Nmyc plays an essential role during lung development as a dosage-sensitive regulator of progenitor cell proliferation and differentiation

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Summary

Understanding how lung progenitor cells balance proliferation against differentiation is relevant to clinical disorders such as bronchopulmonary dysplasia of premature babies and lung cancer. Previous studies have established that lung development is severely disrupted in mouse mutants with reduced levels of the proto-oncogene *Nmyc*, but the precise mechanisms involved have not been explored. We show here that Nmyc expression in the embryonic lung is normally restricted to a distal population of undifferentiated epithelial cells, a high proportion of which are in the S phase of the cell cycle. Overexpression of NmycEGFP in the epithelium under the control of *surfactant protein C* (*Sftpc*) regulatory elements expands the domain of S phase cells and upregulates numerous genes associated with growth and metabolism, as shown by transcriptional microarray. In addition, there is marked inhibition of differentiation, coupled with an expanded domain of expression of Sox9 protein, which is also normally restricted to the distal epithelial compartment. By contrast, conditional deletion of *Nmyc* leads to reduced proliferation, epithelial differentiation and high levels of apoptosis in both epithelium and mesenchyme. Unexpectedly, about 50% of embryos in which only one copy of *Nmyc* is deleted die perinatally, with similarly abnormal lungs. We propose a model in which *Nmyc* is essential in the developing lung for maintaining a distal population of undifferentiated, proliferating progenitor cells.

Key words: Lung development, *Nmyc*, Conditional inactivation, Progenitor cells, Sox proteins, Growth, Differentiation

Introduction

The mammalian lung develops from two primary buds consisting of an inner epithelial layer surrounded by mesenchyme. These buds undergo repetitive outgrowth and branching to give rise to the respiratory tree containing different specialized epithelial cell types organized along the proximodistal axis. These include the ciliated, secretory and neuroendocrine cells of the proximal bronchi, and the type II and type I cells of the distal alveoli (for reviews, see Cardoso, 2000; Warburton et al., 2000). As with other organ systems, lung development involves a balance between expansion in the number of undifferentiated progenitor cells and withdrawal of cells from this pool by terminal differentiation. Understanding how this balance is achieved is relevant to a number of clinical problems; for example, the pulmonary dysplasia of premature babies, lung repair after injury, and the progression of lung cancer. Previous studies had pointed to a crucial role in lung development for the gene *Nmyc1* (hereafter *Nmyc)*, which is specifically expressed in the epithelium; *Myc* (previously *c-Myc*) is transcribed only in the mesenchyme. *Nmyc* is amplified in some lung tumors, and analysis of mutant mouse embryos with reduced levels of *Nmyc* activity suggested a role in cell proliferation and branching morphogenesis (Moens et al., 1992; Moens et al., 1993; Sawai et al., 1993; Stanton et al., 1992). However, since these initial studies there has been no new information about the precise role of *Nmyc* in lung development.

Nmyc is a member of a small family of proto-oncogenes (*Myc*, *Lmyc* and *Nmyc*) encoding basic helix-loop-helix-leucine zipper (bHLHZ) proteins. Myc proteins can either activate or repress transcription by interacting with specific binding partners, and by recruiting cofactors, including histone and chromatin protein modifying enzymes, to the vicinity of a very large number of target genes (for reviews, see Eisenman, 2001; Levens, 2003; Patel et al., 2004; Zeller et al., 2003) (see also http://www.myccancergene.org/site/mycTargetDB.asp). Myc proteins appear to coordinate many interdependent processes, including cell growth (increase in cell mass), cell proliferation (DNA replication and cell cycle progression), differentiation and apoptosis. Recent gain- and loss-of-function studies in the embryonic nervous system have highlighted roles for *Nmyc* both in promoting cell cycle progression in undifferentiated progenitor cells and in inhibiting their differentiation in response to specific signaling pathways. In mouse cerebellar granule neuron progenitors, for example, *Nmyc* is directly upregulated by sonic hedgehog acting as a mitogen, and *Nmyc* overexpression in the same cells stimulates cyclin D1 accumulation and cell cycle progression (Kenney et al., 2003). Moreover, recent studies have shown that Nmyc protein is partially stabilized in neuronal cells by activity of the PI3K pathway (Kenney et al., 2004). By contrast, conditional

deletion of *Nmyc* in the embryonic nervous system results in a decrease in the pool of granule cell precursors, largely due to an increase in their expression of cell cycle inhibitors and premature differentiation (Knoepfler et al., 2002).

As indicated earlier, it is well established that *Nmyc* is required for lung development (Moens et al., 1992; Moens et al., 1993; Sawai et al., 1993; Stanton et al., 1992). Embryos homozygous for a hypomorphic mutation ($Nmyc^{9a/9a}$) die at birth with lungs that are about half the normal size, and that contain fewer branches and highly enlarged air spaces. Compound mutants between $Nmyc^{9a}$ and a null allele ($Nmyc^{BRP}$) have an even more severe reduction in lung development. To further explore how *Nmyc* functions in the lung, we have exploited transgenic and conditional gene deletion techniques to overexpress *Nmyc*, or, conversely, to remove one or both copies of the gene specifically in the epithelium of the developing lung.

Materials and methods

Transgenic and $Nmyc^{flox/flox}$ mice

To generate Nmyc1EGF fusion protein, mouse *Nmyc1* cDNA was inserted into the *Sma*I site of pEGFP (BectonDickinson), excised by *Sal*I and *Eco*RI, and inserted into a vector containing a 3.7 kb promoter/enhancer of the human *SFTPC* gene (Wert et al., 1993). Transfection of 293 cells shows that the fusion protein localizes to the nucleus (see Fig. S1 in supplementary material). Four transgenic embryos were generated by pronuclear injection into (C57BL/6×DBA)F2 fertilized eggs. They were collected at E18.5, so it is not known if they would have survived postnatally.

The *Sftpc-cre* transgene was constructed by cloning the same *SFTPC* promoter upstream of the rabbit β-globin second intron, followed by a modified *cre recombinase* gene containing a Kozak consensus sequence, an N-terminal nuclear localization signal, and the β-globin polyadenylation signal. The modified 5′ sequences of the *cre recombinase* gene are: ACC**ATG**GCTCCCAAGAAGAAGAG-GAAGgtg, with the Kozak sequence underlined, the start ATG in bold and the first *cre* recombinase codon shown in lower case. A line was established from a male giving specific temporal and spatial expression of cre in the lung epithelium from E10.5, as judged by crossing with a Rosa26R female and analyzing *lacZ* expression. It was maintained by crossing with ICR mice. $Nmyc^{flox}$ mice were generated as described (Knoepfler et al., 2002), and maintained as homozygotes on the 129×C57BL/6 background. 129S4-Gt(ROSA)26Sor^{tm1Sor} mice from The Jackson Laboratory on the C57BL/6 background were maintained by interbreeding.

β-Gal staining

Lungs were fixed in 4% paraformaldehyde in PBS (pH 7.4), permeabilized in 2 mM $MgCl_2$, 0.01% NaDeoxycholate, 0.02% NP-40, stained with X-gal overnight, embedded in paraffin wax, sectioned at 7 μm, and counterstained with Eosin.

BrdU incorporation

BrdU (Amersham Bioscience, UK) was injected intraperitoneally into pregnant females at a dose of 10 μl per gram bodyweight. After 1 hour, embryonic lungs were fixed in 4% paraformaldehyde in PBS (pH 7.4). For immunohistochemistry, BrdU monoclonal antibody (Sigma, St Louis, MO, USA) was used, in combination with with MOM-blocking solution (Vector Laboratories, Burlingame, CA, USA).

RT-PCR

Total RNA was extracted using the RNeasy Kit (Qiagen, Valencia, USA). The cDNA was synthesized from 1 μg total RNA using SuperScript^{TM} First-strand Synthesis Kit (Invitrogen). Primer sets were:

claudin 6, 5′-ATGGCCTCTACTGGTCTGCAAATC-3′ and 5′-GCATCACACATAATTCTTGGTGGG-3′;

cyclin D2, 5′-TGAGCACATCCTTCGCAAGC-3′ and 5′-CT-CACAGGTCAACATCCCG-3′;

Nmyc, 5′-CGAATTGGGCTACGGAGATGCT-3′ and 5′-TTGT-GCTGCTGATGGATGGG-3′;

Nol5a, 5′-TGAAGGAAGCTGTGGTTCAGG-3′ and 5′-CTAATC-CTCCTGTGCTTTCTG-3′;

Ppan, 5′-ATGGGGCAGTCCGGGCGGTC-3′ and 5′-GCTCACT-GATGTCCTGCAGTC-3′;

Rog, 5′-CCCCACTCCAGGATCTTTTCC-3′ and 5′-AGGTG-GCAGCAGANGAGGTAG-3′;

Scgb1a1, 5′-TGAAGATCGCCATCACAATC-3′ and 5′-ATCT-TGCTTACACAGAGGAC-3′; and

Sox9, 5′-ACGTGTGGATGTCGAAGCAG-3′ and 5′-ACTG-GTTGTTCCCAGTGCTG-3′.

The primers for *Sftpa*, *Sftpb*, *Sftpc*, *Aquaporin 5* (*Aqp5*) and *βactin* were as described (Okubo and Hogan, 2004).

In situ hybridization

Mouse *Sftpc*, rat *Scgb1a1* and mouse *Foxj1* cDNA have been described previously (Weaver et al., 1999). Digoxigenin (DIG) or fluorescein-labeled antisense cRNA probes were made using T7 or SP6 RNA polymerase. Briefly, paraffin wax-embedded sections were dewaxed, pretreated with proteinase K and hybridized with one DIG-labeled and one fluorescein-labeled probe at 60°C overnight. Slides were then washed sequentially in 5×SSC, 2×SSC (50% formamide), 0.1×SSC and blocking solution (supplied in TSA Plus Biotin System, Perkin Elmer). The DIG-labeled probe was detected using POD-coupled anti-DIG (Roche), followed by standard biotin-tyramide and streptavadin-HRP amplification, and visualized using Cy3-tyramide (Perkin Elmer). HRP activity was quenched by treatment with 1% H_2O_2 in methanol (15 minutes). This was followed by detection of the fluorescein-labeled RNA probe with POD-coupled anti-fluorescein (Roche), and similar amplification and visualization with fluorescein-tyramide. Nuclei were counterstained with DAPI.

Immunohistochemistry

The following reagents were used: rabbit polyclonal antibody to Nmyc (SantaCruz); phosphohistone H3 (Upstate Biotechnology); cyclin D1 (clone AM29, Zymed); cleaved caspase-3 (Cell Signaling Technology, Beverly MA); Scgb1a1 (kindly provided by Barry Stripp, University of Pittsburgh); Sox2 (kindly provided by Larysa Pevny, UNC Chapel Hill); and hamster monoclonal anti-Gp38 (University of Iowa Hybridoma Bank). The rabbit Sox9 antibody was kindly provided by Dr Francis Poulat, CNRS, Montpellier, and was used at a dilution of 1:3000. It was raised against amino acids 408-504 of the human protein (Gasca et al., 2002), a region showing significant similarity only with sequences in mouse Sox10. However, RT-PCR analysis indicates that *Sox10* is not expressed in either wild-type or transgenic embryonic mouse lung (data not shown). The specificity of the rabbit antibody for Sox9 in the chick was described previously (Moniot et al., 2004), and was confirmed in this study by specific staining of Sertoli cells in the mouse testis (E18.5; data not shown). The Nmyc antibody showed no staining of neurones in which *Nmyc* was deleted (Knoepfler et al., 2002), and only low-level staining of epithelial cells in conditional mutant lungs (Fig. 7F).

Electron microscopy

Tissue was fixed in 2% glutaraldehyde in PBS, post-fixed in osmium tetroxide, stained en bloc with uranyl acetate and embedded in Spur's embedding medium (EM science). Thin sections were stained with uranylacetate/lead citrate before viewing with a Philips electron microscope.

DNA Affymetrix analysis

Total RNA (10 μg) was extracted from three different transgenic and two wild-type lungs (right caudal lobe) by using RNeasy kit. All subsequent reactions using Affymetrix GeneChip Mouse 430A arrays and statistical analyses were carried out in the Duke Center for Genome Technology Microarray core facility as detailed previously (Okubo and Hogan, 2004). All primary data files are freely available (see Tables S1 and S2 in supplementary material).

Results

Nmyc expression is restricted to distal epithelium containing a high proportion of cells in the S phase of the cell cycle

Levels of *Nmyc* RNA are highest in the early lung, decline significantly before birth and are very low in the adult (Fig. 1A). Immunohistochemistry revealed the previously unreported fact that Nmyc protein is preferentially localized to epithelial cells in the distal regions of the developing lung (Fig. 1B). This restriction is particularly evident at E12.5 to E16.5 as a boundary between the Nmyc-positive cells of the distal tubules and the mostly negative cells of the future proximal airways. Reactivity is both cytoplasmic and nuclear, as previously reported in neuronal cells (Wakamatsu et al., 1993). By E18.5, only low levels of Nmyc staining can be detected in the lung, in both presumptive type II cells and bronchial epithelium.

In the developing cerebellum, high levels of Nmyc expression coincide with distinct domains of the germative neuroepithelium in which a large percentage of the progenitor cells are in S phase, as determined by BrdU incorporation (Knoepfler et al., 2002). Analysis of the embryonic lung revealed a similar overlap between Nmyc expression and BrdU-labeled cells in the distal epithelium (Fig. 1C for E12.5; Fig. 8M for E15.5). At E12.5, almost 75% of distal epithelial cells are labeled with BrdU over a 1-hour time period, whereas only 30% are positive in the proximal epithelium where Nmyc expression is low (Fig. 1C). Analysis of adjacent sections shows that the domain of high Nmyc expression also coincides with high levels of cyclin D1 expression. By contrast, phosphohistone H3-positive cells in late G2 and mitosis are not preferentially localized to either proximal or distal domains (Fig. 1C). These results suggest that the cell cycle kinetics of distal and proximal endoderm are already different at E12.5, and correlate with Nmyc expression.

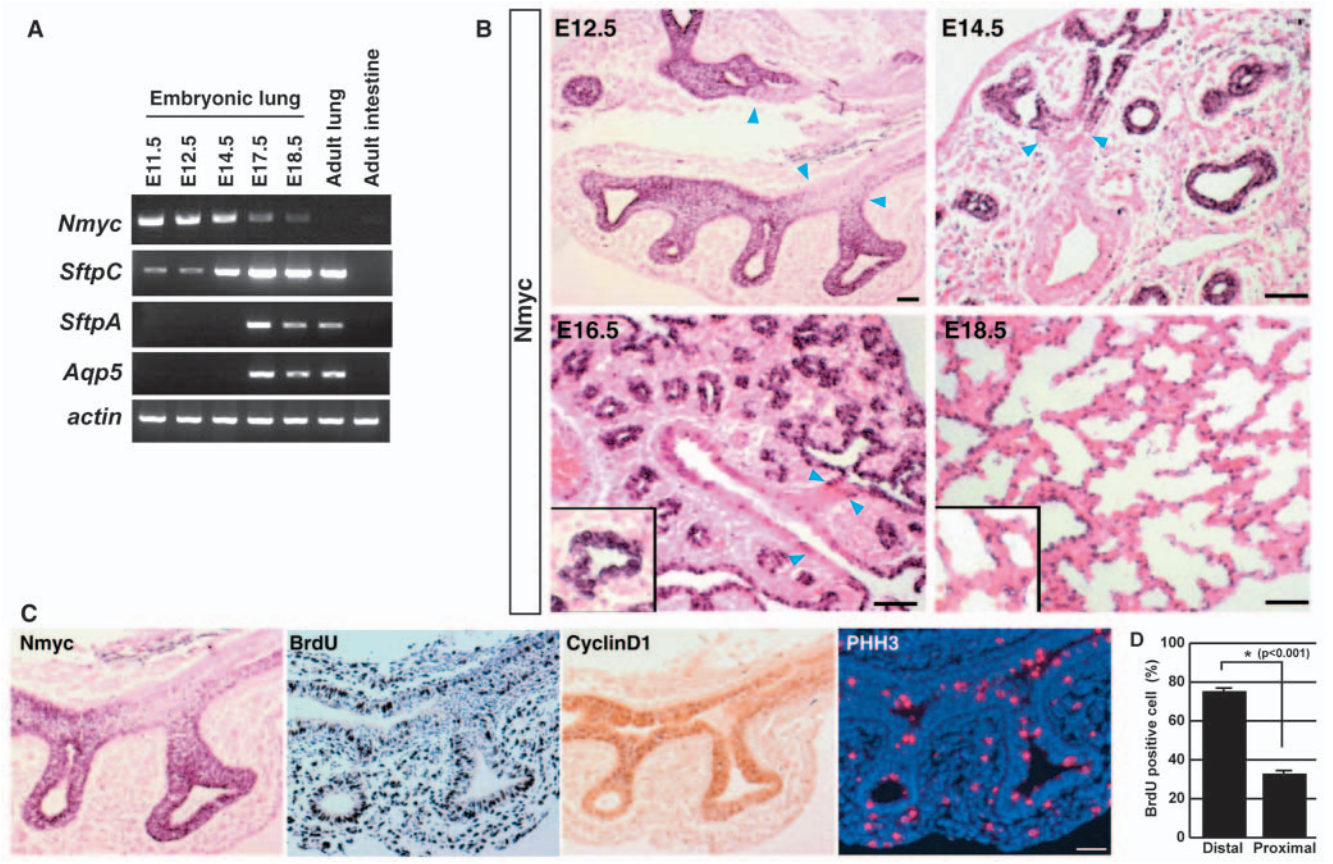

Fig. 1. Nmyc expression and BrdU incorporation in the wild-type lung. (A) RT-PCR shows that *Nmyc* transcripts decrease during lung development, whereas those for differentiated epithelial cell markers (*Sftpc*, *Sftpa* and *Aqp5*) increase. (B) Immunohistochemistry at E12.5, E14.5 and E16.5 shows higher Nmyc expression in the distal compared with the proximal epithelium. Blue arrows mark the boundary between the two regions. At E18.5, low levels are seen in presumed type II cells (insets). (C) Immunostaining for Nmyc, BrdU, cyclin D1 and phosphohistone H3 (PHH3) in adjacent sections of E12.5 lung. (D) Percentage of BrdU-positive nuclei in Nmyc-high distal endoderm and Nmyc-low proximal endoderm of E12.5 lungs, as determined by counting nuclei in cross sections of tubules, where the identification of individual cells is unambiguous. *, difference is significant (P<0.001). Scale bars: 50 μm.

Table 1. Correlation between genotype and phenotype of conditional mutant lungs

Mating genotype	cre(+); $Nmyc^{flox/+}$	cre(+); $Nmyc^{flox/flox}$
*Sftpc-cre×$Nmyc^{flox/flox}$ (F1)	28/52 (53.8%)	–
*cre(+);$Nmyc^{flox/+}$×$Nmyc^{flox/flox}$ (F2)	8/13 (61.5%)	12/12 (100%)
†cre(+);$Nmyc^{flox/+}$×Rosa26R(+) $Nmyc^{flox/+}$	4/16 (25.0%)	9/9 (100%)

The Table shows the proportion of embryos/newborn pups from different crosses with an abnormal phenotype (number of lungs with abnormal phenotype/total number).
*Results of the mating scheme shown in Fig. 6A.
†Results of the mating scheme used to generate the data in Fig. S2 in supplementary material.

Overexpression of Nmyc disrupts lung development

For gain-of-function studies, we used a well-characterized human *surfactant protein C* (*Sftpc*) promoter to express an NmycEGFP fusion protein in the epithelium. Expression begins in primary lung buds from about E10.5 and continues throughout development with highest levels observed distally (Okubo and Hogan, 2004; Wert et al., 1993). The four transgenic lungs obtained at E18.5 resembled control lungs in overall dimensions and lobulation pattern (Fig. 2A). Internally, however, they had an abnormal phenotype that varied with the level of transgene expression, as determined by three independent criteria: fluorescence from NmycEGFP (Fig. 2B); RT-PCR (Fig. 2C); and transcriptional profiling by microarray (see Tables S1 and S2 in supplementary material). Control lungs had numerous primitive alveoli, with well-differentiated type II cells and flattened type I cells closely apposed to blood vessels (Fig. 2D,G,J). Such primitive alveoli were absent from transgenic lungs, which instead contained many distal epithelial tubules surrounded by abundant mesenchyme (Fig. 2E,F,H,I). In the two transgenic lungs with the highest levels of *Nmyc* expression (approximately 12- to 15-fold higher than normal from microarray), electron microscopy revealed large accumulations of

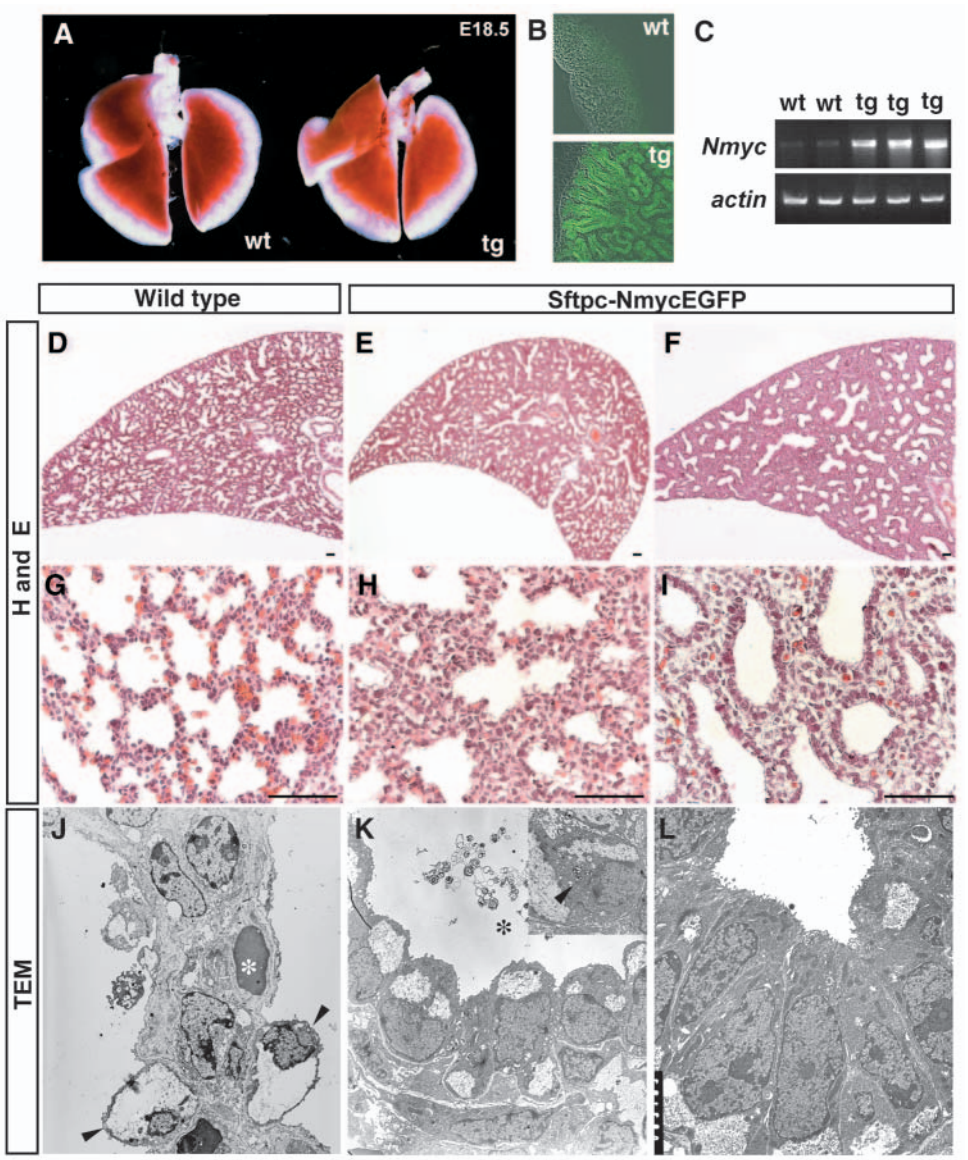

Fig. 2. Morphology of *Sftpc-NmycEGFP* transgenic and normal lungs at E18.5. (A) External appearance of wild-type and transgenic lungs. (B) The NmycEGFP fusion protein is expressed throughout the distal transgenic epithelium (lower panel). Fluorescence in wild type is background (upper panel). (C) RT-PCR analysis confirming *Nmyc* overexpression in E18.5 transgenic lungs. (D-I) Low and high power images of sections stained with Haematoxylin and Eosin from normal (D,G) and transgenic lungs (E,F,H,I) with least severe (E,H) and most severe (F,I) phenotypes. Note the cuboidal undifferentiated epithelium, extensive mesenchyme, and lack of apposition of blood vessels to the epithelium in the transgenic lungs. (J-L) Electron micrographs of wild-type (J) and transgenic (K,L) lungs. In J, note the blood vessels (asterisk) close to the lumen of the alveolar sac, and the thin walled type I alveolar cells and two type II cells that have lost their glycogen (arrows). In K, the epithelial cells resemble immature type II cells, as they contain both glycogen and lamellar bodies (inset, arrowheads). Lamellar bodies are also in the lumen (asterisk). (L) Densely packed undifferentiated epithelial cells with glycogen but no lamellar bodies. Scale bars: 50 μm.

glycogen typical of undifferentiated endoderm (Ten Have-Opbroek, 1981; Ten Have-Opbroek, 1991), but no lamellar bodies characteristic of differentiated type II cells (Fig. 2L). However, some lamellar bodies were present in the cells and lumina of lungs with lower *Nmyc* expression (up to 8-fold higher than normal) (Fig. 2K).

BrdU incorporation and phosphohistone H3 staining showed a significantly higher number of proliferating cells in both the endoderm and mesenchyme of transgenic lungs when compared with wild type (Fig. 3A-D). Staining with anti-cleaved caspase 3 antibody also revealed many apoptotic epithelial cells in the transgenic lungs (Fig. 3E,F).

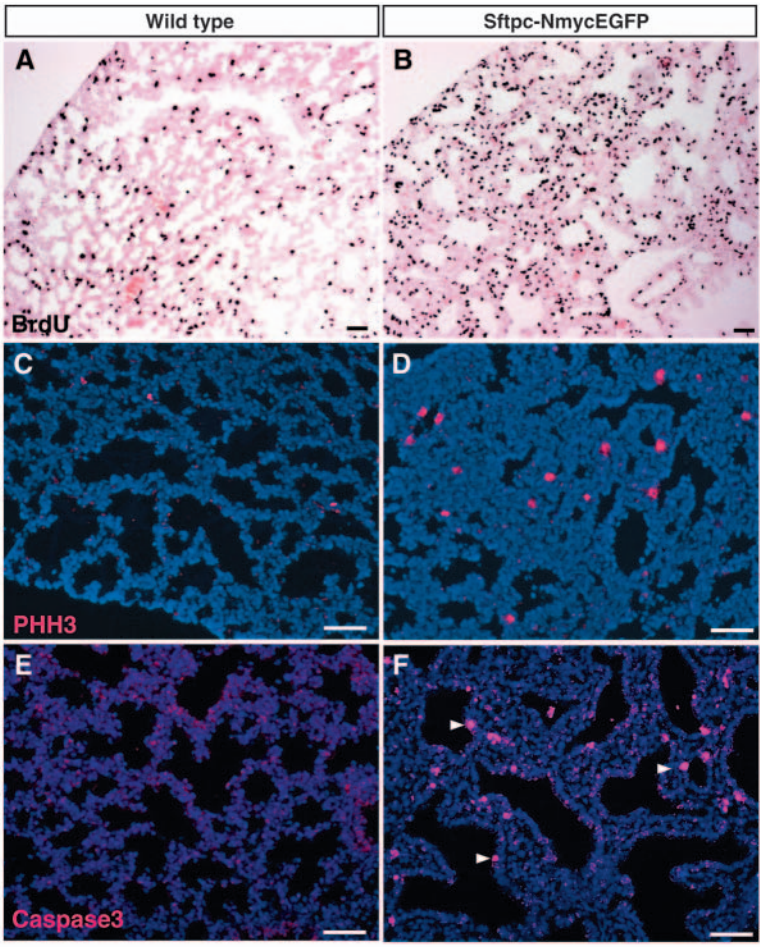

Fig. 3. Cell proliferation and apoptosis in *Sftpc-NmycEGFP* transgenic lungs. Sections of wild-type (A,C,E) and transgenic (B,D,F) E18.5 lung after a 1-hour pulse in vivo with BrdU followed by immunohistochemistry (A,B), or after staining with antibody to PHH3 (C,D) or cleaved caspase 3 (E,F). Note much higher rate of cell proliferation in both mesenchyme and epithelium of the transgenic lung, and the epithelial cells undergoing apoptosis (arrowheads). Scale bars: 50 μm.

Increased levels of *Nmyc* expression inhibit epithelial differentiation

To explore the effect of Nmyc overexpression on endoderm differentiation, we first examined transcripts for *Scgb1a1* and *Foxj1*, markers for Clara and ciliated cells, respectively (Fig. 4A,B, and data not shown for Foxj1). At E18.5, *Scgb1a1* is highly expressed in proximal epithelium, with a sharp boundary between the terminal bronchioles and the future alveoli, which contain type II cells and their precursors expressing high levels of *Sftpc*. This boundary corresponds to the future bronchioalveolar duct junction (BADJ). It is also seen in sections of E18.5 lungs stained with antibody to Sox2, an HMG box transcription factor specifically expressed in proximal lung endoderm from E11.5 (Fig. 4C, and data not shown). By contrast, in E18.5 transgenic lungs, there is no distinct boundary between *Scgb1a1* and *Sftpc* domains (Fig. 4B). Instead, there is an intermediate zone in which expressing cells are interspersed with non-expressing cells. A similar absence of a sharp boundary was seen with Sox2 (Fig. 4D). Taken together, these results suggest that Nmyc overexpression significantly reduces the probability that progenitor cells will differentiate into proximal or distal cell types. This conclusion was supported by RT-PCR analysis that showed a dramatic reduction in the expression of *Aqp5*, *SftpA* and *SftpB*, late differentiation markers for alveolar type I and type II cells (Fig. 4E).

High-level expression of Sox9 in Nmyc overexpressing lung

The idea that Nmyc overexpression inhibits the differentiation of progenitor cells was further supported by immunohistochemistry to Sox9. This HMG box transcription factor is specifically expressed in the distal progenitor cells from E11.5-E16.5 (Fig. 5A,C) (Liu and Hogan, 2002). By E18.5, however, the protein is almost undetectable in normal lungs (Fig. 5E,G). Significantly, in transgenic lungs the downregulation of Sox9 expression does not occur and high levels of nuclear protein are present throughout the E18.5 epithelium, including the bronchiolar region in which Scgb1a1-expressing cells are present (Fig. 5G,H). Double immunohistochemistry shows that a few transgenic cells express both Sox9 and Scgb1a1 (Fig. 5H). Elevated levels of Sox9 protein are seen in transgenic lungs even though RNA levels are not apparently different from wild type, as judged by RT-PCR and microarray (Fig. 4E and see Tables S1 and S2 in supplementary material). This suggests that postranscriptional mechanisms are involved in maintaining the levels of Sox9 protein.

Microarray analysis of gene expression in Sftpc-NmycEGFP transgenic lungs

Previous studies have identified many genes that are directly or indirectly upregulated by *Nmyc* or *Myc* in mammalian cell lines or tumors, or in normal mouse skin (Boon et al., 2001; Frye et al., 2003; Patel et al., 2004; Raetz et al., 2003; Shiio et al., 2002; Zeller et al., 2003). The majority of these genes are associated with the general machinery of cell growth (RNA and protein synthesis and energy metabolism) and proliferation. To see whether a similar set of genes is upregulated by *Nmyc* in embryonic lung epithelium, we used mouse MOE430 Affymetrix gene arrays to analyze transcripts in E18.5 transgenic and wild-type lungs. We found 381 genes (excluding ESTs) that were upregulated twofold or more in transgenic lungs ($P<0.05$), and 391 that were similarly

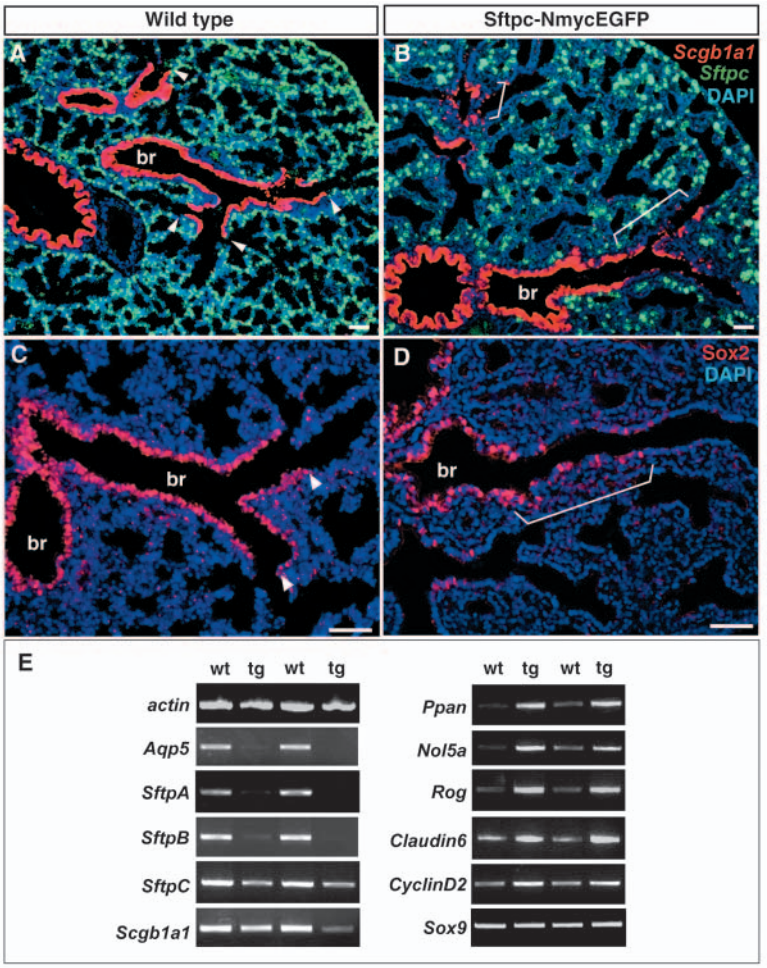

Fig. 4. High levels of *Nmyc* inhibit epithelial cell differentiation. (A,B) Sections of normal and severely affected E18.5 transgenic lungs hybridized with riboprobes for *Scgb1a1* (a marker for differentiated Clara cells) and *Sftpc* (a marker, when expressed at high levels, for type II cells). (A,C) In wild type, a sharp boundary is present (arrowheads) at the BADJ between bronchioles lined with Clara cells (red) and the future alveoli, lined by type II cells (green). In the transgenic lung the boundary is not sharp (white bracket). (C,D) Sections of the same lungs after staining with antibody to Sox2. In normal lungs (C), Sox2 is restricted to nuclei of proximal epithelial cells, with a sharp boundary at the BADJ (arrowheads). In the transgenic lung (D), the boundary is not sharp (white bracket). Nuclei are stained with DAPI. (E) Gene expression assayed by RT-PCR of total RNA extracted from two normal and two transgenic lungs. Representative genes are lung differentiation markers (*Aqp5*, *SftpA*, *SftpB*, *SftpC*, and *Scgb1a1*), upregulated genes from microarray data (*Ppan*, *Nol5a*, *Rog*, *cyclin D2* and *claudin 6*), and *Sox9*. Scale bars: 50 μm.

downregulated. A limitation was that the RNA was extracted from total lung tissue and therefore contained both epithelial and mesodermal cells. Nevertheless, many of the genes expressed at higher level in the transgenic lungs matched those elevated in response to Myc in other cell types, and in some cases were known to be direct Myc targets (see Table S1 in supplementary material). They fell into a number of categories, including RNA metabolism (transcription, pre-mRNA splicing, RNA binding, nucleolar function, nuclear transport and RNA turnover; 14% of total), ribosome biogenesis (4%), protein synthesis, turnover and folding (7%), cell cycle regulators such as cyclin D2 (6%), DNA replication (9%), and energy and cell metabolism, including glycolysis and mitochondrial function (32%). A number of genes encoding transcription and DNA-binding factors were expressed at more than twofold higher levels in transgenic lungs than in wild type. Among them were the genes encoding Mllt10 (formerly, Af10), the homolog of *Drosophila* dAf10, thought to be involved in heterochromatin-mediated gene regulation (Linder et al., 2001), and Repressor of gata (Rog), first identified as a protein that inhibits the transactivating function of Gata3 (Miaw et al., 2000). Gata6, a close family member, is required for the normal differentiation of lung epithelial cells (Yang et al., 2002), thus higher expression of *Rog* could play a role in inhibiting the differentiation of lung progenitor cells. However, as in other studies, there was no overwhelming evidence from the microarray data that increased transcription of tissue-specific regulators is the primary mechanism by which Nmyc controls cell differentiation. Finally, analysis showed that there was an upregulation of genes encoding proteins with a proapoptotic function, for example *Bid* and *Siva*. This correlated with the presence of apoptotic epithelial cells in the transgenic lungs (Fig. 3F).

The genes downregulated in *Sftpc-NmycEGFP* lungs also fell into several categories (see Table S2 in supplementary material). One of the largest (12% of total) encodes specialized products characteristic of type I and II alveolar cells, numerous genes encoding transmembrane proteins involved in the directed movement of ions and molecules into and out of cells, and proteins involved in cellular immunity (complement, immunoglobulin). Another category includes proteins involved in cell adhesion, extracellular matrix production and cell structure (26%). This grouping may reflect the simpler cuboidal morphology of the distal epithelium in transgenic lungs compared with the more complex three-dimensional organization of the primitive alveoli in the non-transgenic lungs. However, a number of the genes in this category are similar to those downregulated in other epithelial cells, such as keratinocytes (Arnold and Watt, 2001), and/or are direct targets of Myc repression (http://www.myccancergene.org/site/mycTargetDB.asp). In the transgenic lungs, we saw no evidence for epithelial cells detaching from the basal lamina, although branching morphogenesis was severely disrupted. RT-PCR confirmed the up- and downregulation of a number of the genes detected by microarray analysis (Fig. 4E).

Conditional deletion of Nmyc leads to abnormal lung development

To study the effect of conditional disruption of *Nmyc*, we used a *Sftpc-cre* transgene, driving cre recombinase specifically in lung endoderm from about E10.5 (Okubo and Hogan, 2004), in combination with a previously described floxed allele of *Nmyc* (Knoepfler et al., 2002).

To generate cre(+);$Nmyc^{flox/flox}$ mice, we initially crossed *Sftpc-cre* and homozygous $Nmyc^{flox/flox}$ mice (Fig. 6A). As expected, about 50% of the F1 offspring were cre(+);$Nmyc^{flox/+}$. It was verified repeatedly that the $Nmyc^{flox}$

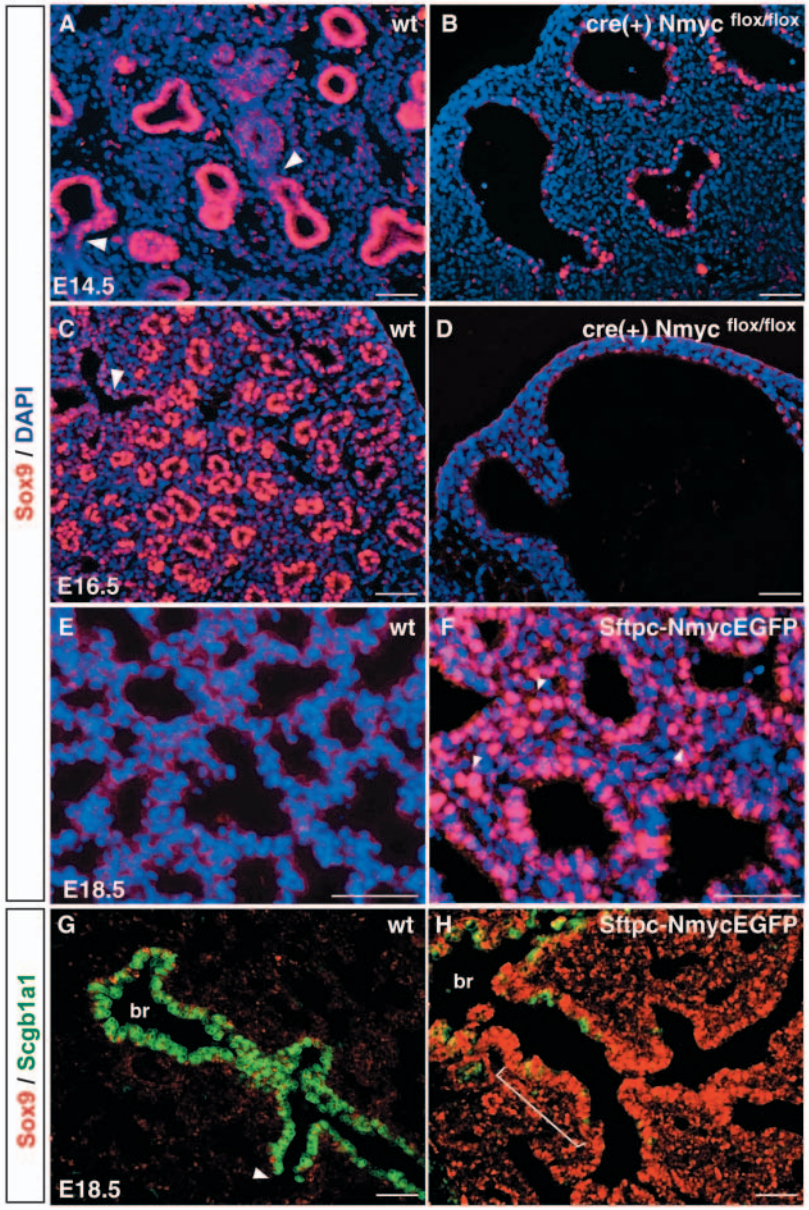

Fig. 5. Expression of Sox9 in normal, transgenic and conditional mutant lungs.
(A,C,E) Immunohistochemistry for Sox9 in wild-type lungs. Note high expression of Sox9 in undifferentiated distal epithelial cells at E14.5 and E16.5, and downregulation in alveolar epithelium at E18.5 (E,G). In cre(+);*Nmyc*$^{flox/flox}$ lungs at E14.5 and E16.5, the number of Sox9-positive cells is greatly reduced (B,D). By contrast, there are numerous Sox9-positive epithelial cells, including small clusters (arrowheads), in the E18.5 *Sftpc-NmycEGFP* lung (F). (G,H) Double immunohistochemistry for Sox9 and Scgb1a1. Note the sharp junction between proximal and distal domains (arrowhead in G), and low Sox9 expression, in wild-type lung. In transgenic lung (H), note the absence of a clear boundary and the high Sox9 expression, even in bronchiolar regions (br) where Scgb1a1 is expressed. DAPI staining marks nuclei. Scale bars: 50 μm.

allele was deleted in the lung epithelium of embryos inheriting *Sftpc-cre* (Fig. 6B and data not shown). Previous studies had shown that mice heterozygous for both a germline null allele of *Nmyc,* and a conditional null allele in the nervous system (Knoepfler et al., 2002), have only a mild phenotype of reduced body or brain mass. It was therefore surprising to observe that about half of the cre(+);*Nmyc*$^{flox/+}$ pups died at or shortly after birth with a very severe lung phenotype (Fig. 7A,B; Table 1). This result was obtained whether the cre transgene was inherited from the mother or father. The remaining pups (both males and females, in normal ratio) developed normally and had no obvious defects in lung morphology when analyzed as adults.

Surviving F_1 cre(+);*Nmyc*$^{flox/+}$ mice were mated with *Nmyc*$^{flox/flox}$ mice (Fig. 6A) giving offspring in approximately Mendelian ratios: cre(–);*Nmyc*$^{flox/+}$, 29.6%; cre(+);*Nmyc*$^{flox/+}$, 24.1%; cre(–);*Nmyc*$^{flox/flox}$, 25.9%; and cre(+);*Nmyc*$^{flox/flox}$, 20.4%. All cre(+);*Nmyc*$^{flox/flox}$ embryos died at birth or had very severe lung phenotypes when examined prenatally. Again, unexpectedly, a significant proportion (8/13; 62%) of the cre(+);*Nmyc*$^{flox/+}$ pups or embryos had abnormal lung phenotypes (Table 1). In these affected lungs there were regions that appeared normal by external morphology and histological analysis (Fig. 7B). In some cases, an entire lobe was normal, but more usually the lung consisted of a mosaic of normal and highly abnormal areas. By contrast, serial sectioning of cre(+);*Nmyc*$^{flox/+}$ lungs that were scored as normal showed a uniform histology throughout, resembling wild-type lungs (data not shown).

To confirm that the cre transgene is active, we crossed cre(+);*Nmyc*$^{flox/+}$ mice with mice compound heterozygous for *Nmyc*$^{flox/+}$ and the *Rosa26R* reporter allele. These compound heterozygotes were generated by crossing *Nmyc*$^{flox/flox}$ mice (129×C57BL/6) with Rosa26R mice (C57BL/6) (see Materials and methods). This showed that recombination had indeed taken place throughout the epithelium in both cre(+);*Nmyc*$^{flox/flox}$ and cre(+);*Nmyc*$^{flox/+}$ lungs at E16.5 and E18.5 (see Fig. S2 in supplementary material). In this set of experiments, 25% (4/16) of the cre;*Nmyc*$^{flox/+}$ lungs were abnormal (Table 1).

Increased apoptosis, reduced proliferation, and evidence for depletion of progenitor pool in conditional mutant lungs

At E18.5, the lobulation pattern and tracheal morphology of cre(+);*Nmyc*$^{flox/flox}$ and affected cre(+);*Nmyc*$^{flox/+}$ lungs were normal. However, they were composed of numerous large fluid-filled sacs, containing cellular debris, lined by highly attenuated epithelial cells, and separated by a thin layer of mesoderm (Fig. 7A,B; Fig. 8F; and data not shown).

When examined earlier (E14.5-E16.5), the external dimensions of the lungs of cre(+);*Nmyc*$^{flox/flox}$ and affected cre(+);*Nmyc*$^{flox/+}$ embryos were approximately normal (Fig. 7C,D). This was in contrast to the report that *Nmyc*$^{9a/9a}$ or *Nmyc*$^{9a/BRP}$ mutant lungs were about half the size of wild type (Moens et al., 1993). However, internally,

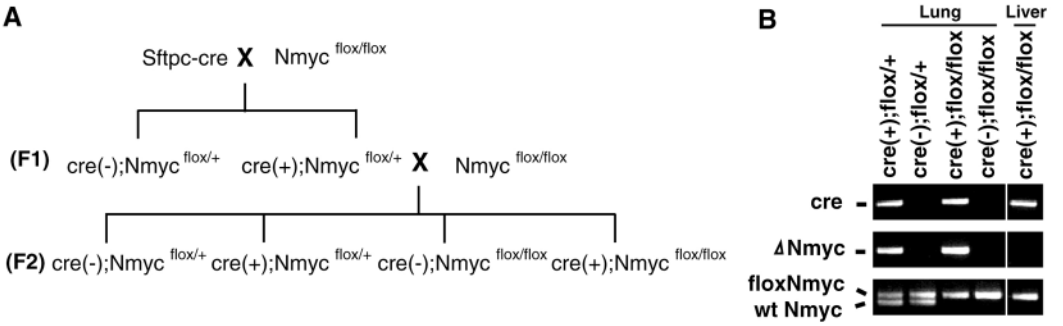

Fig. 6. Strategy for conditional deletion of *Nmyc* in the developing lung. (A) Breeding strategy to generate *Sftpc-cre* transgenic mice either homozygous or heterozygous for *Nmyc*^{flox}. (B) Typical result of genotyping lung and liver DNA. Note that the recombined *Nmyc*^{flox} allele was detected in the lung and not the liver. The unrecombined *Nmyc*^{flox} allele in the cre(+) lungs presumably derives from the mesoderm in which cre is not expressed.

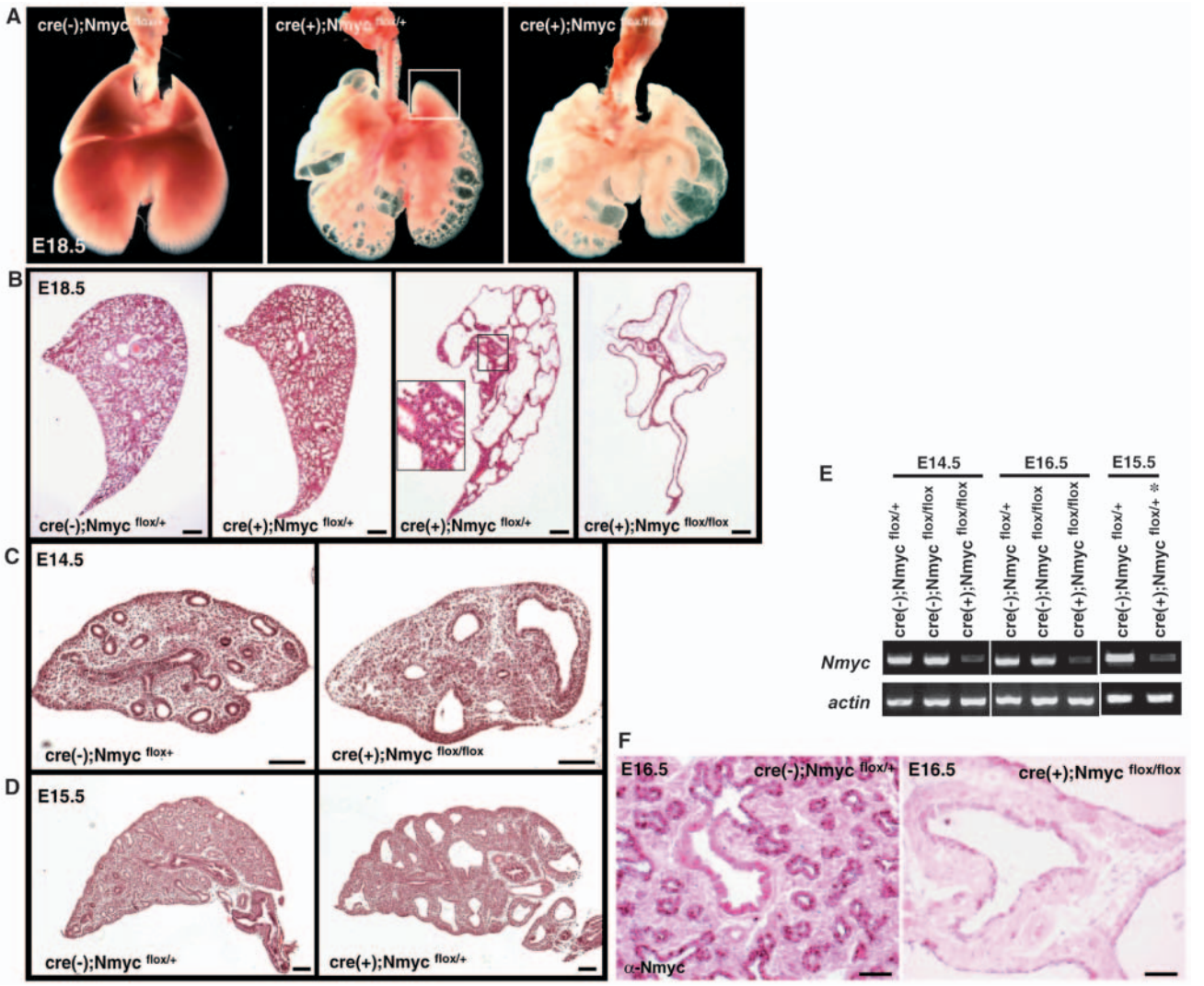

Fig. 7. Conditional deletion of *Nmyc* results in severely abnormal lung development. (A) Gross morphology of lungs at E18.5. The lung that is cre(+);*Nmyc*$^{flox/+}$ (middle panel) has a mosaic phenotype with some normal tissue (white box). By contrast, all cre(+);*Nmyc*$^{flox/flox}$ lungs (right panel) had a much more uniform abnormal phenotype. (B) Sections of E18.5 lungs after staining with Hematoxylin and Eosin. In this case, two examples of cre(+);*Nmyc*$^{flox/+}$ lungs are shown (middle panels): the one on the left has a normal morphology and resembles a cre(–) lung (far left), whereas the one on the right is severely abnormal and resembles a cre(+);*Nmyc*$^{flox/flox}$ lung (far right), except that regions of normal tissue are present (black box enlarged in inset). (C,D) Sections of wild-type and abnormal heterozygous cre(+);*Nmyc*$^{flox/flox}$ or *Nmyc*$^{flox/+}$ lungs at E14.5 (C) and E15.5 (D). (E) Level of *Nmyc* RNA in conditional mutant lungs as measured by RT-PCR of total RNA. Note the significant decrease in RNA levels at E14.5-E16.5. Asterisk indicates abnormal cre(+);*Nmyc*$^{flox/+}$ lung at E15.5. (F) Nmyc antibody staining of wild-type and cre(+); *Nmyc*$^{flox/flox}$ lungs at E18.5. Note there is only weak staining of cells in which *Nmyc* has been deleted. Scale bars: in B, 200 μm; in C,D, 100 μm; in F, 50 μm.

the conditional mutant lungs showed very reduced branching, with a few expanded tubes separated by abundant mesoderm. RT-PCR analysis showed that both the levels of *Nmyc* RNA and protein were significantly reduced at these stages (Fig. 7E,F). Epithelial cell size was irregular, and dead cells were frequently observed in the lumen, suggesting a high level of apoptosis (Fig. 8D-F). This was confirmed by staining sections with an antibody to cleaved caspase 3 (a marker for apoptosis), which revealed many positive cells in both the epithelium and mesenchyme (Fig. 8G-L). BrdU labeling at E15.5 and E16.5 (Fig. 8M-O) showed a striking reduction in the proportion of proliferating cells in the mutant lung endoderm (17% compared with 65% for wild type at E15.5). These results indicate that

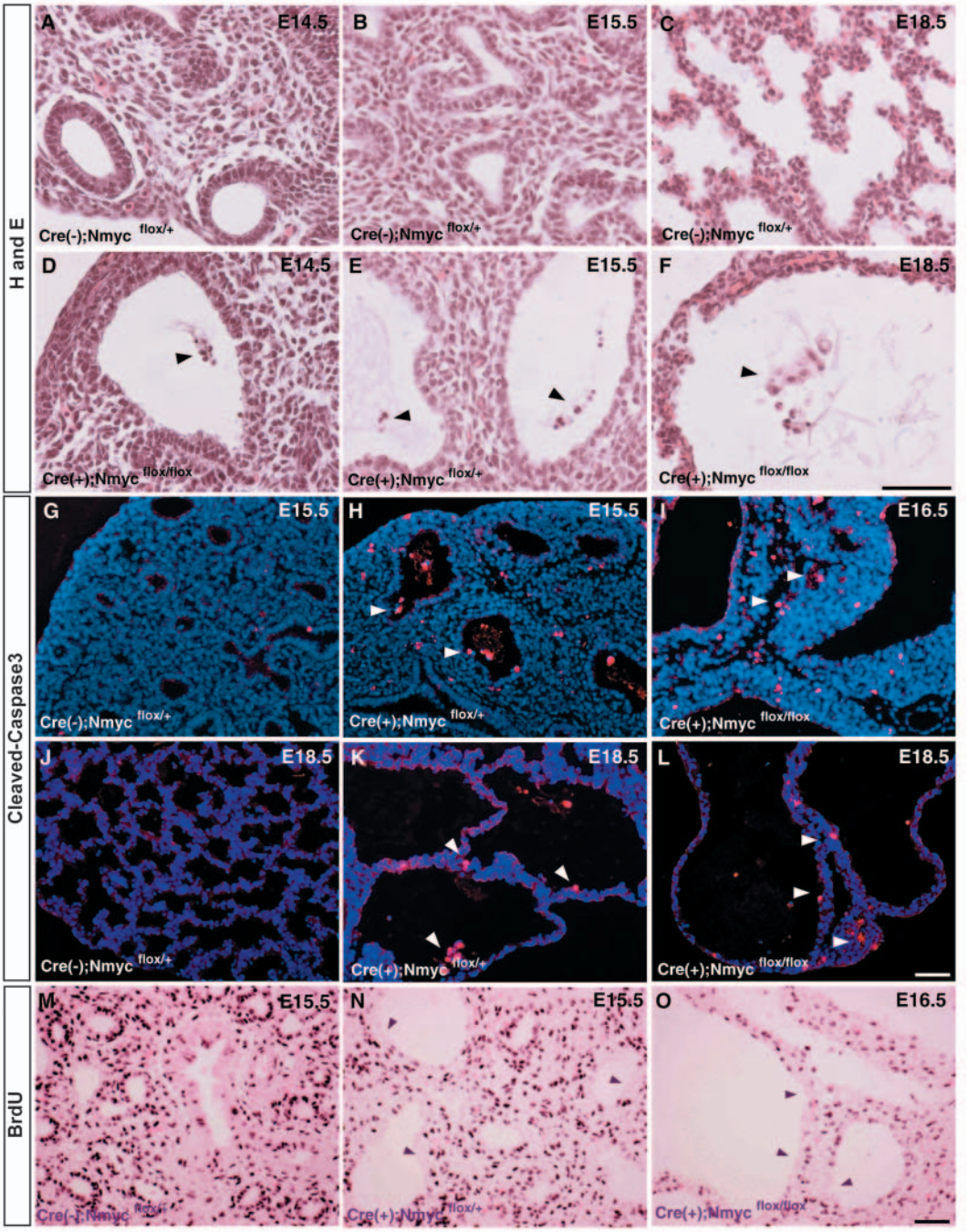

Fig. 8. Evidence for increased apoptosis and reduced proliferation in conditional mutant lungs. (A-F) Sections of cre(–) and cre(+) lungs at E14.5, E15.5 and E18.5. Note the presence of cellular debris (arrowheads), and the irregular shape and smaller size of the epithelial cells in the conditional mutant lungs (D-F). (G-L) Immunofluorescence for cleaved caspase 3 and DAPI (nuclei). Note abundant apoptotic cells in the lumen, epithelium and mesenchyme of cre(+);*Nmyc*$^{flox/+}$ and cre(+);*Nmyc*$^{flox/flox}$ lungs at E15.5, E16.5 and E18.5 (arrowheads). The faint fluorescence seen in J is from red blood cells. (M-O) BrdU labeling. Most distal epithelial cells in the E15.5 wild-type lung are positive for BrdU after a 1-hour pulse. By contrast, note the reduced epithelial labeling of mutant lungs at E15.5 and E16.5 (arrowheads). Scale bars: 50 μm.

Nmyc deletion severely inhibits both cell proliferation and survival.

Finally, we explored the differentiation of epithelial cells in the conditional mutant lungs. There was a clear reduction of the number of Sox9-positive cells in cre(+);*Nmyc*$^{flox/flox}$ lungs at E14.5 and E16.5 (Fig. 5B,D), suggesting that the pool of progenitor cells is not maintained but is lost through apoptosis or differentiation. The presence in E18.5 conditional mutant lungs of both *Sftpc*-positive type II cells and Gp38 (T1α)-positive, attenuated type I cells shows that differentiation does occur (Fig. 9B,D). Evidence that some differentiation may be premature comes from the analysis of lung differentiation markers at E16.5 by RT-PCR. As shown earlier in Fig. 1A, expression of *Aqp5* (a marker for type I cells) is normally not upregulated until E17.5-E18.5. However, *Aqp5* was detected in cre(+);*Nmyc*$^{flox/flox}$ lungs at E16.5, although it was absent from cre(–);*Nmyc*$^{flox/+}$ lungs at the same age (Fig. 9E).

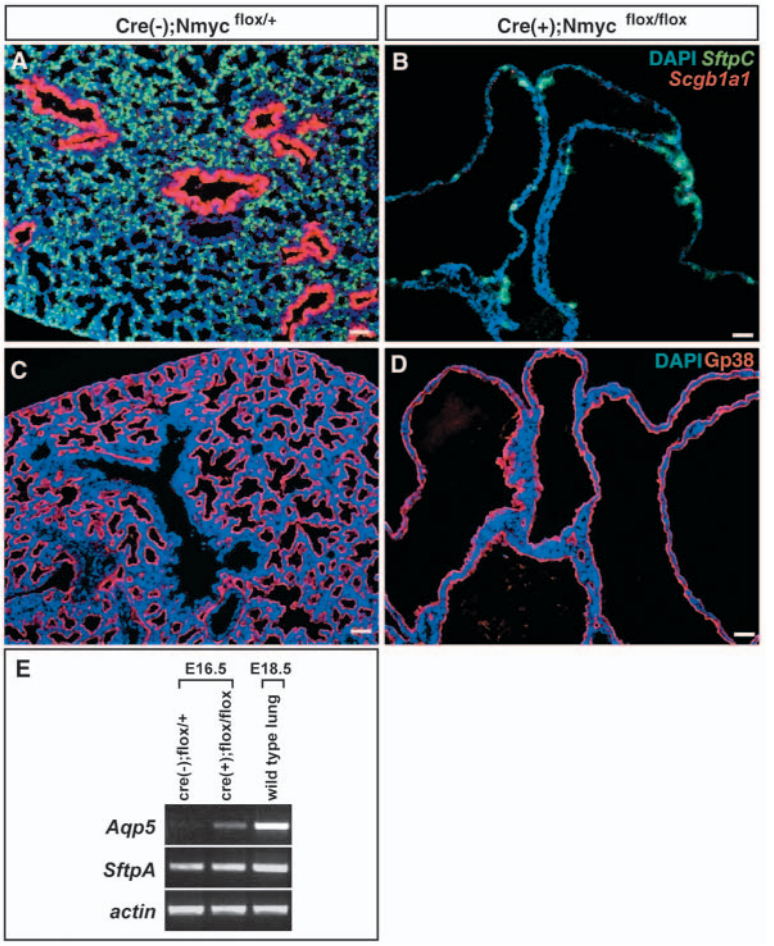

Fig. 9. Differentiation of epithelial cells in conditional mutant lungs. Double in situ hybridization for *Sftpc* and *Scgb1a1* in cre(–);*Nmyc*$^{flox/+}$ (A) and cre(+);*Nmyc*$^{flox/flox}$ (B) lungs at E18.5. Note the paucity of type II cells in the mutant lung. (C,D) Immunofluorescence for Gp38 expressed on the apical surface of type I cells at this stage. Note the presence of attenuated presumptive type I cells lining the large sacs. (E) RT-PCR for *Aqp5, SftpA* and actin. Expression of *Aqp5* is prematurely increased in cre(+);*Nmyc*$^{flox/flox}$ lung at E16.5. Scale bars: 50 μm.

Discussion

Nmyc was shown over 10 years ago to be expressed at high levels in the epithelium of the embryonic mouse lung and to be required for its normal development. However, this paper provides the first detailed analysis of the temporal and spatial distribution of the protein in relation to the proximodistal growth and patterning of the lung endoderm. This reveals a population of distal, immature epithelial cells that is characterized by high levels of Nmyc, Sox9 and cyclin D1 proteins, and contains a high proportion of cells in the S phase of the cell cycle. This population most likely represents a progenitor pool that normally gives rise to differentiated cell types along the proximodistal axis. At present, in the absence of detailed lineage analysis, it is not known whether the pool contains multipotent progenitors that can give rise to either proximal or distal cell types, a mixture of committed cells with a more restricted developmental potential, or progenitors that change their developmental potential from proximal to distal over time (Perl et al., 2002). Independent of these models, our results suggest that high levels of *Nmyc* normally ensure that a progenitor cell in the distal endoderm (regardless of its potential) remains in a cell cycle program with a high probability of re-entering S phase, rather than exiting to a differentiated state with different cell cycle kinetics (Wartiovaara et al., 2002; Westbury et al., 2001).

How do high levels of *Nmyc* perform this function in the lung? Our array analysis provides evidence that overexpression of *Nmyc* leads to the upregulation of many well-documented Myc target genes that promote cell growth (increase in cell mass), RNA processing and nucleolar structure, DNA replication, and transit of the cell cycle. It is therefore tempting to speculate that Nmyc functions not only by controlling positive and negative cell cycle regulators but also by promoting a particular functional organization of the nucleus, including changes associated with S-phase, and/or elevated levels of nucleolar proteins such as fibrillarin and nucleostemin, which are characteristic of embryonic, stem and cancer cells (Newton et al., 2003; Tsai and McKay, 2002). Our results also suggest Nmyc functions in the lung through maintaining high levels the expression of the HMGbox protein Sox9. At present, we do not know the significance of this finding, as the effect of overexpressing or deleting *Sox9* in the lung has not been reported. However, recent studies show that Sox9 protein is also localized in the proliferative compartment of the adult intestine and its overexpression in cell lines inhibits the expression of genes involved in epithelial cell differentiation (Blache et al., 2004).

The increased proliferation seen in *Sftpc-Nmyc* transgenic lungs was also accompanied by apoptosis. The induction of both apoptosis and hyperproliferation in response to Myc overexpression has been well documented in other systems, particularly under conditions in which the availability of cell survival factors becomes rate limiting (for reviews, see Hipfner and Cohen, 2004; Hueber and Evan, 1998). Recent studies have suggested a mechanism by which cells undergoing

apoptosis are extruded from an epithelial layer (Rosenblatt et al., 2001), and this might account for the dead cells seen in the lumen of both transgenic and conditional mutant lungs.

Nmyc is essential for the proliferation and survival of embryonic lung epithelial cells and for normal epithelial-mesenchymal interactions

Previous studies on embryos carrying germline mutations in *Nmyc* had suggested that lung development is particularly sensitive to reductions in Nmyc levels. Our results here support this conclusion and show that conditional deletion of *Nmyc* leads to inhibition of cell proliferation and to extensive cell death. Dying cells drop out of the epithelial layer and the survivors that fill the vacated space may possibly undergo premature differentiation, so that, by E18.5, only attenuated type I cells and a few type II cells line the large sacs. The extensive apoptosis seen in the conditional mutant lungs is in marked contrast to the effect of deleting $Nmyc^{flox}$ in neuronal progenitors in the developing brain. In this organ, there is little cell death of the neuroblasts in which *Nmyc* is deleted, and they appear to undergo cell cycle arrest and precocious differentiation (Knoepfler et al., 2002). There are at least two possible reasons for this discrepancy between lung and brain. First, in the lung, other members of the *myc* gene family apparently cannot compensate for the absence of an essential function normally provided by *Nmyc*. By contrast, in the brain *Myc* can presumably compensate for the absence of *Nmyc* (Knoepfler et al., 2002). Another reason may relate to the different organization of the cell layers in the two organs, and to the recent proposal that absence of Myc places cells at a disadvantage when competing with their neighbors for limited access to growth factors or necessary substrates (de la Cova et al., 2004; Moreno and Basler, 2004). In the case of the developing cerebellum, the progenitor cells in the germinal layer that undergo neurogenic (asymmetric) cell divisions rather than proliferative (symmetric) division generate daughter cells that move into the developing brain and differentiate. There may therefore be relatively little selective pressure in the germinal epithelium against cells with reduced or absent *Nmyc* expression. By contrast, in the lung, all the daughters of epithelial cells normally remain attached to a common, continuous basal lamina. Therefore, since recombination takes place asynchronously, the cells that are the first to lose *Nmyc* will be at a growth disadvantage compared with their neighbors, and may be competed or forced off the substrate on which they depend for their survival. Restriction in access to essential factors for cell growth and survival may also account for the high level of apoptosis seen in the mesenchyme of conditional mutant lungs at E15.5 and E16.5, even though *Nmyc* is not expressed in this cell population. We and others have shown that, in the developing lung, the endoderm produces factors, such as sonic hedgehog, that are necessary for the survival and proliferation of the mesoderm (Gebb and Shannon, 2000; Weaver et al., 2003).

A general conclusion from our gain- and loss-of-function studies is that Nmyc plays a key role in controlling the flow of cells into and out of a distal progenitor pool during lung development. It will therefore be important to determine how factors such as Wnts, Tgfβs and Igfs regulate the expression of *Nmyc* RNA, and the phosphorylation and turnover of the protein, at different stages of embryogenesis (Frederick et al., 2004; Kenney et al., 2004) (our unpublished observations suggesting that *Nmyc* is a direct target of Wnt signaling in the embryonic lung).

Effects of acute *Nmyc* hemizygosity on lung development

A striking finding of this study was the failure in lung development in half (40/81) of the embryos in which only one copy of *Nmyc* is deleted by cre recombination. The precise proportion of affected embryos varied from 53% (28/52) in the F1 generation from crossing Sftpc-cre;$Nmyc^{+/+}$ with $Nmyc^{flox/flox}$ mice, to 62% (8/13) in the F2 generation (Fig. 6), and 25% (4/16) when Sftpc-cre(+); $Nmyc^{flox/+}$ mice were crossed with $Nmyc^{flox/+}$;Rosa26R mice (Table 1). There are two possible explanations for these results, neither of which can be excluded without further extensive experimentation. The first possibility is that the (129×C57BL/6) background on which the $Nmyc^{flox}$ allele is maintained is segregating for a modifier gene that decreases the probability that a lung epithelial cell in which only one *Nmyc* allele is active will continue to proliferate. The second, more speculative, model proposes that the level of *Nmyc* transcription is normally tightly controlled and involves an autoregulatory negative-feedback loop acting early in development (Penn et al., 1990). According to this model, in the developing embryo the probability that *Nmyc* transcription is initiated is low and, once one allele is active in a cell, the transcription of the other allele is inhibited by the feedback loop. This essentially monoallelic expression is then maintained, possibly by epigenetic modification. Consequently, the epithelium of the primary buds of heterozygous cre(+);$Nmyc^{flox/+}$ embryos will be a mosaic of cells in which either one or the other of the *Nmyc* alleles is active. Those cells in which the $Nmyc^{flox}$ allele is active will become functionally null after recombination. Depending on the proportion of cells in the early lung primordium with each allele active, and on the level of competition between normal and mutant cells, the conditional mutant lungs will be either abnormal, a mosaic of normal and abnormal tissue, or completely normal. To further test this model will require analysis of the transcription or epigenetic modification of distinguishable *Nmyc* alleles at the single-cell level in the embryonic lung. Meanwhile, whichever model is correct, our findings raise the interesting possibility that, prior to amplification of Nmyc or aberrant activation of Myc, lung tumor cells should be particularly sensitive to agents downregulating Nmyc expression.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/6/1363/DC1

References

Arnold, I. and Watt, F. M. (2001). c-Myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny. *Curr. Biol.* **11**, 558-568.

Blache, P., van de Wetering, M., Duluc, I., Domon, C., Berta, P., Freund, J. N., Clevers, H. and Jay, P. (2004). SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. *J. Cell Biol.* **166**, 37-47.

Boon, K., Caron, H. N., van Asperen, R., Valentijn, L., Hermus, M. C., van Sluis, P., Roobeek, I., Weis, I., Voute, P. A., Schwab, M. et al. (2001). N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. *EMBO J.* **20**, 1383-1393.

Cardoso, W. V. (2000). Lung morphogenesis revisited: old facts, current ideas. *Dev. Dyn.* **219**, 121-130.

de la Cova, C., Abril, M., Bellosta, P., Gallant, P. and Johnston, L. A. (2004). Drosophila myc regulates organ size by inducing cell competition. *Cell* **117**, 107-116.

Eisenman, R. N. (2001). Deconstructing myc. *Genes Dev.* **15**, 2023-2030.

Frederick, J. P., Liberati, N. T., Waddell, D. S., Shi, Y. and Wang, X. F. (2004). Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element. *Mol. Cell. Biol.* **24**, 2546-2559.

Frye, M., Gardner, C., Li, E. R., Arnold, I. and Watt, F. M. (2003). Evidence that Myc activation depletes the epidermal stem cell compartment by modulating adhesive interactions with the local microenvironment. *Development* **130**, 2793-2808.

Gasca, S., Canizares, J., De Santa Barbara, P., Mejean, C., Poulat, F., Berta, P. and Boizet-Bonhoure, B. (2002). A nuclear export signal within the high mobility group domain regulates the nucleocytoplasmic translocation of SOX9 during sexual determination. *Proc. Natl. Acad. Sci. USA* **99**, 11199-11204.

Gebb, S. A. and Shannon, J. M. (2000). Tissue interactions mediate early events in pulmonary vasculogenesis. *Dev. Dyn.* **217**, 159-169.

Hipfner, D. R. and Cohen, S. M. (2004). Connecting proliferation and apoptosis in development and disease. *Nat. Rev. Mol. Cell. Biol.* **5**, 805-815.

Hueber, A. O. and Evan, G. I. (1998). Traps to catch unwary oncogenes. *Trends Genet.* **14**, 364-367.

Kenney, A. M., Cole, M. D. and Rowitch, D. H. (2003). Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors. *Development* **130**, 15-28.

Kenney, A. M., Widlund, H. R. and Rowitch, D. H. (2004). Hedgehog and PI-3 kinase signaling converge on Nmyc1 to promote cell cycle progression in cerebellar neuronal precursors. *Development* **131**, 217-228.

Knoepfler, P. S., Cheng, P. F. and Eisenman, R. N. (2002). N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. *Genes Dev.* **16**, 2699-2712.

Levens, D. L. (2003). Reconstructing MYC. *Genes Dev.* **17**, 1071-1077.

Linder, B., Gerlach, N. and Jackle, H. (2001). The Drosophila homolog of the human AF10 is an HP1-interacting suppressor of position effect variegation. *EMBO Rep.* **2**, 211-216.

Miaw, S. C., Choi, A., Yu, E., Kishikawa, H. and Ho, I. C. (2000). ROG, repressor of GATA, regulates the expression of cytokine genes. *Immunity* **12**, 323-333.

Moens, C. B., Auerbach, A. B., Conlon, R. A., Joyner, A. L. and Rossant, J. (1992). A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung. *Genes Dev.* **6**, 691-704.

Moens, C. B., Stanton, B. R., Parada, L. F. and Rossant, J. (1993). Defects in heart and lung development in compound heterzygotes for two different targeted mutations at the N-*myc* locus. *Development* **119**, 485-499.

Moniot, B., Biau, S., Faure, S., Nielsen, C. M., Berta, P., Roberts, D. J. and de Santa Barbara, P. (2004). SOX9 specifies the pyloric sphincter epithelium through mesenchymal-epithelial signals. *Development* **131**, 3795-3804.

Moreno, E. and Basler, K. (2004). dMyc transforms cells into super-competitors. *Cell* **117**, 117-129.

Newton, K., Petfalski, E., Tollervey, D. and Caceres, J. F. (2003). Fibrillarin is essential for early development and required for accumulation of an intron-encoded small nucleolar RNA in the mouse. *Mol. Cell. Biol.* **23**, 8519-8527.

Okubo, T. and Hogan, B. L. (2004). Hyperactive Wnt signaling changes the developmental potential of embryonic lung endoderm. *J. Biol.* **3**, 11.

Patel, J. H., Loboda, A. P., Showe, M. K., Showe, L. C. and McMahon, S. B. (2004). Opinion: analysis of genomic targets reveals complex functions of MYC. *Nat. Rev. Cancer* **4**, 562-568.

Penn, L. J., Brooks, M. W., Laufer, E. M. and Land, H. (1990). Negative autoregulation of c-myc transcription. *EMBO J.* **9**, 1113-1121.

Perl, A. K., Wert, S. E., Nagy, A., Lobe, C. G. and Whitsett, J. A. (2002). Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc. Natl. Acad. Sci. USA* **99**, 10482-10487.

Raetz, E. A., Kim, M. K., Moos, P., Carlson, M., Bruggers, C., Hooper, D. K., Foot, L., Liu, T., Seeger, R. and Carroll, W. L. (2003). Identification of genes that are regulated transcriptionally by Myc in childhood tumors. *Cancer* **98**, 841-853.

Rosenblatt, J., Raff, M. C. and Cramer, L. P. (2001). An epithelial cell destined for apoptosis signals its neighbors to extrude it by an actin- and myosin-dependent mechanism. *Curr. Biol.* **11**, 1847-1857.

Sawai, S., Shimono, A., Wakamatsu, Y., Palmes, C., Hanaoka, K. and Kondoh, H. (1993). Defects of embryonic organogenesis resulting from targeted disruption of the N-*myc* gene in the mouse. *Development* **117**, 1445-1455.

Shiio, Y., Donohoe, S., Yi, E. C., Goodlett, D. R., Aebersold, R. and Eisenman, R. N. (2002). Quantitative proteomic analysis of Myc oncoprotein function. *EMBO J.* **21**, 5088-5096.

Stanton, B. R., Perkins, A. S., Tessarollo, L., Sassoon, D. A. and Parada, L. F. (1992). Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop. *Genes Dev.* **6**, 2235-2247.

Ten Have-Opbroek, A. A. W. (1981). The development of the lung in mammals: an analysis of concepts and findings. *Am. J. Anat.* **162**, 201-219.

Ten Have-Opbroek, A. A. W. (1991). Lung development in the mouse embryo. *Exp. Lung Res.* **17**, 111-130.

Tsai, R. Y. and McKay, R. D. (2002). A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. *Genes Dev.* **16**, 2991-3003.

Wakamatsu, Y., Watanabe, Y., Shimono, A. and Kondoh, H. (1993). Transition of localization of the N-Myc protein from nucleus to cytoplasm in differentiating neurons. *Neuron* **10**, 1-9.

Warburton, D., Schwarz, M., Tefft, D., Flores-Delgado, G., Anderson, K. D. and Cardoso, W. V. (2000). The molecular basis of lung morphogenesis. *Mech. Dev.* **92**, 55-81.

Wartiovaara, K., Barnabe-Heider, F., Miller, F. D. and Kaplan, D. R. (2002). N-myc promotes survival and induces S-phase entry of postmitotic sympathetic neurons. *J. Neurosci.* **22**, 815-824.

Weaver, M., Yingling, J. M., Dunn, N. R., Bellusci, S. and Hogan, B. L. (1999). Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. *Development* **126**, 4005-4015.

Weaver, M., Batts, L. and Hogan, B. L. (2003). Tissue interactions pattern the mesenchyme of the embryonic mouse lung. *Dev. Biol.* **258**, 169-184.

Wert, S. E., Glasser, S. W., Korfhagen, T. R. and Whitsett, J. A. (1993). Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. *Dev. Biol.* **156**, 426-443.

Westbury, J., Watkins, M., Ferguson-Smith, A. C. and Smith, J. (2001). Dynamic temporal and spatial regulation of the cdk inhibitor p57(kip2) during embryo morphogenesis. *Mech. Dev.* **109**, 83-89.

Yang, H., Lu, M. M., Zhang, L., Whitsett, J. A. and Morrisey, E. E. (2002). GATA6 regulates differentiation of distal lung epithelium. *Development* **129**, 2233-2246.

Zeller, K. I., Jegga, A. G., Aronow, B. J., O'Donnell, K. A. and Dang, C. V. (2003). An integrated database of genes responsive to the Myc oncogenic transcription factor: identification of direct genomic targets. *Genome Biol.* **4**, R69.