-7 family of tumor suppressor miRs. We further show that the let-7e promoter is a direct target of JARID1B H3K4 demethylase activity. In breast tumor cells, JARID1B-mediated repression of let-7e results in expression of a let-7e target gene, cyclin D1, and subsequent cell cycle progression.

Interestingly, JARID1B and let-7 appear to have apposing roles on the cell cycle. Whereas JARID1B demethylase activity promotes breast tumor cell G_1/S progression, multiple members of the let-7 family, including let-7e, cause a delay in the G_1/S transition (23). Consistent with a role as important cell cycle regulators, let-7 indirectly or directly suppresses the expression of multiple cell cycle-associated proteins including Ras, c-Myc, cyclins A, A2, D1, and D2, multiple cyclin-dependent kinases, $p16^{INK4A}$, CDC25A, and CDC34 (23–26). In contrast, a direct association between JARID1B-regulated genes and the JARID1B role in promoting the G_1/S transition has remained elusive. Therefore, our finding that JARID1B represses expression of let-7e represents the first functional connection between a JARID1B target and JARID1B regulation of cell cycle progression. Similar to other let-7 family members (23), we show that ectopic let-7e expression delays the G_1/S transition in breast tumor cells, and this cell cycle defect is likely a result of let-7e targeting cyclin D1. In corroboration with our results, RNAi-mediated suppression of cyclin D1 also causes a G_1/S delay in MCF-7 cells (22).

Although multiple miRs have been shown to be regulated through epigenetic mechanisms (10, 11), details of the molecular basis of miR epigenetics remain to be established. Our results demonstrating association of JARID1B with the let-7e promoter provide an important mechanistic paradigm for the epigenetic regulation of miRs. Once bound to the let-7e promoter JARID1B catalyzes demethylation of H3K4me3 thereby removing this critical histone mark of active transcription and epigenetically repressing let-7e expression. MiR regulation by JARID1B may not be limited to let-7e. Indeed, in JARID1B-

deficient MCF-7 cells we observed up-regulation of multiple let-7 family members as well as miR-1246, miR-1826, and miR-361–5p. We are currently investigating the direct role of JARID1B in the regulation of miRs in addition to let-7e.

Although our results require clinical verification, JARID1B suppression of let-7e and the resultant enhanced expression of cyclin D1 may have important implications for the most aggressive forms of breast cancer. Of the five recently defined breast cancer molecular subtypes (27), patients with the HER2-positive subtype have the shortest overall survival (28–30). Interestingly, cyclin D1 is an obligate effector of HER2-driven tumorigenesis (31, 32). Our results demonstrating that JARID1B indirectly up-regulates cyclin D1 expression in breast tumor cells suggests that disrupting JARID1B activity may represent a therapeutic strategy to promote expression of tumor suppressor miRs, including let-7e, and suppress cyclin D1 expression. We predict that HER2-positive tumors, which require cyclin D1 expression, would be most sensitive to JARID1B loss of function. Indeed, JARID1B was first described as a HER2-regulated gene (33), suggesting that JARID1B undergoes positive selection in HER2-expressing tumors and may therefore play a critical role in HER2 breast tumorigenesis.

In conclusion, the JARID1B transcriptional repressor promotes tumor cell proliferation, in part, through epigenetic silencing of the let-7e tumor suppressor miR. Our results identify a novel function for JARID1B in the epigenetic regulation of miRs, and our data complement the growing body of evidence indicating that miR and gene expression are regulated by similar epigenetic mechanisms.

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