A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung

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The N-myc proto-oncogene encodes a putative transcription factor that has been postulated to be involved in the control of differentiation in a number of lineages at various stages during mammalian embryogenesis. We have generated a leaky mutation in N-myc by gene targeting in embryonic stem cells. In this allele, the neo' gene was inserted into the first intron of N-myc, in such a way that alternative splicing around this insertion could result in the generation of a normal N-myc transcript in addition to a mutant transcript. Mice homozygous for this mutation died immediately after birth owing to an inability to oxygenate their blood. Histological examination revealed a marked underdevelopment in the lung airway epithelium, resulting in a decreased respiratory surface area. Analysis of N-myc expression in wild-type and homozygous mutant embryonic lungs suggests that N-myc is required for the proliferation of the lung epithelium in response to local inductive signals emanating from the lung mesenchyme. Homozygous mutant embryos were slightly smaller than normal and also had a marked reduction in spleen size, whereas other tissues that normally express N-myc appeared to be unaffected by the mutation. Molecular analysis revealed that normal N-myc transcripts were found in tissues from homozygous mutant embryos. Different tissues expressed the normal N-myc transcript at different levels relative to those observed in wild-type embryos, with the lowest levels being observed in the lungs. These results illustrate one way in which gene targeting can be used to generate partial loss-of-function mutations and support the importance of generating a series of alleles at a given locus to elucidate the various different functions of a gene during development.

[Key Words: Gene targeting; N-myc; lung branching morphogenesis; embryonic stem cells]

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N-myc is a proto-oncogene that is expressed during the differentiation of several lineages during mammalian embryogenesis and, when deregulated, participates in neoplastic transformation. The myc family of proto-oncogenes, which includes N-myc, c-myc, and L-myc, encodes nuclear proteins that share structural motifs with a number of transcription factors that control cell determination (for review, see Lüscher and Eisenman 1990). Myc proteins share a basic region/helix-loop-helix (b-HLH) domain with the products of a number of the myogenic genes (for review, see Olson 1990), as well as with products of genes that function in the specification of the Drosophila central nervous system, such as hairy (Rushlow et al. 1989), daughterless (Caudy et al. 1989), and the genes of the achaete-scute complex (Villares and Cabrera 1987). The Myc proteins also possess a leucine zipper dimerization motif, which is found in transcription factors such as those encoded by CCAAT/enhancer-binding protein (C/EBP) and the jun proto-oncogene, and in Fos, which associates with Jun by this motif (Kouzarides and Ziff 1988; Landschulz et al. 1988). A number of transcription factors possess both a b-HLH domain and a leucine zipper domain, including TFE3 (Beckmann et al. 1990), AP4 (Hu et al. 1990), and USF (Gregor et al. 1990).

The c-Myc protein possesses sequence-specific DNA-binding activity (Blackwell et al. 1990), which is enhanced by association, by complementary HLH–leucine zipper domains, with the Max and Myn proteins in humans and mice, respectively (Blackwood and Eisenman 1991; Prendergast et al. 1991). Although sequence-specific DNA binding has not yet been shown for the N-Myc protein, it has been shown to associate with Max with the same affinity as does the c-Myc protein (Blackwood and Eisenman 1991). Thus, N-myc is also likely to encode a sequence-specific DNA-binding protein. These results, taken together with the similarity N-myc has
with genes encoding known transcription factors, suggest that N-myc also encodes a transcription factor.

The expression pattern of N-myc during mouse development suggests a role in the differentiation of certain embryonic lineages. Unlike c-myc, which is generally expressed in proliferating cells during embryogenesis (Zimmerman et al. 1986; Schmid et al. 1989), and continues to be expressed in some tissues in the adult mouse (Zimmerman et al. 1986; Semsei et al. 1989). N-myc expression is confined to specific lineages during embryonic development and is expressed in the adult only at low levels in the heart (Zimmerman et al. 1986). N-myc is expressed in undifferentiated embryonic stem (ES) and embryonal carcinoma (EC) cells and is down-regulated upon EC cell differentiation in vitro (Jakobovits et al. 1985). Within the embryo, N-myc is expressed in primitive mesoderm cells arising from the primitive streak, and its down-regulation correlates with differentiation into somites (Downs et al. 1989). During organogenesis, N-myc is expressed in the brain, eye, kidney, lung, heart, and intestine (Zimmerman et al. 1986; Hirning et al. 1991). Detailed in situ analyses of N-myc transcripts during organogenesis (Mugrauer et al. 1988; Hirning et al. 1991) have shown that expression is restricted to particular undifferentiated populations of cells within these organs, regardless of their proliferative state. Expression of N-myc within the lung, kidney, and intestine is localized to epithelial cells and is not observed in the surrounding mesenchyme (Mugrauer et al. 1988; Hirning et al. 1991). N-myc is also expressed at higher levels in pre-B-cell lines than in mature B-cell lines (Zimmerman et al. 1986). Thus, in general, N-myc is expressed in subsets of cells that are in an early state of differentiation, and the further differentiation of these cells correlates with N-myc down-regulation. This suggests that N-myc plays a role in the completion of the differentiation pathway in a number of lineages by maintaining cells in an undifferentiated state.

N-myc was originally identified as an amplified gene in neuroblastoma (Kohl et al. 1983, 1984; Schwab et al. 1983), and has also been found often to be amplified or overexpressed in Wilms’ tumor (Nisen et al. 1986), small-cell lung cancer (Nau et al. 1986; Wong et al. 1986), and retinoblastoma (RB) (Lee et al. 1984). This series of tumors correlates with tissue types that normally express N-myc during development: the brain, kidney, lung, and eye, respectively (Zimmerman et al. 1986; Hirning et al. 1991). All of these tumors are thought to arise from embryonic cells or, in the case of small-cell lung cancer, from undifferentiated progenitor cells. These observations support the hypothesis that N-myc normally plays a role in controlling the differentiation of these tissues, and that continued N-myc expression prevents differentiation and thus promotes uncontrolled growth.

To address directly the function of N-myc during development, we attempted to mutate it by homologous recombination in ES cells and to introduce the mutation into the mouse germ line (Capcacci 1989). A replacement-type targeting vector was used to eliminate gene function. However, during our studies we identified a line in which the targeting vector had integrated into the first intron by an insertion-type event. Our analysis has shown this to be a leaky mutation that reduces but does not eliminate normal N-myc transcription. Mice homozygous for this mutation die at birth owing to an inability to oxygenate their blood, a phenotype that apparently arises from a defect in branching morphogenesis in the lung. This mutant phenotype suggests that one role for N-myc is in the epithelial–mesenchymal interactions that are the basis of lung morphogenesis.

Results

Gene targeting

N-myc was mutated in ES cells using a replacement-type targeting vector [Fig. 1b], which included a promoterless neo gene flanked by 0.8 kb of 5’ homology and 3.4 kb of 3’ homology to the endogenous N-myc gene [Fig. 1a, DePinho et al. 1986]. Homologous recombination or gene conversion between the vector and the endogenous N-myc gene was expected to result in the replacement of the coding region of the second N-myc exon with the neo gene and its accompanying SV40 polyadenylation signal. Because N-myc is expressed at high levels in ES cells [Jakobovits et al. 1985], this type of vector was designed to enrich for the detection of homologous recombination events relative to random integration events. A similar targeting strategy for the N-myc gene has been described by others (Charron et al. 1990; Stanton et al. 1990, Sawai et al. 1991).

Eleven targeted G418-resistant colonies were identified by detection of a novel polymerase chain reaction (PCR) fragment at a frequency of 1 targeted colony per 65 G418-resistant colonies, or 1 targeted clone per 1.6 × 10^7 cells electroporated (Table 1). All of the PCR-positive colonies that were established as ES cell lines were confirmed as targeted lines by Southern blot analysis [Fig. 2]. The level of enrichment for N-myc homologous recombinants among G418-resistant colonies was lower than that reported by others [Charron et al. 1990; Stanton et al. 1990; Sawai et al. 1991]. This discrepancy may be the result of differences in the targeting vectors used, as our vector did not include a splice donor in the 5’ region of homology upstream of the neo-containing exon.

Four of the targeted ES cell lines had integrated the targeting vector by homologous recombination in the predicted manner [Fig. 1c], as determined by Southern blot analysis [Fig. 2]. The remaining line, line 9a, had integrated the targeting vector into N-myc in an unexpected manner. Line 9a was initially detected as a homologous recombinant both by PCR and by Southern blot analysis using an upstream probe on DNA digested with an enzyme that cuts within neo [Fig. 2a]. However, Southern blot analysis using an exon 2 probe demonstrated that exon 2 had not been replaced in this targeted cell line [Fig. 2b]. Extensive Southern blot analysis of line
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Figure 1. N-myc targeting scheme. (a) The endogenous N-myc locus, contained within a 7.4-kb EcoRI fragment (DePinho et al. 1986). (b) Targeting vector. A 0.9-kb PvuII–SmaI fragment of the N-myc gene, including all but three codons of the coding region of the second exon, was replaced with the promoterless neo gene, which also had an SV40 polyadenylation signal. The neo gene was flanked upstream and downstream by 0.8 and 3.4 kb of N-myc sequences, respectively. (c) The predicted structure of the N-myc gene after gene replacement via homologous recombination. Primer annealing sites for PCR detection of homologous recombinants are shown (arrows a and b). (d) The structure of the targeted N-myc locus in line 9a, N-myc\textsuperscript{9a}. A probable single homologous recombination event between the endogenous gene and the 5' region of homology of the targeting vector has resulted in the insertion of the majority of the targeting vector into intron I of the endogenous gene. The annealing sites for primers that amplify the junction fragment at the site of integration of the 3' end of the targeting vector are shown (arrows x and y). (e) Possible transcripts encoded by the N-myc\textsuperscript{9a} allele. Alternative splicing to the inserted neo-containing exon or to the endogenous exon 2 downstream of it results, respectively, in a truncated transcript that includes only three N-myc codons or a normal N-myc transcript. (f) Sequence of the PCR-amplified junction fragment between the 3' end of the targeting vector and the endogenous gene. Nucleotides in boldface type are derived from intron II of the targeting vector; underlined nucleotides are from intron I of the endogenous gene. [Solid bars] N-myc-coding regions; [open bars] N-myc-noncoding exon sequences; [lines] intron and flanking regions; [SA] splice acceptor; [SD] splice donor; [B] BamHI; [E] EcoRI; [SII] SstII; [S] SalI; [PvuII] PvuII; [Sm] SmaI.

9a (not shown) demonstrated that most of the targeting vector was inserted within the first intron of the endogenous N-myc gene. The deduced structure of the targeted N-myc gene in line 9a is shown in Figure 1d.

To determine precisely the site of integration of the targeting vector in line 9a, the new junction created between intron II of the inserted targeting vector and intron I of the endogenous N-myc gene was amplified by PCR using primers x and y [Fig. 1d] and then sequenced. The sequence of the junction [Fig. 1f] shows a clear transition from targeting vector to endogenous gene sequences, and the joining occurred without the generation of repeats or deletions. These results suggest the possibility that the electroporated linear targeting vector was partially degraded, recircularized within the cell, and integrated by a single insertion-type recombination event in the 5' region of homology.

The structure of the N-myc allele in line 9a, which will henceforth be referred to as N-myc\textsuperscript{9a}, was such that it would be a null mutation if all transcripts terminated in the neo poly(A) addition site. However, the N-myc\textsuperscript{9a} mutation would be leaky if some primary transcripts were alternatively spliced to the normal second exon, such that the neo-containing exon was removed [Fig. 1e].
Table 1. Frequency of gene targeting

| Experiment | Number of cells electroporated | Number of G418R colonies | Number of homologous recombinants assayed by PCR | Number of homologous recombinants targeted by replacement | Number of homologous recombinants targeted by insertion |
|------------|-------------------------------|--------------------------|--------------------------------------------------|--------------------------------------------------------|------------------------------------------------------|
| 1          | 5 x 10^6                      | 100                      | 2                                                | 2                                                      | 0                                                    |
| 2          | 2.5 x 10^7                    | 180                      | 5                                                | 1                                                      | 0                                                    |
| 3          | 7.5 x 10^7                    | 300                      | 3                                                | 1                                                      | 1                                                    |
| 4          | 3 x 10^7                      | 130                      | 1                                                | 0                                                      | 0                                                    |
| Total      | 1.8 x 10^6                    | 710                      | 11                                               | 4                                                      | 1                                                    |

**Germ-line transmission of the targeted ES cell line**

Cells from three of the targeted lines, including line 9a, were injected into blastocysts from C57BL/6J females. All were able to contribute extensively to the resulting chimeric pups. However only line 9a, which contains N-myc^{9a}, was transmitted through the germ line (Table 2). The four male germ-line chimeras produced by blastocyst injection of mutant line 9a were bred to C57BL/6J and CD1 females to generate stocks of N-myc^{9a} mice that were used for subsequent experiments. These mice had no apparent abnormal phenotype.

**Mice homozygous for the N-myc^{9a} mutation die at birth**

To investigate the effect of the N-myc^{9a} allele in the homozygous condition, N-myc^{9a/+} mice were intercrossed, and DNA isolated from their offspring was tested by Southern blot analysis for the presence of the mutation. A total of 171 pups from such matings were analyzed at 3–5 weeks of age, and none were N-myc^{9a/9a}. To delineate when N-myc^{9a/9a} embryos died, DNA from embryos at various stages of gestation from heterozygote intercrosses was analyzed by Southern blotting or PCR.

**Figure 2.** Southern blot analysis of homologous recombinants. DNA isolated from 5 of the 12 PCR-positive ES cell lines was digested as shown. (a) BamHI digest, probed with probe 1 (Fig. 1a). The lower band in this digest represents the targeted allele (for predicted fragment sizes, see Fig. 1). (b) EcoRI + Sall digest, probed with an exon 2 probe [probe 2, Fig. 1a]. The band present in all lanes represents the normal allele. Note that only line 9a has a new exon 2-containing band, indicating that neo has not replaced the coding region of exon 2 in this line.
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Table 2. Frequency of germ-line transmission of targeted ES cell lines

| Cell line      | Number of blastocysts injected | Number of live progeny | Number of chimeras | Number of males test bred | Number of germ line |
|----------------|--------------------------------|------------------------|---------------------|----------------------------|---------------------|
| D3-N-myc^{ya}  | 119                            | 34                     | 3                   | 5                          | 4                   |
| D3-N-myc^{37}  | 280                            | 48                     | 8                   | 15                         | 0                   |
| D3-N-myc^{38}  | 182                            | 28                     | 1                   | 3                          | 2                   |

Of a total of 372 embryos dissected between 10.5 and 18.5 days of gestation, there were 93 wild-type, 179 N-myc\(^{ya}\) + and 100 N-myc\(^{ya/ya}\) embryos. Thus, the absence of N-myc\(^{ya/ya}\) mice observed at weaning was not the result of fetal death but of early postpartum death. Occasionally, dead pups were found the first morning after delivery from a heterozygote mating. All but one of these pups were subsequently found to be N-myc\(^{ya/ya}\). Thus, N-myc\(^{ya/ya}\) pups died near birth and were generally being cannibalized by the mother before they could be analyzed. On two occasions, a pup that was later found to be N-myc\(^{ya/ya}\) survived as long as 12 hr after birth; these pups were small, did not feed, and had erratic breathing.

To rescue N-myc\(^{ya/ya}\) embryos for further analysis, pups from heterozygote intercrosses were delivered by Caesarian section at 18.5 days postcoitum (p.c.). Of 68 pups, 52 lived, but the remaining 16 attempted to breathe and failed to oxygenate their blood sufficiently. In the 30 min following delivery, while littermates turned pink and began to move and breathe regularly, these 16 pups remained gray, immobile, and only gasped for air sporadically. These pups were always the smallest ones in a litter. Subsequent analysis of DNA isolated from all pups showed that these 16 and no others were N-myc\(^{ya/ya}\).

Germ-line chimeras were also crossed to 129Sv/J females to place the mutation on an inbred background. The phenotype of N-myc\(^{ya/ya}\) embryos produced by heterozygote intercrosses was identical on inbred or hybrid genetic backgrounds [data not shown].

N-myc\(^{ya/ya}\) pups die because of a defect in lung morphogenesis

The abrupt death and the inability of N-myc\(^{ya/ya}\) pups to oxygenate their blood suggested a defect either in their ability to switch blood flow from the placenta to the lungs, or in the lungs themselves. Histological analysis of the lungs showed the latter to be true. We observed that immediately after birth, the air spaces in the lungs of N-myc\(^{ya/ya}\) pups were reduced in number and were individually much larger than the air spaces of their littermates (Fig. 3a,b). Furthermore, in the N-myc\(^{ya/ya}\) pups, the septa that separate the air spaces were thinner and often appeared to have been broken by the pressure of the first breaths of the pups. The outer surface of the lung was very thin and fragile in the homozygotes, whereas in wild-type littermates it was strengthened by progressively smaller air spaces.

To determine the developmental progression of this phenotype, we analyzed the lungs of 12.5- to 16.5-day embryos. Between 15.5 and 16.5 days p.c., the lung mesenchyme of wild-type embryos is regularly interrupted by the organized epithelium that forms the developing airways and air spaces. The lungs of N-myc\(^{ya/ya}\) embryos were smaller and the epithelium was not as extensively branched, giving the mesenchyme a smooth, uninterrupted appearance [cf. Fig. 3c and d]. We quantitated this effect by counting the number of developing air spaces per unit area in matched sections of the same lobe of lungs dissected from N-myc\(^{+/-}\) and N-myc\(^{ya/ya}\) 16.5-day sibling embryos (Table 3), and found that N-myc\(^{ya/ya}\) lungs contained approximately two-thirds the number of developing airways per unit area when compared with wild-type lungs [\(P <0.001\)]. This number is an underestimate of the reduction in the total number of developing airways because N-myc\(^{ya/ya}\) lungs were roughly half the size of wild-type lungs. It further underestimates the phenotypic difference between mutant and wild-type lungs because the developing airways in wild-type lungs were individually larger than in N-myc\(^{ya/ya}\) lungs.

The number and distribution of bronchi and bronchioles, from which the developing airways branch and which are histologically distinct from the more distal airways, appeared to be normal in N-myc\(^{ya/ya}\) embryos at 15.5 and 16.5 days p.c. Thus, the effect of the N-myc\(^{ya}\) mutation in homozygotes was restricted to the more distal airways of the developing lungs. A change in the the overall size and morphology of the lungs in N-myc\(^{ya/ya}\) embryos was detectable as early as 12.5 days p.c., when N-myc\(^{ya/ya}\) lungs were dramatically reduced in size compared to normal lungs, with considerably less epithelial branching (Fig. 6a,b, below). At 13.5 days p.c., when the right lung normally is subdivided into its four constituent lobes, it was often observed that in the N-myc\(^{ya/ya}\) embryo these lobes were fused together, making the single right lung larger than the left lung [not shown].

Analysis of hematoxylin- and -eosin (H&E)-stained sections of embryonic lungs throughout their development did not indicate that any cell types were absent from N-myc\(^{ya/ya}\) lungs. Furthermore, we performed immunohistochemistry on lungs at 14.5 and 18.5 days p.c. using an antibody against the endothelial cell-specific Von Willebrand's factor and did not observe any abnormalities in the vascularization of N-myc\(^{ya/ya}\) lungs [not shown].
Figure 3. Histological analysis of lungs of N-myc<sup>+/+</sup> and N-myc<sup>-/-</sup> embryos. (a,b) Matched sections through the left lung of newborn littermates delivered by cesarian section at 18.5 days p.c. Bar, 250 μm. [a] N-myc<sup>+/+</sup>; (b) N-myc<sup>-/-</sup>. [sep] septum; [tas] terminal air sac. [c,d] Matched sagittal sections of 15.5-day sibling embryos showing the large left lung and the small postcaval lobe of the right lung where it crosses to the left side of the embryo. Bar, 500 μm. [c] N-myc<sup>+/+</sup>; [d] N-myc<sup>-/-</sup>. [aw] airway; [br] bronchus; [rl] right lung (postcaval lobe); [liv] liver.

The effect of the N-myc<sup>9a</sup> mutation on the development of other lineages

The most direct explanation for the abrupt postnatal death of N-myc<sup>9a/9a</sup> pups was that reduced branching and/or proliferation of the lung epithelium during lung morphogenesis led to a severely reduced lung surface area, which did not permit full oxygenation of the blood of the pups after birth. However, it was important to examine fetuses for other possible morphological alterations caused by the mutation. The only other obvious morphological defect in N-myc<sup>9a/9a</sup> embryos was that the spleens of 18.5-day p.c. embryos were reduced to approximately one-fifth of their normal size (not shown). Nevertheless, there were no clear histological abnormalities in the cellular composition of the mutant spleens (not shown), as determined by examination of H&E-stained sectioned spleens. N-myc<sup>9a/9a</sup> embryos were slightly smaller than their littermates from 12.5 days p.c. on, without appearing to be developmentally retarded. This reduction in overall size was not as considerable as the specific reduction observed in the sizes of the lungs and spleen. Surprisingly, we did not observe any obvious defect in the morphology or cytodifferentiation of any other organs in N-myc<sup>9a/9a</sup> embryos as determined by examination of H&E-stained, sectioned embryos and dissected tissues. Unaffected tissues include the brain and kidney (not shown), which normally express N-myc at high levels during their development, but we cannot preclude the possibility that subtle defects exist in these and other tissues that would be manifested postnatally were the lung defect not lethal.

Expression of the normal N-myc transcript in N-myc<sup>9a/9a</sup> embryos

Because many of the tissues that express N-myc were not affected by the N-myc<sup>9a</sup> mutation, it seemed likely that the phenotype observed in N-myc<sup>9a/9a</sup> embryos was the result of a leaky mutation generating some normal N-myc transcripts because of alternative splicing around the neo-containing exon in the targeted locus. Northern blot analysis of RNA isolated from 18.5-day embryonic brain and kidney using an N-myc exon 1 probe showed that normal-length N-myc transcript was indeed present in N-myc<sup>9a/9a</sup> embryos, but at considerably reduced levels relative to those observed in N-myc<sup>+/+</sup> or N-myc<sup>9a/+</sup> embryos [Fig. 4]. In N-myc<sup>9a/</sup> and N-myc<sup>9a/9a</sup> embryos, an additional truncated transcript of
the size expected for an exon 1–neo fusion transcript was also detected with an exon 1 probe. These results indicate that the primary transcript encoded by the N-myc<sup>neo</sup> gene could produce two alternatively spliced secondary transcripts, joining exon 1 either with neo or with the intact exon 2, which is 3' of neo (see Fig. 1e). Thus, the N-myc<sup>neo</sup> mutation is leaky, and the phenotype observed in N-myc<sup>neo</sup> embryos is unlikely to represent the null phenotype.

The exon 1–neo fusion transcript predominated over the normal transcript in N-myc<sup>neo/neo</sup> embryos (Fig. 4), but this does not indicate the extent to which splicing to the neo-containing exon was favored over splicing to the downstream exon, because the mutant transcript also predominated over the normal N-myc message in N-myc<sup>neo</sup> embryos (Fig. 4). Rather, it suggests that the neo-containing transcript was more stable than the normal N-myc transcript.

We wished to determine whether different tissues of N-myc<sup>neo/neo</sup> embryos expressed different relative levels of the normal N-myc transcript compared with levels observed in those tissues in wild-type embryos, and whether there was any correlation between lower relative levels of the normal transcript and mutant phenotype. To this end, RNase protections were performed using an N-myc exon 2 probe that detects the normal N-myc transcript but not the N-myc-neo fusion transcript. Levels of N-myc message in N-myc<sup>neo/neo</sup>, N-myc<sup>neo</sup>, and wild-type kidney, brain, and lung were quantitated by scanning densitometry, and the ratio of transcript levels within each tissue in N-myc<sup>neo/neo</sup> compared with wild type were determined. We observed that N-myc<sup>neo</sup> 18.5-day kidney expressed at 35% of normal levels, 18.5-day brain expressed at 50% of normal levels, and 18.5-day lung expressed at 25% of normal levels [Fig. 5]. Thus, this analysis, which was repeated three times, showed that splicing around the neo-containing exon occurred in all tissue types examined, and that the efficiency with which this splice was made varied from tissue to tissue. We note that splicing around the neo-containing exon was consistently least efficient in the lungs, which, of the tissues examined, were the most severely affected in N-myc<sup>neo/neo</sup> mice.

**Analysis of N-myc expression in normal and N-myc<sup>neo/neo</sup> lungs by in situ hybridization**

To examine the normal N-myc expression pattern and the effect of the N-myc<sup>neo</sup> mutation on the expression of N-myc in earlier embryos, whole-mount RNA in situ hybridization was performed on 12.5-day p.c. lungs dissected from wild-type and N-myc<sup>neo/neo</sup> littermates using an N-myc exon 2 probe (Fig. 6). In situ analysis of wild-type lungs showed that N-myc is normally expressed exclusively in the lung epithelium, with particularly high levels in the distal tips of the developing airways. The primary and secondary bronchi express N-myc at lower levels (Fig. 6a). This was also observed in whole-mount RNA in situ hybridizations of 13.5-day lungs (not shown) and is consistent with the N-myc expression pattern observed in the lung epithelium by RNA in situ hybridization to sectioned 14.5-day lungs [Hirning et al. 1991]. In contrast to the expression pattern observed in 12.5-day wild-type lungs, the normal N-myc transcript was barely detected in the developing epithelium of 12.5-day N-myc<sup>neo/neo</sup> lungs (Fig. 6b). The normal N-myc transcript appears to be present at even lower levels relative to the wild-type levels.

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### Table 3. Quantitation of the lung defect in N-myc<sup>neo/neo</sup> 16.5-day embryos

| Litter | Section | developing airways (no.) | N-myc<sup>+/+</sup> or N-myc<sup>neo/neo</sup> | mean | developing airways (no.) | Chi-square | Degree of freedom |
|--------|---------|--------------------------|-----------------------------------------------|------|--------------------------|------------|------------------|
| 38     | 1       | 68                       | 55                                             | 25   | 24                       | 12.2       | 1                |
|        | 2       | 62                       |                                                | 23   |                          |            |                  |
|        | 3       | 40                       |                                                | 25   |                          |            |                  |
| 57     | 1       | 46                       | 44                                             | 15   | 27                       | 4.1        | 1                |
|        | 2       | 48                       |                                                | 32   |                          |            |                  |
|        | 3       | 39                       |                                                | 35   |                          |            |                  |
| 58     | 1       | 37                       | 38.5                                           | 23   | 25                       | 2.9        | 1                |
|        | 2       | 40                       |                                                | 29   |                          |            |                  |
| 57, 59 | 1       | 40                       | 45                                             | 31   | 34                       | 1.5        | 1                |
|        | 2       | 47                       |                                                | 40   |                          |            |                  |
|        | 3       | 47                       |                                                | 31   |                          |            |                  |

Total of chi-squares: 20.7
Chi-square of totals: 182.5
Heterogeneity chi-square: 110

Population is homogeneous: 0.25 < P < 0.5, therefore, data may be pooled.

Chi-square analysis was performed on these data to determine whether the difference in the number of developing airways in the lungs of N-myc<sup>+/+</sup> and N-myc<sup>neo/neo</sup> embryos was significant or whether it could be due to chance alone.
to wild type than was determined by RNase protection analysis of 15.5-day lungs (Fig. 5), suggesting that the lung phenotype is the result of the particularly low levels of normal N-myc transcripts present during its morphogenesis.

Discussion

We have generated mice carrying a leaky mutation in the N-myc proto-oncogene by gene targeting in ES cells. The phenotype of mice homozygous for this N-myc allele, N-myc<sup>9a</sup>, has indicated an important role for N-myc during organogenesis in the lung. This does not imply that N-myc does not function in controlling the proliferation and/or the differentiation of other lineages in which it is expressed. The nature of the N-myc<sup>9a</sup> mutation is such that the lack of any apparent phenotypic defects in other lineages in N-myc<sup>9a/9a</sup> embryos probably results from expression of sufficient levels of the normal gene product in these lineages.

The N-myc<sup>9a</sup> allele reveals a function in lung morphogenesis

Mice homozygous for the N-myc<sup>9a</sup> mutation died at birth owing to a defect in lung morphogenesis. Specifically, we observed that the pulmonary epithelium, which lines the airways and air spaces of the developing lung and which normally expresses N-myc, failed to proliferate and branch normally in N-myc<sup>9a/9a</sup> embryos. This phenotype was evident as early as 12.5 days of embryogenesis, when N-myc expression in normal lungs is at its highest level as determined by Northern analysis (Mugrauer et al. 1988), and appeared ultimately to result in small lungs with an insufficient surface area to oxygenate the blood of the newborn mouse. Morphologically, the lung phenotype was restricted to the developing airways while the number and distribution of the more proximal bronchi appeared to be normal. This is consistent with the normal expression pattern of N-myc observed in the lung, where there were very low levels of transcripts in the trachea and primary and secondary bronchi but higher levels in the tips of the developing airways.

Many studies have addressed the mechanism of morphogenesis in epitheliomesenchymal organs in general and in the lung in particular. In the lungs there is a mesodermally derived mesenchymal component and an epithelial component that is endodermally derived. The murine lung begins to form at the 25-27 somite stage as two endodermal evaginations from the pharyngeal region of the gut that extend into mesenchyme derived from splanchnic mesoderm (Weston 1984). These endodermally derived buds develop into the bronchial tree and the respiratory surface of the lung while the mesenchyme eventually forms the connective tissue, pulmo-
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Figure 6. Whole-mount RNA in situ analysis of N-myc expression in N-myc$^{+/+}$ and N-myc$^{+/lo}$ 12.5-day p.c. embryonic lungs showing expression (black staining) in the wild-type epithelium, but not in the mesenchyme, and very little expression in the N-myc$^{+/lo}$ epithelium. (a) N-myc$^{+/+}$, (b) N-myc$^{+/lo}$. The probe used was the antisense transcript of an N-myc PvuII-Asall exon 2 fragment. [br] Primary bronchus; [mes] mesenchyme; [ep] epithelium. Bar, 200 μm.

N-myc in branching morphogenesis may help in uncovering other genes that function upstream of N-myc in this epithelial–mesenchymal interaction.

N-myc has been shown to be amplified or otherwise deregulated in small-cell lung cancer (SCLC; Nau et al. 1986; Wong et al. 1986). The role for N-myc in lung morphogenesis revealed by the N-myc$^{+/lo}$ mutation is consistent with N-myc deregulation contributing to the transformation of adult lung cells and to the progression of lung cancer, particularly because SCLC has its origin in the lung epithelium [Andrew et al. 1982, and references therein]. Specifically, SCLC is derived from bronchiolar epithelial stem cells that are committed to differentiate toward neuroendocrine cells of the lung (Gazdar et al. 1981; Gazdar 1984). N-myc amplification is not a first step in the progression of SCLC (Bergh 1990; Noguchi et al. 1990), and the inactivation of tumor suppressor genes, such as p53 or RB—events that are common in lung cancer (Bergh 1990; Kaye et al. 1990; Takahashi et al. 1991)—may initiate the process of tumorigenesis in bronchial epithelial stem cells. Amplification of N-myc may then be one factor that provides a selective growth advantage to transformed cells and that promotes tumor progression.
Phenotypic effects of the N-myc\textsuperscript{9a} mutation on other lineages

A number of tissues other than the lung express N-myc at particular stages during their development. RNA in situ hybridization analysis has shown that subsets of cells within the developing kidney, lung, brain, and intestine express N-myc (Mugrauer et al. 1988; Hirning et al. 1991), and Northern blot analysis has shown that low levels of N-myc transcripts are also detectable in the embryonic spleen and heart (Zimmerman et al. 1986; Semsei et al. 1989). Despite expression at these sites, the only tissue other than the lung that showed a clear defect in N-myc\textsuperscript{9a} embryos was the spleen. The effect of the N-myc\textsuperscript{9a} mutation on the spleen was a reduction in the size of the spleen without an alteration of its morphology. The exact nature of this defect is not yet clear, as little is known about the mechanism of spleen morphogenesis or about the expression pattern of N-myc within the developing spleen. The primary spleen phenotype could not have been the result of an absence of hematopoietic cells, because the phenotype could be observed before hematopoiesis occurs in the spleen. Furthermore, lethally irradiated mice reconstituted with fetal liver cells from N-myc\textsuperscript{9a}\textsuperscript{9a} embryos were healthy and expressed terminally differentiated B-cell antigens, suggesting that later stages of hematopoiesis and lymphopoiesis were not affected by the N-myc\textsuperscript{9a} mutation (C.B. Moens et al., unpubl.).

The N-myc\textsuperscript{9a}\textsuperscript{9a} phenotype provides no information about the function of N-myc in epithelial-mesenchymal interactions other than in the lung. However, it is interesting to note that within the developing kidney and intestine, N-myc is expressed exclusively in epithelial cells (Mugrauer et al. 1988; Hirning et al. 1991). Other N-myc alleles or combinations of alleles may make it possible to study whether N-myc functions more generally in epithelial-mesenchymal interactions during development.

Because N-myc\textsuperscript{9a}\textsuperscript{9a} mice die at birth, we could not determine whether other tissues suffered subtle defects that would manifest themselves postnatally. We note, however, that N-myc\textsuperscript{9a}\textsuperscript{9a} embryos were slightly smaller than their wild-type or N-myc\textsuperscript{9a}\textsuperscript{9a} littermates, suggesting that there were subtle effects in some lineages other than the lungs and the spleen. This observation is unlikely to indicate a role for N-myc in general cell proliferation, because N-myc is expressed in a restricted manner during development.

Molecular basis of the N-myc\textsuperscript{9a}\textsuperscript{9a} phenotype

The phenotypic effects of the N-myc\textsuperscript{9a} mutation are very specific to the lungs and the spleen. Recent results from studies of mice bearing a null mutation at the N-myc locus indicate that N-myc must play a vital role earlier in development, as homozygous embryos die before birth (Sawai et al. 1991). Embryos homozygous for a N-myc null allele generated elsewhere have been found to die prior to organogenesis when neither the lungs nor the spleen are required to function (B. Stanton and L. Parada, pers. comm.). Presumably the difference in phenotypes between homozygotes of N-myc\textsuperscript{9a} and of the null mutation is the result of the leaky nature of the N-myc\textsuperscript{9a} mutation.

The N-myc\textsuperscript{9a} allele contains a splice donor in the endogenous first exon, followed by two identical exon 2 splice acceptors, the first preceding neo and the second in the normal exon 2, which is downstream of the inserted targeting vector (Fig. 1). The neo-containing exon has no splice donor but does contain an SV40 polyadenylation signal. We have observed that both splice acceptors are used, generating two alternate transcripts: a mutant transcript that terminates after neo, and a normal N-myc transcript. It has been shown that the presence of a splice donor is required for exon definition and efficient splicing in vivo, whereas a mutation of the splice donor of an internal exon often results in the skipping of that exon in vitro and in vivo (Robberson et al. 1990; Talerico and Berget 1990). However, the polyadenylation machinery likely functions to define an exon that lacks a splice donor but includes a polyadenylation site (Robberson et al. 1990). Thus, in cells containing the N-myc\textsuperscript{9a} allele, there may be a competition between the RNA-processing machinery: if neo is polyadenylated first, a mutant transcript is generated, and if the flanking exons are defined prior to neo polyadenylation, the neo-containing exon is skipped and a wild-type N-myc transcript is generated.

All tissues tested were able to splice around the neo-containing exon to make a normal N-myc transcript, but with different efficiencies. The lungs, which were severely affected in N-myc\textsuperscript{9a}\textsuperscript{9a} embryos, appeared to splice around neo with the lowest efficiency as judged by RNase protection analysis. Furthermore, RNA in situ hybridizations showed the levels of normal N-myc transcripts in very early N-myc\textsuperscript{9a}\textsuperscript{9a} lungs to be extremely low compared with wild-type lungs. This suggests that the lung phenotype was caused by these low levels of normal transcript, and the absence of a phenotype in other tissues that normally express N-myc was attributable to sufficient levels of the normal transcript. However, the differences in levels of the normal transcript between different tissues in N-myc\textsuperscript{9a}\textsuperscript{9a} embryos was not so significant as to preclude the possibility that the lungs are particularly sensitive to reduced levels of the N-myc transcript. Unfortunately, the small size of the N-myc\textsuperscript{9a}\textsuperscript{9a} spleen and the low levels of N-myc expression in the spleen prevented a similar analysis of N-myc expression in this tissue.
targeting vector. This type of insertion event would only be detected as a homologous recombinant by our PCR screening approach if the short arm of homology were involved in the crossover, as was the case in N-myc\(^{\Delta}\) To N-myc\(^{\Delta}\). Another possible mechanism by which this type of insertion could occur is that the targeting vector concaten- merized prior to integration and then underwent a double crossover or gene conversion event involving the two S' regions of homology. Regardless of the mechanism, the insertion of replacement-type vectors resulting in this type of targeted locus is not uncommon [Hasty et al. 1991].

Given that the integration of the targeting vector results in a duplication of N-myc sequences, the N-myc\(^{\Delta}\) mutation might be expected to be somewhat unstable, with intrachromosomal homologous recombination resulting in either the regeneration of the wild-type N-myc allele or in the generation of an N-myc\(^{\Delta}\)null allele. If this occurred regularly during the development of N-myc\(^{\Delta}\) \(\Delta\) embryos, the ratio of N-myc\(^{\Delta}\)\(\Delta\) to N-myc\(^{\Delta}\)\(\Delta\) embryos detected by PCR in litters from heterozygote inter crosses would be expected to exceed 2:1, because N-myc\(^{\Delta}\)\(\Delta\) embryos in which this had occurred would be scored as N-myc\(^{\Delta}\)\(\Delta\). This was not observed: among 372 embryos dissected during gestation, there were 93 N-myc\(^{\Delta}\)\(\Delta\), 179 N-myc\(^{\Delta}\)\(\Delta\), and 100 N-myc\(^{\Delta}\)\(\Delta\) embryos. Thus, within the limits of detection, the insertion mutation appears to be stable, and mosaicism need not be a concern in interpreting the phenotype.

Among the embryos that were dissected during gestation, the gross phenotypes of very few were inconsistent with their genotype. Three embryos appeared to have a mutant phenotype (small lungs and spleen) upon dissec tion but were N-myc\(^{\Delta}\)\(\Delta\), and one embryo that did not have a mutant phenotype was N-myc\(^{\Delta}\)\(\Delta\). These anom alous embryos may have been mosaics, or more likely are indicative of variable penetrance of the N-myc\(^{\Delta}\) mutation, whereby in some embryos the N-myc\(^{\Delta}\) allele makes slightly more or less of the normal N-myc transcript than in others. Variable penetrance would not be entirely unexpected because the mutation is on an outbred genetic background. An alternative possibility is that the phenotype we have associated with the N-myc\(^{\Delta}\) mutation is a result of an independent, closely linked mutation, which, in anomalous embryos, has segregated from the N-myc\(^{\Delta}\) locus. Because the N-myc\(^{\Delta}\) mutation is unique to the cell line we generated, and it would be very difficult to generate precisely the same mutation in the N-myc proto-onogene. This phenotype has allowed us to propose a function for N-myc in the epithelial–mesenchymal interaction that is the basis of lung morphogenesis. Such a function is consistent with the known expression pattern of N-myc in the lung and with the involvement of N-myc in the progression of lung cancer.

If patterns of expression are a guide, many developmentally important genes play multiple roles at different stages and in a number of different lineages during embryogenesis. A phenotypic series generated by different mutant alleles or combinations of alleles is therefore useful in determining the functions of a gene during development. These alleles would include null mutations, leaky mutations, and more subtle site-directed mutations either in tissue- and stage-specific cis-acting regulatory elements or in the gene itself, which would lead to tissue- or stage-specific defects.

N-myc is one such gene, whose wide expression pattern during embryogenesis indicates a multiplicity of roles. Embryos homozygous for a null mutation in N-myc die at mid-gestation [B. Stanton and L. Parada, pers. comm.], and clearly this mutation is central to defining the earliest essential role for the gene. Two later roles in lung and spleen morphogenesis are revealed by the N-myc\(^{\Delta}\)\(\Delta\) mice. By crossing N-myc\(^{\Delta}\)\(\Delta\) mice with N-myc\(^{\Delta}\)\(\Delta\), we can generate N-myc\(^{\Delta}\)\(\Delta\)\(\Delta\) embryos that should have further reduced levels of normal N-myc transcript and may reveal other sensitive developmental stages. Thus, this kind of leaky mutation can help to achieve the goal of producing an allelic series of mutations for a developmentally interesting gene while strategies for directing mutations to particular stages of embryogenesis are being developed.

Materials and methods

Targeting vector construction

The N-myc genomic clone [DePinho et al. 1986] was the gift of R. DePinho and F. Alt (Columbia University, NY). The targeting vector extends from an SstI site in intron 1 to an EcoRI site downstream of exon 3 but is deleted for a Pvull-Smal fragment, which includes the coding region of exon 2 and 70 bp of intron 3'. Downstream of exon 3 but is deleted for a PvuII-Smal fragment, which includes the coding region of exon 2 and 70 bp of intron 3 and is replaced with a 1-kb promoterless neo [RhlII-Smal] with a 250-bp SV40 polyadenylation signal. There are two in-frame stop codons between the N-myc ATG and the neo ATG, the 5' most being 15 codons upstream of the neo ATG.

Electroporation and selection of ES cells

D3 ES cells (5 \(\times\) 10⁶) [Joyner et al. 1989] per 0.8 ml of PBS were electroporated with 40 \(\mu\)g of EcoRI-digested targeting vector DNA using a Bio-Rad Gene Pulser [500 \(\mu\)F, 250 V]. Treated cells were allowed to recover for 20 min at room temperature before being seeded onto gelatinized 90-mm tissue culture dishes (Nunculon) at a density of 5 \(\times\) 10⁶ cells per plate in Buffalo rat liver cell (BRL)-conditioned medium [Smith and Hooper 1987]. BRL-conditioned medium was replaced 48 hr after electroporation with BRL-conditioned medium containing 100 \(\mu\)g of active G418 [GIBCO]. Cells were maintained under selection for \(\sim\)10 days, with selection medium being changed every other day.

Conclusions

We have described the phenotype of mice that are ho mozygous for a leaky mutation in the N-myc proto-on-
Screening of G418-resistant colonies

After 10 days of selection, one-half of each resistant colony was picked and analyzed by PCR as described (Joyner et al. 1989) to distinguish targeted from random integrations. The remaining half-colony was left under selection until the PCR result was obtained. The 3' and 5' oligonucleotide primers for PCR anneal in the 5' end of the neo gene and in the first N-myc intron outside of the sequences included in the targeting vector (primers a and b, Fig. 1c). PCR was run for 40 cycles of 2 min denaturation (94°C), 2 min annealing (55°C), and 10 min extension (70°C) in a Perkin-Elmer thermal cycler. The remaining halves of those colonies that gave positive signals by PCR were subsequently pooled onto dishes containing a primary embryonic fibroblast layer and were expanded in 15% FCS in the absence of G418 and later used for Southern analysis and blastocyst injection.

Southern blot analysis of ES cell lines and mice

Ten micrograms of DNA purified from ES cells or tails of mutant mice was digested, either with BamHI or EcoRI plus SalI. Digested DNA was transferred onto GeneScreen nylon membrane and was probed either with probe 1 [Fig. 1a (for BamHI digests)] or with probe 2 [Fig. 1a (for EcoRI plus SalI digests)]. This latter probe is deleted in a predicted homologous recombination but is not deleted in N-mycneo. Blots were hybridized in 50% sodium phosphate, 15% formamide, 7% SDS, 100 mg/ml of fatty acid-free BSA, and 1 mM EDTA at 63°C. Blots were washed in 30 mM sodium phosphate at 60°C.

Blastocyst injection

Groups of 10–20 cells from the three mutant cell lines indicated were injected in separate experiments into blastocysts flushed from the uteruses and oviducts of 3.5-day p.c. C57BL/6J (Jackson Lab) mice. Injected blastocysts were transferred into the uterus of 2.5-day p.c. pseudopregnant CD1 (Charles River) recipients. Breeding of chimeric animals

Because the parental D3 ES cell line is derived from 129/1 agouti mice, its descendants in the skin of the chimer will produce agouti hair on an otherwise black background. Male chimeras with extensive ES contribution in the coat were bred with C57BL/6J females to test for germ-line transmission of the dominant Agouti coat color marker. Agouti offspring of chimeric males were then tested for the presence of the N-mycneo mutation by Southern blot analysis of BamHI-digested tail DNA using probe 1 (Fig. 1a).

Typing and fixation of embryos for histology

Dissected embryos were typed by Southern blot analysis or by PCR. For PCR, three 21-mer primers were used to distinguish between N-mycneo, N-mycneo, and N-mycneo embryos. Primer 1 annealed to the antisense strand of N-myc intron 1, upstream of the site of integration of the targeting vector; primer 2, which is the same as primer b in Figure 1, annealed to the sense strand in the 5' end of neo; and primer 3 annealed to the sense strand in the 5' end of N-myc exon 2. Primers 1 and 2 amplified a 475-nucleotide fragment from the N-mycneo allele, and primers 1 and 3 amplified a 583-nucleotide fragment from the wild-type allele. Tissues from N-mycneo, N-mycneo, and N-mycneo embryos were either fixed for histology or pooled according to genotype for extraction of total RNA. Dissected embryos and organs were fixed overnight in 30% ethanol, 10% acetic acid, and 10% formaldehyde and were cleared and embedded in paraffin wax (Tissue Prep). Blocks were sectioned at 5–7 μm and stained with H & E.

RNA preparation, Northern and RNase protection analysis

Fetal brain, kidney, lung, and liver were dissected from embryos at the stages stated in the text. Tissues were frozen immediately in liquid nitrogen until embryos were genotyped. RNA from genotyped embryos was prepared using an acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 1987). For Northern analysis, 10 μg of total RNA was run on a denaturing formaldehyde gel and transferred onto a GeneScreen nylon membrane. Blots were probed under the conditions described for Southern with an exon 1 probe (0.3-kb Smal fragment of the N-myc genomic clone) and were washed in 30 mM sodium phosphate at 62°C. For RNase protection analysis, probes were transcribed in vitro using Promega Riboprobe System II. For N-myc probes, a 215-nucleotide PvuII–AsuII fragment from exon 2 was cloned into pGEM7, and sense and antisense probes of 290 nucleotides were transcribed with T7 and SP6 polymerases, respectively. For β-actin probes, a 425-nucleotide KpnI–Xbal fragment of the mouse β-actin cDNA was cloned into pGem3Z, which was cut with Smal and PvuII with S1 and SP6 polymerase. RNase protections were performed as described (Sambrook et al. 1989). A phosphor screen was exposed to the dried gel and scanned using the Molecular Dynamics PhosphorImager. Band intensities were quantitated using the ImageQuant software (Molecular Dynamics). Levels of N-myc transcripts were corrected for uneven loading by the use of a β-actin control.

Sequence analysis of N-mycneo mutation

Southern analysis of DNA from cell line 9a and from embryos homozygous for N-mycneo localized the point at which targeting vector sequences rejoin endogenous N-myc sequences to be within 200 bp, 5' of the XhoI site in intron 2 of the targeting vector, and within 200 bp, 3' of the BgIII site in intron 1 of the endogenous gene. Primers capable of amplifying a junction fragment in this region were designed (primers x and y in Fig. 1d) and were used to amplify a 170-nucleotide fragment. This fragment was blunt ended and cloned into Smal-digested pGem7. Sequencing was carried out using the universal primer and Sequenase Sequencing Kit (U.S. Biochemical).

Whole-mount in situ hybridization

The in situ hybridization procedure was adapted from Tautz and Pfeife (1989) and from Hemmati-Brivanlou et al. (1990) as described (R. Conlon and J. Rossant, in prep.). The probe used was transcribed from a 215-bp PvuII–AsuII fragment of exon 2.

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References

Alescio, T. and A. Cassini. 1962. Induction in vitro of tracheal buds by pulmonary mesenchyme grafted on tracheal epithelium. J. Exp. Zool. 150: 83–94.

Alescio, T. and T. Colombo Pignoro. 1967. A quantitative assessment of mesenchymal contribution to epithelial growth rate in mouse embryonic lung developing in vitro. J. Embryol. Exp. Morphol. 17: 213–227.

Andrew, A., B. Kramer, and B.B. Rawdon. 1982. The embryonic origin of endocrine cells of the gastrointestinal tract. Gen. Comp. Endocrinol. 47: 249–265.

Beckmann, H., L.-K. Su, and T. Kadesch. 1990. TFE3: A helix-loop-helix protein that activates transcription through the immunoglobulin enhancer MuE3 motif. Genes & Dev. 4: 167–179.

Bergh, J. 1990. Gene amplification in human lung cancer. Am. Rev. Respir. Dis. [Suppl.] 142: 20–26.

Blackwell, T.K., L. Kretzner, E.M. Blackwood, R.N. Eisenman, and H. Weintraub. 1990. Sequence-specific DNA binding by the c-Myc protein. Science 250: 1149–1151.

Blackwood, E.M. and R.N. Eisenman. 1991. Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science 251: 1211–1217.

Capecchi, M.R. 1989. Altering the genome by homologous recombination. Science 244: 1288–1292.

Caudy, M., H. Vassim, M. Brand, R. Tuma, L.Y. Jan, and Y.N. Jan. 1989. Daughterless, a drosophila gene essential for both neurogenesis and sex determination, has sequence similarities to myc and the achaete-scute complex. Cell 55: 1061–1067.

Charron, J., B.A. Malynn, E.J. Robertson, S.P. Goff, and F.W. Alt. 1990. High-frequency disruption of the N-myc gene in embryonic stem and pre-B cell lines by homologous recombination. Mol. Cell Biol. 10: 1799–1804.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156–159.

DePinho, R.A., E. Legouy, L.B. Feldman, N.E. Kohl, G.D. Yancopolous, and F.W. Alt. 1986. Structure and expression of the murine N-myc gene. Proc. Natl. Acad. Sci. 83: 1827–1831.

Downs, K.M., G.R. Martin, and J.M. Bishop. 1989. Contrasting patterns of myc and N-myc expression during gastrulation of the mouse embryo. Genes & Dev. 3: 860–869.

Gazdar, A.F., D.N. Carney, J.G. Guccion, and S.B. Baylin. 1981. Small cell carcinoma of the lung: Cellular origin and relationship to other pulmonary tumors. In Small cell lung cancer (ed. F.A. Greco, R.K. Oldham, and P.A. Bunn), pp. 145–175. Grune & Stratton, New York.

Gazdar, A.F. 1984. The pathology of endocrine tumors of the lung. In The endocrine lung in health and disease (ed. A.F. Gazdar and K.L. Becker), pp. 364–372. Saunders, Philadelphia.

Goldin, G.V. and L.A. Opperman. 1980. Induction of supernumerary tracheal buds and the stimulation of DNA synthesis in the embryonic chick lung and trachea by epidermal growth factor. J. Embryol. Exp. Morphol. 60: 235–243.

Goldin, G.V. and N.K. Wessells. 1979. Mammalian lung development: The possible role of cell proliferation in the formation of supernumerary tracheal buds and in branching morphogenesis. J. Exp. Zool. 208: 337–346.

Goldin, G.V., H.M. Hindman, and N.K. Wessells. 1984. The role of cell proliferation and cellular shape change in branching morphogenesis of embryonic mouse lung: Analysis using aphidicolin and cytochalasins. J. Exp. Zool. 232: 287–296.

Gregor, P.D., M. Sawadogo, and R.G. Roeder. 1990. The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes & Dev. 4: 1730–1740.

Hastry, P., J. Rivera-Perez, C. Chang, and A. Bradley. 1991. Target frequency and integration pattern for insertion and replacement vectors in embryonic stem cells. Mol. Cell Biol 11: 4590–4517.

Hemmatti-Brivanlou, A., D. Frank, M.E. Bolce, B.D. Brown, H.L. Sive, and R.M. Harland. 1990. Localization of specific mRNAs in Xenopus embryos by whole-mount in situ hybridization. Development 110: 325–330.

Hirning, U., P. Schmid, W.A. Schulz, G. Rettenberger, and H. Hameister. 1991. A comparative analysis of N-myc and c-myc expression and cellular proliferation in mouse organogenesis. Mech. Dev. 33: 119–126.

Hu, Y.-F., B. Luescher, A. Admon, N. Mermod, and R. Tjian. 1990. Transcription factor AF-4 contains multiple dimerization domains that regulate dimer specificity. Genes & Dev. 4: 1741–1752.

Jakobovits, A., M. Schwab, J.M. Bishop, and G.R. Martin. 1985. Expression of N-myc in teratocarcinoma stem cells and mouse embryos. Nature 318: 188–191.

Joyner, A.L., W.C. Skarnes, and J. Rossant. 1989. Production of a mutation in mouse En-2 gene by homologous recombination in embryonic stem cells. Nature 338: 153–156.

Kaye, F.J., R.A. Kratzke, J.L. Gerster, and P.S. Lin. 1990. Recesive oncogenes in lung cancer. Am. Rev. Respir. Dis. [Suppl.] 142: 44–47.

Kohl, N.E., N. Kanda, R.R. Schrenck, G. Bruns, S.A. Latt, F. Gilbert, and F.W. Alt. 1983. Transposition and amplification of oncogene-related sequences in human neuroblastomas. Cell 35: 359–367.

Kohl, N.E., C.E. Gee, and F.W. Alt. 1984. Activated expression of the N-myc gene in human neuroblastomas and related tumors. Science 226: 1335–1337.

Kouzarides, T. and E.B. Ziff. 1988. The role of the leucine zipper in the fos-jun interaction. Nature 336: 646–651.

Landschulz, W.H., P.F. Johnson, and S.L. McKnight. 1988. Structure and expression of the murine N-myc gene. Proc. Natl. Acad. Sci. 85: 1827–1831.

Lee, W.H., A.L. Murphree, and W.F. Benedict. 1984. Expression and amplification of the N-myc gene in primary retinoblastoma. Nature 309: 458–460.

Lüschel, B. and R.N. Eisenman. 1990. New light on Myc and the c-myc gene. In Myc (ed. F. Weintraub), pp. 1325–1335. Grune & Stratton, New York.
Nau, M.M., B.J. Brooks, Jr., D.N. Carney, A.F. Gazdar, J.F. Batten, E.A. Sausville, and J.D. Minna. 1986. Human small-cell lung cancers show amplification and expression of the N-myc gene. Proc. Natl. Acad. Sci. 83: 1092–1096.

Nisen, P.D., K. Zimmerman, S.V. Cotter, F. Gilbert, and F.W. Alt. 1986. Enhanced expression of the N-myc gene in Wilms’ tumors. Cancer Res. 46: 6217–6222.

Noguchi, M., S. Hirohashi, F. Hara, A. Kojima, Y. Shimosato, T. Shinkai, and R. Tsuchiya. 1990. Heterogenous amplification of myc family oncogenes in small cell lung carcinoma. Cancer 66: 2053–2058.

Olson, E.N. 1990. MyoD family: A paradigm for development. Genes & Dev. 4: 1454–1461.

Prendergast, G.C., D. Lawe, and E.B. Ziff. 1991. Association of Myn, the murine homolog of Max, with c-Myc stimulates methylation-sensitive DNA binding and Ras cotransformation. Cell 65: 395–407.

Robberson, B.L., G.J. Cote, and S.M. Berget. 1990. Exon definition may facilitate splice site selection in RNAs with multiple exons. Mol. Cell Biol. 10: 84–94.

Rushlow, C.A., A. Hogan, S.M. Pinchin, K.M. Howe, M. Lardelli, and D. Ish-Horowicz. 1989. The drosophila hairy protein acts in both segmentation and bristle patterning and shows homology to N-myc. EMBO J. 8: 3095–3103.

Sambrook, J., E.F. Fritsch and T. Maniatis. Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sawai, S., A. Shimono, K. Hanaoka, and H. Kondoh. 1991. Embryonic lethality resulting from disruption of both N-myc alleles in mouse zygotes. New Biol. 3: 861–869.

Schmid, P., W.A. Schulz, and H. Hameister. 1989. Dynamic expression pattern of the myc protooncogene in midgestation mouse embryos. Science 243: 226–229.

Schwab, M., K. Alitalo, K. Klempnauer, H.E. Varmus, J.M. Bishop, F. Gilbert, G.M. Brodeur, M. Boldstein, and J. Trent. 1983. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305: 245–248.

Semsei, I., S. Ma, and R.G. Cutler. 1989. Tissue and age specific expression of the myc proto-oncogene family throughout the life span of the C57BL/6J mouse strain. Oncogene 4: 465–470.

Smith, A.G. and M.L. Hooper. 1987. Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev. Biol. 121: 1–9.

Stanton, B.R., S.W. Reid, and L.F. Parada. 1990. Germ line transmission of an inactive N-myc allele generated by homologous recombination in mouse embryonic stem cells. Mol. Cell Biol. 10: 6755–6758.

Takahashi, T., H. Suzuki, H. Toyokai, Y. Sekido, Y. Ariyoshi, and R. Ueda. 1991. The p53 gene is very frequently mutated in small-cell lung cancer with a distinct nucleotide substitution pattern. Oncogene 6: 1775–1778.

Talerico, M. and S.M. Berget. 1990. Effect of 5’ splice site mutations on splicing of the preceding intron. Mol. Cell Biol. 10: 6299–6305.

Tautz, D. and C. Pfeifle. 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. Chromosoma 98: 81–85.

Villares, R. and C.V. Cabrera. 1987. The achaete-scute gene complex of D. melanogaster: conserved domains in a subset of genes required for neurogenesis and their homology to myc. Cell 50: 415–424.

Weston, J.A. 1984. The embryonic neural crest: Migration and differentiation and possible contributions to the developing lung. In The endocrine lung in health and disease (ed. A.F. Gazdar and K.L. Becker), pp. 79–97. Saunders, Philadelphia.

Wong, A.L., J.M. Ruppert, J. Eggleston, S.R. Hamilton, S.B. Baylin, and B. Vogelstein. 1986. Gene amplification of c-myc and N-myc in small cell carcinoma of the lung. Science 233: 461–464.

Zimmerman, K., G.D. Yancopoulos, R.G. Collum, R.K. Smith, N.E. Kohl, K.A. Denis, M.M. Nau, O.N. Witte, D. Toran-Allerand, C.E. Gee, J.D. Minna, and F.W. Alt. 1986. Differential expression of myc family genes during murine development. Nature 319: 780–783.
A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung.

C B Moens, A B Auerbach, R A Conlon, et al.

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