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c-myc as a mediator of accelerated apoptosis and involution in mammary glands lacking Socs3

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Suppressor of cytokine signalling (SOCS) proteins are critical attenuators of cytokine-mediated signalling in diverse tissues. To determine the importance of Socs3 in mammary development, we generated mice in which *Socs3* was deleted in mammary epithelial cells. No overt phenotype was evident during pregnancy and lactation, indicating that Socs3 is not a key physiological regulator of prolactin signalling. However, *Socs3*-deficient mammary glands exhibited a profound increase in epithelial apoptosis and tissue remodelling, resulting in precocious involution. This phenotype was accompanied by augmented Stat3 activation and a marked increase in the level of c-myc. Moreover, induction of *c-myc* before weaning using an inducible transgenic model recapitulated the *Socs3* phenotype, and elevated expression of likely c-myc target genes, E2F-1, Bax and p53, was observed. Our data establish *Socs3* as a critical attenuator of pro-apoptotic pathways that act in the developing mammary gland and provide evidence that *c-myc* regulates apoptosis during involution.

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Introduction

Morphogenesis of the mammary gland occurs in distinct stages that are governed by the concerted action of hormones and locally produced cytokines (reviewed in Hennighausen and Robinson, 2005). The mammary gland primarily develops after birth, with ductal branching and elongation resulting in an epithelial tree that fills the mammary fat pad by the end of puberty. During pregnancy, lobuloalveolar units develop and undergo terminal differentiation before parturition, thus allowing milk production during lactation. During involution, structural remodelling of the gland occurs in a two-phase process (Lund *et al*, 1996). The first phase involves extensive apoptosis of the alveolar epithelial cells and is reversible, whereas the second phase involves remodelling of the mammary gland through degradation of the extracellular matrix by matrix metalloproteinases (MMPs), together with macrophages, and is irreversible. In mice, the number of apoptotic cells peaks around days 2–3 of involution and remodelling of the gland is complete by days 6–8 of this process (Lund *et al*, 1996; Li *et al*, 1997).

Signalling mediated by glycoprotein 130 (gp130), the common receptor chain for interleukin (IL)-6-type cytokines, plays an important role in regulating programmed cell death during involution. Involution is delayed in *IL-6*- and *gp130*-deficient glands, accompanied by reduced Stat3 activation in the latter model (Zhao *et al*, 2002, 2004). Conditional deletion of *Stat3* in the mammary epithelium also results in a dramatic delay in mammary gland involution (Chapman *et al*, 1999). LIF appears to be a key regulator of Stat3-dependent apoptosis in the mammary gland as $LIF^{-/-}$ female mice exhibit delayed involution accompanied by a decrease in Stat3 phosphorylation (Kritikou *et al*, 2003). Recent studies have shown that one mechanism by which Stat3 induces apoptosis is by downregulating PI3-K activity via the modulation of its regulatory subunits, p50α and p55α (Abell *et al*, 2005). These findings suggest that crosstalk between multiple signalling pathways determines cell death during involution.

The suppressor of cytokine signalling (SOCS) proteins are critical regulators of cytokine-mediated signalling in multiple organs. These proteins are cytokine-inducible and act in a classical negative feedback loop to inhibit signal transduction along the JAK/STAT pathway (reviewed in Kubo *et al*, 2003; Alexander and Hilton, 2004). Functionally, SOCS proteins interact with JAK kinases and/or cytokine receptors, targeting them for ubiquitination and proteasomal degradation. The eight members of the SOCS family are characterised by an N-terminal region of variable length and limited homology, a central SH2 domain and a conserved SOCS box at the C-terminus. Physiologically, Socs proteins have been shown to have distinct roles. In the mammary gland, both *Socs1* and *Socs2* attenuate prolactin receptor (PRLR) signalling *in vivo*. Mice carrying targeted deletions of *Socs1* and *Ifn-γ* exhibited precocious lobuloalveolar development, and loss of a single *Socs1* allele was sufficient to rescue lactation in *PRLR*-deficient mice (Lindeman *et al*, 2001). In the case of *Socs2*, removal of both alleles was necessary to restore lactogenesis in *PRLR* heterozygous females (Harris *et al*, 2006). Overexpression of *Cis* in transgenic mice led to impaired

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terminal differentiation and failure of lactation (Matsumoto *et al*, 1999), although *Cis*-null mice appeared to lactate normally (Marine *et al*, 1999).

Socs3 has the greatest sequence homology to *Socs1* but binds the receptor rather than directly interacting with JAKs to gain access to the JAK activation loop (Yasukawa *et al*, 1999; Nicholson *et al*, 2000). Within the mammary epithelium, *Socs3* expression is inducible by prolactin (Prl) and epidermal growth factor (EGF) (Tam *et al*, 2001; Tonko-Geymayer *et al*, 2002), and by activation of Stat3 in KIM-2 mammary epithelial cells (Clarkson *et al*, 2006). Overexpression studies in 293T and HC11 mammary epithelial cells have shown that Socs3 inhibits Stat5 activation in response to Prl and EGF signalling (Helman *et al*, 1998; Pezet *et al*, 1999; Tonko-Geymayer *et al*, 2002), and inhibits β-casein expression in response to Prl in SCp2 cells (Lindeman *et al*, 2001). Although the expression of *Socs3* as well as other family members is induced by a plethora of cytokines *in vitro*, gene targeting experiments have revealed that they have more specific roles *in vivo*.

Socs3 nullizygous mice die at mid-gestation owing to placental insufficiency (Marine *et al*, 1999; Roberts *et al*, 2001). However, cell-specific targeting of the *Socs3* locus has established that *Socs3* is a key negative regulator of IL-6 signalling in hepatocytes and macrophages (Croker *et al*, 2003; Lang *et al*, 2003; Yasukawa *et al*, 2003). We sought to elucidate the physiological role of *Socs3* in the developing mammary gland and to identify signalling pathways regulated by *Socs3* in mammary epithelium. Deletion of the *Socs3* locus in alveolar epithelial cells using an improved *WAPiCre* transgene resulted in a marked increase in epithelial apoptosis and accelerated involution. This was accompanied by increased levels of activated Stat3, as well as a marked increase in the level of c-myc and its target genes. We conclude that Socs3 functions as a negative regulator of gp130 signalling during involution and that *c-myc*, a target of Stat3, plays a role in mediating apoptosis in the mammary gland.

Results

WAPiCre-mediated excision of Socs3 in the mammary gland

To examine the physiological role of *Socs3* in the adult mammary gland, we utilised *WAPiCre* transgenic mice (Wintermantel *et al*, 2002). The specificity and timing of *WAPiCre*-mediated recombination during mammary gland development was confirmed by intercrossing with *GtRosa26* reporter mice (Soriano, 1999), which express *lacZ* following *Cre*-mediated deletion. Intense lacZ staining was observed in the majority of alveolar cells in *WAPiCre*;*GtRosa26* bi-transgenic mice at day 10 of lactation and day 2 of involution, but no staining was detectable in the mammary glands of control *GtRosa26* mice (Figure 1A).

Socs3$^{+/-}$ mice bearing a single copy of the *WAPiCre* transgene were crossed with *Socs3*$^{fl/fl}$ mice (Croker *et al*, 2003) to generate offspring in which the conditional (fl) allele was deleted in lobuloalveolar cells. The frequency of excision by *WAPiCre* was determined by Southern blot analysis of genomic DNA, demonstrated by loss of the floxed allele (fl, 9 kb) and appearance of the excised allele (Δ, 4.9 kb). As expected, this was highest in mammary glands from *WAPiCre*;*Socs3*$^{-/fl}$ mice during lactation and involution (Figure 1B). As the mammary gland comprises a heterogeneous cell population that includes stroma, in which the *WAPiCre* transgene is inactive, the extent of *Socs3* inactivation in mammary epithelium is likely to be underestimated (Figure 1B). To confirm the absence of *Socs3* expression in the mammary glands of *WAPiCre*;*Socs3*$^{-/fl}$ mice, we analysed *Socs3* transcript levels by real-time PCR (Figure 1C and Supplementary Figure 1) and Socs3 protein levels (Figure 1D) by Western blot analysis. Taken together, these data demonstrate efficient deletion of *Socs3* in alveolar cells during lactation and involution.

Conditional deletion of Socs3 during lactation leads to increased apoptosis

We examined the effect of deleting *Socs3* in the mammary gland during established lactation using *WAPiCre* transgenic mice. At day 4 of lactation, no differences were observed in the ability of either *Socs3*$^{+/fl}$ or *WAPiCre*;*Socs3*$^{-/fl}$ mothers to maintain their litters, and both *Socs3*$^{+/fl}$ and *WAPiCre*;*Socs3*$^{-/fl}$ mammary glands exhibited well-rounded alveoli with large distended lumens (Figure 2A). At day 10 of lactation, the alveoli in *WAPiCre*;*Socs3*$^{-/fl}$ glands appeared smaller than in control *Socs3*$^{+/fl}$ glands (Figure 2A), but there were no TUNEL-positive cells in either these or control glands (data not shown). By day 14, the lumens had partly collapsed and adipocytes were more conspicuous throughout *WAPiCre*;*Socs3*$^{-/fl}$ mammary glands (Figure 2A). This was accompanied by an increase in the number of TUNEL-positive cells (1.0 versus 0%; Figure 3A). These findings suggest that apoptosis and tissue remodelling are accelerated in *Socs3*-deficient mammary glands during established lactation.

Mammary gland involution is accelerated by loss of Socs3 and is accompanied by increased apoptosis

Given that *Socs3* expression peaks at the onset of mammary gland involution (Supplementary Figure 2), we examined the effect of loss of *Socs3* during involution. Pups were removed from *Socs3*$^{+/fl}$ and *WAPiCre*;*Socs3*$^{-/fl}$ mice after 10 days of lactation, and glands were collected for histological analysis at days 1, 2, 3 and 6 of involution (Figure 2B). *WAPiCre*;*Socs3*$^{-/fl}$ glands exhibited a dramatic increase in apoptotic bodies as early as 12 h (data not shown) and 24 h of involution (Figure 2B). By 48 h, remodelling of the gland had already commenced. Collapsed lobuloalveolar structures, concomitant with the reappearance of stroma, were readily apparent in *WAPiCre*;*Socs3*$^{-/fl}$ mammary glands but not in the glands from *Socs3*$^{+/fl}$ (Figure 2B) or *Socs3*$^{-/fl}$ mice (data not shown), both of which displayed well-ordered alveolar structures with apoptotic cells visible within their lumens. By day 3 of involution, the lobuloalveolar structures in the mutant mammary glands had largely been remodelled, resulting in glands that were typical of mice that had completed involution (Figure 2B).

To determine whether the accelerated involution evident in *Socs3*-deficient mammary glands was due to increased apoptosis, TUNEL assays were performed in mammary gland sections. A significant increase in TUNEL-positive cells was seen at day 1 (4.6±1.6% in *WAPiCre*;*Socs3*$^{-/fl}$ versus 1.0±0.2% in control mammary glands) and day 2 (7.9±1.1% in *WAPiCre*;*Socs3*$^{-/fl}$ versus 1.9±0.1% in control

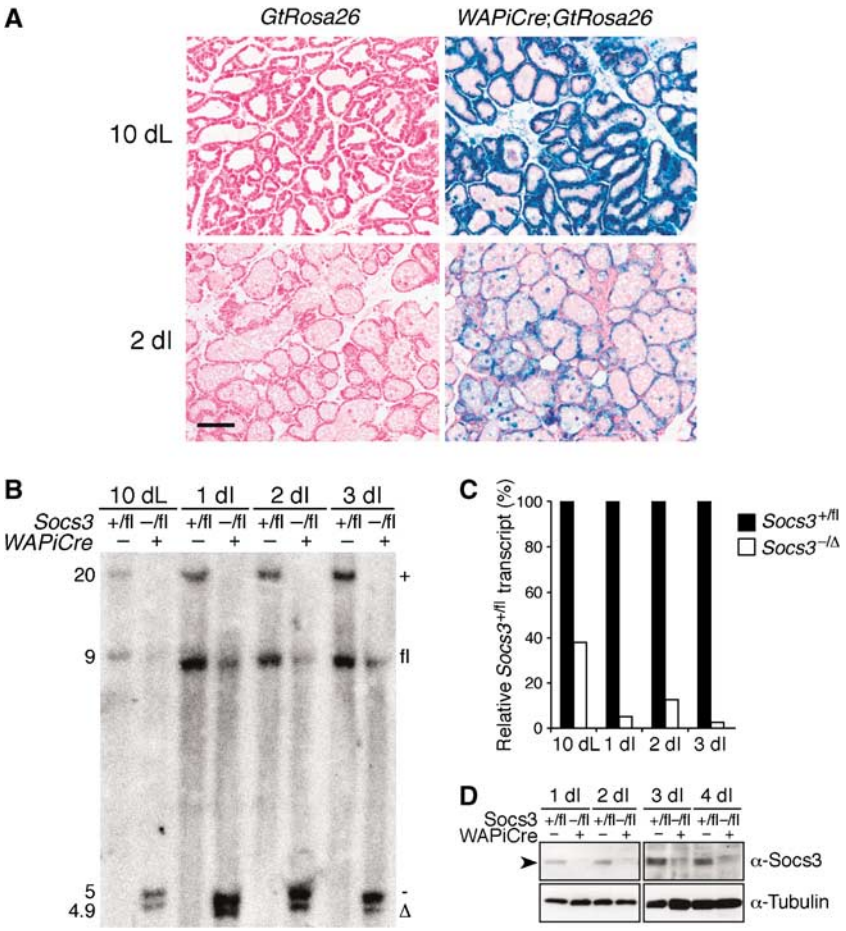

Figure 1 Targeted deletion of *Socs3* in alveolar cells by *WAPiCre*. (**A**) Small portions of the thoracic and inguinal mammary glands from *GtRosa26* mice or *WAPiCre*;*GtRosa26* bitransgenic mice were analysed at day 10 of lactation (L) and day 2 of involution (I) by staining for β-galactosidase activity. Scale bar, 100 μm. (**B**) Genomic DNA derived from $Socs3^{+/fl}$ and *WAPiCre*;$Socs3^{-/fl}$ mammary glands at day 10 of lactation (L) and days 1, 2 and 3 of involution (I) was digested with *Bam*HI. Membranes were hybridised with a 5′ *Socs3* probe that distinguishes the wild-type (+, 20 kb), conditional (fl, 9 kb), null (−, 5 kb) and excised (Δ, 4.9 kb) *Socs3* alleles. (**C**) The expression of *Socs3* transcripts relative to *Hprt* was determined by real-time PCR analysis of RNA derived from $Socs3^{+/fl}$ and *WAPiCre*;$Socs3^{-/fl}$ (depicted as $Socs3^{-/\Delta}$) mice (2–3) at day 10 of lactation (L) and days 1–3 of involution (I) performed in duplicate. (**D**) Mammary gland protein lysates (50 μg) were analysed by Western blotting using an anti-Socs3 antibody and reprobed with anti-tubulin to control for protein loading.

mammary glands) of involution (Figure 3A). In late involution (days 4 and 6), no difference in the number of TUNEL-positive cells was detected between *WAPiCre*;$Socs3^{-/fl}$ and $Socs3^{+/fl}$ glands. Thus, *Socs3* deficiency profoundly accelerates the rate of apoptosis of alveolar cells during the initial phase of involution.

Mammary epithelial cell proliferation was assessed in involuting mammary glands following bromodeoxyuridine (BrdU) injection and immunostaining. Cell proliferation was elevated at least six-fold in *WAPiCre*;$Socs3^{-/fl}$ glands at day 4 of involution compared to control glands (5.5±0.9% in *WAPiCre*;$Socs3^{-/fl}$ versus 0.7±0.4% in $Socs3^{+/fl}$ glands), whereas no difference was found at day 2 (4.3±0.6% in *WAPiCre*;$Socs3^{-/fl}$ versus 4.9±1.9% in $Socs3^{+/fl}$ glands) and day 6 (5.0±1.2% in *WAPiCre*;$Socs3^{-/fl}$ versus 3.4±1.1% in $Socs3^{+/fl}$ glands) of involution (Supplementary Figure 3). These results indicate that *Socs3* deficiency results in increased epithelial cell proliferation during the second but not the first phase of involution. This may represent a compensatory response to the precocious apoptosis that ensues in *Socs3*-deficient glands or reflect a more direct activity of a Socs3-regulated pathway that acts in late involution.

Increased levels of Bax and Bak in Socs3-deficient glands at the onset of involution

Given that *Socs3* deficiency promotes apoptosis of lobulo-alveoli, we examined the expression of members of the Bcl-2 family (Cory *et al*, 2003). Expression of the pro-survival family members Mcl-1 (Figure 3B), Bcl-X_L and Bcl-2 (data not shown) was not altered in *Socs3*-deficient glands relative to $Socs3^{+/fl}$ control glands, as detected by Western blot analysis. It is noteworthy that Bcl-X_L levels also did not change in *Stat3*-deficient mammary glands (Chapman *et al*, 1999). In contrast, expression of the pro-apoptotic regulators Bax and Bak was significantly increased in *WAPiCre*;$Socs3^{-/fl}$ mammary glands at days 1 and 2 of involution (Figure 3B), relative to control glands. In addition, the level of *Bim* mRNA was elevated in *Socs3*-deficient glands at day 1 of involution (Figure 3B). It is therefore likely that Bax, Bak and Bim directly contribute to the increased epithelial apoptosis in *Socs3*-deficient glands.

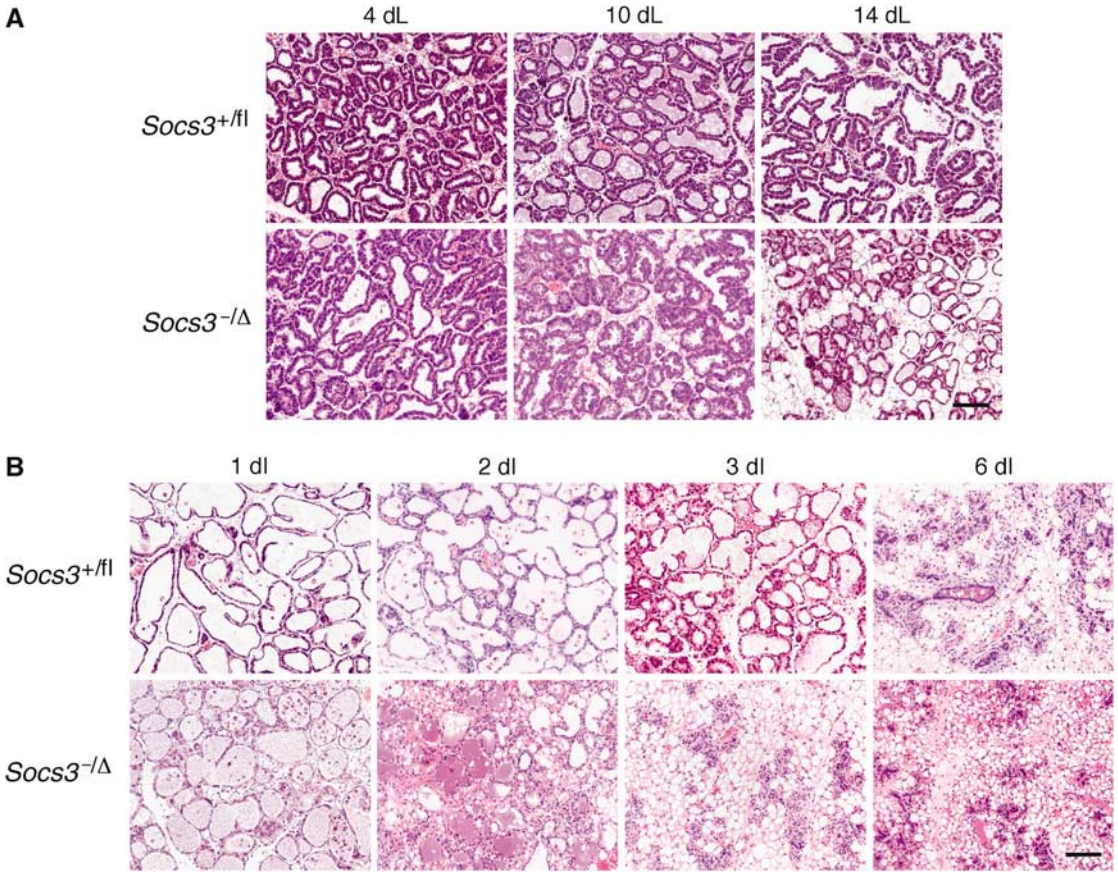

Figure 2 Conditional deletion of *Socs3* in alveolar cells results in accelerated involution. (**A**) Sections of mammary glands from control $Socs3^{+/fl}$ and *Socs3*-deficient mice at days 4, 10 and 14 of lactation (L). Scale bar, 100 μm. (**B**) Sections from $Socs3^{+/fl}$ and *WAPiCre*;$Socs3^{-/fl}$ mice at days 1, 2, 3 and 6 of involution (I). Scale bar, 100 μm.

Perturbed activation of matrix metalloproteinases in Socs3-deficient mammary glands

The second phase of mammary gland involution is characterised by the activation of MMPs, which remodel the gland through degradation of the extracellular matrix (ECM) (reviewed in Mott and Werb, 2004). Gelatinase A (MMP-2) and gelatinase B (MMP-9) are activated during involution and degrade collagen, gelatin and type IV collagen within the ECM. Gelatin zymography of control glands revealed activation of MMP-2 (60 kDa) at day 3 of involution, with high levels of both the latent (pro-MMP-2; 68 kDa) and active forms evident by day 4 (Figure 3C). In comparison, *WAPiCre*;$Socs3^{-/fl}$ glands exhibited activated MMP-2 at day 2 of involution, with high levels of the latent and activated forms at day 3 (Figure 3C). By day 4 of involution (Figure 3C), similar levels were apparent in mutant and control glands. An increase in MMP-9 protein (109 kDa), representing both pro- and active forms, was also observed in *Socs3*-deficient glands at days 1 and 2 of involution (Figure 3C).

MMP activity is inactivated by a small family of inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) (Lafleur *et al*, 2003a), and the ratio of MMP to TIMP expression is critical for tissue remodelling during involution. To examine whether TIMP levels changed in *Socs3*-deficient mammary glands, we determined *Timp-3* expression by real-time PCR analysis. A two- to four-fold reduction in *Timp-3* expression was observed in *WAPiCre*;$Socs3^{-/fl}$ mammary glands throughout involution relative to that in $Socs3^{+/fl}$ mice (Figure 3D). The premature activation of MMP-2 and MMP-9 and decrease in *Timp-3* mRNA expression are compatible with the accelerated tissue remodelling that occurs in *Socs3*-deficient mammary glands (Figure 2B). Furthermore, accelerated apoptosis was observed in *Timp-3*-deficient mammary glands (Fata *et al*, 2001).

Socs3 deficiency leads to increased Stat3 activation in involuting mammary glands and hyper-responsiveness to LIF in Socs3-null mammary epithelial cells

Stat3 phosphorylation is negligible during lactation, but increases at the onset of involution (Liu *et al*, 1996; Philp *et al*, 1996). *WAPiCre;*$Socs3^{-/fl}$ mammary glands displayed substantially elevated levels of Stat3 phosphorylation at days 10 and 14 of lactation, relative to that in control mammary glands (Figure 4A). During involution, higher levels of activated Stat3 were evident in *Socs3*-deficient glands at days 2 and 3, whereas by day 4 they were comparable to that seen in control mammary glands. These data suggest that the increased levels of activated Stat3 in *Socs3*-deficient glands during established lactation (day 10) and early involution trigger apoptosis.

To explore whether the kinetics of Stat3 activation by cytokines was altered in the absence of *Socs3*, we generated mammary epithelial cell (MEC) lines lacking *Socs3*. Primary

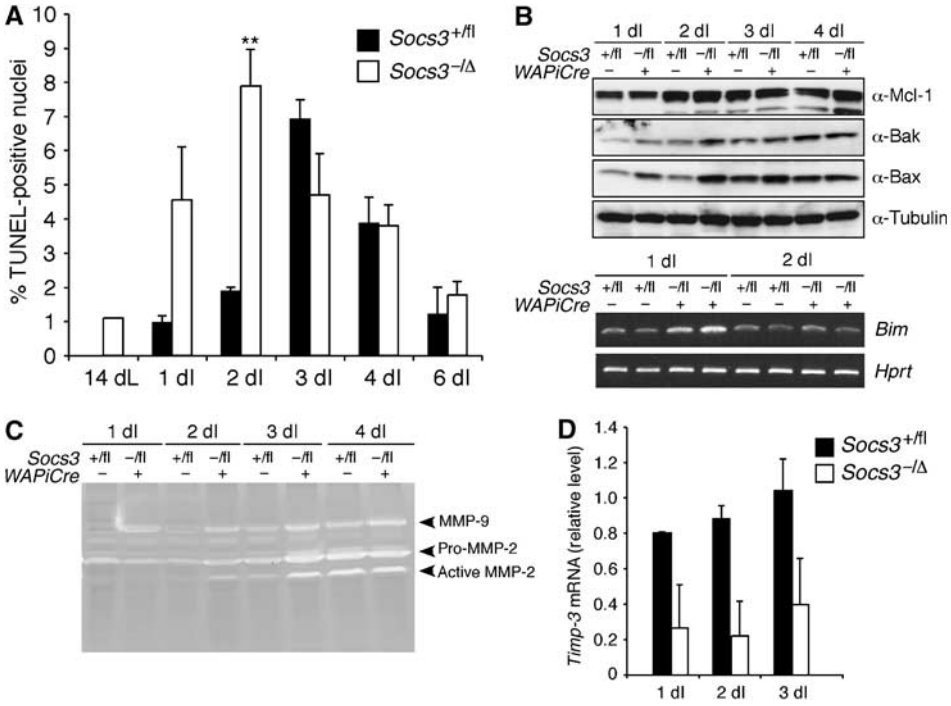

Figure 3 Loss of *Socs3* results in increased epithelial apoptosis during involution. (**A**) TUNEL assays were performed on mammary glands from three mice for each time point. Error bars represent standard error of the mean; $^{**}P<0.05$. (**B**) Top: Western blot analysis of protein lysates derived from $Socs3^{+/fl}$ and *WAPiCre*;$Socs3^{-/fl}$ mammary glands at days 1, 2, 3 and 4 of involution (I), using antibodies specific for Mcl-1, Bak and Bax. Expression of tubulin served as a control for protein loading. Bottom: The level of *Bim* transcripts was determined by reverse transcription–PCR, and products resolved by electrophoresis. (**C**) Gelatin zymography was used to detect MMP-2 and MMP-9 activity. The latent (68 kDa) and activated (60 kDa) forms of MMP-2, and total MMP-9 protein (109 kDa) are indicated. (**D**) Decreased *Timp-3* mRNA expression in *Socs3*-deficient mammary glands during the first stage of involution, as determined by real-time PCR analysis of *Timp-3* mRNA expression in glands from $Socs3^{+/fl}$ and *WAPiCre*;$Socs3^{-/fl}$ ($Socs3^{-/\Delta}$) mice, at days 1, 2 and 3 of involution. Results represent the mean data from at least two mice. Error bars represent standard error of the mean.

MEC cultures were established from $Socs3^{-/fl}$ mid-pregnant mammary glands and then transduced with an E6/E7-expressing retrovirus to immortalise the cells, followed by a *Cre*-recombinase-expressing retrovirus (Krempler *et al*, 2002) to excise the conditional allele (Supplementary Figure 4A). $Socs3^{+/fl}$ and $Socs3^{-/\Delta}$ cells were pulsed with LIF and Stat3 activation was analysed by Western blotting. Stimulation with LIF resulted in the rapid activation of Stat3 in both *Socs3*-deficient and control cells, but the intensity and duration of Stat3 phosphorylation were significantly enhanced in $Socs3^{-/\Delta}$ cells (Figure 4B). No *Socs3* mRNA was detectable in the *Socs3*-null cells in contrast to control lines (Supplementary Figure 4B). Although LIF has also been shown to signal through the MAP kinase pathway, similar patterns of ERK1/2 phosphorylation were observed in $Socs3^{+/fl}$ and $Socs3^{-/\Delta}$ cells (Figure 4B). Prolonged Stat3 activation was also observed in $Socs3^{-/\Delta}$ MECs when stimulated with IL-6 (data not shown). Taken together, these data suggest that Socs3 is a critical negative regulator of Stat3-mediated apoptosis in the mammary gland.

Akt activation is unaltered in glands lacking Socs3

Stat3 has been proposed to inhibit Akt-mediated survival by inducing expression of the PI3-K regulatory subunits p50α and p55α (Abell *et al*, 2005). We found that the level of activated Akt in $Socs3^{+/fl}$ and *WAPiCre*;$Socs3^{-/fl}$ mammary glands was similar at days 1 and 2 of involution (Figure 4C; data not shown), suggesting that Socs3 does not act on the Akt pathway in early involution. The increase in phosphorylated Akt in *WAPiCre*;$Socs3^{-/fl}$ glands around days 2–3 of involution (Figure 4C) may reflect a feedback response to the accelerated epithelial apoptosis that occurs in these mammary glands. In any event, no inhibition of Akt activation was evident in *Socs3*-deficient glands, indicating that Stat3 is acting through other pathways.

Elevated levels of c-myc and its potential target genes in Socs3-deficient glands

The *c-myc* gene is a direct target of Stat3 and is a critical regulator of both cell proliferation and apoptosis (Eisenman, 2001). We therefore examined whether increased *c-myc* expression accompanied enhanced Stat3 activation in *Socs3*-deficient mammary glands. Substantially higher levels of c-myc protein were apparent in *Socs3*-deficient mammary glands at days 1, 2, 4 and 6 of involution (Figure 5A) relative to $Socs3^{+/fl}$ glands. Higher levels of *c-myc* mRNA were also observed in *Socs3*-deficient glands using real-time PCR analysis (Figure 5B). Expression of c-myc was also elevated during lactation (days 10 and 14) in glands lacking *Socs3* (data not shown). Notably, *c-myc* mRNA expression was acutely induced following LIF treatment of *Socs3*-deficient mammary epithelial cells relative to that in control cells, as determined by real-time PCR analysis (Figure 5C). This observation is consistent with *c-myc* being a target of Stat3. The mechanism by which *c-myc* is downregulated at 1 h following LIF treatment is yet to be determined. Interestingly, levels of the pro-apoptotic proteins E2F-1 and p53, whose genes are either direct or indirect targets of c-myc,

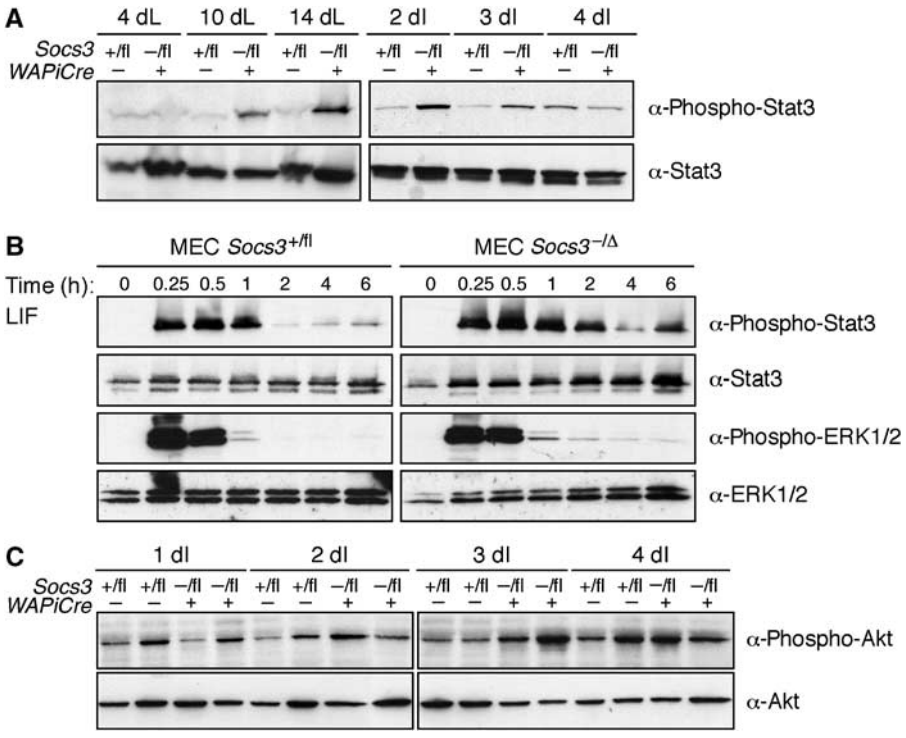

Figure 4 Increased levels of activated Stat3 but not Akt in *Socs3*-deficient mammary glands. (**A**) The levels of phospho-Stat3 and total Stat3 in $Socs3^{+/fl}$ control and *WAPiCre*;$Socs3^{-/fl}$ glands at days 4, 10 and 14 of lactation (L), and days 2, 3 and 4 of involution (I) were determined by Western blot analysis. (**B**) $Socs3^{+/fl}$ and $Socs3^{-/\Delta}$ immortalised mammary epithelial cell (MEC) clones were pulsed with 100 ng/ml leukaemia inhibitory factor (LIF) for 15 min and protein lysates (10 μg) were analysed by immunoblotting using antibodies specific for phospho-Stat3, total Stat3, phospho-ERK1/2 and total ERK1/2. (**C**) Levels of serine^{136} phosphorylated Akt and total Akt were assessed by Western blot analysis at the indicated time points. Two mice of each genotype are shown.

were markedly elevated in involuting mammary glands lacking *Socs3* (Figure 5D).

c-myc promotes alveolar apoptosis and premature involution in the mammary gland

The physiological role of the c-myc proto-oncogene in mammary gland involution could not be addressed in MMTV-*myc* or in MMTV-rtTA/TetO-MYC (MTB/TOM) bi-transgenic mice chronically induced with doxycycline, as they fail to nurture their pups (Stewart *et al*, 1984; Andres *et al*, 1988; Blakely *et al*, 2005). To circumvent this problem, we induced expression of the c-MYC transgene in bitransgenic MTB/TOM females (D'Cruz *et al*, 2001) with doxycycline at day 8 of lactation, before initiating involution on day 10 of lactation via forced weaning, as transient *c-MYC* expression has been shown to be compatible with maintenance of lactation (Blakely *et al*, 2005). Involution was found to be dramatically accelerated in MTB/TOM mice compared to glands from doxycycline-treated MTB mice, as shown histologically in Figure 6A. Furthermore, the number of TUNEL-positive cells in MTB/TOM mice at day 2 of involution was increased at least three-fold compared to control glands from MTB mice (Figure 6B).

The level of c-MYC induction following 4 days of doxycycline administration was determined by Northern blot analysis. Notably, the expression of *c-myc* was comparable between *Socs3*-deficient mammary glands during involution and MTB/TOM bitransgenic glands following doxycycline induction (Supplementary Figure 5), indicating that MYC is not highly overexpressed in the transgenic glands and providing further support that c-myc functions as a mediator of apoptosis in the post-lactational mammary gland. At day 2 of involution, MTB/TOM mice exhibited a marked yet variable increase in *c-MYC* expression compared with MTB control mice (Figure 6C). Moreover, elevated protein levels of Bax, E2F-1 and p53 were apparent in doxycycline-induced MTB/TOM glands at day 2 of involution (Figure 6D), consistent with their corresponding genes being potential downstream targets of c-MYC. No change in activated Stat3 was apparent in *c-MYC*-expressing mice.

Discussion

Here we demonstrate that *Socs3* is an important attenuator of pro-apoptotic pathways in the mammary gland during lactation and involution. Targeted deletion of *Socs3* in alveolar epithelial cells led to increased apoptosis during established lactation with evidence of tissue remodelling. More profoundly, deletion of *Socs3* resulted in increased apoptosis and accelerated involution. Premature degradation and remodelling of the extracellular matrix, which normally occurs in the second phase of involution, was also evident in *Socs3*-deficient mammary glands in early involution. Levels of activated Stat3 as well as activated MMP-2 were substantially elevated in *Socs3*-mutant glands. Our data implicate *c-myc*, a target gene of Stat3, as an effector of apoptosis induced by the Jak1–Stat3 pathway during involution.

Socs3 is not a key attenuator of Prl signalling in the mammary gland

Prl signal transduction via the JAK2/STAT5 pathway is critical for the establishment and maintenance of alveolar cells during pregnancy and lactation (Hennighausen and Robinson, 2005). Given that Prl induces the expression of *Socs3* in the mammary

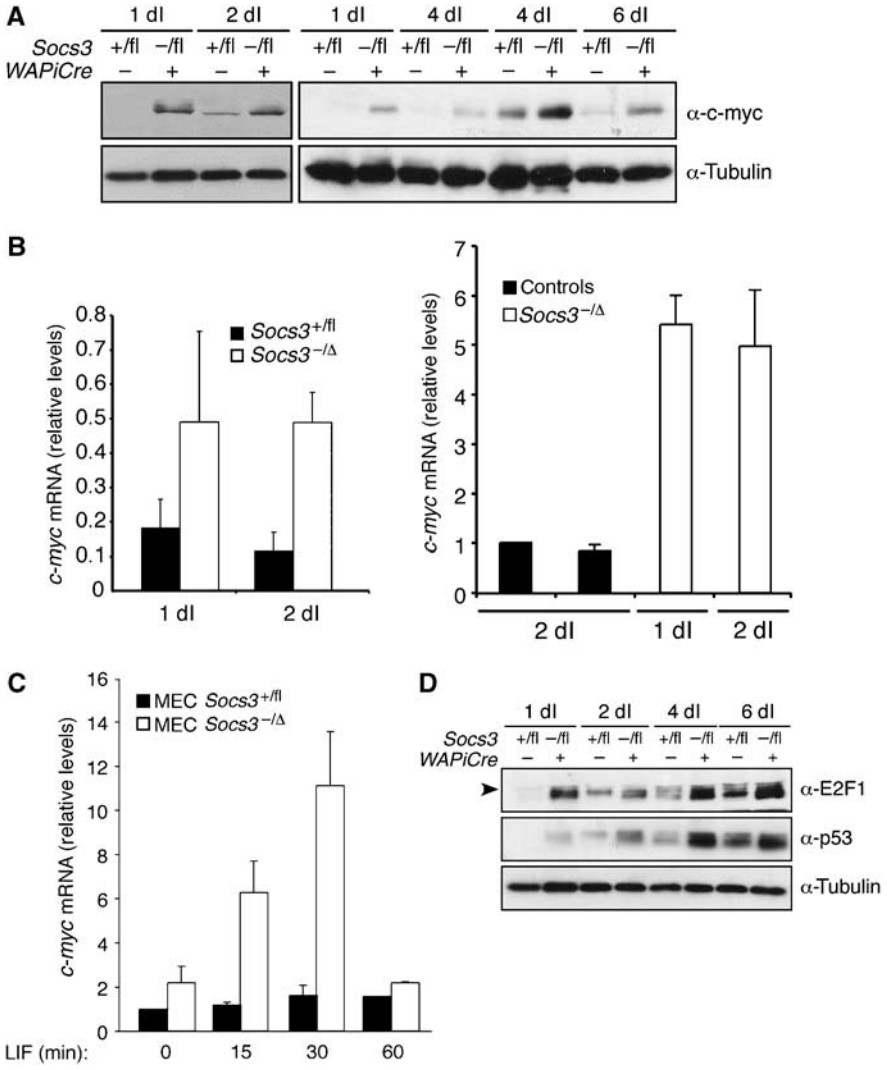

Figure 5 Increased expression of c-myc in the absence of *Socs3*. (**A**) The levels of c-myc in *Socs3*$^{+/fl}$ and *WAPiCre*;*Socs3*$^{-/fl}$ glands at days 1, 4 and 6 of involution (I) were assessed by Western blot analysis using a polyclonal anti-c-myc antibody (AbCam). Two mice for each genotype are shown for day 4 of involution. (**B**) The expression of *c-myc* transcripts relative to 18S rRNA was determined in individual *Socs3*$^{+/fl}$ control and *Socs3*$^{-/\Delta}$ mammary glands by real-time PCR analysis. Error bars represent the standard error of the mean for two to three independent experiments. (**C**) The expression of *c-myc* transcripts relative to 18S rRNA was determined in *Socs3*$^{+/fl}$ and *Socs3*$^{-/\Delta}$ immortalised mammary epithelial cell clones by real-time PCR analysis, following induction with 100 ng/ml LIF. Error bars represent standard error of the mean for two independent experiments. (**D**) The expression of likely c-myc apoptotic targets, E2F-1 and p53, was assessed in *Socs3*$^{+/fl}$ and *WAPiCre*;*Socs3*$^{-/fl}$ glands by Western blot analysis. The higher molecular weight bands represent phosphorylated forms of E2F-1 and the lower band corresponds to free E2F-1. Expression of tubulin served as a control.

gland and that overexpression of Socs3 attenuates PRLR-mediated responses *in vitro*, we examined the consequences of *Socs3* loss during pregnancy using β-*lactoglobulin-Cre* (Selbert *et al*, 1998) and *MMTV-Cre* (Wagner *et al*, 1997) transgenic mice. No histological differences were apparent between control and *Socs3*-deleted glands at days 10.5, 12.5, 16.5 and 18.5 of pregnancy, indicating that *Socs3* is not a key physiological attenuator of Prl signalling in the mammary gland (data not shown). Thus, Socs3 appears to have a distinct role from its closest SOCS family member (Socs1) in the mammary gland.

Socs3 is an important regulator of gp130 signalling in the mammary gland

Socs3 is a critical negative regulator of signalling mediated by the gp130 common receptor subunit in different cell types. Ablation of *Socs3* in hepatocytes and macrophages led to prolonged Stat3 activation and altered gp130 signalling in response to IL-6 (Croker *et al*, 2003; Lang *et al*, 2003; Yasukawa *et al*, 2003), and *Socs3*-null mice show aberrant LIF signalling (Takahashi *et al*, 2003; Robb *et al*, 2005). Our data support a role for *Socs3* as an important regulator of gp130 signalling in the mammary gland. The current model suggests that Socs3 binds with high affinity to the phosphorylated Y757 residue of the gp130 receptor chain, thus diminishing Stat3 activation (Nicholson *et al*, 2000; Schmitz *et al*, 2000). In agreement, augmented levels of phosphorylated Stat3 were observed in *Socs3*-deficient mammary glands, and prolonged Stat3 activation occurred in MECs lacking *Socs3* upon stimulation with either LIF or IL-6.

Other IL-6-type cytokines may contribute to accelerated involution

There is considerable evidence for gp130–Stat3 signalling playing a major role in mammary gland involution, but it is

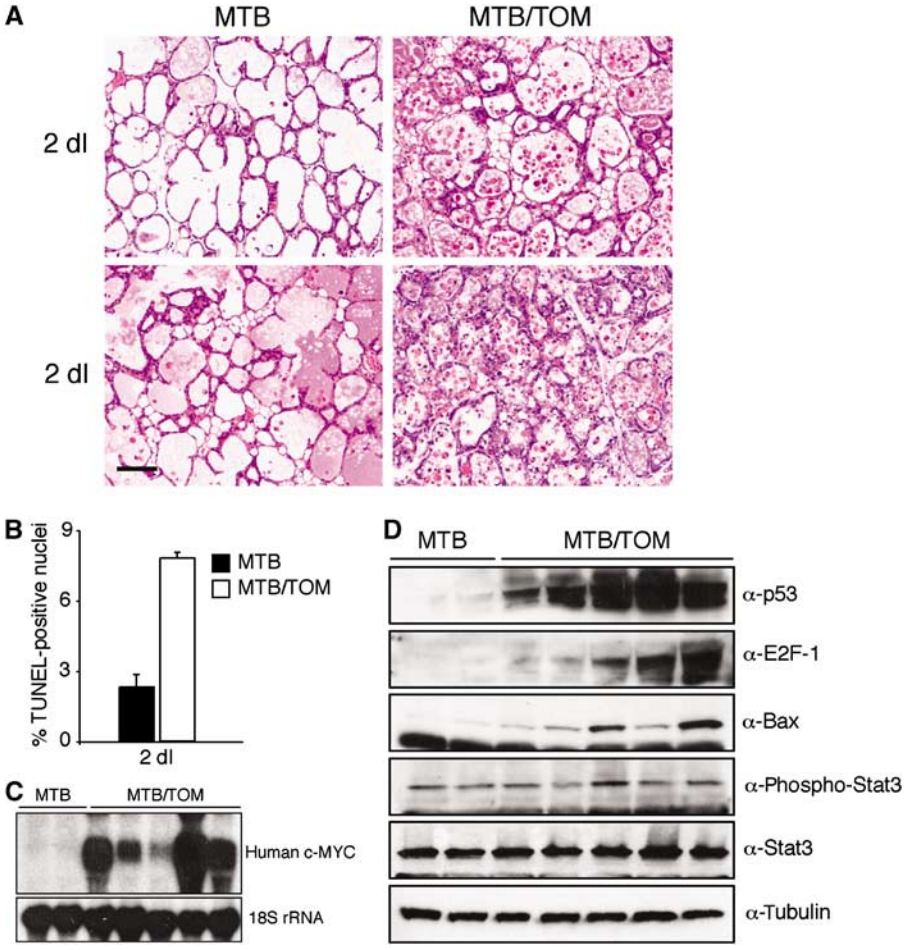

Figure 6 c-myc promotes apoptosis of mammary epithelial cells during involution. (**A**) Mammary gland sections from MTB and MTB/TOM mice at 2 days of involution (I) following treatment with doxycycline (1 mg/ml) for 4 days (day 8 of lactation to day 2 of involution). Two mice of each genotype are shown. Scale bar, 100 μm. (**B**) TUNEL assays were performed on mammary glands from 2 to 4 mice of each genotype. Error bars represent standard error of the mean. (**C**) Northern blot analysis of total RNA from mammary glands taken from independent MTB (#26, 29) and MTB/TOM mice (#66, 65, 69, 133, 122) following treatment with doxycycline for 4 days (day 8 of lactation to day 2 of involution). The filter was hybridised sequentially with a human *c-MYC* cDNA probe, specific for the transgene, and an 18S rRNA oligonucleotide probe, to control for the level of RNA. (**D**) Mammary gland protein lysates from doxycycline-treated MTB and MTB/TOM mice analysed at day 2 of involution were subjected to Western blot analysis using specific antibodies against p53, E2F-1, Bax, phospho-Stat3 and total Stat3. Expression of tubulin served as a control for protein loading.

likely that more than one cytokine is involved in this process. Mammary glands in *gp130*- and *Stat3*-deficient mice, and *LIF*- and *IL-6*-null mice, all exhibit a delay in involution, but differences exist between the affected pathways. It seems unlikely that hyper-responsiveness to LIF fully accounts for the phenotype observed in mammary glands lacking *Socs3*, as Akt signalling plays a prominent role in LIF-mediated activation of Stat3 and apoptosis (Zhao *et al*, 2002, 2004; Kritikou *et al*, 2003), but not in *Socs3*-deficient glands undergoing involution. These data suggest that Socs3 may attenuate signalling by other cytokine-family members during involution. Oncostatin M (OSM) induces heterodimerisation of gp130 with the OSM-specific β receptor (OSMR) subunit, resulting in activation of the JAK/STAT and MAP kinase signalling pathways (Heinrich *et al*, 2003). Interestingly, *Socs3* is induced by OSM, and OSMR has recently been identified as a Stat3 target in KIM-2 cells (Clarkson *et al*, 2006; Stross *et al*, 2006). OSMR signalling has also been implicated in tissue remodelling (Richards *et al*, 1993), suggesting that it may regulate mammary gland remodelling during involution. Indeed, *OSMR*-knockout mice exhibit increased liver destruction, in part due to augmented MMP-9 activity and reduced expression of the *TIMP-1* and *TIMP-2* genes (Nakamura *et al*, 2004).

c-myc appears to be a central effector of apoptosis in mammary glands lacking Socs3

The *c-myc* gene has emerged as a key target of Stat3 in the involuting mammary gland and a central pro-apoptotic regulator during this process. Stat3 has been shown to directly activate *c-myc* gene transcription in a number of different contexts (Kiuchi *et al*, 1999; Shirogane *et al*, 1999; Bowman *et al*, 2001; Cartwright *et al*, 2005). In the mammary gland, *c-myc* expression is induced at the onset of involution, consistent with it representing a Stat3 target gene in mammary epithelium (Blakely *et al*, 2005). Mammary glands from both *Socs3*-deficient and *c-MYC* transgenic mice exhibited a marked increase in apoptosis and accelerated involution. No change in the level of activated Stat3 occurred in *c-MYC* transgenic mice, compatible with Stat3 lying upstream of c-MYC. Premature tissue remodelling was more pronounced

in *Socs3*-deficient glands, consistent with Stat3 regulating multiple target genes in addition to *c-myc*.

A number of potential pro-apoptotic targets of the c-myc transcription factor have been identified, such as Bax, E2F-1, p53 and Bim (Reisman *et al*, 1993; Mitchell *et al*, 2000; Tanaka *et al*, 2002; Egle *et al*, 2004). The expression of Bax was considerably elevated in both *Socs3*-deficient mammary glands and *c-MYC* transgenic mice. These observations are consistent with *bax*-deficient mice exhibiting reduced mammary epithelial apoptosis (Schorr *et al*, 1999). Elevated levels of E2F-1 and p53 were also evident in involuting mammary glands from *Socs3*-deficient and c-*MYC* transgenic mice.

In summary, our data implicate *Socs3* in the regulation of a signalling network that controls the survival of mammary epithelium (Figure 7). *Socs3* acts as a negative regulator of Jak1–Stat3 signalling during involution, with Stat3 as a central mediator. The *c-myc* proto-oncogene, a target gene of Stat3, may act at a nodal point in controlling apoptosis during involution. Perturbed *c-myc* expression, in turn, is likely to activate (either directly or indirectly) the expression of pro-apoptotic genes including E2F-1, p53 and Bax, which leads to premature apoptosis and involution in mammary glands lacking *Socs3*.

Materials and methods

Mouse strains

All animal experiments were conducted according to the Melbourne Health Research Directorate Animal Ethics Committee guidelines. The *Socs3*-null and conditionally targeted *Socs3* mice have been described previously (Roberts *et al*, 2001; Croker *et al*, 2003). Mice expressing the *WAPiCre* transgene (Wintermantel *et al*, 2002) were initially bred onto a background heterozygous for the *Socs3* null allele ($Socs3^{+/-}$) and then mated with $Socs3^{fl/fl}$ mice to produce mice containing one *Cre*-excised allele ($Socs3^{\Delta}$) with either a wild-type or a null *Socs3* allele. Mouse tail DNA was genotyped for wild-type (+) null (−) or conditional (fl) *Socs3* alleles by PCR using the following primers:

5′-GAGTTTTCTCTGGGCGTCCTCCTAG-3′,
5′-TGGTACTCGCTTTTGGAGCTGAA-3′ and
5′-GATAACTGCCGTCACTCCAACG-3′. *WAPiCre* transgenic mice were maintained on an FVB genetic background. *GtRosa26* mice (Soriano, 1999) were generously provided by Dr P Soriano. The generation of the MMTV-rtTA (MTB) transactivator and TetO-MYC (TOM) responder lines has been described previously (D'Cruz *et al*, 2001). Bitransgenic MTB/TOM and littermate MTB control mice were administered doxycycline (1 mg/ml; Sigma) in their drinking water to induce expression of the *MYC* transgene. For the involution studies, adult female mice with litters of at least 6 pups were maintained. Pups were removed after 10 days to initiate involution.

Histology and whole-mount staining for β-galactosidase activity

For histological examination, mammary glands were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in paraffin and sections (1.5 μm) prepared and stained by haematoxylin and eosin. Mammary tissue from *WAPiCre*;*GtRosa26* and *GtRosa26* mice was stained for β-galactosidase activity as described (Sum *et al*, 2005).

Northern blot and reverse transcription–PCR

Total RNA was isolated from mouse mammary glands using TRIzol reagent (Life Technologies) according to the manufacturer's instructions, and then treated with DNA-free™ Kit (Ambion). A human c-MYC cDNA restriction fragment was used to probe the Northern, followed by a ^{32}P-labelled 18S oligonucleotide. Primers used for RT–PCR of *Bim* from cDNA: forward 5′-TCAGGAACCTGAA GATCTG-3′ and reverse

5′-TCAATGCCTTCTCCATACCAGAC-3′. Quantitative RT–PCR was performed as previously described (Sutherland *et al*, 2004). Primer sequences were as follows: *Hprt* 5′-CACAGGACTAGAACACCTGC-3′ and 5′-GCTGGTGAAAAGGACCTCT-3′; *Socs3* 5′-GGCCACCCTCCAGC ATCTTTGTCG-3′ and 5′-GTGGCAGCTCCCCCTCCCCTCAG-3′; *Timp-3* 5′-TGACAGGGCGCGTGTATGAAGG-3′ and 5′-GTGGTAGCGGTAATT GAGGCC-3′; *c-myc* 5′-TGAGCCCCTAGTGCTGCAT-3′ and 5′-AGCCCG ACTCCGACCTCTT-3′; 18S rRNA 5′-TCGGAACTGAGGCCATGATT-3′ and 5′-CCTCCGACTTTCGTTCTTGATT-3′.

Derivation of Socs3-deficient mammary epithelial cells and cytokine stimulation

The thoracic and inguinal mammary glands were excised from day 12.5 pregnant $Socs3^{+/fl}$ and $Socs3^{-/fl}$ mice and MEC suspensions were prepared as described (Shackleton *et al*, 2006). MECs were cultured for 72 h prior to infection with a retrovirus expressing the human papilloma virus 16 proteins E6/E7 and a neomycin resistance cassette for immortalisation (kindly provided by D Galloway). Twenty-four hours post-infection, neomycin (200 μg/ml) was added to the cells, and selection continued for 14 days. To excise the floxed allele, immortalised epithelial cells were infected with a retrovirus expressing *Cre*- and puromycin-resistance cassette, generously provided by K-U Wagner (Krempler *et al*, 2002). Cells were selected in 1.5 μg/ml puromycin for 5 days. *Socs3* MEC clones were genotyped by PCR as described (Croker *et al*, 2003), as shown in Supplementary Figure 4.

$Socs3^{+/fl}$ and $Socs3^{-/\Delta}$ MEC clones (2×10^5) were plated in 6 cm^2 dishes and starved of serum overnight. The following day, cells were stimulated with LIF (100 ng/ml). After harvesting, cells were lysed directly in 100 μl RIPA buffer supplemented with Complete protease inhibitor (Roche), 10 mM NaF, 1 mM Na_3VO_4 and 1 mM PMSF.

Western blot analysis and antibodies

Mammary gland protein lysates were prepared by grinding the tissue in liquid nitrogen and solubilizing in 1% TEB (150 mM NaCl; 5 mM EDTA, 50 mM Tris (pH 7.5); 0.1% NP-40) supplemented with Complete protease inhibitor (Roche), 10 mM NaF, 1 mM Na_3VO_4 and 1 mM PMSF. Protein (50 μg) was resolved on polyacrylamide gels (Novex), before transfer to polyvinylidene

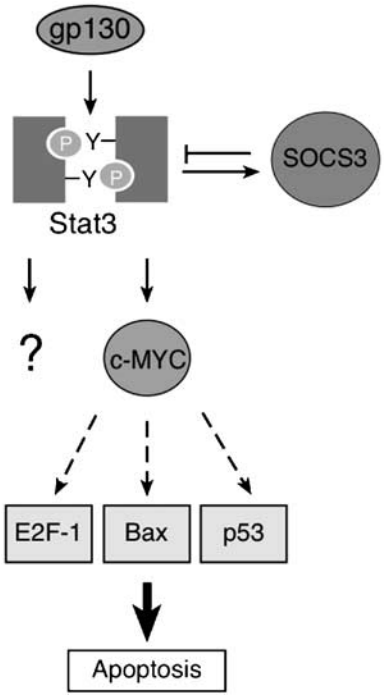

Figure 7 Proposed model by which *Socs3* mediates precocious cell death during involution in mammary glands lacking Socs3. Under normal conditions, Socs3 serves to attenuate Stat3 signal transduction and therefore c-myc induction. There may be other Stat3-regulated pathways that act during involution in *Socs3*-deficient mice.

difluoride membranes (Millipore). Non-specific binding of proteins to membranes was blocked by incubation in PBS containing 0.1% Tween 20 and either 5% skim milk or 5% skim milk and 1% casein, before incubation with the primary antibody. These included α-phospho-STAT3 (Tyr705), α-STAT3, α-phospho-p42/44 (Thr2020/Tyr204) and α-p42/44 from Cell Signaling Technologies, α-Bak (Ab-1; Calbiochem), α-Bax (2D2 and 5B7; Sigma), α-Mcl-1 (Rockland), α-c-myc (AbCam, ab11917; Santa Cruz, N-262), α-E2F-1 (Zymed Laboratories), α-SOCS-3 (Immuno-Biological Laboratories), α-p53 (DO-1, Santa Cruz), and α-tubulin mAb (Sigma). Membranes were then incubated with horseradish peroxidase-coupled secondary antibodies (Amersham Biosciences, Inc.) and developed by ECL (Amersham Biosciences, Inc.).

Zymography

Mammary gland protein lysate (40 μg) was mixed with an equal volume of SDS sample buffer (50 mM Tris–HCl pH 6.8, 1% SDS, 0.025% bromophenol blue and 10% glycerol) and resolved on a 10% polyacrylamide gel containing 1 mg/ml gelatin (Sigma) (Lafleur *et al*, 2003b). Following electrophoresis, the gels were washed twice in buffer A (50 mM Tris–HCl, pH 8.0; 5 mM $CaCl_2$; 2.5% Triton X-100), before incubation in buffer B (50 mM Tris–HCl, pH 7.5; 5 mM $CaCl_2$) overnight at 37°C. Gels were stained with 2.5 mg/ml Coomassie brilliant blue (R) dye (Bio-Rad) in 10% acetic acid; 10% isopropanol for 2–4 h, then destained in 10% acetic acid; 10% isopropanol for 30–60 min. Gelatinolytic activity appeared as a clear band on a blue background.

TUNEL and bromodeoxyuridine immunodetection

Apoptotic nuclei were detected in paraformaldehyde-fixed paraffin sections by TUNEL analysis using Terminal deoxynucleotidyl transferase (TdT; Promega) in the presence of biotinylated dUTP (Roche). Incorporated biotinylated dUTP was revealed by HRP-conjugated streptavidin (LSAB2; Dako), followed by detection with DAB. TUNEL-positive cells were scored by counting greater than 1000 epithelial nuclei in 10 random fields (400× magnification) from each gland.

Mice were injected with BrdU Cell Labelling Reagent (0.5 mg/10 g body weight; Amersham Biosciences Inc.) 1 h before tissue collection and immunodetection was carried out as described (Sum *et al*, 2005).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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