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A Positive Feedback Loop Regulates the Expression of Polycomb Group Protein BMI1 via WNT Signaling Pathway*[S]

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Background: PcG protein BMI1 is transcriptionally regulated by Myc and is up-regulated in cancer cells.
Results: The WNT pathway plays an important role in Myc regulation of BMI1.
Conclusion: BMI1 up-regulates the WNT pathway, which in turn regulates expression of BMI1 via c-Myc.
Significance: The study provides insights into the regulation of BMI1 and suggests that BMI1 expression may be targeted by WNT inhibitors in cancer cells.

Polycomb group protein BMI1 plays an important role in cellular homeostasis by maintaining a balance between proliferation and senescence. It is often overexpressed in cancer cells and is required for self-renewal of stem cells. At present, very little is known about the signaling pathways that regulate the expression of BMI1. Here, we report that BMI1 autoactivates its own promoter via an E-box present in its promoter. We show that BMI1 acts as an activator of the WNT pathway by repressing Dickkopf (DKK) family of WNT inhibitors. BMI1 mediated repression of DKK proteins; in particular, DKK1 led to up-regulation of WNT target c-Myc, which in turn further led to transcriptional autoactivation of *BMI1*. Thus, a positive feedback loop connected by the WNT signaling pathway regulates *BMI1* expression. This positive feedback loop regulating BMI1 expression may be relevant to the role of BMI1 in promoting cancer and maintaining stem cell phenotype.

Polycomb group (PcG)[2] proteins are evolutionarily conserved gene silencers that determine cell fate decisions during development (1). These proteins are often aberrantly expressed in cancer cells. In particular, BMI1 and EZH2 are known to be overexpressed in a number of human malignancies including breast and prostate cancers (2–4). *In vitro* models of cancer development strongly support an oncogenic role of overexpressed BMI1 in cancer and metastasis (5, 6). In addition to its role in cancer, BMI1 is known to be required for self-renewal of neural, hematopoietic, intestinal, and mammary stem cells (7–12). BMI1 is also suspected to play a role in cancer stem cell development in different cancer progression models (13, 14). Consistent with its role in maintenance and self-renewal of stem cells, recently it was demonstrated that BMI1 can replace c-Myc, and together with OCT3/4, it can promote conversion of human fibroblasts into induced pluripotent stem cells (15). It was also shown that the knockdown of BMI1 inhibits conversion of human fibroblasts into induced pluripotent stem cells (16). The overexpression of BMI1 is also thought to promote stemness and therapy resistance in tumor cells (2, 17).

With respect to its role in senescence and aging, it has been shown that the overexpression of BMI1 results in repression of tumor suppressor p16INK4a and bypass of senescence (18, 19). Deficiency of Bmi1 in mouse results in tissue atrophy, developmental and neurological abnormalities, type II diabetes, and accelerated aging (20–22), underscoring its critical role in pathological conditions. Despite its well documented role in cellular senescence, cancer, and stem cell phenotype, at present, very little is known about the regulation of BMI1. Recently, it was reported that BMI1 is transcriptionally regulated by c-Myc (23). Aberrant activation of the WNT pathway is a common feature of many cancers including breast cancer (24, 25). WNT family proteins are secreted signaling proteins that bind specific receptors to activate intracellular signaling via canonical and non-canonical WNT signaling pathways. In the canonical pathway, the interaction of WNT factors and receptors leads to inhibition of phosphorylation of β-catenin and its destruction by ubiquitin/proteasome machinery (24, 25). The β-catenin then activates transcription of target genes after translocating to the nucleus and by complexing with TCF/LEF (24, 25). WNT inhibitors such as members of Dickkopf (DKK), secreted Frizzled-related protein (SFRP), and WNT inhibitory factor families act as brakes of the WNT pathway by complexing with WNT factors and disrupting their interaction with WNT receptors (24, 25). WNT inhibitors can thus function as growth inhibitors and tumor suppressors. Down-regulation of these inhibitors often occurs in cancer cells, amplifying the aberrant WNT signaling to promote growth and survival of cancer cells (24, 25). One of the important downstream targets of the WNT pathway is c-Myc, which is activated in multiple cancers (24, 26). As PcG proteins including BMI1 often target tumor suppressors such as p16INK4a, we investigated potential

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[2] The abbreviations used are: PcG, Polycomb group; DKK, Dickkopf; qRT-PCR, quantitative real time RT-PCR; TCF, T cell factor; LEF, *lymphocyte enhancer factor;* SFRP, secreted *Frizzled*-related protein; ALDH, aldehyde dehydrogenase; PRC, *polycomb* repressive *complex; CSC,* cancer stem cell; PS, proline-serine rich domain.

TABLE 1
Primer sets
F, forward; R, reverse; QPCR, quantitative PCR.

Real time RT-PCR primer sets	
BMI1	(F) 5′-TGGAGAAGGAATGGTCCACTTC-3′
	(R) 5′-GTGAGGAAACTGTGGATGAGGA-3′
DKK1	(F) 5′CCGCGCCGGGAATCCTGTAC-3′
	(R) 5′-GCAGCGTTTTCGGCGCTTCC-3′
DKK2	(F) 5′-CCAGTACCCGCTGCAATAAT-3′
	(R) 5′-CCAGAAATGACGAGCACAG-3′
DKK3	(F) 5′-CTGTGTGTCTGGGGTCACTG-3′
	(R) 5′-GCTCTAGCTCCCAGGTGATG-3′
c-*Myc*	(F) 5′-CCTACCCTCTCAACGACAGC-3′
	(R) 5′-CTCTGACCTTTTGCCAGGAG-3′
Cyclin D1	(F) 5′-TGGGGAAGTTGAAGTGGAAC-3′
	(R) 5′ATCATCGACGGTGGGTACAT-3′
WNT1	(F) 5′-GCAGGCCGTACGACCGTATTC-3′
	(R) 5′-CGGTTGCCGTACAGGACGCG-3′
BMI (3′-UTR)	(F) 5′-CATTCTATGTAGCCATGTCACTGTG-3′
	(R) 5′-CTGATTCTTACAAAAGCGAATCATT-3′
GAPDH	(F) 5′-GCTAACGGGAAGCTCACTG-3′
	(R) 5′-GTGCTCAGTGTAGCCCAGGA-3′
ChIP qPCR primer sets	
Set 1 (−1003, −702)	(F) 5′-CCACTTTGATCTCACGCGTC-3′
	(R) 5′-CCCGGCTTTGAGGTCCTTCA-3′
Set 2 (−712, −409)	(F) 5′-GGAGGGCAACTGAAGGACCT-3′
	(R) 5′-GAACTTGGGTGCCCTTGCCTG-3′
Set 3 (−419, −127)	(F) 5′-ACCCTTACTGCCAGGCAAGG-3′
	(R) 5′-CGCGGCTGCCTTTATACCGC-3′
Set 4 (−140, +155)	(F) 5′-ATGCTCCGGGCCCGCGGTAT-3′
	(R) 5′-GTGGCGCTCACTCCCAGCAG-3′

TABLE 2
Antibodies for Western blot analyses
pAb, polyclonal antibody.

BMI1	F6 mouse mAb (Millipore)
c-Myc	C-33 mouse mAb (Santa Cruz Biotechnology, Santa Cruz, CA)
Cyclin D1	A-12 mouse mAb (Santa Cruz Biotechnology)
GFP	B2 mouse mAb (Santa Cruz Biotechnology)
β-Catenin	E-5 mouse mAb (Santa Cruz Biotechnology)
Phospho-β-catenin	Ser-33/-37/Thr-41 rabbit pAb (Cell Signaling Technology, Danvers, MA)
DKK1	Rabbit pAb (Epitomics, Burlingame, CA)
WNT1	Rabbit pAb (Abcam, Cambridge, MA)
β-Actin	Mouse mAb (Sigma-Aldrich)

regulation of the WNT pathway and expression of DKK1, DKK2, and DKK3 by BMI1. We report that BMI1 negatively regulates expression of these DKK family members, in particular DKK1, and that their negative regulation results in up-regulation of WNT targets such as c-Myc that participate in a positive feedback loop, activating transcription of *BMI1* gene via an E-box present in its promoter.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture Methods—MCF10A, breast cancer cell lines, and 293T cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The cells were cultured as described previously (3, 23, 27).

Expression Vectors and Expression-related Methods—Retroviral vector overexpressing wild type BMI1 and a BMI1 shRNA-expressing vector for knockdown studies and methods for producing retroviruses, transient transfection, proliferation assays, and colony formation in soft agar have been described previously (3, 23, 27). Lentiviral vectors expressing DKK1 (pCS2-hDKK1) and Wnt1 (pHIV-Wnt1) were obtained from Addgene (Cambridge, MA). The DKK1 shRNA-expressing retroviral vectors were obtained from Origene (Rockville, MD).

Quantitative Real Time RT-PCR Assays—The quantitative real time RT-PCR (qRT-PCR) was carried out in a StepOnePlus real time PCR system (Applied Biosystems, Foster, CA) using total RNA and primers specific for a particular gene as recommended by the manufacturer. The cDNA was generated using oligo(dT) primer mixture and 2.0 μg of total RNA and amplified using primers specific for *BMI1*, *DKK1*, c-*Myc*, Cyclin D1, *WNT1*, and *GAPDH* (Table 1). The PCR conditions consisted of an initial activation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 58 °C for 1 min. The real time amplification was detected using SYBR Green dye, and the Ct (threshold cycle) value of each PCR product was normalized to that of GAPDH control.

Analysis of WNT PCR Array—Gene expression was assessed using a gene-specific Human WNT Pathway TaqMan® Array from Applied Biosystems/Invitrogen. The array set has 96 genes related to the WNT pathway including three housekeeping genes, *GAPDH*, *HPRT1*, and *GUSB*. Total RNA was extracted with a RNAqueous PCR kit (Ambion), and 2 μg of RNA was reverse transcribed using High Capacity cDNA RT kits (Invitrogen). Real time PCR was performed using a StepOnePlus RT-PCR system (Applied Biosystems) with TaqMan Gene Expression Master Mix (Invitrogen) as suggested by the manufacturer. Gene expression data analysis and visualization of differentially expressed genes were conducted using online DataAssist software from Applied Biosystems/Invitrogen (version 3.0). Unsupervised hierarchical cluster analysis was performed based on the ΔCT values using Pearson's correlation as a measure of similarity, and Average Linkage and Global View were used for generating a heat map.

Promoter-Reporter Vectors, Luciferase Assays, and Chromatin Immunoprecipitation (ChIP) Methods—The *BMI1* promoter-reporter constructs pGL3-BmiPrWT and pGL3-BmiPrMut have been described previously (23). The WNT reporter was kindly provided by Dr. Ray Wu (George Washington University, Washington, D. C.). The *DKK1* promoter region was amplified by PCR and cloned in the pGL4.18 luciferase reporter vector (Promega, Madison, WI). Luciferase assays were performed as described (23). ChIP assays were performed as described (28). Briefly, cells were treated with 1% formaldehyde for 20 min at room temperature. The cross-linked chromatin was isolated, sonicated to yield 200–500-bp fragments, and immunoprecipitated using a custom-made rabbit polyclonal antibody raised against BMI1, a mouse monoclonal antibody against H3K27me3 (Millipore, Billerica, MA), and a control IgG. The BMI1-, H3K27me3-, and IgG-bound chromatins were amplified by qPCR using *DKK1* primer sets described in Table 1.

Antibodies and Western Blot Analyses—Western blot analyses were done as described previously (23). The various antibodies and their sources are described in Table 2. For DKK1 and Wnt1 detection, cells were plated on a 100-mm dish, and supernatant was collected 48 h after plating the cells. The supernatant was spun down at 4000 rpm at 4 °C and then concentrated using Amicon ultracentrifugal filter units (Millipore). The protein was quantified using the BCA protein assay kit, and

40 μg was run on SDS-PAGE, transferred to a PVDF membrane, and probed with respective antibodies.

Soft Agar and Mammosphere Formation Assays—The soft agar assay to measure the anchorage-independent growth was performed as described previously (5, 29). For the mammosphere formation assay, cells were trypsinized and carefully resuspended in a serum-free mammosphere culture medium supplemented with MammoCult Proliferation Supplements (StemCell Technologies, Vancouver, Canada). Single cells were plated in ultralow attachment 24-well plates (Corning, Lowell, MA) at a density of 10,000 cells/well in triplicates. After 3–7 days of cell plating, the number of mammospheres was counted and photographed under phase-contrast (×10 magnification). Each experiment was done in triplicates, and the numbers of mammospheres/10,000 seeded cells were plotted.

ALDEFLUOR Assay and Flow Cytometry—Aldehyde dehydrogenase (ALDH) activity was detected using the ALDEFLUOR assay kit as described by the manufacturer (StemCell Technologies). Briefly, cells were resuspended in the assay buffer at 1×10^6 cells/ml and added to a tube containing a 1.5 μM concentration of an activated ALDH substrate, BODIPY aminoacetaldehyde. Half of the sample was transferred to a tube containing a specific inhibitor of ALDH, diethylaminobenzaldehyde, and incubated for 45 min at 37 °C, and the fluorescence intensity of ALDH-positive cells was measured by flow cytometry using a FACSCalibur DxP8 analyzer (BD Biosciences). The data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Statistical Analysis—All experiments were performed at least twice in triplicates for each group. The results are presented as the mean ± S.D. Statistical significance was determined using Student's *t* test, and $p < 0.05$ was considered significant.

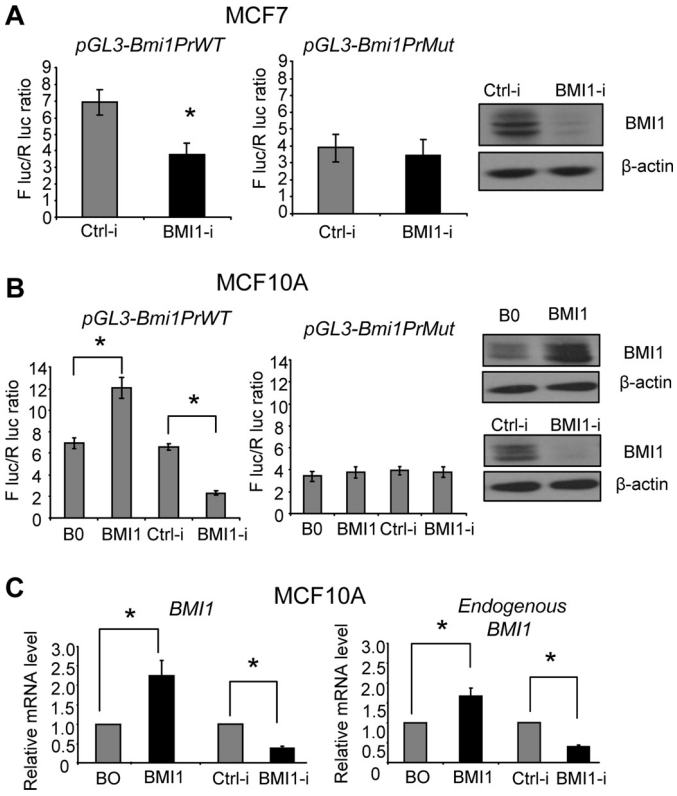

FIGURE 1. **BMI1 autoactivates its promoter.** *A* and *B*, wild type and mutant *BMI1* promoter-reporters constructs (as indicated) with pRL-TK plasmid were transiently transfected into MCF7 (*A*) or MCF10A (*B*) cells, and luciferase (*luc*) assays were performed. *F* and *R* represent Firefly and Renilla respectively. The *error bars* represent the means ± S.D. of three independent experiments. *C*, the qRT-PCR analysis of endogenous BMI1 was performed using primers specific for endogenous BMI1 that amplify the 3′ non-coding region of *BMI1* and RNA isolated from an MCF10A series of cells (*B0* and *Ctrl-i*, controls; *BMI1*, BMI1-overexpressing; *BMI-i*, BMI1 knockdown cells). *Error bars* represent ±S.D. *, $p < 0.05$ (Student's *t* test).

RESULTS

BMI1 Transcriptionally Regulates Its Own Promoter—Many breast cancer cells express high levels of BMI1 (3, 27). We hypothesized that BMI1 can directly or indirectly activate its own expression and thereby provide a positive feedback loop to maintain constitutively high expression in cancer cells. As the *BMI1* promoter contains an E-box, which is evolutionarily conserved in mammalians and other vertebrates (supplemental Fig. S1) (30), we also hypothesized that the E-box to which c-Myc binds may be part of the autoregulation of BMI1. To probe this hypothesis, we performed *BMI1* promoter-reporter assays in control and BMI1 knockdown cells. Cells were transiently transfected with plasmid expressing a luciferase reporter driven by wild type *BMI1* promoter (pGL3-Bmi1PrWT) and *BMI1* promoter with a mutant E-box (pGL3-Bmi1PrMut) (23), and the luciferase activity of the different promoter-reporters was determined. The results showed that cells expressing a BMI1 shRNA exhibited lower activity of the *BMI1* wild type promoter; however, the activity of the E-box mutant promoter remained constitutively low and was not affected by BMI1 knockdown (Fig. 1*A*). Next, we performed promoter-reporter assays in MCF10A control and MCF10A cells that either overexpress BMI1 (MCF10A-BMI1) or underexpress BMI1 (MCF10A-BMI1-i). The results showed that compared with the control cells the activity of the wild type *BMI1* promoter but not the mutant promoter is up-regulated in MCF10A-BMI1 cells, whereas it is down-regulated in MCF10A-BMI1-i cells (Fig. 1*B*). These data therefore suggest that BMI1 can transactivate its own promoter in an E-box dependent manner. To further confirm the autoactivation of *BMI1* transcription, we performed qRT-PCR analysis. The assay was performed using primers specific for endogenous *BMI1* gene and RNA prepared from the MCF10A series of cells (MCF10A-B0, MCF10A-BMI1, MCF10A-Ctrl-i, and MCF10A-BMI1-i). The results confirmed that the expression of endogenous BMI1 is down-regulated in BMI1 knockdown cells, whereas it is up-regulated in cells overexpressing exogenous BMI1 (Fig. 1*C*). Therefore, our data indicate that BMI1 autoactivates its own transcription.

BMI1 Activates WNT Pathway to Up-regulate c-Myc—Because autoactivation of BMI1 is dependent on the E-box to which c-Myc binds and because c-Myc is a target of the WNT pathway, we surmised that c-Myc is a part of positive feedback loop that autoactivates *BMI1* transcription via the WNT pathway. To test this hypothesis, we first determined whether BMI1 can regulate the WNT pathway using a PCR array that contains various WNT pathway-related genes. The PCR array was probed with cDNA prepared from the RNA of MCF10A-Ctrl-i and MCF10A-BMI1-i cells. The results show differential regu-

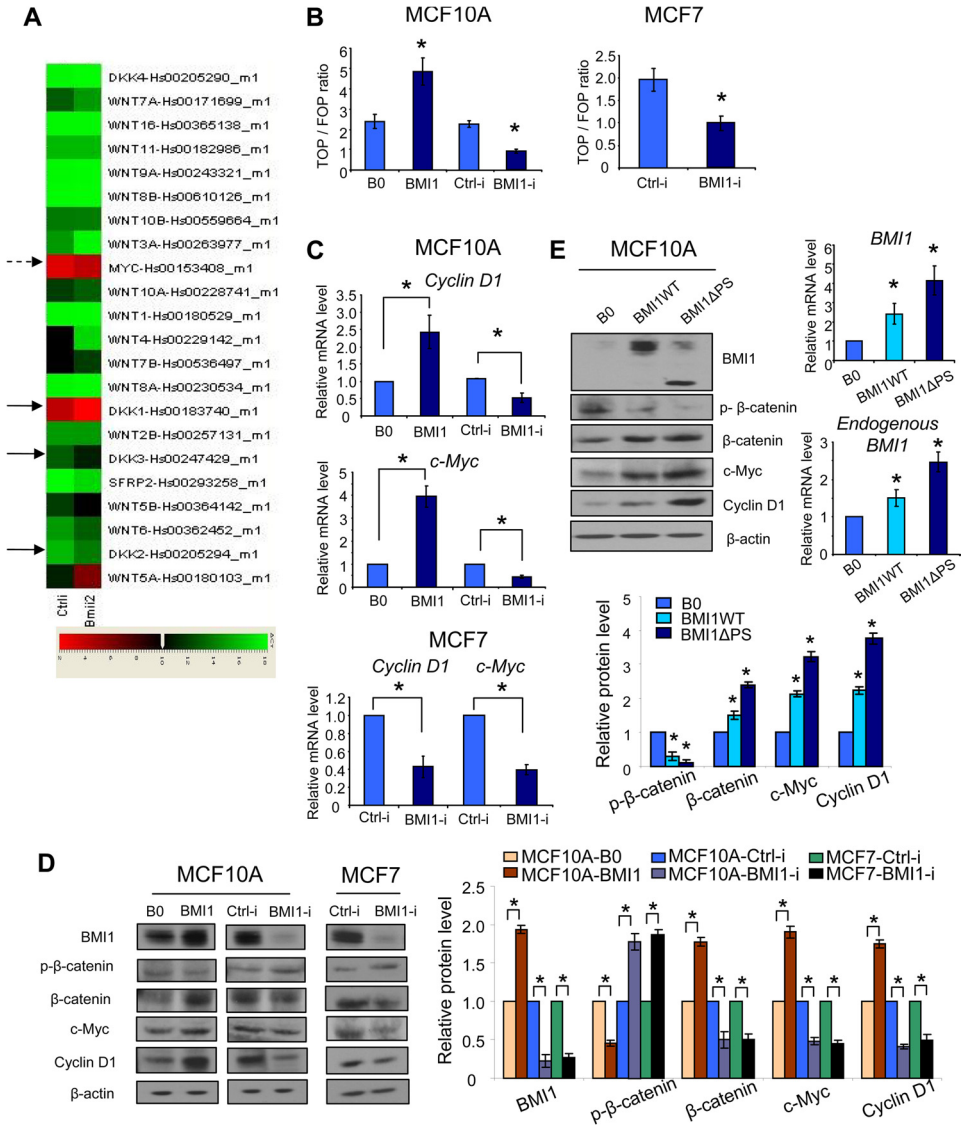

FIGURE 2. **BMI1 autoactivation involves WNT pathway.** *A*, the heat map of the gene expression profile of the human WNT pathway genes in control and BMI1 shRNA-expressing MCF10A cells. The color scale depicts the relative gene expression between the two cell lines: *red* indicates an increase with a ΔCT value below the neutral level, and *green* indicates a decrease with a ΔCT value above the neutral level. The *broken arrow* indicates c-Myc, whereas the *solid arrows* represent DKK1, DKK2, and DKK3. *B*, the LEF/TCF reporter assay was performed to confirm the regulation of the WNT pathway by BMI1. The TOPflash reporter contains wild type LEF/TCF binding sites, whereas the FOPflash reporter contains mutant LEF/TCF binding sites. These constructs were transiently transfected into an MCF10A series, MCF7 control, and MCF7 BMI1 knockdown cells, and the reporter activity was measured as described under "Experimental Procedures." *C*, the qRT-PCR analysis of Cyclin D1 and c-*Myc* was performed using RNA isolated from control, BMI1-overexpressing, and BMI1 knockdown cells as indicated. *D*, Western blot analysis of WNT pathway-related proteins c-Myc, Cyclin D1, β-catenin, and phospho (*p*)-β-catenin in the indicated cell types derived from MCF10A and MCF7 was performed using specific antibodies as described under "Experimental Procedures." *E*, the ΔPS mutant of BMI1 is more active than wild type BMI1 in activating the WNT pathway and up-regulating its target genes, c-Myc and Cyclin D1. The Western blot analysis of WNT pathway genes (as indicated) was performed using total cell extracts of MCF10A-B0, MCF10A-BMI1WT, and MCF10A-BMI1ΔPS cells (*E*, *top panel*). The qRT-PCR analysis of endogenous *BMI1* was performed using MCF10A-derived cells (as indicated) and primers specific for the BMI1 coding region and 3′-UTR (designed for detection of endogenous *BMI*) (*E*, *bottom panel*). A representative Western blot and protein quantification data from three different experiments are shown in *D* and *E*. *Error bars* represent ±S.D. *, $p < 0.05$ (Student's *t* test). *Ctrl*, control.

lation of WNT-related genes by BMI1. The genes that were notably up-regulated in BMI1 knockdown cells were WNT inhibitors such as DKK family of proteins (DKK1, DKK2, and DKK3) and SFRP2 (Fig. 2*A*). Among other factors, WNT factors that are involved in the canonical pathway such as WNT3A, WNT7A, WNT10A, and WNT4 and c-Myc were down-regulated in BMI1 knockdown cells (Fig. 2*A*). WNT5A on the other hand was up-regulated by BMI1 knockdown in MCF10A cells (Fig. 2*A*). Together, these results suggested that BMI1 may down-regulate WNT inhibitors and up-regulate WNT factors that are involved in the WNT canonical pathway. To confirm the array data, we performed a TCF/LEF reporter assay utilizing the widely used TOPflash and FOPflash luciferase reporter plasmids, which measure WNT activity (24, 25). These reporters were transiently transfected into MCF7 and the MCF10A series of cells. The results showed that compared with control cells the reporter exhibited higher activity in BMI1-overexpressing cells, and lower activity of the reporter was seen in BMI1 knockdown cells (Fig. 2*B*). Based on these data, we conclude that BMI1 activates the TCF/LEF reporter,

and thus, BMI1 very likely regulates the WNT canonical pathway.

We further confirmed array and TCF/LEF reporter assay data using qRT-PCR for Cyclin D1 and c-*Myc* in these cells. Our results confirmed that indeed Cyclin D1 and c-*Myc* are transcriptionally regulated by BMI1 (Fig. 2*C*). We also performed Western blot analyses of total and phospho-β-catenin, Cyclin D1, and c-Myc. The results showed down-regulation of total β-catenin, Cyclin D1, and c-Myc in BMI1 knockdown cells and their up-regulation in BMI1-overexpressing cells (Fig. 2*D*, *left panel*). The expression of phospho-β-catenin on the other hand was down-regulated in BMI1-overexpressing cells and up-regulated in BMI1 knockdown cells (Fig. 2*D*, *left panel*). Similar data were obtained with MCF7 cells where we probed control and BMI1 knockdown cells for the proteins indicated above (Fig. 2*D*, *right panel*). We have recently shown that deletion of the PS region of BMI1 results in an increase in BMI1 oncogenic activity (31). Hence, we determined whether the PS mutant of BMI1 is more active in inducing WNT target genes. Our results indicated that compared with wild type BMI1 the PS mutant of BMI1 induced higher expression of c-Myc and Cyclin D1 (Fig. 2*E*). The mutant was also more active in inducing endogenous BMI1 (Fig. 2*E*). Taken together, our data strongly suggest that BMI1 up-regulates the WNT pathway and its target genes.

BMI1 Expression Inversely Correlates with Expression of DKK Family of Proteins in Breast Cancer Cells—As our array data suggested that BMI1 may repress WNT inhibitors, in particular the DKK family of proteins, and BMI1 is known to be overexpressed in many breast cancer cell lines, we examined whether there is an inverse correlation between the expression of BMI1 and DKK1, DKK2, and DKK3 in breast cancer cells. We performed qRT-PCR for *DKK1*, *DKK2*, and *DKK3* using RNA isolated from MCF10A and several breast cancer cell lines. To correlate the expression of *DKKs* with BMI1, we performed Western blot analysis of BMI1. Our data indicated that, consistent with published data, in general BMI1 is overexpressed in breast cancer cell lines, whereas DKK family proteins were either not expressed or expressed at lower levels in most breast cancer cell lines (Fig. 3*A* and supplemental Fig. S2). However, some exceptions were noted; for example, compared with MCF10A cells, *DKK1* was high in MDA-MB-231 cells, whereas *DKK2* was expressed at higher levels in BT474 and HS578t cells (Fig. 3*A* and supplemental Fig. S2). To establish an inverse correlation between the expression of BMI1 and DKK1, we determined the Pearson's correlation coefficient (r) of expression of BMI1 and DKK1; it showed a negative correlation ($r = -0.3$, $p < 0.00001$) (Fig. 3*A*). The negative correlation between BMI1 and DKK1 was further strengthened if MDA-MB-231 was not considered ($r = -0.6$, $p < 0.0001$). We further determined the effect of knockdown of BMI1 on the DKK family of proteins in MCF10A cells. Of four known DKK proteins, DKK4 expression was found to be very low in our cell system, and it did not change significantly between control and BMI1 knockdown cells. Hence, we focused on DKK1, DKK2, and DKK3. The expression of *DKK1*, *DKK2*, and *DKK3* was determined in control and a BMI1 knockdown derivative of MCF10A cells using qRT-PCR analysis. The results showed that BMI1 knockdown up-regulated *DKK1*, *DKK2*, and *DKK3* (Fig. 3*B*). Taken together, our data suggested that BMI1 can regulate members of the DKK family of WNT inhibitors.

We also determined the expression of WNT1, Myc, and DKK1 in an MCF10A tumor progression series consisting of MCF10A-B0 (normal immortal), MCF10A-BMI1 (epithelial to mesenchymal transition-positive and partially transformed), MCF10A-Ras (transformed), and MCF10A-BMI1+Ras (transformed, aggressive, and metastatic) (5, 6). Our data showed an increased expression of WNT1 and c-Myc and correspondingly decreased expression of DKK1, which correlated with an aggressive phenotype of the cells (Fig. 3*D*).

BMI1 Transcriptionally Regulates DKK1—Next, we focused on the potential mechanism of regulation of the WNT pathway by BMI1. Because among all DKKs DKK1 was expressed at higher levels in normal mammary epithelial cells and showed better inverse correlation with BMI1, we further studied potential regulation of the WNT pathway by BMI1 through DKK1. Because PcG proteins function as transcriptional repressors, we hypothesized that BMI1 may act as a transcriptional repressor of the DKK family of WNT inhibitors and that BMI1 could regulate its own expression via down-regulation of DKK1, activation of the canonical WNT pathway, and up-regulation of c-Myc. First, we observed that BMI1 overexpression repressed DKK1 expression, confirming results obtained from BMI1 knockdown studies (Fig. 3*C*). We also confirmed qRT-PCR data suggesting DKK1 regulation by BMI1 using Western blot analyses of control, BMI1-overexpressing, and BMI1 knockdown cells (Fig. 3*C*). Similar to MCF10A cells, BMI1 knockdown up-regulated expression of DKK1 at the RNA level in MCF7 and MDA-MB-453 breast cancer cells lines (supplemental Fig. S3). Based on data from normal immortal human mammary epithelial cells (MCF10A) and breast cancer cell lines, we conclude that BMI1 transcriptionally regulates *DKK1*.

Next, we cloned a 1-kb promoter region of *DKK1* gene into a luciferase vector and performed promoter-reporter assays. The relative promoter activity of *DKK1* was examined in an MCF10A series of cells and MCF7-derived BMI1 knockdown cells using transient transfections. The results showed that indeed BMI1 overexpression repressed *DKK1* promoter, whereas BMI1 knockdown up-regulated BMI1 promoter activity (Fig. 4*A*). To demonstrate that BMI1 directly binds to *DKK1* promoter, we performed a ChIP assay using a set of four primers covering a 1-kb region of *DKK1* promoter. The results of ChIP-qPCR suggested that indeed BMI1 binds to *DKK1* promoter (Fig. 4*B*). The highest binding of BMI1 was noticed in region 1 (further upstream from the transcription initiation site), and the lowest binding was detected near the transcription initiation site (region 4). The binding of H3K27me3 mirrored the binding of BMI1 to *DKK1* promoter sequences, which is consistent with PRC-mediated repression involving PRC1 and PRC2. We further chose to study region 1 and region 4 in MCF7 and MDA-MB-453 cells in which BMI1 is expressed at high levels. The data showed that, similar to MCF10A cells, in cancer cells BMI1 showed high binding to region 1 and low binding to region 4. Our results also showed that relative to MCF10A cells BMI1 binding to *DKK1* promoter is increased severalfold in MCF7 and MDA-MB-453 cells, suggesting that in cancer cells BMI1 overexpression leads to increased binding of

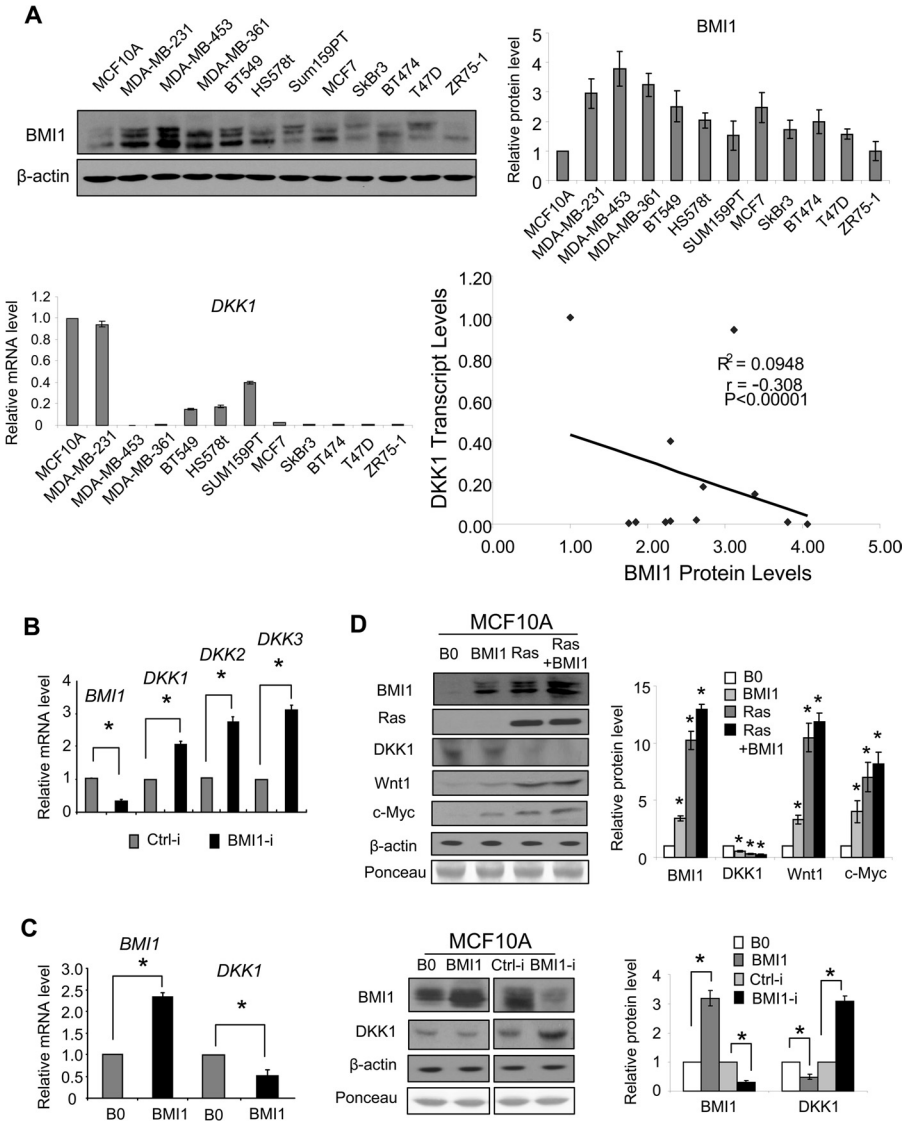

FIGURE 3. **The expression of BMI1 and DKK1 inversely correlates.** *A*, Western blot analysis of BMI1 and qRT-PCR analysis of DKK1 were performed using total cell extract or RNA isolated from MCF10A and various breast cancer cell lines as indicated. The *error bars* represent the means ±S.D. of three independent assays. The Pearson's correlation coefficient (*r*) was calculated using Microsoft Excel data analysis tools. *B*, knockdown of BMI1 up-regulates DKK1, DKK2, and DKK3. qRT-PCR analysis of BMI1 and DKK family members was carried out using specific primers and RNA isolated from an MCF10A series of cells (described in Fig. 1). *C*, BMI1 regulation of DKK1 was studied in MCF10A control and BMI1-overexpressing MCF10A cells using qRT-PCR and Western blot analysis as described under "Experimental Procedures." Each experiment was done in triplicates. *Error bars* represent ±S.D. *, $p < 0.05$ (Student's *t* test). *D*, the expression of BMI1, DKK1, Wnt1, and c-Myc was studied in an MCF10A tumor progression series using a Western blot analysis. A representative Western blot and protein quantification data are shown in *C* and *D*. *Ctrl*, control.

BMI1 to *DKK1* promoter and consequently increased repression of DKK1 (Fig. 4*C*).

WNT Pathway Regulates BMI1 Expression—Because BMI1 is a target of the WNT pathway and DKK1 is a repressor of the WNT pathway, we determined whether DKK1 regulates BMI1 expression via a negative feedback loop. We transiently transfected 293T cells with increasing amounts of DKK1-expressing plasmid and determined the expression of endogenous BMI1 as well as Cyclin D1 and c-Myc (Fig. 5*A*). The results showed that increasing doses of DKK1 proportionally down-regulated c-Myc, BMI1, and Cyclin D1 (Fig. 5*A*). To further confirm our results, we generated MCF10A cells stably expressing DKK1 shRNAs, which showed ~50% knockdown efficiency, and determined the expression of BMI1 and c-Myc. Our results showed that the knockdown of DKK1 up-regulated c-Myc and BMI1 at both mRNA and protein levels (Fig. 5*B*). These data validate our hypothesis that DKK1 can regulate expression of BMI1 via inhibition of the canonical WNT pathway. Taken together, our data suggest that BMI1 and DKK1 can regulate expression of each other via a negative feedback loop.

Next, we determined whether WNT1 can up-regulate BMI1 expression via c-Myc induction. We transiently transfected 293T cells with increasing doses of a mouse Wnt1-expressing plasmid and determined the expression of BMI1, c-Myc, and Cyclin D1. Our data showed a dose-dependent increase in the expression of c-Myc and its target genes Cyclin D1 and BMI1 (Fig. 6*A*). Next, to determine whether exogenous DKK1 and Wnt1 regulated BMI1 via the E-box (c-Myc binding site) in *BMI1* promoter, we performed promoter-reporter assays in 293T and MCF7 cells. Our results indicated that indeed Wnt1

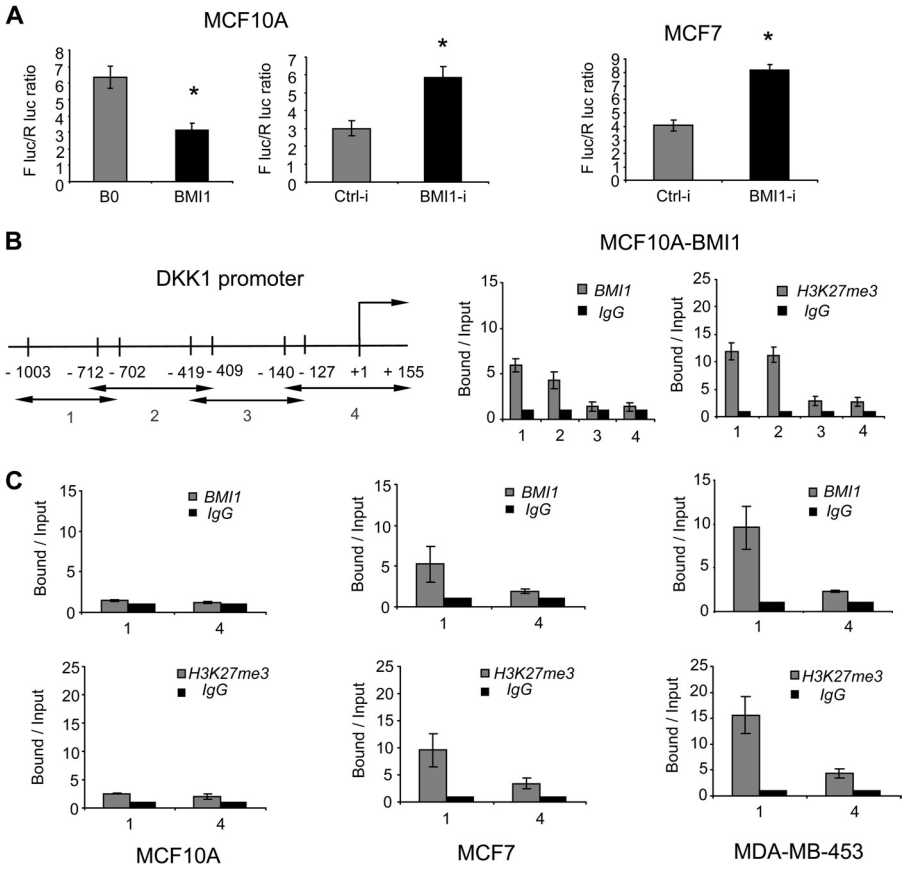

FIGURE 4. **BMI1 regulates *DKK1* promoter and binds to it.** *A*, the *DKK1* promoter (1 kb upstream) was cloned in pGL4.18 luciferase reporter vector, and its activity was determined as described under "Experimental Procedures." *F* and *R* represent Firefly and Renilla, respectively. *Error bars* represent ±S.D. *, $p < 0.05$ (Student's *t* test). *B* and *C*, binding of BMI1 and H3K27me3 to *DKK1* promoter was determined using a ChIP assay in the indicated set of cells and four different sets of primers covering 1 kb of promoter region as described under "Experimental Procedures." *Ctrl*, control.

activate *BMI1* wild type but not the mutant promoter to which c-Myc cannot bind (Fig. 6*B*). Similarly, DKK1 repressed wild type but not the mutant promoter, and Wnt1 up-regulated BMI1 promoter in MCF7 cells (Fig. 6*C*). Thus, Wnt1 induces expression of BMI1 via c-Myc up-regulation, which transcriptionally activates *BMI1* expression, and DKK1 inhibits *BMI1* promoter activity in an E-box dependent manner. Taken together, our data suggest that the WNT pathway regulates BMI1 expression via a positive feedback loop.

BMI1 and Wnt1 Mediate Oncogenic Activity of Each Other—Next, to determine the functional significance of BMI1 autoregulation and the role of BMI1 in the WNT pathway, we determined whether the WNT-dependent increase in oncogenic activity depends on BMI1 and vice versa. We overexpressed Wnt1 in MCF7 cells in which BMI1 expression was reduced using stable expression of a BMI1 shRNA and examined cells for proliferation as well as colony formation in soft agar. The results indicated that BMI1 knockdown inhibited proliferation as well as colony formation in soft agar and that Wnt1 overcomes the inhibitory effect of BMI1 knockdown (Fig. 7*A*). Results also indicated that Wnt1 increased proliferation as well as colony formation activity in soft agar in control cells but not in BMI1 knockdown cells. Similar data were obtained in MDA-MB-453 cells where exogenous Wnt1 reversed proliferation inhibition and decreased colony formation in BMI1 knockdown cells (supplemental Fig. S4).

BMI1 Overrides Tumor Suppressive Activity of DKK1—To further determine the functional role of BMI1 in the WNT pathway, we determined whether BMI1 is a target of the tumor suppressive activity of DKK1 in MCF7 cells. Our results indicated that DKK1 inhibited proliferation of control but not exogenous BMI1-overexpressing MCF7 cells (Fig. 7*B*). Furthermore, the results of the soft agar colony formation assay showed that the exogenous BMI1 expression overcomes DKK1-mediated inhibition of colony formation in soft agar (Fig. 7*B*). Similar data were obtained in 293T and MDA-MB-453 cells, which stably overexpressed exogenous BMI1. In these cells, transient transfection of DKK1 (293T cells) or stable overexpression of DKK1 (MDA-MB-453 cells) did not cause proliferation attenuation or decreased colony formation in soft agar (supplemental Fig. S5, A and B). These data suggest that BMI1 is a target of growth inhibitory activity of DKK1 and that it may be required for DKK1-mediated tumor suppressive activity.

Next, we determined whether DKK1 is required for oncogenic activity of BMI1. We co-expressed shRNAs of BMI1 and DKK1 in MCF7 cells and determined the effect of knockdown of BMI1 alone or knockdown of both BMI1 and DKK1. The results indicated that BMI1 knockdown inhibits proliferation of MCF7 cells and that further DKK1 knockdown can partially rescue the proliferation defect of these cells, suggesting that the BMI1 knockdown effect is mediated via up-regulation of DKK1 (Fig. 7*C*). Next, cells expressing either BMI1 shRNA or both

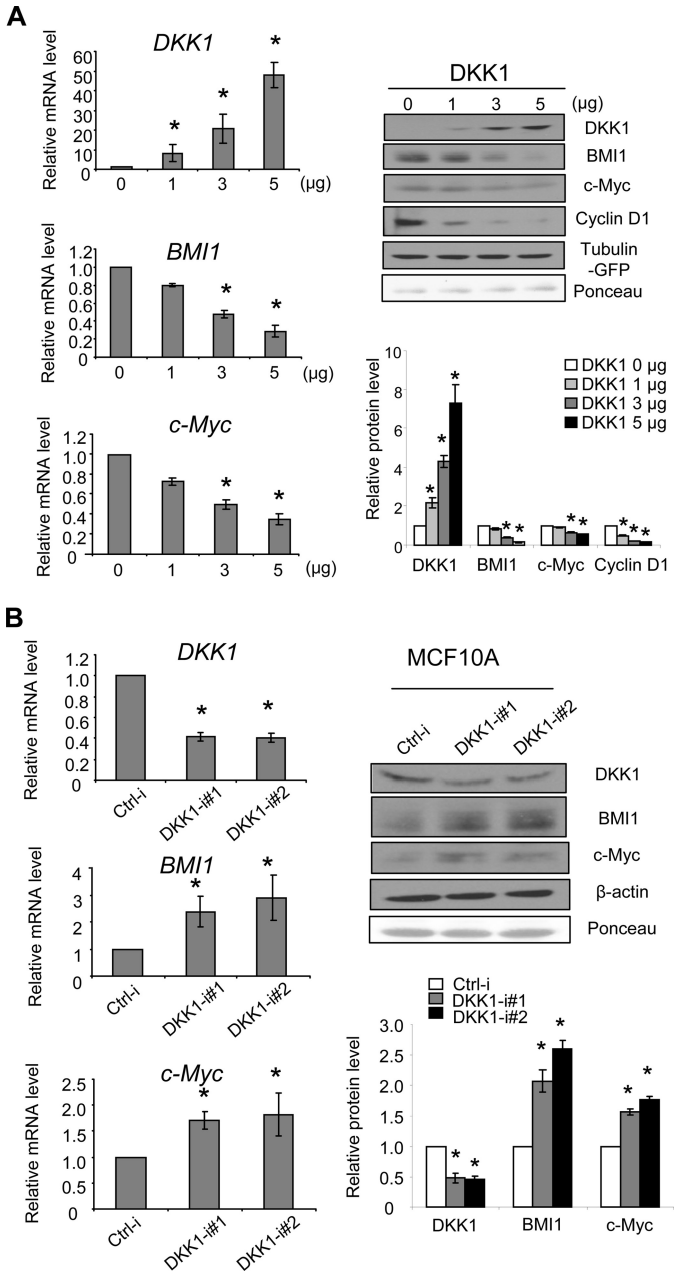

FIGURE 5. **DKK1 regulates BMI1 expression.** *A*, DKK1-expressing plasmid (0–5 μg) was transiently transfected into 293T cells, and the expression of DKK1, BMI1, and c-Myc was determined by qRT-PCR analysis (*left panel*) and Western blot analysis (*right panel*). GFP-tubulin plasmid was transfected as a loading control with DKK1 plasmid. Ponceau staining of the blot was also done to ascertain equal loading of supernatant extract used for DKK1 probing. *B*, MCF10A cells with DKK1 knockdown were generated and analyzed for the expression of DKK1, BMI1, and c-Myc using qRT-PCR and Western blot analysis. In each case, a representative Western blot and protein quantification data are shown. *Error bars* represent ±S.D. *, $p < 0.05$ (Student's *t* test). *Ctrl*, control.

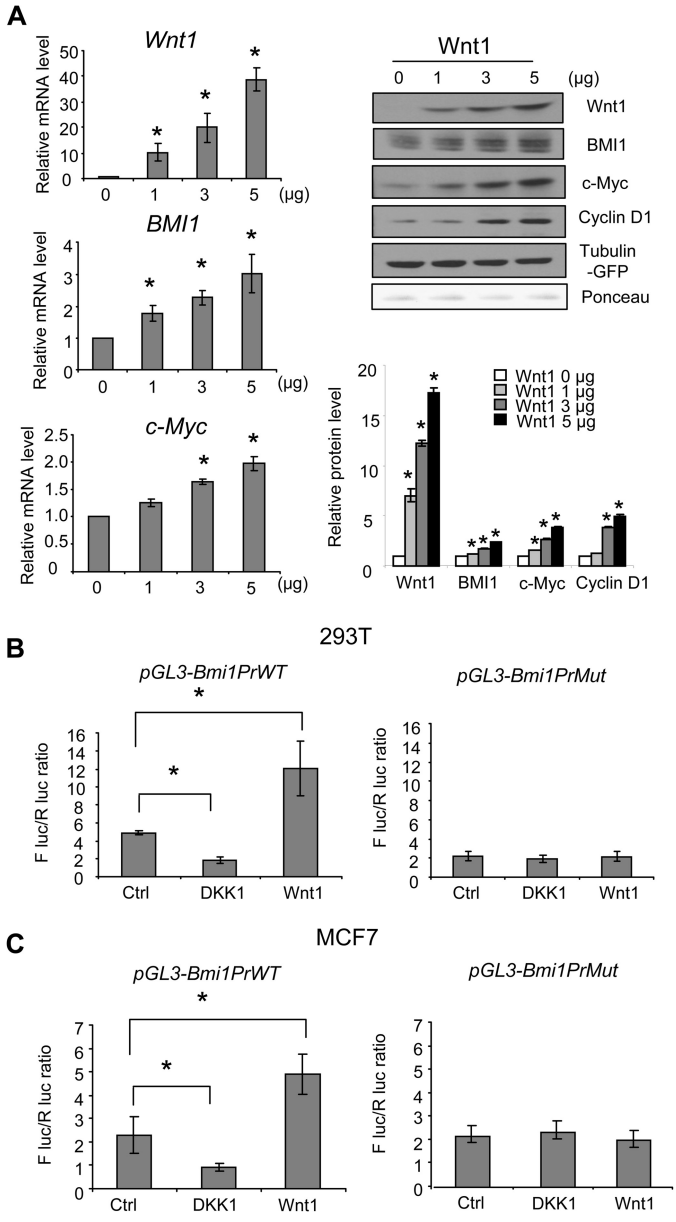

FIGURE 6. **WNT pathway regulates BMI1 expression.** *A*, 293T cells were transiently transfected with a Wnt1-expressing plasmid (0–5 μg), and the expression of Wnt1, BMI1, and c-Myc was determined by qRT-PCR (*left panel*) and Western blot analysis (*right panel*). Controls are described in Fig. 5*A*. A representative Western blot and protein quantification data are shown (*right panel*). *B*, promoter-reporter assays using pGL3-Bmi1PrWT and pGL3-Bmi1PrMut vectors were performed in 293T cells transiently transfected to overexpress DKK1 or Wnt1. *F* and *R* represent Firefly and Renilla respectively. *C*, the reporter activity of wild type and mutant *BMI1* promoters was determined in MCF7 cells stably expressing DKK1 or Wnt1 (as indicated). *Error bars* represent ±S.D. *, $p < 0.05$ (Student's *t* test). *Ctrl*, control.

DKK1 and BMI1 shRNAs with controls were studied for colony formation in soft agar. The results indicated that BMI1 knockdown results in reduced proliferation as well as decreased colony formation in MCF7 cells and that further knockdown of DKK1 can partially reverse the effect of BMI1 knockdown (Fig. 7*C*). Similar data were obtained in MDA-MB-453 cells where DKK1 knockdown partially reversed the effect of BMI1 knockdown on proliferation and colony formation in soft agar (supplemental Fig. S6). Collectively, our data indicate that DKK1 is required for the pro-oncogenic activity of BMI1.

Cross-regulation of BMI1 and DKK1 Plays a Role in Cancer Stem Cell Phenotype—One of the phenotypes that BMI1 is known to be associated with is cancer stem cell (CSC) phenotype. To determine the relevance of cross-regulation of BMI1 and WNT inhibitor DKK1 on CSC phenotype, we carried out mammosphere formation and ALDEFLUOR assays, both of which are widely used to define/identify mammary stem cells and breast CSCs (32, 33). First, we confirmed that indeed over-

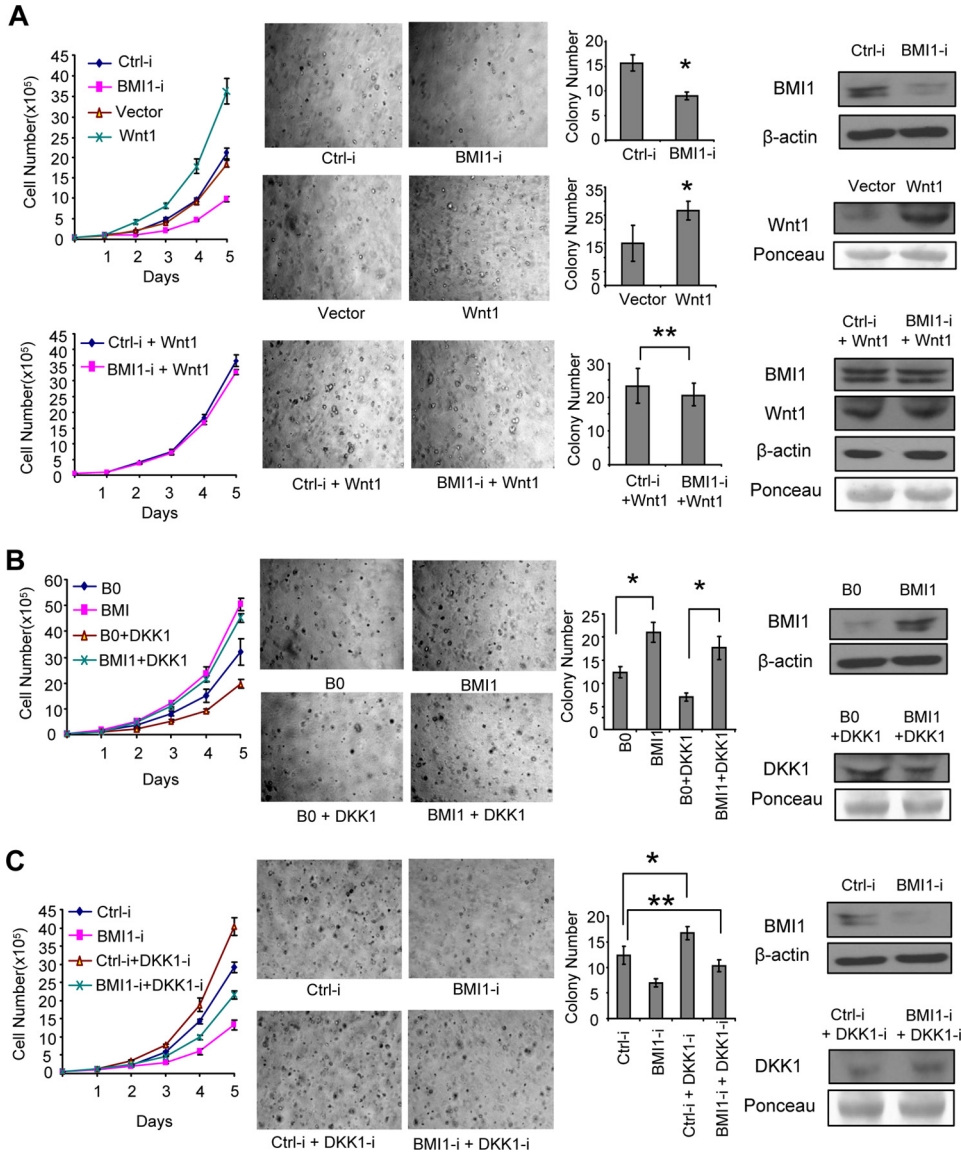

FIGURE 7. **BMI1 and WNT regulators modulate oncogenic activity of each other.** *A*, MCF7 cells expressing a control shRNA (*Ctrl-i*) or BMI1 shRNA (*BMI1-i*) were infected with an empty vector (*vector*) or a Wnt-expressing retroviral vector (*Wnt1*), and resulting cells were studied for cell proliferation (*left panel*) and colony formation in soft agar (*right panel*). *B*, the ability of BMI1 to overcome DKK1 tumor suppressor activity in MCF7 cells was studied using cell proliferation and colony formation assays in soft agar (as indicated). *C*, knockdown of DKK1 restores proliferation and colony formation in soft agar in MCF7 cells that express BMI1 shRNA. The combinations of cells expressing a single shRNA (BMI1 or DKK1) or both shRNAs (BMI1 and DKK1) were generated using respective retroviral vectors. *Error bars* represent ±S.D. *, $p < 0.05$ (significant); **, $p = 0.08$ (not significant) (Student's *t* test).

expression of BMI1 increases and its knockdown decreases the number of mammospheres and the fraction of ALDH-positive cells in the MCF10A cell line (supplemental Fig. S7). Next, mammosphere formation and ALDEFLUOR assays were carried out in MCF7 cells expressing different combinations of overexpression and/or knockdown of BMI1, DKK1, and Wnt1. Our results showed that knockdown of BMI1 decreased whereas Wnt1 overexpression increased the number of mammospheres in MCF7 cells (Fig. 8*A*, *upper panel*). Furthermore, Wnt1 overexpression in BMI1 knockdown cells restored the number of mammospheres, suggesting that BMI1 knockdown modulated the number of mammospheres via the WNT pathway. Similarly, BMI1 knockdown modestly decreased the fraction of ALDH-positive cell population, which was overcome by exogenous expression of Wnt1 (Fig. 8*A*, *lower panel*). Overexpression of DKK1 in MCF7 cells also resulted in a decrease in the number of mammospheres and fraction of ALDH-positive cells that could be restored by the exogenous expression of BMI1 (Fig. 8*B*). Thus, BMI1 knockdown decreases the number of CSCs as determined by mammosphere formation and ALDEFLUOR assays. Based on these results, we hypothesized that BMI1 knockdown may affect CSCs via up-regulation of DKK1. To test this hypothesis, we generated cells expressing shRNAs for both BMI1 and DKK1 and carried out mammosphere and ALDEFLUOR assays. Indeed, our data indicated that knockdown of DKK1 could overcome the effect of BMI1 knockdown on CSC phenotype (Fig. 8*C*). The other most used marker of breast CSCs is $CD44^{high}/CD24^{-/low}$ (34). Because the MCF7 cell line contains undetectable number of such cells, we used MCF10A-Ras transformed cells (5, 6) to determine

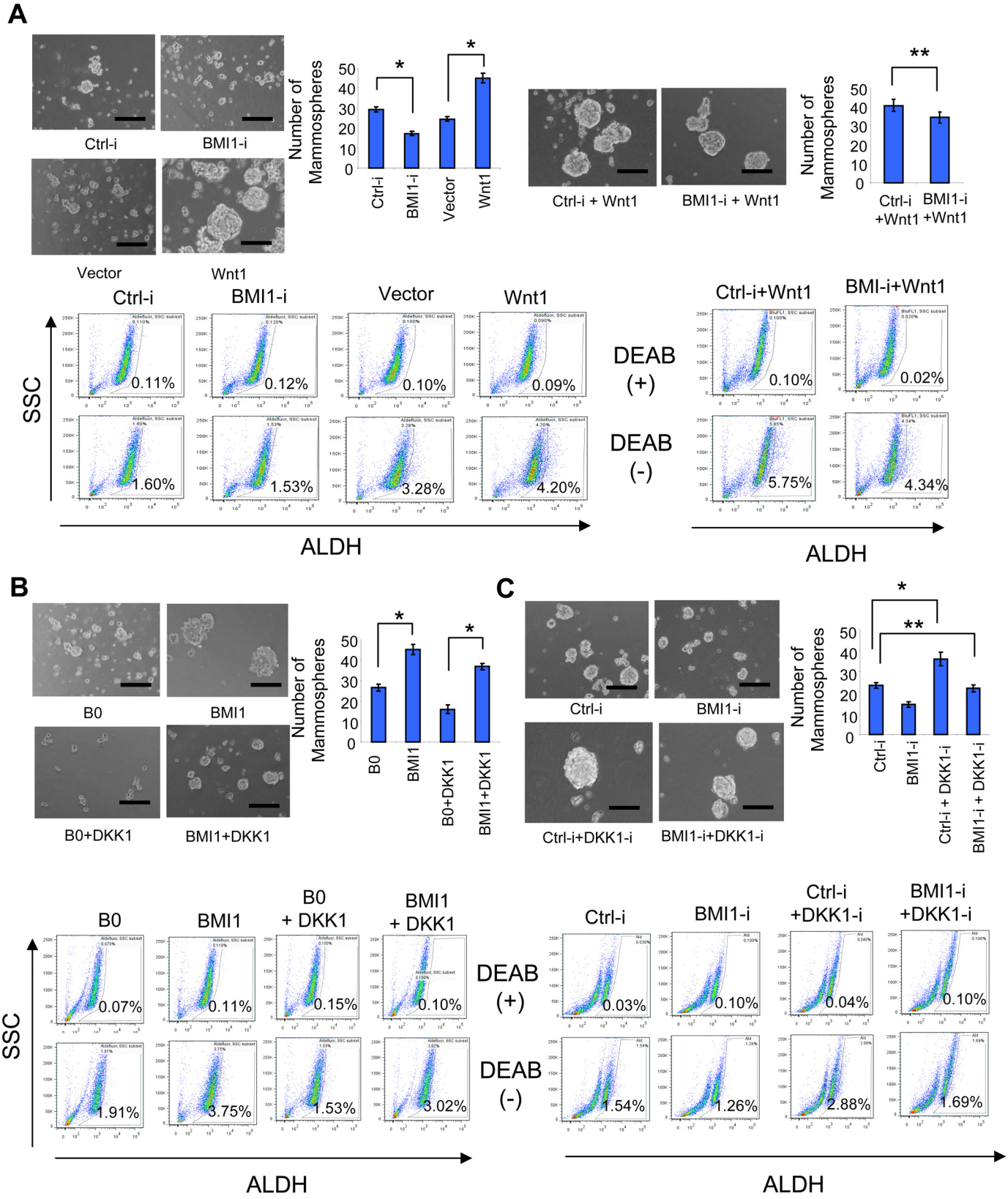

FIGURE 8. **BMI1 and DKK1 regulate breast cancer stem cell phenotype.** *A*, knockdown of BMI1 results in a decrease in the number of CSCs, which can be restored by Wnt1 overexpression. *B*, DKK1 decreases the number of CSCs, which are restored by BMI1 overexpression. *C*, decrease in the number of mammospheres and ALDH-positive fraction of cells in BMI1 knockdown cells is overcome by knocking down the expression of DKK1. Mammosphere formation (*upper panel*) and ALDEFLUOR (*lower panel*) assays were carried out using MCF7-derived cells as indicated (*upper panel*). Assays were performed as described under "Experimental Procedures." In the case of ALDEFLUOR assays, diethylaminobenzaldehyde (*DEAB*; ALDH inhibitor)-treated cells were used to set the background. For the mammosphere formation assay, the average diameter of spheres was >50 μm. *Error bars* represent $\pm$S.D. *, $p < 0.05$ (significant); **, $p > 0.05$ (not significant) (Student's *t* test). *Ctrl*, control.

whether DKK1 overexpression decreased the number of $CD44^{high}/CD24^{-/low}$ cells and whether BMI1 overexpression could restore the number of such cells. Our results suggested that indeed DKK1 overexpression or BMI1 knockdown decreases the number of $CD44^{high}/CD24^{-/low}$ cells, and BMI1 overexpression in DKK1 knockdown cells restores the fraction of $CD44^{high}/CD24^{-/low}$ cells (supplemental Fig. S8). Taken together, our results suggest that BMI1 likely modulates CSC phenotype via regulation of DKK1 and the WNT pathway.

DISCUSSION

The role of PcG proteins in cancer development and stem cell phenotype is well documented (35). The primary mechanism by which PcG proteins promote oncogenesis and metastasis appears to be by senescence bypass (18, 19) and increased cell survival (3, 6). The exact mechanism by which BMI1 exhibits its oncogenic activity is not known. The prime relevant target of PcG proteins, the *p16INK4a* locus, is methylated or deleted in most breast cancer cells. Hence, during breast cancer development, PcG proteins such as BMI1 are likely to target other tumor suppressors and/or growth-regulatory pathways. Indeed, PcG protein EZH2 was recently reported to promote hepatocellular carcinoma via regulation of the WNT pathway (36), and cross-talk between EZH2 and the WNT pathway has been reported (37).

In this study, we examined whether BMI1 can regulate the WNT pathway and up-regulate its target genes such as Cyclin D1 and c-Myc. Indeed, our data suggest that BMI1 can up-regulate the canonical WNT pathway and that its overexpression leads to up-regulation of Cyclin D1 and c-Myc. With respect to the mechanism of regulation of the WNT pathway, BMI1 appears to transcriptionally repress expression of WNT inhibitors such as the DKK family of proteins. Recently, it was reported that in lung cancer cells tobacco smoke can induce PcG-mediated repression of DKK1 (38). Our data are consistent with this finding and suggest that the autoactivation of BMI1 can lead to further down-regulation of DKK1 and potentiation of carcinogenic activity of tobacco smoke. DKK1 is down-regulated in many cancer cells, and it is known to function as a potent tumor suppressor by antagonizing the WNT pathway and inhibiting cell proliferation, migration, and invasion (39). The mechanism of down-regulation of DKK1 in cancer cells is not very well understood. Our data suggest that BMI1, which is often overexpressed in cancer cells, is a direct transcriptional repressor of DKK1. Other WNT inhibitors such as DKK2, DKK3, and SFRP2 also appear to be repressed by BMI1 in breast cancer cells. It is conceivable that tobacco smoke could regulate all these inhibitors by up-regulating BMI1 and other PcG proteins. In addition to repression of WNT inhibitors, our ongoing studies suggest that BMI1 may directly up-regulate the WNT pathway and its target by transcriptionally up-regulating WNT factors such as WNT3A, WNT7A, WNT10A, and WNT4. The mechanism of up-regulation of certain WNT factors by BMI1 is not clear at present.

Because *BMI1* is a transcriptional target of c-Myc, our results led us to examine an interesting possibility that BMI1 may autoregulate its expression via a positive feedback loop involving c-Myc. Indeed, the promoter-reporter assay and the qRT-PCR

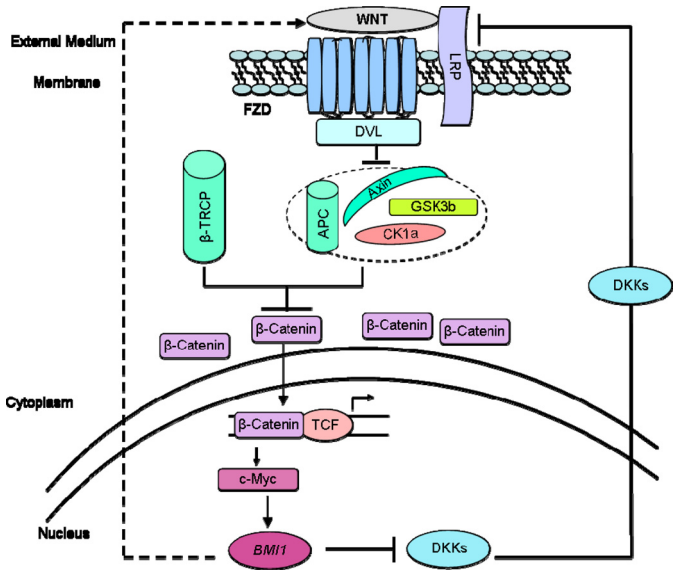

FIGURE 9. **Schematic model of autoregulation of BMI1.** BMI1 activates the WNT pathway by repressing the expression of DKK family members (*DKKs*), which leads to up-regulation of c-Myc. c-Myc in turn up-regulates BMI1 via a c-Myc binding site (E-box) that is present in its promoter. BMI1 can also directly activate expression of certain WNT factors (*broken line*), resulting in up-regulation of c-Myc and ultimately transcriptional up-regulation of *BMI1*. *FZD*, Frizzled; *LRP*, LDL *receptor-related protein*; *DVL*, Dishevelled; *β-TRCP*, *β*-transducin repeat-containing protein; *APC*, anaphase-promoting complex.

assay using primers specific for endogenous BMI1 suggest that BMI1 positively autoregulates its expression and that the autoregulation of BMI1 is mediated via activation of the WNT pathway and its downstream target, c-Myc, by BMI1 (Fig. 9). Recently, it was reported that in colon cancer cells the WNT pathway can regulate expression of BMI1 (40). We confirmed that, similar to colon cancer cells, overexpression of Wnt1 and knockdown of DKK1 lead to BMI1 up-regulation in human mammary epithelial cells. Our new data suggest that although the WNT pathway regulates BMI1 conversely BMI1 also regulates the WNT pathway via direct repression of DKK family members and up-regulation of WNT factors (Fig. 9). Both of these regulations converge at c-Myc (Fig. 9). Interestingly, the promoter region of *DKK1* contains putative binding sites for *β*-catenin·TCF, and it has been shown that *β*-catenin·TCF complexes up-regulate DKK1 expression (41, 42). DKK1 autoregulation presents a negative feed back loop, which controls its expression. In this scenario, up-regulation of the WNT pathway may lead to up-regulation of DKK1, which then will inhibit expression of c-Myc and its target BMI1. Inhibition of BMI1 will likely lead to down-regulation of the WNT pathway and its targets including c-Myc and DKK1. Down-regulation of DKK1 may lead to up-regulation of BMI1 via c-Myc expression. At the same time, BMI1 will likely down-regulate DKK1, further up-regulating the WNT pathway and its target, c-Myc, leading to an increase in *BMI1* transcription (Fig. 9). It is expected that such regulation might favor BMI1 overexpression in cancer cells via increased WNT activity and c-Myc expression. The resulting BMI1 overexpression is likely to increase the fraction of CSCs and impart drug resistance to tumors. On the other hand, increased expression of WNT inhibitors such as DKK1 might favor BMI1 down-regulation in senescent and aged tis-

sues where it is likely to result in up-regulation of p16INK4a and exhaustion of the stem cell pool. Thus, a fine-tuned positive feedback loop controls BMI1 expression via the WNT signaling pathway, and this feedback loop may be relevant to the role of BMI1 in cancer, stem cells, and aging.

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