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CREATING A MOUSE MODEL OF NEUROFIBROMATOSIS TYPE I: PATHOLOGICAL AND FUNCTIONAL ANALYSIS OF A TUMOR SUPPRESSOR GENE

by

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ABSTRACT

Neurofibromatosis Type I (NF1) is one of the most commonly inherited human disorders, affecting one in 3,500 newborns worldwide. The mode of disease transmission is believed to be autosomal dominant, with 100% penetrance. However, the severity of the disease phenotype varies significantly among NF1 patients. Hallmark lesions of NF1 affect several neural crest-derived tissues, includes cafe-au-lait spots, Lisch nodules and numerous cutaneous and/or plexiform neurofibromas. The gene responsible for NF1 was cloned in 1990, and is believed to function as a tumor suppressor. The protein (neurofibromin) shows close sequence homology to a family of GTPase Activating Proteins (GAP), which are negative regulators of the *ras* proto-oncogene signal transduction pathway.

The focus of this work has been to generate mouse models of NF1 by disrupting the murine *Nf1* gene in embryonic stem (ES) cells. We created a strain of mice with a mutant *Nf1* allele, which has been shown to produce no functional protein. Initial analysis indicate that heterozygotes are predisposed to certain NF1-related malignancies, while mice homozygous for the mutation die around 13.5 days of gestation from cardiac abnormalities. Using the double knockout (DKO) chimera system, we were able to provide evidence showing that simple *Nf1*-deficiency may be sufficient for the induction of neurofibromas. Histological characterization revealed that murine neurofibromas show close pathologic similarities to the human tumors.

Another serious consequence of the NF1 disease is the malignant progression of benign neurofibromas into malignant peripheral nerve sheath tumors (MPNST). A potential explanation for this is that somatic gene mutation must occur in conjunction with *Nf1* mutations to cause tumorigenesis in most tissues. To test this model, *Nf1*-deficient mice were crossed with a *p53* mutant strain. Animals harboring mutations in both genes on the same chromosome are susceptible to malignant sarcomas reminiscent of MPNST. All the tumors have incurred simultaneous LOH for both genes, suggesting that chromosomal loss or large deletions are the primary mechanisms for somatic mutations in mice. This could provide a good mouse tumor model for drugs targeting tumorigenic pathways involving *Nf1*, *p53* or both.

The creation of this *Nf1* mutant mouse model has also provided a number of useful biological reagents for the study of *Nf1* function in various affected tissues, including hematopoietic, chromaffin and fibroblastic cells, as well as the brain and PNS.

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Overview of Neurofibromatosis Type 1

Neurofibromatosis Type 1 (NF1, also known as von Recklinghausen neurofibromatosis) is a common inherited human disorder, affecting one in 3,500 births worldwide (Stumpf et al, 1987). It is estimated that over 50% of the *NF1* mutations are novel mutations (with a mutation rate approximately one in 10,000 gametes per generation), making it one of the most mutable genes involved in familial human disorders (Carey et al., 1986). The hallmark lesions of the disease range from subtle skin pigmentation and eye defects to the development of multiple peripheral nerve growths that are often disfiguring and painful. Some of these benign lesions may undergo malignant transformation to become aggressive sarcomas, resulting in poor prognosis of the patient. The mode of inheritance for NF1 is autosomal dominant, having close to 100% penetrance (Riccardi, 1992). However, the variable expressivity of the clinical symptoms also makes NF1 one of the most interesting diseases from a tumor etiological standpoint. In this document, I intend to provide a detailed overview of NF1, including the clinical phenotypes, molecular genetics, and the creation of animal models to study the fundamental aspects of disease.

The NF1 disease.

The diagnostic criteria for the human NF1 disorder include the appearance of cafe-au-lait spots (CLS, hyperpigmentation of skin melanocytes), Lisch nodules (hamartomas of the iris), neurofibromas (benign lesions arising from the cutaneous or plexiform peripheral nerves), and neurofibrosarcomas (malignant growths typically arising from existing neurofibromas, also known as malignant peripheral nerve sheath tumors, or MPNSTs) (Riccardi, 1992). To a lesser extent, some NF1 patients are also predisposed to developing malignancies such as myeloid hyperplasia, pheochromocytoma (tumors of the adrenal medulla), optic glioma and various sarcomas (Hope & Mulvihill, 1981; Seizinger, 1993). In addition, facial and long bone abnormalities, as well as mild learning difficulties are seen with increased frequencies in NF1 patients (Riccardi, 1992). The current clinical definition for an affected NF1 individual requires meeting two or more of the following criteria:

- six or more CLS over 5 mm in diameter
- two or more neurofibromas of any time or one plexiform neurofibroma
- freckling in the axillary or inguinal regions
- optic glioma
- two or more Lisch nodules
- a distinctive bone lesion such as sphenoid dysplasia or thinning of the long bone
- a first degree relative with NF1 by the above criteria.

Given the disease definition, it is possible for an individual afflicted with NF1 to show as little as a few benign skin lesions to over hundreds of palpable neurofibromas. This variability in the disease spectrum has been an intrinsic feature of NF1: Not only is this evident among unrelated NF1 patients, the variability is apparent even between affected family members that supposedly inherited the same *NF1* gene mutation (Riccardi, 1993). In fact, studies have shown that identical twins tend to exhibit a more closely-related set of NF1 phenotypes than fraternal twins and other more distantly-related individuals (Easton et al., 1993; Huson & Hughes, 1994). These observations suggest that other genetic (additional mutations) and environmental (biological or physical) factors must act in conjunction with specific *NF1* mutations in modulating the severity of the disease.

While it is almost impossible to precisely predict the occurrence of complications, there are certain sets of recognized (i.e. empirical) risks for the onset of the NF1 disease. For example, malignant optic gliomas, brain tumors and myeloid leukemia are found almost exclusively between infant and adolescent (up to 15 years of age) NF1 patients. Congenital neurofibromas are usually of the diffuse plexiform variety. Past that stage, patients are typically at-risk of developing increasing numbers of cutaneous and plexiform neurofibromas. The severity of the disorder (i.e. the risk of progression from these existing neurofibromas into MPNSTs) typically increases with age and the onset of hormonal perturbations such as puberty and pregnancy (Riccardi, 1992).

The NF1 clinical heterogeneity story is further complicated by the frequent association of the disease with two other autosomal dominantly inherited disorders, the Noonan (NS) and Watson

syndromes (WS). NS is characterized by CLS, mental retardation and short stature (Collins and Turner, 1973), while WS patients present these same features, as well as neurofibromas and Lisch nodules (Watson, 1967; Allanson et al, 1991). The only WS phenotype not seen in NF1 patients is the 100% penetrance of pulmonary stenosis (valve restriction of the pulmonary artery). It should be noted that variable expressivity is a trait common to both NS and WS. The extensive phenotypic overlaps among these three disorders led researchers to hypothesize that NS and WS either (1) are allelic to NF1 or (2) involve the mutation of genes closely linked to *NF1*. In fact, Tassabehji and colleagues (1993) found a perfect, 42 bp, in-frame tandem duplication mutation of the *NF1* gene in a family showing features of NF1, NS and WS. Currently, studies have shown that the NS gene seems to be excluded from chromosome 17 (Sharland et al., 1992), where the *NF1* gene resides (described below), and later localized to chromosome 12 (Jamieson et al., 1994). The WS disease region has been mapped to chromosome 17 (Allanson et al., 1991), but the large deletion used for this mapping does not exclude the potential involvement of other genes. Lastly, the inability to pinpoint the exact chromosomal location for these disease genes may speak to the multifactorial nature of the disease.

In summary, the variable expressivity of the NF1 disease poses interesting questions about the genetic and ectopic influences. Given the prevalence and impact of this disorder, my goal is to create a murine model of NF1 in an attempt to elucidate such factors.

NF1 neurocristopathy

Many of the affected tissues are derivatives of a transiently-existing cell type during early vertebrate embryogenesis, the **neural crest** (NC) (summarized in Table 1). This specialized cell type arises along the dorsal apex of the newly-fused neural tube in an anterior-posterior fashion. These cells then quickly detach and begin to migrate through spaces between the neural tube, scleroderm and the ectoderm. It is believed that the pathways of NC migration is determined by specific combinations of extracellular matrix (ECM) and cell surface proteins lining each of these spaces,

and that these proteins cause a progressive restriction of developmental fates for each migrating NC cell (Anderson, 1989). At the appropriate destination, the NC cells cease to migrate (some having traveled great distances) and assume their appropriate differentiative states. Tissues derived from the NC include (Le Douarin, 1982):

- melanocytes of the dermis, epidermis and internal organs
- peripheral nerves and nerve sheaths (e.g. sympathetic ganglia)
- adrenal medulla (chromaffin cells)
- the eye (chromaphores of the iris, corneal endothelium)
- the developing heart and connective tissues of large vessels derived from the aortic arches
- cartilage and bones of the face (including nasal and orbital skeleton and the palate)
- connective tissue of the pituitary, thymus, thyroid, parathyroid and salivary glands

Table 1.1. NF1 clinical symptoms involve several neural crest-derived tissues (NC)

Symptoms	Tissues affected
cafe-au-lait spots	melanocytes (NC)
Lisch nodules	iris (NC)
neurofibroma	neurons, schwann, fibroblast, perineurial (NC)
neurofibrosarcoma/MPNST	neurons, schwann, fibroblast, perineurial (NC)
pheochromocytoma	adrenal medulla (NC)
facial bone dysplasia	bone derived from NC
myeloid hyperplasia	hematopoietic -- myeloid lineage
long bone dysplasia	skeletal
mental retardation	CNS

The patterns of migration and fates of NC cells were identified by years of experimentation involving (1) tagging migratory cells with vital dye, radioactivity, antibodies or other genetic markers and (2) ablation of particular populations of NC cells and assessing their developmental impact. For example, the NC ablation studies in avian embryos revealed the role of certain cranial NC cells in the development of embryonic heart. In fact, animals lacking these NC cells developed a variety of cardiac anomalies, including double outlet right ventricle (DORV), persistent truncus arteriosis (PTA), aortic defects, and other cardiac inflow abnormalities (Kirby and Waldo, 1990). However, clinical evidence does not establish a clear correlation between NF1 and congenital heart defects (Lin and Garver, 1988).

Despite a clear involvement of the NC-derived tissues in various NF1 lesions, explaining why only these cell types are predisposed to proliferative disorders is more difficult. The fact that some non-NC cell types are also involved (e.g. mast and endothelial cells) suggest that the mechanism of disease involves extrinsic agents which acts across cell types and lineages. Do these cells hyperproliferate due to loss of their terminally-differentiated state, or have they become more susceptible to growth signal stimulation? This requires an examination of the signals that affect various NC decisions.

Anderson (1993) compared the mechanism of NC cell differentiation to the process of hematopoiesis, where proliferating progenitors are generated from stem cells in a single location and subsequently migrate to peripheral sites of terminal differentiation. The pluripotency of NC cells are further demonstrated by the identification of environmental and growth signals responsible for controlling their proliferation, migration and differentiation. For example, NC cells plated on different extracellular matrix substrates develop into distinctive cell fates (Sieber-Blum and Cohen, 1980), and that these differentiation signals may be further modified by growth factors such as α-melanocyte stimulating factor (α-MSH) (Satoh and Ide, 1987) and glucocorticoid hormones (Unsicker et al., 1978). The current disease model suggested that the specific genetic mutations responsible for NF1 confer some kind of growth advantage for these NC cells derivatives, be it a reversion from their terminally-differentiated state, or acquiring a lower threshold for growth signal stimulation.

Cloning the *NF1* gene.

The cloning of the gene responsible for NF1 was one of the best examples of the positional cloning technique. The first step is to identify, within multiple affected families, chromosomal regions and/or polymorphic markers that consistently segregate with individuals showing the disease symptoms. For *NF1*, the first linkage was reported by Safarazi et al. (1987), when they localized the gene to either chromosome 5 or 17. Subsequent narrowing down of the gene locus to the long

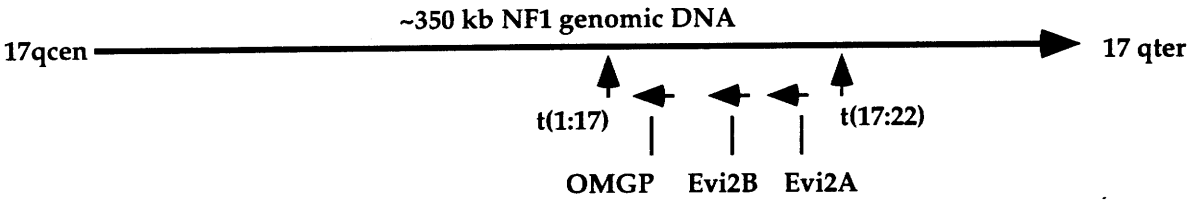
arm of chromosome 17 was provided by the efforts of several groups (Seizinger et al., 1987; Barker et al., 1987; White et al., 1987; Van Tuinen et al., 1987; Skolnick et al., 1987). This multipoint linkage map was summarized by Goldgar et al. in 1989, where the NF1 disease locus was localized to a 3 cM region of 17q11.2.

The detection of two balanced translocation breakpoints from two affected NF1 families was instrumental to the gene cloning. One translocation occurred between chromosome 1p and 17q (Schmidt et al., 1987), and the other between 17q and 22q (Ledbetter et al., 1989). Given the correlation between the physical mapping of these two breakpoints to the linkage analysis to 17q11.2, these balanced translocation regions were believed to reside within -- and therefore disrupted -- the *NF1* gene itself. Using these two guideposts (Figure 1.1), researchers began to analyze the coding sequences from this region. The first genes identified by walking between the breakpoints were: *EVI*2A (Ecotropic viral integration site 2A) (Cawthon et al., 1990), *EVI*2B (Cawthon et al., 1991), *OMGP* (oligodendrocyte-myelin glycoprotein) (Viskochil et al., 1991) and a pseudogene of AK3 (adenylate kinase 3) (Xu et al., 1992). The *EVI*2 genes are human homologs of the mouse *Evi*2 gene, and have been implicated in the retroviral-induction of myeloid disorders (Buchberg et al., 1990a). OMGP encoded a glial cell-specific protein, which was postulated to be a cell adhesion molecule (Mikol et al., 1990). However, these genes were not considered candidates for *NF1*, since none of their coding sequences were interrupted by the two translocation breakpoints, and no *NF1* specific mutations were found in these genes (Cawthon et al., 1990; Cawthon et al., 1991; Viskochil et al., 1991).

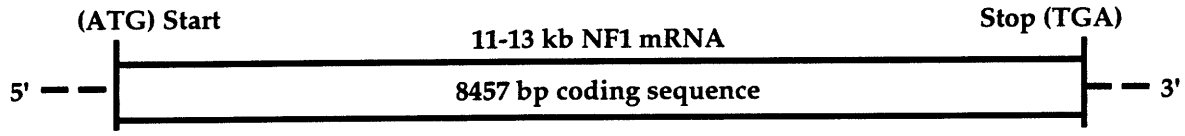
The true *NF1* gene was finally cloned in 1990 by two groups, led by Dr. Francis Collins (Wallace et al., 1990a) and Dr. Ray White (Viskochil et al., 1990). Three probes were used to identify the *NF1* transcript: The first probe was a genomic fragment mapping distal to the t(17;22) breakpoint that was isolated from a cosmid contig that extends through the breakpoint region (Viskochil et al., 1990). A second probe was a fragment from a jump clone that began between the two breakpoints

Figure 1. 1 The NF1 gene structure

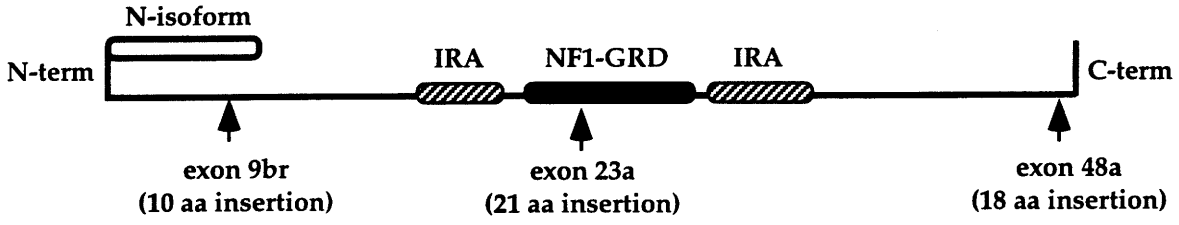
A. **Genomic sequence** (embedded genes, translocation breakpoints)



B. **NF1 mRNA**



C. **Neurofibromin** (isoforms, GRD and IRA-related domains)



Adapted from Shen et al., J Med Genet 33, 1996

and extend distal to the t(17;22) breakpoint (Wallace et al., 1990a). The third probe was an entire yeast artificial chromosome (YAC) encompassing the two breakpoints and extending over 100 kb toward the telomere (Wallace et al., 1990a). Through the screening of several cDNA libraries, mRNAs of 11 kb (Viskochil et al., 1990) and 13 kb (Wallace et al., 1990a) were identified. The coding regions of both these transcripts were found to be disrupted by the t(17;22) translocation breakpoint, as well as several *NF1*-specific mutations (Buchberg et al., 1990b).

The *NF1* gene structure.

NF1 spans over 350 kb of genomic DNA on chromosome region 17q11.2 and encodes an mRNA of 11-13 kb as indicated by its migration on a northern blot, with 59 exons identified to date. The most prevalent allele of the *NF1* transcript (Type I transcript) has an 8454 bp open reading frame, giving rise to a protein of 2818 amino acids, called neurofibromin. The *NF1* protein has an estimated molecular mass of 327 kDa. A large (2.5 to 4 kb) 3' untranslated region (UTR) is also present on the *NF1* transcript (reviewed in Li et al., 1995).

The *NF1* gene sequence predicts a span of 280 amino acid region in the center of the gene that show homology to the catalytic domain of a family of GTPase activating proteins (GAP), including p120 mammalian GAP, budding yeast *IRA*1 and *IRA*2, fission yeast sar1 and *Drosophila* Gap1. This region is known as the *NF1* GAP-related domain (*NF1*-GRD), and spans exons 21 through 27. The importance of this homology will be discussed below. The first alternatively spliced transcript of *NF1* was identified in this region, a 63 bp (or 21 aa) insertion into exon 23 (23a) called the Type II (or GRD II) transcript (Andersen et al., 1993).

Several other alternative splice variants of the *NF1* transcripts have been identified. The summary of the isoforms is provided below:

- Type I, encoding a 2818 aa protein
- Type II, with a 63 bp (21 aa) insertion into exon 23 (Andersen et al., 1993)

- Type III (3'ALT isoform), with an 54 bp insertion into exon 48 (Gutmann et al., 1993)
- Type IV, containing both the Types II and III insertions (Gutmann et al., 1995)
- N-isoform, producing a 2.9 kb truncated transcript (551 aa protein) (Suzuki et al., 1992)
- 5'ALT2, containing a 30 bp insertion between exons 9 and 10a (exon 9br) (Danglot et al., 1995)

The expression patterns and potential function for these various neurofibromin isoforms will be discussed below.

The three genes originally characterized in the search for the true *NF1* gene, *EVI*2A, *EVI*2B and OMGP, were found to be embedded in the 40 kb intron 27 of *NF1* (Figure 1.1). All three genes have the opposite transcriptional orientation relative to the *NF1* gene, and their exact roles in regulating the NF1 disease phenotype are yet unknown. However, some have proposed that the expression of these genes could act to suppress the transcription of *NF1* in the opposite DNA strand by steric hindrance of the RNA polymerase proteins (Wallace et al., 1990b). Along the same line, loss of NF1 express may allow overexpression of these embedded genes, leading to disease development. This would be consistent with the reported role of the *Evi*2 genes in the development of mouse myeloid leukemia (Buchberg et al., 1990a). More recently, Largaespada et al. (1995) reported a finding in which murine myeloid leukemia cell lines showing retroviral integration into *Evi*2 effectively inhibited the production of neurofibromin. This is consistent with human data showing a causal relationship between mutations in the *NF1* gene and the onset of juvenile chronic myelogenous leukemia (Shannon et al., 1994).

In addition, eight pseudogenes showing homology to the *NF1* gene have been detected in the human genome. These pseudogene loci have been mapped to chromosomes 2 (with two separate loci), 20, 21 (Cummings & Marchuk, 1996), 15 (Legius et al., 1992), 14 and 22 (Marchuk et al., 1992). Using fluorescence *in situ* hybridization techniques, Purandare et al. (1995) confirmed these finding and reported an additional *NF1* homologous loci on chromosome 18. The locus on chromosome 12 contains open reading frames homologous to *NF1* in at least two exons and is

expressed in a number of tissues (Gasparini et al., 1993). The evolutionary rationale and history of these pseudogenes are unknown, but many have speculated that they serve as reservoirs of mutations for the *NF1* gene through homologous recombination. However, studies have not conclusively demonstrated this phenomenon.

The *NF1* gene has been found to be evolutionarily conserved (Baizer et al., 1993; Bernards et al., 1993; Schafer et al., 1993; The et al., 1997), with a majority of the studies focusing on the similarities between human and mice. Mouse *NF1* gene shows close homology not only in the protein coding region, but also the 5'- and 3'-UTR regions (Bernards et al., 1993). The neurofibromin proteins for the two species are predicted to have 98.5% sequence identity, with the *NF1*-GRD and the *IRA*-related domains being the most conserved. The 3'-UTR sequences have been suggested to play a role in the regulation of gene expression and mRNA stability. In addition, sequence comparison of the human and murine *NF1* promoter regions showed close sequence homology (over 95% in some regions) as well as the presence of several perfectly conserved transcription factor binding site motifs, including a cAMP response element and serum response elements (Hajra et al., 1994). However, the physical binding of these transcription factors has not been demonstrated *in vitro* or *in vivo*. Furthermore, the different *NF1* transcript isoforms (discussed below) are also conserved across the two species (Danglot et al., 1995). Together, these data suggest not only evolutionary conservation of protein function, but also the control mechanisms for regulation. In addition, given the high degree of sequence homology between the two species, mutations in any of these conserved regions may be potentially significant.

The gene structure for the *Drosphila NF1* homolog has also been determined (The et al., 1997). The gene spans over 13 kb of the fly genome, and produces a transcript of 9750 bp. Further analysis shows that the *Drosophila* gene also has sequence similarities in the *NF1*-GRD and *IRA* domains, while encoding 19 exons, two of which are found to be alternatively spliced. However,

the predicted protein sequence identity is only 60% to human neurofibromin, as opposed to over 90% between human and mouse.

NF1 gene expression.

The *NF1* transcript has been detected in many human tissues using RT-PCR, such as the brain, kidney (Wallace et al., 1990), white blood cell, skin, spleen, lung and muscle (Nishi et al., 1991). In addition, *NF1* expression was found in several normal and tumor cell lines (Wallace et al., 1990; Nishi et al., 1991; Huynh et al., 1992; Nakamura et al., 1994; Takahashi et al., 1994; Tokuyama et al., 1995; Metheny and Skuse, 1996). Early studies showed that in development, the 11-13 kb transcript was is expressed ubiquitously in all tissues. As the embryo progresses through mid-gestation and into adulthood, the expression becomes increasingly localized to several tissues, including the CNS, PNS and adrenal medulla (but not adrenal cortex) (Daston and Ratner, 1992).

The most interesting studies on *NF1* expression in the last few years have focused on the various isoforms (splicing variants) in terms of their tissue specificity, development and induction patterns. As described above, 6 distinct isoforms have been identified in various tissues. Type I transcripts are typically associated with terminally-differentiated CNS tissues, especially in neurons (Suzuki et al., 1991; Gutmann et al., 1995; Tokuyama et al., 1995), in late development (Gutmann et al., 1995), and in tumor cell lines that have been induced to cease proliferating (Mantani et al., 1994; Metheny and Skuse, 1996). Type II transcripts, on the other hand, are expressed at a high level in undifferentiated tumor cell lines (Suzuki et al., 1991; Takahashi et al., 1994; Tokuyama et al., 1995) and early in mammalian development (Huynh et al., 1993; Gutmann et al., 1995). Overall, the available evidence points to the Type II transcript playing a role in promoting cell growth while Type I functions to induce (or maintain) differentiation. However, one study reported a complete set of contradictory data, suggesting that Type II is associated with differentiation and Type I is

found in undifferentiated tumors (Nishi et al., 1991). The discrepancy may have resulted from the different tumor cell lines used in the two experiments.

Type III transcripts are expressed exclusively in muscle tissues as shown by RT-PCR (Gutmann et al., 1993). Type IV, containing both exons 23a and 48a insertions, is expressed in cardiac muscle (Gutmann et al., 1995) as well as other tissues (Mantani et al., 1994). The N-isoform has been found to be equally expressed in normal brain and brain tumor cell lines (Takahashi et al., 1994). The function of this 551 aa protein, which includes the first 547 aa of neurofibromin, is not known. The last, and most recently-identified isoform, 5'ALT2, has been found exclusively in the CNS (Danglot et al., 1995). Since the primary NF1 CNS defects are mental retardation and learning disabilities, this isoform may have a role in the long-term potentiation of signals in the brain.

Mutational spectrum and genetic analysis

As mentioned previously, *NF1* has one of the highest rates of mutation known for any cancer gene, with over 50% of the affected individuals believed to have inherited *de novo* mutation (Carey et al., 1986). Therefore, it is not surprising to find ample medical literature describing these mutations (summarized in Shen et al., 1996; Korf, 1995). Many more have attempted to assign a particular set of NF1 phenotypes to each mutation, with little or no success (Riccardi, 1992; Huson and Hughes, 1994). The most comprehensive correlative studies have documented specific phenotypes associated with large deletions spanning the entire *NF1* gene. These families of patients have consistently shown mild facial dysmorphology, mental retardation, and a large number of cutaneous and plexiform neurofibromas at a young age (Kayes et al., 1994; Cnossen et al., 1997; Wu et al., 1997). Surprisingly, even though these deletions included the three embedded genes in intron 27, none of the patients showed signs of leukemia. As a note, these large deletions (some as large as 700 kb) could potentially involve the loss of other genes adjacent to the *NF1* locus. In contrast, Shen et al. (1993) reported the finding of identical exon 17

mutations in two unrelated NF1 patients that led to different disease features (Shen et al., 1993), suggesting the involvement of other factors.

The over 100 germline mutations in the *NF1* gene covers the spectrum of genetic alterations, including various missense and nonsense point mutations, large and small deletions, insertions, translocations, and tandem duplications (Shen et al., 1996). Some researchers have targeted their mutational search to exons which are believed to be mutational hotspots, including exons in the *NF1*-GRD (Marchuk et al., 1991; Gutmann et al., 1993), *IRA*-related domain (Ballester et al., 1990; Buchberg et al., 1990b) and exons 31 through 34 (Ainsworth et al., 1993; Dublin et al., 1995). Others have used techniques to detect mutations in a more comprehensive fashion, including heteroduplex analysis (HA) and single strand conformation polymorphism analysis (SSCP). Recently, several novel techniques have been used for *NF1* mutation analysis: denaturing gradient gel electrophoresis (DGGE) (Valero et al., 1994), chemical cleavage of mismatch (CCM) (Purandare et al., 1994), and protein truncation test, based on the in vitro transcription-translation of the entire *NF1* coding sequence (Heim et al., 1995). However, NF1's large size (350 kb genomic region), number of exons (59) and non-coding sequence conservation has traditionally prevented a comprehensive mutational screen for the entire gene.

Several lines of evidence provided potential insights into mechanisms responsible for this high mutation rate. First, as previously described, the accumulation of mutations in the various pseudogenes may contribute toward novel *NF1* mutations via homologous recombination (Purandare et al., 1995). Second, CpG dinucleotides show a high mutation rate in the human genome because of their likelihood to deaminate at the 5-methylcytosine (Cooper and Krawczak, 1990). This has led to the identification of nine substitution mutations at the CpG islands in exon 31 (Cawthon et al., 1990; Estiville et al., 1991; Ainsworth et al., 1993; Valero et al. 1994), as well as others in exons 10a (Korf, 1995), 29 (in the *NF1*-GRD; Valero et al., 1994), 42 (Purandare et al., 1994) as well as the 5'- and 3'-UTR (Bernards et al., 1993; Rodenhiser et al, 1993). Third,

Rodenhiser et al. (1997) showed that single nucleotide repeats (homonucleotides), short direct repeats, as well as CpG motifs in *NF1* are common sites of small deletions, insertions and nucleotide substitutions. Lastly, it is possible that the genomic location of the *NF1* locus may be more susceptible to mutations (Krawczak and Cooper, 1991; Wolfe et al., 1989).

The observation that a large number of the germline mutations were inherited from the paternal allele suggests a possible mutation mechanism for *NF1*. Using DNA markers, the original reports suggested that 84%-98% of germline mutations were derived from the paternal chromosome (Jayadel et al., 1990; Stephens et al., 1992). In fact, one report was able to demonstrate that a clinically unaffected father carried *the de novo NF1* mutation in 10% of his sperm (Lazaro et al., 1994). More recently, however, the same group (Lazaro et al., 1996) examined 470 NF1 families in order to refine the paternal allele phenomenon in a way that was specific to the types of mutations. Their report showed that large, intragenic deletions were preferentially detected in the maternal chromosome, while all other types of mutations were more likely to be inherited from the father -- suggesting the existence of different mechanisms for mutations during the production of the egg and the sperm. Rodenhiser et al. (1993) provided evidence showing that specific methylation patterns for the mutable CpG islands on the paternally-derived *NF1* alleles are responsible for the increased mutation rates.

NF1 protein: neurofibromin.

The *NF1* gene product encodes a large (2818 aa) protein with an approximate molecular weight of 220-320 kDa. The protein has been identified both in development -- in a manner consistent with the *NF1* transcript expression pattern (Gutmann et al., 1995 -- and in a variety of human and murine cell lines (DeClue et al., 1991; Gutmann et al., 1991; Daston et al., 1992; Basu et al., 1992; Hattori et al., 1992; The et al., 1993). Traditionally, neurofibromin antibodies have been less than ideal in specifically-recognizing the protein. However, a number of immunoprecipitation and immunohistochemical studies have attempted to determine the cellular and subcellular

localization of neurofibromin. The highest expression of neurofibromin was found in neurons and glial cells such as Schwann cells and oligodendrocytes (Daston et al., 1992), which is consistent with the *NF1* transcript localization patterns in animals. In addition, two reports (DeClue et al., 1991; Hattori et al., 1991) independently demonstrated that neurofibromin co-precipitated with the particulate fraction of cellular proteins in a detergent-extraction/fractionation experiment, while p120 GAP was primarily in the soluble fraction. This is consistent with findings that neurofibromin binds to polymerized cytoplasmic microtubules, and that the critical residues for this interaction is contained within the *NF1*-GRD (Gregory et al., 1993). Other subcellular localization study for neurofibromin found the protein to aggregate upon stimulation of B lymphocytes (Boyer et al., 1994). This aggregation was inhibited by cytoskeletal disrupting agents such as colchicine and cytochalasin D, suggesting that microtubule or microfilament structures may be necessary for the redistribution and normal function of neurofibromin. More recently, Roudebush et al. (1997) showed that neurofibromin co-precipitated with purified mitochondria. The significance of this finding is unclear at present.

NF1 Protein function

Only a few functions have been suggested for neurofibromin based on its homology with known protein motifs. The most obvious similarity is neurofibromin's *NF1*-GRD domain, which shows close sequence homology to a family of GTPase activating proteins (GAPs) (Hall A, 1990). GAP proteins function as negative regulators (and possibly as effectors) for the *RAS* family of small GTPase proteins. GAP physically interacts with its target and stimulates the hydrolysis of the ras-bound guanine nucleotides from GTP to GDP, effectively shutting off the ras signaling pathway (McCormick, 1989).

The ras GTP/GDP cycle

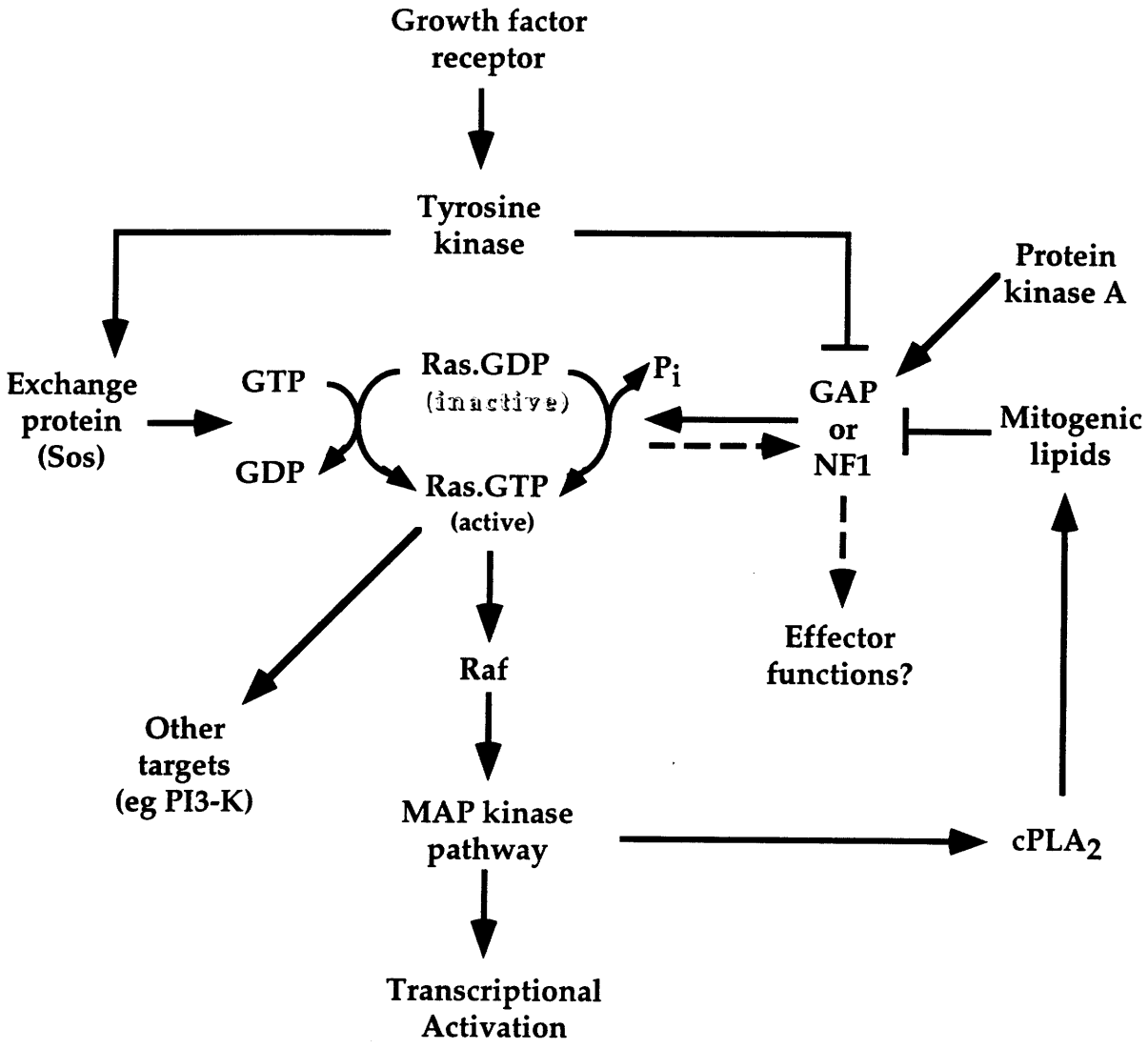
The p21 ras family of proteins function as the master molecular switch in one of the most elegantly-characterized signal transduction pathways in mammalian systems, starting with the

stimulation of cells by extracellular mitogenic molecules, and ending with increased transcriptional activation and synthesis of growth genes in the nucleus (reviewed by Egan and Weinberg, 1993). A variety of extracellular signals induce a rapid and transient increase in the level of active GTP-bound ras proteins, including ones that promote the growth in fibroblasts (e.g. PDGF, EGF, FGF), the cycling and differentiation of hematopoietic cells (e.g. IL-3, GM-CSF), and the differentiation of neuronal cells (e.g. NGF) (Satoh et al., 1992; Khosravi and Der, 1994). All these factors cause dimerization, phosphorylation and subsequent conformational change of receptor tyrosine kinases (e.g. PDGF and FGF receptors) or non-receptor tyrosine kinases (e.g. IL-2 receptor).

This series of events exposes binding sites on the proteins for Grb2, a small adaptor protein whose only function is to link the tyrosine kinases domains to Sos, a guanine nucleotide exchange factor. Sos facilitates the dissociation of the inactive ras from GDP, freeing the protein for binding with a GTP molecule, which causes ras to assume an active conformation (Egan et al., 1993). In effect, Sos is acting as a positive regulator in the ras GTP/GDP cycle (Figure 1.2). The Grb2/Sos complex serves to (1) recruit the ras protein to the plasma membrane and (2) increase the level of active ras-GTP. One of the critical steps to ras signaling is the attachment of the protein to the cell membrane. This is accomplished by modifying the ras protein by the addition of a hydrophobic moiety called the farnesyl group, which serves to anchor ras to the cytoplasmic surface of the cell membrane (Hancock et al., 1991; Kato et al., 1992). The protein which carries out this function is called the farnesyl protein transferase (FPT).

Once localized to the membrane, activated ras causes the co-localization of the serine/threonine kinase raf to the membrane. Raf then continues this rapid burst of phosphorylation of signaling events through a series of proteins called MAP (mitogen-activated protein) kinases, including MEK, ERK1 and ERK2 (Ruderman, 1993; Marshall, 1994), eventually causing the translocation of some of these proteins into the nucleus, where they activate transcription factors such as *c-jun*

Figure 1.2 The ras GTP/GDP cycle



and *c-fos* by phosphorylation (Smeal et al., 1991). This represents the current model explaining the increase in growth-related gene expression upon mitogenic stimulation.

GAP protein functions

The regulation of active ras level in any cell is crucial, because moderate changes of in the ratio of active vs. inactive ras would alter the activity of many critical signal transduction pathways. It is therefore not surprising to find proteins that both positively (e.g. Sos) and negatively (GAP and NF1) regulate ras-GTP levels (Figure 1.2). In fact, this check and balance mechanism is conserved across many species: In *D. melanogaster*, GAP1 regulates *Ras1* activity in eye development (Barbacid, 1987; Gaul et al., 1992); in *S. cerevisiae Ira1* and *Ira2* gene products regulate RAS1 and RAS2 in the production of cAMP as a part of a nutrient-response pathway as well as a mating pathway (Tanaka et al., 1990); the *S. pombe sar1* gene regulates *ras1* also in a mating signaling pathway (Nadin-Davis, 1986).

Mammalian GAPs

In vertebrates, p120 ras-GAP and NF1 share many sequence and functional attributes. The *NF1*-GRD homology with GAP involves a 360 aa stretch of the catalytic domain. The introduction of the NF1 catalytic fragment was able to downregulate the ras pathway in *in vitro* and in *vivo* experiments (Martin et al., 1990; Hattori et al., 1992). Both interact with the same range of ras-related proteins, as they provide GAP function for H-ras, N-ras, K-ras and R-ras (McCormick, 1995). The GAP binding site has been localized to an N-terminal region of ras, called the "effector loop" region, a highly-conserved stretch of 8 aa residues on GTPase proteins. Several mutations in this region drastically reduce GTPase activity without affecting nucleotide binding (Sigal et al., 1986), probably due to the protein's inability to interact with GAP and neurofibromin (Marshall and Hettich, 1993). Stang et al. (1996) reported, however, that the Tyr32His mutation on p21 ras (an effector loop mutation) dissociated p120 GAP but not *NF1*-GRD. In fact, site-directed mutagenesis of the *NF1*-GRD was able to generate variants of the domain that were able to

suppress oncogenic ras (Gutmann et al., 1993; Nakafuku et al., 1993; Poullet et al., 1994). This observation suggest that NF1 and GAP may be regulating ras activity in slightly different manners.

Other lines of study demonstrate that the two proteins are by no means structurally or functionally redundant, and are regulated by different molecular mechanisms. First of all, neurofibromin lacks the SH2 (Src homology 2) domain of GAP, which is crucial for the binding to phosphorylated protein domains (Settleman et al., 1992). Second, *NF1*-GRD has between 20-300 fold higher affinity for ras than GAP, but has approximately 30-fold lower GAP activity (Bollag and McCormick, 1991). Third, neurofibromin has extended homology with Ira1 and Ira 2 beyond the 360 aa GAP catalytic domain, a stretch of over 1500 aa (from residue 834 to 2394) (Ballester et al., 1990). In fact, the *NF1*-GRD is able to functionally rescue *Ira*1 and *Ira*2 yeast mutants (Xu et al., 1990), which function as negative regulators of the yeast-ras cAMP signaling pathway (Ballester et al., 1990; Buchberg et al., 1990b).

Furthermore, GAP proteins are known to have different sensitivities to inhibition by several mitogenic lipids (Tsai et al., 1989). For example, arachidonic acid inhibits the catalytic activity of GAP, *NF1*-GRD and Ira2 while phosphatidic acid (containing arachidonic and stearic acid) only inhibits *NF1*-GRD but not GAP or Ira2 (Golubic, 1992). Interestingly, a major target of MAP kinase protein is cytoplasmic phospholipase A_2 ($cPLA_2$) which, upon mitogenic stimulation, is recruited to the membrane where it cleaves phospholipids to produce arachidonic acid (Lin et al., 1993). Not only is arachidonic acid able to regulate ion channel activation in neurons (Freeman et al., 1991), modulate hormone secretion (Landt et al., 192), and specifically inhibit the catalytic activity of GAP and neurofibromin, but its prostaglandin derivatives are able to selectively stimulate GAP activity for p120 GAP, but not neurofibromin (Han et al., 1991). The prostaglandin stimulatory activity competes with the arachidonic acid inhibitory effects, probably because of a common binding site on the GAP protein. This suggests another mechanism for differentially regulating the two GAPs.

Negative regulator or effector?

Schwann cell culture studies provided evidence for cell-type specific regulation of the two GAPs. In normal cells, 50% of the ras protein is in the GTP-bound form, while only 5% of ras-GTP is found upon GTPase activation. In Schwannoma cell cultures from NF1 patients that lack neurofibromin (but with normal p120 GAP levels), the ras-GTP level again approached 50%, suggesting little to no GAP activity (Bollag and McCormick, 1992). Other reports have supported this increase in active ras levels in Schwannoma (DeClue et al., 1992) and neurofibrosarcoma (Basu et al., 1992) cell lines, as well as in tumor tissues derived from NF1-related neurogenic sarcomas and neurofibromas (Guha et al. 1996).

The strongest association between the catalytic function of neurofibromin and the development of cancer comes from studies of the myeloid cell lineage. The suggestion that the NF1 population are more at-risk of developing myeloid dysplasia received genetic support when Shannon et al. (1994) found no functional NF1 in bone marrow of patients suffering from chronic juvenile myeloid leukemia. This was followed by experiments using mouse hematopoietic cells showing that Nf1-deficiency causes hypersensitivity to GM-CSF (which signals through ras), raised ras-GTP levels as well as aberrant growth (Bollag et al., 1996; Largaespada et al., 1996). It is possible that NF1 may confer the primary GAP protein in Schwann and myeloid cell lineages. The inactivation of NF1 would predispose these cells to malignant growths.

However, this simple model is complicated by experiments in neurofibromin-deficient neuroblastoma (The et al., 1993) and melanoma-derived cells lines (Johnson et al., 1993) showing normal levels of ras-GTP. This may simply suggest that NF1 may not confer the primary GAP activity in these cell types, or that NF1 may have tumor suppressive functions distinct from the GAP catalytic activity.

A further complication of this model arises from the fact that oncogenic ras proteins (or other mitogenic stimulation that results in an accumulation of the active GTP-bound protein) cause growth arrest or differentiation in certain neural cells (Schwann cells and PC12, a pheochromocytoma cell line) (Greene and Tischler, 1976; Kremer et al., 1991; Li et al., 1992). In fact, oncogenic ras mutations are almost never found in most type of neural crest derived tumors (Bos, 1989).

Studies have shown that Type I and Type II *NF1* isoforms interact differently with ras. Type I (typically associated with later developmental stages and differentiated cell types) shows good GAP activity toward ras and is found to associate with microtubules. Type II (involved with primary tumors and early development) on the other hand, is 10-fold less efficient in catalysis and does not associate with microtubules (Andersen et al., 1993; Gutmann et al., 1995). It is quite possible that Type I neurofibromin plays a bigger role as a negative regulator of ras in maintaining differentiation, while Type II proteins serves as an effector for ras signals, be it normal or oncogenic.

Interacting proteins

Other than ras, one of the few proteins known to interact with neurofibromin is tubulin. Aside from the localization studies (Gregory et al., 1993), it has also been shown that the GAP activity of neurofibromin is inhibited during this interaction (Bollag et al., 1993). Since the microtubule binding domain overlaps with the ras-binding region, this may be a simple case of inhibition by steric hindrance, sequestration or binding site competition. However, the functional significance of this interaction is unclear.

Neurofibromin activity may also be regulated by protein phosphorylation. Putative phosphorylation sites were identified during the initial cloning of the *NF1* gene (Marchuk et al., 1991). However, only recently did one group provide *in vitro* evidence showing that protein

kinase A (PKA) is responsible for the constitutive phosphorylation at a C-terminal cysteine/serine-rich domain of neurofibromin (Izawa et al., 1996). This places neurofibromin downstream of the kinase in the PKA signaling pathway. However, experiments in *Drosophila* demonstrated that the loss of *NF1* function was not rescued by Ras1, Sos or Raf, but by the activation of PKA (The et al., 1997). This data argues for a model where PKA is either downstream of neurofibromin, or the two proteins act in parallel signaling pathways.

NF1 as a tumor suppressor gene

The genetic components of tumorigenesis can be categorized into three groups: Oncogenes, tumor suppressor genes and DNA repair genes. Oncogenes normally function as components of pathways that regulate cell growth, and activating mutations of oncogenes cause an excessive level of positive signaling to the cell growth machinery (Bishop, 1995). On the other hand, the classic definition of a tumor suppressor gene is one that normally functions to control cell growth, and the inactivation of which would lead to the promotion of cancer (Weinberg, 1991).

Genetic and molecular studies of *NF1* suggest that its gene is a classic tumor suppressor. Not only does it function as a negative regulator of a major oncogene, p21 ras, loss-of-function mutations in the *NF1* gene have been found in various NF1 and non-NF1 related tumors (reviewed in Seizinger, 1993). At the protein level, some NF1-related neurofibrosarcomas (DeClue et al., 1992), neuroblastomas, Schwannomas (The et al., 1993) and pheochromocytomas (Gutmann et al, 1995) show reduced or the lack of functional neurofibromin expression . As described above, a number of these tumors exhibit abnormally high levels of active ras-GTP.

Consistent with this model, the introduction of full-length *NF1* gene or its catalytic domain *NF1*-GRD was found to suppress growth in a human colon cancer cell line (Li and White, 1996). Interestingly, this study demonstrated that growth suppression was seen even with the introduction

of an *NF1*-GRD mutant that shows minimal GAP activity, but still retained normal binding affinity to ras. Therefore, neurofibromin may function in other non-GAP-related tumor suppressive role.

The most convincing genetic evidence to date in support of *NF1*'s tumor suppressor hypothesis has been the demonstration of loss-of-heterozygosity (LOH) at the *NF1* locus in malignant neurofibrosarcomas (Legius et al., 1993), malignant melanomas (Andersen et al., 1993b), myeloid leukemia (Shannon et al., 1994), pheochromocytomas (Gutmann et al., 1994), and more recently, in benign neurofibromas (Colman et al., 1995; Sawada et al., 1996; Serra et al., 1997). The loss of both alleles is consistent with the two-hit hypothesis first presented by Knudson (1971) to explain the variations between familial and sporadic cases of human retinoblastoma. The two-hit model of oncogenesis stipulates that individuals inheriting one mutated copy of a tumor suppressor gene is predisposed to cancer. However, this cancer will not arise until the second (wild-type) copy of the same gene has been inactivated by somatic gene mutation. Indeed, every human NF1 patient has inherited one mutant allele of the gene (Huson, 1994) and is therefore constitutionally heterozygous for the *NF1* mutation. This allele may be transmitted from an affected parent (familial mutation) or unaffected parent (sporadic germline mutation) (Lazaro et al., 1994).

Traditionally, it is believed that the failure in characterizing *NF1* LOH in neurofibromas resulted from the multiple cell types involved in the benign lesion as well as the difficulty in detecting all *NF1* mutations. For example, if only one cell type is devoid of functional NF1, sensitive assays such as PCR commonly used for such analyses would amplify the wild-type allele from adjacent cells.

The etiology of neurofibromas

One of the hallmark lesions of NF1 is the neurofibroma, a benign growth arising from the peripheral nerve trunks (either cutaneous or deep plexus) that has the potential to undergo malignant transformation. Macroscopically, neurofibromas are seen as focal enlargements along

nerves. One of the unmistakable histological features of neurofibromas is the presence of "onion bulbs," where a neuron or nerve bundle is surrounded by a thick layer of nerve sheath cells. These lesions typically appear hypocellular, with abundant collagen present between the hyperplastic cells. The lesion poses another interesting question in tumor etiology: Contrary to the popular belief that cancer results from the clonal growth of one particular cell type, neurofibromas always include cells showing different terminally-differentiated states:

- neurons (few)
- Schwann cells (60-85%)
- perineurial cells or fibroblasts (10-20%)
- mast cells
- others such as endothelium, pericytes and smooth muscle cells.

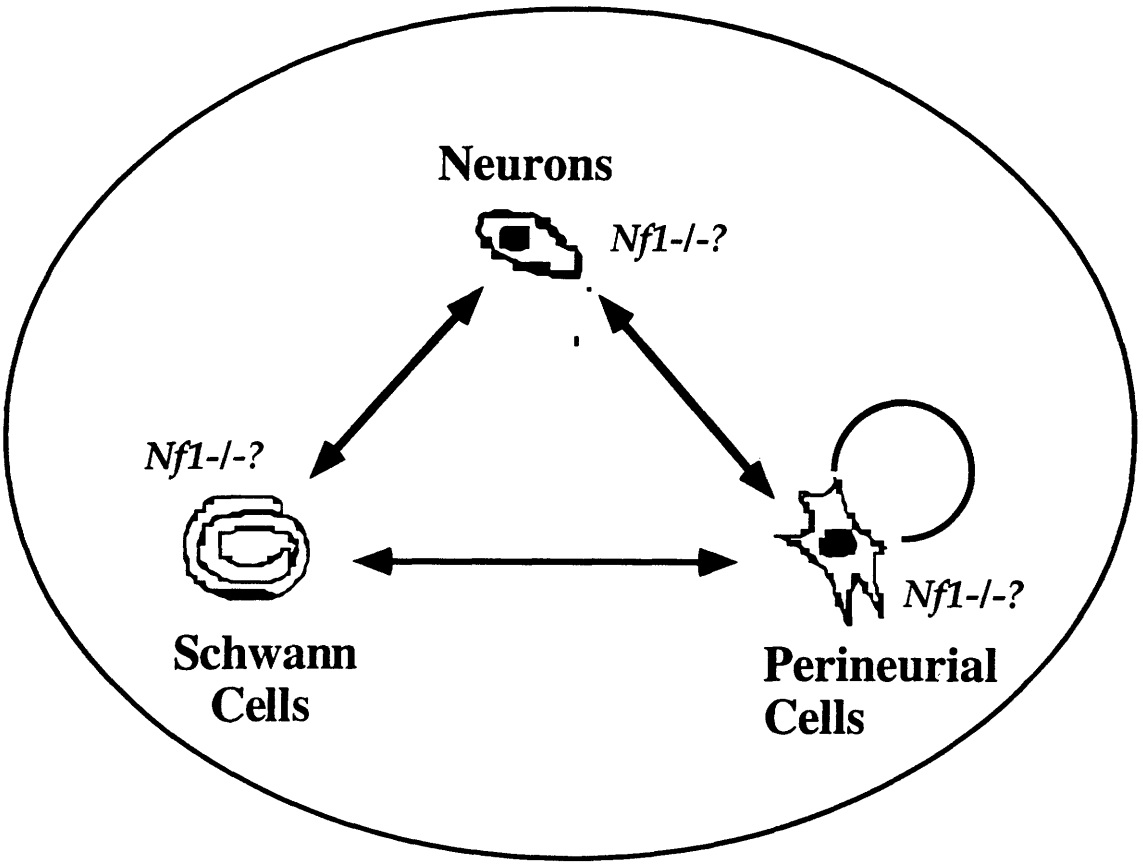
Several genetic and signaling models can be proposed to explain how the multiple cell types interact to promote neurofibroma development (Figure 1.3). For example, a LOH event may induce proliferation by rendering a cell more sensitive to growth factor stimulation. On the other hand, such a mutation may cause a cell to secrete abnormal levels of growth factors that recruits its (genetically heterozygous) neighbors to divide. Both cases may act in an autocrine or paracrine fashion, or both. However, it is currently not known which one of these cell types is responsible for the initiation and growth promotion of the tumor.

Schwann cells

Previous attempts have been made to describe the effects of neurofibromin-deficiency in various cell types, using cell culture from human NF1 tumors or *Nf1*-deficient mouse cell lines. Consistent with previous observation from the introduction of oncogenic ras into these cells, Schwann cells lacking Nf1 function showed increased ras signaling and a tendency to differentiate

Figure 1.3 Mechanisms of neurofibroma formation, neuron, schwann, perineurial (circle with triangle)

Figure 1.3



(Kim et al., 1995). Only when these Schwann cell were provided with increased cAMP pathway signaling did they begin to proliferate (Kim et al., 1997). Despite this apparent contradiction between activated ras pathway and proliferation, most still believe Schwann cells to be the mutant cell type in neurofibromatosis because (1) 40-85% of cells present in these tumors are of this lineage and (2) most of the cells have lost normal contact with neurons, indicating loss of normal function (Stefannson et al., 1982; Sheela et al., 1990)

Perineurial cells

Perineurial cells normally form concentric rings surrounding nerve bundles, and are believed to act as a selective barrier to prevent free diffusion of most macromolecules (e.g. growth factors) from the nerve fascicle (Olsson and Kristensson, 173). It is possible that NF1-deficiency in perineurial cells result in the breakdown of this barrier, exposure of the nerve fascicle to aberrant levels of mitogens, and leading to tumor formation. However, the origin of perineurial cells are still controversial, with the most direct evidence suggesting a fibroblastic origin (Bunge et al., 1989). Recent experiments found that NF1 may play a role in the differentiation of perineurial cells from fibroblasts, since murine fibroblast lacking neurofibromin fail to form perineurial cells *in vitro* (Rosenbaum et al., 1995).

Neurons

The only reports on Nf1-deficient neurons suggested an ability to survive in the absence of neurotrophic factors (Vogel et al., 1995) and a slight growth advantage in development (Brannan et al., 1994). In addition, the lack of neurofibromin in conjunction with other gene mutations implicated in memory has been shown to alter long term potential in the brain (Silva et al., 1997).

Chapter 3 of this thesis describes experiments in which we attempted to identify the critical cell type(s) involved in neurofibroma formation by creating a mouse composed of both Nf1-deficient and wild-type cells.

Existing animal models of NF1

A number of spontaneous, chemically-induced and transgenic animal models showing various phenotypes involving neural crest-derived tissues (i.e. neurocristopathy) have been described. For example, a population of Holstein cows shows predisposition to the development of multiple cutaneous neurofibromas which are histologically similar to the human lesions (Sartin et al., 1994). An NF1 model of the bicolor damselfish (*P. partitus*) has been described by Schmale et al. (1986) to show lesions such as pigmentation defects, neurofibromas, Schwannomas and neurofibrosarcomas. The genetics underlying this set of phenotype is unclear, but the seemingly transmissible pattern of the lesions (Schmale and Hensley, 1988) suggest the involvement of a possible infectious oncogenic agent.

Transplacental and neonatal ethylnitrosourea (ENU) mutagenesis experiments in hamsters (Riccardi, 1988; Nakamura et al., 1989) and rats (Cardesa et al., 1979; Cardesa et al., 1989) have produced animals that develop a number of NF1-related lesions, such as neurofibrosarcomas and Schwannomas. Nakamura et al. (1989) found full-length neurofibromin expression in 7 out of 14 neurofibromas isolated from hamsters. The possibility that ENU mutated genes other than Nf1 was supported by the detection of several activating mutations in the *neu* oncogene (Nikitin et al., 1991; Nakamura et al., 1994; Perantoni et al., 1994).

Mouse models of neurocristopathy have been generated by the ectopic expression of various viral oncogenes, including HTLV-1 tax (Hinrichs et al., 1987; Nerenberg et al., 1987; Green et al., 1992), HIV-1 tat (Corallini et al., 1993) and SV40 large T antigen (Jensen et al., 1993; Messing et al., 1994; Mazarakis et al., 1996). Lesions were found in neural crest tissues such as the peripheral nerve, the iris and adrenal medulla. However, the introduction of viral proteins has a major shortcoming because they usually alter several cellular pathways at once. For example,

SV40 large T antigen and HTLV-1 tax are able to simultaneously transactivate growth factor genes (Green, 1991) and oncogenes (Duyao et al., 1992; Ejima et al., 1993), while inactivating the Rb, *p53* (reviewed in van Dyke, 1994) and *NF1* tumor suppressor genes (Feigenbaum et al., 1996). Therefore, these models do not allow a careful dissection of the minimal genetic pathways responsible for tumor formation.

Knockout mouse models

Using gene targeting and homologous recombination to disrupt mouse genes implicated in human tumor suppression provides several advantages: First of all, the models provide mammalian systems with pre-defined genetic mutations. Not only are these animals useful as *in vivo* models for studying pathways in which these tumor suppressor genes act, the various primary and tumor cell lines that may be derived provide invaluable reagents for detailed *in vitro* molecular assays. Secondly, mouse and human chromosomes have regions of synteny that would allow the conservation of any transcriptional interplay between adjacent genes or regulatory elements (e.g. activation or silencing of gene expression).

Another advantage to using mouse models of cancer is the ability to study various combinations of tumor suppressor and oncogene mutations that may be required for the development of specific types of cancer. This multi-step nature of tumorigenesis has been suggested for many types of cancer, but the most comprehensive model to date is that for colorectal carcinogenesis (Fearon and Vogelstein, 1990). The ability to study multifactorial/multigenic diseases using mouse knockouts is further enhanced by the opportunity to breed mutations onto different inbred genetic backgrounds of mice. This has significant implications for studying the role of modifier genes in carcinogenesis, which has been implicated to modulate the phenotype for tumors of the intestines (Dietrich et al., 1993), mammary (Moser et al., 1995), ovarian (Phelan et al., 1996), skin (Sundberg et al., 1997) and lung (Obata et al., 1996). It is not surprising that most of these

modifier genes have been identified through the use of the densely-mapped chromosomal polymorphic markers in mice.

Tumor suppressor knockout models

Several mouse homologs of human tumor suppressor gene have been disrupted by homologous recombination. A sample of the models are provided in table 1.2:

Table 1.2. Summary of representative mouse tumor suppressor knockouts (adapted from Jacks T, Annu Rev Genet 30, 1996).

Gene	Protein features	+/- phenotype	-/- phenotype
Rb	nuclear phosphoprotein, binds transcription factors	pituitary tumor	embryonic lethality (E13.5-15.5), erythropoiesis defect
p53	nuclear phosphoprotein, transcription factor	sarcoma, lymphoma, others	10% embryonic lethality due to excencephaly, lymphoma, sarcoma
Wt1	zinc fingers, transcription factor	none	embryonic lethality (E13.5), cardiac & urogenital defects
Apc	coiled-coil motif, binds b-catenin	intestinal polyps	pregastrulation lethality (E5-6)
Nf1	ras-GAP activity	**Chapter 2**	**Chapter 2**
Nf2	membrane cytoskeletal attachment	sarcoma	pregastrulation lethality (~E7)
Brca1	zinc finger, secreted granin? nuclear protein?	?	pregastrulation lethality (E5-6)
Brca2	association with RAD51, a DNA repair protein	?	pregastrulation to embryonic lethality (E8.5-9.5) with defects in cellular proliferation; hypomorphic allele mutants survive and show cancer predisposition*
p107	*Rb*-family	none	none
p130	*Rb*-family	none	none
p16	inhibit cyclin D-dependent kinases	?	predisposition to lymphoma and sarcoma

*Hypomorphic Brca2 allele reference (Connor et al., 1997).

Judging from the number of mutant strains exhibiting embryonic lethality, many of the tumor suppressor genes are required for normal mouse development. In many of those cases, inheriting one mutant copy of the gene predisposes the animal to certain lesions, which is consistent with Knudson's "two-hit" model, requiring the inactivation of the second, wild-type tumor suppressor allele (Knudson, 1971).

While the Rb mutant mice show increased risk to pituitary tumors, it is nonetheless an inadequate model for retinoblastoma, the hallmark malignancy associated with the familial disease (Clarke et al., 1992; Lee et al., 1992; Jacks et al., 1992). The *Apc* and *p53* mutant strains, however, do show predisposition to a spectrum of malignancies characteristic of their respective inherited human diseases, i.e. familial adenomatous polypolis coli (Miyaki et al., 1995) and Li-Fraumeni syndrome (Levine , 1992; Greenblatt et al., 1994).

The *p53* mutant mouse strain is worthy of note both in a clinical and mechanistic sense. An estimated 50% of all cancer cases show mutations in this gene (Greenblatt et al., 1994). Furthermore, p53 is responsible for initiating G1-phase cell-cycle arrest (Kastan et al., 1992) or programmed cell death (apoptosis) (Clarke et al., 1993; Lowe et al., 1993) in response to DNA damaging agents or other insults, depending on the cell type involved. The role of p53 in tumor suppression is furthered by experiments demonstrating that many tumor viruses produce proteins that bind and inactivate p53, and that this event is a required step in viral transformation (Moran, 1993).

In the case of the NF1 disease, the inactivation of p53 has been suggested as a required step for malignant transformation from benign lesions (Greenblatt et al., 1994). One study found *p53* mutations in 100% of DNA samples extracted from malignant neurofibrosarcomas associated with NF1 patients (Menon et al., 1990). Therefore, it would be of particular interest to study the effect of combining both the *NF1* and *p53* mutations in a mouse model (Chapter 4).

Lastly, not all tumor suppressor mouse knockout models develop the expected phenotype. However, if the animal model does indeed recapitulate the human disease, then it becomes an invaluable model for further genetic and mechanistic studies on the etiology of the tumor.

Eventually, they may be used as critical preclinical animal models to test the safety and efficacy of potential anti-cancer drugs.

Conditional gene knockout models

Given that many tumor suppressor proteins are required for embryogenesis, it is not surprising to find germline tumor suppressor null mutations to result in embryonic lethality. However, in order to generate mouse models of cancer, we must be able to assay the loss of tumor suppressor function in the context of an adult animal. The question then becomes: Is it possible to create mouse models that maintains gene function throughout development (or only in critical tissues) -- allowing it to bypass the embryonic lethality stage -- yet still be able to show the consequences of losing that particular gene function as an adult animal?

One possible alternative to straight knockouts is the generation of double knockout (DKO) chimeras. In this approach, engineered embryonic stem (ES) cells lacking both copies of a particular tumor suppressor gene are injected into mouse blastocysts and allowed to contribute toward the developing animal. The resulting "chimera" will be composed of cells derived from both the wild-type blastocyst as well as the DKO (tumor suppressor-deficient) ES cells. If the level of mutant cell contribution to critical tissues fall below a threshold range, the animal will be able to escape the developmental bottleneck seen in completely null animals. In fact, the efficacy of this approach in bypassing embryonic lethality has been demonstrated for the *Rb* mutant mice (Maandag et al., 1994; Williams et al., 1994). There is one drawback, however, since the extent of mutant cell contribution cannot be regulated except through the number of ES cells injected, a high level contribution may still cause developmental abnormalities in chimeric embryos.

In recent years, several new techniques have been described which offers better control over the specificity of knockouts.

• Cre-lox conditional knockout: The use of the bacteriophage P1 site-specific recombination machinery has been effectively demonstrated in murine cell culture and animal systems. The two required components are (1) a pair of 34 bp *loxP* (locus of crossing-over) sequences and (2) the 38 kDa *cre* (causes recombination) recombinase (Kilby et al., 1993). The presence of *cre* in cells causes the two *loxP* sites to recombine, excising the sequences between two *loxP* sites in the same orientation, or causes an inversion if the two *loxP* sites are in opposite orientations. One of the first studies utilizing this system in ES cells demonstrated T cell specific deletion of the DNA polymerase β gene (Gu et al., 1994). It is therefore possible to analyze the function of a tumor suppressor gene only in certain cell types by introducing *cre* in a tissue-specific manner. Such an option is beneficial given the fact that many tumor suppressor genes are required for normal mouse development. Therefore, by maintaining normal gene function in all but one tissue type, the mouse embryo is then more likely to survive beyond the developmental bottleneck. Many other uses for the *cre-loxP* recombinase system have been reported. For example, a mouse lens tumor model was created by a targeted activation of oncogenes with *cre* recombinase driven by the aA-crystalline promoter (Lasko et al., 1992).

• Tetracycline inducible gene expression: Control elements of the antibiotic tetracycline-resistance operon from the *E. coli* transposon Tn10 have been utilized to establish a tightly-regulated system in mammalian cells. The primary regulatory mechanism is the tetracycline-controlled transactivator (tTA), which is a hybrid protein containing a tet regulatory element and a viral transactivator domain. This protein drives gene expression from a promoter that specifically recognizes tTA (Gossen and Bujard, 1992). Transgene transcription can then be selectively activated by introducing the antibiotic into cell culture or by feeding the mice. Removal of the antibiotic quickly shuts down gene transcription. This gives researchers to tight control over the temporal induction of gene expression, because tetracycline can be introduced to pregnant females in order to cause transgene expression during various stages of development, or to adult mice at any age.

The work presented in this thesis describes my efforts in characterizing the effects of murine *Nf1* mutations in various genetic and physiological contexts. The primary goals are to develop mouse models that closely recapitulate the various human NF1 disease symptoms. These models will provide valuable biological systems from which to better understand the the genetic and environmental events that are necessary and sufficient for tumor development. Chapter 2 will summarize the effect of the germline *Nf1* mutation in development and in the adult animal. Chapter 3 will describe the first genetic mouse model of neurocristopathy specifically addressing the role of *Nf1* deficiency. Chapter 4 will address the cooperation between the *Nf1* and *p53* tumor suppressor genes in tumor progression into malignant peripheral nerve sheath tumors.

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Chapter 2

Tumorigenic and developmental consequences of a targeted *Nf1* mutation in the mouse

Introduction

Neurofibromatosis type I (NF1) is a dominantly inherited genetic disorder affecting approximately 1 in 3500 individuals (Stumpf et al., 1987). The major symptoms of the disease are benign neurofibromas, pigmentation defects termed cafe-au-lait spots, Lisch nodules (hamartomas of the iris), skeletal abnormalities, and learning disabilities (Riccardi, 1992). NF1 patients are also at increased risk of developing certain malignancies including optic glioma, neurofibrosarcoma, pheochromocytoma and myeloid leukemia (Hope and Mulvihill, 1981), thus classifying the disease as a familial cancer syndrome. Since many of its lesions involve cells of neural crest origin, NF1 has been described as a neurocristopathy (Bolande, 1974).

The *NF1* gene was cloned in 1990 (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990) and shown to encode a member of the GAP family of ras regulatory proteins (Xu et al., 1990). The GAP-related domain of the *NF1*-encoded protein (neurofibromin) can stimulate the GTPase activity of $p21^{ras}$ *in vitro* (Martin et al., 1990; Xu et al., 1990) and functionally complement yeast IRA1 and IRA2 mutants *in vivo* (Xu et al., 1990; Ballester et al., 1990), suggesting that neurofibromin normally functions in a ras-dependent signal transduction pathway. Accordingly, the pathological consequences of inherited mutations in *NF1* may result from defects in ras signaling, particularly in certain cell types derived from the neural crest. However, the expression of *NF1* is not restricted to cells of this lineage. *NF1* mRNA has been detected in a wide range of human tissues and cell lines (DeClue et al., 1991; Basu et al., 1992), and the gene is ubiquitously expressed during early development in the rat (Daston and Ratner, 1992; Huynh et al., 1994). Expression at later stages of gestation and in the adult is largely restricted to neuronal tissues (Huynh et al., 1994; Daston et al., 1992).

Several lines of evidence implicate *NF1* as a tumor suppressor gene. First, the majority of mutant alleles of *NF1* isolated to date from neurofibromatosis patients cause gross alterations in

the gene (reviewed in Seizinger, 1993)) , indicating that the disease is caused by the inheritance of a single loss-of-function allele. Consistent with Knudson's "two-hit" model for inactivation of tumor suppressor genes (Knudson, 1971), some malignant tumors from NF1 patients have shown either the absence of the wild-type *NF1* allele (Legius et al., 1993) or reduced neurofibromin activity (Basu et al., 1992; DeClue et al., 1992). In addition, the *NF1* gene is inactivated in a variety of sporadic tumors which are not normally associated with NF1 (Li et al., 1992; Andersen et al., 1993; Johnson et al., 1993; The et al., 1993).

Here, we describe the construction and characterization of a mouse strain with a germline mutation in the *Nf1* gene. The mouse and human *NF1* genes are highly related (Bernards et al., 1993), suggesting a conservation of function across species. The predicted amino acid sequence of the human and mouse neurofibromin proteins are 98% identical, and there is also significant similarity in the 3' non-coding region of the mRNAs (Bernards et al., 1993). Recently, Brannan et al. (Brannan et al., 1993) described their analysis of homozygous embryos lacking the *Nf1* gene, in which they found widespread developmental abnormalities. We have constructed a similar targeted mutation, provided a detailed characterization of its molecular effects, and examined the resulting phenotype in both homozygous and heterozygous mutant mice. Our extensive analysis of *Nf1* heterozygotes over a period of more than two years reveals an enhanced predisposition to various tumors including phaeochromocytomas and myeloid leukemias, tumors that are also evident in human NF1 patients, and the strongest evidence to date that *Nf1* acts as a classical tumor suppressor gene.

Methods

Nf1 Gene Targeting.

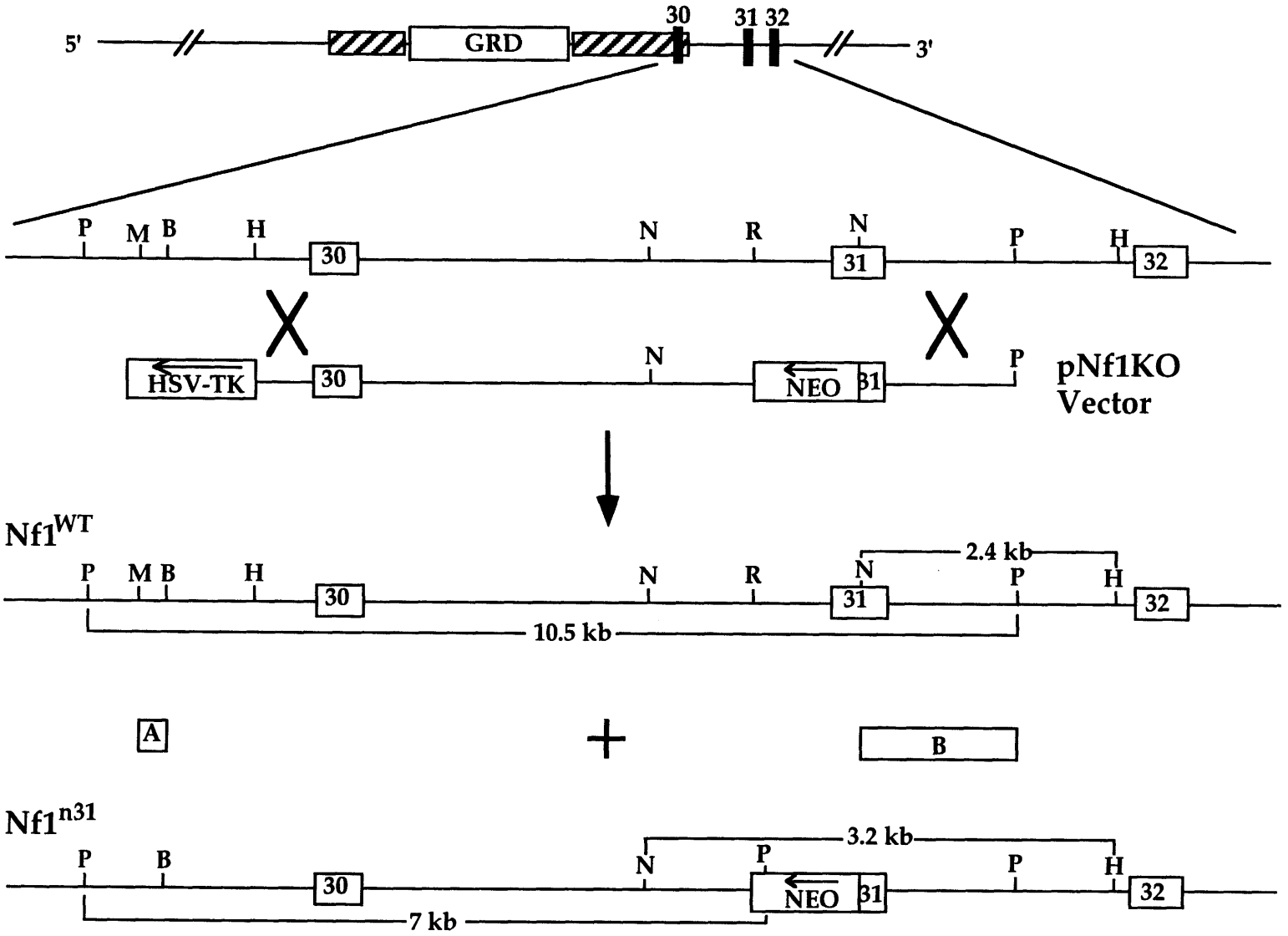
The *Nf1* knock-out vector (pNf1KO) was constructed from a 16.6 kb mouse genomic fragment (isolated from a strain 129/sv genomic DNA library, a generous gift of R. Jaenisch). The library was screened with a probe from human *NF1* exon 31. This DNA fragment was found to span exons 30, 31 and 32, as determined by hybridization with oligonucleotide probes specific for each exon (data not shown). In pNf1KO, the *neo* gene was flanked by two regions of homology: a 5.1 kb fragment extending from an *Hind*III site in intron 29 to a *Eco*RV site in intron 30 and a 1.4 kb fragment from an *Nco*I site in exon 31 to the *Pst*I site in intron 31 (Figure 2.1). The *MC1-neo* expression cassette (Thomas and Capecchi, 1987) replaced a 2 kb fragment between the *Eco*RV site in intron 30 to the *Nco*I site in exon 31. The *HSV-TK* gene (under the control of the *pgk* promoter) and the *MC1-neo* cassette are transcribed in the opposite transcriptional orientation to *Nf1*. The targeting scheme and the restriction map of the wild-type ($Nf1^{wt}$) and mutant ($Nf1^{n31}$) alleles are depicted in Figure 2.1. Electroporation of pNf1KO into D3 ES cells (Gossler et al., 1986), subsequent drug selection, and Southern blot analysis were performed as described previously (Xu et al., 1992). The wild-type and the mutant *Nf1* alleles in ES cells can be distinguished by Southern blotting as described in Figure 2.1.

Generation of chimeras and testing for germline transmission were done essentially as described in Jacks et al. (1992). Genotype analysis was performed by Southern blotting (Figure 2.1) or polymerase chain reaction (PCR). The $Nf1^{wt}$ allele was amplified by PCR using primers directed against exon 31 (5'-GGTATTGAATTGAAGCAC-3') and an intronic sequence (NfX4I: 5'-TTCAATACCTGCCCAAGG-3'), while the $Nf1^{n31}$ allele was amplified using a primer directed against neo (5'-ATTCGCCAATGACAAGAC-3') and NfX4I. The PCR products for $Nf1^{wt}$ and $Nf1^{n31}$ are 230 bp and 350 bp, respectively. Homozygous embryos were generated from heterozygous intercrosses. All animals described here are on a 129/sv x C57BL/6 mixed

Figure 2.1 Targeted disruption of the murine *Nf1* locus.

The relevant position of the *Nf1* gene involved in gene targeting is shown (top), with the open box depicting the GAP-related domain (GRD), and the hatched boxes indicating the IRA-related domains flanking the GRD. The p*Nf1*KO vector consists of a *MCl-neo* cassette in the opposite transcriptional orientation to the *Nf1* gene, flanked by 5' and 3' *Nf1* homologous regions of 5.1 kb and 1.4 kb, respectively. A *HSV-T*K cassette transcribed in the opposite orientation to *Nf1* precedes the *Nf1* sequences. Homologous recombination between the p*Nf1*KO vector and an endogenous *Nf1* allele results in the insertion and partial replacement of intron 30 and exon 31 with the *neo* gene expression cassette. ES cells were screened by Southern blot analysis of genomic DNA digested with *Pst*I and hybridization with probe A. Relevant *BamH*I (B), *Hind*III (H), *Mbo*I (M), *Nco*I (N), *Pst*I (P) and *EcoR*V (R) recognition sites are shown.

Figure 2.1 Targeted disruption of the murine Nf1 locus.



genetic background, but we have observed similar results using animals on an inbred 129/sv genetic background.

Nf1 RNA Analysis.

Total cellular RNA was prepared from 12.5 dpc whole mouse embryos using the RNAzol B RNA isolation kit (Biotecx). Northern blotting was performed essentially as described (Shackleford and Varmus, 1987). Probes used were a 1.3 kb *EcoR*I fragment of mouse *Nf1* cDNA located just upstream of the GAP-related domain (GRD) (The et al., 1993) and a 0.5 kb *Eag*I-*Nco*I fragment from *pMC1neo* (Stratagene).

Nf1 cDNA Sequencing.

Embryonic fibroblast cultures were prepared according to the protocol of Robertson (1987) from 12.5 dpc embryos. Total RNA was isolated from cultures (Xie and Rothblum, 1991) and 3 mg were reverse-transcribed using synthetic random hexamers (Ausubel et al., 1994). A fragment extending from exon 30 to 32 was amplified using primers specific to each exon (NFX35'3': 5'-CTATAATCTCCTGTGTGC-3' and NFX53'5': 5'-ACTCGACACCAATTTCAC-3'), and subsequently cloned into pT7Blue Vector (Novagen). DNA mini-preps were made from colonies containing correct fragment insertions and sequenced using Sequenase (USB) with either the NFX35'3' or the NFX53'5' primer.

Immunoprecipitation Analysis.

Radiolabeling of cellular proteins and immunoprecipitation were performed essentially as described (the et al., 1993). Cell lysates were made from embryonic fibroblast cultures incubated for 16 h with 0.5 mCi of [^{35}S]-EXPRE^{35}S^{35}S (DuPont NEN, specific activity >1000Ci/mmol). Antibodies directed against specific synthetic C-terminal (serum 912) and GRD peptides (serum 1034) were used separately to immunoprecipitate neurofibromin. Proteins were resolved on a 6% SDS-polyacrylamide gel.

Histopathology.

Histological analysis of tumors and embryonic tissues were performed as described (Xu et al., 1992). Tissue samples were fixed in either Bouin's solution or 10% neutral buffered formalin, processed for histology, embedded in paraffin, sectioned at 6 m, and stained with hematoxylin and eosin.

Southern Analysis of Tumor DNA.

Loss of heterozygosity analysis was performed on DNA isolated from fresh tumor samples according to the method of Laird et al. (1991). The genotype of the tumor DNA was determined by Southern blotting following *Nco*I/*Hind*III digestion and hybridization with a *Nco*I-*Pst*I fragment from intron 31 (Figure 2.1).

Results

Nf1 gene targeting.

One allele of the *Nf1* gene was disrupted in the D3 murine ES cell line using the targeting vector p*Nf1*KO shown in Figure 2.1. p*Nf1*KO was designed to create a mutant allele of *Nf1* representative of certain human *NF1* mutations. It carries a portion of mouse genomic *Nf1* DNA surrounding exon 31, which is the site of several mutations in human patients with NF1[5,27,28]. The mutation introduces a neomycin resistance gene (*neo*) expression cassette in place of the first 42 codons of exon 31 and also replaces the exon 31 splice acceptor sequence and approximately 2 kilobases (kb) of intron 30. The direction of transcription of *neo* is opposite to that of *Nf1*. In addition, p*Nf1*KO contains the herpes simplex virus thymidine kinase gene (*HSV-TK*) to provide a means for negative selection according to the protocol of Mansour et al. (1988) (Figure 2.1). However, previous experiments using a related targeting vector indicated that this fragment of *Nf1* genomic DNA underwent homologous recombination at high frequency (data not shown). Therefore, the *HSV-TK* negative selection protocol was not used in the experiment described below.

The p*Nf1*KO targeting vector was introduced into D3 ES cells (strain 129/sv) (Gossler et al., 1986) by electroporation, and, following drug selection for *neo* (with G418), ES cell clones were screened by Southern blotting. As shown in Figure 2.1, homologous recombination between p*Nf1*KO and endogenous *Nf1* creates a mutant allele that can be distinguished from the wild-type gene following digestion of genomic DNA with several different restriction enzymes. DNA samples isolated from ES cell clones were initially screened using *Pst*I digestion and a probe located 5' to the sequences present in p*Nf1*KO which recognizes 10.5 kb and 7 kb fragments from the wild-type and mutant *Nf1* alleles, respectively (Figure 2.1). From approximately 150 G418-resistant ES cell clones examined, two showed the mutant-specific band, indicating that they were heterozygous for the targeted *Nf1* mutation (not shown). Therefore, the targeting efficiency

was approximately 1/75 G418 resistant clones. The structure of the targeted allele in these two clones was confirmed using a probe located 3' to exon 31 (probe B in Figure 2.1; data not shown). We have termed this mutant allele *Nf1*^{n31} to indicate the insertion of the *neo* gene into exon 31.

Both heterozygous ES cell clones were independently injected into C57BL/6 blastocyst-stage embryos in order to create chimeric animals. Each clone yielded chimeras with contribution of the mutant cells to the germline, as evidenced by their ability to produce agouti-colored offspring when bred to C57BL/6 animals. Tail DNA was isolated from these agouti offspring at weaning and tested for the presence of the *Nf1*^{n31} allele using the Southern blot strategy outlined in Figure 2.1. As expected, approximately half of the agouti animals were heterozygous for the *Nf1*^{n31} mutation (data not shown). Germline chimeras were also crossed to 129/sv females in order to establish the mutation on an inbred genetic background. Animals on the mixed (129/sv x C57BL/6) and inbred 129/sv genetic background exhibited the same heterozygous and homozygous mutant phenotypes as described below.

Molecular Effects of the *Nf1*^{n31} mutation.

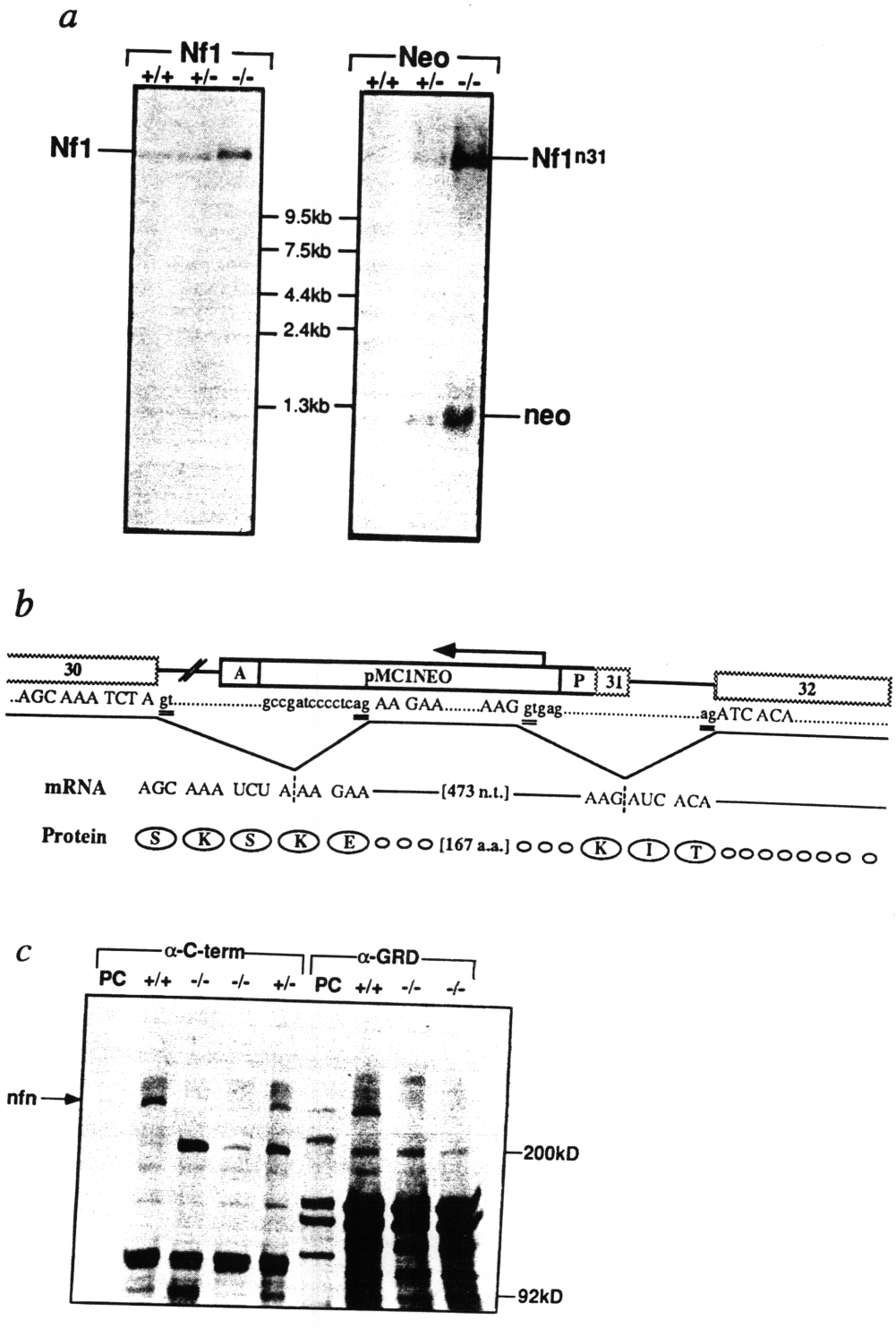
In order to characterize the effects of the exon 31 *neo* insertion on the expression of *Nf1* mRNA and neurofibromin protein, we analyzed tissues and cells isolated from heterozygous and homozygous mutant and wild-type embryos (see below). Total RNA was isolated from *Nf1*$^{+/+}$, *Nf1*$^{+/n31}$, and *Nf1*$^{n31/n31}$ embryos at 12.5 days of gestation and examined by northern blotting using probes derived from mouse *Nf1* cDNA. As shown in Figure 2.2*a*, the *Nf1* probe detects a transcript of similar size and abundance in embryos of all three genotypes. This result indicates that the *Nf1*^{n31} allele is efficiently transcribed and processed and that the resulting mRNA is stable. Based on the structure of the targeted mutation (Figure 2.1), we thought that the mRNA produced from the mutant allele might contain sequences from the noncoding strand of *neo*. Indeed, a double-stranded probe directed against *neo* detects an mRNA

Figure 2.2 Molecular effect of *Nf1*^{n31} disruption.

a, Northern blot analysis of *Nf1* RNA expression in embryos at 12.5 days of gestation. Approximately 30 μg of total RNA isolated from whole embryos was resolved by electrophoresis, transferred to nylon membrane, and hybridized with an *Nf1*-specific probe (left panel, see Methodology). An mRNA of approximately 13 kb was detected in samples from wild-type (+/+), heterozygous (+/-) and homozygous (-/-) embryos. The slightly stronger signal in the -/- sample results from more total RNA in this lane (not shown) rather than increased expression. The double-stranded *neo*-specific probe (right) recognizes a 13 kb *Nf1*^{n31} message as well as the 1 kb message from the *neo* cassette. *b*, Diagram of splicing in the *Nf1*^{n31} transcript. The *pMC1neo* cassette in the opposite orientation (as shown) creates both consensus splice acceptor and donor sequences (intronic splicing sequences are shown in lower cases, with highly conserved nucleotides doubly underlined). As revealed by sequences of a cDNA product of the mutant mRNA, exon 30 splices into the *neo* cassette at position 1531 of the *MC1-neo* sequence (Stratagene), and after 473 nucleotides, splicing occurs from a splice donor located in *neo* at position 1059 of *MC1-neo* (Stratagene) into exon 32. This novel sequence in the *Nf1*^{n31} mRNA contains no stop codons and therefore replaces the entire exon 31 coding region with a larger open reading frame derived from the non-coding strand of *neo*. Amino acid residues adjacent to the splicing junctions are shown. P and A represent the promoter and poly-A addition sequence of *pMC1neo*. *c*, Immunoprecipitation of mouse neurofibromin. Cultured fibroblasts isolated from wild-type (+/+), heterozygous (+/–) and two homozygous mutant embryos (–/–) were radiolabeled, and lysates immunoprecipitated with serum 912, directed against the extreme C-terminal 13 a.a. peptide (a-C-term) of human neurofibromin, or with affinity purified serum 1034, directed against a synthetic 21 amino acid GRD peptide (a-GRD) (The et al., 1993). A neuroblastoma cell line (90-5) (The et al.,1993) expressing high levels of neurofibromin was used as a positive control (PC). Approximately 2 mg of total protein (determined by the Bradford method (Biorad)) was used for each immunoprecipitation. The

position of full-length neurofibromin is indicated (nfn). Note the mutant cells do not produce full-length neurofibromin or a protein of slightly higher mobility which could result from translation of the *Nf1*^{n31} mRNA. Also, the heterozygous cells contain approximately half as much neurofibromin as the wild-type cells. The protein at approximately 200 kD detected by the a-C-term antibody has been seen at variable levels in cells of all genotypes in different experiments and differs substantially between the two –/– samples examined here.

Figure 2.2


a
Nf1
+/+ +/- -/-
Neo
+/+ +/- -/-
Nf1
Nf1n31
9.5kb
7.5kb
4.4kb
2.4kb
1.3kb
neo
b
30
A
pMC1NEO
P
31
32
..AGC AAA TCT A gt.....gccgatcccctcag AA GAA.....AAG gtgag.....agATC ACA.....
mRNA AGC AAA UCU A|AA GAA —— [473 n.t.] —— AAG|AUC ACA
Protein S K S K E [167 a.a.] K I T
c
α-C-term
α-GRD
PC +/+ -/- -/- +/- PC +/+ -/- -/-
nfn
200kD
92kD

in the heterozygous and homozygous mutant samples that co-migrates with the *Nf1* message, as well as the 1 kb transcript from the *neo* expression cassette (Figure 2.2*a*).

In order to better characterize the mRNA product of the $Nf1^{n31}$ allele, we performed reverse transcriptase PCR (RT-PCR) on mRNA isolated from homozygous mutant embryonic fibroblasts using primers surrounding the site of the mutation. As diagrammed in Figure 2.2*b*, the sequence of the RT-PCR product revealed that the mutant transcript contains a stretch of 473 nucleotides derived from the non-coding strand of *neo*, located between *Nf1* sequences encoded by exons 30 and 32. This *neo* insertion and complete replacement of the *Nf1* exon 31 sequences results from mRNA splicing from the exon 30 splice donor to a canonical splice acceptor sequence located in *neo* followed by splicing from a donor sequence in *neo* to the splice acceptor of exon 32 (Fig. 2.2*b*). Quite unexpectedly, the insertion in the mutant mRNA maintains an open reading frame, potentially allowing the translation of an altered *Nf1* protein product which would lack the 64 amino acids encoded by exon 31 and contain an additional 167 amino acids encoded by the non-coding strand of *neo*. This mutant neurofibromin species would be predicted to be approximately 10 kilodaltons (kD) larger than the wild-type protein.

To determine whether the altered neurofibromin species was stably produced, we performed immunoprecipitation of radiolabeled lysates from fibroblasts isolated from embryos of the three genotypes. Secondary fibroblast populations were labeled with ^{35}S-methionine and -cysteine in vitro, and the lysates were precipitated with polyclonal sera raised against two human neurofibromin peptides. Serum 1034 recognizes an epitope located in the GAP-related domain (GRD), N-terminal to the targeted mutation, while serum 912 is directed against the extreme C-terminus of the protein (The et al., 1993). As shown in Figure 2.2, both of these sera precipitate a protein of approximately 250 kD from wild-type mouse fibroblasts which co-migrates with human neurofibromin. Also, fibroblasts from heterozygous animals produced approximately half that of the wild-type cells (Fig. 2.2*c*, data not shown). However, fibroblasts derived from

Nf1^{n31}/*Nf1*^{n31} embryos lack full-length neurofibromin (Fig. 2.2*c*), and we also do not observe a higher molecular weight species that might correspond to the slightly larger mutant neurofibromin protein. These data suggest that the mutant protein product is unstable and does not accumulate in cells. It is also possible that the 1 kb *neo* mRNA transcribed from the *MC1* promoter might hybridize to the complementary *neo* sequences present in the mutant *Nf1* mRNA and block its translation. These data indicate that the *Nf1*^{n31} mutation eliminates the production of stable neurofibromin and, therefore, should act as a null. It is also relevant that the mutation causes skipping of exon 31 all together, since this exon is the site of several missense (Cawthon et al., 1990) and nonsense (Ainsworth et al., 1993) mutations in human NF1 patients.

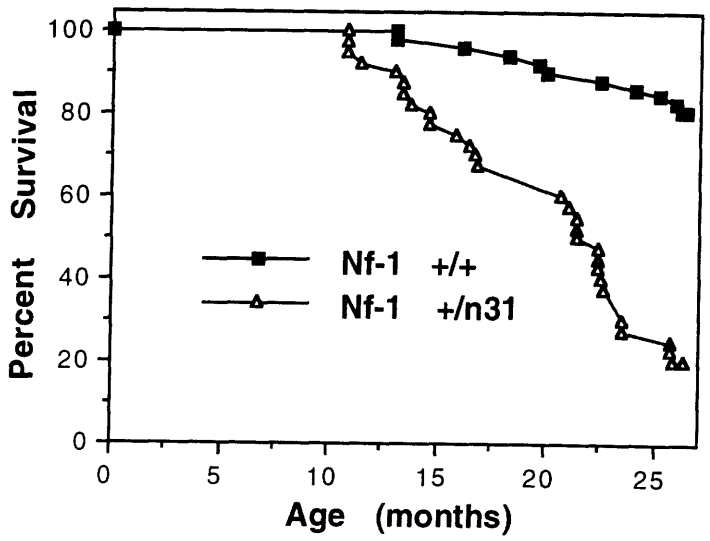
The heterozygous phenotype.

Animals heterozygous for the *Nf1*^{n31} mutation are potential models for human NF1. Therefore, we carefully screened heterozygotes for the common lesions associated with this disease, as well as any other signs of pathology. From a total of more than 250 *Nf1*$^{+}$/*Nf1*^{n31} animals followed for 7 months to over two years of age, none has developed obvious neurofibromas or pigmentation defects resembling human cafe-au-lait spots. Also, we failed to detect Lisch nodules or other pathology of the iris in more than 30 heterozygotes examined microscopically. Therefore, with respect to these and most of the other cardinal features of human NF1, the *Nf1*$^{+}$/*Nf1*^{n31} mice do not represent an accurate animal model for this genetic disease.

Consistent with the putative role of *Nf1* as a tumor suppressor gene, however, heterozygosity for the *Nf1*^{n31} mutation does increase the rate of tumorigenesis in the mouse. As shown in Figure 2.3, in a closely monitored set of 40 *Nf1*$^{+}$/*Nf1*^{n31} animals, 30 (75%) succumbed to tumors over a period of 27 months, as compared to 15% (8/52) in a comparable set of wild-type animals. In a larger series of 62 heterozygous animals, we have observed a variety of tumor types, including lymphoma (14 animals), lymphoid leukemia (2 animals), lung adenocarcinoma (9 animals), hepatoma (4 animals), and fibrosarcoma (3 animals). This spectrum of tumors is similar to that

Figure 2.3 Survival curve of *Nf1*$^{+/n31}$.

Viability of a population with 52 wild-type animals (filled squares) and 40 heterozygotes (open triangles) was monitored for a total of 27 months. Points represent animals that had died or required euthanasia due to ill health. Tumors were observed in 30 heterozygotes and 8 wild-type animals in this population as revealed by necropsy. Tumors found in these heterozygous animals included lymphomas (8), adrenal tumors (9), adenocarcinomas of the lung (8), fibrosarcomas (3), hepatomas (3), leukemias (3), and one neurofibrosarcoma. In addition, two heterozygotes mice not included in this data set also developed adrenal tumors.



seen in older wild-type mice (Bronson, 1990), suggesting that the germline *Nf1* mutation may accelerate the development of tumor types to which these animals are already susceptible. A section through a typical lymphoma from a $Nf1^{+/Nf1^{n31}}$ is shown in Figure 2.4*d*.

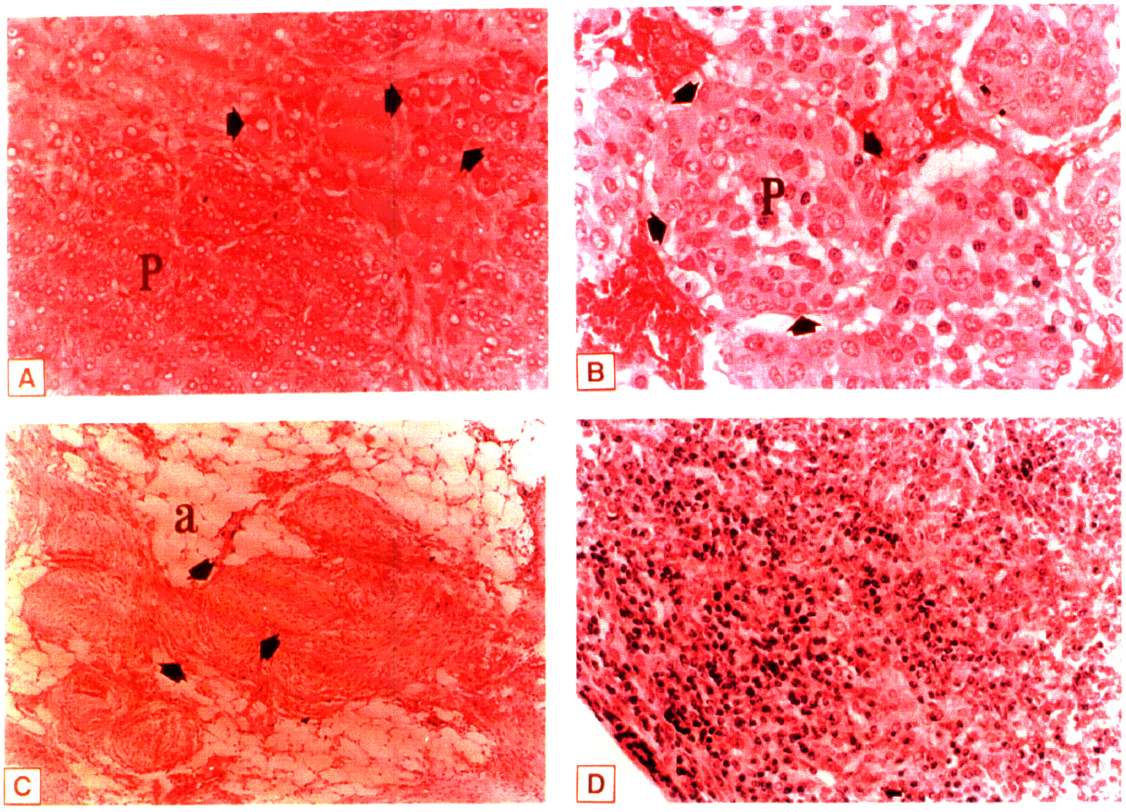
The $Nf1^{+/Nf1^{n31}}$ mice also develop certain tumor types that are characteristic of human NF1. A single heterozygous animal was found to have a neurofibrosarcoma at 21 months of age, and twelve heterozygotes in the total study population developed adrenal tumors at 15 to 28 months of age (Figure 2.3, data not shown). Nine of these adrenal tumors have been classified histologically as pheochromocytoma, with one example of a composite tumor also showing neuronal involvement. In addition, three of these animals developed bilateral pheochromocytomas. Representative sections of these tumors are shown in Figure 4.4. Also, seven heterozygotes ages 17.7 to 27 months developed myeloid leukemia, as evidenced by the overabundance of myeloid cells in the blood and the spleen.

In approximately 10% of human NF1 patients, benign neurofibromas progress into malignant neurofibrosarcomas (Riccardi, 1992). Phaeochromocytoma is diagnosed in approximately 1% of NF1 patients (Riccardi, 1981), and composite pheochromocytomas with areas of ganglioneuroma have been described (Chetty and Duhig, 1993). Pheochromocytoma occurs rarely in mice (Bronson, 1990), and the relatively frequent occurrence of this tumor in the $Nf1^{+/Nf1^{n31}}$ animals (8 of 43) is therefore striking. Indeed, it represents the strongest similarity between the human and mouse phenotypes observed to date. It is also noteworthy that the transformed chromaffin cells of the pheochromocytoma are of neural crest cell origin (Le Douarin, 1982). Moreover, it has been documented that myeloid disorders develop at an abnormally high frequency in children with NF1 (Shannon et al., 1994). Finally, the $Nf1^{+/Nf1^{n31}}$ animals are not apparently predisposed to optic glioma, another tumor type linked to NF1.

Figure 2.4 Histopathology of tumors from Nf1 heterozygous mice.

a, Ganglioneuroma associated with pheochromocytoma. The entire adrenal is replaced by a dimorphic population of cells, including chromaffin medullary cells typical of pheochromocytoma (P) and ganglionic neurons (arrows). *b*, Pheochromocytoma. Polygonal cells (P) forming rounded nodules (arrows) are surrounded by a delicate fibrovascular stroma. *c*, Neurofibrosarcoma found in neck of heterozygote. Note nodules of spindle cells arranged in whorls (arrows), which infiltrate adipose tissue (a). *d*, Follicle center cell type lymphoma in spleen. Sheets of large and small lymphocytes replace normal splenic architecture. Magnifications: *a*, 50x, *b*, 100x, *c*, 25x, and *d*, 100x.

Figure 2.4



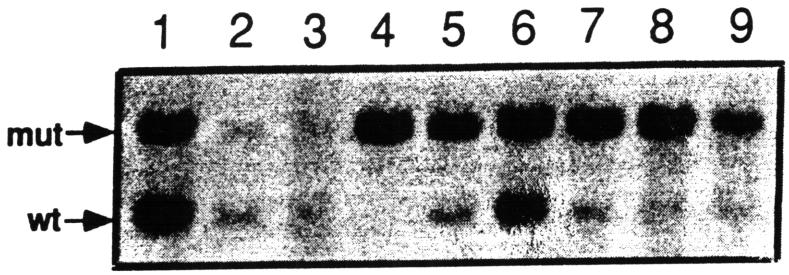
According to Knudson's "two-hit" model for tumor suppressor gene inactivation (Knudson, 1971), tumor development in the *Nf1*$^{+/Nf1^{n31}}$ animals should be accompanied by mutation of the remaining wild-type *Nf1* allele. Therefore, we used Southern blotting to examine the state of the wild-type allele in the DNA of several tumors isolated from heterozygous animals. This assay detects gross mutational events such as large deletion, chromosomal nondisjunction and mitotic recombination. Approximately half of the tumors tested, including all of the pheochromocytomas and all of the myeloid leukemias, showed loss of the wild-type *Nf1* allele (Figure 2.5, data not shown). In addition to the pheochromocytomas, the one lung adenocarcinoma and one of nine lymphomas examined by this assay showed evidence of reduction to homozygosity in the tumor DNA. These data support the view that elimination of residual *Nf1* function can contribute to tumorigenesis. In our experiments, those tumors (such as the one neurofibrosarcoma) that retained the fragment corresponding to the wild-type *Nf1* allele (see Figure 2.5) may have incurred a subtle mutation that was not detectable by our assay. Alternatively, in these tumors, the wild-type allele may have survived intact.

The homozygous phenotype.

To begin to investigate the developmental requirements for *Nf1* function, we intercrossed heterozygous animals and determined the genotypes of the offspring at weaning. Of the first 131 offspring tested, we recorded 39 *Nf1*$^{+/Nf1+}$, 92 *Nf1*$^{+/Nf1^{n31}}$, and 0 *Nf1*$^{n31/Nf1^{n31}}$ animals. This result establishes that *Nf1* function is essential for some aspect of mouse development. The timing of the lethality associated with *Nf1*^{n31} homozygosity was then determined by recovering and genotyping embryos from heterozygous intercrosses at progressively earlier times of gestation. Through 11.5 days post-coitum (dpc), homozygous mutant embryos of normal appearance were recovered at the expected Mendelian value of 25%. Beginning at 12.5 dpc, homozygous mutant embryos were recognizable, being slightly edematous, although all but one

Figure 2.5 Loss-of-heterozygosity (LOH) analysis.

Southern blot analysis of nine representative tumor DNA samples. Note the absence or under-representation of the wild-type allele (wt) in one lung adenocarcinoma (lane 4), two lymphomas (lanes 5-6), and three pheochromocytomas (lanes 7-9). The lymphoma N207 (lane 6) showed only a slight indication of LOH, possibly due to the contamination of surrounding normal cells in the spleen. Tumors showing maintenance of the wild-type allele are a thymic lymphoma (lane 1), a lung adenocarcinoma (lane 2) and a neurofibrosarcoma (lane 3).



of eleven *Nf1*^{n31}/*Nf1*^{n31} embryos recovered at this stage were alive. Approximately 23% (3/13) of the homozygotes recovered at 13.5 dpc were dead upon isolation and the remainder were very edematous and pale (Figure 2.6). No homozygous mutant embryos isolated after day 13.5 dpc were viable. Thus, the lethality associated with homozygosity for the *Nf1*^{n31} mutation occurs between days 12.5 and 14 of gestation.

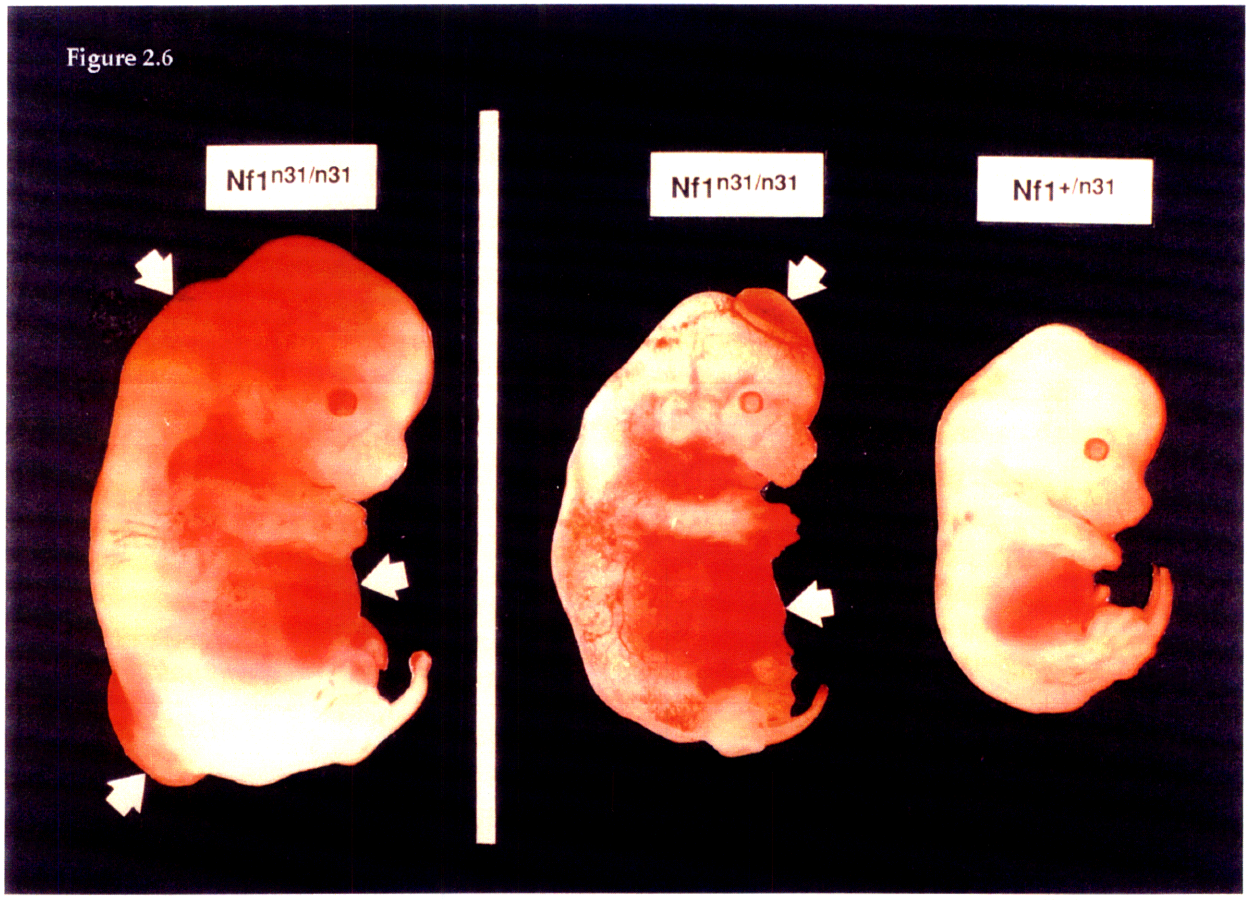
The appearance of the homozygous mutant embryos at day 13.5 of gestation (Figure 2.6) is suggestive of a defect in red blood cell development or circulation. Therefore, we performed gross dissection on several of these animals, concentrating on the liver (the main site of erythropoiesis at this stage of development) and the heart. The livers of the *Nf1*^{n31}/*Nf1*^{n31} embryos were apparently normal (not shown), but the hearts were obviously defective. As shown in Figure 2.7, rather than the proper configuration of the great vessels, with the aorta exiting the left ventricle and the pulmonary artery exiting the right, the hearts of the *Nf1*$^{n31/31}$ embryos had both great vessels emerging from the right ventricle, a condition known as double outlet right ventricle (Figure 2.7*c*, *d*). We have observed this defect in all of the approximately 15 homozygous mutant embryos examined at 13.5 dpc. This is an unexpected finding, since heart abnormalities are not a common feature of human NF1. However, it is known that cells derived from the neural crest contribute to the formation of the outflow tracts of the heart (Kirby et al., 1983). Also, Huynh et al. (1994) have recently reported high levels of neurofibromin expression in the developing mouse heart coincident with the development of the abnormalities observed here.

To further investigate this heart abnormality and to uncover any other pathology, we next analyzed embryos histologically. Homozygous mutant embryos and littermate controls were serially cross sectioned at 6 μ, stained with hematoxylin-eosin, and examined by light microscopy. Given the neuronal manifestations of NF1 and the restricted neuronal expression of neurofibromin at later stages of gestation, we paid particular attention to the developing central

Figure 2.6 Appearance of $Nf1^{n31}/Nf1^{n31}$ embryos at 13.5 days of gestation.

Two typical mutant embryos at 13.5 dpc (left and center) compared with a control $Nf1^{+}/Nf1^{n31}$ embryo (right). Note the generalized edema, particularly in the pericardial space and in various regions below the epidermis (arrows). The two embryos on the right were derived from the same litter; the photograph of the left embryo was taken at a higher magnification.

Figure 2.6



and peripheral nervous system as well as other structures to which neural crest cells contribute (e.g., thymus, thyroid, parathyroid, cranio-facial structures). However, with the exception of the heart (see below), all organs and tissues of the *Nf1*^{n31}/*Nf1*^{n31} embryos were comparable to the controls. A section showing the normal appearance of the spinal cord and a dorsal root ganglion of a mutant embryo is included in Figure 2.7 (*a*, *b*).

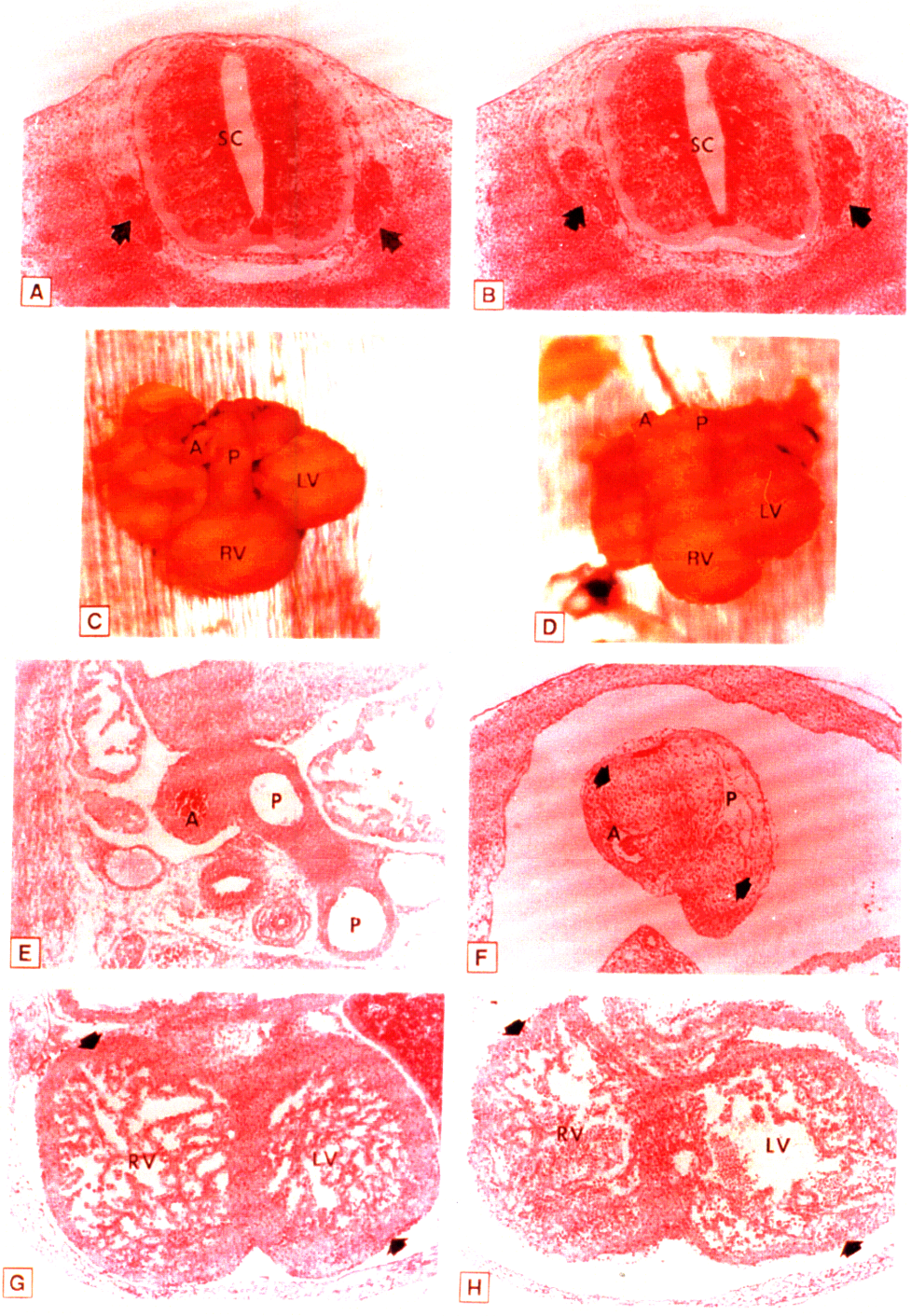
Histological analysis of the mutant hearts confirmed the presence of double outlet right ventricle. In particular, serial cross sections indicated that the truncus arteriosus (the single outflow vessel that gives rise to the aorta and pulmonary artery) was septated into two outflow tracts both of which connected through the right ventricle (Figure 2.7*d*, *f*). In addition, the cells lining the aorta and pulmonary artery were decidedly more abundant in the hearts of *Nf1*^{n31}/*Nf1*^{n31} mutants (Figure 2.7*e*, *f*). This increased cell density results in a partial occlusion of the great vessels and could account for the reduced blood flow, edema, and ultimate death of the mutant embryos. The pericardial sacs of the homozygous mutants were also distended (Figure 2.7*f*), presumably because of the increased fluid in the pericardial space. Finally, in most of the *Nf1*^{n31}/*Nf1*^{n31} embryos examined, the myocardium, particularly of the ventricles, was lacy in appearance and thinner than normal (Figure 2.7*g*, *h*). This could be a primary consequence of the *Nf1* mutation or secondary to the other defects in cardiac development.

In addition to noting the cardiac abnormalities, Brannan et al. (1993) have recently described other developmental phenotypes in embryos homozygous for a similar targeted disruption of *Nf1*. One such phenotype involved the hyperplasia of splanctic ganglia in 13.5 g.d. embryos. We did not observe these defects in our *Nf1*^{n31}/*Nf1*^{n31} embryos, which could be due to subtle differences in the mutant alleles, or the timing at which embryological and histological examinations were performed.

Figure 2.7 Histopathology of *Nf1*^{n31}/*Nf1*^{n31} mutant embryos.

All heterozygous or wild-type control embryos (*a*, *c*, *e*, *g*) and homozygous mutant embryos (*b*, *d*, *f*, *h*) were isolated at 13.5 dpc. *a*, *b*, Cross sections through the lumbar region show normal development of the spinal cord (SC) and dorsal root ganglia (arrows). *c*, *d*, Intact hearts isolated from embryos fixed in Bouin's solution. *c*, The normal heart shows the pulmonary artery (P) and aorta (A) exiting the right (RV) and left (LV) ventricles, respectively. *d*, In contrast, the mutant heart has a single, large vessel exiting the right ventricle. Histological analysis reveals that this vessel is septated into the aorta and pulmonary artery. *e*, *f*, Cross sections through the outflow tracts. Note the large, well-structured lumen of the aorta (A) (filled with blood) and pulmonary artery (P) of the control heart, *e*, as compared to the restricted lumenal space in the mutant, *f*. Arrows point to the overabundant cells lining the outflow tracts in the mutant heart. *g*, *h*, Cross sections through the ventricles of the hearts. Left (LV) and right (RV) ventricles are shown. Note the thinner myocardium in the mutant heart (arrows).

Figure 2.7



Discussion

Although predisposed to malignancy, mice heterozygous for the *Nf1*^{n31} mutation do not develop the hallmark features of human NF1, including neurofibromas, Lisch nodules, or pigmentation defects. Therefore, as with mice mutant for *Rb* (Xu et al., 1992; Jacks et al., 1992; Lee et al., 1992) or *HPRT* (Clarke et al., 1992; Hooper et al., 1987) genes, the *Nf1*-mutant animals do not serve as an accurate model of the cognate human genetic disease. Several factors could account for this difference in phenotype. For example, the various symptoms of human NF1 may all require the somatic inactivation of the wild-type *NF1* allele. Perhaps due to the reduced number of target cells in the mouse, their more rapid development, or an intrinsically lower mutation rate, the wild-type *Nf1* allele is not inactivated at a sufficiently high rate in the relevant cells of the *Nf1*$^{+/}$*Nf1*^{n31} animals. Alternatively, the regulatory mechanisms controlling the growth and differentiation of these cells in the mouse may be coordinated differently than in the corresponding cells in humans.

The phenotypic expression of *NF1* mutations in humans is highly variable, such that the same mutation can cause a distinct array of symptoms in different individuals. Pedigree analysis by Easton et al. (1993) has indicated that the NF1 phenotype is strongly affected by unlinked modifier genes. Therefore, it will be important to cross the *Nf1*^{n31} mutation onto several different genetic backgrounds. In addition, Jay and co-workers (Easton et al., 1993; Hinrichs et al., 1987) have previously described a transgenic mouse line expressing the HTLV-1 *tat* gene that develops various lesions reminiscent of human NF1. Intercrossing this transgenic line with the *Nf1* mutation described here could potentially accentuate this phenotype.

Based on our failure to detect mutant neurofibromin using antisera which recognize two different epitopes on the protein, we believe that the targeted mutation results in destabilization of the protein, and therefore, acts as a null. The instability of the protein could result from the deletion

of exon 31 encoded sequences, insertion of amino acids encoded by the non-coding strand of *neo*, or both. It is also noteworthy that the splice acceptor and donor sites located on the noncoding strand of *neo* used in the production of the $Nf1^{n31}$ mRNA species closely match the different consensus sequences and are used in this context quite efficiently. This, coupled with the fact that the neo sequences located between these sites constitutes an open reading frame, should be considered by others using this general strategy for making mutations by gene targeting.

The acceleration of tumorigenesis in the $Nf1^{+/}Nf1^{n31}$ animals reported here, along with the high frequency of mutation of the wild-type *Nf1* allele, is perhaps the best evidence to date that this gene functions in negative growth regulation and tumor suppression. Since the heterozygous animals develop a wide range of tumors, it appears that the elimination of neurofibromin function can promote transformation of a variety of cell types, perhaps through changes in ras signaling. Mutations in the human *NF1* gene have also been found in a variety of tumor types, including neurofibrosarcoma, astrocytoma, neuroblastoma, melanoma, and colon carcinoma (Legius et al., 1993; Li et al., 1992; Andersen et al., 1993; Johnson et al., 1993).

The most striking aspect of the heterozygous phenotype in the mouse is the relatively high incidence of pheochromocytoma, a tumor of the adrenal medulla consisting of cells of neural crest origin, in mixed background animals (C57BL/6 x 129sv). Indeed, predispositions to pheochromocytoma (mixed background animals) and myeloid leukemia (seen only in 129sv inbred strains) are the closest phenotypic similarity between the $Nf1^{+/}Nf1^{n31}$ mice and human NF1 patients. In addition, from a total of eight pheochromocytomas and seven myeloid leukemias examined from these animals, we have observed the absence of the wild-type *Nf1* allele in the tumor DNA in each case. Pheochromocytomas from human NF1 patients also show loss of heterozygosity on chromosome 17 (Green et al., 1992), and in the three informative cases in this study the markers from the chromosome 17 carrying the presumed wild-type allele of *NF1*

were lost during tumorigenesis. Furthermore, children with NF1 also develop malignant myeloid disorders at an increased frequency, and approximately half of these show loss of heterozygosity for markers in and around the *NF1* locus (Riccardi, 1981).

The strain-specific tumor spectrum in the *Nf1* mouse model suggest the presence of modifier genes which influence the development of particular tumor types in mice. Myeloid leukemia were found exclusively in the 129sv inbred mouse strain, while pheochromocytomas were seen only in mixed background mice. Given the carefully-controlled environmental conditions under which our study population was kept, it is likely that genetic (rather than environmental) variability contributed toward the disparity in phenotype. Modifier genes have been proposed to modulate tumor phenotype in a variety of tumors, and cloning of such genes have been demonstrated in the case of *Mom-1*, a modifier of the *Min* (multiple intestinal neoplasia) gene (Dietrich et al., 1993) in mice. More recently, the phospholipase A2 gene was suggested as the actual modifier gene residing in the *Mom-1* locus (MacPhee et al., 1995). Modifier genes present in various human genetic backgrounds may indeed explain the variable expressivity across the human *NF1* population.

Homozygosity for the $Nf1^{n31}$ mutation leads to mid-gestational embryonic lethality with associated defects in cardiac development. Neural crest cell lineage mapping using chick/quail chimeras has shown that cardiac neural crest cells contribute to the formation of the outflow tracts of the heart (Kirby et al., 1983). Laser ablation of this population of neural crest cells in the chick leads to cardiac abnormalities very similar to those described here (Kirby and Waldo, 1990). In addition, homozygosity for the *Splotch* mutation in the *Pax-3* gene (Epstein et al., 1991) leads to defects in several neural crest cell-derived structures, including the septation of the truncus arteriosus (Franz, 1989). Thus, it is likely that the abnormal cardiac development caused by homozygous mutation of the *Nf1* gene results from either a failure of the cardiac neural crest cells to properly migrate to the heart or to function normally once there.

Interestingly, one of the two known alternatively spliced forms of *NF1* mRNA is restricted to skeletal and cardiac muscle (Gutmann et al., 1993), and there is a dramatic rise in neurofibromin expression in the heart on day 12 of mouse development (Huynh et al., 1994). In addition, a variant form of NF1 known as Watson's syndrome includes the cardiac defect pulmonary valvular stenosis (Tassabehji et al., 1993). The connection between the homozygous mutant phenotype described here and this rare manifestation of NF1 is unclear at present, but it is interesting that a recently described allele of *NF1* from a patient with Watson's syndrome showed an in-frame duplication in exon 28 (Tassabehji et al., 1993). It is possible that this mutant protein could act in a dominant negative fashion leading to partial functional inactivation of wild-type neurofibromin in the developing heart.

We have also noted an overabundance of cells lining the outflow tracts of the homozygous mutant embryos. Given the other associations between neurofibromin function and proliferation/differentiation of neural crest cells, it is tempting to speculate that *Nf1*-deficient cardiac neural crest cells do migrate to the heart but fail to differentiate properly and continue to proliferate. In this way, the growth properties of these cells may provide a model for the various pathological effects in the human disease. We expect that the study of this phenotype as well as the tumor development in heterozygous mice will provide important insights into the pathogenesis of human NF1.

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Chapter 3

Nf1 Double Knock-out Chimeras: Characterization of a Murine Neurofibroma Model

Introduction

The hallmark lesions associated with the human NF1 condition includes the development of benign neurofibromas, Lisch nodules and cafe-au-lait spots. In fact, the presentation of a certain combination of such lesions is usually considered diagnostic of the NF1 disease. The cell types involved in all these lesions have a common, neural crest origin, namely the peripheral nerve sheath, iris and skin melanocytes (Bolande, 1974). Of these lesions, the neurofibroma poses the biggest threat to the patient, since it is precisely the progression of these benign growths (into neurofibrosarcomas, or more recently categorized as a type of malignant peripheral nerve sheath tumors, MPNSTs (reviewed in Fletcher CDM, 1995)) that results in nerve compression, pain, and even death. In fact, treatments for NF1 often focus on the removal or shrinkage of neurofibromas and their malignant outgrowths.

Neurofibromas arise under the skin (subcutaneous) or in the deep plexus of the peripheral nerve trunks (plexiform). It is also widely accepted that neurofibromas are composed predominantly of neurons, Schwann cells, perineurial cells and fibroblasts. A subset of the tumors also include mast cells, although the role such cells play in the formation of neurofibromas is still unknown (Lascano EF, 1958; Johnson et al, 1989).

Unfortunately, the etiology of these neurofibromas has also remained elusive to researchers. There have been long-standing disagreements among pathologists as to the cell types involved, and the role in which each one plays in tumor formation. For example, a survey of the literature describing the composition of plexiform neurofibromas show a wide range of findings, from predominantly Schwannian (as shown by the positive staining with S-100, MBP or P0 antibodies) to predominantly perineurial/fibroblastic (staining positive for EMA and/or negative with S-100) (reviewed in Riccardi, 1992). Some argue that the lack of S-100 protein expression in neurofibromas resulted from the inability of proliferating Schwann cells to produce such cell-

specific markers. Others suggest that Schwann cells, perineurial cells as well as fibroblasts represent distinct differentiation states from a common precursor, and that the hyperplastic cell type in neurofibromas represents an intermediary differentiation state (Hirose et al, 1986). Electron microscopy has been used in an attempt to address this question, but has done little to resolve these issues.

Consistent with the tumor suppressor hypothesis, loss-of-heterozygosity (LOH) at the *NF1* locus has been documented in various hyperplastic/tumor tissues from human patients. However, whereas the malignant growths show a high incidence of *NF1* LOH, benign neurofibromas have rarely shown deficiency in *NF1* function (either by mutational analysis or protein detection) (Huson, 1994). A popular hypothesis to explain this discrepancy assumes that a single cell type in a peripheral nerve trunk initially incurs LOH at the *NF1* locus, and begins to proliferate. Alternatively, this *NF1*-deficient cell could start to secrete some factor to induce other cells to grow, be it in an autocrine (same cell type) or paracrine (other cell types) fashion. Unless the growth is clonal from the *NF1* -/- cell, it would be very difficult to detect the LOH in a small subset of mutant cells using PCR or Southern blot analysis.

Given the prevalence of NF1 (one in 3,500 (Stumpf, 1987)) and the unanswered questions on tumor etiology, we decided to address these issues by expanding upon the mouse model of Nf1 generated in our lab (Jacks et al., 1994). The previous *Nf1* mouse model proved to be an insufficient model due to the early embryonic lethality resulting from *Nf1* deficiency and prolonged tumor latency in the heterozygous cases (Jacks et al., 1994). Instead, we wished to generate a model to study the effect of *Nf1*-deficiency in the context of adult animals. The particular approach that would allow us to artificially create such a model is to generate double knock-out (DKO) chimeras, where *Nf1* -/- embryonic stem (ES) cells are injected into normal blastocysts. The resulting chimeric animals would be partially composed of *Nf1* +/+ and mutant cells (Figure 3.1c). First, the DKO chimera generation approach has allowed researchers to bypass the embryonic

lethality phenotype by limiting the contribution of the mutant ES cells to certain tissue types, as in the case of the retinoblastoma (Rb) gene (Maandag et al., 1994; Williams et al., 1994). Second, if the *Nf1* -/- ES cells differentiate into cell types critical to the formation of neurofibromas (or other diagnostic NF1 lesions), we would be able to (1) suggest a role of *Nf1*-deficiency in the formation of these lesions and (2) identify the cell types that are *Nf1*-deficient in these benign growths. The latter question could be addressed by engineering appropriate molecular markers into the injected mutant ES cells.

One such marker is the *neo* gene inserted as a part of the *Nf1* mutant allele construct. This single copy transgene can be specifically amplified by *in situ* PCR . Alternatively, a radio-labeled riboprobe against *neo* can be used to detect the mRNA produced by the *Nf1-neo* mutant allele, which contains a stretch of the *neo* gene sequence (Jacks et al., 1994). Another ES cell marker is the reverse orientation splice acceptor (*ROSA*) transgene developed by Friedrich and Soriano (1991) as a part of a screen to identify developmentally-regulated genes in mouse embryos. Strains of *ROSA* mice were generated by electroporation of promoterless reporter constructs consisting of fusion β-*gal* and *neo* genes (β-*geo*) into ES cells. Such strains can be categorized into two groups: some strains express the β-*geo* gene product in a spatially- and temporally-regulated fashion, while others show ubiquitous expression throughout development. For our purposes, we used the used the *ROSA-26* strain, which was shown to express strongly and ubiquitously the transgene (Friedrich and Soriano, 1991). Equally important was the observation that mice homozygous for this single-copy insertion showed no phenotypic effect. By introducing the *ROSA-26* transgene into *Nf1* DKO ES cells, we were hoping to provide a specific marker for cells derived from these injected clones, regardless of tissue type. The presence of the β-*geo* transgene can be easily detected by staining the tissues of interest with X-gal. Chimeric tissue sections were stained with X-gal in order to further analyze, at the cellular level, the contribution of *Nf1*-/- ES cells to various tissues and organs. Previous studies have reported the X-gal staining patterns for various tissues from *ROSA-26* animals (Friedrich and Soriano, 1991; Williams

dissertation, 1996). It was found that even though the β-*geo* expression was found in almost all cell types examined, the subcellular localization of the β-galactosidase protein varied widely among cell types. The X-gal staining patterns also differed depending on the type of tissue preparation and staining protocols used. Given the likely localization of Nf1-associated lesions to the peripheral nervous system, we focused our efforts on optimizing the staining pattern analysis for various neuronal tissue sections.

About twenty *Nf1* DKO chimeras were generated using both *ROSA-26* and non-*ROSA*-tagged ES cells. Pathology was performed on animals showing signs of wasting or other phenotypes. A subset of animals with moderate contribution from the *Nf1*-deficient ES cells grew increasingly weak with neuro-motor defects. It was within this population of animals where we found the presence of lesions reminiscent of human neurofibromas as well as myeloid dysplasia. The murine neurofibromas were characterized using immunohistochemistry, electron microscopy and, where available, X-gal staining.

Methods

Generation of *Nf1*-DKO ES Cells

Re-targeting of wild-type allele in heterozygous cells

ES cells heterozygous for the *Nf1* mutation (Jacks et al., 1994) were electroporated with an *Nf1*-targeting vector containing a hygromycin resistance gene (*hygro*) in place of the neomycin resistance gene (*neo*) of the original construct, as described in Jacks et al., 1994. The *hygro* gene is driven by the *PGK* promoter and polyadenylation sequences. Conditions for the *hygro* vector electroporation were identical to the first round of *neo* targeting steps (Jacks et al., 1994). Following electroporation, the cells were plated either in the absence of feeders or in the presence of G418-resistant feeder cells (courtesy of R. Jaenisch), and subsequently selected with 300 μg/ml G418, 125 μg/ml hygromycin and 2 mM gancyclovir (Figure 3.1a). Colonies were picked and expanded for DNA analysis. Confirmed *Nf1* DKO (-/-) ES cell lines were chosen for blastocyst injection.

Increasing drug selective pressure

An alternative protocol for obtaining *Nf1* -/- ES cells was also used (Mortensen, 1992). *Nf1* +/- ES cells were incubated in selection media containing higher levels of G418 (300 μg/ml, 700 μg/ml, 800 μg/ml, 900 μg/ml, 1.1 mg/ml and 1.3 mg/ml). Ten thousand ES cells were used for each ten centimeter plate, and three plates were used per condition. The cells were selected at the lowest selection concentration for one day (300 mg/ml of G418). All plates were pretreated with gelatin and layered with drug-resistant feeder cells. Selection began on the second day and fresh media was added daily. The increased selective pressure may potentially lead to the duplication of the drug resistance gene, be it from nondisjunction, non-reciprocal recombination or chromosomal loss events. Colonies were picked from plates where significant selection has occurred (judged by significantly reduced number of colonies in the plate).

Generation of *ROSA-26* DKO ES Cells

Genotyping of *ROSA-26* animals

ROSA-26 animals were genotyped by colorimetric and PCR methods. Small sections of the ear were removed with an ear punch and placed in a staining solution at 37°C for 2-8 hours. The solution reacts with the β-galactosidase enzyme (present in most tissues of *ROSA-26* animals) to turn the ear tissue blue, identifying the animal as *ROSA-26* (Friedrich & Soriano, 1991). The PCR assay was performed on precipitated tail DNA as described previously using the following primers (ROSA-F=5'-TTTCCACAGCTCGCGGTTG-3' and ROSA-R=5'-GATCCGCCATGTCACAGA-3'). The conditions for the PCR reaction are as follows: 94°C for 3 minutes, followed by 30 cycles of 94°C for 1 minute, 62°C for 2 minutes and 72°C for 2 minutes. It must be noted that the two assays described here only detects the presence of the ROSA-26 single-copy transgene, but do not elucidate whether the animal carries one or two copies of the gene (heterozygous and homozygous for *ROSA-26*, respectively).

Ovariectomization and delayed blastocyst harvest

Timed matings were performed between a 129SV inbred *Nf1* +/- animal and an F1 hybrid (129SV x C57BL/6) *Nf1* +/- animal. At least one of the parents was previously identified as a carrier for the *ROSA-26* transgene. The ovariectomy procedure was performed on the female 2.5 days after the detection of a vaginal plug (equated to day 0.5). In addition, the female is injected with 0.1 ml of 10 mg/ml Depa-Provera subcutaneously in order to inhibit the implantation of the blastocyst into the uterine wall, creating "delayed blastocysts" (Bradley, 1987). At 6.5 days after the vaginal plug, the females are sacrificed and the uterine horns flushed with blastocyst media. Delayed blastocysts (having lost the zona pellucida) are then collected and individually placed over feeder layers in 24-well wells (previously coated with 0.1% gelatin) and fed with ES cell media. The feeder layer is plated 1-2 days prior at the density of $1x10^6$ cells per well. The feeder layers are derived from murine embryonic fibroblast cell lines and irradiated with 6 Grays (Gy) of ionizing radiation to stop cell proliferation. After 3-4 days in culture (or until the delayed blastocysts have

attached to the feeder layer and flattened into a colony of cells grown out from the inner cell mass (ICM)), the well is washed with sterile PBS and the colony is picked using a sterile plastic pipette tip. The cells are then placed into approximately 50 ml of ES-grade trypsin (Robertson, 1987) for 5-10 minutes at 37˚C, after which the cells are gently disaggregated into single cell suspension by pipetting action. The suspension is then placed in gelatinized 24-well wells plated with ES feeder 1-2 days earlier. Over the next 5-10 days, the wells are monitored for the appearance of ES cell colonies. Once the ES colonies have grown to the appropriate size and morphology, they are removed, disaggregated (as described above) and expanded over ES feeders and gelatin. The ES cells over feeder are kept undifferentiated and are subsequently frozen back in ES freezing media (Robertson, 1987) and stored at -80˚C or in a liquid nitrogen freezer. DNA is made from ES cells grown on gelatin-only plates in order to determine the genotype of the ES cells.

Identification of appropriate clones

ES cell lines are genotyped for *Nf1* status by Southern analysis as described in previous chapters. In addition, the presence of the Y chromosome-specific *Zfy* gene was determined using a PCR-based assay (Page et al., 1987). The cell line is determined to be male by the presence of a *Zfy*-specific band, while the absence of such a band suggested that the cell line was female. ES cell lines that are *Nf1*-heterozygous, *ROSA-26* and *Zfy* positive are then re-targeted with the *Nf1-hygro* vector to disrupt the wild-type allele of *Nf1* (as described above).

Preparation of ES cells for blastocyst injection

Nf1 DKO ES cell clones were thawed onto 6-well plates coated with either gelatin or layered with ES feeders 1-4 days prior to the scheduled injection date. On the day of injection, the ES cells were disaggregated into single-cell suspension using trypsin, spun into a pellet at 1000 rpm, and resuspended in blastocyst media (Robertson, 1987) at a concentration of approximately 60,000 cells per ml.

Generation of *Nf1*-DKO mice

Murine chimera generation techniques were used as described in Robertson, 1987 and Bradley, 1987.

Blastocyst isolation

Female mice were prepared in one of two ways for blastocyst isolation. One, C57BL/6 females over 7 weeks of age were mated to males of identical strain. Pregnant females (identified by the detection of vaginal plugs) were sacrificed three days after mating and blastocysts were then harvested by flushing the uterus with blastocyst media. The second method involved superovulating C57BL/6 females over 3 weeks of age (but less than 5 weeks old): First, the females received 5 IU of pregnant mare serum (PMS) intraperitoneally (IP), followed forty-eight hours later by IP injection of human chorionic gonadotrophin (HCG) and subsequent housing with C57BL/6 males. Blastocyst harvest procedures were identical to the above for females in which vaginal plugs were detected on the following day (Figure 3.1c).

For *Nf1* +/- blastocyst harvest, *Nf1* +/- C57BL/6 males were mated naturally to wild-type females of the same strain. Blastocyst collection procedur5 above.

Blastocyst injection and Implantation

The injection and implantation procedures were described by Bradley, 1987. Each blastocyst was injected with between 2-5 ES cells. Typically, between 5-10 blastocysts were implanted into each uterine horn of pseudopregnant Swiss Webster female mice (Taconic Farms).

Assaying for ES cell contribution

Nf1 -/- ES cell contribution was assayed in two ways: First, agouti coat color chimerism was estimated by observation. Second, glucose phosphate isomerase enzymatic isoform analysis was performed on various tissues from the chimeric mice (Robertson, 1987).

Analysis of chimeras generated from heterozygous blastocysts

Tail DNA from potential *Nf1* DKO chimeras was prepared with proteinase K digestion and subsequently purified using phenol-chloroform extraction. 15 μg of DNA was digested with *NcoI* and *HindIII* for Southern Blot analysis. The probe used was an *NaeI-PstI* (449 basepair) fragment of the *NEO* gene from the *pMCI-neo* vector. This *NEO* fragment was first cloned into the *pBS-KS* vector (Stratagene). The probe was isolated for radio-labeling by digesting the *pBS-KS* vector with *PstI* and *XhoI*.

Histology and β-gal staining

Embryonic and neonatal: Embryos prior to mid-gestation (13.5 g.d.) were extracted from euthanized females, washed once in pH 7.3 buffer and fixed with 10% NBF, Bouins fixative or 4% paraformaldehyde for 30 minutes up to one hour (depending on size). Larger embryos and neonates required exposure of the cranium and abdomen prior to fixation to ensure penetration of the fixative. The animals were then washed in the buffer for three times (15 minutes each) and then placed into β-gal staining solution (described above) for overnight to a maximum of a week at room temperature (changing to fresh solution once every two days). The embryos were then photographed under dissecting microscopes for staining patterns.

Adult: The animals were euthanized using CO_2 asphyxiation. The mice were fixed by either intracardial perfusion (Bronson & Lipman, 1991) or whole-mount fixation after the tissues were dissected. The tissues were carried through the β-gal staining protocol identical to that for embryos and neonates. The tissues were sliced as thinly as possible to assure stain penetration, either with a sharp razor blade or with vibratome.

Frozen Section β-gal staining: Adult chimeras were dissected and desired tissues were isolated in sections no larger than 1 cm^3. These tissues were then fixed in 4% paraformaldehyde at 4°C for 30 minutes. The sections were then washed in cold PBS (4°C) for 30-45 minutes using three changes of fresh PBS. After the washes, the sections were placed in 30% (weight/volume) sucrose in PBS for 18 hours or until the tissues have equilibrated and no longer float in the sucrose solution (this equilibration step was performed unless otherwise noted). The sections were then immersed in OCT (Tissue-Tek) media and quickly frozen by dipping into a beaker of isopentane cooled by liquid nitrogen. Frozen sections between 5-10 microns were then cut using a cryostat and stored in -80°C. Immediately prior to staining, the sections were thawed and post-fixed briefly with 4% paraformaldehyde (in PBS), followed by two washes of PBS for 5 minutes each. Sections were then incubated in X-gal staining solution between overnight to a week (depending on the time required for the X-gal crystals to form) in a light-protected humidified chamber. X-gal stain contained the following: (PBS with 1 mg/ml 4-chloro-5-bromo-3-indolyl-β-galactoside (X-gal), 4 mM potassium ferrocyaniade, 4 mM potassium ferricyanide, and 2 mM magnesium chloride).

Blood smears

A drop of blood from the mouse was placed on a glass histological slide. An edge from a clean glass slide was used to apply even pressure in spreading the blood into a mono-layer. After drying for at least thirty minutes, the slide was covered with Wright stain (Sigma Diagnostics) for 3 minutes and then Giemsa stain (Sigma Diagnostics, diluted 1:1 with ddH_2O) for 8 minutes. The slide was subsequently washed with ddH_2O, let dry and cover-slipped with permount. The number and type of blood cells are then counted by taking the average number of cells of 40X magnification

Immunohistochemistry

Immunohistochemistry protocols were essentially as described in Williams et al., 1994. Incubation buffer comprise of 10% normal goat serum (Sigma), 0.05% Tween 20 and 0.02% sodium azide. Antibodies against Neurofilament 200 (Dako, 1:200 dilution in buffer), S-100 (Dako, 1:200), vimentin (courtesy of R. Hynes, 1;100), myelin basic protein (MBP) (Dako, 1:200), epithelial membrane antigen (EMA) (ICN, 1:75 and Dako, 1:200), human milk fat globulin protein (Novocastra, 1:50) and anti-coagulation factor XIIIA (Calbiochem, 1:500) were used. Appropriate secondary antibodies were used against the primary antibodies, including biotinylated anti-mouse (Jackson ImmunoResearch Labs, 1:200), FITC goat anti-rabbit (Vector, 1:100) and biotinylated goat anti-rabbit (Jackson, 1:200). Mast cells were visualized in paraffin sections by staining with 0.5% toluidine blue for up to 10 minutes.

Antigen retrieval/exposure techniques included microwave heating as described by Tischler, 1995 or 15-30 minute digestion with diluted trypsin (0.05%, Sigma type II) in 0.05% calcium chloride at pH 7.8.

Transmission Electron Microscopy

Tissues were prepared for EM in one of two ways. Freshly-dissected tissues were fixed in 4% paraformaldehyde and 2% EM-grade glutaraldehyde (Ted Pella, Inc.) for one hour or longer (depending on the size of the tissue). For tissues that were already paraffin-embedded, the paraffin block was first melted, the tissue re-hydrated to PBS, followed by at least one hour of fixation with 4% paraformaldehyde and 2% glutaraldehyde. If decalcification of bone is required, tissues are then placed into 0.5 M EDTA at 4°C for two weeks prior to sectioning. After proper fixation and necessary decalcification, the tissues are washed thoroughly with three changes of 0.1 M cacodylate buffer (Hayat, 1970), and can be subsequently stored in the 0.1 M cacodylate buffer at 4°C until the next step. This is followed by perfusing the tissues with osmium tetroxide (OsO_4, in 0.1 M cacodylate buffer) for 1 hour on ice, then incubated overnight in cold 0.1 M cacodylate buffer). The sections are then taken through a series of dehydration and staining steps including

50% ethanol for 10 minutes, 2% aqueous uranyl acetate in 70% ethanol for one hour (4°C in the dark), 90% ethanol for 10 minutes, followed by 100% ethanol for three changes of 10 minutes each. The sections were then washed with propylene oxide for 20 minutes (change of fresh solution after the first 10 minutes). The embedding medium used for all EM work was Spurr resin: First, the tissues were placed in a small glass jar with 1:1 resin:propylene oxide and rotated overnight at room temperature. Second, the tissues were transferred to freshly-made resin in embedding capsules and allowed to polymerize at 70°C for 24 hours. Semi-thin sections were cut and stained (1% methylene blue and 1% azure II in 1% borate) to identify the histological regions of interest. Ultrathin sections were then cut on Leica Ultracut S at 60 to 90 nm, embedded in 200 mesh copper EM grids, and stained with 4% uranyl acetate and Reynold's lead citrate. EM images were captured on Kodak EM Film 4489 using a JEOL 1200 EX electron microscope set at 80.0 KV.

In situ PCR

Histologically slides were deparaffinized and hydrated as described for immunohistochemistry, followed by 5 minute washes with 0.1 M Tris (pH 7.4) and then 0.3% Tween20 and 0.5% NP40 in 0.1 M Tris pH 7.4. The sections were then permeabilized with trypsin (2 mg/ml) for one hour at room temperature, followed by 2 washes with 0.1 M Tris 7.4 for 5 minutes each. The slides were then incubated in the appropriate PCR buffer, cover-slipped and sealed with nail polish (PCR buffer consist of 10 mM Tris pH 8.4, 50 mM KCl, 4 mM $MgCl_2$, 0.01% gelatin, 0.05% Tween-20, 0.05% NP40, 100 pmol of each of the Nf1 mutant allele PCR primers NfX4I and neo (Jacks et al., 1994), 62.5 mM of dATP, dCTP, dGTP, and dTTP-biotin and 5 units of AmpliTaq). The slides were then placed inside a HybAid OmniSlide thermocycler pre-heated to 80°C (hot-start). The PCR cycling times are: 92°C for 3 minutes, followed by 40 cycles of 92°C for 1 minute, 55°C for 2 minutes and 75°C for 2 minutes. Sections incubated in PCR buffer without primers served as negative controls for these experiments. Incorporated biotin-conjugated dTTP was visualized with an avidin and biotinylated horseradish peroxidase complex (Vectastain ABC kit). Finally, the

sections were counter-stained with either methyl green or nuclear fast red prior to observations using the microscope.

In situ hybridization

A *PstI-PstI* 200 bp fragment of the *NEO* gene from *pMC1-neo* (Stratagene) was cloned into *pBS-SK*. The sense *in situ* riboprobe against neo was made using the T3 promoter after linearization of the vector with *HindIII*, while the anti-sense probe was made using the T7 promoter after linearizing with *BamH1*. The *in situ* protocol is essentially as described in McClatchey et al., 1997.

Results

Generation of *Nf1* DKO chimeras

ES cell clones heterozygous for the *Nf1* mutation (Jacks et al., 1994) were re-targeted with the *Nf1-hygro* vector (Figure 3.1a). Electroporated clones were genotyped by Southern blot analysis. ES cell lines that included both the *Nf1-neo* and *Nf1-hygro* mutant alleles were selected for blastocyst injection. Two of the 63 ES cell lines established were shown to be *Nf1* -/- (Nfdko6 and Nfdko48). Figure 3.1c graphically depicts the process of generating the DKO ES cells and the subsequent *Nf1* DKO chimeras.

Initially, 5-7 ES cells were injected into each blastocyst. It proved exceedingly difficult to generate adult chimeras, and the yield of pups born per recipient female (versus the number of blastocysts implanted) was consistently low. This suggested that the contribution of *Nf1* -/- ES cells to certain developing tissues resulted in reduced fitness of these embryos *in utero*. To address this question, embryos were taken at various stages of gestation and the level of ES cell contribution was determined using GPI analysis (Figure 3.2). Table 3.1 summarizes the results of that set of experiments.

Table 3.1 Developmental bottlenecks associated with *Nf1* deficiency as seen by high mid-gestational and embryonic lethality in the *Nf1*-DKO chimeric population.

Age	Total	Chimeric	% Chimeric
13.5 g.d.	18	11	61%
15.5 g.d.	22	4	18%
neonates (d)*	42	10	24%

*Dead neonates recovered from litters within 2 days after birth

The results suggested that a percentage of chimeric embryos were not progressing beyond the 13.5 g.d. mid-gestational stage. We speculated that these embryos were dying from the same cardiac defects as the *Nf1*-mutant embryos (Jacks et al., 1994). By examining the developing hearts of

Figure 3.1. Schematic and Southern blot analysis for DKO ES cell generation

a. Two round targeting protocol to inactivate both *Nf1* alleles in ES cells. The *neo* and *hygro* selectable markers were used for each targeting construct. The Southern probe used is indicated by (Pr). b. Southern analysis showing the loss of the wild-type *Nf1* allele in NfDKO6, NR30 and NR47. c. Schematic diagram describing the sequence of events resulting in the generation of DKO chimeras.

Figure 3.1

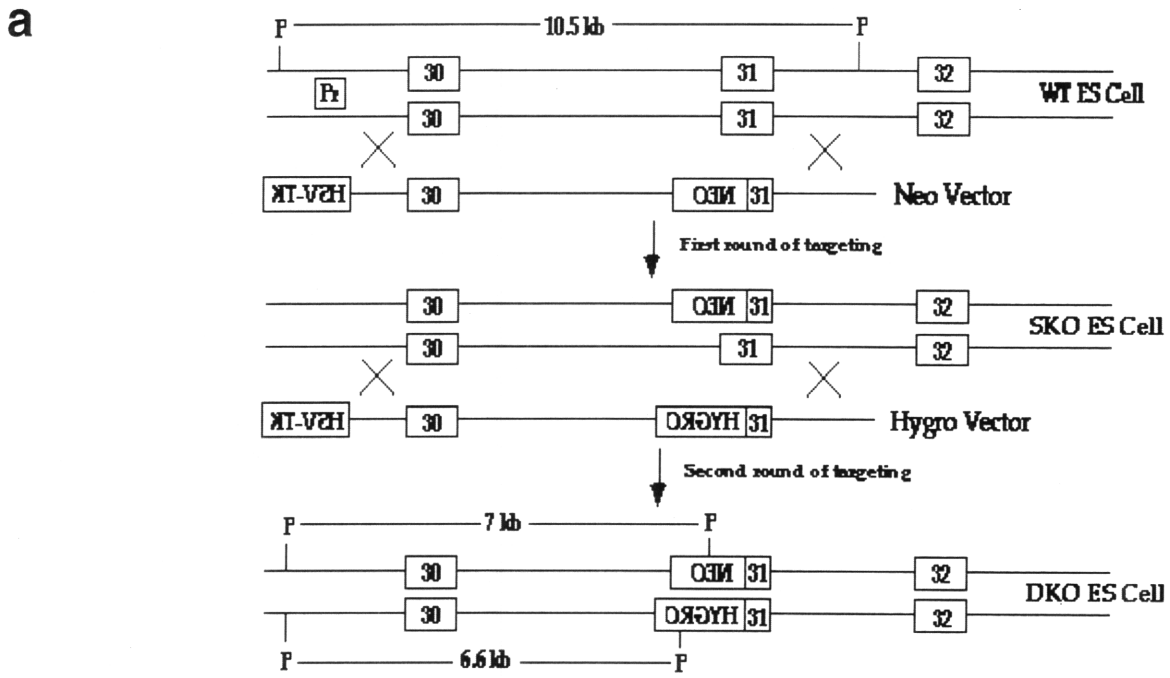
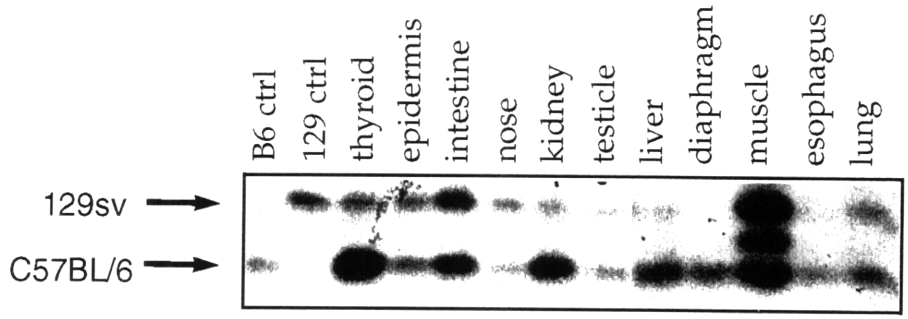

a
P
10.5 kb
P
Pr
30
31
32
WT ES Cell
HSV-TK
NEO
Neo Vector
First round of targeting
SKO ES Cell
HYGRO
Hygro Vector
Second round of targeting
7 kb
6.6 kb
DKO ES Cell
b
neo/neo
+/neo
NfDKO 6
NR 5
NR 7
NR 20
NR 30
NR 35
NR 47
NR 50
+/+ control
Nf1wt
Nf1neo
Nf1hygro
c
WT ES
Nf1 +/+
Nf1(neo31)
vector
sko ES
Nf1 +/-
Nf1(hygro31)
vector
dko ES
Nf1 -/-
Inject into wildtype blastocysts
Implant injected blastocysts into pseudo-pregnant recipient females
dko chimera

Figure 3.2. GPI analysis for ES cell contribution in chimeric animals.

GPI isoforms were separated on a TLC plate electrophoretically. The C57BL/6 isoform (b) migrates faster than the 129sv isoform (a). Chimeras show varying levels of isoform band intensities, from which relative contribution can be determined. In this animal (NfDKO#1), the thyroid gland and kidney received low levels of contribution, while the liver and diaphragm were basically nonchimeric. Muscle tissues exhibit an intermediate band due to the fusion of ES-derived and wild-type muscle cells (syncitium), leading to the heterodimerization of the two different isoforms.

Figure 3.2


B6 ctrl
129 ctrl
thyroid
epidermis
intestine
nose
kidney
testicle
liver
diaphragm
muscle
esophagus
lung
129sv
C57BL/6

some of these chimeric embryos under the dissecting scope, we found a number of the hearts (3/5) exhibited either delayed development or dorsal outlet right ventricle (DORV) at 13.5 g.d.

Furthermore, a number of chimeric neonates were unable to survive past 1-2 days after birth. A few of these deceased neonates were salvaged before cannibalization by the female, and thorough pathology was performed. We found a particular adrenal medullary defect in two of the four dead neonates. This lesion was best described as a "streaming" of cells resembling the adrenal medullary differentiation from the developing adrenal gland (Figure 3.3a). A quick examination on some nonchimeric neonates revealed that the normal neonatal adrenal glands were fully enclosed (medulla within the adrenal cortical layer, Figure 3.3b). Therefore, we hypothesized that the adrenal medullary abnormality was either a result of incomplete migration of neural crest precursors into the developing adrenal primordium, or a hyperplastic outgrowth of these same cells. In either case, such a lesion could cause severe imbalance in the blood epinephrine and norepinephrine levels, resulting in the animals' failure to thrive after birth.

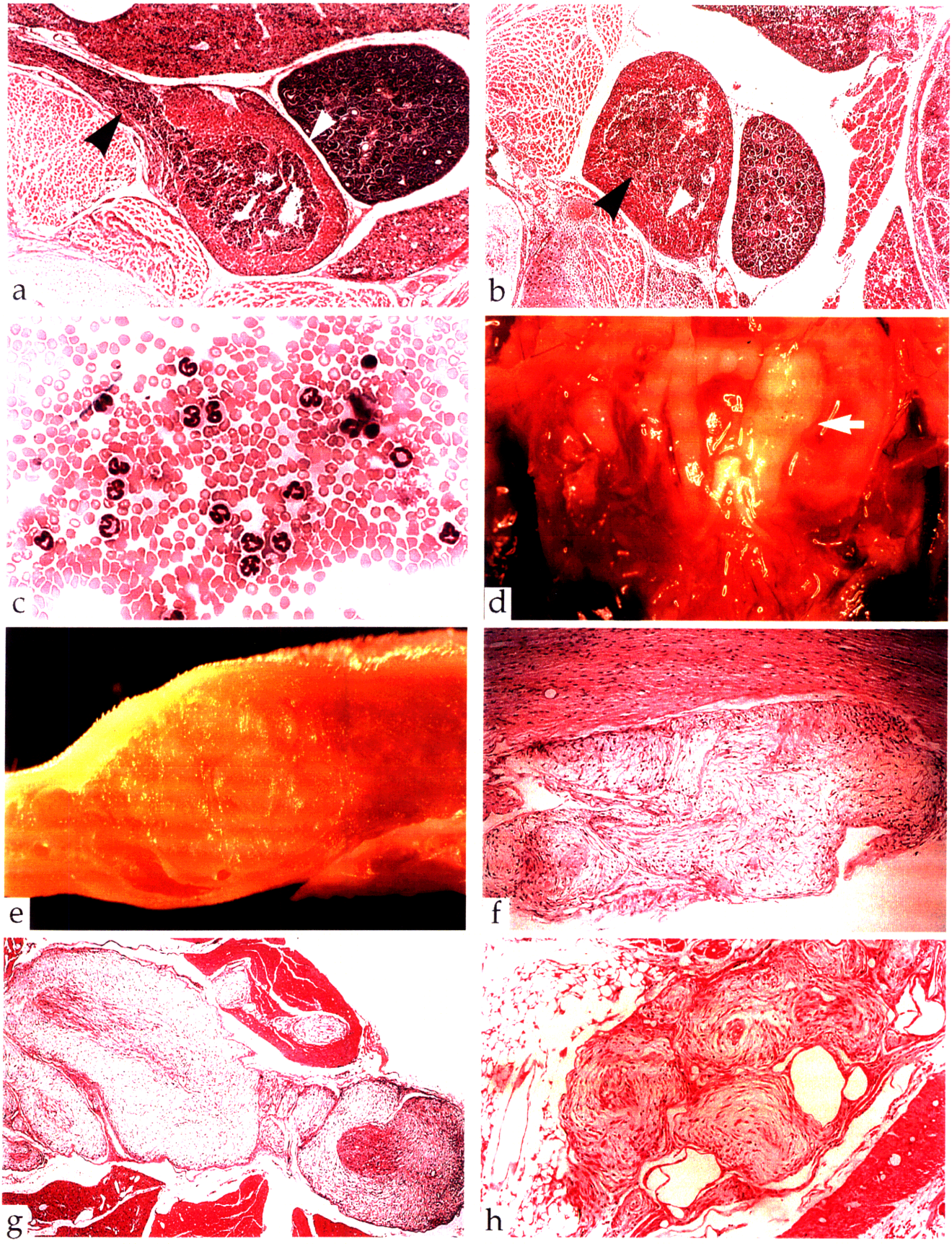
It was hypothesized that the some of the steps involved with re-targeting single knock-out (SKO) ES cells with the *Nf1-hygro* allele (re-electroporation, selection with hygromycin, etc.) might have rendered the resulting *Nf1* -/- cells less effective in generating adult chimeras. To bypass potentially damaging steps, we utilized an alternative protocol in which *Nf1* SKO ES cells were subjected to high G418 selection. The increased selection pressure would select for ES cell clones in which a non-reciprocal recombination has taken place, resulting in the duplication of the *Nf1-neo* mutant allele. These DKO ES cells should be able to tolerate higher G418 selection than cells with only one copy of the neo-resistance gene (Mortensen, 1992).

An *Nf1* SKO ES cell line (Nfsko155) was placed in five concentrations of G418 (300 mg/ml, 700 mg/ml, 800 mg/ml, 900 mg/ml, 1.1 mg/ml and 1.3 mg/ml) for ten to 14 days. Starting at 900 mg/ml, a dramatic reduction in colony numbers was seen relative to 300 mg/ml, which was the

Figure 3.3 Pathological and histological features of *Nf1* DKO chimeras.

(a) Adrenal medullary abnormality as observed by the outgrowth of the medullary tissue (arrow) from within the adrenal cortex (white arrow). (b) A normal neonatal adrenal gland, with the medulla (arrow) fully enclosed (white arrow). (c) Myeloid dysplasia from peripheral blood smears. The ring-shaped nuclei are indicative of terminally-differentiated myeloid cells, or neutrophils. (d) wholemount observation of a neurofibroma on the left 5th cranial (trigeminal) nerve (white arrow). The normal-sized right trigeminal is also present. (e) Sagittal section through the tongue showing the neurofibromas as white, round masses. (f) Neurofibroma found arising from the sciatic nerve (H&E, 100x). Note the diffuse and disorganized pattern of the tumor compared to the normal sciatic nerve above. (g) Multiple neurofibromas growing within muscle (H&E, 40x). Note that the hyperplastic cells surround normal nerves, which is indicative of the nerve sheath origin. (h) Neurofibromas arising from smaller peripheral nerves (H&E, 400x). Note the whorl patterns of the diffuse cells.

Figure 3.3



normal drug concentration for one copy of the *neo* resistance gene. One hundred and sixty colonies were picked (16 from 1.3 mg/ml, 37 from 1.1 mg/ml, 43 from 900 μg/ml and 64 from 700 μg/ml). Eighty-eight of these clones remained undifferentiated after expansion and were genotyped by PCR and Southern analysis. None of the picked colonies showed the expected LOH at the *Nf1* locus. These colonies might have developed increased drug resistance due to hyperactive mutations in the *neo* promoter or within the gene itself.

In the meantime, we continued to use the two characterized DKO ES clones (Nfdko6 and Nfdko47) in chimera generation. Two changes were made in the standard injection protocol: First, the number of ES cells injected per blastocyst was lowered to 2-4 in an attempt to reduce the overall contribution to the chimeric animals (and more specifically, to lower the likelihood of these Nf1 -/- cells from contributing to the developing heart and adrenal medulla). Second, the ES cells were plated on irradiated feeder cells rather than on gelatin only. This treatment has been suggested to maintain the undifferentiated state of the ES cells and therefore facilitate chimera generation (A. McClatchey, personal communication). As a result of these two changes, a total of eighteen chimeras were generated in a year.

Adult chimera phenotype

Table 3.2 below summarizes the relevant results on the eighteen chimeras. As expected, a slight male predominance was seen because the ES cells used contain the XY (male) chromosomes. Animals generated from both ES cells clones exhibited the same sets of phenotype, showing that the lesions were not due to mutations specific to any one clone.

The chimeric phenotype can be divided up into three groups, depending on the level of contribution by the *Nf1*-deficient ES cells. First, the high contributors (usually over 80% coat color chimerism) survive less than one month. Upon necropsy, the only clear observation was a bloating of the intestines, which may be associated with death. No neurofibromas, Lisch nodules or adrenal

Table 3.2 Summary of adult DKO chimera phenotype

Chimerism[1]	Sex	Age[2]	NF?[3]	Location[4]	Lisch[5]	Myeloid[6]	clone	Other lesions
85%	M	< 1	- -	- -	- -	- -	6	
80%	M	< 1	No	- -	No	No	47	
80%	M	< 1	No	- -	No	**Slight**	47	
65%	M	< 1	No	- -	No	- -	47	
75%	F	5.1	Yes	DRG, ovary	No	**Yes**	6	
70%	M	2.3	Yes	DRG, subQ	No	**Yes**	47	
60%	M	4.5	Yes	DRG	No	**Yes**	47	
45%	M	2.4	**Yes**	DRG	No	No	47	
40%	M	3.3	Yes	muscle	No	**Slight**	6	
40%	F	3.7	Yes	DRG, rectal	No	**Yes**	47	Meningioma
35%	F	4.0	Yes	DRG	- -	- -	47	
30%	M	6.9	Yes	DRG	No	**Slight**	6	
25%	F	7.0	Yes	TR, DRG	No	**Slight**	6	
25%	M	11.0	Yes	DRG, lung	No	No	47	Ganglioneuroma
20%	F	11.0	**Yes**	TR, DRG, tongue	No	No	6	
20%	M	26.4	Yes	rectal	No	**Slight**	6	
15%	M	15.0	No	- -	No	No	6	
10%	M	17.6	- -	- -	No	No	6	

[1]Chimerism is determined by approximating the agouti coat color contribution
[2]Age in months
[3]Detection of neurofibromas
[4]Location of neurofibromas: DRG=dorsal root ganglia, subQ=subcutaneous, TR=trigeminal
[5]Irises of chimeras were examined under a dissecting scope while directing a lightsource at the eyeball.
[6]Presence of myeloid hyperplasia as determined by counting the number of cells in the myeloid lineage

medullary hyperplasia were found (Figure 3.3c), possibly due to the lack of time for significant growth. One of these animals developed a slight myeloid dysplasia, a condition found with increased frequency in the human NF1 population. Another animal only had 65% coat color contribution, yet survived less than one month. This may be caused by lesions resulting from the contribution of ES cells to certain critical -- yet unknown -- tissues. A second category was the low contributors (15% or less by coat color). These animals survived for over a year without obvious phenotype.

The third, and most revealing category, involved the mid-range contributors (between 20-75%). These animals developed steadily worsening neuro-motor defects that included tremors, grasping of the hind legs and general wasting. The onset of these symptoms and the age of death generally correlated with the extent of chimerism. None of these animals developed Lisch nodules, yet 2/3 of the animals exhibited various extents of myeloid hyperproliferation in peripheral blood smears. The myeloid disorders were characteristically benign, since most of the cells found were neutrophils (Figure 3.3c), which constitute terminally-differentiated myeloid cells (as opposed to the more undifferentiated, blastic cells).

Furthermore, all of these animals developed peripheral nerve lesions reminiscent of human plexiform neurofibromas. Some of these lesions were large enough to be found using a microscope, including the trigeminal (Figure 3.3d) and tongue (Figure 3.3e). Histologically, these lesions appear diffuse and fairly acellular (predominantly composed of collagen), with distinctive whorl patterns similar to human neurofibromas (Figures 3.3f-h). Normal nerve bundles are often found in the center of these whorls (Figure 3.3g and 3.3h), suggesting that the hyperproliferative cells originated from the nerve sheath.

Characterization of nerve sheath lesions

It was necessary to determine whether the cell types present in these murine lesions correspond to those in their human counterparts. These peripheral nerve lesions were characterized by antibody staining against specific antigens, chemical stains and ultrastructurally.

Twenty-two lesions from eight chimeras were stained with antibodies against neurons (neurofilament-200, NF200), Schwann cells (S-100), fibroblasts and perineurial cells (vimentin). Results are shown in Figure 3.4. All the lesions studied show localized NF200 and S-100 staining in the normal nerve bundles. In the hyperplastic regions of the neurofibromas, however, only spotty NF200 and S-100 staining are seen, suggesting that some neurons and Schwann cells have been displaced by infiltrating tumor growths. The hyperplastic areas stained strongly for vimentin, an intermediate filament protein present on fibroblasts, perineurial cells and Schwann cells. These staining patterns suggest that the abnormally proliferating cell types in these lesions are fibroblastic in nature or perineurial cells. While antibodies such as epithelial membrane antigen (EMA, Coagulation Factor XIIIA and human milk globule protein (HMGP)) have been widely used to detect the presence of perineurial cells in tumors, we have been unsuccessful in showing specific staining with these antibodies despite trying a variety of conditions and treatments.

To further characterize the cell types involved, three of these lesions from two chimeras were studied ultrastructurally. One of these sections had to be removed from paraffin wax and reprocessed for EM analysis. Figure 3.5 shows some of the common features of these lesions. Normal myelinated nerve bundles were frequently found in close proximity (Figure 3.5a), suggesting the relative preservation of normal nerve bundles. The tumor cells exhibit long processes that are often found to run in parallel, covered in continuous basal lamina (Figure 3.5b), and laced with numerous pinocytotic vesicles (caveolae, Figure 3.5b and 3.5c). The tumors cells are also collagen-producing, as shown by the presence of collagen packets (Figure 3.5d). Lastly,

mast cells have been found within the lesions by EM (Figure 3.5e) and by toluidine blue staining (data not shown).

These EM results provided some answers: First, the hyperplastic cells are not fibroblastic, since the cells in question produce either a continuous or disrupted basal lamina, which fibroblasts do not. Beyond this, however, the cellular identify becomes less clear: Some of the features found using EM suggest that the hyperplastic cells exhibit features common to both Schwann cells and perineurial cells, while others show characteristics exclusive to one or the other (see Table 3.3). It is possible that the hyperplastic cells represent proliferating Schwann cells that have lost S-100 expression. It is equally likely that the abnormal cells are dividing perineurial cells that are producing continuous basal lamina (as opposed to a normally discontinuous layer). Lastly, these cells could be an intermediate differentiation cell type between Schwann cells and perineurial cells, since some have argued that both cell types originated from a common precursor (Bolande, 1974).

Table 3.3 Ultrastructural comparison between Schwann cells and perineurial cell

Schwann Cell Features	Perineurial Cell Features
interdigitating processes, sometimes long	long, **parallel** processes
continuous basal lamina	**discontinuous** basal lamina
pinocytotic vesicles	pinocytotic vesicles (caveolae)
normally found ensheathing neurons	normally form barrier layer surrounding nerve bundles

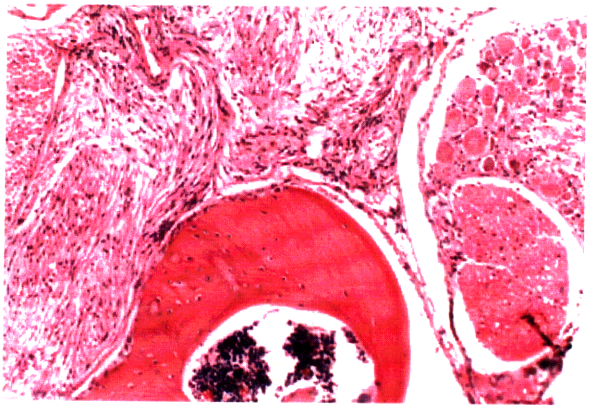
Identification of ES-derived cells

As mentioned previously, one of the unanswered question in neurofibromatosis research is the identity of the cell type responsible for initiating the development of a neurofibroma. In these chimeric models of neurofibromatosis, we have an unique opportunity to answer this question by attempting to identify the *Nf1*-deficient cells in the murine peripheral nerve sheath tumors. To this end, we have attempted to (1) amplify the mutant allele by performing *in situ* PCR on the histological slides and (2) detect the mutant *Nf1* transcript and/or the neo mRNA, which should be specific to only the ES-derived cells.

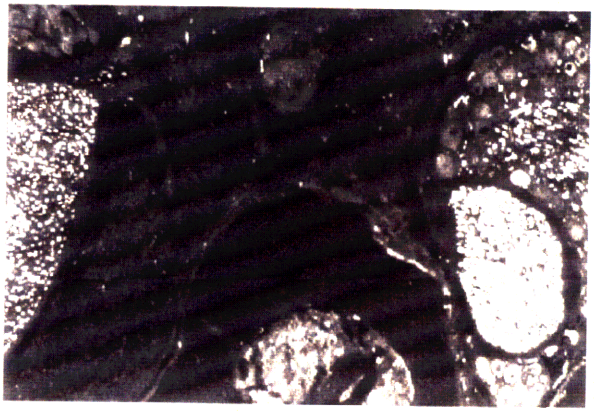
Figure 3.4 Immunohistochemical analysis of murine neurofibromas.

A neurofibroma was found in between normal ganglia (to the right) and a nerve bundle (to the left). Neurofilament (b) and S-100 (c) staining were only sporadic and confined to the nerve from which the lesion arose. However, the entire lesion stains positive with vimentin (d), which suggest the fibroblastic or perineurial nature of the hyperplastic cells. All images were taken at 100x magnification.

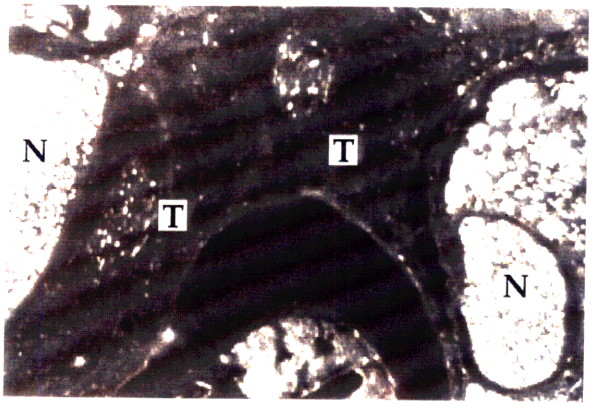
Figure 3.4



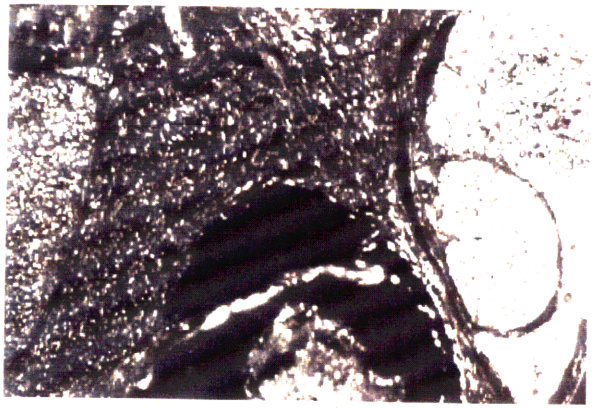
H & E



Neurofilament 200



S-100

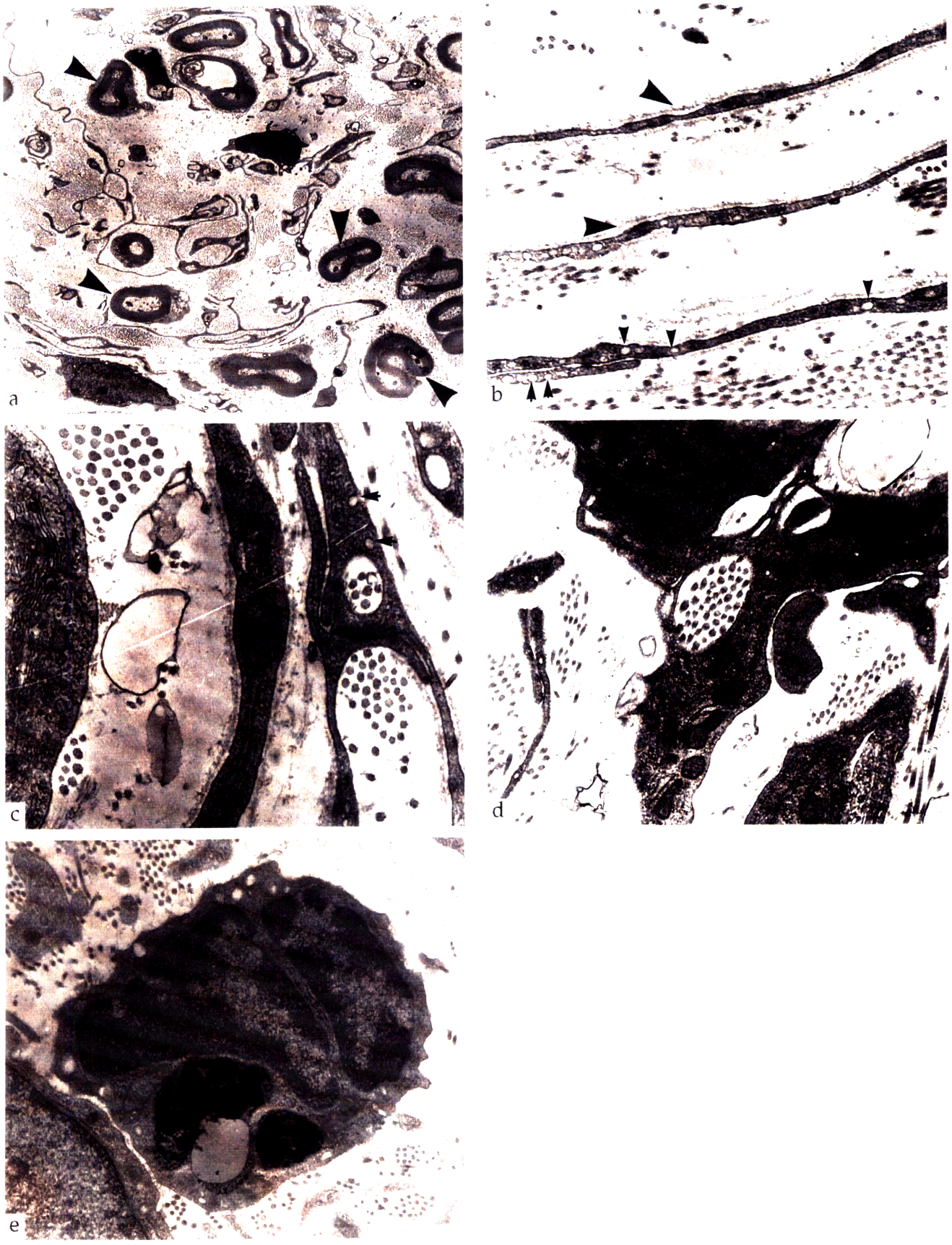


Vimentin

Figure 3.5 EM analysis of murine neurofibromas.

(a) Localized areas of normal neurons ensheathed by myelinating Schwann cells (arrows) (2,500x). (b) Long, parallel processes with continuous (and sometimes interrupted) basal lamina (arrow). Several caveolae can be seen along these processes (small arrowheads) (25,000x). (c) Hyperplastic cells containing caveolae (small arrowheads) and collagen packets (to the lower right). Note the basal lamina covering the cells (25,000x). (d) Enclosed collagen packet (collagen bundles circumscribed by plasma membrane within the cell) showing that the hyperplastic cells produce collagen (15,000x) (e) Mast cell, with the electron-dense granules (12,000x).

Figure 3.5



The primers used in the *in situ* PCR protocol were described in the *Nf1* mouse knock-out manuscript (Jacks et al., 1994). Instead of dTTP, a biotinylated dTTP was used in the reaction solution. The biotin was subsequently detected using the DAB protocol (see Methods). Despite the many trials using various thermocyclers, cycle times/temperature, and reagent concentrations, we could not specifically amplify the nuclear signal in mutant cells (in either positive control sections or neurofibromas). One possible explanation for the failure of the technique is the damage or fragmentation of genomic DNA during the fixation and/or tissue processing steps.

Alternatively, *in situ* hybridization was used. Radiolabeled nucleotides were incorporated into the riboprobes prior to the hybridization step. The advantages of *in situ* hybridization include the presence of multiple copies of the target mRNA available for binding (signal enhancement). We used antisense probes against the *neo* gene as well as the nonsense *neo* sequences present in the mutant Nf1-neo mRNA (Jacks et al., 1994). However, these attempts yielded nothing more than high background hybridization. It is possible that the 200 bp *neo* probe used was particularly sticky (e.g. have sequence similarity to other mammalian genes).

Generation of *ROSA-26 Nf1* DKO chimeras

Data from the DKO chimera experiments suggest that the contribution of *Nf1*-deficient cells to the adult mouse is able to induce the formation of neurofibromas. However, attempts at identifying tumor cells derived from the ES cells have fallen short of a conclusive answer. Therefore, we decided to generate *Nf1* DKO ES cells with a β-gal marker, using the *ROSA-26* transgene (which has been reported to express ubiquitously in mice). Two such *Nf1*-deficient/*ROSA-26* positive clones were generated using the delayed blastocyst protocol (see Methods). To date, seven chimeras were generated and three have been sacrificed and analyzed histologically. The *ROSA-26* chimeras exhibited a slightly different spectrum of lesions associated, which may be attributable to

the ES cell strain differences (129sv x C57BL/6 mixed background for the ROSA-26 chimeras as opposed to the 129sv inbred ES cells for the non-*ROSA-26* experiment).

Generation of ES cells

Thirty-seven females were ovariectomized and 145 delayed blastocysts were collected. From these blastocysts, 38 independent ES cell lines were established. After selection and expansion, 14 undifferentiated ES cell lines were chosen for genotyping. Of these 14 lines, ten were heterozygous for the *Nf1* mutation, while the other four were wild-type. In addition, 9 lines were found to be male (by *Zfy* PCR) and 7 lines were *ROSA-26* positive. We decided to re-target one of the mixed background *Nf1* +/- cell lines (NR3, which was also *Zfy* and *ROSA-26* positive) with the *Nf1-hygro* mutant allele in order to generate *ROSA-26* DKO cells (see Methods). *Zfy*-positive ES cells were used because, for reasons that are still unknown, male ES cells tended to be better in terms of giving rise to chimeras (Robertson, 1987).

Fifty-nine colonies were picked from electroporated ES cells selected with hygromycin. Of these, two clones (NRdko30 and NRdko48) were found to be *Nf1*-deficient using Southern analysis (Figure 3.1b) and subsequently used for the creation of chimeric animals. During initial chimera generation, the NRdko30 ES clone was found to give moderate to high contribution chimeras at an increased frequency. Subsequent analysis of the clone found the presence of heterozygous ES cells intermixed with the DKO cells. Single-colony re-derivation was performed on this ES cell line. Of the 48 colonies picked, 21 undifferentiated colonies were analyzed, and one was found to be *Nf1* -/- by PCR and then confirmed by Southern analysis.

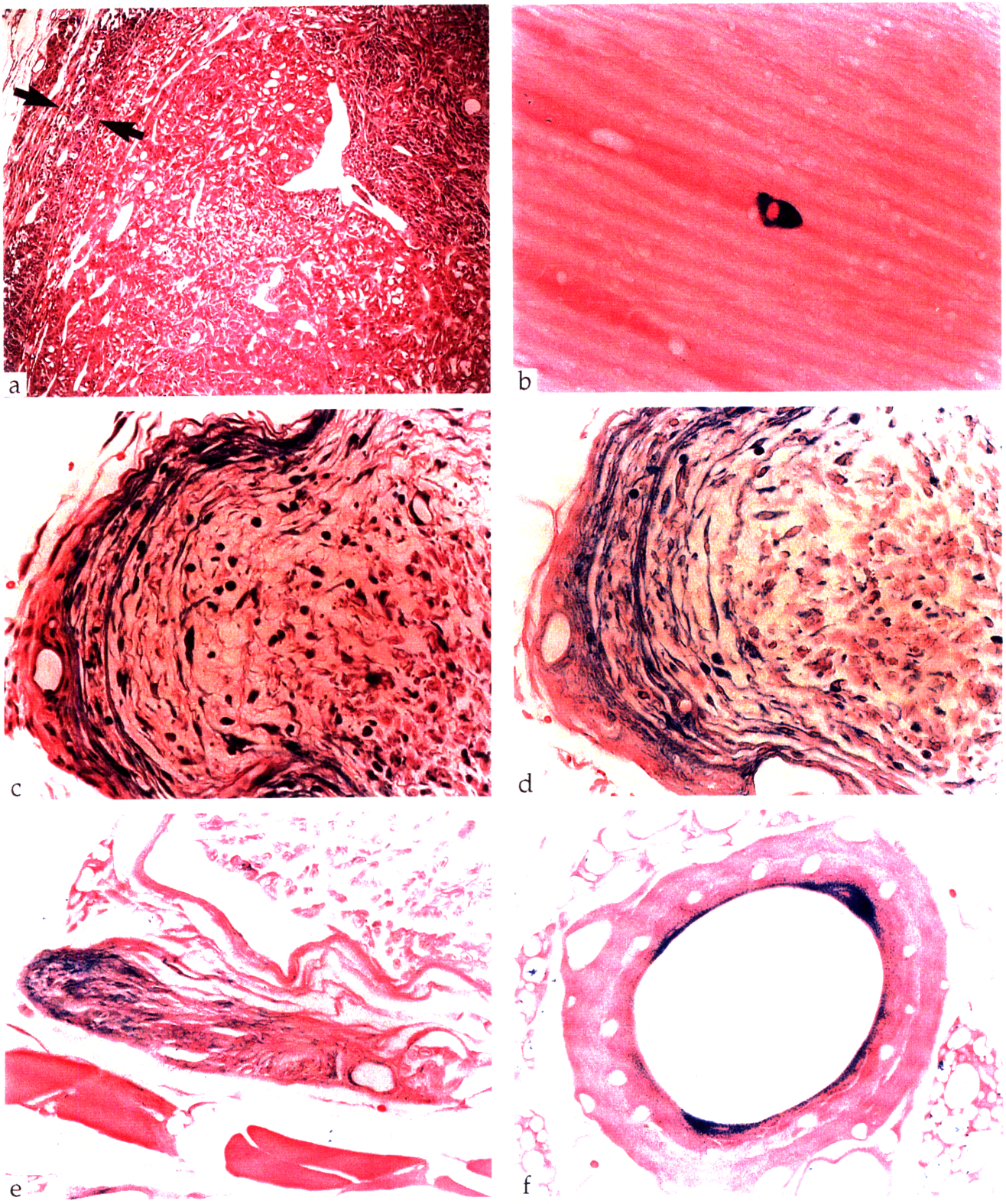
Generation of *ROSA*-26 chimeras

To date, seven adult chimeras have generated using these ES cells from the NRdko48 clone. Only three have been sacrificed and examined histologically (at 5.6, 3.4 and 5.3 months, respectively). All three of these animals developed bilateral pheochromocytomas (Figure 3.6a), but none showed

Figure 3.6 Phenotype and X-gal staining patterns of ROSA-26 chimeras.

(a) Pheochromocytoma (H&E, 40x). Note the compressed adrenal cortical layer (arrows). The tumor exhibits extensive pleomorphism. (b) A normal trigeminal nerve with very low ES cell contribution, as seen by the single blue cell (cytoplasmic staining) (Eosin only, 400x). (c) Neurofibroma arising from the DRG (H&E, 400x), with (d) stained only with eosin to highlight the X-gal staining. (e) Normal nerve with high levels of X-gal staining (400x). (e) Blood vessel wall showing X-gal staining (100x).

Figure 3.6



any signs of Lisch nodules or myeloid hyperplasia. Multiple nerve sheath lesions were found in one of the animals (Figure 3.6c-d). The location (DRG, sciatic nerve) and histological appearance of these tumors resembled those of the neurofibromas found in the non-*ROSA-26* DKO chimeras (Figure 3.3). In the same animal, some normal-looking peripheral nerve sections received very limited ES cell contribution (Figure 3.6b). At the same time, other nerves showed high level contribution (as determined by β-gal staining) yet still appeared normal (Figure 3.6e).

Chimera ES cell contribution assays

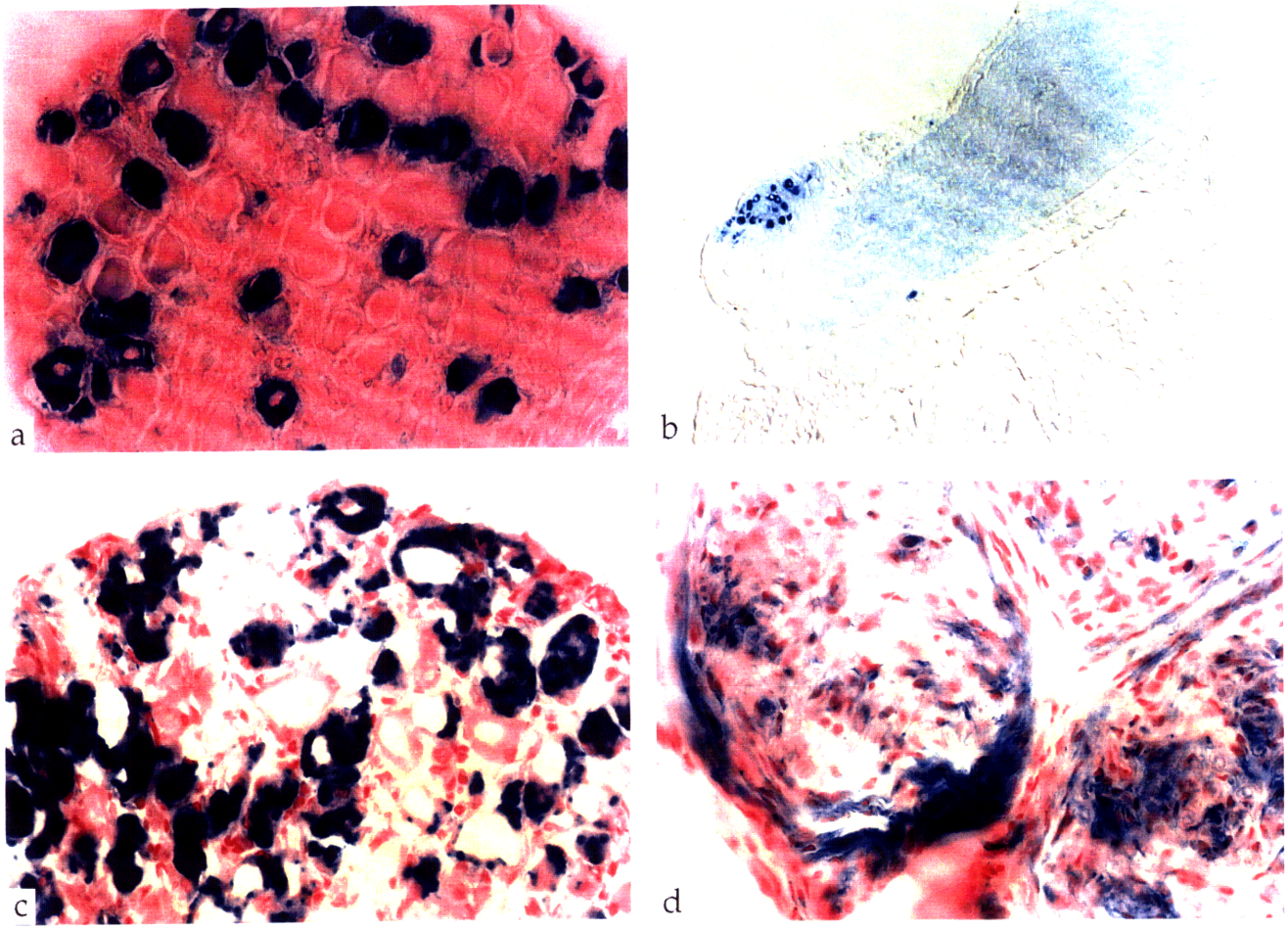
Initially, we needed to develop a consistent X-gal staining protocol for the various murine tissues. Previous studies have found differences in the intensity and subcellular localization patterns of the β-gal protein expression, depending on the cell type studied (Friedrich and Soriano, 1991; B. Williams, personal communication). Because we were looking for NF1-related lesions, we decided to focus on the neural crest derivative tissues -- specifically tissues of the peripheral nervous system. Two X-gal staining protocols were used and the results compared: Wholemount sectioning (with razor blades or vibratome) and frozen (cryostat) sectioning. It was determined that wholemount sectioning produced good staining at the surface of most tissues, but the X-gal staining solution was only able to penetrate 3-5 cell layers deep into the section. Frozen section staining showed intense, uniform staining in only some tissues (B. Williams, personal communication and dissertation). However, frozen section staining patterns tended to be punctate (Figure 3.7c-d) rather than uniform, as in wholemount sections (Figure 3.7a-b).

Cellular components of the nervous system showed a distinct set of X-gal subcellular localization: The blue X-gal crystals are found predominantly in the cell body of neurons and sheath cells, while the axons and cellular processes for Schwann and perineurial cells had only diffuse X-gal staining (Figure 3.7b). Given the relatively long processes of neurons, Schwann cells and perineurium, the majority of the cellular structures seen at any plane of section would be processes rather than cell bodies. The low level/diffuse staining in the cellular processes could potentially make the

Figure 3.7 Comparison of X-gal staining assays.

(a-b) Whole mount staining followed by paraffin sectioning. (a) Trigeminal nerve stained with eosin only (400x). (b) Nerve root with no staining. Note the blue haze in the axons and the intense blue staining in the ganglia (40x). (c-d) Frozen section staining. (c) Trigeminal nerve staining counterstained with nuclear fast red. Note the large, punctuate staining as compared to the relatively uniform staining for (a) (400x). (d) Axons showing hazy X-gal staining (400x).

Figure 3.7



identification of the Nf1-mutant extremely difficult. Preliminary data on the X-gal staining patterns of neurofibromas from the sciatic nerve and DRG (wholemount staining protocol only) show that most of the cells in the hyperplastic regions exhibit light, punctate staining (Figure 3.6c-d).

Discussion

In this chapter, we reported the generation and characterization of a mouse model for NF1. The phenotype observed is consistent with human neurocristopathy, which includes neurofibromas, pheochromocytomas and myeloid leukemia. Furthermore, this model represents the first genetic animal model for type 1 Neurofibromatosis, and the population of chimeric mice has allowed us a glimpse into the role of Nf1-deficiency in the etiology of the most common NF1-related lesion, the benign neurofibroma.

Mouse model of neurocristopathy

The characterization of the murine neurofibromas has revealed several similarities -- as well as differences -- compared to human plexiform neurofibromas (Figures 3.3 and 3.6). While the involvement of nerve sheath cells (including Schwann cells, perineurium and fibroblast) is indisputable, the hyperplastic cells consistently exhibit features indicative of perineurial differentiation (lack of S-100 staining (Figure 3.4), collagen packets, long, parallel processes, pinocytotic vesicles (Figure 3.5) and basal lamina). However, the presence of continuous basal lamina covering some of these cell surfaces seems to indicate a Schwannian differentiation. The presence of features from seemingly diverging cellular lineages on the same cell type may either (1) result from the uncontrolled growth properties of a tumor, or (2) call into question the precise origin of these cells. In fact, it has been reported that a type of Schwann-perineurial "transitional" cells is often found in human neurofibromas, sharing ultrastructural and antigenic features of both cell types (Hirose et al, 1986). Even in the medical literature, there is evidence showing differing extents of Schwannian and perineurial contribution toward plexiform neurofibromas (reviewed in Erlandson, 1991). Regardless, we cannot conclusively determine the cell type identity in this report without performing additional analyses.

To address the ambiguity of the cell types involved in these murine neurofibromas, we are pursuing two independent lines of investigation. First, we are attempting to optimize the perineurial-specific staining using various antibodies against the EMA epitope. Along the same line, we plan to stain the tumors with antibodies against markers expressed only in developing/immature Schwann cells (N-CAM, L1, p75 low affinity NGF receptor or galactocerebroside; reviewed in Jessen and Mirsky, 1992), in addition to the terminally-differentiated Schwann cell antigens (MBP or myelin protein P_0). The use of immature Schwann cell markers poses a problem because it has not been documented whether tumor cells will again turn on the expression of these developmentally-regulated markers. To draw upon additional experience, we are in the process of consulting with medical experts in the areas of human soft tissue and neurogenic sarcomas, including Dr. Christopher Fletcher at the Brigham & Women's Hospital in Boston as well as Dr. David Wolfe at the Mount Sinai Hospital in New York City. It is our hope that their expertise in the EM and immunohistochemical identification of peripheral nerve sheath tumors will help us in identifying the cell types involved.

Perineurial cells

Perineurial cells are normally found as concentric rings surrounding each nerve fascicle (Bennington, 1978). There have been many studies supporting the normal function of the perineurium as a diffusion barrier to most macromolecules (Olsson and Kristensson, 1973; Thomas and Olsson, 1984), with tight junctions providing the seal between two cells. Transport across the perineurium apparently occurs via the abundant pinocytotic vesicles found along the cellular processes (Peters et al, 1991). It has been previously reported that only fibroblasts have the ability to differentiate into perineurium in an *in vitro* differentiation study (Bunge et al, 1989). However, other studies suggest that perineurium may have originated from other cell types, including Schwann cells, arachnoid cap cells of the CNS (reviewed in Erlandson, 1991) and neural crest cells (Bolande, 1974). Rosenbaum et al (1995) reports that *Nf1*-deficient fibroblasts acquired a slight growth advantage *in vitro* and were unable to form normal perineurium in co-culture

experiments with wild-type neurons and Schwann cells. This line of evidence, combined with our data, points to a model in which *Nf1* -/- perineurial cells are sufficient to hyperproliferate into entities known as benign neurofibromas, resulting in the breakdown of normal barriers shielding nerve fascicles from exogenous growth factors. The exposure to altered levels of growth signals may open the door to additional proliferation and novel mutation by other cell types, such as Schwann cells and neurons, to eventually form malignant neurofibrosarcomas.

Schwann cells

Our data do not suggest a major role of proliferating Schwann cells in murine neurofibroma formation. However, many researchers believe that there is an intrinsic role of Schwann cells in the formation of such tumors because studies have estimated that 40-85% of cells in neurofibromas are of the Schwann cell lineage, and that many of these Schwann cells no longer associate with nerves (reviewed in Erlandson 1991; Riccardi, 1992). Moreover, researchers have hypothesized that neurofibrosarcomas are derived from Schwann cells (Riccardi, 1992). In order to further study the role of neurofibromin in Schwann cells, Kim et al (1995) have developed an *in vitro* system where mouse embryonic Schwann cells can be grown and co-cultured with other cell types of interest. They found that mutant Schwann cells become invasive, promote angiogenesis, and become hyperproliferative when exposed to increased levels of cAMP (Kim et al, 1997). However, the same study also noted that exposure to nerve growth factors such as glial growth factor lead to growth arrest in *Nf1* -/- Schwann cells, and that growth is restored only after serum removal (Kim et al, 1995). This indicates that simple *Nf1*-deficiency is not enough to trigger Schwann cell growth, and that additional events may be required -- be it epigenetic (cAMP level increase) or mutational (v-*ras* cooperates in transformation (Kim et al, 1995)). Since our model only provides for mutations in the *Nf1* gene, it was not surprising to find the lack of a Schwann cell component in the murine neurofibromas.

Neurons

Several reports have suggested that *Nf1*-deficiency can alter the behavior of neurons. First, Brannan et al (1994) found enlarged splanctic ganglia in about half of the 13.5 g.d. *Nf1* -/- mouse embryos examined histologically. Furthermore, *Nf1* -/-sympathetic and sensory-derived neurons were able to survive and extend neurites without the presence of neurotrophins, whereas wild-type neurons did not (Vogel et al, 1995). This is consistent with the role of neurofibromin in negatively regulating the neurotrophin/p21ras signaling pathway (Martin et al, 1990). It is possible that neurons lacking *Nf1* secrete factors to cause the growth of nerve sheath cells (paracrine signaling), resulting in the formation of neurofibromas. However, the inability to identify *Nf1* -/- cells effectively in the context of the mouse neurofibroma does not allow us to answer this question.

Mast cells

It was found through chemical staining and EM analysis that mast cells are commonplace in the murine neurofibromas. Mast cell infiltration has been found in a majority of human neurofibromas (Lascano EF, 1958; Johnson et al, 1989). These cells are responsible for the secretion of histamines, heparin and other mucopolysaccharides in areas of wound, inflammation or injury. It has been hypothesized that mast cells were recruited to the site of traumatic injury in NF1 patients, and has a role in promoting the growth of neurofibromas (Riccardi, 1992). However, it must be noted that while mast cells are seldom found in normal human nerves, they are usually abundantly present in healthy murine nerves (Gamble HJ and Goldby S, 1961). Given that the role of mast cells is poorly defined in normal as well as neoplastic nerves, their presence in the murine lesions may represent -- or cause -- inherent etiological discrepancies between human and mouse neurofibromas.

Myeloid cells and Adrenal medullary cells

The role of *Nf1* gene mutations in human and murine lesions involving these two cell types have been discussed in Chapter 2.

Modifier Genes

Given the limited number of *ROSA-26* chimeras analyzed, we nonetheless believe that there may be phenotypic variations caused by genetic differences in the ES cell strain. For example, pheochromocytomas were observed only in chimeric animals made from ES cells which were derived from heterogeneous parental strains (*ROSA-26* ES lines), while none of the 129sv inbred ES cell lines gave rise to such tumors. Conversely, myeloid leukemia only developed in inbred ES chimeras. This data corroborates our previous report on the phenotypic differences between inbred and mixed strains of Nf1 heterozygous mice (see Chapter 2). One study in human NF1 patients have found that identical twins afflicted with the condition tend to develop more similar patterns of NF1 lesions than either fraternal twins or the general NF1 population (Easton et al, 1993). Such epidemiological findings in human and mice suggest the existence of modifier genes that determine the extent, severity or expressivity of various NF1 phenotypes.

Genetic pathway for neurofibroma formation

What we have presented in this chapter represents the first genetic model of neurocristopathy (tumors of neural crest-derived tissues) focusing solely on the role of the *Nf1* tumor suppressor gene. Previous animal models of similar neurocristopathies have been established in several ways, including mutagenesis, naturally-occurring, or by the introduction of viral proteins (reviewed in Riccardi et al, 1994). A summary of the mouse models, strain used, and phenotypes are listed in Table 3.4.

Even though a few of these models were able to demonstrate the development of neurofibromas as well as other lesions arising from neural crest-derived tissues, the usefulness of these models are often limited by (1) the inability to identify specific gene mutations or (2) the introduction of viral gene products, which are known to be multi-functional: Viral proteins such as SV40 Large T antigen and HTLV-1 *tax* are able to transactivate a variety of cellular genes, including those

encoding growth factors (Green, 1991), oncogenes (Duyao et al, 1992; Ejima et al, 1993), and can also sequester/downregulate tumor suppressor gene products, such as the *retinoblastoma* (*RB*) *susceptibility gene*, *p53* (reviewed in Van Dyke, TA 1994) and *NF1* (Feigenbaum et al, 1996). However, none of these models were able to address the early sequence of events necessary for the development of neurofibromas.

Table 3.4 Summary of existing transgenic models of Nf1

Group	Description of vector and strain	Phenotype
Mazaraki, 1996	NGF-driven SV40 T into **C57BL 10 x CBA** F1	neurofibromas (Schwannian component)
Messing, 1994	P0-driven SV40 T into **C57BL/6J x SJL** F1	Schwannomas, hypomyelination
Jensen, 1993	MBP-driven SV40 T into **C57BL/6J x DBA** F1	Schwannomas, osteosarcomas
Corallini, 1993	systemic HIV-1 tat into BDF1 (**C57BL/6 x DBA/2**)	leiomyosarcoma, carcinoma, lymphoma
Green, 1992	HTLV-1 tax into **CD-1** (**C57BL/6 x DBA/2**)	neurofibromas, iris & adrenal lesions
Nerenberg, 1987	HTLV-1 tax into **CD-1** F1 males	spindle cell tumors (mesenchymal)
Hinrichs, 1987	HTLV-1 tax (tat) **CD-1**	neurofibromas (perineurial)

We postulated that *Nf1*-deficiency is necessary -- and in the mouse, sufficient -- for the formation of a benign neurofibromas for two reasons: First, given the early onset of these lesions (between 3 to 7 months of age), it was unlikely that other spontaneous mutations played a significant role in the promotion of the neurofibromas. Second, other lines of tumor suppressor knock-out mice created in our lab never developed similar neurocristopathies, while the phenotype was consistent across the two independently-derived lines of *Nf1* DKO ES cells. This implied that the nerve sheath lesions did not result from unexpected mutations present in the ES cell clones, and was specific to the mutations in the *Nf1* alleles. Lastly, because (1) the number of lesions found per animal and (2) the timecourse for the onset of tumor-induced neurological phenotype both seemed

dependent on the extent of ES cell contribution, we further hypothesized that the neurofibromas were congenital and grew slowly over time. This would indicate that a simple *Nf1*-deficiency (without other genetic events that the animal might have incurred early in life) was the first step toward the formation of neurofibromas, providing an environment that was conducive for hyperproliferative nerve sheath cells.

Making a clear connection between NF1 mutations and neurofibroma formation has been controversial because many studies have failed to find mutations in both *NF1* alleles in benign human neurofibromas. While it is possible that heterozygosity for the *NF1* gene confers some sort of growth advantage to nerve sheath cells (due, for example, to haploinsufficiency), a second hypothesis must be considered: It is possible that the current diagnostic protocols for gene mutations were not be able to pick up LOH in a mixed cell population (where only a some of the cells were deficient for a particular tumor suppressor gene). In fact, only a few reports have been able to document *NF1* LOH in benign neurofibromas (Skuse et al, 1991; Colman et al, 1995; Sawada et al, 1996; Serra et al, 1997). However, such studies were not able to demonstrate a clean, causal relationship between the *NF1* deficiency and tumor formation, nor were they able to identify the critical cell types that have incurred the LOH.

Future experiments

Given the similarities and discrepancies between the human and mouse neurofibromas discussed above, two sets of experiments may help to address some of the underlying mechanisms and differences between the two models.

Nf1 +/- blastocysts

Given that every human NF1 patient is constitutionally heterozygous for the *NF1* mutation (Huson, 1994), it is reasonable to imagine that once a critical cell type loses heterozygosity for the *NF1* function, it is able then to stimulate the surrounding *Nf1* +/- cell type(s) to proliferate and/or

to secrete factors that eventually cause the formation of neurofibromas. The assumption here is that, given the same level of growth signals, *Nf1* +/- cells have a lower threshold for hyperproliferation. In fact, there are *in vitro* studies suggesting that *Nf1* heterozygosity in Schwann cells (Kim et al, 1995) and fibroblasts (Rosenbaum et al, 1995) causes an intermediate phenotype/growth property as compared to the *Nf1* -/- cells. Therein lies a major genetic difference between our murine model and the human disease: in the current DKO chimera scheme, cells that become *Nf1*-deficient are surrounded by wild-type cells (instead of *Nf1*+/- cells, as in the human case). Using *Nf1* +/- blastocysts for chimera generation will allow us to more closely recapitulate the human condition. Such studies are in the preliminary stages.

Generation of tissue-specific knockouts

Another way to identify the critical cell type for the formation of neurofibromas *in vivo* is to generate animals in which the *Nf1* function is ablated in a tissue-specific manner. Several reports have shown that such conditional knock-out mouse strains can be made using the *cre-lox* technique, including the first manuscript by Gu et al (1994) describing experiments in which a fragment of the DNA polymerase β gene was selectively deleted in mouse T cells. In order to inactivate genes, animals are generated where selected regions of DNA are flanked by a 34 bp bacteriophage P1 site called *loxP* (locus of crossing-over). By driving the expression of *Cre* (causes recombination), an enzyme that causes two l*oxP* sequences to recombine, with promoters active only in defined cell types, the gene in question will be inactivated in those cell types. Depending on the promoter used, this approach allows for temporal as well as tissue-specific gene inactivation. By creating a strain of mice containing the *loxP* sequences surrounding crucial *Nf1* coding regions, we will be able to mutate the gene only in Schwann cells, neurons, or other cell types by breeding the animals to pre-established *cre*-expressing mouse lines (databases for existing mouse cre lines are available). The development of neurocristopathy using a particular cre line will help us to identify the cell type responsible for causing the lesions.

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Chapter 4

The effect of the murine *Nf1* mutation on a *p53* mutant background

Introduction

Neurofibromatosis Type I (NF1) is one of the most common hereditary disorders in humans, affecting one in 3,500 individuals (Stumpf et al., 1987). The gene associated with the NF1 disease was identified in 1990 (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990). The normal gene product encoded by this locus, called neurofibromin, contains a region showing close homology to the GTPase Activating Protein (GAP) for the *p21* ras oncogene (Xu et al., 1990) as well as the yeast IRA1 and IRA genes (Xu et al., 1990, Ballester et al., 1990). Neurofibromin was subsequently shown to downregulate the *ras* oncogene signaling function in both *in vitro* and *in vivo* systems (Martin et al., 1990; Xu et al., 1990; DeClue et al., 1992). Other studies on neurofibromin have suggested additional roles as a tubulin-binding protein (Gregory et al., 1993) and a modulator of the cAMP pathway (Guo et al., 1997).

Human patients who inherit a defective allele of *NF1* develop characteristic lesions such as cafe-au-lait spots, Lisch nodules and neurofibromas. At a certain frequency (10%), neurofibromas can progress to become malignant neurofibrosarcomas (Riccardi, 1992). This transformation leads to poor prognosis for many NF1 patients. In addition, mutations in *NF1* have been linked to a variety of human tumors, including malignant peripheral nerve sheath tumors (MPNST), neuroblastomas, sarcomas, myeloid leukemia and pheochromocytomas (Xu et al., 1992; Seizinger, 1993; The et al., 1993; Shannon et al., 1994). We and other have created mouse strains harboring the *Nf1* mutation in order to assess the *in vivo* function of the protein (Brannan et al., 1994; Jacks et al., 1994). The *Nf1* mutation predispose mice to cancer, in particular pheochromocytomas and myeloid leukemia (Jacks et al., 1994).

The multistep nature of human carcinogenesis has been demonstrated in many tumors. Perhaps the best demonstration of this is the ordered nature of mutations which occur during colorectal carcinogenesis (Fearon, 1992). One of the genetic alterations during this progression is the

inactivation of the *p53* tumor suppressor gene. In fact, *p53* mutations have been shown to occur in approximately 50% of all human tumors (Harris & Hollstein, 1993; Malkin, 1993; Greenblatt et al., 1994). The creation by several groups of *p53* deficient murine strains has enabled the examination of how the gene functions in tumor suppression (see below for examples; Armstrong et al., 1995; Donehower et al., 1992; Greenblatt et al., 1994; Harvey et al., 1993; Jacks et al., 1994; Purdie et al., 1994; Tsukada et al., 1994). To date, the *p53* gene product has been implicated primarily to function in the apoptosis pathway (Clarke et al., 1993; Lowe et al., 1993; Merritt et al., 1994; Ziegler et al., 1994), as a checkpoint for genomic stability (Kastan et al., 1991; Kastan et al., 1992), and in the G1 cell cycle arrest pathway (Kastan et al., 1992).

Many groups have reported cases citing cooperativity between a tumor suppressor genes and another tumor suppressor gene or an oncogene. The *p53* gene has been most widely studied with respect to its ability to cooperate with other genes in tumor progression. In NF1 patients, studies found the concomitant loss of *p53* function or mutations in *p53* in most cases where benign neurofibromas progressed into malignant neurofibrosarcomas (Menon et al., 1990; Greenblatt et al., 1994). This suggests a role for *P53* in tumor transformation. In mice, several important studies have supported the role of the mouse *p53* homologue in collaboration with other tumor suppressor genes or oncogenes in tumor progression (Kindblom et al., 1995; Luongo & Dove, 1996). In one of the first studies on tumor suppressor cooperativity, Williams et al. (1994) demonstrated that *p53* and the retinoblatsoma susceptibility gene (*Rb*) cooperated to decrease tumor latency and caused the development of new pathology in mice. Another group found an increase in genomic instability and changes in mammary tumor histopathology when the *Wnt-1* transgene was crossed onto a *p53* mutant background (Donehower et al., 1995). Kemp et al. (1993) found dramatic changes in tumor progression and malignancy, but not initiation, when the *p53* mutation was combined with an activated *H-ras* gene. Moreover, the combination of severe combined immune deficient (*scid*) and *p53* mutations accelerated the development of thymic lymphomas in mice (Bogue et al., 1996; Nacht et al., 1996).

Because of synergistic effect of *p53* with other genes, we decided to test the cooperativity between the *Nf1* and *p53* genes. It is hoped that the combination of these two mutations would exaggerate tumor promotion and/or progression, and reveal novel tumor types previously masked by genetic rate-limiting steps. There is additional difficulty in generating mice with the desired combination of mutations, because the *Nf1* and *p53* genes are very closely linked on the distal arm of mouse chromosome 11. This was done by generating recombinants through breeding (see below).

Mice inheriting different combinations of *Nf1* and *p53* mutations show reduced viability as well as increased susceptibility to tumor formation. A novel mouse phenotype reminiscent of an human NF1-associated lesion was found in two groups of these animals.

Methods

Genotyping of animals.

Animals were genotyped from tail DNA using PCR protocols involving three primer as previously described for the *Nf1*^{n31} (Jacks et al., 1994)and *p53*$^{\Delta}$ (Jacks et al., 1994) mutations.

Southern analysis of tumor DNAs.

Tumor tissues were excised and DNA prepared by standard methods. LOH analysis of *Nf1* status, tumor DNA was digested with *NcoI* and *HindIII* and hybridized with a probe derived from a *NcoI-PstI* genomic fragment from intron 31 of the murine *Nf1* gene (probe B, Jacks et al., 1994). *p53* status was evaluated by digesting tumor DNA with *EcoR1* and *Stu1* and hybridizing with a 3' cDNA probe (probe B, Jacks et al., 1994).

Histopathology.

Analysis was performed as described (Jacks et al., 1992; Jacks et al., 1994). Tissues were surgically removed and fixed in either 10% formalin or 4% freshly-prepared paraformaldehyde, dehydrated, embedded in paraffin, sectioned at 6 mm and stained with hematoxylin and eosin prior to microscopic analysis.

Immunohistochemistry and immunofluorescence.

Analysis was performed on unstained histological sections prepared as described above. For α-sarcomeric actin antibody (Sigma) analysis, sections were permeabilized with IF buffer (0.1% triton X-100 in PBS containing 5% horse serum and 1% goat serum), incubated with dilute antisera (1:500), immunostained using Vectastain ABC kit and visualized by diaminobenzidine (DAB) reaction. For myelin basic protein (MBP) analysis (Dako), sections were treated with blocking buffer (BB: 10% goat serum, 0.05% Tween-20 and 0.02% sodium azide in PBS), hybridized with dilute antisera (1:200), and visualized with Vectastain ABC and DAB kits. For S-

100 (Dako) immunofluorescence detection (1:200 dilution of primary antibody), sections were incubated in BB buffer, and a FITC-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, used at 1:75 dilution) was used as the secondary antibody. Signal was detected using an immunofluorescence scope.

Results

Separately, the *Nf1* and *p53* mutations predispose mice to cancer. *Nf1* heterozygous animals are prone to the development of pheochromocytomas and myeloid leukemia between twelve to eighteen months of age (Figure 4.1) (Jacks et al., 1994). *p53* heterozygous animals exhibit similar kinetics of cancer development (Figure 4.1), but succumb to lesions distinct from the *Nf1* heterozygotes, including sarcomas and lymphomas (Table 4.1) (Jacks et al., 1994, Harvey et al., 1993). Consistent with the tumor suppressor hypothesis, cancerous tissues arising in both mutant strains exhibit loss-of-heterozygosity (LOH) at the *Nf1* (Jacks et al., 1994) or *p53* (Jacks et al., 1994, Harvey et al., 1993) locus. Moreover, p53 null animals show a significantly accelerated rate of tumor formation, with over 90% mortality by 3-6 months of age (Figure 4.1). It is not possible to generate *Nf1*-null adult animals, since *Nf1*-deficiency results in mid-gestation embryonic lethality due to abnormal cardiac development (Brannan et al., 1994; Jacks et al., 1994).

Generation of animals through Mitotic Recombination

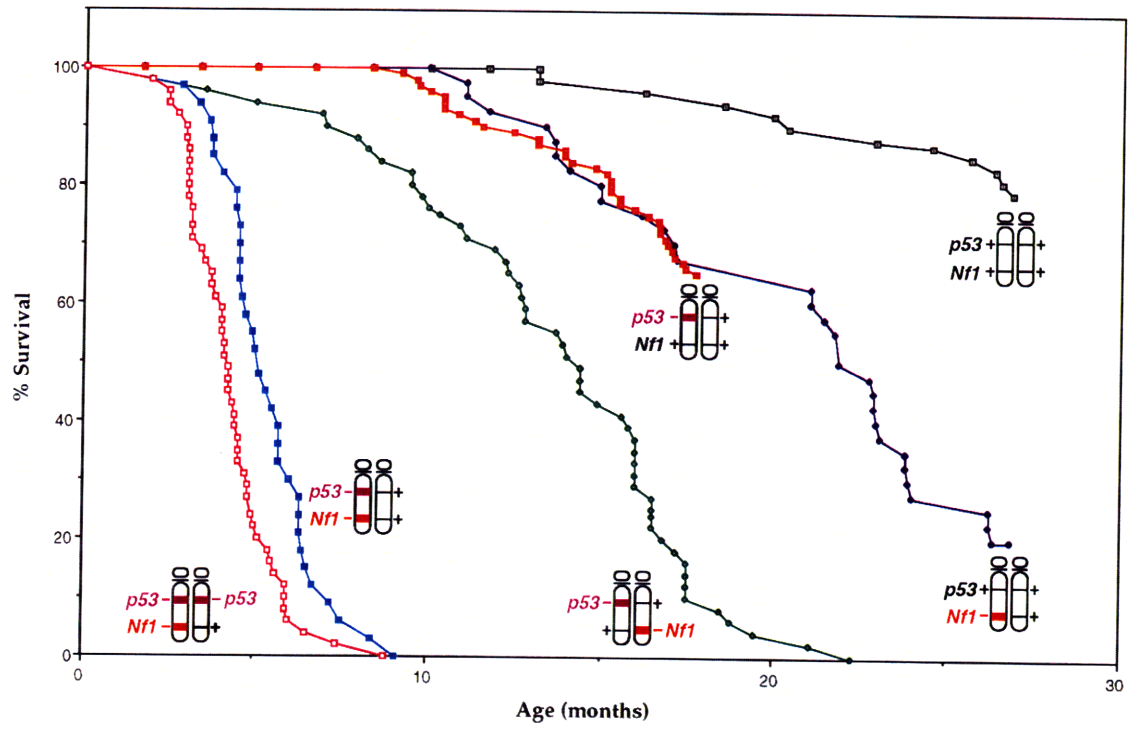
In order to study cooperative effects between *Nf1* and *p53* on tumor development, three novel strains of mice were created. First, mice heterozygous for the two mutations on separate chromosomes (in 'trans') were created. Second, mice constitutionally heterozygous for the two mutations carried on the same chromosome (in 'cis') were generated as described below. Finally, animals heterozygous for the *Nf1* mutation on a *p53* null background ('het-mutant', or 'H-M') were generated from appropriate crosses.

'Trans' animals were generated and identified by PCR genotyping. These animals represent approximately 50% of the progeny derived from matings between *Nf1* +/- and *p53* -/- parents. However, since the two gene loci are tightly linked on mouse chromosome 11 (5 centimorgans (cM) apart), a chromosome containing both mutant genes was needed to generate the 'cis' and 'H-M' strains of mice. Instead of re-targeting single-mutant ES cells, we decided to take advantage of

Figure 4.1. Survival of mice with mutations in *p53* and *Nf1*.

Graph summarizing the lifespan of 51 'trans' animals; 34 'cis' animals; 49 'H-M' animals; 19 wild-type animals; 37 *Nf1* +/- animals; and 41 *p53* +/- animals. The p53 -/- survival curve was not shown, but closely follows that of the 'cis' curve. The mean age of survival (the point at which 50% of the animals had died) was 14 months for the 'trans' animals; 5 months for the 'cis' animals; and about 4 months for the 'H-M' animals. At 20 months, over 50% of the Nf1 and p53 heterozygous animals remained alive and about 90% of the wild-type animals were still alive at 25 months of age.

Figure 4.1


% Survival
100
80
60
40
20
0
0
10
20
30
Age (months)
p53 +
Nf1 +
p53 -
Nf1 +
p53 -
Nf1 -
p53 -
p53
Nf1 -
p53 -
Nf1
p53 +
Nf1 -

the spontaneous meiotic cross-over events that occur between homologous chromosomes in germ cell precursors. If such a reciprocal recombination event took place between the *Nf1* and *p53* loci of a 'trans' parent (as depicted in Figure 4.2), the animal would theoretically produce equivalent numbers of zygotes containing either (1) wild-type copies or (2) mutant copies of both genes. The frequency of this desired cross-over event depends on the chromosomal distance between the two genes. To this end, 'trans' animals were mated to wild-type or *p53* null mice. Genotypic analysis of animals from these matings identified thirty-two (32) cross-over events in the 283 animals generated. This gives an empirical cross-over distance of 4.2 cM, a value consistent with the previously reported 5 (Buchberg et al., 1992). Of the 32 mice inheriting recombinant chromosomes, only 4 were of the 'cis' configuration. The other 28 recombinant mice were wild-type for both tumor suppressor gene mutations. These 4 mice were then used to generate the other 'cis' mice and were mated to p53 mutant mice to generate the 'H-M' mice.

In order to assess the effect of these mutational configurations on the adult mouse, we followed cohorts of different groups over time and analyzed the resulting tumor spectrum. Animals were carefully monitored and sacrificed at first signs of illness or significant tumor burden.

Analysis of Nf1 +/-;p53+/- 'trans' mice

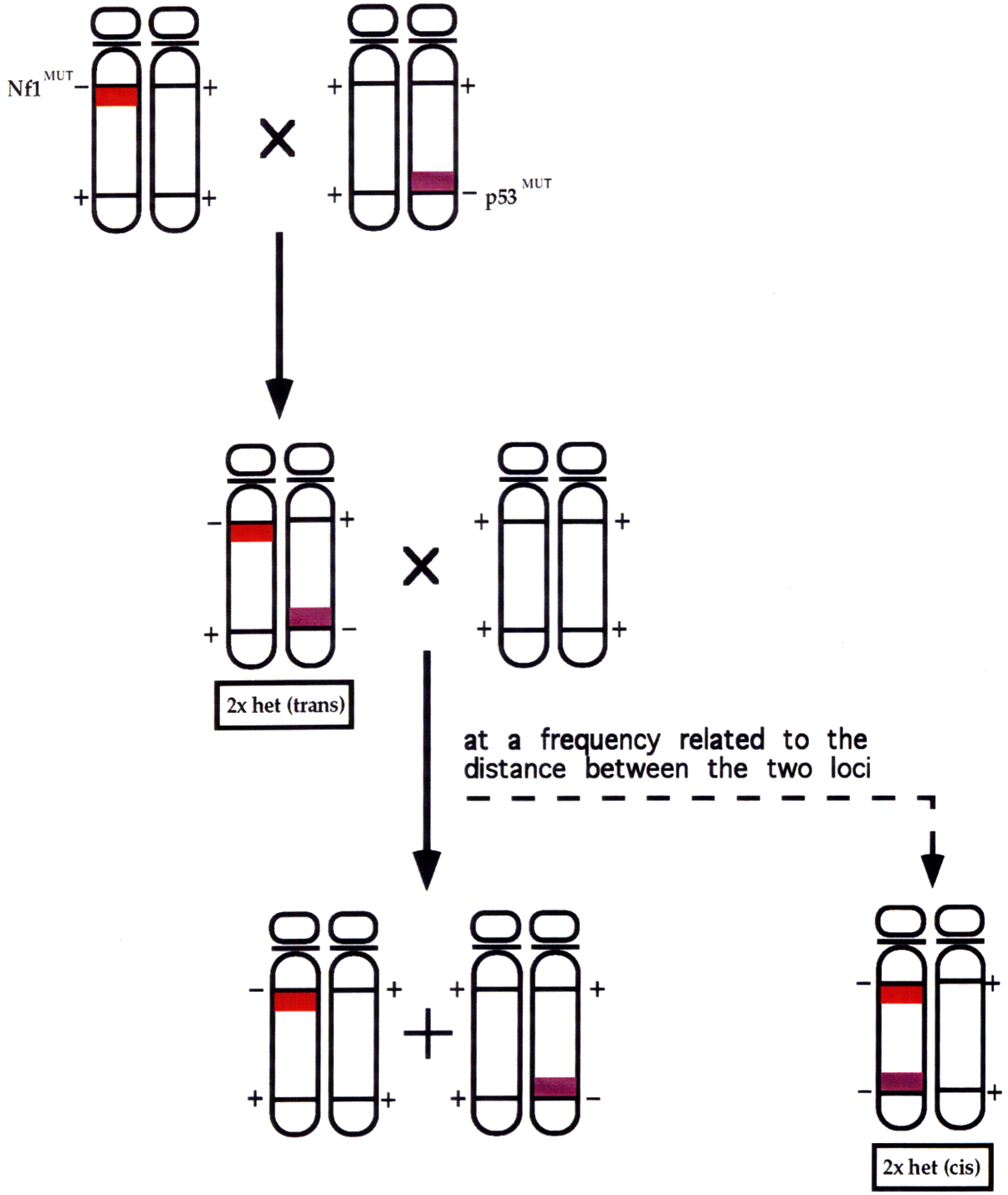
The mean age of survival (defined as the age at which 50% of the animals had died) of the 'trans' animals was slightly reduced compared to *Nf1* or *p53* heterozygous alone (Figure 4.1). The 'trans' animals exhibited similar tumor spectra to those found in either single heterozygotes (Table 4.1), including lymphomas, various sarcomas, myeloid leukemia and hemangiomas.

For each genotype indicated, the number of animals analyzed is found at the bottom of the column. Most of these tumors could be found by gross necropsy as abnormal enlargements or growths. Rhabdomyosarcomas, fibrosarcomas and anaplastic sarcomas are combined into one category due to the variable morphology among the tumors. Note the predominance of sarcoma development in

Figure 4.2. Meiotic cross-over was required between two closely-linked tumor suppressor loci.

Generation of *Nf1-p53* a 'cis' chromosome containing both mutant alleles heterozygotes was achieved by a cross-over event between homologous chromosomes in germ cell precursor cells. The reported chromosomal distance between *Nf1* and *p53* was 5 cM. This study found the chromosomal distance to be 4.3 cM.

Figure 4.2


Nf1 MUT
p53 MUT
2x het (trans)
at a frequency related to the distance between the two loci
2x het (cis)

'cis' animals. 'H-M' animals developed a combination of *p53*-related (thymic lymphoma) 'cis'-related (sarcoma) lesions. An uterine tumor was found in a 'cis' mouse. Due to the gender-specificity of this tumor, the actual incidence could be underestimated.

Table 4.1: Incidence of tumor types in mice with various combination of *Nf1* and *p53* mutations

Tumor/lesion	Nf1 +/-	p53 +/-	p53 -/-	2x trans	2x cis	Nf1 +/-/p53 -/-
lymphomas	25%	25%	71%	36%	5%	42%
rhabdomyosarcoma/fibrosarcoma	5%	57%)	21%	36%	81%	54%
osteosarcomas	0%		0%	18%	0%	4%
myeloid leukemia	16%	0%	0%	9%	0%	4%
hemangioma	0%	0%	0%	9%	14%	17%
epithelial neuroblastoma	0%	0%	0%	0%	0%	4%
lung adenocarcinoma	16%	0%	0%	0%	0%	0%
pheochromocytoma	21%	0%	0%	0%	0%	0%
hepatocarcinoma	9%	0%	0%	0%	0%	0%
brain tumor	0%	0%	0%	0%	5%	0%
uterine tumor	0%	0%	0%	0%	5%	0%
(n=)	57	44	56	11	21	24

Southern blot analysis of the tumor DNA (n=6) revealed LOH of either the *Nf1* mutant locus (with *p53* becoming wild-type at both alleles), or the *p53* mutant locus (with two functional *Nf1* alleles) (Figure 4.3). This result suggests one of two possible mechanisms for LOH: (1) the tight linkage of the two loci does not allow cross-over between *Nf1* and *p53* or (2) the loss of the entire chromosome 11 and its subsequent duplication from the remaining template, which has a mutant allele as well as a wild-type allele for the two tumor suppressor genes, respectively. In fact, in the two 'trans' fibrosarcomas and two osteosarcomas analyzed, one of each showed LOH to *Nf1* while the other was found to have LOH to *p53*. Despite the different genetic events, the tumors did not appear histologically different upon examination. The one case of myeloid leukemia, a condition primarily associated with *Nf1* deficiency, showed LOH at the *Nf1* locus.

Analysis of *Nf1*+/-;*p53*+/- 'cis' mice

As shown in Figure 4.1, mice inheriting both mutations on the same chromosome showed a dramatic increase in tumor predisposition. The mean survival age for this group of animals is 5

months (similar to the *p53* -/- animals, with closely matching survival curves; *p53*-/- survival curve not shown). This is substantially lower than the 14 months for the 'trans' population. Furthermore, none of the 'cis' animals lived past 10 months of age. The 'cis' mice typically develop a single, large subcutaneous tumor, ranging in size from 1 centimeter (cm) to 4 cm in diameter. Overall, 80% of tumors seen in 'cis' animals are of this type (Table 4.1).

Histological analysis of these tumors revealed that they were sarcomas exhibiting varying degrees of differentiation. As shown in Figure 4.4, the tumors range from undifferentiated fibrosarcomas to clear rhabdomyosarcomas with the associated muscular striations. In addition, a new class of pleomorphic 'anaplastic' sarcomas was found, composed of an extremely heterogeneous cell population (Figure 4.4). Many of these tumors contain at least two distinct regions of cellular morphology, one comprised of compact, spindle-shaped cells and the other of pleomorphic, irregularly-shaped cells and nuclei, usually with abundant cytoplasm. The tumors are reminiscent of malignant soft tissue sarcomas found in humans in terms of the high mitotic index, infiltration into adjacent tissues (as seen by the trapped normal muscle fibers in Figure 4.4A), necrotic core, high ploidy nuclei, and rapid rates of growth. Consistent with the tight linkage of *Nf1* and *p53*, 100% of all sarcomas analyzed by Southern blot showed simultaneous LOH at both loci (Figure 4.3). Since none of the 'trans' animals were able to lose both loci, the absence of such anaplastic sarcomas seems to suggest a crucial role for the combined *Nf1*- and *p53*-deficiency in the development of these novel mouse tumors.

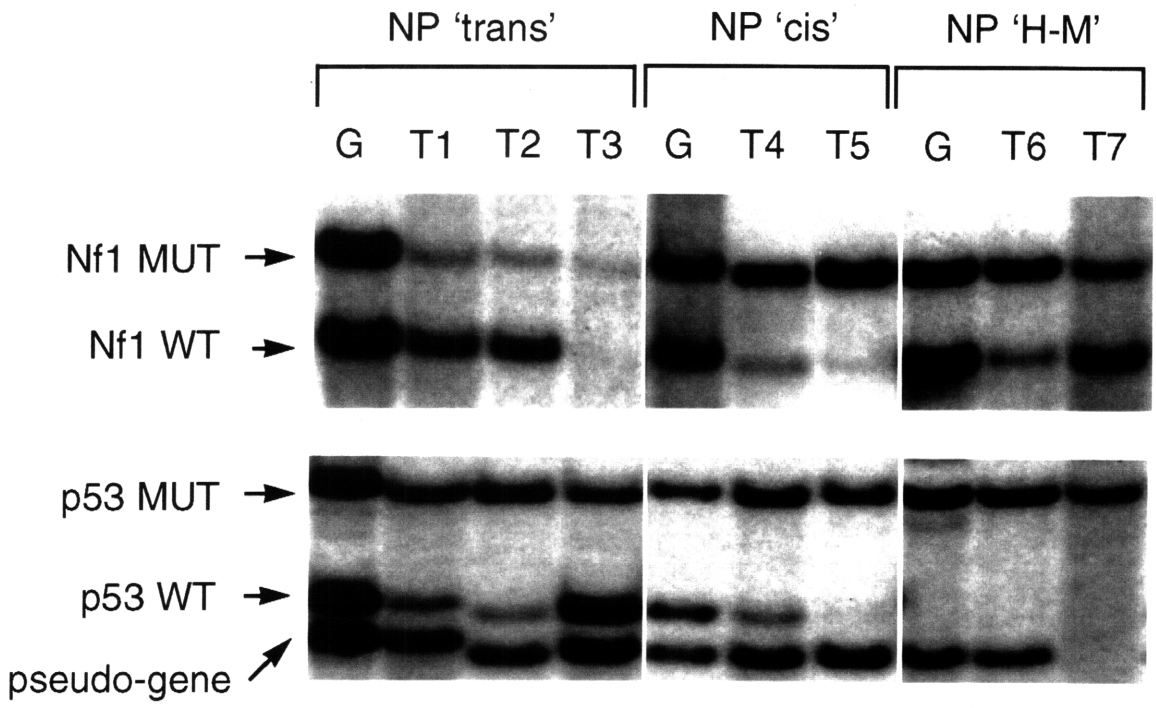
Analysis of *Nf1*+/-;*p53*-/- 'het-mutant' animals

The 'H-M' mice exhibited significant decrease in tumor latency, with a mean survival age of just over 4 months. This represents a shift of survival curve to the left of both the 'cis' (see Figure 4.1) and *p53* -/- curves (previously published data; Jacks et al., 1994). The tumors observed in this population included those associated with p53 deficiency (42% thymic lymphomas) as well as tumors seen in the 'cis' mice (54% sarcomas). These sarcomas exhibited similar ranges of cellular

Figure 4.3. LOH in *Nf1-p53* mouse sarcomas.

Status of *Nf1* and *p53* in genomic (G) and tumor (T) tissues of various mutation combinations. T1 'trans" fibrosarcoma; T2 'trans' osteosarcoma; T3 'trans' myeloid leukemia; T4 'cis' anaplastic sarcoma; T5 'cis' brain tumor; T6 'H-M' fibrosarcoma; T7 'H-M' thymic lymphoma. 'trans' tumors T1 and T2 show LOH to *p53* (with *Nf1* locus reverting back to wild-type), but the myeloid leukemia showed loss to *Nf1*. T4 and T5 show simultaneous loss of both mutant alleles. Note the LOH to *Nf1* in the 'H-M' fibrosarcoma (T6) but not in the 'H-M' thymic lymphoma (T7). Most tumors were well-vascularized, hence the extracted tumor tissues were contaminated with genomic DNA (blood and skin).

Figure 4.3


NP 'trans'
NP 'cis'
NP 'H-M'
G T1 T2 T3 G T4 T5 G T6 T7
Nf1 MUT
Nf1 WT
p53 MUT
p53 WT
pseudo-gene

differentiation and pleomorphism as their 'cis' counterparts (Figure 4.4).

In contrast to the 100% LOH at the *Nf1* and *p53* loci for the 'cis' tumors, only about 50% (4/8) of the H-M fibrosarcomas and rhabdomyosarcomas showed loss of *Nf1* (Figure 4.3, some tumor samples not shown). In contrast, 4 out of 5 (80%) anaplastic sarcomas exhibited loss of the wild-type *Nf1* allele. This suggests that *p53*-deficiency is required for the development of sarcomas, but that loss of *Nf1* function generates a selective growth advantage during tumor progression. The wild-type alleles of *Nf1* were never lost in any of the thymic lymphomas analyzed (Figure 4.3), suggesting that Nf1 deficiency confers no selective advantage in the progression of this tumor type.

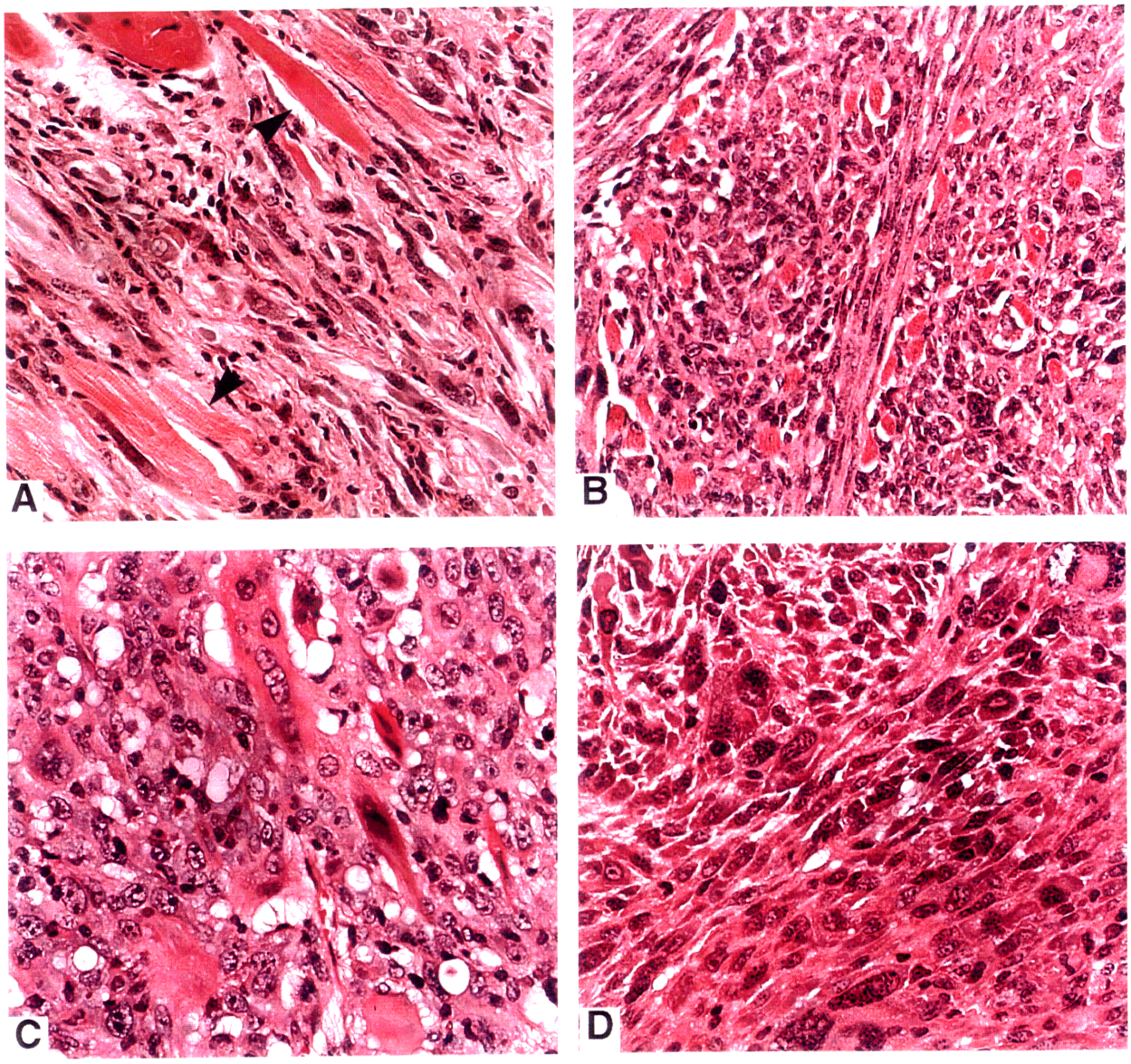
Immunohistochemical analysis of anaplastic sarcomas

In order to better understand the anaplastic sarcomas seen in the 'cis' and 'H-M' mice, we performed extensive immunohistochemical analysis to determine the cell types present in the tumors. An α-sarcomeric actin antibody was chosen to confirm the presence of muscle cells in rhabdomyosarcomas. Their presence was further verified by Mallory's Phosphotungstic Acid Hematoxylin (PTAH) chemical staining method (not shown). In addition, to determine the similarities between these sarcomas and the class of NF1-related tumors called malignant peripheral nerve sheath tumors (MPNSTs), Schwann cell markers (S-100 and myelin basic protein) were used. The results of these analyses are shown in Figure 4.5, where adjacent sections of representative tumors were stained with the panel of antibodies described above. Care was taken to distinguish positive staining from background staining due to endogenous peroxidase activity, particularly in red blood cells and necrotic regions within the lesions (regions of necrosis are often found in the core of these large tumor masses). Results of the immunohistochemical profile are summarized in Table 4.2.

Figure 4.4. Tumor pathology of 'cis' and 'H-M' tumors (H&E).

(A) 'cis' rhabdomyosarcoma (400x magnification). Note the characteristic muscle striations in the tumor (arrowheads). (B) 'cis' fibrosarcoma (400x). Tumor exhibit compact, spindle-shaped cells with "streaming" morphology, indicative of fibroblastic fascicles. (C) 'H-M' anaplatsic sarcoma (400x). Pleomorphic appearance including cells with abundant cytoplasm, irregularly-shaped nuclei and variable ploidy counts. (D) 'H-M' rhabdomyosarcoma (400x). Note the two distinct cell populations in the tumor as distinguished by the intercellular spacing and the variable size of the nucleus (poly-ploidy) in the bottom half of (D). All these tumors are solitary and subcutaneous. Rapid growth and necrosis in the tumor core are common traits among the sarcomas.

Figure 4.4



Approximately a third of the sarcomas stained positive only for skeletal muscle markers. In comparison, 21% of the sarcomas were found to be positive for Schwann cell markers, while only 12% of the lesions stained positive for both markers. These staining patterns are consistent with the varied range of differentiation as seen by H&E staining. In the double positive lesions, a careful comparison between the size and morphology of each cell type showed that these tumors comprised of a mixture of Schwann and muscle cells, rather than cells expressing both markers simultaneously (Figure 4.5). In summary, the 'cis' and 'H-m' sarcomas show a variegated pattern of staining with respect to the various nerve sheath markers, and do not always correspond to the expected morphological features. These lesions are reminiscent of the MPNSTs, with a subset of the tumors resembling "Triton Tumors" with its expression of both Schwann and muscle surface markers. Triton tumors are found to be highly associated with patients constitutionally heterozygous for the *NF1* mutation.

Table 4.2. Immunohistochemical results for 'cis' and 'H-M' sarcomas.

	n=	Schwann (+)	Muscle(+)	**Double (+)**
'cis'	21	4	5	**3**
'H-M'	13	3	6	**1**
Total	34	7	11	**4**
%*		**21%**	**32%**	**12%**

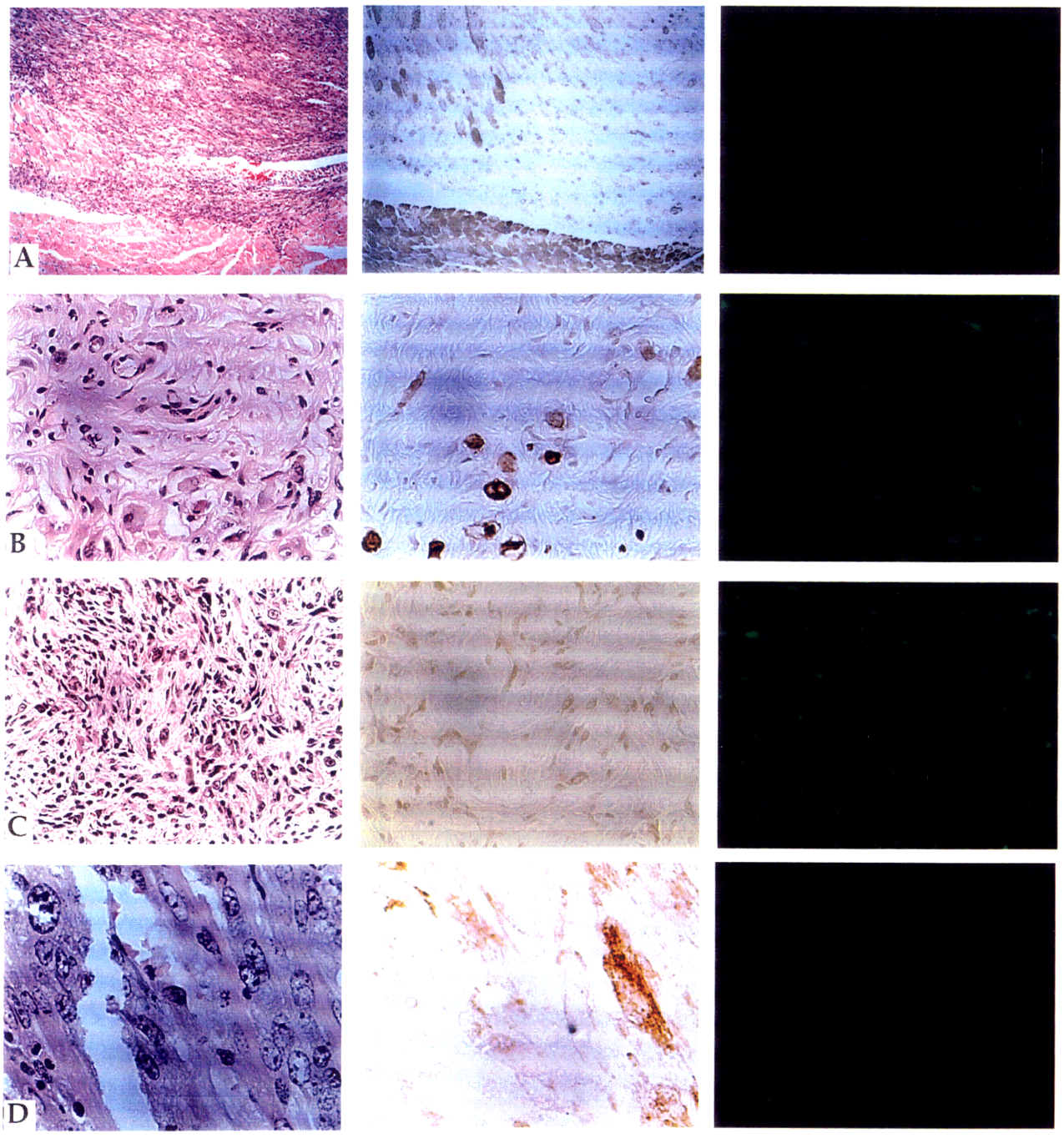
*Some tumors were negative for both markers

Table 4.2. Approximately one-third of the tumors stained positive for α-sarcomeric actin. Twenty-one percent of the tumors stained positive for either S-100 or MBP, or both. In addition, 12% of the tumors were positive for both types of markers. The different combination of *Nf1* and *p53* mutant allele did not seem to bias the development and differentiation of cell types in these tumors. *Some of the tumors did not stain with either marker (9 'cis' and 3 'H-M' tumors).

Figure 4.5. Immunohistochemical analysis of representative sarcomas in 'cis' and 'H-M' animals.

Anaplastic sarcoma sections from each genotype are tested for markers against α-sarcomeric actin, S-100 and MBP (not shown). (A) 'cis' rhabdomyosarcoma. Note positive α-sarcomeric actin staining in normal muscle staining as well as neoplastic regions. Only background S-100 staining is seen (magnification 40x); (B) 'cis' anaplastic sarcoma. Tumor has infiltrated into surrounding muscle, displacing individual muscle fibers. Cells extending processes stain positive for S-100 (magnification 400x); (C) 'H-M' anaplastic sarcoma. H&E staining show combination of spindle-shaped and pleomorphic cells. Note that the tumor comprise of a mixture of S-100 and α-sarcomeric actin-positive cells (magnification 100x); (D) 'H-M' rhabdomyosarcoma. α-sarcomeric actin stains striations in rhabdoid cells (magnification 400x).

Figure 4.5



Discussion

The data presented in this paper illustrates that the *Nf1* and *p53* tumor suppressor genes act synergistically to exacerbate the tumor phenotype and decrease survival. We generated two distinct *in vivo* murine models that were useful in assaying for the effects of simultaneously losing both gene functions. One novel phenotype produced in the models was a set of lesions reminiscent of human malignant peripheral nerve sheath tumors (MPNST), a condition known to be strongly associated with the inherited NF1 disease. This is consistent with data in humans suggesting that *p53* loss contributes toward the progression of neurofibromas to neurofibrosarcomas (Menon et al., 1990). Furthermore, this study represents the first demonstration of the simultaneous loss of two linked tumor suppressor genes and its effect on tumorigenesis.

Concomitant loss of two linked tumor suppressor genes

The concomitant loss of both the *Nf1* and *p53* genes observed in tumor DNA samples was not surprising given the tight linkage of the two loci on mouse chromosome 11 (Figure 4.2). Several molecular mechanisms may explain the LOH event, including inactivating mutation, mitotic recombination, gene conversion, large deletion, or chromosomal loss followed by duplication. However, due to the proximity of the two loci, several of these mechanisms could result in the observed LOH. We favor the chromosomal loss mechanism for several reasons: First, 100% of the LOH observed in the sarcomas demonstrated the loss of the wild-type allele, ruling out the possibility of point mutations or small deletions. Furthermore, by analyzing simple sequence length polymorphism (SSLP) markers that are well-defined for many mouse strains, it has been observed by several groups that LOH associated with neoplasia is usually accompanied by loss of polymorphic markers on the entire chromosome (Luongo & Dove, 1996; McClatchey, submitted). However, since the mouse population used in this study is derived from several generations of backcrossing mixed 129SV and C57BL/6 parents, the resulting randomization of strain-specific

SSLP markers did not allow us to utilize such markers to clarify the mechanism of loss in the sarcomas.

It was documented that the 'trans' and 'cis' mice develop tumors with clear LOH at the tumor suppressor loci (Figure 4.3). However, the fact that 'cis' tumors appear at a much earlier age (mean survival age of 5 months compared to 14 months for the 'trans' population) suggest that the configuration of these two linked tumor suppressor genes may confer great advantage toward tumor transformation. There are two mechanistic explanations for the early onset of tumors: (1) in 'trans' animals, LOH at the tumor suppressor loci occur at the same rate as the 'cis' animals, but additional genetic events are required for tumor progression; (2) the 'cis' configuration increased the likelihood of LOH, possibly through greater genomic instability.

Cooperativity between tumor suppressor genes

Previous studies on genetic cooperativity in mouse models of tumorigenesis provide insights into the development of malignant sarcomas in the *Nf1-p53* mice. For example, by combining these genetic mutations, the sarcomas appear earlier with increased pleomorphic morphology. This is reminiscent of the faster progression to malignancy of skin tumors resulting from breeding the *H-ras* mutation into a mutant *p53* background (Kemp et al., 1993). The development of a novel lesion in the 'cis' and 'H-M' mice which is similar to the human Triton tumor suggests that *Nf1* and *p53* might play a role in the differentiation pathways of such precursor cell types (see below for further discussion on the etiology of Triton tumors). Williams et al. (1994) found the appearance of several novel tumors in *Rb* mutant mice after crossing the mutation onto the *p53*-deficient background. These include the rare pinealoblastomas and islet cell tumors. In addition, studies combining the *Wnt-1* transgene with the *p53*-mutation found an earlier onset of the same tumor type, mammary adenocarcinomas. However, the tumors appear less differentiated with more cells showing aneuploidy (Donehower et al., 1995). This was attributed to *p53*'s role in promoting genomic instability and cellular proliferation. Furthermore, it was found that the tumor

promotion seems to be unrelated to decreased level of apoptosis as a result of *p53* deficiency (Jones et al., 1997). Sometimes, combining specific gene mutations may serve to promote the development of one tumor type but not the others. For example, the *p53* and *Apc* double mutant mice showed higher pancreatic tumor risk but did not exhibit increased intestinal abnormalities (Clarke et al., 1995). Given the predicted involvement of both genes in colorectal cancer progression, this result suggest that *p53* and *Apc* mutations alone may not be sufficient to induce intestinal cancer.

The *ras* gene is often seen as the counterpart of *p53* in the area of oncogenes because of the frequency of mutations (30%) found in human cancers. In fact, *ras* has been implicated in many types of cancer (review Bos, 1989), and most notably has been included as a pre-malignancy mutational event in the multi-step model of colorectal cancer development (Fearon & Vogelstein, 1990). The *ras* signaling pathway is also one of the most well-defined in all of biology, with upstream molecules such as tyrosine kinases, *GRB2* and *mSOS* and downstream effectors including -- but not limited to -- *Raf*, *MEK*, and the rest of the *MAP Kinase* pathway (review Egan & Weinberg, 1993). Neurofibromin is one of two well-defined negative regulators of mammalian *ras* , the other being the *GAP* (review Bollag and McCormick, 1992). It is therefore reasonable to hypothesize that activated *ras* contributed toward the tumor progression seen in the *Nf1-p53* animals. For example, increased levels of *ras* signaling could provide the necessary mitogenic signals to drive tumor cells through the cell cycle (provided that the necessary nutrients are available). Furthermore, the effect of activated *ras* differs widely in different cell types, inducing proliferation in NIH 3T3 and fibroblasts, while promoting differentiation and growth inhibition in cells such as the cultured pheochromocytoma line PC12 (Greene and Tischler, 1976). *Nf1* deficiency in nerve sheath precursor cells might confer growth advantages or survival factors similar to that of an activating *ras* mutation. Interestingly, mice inheriting mutations in *Nf1*, *p53* and *GAP* are predisposed to the development of hemangiosarcomas (T.S. Shih, unpublished results). This could be due to the action of these genes in cells comprising of the vasculature, as

suggested by the development of a vascular phenotype observed in embryos deficient in *Nf1* and *GAP* (Henkemeyer et al., 1995).

Effect of the *Nf1* and *p53* mutations in 'trans'

A statistical comparison revealed that the 'trans' animals had a lower survival curve when compared to a theoretical kill curve (not shown). This hypothesized kill curve was constructed by taking into account the statistical likelihood of 'trans' animals to die from either the *Nf1* or *p53* mutation alone, i.e. without synergistic effects toward tumor progression. Such evidence suggest a slight cooperative effect of combining germline mutations in *Nf1* and *p53*, even in the 'trans' configuration. It is possible that heterozygosity for *Nf1* increases the level of activated ras in certain crucial cell types, or that *p53* heterozygosity predisposes the same cells to chromosomal aberrations, resulting in decreased tumor latency.

Developmental effect of Nf1 and p53 mutations in 'cis'

Furthermore, in our attempt to generate the 'cis' chromosome by breeding, only 12% of the progeny carrying recombinant chromosomes were of the 'cis' configuration. This suggests that either the gametes or the developing animals carrying the 'cis' chromosome are somehow selected against. The mechanism underlying this reduction in developmental fitness is not clear at the present time. However, it is interesting to note that the male/female ratio for the four founder 'cis' mice as well as their subsequent progeny was close to 1:1. This result argues against a selection pressure based on gender.

MPNST and NF1

The histopathology of sarcomas seen in the *Nf1-p53* 'cis' and 'H-M' mice closely resemble those of human tumors classified as malignant peripheral nerve sheath tumors (MPNSTs). MPNSTs account for approximately 5-10% of all soft tissue sarcomas in human (Hashimoto, 1995; Fletcher, 1995), and are defined as aggressive neoplasm typically, but not exclusively, occurring in major

trunks of the peripheral nerve or benign neurofibromas of NF1 patients. Traditionally, Schwannomas, neurofibrosarcomas, perineuriomas, as well as other less-differentiated lesions are included in this category. MPNSTs often contain a heterogeneous cell populations, with the two most common being (1) compact, spindle-shaped cells arranged in interlacing fascicles, usually expressing Schwann cell markers such as S-100 and MBP and (2) pleomorphic cells with varying amounts of eosinophilic cytoplasm. In recent years, the advent of immunohistochemical techniques and electron microscopy have become crucial in the diagnosis of MPNSTs. Ultrastructural studies indicate that the predominant cell types in MPNSTs are Schwann cells, fibroblasts and perineurial cells (reviews Fletcher, 1995; Hirose 1992). Johnson and colleagues (1988) found that approximately 60% of MPNSTs expressed the S-100 protein (17/28). However, the same study stressed that the immunohistochemical profiles of MPNSTs do not always correlate exactly with the cell morphology or pathological patterns. This histopathological description resembles that of the set of sarcomas found in the 'cis' and 'H-M' animals. A subset (approximately 10%) of MPNSTs contain heterologous elements, or cells displaying differentiation distinct from the nerve sheath (Fletcher, 1995). The most commonly observed differentiation pattern is rhabdomyoblastic, called "Malignant Triton tumors," are defined as malignant nerve sheath tumors or Schwannomas with rhabdomyosarcomatous differentiation (Brooks, 1988; Woodruff, 1996). Triton tumors account for approximately 16% of all MPNSTs, and typically represent much worse clinical prognosis than MPNST in general (Brooks, 1988; Woodruff, 1996). The designation of "Triton" came from experiments conducted in Triton salamanders (genus Triturus) where sciatic nerve transplants evolved both neural and muscular elements (Masson, 1932). Hallmark traits of Triton tumors include (1) the presence of two distinct cellular regions (spindle and pleomorphic) in the same tumor, (2) skeletal muscle actin striation in differentiated rhabdoid cells, and (3) immunohistochemical confirmation of muscle-specific markers such as α-sarcomeric actin and myoglobin.

A clear involvement of the *NF1* mutation in MPNSTs and Triton tumors is has been established in clinical studies, as evidenced by studies noting the predominance of Triton tumor patients with family histories of NF1 (Sordillo et al., 1981; DeWit et al., 1986; Ducatman et al., 1986, Hruban et al., 1990). In addition, the human NF1 population exhibit an increased frequency of Triton tumor development (1-4%) (Fletcher 1995; Doorn et al., 1995). Sporadic MPNSTs arise in humans at the mean age of 40 years, while the NF1-associated MPNSTs become apparent on average 10 years earlier in life (Fletcher 1995). Lastly, NF1 mutations have been found in a subset of MPNSTs previously defined as neurofibrosarcomas (Legius, 1993). The correlation of *p53* mutations in MPNSTs has also been demonstrated by the occasional LOH at that genetic locus on chromosome 17p (Legius, 1994; Jhanwar, 1994, Lothe, 1995), loss of *p53* expression in MPNSTs (Lothe et al., 1995) or by the overexpression of the presumed mutant *p53* protein in MPNSTs but not benign PNSTs (Kindblom et al., 1995).

In mice, the effect of the *Nf1* mutation has been studied in both Schwann cells and fibroblasts. *Nf1*-deficient Schwann cells show properties consistent with an activated *ras* mutation, i.e. decreased cell division in response to glial growth factor 2 (Kim et al., 1995). This suggests that mutations in *Nf1* are required, but not sufficient, for abnormal Schwann cell growth in tumors, and that the *p53* mutation may be a necessary second mutation for proliferation. *Nf1*-deficient fibroblasts exhibit only slight increases in growth properties, and a failure to form perineurium in cultured conditions (Rosenbaum et al., 1995). Again, the lack of a drastic effect on nerve sheath proliferation suggest that another mutation is needed in addition to *Nf1* for tumor transformation, as is the case in the 'cis' and 'H-M' animals.

Triton tumors

Several theories have been put forth to explain the divergent differentiation observed in Triton tumors. Some theories such as (1) induction by endoneural cells (Masson et al., 1932), (2) de-differentiation due to some type of teratoid influence (Shuangshoti et al., 1979), or (3) a specific

type of "collision tumor" of Schwann and rhabdoid elements (Naka et al., 1975), all have little scientific support. The most widely accepted theory, called the "ectomesenchymal theory," was developed from the neural crest precursor cell concept initiated by Horstadius (Woodruff, et al., 1973, Karcioglu et al, 1977; Ducatman et al., 1984). According to this theory, neuroectodermal structures evolve from migrating neural crest cells, a transient cell population existing only in early development. These neuroectodermal structures include neurons, nerve sheath cells, melanocytes, ganglion cells, as well as mesenchymal tissues such as bone, cartilage and muscle. A few of these cells, such as the Schwann cell, are known to possess the ability to differentiate into other tissues. The ectomesenchymal theory draws upon such pluripotent characteristics to suggest that neuroectodermal tumors, especially of the peripheral nerve sheath, may recapitulate the developmental lineage of neural crest cells. In fact, it has been suggested that Schwann cells may possess the innate program to de-differentiate and the "trans-differentiate" into rhabdomyoblastic cells (Nathanson, 1986). MPNSTs, therefore, are tumors of the "ectomesenchymal" type dominated by Schwann or rhabdoid elements. This theory corresponds well with the involvement of the *Nf1* mutation in the murine models, because human NF1 lesions occur primarily in tissue types derived from the neural crest lineage (melanocytes, peripheral nerve sheaths, facial bone, etc.) (Le Douarin, 1982). The *p53* mutation may serve to promote tumors in cell types predisposed to abnormal growth by the *Nf1* mutation.

Finally, we believe that the murine models described here provide a reproducible --and previously unavailable -- solid tumor model associated with mutations in not only in the *NF1*, but the*p53*. tumor suppressor gene as well. Such models are suitable for screening therapeutic agents aimed at either (or both) of these molecular targets. Furthermore, this model not only affords the opportunity to study the *in vivo* tumor response, but also *in vitro* assays using cell lines derived from these lesions. This is particularly exciting since the sarcomas found in the *Nf1-p53* animals share growth and histopathological features with the lesions belonging to the important clinical classification of MPNSTs.

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Chapter 5

Summary and Implications

By the time this thesis project was initiated, the human *NF1* gene had been identified and cloned for less than two years. In the years since, relatively little was known about the precise function of the gene and its protein product, despite a flourish of reports documenting the gene's mutational spectrum and patterns of expression. To summarize the findings: It has been established -- across several species -- that *NF1* is expressed ubiquitously in early development, and becomes progressively restricted to various neural crest-derived tissues beginning in mid-gestation and through adulthood. The adult tissues showing high levels of *NF1* expression were consistent with many of the tissues affected in NF1 patients, the most striking of which being neural crest-derived tissues (peripheral nerve sheath, melanocytes, adrenal chromaffin cells, etc.). To date, the only clearly assigned function to the *NF1* protein (neurofibromin) is its ability to downregulate the p21*ras* oncogene signaling pathway. This anti-oncogenic activity, together with the frequent loss of heterozygosity (LOH) at the *NF1* locus in human tumors, suggest that *NF1* normally functions as a tumor suppressor gene.

Yet, the progress toward understanding of the NF1 disease has remained stagnant over the past several years. The lack of progress may be attributed to the large size of the gene's coding domain, the difficulty to express and develop functional assays using the entire protein, and the extremely variable disease severity found across the human patient population. Of these, the inability to address even the most basic functions of *NF1* in normal and diseased cellular processes has been frustrating, given the prevalence of NF1 and the potentially debilitating symptoms associated with the disease. In my efforts to create a mouse model of Nf1, I focused on trying to answer the following questions:

- What role does *Nf1* play in development?
- What are the crucial cell type(s) in neurofibromas?
- What genetic events are required for the development of neurofibromas?
- What additional events are needed for malignant progression of these lesions?
- What genetic and environmental factors modulate the expressivity of the NF1 disease?
- What functional role does neurofibromin play in influencing various disease pathways?

- Are there other functional domains on neurofibromin?
- What are the upstream regulators and downstream effectors of neurofibromin?
- Can we develop a good set of biological reagents to better answer these questions?

This thesis attempts to (1) understand the genetic basis for the NF1 disease and (2) utilizing that knowledge to generate mouse model that accurately recapitulate the various lesions found in human NF1 patients and (3) eventually use that model to help develop effective treatments for NF1 complications. Mouse models are ideal for this type of analysis given the ability to modify and introduce various gene sequences *in vivo* and *in vitro*. Furthermore, clinically-relevant animal disease models are often ideal candidates for pharmaceutical companies to assess the preclinical drug safety and efficacy profiles prior to human trials. I will discuss below one such compound with the potential to treat NF1 patients. Therefore, in this final chapter, I intend to summarize the progress I have made toward each of those goals, describe the implication of those findings, and then raise issues that have not been adequately addressed.

Gene targeting

When the germline *Nf1* mutation was created in mice via homologous recombination (Chapter 2), many other mouse genes have already been functionally disrupted using similar techniques. Most targeting vectors included the insertion of a selectable marker, the neomycin resistance gene (*neo*), in the opposite transcriptional orientation relative to the gene of interest. This selectable marker was important for both disrupting the gene function as well as identifying cells with the appropriate homologous gene integration. In an effort to provide a detailed characterization on the molecular/splicing effect of the *neo* gene insertion, we determined that the *neo* coding sequences, when inserted in the opposite orientation, provides a set of very efficient splice donor and acceptor sites. As a result, the mutant transcript from the *neo*-disrupted *Nf1* allele included a 473 nucleotide stretch of antisense *neo* sequence (Jacks et al., 1994), instead of a series of potential translational

stop codons expected with this type of insertional mutagenesis events. In fact, all mutant alleles constructed in this manner should be expected to generate such aberrant transcripts, which may result in the production of proteins with unexpected functions. This has potentially significant implications in the design of targeting vectors for homologous recombination, given the explosion of knockout mouse strains in the years since the publication of our report.

Role of *Nf1* in development

Neurofibromin is expressed at high levels in every cell type in early embryogenesis, and becomes increasingly restricted to the PNS, white blood cells and the adrenal medulla after mid-gestation. Given the ubiquitous expression early in development, it was surprising to find that *Nf1*-deficient embryos were able to survive up to day 13.5 of gestation. One possible explanation for the lack of an *Nf1* phenotype prior to mid-gestation (despite high levels of gene expression) is that of functional redundancy, i.e. the existence of other proteins which may compensate for the loss of the *Nf1* protein. In fact, based on the proposed function of neurofibromin as a GTPase activating protein (GAP), it is likely that other GAP proteins are active early in embryogenesis in addition to *Nf1*.

The cause of death was a restriction of blood flow due to the combination of abnormal heart development (double outlet right ventricle) and a thickening of endocardial cushion tissues in the cardiac outflow tracts (i.e. the aorta and pulmonary artery). These features provided further support for the involvement of neural crest tissues in the NF1 disease tissues, largely because of previous observations made in avian neural crest experiments: laser ablation of neural crest cells in birds resulted in cardiac defects reminiscent of the mouse *Nf1* mutant phenotype, but the endocardial cushion hyperplasia was noticeably absent. Thus, the cushion defect may be a specific functional consequence of *Nf1* deficiency in these cell types. A second explanation for the cardiac phenotype may be related to the onset of Type III neurofibromin isoform expression in cardiac

muscle during mid-gestation (Gutmann et al., 1995a). Perhaps Type III neurofibromin confers the predominant GAP activity in the embryonic heart, without which the cells lining the outflow tracts fail to cease proliferation (Chapter 2).

A separate developmental phenotype associated with the loss of *Nf1* function was described in neonatal DKO chimeras -- this time affecting another neural crest-derived tissue: the adrenal medulla. It appears that *Nf1*-deficient chromaffin precursors are either unable to migrate completely into the adrenal primordium, and differentiated ectopically into adrenal chromaffin cells (Chapter 3). An alternative scenario is the abnormal growth and metastasis of chromaffin cells into the surrounding tissues. In either case, the adrenal medullary defect most likely resulted in neonatal lethality by causing dysregulation of epinephrine and norepinephrine production in mice. Neurofibromin may play a role in signals required for migration, due to its physiological association with microtubules (Gregory et al., 1993). However, it does not appear that *Nf1* function is required for proper differentiation of adrenal chromaffin cells.

Nf1 in benign lesions

Despite being two of the most common lesions of human the NF1 disease, the development of cafe-au-lait spots and Lisch nodules have not been detected in the mouse mutant *Nf1* models. This was true both in the case of the *Nf1* heterozygous mice as well as the *Nf1* DKO chimeras (positive contribution of *Nf1*-deficient ES cells to the iris and skin has been suggested by GPI analysis and/or X-gal staining). The absence of these two benign phenotypes may be attributable to the inability of the *Nf1* mutant locus to undergo LOH (a possible rate-limiting step required for abnormal tissue growth). To address whether *Nf1* deficiency is necessary in the initiation of benign growths, it would be interesting to quantify the extent of *Nf1* LOH in iris and skin melanocytes of *Nf1* +/- adult mice without cafe-au-lait spots or Lisch nodules (these experiments

are now feasible due to improvements in single-cell PCR and trace DNA isolation techniques). If no LOH is detected even in aging animal tissues, it may suggest iris cells and skin melanocytes have high genomic stability, or that the mouse susceptible "target cell" population is too small for any meaningful LOH to occur during the animals' lifetime.

On the other hand, intrinsic physiological differences between mice and human in these two cell types may preclude the development (or detection) of cafe-au-lait spots and Lisch nodules. For example, mouse skin normally undergoes wave-like patterns of continuous hair follicle and melanocyte regeneration, which then induces high levels of natural melanin production throughout their lifetime. Therefore, it may be impossible to differentiate between normal skin hyperpigmentation and cafe-au-lait spots arising from Nf1-deficient melanocytes.

Role of *Nf1* in tumor suppression

Consistent with the Knudson model of tumor suppressor genes (Knudson, 1971), our work suggests that the loss of both *Nf1* alleles is a step toward tumorigenesis in at least four tumor types: pheochromocytoma, myeloid leukemia, neurofibroma and malignant peripheral nerve sheath tumors (MPNSTs).

Pheochromocytoma

Animals inheriting one mutant copy of *Nf1* are prone to developing tumors (unilateral or bilateral) of the adrenal medulla (pheochromocytoma), albeit late in their normal life expectancy (Chapter 2). Pheochromocytoma is another component of the *Nf1*-related neurocristopathy proposed for the involvement of various neural crest derivatives in NF1 lesions. Our analyses concur with human studies in suggesting that loss of *NF1* function is required for the onset of malignant pheochromocytomas in patients with or without other NF1 clinical features (Xu et al., 1992; Gutmann et al., 1995b). Functionally, this provides an argument for neurofibromin as a

downstream effector of ras: It has been established that transient activation of ras causes proliferation while constitutively activated *ras* induces differentiation and inhibit proliferation of neuronal and PC12 cells, a rat pheochromocytoma cell line (Greene and Tischler, 1976). Therefore, since the loss of *NF1* is expected to result in prolonged *ras* activation, it suggests that neurofibromin is functioning as an effector in the differentiative signaling pathway of *ras*.

Myeloid leukemia

Benign myeloid hyperproliferative disorders were seen in both the DKO chimeras and *Nf1* heterozygous animals (Chapters 3 and 2, respectively). Consistent with the human genotypic analyses of NF1-related juvenile chronic myeloid leukemia (JCML) (Shannon et al., 1994), both of these murine myeloid tumor models are accompanied by clear LOH at the *Nf1* locus (Chapter 2). Pathologically, the murine tumors are reminiscent of human myeloid disorders due to the presence of predominantly well-differentiated cells in the myeloid lineage, suggesting a benign lesion rather than a full blown leukemia. Biochemical assays show these murine cells to have abnormally high ras-GTP levels, and are hypersensitive to GM-CSF stimulation -- a pathway known to signal through ras (Largaespada et al., 1996). Therefore, neurofibromin seems to be a classic negative regulator of ras signaling in response to growth factor stimulation, and that the loss of one or both alleles of *NF1* may accordingly lower the proliferative stimulation threshold for the myeloid lineage.

Modifier Gene Effects?

One of the more curious aspects about the NF1 disease is the variable expressivity of phenotypes across the human population. Two explanations have often been proposed as the underlying causes for such variability, including the presence of modifier genes and other ectopic factors, such as environmental influences.

It has long been assumed that environmental effects, while playing a potentially important role in the regulation of the disease phenotype, are not easily isolated or quantifiable from the study of the human NF1 population (due to the inability to control many such conditions among human patients). On the other hand, attempts to link disease severity to specific gene mutations have revealed subtle correlations at best (Cnossen et al., 1997). In fact, it has been demonstrated consistently that NF1 patients inheriting identical gene mutation can develop vastly different symptoms. The only known genotypic-phenotypic relationship has come from studies of monozygotic twins (with identical DNA contents), who tend to exhibit a more similar (though not the same) spectrum of lesions than more distantly-related NF1 individuals, such as dizygotic twins (Easton et al., 1993). To date, the human data can be used only to implicate the involvement of environmental and genetic components in regulating the onset of various NF1 lesions, but fall well short of suggesting the identity or extent of such influences. Lastly, given the diversity of its genome through randomized breeding, the human model does not provide an easy reagent from which to clone and identify modifier genes involved in the NF1 disease.

We believe that the *Nf1* mouse tumor models may yet prove valuable in providing the necessary *in vivo* tools to elucidate some of the genetic components affecting NF1 disease onset and development. First of all, we were able to minimize the impact of environmental variabilities by maintaining our mouse study population under strictly-controlled laboratory conditions. Secondly, because the *Nf1* mice show tumor development in a strain-dependent manner (Chapters 2 and 3), it therefore suggests the existence of strain-specific modifier genes which serve to modulate the onset of certain tumor types. In fact, myeloid proliferative disorders have been found only in 129sv inbred *Nf1* +/- mice or in DKO chimeras generated from 129sv inbred *Nf1* -/- ES cells. On the other hand, pheochromocytomas were exclusively detected in the 129sv x C57BL/6 mixed background animals (both in animals inheriting germline mutations as well as DKO chimeras).

Evidence supporting a genetic component in (1) the modulation of disease predisposition as well as (2) drug efficacy in patients from various genetic backgrounds has led to the emergence of a field in modern medicine that focuses on establishing genetic "profiles" of patients. The goal of genetic profiling is to identify a set of genetic markers that determines why some patients develop a certain set of malignancies and, more importantly, why only a percentage of patients respond to a particular treatment, while the same treatment show little efficacy on others (also known as "pharmacogenomics"). Researchers in this field intend to use the power of genomics research and high-throughput screening to identify these modifier genes. Eventually, this genetic data will be used to improve disease management protocols in healthcare, be it interventional or prophylactic.

The cancer genetics data shown in Chapters 2 and 3 is critical to this area of research because it provides an *in vivo* situation showing clear-cut divergence of NF1 clinical symptom based on genetic differences. Therefore, such a model may be used to identify and clone individual modifier genes in mice that influence the development of each NF1-related phenotypes -- by extending the same type of genetic/phenotypic analysis across other *Nf1* inbred mouse strains. If modifier gene candidates were confirmed in the *Nf1* murine models, the study of their human homologs may eventually be useful in (1) determining the predisposition of certain patient segments to succumb to various NF1 malignancies and (2) defining other players in the genetic pathways of the disease. A prime example of this type of research is the cloning of *Mom-1*, a modifier locus of the intestinal polyp numbers in *Apcmin* mice (Dietrich et al., 1993). More recently, the phospholipase A2 gene was suggested as the actual modifier gene residing in the *Mom-1* locus (MacPhee et al., 1995).

Mechanistically, the differences in tumor spectrum may be a result of (1) slight variations in how the neurofibromin signaling pathways are regulated (e.g. mixed background animals may have redundant GAP functions in myeloid cell lineages, which prevents the cells from abnormal proliferation) or (2) differential regulation of neurofibromin activity (e.g. lower expression or higher rate of turnover of neurofibromin in 129sv chromaffin cells such that the physiological level

of active p21*ras* remains higher relative to other strains). As opposed to myeloid leukemia and pheochromocytoma, the development of neurofibromas (in timing, size and number) does not seem to vary across these two strains. This either suggests that (1) modifier genes required for neurofibroma development is present in both murine strains examined in this thesis or (2) the presence of an *Nf1* mutation alone may be sufficient to induce neurofibroma formation without the influence of modifier genes. If the latter case was true, it may explain why neurofibromas are so prevalent in the human NF1 population. To address these questions, the *Nf1* mutation is currently being bred onto different inbred mouse backgrounds. If any of these different mutant *Nf1* strains show definitive changes in the presentation of NF1-related lesions, a full-scale search will commence for possible modifier genes, using the densely-mapped (and commercially-available) chromosomal markers.

Neurofibroma associated with simple *NF1* LOH?

Ever since the cloning of the *NF1* gene, the question of whether *NF1* LOH is required for neurofibroma formation has remained unresolved. This is partly because the search for the inactivation of the second *NF1* allele in these benign lesions has produced mostly negative results, with only a few recent reports demonstrating LOH in some lesions (Colman et al., 1995; Sawada et al., 1996; Serra et al., 1997). Despite this ambiguity of data, there is little doubt that *NF1* heterozygosity is a prerequisite toward the development of neurofibromas, given that all NF1 patients are constitutionally heterozygous for the *NF1* mutation. It is worth noting again, however, that in human NF1 clinical studies, the inability to control for genetic and other environmental variables makes it extremely difficult to identify and/or rule out the involvement of other factors which may promote neurofibroma formation.

With this in mind, it was nonetheless a surprising observation that no neurofibromas were ever found in heterozygous *Nf1* mice (Chapter 2). Perhaps the absence of only one *Nf1* allele was insufficient to induce tumor growth -- and that additional genetic events or environmental factors

are required (during the animal's lifetime) for initiation. In Chapter 3, we described the detection and characterization of multiple plexiform neurofibromas in *Nf1* DKO chimeras, a mouse model in which a number of *Nf1* deficient cells are randomly introduced into various adult tissues. In fact, increased numbers and size, as well as earlier onset of neurofibromas are correlated with higher levels of *Nf1* -/- cell contribution. Given the early onset of these peripheral nerve lesions (as early as 2.5 months of age), it is unlikely that the cells responsible for neurofibroma formation have had enough time to incur a number of other mutations. Taken together, these observations extends the current thinking about neurofibroma etiology by implying that simple *Nf1* deficiency may be sufficient to initiate and promote neurofibroma growth. However, additional data will be needed to validate this hypothesis. One such direct proof includes demonstrating the presence of *Nf1* deficient cells in these murine lesions. Hopefully, this evidence will become available in the near future using the *ROSA-26* transgene as a molecular marker in *Nf1* -/- ES cells (Chapter 3).

Cellular composition of neurofibromas

The murine neurofibromas appear to lack the variable cellular composition relative to their human counterparts. In humans, approximately 40-85% of the neurofibroma cells are believed to be of the Schwann cell origin (Stefansson et al., 1982), although the recorded range vary significantly between individuals and from study to study. In contrast, our murine neurofibromas are almost entirely perineurial in nature, as determined by immunohistochemical and ultrastructural evidence (Chapter 3). Does this observation imply intrinsic differences between the human and mouse tumorigenic pathways? Indeed, it is possible that the particular exon 31 mutation we generated in mice specifically caused the hyperplasia of perineurial cells. As mentioned previously, however, such phenotype-genotype correlation has not been conclusively demonstrated in humans. Furthermore, the mouse neurofibromas may lack the cell type variability because, contrary to studies using human subjects, we were able to more closely control (and alter) the genetic background and environmental influences in our mouse population.

The difference in cell type composition may also be explained by a major discrepancy between the genetics of the mouse and human disease systems: In the adult DKO chimeras, *Nf1* null cells are typically surrounded by wild-type (+/+) cells. In the human case, however, these *Nf1* null cells would be surrounded by cells heterozygous (+/-) for the *Nf1* mutation (every human NF1 patient is born constitutionally heterozygous for the *NF1* mutation). A simple and elegant way to recapitulate the human condition in mice is to inject *Nf1* null ES cells into blastocysts heterozygous for the same mutation, hence the resulting chimeras would be strictly composed of *Nf1* +/- or -/- cells. It is possible that haploinsufficiency in *Nf1* function is critical to the development of neurofibromas in addition to the LOH event, for example, in providing appropriate environment cues (e.g. cell surface molecules) or increasing certain cell types' sensitivities to various growth factors.

As mentioned previously, one of the unanswered questions is the identity of the *Nf1*-deficient cell type(s) responsible for the tumor growth. So far, our attempts at identifying the *Nf1* DKO ES cells in the mouse lesions have fallen short (*in situ* hybridization, *in situ* PCR, etc.) However, only recently were we able to observe the development of neurofibromas in a ROSA-26 DKO chimera, which could finally render the visualization of null cells in neurofibromas technically feasible. The potential drawbacks to this approach are (1) the variability in β-gal staining patterns in control PNS tissue sections and (2) the problem of X-gal penetration into the tumor (Chapter 3). However, preliminary data suggest that the current staining protocol may be able to yield specific staining patterns in the cell types involved. This should be one of the top short-term priorities for the project.

The neurofibroma is the only benign lesion associated with NF1 with the potential to progress into MPNSTs. Such malignancies are both painful and generally lead to poor patient prognosis. The findings presented here will hopefully provide a framework that defines the minimal genetic alterations that are necessary and sufficient for the development of neurofibromas, and will

subsequently lead to rational disease management -- and possibly therapeutic -- protocols for the NF1 patients.

MPNST

Malignant lesions associated with NF1 include neurofibrosarcomas, Schwannomas, rhabdomyosarcomas, fibrosarcomas, as well as other undifferentiated sarcomas. There is evidence implicating the *p53* tumor suppressor gene in malignant transformation of peripheral nerve sheath lesions (Menon et al., 1990). This was the underlying rationale for the experiments in Chapter 4, which described three mouse models inheriting various combinations of germline *Nf1* and *p53* mutations. Two of these mouse strains, the 'cis' (*Nf1-p53* double heterozygotes on the same chromosome) and the 'H-M' (*Nf1* +/- and *p53* -/-) were prone to developing large, solitary sarcomas within five months of birth.

In the mouse genome, the *Nf1* and *p53* loci are closely linked (5 cM). Therefore, in both the 'cis' and 'H-M' mice, the LOH of the two mutant alleles was found to occur simultaneously. The data from these mice complement previous studies that simply document the detection of *Nf1* and *p53* mutations in MPNSTs. In fact, the kinetics of tumor formation provide strong (and direct) evidence that *Nf1* and *p53* alone are sufficient to induce malignant transformation of tumors. Since the lack of *p53* confers resistance to a variety of therapeutic protocols, this is a particularly good model for testing cancer drugs because it will also show whether the compound can cause tumor regression in a *p53*-independent manner.

Histological and immunohistochemical analysis showed a wide range of differentiation and pleomorphic features among the tumors -- and frequently within individual tumors -- features that are strongly indicative of MPNSTs (Fletcher, 1995). However, the fact that some tumors are clearly undifferentiated fibrosarcomas while others resemble differentiated rhabdomyosarcomas raises an interesting point: Do these tumors share a common nerve sheath origin, or do they

represent a collection of tumors arising from several different, terminally-differentiated, cell types? This answer to this question is central in understanding the etiology of the tumor model. However, there may not be a clear answer, given (1) the frequent disagreements among pathologists as to what constitutes an MPNST and (2) a lack of strong correlation between genetic mutations and cellular composition in such lesions. The situation is further complicated by the rapid accumulation of chromosomal aberrations and the activation of multiple signaling pathways that usually occur in rapidly-dividing tumor cells. We are hoping to address this question in the short-term by obtaining ultrastructural and additional immunohistochemical data on this set of tumors.

Past and present collaborations

Given the advantages of our mouse model as a well-defined biological reagent, we have initiated collaborative efforts with several principal investigators to study the role of Nf1 in various biological pathways.

- Nf1 signaling in the myeloid lineage to modulate proliferation and differentiation (Dr. Kevin Shannon of UCSF, Dr. Wade Clapp of the University of Indiana Medical School, and Dr. Gideon Bollag of Onyx Pharmaceuticals). Nf1-deficient bone marrow cells were used to reconstitute the hematopoietic cells in lethally-irradiated mice. Results show the development of myeloproliferative disorders in these mice (Bollag et al., 1996), which are currently being treated with the farnesyl transferase inhibitor (described below).

- Immunohistochemical analysis of pheochromocytoma and attempted derivation of cell lines (Dr. Arthur Tischler, New England Medical Center in Boston). Pheochromocytomas derived from the Nf1 mice maintained normal chromaffin differentiation in culture and were found to express epinephrine (Tischler et al., 1995). Since Rb DKO chimeras also tend to develop

pheochromocytomas, we decided to study the cooperative effects of the Nf1 and Rb germline mutations on tumor development. Preliminary data suggest that these animals show an early onset of multifocal pheochromocytoma in the adrenal medulla (unpublished results).

- Cooperative effects between Nf1 and p120 GAP (Dr. Mark Henkemeyer and Dr. Tony Pawson at the Mt. Sinai Hospital, Toronto). The combination of GAP and Nf1 mutations in development led to an early stage embryonic lethality associated with vascular defects and hyperproliferation in the developing nervous system (Henkemeyer et al., 1995). These two mutations cooperated in adult mice to induce a preponderance of hemangiosarcomas (unpublished results).

- A putative role of Nf1 in B cell proliferation mediated through a tyrosine kinases (Dr. Shiv Pillai of the Massachusetts General Hospital in Charlestown). The isolation of bone marrow cells for biochemical studies on the signaling and proliferative effects of Nf1-deficiency.

- The role of Nf1 in learning and memory (Dr. Alcino Silva of Cold Spring Harbor Labs). The combination of Nf1 and other synaptic function mutations (e.g. NMDAR1, a glutamate gated calcium channel) caused deficits in learning and long-term potentiation. Published data suggest a significant alteration of brain signaling and memory, which may begin to explain the mental retardation and learning disability associated with some NF1 patients (Silva et al., 1997).

- The effect of Nf1 deficiency in the neural crest lineage (Dr. Hubert Schorle and Dr. Rudolph Jaenisch at the Whitehead Institute in Cambridge). Nf1-deficient neural crest cells were cultured and transplanted back into murine hosts. In the few animals tested, the detection of melanin-producing cells in lung and muscle tissues suggest that Nf1 null neural crest cells may be defective in migration and differentiation.

• The role of Nf1 heterozygosity in skin melanocytes (Dr. Dieter Kaufmann and Dr. Winfried Krone, Universitat Ulm, Germany). Skin melanocyte cultures were established from Nf1 heterozygous animals for biochemical and functional analysis.

Long-term goals

Despite the progress we have made in understanding the genetic and functional pathways involving *Nf1*, a number of the outstanding questions still remain pertaining to the onset and growth of neurofibromas. Given the limitation of current mouse models, we see a necessity to build better mouse models to address these questions.

Conditional knock-outs

The cell type responsible for the formation of neurofibromas may be identified by using the *cre-loxP* conditional knockout technique described in Chapter 1. Briefly, this model allows for tissue-specific inactivation of gene function by expressing the *cre* recombinase in a restricted set of cell types. Therefore, by disrupting *Nf1* function only in Schwann cells, neurons or perineurial cells in mice, the appearance of neurofibromas can be clearly affiliated with the loss of *Nf1* function in a particular cell type.

Neurofibromin domain-function analysis

Since the cloning of the *NF1* gene in 1990, only a few domains of neurofibromin have been assigned putative functions. Given the large size of the protein and the various signal transduction pathways in which neurofibromin has been implicated, the identification of these domains would move the NF1 field forward considerably. To this end, we suggest using a yeast artificial chromosome to introduce the entire *Nf1* genomic fragment into DKO ES cells. The advantages to this approach include the ability to propagate up to a megabase of mammalian genomic DNA, making it possible to clone the entire mouse *NF1* gene (300 kb genomic region) into a single YAC.

In fact, a YAC encompassing the entire human *NF1* gene has been constructed (Marchuk et al., 1992). However, frequent rearrangements of genes cloned into YACs (especially large fragments) may limit the usefulness of this approach. To avoid this difficulty, a good restriction map/fingerprint of the cloned gene must be available in order to detect a wide range of mutations that might occur during the propagation and transfer of the YACs.

Because the DKO ES cells are functionally null for *Nf1* function, any mutant allele brought in by YACs will become the exclusive source of neurofibromin in these cells. Previous reports have documented successful introduction and expression from YAC DNA fragments in mammalian cells (Pachnis et al., 1990; Strauss and Jaenisch, 1992; Choi et al., 1993; Schedl et al., 1993; Montoliu et al., 1994; Huxley et al., 1996; Matsuura et al., 1996; Li et al., 1997; Maas et al., 1997; Manson et al., 1997). This will constitute a quick and clean system to study the consequences of various NF1 mutations *in vivo*. Mutagenesis and manipulation of the large *Nf1* gene can be greatly facilitated by the powerful recombination machinery in yeast (reviewed in Lamb and Gearhart, 1995). Precise point mutations, insertions and deletions of YAC DNA can be easily made by homologous recombination. Therefore, we envision this approach as an effective screen for functional domains of neurofibromin -- by selectively mutating various regions of neurofibromin (highly-conserved domains as well as the regulatory elements, embedded genes, or the 5'- and 3'-UTR). The resulting phenotype of the YAC chimeras may provide clues to other unknown *Nf1* functions.

Another advantage of this system is the short time frame required to screen for a specific mutation. After generating the mutant *Nf1* YAC construct, it should be a matter of months before a chimera can be obtained and its phenotype analyzed. Furthermore, if a mouse strain reliably succumbs to NF1 lesion (such as neurofibromas, pheochromocytomas, or connective tissue abnormalities), not only have we generated additional mouse models of the NF1 disease, we might also be able to attribute such lesions to a specific domain mutated in the *Nf1* gene. This mutagenesis data may be

helpful in identifying potential binding partners of neurofibromin, and clarifying the biochemical pathways giving rise to NF1 phenotypes as well as the particular cell types in which they act.

Genetic studies of a neurofibromin homolog (60% protein identity) in *D. melanogaster* has provided evidence for an additional ras-independent signaling pathway. Flies deficient for this protein show reduced size and the noticeable absence of a K^+ current upon stimulation by neuropeptides. Interestingly, these phenotypes can be rescued by increased signaling through the cAMP-PKA -- but not the Ras1 -- pathway (The et al., 1997). Such evidence suggest that human neurofibromin may also possess domains which interact with players in the PKA-mediated signal transduction pathway. Clinically, it is possible that *NF1* deficiency may lead symptoms resulting from the abnormal regulation of the PKA pathway, such as learning deficiencies. The YAC transgenesis system may be used to quickly map the protein domains involved in this, as well as other, signaling pathways.

It has been suggested that an expression vector driving the *Nf1* cDNA may be a simpler way to generate such mouse models of NF1. However, several lines of evidence argue for using the YAC approach: First, the YAC vector will supposedly contain the physiological promoter driving Nf1 expression, as well as its nearby regulatory elements (i.e. enhancers and repressors). Second, and probably more importantly, if intronic sequences are crucial to the function and regulation of *Nf1* (e.g. alternatively spliced forms of Nf1 or embedded genes), then a cDNA will not be able to recapitulate normal *Nf1* function.

Therapeutic models of NF1

As mentioned previously, one of the reasons for creating these mouse models of cancer is their utility as animal disease models to be used during preclinical drug testing. Given that the various *Nf1* knockout mice exhibit subsets of the NF1 disease phenotype (within a genetically-defined

system), pharmaceutical companies will be able to pinpoint the design of their drug development trials toward very specific therapeutic indications by using one or more of these models. This is particularly important given the competitive nature of the pharmaceutical industry, the high cost of entering into full-blown clinical trials, and the necessity to demonstrate efficacy toward alleviating well-defined disease symptoms.

One obvious candidate for the treatment of NF1 is a class of compounds called farnesyl protein transferase inhibitors (FPTI) (reviewed in Khosravi-Far et al., 1992). These small molecule drugs act by inhibiting the farnesyl transferase, an enzyme that confers *ras* activity by anchoring active *ras* proteins to the cell membrane. In fact, various forms of FPTI competitively inhibit the active binding site on the protein and, by definition, act as general inhibitor of the ras signaling pathway. Currently, over ten pharmaceutical and biotechnology companies have FPTI research programs, with Merck, Schering Plough and Janssen pharmaceuticals being the closest to filing an investigational new drug application (IND) for the compound (Janssen filed the IND in October of 1997).

This potentially exciting drug has been successfully used to treat activated ras tumor cell lines (Yan et al., 1995) as well as *in vivo* mouse cancer models harboring oncogenic ras mutations (Kohl et al., 1995). Despite early concerns that FPTI may cause serious side effects, given that farnesyl transferase proteins normally function in the retina, skeletal muscle as well as rapidly-dividing tissues (Khosravi-Far et al., 1992), these drugs have shown good clinical efficacy in reducing tumor mass, while including relatively few deleterious features in animal tests (within the therapeutic dose). This lack of serious side effects prompted investigators to examine the mechanism and specificity of FPTI's. In fact, it was recently determined that H-ras protein prenylation is highly sensitive to farnesyl transferase inhibitors, while N- and K-ras are alternatively prenylated by geranylgeranyl transferase-1 (GGTase1) in the presence of FPTIs (Whyte et al., 1997). This was due to the fact that GGTase1 are 100 fold less sensitive to FPTI

inhibition than FTase proteins. In addition, it was found that K-ras4B (the predominant isoform) can be modified by both GGTase1 and FTase *in vivo* and *in vitro* (James et al., 1995; Lerner et al., 1995). That evidence suggest a mechanism where FPTI efficiently shuts down signaling through oncogenic H-ras mutants, but normal cellular signaling through N- and/or K-ras still persists in non-transformed cells at concentrations sufficient to revert malignant phenotypes. Therefore, depending on the type of oncogenic ras (H-, N- or K-ras) proteins found in the tumors, the FPTI specificity toward cancer cells (and non-toxic effects toward normal cells) may be explained by the fact that geranylgeranylation is highly resistant to their inhibitory effects, and may indeed keep physiological ras signaling pathways functioning.

As a side note, it is known that modified Ras is very stable (half-life of 24 hours) (Ulsh and Shih, 1984). Therefore, it is curious that FTase inhibition takes effect much more rapidly than can be explained by simple ras signal depletion alone. A consistent explanation for this type of drug kinetics is an effect on the cellular cytoskeleton, namely the induction of actin stress fiber formation. This observation suggest the Rho-subfamily of proteins as targets of FPTI. In fact, RhoB is modified by both GGTase and FTase with a half life of approximately two hours (Adamson et al., 1992; Lebowitz et al., 1995). Another likely FPTI target could be the RhoE GTPase, which is normally GTP-bound and exclusively farnesylated (Foster et al., 1996). Furthermore, dominant negative mutants of Rho were able to impair transformation by oncogenic ras, similar to FPTI (Qiu et al., 1995). Lastly, Lebowitz et al. (1995) demonstrated that FPTI can suppress ras transformed phenotype by interfering with Rho function.

Together, the effect of FPTI in reducing tumor mass revealed mechanistic implications that were not ras-dependent, as originally assumed by researchers. Subsequent findings on the effect of FPTI showed the reasons behind its efficacy and lack of toxicity, which potentially involves other farnesylated proteins that may be crucial in tumor development. This sort of seredipitous observation, followed by exhaustive mechanistic studies, will eventually help to refine cancer drug

targets. Such detailed understanding of drug action will be especially helpful given the current trend of combining multiple drug therapies (cocktails) simultaneously targeting the various genetic defects implicated in one cancer type.

Biological Models of NF1

In summary, this thesis work had not only been an exercise in the generation of a tumor suppressor mutant mouse strain via homologous recombination. Aside from the creation and detailed characterization of murine myeloid leukemia, pheochromocytoma and neurofibroma models, I believe we have, in fact, opened the door to understanding the tumorigenic and signaling mechanisms of the *Nf1* gene:

First of all, the mouse model has provided powerful *in vivo* and *in vitro* systems to study the function, signaling pathway and mutational consequences of *Nf1*: We now have the ability to systematically manipulate genetic and environmental factors which may be relevant to *Nf1* tumor development. Concrete experimental protocols can be quickly established to test each of these variables alone and in combination. We also have the tools to address the multi-factorial and multi-step nature of neurofibromas, and how such cues would lead to tumor initiation, promotion and progression to malignancy (growth factors, physical trauma, diet, stress, etc.). One way to accomplish this is to study the effect of two or more cancer genes acting in concert, be it in a synergistic or complementary context/pathway. We now have the ability to be able to follow a large number of animals in order to assess the effects of a particular mutation across different genetic backgrounds -- a sort of controlled cancer population study that has previously been deemed unfeasible for the NF1 disease. Such a study can be crucial in defining the complex genetic and environmental factors leading to the variable expressivity of the disease. Finally, using the YAC transgene approach, we now have an efficient screen for functional domains of

neurofibromin, be it as a negative regulator or effector of the ras pathway, for the identification of binding domains and partners, or other yet unrevealed functional protein regions.

Yet, equally important to the scientific pursuits outlined above, these *Nf1* murine models can be applied toward developing more effective therapeutic protocols for human NF1 patients. In contrast to previous *Nf1* animal models, most of which have ill-defined etiology or the inclusion of broadly-acting oncogenes, we now have several clearly-defined mammalian tumor models exhibiting various features of the NF1 disease (both benign and malignant). Expanding the genetic analysis (as described above) on our existing models may eventually help to determine the critical genetic profiles for human NF1 patients -- in order to better define disease predisposition/risk and for drug response profiles.

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