C/EBPδ is a crucial regulator of pro-apoptotic gene expression during mammary gland involution

Muthusamy Thangaraju[1,*], Martina Rudelius[2], Brian Bierie[3,†], Mark Raffeld[2], Shikha Sharan[1], Lothar Hennighausen[3], A-Mei Huang[1,‡] and Esta Sterneck[1,§]

[1]Laboratory of Protein Dynamics and Signaling, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702-1201, USA
[2]Laboratory of Pathology, National Cancer Institute, National Institute of Health, Bethesda MD 20892, USA
[3]Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bethesda, MD 20892, USA

*Present address: Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA 30912-0006, USA
†Present address: Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN 37232, USA
‡Present address: Department of Biochemistry, Kaohsiung Medical University, Kaohsiung 807, Taiwan
§Author for correspondence (e-mail: sterneck@ncifcrf.gov)

Accepted 19 August 2005

Development 132, 4675-4685
Published by The Company of Biologists 2005
doi:10.1242/dev.02050

Summary

The STAT3 transcription factor is an important initiator of mammary gland involution in the mouse. This work shows that the STAT3 target gene CCAAT/enhancer binding protein delta (C/EBPδ) is a crucial mediator of pro-apoptotic gene expression events in mammary epithelial cells. In the absence of C/EBPδ, involution is delayed, the pro-apoptotic genes encoding p53, BAK, IGFBP5 and SGP2/clusterin are not activated, while the anti-apoptotic genes coding for BFL1 and Cyclin D1 are not repressed. Consequently, p53 targets such as survivin, BRCA1, BRCA2 and BAX are not regulated appropriately and protease activation is delayed. Furthermore, expression of MMP3 and C/EBPβ during the second phase of involution is perturbed in the absence of C/EBPδ. In HC11 cells, C/EBPδ alone is sufficient to induce IGFBP5 and SGP2. It also suppresses Cyclin D1 expression and cooperates with p53 to elicit apoptosis. This study places C/EBPδ between STAT3 and several pro- and anti-apoptotic genes promoting the physiological cell death response in epithelial cells at the onset of mammary gland involution.

Key words: Mammary gland, Involution, C/EBP, Apoptosis, Mouse

Introduction

Hormonal stimuli during pregnancy lead to extensive proliferation of the epithelial compartment, culminating in the differentiation of the milk-secreting alveolar units, while adipocytes lose their lipid stores. During lactation, the tissue is relatively static and produces large quantities of milk. Involution, experimentally initiated by removal of the pups at mid-lactation, is caused by accumulation of milk and changes in hormone levels, which trigger cascades of molecular events leading to dramatic tissue reorganization (Green and Streuli, 2004). Our current knowledge on these processes has been summarized in a recent, comprehensive review (Green and Streuli, 2004). Involution can be divided into two morphologically recognizable stages. The first reversible phase constitutes the initiation of mammary epithelial cell (MEC) apoptosis and the second phase includes extensive tissue remodeling (Lund et al., 1996). Furthermore, adipocytes refill during the process of involution. Microarray expression analyses lead to the characterization of several distinct clusters of gene expression patterns, suggesting a number of sub-stages with intricate regulation and communication of multiple signaling pathways occurring in several cell types that fine-tune the tissue remodeling process (Clarkson et al., 2004; Stein et al., 2004). Several hundred genes are dynamically regulated during involution. However, a specific role has been established by gene targeting approaches in mice for only a few. According to such studies, the signals contributing to initiation of epithelial cell apoptosis include death-receptor activation and TGFβ3/SMAD3 signaling (Green and Streuli, 2004). Well characterized is the crucial role of the signal transducer and activator of transcription 3 (STAT3), which is activated by the gp130 cytokine receptor with leukemia inhibitory factor (LIF) as a critical activating ligand (Kritikou et al., 2003; Zhao et al., 2004). *Stat3* null mice are embryonic lethal (Takeda et al., 1997). However, conditional deletion of the *Stat3* gene in secretory MEC leads to a severe delay in involution with expanded, lactation-competent alveoli present for several days. *Stat3* null epithelial cells exhibit significantly less apoptosis than controls and protease activity in the second phase of involution is delayed (Chapman et al., 1999; Humphreys et al., 2002). STAT3 mediates MEC apoptosis in part by inducing expression of regulatory PI(3) kinase subunits and IGFBP5, which lead to inhibition of the pro-survival signals from AKT and IGF1 (Abell et al., 2005; Chapman et al., 1999).

Another gene with reduced expression in both LIF and STAT3-deficient mammary glands encodes the transcription factor C/EBPδ (CEBPD, CRP3, CELF, NF-IL6β) (Kritikou et al., 2003). C/EBPδ is a member of the C/EBP family of transcription factors, which dimerize and bind DNA through a

highly homologous basic region leucine zipper domain. Each of the five C/EBP proteins has unique properties regulating cell-type-specific growth and differentiation. For example, within the hematopoietic system, C/EBPα is required for the development of granulocytes, while lack of C/EBPβ affects differentiation of the B-cell lineage and monocytes (Takiguchi, 1998). Expression of C/EBPδ is typically low to undetectable in most cell types and tissues, but is rapidly induced by a variety of extracellular stimuli, e.g. growth hormone, insulin, IFNγ, IL1, IL6, LPS, TNFα, noradrenalin and glutamate (Ramji and Foka, 2002). In-vitro and in-vivo studies have implicated C/EBPδ in inflammatory responses (Takiguchi, 1998), proliferation of osteoblasts (Umayahara et al., 1997), growth arrest in certain cancer cell lines (Gery et al., 2005; Ikezoe et al., 2005) and differentiation of lung epithelial cells (Cassel et al., 2000). Thus, C/EBPδ appears to have highly diverse functions, depending on cell type and specific physiological stimuli. However, C/EBPδ-deficient mice display no overt phenotype, are fertile and achieve normal life spans. The null mutation leads to altered learning and memory functions (Sterneck et al., 1998) and increased mammary ductal branching, specifically in nulliparous outbred mice (Gigliotti et al., 2003). It also exacerbates the differentiation defect of C/EBPβ-deficient adipocytes in vitro (Tanaka et al., 1997) and causes genomic instability in embryonic fibroblasts (Huang et al., 2004).

In the mammary gland, the C/EBPδ gene is activated transiently at the onset of involution (Gigliotti and DeWille, 1998), but its function in this process has hitherto not been elucidated. Several reports analyzing immortalized mammary epithelial cell lines in vitro demonstrate that expression of C/EBPδ is induced during growth arrest and document that C/EBPδ participates specifically in the growth arrest response of MEC lines in vitro (Dearth and DeWille). One study also reported reduced cell viability under growth arrest conditions when MEC lines were stably transfected with a C/EBPδ expression construct (O'Rourke et al., 1999).

The present study addresses the role of C/EBPδ in mammary epithelial cells in vivo and demonstrates that C/EBPδ participates in the initiation of physiological cell death responses in MEC as a crucial target of STAT3. These data identify a novel role for C/EBPδ in the initiation of cell death, and shed light on the signaling cascade from the activation of STAT3 to tissue remodeling during the second phase of involution.

Materials and methods

C/EBPδ mutant mice

C/EBPδ$^{+/-}$ mice (Sterneck et al., 1998) backcrossed for at least 11 generations into C57BL/6 were intercrossed to generate subjects. Females were mated at approximately 7 weeks of age and males were removed postcoitum. Litters were standardized to six pups at birth. Pups were removed on day 7 of lactation (Strange et al., 1995; Thangaraju et al., 2004). Females were euthanized by CO_2 and the #4 glands removed for analysis. All animals were housed and handled according to approved protocols established by the NCI Animal Care and Use Committee and NIH guidelines.

Stat3 fl/fl;WC mice

Mice carrying a *Stat3* gene with LoxP-flanked exons 16-21 (*Stat3* fl/fl) were bred to mice carrying the Cre transgene under control of the *WAP* gene promoter (Humphreys et al., 2002). *Stat3* fl/fl mice without Cre were used as controls. Litter sizes were maintained at six to eight pups. All animals were housed and handled according to approved protocols established by the NIDDK Animal Care and Use Committee and NIH guidelines.

Cell culture

HC11 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum, 10 ng/ml epidermal growth factor and 10 μg/ml insulin (Invitrogen). For transient transfections, 0.7×10^6 cells were plated per 10-cm dish, transfected the next day with 10 μg expression constructs plus 3 μg pEGFP-N1 and 20 μl Fugene 6 (Roche). Cells were collected 2 days later for analysis.

Constructs

To construct wild-type C/EBPδ fused to EGFP, the full-length mouse C/EBPδ coding region was amplified by PCR from a pMEX expression construct (Colangelo et al., 1998) with specific primers (5′-CAAGCTAGCATGAGCGCCGCGCTTTTC-3′; 5′-CAAGGATCCACCGGCAGTCGGCGCCGG-3′) and the GC-rich PCR system (Roche), inserted into the TOPO TA vector (Invitrogen), sequenced and cloned into pEGFP-N1 (Clontech) by *Nhe*I and *Bam*HI ('filled-in').

Cell-cycle analysis

Cells were fixed in 50% ethanol, lysed with 0.1% sodium citrate, 1 mg/ml RNase A, 50 μg/ml propidium iodide, and subjected to FACS (Becton Dickinson FacsCaliber) analysis. At least 20,000 gated events per sample were quantified (Cellquest software). Cells with DNA content below G1 were scored as apoptotic. For transiently transfected cells, data represent the total unsorted population (transfection efficiencies averaged at 60% by EGFP).

Histological analysis

One abdominal mammary gland per mouse was fixed in 10% neutral buffered formalin and sections were stained with haemotoxylin and eosin. Photographs were taken with an AxioHOME microscope (Zeiss). For quantifications, 10-12 pictures per gland were taken at random across the whole gland at 200-fold magnification. The areas occupied by fat cells were identified manually and quantified by the public domain NIH1.62 Image program (http://rsb.info.nih.gov/nih-image/).

Immunohistochemical staining was performed with the aid of an automated immunostainer (DakoCytomation Inc., Carpinteria, CA). Deparaffinized tissue sections on glass slides were subjected to heat-induced antigen retrieval by placing the slides into target retrieval solution, high pH (DakoCytomation), and steaming them in a commercial vegetable steamer at full temperature for 30 minutes. Following the antigen retrieval procedure the slides were incubated with a C/EBP delta rabbit polyclonal antibody (Santa Cruz Biotechnology, M17; dilution 1:500) overnight at 4°C, and the detection was carried out on the automated system using an HRP/DAB polymer based rabbit detection system (Envision$^+$ DakoCytomation) according to the manufacturer's recommendations.

RNA analysis

Total RNA was isolated using TRIZOL reagent (Life Technologies, Ind.). Ten micrograms of each sample was fractionated and analyzed by standard Northern blotting techniques. Expression of beta-casein mRNA was analyzed using specific oligonucleotides as previously described (Robinson et al., 1996). To generate a DNA probe for BFL1, a fragment was amplified from C57BL/6 mouse genomic DNA (primers: 5′-TTTGCAGTCTTTGCCTCCTT-3′; 5′-TTCTGCCGTATCCATTCTCC-3′), sub-cloned and sequenced. All other radiolabeled DNA probes were prepared from isolated cDNA clones for the indicated genes. The multiprobe ribonuclease protection assay using 10 μg total RNA per sample was performed as described

(Hodge et al., 2002), using the mAPO2 multiprobe template set (PharMingen). The signals were visualized by phosphorimaging and densitometry analysis performed by ImageQuant software (Molecular Dynamics).

Protein analysis

For Western blot analyses, protein was extracted from fresh or frozen mammary gland tissue as described (Li et al., 1997). Briefly, tissue was homogenized in lysis buffer, incubated at 4°C overnight and cleared by centrifugation. HC11 cells were lysed by sonication (3×10 pulses) in lysis buffer and extracts cleared by centrifugation. Fifty micrograms of protein were fractionated on tris-glycine gels and transferred to Protran nitrocellulose membrane (Schleicher & Schull). Membranes were blocked with bovine serum albumin (BSA) in 10 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl and 0.5% Tween 20 (TBST) exposed to primary antibody (1:15,000 in TBST) at 4°C overnight, followed by the secondary antibody (1:50,000 in TBST) at room temperature for 1 hour. Proteins were visualized using the ECL super signal western system (Pierce). Antisera were from Santa Cruz Biotechnology (STAT3, sc-7179; p53, sc-100; PARP, sc-7150; anti-goat, sc-2094), Upstate Cell Signaling (phospho-STAT3 Y705, # 07-391; IGFBP5 # 06-110), R&D Systems (Survivin, AF886), RDI Biotechnology (Caspase 3, RDI-CPP32 Nabg), Promega (anti-rabbit WA401B, #17330901) and Pierce (anti-mouse #31432, Pierce).

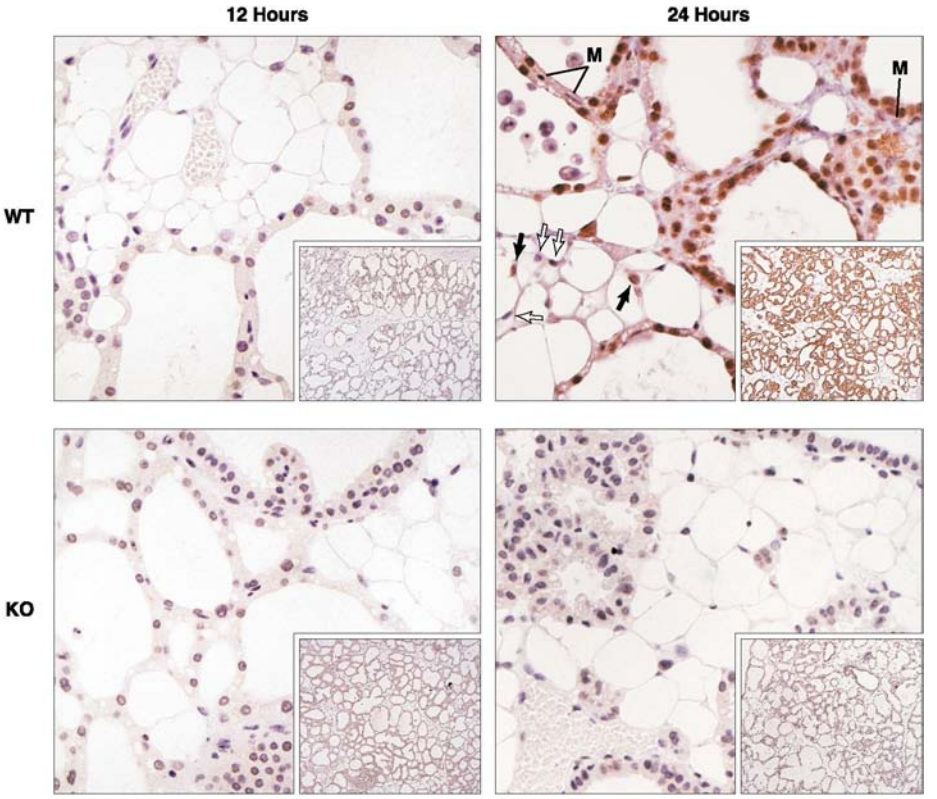

Fig. 1. C/EBPδ is expressed in luminal epithelial cells. Immunohistochemistry of C/EBPδ in wild-type and C/EBPδ null mammary glands at the indicated time points of involution after 7 days of lactation. Representative sections at 400× and 50× original magnification are shown (insets). M, myoepithelial cells; solid arrows, positive stromal cells; open arrows, negative stromal cells.

Results

C/EBPδ is expressed in luminal mammary epithelial cells

Previous analyses of C/EBPδ expression in the mammary gland were based on whole organ RNA levels. Although MEC lines in vitro clearly express inducible C/EBPδ, the gene can also be expressed in many other cell types, including adipocytes, smooth muscle cells and endothelial cells. Thus, we first applied immunohistochemistry to assess the range of cell types in which C/EBPδ is expressed at different stages. By this approach we were unable to detect C/EBPδ protein in any cell type of lactating glands (data not shown) or at 12 hours after removal of the pups (Fig. 1). However, on day 1 of involution nuclear C/EBPδ protein expression was evident in most of the luminal epithelial cells specifically in wild-type mice. Parallel staining for smooth muscle actin suggested that myoepithelial cells do not express C/EBPδ (see Fig. S1 in the supplementary material), similar to endothelial cells (data not shown). However, a subset of stromal adipocytes also expressed nuclear C/EBPδ (Fig. 1). No specific staining for C/EBPδ was detectable in the mammary gland from day 3 of involution (Inv d3) (data not shown), in agreement with the significantly reduced mRNA levels at this stage reported for the same C57BL/6 strain (Thangaraju et al., 2004) as well as FVB/N mice (Gigliotti and DeWille, 1998). The present data confirm massive induction of nuclear C/EBPδ protein in the luminal epithelial layer of the mammary gland specifically in the early phase of involution. Furthermore, a subset of stromal cells expressed C/EBPδ protein on Inv d1.

STAT3 is required for C/EBPδ expression in mammary epithelial cells

The C/EBPδ promoter can be activated by STAT3 in human hepatoma and mouse MEC lines (Cantwell et al., 1998; Hutt et al., 2000). Furthermore, Oncostatin M and STAT3 can activate C/EBPδ in mouse and human MEC lines (Hutt and DeWille, 2002; Zhang et al., 2003), identifying C/EBPδ as a target of STAT3 in vitro. However, mice with a conditional null allele of *Stat3* revealed that STAT3 is not necessary for induced C/EBPδ expression in the liver (Alonzi et al., 2001). By contrast, STAT3- or LIF-deficient mammary glands exhibited approximately 2.5-fold and 4.5-fold reduced C/EBPδ levels on Inv d2 compared with controls (Kritikou et al., 2003). To define the exact extent to which C/EBPδ gene expression is dependent on STAT3 in MEC, mice with a conditional *Stat3* null allele transgenic for Cre recombinase driven by the MEC specific whey acidic protein (WAP) gene promoter (Humphreys et al., 2002) were analyzed on Inv d0-3. In the absence of STAT3, C/EBPδ levels were greatly reduced even in the lactating gland

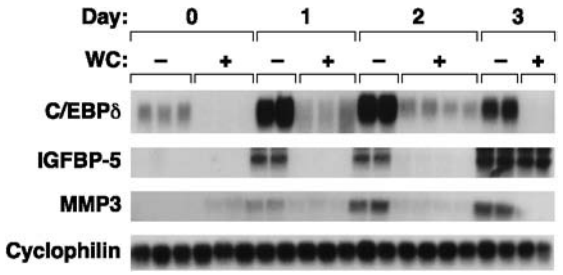

Fig. 2. C/EBPδ and MMP3 expression in the mammary gland are dependent on STAT3. Northern analysis of the indicated genes in total RNA from mammary glands of mice with a floxed *Stat3* allele and with or without the WAP-cre (WC) transgene during lactation (involution day 0) and 1-3 days after removal of the pups (two to four independent mice per group).

(Fig. 2). On Inv d1, C/EBPδ expression was highly induced in wild-type mice, as shown previously (Gigliotti and DeWille, 1998; Thangaraju et al., 2004). However, induction of C/EBPδ was dramatically impaired in the absence of STAT3. The low level induction of C/EBPδ still detectable in involuting mutant glands may stem from luminal cells with inefficient deletion of the *Stat3* gene, or could be due to stromal cells. Expression of C/EBPδ was reduced by Inv d3 in wild-type glands and absent in mutant tissue. IGFBP5, an inhibitor of the pro-survival signal IGF, was induced at early stages of involution by the STAT3 pathway in wild-type tissue but not in mutant tissue, as shown previously (Kritikou et al., 2003). Furthermore, this analysis revealed that expression of matrix metalloproteinase 3 (MMP3, stromelysin 1), a marker for the second phase of involution (Lund et al., 1996; Strange et al., 1992), was not induced in the absence of STAT3 (Fig. 2).

In summary, we have confirmed the previously reported requirement of STAT3 in MEC for C/EBPδ expression during mammary involution in vivo. Furthermore, the expanded kinetic analysis revealed that this requirement extends to the lactation period and Inv d1-3, with no compensatory mechanism developing by Inv d3. Furthermore, STAT3 was found to be required for MMP3 expression during the later phase of involution.

Involution is delayed in the absence of C/EBPδ

Next, the role of C/EBPδ in involution was analyzed by use of C/EBPδ-deficient mice. On Inv d1, alveoli are expanded because of accumulating milk, and the contribution of fat to tissue morphology is low. As in lactating glands (data not shown), there was no difference between mutants and controls (Fig. 3A). On Inv d2, wild-type alveoli began to regress and fill with debris while fat cells started to re-fill. This process was significantly blunted in C/EBPδ-deficient glands compared with controls. On Inv d3, we found heterogeneity in tissue morphology between mice of the same genotype. The sections shown in Fig. 3 are representative of the most and least involuted appearance per group. However, in general wild-type alveoli are almost completely collapsed, while in C/EBPδ null glands significantly expanded alveoli can still be detected. Because of the aforementioned tissue heterogeneity, the proportional contribution of fat to tissue morphology was quantified from several independent glands per time point (Fig. 3B). Mutant and wild-type mice had similar amounts of fat on Inv d1. However, the proportional increase in adipose tissue was significantly delayed on Inv d2-4 in mutant mice, in agreement with delayed regression of the epithelial compartment. These data show that in the absence of C/EBPδ, the kinetics of post-lactational mammary gland involution is delayed at the morphological level.

C/EBPδ is required for the timing of pro-apoptotic events

Analyses of STAT3-deficient glands had demonstrated a defect in the initiation of apoptosis (Chapman et al., 1999; Humphreys et al., 2002). Therefore, we first addressed apoptotic markers in C/EBPδ-deficient involuting mammary glands. Apoptosis is initiated through regulated proteolytic activation events of the caspase system (Marti et al., 2001). By Western analysis, wild-type mice displayed significant levels of activated, cleaved caspase 3 on Inv d3 and 4 (Fig. 4). By contrast, cleaved caspase 3 was not detected before Inv d4 in mutant mice and pro-caspase 3 remains high through Inv d4. In wild-type mice, cleavage of the protease substrate PARP (Marti et al., 2001) was detected from day 1 to 4 of involution, but only by Inv d4 in C/EBPδ null tissue. In wild-type mice, significant cleavage of PARP was detected earlier than caspase 3 activation. This could be because PARP cleavage is a particularly sensitive measure of apoptosis (Duriez and Shah, 1997), or because other caspases or protease activities (Soldani and Scovassi, 2002) may be more important at early stages of involution. Importantly, by the measure of both caspase 3 and PARP cleavage, protease activation is delayed in the absence of C/EBPδ.

The first stage of involution is dependent on the pro-apoptotic tumor suppressor p53 in some mouse strains (Blackburn and Jerry, 2002; Li et al., 1996). p53 protein levels were elevated in wild-type mice on Inv d1-4. In mutant glands, p53 protein failed to accumulate through the first 3 days of involution. Consequently, downregulation of the p53-repressed target survivin (BIRC5 – Mouse Genome Informatics), which inhibits p53-mediated apoptosis (Hoffman et al., 2002; Mirza et al., 2002), was delayed and reduced in mutant glands (Fig. 4).

As seen in *Stat3* null mammary tissue (Fig. 2) (Chapman et al., 1999), IGFBP5 expression was significantly impaired even in the absence of C/EBPδ alone. These data raised the question of whether STAT3 activation itself was affected by the C/EBPδ mutation. However, both the expression levels and kinetics of STAT3 activation as judged by its tyrosine phosphorylation were completely normal in mutant glands. On Inv d8, all results were comparable between wild-type and mutant glands (Fig. 4). These data demonstrate that C/EBPδ is downstream of STAT3 and upstream of several cell death regulators, such as protease activation, and p53 and IGFBP5 protein expression.

C/EBPδ is essential for regulation of pro-apoptotic gene expression

As C/EBPδ is a transcription factor, we next assessed gene expression patterns at the level of RNA. Because cell death was delayed in the absence of C/EBPδ, we first analyzed expression of some members of the BCL2 family of apoptosis regulators, known to be modulated during involution (Furth, 1999). Activation of the pro-apoptotic genes *Bak* on Inv d1 and *Bax*

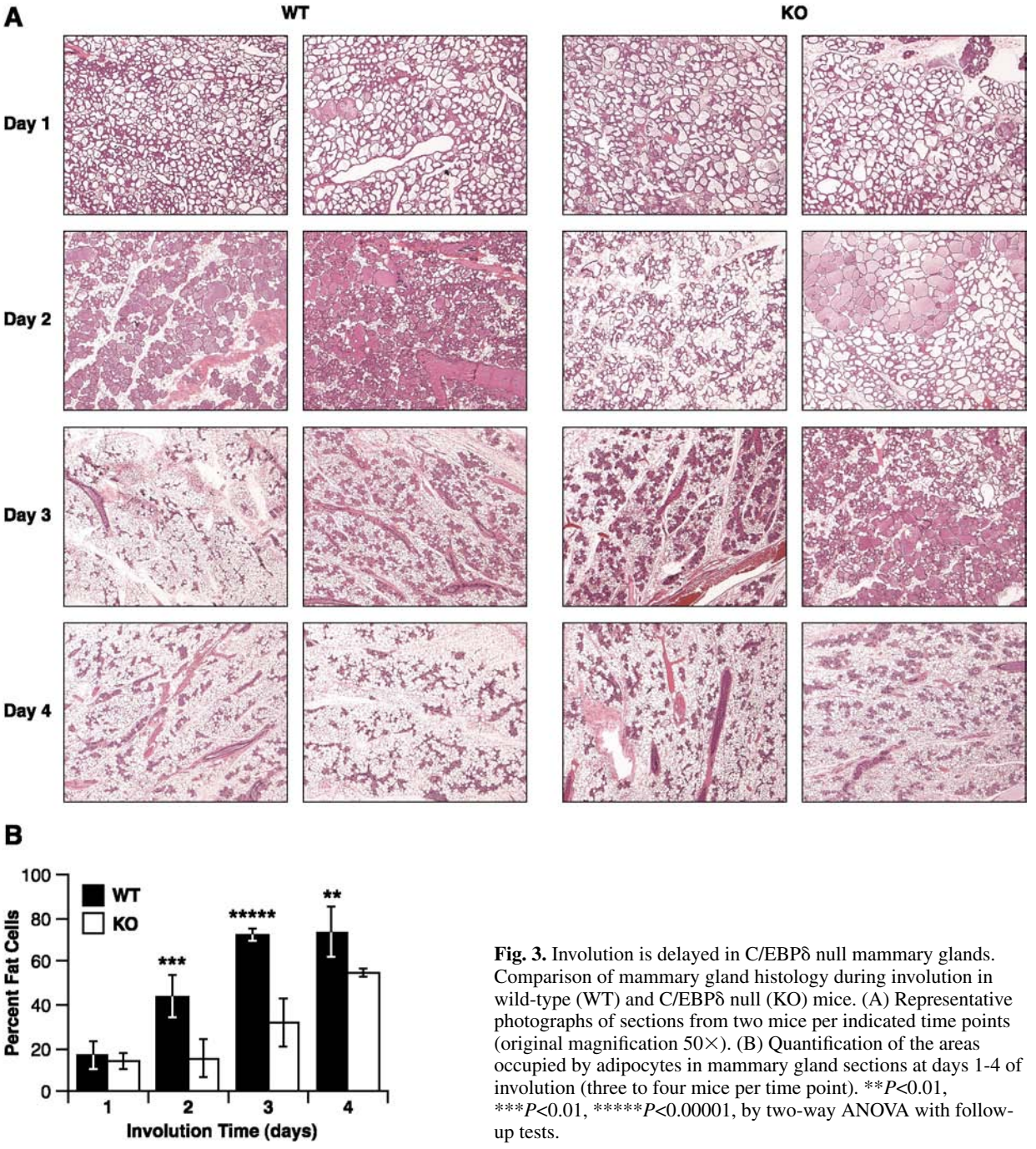

Fig. 3. Involution is delayed in C/EBPδ null mammary glands. Comparison of mammary gland histology during involution in wild-type (WT) and C/EBPδ null (KO) mice. (A) Representative photographs of sections from two mice per indicated time points (original magnification 50×). (B) Quantification of the areas occupied by adipocytes in mammary gland sections at days 1-4 of involution (three to four mice per time point). $^{**}P<0.01$, $^{***}P<0.01$, $^{*****}P<0.00001$, by two-way ANOVA with follow-up tests.

on Inv d2 was impaired in mutant glands (Fig. 5A). Expression of Bcl-x-S/L (Walton et al., 2001) was slightly elevated in mutants on Inv d2 (data not shown). However, the anti-apoptotic genes *Bfl1* (*Bcl2a1*) and *Bcl-w* (*Bcl2l2*) were downregulated in wild-type glands by Inv d1, but remained expressed in the absence of C/EBPδ (Fig. 5A). These data are in agreement with a delay in cell death initiation in C/EBPδ-deficient glands.

Northern analysis (Fig. 5B,C) showed that as described before, C/EBPδ was induced in wild-type glands by Inv d1. In the C57BL/6 strain used here, this activation faded already on Inv d2 (see Discussion). Other genes induced in wild-type glands at the RNA level 24 hours after removal of the pups were p53, SGP2 (clusterin), IGFBP5 and STAT3, while Cyclin D1, BRCA1 and BRCA2 were repressed. The elevated levels of *Stat3* mRNA at the onset of involution are by contrast to unchanged protein levels (Fig. 4), which suggests additional regulation of STAT3 expression at the protein level. On Inv d2, C/EBPβ was induced, and MMP3 was highly expressed on Inv d3 and 4. With the disappearance of epithelial cells, expression of epithelial specific keratin 18 (CK18) was significantly reduced by Inv d2. Surprisingly, β-casein (Fig. 5B) as well as WAP (data not shown) mRNA levels continued to be expressed, decreasing only in parallel with the epithelial

marker CK18. This pattern was also observed by microarray analysis of involution in the same C57BL/6 background (Clarkson et al., 2004), and indicates that metabolic de-differentiation may not be required for involution. As described previously (Allar and Wood, 2004), IGFBP3 mRNA levels did not change during the involution process.

In the absence of C/EBPδ, regulation of most of these genes was significantly perturbed. The proliferation-associated genes *Cyclin D1*, *Brca1* and *Brca2* were not repressed (Fig. 5B), while IGFBP5 and SGP2 induction was delayed and significantly blunted (Fig. 5B,C). Consistent with the delayed tissue regression, CK18 levels continued to be elevated through Inv d2, and induction of the pro-apoptotic p53 gene was delayed to Inv d4 (Fig. 5B,C). As seen in conditionally *Stat3* null mammary glands (Fig. 2), expression of MMP3 on Inv d2-4 was completely eliminated in the C/EBPδ null glands. However, the C/EBP family member C/EBPβ was first activated normally on Inv d2, although this induced expression was not maintained through days 3 and 4 of involution (Fig. 5B).

Because of the cascade of cellular events during involution and the heterogeneity of the tissue after the onset of epithelial cell apoptosis, interpretation of differences in gene expression at later stages of involution are complicated in the absence of cellular localization. Furthermore, the most direct consequences of C/EBPδ-deficiency take place during the early stages of involution. Therefore, we have focused our attention on day 1 of involution, when C/EBPδ levels are highest in wild-type glands, C/EBPδ-expressing luminal epithelial cells represent the majority of the tissue, and no histological differences are detected between mutant and wild-type glands. Four to five mice per genotype and time point were quantified for expression of some of the genes shown in Fig. 5A-B. This approach confirmed the complete block in p53 and IGFBP5 induction in the absence of C/EBPδ. Furthermore, not only was Cyclin D1 expression maintained in C/EBPδ null glands, but BFL1 expression was in fact induced rather than suppressed compared with wild-type glands (Fig. 5D).

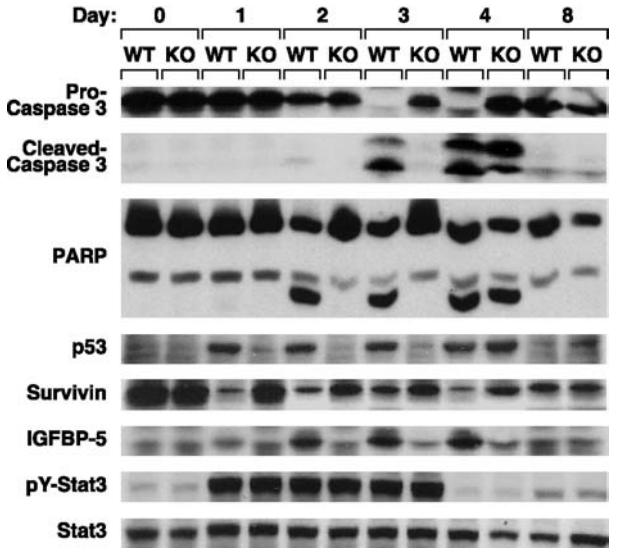

Fig. 4. Pro-apoptotic protein expression and activity are delayed in C/EBPδ null mammary glands. Western blot analysis of the indicated proteins in total protein extracts (50 μg each) from mammary glands of wild-type and C/EBPδ null mice on days 0-4 and 8 of involution.

In summary, expression analyses revealed dramatically impaired activation of pro-apoptotic genes and suppression of anti-apoptotic genes in C/EBPδ null mammary glands. With the exception of *Stat3* mRNA expression and protein phosphorylation, every gene we have analyzed and that showed specific regulation on day 1 of involution in wild-type mice was significantly affected by lack of C/EBPδ. Thus, impaired regulation of genes whose products participate in the regulation and execution of the involution process underlies the morphologically delayed tissue regression in C/EBPδ null mice.

C/EBPδ is sufficient to initiate involution-specific events in HC11 cells

To investigate whether C/EBPδ was sufficient to trigger some of the genetic events impaired in the mutant glands, we transfected the non-transformed mouse MEC line HC11 (Hynes et al., 1990) with various expression vectors. As shown previously (O'Rourke et al., 1999), C/EBPδ elicited a growth arrest response in HC11 cells with concomitant upregulation of Gas1 mRNA levels (Fig. 6). In addition, C/EBPδ alone was able to induce expression of SGP2 and IGFBP5, and inhibited expression of Cyclin D1. By contrast, p53 mRNA (Fig. 6) and protein (data not shown) levels were not induced by C/EBPδ. This result suggests that the activation of p53 expression downstream of C/EBPδ in the mammary gland is indirect and/or requires additional signals. Expression of *Brca1* and *Brca2*, two genes associated with proliferation and differentiation (Kubista et al., 2002), was suppressed by p53 but not by C/EBPδ, suggesting that the decline of BRCA1and BRCA2 on day 1 of mammary gland involution is mediated by p53. This data is in agreement with the ability of p53 to repress the *Brca1* promoter (MacLachlan et al., 2000), and the frequent co-regulation of these two genes (Rajan et al., 1997). Interestingly, p53 activated expression of the endogenous C/EBPδ gene in HC11 cells. Thus, in the mammary gland, C/EBPδ and p53 pathways may engage in a positive feedback loop on expression. HC11 cells harbor p53 alleles with two independent mis-sense mutations (Merlo et al., 1994). Overexpression of wild-type p53 itself triggered apoptosis in HC11 cells (Fig. 6C). Co-transfection of C/EBPδ significantly enhanced this apoptotic response, indicating that p53 and C/EBPδ cooperate in the initiation of MEC apoptosis. Furthermore, this result raises the possibility that growth arrest rather than apoptosis is the predominant response of cell lines to C/EBPδ in vitro (Dearth and DeWille, 2003, and references therein), because most cell lines harbor mutant p53 genes.

Taken together, these data show that C/EBPδ alone is able to initiate involution-specific genetic responses in an in-vitro model and cooperates with p53 to elicit apoptosis, further supporting its active role in regulation of mammary epithelial cell death during involution.

Discussion

This study has identified C/EBPδ as a master regulator of pro-apoptotic gene regulation during initiation of physiological cell death, specifically at the onset of mammary gland involution. Based on the phenotype of involuting C/EBPδ null mammary

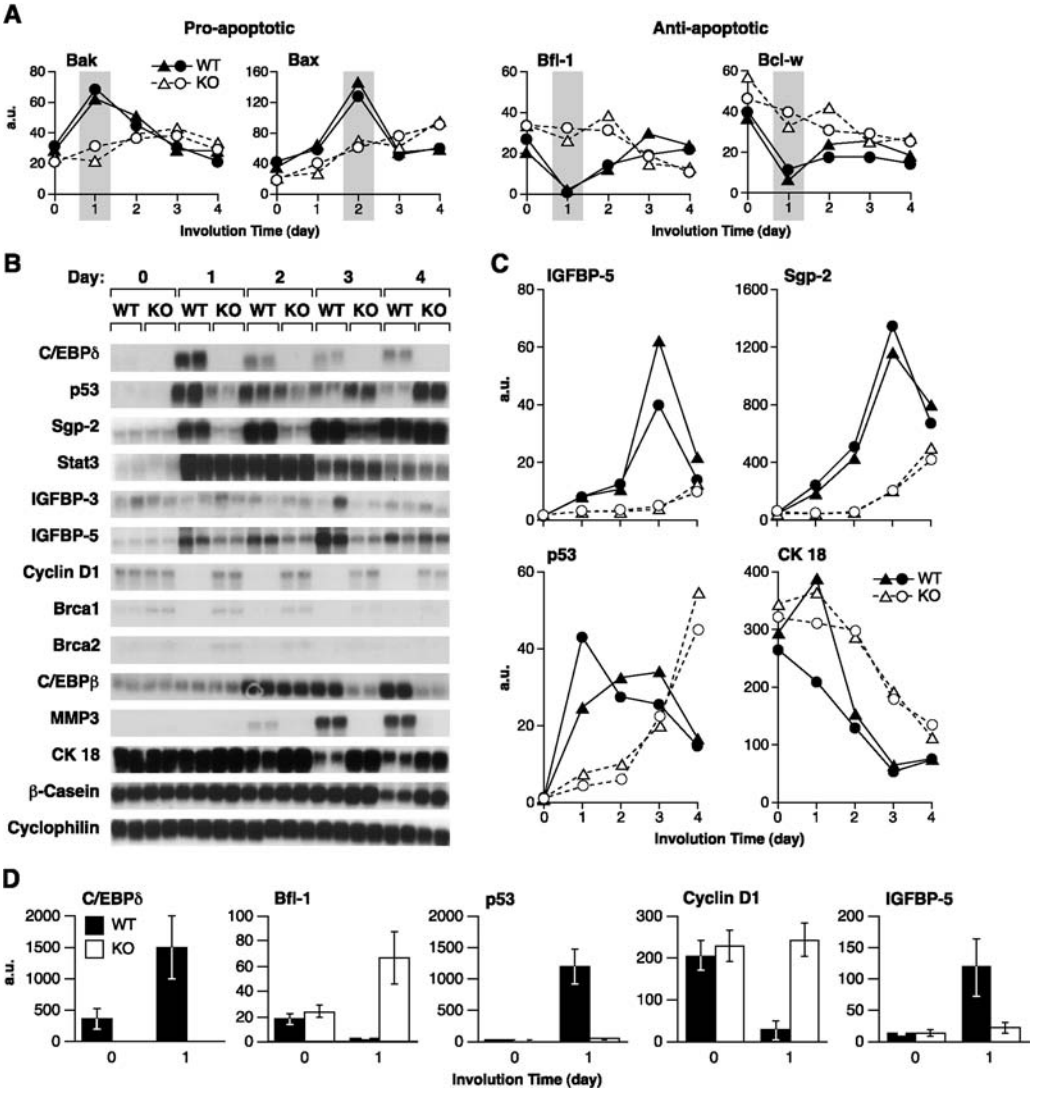

Fig. 5. Involution-associated gene expression patterns are significantly altered in the absence of C/EBPδ. (A) Multiprobe ribonuclease protection assay of the indicated genes in total RNA from mammary glands of two mice per genotype and time point. Connecting lines were drawn as a visual aid. Shaded boxes indicate the time points of most significant differences between wild-type and C/EBPδ null glands. (B) Northern analysis of the indicated genes in total RNA from mammary glands during lactation (Inv d0) and 1-4 days after removal of the pups (two mice per group). (C) A selection of the data from panel B quantified by phosphoimaging and normalized to cyclophilin (CK18) or CK18 (IGFBP5, SGP2, p53) expression. (D) Northern analysis of the indicated genes in total RNA from mammary glands on days 0 and 1 of involution. Data shown are the phosphoimage quantifications of expression levels in four to five mice per data point normalized to cyclophilin expression. a.u., arbitrary units.

glands, the genetic response of HC11 cells to C/EBPδ expression, and known targets of p53, we propose the following model for the signaling cascade in involution of C57BL/6 mice (Fig. 7). C/EBPδ, induced by STAT3, is upstream of and required for induced expression of p53, IGFBP5, SGP2 and BAK. Furthermore, C/EBPδ activity leads to suppression of Cyclin D1 and BFL1 expression. C/EBPδ is necessary but not sufficient to induce expression of p53, which leads to activation of BAX, and inhibition of survivin, BRCA1 and BRCA2 expression. However, p53 and other factors may cooperate also in the regulation of genes downstream of C/EBPδ as well as C/EBPδ itself. In addition, C/EBPδ activity is required for expression of MMP3 during the later phase of involution and for maintenance of C/EBPβ expression. Furthermore, the STAT3 transcription factor is required for significant activation of the C/EBPδ gene in mammary epithelial cells from lactation through at least the first 3 days of involution, by contrast to being dispensable for C/EBPδ expression in the liver (Alonzi et al., 2001).

Taken together, our data place C/EBPδ between STAT3 and multiple pro-apoptotic genetic events during the initial phase of apoptosis initiation, which further affect gene expression during the subsequent phase of tissue remodeling. Interestingly, expression of C/EBPδ also correlates with degeneration of surface mucous cells of the larval stomach during metamorphosis of *Xenopus laevis* (Ikuzawa et al., 2005). Thus, a role in apoptosis during developmental tissue regression may be an evolutionarily 'old' function of C/EBPδ.

In *Stat3* mutant MEC, IGFBP5 activation was initially impaired, but recovered by day 3. However, C/EBPδ levels remained low. Thus, activation of C/EBPδ expression is very specific for the signaling pathways that elicit the physiological first phase of apoptosis in MEC. Many extracellular signals can potentially activate C/EBPδ expression (Ramji and Foka, 2002). Nevertheless, STAT3 alone is essential for high levels of expression in MEC during involution. However, STAT3 alone may not be sufficient, because C/EBPδ expression declines already on Inv d2 before STAT3 activation ceases on Inv d4. Interestingly, STAT3 often exhibits constitutive activation in breast tumor cells (Bromberg, 2002), while C/EBPδ expression is significantly reduced in mammary and breast tumors compared with normal tissue (Kuramoto et al., 2002; Porter et al., 2003). Thus, one can speculate that lack of C/EBPδ as an apoptosis mediator may in part explain why

tumor cells with constitutive STAT3 activity do not undergo apoptosis.

C/EBPδ is also inducible in macrophages, fibroblasts and adipocytes (Ramji and Foka, 2002), cell types that participate in mammary gland involution subsequent to initiation of epithelial cell apoptosis (Monks et al., 2002; Werb et al., 1996). Our data indicate transient induction of C/EBPδ expression in a subset of stromal cells in parallel with its massive expression in the luminal epithelial cell layer. Thus, we cannot rule out that some phenotypes in the C/EBPδ null glands may be caused in part by defective responses of adipocytes in the early phase of involution. However, C/EBPδ null glands and mammary glands with *Stat3* deletion specifically in secretory epithelial cells (Clarkson et al., 2004; Stein et al., 2004) exhibit several overlapping phenotypes, including impaired expression of IGFBP5, MMP3, BAX and SGP2, demonstrating that several significant effects are caused by the role of C/EBPδ as a STAT3 target in secretory epithelial cells.

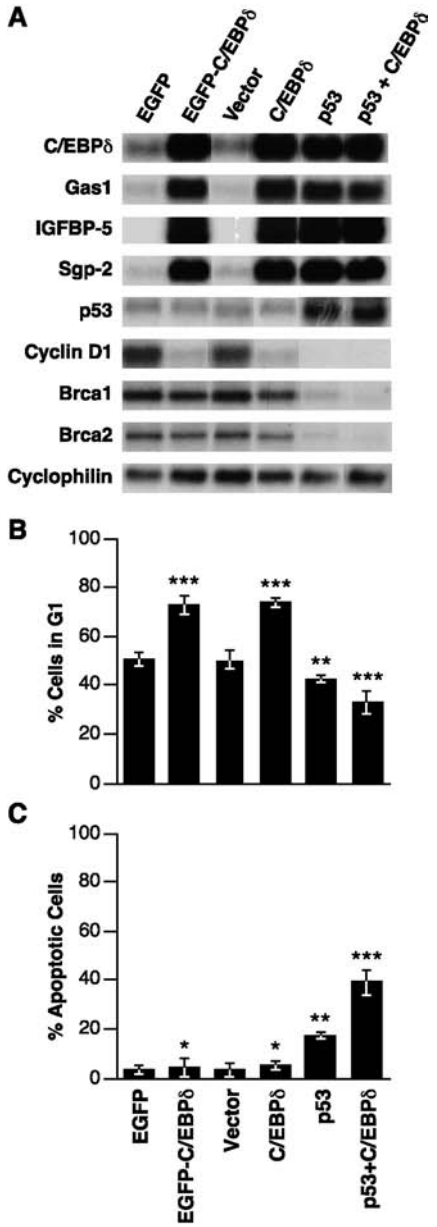

Fig. 6. C/EBPδ induces involution-related genes in HC11 cells and cooperates with p53 in inducing apoptosis. The non-transformed mouse mammary epithelial cell line HC11 was transiently transfected with constructs expressing the indicated proteins, and harvested after 2 days. (A) Northern analysis of the indicated genes in total RNA from representative samples are shown. (B) The flow cytometric quantification of arrested (G1 DNA content) and (C) apoptotic cells (sub-G1 DNA content) represents the means ± s.d. of six to eight samples from three to four independent experiments.

However, there are also differences between the STAT3 null and C/EBPδ null mammary glands, such as in the regulation of p53 expression (Chapman et al., 1999; Humphreys et al., 2002; Kritikou et al., 2003; Zhao et al., 2004). These may be explained in part by the genetic background. We chose to study the C/EBPδ null mutation on the C57BL/6 strain background because these mice do not exhibit increased ductal branching in the absence of C/EBPδ (data not shown) as do outbred C57BL/6×129S1 mice (Gigliotti et al., 2003). In fact, on the mixed strain background C/EBPδ deficiency did not result in a morphological delay of involution, and SGP2 and IGFBP5 expression were impaired on only Inv d1 and 2, respectively (Gigliotti et al., 2003). We had demonstrated that the mRNA expression levels of several genes, including C/EBPδ and SGP2, are not modulated during involution in 129S1 mice. However, the dynamic gene expression patterns we observed in C57BL/6 during involution were similar to those described for FVB/N mice (Thangaraju et al., 2004). By contrast to the 129S1 strain, the C57BL/6 and FVB/N strains are widely used in the study of mammary gland development, rendering them more suitable for the analysis of gene deletions on involution.

Despite the dramatic effects of C/EBPδ-deficiency on gene expression patterns, the mammary gland still involutes, albeit with delayed kinetics. In fact, to our knowledge, no reported genetically engineered mouse model has led to a complete block of involution. This is most probably due to the fact that a cell in distress can find many ways to die (Lockshin and Zakeri, 2004). In the conditional *Stat3* null model, elevated levels of p53 mediate in part the compensatory apoptotic response (Matthews and Clarke, 2005). However, these alternative pathways would not represent the physiological sequence of events. With respect to the morphological

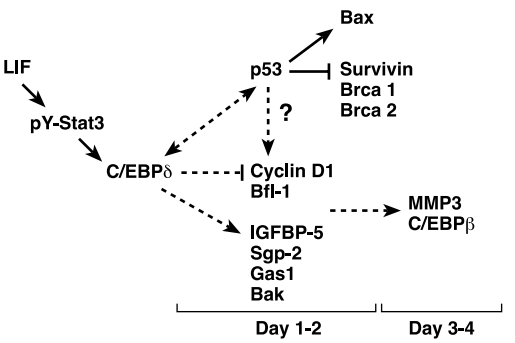

Fig. 7. Schematic representation of the gene expression events following C/EBPδ induction by the STAT3 pathway in involuting mouse mammary glands. Dashed lines indicate interactions that may be either direct or indirect. Solid lines indicate probable direct interactions.

regression and remodeling of the gland, the C/EBPδ null mutation causes a more subtle delay than mutations in *Stat3* or its upstream activators gp130 and LIF (Chapman et al., 1999; Humphreys et al., 2002; Kritikou et al., 2003; Zhao et al., 2004). This is not surprising, as C/EBPδ is surely only one of many target genes of STAT3. Similarly, no single target of C/EBPδ can be responsible for the phenotype of C/EBPδ null mammary glands. Rather, the combined actions of the multiple pro- and anti-apoptotic proteins whose regulation of expression is perturbed in the absence of C/EBPδ underlies the delay in apoptosis and involution.

IGFBP5 is induced by Inv d1 if a functional C/EBPδ gene is present. C/EBPδ can activate IGFBP5 expression through direct promoter regulation (Ji et al., 2003). Thus, the dependence of IGFBP5 expression on C/EBPδ in vivo and the ability of C/EBPδ to induce IGFBP5 in HC11 cells are consistent with IGFBP5 representing a direct target gene. In vitro and in vivo IGFBP5 can cause apoptosis of MEC, involving IGF1 dependent and independent mechanisms (Flint et al., 2003; Marshman et al., 2003; Schneider et al., 2002). Thus, the target gene IGFBP5 is part of the molecular mechanism by which C/EBPδ promotes involution.

C/EBPδ also induced SGP2/clusterin, which is mostly known as a secreted protein whose expression is activated in many examples of developmental tissue regression. Its role in cell survival has been controversial, but recent reports show that nuclear isoforms can elicit growth arrest and apoptosis (Scaltriti et al., 2004). Thus, lack of SGP2 expression in C/EBPδ mutant mice may further contribute to delayed apoptosis.

Cyclin D1 and *Bfl1*, two genes that are normally suppressed at the onset of involution, continued to be expressed in C/EBPδ null glands. *Cyclin D1* promotes G1/S progression through regulation of cyclin-dependent kinases but can also affect histone acetylation and chromatin remodeling (Fu et al., 2004). BFL1, whose promoter also contains a functional C/EBP site (Edelstein et al., 2003), inhibits the pro-apoptotic activity of other BCL2 members (Werner et al., 2002) and of p53 (D'Sa-Eipper et al., 1996). Thus, continued expression of these two genes in the absence of C/EBPδ can contribute to delayed apoptosis and involution.

Remarkably, loss of C/EBPδ had profound effects on the later stages of involution, despite its transient peak expression on Inv d1. A null mutation in *Stat3* null epithelial cells causes delayed activation of MMP9, while MMP2 activity is not affected (Humphreys et al., 2002). The present study shows that MMP3 expression during involution is completely dependent on C/EBPδ as well as its upstream activator STAT3. Expression of the C/EBPβ gene during the second phase of involution is also affected by lack of C/EBPδ. C/EBPβ is essential for proliferation and differentiation of epithelial cells during mammary gland development (Robinson et al., 1998; Seagroves et al., 1998). However, C/EBPβ is also a crucial player during adipocyte differentiation in vitro (Tanaka et al., 1997). While it is known that MMP3 is expressed in stromal cells during mammary development and involution (Lund et al., 1996), it remains to be determined which cell types express C/EBPβ during involution. However, the differences in expression kinetics between C/EBPδ, C/EBPβ and MMP3 suggest that C/EBPδ does not regulate either of them directly.

C/EBPδ expression alone may not be sufficient for activation of its target genes. The C/EBPδ protein requires phosphorylation for efficient transactivation (Ji et al., 2003) and DNA binding, such as by CaseinKinase II (Osada et al., 1996), IKKi (Kravchenko et al., 2003) or IL1 receptor signaling (Lacorte et al., 1997). Expression of IKKi as well as the IL1 ligand receptor system is activated at the onset of involution (Clarkson et al., 2004), and may contribute to C/EBPδ activation. Furthermore, C/EBPδ interacts with many other transcription factors, including Smad3 (Choy and Derynck, 2003), which promotes involution as a mediator of TGFβ3 receptor activation (Nguyen and Pollard, 2000; Yang et al., 2002). Thus, C/EBPδ potentially serves to integrate the pathways from several extracellular signals such as LIF, TGFβ and interleukins to initiate apoptosis of mammary epithelial cells.

Clearly, many genes are dynamically regulated in unique spatial and temporal patterns during the mammary involution process. Data derived from individual gene deletions in mice allow us to place genes into signaling cascades and derive their crucial functions. The present study places C/EBPδ between STAT3 and several pro- and anti-apoptotic genes as an inducer of a physiological cell death response in epithelial cells at the onset of mammary gland involution.

We thank Lori Warg, Margaret Lualdi, Angie Hackley and Barb Shankle for expert technical support. We are indebted to many colleagues for reagents and advice; in particular, Ira Daar, Howard Fearnhead, Yili Yang and Howard Young for antibodies; David Salomon for HC11 cells; Karen Vousden for mouse p53; Shyam Sharan for *Brca1* and *Brca2* probes; Susan Dorsey for help with image analysis; Dough Powel for statistical analysis; and Gertraud Robinson and Peter Johnson for critical reading of the manuscript. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute and National Institute of Diabetes and Digestive and Kidney Diseases.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/21/4675/DC1

References

Abell, K., Bilancio, A., Clarkson, R. W., Tiffen, P. G., Altaparmakov, A. I., Burdon, T. G., Asano, T., Vanhaesebroeck, B. and Watson, C. J. (2005). Stat3-induced apoptosis requires a molecular switch in PI(3)K subunit composition. *Nat. Cell Biol.* **7**, 392-398.

Allar, M. A. and Wood, T. L. (2004). Expression of the insulin-like growth factor binding proteins during postnatal development of the murine mammary gland. *Endocrinology* **145**, 2467-2477.

Alonzi, T., Maritano, D., Gorgoni, B., Rizzuto, G., Libert, C. and Poli, V. (2001). Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation [correction of activation] in the liver. *Mol. Cell. Biol.* **21**, 1621-1632.

Blackburn, A. C. and Jerry, D. J. (2002). Knockout and transgenic mice of Trp53: what have we learned about p53 in breast cancer? *Breast Cancer Res.* **4**, 101-111.

Bromberg, J. (2002). Stat proteins and oncogenesis. *J. Clin. Invest.* **109**, 1139-1142.

Cantwell, C. A., Sterneck, E. and Johnson, P. F. (1998). Interleukin-6-specific activation of the C/EBPδ gene in hepatocytes is mediated by Stat3 and Sp1. *Mol. Cell. Biol.* **18**, 2108-2117.

Cassel, T. N., Nordlund-Moller, L., Andersson, O., Gustafsson, J. A. and Nord, M. (2000). C/EBPalpha and C/EBPdelta activate the clara cell secretory protein gene through interaction with two adjacent C/EBP-binding sites. *Am. J. Respir. Cell Mol. Biol.* **22**, 469-480.

Chapman, R. S., Lourenco, P. C., Tonner, E., Flint, D. J., Selbert, S.,

Takeda, K., Akira, S., Clarke, A. R. and Watson, C. J. (1999). Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev*. **13**, 2604-2616.

Choy, L. and Derynck, R. (2003). Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. *J. Biol. Chem*. **278**, 9609-9619.

Clarkson, R. W., Wayland, M. T., Lee, J., Freeman, T. and Watson, C. J. (2004). Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res*. **6**, R92-R109.

Colangelo, A. M., Johnson, P. F. and Mocchetti, I. (1998). beta-adrenergic receptor-induced activation of nerve growth factor gene transcription in rat cerebral cortex involves CCAAT/enhancer-binding protein delta. *Proc. Natl. Acad. Sci. USA* **95**, 10920-10925.

D'Sa-Eipper, C., Subramanian, T. and Chinnadurai, G. (1996). bfl-1, a bcl-2 homologue, suppresses p53-induced apoptosis and exhibits potent cooperative transforming activity. *Cancer Res*. **56**, 3879-3882.

Dearth, L. R. and DeWille, J. (2003). Posttranscriptional and posttranslational regulation of C/EBPdelta in G0 growth arrested mammary epithelial cells. *J. Biol. Chem*. **278,** 11246-11255.

Duriez, P. J. and Shah, G. M. (1997). Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. *Biochem. Cell Biol*. **75**, 337-349.

Edelstein, L. C., Lagos, L., Simmons, M., Tirumalai, H. and Gelinas, C. (2003). NF-kappa B-dependent assembly of an enhanceosome-like complex on the promoter region of apoptosis inhibitor Bfl-1/A1. *Mol. Cell. Biol*. **23**, 2749-2761.

Flint, D. J., Beattie, J. and Allan, G. J. (2003). Modulation of the actions of IGFs by IGFBP-5 in the mammary gland. *Horm. Metab. Res*. **35**, 809-815.

Fu, M., Wang, C., Li, Z., Sakamaki, T. and Pestell, R. G. (2004). Minireview: Cyclin D1: normal and abnormal functions. *Endocrinology* **145**, 5439-5447.

Furth, P. A. (1999). Introduction: mammary gland involution and apoptosis of mammary epithelial cells. *J. Mammary Gland Biol. Neoplasia* **4**, 123-127.

Gery, S., Tanosaki, S., Hofmann, W. K., Koppel, A. and Koeffler, H. P. (2005). C/EBPdelta expression in a BCR-ABL-positive cell line induces growth arrest and myeloid differentiation. *Oncogene* **24**, 1589-1597.

Gigliotti, A. P. and DeWille, J. W. (1998). Lactation status influences expression of CCAAT/enhancer binding protein isoform mRNA in the mouse mammary gland. *J. Cell Physiol*. **174**, 232-239.

Gigliotti, A. P., Johnson, P. F., Sterneck, E. and DeWille, J. W. (2003). Nulliparous CCAAT/enhancer binding protein delta (C/EBPdelta) knockout mice exhibit mammary gland ductal hyperplasia. *Exp. Biol. Med. (Maywood)* **228**, 278-285.

Green, K. A. and Streuli, C. H. (2004). Apoptosis regulation in the mammary gland. *Cell Mol. Life Sci*. **61**, 1867-1883.

Hodge, D. L., Martinez, A., Julias, J. G., Taylor, L. S. and Young, H. A. (2002). Regulation of nuclear gamma interferon gene expression by interleukin 12 (IL-12) and IL-2 represents a novel form of posttranscriptional control. *Mol. Cell. Biol*. **22**, 1742-1753.

Hoffman, W. H., Biade, S., Zilfou, J. T., Chen, J. and Murphy, M. (2002). Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J. Biol. Chem*. **277**, 3247-3257.

Huang, A. M., Montagna, C., Sharan, S., Yajun, N., Ried, T. and Sterneck, E. (2004). Loss of CCAAT/enhancer binding protein δ promotes chromosomal instability. *Oncogene* **23**, 1549-1557.

Humphreys, R. C., Bierie, B., Zhao, L., Raz, R., Levy, D. and Hennighausen, L. (2002). Deletion of Stat3 blocks mammary gland involution and extends functional competence of the secretory epithelium in the absence of lactogenic stimuli. *Endocrinology* **143**, 3641-3650.

Hutt, J. A. and DeWille, J. W. (2002). Oncostatin M induces growth arrest of mammary epithelium via a CCAAT/enhancer-binding protein delta-dependent pathway. *Mol. Cancer Ther*. **1**, 601-610.

Hutt, J. A., O'Rourke, J. P. and DeWille, J. (2000). Signal transducer and activator of transcription 3 activates CCAAT enhancer-binding protein delta gene transcription in G0 growth-arrested mouse mammary epithelial cells and in involuting mouse mammary gland. *J. Biol. Chem*. **275**, 29123-29131.

Hynes, N. E., Taverna, D., Harwerth, I. M., Ciardiello, F., Salomon, D. S., Yamamoto, T. and Groner, B. (1990). Epidermal growth factor receptor, but not c-erbB-2, activation prevents lactogenic hormone induction of the beta-casein gene in mouse mammary epithelial cells. *Mol. Cell. Biol*. **10**, 4027-4034.

Ikezoe, T., Gery, S., Yin, D., O'Kelly, J., Binderup, L., Lemp, N., Taguchi, H. and Koeffler, H. P. (2005). CCAAT/enhancer-binding protein delta: a molecular target of 1,25-dihydroxyvitamin D3 in androgen-responsive prostate cancer LNCaP cells. *Cancer Res*. **65**, 4762-4768.

Ikuzawa, M., Kobayashi, K., Yasumasu, S. and Iuchi, I. (2005). Expression of CCAAT/enhancer binding protein delta is closely associated with degeneration of surface mucous cells of larval stomach during the metamorphosis of Xenopus laevis. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol*. **140**, 505-511.

Ji, C., Chang, W., Centrella, M. and McCarthy, T. L. (2003). Activation domains of CCAAT enhancer binding protein δ: regions required for native activity and prostaglandin E2-dependent transactivation of IGF-I gene expression in rat osteoblasts. *Mol. Endocrinol*. **17**, 1834-1843.

Kravchenko, V. V., Mathison, J. C., Schwamborn, K., Mercurio, F. and Ulevitch, R. J. (2003). IKKi/IKKepsilon plays a key role in integrating signals induced by pro-inflammatory stimuli. *J. Biol. Chem*. **278**, 26612-26619.

Kritikou, E. A., Sharkey, A., Abell, K., Came, P. J., Anderson, E., Clarkson, R. W. and Watson, C. J. (2003). A dual, non-redundant, role for LIF as a regulator of development and STAT3-mediated cell death in mammary gland. *Development* **130**, 3459-3468.

Kubista, M., Rosner, M., Kubista, E., Bernaschek, G. and Hengstschlager, M. (2002). Brca1 regulates in vitro differentiation of mammary epithelial cells. *Oncogene* **21**, 4747-4756.

Kuramoto, T., Morimura, K., Yamashita, S., Okochi, E., Watanabe, N., Ohta, T., Ohki, M., Fukushima, S., Sugimura, T. and Ushijima, T. (2002). Etiology-specific gene expression profiles in rat mammary carcinomas. *Cancer Res*. **62**, 3592-3597.

Lacorte, J. M., Ktistaki, E., Beigneux, A., Zannis, V. I., Chambaz, J. and Talianidis, I. (1997). Activation of CAAT enhancer-binding protein delta (C/EBPdelta) by interleukin-1 negatively influences apolipoprotein C-III expression. *J. Biol. Chem*. **272**, 23578-23584.

Li, M., Hu, J., Heermeier, K., Hennighausen, L. and Furth, P. A. (1996). Apoptosis and remodeling of mammary gland tissue during involution proceeds through p53-independent pathways. *Cell Growth Diff*. **7**, 13-20.

Li, M., Liu, X., Robinson, G., Bar-Peled, U., Wagner, K. U., Young, W. S., Hennighausen, L. and Furth, P. A. (1997). Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proc. Natl. Acad. Sci. USA* **94**, 3425-3430.

Lockshin, R. A. and Zakeri, Z. (2004). Apoptosis, autophagy, and more. *Int. J. Biochem. Cell Biol*. **36**, 2405-2419.

Lund, L. R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Dano, K. and Werb, Z. (1996). Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* **122**, 181-193.

MacLachlan, T. K., Dash, B. C., Dicker, D. T. and El-Deiry, W. S. (2000). Repression of BRCA1 through a feedback loop involving p53. *J. Biol. Chem*. **275**, 31869-31875.

Marshman, E., Green, K. A., Flint, D. J., White, A., Streuli, C. H. and Westwood, M. (2003). Insulin-like growth factor binding protein 5 and apoptosis in mammary epithelial cells. *J. Cell Sci*. **116**, 675-682.

Marti, A., Ritter, P. M., Jager, R., Lazar, H., Baltzer, A., Schenkel, J., Declercq, W., Vandenabeele, P. and Jaggi, R. (2001). Mouse mammary gland involution is associated with cytochrome c release and caspase activation. *Mech. Dev*. **104**, 89-98.

Matthews, J. R. and Clarke, A. R. (2005). p53 mediates a default programme of mammary gland involution in the absence of STAT3. *Oncogene* **24**, 3083-3090.

Merlo, G. R., Venesio, T., Taverna, D., Marte, B. M., Callahan, R. and Hynes, N. E. (1994). Growth suppression of normal mammary epithelial cells by wild-type p53. *Oncogene* **9**, 443-453.

Mirza, A., McGuirk, M., Hockenberry, T. N., Wu, Q., Ashar, H., Black, S., Wen, S. F., Wang, L., Kirschmeier, P., Bishop, W. R. et al. (2002). Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. *Oncogene* **21**, 2613-2622.

Monks, J., Geske, F. J., Lehman, L. and Fadok, V. A. (2002). Do inflammatory cells participate in mammary gland involution? *J. Mammary Gland Biol. Neoplasia* **7**, 163-176.

Nguyen, A. V. and Pollard, J. W. (2000). Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. *Development* **127**, 3107-3118.

O'Rourke, J. P., Newbound, G. C., Hutt, J. A. and DeWille, J. (1999).

CCAAT/enhancer-binding protein delta regulates mammary epithelial cell G0 growth arrest and apoptosis. *J. Biol. Chem.* **274**, 16582-16589.

Osada, S., Yamamoto, H., Nishihara, T. and Imagawa, M. (1996). DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. *J. Biol. Chem.* **271**, 3891-3896.

Porter, D., Lahti-Domenici, J., Keshaviah, A., Bae, Y. K., Argani, P., Marks, J., Richardson, A., Cooper, A., Strausberg, R., Riggins, G. J. et al. (2003). Molecular markers in ductal carcinoma in situ of the breast. *Mol. Cancer Res.* **1**, 362-375.

Rajan, J. V., Marquis, S. T., Gardner, H. P. and Chodosh, L. A. (1997). Developmental expression of Brca2 colocalizes with Brca1 and is associated with proliferation and differentiation in multiple tissues. *Dev. Biol.* **184**, 385-401.

Ramji, D. P. and Foka, P. (2002). CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem. J.* **365**, 561-575.

Robinson, G. W., Smith, G. H., Gallahan, D., Zimmer, A., Furth, P. A. and Hennighausen, L. (1996). Understanding mammary gland development through the imbalanced expression of growth regulators. *Dev. Dyn.* **206**, 159-168.

Robinson, G. W., Johnson, P. F., Hennighausen, L. and Sterneck, E. (1998). The C/EBPb transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. *Genes Dev.* **12**, 1907-1916.

Scaltriti, M., Santamaria, A., Paciucci, R. and Bettuzzi, S. (2004). Intracellular clusterin induces G2-M phase arrest and cell death in PC-3 prostate cancer cells1. *Cancer Res.* **64**, 6174-6182.

Schneider, M. R., Wolf, E., Hoeflich, A. and Lahm, H. (2002). IGF-binding protein-5: flexible player in the IGF system and effector on its own. *J. Endocrinol.* **172**, 423-440.

Seagroves, T. N., Krnacik, S., Raught, B., Gay, J., Burgess-Beusse, B., Darlington, G. J. and Rosen, J. M. (1998). C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. *Genes Dev.* **12**, 1917-1928.

Soldani, C. and Scovassi, A. I. (2002). Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. *Apoptosis* **7**, 321-328.

Stein, T., Morris, J. S., Davies, C. R., Weber-Hall, S. J., Duffy, M. A., Heath, V. J., Bell, A. K., Ferrier, R. K., Sandilands, G. P. and Gusterson, B. A. (2004). Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Res.* **6**, R75-R91.

Sterneck, E., Paylor, R., Jackson-Lewis, V., Libbey, M., Przedborski, S., Tessarollo, L., Crawley, J. N. and Johnson, P. F. (1998). Selectively enhanced contextual fear conditioning in mice lacking the transcriptional regulator CCAAT/enhancer binding protein delta. *Proc. Natl. Acad. Sci. USA* **95**, 10908-10913.

Strange, R., Li, F., Saurer, S., Burkhardt, A. and Friis, R. R. (1992). Apoptotic cell death and tissue remodelling during mouse mammary gland involution. *Development* **115**, 49-58.

Strange, R., Friis, R. R., Bemis, L. T. and Geske, F. J. (1995). Programmed cell death during mammary gland involution. In *Methods in Cell Biology*, vol. 46 (ed. L. M. Schwartz and B. A. Osborne), pp. 355-368. San Diego: Academic Press.

Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T. and Akira, S. (1997). Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc. Natl. Acad. Sci. USA* **94**, 3801-3804.

Takiguchi, M. (1998). The C/EBP family of transcription factors in the liver and other organs. *Int. J. Exp. Pathol.* **79**, 369-391.

Tanaka, T., Yoshida, N., Kishimoto, T. and Akira, S. (1997). Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. *EMBO J.* **16**, 7432-7443.

Thangaraju, M., Sharan, S. and Sterneck, E. (2004). Comparison of mammary gland involution between 129S1 and C57BL/6 inbred mouse strains: differential regulation of Bcl2a1, Trp53, Cebpb, and Cebpd expression. *Oncogene* **23**, 2548-2553.

Umayahara, Y., Ji, C., Centrella, M., Rotwein, P. and McCarthy, T. L. (1997). CCAAT/enhancer-binding protein delta activates insulin-like growth factor-I gene transcription in osteoblasts. Identification of a novel cyclic AMP signaling pathway in bone. *J. Biol. Chem.* **272**, 31793-31800.

Walton, K. D., Wagner, K. U., Rucker, E. B., 3rd, Shillingford, J. M., Miyoshi, K. and Hennighausen, L. (2001). Conditional deletion of the bcl-x gene from mouse mammary epithelium results in accelerated apoptosis during involution but does not compromise cell function during lactation. *Mech. Dev.* **109**, 281-293.

Werb, Z., Sympson, C. J., Alexander, C. M., Thomasset, N., Lund, L. R., MacAuley, A., Ashkenas, J. and Bissell, M. J. (1996). Extracellular matrix remodeling and the regulation of epithelial-stromal interactions during differentiation and involution. *Kidney Int.* **54**, S68-S74.

Werner, A. B., de Vries, E., Tait, S. W., Bontjer, I. and Borst, J. (2002). Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit is collaboration with pro-apoptotic Bak or Bax. *J. Biol. Chem.* **277**, 22781-22788.

Yang, Y. A., Tang, B., Robinson, G., Hennighausen, L., Brodie, S. G., Deng, C. X. and Wakefield, L. M. (2002). Smad3 in the mammary epithelium has a nonredundant role in the induction of apoptosis, but not in the regulation of proliferation or differentiation by transforming growth factor-beta. *Cell Growth Diff.* **13**, 123-130.

Zhang, F., Li, C., Halfter, H. and Liu, J. (2003). Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates the expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells. *Oncogene* **22**, 894-905.

Zhao, L., Hart, S., Cheng, J., Melenhorst, J. J., Bierie, B., Ernst, M., Stewart, C., Schaper, F., Heinrich, P. C., Ullrich, A. et al. (2004). Mammary Gland Remodeling Depends on gp130 Signaling through Stat3 and MAPK. *J. Biol. Chem.* **279**, 44093-44100.