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Musashi1 Modulates Mammary Progenitor Cell Expansion through Proliferin-Mediated Activation of the Wnt and Notch Pathways▽†

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The RNA-binding protein Musashi1 (Msi1) is a positive regulator of Notch-mediated transcription in *Drosophila melanogaster* and neural progenitor cells and has been identified as a putative human breast stem cell marker. Here we describe a novel functional role for Msi1: its ability to drive progenitor cell expansion along the luminal and myoepithelial lineages. Expression of Msi1 in mammary epithelial cells increases the abundance of $CD24^{hi}$ $Sca\text{-}1^{+}$, $CD24^{hi}$ $CD29^{+}$, CK19, CK6, and double-positive CK14/CK18 progenitor cells. Proliferation is associated with increased proliferin-1 (PLF1) and reduced Dickkopf-3 (DKK3) secretion into the conditioned medium from Msi-expressing cells, which is associated with increased colony formation and extracellular signal-regulated kinase (ERK) phosphorylation. Treatment with the MEK inhibitor U0126 inhibits ERK activation and decreases Notch and β-catenin/T-cell factor (TCF) reporter activity resulting from Msi1 expression. Reduction of DKK3 in control cells with a short hairpin RNA (shRNA) increases Notch and β-catenin/TCF activation, whereas reduction of PLF1 with a shRNA in Msi1-expressing cells inhibits these pathways. These results identify Msi1 as a key determinant of the mammary lineage through its ability to coordinate cell cycle entry and activate the Notch and Wnt pathways by a novel autocrine process involving PLF1 and DKK3.

The regenerative capacity of the mammary gland following postlactation involution resides in multipotent stem cells within the luminal tissue. Mammary stem cells exist as a quiescent and self-renewing population capable of giving rise to ductal, alveolar, and myoepithelial cells. The presence of mammary stem cells was first suggested by the ability of hyperplastic mammary tissue to generate complete mammary outgrowths upon engraftment into the cleared prepubertal mammary fat pad (16) and has been corroborated using normal mammary tissue and primary mammary epithelial cell cultures (15, 17, 33, 57, 59). Mammary stem cells exhibit the ubiquitous feature of either remaining quiescent or undergoing self-renewal in response to their microenvironment (64) and retain the ability to pass on newly labeled DNA to their progeny by asymmetric cell division ("label-retaining cells" [LRC]) (58). LRC express the neuroglial stem cell marker Musashi1 (Msi1) (14), as well as the $CD24^{hi}$ $CD133^{+}$ phenotype (55). Since the mammary gland contains a population of multipotent and early progenitor cells throughout development (10), it is important to understand the pathways regulating their proliferation within the mammary gland, as well as those leading to malignant transformation (24).

Msi1 was identified originally as a positive regulator of Notch and a cell fate determinant for neuroglial stem cells (13, 49). Notch is activated by sequential proteolytic cleavage of its membrane-associated form to a constitutively active intracellular coactivator (2), whose expression is regulated by Numb and Msi1 (45). Numb promotes ubiquitination of intracellular Notch (40) and interferes with its nuclear translocation (68). Msi1 is an RNA-binding protein that associates with the *cis*-acting repressor sequences in the 3′-untranslated region of the Numb mRNA to block its translation (29). Msi1 maintains the proliferation of multipotential neural stem cells (45) and is rapidly downregulated in postmitotic neurons (49). It is highly expressed in central nervous system tumors originating from neural stem cells (62, 75). In a similar context, human breast stem cells are enriched in Notch3 (19) and Notch ligands promote the proliferation of epithelial and myoepithelial progenitor cells. Breast LRC and side population cells express Msi1 and intracellular Notch1 and exhibit a $CK19^{+}$ estrogen $receptor^{+}$ phenotype with progenitor cell morphology (14). Although Msi1 appears to be a putative breast stem cell marker, little is known about its functional role in mammary gland development and in stem/progenitor cell self-renewal.

Here we show that Msi1 drives the proliferation of mammary progenitor cells by an autocrine process associated with increased proliferin-1 (PLF1) and reduced Dickkopf-3 (DKK3) secretion, which results in extracellular signal-regulated kinase (ERK) phosphorylation, downregulation of $p21^{Cip1}$, and enhanced Wnt and Notch signaling. Additionally, Msi1 modulates the expression of a number of genes associated with the cell cycle, development, and cell adhesion. These results are the first to document a mechanistic role for Msi1 in mammary stem/progenitor cell expansion.

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MATERIALS AND METHODS

Cell culture. Mouse mammary epithelial cell line COMMA-1D was obtained from the Tissue Culture Shared Resource, Lombardi Comprehensive Cancer Center. Cells were maintained in Dulbecco's modified Eagle medium (DMEM)-F12 medium (Sigma-Aldrich Chemical Co., St. Louis, MO) supplemented with 5% fetal calf serum at 37°C under 5% CO_2.

Viral transduction. Flag-tagged Msi1 cDNA was prepared and subcloned into retroviral vector pCMV/hyg, which was generated by replacement of the Tet on/off control element in pRevTRE (Clontech, Inc., Palo Alto, CA) with the cytomegalovirus promoter from pRc/CMV (Invitrogen) (76). 293T cells were cotransfected with either pCMV/hyg or pCMV/Msi1 and ecotropic retroviral vector pSV-ψ^--E-MLV. After 48 h, the supernatant was collected, mixed with an equal volume of fresh DMEM-F12 medium plus 2× supplement in the presence of 4 μg/ml Polybrene, and added to COMMA-1D cells. After four rounds of infection, COMMA-1D cells were selected in hygromycin for 2 weeks, and the expression of Flag-Msi1 was confirmed by Western blotting with anti-Flag antibody and anti-Msi1 antibody.

Growth assay. Cells were seeded into a 96-well plate at 3,000 cells/well in 200 μl medium, and growth was determined 24 to 72 h later by sulforhodamine B staining (53, 72).

Colony assay. Colony formation was performed by seeding 3,000 cells into a 100-mm dish containing a 50:50 mixture of fresh medium and conditioned medium from COMMA-1D cells transduced with either empty vector (COMMA/Vect) or Msi1 (COMMA/Msi1) (76). The number of colonies with >50 cells was determined after 8 days.

Western blots. Western blotting was performed using the Criterion system (Bio-Rad, Hercules, CA). Cells were lysed on ice in lysis buffer (76), and protein concentration was determined using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). Protein (20 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad) and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Proteins were detected with the appropriate primary antibody (see Table S1 in the supplemental material) using horseradish peroxidase-linked secondary antibodies and visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ). In some instances, pERK was measured after COMMA/Vect or COMMA/Msi1 cells were treated for 48 h with U0126 (Sigma-Aldrich Chemical Co., St. Louis, MO). COMMA/Msi1 cells were also treated for 4 h with 0.3, 1, and 3 ng/ml pertussis toxin (List Biological Laboratories, Inc., Campbell, CA), and cells were harvested for Western analysis of pERK.

Proliferin depletion from conditioned medium. Conditioned medium from COMMA/Msi1 cells was concentrated 2.5-fold by centrifugation through a Centricon YM-10 spin filter with a 10-kDa cutoff (Millipore Corp.). Concentrated medium was then incubated overnight at 4°C with 4 μl PLF1 monoclonal antibody 209 (kindly provided by Daniel Linzer, Northwestern University, Evanston, IL) or with control immunoglobulin G (IgG). Protein G-Sepharose (40 μl) preequilibrated with phosphate-buffered saline (PBS) was added, and after 30 min, samples were centrifuged at 2,500 × *g* for 10 min and removal of PLF1 was confirmed by Western blotting. Cells were seeded into a 24-well plate in medium containing 0.2 ml PLF1-depleted conditioned medium or control medium, and after 4 h, pERK was measured by Western blotting.

Fluorescence microscopy. Cells were grown in chamber slides (Lab-Tek, Hatfield, PA), fixed in 100% cold methanol for 10 min, and stored at −20°C. Fixed cells were stained with primary antibodies overnight at 4°C, followed by incubation with either fluorescein isothiocyanate- or phycoerythrin-conjugated secondary antibody (Invitrogen, Carlsbad, CA). Cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (Invitrogen). A Nikon E600 Epi-Fluorescent microscope was used for image capture.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted using an RNeasy minikit (Qiagen, Valencia, CA) by following the manufacturer's protocol. Genomic DNA was digested by incubation with RNase-free DNase for 15 min at room temperature. One microgram of RNA was reverse transcribed in a total volume of 20 μl using the Omniscript RT kit (Qiagen). PCR was performed in triplicate in an ABI-Prism 7700 instrument (Applied Biosystems, Foster City, CA) using Sybr green I detection (Qiagen) according to the manufacturer's protocol. Amplification using the appropriate primers (see Table S3 in the supplemental material) was confirmed by ethidium bromide staining of the PCR products on an agarose gel. The expression of each target gene was normalized to the expression of 18S RNA and is presented as the ratio of the target gene to 18S RNA, expressed as $2^{-\Delta Ct}$, where Ct is the threshold cycle and $\Delta Ct = Ct^{Target} - Ct^{18S}$.

Gene microarray analysis. Total RNA was extracted using an RNeasy minikit (Qiagen) by following the manufacturer's protocol. cRNA synthesis was carried out using the Affymetrix (Santa Clara, CA) protocol with minor modifications as described previously (73). Biotin-labeled cRNA was fragmented at 94°C for 35 min and used for hybridization overnight to an Affymetrix mouse 430A2.0 GeneChip representing approximately 14,000 annotated mouse genes by the Macromolecular Analysis Shared Resource. GeneChips were scanned using an Agilent gene array scanner, and grid alignment and raw data generation were performed with Affymetrix GeneChip operating software, version 1.1. The expression levels of genes that were either increased or decreased at least twofold in both experiments were clustered hierarchically by CIMiner software (National Cancer Institute, NIH). Gene ontology analysis was performed by the L2L microarray analysis tool (http://depts.washington.edu/l2l/) (42). For the results of these analyses, see Table S1 in the supplemental material.

Flow cytometry. For surface antigen detection, cells were suspended to a concentration of 0.5×10^6 to 1×10^6 cells/ml in ice-cold PBS containing 3% fetal bovine serum, 2 μg/10^6 cells of primary antibody or control isotype antibody conjugated to fluorescein isothiocyanate or phycoerythrin (see Table S2 in the supplemental material) was added, and cells were incubated on ice for 60 min in the dark. Cells were washed three times by centrifugation at 400 × *g* for 5 min and resuspended in ice-cold PBS containing 3% fetal bovine serum. Stained cells were detected by FACScan and analyzed with FCS Express V3 software (De Novo Software, Ontario, Canada).

Luciferase reporter assays. COMMA/Vect or COMMA/Msi1 cells were grown in 24-well plates in DMEM-F12 medium containing 5% fetal calf serum. After 24 h, medium was replaced with DMEM-F12 containing 5% delipidated fetal calf serum (Sigma-Aldrich Chemical Co.), and cells were transfected using Lipofectamine Plus (Invitrogen) and the reporter plasmids. pTopFlash (Invitrogen) and CBF1-luciferase (provided by Tony Cappobianco, Wistar Research Institute, Philadelphia, PA). pTopFlash was used to measure β-catenin/T-cell factor (TCF)-dependent transcription, and CBF1-luciferase was used to measure Notch/CSL-dependent transcription. Luciferase activity was measured with the Dual-Luciferase assay system (Promega, Madison, WI).

RNA interference assays. Three short hairpin RNAs (shRNAs) targeting either PLF1 or DKK3 were designed in collaboration with Xiao Zeng, SuperArray Biosciences, Frederick, MD, and cloned into an expression vector for either transient expression (reporter assay and qRT-PCR analyses) or stable expression (pERK Western analysis) after selection with G418 for 2 weeks. For reporter assays, 4×10^4 COMMA/Vect or COMMA/Msi1 cells were seeded into each well of a 24-well plate in 1 ml medium 1 day before transfection. When cells were 70% confluent, they were transfected with 0.2 μg shRNA per well using Lipofectamine Plus reagent. After 24 h, cells were transfected with 0.2 μg of either Topflash or CBF1-luciferase plasmid DNA, and luciferase activity was measured after 24 h using the Dual-Luciferase reporter assay system (Promega, Madison, WI). Although all shRNAs tested gave similar results, those with the greatest activity were GCAAGCTTACCTCCCAACTAT for mouse shDKK3 and AAG AAGCCCTGCCTGGTTCTT for mouse shPLF1; GGAATCTCATTCGATGC ATAC was used as a negative control. All experiments were repeated in triplicate.

RESULTS

Msi1 promotes a progenitor cell phenotype. To determine if Msi1 influences stem/progenitor cell expansion in mouse mammary epithelial cells, COMMA-1D cells (15) were retrovirally transduced with Msi1 or an empty virus (Fig. 1A) and examined for expression of differentiation antigens CD24, CD29 (integrin β1), and stem cell antigen 1 (Sca-1), shown previously to be expressed by mammary stem/progenitor cells. COMMA/Msi1 cells were enriched for $CD24^{hi}$/Sca-1^+ and expressed a lower percentage of $CD24^{lo}$ Sca-1^+ cells (Fig. 1B) and a greater percentage of $CD24^{hi}$ $CD29^+$ cells (Fig. 1C) than control cells. Cells were also examined for the presence of cytokeratin markers known to be differentially expressed in stem/progenitor cells. COMMA/Msi1 cells expressed much greater levels of CK6 (Fig. 2A) and CK19 (Fig. 2B), as well as a greater number of CK14/CK18 double-positive cells, than control cells (Fig. 2C).

Msi1 upregulates gene expression associated with proliferation, adhesion, and development. Gene expression profiling

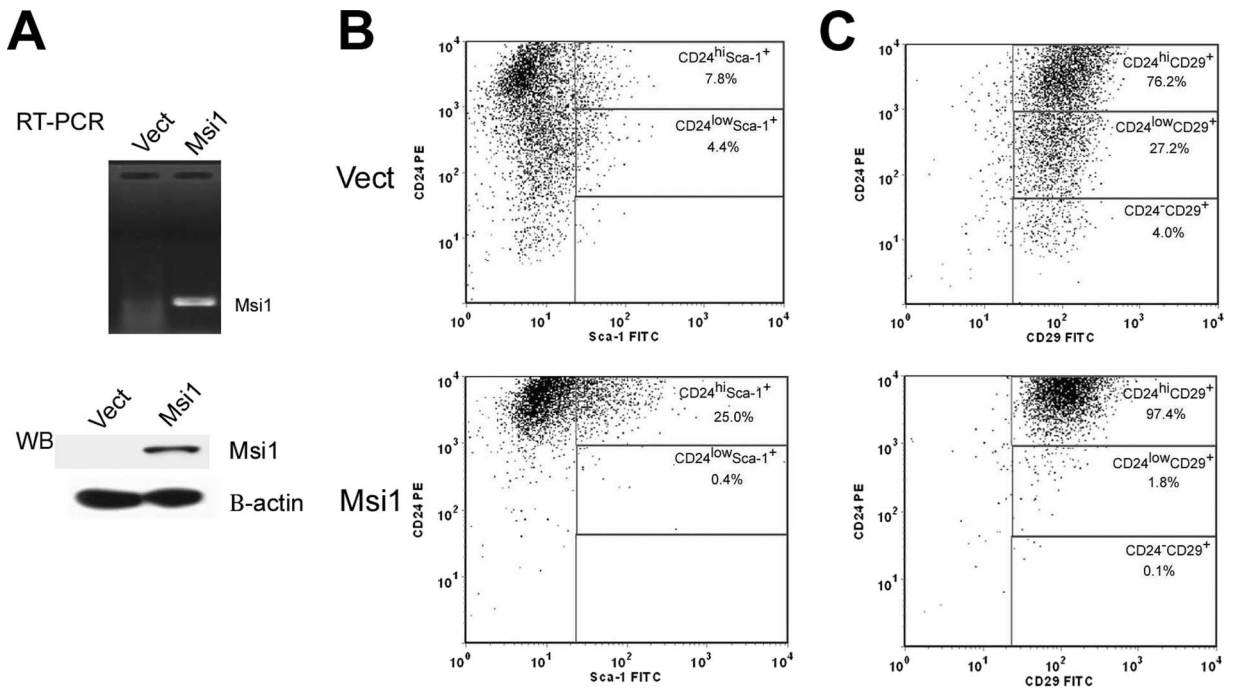

FIG. 1. Msi1 expression leads to progenitor cell expansion. (A) Stable expression of Msi1 in mouse mammary epithelial cell line COMMA-1D (Msi1) versus control cells (Vect). (Top) Increased Msi1 mRNA expression determined by RT-PCR. (Bottom) Increased Msi1 protein expression determined by Western blotting. (B and C) Dual fluorescence-activated cell sorting of cells stained for stem and progenitor cell markers. The percentage of cells expressing the markers is shown in each quadrant. COMMA/Msi1 cells (Msi1) exhibit a higher percentage of $CD24^{hi}$ $Sca\text{-}1^{+}$ cells (B) and $CD24^{hi}$ $CD29^{+}$ cells (C) than control (Vect) cells.

revealed several specific and significant changes associated with proliferation and development (see Fig. S1A in the supplemental material). COMMA/Msi1 cells exhibited enrichment in expression for genes related to proliferation, the cell cycle, development, cell adhesion, motility, and double-stranded DNA break repair (see Table S1 in the supplemental material). Eighteen changes in gene expression were validated by qRT-PCR and included changes in expression of the growth-stimulatory CSF3, PLF1, vascular endothelial growth factor D, mitogen-activated protein kinase 13, and cyclin D2 genes and the growth-inhibitory NOV, DKK3, and GAS6 genes (see Fig. S1B in the supplemental material).

Msi1 stimulates proliferation, increases PLF1 secretion, and reduces DKK3 secretion in an autocrine fashion. The growth of COMMA/Msi1 cells on plastic was far greater than that of control cells (Fig. 3A), but the cells did not exhibit anchorage-independent growth in soft agar (results not shown), suggesting that Msi1 expression per se does not lead to transformation. To determine if an autocrine mechanism was associated with proliferation, levels of colony formation by the two cell lines were compared using a 50:50 mixture of fresh medium and conditioned medium from either COMMA/Msi1 or COMMA/Vect cells (Fig. 3B). After 8 days in culture, COMMA/Msi1 cells exhibited increased colony formation compared to control cells when grown in conditioned medium from control cells; however, the growth of both cell lines was markedly stimulated by conditioned medium from COMMA/Msi1 cells. Since the PLF1 and DKK3 genes exhibited the greatest changes among candidate growth-stimulatory and -inhibitory genes, respectively, changes in their expression were examined by qRT-PCR, as well as Western analysis of the conditioned medium (Fig. 3C). Gene microarray and qRT-PCR analyses indicated 17- and 38-fold increases in PLF1 expression, respectively (Fig. 3C), which coincided with a 15- to 20-fold reduction in expression of DKK3 (Fig. 3C, left). Western blot analysis of concentrates of conditioned medium from COMMA/Msi1 cells indicated an increase in PLF (the antibody does not distinguish between the three PLF homologs, which differ by 3 or 4 amino acids) and the disappearance of DKK3, which was readily detectable in the conditioned medium from control cells (Fig. 3C, right).

Msi1 activates Wnt and Notch signaling. Potential signaling pathways associated with Msi1 were next examined by measuring changes in several protein kinases associated with the Wnt and Notch pathways (Fig. 4). pERK was increased and glycogen synthase kinase 3β (GSK3β) was reduced in COMMA/Msi1 cells, but no changes in mTOR, PDK1, or AKT were evident (Fig. 4A). Both cyclin D1 and D2 were markedly increased, and the Msi1 target $p21^{Cip1}$ virtually disappeared in COMMA/Msi1 cells in the absence of any change in $p21^{Cip1}$ mRNA levels (results not shown). Inhibition of ERK phosphorylation by the highly selective MEK inhibitor U0126 preferentially reduced proliferation in COMMA/Msi1 cells (Fig. 4B).

We next determined if PLF1 was associated with ERK activation by depleting it from conditioned medium with the use of an immobilized anti-PLF1 monoclonal antibody (63) (Fig. 4C). PLF1-depleted medium reduced ERK phosphorylation compared to treatment with nonimmune IgG. To

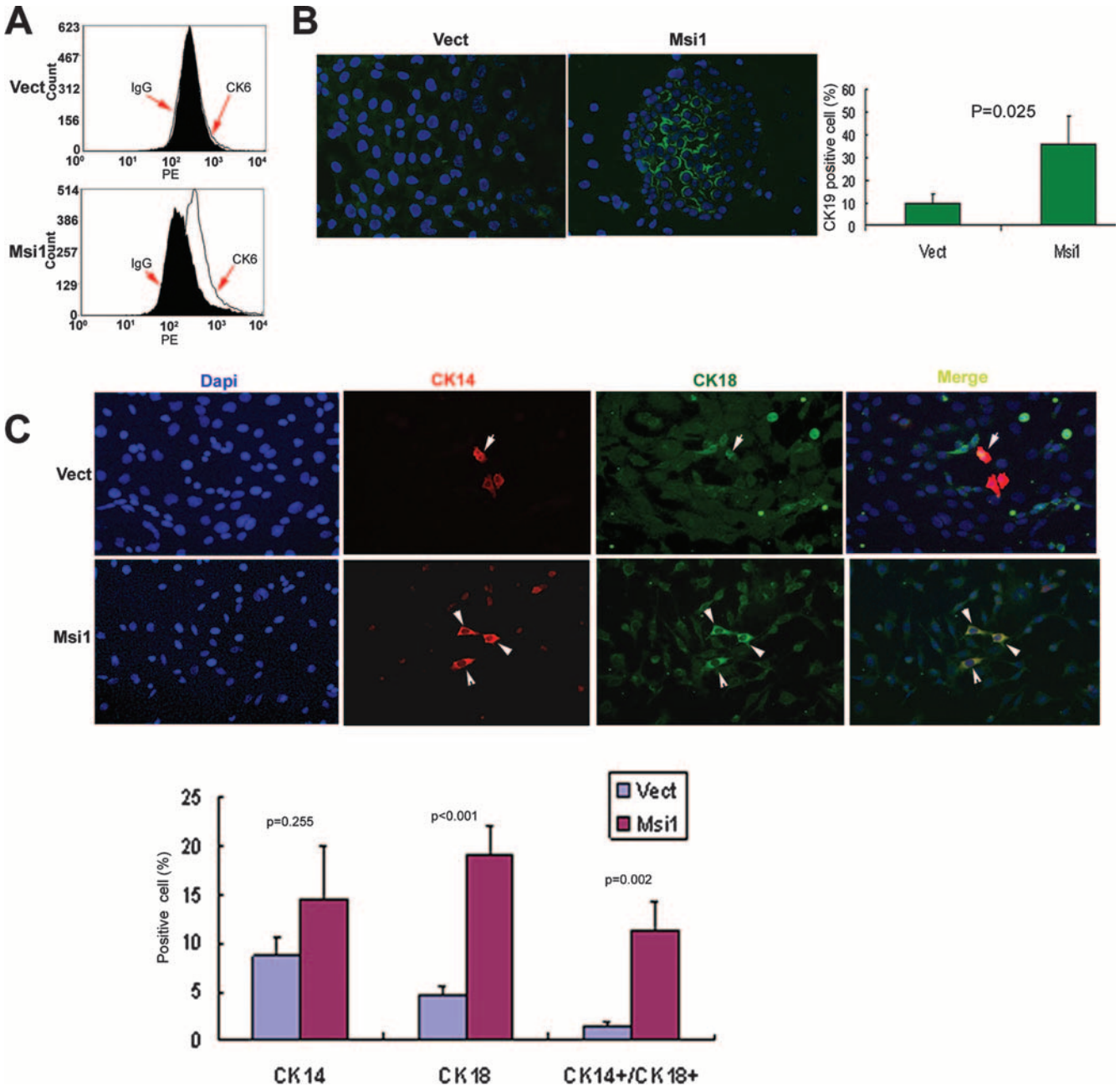

FIG. 2. Msi1 increases expression of multipotential progenitor cells. (A) Fluorescence-activated cell sorting histogram of $CK6^+$ cells. CK6 expression is increased in COMMA/Msi1 (Msi1) versus control (Vect) cells. (B) Immunofluorescence for $CK19^+$ cells. CK19 expression is increased in COMMA/Msi1 cells (Msi1) versus control cells (Vect). (Right) Quantitation of the percentage of $CK19^+$ cells (means ± standard errors [SE]) from five microscopic fields. (C, top) Immunofluorescence for $CK14^+$ $CK18^+$ cells. COMMA/Msi1 cells express an increased percentage of $CK14^+$ $CK18^+$ cells. $CK14^+$ cells are stained green, and $CK18^+$ cells are stained red; the merge shows that COMMA/Msi1 cells (Msi1) express a greater percentage of $CK14^+$ $CK18^+$ cells (yellow) than control cells (Vect). (Bottom) Quantitation of $CK14^+$, $CK18^+$, and $CK14^+$ $CK18^+$ cells. Each value is the mean ± SE of the percentage of positive cells from five microscopic fields.

confirm that ERK was activated by PLF1 through its Gi-coupled insulin-like growth factor 2R (IGF2R) (67), cells were treated with the selective Gi inhibitor pertussis toxin (25) (Fig. 4D). Treatment of control cells grown in conditioned medium from Msi-expressing cells with 1 and 3 ng/ml pertussis toxin dramatically inhibited ERK phosphorylation. As an additional confirmation of the involvement of PLF1 in ERK activation, COMMA/Msi1 cells stably expressing a PLF1 shRNA exhibited a reduction in pERK, particularly pERK1 (Fig. 4E).

COMMA/Msi1 cells expressed greater cyclin D1 and D2 levels than control cells (Fig. 4A), which is consistent with β-catenin/TCF (52) and Notch (48) pathway activation and reduced GSK3β activation, which normally promotes the phos-

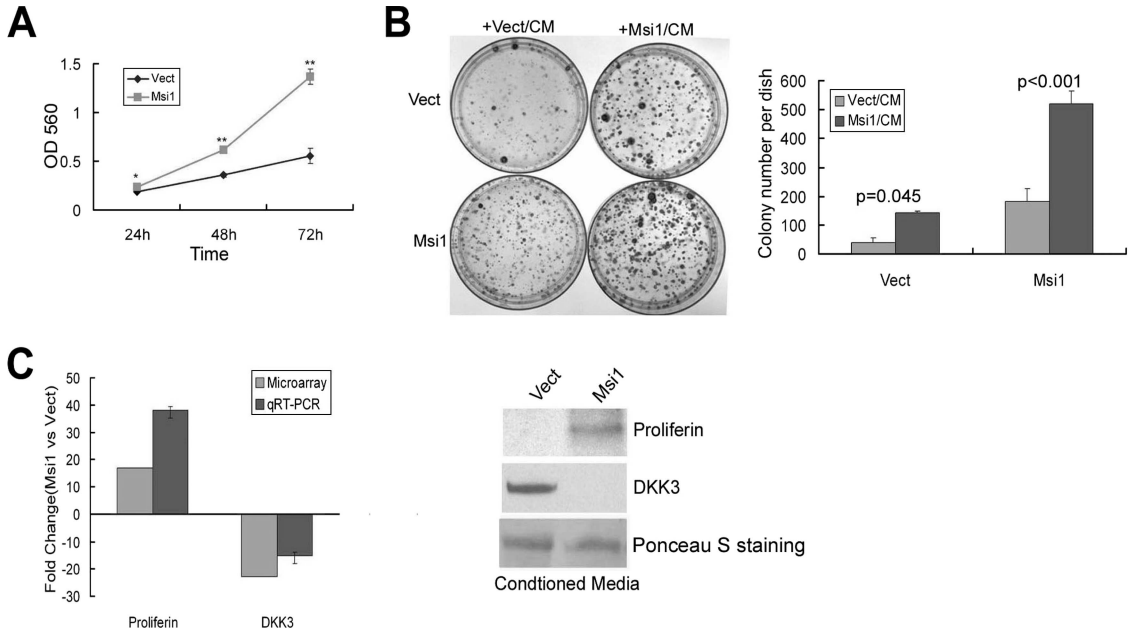

FIG. 3. Msi1 promotes S-phase proliferation and growth factor secretion. (A) Cell proliferation is increased in COMMA/Msi1 (Msi1) cells versus control (Vect) cells. Cells were grown in 96-well plates for 24 to 92 h, and cell density was determined by sulforhodamine B staining (means ± standard errors [SE]; $n = 5$; *, $P < 0.05$; **, $P < 0.001$). OD 560, optical density at 560 nm. (B) Conditioned media (CM) from COMMA/Msi1 cells (Msi1) increases colony formation in control (Vect) cells. Cells were seeded at 3,000 cells/100-mm dish and grown in a mixture containing 50% fresh medium and 50% CM from either COMMA/Vect or COMMA/Msi1 cells. (Left) Colony formation in the conditioned medium from control cells (+Vect/CM) was greater in COMMA/Msi1 (Msi1) cells than in control (Vect) cells; however, both COMMA/Msi1 and control cells exhibited increased colony formation when grown in conditioned medium from COMMA/Msi cells (+Msi1/CM). (Right) Colonies of >50 cells were counted in each dish (means ± SE; $n = 3$). (C, left) Increased PLF1 and reduced DKK3 mRNA expression in COMMA/Msi1 cells versus control cells, as determined by gene microarray and qRT-PCR analyses (means ± SE; $n = 3$). (Right) Increased PLF1 and reduced DKK3 protein levels in the conditioned medium from COMMA/Msi1 cells (Msi1) versus control cells (Vect), as determined by Western blotting. Sample loading was measured by Ponceau S staining.

phorylation, ubiquitination, and proteasomal degradation of β-catenin (66) and intracellular Notch (21). Therefore, Notch and β-catenin/TCF signaling was assessed by reporter gene analysis. CBF1/Notch reporter activity increased sevenfold in COMMA/Msi1 cells (Fig. 5A, top) and correlated with increased expression of Notch1, its target the Hes1 gene, and the Notch ligands Delta1 and Jagged1, as determined by qRT-PCR (Fig. 5A, middle). Importantly, CBF1 activity was dependent on ERK activation, as shown by the inhibitory effect of U0126 (Fig. 5A, bottom). As a measure of β-catenin/TCF activation, nuclear localization of β-catenin was determined by immunofluorescence (Fig. 5B). Increased nuclear localization of β-catenin in Msi1-expressing cells correlated with increased β-catenin/TCF-dependent reporter gene activity (Fig. 5C, top). As with Notch reporter gene activity, β-catenin/TCF reporter gene activity in COMMA/Msi1 cells was preferentially inhibited by U0126 (Fig. 5, bottom), indicating dependence on MEK/ERK activation.

One of the major features of Msi1 expression is the increase in PLF1 and reduction in DKK3 secretion in COMMA/Msi1 cells (Fig. 3C). To determine the direct influence of PLF1 and DKK3 on Wnt and Notch signaling, shRNAs were designed and transiently expressed in the indicated cell lines (Fig. 5D and E). Reduction of DKK3 mRNA resulted in increased Topflash and CBF1 luciferase activity in control cells (Fig. 5D), whereas, reduction of PLF1 mRNA in COMMA/Msi1 cells reduced both reporter gene activities (Fig. 5E). Although mRNA suppression was not complete after transient shRNA expression (transfection efficiency was ~40%), these results provide strong support for the involvement of DKK3 and PLF1 signaling downstream of Msi1-mediated changes in proliferation, progenitor cell expansion, and Wnt and Notch pathway activation.

DISCUSSION

The mammary gland represents a unique tissue in which to study the hierarchy of progenitor cell lineages associated with stem cell self-renewal. Although Msi1 has been identified as a marker in hematopoietic, neuroglial, and breast stem cells (13, 49), there is no information pertaining to its role in mammary gland differentiation. We therefore sought to determine if Msi1 could modulate mammary progenitor cell proliferation to influence lineage specification. Our results indicate that COMMA/Msi1 cells express an increased percentage of $CD24^{hi}$ Sca-1^{+} cells and a lower percentage of $CD24^{lo}$ Sca-1^{+} cells than control cells, which suggests progenitor cell expansion. The literature is controversial with regard to the utility of Sca-1 as a mammary stem cell marker, since in one instance, $CD24^{hi}$ Sca-1^{+} cells were reported to have no repopulating activity compared to $CD24^{hi}$ Sca-1^{-} cells (54, 55), yet studies to the contrary demonstrate that Sca-1^{+}, but not Sca-1^{-}, cells possess mammary outgrowth activity (17, 70). Multipotent self-renewing mammary stem cells capable

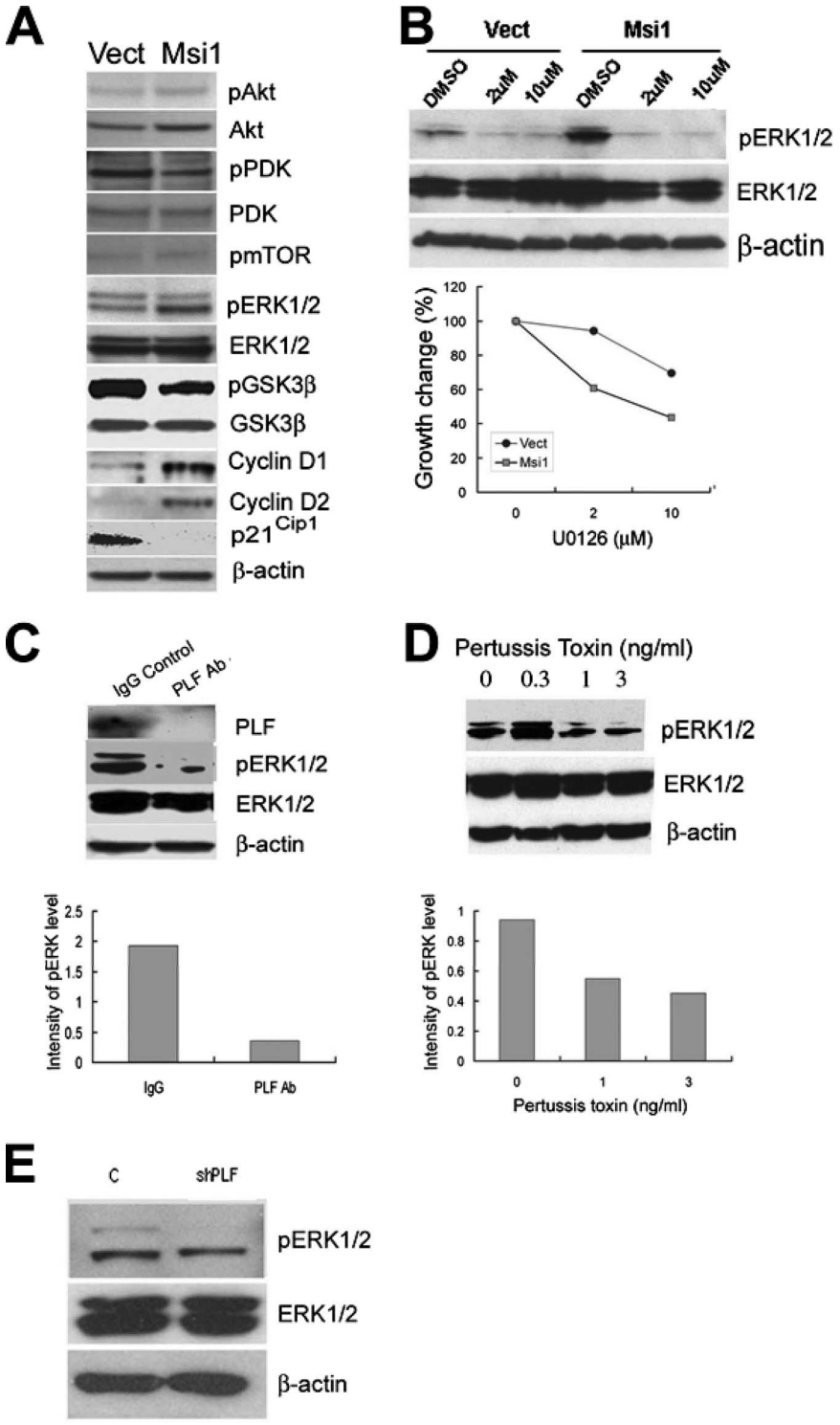

FIG. 4. Msi1 inhibits $p21^{Cip1}$ and GSK3β and activates ERK. (A) Msi increases ERK2 and cyclin D1 and D2 levels and reduces $p21^{Cip1}$ and pGSK3β but does not affect PDK1/Akt activation. Western blots of cell lysates from COMMA/Msi1 cells (Msi1) and control cells (Vect) are shown. (B) MEK inhibitor U0126 preferentially reduces cell growth of COMMA/Msi1 cells. COMMA/Msi1 cells (Msi1) and control cells (Vect) were treated for 48 h with 2 and 10 μM U0126, and pERK1/2 levels (top) and cell growth (bottom) were determined. (C) pERK is reduced in cells grown in PLF1-depleted conditioned medium from COMMA/Msi1 cells. Conditioned medium was concentrated and incubated with a PLF1 monoclonal antibody, and antigen-antibody complexes were removed with protein G-Sepharose. PLF1 and pERK1/2 were reduced in cells grown in PLF1-depleted conditioned medium (PLF1 Ab) versus medium treated with IgG (IgG control). pERK1/2 was quantitated with Scion Image software and normalized to β-actin. (D) Pertussis toxin inhibits ERK phosphorylation in COMMA/Msi1 cells. Cells were treated for 4 h with 0, 0.3, 1, and 3 ng/ml pertussis toxin, and pERK was measured by Western blotting. pERK1/2 was quantitated with Scion Imaging Software and normalized to β-actin. (E) PLF1 shRNA reduces pERK levels. COMMA/Msi1 cells were transfected with a PLF1 shRNA or control inactive shRNA and selected with G418 for 2 weeks. pERK was reduced only in cells expressing the PLF1 shRNA.

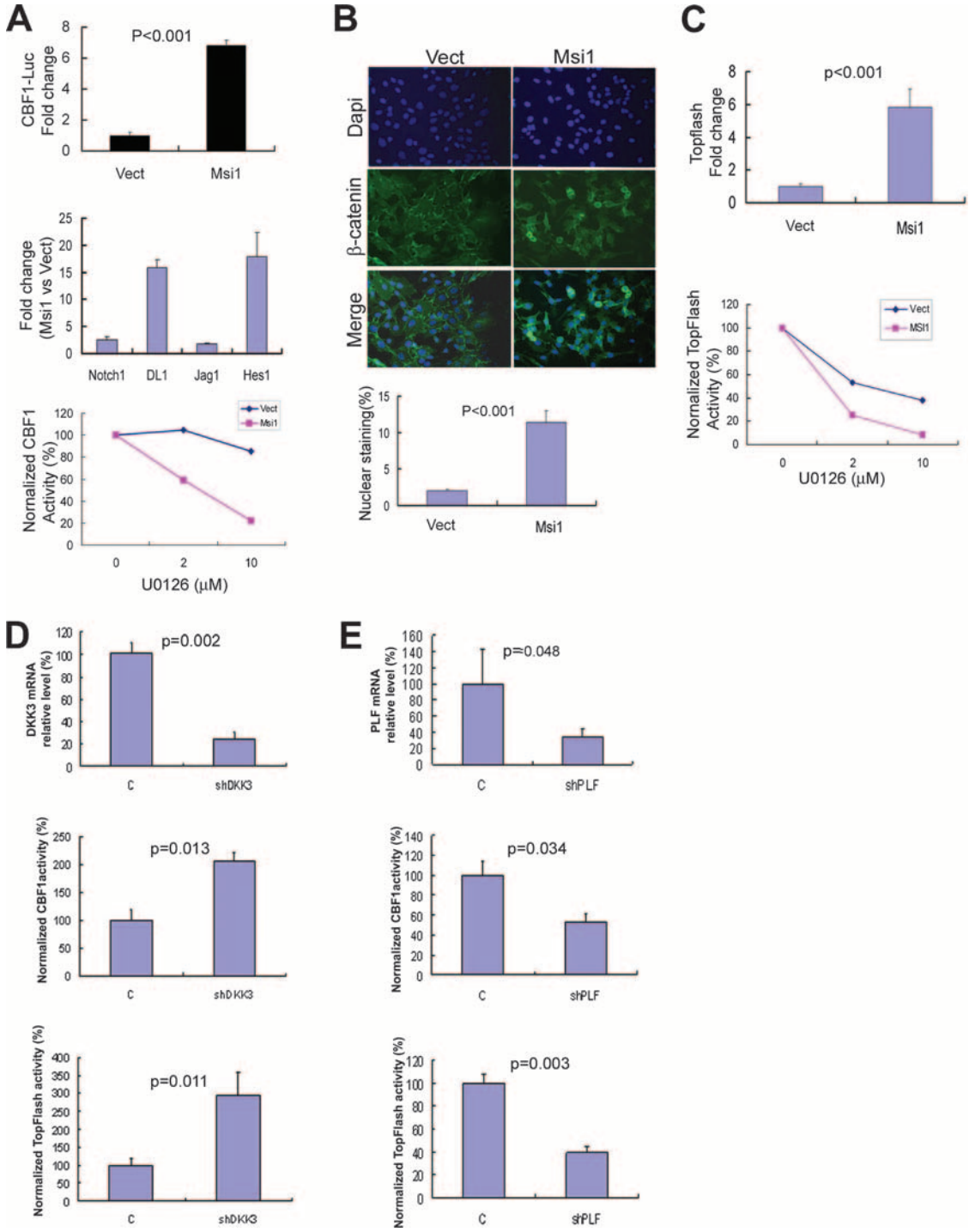

FIG. 5. Msi1 activates the Wnt and Notch pathways. (A, top) Msi1 activates the Notch pathway. CBF1-luciferase reporter gene activity is increased sevenfold in COMMA/Msi1 cells (Msi1) versus control cells (Vect). (Middle) qRT-PCR analysis indicates increased expression of Notch1, Notch ligands Delta1 (DL1) and Jagged1 (Jag1), and the Notch target Hes1 gene (Hes1). Data are means ± standard errors (SE) ($n = 3$). (Bottom) CBF1 reporter gene activity in COMMA/Msi1 cells is preferentially inhibited by U0126. (B, top) Msi1 increases nuclear β-catenin localization. β-Catenin immunofluorescence indicates greater nuclear localization in COMMA/Msi1 cells (Msi1) than in control cells (Vect). Magnification, ×200. (Bottom) Quantitation of nuclear β-catenin based on the number of fluorescent nuclei in three microscopic fields (means ± SE; $n = 3$). (C) Msi1 activates the Wnt pathway. (Top) β-Catenin/TCF reporter gene activity is increased fivefold in COMMA/Msi1 cells (Msi1) versus control cells (Vect) (means ± SE; $n = 3$). (Bottom) β-Catenin/TCF-dependent reporter gene activity in COMMA/Msi1 cells is preferentially inhibited by U0126. (D) Reduction of DKK3 mRNA in control cells increases β-catenin/TCF-dependent and CBF1-luciferase reporter gene activities. (Top) qRT-PCR analysis of DKK3 mRNA in control cells treated with a control (C) or DKK3 (shDKK3) shRNA. (Middle) CBF1-luciferase activity is increased following DKK3 shRNA treatment. (Bottom) β-Catenin/TCF-dependent reporter gene activity is increased following DKK3 shRNA treatment. Statistical significance is indicated by P values of <0.05. (E) Reduction of PLF1 mRNA in COMMA/Msi1 cells reduces β-catenin/TCF-dependent and CBF1-luciferase reporter gene activities. (Top) qRT-PCR analysis of PLF1 mRNA in COMMA/Msi1 cells treated with a control (C) or PLF1 (shPLF1) shRNA. (Middle) CBF1-luciferase activity is reduced following PLF1 shRNA treatment. (Bottom) β-Catenin/TCF-dependent reporter gene activity is reduced following PLF1 shRNA treatment. Statistical significance is indicated by P values of <0.05.

of reconstituting the mammary gland from a single cell have been defined as being $CD24^+$ $CD29^{hi}$ (51), in contrast to the $CD24^{hi}$ $CD29^+$ phenotype upregulated by Msi1 expression. Preneoplastic tissue from mouse mammary tumor virus (MMTV)-Wnt1 mice also exhibits an increased percentage of $CD24^+$ $CD29^+$ cells (51), but other reports have shown that mammary outgrowth capacity segregates with $CD24^{lo}$ rather than $CD24^{hi}$ cells (54). Notwithstanding the disparities of what constitutes a mammary stem cell marker, our data suggest that Msi1 is involved in driving the expansion of multilineage luminal progenitor cells rather than stem cells.

Msi1 cells also express a higher percentage of cytokeratin (CK) markers, indicative of basal cells (a mixture of stem and progenitor cells), such as CK6 and CK19, and are double positive for CK14/CK18. CK6 is abundant in stem and basal cells (7, 17, 60, 69) and has been linked to proliferation of the alveolar epithelium and activation of the Wnt pathway (37). CK19 is expressed in luminal progenitor cells that give rise to $CK14^+$ basal cells (46, 56), and double-positive CK14/CK18 cells exhibit bipotential progenitor cell characteristics (7). These results are also consistent with the contention that Msi promotes the expansion of early progenitor cells.

Msi1 expression increased Wnt pathway activation, as demonstrated by increased β-catenin/TCF-dependent transcription and β-catenin nuclear localization. The Wnt pathway drives alveolar proliferation in MMTV-Wnt1 (5) and MMTV-ΔN89β-catenin (30) transgenic mice, and targeting of ΔN89β-catenin to mammary basal cells by the CK5 promoter produces precocious end bud development (61). Expansion of mammary basal cells with characteristic CK6/CK14 expression was also noted in mice with increased Notch pathway activation (7). These results are consistent with our findings that Msi1 activates progenitor cell expansion via activation of the Wnt and Notch pathways.

Msi1 expression in mammary epithelial cells resulted in downregulation of the cyclin-dependent kinase inhibitor $p21^{Cip1}$, which is in agreement with $p21^{Cip1}$ being a translational target of Msi1 (3). $p21^{Cip1}$ is thought to function as a rheostat to maintain a balance between stem cell quiescence and stem cell exhaustion resulting from increased cell cycle entry (9, 32). Reduced $p21^{Cip1}$ expression due to Msi1 is consistent with actively cycling progenitor cells and their increased proliferation in vitro.

Gene profiling identified increased PLF1 expression, as well as an equally large reduction of DKK3, a secreted Wnt pathway inhibitor, and these changes were confirmed to occur in the conditioned medium of COMMA/Msi1 cells. The PLF1 gene is one of three highly homologous genes related to the prolactin gene family, mapping to a single locus on mouse chromosome 13 (71). PLF1 is a ligand for the Gi protein-coupled IGF2R (25, 36), which mediates prolactin-induced alveolar development in the mammary gland through activation of ERK and Jak2 (6, 27). Receptor activation by PLF1 activates ERK (20, 25) and transcription factor AP-1 (63) and is blocked by pertussis toxin (20, 25), which catalyzes ADP-ribosylation of the Giα subunit to prevent its interaction with cell membrane receptors (8). This mechanism is consistent with the inhibitory effect of pertussis toxin on PLF1-mediated ERK activation by COMMA/Msi-conditioned medium, as well as inhibition of ERK activation by PLF1 depletion from COMMA/Msi1-conditioned medium. This interrelationship was further confirmed by shRNA-mediated reduction of PLF1 in COMMA/Msi1 cells. IGF2R stimulation is known to increase β-catenin nuclear localization and epithelial-mesenchymal transition (41), which are associated with growth and invasion (12). Particularly relevant is the finding that IGF2 signaling increased the number of Msi1-positive intestinal stem/progenitor cells and their susceptibility to tumorigenesis (50). Also pertinent to our findings is the identification of the PLF2 and PLF3 genes as Wnt-1 target genes (38). Although Wnt signaling has not previously been shown to increase PLF1 expression, the fact that the three PLF genes are transcribed from a single locus suggests that they are regulated in a similar manner. We have previously found that PLF1 expression is upregulated in mammary tumors with basal cell characteristics (73, 74), and PLF2 has been shown to expand mouse hematopoietic stem cells ex vivo (11). It is therefore likely that PLF1 plays a similar role in the expansion of mammary progenitor cells through Msi1. This is further suggested by RNA interference, where reduction of PLF1 decreased Wnt and Notch reporter activity. These results therefore support a role for PLF1 in Msi1-mediated activation of the Wnt and Notch pathways and in mammary progenitor cell expansion.

DKK3 was identified as negative regulator downstream of Msi1 signaling. DKK3 (also known as REIC [reduced expression in immortalized cells]) is one of four homologous secreted proteins (34) that function as tumor suppressors to block proliferation (44). DKK1 and DKK2, but not DKK3, block Wnt pathway activation by binding to the Wnt coreceptor LRP5/6 (39), but DKK3 also prevents nuclear localization of β-catenin by an unknown mechanism (26). RNA interference in control cells showed that DKK3 negatively regulated β-catenin/TCF and CBF1 activation, producing a phenotype which resembled the phenotype resulting from Msi1 expression. DKK3 expression in lung, prostate, and liver tumor cells induced apoptosis (1, 28, 65) and disrupted acinar morphogenesis and the growth of prostate tumor cells (31). Reduced expression of DKK3 in melanoma cells correlated with loss of cell adhesion, increased invasion, upregulation of the transcriptional repressor Snail-1 (47), and reduction of E-cadherin (35), suggesting that loss of DKK3 promoted epithelial-mesenchymal transition. However, gene profiling and Western analysis of COMMA/Msi1 cells did not indicate a reduction in E-cadherin expression (results not shown), suggesting that this mechanism is not operative. Overall, our findings suggest that reduction of DKK3 is responsible, in part, for increased proliferation upstream of Wnt and Notch pathway activation.

Notch-mediated transformation was shown previously to be downstream of Ras and ERK activation (22), and our data also suggest such a relationship. Msi1 activated Wnt signaling that was dependent on ERK activation, as shown by the inhibitory effect of U0126 on proliferation and β-catenin/TCF gene transcription. ERK activation occurred downstream of PLF1, and inhibition of PLF1 signaling by pertussis toxin inhibited ERK. One mechanism common to activation of both Notch and Wnt signaling is inhibition of GSK3β by ERK phosphorylation, which primes GSK3β for inactivation (18). GSK3β in its activated state phosphorylates and promotes ubiquitination and proteasomal degradation of β-catenin (66) and intracellular

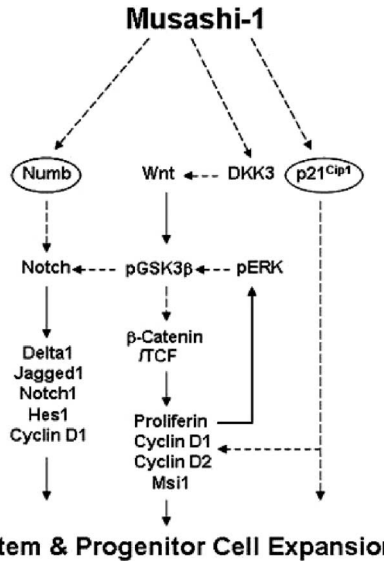

FIG. 6. Musashi1 signaling in mammary progenitor cell proliferation. Msi1 blocks translation of two known targets in mammalian cells, Numb and $p21^{Cip1}$. The present study indicates that Msi1 inhibits DKK3 protein secretion and mRNA levels, but it is unknown if this occurs by interference with nuclear processing and/or translation. Inhibition of Numb expression increases intracellular Notch and Notch-dependent transcription of genes such as the Delta1, Jagged1, Notch1, Hes1, and cyclin D1 genes. Inhibition of $p21^{Cip1}$ relieves its inhibitory effect on cyclin D-dependent protein kinases to increase G_1/S transit through the cell cycle. Loss of DKK3 expression activates the Wnt pathway, resulting in increased nuclear localization of β-catenin and transcription of β-catenin/TCF-dependent target genes such as the cyclin D1 and D2 and PLF1 genes, and possibly the Msi1 gene itself. PLF1 activates ERK, presumably through the IGF2R, which in turn inhibits GSK3β to further increase activation of the Notch and Wnt pathways through an autoregulatory loop. The net result of these processes is the stimulation of mammary progenitor cell proliferation. Dashed lines indicate inhibition; solid lines indicate stimulation.

Notch (21), providing a link between PLF1, ERK, and Wnt and Notch pathway activation.

PLF1-mediated ERK signaling correlated with the $CD24^{hi}$ $CD29^{+}$ progenitor cell phenotype observed in COMMA/Msi1 cells. CD24 is highly expressed in invasive tumor cells (4) and mediates its effects through integrin β1, the subunit expressed by CD29, which itself is upregulated through the Ras/ERK pathway (43). CD24 expression is linked to IGF2 signaling through the IGF2R, the same receptor for which PLF1 serves as a ligand. Importantly, deletion of the IGF2 gene in glioblastoma cells reduced CD24 expression by 90% and suppressed invasion (23).

In summary, our studies suggest a novel signaling mechanism, depicted in Fig. 6, where Msi1 increases PLF1 secretion and reduces DKK3 expression, leading to increased Notch and Wnt pathway activation through ERK activation. Inhibition of $p21^{Cip1}$ expression by Msi1 works cooperatively with Notch and Wnt pathway activation to promote cyclin D1- and D2-dependent protein kinase activation and G_1/S transit through the cell cycle. Inhibition of Numb by Msi1 maintains Notch activity and transcription of its downstream ligand and effector genes, which also promote proliferation. The net result of these processes is the stimulation of progenitor cell expansion and proliferation, conditions likely to promote mammary gland development and provide a new paradigm for progenitor cell signaling in the mammary gland.

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