

Hypoxia Inducible Factor 3 α Plays a Critical Role in Alveolarization and Distal Epithelial Cell Differentiation during Mouse Lung Development

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Abstract

Lung development occurs under relative hypoxia and the most important oxygen-sensitive response pathway is driven by Hypoxia Inducible Factors (HIF). HIFs are heterodimeric transcription factors of an oxygen-sensitive subunit, HIF α , and a constitutively expressed subunit, HIF1 β . HIF1 α and HIF2 α , encoded by two separate genes, contribute to the activation of hypoxia inducible genes. A third HIF α gene, *HIF3 α* , is subject to alternative promoter usage and splicing, leading to three major isoforms, HIF3 α , NEPAS and IPAS. HIF3 α gene products add to the complexity of the hypoxia response as they function as dominant negative inhibitors (IPAS) or weak transcriptional activators (HIF3 α /NEPAS). Previously, we and others have shown the importance of the Hif1 α and Hif2 α factors in lung development, and here we investigated the role of Hif3 α during pulmonary development. Therefore, HIF3 α was conditionally expressed in airway epithelial cells during gestation and although HIF3 α transgenic mice were born alive and appeared normal, their lungs showed clear abnormalities, including a post-pseudoglandular branching defect and a decreased number of alveoli. The HIF3 α expressing lungs displayed reduced numbers of Clara cells, alveolar epithelial type I and type II cells. As a result of HIF3 α expression, the level of Hif2 α was reduced, but that of Hif1 α was not affected. Two regulatory genes, *Rarb*, involved in alveologenesis, and *Foxp2*, a transcriptional repressor of the Clara cell specific *Ccsp* gene, were significantly upregulated in the HIF3 α expressing lungs. In addition, aberrant basal cells were observed distally as determined by the expression of *Sox2* and *p63*. We show that Hif3 α binds a conserved HRE site in the *Sox2* promoter and weakly transactivated a reporter construct containing the *Sox2* promoter region. Moreover, Hif3 α affected the expression of genes not typically involved in the hypoxia response, providing evidence for a novel function of Hif3 α beyond the hypoxia response.

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Introduction

The lung originates from the primitive foregut early in the development of land dwelling organisms, and through a complex interplay of signaling molecules the future airway epithelium and surrounding mesenchyme develop into the highly structured arbor-like bronchial-vascular tree (reviewed in [1,2,3]). Normal development in mammals occurs in a relative hypoxic environment, which is beneficial for lung organogenesis [4,5]. Cellular responses to different levels of oxygen are important for development and homeostasis [6], and the most important oxygen-sensing mechanism to protect cells from oxygen toxicity is the transcriptional response mediated by Hypoxia Inducible Factors (HIF), which are also expressed in the lungs [7].

HIFs are critical mediators of the hypoxic cellular response and regulate cellular adaptation by transactivating genes involved in angiogenesis, metabolism and cellular homeostasis (for recent reviews see [6,8,9]). HIFs are heterodimeric transcription factors which have two structurally related subunits, an oxygen sensitive

HIF α subunit and a constitutively expressed HIF β or ARNT subunit (Aryl hydrocarbon Receptor Nuclear Translocator). Both subunits belong to the transcription factor family containing a basic Helix-Loop-Helix (bHLH) and a Per/ARNT/Sim (PAS) domain at the N-terminus, which mediate heterodimerization and DNA binding [10,11]. HIF β is expressed ubiquitously and as such, the level and expression patterns of the HIF α proteins are mostly determining the activity of the heterodimers [12]. Currently, three genes have been identified in human and mouse that encode HIF α isoforms, *HIF1 α* [10,11], *HIF2 α* or *EPAS1* [13,14,15], and *HIF3 α* [16,17,18,19,20]. Aside from the N-terminal bHLH/PAS domain, the HIF α subunits contain an Oxygen-Dependent Degradation Domain (ODDD) in the center of the protein, an N-terminal transactivation domain (NTAD) and a C-terminal transactivation domain (CTAD) [21,22,23,24,25]. The CTAD is absent in the HIF3 α subunit, which significantly reduces the transcriptional activity of the protein [16,26]. The three α subunits are post-transcriptionally regulated by prolyl hydroxylase domain-contain-

ing enzymes (PHD1-3), which hydroxylate with different specificity the HIF α subunits at two critical prolyl residues in the ODDD under normoxic conditions [22,27]. The PHD proteins are dioxygenases which require oxygen for their function and as such are sensitive to oxygen concentrations, losing their activity under low oxygen concentration [22]. The hydroxylated HIF α proteins are poly-ubiquitinated and targeted for 26S proteasomal degradation through the von Hippel-Lindau (pVHL)/Elongin BC/Cul2 ubiquitin-ligase complex [28,29,30,31,32,33,34]. Under low oxygen conditions, the PHD proteins are inactive, so the HIF α proteins are not hydroxylated and stable. They will translocate to the nucleus and dimerize with HIF1 β , leading to the transcription of target genes, such as EPO and VEGF, through the binding to specific DNA sequences (Hypoxia Responsive Elements, HRE) [8,35,36]. Aside from the regulation of the stability of the HIF α isoforms by PHDs, additional regulatory activities are identified. The oxygen-dependent asparaginyl hydroxylase Factor Inhibiting HIF (FIH), a member of the Fe(II) and 2-oxoglutarate-dependent dioxygenase, hydroxylates a conserved asparaginyl residue in the CTAD, preventing the association of HIF α with the p300 coactivator [37,38,39]. In addition to these hydroxylation dependent regulation of HIF α isoforms, several other posttranslational modifications have been identified (for review, see [8,40,41]).

The regulation and functions of the HIF3 α gene and isoforms is very complex, contrasting HIF1 α and HIF2 α . The *HIF3 α* locus gives rise to different splice variants, resulting in three protein isoforms, HIF3 α , NEPAS (neonatal and embryonic PAS) and IPAS (inhibitory PAS) [19,20,42]. HIF3 α and NEPAS only differ in the first eight N-terminal amino acids due to alternative exon usage. IPAS and NEPAS are hypoxia inducible, whereas HIF3 α is not because of alternative usage of promoters [43,44]. HIF3 α expression is induced under hypoxia in several organs, including cortex, hippocampus, lung, heart, kidney, cerebral cortex [17,45,46]. NEPAS is almost exclusively expressed during late embryonic and neonatal stages of development, especially in the lung and heart, while HIF3 α mRNA is rarely detectable during embryonic and neonatal stages [42]. HIF3 α has a high homology to HIF1 α and HIF2 α at the N-terminus, but only a low degree of sequence similarity across the C-terminus [26]. The HIF3 α /HIF1 β (HIF3) and NEPAS/HIF1 β dimers suppress basal and hypoxia induced reporter gene activation, as well as HIF1 (HIF1 α /HIF1 β) or HIF2 (HIF2 α /HIF1 β) driven expression [16,42]. HIF3 binds to HRE sites in promoter regions, but the transcriptional activity is much weaker than that of HIF1 and HIF2, because it lacks the CTAD [16,26,42]. Therefore, both HIF3 α and NEPAS serve as competitors of HIF1 and HIF2 dependent transcription, not only by occupying identical promoter regions, but also by associating with the same HIF1 β partner [16,42]. The splice variant IPAS lacks both the NTAD and CTAD domains producing a dominant negative regulator of the HIF1 α and HIF2 α dependent pathway [16,18,43]. It was shown that IPAS directly associates with HIF α isoforms, thereby displacing Hif1 β , and the resulting IPAS/Hif α dimer is unable to bind to DNA [18]. Both short HIF3 α isoforms related to IPAS in human and the IPAS in mouse have antagonistic effects on the expression of HIF1 and HIF2 dependent hypoxia regulated target genes [47]. Thus, the *HIF3 α* locus encodes isoforms generally thought to act as negative regulators of the hypoxic response.

The importance of the hypoxia response was shown by the identification of mutations in the VHL-HIF pathway in different human diseases (reviewed in [9]). Specific gene ablation studies in mice also added to the knowledge on the pleiotropic effects of the members of the hypoxia response pathway. Complete ablation of this pathway through inactivation of Hif1 β resulted in a severe

lethal phenotype with defective angiogenesis of the yolk sac and branchial arches, stunted development and embryo wasting [48,49]. Hif1 α knockout mice also died early during development with cardiac malformations and vascular defects [50]. Hif2 α null mice displayed a pleiotropic phenotype ranging from premature death until postnatal abnormalities, depending on the background of the mouse strain [51,52,53,54]. The neonates that survived suffered from breathing problems and did not produce sufficient surfactant phospholipids and surfactant associated proteins [51]. It is interesting to note that the inactivation and ectopic activation of Hif2 α showed comparable phenotypes, suggesting that type II cells require different levels of Hif2 α at distinct phases of type II cell maturation [51,55]. Homozygous mutant NEPAS/Hif3 α ^{-/-} mice were alive at birth, but displayed enlarged right ventricle and impaired lung remodelling, suggesting that NEPAS/Hif3 α is important in lung and heart development during embryonic and neonatal stages [42]. Interestingly, the *Hif3 α* gene contains hypoxia response elements in its promoter region and has been shown to be a transcriptional target of Hif1 α [56].

In order to understand the precise role of Hif3 α during pulmonary epithelium development, we generated transgenic mice with an inducible *HIF3 α* gene. Mice expressing the *HIF3 α* transgene in the developing airways showed a post-pseudoglandular branching defect with a reduced number of airspaces and a clear reduction in the number of alveolar type I and type II cells. Importantly, expression of the HIF3 α transgene did not lead to changes in the levels of Hif1 α , but affected Hif2 α . The lungs of the HIF3 α expressing mice showed an upregulation of genes normally expressed in the proximal parts of the lung, while genes only expressed in distal parts of the lung were downregulated. Specifically, Foxp2, a repressor of distal cell markers, and *Rarb* were induced in the lungs of Hif3 α expressing mice, which may explain the reduction in the number of distal cell types. Furthermore, we showed that Hif3 α binds a conserved HRE in the Sox2 promoter and induces the expression of a Sox2 promoter driven reporter gene, explaining the appearance of aberrant Sox2- and p63 positive cells. Collectively, our results show that Hif3 α is involved in modulating correct development of the lung epithelium.

Materials and Methods

Generation of transgenic animal

The myc epitope encoding sequence (EQKLISEEDL) was cloned directly after the endogenous ATG start codon of the full length human HIF3 α cDNA (GenBank: BC080551) and subcloned into a modified pTRE-Tight vector [55]. Transgenic lines were produced by pronuclear injection of FVB/N fertilized eggs, and tail tip DNA of transgenic lines was initially genotyped by Southern blot analysis, after which positive lines were routinely checked by PCR, using transgene-specific primers (sense: 5'-GTCAAGCTTATGGCGCTGGGGCTGCAGCG; antisense 5'-GCATCTAGATCAGTCAGCCTGGGCTGAGC). Three independent lines were initially analyzed, which all produced the same phenotype as described in this manuscript. Lung-specific expression of the HIF3 α transgene, i-Tg-mycHIF3 α , was obtained by crossing the mycHIF3 α lines with the SPC-rtTA transgenic mice (A generous gift of Jeffrey Whitsett). Administration of doxycycline to pregnant mothers from gestational age 6.5 onward in the drinking water (2 mg/ml, 5% sucrose) resulted in lung epithelium-specific expression. Each experiment was performed with at least three independent litters containing double transgenic, single transgenic and wild type pups. All double transgenic animals receiving doxycycline expressed mycHIF3 α in the pulmonary

epithelium and showed the described phenotype. Mice were housed under standard conditions at 40–50% relative humidity and 21 \pm 1°C (12/12 hour dark/light cycle) with food and water ad libitum. All animal experiments were performed according to the Dutch and European guidelines and approved by the local ethics committee (DEC Nr 1657, 1833 and 2206).

Immunohistochemistry

Immunohistochemistry was essentially performed as previously described [57]. Briefly, lungs were dissected and fixed in formal saline (BDH) overnight at 4°C before processing for paraffin embedding according to routine protocols. Antigen retrieval was performed with microwave treatment in 10 mM citric acid buffer pH 6.0 or Tris-EDTA. Sections were blocked with 5% BSA or 5% ELK in PBS for 10min and incubated with primary antibody diluted in 5% BSA or 5% ELK in PBS overnight at 4. The following antibodies were used: Myc (9E10, Roche; 4A6, Millipore), Hif3 α (Ab2165, Abcam; NBP1-03155, Novus Biologicals), β -tubulin IV (bioGenex), proSP-C (Chemicon), T1 α (University of Iowa Hybridoma bank), Ttf1 (Thermo), Ccsp (seven hills), Sox2 (seven hills), Foxp2 (Abcam), Lpcat1 (Seven hills Bioreagents), α -Sma (Thermo), Ki67, cGRP Secondary antibodies against the correct IgG species were conjugated with peroxidase (Dako).

Lungs were imaged using an Olympus BX41 microscope and DP71 camera (Olympus, Zoeterwoude, The Netherlands). Subsequent airspaces counting were performed with SIS Software Cell D (Olympus). Three independent samples of control and double-transgenic lungs of gestational age E18.5 were used to count the number of airspaces on a selected surface area (140000 μm^2) on those selected lung samples.

Microarray analysis

Lungs of three control and three double transgenic embryos were dissected at E18.5 and the middle and caudal lobes were used for total RNA isolation with Trizol reagent according to the manufacturer's instructions (Invitrogen life technologies, Carlsbad, CA, USA). RNA was purified using the RNeasy MinElute Cleanup kit. (Qiagen, Valencia, CA, USA) and cDNA was synthesized from 3 μg RNA using the GeneChip Expression 3'-Amplification Reagents One-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA, USA). Biotin-labelled cRNA synthesis, purification and fragmentation were performed according to standard conditions. Fragmented biotinylated cRNA was subsequently hybridized onto Affymetrix Mouse Genome 430 2.0 microarray chips. After normalization, the data were analysed with OmniViz software, version 3.6.0 (Omniviz, Inc., Maynard, MA, USA).

Functional annotation of the statistical analysis of microarrays results was done using Ingenuity Pathway Analysis (Ingenuity, Mountain View, CA) and DAVID (<http://david.abcc.ncifcrf.gov>). The results are shown for biological processes, which are significantly ($P < 0.05$) enriched after multiple testing.

RT-PCR

RNA isolation and subsequent quantitative PCR analysis was essentially performed as previously described [7]. Gene-specific primer sets were Abca3: 5'-TTACGGTCCAAGTTCCTGAG-3' and 5'-TAACATCAGCACCTTAGAGCC-3'; Aqp5: 5'-GTGGTCATGAATCGGTTTACAG-3' and 5'-CAAGTAGAAGTAGAGGATTGCAG-3'; Epa1: 5'-CTGTGACGACGAATCTTGG-3' and 5'-GGCATGGTAGAACTCATAGG-3'; Foxp2: 5'-TGTCATCAGAGATTGCC-3' and 5'-ATAGCCTGCCTTATGAGTG-3'; Rarb: 5'-AACTGCGT-

CATTAACAAGGTC-3' and 5'-TCATTCCTAACAGACTCTTTGG-3'; Scd1: 5'-GAGCCACAGAACTTCAAGG-3' and 5'-GTACACGTCATTCTGGAACG-3'; Sfpd: 5'-GGAAGCAATCTGACATGCTG-3' and 5'-GAGGCTCTTCATTCTGCTC-3'. Standard deviations of the duplicates are calculated with the SPSS program (Independent-samples T test), which also generated the P values.

Luciferase reporter activity assays

HEK293T cells were transfected in duplo with Lipofectamine 2000 (Invitrogen) with a total concentration of 500 ng DNA/well, using 9*HREluc (Gift from Manuel Landazuri), pGL3-mpSox2 and pGL3-mpSox2delta (Named Sox2-Luc and Δ Sox2-Luc; Gift from Victoria Moreno), Hif2 α -pcDNA3, (gift from Carole Peyssonnaud), Hif3 α -pcDNA3 or pcDNA3. Cells were lysed with passive lysis buffer (Promega) 24-hours after transfection and processed for luciferase analysis by the addition of the LARII reagent (Promega), which was subsequently quantified with the VICTOR luminometer. A construct containing the renilla gene (10 ng/well) was co-transfected in each well to serve as an internal control for transfection efficiency. The renilla luciferase activity was quantified by addition of Stop&Glo reagent and also detected with the VICTOR luminometer. The experiment was repeated three times, and all samples were measured at least in duplo. The average luciferase activity was calculated and divided by the average of renilla activity. Standard deviations were measured with the SPSS program (Independent-samples T test), which also generated the P values.

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed essentially as previously described [58], with some modifications. Chromatin-protein complexes of confluent A549 cells were fixed by adding 1% formaldehyde to the cultures. Nuclear extracts were made and chromosomal DNA was fragmented by sonication. Equal amounts of DNA was diluted 1:10 with ChIP dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1 and 167 mM NaCl) and the samples were pre-cleared with 80 μl prot A/G agarose beads for 1 hour, after which the sample was split in equal volumes and incubated O/N with 6 μg antibody specific for HIF3 α (NBP1-03155) or control IgG (rabbit). Immune complexes were subsequently purified by adding 80 μl of prot A/G beads, which were washed several times before the immune-precipitated DNA was eluted with elution buffer (1% SDS and 0.1 M NaHCO₃). After de-crosslinking the DNA-protein complexes by incubation at 65°C O/N with 200 mM NaCl, the eluted DNA was phenol-extracted, precipitated and qPCRs were performed to analyze the enrichment of HIF3 α specific binding to the HRE in the SOX2 gene using the following primer set 5'-CAAGTGCATTTTAGC-CACAAAG-3' and 5'-CCCAAGAGGGTAATTTTAGCCG-3', while the primers for the ARRDC3 and EGLN3-D were described previously [36,59]. The data are the average of two independent ChIP assays, which were each analyzed by duplicate qPCRs, and are represented as the fold enrichment of the specific immune precipitation compared to the control IgG precipitation.

Results

Ectopic expression of mycHIF3 α causes late branching defects

Previously, it was shown that homozygous NEPAS/Hif3 α knockout mice were viable, but displayed an enlarged right ventricle and impaired lung remodelling, suggesting that Hif3 α plays an important role during pulmonary development. However,

the precise role of Hif3 α during the formation of the lung is not fully understood. We first analyzed the endogenous expression of Hif3 α in normal fetal lungs isolated at the end of gestation (E18.5) and in lungs of adult mice (8 weeks). Hif3 α positive cells were present in the epithelium of the developing lung, as well as in the type II pneumocytes of the adult lung (arrows in Figure 1A, B). In order to determine the precise role of Hif3 α in the epithelium during lung development, and more specifically in type II pneumocytes, we generated transgenic mice carrying a myc-epitope tagged HIF3 α under the control of a doxycycline-inducible tet-on promoter (i-Tg-mycHif3 α ; Figure 1C). Expression of mycHIF3 α in embryonic lung epithelium was established by crossing the i-Tg-mycHIF3 α transgenic line with the established SPC-rtTA line, which drives the expression of the rtTA gene in epithelial cells of the embryonic lung [60]. Pregnant females from timed matings between SPC-rtTA and i-Tg-mycHIF3 α mice received doxycycline to induce the expression of the HIF3 α transgene in double-transgenic fetuses. Lungs isolated from doxycycline-induced or non-induced single i-Tg-mycHIF3 α or SPC-rtTA transgenic mice, or lungs from non-induced double transgenic i-Tg-mycHIF3 α /SPC-rtTA animals appeared indistinguishable from wild type lungs. Doxycycline-induced, double-transgenic pups were born at Mendelian ratio and did not show obvious external abnormalities compared to their control litter mates.

In order to determine whether expression of mycHIF3 α leads to pulmonary development defects, we analyzed lungs of double-transgenic animals and control lungs at different gestational ages. Macroscopic analysis of isolated lungs did not show clear abnormalities in double-transgenic animals at gestational ages E16.5, E17.5, E18.5 days and postnatal day 1 (PN1) (Figures 1E and F, I and J; Figure S1). Histological examinations at E16.5 did not show clear differences between control and mycHIF3 α transgenic lungs (Figures S1C and D). However analysis of a series of developmental ages clearly showed aberrant alveolar airspaces in mycHIF3 α expressing lungs starting at E17.5 compared to controls (Figure S1G, H). mycHIF3 α expressing lungs contained significant fewer alveolar spaces compared to control ones at E18.5 and PN1 (Figures 1D). Staining with a specific antibody against the myc-epitope confirmed the expression of transgenic mycHIF3 α protein in the epithelium of double-transgenic lungs (Figures 1H and L, Figure S1). The abnormal alveolar spaces remain present in the PN1 stages, but apparently, the mice do not suffer from respiratory distress, indicating that the initial requirements for life are present. So, we conclude that mycHIF3 α expression in epithelial cells leads to aberrant alveolar formation and affects late branching morphogenesis during pulmonary development.

This post-pseudoglandular branching defect prompted us to analyze the expression of the mycHIF3 α at early embryonic stages of development. This showed that the transgene is expressed in a non-uniform manner in the epithelium of early E11.5 lungs (Figure 2A), but gradually all epithelial cells express the transgene (Figure 2B-D). Next, we analyzed whether the primary airway branches appropriately expressed some of the major branch-inducing genes [2]. Therefore, embryonic lungs of controls and double transgenic animals were isolated at gestational age 12.5. At this stage of development, the primary bronchi are already present, and these branches start to form secondary and tertiary branches. The expression of *Fgf10*, the growth factor with a very potent branch-inducing activity, was found in the mesenchymal compartment, alongside the epithelium that is in the process of branching (Figure 2E and I, arrows). Moreover, its receptor, *FgfR2*, was detected at the tips of the epithelium, in close proximity

of the *Fgf10* signal (Figure 2F and J). Next, we also analyzed the expression of two genes known to be induced as a result of the *Fgf10*-*FgfR2* signalling, *Shh* and *Bmp4*. Both genes were also expressed in the epithelium at the same location as the *FgfR2*, indicating that the *Fgf10*-*FgfR2* signalling cascade is intact (Figure 2G and K; H and L). In addition, quantitative PCR analysis of embryonic lungs isolated at E12.5, E15.5 and E17.5 of controls and double transgenic mice using primers specific for *FgfR2*, *FgfR2-IIIb*, *FgfR2-IIIc*, *Bmp4* and *Spry* did confirm the absence of differential expression of these important branch-inducing genes (data not shown). In conclusion, no differences in expression pattern were observed for the early branch-inducing genes between controls and double transgenics, suggesting that the initiation of the branching process occurred normally.

mycHIF3 α expression inhibits Clara cells differentiation

Since we observed significant alveolar changes and aberrant branching morphogenesis, we analyzed the integrity and differentiation potential of fetal transgenic lungs by immunohistochemistry with cell-specific markers. The smooth muscle cell component of the mesenchyme (α -Sma) did not reveal striking differences between control and transgenic lungs (Figures 3A, B). Thyroid transcription factor (Ttf1) was expressed in nearly all epithelial cells in both control and transgenic lungs (Figures 3C, D). Ciliated cells (β -tubulin) and neuroendocrine cells (cGRP) were present in proximal conducting airways of control and transgenic lungs at gestational age E18.5 (Figures 3E, F and 3G, H, arrows). Moreover, both type I (T1 α ; Figure 4A, B) and type II pneumocytes (Lpcat1; Figures 4C, D) were present in the alveolar regions. These results indicate that differentiation into the various epithelial cell types is not hampered by Hif3 α , although the total number of each cell type may be different. In addition, no differences were observed in the proliferation of epithelial or mesenchymal cells between control and transgenic lungs as indicated by Ki67 staining (Figure 4E, F).

Next, three mycHIF3 α -expressing lungs and three control lungs were processed at gestational age 18.5 days for microarray analysis, to elucidate the origin of the aberrant branching morphogenesis. Hierarchical clustering of differentially expressed genes revealed large differences between controls and double transgenic lungs (Figure 5A) and the major biological processes (Figure 5B) and molecular functions (Figure 5C) are indicated. Although mycHIF3 α does not prevent the differentiation of epithelial cells into Clara cells, we noticed that the number of Clara cells was significantly reduced. Both in the microarray analysis as well as the qPCR validation showed downregulation of the *Ccsp* gene in mycHIF3 α transgenic mice. These gene expression results were confirmed by immunohistochemistry, showing that *Ccsp* positive cells were less prominent in the proximal airways of the Hif3 α expressing lungs compared to control lungs (Figures 6A-D). Quantification of the total number of Clara cells revealed a significant reduction in the double transgenic mice (Figures 6H). So, our data show that mycHIF3 α expression inhibits Clara cells differentiation during pulmonary development.

mycHIF3 α induces airway epithelial cells to differentiate into proximal cell types

Analysis of the microarray data revealed that genes associated with proximal cell types of the lung appeared to be upregulated, whereas genes specifically expressed in distal epithelial cells were downregulated (Table 1 and Table 2). The induction of proximal markers is reflected by the significant downregulation of genes specific for the distal lung epithelium. The type 1 pneumocyte cell

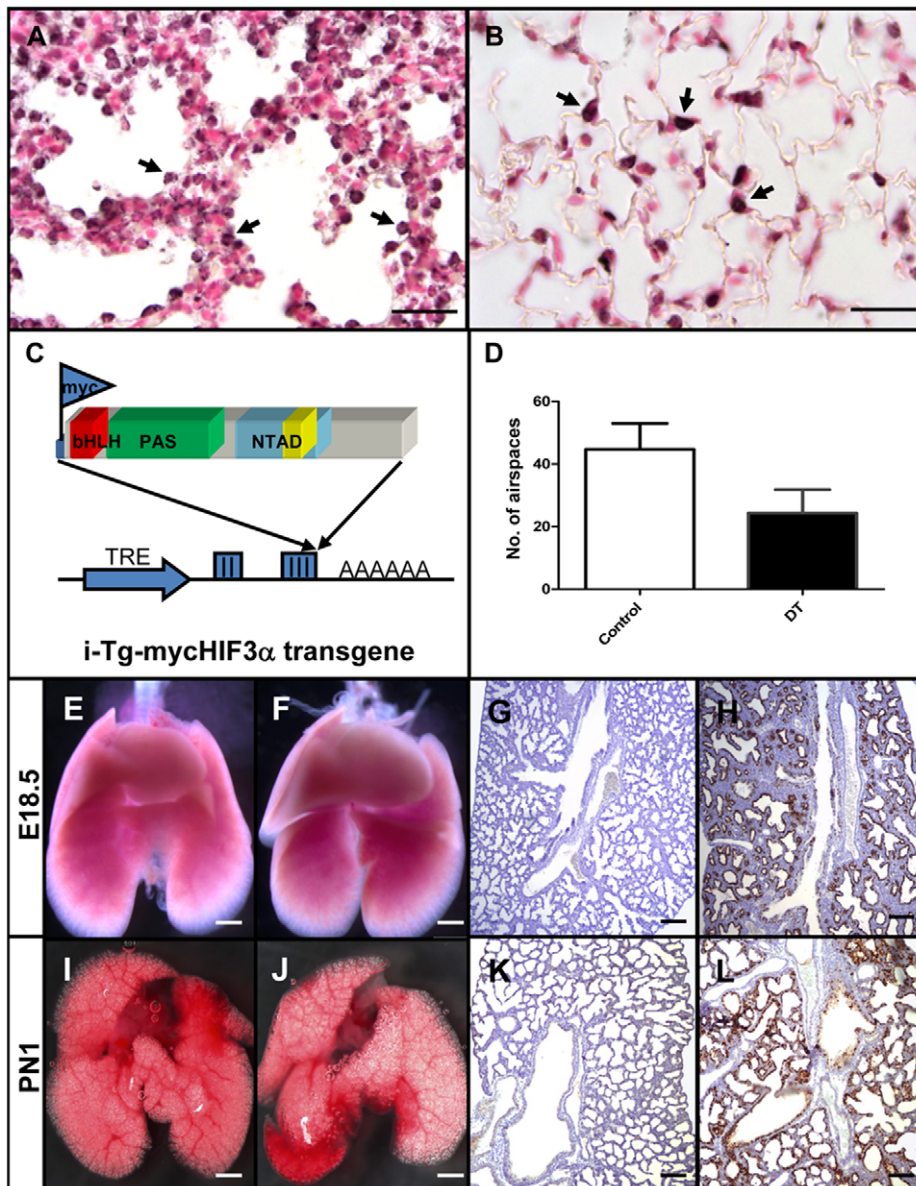


Figure 1. Enhanced expression of HIF3 α results in late branching defect. Endogenous expression of Hif3 α was detected in epithelial cells at gestational age E18.5 (A, arrows) and in type II pneumocytes in adult mice (B, arrows). (C) Graphic representation of the tet-inducible Hif3 α /NEPAS cDNA construct used to generate transgenic mice. TRE is the Tet-responsive element containing minimal promoter, II and III refer to exon 2 and 3 of the β -globin gene and AAAAAA is the poly-adenylation signal. Indicated are the position of the myc-epitope, and the bHLH, PAS and NTAD domains (see text) (D) Quantification of the number of airspaces in the lung. Three independent samples of control and double-transgenic lungs at gestational age E18.5 were used to count the number of airspaces. External appearances of control (E, I) and double transgenic mycHIF3 α (F, J) lungs at E18.5 days of gestation (E18.5) and post natal age 1 (PN1) do not show apparent differences. Histological analysis of control (G, K) and double transgenic lungs (H, L) showed decreased number of alveolar spaces and reduced branching in the double transgenic lungs (H and L). Anti-Myc epitope staining confirmed the expression of the mycHIF3 α transgene in double transgenic lungs (H and L), which is absent in control lungs (G and K). Scale bars: Scale bars: 25 μ m (A, B), 2 mm (E, F, I, J) or 200 μ m (G, H, K, L). doi:10.1371/journal.pone.0057695.g001

marker Aquaporin 5 (*Aqp5*) was downregulated in the Hif3 α expressing mice, as were three genes specifically expressed in type II pneumocytes, stearoyl-coenzyme A desaturase (*Scd1*), surfactant associated protein D (*Sftpd*) and ATP-binding cassette (ABC) subfamily A3 (*Abca3*) (Figure 6E) [61,62,63]. Quantification of the number of type II pneumocytes present in the Hif3 α expressing lungs using *Sftpd* in reference to *Tcf1* confirmed a significant reduction in these cells (Figure 6G). Since we are inducing the Hif3 α family member of hypoxia inducible genes, we analyzed the

expression of Hif1 α and Hif2 α in the transgenic lungs. Although no apparent difference could be detected for Hif1 α (Figure 6F), but we did notice a significant downregulation of Hif2 α (*Epas1*) (Figure 6E). Previously, we showed that Hif2 α is involved in maturation of type II pneumocytes, so the reduction of *Epas1* expression could be directly related to the loss of type II cells.

Among the upregulated genes are two transcription factors known to play important functions during lung development, *Foxp2* and *Sox2* [57,64]. *Foxp2* is important during lung

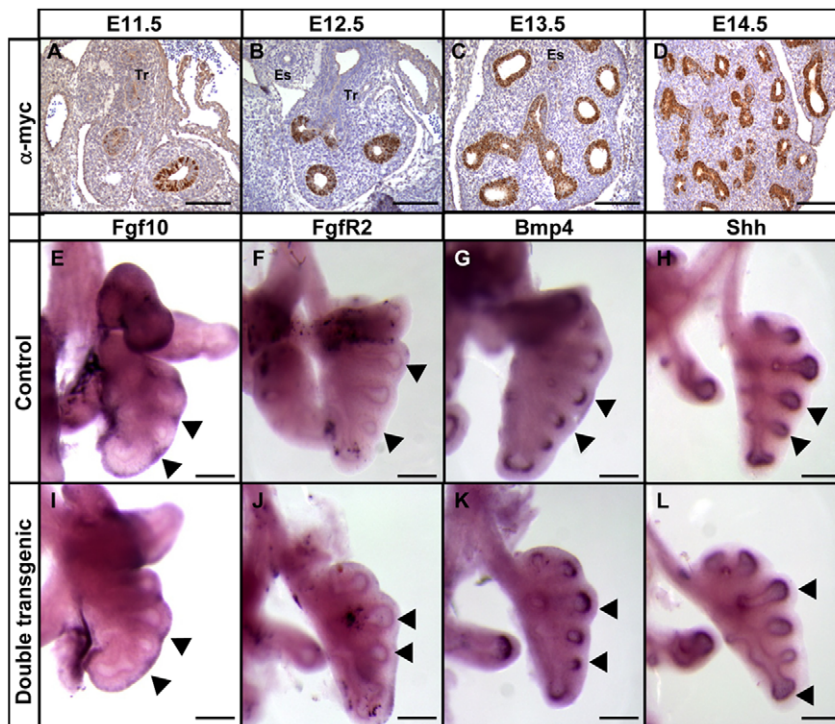


Figure 2. Expression of genes involved in branching morphogenesis. Analysis of the distribution of mycHIF3 α early in lung development in double transgenic animals at E11.5 (A), E12.5 (B), E13.5 (C) and E14.5 (D). Whole mount in situ hybridization to detect the expression and localization of Fgf10 (E and I), FgfR2 (F and J), Bmp4 (G and K) and Shh (H and L) in lungs isolated at gestational age E12.5 from control (E–H) and mycHIF3 α double transgenic animals (I–L). Tr: Trachea; Es: Esophagus. Scale bars: 200 μ m. doi:10.1371/journal.pone.0057695.g002

development and is expressed in the distal parts of the lung. It represses the transcription of several distal cell markers, such as T1 α , Spc, and Ccsp [65]. In our microarray analysis, *Foxp2* was significantly upregulated, which we validated by quantitative PCR (Table 1 and Figure 7G). Staining with a *Foxp2* antibody show that the distribution of *Foxp2* positive cells in Hif3 α double transgenic lungs was expanded compared to control lungs (Figures 7A, D), suggesting that Hif3 α suppressed the transcription of genes specific for alveolar epithelial cells through the induction of *Foxp2*. In addition, *Rar β* , which is expressed at proximal sites in the lung from embryonic day 11 to 12 and not in the distal epithelium of the lung [66,67], was significantly induced in Hif3 α transgenic mice (Figure 7G), confirming the expansion of proximal cell makers in these lungs [64,65].

Sox2 is important for pulmonary branching morphogenesis, epithelial cell differentiation and is exclusively expressed in the proximal parts of the lung [57]. However, in mycHIF3 α expressing lungs, Sox2 is present in epithelial cells of both proximal airways and certain alveoli at postnatal day 1, suggesting that Hif3 α is able to induce proximal cell fate (Figures 7B, E, arrows). The basal cell marker p63 is expressed in the esophageal and tracheal epithelium, and previously we showed that ectopic Sox2 expression induced the appearance of p63 positive cells in the epithelium of the bronchioles and enlarged distal airspaces [57]. Therefore, we analysed the distribution of basal cells in the mycHIF3 α expressing lungs and found that p63 is abnormally expressed in the alveolar epithelial cells of mycHIF3 α expressing lungs, contrasting the unique expression in the trachea (Figures 7C insert, arrows F). Collectively, our data indicate that mycHIF3 α expression leads to the induction of crucial genes, such as Sox2,

Foxp2 and *Rar β* , which cause airway epithelial cells to differentiate into proximal cell types.

Hif3 α binds the promoter region of Sox2 and induces transcription of Sox2

The promoter region of the *Sox2* gene contains two functional HREs, which are bound by Hif2 α [68]. Since Sox2 is upregulated in Hif3 α transgenic lungs, we analyzed whether Hif3 α can directly induce the transcription of Sox2. Therefore, we first performed transcription reporter assays using a luciferase reporter construct under the influence of the Sox2 promoter containing two HREs, or two mutated HREs (Sox2-Luc and Δ Sox2-Luc [68]). Hif3 α induced the expression of the Sox2-Luc promoter about 2 fold, whereas the Δ Sox2-Luc promoter was hardly induced compared to controls (Figure 7H). The positive control, HRE, was considerably induced by Hif2 α , but only mildly by Hif3 α , corresponding with the weak transcriptional activity of Hif3 α [16,42]. Under hypoxia-mimicking conditions, induced by adding CoCl₂ to the medium, which inhibits prolyl hydroxylases by displacement of Fe(II) from their catalytic center [22], Hif3 α could induce the 9*HRE-Luc considerably, and the difference with the Hif2 α induced expression was much reduced (10 times versus 2 times). Moreover, the induction of the Sox2-Luc construct by Hif3 α was 4 times higher than under normoxic conditions, and was comparable between Hif2 α and Hif3 α (Figure 7H). Subsequent analysis of the 1 kilobase region immediately upstream of the Sox2 transcriptional start site revealed that the most upstream of the two putative HRE sites was highly conserved between mice and human [68]. In order to investigate whether Hif3 α could directly bind this conserved HRE site, we performed a chromatin immunoprecipitation of chromatin-protein complexes isolated

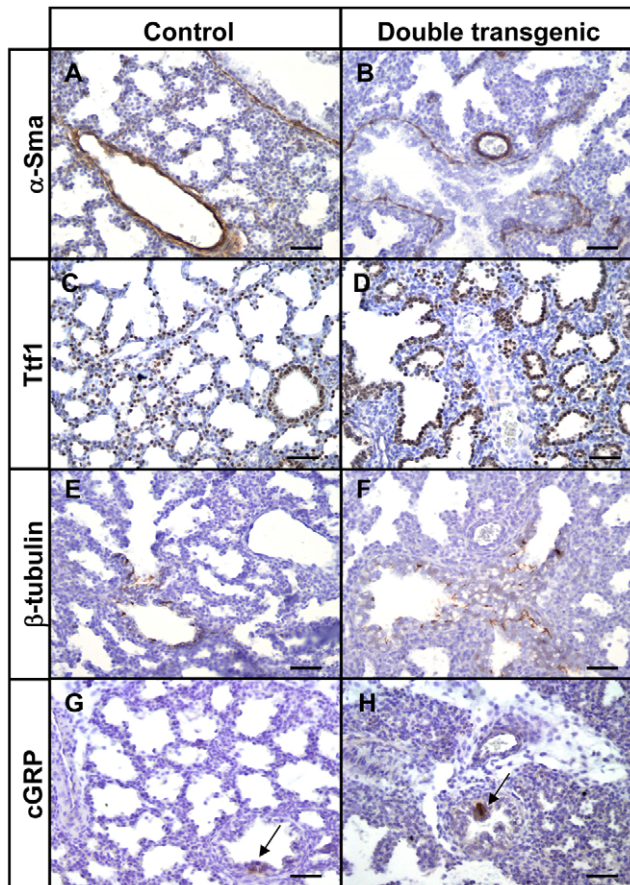


Figure 3. Normal differentiation of proximal epithelial cells in mycHIF3 α transgenic lungs. The site and expression pattern of α -Sma (A and B), Ttf1 (C and D), β -tubulin (E and F) and cGRP (arrows in G and H) are comparable between control and mycHIF3 α double transgenic lungs at gestational age E18.5. Scale bars: 100 μ m. doi:10.1371/journal.pone.0057695.g003

from human A549 cells. Analysis of the HIF3 α precipitated chromatin showed that the region containing the conserved HRE site in the SOX2 promoter region was indeed preferentially enriched compared to the IgG fraction (Figure 7I). ARRDC3 was used as a potential positive control, as it is bound by both HIF1 α and HIF2 α , and the enhancer region D of the EGLN3 gene served as negative control [36,59]. Indeed, HIF3 α did not bind to the EGLN3-D region, but did bind to the ARRDC3-HRE. This indicated that HIF3 α could bind the HRE site present in the Sox2 promoter, suggesting a potential direct regulatory role of Hif3 α in the transcription of Sox2.

So, Hif3 α binds to the conserved HRE in the Sox2 promoter and weakly induces Sox2 expression, resulting in an abnormal Sox2 expression in airway epithelial cells of HIF3 α transgenic lungs.

Discussion

Hypoxia inducible factors are an important family of proteins involved in the regulation of the cellular response to hypoxia. Its functions are required from the earliest steps of mammalian life to the correct development of multiple organs and tissues, like the placenta, trophoblast formation, bone development, heart and vascular development (reviewed in [6,8]). The importance of the hypoxia response was shown by the identification of human

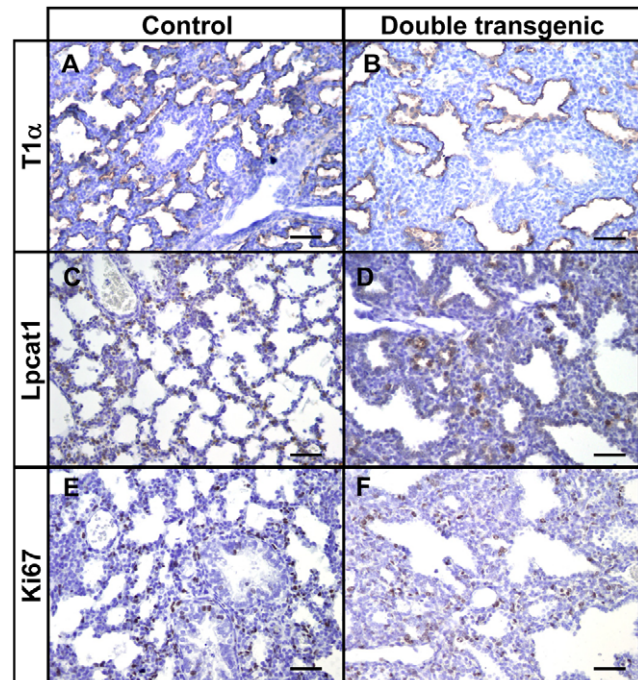


Figure 4. Normal differentiation of distal epithelial cells in mycHIF3 α transgenic lungs. The site and expression pattern of T1 α (A and B), Lpcat1 (C and D) and Ki67 (E and F) are comparable between control and mycHIF3 α double transgenic lungs at gestational age E18.5. Scale bars: 100 μ m. doi:10.1371/journal.pone.0057695.g004

mutations in the VHL-HIF pathway in different diseases [9]. Gene ablation studies in mice have revealed in more detail the specific and important roles of the different subunits of the Hif α /Hif β heterodimers. Inactivation of the stable subunit, Hif1 β , resulted in severe embryonic defects and premature death [48,49]. The disruption of the different Hif α genes identified specific roles for the individual Hif α isoforms. Hif1 α knockout mice die early at gestation, have multiple developmental defects in neural tube-formation, vascularization, heart development, neural crest migration [69,70,71], whereas depending on the genetic background of the mouse strain, Hif2 α knockout mice ranging from early embryonic lethality to adulthood [51,52,53,54].

Hif genes and lung development

The lung is under continuous exposure of external oxygen and several (patho)-physiologic conditions trigger global or local hypoxia in the lung, resulting in pulmonary abnormalities to which HIFs contribute, such as lung cancer, acute lung injury and pulmonary hypertension (reviewed in [72]). Long term changes in oxygen levels, as experienced at high altitude gives rise to lung damage as a result of chronic mountain sickness. Recently, the *EPAS1* gene, encoding for HIF2 α , was shown to be associated with adaptation of living at high altitude [73,74,75,76].

Inactivation of Hif2 α in mice resulted in respiratory distress and surfactant deficiency in newborns on a mixed genetic background [51]. Remarkably, heterozygous Hif1 α ^{+/-} or Hif2 α ^{+/-} mice showed a reduced increase in pulmonary arterial pressure and right ventricular hypertrophy upon exposure to chronic hypoxia in comparison with wild type mice [77,78]. Ectopic expression of an oxygen-insensitive Hif1 α transgene in lung epithelial cells during development resulted in defective branching, impaired epithelial maturation and respiratory distress. Moreover, increased expres-

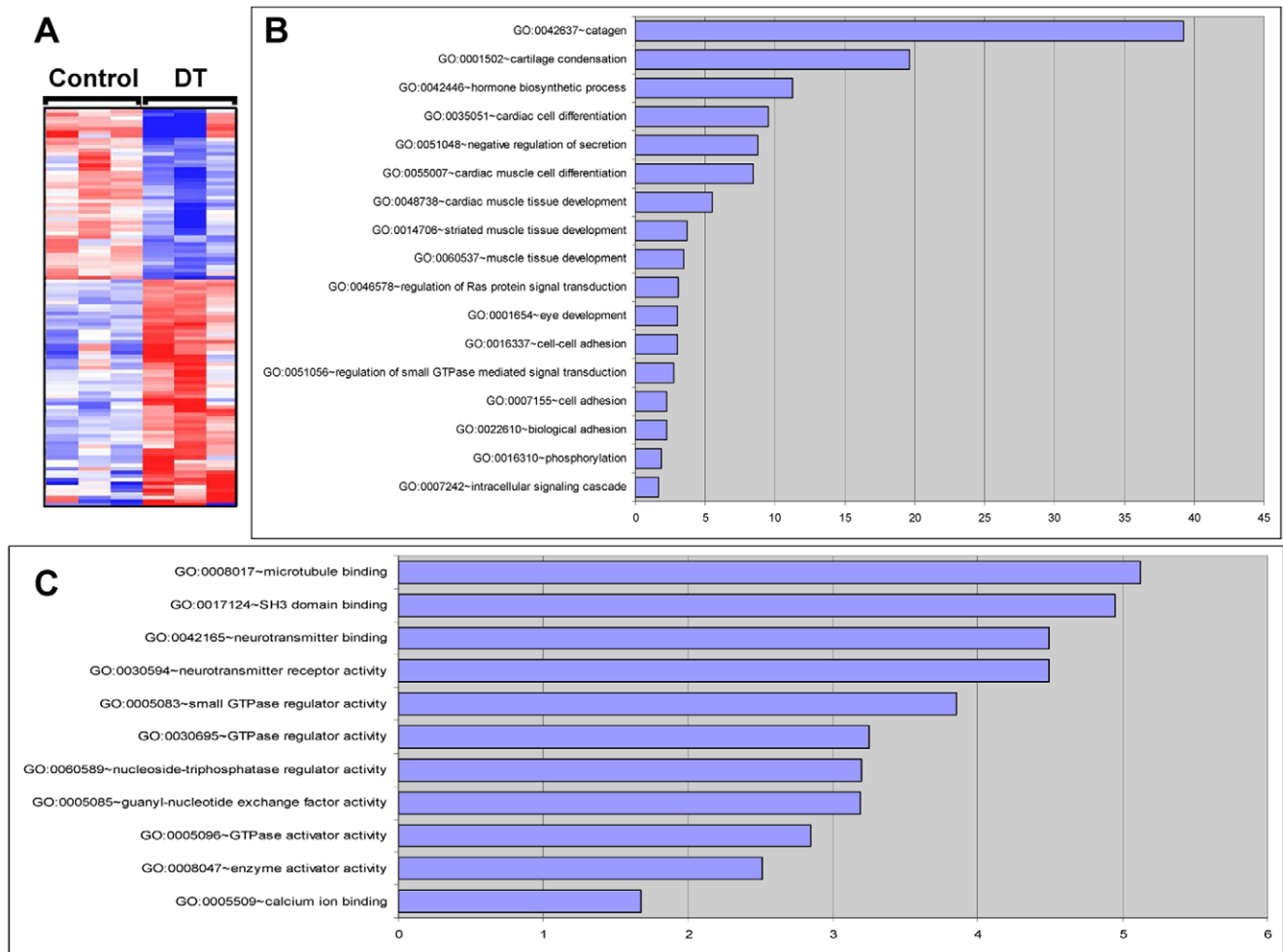


Figure 5. Transcriptome analysis of mycHIF3 α expressing lungs. Treescapes showing that the transcriptome of the lungs of the mycHIF3 α expressing animals are significantly different from that of the control lungs (A). The red color indicates the upregulated genes and the blue color indicates downregulated genes. The expression of the genes presented in the treescapes is at least 1.5 fold changed with a false discovery rate (FDR) of 10%. The top 10 biological processes (B) and molecular functions (C) of the differentially expressed genes are shown. doi:10.1371/journal.pone.0057695.g005

sion of VegfA and VegfC was observed, leading to sub-pleural hemorrhaging [79]. We recently showed that the transgenic expression of an oxygen-insensitive mutant of Hif2 α also lead to a late branching defects with enlarged alveoli and altered epithelial differentiation [55]. Contrasting the Hif1 α transgenic study, we did not find increased levels of VegfA or endothelial abnormalities, even though the transgenes were expressed in the same manner. This indicates that Hif1 α and Hif2 α have different effects. In addition, the expression of Hif1 α had not changed, whereas Hif3 α expression was reduced in our Hif2 α transgenic mice [55]. It seems that the effects of Hif1 α are more widespread, whereas the number of affected genes by Hif2 α is restricted, which is in line with previous reports describing target genes of Hif1 α and Hif2 α [35,36,80,81,82,83,84,85].

The occurrence of the Hif3 α isoforms is well described transcriptionally, but the functional analysis is complicated by the appearance of different splice variants [19,26,42,43,86]. Hif3 α isoforms act as negative regulators of the traditional Hif1 (Hif1 α /Hif1 β) and/or Hif2 (Hif2 α /Hif1 β) driven hypoxia response by functioning as dominant negative modulators, effectively resulting in the transcriptional competition with Hif1 and Hif2 [16,18,26,42,43]. Gene ablation of Hif3 α /NEPAS/IPAS, resulted

in mice that were born alive with enlarged right ventricles and impaired lung remodelling [42]. Furthermore, they showed that expression of endothelin-1 is negatively influenced by Hif3 α /NEPAS, by regulating the binding of Hif1 α and Hif2 α to the HRE sites if the ET-1 promoter, which may contribute to the observed phenotype. Remarkably, the expression of Vegf, a direct target of Hif1 and Hif2, had not changed, even though the expression of Hif1 α and Hif2 α was not affected. This hinted at a selective regulation of target genes by NEPAS/Hif3 α during pulmonary development. Therefore, we conditionally expressed mycHIF3 α in airway epithelial cells during embryonic development in order to further elucidate the role of Hif3 α in pulmonary development.

Cellular effects of mycHIF3 α transgene expression

Since the NEPAS/Hif3 α knockout mice suggested a selective regulation of genes by Hif3 α , and our Hif2 α transgenic mice showed a selective reduction in Hif3 α expression, we conditionally expressed mycHIF3 α in airway epithelial cells during embryonic development in order to further elucidate the role of Hif3 α in pulmonary development. Analysis of mice expression a transgenic mycHIF3 α in lung epithelium revealed a late branching morpho-

Table 1. Significant upregulated genes in the mycHIF3 α expressing lungs.

Gene symbol	Gene name	Entrez ID	Fold Change
Dub2a	deubiquitinating enzyme 2a	384701	6,22
Naaladl2	N-acetylated alpha-linked acidic dipeptidase-like 2	635702	2,16
Cldn6	claudin 6	54419	2,14
Hspa1a	heat shock protein 1A	193740	2,14
Fbn2	fibrillin 2	14119	2
ATP6	ATP synthase F0 subunit 6	17705	1,87
Rimkb	ribosomal modification protein rimK-like family member B	108653	1,83
Sema3e	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3	20349	1,8
Tinag	tubulointerstitial nephritis antigen	26944	1,71
Mia1	melanoma inhibitory activity 1	12587	1,68
Plac1	placental specific protein 1	56096	1,68
Cdh16	cadherin 16	12556	1,64
Cnksr2	connector enhancer of kinase suppressor of Ras 2	245684	1,64
Mthfd2l	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	665563	1,63
Pcgf1	polycomb group ring finger 1	69837	1,61
Pfn2	profilin 2	18645	1,61
Hspe1	heat shock protein 1 (chaperonin 10)	15528	1,58
Fmod	fibromodulin	14264	1,54
Cdh3	cadherin 3	12560	1,54
Maob	monoamine oxidase B	109731	1,54
Rpl23a	ribosomal protein L23a	268449	1,53
Flrt2	fibronectin leucine rich transmembrane protein 2	399558	1,53
Lgals12	lectin, galactose binding, soluble 12	56072	1,53
Nnat	neuronatin	18111	1,53
Rasef	RAS and EF hand domain containing	242505	1,53
Egfl6	EGF-like-domain, multiple 6	54156	1,53
Ctnd2	catenin (cadherin associated protein), delta 2	18163	1,52
LOC674930	similar to suppressor of initiator codon mutations, related sequence 1	674930	1,5
Sox2	SRY-box containing gene 2	20674	1,57
Foxp2	forkhead box P2	114142	1,51

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genesis defect with a reduced number of alveoli and changes in the differentiation of epithelial cell types.

Surprisingly, no apparent defects are observed early during lung development, even though the transgene is expressed. This may be due to the fact that at these stages of development, putative associating factors of Hif3 α , like Hif2 α and Hif2 α , are not expressed yet. After the pseudoglandular stage of lung development, endogenous Hif2 α becomes expressed in the cells positive for mycHIF3 α and the effect of the mycHIF3 α transgene starts to be noticeable. Histological analysis and gene expression profiling revealed changes in the differentiation of the developing pulmonary epithelium. We found reduced numbers of Clara cells, alveolar type I and type II cells, and in addition, basal cells were observed in atypical spatial positions. The expression pattern of diverse sets of genes was affected, and revealed that mycHIF3 α expression mainly affects Hif2-directed transcription, although not all Hif2 target genes are equally affected. We show that expression of mycHIF3 α in epithelial cells results in a down regulation of Hif2 α , but not of Hif1 α . This suggests that Hif3 α is not a global regulator of the hypoxic response, but that Hif3 α may selectively

function to modulate Hif2 α controlled target genes, supporting previous work [42]. The reduction in the expression level of Hif2 α late in gestation may be due directly to the presence of mycHIF3 α , or due to the impaired differentiation of the type II cells. However, it is clear that mycHIF3 α does affect the differentiation of epithelial cells, and this could partly be explained by the aberrant activation of specific genes that are not part of the hypoxic response. Gene expression analysis does not show significant changes in typical hypoxia responsive genes, which indicates that Hif3 α may have specific functions beyond the hypoxia response. Therefore, we provide first evidence for novel Hif3 α functions beyond the hypoxia response.

The apparent increase in the mesenchymal compartment after the pseudoglandular stage does not seem to be induced by proliferation, as we did not observe an increase in mitotic cells in the mycHIF3 α lungs. It may be due to either a delayed development of the double transgenic lungs, or, alternatively, to a specific response in epithelial cells triggered by mycHIF3 α . Lysyl oxidase may be activated, which subsequently activates a cascade of proteins, such as Snail, involved in the repression of E-cadherin,

Table 2. Significant downregulated genes in the mycHIF3 α expressing lungs.

Gene symbol	Gene name	Entrez ID	Fold Change
Olfir767	olfactory receptor 767	258315	0,45
Ass1	argininosuccinate synthetase 1	11898	0,53
Pgam2	phosphoglycerate mutase 2	56012	0,56
Gipr	gastric inhibitory polypeptide receptor	381853	0,58
Olfir6	olfactory receptor 6	233670	0,6
Igfbp6	insulin-like growth factor binding protein 6	16012	0,61
Nppa	natriuretic peptide precursor type A	230899	0,61
Dio3	deiodinase, iodothyronine type III	107585	0,63
Mphosph6	M phase phosphoprotein 6	68533	0,64
Plscr2	phospholipid scramblase 2	18828	0,64
Ccin	calicin	442829	0,65
Fabp5	fatty acid binding protein 5, epidermal	16592	0,65
Nudcd3	NudC domain containing 3	209586	0,65
Olfir171	olfactory receptor 171	258960	0,65
Rtl1	retrotransposon-like 1	353326	0,66
Rasgrf2	RAS protein-specific guanine nucleotide-releasing factor 2	19418	0,66
Fabp12	fatty acid binding protein 12	75497	0,66
Scnn1a	sodium channel, nonvoltage-gated 1 alpha	20276	0,66
<i>Surfactant related genes</i>			
Scd1	stearoyl-Coenzyme A desaturase 1	20249	0,31
Sftpd	surfactant associated protein D	20390	0,65
<i>Clara cells marker</i>			
Scgb1a1(ccsp)	secretoglobin, family 1A, member 1 (uteroglobin)	22287	0,65
<i>Type I pneumocytes marker</i>			
Aqp5	aquaporin 5	11830	0,65

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and ultimately leading to epithelial-mesenchymal transition, as described for metastatic tumors [87,88].

Genes affected by mycHIF3 α

The appearance of proximal cells at the expense of distal cells in the mycHIF3 α lungs is paralleled by transcriptional changes in several genes, such as Sox2, Rar β and Foxp2. At this point, it remains to be seen whether all effects observed are directly related to mycHIF3 α , or that the expression of mycHIF3 α affects Hif1 α and Hif2 α specific complexes, thereby interfering with transcription of specific genes. The increased expression of mycHIF3 α could lead to the formation of complexes that normally are not present in the cell, which would shift the balance between Hif2 α and Hif3 α .

We observed Sox2 positive cells at unusual sites in the lung, which was supported by the aberrant presence of p63 positive basal cells. Previously, we showed that Sox2 directly induces the appearance of basal cells [57]. Since a link was found between Hif2 α and Sox2 transcription [68], we analyzed the putative regulation of the Sox2 gene by Hif3 α . We show that Hif3 α is capable of inducing basal expression of a reporter construct under the control of the Sox2 promoter containing two HRE sites. In addition, we show that HIF3 α binds to the conserved HRE sequence in the Sox2 promoter, which suggests that Hif3 α may contribute directly to the regulation of Sox2 expression. However, the minimal transcriptional activity of Hif3 α , as also shown previously, may explain the appearance of only scattered Sox2

positive cells in the lungs of mycHIF3 α mice [16,26,42]. In addition, depletion of individual HIF α genes by siRNA in human ES cells suggested that HIF3 α upregulates HIF2 α , which subsequently induced the expression of stem cell marker genes, like SOX2 [89]. Although this hypothesis is intriguing, no direct relationship was established, yet. It was also shown that ectopic expression of HIFs in cancer cell lines can induce embryonic stem cell markers, like SOX2 and NANOG [90]. The combination of weak transcriptional activity and the ability to act as a dominant negative modulator of Hif2 α may be responsible for the transcriptional regulation of Sox2. These results directly show that through the expression of HIF3 α , Sox2⁺ and p63⁺ basal cells appear and suggest that the balance between Hif2 α and Hif3 α may function as a modulator of basal cell emergence [68].

Besides the aberrant induction of Sox2 and p63, the expression domain of Rar β was expanded distally in the mycHIF3 α transgenic lungs. Rar β knockout mice exhibited premature septation, and formed alveoli twice as fast as wild-type mice [66,67,91]. So, upregulation of Rar β in mycHIF3 α transgenic mice may in part explain the observed inhibition of pulmonary alveoli formation. We also detected an increase of Foxp2, which is a transcriptional repressor able to inhibit the expression of Ccsp and markers specific for distal epithelial cells, such as Spc and T1 α [64,65,92]. Therefore, the reduced numbers of Clara cells (Ccsp⁺), alveolar type I (Aqp5⁺) and alveolar type II (Sftpd⁺) cells could be directly related to the upregulation of Foxp2. Recent findings showed that depletion of cells with CCSP promoter activity was associated with

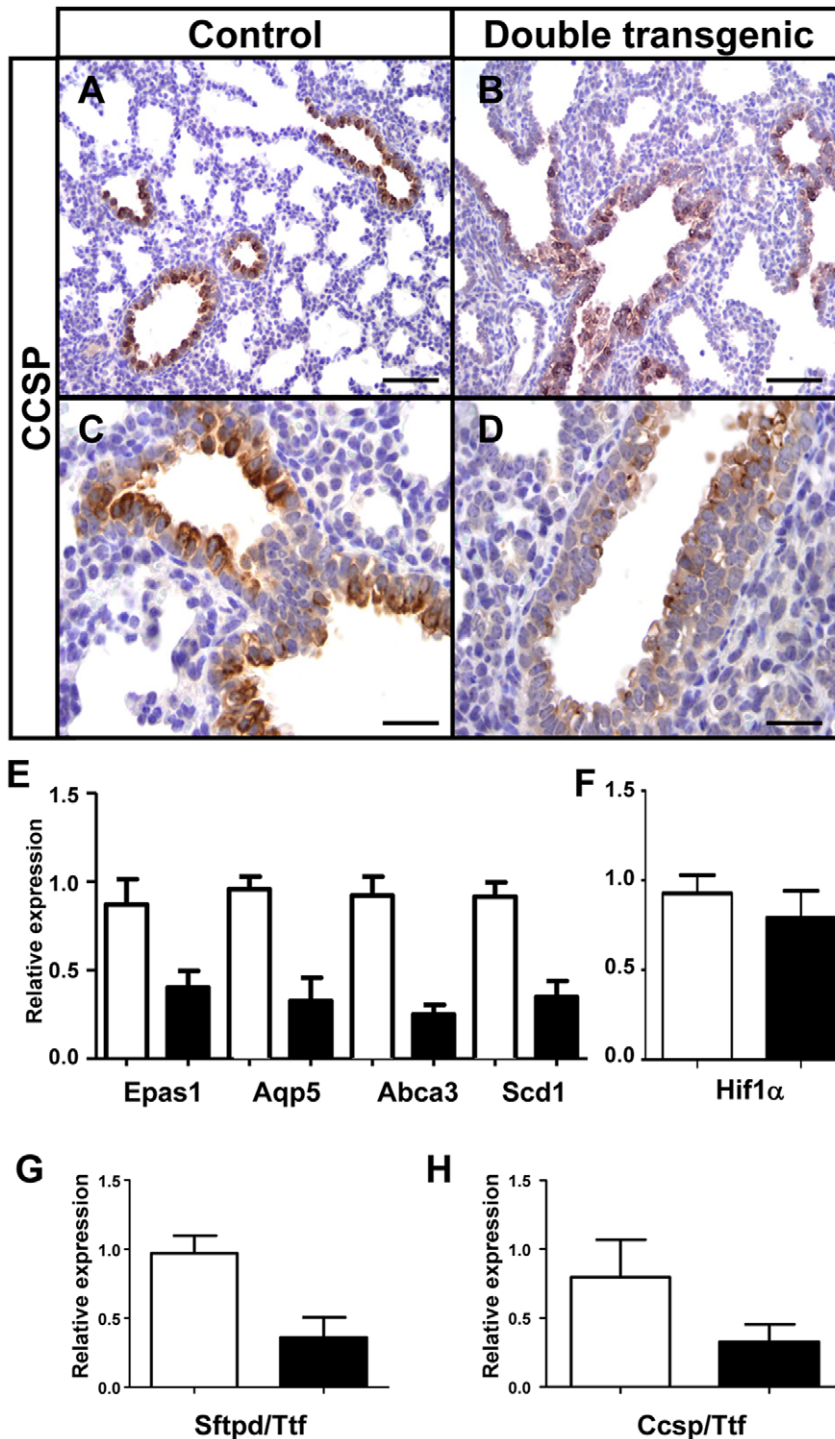


Figure 6. mycHIF3 α reduces the number of Clara cells. The expression of the Clara cell marker, *Ccsp*, was strongly decreased in mycHIF3 α transgenic lungs at gestational age E18.5 compared to controls (A and C versus B and D). (E) Alveolar epithelial cell markers are downregulated in Hif3 α transgenic lungs at gestational age E18.5 as shown by quantitative PCR. *Epas1* (0.4 ± 0.1 versus control 0.96 ± 0.1, n = 3 each, P = 0.005), *Aqp5* (0.33 ± 0.1 versus control 0.96 ± 0.1, n = 3 each, P = 0.001), *Abca3* (0.25 ± 0.1 versus control 0.92 ± 0.1, n = 3 each, P = 0.002), *Scd1* (0.35 ± 0.1 versus control 0.92 ± 0.1, n = 3 each, P = 0.001). (F) There is no significant change in the mRNA expression of *Hif1 α* gene (0.8 ± 0.1 versus control 0.7 ± 0.1, n = 3 each, P > 0.05). Quantification of the number of (G) type II pneumocytes (*Sftpd* over *Ttf1*, 0.36 ± 0.1 versus control 0.9 ± 0.1; n = 3, P = 0.01) and (H) Clara cells (*Ccsp* over *Ttf1*, 0.3 ± 0.1 versus control 0.82 ± 0.1; n = 5, P = 0.01) showed a significant reduction of in the Hif3 α double transgenic animals. White bars represent control lung samples, black bars represent mycHIF3 α double transgenic lung samples. Scale bars: 100 μ m (A, B) and 50 μ m (C, D).

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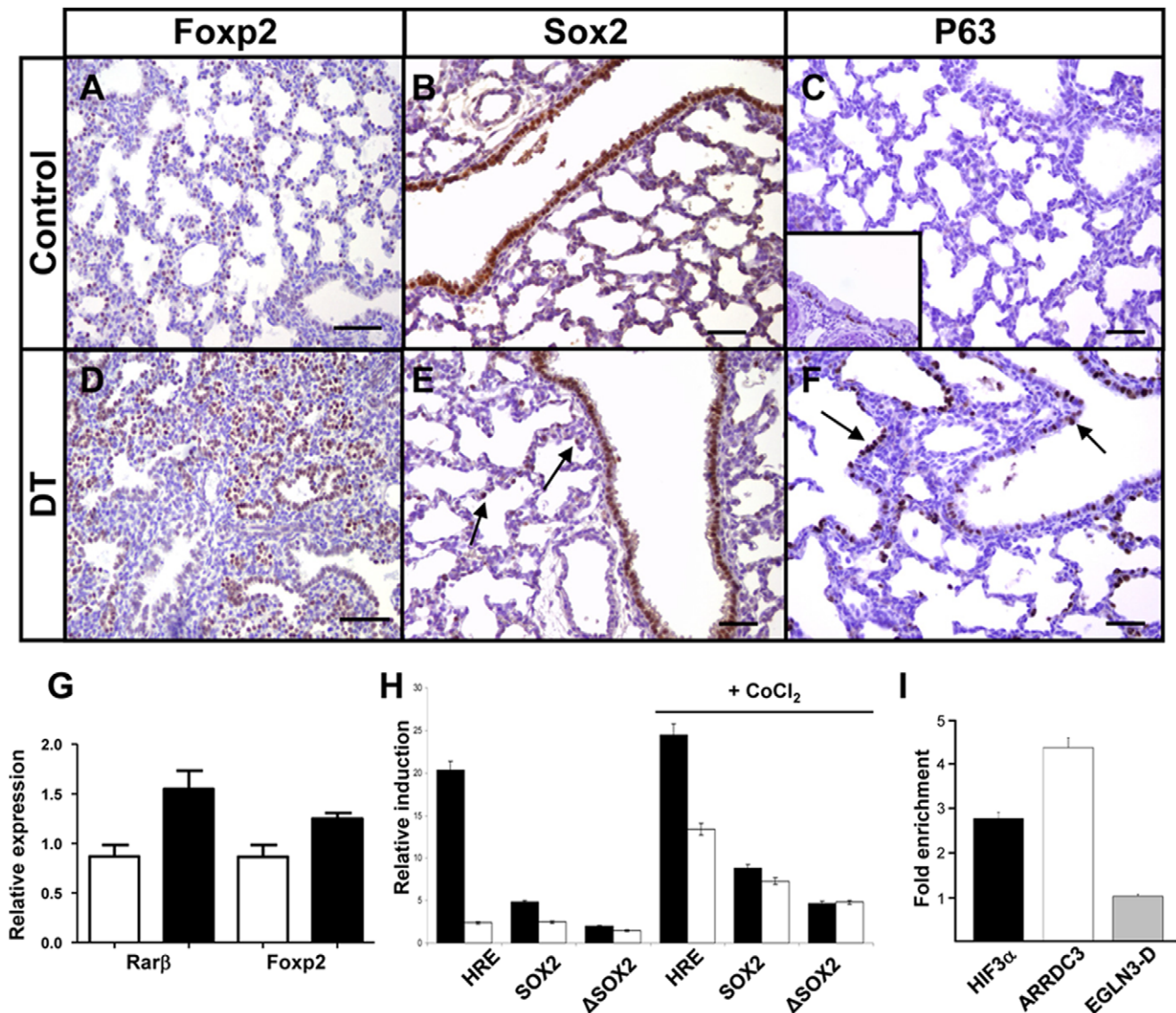


Figure 7. mycHIF3 α induces the expression of proximal differentiation markers. mycHIF3 α induces an expansion of the Foxp2 positive cells in the double transgenic lungs at gestational age E18.5 (A, D), as well as an expansion towards the distal parts of the lungs of Sox2 (B, E) and p63 (C, F). Sox2 was expressed in both proximal airways and alveolar epithelial cells in mycHIF3 α transgenic lungs (arrows, E) at PN1. Basal cells are absent in control lungs (C), but are expressed in basal cells of trachea (C, insert). However, p63 is expressed in the proximal airways and alveolar epithelial cells in mycHIF3 α transgenic lung (arrows, F). Scale bar: 200 μ m (A and D) and 100 μ m (B, C, E, F). (G) Foxp2 and Rar β are significantly upregulated in Hif3 α transgenic lungs at gestational age E18.5 as shown by quantitative PCR. (Foxp2: 1.25 ± 0.1 versus control 0.87 ± 0.1 , $n = 3$, $P = 0.007$; Rar β : 1.55 ± 0.1 versus control 0.87 ± 0.1 , $n = 3$, $P = 0.009$). White bars represent control lung samples, black bars represent mycHIF3 α double transgenic lung samples. (H) Hif2 α (black bars) and Hif3 α (white bars) induce the 9*HRE-Luc (HRE) and Sox2-Luc (Sox2) as measured by the amount of luciferase activity. The fold induction of the HRE promoter is higher with Hif2 α (20,3 fold and 24,5 fold under hypoxic conditions-CoCl₂) than with Hif3 α (2,4 fold and 13,4 fold under hypoxic conditions-CoCl₂). The induction of the Sox2 promoter is higher with Hif2 α than with Hif3 α under normoxic conditions (4,8 versus 2,5), but equally strong under hypoxia mimicking conditions (8,8 versus 7,3). Data are presented as the induction (n-fold) relative to cells transfected with the corresponding reporter plasmid and control vector (pcDNA3). The values are the average of two duplicates, and standard deviations are: 0,04 (HRE-Hif2 α), 0,02 (Sox2-Hif2 α), 0,03 (Δ Sox2-Hif2 α), 0,08 (HRE-Hif3 α), 0,24 (Sox2-Hif3 α), 0,06 (Δ Sox2-Hif3 α), 0,53 (HRE-Hif2 α +CoCl₂), 0,007 (Sox2-Hif2 α +CoCl₂), 0,03 (Δ Sox2-Hif2 α +CoCl₂), 0,88 (HRE-Hif3 α +CoCl₂), 0,02 (Sox2-Hif3 α +CoCl₂), 0,1 (Δ Sox2-Hif3 α +CoCl₂). (I) Chromatin immunoprecipitation (ChIP) using anti-HIF3 α antibody and chromatin isolated from A549 cells. Graph represents the fold enrichment of the HIF3 α -specific binding to the conserved HRE of the SOX2 promoter compared to the IgG control ChIP. HIF3 α also bound the ARRDC3 HRE region, and the enhancer region D of the EGLN3 gene served as negative control (EGLN3-D). doi:10.1371/journal.pone.0057695.g007

alveolar hypoplasia and respiratory failure, adding to the idea that Ccsp downregulation as a result of Hif3 α -mediated Foxp2 upregulation, directly leads to reduced numbers of Clara cells [93].

The increased expression of key genes in lung development, which lead to major changes in epithelial differentiation, was

confirmed by the loss of expression of other cell type specific markers, such as *Sftpd*, *Scd1* and *Abca3* for type II cells. At this point it is not clear if the reduced expression of the type II cell markers is the cause, or the result of the loss of type II cells. Previously, we showed a significant downregulation of *Scd1* and

Abca3 in Hif2 α expressing transgenic mice, which suffered from respiratory distress and surfactant deficiency [55]. However, the mycHIF3 α transgenic mice appeared to produce sufficient levels of *Scd1* and *Abca3* to support respiration, even though the expression of Hif2 α is decreased.

Thus, the increased expression of Sox2, Rar β and Foxp2 in the developing mycHIF3 α lungs may directly contribute to the cellular changes observed and explain the phenotypic abnormalities observed in these lungs. The effects may also be cell type specific, as increased HIF3 α expression in vascular cells resulted in an antagonistic effect on hypoxia induced HIF1/HIF2 target genes [47].

Concluding remarks

Although we cannot conclude that the dominant negative role of Hif3 α as part of the hypoxic response is absent, our previous and current data do suggest that Hif2 α and Hif3 α have different target genes, during pulmonary development [55]. This is in line with previous findings describing common targets, as well as specific genes induced by Hif1 α and Hif2 α [80,81,82]. However, these studies used overexpression of Hif1 α and Hif2 α , which may cause aberrant complexes and loss of target gene specificity, as was reported for certain tumor cells [94]. Using siRNA and chromatin immuno-precipitation approaches, HIF1 and HIF2 target genes were identified [35,36,83,84,85]. Interestingly, it was shown that ETS transcription factors were involved in the regulation of HIF1 and HIF2 driven gene activation in MCF7 cells [83]. Knock down of ELK1 resulted in a reduction of hypoxia induced HIF2 dependent transcription. These data suggested a cooperation between ETS family members and HIF1 and HIF2 in the selection of target genes. An interesting idea is that target selection by HIFs may be cell specifically regulated by additional factors, adding to the complexity of the hypoxic response [8,95]. This is also observed in the analysis of the different transgenic mouse models expressing Hif1 α [79], and our studies with Hif2 α or Hif3 α , showing similarities and differences [55].

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Supporting Information

Figure S1 Expression of mycHIF3 α leads to late branching defect. External appearances of control (A and E) and mycHIF3 α transgenic lungs (B and F) at E16.5 and E17.5 showed no apparent differences. Histological analysis of control (C and G) and mycHIF3 α transgenic (D and H) lungs showed a gradual decrease in the number of air spaces and aberrant, late branching morphogenesis in mycHIF3 α transgenic lungs. Anti-Myc epitope staining confirmed the expression of the mycHIF3 α transgene in double transgenic lungs (D and H), which is absent in control lungs (C and G). Scale bars: 2 mm (A, B, E, F) or 200 μ m (C, D, G, H). (TIF)

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Conceived and designed the experiments: YH FG DT RJR. Performed the experiments: YH J-KO MB-vK AB-dM SS WvIJ RJR. Analyzed the data: YH J-KO MB-vK AB-dM SS WvIJ FG RJR. Contributed reagents/materials/analysis tools: YH MB-vK AB-dM SS WvIJ RJR. Wrote the paper: YH FG SS DT RJR.

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