ID4 regulates mammary gland development by suppressing p38MAPK activity

Jie Dong1, Shixia Huang2,3, Marian Caikovski4, Shaoquan Ji5, Amanda McGrath1, Myra G. Custorio2, Chad J. Creighton2, Paul Maliakkal1,1, Ekaterina Bogoslovskaja1, Zhijun Du1, Xiaomei Zhang1, Michael T. Lewis1,2,3, Fred Sablitzky6, Cathrin Brisken4 and Yi Li1,2,3,*

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; 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 44 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.

#Lester and Sue Smith Breast Center, 2Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA. 3NCCR Molecular Oncology, Ecole polytechnique fédérale de Lausanne (EPFL), ISREC-Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges, Switzerland. 4BioLegend, San Diego, CA 92121, USA. 5Institute of Genetics, The University of Nottingham, Nottingham NG7 2UH, UK.
*Author for correspondence (ily@bcm.edu)
†Deceased

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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 quantification 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 acetate acid/ethanol for 2-4 hours at room temperature and stained with carmine alun. Whole-gland β-galactosidase staining was performed as described (Briskin 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, Novacasta), BrdU (Beckon Dickinson), p21 (F-5, Santa Cruz Biotech), p16 (M-156, Santa Cruz Biotech), cyclin D1 (Ab-4, NeoMarkers), phospho-p38 (AB382, Upstate), p38α (C-20, Santa Cruz Biotech), p38β (11A5, Invitrogen), p38α (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).

Lumina bead antibody assay

Mammary glands were ground in a mortar with liquid N2 and dissolved in protein extraction 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 content was measured using the BCA assay (Pierce). A total of 65 proteins were assayed by combining multiple Lumina bead kits (Millipore) according to the manufacturer’s protocols with 25 μg protein per well, in duplicate. The plates were read using the Lumina 200 system (Lumina 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% CO2. This 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 96AQueous 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 Ct 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 × 10⁴) 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 (555142, 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 (55735, BD) for 20 minutes on ice. All cell sorts were performed using FACSAria (Becton Dickinson), and gates were set according to the isomorph 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 ≤ 0.05 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).
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 mm² 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.
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. S2D,F). This reduced branching morphogenesis persisted into pregnancy: alveoli density in Id4-null glands was half that of wild-type glands (12 dpc; Fig. 2F). BrdU incorporated in 3-week-old cleared #4 fat-pads in 3-week-old Id4-null mice resulted in 72±27% fat-pad filling, the Id4-null TEBs. Immunohistochemical staining was diminished in the cap cell layer, indicating active milk secretion.

Furthermore, side-branching was also significantly reduced (supplementary material Fig. S1B). 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.
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 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 mature ducts of Id4-null mice (supplementary material Fig. S8B,C), although it was undetectable in the ducts of either genotype (data not shown). In addition, fewer cyclin D1-positive cells were found in Id4-null than wild-type TEBS (n=5; P=0.02 and P=0.04, respectively; 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
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
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 (Id4siRNA1 and Id4siRNA2) 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). Knockdown leads to both p38MAPK activation and p38MAPK-dependent apoptosis in cultured mammary tumor cells.

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 Id4siRNA1-treated group than in the 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 Id4siRNA1-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 p38αMAPKsiRNA or p38βMAPKsiRNA, or both. After 48 hours, we first confirmed the knockdown of Id4, p38αMAPKα or p38MAPKβ by immunoblotting (Fig. 6E), and then quantified apoptosis rates by TUNEL staining. Silencing p38αMAPKα (Mapk14) or p38MAPKβ (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 p38αMAPKα and p38MAPKβ (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 (Mailloux 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 Id4siRNA-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 p33MAPK 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 http://dev.biologists.orglookup/suppl?doi=10.1242/dev.0692037-DC1

References


A

consiRNA1  Id4siRNA1

BrdU

B

% BrdU+ cells

consiRNA1  Id4siRNA1

0  5  10  15  20  25

20um
10-week-old

A

B

% epithelial filling of fat pad

C

side branches per gland

1mm

p=0.02

-/-

1mm

p=0.02

1mm

25-week-old

D

E

% epithelial filling of fat pad

F

side branches per gland

1mm

p=0.04

-/-
A  Donor: +/+  Donor: +/-  

[Images of blood vessel structures]

B  Donor: +/-  Donor: -/-

[Images of blood vessel structures at different stages: 5 weeks, 7.5 dpc, 14.5 dpc, L1]

% epithelial filling of fat pad

p<0.001
A) Comparison of colony formation in Luminal and Basal cells between ID4+/+ and ID4-/- conditions. 
B) Bar graph showing the number of colonies formed per 500 cells, with P = 0.005 indicating a significant difference between ID4+/+ and ID4-/- conditions.

C) Comparison of Ki67+ cells in Luminal and Basal cells between ID4+/+ and ID4-/- conditions. 
D) Bar graph showing the percentage of Ki67+ cells, with P = 0.01 for Luminal and P = 0.02 for Basal, indicating significant differences.
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<tr>
<td></td>
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<tr>
<td>Duct</td>
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phospho-p38MAPK

100μm
A

B

% Ki67+ cells

% TUNEL+ cells

Saline
SB239063

Saline
SB239063

p < 0.01

p < 0.01
Table S1. Primers for quantitative real-time PCR

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