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

Muthusamy Thangaraju1,*  Martina Rudelius2,  Brian Bierie3,†  Mark Raffeld2,  Shikha Sharan1,  Lothar Hennighausen3,  A-Mei Huang1‡  and Esta Sterneck1,§

1Laboratory of Protein Dynamics and Signaling, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702-1201, USA
2Laboratory of Pathology, National Cancer Institute, National Institute of Health, Bethesda MD 20892, USA
3Laboratory 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
§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/EBPδ 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-IL6δ) (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, IL-1, IL-6, 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; Ikoezo 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<sup>fl/fl</sup> 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 CO2 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<sup>fl/fl</sup>/WC mice**

Mice carrying a Stat3<sup>fl</sup> gene with LoxP-flanked exons 16–21 (Stat3<sup>fl/fl</sup>) were bred to mice carrying the Cre transgene under control of the WAP gene promoter (Humphreys et al., 2002). Stat3<sup>fl/fl</sup> 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<sup>6</sup> 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′-CAAGCTAGCTGGCCGCGCTTTTC-3′, 5′-CAAGGATCCACCGGCAGTCGGCCGCGC-3′) and the GC-rich PCR system (Roche), inserted into the TOPO TA vector (Invitrogen), sequenced and cloned into pEGFP-N1 (Clontech) by NheI and BamHI (‘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 haematoxylin 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/DA-B polymer based rabbit detection system (Envision<sup>®</sup> 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′-TTTGCAGTTTCCTGCTTCTT-3′, 5′-TTTTCGCGTA-TCCATTCTCC-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.
Development

C/EBP

Results

C/EBPβ is expressed in luminal mammary epithelial cells

Previous analyses of C/EBPβ expression in the mammary gland were based on whole organ RNA levels. Although MEC lines in vitro clearly express inducible C/EBPβ, 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/EBPβ is expressed at different stages. By this approach we were unable to detect C/EBPβ 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/EBPβ 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/EBPβ (see Fig. S1 in the supplementary material), similar to endothelial cells (data not shown). However, a subset of stromal adipocytes also expressed nuclear C/EBPβ (Fig. 1). No specific staining for C/EBPβ 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/EBPβ 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/EBPβ protein on Inv d1.

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

The C/EBPβ 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/EBPβ in mouse and human MEC lines (Hutt and DeWille, 2002; Zhang et al., 2003), identifying C/EBPβ 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/EBPβ 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/EBPβ levels on Inv d2 compared with controls (Kritikou et al., 2003). To define the exact extent to which C/EBPβ 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/EBPβ levels were greatly reduced even in the lactating gland
Next, the role of C/EBP\textsuperscript{a} Involution is delayed in the absence of C/EBP\textsuperscript{a} found to be required for MMP3 expression during the later mechanism developing by Inv d3. Furthermore, STAT3 was lactation period and Inv d1-3, with no compensatory kinetic analysis revealed that this requirement extends to the mammary involution in vivo. Furthermore, the expanded level induction of C/EBP\textsuperscript{a} was dramatically impaired in the absence of STAT3. The low 1998; Thangaraju et al., 2004). However, induction of C/EBP\textsuperscript{a} Stat3 gene, or could be due to stromal cells. Expression of C/EBP\textsuperscript{a} 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/EBP\textsuperscript{a} 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/EBP\textsuperscript{a}

Next, the role of C/EBP\textsuperscript{a} in involution was analyzed by use of C/EBP\textsuperscript{a}-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/EBP\textsuperscript{a}-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/EBP\textsuperscript{a} 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/EBP\textsuperscript{a}, the kinetics of post-lactational mammary gland involution is delayed at the morphological level.

C/EBP\textsuperscript{a} 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/EBP\textsuperscript{a}-deficient 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/EBP\textsuperscript{a} 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/EBP\textsuperscript{a}.

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/EBP\textsuperscript{a} alone. These data raised the question of whether STAT3 activation itself was affected by the C/EBP\textsuperscript{a} 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/EBP\textsuperscript{a} is downstream of STAT3 and upstream of several cell death regulators, such as protease activation, and p53 and IGFBP5 protein expression.

C/EBP\textsuperscript{a} is essential for regulation of pro-apoptotic gene expression

As C/EBP\textsuperscript{a} 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/EBP\textsuperscript{a}, 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.
Role of C/EBPβ/H9254 in mammary involution

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/EBPβ (Fig. 5A). These data are in agreement with a delay in cell death initiation in C/EBPβ-deficient glands.

Northern analysis (Fig. 5B,C) showed that as described before, C/EBPβ 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

Fig. 3. Involution is delayed in C/EBPβ null mammary glands. Comparison of mammary gland histology during involution in wild-type (WT) and C/EBPβ 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<0.0001, by two-way ANOVA with follow-up tests.
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/EBPβ, 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/EBPβ 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/EBPβ-deficiency take place during the early stages of involution. Therefore, we have focused our attention on day 1 of involution, when C/EBPβ levels are highest in wild-type glands, C/EBPβ-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/EBPβ. Furthermore, not only was Cyclin D1 expression maintained in C/EBPβ null glands, but BFL1 expression was in fact induced rather than suppressed compared with wild-type glands (Fig. 5D).

In summary, expression analyses revealed dramatically impaired activation of pro-apoptotic genes and suppression of anti-apoptotic genes in C/EBPβ 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/EBPβ. 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/EBPβ null mice.

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

To investigate whether C/EBPβ 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/EBPβ elicited a growth arrest response in HC11 cells with concomitant upregulation of Gas1 mRNA levels (Fig. 6). In addition, C/EBPβ 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/EBPβ. This result suggests that the activation of p53 expression downstream of C/EBPβ 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/EBPβ, suggesting that the decline of BRCA1 and 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/EBPβ gene in HC11 cells. Thus, in the mammary gland, C/EBPβ 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/EBPβ significantly enhanced this apoptotic response, indicating that p53 and C/EBPβ 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/EBPβ in vitro (Dearth and DeWille, 2003, and references therein), because most cell lines harbor mutant p53 genes.

Taken together, these data show that C/EBPβ 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/EBPβ 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/EBPβ null mammary
Role of C/EBPβ in mammary involution

Involvement of C/EBPβ/H9254 in mammary involution, the genetic response of HC11 cells to C/EBPβ/H9254 expression, and known targets of p53, we propose the following model for the signaling cascade in involution of C57BL/6 mice (Fig. 7). C/EBPβ, induced by STAT3, is upstream of and required for induced expression of p53, IGFBP5, SGP2 and BAK. Furthermore, C/EBPβ activity leads to suppression of Cyclin D1 and BFL1 expression. C/EBPβ 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/EBPβ as well as C/EBPβ itself. In addition, C/EBPβ 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/EBPβ gene in mammary epithelial cells from lactation through at least the first 3 days of involution, by contrast to being dispensable for C/EBPβ expression in the liver (Alonzi et al., 2001).

Taken together, our data place C/EBPβ 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/EBPβ 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/EBPβ.

In Stat3 mutant MEC, IGFBP5 activation was initially impaired, but recovered by day 3. However, C/EBPβ levels remained low. Thus, activation of C/EBPβ 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/EBPβ 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/EBPβ 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/EBPβ 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/EBPβ as an apoptosis mediator may in part explain why
tumor cells with constitutive STAT3 activity do not undergo apoptosis.

C/EBPβ 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/EBPβ 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/EBPβ null glands may be caused in part by defective responses of adipocytes in the early phase of involution. However, C/EBPβ 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/EBPβ as a STAT3 target in secretory epithelial cells.

However, there are also differences between the STAT3 null and C/EBPβ 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/EBPβ null mutation on the C57BL/6 strain background because these mice do not exhibit increased ductal branching in the absence of C/EBPβ (data not shown) as do outbred C57BL/6×129S1 mice (Gigliotti et al., 2003). In fact, on the mixed strain background C/EBPβ 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/EBPβ 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/EBPβ-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

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

\[\text{Fig. 7. Schematic representation of the gene expression events following C/EBPβ induction by the STAT3 pathway in involuting mouse mammary glands. Dashed lines indicate interactions that may be either direct or indirect. Solid lines indicate probable direct interactions.}\]
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 is 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., 2003). 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 ceases 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 Voulsen 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
Chapman, R. S., Lourenco, P. C., Tonner, E., Flint, D. J., Selbert, S.,


Werner, A. B., de Vries, E., Tait, S. W., Bontj, 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.

