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

Cédric Hesling1*, Jonathan Lopez1,2,3,†, Laurent Fattet1, Philippe Gonzalo1,2,3, Isabelle Treilleux1, Daphné Blanchard1, Régine Losson4,‡, Vincent Goffin5, Natascha Pigat5, Alain Puisieux1,2, Ivan Mikaelian1,2, Germain Gillet1,2 and Ruth Rimokh1,2,§

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 phosphorlates 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
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 ectoderm-in, 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 Tif1γ-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 Tif1γ in the mammary gland, allowing us to analyse the impact of Tif1γ inactivation on MG development and function. Using these mouse models and an immortalized mouse epithelial cell line, we demonstrate that Tif1γ 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 Tif1γ, 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 Tif1γ-deficient mice in the mammary gland

Tif1γ<sup>fllox/fllox</sup> 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-HamJ, stock 003553] or Whey Acidic Protein-Cre (WAP-Cre) [H6.Cg-Tg(WapCre)11738Mam, strain number 01X58] transgenic mice to generate MMTV- and WAP-Cre/Tif1γ<sup>flox/cre-deleted</sup> (Tif1γ<sup>cre-deleted</sup>). Heterozygous mice were then mated together to generate MMTV- and WAP-Cre/Tif1γ<sup>fllox/fllox</sup>, Cre/Tif1γ<sup>fllox/fllox</sup> and Cre/Tif1γ<sup>fllox/cre-deleted</sup>. This mating scheme allowed us to generate the MMTV- and WAP-Cre/Tif1γ<sup>cre-deleted</sup> control mice. To study lactation defects, MMTV- and WAP-Cre/Tif1γ<sup>fllox/cre-deleted</sup>, Tif1γ<sup>cre-deleted</sup> and Tif1γ<sup>cre-deleted</sup> females were mated with wild-type males. Mice were housed and bred in the 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 Tif1γ floxed mice was determined by multiplex PCR using three different primers in the same reaction (A, 5'-GGTACAGTCTTGTGAGGAGT-3'; B, 5'-GGTAACTCAGCAGAATGCTCA-3'; and C, 5'-AGCTCTGGAGGAACTCCTGTC-3'). The wild-type and floxed Tif1γ 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 recombinant transgenes were also detected by PCR using primers 5'-GGTACAGCAGAATGCTCA-3' and 5'-CCAGTTACGGATATGCTTCAG-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 wholomeumons were prepared as previously described (Robinion 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 (Razanjajona 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 ΔACT method. Primers used for each gene are: Tif1γ and HPRT (Hesling et al., 2011); β-casein (5'-ACAGCTGAGGCCAGAGAGATG-3'; 5'-AATGTTGTGAGGAGGAGGATG-3'); α-lactalbumin (5'-TCTTTACATCTCTTGTGCAGAATG-3'; 5'-TGGCGGTAGCTTCGGTTCAGATG-3'); mPRLR total (5'-AGTGGAACTCTGGTACTGATGAGAG-3'; mPRLR total (5'-AGGACCTGGGTGGTTGACACTG-3'); mPRLR total (5'-ATAAAAGATTTGAACTCATCTGAGG-3'; mPRLR total (5'-TGTACGCTTTCACAGAAGAGAG-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 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 (Bernicheein et al., 2003). In some experiments, cells were pretreated with TGFβ1 (5 ng/ml, Peprotech) or SB-431542 (10 µM, TGFβ type I receptor antagonist).
To determine Tif1γ expression patterns, we first studied its cellular distribution by immunohistochemistry during developmental stages 4, 12, 24, 48 and 64 weeks of virgin mice. MGs collected from late-pregnancy (day 18), lactating (day 2), involuting (day 7) and 52-week old parous female. 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/+ and Cre+/+) mice were mated to generate litters in which the three resulting genotypes (Cre/Tif1g<sup>mdr-mdm</sup>, Cre/Tif1g<sup>mdr-mdm</sup> and Cre/Tif1g<sup>mdr-mdm</sup>) 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<sup>mdr-mdm</sup> females, whereas intermediate expression was found in MECs of WAP-Cre/Tif1g<sup>mdr-mdm</sup> relative to the control.
Cre/Tif1gmfd/mfd mice developed normally, with body sizes comparable with those of Cre/Tif1g+/+ and Cre/Tif1g+++, 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/Tif1gmfd/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/Tif1gmfd/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 expanded 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
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 Tif1γ strongly decreases expression of genes encoding milk proteins

As PRL levels are not affected by Tif1γ 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 Tif1γ 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 Tif1γ 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 Tif1γ, DIP lane). Because this effect is independent of PRL levels per se, it might be due to alterations in PRL receptor (PRLR) expression upon Tif1γ 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
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 Tyr694 in MGs mediated phosphorylation of STAT5 by JAK2 (Ihle and Kerr, 1995), we checked phosphorylation of STAT5 on Tyr694 in MGs 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 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 means±s.d.

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 silencing cells (Fig. 6B, DIP si-1 Tif1γ). 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
sufficient to activate SMAD signalling in cells in which Tif1γ was inactivated. This might explain the decrease in STAT5 phosphorylation observed in cells depleted of Tif1γ (Fig. 6B; Fig. 7A,B, DIP si-Tif1γ) 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 Tif1γ-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 Tif1γ 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 Tif1γ 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/Tif1γ<sup>±/±</sup> mice is also observed in WAP-Cre/Tif1γ<sup>±/±</sup> mice, in which genetic 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 Tif1γ gene inactivation (Fig. 2 and see below).

![Diagram](DEVELOPMENT)

**Fig. 7. Tif1γ acts as a repressor of SMAD4-mediated effects on STAT5 phosphorylation.** (A) HC11 cells, silenced for Tif1γ (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. DIP indicates TGFβ pre-treatment for 24 hours followed by D, I and P treatment for 30 minutes. (B) HC11 cells, silenced for Tif1γ (si-1 Tif) or both Tif1γ and Smad4 (si-Tif/S4), were treated with D, I and P (as indicated) for 30 minutes. (C) HC11 cells were silenced for Tif1γ (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 Tif1γ and Smad4 knockdown was verified as shown. Mouse tubulin was used as loading control.

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 Tif1γ silencing. It is important to note the absence of a decrease in PRLR expression in HC11 cells in which Tif1γ was depleted, supporting the conclusion that loss of Tif1γ 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, Tif1γ 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 lobuloalveoli 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-Tif1γmfd/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 Tif1γ 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 Tif1γ inactivation.

The lactation defect observed in Tif1γ-null mammary glands hence stands out as the most significant phenotype resulting from the absence of Tif1γ. 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 Tif1γ 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. Languille for excellent technical assistance. The authors thank Dr Emmanuel Käs (LBMé, 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-INSEM U57) 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

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

References


Tif1γ is essential for lactation