Development 140, 167-175 (2013) doi:10.1242/dev.085068

Tif1γ is essential for the terminal differentiation of mammary alveolar epithelial cells and for lactation through SMAD4 inhibition

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SUMMARY

Transforming growth factor β (TGFβ) is widely recognised as an important factor that regulates many steps of normal mammary gland (MG) development, including branching morphogenesis, functional differentiation and involution. Tif1γ has previously been reported to temporally and spatially control TGFβ signalling during early vertebrate development by exerting negative effects over SMAD4 availability. To evaluate the contribution of Tif1γ to MG development, we developed a Cre/LoxP system to specifically invalidate the *Tif1g* gene in mammary epithelial cells *in vivo*. *Tif1g*-null mammary gland development appeared to be normal and no defects were observed during the lifespan of virgin mice. However, a lactation defect was observed in mammary glands of *Tif1g*-null mice. We demonstrate that Tif1γ is essential for the terminal differentiation of alveolar epithelial cells at the end of pregnancy and to ensure lactation. Tif1γ appears to play a crucial role in the crosstalk between TGFβ and prolactin pathways by negatively regulating both PRL receptor expression and STAT5 phosphorylation, thereby impairing the subsequent transactivation of PRL target genes. Using HC11 cells as a model, we demonstrate that the effects of *Tif1g* knockdown on lactation depend on both SMAD4 and TGFβ. Interestingly, we found that the Tif1γ expression pattern in mammary epithelial cells is almost symmetrically opposite to that described for TGFβ. We propose that Tif1γ contributes to the repression of TGFβ activity during late pregnancy and prevents lactation by inhibiting SMAD4.

KEY WORDS: Tif1γ (Trim33), TGFβ, SMAD4, STAT5, Lactation, Mouse

INTRODUCTION

The development of the mammary gland (MG) is a complex process, beginning during fetal life and continuing until pregnancy. At birth, a rudimentary ductal structure forms within the fat pad. During puberty, rapid ductal growth and branching morphogenesis are induced by cyclic production of ovarian hormones. By contrast, during pregnancy, the MG is characterised by the development of lobuloalveolar structures along the existing ductal tree. At the end of pregnancy, alveolar epithelial cells are terminally differentiated and acquire the capacity to synthesise milk components (Hennighausen and Robinson, 2005). Various steroids, polypeptides, hormones and growth factors tightly control all the steps of mammary growth and development. Among them, the lactogenic hormone prolactin (PRL) and the transforming growth factor β (TGFβ) family members play crucial antagonistic roles in the lactation process.

The PRL polypeptide hormone is required for lobuloalveolar formation and functional differentiation of alveolar epithelial cells, allowing the MG to synthesise milk proteins. At parturition, the transition from late-pregnancy to lactation, referred to as the secretory step, is stimulated by a rise in PRL and a decrease in serum progesterone. Copious production of milk during lactation is stimulated and maintained by PRL, which induces expression of milk protein genes, including β-casein, α-lactalbumin and lipid biosynthetic enzymes. Prolactin also maintains the viability of mammary epithelial cells (MECs) throughout lactation and until weaning (Kelly et al., 1991; Guyette et al., 1979). At the beginning of involution, suckling stops and TGFβ fulfills its competing effect on PRL functions, inducing cell apoptosis and inhibition of milk protein expression (Jhappan et al., 1993; Nguyen and Pollard, 2000). Binding of PRL to its receptor activates Janus kinase 2 (JAK2) (Argetsinger et al., 1993; Campbell et al., 1994), which in turn phosphorylates STAT transcription factors (Ihle and Kerr, 1995). STAT5A and STAT5B, hereafter referred to as STAT5, are the main STAT proteins that transduce the effects of PRL in the mammary gland. Deletion of STAT5 causes an attenuation of mammary alveolar development and milk secretion (Hennighausen and Robinson, 2005). STAT5 also promotes cell-cycle progression and suppresses apoptosis, thus contributing to cellular transformation (Yu and Jove, 2004). Interestingly, it was recently demonstrated that STAT3 and STAT5 are activated in a high proportion of breast tumours (Diaz et al., 2006) and both have been shown to be mammary oncogenes in the mouse (Barbieri et al., 2010; Vafaizadeh et al., 2010).

During postnatal MG development and pregnancy, the three TGFβ isoforms (TGFβ1, TGFβ2 and TGFβ3) are abundantly expressed in mammary alveoli, ducts and fat pad. Upon parturition and during lactation, TGFβ is significantly downregulated, whereas all three isoforms are markedly upregulated during involution (Nguyen and Pollard, 2000). In addition to inhibition of ductal and

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Accepted 16 October 2012

branching morphogenesis during puberty, TGFβ also inhibits alveolar formation, disrupts the alveolar structure, inhibits synthesis of milk proteins and induces apoptosis after weaning (Bierie et al., 2009; Serra and Crowley, 2005).

Transcriptional intermediary factor 1γ (Tif1γ; also called ectodermin, Trim33, RFG7 or PTC7) has been recently implicated in the activities promoted by TGFβ superfamily pathways. Tif1γ is a member of the TIF family of transcriptional co-factors. The TIF family is characterised by an N-terminal RING-finger B-box coiled-coil (RBCC/TRIM) motif and a C-terminal bromodomain preceded by a PHD finger (Yan et al., 2004). One study indicates that Tif1γ acts as a co-factor for phosphorylated SMAD2/3 in competition with SMAD4 to promote an alternative SMAD4-independent TGFβ pathway (He et al., 2006). Others studies show that Tif1γ acts as a repressor of TGFβ superfamily responses through mono-ubiquitylation of SMAD4 (Levy et al., 2007; Dupont et al., 2009; Morsut et al., 2010). Recent data suggest a tumour suppressor role in different types of mouse and human tumours, including leukaemia, hepatocellular carcinoma and pancreatic cancer (Vincent et al., 2009; Aucagne et al., 2011; Herquel et al., 2011). By contrast, a recent study has demonstrated that overexpression of Tif1γ occurs during the early stages of colorectal carcinogenesis, suggesting a role in promoting colorectal cancer (Jain et al., 2011). We recently demonstrated that Tif1γ can function as a repressor of SMAD4 in the TGFβ-induced epithelial-to-mesenchymal transition (EMT) in human mammary epithelial cell lines (Hesling et al., 2011). Because EMT favours metastasis (Thiery et al., 2009), our data suggest that Tif1γ might interfere with MG tumour progression. These observations raise the important question of the involvement of Tif1γ in MG development and tumorigenesis, which we have addressed in this work.

As *Tif1g*-null mice are embryonic-lethal at day 9.5 and exhibit a dramatic developmental delay (Kim and Kaartinen, 2008), we used two *in vivo* Cre/LoxP systems that lead to conditional deletion of *Tif1g* in the mammary gland, allowing us to analyse the impact of *Tif1g* inactivation on MG development and function. Using these mouse models and an immortalized mouse epithelial cell line, we demonstrate that *Tif1g* is essential for the terminal proliferation of ducts and differentiation of alveolar epithelial cells. We also show that PRL-induced STAT5 phosphorylation is inhibited upon inactivation of *Tif1g*, an effect that depends on the TGFβ/SMAD pathway. Taking these results together, we propose that Tif1γ, as a negative regulator of SMAD4 function, could be a crucial factor that regulates the crosstalk between PRL and TGFβ signalling during MG development.

MATERIALS AND METHODS

Generation of *Tif1g*-deficient mice in the mammary gland

$Tif1g^{flox/flox}$ mice harbouring floxed exons 2-4 have been previously described (Morsut et al., 2010). These mice were mated with Mouse Mammary Tumor Virus-Cre (MMTV-Cre) [Tg(MMTV-cre)4-MamJ, stock 003553] or Whey Acidic Protein-Cre (WAP-Cre) [B6.Cg-Tg(WapCre)11738Mam, strain number 01XA8] transgenic mice to generate MMTV- and WAP-Cre/$Tif1g^{mammary\text{-}flox\text{-}deleted/+}$ ($Tif1g^{mfd/+}$). Heterozygous mice were then mated together to generate MMTV- and WAP-Cre/$Tif1g^{mfd/mfd}$, Cre/$Tif1g^{mfd/+}$ and Cre/$Tif1g^{+/+}$. This mating scheme allowed us to generate the MMTV- and WAP-Cre/$Tif1g^{+/+}$ control mice. To study lactation defects, MMTV- and WAP-Cre/$Tif1g^{mfd/mfd}$, $Tif1g^{mfd/+}$ and $Tif1g^{+/+}$ females were mated with wild-type males. Mice were housed and bred in the AniCan pathogen-free animal facility (Centre Léon Bérard, Lyon, France). Experiments were performed in accordance with the animal care guidelines of the European Union and French laws and were validated by the local Animal Ethics Evaluation Committee (CECCAPP).

Genotyping analysis

Offspring were genotyped by PCR performed on genomic tail DNA extracted using standard procedures. The genotype of *Tif1g* floxed mice was determined by multiplex PCR using three different primers in the same reaction (A, 5′-GGTAGTACTTGTATGGAGGT-3′; B, 5′-GGTAAG-TCAGCAAGAGCTCA-3′; and C, 5′-AGCTCTGGAGGAACGTCGTC-3′). The wild-type and floxed *Tif1g* alleles were detected using primers A and B located on either side of the LoxP insertion. These primers amplify a 498 bp fragment in wild-type mice and a 531 bp fragment from the floxed allele. The deleted allele was detected using primers C and B. This primer pair amplifies a 360 bp fragment from the deleted allele but yields no amplification products from the floxed and wild-type alleles. MMTV-Cre and WAP-Cre recombinase transgenes were also detected by PCR using primers 5′-TGCCACGACCAAGTGACAGC-3′ and 5′-CCAGGT-TACGGATATAGTTCATG-3′ located within Cre recombinase sequences. The MMTV-Cre and WAP-Cre transgenes produce a 675 bp fragment. All products were separated in 2% agarose gels.

Whole-mount and histological analysis

For each developmental stage, mice were sacrificed by cervical dislocation and the fourth inguinal gland was collected and fixed in 10% neutral buffered formalin for at least 2 hours. Haematoxylin-stained wholemounts were prepared as previously described (Robinson et al., 1991) and were examined for ductal outgrowth using a microscope-mounted camera. For histological analysis, dissected mammary glands were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned (5 μm), and stained with Haematoxylin and Eosin. Immunohistochemical analysis was performed as previously described (Razanajaona et al., 2007) using anti-Tif1γ (TIF-3E9, Euromedex) or anti-pSTAT5 (C11C5, Cell Signaling) antibodies. After washing in PBS, a biotinylated secondary antibody bound to a streptavidin-peroxidase conjugate (LSAB+ kit, Dako) was added. The bound antibody was revealed with 3,3-diaminobenzidine. Slides were counterstained with Haematoxylin after washing.

PRL quantification in serum

Intra-cardiac blood was collected from mice on day 2 of lactation. Sera were collected after blood coagulation followed by a 10-minute centrifugation at 500 ***g***. Serum prolactin (PRL) was quantified using the RayBio Mouse Prolactin ELISA kit according to the manufacturer's instructions (RayBiotech).

Quantitative RT-PCR analysis

Total RNA (1 μg) was used for cDNA synthesis using the SuperScript II Reverse Transcriptase system (Invitrogen). mRNA levels were quantified using the SYBR Green StepOne Plus Real Time PCR system (Applied Biosystems) and normalized relative to mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT). Quantification of gene expression was performed using the comparative ΔΔCT method. Primers used for each gene are: *Tif1g* and HPRT (Hesling et al., 2011); β-casein (5′-ACAGCTGCAGGCAGAGGAT-3′; 5′-GAATGTTGTGGAGTGGCAGG-3′); α-lactalbumin, (5′-TCTGTGGCATCTCCTGTGACAAGT-3′; 5′-TGGGCTTGTAGGCTTTCCAGTAGT-3′); mPRLR total (5′-GTGG-AATCCTGGGTCAGATG-3′; 5′-GGGCCACTGGTTTTGTAGTC-3′); and mPRLR long isoform (5′-ATAAAAGGATTTGATACTCATCTG-CTAGAG-3′; 5′-TGTCATCCACTTCCAAGAACTCC-3′).

In vitro differentiation of HC11

Mouse mammary epithelial cell line HC11 was routinely maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml bovine insulin (Sigma-Aldrich) and 10 ng/ml human recombinant epidermal growth factor (EGF; Upstate Biotechnology). To induce cell differentiation, confluent cell cultures, deprived of EGF for 24 hours, were treated with lactogenic hormone mix (DIP: 1 μM dexamethasone (Sigma-Aldrich), 5 μg/ml insulin and 5 μg/ml mouse recombinant PRL) for the indicated time. Mouse PRL was produced following in-house routine protocols and its activity was validated using a classical STAT5 reporter luciferase gene assay (Bernichtein et al., 2003). In some experiments, cells were pretreated with TGFβ1 (5 ng/ml, Peprotech) or SB-431542 (10 μM, TGFβ type I receptor

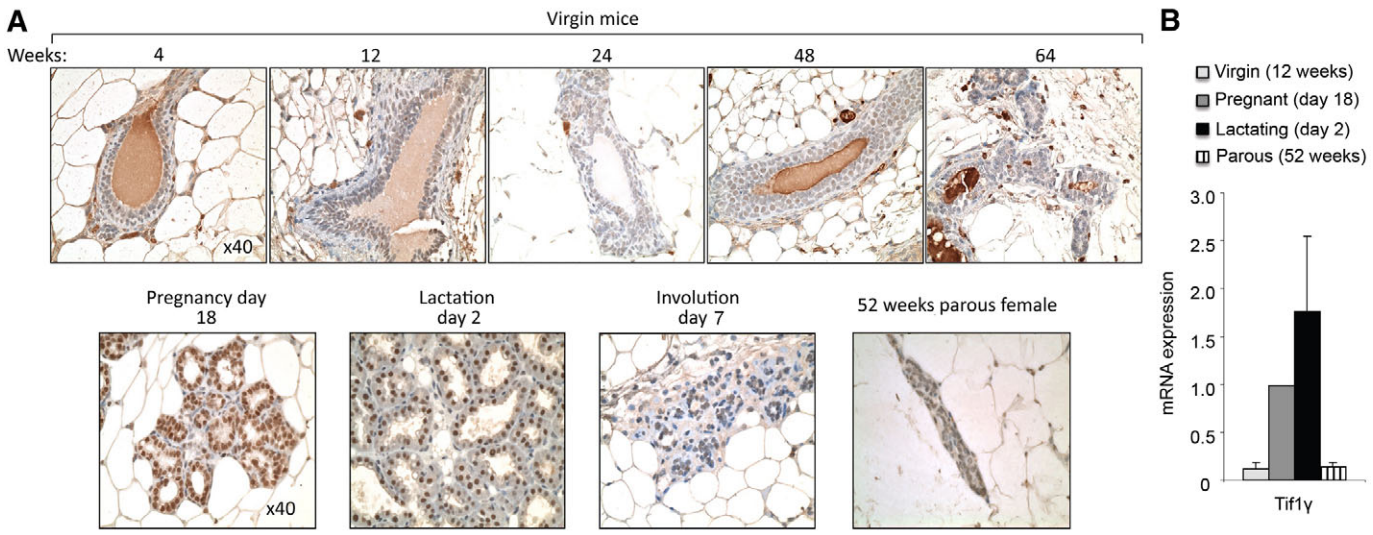

Fig. 1. *Tif1g* expression in epithelial ducts during mammary development. (**A**) Immunohistochemical localization of Tif1γ was performed for each developmental stage from non-transgenic control mice. Pregnancy, lactation and involution were observed in 20-week-old mice (bottom row). Photographs are representative of at least five mice per stage. (**B**) Total RNA was extracted from MGs collected at the indicated developmental stages. *Tif1g* expression was determined by RT-qPCR. Values were normalized to the amount of HPRT mRNA (±s.d.) and expressed relative to the value obtained in pregnant mice.

inhibitor; Sigma) for 24 hours before adjunction of PRL. Cells treated with medium containing dexamethasone and insulin (DI) without PRL were used as controls. mRNAs and/or proteins were then extracted and purified.

For knockdown experiments, 2.5×10^5 cells were transfected with 5 nM siRNA and 0.5 μl/ml lipofectamine RNAiMax (Invitrogen). For double silencing (Tif1γ and SMAD4), cells were transfected with 10 nM siRNAs (5 nM of each siRNA). Controls cells were transfected by 10 nM Scramble siRNA (Qiagen). Cells were plated overnight in antibiotic-free medium and cultured in complete fresh medium until confluence. Two distinct mouse Tif1γ siRNA sequences were used, yielding similar results: siRNA#1 (5′-CCGUCUGUUACAGCAAUAGAAUUAA-3′ and 5′-UUAAUUCUAUUGCUGUAACAGACGG-3′); siRNA#2 (5′-UACCUCUAUUGUCACGAAUUA-3′ and 5′-UAAUUCGUGACAAUAGAGGUA-3′). The mouse SMAD4 siRNA sequences were 5′-CCCACAGCCUUUAGACUGA-3′ and 5′-UCAGUCUAAAGGCUGUGGG-3′. For rescue experiments, 2.5×10^5 cells were transfected with 5 nM siRNA#1 targeting TIF1γ and 0.5 μl/ml lipofectamine RNAiMax (Invitrogen). Cells were plated overnight in antibiotic-free medium and then infected with the pLVX-based lentiviral vector expressing human *TIF1G*. Cells were grown to confluence in complete fresh medium before measuring STAT5 phosphorylation levels.

Immunoblot analysis

Cells were lysed using RIPA buffer. Cell lysates containing equal amounts of proteins were subjected to SDS-PAGE, transferred to a PVDF membrane and probed with antibodies. Proteins were visualized using an ECL kit (Millipore). Phospho-STAT5 (Tyr694) was detected using rabbit monoclonal antibody C11C5 (Cell Signaling). Total STAT5 was semi-quantified using rabbit polyclonal antibody C17 (Sc-835; Santa Cruz). Mouse tubulin was used as a loading control; tubulin was detected using mouse polyclonal antibody DM1A (Sigma).

RESULTS

Expression of Tif1γ during mammary gland development

To determine Tif1γ expression patterns, we first studied its cellular distribution by immunohistochemistry during developmental stages 4, 12, 24, 32, 48 and 64 weeks of virgin mice. MGs collected from late-pregnancy (day 18), lactating (day 2), involuting (day 2) and 52-week old parous mice were also studied. A weak immunoreactivity of the anti-Tif1γ antibody was observed in the mammary epithelia of virgin mice at all developmental stages (Fig. 1A, upper panel). A dramatic increase in Tif1γ expression became apparent in MEC nuclei during pregnancy, peaking during lactation before declining by day 7 of involution (Fig. 1A, lower panel). Comparable staining was observed in 52-week old parous glands. This pattern of expression strongly suggests that Tif1γ is active predominantly during late-pregnancy and lactation, as confirmed by measurements of Tif1γ mRNA levels detected at the indicated stages (Fig. 1B). Intermediary Tif1γ staining indicates that, after parturition, Tif1γ expression does not return to the basal level observed in 48- and 64-week old nulliparous mice. Taken together, these results establish that high Tif1γ expression levels are a hallmark of late-pregnancy and lactation, suggesting a role in at least some aspects of MG development.

Loss of *Tif1g* does not affect duct development in virgin mice

Is Tif1γ a direct actor of MG development, differentiation and, in addition, of tumorigenesis? To address this question, we generated two transgenic lines selectively invalidated for *Tif1g* in MECs: MMTV-Cre/*Tif1g* and WAP-Cre/*Tif1g*, which express Cre recombinase prior to lactation. Heterozygous (Cre/$Tif1g^{mfd/+}$) mice were mated to generate litters in which the three resulting genotypes (Cre/$Tif1g^{mfd/mfd}$, Cre/$Tif1g^{mfd/+}$ and Cre/$Tif1g^{+/+}$) were found among females (supplementary material Fig. S1A). Because *Tif1g* expression is maximal during lactation (Fig. 1B), we verified the deletion of *Tif1g* during the first lactation of MMTV- and WAP-Cre/*Tif1g* females. For the MMTV-Cre/*Tif1g* line, most MECs of homozygous mutants were devoid of *Tif1g* and intermediate staining was observed in MECs of heterozygous mice compared with control (supplementary material Fig. S1B). In the WAP-Cre/*Tif1g* line, mosaic expression was observed in MECs of Cre/$Tif1g^{mfd/mfd}$ females, whereas intermediate expression was found in MECs of WAP-Cre/$Tif1g^{mfd/+}$ relative to the control.

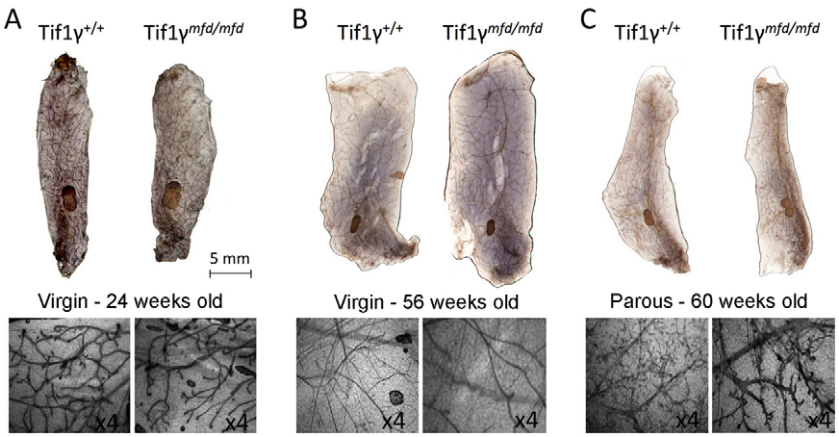

Fig. 2. Knockout of *Tif1g* in mammary epithelium does not affect virgin mammary development. (**A-C**) Wholemounts of control and MMTV-Cre/*Tif1g*$^{mfd/mfd}$ MGs collected from 24-week-old virgin mice (A), 56-week-old virgin mice (B) and parous 60-week-old mice (C). Images are representative of five mice from each genotype. The lower photographs show a higher magnification (×4) of the whole mounts.

Cre/*Tif1g*$^{mfd/mfd}$ mice developed normally, with body sizes comparable with those of Cre/*Tif1g*$^{+/+}$ and Cre/*Tif1g*$^{mfd/+}$, for both MMTV-Cre and WAP-Cre/*Tif1g* lines. To investigate whether *Tif1g* deletion in MECs might promote mammary cancer, virgin and parous mice of the MMTV-Cre/*Tif1g* line were kept alive until 56 and 60 weeks, respectively. Mammary glands from five mutant and five control virgin mice were compared at 24 and 56 weeks after birth using whole-mount analysis. For both stages, no histological difference in MG morphogenesis was observed between mutants and controls, suggesting that *Tif1g* deletion does not influence ductal elongation and branching during puberty (Fig. 2A,B). No tumour or neoplastic lesions could be detected. Ductal trees of MGs collected from 10 mutants (five homozygous and five heterozygous) of 60-week-old parous females were similar to those observed for the five control mice. Once again, no tumours were observed (Fig. 2C). These results show that conditional *Tif1g* knockout neither affects duct development in virgin mice nor favours spontaneous mammary tumour formation.

Lack of *Tif1g* in the mammary epithelium induces lactation failure

In the course of this analysis, we observed that the offspring of homozygous MMTV-Cre/*Tif1g*$^{mfd/mfd}$ females died just after birth and had a very small quantity of milk in their stomach (data not shown). We investigated this phenomenon further by crossing a total of 10 MMTV-Cre/*Tif1g* females of each genotype with wild-type males. Offspring from controls and heterozygous females were fed and gained weight normally, confirming that the presence of the MMTV-Cre transgene in the MG had no effect on lactation efficiency, as previously reported (Robinson and Hennighausen, 2011). By contrast, pups of the 10 Cre/*Tif1g*$^{mfd/mfd}$ females, although born with normal size and weight, all died by 2 days postpartum. Importantly, if pups were exchanged between mutant and control mothers, pups born of a mutant survived when fed by a foster mother. These results show that MG-specific *Tif1g* deletion affects lactation in the mutant mothers. As observed with the MMTV-Cre/*Tif1g* line, pups from WAP-Cre/*Tif1g* homozygous mutants also died within a few days of birth, whereas pups from control mothers survived. Because, in the WAP-Cre line, conditional gene inactivation occurs at mid-pregnancy, i.e. prior to lactation, (Wagner et al., 1997), our results demonstrate that *Tif1g* invalidation just prior to lactation is sufficient to induce lactation failure. These observations strongly suggest that *Tif1g* is required for the latest steps of MG differentiation, such as lactation, which are predominantly controlled by signalling of the lactogenic hormone prolactin.

The absence of *Tif1g* results in a lack of lobuloalveolar development

To characterise lactation failure in more detail, MGs from mutant and control mice from both lines were compared 1 day after parturition and before the death of pups to conserve secretory activation in response to suckling. Whole-mount analysis clearly demonstrated significant morphological differences between mutants and controls at this stage. First, MGs from controls appeared to be optically opaque, revealing the presence of a large amount of milk in lobuloalveolar units, whereas MGs from homozygous mutants retained a clear appearance (Fig. 3A). At higher magnification, we observed that the alveolar-like units of homozygous mutants were dramatically condensed and less developed than those observed in controls, possibly because MECs never acquired the capacity to produce milk components (Fig. 3B). Of note, underdeveloped alveoli were also observed in MGs from WAP-Cre-Tif1g homozygous mutants, confirming that defects in ductal terminal growth and differentiation at parturition are likely to be responsible for lactation failure. Interestingly, we also observed similar but smaller defects in lactating MGs of heterozygous females in both lines, suggesting a correlation between efficiency of lactation and nuclear *Tif1g* expression. Although we observed a similar number of lobuloalveolar units in controls and mutant mice (Fig. 3D), they failed to expand and differentiate in mutant mice. Histological sections of MGs from control lactating mice revealed the presence of large lobuloalveolar units with expended lumina as a consequence of milk secretion, whereas lobuloalveolar units from homozygous mutants contained fewer acini without lactation (Fig. 3). Moreover, controls and heterozygous alveolar cells actively secreted milk, as judged by the presence of lipid droplets in the cytoplasm. By contrast, lobuloalveolar units of homozygous mutant mice exhibited no lipid droplets, confirming the absence of secretory activity. We conclude from these observations that *Tif1g* invalidation results in a failure of terminal proliferation of ducts and differentiation of alveolar epithelial cells, thus impairing lactation.

Pituitary and serum prolactin levels are not altered in *Tif1g* mutant mice

Tif1g inactivation-induced lactation failure could result from a defect in production or secretion of the lactogenic hormone prolactin. The staining intensity of PRL in pituitary glands of homozygous mutants was comparable with that observed in control mice (supplementary material Fig. S2A), demonstrating that PRL synthesis is not altered by *Tif1g* deletion in the MG and/or by the

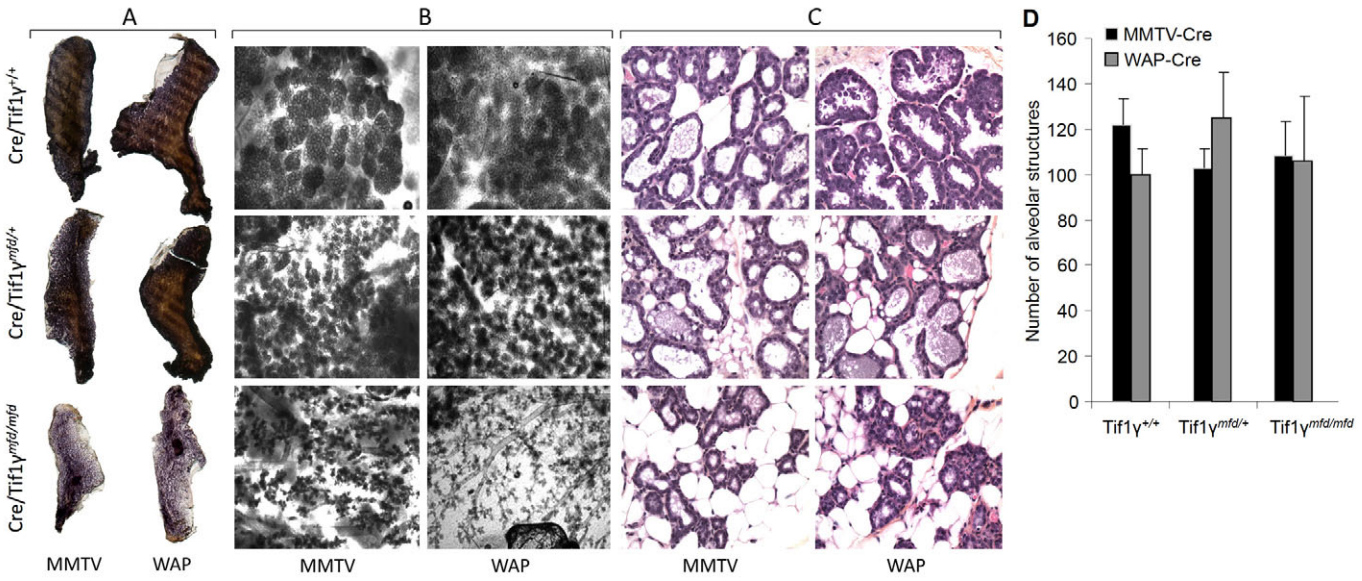

Fig. 3. Lack of Tif1γ in the mammary epithelium leads to alveolar defects during lactation. The fourth inguinal gland was collected 2 days after parturition (10 mice per genotype for the MMTV-Cre/*Tif1g* line and five mice per genotype for the WAP-Cre/*Tif1g* line). (**A**) Whole-mount analysis. (**B**) Wholemounts. (**C**) Histological sections from mammary glands stained with haematoxylin phloxine saffron are also shown for each genotype. Images are representative of 10 mice per genotype for the MMTV-Cre/*Tif1g* line and five mice per genotype for the WAP-Cre/*Tif1g* line. (**D**) The number of alveolar structures were counted on wholemounts of the fourth inguinal gland collected 2 days after parturition for MMTV and WAP lines. Data are mean±s.d.

MMTV-Cre transgene. The amount of PRL was also quantified in the sera of virgin and lactating mice. As expected, a strong increase in PRL concentration was found in sera of lactating mice compared with virgins, but PRL levels were comparable in the three genotypes (supplementary material Fig. S2B). We conclude from these observations that lactation failure does not result from impaired production or secretion of PRL in homozygous mutant mice.

Lack of *Tif1g* strongly decreases expression of genes encoding milk proteins

As PRL levels are not affected by *Tif1g* deletion, we further checked by qRT-PCR the expression of two PRL target genes, β-casein and α-lactalbumin (*Csn2* and *Lalba*), which encode milk proteins. Although both genes were expressed in MGs of control lactating mice, mRNA expression was reduced in heterozygous mutants and was barely detectable in MGs of homozygous mutants (Fig. 4A). These results confirm that inactivation of *Tif1g* in mutant MGs inhibits the production of milk components. Further support for this observation was obtained by using the mouse mammary epithelial HC11 cell line, which can be differentiated upon lactogenic hormone stimulation, as measured by the induction of milk protein synthesis, including β-casein synthesis (Fig. 4B). β-Casein mRNA expression was strongly induced in control cells treated with PRL (si-ctrl, DIP lane). This induction was strongly counteracted by TGFβ1 (si-ctrl, DIPT lane), confirming the antagonistic actions of PRL and TGFβ on target genes encoding milk proteins (Mieth et al., 1990; Cocolakis et al., 2008). We next transiently silenced *Tif1g* expression in HC11 cells by siRNA-mediated knockdown. In this and subsequent experiments, siRNA#1 was used for silencing (see Materials and methods). This, interestingly, also led to the inhibition of β-casein induction upon PRL treatment (si-1 *Tif1g*, DIP lane). Because this effect is independent of PRL levels per se, it might be due to alterations in PRL receptor (PRLR) expression upon *Tif1g* inactivation. We tested this possibility by measuring expression of PRLR in mutant mice by qRT-PCR, using primers allowing detection of either the long PRLR isoform or all PRLR isoforms. High levels of mRNA encoding PRLR were detected in control mice. Mutant mice also expressed PRLR mRNA, albeit at a much lower level than control mice (Fig. 5A). We further confirmed this observation in the HC11 model. Whereas Tif1γ inactivation had no effect on PRLR gene expression in HC11 cells, TGFβ-induced downregulation of all

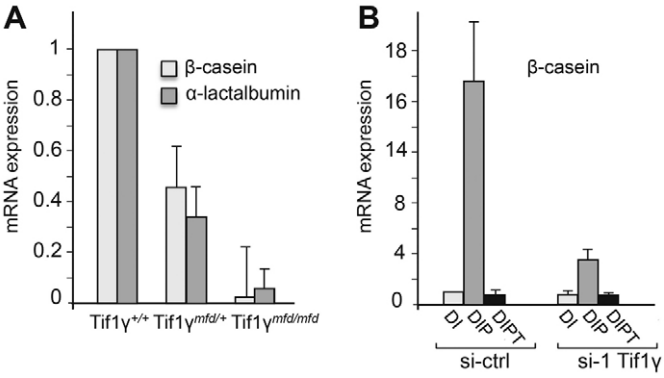

Fig. 4. Lack of *Tif1g* in mammary epithelial cells induces a strong decrease in the expression of milk proteins. (**A**) Total RNA was extracted from MGs collected 2 days after parturition and expression of β-casein, α-lactalbumin and PRLR was determined by RT-qPCR. Values were normalized to the amount of HPRT mRNA and expressed relative to the value obtained in control mice (±s.d.). (**B**) HC11 cells transiently silenced for *Tif1g* (si-1 Tif) or transfected with a control siRNA (si-ctrl) were treated with dexamethasone (D), insulin (I), prolactin (P) and TGFβ1 (T) (as indicated) for 48 hours. Total mRNA was then extracted and RT-qPCR was performed as in A.

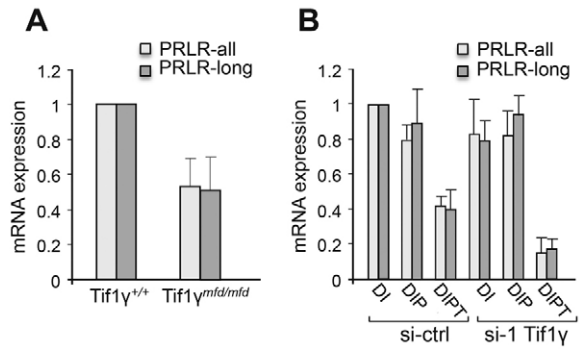

Fig. 5. TGFβ induces a strong decrease in the expression of PRLR. (**A**,**B**) Total RNA was extracted from MGs collected 2 days after parturition (A) and from HC11 cells transiently silenced for *Tif1g* (si-1 Tif) or transfected with a control siRNA (si-ctrl) and treated with dexamethasone (D), insulin (I), prolactin (P) and TGFβ (T) (as indicated) for 48 hours (B). Expression of PRLR (PRL-R all, all isoforms; PRLR-long, long isoform) was determined by RT-qPCR as described in Fig. 4. Data are mean±s.d.

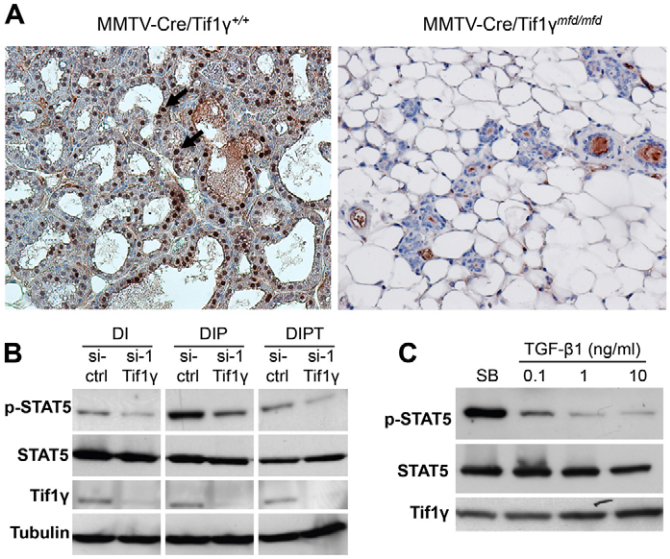

Fig. 6. STAT5-phosphorylation is altered by *Tif1g* deletion. (**A**) Phosphorylation of STAT5 was assayed by immunohistochemistry on MGs collected 2 days after parturition from MMTV-Cre/*Tifg*$^{+/+}$ and MMTV-Cre/*Tifg*$^{mfd/mfd}$ mice. Arrows show the nuclear staining of STAT5 in control mice that almost disappears in mutant alveolar-like units. Images are representative of three mice per genotype. (**B**) HC11 cells, silenced for *Tif1g*, were treated with dexamethasone (D), insulin (I) and prolactin (P) (as indicated) for 30 minutes. DIPT indicates TGFβ1 pre-treatment for 24 hours followed by D, I and P treatment for 30 minutes. STAT5 phosphorylation and expression were assayed by immunoblotting. Mouse tubulin was used as loading control and efficiency of the *Tif1g* siRNA-mediated knockdown (si-1 Tif) was verified as shown. (**C**) HC11 cells were treated for 24 hours with the TGFβ type I receptor inhibitor SB-431542 (SB) or the indicated amount of TGFβ1 followed by D, I and P treatment (as indicated) for 30 minutes. STAT5 phosphorylation and expression were assayed as described in B.

PRLR species was significantly enhanced by Tif1g silencing (Fig. 5B). Taken together, these results demonstrate that TGFβ decreases PRLR expression and that the siRNA-mediated knockdown of *Tif1g* counteracts PRL signalling, as TGFβ does, consistent with the known role of Tif1γ as an antagonist of TGFβ activity (Dupont et al., 2009).

Loss of *Tif1g* decreases STAT5 phosphorylation

Because transcription of milk protein genes requires PRLR-mediated phosphorylation of STAT5 by JAK2 (Ihle and Kerr, 1995), we checked phosphorylation of STAT5 on Tyr^{694} in MGs collected 2 days after parturition of MMTV-Cre/Tif1g$^{mfd/mfd}$ mice. A dramatic decrease in STAT5 phosphorylation was observed in homozygous mutant MGs compared with controls (Fig. 6A). We further confirmed this observation in the HC11 model. As expected, treatment with PRL for 30 minutes induced a strong increase in STAT5 phosphorylation (Fig. 6B, DIP si-ctrl), which, importantly, was not observed in *Tif1g* siRNA-mediated knockdown cells (Fig. 6B, DIP si-1 *Tif1g*). We verified that another siRNA targeting *Tif1g* (si-2 *Tif1g*) yielded similar results and that ectopic expression of *Tif1g* could indeed rescue the effect of *Tif1g* inactivation on STAT5 phosphorylation (supplementary material Fig. S3). Taken together, these results support the hypothesis that loss of *Tif1g* affects PRLR-mediated phosphorylation of STAT5, a possibility we examined in more detail (described below).

The effect of *Tif1g* silencing on STAT5 phosphorylation depends on SMAD4 and TGFβ pathways

TGFβ had previously been shown to inhibit PRL/STAT5 signalling via mechanisms that involve SMAD proteins (Cocolakis et al., 2008; Wu et al., 2008) and Tif1γ is known to inhibit the SMAD4-dependent TGFβ pathway (Dupont et al., 2009; Morsut et al., 2010). Accordingly, we observed that co-treatment with TGFβ1 counteracted STAT5 phosphorylation induced by PRL (Fig. 6B, DIPT lanes), while *Tif1g* siRNA-mediated knockdown potentiated the inhibitory effect of TGFβ (Fig. 6B, DIPT si-1 *Tif1g*). Moreover, increasing the concentration of TGFβ in the culture medium led to a dose-dependent decrease of PRL-induced STAT5 phosphorylation, without affecting STAT5 expression (Fig. 6C). The known inhibitory activity of Tif1γ on the SMAD4-dependent TGFβ pathway (Dupont et al., 2009) led us to suspect that inhibition of STAT5 phosphorylation by *Tif1g* siRNA-mediated knockdown might be attributed to TGFβ and mediated by SMAD4. We tested this hypothesis using HC11 cells transiently silenced for *Tif1g* or *Smad4* and treated for 30 minutes with PRL. Results shown in Fig. 7A confirm that TGFβ1 abolished PRL-induced STAT5 phosphorylation. Inversely, siRNA-mediated knockdown of SMAD4 (si-S4) fully counteracted inhibition by TGFβ, demonstrating the role of SMAD4 in this process. This conclusion is further reinforced by the observation that STAT5 phosphorylation could still be induced by PRL in cells co-silenced for *Tif1g* and *Smad4* expression (Fig. 7B).

In all these experiments carried out in HC11 cells, we observed that Tif1γ inactivation could decrease STAT5 phosphorylation even in the absence of TGFβ treatment, leading us to ask whether this effect was independent of TGFβ. We tested this possibility by treating HC11 cells with SB-431542 before induction by PRL. Interestingly, this TGFβ type I receptor inhibitor fully abolished the effect of *Tif1g* depletion (Fig. 7C), demonstrating its dependence on TGFβ signalling. Of note, the small amount of bovine TGFβ (present in serum) and/or autocrine mouse TGFβ (produced by cells) seemed to be

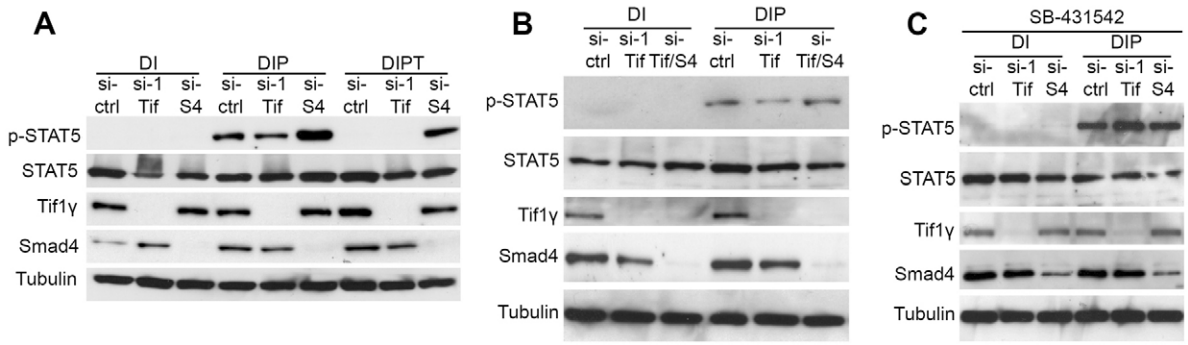

Fig. 7. Tif1γ acts as a repressor of SMAD4-mediated effects on STAT5 phosphorylation. (**A**) HC11 cells, silenced for *Tif1g* (si-1 Tif) or *Smad4* (si-S4), were treated with dexamethasone (D), insulin (I) and prolactin (P) (as indicated) for 30 minutes. Scrambled siRNAs (si-ctrl) were used as controls. DIPT indicates TGFβ1 pre-treatment for 24 hours followed by D, I and P treatment for 30 minutes. (**B**) HC11 cells, silenced for *Tif1g* (si-1 Tif) or both *Tif1g* and *Smad4* (si-Tif/S4), were treated with D, I and P (as indicated) for 30 minutes. (**C**) HC11 cells were silenced for *Tif1g* (si-1 Tif) or SMAD4 (si-S4) and pre-treated with SB-431542 for 24 hours before D, I and P treatment (as indicated) for 30 minutes. STAT5 phosphorylation and expression were assayed by immunoblotting. The efficiency of *Tif1g* and *Smad4* knockdown was verified as shown. Mouse tubulin was used as loading control.

sufficient to activate SMAD signalling in cells in which *Tif1g* was inactivated. This might explain the decrease in STAT5 phosphorylation observed in cells depleted of *Tif1g* (Fig. 6B; Fig. 7A,B, DIP si-1 *Tif1g*) and the increase in STAT5 phosphorylation in cells depleted of *Smad4* (Fig. 7A, DIP si-S4).

These results clearly demonstrate that the inhibition of STAT5 phosphorylation associated with the failure of lactogenic differentiation observed in *Tif1g*-depleted cells depends on TGFβ stimulation and is mediated by SMAD4. They also highlight the antagonistic roles played by SMAD4 and Tif1γ in TGFβ signalling and by the TGFβ and PRL pathways. As discussed below, these observations are consistent with the inverse correlation that exists between the pattern of expression of *Tif1g* during MG development (Fig. 1A) and that of TGFβ, the three isoforms of which show dramatically reduced expression in lactating tissue (Robinson et al., 1991).

DISCUSSION

TGFβ is widely recognised as an important factor that regulates normal mammary gland development and also plays a role in breast cancer. Use of genetically engineered mouse models has helped show that TGFβ regulates many steps of normal mammary gland development, including branching morphogenesis, functional differentiation, cell-lineage decisions and involution (Serra and Crowley, 2005). Tif1γ has been described as a negative regulator of the TGFβ pathway through mono-ubiquitylation of SMAD4 (Dupont et al., 2009). To establish more clearly the possible contributions of Tif1γ in mammary gland development and, possibly, tumorigenesis, we developed a Cre/LoxP system to specifically inactivate the *Tif1g* gene in mammary epithelial cells of mice. We demonstrate that Tif1γ is essential for terminal duct proliferation and differentiation of alveolar epithelial cells at the end of pregnancy (Fig. 3), but does not affect duct development in virgin mice (Fig. 2). Moreover, the lactation failure observed in MMTV-Cre/*Tif1g*$^{mfd/mfd}$ mice is also observed in WAP-Cre/*Tif1g*$^{mfd/mfd}$ mice, in which gene invalidation occurs prior to lactation (Fig. 3), clearly pointing to a key function of Tif1γ during late pregnancy and lactation. Of note is the absence of any detectable effects on tumorigenesis upon *Tif1g* gene inactivation (Fig. 2 and see below).

Importantly, we found an unexpected Tif1γ expression pattern in the epithelium of developing MGs. The protein appeared to be weakly expressed in MECs over the life of virgin mice, whereas a peak of expression was observed during pregnancy and lactation (Fig. 1). Strikingly, this pattern is almost symmetrically opposed to that described for the three isoforms of TGFβ. All three isoforms are downregulated during pregnancy and lactation, whereas they are upregulated during mammary gland involution to suppress lactation (Robinson et al., 1991; Bierie et al., 2009). These results concur with previous work showing that Tif1γ temporally and spatially controls TGFβ signalling during early vertebrate development by reducing SMAD4 availability (Morsut et al., 2010).

This report uncovers a novel mechanism whereby the TGFβ pathway could control PRL signalling. It has previously been shown that TGFβ signalling – via SMAD3/4 – could block the association of STAT5 with its co-activator CBP (CREB-binding protein), leading to inhibition of the transactivation of STAT5 target genes (Cocolakis et al., 2008). We note that the authors of this study did not report a modification of PRL-induced STAT5 phosphorylation by TGFβ. Inversely, and in agreement with our data, Wu et al. clearly demonstrated that TGFβ inhibits both PRL-induced tyrosine phosphorylation of STAT5 and suppression of β-casein expression in primary mouse MECs (Wu et al., 2008), but did not implicate SMAD4 in this inhibition. These discrepancies may be due to the absence of prolonged pre-treatment (24 hours) with TGFβ by Cocolakis et al. Our own results demonstrate that PRL-induced STAT5 phosphorylation decreases upon depletion of Tif1γ, leading to the silencing of PRL target genes (β-casein and α-lactalbumin). We also show that TGFβ-induced downregulation of PRLR is enhanced by *Tif1g* silencing. It is important to note the absence of a decrease in PRLR expression in HC11 cells in which *Tif1g* was depleted, supporting the conclusion that loss of *Tif1g* can also affect PRL-mediated phosphorylation of STAT5 independently of its effect on PRLR levels. Tif1γ can control STAT5 phosphorylation by negatively regulating SMAD4 functions during late-pregnancy and lactation. We show that, as TGFβ does, *Tif1g* knockdown counteracts PRL signalling. This is consistent with the observation that mice overexpressing WAP promoter-driven TGFβ display increased apoptosis in pregnant and lactating mammary

glands that is associated with decreased lobuloaveoli formation and decreased lactation (Jhappan et al., 1993), and that mice lacking SMAD4 in MECs show alveolar hyperplasia and transdifferentiation (Li et al., 2003).

None of the MMTV- or WAP-Cre-*Tif1g*$^{mfd/mfd}$ females, including old multiparous ones, studied as part of our work developed spontaneous mammary tumours. This is consistent with a previous study showing that the conditional knockout of SMAD4 in MG induces cell proliferation, alveolar hyperplasia and transdifferentiation of mammary epithelial cells into squamous epithelial cells (Li et al., 2003), but contrasts with reports that loss of *Tif1g* promotes tumorigenesis in blood, pancreas and liver (Vincent et al., 2009; Aucagne et al., 2011; Herquel et al., 2011; Hesling et al., 2011). To reconcile our apparently contradictory results, we propose that, in these tissues, the potent tumour suppressive effects of Tif1γ could be independent of SMAD4, as shown in the pancreas (Vincent et al., 2012), and may be due to its ubiquitin ligase activity on other substrates. Conversely, in the mammary gland, as well as in the embryo, SMAD4 activity has to be tightly controlled to spatially and temporally restrict TGFβ signalling (Morsut et al., 2010), leading to significantly different effects in response to conditional *Tif1g* inactivation.

The lactation defect observed in *Tif1g*-null mammary glands hence stands out as the most significant phenotype resulting from the absence of *Tif1g*. We conclude from our results that it is due to a loss of balance in the crosstalk between TGFβ/SMADs and PRL/STAT5 pathways in mammary epithelial cells. According to this hypothesis, Tif1γ should play a critical role in the crosstalk between TGFβ and PRL pathways by negatively regulating SMAD4 functions to control STAT5 phosphorylation and subsequent transactivation of PRL target genes. We propose that *Tif1g* expression during late-pregnancy and lactation contributes to the inhibition of residual TGFβ activity by inhibiting SMAD4 during these crucial stages of MG development.

Acknowledgements
We thank S. Léon, A. Colombe and C. Languilaire for excellent technical assistance. The authors thank Dr Emmanuel Käs (LBME, CNRS/Université Paul Sabatier, Toulouse) for help in writing this manuscript. We are grateful to the staff of the animal facility (AniCan, Centre Léon Bérard and ALECS, SFR Lyon-Est, CNRS UMS3453-INSERM US7) for excellent support during the mouse studies.

Funding
This work was supported by grants from Inserm, CNRS, the Ligue Nationale contre le Cancer (Savoie) and Association pour la Recherche sur le Cancer. C.H. is supported by fellowships from the Centre Léon Bérard.

Competing interests statement
The authors declare no competing financial interests.

Author contributions
C.H., J.L., P.G., A.P., G.G. and R.R. designed experiments and analysed data. C.H. performed breeding. C.H., J.L., L.F., D.B. and V.G. performed colony genotyping, real-time PCR, differentiation of HC11 cells, immunoblot, whole-mount and histological studies with P.G., I.T., N.P. and I.M. R.L. designed and generated the *Tif1g* knockout alleles; C.H., J.L. and R.R. wrote the paper.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.085068/-/DC1

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