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Receptor Activator of NF-κB Ligand Regulates the Proliferation of Mammary Epithelial Cells via Id2

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Receptor activator of NF-κB ligand (RANKL) is a key regulator for mammary gland development during pregnancy. RANKL-deficient mice display impaired development of lobulo-alveolar mammary structures. Similar mammary gland defects have been reported in mice lacking Id2. Here we report that RANKL induces the proliferation of mammary epithelial cells via Id2. RANKL triggers marked nuclear translocation of Id2 in mammary epithelial cells. In vivo studies further demonstrated the defective nuclear translocation of Id2, but the normal expression of cyclin D1, in the mammary epithelial cells of *rankl*$^{-/-}$ mice. In vitro studies with nuclear localization sequence-tagged Id2 revealed that the nuclear localization of Id2 itself is critical for the downregulation of p21 promoter activity. Moreover, RANKL stimulation failed to induce cell growth and to downregulate p21 expression in *Id2*$^{-/-}$ mammary epithelial cells. Our results indicate that the inhibitor of helix-loop-helix protein, Id2, is critical to control the proliferation of mammary epithelial cells in response to RANKL stimulation.

Mammary gland development mostly occurs postnatally, by the actions of pregnancy hormones, and proceeds in distinct steps. At birth, the mammary anlage consists of a few rudimentary ducts that occupy a small portion of the mammary fat pad. Pronounced ductal outgrowth and branching commences at puberty. At the onset of pregnancy, increased ductal side branching and extensive epithelial cell proliferation occur, resulting in the formation of lobulo-alveolar structures and the differentiation of secretory epithelia (13, 14, 39). These differentiation steps are essential to form a lactating mammary gland in pregnancy.

The TNF family molecule, receptor activator of NF-κB ligand (RANKL; also known as OPGL, ODF, and TRANCE), is a key factor for osteoclast differentiation and/or activation and dendritic cell survival (1, 20, 41, 42). RANKL-deficient mice exhibit severe osteopetrosis and tooth eruption failure due to a complete absence of osteoclasts (19). In addition to the regulation of bone remodeling and immune functions, we have previously shown that RANKL plays a critical role in mammary gland development during pregnancy. Mice lacking RANKL or its receptor, RANK, show impaired lobulo-alveolar development during pregnancy, owing to intrinsic defects in both the proliferation and survival of mammary gland epithelial cells (9). These mutant mice also display a complete transcriptional block in the β-casein gene. The defect of β-casein gene expression in RANKL-null mice is due to the failure of nuclear translocation of CCAAT/enhancer binding protein β (C/EBPβ) (18). These findings indicate that RANKL and RANK are essential for the development of a lactating mammary gland during pregnancy.

In mammary glands, as well as osteoclasts and dendritic cells, RANKL-RANK interactions induce the phosphorylation and/or activation of IκB kinase (IKK), a complex composed of IKKα, IKKβ, and IKKγ/NEMO (6, 40). This phosphorylation event leads to polyubiquitination and proteasome-mediated degradation of IκB, and the subsequent activation of NF-κB. Using *IKKα*$^{AA/AA}$ knockin mice, it was recently reported that IKKα activity is required for NF-κB activation in mammary gland epithelial cells during pregnancy and in response to RANKL (6). Furthermore, IKKα and NF-κB activation are required for optimal cyclin D1 induction, suggesting a linear signaling cascade: RANKL → RANK → IKKα → IκBα → NF-κB → cyclin D1. In contrast, Brisken et al. found that prolactin induces cyclin D1 expression via insulin-like growth factor 2 (IGF-2) and that *cyclin D1*$^{-/-}$ mammary gland epithelial cells fail to proliferate in response to prolactin. Importantly, in the present study, RANKL failed to induce cyclin D1 expression, suggesting that cyclin D1 is a mediator of the prolactin-induced proliferation of mammary epithelial cells through IGF-2 (4). Thus, the exact biochemical pathway and downstream targets that are essential for the RANKL-induced proliferation of mammary gland epithelial cells via its receptor, RANK, are still elusive.

Transcription factors characterized by the basic helix-loop-helix (bHLH) domain play an essential role in a wide array of developmental processes. The bHLH proteins are two distinct classes, based on their tissue distribution and dimerization capabilities (30). The class A bHLH proteins, exemplified by E2A-encoded E12 and E47, are ubiquitously expressed, whereas the class B bHLH proteins include more tissue-specific transcription factors, such as MyoD and NeuroD (24, 32). The inhibitors of helix-loop-helix (HLH), the Id proteins, lack

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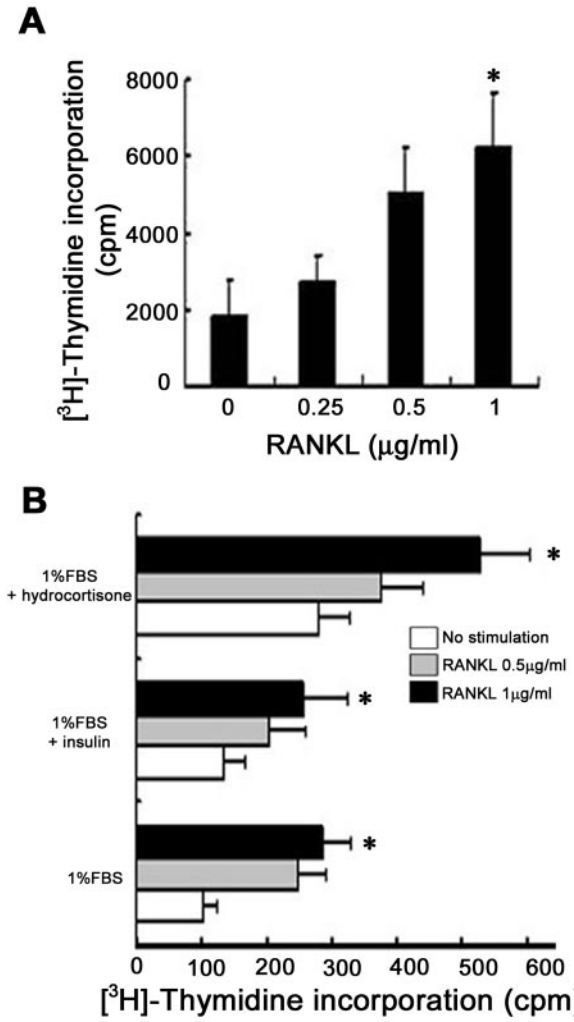


FIG. 1. RANKL induces the proliferation of primary mammary epithelial cells. (A) Serum-starved primary mammary epithelial cells, isolated from 14.5-day pregnant mice, were stimulated with the indicated doses of RANKL in DMEM containing 10% FBS for 24 h. Cells were incubated with 1 μCi of [^{3}H]thymidine/ml for the last 12 h of culture, and [^{3}H]thymidine incorporation was measured. ❋, significant difference ($P < 0.001$). (B) Primary mammary epithelial cells isolated from 14.5-day pregnant mice were stimulated with the indicated doses of RANKL in DMEM containing 1% FBS and growth factors for 60 h. Cells were incubated with 1 μCi of [^{3}H]thymidine/ml for the last 24 h of culture, and [^{3}H]thymidine incorporation was measured. The results are shown as mean values ± the standard error of the mean of three separate experiments. ❋, significant difference ($P < 0.01$).

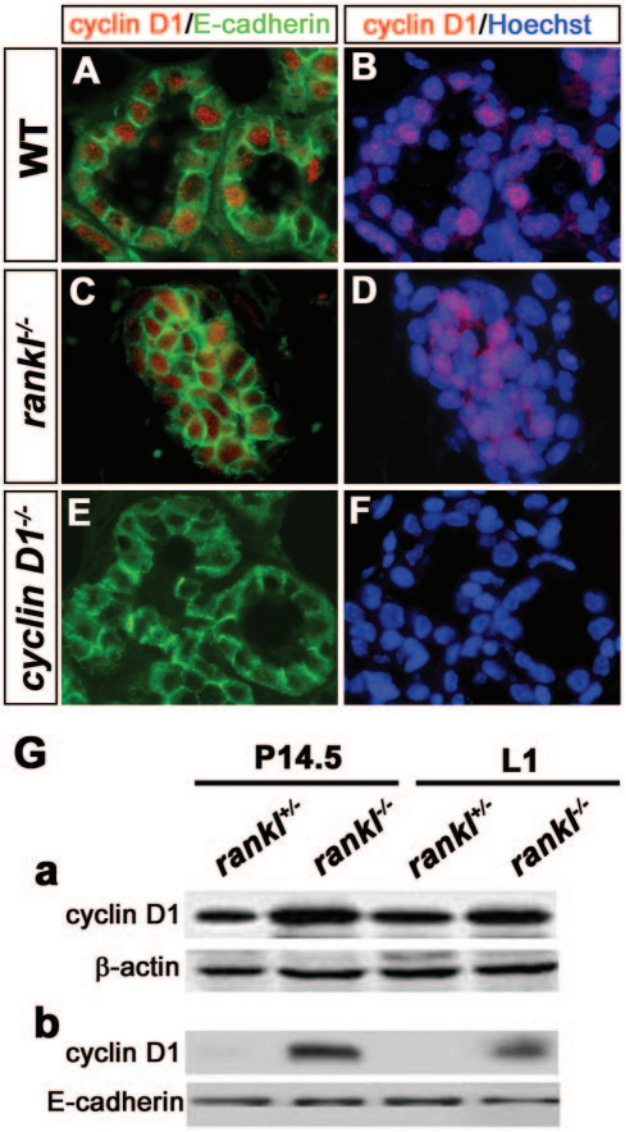


FIG. 2. Cyclin D1 expression in *rankl*$^{-/-}$ mammary glands. (A to F) Immunohistochemistry of cyclin D1 in wild-type (A and B) and rankl$^{-/-}$ (C and D) mammary glands. Tissue sections of mammary glands at 1 day of lactation (L1) from the specified genotypes were stained with anti-cyclin D1 (red)/anti-E-cadherin (green) antibodies (A, C, and E) and Hoechst (B, D, and F). The specificity of antibody staining for cyclin D1 was confirmed by its absence in *cyclin D1*$^{-/-}$ mammary epithelium (E and F). (G) Western blot analysis of lysates from *rankl*$^{+/-}$ and *rankl*$^{-/-}$ mammary tissues at P14.5 and L1. Actin (a) and E-cadherin (b) were used as loading controls. Similar results were obtained from three independent experiments.

a DNA-binding domain but possess a helix-loop-helix motif and hence can inhibit DNA binding and cell differentiation. As a consequence, they act as dominant-negative regulators of bHLH transcription factors through heterodimerization with their bHLH partners (32). The Id proteins also play positive regulatory functions in cell growth and cell cycle progression in several cell culture systems (21, 33). Indeed, Id2 contributes to the cell cycle progression of smooth muscle cells, presumably via the cdk2-dependent inhibition of p21 expression (25). Among the four known mammalian Id proteins, Id2 has been identified as a crucial regulator of mammary gland development. Similar to RANKL-deficient mice, Id2-null mice display impaired lobulo-alveolar development during pregnancy and intrinsic defects in both cell proliferation and survival (26, 28). However, although Id2 seems to be positioned at the convergence of various lactogenic signals, the upstream signals that control mammary epithelial cell growth through the Id2 protein are not known.

The morphological similarity in the mammary glands between RANKL-null and Id2-null mice prompted us to investigate the relationship between RANKL and Id2. We now

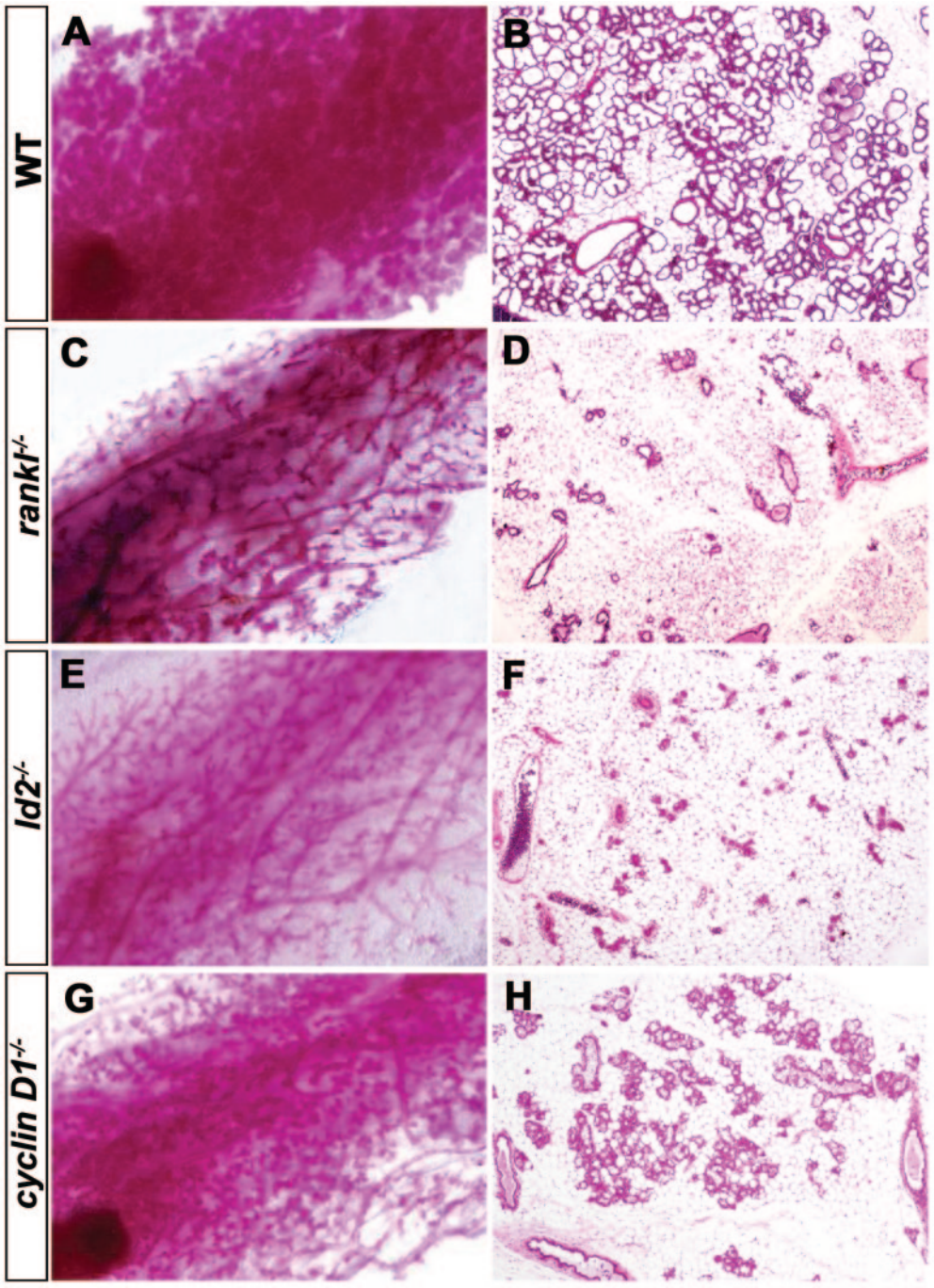


FIG. 3. The mammary gland defects in *rankl*$^{-/-}$ mice resemble those of *Id2*$^{-/-}$ mice but not *cyclin D1*$^{-/-}$ mice. Whole-mount carmine-alum strain (A, C, E, and G) and histological (B, D, F, and H) analysis by hematoxylin-eosin staining of no. 4 abdominal glands from wild-type (WT) (A and B), *rankl*$^{-/-}$ (C and D), *Id2*$^{-/-}$ (E and F), and *cyclin D1*$^{-/-}$ (G and H) mice at L1. Note the substantial alveolar formation in *cyclin D1*$^{-/-}$ mice compared to those in *rankl*$^{-/-}$ and *Id2*$^{-/-}$ mice.

show that RANKL stimulation of mammary epithelial cells triggers marked nuclear translocation of Id2, which is essential for cell cycle progression and the regulation of p21 expression. These results identify RANKL as a novel regulator for the proliferation of mammary epithelial cells via the inhibitor of HLH protein, Id2.

MATERIALS AND METHODS

Mice. Mice genetically deficient for *rankl* or *Id2* were described previously (19, 43). *cyclin D1*$^{-/-}$ mice were purchased from Jackson Laboratories. Mouse genotypes were determined by PCR and Southern blot analysis. For timed pregnancies, male and female mice were mated overnight, and female mice were scored for vaginal plaques the next morning. The presence of vaginal plaques was taken to represent pregnancy day 0.5 (P0.5). The *rankl*$^{-/-}$ and *Id2*$^{-/-}$ mice were

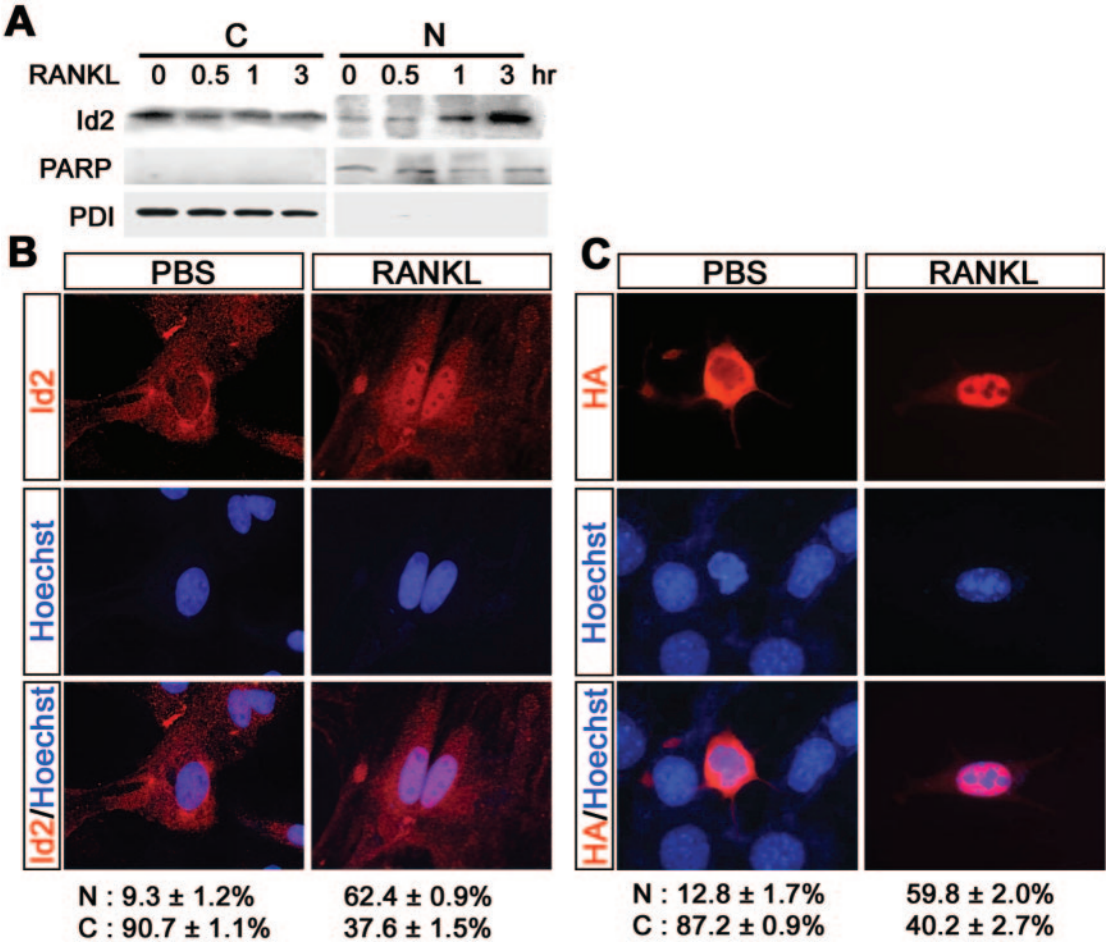


FIG. 4. RANKL induces the nuclear translocation of Id2 in HC11 and primary mammary epithelial cells. (A) Primary MECs from P14.5 mice were treated with 1 μg of RANKL/ml for the indicated times. Cytoplasmic (C) and nuclear (N) proteins were analyzed by Western blotting for the presence of Id2. Protein disulfide isomerase (PDI) and poly(ADP-ribose) polymerase (PARP) are shown as loading controls for cytoplasmic and nuclear proteins, respectively. One result, representative of four independent experiments, is shown. (B) Primary MECs from P14.5 mice were placed in DMEM containing 1% FBS for 24 h. Serum-starved cells were untreated (left panels) or stimulated (right panels) with 1 μg of RANKL/ml for 3 h. Id2 was detected with an anti-Id2 antibody, followed by Alexa 594-labeled anti-rabbit IgG antibody (red; upper panel). Nuclear DNA was stained with Hoechst (blue; middle panel). Lower panels indicate merged images. At the bottom, the numbers indicate the localization of Id2 in the cytoplasm (C) and nucleus (N) of each cell. The values shown are means ± the standard error of the mean of three separate experiments. One result, representative of three independent experiments, is shown. (C) HC11 cells transiently transfected with HA-tagged Id2 were placed in RPMI containing 0.5% FBS for 24 h. Serum-starved cells were untreated (left panels) or stimulated (right panels) with 1 μg of RANKL/ml for 3 h. HA epitopes were detected with an anti-HA antibody, followed by Alexa 594-labeled anti-mouse IgG antibody (red; upper panels). Nuclear DNA was stained with Hoechst (blue; middle panels). Lower panels indicate merged images. One result, representative of four independent experiments, is shown.

of the C57BL/6 (*H-2*$^{b/b}$) and ICR backgrounds, respectively. All mice were maintained at the POSTECH animal facilities.

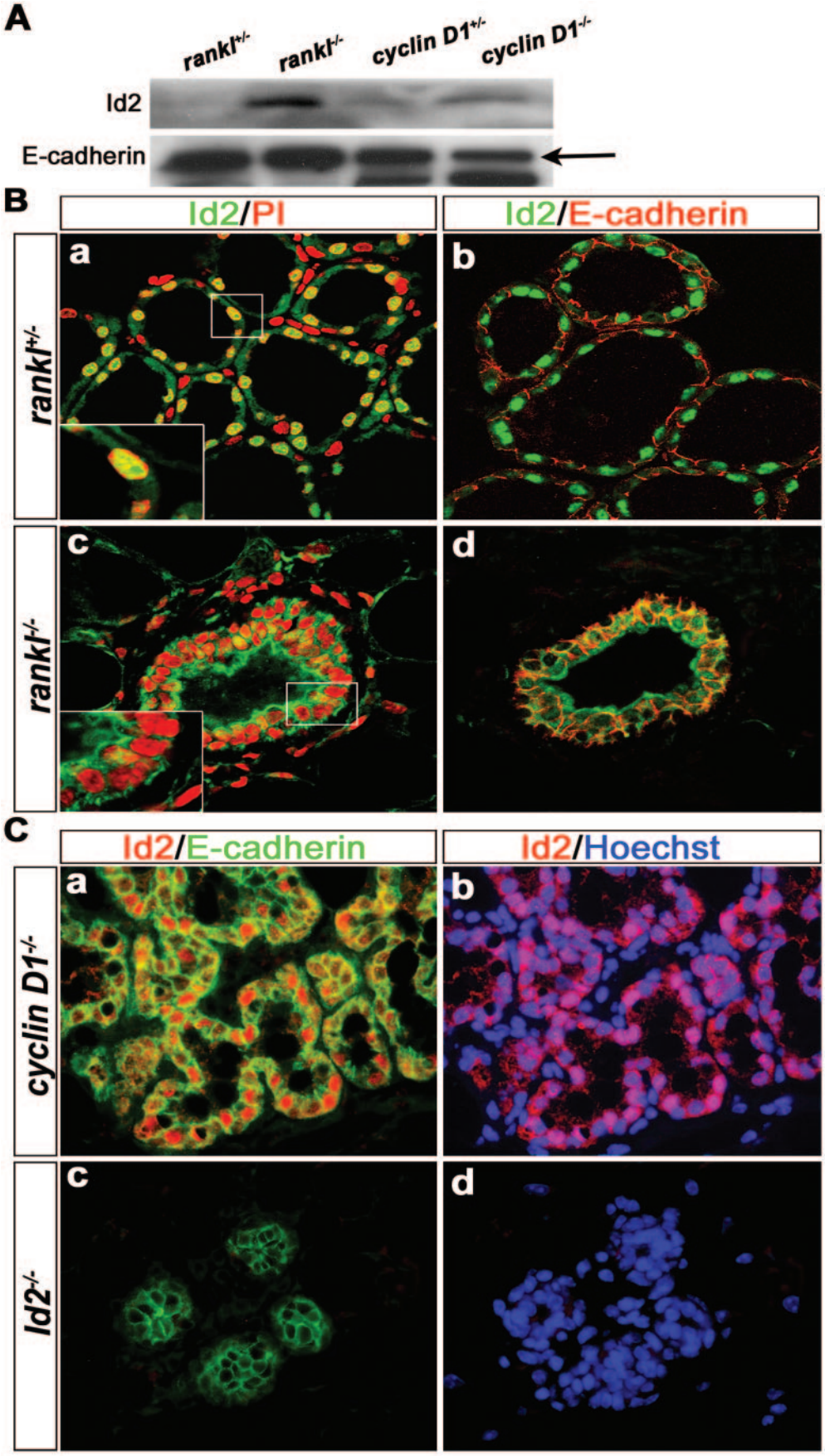
Cell culture and proliferation assays. Primary mammary epithelial cells were obtained as described previously (18). For [^{3}H]thymidine incorporation, cells were plated at low density, allowed to attach overnight in complete media, and then placed in Dulbecco modified Eagle medium (DMEM) containing 1% fetal bovine serum (FBS) for 24 h prior to RANKL stimulation. [^{3}H]thymine was added 12 h before harvest, and the cells were collected 24 or 48 h after RANKL stimulation. Recombinant murine RANKL(158–316) (rRANKL) was produced as described previously (20), and the osteoclastogenic activity of 1 μg of rRANKL/ml was equivalent to that of 100 ng of rRANKL/ml from Peprotech (Rehovot, Israel).

Nuclear and whole-cell protein extraction. For nuclear translocation experiments, cells were placed in RPMI (HC11 cells) or DMEM (primary epithelial cells) containing 10% FBS prior to RANKL stimulation. Nuclear and whole-cell protein extracts were prepared as described previously (18).

Immunoblotting. Equal amounts of whole-cell extracts or nuclear extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were incubated with antibodies to Id2, cyclin D1, p21, ERK1, PDI (Santa Cruz Biotechnology), E-cadherin (BD Biosciences), and PARP (BD Transduction Laboratories). Protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Promoter reporter assays. Primary mammary epithelial cells were transiently transfected by using the Lipofectamine Plus reagent (Life Technologies) with 1 μg of plasmid DNA (p21-Luc) per well in 12-well plates. The *Renilla* reporter construct pRL-TK (Promega) was used for normalizing the transfection efficiency. After transfection, the cells were incubated for 24 h in DMEM containing 1% FBS and harvested 24 h after stimulation with RANKL. Luciferase activity was determined by using the dual-luciferase reporter assay system (Promega).

Immunohistochemistry. For histological analysis, tissues were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin wax for sectioning.



The sections (4 μm) were stained with hematoxylin and eosin. For immunostaining, paraffin-embedded sections were dehydrated, and the antigenic epitopes were exposed by using 10 mM citrate buffer and microwaving. Sections were incubated in blocking solution (3% bovine serum albumin, 3% goat serum, and 0.5% Tween 20 in phosphate-buffered saline) at room temperature for 4 h, followed by an additional incubation with polyclonal antibodies to Id2 (1: 50 dilution; Santa Cruz Biotechnology), cyclin D1 (1:50 dilution; Neomarker) and monoclonal antibodies to E-cadherin (1:200; BD Biosciences). Specific binding was detected with Alexa 488-labeled anti-mouse and Alexa 594-labeled anti-rabbit immunoglobulin G (IgG; Molecular Probes), respectively.

Immunocytochemistry. Primary mammary epithelial cells (MECs) were incubated for 24 h in DMEM containing 1% FBS, prior to RANKL stimulation. Primary MECs, MCF7 cells, and HC11 cells were transiently transfected with plasmid DNA constructs. After 24 h, the cells were placed in RPMI containing 0.5% FBS for 24 h prior to RANKL stimulation. Serum-starved cells were pretreated with kinase inhibitors, cdk2 inhibitor II (Calbiochem, catalog no. 219445, 15 μM), Roscovitine (Calbiochem, catalog no. 557360, 15 μM), mitogen-activated protein kinase (MAPK) inhibitor (SB202190; Calbiochem, catalog no. 559388, 30 μM), PI_3K inhibitor (LY294002; Calbiochem, catalog no. 440202, 20 μM), and c-Jun N-terminal kinase (JNK) inhibitor (Calbiochem, catalog no. 420119, 25 μM) for 3 h and were stimulated with 1 μg of RANKL/ml for 3 h. The cells were then fixed with 4% paraformaldehyde at room temperature for 10 min. Fixed cells were incubated in blocking solution (3% bovine serum albumin, 3% goat serum, and 0.5% Tween 20 in PBS) at room temperature for 4 h, followed by an additional incubation with antihemagglutinin anti-HA) antibody (1:200 dilution; Santa Cruz Biotechnology) and anti-Id2 antibody (1:50 dilution; Santa Cruz Biotechnology). Subsequently, the cells were stained with Alexa 594-labeled anti-mouse and anti-rabbit IgG (Molecular Probes) at room temperature for 1 h and then stained with Hoechst (10 μg/ml) for 5 min.

RESULTS

RANKL directly induces the proliferation of mammary epithelial cells. Both *rankl*$^{-/-}$ and *rank*$^{-/-}$ mice show rudimentary alveolar development, but the proliferation of the alveolar bud epithelium was significantly reduced, as revealed by in situ immunostaining for proliferating cell nuclear antigen (PCNA) (9). Implantation of rRANKL-containing pellets restored PCNA expression in the alveolar epithelial cells of pregnant *rankl*$^{-/-}$ mice, suggesting the critical role of RANKL in the proliferation of mammary epithelial cells (9). However, it is not clear whether RANKL directly regulates the proliferation of mammary epithelial cells or indirectly influences the proliferation via other factors, such as prolactin, progesterone, or IGF-2 (4). Thus, we tested whether RANKL can directly induce the proliferation of mammary epithelial cells in vitro. RANKL stimulation resulted in a significant dose-dependent increase in DNA synthesis by primary mammary epithelial cells from P14.5 pregnant C57BL/6 mice (Fig. 1). In the mouse mammary epithelial cell line HC11, we observed significant proliferation in response to RANKL in a dose-dependent manner, and stimulation of the synchronized HC11 cells with RANKL for 12 h led to a marked increase in the percentage of cells in the S phase, from 18.4% (without RANKL) to 27.7% (with RANKL) (data not shown). Thus, our data clearly show that RANKL directly induces the proliferation of mammary epithelial cells by triggering cell cycle progression.

Normal cyclin D1 expression in *rankl*$^{-/-}$ mammary glands. The underlying molecular mechanism required for the proliferation of mammary epithelial cells in response to RANKL has been controversial. While Cao et al. suggested that RANK signaling leads to NF-κB activation through cyclin D1 in mammary epithelial proliferation, RANKL failed to induce cyclin D1 activity in primary mammary epithelial cells (4, 6). To test whether cyclin D1 expression is regulated by RANKL, we examined cyclin D1 expression in *rankl*$^{-/-}$ mammary glands. As shown in Fig. 2C, immunohistochemistry studies revealed that cyclin D1 was clearly expressed in mammary epithelial cells from L1 (lactation day 1) *rankl*$^{-/-}$ mammary glands (Fig. 2C). Importantly, in Western blots, the levels of cyclin D1 were even increased in P14.5 and L1 *rankl*$^{-/-}$ mammary glands compared to of the level in wild-type mammary tissues (Fig. 2G). Taken together, our results suggest that cyclin D1 expression is not induced in response to RANKL-RANK signaling.

RANKL induces nuclear translocation of the HLH protein inhibitor, Id2. Since RANKL stimulation of mammary epithelial cells does not lead to cyclin D1 induction, the molecular mechanism that leads to the proliferation of mammary epithelial cells by RANKL is not clear. The Id proteins (inhibitor of DNA binding and cell differentiation) are a group of HLH transcription factors that lack a DNA-binding domain. In particular, the mammary gland defects in Id2 mutant mice (26, 28) resemble those of *rankl*$^{-/-}$ mice (9) but not *cyclin D1*$^{-/-}$ mice (36) (Fig. 3). Moreover, both *Id2*$^{-/-}$ and *rankl*$^{-/-}$ mice share phenotypes, such as the lack of lymph nodes (19, 43). When we tested the Id2 protein expression patterns in the mammary tissues from virgin, pregnant, and lactating C57B/L6 mice, the Id2 protein level was relatively low in the mammary tissue from virgin mice. Similar to RANKL (9), the Id2 levels gradually increased during pregnancy (data not shown). Therefore, we speculate that there must be a relationship between RANKL and Id2 in the proliferation of mammary gland epithelial cells and that Id2 might be a downstream mediator in RANK signaling.

Thus, we tested whether RANKL can regulate Id2 gene expression. After the RANKL treatment of primary mammary epithelial cells, the total RNA and protein levels of Id2 were not changed (data not shown), indicating that RANKL does not lead to Id2 induction. Since previous studies suggested that Id2-regulated cell proliferation is controlled via nuclear transport (25), we investigated whether RANKL treatment would cause the translocation of Id2 from the cytosol to the nucleus in mammary gland epithelial cells. RANKL stimulation induced the marked nuclear translocation of Id2 in p14.5 pri-

FIG. 5. Impaired nuclear translocation of Id2 in *rankl*$^{-/-}$ mammary glands. (A) Western blot analysis of Id2 in *rankl*$^{+/-}$, *rankl*$^{-/-}$, *cyclin D1*$^{+/-}$ and *cyclin D1*$^{-/-}$ mammary glands at L1. E-cadherin is shown as a protein loading control (black arrow). (B) Localization of Id2 in *rankl*$^{+/-}$ (upper panels) and *rankl*$^{-/-}$ (lower panels) mammary gland epithelial cells. Tissue sections of mammary glands from *rankl*$^{+/-}$ and *rankl*$^{-/-}$ mice at L1 were stained with anti-Id2 (a to d; green) and anti-E-cadherin (b and d; red) antibodies. Nuclear DNA was stained with PI (a and c; red). Images were acquired by using a confocal microscope (Leica Dmire2). Note the complete absence of Id2 in the nuclei of *rankl*$^{-/-}$ epithelial cells. Magnification, ×400. The magnified images are shown in the insets. (C) Tissue sections of mammary glands from *cyclin D1*$^{-/-}$ (upper panels) and *Id2*$^{-/-}$ (lower panels) mice at L1 were stained with anti-Id2 (a to d; red) and anti-E-cadherin (a and c; green) antibodies. Nuclear DNA was stained with Hoechst (b and d; blue). The specificity of antibody staining for Id2 was confirmed by its absence in *Id2*$^{-/-}$ mammary epithelium (c and d).

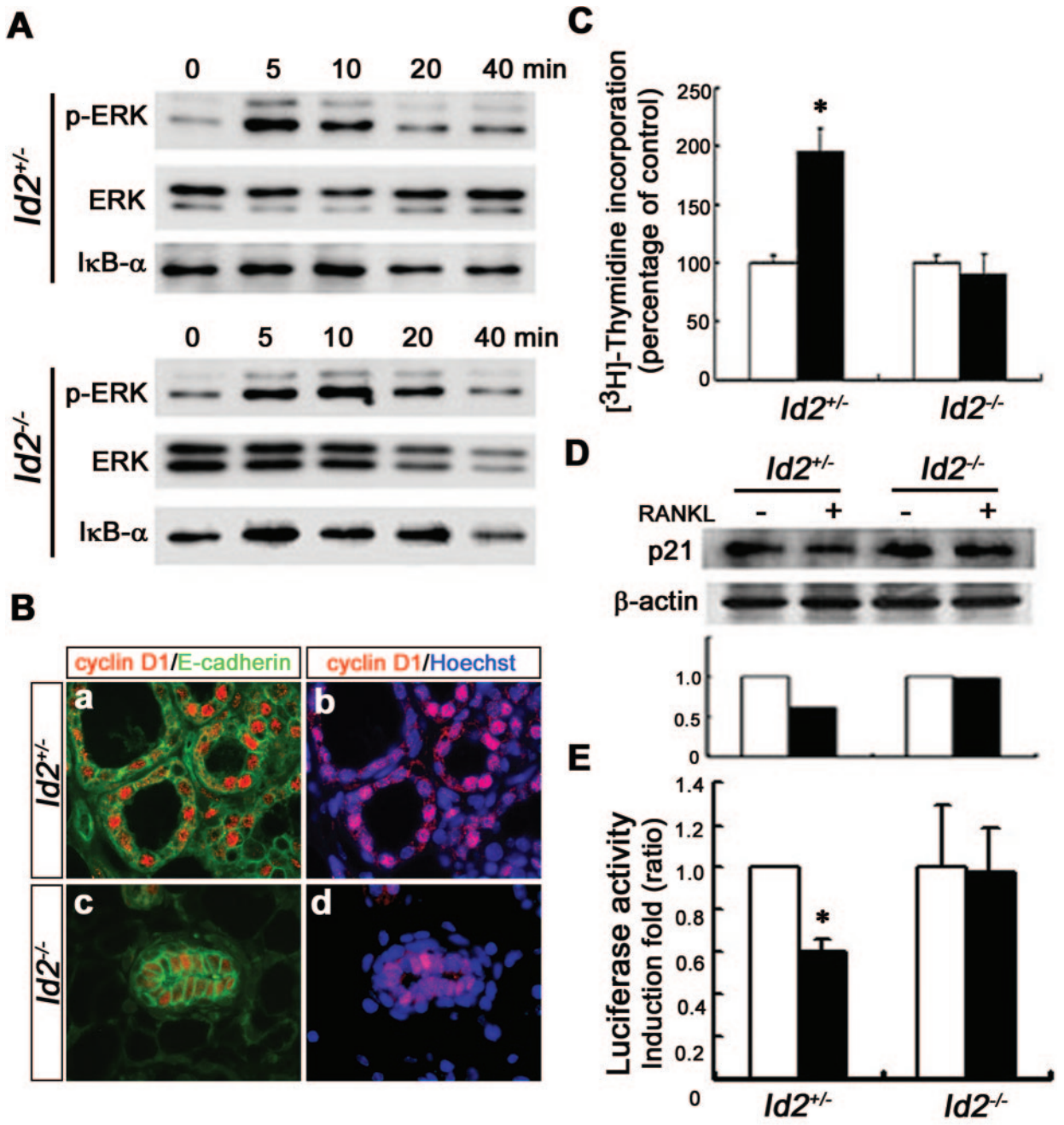


FIG. 6. Defective RANKL-mediated downregulation of p21 expression and proliferation in Id2$^{-/-}$ mammary epithelial cells. (A) Activation of ERK and IκB in P14.5 *Id2*$^{+/-}$ and *Id2*$^{-/-}$ mammary epithelial cells after RANKL treatment. Cells were treated with 1 μg of RANKL/ml for the indicated times. The phosphorylated form of ERK (p-ERK) in whole-cell extracts was detected with the phospho-specific antibody. The membrane was stripped and probed with antibodies against ERK and IκB-α as indicated. One representative of three independent experiments is shown. (B) Immunohistochemistry of cyclin D1 from *Id2*$^{+/-}$ (a and b) and *Id2*$^{-/-}$ (c and d) mice. Tissue sections of mammary glands from *Id2*$^{+/-}$ and *Id2*$^{-/-}$ mice at L1 were stained with anti-cyclin D1 (red)/anti-E-cadherin (green) antibodies (a and c) and Hoechst (b and d; blue). (C) Proliferation of mammary epithelial cells. Primary mammary epithelial cells from P14.5 *Id2*$^{+/-}$ and *Id2*$^{-/-}$ mice were stimulated with the indicated doses of RANKL for 24 h. Cells were incubated with 1 μCi of [^{3}H]thymidine/ml for the last 12 h of culture, and [^{3}H]thymidine incorporation was measured. The results are shown as mean values ± the standard error of the mean of three separate experiments. ✻, significant difference ($P < 0.001$). (D) Western blotting of p21 expression in *Id2*$^{-/-}$ primary mammary epithelial cells after RANKL stimulation. Serum-starved primary mammary epithelial cells from P14.5 *Id2*$^{+/-}$ and *Id2*$^{-/-}$ mice were stimulated without (−) or with (+) 1 μg of RANKL/ml for 12 h. Actin is shown as a protein loading control. Relative expression levels of p21 to actin are indicated in the bar graphs. One result, representative of four independent experiments, is shown. (E) p21 promoter luciferase assays in primary mammary epithelial cells from P14.5 pregnant mice. Mammary epithelial cells transfected with p21-luciferase were left untreated (□) or treated with 1 μg of RANKL/ml (■) for 24 h. Luciferase reporter activity was normalized to *Renilla* luciferase activity. The results are shown as mean values ± the standard error of the mean of five separate transfection experiments. ✻, significant difference ($P < 0.001$).

mary mammary gland epithelial cells (Fig. 4A and B). To directly examine the nuclear translocation of Id2 by RANKL stimulation, we transfected HC11 cells with HA-tagged Id2 (HA-Id2). Without stimulation, most of the HA-Id2 was localized in the cytoplasm. In contrast, we observed the nuclear localization of HA-Id2 upon RANKL stimulation, indicating that RANKL readily induces the nuclear translocation of Id2 in mammary gland epithelial cells (Fig. 4C). Taken together, these data suggested that RANKL may induce proliferation of mammary gland epithelial cells through the nuclear translocation of Id2, a positive regulator of cell cycle progression.

Impaired nuclear localization of Id2 in the mammary glands of *rankl*$^{-/-}$ mice. Since Id2 might be a downstream mediator of the RANKL/RANK signaling pathway, we analyzed the status of Id2 expression in vivo in the mammary glands of pregnant and lactating *rankl*$^{-/-}$ mice. The levels of Id2 protein expression in the mammary glands of lactating (L1) *rankl*$^{-/-}$ females were increased compared to the *rankl*$^{+/-}$ mammary glands (Fig. 5A). Since RANKL did not induce Id2 gene expression (data not shown), this result suggests that other factors may induce the gene expression of Id2 and/or that increased Id2 expression occurs as a compensatory mechanism for the loss of RANK signaling. In the mammary tissues of L1 *cyclin D1*$^{-/-}$ mice, Id2 expression was not decreased, indicating the cyclin D1-independent expression of Id2.

Since Id2 acts as a dominant-negative regulator of bHLH transcription factors through heterodimerization with their bHLH partners (32) and it changes its subcellular localization in cell growth regulation (25, 38), we examined the status of Id2 localization in mammary epithelial cells. In the mammary epithelial cells of P14.5, P18.5, and L1 *rankl*$^{+/-}$ mice, Id2 was localized in the nuclei (Fig. 5Ba and b and unpublished data). In contrast, we could not detect Id2 in the nuclei of mammary gland epithelial cells of P14.5, P18.5, and L1 *rankl*$^{-/-}$ mice, even in the increased expression (Fig. 5Bc and d and unpublished data). Furthermore, in the mammary tissues of L1 *cyclin D1*$^{-/-}$ mice, the nuclear accumulation of Id2 was readily observed (Fig. 5Ca and b). These data reveal that the loss of RANKL impairs both the nuclear accumulation and the translocation of Id2 in vivo, independently of cyclin D1.

Impaired proliferation of *Id2*$^{-/-}$ mammary epithelial cells in response to RANKL. Id2 mutant epithelia display defective proliferation and increased p21, p27, and cyclin D1 expression (27, 28). However, the upstream signals that control mammary epithelial cell growth through the Id2 protein are not known. Since the nuclear translocation of Id2 is induced by RANKL stimulation, it would be interesting to know whether this defective proliferation is due to a defect in RANK signaling. To test whether *Id2*$^{-/-}$ mammary epithelial cells express functional RANK, which is able to activate ERK and NF-κB in response to RANKL, we stimulated primary mammary epithelial cells with RANKL and evaluated the activation of ERK and NF-κB. As shown in Fig. 6A, the phosphorylated forms of extracellular signal-regulated kinase (ERK) 1 and 2 and the degradation of IκB-α were readily detected in the mammary epithelial cells from both P14.5 *Id2*$^{+/-}$ and *Id2*$^{-/-}$ mice after RANKL treatment. Thus, the loss of Id2 expression has no apparent effect on RANKL-mediated ERK phosphorylation and IκBα degradation. In addition, consistent with the previous report that cyclin D1 is expressed during pregnancy, we readily observed cyclin D1 expression in the mammary epithelial cells of L1 *Id2*$^{-/-}$ mice (Fig. 6Bc and d). Importantly, in spite of the intact RANKL-mediated ERK phosphorylation and IκB-α degradation, no increase in [^{3}H]thymidine incorporation was observed in *Id2*$^{-/-}$ mammary epithelial cells after RANKL stimulation, whereas RANKL stimulation induced a marked increase of DNA synthesis in primary mammary epithelial cells from *Id2*$^{+/-}$ mice (Fig. 6C). These data indicate that Id2 is crucial for the RANKL-induced proliferation of mammary epithelial cells.

To further confirm that Id2 is a downstream molecule of RANK signaling in mammary epithelial cell proliferation, we tested the RANKL-induced downregulation of p21 expression in primary mammary epithelial cells from mice lacking Id2. As shown in Fig. 6D, RANKL treatment of *Id2*$^{+/-}$ primary mammary epithelial cells resulted in a 38% decrease in p21 expression. In contrast, RANKL treatment in *Id2* $^{-/-}$ primary mammary epithelial cells failed to downregulate the expression of p21 (Fig. 6D). Consistently, *Id2*$^{-/-}$ primary mammary epithelial cells transfected with p21-luciferase did not downregulate p21-promoter activity after RANKL stimulation, while *Id2*$^{+/-}$ primary mammary epithelial cells transfected with p21-luciferase displayed a 40% decrease compared to the control stimulation (Fig. 6E). These data indicate that Id2 mediates the RANKL-induced downmodulation of p21 expression in mammary epithelial cells. Taken together, these results show that Id2 is a critical downstream target molecule of RANK signaling that regulates RANKL-mediated cell growth and p21 expression in mammary epithelial cells.

Id2 nuclear localization in MEC proliferation. To further examine the importance of the nuclear localization of Id2 in MEC proliferation, we generated an HA-Id2 expressing vector carrying the nuclear localization sequence (NLS) of simian virus 40 (HA-NLS-Id2). In MCF7 cells cotransfected with HA-Id2 and p21-luciferase, HA-Id2 was localized in the cytoplasm in the absence of RANKL stimulation and the p21 promoter activity was unaffected compared to that of the mock transfection (Fig. 7A and C). In contrast, HA-NLS-Id2 was localized in the nuclei (Fig. 7A), and the p21 promoter activity was decreased (ca. 40%) in the MCF7 cells transfected with NLS-HA-Id2 and p21 luciferase (Fig. 7C). Furthermore, when *rankl*$^{-/-}$ MECs from P14.5 mice were transfected with either HA-Id2 or HA-NLS-Id2, HA-NLS-Id2 was localized in the nuclei, whereas HA-Id2 was in the cytoplasm (Fig. 7B). Importantly, the *rankl*$^{-/-}$ MECs transfected with HA-NLS-Id2 displayed about a 50% decrease in p21 promoter activity (Fig. 7D). These results indicate that the nuclear localization of Id2 is important for the reduced p21 promoter activity.

Previous studies reported that cyclin E/cdk2 phosphorylates serine 5 of Id2 (13), which is critical for the proliferation of smooth muscle cells (25). To examine whether the RANKL-induced nuclear translocation of Id2 in mammary epithelial cells is mediated by cyclin E/cdk2, MCF7 cells transfected with HA-Id2 were stimulated with RANKL in the presence of the cdk2 inhibitors cdk2 inhibitor II and Roscovitine. The nuclear localization of Id2 was completely abrogated by cdk2 inhibitors (Fig. 7Ec and d), suggesting that cyclin E/cdk2 is important for the RANKL-induced nuclear translocation of Id2 in mammary epithelial cells. To test what pathways downstream of RANK are involved in the Id2 translocation, MCF7 cells transfected

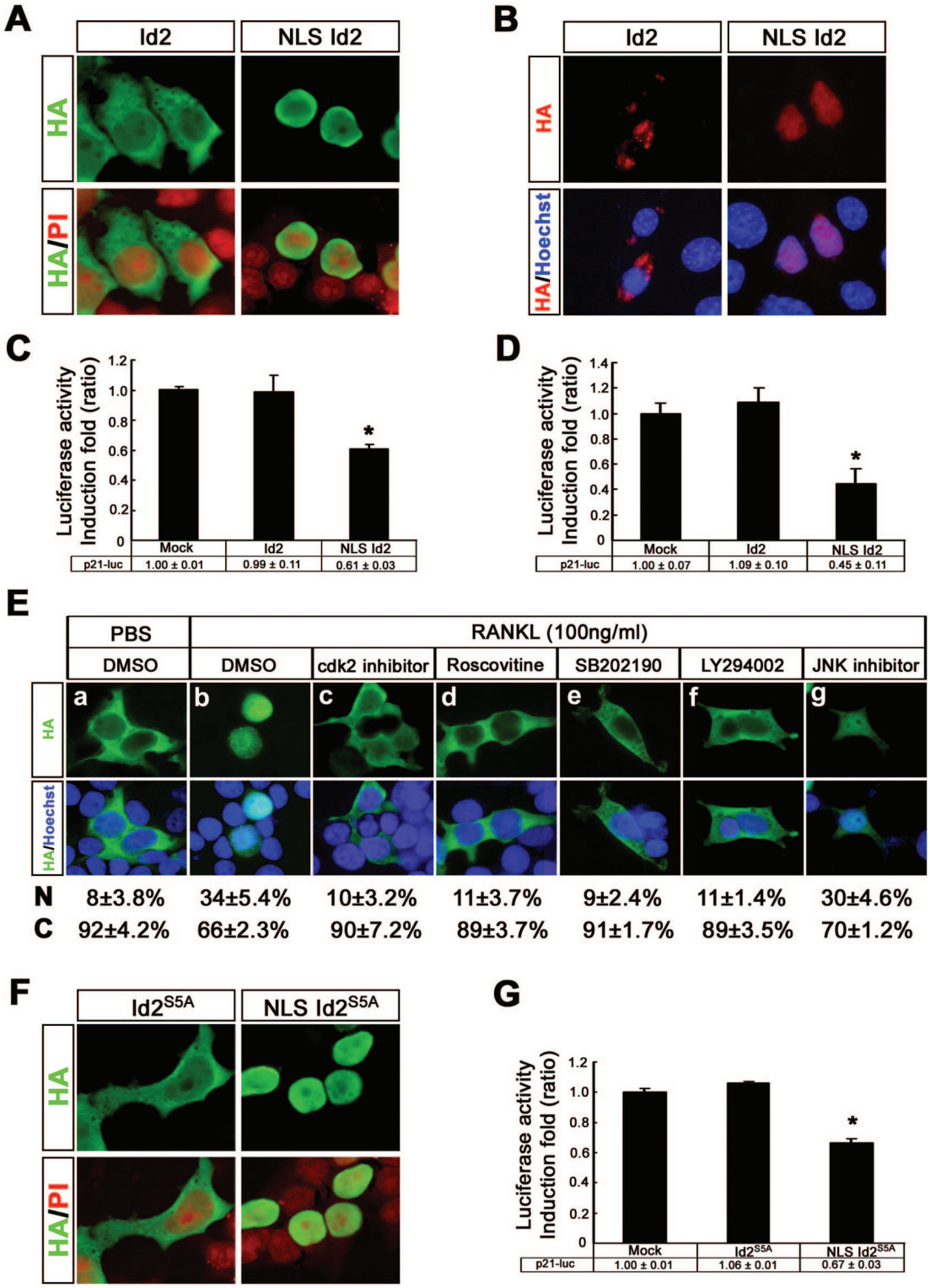


FIG. 7. Nuclear localization of Id2 in mammary epithelial cells. (A and B) Localization and p21 promoter luciferase assays of Id2 and NLS-tagged Id2. MCF7 cells (A) and primary mammary epithelial cells from *rankl*$^{-/-}$ P14.5 mice (B) were transiently transfected with HA-Id2 (Id2) and NLS-HA-Id2 (NLS-Id2) constructs and placed in DMEM containing 10% FBS for 48 h. HA epitopes were detected with an anti-HA antibody, followed by Alexa 488-labeled anti-mouse IgG antibody (A, green) and Alexa 594-labeled anti-mouse IgG antibody (B, red). Nuclear DNA was stained with PI (A, red) and Hoechst (B, blue). One result, representative of three independent experiments, is shown. (C and D) p21 promoter luciferase assays in MCF7 cells (C) and primary mammary epithelial cells from *rankl*$^{-/-}$ P14.5 mice (D). Cells were cotransfected with p21-luciferase and Id2 constructs and, after 48 h, the luciferase reporter activity was measured and normalized to the *Renilla* luciferase activity. The results are shown as mean values ± the standard error of the mean of three separate transfection experiments. ✱, significant difference ($P < 0.001$). (E) Localization of Id2 in MCF7 cells after RANKL treatment in the presence of kinase inhibitors. MCF7 cells transiently transfected

with HA-Id2 were treated with pathway inhibitors prior to RANKL stimulation. MAPK inhibitor (SB202190) and PI_3K inhibitor (LY294002) abrogated the nuclear translocation of Id2 by RANKL stimulation, but JNK inhibitor did not (Fig. 7Ee to g). These data suggest that RANKL stimulation results in the nuclear translocation of Id2 via the activation of cdk2, which is mediated by PI_3K- and MAPK-dependent pathways.

Since cyclin E/cdk2 phosphorylates serine 5 of Id2 (13), we generated an HA-Id2 mutant with an alanine replacing serine 5 (HA-$Id2^{S5A}$) and an NLS-tagged HA-$Id2^{S5A}$ (HA-NLS-$Id2^{S5A}$) to further examine the importance of the nuclear translocation of Id2 in mammary epithelial cells. In MCF7 cells transfected with HA-$Id2^{S5A}$, it was localized in the cytoplasm (Fig. 7F). Neither this cytoplasmic localization of $Id2^{S5A}$ nor the p21 promoter activity were altered, even after RANKL stimulation (data not shown), suggesting that the cdk2 phosphorylation site (serine of the 5) of Id2 is essential for RANKL-induced nuclear localization. In contrast, all HA-NLS-$Id2^{S5A}$ was observed in the nuclei in HA-NLS-$Id2^{S5A}$-transfected MCF7 cells (Fig. 7F). Intriguingly, the p21 promoter activity was also decreased in the MCF7 cells expressing HA-NLS-$Id2^{S5A}$ (Fig. 7G). These results indicate that the nuclear localization of Id2 itself is critical for the downregulation of p21 promoter activity and suggest that RANKL stimulation leads to the phosphorylation of Id2 via cdk2, which results in its nuclear localization.

DISCUSSION

RANKL is a critical molecule for osteoclast development or activation and skeletal calcium release. In addition, *rankl*$^{-/-}$ mice revealed defects in lactating mammary gland formation due to the impaired survival and proliferation of mammary gland epithelial cells (9). In the present study, we investigated the molecular events that lead to the proliferation of mammary gland epithelial cells through RANKL/RANK signaling. Our results demonstrated that RANKL directly induces the growth of mammary gland epithelial cells, a finding consistent with the in vivo data that *rankl*$^{-/-}$ mice displayed impaired proliferation of mammary gland epithelium during pregnancy (9). Since both Id2- and IKKα-mutant mice exhibited defects in mammary gland development that are similar to those in RANKL and RANK deficient mice (6, 9, 28), we speculated that one of these signaling pathways might be a possible mediator of the RANKL/RANK-mediated proliferation of mammary gland epithelial cells. Our results show that the inhibitor of helix-loop-helix, Id2, is a critical downstream mediator of RANKL/RANK-induced mammary epithelial cell growth through the downregulation of p21.

The proliferation and differentiation of mammary alveolar cells during pregnancy is controlled by the prolactin receptor, Stat5a, cyclin D1, p27, Id2, C/EBPβ, and RANKL/RANK (5, 8, 9, 23, 28, 29, 34–36). The prolactin receptor-Jak2-Stat5a signaling pathway has been extensively characterized, and prolactin stimulation leads to cyclin D1 induction through IGF-2 (4). The mammary glands of cyclin D1-deficient mice fail to develop fully during pregnancy (8, 36). P27-null mice also display a decreased proliferation rate and delayed differentiation (29), which is reminiscent of the situation in *cyclinD1*$^{-/-}$ mice. The combined loss of cyclin D1 and p27 results in overtly normal mammary gland development, suggesting that cyclin D1 and p27 function antagonistically (10). Since cyclin D1 and p27 double mutant null mice exhibit normal mammary development, it can be assumed that a second pathway is activated in the absence of both proteins. In the present study, the nuclear translocation of Id2 was absolutely impaired in the *rankl*$^{-/-}$ mice, and the proliferation of *id2*$^{-/-}$ mammary epithelial cells was severely decreased in response to RANKL stimulation, indicating that Id2 is a critical mediator in the RANK signaling pathway. Thus, in addition to the RANK-IKKα-cyclin D1-p27 pathway, we suggest a new critical pathway, RANKL/RANK-Id2-p21, that leads to the proliferation of mammary epithelial cells.

Id proteins function as positive regulators of cell proliferation and negative regulators of cell differentiation (32). Notably, recent evidences have linked inhibitor of HLH factors to cell cycle control in a variety of cell types, including natural killer cells, trophoblasts, oligodendrocytes, and smooth muscle cells (16, 21, 22, 25, 38). Interestingly, several studies reported the change in the subcellular localization of the Id2 protein (25, 38). Especially, in smooth muscle cells, Id2 was translocated to the nucleus, resulting in the enhancement of cell growth (25). In oligodendrocytes, Id2 was translocated out of the nucleus into the cytoplasm during oligodendrocyte differentiation, leading to the promotion of cell differentiation (38). Moreover, the mammary glands of Id2-deficient female mice showed impaired lobuloalveolar development due to the defective proliferation and survival of epithelial cells (28). In our experiments, Id2 expression in mammary tissue was upregulated from midpregnancy until the early phase of lactation (data not shown). Likewise, RANKL expression is observed at midpregnancy (P12.5) and gradually increases to 1 day of lactation (L1) and is induced by pregnancy hormones such as prolactin, progesterone, and parathyroid hormone (9). However, the upstream hormones that cause the upregulation of the Id2 protein in mammary glands have not been identified, except a recent report that Id2 is a direct target of C/EBPβ (17). RANKL stimulation of mammary gland epithelial cells did not trigger Id2 expression (data not shown). Moreover, the Id2 protein levels were increased in the mammary glands of pregnant *rankl*$^{-/-}$ mice, suggesting that other factors related to pregnancy can induce Id2 gene expression independently of RANKL expression (data not shown). Surprisingly, despite the increased levels of Id2 in *rankl*$^{-/-}$ mammary tissue during

with the HA-Id2 construct were placed in DMEM containing 1% FBS for 12 h. Serum-starved cells were treated with dimethyl sulfoxide (a and b) or the indicated kinase inhibitors (c to g) for 3 h. Inhibitor-pretreated cells were untreated (a) or stimulated with 1 μg of RANKL/ml for 3 h (b to g). HA epitopes were detected with an anti-HA antibody, followed by Alexa 488-labeled anti-mouse IgG antibody (green). Nuclear DNA was stained with Hoechst (blue). One result, representative of two independent experiments, is shown. (F and G) Localization (F) and p21 promoter luciferase activity (G) of $Id2^{S5A}$ and NLS-$Id2^{S5A}$ in MCF7 cells. These experiments were performed as described for panels A and C. One result, representative of two independent experiments, is shown. Magnification, ×400.

pregnancy, Id2 was localized in the cytosol but excluded from the nucleus, indicating that RANKL is critical for the nuclear translocation of Id2. This is consistent with our in vitro data that RANKL stimulation results in the nuclear translocalization of Id2 in both HC11 and primary mammary epithelial cells. Taken together, our data show that RANKL/RANK activation results in the translocation of Id2 to the nuclei of mammary epithelial cells through cyclin E/cdk2 activation.

RANKL binding to its receptor, RANK, results in NF-κB activation (1). Recent studies have revealed that NF-κB is induced during mammary gland development (7, 11) and that one of the target molecules is cyclin D1 (12, 15). Indeed, NF-κB positively regulates mammary epithelial proliferation and branching during normal mammary gland development (3). Based on these findings, we speculated that RANKL stimulation of mammary epithelial cells would lead to NF-κB activation and subsequently result in cyclin D1 induction. In fact, constitutive activation of NF-κB is observed in a number of breast cancers (2, 31, 37). However, we could not detect any increase in cyclin D1 induction in in vitro and in vivo experiments, despite NF-κB activation and IκBα degradation after the RANKL treatment in mammary epithelial cells. In accordance with our in vitro results, Brisken et al. also reported that RANKL failed to induce cyclin D1 expression, whereas prolactin stimulation led to cyclin D1 induction through IGF-2 (4). Whether Id2 is involved in the NFκB pathway needs to be determined.

In the present study, we demonstrated that Id2 function is regulated by RANK signaling and links RANK activation to cell cycle progression and p21 modulation. Our findings describe a new signaling pathway that controls mammary epithelial cell proliferation during pregnancy. Our data also suggest that the RANKL-RANK-Id2-p21 pathway may cooperate with the prolactin receptor-IGF2-cyclin D1-p27 pathway to fully develop mammary gland structures during pregnancy.

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REFERENCES

1. **Anderson, D. M., E. Maraskovsky, W. L. Billingsley, W. C. Dougall, M. E. Tometsko, E. R. Roux, M. C. Teepe, R. F. DuBose, D. Cosman, and L. Galibert.** 1997. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature **390:**175–179.
2. **Biswas, D. K., S. C. Dai, A. Cruz, B. Weiser, E. Graner, and A. B. Pardee.** 2001. The nuclear factor κB (NF-κB): a potential therapeutic target for estrogen receptor negative breast cancers. Proc. Natl. Acad. Sci. USA **98:** 10386–10391.
3. **Brantley, D. M., C. L. Chen, R. S. Muraoka, P. B. Bushdid, J. L. Bradberry, F. Kittrell, D. Medina, L. M. Matrisian, L. D. Kerr, and F. E. Yull.** 2001. Nuclear factor-κB (NF-κB) regulates proliferation and branching in mouse mammary epithelium. Mol. Biol. Cell **12:**1445–1455.
4. **Brisken, C., A. Ayyannan, C. Nguyen, A. Heineman, F. Reinhardt, J. Tan, S. K. Dey, G. P. Dotto, R. A. Weinberg, and T. Jan.** 2002. IGF-2 is a mediator of prolactin-induced morphogenesis in the breast. Dev. Cell **3:**877–887.
5. **Brisken, C., S. Kaur, T. E. Chavarria, N. Binart, R. L. Sutherland, R. A. Weinberg, P. A. Kelly, and C. J. Ormandy.** 1999. Prolactin controls mammary gland development via direct and indirect mechanisms. Dev. Biol. **210:**96–106.
6. **Cao, Y., G. Bonizzi, T. N. Seagroves, F. R. Greten, R. Johnson, E. V. Schmidt, and M. Karin.** 2001. IKKα provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. Cell **107:**763–775.
7. **Clarkson, R. W., J. L. Heeley, R. Chapman, F. Aillet, R. T. Hay, A. Wyllie, and C. J. Watson.** 2000. NF-κB inhibits apoptosis in murine mammary epithelia. J. Biol. Chem. **275:**12737–12742.
8. **Fantl, V., G. Stamp, A. Andrews, I. Rosewell, and C. Dickson.** 1995. Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev. **9:**2364–2372.
9. **Fata, J. E., Y. Y. Kong, J. Li, T. Sasaki, J. Irie-Sasaki, R. A. Moorehead, R. Elliott, S. Scully, E. B. Voura, D. L. Lacey, W. J. Boyle, R. Khokha, and J. M. Penninger.** 2000. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. Cell **103:**41–50.
10. **Geng, Y., Q. Yu, E. Sicinska, M. Das, R. T. Bronson, and P. Sicinski.** 2001. Deletion of the $p27^{Kip1}$ gene restores normal development in cyclin D1-deficient mice. Proc. Natl. Acad. Sci. USA **98:**194–199.
11. **Geymayer, S., and W. Doppler.** 2000. Activation of NF-κB p50/p65 is regulated in the developing mammary gland and inhibits STAT5-mediated beta-casein gene expression. FASEB J. **14:**1159–1170.
12. **Guttridge, D. C., C. Albanese, J. Y. Reuther, R. G. Pestell, and A. S. Baldwin, Jr.** 1999. NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol. Cell. Biol. **19:**5785–5799.
13. **Hennighausen, L., and G. W. Robinson.** 2001. Signaling pathways in mammary gland development. Dev. Cell **1:**467–475.
14. **Hennighausen, L., and G. W. Robinson.** 1998. Think globally, act locally: the making of a mouse mammary gland. Genes Dev. **12:**449–455.
15. **Hinz, M., D. Krappmann, A. Eichten, A. Heder, C. Scheidereit, and M. Strauss.** 1999. NF-κB function in growth control: regulation of cyclin D1 expression and G_0/G_1-to-S-phase transition. Mol. Cell. Biol. **19:**2690–2698.
16. **Ikawa, T., S. Fujimoto, H. Kawamoto, Y. Katsura, and Y. Yokota.** 2001. Commitment to natural killer cells requires the helix-loop-helix inhibitor Id2. Proc. Natl. Acad. Sci. USA **98:**5164–5169.
17. **Karaya, K., S. Mori, H. Kimoto, Y. Shima, Y. Tsuji, H. Kurooka, S. Akira, and Y. Yokota.** 2005. Regulation of Id2 expression by CCAAT/enhancer binding protein beta. Nucleic Acids Res. **33:**1924–1934.
18. **Kim, H. J., M. J. Yoon, J. Lee, J. M. Penninger, and Y. Y. Kong.** 2002. Osteoprotegerin ligand induces beta-casein gene expression through the transcription factor CCAAT/enhancer-binding protein beta. J. Biol. Chem. **277:**5339–5344.
19. **Kong, Y. Y., H. Yoshida, I. Sarosi, H. L. Tan, E. Timms, C. Capparelli, S. Morony, A. J. Oliveira-dos-Santos, G. Van, A. Itie, W. Khoo, A. Wakeham, C. R. Dunstan, D. L. Lacey, T. W. Mak, W. J. Boyle, and J. M. Penninger.** 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature **397:**315–323.
20. **Lacey, D. L., E. Timms, H. L. Tan, M. J. Kelley, C. R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, H. Hsu, J. Sullivan, N. Hawkins, E. Davy, C. Capparelli, A. Eli, Y. X. Qian, S. Kaufman, I. Sarosi, V. Shalhoub, G. Senaldi, J. Guo, J. Delaney, and W. J. Boyle.** 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell **93:**165–176.
21. **Lasorella, A., R. Boldrini, C. Dominici, A. Donfrancesco, Y. Yokota, A. Inserra, and A. Iavarone.** 2002. Id2 is critical for cellular proliferation and is the oncogenic effector of N-myc in human neuroblastoma. Cancer Res. **62:**301–306.
22. **Lasorella, A., M. Noseda, M. Beyna, Y. Yokota, and A. Iavarone.** 2000. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. Nature **407:**592–598.
23. **Liu, X., G. W. Robinson, K. U. Wagner, L. Garrett, A. Wynshaw-Boris, and L. Hennighausen.** 1997. Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev. **11:**179–186.
24. **Massari, M. E., and C. Murre.** 2000. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol. Cell. Biol. **20:**429–440.
25. **Matsumura, M. E., D. R. Lobe, and C. A. McNamara.** 2002. Contribution of the helix-loop-helix factor Id2 to regulation of vascular smooth muscle cell proliferation. J. Biol. Chem. **277:**7293–7297.
26. **Miyoshi, K., B. Meyer, P. Gruss, Y. Cui, J. P. Renou, F. V. Morgan, G. H. Smith, M. Reichenstein, M. Shani, L. Hennighausen, and G. W. Robinson.** 2002. Mammary epithelial cells are not able to undergo pregnancy-dependent differentiation in the absence of the helix-loop-helix inhibitor Id2. Mol. Endocrinol. **16:**2892–2901.
27. **Mori, S., K. Inoshima, Y. Shima, E. V. Schmidt, and Y. Yokota.** 2003. Forced expression of cyclin D1 does not compensate for Id2 deficiency in the mammary gland. FEBS Lett. **551:**123–127.
28. **Mori, S., S. I. Nishikawa, and Y. Yokota.** 2000. Lactation defect in mice lacking the helix-loop-helix inhibitor Id2. EMBO J. **19:**5772–5781.
29. **Muraoka, R. S., A. E. Lenferink, J. Simpson, D. M. Brantley, L. R. Roebuck, F. M. Yakes, and C. L. Arteaga.** 2001. Cyclin-dependent kinase inhibitor $p27^{Kip1}$ is required for mouse mammary gland morphogenesis and function. J. Cell Biol. **153:**917–932.

30. **Murre, C., G. Bain, M. A. van Dijk, I. Engel, B. A. Furnari, M. E. Massari, J. R. Matthews, M. W. Quong, R. R. Rivera, and M. H. Stuiver.** 1994. Structure and function of helix-loop-helix proteins. Biochim. Biophys. Acta **1218:**129–135.
31. **Nakshatri, H., P. Bhat-Nakshatri, D. A. Martin, R. J. Goulet, Jr., and G. W. Sledge, Jr.** 1997. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol. Cell. Biol. **17:**3629–3639.
32. **Norton, J. D., R. W. Deed, G. Craggs, and F. Sablitzky.** 1998. Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol. **8:**58–65.
33. **Prabhu, S., A. Ignatova, S. T. Park, and X. H. Sun.** 1997. Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and Id proteins. Mol. Cell. Biol. **17:**5888–5896.
34. **Robinson, G. W., P. F. Johnson, L. Hennighausen, and E. Sterneck.** 1998. The C/EBPβ transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes Dev. **12:**1907–1916.
35. **Seagroves, T. N., S. Krnacik, B. Raught, J. Gay, B. Burgess-Beusse, G. J. Darlington, and J. M. Rosen.** 1998. C/EBPβ, but not C/EBPα, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. Genes Dev. **12:**1917–1928.
36. **Sicinski, P., J. L. Donaher, S. B. Parker, T. Li, A. Fazeli, H. Gardner, S. Z. Haslam, R. T. Bronson, S. J. Elledge, and R. A. Weinberg.** 1995. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell **82:**621–630.
37. **Sovak, M. A., R. E. Bellas, D. W. Kim, G. J. Zanieski, A. E. Rogers, A. M. Traish, and G. E. Sonenshein.** 1997. Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. J. Clin. Investig. **100:**2952–2960.
38. **Wang, S., A. Sdrulla, J. E. Johnson, Y. Yokota, and B. A. Barres.** 2001. A role for the helix-loop-helix protein Id2 in the control of oligodendrocyte development. Neuron **29:**603–614.
39. **Wiseman, B. S., and Z. Werb.** 2002. Stromal effects on mammary gland development and breast cancer. Science **296:**1046–1049.
40. **Wong, B. R., D. Besser, N. Kim, J. R. Arron, M. Vologodskaia, H. Hanafusa, and Y. Choi.** 1999. TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. Mol. Cell **4:**1041–1049.
41. **Wong, B. R., J. Rho, J. Arron, E. Robinson, J. Orlinick, M. Chao, S. Kalachikov, E. Cayani, F. S. Bartlett III, W. N. Frankel, S. Y. Lee, and Y. Choi.** 1997. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J. Biol. Chem. **272:**25190–25194.
42. **Yasuda, H., N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, E. Tsuda, T. Morinaga, K. Higashio, N. Udagawa, N. Takahashi, and T. Suda.** 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc. Natl. Acad. Sci. USA **95:**3597–3602.
43. **Yokota, Y., A. Mansouri, S. Mori, S. Sugawara, S. Adachi, S. Nishikawa, and P. Gruss.** 1999. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature **397:**702–706.