REVIEW ARTICLE

Neurofibromatosis Type 1: Piecing the Puzzle Together

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ABSTRACT: Neurofibromatosis type 1 (NF1) was first described in 1882 and is characterized by a diverse spectrum of clinical manifestations, including neurofibromas, café au lait spots, and Lisch nodules. NF1 is also noted for the higher risk of associated malignancies, making it the most common tumour-predisposing disease in humans. Transmitted in an autosomal dominant manner, the *NF1* gene was cloned in 1990, and belongs to the family of tumour suppressor genes. Since then, there has been an explosion in our understanding of how the gene product, neurofibromin, functions in normal cellular physiology, and how its loss in NF1 relates to the wide spectrum of clinical findings, including NF1-associated tumours. Neurofibromin is a major negative regulator of a key signal transduction pathway in cells, the Ras pathway, which transmits mitogenic signals to the nucleus. Loss of neurofibromin leads to increased levels of activated Ras (bound to GTP), and thus increased downstream mitogenic signaling. Our understanding of neurofibromin's role within cells has allowed for the development of pharmacological therapies which target the specific molecular abnormalities in NF1 tumours. These include the farnesyl transferase inhibitors, which inhibit the post-translational modification of Ras, and other agents which modulate Ras-mediated signaling pathways.

RÉSUMÉ: Neurofibromatose de type I: la solution de l'énigme. La neurofibromatose de type I (NFI) a été décrite pour la première fois en 1882. Elle se caractérise par un spectre varié de manifestations cliniques incluant des neurofibromes, des taches café au lait et des nodules de Lisch. On sait aussi que la NFI confère un risque plus élevé de cancers associés, ce qui en fait la maladie la plus fréquente associée à une prédisposition tumorale chez l'humain. Le gène de la NFI, cloné en 1990, est transmis sur le mode autosomal dominant et fait partie de la famille des gènes suppresseurs de tumeurs. Depuis ce temps, il y a eu une explosion dans notre compréhension de la façon dont le produit du gène, la neurofibromine, fonctionne dans les cellules physiologiquement normales et comment son absence dans la NFI mène à un large spectre de manifestations cliniques dont les tumeurs associées à la NFI. La neurofibromine est un régulateur négatif important d'une voie de transduction d'un signal clé dans les cellules, la voie Ras, qui transmet des signaux mitogènes au noyau. Le déficit en neurofibromine provoque des niveaux élevés de Ras activé (lié au GTP) et donc une augmentation du signal mitogène en aval. Notre compréhension du rôle de la neurofibromine dans les cellules a mené au développement de traitements pharmacologiques qui ciblent les anomalies moléculaires spécifiques aux tumeurs NFI. Parmi ceux-ci, signalons les inhibiteurs de la farnésyl transférase qui inhibent la modification post-traductionnelle de Ras et les autres agents qui modulent les voies de signalisation médiées par Ras.

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Neurofibromatosis type 1: clinical features

The first published case of neurofibromatosis type 1 (NF1) was by von Recklinghausen in 1882,[1] whose name was associated with a diverse group of diseases characterized by multiple peripheral nerve tumours. Recent clinical and genetic understanding has led to the identification of two separate disease entities, termed NF1 (the subject of this paper) and NF2, both grouped together as von Recklinghausen disease in the past. NF1 is the result of alterations of the *NF1* gene on chromosome 17q, clinically characterized by peripheral neurofibromas, café au lait spots (CAL), Lisch nodules, and a myriad of other abnormalities.[2] NF2 results from alterations of the *NF2* gene on chromosome 22,[3,4] and is characterized by bilateral vestibular schwannomas, meningiomas, ependymomas, and astrocytomas.[5] Another point of confusion is the association of NF1 with the Elephant Man Disease, in reference to the elephant man Joseph Merrick who lived from 1862-1890 and suffered from severe disfiguring skin lesions. However, recent re-evaluation of Joseph Merrick's medical history clearly shows that he did not suffer from NF1, but rather suffered from the rare Proteus syndrome.[6]

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The hallmarks of NF1 are the cutaneous neurofibromas, consisting of a mixture of Schwann cells, fibroblasts, and mast cells,[7] which generally do not appear until adolescence. Therefore, other defining features arising earlier in childhood are often the first signs of NF1. These include CAL, which appear during the first year of life and are present in 95% of NF1 adults.[8] Since 3 CAL spots may be present in up to 25% of the normal population,[9] 6 or more CAL spots is considered suggestive of NF1.[10] Freckling in non-sun-exposed regions of the body and Lisch nodules (pigmented hamartomas of melanocytic origin in the iris) are more specific and can be detected early in NF1 patients.[8,11] While these are the most common clinical signs of NF1, a large number of diverse cutaneous, osseous, hematologic, developmental, and nervous system abnormalities are often present.[2] The number and severity of these clinical abnormalities is variable not only between members of different NF1 families, but between members of the same family, leading to occasional confusion in the clinical diagnosis. In an effort to standardize diagnostic criteria, which has tremendously helped both epidemiological and molecular studies on NF1, the National Institutes of Health set uniform diagnostic criteria for NF1, as well as a separate set of criteria for NF2[10] (Table). Despite recent advances in our understanding of the molecular basis of NF1, these clinical criteria continue to be the most reliable means for making the diagnosis.[12]

The two major life threatening complications of NF1 are hypertension and cancer.[13,14] Renal artery stenosis and pheochromocytoma, which occur at a higher frequency in NF1 patients, should be excluded as the cause for hypertension, although most hypertension is primary in nature.[15] Malignancies other than neurofibromas and pheochromocytomas noted at a higher frequency in NF1 patients include malignant peripheral nerve sheath tumours (MPNST), astrocytomas (most notably optic gliomas), and chronic myeloid leukemias of childhood.[16] In contrast, common adult malignancies such as lung, colon and breast cancers are not seen at higher frequency in NF1.[17] Most NF1-related deaths are thus attributed to either hypertension or cancer. In a 12-year prospective population study in Göteborg, Sweden, 22 of 70 NF1 patients died, 10 from hypertension and 12 from malignancies.[14] These NF1 patients thus demonstrated an age and sex-adjusted death rate 4.3 times that of the non-NF1 Swedish population. Nonetheless, the diagnosis of NF1 should not be routinely regarded as life threatening, with a majority of patients surviving well into their adulthood, with a mean survival age of 61.6 years in this study.[14]

Table: Diagnostic Criteria for NF1.[10]

The diagnostic criteria of the National Institutes of Health Consensus Conference on Neurofibromatosis (July, 1987) are met by an individual who demonstrates two or more of the following:
• 6 or more café au lait macules of over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals
• 2 or more neurofibromas of any type or one plexiform neurofibroma
• axillary or inguinal freckling
• optic glioma
• 2 or more Lisch nodules (iris hamartomas of melanotic origin)
• a distinctive osseous lesion such as sphenoid dysplasia or thinning of the long bone cortex with or without pseudoarthrosis
• a first-degree relative (parent, sibling, or offspring) with NF1 by the above criteria.

Genetics

NF1 has a birth incidence of 1:2500-1:3000, with a prevalence of 1:4000-1:5000.[18] The Mendelian inheritance pattern of NF1 is autosomal dominant.[19-21] Although case reports have suggested that NF1 can skip generations, these cases likely represent mildly affected individuals.[22] About 30-50% of NF1 patients represent de-novo germline mutations,[2,20] implying an extremely high spontaneous mutation rate of the *NF1* gene. This mutation rate has been estimated at 1 mutation per 10,000 alleles per generation, a rate 10-100 times higher than the usual mutation rate for a single locus.[20,23,24] Such a high mutation rate may be related to the large size of the *NF1* gene, as the similarly large Duchenne muscular dystrophy (*DMD*) gene[25] also demonstrates a mutation rate of 1/10,000 alleles/generation.[26] However, the size of the gene may not be the full explanation, as evidenced by a similar germ-line mutation rate of the much smaller *NF2* gene, which has a ten-fold lower birth incidence than NF1 (1/33,000-40,000 live births).[5] Germ-line mutations of the *NF1* gene occur preferentially on the paternally-derived allele,[26-29] which has also been noted in achondroplasia, Lesch-Nyhan syndrome, and hemophilia A. However, in each of the latter diseases the incidence of new mutations increases with parental age,[29] implying that paternal bias is related to the high number of cell divisions which occurs during spermatogenesis,[26,29] a pattern not seen in NF1.[2,29] The phenomenon of genomic imprinting has been proposed as an alternate explanation for these preferential paternal allele mutations.[27-31] Classical Mendelian genetics assumes that both alleles (maternal and paternal) of a gene participate equally in determining the phenotype of the offspring. The concept of genomic imprinting has thus been developed to explain situations where one allele is preferentially expressed over the other allele. Hence, genomic imprinting is an epigenetic phenomenon, in which non-genetic modifications of an allele result in altered expression and phenotype. A common mechanism resulting in genomic imprinting involves hypermethylation of the sperm genome;[32,33] a hypermethylated gene is at higher risk for mutations through the spontaneous deamination of 5-methylcytosine (methylated cytosine nucleotides) to thymine.[30] Methylation has also been proposed as a mechanism for gene silencing, in which either the maternally- or paternally-derived allele is active while the other allele does not contribute to gene expression. Methylation may thus explain why children of affected NF1 mothers (mutation of the maternal NF1 allele with gene silencing of the paternal NF1 allele by methylation) often demonstrate a more severe clinical manifestation than children of affected fathers (in which the mutated paternal allele may be silenced by methylation, allowing the non-mutated maternal allele to alone contribute to gene expression).[34]

In addition to the *NF1* allele, some evidence exists that other genetic loci may also contribute to the phenotype of NF1 patients. Analyses of monozygotic twins and more distantly-related family members with NF1 demonstrate that monozygotic twins have very similar phenotypes, while non-twin siblings

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and more distantly-related family members (who presumably carry identical *NF1* mutations) frequently exhibit very different phenotypes.[35] Such a pattern is suggestive of polygenic inheritance, and implies that the expression or mutation of non-*NF1* alleles is important in determining the phenotype of NF1 patients.[35]

Cloning the NF1 gene

The uniform NIH clinical diagnostic criteria led to the identification of a relatively homogenous cohort of patients and families with NF1,[10] which in turn played a pivotal role in the ultimate identification of the responsible gene. Restriction fragment length polymorphism (RFLP) and linkage studies of NF1 families suggested the pericentromeric region of chromosome 17 as the likeliest site for the *NF1* gene.[36-39] Two NF1 families with balanced translocations involving 17q (t(1;17) and t(17;22)),[40,41] helped map the *NF1* gene to chromosome 17q11.2,[42] a region which harboured three candidate genes.[43] Mutations of the first candidate gene, *EVI2A*, the human homologue of a mouse gene implicated in retrovirus-induced murine myeloid tumours, were not found in any NF1 patients, thus ruling it out.[44] The second candidate gene was *EVI2B*, also a human homologue of a murine gene implicated in retroviral insertions, however, it also failed to demonstrate mutations in NF1 patients.[45] The *OMGP* gene, encoding oligodendrocyte-myelin glycoprotein and expressed only in oligodendrocytes and Schwann cells, was the third candidate gene,[46] but also was found not to be mutated in NF1 patients. Subsequent analysis has shown that these three genes are encoded within the large authentic *NF1* gene,[47] but transcribed off the opposite strand, a phenomenon known as "nested genes". The contribution of these three nested genes to the NF1 clinical phenotype, if any, is not known.

The authentic *NF1* gene was cloned in 1990, largely with the help of probes that extended well beyond the previously reported translocation breakpoints (Figure 1).[18,45,48,49] Most of the *NF1* gene maps to a *NotI* restriction fragment of approximately 350 kilobases (kb) in size (all but 15 kb of this fragment is made up of *NF1* genomic DNA), while exon 1 and the 5' untranslated region maps to an adjacent 120 kb *NotI* fragment (Figure 1).[50] Due to the evolution of the exon numbering scheme, the exon containing the 3' stop codon is numbered exon 49, even though the entire gene consists of 59 exons.[50] The second-largest intron is intron 27b (45-50 kb), which contains the three nested genes *OMPG*, *EVI2A*, and *EVI2B* described above.[50] Exons 21-27a encode a 360 amino acid domain in the protein product which demonstrates homology with GTPase-activating proteins, and is termed the GAP-related domain (GRD).[50] The 5' promoter region contains a cAMP response element (CRE), AP2 consensus binding sites, and a serum response element (SRE).[51] The cDNA is 8454 nucleotide base pairs (bp) in length,[18] with two messenger RNA (mRNA) transcripts (11 and 13 kb), which differ in the length of their 3' untranslated region.[50,52]

Expression of neurofibromin

Neurofibromin is composed of 2818 amino acids with a molecular weight of 220 kilo-Daltons (kDa).[18,53,54] While *NF1* mRNA is detectable in most tissues, the expression of the neurofibromin protein is more tightly regulated, with highest levels found in neurons, oligodendrocytes, non-myelinated Schwann

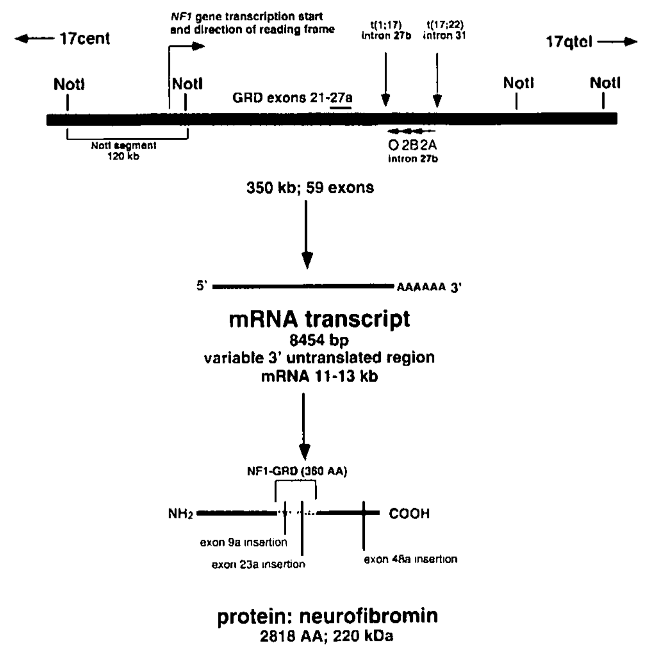

Figure 1: *Schematic representation of the* NF1 *gene, its mRNA transcript, and its protein product, neurofibromin. The* NF1 *gene is a large gene of 350 kb. Its cloning in 1990 was facilitated by the discovery of two NF1 patients with balanced translocations involving chromosome 17q (t(1;17) and t(17;22)). Three attempts at identifying the* NF1 *gene failed to discover the true* NF1 *gene, instead discovering the three nested genes* OMGP (O), EV12A (2A), *and* EV12B (2B), *which are transcribed off the opposite strand. The mRNA transcript is 11-13 kb long, depending on the length of the 3' untranslated region. Neurofibromin is a large protein, with a molecular weight of 220 kDa. The indicated size of the* NF1 *mRNA (8454 bp) and resultant protein (2818 amino acids) do not include the various tissue- and development-specific isoforms, which arise from the insertion of alternatively spliced exons 9a, 23a, and/or 48a.*

cells, adrenal medulla, leukocytes and testis.[55,56] In comparison, it is expressed in low amounts in adult astrocytes, myelinated Schwann cells, lung and kidney.[55,56] Four neurofibromin isoforms, which differ in their developmental and tissue expression, have been identified due to insertions into exons 23a, 48a, and 9a (Figure 1). Type 1 neurofibromin was the original isoform isolated and lacks any of these insertions. Type 2 isoform has an insertion of 63 nucleotides (21 amino acids) in exon 23a within the NF1-GRD,[57,58] which results in a 10-fold lower Ras-GAP activity and lower tubulin binding capacity.[56,57] Type 1 neurofibromin is the predominant isoform expressed in neurons of the CNS (cerebellum, cerebral hemispheres, and brainstem) and dorsal root ganglia, while the Type 2 isoform is predominantly expressed in tissues derived from the neural crest such as Schwann cells and adrenal medullary cells, in addition to glia and anterior horn cells of the adult spinal cord.[56,59]

Insertion of 54 nucleotides (18 amino acids) in exon 48a near the extreme carboxyl terminus results in the Type 3 isoform,[60] which is almost exclusively expressed in skeletal, cardiac, and smooth muscle, with little or no expression in brain, spleen or kidney.[61] This isoform is highly expressed during embryogenesis and declines shortly after birth, suggesting a role in muscle development.[61] Insertion of 30 nucleotides (10 amino acids) in

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exon 9a, within the NF1-GRD yields the Type 4 isoform, which is only expressed in neurons of the CNS, and not in neural crest derived tissues[62] or in glial cells.[63] Within the CNS, there is regional variation in the expression of the Type 4 isoform, which also likely plays a role in neuronal development, with increased expression during late embryogenesis.[63] In addition, this isoform is highly expressed in medulloblastomas and oligodendrogliomas, while it is found in low levels in astrocytomas, meningiomas, and ependymomas.[62,64]

Neurofibromin function: negative regulator of Ras

At the time of its cloning, the function of the *NF1* gene product, since termed neurofibromin, was unknown. Homology screening was undertaken to identify genes with known function that resembled the *NF1* gene, and that would offer clues as to the function of the neurofibromin protein. A small region in the central portion of neurofibromin demonstrated 30% homology with members of the Ras-GTPase Activating Protein (Ras-GAP) family, which includes three other mammalian proteins (p120-GAP, GAP1^{m}, and GAP1^{IP4BP}) plus two yeast proteins (Ira1 and Ira2).[47,65-67] Members of the Ras-GAP family are negative regulators of the key signal transduction protein Ras, as discussed below.[47,67-74]

Ras is a 21 kDa intracellular protein that is involved in the transmission of signals from a variety of upstream activators such as growth factor receptors, to a large number of downstream signaling molecules which eventually alter gene function in the nucleus. Ras is a member of the small G protein family, characterized by being bound to GDP in the basal inactive state, and being activated to the GTP bound state by guanine nucleotide exchange factors, in particular Sos.[69,70,75-79] As schematized in Figure 2, activated growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) bind and activate their cognate receptor, with receptor activation characterized by autophosphorylation of specific tyrosine residues on the intracellular domain of the receptor. Specific phosphotyrosine residues on the activated receptor are recognized by protein modules, such as SH2 (*src* homology-2) and PTB (Phosphotyrosine Binding) domains, on intracellular signaling proteins such as Grb2 and Shc.[75,80,81] Grb2 and Shc, through their interactions with the activated receptors in turn recruit other proteins such as Sos to the receptor in proximity to its substrate (inactive Ras•GDP) at the cell surface.[76] Sos exchanges Ras-bound GDP for GTP, resulting in activation of Ras and initiation of downstream signaling pathways, in particular the mitogenic cascade through Raf, MAPKK (mitogen-activated protein kinase kinase, also known as MEK or ERK kinase), and MAPK (mitogen-activated protein kinase, also known as extracellular signal related kinase or ERK) (Figure 2).

The importance of activation of the Ras pathway and subsequently Raf-MAPKK-MAPK leading to mitogenic signals, is exemplified by oncogenic activating mutations of Ras being found in approximately 30% of all human cancers.[71,82,83] In addition to oncogenic mutations, functional up-regulation of the Ras pathway can also be achieved by increased stimulation from upstream activators of Ras (i.e., signals from activated receptors) or decreased inhibitors of the Ras pathway. With regards to the latter mechanism, activated Ras•GTP is slowly hydrolyzed to GDP by intrinsic GTPase activity in native Ras, a process that is rapidly catalyzed by Ras-GAPs, including neurofibromin.[47,67-74] Several pieces of evidence demonstrate that neurofibromin is an important mammalian Ras-GAP, other than the homology of the GRD domain with other known Ras-GAPs (reviewed above): 1) Expression of only the NF1-GRD portion is capable of hydrolyzing Ras•GTP to Ras•GDP *in vitro*[84] and *in vivo*.[85] 2) Expression of the NF1-GRD is able to rescue Ira1 and Ira2 deficient yeast mutants.[86] 3) Overexpression of neurofibromin or the GRD portion suppresses oncogenic *Ras* transformed cells.[87-91] Another pivotal discovery that supported the Ras-GAP function of neurofibromin was the demonstration that neurofibrosarcoma (malignant peripheral nerve sheath tumour) cell lines established from NF1 patients not only lacked neurofibromin expression but had elevated levels of activated Ras•GTP.[88,89] We have recently adapted a luciferase-based enzymatic assay to quantitate the levels of Ras•GTP in actual tissue specimens from NF1 patients. We have confirmed that in NF1-associated malignant peripheral nerve sheath tumours (MPNST), neurofibromin expression is absent and that levels of Ras•GTP are substantially elevated.[73] Our studies demonstrate that Ras•GTP levels are approximately four and fifteen times higher in NF1-associated neurofibromas and MPNSTs respectively, compared to schwannomas from non-NF1 patients.[73,92]

Neurofibromin function: additional functions

While it is clear that neurofibromin acts as a Ras-GAP, there is also a growing body of evidence that this is not its only function. First, in melanoma and neuroblastoma cell lines levels of activated Ras•GTP are not elevated, despite complete absence of neurofibromin.[93] Second, overexpression of neurofibromin in *Ras* transformed cells revert the tumourigenic phenotype, even though the oncogenic Ras mutant is insensitive to neurofibromin's GTPase activity.[90] Since oncogenic Ras mutants, like normal Ras, can bind neurofibromin (through interactions with the NF1-GRD region) but are resistant to its Ras-GAP activity, it would suggest that in addition to its Ras-GAP role neurofibromin has other tumour suppressor functions. In fact, overexpression of just the NF1-GRD region in these *Ras*-transformed cells is equally successful in reverting the malignant phenotype.[94] It is not clear what these non Ras-GAP related tumour suppressor functions of neurofibromin entail. Neurofibromin does associate with microtubules,[95-97] suggesting a potential role in linking activated Ras•GTP to changes in cellular structure. Additional functions may relate to neurofibromin's six potential serine/threonine cAMP dependent protein kinase recognition sites and one potential tyrosine phosphorylation site, which are phosphorylated in response to PDGF or EGF.[18,98,99] Phosphorylation of these residues however has no effect on neurofibromin's Ras-GAP activity.[100]

The presence of phosphorylation sites as well as neurofibromin's interactions with the cytoskeleton suggest that it is a highly regulated protein. We have investigated how activation of Ras might affect the expression of neurofibromin, and demonstrated that activated Ras•GTP transcriptionally upregulates neurofibromin expression.[101] For instance, human malignant astrocytoma cell lines have increased levels of Ras•GTP due to stimulation from growth factor receptors (A. Guha, unpublished results), with abundant neurofibromin expression. Inhibiting Ras•GTP levels in these cells, using dominant negative Ras mutants, reduced NF1 mRNA and protein

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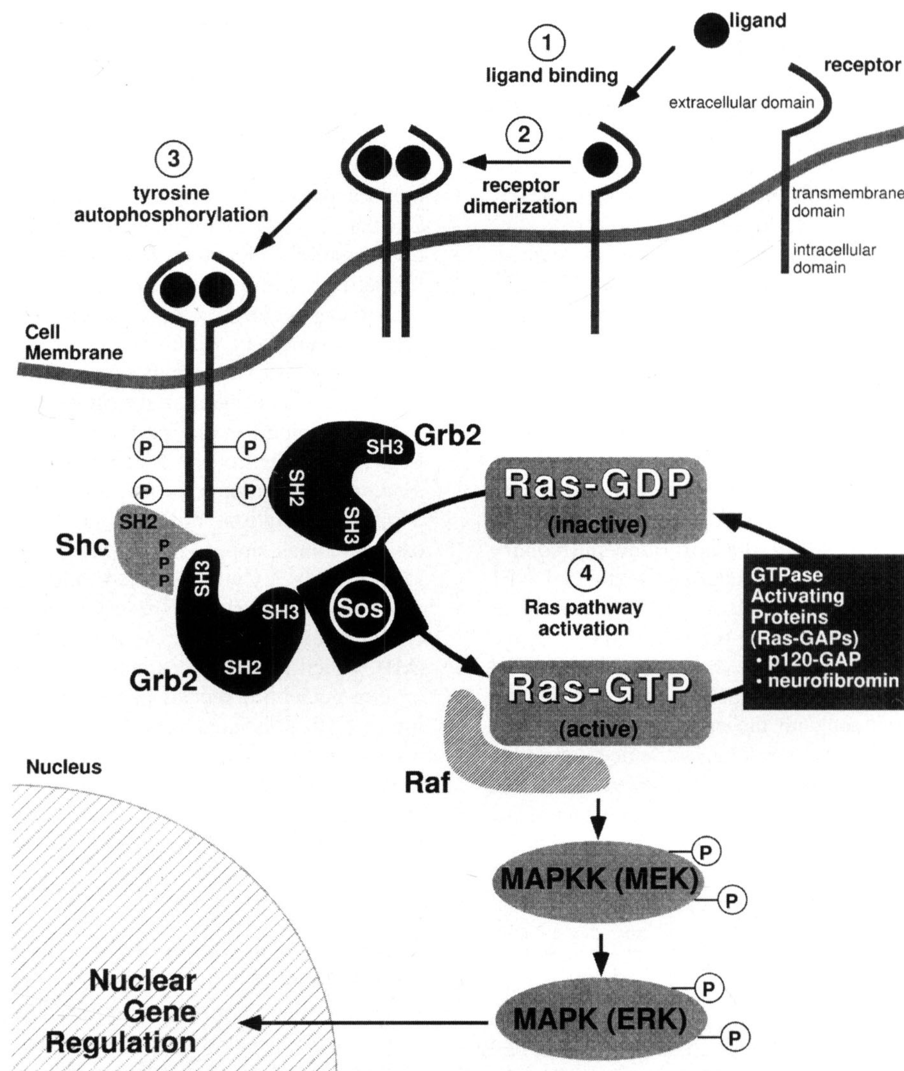

Figure 2: *Schematic representation of the Ras-Raf-MAPK mitogenic signaling pathway. This cascade is activated in normal cells when ligand (a growth factor such as platelet-derived growth factor) binds its cognate surface receptor* (STEP 1). *Ligand:receptor interaction results in receptor dimerization* (STEP 2), *resulting in transautophosphorylation of tyrosine residues on the intracellular domain of the receptor* (STEP 3). *This allows signaling molecules with SH2 domains (Shc, Grb2 or the Shc:Grb2 complex) to interact with the phosphotyrosine residues, bringing the nucleotide exchange factor Sos in proximity to the cell surface, where it exchanges GDP with GTP, activating Ras. Raf interacts with activated Ras•GTP, phosphorylating MAPKK, which subsequently phosphorylates MAPK. MAPK translocates to the nucleus where it participates with other molecules in activating the transcription of the transcription factors fos and jun, resulting in the increased transcription of genes involved in cell division and other functions* (STEP 4).

expression.[101] Neurofibromin expression is also increased in reactive astrocytes surrounding regions of focal ischemia,[102] and in juvenile pilocytic astrocytomas.[64] These results suggest a physiological negative feedback response, whereby activation of the Ras pathway increases neurofibromin expression in order to downregulate the Ras pathway.

Neurofibromin as a tumour suppressor gene

The concept of the "two hit" hypothesis in tumour suppressor genes (TSG) in familial cancer syndromes developed from the work of Knudson in the molecular pathogenesis of retinoblastoma.[103] The first hit involves a germ-line loss of function in one allele present in all cells, with the second hit being a somatic loss of function of the remaining allele in selected cells leading to tumour formation. NF1 patients bear a germ-line loss of function in one *NF1* allele which is either inherited or a de-novo mutation as previously discussed, with loss of function in the second allele resulting in complete absence of neurofibromin expression and the genesis of tumours. There is evidence that this model holds true for a variety of NF1-associated tumours, including benign neurofibromas,[28,73] pheochromocytomas,[104-106] malignant myeloid disorders,[16] and MPNSTs.[73,92,107,108]

Further evidence to support the hypothesis that *NF1* acts as a TSG comes from gene knockout studies, in which heterozygous

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NF1 +/- mice possess a single functional *NF1* allele.[109] While the classical cutaneous and ocular features of NF1 were not detected, 75% of these mice succumbed to tumours compared to 15% in a matched set of wild type animals. Many of the tumours encountered (such as lymphoma) are tumours often seen in older wild-type mice, but in the heterozygous knockout mice these tumours appeared earlier and at a higher frequency. Other tumours identified in the heterozygous mice (particularly pheochromocytoma and myeloid leukemia) are rare in wild-type animals, but are characteristic tumours found at higher frequency in NF1 patients. It is significant to note that all NF1-like tumours (pheochromocytomas and myeloid leukemias) in the mice demonstrated loss of the second *NF1* allele, consistent with the Knudson hypothesis.[109] The importance of neurofibromin in cellular growth and differentiation was exemplified by the homozygous *NF1* -/- knockout mice, which are embryonically lethal between days 12.5 and 14 of gestation.[109,110] Surprisingly, the cause of death in all embryos examined was severe cardiac vessel developmental anomalies, notably a double outlet right ventricle in which the aorta and pulmonary artery are joined, which is not a characteristic feature of NF1 patients.[109] The cardiac anomalies are however not surprising, as neural crest-derived cells contribute to the development of the great vessels.[111] Additional anomalies discovered in some of these homozygous knockout embryos include liver and skeletal muscle hypoplasia (consistent with the proposed role for exon 48a-containing neurofibromin isoforms in muscle development), delayed renal development, hyperplasia of the prevertebral and paravertebral sympathetic ganglia, as well as hyperplasia of the cells lining the aorta and pulmonary artery.[109,110]

The creation of *NF1* -/- knockout mice[109,110] has been exploited in gaining further insights into the functions of neurofibromin. Sensory neurons of the trigeminal and dorsal root ganglia derived from wild-type mouse embryos are dependent on the neurotrophic factor nerve growth factor (NGF) for survival, while nodose ganglion sensory neurons are dependent on brain-derived growth factor (BDNF) for their survival in cell culture. Sensory cells derived from *NF1* -/- embryos on the other hand are capable of surviving and extending neuronal processes in the absence of NGF or BDNF.[112] This extends previous studies in which activated Ras•GTP was able to mimic the survival-promoting effects of neurotrophic factors,[113] and identifies neurofibromin as a major regulator of neurotrophin- and Ras-mediated neuronal survival.[112] Studies on *NF1* -/- Schwann cells confirmed that neurofibromin is a major negative regulator of Ras in these cells, as they demonstrate elevated levels of Ras•GTP.[114] Furthermore, these Schwann cells take on morphological characteristics of *Ras* transformed cells, and are similar to Schwann cells stimulated with trophic factors such as the neuregulins, which activate the Ras pathway through activation of their receptors.[114,115] In contrast to Schwann cells, fibroblasts from the NF1 -/- mice did not demonstrate elevated levels of Ras•GTP, in support of other experimental data previously discussed that the importance of neurofibromin as a Ras-GAP differs between cell types, with perhaps Ras activity in some cells predominantly regulated by other Ras-GAPs such as p120-GAP.[114]

Malignant transformation of neurofibromas in NF1 patients

Benign neurofibromas consist of a heterogeneous population of Schwann cells, mast cells, fibroblasts, neuronal axons, perineural cells and endothelial cells.[2,116] The dermal neurofibromas are generally little more than a cosmetic problem while the larger plexiform neurofibromas, though histologically benign, arise in nerve plexuses and present with neurological deficits. Of greater significance, the plexiform neurofibromas have a 3-4.6% risk for malignant transformation,[13,117] a figure much lower than previously speculated, but one which is much higher than the less than 1% risk of malignant transformation in a non-NF1 neurofibroma.[118,119] Knowledge of these risks for malignant transformation vs. the surgical risks have to be considered for the proper management of NF1 patients presenting with peripheral nerve tumours.

Although activating mutations of Ras are not found in benign or malignant peripheral nerve tumours, several other TSGs have been implicated in the pathogenesis of MPNSTs (Figure 3). Loss or mutations of the *p53* gene on chromosome 17p has been discovered in numerous sporadic and NF1-associated MPNSTs.[120-125] Mutations of the retinoblastoma (*Rb*) gene, found in osteosarcomas, appear not to play a role in the pathogenesis of MPNSTs.[126-128] Cytogenetic analysis suggests that hotspots for additional mutations in NF1-associated MPNSTs are located on chromosomes 1, 11, 12, 14, and 22.[129] Microsatellite instability (MI), which represents expansions or compressions of di-, tri-, or tetra-nucleotide repeats in a genetic locus, are a frequent finding in NF1-associated neurofibromas, with 8/16 patients manifesting alterations at one or more microsatellite loci within the tumour.[130] However, the definition of what is significant MI is evolving, with the current accepted criterion requiring involvement of at least two or more loci.[131] When this more stringent criterion is applied to NF1-associated MPNSTs, only 1/16 patients truly demonstrates MI.[132]

Applying our understanding of molecular biology to improving the diagnosis of NF1

Despite the identification of the *NF1* gene, it has been difficult to apply genetic techniques to the diagnosis of NF1. NF1 patients frequently request prenatal genetic testing to rule out transmission of the defective *NF1* gene to their child. Similarly, NF1 patients who do not desire prenatal testing may still wish to screen their young child for the defective allele, as many phenotypic manifestations of NF1 do not appear until late childhood or early adolescence. Both of these situations lend themselves well to genetic testing, provided that the family *NF1* mutation is known. As the child can be expected to carry the same mutation as the affected parent, genetic testing can be very focussed and specific. In situations where the *NF1* mutation has not been characterized, genetic testing would imply a far more general screening of the entire gene. Such testing would thus be identical to genetic screening techniques applied to patients without a family history of NF1. A large variety of mutations have been discovered in the *NF1* gene in NF1 patients, including single base mutations, deletions of various sizes including the entire gene, insertions, splicing errors, and translocations.[50,133] Only one mutation has been found to be relatively common in NF1 patients, but this R1947X mutation still only accounts for at most 8% of mutations among Japanese patients and at most 2% of *NF1* mutations among Caucasians.[134] Hence, no single specific alteration in high frequency (mutational hotspot) or significant genotype-phenotype correlation (where one can predict the

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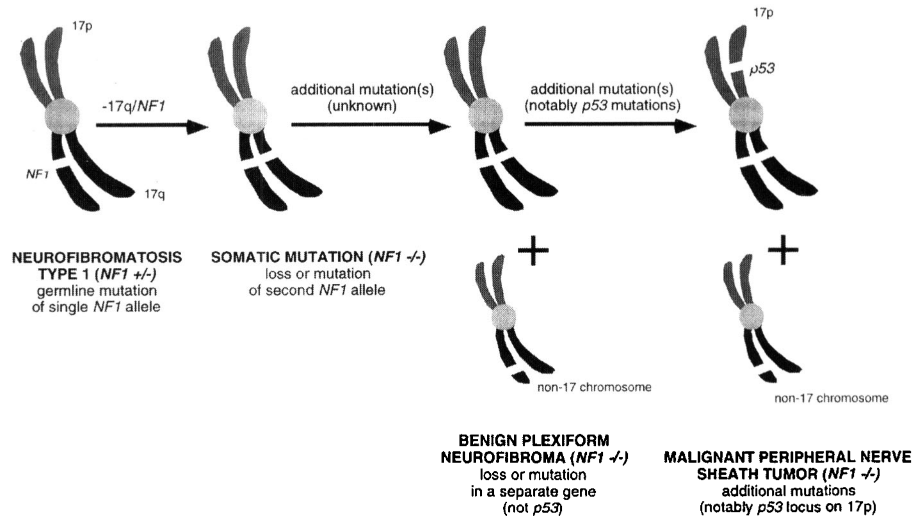

***Figure 3:** Schematic representation of the proposed molecular pathogenesis of benign and malignant NF1-associated peripheral nerve tumours. One allele of the* NF1 *gene on 17q is mutated in all cells in NF1 patients (germline mutation). Subsequent mutation or loss of the second* NF1 *allele in an individual cell (somatic mutation) appears insufficient to result in the formation of a benign neurofibroma, with mutations in one or more additional genes required for the development of the benign NF1-associated neurofibromas. Further additional genetic mutations (such as in the* p53 *locus on 17p) result in the malignant transformation of a benign plexiform neurofibroma into a malignant peripheral nerve sheath tumour. Dermal neurofibromas do not appear to be subject to the risk of malignant transformation.*

clinical presentation based on the location and type of mutation) has been identified.[133] The lack of mutational hot spots, large size of the *NF1* gene, high spontaneous mutation rate and lack of genotype-phenotype correlation have all contributed to the hurdles which still make routine genetic screening impractical.[12,135-137] The NIH clinical diagnostic criteria remains the best method of detecting new patients with NF1 (see Table).

Applying our understanding of molecular biology to improving the clinical management of NF1

Although our understanding of the molecular events in NF1 have not displaced the NIH clinical criteria in making the diagnosis of NF1 (see Table), our expanding understanding of these molecular events has resulted in new efforts to treat the variety of symptoms and tumours associated with this syndrome. Surgery is not always an option for deep-seated tumours or in eloquent regions of the central or peripheral nervous system. Even when surgery is performed, patients with MPNST frequently succumb not to their primary tumour but to metastases (L. Angelov, R. Bell, and A. Guha, unpublished observations), and all patients dying of malignancy in the Swedish prospective study harboured metastases at the time of death.[14] In particular, the pivotal role that neurofibromin plays in the regulation of the Ras pathway suggests that novel pharmacological inhibitors of Ras pathway activators may hold great promise for managing a number of NF1-associated benign or malignant lesions.

Ras pathway activation has been implicated in 30% of human malignancies, on the basis of oncogenic point mutations in *Ras*.[71,82,83] Many pharmaceutical companies and academic research groups have thus developed inhibitors of Ras or of proteins in the Ras-Raf-MAPK pathway. In particular, farnesyl transferase inhibitors (FTIs) have been developed which inhibit the first critical step (farnesylation) in the post-translational modification of Ras, a step which is vital for Ras to be recruited to the inner cell membrane where it may be activated.[138] This post-translational modification is catalyzed by the enzyme farnesyl transferase (FTase), and involves the transfer of a 15-carbon *trans,trans*-farnesyl moiety from farnesyl pyrophosphate (FPP) to the cysteine residue on the CAAX (C = cysteine, A = aliphatic amino acid, X = other amino acid) motif at the C-terminal of Ras. This first step is absolutely essential for Ras to become activated within the cell, while subsequent steps are not critical[139] (Figure 4). Although many other cellular proteins in addition to Ras are farnesylated, no adverse effects have been noted in experimental use of these agents in cell culture and animal models of human tumours bearing oncogenic *Ras* mutations.[140]

However, most studies to date have involved models of human cancer in which oncogenic *Ras* mutations resulted in constitutive activation of *Ras*, as demonstrated by elevated levels of *Ras*•GTP. NF1 patients do not harbour oncogenic mutations of Ras, and yet the downregulation of activated Ras•GTP through hydrolysis of GTP to GDP is slowed in the absence of neurofibromin. We had thus hypothesized that Ras•GTP levels would be elevated in MPNSTs in NF1 patients. We have confirmed that levels of Ras•GTP are substantially elevated in NF1-associated MPNSTs,[73] with Ras•GTP levels being approximately fifteen times higher in NF1-associated MPNSTs than in non-NF1 schwannomas.[73,92] Hence, NF1-associated MPNSTs demonstrate activation of the Ras pathway, providing additional evidence to support a beneficial effect of FTIs in the management of NF1. Indeed, FTIs have been shown to inhibit the proliferation of the human MPNST cell line NF188-14 in tissue culture experiments.[141]

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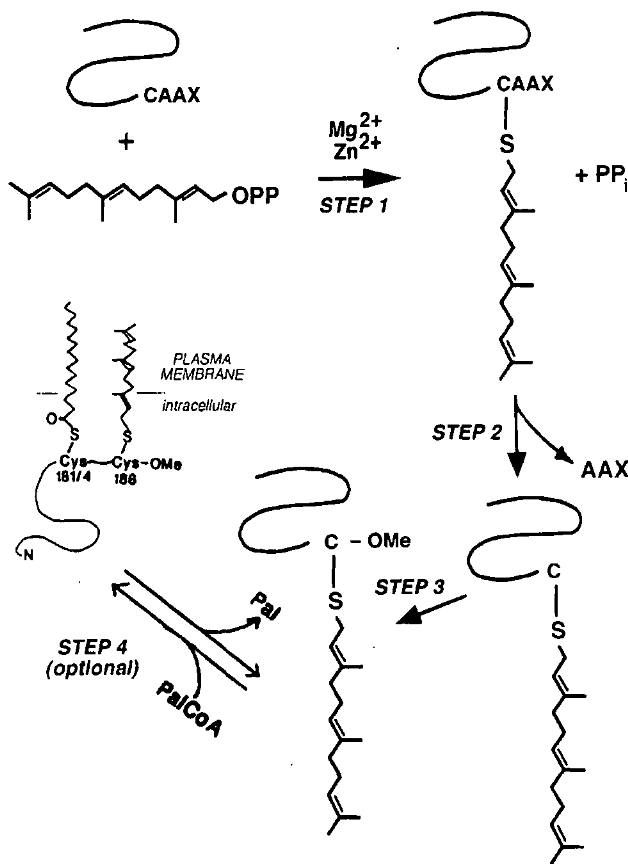

Figure 4: *The post-translational modification of Ras has been targeted in the pharmacological inhibition of Ras pathway activation. Ras undergoes three or four post-translational modifications. The first step, catalyzed by the enzyme farnesyl transferase (FTase), involves the transfer of a 15-carbon* trans,trans-*farnesyl moiety from farnesyl pyrophosphate (FPP) to the cysteine residue on the CAAX (C = cysteine, A = aliphatic amino acid, X = other amino acid) motif at the C-terminal of Ras; this first step is absolutely critical for normal Ras functioning. Subsequent steps are less critical, and these include the cleavage of the terminal three AAX peptides, carboxyl-methylation of the now C-terminal, farnesylated cysteine residue and optional palmitoylation at cysteine residues upstream of the C-terminal cysteine.*

Additional compounds targeting the Ras pathway are not as well developed presently, but include inhibitors of MEK which is involved in mitogenic signaling downstream of Ras in the Ras-Raf-MAPK cascade[142] (Figure 2). While such pharmacological manipulations of the Ras pathway are still many years away from routine clinical use, they represent the progress that can be made in clinical treatment based on a solid understanding of basic cellular physiology and of the molecular mechanisms which result in individual disease processes. Future therapy tailored more specifically to the management of NF1 will benefit from further study and understanding of the role that neurofibromin plays in cells, in particular in its non-Ras-GAP functions. Additionally, the clinical management of NF1 will benefit from progress made in our understanding of other aspects of cellular physiology and gene regulation, as activity in a host of other cellular pathways likely impacts on the function of neurofibromin, through positive and negative regulatory loops. We can thus look upon the future with optimism in our goal of developing and implementing novel, effective, and safe therapeutic strategies for NF1.

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