J. Cell Commun. Signal. (2015) 9:207–215
DOI 10.1007/s12079-015-0299-1

RESEARCH ARTICLE

Interdependent TTF1 - ErbB4 interactions are critical for surfactant protein-B homeostasis in primary mouse lung alveolar type II cells

Elger Marten[1,2] · **Heber C. Nielsen**[1,3] · **Christiane E. L. Dammann**[1,2,3]

Received: 18 February 2015 / Accepted: 14 July 2015 / Published online: 22 July 2015

Abstract ErbB4 receptor and thyroid transcription factor (TTF)-1 are important modulators of fetal alveolar type II (ATII) cell development and injury. ErbB4 is an upstream regulator of TTF-1, promoting its expression in MLE-12 cells, an ATII cell line. Both proteins are known to promote surfactant protein-B gene (*SftpB*) and protein (SP-B) expression, but their feedback interactions on each other are not known. We hypothesized that TTF-1 expression has a feedback effect on ErbB4 expression in an in-vitro model of isolated mouse ATII cells. We tested this hypothesis by analyzing the effects of overexpressing *HER4* and *Nkx2.1*, the genes of ErbB4 and TTF-1 on TTF-1 and ErbB4 protein expression, respectively, as well as SP-B protein expression in primary fetal mouse lung ATII cells. Transient ErbB4 protein overexpression upregulated TTF-1 protein expression in primary fetal ATII cells, similarly to results previously shown in MLE-12 cells. Transient TTF-1 protein overexpression down regulated ErbB4 protein expression in both cell types. TTF-1 protein was upregulated in primary transgenic ErbB4-depleted adult ATII cells, however SP-B protein expression in these adult transgenic ATII cells was not affected by the absence of ErbB4. The observation that TTF-1 is upregulated in fetal ATII cells by ErbB4 overexpression and also in ErbB4-deleted adult ATII cells suggests additional factors interact with ErbB4 to regulate TTF-1 levels. We conclude that the interdependency of TTF-1 and ErbB4 is important for surfactant protein levels. The interactive regulation of ErbB4 and TTF-1 needs further elucidation.

Keywords Alveolar type II cells · ErbB4 · Lung development · SP-B · TTF-1

Abbreviations

ATII cells	Alveolar type II cells
BPD	Bronchopulmonary dysplasia
RDS	Respiratory distress syndrome of the neonate
ErbB4	Receptor tyrosine-protein kinase
HER4	Human ErbB4 encoding gene
TTF-1	Thyroid transscription factor 1
Nkx2.1	TTF-1 encoding gene
SP-B	Surfactant protein B
SftpB	SP-B encoding gene
4ICD	ErbB4 intracellular domain
MLE-12	Mouse lung epithelium 12 cell line
BSA	Bovine serum albumin
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
E	Embryonic day
HBSS	Hank's buffered salt solution
PBS	Phosphate buffered saline solution
$HER4^{heart}(-/-)$	Homozygote ErbB4 negative transgene
$HER4^{heart}(+/-)$	Heterozygote transgene
EGFP	Enhanced green fluorescent protein

Electronic supplementary material The online version of this article (doi:10.1007/s12079-015-0299-1) contains supplementary material, which is available to authorized users.

✉ Christiane E. L. Dammann
cdammann@tuftsmedicalcenter.org

[1] Division of Newborn Medicine, Floating Hospital for Children at Tufts Medical Center, 800 Washington St, Boston, MA 02111, USA

[2] Hannover Medical School, Hannover 30625, Germany

[3] Sackler School for Biomedical Sciences, Tufts University, Boston, MA 02111, USA

HITES	Hydrocortisone, insulin, transferrin, estrogen and selenium
BCA	Bicinchoninic acid

Background

Surfactant deficiency causes neonatal respiratory distress syndrome (RDS) and the development of bronchopulmonary dysplasia (BPD), both severe diseases of immature lungs in preterm born infants (Northway 1967). While the severity of RDS in preterm born infants has decreased in recent decades with better pre- and postnatal care including the application of antenatal corticosteroids, postnatal surfactant supplementation, and more gentle modes of mechanical ventilations (Jobe 1993), the incidence of BPD has only minimally changed (Kair 2012). Despite the growing knowledge of inflammatory (Bose 2008) and oxidative injury (Saugstad 2003) contributing to BPD pathogenesis, treatment options for BPD still have limited success (Beam 2014). Increasing knowledge of the regulation of growth and transcription factor signaling impacting surfactant protein levels in RDS and lung remodeling in BPD should contribute important insights into new therapeutic strategies to prevent or treat BPD.

ErbB receptors are important regulators of cell proliferation, maturation and differentiation (Gassmann 1995). In the fetal lung, ErbB4 protein is highly expressed in fetal alveolar type II (ATII) cells (Liu 2007) and is necessary for the timely initiation of surfactant expression and synthesis (Dammann 2003) and the progression of morphologic lung development (Liu 2010). ErbB4 is unique in the ErbB family of tyrosine kinase receptors, since it can be cleaved by presenillin (Hoeing 2011; Fiaturi 2014), expresses strong nuclear signaling (Williams 2004) and interacts with transcription factors such as Stat5A (Zscheppang 2011) and TTF-1 (Zscheppang 2013) that are important in promoting *Sftpb* expression in ATII cells.

TTF-1 is a nuclear transcription factor that activates transcription of genes in lung, brain and thyroid (Bingle 1997). TTF-1 plays an important role in the developing lung by regulating branching morphogenesis (Minoo 1995) and surfactant protein gene expression (DeFelice 2003). The localization of *Nkx2.1* – the gene encoding for TTF-1 – expression in human fetal lung tissue follows the pattern of distribution of *Sftpb*, and is down regulated in pathologic situations like inflammation (Stahlman 1996). Surfactant Protein B (SP-B), the protein product of the *SftpB* gene, is the most critical functional component of surfactant for lowering surface tension (Perez-Gil 2008). Heterozygous dysfunction of *Nkx2.1* leads to respiratory dysfunction and recurrent pulmonary infections likely due to decreased SP-B levels (Devriendt 1998). The regulation of *Nkx2.1* expression is only partially understood (Hamdan 1998).

Little is known about the regulation of ErbB4 and TTF-1 signaling mechanisms in the fetal lung around the time of initiation of surfactant production or about their interactions with each other. We hypothesized that expression of ErbB4 and TTF-1 proteins are regulated in a feedback loop to coordinate their mutual activity on regulating Sftpb expression. We here show negative feedback regulation between TTF-1 and ErbB4, and speculate that TTF-1 plays a key role in compensating for ErbB4 loss to maintain *Sftpb* expression.

Materials and methods

Materials

The immortalized mouse lung alveolar epithelial cell line MLE-12 was obtained from the American Type Culture Collection (Manassas, VA); time-dated pregnant wild type Swiss Webster Mice were obtained from Taconic (Hudson, NY). Rabbit polyclonal TTF-1 antibody (H-190) was obtained from Abcam (Cambridge, MA), rabbit polyclonal ErbB4 antibody (C-18) and rabbit polyclonal SP-B antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal β-actin antibody, IRDye 680LT goat anti-rabbit IgG antibody (H + L) and IRDye 800CW and goat anti-mouse antibody IgG (H + L) were obtained from Li-Cor (Lincoln, NE). Bovine serum albumin (BSA) was obtained from Sigma (St.Louis, MO); Dulbecco's Modified Eagles Medium (DMEM) and Ham's F12 culture media were obtained from Invitrogen (Grand Island, NY); fetal bovine serum (FBS) was from Thermo Fisher Scientific (Waltham, MA); Plasmid Midi Kit was from Qiagen (Germantown, MD). FuGene® HD Transfection Reagent was obtained from Promega (Madison, WI); Dispase was from BD Bioscience (Franklin Lakes, NJ); Collagenase type 2 was obtained from Worthington (Lakewood, NJ), Trypsin 1:250 was from USB Corporation (Cleveland, OH) and Desoxyribonuclease I was from Sigma Aldrich (St. Louis, MO); Ketamine hydrochloride was from Fort Dodge Animal Health (Fort Dodge, IA) and Rompun® (Xylazine) was obtained from Bayer Agriculture Division (Shawnee Mission, KS). DAPI was from Vector Laboratories (Burlingame, CA). pEGFP N3 (control), and pHER4 (full length human ErbB4 receptor) plasmids (Lee 2002; Williams 2004) were used as previously published (Zscheppang 2011). pRC/CMV/Nkx2.1 (*Nkx2.1* expression plasmid) was kindly provided by Dr. Jeffrey Whitsett (Cincinnati Children's Hospital Medical Center, Cincinnati, OH) (Zhou 2008).

Preparation of primary fetal mouse ATII epithelial cell cultures

All animal use was performed according to an animal research protocol approved by the institutional IACUC. Primary ATII

cells were freshly isolated from time-dated pregnant Swiss Webster mice as previously described (Zscheppang 2013). Briefly, pregnant Swiss Webster mice were sacrificed at E17.5 of gestation by CO_2 inhalation followed by cervical dislocation. Fetal lungs were removed from the isolated fetuses, washed in sterile HBSS, minced with a razorblade, and incubated with collagenase type II diluted in serum-free DMEM for 2 h at 37 °C. The reaction was stopped on ice for 30 min. Cells were centrifuged and resuspended in DMEM. After a second centrifugation the pellet was resuspended in DNase and trypsin and incubated for 12 min at 37 °C. The reaction was stopped by DMEM containing 10 % fetal calf serum (FBS). The cells were filtered through a 40 μm nylon filter, centrifuged, resuspended in DMEM containing 10 % FBS, and plated in culture flasks for 60 min at 37 °C (21 %0_2/5 %$C0_2$) to allow for differential adherence of lung fibroblasts. For ATII cell isolation the supernatant from the first differential adherence was centrifuged, the cell pellet resuspended in DMEM containing 10 % FBS, and plated again in the same conditions for a second differential adherence. Supernatants were removed and centrifuged. The cell pellet was resuspended and cells were plated in 6-well plates in DMEM containing 20 % FBS. After 24 h of incubation 200 μg of cis-4-Hydroxy-L-Proline was added to each well for another 24 h to minimize the proliferation of the residual fibroblasts (Kao 1977). Wells were washed three times with PBS before transfection experiments were started.

Preparation of primary ATII epithelial cell cultures from adult male ErbB4-transgenic mice

$HER4^{heart}(-/-)$ mice (hereafter designated $HER4^{heart}$ mice), a transgenic mouse line in which fetuses homozygous for ErbB4 deletion were rescued from their lethal cardiac defects by expressing a human ErbB4 (*HER4*) cDNA under the cardiac-specific α-myosin heavy chain promoter (*MHC*) (Tidcombe 2003), were used for these experiments. Mice were genotyped by PCR (Supplemental Fig. 1A), and the absence of ErbB4 receptors was confirmed in lung fibroblasts (Supplemental Fig. 1B). All animals were housed in a pathogen-free animal facility at Tufts University. Primary ATII cells were freshly isolated from adult male $HER4^{heart}$ mice as previously published with slight changes (Corti 1996; Warshamana 2001). Briefly, mice were narcotized with a ketamine-xylazine solution (153 mM ketamine, 24.5 mM xylazine). After rinsing the lung blood vessels free from cells with PBS, 1 mL of dispase was installed into the lung via the trachea. The lung was removed from the mouse and incubated in dispase on ice for 40 min, minced with a razorblade, incubated with DNase in DMEM at 37 °C for 10 min, sequentially filtered through 100, 40, and finally 20 μm nylon filters, centrifuged and resuspended in DMEM containing 10 % FBS. For differential adherence of fibroblasts, cells were plated in culture plates for 60 min at 37 °C (21 %0_2/5 %$C0_2$). For ATII cell isolation, the supernatant was collected, centrifuged, and the resuspended pellet plated for a second and third differential adherence under the same conditions for 45 and 30 min, respectively. After the third differential adherence, cells were centrifuged, the pellet was resuspended in DMEM containing 10 % FBS, and cells were plated in one well of a 6-well plate. After 48 h of incubation, 200 μg of cis-4-Hydroxy-L-Proline was added to each well for another 24 h to inhibit the proliferation of the residual fibroblasts (Kao 1977). Cells were grown on plastic in DMEM for another 24 h before harvesting. Growth conditions for fetal and adult ATII cells were similar, since they conserve the expression of ATII cell markers in both cell types (Dey Hazra 2011).

Transfection experiments

To examine the effects of *HER4* and *Nkx2.1* (the genes encoding for ErbB4 and TTF-1) overexpression on SP-B, ErbB4, and TTF-1 protein expression levels, MLE-12 cells and primary fetal ATII cells were transfected with plasmid expressing human ErbB4 (*HER4*), or *Nkx2.1*, or an empty control construct (*EGFP*) (Zhou 2008; Zscheppang 2011, 2013). The MLE-12 cells were only transfected with *Nkx2.1* or *EGFP*, since we have already published the effects of ErbB4 overexpression in MLE-12 cells on surfactant protein expression (Zscheppang 2013). The cells were transfected for 24 h using FuGene® transfection reagent in accordance with the manufacturer's suggestions. Briefly, cells were grown on 6-well plates up to 30–50 % confluence for primary ATII cells and MLE-12 cells, respectively. Two micrograms of DNA were diluted in serum free medium and FuGene® reagent and incubated for 15 min at room temperature before the mixture was added drop-wise into the serum-containing media. Primary ATII cells were grown in DMEM containing 20 % FBS. MLE-12 cells were grown in serum-supplemented (2 %) HITES medium. To quantify the transfection efficiency, the numbers of green fluorescent EGFP positive cells and total DAPI stained cells were counted under fluorescence microscopy. The transfection efficiency was 30–40 % in MLE 12 cells (Supplemental Fig. 2A), and about 20 % in primary fetal ATII cells (Supplemental Figure 2B).

Western blotting

After 24 h of incubation the cells were washed three times with PBS (pH 7.2; 137 mM NaCl, 10.14 mM Na_2HPO_4, 2.7 mM KCl, 1.76 mM KH_2PO_4) and scraped in 100–300 μL lysis buffer per well (20 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 % NP-40, 1 mM Na_3VO_4, 4.5 mM $Na_4P_2O_7$, 10 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 mM $ZnCl_2$, 1 mM NaF, 1 mM PMSF and 10 μg each of aprotinin, leupeptin and pepstatin). After 15 min of

incubation on ice, cells were scraped and lysates were cleared by microcentrifugation for 15 min at 4 °C. Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA) was used to determine the concentration of total protein. To detect individual proteins, cell lysates with an amount of 30 ng of total protein were boiled in Laemmli buffer for 5 min at 100 °C. Proteins were separated by 7 % SDS polyacrylamide gel electrophoresis and transferred to a 0.45 μm nitrocellulose membrane. For SP-B and TTF-1 detection, a 9 % SDS polyacrylamide gel and a 0.2 μm membrane were used. Blots were blocked in 1 % BSA and incubated with antibodies against individual proteins. Proteins were visualized by scanning the membranes with a Li-Cor® Odyssey infrared imaging system. Membranes were stripped in stripping buffer (pH 2; 25 mM glycine and 52 mM SDS) for 30 min at 50 °C and reprobed up to a maximum of 5 times.

Data analysis

All protein expressions of ErbB4, TTF-1, or SP-B were measured by densitometry, normalized to the control condition (set to 100 %) and presented as mean±SEM. The results of all transfection experiments were also expressed as percentage of their *EGFP*-transfected experiment-specific control (set to 100 %). The effect of ErbB4 depletion in adult homozygous mutant HER4^{heart} mice on SP-B and TTF-1 protein expression was expressed as percentage of the expression of the heterozygous controls (set to 100 %). Statistical significance was evaluated using a paired two-tailed *t*-test except for one instance when the a priori hypothesis tested was a one-way hypothesis.

Results

Nkx2.1 overexpression increases SP-B, but decreases ErbB4 protein expression in MLE-12 cells

To determine the effect of *Nkx2.1* overexpression in MLE-12 cells only experiments which showed an adequate (>400 %) increase of TTF-1 protein expression were analyzed. These experiments showed increased TTF-1 protein expression to 647±76 % of controls (N=8, P=0.0002) (Fig. 1a). This was associated with a significant increase of SP-B protein expression to 130±10 % (N=8, P=0.022) and a significant decrease of ErbB4 protein expression to 72±4 % (N=8, P=0.0003) (Fig. 1b) when compared to the specific *EGFP*-transfected control cells.

Nkx2.1 overexpression increases SP-B, but decreases ErbB4 protein expression in primary ATII cells

In fetal mouse ATII cells only experiments which showed an adequate (>150 %) increase of TTF-1 protein expression were analyzed. In these experiments *Nkx2.1* overexpression increased TTF-1 protein expression to 224±43 % (N=8, P=0.023) (Fig. 2a), resulting in a similar significant increase in SP-B protein expression to 151±18 % (N=7, P=0.03) and a significant decrease in ErbB4 protein expression to 75±3 % (N=8, P=0.0001) (Fig. 2b) when compared to *EGFP*-transfected control cells.

HER4 overexpression increases SP-B and TTF-1 protein expression in fetal mouse ATII cells

Only experiments which showed an adequate (>150 %) increase of ErbB4 protein expression were analyzed. *HER4* overexpression increased ErbB4 protein expression in fetal mouse ATII cells to 186±13 % (N=6, P=0.0013) (Fig. 3a). This was associated with a significant increase in SP-B protein expression to 133±1.7 % (N=6, P=0.0001) and in TTF-1 protein expression to 178±28 % (N=5, P=0.05) (Fig. 3b) when compared to *EGFP*-transfected control cells.

ErbB4 deletion increases TTF-1 protein expression but does not affect SP-B protein expression in adult male HER4^{heart} ATII cells

In primary male adult HER4^{heart} ATII cells, genetic deletion of ErbB4 was associated with a significant increase in TTF-1 protein expression to 174±14 % (N=14, P=0.0002) when compared to heterozygote control cells (100±9 %, N=14, 100±11 %, N=8, respectively). However, the loss of ErbB4 did not affect SP-B protein expression (103±15 %, N=8, P=0.86) (Fig. 4).

Discussion

The TTF-1 transcription factor is a crucial regulator of the development of the lung, the brain, and the thyroid (Bingle 1997). In the lung, acute inflammation, edema, hemorrhage, and atelectasis inhibit expression of *Nkx2.1*, the gene encoding for TTF-1, while in regeneration processes (Stahlman 1996) or in compensatory growth (Takahashi 2010), TTF-1 protein levels are increased, indicating the importance of TTF-1 in lung growth and remodeling. In acute inflammation, cytokines like TNF-α have been shown to decrease TTF-1 proteins at the transcriptional level (Das 2011), but little is known about how *Nkx2.1* expression is otherwise regulated and if expression feedback mechanisms are present. We have previously shown that TTF-1 functions as a downstream signal for ErbB4-mediated upregulation of *Sftpb* expression (Zscheppang 2013). Here we show that TTF-1 provides feedback regulation on ErbB4 protein expression. Our results also suggest that elevated TTF-1 levels may maintain

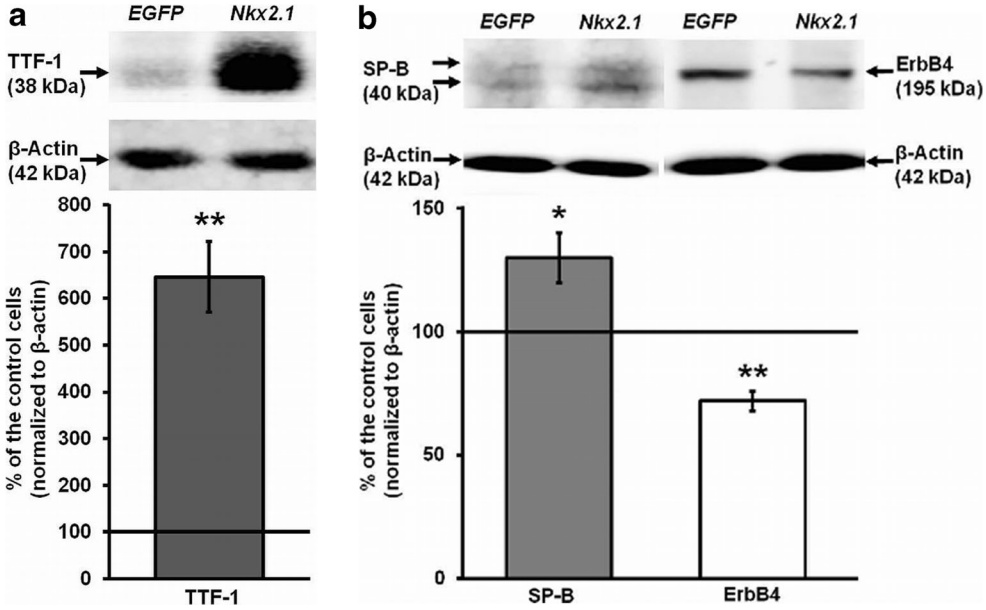

Fig. 1 *Nkx2.1* overexpression in MLE-12 cells increases SP-B protein expression and decreases ErbB4 protein expression. MLE-12 cells were transfected with a vector expressing *Nkx2.1* or a control vector expressing only *EGFP*. **a** *Nkx2.1* overexpression was confirmed by Western blotting for TTF −1 (*upper panel*) and quantified by densitometry (*lower panel*). **b** A representative Western blot (*upper panel*) and densitometry (*lower panel*) shows the effects of *Nkx2.1* overexpression on SP-B (*left*) and ErbB4 (*right*) protein expression. β-actin reprobing was used to control for protein loading. Means of the controls were set to 100 %. Data are presented as means±SEM of 8 independent experiments each. **P*<0.05, ***P*<0.01

SftpB expression to compensate for longstanding ErbB4 deletion.

TTF-1 is a tumor marker for thyroid and lung carcinomas (Moldvay 2004). The role of TTF-1 in lung cancer cells is not fully understood, but TTF-1 is an apparent positive prognostic marker in adenocarcinomas of the lung (Berghmans 2006). In non-small cell lung carcinoma cells, *Nkx2.1* overexpression represses the expression of the cell cycle protein Ki-67 and

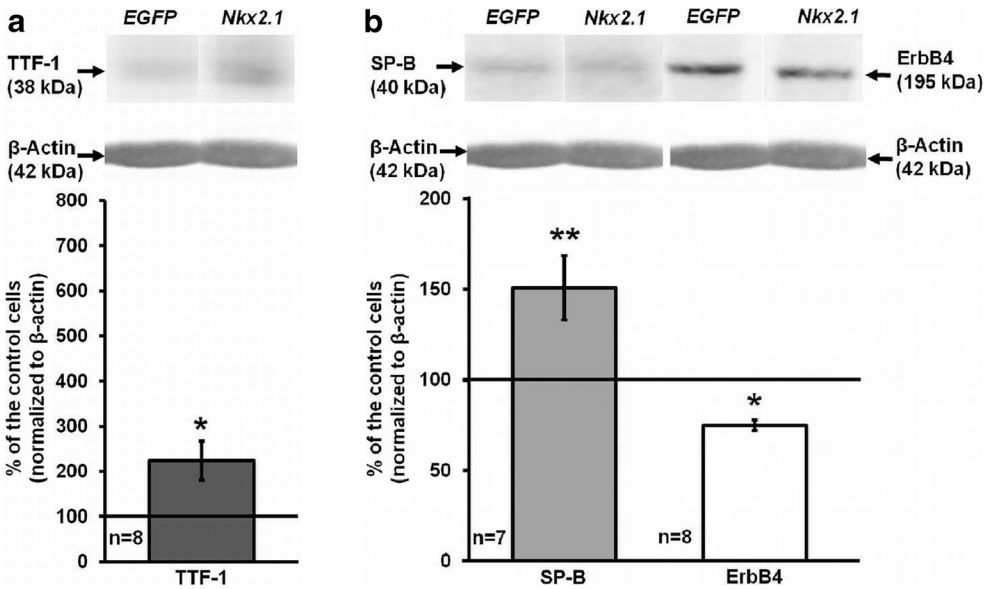

Fig. 2 *Nkx2.1* overexpression in primary fetal ATII cells increases SP-B protein expression while decreasing ErbB4 protein expression. Primary fetal ATII cells were transfected with *Nkx2.1* or a control vector expressing only *EGFP*. **a** *Nkx2.1* overexpression was confirmed by Western blotting for TTF-1 (*upper panel*) and quantified by densitometry (*lower panel*). **b** A representative Western blot (upper panel) and densitometry (*lower panel*) shows the effects of *Nkx2.1* overexpression on SP-B (*left*) and ErbB4 (*right*) protein expression. β-actin reprobing was used to control for protein loading. Means of the controls were set to 100 %. Data are presented as means±SEM of the numbers of independent experiments displayed in the figure. **P*<0.05, ***P*<0.01

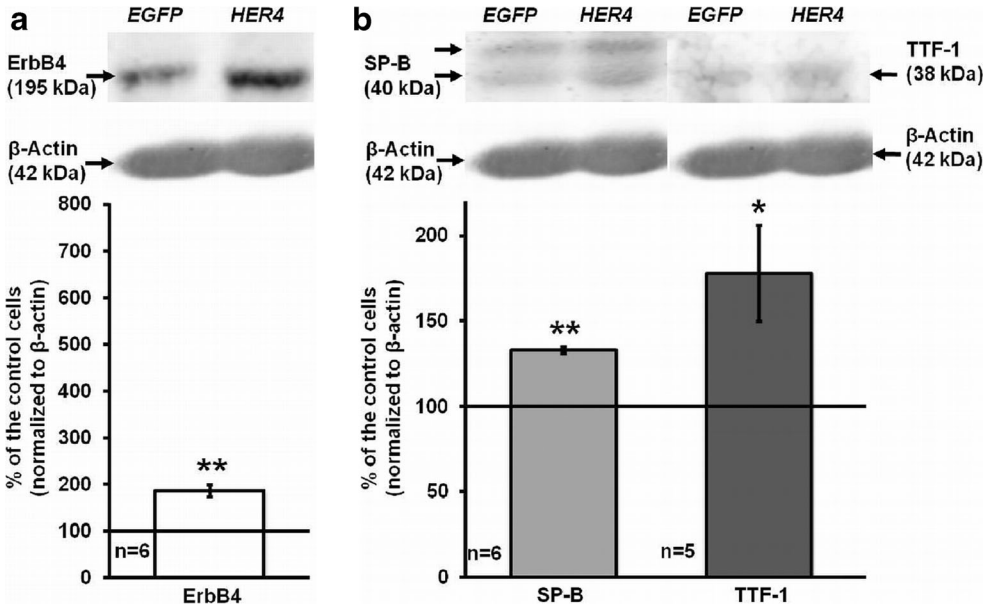

Fig. 3 *HER4* overexpression in primary fetal ATII cells increases both SP-B and TTF-1 protein expression. Primary fetal ATII cells were transfected with a vector expressing *HER4* or a control vector expressing only *EGFP*. **a** *HER4* overexpression was confirmed by Western blotting for ErbB4 protein (*upper panel*) and quantified by densitometry (*lower panel*). **b** A representative Western blot (*upper panel*) and densitometry (lower panel) shows the effects of *HER4* overexpression on SP-B (*left*) and TTF-1 (*right*) protein expression. β-actin reprobing was used to control for protein loading. Means of the controls were set to 100 %. Data are means±SEM of the number of independent experiments displayed in the figure. *$P \leq 0.05$ (one tailed paired *t*-test, based on our *a priori* hypothesis of an increase with *HER4* overexpression), **$P < 0.01$ (two tailed paired *t*-test)

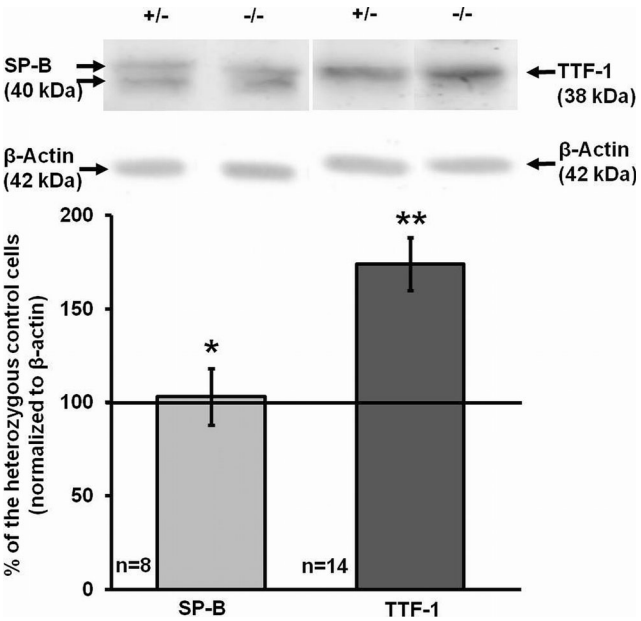

Fig. 4 Genetic deletion of ErbB4 does not affect SP-B protein expression, while it increases TTF-1 protein expression in adult HER4^{heart}(−/−) murine ATII cells. Representative Western blots from adult HER4^{heart} ATII cells isolated from heterozygous controls (+/−) and homozygous negative (−/−) mice. Upper panel shows representative blot of SP-B (*left*) and TTF-1 protein (*right*). Lower panel shows normal SP-B levels and increased TTF-1 protein levels in HER4^{heart} homozygote ATII cells compared to heterozygote controls. β-actin reprobing was used to control for protein loading. The mean of the controls was set to 100 %. Data are means±SEM of the number of independent experiments displayed in the figure. *P=0.859, **P=0.0002

may serve as a tumor suppressor gene (Zu 2012). It also represses Muc5b, one of the major mucin genes of the respiratory tract, which is highly expressed in human lung adenocarcinoma (Jonckheere 2010). On the other hand, *Nkx2.1* promotes survival of cancer cells if the gene is amplified, functioning as a lung cancer specific oncogene (Kwei 2008), while *Nkx2.1* suppression inhibits cell proliferation of lung adenocarcinoma cells leading to apoptosis (Tanaka 2007). This "zwitter function" of TTF-1 is paralleled by similar zwitter functions of ErbB4 in cancer biology, in which ErbB4 expression is associated with either tumor progression or with good prognosis (Gullick 2003). The functions of ErbB4 and of TTF-1 in lung development initiated our focus on their interactions in developing alveolar ATII cells.

In our initial approach, we used the MLE-12 cell line, a mouse adult lung alveolar epithelial cell line that has been frequently used to study the regulation of surfactant protein production (Wikenheiser 1993). We found that the transfection efficiency of MLE-12 cells is twice that of primary fetal ATII cells. However, our data here indicate that despite the lower efficiency of transfection observed in primary fetal ATII cells the effects of *Nkx2.1* and *HER4* overexpression on SP-B protein expression are similar in both cell types. This difference in transfection efficiency is consistent with other reports (Marsh 2009) and is likely due to a higher replication rate seen in cell lines like MLE-12 cells. When comparing the phenotypical behavior of primary adult with fetal ATII cells it seemed to be more difficult to grow primary adult ATII cells,

resulting in an unpredictable and widely variable viability in these different kinds of primary cells (Hamm 2002). We have used fetal primary cells for our transfection experiments, despite the fact that adult primary cells may be closer in their character to the MLE-12 cells, since MLE-12 cells were derived from adult lungs. In addition, fetal cells grow more efficiently and are therefore easier to transfect than their adult primary cell counterparts. To gain cell culture material from adult animals needs to sacrifice a lot of animals and we tried to keep the number of sacrificed animals low.

The mature SP-B protein has a size of 8 kDa. For our experiments we detected the SP-B proprotein (pro SP-B) with a size of 40 kDa due to clearer signals. The pro SP-B can show split (Figs. 1b, 3b and 4) or single (Fig. 2b) signal bands. Depending on the maturational status of the cell cycle the proprotein can show processing intermediates (Brasch et al. 2004). Inconsistent western blot bands can also be caused by spicing variants of SP-B (Chi et al. 1998). This phenomenon is known for TTF-1 as well (Li 2000) even though our experiments showed only single band signals for TTF-1.

ErbB4 is known to be important for differentiation of ATII cells of the developing lung (Dammann 2003), the mammary gland, the nervous system, and the heart (Carpenter 2002). In the lung, both TTF-1 and ErbB4 protein have important roles in promoting surfactant protein expression (Boggaram 2009; Zscheppang 2011). Our previous experiments in fetal murine ATII cells showed that there is an interaction between TTF-1 and ErbB4 (Zscheppang 2013). We here show evidence of a negative feedback loop between ErbB4 and TTF-1, in that overexpression of *Nkx2.1* decreased ErbB4 protein expression. Negative feedback loops are well-documented for several hormones, for example thyroid-stimulating hormone and thyroxine (Shupnik 1989) or the adrenocorticotropic hormone and cortisol (Keller-Wood 1984). However, the feedback relationship of ErbB4 on TTF-1 appears more complex. TTF-1 protein levels were increased by ErbB4 overexpression in fetal ATII cells and MLE-12 cells, consistent with the proposed negative feedback relationship. However, the opposite was seen in HER4^{heart} ATII cells, which showed evidence of positive feedback, i.e., the lack of ErbB4 was associated with increased TTF-1 protein levels. This suggests that the feedback effects of ErbB4 on TTF-1 protein are more complex and likely involve additional components to mediate the full effect. Nevertheless, our data clearly show that ErbB4 levels do affect the levels of TTF-1 protein. More studies are needed, including gene expression studies, in order to further characterize the mechanisms involved in the mutual feedback relationships of TTF-1 and ErbB4 in their roles of orchestrating surfactant protein production.

We used the HER4^{heart} ATII cells in an attempt to gain further insight into the importance of the regulatory feedback between TTF-1 and ErbB4. We have previously shown that pulmonary ErbB4 deletion leads to a lung phenotype in fetal and adult animals, including a delayed onset of fetal surfactant synthesis and *Sftpb* expression (Liu 2010), accompanied by decreased alveolar space and increased thickness of alveolar septae, mirroring the pathologic picture of bronchopulmonary dysplasia. However, these lungs exhibit catch-up expression on the effects of ErbB4 deletion at term, resulting in normal *Sftpb* gene expression in adult animals (Purevdorj 2008). While *Sftpb* gene expression is normalized in ErbB4-deleted adult lungs, other components of lung development remain abnormal, including evidence of alveolar simplification combined with evidence of chronic inflammation, a hyperreactive airway system and surfactant protein D downregulation (Purevdorj 2008). Thus, while our observed relationship between ErbB4 loss and TTF-1 expression was unexpected and differed from that in fetal ATII cells, these results provide more evidence of the importance of normal ErbB4 expression in lung ATII cells for normal cellular function.

We here studied the effect of TTF1-ErbB4 interactions on SP-B protein levels. This is just one element of surfactant homeostasis, and so these studies do not give information on overall surfactant system homeostasis. Surfactant is composed of approximately 12 % protein, 80 % phospholipids and 8 % other lipids (cholesterol, triacylglycerol and free fatty acids) (Frerking 2001) and its homeostasis is regulated not only by synthesis but also by trafficking, secretion, catabolism, and recycling (Whitsett 2015). To elucidate the influence of ErbB4 and TTF-1 on more aspects of the surfactant homeostasis, studies of the metabolism cycle of surfactant proteins, genes, and other components of the surfactant system are needed.

Summary statement

Both ErbB4 and TTF-1 play critical roles in surfactant homeostasis. Our findings show a negative feedback relationship between ErbB4 and TTF-1 expression in fetal ATII cells. While there are a number of additional factors involved in the regulation of TTF-1, ErbB4, and SP-B protein expression, we here present important insights into the lung cell biology involved in the regulation of *SftpB* expression. More research is needed to further elucidate these interactions.

Acknowledgements We thank Drs. M. Gassmann and C. Birchmeier for providing the HER4^{heart} mouse line. The studies were funded by NIH NHLBI R01 HL085648, HL037930, and Hannover Medical School.

Conflicts of interest The authors have no conflicts of interest.

Authors' contributions Elger Marten - experimental design, data generation and analysis, manuscript preparation; Heber Nielsen - experimental design, manuscript preparation; Christiane Dammann - experimental design, manuscript preparation.

References

Beam KS (2014) A systematic review of randomized controlled trials for the prevention of bronchopulmonary dysplasia in infants. J Perinatol 34(9):705–710

Berghmans T (2006) Prognostic role of thyroid transcription factor-1 in stage III non-small cell lung cancer. Lung Cancer 52(2):219–224

Bingle CD (1997) Thyroid transcription factor-1. Int J Biochem Cell Biol 29(12):1471–1473

Boggaram V (2009) Thyroid transcription factor-1 (TTF-1/Nkx2.1/TITF1) gene regulation in the lung. Clin Sci (Lond) 116(1):27–35

Bose CL (2008) Bronchopulmonary dysplasia and inflammatory biomarkers in the premature neonate. Arch Dis Child Fetal Neonatal Ed 93(6):F455–F461

Brasch F, Johnen G, Winn-Brasch A, Guttentag SH, Schmiedl A, Kapp N, Suzuki Y, Müller KM, Richter J, Hawgood S, Ochs M (2004) Surfactant protein B in type II pneumocytes and intra-alveolar surfactant forms of human lungs. Am J Respir Cell Mol Biol 30(4):449–458

Carpenter RH (2002) Reaching out: cortical mechanisms of directed action. Curr Biol 12(15):R517–R519

Chi X, Garnier G, Hawgood S, Colten HR (1998) Identification of a novel alternatively spliced mRNA of murine pulmonary surfactant protein B. Am J Respir Cell Mol Biol 19(1):107–113

Corti M (1996) Isolation and primary culture of murine alveolar type II cells. Am J Respir Cell Mol Biol 14(4):309–315

Dammann CE (2003) Role of neuregulin-1 beta in the developing lung. Am J Respir Crit Care Med 167(12):1711–1716

Das A (2011) Thyroid transcription factor-1 (TTF-1) gene: identification of ZBP-89, Sp1, and TTF-1 sites in the promoter and regulation by TNF-alpha in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 301(4):L427–L440

DeFelice M (2003) TTF-1 phosphorylation is required for peripheral lung morphogenesis, perinatal survival, and tissue-specific gene expression. J Biol Chem 278(37):35574–35583

Devriendt K (1998) Deletion of thyroid transcription factor-1 gene in an infant with neonatal thyroid dysfunction and respiratory failure. N Engl J Med 338(18):1317–1318

Dey Hazra RO (2011) Age-Dependent In Vitro mouse lung type II cell behavior. E-PAS 2011:3825.200

Fiaturi N (2014) Dissociated presenilin-1 and TACE processing of ErbB4 in lung alveolar type II cell differentiation. Biochim Biophys Acta 1843(4):797–805

Frerking I (2001) Pulmonary surfactant: functions, abnormalities and therapeutic options. Intensive Care Med 27(11):1699–1717

Gassmann M (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature 378(6555):390–394

Gullick WJ (2003) c-erbB-4/HER4: friend or foe? J Pathol 200(3):279–281

Hamdan H (1998) Structure of the human Nkx2.1 gene. Biochim Biophys Acta 1396(3):336–348

Hamm A (2002) Efficient transfection method for primary cells. Tissue Eng 8(2):235–245

Hoeing K (2011) Presenilin-1 processing of ErbB4 in fetal type II cells is necessary for control of fetal lung maturation. Biochim Biophys Acta 1813(3):480–491

Jobe AH (1993) Beneficial effects of the combined use of prenatal corticosteroids and postnatal surfactant on preterm infants. Am J Obstet Gynecol 168(2):508–513

Jonckheere N (2010) The mouse Muc5b mucin gene is transcriptionally regulated by thyroid transcription factor-1 (TTF-1) and GATA-6 transcription factors. FEBS J 278(2):282–294

Kair LR (2012) Bronchopulmonary dysplasia. Pediatr Rev 33(6):255–263, **quiz 263–4**

Kao WW (1977) Proline analogue removes fibroblasts from cultured mixed cell populations. Nature 266(5597):63–64

Keller-Wood ME (1984) Corticosteroid inhibition of ACTH secretion. Endocr Rev 5(1):1–24

Kwei KA (2008) Genomic profiling identifies TITF1 as a lineage-specific oncogene amplified in lung cancer. Oncogene 27(25):3635–3640

Lee HJ (2002) Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4. J Biol Chem 277(8):6318–6323

Li C (2000) Two functionally distinct forms of NKX2.1 protein are expressed in the pulmonary epithelium. Biochem Biophys Res Commun 270(2):462–468

Liu W (2007) The ErbB4 receptor in fetal rat lung fibroblasts and epithelial type II cells. Biochim Biophys Acta 1772(7):737–747

Liu W (2010) ErbB4 regulates the timely progression of late fetal lung development. Biochim Biophys Acta 1803(7):832–839

Marsh LM (2009) Surface expression of CD74 by type II alveolar epithelial cells: a potential mechanism for macrophage migration inhibitory factor-induced epithelial repair. Am J Physiol Lung Cell Mol Physiol 296(3):L442–L452

Minoo P (1995) TTF-1 regulates lung epithelial morphogenesis. Dev Biol 172(2):694–698

Moldvay J (2004) The role of TTF-1 in differentiating primary and metastatic lung adenocarcinomas. Pathol Oncol Res 10(2):85–88

Northway WH Jr (1967) Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med 276(7):357–368

Perez-Gil J (2008) Structure of pulmonary surfactant membranes and films: the role of proteins and lipid-protein interactions. Biochim Biophys Acta 1778(7–8):1676–1695

Purevdorj E (2008) ErbB4 deletion leads to changes in lung function and structure similar to bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 294(3):L516–L522

Saugstad OD (2003) Bronchopulmonary dysplasia-oxidative stress and antioxidants. Semin Neonatol 8(1):39–49

Shupnik MA (1989) Molecular biology of thyrotropin. Endocr Rev 10(4):459–475

Stahlman MT (1996) Expression of thyroid transcription factor-1(TTF-1) in fetal and neonatal human lung. J Histochem Cytochem 44(7):673–678

Takahashi Y (2010) Thyroid transcription factor-1 influences the early phase of compensatory lung growth in adult mice. Am J Respir Crit Care Med 181(12):1397–1406

Tanaka H (2007) Lineage-specific dependency of lung adenocarcinomas on the lung development regulator TTF-1. Cancer Res 67(13):6007–6011

Tidcombe H (2003) Neural and mammary gland defects in ErbB4 knockout mice genetically rescued from embryonic lethality. Proc Natl Acad Sci U S A 100(14):8281–8286

Warshamana GS (2001) TNF-alpha, PDGF, and TGF-beta(1) expression by primary mouse bronchiolar-alveolar epithelial and mesenchymal cells: tnf-alpha induces TGF-beta(1). Exp Mol Pathol 71(1):13–33

Whitsett JA (2015) Alveolar development and disease. Am J Respir Cell Mol Biol

Wikenheiser KA (1993) Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. Proc Natl Acad Sci U S A 90(23):11029–11033

Williams CC (2004) The ERBB4/HER4 receptor tyrosine kinase regulates gene expression by functioning as a STAT5A nuclear chaperone. J Cell Biol 167(3):469–478

Zhou B (2008) Foxp2 inhibits Nkx2.1-mediated transcription of SP-C via interactions with the Nkx2.1 homeodomain. Am J Respir Cell Mol Biol 38(6):750–758

Zscheppang K (2011) Neuregulin receptor ErbB4 functions as a transcriptional cofactor for the expression of surfactant protein B in the fetal lung. Am J Respir Cell Mol Biol 45(4):761–767

Zscheppang K (2013) ErbB4 is an upstream regulator of TTF-1 fetal mouse lung type II cell development in vitro. Biochim Biophys Acta 1833(12):2690–2702

Zu YF (2012) Thyroid transcription factor 1 represses the expression of Ki-67 and induces apoptosis in non-small cell lung cancer. Oncol Rep 28(5):1544–1550