Sustained expression of the histone demethylase, KDM2B (Ndy1/FBX110/JHDM1B), bypasses cellular senescence in primary mouse embryonic fibroblasts (MEFs). Here, we show that KDM2B is a conserved regulator of lifespan in multiple primary cell types and defines a program in which this chromatin-modifying enzyme counteracts the senescence-associated down-regulation of the EZH2 histone methyltransferase. Senescence in MEFs epigenetically silences KDM2B and induces the tumor suppressor miRNAs let-7b and miR-101, which target EZH2. Forced expression of KDM2B promotes immortalization by silencing these miRNAs through locus-specific histone H3K36me2 demethylation, leading to EZH2 up-regulation. Overexpression of let-7b down-regulates EZH2, induces premature senescence, and counteracts immortalization of MEFs driven by KDM2B. The KDM2B-let-7-EZH2 pathway also contributes to the proliferation of immortal Ink4a/Arf null fibroblasts suggesting that, beyond its anti-senescence role in primary cells, this histone-modifying enzyme functions more broadly in the regulation of cellular proliferation.

KDM2B encodes a Jumonji domain family histone H3K36me2 and H3K4me3 demethylase that was identified as a common insertion site in rodent lymphoma insertion mutants (1, 2), and its ectopic expression is sufficient to transform hematopoietic progenitors (3). The functions of endogenous KDM2B are most clearly defined in the control of the lifespan of mouse embryonic fibroblasts (MEFs). Levels of KDM2B decline upon serial passage of MEFs, and shRNA-mediated depletion of KDM2B results in premature senescence (1, 2, 4). Reciprocally, KDM2B overexpression immortalizes MEFs in a Jumonji-dependent manner (1, 2). This is associated with a reduction in the senescence-associated up-regulation of p16Ink4a as well as suppression of p15Ink4b (1, 2, 4). Correspondingly, overexpressed KDM2B can bind directly to the Ink4b/Arf/Ink4a tumor suppressor locus (encoding p15Ink4b, p16Ink4a, and p16Ink4a) and demethylate the locus-associated histone H3K36me2 and H3K4me3 (2, 4). KDM2B only modestly suppresses p19Arf expression (2), and thus, because neither ablation of p16Ink4a alone or in combination with p15Ink4b results in immortal growth (5, 6), KDM2B must have additional downstream mediators in the control of proliferation.

The PRC1 and PRC2 (polycomb repressive complexes 1 and 2) are additional candidate targets for mediating the effects of KDM2B on cellular lifespan. These complexes counteract senescence of primary fibroblasts, in part, through silencing the Ink4a/Arf locus (7, 8). Endogenous KDM2B forms a physical complex with Polycomb group (PcG) proteins in both flies and mammals and can facilitate the PRC1-mediated ubiquitylation of H2A, which silences gene expression (2, 9–11). KDM2B also modulates the activity of PRC2 by up-regulating EZH2, which mediates epigenetic gene silencing by trimethylating histone H3 at lysine 27 (2). Levels of EZH2 decline during passage of primary MEFs, whereas knockdown of EZH2 results in premature senescence, in part due to a loss of H3K27 trimethylation of the Ink4a/Arf locus, leading to reduced binding of PRC1 and consequent activation of the locus (7). The regulation of EZH2 in primary cells is incompletely understood, although evidence in cancer cell lines suggest potential roles for transcriptional regulation by the pRB-E2F pathway (12) and post-transcriptional regulation by tumor suppressor miRNAs (13–19). Notably, KDM2B can also positive regulate EZH2 levels through an undefined pathway (2). Overall, although KDM2B appears to modulate PRC1 and PRC2 function, the molecular mechanisms and specific contribution of these processes to growth control downstream of KDM2B in primary cells has not been determined.

Here, we sought to elucidate the functional relationship between KDM2B and EZH2 in primary cells. We show that KDM2B and EZH2 are coordinately down-regulated in a series of primary cell types undergoing senescence. Moreover, we find that up-regulation of EZH2 is a critical component of KDM2B-dependent control of replicative mortality. This is mediated in
part through the direct repression of miRNAs let-7b and miR-101, which are up-regulated during cellular aging and present a barrier to cellular immortalization by down-regulating EZH2 and its transcriptional activator E2F2. Importantly, this pathway is also required for sustained proliferation of immortal Ink4a/Arf null fibroblasts suggesting a broad role of this histone modifying enzyme in cell cycle progression beyond its anti-senescence function in primary cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MEFs were isolated from E13.5 C57BL/6 mouse embryos as described previously (2). For the isolation of murine mouse mesenchymal stem cells, bone marrow cells were collected from 6–8-week-old C57BL/6 mice by crushing femurs and tibias. Nucleated cells were counted using a hemocytometer and seeded in 75 cm² flasks at a density of 1 × 10⁶ cells/cm² with complete medium consisting of high glucose DMEM, 10% (v/v) fetal bovine serum (Hyclone), and 1% penicillin/streptomycin. The non-adherent cell population was removed after 72 h, and fresh medium was added in the culture.

For the isolation and replating at 1000 cells/cm². The human cell lines IMR90 were harvested with trypsin-EDTA (Sigma) at 37 °C for 5 min. Thereafter, the medium was changed every three to 5 days; when 70–80% confluent, adherent cells were harvested with trypsin-EDTA (Sigma) at 37 °C for 5 min and replated at 1000 cells/cm². The human cell lines IMR90 (CCL-186), HEK293T (CRL-11268), BJ human skin fibroblasts (CRL-2522) were bought from the American Type Culture Collection and cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin, and streptomycin.

**Retroviral- and Lentiviral-mediated Gene Expression**—Retroviral vectors to express mmu-let-7b (M101000558), mmu-miR-101 (M10000148), and mmu-miR-26 (M10000573) were generated by cloning into the pGEM-T cloning vector (Promega) the miRNA of interest flanked by ~250 nucleotides upstream and downstream. After sequencing the cloned DNA fragments were subcloned into the EcoRI restriction enzyme sites of the pMSCV retroviral vector (Clontech) to generate pMSCV-let-7b, pMSCV-miR-101a, and pMSCV-26a1 expression vectors. The following set of primers were used: let-7bF (5'-ATATATGATCCGAGGGTACGCCTGACGAGCAAGGTCGTC-3'), and let-7bR (5'-ATATATGATCCGAGGGTACGCCTGACGAGCAAGGTCGTC-3'), miR-101aF (5'-ATATATGATCCGAGGGTACGCCTGACGAGCAAGGTCGTC-3'), and miR-101aR (5'-ATATATGATCCGAGGGTACGCCTGACGAGCAAGGTCGTC-3').

To overexpress wild-type and mutant forms of the KDM2B gene based on the pLKO1 lentiviral system obtained through the RNAi Consortium (Broad Institute). The accession numbers of the hairpins used in this study are as follows: human KDM2B (TRCN0000118440 and TRCN0000118437); mouse KDM2B (TRCN0000092539, TRCN0000092540, TRCN0000092541, and TRCN0000092542); mouse BMI-1 (TRCN0000012563 and TRCN0000012567); and mouse EZH2 (TRCN0000039040).

**Isolation and Quantitation of miRNAs**—miRNAs were quantified as described previously (23). Briefly, total RNA, including miRNAs, was isolated either with TRIzol reagent (Invitrogen; 15596-026) or with the mirVana miRNA Isolation Kit (Ambion; AM1560) and dissolved in diethyl pyrocarbonate-treated water. Total RNA (1 μg) was polyadenylated using poly(A) polymerase (Ambion; AM2030) according to the manufacturer’s instructions and was reverse-transcribed using Superscript III Reverse transcriptase (Invitrogen; 18080-044) in a 20-μl reaction supplemented with 1 μl of random hexamer (New England Biolabs; S1230S), 1 μl of oligo-dT, 1 μl of RNase inhibitor (New England Biolabs; M0370S), and 1 μl of the adapter primer (5'-GGGAGACAGAATTAATACGACTCACTATAGGGATTTTTTTTTTTTTT-3'). The reaction took place at 37 °C for 1 h and was terminated by incubation for 3 min at 95 °C. To quantitate the mature miRNA, a universal reverse primer miRNA-qPCR-3'-3' (5'-GGGAGACAGAATTAATACGACTCACTATAGGGATTTTTTTTTTTTTT-3') was used in conjunction with an exact sequence-specific primer to each miRNA. U6 snRNA (U6-F: 5'-CGGTTGCGGCCAACATATAC-3' and U6-R: 5'-TCCAGAATTTGCGCTTAT-3') was used as internal control.

**Protein Extraction and Western Blotting**—Cells were washed twice in ice-cold PBS and solubilized in the lysis buffer (50 mM Tris (pH 7.5), 200 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10 mM Na₂VO₄, 50 mM sodium fluoride, 1 mM β-glycerophosphate, 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF supplemented with a mixture of protease inhibitors (Roche Applied Science, no. 11836170001)). The lysates were briefly sonicated and subsequently centrifuged for 20 min at 16,000 × g. The supernatant (soluble whole-cell lysate) was analyzed by Western blotting. Endogenous KDM2B was probed with anti-KDM2B goat polyclonal antibody (Ab5199; Abcam), EZH2 (4905S; Cell Signaling), pRB807/811 (2276; Cell Signaling), BMP11 (5856; Cell Signaling), and p16 (M-156, sc-1207; Santa Cruz Biotechnology).

**ChIP—**MEFs from two 20-cm dishes were fixed with 1% formaldehyde for 20 min, followed by two washes with PBS. Cells were lysed in 500 μl of SDS lysis buffer (50 mM Tris-HCl (pH 8.0), 1% SDS, 150 mM NaCl, and 5 mM EDTA) plus protease inhibitor mixture I from Roche Applied Science (no. 11836170001) and were incubated on ice for 10 min. lysates were sonicated in a Bioruptor sonicator three times, 8-min
each, with 30-s off-interval times at the maximum output setting to achieve a chromatin size of 200–700 bp. The sonicated lysates were centrifuged at 14,000 \times g for 30 min at 4 °C and diluted 10 times with dilution buffer (16.7 mM Tris (pH 8.0), 167 mM NaCl, 0.01% SDS, 1.1% (v/v) Triton X-100, and 1.2 mM EDTA). DNA was recovered from immune complexes on protein A- or G-agarose beads with antibodies against H3K27me3 (no. 9733; Cell Signaling), H3K4me3 (no. 9751; Cell Signaling), H3K36me2 (no. 07-274; Millipore), KDM2B (sc-69477; Santa Cruz Biotechnology) overnight at 4 °C on a rocking platform. Subsequently, the beads were washed with low salt immune complex wash buffer (20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), high salt immune complex wash buffer (200 mM Tris-Cl (pH 8.0), 500 mM NaCl, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), LiCl immune complex wash buffer (10 mM Tris-Cl (pH 8.0), 0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 1 mM EDTA), and twice with Tris-EDTA. DNA-protein cross-links were eluted with elution buffer (0.1M NaHCO3 and 1% SDS) at 65 °C, followed by proteinase K treatment for 1 h at 45 °C. The immunoprecipitated DNA was recovered by a PCR purification kit (no. 28106; Qiagen) and analyzed either by real-time PCR with FastStart Universal SYBR Green (no. 4931850001; Roche Applied Science) in an Stratagene MX3005P continuous fluorescence detector or by PCR in a 2% agarose gel. The primer sets used in ChIP assays are listed in the supplemental data.

Senescence-associated-β-galactosidase Activity—Cells were washed twice in PBS and fixed in 0.2% glutaraldehyde for 10 min. After washing twice with PBS, cells were incubated with SA-β-galactosidase staining solution (PBS at pH 6.0 supplemented with 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactosidase, 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride) at 37 °C for 12 h. Senescent cells were identified as blue-stained cells by standard light microscopy and photographed.

Cell Proliferation—Cells were plated in duplicates in 12-well plates at concentrations as indicated in the corresponding figure legends and passed in 1:2 or 1:3 ratio. Cells were counted with an automatic cell counter (Countess, Automated Cell Counter; Invitrogen) according to the instructions of the manufacturer.

Histone Methylation Maps—Chromatin coordinates for the H3K4me3, H3K27me3, and H3K36me3 methylation patterns at the genomic loci of mmu-let-7b and mmu-mir-101a in MEFs were computed through the Epigenomics Initiative supported by the Broad Institute and visualized through the UCSC Genome Browser.

Cell Cycle Analysis—Logarithmically growing cells were washed twice with PBS, fixed overnight with methanol, and stained with propidium iodide (50 μg/ml) in PBS containing 0.05% Nonidet P-40, and RNase A (0.5 mg/ml). After incubation for 30 min at 37 °C, the DNA content of each cell cycle phase was determined by FACS with a FACSCalibur (BD Biosciences) flow cytometer. Raw data were analyzed with the FlowJo software by applying the Dean-Jett-Fox algorithm that fits G0/G1 and G2/M phases with Gaussian curves and S phase with a second-degree polynomial curve.

Bioinformatics Analysis—Raw data for GSE9520 (24), GSE11954 (25), and GSE15161 (26) were downloaded from the Gene Expression Omnibus and analyzed with the dChIP analyzer. The Invariant Set Normalization method was used to normalize arrays, and the model-based method was used for probe selection and computing expression values (27). Background subtraction was performed with the mismatch probe (PM/MM difference) algorithm. Clustering of miRNAs (GSE9664, (28)) took place with the Cluster 3.0 and visualized with the JavaTreeView software. The miRNA TargetScan database (version 4.2) was used to identify EZH2 targeting miRNA. Statistical analysis took place with the GraphPad PRISM (version 5.01).

RESULTS

KDM2B Is Conserved Regulator of EZH2 Activity and of Onset of Senescence in Primary Cells—We have shown previously that KDM2B is the only Jumonji domain containing histone demethylase that is down-regulated in MEFs undergoing senescence (2). Fig. 1, A–C, extends these findings, showing that the down-regulation of KDM2B is a hallmark of senescence in multiple types of primary mouse and human cells and that senescence is accompanied by a concomitant down-regulation of EZH2. To delineate the potential role of epigenetic changes in the silencing of the KDM2B upon cellular aging, we performed ChIP experiments in early and late passage MEFs. We found that cell passage induces a repressive epigenetic signature at the KDM2B promoter characterized by increased tri-methylation of H3K27, a repressive histone mark, and reduced trimethylation of H3K4, a permissive histone mark (Fig. 1D). Thus, cellular aging epigenetically silences KDM2B, and its down-regulation may be a prerequisite for multiple cell types to undergo senescence. Indeed, as in MEFs, knockdown of KDM2B accelerated the onset of senescence across the panel of primary cell lines (Fig. 1E and supplemental Fig. S1A), whereas ectopic expression of KDM2B accelerated cell cycle progression and led to lifespan extension in a Junosnji domain-dependent manner (supplemental Fig. S1, B and C) (1, 2).

Because KDM2B and EZH2 are coordinately down-regulated during senescence, and because KDM2B knockdown and overexpression studies show that KDM2B regulates EZH2 levels (Fig. 1, F and G, and supplemental Fig. S1, D and E), we performed genetic complementation studies to evaluate the functional interactions between KDM2B and PcG proteins in the senescence program. Knockdown of EZH2 and BMI1 counteracted the capacity of KDM2B to promote immortalization of MEFs and to suppress p16(hk44) expression and consequent up-regulation of retinoblastoma-Ser807/811 phosphorylation (Fig. 1H). Concurrent EZH2 overexpression did not enhance the effects of KDM2B on proliferation and p16(hk44), suggesting that KDM2B overexpression maximally activates EZH2, whereas BMI1 and KDM2B acted synergistically (Fig. 1I). Reciprocally, premature senescence caused by KDM2B knockdown was partially reverted by BMI1 overexpression, whereas EZH2 overexpression had only modest effects upon KDM2B depletion (Fig. 1J and data not shown). We conclude that cross talk between...
KDM2B-let-7-EZH2 Axis in Senescence

A. P1 P5 EzH2

B. GSE9520

C. GSE11954

D. KDM2B chr:123,320,685-123,439,101 (NCBI37/mm9 assembly)

E. MEFs mMSCs

F. shControl

G. KDM2B ΔJunC

H. Myc-KDM2B

I. KDM2B+shBmi-1

J. Bmi-1+shControl
KDM2B and PcG proteins is central to lifespan regulation in primary cells. It is important to note that unlike KDM2B or BMI1, the overexpression of EZH2 did not result in immortalization of MEFs (Fig. 1J) (12)). Therefore, although EZH2 function is absolutely required for KDM2B-mediated immortal growth, it cannot independently override additional control mechanisms (12). Collectively, these data indicate that KDM2B and BMI1 function cooperatively as master regulators of immortalization and that KDM2B acts upstream of BMI1 through the induction of EZH2 as well as through additional pathways.

KDM2B Counteracts Senescence-associated Up-regulation of miRNAs That Target EZH2—Next, we sought to explore what triggers the down-regulation of EZH2 in aging cells and how this process is counteracted by KDM2B. Because the epigenetic alterations induced by KDM2B relate to transcriptional repression (2, 4, 9, 10, 29), the role of this factor in EZH2 induction may be indirect. A number of miRNAs that regulate EZH2 abundance by targeting its 3′-UTR have been identified and are summarized in Table 1. Importantly, overexpression of KDM2B potently increased the expression of let-7b (Fig. 2F), as well as that of c-Myc, an additional let-7b target (supplemental Fig. S2B). Notably, E2F2 transcriptionally activates EZH2 (12); thus, induction of let-7b in aging MEFs appears to contribute to down-regulation of EZH2, by targeting of both E2F2 (35) and the 3′-UTR EZH2 (19). Importantly, ectopic expression of BMI1 largely rescued the proliferation of MEFs stably expressing let-7b (Fig. 2K), suggesting that let-7b represses senescence by down-regulating BMI1 and deregulates the cell cycle machinery and that these miRNAs are components of the senescence program in MEFs.

KDM2B Epigenetically Represses Expression of let-7b and mir-101—Next, we sought to determine whether KDM2B directly regulates the expression of the EZH2-targeting miRNAs through binding and histone demethylation of their genomic loci. We first optimized ChIP for endogenous KDM2B using the Ink4a/Arf locus as a control. This locus had previously been shown to be bound by ectopically overexpressed KDM2B (2). Here, we show that endogenous KDM2B strongly bound the Ink4a promoter but not to Arf or Ink6b (Fig. 3A; supplemental Fig. S2C) shows specificity of the antibody and ectopic KDM2B potently repressed the expression of p16Ink4a, and to a lesser extent p19Arf, in a Jumonji domain-dependent manner (Fig. 1G and supplemental Fig. S2D). KDM2B overexpression did not affect the levels of p15Ink4b (supplemental Fig. S2E). Consistently, KDM2B strongly repressed the expression of a luciferase reporter driven by the Ink4a promoter, whereas deletion of the CxxC (ZF-CxxC) domain, which binds nonmethylated CpG islands (36), completely reversed the effect of KDM2B on p16Ink4a (Fig. 1G and supplemental Fig. S2E).

We subsequently found that KDM2B binds the let-7b and mir-101a loci, but not to mir26a-1, consistent with the observed effects of KDM2B on the expression of these miRNAs (Fig. 3B; compare with Fig. 2, B and C). Computational analysis of histone methylation maps in MEFs revealed a common epigenetic signature for let-7b and mir-101a characterized by the

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**FIGURE 1. KDM2B regulates the lifespan of primary cells.** A, serially passaged mouse mesenchymal stem cells (mMSC) were analyzed by Western blotting. B and C, meta-analysis of the relative expression levels of KDM2B and EZH2 in human mesenchymal (hMSC) and hematopoietic stem cells (hHSC). ChIP analysis of the relative tri-methylation levels of histone H3 at Lys27 and Lys4 normalized to histone H3 occupancy at the promoter of the KDM2B locus in passage 1 (P1) and passage 5 (P5) MEFs (n = 2). The regions of the KDM2B loci analyzed (A–D) are shown in the schematic at the top (the nucleotide positions are indicated; image not to scale). E, MEFs and mouse mesenchymal stem cells were infected with lentiviruses to knock down KDM2B. Cells were seeded at a concentration of 1 × 105 cells/well and serially passaged every 4 days and assayed for cumulative cell number. F and G, MEFs were infected with viruses to knock down (F) or to overexpress (G) KDM2B (EV, empty vector). At passage 5, whole cell lysates were analyzed by Western blotting. H, MEFs were infected with the combinations of viruses to overexpress KDM2B or to knock down BMI1 or EZH2 and seeded at a concentration of 1 × 105 cells/well. Left panel, cell number upon serial passage. Right panels, Western blotting analysis for the indicated proteins at passage 5. I, MEFs infected with combinations of viruses to overexpress KDM2B, BMI1, or EZH2 were analyzed as in H. J, MEFs infected with combinations of viruses to overexpress BMI1 or knock down KDM2B were analyzed as in I.
FIGURE 2. KDM2B represses the expression of miRNAs that target EZH2. A, analysis of MEFs by qRT-PCR for the relative levels of the indicated miRNAs at P1, P3, and P8 (n = 2). B and C, MEFs overexpressing KDM2B (B) or shRNA targeting KDM2B (C) were analyzed at P5 for the relative levels of the indicated miRNAs (n = 2). D, MEFs were infected with a lentivirus to overexpress let-7b, seeded at a concentration of 1 × 10^5 cells/well, and serially passaged every 4 days. Left panel, cumulative number of cells upon passaging. Right panel, staining of cells at P5 for SA-β-galactosidase activity. E, MEFs overexpressing KDM2B were engineered to overexpress let-7b and serially passaged every 2 days. F, MEFs infected with retroviruses to overexpress the indicated miRNAs were analyzed by Western blotting for the levels of EZH2. G, MEFs were infected with lentiviruses to knock down endogenous KDM2B (left panel) and subsequently were passaged and transfected twice with anti-let-7b inhibitor (final concentration of 50 nM). Cells were analyzed by Western blotting for the levels of EZH2. H, analysis of MEFs by qRT-PCR for the relative levels of E2F2 at P2 and P8 (n = 2). I, MEFs overexpressing let-7b were analyzed for the relative levels of E2F2 by qRT-PCR. J, MEFs overexpressing KDM2B were analyzed for the relative levels of E2F2. K, cumulative cell number of MEFs engineered to overexpress combinations of let-7b, BMI1, and vector controls. Cells were passaged every 2 days. EV, empty vector.
presence of the H3K36me3 mark, suggesting active transcription by RNA polymerase II but lack of either H3K4me3 or H3K27me3 (supplemental Fig. S3A). We found that overexpression of KDM2B decreased the H3K36 methylation at both the let-7b and miR-101a loci in a JmjC domain-dependent manner (Fig. 3C). Reciprocally, knockdown of endogenous KDM2B in MEFs caused increased methylation of H3K36 at these regions (Fig. 3, D and E). Because H3K36 methylation positively correlates with the recruitment of RNA polymerase II, we conclude that KDM2B-mediated demethylation of H3K36 contributes to the silencing of let-7b and miR-101a.

KDM2B–let-7 Pathway Regulates Proliferation of Ink4a/Arf Null Fibroblasts—Both let-7b and EZH2 have been implicated in growth control independent of their functions in senescence (12, 32, 37). To study whether KDM2B has a comparable role, we proceeded to overexpress and knock down KDM2B in immortal Ink4a/Arf null fibroblasts. Notably, unlike passaged wild-type MEFs, MEFS immortalized by inactivation of either Ink4a/Arf or p53 tumor suppressors or by overexpression of the c-myc oncogene, showed sustained high levels of KDM2B expression (Fig. 4A, supplemental Fig. S3B, and data not shown). Knockdown of KDM2B in Ink4a/Arf null and c-myc-
overexpressing fibroblasts markedly reduced cell proliferation (Fig. 4B and supplemental Fig. S3C) in association with let-7b up-regulation (Fig. 4C) and EZH2 down-regulation (Fig. 4D). Levels of p151nsk4b were unaffected (supplemental Fig. S3D). Knockdown of BMI1 or EZH2 (Fig. 4B) and overexpression of let-7b or a dominant-negative KDM2B Jumonji deletion mutant (1, 2) strongly inhibited cell proliferation (Fig. 4E). Therefore, KDM2B function in growth control and EZH2 regulation extends beyond the bypass of senescence in primary cells through a mechanism associated with regulation of let-7b miRNA.

**DISCUSSION**

Here, we show that epigenetic silencing of KDM2B contributes to the senescence of multiple types of primary embryonic and adult cells, and we define a let-7b-EZH2 pathway as an important component of KDM2B-mediated proliferative control and bypass of the senescence checkpoint. Thus, our data show that KDM2B has a conserved function in replicative lifespan and define a novel mechanism for EZH2 regulation. As both EZH2 and KDM2B contribute to the silencing of the Ink4a/Arf locus by direct chromatin modifications, the KDM2B-let-7b-EZH2 circuit appears to act in a feed-forward manner to reinforce the senescence phenotype (Fig. 4F).

Genetic studies in Drosophila (11) and biochemical purification in mammalian cells (2, 9, 10) have demonstrated interplay between KDM2B and Polycomb-mediated gene repression. Our genetic complementation studies highlighted the role of such functional interactions in the growth control of primary cells. KDM2B is required to maintain EZH2 expression levels in the immortalization process, and knockdown of either EZH2 or BMI1 counteracts cellular immortalization and repression of p161nsk4a driven by KDM2B. BMI1 synergizes with KDM2B in facilitating cell proliferation and p161nsk4a repression, effects that are consistent with the predicted function of EZH2-catalyzed H3K27 trimethylation in facilitating PRC1 recruitment. Whereas KDM2B and BMI1 are master regulators of immortalization, overexpression of EZH2 alone does not immortalize MEFs. Moreover EZH2 is much weaker at rescuing cell growth upon KMD2B knockdown in comparison to BMI1. Thus, KDM2B appears to act upstream of both EZH2 and BMI1 in cellular immortalization and functions both by maintaining EZH2 expression as well as through additional pathways.

KDM2B regulates the Ink4a/Arf locus both indirectly through EZH2 modulation and by direct epigenetic modifications. Our chromatin immunoprecipitation of the endogenous protein and gene expression studies in MEFs showed that KDM2B binds to and potently represses the Ink4a promoter, whereas Arf binding and repression are much weaker (Fig. 3A and supplemental Fig. S2, D and E). We did not detect binding of KDM2B at the Ink4b promoter, and we did not observe reduced
Ink4b levels upon KDM2B overexpression. Although these results differ from a prior report describing Ink4b as a critical target for KDM2B in MEFs (4), they are consistent with recently published data (38) showing that knockdown of the long form of KDM2B in MEFs caused the up-regulation of p16Ink4a, and to a lesser extent p19Arf, whereas no changes were seen in the p15Ink4b.

It should be noted that both Ink4a and Ink4b are dispensable for MEF senescence (6); thus, there are additional critical targets of KDM2B (and EZH2) in cellular lifespan function.

Another important finding of this study is that KDM2B function in growth control and EZH2 regulation extends beyond the bypass of senescence in primary cells through a mechanism that involves the regulation of let-7b. Let-7b is an established tumor suppressor that inhibits the function of a number of onco genes in addition to EZH2, including c-Myc, lin-28b, K-Ras, Hmg2a, and E2F2 (31, 33–35). Thus, the capacity of overexpressed KDM2B to promote immortalization of wild-type MEFs supports a role in tumor initiation, whereas the critical role for KDM2B in proliferation and let-7b regulation in MEFs immortalized by Ink4a/Arf deletion or c-Myc expression suggests sustained function in the maintenance of established cancers.

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