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ID4 regulates mammary gland development by suppressing p38MAPK activity

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SUMMARY

The ID family of helix-loop-helix proteins regulates cell proliferation and differentiation in many different developmental pathways, but the functions of ID4 in mammary development are unknown. We report that mouse *Id4* is expressed in cap cells, basal cells and in a subset of luminal epithelial cells, and that its targeted deletion impairs ductal expansion and branching morphogenesis as well as cell proliferation induced by estrogen and/or progesterone. We discover that p38MAPK is activated in *Id4*-null mammary cells. p38MAPK is also activated following siRNA-mediated *Id4* knockdown in transformed mammary cells. This p38MAPK activation is required for the reduced proliferation and increased apoptosis in *Id4*-ablated mammary glands. Therefore, ID4 promotes mammary gland development by suppressing p38MAPK activity.

KEY WORDS: ID4, p38MAPK (MAPK14, MAPK11), Mammary development, Breast cancer, Wnt, Mouse

INTRODUCTION

The ID (inhibitor of DNA binding) group of proteins comprises four members in vertebrates (ID1-4) that belong to the basic helix-loop-helix (bHLH) family of transcription factors. The ID group of proteins has been reported to promote proliferation and inhibit differentiation in several cell types (Desprez et al., 1995; Norton and Atherton, 1998; Morrow et al., 1999; Kondo and Raff, 2000). For example, ID1 regulates hematopoietic stem cell maintenance (Jankovic et al., 2007; Perry et al., 2007). The gene encoding ID4 is required for neuroprogenitor cell proliferation and proper differentiation (Yun et al., 2004; Bedford et al., 2005), and enforced *Id4* expression causes astrocytes to dedifferentiate into neural stem-like cells (Jeon et al., 2008). Because they lack a DNA-binding domain at the N-terminus, ID proteins are generally thought to exert their function by forming heterodimers with other bHLH proteins, preventing these other proteins from forming transcriptionally active homodimers or heterodimers with the ubiquitous E proteins (Perk et al., 2005).

In the mammary gland, ID1 is not detectable in luminal epithelium during any phase of development (Uehara et al., 2003; Nair et al., 2010). *Id2* is expressed in mammary epithelial cells and is important for terminal differentiation in cultured mammary epithelial cells and for mammary gland alveologenesis during pregnancy (Mori et al., 2000; Parrinello et al., 2001; Itahana et al., 2003). *Id4* is expressed in mammary epithelial cells and basal cells, as assessed by in situ hybridization (de Candia et al., 2006), and can be induced by acute progesterone treatment (Fernandez-Valdivia et al., 2008), but its functions in mammary development are not known.

In this study, we surveyed the expression pattern of *Id4* in mammary glands at puberty and in adulthood, and studied the impact of *Id4* loss on mammary development. Importantly, we discovered p38MAPK as a novel target of ID4 functions.

MATERIALS AND METHODS

Animals

Two lines of *Id4* knockout mice were used. In one line on the CD1 background, 220 bp in the 3′ coding region of *Id4* was replaced by a *lacZ/neo* cassette, leading to the formation of a fusion protein of the N-terminal 65 amino acids of ID4 with β-galactosidase, with concomitant deletion of most of the ID4 C-terminus (Bedford et al., 2005). This line was also backcrossed onto the FVB/N background for a subset of the experiments. In the second line on the 129SV/C57BL6 background, exons 1 and 2 of *Id4* were replaced by a *GFP/neo* cassette (Yun et al., 2004). MMTV-*Wnt1* transgenic mice (on the FVB background) were purchased from the Jackson Laboratory. The DsRed.T3 transgenic mice have been described previously (Vintersten et al., 2004).

Tissue transplantation

The inguinal #4 mammary glands of 3-week-old *Rag*$^{-/-}$, 129SV/C57BL6, or FVB females were cleared of the endogenous epithelium and implanted with a small piece (1-2 mm in diameter) excised from the inguinal glands of 12- to 16-week-old donor mice (placed under the UV lamp when DsRed was present). Tissue pieces prepared from *Id4*$^{-/-}$ and *Id4*$^{+/+}$ donors were transplanted contralaterally.

Hormone treatment

For treatment with either estrogen or progesterone individually, 10-week-old *Id4*-null or wild-type mice (CD1 strain) were ovariectomized. Two weeks later, they were subcutaneously injected daily for 2 days with vehicle (sesame oil), β-estradiol-3-benzoate (1 μg), or progesterone (1 mg; Sigma) in 100 μl sesame oil. For treatment with both hormones, FVB mice bearing transplants from wild-type or *Id4*-null mammary fragments (FVB strain) were subcutaneously injected daily with β-estradiol-3-benzoate (1 μg) and progesterone (1 mg) for 9 days.

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In vivo and in vitro experiments using p38MAPK inhibitors

FVB mice bearing transplants of mammary fragments from wild-type or *Id4*-null FVB mice were injected subcutaneously with β-estradiol-3-benzoate (1 μg) and progesterone (1 mg) daily for 9 days, and on each of the last 3 days were additionally injected with saline or SB203580 (Promega) or SB239063 (Sigma) at 15 mg per kg body weight. Twenty-four hours following the final treatment, the mammary transplants were collected for analysis. For quantitation of cell proliferation (Ki67 immunohistochemistry) or apoptosis (TUNEL), at least 1000 cells were counted for each section per mouse. For in vitro experiments, these inhibitors were added to cell culture at a final concentration of 10 μM.

BrdU incorporation, tissue collection, histology and whole-mount analysis

Two hours before euthanasia, BrdU (100 μg/g body weight; Sigma) was injected intraperitoneally into some of the mice for assaying cell proliferation. Mammary glands were fixed in 10% formalin, embedded in paraffin, sectioned and stained with Hematoxylin and Eosin (H&E). For whole-mount analysis, the inguinal mammary glands were fixed in acetic acid/ethanol for 2-4 hours at room temperature and stained with carmine alum. Whole-gland β-galactosidase staining was performed as described (Brisken et al., 1999).

Immunohistochemistry, immunoblotting and TUNEL assay

Tissue paraffin sections (3 μm) were deparaffinized and heated in a pressure cooker to 145°C in 0.1 M citric acid (pH 6.0) for 10 minutes to retrieve antigen epitopes. Immunoperoxidase staining was performed using the Vectastain Elite ABC System (Vector Laboratories). For immunoblotting, 50 μg protein extracts were separated by SDS-PAGE. Apoptotic cells were determined by the DeadEnd Fluorometric TUNEL System (Promega). DAPI counterstain was used to visualize nuclei. TUNEL-positive cells were scored in at least five fields per section, and at least 1000 cells were counted for each section per mouse.

Antibodies

Antibodies were against: ID4 (L-20, Santa Cruz Biotech), ID2 (C-20, Santa Cruz Biotech), keratin 8 (TROMA1, DSHB), keratin 5 (AF138, Convance), α-smooth muscle actin (DAKO), netrin 1 [11760 (Salminen et al., 2000)], neogenin (H-175, Santa Cruz Biotech), Ki67 (2011-11, Novacastra), BrdU (Beckon Dickinson), p21 (F-5, Santa Cruz Biotech), p16 (M-156, Santa Cruz Biotech), cyclin D1 (Ab-4, Neomarkers), phospho-p38 (AB3828, Upstate), p38α (C-20, Santa Cruz Biotech), p38β (11A5, Invitrogen), p38 (9212, Cell Signaling), phospho-BimEL (AB3579, Upstate), β-casein (H-7, Santa Cruz Biotech), WAP (R-131, Santa Cruz Biotech), β-actin (AC-15, Sigma) and GAPDH (FL-335, Santa Cruz Biotech).

Luminex bead antibody assay

Mammary glands were ground in a mortar with liquid N_2 and dissolved in MPER lysis buffer containing protease inhibitors (Roche). The samples were left on a rotator overnight at 4°C, and then homogenized on ice with a sonicator for 1 minute. The protein supernatant was aliquoted and stored at –80°C. Protein content was measured using the BCA assay (Pierce). A total of 65 proteins were assayed by combining multiple Luminex bead kits (Millipore) according to the manufacturer's protocols with 25 μg protein per well, in duplicate. The plates were read using the Luminex 200 system (Luminex Corporation).

Cell culture, transfection and cell proliferation (MTS) assays

ZD2855 tumor cells were established from a mammary tumor from an MMTV-*Wnt1* mouse by culturing dissociated cells in DMEM/F-12 medium supplemented with 2.5% fetal bovine serum, 10 ng/ml epidermal growth factor, 150 μg/ml insulin and 100 units/ml penicillin-streptomycin at 37°C and 5% CO_2. This same medium was used to maintain these cells. For small interfering RNA (siRNA) transfection, ZD2855 cells were seeded in the growth medium without penicillin-streptomycin in 6-well plates. Twenty-four hours later, they were transfected with 100 nM siRNA oligos in DharmaFECT1 transfection reagent (Dharmacon). The sequences of siRNA oligos against *Id4*, *p38α* and *p38β* are described in supplementary material Table S2. Non-targeted ConsiRNA oligos were siGENOME non-targeting siRNA pool #1 (D-001206-13-05, Dharmacon) and non-targeting siRNA #2 (AM4613, Ambion). For BrdU incorporation, BrdU was added to the cell culture medium to a final concentration of 10 μM for 10-12 hours, and an FITC-conjugated anti-BrdU mouse monoclonal antibody (556028, BD Pharmingen) was used for flow cytometry (FACScan, Becton Dickinson). Cell viability was measured using the CellTiter $96AQ_{ueous}$ One Solution Reagent (Promega).

Quantitative (q) real-time PCR analysis

Primary mammary epithelial cells (MECs) were isolated from 6-week-old $Id4^{+/+}$ or $Id4^{-/-}$ mice as described (Shackleton et al., 2006), and total RNA extracted using the RNeasy Mini Kit (Qiagen). Following reverse transcription to generate single-stranded cDNA, qPCR was performed using the SYBR Green-based system on the ABI 7900HT according to the manufacturer's instruction (Applied Biosystems). Differences in gene expression between $Id4^{+/+}$ and $Id4^{-/-}$ mice were determined by the quantitative comparative C_T method in which keratin 5 served as internal control. Primers for mouse *Id4*, *p21*, *p16*, cyclin D1 and keratin 5 are described in supplementary material Table S1.

In vitro colony assay

Mammary epithelial cells (1×10^6) were prepared from 12-week-old $Id4^{+/+}$ or $Id4^{-/-}$ mice as described (Shackleton et al., 2006). After treatment with 10 μg rat γ-globulin (012-000-002, Jackson Laboratories) and 1 μg Fc (553142, BD Pharmingen) for 10 minutes, the cell suspensions were incubated with lineage (Lin) antibodies (biotinylated CD45/CD31/TER119 and streptavidin-APC; 559971, 554067, BD), CD24-PE (553262, BD) and CD49-FITC (555735, BD) for 20 minutes on ice. All cell sorts were performed using FACSAria (Becton Dickinson), and gates were set according to the isoform control antibody labeled with the corresponding fluorochromes. The sorted cells were mixed with 100 μl Matrigel mix [5% FCS Epicult medium (Stem Cell Technologies) and 50% Matrigel] and plated in Matrigel-coated 8-well chambers. After 8-10 days, the Matrigel culture was fixed in 4% paraformaldehyde, transferred into HistoGel (HG-4000-012, Thermo Scientific) and embedded in paraffin.

Statistical analyses

All comparisons were analyzed using two-tailed Student's *t*-tests. $P\leq0.05$ was defined as statistically significant. Values are given ± s.d.

RESULTS

Expression patterns of *Id4* in mammary glands

We first surveyed the expression pattern of ID4 in mammary glands during pubertal development. We used an *Id4*-knockout mouse line (on the CD1 background) in which the *lacZ* reporter cassette replaced 220 bp of exon 1 and most of the C-terminus of the *Id4* locus (Bedford et al., 2005). Using a β-galactosidase assay to stain whole-mount mammary glands of 6-week-old mice heterozygous for this reporter gene, we observed β-galactosidase activity throughout the ductal tree, including the terminal end buds (TEBs) (Fig. 1A). Specifically, staining was detected throughout the cap cell layer of TEBs (Fig. 1B) and the basal cell layer of the subtending ducts; staining was also detected in some of the body cells and in a minor fraction of the luminal cell layer (Fig. 1B,C). As expected, no β-galactosidase staining was detected in mammary glands from *Id4* wild-type mice (insets in Fig. 1A,B).

Using immunohistochemical staining to detect ID4 in age-matched wild-type CD1 mice, we confirmed ID4 expression in cap and myoepithelial cells as well as in some of the body cells (Fig. 1D,E). However, no convincing signal was detected in luminal epithelial cells. This is not surprising because this immunoassay is less sensitive than the enzymatic reporter assay. Of note, we confirmed the specificity of this antibody using mice that were homozygous for the *lacZ* gene cassette and thus were null for *Id4* (insets in Fig. 1D,E).

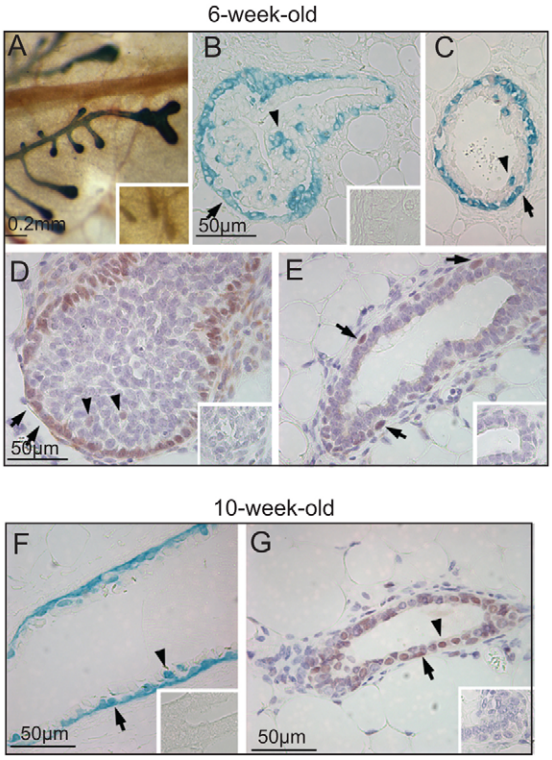

Fig. 1. *Id4* is expressed in mammary cap cells, myoepithelial cells and a subset of luminal epithelial cells. (**A-C**) β-galactosidase staining showing *Id4* expression in mammary whole-mounts (A), terminal end buds (TEBs) (B) and ducts (C) of 6-week-old $Id4^{+/-}$ mice (CD1 strain). Insets show negative staining of $Id4^{+/+}$ TEBs. Arrows highlight stained cap cells and basal cells, and arrowheads body cells and luminal cells. (**D**,**E**) Immunohistochemical staining of ID4 in TEBs (D) and ducts (E) of 6-week-old $Id4^{+/+}$ mice (CD1 strain). Insets show negative staining of $Id4^{-/-}$ mammary TEBs and ducts. (**F**) β-galactosidase staining showing *Id4* expression in mammary ducts of 10-week-old $Id4^{+/-}$ mice (CD1 strain). Inset shows negative staining of $Id4^{+/+}$ ducts. (**G**) Immunohistochemical staining detecting ID4 in ducts in 10-week-old $Id4^{+/+}$ mice (CD1 strain). Inset shows negative staining of $Id4^{-/-}$ ducts.

At 10 weeks of age, basal cells continued to produce ID4, and numerous luminal cells also expressed ID4, based on β-galactosidase staining (Fig. 1F). The specificity of β-galactosidase staining was again confirmed by the lack of signal in age-matched *Id4* wild-type mice (inset in Fig. 1F). This expression pattern was confirmed by immunohistochemical staining for ID4 in mammary glands from 10-week-old wild-type mice (Fig. 1G).

Id4-null mice exhibit impaired mammary development

To investigate the importance of *Id4* in mammary development, we first examined the #4 mammary glands from 6-week-old *Id4*-null mice (CD1 strain) by whole-mount carmine staining. At this developmental stage, the wild-type ductal tree filled 65±12% of the fat-pad, whereas the *Id4*-null ductal tree occupied only 49±10% (P<0.001; n=5; Fig. 2A,B). The *Id4*-null ducts extended only 1.7±1.5 mm beyond the edge of lymph node, which is much less than the wild-type ducts (7.9±1 mm) (Fig. 2A,C; P=0.004). Furthermore, whereas 27±6 side-branches per mm^2 were observed in wild-type glands, only 15±2 were found in *Id4*-null glands (Fig. 2A,D), constituting a significant reduction (P=0.01). In addition, whereas 17±4 TEBs were found in wild-type glands, only 10±5 were observed in *Id4*-null glands (P=0.02; Fig. 2E). Heterozygous glands were similar to those of wild-type mice in all four measurements (Fig. 2B-E), suggesting that one copy of *Id4* is sufficient for ductal development.

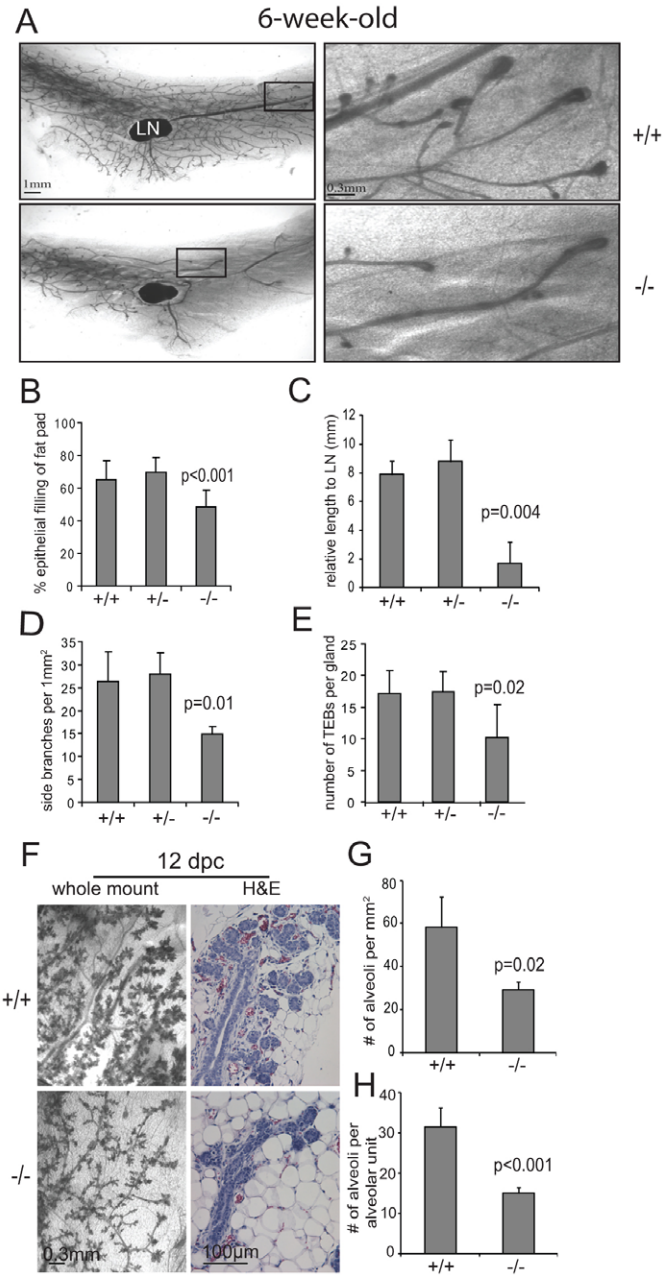

Fig. 2. Impact of *Id4* loss on mammary development. (**A**) Whole-mounts of mammary glands from 6-week-old $Id4^{+/+}$ and $Id4^{-/-}$ mice (CD1 strain). On the right are higher magnification views of the boxed areas. LN, lymph node. (**B-E**) Quantitative analysis of epithelial filling, relative distance to lymph node, side-branches, and average number of TEBs in whole-mounts of the indicated *Id4* genotypes (n=5). (**F**) Whole-mounts and H&E sections of $Id4^{+/+}$ and $Id4^{-/-}$ mammary glands at pregnancy day 12. (**G**,**H**) Quantification of alveoli per mm^2 or per alveolar unit of $Id4^{+/+}$ or $Id4^{-/-}$ mammary glands at pregnancy day 12 (n=3, CD1 strain). Error bars indicate s.d.

By H&E staining, subtending ducts in *Id4*-null glands appeared normal; however, 18 of 50 TEBs in *Id4*-null mice (6 weeks) appeared irregular: the cap cell layer was disorganized and detached from the body cells (supplementary material Fig. S1A), with stroma and collagen wrapping along these defective TEBs (supplementary material Fig. S1A,B). Immunohistochemical staining showed that the α-smooth muscle actin (α-SMA)-stained cap cell layer was discontinuous and detached from the keratin 8-positive body cell compartment (supplementary material Fig. S1A). Keratin 8 and keratin 5 co-immunostaining confirmed this phenotype (supplementary material Fig. S1B).

To determine whether this TEB defect was due to impaired cell adhesion and migration, we first immunostained TEBs for several cell adhesion markers, including E-cadherin (cadherin 1), P-cadherin (cadherin 3), fibronectin and laminin, but failed to detect any alteration in *Id4*-null glands (data not shown). Netrin 1 and its receptor neogenin are crucial for cell migration, especially in the nervous system, and ablation of the gene encoding either protein results in disorganized TEBs (Srinivasan et al., 2003), which appear similar to those in *Id4*-null glands. Therefore, we investigated whether netrin 1 or neogenin expression was altered in *Id4*-null TEBs. Immunohistochemical staining detected netrin 1 in wild-type TEBs (in the majority of body cells as well as the cap cell layer) as reported (Srinivasan et al., 2003), but the intensity of staining was diminished in *Id4*-null TEBs (supplementary material Fig. S1C). Likewise, whereas neogenin was found in the cap cell layer of wild-type TEBs, it was undetectable in the cap cell layer of *Id4*-null TEBs (with the exception of the few cells in the space between the cap cell and body cell compartments) (supplementary material Fig. S1C).

By 10 weeks of age, the wild-type mammary ductal tree had filled the entire fat-pad and TEBs had all regressed (supplementary material Fig. S2A). However, ductal expansion in the *Id4*-null gland was only 70% complete and was accompanied by fewer side-branches (P=0.02; supplementary material Fig. S2A-C); many defective TEBs remained (supplementary material Fig. S2A). By 25 weeks of age, the *Id4*-null ductal tree had filled the entire fat-pad and lost TEBs, but still contained significantly fewer side-branches than the ductal tree in wild-type mice (P=0.04; supplementary material Fig. S2D,F). This reduced branching morphogenesis persisted into pregnancy: alveoli density in *Id4*-knockout glands was half that of wild-type glands (12 dpc; Fig. 2F-H). By parturition, alveolar density was still lower in these knockout glands than in wild-type glands. Nevertheless, functional differentiation occurred normally in *Id4*-null glands: cytoplasmic lipid droplets were observed in many alveolar cells and β-casein and WAP were detected at a similar level as in wild-type cells (supplementary material Fig. S3A,B), indicating active milk synthesis.

Next, we determined whether the developmental defects in *Id4*-null mammary glands resulted from defective ductal cells per se or from stromal or systematic abnormalities as a result of germline deletion of *Id4*. We transplanted small mammary pieces (1-2 mm in diameter) from 12- to 16-week-old wild-type and *Id4*-null mice (n=3; on the CD1 background) contralaterally into epithelium-cleared #4 fat-pads in 3-week-old *Rag1*-null immunodeficient mice (n=16), and examined their outgrowths 8 weeks later. Whereas the *Id4* wild-type donors resulted in 72±27% fat-pad filling, the *Id4*-null donors gave rise to only 38±32% filling. This reduction (47%) is highly significant (P<0.001; supplementary material Fig. S4A). Furthermore, side-branching was also significantly reduced (supplementary material Fig. S4A).

Developmental phenotypes associated with a knockout strain are also modulated by the genetic background, the reporter gene cassette used in targeted gene ablation, and the immune system. Therefore, we confirmed some of the above results using a different line of *Id4*-knockout mice. This line is on the 129SV/C57BL6 background, has had most of the *Id4* coding sequence (exons 1 and 2) replaced by a *GFP/neo* gene cassette, and also carries the transgenic allele of DsRed.T3 (Vintersten et al., 2004), which helps visualize transplant outgrowth. Mammary fragments from this line, as well as from *Id4* wild-type DsRed.T3 transgenic littermate controls, were transplanted into cleared fat-pads of syngeneic (and thus immune-intact) mice. The glands of the recipient mice were analyzed by fluorescent imaging of the ubiquitously expressed DsRed at different developmental stages: 5 and 10 weeks post-transplantation; days 5.5, 7.5, 8.5 and 14.5 of pregnancy initiated at 6 weeks after transplantation; and lactation day 1. At both 5 and 10 weeks post-transplantation, side-branching was reduced in *Id4*-null epithelium in 12 of 18 and 6 of 6 mice, respectively (supplementary material Fig. S4B). Likewise, at pregnancy days 5.5-8.5, side-branching was reduced in *Id4*-null epithelium in 11 of 23 mice. However, at pregnancy day 14.5 and lactation day 1, *Id4*-null epithelium was indistinguishable from wild-type tissue in 4 and 3 mice, respectively. This transplantation experiment using a different knockout allele confirmed the role of ID4 in branching morphogenesis. The lack of alveolar defects during mid-pregnancy and early lactation in this line might be due to a difference in the gene-targeting strategy, genetic background, or systemic or stromal impact. For the remainder of this study, only mice carrying the *lacZ* knock-in cassette was used.

Impaired mammary cell proliferation in *Id4*-null mice

We tested whether reduced ductal and alveolar expansion in *Id4*-null glands was caused by a reduction in cell proliferation. We first compared TEBs and ducts between wild-type and *Id4*-null mice (n=4) at 6 weeks of age by immunohistochemical staining for BrdU and Ki67 (Fig. 3A-C). BrdU incorporation into body cells of *Id4*-null TEBs was similar to that of wild-type TEBs (P=0.4; Fig. 3B), but BrdU incorporation into cap cells of *Id4*-null TEBs was half that of wild-type TEBs (P=0.02; Fig. 3B). In ducts, however, luminal epithelial cell proliferation was reduced by 33% as a result of *Id4* loss (P=0.03; Fig. 3C,D), whereas myoepithelial proliferation was unaltered (Fig. 3D).

To further test the proliferative impact of *Id4* loss on luminal and myoepithelial cells, we isolated by FACS the luminal (Lin^- $CD24^+$ $CD49f^-$) and basal (Lin^- $CD24^+$ $CD49f^+$) cell populations of 12-week-old wild-type and *Id4*-null mammary glands and then performed an in vitro colony assay in Matrigel (Stingl et al., 2006). Luminal cells from *Id4*-null mice were defective in forming colonies (P=0.005; supplementary material Fig. S5A,B). In accordance, fewer cells were positive for Ki67 in the *Id4*-null than in the wild-type luminal colonies (P=0.01; n=3; supplementary material Fig. S5C,D). Basal cells from both genotypes formed very few and generally miniature colonies, which also showed very low proliferation rates (supplementary material Fig. S5).

Moreover, we asked whether *Id4* loss also affected cell proliferation during alveolar expansion in pregnant animals. Ki67 staining was positive in 54±8% of mammary epithelial cells in wild-type mid-pregnant mice, but in only 41±5% of the *Id4*-null mammary cells (P=0.04; n=5; data not shown). We conclude that ID4 promotes cell proliferation in mammary TEBs, ducts and alveoli.

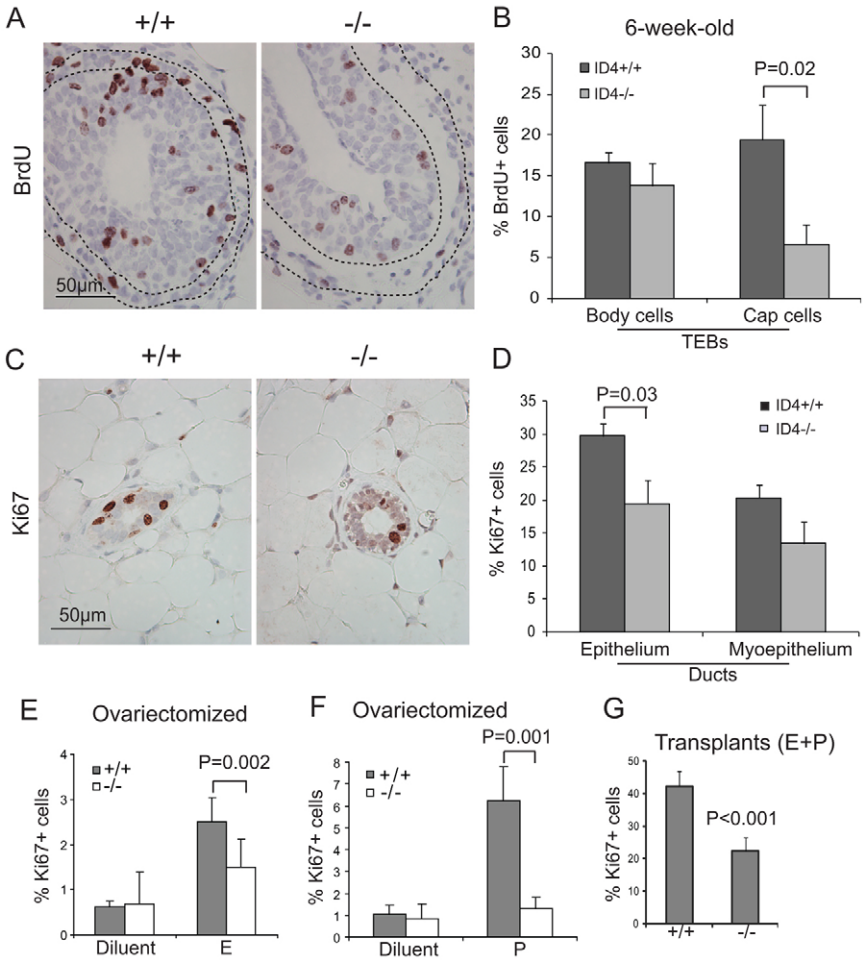

Fig. 3. Impact of *Id4* loss on mammary cell proliferation and hormone-induced proliferation. (**A-D**) Proliferation in TEBs and ducts at 6 weeks of age. Photomicrographs (A,C) and quantitation (B,D) are shown. *n*=4 mice (CD1 strain). Body cells and cap cells in TEBs are outlined. (**E**,**F**) Quantitation of Ki67 staining of mammary ducts. Ten-week-old ovariectomized $Id4^{-/-}$ and $Id4^{+/+}$ mice (CD1 strain) were treated with vehicle (sesame oil), β-estradiol-3-benzoate (1 μg) or progesterone (1 mg) daily for 2 days. Total ductal cells in at least ten 40× views were counted for each mouse (*n*=3). (**G**) Quantitation of Ki67 staining of $Id4^{-/-}$ or $Id4^{+/+}$ mammary transplants (FVB strain) in mice that had been injected daily with β-estradiol-3-benzoate (1 μg) and progesterone (1 mg) for 9 days. At least 1000 cells were counted for each section. *n*=7 transplanted mice. Error bars indicate s.d.

In addition, we tested whether these proliferative defects in *Id4*-null mammary glands were due to an impaired proliferative response to estrogen and/or progesterone signaling. Indeed, stimulation with estrogen or progesterone induced significantly lower mammary cell proliferation in ovariectomized *Id4*-null mice than in wild-type mice (Fig. 3E,F). Likewise, stimulation with both hormones induced significantly less proliferation in *Id4*-null mammary transplants than in wild-type transplants (Fig. 3G). These data suggest that, in the mammary ductal epithelium, ID4 maintains a normal proliferative response to estrogen and progesterone. This transplantation experiment also validated the epithelium-intrinsic role of ID4 in regulating mammary development.

p38MAPK activation mediates cell cycle arrest in *Id4*-null mammary glands

To investigate the molecular mechanism by which ID4 mediates a normal proliferative response to estrogen and progesterone, we used Luminex suspension antibody arrays to compare 65 proteins in mammary total protein lysates extracted from 10-week-old wild-type and *Id4*-null CD1 mice (*n*=3). Only two proteins – PAI1 (serpine 1 – Mouse Genome Informatics) and phosphorylated p38MAPKα/β (T180/pY182; MAPK14/11 – Mouse Genome Informatics) – were found to be significantly different ($P<0.05$) between these two sets of lysates (Fig. 4A; supplementary material S6). Since the absolute difference for PAI1 was small, only p38MAPKα/β (referred to as p38MAPK hereafter) was further investigated. By immunohistochemical staining, we confirmed p38MAPK activation: phospho-p38MAPK-positive cells were much more readily detected in mature ducts and TEBs in *Id4*-null mice than in wild-type mice at 4, 6 or 10 weeks of age (Fig. 4B; supplementary material Fig. S7; *n*=4 for each).

p38MAPK is known to block the cell cycle by phosphorylating and stabilizing p21 (CDKN1A – Mouse Genome Informatics) (Kim et al., 2002), by upregulating *p16* (*Cdkn2a*) expression (Bulavin et al., 2004), and by phosphorylating cyclin D1, resulting in cyclin D1 ubiquitylation and proteosomal degradation (Casanovas et al., 2000). Analysis of mammary gland RNA by qPCR revealed that *p21* and *p16* were increased 3-fold in *Id4*-null as compared with wild-type mammary glands (6 weeks of age; *n*=5; $P=0.02$ and $P=0.04$, respectively; supplementary material Fig. S8A). In accordance, the p21 protein was detected in 2.5-fold more cells in the TEBs of *Id4*-null versus wild-type mice (3.0±1.1% versus 1.3±1.0%; $P=0.03$; supplementary material Fig. S8B,C), although it was undetectable in the ducts of either genotype (data not shown). Likewise, whereas it was nearly undetectable in wild-type TEBs and ducts, p16 was found in approximately half of the cells in the TEBs and some epithelial cells in the mature ducts of *Id4*-null mice (supplementary material Fig. S8B; data not shown). In addition, fewer cyclin D1-positive cells were found in *Id4*-null than wild-type TEBs (6.2±0.7% versus 10.7±1.1%; $P=0.02$; supplementary material Fig. S8D). As expected, the mRNA level of cyclin D1 was unaffected by *Id4* loss (*n*=5; $P=0.79$; supplementary material Fig. S8A). Collectively, these experiments demonstrate that aberrant

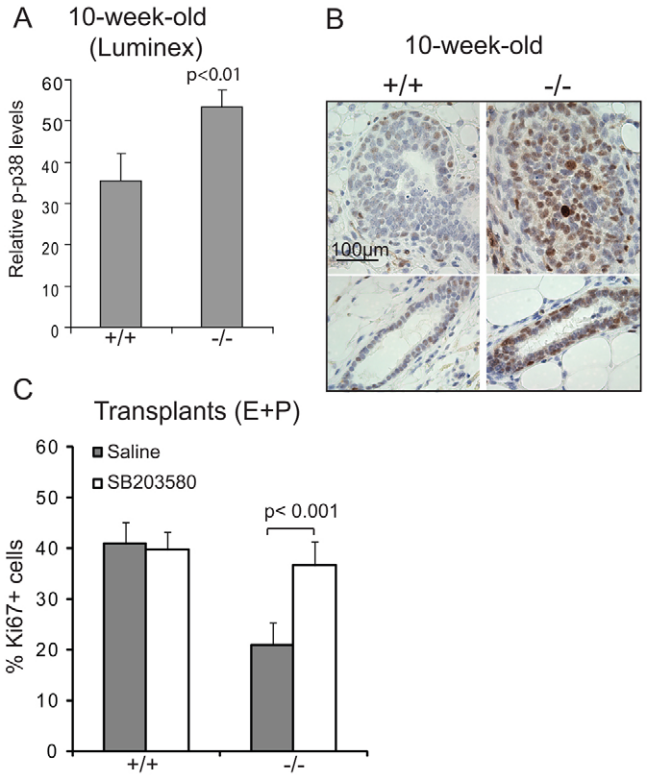

Fig. 4. *Id4* loss activates p38MAPK, and a p38MAPK inhibitor restores cell proliferation in *Id4*$^{-/-}$ mammary glands. (**A**) Relative p38MAPK levels in 10-week-old *Id4*$^{+/+}$ and *Id4*$^{-/-}$ (CD1 strain) mouse mammary lysates as measured by the Luminex bead assay. *n*=3 each. (**B**) Immunohistochemical staining for phospho-p38MAPK in mammary glands of 10-week-old *Id4*$^{+/+}$ and *Id4*$^{-/-}$ mice (CD1 strain). Both TEBs and mammary ducts are shown. (**C**) Levels of proliferation in estrogen and progesterone (E+P)-stimulated mammary epithelial transplants (FVB strain) treated with the p38MAPK inhibitor SB203580. *n*=7 transplanted mice. Error bars indicate s.d.

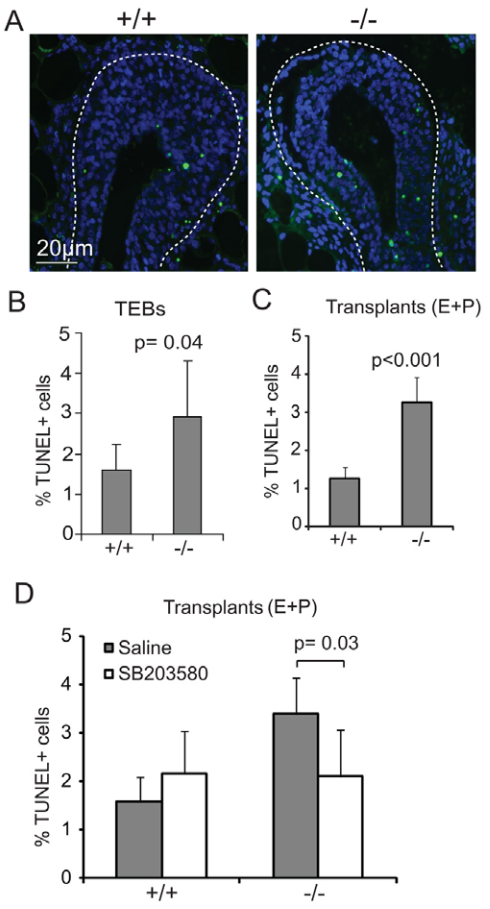

Fig. 5. Apoptosis induction in *Id4*$^{-/-}$ mammary gland is mediated by p38MAPK. (**A**) TUNEL staining showing apoptosis in TEBs of 6-week-old mice (CD1 strain). *Id4* genotypes are indicated. Dotted line highlights the TEB. (**B**) Levels of apoptosis as assessed by TUNEL staining in A. At least 1000 cells were counted per section per mouse (*n*=5). (**C**) Levels of apoptosis in transplanted ductal outgrowths stimulated with β-estradiol-3-benzoate and progesterone. (**D**) Levels of apoptosis in transplanted ductal outgrowths that were stimulated with β-estradiol-3-benzoate and progesterone and were also treated with the p38MAPK inhibitor SB203580 or a saline control. Error bars indicate s.d.

activation of p38MAPK in *Id4*-null mammary glands is associated with alterations of its downstream components that are known to regulate cell cycle progression.

To test whether p38MAPK activation has a causal role in the reduced proliferation in *Id4*-null mammary glands, we used a p38MAPK inhibitor to treat mice that had been transplanted with *Id4*-null or wild-type mammary fragments and stimulated with estrogen and progesterone. Daily treatment for 3 days with SB203580 at 15 mg per kg body weight – a dosage reported to suppress p38MAPK in mammary glands in vivo (Bulavin et al., 2004) – did not affect the proliferation rate of wild-type transplants (39.6±3.5% versus. 40.9±4.1% for the saline control; Fig. 4C), suggesting that basal p38MAPK activity has little effect on estrogen/progesterone-induced proliferation of normal mammary glands. However, this treatment increased cell proliferation of the *Id4*-null transplants from 20.9±4.3% to 36.5±4.8% (P<0.001), a level similar to that in wild-type transplants (40.9±4.1%; P=0.53; Fig. 4C). As expected, SB203580-treated *Id4*-null outgrowths had fewer cells positive for p21 (data not shown) and p16 (supplementary material Fig. S8E), and more cells positive for cyclin D1 (supplementary material Fig. S8F). Since chemical inhibitors such as SB203580 may affect the activity of other protein kinases, we repeated the above inhibitor experiment using SB239063, which exhibits better p38MAPK selectivity than SB203580 (Barone et al., 2001). Again, the level of proliferation in *Id4*-null transplants was fully restored (supplementary material Fig. S9A). Collectively, these data suggest that p38MAPK activity is responsible for the reduction in proliferation caused by *Id4* loss.

Apoptosis induction in *Id4*-null mammary glands is mediated by p38MAPK

ID proteins and p38MAPK have both been associated with the regulation of cell survival; therefore, we asked whether an increase in cell death contributes to the impaired mammary development of *Id4*-null mice. Wild-type TEBs contained 1.6±0.5% apoptotic cells, but the *Id4*-null TEBs harbored 3±1% (*n*=5; P=0.04; Fig. 5A,B). Both wild-type and *Id4*-null mammary ducts in 6-week-old mice had few apoptotic cells as determined by TUNEL staining (data not shown). However, in mammary transplants treated with estrogen and

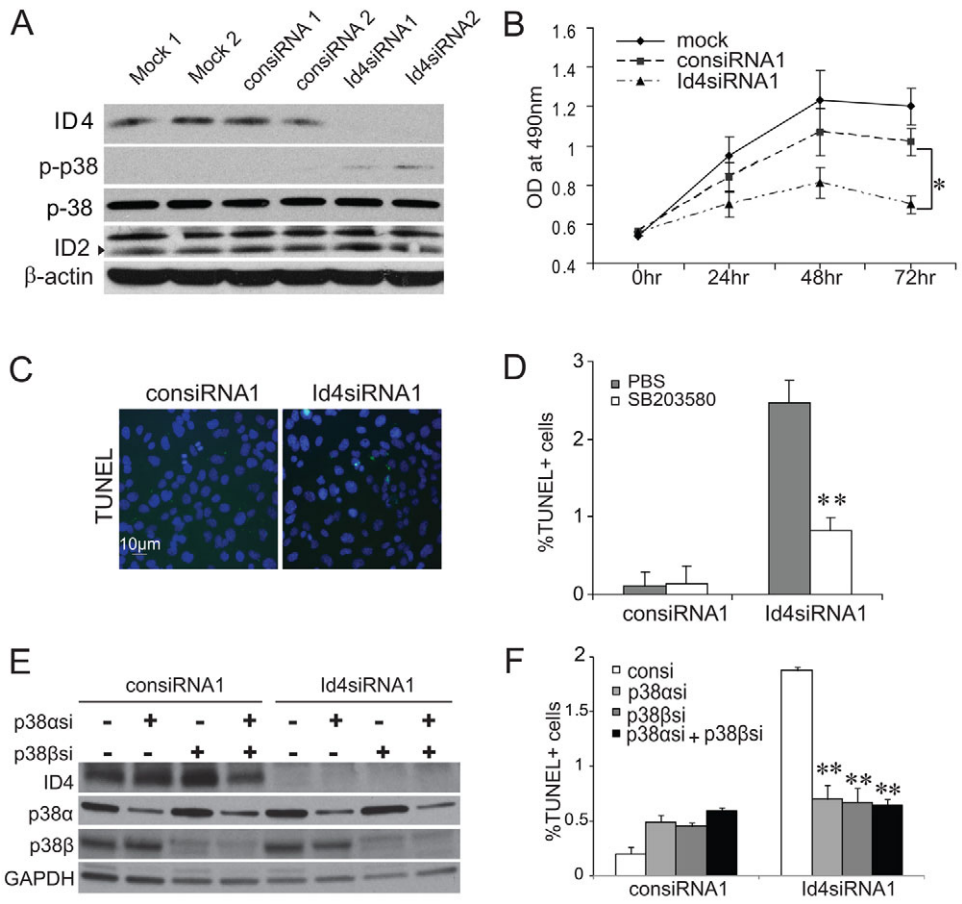

Fig. 6. *Id4* is required for ZD2855 mammary tumor cell survival. (**A**) Western blotting for the indicated proteins in ZD2855 cells 48 hours post-transfection with the indicated siRNA oligos. (**B**) Cell growth as measured by the MTS assay at 0, 24, 48 or 72 hours after treatment with the indicated siRNAs. *, P=0.03, ConsiRNA-treated versus *Id4*siRNA1-treated groups at 48 or 72 hours. (**C**) TUNEL staining of ZD2855 cells 48 hours following treatment with the indicated siRNAs. (**D**) Quantitation of TUNEL-positive cells among ZD2855 cells transfected with the indicated siRNAs. PBS (control) or SB203580 (10 μM) was added 12 hours following transfection; after another 48 hours, TUNEL staining was performed. (**E**) Western blotting for the indicated proteins in ZD2855 cells 48 hours after transfection with the indicated siRNAs. (**F**) Quantitation of apoptosis in ZD2855 cells transfected with ConsiRNA or *Id4*siRNA1, as well as with siRNA oligos against the indicated genes. **, P<0.01. The bar charts (B,D,F) show data from three independent experiments. Error bars indicate s.d.

progesterone for 9 days, the wild-type ductal epithelium had 1.3±0.3% apoptotic cells, whereas the *Id4*-null epithelium contained 3.3±0.7% apoptotic cells (P<0.001; Fig. 5C). Collectively, these observations suggest that ID4 plays a crucial role in maintaining cell survival of both TEBs and the ductal epithelium.

Next, we tested whether p38MAPK also mediates apoptosis in these *Id4*-null mammary cells. In the wild-type epithelium, SB203580 or SB239063 had little effect on apoptosis; however, in the *Id4*-null mammary epithelium, treatment with SB203580 or SB239063 reduced the apoptotic rate from 3.4±0.9% to 2.1±1.0% (P=0.03) or from 1.8±0.3% to 0.8±0.1% (P=0.004), respectively (Fig. 5D; supplementary material S9B). These reduced apoptosis rates are similar to those in the wild-type epithelium treated with these inhibitors. We conclude that ID4 maintains mammary cell survival by suppressing p38MAPK.

p38MAPK has been reported to stimulate apoptosis by phosphorylating and activating BimEL (BCL2L11 – Mouse Genome Informatics) (Cai et al., 2006), one of three gene products of the *Bim* locus. *Bim*-knockout mice fail to activate apoptosis in body cells, thus interfering with lumen clearing during pubertal development (Mailleux et al., 2007). By immunohistochemical staining, we found more pBimEL-positive cells in *Id4*-null than wild-type TEBs (supplementary material Fig. S10A). As expected, SB203580 treatment reduced the frequency of pBimEL-positive cells in *Id4*-null mammary epithelium (supplementary material Fig. S10B). These data suggest that p38MAPK-induced apoptosis in *Id4*-ablated mammary cells is likely to occur by phosphorylation and activation of BimEL.

Id4 knockdown leads to both p38MAPK activation and p38MAPK-dependent apoptosis in cultured mammary tumor cells

To further demonstrate ID4-mediated suppression of p38MAPK, we performed siRNA-mediated knockdown of *Id4* in cultured cells. We have reported that *Id4* is overexpressed in mammary tumors from the MMTV-*Wnt1* transgenic model of breast cancer (Huang et al., 2005). We have since established a cell line, ZD2855, from a tumor of this model, and confirmed ID4 protein expression in this culture by western blotting (Fig. 6A). Transfection of two independent siRNA oligos (*Id4*siRNA1 and *Id4*siRNA2) targeting two different regions of *Id4* mRNA achieved a significant knockdown of ID4 in these cells within 48 hours, and the level of its close family member ID2 did not compensatively rise (Fig. 6A). *Id4* knockdown with either *Id4*siRNA1 or *Id4*siRNA2 caused an increase in the levels of phospho-p38MAPK (Fig. 6A). This observation further established the ID4-mediated suppression of p38MAPK activity.

We also tested whether this ID4-p38MAPK pathway regulates cell proliferation and apoptosis in the ZD2855 cell line. At 48 and 72 hours post-transfection, cell numbers were much lower in the *Id4*siRNA1-treated group than in both control siRNA- and mock-transfected groups (P=0.03; Fig. 6B). This difference in cell expansion was not due to cell proliferation: no difference in BrdU incorporation or cell cycle distribution was detected between control siRNA- and *Id4*siRNA1-transfected cells 48 hours after transfection (supplementary material Fig. S11; data not shown), and levels of p21, p16 and cyclin D1 were also unaltered (data not shown).

However, *Id4*siRNA1-treated and control siRNA-treated cell cultures exhibited a significant difference in apoptosis: very few apoptotic cells were detectable in control siRNA-transfected cultures (0.1±0.2%), whereas the number of apoptotic cells was 2.5±0.3% in *Id4*siRNA1-transfected cultures ($P<0.001$; Fig. 6C,D). Collectively, these observations suggest that ID4 maintains the viability of transformed mammary cells, but does not affect their proliferation.

We next investigated whether this ID4-regulated tumor cell survival was also mediated by p38MAPK activation. We transfected ZD2855 cells with *Id4*siRNA1 and treated the culture 12 hours later with either PBS (control) or SB203580 (at a final concentration of 10 μM). After another 48 hours, we quantified the apoptosis rate by TUNEL staining. SB203580 blocked apoptosis in these cells by 66% ($n=3$; $P=0.001$; Fig. 6D), suggesting a crucial role of p38MAPK in mediating apoptosis caused by *Id4* knockdown. To confirm that the restoration of cell viability in these SB203580-treated cells was indeed due to specific suppression of p38MAPK, and not due to off-target effects of this inhibitor, we transfected ZD2855 cells with ConsiRNA or *Id4*siRNA1, and additionally with *p38αMAPK*siRNA or *p38MAPK*βsiRNA, or both. After 48 hours, we first confirmed the knockdown of ID4, p38MAPKα or p38MAPKβ by immunoblotting (Fig. 6E), and then quantified apoptosis rates by TUNEL staining. Silencing *p38MAPKα* (*Mapk14*) or *p38MAPK*β (*Mapk11*) or both suppressed apoptosis in *Id4*-knockdown cells, down to levels comparable to those seen in *Id4* wild-type cells transfected with corresponding *p38MAPK* siRNA oligos (Fig. 6F). This suppression of apoptosis in *Id4*-knockdown cells was validated using another set of siRNA oligos against different gene regions of *p38MAPKα* and *p38MAPK*β (data not shown). Collectively, these data confirmed a key role of p38MAPK in mediating mammary tumor cell apoptosis caused by *Id4* knockdown.

DISCUSSION

We found that *Id4* is expressed in cap and basal cells as well as in some of the luminal cells in the mammary gland. It partly mediated proliferative responses to estrogen and progesterone signaling, and promoted ductal elongation and branching morphogenesis. ID4 also maintained the survival of normal mammary cells as well as cultured mammary tumor cells. These normal and tumorigenic functions of ID4 were primarily achieved by suppressing p38MAPK activity. This is the first report, to our knowledge, linking the ID family of proteins to p38MAPK signaling.

During puberty, ID4 was expressed in the cap cell layer in TEBs and the myoepithelium in subtending ducts, but was found only sporadically in body cells and luminal epithelial cells. In more mature mammary glands, ID4 continued to be expressed in the myoepithelium, and it was also more frequently detected in the luminal cell population. Using two independent knockout lines and transplantation experiments, we demonstrated that *Id4* loss caused numerous developmental defects in the mammary gland, including impairments in ductal elongation, side-branching and possibly alveologenesis (Fig. 2; supplementary material Figs S2, S3). ID4 appears to be required for the proliferation of cap cells, but not body cells, in TEBs (Fig. 3B). By contrast, ID4 seems to be required for the proliferation of luminal epithelial cells, but not myoepithelial cells, in the subtending ducts (Fig. 3D). The role of ID4 in supporting luminal cell proliferation in more mature ducts was confirmed by an in vitro colony assay using isolated luminal cells (supplementary material Fig. S5), which also established luminal cell-autonomous functions of ID4. Furthermore, we found that ID4 is required for increased mammary cell proliferation in response to estrogen, progesterone, or both (Fig. 3E-G). *Id4* has been reported to be transcriptionally activated by ectopically expressed progesterone receptor in cultured mammary epithelial cells (Fernandez-Valdivia et al., 2008); therefore, ID4 might be an important mediator of cell proliferation induced by progesterone, adding to the small number of genes, such as *RANKL* (*Tnfsf11*), *Elf5* and *Wnt4*, that have also been reported to mediate progesterone signaling in branching morphogenesis and alveologenesis (Brisken and Rajaram, 2006). Although ID4 partly mediated ductal cell proliferation induced by estrogen signaling, *Id4* does not seem to be a direct transcriptional target of estrogen receptor because: (1) *Id4* is expressed in basal cells, which lack estrogen receptor; (2) there is no estrogen-response motif in the promoter region of the *Id4* gene (data not shown); and (3) in MCF7 breast cancer cells, *Id4* is neither induced nor inhibited by estradiol (Beger et al., 2001) (Adrian Lee, personal communication), and is inversely correlated with estrogen receptor in human breast cancer (de Candia et al., 2006; Roldan et al., 2006). We postulate that ID4 functions via suppression of p38MAPK to maintain low levels of the cell cycle inhibitors p21 and p16 and elevated levels of cyclin D1, allowing cells to undergo a proper proliferative response to estrogen-induced cell cycle stimulators arising in cis or via paracrine signaling from a neighboring estrogen receptor$^+$ cell.

Besides regulating the proliferation of cap cells, ID4 also has an important role in the proper organization of TEBs and their penetration of the fat-pad, as *Id4*-null TEBs exhibited cap cell detachment and stromal condensation (supplementary material Fig. S1). The disorganized TEBs are reminiscent of those found in netrin 1 or neogenin knockout mice (Srinivasan et al., 2003), or in mice lacking Slit2 or its receptor Robo1 (Strickland et al., 2006). We have confirmed reduced protein expression of netrin 1 or its receptor neogenin in *Id4*-null TEBs (supplementary material Fig. S1C), suggesting that ID4 might regulate TEB organization through netrin-neogenin signaling. Of note, although members of the ID family have been reported to regulate stem cells in several other tissues by preventing premature differentiation (Perk et al., 2005), the structural and proliferative defects of TEBs, as well as the proliferative blockade of more mature ducts in these *Id4*-null mice, were unlikely to be the result of a dysfunctional stem cell population: we did not detect a defect in either primary or secondary mammosphere-forming potential in *Id4*-null primary mammary cells when compared with wild-type mammary cells (supplementary material Fig. S12). Furthermore, ID4-mediated regulation of TEB organization and migration does not appear to originate from its role in mediating hormone signaling, as these defects were not observed in mice deficient for estrogen receptor or progesterone receptor (Lydon et al., 1995; Bocchinfuso et al., 2000; Feng et al., 2007).

p38MAPK activity was increased in *Id4*-null mammary glands compared with wild-type glands (Fig. 4; supplementary material Figs S6, S7). Suppression of p38MAPK by pharmacological inhibitors reversed the proliferation and survival defects caused by *Id4* loss during normal mammary development (Fig. 4; supplementary material Fig. S9), and the suppression of p38MAPK by either pharmacological inhibitors or siRNA reversed the survival defects caused by *Id4* loss in transformed mammary cells (Fig. 6). We conclude that ID4-mediated suppression of p38MAPK is necessary for the pro-proliferation and anti-apoptosis functions of ID4 in normal mammary glands and for the anti-apoptosis role in transformed mammary cells. These findings are consistent with a recent report that *p38MAPKα* is required for anoikis and lumen formation during mammary development (Wen et al., 2011), and

with earlier studies linking p38MAPK to both cell cycle arrest and apoptosis induction in several other tissues (Thornton and Rincon, 2009).

p38MAPK is known to control cell proliferation and apoptosis by modulating a number of downstream factors. We found that the p38MAPK targets p16, p21 and cyclin D1 were altered in *Id4*-null mammary glands, and that these alterations were reversed in mammary glands treated with the p38MAPK inhibitor SB203580 (supplementary material Fig. S8). As crucial cell cycle regulators, these targets are likely to play a key role in mediating p38MAPK-mediated anti-proliferation. Of note, the *p21* and *p16* promoters harbor the bHLH-binding E box motif and have been reported to be responsive to bHLH proteins (Prabhu et al., 1997; Zheng et al., 2004); therefore, unrestrained activity of tissue-specific bHLH proteins as a result of *Id4* loss might also contribute to the aberrant levels of p21 and p16 in *Id4*-null mammary glands. However, this contribution seems to be minor because p21 and p16 levels in these *Id4*-null mammary cells were highly responsive to a p38MAPK inhibitor (supplementary material Fig. S8E,F) and because proliferation of these *Id4*-null cells was completely restored by a p38MAPK inhibitor (Fig. 4C; supplementary material S9A).

BimEL is a major downstream target of p38MAPK that is implicated in apoptosis. Bim is required for apoptosis in TEBs, which allows lumen clearing during pubertal development (Mailleux et al., 2007). We found that BimEL was activated in *Id4*-null mammary glands, and that treatment of these glands with the p38MAPK inhibitor SB203580 blocked BimEL activation (supplementary material Fig. S10) and also reversed excessive apoptosis in TEBs. Collectively, these data suggest that high levels of apoptosis in *Id4*-null TEBs are likely to be due to disproportionate activation of p38MAPK-BimEL. However, in transformed mammary cells, BimEL does not appear to play a significant role in mediating p38MAPK-induced apoptosis. pBimEL was not activated following *Id4* knockdown-mediated p38MAPK activation in ZD2855 mammary tumor cells (data not shown). Furthermore, ConsiRNA- and *Id4*siRNA-treated ZD2855 mammary tumor cells did not exhibit differences in the levels of p53 and FOXO3 (data not shown), which have also been reported to mediate p38MAPK-induced apoptosis (Cai et al., 2006; Cai and Xia, 2008). Therefore, it is not yet clear how *Id4* loss and the associated p38MAPK activation cause apoptosis in these mammary cancer cells.

In conclusion, in this in vivo study we discovered that ID4 stimulates the proliferation of mammary epithelium during puberty, pregnancy, and under stimulation by estrogen or progesterone or both, and that it maintains the survival of normal and cancerous breast cells. ID4 exerts these functions primarily through suppressing p38MAPK activity. Therefore, blocking the prosurvival function of ID4 or protecting p38MAPK activity might be important in breast cancer prevention and treatment.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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