Meena Upadhyaya · David N. Cooper
Editors

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Molecular and Cellular Biology

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To Lara Devi

Foreword

This book marks a true "coming of age" for our understanding of neurofibromatosis. It is 130 years since the disorder was first accurately described, but only the last 30 of these years have seen real progress in our understanding of this common and important disorder. This 30-year period is exactly divided by the Editors' first book in 1998 (Neurofibromatosis Type 1: From Genotype to Phenotype BIOS Scientific Publishers), itself a landmark that showed how the advent of molecular genetics had not only defined the gene involved, but had also begun to point the way to the understanding of its function in the disorder and in the normal state.

This second book by Meena Upadhyaya and David Cooper shows us how this new understanding has blossomed and broadened, throwing light on a variety of different aspects of this complex and multifaceted condition. We can begin to see how the molecular analyses and the more clinical studies can support and inform each other, so that the book shows us a true community of workers, making different but interacting contributions to the field.

The Editors can be proud not only of their own leading role in this international community, but also of their achievement in bringing together its key members to create a book that represents what will undoubtedly be a lasting as well as an outstanding landmark for one of the most important human genetic disorders. All those in the neurofibromatosis community, whether they are affected individuals, or workers involved in research or the provision of services, will be grateful to them for this achievement.

July 2012 Peter S Harper
Cardiff, UK

Preface

Neurofibromatosis type 1 is a common autosomal dominant cancer predisposition syndrome that is caused by germline mutations in the *NF1* tumour suppressor gene. The aim of this volume is to present in concise fashion, but as comprehensively as possible, current knowledge of the molecular genetics and cellular biology of neurofibromatosis type 1 (NF1). The molecular and cellular biology of NF1 is a burgeoning subject with a large and widely dispersed literature that is difficult to access. This volume comprises 43 chapters, written by internationally recognised experts in the field, on the latest developments in the molecular and cellular biology of NF1. It should be of interest to human geneticists, clinicians, general practitioners, psychologists and genetic counsellors as well as undergraduates and postgraduates in medicine and the biological sciences. It is hoped that this volume will serve as a useful reference source for years to come.

Since the publication of our first edited volume on the topic in 1998 (Upadhyaya and Cooper 1998), there has been an explosion in our knowledge of the molecular and cellular mechanisms underlying the development of the different features of NF1. During this period, remarkable advances have been made in the management of NF1 clinics, molecular diagnosis, the detection of somatic mosaicism, genotype–phenotype correlations, and the identification of *NF1* modifying loci. Intensive research over the last 14 years has greatly expanded our understanding of the functional role of the *NF1* gene and its protein product (neurofibromin), the germline and somatic mutational spectra of the gene and the underlying mutational mechanisms. An apparent relationship between *NF1* and mismatch repair genes has also been explored.

With the emergence of sensitive imaging techniques, including whole-body MRI and PET studies, a wealth of information on the load of internal tumours and their management has been generated. We have also acquired a greatly improved understanding of bone skeletal and cardiovascular abnormalities in NF1 patients. Clinical, molecular and cellular aspects of cognitive impairment have been extensively studied, giving rise to therapeutic possibilities and spawning clinical trials.

Much has been learnt about the pathogenesis of the different types of NF1-associated tumours including optic gliomas, phaeochromocytomas, cutaneous

and plexiform neurofibromas, malignant peripheral nerve sheath tumours (MPNST), glomus tumours and leukaemia. As a result, we are better equipped in terms of our ability to manage these tumours. Identification of the cell(s) of origin of NF1-associated tumours will be important for the design of new therapies. Available evidence suggests that cutaneous neurofibromas arise from skin-derived precursors or their derivatives, whereas bi-allelic inactivation of *NF1* in the Schwann cell lineages leads to plexiform neurofibroma formation. The cell of origin of MPNSTs is not yet known.

We also remain ignorant of the mechanisms underlying the transformation of benign neurofibromas to malignancy. These may involve sequential events involving both genetic and epigenetic alterations, and the order in which these changes occur needs to be elucidated. The tumour microenvironment is now known to play a critical role in NF1 tumorigenesis and this will need to be further explored. Beyond its central role in NF1, the *NF1* gene is also emerging as an important tumour suppressor gene in its own right in sporadic cancers.

We have learned a lot about the biology of NF1 from animal models including *Drosophila,* zebrafish and mouse. These models are helping to advance our knowledge of disease progression and should facilitate the identification of biomarkers, the development of suitable therapies, and the prediction of drug response in clinical trials. Research based on preclinical work has already been translated into therapeutic trials for various NF1 features including plexiform neurofibromas, gliomas, MPNST and neurocognitive disorders. A new class of human developmental syndromes, designated "RASopathies", has recently been recognised that are caused by germline mutations in different genes involved in regulating the Ras/MAPK signalling pathway. Indeed, locus heterogeneity is evident in NF1: one of the RASopathies, Legius syndrome, is caused by mutations in the *SPRED1* gene and has clinical features that overlap with NF1.

Recent reports have indicated that different cancers may be associated with different patterns of micro-RNA (miRNA) expression pattern, and these might provide us with extra prognostic and diagnostic markers, as well as providing new avenues for therapeutic intervention in MPNSTs. Indeed, a subpopulation of cells positive for CD133 (a cancer stem marker) have been detected in human primary MPNST. Functional analyses have confirmed these cells to be cancer stem cells (CSC) and they have been shown to exhibit enhanced chemo-resistance in vitro (Borrego-Diaz et al. 2012).

Worldwide NF1 lay foundations, which provide an important resource for the NF1 community, are also emerging as drivers of NF1 research. A comprehensive account of social problems faced by NF1 sufferers is discussed. Finally, potential future directions for NF1 research are discussed, highlighting novel research avenues and therapeutic targets.

Cardiff, UK David Cooper, Meena Upadhyaya

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Contents

Chapter 1
von Recklinghausen Disease: 130 Years

Vincent M. Riccardi

1.1 Introduction

I am a geneticist and a Recklinologist. In my capacity as a Recklinologist, including some of my genetic wherewithal, I have attempted to understand and explain the disorder, von Recklinghausen disease, that is, neurofibromatosis type 1 (NF1). One problem for the Recklinologist, however, is focus almost exclusively on the disease, that is, the consequences of mutations in or loss of the *NF1* gene—as opposed to studying perhaps the more interesting element, the normal wild-type gene at the *NF1* locus. In this chapter, I will first provide some selected chronological and topical information about various attempts to study *NF1* gene disturbances and their consequences, with some modest emphasis on several developments to which I personally contributed. On the other hand, ultimately I wish to consider the wild-type *NF1* gene and its likely very special place in human evolution and current function. It is very important to understand all the way through this chapter—and actually the whole book—that labeling *NF1* as a tumor suppressor gene is more a statement about the mutation than about the wild-type gene itself.

The more we know about a disease, the more likely we are able to avoid that disease and provide effective treatment. The more we know about the gene that causes a specific Mendelian genetic disease, the more likely we are able to provide effective treatment, either in terms of mollifying the effects of the mutant gene(s) or in terms of replacing or modifying the mutant gene(s). That is, to understand and treat a genetic disease, we must know about the whole panoply of mutations known to occur at the locus of concern. Two questions immediately follow. First, do we focus on the mutant gene or on the normal (wild-type) gene? Second, what is a gene? As we prepare to go beyond the 130 years of studying the disease, NF1, since Friedrich von Recklinghausen attached his name to it, we realize that we know

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some substantial amounts about the natural history, pathogenesis, and cell biology of NF1; only modest amounts about mutant *NF1* genes; and almost nothing about the wild-type *NF1* gene. Part of the problem is that at this same point in time we find our current definitions of "gene" wanting.

As noted already, in this introductory chapter of a book dedicated to sharing our current—early twenty-first century—knowledge and understanding of NF1 the disease and *NF1* the gene, I will proceed from two vantage points: a history of the highlights (and occasional details) of the steps we took to get here and a consideration of what NF1 and *NF1* are teaching us about genes and genetic disease in more general terms.

1.2 History

The human disorder we now call "neurofibromatosis type 1," or "NF1" and previously designated as "von Recklinghausen disease," has been an identifiable entity for centuries, as outlined on multiple occasions, including 1988 (Chap. 1) (Mulvihill 1988) and 1994 (Chap. 1) (Huson and Hughes 1994). The basis for the eponym, von Recklinghausen disease, is Friedrich von Recklinghausen's (1882) treatise, "Ueber die multiplen Fibrome der Haut and ihre Beziehung zu den multiplen Neuromen," translated into English in part for inclusion in the 1981 Volume 29 of *Advances in Neurology* (p. 259) edited by Riccardi and Mulvihill (1981). A biography of von Recklinghausen, authored by Josef Warkany, is also included in that book (p. 251).

Thus, as of 2012, respecting a publication year of 1882, 130 years have elapsed since von Recklinghausen placed his stamp on the disorder we presently consider to be NF1. However, perhaps a more accurate year for the disorder's initial comprehensive characterization is 1849, which, in turn, means that 163 years might be celebrated. In 1849, Irish physician, R.W. Smith, published "A treatise on the pathology, diagnosis and treatment of neuroma." This article was republished in 1989 (Smith 1989), with the notation that Smith focused on the "connective tissue" around the nerves and not on the nerves themselves. A 2003 publication made similar notations (Reynolds et al. 2003), and in 1992, a separate article's title asked a particularly cogent question: "Neurofibromatosis: Why not Smith's disease?" (Lyons and Staunton 1992).

From the middle and end of the nineteenth century through the early twentieth century, there was little comprehensive work on this disorder, NF1, until the 1951 publication of the Borberg study of a very large Danish cohort (Borberg 1951; Mulvihill et al. 1983). His population-based approach clarified the tremendous variability from one person to another within a family, and it emphasized that adults with NF1 are much more numerous than are children and that not all of the patients' clinical problems are tumor-related. In other words, NF1 was seen to be more than a children's tumor disorder. In 1956, the study of Crowe, Schull, and Neel of the University of Michigan (Crowe et al. 1956) emphasized these points,

although they perhaps also compromised matters by subsuming what we now call NF2 into a subtype of NF1. In 1971 and 1972, Brasfield and Das Gupta (Das Gupta and Brasfield 1971; Brasfield and Das Gupta 1972) provided cogent data collected on surgical specialty and institutional bases, nonetheless emphasizing the long-term, that is, adult, morbidity of NF1.

In this context, in *February 1972*, while a genetics fellow at the Massachusetts General Hospital, I was asked to present Pediatric Grand Rounds on a topic of my choosing. My mentor, Lewis B. Holmes, suggested that I select a topic that I would focus on for the rest of my life. My literature searches made von Recklinghausen disease the obvious choice. With this intellectual commitment foremost, when recruited to Baylor College of Medicine 1976, I made clear that my move to Houston, Texas, was contingent on support for establishing a care- and research-based Neurofibromatosis Program. Thus, in *February 1977*, the Baylor NF Program (BNFP) became a reality, and in *August 1978,* the program's first patient was enrolled. Also in *1978*, the National Neurofibromatosis Foundation, Inc. (NNFF) was founded by Lynne Courtemanche, RN, Allan Rubenstein, MD, and attorney Joel Hirschtritt. In *June 1979*, the National Cancer Institute (NCI) and the BNFP hosted the first international conference on neurofibromatosis, the proceedings of which were published as noted below.

In *1980*, the Texas Neurofibromatosis Foundation was established. In *1981*, *Advances in Neurology*, Volume 29, reported the proceedings of the 1979 NCI-BNFP international NF conference (Riccardi and Mulvihill 1981). At the end of that year, the *New England Journal of Medicine* Medical Progress series published the BNFP's overview of NF up to that point in time (Riccardi 1981b). Also in 1981, the initial advances into understanding the cell biology of NF1 were published by Winfrid Krone et al. (1981) and Juha Peltonen et al. (1981). In *November 1983*, in the first of a series of international outreaches by the BNFP, compelling data about compromise of intellectual performance among more than half of patients with NF1 were presented in Tokyo (Riccardi 1984). In *March 1985*, the second major BNFP international NF outreach to five European countries was carried out over a 3-week period, culminating in the signal lecture on NF at the London meeting of LINK (Let's Increase NF Knowledge). In *1986*, the cell biology approach to NF1 was entrenched through the brilliant efforts of Nancy Ratner (Ratner et al. 1986). In *July 1987*, an NIH Consensus Conference adopted the BNFP nomenclature for naming NF1 and NF2 and suggested specific diagnostic criteria for both disorders (1988). Also in *1987*, assignment of the *NF1* gene to the long arm of human chromosome 17 (17q) was made (Van Teinen et al. 1987; Barker et al. 1987; Skolnick et al. 1987; Pericak-Vance et al. 1987), as was the assignment of the *NF2* gene to the long arm of human chromosome 22 (22q) (Rouleau et al. 1987). In *1988*, NF, Inc., was founded by a series of like-minded NF groups organized at state and local levels, the point being to establish an NF organization that allowed local autonomy of multiple individual organizations while affording collaboration on activities that were national in nature. In *June 1989*, portions of the growing international NF community met in Oxford, England, while in *November 1989,* there were also

NF-dedicated meetings in Tokyo, Japan, and Siena, Italy, the latter being a model meeting for the evolving European NF Association.

In *1990*, the *NF1* gene and its gene product, neurofibromin, were identified (Viskochil et al. 1990; Wallace et al. 1990; Daston et al. 1992; Hattori et al. 1992; DeClue et al. 1992; Daston and Ratner 1992). In *1993*, the *NF2* gene and its gene product, merlin (schwannomin), were identified (MacCollin et al. 1993). In *1994,* the first publications of NF1 mouse models were having their impact (Jacks et al. 1994; Brannan et al. 1994), and Parada and colleagues were beginning to exploit the rodent approach in understanding human NF1 mutations (Smullen et al. 1994; Le et al. 2011; Li et al. 2012). In *2005*, NNFF changed its name to the "Children's Tumor Foundation," a puzzle to the many clinicians and affected families and patients who see the burdens of NF1 going far beyond childhood and tumors.

In the meantime, NF specialists were also making substantial headway in Japan, through the leadership of Michihito Niimura; in Canada, through the leadership of Jan Friedman; and in Europe, through multiple active centers under leadership of the European NF Association and numerous Recklinologists such as (in alphabetical order) G.D. Evans, R.E. Ferner, A. Heiberg, S.M. Huson, D. Kaufmann, W. Krone, E. Legius, V.-F. Mautner, L.M. Messiaen, J. Peltonen, B. Samuelsson, R. Tenconi, M. Upadhyaya, and P. Wolkenstein, among many others. Australia, under the leadership of K. North, and Brazil, under the leadership of L.O. Rodrigues, have also contributed substantially to a united and very productive international effort to understand and treat NF1.

Among the most telling consequences to derive from the multiple American, European, and international consortia, seven are particularly remarkable. Of course, additional areas of focus are also worthy of special attention and a perusal of this book's "Table of Contents" will show that no area relevant to understanding NF1 has been ignored or downplayed. We have indeed made substantial progress over the time span from 1882 to 2012.

One area of consequence is the exploitation of computerized *magnetic resonance neuroimaging*, with an emphasis on neurofibroma volumetrics and whole-body scanning to detect and measure over time symptomatic and presymptomatic diffuse and encapsulated plexiform neurofibromas (Dombi et al. 2007; Solomon et al. 2004; Mautner et al. 2008; Jaremko et al. 2012). On the one hand, while clarifying the early stages of plexiform neurofibroma development and assessing responses to specific neurofibroma-compromising treatment, strategies have largely been *successful*. On the other hand, it is of some interest that following neurofibroma growth has relied on volumetrics. Given the fact that diffuse plexiform neurofibromas (Riccardi 1992; Masson 1970) enlarge primarily by budding, as discussed by von Recklinghausen (1882) and portrayed in Fig. 64 of Harkin and Reed (1969), it would seem that measurement of whole tumor surface area might be more sensitive to diffuse neurofibroma growth changes (Lebioda et al. 2008; Andea et al. 2004; Shahar et al. 2002; Videtic et al. 2001) either as a function of age or a function of treatment.

A *second* especially noteworthy of emphasis has been the exploitation of evolving *genomic technology*, with an emphasis on whole gene deletions of *NF1*

(Mautner et al. 2010; Zickler et al. 2011) and increasingly relevant genotype–phenotype correlations for both whole gene deletions (Pasmant et al. 2010; Vogt et al. 2011b) and intragenic mutations (Upadhyaya et al. 2007). Mautner and colleagues in Hamburg, Germany (Kluwe et al. 2004; Mautner et al. 2006, 2010), and Messiaen and colleagues in Birmingham, Alabama (Messiaen et al. 2000, 2010), have been especially contributory. Genomic diagnosis, both for direct patient management and for preimplantation conceptus selection (Verlinsky et al. 2002; Spits et al. 2005), is now uniformly and consistently available.

Third, *clinical heterogeneity and natural history studies* have received special emphasis, in addition to the genotype–phenotype correlations acknowledged above, especially as regards somatic mosaicism (Ruggieri and Huson 2001; Maertens et al. 2007; Kaplan et al. 2010; Bottillo et al. 2010; Messiaen et al. 2010; Vogt et al. 2011a) and the overlap of NF1 and the Legius syndrome (Brems et al. 2007; Messiaen et al. 2009). The compelling contributions in terms of clinical heterogeneity and genotype–phenotype correlations now—in 2012—make it entirely reasonable to establish a complementary genomic diagnosis for every person carrying the clinical diagnosis of NF1. On the other hand, an increased emphasis on natural history and longitudinal studies have not led to a commensurate increase in the availability of clinics and treatment centers for the largest segment of the NF1 population, adults with NF1. As of early 2012, there has been little effort to establish programs focused on NF1 adult patients, and the NF1 cell biology and genomics research programs have continued to focus on the cell biology and biochemistry of the *NF1* gene product, neurofibromin, without considering other genetic mechanisms, such as various types of transcription products, including miRNAs, as, for example, miR-10b (Chai et al. 2010).

Fourth, emphasis on *gliomas of the optic pathway* and other parts of the brain has made it clear that such tumors are key elements of NF1, contributing substantially to the disorder's chronic morbidity and early mortality (Lewis et al. 1984). Special credit in these regards goes to Gutmann and his colleagues (Bajenaru et al. 2002; Gutmann et al. 2000; Listernick et al. 2004; Rodriguez et al. 2008; Gutmann 2011; Banerjee et al. 2011). Their work on the various isoforms of the *NF1* gene product, neurofibromin (Grand et al. 1993; Gutmann et al. 1993a, b, 1995a, b), is also noteworthy.

Fifth, while emphasis on the widespread skeletal involvement in NF1 has been noted by multiple Recklinologists (Riccardi 1992), the Recklinology group at Salt Lake City, Utah, under the leadership of D.A. Stevenson, warrants special mention (Stevenson et al. 2005, 2006, 2008; Kossler et al. 2011; Johnson et al. 2011). Understanding the commonalities of the cells of the skeletal tissues (cartilage and bone) and Schwann cells and melanocytes will be especially rewarding in terms of helping patients with NF1 and for understanding the normal dynamics of bone growth and repair.

Sixth, *mast cell* contributions to understanding neurofibroma pathogenesis and treatment approaches are particularly noteworthy for the ultimate sublime overlap of clinical acumen and benchtop research. Based purely on clinical observations and the pathologist's earlier documentation of mast cells in normal peripheral

nerves and in neurofibromas (from patients with NF1 or otherwise), as early as 1981, V.M. Riccardi established the role of mast cells in neurofibroma development (Riccardi 1981a, 1987, 1990a; 1993; Virchow 1857). Additional pathology data later suggested that the two basic types of neurofibromas (encapsulated versus diffuse; or fascicular versus extra-fascicular) could be differentiated on the bases of the numbers and distributions of mast cells within the neurofibromas (Tucker et al. 2011). In the meantime, multiple mouse models documented at the cell biology level the critical role of mast cells haploinsufficient at the NF1 locus in the initiation and progression of NF1 neurofibromas (Viskochil 2003; Badache et al. 1998; Staser et al. 2010, 2011). The mast cell's modification of the microenvironment of the target Schwann cells is obviously critical in neurofibroma development and growth. Early attempts to treat NF1 neurofibromas with ketotifen, an orally administered mast cell stabilizer, were modestly successful, but did not capture the imagination of the Recklinology community (Riccardi 1990a, b, 1993).

Seventh, *intellectual compromise* by various names and mechanisms has been especially relevant to patients and families, as well as to Recklinologists and geneticists and students of the human brain in general. The BNFP was especially attentive to this aspect of NF1 (Riccardi and Eichner 1986; Riccardi 1984, 1992; Coleman 1987) although many others also contributed substantially (Cutting et al. 2000b; Ozonoff 1999; Descheemaeker et al. 2005; Acosta et al. 2006; Barton and North 2007; Cutting et al. 2000a; Krab et al. 2008a; Said et al. 1996; Park et al. 2009; Pasmant et al. 2010), premier among whom were North and colleagues (North 1993; North et al. 1994, 1997, 2002; Hyman et al. 2003, 2005; Payne et al. 2010; Sangster et al. 2011). Silva and his colleagues focused on the *Nf1*+/− mouse model, relying intensely on the Morris water maze test and, later, the potentially beneficial effects of treatment with statins (Silva et al. 1997; Li et al. 2005; Shilyansky et al. 2010). Krab and coworkers corroborated many of the relevant data and initiated an inconclusive (and ongoing) study directly testing the short-term effect of simvastatin on selected patients with NF1 (Krab et al. 2008a, b, c; van Engelen et al. 2008). Other Recklinologists specifically focused on the NF1 patient's attention and the obverse, inattentiveness (Brown et al. 2010a, 2011) with Ribeiro and coworkers specifically considering visual attentiveness (Ribeiro et al. 2012). More personally, in April 2007, I presented at the European NF Association meeting in Lisbon, Portugal, a paper entitled "Prosody: The Real Learning Disability of NF1." The point was to emphasize that in both sensory and motor skills, a substantial number of persons with NF1 are inattentive: They simply do not avail themselves of all the cues available in the world around them. The other three presenters in the session (W. Li, P. Wolkenstein, and L.C. Krab) were similarly consistent in their emphasis on attention and attentiveness as elements of the school performance and learning problems seen in NF1, in both mice and people. Elsewhere, others have studied the relevance of purine nucleotides, particularly cAMP, ATP, and GTP, in relation to NF1 brain function (Hannan et al. 2006; Tong et al. 2002, 2007; Dasgupta et al. 2003; Guo et al. 2000; The et al. 1997; Ho et al. 2007; Hegedus et al. 2007; Xu et al. 2002; Kim et al. 1997; Brown et al. 2010b, 2012; Park et al. 2009; Bland and Birnbaum 2011). The interrelation of

oxidative stress, cell biology stress, and organismal/patient stress as a key element of NF1 pathogenesis and wild-type *NF1* gene function was one of my main areas of interest during the earliest stages of development of the BNFP. Specifically, the ability of NF1 haploinsufficient fibroblasts to accommodate readily to the metabolic stress of exposure to 3-nitrotyrosine encouraged us along these lines (Riccardi and Maragos 1980).

In this context, it is especially worthwhile to consider the fact that there are no populations of feral animals with *Nf1* mutations. A wild animal born with a constitutional *Nf1* mutation that survived into adulthood has never been documented, even though domesticated cattle with multiple neurofibromas are well known (Doughty 1977; Canfield 1967; Canfield and Doughty 1980; Sartin et al. 1994; Omi et al. 1994). One possible exception is the documentation of one or more populations of the bicolor damselfish that manifest multiple individuals with pigmented neurofibromas consistent with a piscine equivalent of NF1 (Schmale et al. 1983, 1986; Schmale and Udey 1983; Lacson et al. 1988, 1989; Fieber and Schmale 1994). However, the NF1-like disease in these fish (*Stegastes partitus* presently, though previously specified as *Pomacentrus partitus* or *Eupomacentrus partitus*) appears to result from an infection with a virus or "virus-like" organism (Schmale and Hensley 1988; Campbell et al. 2001; Schmale et al. 2002). I have repeatedly suggested that this lack of a feral NF1 disease is that the attendant compromise of attentiveness severely compromises both predators and prey: Figuratively speaking, the prey prematurely "become lunch" and the predators "don't get lunch" in consistent and timely ways. To survive as predator or prey requires the highest level of attentiveness, which "highest level" requires an intact, wild-type *NF1* gene.

1.3 Gene

My starting place was NF1 fibroblast resistance to 3-nitrotyrosine (Riccardi and Maragos 1980; Ma et al. 2007), the multiple ways that purine nucleotides participate in the pathogenesis of NF1, and the pivotal role of ATP in the origin and maintenance of life (Table 1.1) (Saygin 1981; Galimov 2004, 2009; Shelly et al. 2010; Bland and Birnbaum 2011). I then reasoned that the human *NF1* wild-type gene, like its *Saccharomyces cerevisiae* counterpart (inhibitor of Ras activity; *IRA*), is a critical sensor gene, balancing the nutritional/energetics demands of the organism (particularly the central nervous system and peripheral nerves) with the available nutritional/energy supplies (Daston et al. 1992; Gutmann et al. 1993b; Russell et al. 1993; Dechant and Peter 2008; Hertz et al. 2007; Wallace and Fan 2010).

With these considerations in mind, at the November 2009 inaugural meeting of the *Japanese Society of Recklinghausen Disease*, I proposed the following (Riccardi 2010): "... we must consider the possibility that *NF1* is not simply a 'tumor suppressor' gene, but actually a Purine Nucleotide Balance (PNTB) gene."

Table 1.1 ATP is a critical purine nucleotide

ATP is the hub of purine interconversions (ATP, cAMP, GTP, cGMP) and a critical contributor to nucleic acid synthesis
ATP is critical in protein activation, the prototypic kinase cofactor (complemented by a reciprocal phosphatase modulation)
ATP is the key component of biological energy transfer, as a function of O_2
Even though the brain represents only 2–3 % of the body weight, it consumes 20 % of total body oxygen and 25 % of total body glucose
The peripheral nervous system has similar requirements and vulnerabilities
The human body's ATP is constantly being broken down into ADP, which then is converted back into ATP
Although the total amount of ATP + ADP is constant, the energy need of a human's cells requires the turnover of 50–75 kg of ATP daily, each ATP molecule recycling 1,000–1,500 times

That is, while consequences of *NF1* mutations may include various types of tumors, the primary function of wild-type *NF1* is generation and maintenance of the combination of purine nucleotides most likely to afford optimal central nervous system and peripheral nerve function over very short time intervals (seconds). This gene ensures that the brain, spinal cord, and peripheral nerves have the optimal balance of purine nucleotides to afford peak performance—with particular regard to attentiveness and the appropriate motor responses thereto.

In short, the geneticist in me suggests that the wild-type *NF1* gene is best thought of in terms of brain and nerve functions that enhance moment-to-moment survival. It is now time to begin studying how to exploit this aspect (or these aspects) of the *NF1* wild-type gene. Can we enhance its function? What do we need to know to answer this latter question most assiduously?

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Chapter 2
Clinical Diagnosis and Atypical Forms of NF1

Sirkku Peltonen and Minna Pöyhönen

2.1 Clinical Diagnosis of Neurofibromatosis Type 1

The clinical diagnosis of NF1 (MIM 162200) is based on the diagnostic criteria outlined in NIH Consensus Development Conference in 1987 (Table 2.1) (Stumpf et al. 1988). Nearly all (95 %) NF1 patients can be diagnosed using these criteria by the age of 8 years (DeBella et al. 2000).

The diagnosis of NF1 relies on a slightly different set of diagnostic signs in different age groups. Diagnosing small children is often challenging since about half of sporadic NF1 cases fail to fulfill the NIH Diagnostic Criteria at the age of 1 year (DeBella et al. 2000). In rare cases, such as mosaic NF1 (Chap. 12) or Legius syndrome (Chap. 31), the NIH diagnostic criteria may lead to false NF1 diagnoses. Thus, there is a clear need for molecular diagnostics. Mutation analysis of the *NF1* gene may be helpful in the diagnosis of patients if NF1 is suspected but where the patient does not fulfill the clinical diagnostic criteria.

Two of the diagnostic criteria are readily visible on the skin: six or more café-au-lait macules and axillary/inguinal freckling. Skinfolds are however easily ignored in routine clinical examination. Cutaneous neurofibromas can resemble other benign cutaneous tumors such as intradermal naevi. Diagnosis of cutaneous neurofibromas may thus require histology of one or two nodules. Plexiform neurofibromas may be anticipated in clinical examination as asymmetric outlines of the face, limb, or trunk. In particular, facial masses become detectable during the

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Table 2.1 NIH diagnostic criteria of NF1. Two or more of the following are required for the diagnosis

1. Six or more café-au-lait macules over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals
2. Two or more neurofibromas of any type or one plexiform neurofibroma
3. Freckling in the axillary or inguinal regions
4. Optic glioma
5. Two or more Lisch nodules in iris
6. A distinctive osseous lesion such as sphenoid dysplasia or thinning of the long bone cortex with or without pseudarthrosis
7. A first-degree relative with NF1 by the above criteria

first years of life. Identification of Lisch nodules requires biomicroscopic examination, and referral to an ophthalmologist with this specific question is recommended. Pseudarthrosis can be presumed in small children based on anterolateral bowing of the tibia. Thus, most diagnostic signs can be found by careful clinical examination. Since the development of different manifestations of NF1 follow different timetables, it is practical to consider NF1 diagnosis in different age groups.

2.1.1 NF1 Diagnosis in Children Under 5–6 Years

2.1.1.1 Café-au-Lait Macules

Café-au-lait spots are the most common first sign of NF1, and they are present in 95 % of patients by the age of 1 year (DeBella et al. 2000). Café-au-lait spots may be visible at birth, but usually begin to appear during the first months and are evident by the third birthday. Early appearance of café-au-lait macules is a very important diagnostic feature when differential diagnosis is considered (see Table 2.2). Café-au-lait spots are slightly darker than the person's skin, and they have even color. Because they are macules, meaning that they differ from the surrounding skin only in terms of color, they cannot be felt by palpation. Typical café-au-lait macules in NF1 are over 0.5 cm in diameter, oval in shape, and have well-defined smooth borders (Fig. 2.1). The delineation may also be rough. Solitary café-au-lait macules, common "birth marks," however occur in 10–15 % of the general population.

Café-au-lait macules are easily detectable by any health-care professional who understands them as a diagnostic sign. Since about half of all NF1 cases are sporadic, the diagnosis may be delayed. Four to six macules in a child should raise the suspicion of NF1, but if neither of the parents has NF1, café-au-lait macules are too often ignored by primary health care as a sign of a syndrome. Differential diagnosis of café-au-lait macules is not always simple since other lesions may mimic these spots (Shah 2010). If the suspicion of NF1 emerges, the parents need to be examined carefully for café-au-lait macules, axillary or inguinal freckles, and cutaneous neurofibromas, since a very mild NF1 may have remained

Table 2.2 Differential diagnosis of café-au-lait macules

Diagnosis	Clinical features
Melanocytic nevus	Color may be light brown to dark brown. Areas of different color and hypertrichosis can be seen. Congenital nevus may first be smooth and become elevated within the first years
Lentigo	Found mainly in sun-exposed areas, very seldom present in small children
Becker nevus	Light brown, usually appears in adolescence in the upper trunk or shoulder areas. Hypertrichosis is common
Nevus spilus	Hyperpigmented macule, may have sharp or poorly defined outline, with dark small nevi within the macule
Postinflammatory hyperpigmentation	May follow various inflammatory skin diseases such as atopic dermatitis or varicella. The borders are not as sharp as in café-au-lait macules
Pityriasis versicolor (tinea versicolor)	Superficial yeast infection. Light-brown macules localize to trunk and slight scaling appears if scratched
Urticaria pigmentosa	Light-brown macules, delineation may be quite sharp. In addition to macules, light-pink papules and maculopapules are seen. Histology of a papule confirms the presence of mast cells

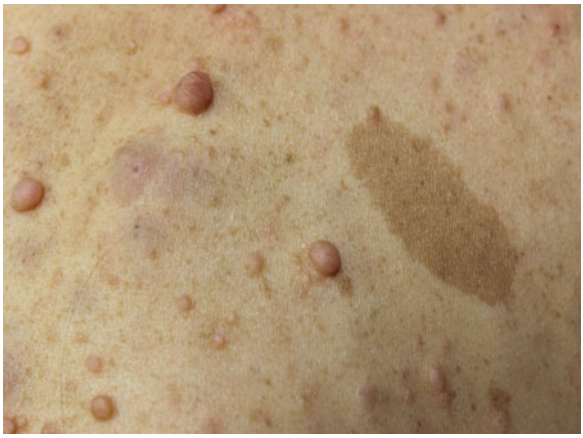

Fig. 2.1 Café-au-lait macule and cutaneous neurofibromas. Typical café-au-lait macules are evenly pigmented and have a clear outline. Neurofibromas can be elevated or appear as purplish macules (photo, Eeva-Mari Jouhilahti)

unrecognized even in an adult. Thus, sole anamnestic data on the parents not having NF1 does not exclude an inherited NF1 in a child. Differential diagnosis of café-au-lait macules is presented in Table 2.2.

2.1.1.2 Plexiform Neurofibromas

About 25–30 % of NF1 patients have a visible or symptomatic plexiform neurofibroma, but whole-body MRI reveals more plexiform tumors: over 50 % of 65

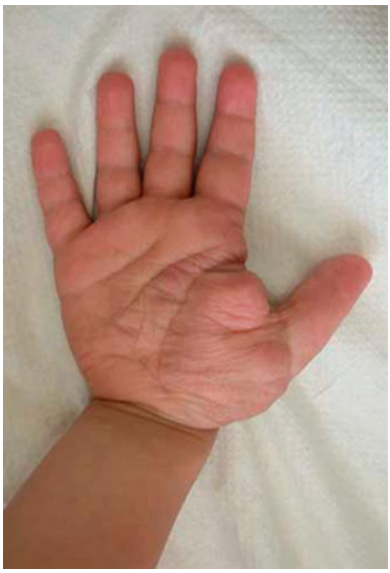

Fig. 2.2 Plexiform neurofibroma in the palm of a child

pediatric NF1 patients studied had one or more plexiform tumor (Nguyen et al. 2011; Chap. 7). Plexiform tumors are most common in the lower extremity, followed by thoracic, paraspine, and pelvic tumors (Nguyen et al. 2011). The facial tumors which will be symptomatic usually manifest during the first years of life (Ferner et al. 2007). Head and neck tumors may be readily visible in a newborn but may as well first cause only a slight asymmetry of cheek or lip. Plexiform tumors may be anticipated in clinical examination by paying attention to body asymmetries, not forgetting the palms and soles (Fig. 2.2). Plexiform neurofibromas growing from spinal roots through the abdominal wall may be visible as an asymmetric curvature on the waistline, or a limb lesion leads to a bulkier appearance on one side. The thin skin overlying a tumor may look slightly purplish and mislead diagnosis to a vascular malformation. The skin over plexiform neurofibromas is sometimes hairy and/or hyperpigmented, with a hypopigmented halo encircling the tumor. Plexiform neurofibromas carry a risk for malignant transformation already in childhood (Evans et al. 2002; Chaps. 28 and 29).The size and distribution of plexiform tumors are best visualized by MRI, but whole-body MRI is not used as a diagnostic procedure.

2.1.1.3 Pseudarthrosis

Long bone dysplasia complicates NF1 in 3–5 % of patients (Friedman and Birch 1997, Stevenson et al. 1999; Chap. 21). Pseudarthrosis is most common in the tibia and becomes evident by the time when the child learns to stand up and walk. The tibia or both the tibia and fibula bend anterolaterally and may fracture. Pseudarthrosis

may in rare cases develop to other long bones such as the ulna or radius. The surgical treatment of a fractured pseudarthrosis is challenging, and there are not enough large studies to allow any consensus regarding therapeutic approaches (Elefteriou et al. 2009). Since at least half of all the pediatric patients with pseudarthrosis have NF1, the skin of all pediatric pseudarthrosis patients should be searched for café-au-lait macules.

2.1.1.4 Glioma of the Optic Nerve

Of children with optic glioma, over half have NF1 (Nicolin et al. 2009). In NF1 patients, optic nerve gliomas are histologically grade I pilocytic astrocytomas (Listernick et al. 2007). They occur in about 15 % of children with NF1 (Listernick et al. 1994). Most optic gliomas appear during the first years of life but appearance in adolescence or adulthood is possible. The risk of developing a symptomatic optic glioma is greatest in children under 7 years, the mean age of diagnosis being 5 years (Nicolin et al. 2009). The most common symptoms caused by optic gliomas and leading to MRI are decreased visual acquity, proptosis, and headaches combined with vomiting. Optic glioma may also cause precocious puberty by interfering with the pituitary gland. Optic gliomas related to NF1 are, however, most often asymptomatic, and their natural history is more indolent than in patients without NF1. Optic gliomas can be detected by MRI which requires anesthesia in small children. Routine MRI in an asymptomatic child is not indicated, but visual assessment should be carried out even in the youngest children. If, however, MRI is carried out, T2-weighted images often reveal hyperintense lesions, sometimes misleadingly be called hamartomas, in various locations in the brain. This finding can assist the NF1 diagnosis, but MRI under anesthesia is not warranted for this purpose.

2.1.1.5 Other Features of NF1 in Small Children

Table 2.3 lists clinical features of NF1 which can be present in children and require evaluation.

2.1.1.6 Mutation Analysis

If the parents do not have NF1, and café-au-lait macules are the only finding of a small child, the NF1 diagnosis cannot be made until additional clinical features appear. This may take up to 5 years during which follow-up visits and ophthalmologic evaluations need to be carried out. Living in uncertainty is also stressful for the family. Mutation analysis of the *NF1* gene may clarify the diagnosis earlier and can be carried out from the probands' blood sample. Current multistep mutation detection protocol allows the identification of pathogenic mutations in well over

Table 2.3 Additional clinical features of NF1 which may require attention in children

Symptom	Clinical finding
Short stature and macrocephaly[a]	13 % of patients have short stature (>2 standard deviations below the population mean), and 24 % have macrocephaly (occipitofrontal circumference >2 standard deviations above the population mean)
Cognitive deficits, learning difficulties[b]	Most common NF1 complication in children (over 50 %). Should be assessed in all children with NF1
Dystrophic scoliosis	Sharp angulation over a short segment of the spine, develops over a period of a few months in about 5 % of children. Potentially debilitating, it requires surgical intervention
Delays in motoric and speech development[c]	Should be assessed in all children with NF1
Precocious puberty	Signs of puberty under the age of 8 years. A sign of an optic glioma
Skin color	Generalized mild hyperpigmentation
Juvenile xanthogranuloma	Yellowish-red nodules with predilection for the head and neck. Usually arise during the first 6 months of life. Increased risk for juvenile myelomonocytic leukemia has been reported[d]
Congenital glaucoma	Unilateral buphthalmos is a rare complication in the newborn period
Epilepsy	Seizures and infantile spasms are rare in NF1
Renal artery stenosis	High blood pressure
Pulmonary arterial stenosis and hypertension	Children with unexplained murmur and dysmorphic features should undergo cardiological evaluation

[a]Szudek et al. (2000).
[b]Hyman et al. (2006).
[c]Alivuotila et al. (2010).
[d]Raygada et al. (2010).

90 % of NF1 patients (Chap. 10). A negative diagnosis virtually rules out NF1 and eliminates the need of specialist follow-up. The *NF1* mutation analysis has been calculated to be cost-effective, when all the costs of health care are taken into account (Tsang et al. 2012). In addition, the improvement of quality of life upon ruling out NF1 is of great significance.

2.1.2 NF1 Diagnosis in Children over 5–6 Years

2.1.2.1 Axillary and Inguinal Freckling

Typical NF1 features, axillary and inguinal freckles, most often begin to appear at the age of 3–5 years, and freckling is visible in about 80 % of patients with NF1 by 7 years of age. Thus, freckles serve as a useful diagnostic sign in children over 5–6 years. More widespread freckling in several skin areas such as neck and trunk is also common in persons with NF1.

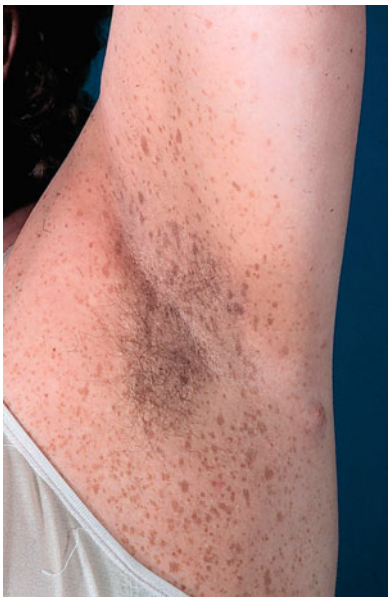

Fig. 2.3 Axillary freckling in an adult NF1 patient

Freckles in axillary and inguinal areas are the same color as café-au-lait macules, but smaller (Fig. 2.3), and they cannot be found in general population. Skinfolds are easily ignored in general medical examination, but for NF1 diagnosis the folds require careful inspection.

2.1.2.2 Lisch Nodules

Lisch nodules (Fig. 2.4) are asymptomatic lesions of the iris which usually appear by the age of 5–6 years. Over 90 % of adults with NF1 have Lisch nodules. They cannot be reliably diagnosed without a slit lamp examination, and their diagnosis should therefore be left to an ophthalmologist.

2.1.3 Diagnosis of NF1 in Puberty and in Adults

2.1.3.1 Neurofibromas

Two or more neurofibromas are a diagnostic sign of NF1, but it should be recognized that single neurofibromas are a common finding in otherwise healthy adults. Neurofibromas are benign tumors which contain the elements of normal peripheral nerve (Peltonen et al. 1988; Chap. 26). Cutaneous neurofibromas start to grow during puberty, and they are usually first seen on the abdomen and back as

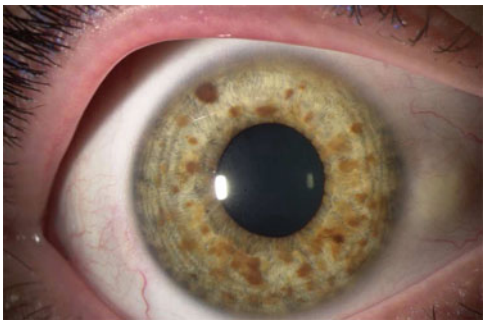

Fig. 2.4 Diagnosis of Lisch nodules in the iris requires examination by an ophthalmologist (photo, Vesa Aaltonen)

slightly elevated soft nodules. When they grow further, they become more visible and protrude above the skin level (Fig. 2.1).They may also appear as purplish macules and be mistaken for bruises. These neurofibromas are hence not elevated, and when palpated they feel softer than the surrounding skin (buttonhole sign). If the diagnosis of NF1 is not certain, removing a cutaneous neurofibroma for histology is indicated. Otherwise, cutaneous tumors need not to be operated upon unless they cause disturbance for the patient, since these tumors do not undergo malignant transformation. The number of cutaneous neurofibromas in adult NF1 patients varies from a few to thousands. When numerous, cutaneous neurofibromas cause esthetic and social disability and give rise to the major disease burden in adult patients. Patients with NF1 microdeletions tend to have heavier tumor loads (see below).

Subcutaneous neurofibromas also start to grow during puberty. They are firm nodules which move under the skin and may be tender to the touch. When touched, subcutaneous neurofibromas may also cause pain or tingling in the affected nerve. Careful consultation of, and consideration by, a specialist is needed before operative removal of subcutaneous neurofibromas since careless surgery may injure the nerve traversing the tumor.

2.1.4 Differential Diagnosis of NF1

Café-au-lait macules are associated with several other syndromes in addition to NF1. Table 2.4 lists various syndromes which are characterized by café-au-lait macules.

The most important differential diagnosis of neurofibromas includes common melanocytic nevi, lipomas, and schwannomas. When making an NF1 diagnosis in a patient with only a few skin tumors, a histological diagnosis for at least one or two neurofibromas is warranted. Subcutaneous neurofibromas clinically resemble lipomas which are far more common in the general population than neurofibromas. Table 2.5 lists syndromes with tumors which can be confused with neurofibromas.

Table 2.4 Syndromes with café-au-lait macules

Syndrome	Symptoms
Legius syndrome (MIM 611431)	Café-au-lait macules similar to those in NF1, axillary freckling but no tumors[a]
NF2 (MIM 101000)	Café-au-lait macules similar to those in NF1 in 40 % of patients. Macules usually less numerous than in NF1
McCune–Albright syndrome (MIM 174800)	Large café-au-lait macules with jagged borders. May follow lines of Blaschko and respect midline. In addition, fibrous dysplasia of bone, precocious puberty, and other endocrinopathies[b]
LEOPARD (MIM 151100) syndrome	Café-au-lait macules, lentigines, pulmonary stenosis, hypertelorism, hearing loss[c]
Mismatch repair cancer syndrome (MIM 276300)	Café-au-lait macules, childhood cancers, hematological malignancies, brain tumors, early-onset colorectal cancers[d]
Neurocutaneous melanosis (MIM 249400)	Large bilateral hairy dark nevus with satellite nevi over the trunk and neck in the neonatal period[e]
Peutz–Jeghers syndrome (MIM 175200)	Lentigines in perioral region, conjunctivae, and genital mucosa. GI polyps, neoplasias of GI tract, pancreas, breasts, ovaries, and uterus
Carney complex (MIM 160980)	Lentigines, primary pigmented nodular adrenocortical disease, cardiac, breast and skin myxomas, schwannomas, and testicular tumors

[a]Brems et al. (2007).
[b]Dumitrescu and Collins (2008).
[c]Digilio et al. (2002).
[d]Chap. 16.
[e]Lodish and Stratakis (2011).

2.2 Atypical Forms of NF1

Phenotype–genotype correlations of NF1 have been sought based on unusual phenotypes or similarities in the clinical outcome between family members in more than one or two generations. However, only a few mutation types have been linked to a typical combination of clinical characteristics (Chap. 43).

2.2.1 Microdeletion

NF1 microdeletions are large deletions which cover the whole *NF1* gene and multiple neighboring genes. Three different NF1 microdeletion types are known (Chap. 14), and altogether they comprise about 10 % of NF1 mutations. The majority of microdeletions are maternal in origin, and they cause a more severe NF1 phenotype than other types of mutations (De Raedt et al. 2003, Pasmant et al. 2010, Messiaen et al. 2011). Patients with NF1 microdeletions have craniofacial dysmorphism with coarse facial appearance and large hands and feet. Sometimes the dysmorphic features are described as resembling Noonan syndrome (Chap. 32).

Table 2.5 Syndromes with multiple tumors

Tumor type	Syndrome	Clinical finding
Neural tumors	NF2 (MIM 101000)	Schwannomas: cutaneous, intradermal, subcutaneous, and bilateral vestibular. Neurofibromas may be found. Ependymomas and meningiomas
	Schwannomatosis (MIM 162091)	Multiple schwannomas in skin and spinal and peripheral nerves
Lipomas	Multiple lipomatosis (MIM 151900)	Numerous encapsulated lipomas in trunk and extremities
	PTEN hamartoma tumor syndromes (PHTS)[a] Cowden syndrome (MIM 158350)	Macrocephaly, multiple lipomas, facial trichilemmomas, acral keratoses, papillomatous papules, and an increased risk for the development of breast, thyroid, and endometrial carcinoma
	Bannayan–Riley–Ruvalcaba syndrome (MIM 153480)	Lipomas, hemangiomas, and pigmented speckled macules of the glans penis in males, macrocephaly, developmental delay
Lipomas and/or other nodular skin or mucosal lesions	MEN1 (MIM 131100)	Café-au-lait macules (1–3), facial angiofibromas, collagenomas, lipomas, various endocrine tumors (parathyroid hyperplasia, pancreatic, and pituitary adenomas)[b]
	MEN2A (MIM 171400)	Café-au-lait macules (1–3), cutaneous lichen amyloidosis, medullary thyroid carcinoma with or without pheochromocytoma, and hyperparathyroidism
	MEN2B (MIM 162300)	Mucosal and intestinal neuromas, marfanoid habitus, aggressive medullary thyroid carcinoma, pheochromocytoma
	FAP + extracolonic features (Gardner syndrome) (MIM 175100)	Cystic nodules in skin.[c] Lipomas, fibromas, desmoid tumors, osteomas, FAP
Overgrowth syndromes	Proteus syndrome (MIM 176920)	Asymmetric overgrowth in limbs. Linear verrucous epidermal nevus, connective tissue nevus typically on the sole of the foot, a few small café-au-lait macules[d]
	Klippel–Trénaunay–Weber (MIM 149000)	Cutaneous capillary malformations (port-wine stain), varicose veins, or venous malformations, and bony or soft tissue hyperplasia of an extremity
	Congenital generalized fibromatosis (MIM 228550)	Multiple fibroblastic tumors of various tissues

[a]Blumenthal and Dennis (2008).
[b]Almeida and Stratakis (2010).
[c]Juhn and Khachemoune (2010).
[d]Nowak (2007).

NF1 microdeletion patients are taller, typically have a heavy tumor burden, and have an elevated risk for MPNST compared to other NF1 patients. Cognitive deficiency ranging from learning disability to mental retardation is more common in microdeletion patients, and their average IQ is slightly lower than in NF1 patient groups having other types of mutations (Descheemaeker et al. 2004).

2.2.2 Spinal NF

Familial spinal NF or hereditary spinal neurofibromatosis (FSNF, MIM 162210) is a rare form of NF1 (Messiaen et al. 2003). All affected adult family members have multiple symmetrically distributed spinal nerve root neurofibromas and café-au-lait macules, but no other diagnostic signs of NF1. Only 12 two- or three-generation families with spinal NF and an identified *NF1* mutation have been reported. *NF1* mutations reported were splice or missense mutations, but there are no clear genotype–phenotype correlations (Chap. 10). It should however be noted that isolated, asymptomatic spinal tumors are common findings in NF1 patients, but only a few of them are symptomatic (Chap. 7).

2.2.3 3-bp Deletion in Exon 17

Some families have very mild NF1 with only café-au-lait macules, skinfold freckling, and Lisch nodules but no neurofibromas in multiple generations (Upadhyaya et al. 2007; Chap. 10). In these families, a 3-bp AAT deletion in exon 17 has been found. In this patient group, the NF1 symptoms are so mild that it would not be surprising if many of these families would still be undiagnosed. On the other hand, the 3-bp deletion in exon 17 cannot be found in all families with this phenotype.

2.2.4 Mosaic NF1

Mosaic NF1 is considered to be caused by a somatic mutation during embryonic development. Mosaic NF1 patients do not have symptoms such as pigmentary lesions and neurofibromas in all skin areas, although they may fulfill the NF1 diagnostic criteria. Segmental neurofibromatosis refers to mosaic NF1 in which neurofibromas and/or pigmentary changes are only found in a localized area unilaterally (Fig. 2.5). Based on the clinical symptoms, it is not possible to predict whether the *NF1* gene mutation is found in germ cells, and patients with segmental NF1 have been shown to transmit the mutation to the next generation (Poyhonen 2000; Chap. 12). This possibility needs to be considered in genetic counseling.

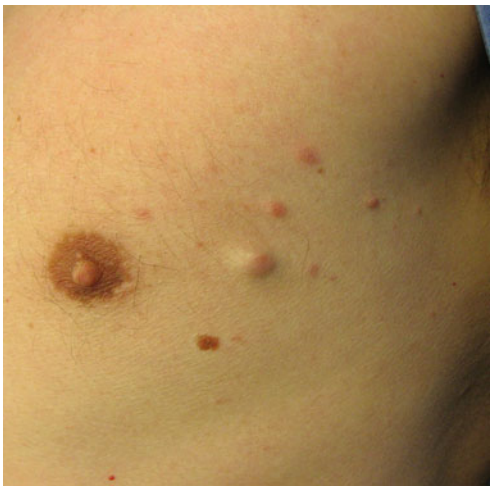

Fig. 2.5 A group of neurofibromas in the chest of a patient with segmental NF

The prevalence of mosaic NF has been estimated to be 1/36,000–1/40,000 (Ruggieri and Huson 2001), but it is likely to be an underdiagnosed condition.

2.2.5 Watson Syndrome

Watson syndrome (MIM 193520) is an extremely rare autosomal dominantly inherited syndrome with café-au-lait macules, pulmonar stenosis, and learning difficulties. It has been reported only in a few families, and in most of them an *NF1* mutation has been found.

In summary, this chapter reviews the diagnosis of NF1 at different ages since the diagnostic signs of the disease become manifest to different timetables. Although the first signs of NF1 manifesting in childhood are café-au-lait macules, plexiform neurofibromas, optic gliomas, freckles of the flexural areas and Lisch nodules, cutaneous neurofibromas usually begin to grow only in adolescence. This chapter presents the main differential diagnoses of café-au-lait macules and syndromes with cutaneous tumors. The role of mutation analysis in NF1 diagnostics is also discussed.

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Chapter 3
Management and Treatment of "Complex Neurofibromatosis 1"

Rosalie E. Ferner and Susan M. Huson

3.1 Introduction

There are approximately 17,000 patients in England with neurofibromatosis 1 (NF1), an inherited tumour suppressor disorder with a birth frequency of 1 in 2,500–3,000 and a minimum prevalence of 1 in 4,000 (Huson et al. 1989). NF1 has a major impact on the skin, nervous system and bone (National Institutes of Health Consensus Development Conference Statement Neurofibromatosis 1988; Huson et al. 1988; Ferner 2007). The disease complications are numerous, widespread and variable even within families. They range from cognitive problems, hypertension and gastrointestinal problems to disfigurement and malignancy including nervous system tumours (Huson et al. 1988; Ferner 2007). Many individuals do not have serious health problems related to NF1 and manage well with support and education from local clinicians and lay organisations. However, some NF1 patients suffer from rare disease complications that are associated with significant morbidity and may be life-threatening (Ferner 2011; Huson and Evans 2011). The needs of these individuals are best served by a specialised multidisciplinary team of clinicians and nurses conversant with the diagnosis and management of unusual manifestations of NF1 and with developments in novel therapies.

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3.2 National Health Service Commissioning

National Health Service (NHS) Commissioning is undertaken in England to assess the requirements of the population and to tailor the health services to their needs (Scotland, Wales and Northern Ireland have different commissioning systems). The remit of the selected health service providers is to deliver optimal, safe and effective patient-centred care. Highly specialised services are commissioned nationally by the NHS Specialist Services to provide outstanding quality of care and management for conditions that affect about 500 people in England. The goal is for all patients to have access to the resources, expertise and range of services provided in designated national centres for rare conditions.

3.3 Development of a Nationally Commissioned "Complex NF1" Service

NF1 is a common disorder, but affected individuals are at risk of developing rare "complex" manifestations which may affect many of the body systems. These include extensive, disfiguring and symptomatic plexiform neurofibromas, peripheral and central nervous system complications, atypical NF1 phenotypes and bone dysplasia (Table 3.1) (Ferner et al. 1995, 2004; Ferner and Gutmann 2002; Ferner 2007; Ferner and Jackson 2011; Leonard et al. 2007; Listernick et al. 2007; Ferner, Huson and Evans 2011). Individuals with complex NF1 do not necessarily fall within the remit of one medical speciality, monitoring and management are frequently disparate and fragmented and patients are obliged to attend multiple clinical services in different hospitals.

In 1990, the Department of Neurology, Guy's and St. Thomas' Hospitals NHS Foundation Trust London (GSTT) and the Department of Medical Genetics, St. Mary's Hospital, Central Manchester University Hospitals Foundation Trust (CMFT) established clinical services for people with NF1 in the London and Manchester regions. As specialist NF centres GSTT and CMFT were instrumental in formulating and publishing National Consensus Guidelines for Diagnosis and Management of NF1 (Ferner et al. 2007). The services developed links with local genetics and neurology units, community paediatricians and the Neuro Foundation (lay NF organisation). However, they identified that the needs of patients with complex NF1 were not met by all existing local services in England. On the one hand, clinicians and patients reported cases of delay in diagnosis and treatment of serious NF1 complications like malignant peripheral nerve sheath tumour and misdiagnosis of pseudarthrosis as non-accidental injury. On the other hand, instances were recorded of unwarranted surgical intervention for asymptomatic cervical neurofibromas and chemotherapy treatment for indolent optic pathway gliomas.

After extensive discussion and an application to the National Specialist Commissioning Team, the national service was funded and designated in April

Table 3.1 NFI manifestations of complex NF1 (Ruggieri and Huson 2001; Ferner 2007)

Extensive, symptomatic plexiform neurofibroma involving face, limb, thorax, abdomen or pelvis
Spinal cord compression arising from cervical neurofibroma
Neurofibromatous neuropathy
Atypical neurofibroma/malignant peripheral nerve sheath tumour
Symptomatic optic pathway glioma
Brain and spine glioma
Refractory epilepsy due to underlying structural lesion
Complex neurovascular disease
Multiple sclerosis
Sphenoid wing dysplasia
Pseudarthrosis of long bone
Atypical phenotypes
Prenatal counselling for segmental NF1

2009 in GSTT and CMFT to assess, review and manage the care of patients with complex NF1 in England.

Individuals with uncomplicated NF1 continue to be seen within the existing clinical setup, requiring diagnosis, genetic counselling, monitoring of education, blood pressure, bone health and neurofibromas (Ferner et al. 2007). Flexibility is required within the system as some individuals with non-complex disease will develop complex NF1 during the course of follow-up; conversely, the complex manifestations of NF1 may resolve after treatment, and patients will transfer to a non-complex service either at GSTT/CMFT or in their local area.

3.3.1 Aims of National Complex NF1 Service

The National Complex 1 Services in England work in partnership with clinicians, allied health professionals and with the Neuro Foundation to provide the best possible diagnosis, treatment, education and support for patients with NF1 and their families.

3.4 Clinical Manifestations of NF1 Included in Complex NF1 Service: Role of the Nationally Commissioned Specialist Teams (Table 3.1)

3.4.1 Extensive Plexiform Neurofibromas (Huson et al. 1988; Ferner et al. 2007)

Symptomatic plexiform neurofibromas that cause disfigurement of the face, diffusely involve a limb, thorax, abdomen or pelvis are included. The team ensure

that patients are seen by surgeons with expertise in neurofibroma surgery; the possible risks of haemorrhage, tumour regrowth, delayed wound healing and neurological, respiratory, or sphincter problems require discussion. People with symptomatic neurofibromas that are negative on [18F]-2-fluoro-2-deoxy-D-glucose positron emission tomography computerised tomography (FDG PET CT) are monitored twice yearly for 5 years to ensure that there is not malignant change (see Sects. 3.4.4 and 3.4.5).

3.4.2 *Spinal Cord Compression Arising from cervical Plexiform Neurofibromas (Leonard et al. 2007)*

NF1 individuals with neuroimaging evidence of spinal cord compression arising from cervical neurofibromas may remain asymptomatic for long periods without surgery. Patients should be assessed jointly by a neurologist and neurosurgeon with input from an experienced neuroradiologist. Decisions regarding the need for surgical intervention must be based on a combination of neurological deficit and neuroimaging. Assessment of pulmonary function is recommended as respiratory compromise may result from high cord lesions and be compounded in patients with significant scoliosis.

3.4.3 *Neurofibromatous Neuropathy (Ferner et al. 2004; Ferner 2007)*

Patients present with thickened nerves and symmetrical, length-dependent axonal neuropathy. Neurological examinations are undertaken on all NF1 patients and nerve conduction studies are performed on individuals with motor, sensory or reflex change that is not attributable to spinal nerve root, brachial or lumbosacral plexus disease, and common causes of neuropathy are excluded. NF1 neuropathy requires surveillance at least yearly because of the possible risk of developing malignant peripheral nerve sheath tumour (MPNST).

3.4.4 *Atypical Neurofibromas and Malignant Peripheral Nerve Sheath Tumour (Ferner and Gutmann 2002; Ferner 2007)*

MPNSTs are difficult to diagnose and treat, and high-grade tumours are associated with significant morbidity and mortality. Atypical neurofibromas are pleomorphic and hypercellular tumours, without evidence of mitosis, and they may have

malignant potential, as both atypical neurofibromas and MPNST have chromosomal aberrations and are positive on FDG PET CT (Beert et al. 2011; Ferner et al. 2008).

Persistent pain, rapid growth, hard texture or unexplained neurological deficit in association with a plexiform neurofibroma should prompt urgent referral and assessment in collaboration with specialist sarcoma units.

The remit of the complex NF1 service is to shorten the time between onset of symptoms and diagnosis of MPNST by facilitating rapid access to specialist sarcoma units and educating patients about MPNST. It is recognised that the greatest influence is likely to be on low-grade tumours as high-grade tumours declare themselves rapidly. To this end, the complex NF1 clinics have instituted joint meetings with the sarcoma units, and the major role for the clinical nurse specialist is to provide telephone advice and triage for patients with symptomatic neurofibromas.

3.4.5 Optic Pathway Glioma (Ferner 2007; Listernick et al. 2007)

Optic pathway gliomas (OPGs) are detected in 15 % of NF1 children but only 5–7 % of tumours are symptomatic, and many do not cause progressive visual deterioration. Reasons for instituting chemotherapy vary across different centres and include progressive visual loss, severe visual impairment at outset, risk of visual loss to the other eye, inability to obtain visual function in infants and tumour growth on neuroimaging. Currently, the paediatric oncologists in England do not use visual function as an outcome measure and outcomes in NF1 are combined with sporadic OPG.

The aim is to liaise with the national paediatric oncology group to ensure that indications and protocols for treatment for NF1–OPG are standardised in all centres and that and that visual outcome measures recorded systematically. The role of the complex NF1 service is to work with the paediatric ophthalmologist and orthoptist to ensure that NF1 children attending the clinic have at least annual visual screening. Community paediatricians are encouraged to perform yearly visual screening for all NF1 children, advised to avoid magnetic resonance imaging as a screening tool for OPG, and to refer patients with OPG to the multidisciplinary complex NF1 service. The team recommend that children with known OPG are managed jointly by a paediatric oncologist and paediatric ophthalmologists in collaboration with an NF1 team.

3.4.6 Brain and Spine Glioma (Créange et al. 1999; Ferner 2007)

Gliomas may occur in all parts of the central nervous system, and are frequently indolent, but tumours presenting in adulthood and sited outside the optic pathway

are associated with a worse prognosis. The role of the nationally commissioned service is to link with general neurologists and neurosurgeons to highlight the association of these complications with NF1 and to discourage intervention in the absence of clinical or neuroimaging progression.

3.4.7 Refractory Epilepsy Due to Structural Lesion (Ferner 2007; Ferner and Jackson 2011)

NF1 increases the lifetime risk of epilepsy about tenfold: the seizures are usually mild, focal epilepsies predominate and potentially present from infancy to late middle age. Refractory epilepsy may be associated with a structural abnormality including hippocampal sclerosis, glioma or dysembryoblastic neuro-epithelial tumour (DNET) and in some cases responds to neurosurgical intervention. The role of the complex NF1 service is to link with epilepsy specialists to emphasise that patients with NF1 may have cognitive impairment and an increased risk of osteoporosis. Monitoring of 25-hydroxy vitamin D is required because the increased risk of osteoporosis in NF1 is compounded by anticonvulsant medication. Caution should be exercised in prescribing antiepileptic drugs that may exacerbate cognitive impairment and mood disturbance. If neuropsychological testing is undertaken prior to surgery, the possibility that cognitive impairment is related to the NF1 phenotype should be borne in mind. The remit of the specialist team is to improve seizure control by appropriate medication and make a diagnosis of epilepsy when the symptoms have been attributed to other NF1-related problems.

3.4.8 Multiple Sclerosis (Ferner et al. 1995; Ferner 2007)

All forms of multiple sclerosis (MS) are encountered in NF1, but unlike the general population the commonest presentation is with primary progressive MS. As NF1 is a tumour suppressor condition, there is a theoretical increased risk of malignancy associated with immunosuppressant therapy and an NF1 specialist should be contacted before instituting treatment in NF1 individuals with multiple sclerosis. People who have demyelinating disease and present with spinal cord pathology should have neuroimaging to exclude a symptomatic spinal neurofibroma.

3.4.9 Complex Neurovascular Disease (Leschziner et al. 2012; Rea et al. 2009; Rosser et al. 2005)

Hypertension is reported in NF1 individuals and increases the risk for neurovascular complications. Neurovascular disturbances are detected on neuroimaging in about 6 % of children and are associated with intrinsic abnormalities of the

intracranial blood vessels. Manifestations range from stenosis and/or occlusion of internal carotid and cerebral arteries, moyamoya disease, intracranial haemorrhage and aneurysm to post radiation vasculopathy and vertebro-vertebral fistula formation. Superficial siderosis is reported rarely in the general population and in NF1 (Leschziner et al. 2012). It is characterised by haemosiderin deposition in the subpial layers of the spinal cord and the brain. The presentation is with inexorably progressive ataxia, deafness and tinnitus. The condition is detected in people who have had previous neurosurgery for high cord lesions and is also associated with meningoceles, brain tumour and trauma.

The specialist NF1 team should be alert to the possibility neurovascular disease in people with unusual neurological symptoms, particularly in young patients, and rapid access to a neurovascular specialist should be facilitated.

3.4.10 *Pseudarthrosis of the Long Bones (Crawford and Bagamery 1988; Huson et al. 1988; Ferner et al. 2007)*

Bowing of the long bone is detected in about 2 % of children with NF1 and affects the tibia predominantly, but may also involve the ulna, radius and fibula. Fracture may occur after trivial trauma, healing is delayed and the consequence may be a false joint or pseudarthrosis.

The aim of the complex NF1 team is to educate paediatricians to ensure pseudarthrosis is not misdiagnosed as non-accidental injury. Prompt referral to a specialist unit orthopaedic unit conversant with this rare complication in NF1, is essential. The 25-hydroxy vitamin D status and bone chemistry should be checked and corrected if necessary.

3.4.11 *Sphenoid Wing Dysplasia (Ferner 2007)*

About 1 % of NF1 individuals have an absent sphenoid wing which causes the temporal lobe to push forward into the orbit, causing pulsating exophthalmos. The major problem is the cosmetic appearance and surgery is rarely required for visual impairment. The role of the complex NF1 service is to discourage major neurosurgical intervention in asymptomatic patients and to ensure that individuals contemplating surgery are assessed by a specialist craniofacial team.

3.4.12 *Atypical NF1 Phenotypes (Ferner, Huson and Evans 2011)*

In most patients, the clinical diagnosis of NF1 is straightforward, but about 2 % of referrals to a specialist NF clinic will be diagnosed with a disorder that is not NF1,

and about 5 % of patients will have a subtype of NF1, caused by germline or somatic mutations in the *NF1* gene or the *RasMapK* gene (Legius syndrome), (Brems et al. 2007). The aim of the complex NF1 service is to ensure that individuals with unusual clinical presentations receive appropriate clinical assessment and genetic investigation to facilitate the correct diagnosis, counselling and management.

3.4.13 *Genetic Counselling for Segmental NF1 (Ruggieri and Huson 2001; Maertens et al. 2007; Ferner, Huson and Evans 2011)*

In segmental NF1, the genetic mutation occurs after fertilisation and the signs and symptoms of the disease are restricted to one or more body segments. Regardless of the segment of the body affected, there is a small but definite risk of gonadal involvement and therefore full-blown NF1 being transmitted to the children. The empiric risk of this happening is no more than 5 %. Some patients with segmental NF1 find this risk unacceptable. CMFT offers prenatal genetic counselling and genetic testing with RNA blood testing to detect low levels of mosaicism and if this is normal mutation detection on melanocytes or schwann cells cultured from a skin biopsy from a CAL macule or neurofibroma, respectively.

3.5 The Complex NF1 Team (Table 3.2)

The NF1 group comprises a multidisciplinary team of clinicians, nurses, psychologists, coordinators and patient representatives. There are close links at all levels between GSTT and CMFT with regular face to face meetings, telephone and video conferencing. Multidisciplinary team meetings in neurosurgery, adult and paediatric neurology, craniofacial surgery, oncology and respiratory medicine facilitate decision making on complex clinical cases and ensures patient access to appropriate specialist expertise. Joint clinics between different specialities improve communication, education and governance. Referrals to the GSTT and CMFT are received from general practitioners, specialist nurses and hospital clinicians; patients from the south of England are assessed in London and from the north of England in Manchester. The aim is standardisation of care of complex NF1 patients throughout England and development of evidence-based guidelines to improve clinical outcomes.

The National Specialist Commissioning Team (NSCT) visits each centre twice yearly to audit patient numbers, geographical access to the services, staff recruitment, succession planning and patient involvement with the service. It is envisaged

Table 3.2 Complex NF1 staff roles

Staff member	Role
Lead clinician (Neurology GSTT; Genetics at CMFT)	Lead, coordinate and represent complex NF1 team at national level. Clinical care and decision making on patients attending complex NF1 service
Adult neurologist/paediatric neurologist	Diagnose, treat neurological complications, liaise with surgical teams Transitional clinics for young people
Geneticist	Diagnose, assess complex NF1, atypical phenotype; counselling for segmental NF1
Ophthalmologist and orthoptist	Assess vision in all complex NF1children, monitor vision in children with OPG
Respiratory physician	Monitor patients with symptomatic high cervical/thoracic neurofibromas
Adult/paediatric neurosurgeon	Neurosurgical assessment and surgery—cord compression, extensive nerve root neurofibromas, symptomatic epilepsy, brain gliomas, sphenoid wing dysplasia
Craniofacial surgeon	Assessment, surgery for patients with extensive craniofacial neurofibromas
Sarcoma surgeon	Assessment, management of MPNST
Oncologist	Assessment, treatment of symptomatic OPG and MPNST
Orthopaedic surgeon	Assessment, management of patients with pseudarthrosis
Plastic surgeon	Assessment, surgery for extensive plexiform neurofibromas
PET physician	Assessment of symptomatic neurofibromas for malignancy
Neuroradiologist	Neuroimaging neurological problems
Musculoskeletal radiology	Imaging plexiform neurofibromas, bone dysplasia
Neurophysiologist	Nerve conduction studies NF1 neuropathy
Clinical nurse specialist	Education, information and support of NF1 patients. Telephone clinics
Adult/paediatric psychologist/ psychiatrist	Assessment of cognition, clinical psychology assessment/ psychiatric evaluation, treatment

that the majority of specialist services require 5 years to reach full development and to be capable of measuring improvements in disease outcomes.

3.6 The NSCT Complex NF1 Service at GSTT 2009–2011

3.6.1 Demographics of Patients Seen at GSTT

In 2009–2010, the focus was directed to recruitment of the team and set-up of the clinics and multidisciplinary meetings. Between April 2010 and April 2011, 326 new and follow-up patients were assessed in 441 clinic appointments and the age range was 1–75 years (mean 30 years, median 29 years, SD 15.9 years). The clinical service is gradually expanding (see Table 3.3 for patient population April

Table 3.3 Demographics of patients attending GSTT complex NF1 clinic April 2010–October 2011

n = number of patients	April 2011–October 2011
Total number of patients (new and follow-up)	208
Males	102
Females	106
Patients >50 years	23
Patients <18 years	55
Deaths	1

2011–October 2011), but some patients will be transferred to the non-complex clinic when their problems have been treated.

3.6.2 Deaths April 2009–October 2011 GSTT Complex NF1 Service

Since April 2009, there have been seven deaths: four females and three males (age range 31–66 years; median 40 years). The cause of death was malignant peripheral nerve sheath tumour (4), brain glioma (1), peritoneal carcinoma (1) and brainstem exacerbation of multiple sclerosis (1). Although cardiovascular disease is postulated as a significant cause of death in NF1, no patient died from vascular disease in either the complex or non-complex services (Friedman et al. 2002).

3.7 Clinical Manifestations of Complex NF1: GSTT Experience 2009–2011

Many patients with serious NF1 complications are already under the care of GSTT and CMFT, but the challenging task ahead is to ascertain all patients in England with complex NF1 disease manifestations. This will require a combination of strategies including access to genetic registers, communication with specialist groups (neurologists, paediatricians, ophthalmologists and sarcoma surgeons) and educational meetings.

3.7.1 Extensive Plexiform Neurofibromas

The GSTT clinic has assessed 71 patients, age range 4–71 years, with symptomatic or disfiguring neurofibromas. Eighteen patients were referred to the service after April 2009, and 17 individuals had multiple symptomatic lesions. The plexiform

neurofibromas were located in the head and neck (17) limb (17), pelvis (8) and abdomen (4). Twenty patients were referred for surgery including one individual who required an above-knee amputation with division of the sciatic nerve, for intractable pain.

3.7.2 High Cord Compression Due to Cervical Neurofibroma

Twenty-six patients, age range 15–67 years, were identified with radiological signs of cord compression due to cervical neurofibromas, and 22 patients had previous surgery. Four patients were diagnosed with cord compression after April 2009, two required intervention and the team did not recommend surgery in two patients. One individual had non-organic deficit, and in the second case, the patient's symptoms were related to neurofibromatous neuropathy and not to cervical cord compression.

3.7.3 Neurofibromatous Neuropathy

Nineteen patients, age range 14–74 years, have been diagnosed on clinical assessment and neurophysiology with an axonal neuropathy that is related to NF1, and six new patients have been diagnosed since April 2009. Four out of 19 individuals have developed MPNST, and one has an atypical neurofibroma. No new cases of malignancy have been diagnosed since April 2009, and the patients with MPNST remain stable.

3.7.4 Atypical Neurofibroma

Twenty patients, age range 15–57 years, have atypical neurofibroma on biopsy, and seven have been diagnosed after April 2009. Eight patients have a history of malignancy including optic pathway glioma and malignant peripheral nerve sheath tumour. The sites of the lesions diagnosed since 2009 included abdomen and pelvis (2), limb (3), brachial plexus and neck (2). All but one of these patients underwent FDG PET CT and the lesions were all positive, suggesting malignant potential.

3.7.5 Malignant Peripheral Nerve Sheath Tumour

Since 2000, 70 patients have been referred to GSTT with presumed NF1 and MPNST, and these have been diagnosed as NF1–MPNST (57), sporadic MPNST (1), malignant sporadic schwannoma (1), gastrointestinal tumour (7), soft tissue sarcoma (3) and bone sarcoma (1). Twenty-three people have had MPNST for more

than 5 years, 17 have been diagnosed for less than 5 year and 30 out of 70 patients have died. Since 2009, four patients have died of metastatic disease 4 months, 13 months, 19 months and 37 months after diagnosis. Since April 2009, nine patients have been diagnosed with MPNST, age range 11–55 years. Seven of these patients have high-grade malignancy, two have a previous history of MPNST and six individuals have recurrent or metastatic disease.

3.7.6 Optic Pathway Glioma

The GSTT team look after 72 children and adults with NF1-related OPG, age range 5–43 years, and eight patients were diagnosed after April 2009. Five OPG were detected on visual screening and although initially asymptomatic, two of these children eventually required chemotherapy. Five patients had been treated previously with surgery and eight with radiotherapy, and two subsequently developed MPNST and one a brain glioma. Fifteen of 72 patients required chemotherapy.

3.7.7 Brain and Spine Glioma

Forty-nine patients with definite or presumed brain glioma, age range 6–56 years, attend the GSTT clinic. Since April 2009, there have been three new diagnoses of glioma including one temporal lobe and two in the brainstem. There was one death due to progressive neurological deficit arising from an astrocytoma, two patients required surgery for increased tumour growth and neurological deficit and a ventriculo-peritoneal shunt was inserted 10 years after presentation to treat progressive hydrocephalus. Two patients with spinal cord gliomas were treated with chemotherapy prior to 2009 and are stable.

3.7.8 Refractory Epilepsy

Fifty NF1 patients at GSTT, age range 5–75 years, have a previous or ongoing history of epilepsy, including 13 children and 37 adults, and most have cryptogenic focal epilepsies. Since April 2009, 16 patients have been diagnosed with refractory epilepsy due to DNET (5), glioma (2), hippocampal sclerosis (1) and related to intracranial shunt (1). One has improved after surgery for DNET and the majority have been stabilised on appropriate medication.

3.7.9 Complex Neurovascular Disease

Six patients aged 3–40 years have been referred with a history of complex neurovascular problems including perinatal intraventricular haemorrhage with arrested hydrocephalus, middle cerebral artery aneurysm requiring clipping and radiation-related vasculopathy. Two patients were diagnosed after April 2009; vertebro-vertebral fistula was diagnosed in a patient who complained of tinnitus, headache, speech disturbance and a loud bruit and was referred for neurosurgical intervention. A 52-year-old male reported progressive ataxia and deafness several years after spinal decompression for a cervical cord neurofibroma and was diagnosed with superficial siderosis. His symptoms have been helped by auditory rehabilitation (Leschziner et al. 2012).

3.7.10 Multiple Sclerosis

Fourteen patients (age range 29–69 years; median 45 years) have been assessed and diagnosed with primary progressive multiple sclerosis (4), relapsing remitting MS (3), secondary progressive MS (3), unclassified MS (1) and transverse myelitis (1). Six patients were diagnosed after April 2009, and there has been one death related to progressive brainstem disease. Two patients have MRI changes of demyelination but are currently asymptomatic, and two patients have required immunosuppressant therapy. Three patients have developed malignancies, but the diagnosis either predated immunosuppressant therapy or the patients did not receive specific treatment for MS.

3.7.11 Sphenoid Wing Dysplasia

Eight patients, age range 16–40 years, have been diagnosed with sphenoid wing dysplasia, one new referral was received after 2009 and underwent orbital decompression for cosmetic reasons. Two patients who referred for a second opinion elected not to have treatment when they had fully understood the extent of the proposed procedures.

3.7.12 Pseudarthrosis of the Long Bone

Twenty-nine patients, age range 3–48 years, have been assessed with pseudarthrosis of the long bone and there have been three new cases since 2009, including one erroneous diagnosis of non-accidental injury. The tibia is involved predominantly, but 4 patients have ulna pseudarthrosis.

3.7.13 Atypical Phenotypes (Huson 2011 in Ferner and Jackson 2011)

Since 2009 at GSTT, there have been five patients, age range 3–24 years, who were referred with a putative diagnosis of NF1 and have been diagnosed subsequently with Legius syndrome (personal communication Guy Leschziner), Beckwith–Wiedemann syndrome, ataxia telangiectasia, Albright's disease, PTEN–Cowden's disease (personal communication Dragna Josifova).

3.7.14 Genetic Counselling for Segmental NF1

The major expertise for genetic counselling of segmental NF1 is at CMFT and one couple where the man had segmental disease have had prenatal testing.

3.8 Conclusion

The complex NF1 service at GSTT and CMFT was established by the National Commissioning Service Team in April 2009. It has been complicated to set up because of the great variability and unpredictability of the clinical phenotype. The natural history of the disease can only be determined by ensuring data are collected on patients who move between complex and non-complex services. The strengths of the service are that they permit a solid infrastructure of clinicians and nurses with specialist expertise in diagnosing and managing neurofibromatosis 1. Standardised protocols for clinical care and outcome measures coupled with meticulous interrogation of data will provide the baseline for evaluating clinical trials. The goal for our NF1 patients is to provide information, to foster integration in the community and to promote independence in decision making. The task of raising awareness of NF1 in the general public is of paramount importance, so that people with NF1 are treated with respect and dignity and are permitted to reach their full educational, emotional, social and economic potential within society.

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Chapter 4
Mortality in Neurofibromatosis 1

Gareth R. Evans

4.1 Introduction

A small number of studies have confirmed a generally shortened life expectancy in both forms of the neurofibromatoses. Most studies have reported on mortality and life expectancy in NF1. Essentially two types of study have assessed this: cohort studies and death certification studies. Both types of studies have shown around an 8–15-year decrease in life expectancy in NF1 patients.

4.2 Mortality Studies

4.2.1 Cohort Studies

There have been four previous NF1 cohort studies. Sorensen et al. (1986) followed up a nationwide cohort of 212 NF1-affected patients in Denmark. They obtained follow-up information on 99 % over a 42-year period. In comparison with the local general population, survival rates were significantly reduced in relatives with NF1, worse in probands, and worst in female probands. Malignant neoplasms or benign central nervous system tumours occurred in 45 % of the probands, giving a relative risk of 4.0 (95 % CI 2.8, 5.6). Compared with the general population, male relatives with neurofibromatosis had the same rate of neoplasms, whereas female relatives had a nearly twofold higher rate (relative risk, 1.9; 95 % CI 1.1–3.1). Nervous system tumours were disproportionately represented.

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Zöller et al. (1995) conducted a 12-year follow-up study of 70 adult NF1 patients in the city of Göteborg, Sweden. Life expectancy, mortality, and cause of death were investigated. Survival in the NF1 cohort was compared to that in the general Swedish population. Twenty-two deaths occurred in the NF1 group, whereas 5.1 deaths were expected in the general Swedish population ($p < 0.001$). The mean age at death was 61.6 years. Malignancy was found in 12 (55 %) of the deceased (soft tissue sarcomas in 3, and carcinomas in 9). Mean age at death was 15 years younger than expected in the general population.

Evans et al. (2011) reported a population-based cohort from North West England (McGaughran et al. 1999). This differed from the previous two cohort studies in that it ascertained all individuals with NF1 rather than just adults. As such, the population still remains quite young and most deaths are yet to occur. Deaths were therefore biased towards a younger age where MPNSTs and gliomas predominate. This is reflected in 26 % (34/130) of deaths even in the unbiased regional cohort being due to MPNST when only 8–13 % of patients would be expected to develop this complication in their lifetime (Evans et al. 2002). Nonetheless, Kaplan–Meier analysis reduces the bias as living individuals are included. The median survival was substantially higher at 71.5 years, only 8 years less than the population mean survival. This would suggest that, despite ascertaining a younger NF1 population, NF1 individuals are living closer to the population norm than estimated from the previous cohort studies. Cause of death from death certification was established in 129/130 (99 %) cases (an updated analysis appears in Table 4.1). Of those living within the strict regional boundaries, 109/1,023 (10 %) had died. The most common cause of death was MPNST with 34/130 (26 %). Glioma was the second most common cause of death and the most common cause in those <20 years of age (Table 4.1). Conversely, cardiovascular deaths were not particularly common with only 10/80 deaths in those aged <50 years being attributed to this cause, and only six of these deaths have a clear vascular origin. However, 8/10 cardiovascular deaths <50 and 19/26 (73 %) of all cardiovascular deaths occurred in male NF1 cases. Two deaths aged 34 and 44 years were due to blood vessel disease secondary to radiotherapy in childhood. The clearest indication of a vascular death unrelated to radiotherapy was a ruptured thoracic aneurysm in a 20-year-old male. The mean and median age at death in the regional cohort from all causes was 43.55 and 44.13 years of age.

Comparing the NF1-associated deaths against deaths within the local North West England population, cardiovascular disease was fourfold more frequently recorded in NF1 males (proportionate mortality ratio (PMR) 4.1; 95 % CI 1.4–2.6) than the general population. Similarly, breast cancer death was recorded 3.5 times more commonly in NF1 females (PMR 3.5; 95 % CI 1.3–7.7). Brain tumours were also a more frequent cause of death in NF1 females than in the local population (PMR 29.5; 95 % CI 12.7, 58.1), although not statistically significantly so in males (PMR 6.7; 95 % CI 0.8–24.1). The most notable result was that of death attributable to MPNST: MPNST was reported as the cause of death significantly more often than expected in both NF1 males and NF1 females (male PMR 3,819.6; 95 % CI 1,971.4–6,672.5) (female PMR 7,788.2; 95 % CI 4,355.7–12,846.2).

Table 4.1 Cause of death by age cohort in NF1 patients in the Manchester cohort updated from Evans et al. (2011)

	0–10	11–20	21–30	31–40	41–50	51–60	60+	Total	NF on death cert
Glioma	4	2	3	4	1			14	8/14
MPNST		4	11	14	2	3	2	36	21/36
JCML	2							2	1/2
Rhabdomyosarcoma		1						1	0/1
Breast cancer			1	2	2	3	1	9	1/9
Colorectal cancer			1		1	1		3	0/3
Ovarian cancer					1		1	2	0/2
Lung cancer					1	1	1	3	0/3
Lymphoma						1		1	0/1
Other cancers						1	2	3	0/3
Post-op benign tumour		1			1		1	3	2/3
Tumour-related	**6/7**	**8/10**	**16/19**	**20/28**	**9/20**	**10/23**	**8/31**	**78/138**	**35/78**
Cerebrovascular				1	3	2	4	10	3/10
MI					2	3	7	12	0/12
Cardiomyopathy				1	1			2	0/2
Pulmonary hypertension						1		1	0/1
Cardiac failure					1	2	1	4	0/4
Ruptured aneurysm		1	1	1				3	0/3
Cardiovascular		**1/10**	**1/19**	**3/28**	**7/20**	**8/23**	**12/31**	**32/138**	**3/32**
Pneumonia	1			1		1		3	2/3
Quadriparesis/cord compression respiratory failure				1		1		2	½
Kyphoscoliosis respiratory failure					1	1	2	4	3/4
Pulmonary fibrosis				1		1		2	2/2
COAD						1	2	2	1/3
Respiratory	**1/7**	**0/10**	**0/19**	**3/28**	**1/20**	**4/23**	**3/31**	**12/139**	**8/12**
Epilepsy		1	1					2	1/2
Hepatorenal syndrome					1			1	0/1

(continued)

Table 4.1 (continued)

	0–10	11–20	21–30	31–40	41–50	51–60	60+	Total	NF on death cert
Septicaemia						1	1	2	0/2
Multiple organ failure							1	1	0/1
Renal failure				1			1	2	0/2
Suicide			1				1	2	0/2
Accident							2	2	0/2
Dementia							2	2	0/2
Mesenteric infarct small bowel strangulation						1		1	1/1
Other total	**0/7**	**1/10**	**2/19**	**1/28**	**1/20**	**2/23**	**8/31**	**14/138**	**2/14**
Unknown					2			2	
Total	**7**	**10**	**18**	**29**	**20**	**23**	**31**	**138**	**48/136(36 %)**

The fourth cohort study was from France (Duong et al. 2011). Consecutive NF1 patients referred to the National French Referral Center for Neurofibromatoses were included. Between 1980 and 2006, 1,895 NF1 patients were seen. Median follow-up was 6.8 years (range, 0.4–20.6). Vital status was available for 1,226 (65 %) patients, of which 1,159 (94.5 %) survived and 67 (5.5 %) died. Overall mortality was significantly increased in the NF1 cohort (PMR 2.02; CI 1.6–2.6; $p < 10^{-4}$). The excess mortality occurred among patients aged 10–20 years (PMR 5.2; CI 2.6–9.3; $p < 10^{-4}$) and 20–40 years (PMR 4.1; CI 2.8–5.8; $p < 10^{-4}$). Significant excess mortality was found in both males and females. In the 10–20-year age group, females had a significant increase in mortality compared to males (PMR 12.6; CI 5.7–23.9 and PMR 1.8; CI 0.2–6.4, respectively). The cause of death was reported for 58 (86.6 %) patients; MPNST was the most frequent cause of death (60 %). They found significantly increased PMRs indicating excess mortality in NF1 patients as compared to the general population. Overall mortality was significantly increased in NF1 patients aged 10–40 years and tended to be higher in females than in males. There was no excess mortality >40 years of age which may have been due to limited numbers in the older age groups.

4.2.2 Death Certificate Studies

There have been three published death certificate studies on NF1.The first death certificate study used data from Japanese vital statistics from 1968 to 1992 (Imaizumi 1995). This reported 605 deaths for which neurofibromatosis was listed as the underlying cause of death. The mean age at death in this study was 43 years. However, the authors did not distinguish between NF1 and neurofibromatosis 2 (NF2), and no data were available on causes of death other than neurofibromatosis. Additionally, because the only individuals included in the study were those who had NF1 listed as the underlying cause of death, neurofibromatosis was again believed to have been under-ascertained even by the author (Imaizumi 1995). Mean age of death in the Japanese study would be expected to be biased to a younger age as NF1 is less likely to be an attributable cause of death in older patients.

A second death certificate study from USA (1983–1997) established cause of death of 3,770 NF1 patients, and again the mean age of death was around 15 years lower than in the general population (Rasmussen et al. 2001). Mean and median ages at death for NF1 males and females were 54.4 and 59 years, respectively, compared with 70.1 and 74 years in the general population. Individuals with NF1 were 34 times more likely (PMR 34.3; 95 % CI 30.8–38.0) to have a malignant connective or other soft tissue neoplasm listed on their death certificates than the general population. Overall, NF1 cases were 1.2 times more likely than expected (PMR 1.21; 95 % CI 1.14–1.28) to have a malignant neoplasm listed on their death certificates, but the PMR was 6.07 (95 % CI 4.88–7.45) for persons who died at 10–19 years of age and was 4.93 (95 % CI 4.14–5.82) for those who died at 20–29

years of age. Similarly, cardiovascular disease was recorded more often than expected on death certificates of people with NF1 who died at <30 years of age (PMR 3.26; 95 % CI 1.31–6.71 at age <10 years; PMR 2.68; 95 % CI 1.38–4.68 at age 10–19 years; and PMR 2.25; 95 % CI 1.46–3.32 at 20–29 years) but not in older persons. These deaths were amongst 32,722,122 deaths in the United States in the study period representing 1 in 8,700 deaths.

The most recent death certificate study is from Italy (Masocco et al. 2011). These authors used the National Mortality Database and individual multiple-causes-of-death records to estimate NF1-associated mortality from 1995 to 2006. Of 6,753,000 deaths in the study period, 632 had a diagnosis of NF1 recorded, but in only 25 % was the underlying cause given as neurofibromatosis. The mean age for NF1-associated death was approximately 20 years lower than that for the general population. The gender differential may suggest that women are affected by more severe NF1-related complications, or they may simply reflect a greater tendency for NF1 to be reported on the death certificates of young women. They found an excess for malignant neoplasm of connective and other soft tissue (PMR 22.3; 95 % CI 15.50–30.95) and brain (PMR 4.2; 95 % CI 2.69–6.15), as the underlying cause of death but not for other sites, or for malignancy as a whole. They also found an excess for obstructive chronic bronchitis and musculoskeletal system diseases among elderly individuals with NF1. However, all PMRs for cardiovascular disease were below 1.0.

4.3 Discussion

There have now been seven major attempts to assess mortality in NF1.These provide life expectancy estimates that are reduced by 8–20 years. The main causes of early death in these studies were MPNST and glioma. The finding of early vascular deaths by Rasmussen et al. (2001) is supported by Evans et al. (2011) who found cardiovascular deaths were four times more common in males with NF1 than expected overall. However, this was not supported by the recent Italian study (Masocco et al. 2011). Malignancy risk in NF1 outside MPNST is probably not substantially increased in common tumours apart from gliomas, which are usually low grade and not considered malignant. Nonetheless, Evans et al. (2011) found that 8 NF1 patients died from glioma disease.

Although death certificate studies might be expected to overcome the ascertainment bias of cohort studies, they are dependent on NF1 being accurately recorded on the death certificate. In the Evans et al. (2011) study, only 35 % of NF1 patients with available death certification listed NF1 as a contributing cause on the death certificate. Even with complications such as glioma and MPNST, which would have clearly been secondary to NF1, only 29/48 (60 %) had NF1 or neurofibromatosis recorded on the death certificate. This could be a particular problem in assessing risk of death from common cancers. The recently identified increased risk of breast cancer (Sharif et al. 2006; Walker et al. 2006) is a case in point. Evans et al. (2011)

found an increased PMR for breast cancer of 3.5 (95 % CI 1.3–7.7), but only 1/9 had NF1 recorded on the death certificate. The use of death certificates by Rasmussen et al. (2001) and Masocco et al. (2011) therefore needs to be assessed against the likelihood that all deaths associated with NF1 were recorded. The Rasmussen et al. study found that NF1 was only recorded on 1 in 8,679 deaths. In the Masocco et al. (2011) study, this was even lower at 1 in 10,685. The estimated birth incidence for NF1 in most reports varies between 1 in 2,500 and 1 in 3,000 (Huson et al. 1989; Lammert et al. 2005; Evans et al. 2010). If it is assumed that incidence and death rates remain constant, then both death certificate studies are likely to have a substantial bias, with only 23–35 % of expected NF1 deaths being recorded on the death certificate. Under representation of NF1 deaths could also lead to an exaggeration in the degree of reduction in life expectancy of NF1 patients.

Several studies have suggested a higher mortality rate from malignancy in women with NF1 (Sorensen et al. 1986; Rasmussen et al. 2001; Duong et al. 2011). Evans et al. (2011) also reported a higher number of female deaths due to malignancy, but this was not significant. Indeed, the difference was accounted for almost entirely by the newly identified association between NF1 and breast cancer (Sharif et al. 2006; Walker et al. 2006).

Nearly all studies have shown that most of the excess mortality in NF1 exists prior to 50 years of age and that NF1 does not clearly contribute to more than a small minority of deaths after that age. Therefore, individuals living beyond 50 years without a serious NF1 complication could expect to live a near-normal life expectancy. Nonetheless, no study has yet circumvented completely the ascertainment biases inherent in such research, and the ideal study has not yet been performed. This would need to cover a cohort of NF1 patients identified with complete ascertainment and followed until every patient had died. The 212 nationwide cohort of NF1 patients identified by Sorensen et al. (1986) is close to maturation, but this clearly was not a fully ascertained population.

4.4 Conclusion

Whilst there are clearly excess deaths from malignancy in NF1 in the first 40–50 years of life and overall life expectancy is reduced, it is likely that life expectancy overall is not reduced by the 15–20 years suggested in many studies.

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Chapter 5
The Cognitive Profile of NF1 Children: Therapeutic Implications

Natalie A. Pride and Kathryn N. North

5.1 Human Cognitive Studies in NF1 Children

5.1.1 Introduction

Cognitive impairment is one of the most common complications of NF1 in childhood; approximately 70 % of affected individuals have learning difficulties and/or neuropsychological deficits. Cognitive dysfunction is an important cause of lifetime morbidity as it impacts on an individual's scholastic achievement, employment opportunities, and overall quality of life. Over the past 15 years, a great deal of research has been devoted to characterising the cognitive phenotype of NF1. Although there is marked variability between individuals with NF1, a number of core neuropsychological features have been identified—which, in turn, provide a basis for studies of disease mechanism and targets for therapy. The purpose of this chapter is to summarise the features of the NF1 cognitive phenotype and current knowledge concerning pathogenesis and potential therapies.

5.1.2 General Intellect and Academic Functioning

Mental retardation was once thought to be frequent in NF1, with early studies reporting that approximately 30 % of individuals with NF1 have an intellectual disability (Samuelsson and Axelsson 1981). However, these studies focused

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predominately on severely affected individuals and did not use quantitative psychometric assessment, resulting in ascertainment bias and gross overestimation of the frequency of mental retardation. Based on quantitative data from large cohorts of patients with a range of physical manifestations, it is now accepted that between 4 % and 8 % of individuals with NF1 fall into the intellectually impaired range (IQ < 70) compared to approximately 3 % in the general population (Hyman et al. 2005; North et al. 1997). Typically, full-scale IQ tends to fall within the average to low average range (high 80s to low 90s) (Hyman et al. 2005; Levine et al. 2006). Early studies reported a profile of better verbal skills and poorer perceptual organisational skills (Eliason 1986; Legius et al. 1995), although most studies have found a similar pattern of verbal and non-verbal skills (Ferner et al. 1996; Hyman et al. 2005; Moore et al. 2000; North et al. 1995). Some authors have proposed a particular neuropsychological model, non-verbal learning disorder (NVLD), to describe some aspects of the NF1 cognitive phenotype (Eliason 1986; Wang et al. 2000). The NVLD profile involves a pattern of poor mathematical ability; visuospatial, fine motor, and handwriting deficits; and social problems in the presence of sound verbal skills (Harnadek and Rourke 1994). Although deficits in mathematical ability and visuospatial skills are extremely common in NF1, there has been movement away from the conceptualisation of NF1 as an NVLD as recent studies have documented language, spelling, and reading impairments. Dyslexia, a specific reading disability, appears to be common in NF1 (Hofman et al. 1994; Mazzocco et al. 1995); one study found that approximately 50 % of their cohort met the diagnostic criteria for phonological dyslexia, i.e. impaired non-word reading and a specific difficulty in utilising spelling to sound rules to read (Watt et al. 2008). Specific impairments in phonological processing, including phoneme segmentation, rapid naming, phonological memory, word recognition, and decoding, have also been reported (Cutting and Levine 2010; Mazzocco et al. 1995). This pattern of deficits is comparable to the pattern of deficits seen in children with idiopathic reading disorders (Cutting and Levine 2010).

The reported frequency of learning disorder (LD) in NF1 varies across studies with estimates ranging between 30 % and 65 % (Brewer et al. 1997; Clements-Stephens et al. 2008; Ferner et al. 1996; Huson et al. 1988; Hyman et al. 2006; North et al. 1997). This variability in frequency is due to the use of different definitions of LD by different researchers. According to the current version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV TR) (American Psychiatric Association 2000), an LD is diagnosed when the individual's achievement on individually administered, standardised tests in reading, mathematics, or written expression is substantially below that expected for age, schooling, and level of intelligence. A variety of statistical approaches can be used to establish that a discrepancy is significant. "Substantially below" is usually defined as a discrepancy of more than 2 standard deviations (SD) between achievement and IQ; however, a smaller discrepancy (between 1 and 2 SD) has also been used, resulting in inconsistency between studies. In one study involving a large cohort of 81 children with NF1 (and 49 unaffected controls), Hyman et al. (2006) examined the frequency of specific learning disabilities (SLD) as defined by a discrepancy of 2 SD between IQ

and achievement on co-normed standardised tests (WISC III and WIAT) and found that 20 % of their cohort presented with an SLD, 32 % had a general learning difficulty (impaired academic achievement commensurate with low IQ), and 48 % had age-appropriate academic abilities. A strong gender effect for SLD was also reported with the bulk of this group being male. This has important implications for assessment and remediation and suggests that females with NF1 are at no greater risk of SLD than those in the general population.

5.1.3 Visual Spatial Function

One of the hallmark features of the NF1 cognitive phenotype is visual spatial impairment, characterised by a problem accurately perceiving and interpreting visual information. The Judgement of Line Orientation Task (JLO) is a test that is consistently used to measure this ability in the NF1 literature, and approximately 80 % of studies have documented deficits on this test in children with NF1 (Dilts et al. 1996; Hofman et al. 1994; Hyman et al. 2005, 2007; Levine et al. 2006; Mazzocco et al. 1995; Schrimsher et al. 2003). Many of these studies report impairment on this measure in a large majority of their NF1 cohort. For example, Hyman et al. (2005) compared 81 children with NF1 to 49 unaffected siblings and found 56 % of NF1 children performed within the impaired range on the JLO (greater than 1 SD below the general population mean). Deficits on a range of other tests that are sensitive to visual spatial and visual perceptual function such as the Beery-Buktenica Visual-Motor Integration Test, the Rey Complex Figure Test, block design subtest of the Wechsler Intelligence Scales, and the Test of Visual Perceptual Skills have also been identified in NF1 (Dilts et al. 1996; Hyman et al. 2005).

5.1.4 Attention

Attention deficit hyperactivity disorder (ADHD) is a neurobiological disorder that is characterised by persistent and pervasive symptoms of inattention, hyperactivity, and impulsivity. Although the exact incidence of ADHD in NF1 is unknown, estimates have ranged from 33 % to 49.5 % of study cohorts (Hofman et al. 1994; Kayl et al. 2000; Koth et al. 2000; Mautner et al. 2002; Payne et al. 2011), a marked increase above the estimates of ADHD in the general population (5 %) (Polanczyk et al. 2007). The majority of studies have shown that children with NF1 are more likely to meet the criteria for predominately combined or inattentive subtype of ADHD, and the frequency of the ADHD diagnosis across genders in NF1 is generally equal. This is in contrast to ADHD in the general population where the incidence rate is 2.5–9.0 times higher in males than females (Durston 2003).

Attention is a not a unitary process but refers to a multifaceted range of cognitive processes that operate through a variety of neural networks. It is central to the process of information reduction, response selection, and planning for eventual actions. Deficits on a number of tasks designed to measure distinct facets of attention have been documented in NF1. Continuous Performance Tests (CPTs) such as the Test of Variables of Attention (TOVA) and the Kiddie's CPT are common measures used to assess sustained attention (using errors of omission) and are frequently found to be impaired in NF1. Higher rates of errors of omission have been documented in children with NF1 when compared to unaffected siblings (Mazzocco et al. 1995; Sangster et al. 2011) and normative data (Ferner et al. 1996). Conversely, normal levels of CPT errors of omission have been reported elsewhere (Dilts et al. 1996; Mautner et al. 2002). Although there is inconsistency in these findings, there is sufficient evidence from studies using alternative measures of sustained attention to support a true sustained attention deficit in this population (Hyman et al. 2005). Studies investigating other areas of attention such as selective attention have offered inconsistent findings. Ferner et al. (1996) administered the Stroop Task to 98 children with NF1 and 105 matched controls and found this task was consistently impaired in children with NF1. Nonetheless, this conclusion should be viewed with caution given that this task relies on other cognitive processes to be completed successfully including response inhibition and single-word reading. In a recent study, which used the Sky Search subtest from the TEA-Ch to assess selective attention, Payne et al. (2011) found that NF1 patients ($n = 199$) performed significantly worse than unaffected controls ($n = 55$). In contrast, Hyman et al. (2005) did not find any differences between children with NF1 ($n = 81$) and unaffected siblings ($n = 49$) on this same measure. Inconsistencies between studies regarding the presence of selective attention deficits are likely a result of variability in subject selection and choice of control group (i.e. unaffected siblings versus matched controls) further highlighting the need for well-characterised samples and appropriate controls. Nevertheless, attention deficits are one of the most common manifestations of the NF1 cognitive phenotype. Studies that identify the underlying neurobiology and biochemical mechanisms involved are likely to provide important insights into the best targeted therapies for ADHD in children with NF1 as well as the pathogenesis of ADHD in the general population.

5.1.5 Executive Function

Several behavioural characteristics, including an unstructured learning style, distractibility, impulsivity, failure to plan, and poor problem-solving skills, were identified in early studies of children with NF1; these characteristics are reminiscent of the kinds of impairments seen in patients with prefrontal cortical damage (Bawden et al. 1996; Eliason 1986; North et al. 1995). During recent years, there has been a significant increase in the body of literature supporting a primary role for

executive function deficits in the NF1 cognitive phenotype. Executive functions refer to a set of abilities that regulate and control other abilities and behaviour, e.g. the ability to problem-solve, shift attentional flexibility, monitor and change behaviour, and plan future behaviour when faced with novel tasks and situations. Executive dysfunction is thought to be a result of damage to the frontal regions of the brain, in particular, the prefrontal cortex, as well as subcortical regions. Deficits in a wide range of executive functions have been reported in NF1 including cognitive flexibility (Hyman et al. 2005; Joy et al. 1995; Payne et al. 2011; Zoller et al. 1997), set-shifting (Hofman et al. 1994; Mazzocco et al. 1995), abstract concept formation (Hyman et al. 2005; Payne et al. 2011), working memory (Huijbregts et al. 2010; Rowbotham et al. 2009), response inhibition (Ferner et al. 1996; Mautner et al. 2002), divided attention (Ferner et al. 1996; Payne et al. 2011), and planning (Bawden et al. 1996; Hofman et al. 1994; Hyman et al. 2005; Mazzocco et al. 1995; Payne et al. 2011; Roy et al. 2010). For the school-aged child with NF1, executive dysfunction may cause problems in several areas in the classroom. A rigid work style and cognitive inflexibility can contribute to difficulty adjusting to the school environment at an age-appropriate level, such that for some children a small departure in expected routine may result in adjustment difficulties and feelings of anxiety. Executive deficits can also make it difficult to start and finish work, keep track of assignments, and allocate their time. Executive dysfunction is associated with behavioural disturbance, social dysfunction, and reduced quality of life (Baron 2004; Lezak et al. 2004).

5.1.6 Memory and Learning

The terms “learning” and “memory” are often used interchangeably—even though they represent a range of diverse cognitive processes. Learning refers to how we acquire new information, whereas memory is the process by which that information is encoded, stored, and later retrieved. The hippocampus, located in the medial temporal lobe of the brain, has been linked to learning and memory function (Kandel et al. 2000). Distinct from these memory systems is working memory, which is heavily associated with the dorsolateral prefrontal cortex. Working memory is the ability to temporarily store and manipulate information in mind and is considered an executive function rather than part of the memory system. Although working memory impairment has been well documented in NF1 (see Sect. 5.1.4), controversy exists surrounding the presence of learning and memory deficits. While some studies report that the ability to learn and retrieve new information is intact in children with NF1 (Hyman et al. 2005; Joy et al. 1995; Moore et al. 1996), others do not (Ferner et al. 1996; Payne et al. 2012; Ullrich et al. 2010). The majority of these studies make it difficult to ascertain whether children with NF1 experience true memory impairment as the tests used to assess memory also require other cognitive functions such as language or visuospatial abilities. Recently, Payne et al. (2012) examined visuospatial learning in 71 children with NF1 and 29 unaffected controls

using a measure (Paired Associate Learning from the Cambridge Neuropsychological Test Automated Battery) that has been shown to be sensitive to hippocampal-based function in non-human primate studies (Taffe et al. 2002) and in clinical populations (Fowler et al. 2002). They found that these deficits were present even after accounting for sustained attention, visuospatial abilities, and intelligence, thus providing convincing evidence for a hippocampal-based learning impairment in NF1.

5.1.7 Language

Language can be divided into two general categories; expressive and receptive. Expressive language disorder is a communication disorder in which there are difficulties with verbal an written expression. It is characterised by a difficulty in expressing spoken language at the level expected for one's developmental age. Receptive language disorder occurs when a child has difficulty understanding what is said to them. Deficits in language are characteristic of the school-aged child with NF1. Almost half of the studies that have examined language in NF1 reveal a delay in some aspect of language (Dilts et al. 1996; Eldridge et al. 1989; Hofman et al. 1994; Hyman et al. 2005; Mazzocco et al. 1995; North et al. 1994). Dilts et al. (1996) examined broad language abilities using the Clinical Evaluation of Language Fundamentals Screening Test (CELF) in 19 children with NF1 (aged 6–17 years of age) and compared their performance on this screener to matched unaffected siblings; 58 % of their sample failed the expressive language screening test, 26 % of whom also exhibited receptive language deficits. Hyman et al. (2005) examined the receptive and expressive language abilities of 81 children with NF1 and found language delays are present to a lesser degree than initially reported. Compared to normative data, 15 % and 2.5 % of the NF1 group (aged 8–16 years) demonstrated receptive and expressive language delays, respectively. Deficits on tests of picture naming, receptive grammar, written language, and phonological processing have also been identified in NF1 (Eldridge et al. 1989; Hofman et al. 1994; Hyman et al. 2005; Mazzocco et al. 1995). Recent evidence suggests that delays in language development can be detected in children as young as 21 months. Lorenzo et al. (2011) used the MacArthur Communicative Development Inventories and Sentences to provide parental assessments of 39 toddlers with NF1 (aged 21–30 months) and found more than 70 % were rated as falling below age expectations. Language delays have also been found in children with NF1 aged 3–5 years with around 37 % demonstrating deficits on both expressive and receptive measures of language (Thompson et al. 2010). These findings have important implications for the management of children with NF1 in that they highlight the importance of conducting language assessments and intervention in children as young as 2.5 years of age, thus decreasing the risk of later problems with literacy.

5.2 Neurobiology of NF1 Cognitive Deficits: Human Imaging Studies

NF1 is a highly heterogeneous disorder in multiple respects, both behaviourally and biologically. The high frequency of cognitive deficits in individuals with NF1 undoubtedly reflects a perturbation of central nervous system functioning—and recent studies in both humans and in animal models are beginning to provide insights into the underlying neuroanatomical and biochemical mechanisms involved.

5.2.1 Structural Brain Abnormalities

Structural imaging studies have provided evidence of early abnormalities in brain development that coincide with, if not precede, the onset of cognitive deficits in NF1.

The single most replicated finding in NF1 has been an increase in brain size associated with *macrocephaly*, documented through head circumference and imaging measures of brain volume (Greenwood et al. 2005; Moore et al. 1996; Said et al. 1996). Several studies suggest that increased brain volume in children with NF1 appears to be largely driven by an increase in white matter; predominately in frontal regions and the corpus callosum, albeit, there is suggestion of increased grey matter volume in posterior regions (Greenwood et al. 2005). A number of studies have examined the relationship between brain volume and cognitive function in NF1 with mixed findings reported. Moore et al. reported that increased grey matter in NF1 is associated with a larger discrepancy between intelligence and academic achievement. In contrast, Said et al. found a relationship between decreased grey matter and poor visuospatial functioning.

Because neural connectivity is a central issue in NF1, the corpus callosum as the main white matter structure connecting the two hemispheres has become of particular interest. A number of studies have reported an *enlarged corpus callosum* in individuals with NF1 (Kayl et al. 2000; Moore et al. 2000), some finding an enlargement relative to total brain volume (Dubovsky et al. 2001; Pride et al. 2010). Three studies have found a correlation between this abnormality and cognition. Specifically, larger corpus callosum volume has been associated with academic underachievement, lower intelligence, executive functioning, and visual spatial impairment (Moore et al. 2000; Pride et al. 2010), while reduced corpus callosum volumes have been associated with attention problems (Kayl et al. 2000).

A number of other studies have pursued the identification of *other regional abnormalities* in the brain that may underlie specific cognitive impairments. Atypical size or asymmetry of several structures in the language cortex, including the planum temporale (Billingsley et al. 2002), the inferior frontal gyrus, and Heschl's gyrus (Billingsley et al. 2003b), has been documented in NF1 and has been found to be associated with poor cognitive or academic performance, particularly language

or academic ability. This is an interesting finding, given that similar findings have been found in dyslexic populations. These reports of abnormalities in the symmetry patterns of the language cortex in NF1 may reflect a lack of normal left hemisphere specialisation; however, further research into the functional organisation of language in the NF1 brain is needed.

One of the most frequently occurring brain abnormalities in NF1 are areas of increased signal on T2-weighted sequences of brain MRI. Occurring in approximately 55–90 % of children, these *T2 hyperintensities (T2H)* appear most commonly in the basal ganglia, cerebellum, thalamus, brain stem, and subcortical white matter. Many studies have examined the relationship between these lesions and cognitive function. While some have found a correlation between intelligence levels and the presence and number of T2H (Moore et al. 1996; North et al. 1994), many studies have failed to find an association between T2H and cognitive status (Bawden et al. 1996; Ferner et al. 1993; Legius et al. 1995). More recent studies suggest that T2H in the thalamus or thalamo-striatal region may have an influence on cognitive performance (Chabernaud et al. 2009; Goh et al. 2004; Hyman et al. 2007). The thalamus has reciprocal connections with nearly all major brain structures and is particularly interesting with regard to synchronisation of information processing. It has been proposed that T2H in the thalamo-striatal region could cause an alteration of the cortico-subcortical loop (which plays a major role in executive functions), resulting in cognitive impairment (Chabernaud et al. 2009).

5.2.2 Functional Brain Abnormalities

Whereas conventional MRI provides imaging of the structure of the brain, functional MRI (fMRI) allows dynamic interrogation of the brain at work (i.e. neural activity) and permits the identification of cognitive systems that are abnormal in NF1. Despite the minimal risk and non-invasiveness associated with this technology, there has been surprisingly little work done in NF1 using fMRI. Functional MRI has been used to investigate the neural basis for phonological processing in NF1. Billingsley et al. (2003a) studied 15 individuals with NF1 and 15 controls during a written and auditory rhyming task assessing phonological processing. Compared to controls, the NF1 group showed significantly more activation in the inferior frontal regions relative to temporoparietal regions on the auditory task. This differential pattern of activation is similar to the pattern observed in dyslexic patients from the general population (Shaywitz et al. 1998); however, Billingsley et al. report increases predominately in the right hemisphere rather than the left. In contrast, during the written task, the NF1 group showed relatively less frontal compared with temporal and occipital activity than the control group (Billingsley et al. 2003a). The authors speculated that this pattern of results (reduced frontal activity and increased posterior activity) could reflect compensatory neuronal recruitment whereby children with NF1 utilise more posterior than frontal regions as a result of functional inefficiency of the frontal lobe. Given aberrations in the

white matter connectivity of the temporoparietal region are associated with reading ability in dyslexic individuals in the general population, it will be important to determine whether a reduction in white matter interconnectivity also occurs in NF1. Diffusion tensor imaging should add important information on the nature of the white matter and potentially on the nature of specific fibre tracks within the NF1 brain and its relationship to impaired reading.

Functional MRI has also been used to better understand the neural systems that mediate visual spatial processing in NF1 (Billingsley et al. 2004; Clements-Stephens et al. 2008). Billingsley and colleagues performed fMRI on 15 individuals with NF1 compared to 15 unaffected individuals and showed that subjects with NF1 had relatively less task-related frontal activation to posterior cortical activation (parietal, occipital, and temporal) compared with healthy subjects during a visual spatial task. This pattern was related to their behavioural performance on the fMRI task, which was also positively correlated with reading scores. These data support earlier findings that suggest functional abnormalities are present in frontal regions of the NF1 brain. In a more recent fMRI study, Clements-Stephens et al. (2008) studied the neural basis of the JLO task in 13 children with NF1 and 13 unaffected controls and found the NF1 group showed significantly greater right than left hemisphere activation. They attributed this finding to an inefficient right hemisphere network in NF1.

The dorsolateral prefrontal cortex (PFC) is critical for working memory and executive functioning. One recent fMRI study of 14 young adults with NF1 compared to 12 controls showed that subjects with NF1 had significantly less task-related activation in the dorsal lateral PFC, the parietal cortex, and the striatum in comparison with healthy subjects during a spatial working memory task (Shilyansky et al. 2010a). The degree of dorsolateral PFC activation correlated with task performance. These data suggest that abnormalities in the frontal–striatal pathway may underlie executive deficits in NF1.

Although neuroimaging studies have provided important insight into the structural and functional brain abnormalities present in NF1, how these anatomical findings relate to cognitive function has been much more challenging. Many relationships between anatomical findings and cognition in NF1 have been inconsistently replicated, which may be a function of the insensitivity of the anatomic method, variable subject ascertainment and sample size, or the intrinsic biological heterogeneity of the disorder. Early childhood also offers only limited opportunities for anatomical studies since children in this age range are unlikely to remain still unsedated (hindering the acquisition of sufficient quality brain imaging studies); yet these early years are presumably the ones during which the most influential brain changes occur. Another important question to consider is whether specific cognitive deficits seen in patients with NF1 are due to regional anatomical and/or neurochemical abnormalities or more widely distributed abnormalities, such as altered connectivity. Studies in animal models of NF1 are likely to provide novel insights into the developmental time course and neuroanatomical correlates of NF1-related cognitive deficits.

5.3 Neurobiology of NF1 Cognitive Deficits: Animal Studies

NF1 is caused by a mutation in the *NF1* gene, which encodes the protein, neurofibromin. This protein is highly expressed in the brain and skin and has several biochemical functions, including activation of Ras GTPase. The absence of neurofibromin results in overactive Ras which drives abnormal cell proliferation. It has been suggested that aberrant Ras signalling impacts on neuronal development, migration, and apoptosis within the brain (Billingsley et al. 2003b; North 2000; North et al. 1997). Neurofibromin also increases adenylyl cyclase/cyclic AMP (AC/cAMP). A number of transgenic mouse and fly models have been developed to help understand the NF1 cognitive profile (see Chap. 37). Mice heterozygous for a null mutation in the *Nf1* gene ($Nf1^{+/-}$ mice) are at increased risk for tumour formation and display spatial learning, attention, and working memory impairments that mimic the human NF1 phenotype (Costa et al. 2002; Li et al. 2005; Shilyansky et al. 2010a; Silva et al. 1997). It has been proposed that these cognitive deficits are a result of hyperactivation of the Ras–MAPK signalling cascade which causes an increase in activity-dependent GABA release and reduced long-term potentiation (LTP) resulting in an imbalance between inhibitory and excitatory processes (Cui et al. 2008; Li et al. 2005; Shilyansky et al. 2010b; Silva et al. 1997). A recent study by Li and colleagues (2005) found that targeting this pathway with therapeutic interventions designed to inhibit Ras function, such as the drug lovastatin, decreased Ras activity and LTP in $Nf1^{+/-}$ mice and rescued the cognitive phenotype. Consequently, a phase I study examining the safety and tolerability of lovastatin was conducted in 24 children with NF1. Results suggested improvement in measures of verbal and non-verbal learning and memory (Acosta et al. 2011). Currently, a phase II trial of this drug in children with NF1 is underway.

More recent studies using $Nf1^{+/-}$ mice with homozygous inactivation of the *Nf1* gene in GFAP-positive glial cells have uncovered behavioural abnormalities in attentional system function and reduced dopamine levels in the striatum (Brown et al. 2010a, b). Further, these studies have revealed that the dopamine uptake inhibitor, methylphenidate, ameliorates attention deficits in these mice. Together, these findings establish a mechanistic connection between *Nf1* gene expression, attention, and the dopaminergic pathway and offer relevant implications for future human clinical trials. Only one study to date has investigated the effects of methylphenidate in this population. Mautner et al. (2002) treated 20 children with NF1 who had ADHD with methylphenidate over a 12-month period. Improvements in attention, social competence, and behaviour were reported. While this study provides some promising results, the design of this study, lack of appropriate control group, and small sample size limit its interpretation. Furthermore, the authors limited their investigation to the effect of the medication in children with ADHD (and NF1) rather than targeting attention deficits in the wider NF1 group. A randomised placebo controlled trial of methylphenidate as a treatment for attention deficits in NF1 is warranted.

5.4 Implications for Assessment and Management and Future Directions

We can draw several conclusions from the above studies of the NF1 cognitive phenotype to guide the assessment and management of children with NF1. Given the heterogeneity of the cognitive presentation and the frequency of deficits that impact on academic performance, it is important that a thorough neuropsychological assessment of the child's cognitive abilities is performed so that support can be offered. This should include measures of intelligence, attention, executive function, visuospatial ability, language, and academic achievement and should be performed prior to school entry to determine classroom placement. Recent evidence suggests that more severe cognitive and learning vulnerabilities may be detected as early as 2.5 years of age highlighting the importance of conducting these types of assessment in children before they enter school (Lorenzo et al. 2011). Since children with NF1 are at risk for LD, a review of school progress and a thorough characterisation of the LD are vital. Children should be followed throughout their school years, as vulnerabilities identified may present at later ages, such as high school, when demands on performance increase. Previous research suggests that children with NF1 who meet criteria for ADHD do benefit from stimulant medication (Mautner et al. 2002); thus, treatment and management by a paediatrician should be considered.

There have been no systematic studies to elucidate the best way to manage cognitive deficits once identified. The approach often taken is the recommendation of a range of strategies that can be implemented at home and in the classroom that help manage specific cognitive difficulties. The majority of these strategies are not evidence-based, and it is unclear if they are being implemented correctly or once implemented whether they are effective in this population. A systematic study evaluating the effectiveness of current management systems for cognitive deficits in NF1 would be beneficial to psychologists, teachers, and families involved with the care of a child with NF1.

Today, we have a much greater understanding of the clinical aspects of cognitive impairment in NF1. Significant advances have also been made in understanding the genetic and biochemical defect underlying cognitive dysfunction in NF1 which have resulted in potential treatments currently being evaluated such as lovastatin and methylphenidate. The introduction of improved MRI technology and developmental neuroscience has also provided us with some insight into the steps in brain development that may be abnormal in NF1. For example, functional MRI and diffusion tensor imaging are opening up entirely new and valuable views of white matter connectivity and neural activity in NF1 that promise exciting insights into the pathophysiology. Nevertheless, it will be a combination of both human and animal studies that will eventually provide precise data concerning the cognitive and neural basis for all the features of the disorder and the developmental neurobiology of the structural and functional abnormalities of the brain. This knowledge will result in a substantial improvement in all phases of intervention and will pave the way for the development of biologically targeted interventions for cognitive deficits in NF1.

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Chapter 6
Clinical Expression of NF1 in Monozygotic Twins

Elizabeth K. Schorry and Emily Sites

6.1 Historical Use of Twin Studies

Monozygotic (MZ) twinning occurs when a zygote or blastocyst splits into two halves, resulting in two individuals with a (presumably) identical genotype. MZ twinning occurs in about 1/260 naturally conceived births in all ethnic groups (reviewed by Machin 2009). Twin studies have historically been a valuable tool for studying genetic disorders. Early studies assumed that MZ twins were genetically identical, and any phenotypic difference between MZ twins was related to environmental factors. Studies of MZ twins raised in different environments were thus used to understand the roles of genetics versus environment. Heritability of many traits was determined by comparing concordance of monozygotic versus dizygotic (DZ) twins. In earlier studies, zygosity was often determined based on fetal membranes (with monoamniotic, monochorionic membranes being defined as MZ); however, it subsequently became clear that there are multiple exceptions to the validity of membranes in predicting zygosity. Most studies currently use highly variable microsatellite markers to determine zygosity, with identity on a panel of 15 polymorphic markers considered adequate to determine monozygosity with greater than 98 % certainty (Frankel et al. 1996).

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6.2 MZ Twins Are Not as Genetically Identical as Previously Assumed

Every family of MZ twins realizes that, although they are phenotypically similar in many respects, MZ twins have enough subtle differences that they can be easily distinguished by those who are close to them. Inspection of the literature reveals that there can be major phenotypic differences among MZ twins—for example, size discrepancy which may be related to twin-to-twin transfusion or to one twin receiving a larger number of stem cells than the other. It is well documented that MZ twins are often discordant for major congenital anomalies such as congenital heart disease (Machin 2009). Many traits with multifactorial etiology such as neural tube defects, schizophrenia, bipolar disorder, Parkinson disease, and multiple sclerosis can be either concordant or discordant in an MZ twin pair (Bruder et al. 2008; Petronis et al. 2003).

As our understanding of the genome and epigenome has grown, it has become clear that MZ twins may have significant genetic and epigenetic differences. Postzygotic non-disjunction events and postzygotic mutations can result in MZ twins discordant for trisomies or single-gene disorders. MZ twins who are heteroplasmic for mitochondrial DNA mutations may be discordant for mitochondrial disorders such as Leber Hereditary Optic Neuropathy (Biousse et al. 1997). X-inactivation and imprinting can also vary within MZ twin pairs, as in the well-known discordance for Beckwith–Wiedemann syndrome in MZ twin pairs (Smith et al. 2006). Discordance for copy-number variations (CNVs) may occur in as many as 10 % of MZ twin pairs, although the significance of this is not yet known (Bruder et al. 2008).

6.3 Twin Studies in NF1

Twin studies can also be of value in our understanding of the complexities of neurofibromatosis 1 (NF1). The mutated *NF1* gene displays almost complete penetrance but extreme variability of expression, with features ranging from mild cutaneous findings to life-threatening complications such as malignancy. There is significant inter- and intra-familial variability, making it challenging to predict patients at risk for serious complications and to appropriately manage those at higher risk. There are limited genotype–phenotype correlations for NF1, with the only examples to date being a more severe phenotype in patients with a full gene deletion (Kayes et al. 1994) and a milder phenotype lacking neurofibromas related to an in-frame deletion in exon 17 (Upadhyaya et al. 2007). This lack of clear genotype–phenotype correlations has made it obvious that most disease variability is not accounted for by the nature of the specific *NF1* germline mutation.

Various mechanisms have been proposed to explain the variability of expression of NF1, including germline modifying genes, second-hit somatic mutational events,

Table 6.1 Concordance in 41 NF1 twin pairs in the literature

Feature	# Concordant pairs	# Discordant pairs	Reported by
Café-au-lait numbers	24	4	A, F, J, O, P, Q, R
Cutaneous neurofibroma numbers	9	5	A, O, P, R
Plexiform neurofibroma present	7	13	A, H, I, J, K, O, P, Q, R
MPNST	1	2	J, P, Q
Optic path glioma	4	11	A, B, C, D, E, F, G, P, R
Learning disability	7	0	K, M, O, P
Speech disorder	9	0	P
ADHD	6	0	P, Q
Chiari I malformation	1	0	P
Aqueductal stenosis	1	0	B
Dandy–Walker malformation	1	0	M
Scoliosis	4	8	A, O, P, R
Pseudarthrosis	0	1	N, Q
Pectus deformity	4	3	P, Q
Seizure disorder	2	0	A

MPNST malignant peripheral nerve sheath tumor; *ADHD* attention deficit/hyperactivity disorder
Authors: *A* = Easton et al. (1993), *B* = Pascual-Castroviejo et al. (1988), *C* = Cartwright (1982), *D* = Crawford and Buckler, 1983, *E* = Kelly et al. (1998), *F* = Vaughn et al. (1981), *G* = Tubridy et al. (2001), *H* = Lubinsky (2006), *I* = Payne et al. (2003), *J* = Akesson et al. (1983), *K* = Bauer et al. (1988), *L* = *M* = Koul et al. (2000), *N* = Craigen and Clarke (1995), *O* = Sabbagh et al. (2009), *P* = Rieley et al. (2011), *Q* = Melean et al. (2010), *R* = Harder et al. (2010)

environmental agents, epigenetic modification, or postzygotic mutations (Rieley et al. 2011). Second-hit events have been well documented in multiple tumor types associated with NF1 (Upadhyaya et al. 2009), but there is yet limited evidence for other specific factors modifying the NF1 phenotype. Study of MZ twins with NF1, who are presumed to be genetically identical for germline modifying genes as well as for the NF1 mutation, can be a valuable tool in determining which NF1 features are influenced by the *NF1* mutation, which by germline modifying genes, and which may be due to postzygotic changes.

There have been at least 45 case reports of MZ twins with NF1 in the medical literature (Table 6.1). Ages of twins reported have ranged from 6 years to 43 years, with the majority being children or young adults. The largest series reported by Rieley et al. (2011) included 9 sets of MZ twins and 1 set of MZ triplets, from a patient population of 1,100 patients. They noted that the incidence of MZ twinning in their patient population (1/110) was significantly higher than the 1/260 rate in the general population and suggested that activation of Ras in the early zygote might increase the chance of MZ twinning. Other large series included a study by Easton et al. (1993) which reported 6 twin pairs together with 175 family members and noted that NF1 features varied to a greater degree with increasing distance from a proband, suggesting that modifier genes play a role in the phenotypic variability of NF1. A recent study (Sabbagh et al. 2009) investigated a group of 750 NF1 patients

including 6 sets of MZ twins and was also highly suggestive of the existence of genetic modifiers. Harder et al. (2010) reported 8 pairs of NF1 twins with various concordant and discordant features. Gender skew has also been noted in the reported NF1 twin sets, with a female–male ratio of 2:1 in those 31 twin pairs where gender was reported. This is similar to a female preponderance among MZ twins in the general population, which could be related to improved survival of female twins or other as yet undetermined factors (Machin 2009).

Other than these four larger series, most other reports of MZ twinning in NF1 have been single-case reports which commented on either concordance or discordance for NF1 traits in the pairs. The most extreme examples of discordance are MZ twins who are phenotypically discordant for clinical diagnosis of NF1. There have been two reports of this occurring in twins whose monozygosity had been confirmed by microsatellite marker testing. Vogt et al. (2011) reported a discordant set of MZ twins where the affected twin had 30–40 % mosaicism for a nonsense mutation in *NF1*, and the unaffected twin had no mutation detected; they concluded that the *NF1* mutational event occurred during the blastocyst stage, after twinning had occurred. In a second case, a twin diagnosed with NF1 was found to have a pathogenic *NF1* mutation in three cell types tested, and the clinically unaffected twin was mosaic for the same mutation (Kaplan et al. 2010).

Although there may be some bias in which twin pairs are chosen to present in the literature, when taken as a whole, the data from these multiple twin sets can be analyzed to give us further insight on genetic control of specific NF1 traits.

6.3.1 Cutaneous Features

Several of the studies of NF1 twins carefully assessed the cutaneous features, such as presence and numbers of café-au-lait (CAL) spots, skin-fold freckling, and cutaneous neurofibromas (Rieley et al. 2011; Easton et al. 1993; Sabbagh et al. 2009; Harder et al. 2010). Although these age-dependent cutaneous manifestations are seen in the large majority of individuals with NF1, all four of these studies found that the overall number (but not location) of CAL spots and cutaneous neurofibromas was quite consistent within pairs of twins. In distinction, siblings and other relatives were less concordant in these traits as genetic distance increased, supporting the existence of a strong genetic component separate from the constitutional *NF1* mutation (Sabbagh et al. 2009). Caution must be raised about the interpretation of cutaneous neurofibroma numbers, however, as many of the twins reported were children or young adults and had low numbers of this age-dependent feature.

6.3.2 Tumors

Neoplasms in MZ twins with NF1 have shown significantly lower concordance than many of the other features. As the majority of NF1-associated neoplasms are known

to require a second-hit event in the *NF1* allele, the sporadic nature of this second hit could satisfactorily explain the frequent discordance of tumors in MZ twins with NF1. Plexiform neurofibromas, which are thought to be congenital lesions or to become apparent in the first few years of life, were highly discordant both in their presence and their specific location in the body. However, there are limitations to the data on plexiforms that has been collected to date. Recent studies using whole-body MRI have shown that over 50 % of children with NF1 have plexiform neurofibromas, many of which are not apparent externally (Nguyen et al. 2011). None of the twin series reported have performed whole-body MRI on the twins, and therefore, there is certainly incomplete ascertainment of plexiforms in many of the twin pairs, with symptomatic or externally visible plexiforms most likely to be reported. At least one study (Rieley et al. 2011) has noted twins concordant for extensive involvement with paraspinal neurofibromas, suggesting that there may be genetic factors modifying the development of multiple paraspinal tumors.

Optic pathway glioma (OPG) occurs in about 15 % of children with NF1 and remains asymptomatic in the majority of cases. Of NF1 twins reported in the literature to date, there have been 4 pairs concordant for presence of optic glioma and 11 pairs discordant with only one twin affected (Easton et al. 1993; Cartwright 1982; Crawford and Buckler 1983; Pascual-Castroviejo et al. 1988; Kelly et al. 1998; Harder et al. 2010; Rieley et al. 2011). Although there may be bias in which pairs are chosen to publish, it appears that development of OPG is more likely related to sporadic or non-germline modifying factors.

6.3.3 Malignancy

Malignant peripheral nerve sheath tumor (MPNST) is a malignancy occurring in between 8 and 13 % of individuals with NF1, primarily arising from plexiform neurofibromas (Evans et al. 2002). Only 3 pairs of MZ NF1 twins with MPNST have been reported to date, 2 of which were discordant and 1 concordant for malignancy. Akesson et al. (1983) reported a pair in which one twin developed MPNST of the leg; another group reported teenaged twins with extensive paraspinal neurofibromas, only one of which developed MPNST (Rieley et al. 2011). Interestingly, one pair of MZ twins was reported to have developed MPNST at similar ages (22 and 24 years) and at the same anatomic site (left sciatic nerve) (Melean et al. 2010). None of the above twins had an *NF1* full gene deletion. As malignancy in NF1 is known to require bi-allelic inactivation of *NF1* as well as additional genetic alterations in other genes such as *TP53*, *RB1*, and *CDKN2A* (Spurlock et al. 2010), it is surprising that twins would develop the requisite second mutations in identical body sites. The authors speculated that events involved in the transformation of plexiform neurofibromas to MPNST are influenced by heritable factors other than *NF1* mutation.

Likewise, if there are germline genetic factors increasing the risk for development of multiple large plexiform neurofibromas, those could also directly increase

the risk for MPNST. Obviously, the sample size with only 3 pairs of twins with malignancy is very small, and additional data will need to be collected over time to determine the concordance of malignancy in MZ twins with NF1.

6.3.4 Learning Disabilities and Cognition

In most of the twin series reported, comment was made on features such as learning disability and attention deficit/hyperactivity disorder (ADHD). However, in most cases, formal neuropsychological testing results were not reported. Rieley et al. (2011) reported that in their 10 sets of multiples, 4 pairs were concordant for presence of learning disabilities (LD), although there was variability in the type of LD and degree of involvement. Four additional pairs who were old enough for testing were concordant for lack of LD. Of the 3 pairs for whom formal IQ scores were available, none differed by more than 10 points.

Presence or absence of ADHD has also been highly concordant in NF1 twin pairs, with no pairs reported to be discordant for ADHD (see Table 6.1) (Rieley et al. 2011; Melean et al. 2010). This high concordance for cognition and ADHD attests to a strong heritable component for these traits, which has been well documented in normal populations. In fact, given the multiple environmental factors associated with twinning, including effects of prematurity, birth asphyxia, and twin–twin transfusion, it is somewhat surprising that NF1 twins are as highly concordant in terms of cognition as they are.

6.3.5 CNS Manifestations

Structural malformations of the central nervous system such as Chiari malformation, Dandy–Walker malformation (DWM), and aqueductal stenosis (AS) are rare features of NF1 but do occur more commonly than in the general population. There were single pairs of twins reported to be concordant for each of these malformations, with very similar findings on MRI (Rieley et al. 2011; Koul et al. 2000; Pascual-Castroviejo et al. 1988), attesting to strong heritable factors. This is consistent with known genetic factors and familial cases of these malformations in non-NF1 populations (Schanker et al. 2011; Jalali et al. 2008; Haverkamp et al. 1999). The genetic etiologies of isolated DWM and AS are largely unknown beyond X-linked *L1CAM* disorders in AS, but population-based studies suggest the existence of other genetic influences. The recurrence risk in siblings of patients with non-syndromic DWM or AS is approximately 5 % in the general population (Murray et al. 1985; Burton 1979).

6.3.6 *Orthopedic Manifestations*

Bone manifestations of NF1 are intriguing features whose underlying pathophysiology has not yet been adequately understood. Long bone dysplasia, which occurs in 3–5 % of NF1 patients, was reported in only 2 MZ twin pairs, both of which were discordant for the feature (Craigen and Clarke 1995; Melean et al. 2010). Given the rare and usually unilateral occurrence of this condition as well as recent data that second-hit events in NF1 have been documented in some pseudarthrosis tissue (Stevenson et al. 2006), the discordance in twins affirms that long bone dysplasia/pseudarthrosis is likely triggered by a sporadic, nonhereditary event.

Scoliosis in NF1 is also complex; it occurs in 15–30 % of NF1 patients and can occur in either a dystrophic form (with dystrophic bone changes and sharply angulated curve) or a non-dystrophic form (with an S-shaped curve similar to idiopathic scoliosis) (Crawford and Schorry 1999). Since idiopathic scoliosis in the general population has a strong genetic component (Miller 2007), it is reasonable to predict a genetic component and possibly modifying genes affecting scoliosis in NF1 as well. Looking at MZ twins pairs with NF1, there have been 4 pairs reported to be concordant for scoliosis and 8 pairs discordant (Easton et al. 1993; Sabbagh et al. 2009; Harder et al. 2010; Rieley et al. 2011). Of interest, however, is that the concordant pairs were actually *discordant* for degree of curvature, presence of dystrophic features, and need for surgery. This implies that both heritable and non-heritable factors contribute to the pathogenesis of scoliosis in NF1. One can speculate that there is a heritable tendency toward non-dystrophic scoliosis in NF1, but with progression to dystrophic curves likely requiring a nonhereditary event such as a closely located tumor or a second-hit genetic event in local bone cells (Rieley et al. 2011).

A final osseous feature assessed was pectus deformity of the chest. Again, there was mixed concordance and discordance for this trait, with 4 pairs reported to be concordant and 3 pairs discordant for pectus excavatum or carinatum (Rieley et al. 2011; Melean et al. 2010). Of interest is one pair of MZ twins who were reported to be "mirror images," one with pectus excavatum and the other with pectus carinatum (Rieley et al. 2011). Pectus deformities are likely underascertained in NF1, as careful reviews show that as many as 50 % of individuals may have mild degrees of chest wall deformity (Riccardi 1999; Stevenson and Yang 2011). Therefore, although there are certainly hereditary and nonhereditary factors influencing development of pectus deformities, data about concordance of pectus needs to be reascertained after more thorough chest exam of twins.

6.4 New Directions

Recent studies have begun to look for epigenetic and mitochondrial differences among twins with NF1 to attempt to explain their phenotypic differences. Detjen et al. (2007) investigated differences in mitochondrial DNA (mtDNA) as an

explanation for variable expression in MZ twins with NF1. They investigated 4 pairs of MZ twins discordant for various NF1 complications by sequencing the entire mitochondrial genome. They did not find any evidence of mtDNA sequence differences or different degrees of heteroplasmy between co-twins, thereby ruling out mtDNA differences as the explanation for discordance in their small group of twins.

Another recently proposed mechanism for the phenotypic variability among MZ twins is difference in *NF1* promoter methylation. Promoter methylation has been shown to alter *NF1* expression in luciferase assays (Zou et al. 2004), with methylation and gene expression inversely correlated. Harder et al. (2010) hypothesized that changes in promoter methylation could modulate the residual activity of the wild-type *NF1* allele, thereby affecting phenotype. *NF1* promoter methylation was measured in lymphocytes from 8 sets of MZ twins with varying concordance and discordance for NF1 features, revealing significant intra-pair differences at several sites, including the 5′UTR. Increased methylation at one site, overlapping a transcription factor binding site, correlated with the incidence of optic pathway glioma both within and between twin pairs. Twins with symptomatic OPGs had higher methylation levels in this region compared to twins who had no OPG. The small sample size precluded statistically significant conclusions regarding other disease manifestations. Further investigation including additional tissue types is needed to better characterize the relationship between *NF1* promoter methylation and phenotypic variability.

6.5 What Have We Learned from Twins with NF1?

Studies of MZ twins with NF1 are a valuable tool to investigate causes of variable expression of this complex disorder. Although presumed identical in their germline DNA, MZ twins are never truly "identical." Phenotypic differences may be as extreme as complete discordance for NF1 diagnosis due to somatic mutation occurring after the time of twinning. Multiple studies have shown that MZ twins are much more similar in traits such as number of CAL spots and cutaneous neurofibromas than are more distant relatives in the same families, suggesting that germline modifying genes play a significant role in regulating these features. Learning disabilities, ADHD, and speech disorder were highly concordant in MZ twins, again implying a major role for germline genes.

Some of the most valuable lessons have come from traits that are discordant in co-twins. The majority of neoplasms, including OPG and MPNST, fell into this category, consistent with sporadic second-hit events that are known to occur in those tumors. Plexiform neurofibromas were somewhat more difficult to analyze, since they were not completely ascertained in all studies. With only a few exceptions, plexiforms occurred in different areas of the body when concordant in co-twins. The occurrence of a few pairs of twins with similar location or extensive tumor burden of plexiforms raises a speculation of genetic modifying

factors affecting overall tumor burden. Most dystrophic skeletal complications, such as dystrophic scoliosis or tibial pseudarthrosis, were discordant in twins, suggesting nonhereditary events; however, known genetic etiology to idiopathic scoliosis and pectus excavatum in the general population may also contribute to those features in NF1.

Other possible causes of variable *NF1* gene expression suggested by twin studies include epigenetic differences, *NF1* promoter methylation differences, and postzygotic mutations (of *NF1* or other genes).

There are a number of limitations to NF1 twin studies that have been published to date. First, there may be some bias as to which twins authors choose to publish. A set of MZ twins with identical location of MPNST (or other rare feature) may be seen as uniquely interesting and more likely to be publishable than twins who are discordant for the feature. Secondly, the numbers are small, and large series of MZ twins with NF1 are difficult to obtain. Finally, many NF1 features are age dependent, leading to incomplete ascertainment of some of the NF1 complications such as tumors and malignancy among the relatively young patients that have been reported to date. The MZ twins that have been reported are clearly a valuable asset for NF1 research, and longitudinal data on these and other twins, as well as innovative investigations in this population, may ultimately help unravel the variable expression of NF1.

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Chapter 7
Value of Whole Body MRI in Patients with NF1

Victor-Felix Mautner

7.1 Introduction

Neurofibromatosis type 1 is characterized by the occurrence of skin neurofibromas in almost all patients, whereas visible plexiform neurofibromas (PNF) are detectable in about 30 % of patients (Huson et al. 1989). Both tumor types can lead to significant disfigurement. Skin neurofibromas rarely cause pain or functional deficits, whereas plexiform neurofibromas exhibit an infiltrating growth pattern and may cause functional deficits in NF1 (Mautner et al. 2006).

External PNF lead to functional deficits in about 10 % of patients and pain in 5 % of patients (Mautner et al. 2006). However, PNF growth can potentially take place at any neural structure within the body (Tonsgard et al. 1998). Internal plexiform neurofibromas are rarely identifiable by physical examination. Analysis of growth patterns of single PNF by magnetic resonance imaging (MRI) revealed internal PNF as being responsible for neurological dysfunction, anatomic impairment and pain in the majority of patients (Mautner et al. 2006). Even though some studies have been carried out to investigate the natural history of single PNF, no systematic evaluation has been performed yet (Dombi et al. 2007; Tucker et al. 2009).

Importantly, PNF are also premalignant lesions with the potential for transformation into malignant peripheral nerve sheath tumors (MPNST), a leading cause of death in patients with NF1 (Evans et al. 2002; Tucker et al. 2009; Duong et al. 2011). Some NF1 patients appear to be at a higher risk of developing MPNST than others, but the factors that distinguish the high- and low-risk groups have not yet been fully defined.

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7.2 Methods of MRI and Estimation of Whole Body Tumor Volume

In the studies of Mautner and coworkers whole-body MRI protocols have been carried out since 2003 and have provided adequate data both, to accurately quantify tumor burden in patients with nerve sheath tumors and to study internal tumor growth in NF1.

The whole-body protocol utilized different 1.5 T magnets and sequences with and without intravenous contrast enhancement (Mautner et al. 2008). The subjects were imaged in a supine position from head to knee in four or five steps (head, thorax, abdomen and legs) in accordance with the maximum range of table movement and body size. The head was imaged with contrast enhancement using T1 and T2 turbo spin echo sequences and T1 sequences with and without spectral fat saturation. The body was imaged without contrast enhancement using T1 gradient echo sequences and T2 STIR (short T1 inversion recovery) technique in the axial plane. Slice thickness was 5–10 mm with no skips between slices.

MRI analysis was performed by a physician trained in image analysis of NF1-associated tumors. The analysis was done without any other information about the patients, all of whom were unknown to the image reader. Plexiform neurofibroma volume was determined using a previously described method on the MEDx software platform (Dombi et al. 2007). The method is based on:

1. Contrast, defined by intensity in the tumor (high-signal intensity) compared with the surrounding tissue (low signal intensity)
2. Intensity gradient, defining the outside border (margin) of the lesion
3. Size of the lesion: plexiform neurofibromas are usually substantial in size and small isolated areas of high signal intensity can be ignored because their contribution to the total plexiform neurofibroma volume is not significant.

The method used for this automated volumetric analysis is sensitive (it detects volume changes as small as 10 %), reproducible (coefficient of variation 0.6–5.6 %) and it produces results similar to manual tumor tracings ($R = 0.999$). When automated tumor volume measurement was not feasible, the reason was recorded and manual tumor tracings with the MEDx software drawing tool were used to define tumor volume as previously described (Dombi et al. 2007). Using this method, we could reliably measure plexiform neurofibromas larger than 3 cm at maximal diameter. The volumes of all those plexiform neurofibromas (3 cm maximal diameter) were summed to obtain the total body plexiform neurofibromas volume. Volumetric analysis omitted lesions smaller than 3 cm in maximal diameter.

The resolution of the whole-body scan allowed for only limited evaluation of spinal neurofibromas. When visible by manual tracing, spinal neurofibromas were classified by location (cervical, thoracic or lumbar spine) and size. The volume of spinal neurofibromas larger than 3 cm in greatest diameter was automatically calculated and included in the total plexiform neurofibroma volume.

7.3 Neurofibromatosis and Malignant Peripheral Nerve Sheath Tumors

NF1 patients have a 100-fold increased risk of developing an MPNST than expected (Walker et al. 2006) in the normal population. The lifetime risk of MPNST among NF 1 patients has been estimated to be about 10–13 % (Evans et al. 2002). Most MPNSTs in people with NF1 are diagnosed in adolescence or early adulthood. People with NF1 whose pathogenic mutation is a deletion of the entire *NF1* gene are thought to have a 16–25 % chance of developing MPNST (De Raedt et al. 2003). Other factors that may be associated with a higher risk of developing MPNST in people with NF1 are the occurrence of neurofibromatous neuropathy (Drouet et al. 2004; Ferner et al. 2004), exposure to therapeutic radiation (Evans et al. 2002), previous occurrence of MPNST (Ferner et al. 2004; Doorn et al. 1995) and the occurrence of MPNST in a relative with NF1 (Dales et al. 1983; Poyhonen et al. 1997; Shearer et al. 1994).

An association of MPNSTs with the presence of subcutaneous neurofibromas was found in a clinical study of 464 subjects with NF1 (Tucker et al. 2005). This finding was confirmed by Sbidian and coworkers, who developed a score, which enables the prediction of internal tumor load (Sbidian et al. 2011).

7.4 Studies

Different whole body MRI studies were carried out to examine the nature of PNF in children and adults and to investigate whether there are any predictive measures that allow those patients who are at risk of developing MPNST to be identified. The identification of patients at high risk of malignancy is of critical importance because complete surgical excision, currently the only hope of cure, is generally impossible without early detection.

- **Whole body tumor burden in children and adolescents with NF1**
- **Whole body tumor burden in patients with NF1**
- **Whole body tumor burden in patients with NF1 and large *NF1* microdeletions**

Sixty-five children aged between 1.7 and 17.6 years underwent clinical investigation and whole-body MRI consecutively as part of routine clinical care. A total of 73 PNF were identified in 37 (57 %) of the 65 children; 20 (31 %) of the children had more than one tumor. The majority of PNF was detected in the legs (23/73), followed by thoracic (18/73) and paraspinal PNF (14/73). Regarding the total of 73 individual tumors, 18 PNF were symptomatic (25 %), whereby the ratio of symptomatic to asymptomatic tumors varied between body regions. Thus, 7 of 11 tumors in the head/neck region were symptomatic, but none of the 11 abdominal tumors. Seventeen of 37 (46 %) child patients with PNF had tumor-related complications

such as motor or neurological complications and/or pain. It is important to note that these figures may underestimate the true tumor burden as lesions smaller than 3 cm in diameter cannot be diagnosed as PNF by MRI.

In this pilot study of children, PNF were detected in a remarkably high frequency (57 %) thereby confirming that internal PNF are a characteristic feature which is present in both children and adolescents. Children with preexisting PNF are at risk of developing neurological deficits, organ dysfunctions, pain and skeletal abnormalities. Whole-body MRI investigation in children enables identification of those individuals with internal PNF and would therefore require regular monitoring of the tumor progression.

Size and location are important factors which might predict future complications. On the one hand, larger PNF generally have a higher risk of complications than smaller ones. On the other hand, certain body regions are more prone to complication, e.g. palpebral region. Such body regions are also sensitive to small tumors.

The median age of the children with PNF was 11.5 years old. When dividing the cohort by the median age, symptomatic PNF were found in 7 of 19 children in the younger group (37 %) and in 10 of 18 children in the older group (56 %). PNF tend to cause deficits more frequently in older children, although this difference was not statistically significant (x^2, $P = 0.25$).

An inverse growth correlation with age was found in two studies that focussed on single PNF growth, although these investigations had methodical limitations (Dombi et al. 2007; Tucker et al. 2009). About 60 % of the children present during the first examination had internal tumor manifestations. First results in an ongoing follow-up in both adults and children detected PNF in 50 % of adults, similar to the 57 % in children with NF1 in the study described here. These data suggest that patients without internal tumors during the initial examination will be unlikely to develop new tumors later in life. Hence, children not burdened with internal tumors during the first presentation probably belong to the low risk group with regard to the development of PNF-associated deficits or the development of MPNST. In this sense, the findings indicate that whole-body MRI may potentially serve as a prognostic marker for lifetime disease severity in NF1 (Nguyen et al. 2011).

7.5 Tumor Burden and MPNST

To fully evaluate the relationship between the total body burden of internal neurofibromas and MPNST, whole-body MRI was performed on 13 NF1 patients with MPNST and 26 controls, matched for age and sex who had NF1 but lacked MPNST.

The study showed that the numbers of cutaneous neurofibromas and external plexiform neurofibromas among the NF1 patients with MPNST and controls were similar, but the median number of subcutaneous neurofibromas was significantly greater among NF1 patients with MPNST than among controls. However, the presence of internal neurofibromas, the median number of measurable internal

neurofibromas, and the total volume of internal neurofibromas on whole-body MRI did not differ significantly between NF1 patients with MPNST and those without MPNST.

Many NF1 patients who develop MPNST do so at an extraordinarily young age (Friedrich et al. 2007; Rasmussen et al. 2001): the median age of NF1 patients with MPNST in this study was only 30 years. By contrast, the median age of diagnosis of MPNST in people who do not have NF1 is 62 years (Evans et al. 2002). Rapid growth of PNF in NF1 patients may occur in children but is unusual in adults (Friedmann and Riccard 1999; Riccardi 2007), so our consideration was whether the burden of PNF seen on whole-body MRI might be associated with the development of MPNST in younger NF1 patients. We therefore compared NF1 patients with MPNST whose age was less than the median in this study (30 years) to NF1 controls without MPNST who were younger than 30 years old. All six of the NF1 patients with MPNST aged younger than 30 years had internal neurofibromas as visualized by whole-body MRI and both their median number and their median volume were significantly greater among these patients than among the NF1 controls younger than 30 years of age. No significant differences in whole-body MRI findings were observed between NF1 patients with MPNST and controls without MPNST who were older than 30 years. This might be due to the fact that patients in this group who developed an MPNST, exhibited only a single internal PNF which became transformed to an MPNST which was then removed by surgery.

Thus, we found an overall association between the median number of subcutaneous neurofibromas in NF1 patients and the occurrence of MPNST. By contrast, we found no difference in the median number of cutaneous neurofibromas or of external visible PNF between the NF1 patients with MPNST and their matched controls without MPNST. Our findings were similar to those of Tucker et al. (2005), who studied 476 French NF1 patients, 25 of whom had MPNST. They found that the presence of subcutaneous neurofibromas was associated with the occurrence of MPNST, but not the presence of superficial PNF or the number of cutaneous neurofibromas. Our findings are also consistent with a cohort study performed by the same investigators, in which subcutaneous, but not cutaneous, neurofibromas were associated with a higher risk of morbidity among adults with NF1 (Khosrotehrani et al. 2005).

The most striking associations we observed were between NF1 patients with MPNST younger than 30 years and the presence of internal neurofibromas, their median number and median total volume. The occurrence of these statistically significant associations is remarkable, despite the small number of MPNST patients in this younger subset. Independent confirmation of these findings is however necessary (Mautner et al. 2008).

7.6 Whole-Body MRI in Patients with Microdeletions

Patients with large deletions are also at risk of developing MPNST with increasing frequency as compared to normal NF1 patients. Furthermore, patients with microdeletions show an increased frequency of associated disease features such as external tumor load (De Raedt et al. 2003; Mautner et al. 2010; Pasmant et al. 2010). Therefore we explored whether internal tumors may contribute to the increased risk of MPNST in NF1 patients with large deletions.

We performed whole-body MRI on 30 NF1 patients with large deletions (Kluwe et al. 2012) (patients with mosaic NF1-deletions were excluded). Each patient was compared with 3 matching patients (by gender and age) without large deletions. Total tumor volume per patient was higher in the 30 deletion patients than in the 67 non-deletion patients (median 260 ml vs. 168 ml, geometric mean 211 ml vs. 125 ml). However, the difference was not significant ($P = 0.29$, Mann–Withney U test, two-tailed unpaired t-test, equal variance assumed). We even found a nonsignificant tendency for higher tumor burden in NF1 deletion patients. We cannot resolve whether this contributes to the higher frequency of MPNST in the patients with these deletions. In opposition to this, it might be true on an individual basis, because several deletion patients manifested an extremely high internal tumor burden (4,000–8,000 ml) which was not observed in non-deletion patients. Thus, at an individual level, a high internal tumor burden is indeed characteristic of some deletion patients. However, as a group, these patients may not be considered as generally having larger higher internal tumor volume than NF1 patients without large deletions. By contrast, both the frequency and volume of internal tumors are low in mosaic deletion patients. These patients are generally not in need of intensive follow-up investigations (Kluwe et al. 2012).

Neurofibromatosis type 1 is characterized by a multiplicity of symptoms and variation in the severity of clinical symptoms. Patients with different disease manifestations are in need of individualized management strategies. However, so far the only marker for disease severity is the occurrence of microdeletions in NF1 patients, as this cohort shows a significantly more severe clinical course of the disease. The presented cross-sequential data of whole body MRI studies were performed to prove whether this method can be employed as another marker of disease severity with regard to internal tumor manifestations and the occurrence of MPNST in NF1.

First, whole body MRI enables us to determine the frequency of PNF and associated clinical deficits in the pediatric age group. Those children and adolescents presenting with internal congenital PNF are in need of regular MRI monitoring to estimate growth and upcoming complications. As opposed to children presenting without internal tumors, these are the ones with a potential future risk of developing MPNST. First, follow-up whole body observations indicate that children without internal tumors do not develop internal tumor manifestations during longer follow-up periods (up to 5 years), although there remains a risk of developing MPNST later in life.

Second, internal tumor load is associated with the occurrence of MPNST especially in the age group younger than 30 years. Moreover, while the median number of skin neurofibromas or visible PNF does not reflect the risk of developing MPNST in NF1, there is a clear association between the number of subcutaneous neurofibromas in NF1 and the occurrence of MPNST. Therefore, patients presented with multiple subcutaneous tumors should undergo investigation of internal tumor load. Close clinical monitoring and serial MRI examination for changes in the appearance or growth of internal tumors and PET-CT may allow earlier diagnosis and more effective treatment of MPNST in these high-risk patients.

About 56 % of NF1 patients show internal tumor manifestations by whole body MRI. Follow-up investigations are needed to confirm that internal tumors stop growing at an older age and that growth of tumors—calculated by growth thresholds—indicates the transformation from a benign PNF to an MPNST. Our initial whole body follow-up observations indicate that adults without internal tumors will not develop new tumors in future and are not at-risk patients in this regard.

Third, NF1 microdeletion patients are at a significantly higher risk of developing an MPNST in general; only a higher "nonsignificant" tendency was found for a higher internal tumor burden measured on the basis of whole body MRI. This might be due to the small number of microdeletion patients investigated in this study. However, patients with microdeletions and internal tumor burden require closer clinical and MRI follow-up examinations.

The first whole body MRI studies discussed in this chapter do not currently allow evidence-based recommendations regarding the use of MRI in the routine care of NF1 patients with internal tumors. Whole body MRI might, however, be used as a potential prognostic stratifier for NF1 risk groups to assess internal tumor burden and the progression of these tumors to MPNST. Longitudinal studies will be necessary to assess the potential value of this approach for clinical care.

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Chapter 8
Quality of Life in NF1

Patricia Birch and J.M. Friedman

8.1 Introduction

Quality of life (QoL) is a personal, subjective assessment of the physical, psychological, and social aspects of one's life that are unique to each individual (Mandzuk and McMillan 2005). QoL may vary throughout life in response to each person's situation and developmental stage (Taylor et al. 2008).

For individuals and families affected by NF1, QoL may be altered by the medical impact of the disease as well as by psychological and societal response to the signs and symptoms of NF1. Other factors influencing QoL in people with NF1 may include the very fact of having a chronic illness or a genetic condition (Mauger et al. 1999).

Quality of life research in NF1 has been performed in various countries and in people of different ages. Assessment of well-being, as opposed to assessment of illness, reflects a more patient-centred and holistic view and one that supports the recognition that patients have a distinctive view of their own health (O'Connor 2004, p. 4). QoL provides an alternative to the traditional clinical outcome measures in treatment research (Stevenson and Carey 2009; Spuijbroek et al. 2011) or for investigating the course of a progressive condition such as NF1. The effect of governmental or health policies can also be assessed by the impact they have on the QoL of affected individuals, their families, and the populations in which they live.

Results from various studies of QoL in individuals with NF1 demonstrate a generalized, decreased QoL when compared to control subjects, as well as specific deficits and strengths in certain domains of QoL. These latter studies suggest directions for intervention and reinforcement to improve QoL as well as avenues for future research.

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8.2 Measuring QoL

Measurement of health-related QoL enables researchers to quantify the impact of a condition and to allow for comparisons between different conditions (Spuijbroek et al. 2011). Further, QoL can be compared in individuals with the same condition but with variable symptoms, as is often the case in NF1. Importantly, QoL is an appropriate end point in situations where a cure is not possible or the impact of the treatment may outweigh its benefits from the patient's point of view. QoL can also be used to monitor the impact of a disease on caregivers as well as to measure the effects of more abstract concepts such as social and family support.

Various age-appropriate scales have been developed in order to assess health-related QoL. None are specific to NF1. The most commonly used general scale (O'Connor 2004, p. 186) is probably the SF-36 (Ware and Sherbourne 1992). It measures health concepts of pain, social functioning, psychological distress/well-being, role limitations, energy/fatigue ("vitality"), and general health perceptions. The reliability and validity of this measure have been extensively tested (O'Connor 2004, pp. 186–191); it is available in multiple languages, and control and research data exist for various populations that provide useful comparisons to individuals with other particular conditions, such as NF1.

Beyond general measures of QoL, several studies have used symptom-specific QoL instruments. For example, a skin-disease-specific measure has been used in NF1 research on the premise that the visibility of skin manifestations has potential to affect functioning, emotions, and physical symptoms (Page et al. 2006). Further, the QoL of children with tibial dysplasia has been assessed by Stevenson and Carey (2009) using a scale appropriate to capture orthopaedic concerns in that patient group, emphasizing factors such as mobility and physical function. Likewise, the well-recognized impact of NF1 on speech (Cosyns et al. 2010) has led a Dutch group to study voice-related quality of life and the impact of voice disorder on functional, emotional, and physical health in people with NF1 (Cosyns et al. 2011). Often, two or more scales are used in combination. For example, a general QoL scale such as the SF-36 may be used with a scale focussed on specific manifestations of NF1 (Wolkenstein et al. 2001).

8.3 Quality of Life Research in Adults with NF1

Wolkenstein et al. (2001) used the generic SF-36 as well as a validated French translation of the Skindex, a QoL instrument for skin diseases, to evaluate the effect of severity and disease visibility on QoL in adults with NF1. The authors estimated severity by the Riccardi scale (Riccardi 1992) and visibility by Ablon's scale (Ablon 1999, p. 178). 128 individuals between 15 and 73 years old completed the two self-administered questionnaires. Results on the SF-36 showed that the mean QoL for individuals with NF1 was lower for all domains in comparison to a French

comparison population. Controlling for age and sex, patients with more severe NF1 tended to have lower SF-36 QoL scores, in particular on the domains of physical function, bodily pain, general health perception, and vitality. Patients with more visible NF1 scored lower in Skindex QoL scores in the three domains of emotions, physical symptoms, and functioning, whereas the severity score was not associated with the Skindex score. This study demonstrates the value of both a general and symptom-specific QoL tool in the assessment of people with NF1. Further, use of the Skindex allowed the authors to compare results to published scores for other skin conditions. For example, the impact of NF1 skin findings was found, on average, to be similar in magnitude to that of psoriasis.

The study was replicated in a dermatology clinic at the University of Rome using Italian versions of the SF-36 and the Skindex (Kodra et al. 2009). The impact of NF1 on general QoL and the cosmetic impact on Skindex scores in this study were very similar to the French study. Another study (Page et al. 2006), using a slightly different study design but the same QoL instruments in English, replicated the findings of the French study in a US population. Together, these studies emphasize the importance of appearance in influencing QoL in NF1 and the importance of cosmetic correction as a means of improving QoL of patients with NF1 (Kodra et al. 2009).

About two-thirds of adults with NF1 have some type of speech or voice problem, often related to laryngeal differences resulting in decreased frequency and intensity range (Cosyns et al. 2012). 29 adults with NF1, mostly recruited from Ghent University Hospital's Medical Genetics Center, completed the Voice Handicap Index (VHI) with a functional subscale designed to measure impact of voice disorder on daily activities, an emotional subscale to measure patients' affective responses to a voice disorder, and a physical subscale to document perceptions of laryngeal discomfort. In addition, the Dysphonia Severity Index (DSI), an objective measure of voice characteristics, and the Riccardi severity scale, a measure of the overall severity of NF1, were used. The authors found significantly poorer results on the functional and emotional subscales of VHI but no association of these values with the Dysphonia Severity Index, suggesting that the subjective QoL measure is indeed quantifying something different than the objective physical measure of dysphonia. Neither the VHI nor the DSI was associated with severity of NF1, as measured by the Riccardi scale.

Although not formally a QoL study, Langenbruch and colleagues (Langenbruch et al. 2011) asked 132 adult NF1 patients who were cared for by NF1 specialists and 92 patients cared for by non-specialists to rate their "health-care quality" with respect to a number of nonmedical treatment benefits. Individuals treated by NF1 specialists were significantly more likely to have experienced greater enjoyment of life and to have been able to lead a normal everyday life. However, the two groups did not differ with respect to other items such as being able to engage in normal leisure activities or to have a normal working life. Further research is needed to clarify the importance of access to NF1 specialists in enhancing QoL of individuals with NF1.

8.4 Quality of Life Research in Children with NF1

A number of studies of toddlers, school-age children, and teenagers with NF1 have been reported. These studies, which used a variety of health-related QoL scales, are summarized below, beginning with studies of children in the youngest age group.

Oostenbrink et al. (2007) studied 34 children aged 1–6 years from an NF1-outpatient clinic of the Erasmus MC-Sophia Children's Hospital, Rotterdam. The children were rated by the authors on the Ablon visibility scale, a measure of skin disease (Ablon 1999, p. 178). Using the Infant Toddler Quality of Life Questionnaire (ITQOL), the authors described the QoL in these children. In a related study, Spuijbroek et al. (2011) compared these children with NF1 to children with one of four other paediatric conditions and to a control group of 410 healthy Dutch children. The instrument used has subscales that pertain directly to the child's physical functioning, growth and development, pain, mood, behaviour, "getting along", and general health. In addition, three subscales measured parental emotional impact, parental time impact, and family cohesion. A final question, distinct from the main scale, assessed parents' perceptions of "change in health", which is defined as present health status relative to the previous year. Oostenbrink et al. (2007) report that the children with NF1 scored lower on all ITQOL subscales with statistically significant moderate to large effect sizes for the subscales of growth and development, general health perceptions, and parental emotional impact.

The 13 children with major features of NF1 (including 6 with plexiform neurofibromas and 3 with pseudarthrosis) had lower scores on all subscales, particularly the pain scale, than children with NF1 who did not have these manifestations. The study was too small for extensive multivariate analysis, but the results suggested that the educational level of the respondent parent and familial NF1 are two factors that independently had a positive effect on several ITQOL subscales, whereas visibility and parental perceived severity of the condition were associated with lower ITQOL scores on several subscales. The authors propose that families with a higher educational level, which is a proxy for income and socio-economic status, may be better able to support the needs of their children with NF1. The surprising finding of the positive effect on QoL of having an affected parent is hypothesized to be due to better knowledge of prognosis and established social supports. However, there is no comment on the severity of parents' NF1, and further study would be needed to determine whether other factors, such as learning disabilities in some affected parents, decrease their ability to recognize areas of concern in their children. Figure 8.1 from Spuijbroek and associates' study (2011) shows the ITQOL profile for the 34 children with NF1 in comparison to 410 children from the Dutch population for the study described above. The ITQOL is typical of scales that allow a profile to be developed for the child and his or her family at a given point in time.

Krab et al. (2009) used similar methods to study 43 children aged 10 years and over and their parents. The Child Health Questionnaire (CHQ) was used in two versions—one for parents and one for children to complete. Subscales are similar,

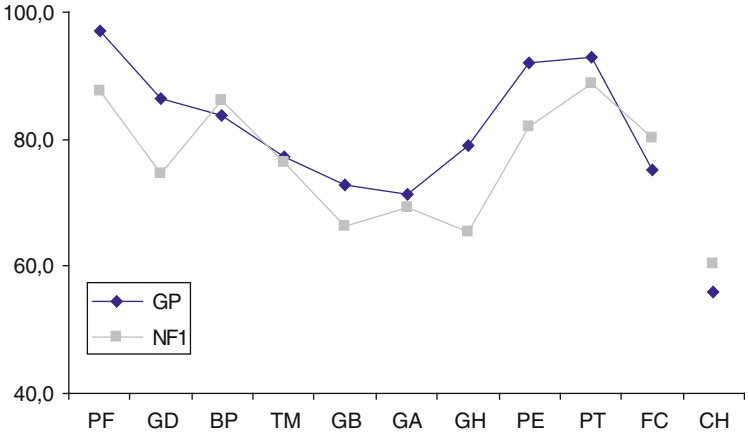

Fig. 8.1 ITQOL scale scores for the general population versus children with NF1. *GP* general population, *NF1* neurofibromatosis type 1. *X*-axis are ITWOL subscales: *PF* physical functioning, *GD* growth and development, *BP* bodily pain, *TM* temperament and moods, *GB* general behaviour, *GA* getting along, *GH* general health, *PE* parental emotional impact, *PT* parental time impact, *FC* family cohesion. *CH* change in health, is a separate item that measures parents' perception of health compared to the previous year (Adapted from Fig. 1, Spuijbroek et al. (2011) Qual Life Res 20:779–786)

but not identical, to those of the ITQOL. In addition, Ablon's visibility scale (Ablon 1999, p. 178) and Riccardi's severity scale (Riccardi 1992, p. 286) were completed, and behavioural and school performance were rated by children's teachers using a validated Dutch measure. Children's self-ratings and parents' ratings of their children were compared. The dramatic difference between children's and parents' scores is shown in Fig. 8.2. In particular, the children's scores on the general behaviour subscale are almost universally above the reference sample mean, whereas their parents' perception is well below reference values. Despite the large difference between mean parental and child self-assessment CHQ scores, both parents' and children's scores deviated from reference data in the same direction. The authors point out that teachers' measures demonstrate substantial behavioural problems in these children, thereby supporting the parental assessments and contradicting the validity of the children's self-assessments. Further research would be necessary to determine if this low self-awareness relates to "factors observed more generally in children with learning disabilities or ADHD" (Krab et al. 2009) or if it is something specific to NF1. This notwithstanding, the lack of ability to accurately self-assess could be seen as protective in terms of quality of life of the child. Krab et al. (2009) point out that in their exploratory regression analysis of the relatively small sample of children, the association between several domains of the CHQ and their teachers' behavioural scores may indicate that treatment to improve children's behaviour has potential to increase children's QoL as well as that of their families.

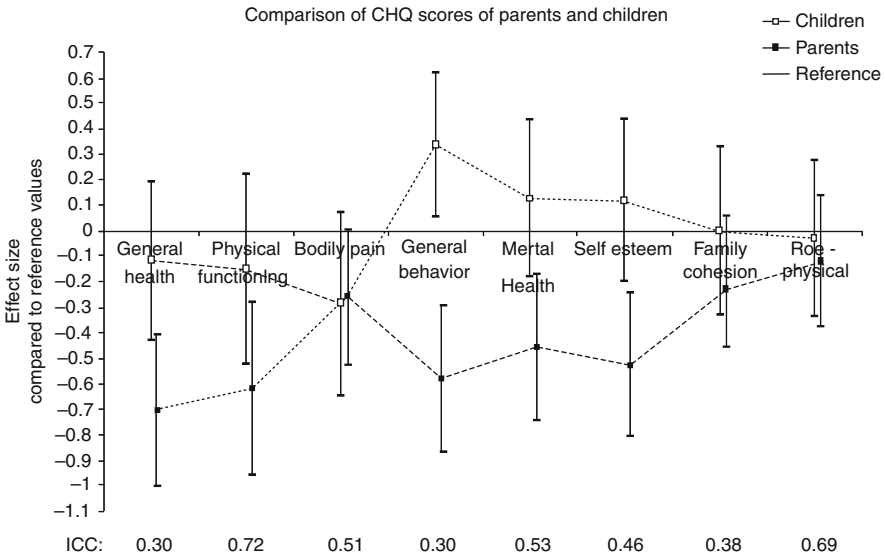

Fig. 8.2 Comparison of CHQ scores of parents and children with NF1. *Error bars* represent 95 % confidence intervals (Reproduced with permission. Krab et al. (2009) J Pediatr 154:420–425)

Although not a QoL study, the research reported by Barton and North (2006) also demonstrated that self-concept in children and adolescents aged 8–16 years was generally positive. Children tended to overestimate their academic abilities in comparison to objective criteria, something that was observed if they had NF1, whether or not they also had ADHD or low academic achievement. Barton and North comment that this may be consistent with Zoller and Rembeck's findings of positive self-perceptions in adults with NF1, which was proposed to be a psychological compensatory mechanism (Zoller and Rembeck 1999).

Several other studies have supported some of the findings discussed above. Vardarinos and colleagues (2009) used the CHQ parent report scale to assess QoL in 43 Greek children aged 6–14 years in comparison to 61 age-matched controls. Parents rated their children with NF1 significantly lower on all domains except for physical functioning. In particular, behaviour, general health, and domains relating to impact on family life showed the biggest differences. A Swiss study by Graf et al. (2006) assessed 46 children between 7 and 16 years of age on various measures: the TACQoL (a generic QoL scale), the CBCL (Child Behaviour Checklist), the FRI (Family Relationship Index), the Riccardi severity scale (physical components only), Ablon's visibility scale, and a calculation of each family's socio-economic status. This study demonstrated low QoL in motor, cognitive, social, and emotional domains, but the difference between the children's self-assessments and their parents' assessments was not significant. As with the later study by Krab et al. (2009), the correlation between the parental and children's assessments was not high. The authors demonstrated a relationship between

positive family relationships and higher parentally reported TACQoL scores, thereby emphasizing the need to support the entire family of children with NF1.

Wolkenstein et al. (2008) used a general paediatric health-related QoL tool, DISABKIDS, which enables children between 8 and 16 years old to self-assess their QoL and their parents to complete the scale by proxy. In addition, Wolkenstein and his colleagues used a skin-disease-specific measure, the Children's Dermatology Life Quality Index (CDLQL), analogous to the adult Skindex used in several earlier studies. 79 children were assessed with these two questionnaires, and the scores were compared to phenotype measures. Unlike the adult Skindex scores, the children's scores on the CDLQL were not dramatically lower than controls, presumably reflecting the milder skin findings of most children with NF1. An exception occurred in the small subset of children with superficial plexiform neurofibromas, as might be expected. The presence of café-au-lait macules or freckling was not an important concern for most paediatric patients. The study demonstrated that, on average, parents scored their children worse on the DISABKIDS instrument than did the children themselves, particularly in social inclusion and exclusion sub-scores. Further, consistent with the findings of Spuijbroek and others, the average proxy score was lower (worse) in parents of new mutation cases than it was for inherited cases. Three specific aspects of NF1 had significant and independent negative effects on QoL. These were orthopaedic manifestations, learning disabilities, and plexiform neurofibromas. The authors suggest that learning disabilities may be one of the major determinants of QoL because of their global effect on self-image in the home, school, and play settings. This study provides additional evidence for the importance of strategies to improve children's functioning and the need for treatment of learning disabilities. Wolkenstein and his associates also compared general QoL scores in the NF1 group to European data for children with other conditions. Their assessment demonstrated that NF1 had a higher impact on general QoL than childhood asthma and was similar to that of generalized atopic dermatitis.

Stevenson and Carey (2009) presented preliminary data on a group of children with NF1, with and without tibial dysplasia. They used the Paediatric Outcome Data Collection Instrument (PODCI) to demonstrate a significant difference in QoL between these two groups of children in the areas of global functioning, transfers/mobility, sports/physical function, and pain/comfort.

Furthermore, the authors suggest use of outcome measures such as QoL to quantify benefit to the patient in a different way from traditional clinical outcome measures. In the case of tibial dysplasia, research and clinical procedures are focussed on measures to promote bone healing and prevent further bone damage. Despite extensive medical efforts, about 20 % of children with pseudarthrosis have an amputation as their clinical end point (Stevenson and Carey 2009). Clinically, this outcome may be seen as a failure; however, a more patient-centred outcome measure may have a different result. The effect of such an amputation on QoL after many failed attempts at surgical correction was beautifully articulated by the parent of an adolescent with tibial dysplasia:

> For the first time in years, he is pain free; he can look forward to walking down to the river, to getting his driver's license, to finishing school and to getting a job. His future has become more positive by this surgical act that was a huge shadow that hung over us for so long. Neither my son nor I have any regrets in trying all options possible—but—had we realized the impact of trying to save his limb—that it would result in so many years lost, long term physical and emotional pain, loss of self worth (and friends), depression, etc., the thought of amputation might not have seemed so overwhelming and defeating. The truth is, this surgery gave him back his life and diminished nothing, except continued suffering.

From the clinician's view, the treatment of tibial dysplasia, with the goal of saving the limb, was a failure, but from the patient's perspective, the amputation represented a significant increase in his quality of life.

8.5 Improving Quality of Life for People with NF1

"The image an affected person has of his/her illness and future is closely tied to their perceptions of their doctors', family's, friends' and society's views of their condition" (Ablon 1999, p. 8). The research summarized above identifies both general and specific ways in which NF1 impacts QoL.

8.5.1 The Role of Support Groups

Support groups offer a "caring community" that provides a social outlet to share information, experiences, and solutions, which may increase quality of life by empowering individuals and enabling them to cope more effectively with such stressors as unemployment or illness (Reissman 1986, pp. 84–85). Support group members offer friendship and social networking and act as mentors, role models, educators, and advocates.

Support groups are also able to effect change in societal and governmental attitudes that may result in immediate or long-term improvement in quality of life. Lobbying by autism support groups is just one example that, in many countries, has resulted in large amounts of specific funding for early behavioural intervention (Autism Society of Canada 2009) and considerable additional research funding (Wadman 2007). Many neurofibromatosis support groups are working hard to increase awareness, provide funding for research, and support for families with NF1.

8.5.2 Governmental Actions

Protection of individuals with genetic conditions has the potential to improve quality of life for many adults with NF1 via access to health insurance and through equal opportunities for employment. Nondiscrimination can be enacted at a

governmental level: In the United States, the Genetic Information Nondiscrimination Act (GINA), which came into effect in 2009, prohibits the use of genetic information for discrimination regarding employment or access to health insurance (GinaHelp 2010). However, GINA does not apply to life, disability, or long-term-care insurance. Many adults with NF1, irrespective of their health and risk factors, are unable to obtain life or disability insurance and the security that affords. Use of current data on the effect of NF1 on mortality (e.g. Evans et al. 2011, Rasmussen et al. 2001) should supersede inaccurate and incomplete information in the life and health insurance literature (e.g. Williams 2006) to enable individuals with NF1 to obtain insurance in a fair manner.

8.5.3 Quality of Life Research in NF1

As yet, there are no specific QoL measures developed for use in NF1 for either children or adults, but combinations of other QoL measures have revealed a number of areas for future research. None of the studies on NF1 to date have investigated the magnitude of the effect of living with a genetically transmissible disease on quality of life. In other genetic conditions, researchers have identified feelings of embarrassment, guilt, and the perception that society is judgemental towards affected adults who have passed onto their children a genetic condition for which prenatal diagnosis is possible (Arnold et al. 2005). Benjamin and colleague's study of 81 adults included information on reproductive decision-making. She demonstrated that about one-third of individuals who did not know that they had NF1 when starting a family stated that their reproductive plans would have been influenced if they had known they had NF1. In this study, some individuals still considering children wished to have prenatal diagnostic testing, but others preferred to have presymptomatic testing at birth (Benjamin et al. 1993). Whereas the health economics of such testing in childhood cannot be supported in terms of the direct cost of care (Tsang et al. 2011), this strictly financial cost has not been examined for prenatal diagnostic testing or for presymptomatic testing in neonates. The effect of having a confirmed diagnosis on QoL has not been measured for any age group. The variability of NF1, and its unpredictability, makes for particularly challenging decisions for future parents and represents an important area for further QoL research.

No specific research to date has quantified the effect of employment and economic difficulties for adults with NF1, nor has impact on QoL of the stress of being uninsurable been measured. Appropriate QoL studies might provide evidence that the stress from lack of insurability and economic disadvantage may justify governmental intervention. Other emerging issues with anecdotal impact on QoL include sleep disorders, lack of family physicians who are knowledgeable in NF1, scarcity of specialized NF1 clinics for adults, and the effect of accelerated appearance of aging on people with NF1. Surprisingly, there no specific research has been undertaken on the effect of chronic pain in NF1 on QoL, although such pain is

recognized to be a major determinant of QoL in other populations (Fine 2011). In children, the outstanding area for research is to measure the effect of educational and behavioural interventions on QoL in children and their parents.

8.6 Conclusion

Use of QoL measures has already identified a number of areas where support, guidance, and specific treatment interventions may make a positive difference for people with NF1. Proactive interventions such as cosmetic surgery, speech therapy, educational, and behavioural modification in childhood have the potential to make a lifelong impact on QoL for the patient and his or her family. Other areas, such as the effect of sleep disorders on QoL, are topics for further research. QoL is a means to monitor treatment, to follow the progress of an individual, and to assess the impact of a health care or social strategy. The progressive nature of NF1 and the lack of treatment options mean that for many people with this condition, QoL may be the most valid outcome measure by which to assess success of a treatment because QoL is an end in itself.

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Chapter 9
NF1 Gene: Promoter, 5′ UTR, and 3′ UTR

Hua Li and Margaret R. Wallace

9.1 Introduction

The *NF1* gene spans 289,701 bp on human chromosome 17q11.2. NIH NCBI (accession NG_009018) genomic sequence starts 4,951-bp upstream of transcription start, 5,334-bp upstream of the start of translation (which is in exon 1), with the 5′ UTR being 484 bp in length. The *NF1* gene has a classic "CpG island" that extends from the proximal promoter into exon 1 (Fig. 9.1). Vertebrate CpG islands are C/G-rich sequences with high frequency of CG dinucleotides, through which cytosine methylation and chromatin structure can be involved as part of gene regulation (Deaton and Bird 2011). The average size is 1 kb. Most are typically unmethylated and associated with active transcription. Most CpG islands are located at the 5′ ends of genes. Currently, there is only one known transcription start site and one translational initiation codon for the *NF1* gene. The *NF1* gene has 58 exons which are generally thought to be constitutive, plus three well-validated small alternatively spliced exons (named 9a (9brain), 23a, and 48a based on original naming convention; based on NCBI numbering, these are between exons 11 and 12, 25 and 26, and 57 and 58, respectively). There is also a report of another alternatively spliced exon, called 10a-2, between exons 10a and 10b (original numbering system) which appears to encode an intracellular transmembrane domain within the 15 amino acids (Kaufmann et al. 2002). The 58 exons encode a 2,818-amino-acid peptide (mRNA RefSeq accession number NM_001042492.2), which appears to be expressed ubiquitously. The 3,522-bp 3′ untranslated region (UTR), described further in Sect. 9.3, contains two polyadenylation sites that correlate with evidence of two transcripts on Northern blot (~11 and 13 kb). Thus, the larger *NF1* transcript (without alternatively spliced exons) is 12,359 bp in length. To date, there is no

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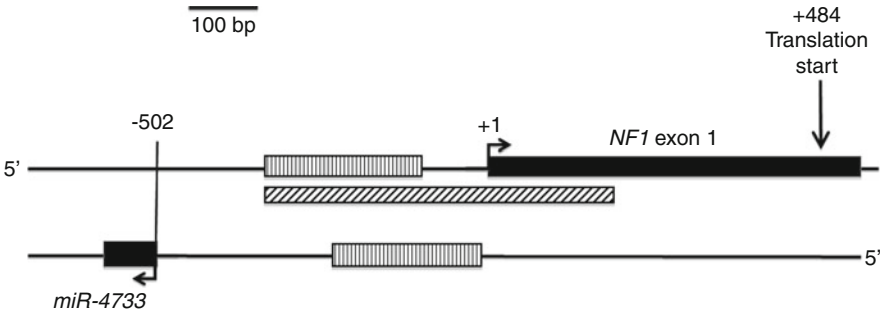

Fig. 9.1 Schematic of the 5′ end of the *NF1* gene on chromosome 17q11.2, drawn to scale. The *NF1* exon 1 and the entire *miR-4733* gene are shown as *solid black boxes*, in their relative orientation on each DNA strand. The *diagonally striped box* represents the CpG island at the 5′ end of *NF1*, as delineated by the University of California Santa Cruz genome browser. The *boxes with vertical stripes* indicate the predicted promoter sequences from the PROSCAN analysis, on the same strand as their target gene. The *NF1* transcriptional start site is indicated at the beginning of exon 1 (+1) and *miR-4733*'s transcriptional start site is 502-bp upstream of the *NF1* transcription start, on the other strand. *NF1* translation starts at 484 bp into the transcript, with the 5′ UTR falling between +1 and +484

evidence that translation can initiate from anywhere other than the currently known translation AUG start site.

There are seven partial duplications of *NF1* on other chromosomes, clustered primarily in the pericentromeric regions, with evidence of transcription from the loci on chromosomes 2, 15, and 21 (the latter of which shows testis-specific expression) (Yu et al. 2005). These loci and transcripts have the potential to interfere with molecular analyses of the *NF1* gene.

There are three two-exon genes that lie within another 60-kb *NF1* intron (traditional nomenclature intron 27b): *EVI2B*, *EVI2A,* and *OMG*, encoded on the opposite strand and transcribed in the opposite direction relative to *NF1*. These are more limited in tissue expression than *NF1*, but clearly both strands of DNA are concurrently transcribed in some tissues, suggesting a possible relationship. As an aside, our recent analysis of the large intron 1 did not find sequences indicating transcribed loci; this intron appears devoid of "embedded" genes (S. Oden and M. Wallace unpublished data).

9.2 Promoter

The first two studies of the *NF1* promoter showed that there is substantial sequence homology between the human and rodent *NF1* promoters (Bernards et al. 1993; Hajra et al. 1994). Hajra et al. (1994) studied a larger sequence (~900-bp upstream of translation start). Using RNAse protection, they confirmed the major transcription start site (−484) but also found two minor transcription start sites (−495 and −483)

in both human and mouse genes. Neither group found evidence of TATA or CCAAT boxes, but identified consensus binding sequences for a number of transcription factors such as SP1 and AP2, included in the 5′ UTR. With the major transcription start at −484, the *NF1* exon 1 is 544 bp in length, containing the 5′ UTR and encoding the first 20 amino acids of neurofibromin. The CpG island, as defined by the UCSC Genome Browser, contains 43 CpG dinucleotides in a 472 bp span (Fig. 9.1). The rest of exon 1 and the beginning of intron 1 are also C–G-rich, albeit not as rich in CpG dinucleotides.

In 2003, Jenne et al. performed a comparison of the genomic *NF1* gene and extended flanking sequence between human and mouse. This substantial physical map indicated that there were no genes within 50 kb of the 5′ end of human *NF1*, and the nearest 3′ gene was *KIA1821* (*RAB11FIP4*, approximately 10 kb downstream). A methylation map of five regions of the promoter (mouse and human) was published by Mancini et al. (1999), showing that three of these regions (several hundred bp at approximately −1000, –3000, and −4000, where +1 is the transcription start site) were consistently methylated, but that the cytosines (in CpG or CpNpG) near the transcription start site (flanked by SP1 sites) were unmethylated, in all tissues examined. Methylation was also shown to functionally impact SP1 sites and a CREB binding site. In 2004, this group used reporter assays to identify the relative levels of promoter activity of overlapping sequences from the promoter, which revealed particularly strong activity in a 500-bp fragment (from −270 to +230 relative to the transcriptional start) (Zou et al. 2004). In addition, the sequences between +230 and translation start codon (+484) appeared to have a transcriptional repressor activity. Gel shift data indicated that the CREB site must be intact in order to achieve normal promoter activity. They also showed that SP1 (as part of a protein complex) bound to the SP1 sites at −141, +415, and +460. These data suggest that methylation at these transcription factor binding sites could influence promoter activity. The authors propose that the four AP2 sites (from +230 to +434) might be in part responsible for the repressor activity of this region. Another group used a bioinformatics approach (Promoter Inspector, Dragon Promoter Finder, MATCH, MatInspector) to predict regulatory elements in the promoter through the front part of intron 1, via comparisons of human, mouse, rat, and pufferfish (*Fugu rubripes*) *NF1* genes (Lee and Friedman 2005). A 24-bp sequence was perfectly conserved except for a 1-bp difference in pufferfish, located 310-bp upstream of translation start, in the 5′ UTR. There were two additional transcription start sites predicted with 80 % sensitivity, closer to the translation start codon (−116 and −384 relative to the translation start). However, these are not the same minor transcription start sites identified by Hajra et al. (1994). Yang et al. (2005) found that an AML-related fusion protein, from translocation-derived juxtaposition of genes *RUNX1* and *MTG8*, binds to the *NF1* gene promoter and represses transcription in myeloid progenitor cells.

NF1 expression is likely controlled with somewhat different regulatory systems in different tissues (and possibly temporally as well). For example, a study by Huang et al. (2007) showed that the transcription factor PU.1 binds to a composite ets/IRF element (also known as EICE sequences), recruiting the IRF2 protein and

subsequently the ICSBP protein (aka IRF8), through cytokine signaling in differentiating myeloid cells. This regulatory system is typically found in genes involved in the inflammatory response. In the *NF1* gene, this regulatory element is at −320 to −336 (upstream of the transcriptional start site), in the proximal promoter where many other key promoter elements lie.

Several groups have looked at proximal *NF1* promoter/5′ UTR methylation in tissues from patients with NF1, with the concept that reduced *NF1* transcription due to methylation could be a "second hit" mechanism equivalent to somatic mutation. Horan et al. (2000) did not observe global hypermethylation in neurofibromas or MPNSTs, but there were some specific sites that were much more frequently methylated in tumor tissues than normal tissues; this same observation was reported by Harder et al. (2004) and Fishbein et al. (2005), although Luijten et al. (2000) did not find evidence of methylation differences. The methylated nucleotides were mostly cytosines in CpG dinucleotides, but Harder et al. (2004) reported some non-CpG methylation. Interestingly, these sites mostly lie within predicted or proven-functional transcription factor binding sites. Most of the sites were within the proximal promoter, although some were in the 5′ UTR. Not unexpectedly, since methylation is a dynamic process affected to environmental influences, these studies and a recent NF1 monozygotic twin study (Harder et al. 2010) showed that there is some interindividual variability in *NF1* promoter (and 5′ UTR and intron) cytosine methylation. This latter study also found that there tended to be greater methylation in the individuals who had pilocytic astrocytoma (leukocyte DNA analysis). However, according to a previous report from another group, these astrocytomas themselves do not show increased methylation (Ebinger et al. 2005).

Ling et al. (2006) found evidence in mouse studies that the imprinting control region of the maternal allele of the *Igf2*/*H19* imprinted locus (mouse chromosome 7) colocalizes with the paternal allele of the *Nf1*/*Wsb1* region (chromosome 11) in the intermitotic nucleus, a phenotype that the CTCF transcriptional repressor appears to influence. When this colocalization is disrupted, expression of *Igf2*, *Nf1*, and *Wsb1* are reduced. The site on chromosome 11 is ~70-kb upstream of *Nf1* and 20 kb downstream of *Wsp1*. Extending their studies to the human genome, this group found several CTCF binding sites between *NF1* and the upstream *RNF135*, as well as two sites within the *NF1* locus (Ling et al. 2011). This implicates the contribution of interchromosomal interactions in regulating *NF1* transcription. It is also known that signals at some distance from well-distant genes (even several megabases) can also affect expression ("position effect"); an example is the *SOX9* gene and upstream deletions (e.g., Hill-Harfe et al. 2005).

Our group performed a promoter search in the 3 kb immediately upstream of *NF1* transcription start, using the programs PROSCAN (http://www-bimas.cit.nih.gov/molbio/proscan/) and JASPAR (Bryne et al. 2008). PROSCAN predicted the strongest promoter sequence (score 68.33, cutoff = 53) between −362 and −112 upstream of the *NF1* transcriptional start but also detected a novel overlapping promoter on the opposite (antisense) strand, from −4 to −254 (score 77.62) (Fig. 9.1). Analysis of the novel promoter using JASPAR (Bryne et al. 2008) to screen for regulatory binding sites found 12 strong positives (score >8.0) on the

NF1 sense strand and 12 positives on the antisense strand. No CCAAT or TATA signals were found. Consistent with presence of an antisense promoter, we noted the presence of a microRNA (*miR-4733*, Gene ID: 100616266, MI0017370 in miRBase, http://www.mirbase.org) encoded on the strand opposite *NF1*, with the direction of transcription away from *NF1* (Fig. 9.1). This microRNA was discovered in a study of normal and cancerous human breast tissue (Persson et al. 2011), but there are no other publications or information about the significance of this microRNA, such as its targets. However, one could speculate that methylation alterations in the *NF1* promoter might influence *miR-4733*'s promoter and expression as well, complicating interpretation of *NF1* promoter methylation data. Deletions spanning this region would, in addition to creating a null *NF1* allele, make such cells effectively hemizygous for *miR-4733*, which may have a functional effect.

There have not yet been any proven-pathogenic *NF1* mutations in the *NF1* promoter or untranslated regions. The Human Gene Mutation Database (http://www.hgmd.org) contains 1,347 *NF1* mutations, but none in the "regulatory" category, despite the gene having a relatively high mutation rate. However, researchers have been screening these regions. Horan et al. (2004) analyzed several novel promoter/5′ UTR variants from NF1 patients (Osborn et al. 2000) and found that one of them slightly altered luciferase reporter function. If a novel promoter or 5′ UTR variant was found in an NF1 patient, the best proof of its pathogenicity would be lack of the variant in the unaffected biological parents. Functional studies are not always straightforward for regulatory region variants, although in cases where there is dramatic effect on transcription, splicing, translation, or binding of a predicted protein, it would be convincing. Any regulatory region variants are of interest, given the strong cross-species conservation of these sequences. In fact, there are no reported 5′ UTR polymorphisms in dbSNP (http://www.ncbi.nlm.nih.gov/snp), but there are ten validated SNPs reported in the 5-kb upstream of the translation start site.

9.3 3′ UTR

In 1993, Bernards et al. compared the mouse *Nf1* cDNA with the human transcript and found that the untranslated regions were highly conserved as well as the coding regions. In 1998, this group verified that there were two *NF1* polyadenylated transcripts (~11 and 13 kb), differing in size due to the length of the 3′ UTR, consistent with two polyadenylation sites already found in mouse *Nf1* gene (Cowley et al. 1998). Both transcripts were found in all tissues examined, except that testis RNA also showed a 9-kb band on Northern blot. They also found that it was difficult to obtain human cDNA clones containing complete 3′ UTR sequences. Several polymorphisms were identified, primarily in the areas not conserved in mice. Haeussler et al. (2000) examined the 3′ UTR, with the notion that its involvement in posttranscriptional gene regulation was one mechanism by which the *NF1* transcript varied in quantity both temporally and spatially. Their study found five regions of the 3′ UTR, which appear to bind proteins, and identified that

one of the proteins was tumor antigen HuR. HuR bound to an AU-rich element (ARE), thought to be a negative regulator of transcript stability. These sites were scattered across the 3′ UTR, and it appears that other proteins besides HuR are involved at these other sites. This supports the hypothesis that posttranscriptional mechanisms influence the levels of *NF1* transcript.

Another mechanism affecting transcript stability, studied predominantly within the past 10 years, is microRNA binding. MicroRNAs are ~22 nucleotide RNAs processed from larger transcripts. These bind to the 3′ UTR of transcripts with seed sequences of 8 nt, causing transcript degradation if the sequence is a perfect match, or attenuating translation if the sequence is an imperfect match (Calin and Croce 2006). The first study implicating miRNA regulation of *NF1* proved that *miR-10b* binds to the 3′ UTR, and found elevated levels of *miR-10b* in NF1 tumor-derived Schwann cells (Chai et al. 2010). Further, it was shown that inhibition of *miR-10b* was associated with reduced cell proliferation, invasion, and migration in MPNST cells. Although there was reduced RAS signaling, there was no detectable increase in neurofibromin expression, suggesting that other predicted *miR-10b* RAS-pathway targets might be involved or that cooperation with other miRNAs was involved in tumorigenesis. Subramanian et al. (2010) also found decreased *miR-34a* in MPNSTs, which they suggest is related to p53 inactivation. *miR-34a* is a fairly well-studied microRNA, and a recent study found that it can inhibit RAS-pathway signaling (Kim et al. 2012). A miRBase (http://www.mirbase.org) analysis of the *NF1* 3′ UTR yielded a list of over 100 putative miRNA binding sites, not unexpected given the size of this region.

Although there are as yet no proven pathogenic mutations in the *NF1* 3′ UTR, it is feasible that such mutations could exist, as has been the case in other inherited disorders via mechanisms such as altered miRNA binding, cryptic splicing, altered stop codon, or altered polyadenylation sites. There are some known polymorphisms and rare variants in the *NF1* 3′ UTR, but their effects, if any, on *NF1* gene regulation are not known. Purandare et al. (1996) reported one of the first 3′ UTR variants, an A to G change at base 10647 in the mRNA. Cowley et al. (1998) reported five 3′ UTR polymorphisms (3 common, 1 with 5 % heterozygosity, and one with a minor allele frequency less than 1 %). Upadhyaya et al. (1995) found one variant in the 3′ UTR (c.11715 A > G) in a patient with mild NF1, and although the patient was the first case in the family, her parents were not available for analysis. The dbSNP database lists 12 validated SNPs (some are single-nucleotide polymorphisms, some are indels) in the 3′ UTR of *NF1*. Future studies could align the location of these SNPs, and any novel variants, with putative miRNA binding sites or other predicted regulatory elements.

9.4 Conclusion

The *NF1* gene is large, with a large 3′ UTR, and the functions of the encoded protein are still being identified, including specific functions of isoforms. The promoter has not been studied extensively, but the gene is active in all tissues

examined (and in development) with a classic CpG island spanning the promoter and 5′ UTR, but no TATA or CCAAT box. The significance of the alternative polyadenylation is not known. The large 3′ UTR is probably involved in posttranscriptional regulation via microRNAs, but this area has only recently begun to be analyzed. There is one major transcriptional start site, although there is evidence suggesting that there may be minor amounts of transcription from other, nearby sites. There is a microRNA gene on the opposite strand, head-to-head with the *NF1* gene (transcription start sites separated by 502 bp, bidirectional promoter). This feature has not been previously addressed in the literature, as this element only appeared in the genomic data within the past year. It is unclear whether these two genes are coordinately regulated. Despite the high *NF1* mutation rate, based on mutations in the body of the gene that result in NF1, there have been none conclusively identified in the promoter or untranslated regions. It could be that such mutations are very rare or that they are of insufficient effect to result in a recognizable phenotype. Several groups have reported data suggesting that moderate, site-specific methylation of the *NF1* promoter may be present in some NF1 tumors, although there is no evidence yet that such methylation can silence the locus. Control of *NF1* expression via its regulatory regions needs further investigation to understand more fully. This will also shed light on understanding the variable expression/stability of mutant transcripts, which seem to defy standard nonsense-mediated decay. The ability of mutant transcripts to produce functional protein is not well characterized and may be heterogeneous depending on the exact mutation and environmental factors. Better understanding of *NF1* gene regulation will lend itself to developing therapies based on compensating for specific mutations. Similarly, better understanding of specific promoter and UTR elements will be key to evaluating variants or aberrant methylation found at these sites as well as for therapies aimed at upregulating *NF1*.

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Chapter 10
The Germline Mutational Spectrum in Neurofibromatosis Type 1 and Genotype–Phenotype Correlations

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10.1 Introduction

The neurofibromatosis type 1 (*NF1*) gene, spanning 283 kb on chromosome 17q11.2, contains 61 exons that give rise to a 12-kb mRNA transcript encoding neurofibromin [reviewed by Upadhyaya (2008)]. The 327-kDa (2,818 amino acid) neurofibromin protein is expressed in most tissues (although it is expressed at its highest level in the brain and central nervous system) and has a number of alternative isoforms [reviewed by Trovó-Marqui and Tajara (2006)]. Neurofibromin is a tumour suppressor protein, reflecting its role as a key negative regulator of the cellular Ras signalling pathway [reviewed by Bennett et al. (2009)]. More specifically, it is a Ras-specific GTPase-activating protein (GAP), with strong structural and sequence homologies to the GAP superprotein family (Scheffzek et al. 1998). It functions by greatly increasing the very low intrinsic GTPase activity of Ras proteins, thereby significantly accelerating the conversion of the active GTP-bound form of Ras into the inactive Ras-GDP-bound state, resulting in an overall reduction in cellular mitogenic signalling via the Ras pathway. Thus, any *NF1* gene mutation that serves to inactivate neurofibromin function may be expected to increase cellular levels of active Ras-GTP significantly, leading to uncontrolled cell growth and potentially tumorigenesis (Klose et al. 1998; Arun and Gutmann 2004).

Heritable mutations of the *NF1* tumour suppressor gene give rise to the tumour predisposition syndrome neurofibromatosis type 1 (NF1) which affects 1/3,000–4,000 individuals worldwide. Consistent with Knudson's two-hit hypothesis, NF1 patients harbouring a heterozygous germline *NF1* mutation develop neurofibromas upon somatic mutation of the second wild-type *NF1* allele. The somatic loss of the second *NF1* allele in the progenitor cell (either the Schwann cell

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or its precursor), combined with haploinsufficiency in a variety of supporting cells (Gutmann et al. 1999; Ingram et al. 2000; Kemkemer et al. 2002; McLaughlin and Jacks 2002; Pemov et al. 2010), is then required for tumour formation. For as yet unknown reasons, the mutation rate at the *NF1* locus is one of the highest reported in any human disorder (Huson et al. 1989; Kehrer-Sawatzki and Cooper 2008; Evans et al. 2010); this is reflected in the finding that almost 50 % of all NF1 patients exhibit a de novo *NF1* mutation. Here, we review the known germline *NF1* mutational spectrum and show how the nature, location and frequency of different types of inherited *NF1* mutation are shaped in large part, and often in remarkably predictable ways, by the local DNA sequence environment.

10.2 The Germline Mutational Spectrum of the *NF1* Gene

Nearly 1,300 different inherited mutations of the *NF1* gene have been reported as a cause of NF1 (Table 10.1). These vary in size from deletions spanning more than a megabase to subtle single base-pair substitutions that alter an encoded amino acid or the function of a splice junction. Below we review the different types of germline mutation so far identified in the *NF1* gene.

10.3 Missense and Nonsense Mutations

Tumour suppressor genes characteristically exhibit a disproportionate number of nonsense mutations (Mort et al. 2008; Ivanov et al. 2011), and the *NF1* gene is no exception. More than 54 % of all known inherited pathological single base-pair substitutions within the coding region of the *NF1* gene result in nonsense mutations. Many of these nonsense mutations are located within CpG dinucleotides, a known hotspot for mutation. The hypermutability of the CpG dinucleotide is related to its role as the major site of cytosine methylation in the human genome. While cytosine spontaneously deaminates to uracil (which is efficiently recognised as a non-DNA base and removed by uracil-DNA glycosylase), the spontaneous deamination of 5 mC yields thymine, thereby creating G•T mismatches whose removal by methyl-CpG binding domain protein 4 (MBD4) and/or thymine DNA glycosylase followed by base excision repair (BER) is inherently less efficient; the rate of CG > TG (and CG > CA on the other strand) transitions within CpG dinucleotides has been estimated to be five times that of the base mutation rate (Krawczak et al. 1998). In the context of the *NF1* gene, some 18 of the 23 CG > TG/CA mutations within the gene coding region are CGA > TGA (Arg > Term) [see HGMD]; such nonsense mutations are known to be more likely to come to clinical attention than missense mutations on account of their greater average phenotypic effect (Krawczak et al. 1998).

Table 10.1 Summary of different germline mutations reported in the *NF1* gene (from the Human Gene Mutation Database (HGMD; http://www.hgmd.org; Stenson et al. 2009) as of November 2011)

Mutation type	Number of different mutations
Missense	142
Nonsense	167
Splicing	285
Microdeletions (≤20 bp)	349
Microinsertions (≤20 bp)	167
Indels (≤20 bp)	28
Gross deletions (>20 bp)	129
Gross insertions (>20 bp)	12
Complex rearrangements	8
Total	1,287

It has become evident that cytosine methylation also occurs in the context of CpNpG sites (where N represents any nucleotide) in mammalian genomes (Lister et al. 2009; Lee et al. 2010). The intrinsic symmetry of the CpNpG trinucleotide supports a semi-conservative model of replication of the methylation pattern (as with the CpG dinucleotide). It therefore comes as no surprise to find that ~5 % of missense/nonsense mutations causing human inherited disease are potentially attributable to methylation-mediated deamination of 5 mC within CpHpG trinucleotides (Cooper et al. 2010). In the context of the *NF1* gene, CpHpG trinucleotides also represent a hotspot for mutation (Rodenhiser et al. 1997); indeed, there are now 24 known examples of CpHpG > TpHpG/CpHpA mutations, all CAG > TAG transitions (Gln > Term) that are compatible with a model of methylation-mediated deamination of 5 mC [see HGMD].

Some clustering of missense mutations is evident in the *NF1* gene. In the absence of any localised hypermutability, the most likely reason for this phenomenon is clinical selection for inactivation of a functional domain in the protein. The best characterised functional domain in neurofibromin is the GTPase-activating protein (GAP) domain encoded by exons 20–27a (Welti et al. 2008). A number of studies have been performed to assess the functional impact of the ~25 known naturally occurring missense variants within the GRD (Skinner et al. 1991; Li et al. 1992; Poullet et al. 1994; Morcos et al. 1996; Scheffzek et al. 1997, 1998; Upadhyaya et al. 1997; Klose et al. 1998; Ahmadian et al. 2003). In principle, the biochemical study of key residues within the GRD has the potential to yield new insights into the role of these residues in Ras–Ras–GAP interaction. However, only four naturally occurring NF1-GRD mutants (K1423E, R1391K, R1391S and R1276P) have so far been functionally analysed (Ahmadian et al. 2003). Li et al. (1992) found that the K1423E mutation resulted in a 200- to 300-fold reduction in GAP activity. It is thought that the K1423 residue forms an intramolecular salt bridge with E1437 in the α7/variable loop, thereby establishing a key component of the Ras–RasGAP interaction; disruption of this salt bridge by mutation of the K1423 residue would be likely to result in negative charges which could interfere with the interaction of the Ras–RasGAP interaction (Klose et al. 1998). Since K1423 is located at some distance from the Ras active site, it is unlikely to be directly involved in catalysis but could be involved in determining the specificity of

neurofibromin for Ras-GTP (Ahmadian et al. 2003). Mutations at the R1391 residue have been studied in two different contexts, viz. R1391K (Skinner et al. 1991) and R1391S (Upadhyaya et al. 1997). The GAP-related domain harbouring the R1391S missense mutant was found to be ~300-fold less active than wild-type NF1 (Upadhyaya et al. 1997). Finally, Klose et al. (1998) determined that although the R1276P substitution only results in a slight reduction in binding affinity for Ras (6.6-fold), it severely compromises GTP hydrolysis (by some 8,000-fold). Mutations in the GAP domain are known to impact upon microtubule binding as well as p21-ras regulation (Xu and Gutmann 1997; Upadyhaya et al. 1997; Klose et al. 1998; Scheffzek et al. 1998). Recently, Thomas et al. (2012) have described 10 NF1-GRD variants which were deemed to be potentially pathogenic based on genetic and bioinformatic analysis and molecular modeling.

A second domain, encoded by exons 11–17 of the *NF1* gene, comprises a cysteine/serine-rich domain with three cysteine pairs suggestive of ATP binding, as well as three potential cAMP-dependent protein kinase (PKA) recognition sites that are phosphorylated by PKA (Fahsold et al. 2000; Mattocks et al. 2004; Welti et al. 2008). Finally, D'Angelo et al. (2006) identified a third domain, comprising a Sec14p-homologous segment (residues 1,560–1,699) closely followed by a region with pleckstrin homology (residues 1,713–1,818). This domain binds phospholipid (Welti et al. 2007), but naturally occurring mutations within this domain do not appear to have a major effect on lipid binding even although protein structure may be perturbed (Welti et al. 2011).

10.4 Gene Conversion

Gene conversion refers to the unidirectional transfer of genetic material from a "donor" sequence to a highly homologous "acceptor". The human genome harbours a number of different sequences paralogous to the *NF1* gene that could in principle be capable of introducing mutations into the *NF1* gene via gene conversion (Luijten et al. 2001a; Yu et al. 2005). Such paralogous sequences have diverged from the parent *NF1* gene at multiple locations and hence contain a large pool of potential disease alleles that could be introduced by interlocus gene conversion (Luijten et al. 2001b). During a recent survey of human disease genes, we noted at least nine known missense mutations in the *NF1* gene (Table 10.2) that could have been templated by gene conversion using one of these *NF1* paralogs as a donor (Casola et al. 2012).

10.5 Splicing Mutations

It has been known for some time that a high proportion (at least 20 %) of *NF1* gene lesions affects mRNA splicing (Ars et al. 2000; Messiaen et al. 2000). This is likely to be a consequence of the large size of the *NF1* gene and the above average number of introns (60) that it contains. However, the number of splicing-relevant mutations

Table 10.2 Single base-pair substitutions in the *NF1* gene, putatively templated by gene conversion mediated by paralogous NF1-related donor sequences with ≥200 bp and ≥92 % sequence identity to the *NF1* gene (from Casola et al. 2012)

HGMD_ID	Wild-type nucleotide	Mutant nucleotide	Mutation position	% Identity	Chromosome location of *NF1* paralogous donor sequence	Donor start	Donor end	CpG
CM000796	A	G	26583996	93.03	15	19399113	19399313	0
CM032012	C	T	26587083	93.03	15	19396329	19396529	0
CM087437	C	T	26551587	94.53	18	14147219	14147419	1
CM087525	A	G	26565668	96.52	2	131671876	131672076	0
CM087525	A	G	26565668	96.52	14	18565252	18565452	0
CM087525	A	G	26565668	96.52	14	19148007	19148207	0
CM087525	A	G	26565668	95.02	22	14725888	14726088	0
CM087526	C	T	26580376	96.02	15	19402801	19403001	1
CM087526	C	T	26580376	95.02	12	36886955	36887154	1
CM087527	A	G	26580422	95.02	15	19402755	19402955	0
CM096910	T	C	26551576	94.03	18	14147230	14147430	0
CM973234	C	G	26584259	93.03	15	19398854	19399050	0

In the CpG column, "1" and "0" denote that the mutation occurs or does not occur in a CpG site, respectively. For the three C > T transitions that occur in CpG dinucleotides, it should be noted that recurrent mutation through methylation-mediated deamination of 5-methylcytosine could be an alternative explanation for these lesions

in the *NF1* gene has almost certainly been underestimated. Thus, it is increasingly being realised that "deep intronic" mutations which impact upon the mRNA splicing phenotype have been routinely overlooked (Raponi et al. 2006; Wimmer et al. 2007; Pros et al. 2008; Cooper 2010). These mutations may, however, exert a variable effect on the clinical phenotype. Thus, for example, Fernández-Rodríguez et al. (2011) have reported a deep intronic *NF1* mutation (c.3198-314G > A) associated with a relatively benign clinical phenotype, in part as a consequence of "leaky splicing" that served to generate a proportion of correctly spliced transcripts and hence, potentially, a sufficient level of neurofibrominfor near-normal function.

Wimmer et al. (2007) classified *NF1* splicing-relevant mutations into five distinct groups: exon skipping resulting from mutations at authentic splice sites (type I), cryptic exon inclusion caused by deep intronic mutations (type II), creation of de novo splice sites causing loss of exonic sequences (type III), activation of cryptic splice sites upon authentic splice-site disruption (type IV), and exonic sequence alterations causing exon skipping (type V). In the latter category (type V) are mutations that disrupt exonic splice enhancers (Colapietro et al. 2003; Zatkova et al. 2004; Baralle et al. 2006; Pros et al. 2008), a type of mutation that has almost certainly been underestimated in terms of its likely impact upon human inherited disease (Sterne-Weiler et al. 2011).

10.6 Regulatory Mutations

As yet, no regulatory mutations have been reported for the *NF1* gene. Despite the presence of a number of functional elements in the proximal promoter region of the *NF1* gene (Zou et al. 2004), no pathological lesions have been identified in mutation screens involving up to 987 bp DNA sequence spanning the promoter and 5′ flanking sequence of the gene (Osborn et al. 2000; Horan et al. 2004).

10.7 Microdeletions, Microinsertions and Indels

Microdeletions, microinsertions and indels comprise 31 %, 15 % and 2.5 % of all microlesions in the *NF1* gene, respectively. The majority of microdeletions and microinsertions in the *NF1* gene occur within mononucleotide tracts and short direct repeats (Rodenhiser et al. 1997). These microdeletions, microinsertions and indels are therefore potentially explicable in terms of slippage mutagenesis, involving the addition or removal of one copy of a mono-, di-, or trinucleotide tandem repeat (Chuzhanova et al. 2003; Ball et al. 2005). Consistent with this interpretation, several mutation hotspots are evident that harbour both microdeletions and microinsertions; these invariably involve the deletion or insertion of a single nucleotide within a mononucleotide tract [e.g. A in $(A)_7$ at codon 76, T in $(T)_4$ at codon 1303 and C in $(C)_5$ at codon 1818]. It should be noted that at least10 intra-exonic deletions of >20 bp but <70 bp have also been reported (see HGMD for details).

10.8 Gross Deletions of the *NF1* Gene

Gross deletions in 17q11.2 that encompass the *NF1* gene and its flanking regions constitute the most frequently recurring mutations causing NF1. Large deletions of the *NF1* gene and its flanking regions (also termed *NF1* microdeletions) are observed in ~5 % of all patients with NF1 (Kluwe et al. 2004). These deletions are discussed briefly here for the sake of completeness but in much more detail in Chap. 15.

Two recurrent subtypes of gross *NF1* deletion (type 1 and type 3) have been noted to occur in the germline of NF1 patients; these deletions differ in terms of their size, relative frequency and the positions of their respective breakpoints. The most common of these are type 1 deletions which encompass 1.4 Mb and lead to the loss of 14 genes including the *NF1* gene. Type 1 deletions are mediated by nonallelic homologous recombination (NAHR) between low-copy repeats (LCRs) flanking the *NF1* gene region, specifically NF1-REPs A and C. Two preferred regions of NAHR have been noted within the NF1-REPs: the paralogous recombination sites PRS2 and PRS1 (Forbes et al. 2004; Raedt et al. 2006). The majority of type 1 deletions have breakpoints within a 3.4-kb region spanning PRS2, whereas the remainder have breakpoints within a 1.8-kb region spanning PRS1 (Raedt et al. 2006). The NAHR events underlying type 1 deletions occur preferentially during maternal meiosis (Upadhyaya et al. 1998; López-Correa et al. 2000).

Type 3 *NF1* deletions are mediated by NAHR between NF1-REPs B and C; they encompass only 1 Mb and occur much less frequently than type 1 deletions; indeed, only 11 patients with germline type 3 deletions have so far been identified (Bengesser et al. 2010; Messiaen et al. 2011; Zickler et al. 2012). Thus, both type 1 and type 3 deletions serve to demonstrate the high recombinogenic potential of the LCRs within the *NF1* gene region.

By contrast to type 1 and type 3 deletions, type 2 deletions frequently arise during postzygotic cell division and hence lead to somatic mosaicism with normal cells alongside cells harbouring the deletion (Petek et al. 2003; Kehrer-Sawatzki et al. 2004; Steinmann et al. 2007; Roehl et al. 2010). The breakpoints of type 2 deletions are located within *SUZ12* and its pseudogene *SUZ12P* (Petek et al. 2003; Kehrer-Sawatzki et al. 2004; Steinmann et al. 2007; Roehl et al. 2010). With an estimated frequency of 9–20 %, type 2 deletions occur less frequently than type 1 *NF1* deletions (Kehrer-Sawatzki et al. 2004; Messiaen et al. 2011; Pasmant et al. 2010).

In contrast to the aforementioned recurrent *NF1* deletions mediated by NAHR, a number of "atypical" germline deletions of the *NF1* gene have also been reported that are not necessarily mediated by homology-based mechanisms or recurrent with respect to the localisation of their breakpoints (Venturin et al. 2004; Mantripragada et al. 2006). Consequently, atypical *NF1* deletions can differ quite markedly from each other in terms of the number of deleted genes (Kehrer-Sawatzki et al. 2008 and references therein; Pasmant et al. 2010). One atypical 1.5 Mb deletion, with a

breakpoint within IVS23a of the *NF1* gene, has been reported to be mediated by *Alu* repeats (Gervasini et al. 2005).

In addition to the above gross deletions involving and encompassing the entire *NF1* gene, it should be noted that there are in excess of 70 known intragenic deletions that serve to remove one or more exons within the gene (e.g. Wimmer et al. 2006). Multi-exonic deletions appear to constitute between 2 % and 7 % of *NF1* gene mutations (De Luca et al. 2007; Orzan et al. 2008). Wimmer et al. (2006) reported the occurrence of a recurrent *Alu*-mediated deletion involving exon 2 of the *NF1* gene in 2 of 25 unrelated NF1 patients with single- or multi-exon deletions.

10.9 Duplication of the *NF1* Gene

A 1.5-Mb gross duplication of the region encompassing the *NF1* gene and at least 12 other genes has been reported by Grisart et al. (2008) in two brothers characterised by mental deficiency, early-onset (age 15) baldness, dental enamel hypoplasia and minor facial dysmorphism. The site of the duplication corresponds perfectly to the NF1 microdeletion region described above. Although NAHR between the homologous repeats flanking the *NF1* gene would be expected to generate reciprocal products (i.e. a deletion and a duplication) with equal frequency, this is the only properly characterised example of a duplication involving the *NF1* gene.

10.10 Gross Insertions

The most prominent examples of the insertional inactivation of the *NF1* gene involve the retrotranspositional insertion of *Alu* elements. Such insertions have been noted to occur either within an exon (Wimmer et al. 2007; Valero et al. 2011) or an intron (Wallace et al. 1991) of the *NF1* gene, with the insertions characteristically tending to occur preferentially in AT-rich regions (Wallace et al. 1991).

Using cDNA sequencing to screen for *NF1* gene mutations, Wimmer et al. (2011) identified abnormally spliced *NF1* transcripts in 18 unrelated NF1 patients which were not explicable at the genomic level in terms of an underlying microlesion. Further characterisation revealed the causative mutations to be 14 examples of *Alu* element insertions, three examples of L1 element insertions and the insertion of a ~120 bp poly(T) stretch (possibly also a highly truncated *Alu* sequence). These 18 pathogenic L1 endonuclease-mediated de novo insertions represent the largest number of insertion-type mutations so far characterised in any human gene. Indeed, these findings demonstrate that retrotransposon insertions may account for as many as ~0.4 % of all *NF1* gene mutations. Further, six different insertions were found to be clustered within a 1.5-kb region (*NF1* exons 21, 22 and 23). Remarkably, three different integration sites, one of them located

within the cluster region, were each used twice (i.e. c.1642-1_1642 in intron 14, c.2835_2836 in exon 21 and c.4319_4320 in exon 33), consistent with the comparatively frequent and highly non-random integration of retrotransposons into the *NF1* gene. It is as yet unclear precisely how these insertions interfered with splicing of the *NF1* transcript, but it is likely to be by a variety of mechanisms including the simple introduction of novel splice sites as well as interference with exon definition either through the disruption of exon splice enhancers or by increasing the distance between exonic *cis* regulatory elements and their cognate splice sites (Wimmer et al. 2011).

10.11 Other Rearrangements of the *NF1* Gene

Two nonidentical t(17;22) translocations that disrupt the *NF1* gene have been reported (Viskochil et al. 1990; Kehrer-Sawatzki et al. 1997). In both cases, the 22q11 breakpoints were found to reside within a palindromic AT-rich repeat (PATRR), while the chromosome 17q11 breakpoints lay close to the centre of a 195-bp PATRR within intron 31 of the *NF1* gene (Kehrer-Sawatzki et al. 1997; Kurahashi et al. 2003). These findings are consistent with a palindrome-mediated mechanism for the generation of the recurring t(17;22) translocations.

A (17)(q11.2q25.1) inversion that disrupted the *NF1* gene has also been reported (Asamoah et al. 1995).

10.12 Genotype–Phenotype Relationships

It is clear that the identification of a specific inherited *NF1* gene mutation only rarely permits the prediction of the likely severity or outcome of the disease in the affected individual. For many heritable conditions, the series of events and processes that separate in time and space a specific change in DNA sequence from the eventual expression of associated disease features is usually too complex to permit accurate correlation. In any attempt to interpret potential genotype–phenotype relationships, the combinatorial effects of many different mutations and polymorphisms, both allelic and nonallelic, need to be considered.

In the context of NF1, only two clear genotype–phenotype associations have so far been reported. Firstly, NF1 patients with gross deletions tend to exhibit a more severe clinical phenotype (Kayes et al. 1994; Upadhyaya et al. 1998; Spiegel et al. 2005; López-Correa et al. 2001; Mautner et al. 2010; Pasmant et al. 2010). Secondly, the absence of cutaneous neurofibromas in NF1 patients harbouring a constitutional 3-bp in-frame deletion in exon 17 of the *NF1* gene (c.2970–2972 delAAT) has also been reported (Upadhyaya et al. 2007); approximately 100 NF1 patients harbouring the 3-bp deletion but lacking cutaneous neurofibromas are now known (unpublished data).

In addition, Upadhyaya et al. (2009) studied 22 spinal neurofibromas derived from 14 unrelated NF1 patients. Seven of these patients satisfied the diagnostic criteria for NF1, whereas the remaining seven patients exhibited only a few clinical features of NF1. The latter group, defined as familial spinal neurofibromatosis, harboured a significantly higher number of constitutional missense and/or splice-site mutations than the group with classical NF1 (Kluwe et al. 2003).

A further potential genotype–phenotype relationship reported in the literature has been the preponderance of mutations noted in the 5′ third of the *NF1* gene in NF1 patients harbouring optic pathway gliomas (Sharif et al. 2011). Confirmation of the validity of these results may allow a more targeted approach to screening for optic pathway gliomas by allowing the early identification of those patients most likely to be at risk.

Several other, as yet largely unconfirmed, genotype–phenotype associations have been suggested, including some unusual NF1 patients recently identified with a 1.4-Mb duplication of the *NF1* gene region (Grisart et al. 2008). Two of these *NF1* duplication cases were apparently phenotypically normal. At present, it is difficult to comment on the phenotype associated with *NF1* duplications because of the omnipresent intra-familial variability and the fact that this *NF1* locus duplication has so far only been reported in a single family. Further studies are clearly required (Grisart et al. 2008).

In a recent study, a five-generation family with Noonan syndrome and NF1 was described in whom a germline *NF1* missense mutation was identified in exon 24 of the gene (Nyström et al. 2009). None of the affected members in this family had cutaneous neurofibromas. The authors suggested that, in future, Noonan-NF1 families should be considered for mutational screening of exon 17 for the c.2970–2972 delAAT deletion and exon 24 for this missense mutation.

Finally, a tentative genotype–phenotype correlation between non-truncating disease causing mutations in the *NF1* gene and NF1 patients with pulmonary stenosis has been identified (Ben-Sachar et al. 2012).

It is likely that the genotype–phenotype relationship in NF1 is influenced by a number of different factors including allele-specific differences in *NF1* gene expression (Jentarra et al. 2012), allele-specific differences in the methylation status of the *NF1* gene (Harder et al. 2010), alternative splicing (Barron and Lou 2012) and the action of unlinked modifier genes (Sabbagh et al. 2009; Pasmant et al. 2011).

10.13 Parental Origin of New Mutations

The new mutation rate for NF1 is one of the highest known for any human inherited disorder (Huson et al. 1989; Takano et al. 1992). Early studies revealed that ~90 % of sporadic *NF1* gene lesions tended to occur on paternally derived chromosomes (Jadayel et al. 1990; Stephens et al. 1992; Lázaro et al. 1996). However, such conclusions were drawn on the basis of the parental origin of mutation being

ascertained by linkage analysis rather than direct detection of the underlying *NF1* gene lesions. It subsequently turned out that there is a tendency for gross *NF1* gene deletions (identified simply by means of intragenic and flanking polymorphic markers) to originate on maternally derived chromosomes (Lázaro et al. 1996; Ainsworth et al. 1997; Upadhyaya et al. 1998). Thus, the origin of a given *NF1* gene lesion is highly likely to be influenced by its type, with gross deletions tending to originate during oogenesis and microlesions including single base-pair substitutions tending to occur during spermatogenesis.

10.14 Germline Mosaicism

Germline mosaicism (Zlotogora 1998) appears to be rare in NF1 since very few families have been reported with more than one affected child born to unaffected parents (Lázaro et al. 1994). Confirmation of germline mosaicism nevertheless requires molecular analysis of the individuals involved because instances of two or more nonidentical *NF1* gene lesions segregating (or occurring de novo) in the same family have been reported (Klose et al. 1999; Upadhyaya et al. 2003). In cases where the mosaicism has originated in the paternal germline, it has been estimated that between 10 % and 17 % of paternal sperm harbour the pathological *NF1* gene mutation (Lázaro et al. 1994, 1995; Bottillo et al. 2010).

The occurrence of classical NF1 in the daughter of a mother with segmental neurofibromatosis type 1 (SNF1), a type of NF1 characterised by the regionally limited distribution of NF1 features, suggested to Consoli et al. (2005) that cutaneous mosaicism might be accompanied by gonadal mosaicism (i.e. combined somatic and germline tissue mosaicism). These authors identified a nonsense mutation in exon 31 (R1947X) of the *NF1* gene in the lymphocyte DNA of the affected child. DNA sequence analysis failed, however, to identify this mutation either in peripheral lymphocytes or in keratinocytes and fibroblasts cultured from affected and unaffected skin in the mother. DNA fragments containing exon 31 of the *NF1* gene were then cloned from each maternal cell line, and these clones were screened using allele-specific PCR. The R1947X mutation was identified in 20 % of clones derived from keratinocytes and in 8.8 % clones derived from fibroblasts from the affected region, but not in clones derived from clinically unaffected tissues. These findings indicate that gonosomal mosaicism can occur in SNF1, with consequent important implications for genetic counselling.

10.15 Copy-Number Variation

Wong et al. (2007) recently claimed to have identified copy-number gains involving the *NF1* gene in 5 individuals and a deletion in one individual out of 95 human genomic DNAs investigated (i.e. 6 CNVs per 190 chromosomes). These *NF1* CNVs

were originally detected on the basis of aberrant fluorescence intensity ratios for BAC RP11-518B17, which spans the distal part of the *NF1* gene. However, Khaja et al. (2006) also noted these CNVs within the *NF1* gene region by means of direct alignment of the human genomic reference sequence with that of the Celera assembly. Since CNVs have been suggested to trigger nonallelic homologous recombination (NAHR) in regions characterised by genomic disorders, Steinmann et al. (2008) wondered whether CNVs within the *NF1* gene region could have facilitated the formation of the gross *NF1* deletions. To this end, these authors investigated whether CNVs within the *NF1* gene region occur at an increased frequency in the transmitting parents of patients with type 1 deletions, the most common type of gross *NF1* deletion. However, a negative finding appeared to exclude such variants from being frequent mediators of NAHR giving rise to type 1 deletions. Moreover, using multiplex ligation-dependent probe amplification (MLPA), Steinmann et al. (2008) were unable to confirm CNVs at the *NF1* locus in 167 chromosomes from healthy controls.

10.16 Molecular Diagnosis of NF1

The initial diagnosis of most NF1 patients is usually made on a clinical basis following careful medical examination by an expert physician specialised in identifying neurofibromatosis. The identification of an *NF1* mutation may direct overall clinical assessment of the patient through understanding of its impact upon the *NF1* gene (Messiaen and Wimmer 2008; Griffiths et al. 2007). Indeed, early diagnosis of the disease is essential to allow such patients and their families to be appropriately counselled and for the affected child to be regularly monitored for any complications, such as learning difficulties, development of optic gliomas and hypertension. It is, however, worth noting that the demand for NF1 prenatal diagnosis has been limited, probably because identification of the causative *NF1* mutation is often of little help in predicting either the severity or the progress of the disease in any future child. There is still, however, a need to develop a more rapid, accurate and cost-effective DNA-based test for NF1. Current NF1 routine molecular diagnostic testing of the NF1 gene takes on average 3–4 weeks. Fortunately, testing the much smaller *SPRED1* gene (Brems et al. 2007) takes only 2–3 days. For most average-sized genes, molecular results can be generated within a week. In the UK, the cost of the NF1 test performed on genomic DNA can range from £700 to £1,000+, whereas an RNA-based test is approximately £600. It is a costly test because it requires the screening of 57 exons in genomic DNA. When this test is fully automated, the cost should come down, hopefully accompanied by an increase in sensitivity and specificity. The reporting time for results should also be significantly reduced.

A suitable preimplantation genetic diagnosis (PGD) protocol for NF1 has also been developed (Spits et al. 2005). PGD is an alternative to prenatal diagnosis that circumvents therapeutic abortion. Diagnosis is carried out on single cells obtained from 3-day-old embryos; only those free of the disease in question are implanted into the mother.

10.17 Conclusions

As mentioned above, the new mutation rate for NF1 is one of the highest known for any human inherited disorder (Huson et al. 1989; Takano et al. 1992). The challenge facing us is to try to account for this high new mutation rate in terms of what we know about the germline mutational spectrum of the *NF1* gene. It is thought that a number of different features of a gene could serve to influence its mutation frequency, for example, chromosomal location, length of coding sequence, number and length of introns, nucleotide composition and sequence repetitivity both within and flanking the gene.

The *NF1* mutational spectrum is still far from complete; indeed, screens for *NF1* gene lesions routinely identify no more than about 80–95 % of pathological mutations assumed to be present in NF1 patient samples (Fahsold et al. 2000; Ars et al. 2000; Messiaen et al. 2000; Mattocks et al. 2004; De Luca et al. 2007). The reason for this is unclear. It is likely that a sizeable proportion of the missing lesions reside in far upstream or downstream regulatory elements or alternatively deep within the introns. While the latter could in principle be detectable using RNA-based mutation screening methods, the former could pose a formidable challenge in terms of their identification. Irrespective of the location(s) of the missing pathological mutations, inspection of the germline mutational spectrum of the *NF1* gene allows us to conclude that, in common with many human genes (Cooper et al. 2011), the nature, location and frequency of the different types of *NF1* gene mutation underlying NF1 are shaped in large part, and often in remarkably predictable ways, by the local DNA sequence environment.

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Chapter 11
Splicing Mechanisms and Mutations in the *NF1* Gene

Marco Baralle and Diana Baralle

11.1 Introduction

Pre-messenger RNA (pre-mRNA) splicing is the process whereby non-coding segments (introns) are removed from primary transcripts within the nucleus, with concomitant joining of the coding segments (exons) for export to the cytoplasm. In the specific case of the NF1, whose messenger RNA (mRNA) is approximately 11–13 kb in length, the most common of which encodes for neurofibromin, a 2818 amino acid polypeptide, this means identifying 60 exons (57 constitutive and three alternatively spliced) that make up the *NF1* mRNA spread over a region of approximately 350 kb. The splicing machinery not only has to recognise the exons but also join them together correctly.

The importance of understanding the splicing process is emphasised by the fact that mutations resulting in aberrant pre-mRNA splicing defects are at the root of a significant fraction of human genetic disorders, being possibly the most frequent cause of hereditary disorders (Tazi et al. 2009; Wang et al. 2008). The *NF1* gene with one of highest mutation rates known for human genes, with 50 % of all NF1 patient cases being classified as sporadic, has emerged as a much quoted example of the frequency of splicing mutations associated with disease, with estimates of how often this occurs ranging from 20% to 50% (Ars et al. 2000b; Valero et al. 2011). Indeed, within the Human Gene Mutation Database (HGMD; http://www.hgmd.org), 286 out of 1,300 mutations listed for the *NF1* gene (22 %) are identified as splicing mutations. This number is more than likely to be an underestimate, as many

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of the missense, nonsense, and possibly even silent sequence variants may also affect the splicing process (Baralle et al. 2006).

11.2 A Brief Overview of Pre-mRNA Splicing

Intron removal is carried out by the spliceosome, a dynamic multi-subunit particle containing five small ribonuclear proteins snRNPs, U1, U2, U4, U5, and U6, that function in conjunction with over 200 distinct non-snRNP auxiliary proteins (Nilsen 2003; Wahl et al. 2009). To dictate splicing, each intron is marked by a GU dinucleotide at the 5′ end and an AG dinucleotide at the 3′ end, although exceptions exist (Zhang 1998). These nearly 100 % conserved dinucleotides at either end of the intron, in reality, form part of more loosely defined sequences known respectively as the 5′ and 3′ splices sites (Zhang 1998). The 5′ splice site is a degenerate 8-nucleotide motif with a consensus motif MAG/GURAGU (where M is A or C and R is A or G) and the holo-3′ splice site which is even more loosely defined being composed of three elements: the 3′ splice site, that has a consensus CAG among which again only dinucleotide AG is universally conserved, a branch point (BP) consensus in which an A nucleotide is universally conserved, and a polypyrimidine tract which is usually spread across the last 40 nucleotides of the intron but can take up considerably more space (Fig. 11.1). Initial recognition of the exon/intron junction is based on direct interactions between the U1 snRNP and the 5′ splice site, the U2 auxiliary factor (U2AF65/35) with the polypyrimidine tract, and U2 snRNP with the branch point sequence (Fig. 11.1). These factors, together with additional proteins, form a first discrete functional spliceosome complex called the E or commitment complex, which bridges the intron and brings the splice sites that are to be cleaved together. Subsequently, a further series of rearrangements and protein complexes occur that are beyond the scope of this chapter and described abundantly in the literature (Will and Luhrmann 2011), resulting in two consecutive transesterification reactions (Lamond 1993) (Fig. 11.1). During step I, the adenosine residue of the branch point sequence (BPS) carries out a nucleophilic attack on the 5′ splice site. This reaction generates the splicing intermediates (free exon 1 and lariat-exon 2). During step II, exon 1 attacks the 3′ splice site to generate splicing products (spliced exon and lariat intron).

Aside from the consecutive recognition and joining of exons, the spliceosome also carries out alternative splicing, a process where spliceosome assembly is altered so that a splice site is optionally used in a tissue-specific and developmental manner resulting in the inclusion or exclusion of alternative exon sequences in the mature mRNA. In addition to cell type-, developmental stage-, and sex-specific regulation, alternative splicing can also be dynamically regulated in response to extracellular stimuli such as cytokines, hormones, or neurotransmitters, adding a further dimension to the control of the flow of genetic information. However, it is not clear in many cases how alternative splicing is controlled by these external stimuli and intracellular signalling pathways (Blaustein et al. 2007).

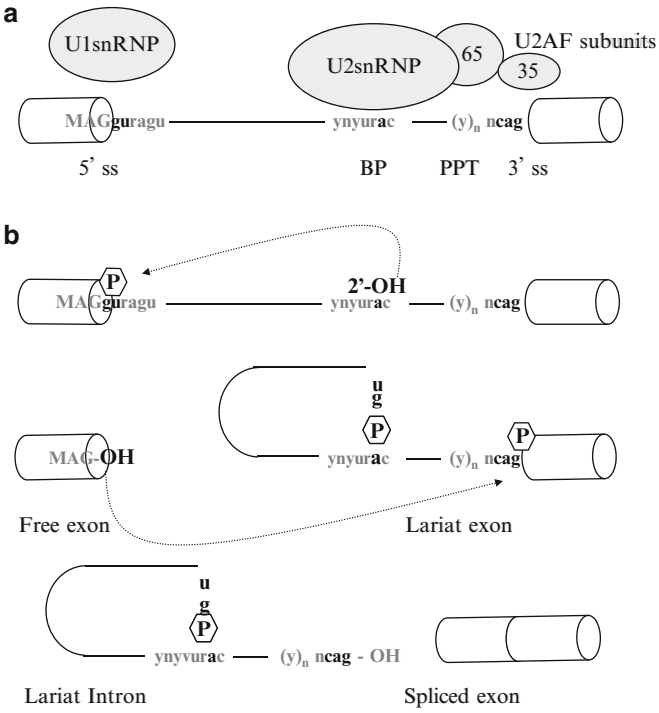

Fig. 11.1 (**a**) Schematic representation of conserved motifs surrounding the exon (*cylinders*) and intron (*black line*) borders: 5′ splice site, branch point, polypyrimidine tract, and 3′ splice site, together with the proteins and the snRNA that recognise them which when bound make up the E complex of the spliceosome. Only the GU and AG dinucleotides, respectively, at the exon–intron and intron–exon junctions and an A residue in the branch point in the motifs are universally conserved (highlighted in *black*). (**b**) Splicing reaction: Two consecutive transesterification reactions occur. During step I, an adenosine residue generally located within the branch point carries out a nucleophilic attack on the 5′ splice site. This reaction generates the splicing intermediates (free exon 1 and lariat-exon 2). During step II, exon 1 carries out a nucleophilic attack on the 3′ splice site to generate splicing products (spliced exon and lariat intron)

The consequence of reconnecting the exons of the RNA in a number of different ways means that several different transcripts can arise from a single gene, each with a potentially distinct function/property. These include altered mRNA stability or subcellular localisation and the addition or deletion of specific protein coding sequences. Functional differences among protein isoforms range from subtle modulations to on/off switches or even antagonistic effects. Typical types of alternative splicing are the inclusion or skipping of one or more exons (cassette exons), shortening or lengthening of an exon by alternative 5′ and 3′ splice sites, mutual exclusion of two or more exons, and retained introns (Matlin et al. 2005). Different promoters and different polyadenylation sites may specify alternative 5′ and 3′ terminal exons, respectively (Licatalosi and Darnell 2010) (Fig. 11.2).

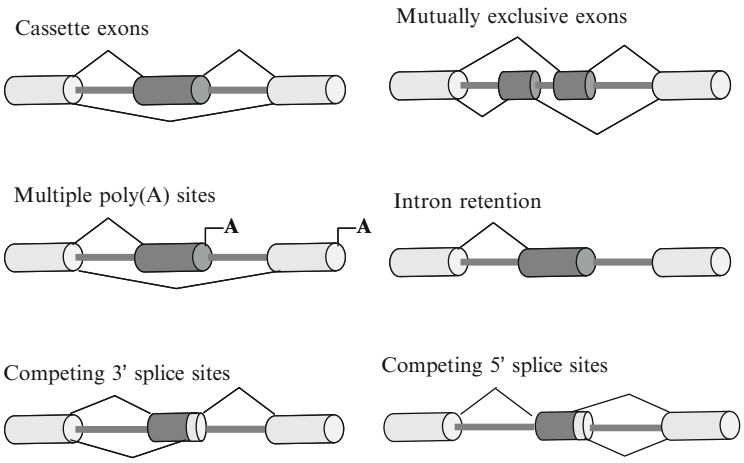

Fig. 11.2 Basic alternative splicing events. The possible events of the alternative exon (represented in *darker shade*) can be followed by following the *diagonal lines*. Cassette exons can be included or excluded from the mRNA. Mutually exclusive splicing involves the selection of only one from of two or more exons. Multiple Poly(A) sites represent alternative 3′ end processing. Intron retention is a form of alternative splicing in which the intron is retained in the mRNA. Competing 3′ or 5′ splice sites represent alternative splicing events where the size of the exon is modified. Variations on these themes can also occur, leading to more complex forms of alternative splicing

As previously mentioned, the *cis*-acting elements that the spliceosome recognises (5′ and 3′ splice sites) are highly degenerate, resulting in the paradox that such an important and precise process as splicing, be it consecutive or alternative, is dictated by redundant sequences (Zhang 1998). One of the more important realisations over the past decade however is that although necessary, these consensus elements are by no means sufficient to define exon/intron borders (Buratti et al. 2006). The correct recognition of the 5′ splice site and 3′ splice site by the spliceosome has to be aided by other *cis*-acting elements. This is especially important if one considers that within a gene, due to the high degeneracy of splice sites (ss), there are hundreds of pseudo-splice sites that are close to or even resemble better the consensus sequence than authentic splice sites (Sun and Chasin 2000). In addition, numerous auxiliary splicing *cis*-acting regulatory elements are now known to exist that can either enhance or repress splicing.

Originally discovered in alternatively spliced exons (Mardon et al. 1987), exon splice enhancers (ESEs) are today also known to be common components of constitutively spliced exons necessary to stimulate exon splicing (Pagani and Baralle 2004). ESEs assist early spliceosomal complex formation by interacting with components of the splicing machinery that make up the previously described E complex. This is done generally, but not universally, through the serine/arginine-rich proteins (SR proteins) that assemble on ESE elements to promote both regulated and constitutive splicing by forming networks of interaction with each other (Blencowe 2000). The SR proteins have a common domain structure of one or

two RNA-binding domains followed by an RS domain containing repeated arginine/serine dipeptides, which can be highly phosphorylated. This phosphorylation modulates protein–protein interaction that serves as a bridge between the 5′ and 3′ splice sites across the introns and across the exons and/or between enhancers and adjacent splice site, within the spliceosome (Long and Caceres 2009). Intronic splice enhancer elements (ISE) also exist and function in much the same way; for example, they can enhance 3′ splice site recognition by recruiting U2AF to the upstream polypyrimidine tract and 5′ splice site recognition by recruiting U1 snRNA to the 5′ splice site (Izquierdo et al. 2005).

While the positive regulation of splicing is thought to occur as a result of protein–protein interactions strengthening the recognition of the splice site, the negative regulation of splice site choice often results from the prevention of recognition of the splice site. These negative regulatory sequences can also be found, either in exonic or intronic sequences, and are called respectively exonic splicing silencers (ESS) and intronic splicing silencers (ISS). In the majority of cases, they are bound by members of the hnRNP family proteins. hnRNPs constitute a large group of molecules identified by their association with pre-mRNA or hnRNAs (heterogeneous nuclear RNAs). At least 20 hnRNPs have been identified and are designated hnRNP A1 to hnRNP U (Dreyfuss et al. 2002). Although much remains to be learned about how these function, they can be thought of as generally repressing splicing through steric hindrance. For example, in the case of hnRNP A1, the most studied of these negative splicing regulatory proteins, positional overlap with an ESE (Cartegni et al. 2006) is thought to block the function of the ESE in SMN2 exon 7. A similar mode of action for hnRNPA1 has also been postulated in the case of an ISS where positional overlap between the ISS element and the branch point occurs, blocking U2 snRNP binding (Tange et al. 2001).

In addition to these more common splicing factors, binding to ESE, ISE, ISS, and ESS, pre-mRNA splicing can also be controlled through an array of other splicing factors that have a more restricted and dynamic expression pattern. These can potentially play a greater role in tissue-specific or developmentally regulated splicing. The mechanisms and impact of these factors are much less characterised and are beyond the scope of this brief overview, but the reader should be aware that one could have involvement of factors such as Nova-1/2, PTB/nPTB, Fox-1/2, muscleblind-like (MBNL), and CELF family proteins, Hu proteins, TIA1/TIAR, and probably many more factors yet to be characterised (David and Manley 2008; Li et al. 2007).

The processing of the constitutive exons in the *NF1* pre-mRNA transcript makes use of all of the types of splicing regulatory elements mentioned above. However, only some of the auxiliary *cis*-acting elements (ESE, ESS, ISE, and ISS) present in the *NF1* gene are known to date as these are generally only discovered when a mutation in one of these occurs in a patient resulting in aberrant splicing and consequently neurofibromatosis type 1. Even then, they generally tend not to be fully characterised at the molecular level as this can be beyond the scope of diagnostic laboratories. However, a few significant cases have been studied in detail and have provided interesting insights into the splicing process. These are discussed below in Sect. 11.4.

Table 11.1 Principal *NF1* alternative transcripts studied in depth to date from Ars et al. (2000a), Messiaen et al. (1999), Park et al. (1998), and Vandenbroucke et al. (2002a, b)

Exon	Notes
9br	In-frame insertion, present CNS
23a	In-frame insertion, predominantly neurons
48a	In-frame insertion, cardiac and skeletal muscle
23a48a	Alternative exon present in adrenal glands, kidney
7	In-frame deletion
43	In-frame deletion
45	Out-of-frame deletion
29	Out-of-frame deletion
30	Out-of-frame deletion
29/30	Out-of-frame deletion
11 most of 49 (N-isoform)	Out-of-frame deletion in brain
4b	Out-of-frame deletion
37	In-frame deletion

11.3 NF1 Alternative Splicing

Estimates of how commonly alternative splicing occurs in human protein coding genes have increased over the years, from an initial 5 % to being nearly ubiquitous (Wang et al. 2008), and the *NF1* gene is no exception to the rule (Table 11.1). NF1 can be thought to have three classically alternative exons that do not alter the reading frame: 9a/9br (Danglot et al. 1995), 23a (Nishi et al. 1991), and 48a (Cawthon et al. 1990). Alternative exon 9a/9br inclusion is restricted to the central nervous system, adding ten codons to the *NF1* transcript (Danglot et al. 1995). Alternative exon 48a adds 18 codons to the *NF1* transcript, and its inclusion is highest in cardiac and skeletal muscle tissues (Geist and Gutmann 1996). Although these isoforms have also been detected at the protein level, the precise difference in NF1 function resulting from the inclusion of either exon in the *NF1* transcript has yet to be fully determined; although in the case of exon 48a, a role in the development and differentiation of heart and skeletal muscles is probable (Gutmann et al. 1995). Alternative splicing of *NF1* exon 23a that adds 21 amino acids to the protein is perhaps the more interesting of the three due to its location within the GRD and the observation that the two protein isoforms: NF1 type I if the exon is skipped and NF1 type II if the exon is included, differ tenfold in their capacity to downregulate Ras activity with the type I isoform (exon 23a is skipped), expressed predominantly in neurons being the more active of the two in this regard (Andersen et al. 1993).

Today, the picture of alternative splicing of NF1 is much more complex and includes several other alternative splicing events (Table 11.1), among which is an isoform termed the N-isoform that excludes exon 11 and most of 49, a potentially regulatory pseudoexon inclusion event between exons 4a/4b, and the use of alternative splice sites in exons 12b and 43 (Thomson and Wallace 2002; Vandenbroucke et al. 2001, 2002a, b). However, the significance of the majority of these still needs to be characterised as does their expression at the protein level.

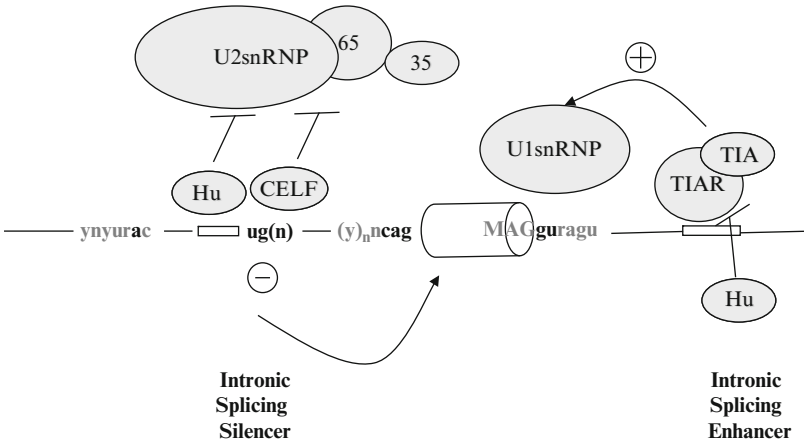

Fig. 11.3 Schematic representation of mechanism behind alternative splicing of exon 23a, represented by *cylinder*. *Black straight lines* are the introns and *small rectangle squares* in introns are binding sites for splicing factors represented in *circles*. Binding of TIAR and TIA aids 5′ splice site definition by helping binding of U1snRNA to the 5′ splice site. Hu proteins may inhibit binding of these to the intronic splicing enhancer. CELF and Hu proteins binding to intronic splicing silencers upstream of the exon inhibit 3′ splice site definition by blocking binding of U2AF65

The mechanisms that control the tissue- and developmental-specific regulation of these alternative exons are mostly unknown, with the only exception being that of 23a (Barron and Lou 2012; Barron et al. 2010; Zhu et al. 2008). This represents a perfect example of combinatorial control of alternative splicing where several different regulatory elements and associated factors provide the cell type-specific regulation of the alternative exon (Fig. 11.3). An ISE composed of a UG-rich sequence downstream of *NF1* exon 23a promotes its inclusion through the binding of TIA-1 and TIAR proteins that aid the binding of the U1 snRNA. Two families of proteins (Hu and CELF proteins) act as negative regulators of the exon. Hu proteins bind to U and AU-rich sequences flanking both sides of *NF1* exon 23a, competing for the binding sites of the TIA-1 and TIAR proteins and blocking the binding of the spliceosome components U1 and U6 to the 5′ splice site and U2AF65 at the 3′ splice site. CELF proteins promote exon 23a skipping by binding to UG-rich elements upstream of *NF1* exon 23a where they also compete for U2AF65 binding. The splicing outcome is therefore dependent upon which factors are more abundant in a particular cell type. TIA-1 and TIAR are generally considered to be widely expressed, whereas the Hu family of proteins are composed of four members, three of which are brain specific (Okano and Darnell 1997), and the CELF family of proteins are composed of six members, three of which are brain-specific and two thought to be enriched in the brain (Dasgupta and Ladd 2012). The higher levels of negative-regulating splicing factors in the brain result in the splicing outcome of *NF1* exon 23a exclusion noted for this tissue.

11.4 Aberrant mRNA Splicing and Neurofibromatosis Type 1

As mentioned above, the *NF1* gene is subject to one of highest mutation rates known for any human gene. Although some of these mutations may not necessarily affect protein function, due to the fact that the pre-mRNA is embedded with splicing regulatory elements responsible for the correct control of splicing, these mutations may instead affect splicing function if they occur within a splicing regulatory element and therefore give rise to one of several types of aberrant splicing of the *NF1* transcript and subsequent pathology. Identification of this class of mutation has been a focal point in clinical diagnostics.

The most common form of splicing mutation that affects the correct processivity of the mRNA occurs within the canonical splicing signals, disrupting the splice donor and acceptor sites. Using the freely accessible section of the Human Gene Mutation Database (HGMD; http://www.hgmd.org) in which 222 splicing mutations of the *NF1* gene are reported, approximately 78 % of these affect splice sites themselves. When these mutations occur within the 100 % conserved dinucleotides at the 5′ and 3′ splices, as is the case of approximately 81 % of splice site mutations found to affect the *NF1* splice sites, the fact that these will result in aberrant splicing is a forgone conclusion. However, it can be difficult to be certain of the type of aberrant splicing that arises, either exon skipping, intron retention, cryptic splice site utilisation with associated inclusion of a cryptic exon, or indeed alternative splicing, although fairly accurate predictions may be made attempted. However, a significant number of sequence variants also fall into the more loosely defined regions of the splice sites, and predicting if these mutations result in aberrant splicing or not represents a challenge. Due to the widespread nature of these mutations, bioinformatic programmes have been developed that try to predict the potential effect of nucleotide variations on splicing. Examples are MaxEntScan (Yeo and Burge 2004), NNsplice (Reese et al. 1997), AST (Carmel et al. 2004), Spliceport (Dogan et al. 2007), Spliceview (Rogozin and Milanesi 1997), HBond (Freund et al. 2003), ASSA (Nalla and Rogan 2005), NetGene2 (Hebsgaard et al. 1996), and Human Splicing Finder (Hubbard et al. 2007). These in silico methods are based on nucleotide frequency matrices, neural networks, hydrogen bond formation, and interdependencies between nucleotides at different positions of the consensus. Obviously, being in silico predictions, care needs to be taken when evaluating the effect of a nucleotide variation as a degree of uncertainty exists. For example, the intronic +5 G>C substitution in exon 3 of the *NF1* gene was suspected to be responsible for neurofibromatosis in a family (Baralle et al. 2003). This nucleotide change decreased the splice site strength significantly but was still higher than other splice sites that are naturally used, for example, those in *NF1* exon 34 (using NNSplice the mutation in *NF1* exon 3 decreased the score for this splice site from 1 to 8.3 and the score for the splice site *NF1* exon 34 is 0.41). The use of several in silico programmes however indicates strongly that this nucleotide substitution is pathogenic and also highlights the utility of using as many prediction programmes as possible when evaluating mutations for a possible role in aberrant

splicing (MAXENT, e.g. predicts a decrease in score for the mutation in the exon 37 splice site from 9.65 to 4.99 and a score of 7.13 for the *NF1* exon 34 5′ splice site). Indeed, using a minigene system (Baralle and Baralle 2005), this was shown to be the pathogenic. Furthermore, the experiment showed that the effect of the +5 G>C mutation was context-specific as it depended upon the presence of a G run at the 5′ splice site, disruption of which resulted in correct MRNA processing even in the presence of the mutation. The functional reason for this was that the hnRNP H protein was shown to bind to this 5′ splice site at the poly-G sequence, thereby competing with the U1 base pairing (Buratti et al. 2004). This example highlights that different RNA fragments encompassing 5′ss are bound by common as well as distinct proteins, and it is the combinatorial effect of these that determines whether the 5′ss is recognised. Notwithstanding that the role of 5′ss is the same for all exons (recognition by U1snRNA), the different combinations of proteins binding to it mean that a nucleotide variation that affects the recognition of the splice site in one exon's 5′ss may be harmless in another, emphasising that each 5′ss should be assessed in their own sequence context.

Another study that also provides further evidence of the need to consider each potential splice affecting mutation in the *NF1* gene carefully is that assessing *NF1* exon 29 (Raponi et al. 2009). As mentioned, 5′ splice site strength predictions can rely on complementarity to U1snRNP; in this study, a reduced need of U1snRNP presence for the correct recognition of the exon 29 donor site was observed. Mutations within the 5′ splice site need not only result in complete or partial exon skipping of the exon in which they are found but may also result in other forms of aberrant splicing such as cryptic exon inclusion due to the use of pseudo or cryptic splice sites as is the case with the *NF1* c.7675+1G>A mutation (Wimmer et al. 2007). Through an analysis of several 5′ splice site mutations, this study also suggests that this outcome is more likely in the presence of a good potential cryptic 5′ splice site close to the authentic 5′ splice site (which could be searched for using the in silico tools mentioned above) combined with a strong 3′ splice site of the exon.

In cases in which canonical splicing sequences are not affected and aberrant splicing occurs, these sequence variants are more than likely to affect an ESE. In the HGMD, these represent approximately 14 % of *NF1* splicing mutations. A lot of work has gone into bioinformatic prediction of the *cis*-acting elements involved in splicing and in particular of ESEs in order to be able to identify if a nucleotide variation would disrupt one of these or not and thus represent a pathogenic mutation. These prediction programmes are developed on either functional consensus motifs for SR proteins identified through a functional SELEX (systemic evolution of ligands by exponential enrichment) method "ESE-finder" (Cartegni et al. 2003), on the statistical analysis of differences in hexamer frequencies between exons and introns and between exons with weak and strong splice sites based on the presumption that the ESE will be more common in exons than in introns and are more common in exons with weak splices sites "ESE rescue"

(Fairbrother et al. 2002), or on consensus sequences derived from known regulatory elements and corresponding *trans*-acting factors (Paz et al. 2010). Recently, integrated interfaces such as Sroogle have been developed to analyse several of these programme outputs together (Schwartz et al. 2009). Although extremely useful as a starting point to investigate the splicing mechanism and the analysis of mutations, the presence of a high-score motif within a sequence does not necessarily identify that sequence as a splicing *cis*-acting element in its native context nor does disruption of the sequence necessarily result in aberrant splicing. This has been demonstrated in the *NF1* gene where disruption of high-score motifs by nonsense, missense, synonymous, and intronic variations analysed using a minigene clearly altered exon 29 inclusion/exclusion levels but did not necessarily result in aberrant splicing with bioinformatic programmes 29 (Raponi et al. 2009). The reality is that the outcome of splicing is dependent on a fine "balance of power" that is often present between all the various regulatory elements that define exon boundaries (Buratti et al. 2006).

As mentioned, only a few ESEs have been completely characterised in the NF1 gene, particularly with regard to their mode of action, but the presence of many more is highlighted by exonic mutations in the *NF1* gene known to result in exon skipping. A further example exists within *NF1* exon 7, where the presence of an alternative transcript has being demonstrated (Vandenbroucke et al. 2002b). A synonymous mutation (c.945G>A/Q315Q) predicted by ESE-finder [http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home] to disrupt an ESE has been shown to result in more than 70 % exon skipping, highlighting the importance of analysing even silent variants for their effect on splicing (Bottillo et al. 2007). Indeed, within the same study a second silent mutation (c.1005T>C/N335N) was observed to actually increase the inclusion of this exon in a minigene system, emphasising how each nucleotide change within the *NF1* coding sequence should be investigated for their potential effect on splicing. Although the clinical significance of the levels of exon 7 inclusion or exclusion is still unknown, minor changes in the levels of alternative exons may ultimately result in pathology.

An example of a more fully characterised ESE in the *NF1* gene comes from the investigation into disease mechanisms caused by the mutations c.6792C>G (p.Tyr2264X) and c.6792C>A (p.Tyr2264X) that were previously thought to result in premature termination codons (PTCs), inducing skipping of *NF1* exon 37 but preserving the open reading frame. However, mutations from other patients located in the same region of the gene, a c.6790_6791TTins and a c.6789_6792TTACdel, both of which create PTCs a few base pairs further downstream, did not result in skipping of this exon. Through the use of minigene analysis, the pathological mutations c.6792C>G and c.6792C>A were actually found to disrupt an A-/C-rich splicing regulatory element found to be important not only for the definition of *NF1* exon 37 but also for the inclusion of the neighbouring *NF1* exon 36 (Baralle et al. 2006). RNA–protein complex studies subsequently showed that the pathological mutation c.6792C>G mutation concurrently decreases the affinity for positive

splicing factors like YB-1, thereby disrupting an ESE and increasing the affinity for negative splicing factors like hnRNPA1, hnRNPA2, and DAZAP1, creating an ESS (Skoko et al. 2008). The inherent necessity of an ESE in this exon was to offset a non-consensus 3′ splice site at *NF1* exon 37. We know this because experiments mutating the exon 37 3′ splice site, to conform to the consensus, eliminate the need for this ESE, with minigenes resulting in full inclusion of the exon even in the presence of these previously pathological mutations.

Intronic mutations that fall outside the splice sites are the third most common type of splicing mutation in NF1, with approximately 5 % of splicing mutations in the HGMD falling into this category. These, as was observed to be the case with the deep intronic c.31-279A>G variation, can create a splice site. In this particular case, the use of this 3′ splice site is associated with the activation of a pseudo 5′ splice site already present in the *NF1* transcript resulting in the exonization of a fragment of intronic sequence (Raponi et al. 2006). An alternative scenario is that of the creation of a cryptic splice site, as occurs with the 3214 del 111 mutation which results in the creation of a cryptic 5′ splice site 104 nt upstream of the authentic one (Ars et al. 2000b).

11.5 Splicing and Phenotypic Variability

NF1 shows great clinical variability between individuals with regard to tumour burden and the severity of disease, even within family members with the same mutation. The mechanisms of splicing and alternative splicing are so tightly controlled that even subtle defects in alternative splicing factors or aberrant inclusion of alternative exons can result in phenotypic differences (Pagani and Baralle 2004). It has been hypothesised that variation in the population in terms of the different aberrant transcripts among patients bearing the same *NF1* splice mutation could be one of the factors partly responsible for the phenotypic variation observed in the disease (Ars et al. 2000b). However, the exploration of genotype–phenotype correlations in this regard has yet to yield conclusive results (Pros et al. 2006). The extent of aberrant splicing due to a mutation in a *cis*-acting regulatory element is often only partial and may vary within tissues and between individuals owing to a variety of factors such as differences in that individual's cells in terms of splicing regulatory proteins as well as differences between tissues within the same individual. For example, it is possible that splicing could play a role in the neurological phenotype of severe spinal neurofibromas (Raponi et al. 2006). In this study, the splicing defect observed involved an intronic regulatory element binding PTB and nPTB, the latter being restricted to neurons. One could hypothesise that differences in the levels of this splicing factor could cause a more severe spinal phenotype in those patients with this mutation.

11.6 Splicing-Based Therapeutic Approaches

Dysregulation of pre-mRNA splicing and alternative splicing is associated with the molecular pathology of a large number of human diseases. Consequently, a great deal of effort has been invested into eventual therapeutic strategies targeting pre-mRNA as splicing therapies, for recent reviews see (Douglas and Wood 2011; Kole et al. 2012). The approaches used depend on the targeted mode of modulation of pre-mRNA splicing required as well as the type of mutation present. One of the more promising therapeutic strategies is the use of antisense oligonucleotides to skip specific mutated exons during pre-messenger RNA splicing such that a functional protein is produced. This has been particularly successful in Duchene muscular dystrophy (Wood et al. 2010). Other methods attempt to include specific exonic sequences as in the case of SMN, to block the inclusion of pseudoexons and to modulate the generation of alternatively spliced protein isoforms (Kole et al. 2012).

Unfortunately, in the case of NF1, with no mutational hotspot, the large spectrum of splicing mutations hampers the development of a treatment. At the present time, developing a therapy specifically for splicing mutations represents too large an investment, bearing in mind that regulatory requirements indicate that each therapy would need to be developed separately for each different mutation. Furthermore, the number of patients carrying a specific mutation is too small for the large trials required. This notwithstanding, studies show that the possibility of correcting defective NF1 exists. Ideal candidate mutations for therapeutic correction lie outside the splice sites, not located in the coding region, and the possibility of therapeutic correction with antisense morpholino oligos (AMO) has been demonstrated for three of these intronic variants (c.288+2025T>G, c.5749 +332A>G, and c.7908-321C>G), each of which was observed to create a cryptic 5′ splice site and result in the inclusion of a cryptic exon (Pros et al. 2009). The AMO were designed to sterically block the recognition of the newly created 5′ splice sites by the splicing machinery thereby forcing the use of the correct splice sites and preventing the inclusion of the cryptic exon.

Another method of restoring normal transcript splicing in the presence of splicing mutations is the use of chemical substances that affect the splicing process in a variety of ways; principally, these substances act by either blocking histone deacetylases or by interfering with the phosphorylation of splicing factors (Sumanasekera et al. 2008). However, owing to the general effect these drugs have, care has to be taken with regard to their effect on splicing of all transcripts, not just NF1. In the specific case of NF1, kinetin and puromycin were observed to be able to partially correct aberrant splicing caused by 4 *NF1* splicing mutations out of 19 tested in a dose-dependent fashion (c.910C>T, c.3113G>C, c.6724C>T and c.6791dupA) (Pros et al. 2009). In this study, the mutations were nonsense, missense, and frameshift mutations. Hence, correction of aberrant splicing would still then produce aberrant transcripts and would therefore not have a therapeutic effect except possibly in the case of missense mutations. However, the approach could be of therapeutic interest when skipping reversion is produced by intronic mutations, or eventually for exonic silent mutations.

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Chapter 12
NF1 Germline and Somatic Mosaicism

Ludwine Messiaen and Jing Xie

12.1 Introduction

Neurofibromatosis type 1 (NF1, MIM 162200) is a common autosomal dominant genetic disorder affecting ~1/3,000 individuals worldwide (Huson et al. 1989). NF1 is a progressive disorder, with more symptoms typically developing with time. NF1 shows complete penetrance, although the disease presents with a high degree of phenotypic variability even within families and within monozygotic twins carrying the same mutation (Rieley et al. 2011). Patients present with multiple café-au-lait macules (CALM), skinfold freckles, iris Lisch nodules, and neurofibromas. Neurofibromas can be subdivided into dermal neurofibromas, benign tumors that increase in number and size with age but do not undergo malignant transformation, and congenital plexiform neurofibromas, tumors involving multiple fascicles which in ~5 % of cases may progress to malignant peripheral nerve sheath tumors (MPNSTs) (Korf 1999). Optic pathway gliomas and specific skeletal abnormalities of the sphenoid wing, long bones, and vertebrae are also typical clinical signs associated with NF1. In addition, macrocephaly, short stature, learning disabilities, and attention difficulties are frequently observed in NF1 patients. NF1 patients also have an increased risk of developing specific malignancies (reviewed by Brems et al. 2009).

NF1 is due to mutations in the 282-kb-long *NF1* gene located on 17q11.2, comprising 57 constitutive and at least three alternatively spliced exons. The 8,454 nucleotides of the open reading frame of the *NF1* transcript encode neurofibromin, which negatively regulates Ras-GTPases (Ballester et al. 1990; Xu et al. 1990). *NF1* functions as a tumor suppressor, and NF1-associated neoplasias, such as neurofibromas, gastrointestinal stromal tumors, glomus tumors,

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juvenile myelomonocytic leukemia, astrocytomas, and pheochromocytomas, have been shown to carry 2 mutant *NF1* copies (reviewed by Brems et al. 2009). *NF1* inactivation of both alleles has been documented in dermal as well as in plexiform neurofibromas, but additional genetic changes, including complex karyotypes, have only been identified in MPNSTs.

In addition, somatic "second hit" *NF1* mutations have also been identified in tissues from tibial pseudarthrosis (Stevenson et al. 2006) and CALMs (De Schepper et al. 2008; Maertens et al. 2007).

Biallelic *NF1* inactivation follows the Knudson "two-hit hypothesis". According to this model of tumorigenesis, one mutant allele (the "first hit" mutation) is inherited from an affected parent (for familial cases) or from a mutant germ cell of an otherwise unaffected parent. Alternatively, the first hit mutation results from a de novo mutation in the fertilized egg or occurs in very early stages of the developing embryo. By contrast, the "second hit" mutation affecting the other (wild-type) allele is then specifically present only in those somatic cells which represent the tumor or CALM initiating cells.

12.2 Mosaicism

NF1 affects ~1/3,000 individuals irrespective of gender or ethnic background and as many as 30–50 % of these patients present as "sporadic" or "founder" patients, that is, have no affected parent from whom the disorder was inherited (Friedman 1999; Evans et al. 2010). It follows that the *NF1* gene has a new mutation rate of $3.7–26 \times 10^{-5}$, that is, 10–100-fold higher to that which is observed in most other known human disease-associated genes (reviewed by Poyhonen et al. 2000 and references therein). The cause of this unusually high mutation rate is still unknown, the large size and complexity of the gene being possibly only partially contributory. Zlotogora proposed in 1993 that a proportion of the sporadic or "founder" NF1 patients may have the disease as the consequence of a somatic mutation after fertilization and the first molecularly proven somatic mosaic NF1 patient was reported in 1996: a multi-exon deletion of at least 100 kb encompassing exons 4–39 was identified in a 31-year-old patient with seven CALMs, bilateral axillary freckling, and multiple small cutaneous neurofibromas (Colman et al. 1996).

Genetic mosaicism refers to a condition where two or more genetically distinct cell populations, derived from the same fertilized egg, coexist in the same individual. Mosaicism in genetic disorders with significant skin manifestations, such as neurofibromatosis type 1, will be more readily visible than in disorders mainly affecting, for example, internal organs. With respect to NF1, mosaicism as discussed here refers to the fact that (only) a subpopulation of the body cells in a patient carries a "first hit" *NF1* mutation.

The following types of mosaicism for a "first hit" *NF1* mutation may be encountered. *Gonadal or germline mosaicism* occurs when a fraction of the germ cells (sperm or ova) carry a mutation, which may then lead to more than one affected offspring from clinically normal parents. Pure gonadal mosaicism is likely

to be extremely rare in NF1, as only two such families have been reported to date (Lázaro et al. 1994; Bottillo et al. 2010). Mutations occurring early during embryonic development, before gastrulation and the formation and separation of the primordial germ cells, may lead to *gonosomal mosaicism* where somatic as well as germ cells can be mosaic. If mutations arise after the primordial germ cells were formed and set apart, patients may present solely with *somatic mosaicism*, implying that they would not be at risk of transmitting the disorder to the next generation. Ruggieri and Huson (2001) proposed the terms *mosaic-generalized* NF1 and *mosaic-localized* NF1 (or *segmental* NF1), reflecting the anticipated timing of the mutational event. Patients with *mosaic-generalized* NF1 present with typical NF1-related symptoms such as pigmentary changes and neurofibromas, not restricted to a few body segments. It may be particularly daunting to predict, solely from clinical observation, whether a sporadic patient has *constitutional* NF1 (i.e., with an *NF1* mutation in all body cells) versus *mosaic-generalized* NF1 and if in the latter patients, whether or not the gonads are involved. Patients with "*mosaic-localized*" or "*segmental*" NF1 present with NF-related symptoms confined to a limited body area. The affected area may present with pigmentary changes only, with neurofibromas only, or with both pigmentary changes and neurofibromas. Further, isolated plexiform neurofibromas also occur and recently the first patient to be described was proven to carry biallelic *NF1* inactivation in this isolated lesion (Beert et al. 2012).

In segmental NF1, the causative mutational event is assumed to have occurred at a later developmental time, offering an explanation for the clinically observed phenotype restricted to a specific body segment. It is most likely to assume that the germ cells of patients with segmental NF1 do not harbour the (first hit) *NF1* mutation. However, apart from the timing of the mutational event, the types of progenitor cells affected as well as the nature of the *NF1* mutation (hypomorphic versus, e.g., a total *NF1* gene deletion) and the age of the patient at clinical evaluation will all equally contribute to the observed widely variable phenotypic outcome in the patient at a given time point. Segmental NF1 is rare, with a frequency estimated to be at least 15 times less prevalent than NF1, between 0.0014 % and 0.002 % (Huson and Ruggieri 2000; Wolkenstein et al. 1995; Ingordo et al. 1995; Listernick et al. 2003). The first proof at the molecular level that *segmental NF1* is indeed due to postzygotic *NF1* mutations was provided through identification of an *NF1* microdeletion in ~15–24 % of the fibroblasts cultured from the CALMs of the affected, but not in the fibroblasts from the unaffected region, from an 18-year-old male with pigmentary lesions scattered over the left upper quadrant of the body (Tinschert et al. 2000). Importantly, however, thereafter some patients have been reported who phenotypically presented as having "*mosaic-localized*" or "*segmental*" NF1 disease, yet were confirmed as being gonosomal mosaic by molecular analysis and as ascertained after birth of their affected offspring (Consoli et al. 2005; Callum et al. 2012).

In the clinical literature, a number of segmental NF1 patients have been described having offspring with segmental NF1 (Rubenstein et al. 1983; Huson and Ruggieri 2000; Oguzkan et al. 2004); such vertical transmission is very difficult

to explain as a result of mosaicism for a mutation shared between parent and child. None of these reported cases had molecular analyses performed and hence the underlying cause remains obscure. In addition, apart from a number of clinical "*mosaic-localized*" or "*segmental*" NF1 cases which are very likely to carry *NF1* mutations in the affected cells, though it was not molecularly investigated and hence the precise underlying cause remains unknown (e.g., Hager et al. 1997; Ruggieri and Huson 2001; Listernick et al. 2003; Morais et al. 2010), some patients have been reported in the literature as having "segmental" NF1 even though they presented with, in addition to pigmentary abnormalities, some features clearly not associated with NF1 (e.g., Castori et al. 2008; Pascual-Castroviejo et al. 2008). As these patients were not analyzed at the molecular level, and hence not proven to carry an *NF1* mutation, it must be realized that their phenotype may well be due to a concurrent defect in more than one gene, including the *NF1* gene, or even might be due altogether to a defect in one or more other genes, not even involving *NF1*. As the molecular tools to investigate the genetics associated with NF1 disease have become more widely available, clinical reports of "segmental NF1" cases should be accompanied by appropriate supportive data using state-of-the-art molecular genetic analyses to prove *NF1* involvement, especially when patients present with features not associated with NF1.

Finally, *revertant mosaicism* refers to the situation where a mutant cellular phenotype is reversed back to normal in some somatic cells by, for example, true reverse point mutation, gene conversion, nondisjunction, or mitotic recombination. Revertant mosaicism has been demonstrated in many genetic conditions involving the skin, including epidermolysis bullosa, Bloom syndrome, and Fanconi anemia amongst others (reviewed by Lai-Cheong et al. 2011), yet has never been demonstrated so far in NF1. To date, only three cases have been specifically investigated for the presence/absence of revertant mosaicism: a woman with NF1 signs throughout the entire body with a few sharply delineated segments of the skin *un*affected, suggestive of revertant mosaicism (Vandenbroucke et al. 2004), and two pairs of monozygotic twins discordant for neurofibromatosis type 1 (Kaplan et al. 2010; Vogt et al. 2011).

All cases *with* molecular data supporting mosaicism for a "first hit" *NF1* mutation, reported as of May 2012, are summarized in Table 12.1. Highlights from these studies and case reports include the following:

- The frequency of mosaicism in sporadically affected NF1 patients is currently still largely unknown. A first estimate was obtained for patients carrying specifically a total gene deletion (TGD) interphase FISH analysis in 146 patients carrying a TGD showed mosaicism in blood in 9.6 % (14/146) (Messiaen et al. 2011). At least 10/14 carried a 1.2-Mb NF1 type 2 *or* atypical TGD deletion (see Chap. 14).
- The typical 1.4-Mb type 1 TGD, previously uniquely found as a result of nonallelic homologous recombination during maternal meiosis between the paralogous recombination sites 1 and 2 (PRS 1 and 2), may also rarely result

Table 12.1 Overview of the mutational and clinical data in all published patients in whom molecular genetic studies were performed

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
GO	XAT27: I1	–	M	No signs, but has two affected children	Del ivs31-ivs39 and Ins 30 bp	Haplotype analysis, BP cloning, sequencing, cDNA analysis, q-PCR, southern blot	–	Mutation detected	N/D	~10 % in sperm cells	Lázaro et al. (1994, 1995)
GEN	UF161[a]	31	F	7 CALMs, bilateral axillary freckling, multiple small cutaneous NFs on face, trunk and extremities, first NFs developed at age 26 after 2nd pregnancy, macrocephaly, normal development; no segmental distribution of features	Del ex4-ivs39 (~100 kb)	LOH analysis, cloning	Mat (UF162)	2 sons: UF394:1CALM; UF395:no signs)	~98.5 %	Mosaic in NFs	Colman et al. (1996) and Rasmussen et al. (1998)
GEN	867	21	F	Multiple CALMs, axillary freckling, multiple small NFs, and bilateral Lisch nodules; cranial CT and MRI scans normal	Del ~ivs27-ivs41	Haplotype analysis	Mat	–	Mosaic	–	Ainsworth et al. (1997)
GEN	5b	40.4	F	>100 cutaneous/ subcutaneous NFs, normal IQ	Microdeletion	FISH	–	Daughter 5a: severely affected	83 %	N/D in fibroblasts from normal skin	Tonsgard et al. (1997)

(continued)

Table 12.1 (continued)

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
GEN	95-870-P[a]	0.6	F	Large congenital cervical PNF and multiple CALMs in a generalized distribution, facial dysmorphism with facial nerve palsy	Microdeletion (~700 kb)	FISH	–		77–84 %	N/D in fibroblasts	Wu et al. (1997) and Riva et al. (2000)
GEN	UF113	10	?	Multiple CALMs, 2 cutaneous NFs, axillary freckling, no macrocephaly, normal development, mild hemihypertrophy, bilateral sensorineural hearing loss	Intragenic Del	LOH analysis, Southern blot	Mat	–	Small fraction	–	Rasmussen et al. (1998)
GEN	–	6	M	Severe NF1: multiple CALMs of up to 8 × 5 cm in a generalized distribution, axillary freckling, pigmentation of the genitalia, PNF, growth retardation, unable to crawl, walk, talk. Subdermal NFs below the left nipple and at the right neck. Facial anomalies, severe and early-onset psychomotor retardation, seizures, spasticity, microcephaly	Microdeletion (>1.7 Mb)	FISH	–	–	33 %	58 % in fibroblasts	Streubel et al. (1999)

GEN	N.R.	47	M	CALMs, freckling, Lisch nodules, NFs, macrocephaly, no PNF	Microdeletion	FISH (locus-specific probes)	–	–	Mosaic	–	Riva et al. (2000)
SEG	–	18	M	Freckling and CALMs on the left upper quadrant: side of the neck, upper trunk, axilla and left arm. No NFs and Lisch nodules	Microdeletion	FISH	–	–	~0–2 %	~15–24 % in fibroblasts from CALMs, N/D in normal skin, N/D in hair follicles	Tinschert et al. (2000)
SEG	–	50	M	Multiple soft, skin-colored tumors on the left front, glabella, upper eyelid, nose, nostril. No other symptoms of NF1	N/D (fibroblasts cultured from NFs)	PTT, enzymatic mutation detection, FISH	–	–	N/D	N/D in fibroblasts from NFs and normal skin	Schultz et al. (2002)
GEN	IL39[a]	60	F	4 CALMs on her thighs and forearms; minor freckling in the left axilla; no dermal NFs or Lisch nodules	Microdeletion (type 2)	FISH, BP PCR, haplotype analysis	–	2 severely affected sons	70 %	15 % in fibroblast	Petek et al. (2003) Kehrer-Sawatzki et al. (2004) and Steinmann et al. (2007, 2008)
GEN	WB[a]	65	F	>20 NFs, no facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, polymorphic markers analysis	–	Daughter SB: severely affected	94 %	–	Kehrer-Sawatzki et al. (2004) and Steinmann et al. (2007, 2008)
GEN	659	47	F	>1,000 NFs, no mental retardation	Microdeletion (atypical HKS, personal communi- cation)	FISH, BP cloning, polymorphic markers analysis	–	–	96 %	52 % in buccal smear	Kehrer-Sawatzki et al. (2004)
GEN	928[a]	35	F	>6 CALMs, freckling, Lisch nodules, 9 subcutaneous and 20 cutaneous NFs, 1 PNF. No facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, polymorphic markers analysis	–	–	97.2–99.8 %	80 % in NFs, 55 % in buccal smear, 61.3 % in urine	Kehrer-Sawatzki et al. (2004, 2012), Steinmann et al. (2007, 2008), and Roehl et al. (2012)
GEN	697[a]	11	F	No NFs, no facial dysmorphism or mental	Microdeletion (type 2)	FISH, BP cloning,	Mat	–	95.4–98.7 %	59 % in buccal smear, 27.6 % in urine	Kehrer-Sawatzki et al. (2004), Steinmann et al.

(continued)

Table 12.1 (continued)

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
				retardation. Delayed in writing/reading		polymorphic makers analysis					(2007, 2008), and Roehl et al. (2012)
GEN	488[a]	33	F	>6 CALMs, freckling, Lisch nodules, 80 subcutaneous and 140 cutaneous NFs, no facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, [olymorphic makers analysis	–	–	96.8–100 %	56 % in buccal smear, 46.5 % in urine	Kehrer-Sawatzki et al. (2004, 2012), Steinmann et al. (2007, 2008), and Roehl et al. (2012)
GEN	938[a]	31	F	>6 CALMs, freckling, Lisch nodules, 1 subcutaneous and 20 cutaneous NFs. 1 internal tumor and MPNST, no facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, polymorphic makers analysis	–	–	93.5–99.6 %	80 % in buccal smear, 23.9 % in urine	Kehrer-Sawatzki et al. (2004, 2012), Steinmann et al. (2007, 2008), and Roehl et al. (2012)
GEN	KCD[a]	34	F	>100 NFs, no facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, polymorphic makers analysis	–	–	92 %	51 % in fibroblasts	Kehrer-Sawatzki et al. (2004) and Steinmann et al. (2007, 2008)
GEN	736[a]	68	F	>6 CALMs, freckling, Lisch nodules, ~2,000 cutaneous NFs, no facial dysmorphism, no mental retardation	Microdeletion (type 2)	FISH, BP cloning, polymorphic makers analysis	–	–	93.8–99.6 %	59 % in buccal smear, 26.7 % in urine	Kehrer-Sawatzki et al. (2004, 2012), Steinmann et al. (2007, 2008), and Roehl et al. (2012)
GEN	NF296-UHG[a]	38	F	NF1 disease manifestations throughout the whole body, but leaving a few sharply delineated segments of the skin	Del ex13-ex28	Long-range RT-PCR, FISH, microsatellite analysis, q-PCR, MLPA	– (father unknown—mother deceased)	–	20.5 %	2.4 % in fibroblasts from CALMs, 0.9 % in fibroblasts from normal skin	Vandenbroucke et al. (2004) and Wimmer et al. (2006)

				unaffected (segmental *absence* of symptoms. Multiple CALMs, NFs, axillary and inguinal freckling, bilateral Lisch nodules, congenital pseudarthrosis of the right tibia						
SEG/GOSO	–	–	F	An area of freckling and CALMs confined to the left upper quadrant, with ipsilateral Lisch nodules in one eye	p.Arg1947*	dHPLC, DNA sequencing, cloning, allele-specific PCR	–	1 child with classic NF1	N/D	~17.6 % in fibroblasts and ~40 % in keratinocytes from CALMs, N/D in normal skin	Consoli et al. (2005)
SEG	–	40	M	Regional presentation of NF restricted to the right chest wall	N/D	Comprehensive NF1 testing	–	–	N/D		Fortino et al. (2005)
N/A	NF362-UAB	–	–	–	Del ex10b-ex49	MLPA, RT-PCR, microsatellite analysis	–	–			Wimmer et al. (2006)
N/A	NF467-UHG	–	–	–	Del ex28-ex40	MLPA, RT-PCR, microsatellite analysis	–	–			Wimmer et al. (2006)
Atypical (mosaic Ring chr17)	–	9.5	F	Mild scoliosis, gait wide-based. Multiple CALMs on the skin, mild axillary and inguinal freckling. Mental retardation, autistic features, cognitive and language delay	Mosaic non-supernumerary ring chr17 (0.6–2.5 Mb on 17p, and up to about 10 Mb on 17q)	FISH, MLPA	–	–	12 %		Havlovicova et al. (2007)
SEG	SNF1-1	46	F	*NFs only*: several small (1–4 mm) cutaneous NFs clustered within a limited body region on her trunk	c.2041C>T (p. Arg681*) identified in 3 different NFs	Comprehensive NF1 testing, q-PCR, FISH	–	(2 unaffected sons)	3.7 %	8–19.4 % in fibroblasts and 41–47.4 % in Schwann cells	Maertens et al. (2007)

(continued)

Table 12.1 (continued)

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
				and neck. Intestinal ganglioneuroma. A glomus tumor at the distal falanx of the right middle finger						from NFs, 1.8 % in hair follicles	
SEG	SNF1-2	23	M	*Pigmentary defects only*: several CALMs within a background of hyperpigmented skin involving right leg, hip, and lower back. Inguinal freckling in the affected segment. no NFs, no Lisch nodules, no LD	Microdeletion (atypical) in CALM and hyperpigmented skin	Comprehensive NF1 testing, q-PCR, FISH	–	–	2 % (400 interphase cells analyzed)	Mosaic in melanocytes from CALMs, N/D in normal skin	Maertens et al. (2007)
GEN	SNF1-3	15	F	>6 CALMs over her body, several small cutaneous and subcutaneous NFs on her right hand within an overlying CALM, a putative NF on her left shoulder identified by total body MRI, no LD, no skinfold freckling	Microdeletion (1.84–2.8 Mb) or c.2325+1G>A in multiple NFs and CALMs	Comprehensive NF1 testing, q-PCR, FISH	–	–	~4 % N/D	Mosaic in melanocytes from CALMs and Schwann cells from NFs 54.9–97.1 % in melanocytes from CALMs, 12.3 % in fibroblasts and 67.2–88.8 % in Schwann cells from NFs	Maertens et al. (2007)
GEN	1630[a]	15	F	>6 CALMs, freckling, Lisch nodules, no NFs, no large hands and feet,	Microdeletion (type 2)	Polymorphic markers analysis, BP	–	–	94.1–98.2 %	29.3 % in urine	Steinmann et al. (2007, 2008), Kehrer-Sawatzki

				none of the other features frequently seen in patients with large deletions		analysis, MLPA, aCGH					et al. (2012), and Roehl et al. (2012)
GEN	1104[a]	36	F	Hyperpigmented brownish areas with chessboard pattern on upper back, left shoulder, left arm, left breast, right shoulder, lumbar region, right side of the abdomen; No axillary and inguinal freckling, no Lisch nodules, 4–6 NFs on abdomen, thighs, forearms	Microdeletion (type 2)	Polymorphic markers analysis, BP analysis, MLPA, aCGH	–	–	84–94 %	8 % in buccal smear, 15 % in urine	Steinmann et al. (2007, 2008)
GEN	1502[a]	26	F	Multiple CALMs, axillary and inguinal freckling, Lisch nodules, <10 subcutaneous NFs, IQ90, cerebral and whole-body MRI did not reveal any tumors or other anomalies	Microdeletion (type 2)	Polymorphic markers analysis, BP analysis, MLPA, aCGH	–	–	95.9–99.3 %	70 % in buccal smear, 54.6 % in urine	Steinmann et al. (2007, 2008), Kehrer-Sawatzki et al. (2012), and Roehl et al. (2012)
GEN	811-M[a]	–	F	–	Microdeletion (type 2)	Polymorphic markers analysis, BP analysis, MLPA, aCGH	–	1 affected daughter	93 %	–	Steinmann et al. (2007, 2008)
GEN	HC[a]	–	M	–	Microdeletion (type 2)	Polymorphic markers analysis, BP analysis, MLPA, aCGH	Mat (PH)	–	100 %	Mosaic in buccal smear	Steinmann et al. (2007, 2008)
GEN	1860-M[a]	28, 54	F	LD, developmental delay. Axillary and inguinal freckling, Lisch	Microdeletion (Atypical)	MLPA, haplotype analysis, FISH	Mat (1860-M)	Affected son	94.4–95.9 %	50.9 % in urine	Steinmann et al. 2008 and Roehl et al. 2012

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Table 12.1 (continued)

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
				nodules. ~1,000 cutaneous and subcutaneous NFs, internal tumors in the brachial plexus and lumbar region							
GO	NF-307: I1	–	M	NO signs of NF1	Del ivs27b-ex30 (c.4773-3622-?_5749+?)	q-PCR, microsatellite analysis, MLPA, RNA-based analysis	–	2 affected daughters and 1 granddaughter	N/D	10–17 % in sperm cells	Bottillo et al. (2010)
GOSO (affected twin)/? (discordant MZ twin)	–	57	F	Affected twin: CALMs, cutaneous NF, PNF, Lisch nodules, freckling. Implication of gonosomal mosaicism	p.Arg1968*	Sequencing, STR markers, aCGH	Mat	2 affected children, 1 unaffected child	Present	Present in fibroblasts and buccal smear	Kaplan et al. (2010)
				Unaffected twin: No signs of NF1; has 2 unaffected children				No	Present	Present in buccal smear, N/D in fibroblasts	
GEN	#3	36	M	9 CALMs and macrosomia, bilateral axillary freckling; Normal cognition without LD, NO segmental distribution. Possibly 1 NF	c.2866dupA	SNaPshot, pyrosequencing	–	1 affected son, 2 unaffected children	24 %	–	Muram-Zborovski et al. (2010)
GEN	–	34	F	20 CALMs on trunk and upper extremities, axillary and submammary freckling,	c.3198-314G>A (leaky splice mutation)	DNA-based sequencing, SNaPshot	–	–	~100 %	~100 % in urine, ~20–35 % in buccal smear,	Fernández-Rodríguez et al. (2011)

				mild scoliosis, <50 minuscule NFs which developed since age 18 years, no Lisch nodules, no dysmorphism, no LD						hair follicles and skin	
GEN	UAB-r3302	15	M	>6 CALMs, axillary freckling, cutaneous NFs on left neck, arm, trunk and PNF on left arm. A few freckles and CALMs on right arm. Bilateral Lisch nodules. No spinal NFs or optic gliomas	Microdeletion (type 1)	MLPA, microsatellite, FISH, BP spanning PCR, SNP analysis	–	–	~80 %	–	Messiaen et al. (2011)
GEN	UAB-r7332	10	M	>6 CALMs, bilateral axillary and inguinal freckles. Bilateral Lisch nodules. 2–6 cutaneous and 2–6 subcutaneous NFs, symptomatic optic glioma on left optic nerve. Scoliosis	Microdeletion (likely type 1 according to MLPA)	MLPA, microsatellite, FISH, BP spanning PCR, SNP analysis	–	–	~97 %	–	Messiaen et al. (2011)
GEN	UAB-r3222	27	F	2 CALMs, no freckles. >100 cutaneous NFs, no PNF. Bilateral Lisch nodules. No optic gliomas or skeletal abnormalities. Normal development	Microdeletion (type 1)	MLPA, microsatellite, FISH, BP spanning PCR, SNP analysis	–	–	50 %	–	Messiaen et al. (2011)
SEG	P067	45	F	~20 small dermal NFs, freckling on upper right back and under right breast. Inguinal freckling. No CALMs or Lisch nodules. No facial dysmorphy, LD	Microdeletion (type 1)	MLPA, microsatellite, FISH, BP spanning PCR, SNP analysis	–	–	N/D	Present in Schwann cells from NFs	Messiaen et al. (2011)
SEG	UAB-M1	14	M	3 CALMs, freckling hyperpigmentation on left upper thigh, left	Microdeletion (likely type 1	MLPA, microsatellite, FISH, BP	–	–	N/D	Present in melanocytes from CALMs,	Messiaen et al. (2011)

(continued)

Table 12.1 (continued)

		Phenotype			Molecular analysis				Percentage of cells carrying the mutation in		
Type	Patient ID	Age Years	Gender	Clinical features	NF1 first hit mutation	Methods	Parental origin[b]	Offspring affected	Peripheral blood/ cultured lymphocytes	Others	References
				inguinal region, no NFs or PNFs, no Lisch nodules	according to MLPA)	spanning PCR, SNP analysis				N/D in fibroblasts	
GEN (discordant MZ twins)	–	3	M	Affected twin: >6 CALMs, axillary and inguinal freckling	c.4108C>T (p. Gln1370*)	SNP/allelic marker analysis, SNaPshot, Cloning	Mat	–	~30–40 %	~8 % (4 % allele) in buccal smear, N/D in urine	Vogt et al. (2011)
				Unaffected twin: 2 CALMs	N/D			–	N/D	N/D in buccal smear, urine	
SEG	–	13	M	An isolated plexiform neurofibroma on the lumbosacral area. No CALMs, no Lisch nodules, and no other NF1 features	1st hit: ins(17;1) (q11.2;p35p36) 2nd hit: 8.28 Mb del on 17q11.2q12	Karyotyping, FISH, aCGH	–	–		61 % in Schwann cells (21 % by FISH) 13 % in Schwann cells by FISH, 15–20 % by aCGH	Beert et al. (2012)
GOSO	–	–	M	Sperm donor: only 4 hyperpigmented spots, midline back. No developmental disability, no Lisch nodules, no freckling, no NFs	Del ex11-23.1	RT-PCR, breakpoint cloning MLPA, aCGH	–	–	Present (detected by reverse-transcriptase PCR)	~20 % in sperm cells	Callum et al. (2012)
GEN	1956[a]	9	M	>6 CALMs, axillary freckling, Lisch nodules, scoliosis, normal development	Microdeletion (type 2)	FISH	–	–	96.7–99.1 %	60 % in urine	Kehrer-Sawatzki et al. (2012) and Roehl et al. (2012)
GEN	2442[a]	40	F	>6 CALMs, axillary freckling, Lisch nodules, ~250 cutaneous NFs,	Microdeletion (type 2)	FISH	–	–	98.6–100 %	60 % in urine	Kehrer-Sawatzki et al. (2012) and Roehl et al. (2012)

				foraminal tumors, normal brain, optic pathway and orbital MRI, scoliosis, short stature (<5th PC)							
GEN	UC172	–	–	–	Microdeletion (type 2)	FISH, Microsatellite analysis, breakpoint cloning	–	–	91.9–97.9 %	64.5 % in urine	Roehl et al. (2012)
GEN	585	–	–	–	Microdeletion (type 2)	FISH, microsatellite analysis, breakpoint cloning	–	–	94.3–96.7 %	81.9 % in urine	Roehl et al. (2012)
GEN	3304	47	M	Multiple CALMs, >1,000 cutaneous NFs, muscular hypotonia, large soft hands, funnel chest, difficulties with reading/writing, developmental delay	Microdeletion (atypical or type 1)	FISH, microsatellite analysis, breakpoint cloning	–	Yes affected daughter	16.9–19.3 %	25 % in urine	Roehl et al. (2012)

GO gonadal mosaicism, *GOSO* gonosomal mosaicism, *GEN* generalized mosaicism, *SEG* segmental mosaicism, *NF* neurofibroma, *PNF* plexiform neurofibroma, *CALM* café-au-lait macule, *Del* deletion, *Ins* insertion, *N/D* not detected, *M* male, *F* female, *Mat* maternally derived, *Pat* paternally derived, *LD* learning disability, *BP* breakpoint

[a]Described in multiple studies, as indicated.

[b]Parental origin of the allele carrying the mutation in the mosaic proband.

from recombination during mitotic post-zygotic cell divisions, as now proven in three patients carrying a mosaic TGD type 1 in blood (Messiaen et al. 2011).

- Mosaicism in sporadic patients carrying a TGD type 2 is very high, estimated to occur at least in 70 % of founders (Messiaen et al. 2011). There seems to be a clear preponderance of females amongst mosaic founder patients with a TGD type 2 (Kehrer-Sawatzki et al. 2004; Steinmann et al. 2007).
- Several studies found the percentage of hematopoietic cells carrying an *NF1* mutation, especially when carrying a TGD type 2, to be higher as compared to urine and buccal epithelial cells or fibroblasts from affected as well as unaffected regions in patients with generalized-mosaic NF1 (Kehrer-Sawatzki et al. 2004, 2012; Vandenbroucke et al. 2004; Steinmann et al. 2007; Kehrer-Sawatzki and Cooper 2008; Roehl et al. 2012). Two mosaic patients were, however, found by FISH analysis in blood to carry the deletion in all cells, whereas a lower percentage was found in the buccal cells (Steinmann et al. 2007; Roehl et al. 2012). These tissue-specific differences suggest a selective growth advantage of hematopoietic stem cells carrying an *NF1* deletion (Roehl et al. 2012).
- Patients with mosaic type 2 TGD have milder disease manifestations, with a significantly lower external and internal plexiform neurofibroma burden, no facial dysmorphisms, and no delayed cognitive development compared to patients with a type 1 TGD (which typically is constitutional) (Kehrer-Sawatzki et al. 2012). This can probably be attributed to the presence of normal cells in the mosaic patients. However, since the risk of malignant transformation remains in any plexiform lesions present, special clinical management to allow early detection is still required.
- The line separating generalized mosaic NF1 from segmental NF1 may not always be clear. In general, a mutation in blood is not detected in patients with clear segmental presentation. Gonosomal mosaicism has, however, been proven in an adult male with four pigmentary lesions on the midline of his back as the sole NF1-related manifestation (Callum et al. 2012). This intragenic multi-exon deletion, present in <20 % of the blood cells and in ~20 % of the sperm, was detected because of the preferential amplification of the shorter transcript by reverse-transcriptase PCR as part of a comprehensive *NF1* testing (Callum et al. 2012). Low-level mosaic mutations typically escape detection by Sanger sequencing and array comparative genome hybridization (aCGH) or multiplex ligation-dependent probe amplification (MLPA).
- The aggregated data obtained from all patients with mosaic NF1 ("*generalized*" or "*segmental*") clearly indicate that the percentage of cells carrying the *NF1* "first hit" mutation often is too low to be reliably detected in blood (or even is absent) and hence may be missed (Tinschert et al. 2000; Consoli et al. 2005; Maertens et al. 2007; Callum et al. 2012).
- A subpopulation of the Schwann cells within the neurofibromas carries the second *NF1* hit, in accordance with the two-hit tumor-suppressor hypothesis (Serra et al. 2000; Maertens et al. 2006). In the CALMs of NF1 patients, *melanocytes*, but *not* keratinocytes or fibroblasts, carry a first *and* second hit in the *NF1* gene (De Schepper et al. 2008). Maertens et al. (2007) demonstrated

that accurate diagnosis of mosaic or segmental NF1 necessitates comprehensive mutation analysis specifically of those neural-crest derived cells that are relevant, that is, the Schwann cells in the neurofibromas and the melanocytes in the CALMs. In these specific cells, but not (or only at too low a level) in blood, fibroblasts, or keratinocytes, a common first *NF1* hit can be found in the different lesions (neurofibromas or CALMs) of segmental NF1 patients. This may also explain why no mutations were found in the fibroblasts of the patient described by Schultz et al. (2002).

- In total, 49 unrelated patients, mosaic for a "first hit" post-zygotic *NF1* mutation in the gonads and/or somatic cells, were reported and only 6/49 carried a minor lesion mutation affecting 1 nucleotide, all resulting in a premature stop codon. This probably reflects the technical difficulties associated with the detection of mosaic point mutations.
- In 43/49 of these cases, the mutation was either an intragenic multi-exon deletion or a large deletion. At least 17 of these deletions were type 2 TGD spanning 1.2-Mb.
- Finally, one pair of discordant twins was reported by Kaplan et al. (2010), with a woman carrying an *NF1* nonsense mutation in both the Epstein–Barr virus-transformed lymphoblastoid cells propagated from her B-lymphocytes and her buccal epithelial cells, but not in her skin fibroblasts, yet being asymptomatic as of the age of 57 years. In another pair of discordant twins (Vogt et al. 2011), the affected 3-year-old twin was shown to be mosaic in blood and buccal cells for a nonsense mutation p.Gln1370*, which was not found in the unaffected twin. This can be explained by a post-zygotic mutation occurring after the twinning event took place, that is, after day 3–4 of embryonic life.

12.3 Molecular Diagnosis of NF1

Molecular diagnosis of NF1 is challenging owing to the large size of the gene, the existence of multiple highly homologous non-processed pseudogenes, the lack of mutational hotspots, and the complex mutational spectrum including a significant fraction of unusual splice mutations such as deep-intronic splice mutations or mutations affecting exonic splice enhancers, mimicking nonsense, missense, or even silent mutations at the genomic level (Messiaen and Wimmer 2008). The highest sensitivity and specificity are obtained using a multistep approach including an RNA-based center assay (Messiaen et al. 2000; Wimmer et al. 2006; Valero et al. 2011).

Clinical applications of mutational analysis have increased in relevance since some clear genotype–phenotype correlations have unfolded, with large deletions resulting in a more severe clinical phenotype (Upadhyaya et al. 1998; Riva et al. 2000; Mautner et al. 2010) and with the 3-bp in-frame deletion of 1 amino acid in exon 17 resulting in a milder phenotype without cutaneous or superficial plexiform neurofibromas (Upadhyaya et al. 2007). Further, limited genetic heterogeneity has

been found by identification of a novel disorder, Legius syndrome, caused by mutations in the *SPRED1* gene (Brems et al. 2007). Patients with Legius syndrome have multiple CALMs with or without skinfold freckling and macrocephaly, but do not develop the typical NF1-associated tumors (Brems et al. 2012). In patients presenting solely with CALMs with/without freckling, a correct diagnosis of constitutional NF1 versus mosaic NF1 (for sporadic patients) versus Legius syndrome cannot be made exclusively based on the clinical manifestation.

In ~95% of patients presenting with "classic NF1", including CALMs, skinfold freckling and neurofibromas, the *NF1* mutation is identified if a comprehensive approach including RNA-based sequencing is applied (Messiaen et al. 2009). However, the detection rate in the blood lymphocytes drops in sporadic patients, even if they present with CALMs, skinfold freckling and neurofibromas probably because some of them present with mosaicism for an *NF1* mutation undetectable/absent in the blood.

The majority of generalized mosaic patients reported to date present with a type 2 or atypical TGD, apparently detectable in a higher percentage of blood lymphocytes compared to urine-derived or buccal epithelial cells (Kehrer-Sawatzki et al. 2004, 2012; Vandenbroucke et al. 2004; Steinmann et al. 2007; Kehrer-Sawatzki and Cooper 2008; Roehl et al. 2012). Hence, blood represents a good sample from which to start analyses. In order to ascertain mosaicism, it seems to be sensible to pursue quantitative testing such as FISH in urine-derived epithelial cells in sporadic patients carrying a type 2 or atypical TGD identified in blood. Establishing a diagnosis of *mosaic* versus *constitutional* NF1 has important repercussions for counseling the patients and family with regard to recurrence risk.

Identification of the cell of origin in the CALMs and neurofibromas, that is, the melanocyte (De Schepper et al. 2008) and Schwann cell (Serra et al. 2000; Maertens et al. 2006), can now be applied to the precise identification of the common "first hit" *NF1* mutation in the melanocytes or Schwann cells cultured from the affected regions of segmental patients (Maertens et al. 2007) or suspected mosaic patients in whom no mutation could be identified in the blood after comprehensive testing. Identification of such a common "first hit" establishes the unambiguous diagnosis of segmental or mosaic NF1. Moreover, it provides a marker that can be used for family planning, if desired, as the mutation may be at risk of being transmitted to the next generation and, if so, would result in constitutional NF1 (with all body cells carrying the inherited mutation). Refinement of the risk for transmission can be pursued in mosaic/segmental male patients, through analysis of the sperm, but cannot be provided in female patients, given the inaccessibility of the eggs for screening.

Future progress in diagnosis of founder patients is likely to come from novel technologies such as deep sequencing and may provide a means to detect low-level mosaicism in a reliable and sensitive way.

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Chapter 13
Deep Intronic *NF1* Mutations and Possible Therapeutic Interventions

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13.1 Deep Intronic Mutations: A Specific Type of Splicing Mutation

Splicing is a complex and fine-tuned cellular mechanism consisting of the removal of introns from pre-mRNA transcripts to generate mature messenger RNAs. This essential process is completed by the spliceosome in different reactions that require the participation of several *cis* elements and *trans*-acting factors (reviewed in Hammond and Wood 2011). The recognition of intron–exon boundaries is crucial for correct splicing, so they are marked by highly conserved, almost invariant dinucleotides of the donor or 5′ intronic splice site (GT, in genomic DNA) and the intronic acceptor or 3′ splice site (AG, in genomic DNA). Other important genomic sequences for splice site identification are the branch site and the polypyrimidine tract, both of which are located upstream of the intronic acceptor site. Other relevant *cis* elements include nucleotides motifs representing splice site enhancers (SE) and silencers (SS) that are located within both intronic and exonic regions and participate in the recruitment of enhancer or silencer *trans*-acting factors to the splicing machinery. Genomic variants in the DNA sequences of these elements can alter the correct recognition of a bona fide exon or, alternatively, create a new cryptic splice site that can be identified by the splicing machinery. Hence, alterations in these DNA sequences produced by mutations can lead to errors in the splicing process, leading to exon skipping, partial exon deletion, or

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incorporation of an intronic region into the mature RNA. In all of these cases, either *in-frame* or *out-of-frame* abnormal transcripts may be produced by the mutated allele.

Classically, it was thought that splicing mutations were restricted to DNA changes that disrupt the invariant canonical donor and acceptor splice sites, so only these mutations were reported as splicing mutations in mutation databases (currently fewer than 10 % of all point mutations reported in the *Human Gene Mutation Database* (HGMD Professional 2011.4; http://www.hgmd.org). However, over the last decade, a large number of studies have identified other types of mutation that also affect the correct splicing of a gene, particularly in genetic analyses that have been focused on large genes, using RNA-based approaches for constitutive mutation detection (Teraoka et al. 1999; Ars et al. 2000). These findings have served to extend the group of disease-causing mutations that impact splicing to a range of different mutation types (nonsense, missense, frameshift, etc.) affecting any sequence that is important for splice site recognition and mRNA processing. For example, the largest unbiased comprehensive study of >2,000 unrelated NF1 patients found that approximately 30 % of *NF1* point mutations affected correct mRNA splicing (L Messiaen, personal communication). A small subset of these mutations (about 2 %) (Messiaen and Wimmer 2008; Pros et al. 2008; Wimmer et al. 2007) comprises single-nucleotide changes in sequences residing deep within introns, which create novel donor or acceptor sites that, in conjunction with a nearby cryptic splice counterpart, define a new cryptic exon that the spliceosome then incorporates into mature messenger RNA. Most of these mutations are missed by conventional DNA-based mutation detection techniques because they are located deep within intronic sequences, distant from intron–exon boundaries, and hence are not scanned when this type of mutation detection approach is employed. Moreover, a high proportion of these mutations create mRNAs with premature termination codons (PTC), rendering the transcripts susceptible to degradation by the nonsense-mediated mRNA decay (NMD) machinery. It is therefore desirable to use an NMD inhibitor prior to RNA extraction to be able to detect the mis-splicing effects characteristic of deep intronic mutations (Messiaen et al. 2000). Together, these observations suggest that the frequency of this type of mutation has probably been underestimated for most studied genes.

13.2 Use of Antisense Oligonucleotides to Modulate Aberrant Splicing

In recent years, antisense oligonucleotide (AON)-mediated therapies have been increasingly used to restore gene function by modulating aberrant splicing in different scenarios created by disease-causing mutations. Correction of altered RNA splicing can be achieved by masking a splice motif with AONs complementary to a specific sequence of the pre-mRNA of interest, thereby inhibiting by steric

hindrance the recognition of this region by the splicing machinery. The first indication that AONs could be used as therapeutic agents for genetic diseases was obtained from studies in β-thalassemia, in which abnormal splicing of the beta-globin (*HBB*) gene due to activation of intronic cryptic sites was corrected using an AON approach (Dominski and Kole 1993). Different applications of AON-dependent splicing modulation have been described in the literature, including forcing the skipping of one or more exons flanking a frameshift in order to restore the open reading frame of a gene (Aartsma-Rus et al. 2003, 2004), forcing the selection of an alternative splice site in order to prevent the synthesis of pathogenic transcripts (Mercatante and Kole 2002), preventing the inclusion of an aberrant cryptic exon inserted into the mRNA due to a deep intronic mutation (Du et al. 2007; Rincon et al. 2007; Pros et al. 2009; Rodriguez-Pascau et al. 2009; Vega et al. 2009), and inducing the elimination of in-frame exons that contain a pathogenic mutation (Aartsma-Rus et al. 2003, 2004; reviewed in Perez et al. 2010).

Deep intronic mutations are ideal targets for AON function because they are located within intronic regions, leaving bona fide splice sites intact. Steric blockage by AONs of a newly created splice site prevents the splicing machinery from recognizing the cryptic exon and promotes normal splicing.

13.2.1 Types of AON and Delivery Vehicles

The development of AON technology as a successful means of applying antisense therapy had to overcome a range of obstacles and continues to face several technical challenges, some related to AON stability and cellular delivery.

To avoid degradation by cellular nucleases, different analogs have been used for AON design, including phosphorodiamidate morpholino oligomers (PMO) (Summerton 1999), locked nucleic acids (LNA) (Koshkin and Wengel 1998), peptide nucleic acids (PNA) (Larsen et al. 1999), and 2′-*O*-methyl phosphorothioate (2′OMe) (Manoharan 1999). All have shown stability against degradation, high target affinity, and good biological activity (reviewed in (Kurreck 2003). Antisense phosphorodiamidate morpholino oligomers (PMOs) have been used successfully for splicing modulation in neurofibromatosis type 1 (Pros et al. 2009; Fernandez-Rodriguez et al. 2011). PMOs are analogs of oligonucleotides with a six-membered morpholinoring, replacing the ribose or deoxyribose backbone, and uncharged phosphorodiamidate intersubunit linkages. In addition to high binding specificity, stability, and resistance to nucleases, PMOs exhibit highly durable activity (Summerton and Weller 1997). Due to these advantageous properties, PMOs have been used for therapeutic purposes in several disorders, including β-thalassemia (Lacerra et al. 2000; Suwanmanee et al. 2002), Duchenne muscular dystrophy (McClorey et al. 2006), Hutchinson–Gilford progeria syndrome (Scaffidi and Misteli 2005), ataxia-telangiectasia (Du et al. 2007), and propionic and methylmalonic acidemias (Rincon et al. 2007).

Another important aspect in AON technology is the delivery of the antisense oligomers into target cells. Initially, different techniques were developed to transfect in vitro cell cultures, including electroporation, liposomes, cationic polymers, and other endosomal escape reagents (Thierry et al. 2003; Merdan et al. 2002). However, for AON delivery in vivo, these procedures were characterized by low efficiency and inadequate levels of toxicity. In this context, modification of AON ends to facilitate cellular delivery has been found to be a more effective solution than the use of unmodified forms. Two promising examples are Morpholino oligos linked to cell-penetrating peptides (PPMOs) and Vivo-Morpholinos (Moulton and Jiang 2009). PPMOs are typically an arginine-rich cell-penetrating peptide linked to a Morpholino oligo. Cell-penetrating peptides offer two advantages over unmodified Morpholinos: enhanced uptake into endosomes and enhanced endosomal escape (Abes et al. 2008). Vivo-Morpholinos are eight guanidinium groups on a dendrimeric scaffold linked to a Morpholino oligo (Li and Morcos 2008) that facilitates efficient delivery into most mouse tissues (Morcos et al. 2008) and has been used successfully as a therapeutic agent in different animal models (e.g., Osorio et al. 2011).

13.3 Use of Antisense Oligonucleotides to Reverse the Effect of Deep Intronic *NF1* Mutations

As mentioned previously, approximately 2 % of germline *NF1* mutations are deep intronic nucleotide changes that activate or create novel splice sites, causing the pathogenic inclusion of cryptic exons in mRNA (Messiaen and Wimmer 2008; Pros et al. 2008) (summarized in Table 13.1). These mutations are an ideal target for antisense therapies since the bona fide splice sites remain intact, conserving their potential for normal splicing. Next, a summary of the results obtained to date on the use of AONs to reverse the effects of *NF1* deep intronic mutations is presented (Pros et al. 2009; Fernandez-Rodriguez et al. 2011), focusing on the differences between distinct mutations and cell-type specificities, the required doses and duration of AON effects (in our case, PMOs), the mode of action, and the impact on neurofibromin function.

13.3.1 Effects of PMOs Are Mutation-Dependent and Variable Between Cell Types

To test the efficiency of PMO treatment in restoring normal splicing for this type of mutation, samples were obtained from seven patients with four independent *NF1* germline deep intronic mutations (Table 13.1, Fig. 13.1). Primary lymphocyte and fibroblast cell lines were derived from these patients, and specific PMOs were

Table 13.1 Details of the *NF1* deep intronic mutations reported in the literature

Mutation	Intron	mRNA effect	# Patient present study	References
c.288 +2025T>G[a]	3	r.288_289ins288+1917_288 +2024	1	Pros et al. (2008)
c.889 -942G>T	6	r.888_889ins889-931_889-873	–	Pros et al. (2008)
c.1393-592A>G	10a	r.1392_1393ins1393-673_1393-597	–	Pros et al. (2008)
c.1527 +1159C>T	10b	r.1527_1528ins1527 +1103_1527+1157	–	Spits et al. (2005), Wimmer et al. (2007), and Pros et al. (2008)
c.1642-449A>G	10c	Not described	–	Jeong et al. (2006)
c.3198-314G>A[a]	19a	r.3197-3198ins3198-214-3198-312, r.3197-3198ins3198-245-3198-312	7	Fernandez-Rodriguez et al. (2011)
c.5749 +332A>G[a]	30	r.5749_5750ins5749 +155_5749+331	2, 3	Perrin et al. (1996), Ars et al. (2000), and Wimmer et al. (2007)
c.5750-2792A>G	30	r.5749_5750ins5750-278_5750-108	–	Raponi et al. (2006)
c.7908-321C>G[a]	45	r.7907_7908ins7908-322_7908-391	4[b], 5[b], 6	Pros et al. (2008)

Sequence changes are described at the cDNA level (indicated by "c.")
The +1 nucleotide corresponds to the A of the ATG translation initiation codon in the reference sequence NM_000267.2
[a]Mutations in which PMO treatment has been performed.
[b]Patients from the same family.

designed to block the effects of mutations causing the inclusion of cryptic exons in the *NF1* gene. Three of the studied mutations generated a cryptic donor splice site (c.288+2025T>G, c.5749+332A>G and c.7908-321C>G), whereas the fourth generated a cryptic acceptor site (c.3198-314G>A). All mutations used a wild-type counterpart to insert a cryptic exon into the mature mRNA (Fig. 13.1), which either led to the generation of out-of-frame transcripts susceptible to degradation by the NMD machinery or produced a truncated form of neurofibromin. All PMOs were designed, synthesized, and purified by Gene Tools (Philomath, OR). For one of the mutations (c.3198-314G>A), PMOs were also designed to block the recognition of the two wild-type counterpart sequences used as donor sites for cryptic exon inclusion (Fig. 13.1). Optimal conditions for PMO treatment were established using fibroblasts, and, when possible, PMO treatment was subsequently studied using EBV-transformed lymphocytes.

To determine the effect of PMO concentration on aberrant splicing correction, a dose–response experiment was performed for different mutations, in which

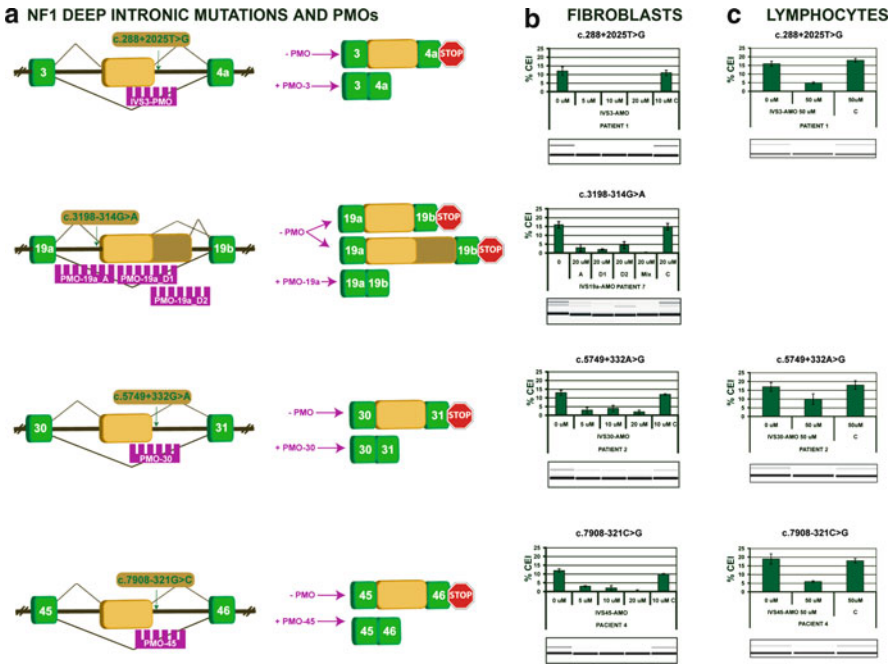

Fig. 13.1 Panel (**a**): Schematic representation of the four mutations examined in the present study and the antisense morpholino designed to treat them. Panel (**b**): Correction of aberrant splicing by PMO treatment of fibroblast cultures. RT-PCR analysis of total RNA was performed using specific primers to analyze both transcripts (wild-type and cryptic exon-containing transcripts). The *y*-axes of each graph show the proportions of cryptic exon-containing transcripts vs. the total. The data are represented by a bar consisting of the mean ± SD for at least three independent experiments. The corresponding Agilent electrophoresis gel is shown below each graph. For mutations in introns 3, 30, and 45, a dose response after 24 h of treatment using different PMO concentrations (5, 10, and 20 μM) is shown. Controls for PMO specificity (C) were as follows: IVS30-PMO, IVS45-PMO, and IVS3-PMO for c.288+2025T>G, c.5749+332A>G, and c.7908-321C>G mutations, respectively. For mutation c.3198-314G>A (intron 19a), PMO treatment is shown using three different antisense oligonucleotides and the sum of all of three. Correction is observed in all cases and is complete when a mixture of the three is used. *CEI* cryptic exon inclusion, *WT* wild-type. Panel (**c**): PMO treatment in EBV-transformed lymphocytes cell lines for mutations c.288+2025T>G, c.5749+332A>G, and c.7908-321C>G. EBV-immortalized lymphocytes were treated at 72 h with 50 μM PMO. Controls for PMO specificity (C) were: IVS30-PMO, IVS45-PMO, and IVS3-PMO for c.288+2025T>G, c.5749+332A>G, and c.7908-321C>G mutations, respectively.*CEI* cryptic exon inclusion, *WT* wild-type

fibroblasts were treated with three different PMO concentrations (5, 10 and 20 μM) for 24 h (Fig. 13.1). In the absence of PMO, the percentage of aberrantly spliced transcripts (containing the cryptic exon) was approximately 10–15 % of the total *NF1* mRNA (wild-type + aberrantly spliced transcripts). As envisaged, the low percentage of cryptic exon-containing *NF1* mRNAs was the result of partial degradation by the NMD machinery and the production of wild-type transcripts from the mutated allele, due to partial recognition of bona fide intron–exon

boundaries by the splicing machinery. When cells were treated with the specifically designed PMO, either complete correction or a dose-dependent correction of aberrant splicing was observed, depending upon the mutation. 20 μM was found to be the optimal concentration of PMO for most mutations tested and was therefore used for the remaining mutations and experiments (Fig. 13.1). The response to PMO treatment was clearly mutation-dependent. By contrast, cells from different patients carrying the same *NF1* mutation (e.g., c.5749+332A>G and c.7908-321C>G) exhibited similar results upon addition of PMO (Pros et al. 2009).

To evaluate the effect of time on mutation correction after Morpholino treatment, a time course was performed for three of the mutations using fibroblast cell lines (Pros et al. 2009). In general, the efficiency of *NF1* splicing correction in fibroblasts after 24 h of treatment ranged from 87 to 100 % for the different mutations.

Several factors could account for the variance in PMO activity between the mutations studied, for example, differences in the strength of the cryptic acceptor/donor splice sites or variations in the extent to which PMO accesses the pre-mRNA secondary structures. Different strategies could be used to enhance the PMO-dependent restoration of normal splicing for these mutations, including the following: designing a different PMO for blocking the mutation site; using a combination of Morpholinos, one directed at the specific mutation site and the other blocking the wild-type cryptic site used as a counterpart; using a Morpholino to target only the latter; designing Morpholinos to target exon splicing enhancer (ESE) elements. Reports have indicated that the use of PMOs may, in some cases, be more efficient for one of the cryptic splice sites than the other (Du et al. 2007). In this sense, our results with mutation c.3198-314G>A, in which three PMOs were designed to block all of the cryptic splice sites used, indicated that although each PMO was able to reduce the levels of mutant transcripts, complete correction was only observed when a combination of the three PMOs was used (Fig. 13.1), as has also been described for other genetic disorders (Gurvich et al. 2008).

To gain insight into the cell-type specificities of Morpholino treatment, in addition to fibroblasts, transformed lymphocytes derived from the same patients were also analyzed. The highest degree of normal splicing restoration (although not complete) in EBV-transformed lymphocyte cell lines was observed at 72 h, following treatment with 50 μM PMO (Fig. 13.1). Lower concentrations of Morpholino were also tested but were found to be less effective. In general, a lower degree of aberrant splicing correction was observed in transformed lymphocyte cell lines (30–70 % depending upon the mutation), and a higher concentration of Morpholino, together with a longer exposure time, was needed to produce similar effects to those observed in fibroblasts. The differences between the effects observed in fibroblasts and lymphocytes could be explained by the inherent difficulty of transfecting lymphocyte cell lines (Galletti et al. 2007; Seiffert et al. 2007). The authors of another comparative study, using electroporation to deliver Morpholino into cells, achieved similar transfection efficiencies in dermal fibroblasts and B-lymphocyte cell lines. However, different electroporation protocols were required for the two cell types. It was shown that a higher number of electroporation cycles and a higher

Morpholino concentration were needed to achieve the same efficiency in lymphocytes as in fibroblasts (Scaffidi and Misteli 2005). The different conditions required for the two cell types were consistent with findings presented by other groups using the same delivery systems in lymphocytes (Du et al. 2007) and fibroblasts (Rincon et al. 2007).

13.3.2 Effects of PMOs Are Durable, Specific, and Impact on the Function of Neurofibromin

The parallel use of a different control PMO in all of the assays revealed the specificity of the mutation-specific PMOs (Fig. 13.1). Only mutation-specific PMOs had a corrective effect on aberrant splicing in all cases, indicating that the effect of PMO treatment was sequence-specific. The stability and duration of the effect of PMO treatment on aberrant splicing correction were assessed in primary fibroblast cell cultures. Although differences were observed for distinct mutations, near-complete correction was maintained for 20 days after PMO administration in all cases (Pros et al. 2009).

The mode of action of Morpholinos on *NF1* mutations was also investigated. To rule out the possibility that the decreased aberrant transcript levels after PMO treatment was caused by an enhancement of the NMD pathway, fibroblasts from different patients were treated with puromycin (an NMD inhibitor) in the presence or absence of PMO. A total or considerable correction of aberrant splicing after PMO treatment (depending on the mutation) was observed in the presence of puromycin, which indicated that the PMOs acted directly on *NF1* splicing, independently of the NMD mechanism (Pros et al. 2009). Moreover, an increase in wild-type transcript levels (relative to control genes) was observed after PMO treatment, which confirmed that PMO induced correct splicing by blocking the recognition of the new splice site created by the mutation (Pros et al. 2009). Taken together, these results indicate that PMO acts in a sequence-specific manner by preventing the splicing machinery from recognizing the newly created aberrant splice sites in the *NF1* gene.

Finally, since neurofibromin function cannot be assessed directly, an indirect functional analysis was performed to confirm that correction of aberrant splicing by PMO treatment was also attained at the functional level. Since neurofibromin is a well-characterized negative regulator of Ras, Ras-GTP levels in patient-derived primary fibroblast cultures were measured. Fibroblast cultures carrying four different deep intronic mutations were evaluated by comparing Ras-GTP levels before and after specific PMO treatment. Cell lysates were prepared, and a Ras activation assay was performed (Fig. 13.2). Levels of active Ras-GTP in untreated fibroblast cultures from all patients were higher than the levels found in the wild-type control fibroblasts, which was consistent with the observation of lower neurofibromin activity in mutant fibroblasts. However, Ras-GTP levels of mutant fibroblasts

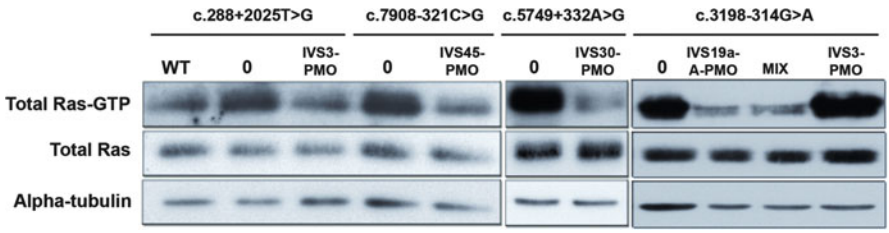

Fig. 13.2 Reduction of Ras-GTP levels after PMO treatment of patient fibroblasts. Neurofibromin GTPase activity was indirectly assessed by quantifying Ras-GTP levels in cell lysates. In all cases, mutation-specific PMO treatment decreased active Ras to levels comparable to those of WT control fibroblasts, presumably by restoration of neurofibromin function. The use of a nonspecific PMO for mutation g.3198-314G>A (IVS45-PMO 20 μM) showed no effect on the levels of Ras-GTP

decreased significantly with PMO treatment, reaching levels comparable to those of wild-type control fibroblasts. This result not only suggested that PMO treatment corrected the aberrant splicing but also indicated that this correction led to the restoration of WT neurofibromin, increasing its overall GAP activity toward Ras proteins.

13.4 AON Therapeutics: From Bench to Bedside

No definitive conclusions have been reached about the in vivo applicability of AON technology for the treatment of NF1 patients with deep intronic mutations; to date, no preclinical animal model or a clinical trial has been performed using this antisense technology for NF1. However, the success of recent clinical trials using AON-directed exon 51 skipping in patients with Duchenne muscular dystrophy (DMD) has created reasonable expectations of eventual success (trials are currently being extended to other exons) (reviewed in Muntoni and Wood 2011; van Putten and Aartsma-Rus 2011). The path from bench to bedside using AON technology is subject to the step-by-step fulfillment of certain requirements, as is the case for any therapeutic agent to treat human disease. The steps comprise in vitro proof of concept followed by the successive phases of clinical trials. The best example of AON technology as a therapeutic strategy is DMD. First, a proof of principle was obtained using cultures of healthy and patient-derived primary human myoblasts (Aartsma-Rus et al. 2002, 2003, 2004). A mouse model of DMD, *mdx* mice, was used for in vivo preclinical trials using different AON chemistries (Goyenvalle et al. 2010; Heemskerk et al. 2009; Wu et al. 2008; Yin et al. 2009). Two exploratory clinical trials using AONs were performed, with the aim of correcting the reading frame of mRNAs encoding a truncated form of dystrophin and producing transcripts similar to those found in patients with Becker muscular dystrophy (a milder form of the disease). The approaches in both trials consisted in forcing the

skipping of exon 51 of the *DMD* gene. One used 2′OMePS (PRO051) (van Deutekom et al. 2007) and the other morpholino oligomers (AVI-4658) (Kinali et al. 2009). In both cases, the direct injection of AON into the muscle corrected significant levels of dystrophin expression, proving the feasibility of the technology without generating clinically important side effects. In a recent phase, I–IIa clinical trial, systemic administration by subcutaneous injection of PRO051 was found to correct dystrophin expression, with no serious adverse events and a modest improvement in the walking capacity of treated patients, although with considerable variance between patients (Goemans et al. 2011). The first systemic clinical trial with PMOs also showed satisfactory levels of dystrophin restoration after treatment (Cirak et al. 2011). More clinical trials for DMD are currently in progress, including phase III trials and some studies involving other exons of the *DMD* gene (Muntoni 2010; van Putten and Aartsma-Rus 2011). For other muscular dystrophies and other diseases, AON therapy is at different points on the path from bench to bedside (Muntoni 2010; van Putten and Aartsma-Rus 2011).

Certain challenges remain in the development of AON therapies (Goyenvalle et al. 2010), such as ensuring efficient AON delivery to all affected tissues or even directing tissue-specific targeting. The first AON chemistries used in clinical trials exhibited poor cellular uptake and a relatively rapid clearance from the circulation, requiring repeated administration. However, recent developments using cell-penetrating peptides conjugated to PMOs (PPMO) or Vivo-Morpholinos have addressed most of these delivery issues, representing a major step forward and revealing what could prove to be an effective strategy, although with room for improvement (Moulton and Jiang 2009; Muntoni 2010).

There are certain limitations to the development of AON therapies, mainly as a result of regulatory constraints and safety considerations. Data collected from the first DMD clinical trials are encouraging, but the number of patients involved has been low in number, the final dosing regimens need to be properly established, and data for treatments longer than 1 year must be provided. It has not yet been confirmed that long-term AON treatment is safe and improves the DMD patient's quality of life, so a cautious view should be taken for now. Given that each AON is mutation-specific (or exon-specific, in the case of forcing skipping), one of the *caveats* of clinical trials using this technology is the low number of patients that could be recruited, since a personalized AON approach will be required for most diseases. Under current regulations, each AON is considered a new drug, so each AON targeting a different exon or mutation would have to undergo the many different steps of clinical development, with all the costs that this process entails (Muntoni 2010). To overcome this hurdle, and since AONs of a certain chemical class generally show more similarities than differences, the same type of chemically identical AONs should be treated as a single drug class, irrespective of the mutation or exon involved, as this would enable sufficient clinical data to be accumulated to develop the technology as a valid therapy for deep intronic mutations and other RNA mis-splicing diseases.

Much work remains to be done in the case of NF1, starting with preclinical animal models. If successful, the foundations provided by DMD AON therapeutics

may facilitate clinical trials with NF1 patients, potentially leading to reasonable treatment opportunities for NF1 patients with deep intronic alterations causing missplicing and possibly also for patients with other AON-treatable *NF1* mutations.

13.5 Conclusions

Some NF1-causing mutations are located well inside intronic regions of the *NF1* gene. These deep intronic mutations lead to the inclusion of a cryptic exon in the *NF1* mRNA, eventually generating an aberrant neurofibromin. We reviewed a body of results that prove the effectiveness of PMOs in correcting abnormal splicing produced by these mutations in the *NF1* gene. Lymphocyte and fibroblast primary cultures derived from seven NF1 patients, bearing four independent *NF1* mutations, were tested. In all cases, PMOs restored the correct splicing of the gene and neurofibromin function, indirectly evaluated by the capacity to downregulate Ras-GTPase. Overall, these results represent a proof of concept for the in vitro capacity of PMOs to correct the effect of the NF1-causing mutations examined. The success of current clinical trials using AON technology to treat DMD patients should spur the development of NF1 preclinical animal models. Deep intronic mutations account for approximately 2 % of all *NF1* constitutional mutations reported to date. AON technology could be applied to patients carrying this type of mutation and perhaps also other types of mutation that generate aberrant *NF1* transcripts.

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Chapter 14
NF1 Microdeletions and Their Underlying Mutational Mechanisms

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14.1 Types of *NF1* Microdeletions and Their Estimated Frequency

The most common type of recurrent *NF1* microdeletion is the type 1 *NF1* deletion which spans 1.4 Mb. The breakpoints of type 1 deletions are located within LCRs termed NF1-REPa and NF1-REPc (Dorschner et al. 2000; Jenne et al. 2001; López-Correa et al. 2001). Type 1 deletions are associated with the loss of 14 protein-coding genes including *NF1* and two microRNA genes (*MIR365-2* and *MIR193a*) (Fig. 14.1). The majority of type 1 *NF1* microdeletions are maternally inherited germ-line deletions (Upadhyaya et al. 1998; López-Correa et al. 2000; Steinmann et al. 2008) whose breakpoints are frequently located within two hotspot regions of meiotic nonallelic homologous recombination (Jenne et al. 2001; López-Correa et al. 2001; Forbes et al. 2004; De Raedt et al. 2006). It has been estimated, from the study of large cohorts of NF1 patients, that 70–80 % of all *NF1* microdeletions are type 1 (Messiaen et al. 2011; Pasmant et al. 2010).

Type 2 *NF1* microdeletions encompass 1.2 Mb and are associated with the loss of 13 genes including *NF1*. Their breakpoints are located within the *SUZ12* gene and its pseudogene *SUZ12P* which immediately flank the NF1-REPs (Fig. 14.1). Type 2 *NF1* deletions are less frequently encountered than the type 1 *NF1* deletions, only 10–20 % of all *NF1* microdeletions being of type 2 (Kehrer-Sawatzki et al. 2004; Messiaen et al. 2011). Both type 2 and type 1 *NF1* deletions are considered to be recurrent because the breakpoints of the deletions in unrelated patients are located within highly homologous sequences. In the case of the type 2 deletions,

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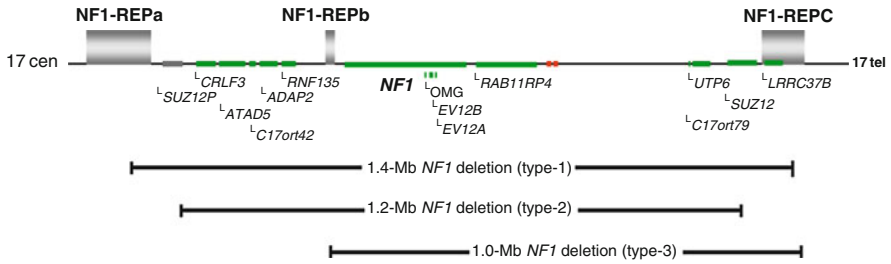

Fig. 14.1 Schematic representation of the *NF1* gene region. The relative positions of the three NF1-REPs are indicated, together with the protein-coding genes located within this region (*green bars*) and the two microRNA genes (*MIR365-2* and *MIR193a*), which are shown as *red bars*. The relative extents of the type 1, type 2 and type 3 *NF1* microdeletions are also shown

these breakpoints reside within the *SUZ12* gene and its derived pseudogene, whereas the breakpoints of type 1 *NF1* microdeletions are located within the NF1-REPa and NF1-REPc.

Also recurrent are the type 3 *NF1* microdeletions which are characterized by breakpoints located within NF1-REPb and NF1-REPc (Bengesser et al. 2010; Pasmant et al. 2010; Zickler et al. 2012). Type 3 deletions span 1.0 Mb and encompass 9 protein-coding genes (Fig. 14.1). Compared with type 1 and type 2 deletions, type 3 deletions occur much less frequently. Indeed, type 3 *NF1* microdeletions account for only 1.4–4 % of all large *NF1* deletions (Messiaen et al. 2011; Pasmant et al. 2010).

In addition to the three basic types of recurrent *NF1* microdeletion, atypical *NF1* deletions have been described which do not have recurrent breakpoints. Extended sequence homology is not evident at the breakpoints of these deletions. Indeed, atypical *NF1* microdeletions are heterogeneous in terms of their size, breakpoint position and the number of genes located within the deleted region (Mantripragada et al. 2006; Kehrer-Sawatzki et al. 2008 and references therein; Pasmant et al. 2008, 2010). An estimated 8–10 % all *NF1* microdeletions are atypical (Messiaen et al. 2011; Pasmant et al. 2010).

14.2 Mosaicism and *NF1* Microdeletions

The postzygotic occurrence of mutations, which leads to mosaicism with normal cells, can influence the clinical manifestation of disease as well as the transmission risk (Erickson 2010; Gottlieb et al. 2001; Kehrer-Sawatzki and Cooper 2008; Youssoufian and Pyeritz 2002). Mosaicism with normal cells is observed in at least 10 % of all patients harbouring *NF1* microdeletions (Messiaen et al. 2011). A certain bias has been noted with regard to the type of *NF1* microdeletion and the frequency of mosaicism. Thus, in individuals with type 2 *NF1* deletions, mosaicism with normal cells is quite frequently observed (Petek et al. 2003; Kehrer-Sawatzki et al. 2004; Roehl et al. 2010,

2012; Steinmann et al. 2007), implying that these type 2 deletions must have occurred postzygotically. At least 44 % of all patients with type 2 *NF1* deletions exhibit somatic mosaicism (Messiaen et al. 2011) although, in practice, the proportion of patients with mosaic type 2 *NF1* deletions may well be considerably higher (Kehrer-Sawatzki et al. 2004). Conspicuously, the proportion of cells harbouring the deletion in blood samples of patients with type 2 deletions is very high, in most cases exceeding 90 % (Kehrer-Sawatzki et al. 2004; Roehl et al. 2010; Steinmann et al. 2007). Significantly lower proportions of cells with the type 2 *NF1* microdeletions have been noted in urine-derived and buccal epithelial cells (Roehl et al. 2012). Thus, at an early developmental stage, it would appear that hematopoietic stem cells carrying the *NF1* microdeletion exhibit a selective growth advantage over normal cells lacking the deletion, leading to a high proportion of cells with the deletion in peripheral blood from the affected patients (Roehl et al. 2012).

In stark contrast to type 2 deletions, only 2–4 % of all patients with de novo type 1 *NF1* microdeletions exhibit mosaicism (Messiaen et al. 2011). The frequency of somatic mosaicism, indicative of a postzygotic origin for type 3 *NF1* deletions, is unknown; indeed, it is not known whether it occurs at all. Only 11 patients with this deletion type have been analysed to date, and none of them has been found to exhibit mosaicism with normal cells (Zickler et al. 2012).

14.3 Mechanisms Underlying *NF1* Microdeletions

14.3.1 NAHR and Recurrent NF1 Microdeletions

Nonallelic homologous recombination (NAHR) is the major mechanism underlying the recurrent type 1, type 2 and type 3 *NF1* microdeletions. NAHR is one of the main mechanisms underlying polymorphic and disease-associated copy number variants (CNVs) in the human genome (Conrad et al. 2010; Kidd et al. 2010; Mills et al. 2011; Neumann et al. 2010; Stankiewicz and Lupski 2010). The molecular processes and determinants underlying NAHR are assumed to be similar to those of meiotic allelic homologous recombination (AHR), but, instead of an allelic homologous template, NAHR employs a very similar yet nonallelic template to repair the initiating double-strand break (DSB) (reviewed by Sasaki et al. 2010). During meiosis as well as mitosis, homologous recombination represents a very precise repair mechanism for DNA lesions such as DSBs (Mao et al. 2008). Consequently, small additional aberrations, such as insertions of a few nucleotides, are not detected at the breakpoint junctions repaired by homologous recombination, using either an allelic or a nonallelic template. Chromosomal regions harbouring multiple duplicated sequences, such as the *NF1* gene region, are inherently prone to recurrent NAHR-mediated rearrangements. Among the duplicated sequences located within the *NF1* gene region are low-copy repeats termed NF1-REPs which disclose a modular structure of different duplicated subunits or paralogous

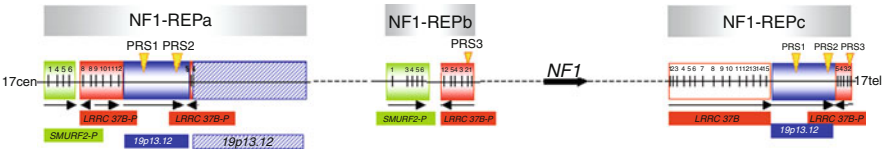

Fig. 14.2 Sequence composition of the NF1-REPs which contain different segmental duplications (subunits) highlighted in different colours. The *LRRC37B*-P sequences in NF1-REPa, NF1-REPb and NF1-REPc are marked in *red*. The functional *LRRC37B* gene is located within the centromeric portion of NF1-REPc and comprises 15 exons. The sequences with homology to chromosome 19p13.2 are indicated in *blue*. The relative positions of the NAHR hotspots, PRS1, PRS2 and PRS3, located within the NF1-REPs, are indicated by *yellow arrowheads*

sequences (Fig. 14.2). NF1-REPa spans ~130 kb and is located 370 kb centromeric to the *NF1* gene, whereas NF1-REPc is 75 kb in length and lies 640 kb telomeric to the *NF1* gene. NF1-REPb, located 44 kb centromeric to *NF1,* is the shortest of the three LCRs, spanning only ~43 kb. NF1-REPs a and c contain paralogous sequences with high similarity to sequences located at 19p13.12 (Jenne et al. 2003; Forbes et al. 2004; De Raedt et al. 2004). These paralogous sequences derive from the latrophilin-1 precursor gene (*LPHN1*) gene located at 19p13.12 (Forbes et al. 2004). By contrast, no sequences with homology to 19p13.12 are present within NF1-REPb as schematically shown in Fig. 14.2. Another group of paralogous sequences located within the NF1-REPs are pseudogene copies of the functional *LRRC37B* gene, which is located within NF1-REPc. *LRRC37B* pseudogene fragments are present within all three NF1-REPs (Fig. 14.2; Jenne et al. 2003; Forbes et al. 2004; De Raedt et al. 2004).

In addition to the *LRRC37B* sequences, NF1-REPs a and b also contain pseudogene fragments of the *SMURF2* (SMAD-specific E3 ubiquitin protein ligase 2) gene, which are absent from NF1-REPc (Fig. 14.2). Taken together, the NF1-REPs comprise different paralogous sequence blocks that form the characteristic structures of each NF1-REP. Importantly, the sequence homology between some of these paralogous sequences exceeds 95 % over more than 9 kb. This high degree of homology, as well as the distance between the LCRs, appears to allow for ectopic pairing of the paralogous sequences and may elicit NAHR between them.

It is widely accepted that NAHR is best explained by the double-strand-break (DSB) repair model which was first described in yeast (Szostak et al. 1983). According to this model, the recombination event (allelic or nonallelic) is initiated in the prophase of meiosis I by a DSB (Fig. 14.3A). A heteroduplex DNA structure that can migrate is then generated and converted to a double Holliday junction which is subsequently resolved. Depending upon whether or not this resolution is associated with a crossover, NAHR or nonallelic gene conversion is observed (NAHGC; Fig. 14.3). Hence, NAHGC and NAHR represent alternative processes, but only NAHR generates rearrangements such as deletions or duplications. NAHR can occur between chromosomes (interchromosomal NAHR) or within one chromosome or chromatid (intrachromosomal NAHR). Type 1 *NF1* deletions have been shown to arise by interchromosomal NAHR during maternal meiosis I (López-Correa et al. 2000; Steinmann et al. 2008). By contrast, mosaic type 2 *NF1* deletions are invariably caused

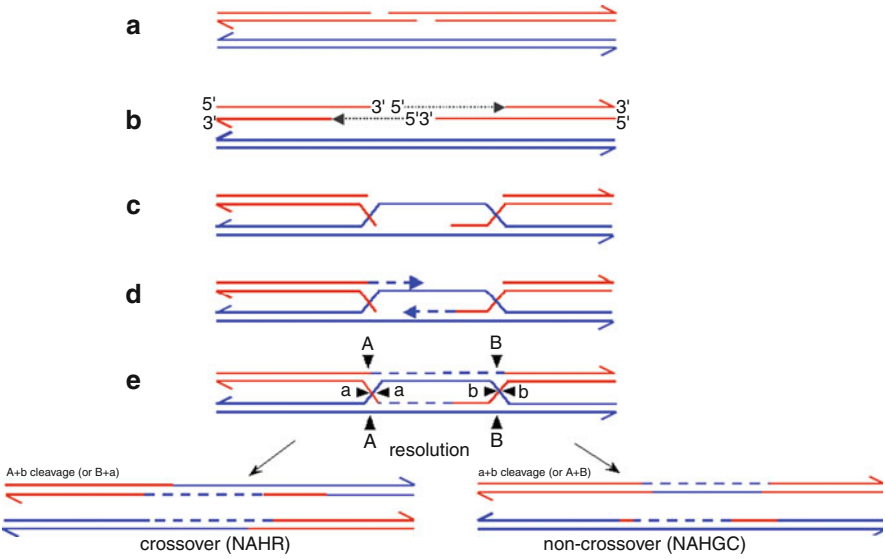

Fig. 14.3 Nonallelic homologous recombination (NAHR) between highly homologous sequences such as the NF1-REPs according to the double-strand-break repair model as outlined for allelic homologous recombination by Szostak et al. (1983). (A) The paralogous sequences involved in the recombination event are indicated by *red* and *blue lines*. The paralog indicated in *red* undergoes a double-strand break (DSB) which initiates recombination. (B) The open ends are further resected in a $5' \rightarrow 3'$ direction. (C) Invasion of the broken strand into the unbroken double strand leads to the formation of a heteroduplex DNA. (D) DNA synthesis and second-end capture lead to the formation of a double Holliday junction (dHJ). (E) Enzymes cutting the DNA strands at two positions resolve the dHJ. Depending upon which strand is cut, a crossover of the chromatids either occurs or does not occur. The crossover resolution is associated with a deletion on one chromosome and a duplication on the other. The resolution of the noncrossover, as well as the subsequent repair of sequence mismatches, results in nonallelic homologous gene conversion (NAHGC) which can be detected by the nonreciprocal transfer of paralogous sequence variants (PSVs). PSVs are non-polymorphic sites specific to each LCR. During recombination, PSVs specific for one LCR can be transferred to its paralog, leading to the introduction of a SNP at the paralogous position of the recipient LCR

by intrachromosomal NAHR, and no preponderance of the maternal or paternal chromosome was observed; this is not completely unexpected if we consider the postzygotic origin of these deletions (Roehl et al. 2010). The preponderance of interchromosomal NAHR between NF1-REPa and NF1-REPc during maternal meiosis may be causally associated with the long dictyotene stage, a unique feature of female meiosis I. The dictyotene stage follows the diplotene stage, which is common to both female and male meiotic prophase I. During the diplotene stage, the synaptonemal complex between homologous chromosomes degrades, and homologous chromosomes slightly separate from one another but nevertheless remain tightly bound at the chiasmata, the regions where crossover occurred. The chiasmata between homologous chromosomes persist until they are separated during anaphase I. In human fetal oogenesis, all oocytes develop up to this stage before entering into the dictyotene

stage. Female meiosis arrests at the dictyotene stage for many years until meiosis continues after puberty (Vogel and Motulsky 1996). By contrast, such an extended arrest in the dictyotene stage is not observed during male meiosis I, which lasts for only a few days (Vogel and Motulsky 1996). It may well be that the extended dictyotene stage during female meiosis is causally associated with the preponderance of interchromosomal NAHR between NF1-REPa and NF1-REPc causing type 1 *NF1* microdeletions. Although ectopic pairing between the NF1-REPs may occur at similar frequencies in male and female meiosis, DSBs triggering NAHR are more likely to occur during the extended dictyotene stage of oocytes. Another hypothesis to explain the preferential occurrence of interchromosomal NAHR between NF1-REPa and NF1-REPc during female meiosis is that oocyte-specific DNA sequence and/or histone modifications could trigger NAHR. It may also be that sperm carrying type 1 *NF1* deletions are either less viable or less frequently involved in fertilization; this could explain why paternally inherited type 1 *NF1* deletions are much less frequently observed than maternally inherited deletions.

As yet, nothing is known about the chromosomal origin of the NAHR events underlying type 3 *NF1* deletions. This is due to the fact that only 11 patients with type 3 deletions have so far been identified, and family studies, using microsatellite markers to determine whether the deletions occurred by inter- or intrachromosomal recombination, could not be performed (Zickler et al. 2012). The parental origin was identified in 5 of these 11 type 3 *NF1* deletions. In four of them, the deletion had occurred on the paternal chromosome 17. Since mosaicism with normal cells was not evident in these patients, the type 3 deletions are assumed to have occurred during paternal meiosis (Zickler et al. 2012). However, the occurrence of these type 3 deletions during premeiotic mitotic cell cycles cannot yet be unequivocally excluded. In contrast to type 3 deletions, most type 1 *NF1* deletions are maternally inherited (López-Correa et al. 2000; Steinmann et al. 2008).

14.3.2 NF1 Microdeletions and NAHR Hotspots

The analysis of the recurrent type 1 and type 3 *NF1* microdeletions, together with the analysis of several other known NAHR-mediated rearrangements, revealed that the respective breakpoints generally occur within hotspots of a few hundred base pairs (Lindsay et al. 2006; Reiter et al. 1996, 1998; Turner et al. 2008; Visser et al. 2005). The breakpoints of type 1 *NF1* microdeletions cluster within NAHR hotspots termed paralogous recombination sites 1 and 2 (PRS1 and PRS2) (De Raedt et al. 2006; Forbes et al. 2004; Jenne et al. 2001; López-Correa et al. 2001). PRS1 and PRS2 are located within the region of NF1-REPa and NF1-REPc with homology to chromosome 19p13.12 (Fig. 14.2). The majority of type 1 *NF1* deletions (67 %) have breakpoints within the PRS2 NAHR hotspot, whereas only 22 % of type 1 deletion breakpoints cluster within PRS1 (De Raedt et al. 2006).

Type 3 *NF1* microdeletion breakpoints also cluster within an NAHR hotspot. So far, 11 patients with type 3 deletions have been identified, and the breakpoints of 10

of these 11 patients are located within this 1-kb hotspot, termed PRS3 (Bengesser et al. 2010; Zickler et al. 2012). PRS3 is located within the *LRRC37B* pseudogene of the NF1-REPb and NF1-REPc low-copy repeats. The latter exhibit 97.8 % sequence identity over a 9.2-kb region and are located in direct orientation (Fig. 14.2). The *LRRC37B*-P paralogs are pseudogenes of the functional *LRRC37B* gene which is located within the proximal portion of NF1-REPc, albeit in inverted orientation as compared with the *LRRC37B* pseudogenes. The *LRRC37B*-P paralogs, as well as the sequences with homology to chromosome 19 located within the NF1-REPs, are readily identifiable in the orangutan genome, and hence, their amplification must have preceded the divergence of the orangutan lineage from the common ancestor of the other great apes 12–16 Mya. Within NF1-REPc, PRS3 lies 12-kb telomeric to PRS2 and 31-kb telomeric to PRS1. The *LRRC37B* gene also contains a region paralogous to PRS3 with sequence homology of >98 % to NF1-REPb and NF1-REPc. The region within *LRRC37B* with homology to PRS3 encompasses parts of intron 1 and exon 2 (Zickler et al. 2012).

Currently, it is not entirely clear what kind of sequence features determine the position and activity of NAHR hotspots. Both NAHR- and AHR-associated crossovers cluster within narrow hotspot regions of 500 bp to 2 kb during meiosis (Jeffreys et al. 2001; Crawford et al. 2004; McVean et al. 2004; Coop et al. 2008; Webb et al. 2008). Consequently, clustering of breakpoints within specific genomic regions would appear to be a general feature of meiotic homologous recombination, and it is reasonable to assume that AHR and NAHR are related processes. Hence, conclusions concerning the sequence features pertaining to NAHR may also be deduced from the detailed analysis of AHR hotspots. *Cis*- and *trans*-acting factors or sequences have been identified that activate AHR hotspots (Jeffreys and Neumann 2002, 2005; Peters 2008; Ubeda and Wilkins 2011; Zheng et al. 2010). The best characterized among them is the 13-mer degenerate sequence motif (CCNCCNTNNCCNC) which is thought to play a crucial role in recruiting crossover events to 40 % of all AHR hotspots (Myers et al. 2008). The histone methyltransferase PRDM9 binds to this 13-mer motif via its array of zinc fingers and acts in *trans* as a recombination-initiating factor at AHR hotspots (Baudat et al. 2010; Berg et al. 2010; Parvanov et al. 2010). The *PRDM9* gene is highly polymorphic in humans, particularly with respect to its encoded zinc-finger array domain, and this genetic variation serves to influence AHR hotspot activity (Baudat et al. 2010; Berg et al. 2010). However, on their own, *PRDM9* variants only account for 18 % of the population variance in hotspot utilization (Baudat et al. 2010). Consequently, additional regulators of AHR hotspot activity must exist.

Genetic variation at the *PRDM9* locus also affects NAHR activity as shown for the CMT1A-REP-mediated duplications and deletions in 17p11.2–p12 (Berg et al. 2010). In the sperm of healthy donors homozygous for the *PRDM9* A allele, de novo rearrangements between the CMT1A-REPs have been observed to occur with a >20-fold higher frequency than in individuals homozygous for non-A alleles (Berg et al. 2010). Furthermore, the 13-mer sequence motif, CCNCCNTNNCCNC, has been found in a total of five different well characterized NAHR hotspots including PRS2 responsible for type 1 *NF1* deletions (Myers et al. 2008). However,

this 13-mer motif, CCNCCNTNNCCNC, is absent from both PRS1 and PRS3. Estimated from the relative frequencies of type 1 and type 3 deletions with breakpoints located in their respective hotspots, PRS2 represents a much more intense hotspot than either PRS1 or PRS3. Hence, the presence or absence of the 13-mer motif may, to some extent, determine NAHR hotspot intensity. However, it is unlikely that the 13-mer motif is the sole determinant for NAHR hotspot activity. Other as yet unknown sequences or binding factors that influence NAHR frequency and breakpoint localization are likely to exist.

PRS1, 2 and 3 hotspots mediating *NF1* microdeletions may be distinguished not only by the frequency of the NAHR events that occur within these hotspots but also by their variation patterns as measured by the number of SNPs per kb (De Raedt et al. 2006; Zickler et al. 2012). Gene conversion is often increased within NAHR hotspots as well as AHR hotspots (Baudat and de Massy 2007). Importantly, gene conversion caused by homologous recombination without crossovers would appear to be even more frequent than recombination associated with crossovers. Indeed, allelic gene conversion events are thought to occur 4–15 times more frequently than AHR events associated with crossovers (Jeffreys and May 2004). The consequence of frequent gene conversion is an increase in the number of SNPs because sequence differences between the LCRs involved are exchanged during the recombination process (Fig. 14.3). By these means, paralogous sequence variants are transformed into SNPs. In the PRS1 and PRS2 hotspots within NF1-REPa and NF1-REPc, a SNP frequency of 5–11 SNPs/kb has been observed which is indicative of frequent nonallelic homologous gene conversion (NAHGC) (De Raedt et al. 2006). A high number of SNPs (7 SNPs/kb) was also noted within PRS3 of NF1-REPc; this represents a ~7-fold increase in SNP density as compared with the genomic average (Sachidanandam et al. 2001; Frazer et al. 2007). By contrast, the SNP frequency within PRS3 in NF1-REPb and *LRRC37B* was much lower (~2 SNPs/kb) (Zickler et al. 2012). This difference in SNP density observed for PRS3 located in NF1-REPb as compared to PRS3 located in NF1-REPc may be explicable in terms of the polarity of the gene conversion events involved. Unidirectional sequence transfer may occur during recombination from NF1-REPb to NF1-REPc, resulting in a higher SNP density in NF1-REPc but not in NF1-REPb. According to this hypothesis, NF1-REPc should be the recipient disproportionately often during recombination (Zickler et al. 2012). As depicted in Fig. 14.2, the recombination-initiating double-strand break occurs on the recipient sequence (i.e. NF1-REPc), which receives a "patch" of DNA from the intact donor sequence used as a template for the DSB repair (i.e. NF1-REPb) during homologous recombination without crossover. The consequence of unidirectional sequence transfer from NF1-REPb to NF1-REPc would be that the recipient sequence (NF1-REPc) contains a higher number of SNPs than the unbroken donor strand (NF1-REPb), as is observed. By contrast, bidirectional sequence transfer during NAHGC implies the involvement of both LCRs, with an equal probability, in DSB induction before invasion of the other strand. Consequently, both strands would be expected to acquire SNPs at similar

rates during the process of NAHGC, leading to high SNP frequencies in both recombining LCRs. Thus, bidirectional gene conversion events most likely account for the observed elevated SNP frequency within PRS1 and PRS2 of both NF1-REPa and NF1-REPc (De Raedt et al. 2006).

In addition to differences in the directionality of gene conversion events within PRS1 and PRS2 as compared with PRS3, further differences between these hotspots have been noted which are likely to have affected ongoing sequence exchange with their progenitor loci. PRS1 and PRS2 within NF1-REPa and NF1-REPc are not involved in sequence exchange with their progenitor locus located on chromosome 19 (De Raedt et al. 2006). By contrast, the sequence of origin of these *LRRC37B* pseudogenes harbouring PRS3, the *LRRC37B* gene, is actively involved in NAHGC with PRS3 within NF1-REPc (Zickler et al. 2012). Hence, sequence exchange with its progenitor locus and unidirectional sequence transfer may account for the unusual variation pattern at PRS3 within NF1-REPc. Additionally, the proximity to two active NAHR hotspots may have influenced the emergence of PRS3 within NF1-REPc (Fig. 14.2). The spatial proximity of PRS3 to PRS1 and PRS2 within NF1-REPc (but not in NF1-REPb) may have facilitated the recent emergence of this NAHR hotspot PRS3 as well as its characteristic feature of an increased level of polymorphic variation (Zickler et al. 2012).

As mentioned above, AHR and NAHR are assumed to be mechanistically very similar. Hence, it is reasonable to suppose that regions of increased NAHR activity will also experience frequent AHR. In agreement with this prediction, the PRS1 and PRS2 hotspots, as well as the NAHR hotspots located within the CMT1A-REPs, overlap with pre-existing AHR hotspots (De Raedt et al. 2006; Lindsay et al. 2006). PRS1 and PRS2 are located within NF1-REPa and NF1-REPc within regions of homology to chromosome 19p13.12 (Fig. 14.2). The progenitor locus of these sequences, termed REP19, contains strong AHR hotspots within the regions paralogous to the NAHR hotspots PRS1 and PRS2 (De Raedt et al. 2006). Moreover, the NAHR hotspots PRS1 and PRS2 are themselves located within NF1-REPa and NF1-REPc and overlap with weak AHR hotspots. These findings provide good evidence for the conservation of recombination hotspot patterns within paralogous sequences (De Raedt et al. 2006). However, it may also be that NAHR hotspots can emerge de novo rather than invariably originating within regions of pre-existing AHR breakpoints. In favour of this hypothesis is the observation that the PRS3 NAHR hotspot mediating type 3 *NF1* microdeletions has not developed on a pre-existing AHR hotspot (Zickler et al. 2012). PRS3 represents a weak and probably evolutionarily rather young NAHR hotspot with unique sequence properties distinct from PRS1 and PRS2.

Taken together, the analysis of the breakpoints of recurrent *NF1* microdeletions has yielded important new information on the biology and sequence characteristics of meiotic NAHR hotspots of differing intensities.

14.3.3 Mitotic and Meiotic NAHR Mediate NF1 Microdeletions

Whereas type 1 and probably also type 3 *NF1* microdeletions are mostly of meiotic origin, the majority of type 2 deletions occur during early embryonic development (before gastrulation) and are mediated by mitotic NAHR (Roehl et al. 2010, 2012). Interestingly, a marked female preponderance has been observed among patients with mosaic type 2 deletions which contrasts with the equal sex distribution noted for the other types of *NF1* microdeletion. The reasons for this female preponderance are unknown.

The analysis of the mechanisms underlying the recurrent *NF1* microdeletions has the potential to indicate differences and similarities between mitotic and meiotic NAHR in the *NF1* gene region. The most striking difference would appear to be that the mitotic NAHR breakpoints of type 2 *NF1* microdeletions do not cluster within narrow regions of only a few hundred base pairs which would be clearly identifiable as NAHR hotspots. By contrast, the breakpoints of type 1 and type 3 deletions unequivocally cluster in hotspots of NAHR activity (López-Correa et al. 2001; De Raedt et al. 2006; Zickler et al. 2012). Mosaicism with normal cells, and hence a postzygotic origin of type 1 *NF1* microdeletions, would appear to be rare. It has been estimated that only 2 % of all type 1 *NF1* deletions occur via postzygotic NAHR, with breakpoints located within the PRS2 and PRS1 hotspots (Messiaen et al. 2011). Other NAHR hotspots in the human genome have been shown to operate exclusively during meiosis and not at all during mitosis (Turner et al. 2008).

Although type 2 *NF1* deletion breakpoints do not cluster within distinctive hotspots, they are non-randomly distributed. A disproportionate number of short sequences capable of forming non-B DNA were noted within the recombination regions (RRs) of type 2 deletions (Roehl et al. 2010). These repeats may well have contributed to the formation of DSBs that triggered the NAHR events which ultimately gave rise to the type 2 *NF1* microdeletions. Furthermore, *Alu* elements were noted at the breakpoints of 60 % of the type 2 deletions mediated by intrachromosomal NAHR (Roehl et al. 2010). *Alu* elements are well-known recombinogenic sequences (reviewed by Konkel and Batzer 2010) and hence are likely to have contributed to local DSB formation and subsequent NAHR underlying the type 2 deletions.

The detailed analysis of the sequences involved in meiotic and mitotic NAHR causing *NF1* microdeletions indicated differences in GC content and in the DNA duplex stability of these sequences. Whereas sequences flanking meiotic NAHR breakpoints located within NF1-REPa and NF1-REPb are characterized by high GC content and high DNA duplex stability, the type 2 deletion breakpoints associated with the mitotic NAHR events are located within regions of significantly lower GC content and DNA stability (Roehl et al. 2010).

The chromosomal mechanisms underlying type 2 and type 1 *NF1* microdeletions also appear to differ considerably. Whereas type 1 deletions are caused by interchromosomal NAHR, type 2 deletions occur within on chromosome 17, either by intrachromosomal NAHR within one chromatid or between the sister chromatids

(Roehl et al. 2010). It is reasonable to assume that differences in chromosomal pairing and chromatin structure are causally associated with the preferential occurrence of intrachromosomal postzygotic NAHR which causes the type 2 *NF1* microdeletions. In contrast to the tight and stable pairing of homologous chromosomes within the meiotic synaptonemal complex, the pairing of homologous chromosomes during the mitotic cell cycle is different in nature and is probably much less stable (reviewed by Meaburn and Misteli 2007). Nevertheless, the processes of meiotic and mitotic recombination are likely to share at least some mechanistic similarities. In yeast, homologous recombination during mitosis is mediated by joint molecule intermediates whose strand composition and size are identical to those of the meiotic double Holliday junctions (Bzymek et al. 2010). Double Holliday junctions form preferentially between yeast sister chromatids during mitotic DSB repair, whereas during meiosis, a preference for interhomologue recombination is evident (Bzymek et al. 2010). These observations are analogous to the observed differences in the chromosomal mechanisms underlying *NF1* microdeletions: intrachromosomal NAHR is the major mechanism causing type 2 *NF1* deletions of postzygotic origin, whereas interchromosomal NAHR during maternal meiosis is responsible for the germ-line type 1 *NF1* microdeletions (López-Correa et al. 2000; Roehl et al. 2010).

Mitotic NAHR underlying the type 2 *NF1* microdeletions is likely to occur during the blastocyst stage or even earlier, certainly before embryonic day 12 and the onset of gastrulation. This conclusion may be drawn from the observation that in patients with type 2 *NF1* microdeletions, cells harbouring the deletion were detectable in derivatives of all three germ cell layers, the endoderm (epithelial cells from the urinary system), mesoderm (blood cells) and ectoderm (skin fibroblasts, neurofibroma-derived cells and buccal epithelium) (Roehl et al. 2012). Mitotic NAHR causing *NF1* microdeletions may however also occur somatically later in life, resulting in tumorigenesis. In a dermal neurofibroma from a patient with a germ-line intragenic *NF1* mutation, loss of the wild-type *NF1* allele due to a somatic type 2 deletion has been identified (Garcia-Linares et al. 2011). The analysis of larger series of dermal as well as plexiform neurofibromas has however indicated that somatic inactivation of the *NF1* gene by NAHR-mediated *NF1* microdeletions is not a frequent event causing loss of heterozygosity (Garcia-Linares et al. 2011; Steinmann et al. 2009).

The reason why the *SUZ12* sequences and not the flanking NF1-REPs are the preferred target for NAHR causing *NF1* microdeletions during postzygotic cell cycles has not yet been elucidated. The largest sequence block of high homology (97.5 %) shared by NF1-REPa and NF1-REPc extends over 51 kb (Forbes et al. 2004). The sequence homology between *SUZ12* and *SUZ12P* extends over 45 kb and amounts to 96 % (Roehl et al. 2010). It seems unlikely that these relatively minor differences in the length of the homologous region or the average degree of sequence homology could be responsible for the observed preference in breakpoint position of meiotic versus mitotic NAHR in the *NF1* gene region. Other yet unknown features are assumed to be responsible for causing the differences in breakpoint localization.

14.3.4 *Atypical* NF1 *Microdeletions and Their Causative Mechanisms*

The mutational mechanisms underlying atypical *NF1* microdeletions with non-recurrent breakpoints are less well characterized than those mediated by NAHR. So far, 41 atypical *NF1* microdeletions have been reported in the literature (Cnossen et al. 1997; Dorschner et al. 2000; Jenne et al. 2001; Kayes et al. 1992, 1994; Kehrer-Sawatzki et al. 2003, 2005, 2008; Mantripragada et al. 2006; Pasmant et al. 2008, 2009, 2010; Riva et al. 2000; Venturin et al. 2004a, 2004b; Upadhyaya et al. 1996). However, only in six of these 41 deletions have the breakpoints been characterized at the base-pair level (Table 14.1). The sequences at the centromeric and telomeric breakpoints of these six deletions did not exhibit extended sequence homology over several hundred base pairs, a finding which appears to exclude homologous recombination as the mechanism underlying these deletions. Furthermore, small insertions of a few base pairs or other rearrangements such as inversions were not noted at the respective deletion breakpoints. Hence, it is reasonable to assume that non-homologous end joining (NHEJ) has mediated these six atypical *NF1* deletions. However, since only six *NF1* microdeletions with non-recurrent breakpoints have so far been analysed, it cannot be excluded that replication-based mechanisms such as "fork stalling and template switching" (FoSteS) or aberrant firing of replication origins could also account for the occurrence of atypical *NF1* microdeletions. Indeed, replication-based errors have been suggested to be responsible for other non-recurrent genome rearrangements associated with various diseases (Ankala et al. 2012; Bauters et al. 2008; Lee et al. 2007; Liu et al. 2011; Vissers et al. 2009; Zhang et al. 2009a, b, 2010). The detailed analysis of the breakpoints of a large series of atypical *NF1* microdeletions will be required to elucidate the role of replication-based errors as the putative mechanism underlying these deletions.

14.4 Genotype/Phenotype Correlations and *NF1* Microdeletions

The analysis of *NF1* microdeletions is of interest not only in the context of the underlying mutational mechanisms but also with regard to genotype/phenotype correlations in NF1. Many studies have suggested that patients with *NF1* microdeletions suffer from a more severe clinical phenotype than patients with intragenic *NF1* mutations (De Raedt et al. 2003; Descheemaeker et al. 2004; Kluwe et al. 2003; Mautner et al. 2010; Mensink et al. 2006; Pasmant et al. 2010; Upadhyaya et al. 1998; Venturin et al. 2004b; Wu et al. 1995, 1999). Although, as a group, patients possessing *NF1* microdeletions would appear to exhibit a more severe form of NF1 than patients with intragenic *NF1* mutations, clinical variability has been observed between some individual patients with large *NF1* deletions (Tonsgard et al. 1997).

Table 14.1 Sequences detected at the breakpoints of the six atypical *NF1* microdeletions characterized to date

Patient	Centromeric breakpoint	Telomeric breakpoint	Length of the deletion	Mutational mechanism	Reference
6[a]	Located within a unique sequence in intron 1 of the *BLMH* (bleomycin hydrolase) gene	Located within a unique sequence within intron 1 of the *ACCN1* (amiloride-sensitive cation channel 1) gene	3 Mb	NHEJ	Venturin et al. (2004a)
442	Located within an intronic partial LINE-element in the *EFCAB5* (EF-hand calcium-binding domain 5) gene	Located within a unique sequence in intron 15 of the *SUZ12* (suppressor of zeste 12) gene	2 Mb	NHEJ	Kehrer-Sawatzki et al. (2005)
552	Located within a unique sequence within intron 21 of the *NF1* gene	Located within a unique sequence within intron 1 of the *ACCN1* (amiloride-sensitive cation channel 1, neuronal) gene	2.7 Mb	NHEJ	Kehrer-Sawatzki et al. (2008)
DUB	Located within a 305-bp AluSx within intron 4 of the *PIPOX* (pipecolic acid oxidase) gene	Located within a 47-bp MIRb (mammalian-wide interspersed repeat type b) within intron 5 of the *GGNBP2* (gametogenin-binding protein 2) gene	7.6 Mb	NHEJ	Pasmant et al. (2008)
NF00028	Located within a 116-bp LINE-element within intron 1 of the *LRRC37B* pseudogene within NF1-REPa	Located within a 96-bp AluSq in intron 1 of the *RAB11FIP4* (RAB11 family-interacting protein 4) gene	837 kb	NHEJ	Pasmant et al. (2009)
NF00358[a]	Located within a unique sequence in intron 1 of the *BLMH* gene	Located within a 307-bp AluSx in intron 3 of the *RAB11FIP4* gene	1.2 Mb	NHEJ	Pasmant et al. (2009)

[a]The centromeric breakpoints in these two patients were nonidentical

Descheemaeker et al. (2004) analysed 11 patients with type 1 *NF1* microdeletions and ascertained their mean full scale IQ (FSIQ) to be 76.0. By contrast, the mean FSIQ in 106 NF1 individuals exhibiting intragenic *NF1* mutations was 88.5. Thus, the mean FSIQ in patients with type 1 *NF1* microdeletions was found to be significantly lower than the mean FSIQ in NF1 patients without such a deletion. Although the average intelligence of the group of patients with a type 1 *NF1* deletion is generally lower than that of the group of patients without an *NF1* microdeletion, a substantial overlap between the two groups has been noted (Descheemaeker et al. 2004). The study of a further 21 patients with molecularly ascertained type 1 *NF1* microdeletions also indicated that type 1 *NF1* microdeletions are disproportionately associated with delayed cognitive development and/or learning disabilities as compared with the general NF1 population (Mautner et al. 2010). Further, mental retardation (IQ < 70) was noted in eight (38 %) of these 21 NF1 patients with type 1 deletions (Mautner et al. 2010). Other features noted to occur disproportionately in patients with type 1 *NF1* microdeletions as compared with NF1 individuals without such deletions were dysmorphic facial features, tall stature, macrocephaly, large hands and feet, hyperflexibility of joints and malignant peripheral nerve sheath tumours (MPNSTs) (Mautner et al. 2010). Whereas the lifetime risk of an MPNST in all NF1 individuals is 8–13 % (Evans et al. 2002; Ingham et al. 2011), patients with *NF1* microdeletions are estimated to have a lifetime risk for an MPNST of 16–26 % (De Raedt et al. 2003; Mautner et al. 2010; Pasmant et al. 2010). Since MPNSTs (and glioma) are the two most common causes of reduced life expectancy among NF1 patients (Evans et al. 2011), it follows that patients with *NF1* microdeletions require intensive clinical care and supervision.

Studies on genotype/phenotype correlations in patients with *NF1* microdeletions also suggested that these patients suffer from a high load of dermal neurofibromas and externally visible plexiform neurofibromas (Kayes et al. 1994; Dorschner et al. 2000; Mautner et al. 2010). Volumetric assessment of the internal tumour load as determined by whole-body MRI in patients with *NF1* microdeletions indicated that, as a group, patients with *NF1* microdeletions do not suffer from a higher internal tumour load than patients lacking *NF1* microdeletions (Kluwe et al. 2012). However, at the level of the single patient, an extremely high tumour burden ($>$3,000 ml of total tumour volume) was significantly more frequent in patients with non-mosaic *NF1* microdeletions than in NF1 patients without *NF1* microdeletions.

Accelerated height growth and carpal bone age have been reported to occur frequently in patients with large *NF1* deletions, especially in pre-school children aged 2–6 years in whom overgrowth is most evident (Spiegel et al. 2005). By contrast, growth retardation and short stature are common in the general NF1 population (Riccardi 1992, 1999; Huson and Hughes 1994). The RING finger protein 135 (*RNF135*) gene located within the type 1 *NF1* microdeletion interval (Fig. 14.1) may well be a good candidate for a modifier gene responsible for the overgrowth observed in patients with *NF1* microdeletions. This assertion stems from the observation that five patients without NF1, but with an overgrowth syndrome associated with dysmorphic facial features and a variable degree of learning disability, were found to harbour mutations within the *RNF135* gene

(Douglas et al. 2007). These five mutations included four different *RNF135* nonsense mutations and one *RNF135* missense mutation. Douglas et al. (2007) identified a sixth patient with an overgrowth syndrome and a deletion of the entire *RNF135* gene as well as its flanking sequences which was mediated by aberrant recombination between NF1-REPa and NF1-REPb, leading to the loss of the complete *RNF135* gene as well as other genes located proximal to the *NF1* gene (but not the *NF1* gene itself). These findings suggest that *RNF135* haploinsufficiency may be responsible also for the overgrowth observed in patients with *NF1* microdeletions (Douglas et al. 2007). Importantly, the dysmorphic facial features observed in the six patients with *RNF135* mutations resembled those features observed in patients with *NF1* microdeletions (Douglas et al. 2007). *RNF135* is widely expressed, but its functions are not yet fully characterized. The encoded protein has a RING finger domain at its N-terminus and a B30.2/SPRY domain at its C-terminus. Via its RING finger domain, RNF135 binds to retinoic acid-inducible gene-I (RIG-I), a cytoplasmic RNA helicase that interacts with a protein on the outer membrane of mitochondria in order to signal the presence of virus-derived RNA. RNF135 facilitates RIG-I-mediated type I interferon induction by ubiquitinating RIG-I, thereby regulating the RIG-I response to minimal copies of viral RNA (Oshiumi et al. 2009). As yet, it is unclear how *RNF135* haploinsufficiency could cause the overgrowth phenotype observed in patients with *NF1* microdeletions and patients with intragenic *RNF135* mutations. In Schwann cells from MPNST biopsies and in MPNST cell lines, both *RNF135* and centaurin-alpha 2 (*CENTA2*), which is also located within the *NF1* microdeletion interval, were found to be downregulated (Pasmant et al. 2011). Hence, Pasmant et al. (2011) suggested that both genes might be involved in the increased risk of malignancy observed in patients with *NF1* microdeletions.

It is tempting to speculate that haploinsufficiency of the *MIR193a* microRNA gene located within the *NF1* microdeletion region may also contribute to the increased tumour risk in patients with large *NF1* deletions. miR-193a inhibits cellular transformation by directly targeting the 3′ untranslated regions of oncogenes such as K-Ras, and miR-193a overexpression inhibits the tumorigenicity of different types of cancer cells (Iliopoulos et al. 2011). Furthermore, miR-193a represses the expression of the proto-oncogene c-kit (Gao et al. 2011). However, the expression level of mi-R193a appears unaltered in plexiform neurofibromas and MPNSTs as compared with dermal neurofibromas (Pasmant et al. 2011). Nevertheless, it cannot be excluded with any degree of certainty that miR-193a is not involved in malignant transformation in NF1, since miR-193a expression is regulated by CpG methylation which may well occur transiently during malignant transformation.

It is reasonable to suppose that the size of the *NF1* microdeletion has an influence on the associated clinical phenotype. Since the different types of *NF1* microdeletion are distinguishable in terms of the number of genes included in the deleted regions, the deletion type may well be of importance with regard to the clinical expression of the disease. However, most of the patients that have been investigated with regard to the associated clinical phenotype have harboured type 1

NF1 deletions. This is presumably because type 1 *NF1* microdeletions are by far the most common type of *NF1* microdeletion. Only two non-mosaic type 2 *NF1* microdeletions have been clinically investigated in greater detail (Vogt et al. 2011). These patients exhibited clinical features (such as high tumour load, learning disabilities, large hands and feet, hyperflexibility of the joints and macrocephaly) which were also reported to occur disproportionally frequently in patients with germ-line type 1 *NF1* deletions. Thus, the severe clinical manifestations frequently observed to be associated with type 1 *NF1* microdeletions may extend to type 2 *NF1* deletions. However, further studies on much larger numbers of patients with non-mosaic type 2 *NF1* microdeletions will be necessary to confirm this assertion.

14.5 Conclusion

The detailed analysis of the breakpoints of many *NF1* microdeletions has indicated that NAHR is the major mechanism underlying recurrent *NF1* microdeletions, whereas those deletions with non-recurrent breakpoints would appear to be frequently caused by NHEJ. However, the full spectrum of mechanisms underlying *NF1* microdeletions and the specific features of these mechanisms are far from being fully understood. For example, it is unclear why meiotic NAHR breakpoints cluster within the NF1-REPs and why the breakpoints of mitotic NAHR events are preferentially located within the *SUZ12* sequences. Further, it is as yet unclear to what extent replication-based errors contribute to the occurrence of atypical non-recurrent *NF1* deletions. It is also unknown whether specific polymorphic structural variants might exist that would predispose to the occurrence of *NF1* microdeletions. Finally, the impact of modifying genes and their potential to account for the more severe clinical phenotype observed in patients with *NF1* microdeletions need to be investigated in greater detail.

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Chapter 15
The Somatic Mutational Spectrum of the *NF1* Gene

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15.1 Introduction

Neurofibromatosis type 1 (NF1) is a common autosomal dominantly inherited tumour predisposition syndrome affecting 1/3,000–4,000 individuals worldwide (Huson et al. 1988; Lammert et al. 2005). NF1 manifests a variety of characteristic features that include hyperpigmentary abnormalities of the skin (café-au-lait macules and inguinal/axillary freckling), iris hamartomas (Lisch nodules) and growth of benign peripheral nerve sheath tumours (neurofibromas) in the skin. Neurofibromas display many different subtypes and are associated with a variety of different clinical complications. Cutaneous neurofibromas are present in almost all adult NF1 patients (Upadhyaya et al. 2007). Plexiform neurofibromas (PNFs), a more diffuse type of tumour, are present in 30–50 % of NF1 patients, and some 10–15 % of these benign tumours become transformed to malignant peripheral nerve sheath tumours (MPNSTs), the main cause of morbidity in NF1 (Walker et al. 2006; Bennett et al. 2009; Upadhyaya 2011).

Cancer represents the transformation of a cell whose growth is normally tightly controlled into one that is no longer under strict regulation, allowing the cell to multiply uncontrollably and even metastasise. This dramatic alteration in cellular control arises as a consequence of the accumulation of both genetic and epigenetic changes; activated oncogenes promote cell growth as a consequence of the acquisition of gain-of-function mutations, whereas tumour suppressor genes (TSGs)

Some of the unpublished mutations in Supplementary Table 2 of Laycock-van Spyk et al. (2011) have now been published by Thomas et al. (2012a, b).

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promote cellular proliferation by acquiring loss-of-function mutations. TSGs typically encode proteins involved in growth regulation, apoptosis initiation, cellular adhesion and DNA repair. In accordance with Knudson's two-hit hypothesis (Knudson 1971; Stratton 2011; Pao and Girard 2011), both alleles of a TSG must be inactivated for cellular transformation to occur. Typically, a patient will inherit a germline mutation in one TSG allele; a second hit or somatic mutation then occurs post-fertilisation, thereby inactivating the remaining wild-type allele. Somatic mutation is therefore a key event in cancers associated with TSG inactivation. Analysis of cancer genomes in their entirety has allowed the detection of many somatic gene lesions as well as epigenetic changes. It should however be noted that only a subset of somatic lesions harboured by each tumour actually contribute to tumorigenesis. Recurrent mutations are more likely to have a direct role in the causation of cancer (driver mutations) in contrast to the majority of somatically acquired lesions in cancer genomes ("passenger" mutations) which are unlikely to be of any pathological significance (Ivanov et al. 2011; Stratton 2011; Pao and Girard 2011).

The neurofibromatosis 1 (*NF1*) gene spans 283 kb of genomic DNA at chromosome 17q11.2 (Ballester et al. 1990) and contains 61 exons (Upadhyaya et al. 2007; Upadhyaya 2008). Neurofibromin, the *NF1* gene product, functions as a negative regulator of active Ras and of the associated Ras-mitogen-activated protein kinase (Ras/MAPK) signalling pathway. *NF1* is a tumour suppressor gene, and it may therefore be inferred that any tumours that arise will have acquired a second, somatic "hit" that serves to inactivate the normal *NF1* allele, resulting in the complete loss of functional neurofibromin; a double hit ($NF1^{-/-}$) is critical for NF1 tumorigenesis to occur (Sawada et al. 1996). A variety of benign and malignant tumours are associated with NF1, and all are likely to involve tumorigenesis of neural crest-derived cells (Carroll and Ratner 2008). Several murine models of neurofibromatosis have both successfully recapitulated much of the NF1 human phenotype and shown that *NF1* is indeed a classical tumour suppressor gene (Cichowski et al. 1999; Cichowski and Jacks 2001). The question as to why only a relatively small proportion of the benign tumours eventually go on to become malignant is however still puzzling. Consistent with a central role for neurofibromin in cellular function, recent cancer genome sequencing studies have found that somatic *NF1* gene mutations occur not only in association with NF1 but also in a number of other common cancers that are not usually associated with NF1 (Parsons et al. 2008; Ding et al. 2008; Sangha et al. 2008; Brennan et al. 2009; McGillicuddy et al. 2009; Haferlach et al. 2010; Hölzel et al. 2010).

In the context of NF1, few genotype–phenotype correlations are apparent, and marked intrafamilial variation in terms of the underlying clinical phenotype is common (Easton et al. 1993; Upadhyaya 2010). This intrafamilial phenotypic variation serves to indicate the importance of the second hit since differences in the type and timing of the somatic *NF1* mutation may help to explain the observed variability in the patient phenotype (Kehrer-Sawatzki and Cooper 2008). An appreciation of the spectrum of somatic mutations in NF1-associated tumours is therefore essential if we are to understand the molecular pathways involved, itself a prerequisite for improvements in clinical treatment and the

development of new therapeutics. Here we review the spectrum of somatic *NF1* mutations so far reported in NF1-associated tumours, with a view to assessing how they may serve to induce tumour growth and whether or not any genotype–phenotype correlation may be discerned.

15.2 NF1-Associated Tumours

15.2.1 Cutaneous Neurofibromas

Benign peripheral nerve sheath tumours (neurofibromas) are a characteristic feature of NF1. Tumours derived from skin sensory nerves are designated dermal or cutaneous neurofibromas and usually present as discrete tumours that remain associated with a single nerve ending. Cutaneous neurofibromas exhibit extensive cellular heterogeneity, being composed of hyperproliferative Schwann cells (SC), fibroblasts, mast cells and perineural cells. The SCs have been identified as the initiating cell type in neurofibromas, and it is only in these cells that the *NF1* gene becomes biallelically inactivated (Serra et al. 2000). SCs are also the target for various growth factors known to stimulate neurofibroma formation and growth.

Cutaneous neurofibromas are thought to arise from skin-derived precursor cells (Le et al. 2009), and these cells have neural crest-like properties and may well be under hormonal control since the majority of such tumours develop only during puberty (McLaughlin and Jacks 2003). An increase in tumour size and number has also been noted during pregnancy, with some evidence for a postnatal decrease in tumour size (Dugoff and Sujansky 1996; Roth et al. 2008).

Approximately 20–50 % of cutaneous neurofibromas exhibit loss of heterozygosity (LOH) at the *NF1* locus, and the majority of these lesions appear to be due to mitotic recombination (Eisenbarth et al. 2000; Serra et al. 2001; Wiest et al. 2003; Upadhyaya et al. 2004; Spurlock et al. 2007; Thomas et al. 2010; Garcia-Linares et al. 2011). Comparison of the relative frequencies of the different types of somatic *NF1* mutation observed in cutaneous neurofibromas with those of their previously reported germline counterparts reveals significant ($p = 0.001$) differences (Thomas et al. 2012a) (See chapter 26 for more in-depth coverage).

15.2.2 Plexiform Neurofibromas

Tumours associated with larger nerves within the skin may spread within the dermis and appear as a diffuse mass. Plexiform neurofibromas (PNFs) are large tumours, usually associated with major nerve trunks and nerve plexus; they are generally slow growing, may develop at both internal and external body locations and can result in major disfigurement. PNFs occur in some 30–50 % of NF1 patients, and, although

these tumours generally remain benign, some neurological impairment may result from their growth. Some 10–15 % PNFs may become malignant (Evans et al. 2002).

While the genetic basis of neurofibroma development is still not fully understood, biallelic *NF1* inactivation does seem to be required, as all tumour cells harbour both a constitutional and a somatic *NF1* gene mutation (Upadhyaya 2010). The cellular composition of plexiform neurofibromas is similar to that of cutaneous neurofibromas. About 70 % of PNFs have been reported to display LOH at the *NF1* locus (John et al. 2000; Upadhyaya et al. 2008b). However, there is no obvious correlation between the type or location of germline *NF1* mutations in NF1 patients and that of their somatic counterparts arising in their tumours (Upadhyaya et al. 2008b). Despite inactivation of the *NF1* gene in Schwann cells, non-tumorigenic cells contribute to tumour development. In a mouse model of plexiform neurofibroma, *NF1*-haploinsufficient mast cells promote inflammation and accelerate tumour formation and growth (Staser et al. 2012). Thus, it would appear that the key to understanding neurofibroma formation lies in the elucidation of the precise molecular interactions of the haploinsufficient tumour microenvironment within the initial cell type harbouring the biallelically inactivated *NF1* gene.

The precise stage of Schwann cell differentiation that gives rise to plexiform neurofibromas is still debatable. Plexiform neurofibromas are considered to be congenital, and therefore, neural crest stem cells or other progenitor cells may give rise to these tumours during embryogenesis, although several studies have indicated that NF1-deficient foetal neural crest stem cells cannot directly form a plexiform neurofibroma (Joseph et al. 2008; Carroll and Ratner 2008). Studies from different mouse tumour models indicate differentiated glia including mature non-myelinating Schwann cells (i.e. Remak bundles) as a probable tumour cell of origin (Zheng et al. 2008; Yang et al. 2008; Zhu et al. 2002; Joseph et al. 2008; reviewed by Staser et al. 2012). Wu et al. (2008) have shown that widespread deletion in glial precursor cells during embryogenesis at approximately day 12.5, as driven by the NF1(flox/flox); DhhCre mouse model allows plexiform neurofibroma development despite the presence of wild-type cells in the microenvironment.

An interesting, although as yet unexplained, observation is that a few mildly affected NF1 patients, who never develop any cutaneous or plexiform neurofibromas, carry the same germline *NF1* mutation (c.2970-2972delAAT), viz. an in-frame 3 bp deletion that leads to the loss of a methionine residue (Upadhyaya et al. 2007) (See chapter 36 for more in-depth coverage).

15.2.3 Malignant Peripheral Nerve Sheath Tumours

Cells derived from within some 10–15 % of PNFs may eventually undergo malignant transformation into a malignant peripheral nerve sheath tumour (MPNST). MPNSTs are aggressive and highly invasive soft tissue sarcomas with an annual incidence of 0.16 % in NF1 patients, as compared to only 0.001 % in the normal population (Ducatman et al. 1986), and with a lifetime risk of 8–13 % in NF1

individuals (Evans et al. 2002; McCaughan et al. 2007; reviewed by Upadhyaya 2011). This form of malignancy represents a major cause of morbidity and mortality in NF1. Malignant transformation usually appears to evolve from within a pre-existing plexiform neurofibroma or atypical neurofibroma (Spurlock et al. 2010; Beert et al. 2011). The distinction between benign PNFs and MPNSTs has been sensitively visualised by non-invasive [^{18}F]-2-fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) imaging (Benz et al. 2010), suggesting a potential role for FDG-PET-based non-invasive imaging in future diagnostic tests. Atypical neurofibromas are symptomatic, hypercellular peripheral nerve sheath tumours composed of cells with hyperchromatic nuclei in the absence of mitoses. In a recent study, Beert et al. (2011) have shown that atypical neurofibromas are premalignant tumours, with a *CDKN2A*/*2B* deletion as the first step in the progression towards an MPNST. In one tumour, the authors observed a clear transition from a benign-atypical neurofibroma towards an intermediate-grade MPNST which was subsequently confirmed by both histopathology and array CGH analysis. The aberrant molecular pathways that underlie this malignant transformation are still largely unknown, and considerable effort is being directed to elucidate the molecular defects involved.

NF1 patients carrying large (usually 1.4 Mb) genomic deletions (that remove the entire *NF1* gene plus a variable number of flanking genes) have an increased risk of MPNST development in certain patient groups (De Raedt et al. 2003; Upadhyaya et al. 2006). Indeed, over 90 % of MPNSTs have been found to harbour large *NF1* somatic deletions (Upadhyaya et al. 2008a). More recently, significantly increased frequencies (relative to the general NF1 population) of plexiform neurofibromas, subcutaneous neurofibromas, spinal neurofibromas and MPNSTs have also been reported in association with molecularly ascertained 1.4-Mb type 1 *NF1* deletions (Mautner et al. 2010). The MPNST-associated deletion breakpoints have not been found to involve the paralogous repetitive sequences that are involved in mediating the majority of germline *NF1* deletions (Kehrer-Sawatzki 2008). The smallest common region of somatic deletion overlap is, however, restricted to approximately the same ~2.2-Mb interval that contains most of the genes deleted in recurrent constitutional *NF1* deletions (Pasmant et al. 2010).

Although it is clear that biallelic *NF1* gene inactivation is required for transformation to occur, mutations at the *NF1* locus are insufficient to explain the process of tumorigenesis since most benign neurofibromas also exhibit such biallelic *NF1* inactivation. The best evidence for the involvement of other loci relates to the *TP53* gene for which several different mutations have been found in MPNSTs but not in benign neurofibromas (Menon et al. 1990; Legius et al. 1994; Upadhyaya et al. 2008a; Upadhyaya 2011). Mice with heterozygous mutations in both their *Nf1* and *Tp53* genes developed malignancy (Cichowski et al. 1999; Vogel et al. 1999), an indication perhaps that *Tp53* loss is critical for transformation. The homozygous loss of the *CDKN2A* gene, which encodes p16INK4A and p14ARF, has also been associated with NF1 malignancy (Kourea et al. 1999; Mantripragada et al. 2008; Nielsen et al. 1999). *PTEN* gene dosage and/or PI3K/AKT pathway activation may be rate-limiting steps in NF1 malignant transformation (Gregorian et al. 2009). As

yet, however, no characteristic gene expression signature has been defined for MPNST development, although several cell cycle and signalling regulation genes (*CDKN2A*, *TP53*, *RB1*, *EGFR*, *CD44*, *PDGFRA*, *HGF*, *MET* and *SOX9*) are frequently deregulated.

Mutational heterogeneity within these malignant tumours greatly complicates the study of the underlying mechanisms of tumorigenesis. We have explored this molecular heterogeneity by performing loss of heterozygosity (LOH) analysis of the *NF1*, *TP53*, *RB1*, *PTEN* and *CDKN2A* genes on sections of ten MPNSTs derived from ten unrelated NF1 patients (Thomas et al. 2012b; Gerlinger et al. 2012). LOH data for the *TP53* gene were found to correlate with the results of p53 immunohistochemical analysis in the same tumour sections (Thomas et al. 2012b). Further, ~70 % of MPNSTs were found to display intra-tumoral molecular heterogeneity as evidenced by differences in the level of LOH between different sections of the same tumour samples (Thomas et al. 2012a, b). This study constitutes the first systematic analysis of molecular heterogeneity within MPNSTs derived from NF1 patients. Appreciation of the existence of molecular heterogeneity in NF1-associated tumours is important not only for optimising somatic mutation detection but also for understanding the mechanisms of NF1 tumorigenesis, a prerequisite for the development of specifically targeted cancer therapeutics (See chapters 29 and 38 for more in-depth coverage).

15.2.4 Spinal Neurofibromas

About 40 % of NF1 patients present with tumours involving their spinal nerves. This is especially marked in individuals affected with familial spinal neurofibromatosis, in which bilateral tumours involving multiple spinal nerve roots are often the only manifestation of NF1. Some NF1 patients have familial spinal neurofibromatosis (FSNF), a variant form of NF1 in which patients present with multiple bilateral spinal tumours but with few other clinical features of the disease (Pulst et al. 1991; Poyhonen et al. 1997; Ars et al. 1998). Patients with FSNF have been reported to be significantly more likely to harbour germline missense or splice-site mutations as compared to patients with classical NF1 (Upadhyaya et al. 2009). A recent study of the *NF1* locus found LOH in 8 of 22 spinal tumours analysed, with most (75 %) of this LOH being due to mitotic recombination rather than genomic deletions (Upadhyaya et al. 2009).

15.2.5 Low-Grade Pilocytic Astrocytomas

Low-grade pilocytic astrocytomas are found in ~15 % of paediatric NF1 patients (Listernick et al. 1994). These benign tumours involve the visual pathways (optic pathway gliomas) or some other regions of the brain, with the complete loss of neurofibromin (Gutmann et al. 2003). NF1-associated optic pathway gliomas (OPGs) occur predominantly in early childhood. However, they are only

symptomatic in around 5 % of NF1 cases. Debate exists as to the role and nature of visual screening in the detection of NF1 OPGs, and the presence of cognitive impairment often makes it difficult for children to be cooperative with regard to visual testing. It is difficult to determine, both clinically and radiologically, which tumours will behave more aggressively and hence will require intervention. In a recent study, Sharif et al. (2011) identified 80 patients with NF1 OPGs, and molecular analyses were performed on a subset of 29 patients. A clustering of pathological mutations in the 5′ tertile of the *NF1* gene was found. The authors combined these results with those for another two NF1 OPG cohorts and collectively found the same trend. The apparent localisation of mutations at the 5′ end of the *NF1* gene therefore appears to be a bona fide feature of mutations in NF1 patients with OPGs when compared with NF1 patients without OPGs (mutations in the 5′ third of the gene, OR $= 6.05$, $p = 0.003$).

Loss of *NF1* expression is an important primary genetic event in the pathogenesis of NF1-associated pilocytic astrocytomas. LOH and *NF1* inactivations occur in both early- and late-onset NF1-associated pilocytic astrocytomas (Gutmann et al. 2000; 2003) (See chapter 22 for in-depth coverage).

15.2.6 Gastrointestinal Stromal Tumours

Gastrointestinal stromal tumours (GISTs) are the most common mesenchymal tumours of the gastrointestinal tract. Although most GISTs harbour activating somatic mutations of *KIT* and *PDGFRA*, the absence of such mutations from NF1-associated GISTs (NF1-GISTs) is probably indicative of a different pathogenetic mechanism. In NF1, the majority (60 %) of GISTs develop in the small intestine, whereas sporadic non-NF1 GISTs usually involve the stomach (Miettinen et al. 2006).

Somatic *NF1* mutations have been identified in the interstitial cells of Cajal (ICC), throughout the GI tract and in NF1-GISTs lacking *KIT* or *PDGRA* mutations. Increased signalling through the Ras/MAPK pathway has also been shown to occur in NF1-GISTS as opposed to sporadic GISTs. This would seem to indicate that a decrease in neurofibromin level, in the presence of normal c-KIT and PDGFRA levels, leads to tumour formation. It also suggests that *NF1* haploinsufficiency is required for ICC hyperplasia, again demonstrating that, although a somatic *NF1* mutation is absolutely necessary, it is not sufficient to permit tumorigenesis, with additional genetic events being required. These observations concur with Knudson's two-hit hypothesis. Somatic inactivation of the *NF1* gene through gene deletion, intragenic deletion and LOH through mitotic recombination have also been described (Maertens et al. 2006; Stewart et al. 2007b).

15.2.7 Gastric Carcinoid

Gastric carcinoid tumours are associated with multiple endocrine neoplasia, atrophic gastritis and pernicious anaemia but are very rare in NF1. LOH at the *NF1* locus has been demonstrated in a gastric carcinoid tumour derived from an NF1 patient (Stewart et al. 2007a).

15.2.8 Juvenile Myelomonocytic Leukaemia

Young NF1 patients are at particular risk of developing juvenile myelomonocytic leukaemia (JMML) (Stiller et al. 1994), a clonal haematopoietic disorder characterised by hypersensitivity (at least in vitro) to granulocyte–macrophage colony-stimulating factor (GM-CSF). Moreover, some 15–20 % of JMML patients harbour a somatic *NF1* inactivating mutation, even although most exhibit no other NF1 symptoms (Flotho et al. 2007). Patients may also carry inactivating mutations of other genes, with a recent study identifying 70–80 % of mutations involving genes in the Ras/MAPK pathway, including *PTPN11*, *NRAS* and *KRAS*, as well as *NF1* (Yoshimi et al. 2010). Additional somatic mutations have also been reported in *CBL* and *ASXL1* (Sugimoto et al. 2009). In most cases, the *NF1* gene is lost either via LOH or by compound heterozygous micro-lesions (Steinemann et al. 2010) that lead to a complete loss of neurofibromin and hyperactive signalling through the Ras/MAPK pathway. LOH may occur through 1.2–1.4-Mb interstitial deletions mediated by low copy number repeat (LCR) elements that flank the *NF1* gene (Chen et al. 2010). LOH through uniparental interstitial isodisomy (50–52.7 Mb) of chromosome 17 through double mitotic recombination, in an as yet unknown initiator cell, has also been reported (Steinemann et al. 2010). The rarity of such events may indicate the existence of a selective advantage, conferred upon the $NF1^{-/-}$ cells, which could explain the propensity of NF1 patients to develop leukaemia (Stephens et al. 2006). Tissue-specific differences in the proportion of mosaic large *NF1* type 2 deletions are suggestive of a selective growth advantage of hematopoietic del($^{+/-}$) stem cells (Roehl et al. 2012) (See chapter 30 for in-depth coverage).

15.2.9 Neuroblastomas

Neuroblastoma is a neuroendocrine tumour that originates from neural crest cells of the sympathetic nervous system. Most such tumours develop in the abdomen. Associations between neuroblastoma and NF1 are rare (Kushner et al. 1985). Somatic mutations of the *NF1* gene in neuroblastoma cell lines and primary tumours have been described, but this does not result in elevation of the Ras-GTP level (The et al. 1993; Hölzel et al. 2010).

15.2.10 Phaeochromocytomas

Phaeochromocytomas (PCs) are extremely rare tumours, with only 1–6 cases observed per million individuals. PCs develop from neural crest-derived chromaffin cells, and the tumour cells produce and release catecholamines that cause hypertension and flushing. These are tumours of the adrenal medulla and are primarily associated with mutations of the *RET*, *VHL*, *SDHB*, *SDHC* and *SDHD* genes, although LOH in the *NF1* region (as well as of other loci on both 17q and 17p) has been observed. About 3 % NF1 patients develop phaeochromocytomas. Absence of neurofibromin expression and LOH involving several NF1-associated phaeochromocytomas has been reported (Gutmann et al. 1994, 1995; Bausch et al. 2007). In another study, both LOH and copy-neutral LOH have been reported in NF1-associated phaeochromocytomas (Burnichon et al. 2011). The frequent LOH surrounding the *NF1* locus and the lack of neurofibromin expression in these tumours suggest that *NF1* gene mutations contribute to the development of adrenal gland neoplasms in patients with NF1 (See chapter 25 for more in-depth coverage).

15.2.11 Glomus Tumours

Glomus tumours are small (<5 mm) benign but often very painful tumours that develop specifically within the highly innervated glomus body located at the end of each digit. These tumours appear to develop from α-smooth muscle actin-positive cells that have undergone biallelic *NF1* inactivation, resulting in increased Ras/MAPK activity (Brems et al. 2009). The somatic *NF1* mutations often differ between glomus tumours, indicating highly specific tumorigenic events. Brems et al. (2009) have suggested that glomus tumours, although rare, should now be recognised as an integral component of the NF1 spectrum of disease (See chapter 24 for more in-depth coverage).

15.2.12 Rhabdomyosarcoma

Rhabdomyosarcoma occurs during childhood and is the most common soft tissue sarcoma in children. The prevalence of rhabdomyosarcoma associated with NF1 is about 20 times higher than in the general population (0.02–0.03 %) (Friedman et al. 2002). In NF1 children, rhabdomyosarcomas tend to occur in bladder and prostate (Sung et al. 2004; Bien et al. 2007). No data are available on *NF1* somatic mutations in rhabdomyosarcoma.

15.2.13 Breast Cancer

An increased risk for breast cancer has been reported in female NF1 patients (Sharif et al. 2007). The lifetime risk of breast cancer for women with NF1 who are under

the age of 50 years is 8.4 %, approximately four times higher than the cumulative risk in the general population. In an array-based study, Lee et al. (2010) reported copy number loss at the *NF1* locus in malignant phyllodes tumour of the breast. However, no *NF1* somatic mutations have so far been reported in NF1-associated breast cancer, although LOH has been observed in one NF1-associated breast tumour (Upadhyaya et al. unpublished work).

15.3 NF1-Like Features Associated with DNA Mismatch Repair Deficiency

In a small number of families with DNA mismatch repair (MMR) deficiency, some rare affected individuals, who have either a biallelic (homozygous or compound heterozygous) MMR mutation involving the *MLH1*, *MSH2*, *MSH6* or *PMS2* genes or a more complex constellation of heterozygous MMR mutations, may have several NF1-like features, often displaying café-au-lait (CAL) spots and skinfold freckling and very occasionally a few dermal neurofibromas. Such individuals and any affected siblings with the same biallelic mismatch repair gene mutations normally have unaffected parents and may develop a few large CAL spots that satisfy the NF1 diagnostic criteria, although no germline *NF1* gene mutations have so far been reported (Wimmer and Etzler 2008; Bandipalliam 2005).

15.4 The Somatic Mutational Spectrum of NF1-Associated Tumours

A meta-analysis of all published and many unpublished (from our own laboratory) somatic *NF1* alterations associated with NF1 tumours has recently been undertaken to acquire a better understanding of NF1 tumorigenesis (Laycock-van Spyk et al. 2011). As of November 2011, approximately 600 different somatic *NF1* gene changes had been reported in different NF1-associated tumours, with more than half corresponding to LOH (Laycock-van Spyk et al. 2011). The level of LOH detected differs between cutaneous neurofibromas, PNFs and MPNSTs, 40 %, 79 % and 85 %, respectively (Table 15.1). Seventy-eight percent (28/36) of cutaneous neurofibromas, 44 % (11/25) of PNFs and 16 % (5/31) of MPNSTs display LOH resulting from mitotic recombination. Some 79 % (15/19) of the JMML samples that exhibited LOH appear to have lost the entire 17q arm through mitotic recombination, perhaps indicating a significant correlation with this tumour type.

The main focus of this chapter will be placed on *NF1* somatic micro-lesions. For the precise details of LOH studies, the reader is referred to our recent review article (Laycock-van Spyk et al. 2011). Tumour DNA analysis has also identified 254 somatic *NF1* gene lesions including nonsense, missense, splice site, micro-deletions,

Table 15.1 (**a**) The spectrum of somatic *NF1* micro-lesions reported in different NF1-associated tumours. (**b**) The percentile distribution of somatic *NF1* micro-lesions in different NF1-associated tumours

(a)

	Mutation type							
Tumour type	Deletion	Insertion	Indel	Nonsense	Splice site	Missense	Truncating	Total
Dermal neurofibroma	82	15	2	59	32	21	158	211
Plexiform neurofibroma	6	1	–	7	2	2	14	18
Spinal neurofibroma	–	–	–	–	2	1	0	3
MPNST	7	1	1	1	–	–	10	10
GIST/gastric carcinoid	1	–	–	3	1	–	4	5
JMML	[a]	[a]	[a]	1	[a]	[a]	1	1
Glomus tumour	2	1	–	1	2	–	4	6
Overall	98	18	3	72	39	24	191	254

(b)

	Mutation type (%)							
Tumour type	Deletion	Insertion	Indel	Nonsense	Splice site	Missense	Truncating (%)	Total
Dermal neurofibroma	38.9	7.1	0.9	28.0	15.2	10.0	75	211
Plexiform neurofibroma	33.3	5.5	–	38.9	11.1	11.1	78	18
Spinal neurofibroma	–	–	–	–	66.6	33.3	0	3
MPNST	70	10	10	10	–	–	100	10
GIST/gastric carcinoid	20	–	–	60	20	–	80	5
JMML	[a]	[a]	[a]	100	[a]	[a]	100	1
Glomus tumour	33.3	16.7	–	16.7	33.3	–	67	6

[a]Compound heterozygosity of *NF1* mutations in several JMML tumours (distinguishing between the germline and somatic mutations was not possible) (see Laycock-van Spyk et al. 2011).

micro-insertions and indels (combined insertion–deletion events) of <20 bp and larger (>20 bp) deletions/insertions (Table 15.1a, b). The consequences of all micro-deletions and micro-insertions for the *NF1* reading frame were also determined. About 75 % (191/254) of the somatic *NF1* mutations identified in NF1 tumours comprised mutations that are predicted to give rise to truncated proteins. Of these 191 changes, only 18 resulted from the insertion or duplication of bases; the remaining 173 truncations arose from micro-deletion, nonsense mutation or other frameshift events (Table 15.1a). Splice-site mutations comprised a considerable proportion (39/254; 15.4 %) of the mutational spectrum, whilst missense changes only accounted for 9.4 % (24/254) of the somatic *NF1* mutations detected.

Any attempt to make direct comparisons between the various tumour types would be unwise at this stage owing to the paucity of somatic mutation data, especially for the less commonly encountered tumours. Table 15.1a, b nevertheless attempts to summarise the available data. The bias inherent in the data is immediately evident, with 211/254 (83 %) mutational changes originating from the analysis of cutaneous neurofibroma DNA. Hence, the relative frequencies of the various mutation types in cutaneous neurofibromas are essentially comparable to the overall

Table 15.2 Contribution of LOH and *NF1* micro-lesions to the somatic *NF1* mutational spectrum in different types of NF1-associated tumour

Tumour type	LOH	Point mutations	Total
Dermal neurofibroma	144 (40 %)	211 (60 %)	355 (100 %)
Plexiform neurofibroma	67 (79 %)	18 (21 %)	85 (100 %)
Spinal neurofibroma	7 (70 %)	3 (30 %)	10 (100 %)
MPNST	55 (85 %)	10 (15 %)	65 (100 %)
Low-grade pilocytic astrocytoma	18 (100 %)	0 (0 %)	18 (100 %)
GIST/gastric carcinoid	3 (38 %)	5 (62 %)	8 (100 %)
JMML	18 (95 %)	1[a] (5 %)	19 (100 %)
Phaeochromocytoma	10 (100 %)	0 (0 %)	10 (100 %)
Glomus tumour	1 (14 %)	6 (86 %)	7 (100 %)
Overall	323 (55 %)	254 (44 %)	577 (100 %)

[a]Compound heterozygous *NF1* mutations found in 5 haemopoietic tumours. Since no other tissue was analysed, it was not possible to distinguish between the germline and somatic *NF1* point mutations identified.

mutational spectrum, with nonsense mutations, splice-site mutations and missense alterations found in cutaneous neurofibromas at frequencies of 28 % (59/211), 15 % (32/211) and 10 % (21/211), respectively (Table 15.1a). Tables 15.1a and 15.2 do, however, serve to highlight the high proportion of truncating mutations 191/254 (~75 %) involved in the somatic inactivation of the *NF1* gene in all tumour types, especially cutaneous neurofibromas.

An additional comparison between the frequency distributions of somatic micro-lesions and LOH is made in Table 15.2. There appears to be a marked difference between cutaneous neurofibromas, PNFs and MPNSTs represented by LOH. This may be explained in part by the extent of the molecular rearrangements in each tumour type; MPNSTs, for example, would be predicted to display a larger number of genetic aberrations than a benign dermal neurofibroma. However, the types of analyses performed will also have a direct bearing on conclusions drawn because either micro-lesions or LOH may not have been screened for in some studies.

In summary, the more severe MPNSTs manifest a greater degree of genetic abnormality than other tumour types, with LOH constituting a much more frequent event in these tumours. Further comparison within and between the rarer tumour types would not be valid owing to the relative paucity of mutation data currently available for analysis.

15.5 Mutational Mechanisms Underlying the Known Somatic *NF1* Gene Lesions

Somatic inactivation of the *NF1* gene may involve intragenic mutations resulting from different mutational mechanisms, as well as LOH and epigenetic modification of the promoter region. Among the 254 somatic *NF1* micro-lesions listed by

Laycock-van Spyk et al. 2011, 72 were nonsense mutations, 36 of which were located in just 15 codons in different tumours (codons 192, 304, 426, 440, 816, 1241, 1306, 1362, 1513, 1569, 1604, 1748, 1939, 1976, 2429). Ten of these 15 different recurrent nonsense mutations involve C>T or G>A transitions within CpG dinucleotides and are therefore compatible with the endogenous mutational mechanism of methylation-mediated deamination of 5-methylcytosine (5mC). Of these 72 nonsense mutations, 28 have also been reported as germline mutations in NF1 patients [Human Gene Mutation Database (HGMD); http://www.hgmd.org; Stenson et al. 2008], indicating that the same mutational mechanism is operating in both the soma and the germline. The importance of this mutational mechanism is evidenced by the finding that 12 of the 15 recurrent somatic nonsense mutations have also been independently reported in the germline (codons 192, 304, 426, 440, 816, 1241, 1306, 1362, 1513, 1569, 1748, 2429). For the 10 of these 15 nonsense mutations that correspond to C>T or G>A transitions within CpG dinucleotides, we may infer that the mutated cytosine must be methylated both in the soma and in the germline, thereby explaining the vulnerability of these sites to methylation-mediated deamination in both cell lineages.

Among the somatic *NF1* mutations listed in Laycock-van Spyk et al. (2011) are 21 different missense mutations. Of these mutations, two (in codons 519 and 776) have been reported more than once in different tumours or different studies, although neither is compatible with methylation-mediated deamination of 5mC. Of the 21 missense mutations, only one (in codon 176) has also been reported in the germline (see HGMD). Since this Asp176Glu mutation has also been reported more than once in NF1-associated tumours, it may well be that this residue is of importance for the function of neurofibromin in both the soma and the germline. This residue has been evolutionarily conserved in a number of different species including *Drosophila* and *Fugu*, and no polymorphism at this residue has been identified in 250 unrelated normal individuals.

Nonsense mutations are not the only type of *NF1* mutation to occur recurrently in the soma. Among the somatic *NF1* micro-deletions listed by Laycock-van Spyk et al. (2011) are six that have been reported more than once in different tumours (c.1888delG, c.2033delC, c.3058delG, c.4374_4375delCC and c.5731delT). Four of these micro-deletions occurred in mononucleotide tracts (G_4, C_5, C_7 and T_3, respectively), suggestive of a model of slipped mispairing at the DNA replication fork. Importantly, c.2033delC has also been reported in the germline (see HGMD) indicating that this C pentanucleotide stretch is a hotspot for mutation in both the germline and the soma. A micro-insertion (c.1733insT, located within a T_6 tract) has also been found to occur recurrently in the soma, but this has not so far been reported in the germline. The reader interested in a detailed comparative analysis of germline and somatic mutations in human tumour suppressor genes is referred to Ivanov et al. (2011).

NF1 LOH, secondary to somatic *NF1* mutations, has been recently reported in NF1-associated tumours (Laycock-van Spyk et al. 2011). Several mechanisms can lead to LOH, including deletion, nondisjunctional chromosome loss with or without reduplication, gene conversion, point mutation, epigenetic inactivation and

Table 15.3 Mechanistic basis of the *NF1* gene-associated LOH observed in different NF1-associated tumours

Tumour type	Mitotic recombination	Genomic deletion
Dermal neurofibroma	28	8
Plexiform neurofibroma	11	14
Spinal neurofibroma	7	1
MPNST	5	26
Low-grade pilocytic astrocytoma	0	2
GIST/gastric carcinoid	1	1
JMML	15	4
Phaeochromocytoma	0	0
Glomus tumour	2	0

Information pertains only to those cases where the precise mechanism of LOH could be identified (see Laycock-van Spyk et al. 2011)

mitotic recombination. Mitotic recombination is important for inactivating tumour suppressor genes by copy-neutral loss of heterozygosity (LOH). Table 15.3 summarises mitotic recombination observed in different NF1 tumours. It appears to be more prevalent in cutaneous neurofibromas and JMML (Serra et al. 2001; Stewart et al. 2007a, b, 2012; Steinmann et al. 2010; Upadhyaya et al. 2008b, 2009; Garcia-Linares et al. 2011; Laycock-van Spyk et al. 2011).

15.6 Comparison of the Germline and Somatic *NF1* Mutational Spectra

Analysis of the combinations of somatic and germline mutations observed in the same tumour (Table 15.4) revealed that the type of somatic mutation present in the tumour is independent of the type of germline mutation occurring in the same tumour ($p = 0.366$). Only, somatic missense, nonsense/frameshift mutations and splice-site mutations were compared with long deletions and nonsense/frameshift germline mutations. The proportions of intronic, missense and nonsense mutations, micro-deletions and micro-insertions were not found to differ significantly between the soma and the germline ($p = 0.498$). Comparison of the relative frequencies of observed somatic missense mutations, nonsense mutations, micro-deletions/micro-insertions and splicing mutations with the corresponding frequencies of the germline mutations (data retrieved from the HGMD; Stenson et al. 2008) indicated significant differences (Fisher's exact test, $p = 0.00014$). A greater proportion of nonsense mutations (25 %) was observed in the soma as compared to the germline (15 %), whereas a smaller proportion (15 %) of splice-site mutations was observed in the soma as compared to the germline (26 %). Thirty-three percent of all somatic missense/nonsense mutations were C>T and G>A changes in CpG- and CpHpG-oligonucleotides (where H is an A, T or C nucleotide). The proportion of somatic mutations in these oligonucleotides was significantly overrepresented

Table 15.4 Relative frequency of occurrence of specific types of somatic mutation in relation to the type of germline mutation identified in the same tumours

	Somatic mutation type				
Germline mutation type	Missense	Nonsense/ frameshift	Gross deletions	Splice-site mutations	Total
Gross deletions	3	41	4	3	51
Missense	1	8	0	1	10
Nonsense/frameshift	14	78	3	14	109
Splice-site mutations	1	11	0	2	14
Total	19	138	7	20	184

($p = 0.007$) as compared to the germline mutations. No difference (Fisher's exact test, $p = 0.334$) was observed in the location of somatic and germline mutations identified in the intronic regions of the *NF1* gene; a small proportion of these mutations occur outside of a ±15 bp region flanking exon/intron and intron/exon junctions. It was also noted that all somatic mutations (with one exception) identified in the intronic regions of the *NF1* gene occurred within, respectively, +6 bp and −12 bp of the exon/intron and intron/exon junctions, whereas all intronic changes observed in normal population occurred outside of these regions (unpublished data).

15.7 *NF1* Gene Somatic Mutations in Non-NF1-Associated Tumours

Various studies have identified somatic *NF1* gene mutations in non-NF1-associated cancers. Thus, somatic *NF1* aberrations have been identified in glioblastoma multiforme (GBM) tumours, lung adenocarcinomas, malignant breast tumours, leukaemia, ovarian serous carcinomas (OSC) and neuroblastoma (Ding et al. 2008; Parsons et al. 2008; Sangha et al. 2008; McGillicuddy et al. 2009; Haferlach et al. 2010; Hölzel et al. 2010; Lee et al. 2010). Some of the *NF1* gene changes are relatively frequent in these tumours and therefore have the potential to represent specific prognostic and diagnostic markers. Thus, for example, 23 % of sporadic GBMs harbour an inactivating *NF1* somatic mutation, and this may enable such GBM tumours to differentiate into the mesenchymal molecular subclass (Brennan et al. 2009). Similarly, in 22 % (9/41) of primary OSCs, an *NF1* mutation was detected, six of which exhibited biallelic inactivation (Sangha et al. 2008). Interestingly, all nine of these OSC samples also contained a *TP53* mutation, highlighting the likely involvement of this tumour suppressor gene in OSC pathogenesis (Sangha et al. 2008).

Given the pivotal role that neurofibromin plays in several cell signalling pathways, it is not surprising that its loss will affect distinct molecular subtypes in different cancers. Indeed, the efficacy of any future therapeutic intervention for

many tumours will almost certainly hinge upon our ability to successfully identify such molecular subclasses of tumour.

15.8 Concluding Remarks

We here report on the somatic mutational spectrum in the *NF1* gene. We have sought to examine how the somatic *NF1* lesion interacts with its germline counterpart and explore how this might improve our understanding of the pathophysiological mechanisms underlying tumorigenesis. Copy number changes in NF1 tumours are covered in Chap. 27.

Biallelic inactivation of the *NF1* gene, resulting in the complete loss of functional neurofibromin, initiates the pathogenic process that eventually results in the formation of nerve sheath tumours. *NF1* gene inactivation may occur through relatively subtle lesions that affect just a few DNA bases or may involve gross genomic changes that affect large chromosomal regions or even the entire chromosome 17. In MPNSTs, additional mutations at different gene loci are involved in the progression of the tumour.

In terms of the molecular mechanisms of mutagenesis, both methylation-mediated deamination of 5-methylcytosine and slipped mispairing within polynucleotide tracts appear to be responsible for the occurrence of mutation hotspots that are shared between the germline and the soma. For some types of tumour, it would appear that there is some interplay between the soma and the germline in that the location of the germline mutation can influence the nature, frequency and location of the subsequent somatic mutation (Lamlum et al. 1999; Dworkin et al. 2010). As yet, however, there is no evidence for this phenomenon in the context of NF1 tumorigenesis.

Although our knowledge of the role of the *NF1* gene in tumorigenesis is continuing to grow, definitive markers of malignant transformation still remain to be discovered. Mouse and other animal models, including zebrafish (Padmanabhan et al. 2009), have provided new perspectives for research with various knockout and mutagenesis studies potentiating functional studies. It is already clear that in order to clarify the role of the *NF1* gene in NF1-associated tumours, we must improve our understanding of the significance of the somatic (second hit) mutations. Our meta-analysis of known somatic *NF1* mutations in various NF1-associated tumour types reported here failed to unearth any specific genotypic correlations. The limited size of the mutation dataset meant that reliable conclusions were hard to draw, and larger and better defined patient groups will be required to allow more reliable comparisons to be made. Additionally, there is an urgent need to identify prognostic markers that could reliably permit differentiation between benign neurofibromas that are likely to progress to malignancy and those that are unlikely to progress to malignancy.

Our review has nevertheless emphasised that NF1 is a highly individualised clinical entity that exhibits extreme somatic mutational heterogeneity both within

and between patients. These are the mutations which are ultimately responsible for the molecular changes that can lead to tumour formation. If we can come to understand how these changes bring about tumorigenesis, we shall be better placed not only with respect to the provision of genetic counselling but also in terms of exploring new avenues for the development of new drug-based therapies.

Mutation analysis combined with functional analysis enhances our knowledge of the biological basis of cancer and can help to identify biological pathways affected by somatically altered genes, leading to the development of novel therapeutic strategies. Owing to the inherent heterogeneity characteristic of MPNSTs, a larger study is required to achieve statistically meaningful results. Considering the pace at which sequencing capacity is increasing whilst the cost is steadily decreasing, it is highly likely that genome-wide profiling will soon be applicable to most NF1-associated tumours. The integration of both structural and functional data promises to provide us with a better understanding of the somatic *NF1* alterations that programme tumour initiation, maintenance and progression.

Future developments should focus on collecting accurate clinical information on tumour samples and improving imaging techniques. In addition, tumour genetic heterogeneity should be considered for the interpretation of results, how such heterogeneity affects tumour biology and the response to treatment. The scarcity of systematic approaches to study solid tumours with complex karyotypes is also being addressed by whole-genome sequencing that will shortly provide the most comprehensive characterisation of the *NF1* tumour genome including the gross chromosomal rearrangements.

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Chapter 16
Relationship Between NF1 and Constitutive Mismatch Repair Deficiency

Katharina Wimmer

16.1 Introduction: The DNA Mismatch Repair System

Genomic DNA is constantly exposed to endogenous and exogenous DNA damage. Failure to repair this damage leads to mutations, rearrangements, and other deleterious events that can cause cellular transformation. Therefore, all living organisms have evolved efficient DNA repair pathways that safeguard their genomes. A key guardian of genomic integrity is the highly conserved mismatch repair (MMR) system. It corrects replication errors that are caused by DNA polymerase and skipped by its proofreading capacity (Jiricny 2006a). In humans, five MMR genes, *MSH2*, *MSH6*, *MSH3*, *MLH1,* and *PMS2*, play a crucial role in this process. Base–base mismatches and small insertion–deletion loops (IDLs) are detected by one of two heterodimers, MSH2·MSH6 (MutSα) or MSH2·MSH3 (MutSβ). MutSα, which is the more abundant, participates in the repair of base–base mismatches and misalignments of one or two nucleotides while MutSβ recognizes larger IDLs. Mismatch-bound MutSα (or MutSβ) recruits a second heterodimer, MLH1·PMS2 (MutLα). MutLα possesses endonuclease activity located in the PMS2 subunit that introduces random nicks at distal sites spanning the mismatch (Kadyrov et al. 2006). Subsequent loading of EXO1 at the 5′ side of the mismatch activates its 5′-to-3′ exonuclease activity leading to the removal of the error-containing DNA fragment. The repair process is finalized by polymerase δ and its cofactors' proliferation cell nuclear antigen (PCNA) and replication factor C (RFC) that fill in the single-stranded gap. Ligase I then seals the remaining nick (reviewed in Jiricny 2006b).

In the absence of MMR, base–base mismatches and IDLs remain uncorrected, which results in a mutator phenotype. Repeated-sequence motifs comprising units of one, two, or a few more nucleotides, i.e., microsatellites, are frequent targets of

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polymerase slippage errors, and uncorrected IDLs at these sites lead to shortening and lengthening of these sequences. Hence, tissues derived from clonally proliferating MMR-deficient cells exhibit *microsatellite instability* (MSI) which is easily detected by PCR amplification and fragment analysis of a set of microsatellite markers (Boland et al. 1998).

In addition to DNA repair activity, the MMR system is also involved in a number of other cellular processes. It is involved in apoptotic response to a variety of DNA-damaging agents. In particular, MMR-deficient cells are up to 100-fold more resistant to death induced by methylating agents than matched MMR-proficient cells (reviewed in Stojic et al. 2004). Furthermore, the MMR system is involved in immunoglobulin class switch recombination and somatic hypermutation of variable regions of immunoglobulin genes (Durandy 2009).

16.2 The Constitutional Mismatch Repair Deficiency Syndrome

The contribution of defective mismatch repair (MMR) to the development of human cancer has been acknowledged now for almost two decades (for a recent review, see Peltomaki (2003)). Heterozygous (monoallelic) germ line mutations in the *MMR* genes *MSH2*, *MSH6, MLH1*, and *PMS2* are linked to the autosomal dominant Lynch syndrome (Aaltonen et al. 1993), which predisposes primarily to colorectal and endometrial cancers but also to other types of cancer, including urinary tract, stomach, small bowel, ovarian, and brain tumors (Lynch and de la Chapelle 2003; Lynch et al. 2009). Affected individuals generally present during the fourth or fifth decade of life. In Lynch syndrome-associated tumors, MMR deficiency results from loss of the wild-type MMR allele through somatic mutations or loss of heterozygosity.

In 1999, two reports described the phenotype of the offspring, from consanguineous marriages within Lynch syndrome families, who carry homozygous *MLH1* germ line mutations (Ricciardone et al. 1999; Wang et al. 1999). These individuals developed hematological malignancies (and a medulloblastoma in one individual) in early childhood (age range 14 months to 6 years). Of note, they also displayed clinical features reminiscent of neurofibromatosis type 1 (NF1). Since then, more than a hundred pediatric and young adult patients have been reported to carry biallelic (homozygous or compound heterozygous) mutations in one of the four MMR genes involved in Lynch syndrome. OMIM has assigned a number (#276300) to this distinct childhood cancer syndrome which has received different names such as mismatch repair cancer syndrome (OMIM) and mismatch repair deficiency syndrome (Scott et al. 2007b). We have adopted the latter name and extended it to constitutional mismatch repair deficiency (CMMR-D) (Wimmer and Etzler 2008) to avoid possible confusion with somatic mismatch repair deficiency (MMR-D) as seen in tumors in Lynch syndrome patients (and in sporadic tumors exhibiting *MLH1* hypermethylation).

My own knowledge of the clinical phenotype of CMMR-D originates primarily from the medical literature (as of Jan 2012, 56 articles reporting 114 patients) and

14 so far unpublished cases (for most of whom mutation analysis, primarily in the *PMS2* gene, was performed at our institution); consequently potential bias must be kept in mind as I interpret these data.

The tumor spectrum of this cohort of CMMR-D patients is very broad and can be divided into four main groups: (1) hematological malignancies with a predominance of lymphoid malignancies, i.e., non-Hodgkin's lymphoma (NHL), especially T-cell NHL and T-cell as well as B-cell acute lymphoblastic leukemia but also including leukemia of the myeloid lineage (reviewed by Ripperger et al. 2010); (2) brain (central nervous system) tumors including glioblastoma and other high-grade astrocytic tumors, medulloblastoma, and supratentorial primitive neuroectodermal tumors (reviewed by Johannesma et al. 2011); (3) Lynch syndrome-associated carcinomas primarily in the colon and rectum but also in the small intestines, the endometrium, the ovaries, the upper urinary tract, and the bladder, as well as multiple intestinal polyps (reviewed by Durno et al. 2010 and Herkert et al. 2011); and (4) embryonic tumors and other malignancies including rhabdomyosarcoma. Table 16.1 summarizes the full tumor spectrum deduced from 128 patients in 80 kindreds.

The distribution of MMR gene mutations among biallelic patients is different from Lynch syndrome. Heterozygous *MLH1* and *MSH2* mutations account for the vast majority (~90 %) of Lynch syndrome cases (de la Chapelle 2004; Liu et al. 1996; Peltomaki 2003), whereas *MSH6* has been found to be mutated in ~10 % of cases (Berends et al. 2002; Hendriks et al. 2004; Plaschke et al. 2004). *PMS2* mutations appear to play only a very minor role in Lynch syndrome. By contrast, ~62 % (79/128) of CMMR-D patients carry biallelic *PMS2* mutations, ~17 % (22/128) *MSH6* mutations, and only ~21 % (27/128) *MLH1* or *MSH2* mutations. Several reasons may account for this difference. (1) The penetrance of monoallelic *PMS2* mutations appears to be lower than that of mutations in the other MMR genes. This may explain, at least in part, the lower prevalence of heterozygous *PMS2* mutation carriers among Lynch syndrome patients which are frequently selected using the revised Bethesda criteria (Umar et al. 2004) and also the frequent lack of a clear Lynch syndrome family history in biallelic *PMS2* mutation carriers. (2) The comparison of the overall tumor spectrum and age of malignancy onset in *MLH1* and *MSH2* vs. *MSH6* and *PMS2* biallelic mutation carriers (Table 16.2) shows also a trend towards a genotype–phenotype correlation. CMMR-D patients harboring biallelic *MLH1* or *MSH2* mutations tend to develop malignancies and premalignant tumors earlier than *MSH6* and *PMS2* mutation carriers (mean age at first diagnosed tumor, 4.5 vs. 9 years). *MLH1/MSH2* biallelic mutation carriers have more frequently hematologic malignancies when compared to *MSH6* and *PMS2* mutation carriers, who appear to have a higher prevalence of brain tumors and Lynch syndrome-associated tumors. Hence, CMMR-D patients with biallelic *MSH6* and *PMS2* mutations frequently exhibit the phenotype of Turcot syndrome which has historically been considered to be a subentity of Lynch syndrome. Patients with biallelic mutations in *MSH6* and *PMS2* are more likely to survive their first tumors and develop a second malignancy (28 % vs. 40 %). These factors may facilitate the clinical diagnosis of CMMR-D in patients with *MSH6* and *PMS2* mutations. (3) In

Table 16.1 The tumor spectrum of CMMR-D syndrome (updated Jan 2012)

Malignancies	No. of tumors	Median age at diagnosis	Range
Hematological malignancies			
Non-Hodgkin's and other lymphoma	28	6	0.4–17
Acute lymphoblastic leukemia	7	6	2–21
AML	3	9	6–10
Atypical CML	1	1	
Nonspecified leukemia	1	n.r.	
Total	40	6	0.4–17
Brain tumors			
Glioblastoma and other astrocytic tumors	51	9	2–35
Supratentorial primitive neuroectodermal tumors (SPNET)	7	8	4–14
Medulloblastoma	4	7	6–7
Cerebral angiosarcoma	1	12	
Nonspecified brain tumor	5	8	4–14
Total	68	9	2–35
HNPCC-associated tumors			
Colorectal cancer	45	16	8–48
Duodenum/jejunum/ileum cancer	13	17	11–41
Endometrial cancer	5	24	23–35
Ureter/renal pelvis cancer	3	19	15–22
Papillary transitional cell cancer of bladder	1	21	
Ovarian cancer	1	17	
Total	68	17	8–48
Others			
Neuroblastoma	1	13	
Wilms' tumor	1	4	
Ovarian neuroectodermal tumor	1	21	
Infantile myofibromatosis	1	1	
Rhabdomyosarcoma	1	4	
Basal cell carcinoma	1	n.r.	
Mucoepidermoid cancer of parotid	1	11	
Total	7		

addition to a possible ascertainment bias, it may be speculated that certain *MLH1* and *MSH2* mutations are not viable in the homozygous state, whereas this is less likely to be the case for *PMS2* and *MSH6* mutations.

Clinical signs reminiscent of NF1 can be found in most CMMR-D patients and will be discussed below (Sect. 16.3). Another cutaneous feature recurrently reported (in at least nine CMMR-D patients) is areas of skin hypopigmentation (ash leaf spots, vitiligo). The analysis of patients with biallelic *PMS2* and *MSH6* mutations (three and eight patients, respectively) has shown that constitutional deficiency of these MMR genes leads to impaired immunoglobulin (Ig) class switch recombination characterized by a decrease or absence of IgG2, IgG4, and IgA concomitant (particularly in young patients) with increased IgM levels,

Table 16.2 Differences in overall tumor spectrum and the age of malignancy onset between carriers of biallelic *MLH1*/*MSH2* and *MSH6*/*PMS2* mutations, respectively

		Tumor type											
		Hematologic		Brain		LS-associated		Other		Premalignant gastrointestinal and hepatic neoplasias[a]		Median age at first diagnosed tumor (range)	No. of patients with more than one malignancy (%)
Genes	No. of patients (families)	No. of patients (%)	Median age of diagnosis (range)	No. of patients (%)	Median age of diagnosis (range)	No. of patients (%)	Median age of diagnosis (range)	No. of patients (%)	Median age of diagnosis (range)	No. of patients (%)	Median age of diagnosis (range)		
MLH1	16 (10)	6 (37)	2.6 years (1–6 years)	7 (44)	4.5 years (3–14 years)	5 (31)	12 years (9–22 years)	1 (6)	4 years	2 (12)	9.5 years (9–10 years)	4.5 years (1–22 years)	4 (25)
MSH2	12 (6)	5 (42)	2 years (0.4–2.5 years)	3 (25)	13 years (3–14 years)	5 (42)	14 years (11–39 years)	0	–	6 (50)	13 years (6–46 years)	7 years (0.4–39 years)	4 (33)
MLH1/ *MSH2*	28 (16)	11 (39)	2 years (0.4–6 years)	10 (36)	6 years (3–14 years)	10 (36)	13 years (9–39 years)	1 (3.5)	4 years	8 (29)	11.5 years (6–46 years)	4.5 years (0.4–39 years)	8 (29)
MSH6	22 (14)	8 (36)	8.5 years (5–11 years)	11 (50)	9 years (2–17 years)	9 (41)	13 years (8–31 years)	0	–	5 (23)	11 years (9–17 years)	9 years (2–31 years)	9 (41)
PMS2	78 (49)	19 (24)	7.5 years (2–21 years)	46 (59)	9 years (2–35 years)	35 (45)	16 years (9–32 years)	6 (8)	11 years (1–21 years)	27 (35)	15 years (7–24 years)	9.5 years (1–28 years)	31 (40)
MSH6/ *PMS2*	100 (63)	27 (27)	7 years (2–21 years)	57 (57)	9 years (2–35 years)	44 (44)	16 years (8–32 years)	6 (6)	11 years (1–21 years)	32 (32)	13.5 years (7–24 years)	9 years (1–31 years)	40 (40)

[a]Only two cases reported so far with hepatic adenomatosis; *LS* Lynch syndrome; *No*. number

i.e., hyper-IgM syndrome (Gardes et al. 2012; Péron et al. 2008). IgA deficiency indicative of this humoral defect was also observed in a patient with a homozygous *MSH2* mutation (Whiteside et al. 2002). Gardes et al. (2012) reported that *PMS2* deficiency leads to a more profound Ig class switch recombination defect than a *MSH6* deficiency and may cause clinical susceptibility to infection. Two patients developed Lupus erythematosus (Plaschke et al. 2006; Rahner et al. 2008). Agenesis of the corpus callosum has been observed in four CMMR-D patients (Baas et al. 2012 and Gururangan et al. 2008). Other congenital malformations have so far been described only in individual patients; congenital asplenia, left isomerism, and ventricle septum defect in one patient (Herkert et al. 2011); renal cortical cyst in one patient (Durno et al. 2012; Gallinger et al. 2004); and hemangioma in two patients (Leenen et al. 2011; Baas et al. 2012).

16.3 Clinical Overlap of CMMR-D and NF1

NF1-associated features have been reported to be present in 83 of the 128 CMMR-D patients listed in Table 16.1. Only three patients (Kets et al. 2009; Rahner et al. 2008; Sjursen et al. 2009) have been explicitly reported to lack signs of NF1. All 83 patients showed more than one café-au-lait macule (CALMs). However, in not all of the patients did they reach the critical number of $\geq$6 which is diagnostic for NF1. In some patients, a hemicorporal or segmental distribution of CALMs has been reported (Auclair et al. 2007; Wang et al. 1999) (Fig. 16.1a, b shows the segmental distribution of CALMs and freckling in a patient compound heterozygous for two *MSH6* mutations). Furthermore, several reports stress that CALMs present in CMMR-D patients vary in their degree of pigmentation and have irregular margins and, therefore, differ from those seen in NF1 patients (De Vos et al. 2006; Kruger et al. 2008; Scott et al. 2007a; Tan et al. 2008). However, these subtle differences (1) may be recognized only by experienced clinicians and they (2) may not be discernable in all patients (Fig. 16.1c shows the only two CALMs present in a patient who is compound heterozygous for two *PMS2* mutations). Other NF1-associated signs were seen less frequently. Lisch nodules and neurofibromas were reported each in four patients (Auclair et al. 2007; Gallinger et al. 2004; Ricciardone et al. 1999; Wang et al. 1999). Tibia pseudarthrosis (Wang et al. 1999) and unilateral dysplasia of the sphenoid wing bone (Dr. Shenkmann, personal communication) were each reported in one patient. However, only a minority of patients will actually fulfill the NIH criteria for NF1 in terms of their skin manifestations. Taken together, the presence of CALMs and other features of NF1 are a hallmark of CMMR-D that should be used as diagnostic criteria (see also Sect. 16.5).

Owing to the clinical overlap of CMMR-D with NF1, a number of CMMR-D patients have been misdiagnosed as NF1 in the past. This had not only confounded proper diagnosis in these patients and the wider family but it may also have influenced our knowledge of NF1 and its association with rare tumor entities.

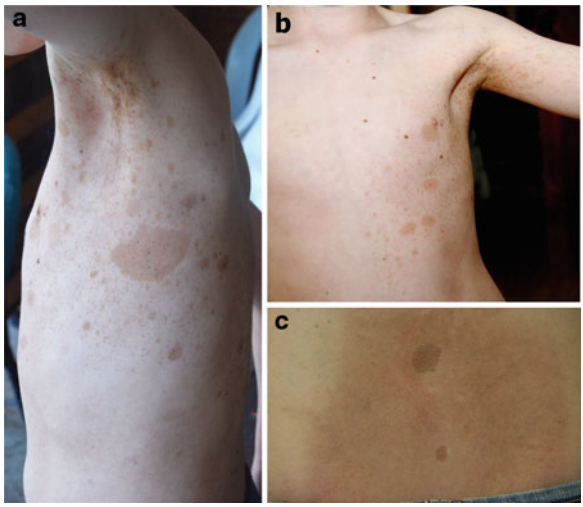

Fig. 16.1 Pigmentary manifestations in two CMMR-D patients. Note the segmental distribution in the first patient (**a** and **b**) carrying compound heterozygous *MSH6* mutations. The second patient (**c**), who was compound heterozygous for *PMS2* mutations, had only two café-au-lait macules on the lower back

Most brain tumors in children with NF1 are low grade and are classified as WHO grade I pilocytic astrocytomas; they do not typically progress to malignancy. However, a few case reports (Distelmaier et al. 2007; Glover et al. 1991; Uyttebroeck et al. 1995) and a retrospective clinic-pathological study (Huttner et al. 2010) have provided evidence for an association of childhood glioblastoma and NF1. The fact that high-grade astrocytic tumors, mainly WHO grade IV glioblastomas, are the most prevalent tumor entity found in CMMR-D patients (so far >50 patients) challenges the diagnosis of NF1 at least in some of these patients. Therefore, the association of NF1 and pediatric high-grade astrocytoma remains to be reevaluated in further studies to clearly exclude CMMR-D as the underlying disorder in childhood patients with high-grade astrocytomas and signs of NF1.

Overlap in the clinical presentation of CMMR-D and NF1 should also be kept in mind when interpreting data from patients with embryonic tumors. In particular, rhabdomyosarcoma (RMS), the most common soft tissue sarcoma in children, has been reported to be associated with NF1 (Sung et al. 2004). So far, rhabdomyosarcomas were reported in three CMMR-D patients (Kratz et al. 2009; Wang et al. 1999); in two of them, the inferred mutations in the *PMS2* and *MLH1* gene, respectively, were not genetically confirmed, and therefore, these two patients are not included in Table 16.1. Other embryonic tumors found in CMMR-D patients include neuroblastoma (De Rosa et al. 2000) and Wilms' tumor (Poley et al. 2007; Wagner et al. 2003). Their association with NF1 is still under debate.

The increased risk for NF1 children to develop juvenile myelomonocytic leukemia (JMML) is well established (see Chap. 30). So far, no CMMR-D patients with this rare myeloproliferative disease have been described. NF1 children can also develop myelodysplastic syndromes (MDS) with monosomy 7 (Shannon et al. 1992). Secondary therapy-related MDS with monosomy 7 was reported to be associated with NF1 in two reports of a total of five patients who developed

different primary malignancies and clinically presented as NF1 patients (Maris et al. 1997; Perilongo et al. 1993). In none of these patients was LOH found at the *NF1* locus in the neoplastic cells although this is the most frequent second hit mechanism of NF1 inactivation in myeloid malignancies (Stephens et al. 2006). The primary malignancies in these five patients included high-grade astrocytomas, lymphoblastic leukemia, and Wilms' tumor—all malignancies that are also observed in CMMR-D patients (see Table 16.1). Furthermore, MDS/AML was also the second malignancy in two CMMR-D patients (Etzler et al. 2008; Scott et al. 2007b), and at least in one of these patients was associated with partial loss of chromosome 7q due to a translocation t(2;7). Taken together, it is conceivable that at least some of the reported NF1 patients who developed MDS with monosomy 7 as second malignancy indeed suffered from CMMR-D, rather than from NF1.

To avoid the inclusion of CMMR-D patients misdiagnosed with NF1 in prospective studies and future case reports, a MMR defect should be excluded in all pediatric patients who show features reminiscent of NF1 without an affected parent and a malignancy other than a clearly NF1-associated one such as MPNST or JMML.

16.4 The *NF1* Gene Is a Target of MMR-D

Although the vast majority of CMMR-D patients exhibit CALMs, and in some cases, also other signs of NF1, an underlying *NF1* germ line mutation was not identified (even when applying comprehensive mutation analysis) in most of the patients for whom this analysis was performed (Auclair et al. 2007; Etzler et al. 2008; Hegde et al. 2005; Menko et al. 2004; Ostergaard et al. 2005; Trimbath et al. 2001). It has been proposed that the signs of NF1 in CMMR-D patients result from postzygotic *NF1* mutations that occur at an increased frequency early in development in these patients due to the constitutive DNA repair defect. Because of their mosaic status, such mutations will often escape detection in blood cells (see Chap. 12). This notion is supported by the hemicorporal or segmental distribution of NF1 features observed in several CMMR-D patients. So far, a deleterious *NF1* mutation has been found in only one patient (Alotaibi et al. 2008) who was originally described by Ricciardone et al. (1999). Since this mutation, a recurrent C to T transition at a CpG dinucleotide (c.3721C>T), was found in the heterozygous state in blood cells of the patient (there was no evidence of mosaicism), the authors speculated that it was a very early somatic mutation that occurred in embryonic cells. Furthermore, they hypothesized that the MMR defect due to the homozygous *MLH1* mutation and the heterozygous *NF1* mutation could have cooperated in causing the phenotype observed in this patient, i.e., >10 abdominal CALMs and two fibromatous skin tumors at the age of 12 months and an atypical chronic myeloid leukemia. This hypothesis is supported by mouse models that show that Mlh1 deficiency accelerates myeloid leukemogenesis in *Nf1* heterozygous mice (Gutmann et al. 2003).

Evidence that the *NF1* gene is a target of MMR deficiency also comes from a study of Wang and colleagues (Wang et al. 2003). In 4/10 tumor cell lines exhibiting MSI, a screen for *NF1* mutations was successful in identifying seven *NF1* alterations (one of the identified alterations, i.e., p.D176E, is a known *NF1* polymorphism, and therefore, the cell line carrying this alteration was not taken into account here), whereas in none of the five MMR-proficient tumor cell lines, a *NF1* mutation was found. This study uncovered two *NF1* alterations in five primary tumors exhibiting MSI, and the analysis of subclones revealed a mosaic *Nf1* mutation in an Mlh1-deficient mouse embryonic fibroblast cell line. Five of the ten *NF1* alterations were frameshift mutations, and four of them were located at mononucleotide repeat sequences within the coding sequence of *NF1*. This reflects the typical mutational footprint of an MMR defect and, therefore, supports the notion of an accelerated *NF1* mutation rate in MMR-deficient cells. Furthermore, it may be speculated that neurofibromin inactivation could have conferred a tumorigenic advantage which led to the clonal expansion of cells harboring *NF1* mutations in these mainly Lynch syndrome-associated tumors and tumor cell lines (Bertholon et al. 2006).

Although there is strong evidence for *NF1* mutations being the underlying cause of NF1 clinical features in CMMR-D patients, it is possible that CALMs and freckling as well as non-NF1-associated cutaneous features, such as hypopigmented skin areas, in these patients represent "isolated" skin manifestations rather than a clinical feature of mosaic or segmental NF1. In fact, CALMs are also found in other hereditary syndromes like Legius syndrome, LEOPARD syndrome, Nijmegen breakage syndrome, and Fanconi anemia. Hence, it is conceivable that somatic genetic alterations in other genes may also account for the development of the cutaneous manifestations observed in CMMR-D patients.

16.5 Diagnostic Strategies in CMMR-D Patients

In view of the wide tumor spectrum, CMMR-D syndrome should be suspected in all pediatric cancer patients showing multiple CALMs with or without other signs of NF1. Since NF1 signs in CMMR-D patients are not caused by an inherited germ line *NF1* mutation, parents of CMMR-D patients typically do not show signs of NF1. However, equally affected siblings may also present with NF1-associated features, and therefore, another NIH criterion for the clinical diagnosis of NF1 would be fulfilled in a CMMR-D patient showing >6 CALMs and/or freckling. In view of this potential pitfall, it has been suggested to change the wording of the NIH diagnostic criterion from "first-degree relative" (which includes siblings) to "parent or offspring" (Huson 2008).

In over 50 % of the reported families, the affected children carried a homozygous MMR gene mutation, suggesting consanguinity of the parents (in most of these patients) or presence of a founder mutation (De Vos et al. 2006).

As monoallelic mutations in the mismatch repair genes cause Lynch syndrome, CMMR-D patients will often have relatives with Lynch syndrome-associated cancers in their wider family. Nevertheless, it has to be kept in mind that monoallelic *MSH6* and, particularly, *PMS2* mutations have a reduced penetrance and, therefore, the family history may be unsuspicious in biallelic *MSH6* or *PMS2* mutation carriers.

Taken together, it has been suggested that a high index of suspicion for CMMR-D syndrome is expedient in any pediatric cancer patient who shows one or more of the following features:

- Café-au-lait macules and/or other signs of NF1 and/or hypopigmented skin lesions
- Consanguineous parents
- A family history of Lynch syndrome-associated tumors
- A second malignancy or a sibling with a childhood cancer

The diagnostic steps in patients suspected to suffer from CMMR-D syndrome largely follow the protocols developed for Lynch syndrome. Typically, the first step involves analysis of MSI and assessment of loss of the affected MMR gene in neoplastic tissue. MSI analysis following current protocols uses a panel of 5–6 dinucleotide (Umar et al. 2004) and/or mononucleotide (Goel et al. 2010) repeat markers. This approach is a reliable tool to diagnose MMR deficiency in gastrointestinal tumors of CMMR-D patients. However, this analysis may fail to show MSI in brain tumors (Leenen et al. 2011; Wimmer and Etzler 2008). One hypothesis put forward to explain the lack of MSI in brain tumors of CMMR-D patients is that in brain tissue MMR-D could lead to tumorigenesis through a different mechanism than postreplicative DNA repair (Bougeard et al. 2003; Poley et al. 2007). In addition, the extent and pattern of MSI may differ between gastrointestinal and brain tumors making MSI analysis that is routinely used for Lynch syndrome-associated tumors less reliable for brain tumors (Giunti et al. 2009). Immunohistochemistry (IHC) is effectively employed in all solid tumors of CMMR-D patients and guides subsequent mutation analysis in the four MMR genes. In general, a truncating mutation in *PMS2* or *MSH6* will result in the isolated loss of these proteins, whereas a mutation in *MLH1* or *MSH2* will lead to concurrent loss of MLH1/PMS2 or MSH2/MSH6, respectively, since MLH1 and MSH2 are the obligatory partners in the formation of MLH1/PMS2 and MSH2/MSH6 heterodimers. Notably, in the case of an underlying missense mutation, IHC may show normal expression of the affected MMR gene. In contrast to Lynch syndrome where expression loss is observed only in neoplastic cells, IHC detects expression loss of one (or two) of the MMR genes in both neoplastic and non-neoplastic tissues of CMMR-D patients. Expression loss of one of the MMR genes can also be demonstrated in blood lymphocytes of CMMR-D patients, e.g., by Western blotting (Péron et al. 2008). Similarly, it has been shown that MSI can be determined in normal non-neoplastic tissue of CMMR-D patients by analyzing DNA samples diluted to approximately 1–3 genome equivalents per PCR reaction (Agostini et al. 2005; Felton et al. 2007). Nonetheless, standardized procedures for the detection of

MMR expression loss and MSI in non-neoplastic tissue from CMMR-D patients have not been developed to date. The diagnosis of CMMR-D should be confirmed by gene-specific mutation analysis. Reliable and robust comprehensive analysis now exists for all MMR genes including the historically difficult *PMS2* gene (Etzler et al. 2008; van der Klift et al. 2010; Vaughn et al. 2011; Wernstedt et al. 2012).

16.6 Counseling, Surveillance, and Treatment of CMMR-D Patients and Their Relatives

The diagnosis of CMMR-D syndrome in an affected child also has important implications for the wider family. Therefore, genetic counseling should be offered to the parents prior to testing of the affected child and should include information on the potential 25 % recurrence risk and on the clinical consequences of a possible heterozygous mutation in both parents. Once a mutation has been identified, predictive testing following the established interdisciplinary counseling guidelines should be offered to all family members at risk of being heterozygous carriers of the mutation. Heterozygous mutation carriers should be followed according to current Lynch syndrome guidelines (Vasen et al. 2007). Since the penetrance of monoallelic *PMS2* mutations may be reduced as compared to *MLH1* and *MSH2* mutations, the standard guidelines may be somewhat relaxed in this case. Senter et al. (2008) have suggested that *PMS2* mutation carriers should probably follow an intermediate screening regimen such as beginning colonoscopy every 1–2 years from the age of 30 years, as was recommended for individuals with *MSH6* mutations (Bonadona et al. 2011; Lindor et al. 2006). Counseling and surveillance recommendations for individuals with heterozygous *PMS2* mutations may be further modified as we learn more about their cancer risks and spectrum of cancers through future studies.

Because of the striking cancer risk and the wide spectrum of malignancies associated with CMMR-D, drawing up recommendations for treatment and surveillance of affected patients remains a challenge. Recently, Durno et al. (2012) presented a screening protocol focused on the detection of brain tumors, leukemias, lymphomas, and gastrointestinal malignancies in CMMR-D patients. This protocol included (starting from birth) complete blood count, erythrocyte sedimentation rate and lactate dehydrogenase every 4 months, and brain ultrasound at birth and then MRI every 6 months. According to their recommendations, colonoscopy and esophagogastroduodenoscopy (video-capsule endoscopy) should be performed annually starting at the age of 3 or at diagnosis. Herkert et al. (2011) have suggested starting with annual colonoscopy at the age of 6 and with video-capsule endoscopy from the age of 8. Upon reaching adulthood, the protocol developed by Durno and colleagues added the general recommendations for individuals with Lynch syndrome to include gynecological and urinary tract screening (Bonadona et al. 2011; Lindor et al. 2006). Currently, there are no recommendations for optimal

treatment of malignancies in these patients. Nevertheless, it should be mentioned that several reports stress that MMR-deficient cells are profoundly resistant to the cytotoxicity of O^6 methylators such as temozolomide (Allan and Travis 2005; Fedier and Fink 2004). Since this suggests that O^6 methylators are likely to be highly mutagenic as well as ineffective in individuals with MMR-D syndrome, their use may increase the risk of tumor relapse and/or the development of second primary tumors (Scott et al. 2007a, b).

16.7 Conclusion

CMMR-D syndrome is an important differential diagnosis in all children who present with CALMs with or without other signs of NF1 and a malignancy that is not typical of those associated with NF1, such as MPNST or JMML. Identification of patients suffering from this childhood cancer syndrome is important since it has far-reaching implications also for the wider family.

The overlap of CMMR-D and NF1 may have lead in the past to misdiagnosis of CMMR-D patients. A critical reevaluation of the frequency of rare tumor entities in NF1 patients may therefore be desirable.

There are strong indications that signs of NF1 in CMMR-D patients result from postzygotic *NF1* mutations which are likely to occur in these patients at higher frequency owing to the inherent MMR defect. However, this finding does not exclude the possibility that other genetic alterations may account for the development of pigmentary alterations in CMMR-D patients.

It has been speculated that a heterozygous *NF1* and biallelic MMR gene mutations cooperate in promoting tumorigenesis in CMMR-D patients. The finding that the *NF1* gene is a target of MMR deficiency may also feed speculation that interindividual differences in MMR capacity could have a role in modifying the NF1 phenotype, e.g., by influencing the rate of second hit *NF1* mutations.

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Chapter 17
Insights into *NF1* from Evolution

Britta Bartelt-Kirbach and Dieter Kaufmann

17.1 Introduction

The *NF1* gene encodes the protein neurofibromin, whose main isoform contains 2818 amino acids and is 320 kDa in size. It harbours a centrally located GAP-related domain, making it a member of the Ras GTPase-activating protein superfamily. This region, termed the "GAP-related domain", (GRD) spans amino acids 1172–1538 of the protein sequence. Two additional domains of this large protein have recently been found (D'Angelo et al. 2006). Amino acid residues 1560–1698 contain a Sec14 homology domain, and a pleckstrin homology (PH)-like domain is found at residues 1715–1816. Altogether, these three domains cover only about 20 % of the whole protein.

Even today, the function of the remaining protein is still more or less unclear. However, looking at the evolutionary conservation can give us insights about functionally relevant regions of neurofibromin.

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17.2 Evolution of *NF1* Coding Region

17.2.1 The Origin of the NF1 Gene

Once the sequence of the *NF1* gene was known, it was compared to the already known sequences of other genes to identify functional domains. This initially revealed homology with the mammalian Ras GTPase-activating protein, GAP (Gutman et al. 1991), but also that this "GAP-related domain" only accounted for 10 % of the whole neurofibromin. In a search for other significant parts of the protein, homologous sequences from other species were analysed. Comparisons with other GTPase-activating proteins revealed that human neurofibromin shared more extended homology with the *Saccharomyces cerevisiae* proteins IRA1 and IRA2 (Ballester et al. 1990). The N-terminal parts of neurofibromin, IRA1, IRA2 and GAP, have nothing in common with each other. Interestingly, in the remainder of the proteins, 16–17 blocks of homology between *NF1* and the two *IRA* proteins were identified which cluster into a central (block 1–10) and a C-terminal (block 11–17) domain. The GAP sequence, however, showed only limited homology to the *NF1* and *IRA* proteins over 223–232 residues (block 7–10) within the catalytic domain. Ballester et al. (1990) therefore concluded that NF1 is more closely related to the yeast *IRA* proteins than to GAP and that it is closest to *IRA2*, especially in the C-terminal domain. In 2006, the group of Klaus Scheffzek identified two novel domains of the *NF1* gene located downstream of the GRD which lie within the C-terminal domain found by Ballester et al. (D'Angelo et al. 2006). The Sec14 homology domain and the pleckstrin homology (PH)-like domain show high homology between human *NF1*, *Drosophila Nf1*, *Neurospora crassa Nf1* homologue (about 21 % residue identity to the human sequence), *Dictyostelium discoideum Nf1* homologue and *Saccharomyces cerevisiae IRA1* and *IRA2* (D'Angelo et al. 2006).

Golovnina et al. (2006) addressed the issue of *NF1* origin and evolution systematically in 2006. They conducted BLAST (basic local alignment) searches with the human and *Drosophila Nf1* sequence against various databases and identified 22 *Nf1*-like sequences from Deuterostomia (vertebrates, echinoderms, tunicates), Arthopoda (insects, crabs) and Platyhelminthes (flat worms, tapeworms). In addition, they found four fungal proteins with less strong similarity to *NF1* (*Neurospora crassa*, *Cryptococcus neoformans*, *Chaetomium globosum*). Together, they concluded that *NF1* homologues are distributed across all major metazoan and fungal lineages. Interestingly, no *NF1* homologous sequence was identified in round worms (*Caenorhabditis elegans*, *C. briggsae*, *C. remanie*, *B. malayi*) and mollusc (*B. glabrata*). This could be explained on the one hand by the *NF1* gene arising after the separation of the bilaterian clades approximately 580 million years ago. On the other hand, it might have been lost in these two groups. The Bilateria separated into the Deuterostomia on the one hand and into the Protostomia on the other hand. The Arthropoda (of which the insects definitely have an *NF1* gene) belongs to the same branch of Protostomia as the Nematoda (round worms), while

the molluscs belong to another branch. Therefore, it seems more likely that the *NF1* gene was lost in round worms and molluscs.

In the light of ongoing sequencing of complete genomes from all lineages, the four fungi putative *NF1* homologues mentioned by Golovnina et al. (2006) deserve more attention. Two of the proteins show very little homology to neurofibromin. The other two proteins are quite similar to human neurofibromin across 2005 amino acids (*Neurospora crassa* protein related to neurofibromin) and 2452 amino acids (*Cryptococcus neoformans* hypothetical protein CNBF2880), including GRD, Sec14 and PH-like domains.

BLAST alignment (Stephen et al. 1997, 2005) of the *Neurospora crassa* protein related to neurofibromin (Q8WZX6, 2735 aa) to human neurofibromin (2818 AA isoform) reveals three blocks of homology. The first block spans amino acid 185–413 of human neurofibromin and shows 24 % identity. The second and largest block (amino acid residues 683–2419) has an identity of 29 % and includes GRD, Sec14 and PH-like domains. The third block is found between amino acid 2672 and 2713 and shows 27 % identity. *C. neoformans* hypothetical protein CNBF2880 (XP_774608) shows a main block of homology to human neurofibromin between amino acid residues 123 and 2468 with an identity of 27 % and a small block with 22 % identity (amino acid residues 2635–2742).

A new BLAST search of human neurofibromin (2818 amino acid isoform) against the current 85 fungal genomic databases (releases of May 22, 2012) yields a list of 134 matches, most of which correspond to Ras GTPase-activating proteins. Thirty-one of these proteins cover 60 % or more of the neurofibromin query sequence with identities ranging from 22% to 31 % (Fig. 17.1). Thirty-six of the matches correspond to only 6 % of the neurofibromin sequence in the range of the GRD and show 32–35 % identity over this region. Two proteins of this list, hypothetical protein FOXB_00813 of *Fusarium oxysporum* and Ras GTPase-activating protein of *Trichoderma reesei*, cover 79 % of the human neurofibromin sequence with 27 % identity. Four proteins are already annotated as neurofibromins (Table 17.1a). These are putative neurofibromin of *Metarhizium anisopliae* and *Metarhizium acridum* (Gao et al. 2011), Ras GTPase-activating protein RasGAP/neurofibromin of *Scheffersomyces stipitis* (Jeffries et al. 2007) and Ras GTPase-activating protein RasGAP/neurofibromin of *Spathaspora passalidarum* (Wohlbach et al. 2011). The above-mentioned *Saccharomyces cerevisiae* proteins IRA1 and IRA2 show 52 % and 49 % coverage, respectively, with 23 % and 21 % identity.

Also, BLAST of human neurofibromin against 39 current Protozoan databases yields, among many other matches, several annotated neurofibromin homologues with even higher coverage (Table 17.1b). All homologues contain the GAP-related domain and Sec14/PH-like domain.

A search against plant databases revealed that plants definitely do not show homologous sequences to human neurofibromin.

So the root of the *NF1* gene indeed seems to be as early as yeast and other fungi. The common ancestor of plants, fungi and animals lived approximately 1,200 million years ago (Feng et al. 1997) (Fig. 17.2), making *NF1* a very old and essential gene.

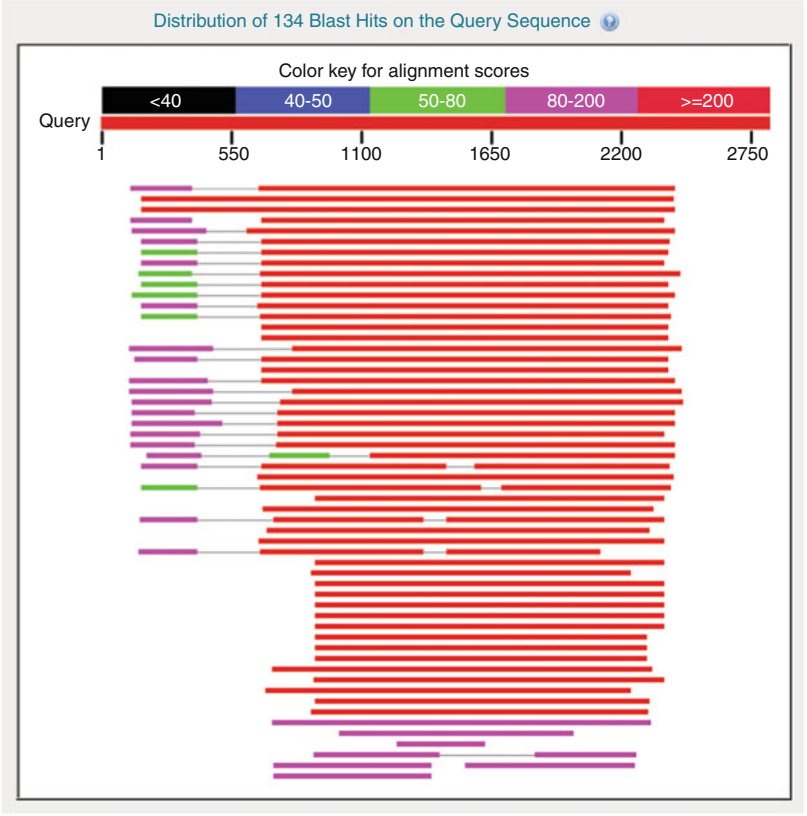

Fig. 17.1 BLAST search with human neurofibromin (2818 AA) as query sequence against fungi genomic databases. Each row represents one homologous fungal protein

Interestingly, the homology between fungal and human neurofibromin extends even beyond GRD, Sec14 and PH-like domain. This makes it very probable that there are neurofibromin functions in this region yet to be uncovered.

17.2.2 The Gene Structure of NF1 Has Also Been Conserved Over a Long Time

The insects (belonging to the Arthropoda clade) present with a clearly recognisable *Nf1* whose coding region is already almost the same size as the human counterpart. The *Drosophila* homologue was identified by the group of Bernards in 1997 (The et al. 1997). It contains 17 constitutive and two alternatively spliced exons, giving rise to two proteins of 2764 and 2802 amino acids, respectively, that are 60 % identical to human neurofibromin. The human *NF1* gene consists of 60 exons, three

Table 17.1 Earliest homologues of neurofibromin. (**a**) Fungal homologues of human neurofibromin. (**b**) Protozoan homologues of human neurofibromin

Protein	Species	Accession no.	Coverage (%)	Identity (%)
(a)				
FOXB_00813	*Fusarium oxysporum*	EGU88669	79	27
Ras GTPase-activating protein	*Trichoderma reesei*	EGR44283	79	27
CNBF2880	*Cryptococcus neoformans*	XP_774608	70	29
Putative neurofibromin	*Metarhizium anisopliae*	EFY99233	69	28
Putative neurofibromin	*Metarhizium acridum*	EFY84834	66	28
Protein related to neurofibromin	*Neurospora crassa*	Q8WZX6	60	29
Ras GTPase-activating protein RasGAP/neurofibromin	*Spathaspora passalidarum*	EGW32510	54	21
IRA1	*Saccharomyces cerevisiae*	EDV11970	52	23
IRA2p	*Saccharomyces cerevisiae*	NP_014560	49	21
Ras GTPase-activating protein RasGAP/neurofibromin	*Scheffersomyces stipitis*	XP_001386919	47	22
(b)				
Neurofibromin	*Polysphondylium pallidum*	EGG19811	91	31
Neurofibromin	*Dictyostelium fasciculatum*	EFA82125	91	30
Neurofibromatosis 1	*Capsaspora owczarzaki*	EFW43762	84	44
Neurofibromin-like protein	*Polysphondylium pallidum*	EFA86557	56	46

of which are alternatively spliced, and its main isoform is 2818 amino acids in length. Remarkably, the sequence similarity was observed over the entire length of the sequence. The most striking difference between the human and *Drosophila NF1* gene therefore is the number of exons while the protein size is almost equal.

If we step up the tree of life to the vertebrates, the next *NF1* homologue that was analysed in detail was that of the pufferfish, *Takifugu rubripes*. It is special because it has a very compact genome of only about 400 Mb compared to the 3,000 Mb of the human genome. This is due to the reduction of intron and intergenic sequence sizes and a paucity of repetitive sequences. The group of Kehrer-Sawatzki revealed in 1998 that due to this fact the Fugu *Nf1* is 13 times smaller than the human homologue. When they looked in detail on the gene structure, they found that it comprised 57 exons 51 of which were of the same size as the corresponding human ones. Even the intron–exon boundaries were exact matches with the exception of

exon 16. The Fugu *Nf1* gene lacks only exon 12b and the alternatively spliced exons 9br and 48a. Comparison of the amino acid sequences showed an overall similarity of 91.5 % and identity of 88.5 % between Fugu and human neurofibromin. The minimal catalytic fragment of the GAP-related domain (230 amino acids) showed 93.5 % similarity and 90.9 % identity. This region of higher homology extended beyond the GRD to the Sec14 domain.

So, remarkably, the gene structure is already the same in the Actinopterygii (to which *Takifugu* belongs) and the Sarcopterygii (to which land-living vertebrates belong) clades which separated approximately 420 million years ago (Fig. 17.2).

17.2.3 Almost No Further Change in the NF1 Coding Sequence Is Observed

Mouse neurofibromin, 2841 amino acids in size, is nearly identical to its human counterpart (98.5 %). They differ only in 45 amino acids, of which 22 are found between amino acids 625 and 900, immediately upstream of the IRA-related segment (AA 900–2350) (Bernards et al. 1993). This region therefore might serve as a linker between functional domains.

A 100 % identity of the amino acid sequences of human (2839 AA) and chimpanzee (*Pan troglodytes*) neurofibromin (2939 AA) indicates a strong functional and structural constraint on the neurofibromin protein in primates (Assum and Schmegner 2008). The divergence time is suggested to be 6 million years. The identity of human neurofibromin to its orthologues is 100 % in gorilla (*Gorilla gorilla*), in orangutan (*Pongo abelii*) and in gibbon (*Nomascus leucogenys*). In the Old World monkey rhesus macaque (*Macaca mulatta*) it is 96 % (2690 AA), in New World monkey marmoset (*Callithrix jacchus*) 100 % and in lemuriforme mouse lemur (*Microcebus murinus*) 83 % (Ensembl database) (Fig. 17.2).

17.3 The Bigger Picture: Changes in the Genomic *NF1* Region

That the evolution of *NF1* is dominated by a strong selective pressure on the correct function of neurofibromin can also be demonstrated by the more recent evolution of the region in and around the *NF1* gene in primate species and in human populations.

17.3.1 Rearrangement in the NF1 Region in Primate Evolution

During primate evolution, a chromosomal rearrangement has occurred in the immediate neighbourhood of the *NF1* region resulting in an altered gene order

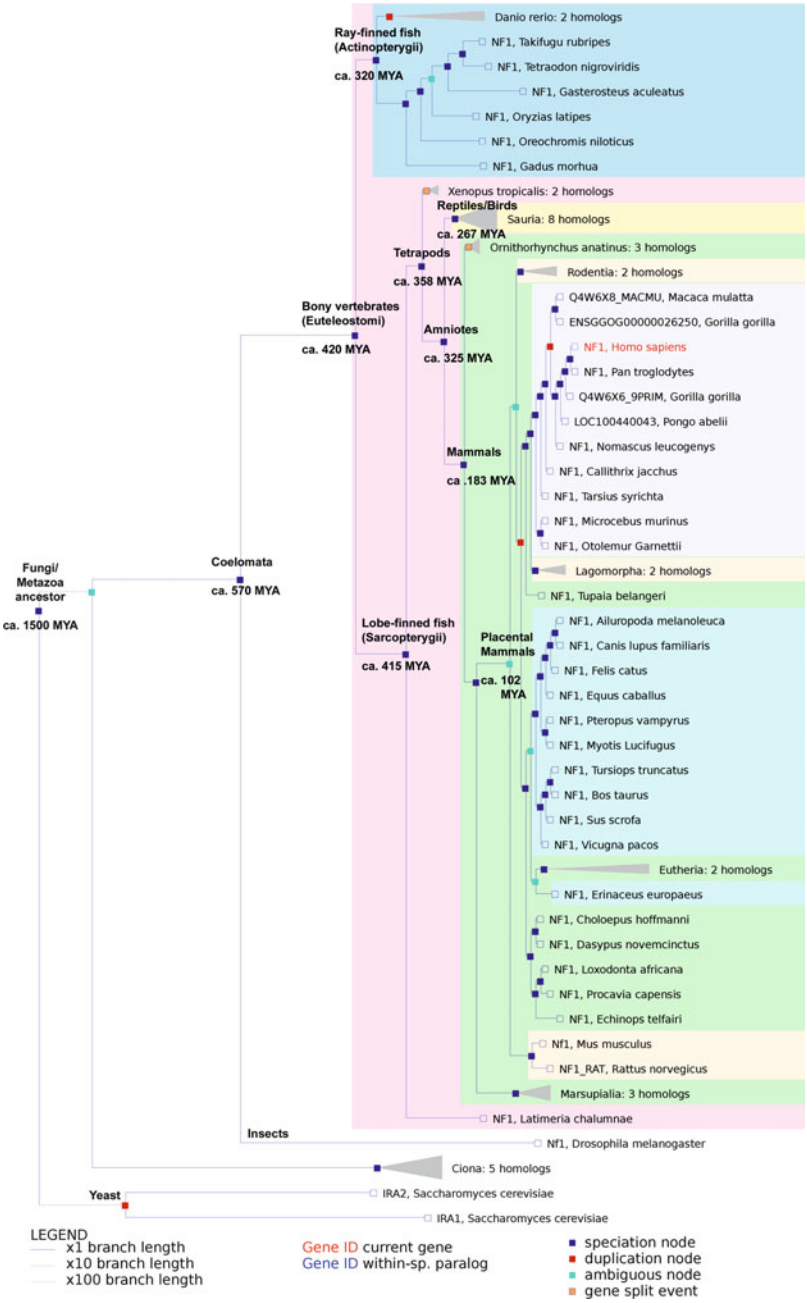

Fig. 17.2 GeneTree of *NF1* (Ensembl)

Table 17.2 Immediate genomic neighbourhood of *NF1*

A	*GOSR1*	*WSB1*	***NF1***	***SUZ12***	*CLRF3*	*RNF135*	*RHOT1*
B	*WSB1*	*GOSR1*	***NF1***	***SUZ12***	*CLRF3*	*RNF135*	*RHOT1*
C	*WSB1*	*GOSR1*	*CLRF3*	*RNF135*	***NF1***	***SUZ12***	*RHOT1*

A, Mouse (chr. 11)/dog (chr. 9); B, macaque (chr. 16); C, human (chr.17)/chimpanzee (chr. 17) (Assum and Schmegner 2008)

(Table 17.2). On macaque chromosome 16, a region of approximately 3 Mb between *GOSR1* and *WSB1* is located immediately upstream of *NF1*. This region was found to be inverted in mouse chromosome 11 and dog chromosome 9 (Assum and Schmegner 2008). The macaque gene order is altered in the chimpanzee and human *NF1* region on chromosome 17. By three inversion events, an exchange of a 700-kb region located between *NF1* and *SUZ12* and a 200-kb region between *CLRF3* and *RNF135* resulted in a complex rearrangement.

17.3.2 Conservation of the NF1 Palindromic AT-Rich Repeat During Primate Evolution

Palindromic sequences are dispersed in the human genome and cause chromosomal translocations. In humans, a palindromic AT-rich repeat was detected in *NF1* intron 40 (17PATRR) (Kehrer-Sawatzki et al. 1997; Kurahashi et al. 2003). An analysis of the 17PATRR in chimpanzees and gorillas revealed that their 17PATRRs are almost identical to those in humans (Inagaki et al. 2005). Similar short inverted repeat sequences were also found in the Old World monkeys, whereas the sequences in mice, rats, dogs and in New World monkeys showed no palindromic sequence in this region. Inagaki et al. (2005) suggested that the 17PATRR was generated accidentally in the primate lineage as a small inverted repeat sequence and developed into a large PATRR during anthropoid evolution. In this view, the palindromic region appeared approximately 25 million years ago. In eukaryotic genomes, long palindromes are often unstable, adopt secondary structures and are susceptible to deletion (Farah et al. 2002; Cunningham et al. 2003). The conservation of the 17PATRR in both humans and great apes was unexpected. The group of Inagaki suggests that this palindromic sequence is necessary for a specific *NF1* function in primates, since dogs and rodents do not have such a structure.

17.3.3 NF1 Region is GC-Poor

The GC content varies at different positions in the human genome. Stretches of a few hundred kb of DNA with a relatively homogenous GC content are called isochores. They are tightly linked to basic biological properties such as recombination (Bernardi

2000; Costantini et al. 2006; Schmegner et al. 2007). The isochore structures can be conserved throughout mammalian evolution. Schmegner et al. (2005a) found that the *NF1* region is an example of such conservation. In human, mouse and dog, the *NF1* gene in its whole length is GC-poor with less than 40 % GC. In these species, the GC content of the DNA sequences increases to 45 % downstream of *NF1* and to over 50 % immediately upstream of *NF1*. There is a defined transition zone of only five kb between the neighbouring isochores (Assum and Schmegner 2008).

17.3.4 Very High Degree of Linkage Disequilibrium Throughout the Whole NF1 Gene

Linkage disequilibrium (LD) is the non-random association of alleles at two or more loci. The extent of LD is not uniform within the human genome. In 2000, Eisenbarth found that the entire *NF1* gene is located in an approximately 300-kb region with long-range high LD (Eisenbarth et al. 2000). In a centromeric direction, the long-range LD ends within the 87 kb of intergenic sequences between *NF1* and *RNF135*; the telomeric boundary is located in the intergenic region between *NF1* and the *RAB11FIP4* gene. In addition to *NF1*, three further genes [ecotropic viral integration site 2A (*EVI2A*), ecotropic viral integration site 2B (*EVI2B*) and oligodendrocyte myelin glycoprotein (*OMG*)] that are embedded within intron 27b of *NF1* are located in the region of high LD. The boundary between these regions coincides with a transition in the GC content of the sequences indicating a correlation between the LD pattern and the isochores (Schmegner et al. 2005a).

17.3.5 NF1 SNPs in Human

SNP analysis of human *NF1* coding sequences revealed a high degree of linkage disequilibrium. At least 32 synonymous and 8 nonsynonymous SNPs are known in *NF1* and validated by frequency (NCBI, dbSNP database). The nonsynonymous SNPs show allele frequencies minor 1 % in the investigated human populations [rs112306990 (0.005 %); rs145891889 (0.005 %); rs17881753 (0.001 %); rs9907627 (0.014 %); rs140523180 (0.001 %); rs17884349 (0.022 %); rs2230850 (0.007 %); rs148154172 (0.001 %)]. This indicates an extremely low recombination rate in the *NF1* coding region. The neighbouring sequences show recombination rates comparable to the genomewide average (Assum and Schmegner 2008). These data support the strong selective pressure on the correct function of neurofibromin in human.

17.3.6 Two NF1 *Haplotypes in a European Population*

In 2005, genetic variability data derived from the *NF1* gene were used from Schmegner et al. (2005b) to infer the demographic history of the anatomically modern human in Europe. The investigation on the haplotype structure in a European population revealed no signs of positive selection on *NF1* during the recent evolution of anatomically modern humans. In a phylogenetic tree, two subgroups appear as two well-separated, old branches with relatively little variability. Haplogroup 1 sequences, found in the German gene pool, show very little intragroup variability which is typical for a population which went through a severe bottleneck followed by a rapid expansion. Deeper splits into a number of subgroups were found in haplogroup 2 sequences, representing 33 % of analysed chromosomes. This is a typical pattern for a population with a stable size for a longer period of time. Therefore, Schmegner et al. (2005b) suggested that this population is a mixture of two ancient subpopulations. The recent population went through a bottleneck followed by a population expansion that started 130,000–150,000 years before present (Assum and Schmegner 2008). The larger part of the gene pool, haplogroup 1, may represent a later immigration and expansion of a population of neolithic farmers; the rest may be derived from the first settlements of modern humans in Europe. The European *NF1* haplotypes represent an admixture of two ancient African populations. A higher diversity of haplotypes can be found in Africa, and the most ancestral haplotypes of both European subgroups are found exclusively in Africa (Assum and Schmegner 2008).

17.4 Genes Within the *NF1* Gene and Their Origin

The human *NF1* gene harbours a processed pseudogene of the adenylate kinase isozyme 3 (*AK3*) in intron 37 (Xu et al. 1992). It shows only three silent base-pair changes and therefore seems to be phylogenetically young. In accordance with this postulate, it was not found in the Fugu *Nf1* intronic sequences (Kehrer-Sawatzki et al. 1998).

In *NF1* intron 27b, three additional genes are located on the reverse strand: *OMG*, *EVI2A* and *EVI2B* (Cawthon et al. 1990, 1991; O'Connell et al. 1990; Viskochil et al. 1991). They deserve more attention as they are also quite old.

17.4.1 OMG

OMG is a cell surface protein of 440 amino acids expressed in glial cells during CNS myelination. Compact myelin appeared with the advent of the vertebrates (Waehneldt 1990). This allowed the formation of thin axons, arising first about 440

million years ago within the clade of the cartilaginous fish (sharks and rays) (Saavedra et al. 1989; Yoshida and Colman 1996).

Vourc'h et al. (2003) sequenced the *OMG* gene from 12 eutherian mammal species from seven orders and compared their protein sequences with those of mice and humans (Mikol et al. 1990, 1993). The protein from all species was 440 amino acids in size and harboured five domains. A sequence identity of 71 % was evident among the 14 mammalian species.

The Ensembl database (http://www.ensembl.org, release 67, May 2012) lists 37 OMG genes. They all belong to species of the Euteleostomi (bony vertebrates) clade which is about 420 million years old. *OMG* genes are found in the taxon of the ray-finned fish (Actinopterygii), to which *Takifugu rubripes* and *Danio rerio* belong, in the lobe-finned fish (*Sarcopterygii*) exemplified by the coelacanth *Latimeria chalumnae*, in tetrapods (as the amphibian *Xenopus tropicalis*), in reptiles and birds and of course in mammals.

Therefore, the origin of *OMG* coincides with the appearance of myelinated axons.

17.4.2 EVI2A

Human *EVI2A* (previously named *EVI2*) was identified in the course of searching for the *NF1* gene (Cawthon et al. 1990; O'Connell et al. 1990). The murine gene had previously been implicated in murine myeloid leukemogenesis (Buchberg et al. 1990). It encodes a transmembrane protein of 236 amino acids.

The Ensembl database shows 37 genes predominantly in the amniote clades (reptiles, birds and mammals). But also an amphibian species (*Xenopus tropicalis*) and one of the Sarcopterygii (the coelacanth *Latimeria chalumnae*) have an *EVI2A* gene homologue. However, no such homologue is listed for the ray-finned fish (Fugu, Zebrafish). Therefore, this gene appeared presumably within the Sarcopterygii clade which is approximately 415 million years old and also gave rise to the terrestrial vertebrates.

17.4.3 EVI2B

The *EVI2B* gene was first described in 1991 (Cawthon et al. 1991). It encodes for a 448 amino acid protein which also has a transmembrane domain.

The Ensembl database contains 32 *EVI2B* genes, only in mammalian species. The oldest homologue according to this dataset is found in platypus (*Ornithorhynchus anatinus*) with a homology of about 40 % to human *EVI2B* protein. According to this database, *EVI2B* is about 183 million years old.

17.4.4 When Do These Three Genes Appear in the NF1 Intron?

The Fugu *Nf1* intron 27b showed no similarity to *EVI2A* or *OMG* sequences, suggesting that these genes were inserted into this intron after the teleost and mammalian lineages separated about 400 million years ago. However, a single exon gene was predicted by the group of Kehrer-Sawatzki on the reverse strand of this intron with an open reading frame of 1,095 bp and a putative protein of 365 amino acids. This protein shows 42.7 % similarity and 27 % identity to 302 amino acids of the human *EVI2B* protein. The Fugu *EVI2B* gene was shown to be expressed in liver and kidney but not in brain. This Fugu *EVI2B* homologue, however, is not listed in the Ensembl database. Using BLAST with the human *EVI2B* protein in the nucleotide database, this sequence is detected (24 % coverage, 33 % identity), but only one other homologous sequence in birds (*Gallus gallus*, 20 % coverage, 44 % identity) is found before the appearance of EVI2B genes with almost 100 % coverage in mammals. It is therefore unclear how functional the Fugu *EVI2B* homologue is.

If one looks at the Ensembl database listings of the *NF1* genomic regions of the different species, it becomes clear that the three genes appear to be inserted into the *NF1* intron shortly after their appearance (Table 17.3). In the case of *OMG*, it is not located in the *Nf1* intron of Fugu and several other ray-finned fish, but it appears at this location in the zebrafish *Danio rerio* of the same clade. *EVI2A* appears within the *Nf1* gene of the coelacanth *Latimeria*; *EVI2B* is, according to Ensembl, first present in the *Nf1* intron of wallaby.

Interestingly, even after the appearance of these three genes within the *Nf1* gene, there seems to be quite some variation. In some species, one or more of the genes is lost again from the *NF1* intron or becomes a pseudogene (Table 17.3). In the light of the marginal change in the *NF1* gene itself over a long period of time, this is quite remarkable.

17.5 Summary

The *NF1* gene is very old. *NF1* homologous sequences have been found in fungi, which separated from the animals approximately 1,200 million years ago. Comparison of the neurofibromin amino acid sequence from fungi to mammals demonstrates a very high degree of structural and functional constraint. Despite several rearrangements of the *NF1* region in various mammalian species over evolutionary time, the isochore structure at the locus remained conserved. No signs of positive selection of *NF1* are evident during the evolution of anatomically modern humans. The strong selective pressure on the correct structure and function of neurofibromin is also represented by the small number of SNPs in different human populations. The three genes *OMG*, *EVI2A* and *EVI2B* were inserted into an

Table 17.3 Presence of *OMG*, *EVI2A* and *EVI2B* within *NF1* intron

Group	Species	OMG	EVI2A	EVI2B
Placental mammals	Human (*Homo sapiens*)	+	+	+
	Gibbon (*Nomascus leucogenys*)	+	+	+
	Gorilla (*Gorilla gorilla*)	−	+	+
	Macaque (*Macaca mulatta*)	+	+	+
	Marmoset (*Callithrix jacchus*)	+	+	+
	Mouse lemur (*Microcebus murinus*)	+	+	+
	Alpaca (*Vicugna pacos*)	+	+	+
	Elephant (*Loxodonta africana*)	+	+	+
	Cow (*Bos taurus*)	+	+	+
	Horse (*Equus caballus*)	+	+	+
	Dolphin (*Tursiops truncatus*)	+	+	+
	Pig (*Sus scrofa*)	+	+	+
	Dog (*Canis familiaris*)	+	−	+
	Cat (*Felis catus*)	−	−	+
	Panda (*Ailuropoda melanoleuca*)	+	+	+
	Mouse (*Mus musculus*)	+	+	+
	Shrew (*Sorex araneus*)	+	−	+
	Pika (*Ochotona princeps*)	(+)	+	−
	Rat (*Rattus norvegicus*)	+	+	+
	Kangaroo rat (*Dipodomys ordii*)	+	+	+
	Megabat (*Pteropus vampyrus*)	+	+	+
	Microbat (Myotis lucifugus)	+	+	+
	Tarsier (*Tarsius syrichta*)	−	+	−
	Rabbit (*Oryctolagus cuniculus*)	+	+	+
	Guinea pig (*Cavia porcellus*)	+	+	+
	Hedgehog (*Erinaceus europaeus*)	−	−	−
	Lesser hedgehog tenrec (*Echinops telfairi*)	−	+	(+)
	Hyrax (*Procavia capensis*)	(+)	(+)	(+)
	Tree shrew (*Tupaia belangeri*)	+	+	+
	Bushbaby (*Otolemur garnettii*)	−	−	−
	Sloth (*Choloepus hoffmanni*)	−	−	−
	Armadillo (*Dasypus novemcinctus*)	−	+	−
Marsupials	Tasmanian devil (*Sarcophilus harrisii*)	+	+	+
	Opossum *(Monodelphis domestica*)	−	+	−
	Wallaby (*Macropus eugenii*)	+	(+)	+
Birds	Chicken (*Gallus gallus*)	+	+	o
	Turkey (*Meleagris gallopavo*)	+	+	o
	Zebra finch (*Taeniopygia guttata*)	+	+	o
Lobe-finned fish	Coelacanth (*Latimeria chalumnae*)	+	+	o
Amphibians	*Xenopus laevis*	+	+	o
Ray-finned fish	Zebrafish (*Danio rerio*)	+	o	o
	Medaka (*Oryzias latipes*)	−	o	o
	Stickleback (*Gasterosteus aculeatus*)	−	o	o
	Tetraodon nigroviridis	−	o	o
	Fugu (*Takifugu rubripes*)	−	o	+ ?
	Tilapia (*Oreochromis niloticus*)	−	o	o
	Cod (*Gadus morhua*)	−	o	o
Insects	Fruit fly (*Drosophila melanogaster*)	o	o	o

+, gene present in *NF1* intron; −, gene present but not in *NF1* intron; (+), pseudogene in *NF1* intron; o, gene not present in genome; +?, data on gene presence in *NF1* intron inconsistent

NF1 intron shortly after their respective appearances. However, there would appear to be more genetic variation in this region than in the rest of the *NF1* gene.

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Chapter 18
Modifier Genes in NF1

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18.1 Introduction

Although neurofibromatosis 1 (NF1) is a common Mendelian condition with an autosomal dominant pattern of inheritance, its expression is highly variable and unpredictable. Many NF1 patients have been genotyped, but little evidence of a genotype–phenotype correlation has been observed. It is possible that important NF1 genotype–phenotype correlations exist but have not been recognized because of the complexity of the NF1 phenotype, its strong dependence on age, the nonindependence of many clinical features, and the huge allelic heterogeneity of pathogenic *NF1* mutations. However, NF1 patients with the same mutation may develop a very severe form of disease, or a mild form. Among the several causes of variable phenotypes (including intrafamilial variability) are unlinked genes (modifier genes) and environmental effects (including chance events). Family studies have first suggested that the variation in expression seen in the majority of NF1 families may be caused by the influence of modifier genes. The first evidence for the existence of modifier genes came from large family studies. NF1 mouse models have reinforced these assumptions. Recent targeted gene strategies have allowed the identification of relevant candidates and the genomic revolution may lead to dramatic progress.

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18.2 Phenotypic Approach: First Evidences of Modifier Genes in NF1

The mechanisms underlying NF1 clinical variability remain poorly understood, probably because of the involvement of complex pathophysiology and multiple factors. The major clinical features of the disease include multiple café-au-lait (CAL) spots, axillary freckling, Lisch nodules, and neurofibromas, the occurrence and the number of which vary greatly from one patient to another, even within the same family. In addition, about one-third of the patients develop one or more complications that can affect almost any organ and which seem to occur in a very unpredictable way. Development of malignant peripheral nerve sheath tumors (MPNSTs) is one of the most serious complications of NF1.

NF1 has historically been described as a very variable disease. It has rapidly become clear that the nature of the *NF1* mutation was not the only source of variation, because NF1 also showed considerable differences in expression between members of the same family. Carey et al. (1986) observed that three-quarters of families showed marked differences in the clinical severity of NF1 between individuals. The remainder of the phenotypic variation could be due to modifier genes, environmental factors, or their combination. The term modifier gene is used here to denote any gene unlinked to the *NF1* locus whose genotype modulates one or several features of the NF1 phenotype. In principle, variations in the NF1 phenotype could be determined by a single modifier gene locus, or there could be several interacting modifier genes. However, several factors other than modifier genes, such as environmental factors, might explain disease expression variability. Before planning the search for genetic factors, it was therefore important to verify that these other factors did not explain all the variability in disease expression and to assess the evidence for a genetic control of the clinical phenotype. For this purpose, the first step was to show the role of familial factors by comparing the correlation of the clinical phenotype in related and unrelated NF1 patients. It is possible to determine if genetic factors contribute significantly to the overall phenotypic variation for each NF1 trait by examining the phenotypic correlations between different types of relatives. Thus, if variation in expression is primarily determined by *NF1* mutations, the phenotype correlation for different individuals within the same family should be the same between close relatives as between distant relatives. By contrast, if the phenotypic variation is determined primarily by alleles in genes unlinked to the *NF1* locus (modifier genes), the phenotypic correlation will decrease with the degree of relationship. Higher correlations between close relatives than between distant relatives could also be the result of shared environmental effects. This possibility can be examined specifically by comparing phenotypic correlations in monozygotic twins and siblings.

18.2.1 First Clues: Study of Monozygotic Twins with NF1 and Associations Between Clinical Features in NF1

Twin studies have historically been a valuable tool for studying genetic disorders. Collection of data on twins can be especially useful in obtaining estimates of the heritability associated with the clinical phenotype. Heritability is defined as the proportion of the phenotypic variance due to genetic variance (Vineis and Pearce 2010, 2011). Twins are usually considered to share the same environment independently of their zygosity. If monozygotic (MZ) twins are more similar than dizygotic twins for the clinical phenotype, this should be explained by the effect of genetic modifiers on the clinical phenotype. MZ twins with NF1 have therefore been intensively studied as a means to investigate the genetic components of variable expressivity in NF1. There are at least 30 case reports of MZ twins with NF1 in the literature. The majority of the NF1 twin literature has focused on case reports. A high degree of concordance with respect to clinical symptoms (CAL spots, axillary and inguinal freckling, Lisch nodules, epilepsy, non-dysplastic scoliosis, renal vascular hypertension, unilateral ptosis, and cutaneous neurofibromas) has been observed in many MZ with NF1, suggesting that genetic factors exert a major influence on the inter- and intra-familial clinical variability (Easton et al. 1993; Sabbagh et al. 2009; Melean et al. 2010). In principle, this concordance is readily explicable, not simply in terms of the presence of identical *NF1* mutations in the MZ, but also as a consequence of the virtual genetic identity of the MZ genome wide, and their shared experience of very similar pre- and perinatal environments. A few cases of MZ twins with NF1 who differ quite markedly with respect to the clinical manifestations of the disease have also been reported (Rieley et al. 2011), testifying to the likely complexity of the underlying biology. However, the causative *NF1* mutation has not always been identified in such cases and the nature of the factors responsible for bringing about the discordance of clinical symptoms in these MZ twin pairs has remained enigmatic. Interestingly, a recent study reported a unique pair of MZ twins discordant for NF1 in which mutational analysis implied the occurrence of a postzygotic *NF1* gene mutation exclusively in the affected twin, leading to somatic mosaicism for the *NF1* mutation (Vogt et al. 2011). Tumors in twins with NF1 have shown significantly less concordance than other features in some reports. Since many NF1-related tumors have been found to require a second hit in the other *NF1* allele, the sporadic nature of the second hit event has been suggested to explain the discrepancy of tumors in twins. Alternatively, there could be other nonhereditary factors influencing tumor initiation and growth such as epigenetic changes, somatic mutations in other tumor-related genes, or environmental events. Inversely, a recent report has described a pair of MZ twins affected by NF1 resulting from a de novo mutation with both twins developing a left sciatic plexiform neurofibroma that evolved into MPNST at a similar age with pulmonary metastasis at the same age (Melean et al. 2010). Data from MZ twins, although precious, should be considered with care because of several limitations. First, the sample sizes of MZ twin pairs are always small (ten pairs in the largest study).

In addition, there has probably been incomplete ascertainment for certain complications of NF1 which would have required routine imaging (like plexiform neurofibromas). Finally, twins have often been of a young age, and it was therefore unclear which additional NF1 complications would develop in the future.

Clues to the existence of modifier genes in NF1 were also obtained by the study of associations between NF1 symptoms. Several statistically significant associations were shown to exist between the occurrences of individual clinical features. Szudek et al. found significant associations in the occurrence of Lisch nodules, optic glioma, learning disability, macrocephaly, and short stature in affected parent–child pairs, but made no attempt to adjust for the nonindependence of multiple relative pairs from the same family or for associations between clinical features in individuals (Szudek et al. 2000). In a following study, analysis was extended to measure correlations of NF1 features among relatives of various classes. By comparing the observed correlations, evidence that genetic sources of variation are generally important in NF1 and vary for different clinical features was provided (Szudek et al. 2002).

18.2.2 Evaluation of the Inherited Component of Variable Expression in NF1

In order to assess the contribution of genetic factors to the phenotypic variation in NF1, several studies have examined a number of NF1-related traits in a large series of multiple-case NF1 families. Since the patterns of variable expressivity are subtle, data are required on a very large number of patients and/or on very large families to identify modifier genes that affect the NF1 phenotype. Actually, the most important confounding factor in familial analyses of NF1 is age. Given the progressive nature of many NF1 disease features and the potentially confounding effects of age on the analysis, it is essential that the data be representative of all age groups. Many disease features are more prevalent in older NF1 patients (Cnossen et al. 1998), and if not appropriately controlled, age might produce a correlation between affected relatives of similar age (sibs) or obscure a correlation between relatives of very different ages (parents and children). Mosaicism is another parameter that must also be taken into account. Somatic mosaicism in de novo NF1 cases has to be considered because it may lead to milder or atypical NF1 phenotypes (Kehrer-Sawatzki and Cooper 2008; Messiaen et al. 2011).

By examining the phenotypic correlations between different types of relatives, evidence for a strong genetic component in NF1 has been provided and the involvement of unlinked modifier genes in the variable expression of the disease has been suggested. To date, only three studies have assessed the inherited component of variable expression in NF1 on large cohorts of well phenotyped NF1 families (Easton et al. 1993; Szudek et al. 2002; Sabbagh et al. 2009).

In a study published in 1993, Easton et al. studied 175 individuals with NF1 from 48 families, including six pairs of MZ twins, 76 pairs of sibs, 60 parent–offspring pairs, 54 second-degree relative pairs, and 43 third-degree relative pairs (Easton et al. 1993). Eight NF1 clinical features were scored: three quantitative traits (number of café-au-lait spots, cutaneous neurofibromas, and head circumference) and five binary traits (presence or absence of plexiform neurofibromas, optic gliomas, scoliosis, epilepsy, and referral for remedial education). Significant intrafamilial correlations were found for three quantitative variables: number of CAL spots, number of cutaneous neurofibromas, and head circumference: correlation was highest between MZ twins, less high between first-degree relatives, and lower still between more distant relatives. The high correlation between MZ twins suggested a strong genetic component in variation of expression, but the low correlation between distant relatives suggested that the type of mutation at the *NF1* locus itself played only a minor role. Easton et al. concluded that the phenotypic expression of NF1 was to a large extent determined by the genotype at other modifier loci and that these modifier genes were trait specific.

About 10 years after the study by Easton et al., a second large family phenotypic correlation study was published (Szudek et al. 2002). Szudek et al. examined familial aggregation of NF1 features among 904 affected individuals in 373 families with two or more members with NF1 (346/373 were nuclear families that included either an affected parent and one or more affected children or two or more affected sibs). This sample size was five times larger than in Easton et al., and ten clinical features were examined (CAL spots, intertrigous freckling, Lisch nodules, cutaneous neurofibromas, subcutaneous neurofibromas, plexiform neurofibromas, seizures, scoliosis, optic glioma, and other neoplasm). All phenotype traits were treated as binary variables: CAL spots and dermal neurofibromas were not counted, unlike in the Easton et al. study. Multivariate regression was used to measure the associations between various classes of relatives for each of the ten clinical features of NF1, while simultaneously adjusting for covariates including related features, age, and gender. As previously described by Easton et al., the familial patterns suggested that unlinked modifier genes and the normal *NF1* allele may both be involved in the development of particular clinical features of NF1, but that the relative contributions vary for different features. Szudek et al. suggested that most NF1 clinical features had important genetic components but more than one genetic factor may be involved, and the relative importance of various genetic and nongenetic effects may vary for different features.

These two studies have been crucial as together they demonstrated a strong genetic component in NF1 expression variability. However, certain limitations have to be taken into account. The study by Easton et al. examined a limited number of patients while the study by Szudek et al., although dealing with a larger number of patients, could not investigate many distant relatives owing to the low number of extended families. Moreover, this study did not analyze CAL spots and dermal neurofibromas (the main manifestations of NF1) as quantitative variables, and these features were instead treated as binary traits. In a third large familial phenotypic study, Sabbagh et al. used variance components analysis based on maximum likelihood procedures to

estimate the proportion of phenotypic variation that was attributable to genetic effects (Sabbagh et al. 2009). Patterns of familial correlations were examined for 12 NF1-related clinical features, including 5 quantitative traits (number of small and large CAL spots, and number of cutaneous, subcutaneous, and plexiform neurofibromas) and 7 binary ones. These phenotypic traits were scored in 750 NF1 patients from 275 multiplex families. All clinical features studied, with the exception of neoplasms, showed significant familial aggregation after adjusting for age and sex. For most of them, patterns of familial correlations indicated a strong genetic component with no apparent influence of the constitutional *NF1* mutation. In accordance with the previous findings by Szudek and Easton, several statistically significant associations between combinations of clinical features of NF1 were found, suggesting that some NF1 features may share common genetic determinants. Such results indicated a possible common repertoire of genetic modifiers for some combinations of traits.

By studying phenotypic correlation in well phenotyped NF1 families, these three large studies have provided evidence that genetic modifiers, unlinked to the *NF1* locus, contributed to the variable expressivity of NF1 (Easton et al. 1993; Szudek et al. 2002; Sabbagh et al. 2009). This assumption has been confirmed by NF1 mouse models that were developed since the identification of the *NF1* gene.

18.3 Modeling NF1 in Mouse: Confirming Existence of Modifier Genes

Humans are typical of a natural population, showing greater genetic diversity and a wider variety of environments than in laboratory animals, so it is not surprising that genotype–phenotype correlations are much looser in humans than in laboratory mice. In laboratory animals, modifier effects are usually attributed to genetic background and can be inherited as Mendelian or polygenic traits. The genetic background of a mouse describes the genetic constitution (all alleles at all loci) except for the mutated gene of interest. Evidence for modifier effects can come from a range of phenotypes among different inbred (consanguineous) strain backgrounds that cannot be explained by alleles of the disease gene or by environmental factors (that are controlled in laboratory mice).

The identification of the *NF1* gene has made it possible to generate mice with null mutations of the murine orthologous gene (*Nf1*) that were expected to recapitulate the clinical features of human NF1, including the development of neurofibromas and MPNSTs (Carroll and Ratner 2008; Parrinello and Lloyd 2009; Staser et al. 2012). Since NF1 patients inherit one defective copy of the *NF1* gene, the first approach was to generate mice carrying a targeted disruption in one *Nf1* allele. Mice heterozygous for an *Nf1* "knockout" mutation ($Nf1^{+/-}$) were viable, fertile and cancer-prone, like their human counterparts. However, they disappointingly developed only a limited subset of the findings seen in human NF1 patients (Jacks et al. 1994). These animals failed to develop some hallmark

features of the human disease, including neurofibromas and MPNSTs. These observations suggested that neurofibromas did not develop in *Nf1*$^{+/-}$ mice because inactivation of the remaining functional *Nf1* allele in murine Schwann cells occurred at a very low frequency, suggesting a possible implication of mutating phenotype. The frequency of a second hit mutation has therefore been proposed to be the rate-limiting event in mice. Given that a mutation in the second *Nf1* allele appeared to be an obligate step in tumor formation, it could be possible that this event did not occur with sufficient frequency in the relevant cell type to initiate the development of neurofibromas and MPNSTs in mice. This mutation rate hypothesis has also been invoked in human, as mismatch repair genes (*MMR*) have been proposed to be putative modifier genes of the NF1-associated tumor rate (see below). Interestingly, another animal model has pointed to a role for the *MMR* genes in neurofibroma formation (Feitsma et al. 2008): low frequency of neurofibromas was observed in zebrafish knockout mutants of the three major *MMR* genes (*mlh1*, *msh2*, and *msh6*).

18.3.1 Mouse Genetic Backgrounds and NF1-Associated Tumor Susceptibility

The development of NF1 murine models provided a unique opportunity to gain insight into NF1-associated tumor initiation and progression. Mouse genetic backgrounds and NF1-associated tumor susceptibility have been connected (Reilly et al 2000, 2004, 2006). These aspects are developed in another chapter of this book. Mouse models have recapitulated, to various degrees, the human counterparts of NF1. However, limitations of mouse models to mimic the complete human NF1 phenotype should also be underlined. For example, mice lacking the *Nf1* alternatively spliced exon 23a showed specific learning impairments (Costa et al. 2001) whereas in human, exon 23a is specifically skipped in central nervous system neurons. Such observations support a specific human genetic approach for identification of NF1 modifier genes in humans.

18.4 Genetic Approach: Proof of Existence of Modifier Genes in NF1, First Identification by Targeted Strategies and Promises of Genome-Wide Techniques

Phenotypic approaches with studies of large cohorts have suggested that the type of mutation in the *NF1* gene did not account for the phenotype variability. Genotype–-phenotype correlations in NF1 have confirmed these observations.

18.4.1 Genotype–Phenotype Correlations in NF1: No Influence of the NF1 Mutation Type

NF1 phenotypic variability could be due to the existence of modifier genes but the allelic heterogeneity of the constitutional *NF1* mutation could also be one of the factors explaining the disease variability. Almost half of all NF1 cases are a result of sporadic mutations, and a huge number of different *NF1* pathogenic mutations have been reported. This extreme variability renders genotype–phenotype correlations difficult. Among the different pathogenic mutations, 5-10% are large 17q11 deletions encompassing the entire *NF1* locus and neighboring genes. Since their initial description in 1992, many studies—sometimes in contraction—have reported a more severe clinical phenotype in NF1 patients carrying genomic *NF1* deletions in comparison with patients with intragenic *NF1* mutations. This "contiguous gene syndrome" does appear to include dysmorphic features, learning disabilities, cardiovascular malformations, childhood overgrowth, a higher tumor burden and earlier onset of benign neurofibromas, and probably, a higher incidence of MPNSTs (Castle et al. 2003; Mautner et al. 2010; Pasmant et al. 2010). It has been suggested that co-deletion of several other genes should be responsible for this more severe phenotype. *OMG* and *RNF135* haploinsufficiency may be involved in the learning disability, *RNF135* gene in the facial dysmorphism, and the *SUZ12* and *CENTA2* genes in the cardiovascular malformations. Some studies have speculated that increased malignancy risk may be explained by variation in the expression of one or more genes (tumor suppressor genes rather than oncogenes) also located in the *NF1* deletion intervals (Bartelt-Kirbach et al. 2009; Pasmant et al. 2011a).

On the other hand, for patients with intragenic *NF1* mutations (>90 % of cases), no clear-cut allele–phenotype correlations have been established to date, with the exception of a 3-bp in-frame deletion (c.2970–2972 delAAT) in exon 17 of the *NF1* gene which has been associated with a particular clinical phenotype characterized by the absence of cutaneous neurofibromas (Upadhyaya et al. 2007). A few other studies have attempted to specify genotype–phenotype correlations in the context of atypical NF1 phenotypes and/or mutation types. Patients with multiple spinal tumors but very few or no additional clinical symptoms of NF1 have been reported, suggesting a subgroup or a distinct genetic form of NF1, called spinal neurofibromatosis, associated with milder *NF1* mutations or other genetic alterations (Kaufmann et al. 2001; Wimmer et al. 2002; Kluwe et al. 2003). However, several studies have shown that patients with spinal tumors can have various NF1 symptoms and *NF1* mutations. Recently, a publication observed a trend of clustering of pathogenic changes in the 5′ tertile of the *NF1* gene in patients with optic pathway gliomas (Sharif et al. 2011). However, further evidence is still required to confirm these suggestions and other potential genotype–phenotype correlations remain to be found. Nonetheless, since different affected members of the same family with NF1 often have quite different disease phenotypes, despite sharing an identical *NF1* mutation, it was already clear that variation in the mutant *NF1* allele itself did not account for all of the disease variability, as suggested by phenotypic studies.

18.4.2 No Influence of the Normal (Nonmutated) NF1 Allele

By examining the phenotypic correlations between different types of relatives, studies of vast collections of well-phenotyped families have provided evidence for a strong genetic component in NF1 expressivity and have suggested the involvement of unlinked modifier genes, and perhaps also of the normal *NF1* allele in the variable expression of the disease. NF1 phenotypic variability could actually be due to the effect of another variant on the normal allele of the *NF1* gene, in the *trans* position to the primary mutation. In a recent study, the role of the normal *NF1* allele in the variable expression of NF1 was investigated through a family-based association study (Sabbagh et al. 2009). Nine tag single-nucleotide polymorphisms (SNPs) in *NF1* were genotyped in 1,132 individuals from 313 NF1 families. No significant deviations of transmission of any of the *NF1* variants to affected offspring was found for any of the 12 clinical features examined, based on single marker or haplotype analysis. This study provided evidence for a strong genetic component in most NF1 clinical features with no apparent influence of the *NF1* gene on disease variation since neither the constitutional *NF1* mutation nor the normal *NF1* allele seemed to contribute significantly to the overall phenotypic variation for each trait, confirming the previous phenotypic observations.

18.4.3 Strategies of Genetic Approach for Identification of Modifier Genes

Strategies used to show the role of genetic factors in phenotypic expression are often classified into two categories depending upon the type of data available: linkage studies and association studies. If family data are available, one may consider performing a linkage analysis by following the segregation of the clinical phenotype and of markers in families. Linkage analysis is usually performed with random markers spanning the whole genome in a systematic approach. It was initially presented as a method to test for linkage in the presence of genetic heterogeneity but it can also be useful for determining the role of modifier genes in Mendelian diseases. An alternative strategy to linkage consists in testing for association with the clinical phenotype in samples of individuals who carry the primary mutation involved in the disease. For qualitative clinical phenotypes, the distribution of marker genotypes is compared in patients with and without the clinical phenotype to detect markers that show differences. These may be involved in phenotype expression or associated with loci involved in phenotype expression. For quantitative clinical phenotypes, the average value of the phenotype for the different genotypes can be compared with ANOVA or *t* tests (Génin et al. 2008). New methods have been developed to account and correct for population stratification in association. These methods however have a cost in terms of power and might thus be difficult to use when searching for modifier genes. Another strategy consists

in using family-based association tests with case–parent trio designs and the transmission disequilibrium test (TDT). The advantage of this approach is that it tests for both linkage and association and hence ensures that any significant results are not due to population admixture (Ott et al. 2011). The basic idea of these tests is to compare the alleles that parents do and do not transmit to their affected children. Thus, the search for modifier genes must examine whether or not there is a difference in parental transmissions according to the phenotypic categories of the affected children.

Another distinction often made in terms of strategy is based on the approach, which can be either a systematic approach where the whole genome is scanned or a more focussed approach, where candidate genes or candidate pathways are selected (Génin et al. 2008).

18.4.3.1 Hypothesis-Driven Approach: Biology-Driven Candidate Genes

Instead of blind searches for modifier genes over the whole genome, an acceptable strategy for the identification of modifier genes would be to focus on a limited number of carefully chosen genes, the so-called candidate genes. The candidates would of course differ depending on the phenotype of interest. The candidate-gene approach can be defined as the study of the genetic influences on a complex trait by (1) generating hypotheses and identifying candidate genes that might have a role in the etiology of the disease; (2) identifying variants (SNPs) in or near those genes; (3) genotyping the variants in a population; and using statistical methods (linkage or association) to determine whether there is a correlation between those variants and the phenotype (Tabor et al. 2002). Testing variants from carefully selected candidates is attractive for several reasons. The number of variants tested is generally small, thereby avoiding severe penalties for multiple comparisons during statistical analysis. Detailed understanding of the candidate gene product and its variants provides mechanistic insight and facilitates experimental studies to evaluate modifier effects.

Different approaches can be used to select candidate genes. One might look first at the genes involved in the same pathway as the primary mutation involved in the disease. Alternatively, one might decide to focus on genes located in another pathway and involved in somewhat more indirect disease consequences. Approaches have also been used based on animal models. Improved understanding of neurofibromin's biochemical functions may permit the discovery of interacting proteins and of upstream and downstream effectors that are critical for the development of particular phenotypic features. Dealing with this biology-driven approach, several modifier genes of NF1-associated tumorigenesis have been suggested because of the importance of tumors in NF1 morbidity and mortality. In humans and in mice, tumor development results from a combination of ubiquitous *NF1* heterozygosity and unpredictable *NF1* loss of heterozygosity in different cell lineages (Staser et al. 2010). Neurofibroma-derived Schwann cells bearing both *NF1*-mutated alleles ($NF1^{-/-}$) have been isolated from different neurofibromas

with mitotic recombination as the preferential mechanism underlying this loss of heterozygosity (Serra et al. 2001). As mitotic recombination shows inter-individual variation, genes that control this phenomenon have been suggested to participate in the wide variability of neurofibroma number in NF1 patients by influencing the somatic mutation rate. Variants of the mismatch repair (*MMR*) genes have also been speculated by several authors to modify the somatic mutation rate in NF1-associated neurofibromas.

18.4.3.2 Mismatch Repair Genes Hypothesis

The number of NF1-associated neurofibromas varies to a huge degree in NF1 patients and could be due to a variable amount of accumulating somatic *NF1* gene mutations. Second hit and loss of heterozygosity events have been well documented in several NF1 tumor types (Upadhyaya et al. 2008a, b; Thomas et al. 2010) and even in tibial dysplasia (Stevenson et al. 2007), but are unlikely to explain the entire spectrum of variable NF1 features. Two research groups described the role of DNA MMR for neurofibroma development in NF1 (Wiest et al. 2003; Wang et al. 2003). Both provided evidence that a reduction of the MMR capacity can result in *NF1* mutations in a high percentage of neurofibromas. From these findings, it has been speculated that early or constitutional alterations of *MMR* genes in NF1 patients may lead to an accumulation of second hits in *NF1*, which exhibits one of the highest mutation rates known for human genes. However, apart from one solitary report, constitutional mutations in human *MMR* genes could not be detected in NF1 patients (Alotaibi et al. 2008). Alotaibi et al. described the unique co-occurrence of a constitutional *NF1* germline mutation and a constitutional homozygous *MLH1* defect. Thus far, all other cases carrying *MMR* gene mutations and presenting NF1 features were not found to carry *NF1* germline mutations. Methylation has therefore been speculated to be a mechanism leading to reduced MMR activity in NF1 patients with a high tumor burden as constitutional mutations of *MMR* genes seem to be very rare. In this hypothesis, downregulation of *MMR* gene promoter regions through methylation would represent an important modifying factor for NF1 phenotypic severity. A recent study analyzed whether an increased tumor load in NF1 (defined by the number of cutaneous neurofibromas) was associated with the methylation of *MMR* genes (Titze et al. 2010). Titze et al. performed methylation-specific PCR (MSP) of *MMR* gene promoters most frequently involved in human cancers (*MLH1*, *MSH6*, *PMS2* and *MSH2*) in leukocytes of NF1 patients. However, they only found evidence for enhanced *MSH2* promoter methylation in some NF1 patients' blood leukocytes.

Several studies have also suggested other candidate modifier genes that may modulate NF1 phenotypic expression. Recent reports concerning the functional structure of the human genome showed that differences in transcription could also explain disease expression variability and that the transcriptional domains of genes might extend very far beyond the usual regulatory sequences. These considerations are in accordance with NF1 mouse model observations showing differing levels of

Nf1 expression in mouse strain backgrounds with specific phenotypes. However, determinants of *NF1* transcript levels, which could be regarded as relevant NF1 modifier genes, are still to be found.

An alternative strategy for selection of candidate modifier genes of specific NF1 phenotype trait would involve using "without a priori" genetic approaches.

18.4.4 First Identification of a Modifier Gene in NF1: A Proof of Concept

In a recent study, Pasmant et al. (2011b) opted for this strategy, in which they used whole genome high-resolution array-comparative genomic hybridization in NF1-associated plexiform neurofibromas to identify candidate modifier genes of the development of these tumors in NF1. For the first time, 9p21.3 deletions were found as the only recurrent somatic alterations in NF1-associated plexiform neurofibromas. The smallest common deletion region in 9p21.3 included the *CDKN2A-CDKN2B-ARF* gene cluster as well as the *ANRIL* gene, specifying a large noncoding RNA. A family-based association study was then carried out using tag SNPs located in region 9p21.3 in 1,105 subjects from 306 families. A strong association of allele T of SNP rs2151280 (located in *ANRIL*) with a higher number of plexiform neurofibromas was found. This association was only observed with plexiform neurofibromas and not with dermal neurofibromas, suggesting a specific role for SNP rs2151280 in the genesis of plexiform neurofibromas but not in dermal neurofibromas. To confirm the functional role of rs2151280, *CDKN2A*, *CDKN2B, ARF* and *ANRIL*, expression was analyzed in 124 NF1 patients' peripheral blood. Allele T of rs2151280 (associated with a higher number of plexiform neurofibromas compared with the other tag SNPs) was statistically significantly associated with reduced *ANRIL* transcript levels.The identification of the first NF1 modifier gene associated with the number of plexiform neurofibromas opened new perspectives in the molecular pathogenesis of the disease, particularly neurofibroma formation. This study demonstrated the relevance of whole genome characterization for the identification of candidate modifier genes involved in the genesis of plexiform neurofibromas. The proof of concept of candidate gene studies for the identification of genetic modifiers underlying NF1 phenotypic traits has therefore been achieved. Targeted strategies hold great promise for the identification of novel genetic variants responsible for the heritable features and complications of NF1. However, this candidate gene approach has been criticized because of the lack of reproducibility of results, and limits on its ability to include all possible causative genes and polymorphisms (Tabor et al. 2002). An alternative strategy is currently emerging with applications of new genome-wide techniques.

18.4.5 From Genetics to Genomics: Promises of Genome-Wide Association Studies and Next Generation Sequencing in NF1

Low-cost genotyping arrays allow researchers to move away from candidate gene-based association studies and perform genome-wide association studies (GWAS) in an unbiased manner. GWAS can interrogate millions of common SNPs for association with human complex traits (Hindorff et al. 2009; Manolio 2010). The extensive repertoire of common human DNA sequence variants are now available that provide good coverage of all common variant in the human genome (Altshuler et al. 2008). By 2011, the public catalogue of variant sites (dbSNP 135) contained approximately 52 million SNPs. Databases of structural variants (for example, dbVAR) indexed the locations of large genomic variants. The International HapMap Project catalogued both allele frequencies and the correlation patterns between nearby variants (a phenomenon known as linkage disequilibrium) across several populations. The 1000 Genomes Project was launched in 2008 with the goal of creating a public reference database for DNA polymorphisms at allele frequencies of at least 1 % in each of multiple human population groups.

The GWAS approach has proven extremely useful in finding robust genetic associations, highlighting promising and often unanticipated underlying biology. With the imminent availability of ultra-high-volume genotyping platforms (of the order of 100,000–1,000,000 genotypes per sample) at a manageable cost, there is growing interest in the possibility of conducting GWAS for the identification of modifier genes in NF1. A broad consensus emerged that the time was now ripe for launching such studies, with notably the considerable efficiency gains of multistage sampling design, specifically those made by testing only a portion of the subjects with a high-density genome-wide technology, followed by testing additional subjects and/or additional SNPs at regions identified by this initial scan. After the identification of a disease-associated region by GWAS, comprehensive study of sequence variation in the region is required to identify the full set of variants that might explain the association signal. Because GWAS arrays incompletely capture DNA variation in each region, it has been hypothesized that causal variants partially captured by linkage disequilibrium might show stronger association with phenotype than the tag SNPs used in GWAS. Thus, an important next step after GWAS is to assemble a more complete catalog of variation present in an associated region (including variants of lower frequency) and to test it for association with the phenotype of interest. With the advent of next-generation sequencing and the emergence of data from the 1000 Genomes Project Consortium (2010), investigators must choose among (or combine) multiple strategies for creating and testing a reference panel of polymorphic sites, notably including next generation sequencing (Lander 2011). Next generation sequencing technology supports high-throughput sequencing and has reduced costs to the point where a genome can be sequenced for $5,000, and it is estimated that these costs will fall further, perhaps to the $1,000 level within a year. Studying rare variants (that would also play role)

will require sequencing protein-coding (or other) regions in genes to identify those in which the aggregate frequency of rare variants is higher in cases than controls. Whether rare variants will reveal modifier genes must await next generation sequencing. The identification of modifier genes using these genome-wide methods therefore appears to be highly promising in NF1.

The huge number of markers tested in GWAS makes it necessary to correct for multiple testing and thus to use very stringent criteria to conclude a significant association. Large cohorts of well phenotyped NF1 patients will therefore be necessary to detect a genetic modifier with a weak effect. The quality of recorded information in clinical files is often unsatisfactory and clinicians have already started to construct very large, high quality clinical databases stored in electronic format. Outcome research based on high quality data retrieved from well organized and exhaustive clinical files is bound to become increasingly important. Standardization is fundamental for these projects to be successful. Clearly, the future appears to be quite fruitful and will require an integrated approach by clinicians caring for patients with NF1, epidemiologists studying its global burden, geneticists interested in understanding its variability, clinical researchers designing studies to better understand the nature of this variation, and basic scientists who will study the mechanisms that underlie the poorly understood inter-individual variability of NF1.

18.5 Conclusion

Cohort studies have shown that the expression of NF1 tends to be similar in close relatives, but unfortunately the extent of this similarity falls well short of that required for useful prediction of clinical severity. In order to provide more precise predictions, it will be necessary to identify more of the relevant modifier genes. The relatively minor contribution of variation at the *NF1* locus suggests that knowledge of the precise *NF1* mutation will generally be unhelpful. The proof of concept has been demonstrated with the identification of a modifier gene which impacts on the number of plexiform neurofibromas (Pasmant et al. 2011a, b). The first and probably the most important steps in the study of modifier genes are still to define the clinical phenotype for which one seeks modifier genes and to select the study population. Modifier genes often have at least two alleles, one of which exacerbates disease, and one that suppresses disease. These alleles can be thought of as being "disease promoting" and "disease suppressing," respectively. These disease-suppressing modifier genes move the phenotypic threshold for expression of traits so that fewer carrier individuals are affected. New disease therapeutics could be based on mimicking and perhaps enhancing the effects of naturally occurring genetic modifiers. Understanding the basis for variable disease presentation in general, and for the suppression of disease in particular, could improve the prediction, treatment and perhaps even the prevention of several NF1 complications (Nadeau 2001).

Elucidating the genetic causes of variation in the inherited component of phenotypic expression of NF1 may provide important clues as to how the disease gene operates and may also provide alternative approaches to treatment by modifying these effects. Since the genetic modifiers are known to alter the course of disease, their protein products become immediate targets for therapeutic intervention.

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Chapter 19
Dissection of Complex Genetic and Epigenetic Interactions Underlying NF1 Cancer Susceptibility Using Mouse Models

Georgette N. Jones and Karlyne M. Reilly

19.1 Introduction

NF1 is notable for the variable expressivity of the disease. Although NF1 is fully penetrant, patients with the disease show a broad range of phenotypes. This presents a problem for patients and their doctors, because it is difficult to predict which disease complications an individual will develop. This has led to an interest in identifying the sources of phenotypic variation associated with the disease. Inherited factors, environmental factors, and diet can all influence phenotypic variation and differential susceptibility in many diseases, including NF1. Comparison of both inter- and intra-familial phenotypic variance in patients has implicated heritable factors independent of the inherited NF1 allele, as well as environmental factors. In this chapter, we will focus on what is known about heritable factors affecting NF1 variable expressivity in both humans and mouse models. These factors, once characterized, can be used by patients and clinicians to predict the future course of the disease.

Despite our growing understanding of the mechanisms of tumor initiation and progression in NF1, it is still unclear why NF1 patients exhibit widely varying susceptibility to tumor development. Mouse models are a powerful tool to address this question, and we will summarize in this chapter how mouse models are informing our understanding of genetic complexity and epistatic interactions in NF1. Studies in a mouse model of NF1 malignancies have demonstrated that modifier genes unlinked to NF1, as well as the sex of the individual and the inheritance of the disease from the mother or father, affect the risk for developing different types of tumors. Taken together, these data suggest that subtle differences

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in the genetic and epigenetic background of NF1 patients can have far reaching implications on the risk of developing NF1-associated tumors.

19.2 Phenotypic Variation in NF1

19.2.1 Variable Expressivity in Patients

Because NF1 is highly pleiotropic, studying its expressivity requires an in depth understanding of the associations between individual or collective phenotypes. For instance, expressivity of the pigmentation features (e.g., café-au-lait spots, skin fold freckling, and Lisch nodules) are highly associated with each other such that if an individual presents with a large number of one trait, he or she is likely to have an increased number of the associated traits (Szudek et al. 2003). The incidence and severity of all three forms of neurofibroma (cutaneous, subcutaneous, and plexiform) are also highly associated with each other, and to some degree subcutaneous neurofibromas may correlate with the pigmentation group (Szudek et al. 2003). In a third association group, optic pathway glioma (OPG), macrocephaly and other neoplasms were also correlated with each other (Szudek et al. 2003). Family studies of these associations revealed that affected first degree relatives had a greater associated expressivity of the pigmentation features than did affected second degree relatives, suggesting a role of increased genetic similarity or more similar environmental/dietary effects, rather than the effect of the inherited *NF1* mutation (Szudek et al. 2002). Expressivity of both pigmentation and neurofibroma features in affected first degree relatives was more highly correlated in siblings than between parent and child, and interestingly, there was higher association in neurofibroma and Lisch nodule expressivity between fathers and children (paternal inheritance) than between mothers and children (maternal inheritance), indicating that not only genetic but also epigenetic modifiers can influence the phenotype (Szudek et al. 2002). In a separate cohort of patients, a high intra-familial association of the non-neoplastic features (including neurofibromas) was observed after controlling for age and sex, but interfamilial expressivity was not influenced by the type of *NF1* mutation (Sabbagh et al. 2009). Although it had initially been thought that interfamilial variations were related to the many mutations identified in the *NF1* gene, collectively these family association studies imply that the specific mutation appears to play a very small role in influencing expressivity. Very few *NF1* mutations actually do correlate with phenotype, suggesting that variable expressivity is primarily caused by genetic factors unlinked to *NF1* or by environmental/dietary factors.

19.2.2 Mouse Models of NF1 and Strain-Dependent Effects

Genetic modifiers can be identified in patients by genome-wide association studies (GWAS) or by testing candidate modifiers in case–control studies. These studies

require testing very large cohorts to overcome the inherent heterogeneity of human populations. In the case of NF1, an even larger number of patients must be identified to study a given phenotype (such as tumor number), due to the variable expressivity of NF1 phenotypes. Mouse models of NF1 can be used to breed large numbers of individuals with reduced heterogeneity, allowing researchers to control factors such as environment, diet, sex, parental inheritance, and genetic background to ask focused questions on how these different factors contribute to variability in NF1.

Identifying candidate modifier genes in mice uses statistical analysis of the linked association between observed phenotypes and genome-wide genotypes through a variety of experimental designs (see Reilly (2009) for additional review). This linkage analysis can identify variable regions of the genome that account for strain-specific differences in phenotypes. Within these regions, natural genetic variations, such as single nucleotide polymorphisms (SNPs), can affect the function of modifier proteins by changing the protein sequence, affecting transcription level or stability, altering mRNA splicing, or changing the sequence of microRNAs that can globally affect the expression of genes. In addition to their potential effects on genes, naturally occurring genetic variations could contribute to phenotypic variance by epigenetic mechanisms, such as by altering imprinting marks, DNA methylation sites, or binding sites for chromatin regulators. Although these naturally occurring variations may be tolerated over the course of evolution within the population, within individuals these genetic or chromosomal modifications can have magnified impacts on tumorigenesis in the setting of an *NF1* mutation. By identifying genomic regions that contribute to phenotypic variability in mouse models, and studying the molecular mechanisms responsible for these effects, one can develop testable hypotheses that can be further studied in patient populations to explain the variable expressivity of NF1.

Numerous NF1 mouse models have been developed to better understand the mechanisms of tumor initiation, the role of the microenvironment, and to identify signaling networks involved in NF1 tumorigenesis, but many models are not useful for studying strain-specific effects on tumor susceptibility due to heterogeneous (and thus uncontrolled) genetic background, as well as the requirement for breeding together multiple mutant mouse lines to obtain the desired phenotypes. This can make the breeding of large cohorts of animals to identify modifiers prohibitively expensive and long-term. For identifying modifiers, simpler mouse models that recapitulate aspects of the disease are more powerful for setting up cross designs.

19.2.2.1 The *Nf1*$^{+/-}$ and *Nf1*$^{-/-}$ Mouse Models of NF1

Nf1$^{+/-}$ mice have been engineered on both an inbred 129S4/SvJae background as well as a 129S4/SvJae;C57BL/6J mixed background. These animals were not subject to the pathognomonic neurofibromas or pigmentation features of NF1, but did develop neurofibrosarcoma, leukemia and pheochromocytoma (Jacks et al. 1994). Whilst F1 hybrid (129S4/SvJae × C57BL/6J)-*Nf1*$^{+/-}$ mice developed pheochromocytoma, inbred 129S4/SvJae-*Nf1*$^{+/-}$ animals never developed these adrenal

tumors (Tischler et al. 1995). This strongly suggested that *Nf1* mutant mice are subject to strain-specific phenotypic variability. However, due to long latency and low penetrance of NF1-related phenotypes, this model has limited use in NF1 modifier studies. Chimeric mice from $Nf1^{-/-}$ 129S4/SvJae ES cells injected into C57BL/6J blastocysts resulted in neurofibroma development that was correlated with the degree of chimerism (Cichowski et al. 1999), but strain-specific effects have not been examined in this model. Furthermore, the need to microinject each blastocyst to create individual mice makes this model impractical for generating the large numbers of animals needed for mapping modifiers. Finally, *Nf1* tissue-specific knockout in various nervous system compartments results in robust models for studying NF1-associated tumor biology (Bajenaru et al. 2003; Wu et al. 2008; Zhu et al. 2002). Because these conditional $Nf1^{-/-}$ models involve combining a minimum of three mutant alleles, studies have not been performed to compare the effects of strain background on the phenotypes, and these models would be difficult to adapt to modifier mapping studies.

19.2.2.2 *NPcis* Mouse Model of NF1

Since $Nf1^{+/-}$ mice do not develop common lesions associated with NF1, and tissue-specific knock-out mice require a minimum of three mutant alleles to initiate tumorigenesis, NF1 modifier studies in mice have focused on using the *NPcis* model of NF1-associated malignancies. *NPcis* mice were engineered to transmit mutations in both *Nf1* and *Trp53* as a single allele. *TP53* tumor suppressor mutation has previously been described as a common second hit in the progression of human NF1 MPNSTs (Menon et al. 1990). Additionally, *TP53* loss is often reported in spontaneous astrocytoma (van Meyel et al. 1994; Watanabe et al. 1997). Mating $Nf1^{+/-}$ and $Trp53^{+/-}$ mice resulted in $Nf1^{+/-};Trp53^{+/-}$ *trans* (*NPtrans*) mice which have an increased susceptibility to MPNSTs (Cichowski et al. 1999). However, this susceptibility further increased when *NPtrans* mice were mated with wild-type animals to yield progeny with $Nf1^{+/-};Trp53^{+/-}$ *cis* (*NPcis*) mutations after meiotic recombination to join the two mutant alleles onto the same chromosome (Cichowski et al. 1999). In mouse, *Nf1* and *Trp53* are linked approximately 5 cM apart from each other on chromosome 11 (Buchberg et al. 1992). Given their close proximity, co-inheritance of both mutant alleles in *cis* without meiotic recombination between them is fairly high, and LOH events typically result in loss of wild-type alleles for both *Nf1* and *Trp53* thereby initiating tumorigenesis in this model (Cichowski et al. 1999; Reilly et al. 2000) (Fig. 19.1).

NPcis mice develop spontaneous cancers associated with the human NF1 syndrome. Most commonly, they develop MPNST or astrocytoma of the brain, and to a lesser extent they are prone to spinal cord astrocytoma, pheochromocytoma, histiocytic sarcoma, and lymphoma (Reilly et al. 2000, 2004). MPNST occurs in up to 80 % of *NPcis* mice beginning at around 5 months of age (Cichowski et al. 1999), although as will be described below, the incidence of these tumors is dependent upon strain background and parental inheritance of the mutant allele (Cichowski

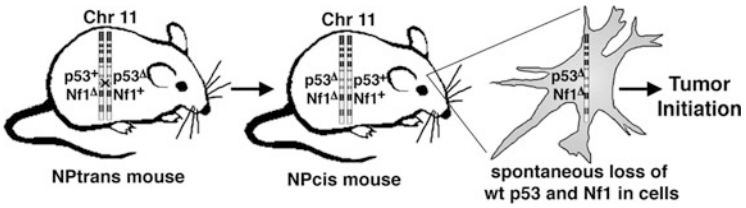

Fig. 19.1 The *NPcis* mouse model was originally made by a rare recombination event between the *Nf1* gene and the *Trp53 (tp53)* gene in *NPtrans* mice (*left*) such that the mutation in *Nf1* (*Nf1Δ*) and the mutation in *Trp53* (*p53Δ*) become linked on the same copy of chromosome 11 in *cis* (*center*). The wild-type copy of *Nf1* (*Nf1+*) and *Trp53* (*p53+*) can be spontaneously lost in cells as the mouse ages, leading to tumor initiation

et al. 1999; Reilly et al. 2006). These tumors typically arise from the peripheral nerves of the limbs and trunk and they exhibit histological features similar to human tumors such as spindle cells, mitotic figures, and S100 and p75 immunoreactivity (Reilly et al. 2006). As with MPNST, astrocytoma incidence is dependent upon strain and inheritance; however, depending on the study, up to 70–100 % of *NPcis* mice can develop astrocytoma by 6 months of age (Reilly et al. 2000, 2004). The World Health Organization (WHO) has described four grades of astrocytoma (I–IV) in human that are histologically distinct and indicative of severity (Kleihues et al. 1993). *NPcis* mice develop astrocytomas resembling WHO grade II–IV astrocytomas (Reilly et al. 2004), with diffuse borders, increased numbers of mitotic figures, and elongated glial cell nuclei. In rare cases, *NPcis* astrocytomas also displayed areas of necrosis, neovascularization, and multinucleated giant cells signifying progression of lower grade lesions to malignant WHO grade IV tumors, also known as glioblastoma multiforme (GBM) (Reilly et al. 2000). In addition to the brain, astrocytomas have been observed in the spinal cords of *NPcis* mice. Similar to the WHO grade II and III tumors of the brain, these spinal cord tumors are diffusely infiltrative and exhibit numerous mitotic figures (Amlin-Van Schaick et al. 2012c). Lower grade spinal cord astrocytomas can progress to a GBM-like pathology, and at times they will spread into the brain to form secondary tumors (Amlin-Van Schaick et al. 2012c).

19.3 Causes of Phenotypic Variation

The advantages of the *NPcis* model for modifier studies are not only the simplicity of genetic transmittance, the high penetrance of multiple tumor types, and similarity to the human condition. Importantly, *Nf1* allele status, sex, strain specificity, and parental inheritance can all be tested independently in *NPcis* mice and have been shown to play varying roles in determining susceptibility to tumorigenesis. Combining data from human studies and the *NPcis* model demonstrates how these different heritable factors all contribute to variable expressivity in NF1.

19.3.1 NF1 Gene Mutations and Alleles

Of the hundreds of identified *NF1* mutations, two have been shown thus far to exhibit genotype–phenotype correlations in patients. One involves large deletions of the *NF1* allele (often maternally inherited), giving rise to NF1 micro-deletion syndrome where patients exhibit severe learning deficits, and dysmorphic growth (Mensink et al. 2006; Riva et al. 2000; Spiegel et al. 2005; Upadhyaya et al. 1998; Venturin et al. 2004). The other mutation is an in-frame deletion of 3 base-pairs in exon 17, termed delAAT, which results in a complete lack of cutaneous neurofibromas (Upadhyaya et al. 2007). Since the other *NF1* mutations have generally not been linked to a clearly defined phenotype, and because susceptibility factors in NF1 patients do not increase overall cancer risk in unaffected family members (Airewele et al. 2001), it led to the hypothesis that both unlinked modifiers and subtle alterations in the wild-type *NF1* allele can influence the phenotype, although the effect of each factor may be dependent upon the specific clinical feature (Szudek et al. 2002).

Differences in endogenous expression levels of normal *NF1* have been observed in both humans and mice and have the potential to modify NF1 phenotypes. One study found that approximately 30 % of unaffected individuals exhibited a skewed ratio of *NF1* allele expression from the normal 50:50 (maternal:paternal) ratio (Jentarra et al. 2011). Healthy individuals could naturally have up to a 25 % difference in expression between the two alleles, which could potentially modify expressivity based on an inherently altered expression of the wild-type *NF1* gene copy in an individual with NF1 (Jentarra et al. 2011). Another study showed that monozygotic twins with discordant disease phenotypes displayed varied levels of *NF1* promoter, 5′-UTR, and exon 1 methylation (Harder et al. 2010), suggesting that *NF1* gene expression may be regulated at both the genetic and epigenetic level. Both wild-type and *NPcis* mice show differential expression of *Nf1* on different strain backgrounds prior to tumor formation. *Nf1* is expressed at higher levels in brains and sciatic nerves on the C57BL/6J background than the 129S4/SvJae background (Hawes et al. 2007; Tuskan et al. 2008). The difference in *Nf1* expression level in the different strains did not correlate with tissue-specific tumor susceptibility. In the central nervous system, the C57BL/6J strain is more susceptible to astrocytoma and expresses higher levels of *Nf1* in the brain compared to the 129S4/SvJae strain. However, in the peripheral nervous system, both C57BL/6J and 129S4/SvJae are equally susceptible to MPNSTs, but the level of C57BL/6J is still higher than 129S4/SvJae in normal nerve (Hawes et al. 2007). In patients, analysis of SNPs in the wild-type *NF1* allele showed no association with expressivity, further supporting the role of modifiers independent of the *NF1* locus (Sabbagh et al. 2009). Mice show relatively few heterogeneous SNPs in *Nf1* when comparing strains that show variable phenotypes in *NPcis* mutant mice. Taken as a whole, these studies demonstrate that while variation in *NF1* expression exists between individuals, its role in variable expressivity of the disease remains in question, and

is likely to be caused by variable *trans*-acting factors, rather than being linked directly to *NF1*.

In addition to variation in *NF1* expression level, differences in the expressed neurofibromin isoform could potentially influence the variable expressivity of NF1. For example, several groups have shown that RNA processing of the *NF1* transcript probably influences expressivity, where certain point mutations cause changes in alternative splicing that can lead to altered functions of the gene product [reviewed in Skuse and Cappione (1997)]. While it is known that CELF, Hu, and TIA-1 can all regulate *NF1* splicing, those genes themselves have not been reported to be altered in NF1 tumorigenesis [reviewed in Barron and Lou (2012)]. It was consequently suggested that unnamed modulators of these splicing factors may be responsible for affecting *NF1* splicing and therefore variable expressivity of the syndrome. As with variation in *NF1* expression levels, variation in *NF1* splicing is likely to be due to variable *trans*-acting factors, unlinked to the *NF1* gene.

Since many NF1 phenotypes require inactivation or loss of the wild-type copy of *NF1*, variability in how the wild-type copy is lost could potentially affect phenotypic variation. One study found that each neurofibroma in a NF1 patient can result from a different secondary somatic hit to *NF1*, and that factors that affect the rate of these mutations (and presumably the cell's ability to repair the damage) are all potential modifiers of tumorigenicity—however, again no candidates have yet been identified (Wiest et al. 2003). Chromosomal instability has also been implicated in tumor modification, most notably related to loss of the wild-type copy of *NF1* (Bartelt-Kirbach et al. 2009; Kehrer-Sawatzki et al. 2008; Stephens et al. 2006). While several specific candidate genes have been hypothesized (mostly based on expression changes in tumors), further analysis of the candidates is needed (Bartelt-Kirbach et al. 2009; Kehrer-Sawatzki et al. 2008). In *NPcis* mice, our studies have found differences in tumorigenesis based on whether the mutant chromosome is inherited from the mother or father (see below) and preliminary data suggests that this is due to different mechanisms of loss of the wild-type copy of *Nf1* and *Trp53* on the maternal and paternal allele (K.M. Reilly unpublished data). Additional studies are needed to determine how important variation in mutational frequency of the *NF1* gene is to variable expressivity in the NF1 disease.

19.3.2 Sex of the Individual

One of the clearest heritable sources of NF1 phenotypic variation is the sex of the individual. Neurofibromas are known to increase during puberty and pregnancy (Huson and Hughes 1994) and respond to steroid hormones to varying degrees in mouse models (Li et al. 2010), emphasizing the role of sex hormones in NF1. Women have a significantly increased risk for developing at least one invasive malignancy or brain tumor in their lifetime compared to males (Airewele et al. 2001). In contrast, female NF1 patients survive longer than male NF1 patients (Evans et al. 2011; Ingham et al. 2011). However, females in the general population

survive longer than males and both male and female NF1 patients have shorter survival relative to the NF1-free population. Thus, NF1 females may be more severely affected by reduced survival with NF1 than males relative to the NF1-free population (Airewele et al. 2001; Masocco et al. 2011; Rasmussen et al. 2001).

A similar complex relationship between sex and tumor susceptibility is seen in *NPcis* mice. In the case of astrocytoma, depending on the cross design, males develop astrocytoma more frequently, but the astrocytomas are lower grade than those seen in females (Reilly 2010). This has been shown to be dependent upon the strain background and the inheritance of the disease from the mother or father (Amlin-Van Schaick et al. 2012b). In the case of MPNST, again depending on the cross design, males develop more MPNST with a shorter survival time than females, and males and females are affected differently by strain background (Walrath et al. 2009). In addition, our preliminary data suggest large differences in the modifier mechanisms underlying phenochromocytoma in females compared to males (G.N. Jones and K.M. Reilly, unpublished data).

19.3.3 Genes Unlinked to NF1

Studies of inter- and intra-familial variation in expressivity (Carey et al. 1979; Easton et al. 1993; Sabbagh et al. 2009; Szudek et al. 2002) support the role of modifier genes unlinked to NF1. In one report, nearly 75 % of the families studied exhibited intra-familial phenotypic variance (Carey et al. 1979). Within families, the highest similarity in expressivity was found in monozygotic twins, which decreased when comparing these probands to first degree relatives (i.e., other siblings or parents), and even more so when compared to second degree relatives (e.g., cousins, aunts/uncles, or grandparents) (Easton et al. 1993). Considering that the heritable *NF1* mutation within a given family is fixed and the family environment is relatively uniform, these data suggest that genetic alterations beyond the environment and the particular *NF1* mutation are responsible for phenotypic variance.

As described above, mouse models can be used for dissecting genetic effects by controlled breeding experiments. Incidence, latency and size of particular tumors, as well as overall tumor spectrum, have all been compared with respect to strain background in *NPcis* mice (Amlin-Van Schaick et al. 2012a, b; Reilly et al. 2000, 2004, 2006; Walrath et al. 2009). The greatest difference in astrocytoma susceptibility was observed between the C57BL/6J-*NPcis* and 129S4/SvJae-*NPcis* inbred strains, where nearly 75 % of C57BL/6J-*NPcis* and approximately 20 % of 129S4/SvJae-*NPcis* mice developed brain lesions (Reilly et al. 2004). The same trend is also observed in spinal cord astrocytomas between C57BL/6J-*NPcis* and 129S4/SvJae-*NPcis* animals (Amlin-Van Schaick et al. 2012c). In addition, *NPcis* mice on (C57BL/6J × 129S4/SvJae) F1 hybrid backgrounds show different susceptibility to astrocytoma depending on how the C57BL/6J and 129S4/SvJae strains are introduced from the parents (Reilly et al. 2004), and can develop astrocytoma

more frequently than either parental inbred strain (Amlin-Van Schaick et al. 2012b). These data show that susceptibility to astrocytoma is not a simple Mendelian trait, but rather is controlled by many genes with complex epistatic interactions in the genome.

As with astrocytomas, the incidence of MPNST in *NPcis* mice is also affected by strain and the C57BL/6J strain is highly susceptible (Reilly et al. 2006). Importantly, the strains that alter the incidence of astrocytoma are not the same as those that alter the incidence of MPNST (Reilly et al. 2004, 2006), demonstrating that modifier mechanisms are likely to be specific to different tissue types (e.g., the peripheral and central nervous system). F1 hybrids of C57BL/6J and the A/J strain are resistant to developing MPNSTs (Reilly et al. 2006), suggesting that dominant modifiers of MPNST are carried by the A/J strain. These F1 (C57BL/6J × A/J) *NPcis* hybrids develop more astrocytoma than C57BL/6J-*NPcis* mice; however, because MPNSTs tend to arise at a younger age than astrocytomas, this shift in tumor spectrum may be due to mice surviving longer in the absence of MPNSTs to develop astrocytoma later in life. Because many of the NF1 phenotypes in patients arise during specific time periods of life (e.g., optic gliomas in young children, neurofibromas at puberty, and MPNSTs in adults), it is important to consider that modifiers that appear to affect one phenotype, may be acting more directly on an earlier phenotype. Studies in mouse models have highlighted the complex interaction of genetic polymorphisms on NF1 phenotypic variation.

19.3.4 Inheritance of NF1 from the Mother or Father

Patient data suggest phenotypic differences in NF1 depending on whether the disease is inherited from the mother or the father. Many of these data can be attributed to differences in the mechanism of mutation in the male and female germline during meiosis that give rise to different types of *de novo NF1* mutations (Lazaro et al. 1996; Steinmann et al. 2007). Large deletions that include *NF1* are more likely to arise in the maternal germline and are associated with a more severe disease, such as NF1 micro-deletion syndrome. As described above, neurofibromas and Lisch nodules are more highly associated between parent and child when the *NF1* mutation is inherited from the father than when it is inherited from the mother (Szudek et al. 2002).

NPcis mice show a strong effect of parental inheritance on the tumor phenotype. *NPcis* mice that inherit the mutations from the mother ($NPcis^{mat}$) are significantly less susceptible to MPNST development but more susceptible to astrocytoma than *NPcis* mice that inherit the mutation from their father ($NPcis^{pat}$) (Reilly 2009, 2010; Reilly et al. 2006). Because these comparisons can be made in inbred strains, where the only difference between the two groups of mice is the inheritance of the mutant allele, these data suggest that the cause of this phenotypic variation is directly linked to mouse chromosome 11 where the *Nf1* and *Trp53* mutations are located, rather than other variable inherited factors, such as mitochondrial variation

or imprinted genes on other chromosomes. Furthermore, this effect of parental inheritance acts independently of the strain background, such that it can be observed on a variety of inbred and F1 hybrid backgrounds (Reilly et al. 2004, 2006). Because mice can be crossed together in controlled ways, the relative contribution of parental inheritance and genetic variation to tumor variation can be studied and candidate modifier genes identified.

19.4 Identified Modifiers of NF1

Only a few specific NF1 candidate modifier loci or genes have been identified thus far, and although more work is needed to fully uncover their mechanisms of action, the evidence to support their role as modifiers is compelling. In studies of NF1 families, paternal inheritance of a SNP variant of *GDNF* interacted with maternal inheritance of *NF1* to increase susceptibility to megacolon, a gastrointestinal feature associated with NF1 (Bahuau et al. 2001). Similarly, a single allele variant in the noncoding RNA, *ANRIL*, was correlated with incidence of plexiform neurofibromas in NF1 patients (Pasmant et al. 2011). In another study, methylation of the promoter for the mismatch repair gene *MSH2* was found to vary from patient to patient and be positively correlated with neurofibroma burden (Titze et al. 2010). Decreased expression of *MSH2* as a result of increased promoter methylation may result in defective DNA damage repair and therefore a greater propensity for tumor initiation.

Mapping of modifiers in *NPcis* mice has led to the identification of several loci responsible for susceptibility to MPNST and astrocytoma. In comparison to the C57BL/6J strain, the A/J strain shows dominant resistance to MPNST in *NPcis* mice (Reilly et al. 2006). Through mapping of modifier loci in backcross populations, two *Nerve sheath tumor resistance* (*Nstr*) loci were identified: one near the centromere of chromosome 19 (*Nstr1*, LOD = 3.0) which is specific to susceptibility in $NPcis^{pat}$ mice, and one on the proximal end of chromosome 15 (*Nstr2*, LOD = 2.6) that is specific to $NPcis^{mat}$ mice (Reilly et al. 2006). In the case of both *Nstr* loci, the presence of A/J markers was associated with a more resistant phenotype, whereas B6 markers were associated with greater susceptibility to MPNST. The localization of *Nstr1* to chromosome 19 has been independently confirmed using chromosome substitution strains in which chromosome 19 is from the A/J strain and the rest of the genome is from the C57BL/6J strain (Walrath et al. 2009). *Nstr1* is syntenic with human chromosome 11q13-12, a region involved in translocations in human MPNSTs (Jhanwar et al. 1994; Mertens et al. 1995, 2000). *Nstr2* is syntenic with human chromosome 5p13-15 and 8q22-24. Human chromosome 8q22-23 is amplified in MPNST and also undergoes translocation (Mertens et al. 2000; Rey et al. 1993; Schmidt et al. 2001). Interestingly, *Nstr2* overlaps *GDNF* on mouse chromosome 15 and human chromosome 5 (Fig. 19.2). Because both *Nstr1* and *Nstr2* show linkage specifically in $NPcis^{pat}$ or $NPcis^{mat}$ mice, respectively, it suggests that these modifiers act epistatically with inheritance

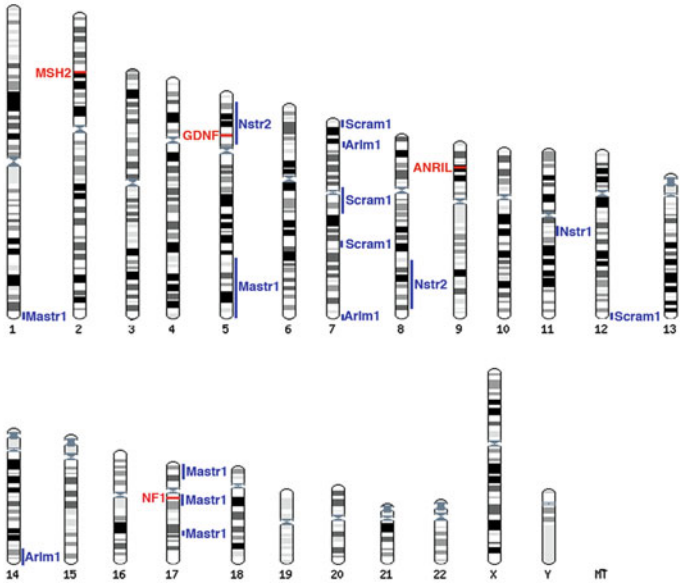

Fig. 19.2 Modifiers of NF1 mapped to the human genome. Genes identified as potential NF1 modifiers in humans are shown in *red* and indicated to the *left* of the human chromosome. Mouse modifier loci are shown in *blue* to the *right* of the human chromosome and mapped to the syntenic regions of the human genome. Because the modifier loci represent broad regions of the genome, they often map to multiple human chromosomes. Cross-species comparisons may help narrow these loci in the future

of the mutant chromosome 11 from the mother or father. Once the modifier gene(s) is identified for these two loci, it will be important to take this epistasis into account when validating these candidates in patients.

In the case of astrocytoma in *NPcis* mice, the 129S4/SvJae strain is resistant overall, whereas the C57BL/6J strain is susceptible. However, F1 hybrids between C57BL/6J and 129S4/SvJae can have higher susceptibility to astrocytoma than either parental inbred strain (Amlin-Van Schaick et al. 2012b), suggesting that recessive resistance modifiers play a role in the astrocytoma phenotype. Crosses between C57BL/6J and 129S4/SvJae mice have suggested that a modifier exists in a 30 Mb region surrounding *Nf1* and *Trp53* (Reilly et al. 2004), termed *Mastr1*. This modifier may be directly related to the differential expression of *Nf1* in C57BL/6J and 129S4/SvJae (see Sect. 19.3.1), or may be another linked gene in the region that modifies the astrocytoma phenotype. To identify modifiers of astrocytoma genome-wide, backcross mapping is being performed between the C57BL/6J and 129S4/SvJae strains. Two modifier loci have been identified thus far that show sex specificity or tissue location specificity (Amlin-Van Schaick et al. 2012a, b).

In a *NPcis*^{pat} backcross between the C57BL/6J and 129S4/SvJae strain, a modifier locus was identified on distal chromosome 12 that showed linkage to astrocytoma in males, but not in females (Amlin-Van Schaick et al. 2012b). This

locus, *Astrocytoma resistance locus in males 1 (Arlm1)*, exhibits recessive resistance to astrocytoma in C57BL/6J. Although the *Arlm1* locus is large, covering 503 genes, cross-species comparison of mouse and human normal brain and brain tumor data were used to narrow the candidate modifier genes in the region to fewer than 15. *Arlm1* is syntenic with human chromosome bands 7p15-21, 7q36, and 14q32 that have been found to be amplified or deleted in human glioma, and candidate genes within the region show strong correlation to survival time in male glioma patients, but not female glioma patients. In addition to the interaction between *Arlm1* and the sex of the individual, comparison between backcrosses suggests that *Arlm1* interacts with polymorphisms on the Y chromosome. In backcross males carrying the 129S4/SvJae allele of chromosome Y, *Arlm1* shows linkage to astrocytoma incidence. By contrast, in backcross males carrying the C57BL/6J allele of chromosome Y, there is no linkage seen with *Arlm1*, similar to females (K.M. Reilly, unpublished data). These data illustrate the epistasis that determines overall susceptibility to astrocytoma, with interactions between strain, sex, and parental inheritance of the disease.

NPcis mice develop astrocytoma in both the brain and the spinal cord. It is an open question whether spinal cord astrocytomas originate within the spinal cord, or whether tumor cells spread from initiating regions in the brain. In rare cases, astrocytomas are only observed in the spinal cord of the mouse, suggesting that the spinal cord is the primary site of tumorigenesis. To better understand the mechanisms governing astrocytoma location within the central nervous system, modifiers of spinal cord astrocytoma were mapped in backcrosses of C57BL/6J and 129S4/SvJae. The *Spinal cord resistance to astrocytoma modifier 1* (*Scram1*) modifier locus was identified in $NPcis^{mat}$ backcross mice on the distal end of chromosome 5 (LOD score > 5.0) (Amlin-Van Schaick et al. 2012c). Similar to the *Arlm1* locus, the C57BL/6J allele is associated with resistance to spinal cord astrocytoma, although the C57BL/6J strain is more susceptible to astrocytoma overall. Mice homozygous for the 129S4/SvJae allele of *Scram1* develop more spinal cord astrocytomas in a shorter period of time, with very little effect on astrocytoma in the brain. The *Scram1* locus is syntenic to human chromosomes 12q24, 7p11, 7q22-21, and 7p22, all of which contain regions that are reported to be either amplified or deleted in human astrocytoma, although not specifically in tumors of the spinal cord (Amlin-Van Schaick et al. 2012c). Very little is known about human spinal cord astrocytomas because they are extremely rare; therefore, studying these tumors in mouse models will be critical to developing testable hypotheses for further examination in the small number of available patient samples.

Studies to identify modifier of NF1-associated tumors in mice are highlighting the complexity of interactions between genetic background, sex, and parental inheritance. Thus far, all polymorphic modifiers identified in the *NPcis* mouse model are specific to either $NPcis^{mat}$ or $NPcis^{pat}$ mice, suggesting fundamental differences in the mechanisms of tumorigenesis depending on inheritance of mouse chromosome 11. In addition, the different tumor types (astrocytoma or MPNST) are modified by different loci and different strain backgrounds, suggesting that the

mechanisms of susceptibility (or resistance) are specific to the tissue type. Finally, males and females show linkage to different loci for a given tumor type, emphasizing the need to analyze males and females separately and suggesting that tumorigenesis may be fundamentally different between the two sexes. Figure 19.2 presents a summary of the modifier genes identified in humans and the modifier loci indentified in mouse relative to the human genome.

19.5 Modeling Human Diversity in the Mouse: The Collaborative Cross

Despite all the advances in cancer research achieved in the mouse over the past several decades, the main limitation for modifier studies in these models is the inbred nature of the species. Stable inbred mice serve as robust tools for identifying quantitiative trait loci (QTLs), but they limit our understanding of how human diversity affects cancer risk. Although there are numerous mouse strains available for laboratory use, there are often large regions of the genome that are essentially unchanged between strains resulting in an overall lack of diversity even in mixed backgrounds involving 2 or 3 strains. To combat these issues, a genetic reference population of approximately 500 recombinant inbred (RI) mouse lines is being developed, representing highly diverse genomes that can be manipulated for rigorous modifier studies (Collaborative Cross Consortium 2012; Threadgill and Churchill 2012a, b).

The Collaborative Cross (CC) originated from the Complex Trait Consortium with the idea that mouse research would benefit from the use of highly diverse and highly reproducible RI lines for use in systems biology and identification of complex traits such as NF1 modifiers (Churchill et al. 2004; Threadgill et al. 2002). As opposed to current linkage mapping which often identifies large QTLs with hundreds of possible candidates, QTL mapping in CC lines would be much more precise and map fewer false positive loci due to the controlling of long distance linkage disequilibrium in the CC (Collaborative Cross Consortium 2012). Comparisons across such a large panel of mice would point to narrower genomic intervals involved in phenotypic variation, leading to smaller numbers of candidate modifiers that are more likely to play significant role in modifying the phenotype.

To establish controlled diversity in the mouse, eight inbred mouse strains were used in the Collaborative Cross. Five of the founder strains (A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ, and NZO/HlLtJ) were laboratory-derived inbred strains, and three (CAST/EiJ, PWK/PhJ, and WSB/EiJ) were inbred wild-derived mice; in combination, they carry genetic diversity evenly spread across the entire genome. Systematically outcrossing these eight founder strains for two generations, followed by random brother–sister inbreeding for at least 20 generations has established approximately 500 RI lines for testing. Within each mouse, it is estimated that over 100 recombination events will take place, thereby providing

countless combinations of genomic contributions from each founder strain. In total, the entire panel will then be likely to have over 100,000 recombination events that will be fully genotyped for each line and made publicly available for research use (Churchill et al. 2004; Threadgill et al. 2002). Several groups thus far have undertaken the arduous task of developing these lines, and so far the results of founder distribution across the RI panel indicate that all eight founder strains are represented in each CC line in varying amounts with high rates of diversity between each line (Aylor et al. 2012; Durrant et al. 2011; Philip et al. 2011). Early studies to identify complex traits have shown that QTLs in CC mice can be mapped to regions as small as 0.5–1 Mb (Aylor et al. 2012; Durrant et al. 2011). This precise mapping is nearly impossible to accomplish using a two-strain approach, which typically yields QTLs that are several megabases in size at best. The resources provided by these crosses will prove invaluable to studies aimed at identifying candidate modifiers of tumorigenesis that can then be applied to ongoing NF1 research.

19.6 Summary

To date, significant progress has been made to determine the various mechanisms of tumor initiation and progression, but much less is known about inherited susceptibility to tumorigenesis. Identifying modifier genes and pathways in humans is confounded by our underlying diversity, although some advances have been made in human studies. Using state of the art mouse genetics approaches, we are able to identify regions and genes of interest that can potentially modify cancer risk. Relating the findings from diversely inbred mouse models to human patients is becoming increasingly feasible, providing a powerful tool in the hunt for NF1 modifiers.

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Chapter 20
Neurofibromin: Protein Domains and Functional Characteristics

Klaus Scheffzek and Stefan Welti

20.1 Introduction

The tumor suppressor gene *NF1* encodes the giant cytoplasmic protein neurofibromin (320 kDa) (DeClue et al. 1991; Gutmann et al. 1991) that is nonfunctional in NF1 patients due to gene alterations (Viskochil et al. 1990; Cawthon et al. 1990; Wallace et al. 1990). Neurofibromin is ubiquitously expressed during development (Daston and Ratner 1992) but at its highest level in adult neurons, Schwann Cells and oligodendrocytes (Daston et al. 1992). The cellular distribution of neurofibromin has been studied using immunostaining/labeling and EM techniques and varies by tissue as well as by cell type (reviewed by Sherman et al. 1998). The significance of observations according to which neurofibromin or fragments of it localize to the cell nucleus (Vandenbroucke et al. 2004; Leondaritis et al. 2009; Li et al. 2001; Godin et al. 2012) under certain conditions is currently unclear.

Neurofibromin (Trovo-Marqui and Tajara 2006) encodes a Ras-specific GTPase activating protein (RasGAP) (Ballester et al. 1990; Martin et al. 1990; Xu et al. 1990a) that downregulates the biological activity of the small guanine nucleotide binding protein (GNBP) Ras by accelerating the hydrolysis of Ras-bound GTP, the signaling active form of Ras. The physiological impact of Ras regulation is underscored by the notion that Ras is mutated in a large number of human malignancies and many of the respective mutants are unable to hydrolyse GTP at a rate sufficient to terminate downstream signaling and are GAP-insensitive (Trahey and McCormick 1987; Bos et al. 1987; Prior et al. 2012; Pylayeva-Gupta et al. 2011). Conversely, some *NF1*-deficient tumor types exhibit elevated levels of activated Ras, suggesting that neurofibromin is a major RasGAP in the respective cell types (Basu et al. 1992; Bollag et al. 1996b; DeClue et al. 1992; Feldkamp et al. 1999; Guha et al. 1996;

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Kim et al. 1995; Lau et al. 2000; Sherman et al. 2000). Beyond its impact in NF1 pathophysiology, the *NF1* gene has been found to be altered in a variety of highly aggressive malignancies such as glioblastoma (McLendon et al. 2008; Parsons et al. 2008), adenocarcinoma (Ding et al. 2008) and ovarian cancers (Bell et al. 2011; Sangha et al. 2008), underscoring its importance in fundamental aspects of cell growth control. Neurofibromin interacts productively with the major Ras isoforms H-, K-, and N-Ras (Ahmadian et al. 1997a) but not with oncogenic/transforming Ras mutants (Bollag and McCormick 1991).

Consistent with the role of neurofibromin in the regulation of the Ras/MAPK pathway, NF1 deficits affect cellular growth control (Johannessen et al. 2005; Grewal et al. 2011), neural development (Hegedus et al. 2007; Romero et al. 2007), cognition (Ferner et al. 1996; Shilyansky et al. 2010) and other cellular/organismal processes.

Neurofibromin is highly conserved in vertebrates with sequence identities commonly >90 % (Bernards et al. 1993; Bernards 2003) including the evolutionarily early *Fugu rubipres* (Kehrer-Sawatzki et al. 1998). A neurofibromin orthologue has been identified in *Drosophila melanogaster* (The et al. 1997) sharing ~60 % sequence identity with the vertebrate versions. Its deletion/truncation is associated with growth and escape behavioral defects (The et al. 1997). In addition to the historical examples of IRA1/IRA2 (Buchberg et al. 1990), orthologues of neurofibromin have also been found in other lower eukaryotes, particularly in fungi including *Dictyostelium discoideum* (Zhang et al. 2008), *Neurospora crassa* (Galagan et al. 2003) and the thermophilic *Chaetomium thermophilum* (Amlacher et al. 2011).

The catalytic RasGAP activity resides in a central portion of neurofibromin, termed the GAP-related domain (GRD), that has been defined as the region closely homologous to p120GAP (Fig. 20.1). Using structural biology approaches, we have previously identified a bipartite structural module composed of a Sec14-like lipid binding domain (Aravind et al. 1999) and a hitherto undetected pleckstrin homology (PH)-like domain, the specific physiological function of which is currently unclear (D'Angelo et al. 2006; Welti et al. 2007) (Fig. 20.1). Although bioinformatics analyses predict neurofibromin to be largely ordered and highly helical, the domain scheme of the remaining portions of neurofibromin is still poorly defined. While a number of regions have been operationally defined as "domains" (Izawa et al. 1996), the justification for such definitions in the sense of a structural entity is questionable and will require further analysis including structure determination.

A number of proteins other than Ras family members have been reported to interact with neurofibromin including protein kinase A (PKA) (Izawa et al. 1996), protein kinase C (PKC) (Mangoura et al. 2006; Leondaritis et al. 2009), caveolin-1 (Boyanapalli et al. 2006; Patrakitkomjorn et al. 2008; Lin and Hsueh 2008), focal adhesion kinase (Kweh et al. 2009), tubulin (Bollag et al. 1993), amyloid precursor protein (De Schepper et al. 2006), syndecan (Hsueh et al. 2001), kinesin-1 (Hakimi et al. 2002), nuclear PML-bodies (Godin et al. 2012), the UBX-UBD protein ETEA (Phan et al. 2010) and p97/VCP (Wang et al. 2011). While some of these proteins are consistent with the notion of neurofibromin being involved in the regulation of

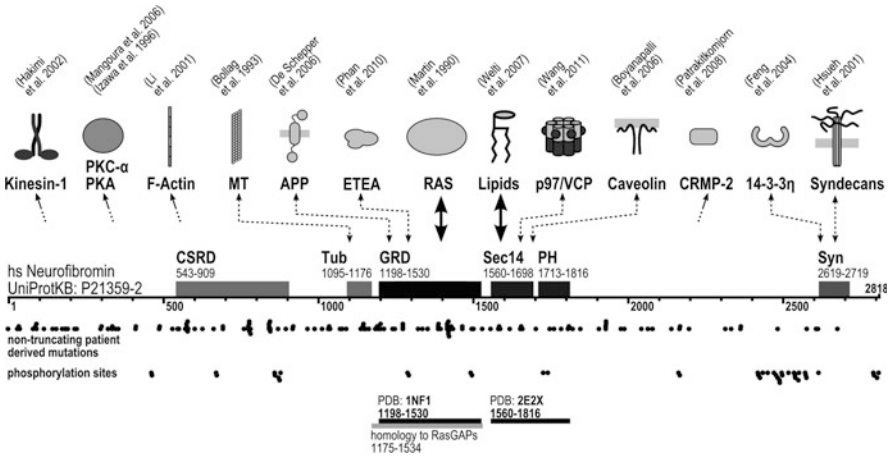

Fig. 20.1 Domain scheme of human neurofibromin including reported interaction partners. *Straight arrows* indicate the availability of structural data as outlined at the bottom of the figure. The approximate positions of non-truncating, patient-derived mutations (Tables 20.1 and 20.2) (Bausch et al. 2007; Brinckmann et al. 2007; Boyanapalli et al. 2006; Cai et al. 2005; De Luca et al. 2003; Hudson et al. 1997; Kaufmann et al. 2001; Kluwe et al. 2003a, b; Messiaen et al. 2000; Pascual-Castroviejo et al. 2007; Upadhyaya et al. 2008; Wimmer et al. 2007) and experimentally verified phosphorylation sites (*Phosphosite Plus*—http://www.phosphosite.org) are indicated with *black dots* below the domain scheme as labeled. The numbering of the domain boundaries is given in amino acids

the cytoskeletal architecture, the physiological significance of the respective interactions is however largely unclear and requires further in-depth analysis.

20.2 The GAP-Related Domain: Core Functional Module

The catalytic GAP domain, commonly known as the GAP-related domain (GRD) comprises a central segment that has initially been found homologous to the catalytic domain of p120GAP (Ballester et al. 1990; Martin et al. 1990; Xu et al. 1990b). Fragments in the residue range between 1095 and 1577 have commonly been studied as "GAP" but the catalytic activity can be narrowed down to a currently minimal segment of 229 amino acids (residues 1248–1477) (Ahmadian et al. 1996). The structure of the GRD displays an elongated protein molecule that is composed of largely helical elements (Scheffzek et al. 1998a) (Fig. 20.2). It consists of a central portion consistent with the minimal central catalytic domain (GAPc) (Ahmadian et al. 1996) and an extra domain (GAPex) formed by a coiled helical arrangement of about 50 residues from the N- and C-terminus, respectively. A shallow pocket in the surface of GAPc is lined by conserved residues and forms the Ras-binding region (Scheffzek et al. 1998a). The structure is very similar to that of the catalytic domain of p120GAP (Scheffzek et al. 1996) that has been

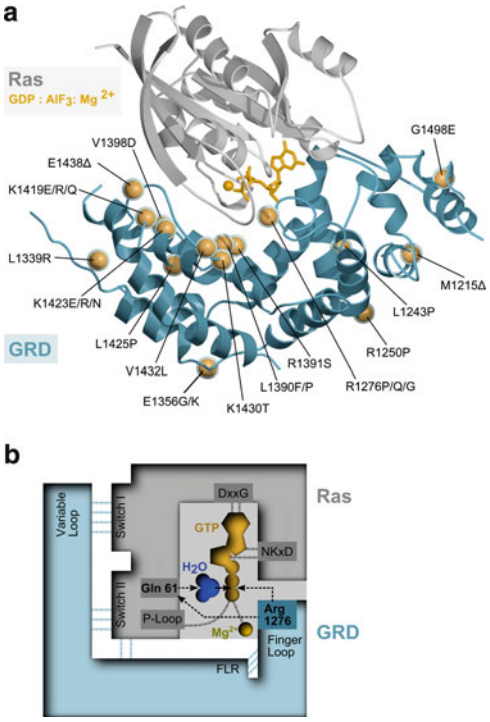

Fig. 20.2 Model of the GRD–Ras complex and the GAP mechanism. (**a**) Ribbon representation of GRD and Ras. GRD (PDB: 1NF1) was superimposed with the p120GAP component from the Ras: RasGAP complex (PDB: 1WQ1). GDP, AlF_3, and Mg^{2+} are depicted as *yellow stick models*. The positions of non-truncating, patient-derived mutations are visualized as *yellow spheres*. (**b**) GRD strongly accelerates the hydrolysis of GTP to GDP + P_i by Ras. The P-Loop, Mg^{2+} ion, and NKxD and DxxG motifs stabilize bound GTP in a favorable orientation for the hydrolysis reaction. The reaction itself is initiated by a water molecule, positioned by Ras-Gln61. Only after binding of GRD can the reaction proceed efficiently. GRD stabilizes the switch I and II regions of Ras and complements the Ras active site with Arg1276. Arg1276 stabilizes the transition state of the reaction by neutralization of developing negative charges and helps positioning of Ras-Gln61 (Bos et al. 2007; Scheffzek and Ahmadian 2005; Sondek et al. 1994)

co-crystallized with Ras to elucidate the structural basis for GTPase activation and its loss in oncogenic Ras mutants (Scheffzek et al. 1997). The respective Ras–RasGAP complex shows Ras bound to the abovementioned pocket in GAPc (Fig. 20.2). As expected, the GTP/GDP sensing switch I and II regions along with regions of the nucleotide binding pocket form major components of the Ras–RasGAP interface, that is dominated by largely polar interactions (Scheffzek et al. 1997). The active site is bound to GDP–AlF_3, a presumed transition state analog of the phosphotransfer reaction in which the planar AlF_3 moiety is believed to mimic the terminal γ-phosphate group during its transfer to the hydrolytic water molecule or the nucleophile derived from it (Wittinghofer 1997). The observation that a GDP–AlFx complex with Ras is only formed in the presence of the GRD but not

with isolated Ras, represents a key contribution to the notion that RasGAPs (and presumably GAPs in general) act to stabilize the active site of GTP-bound Ras (Mittal et al. 1996) in a heterodimeric protein complex (Scheffzek et al. 1998b). In the Ras–RasGAP complex, the chemical arrangement is stabilized by the interaction with the GAP domain featuring a highly conserved arginine residue (Arg1276 in neurofibromin), also called the arginine "finger", that interacts with the presumed GDP–AlFx transition state and additional residues partly derived from the FLR finger print motif that stabilize the catalytically important switch I/II regions (Scheffzek et al. 1997). A key component in the catalytic process is the highly conserved Gln61 that stabilizes the hydrolytic water molecule. Its conformation is stabilized by Arg1391 along with switch II contributions (Fig. 20.2). Mutational analysis has confirmed the importance of Arg1276 and Arg1391 for GAP catalysis, leading to a 2,000- and 50-fold reduction of GAP activity, respectively (Ahmadian et al. 1997b; Sermon et al. 1998). Importantly, both arginines have been found to be mutated in NF1 patients with partially severe symptoms (Upadhyaya et al. 1997b; Klose et al. 1998) (see below).

The observation that RasGAPs provide an arginine residue to complement the otherwise rather inefficient active site of Ras, supports the idea that small molecules may be designed that help to restore the GTPase activity of oncogenic Ras (Scheffzek et al. 1998b; Wittinghofer et al. 1997). Although no such compounds are currently available, it is interesting to note that the contacts between the finger loop (carrying the catalytic arginine) and Ras involve primarily main chain contacts (Scheffzek et al. 1997). Therefore it appears intrinsically difficult to design peptides that mimic specifically a putative finger loop equipped with an associated arginine.

20.3 Patient-Derived Mutations in the GRD

Consistent with the importance of GAP activity for the cellular functions of neurofibromin, one of the two regions where a clustering of non-truncating, patient-derived mutations was observed is located in the GRD (Fahsold et al. 2000). As outlined in Fig. 20.2, different loops and motifs coming from both the GRD and Ras are necessary for efficient GTP hydrolysis, and mutations leading to an impaired GAP activity have been found in all of these regions.

Concerning neurofibromin, the most dramatic effect on GAP activity was observed for mutations of the arginine finger Arg1276. The most prominent example is Arg1276Pro, where an 8,000-fold reduction of the GTP hydrolysis rate was measured *in vitro*, without the Ras-binding affinity being affected. This mutation was found in a patient with a severe malignant schwannoma and further genetic analysis confirmed that loss of GAP activity is sufficient to cause NF1 (Klose et al. 1998). Consequently, stabilization of the finger loop is of major importance as well, indicated by the mutation Arg1391->Ser in the FLR motif, which leads to a 300-fold decrease of the GTPase activity (Upadhyaya et al. 1997b). This mutation has

also been detected in recent cancer genome analyses of glioblastoma underscoring the general role of neurofibromin in Ras regulation.

A number of other mutations can be found in the Ras-binding groove of GRD, probably leading to changes in the binding surface of GRD and in turn causing a reduced affinity of GRD for Ras. Furthermore, stabilization of the Ras switch I and II regions, another prerequisite for efficient GTP hydrolysis, may be impaired by such mutations. For example the Lys1423->Glu mutation leads to the disruption of an intramolecular salt-bridge of the GRD, which appears sufficient to prevent any binding (Ahmadian et al. 2003; Li et al. 1992).

Other mutations do not directly affect the protein motifs highlighted in Fig. 20.2 but are located in close proximity and have therefore probably an indirect effect as may be expected for Leu1339->Arg (Fahsold et al. 2000). For a last group of mutations it seems unlikely that they affect GAP activity, suggesting that potentially other functions of neurofibromin associated with a spatial neighboring domain might be affected (Table 20.1).

20.4 The Sec14-PH Module

Exploring the coding sequence of the *NF1* gene for additional domains, we have previously identified another soluble fragment that could be crystallized (Bonneau et al. 2004). The crystal structure of that fragment defined the Sec14 homology-like region (Aravind et al. 1999) followed by a pleckstrin homology (PH) like domain (D'Angelo et al. 2006). While the former had been previously predicted by bioinformatics means (Aravind et al. 1999), the latter was only detected by the crystal structure determination of the respective fragment and had previously evaded detection. The structural arrangement of the two domains defines a bipartite module that suggests regulatory features (Fig. 20.3).

Sec14 domains are characterized by a cage-like lipid binding pocket that is covered by a helical lid segment believed to control ligand access to the interior (Bankaitis et al. 2010) (Fig. 20.3). While they occur as stand-alone proteins in a variety of examples including Sec14p (Sha et al. 1998), α-tocopherol binding protein (Meier et al. 2003; Min et al. 2003) and supernatant protein factor (Stocker 2004), Sec14-like domain can be found as modules in signal regulators such as RhoGEF, RhoGAPs, PTPases (Phillips et al. 2006) and neurofibromin (Aravind et al. 1999; D'Angelo et al. 2006). They commonly bind lipid ligands, although the identity of the physiologically relevant lipid binders is not clear in all cases. Similarly, the physiological/biochemical functions remain to be elucidated in a variety of Sec14-like examples. In the cases of RhoGAP, Dbs and PTP-MEG2, the Sec14-like domains were reported to affect localization of the proteins although the mechanism is unclear (Sirokmany et al. 2005; Kostenko et al. 2004; Huynh et al. 2003). The importance of Sec14 modules in higher eukaryotes is underscored by the observation that a number of diseases are associated with alterations in the

Table 20.1 Patient-derived, non-truncating mutations in the GRD region

Mutation	Location	Potential or demonstrated effect	Reference
R1204G/W	GAPex	Not visible in the structure. Introduction of higher flexibility (G) or a bulky residue (W). Likely impaired domain fold	Ars et al. (2003), Krkljus et al. (1998)
ΔM1215	GAPex	Impaired domain fold	Fahsold et al. (2000)
L1243P	Core	Likely disruption of helix, impaired domain fold, destabilization of Arg finger	Ferner et al. (2004)
R1250P	Surface, distant from Ras-binding groove	Impaired domain fold, destabilization of finger loop	Fahsold et al. (2000)
R1276G/P/Q	Arg finger, active site residue	GAP assisted GTP hydrolysis by Ras 500–8000x reduced	Mattocks et al. (2004), Klose et al. (1998), Fahsold et al. (2000)
R1325G	Close to Ras-binding groove	Not visible in the structure. Impaired domain fold, destabilization of Ras-binding surface	Lee et al. (2006)
L1339R	Core, close to Ras-binding groove	Introduction of positive charge, likely impaired domain fold, and destabilization of Ras-binding surface	Fahsold et al. (2000)
E1356G/K	Surface, distant from Ras-binding groove	Eventually affecting other neurofibromin domain	Upadhyaya et al. (2009), Trovó et al. (2004)
L1390F/P	FLR motif	Stabilization of finger loop impaired, reduction of GAP assisted GTP hydrolysis by Ras	Nystrom et al. (2009), Lee et al. (2006)
R1391S	FLR motif	Stabilization of finger loop impaired, reduction of GAP assisted GTP hydrolysis by Ras	Upadhyaya et al. (1997b)
V1398D	Ras-binding groove	Significant change of Ras-binding contact surface likely	Upadhyaya et al. (1998)
K1419E/Q/R	Ras-binding groove	Significant change of Ras-binding contact surface, likely ionic interaction with Ras-Glu37 impaired	Mattocks et al. (2004), Upadhyaya et al. (1997b), Purandare et al. (1994)
K1423E/N/R	Ras-binding groove	Fails to stabilize switch II region of Ras, GAP assisted GTP hydrolysis by Ras 200–400× reduced	Li et al. (1992), De Luca et al. (2003), Han et al. (2001)
L1425P	Ras-binding groove	Disruption of helix, major alteration of Ras-binding contact surface likely	Peters et al. (1999)

(continued)

Table 20.1 (continued)

Mutation	Location	Potential or demonstrated effect	Reference
K1430T	Variable loop	Improper stabilization of Switch I region of Ras likely, might disturb Arg finger	De Luca et al. (2005)
V1432L	Variable loop	Improper stabilization of Switch I region of Ras likely might disturb Arg finger‘	De Luca et al. (2005)
ΔE1438	Variable loop	Switch I region of Ras not stabilized, improper Ras binding likely	Ars et al. (2003)
S1468G	Distant from Ras-binding groove	Not visible in the structure. Eventually affecting other neurofibromin domain. Unlikely to affect GTP hydrolysis	Li et al. (1992)
G1498E	Distant from Ras-binding groove	Not visible in the structure. Eventually affecting other neurofibromin domain. Unlikely to affect GTP hydrolysis	Ars et al. (2003)
N1504S	Surface, distant from Ras-binding groove	Not visible in the structure. Eventually affecting other neurofibromin domain. Unlikely to affect GTP hydrolysis	Fahsold et al. (2000)

respective gene loci, including Cayman ataxia (Bomar et al. 2003), ataxia with vitamin E deficiency (Ouahchi et al. 1995), retinal degeneration syndromes associated with the loss of retinal binding proteins (Maw et al. 1997; Fishman et al. 2004) and Bothnia dystrophy (He et al. 2009). Structural and biochemical analysis of a missense mutation detected in the *CRALBP* gene of a Bothnia dystrophy patient suggested that reduced retinal binding due to partial blockage of the lipid binding pocket by the mutated residue was a major contributor to the pathogenicity of the alteration (He et al. 2009).

The structure of the Sec14-PH protein purified and crystallized in the absence of detergent revealed the presence of a glycerophospholipid bound to the interior of the Sec14 portion, presumably taken up from the lipid pool of the bacterial expression host (D'Angelo et al. 2006). In-depth biochemical and crystallographic studies along with mass spectrometry defined phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) as major binders of the Sec14-PH module (Welti et al. 2007). Since PE is abundant in neurofibromin-containing cells and PG is hardly present, PE was proposed as a major candidate physiological lipid ligand of the Sec14 portion (Welti et al. 2007).

The precise function of lipid binding in Sec14 modules of signal regulatory proteins including neurofibromin remains to be established. While one attractive hypothesis points to roles in sensing the lipid environment, a sensing mechanism is not obvious from the structure of the protein, as the bound lipid is essentially shielded from its membrane environment.

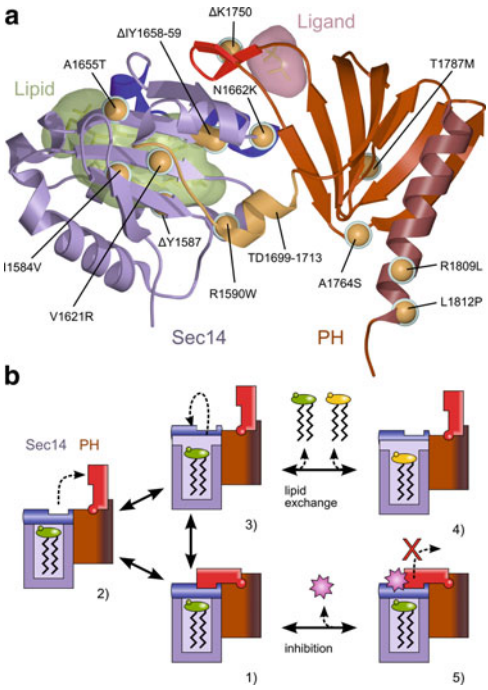

Fig. 20.3 The Sec14-PH module and mechanistic model of lipid exchange. (**a**) Ribbon representation of the Sec14-PH module of neurofibromin (PDB: 2e2x). The PH domain is colored in *red/brown*, the Sec14 domain in *blue/violet*. The positions of non-truncating patient-derived mutations are depicted as *yellow spheres*. Both the lipid in *green* and the regulatory ligand (colored *violet*) are shown as surface and stick representations. (**b**) Mechanistic model of lipid exchange. (1–3) The PH-derived beta-protrusion "lock" (*red*) and the Sec14 "lid" helix (*blue*) closing the Sec14 lipid binding cage can change their conformations to admit lipid exchange (4). (5) Regulatory binding- or interaction partners docking at the lid–lock interface can prevent lipid exchange reactions (D'Angelo et al. 2006; Welti et al. 2007, 2011)

The PH portion of the bipartite module shares its fold with a number of functionally distinct protein domains involved in signaling. These include the phosphotyrosine binding (PTB) domains, phospholipid-binding PH domains, polyproline binding-enabled Vasp-homology domains and PH-like modules that have been found to be involved in protein–protein interactions (Lemmon and Ferguson 2000; Lemmon 2004; Peterson and Volkman 2009). The most distinct feature of the PH-like portion of neurofibromin shows a protrusion connecting two β-strands from the PH core that extends from the domain core to interact with the helical lid of the Sec14 portion, which covers the presumed entrance of the lipid binding cavity (D'Angelo et al. 2006).

While the precise function of the Sec14-PH module is currently unclear, its structure suggests a regulatory interaction between the two portions, in which the abovementioned β-protrusion influences the conformation of the helical lid of Sec14 to control ligand access to the lipid binding cage.

20.5 Patient-Derived Mutations in the Sec14-PH Module

Although the studies investigating the function of the Sec14-PH module point to the presence of a protein interaction surface, the precise function of the module remains unclear (D'Angelo et al. 2006; Welti et al. 2007, 2011). Patient-derived mutations located in this neurofibromin region may therefore give valuable information about the location and nature of potential novel functions of the module. For such studies however, it is important to verify that the described alterations do not impair translation of the protein nor lead to insolubility or aggregation, but instead solely affect a specific protein function. Along this line of reasoning, it could be demonstrated that altered full-length neurofibromin carrying the mutations ΔK1750 and I1584V was present in normal amounts in cultured peripheral blood cells from patients. While the structural analysis of I1584V did not show large changes, deletion of K1750 leads to a local rearrangement resulting *in vitro* in impaired binding of regulatory ligands. Both mutations would appear to point to a novel function in the Sec14-PH module (Welti et al. 2011). Another deletion mutation (ΔY1587) was reported to disrupt an interaction observed between neurofibromin and p97/VCP (Wang et al. 2011). Interestingly, the deleted residue is not located at the surface of the protein as expected, but is instead shielded from the surrounding inside of the lipid binding cage of the Sec14 domain. Its position in the center of a large β-sheet suggests however, that upon removal of Y1587 the neighboring residues in the same β-strand would be flipped by 180°. This would lead to a massive rearrangement of the whole Sec14 domain and the occurrence of a significant alteration of a surface patch involved in a protein–protein interaction is imaginable if not to be expected. In a reported tandem duplication (TD1699-1712) (Tassabehji et al. 1993), the linker helical peptide connecting the Sec14- and PH-like domains is essentially duplicated. The crystal structure of this alteration shows that both the fold and orientation of the two domains towards each other remain unchanged whereas the duplicated linker region appears to be flexible in the crystal (Welti et al. 2011). The inserted residues might therefore occlude a protein interaction surface or lead to steric clashes with surrounding neurofibromin domains. For the remaining mutations, it can be speculated that they either introduce changes which impair the fold of the module or affect a neighboring neurofibromin domain instead of the Sec14-PH module (Table 20.2).

20.6 Functional Characteristics

Neurofibromin has been reported to be involved in a variety of cellular processes, although the sequence of reactions invoking certain responses is largely unclear. Given the prevailing role of neurofibromin in Ras regulation, it is not surprising that a number of functional features emerge in organismal or cellular contexts associated with Ras signaling. These include a variety of cell proliferative

Table 20.2 Patient-derived, non-truncating mutations in the Sec14-PH region

Mutation	Location	Potential or demonstrated effect	Reference
I1584V (PDB 3P7Z)	Sec14—cage backside, core	Structure virtually unchanged, eventually increased flexibility of a close surface patch Altered protein present in normal amounts in cell samples from patients	Fahsold et al. (2000)
ΔY1587	Sec14—cage inside	Reported to be necessary for interaction with p97/VCP. Leads to flipping of many β-strand residues, major structural rearrangement of protein core and lipid binding cage	Wang et al. (2011)
R1590W	Sec14—interface, surface	Introduction of bulky residue into domain interface, impaired domain interaction	Upadhyaya et al. (1997a)
V1621R	Sec14—cage backside, core	Introduction of charged residue into core, impaired Sec14 cage structure	Jeong et al. (2006)
A1655T	Sec14—cage backside, surface	Volume increase, changed surface or impaired Sec14 cage structure	Wang et al. (2011)
ΔIY1658/59	Sec14—cage inside/ backside, core	Shortened beta-strand, impaired Sec14 cage structure	Wu et al. (1999)
N1662K	Sec14—interface, core	Impaired domain ineraction	Wu et al. (1999)
TD1699-1712 (PDB 3PEG)	Sec14—PH linker	Structure similar, linker region flexible, large volume increase might impair other domains or prevent protein binding	Tassabehji et al. (1993)
ΔK1750 (PDB 3PG7)	PH—beta protrusion/lock	Local changes in the lid–lock region, reduced capability to bind regulatory ligand Altered protein present in normal amounts in cell samples from patients	Fahsold et al. (2000)
A1764S	PH—core	Impaired PH fold	Han et al. (2001)
T1787M	PH—surface	Eventually affecting other neurofibromin domain	Lee et al. (2006)
R1809L	PH—surface	Eventually affecting other neurofibromin domain	Griffiths et al. (2007)
L1812P	PH—surface	Eventually affecting other neurofibromin domain	Griffiths et al. (2007)

phenotypes (see reviews Cichowski and Jacks 2001; Zhu and Parada 2001, 2002; Harrisingh and Lloyd 2004; Trovo-Marqui and Tajara 2006; Grewal et al. 2011) although potential RasGAP independent tumor suppressor functions of neurofibromin have also been suggested (Johnson et al. 1993). In addition to the

classic Ras signaling cascades, it has been shown that the mTOR pathway is tightly regulated by neurofibromin in a Ras/PI3K-dependent manner and is constitutively activated in several *NF1*-deficient tumors or cell cultures (Johannessen et al. 2005; Dasgupta and Gutmann 2003). These findings have suggested the mTOR pathway to be an attractive drug target for the treatment of neurofibromin-deficient tumors (Johannessen et al. 2005; Dasgupta and Gutmann 2003).

Learning disabilities occur in a high percentage of NF1 adolescents (North 2000; Ozonoff 1999; Acosta et al. 2006; Ferner et al. 1996). Animal model studies in NF1+/− mice have demonstrated that the differentially spliced exon 23a is critical for learning (Costa et al. 2001) and that genetic or pharmacological inhibition of Ras activity could restore the learning deficits in the spatial learning Morris water maze assay (Costa et al. 2002). Exon 23a encodes a 21-residue peptide segment inserted (Nishi et al. 1991; Andersen et al. 1993) into a helical segment of the GRD (Scheffzek et al. 1998a) and is associated with an approximately tenfold reduction of GAP activity (Uchida et al. 1992). While the structure of the GRD does not suggest an immediate mechanism as to how the presence of the insert may affect GAP activity, intramolecular and indirect interactions potentially modulating Ras–GRD interactions have to be considered also in the full-length neurofibromin (Scheffzek et al. 1998a). The observation that learning phenotypes can be restored by manipulation, i.e. decrease of Ras activity either genetically or pharmacologically (Costa et al. 2002) support a prominent role of neurofibromin-mediated Ras regulation in learning. The involvement of cognitive deficits in NF1 is reflected by the role of Ras signaling in processes associated with learning (Weeber and Sweatt 2002). Detailed molecular and cellular investigations have revealed that neurofibromin-mediated regulation of ERK/synapsin I signaling modulates the release of GABA, synaptic plasticity and learning in mice (Cui et al. 2008). Given the involvement of Ras signaling in cognition processes (Weeber and Sweatt 2002), it is probably not surprising that neurofibromin also has roles in neuronal development (Hegedus et al. 2007; Lush et al. 2008; Romero et al. 2007; Zhu et al. 2001; Yunoue et al. 2003).

Roles of neurofibromin in the regulation of cytoskeletal architecture have been reported to involve the Rho–Rock–LIMK2–cofilin pathway in a process requiring GAP activity (Ozawa et al. 2005) but also by potentially GAP-independent mechanisms (Starinsky-Elbaz et al. 2009). Neurofibromin has also been found associated with microtubules (Gregory et al. 1993) and copurifies with tubulin when overexpressed in insect cells (Bollag et al. 1996a). The tubulin binding region overlaps with the GRD and requires 80 additional residues at the N-terminus of the GRD outer core (residues 1095–1569) (Bollag et al. 1996a). Some missense mutations in the GRD core interrupt the interaction with tubulin while others do not interfere with tubulin binding, which the authors suggested points to a role in differential localization (Xu and Gutmann 1997).

Interaction of neurofibromin with caveolin-1 has been reported (Boyanapalli et al. 2006) and is implicated in modulating ras, Akt and focal adhesion kinase (FAK), the N-terminal region of which has been demonstrated to directly interact with a C-terminal segment of neurofibromin (Kweh et al. 2009). Presumed caveolin-binding motifs partly overlap with the Sec14-PH module (D'Angelo et al. 2006). However, the structural arrangement along with the location of the

motif is difficult to rationalize to form a protein–protein interface. Neurofibromin has also been demonstrated to modulate cAMP signaling although the molecular details remain elusive (Tong et al. 2002). In *Drosophila*, the respective pathways appear to be important for the control of body size as well as of learning (The et al. 1997; Guo et al. 2000; Ho et al. 2007).

20.7 Regulatory Aspects

The activity of neurofibromin seems to be influenced by a number of factors. Lipids have been reported to have inhibitory effects on RasGAP-dependent downregulation of Ras (Bollag and McCormick 1991; Han et al. 1991) with partly controversial results and interpretations (Sermon et al. 1996). The significance of the observed inhibition has not been established *in vivo*.

Differential splicing is a common mechanism to regulate cellular activity of proteins. For NF1-pre-mRNA, four different splice variants have been reported (Barron and Lou 2012). Of particular importance is the alternative splice variant including exon 23a within the GRD (Nishi et al. 1991; Andersen et al. 1993) (see above). Biochemical analysis of the two variants indicates a tenfold lower GAP activity for the type 2 transcript encoded variant including exon 23a with respect to the type 1 variant (Uchida et al. 1992). While it is currently unclear to what extent this biochemical difference translates into physiological significance, it is intriguing that the exon 23a-containing variant can restore learning deficits in otherwise $NF1^{+/-}$ mice (Costa et al. 2001).

Neurofibromin can be S/T-phosphorylated by PKA in the C-terminal portion with the modification being important for the interaction with 14-3-3 proteins, although the relevance of this finding is currently unclear (Izawa et al. 1996; Feng et al. 2004). In addition, PKCα has been shown to phosphorylate neurofibromin, thereby enhancing RasGAP activity and interaction with the actin cytoskeleton (Mangoura et al. 2006; Leondaritis et al. 2009). Whole cell proteomics studies have identified a number of phosphopeptides derived from neurofibromin (http://www.phosphosite.org and references therein). However, the biological relevance of the individual modifications remains to be established.

Proteolysis of neurofibromin by the ubiquitin/proteasome pathway after growth factor stimulation has been shown to dynamically regulate Ras signaling in both amplitude and duration (Cichowski et al. 2003). Priming of degradation requires residues of the extended GRD (Martin et al. 1990) including the N-terminal region important for tubulin binding (Cichowski et al. 2003; Bollag et al. 1993). Proteomics studies using pull-down experiments identified the UBX-UBD protein ETEA as directly interacting with the GRD, with the UBX domain being essential for mediating ubiquitination of neurofibromin (Phan et al. 2010), i.e. downregulation of Ras signaling (Cichowski et al. 2003).

Taken together, a variety of strategies may be employed to control the cellular activities of neurofibromin. Given the crucial roles of neurofibromin in the regulation of Ras signaling and the compartmental organization of Ras isoforms (Rocks

et al. 2005, 2006), mechanisms of differential localization are likely to exist and ensure targeting to the appropriate locations.

20.8 Concluding Remarks

Neurofibromin is the major player in NF1, since its malfunction due to gene alteration is responsible for the disease pathology. Understanding the protein in terms of its biochemical and physiological functions is essential to understand NF1 and requires knowledge of the protein structure and how it interacts with binding partners. With structures available for the known GRD, Sec14- and PH-like domains, the structurally validated segments currently cover about 25 % of the whole protein. Non-truncating mutations that are compatible with a translated protein in the cell are invaluable for analyzing functional aspects at high sequence resolution. In light of the structure, their effect may be estimated if they are in regions of obvious mechanistic impact and may assist in the validation of derived functional mechanisms. Future work will have to concentrate on the structural analysis of the unknown regions of neurofibromin and on the validation of reported interaction partners for their significance. The goal is defining the full functional spectrum of neurofibromin in greatest possible detail.

Note added in the proof Since this chapter was accepted for publication, Thomas et al. (2012) have reported the functional assessment of the potential pathogenicity of 15 constitutional NF1 missense mutations (11 novel and 4 previously reported but not functionally characterized) identified in the NF1-GRD (p.R1204G, p.R1204W, p.R1276Q, p.L1301R, p.I1307V, p.T1324N, p.E1327G, p.Q1336R, p.E1356G, p.R1391G, p.V1398D, p.K1409E, p.P1412R, p.K1436Q, p.S1463F). Ten NF1-GRD variants were considered to be potentially pathogenic by virtue of significantly elevated levels of activated GTP-bound Ras in comparison to wild-type NF1 protein. The remaining five NF1-GRD variants were deemed less likely to be of pathological significance as they exhibited similar levels of activated Ras to the wild-type protein. These conclusions were in concordance with both bioinformatic analysis and molecular modeling data.

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Chapter 21
Molecular Basis of Bone Abnormalities in NF1

David A. Stevenson and Florent Elefteriou

21.1 Background

Neurofibromatosis type 1 (NF1) is caused by inactivating mutations in *NF1* resulting in increased RAS signaling and subsequently impacting cellular proliferation, differentiation and function in bone cells and their progenitors. Although the neurocutaneous manifestations of NF1 are most frequently studied and highlighted, the musculoskeletal system is also impaired in NF1 and has been receiving more focus recently given the morbidity of many of the skeletal findings, the increasing availability of animal models and understanding of the complex mechanisms underlying these skeletal manifestations. In recent years, both human and animal studies have indeed helped to better elucidate the molecular basis for the skeletal problems in NF1. The molecular underpinnings of the effects of neurofibromin loss of function on bone have not yet been well extended to human studies, and much of what is known is based on animal models. In this chapter, we shall review the clinical skeletal phenotype of NF1, the molecular and cellular findings of bone in NF1 humans, and the correlative animal models and findings.

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21.2 Clinical Skeletal Phenotype

The skeletal abnormalities of NF1 have long been known, and several reviews are available (Elefteriou et al. 2009; Stevenson and Yang 2011; Alwan et al. 2005; Crawford and Schorry 1999; Vitale et al. 2002; Ruggieri et al. 1999). In particular, tibial pseudarthrosis and sphenoid wing dysplasia are relatively specific to NF1 and were initially mentioned as one of the examples of the distinctive osseous lesions in NF1 that help fulfill the NF1 diagnostic criteria (Gutmann et al. 1997). Skeletal findings associated with NF1 include bowing (Fig. 21.1) and pseudarthrosis of the long bones (particularly the tibia), dystrophic and non-dystrophic scoliosis, kyphosis, sphenoid wing dysplasia, osteopenia, increased fracture rates in adults, bone cysts, bony overgrowth, anterior chest wall anomalies, and short stature (Crawford and Schorry 1999; Vitale et al. 2002; Elefteriou et al. 2009; Friedman and Birch 1997; Ruggieri et al. 1999; Stevenson et al. 1999, 2005, 2007a; Illes et al. 2001; Kuorilehto et al. 2005; Yilmaz et al. 2007; Lammert et al. 2005; Dulai et al. 2007; Brunetti-Pierri et al. 2008; Tucker et al. 2009; Caffarelli et al. 2010; Seitz et al. 2010). However, some of the orthopedic problems probably include abnormalities that could in part be secondary to the joints, muscle, and ligaments rather than defects in bone cellular processes (e.g., pes planus, non-dystrophic scoliosis, kyphosis, anterior chest wall anomalies) (Johnson et al. 2010; Kossler et al. 2011; Souza et al. 2009). It is likely that the variety of musculoskeletal abnormalities is due to a combined effect of abnormalities of multiple cells types from increased RAS signaling on multiple organ systems. Although there are a large number of orthopedic manifestations, this chapter will focus on some of the typically associated skeletal findings with a more obvious bone involvement.

21.2.1 Long Bone Dysplasia

Long bone dysplasia or osteopathy most often affects the tibia although other long bones can be involved. Typically, the radiographic findings of tibial dysplasia include anterolateral bowing with medullary canal narrowing and cortical thickening at the apex of the bowing (Stevenson et al. 2007b). When the tibia is affected, the fibula is often also affected (Stevenson et al. 1999) (Fig. 21.2). We hypothesize that in some instances the associated bowing of the fibula is in part a mechanical effect of the bowed tibia. However, pseudarthrosis of the bowed fibula can also occur as can isolated fibular pseudarthrosis without tibial involvement in the same limb. It is possible that a combination of mechanical effects in combination with the local microenvironment contributes to the association of tibial and fibular dysplasia. Long bone bowing and pseudarthrosis are not universal findings in NF1 and occur in approximately 3–5 % of NF1 individuals (Friedman and Birch 1997). In addition, the finding is typically unilateral. Long bone bowing with subsequent pseudarthrosis when observed is highly associated with NF1. These findings suggest that *NF1* haploinsufficiency contributes to the development of long bone bowing and pseudarthrosis but probably requires additional modifiers such as the double inactivation of *NF1*.

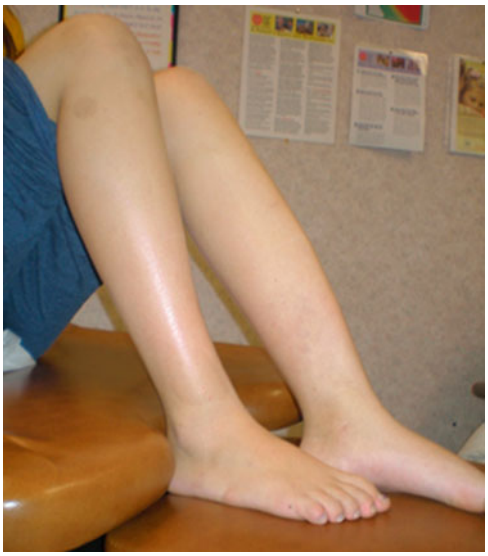

Fig. 21.1 Photograph of individual with NF1 with anterolateral bowing of the left leg without pseudarthrosis

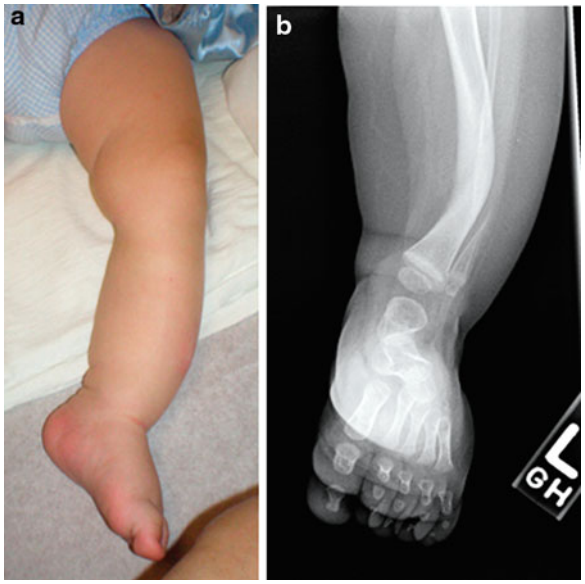

Fig. 21.2 (**a**) Photograph of child with NF1 with anterolateral bowing of the left leg. (**b**) Radiographs of the leg show anterolateral bowing of the tibia without pseudarthrosis but pseudarthrosis of the fibula

NF1 loss of heterozygosity in the pseudarthrosis tissue of two individuals with tibial pseudarthrosis and NF1 has been reported, and double inactivation of *NF1* with retention of the mutant allele in the pseudarthrosis tissue in one of the patients

has been confirmed (Stevenson et al. 2006). However, loss of heterozygosity has not been observed in other cases (Sakamoto et al. 2007) suggesting either a mixed cellular population, other tissue or cell lineages harboring double inactivation of *NF1*, or more plausibly other modifiers.

The tissue found between the distal and proximal fractured tibial bone segments, which is typically described as "pseudarthrosis tissue," has been difficult to characterize. It is a highly cellular tissue and has been given various descriptions (e.g., "fibrous hamartoma," "fibromatosis-like tissue") (Mariaud-Schmidt et al. 2005; Cho et al. 2008; Ippolito et al. 2000). Histologically, the proliferative tissue has a fairly uniform dense fibrocartilaginous appearance and is S100 negative (Stevenson et al. 2006; Sakamoto et al. 2007; Briner and Yunis 1973). Mariaud-Schmidt et al. (2005) evaluated the pseudarthrosis sites of two patients and showed that the tissue was composed of fibrillar cells and suggested that the "hamartomatous" tissue is formed by an undifferentiated type of membranous tissue. Blauth et al. (1984) performed histological and electron microscopy on ten cases of pseudarthrosis revealing a cellular fibrous tissue with elongated cells possessing spindle-shaped nuclei. Cho et al. (2008) cultured "fibrous hamartoma" tissue from seven patients with NF1 and showed that the cells were highly osteoclastogenic and maintained a mesenchymal lineage immunophenotype (i.e., CD44+/CD105+/CD45−/CD14−). The cells appeared to be at different stages of osteoblastic differentiation with low osteogenicity (Cho et al. 2008). The anatomic site of the pseudarthrosis, however, is not composed solely of this proliferative fibrous material as there still remains the proximal and distal bone fragments, periosteum, and an admixture of other cells. Sakamoto et al. (2007) reported that the bone trabeculae of the pseudarthrosis region looked similar to osteofibrous dysplasia with prominent osteoblastic rimming. Heervä et al. (2010) analyzed samples taken from the pseudarthrosis region of three individuals with NF1 and showed numerous multinucleated tartrate-resistant acid phosphatase (TRACP)-positive osteoclasts in contact with bone and bone indentations, but also noted that many (≈50 %) of the osteoclasts were located away from the bone surfaces, embedded in the fibrous pseudarthrosis tissue. There is probably a complex interaction of the various cellular components on the function of the various bone cells within the pseudarthrosis site. Of note is that histological analyses have typically been performed on a region that has developed a pseudarthrosis and fractured, and it is likely, based on knowledge from preclinical NF1 murine models, that the composition of the bone and marrow involved is abnormal prior to the development of the fracture and pseudarthrosis.

21.2.2 Scoliosis

Scoliosis is one of the more common skeletal abnormalities associated with NF1 although the frequency of scoliosis varies widely based on different reports (Young et al. 2002). This variability of frequency is likely to be a direct reflection of the variability inherent in what is termed scoliosis. Scoliosis refers to curvature of the

spine typically based on radiographs taken in the anterior to posterior view but the spine is multidimensional and made up of multiple bony elements. Laterally there are normal degrees of kyphosis and lordosis and mild curvature in the anterior–posterior view (i.e., between 9 and 20°) may not be readily identifiable clinically. In NF1, scoliosis has typically been classified as either "dystrophic" or "non-dystrophic". Durrani et al. (2000) tried to define "dystrophic" scoliosis in NF1 as scoliosis in which there was the presence of a specific radiographic feature (e.g., rib penciling, vertebral scalloping, spindling of the transverse process). In the context of trying to understand the role of NF1 directly on the bony elements, dystrophic scoliosis would be the type to consider although some of the radiographic features listed by Durrani et al. (2000) are not necessarily primary osseous dysplasias (Alwan et al. 2005). It is possible that mechanical forces from adjacent neurofibromas or secretory factors from tumors impact the development and progression of scoliosis. Although non-dystrophic scoliosis is likely not a primary osteopathy, it is possible that an underlying disorder of bone homeostasis on the vertebral elements, that cannot be appreciated on radiographs, could contribute to the development of non-dystrophic scoliosis.

Brunetti-Pierri et al. (2008) reviewed vertebral samples from three NF1 patients who had reduced bone mass and were undergoing surgery for scoliosis and compared them to autopsy control samples and a sample from a patient with osteogenesis imperfecta. The NF1 vertebral samples displayed a reduction in the number and thickness of bony trabecula, appropriate cement lines, and viable osteocytes but the appearance of normal mineralization of the bone was comparable to matched controls. Upon electron microscopy there was a severe reduction in mineral content. On the bone surface, numerous lining cells that replaced well-differentiated osteoblasts were observed. When comparing the NF1 bones to a bone sample from an individual with osteogenesis imperfecta, the lining cells in the NF1 vertebrae were not well differentiated with few cytoplasmic structures resembling active osteoblasts. It was not described whether these individuals had dystrophic scoliosis or non-dystrophic scoliosis or if the vertebral elements analyzed were from dysplastic-appearing vertebrae. Future studies examining vertebral elements from individuals with non-dystrophic vs. dystrophic scoliosis or vertebral elements from different regions of the spine in an individual with dystrophic short-angle scoliosis would be helpful.

21.2.3 Sphenoid Wing Dysplasia

The etiology of sphenoid wing dysplasia is not understood. The severity of the sphenoid wing dysplasia varies, and it is likely that subtle dysplasia is not readily recognized. Individuals with severe sphenoid wing dysplasia can have ophthalmologic complications and obvious cosmetic issues relating to facial asymmetry. In many instances, a plexiform neurofibroma is adjacent to this facial region. The frequency, unilaterality, and intra/interfamilial variability are similar to long bone dysplasia suggesting again the requirement of second hits and other modifiers.

21.2.4 Osteoporosis/Osteopenia

Decreased bone mineral density (BMD) has been reported in many studies in both children and adults with NF1 (Illes et al. 2001; Kuorilehto et al. 2005; Lammert et al. 2005; Stevenson et al. 2005, 2007a; Dulai et al. 2007; Yilmaz et al. 2007; Brunetti-Pierri et al. 2008; Tucker et al. 2009; Caffarelli et al. 2010; Seitz et al. 2010). Unlike long bone dysplasia, sphenoid wing dysplasia, and dystrophic scoliosis in which the clinical presentation suggests somatic events or modifying effects, the generalized and more frequent osteopenia and osteoporosis in NF1 suggest that *NF1* haploinsufficiency alone may impact bone cellular functions.

Although a large number of independent studies from multiple nationalities confirm decreases in BMD, typically as measured by dual energy X-ray absorptiometry, there are inherent problems in the interpretation of areal BMD clinically, particularly in growing individuals with short stature and potentially smaller bones. The precise clinical consequence of decreased BMD in NF1 is debatable, particularly in the apparently non-dysplastic bones. Some may argue that the BMD is appropriate for the shape and size of the bone. Stevenson et al. (2009) assessed volumetric parameters using peripheral quantitative computed tomography of the leg and showed that the strength strain index of the tibia was decreased, suggesting a propensity to fracture. In addition, Tucker et al. (2009) reported that NF1 adults had an increased fracture rate.

Bone resorption markers can provide some evidence that bone is being more actively resorbed. Some studies have shown increases in bone resorption markers in NF1 individuals (Stevenson et al. 2008; Tucker et al. 2009), whereas one cohort did not (Duman et al. 2008). Stevenson et al. (2011a) showed similar increases of markers of bone resorption using urinary pyridinium cross-links in other RASopathies (i.e., Noonan syndrome, Costello syndrome, and cardio-facio-cutaneous syndrome) giving more credence to the view that activation of the RAS signal transduction pathway results in increased bone resorption.

Several investigators have performed in vitro assays from human bone cells cultured from hematopoietic lineages, further substantiating the in vivo findings of increased bone resorption markers (Yang et al. 2006; Heervä et al. 2010; Stevenson et al. 2011a, b). Yang et al. (2006) utilized methods performed in the correlative NF1 mouse model on cultured osteoprogenitor cells in a few NF1 individuals showing multiple increased osteoclast functions, which was later substantiated in a larger cohort of NF1 individuals (Stevenson et al. 2011a, b). The cultured monocytes from individuals with NF1 showed increased osteoclast formation, increased osteoclast migration, and increased activity based on pit resorption assays. Heervä et al. (2010) showed similar findings from human in vitro studies, in addition to documenting that the survival rate of the mononuclear cells was higher in cultures from NF1 individuals as compared to healthy controls upon serum deprivation. *NF1* haploinsufficiency thus impacts at least osteoclast function. These investigations were in vitro studies of cultured cells exposed to various growth factors, and hence, conclusions on the effects of $NF1^{+/-}$ osteoclasts in vivo are still not well known.

Seitz et al. (2010) obtained iliac crest biopsies in 14 individuals with NF1 for histologic and histomorphometric analysis compared to age- and sex-matched autopsy controls. They observed an increase in osteoblast and osteoclast numbers suggesting increased bone turnover. Other findings included decreases in trabecular thickness and defects of bone matrix mineralization with increased osteoid volume and thickness. Some of the findings were suggestive of osteomalacia, and these individuals comprised a cohort from Germany in which vitamin D deficiency has been consistently reported in NF1 individuals from this region (Lammert et al. 2005; Tucker et al. 2009). Hence, it is possible that other factors besides direct effects of NF1 on bone cellular function (e.g., vitamin D deficiency, hormonal imbalances, inactivity, hypotonia) could contribute to the osteopenia and osteoporosis observed in NF1.

Although the knowledge of the clinical skeletal phenotype in addition to some in vitro human cellular studies have helped in some respects to understand the molecular basis for the bone abnormalities in NF1, there is much that is still not understood. Recently, animal models investigating bone in NF1 have provided many more insights.

21.3 Animal Models

Animal models are necessary to understand the role of NF1 in the biology of bone remodeling and repair and to characterize critical molecules or processes that can be targeted to prevent or cure the skeletal manifestations of NF1. Such genetic models may also serve as preclinical models that can be used to address the efficacy of selected and targeted pharmacological approaches, keeping in mind the limitations of such models and that mice are not humans.

21.3.1 Effect of Nf1 Haploinsufficiency in Mice

Neurofibromin is expressed in the bone mesenchymal and hematopoietic lineages and its loss of function can therefore affect bone remodeling and repair through a number of non-mutually exclusive mechanisms. Neurofibromin is expressed in osteoprogenitor cells, chondrocytes, osteoblasts, and osteoclasts, and the loss of one or both copies of *Nf1* triggers continuous RAS and ERK1/2 activation in these cells (Yu et al. 2005; Kuorilehto et al. 2004; Elefteriou et al. 2006; Wu et al. 2006; Yang et al. 2006; Kolanczyk et al. 2007; Wang et al. 2011).

$Nf1^{-/-}$ mice are embryonic lethal but $Nf1^{+/-}$ mice are viable (Brannan et al. 1994; Lakkis and Epstein 1998). $Nf1^{+/-}$ mice are not osteopenic under normal conditions but lose more bone relative to wild-type mice upon ovariectomy. This is caused, at least in part, by an increase in osteoclast-mediated bone resorption (Yang et al. 2006). $Nf1^{+/-}$ osteoblasts extracted from these mice exhibit increased proliferation and impaired differentiation in vitro (Yu et al. 2005) and secrete increased amount of

osteopontin, a matrix protein known to promote osteoclastogenesis and osteoclast migration. On the other hand, *Nf1*$^{+/-}$ osteoclast progenitors show a cell-autonomous defect in adhesion, migration, differentiation, and activity, which is RAC1 and PI3K-dependent (Yan et al. 2008; Yang et al. 2006). These data suggest that *NF1* haploinsufficiency contributes to the osteopenia observed in NF1 patients. Why ovariectomy is required to observe a bone phenotype in *Nf1*$^{+/-}$ mice remains unclear. It is also unclear whether or to what extent *Nf1* haploinsufficiency in other bone cells or tissues contributes to this phenotype.

Nf1$^{+/-}$ mice have been used as a preclinical model of NF1 bone healing following distal tibial fracture. Mice are well known to display robust bone healing and accordingly *Nf1*$^{+/-}$ mice did not show pseudarthrosis, but were characterized by delayed callus formation and healing, delayed cartilage removal, fibrous invasion, insufficient new bone anabolism, and excessive catabolism (Schindeler et al. 2008a, b). This study therefore suggests that NF1 haploinsufficiency contributes, at least in part, to NF1 pseudarthrosis.

21.3.2 Effect of Nf1 Loss-of-Function in Mice

The focal and unilateral nature of the most severe NF1 manifestations, along with the lethality caused by lack of *Nf1* during development, lend credence to the hypothesis that somatic *NF1* loss-of-function may underlie these bony defects. Various conditional mouse mutant models based on the use of the floxed *Nf1* mice generated by Dr. Parada's group (Zhu et al. 2001) and *cre* transgenic mice were thus generated, using selected promoters driving *cre*-recombination (hence *Nf1* deletion) in bony tissues and cells.

The first conditional model generated is characterized by *Nf1* loss of function in mature osteoblasts, using the 2.3 kb collagen type I promoter-*cre* mice (Dacquin et al. 2002). This model is the most specific for bone, but given that *Nf1* recombination is restricted to mature osteoblasts, the effect of *Nf1* ablation in early osteoprogenitor commitment or differentiation is not reflected. Nevertheless, *Nf1*$^{ob-/-}$ mice display several vertebral bone abnormalities, including the generation of a poorly mineralized bone matrix caused by an increase in collagen production and a delay of mineralization, and increased formation of osteoclasts (Elefteriou et al. 2006). These findings are also observed in tibial biopsies from NF1 patients with tibial pseudarthrosis (Wang et al. 2011) and indicated that *Nf1* regulates osteoblast function, i.e., bone matrix production and mineralization, as well as the support of osteoclastogenesis. *Nf1*$^{ob-/-}$ mice also display reduced vertebral cortical and trabecular BMD, as well as weaker bone mechanical properties than wild-type mice (Zhang et al. 2011), but no significant change in BMD in long bones (at least in young mice) (Wu et al. 2011). *Nf1*$^{ob-/-}$ mice have been subjected to distal tibial fracture and displayed, like *Nf1*$^{+/-}$ mice, no pseudarthrosis but delayed healing and bridging, high cortical porosity and exuberant osteoidosis, accompanied by weakened mechanical properties. No fibrous hamartoma was observed (Wang et al. 2010).

Transferring the *Nf1*$^{ob-/-}$ mice on a *Nf1*$^{+/-}$ background did not significantly change any of these bone parameters, but led to an increase in spinal canal area and to a reduction in osteoblast number compared to *Nf1*$^{ob-/-}$ mice (Zhang et al. 2011).

The second NF1 model generated is based on the use of the *Prx* promoter-*cre* mice, which allows *Nf1* loss-of-function very early on in the mesenchymal lineage. In this model, the effect of lack of *Nf1* in commitment and differentiation can be assessed, as opposed to the *Nf1*$^{ob-/-}$ mice, but some degree of specificity is lost, since the floxed *Nf1* allele is recombined in chondrocytic, muscle, and endothelial cells as well, which complicated the interpretation of the observed bony phenotypes. In addition, the *Prx* promoter drives *cre* expression in limbs only, and hence the role of *Nf1* in the axial skeleton and in the development of scoliosis cannot be assessed in this model. Despite these complications, this model recapitulated many of the NF1 skeletal manifestations, including osteopenia, osteoidosis, and bowing of tibiae. The severely reduced size of *Nf1*$^{prx-/-}$ mice stems from defects in growth plate development and is probably not relevant to the relative short stature of NF1 patients. Bone healing is impaired too in the *Nf1*$^{prx-/-}$ model. Using a cortical injury and intramembranous bone repair model, Kolanczyk et al. (2007, 2008) reproduced many facets of the NF1 pseudarthrosis in these lesions, including delayed healing, the persistence of fibrocartilaginous tissue, and impaired bone extracellular matrix mineralization.

The *Nf1*$^{col2-/-}$ model, based on the use of the type II collagen promoter-*cre* mice, is similar to the *Nf1*$^{prx-/-}$ model (Wang et al. 2011). Although mostly used to study cartilage development, the type II collagen *cre* mice recombine genes in osteochondroprogenitor cells in the perichondrium during embryological development, that are precursor cells for bone forming osteoblasts later in life. In contrast to the *Nf1*$^{prx-/-}$ model, *cre* is expressed in the vertebral axis in the *Nf1*$^{col2-/-}$ model. These mice exhibit bowed tibia, macrocephaly, dystrophic scoliosis, kyphosis, osteopenia, increased bone cortical porosity, impaired bone mineralization, and increased osteoclastogenesis, all features of NF1. The bones of these mice are also mechanically weak. They are, however, severely growth deficient (similar to the *Nf1*$^{prx-/-}$ mice and due to lack of *Nf1* in chondrocytes), and a high proportion of these mice die soon after weaning. This model also exhibits a defect in the formation of the intervertebral disc whose contribution to the observed scoliosis is unclear.

The periostin *cre* mice have also been used to inactivate *Nf1* in endosteal bone surfaces. Surprisingly, PeriCre+;*Nf1*$^{flox/-}$ mice but not PeriCre+;*Nf1*$^{flox/flox}$ mice displayed reduced bone mass, BMD and osteoblast number, suggesting that the haploinsufficient hematopoietic microenvironment is required for the skeletal manifestations observed in this model. A reduction in bone volume and BMD has been measured in the callus of gamma-irradiated PeriCre+;*Nf1*$^{flox/flox}$ tibiae of mice transplanted with *Nf1*$^{-/-}$ hematopoietic stem cells (HSC) compared to WT HSC, suggesting again that the *Nf1*$^{+/-}$ microenvironment affects bone cell differentiation or function in fractured bones (Wu et al. 2011). Whether bone healing per se is impaired in this model remains to be addressed by mechanical testing and quantitative bone healing measurements.

Another approach to achieve *Nf1* loss of function in bone cells is to deliver *cre*-adenovirus locally. This approach has been successfully used by the group of Dr. Schindeler and Dr. Little to model NF1 pseudarthrosis (El-Hoss et al. 2011). The advantage of this strategy is that no extensive breeding is required, thus saving substantial amounts of time. It is also very focal, in contrast to the other models based on *cre* overexpression that target multiple bone elements. It has been applied as a model of NF1 pseudarthrosis, and might theoretically be used as a model for dystrophic scoliosis. Focal *Nf1* inactivation using this *cre*-adenovirus approach reduced tibial fracture union, as assessed radiographically, irrespective of whether the *Nf1*flox/flox mice were on a $Nf1^{+/-}$ background or not. The presence of a "fibrous hamartoma," similar to what is observed in human pseudarthrosis biopsies, was observed in this model, along with giant multinucleated TRAP-positive osteoclasts, many of which were not lining bone surfaces (El-Hoss et al. 2011).

21.4 Molecular Targets and Treatments

The characteristic activation of RAS and ERK in $Nf1^{+/-}$ and $Nf1^{-/-}$ cells, including osteoblasts, led to the trial of lovastatin in NF1 mouse models characterized by bone healing delay. Lovastatin inhibits the mevalonate pathway and hence the prenylation and activity of RAS, making it, despite its non-selectivity to RAS inhibition, a reasonable choice to treat NF1 manifestations. High doses of lovastatin given orally and low doses delivered locally at the fracture site via slow release microparticles both had beneficial effect on bone healing and mechanical properties of the callus in the $Nf1^{prx-/-}$ and $Nf1^{ob-/-}$ mouse models (Kolanczyk et al. 2008; Wang et al. 2010).

However, since lovastatin is not approved to treat bone conditions yet, less targeted—but approved—drugs have been tested as well, including bone morphogenetic protein-2 (BMP-2) and bisphosphonates. Using the $Nf1^{+/-}$ mice as a model in an open fracture healing study, Schindeler et al. (2011) showed that BMP-2 alone, used as anabolic stimulus, does not improve bone healing. By contrast, BMP-2 + zoledronic acid improved bone healing, possibly by limiting the increased osteoclastogenesis induced by BMP-2 (Schindeler et al. 2011).

21.5 Conclusions

Both the human NF1 phenotype and associated experimental models provide evidence that neurofibromin is critical in bone cellular functions. The skeletal abnormalities in NF1 are not trivial and cause significant morbidity, and the current lack of optimal interventions is obvious. Although the presently available mouse models do not fully recapitulate the natural history and phenotype of the NF1 skeletal complications, they have provided a solid framework to better understand the molecular basis of the bone abnormalities in NF1 and to test therapeutics.

Although the beneficial effects of statins, bisphosphonates, and BMPs in experimental models of NF1 pseudarthrosis are encouraging, these agents have their limitations, and it is likely that new therapeutic targets or combination treatments will be tested and developed. Although much more needs to be learned, we are at the forefront of translating what is known into clinical trials (Elefteriou et al. 2009), and it is clear that coordinated multicenter and multidisciplinary efforts in designing therapeutic strategies to prevent or treat NF1 musculoskeletal manifestations will be needed.

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Chapter 22
NF1-Associated Optic Glioma

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22.1 Introduction

Neurofibromatosis type 1 (NF1) is a common autosomal dominant tumor predisposition syndrome in which affected individuals are at risk for the development of benign and malignant cancers.

Within the central nervous system (CNS), astrocytic (glial) tumors involving the optic pathway (optic pathway gliomas, OPGs) are the most common, occurring in 15–20 % of individuals with NF1 (Fig. 22.1). These World Health Organization (WHO) grade I gliomas can be located anywhere along the optic pathway, from the retro-orbital optic nerve to the post-chiasmatic optic tract (Listernick et al. 1995). The majority of affected individuals are children under the age of 7 years (Listernick et al. 2007). In contrast to their sporadic counterparts, most OPGs do not cause progressive visual decline. Nearly half of children with NF1-associated OPGs will have visual impairment at initial presentation, but less than one-third of all children with these tumors will experience continued visual decline (Listernick et al. 1997). While the majority of OPGs involving the prechiasmatic optic nerves and chiasm will not clinically progress, OPGs involving the optic tracts (posterior to the optic chiasm) behave more aggressively (Balcer et al. 2001) and require treatment. In all cases, the first-line therapy for these tumors is carboplatin/vincristine chemotherapy (Demaerel et al. 2002; Kato et al. 1998).

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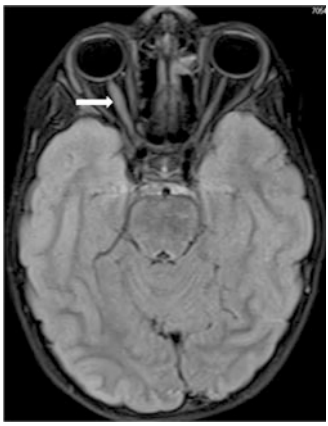

Fig. 22.1 NF1-associated OPG. Magnetic resonance imaging reveals a right optic nerve glioma. The *arrow* points to the glioma expanding the right optic nerve

22.2 Determinants of Gliomagenesis

The unique predilection for NF1-associated gliomas to form in the optic pathway in young children supports a model in which gliomagenesis requires the confluence of multiple obligatory conditions, including susceptible cell types with distinct growth regulatory pathways, a permissive microenvironment, and genomic modifiers (Fig. 22.2).

22.2.1 Neurofibromin Growth Control Pathways

The *NF1* gene product (neurofibromin) was originally identified as a large 220 kDa cytoplasmic protein containing a domain shared with proteins that function as negative Ras regulators (Xu et al. 1990b). Similar to other GTPase-activating protein (GAP) molecules, neurofibromin accelerates the inactivation of Ras by converting active GTP-bound Ras to its inactive GDP-bound form (Xu et al. 1990a). In some cell types, loss of neurofibromin expression leads to high levels of Ras activity, deregulated cell growth, and oncogenic transformation, consistent with its role as a tumor suppressor protein.

While neurofibromin negatively regulates cell growth through suppression of Ras pathway signaling, the specific downstream Ras effectors involved differ between cell types. For example, neurofibromin growth regulation in hematopoietic cells is Ras/MEK dependent (Bollag et al. 1996), whereas astrocyte growth is dependent on Akt-mediated activation of the mammalian target of rapamycin (mTOR) pathway (Dasgupta et al. 2005). Moreover, the mechanism by which neurofibromin regulates Akt/mTOR growth regulation varies between different cell types. In established cell lines and malignant peripheral nerve sheath tumor (MPNST) cells, *NF1* gene inactivation results in Akt phosphorylation of the tuberous sclerosis complex (TSC) protein tuberin and increased activation of Ras

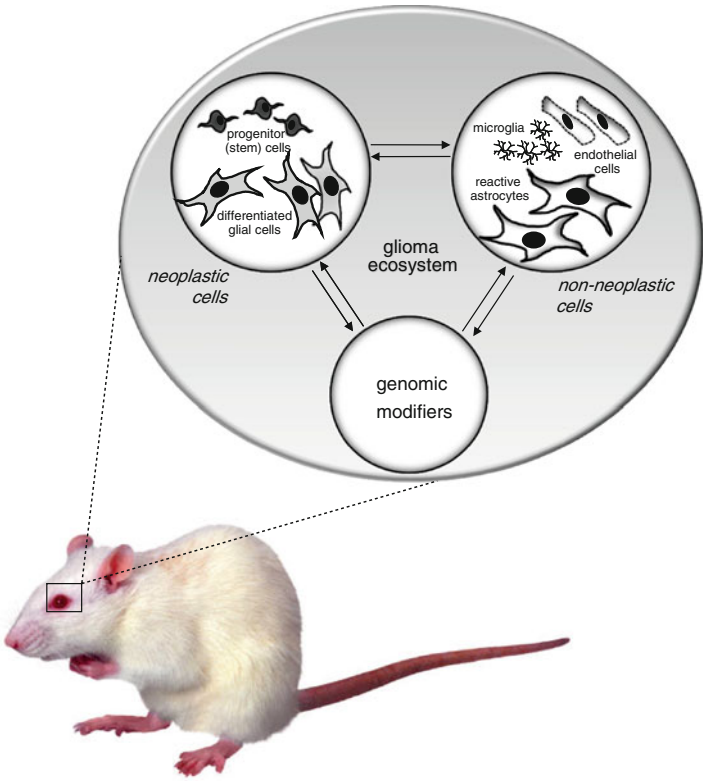

Fig. 22.2 Necessary conditions for NF1-associated gliomagenesis. Optic gliomagenesis results from cooperative molecular changes influenced by modifier genes in the genome, non-neoplastic cells in the tumor microenvironment, and preneoplastic/neoplastic cells. Tumor initiation in susceptible preneoplastic/neoplastic cells accompanies loss of *Nf1* tumor suppressor gene expression. These *Nf1*-deficient glial cells elaborate molecules that recruit or activate microglia and other stromal cell types (endothelial cells and reactive astrocytes) to create a microenvironment permissive for tumor evolution. The non-neoplastic cells in the tumor microenvironment in turn produce additional molecules ("gliomagens") that further promote neoplastic glial cell growth, neoplastic transformation, and tumor progression. Genomic modifiers contribute to tumor susceptibility and growth in currently undetermined ways

homolog enriched in brain (Rheb) and Rheb-mediated mTOR (Johannessen et al. 2005). By contrast, neurofibromin astrocyte growth regulation does not require TSC/Rheb signaling (Banerjee et al. 2011) (Fig. 22.3a).

mTOR is a critical serine/threonine kinase that exists as a complex with numerous other proteins to regulate cell growth. When complexed with the protein raptor (Kim et al. 2002), mTOR complex 1 (mTORC1) phosphorylates ribosomal S6 kinase and 4EBP1 to regulate protein translation and ribosomal biogenesis (Hara et al. 1997). When mTOR binds to rictor (Sarbassov et al. 2004), mTOR complex 2 (mTORC2) regulates Akt activation as well as actin cytoskeletal function through Rac1 (Sarbassov et al. 2005). In astrocytes, neurofibromin regulates cell growth in a

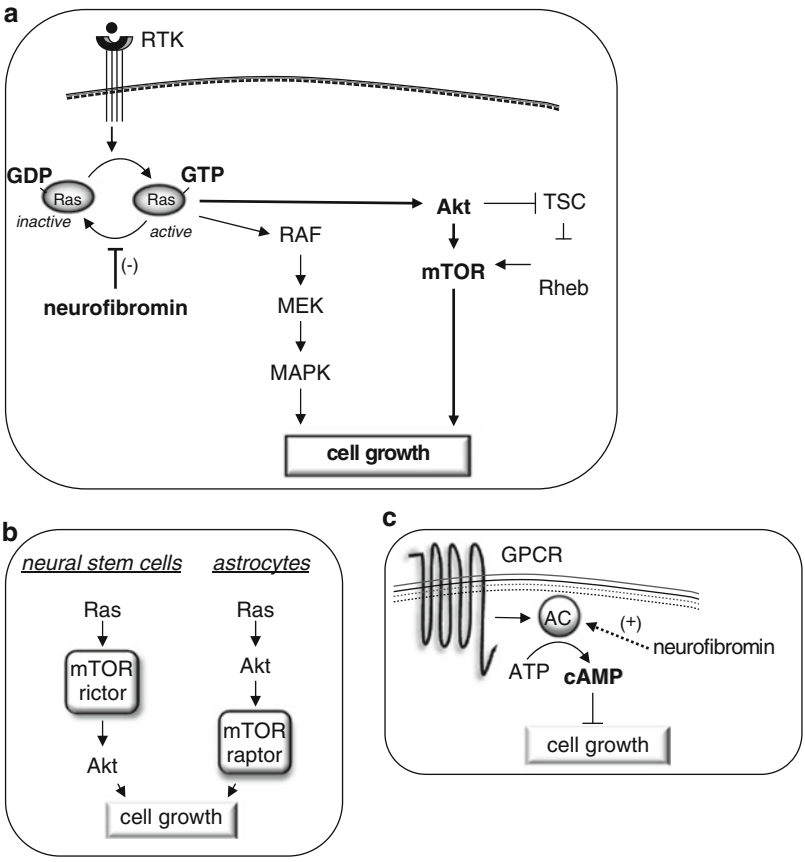

Fig. 22.3 Neurofibromin signaling pathways. Neurofibromin regulates intracellular signaling originating from both receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs). (**a**) The *NF1* gene product, neurofibromin, functions as a negative regulator of the Ras proto-oncogene by accelerating the conversion of active GTP-bound Ras to its inactive GDP-bound form. Active Ras initiates downstream signaling by activating the RAF/MEK/MAPK and the Akt/mTOR pathways. (**b**) In brainstem NSCs, cell growth is regulated by rictor (TORC2)-mediated Akt activation, while, in astrocytes, it is dependent on Akt-mediated activation of TORC1 complex (raptor). (**c**) Neurofibromin also positively regulates adenylate cyclase (AC) activity, leading to increased intracellular cyclic AMP (cAMP) levels. In astrocytes, cAMP inhibits cell proliferation and survival

Rac1- (Sandsmark et al. 2007) and STAT3-dependent manner (Banerjee et al. 2010), whereas in neural stem cells (NSCs), neurofibromin regulates cell growth in an Akt-dependent manner (Lee et al. 2010) (Fig. 22.3b).

Since the Ras-GAP domain comprises only 10 % of the entire neurofibromin polypeptide, it is highly likely that other regions of the molecule are also important for its function. Initial studies in *Drosophila melanogaster* suggested that neurofibromin regulates cyclic AMP (cAMP) signaling (The et al. 1997;

Guo et al. 1997). The ability of neurofibromin to positively regulate intracellular cAMP levels has also been reported for mouse neurons and astrocytes (Tong et al. 2002; Dasgupta et al. 2003). In astrocytes, neurofibromin is a positive regulator of cAMP generation (Dasgupta et al. 2003), such that *Nf1* loss results in reduced intracellular cAMP levels and inappropriate cell survival (Fig. 22.3c). Similarly in neurons, reduced neurofibromin expression leads to decreased cAMP/PKA activation of Rho-associated kinase (ROCK) and myosin light chain (MLC) phosphorylation and results in impaired neurite outgrowth (Brown et al. 2010, 2011). Although it is currently unclear how neurofibromin activates AC, at least two distinct mechanisms may be involved. The first involves receptor tyrosine kinase activation, which acts independently of G-protein-coupled receptors (GPCRs) and probably also involves Ras signaling. The second involves GPCR signaling and is Ras independent (Hannan et al. 2006).

22.2.2 Cellular Heterogeneity of Optic Gliomas

Similar to other solid tumors, OPGs contain a mixture of non-neoplastic and preneoplastic/neoplastic cells. The non-neoplastic (stromal) compartment is composed of immune system cells (microglia), entrapped normal cells (neurons), reactive astrocytes, and tumor-associated blood vessels (endothelial cells). The neoplastic compartment contains both progenitor (stem) cells and differentiated glial cells. It is likely that each of these distinct cell types contributes to glioma formation and growth.

22.2.2.1 Neoplastic Cell Compartment

The contribution of preneoplastic/neoplastic cellular heterogeneity to the pattern of gliomagenesis in NF1 is supported by recent studies demonstrating that mouse astrocytes and neural stem cells (NSCs) from different brain regions differ in their ability to hyperproliferate in response to *Nf1* gene inactivation. Whereas astrocytes from the mouse optic nerve and brainstem express neurofibromin, neocortical astrocytes (a region where gliomas rarely form in children) have low levels of *Nf1* gene expression (Yeh et al. 2009). Consistent with this differential pattern of neurofibromin expression, astrocytes from the brainstem and optic nerve increase their proliferation in vitro and in vivo following *Nf1* gene loss, whereas those from the neocortex fail to increase their growth (Yeh et al. 2009).

Similarly, *Nf1*-deficient mouse NSCs from the brainstem exhibit increased proliferation in vitro and an increased capacity to form differentiated glial cells in vivo. In contrast, *Nf1*-deficient mouse neural stem cells from the cortex do not. This differential response to *Nf1* loss results from increased rictor expression in brainstem NSCs, leading to TORC2-mediated Akt phosphorylation and increased NSC growth and glial differentiation (Lee da et al. 2010).

Taken together, while *Nf1* inactivation is an obligate step in gliomagenesis, it must occur in a cell type capable of expanding in response to loss of neurofibromin expression.

22.2.2.2 Non-neoplastic Cell Compartment

In addition to susceptible preneoplastic cell types, glioma formation and growth requires a supportive non-neoplastic tumor microenvironment (stroma). This stromal dependence was first revealed by genetically-engineered mouse (GEM) modeling experiments, in which mice lacking *Nf1* gene expression in glial fibrillary acidic protein (GFAP)-positive glial progenitors alone do not develop optic glioma (Bajenaru et al. 2002). Glioma formation only occurred in mice where *Nf1* loss in glial progenitor cells was coupled with *Nf1* heterozygosity, similar to children with NF1 (Bajenaru et al. 2003). The requirement for reduced *Nf1* gene expression in non-neoplastic cells supports a model in which gliomagenesis requires the productive interplay between *Nf1*+/− stromal cells and *Nf1*-deficient preneoplastic/neoplastic cells.

One of these stromal cell types are microglia (monocyte-like cells) (Markovic et al. 2009), which comprise 30 % of the total cells in human low-grade gliomas and are also found in *Nf1* mouse optic gliomas (Simmons et al. 2011). Several experimental lines of evidence have established a critical role for microglia in *Nf1* optic glioma growth. First, *Nf1*+/−, but not wild-type, microglia increase the proliferation of *Nf1*−/− astrocytes *in vitro*. Second, inhibition of microglia function using the tetracycline analog, minocycline, reduces optic glioma growth in vivo (Daginakatte and Gutmann 2007). Third, pharmacologic blockade of the c-Jun N-terminal Kinase (JNK) signaling pathway critical for *Nf1*+/− microglia function results in reduced optic glioma growth in vivo (Daginakatte et al. 2008). Fourth, genetic silencing of microglia results in attenuated optic glioma growth in vivo (Simmons et al. 2011).

Glioma-associated microglia produce numerous growth factors and cytokines, including tumor necrosis factor α (TNF-α), epidermal growth factor (EGF), and chemokines (e.g., CXCL12) that bind to receptors on glial cells to stimulate their growth and survival (Sawada et al. 1989). In particular, CXCL12 increases *Nf1*−/− astrocyte survival in a cAMP-dependent manner. The importance of CXCL12 and cAMP to NF1 gliomagenesis is underscored by recent studies in which murine optic glioma growth is reduced by pharmacologic manipulations that restore normal cAMP levels. Conversely, ectopic expression of an enzyme that degrades cAMP (phosphodiesterase-4 PDE4) in the cortex of *Nf1* optic glioma mice results in glioma formation in vivo (Warrington et al. 2010).

22.2.3 Genomic Contributions to Gliomagenesis

The importance of genetic modifiers is highlighted by several studies. First, Easton and colleagues showed that four NF1 clinical traits, including optic gliomas, had significant familial clustering, suggesting segregation of epistatic genes unlinked to

the *NF1* locus (Easton et al. 1993). Second, microarray data from the GeneNetwork database (Chesler et al. 2005) revealed that the levels of *Nf1* expression vary between mouse strains. Third, studies by Reilly and associates showed that astrocytomas form in GEM strains with high frequency on the C57BL/6 background, whereas mice on the 129S4 background are relatively resistant to glioma formation (Reilly et al. 2000). While the identity of these glioma modifier genes remains to be elucidated, the natural variations in *NF1* gene expression coupled with potential genomic loci must be included in the necessary conditions that influence glioma formation in children with NF1.

22.3 *Nf1* GEM Strains for Preclinical Drug Studies

The decision to initiate treatment is largely based on clinical progression, most commonly visual decline. Treatment of OPGs in children with NF1 typically involves chemotherapy, rather than surgery or radiation. While carboplatin/vincristine represents the initial therapy for OPGs, additional genotoxic therapies are often required for children with clinically progressive disease, which may result in secondary damage to proliferative zones (stem cell niches) important for learning and behavior.

While the initial evaluation of promising antineoplastic drugs typically involves the use of xenografts or cell culture models, these do not exist for low-grade gliomas. For this reason, *Nf1* optic glioma GEM strains have been employed. These preclinical models provide unprecedented opportunities to evaluate new drug treatments in the intact animal prior to the initiation of human clinical trials.

22.3.1 Targeting Tumor Cells

Because neurofibromin functions as a negative Ras regulator, initial treatment studies focused on Ras inhibitors. In order to signal to its downstream effectors, Ras must localize to the plasma membrane through post-translational isoprenylation. Early NF1 clinical trials focused on farnesyltransferase inhibitors, which unfortunately did not result in reduced tumor growth (Widemann et al. 2006).

The limited success of Ras inhibitors in clinical trials prompted additional searches for neurofibromin-regulated targets. One such discovery effort involved a proteomic-based screen for proteins differentially expressed in *Nf1*-deficient astrocytes, which led to the identification of mTOR as a downstream effector of neurofibromin growth regulation (Dasgupta et al. 2005). Combined with similar work by others (Johannessen et al. 2005), preclinical studies using the mTOR inhibitor rapamycin have been performed. Rapamycin treatment of *Nf1* optic glioma mice resulted in a dose-dependent decrease in tumor cell proliferation and tumor volume (Hegedus et al. 2008). These encouraging results precipitated current clinical trials using rapamycin analogs for the treatment of NF1-associated plexiform neurofibromas and low-grade gliomas.

Further analysis of mTOR regulation of *Nf1*-deficient astrocyte growth revealed that mTOR-dependent growth control requires Rac1 activation in cells (Sandsmark et al. 2007). Using a high-throughput compound screen, cucurbitacin-I was identified as an inhibitor of *Nf1*-deficient astrocyte growth. Cucurbitacin-I inhibits STAT3 activation, which was shown to be a downstream effector of mTOR (Banerjee et al. 2010). Future preclinical studies are underway to evaluate drugs that inhibit mTOR function in *Nf1* optic glioma mice.

22.3.2 Targeting the Tumor Microenvironment

For decades, chemotherapy has been directed predominantly at the neoplastic cells in cancers. Pioneering studies by Judah Folkman first suggested that the interplay between tumor and endothelial cells could be leveraged to develop therapies that reduce angiogenesis (Folkman 1972), thereby disrupting the stem cell niche within the tumor (Calabrese et al. 2007). Recent chemotherapy approaches for low-grade gliomas entail the use of Avastin to inhibit vascular endothelial growth factor (VEGF) and its receptor (Packer et al. 2009). In addition to targeting the endothelial cells, microglia may be a logical target for future stroma-directed therapies for NF1-associated optic glioma (see Sect. 22.2.2).

22.3.3 Neuroprotective Strategies

While standard chemotherapy results in glioma stabilization in 60–80 % of children with NF1-associated OPG, few patients (20 %) actually exhibit improved visual acuity (Dalla Via et al. 2007). In *Nf1* optic glioma mice, ultrastructural damage to the optic nerve axons and irreversible loss (apoptosis) of retinal ganglion cells (RGCs) are observed early during gliomagenesis (Hegedus et al. 2009). This *Nf1*+/− RGC neuronal cell death results from reduced cAMP levels, such that pharmacologically elevation of cAMP levels leads to attenuated apoptosis in vivo (Brown et al. 2010). These findings raise the exciting possibility that neuroprotective strategies, in combination with new chemotherapeutic approaches that target both the neoplastic and non-neoplastic cells, may improve the outcomes in children with NF1-associated OPG.

22.3.4 Targeting the Tumor Ecosystem

The challenges to treating low-grade gliomas in children suggest that alternative strategies might be considered. Given the complex interactions between cell types and genomic factors, such alternative approaches might consider gliomas as ecological systems.

An ecosystem is an environment composed of interacting living organisms and nonliving, physical components of that environment. Cooperation and division of labor between the individual components are one of the key features of ecosystems. This is well illustrated by social insects, whose nests comprise hundreds or thousands of individual specialized animals—reproductive (queens) and nonreproductive castes (Thorne et al. 2003; Amdam and Page 2010; Hartmann and Heinze 2003).

In an analogous fashion, gliomas are highly heterogeneous tissues with a hierarchical cellular organization and distinct cell types ("castes") specialized for particular tasks. Some cells are primarily responsible for reproduction (neoplastic cells), whereas others function in housekeeping capacities (non-neoplastic cells). The cooperative (inter)action of all these cell types is essential for the fitness of the tumor as a whole.

Using this conceptual framework, future OPG therapies will require a more detailed understanding of the individual and collective contributions of each cell type. Moreover, since ecosystems operate as a network of dependencies, it is necessary to develop treatments that disrupt these relationships and restore a new state of homeostasis to the tumor tissue.

This ecosystems approach might reduce the toxicity of current treatments on the developing brain and thus decrease the long-term cognitive sequelae associated with successful glioma therapy.

22.4 The Future of NF1-Associated OPG Management

Over the past decade, there has been an explosion in our understanding of the genetic basis of optic glioma formation and the contributions of select non-neoplastic and neoplastic cells to gliomagenesis and maintenance. The accelerated pace of scientific discovery has led to the identification, preclinical evaluation, and clinical assessment of several promising drugs for NF1-associated tumors, including OPG. With the availability of high-throughput technologies for rapid drug discovery, robust preclinical models for drug testing and a multicenter NF clinical trials consortium, the future holds great promise for the efficient translation of basic science discoveries to the clinical workplace. Although speculative at present, we stand on the brink of individualized medicine in which genomic profiling, advanced neuroimaging studies, and predictive biomarkers may stratify patients into clinically relevant subgroups with high probabilities of responding to specific targeted therapies. The confluence of faithful small-animal models, advanced multiplex, high-throughput technologies, and specialized treatment centers will enable researchers and clinicians to apply cutting-edge approaches to the management of brain tumors, not only in children with NF1, but perhaps for both pediatric and adult patients with sporadic brain tumors.

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Chapter 23
Molecular Basis of Cardiovascular Abnormalities in NF1

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Most people with NF1 vasculopathy have involvement of multiple arteries in a spotty distribution. NF1 vasculopathy is usually asymptomatic, although the first clinical manifestation may be a life-threatening or fatal event. Vascular lesions in NF1 patients may occur anywhere but are most often identified in the renal arteries, usually in association with hypertension. Cerebrovascular disease typically produces stenosis of the internal carotid artery, middle cerebral artery, or anterior cerebral artery and is often associated with moyamoya.

No cohort of NF1 patients has been systematically screened for vasculopathy of any site, and the overall prevalence among people with NF1 is unknown. Vasculopathy is an unexpectedly frequent cause of death among people with NF1 who die in childhood, adolescence, or early adulthood.

Most of our understanding of the role that neurofibromin plays in cardiovascular system development comes from studies in targeted mouse mutants. Spontaneous cardiovascular disease is not seen in heterozygous $Nf1^{+/-}$ mice, but $Nf1^{-/-}$ mutants die in mid-gestation as a result of double outlet right ventricle, membranous ventricular septal defect, and enlarged endocardial cushions. Studies in lineage-restricted homozygous knockout mice demonstrate critical involvement of endothelial cells in the pathogenesis of these cardiac malformations, with pERK activation leading to hyperproliferation of the endocardial cushions.

Murine models show a normal phenotype and normal function for heterozygous $Nf1^{+/-}$endothelial cells, but these cells exhibit an exaggerated response to environmental changes and growth factors. Adult humans with NF1 have normal numbers

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of endothelial colony-forming cells in their peripheral blood, but these cells exhibit increased ERK activation and increased proliferation and migration in response to angiogenic growth factors.

Hyperproliferative potential has been demonstrated in each of the cell types that comprise the vascular wall—endothelial cells, vascular smooth muscle cells (VSMCs), and pericytes. The characteristic lesions of NF1 vasculopathy in humans are intima hyperplasia and neointima formation, and analogous proliferation of intima VSMCs has been demonstrated in murine models using a variety of techniques. Studies in lineage-restricted knockout mice indicate that Ras–Mek–ERK signaling is amplified in VSMCs, and this appears to play an important role in neointima formation. Experiments using adoptive bone marrow transplantation indicate that heterozygous inactivation of *Nf1* in the marrow is also required for neointima formation in response to injury. A key factor may be stimulation of VSMC proliferation by cytokines produced by marrow-derived macrophages.

Although cardiovascular abnormalities have been recognized as a feature of NF1 for over 65 years (Reubi 1945; Feyrter 1949), the frequency and natural history of these lesions are still incompletely understood. Nevertheless, recent research has provided important insights into the molecular pathogenesis of the congenital heart defects and vasculopathy that occur in some people with NF1.

23.1 Congenital Heart Defects

The prevalence of clinically apparent congenital heart defects (CHD) ranges from 0.4 % to 6.4 % in published series of NF1 patients (Lin et al. 2000). Differences in ascertainment, patient age, and diagnostic criteria for cardiac defects probably account for the wide variation in reported frequencies. Tedesco et al. (2002) performed two-dimensional echocardiograms and color Doppler studies on 48 unselected children and adolescents with NF1 and found cardiovascular disease in four (8 %): one case of pulmonic stenosis, one case of aortic coarctation, and two cases of hypertrophic cardiomyopathy. No abnormalities were found in a control group of 30 children of comparable age.

The strongest evidence that NF1 and CHD are, in fact, associated is the extraordinarily high proportion of these patients in whom the CHD is pulmonic stenosis, usually of the valvar type. Pulmonic stenosis accounts for approximately one-quarter of all CHD among NF1 patients (Lin et al. 2000) and is a recognized feature of three clinical subtypes of NF1-Watson syndrome, NF1-Noonan syndrome, and individuals with large deletions of the *NF1* gene (Lin et al. 2000). Valvar pulmonic stenosis is also a common feature in patients with Noonan syndrome, LEOPARD syndrome, or cardio–facio–cutaneous syndrome, conditions that, like NF1, are genetic disorders that produce dysregulation of the Ras/MAPK pathway (Zenker 2011).

Coarctation of the aorta has been reported repeatedly in patients with NF1 (Lin et al. 2000), although the narrowing in these patients is usually of a long fusiform type. This differs anatomically from the abrupt segmental constriction that is most often seen in other patients with coarctation of the aorta. Hypertrophic cardiomyopathy has also been reported in several NF1 patients, but this appears to be an infrequent occurrence (Friedman et al. 2002).

Double outlet right ventricle, a conotruncal defect, occurs in homozygous *Nf1* "knockout" mouse embryos (Brannan et al. 1994; Jacks et al. 1994) but appears to be quite rare in people with NF1. In fact, the small number of NF1 patients who have been reported to have complex CHD of any kind is striking (Lin et al. 2000; Friedman et al. 2002).

23.2 NF1 Vasculopathy

NF1 vasculopathy may affect arteries that range in size from small arterioles to the proximal aorta. The systemic circulation is affected much more often than the pulmonary arteries, although the latter may also be involved. The outflow tract of the heart or the veins may also be affected. Most NF1 patients with vasculopathy have involvement of multiple vessels (Friedman et al. 2002; Oderich et al. 2007).

NF1 vasculopathy is usually asymptomatic, and the first clinical manifestation may be a life-threatening or fatal event. Sudden death has been reported as a result of rupture of a major artery, cerebral ischemia or hemorrhage, or myocardial infarction in young patients with NF1 vasculopathy (Friedman et al. 2002). Pathological consequences of NF1 vasculopathy may include arterial stenosis, occlusion, aneurysm, pseudoaneurysm, and rupture or fistula formation. The condition is sometimes progressive, with lesions becoming more severe with time or recurring after treatment that initially appeared to be effective (Criado et al. 2002; Rea et al. 2009; Ghosh et al. 2012).

Vascular lesions in NF1 patients are most often identified in the renal arteries: 1–5 % of people with NF1 have renal artery disease (Friedman et al. 2002). High blood pressure may occur as a result of proximal renal artery stenosis, intraparenchymal renal arterial abnormalities, or both. Renovascular hypertension in NF1 usually presents in childhood, adolescence, or early adulthood; it is sometimes recognized as eclampsia or accelerated hypertension during pregnancy (Dugoff and Sujansky 1996).

Cerebrovascular abnormalities in people with NF1 usually result from stenosis or occlusion of the internal carotid artery, middle cerebral artery, or anterior cerebral artery (Friedman et al. 2002). Small telangiectatic vessels may form around the area of the stenosis and look like a puff of smoke on cerebral angiography. Patients with NF1 appear to be unusually prone to this finding, which is called "moyamoya," in response to cerebral hypoxia (Wu et al. 2006) or radiation damage (Ullrich et al. 2007; Milewicz et al. 2010).

23.3 Prevalence of NF1 Vasculopathy, Morbidity, and Mortality

No population-based cohort of NF1 patients has been systematically screened for vasculopathy, but a few studies have assessed various aspects of vascular disease among clinical series. Fifteen (3.8 %) of 398 children with NF1 who underwent MRI were found to have stenosis or occlusion of an intracranial artery or moyamoya in one series (Ghosh et al. 2012). Rosser et al. (2005) examined brain MRIs of 316 unselected children with NF1 and found that 8 (2.5 %) had cerebrovascular abnormalities. In another series, 7 (4.9 %) of 144 patients with NF1 who had undergone head MRI were found to have dysplasia of the cerebral arteries (Cairns and North 2008). The arterial lesions were asymptomatic in most of the patients studied.

Seventeen (6.4 %) of 266 children with NF1 who underwent MRI in another series had stenosis of one or more intracranial arteries, moyamoya, or aneurysms (Rea et al. 2009). Most of these patients were imaged because of central nervous system symptoms. Fifteen of the 17 affected patients also had optic gliomas, but no information is provided regarding radiotherapy.

Patients who have had radiation therapy for intracranial tumors are at increased risk of developing moyamoya (Milewicz et al. 2010), and this is especially frequent among patients with NF1 (Ullrich et al. 2007). The pathogenic basis for this association is unknown, but possibilities include increased vascular damage, abnormal vascular repair, or increased neovascular proliferation in response to radiation injury in NF1 patients.

Fossali et al. (2000) screened 27 unselected children with NF1 and found hypertension in three (11 %) and borderline hypertension in another two. Thirteen of these patients had venous digital subtraction angiography, and renal artery stenosis was seen in seven and aortic coarctation in one. 24-h blood pressure monitoring has been performed in two series of young NF1 patients, which included 20 and 73 subjects, respectively (Fossali et al. 2000; Tedesco et al. 2005). Abnormal 24-h blood pressure measurements were observed in 40 % of the smaller series and in 16 % of the larger series. Some of the cases with abnormal 24-h monitoring had normal basal blood pressure measurements.

Pulmonary hypertension may also occur in NF1, although it is much less frequent than arterial hypertension (Stewart et al. 2007; Montani et al. 2011). Unlike arterial hypertension, pulmonary hypertension is rarely seen in NF1 patients under 50 years of age, and it is often associated with parenchymal lung disease.

Vasculopathy appears to be an unexpectedly frequent cause of death among people with NF1 who die in childhood, adolescence, or early adulthood (Rasmussen et al. 2001). There is a large excess of deaths before 50 years of age among people with NF1 (Rasmussen et al. 2001). Most of this excess mortality is attributable to neoplasms, especially malignant peripheral nerve sheath tumors, but deaths associated with vascular disease also occur more often than expected among young adults with NF1 (Sørensen et al. 1986; Rasmussen et al. 2001; Evans et al. 2011).

In an unselected autopsy series, Salyer and Salyer (1974) found histological lesions of vasculopathy in 8 of 18 NF1 patients who died at a median age of

24 years (range, 4–69 years). Only one of these patients died of vascular disease; the vasculopathy was asymptomatic in the other seven affected patients. The kidneys were involved in six of the eight NF1 patients, but multiple arteries were affected, including those of the heart, pancreas, thyroid, ileum, spleen, and meninges.

23.4 Embryology

Neurofibromin plays a critical role in embryogenesis and cardiovascular development. In animal models, cardiovascular malformations result from substantial or complete loss of neurofibromin activity and constitutive Ras stimulation of aberrant proliferation within key cardiovascular lineages in the embryo (Viskochil 2002). However, the role of haploinsufficiency for neurofibromin in heart development and function is not yet well understood.

Most of our understanding of the in utero role of *NF1* has come from animal studies, principally targeted mouse mutants and more recently zebra fish results (Brannan et al. 1994; Jacks et al. 1994; Gitler et al. 2003; Ismat et al. 2006; Xu et al. 2007; Padmanabhan et al. 2009). Somewhat surprisingly, *Nf1* heterozygous mice are viable postnatally and do not exhibit any in utero cardiovascular abnormalities. The reason underlying the disconnect between the mouse and human *NF1* heterozygous cardiovascular developmental phenotypes remains unclear but may be due to the inbred nature of mouse genetic backgrounds used or the lack of sophisticated experimental tools to analyze and map partially penetrant phenotypes.

However, systemic *Nf1* null mutants exhibit mid-gestation lethality and outflow tract septation defects (double outlet right ventricle/membranous ventricular septal defect) and concomitant enlarged endocardial cushions (Brannan et al. 1994; Jacks et al. 1994). Although the precise cause of the *Nf1* null mid-gestation lethality remains unclear, significant anasarca of the embryo suggests either poor cardiac function or abnormal regurgitant blood flow, and diastolic dysfunction may cause in utero death (Conway et al. 2003; Phoon et al. 2004).

Given the demonstrated role of the neural crest within both NF1 tumor formation and the pathogenesis of outflow tract septation defects, as well as the fact that neural crest-derived cells in the peripheral nervous system have the highest neurofibromin expression levels, these cardiovascular defects were presumed to have a cardiac neural crest cell origin (Kirby et al. 1983; Daston and Ratner 1992; Zhu et al. 2002; Hutson and Kirby 2003; Snider et al. 2007; Yang et al. 2008). Nevertheless, more recent lineage-restricted mouse knockout approaches surprisingly revealed that tissue-specific inactivation within the endothelial lineage using *Tie2–Cre*-mediated *Nf1* inactivation reproduced the in utero *Nf1* null cardiac abnormalities, although neural crest-restricted inactivation resulted in overgrowth of peripheral nervous tissue (Gitler et al. 2003; Ismat et al. 2006).

Moreover, these transgenic mice showed that loss of *Nf1* in the embryonic endocardium, a precursor of the heart valves, is directly linked to an increase in activated GTP-bound Ras. The resulting stimulation of MAPK signaling resulted in abnormal

enlargement of the outflow tract septum. Restoration of the neurofibromin GTPase-activating protein (GAP)-related domain in endothelial cells is sufficient to regulate Ras activity and rescue the CHDs observed in *Nf1* null embryos (Ismat et al. 2006).

Although these elegant transgenic data suggest a cell-autonomous role for *NF1* within the embryonic endothelium and may also provide potential therapeutic targets, it should be noted that the *Tie2* promoter used in these studies is expressed throughout the embryonic and extraembryonic endothelial lineage and is also known to be active in hematopoietic stem cells (Forde et al. 2002). Thus, given the myeloproliferative features of NF1 and the importance of the haploinsufficient hematopoietic microenvironment to NF1 disease progression, the precise role of the cardiovascular endothelium within NF1-deficient CHD pathogenesis remains to be verified. Similarly, the potential modifying role and in utero effects of the *Nf1*-deficient hyperplastic neural crest lineage and its interactions with adjacent endocardial and endothelial lineages during cardiovascular morphogenesis also remain unknown (Gitler et al. 2002; Wang et al. 2005).

Consistent with neurofibromin's function as a regulator of the Ras pathway and the demonstration of ERK hyperactivation, parallels have been drawn with Noonan syndrome (Mangues et al. 1998; Friedman et al. 2002; Araki et al. 2004; Lasater et al. 2008). Noonan syndrome is an autosomal dominant condition characterized by short stature, facial abnormalities, heart defects, and possibly increased risk of leukemia (Araki et al. 2004). Mutations of *PTPN11* occur in approximately 50 % of individuals with Noonan syndrome and lead to pulmonary stenosis, hypertrophic cardiomyopathy, atrial septal defect, tetralogy of Fallot, aortic coarctation, and anomalies of the mitral valve (Marino et al. 1999). In addition to exhibiting similar range of cardiovascular phenotypes, mouse models of both NF1 and Noonan syndrome have enlarged endocardial cushions with increased expression of ERK coincident with double outlet right ventricle and hyperplastic outflow tract cushions (Araki et al. 2004). Moreover, there is a recent report of a child who died early in infancy with severe cardiovascular defects and who was found to carry both an *NF1* missense mutation and a pathogenic mutation of the *PTPN11* gene (Prada et al. 2011).

Thus, the in utero cardiovascular manifestations of *Nf1* null mice appear to result from a pathway mediating CHDs in which endothelially driven hyperproliferation of the endocardial cushions occurs in association with pERK activation. How this mechanism relates to the pathogenesis of CHD in haploinsufficient NF1 patients remains to be seen, but CHD in humans is likely also to involve multiple cell lineages responding in gene dosage-dependent and pathway-selective manners.

23.5 Angiogenesis

Vessel formation and proliferation is a critical component of solid tumor growth and has become an important target for cancer treatment. The ability to form discrete, independent vascular networks, termed "angiogenic switch," is necessary for tumor propagation and has been demonstrated in a variety of

NF1-related tumors. Schwann cells, the primary cell type in neurofibromas, have been shown to undergo angiogenic switch in a number of animal models and human tumor samples (Sheela et al. 1990; Kim et al. 1997; Mashour et al. 1999; Munchhof et al. 2006). Using tumor samples from NF1 patients, Sheela et al. (1990) first demonstrated the ability of neurofibromas to stimulate vascular development independent of additional growth factors. Incubation of single cell colonies on chorioallantoic membranes (CAM) of developing chick embryos showed that wild-type (WT) Schwann cells, fibroblasts, and tumor fragments from non-NF1 patients failed to induce an angiogenic response. However, fragments of tumor from NF1 patients stimulated a robust proliferation of new blood vessels that were easily visualized by 48 h and highly prevalent by 10 days. Addition of fibroblast growth factor (bFGF), a known angiogenic growth factor, greatly enhanced this response. Application of single cell lines of Schwann cells and fibroblasts from neurofibromas demonstrated that Schwann cell-enriched cultures were highly angiogenic and often invaded the mesoderm of the CAM, while fibroblasts, another common cell population within neurofibromas, failed to induce vessel formation (Sheela et al. 1990).

Examination of conditioned media from $NF1^{-/-}$ Schwann cell cultures demonstrated an alteration in the balance of angiogenic growth factors to favor proliferation. Thrombospondin-1, platelet-derived growth factor beta, and midkine mRNA expression were elevated in $NF1^{-/-}$ Schwann cell-conditioned media compared to WT-conditioned media (Mashour et al. 2001). In addition, incubation of endothelial cells (ECs) with $NF1^{-/-}$ Schwann cell-conditioned media produced a hyperproliferative endothelial cell phenotype similar to that seen with stimulation by vascular endothelial growth factor (VEGF) or fibroblast growth factor-2 (FGF-2). This response is mediated through hyperactivation of Ras–Mek–ERK signaling and is abrogated with use of a specific inhibitor of Mek–ERK signaling in vivo (Munchhof et al. 2006). This is a critical observation because alteration in endothelial cell phenotype has been observed in several models of tumor angiogenesis. The contribution of each or all of these growth factors in promoting angiogenesis within the developing neurofibroma and the mechanism through which angiogenic switch occurs to promote tumor growth remains an area for future study.

Although early experiments focused on mutant cell lines with homozygous loss of *Nf1*, a growing body of evidence suggests that haploinsufficiency of stromal cells has important implications for NF1 tumor propagation and angiogenesis. Murine models of neovascularization have demonstrated an exaggerated proliferation of heterozygous endothelial cells, pericytes, and abnormal neovascular networks in response to hypoxia, which are not present in littermate controls in a normoxic environment (Wu et al. 2006; Ozerdem 2004). Furthermore, $Nf1^{+/-}$ mice showed markedly increased neovascularization and endothelial proliferation and migration in response to corneal implantation of micropellets impregnated with bFGF or VEGF compared to controls (Munchhof et al. 2006; Wu et al. 2006).

Importantly, $Nf1^{-/-}$ Schwann cell-conditioned media, which contain increased concentrations of several proangiogenic peptides, further amplify angiogenesis through amplification of Ras–Mek–ERK signaling in heterozygous mice when compared to WT controls (Mashour et al. 2001; Munchhof et al. 2006). Of particular

interest, Wu et al. (2006) noted a nearly fivefold increase in the number of macrophages and a significant mast-cell infiltration in the neovascular zone of *Nf1*$^{+/-}$ corneas compared to controls. This is an important observation since macrophages and mast cells play a critical role in tumor growth and neoangiogenesis. These studies, when taken together, demonstrate a normal phenotype and function for heterozygous endothelial cells but an exaggerated response to environmental changes and growth factors as compared to wild-type controls.

Overall, there are significant murine and human data that demonstrate an increased angiogenic potential in neurofibromas. Additionally, circulating progenitor cells, which are important in vascular development and angiogenesis, are sensitive biomarkers in several tumor types. Specifically, endothelial progenitor cells can be isolated from adult peripheral blood and contain a novel population of endothelial colony-forming cells (ECFCs) with proliferative potential and the ability to form vessels de novo (Ingram et al. 2004). Although they have normal numbers of ECFCs in the peripheral blood, NF1 patients exhibit increased proliferation and migration in response to angiogenic growth factors VEGF and bFGF compared to age and sex-matched controls. Also, ECFCs isolated from NF1 patients demonstrate increased ERK activation, but their proliferative potential is reduced to non-NF1 levels when incubated with a specific inhibitor of Mek–ERK signaling. This is similar to in vitro and in vivo studies using transduced human cell lines and provides validation to previous model systems.

23.6 Models of NF1 Cardiovascular Disease

Expression of neurofibromin, the gene product of *NF1*, has been demonstrated in each cell type that composes the vascular wall, including endothelial cells, vascular smooth muscle cells (VSMCs), and pericytes (Norton et al. 1995; Ozerdem 2004; Li et al. 2006; Munchhof et al. 2006). The hyperproliferative potential of each of these cell lines, along with circulating bone marrow-derived cells, which contribute growth factors and cytokines that maintain vessel wall homeostasis, has been demonstrated using in vitro assays (Ozerdem 2004; Li et al. 2006; Munchhof et al. 2006; Xu et al. 2007). However, murine models of subtle or preclinical NF1-related cardiovascular disease have proved difficult because of the lethality of the homozygous mutant mouse (Friedman et al. 2002). In addition, homozygous loss of *NF1* is an inaccurate representation of arterial disease in humans because it is likely that homozygous loss of *NF1* in patients would result in overt, rather than subtle, disease. Therefore, focus on murine models utilizing heterozygous backgrounds and lineage-restricted inactivation of *Nf1* provide more feasible and accurate representations for investigation.

The characteristic phenotype of NF1-related cardiovascular disease is arterial stenosis resulting from intima hyperplasia (or neointima formation) (Hamilton and Friedman 2000; Friedman et al. 2002). Proliferation of VSMCs in the intima layer using murine models has been demonstrated using a variety of techniques.

Two mechanisms of injury, endothelial denudation using a beaded guidewire and ligation of the common carotid artery proximal to the bifurcation, have been particularly useful in studies of NF1 neointima formation. In addition, the use of lineage-restricted transgenic mice provides a tractable platform to test the contribution of heterozygous and homozygous loss of neurofibromin to vascular remodeling in a cell-specific manner.

To generate the initial model of neointima hyperplasia, Xu et al. (2007) cross-bred alpha-smooth muscle *cre (SM22α-Cre)* mice and $Nf1^{fl/-}$ mice (heterozygous mice with a single floxed allele) to generate offspring with lineage-restricted homozygous deletion of *Nf1* in VSMCs superimposed on an *Nf1* haploinsufficient background. These mice were born at the expected Mendelian ratio, were phenotypically normal at birth, and developed appropriately (Xu et al. 2007). Examination of the cardiovascular system revealed phenotypically normal large arteries, similar to wild-type mice (Xu et al. 2007). In response to ligation of the carotid artery, $Nf1^{fl/-}$;*SM22α-Cre* mice displayed a 2.5-fold increase in intima thickening and intima-to-media ratio compared to WT mice, signifying that proliferation was restricted to the intima layer without simultaneous proliferation of the medial layer (Xu et al. 2007). Smooth muscle cells were the most common cell type in the neointima and demonstrated a profound increase in number and proliferative index compared to WT controls (Xu et al. 2007). However, the phenotype produced in this model was only evident on a haploinsufficient background, suggesting the role of another cell lineage as the primary effector of NF1 vasculopathy.

The accumulation of VSMCs in the neointima suggests that *NF1* plays a critical role in VSMC proliferation, migration, and survival (Li et al. 2006; Xu et al. 2007). Several studies have demonstrated marked increases in murine and human VSMC proliferation and migration compared to WT and in response to growth factor (Xu et al. 2007; Munchhof et al. 2006; Li et al. 2006; Lasater et al. 2010). Interestingly, stimulation of *Nf1* haploinsufficient murine VSMCs with PDGF-BB, which excites both the Ras–ERK and PI-3 kinase-AKT pathways, resulted in a twofold increase in proliferation and fourfold increase in migration compared to WT VSMCs. These phenotypic changes are probably mediated through Ras–ERK signaling in as much as use of a specific Mek inhibitor and restoration of the *Nf1* GAP-related domain resulted in demonstrable ERK inactivation and restoration of *Nf1* heterozygous VSMCS proliferation and migration to that of WT controls.

Examination of the neointimas from $Nf1^{fl/-}$;SM22α-Cre mice showed significantly more pERK-positive cells than WT mice, indicating that Ras–Mek–ERK signaling was amplified and probably plays an important role in neointima formation and vascular repair (Xu et al. 2007). In addition, restoration of the *Nf1* GAP-related domain reduced neointima formation to that of WT mice (Xu et al. 2007). These observations are intriguing and provide insights into the evolution of NF1 vasculopathy because mice harboring genetic mutations that increase signaling through the PDGF-BB–Ras–ERK axis develop exaggerated neointima formation and arterial occlusion, reminiscent of patients with NF1 vasculopathy (Jin et al. 2000; Zhang et al. 2008).

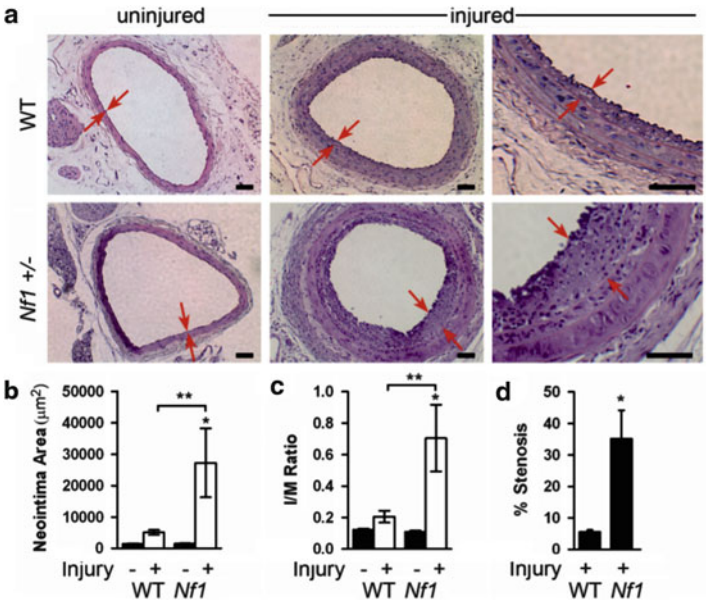

Fig. 23.1 Histological and morphometric analysis of injured carotid arteries from WT and *Nf1*$^{+/-}$ mice. (**a**) Representative photomicrographs of carotid arteries from WT (*top panels*) and *Nf1*$^{+/-}$ (*bottom panels*). *Red arrows* indicate boundary of neointima. (**b**) Neointima area of uninjured and injured carotid artery cross sections from WT and *Nf1*$^{+/-}$ mice. (**c**) I/M ratio of uninjured and injured carotid artery cross sections from WT and *Nf1*$^{+/-}$ mice. (**d**) Percentage of carotid artery stenosis 21 days following injury in WT and *Nf1*$^{+/-}$ mice

To examine the role of *Nf1* heterozygosity alone, Lasater et al. (2008) performed endothelial denudation via a guidewire of the common carotid artery in *Nf1*$^{+/-}$ and WT mice. In response to injury, *Nf1*$^{+/-}$ mice had a significant increase in intima area and intima-to-media ratio similar to other models and to patients with NF1 vasculopathy (Fig. 23.1). As in previous reports, proliferating VSMCs accounted for more than 75 % of the cells in the neointima, and significant pERK staining was noted (Lasater et al. 2008). Interestingly, pretreatment with Gleevec, an inhibitor of PDGF signaling, prior to injury, completely reduced neointima formation in *Nf1*$^{+/-}$, signifying that neointima formation or, more specifically, VSMC proliferation may be mediated through the Ras–Mek–ERK signaling pathway (Lasater et al. 2008). This is an important model of cardiovascular disease and demonstrates that manifestations of abnormal vascular remodeling may be present in asymptomatic NF1 patients without known risk factors.

To further define the role of neurofibromin in vessel wall homeostasis and remodeling, Lasater et al. (2010) generated mice with loss of a single *Nf1* allele in ECs and VSMCs alone. In response to carotid artery ligation, *Nf1*$^{fl/+}$*;Tie2Cre* (ECs) mice, *Nf1*$^{fl/+}$*;SM22Cre* mice (VSMCs), and *Nf1*$^{fl/+}$*;Tie2;SM22Cre* mice (ECs and VSMCs) had similar neointima formation to WT mice, and intima proliferation in these genotypes was greatly reduced when compared to *Nf1*$^{+/-}$

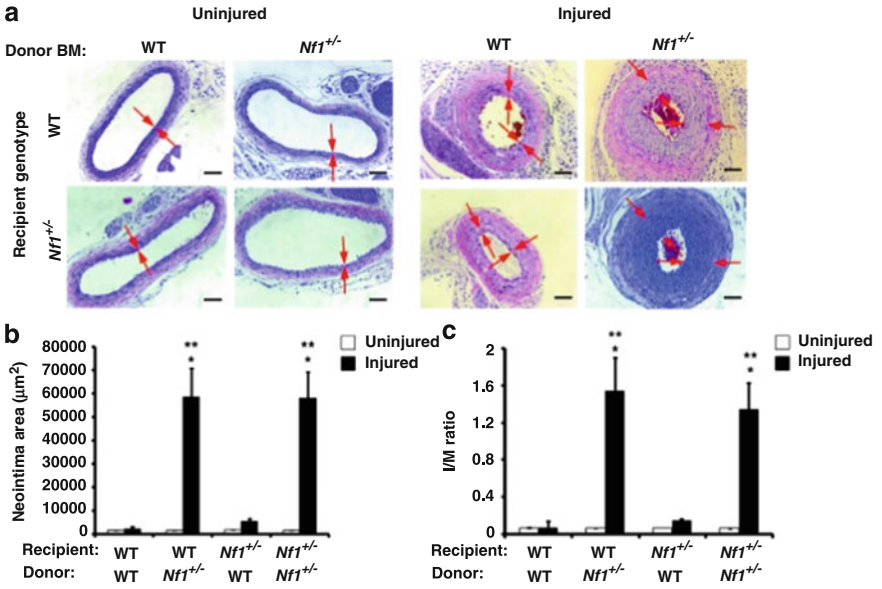

Fig. 23.2 Histological and morphometric analysis of WT and *Nf1*$^{+/-}$ mice transplanted with WT and *Nf1*$^{+/-}$ bone marrow. (**a**) Representative hematoxylin and eosin-stained cross sections of uninjured and injured carotid arteries from WT and *Nf1*$^{+/-}$ mice transplanted with WT or *Nf1*$^{+/-}$ bone marrow. *Red arrows* indicate boundaries of neointima. *Scale bars*: 50 μm. (**b** and **c**) Quantification of neointima area (**b**) and I/M ratio (**c**) of uninjured and injured carotid arteries from WT or *Nf1*$^{+/-}$ recipients transplanted with WT or *Nf1*$^{+/-}$ marrow

mice, providing evidence that neointima formation and VSMC proliferation are not primarily mediated through heterozygous loss of *Nf1* within the vessel wall itself (Lasater et al. 2010). However, using adoptive hematopoietic stem cell transfer techniques, *Nf1*$^{+/-}$ mice reconstituted with WT bone marrow showed a tenfold reduction in neointima area and a ninefold reduction in I/M ratio compared to *Nf1*$^{+/-}$ mice reconstituted with *Nf1*$^{+/-}$ bone marrow (Lasater et al. 2010) (Fig. 23.2). Further, WT mice transplanted with *Nf1*$^{+/-}$ bone marrow had increased neointima formation, comparable to *Nf1*$^{+/-}$ mice transplanted with *Nf1*$^{+/-}$ bone marrow (Lasater et al. 2010) (Fig. 23.2).

These experiments using adoptive bone marrow transplantation strongly indicate that heterozygous inactivation of *Nf1* in the bone marrow plays a critical role in neointima formation in response to injury (Lasater et al. 2010). As noted in previous studies of vascular remodeling, macrophages were the most common bone marrow-derived hematopoietic cell identified within the neointima (Wu et al. 2006; Lasater et al. 2010). This is an important observation because macrophages within the neointima produce cytokines that stimulate VSMC proliferation and generate metalloproteases, which are required for extracellular matrix remodeling in neointima formation (Bendeck et al. 1994).

Mouse modeling has made important contributions to our understanding of the pathogenesis of NF1 cardiovascular disease, although important questions remain unanswered. The primary bone marrow-derived hematopoietic cell involved in VSMC proliferation and neointima formation has yet to be identified formally. Lineage-restricted transgenic mice will continue to play a central role in the identification of this primary cell in as much as macrophages, mast cells, and lymphocytes have all been implicated as significant contributors to vascular remodeling (Strom et al. 2007; Swirski et al. 2007).

The nature of the signaling pathway mediating VSMC proliferation within the neointima remains poorly understood. ERK staining is significantly increased in neointimas, and cell culture experiments suggest that Ras–Mek–ERK signaling is an important mediator of VSMC proliferation and migration, although inactivation of ERK signaling through molecular therapeutics or the use of transgenic mice has yet to be demonstrated in vivo. These experiments are needed for understanding disease pathogenesis, rational design of biomarkers, and developing potential therapeutic strategies for patients with NF1 vasculopathy.

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Chapter 24
Molecular Basis of Glomus Tumours

Hilde Brems, Eric Legius, and Douglas R. Stewart

24.1 Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder with a birth incidence of 1/3,000 and with an increased tumour predisposition (Huson et al. 1989). NF1-associated tumours arise secondarily to somatic mutations in the tumour suppressor *NF1*. Individuals with NF1 have an increased risk of developing benign and malignant tumours from both nervous as well as non-nervous system-derived tissues (Brems et al. 2009a). Glomus tumours of the fingers and toes arise secondarily to the biallelic inactivation of *NF1* and are part of the NF1 tumour spectrum (Brems et al. 2009b).

Glomus tumours are small (<5 mm) benign neoplasms that arise from the glomus body, a thermoregulatory shunt in fingers and toes. Glomus tumours most frequently occur in the extremities. The glomus body is an arterio-venous anastomosis involved in temperature regulation and is comprised of vascular structures, nerve cells and smooth muscle cells. The smooth muscle cells originate from the neural crest. Multipotent neural crest-derived stem cells can differentiate into neurons, Schwann cells and smooth muscle-like myofibroblasts positive for alpha-smooth muscle actin (αSMA) (Morrison et al. 1999). Heat-induced contraction of the smooth muscle layer results in closure of the shunt and forces the blood flow in the capillary network to cool down. When exposed to cold temperatures, relaxation of the muscle layer shunts away blood from the skin to prevent heat loss. Glomus tumours arising from the glomus body should be distinguished from adrenal and extra-adrenal paragangliomas, historically called “glomus tumours” (Strauchen 2002).

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Glomus tumours typically present with a classic triad of symptoms: severe pain, point tenderness and cold hypersensitivity. The only cure is surgical excision, and complete removal of digital glomus tumours is necessary to prevent recurrence (Lin et al. 2010). The diagnosis of glomus tumours is often delayed. One large series of sporadic glomus tumours of the fingers found that an average of 2.5 physicians (range: 0–7), including psychiatrists, were consulted before the correct diagnosis was made. The duration of symptoms averaged 10 years (range: 1–40 years) (Van Geertruyden et al. 1996).

Sporadic glomus tumours of fingers and toes typically are solitary and affect women of middle age (Rettig and Strickland 1977; Tsuneyoshi and Enjoji 1982; Van Geertruyden et al. 1996). There were no examples of multifocal glomus tumours in two large (86 cases) retrospective reviews of sporadic glomus tumours (Tsuneyoshi and Enjoji 1982; Van Geertruyden et al. 1996).

Klaber published the first report of a 13-year-old girl with features consistent with NF1 and glomus tumours of her right neck, bilateral lower extremities and left heel (Klaber 1938). In this chapter, we provide an update on NF1-associated glomus tumours located in the digits. To date, 24 individuals with NF1 and glomus tumours in fingers or toes have been published in the English-language literature (Table 24.1) (Oughterson and Tennant 1939; Park et al. 1994; Sawada et al. 1995; Okada et al. 1999; Kim 2000; De Smet et al. 2002; Brems et al. 2009b; Stewart et al. 2010, 2012; Leonard and Harrington 2010).

24.2 Diagnosis and Clinical Presentation

In individuals with NF1, a glomus tumour can be confused with a neurofibroma. In general, the diagnosis of a glomus tumour is based on clinical suspicion in the context of localised tenderness, cold sensitivity and severe paroxysmal pain. Surprisingly, patients may have lived with the pain so long that they need to be specifically asked about these symptoms. The pain is often continuous, and patients are protective of their fingers and may be reluctant to shake hands. The paroxysms of pain can be prompted by cold temperatures (reaching into the refrigerator) or minor vibrations (turning a doorknob). The paroxysm can be short-lived (minutes) but debilitating and frightening and may take the patients hours to recover. Love's test is useful in making the diagnosis; to perform it, a fine instrument (a pencil tip or a pin) is used to apply gentle pressure and elicit point tenderness in the affected region. The manoeuvre causes no pain in the area immediately adjacent to the pinpoint area (Love 1944). In a positive Love's test, the patient will experience severe pain at the site of the glomus tumour and will withdraw the hand (or foot). According to Bhaskaranand and Navadgi (2002), Love's test is 100 % sensitive and 78 % accurate. In addition, Hildreth's test and the cold sensitivity test can be used to facilitate the correct diagnosis. In Hildreth's test, a tourniquet is applied to the base of the affected digit; during a repeated (previously positive) Love's test, pain should be abolished. Hildreth's test is 71 % sensitive 100 % specific (Bhaskaranand and

Table 24.1 Summary of published NF1-associated glomus tumours in fingers and toes

Individual	Reference	Sex	Age	NIH NF1 diagnostic criteria	Clinical symptoms	# Years symptoms	# Affected digits	Affected digits	Confirmed glomus tumours	Removal	Reduction pain/tenderness	Recurrence	Germline *NF1* mutation	Somatic *NF1* mutation
1	Oughterson (1939) case 2	M	28	CALs	Tenderness + progressive pain	16	1	LF1	LF1	LF1	Yes	No	NA	NA
2	Park (1994) case 2	F	17	CALs, cut neur, freckling	Tenderness + pain	3	6	RF2, RF3, RF4, RF5, LF3, RT1	RF2, RF3, RF4, RF5, LF3, RT1	Biopsy	NM	NM	NA	NA
3	Sawada (1995) case 1	M	45	CALs, cut neur	Tenderness + pain	20	5	RF2, RF4, RF5, LF1, LF4	RF2, RF4, RF5, LF1, LF4	RF2, RF4, RF5, LF1, LF4	Yes	NM	NA	NA
4	Sawada (1995) case 2	F	28	CALs, cut neur	Tenderness	NM	5	RF3, RF4, RF5, LF2, LF5	LF5	LF5	Yes, partial reduction tenderness	NM	NA	NA
5	Sawada (1995) case 3	F	39	Family history NF1	Severe pain + tenderness	NM	1	RF3	No	No	Yes, treated with carbamazepine + stellate block	NM	NA	NA
6	Okada (1999) case 1	F	22	CALs	Pain	3	6	RF4, LF1, LF2, LF3, LF4, LF5	LF3	RF4, LF1, LF2, LF3, LF4, LF5	Yes	No	NA	NA
7	Kim (2000) case 1	F	34	CALs, cut neur	Severe pain + tenderness	NM	1	RT2	RT2	Biopsy	NM	NM	NA	NA
8	De Smet (2002) case 1 = Brems (2009b) NF1-G3 = Stewart (2010) Leu-3 = Stewart (2012)	F	53	> 6CALs, Lisch nodules, cut neur, freckling, 1st degree	Progressive and localised pain in 2 phalanges (LF3, RF4); split nail and progressive and localised pain (LF4)	1	2 + 1	2: LF3, RF4 2:LF4	LF3, RF4, LF4	Yes, 2 times: LF3, RF4 first after 6 years 2 tumours in LF4	Yes, 2 surgeries required	No	c.2546dupG	LF3 NA; RF4 NA; LF4 c.5545C > A, c.5539_5546dup8
9	De Smet (2002) case 2 = Brems (2009b) NF1-G2 = Stewart (2010) Leu-2	M	35	> 6CALs, Lisch nodules, cut neur, freckling, 1st degree	Progressive + localised pain, extreme pain	2–3	2	RF3, RF4	RF3, RF4	RF3, RF4	Yes	No	c.7395_7404del10 c.7395-2A > G	RF3 LOH intron 27–38; RF4 NA
10	Brems (2009b) NF1-G1 = Stewart (2010) Leu-1 = Stewart (2012)	F	42	+ (including neurofibromas)	Progressive + localised pain	1–2	2	RF4, RF5	RF4, RF5	RF4, RF5	Yes	No	Partial skip exon 29	RF4 c.403 del; RF5 NA
11	Brems (2009b) NF1-G4 = Stewart (2010) Leu-4	M	57	+ (including neurofibromas)	Progressive + localised pain, exacerbated by cold temperature. Mild distortion of the nail bed with increased curvature of nail	>5	1	RF4	RF4	RF4	Yes	No	c.2252-11 T > G c.2256A > G	ND
12	Brems (2009b) NF1-G5 = Stewart (2010) Leu-5	F	41	+ (including neurofibromas)	Progressive and localised pain in distal phalanx, worse in cold temperature	4	1	LF3	LF3 2 times	LF3 2 times 13 months interval at same location	Yes, 2 surgeries required	Yes	c.4515-2A > T	LF3 (1st) c.3113 + 1 G > C; LF3 (2nd) NA
13	Brems (2009b) NF1-G6 = Maertens (2007) SNF1-1 = Stewart (2010) Leu-6	F	46	Neurofibromas and only 3 CALs, not fulfilling criteria	Progressive + localised pain in distal phalanx, worse in winter, with few cutane-ousneurofibromas. Segmental NF1	1–2	1	RF3	RF3	RF3	Yes	No	c.2041C > T (<50 %) mosaic	ND

(continued)

Table 24.1 (continued)

Individual	Reference	Sex	Age	NIH NF1 diagnostic criteria	Clinical symptoms	# Years symptoms	# Affected digits	Affected digits	Confirmed glomus tumours	Removal	Reduction pain/tenderness	Recurrence	Germline *NF1* mutation	Somatic *NF1* mutation
14	Brems (2009b) NF1-G7 = Stewart (2010) Leu-7	F	11	+	Severe pain in distal phalanx + swelling of distal phalanx, mild shortening of LF5	2–3	1	RF5	RF5	RF5	Yes	No	c.2304dupT	ND
15	Brems (2009b) NF1-G8 = Stewart (2010) Hamburg-1 = Stewart (2012)	F	26	+ (including CALs, neurofibromas)	Unexplained pain, patient developed depression	5	1	LF4	LF4	LF4	Yes	No	c.311 T > G	c.7727C > A
16	Brems (2009b) NF1-G9 = Stewart (2010) Hamburg-2	F	57	+ (including CALs, neurofibromas)	Pain	1	1	RT1	RT1	RT1	Yes	No	c.1541_1542delAG	ND
17	Brems (2009b) NF1-G10 = Stewart (2010) NIH-1 = Soto (2012) NF1-G10	F	35	+ (including CALs, neurofibromas)	Severe, debilitating pain in both hands with complex regional pain syndrome	5	6	LF3, LF4, LF5, RF3, RF4, RF5	LF4(2 synchronous), LF5, RF3	Surgery 1 LF4, LF5, RF3; surgery 2 LF4, LF3, RF4, RF5; surgery 3 LF4, LF3, LF5	Yes, 3 surgeries required, also treated with ketamine for pain	Yes	c.6789_6792delTTAC	RF3 ND; LF5 c.204 + 1 G > A; LF4 c.7600_7621del22
18	Brems (2009b) NF1-G11 = Stewart (2010) NIH-2	M	50	+ (including CALs, neurofibromas)	Severe, progressive pain in left hand + right thumb with complex regional pain syndrome in left hand + arm	20 (left hand), 5 (right thumb)	6	LF2, LF3, LF4, RF1, RF4, RF5	LF2, LF4, RF1, RF4	Surgery 1 LF2, LF4, RF1; surgery 2 LF2, LF4, RF1, LF3; surgery 3 RF1, RF4, RF5	Required 3 surgeries to remove all glomus tumours but had persistent neuropathic pain. Died secondary to glioblastoma shortly after last surgery	Yes	c.7723_delG	LF2 NA; LF4 NA; RF1 NA; RF4 NA
19	Stewart (2010) NIH-3	F	28	+ (including CALs, neurofibromas)	Complex regional pain syndrome	4	3	LF3, LF4, RF4	LF3	LF3, LF4, RF4	Yes	No	NA	NA
20	Stewart (2010) NIH-4	F	49	+ (including CALs, neurofibromas)	Pain	>40	1	LF3	LF3	LF3	Yes, pain-free after 18 months follow-up	No, but short follow-up	NA	NA
21	Stewart (2010) NIH-5	F	35	+ (including CALs)	Pain	18	1	LF3	LF3	LF3	Yes, pain-free after 18 months follow-up	No, but short follow-up	NA	NA
22	Stewart (2010) Leu-8 = NF1-G12 Stewart (2012)	F	34	+ (including neurofibromas)	Pain	4	1	LF4	LF4	LF4	Yes	No but short follow-up	c.4368-1 G > C	Mitotic recombination 17q
23	Leonard (2010) case 1	M	11	>6 CALs, Lisch nodules, cut neur, freckling, 1st degree	Progressive pain + sensitive thumb	1.5	1	LF1	LF1	LF1	Yes	NM	NA	NA
24	Stewart (2012) NF1-G13	M	40	+	Severe progressive pain	8	1	RF5	RF5	RF5	Yes, but short follow-up	No, but short follow-up	c.3113 + 1 G > A	Mitotic recombination 17q

Legend: *M* male, *F* female, *CALs* café-au-lait spots, *cut neur* cutaneous neurofibromas, *L* left, *R* right, *F* finger, *T* toe, *NM* not mentioned, *ND* not detected, *NA* not analysed, *NIH NF1 diagnostic criteria* + fulfil these criteria

Navadgi 2002). The cold sensitivity test is performed by placing the affected hand (or foot) in cold water, precipitating severe pain in the digit(s) harbouring a glomus tumour. Remarkably, the cold sensitivity test has a reported sensitivity, specificity and accuracy of 100 % (Bhaskaranand and Navadgi 2002).

Table 24.1 summarises the clinical findings from NF1 individuals with glomus tumours in fingers or toes (Oughterson and Tennant 1939; Park et al. 1994; Sawada et al. 1995; Okada et al. 1999; Kim 2000; De Smet et al. 2002; Brems et al. 2009b; Stewart et al. 2010, 2012; Leonard and Harrington 2010). Twenty-four cases are reported with a female predominance (17/24, 71 %), similar to that observed in studies of sporadic digital glomus tumours (134/161 = 83 %) (Van Geertruyden et al. 1996; Lee et al. 2011). The average age of diagnosis among the individuals with NF1 is 36 years, comparable to that observed in sporadic patients (44 years and 42 years) (Van Geertruyden et al. 1996; Lee et al. 2011). Of note, two children with NF1 (individuals #14 and #23) were diagnosed with glomus tumours at the age of 11. Individual #13 was diagnosed with segmental NF1 and did not fulfil the NIH diagnostic criteria for NF1. Stewart et al. (2010) did not find a correlation between the café-au-lait macule and neurofibroma burden and the development of glomus tumours in fingers and toes.

Consistent with published experience on sporadic glomus tumours, most NF1 patients with glomus tumours fulfil two or more of the glomus triad criteria (localised tenderness, cold sensitivity and severe paroxysmal pain) (Stewart et al. 2010) and may suffer for many years (varying from 1 to more than 40 years) before the diagnosis is made (Van Geertruyden et al. 1996) (Table 24.1). The appearance of the finger or toe is often normal, although bluish discoloration of the nail is occasionally seen. Tumour nodules are infrequently palpable on examination. Since glomus tumours are under-recognised, cause considerable morbidity and unnecessary surgery (Sawada et al. 1995) and are likely more common than previously suspected, we have advocated for screening efforts to be established (Stewart et al. 2010). A simple question ("Do the tips of your fingers ever hurt, especially when cold or bumped?") should be asked as part of the routine care of adults and children with NF1.

Glomus tumours of the toes were detected in two individuals (individuals #2 and #16, Table 24.1) whereas glomus tumours of the fingers were noticed in 23/24 individuals with NF1. Similarly, in individuals without NF1, glomus tumours are more frequently located in fingers (106/110) than in toes (4/110) (Lee et al. 2011). Consistent with a tumour predisposition syndrome, multiple digits were affected in 42 % of the individuals with NF1 (10/24), but this was not observed in individuals with sporadic glomus tumours (Tsuneyoshi and Enjoji 1982; Van Geertruyden et al. 1996). In Table 24.1, there was an equal distribution of glomus tumours between the left and right hand, and the fourth and fifth digits were more commonly affected compared to the other digits.

Different imaging techniques have been used in the past to visualise the localisation of glomus tumours. Radiography, magnetic resonance imaging (MRI) and ultrasound have all been used with variable degrees of success depending on the size of the lesion. In classic uncomplicated presentations of glomus tumour

symptoms, imaging may not be needed. However, in more complicated scenarios (e.g. recurrence of symptoms, chronic pain), the predictive power of the physical exam declines and the usefulness of imaging, especially MRI, increases (Stewart et al. 2010). In particular, glomus tumours appear nodular and hyperintense on T2-weighted MRI imaging (Fig. 24.1); T1 post-contrast sequences often show mild to moderate enhancement of the tumours. Bony erosion from glomus tumours is occasionally observed on plain radiography (5/24 individuals; Table 24.1). Overall, radiography is less sensitive than MRI and detected glomus tumours in only 2/8 fingers of 4 patients, whereas 6 lesions were missed (Stewart et al. 2010).

24.3 Treatment, Complications and Prognosis

In the published literature, glomus tumours were surgically excised from 21/24 individuals with NF1 (Table 24.1). A biopsy (only) was performed in two (individuals #2 and #7). One patient (individual #5) did not undergo surgery but was treated with oral carbamazepine in combination with a stellate block. Reduction of pain or tenderness was obtained in 21 patients after surgery (surgeries) and/or after medical therapy (Table 24.1).

In our experience, surgical excision is the only treatment for glomus tumours (Fig. 24.2). Most patients experience dramatic improvement of pain and tenderness post-operatively. Depending on the location of the glomus tumour, different approaches are warranted. The direct transungual resection is appropriate for subungual glomus tumours. The lateral subperiosteal approach is for subungual glomus tumour or glomus tumours located in the pulp. The lateral incision may reduce the risk of nail deformity but has a higher risk of incomplete excision and thus recurrence (Vasisht et al. 2004). Glomus tumours are often surrounded by a pseudocapsule, facilitating excision. When apposed to the bone, bony curettage is needed to reduce the chance of recurrence.

Most glomus tumours are surgically excised without trouble and without recurrence. A retrospective study of 75 sporadic cases of digital glomus tumours showed that the tumour recurred in 17 % of the individuals (Lin et al. 2010). In our experience, glomus tumour recurrence can be early (weeks/months, presumably secondary to inadequate excision) or later (years, presumably secondary to the development of a new tumour). Recurrence of tumour is not noted in 6/24 individuals with NF1 (Table 24.1). Of the 18 individuals in which recurrence status is known, recurrence of tumour was described in a minority (3/18; 17 %) and no recurrence occurred in 15/18 (83 %). However, four of these 15 patients were only followed up for a short-term period.

A less common but potentially more devastating complication is the recurrence of pain, despite adequate surgical resection of tumour, secondary to the complex regional pain syndrome (CRPS). CRPS is a poorly understood, clinically heterogeneous chronic pain syndrome affecting one or more limbs. Formerly known as reflex sympathetic dystrophy (RSD) or causalgia, CRPS presents with allodynia,

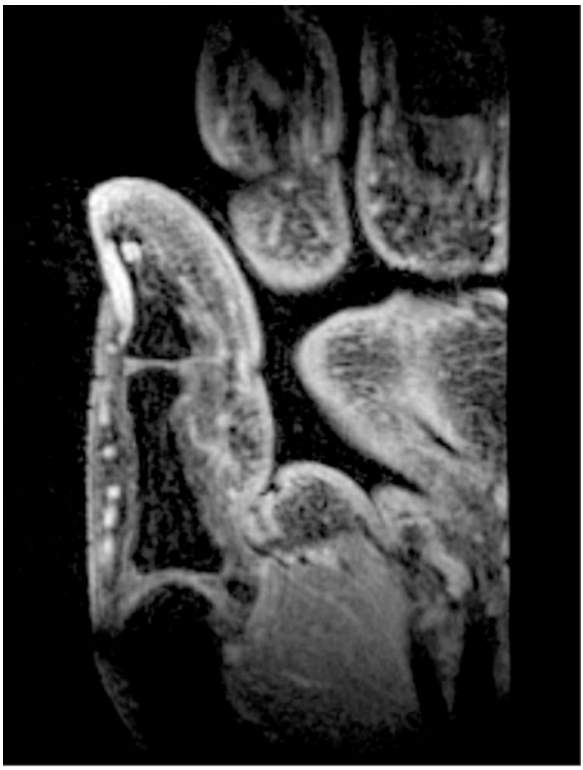

Fig. 24.1 Magnetic resonance imaging of individual #17 (Table 24.1), a 50-year-old man with neurofibromatosis type 1 and a 20-year history of severe, episodic pain in the distal phalanx of his left second and left fourth digits and a 5-year history of similar pain in the distal phalanx of his right thumb (shown). He had minimal use of his left hand and had retired due to disability; physical exam of both hands showed evidence of disuse atrophy and allodynia consistent with the complex regional pain syndrome. A coronal T1-weighted post-gadolinium MRI of the right thumb revealed a plainly visible ~5-mm subcortical, intraosseous lesion. On a plain radiograph (not shown), there was a 3–4-mm well-defined lucent lesion with a sclerotic margin at the location of the lesion, likely secondary to bony erosion. Subsequent surgery (Fig. 24.2) and pathologic review was consistent with a glomus tumour

hyperesthesia and/or dysesthesia and arises secondary to acute or chronic exposure from an inciting "pain generator". In the summarised series of 24 patients, three individuals with NF1 with glomus tumours (individuals #17, 18 and 19) developed CRPS (Table 24.1) (Stewart et al. 2010). Two of the three patients with CRPS required three surgeries to remove all glomus tumours but still had persistent neuropathic pain. One NF1 patient died secondary to a glioblastoma shortly after the last surgery. The chronic pain in individual #17 was resistant to a variety of medical therapies but finally responsive to oral ketamine (Soto et al. 2012).

The best treatment for CRPS is avoidance or elimination of pain generators; this is a challenge since glomus tumours are clinically under-recognised and patients can suffer for many years before proper diagnosis. Thus, we advocate prompt

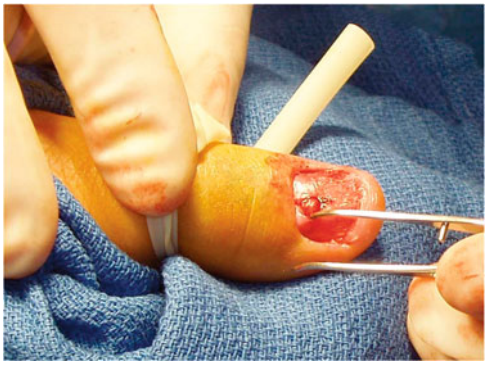

Fig. 24.2 Intraoperative photograph of right thumb of 50-year-old man described in Fig. 24.1. The nailbed has been removed to reveal a midline ~5-mm tumour next to right forceps tip. Pathologic examination of the lesion was consistent with a glomus tumour. Glomus tumours typically have a pseudocapsule, as in this case, which can make excision easier. Despite excision and bony curettage, the patient's tumour recurred, prompting a second surgery 8 months later. The photograph has been digitally altered to remove a patient identifier

resection of symptomatic glomus tumours. In patients with previous resection of glomus tumours and recurrent pain, MRI is useful in distinguishing tumour recurrence and the development of CRPS. Sporadic patients with long-standing symptomatic glomus tumours may or may not develop CRPS (Nebreda et al. 2000; Longdon et al. 2007; Cooke et al. 1995). Individuals with NF1 may be at increased risk of developing chronic pain syndromes such as CRPS since neurofibromin plays a key role in the excitability regulation of nociceptive sensory neurons (Hingtgen 2008).

24.4 Pathology

Table 24.1 lists 43 histologically confirmed glomus tumours. NF1-associated glomus tumours are pathologically indistinguishable from sporadic glomus tumours (Stewart et al. 2012). The diagnosis can typically be made with standard staining with haematoxylin and eosin (Fig. 24.3a, b). The tumour consists of branching vascular channels, surrounded by masses or sheets of specialised glomus cells. The glomus cell is cuboidal with a benign-appearing rounded nuclei (the term "glomus" is derived from the Latin word for "ball", in reference to the nuclei) and moderate-to-abundant eosinophilic cytoplasm. Immunostaining with an αSMA antibody typically shows positive cytoplasmic staining of glomus cells (Fig. 24.4). Only a minority of glomus tumours shows atypical or malignant features; mitosis, atypia or necrosis are typically absent (Folpe et al. 2001).

24.5 Molecular and Functional Characterisation

Germline *NF1* mutation analysis was performed in 13/24 individuals with NF1 and glomus tumours (Table 24.1). A pathogenic germline *NF1* mutation was found in DNA from white blood cells of 12/13 individuals. One patient had a segmental NF1 and a pathogenic *NF1* mutation observed in less than 50 % of the white blood cells (Table 24.1 individual 13). An identical *NF1* mutation was identified in Schwann cell cultures from two different neurofibromas from the same individual (Maertens et al. 2007). From all identified mutations, no *NF1* microdeletions were observed. The mutations are distributed over the whole gene and are typically truncating mutations. This is very similar to what is expected for a group of random NF1 individuals. There is no known genotype–phenotype correlation, although the number of reported germline mutations is small.

Since glomus tumours are typically small lesions, it is frequently a challenge to obtain sufficient quantities of DNA, RNA or protein to perform somatic *NF1* mutation detection and functional analysis. In addition, no animal model of NF1 has been reported to harbour glomus tumours. In the study that demonstrated biallelic inactivation of *NF1* in human glomus tumours, two different strategies were used (Brems et al. 2009b) to collect tumour DNA. First, surgically excised fresh glomus tumour tissue was collected, dissociated and glomus cells were cultured, expanded and DNA or RNA extracted. The glomus cells were αSMA-positive on immunostaining. Second, tumour cells from paraffin-embedded tissue were microdissected via laser capture microscopy (LCM); if needed, extracted DNA was then subject to whole genome amplification.

Combining these two techniques, 14 histologically confirmed glomus tumours were subject to somatic *NF1* mutation analysis (Table 24.1). An *NF1* somatic mutation was not detected in 5/14 tumours. Of the nine tumours with a somatic *NF1* mutation or loss of heterozygosity and a pathogenic *NF1* mutation, there were six with point mutations or small insertion or deletion events (tumours from individuals #8, 10, 12, 15, 17) [individual #17 harboured two glomus tumours in two fingers with two different somatic *NF1* mutations (Brems et al. 2009b)]. These mutations were predicted to result in complete inactivation of *NF1*, from the creation of a premature termination codon (4/14) or an alternative splice-site (2/14). One tumour (from individual #9) featured a loss of heterozygosity spanning introns 27 through 38, which was predicted to result in the loss of the *NF1* allele. Two tumours (from individuals #22 and 24) underwent mitotic recombination of chromosome arm 17q, resulting in duplication of the germline mutant *NF1* allele and hence biallelic inactivation of *NF1*. Mitotic recombination has been reported in many other NF1-associated tumours and, in this series, was observed in 22 % of glomus tumours (Stewart et al. 2012). In two NF1-associated glomus tumours, segregation analysis established biallelic inactivation of *NF1* by proving that the germline and somatic *NF1* mutations had arisen on two different chromosomes (Brems et al. 2009b). If NF1-associated glomus tumours arise from biallelic inactivation of *NF1*, then there should be evidence of monoclonal expansion from

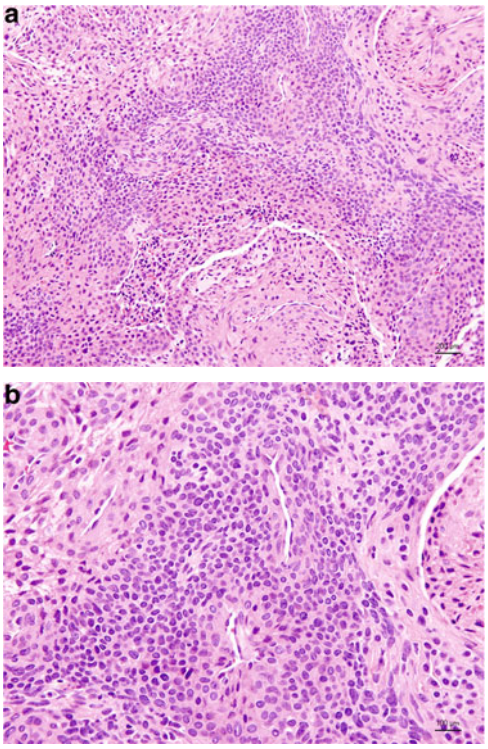

Fig. 24.3 Photomicrograph of glomus tumour stained with haematoxylin and eosin. Glomus tumours are composed of moderately-to-highly cellular populations of cuboidal cells arranged in sheets and aggregates and partially surrounding blood vessels. Lesional cells show rounded nuclei and inconspicuous nucleoli and contain moderate-to-abundant eosinophilic cytoplasm. There is minimal cytologic atypia; there is no necrosis or increased mitotic activity. There is also a variable amount of loose stromal tissue among the lesional cells. Original magnification 20× (**a**) and 40× (**b**). Scale bar = 200 μm (**a**) and 100 μm (**b**). Photomicrographs courtesy of C. Richard Lee, M.D., Ph.D. (Laboratory of Pathology, National Cancer Institute)

a single cell. Consistent with this prediction, in three different glomus tumours from three fingers of one female (individual #17), a single allele was detected using methylated- and unmethylated-specific primer pairs (Brems et al. 2009b). No somatic *NF1* mutation was identified in glomus cells or fibroblasts from two sporadic glomus tumours, suggesting a different pathogenic mechanism (Brems et al. 2009b).

Since complete *NF1* inactivation leads to loss of neurofibromin and overactivation of the RAS-MAPK pathway, NF1-associated glomus-tumour-derived glomus cells ($NF1^{-/-}$), NF1-associated glomus-tumour-derived fibroblasts ($NF1^{+/-}$), sporadic glomus-tumour-derived glomus cells ($NF1^{+/+}$) and control skin fibroblast ($NF1^{+/+}$) were studied for such hyperactivation (Brems et al. 2009b). The effect of acidic fibroblast growth factor on the cultured cells was monitored. Stronger and longer activation of MEK and ERK was found in the $NF1^{-/-}$ glomus

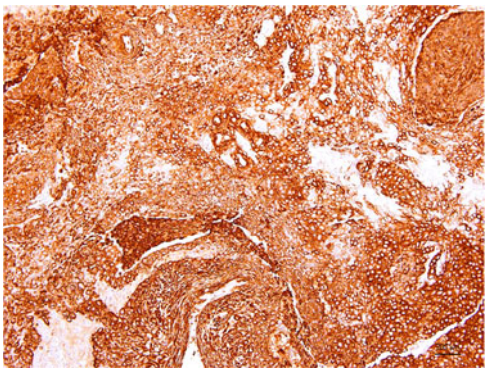

Fig. 24.4 Photomicrograph of glomus tumour stained for alpha-smooth muscle actin. Glomus tumours typically show uniformly positive αSMA cytoplasmic staining in the tumour cells. Original magnification 20×. Scale bar = 200 μm. Photomicrograph courtesy of C. Richard Lee, M.D., Ph.D. (Laboratory of Pathology, National Cancer Institute)

cells compared to the $NF1^{+/-}$ fibroblasts, $NF1^{+/+}$ glomus cells and $NF1^{+/+}$ fibroblasts, consistent with the loss of NF1 regulation on the RAS-MAPK pathway.

One study of the karyotype and copy number of five NF1-associated glomus tumours with proven biallelic inactivation of *NF1* found diploidy and few cytogenetic abnormalities in four of the tumours, consistent with their benign histology and natural history (Stewart et al. 2012). The fifth tumour (from individual #22) featured extreme polyploidy (near-tetraploidy, near-hexaploidy or near-septaploidy) across all chromosomes, but no rearrangements or abnormalities in morphology. That same tumour arose secondary to mitotic recombination of chromosome arm 17q; the authors hypothesise that mitotic recombination also "unmasked" (i.e. reduced to homozygosity) a hypomorphic germline allele in a gene on chromosome arm 17q associated with chromosomal instability, resulting in the extreme polyploidy.

To identify other genomic events underlying tumourigenesis, two studies used comparative genomic hybridisation (SNP array) to determine copy number in glomus tumours (Brems et al. 2009b; Stewart et al. 2012). Amplification of the 3′-end of *CRTAC1* and a deletion of the 5′ end of *WASF1* was identified in LCM-obtained DNA in two NF1-associated glomus tumours (from individual #17) and confirmed by quantitative PCR (Brems et al. 2009b). *WASF1* forms a bidirectional gene pair with the 5′ gene *CDC40*; the bidirectional promoter of *WASF1* and *CDC40* is located within the putative deletions in the two studied glomus tumours. Bidirectional promoters are associated with cancer-related genes, although their role in benign tumourigenesis is unknown. *WASF1* is downregulated in ovarian cancer, and the yeast orthologue of *CDC40* is a controller of cell cycle arrest; both genes are candidates for further investigation in glomus tumours. The second study, using cultured glomus cells, identified deletions in two genes (*WDR17* and *C16orf11*) and one microRNA (*MIR1267*) in at least three of six tumours studied using two different methods of analysis (Stewart et al. 2012).

24.6 Unanswered Questions and Future Directions

The recognition of glomus tumours as part of the NF1 tumour spectrum over 100 years after von Recklinghausen's description is a reminder of the role of astute clinical observation, even when faced with a familiar phenotype. It remains unknown why such small lesions can inflict such severe pain and why some patients develop CRPS and others do not. The reason for female predominance, true incidence and prevalence of glomus tumours in the NF1 population are also unknown. Although surgery is curative for many, glomus tumours impose substantial morbidity and undue suffering as patients wait for a proper diagnosis. Much of this can be avoided with prompt clinical recognition of the signs and symptoms of glomus tumours. As we anticipate future molecular characterisation of these unique tumours (e.g. tumour sequencing), we encourage caregivers to ask about fingertip pain as part of the routine care of adults and children with NF1.

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Chapter 25
Pheochromocytoma and NF1

Birke Bausch and Hartmut P.H. Neumann

25.1 Introduction

Neurofibromatosis type 1 (NF1), or von Recklinghausen disease, is a common autosomal dominant inherited disorder associated with pheochromocytoma, a rare neuroendocrine, catecholamine-producing tumor. Pheochromocytomas occur sporadically but are also a feature of numerous familial cancer syndromes. Neurofibromatosis type 1 is the oldest known classic inherited pheochromocytoma-associated syndrome, a group of disorders that also includes multiple endocrine neoplasia type 2 (MEN2), von Hippel–Lindau disease (VHL), the pheochromocytoma/paraganglioma syndromes (PGL1–4), and the familial pheochromocytoma syndromes. They are characterized by their typical clinical features and their molecular genetic basis. Pheochromocytomas associated with these syndromes differ in terms of their age at diagnosis, their tumor localization, and their malignant potential. Sustained or intermittent hypertension, which can be life-threatening, is the classic symptom of pheochromocytoma. Hypertension is also a frequent finding in NF1 patients and in half of these patients pheochromocytomas are causative. Neurofibromatosis type 1 is a rare cause of pheochromocytomas which are only found in a small number of NF1 patients. However, NF1 and pheochromocytomas have a long history and share some striking features.

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25.2 Historical Background

NF1 was the first described pheochromocytoma-associated syndrome. The first clinical description of a patient with neurofibromatosis and the first clinical description of a patient with pheochromocytoma were not only published in the same century but also in the same decade. Friedrich Daniel von Recklinghausen (1833–1910), a German pathologist, published a detailed description of neurofibromatosis in 1882 and lent his name to the disease (von Recklinghausen 1882). Four years later, in 1886, Felix Fränkel published an article about the clinical history of the 18-year-old Minna Roll with "bilateral adrenal sarcoma and angiosarcoma" (Fränkel 1886). This article has traditionally been considered to be the first description of a pheochromocytoma, but 121 years later, that case has been identified with current knowledge and technology to be a patient with MEN2 (Neumann et al. 2007). In 1910, Suzuki recorded the association of pheochromocytoma with neurofibromatosis type 1. He described a 60-year-old patient with a small chromaffin cell tumor of the right adrenal gland and cutaneous neurofibromas (Suzuki 1910). Since 1911, pheochromocytomas have been found to be a rare but persistent feature of neurofibromatosis type 1.

25.3 Epidemiology and Etiology

Neurofibromatosis type 1 is one of the most common autosomal dominant genetic disorders with an incidence ranging from 1:2,600 to 1:3,000 and complete penetrance (Lammert et al. 2005). Neoplastic proliferation of neural crest-derived cells is the predominant origin of tumor formation in NF1 and pheochromocytoma. In general, the term pheochromocytoma refers to endocrine-active, catecholamine-producing tumors of the adrenal gland and of the extra-adrenal, sympathetic paraganglia, whereas the term paraganglioma refers to endocrine-inactive tumors of parasympathetic paraganglia, mainly of the head and neck region (Neumann 2008). The incidence of pheochromocytomas is far lower than that of NF1 with approximately 2–8:1,000,000 (Beard et al. 1983). Between 25 % and 30 % of these tumors occur in the context of inherited cancer syndromes including NF1, multiple endocrine neoplasia type 2 (MEN2), von Hippel–Lindau disease (VHL), the pheochromocytoma/paraganglioma syndromes (PGL1–4), and the familial pheochromocytoma syndromes (Neumann et al. 2002; Karasek et al. 2010). In about 2–4 % of pheochromocytomas, NF1 constitutes the genetic basis of the disease (Bausch et al. 2006a, b; Mannelli et al. 2009). The estimated prevalence of pheochromocytomas in NF1 is 0.1–5.7 % (Walther et al. 1999).

25.4 Clinical Characteristics

Neurofibromatosis type 1 and pheochromocytoma are both characterized by a wide range of clinical signs and symptoms. NF1 is known for its extreme clinical variability among unrelated patients, among patients within the same family and

even within a single person at different times in life. Pheochromocytomas are referred to be "the great masquerader" owing to their variable clinical presentation. They are characterized by their excessive secretion of the catecholamines epinephrine, norepinephrine, and dopamine, resulting in about 24 % of patients in the classic triad of palpitation, headache, and sweating (Plouin et al. 1981; Mannelli et al. 1999). Severe catecholamine crises can be life-threatening due to the associated heart failure and arrhythmias. Even though the classic triad occurs in only 24 % of patients, almost all suffer from sustained or intermittent hypertension.

The hallmarks of NF1 are pigmentary abnormalities such as axillary or inguinal freckling, café au lait spots, neurofibromas or plexiform neurofibromas, optic gliomas, and Lisch nodules of the iris. About 50 % of the patients have learning disabilities and may develop skeletal abnormalities and vascular disease. Hypertension is a frequent finding and more common than in the general population (Lynch et al. 1972; Walther et al. 1999). In most cases, its origin is either unknown or caused by vasculopathies such as renal artery stenosis and coarctation of the aorta. Pheochromocytomas are a much less common cause with about 0.1–5.7 % in all NF1-affected patients but in 20–50 % of hypertensive NF1 patients (Lynch et al. 1972; Kalff et al. 1982; Walther et al. 1999).

25.5 Molecular Basis

Neurofibromatosis type 1 and the associated pheochromocytomas are caused by heterozygous, inactivating mutations of the *NF1* gene, a tumor suppressor gene, located on chromosome 17q11.2. Mutation analysis in patients with NF1 and pheochromocytoma has been performed rarely. The analysis is time consuming, complicated, and complex and remains a considerable challenge because of the large size of the gene and the low prevalence of pheochromocytomas in NF1 patients (0.1–5.7 %). The mutational spectrum in these patients has been found to be similar to that found in NF1 patients in general. More than 80 % of the mutations are either nonsense or frameshift mutations, likely to yield a truncated nonfunctional neurofibromin protein (Fig. 25.1). About 10 % are deletions or duplications which affect the length of one to >50 exons (Bausch et al. 2007) (Fig. 25.1). Whole gene deletions and large-scale rearrangements have not been described. Specific mutations associated with the development of pheochromocytomas have also not been identified.

In about 2–4 % of patients with pheochromocytomas, NF1 has been found to be the genetic background. So far, *NF1* mutation analysis is uncommon among patients with apparently sporadic pheochromocytomas. One study identified a pathogenic nucleotide substitution resulting in a missense mutation in a patient with benign, bilateral pheochromocytoma. The clinical reevaluation of the patient disclosed faint but characteristic features of neurofibromatosis type 1. Thus, the disease was not only identified by molecular genetic analysis but also by its typical clinical signs (Fig. 25.2) (Bausch et al. 2006a, b).

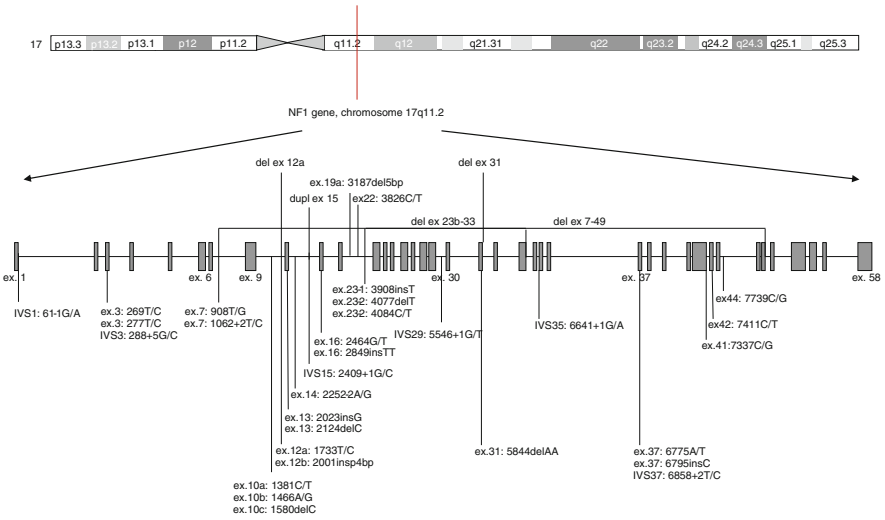

Fig. 25.1 Mutation spectrum in patients with NF1 and pheochromocytoma

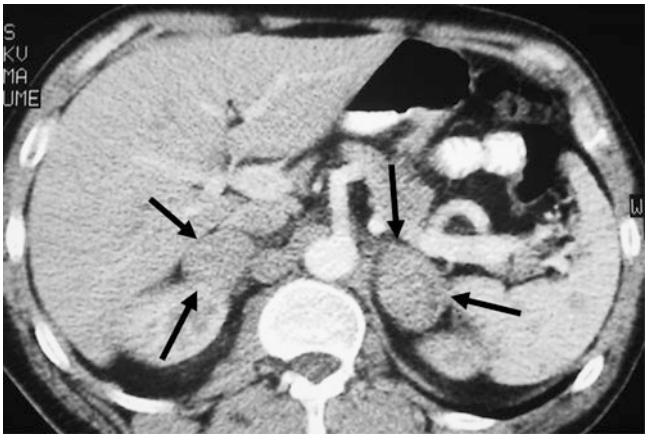

Fig. 25.2 CT scan of a bilateral pheochromocytoma

As the oldest known pheochromocytoma-associated syndrome, NF1 has to be seen in context with the different pheochromocytoma-associated syndromes, multiple endocrine neoplasia type 2 (MEN2), von Hippel–Lindau disease (VHL), the four pheochromocytoma/paraganglioma syndromes (PGL1, 2, 3, 4), and the familial pheochromocytoma syndromes, respectively. They are characterized by their clinical features and their molecular genetic basis. The *NF1* gene is only one of 10 tumor susceptibility genes associated with the pathogenesis of pheochromocytomas (Neumann et al. 2004; Benn and Robinson 2006; Mannelli et al. 2007; Bayley et al. 2010; Burnichon et al. 2010; Qin et al. 2010; Comino-Mendez et al. 2011)

(Table 25.1). Heterozygous, inactivating mutations of the *VHL* gene and of the genes encoding the SDH (succinate dehydrogenase) complex subunits (*SDHA*, *SDHB*, *SDHC*, *SDHD,* and *SDHAF2*) of the *MAX* gene and the *TMEM127* gene cause von Hippel–Lindau disease, the paraganglioma syndromes type 1, 2, 3, and 4, and the familial pheochromocytoma syndromes (Table 25.1). Activating, gain-of-function mutations in the *RET* proto-oncogene are responsible for MEN types 2A and 2B (Table 25.1). Their common pattern of inheritance is autosomal dominant with the exception of paraganglioma syndromes type 1 and 2 and the *MAX* gene associated familial pheochromocytoma syndrome which are autosomal dominant inherited disorders with maternal genomic imprinting, also known as a parent-of-origin effect. Tumors only develop if an individual inherits the mutation from the father (Hensen et al. 2004; Bayley et al. 2010; Comino-Mendez et al. 2011). In contrast to NF1, the molecular basis of these syndromes are genes that are smaller in size than the *NF1* gene and a clear genotype–phenotype correlation has been identified. Owing to the clear genotype–phenotype correlations in VHL, MEN2, and the paraganglioma syndromes and the consequent possibility of clinically significant risk stratification, mutation analysis is recommended and performed if one of these syndromes is suspected. The main phenotype of the familial pheochromocytoma syndromes caused by *TMEM127*, *MAX,* and *SDHA* gene mutations has, yet to be clearly defined, and genetic screening is therefore neither available nor recommended. The diagnosis of NF1 in current clinical and scientific work is still based on clinical criteria, although mutation analysis is possible. Molecular genetic analysis is usually not required for diagnosis and is complex and time-consuming owing to the large size of the gene and the lack of a distinct genotype–phenotype correlation.

25.6 Tumorigenesis

Mutations of *NF1*, a tumor suppressor gene, result predominantly in a loss of function of the protein product neurofibromin. Notably, pheochromocytoma tumorigenesis follows Knudson's "two-hit theory" put forward in 1971: loss of heterozygosity of the *NF1* gene due to a second somatic hit together with the loss of the remaining wild-type allele has been identified in pheochromocytomas (Knudson 1971; Bausch et al. 2007). Thus, the lack of neurofibromin expression seems causative for the development of these tumors. Neurofibromin is part of a family of GTPase-activating proteins (GAPs) that downregulate the cellular proto-oncogene p21–ras. p21–ras is important for cell growth and cell regulation via the activation of numerous different pathways such as stem cell factor (SCF)/c-kit signaling, mTOR, and MAP kinase pathways (Martin et al. 1990; Cichowski et al. 1999; Weiss et al. 1999). The loss of neurofibromin leads to the activation of p21–ras, which in turn stimulates cell proliferation. New insights into the tumorigenesis of hereditary pheochromocytomas were achieved via genome-wide expression studies. Based on their transcriptomes, hereditary pheochromocytomas/paragangliomas comprise two major subgroups (Burnichon et al. 2011). Tumors

Table 25.1 Molecular basis of hereditary pheochromocytoma-associated syndromes

	NF1	VHL	MEN2	PGL1	PGL2	PGL3	PGL4	Familial pheochromocytoma syndromes		
Mode of inheritance	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal dominant + maternal imprinting	Autosomal dominant + maternal imprinting	Autosomal dominant	Autosomal dominant	Autosomal dominant		Autosomal dominant + maternal imprinting
Gene type	Tumor suppressor gene	Tumor suppressor gene	Proto-oncogene	Tumor suppressor gene	Tumor suppressor gene	Tumor suppressor gene	Tumor suppressor gene	Tumor suppressor gene		
Gene	*NF1*	*VHL*	*RET*	*SDHD*	*SDHAF2*	*SDHC*	*SDHB*	*TMEM127*	*SDHA*	*MAX*
Chromosome location	17q11.2	3p25.5	10q11.2	11q23	11q13.1	1q21	1p35-p36	2q11.2	5p15.33	14q23
Exons	60	3	21	4	4	6	8	4	15	5
Mutation type	Loss of function	Loss of function	Gain of function	Loss of function	Loss of function	Loss of function	Loss of function	Loss of function		

caused by *VHL* and *SDHx* mutations belong to group one or cluster one and are characterized by a hypoxic transcriptional signature indicating reduced oxidoreductase activity and increased hypoxia and angiogenesis. Tumors caused by mutations of the *RET*, *NF1,* and *TMEM127* genes belong to group two or cluster two and are characterized by the activation of p21–ras-mediated MAP kinase pathway leading to uncontrolled cell proliferation.

25.7 Clinical Characteristics of Pheochromocytoma in NF1

Pheochromocytomas occur sporadically or are part of an increasing number of familial cancer syndromes. Sporadic pheochromocytomas are mainly diagnosed between the age of 40 and 50 years and are characterized by the "rule of tens": 10 % are bilateral, adrenal tumors; 10 % are extra-adrenal tumors of thoracoabdominal, sympathetic paraganglia; and 10 % are malignant. The differentiation of benign and malignant pheochromocytomas is difficult owing to their identical histological and biochemical signs. The only reliable proofs of malignant pheochromocytomas are lymph node metastases and distant metastases (Tischler 2008).

Between 25 % and 30 % of pheochromocytomas are familial and associated with one of the classic pheochromocytoma syndromes such as NF1, VHL, MEN2, the paraganglioma syndromes, and the familial pheochromocytoma syndromes (Table 25.2). The prevalence is about 0.1–5.7 % in NF1, 10 % to 20 % in VHL, and 50 % in MEN2 (Walther et al. 1999; Dluhy 2002). As with NF1, these hereditary disorders present some striking additional clinical features (Table 25.2). Patients with von Hippel–Lindau disease develop retinal and cerebellar hemangioblastomas, clear cell renal carcinomas, and pancreatic islet cell tumors. MEN type 2A is associated with medullary thyroid carcinoma (MTC) and hyperparathyroidism; MEN type 2B is, in addition to MTC, associated with multiple mucosal neuromas and a Marfanoid constitution. Typical clinical features of paraganglioma syndromes type 1–4 are parasympathetic head and neck paragangliomas.

Paragangliomas, referred to as endocrine-inactive tumors of parasympathetic paraganglia mainly of the head and neck region, are not only associated with PGL syndromes type 1–4 but have a close connection to pheochromocytomas in general. Both tumors arise from neural crest-derived cells and hence share the same histopathologic origin. Nowadays they are usually regarded in the context of pheochromocytomas. Further, they are a striking clinical feature of pheochromocytoma-associated syndromes with a characteristic prevalence depending upon the underlying familial pheochromocytoma syndrome. The vast majority of head and neck paragangliomas are caused by mutations of the *SDHx* genes and hence are associated with paraganglioma syndromes type 1–4. The prevalence of these tumors among VHL patients is about 8 in 1,000. Only single cases were identified among MEN type 2 and NF1 patients (DeAngelis et al. 1987; Boedeker et al. 2009). In 1987, DeAngelis described a NF1 patient with pulmonary paragangliomas, a glomus jugulare tumor, and a pheochromocytoma.

Table 25.2 Clinical characteristics of hereditary pheochromocytoma-associated syndromes

	Sporadic	NF1	VHL	MEN2	PGL1	PGL2	PGL3	PGL4	Familial pheochromocytoma syndromes		
Gene	–	*NF1*	*VHL*	*RET*	*SDHD*	*SDHAF2*	*SDHC*	*SDHB*	*TMEM127*	*SDHA*	*MAX*
Prevalence of pheochromocytoma	100 %	0.1–5.7 %	10–20 %	50 %	34 %	0 %	Single case	37 %	100 %	Single case	100 %
Mean age at diagnosis of pheochromocytoma	40–50	43	16	34	26	23	41	34	43	–	–
Adrenal location	90 %	84–95 %	92 %	97 %	86 %	–	–	42 %	100 %	Single case	100 %
Bilateral location	10 %	5–15 %	55 %	65 %	48 %	–	–	12 %	35 %	–	67 %
Extra-adrenal location	10 %	6 %	17 %	3 %	57 %	–	Single case	58 %	–	Single case	–
Malignant pheochromocytoma	10 %	3–12 %	4 %	3 %	0 %	–	0 %	24 %	5 %	Single case	37 %
Head and neck paraganglioma	–	Single case	Rare	Single case	48 %	100 %	>90 %	6 %	Single case	Single case	–
Additional clinical features	–	Cafe au lait spots, axillary or inguinal freckling, neurofibromas, plexiform neurofibromas, optic gliomas, Lisch nodules	Hemangioblastomas of the eye and CNS, clear cell renal carcinomas, pancreatic islet cell tumors, endolymphatic sac tumors	Medullary thyroid carcinomas, type A: hyperparathyroidism, type B: multiple neuromas, marfanoid habitus	Gastrointestinal stromal tumors		Gastrointestinal stromal tumors	Renal cell carcinomas, Gastrointestinal stromal tumors			

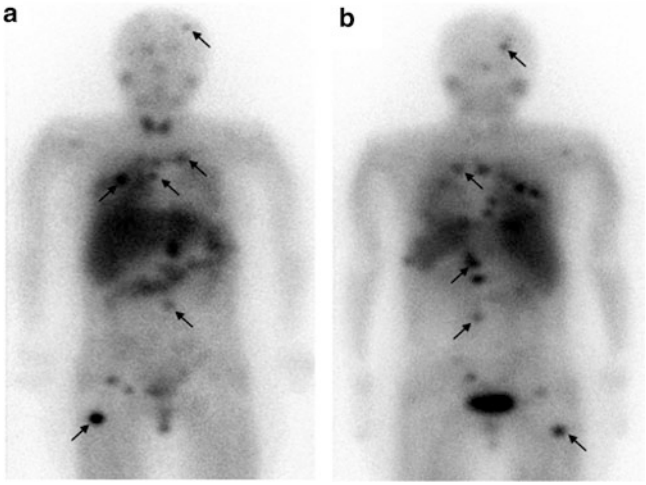

Fig. 25.3 MIBG scintigraphy of a malignant pheochromocytoma

Hereditary pheochromocytomas differ in their age at diagnosis, their tumor localization, and their malignant potential (Neumann et al. 2002; Bausch et al. 2006a, b) (Table 25.2). Compared to sporadic pheochromocytoma, the age at onset is lower, a bilateral and extra-adrenal tumor localization is more common, and a higher malignancy rate is evident. The age at onset is about 15 years younger than that in sporadic tumors. The youngest age at onset (16 years) is found in patients with von Hippel–Lindau disease. *MAX* mutation-associated pheochromocytomas are characterized by bilateral tumor development, identified in at least 67 % of patients. Familial pheochromocytomas are evident in more than 10 % located extra-adrenally with the highest rate of 58 % in patients affected by paraganglioma syndrome type 4 and the lowest rate of 3 % in patients affected by MEN type 2. The frequency of malignant transformation is high. *MAX* mutation-associated tumors and PGL 4-associated tumors show the highest malignancy potential with 37 % and 24 %, respectively. In contrast to these findings, NF1-associated pheochromocytomas share many features with sporadic pheochromocytomas (Walther et al. 1999; Bausch et al. 2006a, b). The mean age at diagnosis is relatively late with 43 years. 84 % to 95 % of them are localized in the adrenal gland with bilateral tumor growth in 5–15 % of patients. Extra-adrenal tumors have been found in up to 6 %; a malignant transformation has been identified in 3–12 % (Fig. 25.3), one of the highest rates among hereditary pheochromocytomas, but similar to those described in sporadic tumors.

25.8 Diagnostic Approach

The diagnosis of NF1 is based on clinical criteria developed by the NIH Consensus Conference in 1987 and updated in 1997 (Anonymous 1988; Gutmann et al. 1997). According to these criteria, at least two of the following clinical features must be

present in the absence of another diagnosis to make the diagnosis of NF1: (1) six or more café au lait spots >5 mm in diameter in prepubertal and >15 mm in diameter in postpubertal individuals, (2) two or more neurofibromas of any type or one plexiform neurofibroma, (3) axillary or inguinal freckling, and (4) optic glioma and two or more Lisch nodules. The diagnosis of pheochromocytoma is based on biochemical tests and imaging modalities. Pheochromocytomas are characterized by the excretion of the catecholamines, norepinephrine, epinephrine, and dopamine, and the predominant intratumoral metabolism of these catecholamines with the formation of metanephrine and normetanephrine. The measurement of 24-h urinary fractionated catecholamines and total metanephrines with a sensitivity of 90 % and the measurement of plasma-fractionated metanephrines with a sensitivity of 97 % are recommended as the first diagnostic step in pheochromocytomas. Biochemical testing should be followed by radiological evaluation. CT scan (computed tomography) and MRI (magnetic resonance imaging) are both sensitive (98–100 %) and quite specific (70 %). MIBG scintigraphy (metaiodobenzylguanidine scintigraphy) and 18-Fluor-DOPA PET (18-Fluor-DOPA positron emission tomography) are recommended in patients with strong clinical and biochemical evidence of pheochromocytoma but negative MRI and CT scan results and in patients with atypical tumor localization, multiple tumors, and malignant pheochromocytomas.

In 20–50 % of hypertensive NF1 patients, pheochromocytomas are causative for elevated blood pressure levels, which can be life-threatening with the possibility of a curative therapy. Owing to the clinical characteristics of pheochromocytomas in NF1, NF1 patients with hypertension and a mean age of 43 years should be screened for pheochromocytomas. The measurement of 24-h urinary fractionated catecholamines and total metanephrines and the measurement of plasma-fractionated metanephrines should be performed, followed by a CT scan or MRI. In the vast majority, the tumor is localized in the adrenal gland with a suspected malignancy rate of up to 12 %. A molecular genetic analysis of the *NF1* gene is possible but usually not required because of a lack of a relevant genotype–-phenotype correlation and high variability of the disease even within one family.

NF1 is associated with pheochromocytomas in only about 2–4 % of cases. The diagnosis of NF1 in both current clinical and scientific work is based on clinical findings, and patients with pheochromocytomas should be carefully examined for the striking and diagnostic features of the disease.

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Chapter 26
Molecular and Cellular Basis of Human Cutaneous Neurofibromas and Their Development

Juha Peltonen, Eeva-Mari Jouhilahti, and Sirkku Peltonen

26.1 Introduction

Multiple cutaneous neurofibromas are one of the two classical characteristics of neurofibromatosis type-1, being also the basis of the name for the syndrome. Even though cutaneous neurofibromas do not pose any threat to life, they create the main disease burden in most adult patients with NF1. Cutaneous neurofibromas are benign tumors which never undergo malignant transformation unlike the plexiform neurofibromas which may turn malignant. Cutaneous neurofibromas most commonly begin to grow during puberty, and they increase in number throughout life so that eventually the number of these tumors may reach into the thousands (Fig. 26.1). Neurofibromas are most common in the trunk but can be found in all skin areas. Cutaneous neurofibromas have however a limited growth potential, ensuring that the final size of tumors seldom exceeds 2–3 cm. The tumors may be sessile, pedunculated, or intradermal, with purple color and buttonhole sign when pressed. They may be itchy or painful, but most of them are symptomless.

Cutaneous neurofibromas are mixed cell tumors composed of Schwann cells, fibroblasts, perineurial cells, mast cells, and axonal processes. The cells are embedded in an abundant collagenous extracellular matrix which gives neurofibromas a characteristic rubbery consistency. A subpopulation of Schwann cells cultured from human neurofibromas carries a second hit in the *NF1* gene. The traditional view sees neurofibroma development as a process of disruption of small nerve tributaries of the skin and subsequent proliferation of the resident cells. Recent studies utilizing gene-manipulated mice and cells cultured from human neurofibromas have revealed evidence suggesting that multipotent precursor cells may play a

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Fig. 26.1 An example of a relatively high tumor burden of cutaneous neurofibromas

crucial role in tumor initiation and in the development of neurofibromas with their astonishingly numerous cellular phenotypes.

26.2 Molecular and Cellular Composition of Neurofibromas

Molecular analysis of neurofibromas can be used to explain the physical characteristics of these tumors and to identify cellular phenotypes according to their specific gene expression profiles. In fact, identification of individual cells in neurofibroma tissue is difficult, if not possible, without molecular markers. It is worth noting that neurofibromin, the protein product of the *NF1* gene, has little value as a biomarker for the following reasons: It is ubiquitously expressed and not cell type specific. Furthermore, in vitro studies have shown that *NF1* expression is under strict temporal control and negative cells may turn into positive ones and vice versa within a short space of time (Cichowski et al. 2003; Kaufmann et al. 1999; Pummi et al. 2000).

26.2.1 Extracellular Matrix of Cutaneous Neurofibromas

Neurofibromas contain an abundant extracellular matrix with 30–45 % collagen of the lipid-free dry weight (Peltonen et al. 1986). That is about half of the corresponding value of skin, but twice that described for human endoneurium.

The classical fibrillar collagen types I and III are the most common in neurofibromas, and the relative amount of type III collagen is high. This is analogous to findings on developing or healing connective tissues.

Collagen fibrils in neurofibromas form loosely oriented networks and do not organize into prominent bundles. This is in contrast to, for example, the overlying skin which harbors thick bundles of collagen observed in routine histological evaluation by light microscopy. Electron microscopy reveals that the diameter of collagen fibrils is typically 30–40 nm in neurofibromas (Lassmann et al. 1976) which is less than in the human skin (60 nm), or the epineurium (80–100 nm), but comparable to that of the endoneurium.

Type VI collagen protein and mRNAs are also readily detectable in neurofibromas (Peltonen et al. 1990). Type VI collagen appears as thin-beaded filaments, and characteristically in neurofibromas, collagen VI can organize into so-called Luse bodies with typical zebra-like appearance observed under electron microscopy. Fibroblasts, Schwann cells, and perineurial cells all have the potential to express collagens I, III, and VI (Jaakkola et al. 1989a, b). Fibroblasts and perineurial cells are likely sources of fibronectin in neurofibromas. The basement membrane-specific type IV collagen is readily detectable in neurofibromas since most cells of the tumor are covered by basement membranes. Here, type IV collagen appears more like a cellular biomarker rather than a component of the extracellular matrix. For instance, Schwann cells and the perineurial cells are ensheathed by basement membranes and can therefore be detected by immunolabeling for type IV collagen, selected laminins, and nidogen.

The proteoglycan/collagen ratio is 4–10 times higher in the neurofibromas than in the surrounding dermal tissue (Peltonen et al. 1986). This in part explains the typical soft consistency of the neurofibromas and may contribute to a favorable milieu for tumor growth.

Most of the volume of a neurofibroma is composed of the extracellular matrix. Thus, downregulation of relevant genes might serve the purpose of limiting the tumor size.

26.2.2 Cellular Differentiation in Cutaneous Neurofibromas

The development of neurofibromas results in a tumor composed of cells with the phenotypic characteristics of Schwann cells, perineurial cells, fibroblasts, and mast cells (Fig. 26.2), which is analogous to the connective tissue of peripheral nerves (Peltonen et al. 1988). Scattered macrophages and lymphocytes are not uncommon. In addition, appendages of skin, such as elements of hair follicles and glandular structures, are typical findings.

26.2.2.1 Schwann Cells

Based on light and electron microscopic examination, many of the neurofibroma cells display morphology consistent with a Schwann cell phenotype. At the ultrastructural level, neoplastic Schwann cells have complex, branched cytoplasmic

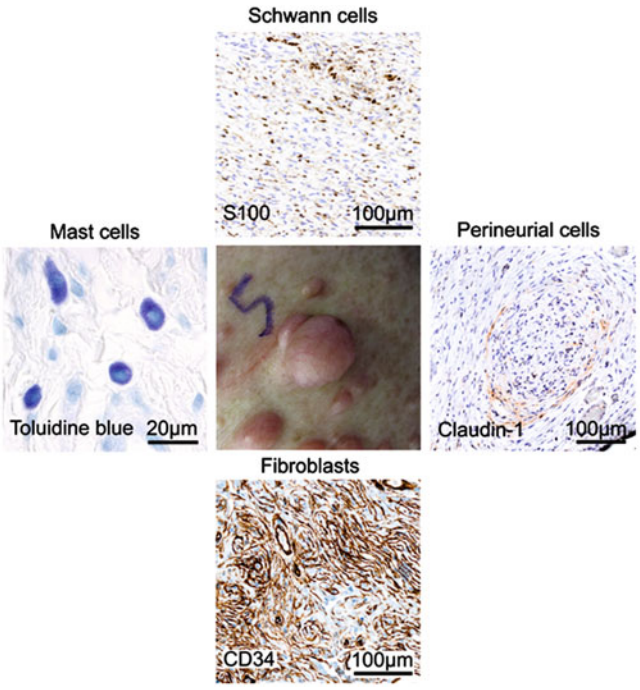

Fig. 26.2 Staining for S100, Claudin-1, and CD34, and toluidine blue visualizes Schwann cells, perineurial cells, fibroblasts, and mast cells in cutaneous neurofibroma

processes which are covered by a continuous basement membrane (Hirose et al. 1986; Lassmann et al. 1976). Tumor Schwann cells express S100beta, and as estimated by immunolabeling for the S100 protein, Schwann cells account for 60–80 % of the cells in neurofibromas (Peltonen et al. 1988). However, there is marked variation in the intensity of S100 labeling signal within the tumor, and neoplastic Schwann cells express S100 at a lower level compared to nerve-associated Schwann cells (Peltonen et al. 1988). Schwann cells display also a positive immunosignal for basement membrane components, such as type IV collagen and laminin chains. It is worth noting that the number of type IV collagen-positive cells in neurofibromas easily exceeds the number of S100-positive cells. This finding has been interpreted as evidence that the perineurial cells can, at least in part, account for the S100-negative, collagen IV-positive cells. In contrast to normal Schwann cells, neurofibroma Schwann cells express nerve growth factor (NGF) receptors (Ross et al. 1986).

A crucial in vitro finding has shown that a subpopulation of cells cultured from cutaneous neurofibromas carries double inactivation of the *NF1* gene (Serra et al. 2000). These cells are positive for S100beta and have a morphology consistent with that of Schwann cells.

26.2.2.2 Perineurial Cells

In normal nerve, perineurial cell layers form a tight sheath around groups of axon-Schwann cell units thereby delineating nerve fascicles. At the ultrastructural level, perineurial cells are covered by a continuous basement membrane, and have long slender cell processes and numerous intracellular vesicles. Solitary perineurial cells in neurofibromas may have a discontinuous basement membrane, and the pinocytotic vesicles may be less numerous as compared to normal perineurial cells (Erlandson and Woodruff 1982; Lassmann et al. 1976). The presence of basement membranes on perineurial cells makes a clear distinction between perineurial cells and fibroblasts. The latter, by definition, do not have a basement membrane. The search and identification of perineurial cells in neurofibromas has been difficult due to the lack of a single characteristic biomarker. A panel of molecular markers such as glucose transporter 1 (Glut-1), epithelial membrane antigen (EMA), and type IV collagen has been used to detect perineurial cells in neurofibromas (Hirose et al. 2003; Peltonen et al. 1988; Perentes et al. 1987). A promising marker for perineurial cells is claudin-1, a tight junction protein (Pummi et al. 2006). The expression of tight junction proteins by perineurial cells is in line with previous ultrastructural observations showing that overlapping processes of adjacent perineurial cells are occasionally joined by tight junctions (Erlandson 1985).

Studies with combinations of antigenic markers suggest that perineurial cells exist in all neurofibromas while their number varies between individuals and tumor areas (Peltonen et al. 1988; Pummi et al. 2006). All neurofibromas show localized areas with perineurial cell differentiation. About 10 % of neurofibromas show a major subpopulation of perineurial cells, as estimated by lack of immunoreaction for S100beta protein and positive reactions to EMA and claudin-1 (Peltonen et al. 1988; Pummi et al. 2006). The role of perineurial cells in the development of neurofibromas has traditionally been considered as permissive, since the neoplastic Schwann cells have been assumed to penetrate an afflicted perineurial barrier and proliferate outside of disrupted nerve fascicles in neurofibromas.

26.2.2.3 Fibroblasts

Endoneurial fibroblasts have been considered to be the origin of neurofibroma fibroblasts. They apparently contribute to the synthesis and accumulation of fibrillar collagens and fibronectin in the tumor. Fibroblasts have abundant cytoplasm with numerous cell organelles, but lack the basement membrane. Immunohistochemically, fibroblasts can be detected by the expression of CD34 (Hirose et al. 2003; Lassmann et al. 1976).

26.2.2.4 Mast Cells

Mast cell precursors leave the bone marrow and migrate into most tissues where they mature. Mast cells are involved in numerous immune, autoimmune, and allergic mechanisms. They contribute to these processes by carrying a variety of bioactive mediators, and cytokines that activated mast cells are able to selectively secrete from their granules. Mast cells are abundant in normal skin and are found in close proximity to hair follicles. They are also known to have a functional association with sensory nerves in the skin and can also be seen within nerve trunks. Mast cells are also numerous in neurofibromas and other peripheral nerve sheath tumors. Mast cells have a specific Kit ligand-mediated mechanism for migration to neurofibromas (Yang et al. 2003). Mast cells can be recognized in light microscopy by their typical cytoplasmic granules and can be visualized by Alcian blue staining at pH 1, toluidine blue staining, or by immunodetection of factor VIII-related antigen (Peltonen et al. 1988). The molecular mechanisms explaining how *NF1* haploinsufficient mast cells may contribute in neurofibroma growth are discussed below.

26.3 The Development of Cutaneous Neurofibromas

Our current understanding is that Schwann cells have a key role in neurofibroma development. This is based on studies with conditional knockout mice and the genetic analysis of cells cultured from human neurofibromas (Maertens et al. 2006; Serra et al. 2000; Zhu et al. 2002). These studies have together shown that the bi-allelic inactivation of the *NF1* gene is a prerequisite for neurofibroma formation, but that tumorigenesis can only occur on an $NF1^{+/-}$ background.

26.3.1 Neurofibroma Cells In Vitro as Keys to Pathoetiology of Neurofibromas

NF1-related cell cultures initiated from neurofibromas and apparently healthy tissues of the patients have been a target of interest for decades but with a limited number of conclusive results. Standard culture procedures using DMEM supplemented with FCS result in an enrichment of fibroblast cell growth. The results have suggested that *NF1*-deficiency can result in the excessive production of fibrillar collagens type I and III by the fibroblasts (Peltonen et al. 1986). The early passages of these cultures have been shown to harbor cells with characteristics of Schwann cells, fibroblasts, and perineurial cells (Jaakkola et al. 1989a). Regardless of the abundant evidence that the tumor suppressor model (with double inactivation in the *NF1* gene) is relevant to neurofibroma development, the mixed cellular composition has hampered the definition of a certain cell type with the *NF1* second hit.

A method designed to enrich two distinct populations of neurofibroma-derived Schwann cells has yielded the key finding that a subpopulation of neurofibroma-derived cells with characteristics consistent with Schwann cell identity carries a second hit in the *NF1* gene, resulting in an $NF1^{-/-}$ genotype (Serra et al. 2000). A second hit on the *NF1* gene has been detected in a subpopulation of tumor Schwann cells, but not in other cell types cultured from human neurofibromas (Maertens et al. 2006; Serra et al. 2000). Further results have revealed that the second hit in the *NF1* gene is unique in individual cutaneous neurofibromas (Maertens et al. 2006). It should also be noted that only one second hit is found in each tumor indicating that the Schwann cell population with *NF1* double hit is clonal (Maertens et al. 2006).

Another line of research on neurofibroma-derived cells has revealed that neurofibromas harbor multipotent cells (neurofibroma-derived precursor cells; NFPs) with an $NF1^{+/-}$ genotype. The NFPs have a high proliferation rate and the potential of differentiation into, for example, Schwann cells, neurons, epithelial cells, and fat (Jouhilahti et al. 2011).

Based on the observations of Schwann cell cultures derived from different cutaneous neurofibromas, it has been suggested that the relative proportions of $NF1^{-/-}$ and $NF1^{+/-}$ Schwann cells within the tumor vary between separate neurofibromas (Serra et al. 2000). The $NF1^{-/-}$ genotype obviously provides these cells with a growth advantage, but it is not known what proportion of the Schwann cells within a cutaneous neurofibroma carry an $NF1^{-/-}$ genotype and how much of the tumor growth can be explained by the increase in the number of other cells with an $NF1^{+/-}$ genotype.

26.3.2 Targeting Neurofibroma Development in Mice

Tumorigenesis has also been investigated in several Nf1 mouse models. The neurofibromas developed in $Nf1^{flox/flox}$ mice with Cre placed downstream of the promoter element for *Krox20*, a peripheral nerve myelination-related gene expressed in 5–10 % of Schwann cells and Schwann cell precursors (Staser et al. 2012; Zhu et al. 2002). In this mouse model, the tumorigenesis occurred only on an *Nf1* heterozygous background. The mouse studies indicate the importance of the tumor microenvironment in the development of neurofibromas. More specifically, further experiments with this mouse model demonstrated that plexiform neurofibroma formation requires an *Nf1* haploinsufficient and c-kit-competent hematopoietic system, including mast cells (Yang et al. 2008). It is, however, worth noting that the majority of mouse studies have focused on plexiform neurofibromas and that the findings on mice may be only partially applicable to human neurofibromatosis type-1 in general and to human cutaneous neurofibromas in particular.

26.3.3 Alternative Explanations for Neurofibroma Development

Previous knowledge has suggested that cutaneous neurofibromas arise from small peripheral nerves during adolescence and adulthood. This view was supported by the fact that neurofibromas contain all the cell types of normal peripheral nerve organized in a haphazard manner. However, adult Schwann cells do not represent a continuously dividing cell population, which makes them unlikely hosts for the second hit. The fact that cutaneous neurofibromas start to grow during puberty may reflect the developmentally late onset of the *NF1* second-hit mutation. Furthermore, the $NF1^{-/-}$ Schwann cells may have a restricted potential for clonal cell divisions which could explain the limited growth of cutaneous neurofibromas.

26.3.3.1 Multipotent Precursor Cells in Neurofibroma Development

Recent findings have elucidated multipotent precursor cells as a novel component of cutaneous neurofibromas and a potential cell of origin of these tumors, both in humans and in the Nf1 mouse model (Jouhilahti et al. 2011; Le et al. 2009). The question of the cellular origin of cutaneous neurofibromas has been approached using a mouse model with an $Nf1^{+/-}$ background and a tamoxifen-inducible second hit in dermal stem/progenitor cells (Le et al. 2009). These mice developed cutaneous neurofibromas upon topical application of tamoxifen and the subsequent double *Nf1* gene inactivation.

Human cutaneous neurofibromas contain multipotent precursor cells, NFPs, which have the potential to differentiate into various cell types found in neurofibromas (Jouhilahti et al. 2011). The NFPs, however, have an $NF1^{+/-}$ genotype. We speculate that the $NF1^{-/-}$ Schwann cells in human cutaneous neurofibromas may also arise from multipotent progenitor cells which have gained a second *NF1* mutation and consequently differentiated into Schwann-like cells. Considering the fact that only one second hit is found in each tumor, a single cell gives rise to a clonal Schwann cell population with an $NF1^{-/-}$ genotype. With human tissue material it is, however, challenging to restore the initial stage of tumor formation and isolate a single multipotent cell with an $NF1^{-/-}$ genotype.

To further specify the potential niches for neurofibroma-derived precursor cells, we examined the structure of cutaneous neurofibromas. The histological analyses invariably revealed the hair follicular structures embedded in cutaneous neurofibromas; even the apparently normal skin from NF1 patients revealed minute neurofibromas, presumably in the early stages of development, in the immediate vicinity of the hair follicular apparatus (Jouhilahti et al. 2011; Karvonen et al. 2000). Earlier studies had characterized skin-derived precursor cells (SKPs) which potentially reside in the hair roots of mice and have the potential to generate subpopulations of cells expressing neuronal, glial, smooth muscle, and adipocyte

markers (Fernandes et al. 2004, 2008; Toma et al. 2005). Together these results suggest that the multipotent cells present in the hair follicles may contribute to the development of cutaneous neurofibromas.

26.3.4 Inflammation

In cancer biology, the role of inflammatory cells in tumor development has been recognized (Hanahan and Weinberg 2011). In the context of neurofibroma development, the presence of mast cells within tumor stroma is an established fact, evidenced by both human and mice studies (Jouhilahti et al. 2011; Peltonen et al. 1988; Yang et al. 2008; Zhu et al. 2002). It has been speculated that by secreting mediators of inflammation and angiogenesis-promoting factor, VEGF, mast cells have an active role in tumor development and maintenance (Yang et al. 2008). Furthermore, mast cells also release PDGF-BB and TGF-β. Together these factors promote the proliferation and survival of Schwann cells, fibroblasts, and pericytes and the synthesis of collagen by fibroblasts.

Our recent results have revealed another inflammatory component which has a potential role in neurofibroma development. The gene expression profiling of neurofibroma-derived cultured Schwann cells revealed that Schwann cells with $NF1^{-/-}$ genotype express HLA class II genes. Furthermore, immunohistochemical analysis showed that neurofibromas harbor scattered T lymphocytes. Previous studies have shown that Schwann cells have the potential to display the entire spectrum of immune response including antigen presentation and recognition, regulation of immune response by secreting soluble factors, and termination of immune response via the interaction of Fas and FasL (Meyer zu Hörste et al. 2008). We suggest that tumor Schwann cells with an $NF1^{-/-}$ genotype may act as dendritic or nonprofessional antigen-presenting cells, and as such they may be involved in immunological tolerance which is mediated by regulatory T cells.

26.4 Conclusion

To conclude, cutaneous neurofibromas are benign tumors which never undergo malignant transformation. Even though they do not pose any threat to life, due to their high number, cutaneous neurofibromas constitute the main disease burden in most adult patients with NF1. It has now been recognized that the development of a cutaneous neurofibroma requires interplay between multiple cell types with $NF1^{+/-}$ genotypes and $NF1^{-/-}$ Schwann cells. The multipotent precursor cells are a potential cell of origin for these tumors. Furthermore, the inflammatory component contributing to the tumor development increases the complexity of the process of pathogenesis.

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Chapter 27
Somatic Copy Number Alterations: Gene and Protein Expression Correlates in NF1-Associated Malignant Peripheral Nerve Sheath Tumors

Meena Upadhyaya and David N. Cooper

27.1 Introduction

Neurofibromatosis type-1 (NF1) is associated with the growth of benign and malignant tumours (Upadhyaya and Cooper 1998; Bennett et al. 2009; reviewed by Upadhyaya 2010). About ~10–15 % of NF1 patients develop malignant peripheral nerve sheath tumours (MPNSTs) (Evans et al. 2002). MPNSTs are soft tissue sarcomas (Barretina et al. 2010), and half of all MPNSTs diagnosed occur in association with NF1, with affected patients exhibiting a poor prognosis. NF1-MPNSTs usually develop in association with a pre-existing plexiform neurofibroma (PNF) or a focal subcutaneous neurofibroma. Patients with multiple internal PNFs, identified by a whole-body MRI scan, exhibit a 20-fold increased risk of MPNST development (Tucker et al. 2005), underlining the need for regular clinical surveillance in such individuals. Sporadic MPNSTs not associated with NF1 appear to be de novo malignant tumours (reviewed by Upadhyaya 2011). Many MPNSTs are not diagnosed until they reach an advanced stage, or when primary tumours have metastasised, most commonly affecting the lungs and less frequently the liver and brain.

The current state of diagnosis and clinical management of NF1-MPNSTs is unsatisfactory, and the molecular mechanism underlying transformation to malignancy is not yet fully defined. In order to identify genes with causal roles in NF1 tumorigenesis and to identify specific diagnostic and prognostic biomarkers associated with MPNST development, it is important to identify chromosomal regions that have undergone frequent alterations in the genome.

Until recently, it was generally assumed that two copies of every gene were almost invariably present in every human genome**.** However, recent discoveries have revealed that large segments of DNA, ranging in size from thousands to millions of bases, can vary in terms of their copy number. Since such copy number

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variations (or CNVs) can encompass entire genes, this can lead to significant interindividual differences in gene number and gene expression. Genes that were once thought to occur in two copies per genome have now been found to be present in one or alternatively three or more copies (Beroukhim et al. 2010).

Constitutional CNVs can represent benign polymorphic variants or may be associated with disease, including predisposition to cancer. Somatic copy number alterations (SCNAs) are distinct from germline copy number variations as they are present exclusively in somatic tissue. SCNAs are extremely common in cancer (Kuo et al. 2009; Rothenberg and Settleman 2010; Kuiper et al. 2010; Shlien and Malkin 2009, 2010; Baudis 2007; Henrichsen et al. 2009a, b; Database of Chromosome Aberrations in Cancer, 2000 [http://cgap.nci.nih.gov/Chromosomes/Mitelman]). As with other tumour-associated genetic changes, such SCNAs (by virtue of the genes they contain) can confer a selective advantage with respect to cellular proliferation, survival and clonal expansion. SCNAs have also been reported in noncancer studies. Thus, Bruder et al. (2008) found copy number differences at several loci between members of each of the 11 pairs of monozygotic twins without any known disease. Subsequently, this group detected copy number differences between different tissues from a single individual sampled at post-mortem, but these copy number differences were not thought to be associated with disease (Piotrowski et al. 2008). From this finding, it is clear that some diseases may well be caused by copy number changes that are specific to the affected tissue but which are not present in the blood or buccal cells, two tissues which are regularly used for genomic analysis (Dear 2009).

The genomes of tumours are genetically heterogeneous (Ding et al. 2008; Kan et al. 2010) with different rearrangements (Campbell et al. 2008) and SCNA (Beroukhim et al. 2010) distinguishing them. SCNA analysis allows the identification of recurrent aberrations in different tumours. In some cases, SCNAs have led to the identification of novel cancer-causing genes which have in turn suggested specific therapeutic avenues (Beroukhim et al. 2010; Valsesia et al. 2011).

Various microarray-based methodologies have been developed to detect both CNVs and SCNAs. Comparative genomic hybridisation arrays (array-CGH) constructed from overlapping human BAC clones have proved to be successful in earlier studies. More recently, combined high-density SNP and CNV genotyping arrays have been employed. Such high-density microarrays, often containing several million individual markers, can now be used to simultaneously assess both heritable copy number variation and somatic SCNA-based expression changes associated with a specific tumour type (Lebron et al. 2011). Other methods available for CNV/SCNA analysis include microscopy, fluorescence in situ hybridisation (FISH), Southern blotting, PCR-based methods including real-time PCR, multiplex ligation-dependent probe amplification (MLPA), molecular copy number counting (Daser et al. 2006), high-resolution array technology and massive parallel sequencing (Campbell et al. 2008; Meyerson et al. 2010).

This chapter discusses the potential role of somatic copy number alterations (SCNAs) in the transformation of benign neurofibromas to malignant peripheral nerve sheath tumours and focuses on expression and protein-based studies in MPNSTs and related cancers.

27.2 How Has the Consideration of SCNAs Improved Our Knowledge of the Molecular Basis of MPNSTs?

Although the functional loss of neurofibromin represents the primary event in NF1 tumourigenesis (reviewed by Upadhyaya 2011), other genetic lesions clearly play a role in influencing cell growth, differentiation and apoptosis which are required to promote the malignant transformation of plexiform and atypical neurofibromas. Microarray-based technologies have made possible the simultaneous analysis of multiple genes and many different genomic regions in RNA or DNA from MPNSTs and other cancers in a single experiment. Such array-based analyses allow us to globally profile all gene expression and copy number changes that may be associated with tumorigenesis. Several studies have already analysed tumour DNA and RNA to search for potential molecular signatures that are capable of differentiating between MPNSTs and benign neurofibromas (Table 27.1).

27.3 Cytogenetic and Chromosomal Comparative Genomic Hybridisation Studies

Cytogenetic and FISH studies of NF1-MPNSTs have revealed a wide spectrum of chromosomal aberrations including translocations, duplications and numerical gains and losses. Most MPNSTs have a complex karyotype (Glover et al. 1991; Plaat et al. 1999; Mechtersheimer et al. 1999; Mertens et al. 2000), making it difficult to identify which of these alterations are of true biological significance. In earlier studies, candidate amplified regions containing potentially relevant proto-oncogenes included chromosomal bands 17q24–q25, 7, 7p11–p13, 8, 5p15, 8q22–q24, 12q21–q24, 8/8q and 15q (Mertens et al. 2000; Frank et al. 2003; Bridge et al. 2004; Lothe et al. 1995, 1996) while those deleted regions with putative tumour suppressor genes were 9p21–p24, 13q14–q22, 1p, 10q, 11q1, 15, 17p1, 17q, 18q1–q2, 19p1, 22q1, X and Y (Mechtersheimer et al. 1999; Lothe et al. 1996; Plaat et al. 1999; Schmidt et al. 2000; Koga et al. 2002) (Table 27.1). In contrast to the specific chromosomal translocations found in many synovial and other sarcomas (Barretina et al. 2010), no consistent pathogenic chromosomal aberrations are known to be associated with MPNSTs, although a number of small recurrent chromosomal alterations have been reported (described below Table 27.1). Cytogenetics and FISH-based studies were initially used to identify large genomic alterations in NF1-MPNSTs (Mechtersheimer et al. 1999; Lothe et al. 1995; Plaat et al. 1999; Mertens et al. 2000; Skotheim et al. 2003; Frank et al. 2003; Perrone et al. 2003; Bridge et al. 2004; Tabone-Eglinger et al. 2008) with subsequent studies (Table 27.1) using chromosomal comparative genomic hybridisation (Schmidt et al. 2000; Koga et al. 2002; Lothe et al. 1996), and BAC arrays (Adamowicz et al. 2006; Storlazzi et al. 2006; Kresse et al. 2008; Mantripragada et al. 2009). Many of these DNA dosage studies had, however, either limited resolution or only

Table 27.1 Reported CNAs and expression analyses in different NF1-associated MPNSTs

			Gene or chromosomal changes	
Reference	Tumours studied	Analytical methods used	Gains	Losses
Lothe et al. (1995, 1996)	Neurofibromas (15) MPNSTs (9)	17q LOH study FISH analysis	–	17q allelic imbalance 4/6 NF1-associated MPNSTs
Lothe et al. (1996)	MPNSTs (7)	Chromosome CGH	Increased 17q24-ter copy number in 5 of 7 tumours	Loss of 13q14–q21 in 6/10 MPNSTs
Mechtersheimer et al. (1999)	MPNSTs (19) Neurofibromas (10)	Chromosome CGH	17q24–q25, 7p11–p13, 5p15, 8q22–q24, 12q21–q24	9p21–24, 13q14–q22, 1p
Kourea et al. (1999)	MPNSTs (11) Primary tumours (8) Recurrent tumours (3)	INK4A cDNA probe on Southern blot	–	INK4A *(CDKN2A)* deletions in many MPNSTs
Nielsen et al. (1999)	MPNSTs (6)	Multiplex PCR	–	Homozygous *CDKN2A* deletions in 3 MPNSTs
Plaat et al. (1999)	MPNSTs (7)	Computer-assisted cytogenetic analysis	7q1	1p3, 9p1, 11q1, 12q2, 17p1, 18q1–q2, 19p1, 22q1, X, Y
Mertens et al. (2000)	MPNSTs (20) Benign Neurofibromas (46)	Chromosomal banding	Chromosome 7	Chromosome 22
Schmidt et al. (2000)	MPNSTs (14)	Chromosome CGH	Gains on Chr 7; 8q; 15q; 17q	–
Koga et al. (2002)	MPNSTs (9) Neurofibromas (27) Schwannomas (14)	Chromosome CGH	17q & X	
Mawrin et al. (2002)	MPNSTs (12)	Loss of heterozygosity		*TP53*, *RB1*

Frank et al. (2003)	MPNSTs (3)	Karyotype	7, 8	1p, 10q, 9, 15
Perrone et al. (2003)	MPNSTs (20) Neurofibromas (14) Sporadic MPNSTs (12)	PCR-based molecular analysisFISH		*CDKN2A* and its variants ($P15^{INK4b}$, $p14^{ARF)}$
Skotheim et al. (2003)	MPNSTs (14) MPNSTs(16) MPNSTs (44)	Combined analyses (1) Chr:17-specific cDNA microarray (2) FISH analysis (3) Tissue microarray	*TOP2A* overexpressed in most MPNSTs	–
Bridge et al. (2004)	MPNSTs (21)	Cytogenetics and FISH	7/7q, 8/8q, *EGFR*	1p36, 3p21–pter, 9p23–pter, 10, 11q23–qter, 16/16q24, 17, 22/22q
Lévy et al. (2004)	MPNSTs (9) PNF (16) Dermal NF (10)	Real-time quantitative PCR on tumour RNA	*MK167, BIRC5, MMP13, SPP1, MMP9, TERT, TERC, TOP2A, SPP2, FOXM1, FOXA2/ HNF3B, HMMR/RHAMM, CXCL5, OSF2, CCNE2, EPHA7* and *TP73* only upregulated in MPNSTs	*ITGB4, CMA1, LICAM, MPZ DHH, S100B, ERBB3, RASSF2, PTCH2, TPSB, TIM4* and *SOX10* all downregulated only in MPNSTs
Watson et al. (2004)	MPNSTs (25) Sporadic MPNSTs (25)	Oligonucleotides Microarray analyses	*NCAM, MBP, L1CAM, PIP*	*IGF2, FGFR1, MDK, MKI67*
Adamowicz et al. (2006)	Soft-tissue sarcomas (34) including MPNSTs (10)	Chr:5p BAC microarray	*TRIO*, *IRX2* and *NKD2* all located at 5p15.3 are amplified	–
Miller et al. (2006)	Primary MPNSTs (44) MPNST cell lines (8) Normal Schwann cells (7)	Gene expression profiling (Affymetrix chip)	*EDGRE, S0X9, TWIST1*	*S0X10, CNP, PMP2*

(continued)

Table 27.1 (continued)

Reference	Tumours studied	Analytical methods used	Gene or chromosomal changes	
			Gains	Losses
Storlazzi et al. (2006)	MPNSTs (28) Primary tumours (25) Recurrent tumours (2) Metastatic tumour (1)	Interpase FISH analysis of 17q with BAC probes	*ERBB2* and *TOP2A* in 17q12–17q25 amplicon that also contains *BIRC5*	–
Lévy et al. (2007)	CNFs PNFs MPNSTs	Agilent 22K	*TNXC*	*TNXB*
Kresse et al. (2008)	High-grade MPNSTs (7)	Array CGH	*LOXL2*, *TOP2A*, *ETV4* and *BIRC5*	–
Tabone-Eglinger et al. (2008)	MPNSTs (42)	FISH	*EGFR*	–
Mantripragada et al. (2008)	MPNSTs (35) PNF s (16) CNF s (8)	Targeted gene array	*ITGB4*, *PDGFRA*, *MET*, *HGF* and *TP73*	*NF1*, *HMMR*, *MMP13*, *INK4A* and *CDKN2B*
Fang et al. (2009)	MPNST cell lines (3)	Molecular cytogenetics	17q25, 17p	9p, 12q21–32, X
Perrone et al. (2009)	Sporadic MPNST (11) NF1 MPNST (16)	Combined analyses (1) FISH (2) Gene activation (3) Mutational analysis	*PDGFRA* and *PDGFRB EGFR* copy number	–
Mantripragada et al. (2009)	MPNSTs (24)	32K BAC genome-wide array	*NEDL1*, *AP3B1* and *CUL1*	*CDKN2A* and *CDKN2B*

Miller et al. (2009)	MPNST cell lines (13) Neurofibromal Schwann cells (22) Primary MPNSTs (6)	Gene expression profiling (Affymetrix chip)	*S0X9, TWIST1*	–
Brekke et al. (2010)	Neurofibromas (10) MPNSTs (48)	Chromosomal and Array CGH	8q, 17q, 7p, 10, 16, X	9p, 11q, 17p
Yang et al. (2011)	MPNSTs (51)		*IGF1R, EGFR, BRAF, ETV1, MET, AKAP*	*CDKN2A, CDKN2B*
Yu et al. (2011)	MPNSTs (125)	Affymetrix genome-wide 500K SNP	*CDK4, FOXM1, SOX5, NOL1, MLF2, FKBP4, TSPAN31*	*ERBB2, MYC, TP53*
Beert et al. 2011	MPNSTs (34) Benign neurofibromas (15)	Agilent 244K microarray	*PDGFRA/KIT,TWIST1, EGFR, MET, MYC region, CCND2 MDM2*, 14q, 15q, 17q, 20q and 21q	*CDKN2A, CDKN2B, TP53 PRDM2, CDKN2C, RNF11, EPS15, NF1A, PGM1, ROR1, UBE2U, CACHD1, RAVER2* and *JAK1. LSAMP* (3q13.31), *EPHB1* (3q22.2) and *STAG1* (3q22.3). *SMARCD1* (4q22.3) and *SPRY1* (4q28.1), the *RB1* (13q14.2), *SPRY2* (13q31.1) and *SUZ12 gene* (17q11.2) was deleted in 12, 14 and 17 high-grade *MPNSTs*, *CHEK2* (22q12.1) and *NF2* (22q12.2), *PTEN, RB1*
Upadhyaya et al. (2012)	MPNSTs (15)	Affymetrix genome-wide SNP 6.0	*RAC1, ROCK2, PTK2, LIMK1, PRKCA, ACTB, ACTG1, TRID*	*MMP12, TP53, CDKN2A, HMMR, RB1*

PNFs plexiform neurofibromas, *CNFs* cutaneous neurofibromas

assessed a few gene regions, and more recently, high-resolution oligonucleotide microarray analysis, screening for specific DNA copy number changes in the NF1 tumours, has been employed (Mantripragada et al. 2008; Yu et al. 2011; Beert et al. 2011; Upadhyaya et al. 2012).

27.4 Molecular Studies

MPNSTs are molecularly complex tumours. About 95 % of MPNSTs harbour somatic large deletions at the *NF1* locus (Upadhyaya et al. 2008). Differently sized *NF1* locus rearrangements in NF1 MPNSTs have been reported (Pasmant et al. 2011a). However, in this study, the MPNST-associated deletion breakpoints did not involve either paralogous repetitive sequences or the sequences from *SUZ12* and *SUZ12P* that are involved in most germline *NF1* deletions.

Deletion of three tumour suppressor genes, *CDKN2A*, *TP53* and *RB1*, has been reported to be associated with MPNSTs for more than 20 years (reviewed by Upadhyaya 2011). Frequent deletions of the 9p21 region, associated with downregulation of the *CDKN2A* (p16) gene located at 9p21, have been reported in several NF1-associated MPNSTs using both low- and high-resolution techniques (Cairns et al. 1995; Kourea et al. 1999; Nielsen et al. 1999; Perrone et al. 2003; Frahm et al. 2004; Sabah et al. 2006; Mantripragada et al. 2008, 2009; Beert et al. 2011; Yu et al. 2011).

Loss of the 17p13 region, which encompasses the *TP53* gene, has been noted in many NF1-MPNSTs (Legius et al. 1994; Upadhyaya et al. 2008; Mantripragada et al. 2008). Whereas the *TP53* gene is often inactivated by allelic loss or by more subtle lesions, biallelic *TP53* inactivation rarely occurs in NF1-associated MPNSTs. Functional loss of p53 leads to an overall increase in genomic instability, manifesting as increased gene amplification, aneuploidy or other chromosomal rearrangements. Mouse NF1 models confirm an active role for *TP53* loss, in conjunction with *NF1* inactivation, in MPNST development (Cichowski et al. 1999; Vogel et al. 1999), although it is still not known whether other definite genetic modifiers may also predispose to NF1-MPNST development.

Disruption of the retinoblastoma (Rb) pathway is common in NF1-associated MPNSTs. In a study based on 12 MPNSTs, it was found that disruption of the *RB1* gene was common in NF1-MPNSTs; loss of heterozygosity involving the *RB1* gene was reported in a substantial number of NF1-MPNSTs (Mawrin et al. 2002; Berner et al. 1999). In an array CGH study, *RB1* gene was deleted in 8/35 MPNSTs (Mantripragada et al. 2008).

Thus, deletion of *p16INK4A*/*CDKN2A*, *RB1* and *TP53* genes in MPNSTs corroborates the model of co-inactivation of the *NF1*, *TP53* and/or *RB1* pathways with functional consequences on cell growth control and apoptosis. Holtkamp et al. (2008) reported that the likely candidate genes involved in the malignant transformation of plexiform neurofibromas included *PDGFRA*, *PDGF* and *KIT*. The upregulation of both *BIRC5* and *TNC* has been described in NF1-associated

MPNSTs, but this was not found in neurofibromas (Karube et al. 2006). Survivin, the protein encoded by *BIRC5*, inhibits apoptosis, and *BIRC5* upregulation is a frequent alteration in many cancers, including NF1-associated MPNSTs (Mantripragada et al. 2009; Storlazzi et al. 2006). Amplifications of two apoptotic genes, *BIRC5* and *TP73*, were also identified in our own study (Mantripragada et al. 2008). The *TP73* gene amplification observed in MPNSTs implies that the encoded DNp73 protein, an apoptosis inhibitor, may be upregulated in malignant Schwann cells (Mantripragada et al. 2008). The topoisomerase II alpha (*TOP2A*) gene is also upregulated as a consequence of the amplification of the 17q21-q22 region in many NF1-associated MPNSTs (Latres et al. 1994; Skotheim et al. 2003; Kresse et al. 2008). The amplification of *TRIO* (5p15.2), *NKD2* (5p15.33) and *IRX2* (5p15.33) has been reported in soft tissue sarcomas including NF1-MPNSTs (Adamowicz et al. 2006).

We initially used a targeted gene array to screen for possible gene alterations in 57 unrelated MPNST DNAs and found concomitant amplification of the hepatocyte growth factor gene (*HGF*) at 7q21.11, the c-MET proto-oncogene/hepatocyte growth factor receptor (*MET*) at 7q31.2 and the platelet-derived growth factor receptor alpha polypeptide gene (*PDGFRA*) at 4q12 (Mantripragada et al. 2008). *MET* is a tyrosine-kinase receptor, whereas HGF is the only known ligand of the *MET* receptor. Normally, only stem cells express *MET*, allowing these cells to grow and generate new tissues in an embryo or regenerate damaged tissues in an adult. However, cancer stem cells are thought to acquire the ability of normal stem cells to express *MET*, thereby resulting in the progression and metastasis of the tumour. Following activation by HGF binding, c-Met auto-phosphorylates, recruiting other downstream proteins which result in the activation of multiple signalling pathways (e.g. Ras-MAPK, PI3K, AKT and STAT3/5) thereby regulating cell growth, motility and morphogenesis.

The hemizygous deletion of the hyaluronan-mediated motility receptor (*HMMR*) gene at 5q34 was also found in about half the MPNSTs analysed (Mantripragada et al. 2008). *HMMR* overexpression has been implicated in several cancers, including NF1-MPNSTs (Kalmyrzaev et al. 2008). However, the hemizygous deletion of the *HMMR* gene is unlikely to result in the overexpression of that gene. The identification of MPNST-specific *HMMR* deletions makes *HMMR* a strong candidate gene for the initiation and/or development of malignancy in NF1 individuals. Subsequent analysis of 25 unrelated MPNST DNAs with a 32K BAC array identified a common ~540 kb deletion at 9p21.3, the region which contains the *CDKN2A, CDKN2B* and *MTAP* genes (Mantripragada et al. 2009). Biallelic loss of all three genes was found in our study of 3 tumour samples, with single copy deletions in 8 MPNSTs (Mantripragada et al. 2009).

A recent study by Yu et al. (2011) examined 38 patients with MPNSTs, 23 (60 %) of whom were affected with NF1 whilst the remaining 15 (40 %) were sporadic cases. These workers identified a number of loci already known to be important in *NF1*-MPNST development and its progression. These included losses of *NF1* on 17q11.2 and *CDKN2A* on 9p21, as well as common gains on 7p, 8q, 12 and 17q. *CDK4* (12q14) gains/amplification and increased FOXM1 protein expression were found to be significant independent predictors of poor survival in MPNST patients.

27.5 Transition from Atypical Neurofibroma to MPNST

Atypical neurofibromas are symptomatic, hypercellular peripheral nerve sheath tumours, composed of cells with hyperchromatic nuclei in the absence of mitoses (Beert et al. 2011). Little is known about the origin, nature and pathology of atypical neurofibromas in NF1 patients. In their elegant study, Beert et al. (2011) showed that atypical neurofibromas are premalignant tumours, with the *CDKN2A*/*B* deletion being the first step in the progression towards MPNST. In this study, deletion of a minimal overlapping region (MOR) in chromosomal band 9p21.3, which includes *CDKN2A* and *CDKN2B,* was identified in 15 of 16 atypical neurofibromas (Beert et al. 2011) but was absent in benign neurofibromas. In another study (Spurlock et al. 2010), genetic processes involved in the transformation from a benign plexiform neurofibroma through atypical plexiform neurofibroma to an aggressive malignant tumour revealed losses of *TP53*, *RB1*, *CDKN2A* as well as several oncogenes and cell-cycle genes (Spurlock et al. 2010).

27.6 Potential MPNST-Specific Pathways

Yang et al. (2011) genomically characterised 51 NF1-associated MPNST tissue samples and identified frequently amplified regions harbouring a total of 2,599 genes plus multiple regions of deletion that together included 4,901 genes. They also identified a significant increase in copy number alteration events involving genes encoding proteins in the insulin-like growth factor 1 receptor (*IGF1R*) pathway, including frequent amplifications of the *IGF1R* gene itself. To validate the IGF1R pathway as a potential target in MPNSTs, Yang et al. (2011) used immunohistochemical techniques to confirm that high IGF1R protein correlated with worse tumour-free survival in an independent set of samples. Using small interfering RNAs or an IGF1R inhibitor, they were able to demonstrate a significant decrease in cell proliferation, invasion and migration accompanied by attenuation of the PI3K/AKT and MAPK pathways.

In a recent study of NF1 tumours, Upadhyaya et al. (2012) employed SNP arrays that were denser than array CGH and offered the added advantage of being able to simultaneously detect chromosomal loss of heterozygosity (LOH) and uniparental disomy (UPD) in addition to copy number changes. The genome-wide Human SNP-Array-6.0 was used to perform single nucleotide polymorphism genotyping and copy number alteration (SCNA), loss-of-heterozygosity (LOH) and copy number neutral-LOH (CNN-LOH) analysis of DNA isolated from 15 MPNSTs, 5 benign plexiform neurofibromas (PNFs) and patient-matched lymphocyte DNAs (Upadhyaya et al. 2012). MPNSTs exhibited high-level LOH, with recurrent changes occurring in MPNSTs but not PNFs. CNN-LOH was evident in MPNSTs but occurred less frequently than genomic deletions. SCNAs involving the *ITGB8*, *PDGFA, RAC1* (7p21-p22), *PDGFRL* (8p22-p21.3) and *MMP12* (11q22.3) genes

were specific to MPNSTs. Pathway analysis revealed the MPNST-specific amplification of seven Rho-GTPase pathway genes and several cytoskeletal remodelling/cell adhesion genes. In knockdown experiments employing short-hairpin *RAC1*, *ROCK2*, *PTK2* and *LIMK1* RNAs to transfect both control and MPNST-derived cell lines, cell adhesion was significantly increased in the MPNST cell lines whereas wound healing, cell migration and invasiveness were reduced, consistent with a role for these Rho-GTPase pathway genes in MPNST development and metastasis. These results have suggested new targets for therapeutic intervention in MPNSTs (Upadhyaya et al. 2012).

Taken together, the results of the above-mentioned molecular studies appear to suggest that no single gene, other than *NF1*, appears to be consistently altered in all NF1-associated MPNSTs.

27.7 Gene Expression and Protein Analysis in NF1-MPNSTs

Evidence that DNA copy number changes can directly affect the expression of genes involved in these genomic changes (Riddick and Fine 2011) has led to the application of array-based gene expression studies to many different cancers. In the context of NF1, a quantitative RT-PCR study measured the expression level of 489 selected genes in NF1-MPNSTs and plexiform neurofibromas and found that 28 genes exhibited significantly different expression levels in MPNSTs, compared to PNFs, with 16 genes being upregulated and 12 downregulated (Lévy et al. 2004). Of the altered genes, two were involved in the Ras signalling pathway (*RASSF2* and *HMMR*) and two in the Hedgehog-Gli signalling pathway (*DHH* and *PTCH2*), whereas the other genes were variously involved in cell proliferation (*MKI67, TOP2A, CCNE2*), senescence (*TERT, TERC*), apoptosis (*BIRC5, TP73*) and extracellular matrix remodelling (*MMP13, MMP9, TIMP4, ITGB4*). Active growth of MPNSTs requires increased angiogenesis, and activated Ras is known to upregulate *VEGF* (vascular endothelial growth factor) expression (Gesundheit et al. 2010). Both *VEGF* expression and tumour vascularisation are significantly increased in MPNSTs (Angelov et al. 1999; Kranenburg et al. 2004).

Although a similar comparison of the RNA "transcriptomes" of both sporadic and NF1-associated MPNSTs failed to detect any differences, this gene expression profiling study did succeed in identifying a 159-gene molecular signature that served to distinguish MPNST cell lines from normal Schwann cells (Miller et al. 2006). Schwann cell differentiation markers (*SOX10, CNP, PMP22* and *NGFR*) were found to be downregulated in MPNSTs, whereas the neural crest stem cell markers, *SOX9* and *TWIST1,* were overexpressed. The *TWIST1* gene is implicated in apoptosis inhibition, resistance to chemotherapy and metastasis (Maestro et al. 1999). However, silencing *TWIST1* expression in MPNST cells, with small interfering RNA, did not affect either apoptosis or chemoresistance, although cell chemotaxis was inhibited.

Another related gene expression profiling study by the same group analysed normal Schwann cells (10), Schwann cells from primary benign (both dermal and plexiform) neurofibromas (22), MPNSTs (6) and cell lines derived from MPNSTs (13) (Miller et al. 2009). No difference was found between benign cutaneous and plexiform neurofibromas. Comparison of gene expression profiles of *NF1* with cultured normal primary Schwann cells identified 1,108 unique gene signatures that served to distinguish NF1 tumour samples from normal Schwann cells. Strong expression of the *SOX9* gene was found in both neurofibroma and MPNST tissue; schwannomas expressed low levels of *SOX9* whilst synovial sarcomas, tumours histologically similar to MPNSTs, were completely negative for *SOX9*. *SOX9* encodes a neural crest transcription factor required for stem cell survival (Cheung et al. 2005). Average *SOX9* expression values were at least twofold higher in MPNST samples relative to neurofibroma samples. The in vitro reduction of *SOX9* expression with small hairpin RNA (shRNA) in MPNST cell lines was found to result in rapid cell death.

A recent study by Miller et al. (2010) reported a decrease in *DACH1* expression and an increase in *PAX6, EYA1, EYA2, EYA4* and *SIX1-4* expression in MPNSTs. *EYA4* expression was elevated 20-fold in tumour cell lines; following its suppression with *EYA4*-specific small interfering RNA (siRNA), the treated cells exhibited reduced adhesion and migration and increased cell death, without apparently affecting either cell proliferation or apoptosis, an indication that the *EYA4* pathway may be a potential therapeutic target.

Differential expression of genes within the *NF1* microdeleted region has been reported in NF1-associated tumours. Thus, Pasmant et al. (2011b) investigated the expression of *NF1*, the other 16 protein-coding genes and the 2 microRNAs located within the 1.4-Mb microdeletion by means of real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) in a large series of human dermal and plexiform neurofibromas and MPNSTs. Five genes were found to be significantly upregulated: *OMG* and *SUZ12* in plexiform neurofibromas and *ATAD5*, *EVI2A* and *C17orf79* in MPNSTs. Two genes (*RNF135* and *CENTA2*) were significantly downregulated in tumour Schwann cells derived from MPNST biopsies and in MPNST cell lines. Further investigation will however be required to confirm these findings.

One major problem with all microarray-based analyses of tumour tissues is the very large number of genes and genomic regions often found to be altered in terms of both copy number and gene expression. Consequently, this makes the identification of the relatively few causative "driver" gene changes, amongst the mass of alterations to other "passenger" genes, difficult. This emphasises the need for rigorous functional analyses of these changes to support the microarray data.

A sarcoma-based study, that included MPNSTs from NF1 patients with moderate to strong expression of HIF1A protein (determined immunohistochemically) in their tumours, revealed a significantly shorter survival rate compared to patients whose tumours showed little HIF1A expression (Shintani et al. 2006).

RalA is reported to be strongly activated in both human and mouse MPNST cell lines and tumour samples, as compared to non-transformed Schwann cells

(Bodempudi et al. 2009). RalA, a key member of the family of Ras-like (Ral) proteins, has a pivotal role in tumourigenesis, and its inactivation in MPNST cells has been found to significantly reduce cell proliferation and tumour invasiveness. Since Ral guanine nucleotide exchange factors are direct effectors of Ras, the Ral signalling pathway may be considered to be a Ras-effector pathway. The study of Bodempudi et al. (2009), which showed consistent over-activation of RalA in mouse and human NF1-MPNSTs, does however require confirmation in a much larger NF1 patient study.

Cabibi et al. (2009) have shown that immunoexpression of CD10 occurs in NF1-associated MPNSTs and atypical neurofibromas but not in neurofibromas.

In summary, a limited number of studies so far have examined the role of different proteins in MPNST development.

27.8 The Role of MicroRNAs in NF1-MPNST Formation

MicroRNAs (miRNAs) are known to regulate the expression of many cancer genes (Calin et al. 2002; Bottoni et al. 2005; Lamy et al. 2006; Zhang et al. 2006; Weiler et al. 2006; Wu and Mo 2009; Wu et al. 2009; Marcinkowska et al. 2011). Many protein-coding genes are known to be regulated by miRNAs, with many miRNAs able to silence multiple target mRNAs (Lim et al. 2005). It is estimated that the expression of at least one third of all protein-coding genes is regulated by approximately 1,000 miRNAs (Lewis et al. 2005). There are nearly 2,000 known miRNA genes in the human genome (Kozomara and Griffiths-Jones 2011). Many of these miRNA genes are expected to experience copy number gains or losses leading to their upregulation or downregulation.

miRNA genes recurrently amplified or deleted in cancer genomes can represent either oncogenes or tumour suppressor genes whose gain or loss will promote cellular proliferation (Bottoni et al. 2005; He et al. 2005). Dong et al. (2010) integrated miRNA, somatic mutation, SCNA and gene expression data to reconstruct networks associated with tumour initiation and progression in glioma.

Since miRNAs play a significant role in the development of cancer, it is therefore likely that the assessment of miRNA profiles in neurofibromas and MPNSTs will provide useful diagnostic markers and will help to identify additional therapeutic targets. Several different histological subtypes of sarcoma are known to exhibit distinct miRNA expression profiles (Subramanian et al. 2008), and recent studies of NF1-MPNSTs have shown miRNA-34a to be downregulated but not in the NF1-associated neurofibromas (Subramanian et al. 2010), while miRNA-10b was found to be upregulated in primary MPNST tissues and derived cell lines, as well as in neurofibroma-derived Schwann cells (Chai et al. 2010). Further studies should highlight the many miRNAs involved in the regulation of MPNST development in order to identify potential therapeutic targets.

27.9 Integrating Copy Number Alteration and Gene Expression Data in Tumorigenesis

Numerous studies have shown that CNVs/SCNAs can influence the expression of protein-coding genes in a copy number-dependent manner (Pollack et al. 2002; Zheng et al. 2004; Perry et al. 2007; Gonzalez et al. 2005; Bergamaschi et al. 2006; Stranger et al. 2007; Lee et al. 2008; Barretina et al. 2010; Beroukhim et al. 2010; Huang et al. 2011). The National Cancer Institute's Cancer Genome Atlas project (http://cancergenome.nih.gov/) is generating multiple data types (including gene expression and copy number data) for a variety of different cancers. For a number of cancers, it has been reported that gene copy number and expression levels correlate with each other (Santarius et al. 2010; Schlattl et al. 2011). It is estimated that ~60 % of genes exhibit differential expression concordant with their copy number status (Huang et al. 2012), depending upon the type of cancer and the analytical methodology employed. Although the effect of copy number on gene expression is being explored (Goh et al. 2011), there is still insufficient information to assess what effect LOH and UPD might have on gene expression. Using these strategies, it is possible to identify patterns of gene deletions and amplifications that are specific to benign, premalignant and malignant states (Nigro et al. 2005; Yi et al. 2005; Lu et al. 2011; Chu et al. 2011).

A combination of SCNA and gene expression data might provide information useful for differentiating between driver and passenger mutations. Indeed, concordance between SCNA and gene expression should reduce the number of false positives (Riddick and Howard 2011). In NF1, there appears to be some agreement between copy number changes for several genes identified in the MPNST microarray and expression data derived from previous studies. For instance, the amplification of the *BIRC5, CCNE2, FOXA2, MMP9, SOX10, SPP1, TERT* and *TP73* genes correlates with the upregulation of these genes noted in expression studies (Mantripragada et al. 2008, 2009; Lévy et al. 2004). Similarly, deletions of *L1CAM2, PTCH2, RB1* and *TIMP4* concur with previous reports showing the downregulation of these genes in MPNSTs (Mantripragada et al. 2008; Mawrin et al. 2002). However, neither array CGH (Mantripragada et al. 2008, 2009) nor expression studies (Miller et al. 2009) have identified any difference in terms of both SCNA and gene expression between cutaneous and plexiform neurofibromas.

It is hoped that the rapidly diminishing cost of next generation sequencing will further enhance gene expression/SCNA studies. Next generation RNA-based sequencing will also provide information on alternative splicing and mRNA expression.

27.10 Somatic Copy Number Changes in Mitochondrial DNA

The identification of either increased or reduced mitochondrial DNA (mtDNA) copy number has been increasingly reported in a range of primary human cancers, suggesting that alterations in mtDNA number may be a critical factor in cancer

pathogenesis and progression (Carew and Huang 2002; Yu 2011). However, the precise role of mtDNA copy number changes in driving the tumourigenic process remains largely unknown. Although it is possible that mtDNA copy number alterations could exert a role in the pathogenic mechanisms of MPNST development, as yet, this has not been addressed. Somatic mtDNA mutations have been sought in benign NF1-associated tumours (Kurtz et al. 2004). In this study, mitochondrial mutations were identified in both cutaneous and plexiform neurofibromas of NF1 patients. Detjen et al. (2007) studied nucleated blood cells of four pairs of discordant MZ twins with NF1 and from cutaneous neurofibromas of one twin pair. They failed to detect evidence for mtDNA sequence differences or for different degrees of heteroplasmy between individuals of the same twin pair. In order to evaluate the role of mitochondrial DNA in MPNST development, a comparative study based on mitochondrial copy number changes between NF1-MPNSTs and benign neurofibromas is ongoing (Upadhyaya et al. unpublished data).

27.11 Future Developments and Challenges

We still do not know how many driver mutations are involved in NF1 tumorigenesis (Stratton et al. 2009). Many of the common focal deletions in human cancers have features suggesting that they are passenger mutations. Thus, these alterations are usually hemizygous and appear to target genes that lack tumour suppressor functions (Beroukhim et al. 2010; Bignell et al. 2010). Although the amplification of a number of oncogenes has been reported in many cancers (Santarius et al. 2010), amplified SCNA contain many other genes whose function is not well defined. In a previous study of NF1-associated MPNSTs, copy number gains were reported at 1q25, 3p26, 3q13, 5p12, 5q11.2-q14, 5q21-23, 5q31-33, 6p23-p21, 6p12, 6q15, 6q23-q24, 7p22, 7p14-p13, 7q21, 7q36, 8q22-q24, 14q22 and 17q21-q25 (Mantripragada et al. 2009). Several oncogenes map to these regions including *NEDL1* (7p14), *AP3B1* (5q14.1) and *CUL1* (7q36.1), and copy number gains involving these genes were identified in >63 % of MPNSTs. However, it is not clear what proportion of genes mapping to this region represent driver mutations.

In 2008, the International Cancer Research Genome (ICGC) was founded with the aim of standardising the approaches by which genome alterations are identified in human cancers (http://www.icgc.org), including cancers of the pancreas, ovary, stomach, liver, breast, oral cavity and chronic lymphocytic leukaemia. It should be noted that NF1-associated tumours have not received similar visibility as, by definition, they are relatively rare because they are confined to NF1 patients. For this reason, there is an urgent need for NF1 workers to formulate a consortium to scrutinise the genomic landscape of these tumours.

MPNSTs, like many other tumours, exhibit markedly greater cellular heterogeneity than their corresponding lymphocyte DNAs (Thomas et al. 2012; Gerlinger et al. 2012) which are used for germline genome analysis. Indeed, a given MPNST

sample invariably contains a mixture of malignant and non-malignant cells (Thomas et al. 2012). This has important implications for the detection of SCNA/gene expression analysis in MPNSTs.

It has therefore come to be appreciated that the somatic alterations in MPNSTs represent both small and large scale changes (Upadhyaya et al. 2008; Bottillo et al. 2009). The application of next generation sequencing strategies to the entire genome, the exome and the transcriptome have resulted in enhanced resolution and detection of different lesions associated with tumorigenesis (Campbell et al. 2008). Hence, a similar approach is required for NF1-associated tumours. The other advantage of next generation sequencing is that its digital nature allows us to estimate the tumour-to-normal copy number ratio at a genomic locus. Integrated analysis of DNA copy number data with gene expression, protein expression and methylation data as revealed for glioblastoma, leukaemia, lung cancer, ovarian cancer and neuroblastoma (Parsons et al. 2008; Ding et al. 2008; Sangha et al. 2008; Haferlach et al. 2010; Wang et al. 2011) is warranted for NF1-associated tumours. Multidisciplinary collaborative efforts are clearly essential to fully decipher the complex molecular basis of NF1-MPNST development. This may aid in the identification not only of driver genes associated with NF1 tumourigenesis but also novel tumour-specific biological pathways and therapeutic targets.

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Chapter 28
Pathologic and Molecular Diagnostic Features of Peripheral Nerve Sheath Tumors in NF1

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28.1 Pathology of Neurofibromas

Neurofibromas are common, benign tumors that may occur as solitary lesions unrelated to a genetic syndrome or as multiple lesions in association with neurofibromatosis type 1 (NF1). Neurofibromas are the hallmark and defining feature of NF1.

28.1.1 Gross and Clinical Characteristics of Neurofibromas

There are multiple classification schemes for neurofibromas based on their clinical, radiological and/or histological features (Riccardi 1992; Scheithauer et al. 1999a, b; Barbarot et al. 2007; Weiss and Goldblum 2008). Different subspecialties and groups (dermatology, neurology, neuropathology, radiology, and basic scientists) have adopted or developed their own terminology. The confusing terminology is problematic and hinders communication between different specialties and between clinicians and basic scientists (when attempting to integrate data from human and mouse model lesions).

Neurofibromas have three distinct growth patterns: localized (nodular, discrete), diffuse, and plexiform.

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28.1.1.1 Localized (Nodular; Discrete) Neurofibroma

The localized pattern is the most common form of neurofibroma encountered in non-NF1 patients (sporadic). Localized neurofibromas are distinct well-circumscribed (but not encapsulated) nodules (Fig. 28.1). Localized neurofibromas most often arise in dermis (cutaneous) or in the subcutaneous tissue from small nerves and may occur anywhere in the body but are most numerous on the trunk. Their number increases with age, with most patients over 30 having more than 100 tumors (Huson et al. 1988)

Localized neurofibromas that arise deeper, in a larger nerve (intraneural, localized neurofibroma) have a fusiform shape and the entrance and exit points for the nerve can be identified in the proximal and distal end of the lesion. Since localized neurofibromas are within the nerve, these are encapsulated by the epineurium.

In the NF1 patient, cutaneous and subcutaneous neurofibromas can be a major cause of morbidity. When numerous, these tumors cause disfigurement and psychological distress. At times they are associated with local, persistent itching (Riccardi 1981). Cutaneous and subcutaneous neurofibromas often become apparent only at puberty and increase in number and size with age. Exacerbated growth may occur during pregnancy (Huson et al. 1988).

28.1.1.2 Diffuse Neurofibroma

Diffuse neurofibromas may occur in young non-NF1 patients, but are more common in the NF1 patient. Diffuse neurofibromas present as ill-defined firm plaques and the areas involved appear diffusely expanded (Fig. 28.2). Diffuse neurofibroma may occur in the skin, in the soft tissue, or in the viscera. The gastrointestinal tract is the most common visceral type involved (Hochberg et al. 1974)

28.1.1.3 Plexiform Neurofibroma (Internal; Deep Neurofibromas)

Plexiform neurofibromas occur almost exclusively in association with NF1. Plexiform neurofibromas develop in early childhood and are thought to be congenital. These are large lesions that grow along large nerves, expanding and distorting the nerves so that they acquire a gross appearance similar to a rope (plexiform) or when multiple nerves are involved to "a bag of worms" (Fig. 28.3). Large plexiform neurofibromas may arise in spinal, cranial or peripheral nerves. Large plexus (brachial, lumbar) may be diffusely involved with the tumor extending along the entire plexus. When superficial, skin overlying the plexiform neurofibroma is hyperpigmented and thickened. In the "spinal neurofibromatosis" form of NF1, multiple spinal nerve roots are involved, bilaterally.

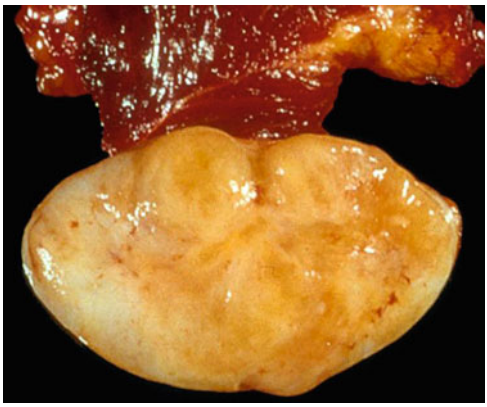

Fig. 28.1 Localized (nodular) neurofibroma, gross. The tumor is well circumscribed and distinct from adjacent soft tissue. It has a homogenous, fleshy cut surface

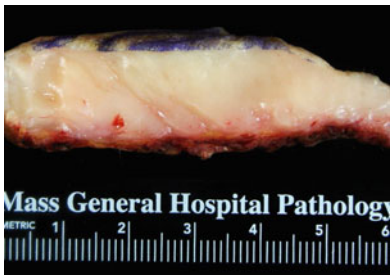

Fig. 28.2 Diffuse neurofibroma (cutaneous), gross. The tumor is diffusely infiltrating the dermis which is thickened and firm

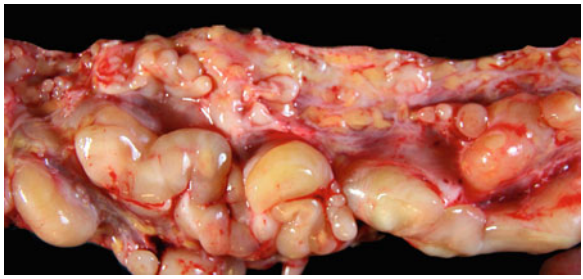

Fig. 28.3 Plexiform neurofibroma, gross. The tumor involves a number of nerves which appear enlarged with a multinodular, entangled appearance, reminiscent of "a bag of worms"

Plexiform neurofibromas occur in 30 % of the patients with NF1 when tumors are identified clinically (Huson et al. 1988). Not all plexiform neurofibromas are symptomatic and the number rises to 40 % when tumors are identified by imaging.

When large, plexiforms may cause pain, neurological deficit or compression of adjacent structures. In rare cases, the tumor provokes peritumoral massive growth of adjacent soft tissue and bone, inducing disproportionate growth of an extremity (massive soft tissue neurofibroma; elephantiasis neuromatosa). Massive soft tissue neurofibromas are only seen in the context of NF1.

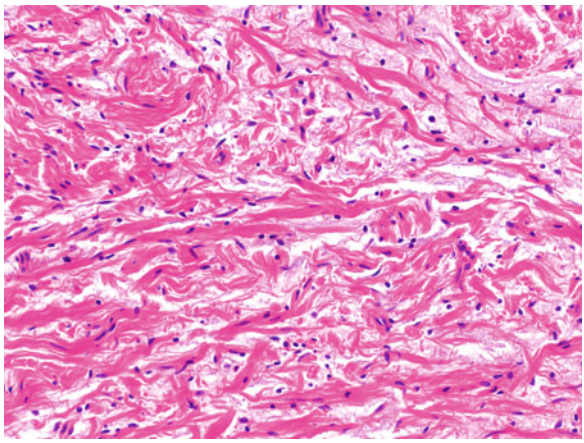

Fig. 28.4 Conventional (classic) neurofibroma, histology. The tumor is hypocellular, with myxoid stroma, collagen strands and cells with small, comma shaped nuclei

Plexiform neurofibromas are the neurofibroma subtype with the highest risk of malignant transformation. The cumulative lifelong risk of MPNST in an NF1 patient has been calculated as 4–5 % (Ducatman et al. 1986) but more recent studies show a higher risk of approximately 8–12 % (Evans et al. 2002).

28.1.2 Histopathology of Neurofibromas

Solitary neurofibromas and plexiform neurofibromas share common histological features. The lesions are composed of two elements: a stroma and a cellular element. The stroma may contain variable proportions of extracellular myxoid matrix and collagen bands. The cellular component is heterogenous and complex, consisting of different cell types and mixture of neoplastic Schwann cells and a variety of non-neoplastic cells (haploinsufficient for NF1). The majority of cells in neurofibromas are Schwann cells and fibroblasts but other cell types contribute to the lesion including perineurial cells, endothelial cells, smooth muscle cells, pericytes, mast cells, lymphocytes, and nerve axons. The histological appearance of the tumor can be variable depending on the type and amount of stroma and the ratio of stroma to cellular element. The classic appearance of a neurofibroma is that of a hypocellular tumor with abundant mucoid stroma, floating strands of collagen ("shredded carrots"), and scarce cells (Fig. 28.4). The cells include cells with wavy cytoplasm and elongated nuclei, cells with small comma shaped nuclei, and scattered mast cells. In some tumors, the stroma is minimally mucoid and is mostly composed of dense collagen bundles. When the stroma is minimal and the cellular component is prominent, the tumor is a "cellular neurofibroma." Histologically, solitary (sporadic) neurofibroma and NF1-associated neurofibromas are histologically similar. There are no histological features that are unique to NF1.

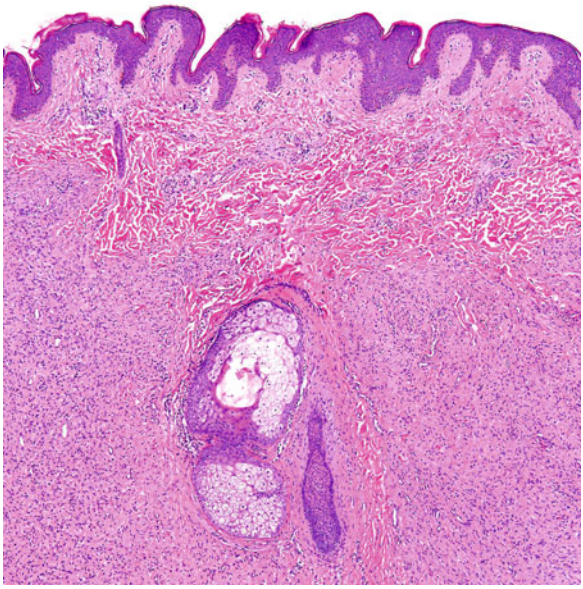

Fig. 28.5 Diffuse neurofibroma, micro. Diffuse neurofibroma infiltrating skin. The tumor is infiltrating around skin adnexa (hair follicle) without destroying them

Diffuse neurofibromas permeate and diffusely infiltrate soft tissues, surrounding (without destruction) hair follicles and glands and infiltrating between adipose cells or muscle fibers (Fig. 28.5).

The histological appearance of a plexiform neurofibroma is different from the diffuse and localized neurofibromas. The plexiform tumor is within the nerve fascicles, expanding and distorting the nerve. The early stages of tumor involvement of a plexiform neurofibroma are characterized by expansion of the endoneurium by increased stroma and widely separated axons within the fascicles of the affected nerve.

A plexiform neurofibroma may be contained within the nerve (intraneural) or can spill out beyond the epineurium, diffusely infiltrating adjacent soft tissue (diffuse, extra-fascicular) (Fig. 28.6). The infiltrating extra-fascicular portion of a plexiform neurofibroma is histologically indistinguishable from a diffuse neurofibroma (unassociated with a plexiform neurofibroma), so the histologic diagnosis of a plexiform neurofibroma depends on sampling; it requires a sample that includes the intrafascicular plexiform part of the tumor.

28.1.3 Molecular Pathology of Neurofibromas and MPNST

Although the different subtypes of neurofibromas share similar histological features and cellular composition, clinical observations and experimental mouse data suggest that they differ in their biological behavior. These differences are reflected in the tumors' rate of malignant transformation and response to hormones and may reflect an underlying difference in their respective progenitor cells or microenvironments.

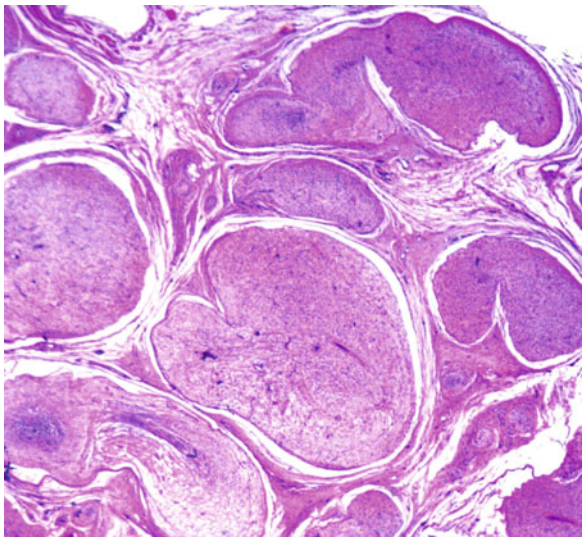

Fig. 28.6 Plexiform neurofibroma, micro. The nerves are greatly expanded by the tumor which infiltrates and replaces the nerve along its course. Tumor has extended beyond the epineurium and is present in the soft tissue surrounding the nerves; encasing the nerves

28.1.3.1 Malignant Transformation

Cutaneous neurofibromas do not undergo malignant transformation. Malignant transformation may occur (rarely) in intraneural localized tumors and in subcutaneous tumors (Scheithauer et al. 1999a, b). The highest risk of malignant transformation is seen with plexiform neurofibromas (8–13 %) (Evans et al. 2002). It is not clear why some subtypes of neurofibromas progress to MPNST.

The development of MPNST from neurofibromas is thought to involve a sequence series of alterations in other tumor suppressor genes and aberrant signaling by growth factors or their receptors. Some of these molecular changes can be used for pathological diagnosis by employing the corresponding immunohistochemical stains. Molecular alterations of *TP53* are common, being found in approximately 75 % of MPNSTs (Birindelli et al. 2001; Menon et al. 1990; Holtkamp et al. 2007; Upadhyaya et al. 2008). Alterations of *TP53* can also be identified by expression of p53 in tumor cells and p53 is expressed (by immunohistochemistry) in 68 % of MPNSTs (Hlling et al. 1996). Another alteration common in MPNST that can be identified by immunohistochemistry is inactivations of $p16^{INK4A}$ (*CDKN2A*), seen in 50 % of MPNSTs (Nielsen et al. 1999; Kourea et al. 1999). In this case, the inactivation of p16 is identified as loss of expression of the protein in the tumor.

Immunostaining for p53 and p16 in tumors can aid in the pathological evaluation of peripheral nerve sheath tumors in which the differential diagnosis is cellular or atypical neurofibroma versus a low-grade MPNST. Expression of p53 and/or loss of expression of p16 support the diagnosis of early malignant transformation in suspected lesions.

28.1.3.2 Hormonal Effect on Growth

Clinical observation that dermal neurofibromas appear in puberty and grow in number during pregnancy suggests a role for hormone-driven growth (Ferner 2007; Lakkis and Tennekoon 2000). Additional supporting evidence for a hormonal effect on growth of dermal neurofibromas include the expression of progesterone receptor in high percentage of cells in dermal neurofibromas and elevated proliferation rates of Schwann cells in vitro in response to progesterone (McLaughlin and Jacks 2003). The growth-promoting effect of hormones on dermal neurofibromas was recently recapitulated in an NF1 mouse model in which the number of cutaneous neurofibromas was increased in pregnant mice versus nonpregnant mice (Zhu et al. 2002).

By contrast, plexiform neurofibromas do not show increased growth in puberty or pregnancy implying insensitivity to hormonal levels.

28.1.3.3 Cell of Origin of Neurofibroma (Mouse Models)

Plexiform neurofibromas and MPNST arising in genetically engineered mouse models (GEMS) for NF1 have similar histological features to their human counterparts (Stemmer-Rachamimov et al. 2004).

There is controversy in the field regarding the cell of origin of plexiform neurofibromas but most mouse studies point to immature Schwann cells (Wu et al. 2008) or non-myelinating Schwann cells (Zheng et al. 2008). However, although NF1 mouse models developed plexiform neurofibromas and MPNSTs, most did not develop cutaneous neurofibromas. Le and coworkers have proposed that different progenitor cells give rise to dermal and plexiform neurofibromas and identified in the mouse a population of stem cell in the dermis (skin-derived precursors) that form dermal neurofibromas following loss of Nf1 (Le et al. 2009). However, the microenviroment (intraneural or extraneural) may also have a determining effect on the biology, pattern of growth and subtype of neurofibroma formed (Le et al. 2009).

28.1.4 Diagnostic Challenges with Neurofibromas

28.1.4.1 Schwannoma—Neurofibroma—Hybrid Tumor?

Conventional schwannomas and neurofibromas are histologically distinct and have different underlying genetic events. Whereas neurofibromas are associated with NF1 and have biallelic alterations of the *NF1* gene, schwannomas are associated with NF2 and schwannomatosis, and are caused by biallelic loss of the *NF2* gene. The distinction of the two forms is particularly important in the patient with multiple nerve sheath tumors (syndromic) as the pathological diagnosis may

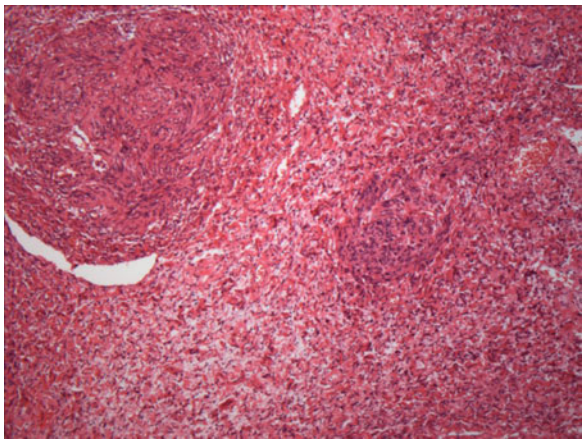

Fig. 28.7 Neurofibroma with Schwann cell nodules, micro. The presence of Schwann cell nodules in a neurofibroma may be prominent; raising the differential with a Schwannoma. In cases that the tumor has characteristics of both schwannoma and neurofibroma, it is described as a "hybrid tumor"

determine the clinical diagnosis for NF1 or NF2 (or schwannomatosis). This is particularly important in cases in which other diagnostic clinical signs/symptoms are equivocal and the patient is difficult to classify (for example in young patients in which the disease is not yet fully manifest or in mosaic patients).

Schwannomas and neurofibromas can be distinguished histologically by several features which reflect the tumors' composition: the type of stroma, the cellular makeup and the relationship of the tumor to the nerve from which it originates. In contrast to the abundant extracellular myxoid matrix seen in neurofibromas, extracellular matrix is minimal in conventional schwannomas, in which tumor cells form compact masses. The cell composition in schwannomas is homogenous. The tumor is composed only of one cell type—Schwann cells. On the other hand, neurofibromas are composed of a complex heterogenous mixture of cells. This characteristic can be highlighted by S100 immunostaining which highlights the Schwann cell population in the tumor. In addition, neurofibromas grow within the nerve, between nerve axons; splaying them apart within the tumor while schwannomas grow as an expansive mass pushing the axons to the periphery of the tumor. Immunostaining for neurofilament highlights the axons within, or at the periphery of, the tumor.

However, some types of schwannomas mimic neurofibromas and vice versa. The myxoid schwannomas, which are often seen in association with schwannomatosis, may be difficult to distinguish from neurofibroma. Similarly, in some neurofibromas, designated as "Schwann cell-rich," there are nodules of proliferating Schwann cells which can form compact nodules (Fig. 28.7). These tumors may be difficult to distinguish from schwannomas.

Hybrid neurofibroma/schwannomas tumors are benign peripheral nerve tumors that are a composite of the two: one part with histological features of neurofibroma and one part with histological features of schwannoma. The term was introduced by Feany et al. (1998). It is unclear if the hybrid tumors represent a true third entity of benign Schwann cell tumors or are peculiar variants of schwannomas and neurofibromas. A recent study of hybrid tumors found them to be most common in the context of NF2 and schwannomatosis. However, a small number of hybrid tumors were also associated with NF1 (Harder et al. 2012).

28.2 Pathology of Malignant Peripheral Nerve Sheath Tumor

A malignant peripheral nerve sheath tumor is a malignant tumor arising from a peripheral nerve or from a pre-existing benign nerve sheath tumor (usually neurofibroma) or a malignant tumor displaying Schwann cell differentiation by histologic, immunohistochemical and ultrastructural features. Other terms used in the literature for MPNST include malignant schwannomas, neurogenic sarcoma, and neurofibrosarcoma.

28.2.1 Gross and Clinical Characteristics of MPNST

MPNSTs are rare in the sporadic (non-NF1) population, accounting for only 5 % of all soft tissue sarcomas (Ducatman et al. 1984). Half of MPNSTs are associated with NF1 and arise from preexisting neurofibromas, 10 % are radiation induced, and the remainder is idiopathic (Ducatman et al. 1986). The lifetime risk of a NF1 patient for developing a MPNST is about 8–13 % (Evans et al. 2002). MPNSTs occur in adults (ages 20–50) but earlier presentation may occur (Ducatman et al. 1984). The most important presenting symptom of malignant change in a preexisting neurofibroma is new onset of pain (Korf 1999). Other indicators are new onset of neurological deficit or rapid growth in the plexiform neurofibroma.

MPNST most commonly arise in large nerves of the extremities (brachial plexus, sciatic nerve). Cranial nerves are rarely involved.

The tumors are fusiform masses and their gross appearance is similar to other sarcomas of soft tissues. They are usually large with heterogeneous cut surface, tan white and firm with foci of necrosis and hemorrhage (Fig. 28.8).

28.2.2 Histopathology of MPNST

MPNSTs are cellular tumors composed of spindled, pleomorphic cells often with areas of geographic necrosis and hemorrhage. Densely cellular areas often alternate with less cellular areas. Some MPNSTs are composed of dense fascicles of spindle cells with an overall appearance resembling fibrosarcomas. In some cases, MPNST display extensive pleomorphism, simulating a high-grade undifferentiated pleomorphic sarcoma.

In contrast to neurofibromas, MPNST is negative for S100 in more than 50 % of cases. When S100 staining is present, it is very focal. Most MPNST are p53 positive and negative for p16.

Divergent differentiation may be seen in MPNST and contributes to its heterogenous histological appearance. 15 % of MPNST show mesenchymal differentiation and contain heterologous elements, such as skeletal muscle, bone, and cartilage.

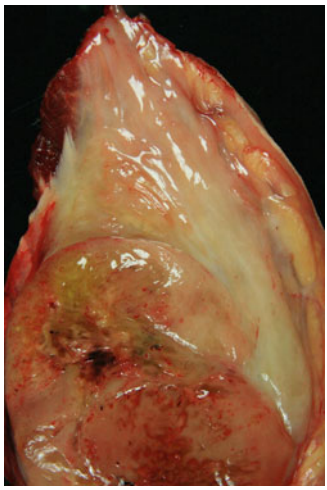

Fig. 28.8 MPNST, gross. A large mass (MPNST) is arising in a preexisting plexiform neurofibroma. In contrast to the neurofibroma which appears homogenous, the MPNST has areas of hemorrhage and necrosis

28.2.2.1 Malignant Triton Tumor

The term Malignant Triton tumor refers to the presence of skeletal muscle differentiation in an MPNST. These tumors are rare and often occur in the setting of NF1 (Ducatman and Scheithauer 1984). The most common sites are head, neck, and trunk. Histologically, the defining feature is the presence of variable number of rhabdomyoblasts with abundant eosinophilic cytoplasm in the MPNST stroma. Occasionally, cross striations may be observed (Fig. 28.9). The rhabdomyoblasts can be highlighted with immunostaining for desmin, MyoD1 and myogenin (Fig. 28.10). Malignant triton tumors are particularly aggressive and have poor prognosis.

28.2.2.2 Glandular MPNST

Glandular MPNST contains scattered well differentiated glands in a spindle cell background. The glands are lined by cuboidal epithelium and mucin may be present. This variant is very rare and almost all of these tumors arise in patients with NF1 (Woodruff and Christensen 1993) (Fig. 28.11). The epithelial cells can be highlighted with cytokeratin and CEA immunostains.

28.2.2.3 Epithelioid MPNST

Epitheloid MPNST is a rare variant of MPNST (less than 5 %), which is composed of plump, epithelioid cells with abundant eosinophilic cytoplasm. Epithelioid MPNST has no association with NF1.

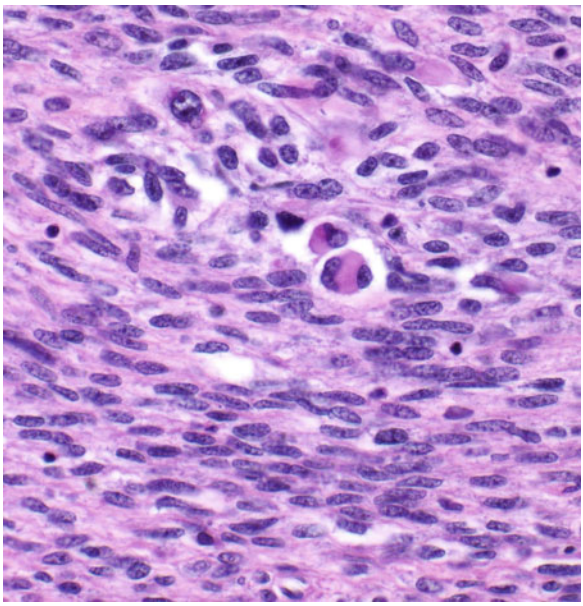

Fig. 28.9 Triton tumor, micro. MPNST with divergent (muscle) differentiation. There are scattered large cells with abundant eosinophilic cytoplasm. These are rhabdomyoblasts

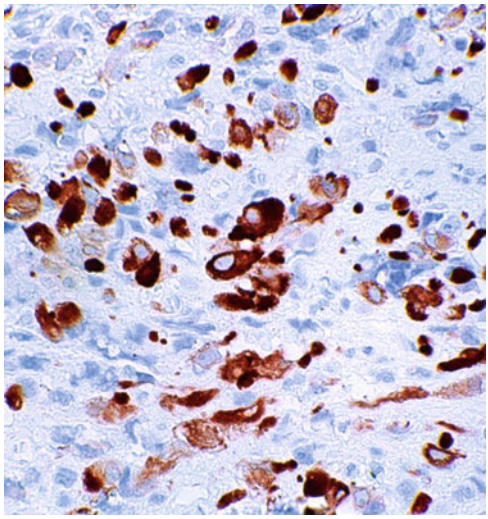

Fig. 28.10 Triton tumor, micro. Immunostaining for desmin highlights rhabdomyoblasts in a Triton tumor

28.2.2.4 MPNST with Angiosarcoma

A rare composite tumor with two components: MPNST and angiosarcoma. Most cases develop in association with NF1 and occur in young individuals (Fig. 28.12).

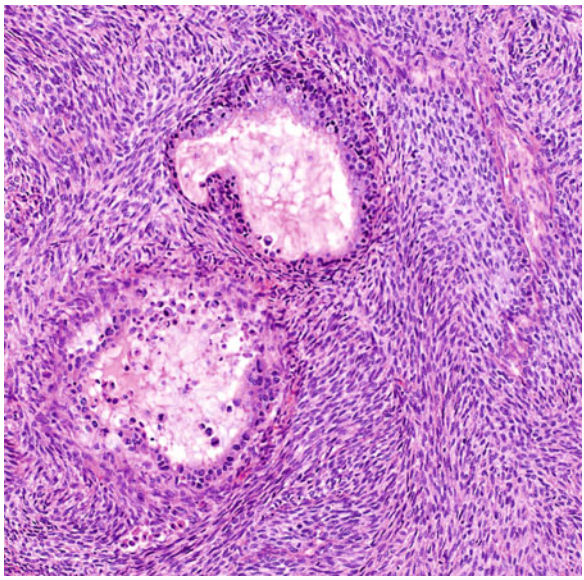

Fig. 28.11 Glandular MPNST, micro. MPNST containing well differentiated glands. These rare tumors are seen mostly in the context of NF1

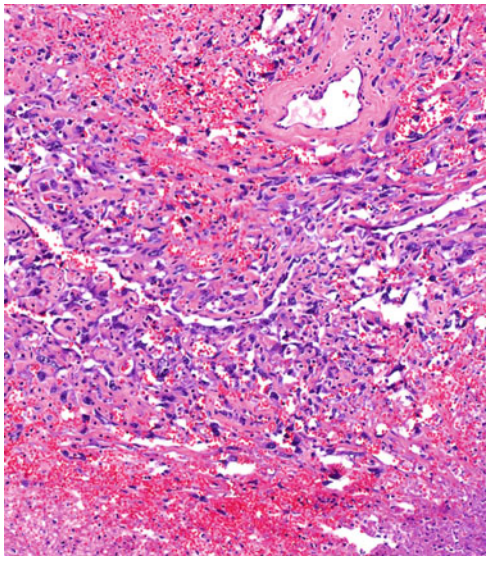

Fig. 28.12 MPSNT with angiosarcoma, micro

28.2.2.5 Grading of MPNSTs

MPSNTs are graded using the same system used for grading other sarcomas which is based on several microscopic criteria. Soft tissue sarcomas are graded on a three-grade system, with grade 1 being the lowest grade and grade 3 being the highest grade (poorly differentiated). The two systems that are most commonly used are the

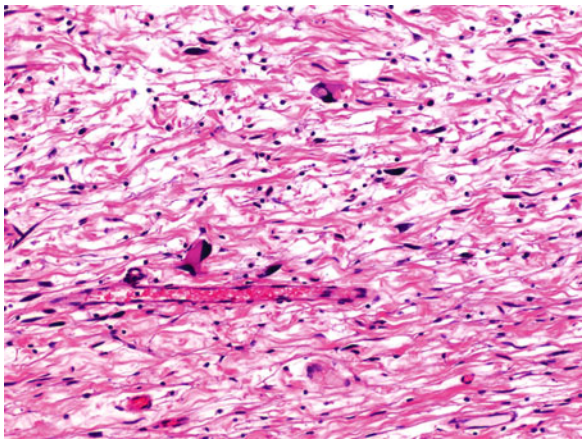

Fig. 28.13 Neurofibroma with large, hyperchromatic nuclei. In the absence of any other atypical features (mitosis, hypercellularity, necrosis), this is not indicative of malignant transformation and may represent degenerative changes

National Cancer Institute (NCI) grading system and the French Federation of Cancer Centers Sarcoma Group (FNCLCC). They take into the consideration the histologic diagnosis, cellularity of the tumor, cellular pleomorphism and mitotic rate. These grading systems are of prognostic value in predicting the metastatic rate of the tumors and overall survival and the grading should be included in the diagnosis of any sarcoma (Trojani et al. 1984; Guillou et al. 1997; Costa et al. 1984).

28.2.3 Diagnostic Challenges with MPNST

28.2.3.1 Neurofibroma—Atypical Neurofibroma—MPNST?

Malignant transformation in a plexiform neurofibroma represents a spectrum of histological changes that are a continuum, which makes it difficult to draw a line between benign and malignant. Atypical features that are associated with malignant transformation are: increased cellularity, mitotic figures and nuclear atypia.

The presence of only one atypical feature is not sufficient for the diagnosis of MPNST. Increased cellularity may be seen in cellular neurofibromas; and nuclear atypia when present in isolation may be secondary to degenerative changes. When all three features are present, the diagnosis of MPNST is warranted. However, the presence of two features only is more problematic and there is controversy weather this constitutes a neurofibroma with atypical features or an MPNST (Scheithauer et al. 1999a, b; Weiss and Goldblum 2008) (Fig. 28.13).

Immunostaining for proliferating cells (Ki67 or MIB1 labeling index) may be helpful in such cases, providing a more sensitive measure than mitotic figures on the rate of proliferation of tumor cells. Additional supportive evidence for malignant transformation is the loss of expression of S100 in tumor cells, as it indicates dedifferentiation. As mentioned above, immunostaining for p53 and/or loss of p16 expression are additional tools for the diagnosis of malignant transformation (Nielsen et al. 1999).

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Chapter 29
Malignant Peripheral Nerve Sheath Tumors: Prognostic and Diagnostic Markers and Therapeutic Targets

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29.1 Introduction

Malignant peripheral nerve sheath tumors (MPNSTs) are the most common NF1-associated malignancy. At present, complete surgical removal is the only successful treatment of MPNST, and the outcome for unresectable, recurrent, or metastatic MPNST remains poor. Half of all MPNSTs arise in individuals with NF1, especially from plexiform neurofibromas through atypical neurofibroma intermediates. Because the prognosis of NF1-associated MPNST appears to be worse than that of sporadic tumors, many studies of MPNST have used NF1 models and compared sporadic and NF1 MPNST. Recent molecular analyses have begun to aid in the identification of genomic abnormalities in MPNST. Importantly, preclinical testing has begun to identify promising therapies to target these aggressive neoplasms. In combination with genome-based discovery and preclinical testing, defining the epidemiology, clinical presentation, diagnosis, and prognostic factors for MPNST is anticipated to allow earlier detection and more successful treatment of MPNST.

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29.2 Epidemiology and Clinical Presentation of NF1 and Sporadic MPNST

MPNSTs account for 5–10 % of all soft-tissue sarcomas and are one of the most common non-rhabdomyosarcoma soft-tissue sarcomas (Ferrari et al. 2011). MPNSTs carry the greatest risk for sarcoma-specific death among all soft-tissue sarcoma histologies (Kattan et al. 2002). Approximately 50 % of MPNSTs are diagnosed in patients with NF1; the lifetime risk of MPNST in NF1 is 8–13 % as compared to 0.001 % in the general population (Ducatman et al. 1986; Evans et al. 2002). MPNSTs are a leading cause of death in NF1: analysis of proportionate mortality ratios demonstrated that those with NF1 were 34 times more likely to have a malignant connective or other soft-tissue neoplasm listed on their death certificate than those without NF1 (Rasmussen et al. 2001). Whereas sporadic MPNSTs are typically diagnosed in late adulthood, MPNST diagnosis peaks at a younger age in patients with NF1, generally in early adulthood (20–50 years) with 10–20 % of cases reported at even younger ages (1–19 years). At diagnosis, NF1-associated MPNSTs tend to be large (>5 cm), invasive, and unresectable (Carli et al. 2005). Sporadic MPNSTs are commonly located in the trunk (34–55 %), extremities (30–45 %), and head/neck (14–19 %), while NF1-MPNSTs are more frequent in the trunk (55 %) compared to sporadic MPNST (35 %) (Stucky et al. 2012; Zou et al. 2009a). This is important and may affect outcome, as central lesions may be less amenable to surgery than are lesions in the extremities (Ducatman et al. 1986; Sordillo et al. 1981).

MPNSTs are at high risk for local recurrence (43 %) and distant metastasis (40 %) (Wong et al. 1998). Another possible reason why NF1-MPNSTs have a poorer prognosis than sporadic MPNSTs is that they may have greater propensity to metastasize. In 62 patients with NF1-MPNSTs, 39 % developed metastatic disease while 58 with sporadic MPNSTs, only 16 % developed metastases (Ducatman et al. 1986). The majority of MPNST metastases in adults are pulmonary, followed in decreasing order of frequency by soft tissue, bone, liver, abdominal cavity, adrenal gland, diaphragm, mediastinal, brain, ovary, kidney, and retroperitoneal (Ducatman et al. 1986). Similar sites of metastasis (lung, lymph nodes, liver, bone, soft tissue, and brain) have been described in children with MPNST (Meis et al. 1992).

A third reason why NF1-MPNST may have poorer prognosis than sporadic MPNST is that most (65–88 %) NF1-MPNSTs arise in preexisting plexiform neurofibromas (Ducatman et al. 1986; Hruban et al. 1990; Meis et al. 1992; King et al. 2003). Symptoms related to an MPNST may overlap with and be difficult to distinguish from those of a benign plexiform neurofibroma as in both conditions patients often present with an enlarging mass, radicular pain, paresthesia, motor weakness or other neurologic symptoms. Pain associated with a mass in NF1 has been described as the greatest risk factor for MPNST (King et al. 2003).

29.3 Diagnosis and Risk for Development of MPNST

Most MPNSTs develop in individuals with NF1 and plexiform neurofibromas. Longitudinal evaluation of individuals with plexiform neurofibromas using MRI demonstrated that growth of plexiform neurofibromas in adults is uncommon and should raise suspicion for malignant degeneration (Tucker et al. 2009). Prospective, longitudinal evaluation of whole body MRI in individuals with NF1 is ongoing in the context of research studies and may allow determining its utility in early detection (e.g. growth) reflecting of malignant degeneration of plexiform neurofibromas. The presence of internal plexiform neurofibromas is also strongly associated with development of MPNST (Tucker et al. 2005). High total tumor burden in patients with NF1 may also be a risk factor for MPNST. Other important risk factors for development of MPNST in NF1 include microdeletion of the *NF1* locus (De Raedt et al. 2003; Leppig et al. 1997) and prior radiation (Ducatman and Scheithauer 1983; Foley et al. 1980; Meadows et al. 1985).

29.3.1 Early Diagnosis of MPNST

Early diagnosis of MPNST is crucial, as only complete surgical resection has been shown to be curative. The biopsy site in NF1-associated MPNST has to be carefully chosen, given that a diagnosis of MPNST in NF1 may be difficult to establish and/or delayed when MPNSTs arise within histologically benign plexiform neurofibromas, and clinical indicators of malignancy may also be features of preexisting benign plexiform neurofibromas. In individuals with NF1 and substantial tumor burden, FDG-PET scanning prior to initiation of treatment may be of value (see below). While imaging modalities can be used to aid in the diagnosis of MPNST and in the selection of the most appropriate tumor area for biopsy, the diagnosis of MPNST requires histopathological confirmation. An oncologic surgeon must approve the biopsy approach in order not to compromise subsequent surgery.

Commonly employed histopathological grading systems (Costa et al. 1984; Parham et al. 1995; Trojani et al. 1984) evaluate a tumor's histologic type or subtype, amount of necrosis, cellularity, numbers of mitoses, and nuclear pleomorphism. The pathology of peripheral nerve tumors has recently been reviewed in detail (Rodriguez et al. 2012). Most MPNSTs are high-grade tumors characterized by high cellularity, mitoses, and necrosis. The American Joint Committee on Cancer (AJCC) soft-tissue sarcoma staging system (Scaife and Pisters 2003) is most commonly used to classify MPNST and utilizes a combination of clinical and pathologic features based on tumor size ($\leq$5 cm versus >5 cm), depth (superficial versus deep), grade (one to four), and the presence or absence of metastasis, resulting in 4 stages (I–IV). Expression of the Schwann cell marker S100β or association of the tumor with nerve bundles can aid in diagnosis. A diagnostic evaluation should include a CT scan of the chest to evaluate for pulmonary parenchymal metastases in addition to detailed imaging of the primary tumor.

Magnetic resonance imaging (MRI) is a frequently utilized imaging modality to evaluate both benign plexiform neurofibromas and MPNSTs. The presence of inhomogeneous lesions (due to necrosis and hemorrhage) and patchy contrast enhancement have been described as characteristic for MPNST (Friedrich et al. 2005). However, the imaging characteristics of MPNST on MRI are not consistent. Malignant lesions can have irregular tumor shapes, infiltrative margins, peripheral enhancement patterns, perilesional edema-like zones, intratumoral cystic lesions or lobulation, presence of high signal-intensity area with heterogeneity on T1-weighted images, lack a target sign on T2-weighted images, or have heterogeneous enhancement after contrast (Bhargava et al. 1997; Chhabra et al. 2011; Li et al. 2008; Matsumine et al. 2009; Wasa et al. 2010). A recent multivariate analysis concluded that intra-tumoral lobulation and the presence of a high signal-intensity area on T1-weighted images were diagnostic of MPNST (Matsumine et al. 2009).

Benign neurofibromas are part of peripheral or cranial nerves, and therefore have well-defined margins, a split fat sign, and the central dot sign [central areas of nerve with reduced signal intensity (Korf 1999; Li et al. 2008). The presence of well demarcated (nodular) lesions $\geq$3 cm on MRI lacking the central dot sign also require attention. Histological analysis of biopsies of nodular rapidly growing lesions [exceeding the growth rate of the plexiform neurofibroma in which they arise] demonstrated areas of benign neurofibroma, but also atypical neurofibroma, and/or MPNST (Meany et al. 2012). These extensive studies demonstrate that MRI often cannot reliably separate a MPNST from a plexiform neurofibroma. Therefore, an increase in the growth rate of a plexiform neurofibroma, particularly in adults, should raise suspicion of malignant degeneration (Dombi et al. 2007; Tucker et al. 2009).

Another valuable albeit imperfect tool for the detection of MPNST in patients with NF1 is 18-fluorodeoxyglucose positron emission tomography (FDG-PET). The sensitivity of this modality for MPNST diagnosis is 89–100 %, with a specificity of 72–95 %, negative predictive value 95–100 % and positive predictive value 50–71 % (Bensaid et al. 2007; Bredella et al. 2007; Ferner et al. 2008). Several studies concur that an elevated SUV_{max} can predict malignancy; mean values of 5.4–7.0 g/mL are associated with malignant lesions and 1.5–2.0 g/mL measured in benign tumors (Benz et al. 2010). However, there is a range of SUV_{max} values (2.5–3.5 g/mL) in which both benign and malignant lesions are detected (Ferner et al. 2000, 2008; Karabatsou et al. 2009; Warbey et al. 2009). For example, in 105 patients with NF1 and symptomatic plexiform neurofibroma, utilizing FDG-PET identified 34 FDG avid sites, 28 malignant lesions were reported (26 MPNST and 2 other malignancies) (Ferner et al. 2008) and the SUV_{max} for all malignant lesions was $\geq$2.5 g/mL. However, of the remaining 6 lesions, on biopsy 4 were benign plexiform neurofibromas, two were atypical neurofibromas a SUV_{max} >3.5 g/mL. The high uptake of FDG in atypical neurofibromas is in agreement with the recent description that these lesions are premalignant and at high risk for transformation to MPNST (Beert et al. 2011). A recent study confirms that atypical neurofibromas and MPNST in NF1 patients with growing or painful nodular plexiform neurofibromas have a higher mean SUV_{max} on FDG-PET compared to patients with a diagnosis of a benign neurofibroma on biopsy (Meany et al. 2012).

29.4 MPNST Serum Biomarkers

The difficulty in definitive MPNST diagnosis by current imaging modalities combined with poor survival rates has led to attempts to identify biomarkers that might foreshadow MPNST growth within plexiform neurofibromas. This analysis is in its infancy. Adrenomedullin, a 52-amino acid secreted peptide that belongs to the calcitonin superfamily of peptides, was differentially expressed in serum of patients with NF1 with more profound elevation in a small number of individuals with MPNST (Hummel et al. 2010), but this has not yet been confirmed in a large cohort of patients. In another study, the growth factor midkine was elevated in individuals with NF1 compared to normal controls, and further increased in a small number of NF1 patients with optic gliomas and MPNSTs (Mashour et al. 2004).

29.5 Prognostic Factors

Negative prognostic factors for disease-specific survival in patients with MPNST include presence of NF1, tumors >10 cm in diameter at diagnosis, primary truncal tumor location, positive surgical margins, high-grade tumors, S100β negative staining, and p53-positive tumors. The association of p53 immunoreactivity and poor outcome was particularly strong for NF1-associated MPNST (Brekke et al. 2009). In most series, the 5-year overall survival rates for patients with a MPNST ranged from 21 % to 52 % and was worse for those with NF1 (Carli et al. 2005; deCou et al. 1995; Ducatman et al. 1986; Moretti et al. 2011; Widemann 2009; Wong et al. 1998; Zou et al. 2009a). However, other studies failed to identify differences in outcome for sporadic versus NF1-associated MPNST (Cashen et al. 2004; Hruban et al. 1990). Tumor stage was also prognostic and patients with metastatic disease at presentation had 2-, 5-, and 10-year disease-specific survival rates of 28 %, 23 %, and 8 % respectively as compared to 54 %, 39 %, and 26 % for those with localized disease ($p = 0.0025$) (Zou et al. 2009a). On a molecular level, gain/amplification of the *CDK4* gene on chromosome 12q14.1 and upregulation of the *FOXM1* gene on chromosome 12p13.3 were significant independent predictors of poor survival in 87 MPNST patients based on array comparative genome hybridization data from 38 MPNST (Yu et al. 2011). Chromosomal losses of 10q and Xq, and gain of 16p, were also associated with reduced MPNST patient survival (Brekke et al. 2010).

While a significant difference in risk of MPNST in females versus males with NF1 has not been noted, a recent study found that survival was better in female patients (46 % and 41.5 %) versus male MPNST patients (22 % and 8.2 %) at 5 and 10 years after diagnosis ($p = 0.05$) (Ingham et al. 2011). The loss of X chromosome loci preferentially in females may contribute to this difference (Brekke et al. 2010). Finally, substantially worse response to chemotherapy in NF1-associated MPNST compared to sporadic MPNST was described in two large studies (Carli et al. 2005; Ferrari et al. 2011). It remains unclear if the outcome for NF1-associated MPNST is worse compared to sporadic MPNST after accounting for prognostic factors.

29.6 Treatment of MPNST

Complete surgical resection is the only curative treatment for sporadic and NF1-associated MPNST (Carli et al. 2005; Ferner and Gutmann 2002; Longhi et al. 2010). The surgical goal is to resect the MPNST with wide negative margins. If complete surgical resection is not achieved, postoperative radiation therapy has a role in improving local control (Wong et al. 1998). Clinical trials have shown that external beam radiation or brachytherapy in addition to limb sparing surgery can improve local control in patients with soft-tissue sarcomas (Pisters et al. 1996; Yang et al. 1998). Adjuvant radiotherapy is therefore recommended to improve local control for intermediate to high-grade lesions >5 cm after a marginal excision (Pisters et al. 1996; Wong et al. 1998; Yang et al. 1998; Ferner and Gutmann 2002). Responses to standard chemotherapy agents used to treat pediatric and adult soft-tissue sarcomas have been reported (Carli et al. 2005; Edmonson et al. 1989; Ferrari et al. 2011; Raney et al. 1987; Valdes and Maurer 1970), but the response rate to chemotherapy in MPNST-specific trials has not yet been evaluated prospectively. Therefore, an ongoing clinical trial of chemotherapy for high-grade unresectable MPNST stratifies patients for sporadic and NF1-associated MPNST, given the substantially worse response to chemotherapy in NF1-associated MPNST compared to sporadic MPNST in retrospective studies (Ferrari et al. 2011; Carli et al. 2005).

Based on evidence that atypical neurofibromas are precursors of MPNST (Beert et al. 2011; see below), consideration should be given to surgical removal of these lesions, if feasible without excessive morbidity. Even close monitoring of atypical neurofibromas may not be sufficient to detect malignant degeneration in time to allow for complete surgical resection. This is exemplified by a patient with NF1 and a long-standing history of an asymptomatic pre-sacral atypical neurofibroma (Fig. 29.1). After sudden growth of the atypical neurofibroma, surgical resection showed positive margins.

29.7 NF1 Mutations Contribute to the Pathogenesis of MPNST

The devastating effect of MPNSTs on NF1 patient survival has provided a powerful impetus to identify the molecular abnormalities responsible for MPNST initiation and progression. Primary among initiating mutations are mutations in the *NF1* gene itself, as at least 95 % of NF1 patients carry a constitutional mutation of the *NF1* tumor suppressor gene located on the long arm of chromosome 17 (17q11.2) together with a functional copy of this same gene (Messiaen et al. 2000), and mutation or loss of the functional second allele are found in at least 40–90 % of NF1 MPNST (Upadhyaya et al. 2008, Bottillo et al. 2009). Although constitutional mutations in NF1 patients occur throughout the *NF1* locus (Messiaen et al. 2000), MPNSTs are particularly prevalent in the 5 % of NF1 patients whose constitutional mutations involve total gene deletion (Wimmer et al. 2006, De Raedt et al. 2003).

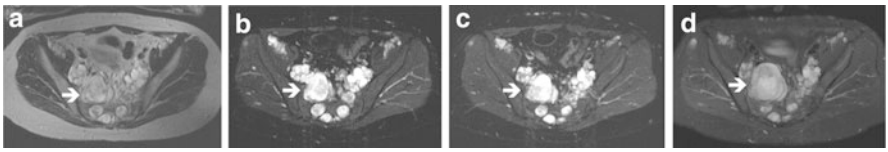

Fig. 29.1 Progression of an atypical neurofibroma to MPNST. Sequential axial MRI of the pelvis of a 23-year-old male with NF1 and extensive plexiform neurofibromas. A right presacral biopsy-proven atypical neurofibroma (*arrow*) remained stable and showed similar imaging characteristics on volumetric MRI analysis over 8 years (**a**) 2003, 47 mL; biopsy-proven atypical neurofibroma (*arrow*) (**b**) 2009, 50 mL; biopsy again confirmed atypical neurofibroma (*arrow*) (**c**) 2011, 47 mL; (**d**) 6 months later in 2012, the patient complained of pain in the left lower leg, the tumor had doubled in size to 98 mL, and a biopsy confirmed high-grade MPNST. The patient underwent surgical resection with positive margins. Importantly, over the 8-year time period, FDG uptake by FDG-PET fluctuated

The possibility that co-deletion of *NF1*-adjacent genes contributes to risk of malignant transformation is being studied; two genes, *RNF125* and *CENTA2*, in the common deleted region show diminished expression in MPNST cells compared to normal Schwann cells (Pasmant et al. 2011a).

Importantly, *NF1* mutations have recently been identified in sporadic MPNST, at least in part explaining why the expression signatures and genomic changes overlap in *NF1* and sporadic tumors (Brekke et al. 2010; Miller et al. 2006; Watson et al. 2004). In the largest study to date, Bottillo et al. (2009) identified *NF1* mutations in 9/22 (41 %) of sporadic MPNST. Somatic *NF1* mutations in NF1 patient and sporadic MPNST are frequently large genomic copy number changes in *NF1* that can include co-deletion of adjacent genes (Upadhyaya et al. 2008; Pasmant et al. 2011c).

29.8 Other Pathways to Formation of MPNST

Mutations in Ras or Ras pathway genes may cause MPNST tumor initiation in some sporadic MPNSTs that lack *NF1* mutations. The quantification of Ras mutations in a large MPNST series of sporadic MPNST has not yet been reported, but in a few cases activating mutations in Ras (N-Ras (1/11) or K-Ras (1/11)) (Perrone et al. 2009) or B-Raf (1/13 MPNST; (Bottillo et al. 2009) mutations have been identified in sporadic MPNST. The microRNA miR-10b can target *NF1* mRNA (Chai et al. 2010); in principle miRs that target *NF1* might also contribute to NF1 tumorigenesis.

29.9 An Intermediate Between Neurofibroma and MPNST: Atypical Neurofibromas

Genomic abnormalities (other than those at the *NF1* locus) have not been detected in neurofibromas, but are numerous in MPNST. Therefore progression of neurofibroma to MPNST must involve a series of genomic alterations. Importantly,

recurrent homozygous loss of the *CDKN2A* locus on chromosome 9p21.3 was detected in 15/16 atypical neurofibromas (Beert et al. 2011). By contrast, the adjacent *CDKN2B* locus was not recurrently deleted. The *CDKN2A* gene encodes $p16^{INK4A}$, a cell cycle inhibitor that inhibits the action of cyclin-dependent kinases 4 (CDK4) and 6 (CDK6). The *CDKN2A* gene also encodes $p14^{ARF}$, which binds to and inhibits the MDM2 ubiquitin ligase, resulting in stabilization of p53. A report that the long noncoding RNA (LncRNA) at the *CDKN2A/B* locus is lost in some neurofibromas may indicate that some surgically removed plexiform neurofibromas contain areas of atypical neurofibroma, reinforcing the critical importance of histopathological analyses of neurofibroma (Pasmant et al. 2011b). Early loss of the *CDKN2A* locus, in the absence of other genomic changes [apart from the *NF1* locus itself] strongly supports the idea that atypical neurofibromas are intermediates between benign neurofibroma and MPNST and suggests that *CDKN2A* loss is a critical early step in neurofibroma progression toward MPNST. Not surprisingly, deletions of the *CDKN2A* locus are present in about 50 % of MPNSTs (Kourea et al. 1999; Nielsen et al. 1999). Mouse models support the importance of this locus in MPNST, as *Nf1+/−;Ink4a/Arf−/−* mice developed MPNSTs, not neurofibroma (Joseph et al. 2008).

29.10 Whole Genome Analysis of MPNST

As in most sarcomas, chromosomal gains, losses, and rearrangements in MPNSTs are highly variable (Wallace et al. 2000). Chromosomal gains and losses are highly variable from tumor to tumor, suggesting that there is more than one series of mutations that lead to MPNST. MPNSTs commonly have hypodiploid or near-triploid karyotypes, with frequent gains on chromosome 7, 8q, and 15q and losses that affect numerous chromosomal regions (1p, 9p, 11, 12p, 14q, 17q, 18, 22q, X, and Y) (Forus et al. 1995; Lothe et al. 1996; Mechtersheimer et al. 1999; Mertens et al. 1995; 2000; Plaat et al. 1999; Schmidt et al. 1999, 2000, 2001). At present, significant effort is being devoted to whole genome analyses of MPNST cell lines and primary tumors, in order to more fully delineate oncogenes and tumor suppressors relevant to MPNST pathogenesis. We anticipate the publication of several studies using array CGH, large scale SNP analyses, and whole genome sequencing to aid in identification of driver mutations in MPNST. Several data series are already available on GEO. These include human mRNA gene expression analysis, DNA array comparative genomic hybridization, and BAC arrays and an MPNST methylome dataset, as well as data from rat and zebrafish MPNST-like tumors (Table 29.1).

Of note, combined genomic somatic copy number alteration (CNA) and Loss of Heterozygosity (LOH) analysis on sets of neurofibromas and MPNST confirmed that recurrent or overlapping CNAs are absent from neurofibromas, while MPNST showed 232 CNAs (encompassing >2,900 genes) and more than 500 genes showed consistent LOH (Upadhyaya et al. 2012).

Table 29.1 Publically available MPNST datasets

Data type	Data series	Species	Sample number	Experiment summary	Citation
Methylome	GSE21714	*Homo sapiens*	3	Comparative methylome analysis of benign and malignant peripheral nerve sheath tumors	Feber et al. (2011)
Gene expression	GSE36144	*Rattus norvegicus*	19	ENU-induced MPNSTs and normal tissue from rat Nervus trigeminus	N/A
Gene expression	GSE14038	*Homo sapiens*	86	86 microarrays, consisting of 77 samples and 9 batch reference samples: 10 NHSC,11 dNFSC,11 pNFSC, 13 MPNST cell lines, 13 dNF,13 pNF, 6 MPNST	Miller et al. (2009)
Gene expression	GSE11493	*Danio rerio*	11	mRNAs from 6 zebrafish MPNSTs arising from rp mutations (3 from rpL35, 1 from rpL14, 1 from rpS5, 1 from rpS11) compared mRNA from 3 MPNSTs arising from p53 M214K/M214K mutation	MacInnes et al. (2008)
Gene expression	GSE6481	*Homo sapiens*	105	105 sarcomas, consisting of 3 MPNST, 16 synovial sarcoma, 19 myxoid liposarcoma, 3 lipoma, 3 well-differentiated liposarcoma, 15 dedifferentiated liposarcoma, 15 myxofibrosarcoma, 6 leiomyosarcoma, 4 fibrosarcoma, and 21 malignant fibrous histiocytoma using an Affymetrix HG-U133A array	Nakayama et al. (2007)
Copy-number alteration	GSE33881	*Homo sapiens*	51	A microarray-based comparative genomic hybridization profiling of MPNST tissue samples	Yang et al. (2011)
Copy-number alteration	GSE16041	*Homo sapiens*	27	Comparison of copy number changes in 24 MPNST samples against 3 benign neurofibromas from NF1 patients, hybridized to a human 32K BAC tiling path array	Mantripragada et al. (2009)

29.11 Common Genomic Changes in Cell Cycle Genes in MPNST

It is generally agreed that *TP53* function is lost in most MPNST. Importantly, an "inactivated p53-associated proliferation" gene expression signature was identified in 18/20 MPNST samples, and p53 inactivation resulted in downregulation of miR-34a, thereby preventing MPNST cell apoptosis in tissue culture (Subramanian et al. 2010). Biallelic inactivation of the *TP53* locus is rare in MPNSTs (Lothe et al. 2001), which led to a suggestion that hemizygous *TP53* mutations might enable progression to MPNST (Upadhyaya et al. 2008). However, in mouse models, only complete loss of *Tp53* correlates with MPNST formation (in the additional absence of *Nf1*; Cichowski et al. 1999; Vogel et al. 1999).

Several mechanisms appear to contribute to *TP53* inactivation at the chromosomal level, with deletions and mutations resulting in loss of function of the *TP53* tumor suppressor gene at chromosome 17p13.1. These are not identified in neurofibromas or in atypical neurofibromas, but are common in MPNSTs (Birindelli et al. 2001; Legius et al. 1994; Menon et al. 1990). Estimates of MPNST with *TP53* genomic alterations vary widely, with some estimates reporting *TP53* mutations in up to 75 % of MPNSTs (Holtkamp et al. 2007; Upadhyaya et al. 2008), while others have much lower estimates (24 %, Verdijk et al. 2010). These differences may be due to the small numbers of tumors often analyzed in each study, the fact that different antibodies have been used to detect p53 expression as a surrogate for DNA-based methods, and/or regional differences within tumors (Brekke et al. 2009; Riddle et al. 2010; Verdijk et al. 2010; Zhou et al. 2003). The stability of p53 can be regulated by MDM2 through $p14^{ARF}$. As $p14^{ARF}$ is retained in some MPNST, this mechanism may in part explain high expression of p53 in the absence of stabilizing *TP53* mutations.

Expression of the Retinoblastoma (Rb) protein, a molecule that impedes cell cycle progression, is lost in 25 % of MPNSTs (Mantripragada et al. 2008; Mawrin et al. 2002). The *PTEN* gene that acts as an off signal for PI3K signaling is the second most frequently mutated tumor suppressor in human cancers. Frequent monosomy of the *PTEN* locus was identified in MPNST (Holtkamp et al. 2008; Perrone et al. 2009), and co-deletion of *Nf1* and *Pten* in mice resulted in formation of neurofibroma and MPNST (Keng et al. 2012); activation of K-RasG12D in combination with *Pten* deletion also resulted in GEM-PNST (Gregorian et al. 2009). It remains unclear how and at what stage in tumor formation the PI3K pathway is de-regulated in human MPNST; (Perrone et al. 2009) failed to identify *PI3KCA* or *PTEN* mutations in MPNST.

29.12 Cell of Origin and Markers of MPNST

The precise identity of the cell type that gives rise to MPNSTs remains unknown. The Schwann cell marker S100β is expressed in about half of all MPNST. Neural crest cells, Schwann cell precursors, immature Schwann cells or tumor cells that de-differentiate from mature Schwann cells all remain possible cells-of-origin. An examination of permanent cell lines derived from murine NPCis (GEM-PNST)

tumors showed that the tumor cells expressed markers characteristic of neural crest cells, Schwann cells, and myogenic cells (Vogel et al. 1999). Expression of markers of neural crest cells is a prominent theme after analysis of human MPNST tumors and MPNST cell lines by gene expression analysis (Miller et al. 2006, 2009). Several gene expression studies have confirmed that the neural crest markers *SOX9* and *TWIST1* are dramatically upregulated in MPNST (Carbonnelle-Puscian et al. 2011; Miller et al. 2006, 2009; Pytel et al. 2010). In an MPNST, cells appear to be dependent on expression of these neural crest genes, as downregulation of *SOX9* caused cell death and downregulation of *TWIST1* decreased cell migration (Miller et al. 2006, 2009). The increased expression, based on immunoreactivity of the lineage markers FOXD3, PAX7, SOX5, and AP-2α in MPNST compared to neurofibroma, has also been described in a series of 34 MPNSTs (Pytel et al. 2010). Placodal markers *EYA*/*SIX* are also upregulated in MPNST cells and tumors (Miller et al. 2010). Using shRNA to diminish *EYA4* expression prevented tumor formation and caused necrosis. This is of interest because EYAs are phosphatases that could in principle be targeted therapeutically.

In addition to lineage markers, other markers of MPNST have been described. Using two-dimensional gels and examination of 1,500 protein spots, MPNST most closely resembled synovial sarcoma and clear cell carcinoma (Kawai et al. 2008). Wilms' tumor 1 (WT1) was expressed in all nerve Schwann cells, neurofibroma, and MPNST cells (Schittenhelm et al. 2010). Using antibody staining, TLE marked 30 % of MPNSTs (Kosemehmetoglu et al. 2009). Tenascin-C (an extracellular matrix glycoprotein) and NNAT (neuronatin) marked 6/6 MPNST, and these markers did not label neurofibromas (Dugu et al. 2010). Cathepsin K, cysteine proteinase that degrades collagens and elastin similarly showed expression in 6/6 MPNST, but not in neurofibroma (Yan et al. 2010). In a single case, an MPNST but not the adjacent neurofibroma showed markers of an angiogenic switch: expression of SMA, vWF, VEGF, and VEGF receptors Flt1 and Flk1 were all upregulated (Gesundheit et al. 2010). Many growing or atypical neurofibromas, and MPNST, stained with anti-CD10 (Cabibi et al. 2009). Some neurofibromas and MPNSTs express hTERT (Patel and Folpe 2009). Finally, c-Fos was expressed in mouse PNST, and required for GEM-PNST formation, and expressed highly in a single human MPNST (Silvestre et al. 2010). As AP-1 transcription factors, including c-fos, act downstream of Ras signaling, this finding is consistent with neurofibromin's major role as a Ras-GAP. A goal remains the identification of markers that distinguish MPNST from synovial sarcoma and clear cell sarcoma, and definitively from surrounding neurofibroma.

29.13 Growth Factor Receptor Amplification in MPNST

A significant body of evidence indicates that aberrant signaling by growth factors and growth factor receptors contributes to MPNST pathogenesis. Amplification of the gene encoding EGFR are frequent (Perry et al. 2002; Holtkamp et al. 2008), EGFR overexpression was correlated with worse prognosis in one study (Keizman et al. 2009), but not another (Tabone-Eglinger et al. 2008). Perrone et al. (2009) studied

27 MPNSTs and found that *EGFR* was amplified in all sporadic MPNST and half of *NF1* MPNST. Importantly, in a number of independent studies, no activating mutations in *EGFR* have been detected, indicating that amplification of the receptor coupled with autocrine and paracrine expression of EGFR ligands including TGFα and HBEGF are likely to facilitate tumor growth. Ligands that activate the EGFR are expressed in 90 % of MPNSTs, suggesting the presence of an autocrine loop in MPNST cells (Holtkamp et al. 2008). By contrast, amplification of the related neuregulin-1 co-receptor erbB2 is rare (Storlazzi et al. 2006; Holtkamp et al. 2008).

An amplicon at chromosome 4q12 encodes three adjacent tyrosine kinase receptor genes: *PDGFRA*, *KIT*, and *VEGFR-2*/*KDR*. In 15 % of a small series of MPNST, the amplicon was present (Zietsch et al. 2010). Among the three genes, *PDGFRA* is most frequently amplified (Badache and De Vries 1998; Holtkamp et al. 2006; Mantripragada et al. 2008) and rarely mutant (Holtkamp et al. 2006). *PDGFRA* and *PDGFRB* were co-amplified in 6/24 MPNST; *PDGFRA* only was amplified in 2/24 (Holtkamp et al. 2008). While amplification of c-Kit can occur, it is a rare event (Holtkamp et al. 2006).

Hepatocyte growth factor is expressed and its c-Met receptor (Mantripragada et al. 2008) is amplified in MPNST. A recent study by Torres et al. (2011) showed that both short hairpin RNAs targeting *MET* and XL184, a compound targeting MET and VEGFR2, decreased MPNST cell growth in tumor xenografts, albeit only if given prior to significant tumor growth.

In vitro studies with MPNST cell lines support functional roles for the molecules encoded by these amplified genes. The proliferation of MPNST cells grown in the presence of limiting amounts of serum is dependent on exogenous EGF; in higher serum concentrations, these cells do not require exogenous EGF, but their proliferation is still inhibited by small molecular inhibitors and neutralizing antibodies targeting the EGFR (DeClue et al. 2000; Johansson et al. 2008; Dilworth et al. 2008). Pharmacologic inhibitors targeting neuregulin-1 receptors (Stonecypher et al. 2005), c-Kit (Badache and De Vries 1998), and PDGF receptors (Holtkamp et al. 2006) similarly inhibit MPNST mitogenesis. PDGF (Aoki et al. 2007) and c-Met (Su et al. 2004) have also been implicated in MPNST cell invasion. At present, it is not clear whether all of these growth factors and growth factor receptors are uniformly required for MPNST pathogenesis or whether distinct subsets of MPNSTs exist in which tumorigenesis is dependent on only some of these molecules. In addition, several histology-specific trials with agents targeting PDGFR, C-KIT, and EGFR were completed, all without achieving responses or meaningful disease stabilization as single agents (Albritton et al. 2006; Chugh et al. 2009; Maki et al. 2009; Schuetze et al. 2010).

29.14 Preclinical Therapeutics in MPNST: Intracellular Pathways

The signaling pathways downstream of activated receptors, including Ras, PI3K, ERK, mTOR, Akt, and Ral have been explored. This is especially important as Nf1 loss is expected to activate signaling pathways downstream of Ras-GTP. An

excellent review (Katz et al. 2009) summarized several years of findings; in no case did any single agent more than transiently block MPNST growth in GEM or xenograft models (Zou et al. 2009b). Newer studies have been more promising found that the PI3K/mTOR inhibitor PI-103 induced G1 cell cycle arrest and autophagy in MPNST cells. The use of hyaluronan oligomers suppressed drug transporter activity and inhibited growth of MPNST xenografts, with synergy between oligomers and doxorubicin (Slomiany et al. 2009). Exposure of MPNST cells to 4-OH-tamoxifen or the calmodulin inhibitors fluoperazine and W-7 mimic this effect on MPNST proliferation and apoptosis in an MPNST xenograft into mouse sciatic nerve in an 18-day assay (Byer et al. 2011). Inhibition of aurora kinases using MLN2036 caused prolonged MPNST growth arrest in 9/10 mice in a xenograft model, leading to cell arrest in the G2/M phase of the cell cycle (Patel et al. 2012). The combination of histone deacetylase inhibitor PC-24791, which promotes autophagy, and block of autophagy with chloroquine abrogated MPNST xenograft graft growth and promoted cell apoptosis; however, very short trials were conducted so the durability of the response is not yet known (Lopez et al. 2011). Using rapamycin, or its analog RAD001, only transiently blocked MPNST growth in the NPCis model of GEM-PNST, a xenograft MPNST model, and an explant model (Johannessen et al. 2008; Johansson et al. 2008; Bhola et al. 2010). In an exciting new development, the cytostatic effect of rapamycin was converted to a cytotoxic effect by combination with agents that promote proteotoxic/endoplasmic reticulum (ER) stress, a type of stress that cancer cells exhibit due to accumulation of unfolded proteins in the ER (De Raedt et al. 2011). Further enhancing ER stress using HSP90 inhibitors, coupled with rapamycin led to dramatic tumor regression in this model, correlated with profound damage to the ER and mitochondria, and cell death. This was prevented by antioxidant treatment indicating a dependence on reactive oxygen species. Based on these important data, combinatorial clinical trials are being considered.

29.15 Conclusions

MPNST continue to be associated with poor outcome, especially in individuals with NF1. Efforts aiming at earlier diagnosis of MPNST, such as (1) identification of biomarkers, which allow separation of plexiform neurofibromas from MPNST, (2) use of longitudinal MRI to monitor individuals with NF1 and plexiform neurofibromas for changes suggestive of malignant degeneration, and (3) evaluation of neurofibroma burden and metabolism using FDG-PET combined with heightened awareness of the potential for malignant degeneration in patients with a growing mass or pain, may allow for more successful surgical treatment. Detailed description of clinical findings in patients enrolled in MPNST-specific trials including details on NF1 manifestations such as internal tumor burden, gender, and treatment responses separately for NF1 and sporadic tumors, will allow further understanding of sporadic and NF1-associated tumors. A complete catalogue of

genome-level alterations in MPNST is evolving, allowing an increasing understanding of the molecular pathogenesis of MPNST. The preclinical evaluation of targeted agents in meaningful preclinical models supports the potential utility of combinatorial therapies to benefit patients who participate in MPNST clinical trials.

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Chapter 30
NF1 Mutations in Hematologic Cancers

Tiffany Chang and Kevin Shannon

30.1 Introduction

In 1958, a paper describing a young child with café au lait macules and an unusual myeloproliferative neoplasm (MPN) raised the possibility of a potential interaction between neurofibromatosis type 1 (NF1) and hematologic cancer (Royer et al. 1958). This report proved prescient as subsequent clinical and epidemiologic research firmly established that the incidence of this aggressive MPN, which is now known as juvenile myelomonocytic leukemia (JMML), is increased 200–500-fold in children with NF1 (Bader and Miller 1978; Stiller et al. 1994). Together with the dominantly inherited cancer predisposition in NF1, the discoveries that affected individuals who inherit inactivating germ line mutations in the *NF1* gene (Marchuk et al. 1991; Viskochil et al. 1990; Wallace et al. 1990) and that the *NF1* gene product, neurofibromin, encodes a GTPase-activating protein (GAP) for Ras (Basu et al. 1992; DeClue et al. 1991; Xu et al. 1990a, b) suggested that *NF1* was a tumor suppressor gene (TSG). Indeed, genetic analysis of JMML bone marrows revealed loss of constitutional heterozygosity (LOH) at the *NF1* locus in multiple children with NF1, which invariably involved deletion of the normal parental allele in familial cases (Miles et al. 1996; Shannon et al. 1994). A subsequent study demonstrating biallelic inactivation of *NF1* in JMML cells provided genetic proof that the *NF1* gene is a "classic" recessive TSG that undergoes somatic loss of function in cancer cells (Side et al. 1997). Importantly, biochemical analysis of leukemia cells from children with NF1 revealed reduced neurofibromin-specific GAP activity, elevated levels of Ras-GTP, and aberrant activation of the Raf/MEK/ERK kinase effector pathway

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(Bollag et al. 1996). Together, these data identified *NF1* as a TSG in hematopoietic cells and suggested that deregulated Raf/MEK/ERK signaling contributes to leukemogenesis. As described below, recent preclinical studies in genetically accurate mouse models of JMML strongly support this hypothesis and provide a rationale for implementing clinical trials of MEK inhibition in this aggressive cancer.

Defining aberrant Ras signaling as the molecular mechanism linking NF1 to JMML also provided new insights into leukemogenesis. In particular, it is now clear that JMML is fundamentally a disease of hyperactive Ras with germ line or somatic mutations in the *NRAS*, *KRAS*, *PTPN11,* and *CBL* genes found in >90 % of cases (Lauchle et al. 2006; Loh 2011). Individual patients almost invariably have a mutation in only one of these genes, which is consistent with the idea that the genes mutated in JMML encode protein components of a conserved biochemical network regulating the proliferation, differentiation, and survival of hematopoietic stem cells (HSC) and their progeny.

The association of NF1 with JMML also provides one of the clearest links between development and cancer. As discussed in Chap. 32, NF1 is the founding member of a group of inherited disorders characterized by overlapping phenotypic features and germ line mutations that deregulate Ras/Raf/MEK/ERK signaling. Constitutional mutations in each of the other known JMML genes cause developmental disorders of the Noonan syndrome spectrum (Cirstea et al. 2010; Niemeyer et al. 2010; Schubbert et al. 2006; Tartaglia et al. 2001). Interestingly, however, these germ line *PTPN11*, *NRAS,* and *KRAS* mutations typically encode proteins with weaker biochemical gain-of-function than the corresponding somatic mutations found in JMML (Cirstea et al. 2010; Keilhack et al. 2005; Kratz et al. 2005; Schubbert et al. 2006). The fact that only young children with NF1 are at greatly increased of hematologic cancer is another intriguing developmental observation, particularly since hematopoiesis is a highly dynamic process with proliferation and maturation of functionally immature cells required to replace billions of leukocytes, erythrocytes, and platelets each day. This observation suggests a fundamental interaction between "seed and soil" with somatic *NF1* inactivation only capable of causing leukemia in specific developmental contexts or under exceptional circumstances.

Somatic mutations represent key events in malignant transformation of human cells. Patients with NF1 inherit a germ line mutation in one allele and frequently sustain a "second hit," either through LOH or a somatic mutation that inactivates the remaining normal allele that confers a growth advantage in cell lineages that can initiate tumorigenesis. While the identification of somatic "second hit" mutations in tumors arising in NF1 patients has been problematic due to the large size of the *NF1* gene and low levels of expression in many tissues, the classification of somatic mutations is essential for understanding the complex molecular mechanisms underlying tumorigenesis. Furthermore, consistent with a central role for neurofibromin in cellular function, emerging data from recent cancer genome sequencing studies has implicated *NF1* in the pathogenesis of sporadic malignancies in patients without neurofibromatosis (Cancer Genome Atlas Network 2008).

Comprehensive review articles addressing the interaction of inherited predispositions with aberrant Ras signaling and leukemia as well as the biology and treatment of JMML have appeared recently (Lauchle et al. 2006; Loh 2011; Schubbert et al. 2007). Here, we focus on the role of *NF1* mutations in hematologic cancers and emphasize recent advances in the field, therapeutic possibilities, unanswered questions, and future directions.

30.2 Hematologic Malignancies in NF1

JMML is a relentless myeloid malignancy of young children with an estimated incidence of 1.2 cases per million per year with a median age of 2 years at diagnosis (Lauchle et al. 2006; Loh 2011). Early reports refer to JMML as "juvenile chronic myeloid leukemia" (JCML) due to the low percentage of blasts in the bone marrow, the presence of immature monocytic cells in the blood, and prominent splenomegaly as a result of leukemic infiltration. Over time, this name was reconsidered once chronic myeloid leukemia in all age groups emerged as a distinct molecular and clinical entity driven by the *BCR-ABL* fusion (Sawyers 1999). Among the adult hematologic cancers, JMML most closely resembles the proliferative subtype of chronic myelomonocytic leukemia (CMML), and these two disorders are now classified together as "overlap" syndromes by the World Health Organization because they demonstrate clinical and pathologic features of both MPN and myelodysplastic syndrome (MDS). Studies demonstrating frequent mutations in *NRAS* and *CBL* in JMML and CMML underscore their common molecular basis (Dunbar et al. 2008; Kalra et al. 1994; Loh et al. 2009; Miyauchi et al. 1994; Onida et al. 2002; Sanada et al. 2009).

In 1991, Emanuel and coworkers (1991) reported that JMML cells selectively form abnormal numbers of colony-forming-unit granulocyte-macrophage (CFU-GM) colonies in methylcellulose cultures containing low concentrations of the growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF). CFU-GM hypersensitivity remains one of the tests used to establish a diagnosis of JMML, which can be problematic due to potential overlap with chronic infections, inherited or acquired immunodeficiency syndromes, and other hematologic malignancies. Molecular testing has also assumed an important role as detecting a mutation in *NF1*, *NRAS*, *KRAS*, *PTPN11,* or *CBL* strongly supports a diagnosis of JMML in patients with suggestive clinical findings (Loh 2011). Kotecha et al. (2008) recently reported that $CD33^{+}CD14^{+}CD38^{lo}$ cell subpopulation of bone marrow cells from patients with JMML, CMML, and the M4 or M5 subtypes of an acute myeloid leukemia (AML) displays a distinct pattern of STAT5 hyperphosphorylation in response to GM-CSF. This biochemical signature was observed in patients with mutations in multiple different JMML genes, including *NF1*.

Although a family history of NF1 is helpful is establishing a diagnosis of JMML, many cases arise in patients with new mutations, and the cardinal phenotypic features of NF1 may be subtle or absent in young children. Furthermore,

developmental disorders of the Noonan syndrome spectrum may manifest some features of NF1 (Schubbert et al. 2007). Demonstrating a germ line mutation (including the precise amino acid substitution) is highly relevant for clinical management as spontaneous regression of MPN is likely in patients with constitutional *PTPN11* mutations and has also been observed in the context of *KRAS* and *CBL* mutations (Loh 2011; Niemeyer et al. 2010; Schubbert et al. 2006). These patients frequently present with a JMML-like myeloid disease in the first few weeks of life and currently receive aggressive supportive care without cytotoxic chemotherapy or hematopoietic stem cell transplantation (HSCT). By contrast, children with NF1 who are diagnosed with JMML tend to be older, and we are not aware of any cases with loss of the normal *NF1* allele that resolved without treatment.

Simple mathematics indicates that the interaction between NF1 and JMML is complex as the overall incidence of JMML (~1 in 1,000,000) is much lower than the frequency of NF1 (1 in 3,000). Furthermore, *NF1* mutations account for only 10–15 % of JMML cases. Based on these data, the *individual* risk of JMML is low in NF1—perhaps 1 in 2,500. This is vastly different from TSGs such as *RB1* or *WT1* where the incidence of retinoblastoma or Wilms' tumor in patients with germ line mutations is 80 % or higher and raises the question as to what additional factors might contribute to JMML susceptibility. About 75 % of JMML patients are boys (Loh 2011). This male predilection persists in NF1 (Shannon et al. 1992) and has also been observed in other developmental disorders. For example, Niemeyer et al. (2010) recently reported a fascinating multigenerational pedigree with a germ line *CBL* mutation in which only males succumbed to JMML. Beyond male sex, we are not aware of any evidence supporting the existence of modifier mutations that cooperate with *NF1* in leukemogenesis. In particular, there are no reports of NF1 pedigrees in which multiple children developed JMML. Alternative models that might explain the relatively low risk of JMML in NF1 include (1) a very small and developmentally restricted population of susceptible target cells that can initiate JMML after somatic loss of the normal *NF1* allele and/or (2) cooperating mutations in addition to *NF1* inactivation might be required for full leukemic transformation. The existence of somatic chromosome 7 deletions (monosomy 7) in ~30 % of JMML is consistent with the second possibility (Loh 2011). The recent widespread availably of whole genome and exome sequencing will allow investigators to pursue this question in greater depth over the next few years.

As noted above, molecular analysis of JMML specimens from children with NF1 frequently reveals loss of constitutional heterozygosity (LOH), which invariably involves the *NF1* allele inherited from the unaffected parent in familial cases (Miles et al. 1996; Shannon et al. 1994; Side et al. 1997). Whereas initial reports did not uncover biallelic inactivation of *NF1* in some cases (Miles et al. 1996; Shannon et al. 1994; Side et al. 1997), a recent study of 10 patients by Steinemann et al. (2010) that deployed state-of-the-art technologies for sequencing and mapping small deletions identified a *NF1* mutation with loss of the normal allele in five cases, compound heterozygous mutations in three, and interstitial heterozygous deletions in two others. These data, which indicate that biallelic *NF1* inactivation is a consistent feature of JMML in NF1 patients, also raise the possibility that occasional cases without *NF1* alterations represent young children with other

developmental disorders who are incorrectly diagnosed with NF1. Surprisingly, uniparental disomy (UPD) affecting the *NF1* locus and a large segment of flanking sequences on chromosome 17q underlies somatic loss of the normal *NF1* allele in JMML (Flotho et al. 2007; Stephens et al. 2006). Interestingly, a similar genetic mechanism is operative in CMML and JMML patients with *CBL* mutations (Dunbar et al. 2008; Loh et al. 2009; Sanada et al. 2009).

Current treatments for JMML are unsatisfactory. Cytotoxic chemotherapy regimens used to treat AML are largely ineffective, and hematopoietic stem cell transplantation (HSCT) is the current standard of care (Loh 2011). While curative in ~50 % of patients, HSCT carries substantial risks of adverse late effects, particularly in young children. Patients who do not enter remission after HSCT or who relapse later have a dismal prognosis with the great majority dying from accelerated JMML or after evolution to AML. Monosomy 7 may be detected after progression to AML (Kaneko et al. 1989). Given our current understanding of JMML pathogenesis, small molecule inhibitors of kinase effector pathways that are activated by Ras represent an appealing therapeutic approach.

Clinical observations suggest that children and adults with NF1 are at increased risk of developing MDS and AML after genotoxic treatment for a different primary cancer. Maris and coworkers (Maris et al. 1997) reported five pediatric patients who developed MDS or AML after receiving aggressive, multimodal therapy for a primary solid tumor. Interestingly, the bone marrows of each patient showed monosomy 7 and retained the normal *NF1* allele. Similarly, two adults with NF1 rapidly developed MDS after being treated for AML (Papageorgio et al. 1999). While inconclusive, these provocative observations in NF1 patients are congruent with studies in mice summarized later in this chapter showing that heterozygous *Nf1* inactivation cooperates strongly with radiation to induce MDS and other common therapy-induced human cancers.

30.3 Somatic *NF1* Mutations in Hematologic Cancer

Somatic *NF1* mutations have recently been reported in lung adenocarcinomas, breast cancer, ovarian serous carcinomas (OSCs), neuroblastoma, and leukemias (Ding et al. 2008; Haferlach et al. 2010; Holzel et al. 2010; Laycock-van Spyk et al. 2011; Parsons et al. 2008; Sangha et al. 2008). Some *NF1* aberrations are relatively frequent in these tumors and therefore may hold specific prognostic and diagnostic importance. For example, the Cancer Genome Atlas Network identified a high incidence of *NF1* mutations in de novo glioblastoma multiforme (GBM), with at least 23% (47/206) of patient samples analyzed harboring somatic *NF1* mutations or deletions (Cancer Genome Atlas Network 2008; Parsons et al. 2008). Similarly, somatic *NF1* mutations were detected in 22% (9/41) of primary OSCs, including six cancers that exhibited biallelic inactivation (Sangha et al. 2008). Interestingly, all nine of the OSC samples also contained a *TP53* mutation, which is consistent with studies of malignant peripheral nerve sheath tumors arising in NF1 patients (Menon et al. 1990).

Most *NF1* mutations are intragenic and have been found over the entire gene, commonly leading to splicing mutations that result in truncation and inactivation of the encoded protein, neurofibromin. Another genetic aberration involves microdeletions that affect the whole *NF1* locus, leading to a more developmental phenotype associated with increased risk of developing JMML with risk of progression to AML. Mechanisms include point mutations, deletions, and an effect of recombinational events leading to UPD (Ding et al. 2008; Haferlach et al. 2010; Holzel et al. 2010; Laycock-van Spyk et al. 2011; Parsons et al. 2008; Sangha et al. 2008; Side et al. 1998). A previous study reported biallelic *NF1* inactivation in patients with JMML lacking clinical evidence of NF1, although the early age at disease onset does not preclude the existence of a germ line mutation in these cases (Side et al. 1998).

Perhaps the best data describing somatic *NF1* mutations in hematologic malignancies come from a recent study by Parkin et al. (2010) who identified a subset of adult AML patients in which *NF1* is functionally inactive, resulting in increased Ras signaling and sensitivity to mTOR inhibition. These investigators utilized high-density SNP microarrays to analyze purified blast and paired buccal DNA samples from 95 adult AML patients. They identified recurrent, somatically acquired microdeletions on chromosome 17q with a ~0.9 Mb minimally deleted region that included the *NF1* locus. Somatic copy number alterations in *NF1* were identified in 11 out of 95 (12%) of leukemia specimens in this cohort (10 patients with heterozygous deletions and 1 with copy gain). The authors found reduced *NF1* expression in AML blasts from 3 out of 10 patients with heterozygous deletions and absent *NF1* expression in three samples that had normal *NF1* copy number. Accordingly, Ras-GTP was substantially increased in AML cases with absent NF1 expression. The authors found *NF1* null AML CD34+/CD38− cells to be sensitive to treatment with rapamycin, thereby implicating mTOR as a potential therapeutic target for this subgroup of adult AML patients and potentially shedding light on the pathogenic role of *NF1* in adult AML. Consistent with a role of somatic *NF1* mutations in adult myeloid malignancies, Kolquist and coworkers (2011) used comparative genomic hybridization microarrays to interrogate 35 bone marrows from adults with MDS and identified recurrent cryptic changes or deletions involving the *NF1* locus on chromosome band 17q11.2 in three cases.

Studies of pediatric patients have provided additional insights into the role of somatic *NF1* in hematologic cancers. A recent report described leukemia-associated *NF1* inactivation in children with T-cell acute lymphoblastic leukemia (T-ALL) and *MLL*-rearranged AML (Balgobind et al. 2008). High-resolution genomic screening of 103 patients with pediatric T-ALL and 71 patients with *MLL*-rearranged AML uncovered a recurrent cryptic deletion, del(17)(q11.2), in 3 patients with T-ALL and 2 patients with *MLL*-rearranged AML. *NF1* expression in the del(17)(q11.2)-positive patients with T-ALL and *MLL*-rearranged AML was significantly reduced compared with leukemias that retained both *NF1* alleles. T-ALL specimens with somatic *NF1* mutations also harbored cooperating mutations in *NOTCH1* and other genes. Mutation analysis revealed small frameshift mutations that disrupted the *NF1* coding region in 3 of 4 del(17)(q11.2)-positive patients, leading to biallelic

inactivation of *NF1* in these cases. There were no homozygous somatic *NF1* mutations identified in these patients, suggesting that the frequency of biallelic inactivation is low. The authors, however, comment that other mechanisms of *NF1* inactivation including UPD could have been missed (Balgobind et al. 2008). Interestingly, a recent SNP analysis of pediatric ALL and JMML without underlying NF1 showed that there was no UPD involved in the *NF1* region. Additionally, approximately 20% of adult AMLs have large regions of UPD, although none involve the *NF1* locus (Mullighan et al. 2007). These studies suggest that UPD of the *NF1* locus may be a rare finding in somatically derived leukemias.

30.4 Insights from Animal Models

In 1994, two groups developed "first-generation" mouse models of NF1 by gene by disrupting exon 31 of the *Nf1* gene (Brannan et al. 1994; Jacks et al. 1994). Homozygous *Nf1* mutant (*Nf1*−/−) embryos died in utero with cardiac defects. About 10% of heterozygous *Nf1* mutant mice (*Nf1*+/−) on a mixed C57Bl/6 × 129Sv strain background spontaneously developed a JMML-like MPN during the second year of life, which was associated with somatic loss of the wild-type allele (Jacks et al. 1994). Importantly, *Nf1*−/− fetal hematopoietic cells showed a similar pattern of hypersensitive CFU-GM colony growth in response to GM-CSF as human JMML bone marrow cells (Bollag et al. 1996; Largaespada et al. 1996), and transplanting fetal liver cells from *Nf1*-deficient embryos into irradiated recipients rescued the hematopoietic compartment and efficiently induced a JMML-like MPN (Largaespada et al. 1996; Zhang et al. 1998). Studies in this mouse model showed that *Nf1* inactivation leads to deregulated growth in multiple hematopoietic compartments and confers a durable proliferative advantage in competitive repopulation assays (Zhang et al. 1998, 2001). Intercross and adoptive transfer experiments also demonstrated that GM-CSF signaling plays a central role in driving the aberrant growth of *Nf1* mutant hematopoietic cells in vivo (Birnbaum et al. 2000; Kim et al. 2007). Together, these studies provide compelling evidence that neurofibromin restrains myeloid cell growth by negatively regulating Ras output in response to cytokine growth factors and demonstrated that *Nf1* inactivation is sufficient to recapitulate the cardinal features of JMML both in vitro and in vivo.

Nf1+/− mice have been used to test the biologic significance of clinical observations suggesting that individuals with NF1 are susceptible to myeloid malignancies and other secondary cancers that arise after treatment with genotoxins (Maris et al. 1997; Papageorgio et al. 1999). An initial study by Mahgoub et al. (1999a) showed that an alkylating agent (cyclophosphamide) or a topoisomerase II inhibitor (etoposide) cooperated with heterozygous *Nf1* inactivation in leukemogenesis. A majority of these chemotherapy-associated leukemias showed loss of the wild-type *Nf1* allele, and fluorescence in situ hybridization (FISH) implicated

acquired uniparental disomy as the underlying genetic mechanism (Mahgoub et al. 1999a). Cyclophosphamide was more potent in this model, and alkylating agents are closely associated with monosomy 7 in cases of therapy-induced leukemia in patients with and without NF1 (Le Beau et al. 1996; Smith et al. 2003). Based on these data, Chao et al. (2005) randomized *Nf1*+/− mice and their wild-type littermates to receive cyclophosphamide only, low-dose whole-body irradiation only, low-dose whole-body irradiation + cyclophosphamide, or no treatment. This study found that heterozygous *Nf1* inactivation cooperated strongly with irradiation and with irradiation + cyclophosphamide to generate a diverse spectrum of secondary cancers that model treatment-induced cancers in humans. MDS and MPN were common in these mice (Chao et al. 2005). Most recently, Nakamura and coworkers (2011) developed a novel technique for administering focal doses of radiation to mice in multiple 300-cGy fractions. This protocol accurately models current clinical radiation oncology practice for human cancer. As in the previous study, *Nf1*+/− mice were highly susceptible to a variety of radiation-induced cancers including squamous cell carcinomas, sarcomas, and MDS. Interestingly, this study identified a strong relationship between radiation dose and tumorigenesis with hematologic cancers showing a high incidence in mice that received a total of 1,500 cGy and sold tumors predominating at 3,000 cGy. Most of the solid tumors generated by Chao et al. and Nakamura et al. demonstrated somatic loss of the normal *Nf1* allele; however, LOH was uncommon in hematologic cancers. At this time, it is uncertain if *Nf1* haploinsufficiency cooperates with other genotoxin-induced mutations in leukemogenesis or if the normal *Nf1* allele is inactivated by point mutations or other genetic mechanisms. It is nevertheless intriguing that none of the human therapy-induced myeloid malignancies studied to date have shown LOH at the *NF1* locus. Together, these studies establish *Nf1*+/− mice as a tractable in vivo platform for probing mechanisms of treatment-induced cancer in humans and for testing preventive strategies.

To overcome the embryonic lethal phenotype resulting from homozygous *Nf1* inactivation, Zhu and colleagues (Zhu et al. 2001) generated a conditional mutant *Nf1* allele by introducing LoxP sites into the *Nf1* locus. In this "second-generation" mouse model, the $Nf1^{flox}$ allele is functionally wild-type in the basal state. Tissue-specific somatic *Nf1* inactivation is achieved by expressing Cre recombinase in discrete cell types (Zhu et al. 2001, 2002). Exploiting the interferon-inducible *Mx1-Cre* strain (Kuhn et al. 1995) to ablate *Nf1* in hematopoietic cells consistently results in MPN, which is characterized by leukocytosis, splenomegaly, hyperproliferation, impaired apoptosis, and cytokine hypersensitivity of bone marrow CFU-GM progenitors (Le et al. 2004).

Lauchle et al. (2009) performed insertional mutagenesis with the MOL4070LTR retrovirus in *Nf1* mutant mice to model progression from JMML to AML. The cooperating mutations provided by retroviral insertions are selected for in cells that have inactivated *Nf1* due to *Mx1-Cre* expression. In this screen, *Mx1-Cre*, $Nf1^{flox/flox}$ mice that were injected with MOL4070LTR demonstrated a higher incidence of AML as well as reduced latency. Whereas the MPN was only transplantable into lethally irradiated hosts (Le et al. 2004), recipient mice that were injected with

AML cells after a sublethal dose of irradiation (450 cGy) developed leukemia 3–6 weeks later. Southern blot analysis with a probe that detects MOL4070LTR sequences demonstrated a pattern of clonal integrations that was stable in transplant recipients. Furthermore, cloning genes that were disrupted or activated by retroviral insertions uncovered candidate genes that might cooperate with *Nf1* inactivation in leukemogenesis and also demonstrated that MOL4070LTR-induced AML models the genetic diversity found in advanced human cancers.

30.5 Preclinical Trials and Therapeutic Implications

Genetic, cell biologic, and biochemical studies of human hematologic cancers with *NF1* mutations and extensive data from mouse models provide compelling evidence that hyperactive Ras signaling is a "driver" of aberrant growth in these cancers. Although restoring neurofibromin function through gene therapy or other strategies is appealing in principle, this approach is not feasible at this time. As a result, there is intense interest in testing treatments directed at inhibiting Ras or its downstream effectors.

In the first preclinical trial performed in genetically engineered *Nf1* mutant mice, the JMML model that was developed by transplanting *Nf1*-deficient fetal liver cells into irradiated recipient mice (Largaespada et al. 1996) was used to evaluate the efficacy of the farnesyltransferase inhibitor L-744,832 (Mahgoub et al. 1999b). Farnesyltransferase is a target for rational drug design in *NF1* mutant tumors because it catalyzes a posttranslational modification of Ras that is essential for membrane targeting and biologic activity (Gibbs et al. 1994). Whereas L-744,832 abrogated CFU-GM colony growth in a dose-dependent manner, this inhibition was not selective for *Nf1* mutant progenitor colonies. In vivo treatment at the maximally tolerated dose (MTD) inhibited H-Ras prenylation in mice; however, *Nras* and *Kras* remained fully processed. Twenty-two recipients of fetal liver cell transplants were evaluated in two controlled preclinical studies that tested L-744,832 at a daily dose of 40 or 80 mg/kg. Recipients that were repopulated with *Nf1*−/− cells and developed MPN showed no improvements in white blood cell counts or splenomegaly. The observed lack of therapeutic benefit was almost certainly due to the existence of an alternative enzyme (geranylgeranyl transferase) that efficiently prenylates N-Ras and K-Ras when farnesyltransferase is blocked (Downward 2003; James et al. 1996). These preclinical data provided early evidence that farnesyltransferase inhibitors do not reduce Ras signaling in vivo and suggested that this would be problematic in treating cancer and tumors in NF1 patients (Mahgoub et al. 1999b). This prediction was later borne out in the clinic (Downward 2003). The challenges of using transplanted fetal liver cells to perform this trial also underscored the need to develop better a better preclinical model of JMML, and this goal was achieved by using the *Mx1-Cre*, $Nf1^{flox/flox}$ strain for subsequent studies (Le et al. 2004).

Lauchle et al. (2009) obtained the MEK inhibitor CI-1040 from Pfizer, Inc. and found that 25–50 μM of CI-1040 abrogated CFU-GM colony formation from *Mx1-Cre*, $Nf1^{flox/flox}$ bone marrow in response to GM-CSF. As in the farnesyltransferase trial, there was no beneficial therapeutic index in vitro, as CFU-GM growth from WT bone marrow was inhibited at similar concentrations. In a randomized preclinical trial, *Mx1-Cre*, $Nf1^{flox/flox}$ mice with MPN that received CI-1040 at the MTD (100 mg/kg twice a day) showed no improvement in leukocyte counts or splenomegaly. Pharmacodynamic analysis demonstrated transient inhibition of MEK kinase activity in bone marrow cells from CI-1040-treated mice, which was assessed by measuring the ability of GM-CSF to increase the levels of phosphorylated ERK (pERK) (Lauchle et al. 2009).

Using *Mx1-Cre* to activate endogenous oncogenic $Kras^{G12D}$ expression causes a JMML-like MPN in mice, which progresses rapidly, leading to death by 4 months of age (Braun et al. 2004; Chan et al. 2004). In contrast to the failure of CI-1040 in *Mx1-Cre*, $Nf1^{flox/flox}$ mice with MPN (Lauchle et al. 2009), treatment with 901 unexpectedly induced hematologic improvement and enhanced survival in the *Mx1-Cre*, $Kras^{G12D}$ model of JMML (Lyubynska et al. 2011). Interestingly, this beneficial therapeutic index was associated with persistence of $Kras^{G12D}$ hematopoietic cells, indicating that MEK inhibition altered the growth of mutant cells but did not eliminate them. To address the question of whether differential efficacy of CI-1040 and 901 was due to the underlying genotype (*Nf1* versus *Kras*) or to the longer duration of inhibition in mice treated with 901, Chang et al. (2011) recently tested 901 in *Mx1-Cre*, $Nf1^{flox/flox}$ mice with MPN. They observed dramatic hematologic improvement with 901, indicating that sustained MEK inhibition is essential for response in this model. As in Kras mutant mice that were treated with 901, *Nf1* mutant cells persisted in the bone marrows of these animals (Chang et al. 2011).

In addition to testing CI-1040 in *Mx1-Cre*, $Nf1^{flox/flox}$ mice with MPN, Lauchle and coworkers (2009) evaluated the effects of MEK inhibition on AMLs that were generated by injecting *Mx1-Cre*, $Nf1^{flox/flox}$ mice with the MOL4070LTR retrovirus. Unexpectedly, in vitro blast colony growth was completely abrogated at much lower drug concentrations (0.25–5 μM) than in WT or MPN bone marrows, suggesting that *Mx1-Cre*, $Nf1^{flox/flox}$ AMLs are more dependent on Raf/MEK/ERK signaling. To test this hypothesis in vivo, 25 recipient mice that were transplanted with four independent leukemias were randomized to receive CI-1040 at the MTD or control vehicle. CI-1040 had dramatic effects on AML growth, with treated mice showing a reduction in circulating with blood cell counts and markedly prolonged survival (24 versus 7 days). These data demonstrated that the biologic response to a molecularly targeted inhibitor is strongly modulated by the genetic context in which a disease-initiating mutation occurs. Specifically, although the MPN induced by *Nf1* inactivation was unresponsive to CI-1040, progression to AML was associated with enhanced sensitivity to this drug.

Although mice with AML that received CI-1040 consistently responded to treatment, all ultimately relapsed and died of AML. Interestingly, these recurrent leukemias were remarkably less sensitive to CI-1040 in vitro and did not respond to treatment in secondary recipients. Subsequent genetic analysis revealed retroviral

integrations in CI-1040-resistant leukemias that were not seen in the drug sensitive, parental AML. These indicate a process of clonal selection during treatment. The authors went on to identify and validate members of the *RasGRP* family and *Mapk14* (which encodes p38) as underlying resistance to CI-1040. Interestingly, and in contrast to *Mx1-Cre*, $Nf1^{flox/flox}$ mice with MPN, PD0325901 was no better than CI-1040 in controlling the growth of *Nf1* mutant AML (Lauchle et al. 2009).

30.6 Summary and Future Directions

The association of NF1 with JMML proved crucial for elucidating the central role of hyperactive Ras in the molecular pathogenesis of this MPN and, more broadly, in myeloid leukemogenesis (Sawyers and Denny 1994; Van Etten and Shannon 2004). Given this, why the low risk of leukemia in patients with NF1 is somewhat surprising and the reason it is restricted to a narrow developmental window is unknown. Addressing this question by characterizing different mechanisms of growth control in fetal and adult HSC may shed light on plexiform neurofibroma and other complications of NF1 that are initiated early in life.

Until recently, the large size of the *NF1* gene and its low expression levels in many tissues were substantial barriers to characterizing the role of somatic mutations in sporadic human cancer. The recent availability of high sensitivity SNP arrays and efficient methodologies for sequencing cancer genomes have been "game changers" in this arena, and *NF1* has emerged as a common target of inactivating mutations in a wide range of human cancers, including leukemia. The prevalence of somatic *NF1* mutations in hematologic malignancies is certain to rise over the next few years as more samples are sequenced. As this work proceeds, an important question will be to clarify whether somatic mutations inactivate both alleles or whether *NF1* contributes to malignant growth by haploinsufficiency. Based on the precedent of other tumor suppressor genes, the answer to this question is likely to be "both." Whereas *NF1* inactivation initiates tumorigenesis in patients with NF1, it likely represents a cooperating mutation that is acquired as a late event in leukemia and other sporadic cancers. This, in turn, will almost certainly modulate the effects of NF1 loss on tumor biology and drug response.

From a therapeutic perspective, the existing genetic, biochemical, and preclinical data suggest that aberrant Raf/MEK/ERK signaling plays a fundamental role in driving the abnormal growth of *Nf1* deficient hematopoietic cells. In particular, the impressive responses of *Kras* and *Nf1* mutant mice with MPN to PD0325901 and early data strongly support initiating clinical trials of MEK inhibitors in NF1 and non-NF1 patients with JMML. In addition, while treatment with 901 induced transient remission in *Nf1* mutant AML, these leukemias invariably relapsed. These data, which are generally consistent with the results of preclinical and clinical trials of signal transduction inhibitors in other contexts, suggest that developing effective treatments for advanced cancers characterized by germ line or somatic

NF1 mutations will require drug combinations. Finally, while neurofibromin plays an integral role in regulating the Ras GTPase switch, the biochemical consequences of *NF1* inactivation are distinct from oncogenic *NRAS* and *KRAS* mutations. In particular, oncogenic Ras proteins are "locked" in the GTP conformation due to a combination of defective intrinsic hydrolysis and resistance to GAPs. By contrast, the Ras proteins in *NF1*-deficient cells are intrinsically normal, cycle in response to guanine nucleotide exchange factors (GNEFs), and can be regulated by other cellular GAPs. These differences may influence therapeutic responses and perhaps even the types of drugs that will be useful. For example, researchers at Genentech recently reported a class of small molecules that inhibit Ras signaling by interfering with the SOS GNEF (Maurer et al. 2012). Compounds of this nature should prove more efficacious in *NF1* mutant cancers, where hyperactive Ras signaling is more dependent on growth-factor-mediated nucleotide exchange than in malignancies with mutations in *NRAS* or *KRAS*. Thus, the distinct consequences of *NF1* inactivation as well as similarities to oncogenic Ras signaling networks will be likely to modulate the responses of hematologic cancers and other malignancies to targeted anticancer drugs.

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Chapter 31
Legius Syndrome: Diagnosis and Pathology

Hilde Brems, Ludwine Messiaen, and Eric Legius

31.1 Introduction

Neurofibromatosis type 1 (NF1) (MIM#162200) is a common genetic disorder with an incidence of 1/3,000 births (Huson et al. 1989). Typical clinical manifestations are multiple café-au-lait macules (CALM), freckles in the armpits or groin, iris Lisch nodules, neurofibromas and optic pathway gliomas. Other NF1-associated characteristics include macrocephaly, short stature, scoliosis, learning disabilities and specific skeletal abnormalities. In addition, individuals with NF1 have an increased risk of specific malignancies (Brems et al. 2009). Individuals diagnosed with NF1 fulfil the diagnostic National Institutes of Health (NIH) criteria (No Authors 1988). NF1 belongs to the group of RASopathies which are genetic syndromes with germline mutations in genes coding for proteins affecting the RAS–MAPK pathway (Zenker 2011). Several syndromes belong to this group: Noonan, cardio-facio-cutaneous (CFC), LEOPARD, Costello, capillary malformation–arteriovenous malformation (CM-AVM), *CBL*-mutation associated syndrome and hereditary gingival fibromatosis. These syndromes have an overlap in phenotype although distinct clinical features can often be distinguished. In 2007, a new syndrome was identified with germline inactivating mutations in *SPRED1*. Initially the syndrome was named as neurofibromatosis type 1-like syndrome (MIM #611431), but later it was renamed Legius syndrome (Upadhyaya 2008; Stevenson and Viskochil 2009). Legius syndrome also belongs to the group of RASopathies.

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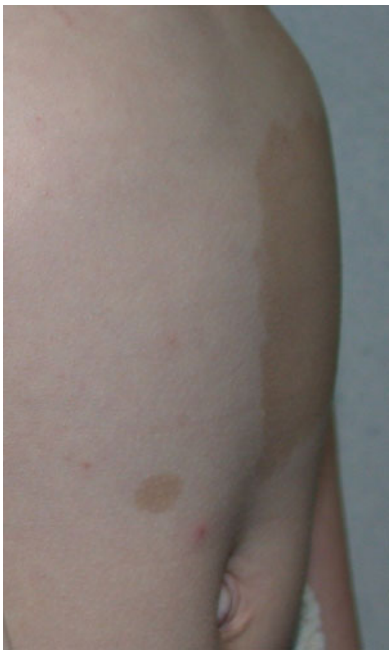

Fig. 31.1 Typical abdominal café-au-lait spots in a child with Legius syndrome

31.2 Clinical Presentation

Neurofibromatosis type 1-like syndrome (Legius syndrome) was initially reported in five families (37 individuals) and in six unrelated individuals (Brems et al. 2007). Because of the important clinical overlap with NF1, it is impossible to diagnose the syndrome based on clinical features alone. Individuals with Legius syndrome exhibit multiple café-au-lait macules (CALM) with or without axillary or inguinal freckling (Fig. 31.1). In the initial report, macrocephaly was present in 13/31 individuals and Noonan-like features were mentioned in five individuals. Learning problems and/or attention deficit were found in six children and developmental delay in one child. Lipomas were present in 14 individuals and a mild pectus excavatum in three individuals. A childhood renal cancer, lung cancer and a colonadenoma developed in one individual each. Other typical NF1-associated features were systematically absent, i.e. Lisch nodules, neurofibromas, specific bone lesions, optic pathway gliomas and malignant peripheral nerve sheath tumours.

Seven subsequent papers confirmed the findings of the first report. CALM (108/119) with (53/115) or without freckles in the armpit or groin were reported as well as macrocephaly (15/93) and Noonan-like features (14/89) (Pasmant et al. 2009a, b; Spurlock et al. 2009; Messiaen et al. 2009; Muram-Zborovski et al. 2010; Denayer et al. 2011a; Laycock-Van Spyk et al. 2011; Spencer et al. 2011). Learning

disabilities (24/115), developmental delay (20/85) and attention deficit (15/87) were frequently reported (Pasmant et al. 2009a, b; Messiaen et al. 2009; Muram-Zborovski et al. 2010; Denayer et al. 2011a; Laycock-Van Spyk et al. 2011; Spencer et al. 2011). A cognitive phenotype was suggested in 15 individuals with Legius syndrome (Denayer et al. 2011b). Children with Legius syndrome showed a lower average performance IQ compared to unaffected family members. The cognitive phenotype is milder in Legius syndrome as compared to NF1.

Additional clinical features were mentioned: pectus excavatum or carinatum 12/87 (Messiaen et al. 2009; Denayer et al. 2011a; Laycock-Van Spyk et al. 2011; Spencer et al. 2011) and unilateral postaxial polydactyly (3/159) (Messiaen et al. 2009; Denayer et al. 2011b). In some individuals with Legius syndrome, lipomas were present (Pasmant et al. 2009a; Messiaen et al. 2009; Denayer et al. 2011a, b; Spencer et al. 2011).

It is not yet clear if malignancies are associated with Legius syndrome. Some events were reported only once: monoblastic acute leukaemia (Pasmant et al. 2009a, b; same individual), giant cell tumour, dermoid tumour of the ovary, breast cancer (Messiaen et al. 2009), vestibular schwannoma and desmoid tumour (Denayer et al. 2011a). No second hit was identified in *SPRED1* in the monoblastic acute leukaemia (Pasmant et al. 2009a, b; same individual) and no evidence was found for the involvement of *SPRED1* in a small set of juvenile myeloblastic leukaemia cases (Batz et al. 2010).

There is currently no evidence that these manifestations are part of the clinical spectrum of Legius syndrome. Since the total number of reported cases is relatively small (159: all ages) and since a large subset of diagnosed individuals are children, rare complications in adults could have been missed. To detect a rare complication with a prevalence of 1 %, at least 250 adults with Legius syndrome would have to be investigated (Messiaen et al. 2009). Therefore it is important to keep collecting detailed clinical information from newly diagnosed individuals with Legius syndrome.

31.3 Molecular Genetics of *SPRED1*

31.3.1 Genomic Organisation of SPRED1 and Molecular Testing

SPRED1 is located on chromosome band 15q13.2 and spans 104.4 kb of genomic sequence. It contains seven exons. The transcript is 7255 bp in length with an open reading frame coding for 444 amino acids. Molecular genetic testing can be performed on DNA or RNA extracted from peripheral white blood cells (Brems et al. 2007; Messiaen et al. 2009). Copy number changes (CNCs) such as multi-exon deletions and whole *SPRED1* gene deletions are detected by multiplex ligation-dependent probe amplification (MLPA), array comparative genomic hybridisation (arrayCGH) or other techniques to detect deletions (Spencer et al. 2011).

SPRED1 mutations are rare compared to *NF1* mutations. A pathogenic *NF1* mutation is found in 43 % of sporadic cases with CALM with or without freckling and no other NF1 diagnostic criteria. Only in 1.3 % of the sporadic cases with this phenotype has a pathogenic *SPRED1* mutation been found. In the cohort of familial cases with the same phenotype, a pathogenic *NF1* mutation was found in 73 % of cases and a pathogenic *SPRED1* mutation in 19 % of the cases (Messiaen et al. 2009). No pathogenic *SPRED1* mutations have been reported in individuals with Lisch nodules, neurofibromas or optic pathway gliomas.

31.3.2 Mutational Spectrum and Polymorphisms

Familial as well as sporadic cases are reported, the largest study described 39 % (13/33) sporadic cases in the *SPRED1* positive group (Messiaen et al. 2009), but this study was biased towards sporadic cases. All identified mutations and polymorphisms in *SPRED1* are summarised in the Leiden Open Variation Database (LOVD) and will be updated regularly. This database is accessible online at http://www.lovd.nl/SPRED1 and contained, at the start in April 2012, 89 different mutations identified in 146 unrelated individuals (Brems et al. 2012). Mutations follow the HGVS nomenclature and are numbered in relation to the *SPRED1* cDNA reference sequence (NM_152594.2) with A as the start codon +1.

The database contains a large spectrum of mutations (Brems et al. 2012): 29 missense (33 %), 28 frameshift (31 %), 19 nonsense (21 %), eight CNCs (9 %), two splicing (2 %), one silent (<1 %), one in-frame deletion (<1 %) and a mutation affecting the initiation codon (<1 %). The mutations are spread over the entire gene. Most of the mutations are truncating or expected to result in the absence of SPRED1 protein synthesis (28 frameshift, 19 nonsense, eight CNCs, two splicing, one mutation affecting the initiation codon). From the 29 missense mutations, eight are probably benign rare variants, three clearly pathogenic, and one was suggested to be pathogenic (based on amino acid conservation and segregation within a large family). Seventeen missense mutations are still unclassified and need further investigation. Only a minority of missense mutations have been classified as pathogenic.

Polymorphisms are described in the public dbSNP135 database with three validated neutral missense variants (p.Asp274Gly, p.Val309Ala and p.Pro315Leu). p.Asp274Gly and p.Pro315Leu are probably benign rare variants since they were not detected in individuals suspected with Legius syndrome (L.M., personal communication). Functional analysis of the missense change p.Val309Ala confirmed its neutral role (H.B., personal communication).

Only one somatic mutation has been published so far, in the original paper of Brems et al. (2007).

Melanocytes were cultured from a CALM from an individual with Legius syndrome. A somatic frameshift *SPRED1* mutation (c.304dupA; p.Thr102Asnfs*7) was identified in addition to the germline *SPRED1* mutation (p.Arg24*).

31.3.3 Genotype–Phenotype Correlation

There is no specific genotype–phenotype correlation reported for *SPRED1* mutations. Spencer et al. (2011) published seven individuals with multi-exon or whole *SPRED1* deletions. The phenotype of these individuals did not differ from that of individuals with a point mutation in *SPRED1*.

31.4 Biological Basis

31.4.1 Protein Structure and Function

SPRED1 is a 55 kDa member of the Sprouty/SPRED family of proteins. The SPRED family contains three members (SPRED1–3) and the Sprouty family has four members (SPRY1–4). Human SPRED1 contains 444 amino acids. Three functional domains have been identified, i.e. an N-terminal EVH-1 domain, a central c-KIT binding domain (KBD) and a C-terminal SPRY related (SPR) domain (reviewed by Bundschu et al. 2007).

Most reports focus on mouse Spred1 and only limited data are available on human SPRED1. Mouse Spred1 is predominantly expressed in brain and in some foetal tissues. It is enriched in the germinal zones of the central nervous system during neurogenesis (Kato et al. 2003; Engelhardt et al. 2004; Phoenix and Temple 2010). Spred1 inhibits cortical progenitor self-renewal and proliferation and maintains the ventricular zone structure (Phoenix and Temple 2010). Spred1 is highly expressed in interleukin-3-dependent mouse hematopoietic cell lines and bone marrow-derived mast cells (Nonami et al. 2004).

Spred1 is a negative regulator of the Ras–MAPK signalling pathway (Wakioka et al. 2001). Growth factor-mediated Spred1 expression inhibits the activation of MAPK by suppressing phosphorylation and activation of Raf after growth factor stimulation (Fig. 31.2). The KBD is not essential for ERK suppression, but contributes to it (Wakioka et al. 2001; Nonami et al. 2004). There is no effect on Akt or Rac activation. Spred1 localises to the lipid raft/caveoli and collaborates with caveolin in inhibiting Erk activation (Nonami et al. 2005). The EVH-1 domain and KBD have an effect on the actin cytoskeleton and the EVH-1 and SPR domains on stress fibre formation (Miyoshi et al. 2004).

Several Spred1 interaction partners have been identified: i.e. microtubule-associated protein/microtubule affinity-regulating kinase-activating kinase (MARKK), testis-specific protein kinase (TESK1) (Johne et al. 2008), fibroblast growth factor receptor like-1 (FGFRL1) (Zhuang et al. 2011), DYRK1A (Li et al. 2010) and SHP2 (Quintanar-Audelo et al. 2011).

Two different microRNAs have been linked to *Spred1*, miR-126 and miR-212. MicroRNA-126 promotes angiogenesis by repressing Spred1 expression (Wang et al. 2008). Spred1 negatively regulates mast cell activation, which is modulated by miR126 (Ishizaki et al. 2011). MicroRNA-212 is upregulated in the dorsal

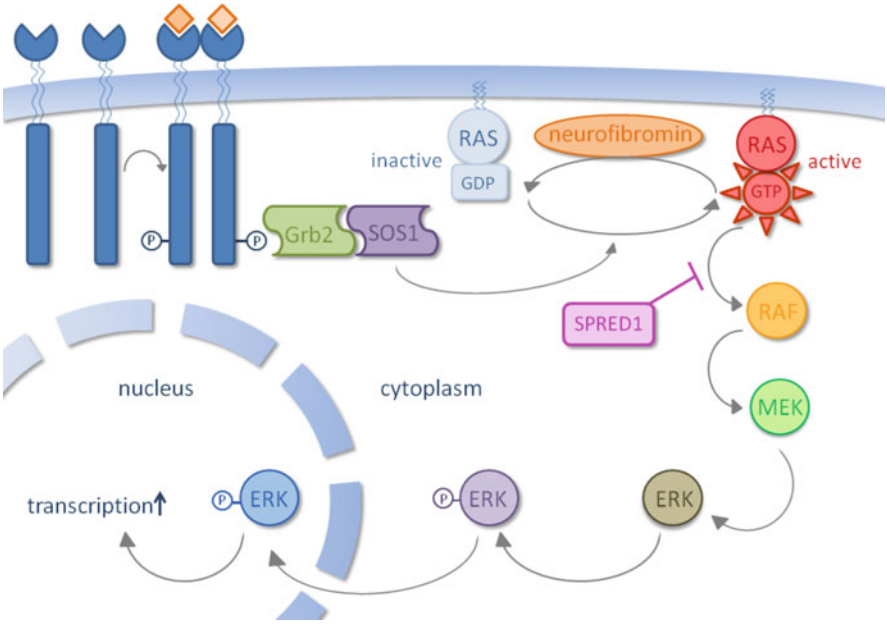

Fig. 31.2 Growth factor binding results in dimerisation of the receptor tyrosine kinase growth factor receptors. This results in phosphorylation of the receptors and activation of the adaptor proteins (GRB2, SOS1). Active RAS is bound to GTP and inactive RAS to GDP. Neurofibromin stimulates the intrinsic GTP-ase activity of RAS and stimulates the transition to the inactive GDP-bound form of RAS. SPRED1 inhibits the activation of RAF by active GTP-bound RAS

striatum in rats with extended access to cocaine (Hollander et al. 2010). MicroRNA-212 activates Raf1 and amplifies the CREB–TORC cascade by repressing Spred1.

Human wild-type SPRED1 inhibits growth factor-induced RAS–MAPK activation. Both the EVH-1 and SPR domains appear to be necessary for this inhibitory effect (Brems et al. 2007). *SPRED1* is expressed in human melanocytes (Brems et al. 2007). Melanocyte cultures from control skin ($SPRED1^{+/+}$), from normal skin ($SPRED1^{+/-}$) and a café-au-lait spot ($SPRED1^{-/-}$) from the same patient with Legius syndrome were compared (Brems et al. 2007). After stimulation with stem cell factor, the highest levels of activated MEK and ERK were seen in the $SPRED1^{-/-}$ melanocytes. The $SPRED1^{+/+}$ melanocytes showed the lowest levels of MEK and ERK activation, with intermediate levels in the $SPRED1^{+/-}$ melanocytes.

31.4.2 Functional Analysis of Missense Mutations

Only a small proportion of detected missense mutations could be classified as pathogenic. We propose combining familial segregation data, amino acid evolutionary conservation and two different functional MAPK pathway assays to

classify *SPRED1* missense mutations (for classification see online database, http://www.lovd.nl/SPRED1). Some missense mutations that do not affect the MAPK pathway in two different functional tests might still be pathogenic by affecting another pathway. However, at this moment, all known familial *SPRED1* missense mutations segregating with the phenotype and all *de novo* missense mutations show functional defects in at least two MAPK-pathway functional tests.

31.4.3 Mouse Model

The Spred1 protein is highly conserved during evolution with a 93.5 % protein identity between human and mouse. The mouse *Spred1* mRNA is expressed in foetal brain, heart, lung, liver and bone (Engelhardt et al. 2004). In adult tissue, *Spred1* is predominantly expressed in brain (Kato et al. 2003; Engelhardt et al. 2004). *Spred1* knockout mice are fertile but they have a lower weight and a shortened face (Inoue et al. 2005, Brems et al. 2007).

The Spred1 mouse model was investigated for hippocampus-dependent learning and memory in several learning tasks (Denayer et al. 2008). In the hidden version of the Morris water maze, $Spred1^{-/-}$ mice showed decreased performance in learning and memory. In the visual discrimination task (T-maze), $Spred1^{-/-}$ mice performed significantly worse in nearly all stages of visual discrimination training. During the final mixed-trial presentations, $Spred1^{-/-}$ mice did not show any learning. The $Spred1^{+/-}$ mice performed at an intermediate level between the $Spred1^{-/-}$ and $Spred1^{+/+}$ mice.

Electrophysiological recordings on hippocampal brain slices from *Spred1* knockout mice identified short- and long-term synaptic plasticity defects (Denayer et al. 2008), including a deficiency in long-term potentiation (LTP) and long-term depression (LTD) in Schaffer collaterals in the CA1 region.

These findings are comparable to the learning and synaptic plasticity defects in the $Nf1^{+/-}$ mice (Cui et al. 2008), and confirm the importance of the RAS–MAPK pathway in learning and memory. The learning and synaptic plasticity defects in adult $Nf1^{+/-}$ mice can be acutely rescued with lovastatin treatment (Cui et al. 2008). Similar experiments have not yet been reported in *Spred1* mice.

31.5 Management and Follow-Up

Individuals with Legius syndrome do not display tumour complications typically reported in NF1. Hence a different and less intense medical follow-up seems to be more appropriate. However, clinical data were only available from 159 individuals with Legius syndrome as this chapter was being written. Therefore, it is essential to collect information on more patients and to offer clinical surveillance for patients with Legius syndrome. Rare complications would have been missed due to the limited amount of clinical data available. A correct diagnosis has important

implications for prognosis, counselling and prenatal diagnosis. The diagnosis of Legius syndrome may relieve the psychological burden in families who are otherwise expecting more serious NF1-related tumour complications. We however recommend paying specific attention to potential learning and behavioural problems in children with Legius syndrome. We hope that clinical trials will show benefit of specific treatments for the cognitive and behaviour problems in children with NF1. Hopefully, such specific treatments will become available for both children with NF1 and Legius syndrome.

In cohorts negative for a pathogenic *NF1* and *SPRED1* mutation, there is still a small group of unclassified individuals with familial multiple café-au-lait spots. In sporadic cases, mosaicism for an *NF1* mutation can be present, explaining the NF1 features in the absence of a detectable *NF1* mutation in white blood cells (Maertens et al. 2007). It is possible that some patients with multiple café-au-lait spots without an *NF1* or *SPRED1* mutation harbour mutations in genes with a link to the RAS-MAPK pathway, as was recently shown in *CBL*-mutation associated syndrome (Niemeyer et al. 2010; Pérez et al. 2010).

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Chapter 32
The RASopathies: Syndromes of Ras/MAPK Pathway Dysregulation

William E. Tidyman and Katherine A. Rauen

32.1 The RASopathies

The RASopathies are a newly defined class of developmental disorders that are caused by germline mutations in genes which encode components of the Ras/mitogen-activated protein kinase (MAPK) pathway. The Ras/MAPK pathway, one of the best studied signal transduction pathways, is critical in cell cycle regulation, differentiation, growth, and cell senescence, all of which are essential to normal mammalian development (Fig. 32.1). Therefore, it is not surprising that its dysregulation may have profound consequences in development. These RASopathies each exhibit unique phenotypes. However, owing to the common mechanisms of Ras pathway dysregulation, they share many overlapping characteristics, including craniofacial dysmorphology, cardiac malformations, cutaneous, musculoskeletal, and ocular abnormalities, neurocognitive impairment, hypotonia, and an increased cancer risk. Taken together, the RASopathies are one of the largest groups of malformation syndromes known, affecting ~1:1,000 individuals. Neurofibromatosis type 1 (NF1) was the first syndrome to be identified as being caused by germline mutations in the Ras/MAPK pathway (Cawthon et al. 1990; Viskochil et al. 1990; Wallace et al. 1990) and since then, numerous other syndromes have been identified as well. These syndromes include (1) Noonan syndrome (NS) caused by activating mutations in *PTPN11* (Tartaglia et al. 2001), *SOS1* (Roberts et al. 2007; Tartaglia et al. 2007), *RAF1* (Pandit et al. 2007; Razzaque et al. 2007), *KRAS* (Schubbert et al. 2006), *NRAS*

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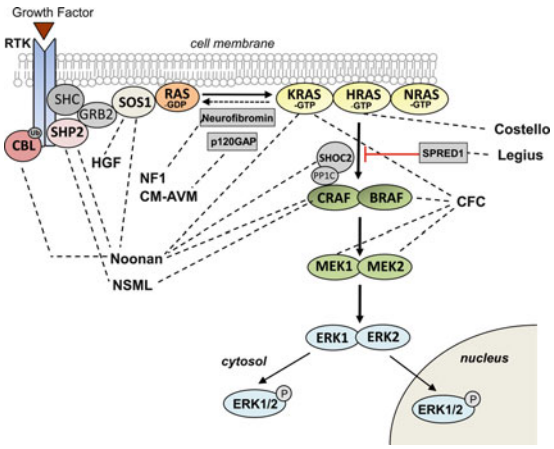

Fig. 32.1 The Ras/mitogen-activated protein kinase (MAPK) signal transduction pathway. The MAPK signaling pathway of protein kinases is critically involved in cell proliferation, differentiation, motility, apoptosis, and senescence. The RASopathies are genetic syndromes caused by mutations in various genes within the Ras/MAPK pathway (indicated by *dashed lines*). These syndromes include: Noonan, Noonan syndrome with multiple lentigines (NSML), hereditary gingival fibromatosis 1 (HGF), neurofibromatosis type 1 (NF1), capillary malformation–ateriovenous malformation (CM–AVM), Costello, cardio-facio-cutaneous (CFC), and Legius

(Cirstea et al. 2010), *SHOC2* (Cordeddu et al. 2009), and *CBL* (Martinelli et al. 2010; Niemeyer et al. 2010); (2) NS with multiple lentigines caused by mutations in *PTPN11* (Digilio et al. 2002) and *RAF1* (Pandit et al. 2007); (3) capillary malformation–AV malformation caused by haploinsufficiency of the *RASA1* gene (Eerola et al. 2003); (4) Costello syndrome (CS) caused by activating mutations in *HRAS* (Aoki et al. 2005); (5) cardio-facio-cutaneous syndrome (CFC) caused by alteration of MAPK pathway activation by activating mutations in *BRAF* (Niihori et al. 2006; Rodriguez-Viciana et al. 2006b) and *MEK1*/*MEK2* (Rodriguez-Viciana et al. 2006b); and (6) Legius syndrome caused by inactivating mutations in the *SPRED1* gene (Brems et al. 2007).

The Ras/MAPK pathway plays a critical role in development and is activated by extracellular input in the form of growth factors (Fig. 32.1). Ras genes exist as a multi-gene family that includes *HRAS*, *NRAS,* and *KRAS*. Ras proteins are small guanosine nucleotide-bound GTPases which comprise a critical signaling hub within the cell. They are activated through growth factors binding to receptor tyrosine kinases (RTKs), G-protein-coupled receptors, cytokine receptors, and extracellular matrix receptors. Ras proteins cycle between an active GTP-bound form and an inactive GDP-bound form. Activation through RTK occurs with the binding of a growth factor causing RTK auto-phosphorylation and interaction with the adaptor protein GRB2. GRB2 is bound to SOS which is then recruited to the plasma membrane. SOS proteins are guanosine nucleotide exchange factors (GEF) that increase the Ras nucleotide exchange rate of GDP for GTP, resulting in an increase of Ras in the active GTP-bound form. The MAPK pathway is one of several critical downstream signaling cascades of Ras. Activated Ras leads to the

activation of Raf (ARAF, BRAF, and/or CRAF), the first MAPK kinase kinase of the pathway. Raf phosphorylates and activates MEK1 and/or MEK2 (MAPK kinase), which in turn phosphorylates and activates ERK1 and/or ERK2. ERK1 and ERK2 are the ultimate effectors and exert their function on a large number of downstream molecules, both nuclear and cytosolic. ERK1/2 substrates include nuclear components, transcription factors, membrane proteins, and protein kinases that in turn control vital cellular functions including cell cycle progression, differentiation and the control of cellular growth (Yoon and Seger 2006). The Ras/MAPK pathway has been studied extensively in the context of oncogenesis since its dysregulation is one of the primary causes of cancer. Ras has been found to be somatically mutated in approximately 20 % of malignancies (Bos 1989). Because of this, the RASopathies as a group are considered cancer syndromes with the majority of mutations associated with the RASopathies resulting in enhanced pathway activation or dysregulated signaling. However, biochemical studies have demonstrated that a large fraction of the novel germline mutations identified in the pathway are not as robustly activating as those associated with oncogenesis. This is likely due to embryonic lethality when occurring in the germline.

32.2 Neurofibromatosis Type 1: The First RASopathy

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder affecting approximately 1 in 3,000 newborns with approximately half of all NF1 individuals inheriting the mutation from a parent [for review see Williams et al. (2009)]. The clinical diagnosis of NF1 is based on the presence of café-au-lait maculae, intertriginous freckling, neurofibromas and plexiform neurofibromas, iris Lisch nodules, osseous dysplasia, optic pathway glioma, and/or a first-degree relative with NF1 (Fig. 32.2). Although these are the signs and symptoms that are most commonly associated with NF1, individuals with NF1 may have other manifestations of their disorder including cardiac malformations, cardiovascular disease, vasculopathy, hypertension, vitamin D deficiency, brain malformations, and seizures. In addition, individuals may have dysmorphic craniofacial features that are reminiscent of Noonan syndrome (Huffmeier et al. 2006; Stevenson et al. 2006), mild neurocognitive impairment, and a predisposition to developing certain malignancies. Segmental and mosaic forms of NF1 are not uncommon and gonadal mosaicism has been documented.

NF1 was the first multiple congenital anomaly syndrome to be associated with a germline mutation within the Ras/MAPK pathway. NF1 is caused by mutations in the *NF1 gene*, with approximately half of the mutations occurring de novo (Cawthon et al. 1990; Viskochil et al. 1990; Wallace et al. 1990). *NF1* encodes neurofibromin, a "RasGAP" which is a GTPase activating protein that is a negative regulator of Ras (Fig. 32.1; Table 32.1). The *NF1* missense mutations result in the loss-of-function of neurofibromin causing haploinsufficiency within the cell. This, in turn, reduces RasGTPase activity and, therefore, results in an overall increase in active GTP-bound Ras.

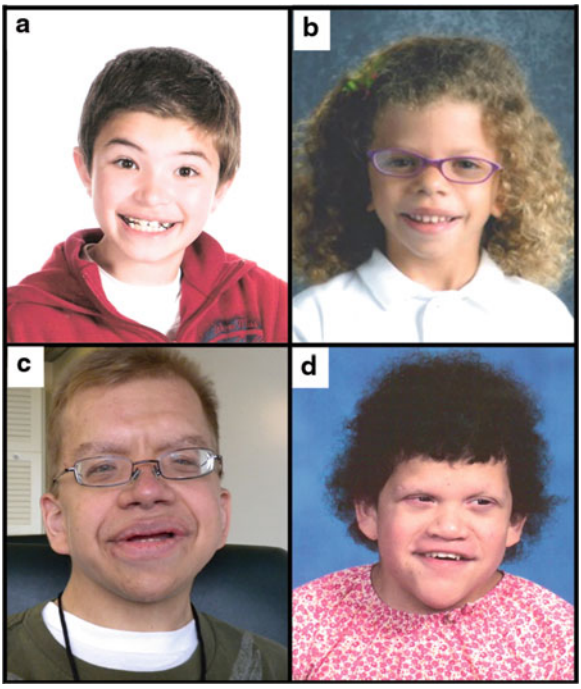

Fig. 32.2 Clinical images of patients with RASopathies. (**a**) 7-year-old boy who has a clinical diagnosis of NF1. (**b**) 7-year-old girl with Noonan syndrome who has a mutation in the *PTPN11* gene. (**c**) 27-year-old man with Costello syndrome who has the common p.G12S *HRAS* mutation. (**d**) 14-year-old girl with cardio-facio-cutaneous syndrome who has a novel *BRAF* mutation

NF1 is an inherited cancer syndrome [for review see Brems et al. (2009)]. Individuals with NF1 are at greater risk for developing malignancies than the general population. Pediatric malignancies include optic pathway glioma, rhabdomyosarcoma (RMS), neuroblastoma, and juvenile myelomonocytic leukemia (JMML); whereas, adult tumors include malignant peripheral nerve sheath tumors, gastrointestinal stromal tumors, somatostatinomas, pheochromocytomas, and breast cancer.

32.3 Legius Syndrome

Legius syndrome (formally called NF1-like syndrome) is a newly described autosomal dominant disorder. It shares many phenotypic features with NF1, as well as with other RASopathies. Individuals may have café-au-lait maculae, intertriginous freckling, mild neurocognitive impairment, and macrocephaly with some having dysmorphic craniofacial features that are reminiscent of Noonan syndrome. However, the neoplastic features common in NF1 such as neurofibromas, plexiform neurofibromas, iris Lisch nodules, and central nervous system tumors do not seem to be associated with this syndrome. Legius syndrome is caused by heterozygous inactivating mutations in *SPRED1* (Brems et al. 2007) (Fig. 32.1; Table 32.1). *SPRED1* encodes SPRED1 which has a SPROUTY-related N-terminal enabled/

Table 32.1 Genetic syndromes of the Ras/MAPK pathway

Syndrome	Ras pathway gene	Chromosome location	Protein	Protein function	Reference
Neurofibromatosis 1	*NF1*	17q11.2	Neurofibromin	RasGAP	Cawthon et al. (1990), Viskochil et al. (1990), Wallace et al. (1990)
Noonan	*PTPN11*	12q24.1	SHP2	Phosphatase	Tartaglia et al. (2001)
	SOS1	2p22.1	SOS1	RasGEF	Roberts et al. (2007)
	RAF1	3p25.1	CRAF	Kinase	Tartaglia et al. (2007)
	KRAS	12p12.1	KRAS	GTPase	Pandit et al. (2007)
	NRAS	1p13.2	NRAS	GTPase	Cirstea et al. (2010)
	SHOC2	10q25.2	SHOC2	Scaffolding	Cordeddu et al. (2009)
	CBL	11q23.3	CBL	E3 ubiquitin ligase	Martinelli et al. (2010), Niemeyer et al. (2010)
Multiple Lentigines-related Noonan	*PTPN11*	12q24.1	SHP2	Phosphatase	Digilio et al. (2002)
	RAF1	3p25.1	RAF1/CRAF	Kinase	Pandit et al. (2007)
Gingival fibromatosis 1	*SOS1*	2p22.1	SOS1	RasGEF	Hart et al. (2002)
Capillary malformation–AV malformation	*RASA1*	5q14.3	p120Gap	RasGAP	Eerola et al. (2003)
Costello	*HRAS*	11p15.5	HRAS	GTPase	Aoki et al. (2005)
Cardio-facio-cutaneous	*BRAF*	7q34	BRAF	Kinase	Niihori et al. (2006), Rodriguez-Viciana et al. (2006b)
	MAP2K1	15q22.31	MEK1	Kinase	Rodriguez-Viciana et al. (2006b)
	MAP2K2	19p13.3	MEK2	Kinase	Rodriguez-Viciana et al. (2006b)
	KRAS	12p12.1	KRAS	GTPase	Niihori et al. (2006)
Legius	*SPRED1*	15q14	SPRED1	Sprouty-related	Brems et al. (2007)

VASP homology 1 (EVH1) domain. SPRED1 functions as a negative regulator of Ras by inhibiting phosphorylation of Raf (Wakioka et al. 2001). The vast majority of *SPRED1* mutations associated with Legius syndrome cause truncation of the protein resulting in a loss of SPRED1 function and dysregulated signaling of the Ras/MAPK pathway. Although several patient series have been published, it remains unclear as to whether individuals with germline *SPRED1* mutations are at increased risk for developing cancer (Denayer et al. 2011).

32.4 Noonan Syndrome

Noonan syndrome (NS) is an autosomal dominant disorder that affects approximately 1 in 1,000–2,000 newborns. NS exhibits a widely variable clinical phenotype. However, it is typically characterized by distinctive craniofacial features,

including a broad forehead, hypertelorism, down-slanting palpebral fissures, and low-set posteriorly rotated ears (Fig. 32.2). Other important phenotypic features include congenital cardiac defects, reduced growth, bleeding disorders and a variable degree of neurocognitive delay [for review see Romano et al. (2010)]. In addition, individuals with NS have been shown to have an increased risk of developing cancer. At present, seven genes have been shown to be associated with NS: *PTPN11* (Tartaglia et al. 2001), *KRAS* (Schubbert et al. 2006), *NRAS* (Cirstea et al. 2010), *SOS1* (Roberts et al. 2007; Tartaglia et al. 2007), *RAF1* (Pandit et al. 2007; Razzaque et al. 2007), *SHOC2* (Cordeddu et al. 2009), and *CBL* (Martinelli et al. 2010; Niemeyer et al. 2010). All of the genes harbor heterozygous germline mutations and encode various components of, or proteins associated with, the Ras/MAPK pathway (Fig. 32.1; Table 32.1).

The most common gene associated with NS is *PTPN11*, which accounts for approximately half of all cases (Tartaglia et al. 2001). SHP2, the protein product of *PTPN11*, is a non-receptor protein tyrosine phosphatase composed of N-terminal and C-terminal SH2 domains and a catalytic protein tyrosine phosphatase (PTP) domain. The majority of NS-causing missense mutations in *PTPN11* cluster in residues involved in the interaction between the N-SH2 and PTP domains. Mutations in this region disrupt stability of the catalytically inactive form of SHP2 resulting in impairment in the protein's ability to switch from the active to the inactive protein conformation (Keilhack et al. 2005; Tartaglia et al. 2006) causing increased signaling of the Ras/MAPK pathway.

SOS1 missense mutations are the second most common cause of NS and account for approximately 15 % of cases (Roberts et al. 2007; Tartaglia et al. 2007). *SOS1* encodes the RasGEF (guanine nucleotide exchange factor) protein, SOS1, which is responsible for stimulating the conversion of Ras from the inactive GDP-bound form to the active GTP-bound form. The majority of SOS1 missense mutations are located in codons encoding residues that are responsible for stabilizing the protein in an inhibited conformation. These mutations disrupt the auto-inhibition of SOS1 RasGEF activity resulting in a gain-of-function of SOS1 and a subsequent increase in the active form of Ras and increased Ras/MAPK pathway signaling.

KRAS mutations are a rare cause of NS (Schubbert et al. 2006). *KRAS* encodes two splice variants with KRASA expressed in a tissue-specific and developmentally restricted fashion and KRASB being ubiquitously expressed. The novel *KRAS* mutations that cause NS increase signaling of the Ras/MAPK pathway through two distinct mechanisms: either by mutations that reduce the intrinsic and GAP-stimulated GTPase activity (Schubbert et al. 2006, 2007) or by mutations that interfere with the binding of KRAS and guanine nucleotides. The resultant increased signaling of the Ras/MAPK pathway is, however, less than that which occurs with oncogenic *KRAS* activating mutations (Schubbert et al. 2006).

Mutations in *NRAS* have also been found in a very small number of individuals with the clinical phenotype of NS (Cirstea et al. 2010). Mutations have been identified within or near the switch II region of NRAS and are thought to interfere

with GTPase function. Mutations have been shown to cause enhanced phosphorylation of MEK and ERK.

Mutations in *RAF1* also cause NS (Pandit et al. 2007; Razzaque et al. 2007). *RAF1* encodes the protein RAF1 (aka CRAF), a serine/threonine kinase that is one of the direct downstream effectors of Ras. The majority of *RAF1* mutations associated with NS cluster in two regions: in conserved region 2 flanking S259 and in conserved region 3, surrounding the activation segment. These mutations result in a CRAF gain-of-function since the phosphorylation of residues S259 and S621 are responsible for regulation of CRAF.

A rare subset of NS individuals with a unique phenotypic feature of loose anagen hair has recently been identified to be caused by a single mutation in *SHOC2*, which results in a p.S2G substitution (Cordeddu et al. 2009). *SHOC2* is a homologue of suppressor of clear (SOC-2) in *C. elegans* which encodes a protein whose primary structure consists almost entirely of leucine-rich repeats. SHOC2 functions as scaffold protein linking Ras to RAF1, its downstream effector in the Ras/MAPK pathway. SHOC2 is ubiquitously expressed and serves as the regulatory subunit of protein phosphatase 1 (PP1C) (Rodriguez-Viciana et al. 2006a). SHOC2 binds GTP-Ras and mediates PP1C translocation to the cell membrane. This enables PP1C dephosphorylation of residue Ser259 of RAF1 which is required for RAF1 translocation to the cell membrane and catalytic activity. The unique p.S2G mutation is proposed to cause the abnormal addition of a 14-carbon saturated fatty acid chain, myristate, to the N-terminal glycine of SHOC2. This results in the aberrant translocation of SHOC2 to the cell membrane, prolonged PP1C dephosphorylation of RAF1 and sustained MAPK pathway activation (Cordeddu et al. 2009).

Another rare cause of NS includes mutations in the gene *CBL*, the tumor suppressor gene casitas b-lineage lymphoma (Martinelli et al. 2010; Niemeyer et al. 2010). Individuals may also have myeloproliferative diseases, including JMML which is seen in individuals with NF1 as well as NS. *CBL* encodes an E3 ubiquitin ligase that negatively regulates the Ras/MAPK signaling downstream of RTK (Dikic and Schmidt 2007). CBL mediates the association of ubiquitin with activated RTK, which is necessary for receptor internalization and degradation (Dikic and Schmidt 2007). Mutations in *CBL* reduce the turnover of activated RTK; hence, these mutations have an overall effect of increased ERK activation.

32.5 Noonan Syndrome with Multiple Lentigines (LEOPARD Syndrome)

Noonan syndrome with multiple lentigines (NSML), formerly referred to as "LEOPARD" syndrome, is a rare autosomal dominant disorder. Individuals have craniofacial features of NS as well as multiple lentigines, EKG abnormalities,

ocular hypertelorism, pulmonary valve stenosis, abnormal genitalia, growth retardation, and deafness. NSML and NS are allelic disorders, caused by different heterozygous missense mutations in the same genes, *PTPN11* (Digilio et al. 2002; Legius et al. 2002) and *RAF1* (Pandit et al. 2007) (Fig. 32.1; Table 32.1). The most common NSML associated *PTPN11* mutations affect amino acids in the catalytic PTP domain which result in reduced SHP2 catalytic activity in vitro causing a loss of function (Digilio et al. 2002; Kontaridis et al. 2006). However, an *in vivo Drosophila* model has demonstrated that the residual catalytic activity in the NSML mutant SHP2 protein is sufficient to produce a gain-of-function phenotype due to dysregulation of the protein causing continuous MAPK pathway activity during development (Oishi et al. 2009).

32.6 Capillary Malformation–Arteriovenous Malformation Syndrome

Capillary malformation–arteriovenous malformation syndrome (CM–AVM) is also a RASopathy and is an autosomal dominant inherited disorder. It is characterized by multifocal capillary malformations which may be associated with arteriovenous malformations and fistulas [for review see Boon et al. (2005)]. CM–AVM syndrome is caused by heterozygous inactivating mutations in the gene *RASA1* (Eerola et al. 2003), which like *NF1*, encodes a RasGAP, more specifically the p120-RasGTPase-activating protein (p120-RasGAP) (Fig. 32.1; Table 32.1). The N-terminus contains a Src homology region and the C-terminus contains a pleckstrin homology domain and the RasGTPase-activating domain. Like neurofibromin, the protein product of *NF1*, RASA1 switches the active GTP-bound Ras to the inactive GDP-bound form. It is a negative regulator of the Ras/MAPK signal transduction pathway which is important for cellular growth, differentiation, and proliferation. The major feature of this syndrome is the multifocality of the malformations. AVMs can occur in many tissues including skin, muscle, bone, and in various internal organs including the heart and the brain. In addition, *RASA1* mutations have been associated with individuals diagnosed with Parkes–Weber syndrome and vein of Galen malformations (Revencu et al. 2008). Haploinsufficiency of p120-RasGAP causes a reduction in the hydrolysis of Ras-GTP and, therefore, increases Ras/MAPK pathway signaling. CM–AVM is an autosomal dominant disorder with most individuals having an affected parent. However, ~30 % of cases are caused by a de novo mutation. The types of mutations are varied with the majority being nonsense, frame-shift, or splice mutations. In addition to AVM, individuals may have cardiovascular malformations including tetralogy of Fallot, septal defects, and valve anomalies. Although still unclear, CM–AVM patients may be at increased risk of developing tumors similar to that seen in NF1 and NF2.

32.7 Costello Syndrome

Costello syndrome (CS) is rarer than NF1, but is a multiple congenital anomaly syndrome that does have many overlapping features with NF1, although the diagnosis of CS would never be confused with NF1. CS individuals have characteristic dysmorphic craniofacial features, failure to thrive especially in the newborn period, cardiac, musculoskeletal, ectodermal and ocular abnormalities, hypotonia, and neurocognitive delay [Fig. 32.2; for review see Rauen (2007)]. Phenotypic features become apparent in the perinatal period with polyhydramnios in utero and many children are born prematurely with increased birth weight. Facial features are coarse and typically include macrocephaly with a prominent forehead, epicanthal folds, down-slanting palpebral fissures, short nose with a depressed nasal bridge and broad base, low-set, posteriorly rotated ears with thickened helices and lobes. The cheeks may be full and the mouth large with full lips. As with NF1, dermatologic manifestations aid in the clinical diagnosis of CS, and include soft skin with excessive wrinkling and redundancy over the dorsum of the hands and the feet, along with deep plantar and palmar crease (Siegel et al. 2012). The majority of individuals have cardiac anomalies which may include hypertrophic cardiomyopathy (HCM), valve anomalies, septal defects, and arrhythmia (Lin et al. 2011). Failure to thrive along with gastrointestinal dysfunction such as reflux, oral aversion, and constipation are typical in early infancy; most require a G-tube for feeding.

Heterozygous activating germline mutations in *HRAS* cause CS (Aoki et al. 2005) (Fig.32.1; Table 32.1). The distribution of mutations reveals that more than 80 % of individuals have a p.G12S substitution, followed by the second most common, p.G12A [for review see Tidyman and Rauen (2008)]. These substitutions disrupt guanine nucleotide binding and cause a reduction in intrinsic and GAP-induced GTPase activity resulting in Ras remaining in the active state (Gibbs et al. 1984). In addition, less frequently observed mutations in *HRAS* may also occur. Some of these novel mutations include p.K117R (Kerr et al. 2006) and p.A146T (Pandit et al. 2007) and may result in an atypical phenotype. It is interesting that amino acid positions 12 and 13, the two most common positions mutated in CS, are also the most frequently mutated positions in oncogenic Ras. Ras mutations in codons 12, 13, or 61 are present in approximately 20 % of all tumors (Bos 1989). As in NF1, individuals with CS are at increased risk of developing neoplasms, both benign and malignant. Benign growths commonly seen in CS are cutaneous papillomas (Siegel et al. 2012). Cutaneous papillomas are observed in ~72 % of CS individuals with age of onset ranging from infancy to 22 years. The most commonly reported location of papillomas is the nose, although papillomas may occur anywhere on the body. Importantly, these neoplasms are not seen in other RASopathies. Of great concern, approximately 15–20 % of individuals with CS develop malignancies with the most common being RMS, transitional cell carcinoma (TCC) and neuroblastoma (Gripp 2005). Interestingly, RMS and neuroblastoma are common childhood malignancies, whereas TCC is not. The most

commonly reported malignancy in CS is embryonal RMS where loss of heterozygosity of wild-type HRAS has been reported (Estep et al. 2006).

The origin of constitutional germline mutations causing CS reflects a paternal bias (Sol-Church et al. 2006; Zampino et al. 2007). Like NF1, although much rarer, somatic mosaicism has been identified in Costello syndrome (Gripp et al. 2006). Also, there has been one case of reported autosomal dominant transmission (Sol-Church et al. 2009) and gonadal mosaicism (Gripp et al. 2011).

32.8 Cardio-facio-cutaneous Syndrome

Cardio-facio-cutaneous syndrome (CFC), like CS, is rare, and has many overlapping phenotypic features with NS and CS, and to some extent with NF1. CFC individuals have Noonan-like facies, including macrocephaly, broad forehead, bitemporal narrowing, hypoplasia of the supraorbital ridges, down-slanting palpebral fissures with ptosis, short nose with depressed nasal bridge and anteverted nares, a high-arched palate and low-set, posteriorly rotated ears with prominent helices (Fig. 32.2). Ectodermal findings typically consist of sparse, curly hair with sparse eyebrows and eyelashes, hyperkeratosis, keratosis pilaris, hemangioma, ichthyosis, and progressively forming nevi (Siegel et al. 2011). Cardiac anomalies are similar in frequency to NS and CS with the most prevalent being pulmonic stenosis, septal defects, and HCM. Musculoskeletal abnormalities are common, as well as ocular abnormalities, including strabismus, nystagmus, myopia, hyperopia, and astigmatism. Failure to thrive is typical in infancy, as is gastrointestinal dysfunction such as reflux, vomiting, oral aversion, and constipation. Neurologic abnormalities are universally present to varying degrees and include hypotonia, motor delay, seizures, speech delay, and/or learning disabilities (Yoon et al. 2007).

Four genes that encode proteins in the Ras/MAPK pathway downstream of Ras have been associated with CFC syndrome: *BRAF* (Niihori et al. 2006; Rodriguez-Viciana et al. 2006b), *MAP2K1* and *MAP2K2* (Rodriguez-Viciana et al. 2006b), and *KRAS* (Niihori et al. 2006) (Table 32.1). The role of KRAS in CFC remains unclear because *KRAS* mutations were also identified in individuals clinically diagnosed with NS (Schubbert et al. 2006). Heterozygous *BRAF* mutations are found in approximately 75 % of mutation-positive CFC individuals [for review see Tidyman and Rauen (2009)]. BRAF is a serine/threonine protein kinase and one of the direct downstream effectors of Ras. BRAF is a known onco-protein with somatic mutations reported in several different types of malignancies including thyroid, lung, ovarian, and colorectal; however, the majority of CFC-associated mutations are novel. Unlike the mutations associated with cancer, the majority of CFC *BRAF* mutations cluster in the cysteine-rich domain in exon 6 and in the protein kinase domain with the most common CFC BRAF mutation being p.Q257R. In vitro functional analyses of the BRAF mutant proteins have demonstrated that most have increased kinase activity; however, a few mutant proteins have kinase-impaired activity (Niihori et al. 2006; Rodriguez-Viciana

et al. 2006b). BRAF kinase impairment has also been shown to increase signaling of the MAPK pathway through CRAF (Heidorn et al. 2010). Further in vivo studies of CFC mutations demonstrate that both kinase active and kinase-impaired mutations result in similar phenotypic dysregulation of MAPK signaling in a zebrafish model (Anastasaki et al. 2009). Heterozygous missense mutations in *MAP2K1* (MEK1) and *MAP2K2* (MEK2) are present in approximately 25 % CFC individuals in which a gene mutation has been identified [for review see Tidyman and Rauen (2009)]. MEK1 and MEK2 are threonine/tyrosine kinases, with both isoforms having the ability to phosphorylate and activate ERK1 and ERK2. Functional studies of MEK mutant CFC proteins have shown that all are activating (Rodriguez-Viciana et al. 2006b; Anastasaki et al. 2009).

CFC syndrome is transmitted in an autosomal dominant manner (Rauen et al. 2010). However, in most cases, it is the result of a *de novo* dominant mutation since it is very rare for individuals with CFC to reproduce. The vast majority of individuals with CFC syndrome have the disorder as the result of a de novo mutation. Although the mutations that cause CFC are in a well-known oncogenic pathway, it is as yet unclear if individuals with CFC syndrome are at an increased risk for malignancies. CFC certainly does not appear to have the malignancy risk of NS, CS, and NF1 (Rauen et al. 2010; Kratz et al. 2011).

32.9 Hereditary Gingival Fibromatosis Syndrome

Hereditary gingival fibromatosis (HGF) is included as a RASopathy for completeness although it is very different from the syndromes described above. HGF is a rare, slowly progressive, benign fibrous overgrowth of the keratinized gingiva (Hart et al. 1998). HGF has been reported to be transmitted in both an autosomal dominant and recessive fashion due its genetic heterogeneity. One very rare autosomal dominant form, HGF Type 1, is caused by an insertion mutation in the *SOS1* gene (Hart et al. 2002) (Fig. 32.1; Table 32.1). This rare *SOS1* insertion mutation causes a frame-shift that produces 22 novel amino acid residues prior to the production of a premature stop codon which abolishes four proline-rich SH3 binding domains required for GRB2 binding in the C-terminus (Hart et al. 2002). In vitro expression of the mutant SOS1 protein shows that the truncated protein localizes to the plasma membrane without growth factor binding. This causes Ras activation and sustained signaling of the Ras/MAPK pathway (Jang et al. 2007). To date, no developmental effects have been reported with the novel *SOS1* mutation associated with HGF1 as there are with the activating *SOS1* mutations associated with NS. However, it is interesting to note that many individuals we have seen with a RASopathy have non-drug induced gingival hyperplasia (Rauen, personal observation).

32.10 Conclusions

The RASopathies, which are caused by germline mutations in genes encoding components of the Ras/MAPK pathway, emphasize the essential role this critical signal transduction pathway plays in embryonic and postnatal development. Mutations that have been identified in these syndromes result in dysregulation of the Ras/MAPK pathway, with functional studies determining that the vast majority enhance pathway signaling. Therefore, it is not surprising that many of these syndromes exhibit overlapping phenotypic features and share a predisposition for certain malignancies. The number of different genes affected and the variety of mutations within each gene are reflected in the wide spectrum of phenotypic variability associated with these syndromes. Although many of the activating mutations are similar to somatic mutations associated with cancer, they tend not to be as strongly activating. It is likely that the strongly activating oncogenic mutations cannot be tolerated within the germline, or in early development.

The initial diagnosis for a patient with a given RASopathy is based on the clinical recognition of phenotypic features. Currently, molecular genetic testing is used to confirm the clinical diagnosis. The correlation between the clinical and molecular diagnosis is often dependent on the clinical diagnostic criteria used. In addition, not all of the genes associated with these syndromes have been identified. The progressive accumulation of genotype–phenotype correlations will increase the importance of the molecular diagnosis and help overcome the intrinsic limitations of the clinical diagnosis. This will not only improve patient management, but will aid in the design of clinical trials for the development of potential treatment for these syndromes.

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Chapter 33
Advances in NF1 Animal Models and Lessons Learned

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33.1 Early Mouse Models of NF1

Initial attempts to model NF1 in the mouse employed classic gene knock-out technology. Interestingly, a role for *NF1* in early embryonic development was revealed by this approach, as homozygous mutant embryos died in mid-gestation due to heart malformation (Brannan et al. 1994). However, the observation that *Nf1* heterozygous mice developed several malignancies and that the wild-type *Nf1* allele was lost in these tumors, provided important evidence that *NF1* acted as a classical tumor suppressor gene (Jacks et al. 1994). Later studies using chimeric mice, generated by injecting $Nf1^{-/-}$ embryonic stem cells into $Nf1^{+/+}$ blastocysts, confirmed that loss of the wild-type *Nf1* allele is also the rate-limiting step for neurofibroma development, which at the time was an unresolved question (Cichowski et al. 1999). To test whether additional mutations would enhance tumor susceptibility, mice were generated carrying compound mutations in the *Nf1* and *p53* tumor suppressor genes on the same chromosome (abbreviated NP cis mice) (Cichowski et al. 1999; Vogel et al. 1999). These mice developed aggressive malignant peripheral nerve sheath tumors (MPNSTs) that were histologically and genetically indistinguishable from their human counterparts (Stemmer-Rachamimov et al. 2004). In addition to demonstrating a causal role for *p53* mutations in MPNST formation, the success of this model underscored the feasibility of generating tractable mouse models of different tumor types associated with NF1. As will be discussed, NP cis mice have been extensively used to study MPNST pathogenesis and for preclinical studies.

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33.2 Lessons Learned from Second-Generation Neurofibroma Models

To develop more tractable and precise models for NF1-associated neurofibromas, mice were generated bearing conditional *Nf1* alleles, which can be rendered neurofibromin-deficient in specific cell types and at specific timepoints by *Cre*-mediated recombination (Zhu et al. 2001). The development of a wide spectrum of conditional NF1 mouse neurofibroma models (Table 33.1) has permitted the identification of cells of origin, environmental cues, and molecular pathways underlying the pathophysiology of these tumors. The details of many of these models will be discussed in other chapters. However, an overall summary of these models and how they have collectively contributed to our understanding of neurofibroma pathogenesis will be summarized below.

Although *Nf1* heterozygous mice succumbed to various malignancies during late adulthood, they did not develop neurofibromas, which are characteristic for NF1 (Jacks et al. 1994). To test the possibility that this absence of neurofibromas could be explained by a limited ability to acquire second hit *Nf1* mutations in mice, chimeric mice were generated which partially comprised $Nf1^{-/-}$ cells (Cichowski et al. 1999). The observation that these mice developed numerous neurofibromas indicated that loss of the wild-type *Nf1* allele is indeed the rate-limiting step for neurofibroma development. It was however difficult to determine which cell type(s) are involved in the formation of neurofibromas using the chimeric mouse model since several cell types are homozygous mutant for *Nf1* in these mice.

Neurofibromas are composed of multiple cell types including Schwann cells, mast cells, perineural cells, fibroblasts, and endothelial cells (Cichowski and Jacks 2001). Schwann cells are the most prevalent cell type in this complex and had been proposed to be the tumorigenic cell population (Rutkowski et al. 2000; Serra et al. 2000). A breakthrough came from conditionally ablating *Nf1* in the Schwann cell lineage, using a *Cre* transgene under the control of the precursor/immature Schwann cell-specific promoter *Krox20* (Zhu et al. 2002). Zhu et al. found that plexiform neurofibromas could be driven by ablating *Nf1* in this precursor cell population. Interestingly, these investigators also found that *Nf1* haploinsufficiency was required in surrounding cell types in this model, as neurofibromas only arose in $Nf1^{flox/-}$;*Krox-20-cre* mice in which all neighboring cells were heterozygous for *Nf1*, and not in $Nf1^{flox/flox}$;*Krox-20-cre* mice in which neighboring cells were wild-type for *Nf1* (Zhu et al. 2002). The requirement for haploinsufficiency of surrounding cells will be briefly discussed below and in detail in another chapter.

Plexiform neurofibromas arising in NF1 patients are thought to be congenital (Riccardi 1992). The observation that neurofibromas developed in mice when *Nf1* is ablated during embryonic development in a Schwann cell precursor population is consistent with this hypothesis (Zhu et al. 2002). However, in NF1 patients it is possible that second hit *NF1* mutations may occur at any stage of development (e.g. ranging from neural crest cells to mature Schwann cells, see Table 33.1) and that different cells of origin might result in differences in tumor pathogenesis.

Table 33.1 Conditional NF1 mouse models of plexiform neurofibroma development

Reference	Genotype	Cre-driver promoter	Stage of Schwann cell development[a]	Plexiform neurofibromas	Additional manifestations
Joseph et al. (2008)	*Nf1*$^{flox/-}$	*Wnt1-Cre*	NCSC	No	
Zheng et al. (2008)	*Nf1*$^{flox/-}$	*P0A-Cre*	SC precursor (early)	Yes, throughout peripheral nerve system	
Wu et al. (2008)	*Nf1*$^{flox/flox}$	*Dhh-Cre*	SC precursor (late)	Yes, near DRG[b], paraspinal	Dermal neurofibromas, pigmentation
Zhu et al. (2002)	*Nf1*$^{flox/flox}$	*Krox20-Cre*	SC precursor + immature SC	No	
	Nf1$^{flox/-}$			Yes, near DRG, paraspinal	
Le et al. (2011)	*Nf1*$^{flox/-}$	*PLP-Cre/ERT*[c]	SC precursor	Yes, near DRG, paraspinal	
			Immature SC	Yes, near DRG, paraspinal	
			Mature SC	Rare	
Mayes et al. (2011)	*Nf1*$^{flox/flox}$	*PLP-Cre/ERT*[c]	Immature SC	Yes, throughout neuroaxis	Hematopoietic expansion, splenomegaly
			Mature SC	Yes, throughout neuroaxis and larger tumors	

[a]The different stages of Schwann cell development: migrating neural crest stem cells (NCSCs) move through immature connective tissue before the time of nerve formation at mouse embryonic days E9–11, and then differentiate into Schwann cell (SC) precursors between E12 and 13. These SC precursors then become immature Schwann cells, which are generated from E14 until early neonatal stages. The immature Schwann cells eventually differentiate into mature Schwann cells in the postnatal period (Carroll and Ratner 2008; Jessen and Mirsky 2005).
[b]DRG: dorsal root ganglia.
[c]Tamoxifen-inducible *Plp-Cre* driver line.

To identify the range of cell types that might serve as a cell of origin for neurofibromas, investigators have now generated mouse models with controlled *Cre* driver transgenes that ablate *Nf1* function in the Schwann cell lineage at various developmental time points (Table 33.1). Conditional deletion of *Nf1* from fetal neural crest stem cells (NCSCs) using the *Wnt-1* promoter led to a transient increase in NCSC frequency and self-renewal, but no tumorigenicity, indicating that neurofibromas cannot emanate from this undifferentiated cell population (Joseph et al. 2008). However, in addition to the *Krox20*-Cre model, mice in which *Cre* recombinase expression was driven by promoters expressed at various stages of Schwann cell development such as *P0, Dhh,* and inducible *Plp* also promoted plexiform neurofibroma formation (Zhu et al. 2002; Wu et al. 2008; Zheng et al. 2008; Le et al. 2011; Mayes et al. 2011). These results suggest that neurofibromas can develop from a range of cell types in the Schwann cell lineage. Accordingly, each of these models may represent a distinct subset of neurofibromas that develop in NF1 patients. Importantly, there appear to be some differences in tumor pathogenesis between these models. For example, neurofibromas driven by *Dhh-Cre* are relatively large and do not appear to require haploinsufficiency of surrounding cell types (Wu et al. 2008), whereas *Krox20-Cre*-driven tumors do require this haploinsufficiency (Zhu et al. 2002). These differences should therefore be taken into consideration when designing and interpreting preclinical studies aimed at identifying potential therapies. Nevertheless, the field will benefit from studying several different neurofibroma models, which collectively may recapitulate the broad spectrum of human tumors. It should be noted that dermal neurofibromas did not develop efficiently in any of these models, suggesting that the cell of origin for these tumors is distinct from the population that gives rise to plexiform neurofibromas. Work from the Parada lab has now provided strong evidence that skin-derived precursors (SKP), a neural crest-like neural stem cell residing in the dermis, can be the cell of origin for this distinct tumor type (Le et al. 2009).

33.3 Using Mouse Models to Identify Cooperating Genetic Events

Individuals with NF1 are predisposed to the development of malignancies such as MPNST, optic pathway glioma, malignant astrocytoma, pheochromocytoma, and JMML (Ferner 2007). Cancer is a complex process that requires a series of successive genetic and epigenetic events in a permissive microenvironment to acquire an essential set of functional capabilities (Hanahan and Weinberg 2011). In line with this multi-step cancer paradigm, additional mutations in the *p53* and/or *INK4a*/*ARF* tumor suppressor genes are often found in *NF1*-deficient MPNST (Kourea et al. 1999; Legius et al. 1994; Menon et al. 1990; Nielsen et al. 1999). To faithfully recapitulate these mutations in human tumors, mouse models were generated carrying compound mutations in *Nf1* and *p53* or *Ink4a*/*Arf* (Cichowski et al. 1999; Vogel et al. 1999; Joseph et al. 2008). Importantly, the mouse tumors

displayed alterations of signaling pathways described in the human counterparts of these neoplasms (Miller et al. 2009) and are histologically indistinguishable from the latter (Stemmer-Rachamimov et al. 2004), illustrating how accurately these mouse models mimic the human condition and establishing a causal role for these genes in tumor progression. As we learn more about the biology and genetics of human MPNSTs, we can use these models to evaluate the contribution of other genes in this process. For example, by crossing *NP cis* mice with mice that harbor mutations in *EGFR*, investigators have shown that *EGFR* contributes to MPNST development (Ling et al. 2005), which may ultimately be harnessed to develop future therapies. In addition, a hallmark for NF1 is the extreme clinical heterogeneity, even among related individuals carrying the same constitutional *NF1* mutation. Epidemiologic studies suggest that the molecular basis underlying this phenotypic variability is determined to a large extent by the genotype at modifying loci (Easton et al. 1993). Genetic studies in the NP cis model have demonstrated a modifying effect of the mouse genetic background strain on tumor phenotype (Reilly et al. 2000, 2004). These mice are now being utilized to identify genetic modifiers that may ultimately prove to mediate the expressivity of the disease in NF1 patients (Reilly et al. 2006). Thus, these models can be used for both genetic as well as therapeutic studies, which will be discussed further below.

It should be noted that there are a subset of NF1 patients that may provide some genetic hints about additional genes that are involved in neurofibroma and MPNST development. Microdeletion patients are a subset of NF1 patients that carry a deletion of *NF1* and 12 surrounding genes (De Raedt et al. 2004). Notably, these patients develop hundreds of neurofibromas and have a more than twofold increased likelihood of developing an MPNST (De Raedt et al. 2003), suggesting that an additional tumor suppressor(s) lies in this region. Thus, the neurofibroma and MPNST mouse models can be used to interrogate the potential involvement of these surrounding genes.

33.4 Using Mouse Models for Therapeutic Development

The availability of mouse models that faithfully recapitulate NF1-associated tumorigenesis has the potential to provide insight into the signaling pathways that become altered in the processes of tumor initiation and progression. At the same time, these mouse models can serve as preclinical tools to start evaluating the most promising targeted therapies for NF1. Notably, in the last 5 years, preclinical studies in these models have begun to directly impact the development of clinical trials in NF1 patients.

Based on the discovery that neurofibromin critically regulates the mTOR pathway and that mTOR is hyperactivated in *Nf1*-deficient tumors (Dasgupta et al. 2005; Johannessen et al. 2005), the therapeutic potential of rapamycin, a potent mTOR inhibitor, was assessed in the *Nf1*/*p53* mutant MPNST model (Johannessen et al. 2008) and the $Nf1^{flox/-}$;*GFAP-Cre* glioma model (Hegedus et al. 2008).

Remarkably, rapamycin rapidly and potently suppressed tumor growth in both models (Johannessen et al. 2008; Hegedus et al. 2008). Notably, these studies inspired the development of four clinical trials using mTOR inhibitors in NF1 patients (clinicaltrials.gov). Although these trials are still ongoing, these efforts underscore how mouse models can be used to guide the development of clinical trials with targeted agents. As current treatments for most NF1-related symptoms have limited efficacy, animal models represent an important tool in identifying and developing appropriate clinical trials.

Clinical trials can also be improved/refined by studying the therapeutic response to specific agents in mouse models. For example, Johannessen et al. found that in addition to promoting a potent cytostatic response in mouse MPNSTs, prolonged exposure to rapamycin resulted in a complete disruption of the tumor microvasculature in the *Nf1*/*p53* MPNST model (Johannessen et al. 2008). Moreover, tumors that had acquired resistance to rapamycin had circumvented this anti-angiogenic block and had re-established their microvasculature (CMJ, KC unpublished observations). It was therefore reasoned that therapeutic strategies aimed at preventing this re-vascularization might delay or prevent the emergence of resistant tumors. To assess this possibility, the anti-angiogenic agent sunitinib was combined with rapamycin in the NP cis model. Whereas sunitinib alone had no effect in these animals, rapamycin and sunitinib potently suppressed tumor growth and significantly extended survival compared to rapamycin monotherapy (Cichowski laboratory, unpublished data). Notably, based on these promising preclinical data, a phase II clinical trial evaluating the oral mTOR inhibitor everolimus in combination with the anti-angiogenic agent bevacizumab has been launched in patients with unresectable and metastatic MPNST. Based on the observations in the NP cis model, it is expected that this combination, while probably not curative, will delay progression and prolong survival.

A second exciting example of how mouse models can be used to develop new promising therapies has now emerged. In an attempt to convert the cytostatic effects of rapamycin into a cytotoxic response, mTOR inhibitor-based combination therapies were further evaluated in NP cis mice. Because of the high aneuploidy and constitutive activation of *Ras*, *Nf1*-deficient MPNSTs exhibit high levels of proteotoxic stress (De Raedt et al. 2011). A number of adaptive mechanisms are known to be activated in such tumor cells to accommodate this stress, which if left unchecked can trigger cell death (Kim et al. 2008). It follows that this cancer cell-specific vulnerability could be exploited in the context of therapeutic development. Indeed, agents that block these adaptive pathways, either alone or in combination with anti-oncogenic agents (e.g. rapamycin), could trigger a cytotoxic response in MPNSTs.

To investigate the potential of this therapeutic strategy in aggressive MPNSTs that are often refractory to conventional therapies, several agents that enhance proteotoxic stress were evaluated *in vivo* (De Raedt et al. 2011). Importantly, the Hsp90 inhibitor IPI-504 induced dramatic tumor regression in the *Nf1*/*p53* MPNST mouse model, but only when combined with rapamycin. This synergistic effect was dependent on the accumulation of reactive oxygen species (ROS), as rapamycin abolished the cell's capacity to neutralize the ROS induced by IPI-504, resulting in

catastrophic endoplasmic reticulum and mitochondrial damage in the tumors (De Raedt et al. 2011). The efficacy of this combination therapy could also be extended to a *Kras* driven mouse model of non-small cell lung cancer (NSCLC) (De Raedt et al. 2011). No targeted therapy had been shown to promote tumor regression in either of these aggressive tumor models, underscoring the significance of these findings and the potential impact on therapeutic development. A phase I clinical trial to evaluate the activity of IPI-504 and the mTOR inhibitor everolimus has been launched for *KRAS* mutant NSCLC, which if successful would evolve into phase II studies in NCSCL as well as MPNSTs.

Finally, as will be detailed in another chapter, mouse models have also been useful for developing potential therapies for neurofibromas. As mentioned earlier Zhu et al. have found that haploinsufficiency of the surrounding microenvironment plays a critical role in the development of neurofibromas in the *Krox20-Cre* driven model (Zhu et al. 2002). Through a series of elegant genetic studies the investigators found that mast cells play a critical role in neurofibroma development (Yang et al. 2008). Therefore the investigators reasoned that targeting the tumor microenvironment could represent a valid therapeutic approach for NF1-associated neurofibromas. Because mast cells are critically regulated by the c-kit receptor, the therapeutic potential of c-kit inhibition was evaluated in this mouse neurofibroma model (Yang et al. 2008). $Nf1^{flox/-}$;*Krox-20-cre* mice were treated with imatinib mesylate, a potent inhibitor of several tyrosine kinases including c-kit, and the effect on neurofibroma burden was assessed by FDG-PET imaging (Yang et al. 2008). Overall, the mice treated with imatinib mesylate had a mean 50 % reduction in FDG-PET uptake after treatment (Yang et al. 2008). Strikingly, and consistent with the preclinical studies in the mouse model, imatinib mesylate treatment of a 3-year-old child with an unresectable plexiform neurofibroma revealed an approximate 70% reduction in tumor volume after 3 months (Yang et al. 2008).

To further promote and accelerate the development of effective new treatments for NF1, the Children's Tumor Foundation developed a Neurofibromatosis Preclinical Consortium (NFPC). This multi-center cooperative group assembles a set of NF1-associated mouse models representing a diverse set of NF1 disease symptoms for which there is currently no treatment available. To date, the NFPC has successfully completed numerous preclinical trials in partnership with four pharmaceutical companies. Several of these preclinical studies have already resulted in clinical trials in NF1 patients. Based on the power of the animal models being utilized and the current rate of throughput from this consortium, these efforts are likely to make a significant impact on the clinical care of NF1 patients.

33.5 Conclusion and Future Directions

It is clear that the development of sophisticated NF1 mouse models has permitted the dissection of the molecular mechanisms underlying the development of a wide range of NF1 disease manifestations. It has provided concrete insights into the genetic defects and cell autonomous as well as non-cell autonomous factors involved in the complex pathophysiology of NF1. Moreover, the design of these

mouse models faithfully recapitulating seminal features of the human condition has allowed investigators to start using these as accurate preclinical tools for testing the most promising targeted therapies for NF1.

One of the most exciting applications for the future is the use of these genetically engineered NF1 mouse models for translational research. The described models represent a powerful experimental tool because it allows the evaluation of candidate drugs in genetically well-defined systems which develop symptoms in the appropriate environment and in the presence of an intact immune system. Moreover, the ability to rapidly assess the biological effects of a given agent can be exploited to determine the best clinical candidate against the most promising therapeutic target. Ultimately, and as illustrated in the MPNST model, the benefits gained will go beyond evaluating responses to targeted monotherapies, and can be used to rapidly evaluate the efficacy of diverse combination therapies, identify biomarkers of sensitivity, and develop therapies that overcome resistance to specific treatments. As such, these models have the potential to serve as an excellent platform to successfully predict drug response in clinical trials.

At the same time, expanding the complexity of NF1 mouse models will undeniably contribute to an even more profound understanding of the basic mechanisms underlying the disease. As such, it can be expected that the development and study of relevant NF1 mouse models will continue to translate into major advances for both basic and clinical research.

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Chapter 34
Drosophila: An Invertebrate Model of NF1

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34.1 Introduction

As discussed in preceding chapters, NF1 is a multisystem chronic genetic disease associated with a variety of symptoms. Another oft-cited hallmark of NF1 is its variability and unpredictability. Thus, patients typically only develop a subset of symptoms, and the severity of individual symptoms can vary dramatically between patients. Variable expressivity of a single-gene genetic disorder, like NF1, can be due to several different factors, including incomplete genetic penetrance, the nature of the culprit gene defect, whether or not a patient is a somatic mosaic, and whether or not a given patient carries genetic modifiers or has been exposed to different environments. In the case of NF1, all these factors are believed to play a role. Thus, although individuals carrying *NF1* mutations develop at least some symptoms with complete penetrance, stochastic genetic or epigenetic events may result in partial penetrance of individual symptoms. An example is provided by plexiform neurofibromas, most of which are believed to develop congenitally. The fact that no more than about a third of patients develop these tumors may reflect the limited probability of loss of the wild-type *NF1* allele in tumor progenitors during a restricted developmental period. Secondly, while genotype–phenotype correlations are uncommon in NF1, some notable exceptions exist. First, 5–10 % of patients harbor recurring 1.2–1.4 Mb microdeletions that include the *NF1* gene and several flanking protein-coding and microRNA genes. Microdeletion patients often develop particularly severe symptoms, including mental retardation, dysmorphism, childhood overgrowth, and high numbers of unusually early onset neurofibromas (Kayes et al. 1994; Pasmant et al. 2010). In support of the view that the loss of contiguous genes may cause or modify these defects, patients carrying deletions

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that remove just the *NF1* gene do not exhibit this more severe phenotype. Another example is provided by the finding that 21 unrelated NF1 patients carrying the same single amino acid deletion lacked obvious neurofibromas (Upadhyaya et al. 2007). Beyond effects attributable to the *NF1* gene itself, variable expressivity can also reflect somatic mosaicism in sporadic patients who acquired their *NF1* mutation post-zygotically (Bernards and Gusella 1994). The hypothesis that symptom-specific genetic modifiers play important roles in determining disease outcome received early support from a landmark study, which analyzed the presence or absence of five binary symptoms, as well as the severity of three quantitative symptoms amongst 175 patients in 48 NF1 families, including six monozygotic (MZ) twin pairs. This study found high correlation coefficients for four binary and two quantitative traits (neurofibroma and café-au-lait spot burden) for the MZ twins, lower scores for first-degree relatives, and lower values still for more distant family members (Easton et al. 1993). The hypothesis that unlinked modifier genes control specific NF1 symptoms has received additional support from subsequent work (Rieley et al. 2011; Szudek et al. 2002). Environmental factors are another potential cause for variable expressivity. In the case of NF1, the clearest example of a nongenetic factor affecting disease progression is the observation that neurofibromas increase in size and/or number during pregnancy (Roth et al. 2008).

The identity of modifier genes may provide clues to mechanisms responsible for disease symptoms, and since modifier genes perform rate-limiting functions, human modifiers represent prevalidated therapeutic targets. However, when the *NF1* gene was identified in 1990, the human genome sequence and other tools to efficiently survey cohorts of differentially affected patients for potential modifiers were not available. Thus, our strategy to identify NF1 modifiers involved the identification of a highly conserved *Drosophila NF1* (*dNF1*) ortholog, the generation of mutants, the analysis of phenotypes, structure–function studies, and genetic screens. The remainder of this chapter summarizes what has been learned so far and how this knowledge may be relevant to those interested in human NF1.

34.2 Identification and Structure of *dNF1*

The human *NF1* gene sequence immediately suggested a function for the encoded protein as a GTPase-Activating Protein (GAP) for Ras (Xu et al. 1990). It also became apparent that, whereas neurofibromin shares a functional ~360 amino acid catalytic domain with RasGAPs from several species, it exhibited more extensive similarity over almost half of its length with the budding yeast *Inhibitor of Ras Activity-1* and *-2* (IRA1/2) proteins (Ballester et al. 1990). These findings prompted us to test whether *NF1* orthologs existed in invertebrate species amenable to genetic analysis. No *NF1* ortholog exists in the nematode, *Caenorhabditis elegans*. However, the *dNF1* gene of the fruit fly, *Drosophila melanogaster,* was found to predict a protein 55 % identical and 69 % similar to human neurofibromin over its entire 2,802 amino acid length (The et al. 1997). As shown in Fig. 34.1, the IRA-related central segment of *Drosophila* neurofibromin is most similar to the human protein, but conserved regions also exist both up- and downstream. Reflecting the smaller

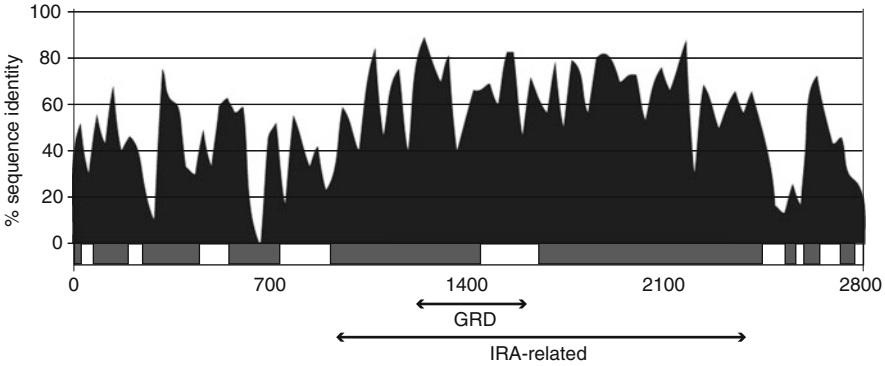

Fig. 34.1 Diagram showing the degree of amino acid sequence identity between *Drosophila* and human neurofibromin. The alternate *gray/white boxes* below the graph indicate the size and location of 18 *dNF1* exons. GRD: GAP-related domain. The extent of the protein segment related to *S. cerevisiae* IRA1 and IRA2 proteins is also indicated

size of the *Drosophila* genome, the *dNF1* gene is more compact than its human counterpart. Thus, while human *NF1* consists of 60 exons distributed over ~283 kb of DNA and harbors three other genes in its introns, *dNF1* comprises 18 exons (alternate white/gray boxes in Fig. 34.1) and spans only 13 kb. At two locations, *dNF1* mRNA is alternatively spliced. The position of alternatively spliced *dNF1* exon 14 corresponds closely to where exon 43 is alternatively spliced in man (Thomson and Wallace 2002). However, while human exon 43 has been suggested to provide a nuclear localization signal (Vandenbroucke et al. 2004), whether alternative splicing of the (not obviously related) 30 amino acids predicted by *dNF1* exon 14 has functional consequences, remains to be determined. At the exact location of alternatively spliced human exon 48a, the penultimate *dNF1* coding exon 17 splices to three different final exons, yielding mRNAs predicting proteins with three distinct C-termini. Again, whether these C-terminal isoforms are functionally different remains unknown. In human NF1, inclusion of alternatively spliced exon 23a in the GAP-related domain (GRD) reduces the catalytic activity of the encoded protein (Andersen et al. 1993). However, the location of exon 23a does not correspond to a splice site in *dNF1*. Finally, we note that disease-associated missense mutations disproportionally affect residues conserved in *Drosophila* NF1. For example, the approximately 1,200 amino acid segment upstream of the GRD shares 46 % overall sequence identity between man and fly, but 35 of 47 missense mutations in this region (74 %) affect residues conserved in *Drosophila*.

34.3 *dNF1* Mutants and Phenotypes

No classical *dNF1* mutations existed. In their absence, we mobilized a flanking *P* transposon to generate two *dNF1* disrupting de novo integrations. Both $dNF1^{P1}$ and $dNF1^{P2}$ alleles behave as molecular and genetic nulls. Given that expression of

oncogenic Ras in fly eyes causes severe defects (Bishop and Corces 1988), it seemed puzzling at first that homozygous loss of *dNF1* did not cause any abnormal patterning. However, both mutants exhibited a 15–20 % reduction in linear dimensions during all phases of postembryonic development, and adult flies had a reduced tendency to fly away when released (The et al. 1997). Since these and other *dNF1* phenotypes may be affected by the genetic background, and because the $dNF1^{P1}$ allele also deleted adjacent *E*(*spl*) complex genes, we made additional alleles by chemical mutagenesis of isogenized flies. Among three new alleles, $dNF1^{E4}$ is a C1045Y missense mutant, and $dNF1^{E1}$ and $dNF1^{E2}$ are Q370* and Q1062* nonsense mutations (Walker et al. 2006).

Beyond invoking Ras-independent functions, at least two other factors may explain why loss of *dNF1* and expression of constitutively active Ras do not cause similar defects. First, the *Drosophila* genome contains five RasGAP genes (*Gap1*, *Vap*, *dNF1*, *CG1657*, *CG42684*) and two plexin-related potential RasGAPs. The relatively subtle *dNF1* phenotypes may also reflect the apparent restriction of *dNF1* expression to the nervous system (Walker et al. 2006). While loss of *dNF1* does not obviously affect viability, fertility, or patterning, several macroscopic, behavioral, and biochemical phenotypes have been identified (Fig. 34.2). Interestingly, the initially identified *dNF1* defects were not sensitive to genetic manipulations that affect Ras signaling strength. Rather, they were restored by increasing, and enhanced or mimicked by reducing, signaling through the cAMP-dependent Protein Kinase A (PKA) pathway (Guo et al. 1997; The et al. 1997). The suggested link between *dNF1* and adenylyl cyclase (AC)/PKA signaling has motivated much subsequent research, and while there is no doubt that loss of *dNF1* somehow affects AC/PKA signaling, we and others have reached conflicting conclusions as to whether *dNF1* affects this pathway directly or indirectly. Before discussing what might explain this discrepancy, the following sections first briefly describe the *dNF1* phenotypes illustrated in Fig. 34.2.

34.3.1 Postembryonic Growth Deficiency

dNF1 embryos are of normal size, but mutants are 15–20 % smaller than controls during all subsequent larval, pupal, and adult stages. By measuring adult wing cell densities, this defect was shown to reflect a reduction in cell size rather than in cell number. However, smaller mutant eyes consist of fewer normal-sized ommatidia, indicating that different tissues respond differently to loss of *dNF1*. Mosaic analysis provided the first indication that reduced wing growth involves a non-cell-autonomous mechanism (The et al. 1997). This conclusion is further supported by evidence that *dNF1* expression is largely restricted to the nervous system (Walker et al. 2006) and by findings that neuronal re-expression of either fly or human NF1 sufficed to restore *dNF1* growth (Tong et al. 2002; Walker et al. 2006). The *dNF1* growth defect was not suppressed by heterozygous loss of *Ras1* or the Ras exchange factor *Son-of-sevenless* and not enhanced in mutants carrying a gain-of-function *Raf*

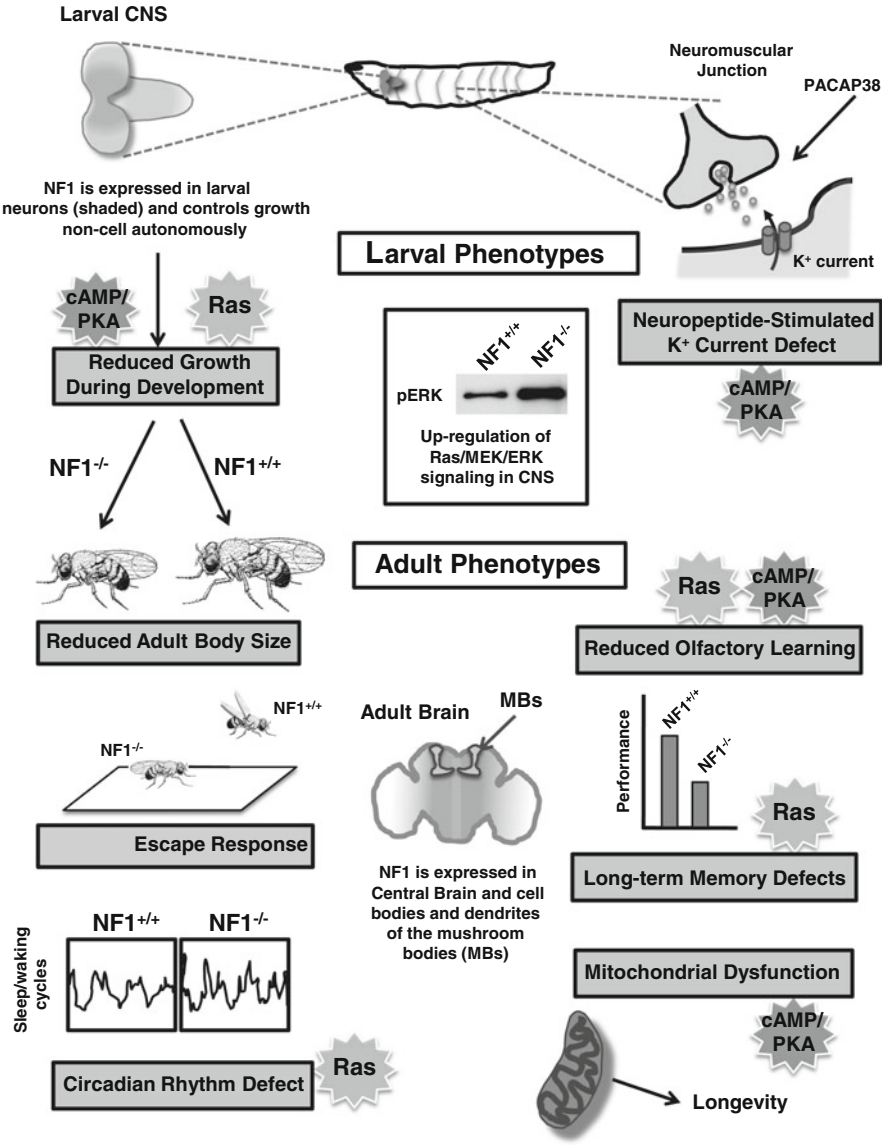

Fig. 34.2 Larval and adult homozygous null *dNF1* phenotypes. *dNF1* larvae lack a neuropeptide-induced rectifying K^+-current at the body wall neuromuscular junction. Larvae, pupae, and adults are 15–20 % smaller than isogenic wild-type controls, and larval and adult *dNF1* brain phospho-ERK levels are approximately threefold higher than in controls. Adult dNF1 flies lack normal day/night rhythmic locomotor behavior and exhibit a reduced olfactory associative learning/short-term memory performance, as well as deficits in middle-term and long-term memory. The diagram depicting the adult brain shows the location of the mushroom bodies (MBs), considered the insect equivalent of the vertebrate hippocampus. MBs play essential roles in olfactory learning and preferentially express several AC/PKA pathway proteins. Although *dNF1* is expressed in MBs, whether its learning/memory-related functions involve cells within or outside of MBs remains controversial (Buchanan and Davis 2010; Gouzi et al. 2011)

allele. Rather, hypomorphic PKA catalytic subunit mutations phenocopied the *dNF1* size defect and expression of a constitutively active PKA transgene throughout development partially restored *dNF1* growth (The et al. 1997).

Organism size is a function of growth rate and duration. In insects, whose growth occurs mostly during larval development, the former is controlled by insulin-like peptides and the latter by a hormonal cascade that culminates in the timed release of the molting hormone, ecdysone. No conclusive evidence implicating *dNF1* in either pathway has yet been found (Walker et al. 2006). Among the various *dNF1* phenotypes, the growth defect is the only one readily amenable to genetic analysis. Another reason for focusing on this defect is that reduced growth is a common symptom of human NF1 and related RASopathies (Szudek et al. 2000).

34.3.2 Behavioral Deficits

dNF1 mutants exhibit subtle behavioral defects. When flies are released, ~15 % do not fly away, even after repeated prodding (The et al. 1997). When adult flies are tapped down in their vials, mutants take longer to climb back up (Tong et al. 2007). No anatomical basis for these defects has been identified; mutants responded normally to visual or olfactory cues (The et al. 1997) and display normal locomotor activity (Williams et al. 2001). Like the reduced growth phenotype, the abnormal climbing response has been attributed to an AC/PKA signaling defect (Tong et al. 2007).

34.3.3 Neuropeptide-Stimulated K^+-Current Defect

Loss of *dNF1* does not affect *sevenless*-mediated Ras signals controlling photoreceptor cell development or torso/Ras signaling in the embryo (The et al. 1997). Hence, to analyze whether *dNF1* affects other Ras pathways, we collaborated with Dr. Yi Zhong, who had reported that a rectifying K^+ current elicited in *Drosophila* larval body wall neuromuscular junction preparations by the mammalian neuropeptide PACAP38 required intact Ras and *rutabaga* AC pathways (Zhong 1995). Electrophysiological analysis showed that *dNF1* mutants lack the PACAP38-elicited current, which was restored by manipulating AC rather than Ras signaling (Guo et al. 1997). An unanswered question remains as to how mammalian PACAP38 stimulates a response in the absence of an obvious *Drosophila* PACAP receptor ortholog.

34.3.4 Circadian Rhythm Defect

The circadian clock controls various rhythmic behaviors, including daily changes in locomotor activity. When analyzed in constant darkness, *dNF1* mutants showed normal cycling of the *period* and *timeless* clock genes but were either completely

($NF1^{P1}$) or nearly completely ($NF1^{P2}$) arrhythmic. Normal rhythmic behavior was restored upon *elav*-GAL4-driven pan-neuronal expression of UAS-*dNF1*, but not by restricted expression in central brain lateral neurons, the site of the circadian clock. Antibody staining revealed a circadian oscillation in phospho-ERK near nerve terminals containing Pigment Dispersing Factor, a secreted output of clock cells, and *dNF1* arhythmicity was restored by mutations that attenuate Ras/ERK signaling. Thus, excess Ras/ERK signaling in *dNF1*-deficient neurons affects a circadian clock output pathway and circadian locomotor activity (Williams et al. 2001).

34.3.5 Associative Olfactory Learning and Short/Middle-Term Memory Defects

Drosophila is widely used to study molecular and cellular circuits responsible for learning and memory (Davis 2005). Because analysis of the neuromuscular signaling defect implicated dNF1 in a pathway involving the *rutabaga* AC, a well-known learning mutant, and because NF1 is associated with learning deficits in both man and mouse (Hyman et al. 2005; Silva et al. 1997), the Zhong group analyzed *dNF1* mutants in a Pavlovian associative learning assay that uses electrical shocks to teach adult flies to choose between two equally aversive odors. In this test, $NF1^{P1}$ and $NF1^{P2}$ mutants both exhibited a significantly reduced learning/short-term memory performance. Arguing that developmental defects do not explain this deficiency, adult expression of a hsp70-*dNF1* transgene suppressed the learning deficit. Further work showed that beyond short-term (3 min) memory, 3- and 8-h middle-term memory retention was also affected in flies lacking *dNF1*. Again implicating abnormal AC/PKA signaling, both learning and memory defects were suppressed by increased PKA expression (Guo et al. 2000).

34.3.6 Long-Term Memory Defect

Excess Ras/ERK signaling underlies the reduced performance of $Nf1^{+/-}$ mice in the Morris water maze, which assesses spatial learning ability (Costa et al. 2002; Cui et al. 2008). By contrast, defective AC signaling appears responsible for the *dNF1* olfactory learning deficit (Guo et al. 2000). Unlike the short-term olfactory learning assay, performance in the water maze requires protein synthesis-dependent long-term memory (LTM). Arguing that this difference may explain the different conclusions reached, *dNF1* null mutants were found to have an LTM defect that, unlike the short-term learning and memory deficits, appeared Ras dependent (Ho et al. 2007). More recent work by others further supports roles for dNF1 in short-term (3 min), middle-term (3 h), and 24-h LTM, and specifically in memory acquisition but not decay (Buchanan and Davis 2010).

34.3.7 Mitochondrial Dysfunction

dNF1 mutants have reduced life spans and increased vulnerability to heat or oxidative stress, in association with reduced mitochondrial respiration and elevated reactive oxygen species (ROS) production. By contrast, *dNF1* overexpression increased life span and improved reproductive fitness, increased resistance to heat and oxidative stress, accompanied by increased mitochondrial respiration and a 60 % reduction in ROS production. Like other *dNF1* defects, these phenotypes were restored by pharmacological or genetic manipulations that increase cAMP/PKA signaling. Counteracting ROS with catalytic antioxidants also restored normal life span to homozygous *dNF1* null mutants (Tong et al. 2007).

34.4 Does *dNF1* Affect AC/PKA Signaling Directly or Indirectly?

As summarized above, genetic evidence strongly supports a link between dNF1 and AC/PKA signaling. A functional link is further supported by biochemical evidence, including the observation that GTPγS-stimulated but not basal AC activity is reduced in $dNF1^{P1}$ and $dNF1^{P2}$ brain membrane preparations (Guo et al. 2000). Moreover, GTPγS-stimulated AC activity and cAMP levels were also found to be reduced in day E12.5 $Nf1^{-/-}$ murine brain extracts (Tong et al. 2002). Thus, while defective AC signaling appears to be an evolutionarily conserved, albeit recessive, *NF1* phenotype, these results do not reveal how neurofibromin affects AC activity. Consistent with the view that non-cell-autonomous mechanisms may be involved, GTPγS-stimulated AC activity was not only reduced in *Drosophila* brain but also in abdominal tissue (Guo et al. 2000), where *dNF1* may not be expressed (Walker et al. 2006). Also compatible with a non-cell-autonomous mechanism, the dNF1/PKA-mediated effect on mitochondrial respiration was detected in experiments with mitochondria isolated from whole *dNF1* mutant flies (Tong et al. 2007; Walker and Bernards 2007).

In experiments aimed at identifying how dNF1 affects AC/PKA signaling, we and others have reached conflicting conclusions. Thus, work by the Zhong group led them to postulate the existence of two dNF1-dependent AC pathways, beyond the canonical Gαs-dependent pathway. The first NF1-requiring pathway, stimulated by serotonin and histamine, depended upon Gαs, whereas a novel AC pathway involved the EGF receptor, NF1, and Ras, but not Gαs. Using transgenic flies expressing human *NF1* deletion and point mutants, the authors reported that NF1-mediated Ras regulation was essential for the novel EGF receptor-stimulated AC pathway, but not for NF1/Gαs-dependent neurotransmitter-stimulated AC activity. Moreover, providing the clearest evidence that NF1 may have Ras-independent functions, a C-terminal segment of human neurofibromin that did not include the GRD was capable of rescuing the AC-dependent organismal growth (Hannan et al. 2006) and learning defects (Ho et al. 2007).

Our conflicting conclusion that excess neuronal Ras/ERK activity is the proximal cause of *dNF1* growth and learning phenotypes is based on the results of two studies. The first focused on the *dNF1* growth defect. In structure/function studies similar to those performed with human *NF1* transgenes (Hannan et al. 2006), we found that large *dNF1* segments other than the GRD were dispensable for growth regulation, that several GAP deficient *dNF1* point mutants did not restore growth, and that expression of a truncated protein representing just the dNF1 GRD was sufficient to suppress the growth defect. Moreover, the growth detect was also suppressed by neuronal expression of a *Drosophila* p120RasGAP ortholog. To investigate why in earlier studies, loss of *Ras1* or other canonical Ras pathway components did not modify the *dNF1* growth defect, we tested a comprehensive set of Ras pathway single and double mutants for their ability to restore *dNF1* growth. As a molecular correlate, we also analyzed the ability of these mutants to restore the elevated larval and adult brain phospho-ERK level. None of the single mutants modified either phenotype, arguing that the tested Ras pathway components are either not involved, or not rate limiting in the pathway leading to ERK activation in *dNF1* brain. Supporting the latter conclusion, some double mutants that did restore normal ERK activity also rescued the growth deficiency at least partially (Walker et al. 2006). Thus, the proximal cause of the non-cell-autonomous *dNF1* organismal growth defect is excessive neuronal Ras/ERK signaling (Walker et al. 2006).

34.4.1 Drosophila *Alk/Ras Signaling May Be Responsible for* dNF1 *Growth and Learning Defects*

Further evidence implicating abnormal Ras/ERK signaling as the primary cause of *dNF1* defects comes from work that began with the observation that overexpression of either the *Drosophila Alk* (*dAlk*) receptor tyrosine kinase or of its activating ligand *jelly belly* (*jeb*) phenocopied both *dNF1* growth and olfactory learning phenotypes. This result was not unexpected, since previous work had identified *dAlk* as an activator of Ras/ERK signaling in vivo (Loren et al. 2001). However, attenuating dAlk expression or activity, either through the use of *dAlk* mutant alleles, *dAlk* shRNA constructs, expression of a dominant-negative *dAlk* transgene, or through pharmacological inhibition, rescued *dNF1* growth, olfactory learning, and brain ERK over-activation phenotypes. In support of the hypothesis that *dAlk* is a rate-limiting activator of *dNF1*-regulated Ras/ERK signals that promote organismal growth and that limit olfactory learning, *dNF1* and *dAlk* expression overlaps extensively in both larval and adult brain. Moreover, *dAlk*-GAL4 driven neuronal UAS-*dNF1* expression sufficed to restore all tested defects (Gouzi et al. 2011). Finally, although a role for dAlk as a negative regulator of learning appears unusual, recent work indicates that murine Alk may have a similar role (Weiss et al. 2012).

Whether excess Alk/NF1/Ras signaling contributes to human NF1 defects is an obvious next question. Intriguingly, loss of *NF1* expression is common and

associated with a worse prognosis in neuroblastoma (Holzel et al. 2010), a significant proportion of which shows *ALK* amplification or gain-of-function *ALK* mutations [reviewed by Azarova et al. (2011)]. ALK overexpression and *NF1* loss have similarly been implicated in glioblastoma (Powers et al. 2002; Verhaak et al. 2010). Finally, mammalian ALK is activated, either directly (Stoica et al. 2001, 2002) or indirectly (Perez-Pinera et al. 2007) by two related secreted ligands, pleiotrophin and midkine. Suggesting the intriguing possibility that inappropriate ALK signaling may contribute to NF1 tumorigenesis, midkine is overexpressed in human NF1 and has been shown to act as a mitogen for NF1 tumor cells (Mashour et al. 2001, 2004).

34.5 Conclusions

Our results have led us to conclude that excess neuronal Ras/ERK signaling is the root cause of most, if not all, *dNF1* defects. However, several important questions remain. Perhaps the most vexing unresolved issue is how *dNF1* affects AC/PKA signaling. One possible mechanism is that loss of *dNF1* precipitates one or more neuroendocrine or neurotransmitter signaling defects. Since hormones and neurotransmitters often signal through AC-coupled receptors, such defects might be restored by increased AC/PKA signaling. We note that in this model, defective AC/PKA signaling does not necessarily involve *dNF1*-requiring neurons. However, although no results to date formally exclude the possibility that *dNF1* affects AC/PKA signaling cell autonomously, our working hypothesis remains that cross talk between distinct neuronal populations may be involved in generating the various Ras/ERK- and AC/PKA-dependent *dNF1* phenotypes. Ongoing genetic screens for dominant genetic modifiers of *dNF1* phenotypes, beyond *dAlk* and *jeb*, experiments to identify the site of action of modifiers, and studies to pinpoint the cells in which increased AC/PKA signaling restores *dNF1* defects, may eventually reveal the neuronal circuits via which *dNF1* exerts its various functions, and provide further clues to molecular and cellular pathways responsible for human NF1 symptoms.

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Chapter 35
Zebrafish Model for NF1

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35.1 Introduction

A number of unique biological and genetic features of the zebrafish (*Danio rerio*) model offer advantages over its mammalian and invertebrate counterparts. These small fish can be stocked at high densities, have a short generation time, and generate large progeny sizes. These characteristics, coupled with external fertilization and rapid development, have made the zebrafish an attractive tool for performing invertebrate-style forward genetic screens in a vertebrate model (Driever et al. 1996; Haffter et al. 1996; Stainier et al. 1996). The optical clarity of the embryos and larvae, coupled with the ability to genetically label specific cell populations by transgenesis, allows for serial *in vivo* visualization of such complex processes as primordial germ cell migration or early vascular development (Köprunner et al. 2001; Lawson and Weinstein 2002). In addition, the zebrafish has emerged as an effective whole-organism vertebrate model for screening large libraries of small molecules with a proven capacity for identification of compounds that suppress disease phenotypes or pathological mechanisms (Peterson et al. 2004; Stern et al. 2005; Hong et al. 2006). Rapidly evolving methods offer increased precision for gene targeting and gene replacement in the zebrafish system (Meng et al. 2008; Doyon et al. 2008; Foley et al. 2009; Zhu et al. 2011). To exploit these

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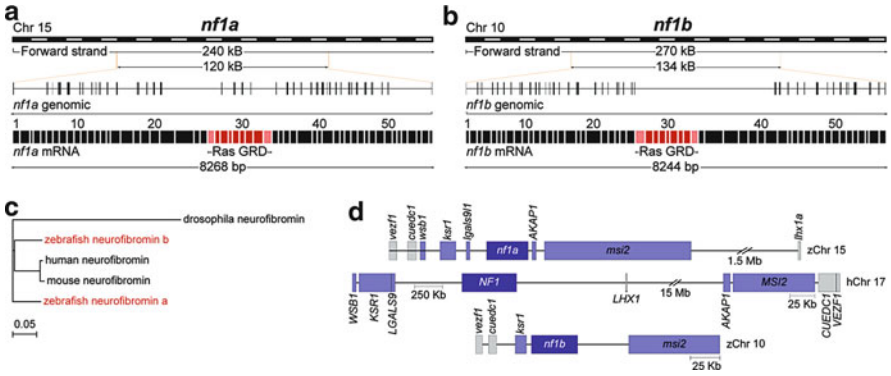

Fig. 35.1 Zebrafish have two orthologues of human *NF1*. (**a**, **b**) Genomic and mRNA structures of the two orthologous zebrafish genes corresponding to human *NF1*. (**c**) Phylogenetic tree comparison of zebrafish, human, mouse, and *Drosophila* neurofibromin. (**d**) Analysis of syntenic relationships between human chromosome 17 (*NF1*) and zebrafish chromosomes 15 (*nf1a*) and 10 (*nf1b*). Relative genomic positions are to scale as indicated

advantages for the study of NF1, we have developed a zebrafish model of this human disorder through identification and characterization of the zebrafish orthologues of the human *NF1* gene, analysis of the phenotypes that arise following their transient loss, and the development of lines of zebrafish harboring stable loss-of-function mutations in these genes (Zhu et al. 2011; Padmanabhan et al. 2009; Lee et al. 2010).

35.2 The Zebrafish Genome Harbors Two Orthologues of Human *NF1*

Bioinformatics analysis of the zebrafish genome identifies two genes orthologous to human *NF1* that are highly similar to it at the amino acid level (90.4 % and 90.7 % respectively). These genes, named *nf1a* and *nf1b*, are highly related to one another sharing 87.4 % identity and 93.7 % similarity as well as similar genomic structures, with each containing 57 exons (Fig. 35.1a, b). *nf1a* is located on chromosome 15 (Fig. 35.1a) and predicts a 311 kDa protein composed of 2,755 amino acids while *nf1b* is located on chromosome 10 (Fig. 35.1b) and predicts a 310 kDa protein composed of 2,747 amino acids. A phylogenetic tree (Fig. 35.1c) demonstrates a tight clustering of the zebrafish neurofibromin orthologues with other mammalian neurofibromins and a divergence from the *Drosophila* neurofibromin orthologue. Analyses of human/zebrafish synteny maps suggest that *nf1a* and *nf1b* likely arose via gene duplication (Fig. 35.1d), consistent with the well-described chromosomal doubling event occurring early in teleost evolution (Amores et al. 1998). Upstream of the human *NF1* gene on chromosome 17 are genes encoding WD repeat and

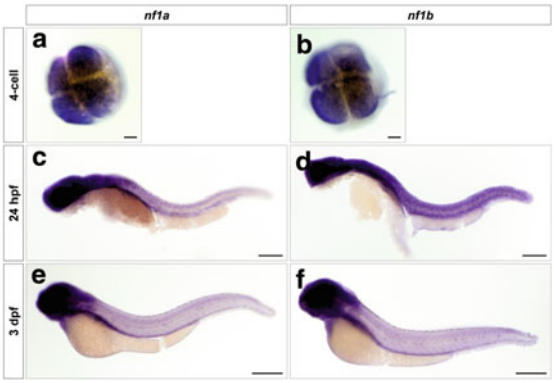

Fig. 35.2 Zebrafish *nf1a* and *nf1b* are expressed during embryonic development. (**a**–**f**) Whole-mount *in situ* hybridization of 4-cell, 24 hpf, and 3 dpf zebrafish embryos shows temporal and spatial expression patterns of *nf1a* and *nf1b*. Scale bars: 25 μm

SOCS box-containing 1 (*WSB1*), Kinase suppressor of ras 1 (*KSR1*), and Galectin-9 (*LGALS9*) while A kinase anchor protein 1 (*AKAP1*) and RNA-binding protein Musashi homolog 2 (*MSI2*) both lie downstream of *NF1*. Similar genes flank *nf1a*, whereas *nf1b* is flanked only by orthologues of *KSR1* and *MSI2*. These genes are expressed maternally (Fig. 35.2a, b) and ubiquitously during early development with later restriction to regions of the head and anterior central nervous system (Fig. 35.2c–f).

35.3 Transient *nf1a* and *nf1b* Loss-of-Function Phenotypes Mimic Those of Murine NF1 Models and Human NF1

Morpholino phosphorodiamidate antisense oligonucleotides (MOs) are a commonly used tool to study gene function in zebrafish by sequence-specific knockdown (Nasevicius and Ekker 2000). The development and validation of multiple MOs directed against *nf1a* and *nf1b* have allowed for assessment of the phenotypic consequences following their loss during early zebrafish development (Padmanabhan et al. 2009; Lee et al. 2010).

35.3.1 Cardiovascular Defects Arising from nf1a and nf1b Knockdown

nf1a and *nf1b* morphant zebrafish embryos display gross abnormalities of cardiovascular development by 48 hours post-fertilization (hpf) including functional deficits in the atrioventricular valve, pooling of blood in the common cardinal vein, and paucity of blood flow along the dorsal aorta and posterior cardinal vein.

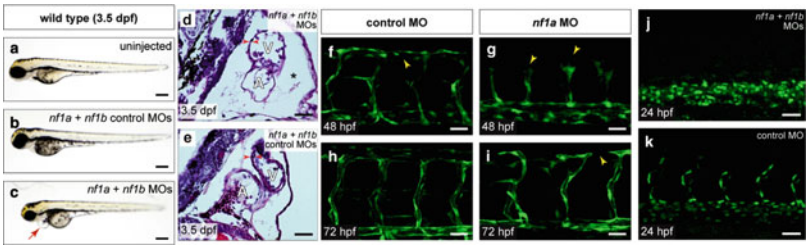

Fig. 35.3 Transient knockdown of *nf1a* and *nf1b* results in cardiovascular defects. (**a**–**c**) *nf1a* + *nf1b* MO-injected embryos reveal no apparent defects in gross morphology, but demonstrate dilation of the pericardial space (**c**) when compared with control-MO injected (**b**) or uninjected (**a**) embryos. (**d**, **e**) Transverse sections of 3.5 dpf *nf1a*/*nf1b* compound morphant embryos reveals a thinning of the ventricular myocardium and pericardial effusion (*asterisk*) when compared with controls (*A*, atrium; *V*, ventricle). (**f**–**i**) At 48 hpf, *nf1a* morphant endothelial-specific GFP transgenic zebrafish embryos display abnormal claw-like projections at the leading edge of the developing intersomitic vessels (**g**) when compared with control-MO treated samples (**f**). By 72 hpf, *nf1a* morphant embryos display only rudimentary DLAVs and a general disorganization of the trunk vasculature (**i**) when compared with control-MO treated embryos (**h**). (**j**, **k**) Development of intersomitic vessels is deficient at 24 hpf in *nf1a*/*nf1b* combined morphant endothelial-specific nuclear GFP transgenic zebrafish embryos (**j**) when compared to controls (**k**). Scale bars: 0.25 mm (**a**–**c**); 25 μm (**d**–**k**)

Gross morphological analysis demonstrates an increased incidence of pericardial effusions beginning at 48 hpf, reflecting cardiac dysfunction, in *nf1a* and *nf1b* morphants when compared with controls (Fig. 35.3a–c). In addition, histological analysis of *nf1a* and *nf1b* morphants at 3.5 days post-fertilization (dpf) demonstrates a thinned ventricular myocardium and large pericardial effusions (Fig. 35.3d, e). These findings are consistent with those of murine models of NF1. *Nf1*$^{-/-}$ mice die during midgestation due to severe cardiac failure and display gross cardiovascular defects including a thinning of the ventricular myocardium, abnormal cardiac valve morphogenesis, and pericardial effusions which have been shown to result from a cell autonomous role for *Nf1* in endothelial cells (Brannan et al. 1994; Jacks et al. 1994; Gitler et al. 2003). Despite these cardiac defects, overall development of the embryos is relatively preserved through the first 3 days.

Knockdown of *nf1a* and *nf1b* in zebrafish embryos harboring a transgene expressing green fluorescent protein (GFP) in the cytoplasm of endothelial cells reveals dramatic abnormalities of vascular patterning in the intersomitic vessels of morphants at 48 and 72 hpf when compared with controls (Fig. 35.3f–i). The leading edge of the sprouting vessels in *nf1a* MO-treated embryos displays claw-like projections at 48 hpf (Fig. 35.3g) and fail to pattern normally such that the dorsal longitudinal anastomotic vessel (DLAV) fails to form, or does so only in rudimentary fashion at 72 hpf (Fig. 35.3i). These abnormalities are evident in embryos that otherwise appear normal in overall size and maturity, with similar defects manifested following compound administration of *nf1a* and *nf1b* MOs or with *nf1b* MO alone, albeit to a lesser degree. There is little or no apparent correlation between these vascular patterning defects and the observed cardiac

defects, as embryos with vascular abnormalities in the absence of pericardial effusions or valvular insufficiency are often observed. Embryos noted to have isolated vascular abnormalities continue to have intact blood flow within both the dorsal aorta and the posterior cardinal vein. At 24 hpf, analysis using zebrafish embryos expressing a nuclear-localized GFP in endothelial cells indicates that morphants display a complete (Fig. 35.3k) or partial absence of intersomitic vessels emanating from the dorsal aorta when compared with controls (Fig. 35.3j). This phenotype was apparent following MO-mediated knockdown of *nf1a* or *nf1b* alone (29 % and 33 % of injected embryos respectively) while knockdown of both in concert had an additive effect (51 % of injected embryos), suggesting partial functional redundancy. Additional confirmation of the role of *nf1a* and *nf1b* in vascular development derives from studies using a genetic background sensitized to vascular insult. Previous studies have employed MOs directed against *flt4*, the zebrafish VEGF receptor-3 orthologue, to investigate genetic interactions during zebrafish artery development (Covassin et al. 2006). Zebrafish embryos injected with *flt4* MO alone or in combination with a MO directed against *nf1a*, *nf1b,* or both display a vascular shunting phenotype that is absent in controls. These shunts occur between the dorsal aorta and the dorsal longitudinal anastomotic vessel with retrograde flow through segmental arteries back into the dorsal aorta or through intersegmental veins into the posterior cardinal vein. In some cases, interruptions were noted along the dorsal aorta. Importantly, abnormalities in vascular patterning have not been previously identified in mouse embryos lacking *Nf1*. In light of the observation of peripheral vascular patterning defects in *nf1a/nf1b* morphant zebrafish embryos, subsequent reevaluation of murine *Nf1* knockouts at time-points prior to overt cardiac failure revealed vascular abnormalities including an increase in overall vascularity and a failure of the primitive vascular plexuses in the somitic region and head to appropriately remodel (Padmanabhan et al. 2009).

These findings underscore the benefit of developing a zebrafish model for NF1. The ability to distinguish a primary vascular defect from a phenotype arising secondary to cardiac failure is possible because early zebrafish vascular development does not require an intact circulation as adequate oxygenation is achieved via passive diffusion (Vogel and Weinstein, 2000). In mice, this is not the case, and it is much more difficult to distinguish a primary vascular defect from one secondary to cardiac dysfunction in a disease model in which both cardiac and vascular abnormalities are present. Interestingly, vascular patterning defects represent a well-recognized component of the pleiotropic spectrum of NF1 disease phenotypes in affected individuals (Friedman et al. 2002). NF1 patients often exhibit a characteristic vascular lesion known as moyamoya, a name that derives from its appearance as a “puff of smoke” on computed tomography scans of the head, due to abnormal small vessel patterning in the brain (Norton et al. 1995; Cairns and North 2008). Other vascular defects, including hypertension and renal artery stenosis, have also been documented. Neurofibromin is known to modulate the activity of *ras* proto-oncogenes through its GAP-related domain (GRD) and multiple lines of evidence support a role for Ras signaling in normal vascular patterning and development (Henkemeyer et al. 1995; Eerola et al. 2003; Liu et al. 2008;

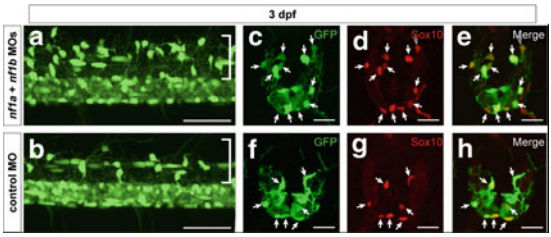

Fig. 35.4 Knockdown of *nf1a* and *nf1b* leads to increased numbers of OPCs. (**a**, **b**) Confocal projections of lateral views along the spinal cord of live 3 dpf *nf1a* + *nf1b* MO-injected *olig2*:GFP transgenic embryos, which also harbor a *p53* mutation to circumvent issues with MO toxicity, demonstrate an increased number of dorsally positioned OPCs in the spinal cord (**a**) when compared with age-matched controls (**b**). (**c**–**h**) Immunohistochemical staining of transverse sections through the spinal cord of 3 dpf *olig2*:GFP transgenic, *p53* mutant zebrafish embryos injected with *nf1a* + *nf1b* MOs (**c**–**e**) show an increase GFP (*green*)/Sox10 (*red*) double-positive cells when compared with age-matched control morphants (**f**–**h**). Scale bars: 50 μm (**a**, **b**); 20 μm (**c**–**h**)

Revencu et al. 2008). Collectively, these data suggest that tight regulation of Ras signaling is required for normal vascular development. The various vascular patterning defects observed in the *nf1*a/*nf1b* morphants may reflect distinct functions of neurofibromin in the vasculature, or may be related by common underlying mechanisms. The genetic interaction between *flt4* and *nf1a/nf1b* suggests that these molecules may function in a common molecular pathway, perhaps related to VEGF receptor signaling through Ras, although alternative interpretations cannot be ruled out.

35.3.2 Oligodendrocyte Progenitor Cell Defects Arising from nf1a and nf1b Knockdown

Oligodendrocytes arise from the motoneuron progenitor (pMN) domain of the ventral spinal cord where the expression of *olig2*, a transcription factor critical for oligodendrocyte specification, is first observed (Lu et al. 2002; Rowitch 2004). Transgenic zebrafish expressing GFP driven by *olig2* regulatory sequences contain two populations of labeled oligodendrocyte progenitor cells (OPCs) in the ventral spinal cord, those that migrate dorsally and differentiate into oligodendrocytes and others that remain in their ventral position intermingled with GFP-positive motoneurons and interneurons (Shin et al. 2003; Park et al. 2004). Ventrally positioned OPCs, but not motoneurons or interneurons, express Sox10 at this stage. At 3 dpf, knockdown of *nf1a*, *nf1b*, or both together in *olig2*:GFP transgenic zebrafish leads to an increase in OPC numbers, as assessed by *in vivo* visualization of dorsally migrating GFP-positive cells (Fig. 35.4a, b) or quantification of Sox10/GFP double-positive cells by immunohistochemical analysis of transverse sections through the spinal cord (Fig. 35.4c–h). Again, knockdown of *nf1a* and *nf1b* together exerted a greater effect than either gene alone, consistent with partial functional redundancy

in their negative regulation of OPC number during development. No appreciable differences are apparent in motoneurons derived from the GFP-positive common precursor cells in the pMN domain of the ventral spinal cord following *nf1a/nf1b* knockdown, suggesting that these genes act specifically on the oligodendrocyte lineage. BrdU pulse-labeling at 80 hpf followed by analysis of BrdU incorporation in GFP/Sox10 double-positive OPCs demonstrates enhanced OPC proliferation in *nf1a/nf1b* morphants when compared to controls, thus accounting for the observed increase in OPC numbers. These data are consistent with murine *in vitro* and *in vivo* studies that show hyperproliferation of astrocytes and oligodendrocytes following heterozygous or homozygous loss of *Nf1* (Gutmann et al. 1999; Bennett et al. 2003; Zhu et al. 2005; Hegedus et al. 2007). In addition, NF1 patients are prone to develop glia-derived tumors including optic pathway gliomas and astrocytomas (Gutmann et al. 2003; Listernick et al. 2007).

Assessment of the dependence of this phenotype upon the well-described negative regulatory activity of neurofibromin on Ras signaling, conferred by virtue of its GAP-related domain (GRD) (Ballester et al. 1990; Xu et al. 1990; Ismat et al. 2006), is readily addressed in the zebrafish system *in vivo* wherein RNA encoding the isolated human *NF1* GRD can be co-injected into *nf1a/nf1b* morphant embryos followed by assessment of OPC numbers in the dorsal spinal cord. Indeed, *nf1a/nf1b* compound morphant embryos injected with human *NF1* GRD RNA exhibited a reduction in dorsally positioned spinal cord OPCs (67.7 per embryo versus 48.5 per embryo) similar to levels seen in embryos injected with control MO or control RNA (Fig. 35.5a–e). These data demonstrate that the isolated GAP activity of neurofibromin is sufficient to rescue the increase in OPCs that results from *nf1a/nf1b* knockdown, indicating that the GAP activity of *nf1a* and *nf1b* regulate OPC proliferation. This finding is notable as murine *Nf1* has been observed to have tissue-specific roles in development that may be independent of GRD activity in some cases (Ismat et al. 2006).

Distinct traits specific to the zebrafish model system allow for a more in-depth analysis of the increase in OPC numbers that follows *nf1a/nf1b* knockdown, namely the ability to assess complex developmental processes *in vivo* by directly visualizing the behavior of fluorescently labeled cell populations in live transgenic embryos. Time-lapse imaging of *nf1a/nf1b* compound morphant *olig2*:GFP embryos over a 12-h period, beginning at 60 hpf followed by quantitative analysis, demonstrates increased numbers of OPCs migrating into the dorsal spinal cord, with morphant OPCs traveling longer distances with shorter pauses when compared with controls (Fig. 35.6). The movements of individual OPCs can be comprehensively analyzed, allowing for construction of migratory cell traces (Fig. 35.6a, b) as well as measurements of total distance traveled (Fig. 35.6c), time spent actively migrating or pausing (Fig. 35.6d), and migration velocity (Fig. 35.6e). OPC migration is characterized by repeated cycles of active migration separated by pauses of variable duration. Knockdown of *nf1a/nf1b* results in abbreviated pauses with no difference in the frequency of pausing or migratory velocity when compared with controls. These data are consistent with *in vitro* studies showing enhanced astrocyte motility (Gutmann et al. 2001) and Schwann cell invasiveness (Kim et al. 1997) in

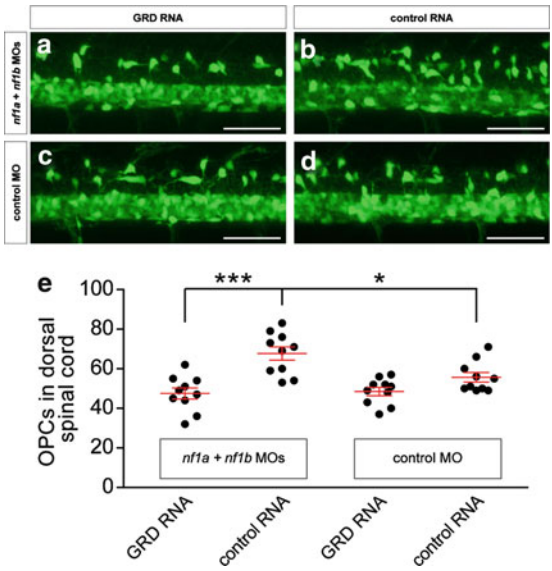

Fig. 35.5 The isolated human *NF1* GRD is sufficient to rescue the OPC proliferation following *nf1a/nf1b* knockdown. (**a**–**d**) Projected confocal images of lateral views along the spinal cord of live 3 dpf *olig2*:GFP transgenic, *p53* mutant zebrafish embryos. Human *NF1* GRD expression in *nf1a* + *nf1b* MO-injected embryos (**a**) attenuates OPC numbers to levels similar to those seen in control MO-injected samples (**c**, **d**), as compared with the increased OPC numbers identified in samples injected with *nf1a/nf1b* MOs and control RNA (**b**). (**e**) Quantitative assessment of OPC numbers in the dorsal spinal cord of individual embryos for the various experimental groups at 3 dpf (*$p < 0.05$; ***$p < 0.001$). Scale bars: 50 μm

the context of haploinsufficiency or homozygous loss of *Nf1*. The *in vivo* motility of these cells, however, has not previously been addressed. The demonstration of enhanced OPC migration following *nf1a/nf1b* knockdown represents a novel phenotype made accessible by modeling NF1 in the zebrafish.

35.4 Generation of Stable Zebrafish Lines Harboring Loss-of-Function Mutations in *nf1a* and *nf1b*

The aforementioned studies utilizing transient knockdown of *nf1a* and *nf1b* showcase the experimental accessibility of the zebrafish embryo and demonstrate its utility as a platform for further mechanistic analyses of the observed phenotypes. However, fully harnessing the power of the zebrafish system necessitates the development of *nf1a*- and *nf1b*-deficient zebrafish lines. Application of cutting-edge reverse genetics strategies in the zebrafish has achieved heritable targeted gene disruption for both *nf1a* and *nf1b* (Zhu et al. 2011). Although classic gene-targeting approaches using homologous recombination in embryonic stem cells has

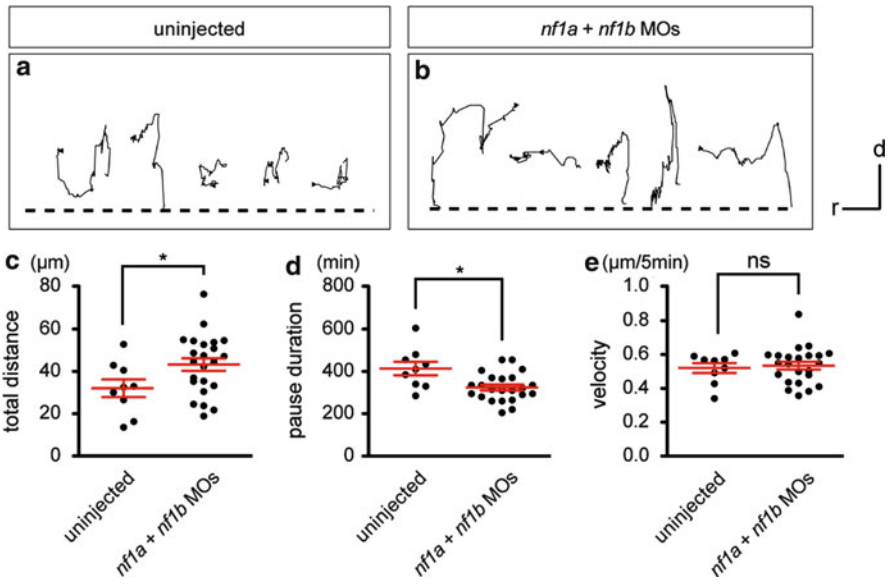

Fig. 35.6 *nf1a/nf1b* loss affects OPC migration. (**a**, **b**) Representative illustrations of the dynamic cell migration paths for five individual OPCs that traveled the greatest distance during the observation period in uninjected (**a**) or *nf1a* + *nf1b* MO-injected (**b**) *olig2*:GFP, *p53* mutant zebrafish embryos. *Arrowheads* indicate the endpoint of cell migration and *dashed line* represents the dorsal-most portion of the GFP-labeled domain in the ventral spinal cord (*top*, dorsal; *left*, rostral). (**c**) Quantitative assessment of total OPC distance traveled in individual embryos shows a 35 % increase in *nf1a* + *nf1b* MO-injected samples when compared with uninjected controls ($*p < 0.05$). (**d**) Quantitative assessment of OPC pause duration in individual *nf1a* + *nf1b* MO-injected embryos shows a 22 % decrease in the total time spent pausing when compared with uninjected controls ($*p < 0.05$). (**e**) No significant difference in velocity of individual OPCs was appreciated in *nf1a* + *nf1b* MO-injected embryos when compared with uninjected controls (*ns* not significant). Scale bars: 10 μm (**c**, **d**; *x*-axis); 5 μm (**c**, **d**; *y*-axis)

not been possible in zebrafish, alternative approaches for targeted mutagenesis using zinc-finger nucleases have been recently described. We have employed this approach to generate multiple independent alleles of *nf1a* and *nf1b* mutants. In addition, random mutagenesis, using chemical mutagens or insertional mutagenesis, followed by gene sequencing has allowed the isolation of zebrafish lines with specific gene mutations. For example, an insertional mutation in zebrafish *nf2* has been described (Amsterdam et al. 2004). Using a TILLING (Wienholds et al. 2002; Wienholds et al. 2003) approach, we have identified several missense mutations in *nf1a* and *nf1b* that will require further characterization. Preliminary studies with the various stable mutant *nf1a* and *nf1b* alleles that are becoming available validate findings produced by *nf1a/nf1b* MO-mediated knockdown and are consistent with functional redundancy of *nf1a* and *nf1b* in the elaboration of NF1-associated phenotypes. Furthermore, analysis of stable mutant *nf1a/nf1b* embryos beyond the time period of MO persistence identifies novel defects in chromatophore

lineages, thus establishing the first animal model of NF1 to recapitulate defects corresponding to the pathognomonic café-au-lait macules of NF1 patients.

35.5 Opportunities for Utilizing the Zebrafish Model of NF1

Modeling NF1 in the zebrafish may now allow us to tackle difficult questions in the NF1 field that are refractory to presently available tools. One such pressing question revolves around the extent to which neurofibromin function in regulating Ras activity accounts for its role in disease. Multiple lines of evidence strongly suggest that functional domains outside the GRD mediate important aspects of neurofibromin biology (Ismat et al. 2006; Upadhyaya et al. 1997; Fahsold et al. 2000; Mattocks et al. 2004; Hannan et al. 2006; Ho et al. 2007). *nf1a/nf1b* stable mutant zebrafish lines will serve as tractable models in which to identify and characterize additional functional domains. Taking advantage of the ease with which proteins or protein fragments can be overexpressed in the zebrafish embryo, functional rescue experiments may be performed using zebrafish or human *NF1* cDNA constructs encoding various putative functional domains. Indeed, a human *NF1* cDNA has been shown to rescue the mutant *Drosophila* phenotype suggesting sufficient evolutionary conservation so as to permit functional replacement (The et al. 1997).

The stable zebrafish lines also promise to serve as a high-throughput discovery platform for both forward genetic approaches as well as small molecule screening. Forward genetic screens performed in the background of *nf1a/nf1b* loss will provide an unbiased approach for identifying novel genetic loci that can enhance or suppress NF1-associated phenotypes. This is critically important as the tremendous clinical variability in the phenotypic spectrum seen among families with the same molecular *NF1* lesion posits the role of unlinked modifier loci in regulating the expressivity of disease characteristics (Sabbagh et al. 2009). Identifying these modifiers would be of great diagnostic and prognostic value, and may further represent new targets for therapeutic development. Whole animal screens, such as those possible using zebrafish, are particularly informative, as they include an inherent requirement for bioavailability and can detect toxicity and off-target effects. Chemical screens performed in the background of *nf1a/nf1b* loss will be informative for the identification of novel lead compounds that may be therapeutically efficacious, and for the identification of molecular pathways that may augment or ameliorate NF1 phenotypes.

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Chapter 36
Cell of Origin and the Contribution of Microenvironment in NF1 Tumorigenesis and Therapeutic Implications

Johanna Buchstaller, D. Wade Clapp, Luis F. Parada, and Yuan Zhu

36.1 Introduction

Neurofibromatosis type 1 (NF1) is one of the most common human inherited diseases affecting 1 in 3,500 newborns in every ethnic background worldwide (Riccardi 1992). Individuals afflicted with NF1 are predisposed to various benign and malignant tumors in different tissues. NF1 is dominantly inherited, and affected individuals carry germline mutations in one *NF1* allele. Because *NF1* germline mutations are inactivating or "loss-of-function" mutations, the *NF1* gene has been classified as a tumor suppressor gene. Consistent with this notion, loss of heterozygosity (LOH) characterized as loss of the inherited wild-type allele or the presence of a mutation in the second *NF1* allele has been found in many NF1-associated benign and malignant tumors (Legius et al. 1993; Side et al. 1997; Xu et al. 1992). One hallmark feature of NF1 is the development of multiple benign peripheral nerve sheath tumors, termed neurofibromas (Cichowski and Jacks 2001; Riccardi

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1992; Zhu and Parada 2001). Some subtypes, particularly plexiform neurofibroma, can acquire additional genetic alterations and progress to malignant peripheral nerve sheath tumors (MPNSTs) (Woodruff 1999).

36.2 Dermal and Plexiform Neurofibromas

Neurofibromas exhibit two characteristic features. First, they arise within peripheral nerves or branches; second, they are heterogeneous masses containing each of the cellular components present in normal peripheral nerves: Schwann cells, neuronal processes, perineurial cells, fibroblasts, and mast cells (Krone et al. 1983; Peltonen et al. 1988). There are mainly two subtypes of neurofibromas associated with NF1, dermal and plexiform neurofibromas (Korf 1999). Dermal neurofibroma is the most common type, arising in association with small, peripheral nerve twigs within the dermis or epidermis. Nearly all individuals with NF1 will eventually develop multiple dermal neurofibromas at some time in their lives. Although potentially disfiguring, dermal neurofibromas are rarely life threatening. Because neurofibromas are comprised of multiple cell types and not all of them are *NF1*-deficient (Muir et al. 2001), LOH has been detected in only a minority of dermal neurofibromas ranging from 13 to 36 % (Colman et al. 1995; Rasmussen et al. 2000; Serra et al. 1997; Thomas et al. 2012). Genetic analysis in mice has demonstrated that neither *Nf1* heterozygous ($Nf1^{+/-}$) mice nor chimeric mice comprised of both $Nf1^{-/-}$ and $Nf1^{+/+}$ cells ($Nf1^{-/-}/Nf1^{+/+}$) developed dermal neurofibromas (Brannan et al. 1994; Cichowski et al. 1999; Jacks et al. 1994b). The mouse studies suggest that additional genetic factors, nongenetic factors, and/or potentially species-specific differences between human and mouse skin could contribute to the development of dermal lesions in humans. Consistently, clinical studies suggest that hormonal changes may play a role in dermal neurofibroma formation, as individuals with NF1 usually develop dermal neurofibromas at the time of puberty and pregnancy often increases tumor size and number (Ferner 2007, 2010).

Approximately 30–50% of individuals with NF1 develop plexiform neurofibromas, which arise along peripheral nerves; the lesions may involve multiple fascicles of a nerve or multiple branches of a large nerve (Korf 1999). Although plexiform neurofibromas share the same cellular composition as dermal lesions, their growth properties and propensity for malignant progression are distinct. Dermal neurofibromas are typically small, localized, and do not progress to malignancy. In contrast, plexiform neurofibromas are typically much larger and grow in a diffuse infiltrative pattern, causing expansion of nerve trunks and their branches. Due to their larger size and deeper location in the body, these tumors often physically impede normal neurological function. In addition, a significant portion of plexiform neurofibromas (5–10 %) eventually undergo malignant transformation and progress to MPNSTs, which is the most common malignancy associated with NF1 (Woodruff 1999). More importantly, MPNST is the most significant cause of death for NF1 patients younger than 40 years of age. Current treatment of plexiform neurofibroma is surgical resection. However, because

plexiform neurofibromas often arise in functionally critical nerves and diffusely infiltrate through the nerves, surgical resection is rarely complete and recurrence is almost inevitable. Therefore, plexiform neurofibroma represents a major source of NF1-associated morbidity.

Thus far, genetic analyses have not revealed obvious differences between plexiform and dermal neurofibromas. In three independent studies, LOH at the *NF1* locus was identified in 40–50 % of plexiform neurofibromas studied, which is comparable to the LOH frequency found in the dermal lesions (Daschner et al. 1997; Kluwe et al. 1999; Rasmussen et al. 2000. Laycock-van Spyk et al. 2011). It is worth noting that with improved techniques, the detection of somatic "second-hit" *NF1* mutations have been found in most, if not all, human neurofibromas (Maertens et al. 2006, 2007). These results suggest that bi-allelic *NF1* inactivation drives both dermal and plexiform neurofibroma formation in humans. Although cytogenetic abnormalities were identified more frequently in cells derived from plexiform tumors than those from dermal lesions, no consistent chromosomal regions with abnormal karyotypes have been identified in plexiform neurofibromas (Wallace et al. 2000). Furthermore, study of chimeric $Nf1^{-/-}/Nf1^{+/+}$ mice demonstrated that bi-allelic inactivation of *Nf1* is sufficient to induce the development of plexiform neurofibromas (Cichowski et al. 1999). These observations support the notion that additional factors other than *NF1*/*Nf1* inactivation contribute to the fundamental differences underlying the development of these two neurofibromas. One well-documented difference between dermal and plexiform neurofibromas is the timing of tumor development and, consequently, the normal cellular target(s) or cell of origin of these benign tumors (Korf 1999; Riccardi 1992).

A critical issue for debate is the cell of origin or the cell type that undergoes *NF1* inactivation and, hence, initiates neurofibroma and subsequently MPNST formation. Because Schwann cells represent the major cell population within neurofibromas (40–80 %) and these cells exhibit abnormal invasive and angiogenic properties, Schwann cells were early on considered to be the most likely cell of origin for neurofibromas (Sheela et al. 1990). Subsequently, genetic studies determining the cell type(s) isolated from human neurofibromas have provided important insights into the nature of the cell of origin (Rutkowski et al. 2000; Serra et al. 2000; Sherman et al. 2000). In addition, the recent development of mouse models with inactivation of *Nf1* at different developmental time points in the Schwann cell lineages has allowed investigation of the nature of the cell of origin of dermal and plexiform neurofibromas as well as MPNSTs (Joseph et al. 2008; Le et al. 2009, 2011; Mayes et al. 2011; Wu et al. 2008; Zheng et al. 2008; Zhu et al. 2002).

36.3 Cell of Origin of Dermal Neurofibromas

By establishing Schwann cell and fibroblast cultures from dermal neurofibromas, several groups have demonstrated that Schwann cells, but not fibroblasts isolated from neurofibromas, typically lack NF1 expression (Rutkowski et al. 2000), have higher levels of Ras-GTP activity (Sherman et al. 2000), and more convincingly harbor bi-allelic *NF1* mutations (Serra et al. 2000). Clinical studies suggest that

hormonal changes may play a role in tumor formation, as individuals with NF1 usually develop dermal neurofibromas at the time of puberty, and pregnancy often increases tumor size and number (Ferner 2007, 2010; Lakkis and Tennekoon 2000). The observation that dermal neurofibromas arise later in life, during adolescence, has led to the speculation that dermal neurofibromas could arise from multipotent stem cells found in the adult organs. Multipotent neural crest-like stem and precursor cells called skin-derived precursor cells (SKPs) have been identified in human and mouse dermis and can be propagated in suspension culture (Fernandes et al. 2004; Sieber-Blum et al. 2004; Wong et al. 2006). Mice in which *Nf1* has been conditionally inactivated in the skin develop dermal neurofibromas, and inactivation of *Nf1* in cultured SKPs leads to neurofibroma formation after transplantation in peripheral nerves (Le et al. 2009). These data suggest that dermal neurofibromas might arise from SKPs or their derivatives.

36.4 Cell of Origin of Plexiform Neurofibromas

In contrast to dermal neurofibromas that usually develop during adolescence and adulthood, plexiform lesions are often identified during early childhood. In one clinic-based study (Waggoner et al. 2000), 44 % (32/72 patients) of plexiform neurofibromas were diagnosed before 5 years of age. Recent longitudinal studies using whole-body volumetric magnetic resonance imaging (MRI) indicate that the number of plexiform neurofibromas does not change significantly over time in individual adult NF1 patients (Cai et al. 2009; Mautner et al. 2008). These observations have led to the hypothesis that plexiform neurofibromas are congenital lesions that arise from developing Schwann cell lineages during development (Riccardi 1992).

36.4.1 Schwann Cell Development

Schwann cell development is a complex process that includes multiple transition phases from neural crest stem cells, Schwann cell precursors, committed Schwann cells, and two mature cell types – myelinating and nonmyelinating Schwann cells (Jessen and Mirsky 2005) (Fig. 36.1). Recent work has been directed at narrowing down the critical developmental stages during which *NF1* inactivation must occur in the Schwann cell lineage to efficiently initiate plexiform neurofibromas.

36.4.1.1 Neural Crest Stem Cells

Schwann cells arise from neural crest stem cells (Fig. 36.1). In the vertebrate embryo, neural crest cells detach from the tips of the dorsal neural folds after neural

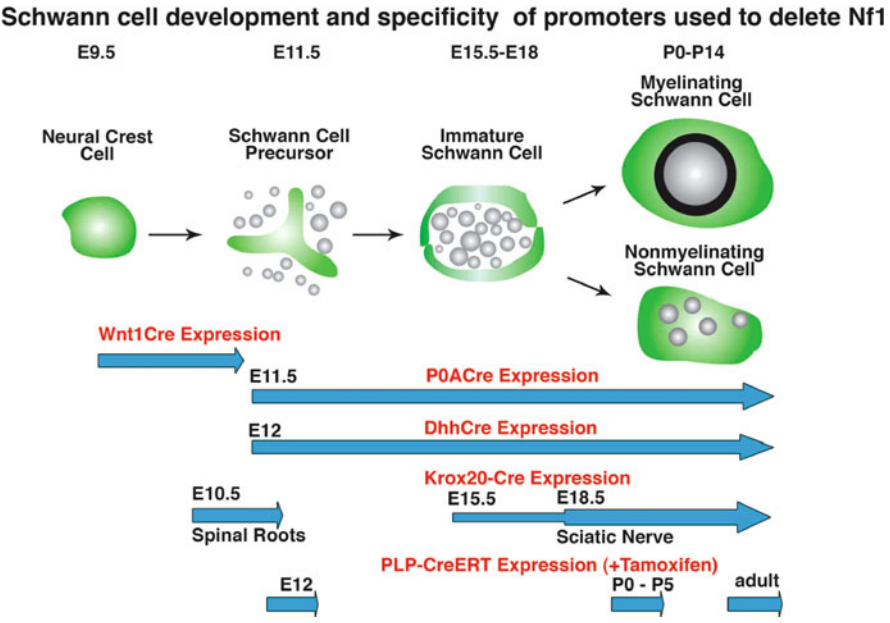

Fig. 36.1 Schwann cell development and promoters used to delete *Nf1* in the Schwann cell lineage [adapted from Jessen and Mirsky (2005)]. Schwann cell arises from neural crest stem cells that migrate out of the dorsal part of the neural tube at embryonic (E) day 9 in the mouse. They travel through the somites and associate with growing sensory and motor nerves at E11.5 where they become Schwann cell precursor cells, although some cells have been shown to remain multipotent (Morrison et al. 1999). Schwann cell precursor cells migrate along the axon bundles, proliferate and progress in their lineage under the influence of axonal signals to yield immature Schwann cells, which invade the axon bundles, undergo proliferative arrest, and proceed to sort the axons by caliber (Jessen and Mirsky 2005). Schwann cells associate with large-caliber axons in a one-to-one relationship at around birth (postnatal (P) day 0), and this interaction triggers the formation of the myelin sheath during the first 2 weeks of life (P0–P14). Several small-caliber axons are engulfed by a Schwann cell which will remain nonmyelinating. *Nf1* has been ablated in the Schwann cell lineage using mice that express Cre recombinase at different developmental time points. The use of Wnt1-cre led to deletion of *Nf1* in migrating neural crest cells (Joseph et al. 2008), whereas P0-cre and Dhh-cre expression leads to deletion of *Nf1* at the earliest stages of Schwann cell development in the peripheral nerve (Joseph et al. 2008; Wu et al. 2008; Zheng et al. 2008). Krox-cre is expressed during development in multipotent boundary cap cells in peripheral nerve roots, at low levels in immature Schwann cells from E15.5 to E18 and finally at high levels in pro-myelinating Schwann cells starting at E18 (Zhu et al. 2002). Mice in which *Nf1* was conditionally inactivated upon the administration of tamoxifen during more narrow-time windows were used by Le et al. (2011) and Mayes et al. (2011). Except for the Wnt1-cre;$Nf1^{flox/flox}$ mice that die at birth and could not be further analyzed, all the other Nf1 mutant strains developed plexiform neurofibromas with a latency of 6 to 12 months

tube closure and migrate along defined pathways to sites in the developing embryo, where they generate a great variety of cell types depending on the anterior–posterior location of the neural tube from which they came (Dupin et al. 2006; Dupin and Sommer 2012; Morales et al. 2005). The neural crest cells of the trunk

differentiate into melanocytes of the skin, neurosecretory cells of the adrenal and thyroid glands, as well as peripheral neurons and glia in sensory, autonomic, and enteric ganglia. Early experiments in which single pre-migratory and migratory cells in avian and mouse embryos were labeled and followed in their target organs have suggested that migrating neural crest cells are a mixture of multipotent stem cells and cells that are already committed to a certain fate (Bronner-Fraser and Fraser 1988, 1989, 1991; Serbedzija et al. 1994). However, more recent in vivo lineage tracing studies have suggested that the fate of neural crest cells might already be determined prior to delamination and depends on the time of delamination (Krispin et al. 2010). Even if neural crest cells appear to be restricted in their fate in vivo, at least a subset of cells appears to retain the potential to self-renew and differentiate into various cell lineages when isolated and challenged with appropriate growth factors in vitro (Dupin et al. 1990; Shah et al. 1994; Sieber-Blum and Cohen 1980; Trentin et al. 2004) or transplanted to a different region of the embryo in vivo. After transplantation to different regions of the anterior–posterior axis, migrating neural crest cells can adopt the fate of the region into which they are transplanted (Le Douarin and Kalcheim 1999), and late-migrating cells can replace early migrating ones in transplantation experiments (Baker et al. 1997). Taken together, these data suggest that neural crest cells have the potential to differentiate into various cell types even though their fate might be more restricted in vivo. It is likely that during their journey and in their target organs, a combination of positive and negative signals allows for the maturation of neural crest cells into various lineages (Jessen and Mirsky 2005).

36.4.1.2 Multipotent Cells Persist in Embryonic and Adult Target Organs

Stem cells that have the potential to self-renew and differentiate into several neural crest lineages after in vitro culture and in vivo transplantation assays have also been isolated from various embryonic and adult neural crest target organs, such as the dorsal root ganglia (DRG) (Hagedorn et al. 1999; Nagoshi et al. 2008), gut (Bixby et al. 2002; Kruger et al. 2002; Lo and Anderson 1995), skin (Fernandes et al. 2004; Wong et al. 2006), and embryonic sciatic nerves (Bixby et al. 2002; Morrison et al. 1999). The in vivo fate of neural crest cells from the peripheral nerve appears to be more restricted, but these cells can still give rise to a surprising variety of cell types. Neural crest-derived cells from the early embryonic peripheral nerve give rise to glia, endoneurial fibroblasts (Joseph et al. 2004), and melanocytes in the skin (Adameyko et al. 2009), but do not form neurons in vivo. Neural crest-derived cells found in the adult gut give primarily rise to glia, and adult neurogenesis in the adult gut is a rare phenomenon even during regeneration after injury or bacterial infection (Joseph et al. 2011). To date, cells with self-renewing capacity and multilineage differentiation potential have not been isolated from the adult sciatic nerves (Kruger et al. 2002). It could be that this neural crest target organ is devoid of adult stem-like cells, but it could also be that the right growth and dissociation conditions remain to be established.

36.4.1.3 Schwann Cell Lineage Restriction

How and exactly at what time point(s) the glial lineage arises and separates from multipotent neural crest stem cells is still poorly understood (Jessen and Mirsky 2005). After trunk neural crest cells reach the somites, they aggregate to form the dorsal root ganglia (DRG). Neural crest-derived cells associate with the sensory nerves growing out of spinal and cranial nerves and differentiate into Schwann cell precursor cells, which travel along the axon bundles, closely following the tip of the growth cone (Wanner et al. 2006). Although not necessary for the nerves to grow and reach their targets (Grim et al. 1992; Riethmacher et al. 1997; Woldeyesus et al. 1999), Schwann cell precursor cells provide trophic support for the survival of sensory and motor neurons and are important for nerve fasciculation (Garratt et al. 2000). Schwann cell precursor cells proliferate and progress in their lineage under the influence of axonal signals to yield immature Schwann cells, which invade the axon bundles and sort the axons by their caliber. Schwann cells associated with large-caliber axons myelinate the axons in a 1:1 Schwann cell–axon relationship. Small-caliber axons ranging from single to >50 axons are engulfed by a Schwann cell that remains nonmyelinating, in a structure called the Remak bundle (Jessen and Mirsky 2005) (Fig. 36.1).

36.4.1.4 Plasticity of Schwann Cells After Injury

When Schwann cells are separated from axons, during an injury, for example, they can dedifferentiate and gain a molecular and morphological phenotype that is similar but not identical to immature Schwann cells before myelination (Jessen and Mirsky 2008). These Schwann cells proliferate and together with infiltrating macrophages clear the axonal and myelin debris. They form tubes that allow the axons to regrow from upstream of the site of damage. The Schwann cells then redifferentiate and remyelinate the axons (Jessen and Mirsky 2008; Mirsky et al. 2008; Zochodne 2012). Despite their ability to reenter the cell cycle after injury, Schwann cells in mature nerves are generally post-mitotic. This feature, along with the fact that no adult stem cell population has been identified in peripheral nerves, may explain the fact that plexiform neurofibroma is extremely rare in the general population (Korf 1999). A critical question then arises of how germline *NF1* heterozygous mutations render a relatively tumor-resistant organ, the peripheral nerve, highly susceptible to benign and malignant tumor formation. At least some of the answers to this fundamental question lie in the identification of the cell(s) of origin of benign neurofibromas and MPNSTs.

36.4.2 Cell of Origin of Plexiform Neurofibroma

A fundamental question in cancer biology is which cell type is at the origin of cancer development (Visvader 2011). Many adult tissues contain stem cells, which self-renew and undergo multilineage differentiation for life in order to maintain the tissue. These

stem cells persist throughout life, although they decrease in number, whereas restricted progenitors and differentiated cells usually have a short life span. Since multiple mutations are typically necessary for a cell to become cancerous (Knudson 1993), it has been hypothesized that mutations must accumulate in the long-lived stem cells with substantial self-renewal capacities for cancer to develop (Clevers 2011). Lineage tracing experiments have revealed this to be the case for crypt stem cells in the intestine (Barker et al. 2009). Other cancers, however, appear to arise from lineage-restricted progenitors or differentiated cells which are due to mutations conferring self-renewal properties usually found in stem cells (Cozzio et al. 2003; Huntly et al. 2004; Jamieson et al. 2004; Krivtsov et al. 2006; Magee et al. 2012; Visvader 2011; Yang et al. 2008b). Another possibility is that since cancer is a multistep process, mutations could start to accumulate in stem cells, but it is the progenitor that starts to proliferate aberrantly (Pardal et al. 2003; Reya et al. 2001; Yang et al. 2008b). The nature of the cell of origin influences which types of mutations can induce cancer in experimental mouse models (Heuser et al. 2009; Somervaille et al. 2009). These data are, however, based on experimentally induced mouse models, and it is unclear in humans which biological differences among human cancers reflect differences in cell of origin (reviewed in Magee et al. 2012). In addition, evidence suggests that differences in the cell of origin might lead to differences in the biological properties of the cancer (Magee et al. 2012; Visvader 2011).

Although it had been shown that loss of Nf1 function in neurofibromas and MPNSTs occurs in Schwann cells in humans and mice, it remained unclear at what time point(s) during Schwann cell development tumor formation is initiated (Cichowski et al. 1999; Serra et al. 2000). The development of mouse models in which it is possible to conditionally inactivate genes at specific time points in different cellular lineages allowed us to investigate this question (Fig. 36.1). To this end, we developed a mouse model of plexiform neurofibroma in which we targeted an *Nf1* mutation to the Schwann cell lineage by using Krox20-cre transgenic mice (Zhu et al. 2002). Krox20-cre is expressed during early embryonic development, at embryonic day 10.5 (E10.5) in nerve roots of cranial and spinal nerves, where multipotent NCSC cells (boundary cap cells) are found (Maro et al. 2004). It is expressed at low levels at later stages (E15.5–E18.5) in immature Schwann cells, until the start of myelination when expression levels go up in pro-myelinating Schwann cells (Ghislain et al. 2002). Krox20-cre-driven *Nf1* conditional knockout mice (Krox20-cre+;$Nf1^{flox/-}$) developed plexiform neurofibromas along nerve roots of cranial and spinal nerves with 100 % penetrance, but neurofibroma formation was never detected in sciatic nerves despite the presence of cells that had undergone loss of *Nf1* (Zhu et al. 2002). Plexiform neurofibromas observed in this model exhibit every discernible molecular, histological, and ultrastructural characteristics of their human counterparts. These data suggest that neurofibroma does not arise from intact myelinating Schwann cells but that the cell of origin appears to be another cell type in the NCSC/SC lineage during developing stages, which is consistent with the hypothesis that plexiform neurofibroma is a congenital lesion in humans (Korf 1999; Riccardi 1992).

To further investigate the cell of origin of neurofibroma, we and others inactivated *Nf1* in migrating neural crest cells and Schwann cell precursor cells in the developing nerves (Joseph et al. 2008; Le et al. 2011; Mayes et al. 2011; Wu et al. 2008; Zheng et al. 2008). *Nf1* deletion in migrating neural crest cells led to a transient expansion and increased self-renewal capacity of neural crest stem cells from the peripheral nerves during embryonic stages. However, no tumors were observed at birth or early postnatal stages, and transplanting *Nf1*-deficient neural crest stem cells into the peripheral nerves of wild-type or *Nf1* heterozygous mice did not lead to tumor formation (Joseph et al. 2008). Early postnatal lethality precluded the analysis of those animals at later stages in this study (Joseph et al. 2008). After circumventing the early postnatal lethality by using different Cre driver animals with a more localized transgene expression, it has been shown that *Nf1* deletion in NCSC/SCP early in the developing nerves led to neurofibroma formation with 100 % penetrance with a latency of 4–12 months (Wu et al. 2008) or 12–24 months (Zheng et al. 2008). In one study, analysis of the nerves of these animals after myelination was completed at postnatal day 22 revealed no difference in the number of Schwann cells present in mutant nerves compared to control and normal myelination (Zheng et al. 2008). However, one subtle phenotypic alteration was observed: some mutant Remak bundles exhibited a Schwann cell pocket defect, containing large numbers of unseparated axons, which was rarely seen in normal nerves (Fig. 36.2A–C). At 3 months of age, abnormally differentiated Remak bundles in mutant nerves were degenerated, leading to Schwann cell dissociation and axonal loss (Fig. 36.2D–G). The number of dissociated and unassociated nonmyelinating Schwann cells gradually increased during neurofibroma formation (Zheng et al. 2008). These data suggest that the nonmyelinating Schwann cell is a cell of origin of neurofibroma in mice. Deletion of *Nf1* in the embryonic Schwann cell lineage thus might lead to developmental defects in axonal sorting and aberrant interactions of axons and non-myelinating Schwann cells, which could lead to degeneration of Remak bundles and to tumor initiation in peripheral nerves by proliferating nonmyelinating Schwann cells. These data, however, do not exclude the possibility that loss of *Nf1* in adult Schwann cells could lead to tumor formation as well, perhaps under different conditions such as injury. More recently, *Nf1* was conditionally deleted at different stages of Schwann cell development using temporally and spatially controlled Cre driver transgenes (Fig. 36.1). Loss of *Nf1* in Schwann cell precursor cells or immature Schwann cells before the beginning of myelination led to a tumor incidence of 80–100 % (Le et al. 2011; Mayes et al. 2011). Tumor formation was also observed when *Nf1* was deleted in adult Schwann cells; however, in one study, only 2 % of mice developed tumors (Le et al. 2011), whereas in the other study, 100 % of mice developed tumors (Mayes et al. 2011). These data suggest that the susceptibility to neurofibroma formation is at its greatest when loss of *Nf1* occurs in Schwann cells during embryonic and early postnatal development but that adult cells can also be affected. It remains to be determined which cell type is affected when loss of *Nf1* occurs in the adult nerves. It could be a fully differentiated mature Schwann cell (nonmyelinating or myelinating), a dedifferentiated Schwann cell, or a rare more immature cell type that has persisted

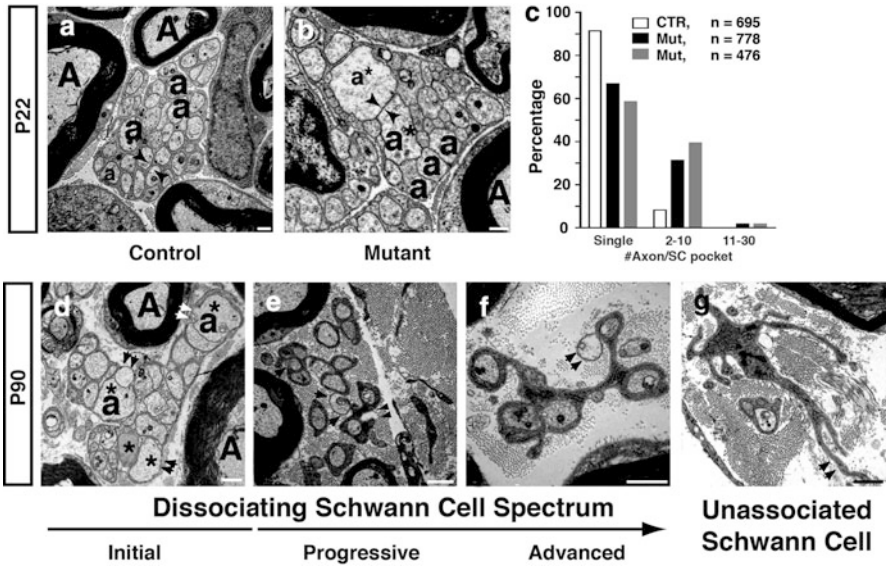

Fig. 36.2 Abnormal Remak bundle formation and degeneration in *Nf1* mutant sciatic nerves [from Zheng et al. (2008)]. Transmission electron microscopy analysis of Remak bundles in sciatic nerves at postnatal (P) day 22 shows abnormal segregation in *Nf1*-deficient mice (**B**) compared to controls (**A**). Scale bars are 1 μm in **A**–**B**. Mutant Schwann cell bundles contain axons (a) which remain directly opposed to each other (*arrowheads* in **B**), while in control mice, they are separated by Schwann cell cytoplasm (*arrowheads* in **A**). Abnormally dilated axons are also observed (a*). (**C**) The percentage distribution of axons per Schwann cell pocket in control and mutant nerves is shown. There is a significant reduction in the number of properly segregated axons between mutant and control Remak bundles and a significant increase in axon bundles which contain >2 unsegregated axons ($p < 0.0001$ in chi-square test). (**D**–**G**) Electron micrographs show various stages of degenerating axon bundles at P90 on cross sections of *Nf1*-deficient sciatic nerves. *Arrows* in (**D**) point to unsegregated axons (*asterisk*) dissociating from each other and to naked axons in (**E**) and (**F**), devoid of Schwann cell cytoplasm. Axonal degeneration likely leads to the presence of free or dissociated Schwann cells found at advanced stages of degeneration (**G**). These free Schwann cells proliferate and lead to neurofibroma formation. Scale bars are 1 μm in all *panels*

in adult nerves. In response to nerve injury, Schwann cells can dedifferentiate and adopt a more undifferentiated state that resembles the immature Schwann cell state. It has been shown that activation of the Raf/MEK/ERK signaling pathway is sufficient to induce dedifferentiation of myelinated Schwann cells in vitro and in vivo (Harrisingh et al. 2004; Napoli et al. 2012). Lloyd and colleagues recently showed that short activation of Raf-kinase in uninjured nerves was sufficient to drive the dedifferentiation of myelinating Schwann cells to a progenitor-like state in peripheral adult nerves and neurofbromas could form from myelinating Schwann cells when loss of *Nf1* was induced in the sciatic nerve in concert with an injury (Kalamarides et al. 2012). Taken together, these data show that bi-allelic inactivation of *Nf1* in the Schwann cell lineages leads to neurofibroma formation. Plexiform neurofibroma penetrance is highest when *Nf1* is deleted in embryonic Schwann

cells in mouse models of the disease, but deletion of *Nf1* in adult Schwann cells could also lead to tumor formation. This shows that cell types at different stages of lineage development have the potential to form tumors, although this might occur via different mechanisms (aberrant axonal sorting, Remak bundle defects, dedifferentiation, and aberrant proliferation after injury).

36.4.3 Cell of Origin of MPNST

The frequency of MPNST in the general population is extremely low with an incidence of 0.001 % whose peak is between the ages of 60 and 70 (Evans et al. 2002). However, individuals with NF1 are estimated to have a 7–13 % lifetime risk of developing MPNST, which usually occurs at much younger ages between 20 and 40 years (Evans et al. 2002). Although the mechanisms underlying this clinical observation is not fully understood, the presence of a benign precursor lesion of MPNST, plexiform neurofibroma, in peripheral nerves of NF1 patients but rarely in normal individuals, provides a mechanistic basis for NF1-associated tumor susceptibility in peripheral nerves. Clinical studies indicate that the majority of MPNSTs arise from preexisting plexiform neurofibromas in individuals with NF1 (Woodruff 1999). Among individuals with NF1, the patients with internal plexiform neurofibromas are 20 times more likely to develop MPNSTs than those without such plexiform lesions (Szudek et al. 2003; Tucker et al. 2005). These observations support the notion that the predisposition to plexiform neurofibroma is the major underlying cause for which *NF1* germline mutations render peripheral nerves highly susceptible to MPNST in individuals with NF1. Furthermore, these clinical results also suggest that plexiform neurofibroma is a source of MPNST. A recent study using array-based comparative genomic hybridization (aCGH) revealed an identical genomic microdeletion observed in an autologous pair of plexiform neurofibroma and MPNST, thereby providing definitive evidence that plexiform neurofibroma and MPNST could share a common tumor progenitor cell (Beert et al. 2011). Future studies using next-generation sequencing technologies should provide comprehensive genetic profiles that will more definitively establish the lineage relationship between plexiform neurofibroma and MPNST. Consistent with the notion that plexiform neurofibroma arises from a fully differentiated nonmyelinated Schwann cell, a recent study using mouse models also suggests that MPNST derives from differentiated Schwann cells, but not from neural crest stem cells (Joseph et al. 2008).

It has been established that bi-allelic inactivation of *NF1/Nf1* underlies plexiform neurofibroma formation in humans and mice (Cichowski et al. 1999; Zhu et al. 2002). However, the molecular mechanisms responsible for malignant transformation of MPNST are less clear. Thus far, only two genetic alterations have been identified as important elements of malignant transformation pathways in MPNST development. Mutations in the *p53* gene have been identified exclusively in MPNST but not in benign neurofibroma, suggesting that the p53-mediated pathway may be involved in the malignant transformation of peripheral nerve sheath tumor

(PNST) (Legius et al. 1994; Menon et al. 1990). Consistent with the role of p53 in the later stages of PNST development, neither homozygous $p53^{-/-}$ nor heterozygous $p53^{+/-}$ mutant mice develop neurofibroma or MPNST (Donehower et al. 1992; Jacks et al. 1994a). In contrast, mice harboring both *Nf1* and *p53* mutations on the same chromosome (so-called "*Nf1*/*p53* cis" mice) develop MPNSTs with high frequency (Cichowski et al. 1999; Vogel et al. 1999). Therefore, both human genetic studies and mouse models support a stepwise genetic model for the development of PNST: (1) loss of *NF1* initiates neurofibroma formation and (2) malignant transformation of PNST requires additional genetic lesions such as those in the p53-mediated pathway. Genetic alterations in the *CDKN2A*/$p16^{INK4a}$ locus are also frequently identified in MPNST but not in neurofibroma (Kourea et al. 1999b; Nielsen et al. 1999), suggesting that defects at this locus may also contribute to malignant transformation of neurofibroma. The *CDKN2A*/$p16^{INK4a}$ locus encodes two independent tumor suppressor genes, $p14^{ARF}$ and $p16^{INK4a}$. The $p14^{ARF}$ gene product ($p19^{Arf}$ in mice) positively regulates p53 function by antagonizing Mdm2-mediated degradation of p53 protein (Sherr 2001). Thus, loss of $p14^{ARF}$ function in MPNST is consistent with the hypothesis that the p53-mediated tumor suppressor pathway plays a pivotal role in malignant transformation of PNST. However, a high frequency of homozygous deletions (50–60 %) disrupting both $p14^{ARF}$ and $p16^{INK4a}$ has been identified in MPNST (Kourea et al. 1999b; Nielsen et al. 1999), suggesting that loss of $p16^{INK4a}$ may also contribute to malignant transformation of PNST. Consistently, loss of $p16^{INK4a}$ is exclusively found in MPNST but not in neurofibroma in human tumors. In addition, genetic alterations of *RB* and amplification of *CDK4* are detected in MPNST lacking $p16^{INK4a}$ mutations (Berner et al. 1999), suggesting that the Rb-mediated G1/S checkpoint pathway may also play a critical role in malignant transformation of PNST. Furthermore, loss of expression of another component of the Rb pathway, $p27^{KIP1}$, has been identified in most MPNSTs (91 %), as compared to only 6 % of neurofibromas (Kourea et al. 1999a). In mice, neither $p16^{Ink4a}$ loss nor $p19^{Arf}$ loss in the *Nf1* heterozygous background induces neurofibroma or MPNST formation (Joseph et al. 2008; King et al. 2002). However, about 26 % of *Ink4a*/$Arf^{-/-}$ double mutant mice in the *Nf1* heterozygous background develop MPNSTs (Joseph et al. 2008). Thus, these studies indicate that loss of both $p16^{Ink4a}$ and $p19^{Arf}$ function is required for *Nf1*-mediated MPNST formation in mice, which further emphasizes the importance of the p53-mediated pathway in the development of MPNST. Recent studies using aCGH on human tumors have shown that while plexiform neurofibromas exhibit few or no chromosomal abnormalities, MPNSTs have complex genomes with widespread chromosomal loss or gain (Mantripragada et al. 2008, 2009). Some of the chromosomal alterations are recurrent, suggesting that potential oncogenes and tumor suppressor genes may reside in these chromosomal regions responsible for the transformation of plexiform neurofibroma to MPNST. Finally, given a sizable number of MPNSTs arising without evidence of plexiform neurofibroma, it is clear that cells in the Schwann cell lineages are capable of undergoing malignant transformation without going through a benign tumor stage (Woodruff 1999). Whether neural crest stem cells, Schwann cell precursors, and mature nonmyelinating or

myelinating Schwann cells can serve as cell(s) of origin of MPNST remains to be determined. The answer to this question requires the development of new highly penetrant MPNST models with targeted mutations at different stages of Schwann cell development.

36.4.4 Tumor Microenvironment and Therapeutic Implications

Research efforts to identify the cell of origin of NF1-associated tumors not only will reveal fundamental mechanisms underlying the role of NF1 in normal Schwann cell development and tumorigenesis but also will have important therapeutic implications. As discussed above, NF1-associated MPNST may represent the only soft tissue sarcoma (STS) subtype with a defined developmental basis and a critical benign precursor lesion. As such, it is a realistic expectation to design chemoprevention strategies to target plexiform neurofibroma and MPNST at the earliest stages. Because plexiform neurofibroma arises from abnormal interactions between nonmyelinating Schwann cells and axons during early postnatal development (Zheng et al. 2008), one possible strategy is to stabilize the axon–Schwann cell interaction as a therapeutic approach to inhibit neurofibroma initiation. Furthermore, compared to MPNST, plexiform neurofibroma appears to have a relatively stable genome with few genetic alterations (Mantripragada et al. 2008, 2009). These features suggest that plexiform neurofibroma may highly depend on its tumor microenvironment. Indeed, studies using mouse models demonstrate that plexiform neurofibroma arises from a nerve environment with nerve degeneration and mast cell infiltration, and more importantly, the development of plexiform neurofibroma is critically dependent on the *Nf1* heterozygous cells in the tumor microenvironment (Zheng et al. 2008; Zhu et al. 2002). These observations are supported by both in vitro and in vivo studies showing that the cell types in the tumor microenvironment including fibroblasts, endothelial cells, and mast cells promote the proliferation and tumorigenic capacities of *Nf1*-deficient Schwann cells more efficiently when they are heterozygous for *Nf1* (Ingram et al. 2000, 2001; Staser et al. 2012; Yang et al. 2003, 2006) (see review in Staser et al. 2012). A recent elegant study using reciprocal bone marrow transplant and compound *Nf1*/c-Kit receptor mutant mice has implicated bone marrow-derived c-Kit-expressing *Nf1* heterozygous mast cells as a key mediator of plexiform neurofibroma formation (Yang et al. 2008a). More importantly, the use of Imatinib (also known as gleevec) has shown therapeutic benefits on an inoperable plexiform neurofibroma in an NF1 patient. Given the striking role of c-kit/kit-L in preventing plexiform neurofibroma formation, a simplistic hypothesis suggests that imatinib disrupts SCF/c-kit-mediated mast cell cytopoiesis and inflammation, thereby interrupting a key link between the tumorigenic Schwann cell and the mitogenic cues required to sustain itself and its microenvironment. However, cooperating mechanisms of action may also exist (Staser et al. 2012). For example, imatinib's well-characterized inhibitory action on the c-abl nonreceptor tyrosine kinase may additionally disrupt TGF-β-mediated signaling in biochemically deregulated fibroblasts comprising the tumor stroma, as has been suggested experimentally in

studies of mouse- and patient-derived cells. Further, imatinib can inhibit signaling at the PDGF receptor, which Schwann cells also express in high concentration (Demestre et al. 2010). Similarly, vascular cells, which are prevalent in the tumor microenvironment, may also depend on PDGF receptor signaling as may angiogenic macrophages that facilitate neoangiogenesis (Li et al. 2006; Munchhof et al. 2006). Accordingly, imatinib may operate on several cell-receptor systems that are important for tumor growth, although the data from transplantation-based and other studies compellingly suggest that disruption of the SCF/c-kit signaling axis may be of primary importance (Staser et al. 2012).

In summary, we believe that cell-of-origin research on NF1-associated tumors will provide important insights into the design of therapies for preventing and treating these incurable human tumors.

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Chapter 37
Molecular and Cellular Approaches to Cognitive Impairments Associated with NF1 and Other Rasopathies

Yong-Seok Lee and Alcino J. Silva

37.1 Introduction

Neurofibromatosis type 1 (NF1) is a common genetic disorder which affects multiple systems including the nervous system, eyes, skeleton, endocrine system, blood vessels, and skin (Ratner and North 2003). Pigmentation abnormalities such as café-au-lait spots are one of the characteristic features. Importantly, NF1 patients show various neuropsychological phenotypes such as learning disabilities.

NF1 is an autosomal dominant disorder caused by loss-of-function mutations in a chromosome 17 gene (*NF1*) which encodes neurofibromin. Neurofibromin is involved in multiple signaling pathways, including Ras-MAPK and the adenylate cyclase-cAMP cascades. Its Ras-GAP (GTPase-activating protein) function has been intensely studied, and it seems to play a key role in brain phenotypes. The NF1 Ras-GAP negatively regulates Ras-MAPK signaling by converting GTP-bound active Ras to its GDP-bound inactive form (Weiss et al. 1999). Accordingly, the loss of NF1 function causes an abnormal increase in Ras signaling in many tissues including brain tissue.

There are several other developmental disorders that share many symptoms with NF1 such as congenital heart deficits, growth delay, and cognitive problems. These include Noonan syndrome (NS), Costello syndrome, LEOPARD syndrome, CFC syndrome, and Legius syndrome. Genetic studies have found that these disorders are caused by mutations in various components of Ras-MAPK signaling. Consequently, these disorders have recently been referred to as rasopathies (Zenker 2011). The emergent similarities in molecular pathogenesis account for the parallels in their clinical symptoms. Advances in clinical genetics not only enable us to make a more precise diagnosis on this group of patients, they also provide us with a great

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opportunity to study the molecular and cellular mechanisms underlying these diseases, which we hope will eventually translate into more effective treatments for this set of conditions.

During the last decade, emerging molecular and cellular findings have brought us closer than ever to understanding the cognitive deficits associated with NF1 and other rasopathies. Animal models, especially mouse models, have played a central role in generating these exciting new findings. In this chapter, we will review molecular, cellular, and behavioral studies of mouse models of NF1 and other rasopathies that have dramatically changed our understanding of the mechanisms and treatments for these conditions. In addition, we shall discuss how the findings from mouse models are being translated to humans.

37.2 Cognitive Symptoms Associated with NF1 and Other Rasopathies

NF1 and other rasopathies affect the same multiple organs, and the brain is one of them. However, each disease displays a different pattern of cognitive impairment. For example, a global cognitive impairment such as an intellectual disability is relatively uncommon in NF1, whereas it is more frequent in CFC syndrome (Wieczorek et al. 1997). Even within one disease, the phenotypic expression is highly variable. We shall review the cognitive symptoms associated with rasopathies with a focus on NF1 and NS.

37.2.1 NF1

Executive functions are associated with goal-directed behaviors that require attention, planning, organizational skills, and inhibitory control. Although global intellectual impairment is uncommon in NF1, specific executive functions are often associated with this condition. Additionally, several studies found discrepancies between verbal IQ and performance IQ (Eliason 1986), suggesting dysfunction in the visual–spatial domain in NF1. Indeed, visual–spatial impairments have been reported in NF1 (Kelly 2004). Children with NF1 who exhibit visual–spatial problems often also display memory deficits (Eliason 1988).

Attention deficit disorder is prominent in NF1. Approximately 40 % of children with NF1 are estimated to have an attention problem. Problems with sustained attention, selective attention, and divided attention have been found in people with NF1 (Hyman et al. 2005; Mautner et al. 2002). Children with NF1 who have attention problems have been treated with stimulants with some positive effects (Aron et al. 1990; Mautner et al. 2002). However, more longitudinal follow-up

studies are required to precisely evaluate the beneficial as well as negative effects of stimulants in this population.

Learning disability is very common in children with NF1. Studies showed that the frequency of learning disability associated with NF1 ranges from 40 % to 60 % (Ratner and North 2003). Learning disabilities are largely divided into verbal (language-based) and nonverbal types. Verbal-type disabilities include reading and writing problems, and nonverbal-type disabilities include visual–spatial deficits, spatial learning difficulties, and social problems. It is important to note that both verbal and nonverbal learning disabilities are associated with NF1, suggesting that learning disabilities in this population do not fall into traditional diagnostic categories. This challenges the traditional view that different learning disabilities reflect distinct pathological causes. In the case of NF1, mutations in a single gene can cause multiple types of learning disabilities (Shilyansky et al. 2010b).

In addition to deficits in higher-order cognitive functions, abnormalities in motor coordination have also been described in the NF1 population. For example, children with NF1 show problems with balance and gait (Hofman et al. 1994; North et al. 1994), which can be associated with learning difficulties in nonverbal tasks.

37.2.2 Noonan Syndrome

Noonan syndrome (NS) is the most common (1 in 2,500 live births) genetic disorder among rasopathies. It is characterized by facial abnormalities, short stature, motor delay, cardiac defects, and cognitive problems (Noonan 1994; Romano et al. 2010; Tartaglia and Gelb 2005). Mutations in the *PTPN11* gene, which encodes the non-receptor protein tyrosine phosphatase SHP-2, account for ~50 % of NS cases. Other germline mutations in *SOS1*, *KRAS*, *RAF1*, *BRAF, SOS1, SHOC2, MEK1, and CBL* have also been reported to cause NS. These mutations affect positive regulators of the Ras-MAPK cascade. As in NF1, they result in increases in Ras-MAPK signaling.

IQ studies in NS have shown that they are highly heterogeneous, ranging from low scores characteristic of mental retardation to the normal range. Average IQ scores are lower in NS individuals than in the general population. Although common (affects approximately 30 % of individuals), mental retardation is usually mild in NS (Lee et al. 2005; van der Burgt et al. 1999). Recent studies have suggested that this heterogeneity in cognitive ability among NS can be partially ascribed to the specific mutations and genes affected (Cesarini et al. 2009; Pierpont et al. 2009).

Discrepancies between verbal and nonverbal IQs have also been reported by some NS studies (van der Burgt et al. 1999; Pierpont et al. 2009; but see Lee et al. 2005). Problems in spatial function, attention, and other executive functions such as planning ability have also been reported in NS. However, many of these

observations are from studies with relatively small numbers of subjects and need to be confirmed by larger studies.

Learning disabilities are quite common in NS. An estimated 30–50 % of patients have special educational needs (Wingbermuehle et al. 2009; Zenker et al. 2004). Impairments in language development (Pierpont et al. 2010) and a higher rate of motor delay, such as clumsiness and poor coordination, have also been reported in NS (Romano et al. 2010). These deficits can affect both verbal- and nonverbal-type learning.

Alfieri and colleagues evaluated long-term memory in individuals affected by NS and LEOPARD syndrome (Alfieri et al. 2011). Hippocampus-dependent long-term memory assessed by a delayed verbal free recall task is impaired in most of the patients. However, recognition memory is largely spared, suggesting that patients with NS show a distinct pattern of memory deficits.

In addition, several studies have suggested that patients with NS have problems in social cognition such as alexithymia (inability to express feelings with words), which can cause delays in social adaptation (Verhoeven et al. 2008; Wingbermuhle et al. 2011).

37.2.3 Cognition in Other Rasopathies

Costello syndrome is a rare disorder resulting from germline mutations in *HRAS*. As with NF1 and NS, Costello syndrome is associated with cardiac deficits, growth delay, and cognitive impairments. Individuals with Costello syndrome show mild to borderline intellectual disabilities (Axelrad et al. 2011). Patients are also characterized by problems in both verbal and nonverbal cognition. Memory impairments on tests of verbal, visual, and spatial learning have also been reported (Dileone et al. 2010). However, other studies showed that learning and memory skills fall in the mildly disabled to borderline range (Axelrad et al. 2011). Problems in visuomotor skills, adaptive behavior, and anxiety have been also reported in Costello syndrome (Axelrad et al. 2011).

The recently named Legius syndrome (an NF1-like syndrome) is caused by loss-of-function mutations in *SPRED1*, another negative regulator of Ras-MAPK signaling (Brems et al. 2007). As with NF1, loss-of-function mutations of *SPRED1* also result in hyperactivity in MEK/MAPK signaling. Patients with Legius syndrome show similar phenotypes to NF1, but they do not develop neurofibromas and central nervous system tumors (Brems et al. 2007). Importantly, affected children display attention deficit and learning disabilities (Brems et al. 2007; Pasmant et al. 2009).

Cardio-facio-cutaneous (CFC) syndrome is another rare rasopathy caused by mutations in *BRAF*, *MEK1,* or *MEK2*. Most of the affected individuals have mild to severe intellectual disabilities (Papadopoulou et al. 2011).

37.3 Testing Cognition in Mouse Models

Discovery of the genes underlying the rasopathies has led to animal model studies in mice. The hope is that these studies will lead to a better understanding of the mechanisms affected and consequently to targeted treatments. Mutant mice are useful tools in studies of cognitive disorders for several reasons. First, behavioral tasks can be designed to model human cognitive deficits. Importantly, behavioral tests can be designed to specifically test function in defined brain regions, including the hippocampus and prefrontal cortex. Second, information about molecular and cellular mechanisms underlying certain behaviors, such as learning and memory, could be used to probe the mechanisms affected by specific mutations, such as those responsible for the rasopathies. This can provide much needed mechanistic links between behavioral phenotypes and molecular and cellular mechanisms. Despite the obvious differences, there is a great deal of evolutionary conservation between mice and men. This is important because there are critical studies that can be done in mice that would be ethically unthinkable in human subjects. However, a word of caution is also in order: there is a significant gap between mice and men, and this could help to explain why not all therapies developed in mouse models have been useful in patients.

37.3.1 Hippocampus-Dependent Learning and Memory

The hippocampus has a well-known role in spatial learning in human subjects (Squire 1992). Hippocampus-dependent phenotypes, such as spatial learning deficits, can be tested in the hidden-platform version of the Morris water maze task (Morris et al. 1982). In this task, mice are trained to find and remember the location of a platform in a round pool of water. Since the platform is hidden just underneath the surface of opaque water, mice have to use spatial cues placed around the pool to learn where the platform is located. Following training, memory can be assessed in a probe trial where the platform is removed from the pool and the mice are allowed to look for it during a small interval of time (usually 60 s). Generally, more accurate and persistent searches for the platform are thought to reflect better learning and memory. A version of this task, where the position of the escape platform is visibly marked, is used to control for other brain functions needed for spatial learning such as vision, motor function, and motivation. Deficits in these other functions affect both versions of the water maze task.

Contextual fear conditioning is another task widely used to examine hippocampus-dependent learning and memory (Phillips and LeDoux 1992). In this task, mice are placed in a conditioning chamber for training. After a few minutes, mild electric shocks are delivered from the floor of the chamber, and consequently, the mice learn to fear the chamber. When returned to the conditioning chamber,

the mice show freezing responses (a reflection of fear). These and other tasks have been shown to be sensitive to hippocampal damage.

Importantly, mechanistic studies of hippocampal-dependent learning and memory (including studies with the water maze and contextual conditioning) have identified long-term potentiation (LTP) as a key cellular mechanism. Deficits in hippocampal LTP usually lead to hippocampal-dependent learning and memory deficits, while most mutant mice with enhancements in hippocampal learning and memory have been shown to have enhancements in LTP. Additionally, hippocampal learning is usually accompanied by increases in hippocampal synaptic function that mimic the properties of LTP. It is noteworthy that impairments in LTP have been reported in many mouse models of cognitive disorders (Matynia et al. 2002).

37.3.2 Attention

Attention deficits are common in NF1 (Ferner et al. 1996). The lateralized reaction time task is used to test attention deficits in rodents (Jentsch et al. 2009a). Prefrontal deficits affect performance in this task in rodents and in humans. In this task, mice are placed in an operant chamber and trained to fixate on central nose-poking ports while paying attention to two other ports on either side of the central port. For brief (but variable) intervals, a light cue is shown in one of the lateral ports, and to get a reward, the subject needs to poke its nose onto the port that just lit up. Attention problems lead the animals to choose the wrong port or more frequently they fail to respond to the light. Animals with attention problems can learn the task and respond normally when the light is on for long periods of time (i.e., 10 s). However, these same animals will show clear deficits compared with normal animals when the light is on for only very brief periods of time (i.e., 1 s).

37.3.3 Working Memory

Working memory systems hold and update information transiently. Corticostriatal networks are thought to be critically involved in working memory. This form of memory can be tested in rodents with the delayed win-shift radial 8-arm maze task. During training in this task, only 4 of the 8 arms of the maze are open and the subjects have to remember this information so that they can enter only unvisited arms during both the training session and the subsequent test session with all arms open. The pattern of open and closed arms changes after each pair of training and testing sessions, thereby forcing subjects to use working memory systems. There are two types of errors that subjects can commit during testing: (1) within-phase errors reflect reentries into the arms already visited during the same test session (affected by corticostriatal lesions), and (2) across-phase errors refer to reentries

into arms that were visited during the previous training session (affected by hippocampal damage).

A delayed nonmatch-to-position test is also used often to examine working memory in rodents (Jentsch et al. 2009b). Each trial consists of a sample phase and a choice phase with a short interval (3–6 s). In the sample phase, a subject is placed in an operant chamber with multiple nose-poke holes, and one of the holes is illuminated. Subjects are supposed to poke their nose in that hole, and they are then rewarded in the other side of the chamber. During the choice phase, in addition to the hole that is lit during the sample phase, another hole (nonmatch position) is illuminated. Response (i.e., nose poking) to the nonmatch hole will result in a reward (hence, the name nonmatch to sample). Subjects with working memory problems make more incorrect choices and more omissions (failure to respond).

37.4 Mouse Models for NF1

The majority of *NF1* mutations are loss-of-function mutations causing truncated, nonfunctional forms of neurofibromin (Shen et al. 1996; Thomson et al. 2002). The sequence, transcriptional regulation, and downstream targets of *NF1* are highly conserved between mouse and human (Bernards et al. 1993; Hajra et al. 1994). To study NF1, mice with a heterozygous null mutation of the *Nf1* gene ($Nf1^{+/-}$) were generated by inserting a *neo* gene in exon 31 of the *Nf1* gene, which results in an unstable, quickly degraded transcript (Jacks et al. 1994). This mutant shows cognitive deficits parallel to human NF1 and has been widely used to study the cellular and molecular mechanisms underlying those deficits (see below).

Among 60 exons in the murine *Nf1* gene, exon 23a is required for efficient Ras-GAP activity. The isoform with *Nf1 23a* is predominantly expressed in neurons. To examine the role of this neuron-specific isoform of *Nf1*, Costa and colleagues generated a mutant mouse harboring a homozygous deletion in exon 23 of the *Nf1* gene (Costa et al. 2001). This mutant showed learning deficits without other consequences of the *Nf1* mutation, such as increased tumor predisposition (Costa et al. 2001), providing evidence that altered NF1 signaling, not tumor formation, for example, is responsible for the learning deficits observed in NF1.

To specifically examine the effect of *Nf1* deletion in specific cell types, conditional knockout mutants have been generated using the Cre-loxP system. A mouse line was engineered with loxP sites flanking exons 31–32 of the *Nf1* gene ($Nf1^{flox/+}$) (Zhu et al. 2001). Temporal and cell type-specific deletion of *Nf1* can be achieved by crossing with a line expressing Cre recombinase under a specific promoter. This system has been extensively used to identify the cellular mechanism underlying the cognitive deficit as we reviewed below. For example, recently, Brown and colleagues developed a mouse model that takes advantage of this Cre system and yields an $Nf1^{+/-}$ mutant harboring complete *Nf1* gene loss in GFAP-expressing glial cells (*Nf1* OPG mouse) (Brown et al. 2010). Unlike the $Nf1^{+/-}$ mice, *Nf1* OPG

developed optic gliomas. Importantly, this mutation also seems to cause reduced striatal dopamine and attention deficits.

37.4.1 Behavioral Phenotypes of *Nf1*$^{+/-}$ and Other NF1 Mouse Models

Nf1$^{+/-}$ mice display multiple behavioral deficits that are analogous to cognitive symptoms in human NF1. First, *Nf1*$^{+/-}$ mice exhibited deficits in the hidden-platform version of the Morris water maze which is a hippocampus-dependent spatial learning task (Costa et al. 2002; Cui et al. 2008; Li et al. 2005; Silva et al. 1997) (see Sect. 37.3.1). During the probe trial, heterozygous mutants spent significantly less time in the target quadrant where the platform was located during the training. Importantly, this memory deficit could be overcome by additional training trials. Additional training also ameliorates NF1 learning deficits (Silva et al. 1997). In addition to the water maze, *Nf1*$^{+/-}$ mice showed deficits in contextual fear conditioning when a mild training condition was applied (Cui et al. 2008). Park and colleagues used *Nf1*$^{+/-}$ mice in a different genetic background (C57Bl/6) from Cui et al. (C57Bl/6 × 129T2/SvEmsJ F1 hybrid) and also found impaired contextual fear conditioning in *Nf1*$^{+/-}$ mice at 7 days after training (Park et al. 2009). Interestingly, learning deficits in *Nf1*$^{+/-}$ mice are restricted to specific domains. For example, *Nf1*$^{+/-}$ mice were not impaired in either the visible-platform version of the water maze or in auditory fear conditioning. Unlike contextual conditioning and the spatial version of the water, these two other tasks are not hippocampal-dependent. The same pattern of cognitive deficits in specific domains has been observed in NF1 patients. Moreover, a similar pattern of hippocampal learning deficits was also found in *Nf1*$^{23a\ -/-}$ mice (Costa et al. 2001).

It has been known for some time that the expressivity of NF1 is highly heterogeneous (i.e., incomplete penetrance). This issue was also addressed in mouse models. Under conditions in which a heterozygous null mutant for NMDA receptor subunit NR1 (*NR1*$^{+/-}$) showed no deficits, mice that are both *Nf1*$^{+/-}$ and *NR1*$^{+/-}$ showed profound spatial learning and contextual discrimination deficits (Frankland et al. 1998; Silva et al. 1997). These results demonstrate that genetic modifiers (e.g., *NR1*$^{+/-}$) could have profound effects on the penetrance of learning phenotypes in NF1. It is conceivable that NF1 patients may have mutations that by themselves do not cause learning deficits (the case of the *NR1*$^{+/-}$ mutation) but that exacerbate the learning and memory phenotypes of NF1.

Attention in *Nf1*$^{+/-}$ mice has been assessed in several tasks (Brown et al. 2010), including the lateralized reaction time task (Li et al. 2005). *Nf1*$^{+/-}$ mice made significantly more errors and omissions than wild-type littermates when the light cue presentation time was less than 0.5 s, meaning *Nf1*$^{+/-}$ mice have the same attention deficit as NF1 patients (Li et al. 2005). As mentioned above, the finding

that *Nf1* mutations affect attention systems in mice is in agreement with recent findings from Brown et al. (2010).

Shilyansky and colleagues tested working memory, one element of executive function in *Nf1*$^{+/-}$ mice (Shilyansky et al. 2010a). *Nf1*$^{+/-}$ mice made more within-phase errors during the test session in the delayed win-shift radial arm maze task, suggesting that *Nf1*$^{+/-}$ mice have cortical-dependent working memory deficits. To confirm the working memory deficit, *Nf1*$^{+/-}$ mice were also tested in the delayed nonmatch-to-position test. *Nf1*$^{+/-}$ mice showed comparable performance to wild-type littermates during the sample phase. However, *Nf1*$^{+/-}$ mice made significantly more incorrect choices during the choice phase, supporting the view that *Nf1*$^{+/-}$ mice have deficits in working memory (Shilyansky et al. 2010a).

Nf1 OPG exhibited similar phenotypes to *Nf1*$^{+/-}$ mice. *Nf1* OPG mice showed deficits in hippocampus-dependent behavioral tasks such as the hidden-platform version of Morris water maze task and the object-placement test (Brown et al. 2010). Moreover, this mouse model showed significant reduction in rearing frequency in response to a novel environment and to an object in a novel position, which suggests that *Nf1* OPG mice show deficits in both nonselective and selective attention (Brown et al. 2010).

It is important to note that the different *Nf1* mouse models discussed above made distinct but important contributions to our understanding of the CNS phenotypes associated with this condition. These studies suggest that a single mouse model does not have to recreate all of the phenotypes associated with a given disorder to be useful in studies of that disorder. In some respects, it may be best that some mouse models only show subcomponents of the phenotypic complexity seen in patients, since this simplification can help to elucidate the multiple causal relationships underlying complex clusters of deficits associated with that disorder. For example, the various *Nf1* mouse mutants mentioned already do not have the significant tumor load expressed in many Nf1 patients, demonstrating that tumors alone cannot explain learning and other cognitive deficits in NF1. Consistent with this view, studies in patients did not find a compelling connection between tumor load and cognitive deficits.

37.4.2 Cellular and Molecular Mechanisms Underlying Behavioral Deficits in Nf1$^{+/-}$

37.4.2.1 Increased Ras Function and Impaired Plasticity

Since NF1 is caused by loss-of-function mutations in neurofibromin, a negative regulator of Ras signaling, it is not surprising that increased Ras signaling is a key mechanism underlying the cognitive impairment in *Nf1* mice. Indeed, increases in Ras activity and its downstream signaling components (MEK/ERK) have been observed in both the hippocampus and cortex of *Nf1*$^{+/-}$ mice (Li et al. 2005).

The idea that increased Ras signaling underlies the cognitive deficits of *Nf1*$^{+/-}$ mice has also been tested by genetic and pharmacological approaches. Costa and colleagues crossed *Nf1*$^{+/-}$ mice to heterozygous Ras knockout mice (*K-ras*$^{+/-}$ or *N-Ras*$^{+/-}$) to genetically decrease Ras activity (Costa et al. 2002). Both the *Nf1*$^{+/-}$ and *K-ras*$^{+/-}$ mutations impair spatial learning in the Morris water maze. However, mice with both mutations have normal spatial learning! In addition, the spatial learning deficits in *Nf1*$^{+/-}$ mice were also rescued by treatment with a farnesyl transferase inhibitor (FTI) (Costa et al. 2002). FTI reduces Ras signaling by blocking farnesylation, a posttranslational modification that is required for Ras activation. These data show that adult increases in Ras signaling are responsible for the hippocampus-dependent cognitive deficits in *Nf1* mutant mice.

Importantly, the ability to rescue NF1 cognitive phenotypes with pharmacological interventions in adults (see also Brown et al. 2010; Li et al. 2005) demonstrated that these behavioral deficits were not solely caused by irreversible developmental changes, but can instead be treated in adults. Neurofibromin regulates trophic factor signaling, and this and other effects of *Nf1* mutations in development were thought to be at the heart of cognitive deficits in *Nf1* mutant mice. Since the Nf1 studies, other groups have also been able to rescue, in adults, phenotypes previously believed to be exclusively neurodevelopmental in origin, including those associated with other developmental disorders such as fragile X syndrome, Down syndrome, Rett syndrome, and tuberous sclerosis (Dolen et al. 2007; Ehninger et al. 2008a, b; Fernandez et al. 2007; Guy et al. 2007). Thus, work that started with animal models of NF1 led to a dramatic change in the neuropathology and treatment development for neurodevelopmental disorders. These studies have shown that although neurodevelopmental disorders have clear developmental components (e.g., deficits that originate in prenatal development), addressing adult molecular and cellular phenotypes (e.g., increased Ras signaling in adult *Nf1* mice) can have an unexpected positive impact on the cognitive phenotypes associated with these disorders.

Searching for a clinically sustainable and FDA-approved treatment, Li and colleagues tested the effect of statins (i.e., lovastatin) on *Nf1*$^{+/-}$ mice. Lovastatin not only lowers cholesterol levels but also reduces Ras activation by blocking isoprenylation of Ras (Li et al. 2005). Similar to FTI, lovastatin treatment rescued the water maze deficit in *Nf1*$^{+/-}$ mice. Moreover, lovastatin treatment also rescued the deficits in attention in *Nf1*$^{+/-}$ mice, suggesting that this treatment has the potential to target different domains of behavioral deficits associated with NF1, including attention and spatial cognition (Li et al. 2005).

What is the physiological mechanism underlying learning and memory deficits in *Nf1*$^{+/-}$ mice? There is a considerable amount of convergent evidence that demonstrates that long-term synaptic plasticity, especially hippocampal long-term potentiation (LTP), is a key physiological mechanism underlying spatial learning and memory (Lee and Silva 2009; Matynia et al. 2002). Manipulations that decrease LTP usually cause spatial learning deficits, whilst the vast majority (>90 %) of mutant mice with enhanced spatial learning also show enhanced LTP in the hippocampal CA1 region (Lee and Silva 2009). Importantly, LTP-like changes are observed in the hippocampus following learning (Whitlock et al. 2006).

Importantly, the Ras-ERK signaling pathway is known to play important roles in hippocampal LTP (Sweatt 2001). Indeed, *Nf1*$^{+/-}$ mice showed deficits in hippocampal LTP induced by theta burst stimulation (TBS) (Costa et al. 2002; Cui et al. 2008; Li et al. 2005). Strikingly, the very manipulations that reverse behavioral deficits in *Nf1*$^{+/-}$ mice were also able to reverse LTP deficits in these mice. For example, the K-Ras knockout mutation, FTI, and lovastatin treatments all rescued LTP deficits in *Nf1*$^{+/-}$ mice (Costa et al. 2002; Cui et al. 2008; Li et al. 2005). These data demonstrate that increased Ras activity leads to LTP impairments that underlie the learning and memory deficits in *Nf1*$^{+/-}$ mice.

37.4.2.2 Increased Inhibition: A Bridge Between Increased Ras and Impaired LTP

How does increased Ras activity result in LTP deficits in *Nf1*$^{+/-}$ mice? What is the cellular consequence of increased Ras signaling? Several lines of evidence suggest that increased Ras signaling results in increased GABA-mediated inhibition in *Nf1*$^{+/-}$ mice (Costa et al. 2002; Cui et al. 2008). First, the hippocampal LTP deficit was revealed only by TBS which is more sensitive to changes in GABA-mediated inhibition. Second, LTP deficits were evident in *Nf1*$^{+/-}$ mice at synaptic stimulation strength (60 mA) that cause an increase in GABA-mediated inhibition, but were absent at synaptic stimulation strength (35 mA) that do not cause increases in inhibition. Third, evoked IPSC measured in the presence of an AMPA and NMDA receptor blocker was larger in *Nf1*$^{+/-}$. Finally, picrotoxin, a $GABA_A$ receptor antagonist, rescued the hippocampal LTP deficit in *Nf1*$^{+/-}$.

To address directly the GABA hypothesis, Cui and colleagues used genetic approaches to examine the effect of deleting *Nf1* in specific types of neurons. Using the Cre-loxP system, *Nf1* was deleted in either excitatory neurons, inhibitory neurons, or glial cells (Cui et al. 2008). Heterozygous deletion of *Nf1* only in inhibitory neurons caused deficits in Morris water maze, whereas deletion in either excitatory neurons (heterozygous or homozygous) or glial cells (heterozygous) did not have the same impact on learning (no spatial learning deficits). Accordingly, the LTP deficit was found only when *Nf1* was deleted from inhibitory neurons (not when *Nf1* was deleted from excitatory neurons). Activity-dependent increases in GABA release, but not glutamate release, were found in the hippocampus of *Nf1*$^{+/-}$ mice, confirming that the balance between inhibition and excitation is altered in *Nf1*$^{+/-}$ mice. Importantly, the increased GABA release was blocked by Ras/MEK/ERK signaling inhibitors (Cui et al. 2008). These studies also showed that *Nf1*$^{+/-}$ mice have increases in the phosphorylation of synapsin I at the ERK site. Since this phosphorylation is known to lead to higher neurotransmitter release, this finding provides another important molecular step for the mechanism underlying the learning deficits in *Nf1*$^{+/-}$ mice. Thus, these mice have higher Ras/ERK signaling, higher synapsin I ERK phosphorylation, and consequently higher GABA release, which then results in deficits in LTP underlying their learning deficits. Importantly,

hippocampal behavioral deficits in *Nf1*$^{+/-}$ mice (e.g., spatial learning) could be rescued by treatments with a subthreshold dose of picrotoxin (Cui et al. 2008).

37.4.2.3 Increased Inhibition and Corticostriatal Functions

Inhibition also plays important roles in other brain areas such as the cortex and striatum. These areas are involved in attention and working memory which are also affected in NF1. Shilyansky and colleagues examined if the same mechanism (increased inhibition) is responsible for working memory deficits in *Nf1*$^{+/-}$ mice (Shilyansky et al. 2010a). Electrophysiological analysis showed that activity-dependent GABA release is increased in both the medial prefrontal cortex (mPFC) and striatum of *Nf1*$^{+/-}$ mice compared to wild-type littermates. As in the hippocampus, there was no difference in glutamate release. This increase in inhibition was also found in the medial prefrontal cortex (mPFC) of *Nf1* mutants with an inhibitory neuron-specific deletion mediated by Cre, indicating that *Nf1* regulates GABA release in mPFC interneurons (Shilyansky et al. 2010a). Similar to the hippocampus, the MEK inhibitor U0126 reverses increased inhibition in the mPFC of *Nf1*$^{+/-}$ mice, showing that increased Ras/MEK/ERK signaling mediates the higher GABA inhibition observed in these mice.

To examine whether increased inhibition underlies working memory deficits in NF1, *Nf1*$^{+/-}$ mice were treated with picrotoxin and tested in the delayed win-shift radial arm maze and delayed nonmatch-to-sample task (Shilyansky et al. 2010a). Remarkably, a dose of picrotoxin that triggered working memory deficits in these tasks in WT mice rescued working memory impairments found in both tasks in *Nf1*$^{+/-}$ mice.

Mouse models of Down syndrome, fragile X, and Rett syndrome also showed altered regulation in inhibition (Dani et al. 2005; Fernandez et al. 2007; Gibson et al. 2008). Consistent with the findings in *Nf1*$^{+/-}$ mice, behavioral deficits in a mouse model of Down syndrome, such as deficits in spatial learning in the Morris water maze, were rescued by picrotoxin, a $GABA_A$ antagonist (Fernandez et al. 2007; Rueda et al. 2008).

37.4.2.4 Other Mechanisms

In addition to its role as a negative regulator of Ras signaling, neurofibromin also acts as a positive regulator for adenylate cyclase (AC) in both mice and *Drosophila* (Guo et al. 2000; Tong et al. 2002). *Nf1* null *Drosophila* mutants showed reduced AC activity as well as deficits in olfactory learning. The learning deficits were rescued by expression of *rutabaga,* a constitutively active AC, showing that decreased AC activity causes learning deficits in this *Nf1* mutant fly (Guo et al. 2000). Neurofibromin also regulates neuronal structure through the AC-PKA pathway. Hippocampal and retinal ganglion cell neurons from *Nf1*$^{+/-}$ mice have decreased growth cone areas and neurite lengths, and increased apoptosis, which can be

reversed by elevating the cAMP level (Brown et al. 2012). However, it is unclear if attenuated AC-PKA signaling is associated with cognitive deficits in NF1.

37.5 Cognitive Studies in Animal Models for Other Rasopathies

37.5.1 NF1-Like (Legius) Syndrome

SPRED1 knockout mice (*Spred1*$^{-/-}$) showed multiple phenotypes seen in individuals affected with the *SPRED1* mutation such as lower body weight, a shortened face, and melanin deposits in the spleen (Brems et al. 2007). Importantly, *Spred1*$^{-/-}$ showed deficits in the Morris water maze and visual-discrimination T-maze, which are hippocampus-dependent tasks (Denayer et al. 2008). This mutant also showed abnormally activated Ras-MAPK signaling and hippocampal LTP impairments, suggesting that LTP deficits caused by altered Ras signaling underlie the learning deficit in Legius syndrome.

37.5.2 Noonan Syndrome

Several mouse models for NS have been generated by knocking-in NS-associated mutations of *Ptpn11*, *Sos1*, or *Raf1* (Araki et al. 2004, 2009; Chen et al. 2010; Wu et al. 2011). Alteration in neural cell fate was observed in the neonatal hippocampus and cortex of *SHP-2*$^{D61G/+}$ mutant mice, suggesting that this developmental perturbation may underlie cognitive problems in NS (Gauthier et al. 2007). However, the impact of this developmental deficit on behaviors needs to be tested.

Effects of an NS-associated mutation on learning and memory have been studied in *Drosophila* (Pagani et al. 2009). Overexpression of gain-of-function *corkscrew* (*csw*), a fly ortholog of *Ptpn11* (SHP-2), caused a long-term memory deficit after spaced training. This deficit could be rescued by either treatment with a subthreshold dose of a phosphatase inhibitor NSC-87877 or a longer inter-training interval. This study showed that *csw* regulates how the duration of resting intervals affect learning by regulating ERK activation dynamics (Pagani et al. 2009).

37.5.3 Costello Syndrome

A *H-Ras*^{G12V} knock-in mutant has been used as a mouse model for Costello syndrome (Schuhmacher et al. 2008). This mutant showed some of the symptoms shown in individuals affected with Costello syndrome, such as facial abnormalities

and cardiomyopathies (Schuhmacher et al. 2008). Homozygous knock-in ($H\text{-}Ras^{G12V/G12V}$) mice showed deficits in the hippocampal-dependent Morris water maze and contextual fear conditioning tasks, but they were normal in the cortical-dependent novel object recognition task. Heterozygous mice did not show any learning deficits. Thus, as in patients, the cognitive deficits of these mice are mild to moderate (Viosca et al. 2009).

37.6 Beyond the Mice: Mechanism and Treatment in Human

Insights from studies using animal models provide opportunities for translational studies to test whether (a) the same mechanisms underlie cognitive deficits in humans and (b) similar treatment strategies can be applied to human NF1 patients.

37.6.1 Corticostriatal Dysfunction in Human NF1

Studies from $Nf1^{+/-}$ mice showed that increased inhibition in the cortex and striatum is responsible for working memory deficits. Using behavioral tasks and fMRI, working memory and corticostriatal function have been examined in human NF1 patients (Shilyansky et al. 2010a). Individuals affected with NF1 showed significant deficits in two working memory tasks, a spatial working memory maintenance and manipulation (stMNM), and a parametric probe of spatial working memory capacity (SCAP), which are visuospatial working memory tasks parallel to the operant delayed nonmatch-to-sample task used in $Nf1^{+/-}$ mice. fMRI analysis focused on DLPFC, which is also homologous to rodent mPFC and also on striatal regions. NF1 individuals showed significantly reduced neural activity as compared to controls in both cortical and striatal regions in stMNM. Consistently, NF1 patients also showed reduced neural activation in cortical areas critically involved in maintenance of spatial information in working memory in SCAP. Moreover, the degree of DLPFC hypoactivation significantly correlated with working memory performances, indicating that corticostriatal dysfunction is directly related to working memory impairments in human NF1 (Shilyansky et al. 2010a). It is highly possible that working memory deficits in mouse and human NF1 have a parallel mechanism: the reduced neural activation caused by increased inhibition.

37.6.2 Effect of Statin Treatment on Cognition in Human NF1

Although it has long been believed that neurodevelopmental disorders cannot be reversed in adults, the studies reviewed above show that both behavioral and physiological phenotypes in $Nf1^{+/-}$ mice can be treated in adults by reversing the

underlying signaling deficits. Although NF1 may contribute to development, it also affects adult function, and reversing adult deficits in mice is sufficient to reverse learning and memory phenotypes. Remarkably, a fragile X syndrome study in *Drosophila* showed that pharmacological treatments could reverse key phenotypes in this mutant fly (McBride et al. 2005). Will similar adult treatments be effective in either fragile X syndrome or NF1 patients? Although it is too early to know, preliminary clinical studies in both fragile X and NF1 are encouraging.

Krab and colleagues conducted a randomized, double-blind, placebo-controlled clinical trial with 62 NF1 children (Krab et al. 2008). To examine if the statin finding in mice can be translated to NF1 patients, children with NF1 were treated either with simvastatin or placebo for 12 weeks and tested before and after treatment. Importantly, the results show that this treatment did not result in negative side effects in this population. Cognitive function tests focused on nonverbal long-term memory, and attention and cerebellar function were used as primary outcome measures. In addition, secondary outcome measures were assessed in individuals with baseline scores of 1 SD or below average in tests for attention and visual–spatial skills. Compared to the significant effects of the placebo, simvastatin treatment seemed to have no additional effect on the primary outcome measures. However, the simvastatin group did show significantly improved scores in the object assembly test, one of the secondary outcome measures. This test reflects multiple cognitive domains including visual synthesis and even working memory. The strong improvement over placebo observed in the object assembly test was seen only in children who showed initial deficits in this task. The statin treatment did not affect NF1 children who initially scored within the normal range.

Additionally, a phase I study primarily designed to test the safety and tolerability of lovastatin in 24 children with NF1 also included cognitive evaluations before and after treatment (Acosta et al. 2011). Besides showing the safety of lovastatin in this pediatric population, the results suggested that the treatment resulted in improvement in areas of verbal and nonverbal memory and in visual attention and efficiency (Acosta et al. 2011). However, it is important to note that the two studies just mentioned were not designed as efficacy trials. However, several other statin clinical trials are being conducted in the USA and abroad designed specifically to test efficacy in NF1 cognitive phenotypes. Results from these additional studies may provide much anticipated information concerning the efficacy of statin treatments in NF1 children and hopefully, adults. If successful, a natural continuation of these studies would be to test whether statins can also help children affected with other rasopathies.

37.7 Concluding Remarks

Animal models are an invaluable tool to investigate the molecular and cellular mechanisms underlying cognitive impairments associated with NF1 and other rasopathies. Over the last 20 years, NF1 research has progressed from identification

of the gene responsible for this disorder to unraveling the molecular and cellular mechanisms underlying associated cognitive deficits to clinical trials. The paths of discovery followed by this research are having an impact in NF1 and in other genetic disorders with cognitive phenotypes. For example, studies in NF1 suggested that it is possible to develop adult treatments for neurodevelopmental disorders. This discovery has changed the way we envision treating neurodevelopmental disorders, and there is now evidence from animal models of many other neurodevelopmental disorders that adult treatments can have an unpredicted large impact on cognitive phenotypes associated with this general class of conditions. Moreover, it is extremely exciting that in some cases these animal model studies have been translated into clinical interventions, and although it is still too early to know, the results so far are very encouraging. There is a great need for close collaborations and interactions between bench neuroscientists, clinicians, and patient organizations in understanding and developing treatments for cognitive disorders. Rasopathies are ideally suited for this collaborative approach because many of the genes are known, there are good animal models, and there are committed and effective patient organizations that facilitate recruitment and are effective at informing and educating both parents and affected individuals. Most importantly, the relatedness of this set of conditions includes an unparalleled level of scientific and clinical synergy that will be critical to solve the many difficult challenges facing the treatment of cognitive disorders. Emerging new technologies such as iPS from human tissues are providing new insights (Carvajal-Vergara et al. 2010) that complement and strengthen current animal model approaches. In addition, the genomics revolution has provided powerful tools and much needed access to the genetic information required to unravel the complexity of these conditions. Despite the many challenges, the path ahead is full of promise: we are closer than ever to developing treatments to one of medicine's greatest challenges; the understanding and treatment of disorders that affect the inner core of our humanity, our cognitive selves.

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Chapter 38
Ras Signaling Pathway in Biology and Therapy of Malignant Peripheral Nerve Sheath Tumors

Faris Farassati

38.1 Introduction to Ras Proto-oncogene and Its Signaling Pathway

The Ras family of guanine-nucleotide bound proteins (G-proteins) has been portrayed in the last three decades as the most important proto-oncogene family with significant involvement in more than 35 % of human malignancies (Bollag et al. 2003; Campbell and Der 2004; Cox and Der 2002; Downward 2003; Jones et al. 2001). While mutations in genes involved in Ras pathway have been identified in a number of human cancers (Bodemann and White 2008; Fernandez-Medarde and Santos 2011; Hamad et al. 2002; Lundquist 2006; van Dam and Robinson 2006; Yamamoto et al. 1999), overactivation of signaling proteins in this pathway can lead to neoplastic progression in the absence of gene alterations (Bodempudi et al. 2009; De Luca et al. 2012). Downstream of Ras proteins, an expanded family of kinases referred to as mitogen-activated protein kinases (MAPKs) (Buday and Downward 2008; Lawrence et al. 2008; Molina and Adjei 2006; Sundaram 2006) transfer the signal through cytoplasm resulting in the activation of a series of transcription factors in the nucleus. Upon such action, a wide continuum of gene expression events leads to a drastic change in the biological characteristics of the cell (Fig. 38.1). Ras activation leads to the stimulation of different MAPKs that are involved in a series of cellular functions such as proliferation, cell cycle progression, angiogenesis, cell migration, and survival (Alvarado and Giles 2007; Chambard et al. 2007; McCubrey et al. 2007; Meloche and Pouyssegur 2007; Murphy and Blenis 2006; Roberts and Der 2007; Shelton et al. 2003; Sundaram 2006; Zebisch et al. 2007). Ras/MAPK machinery is responsive to external stimuli via activation of a series of different plasma membrane receptors such as receptor tyrosine kinases

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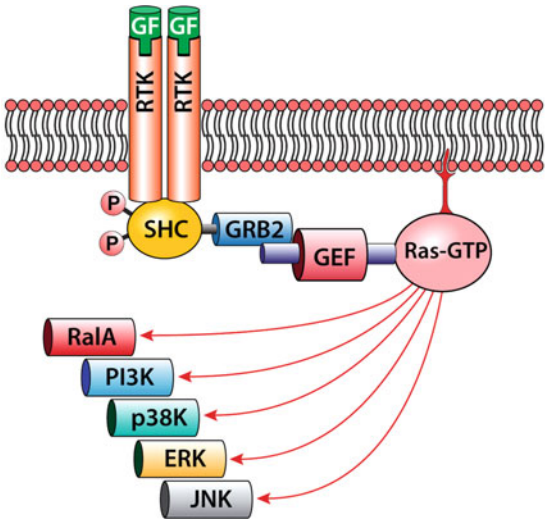

Fig. 38.1 *Activation of Ras downstream of RTKs*: Activation of receptor tyrosine kinases (RTKs) leads to activation of adaptor proteins such as GRB2 to activate Ras-GEFs leading to the activation of Ras. Ras activation, in turn, leads to the activation of a number of effector pathways such as ERK, JNK, p38 kinase, PI3K, and RAL

(RTKs) (Daub et al. 1997) or G-protein coupled receptors (GPCRs) (Chiariello et al. 2010; Daub et al. 1996). Additionally, physical signals such as ultraviolet (UV) can also induce Ras downstream effectors such as p38 kinase pathway (Muthusamy and Piva 2010) (Fig. 38.2). Major effector signaling pathways downstream of Ras include extracellular signal-related kinase(ERK) (Brannan et al. 1994), Jun amino-terminal kinase (JNK), p38 kinase, and phosphatidylinositol 3-kinase (PI3K), Ras like (Ral) (Downward 2003; Giehl 2005; Goldfinger 2008; Harrisingh and Lloyd 2004; Harrisingh et al. 2004; Rajalingam et al. 2007) and phospholipase-C epsilon (PLCε) (Gresset et al. 2012; Song et al. 2001) (Fig. 38.3). Activation of these effectors pathways results in the induction of an array of transcription factors such as FOS, ELK, JUN, STAT, ATF2, CREB, SP-1, and MYC (Esfandyari et al. 2009; Hazzalin and Mahadevan 2002; Keren et al. 2006; O'Donnell et al. 2012; Plotnikov et al. 2011; Terada et al. 1999) initiate a wide spectrum of gene expression activities (Fig. 38.2). For example, the Ras/Raf/ERK1/2 pathway results in serine/threonine kinase ERK1/2 phosphorylating (among other substrates) the nuclear transcription factor, ELK (Davis 1995; Kyosseva 2004). Phosphorylated-ELK, in cooperation with serum response factor (SRF), binds to the specific binding element termed serum response element (SRE) in the c-fos promoter, thereby inducing the transcription of many genes involved in important cell functions such as proliferation and differentiation (Cruzalegui et al. 1999; Whitmarsh et al. 1995) (Fig. 38.4).

38.2 Neurofibromatosis, MPNST, and Ras Signaling

Within the familial cancer predisposing syndromes, neurofibromatosis 1 (NF1) ranks first with 1:2,500–1:3,000 birth incidence (Cawthon et al. 1991). NF1 is an autosomally dominant genetic disease with an elevated rate of malignancy

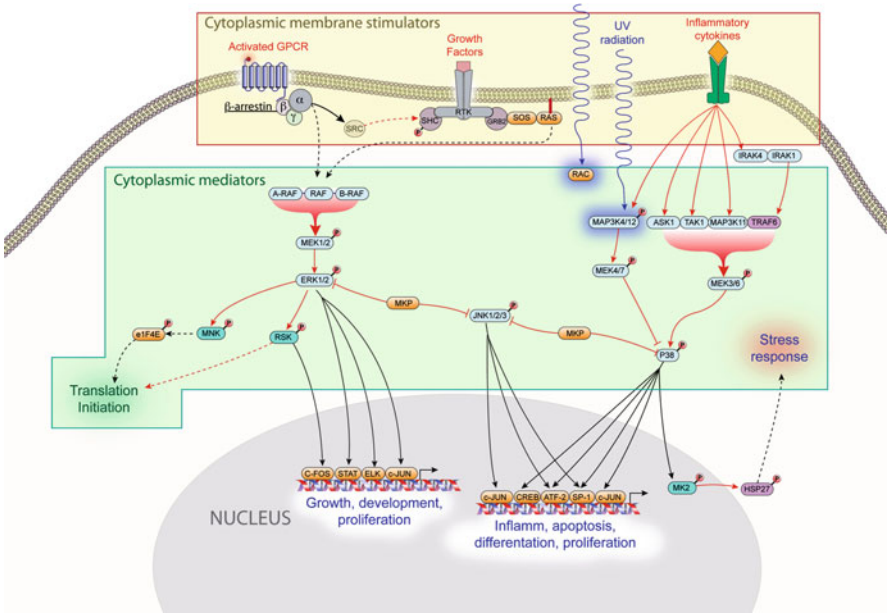

Fig. 38.2 *Ras signaling pathway: A flow of information from plasma membrane to the nucleus*: Different receptors at the level of plasma membrane such as G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) and inflammatory cytokine receptors can stimulate the activation of Ras signaling pathway. At the level of the cytoplasm, ranges of cytoplasmic mediators transfer the signal to nucleus. Activation of a number of transcription factors then influences the biological condition of the cell. Although many pathways are activated downstream of Ras, this figure mainly depicts some of signaling molecules involved in three modules in Ras signaling (plasma membrane inducers, cytoplasmic mediators, and nuclear transcription factors)

(Sorensen et al. 1986; Zoller et al. 1995). A characteristic of NF1 patients is the increased rate of different malignancies such as malignant peripheral nerve sheath tumor (MPNST), astrocytomas, pheochromocytomas, and chronic myeloid leukemias of childhood (Shannon et al. 1994).

The molecular events underlying the malignant transformation of benign neurofibromas to MPNST are not fully understood. These tumors are formed of tightly packed hyperchromatic spindle-shaped cells with an elevated mitotic fraction and frequently arise between the third and sixth decade of life (Laycock-van Spyk et al. 2011; Parrinello and Lloyd 2009). Inactivation of both copies of the neurofibromatosis-1 gene (*NF1*) has been demonstrated in benign human neurofibromas and shown to cause tumors in murine models (Serra et al. 1997). Loss of heterozygosity (LOH) of *NF1* and *P53* has frequently been observed in cases of human MPNST (Legius et al. 1994; Menon et al. 1990; Rey et al. 1992). In confirmation of this, transgenic mouse strains which harbor inactivated *nf1* and *p53* alleles (*cis-nf1*$^{+/-}$:*p53*$^{+/-}$), demonstrate the cumulative effects of the loss of both *nf1* and *p53* genes in the etiology of MPNST (Cichowski et al. 1999; Vogel et al. 1999).

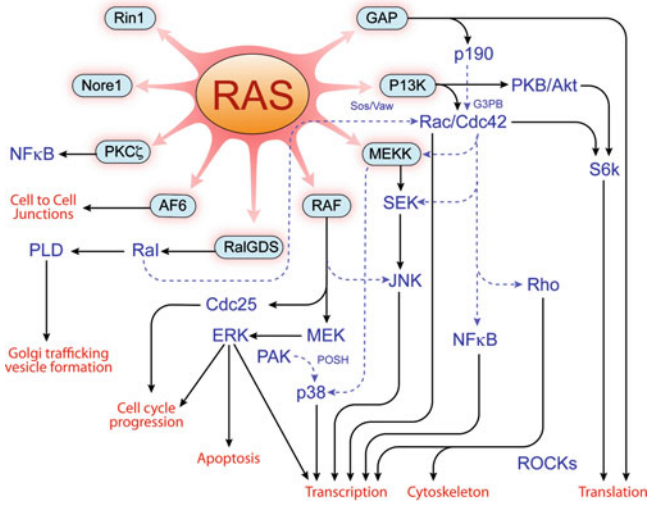

Fig. 38.3 *Ras effector partners*: A myriad of effectors are induced downstream of Ras influencing different facets of cellular biology. With such vast involvement in cell regulation, deregulation of the Ras signaling pathway in cancer can serve as an important step toward the achievement of malignant phenotype

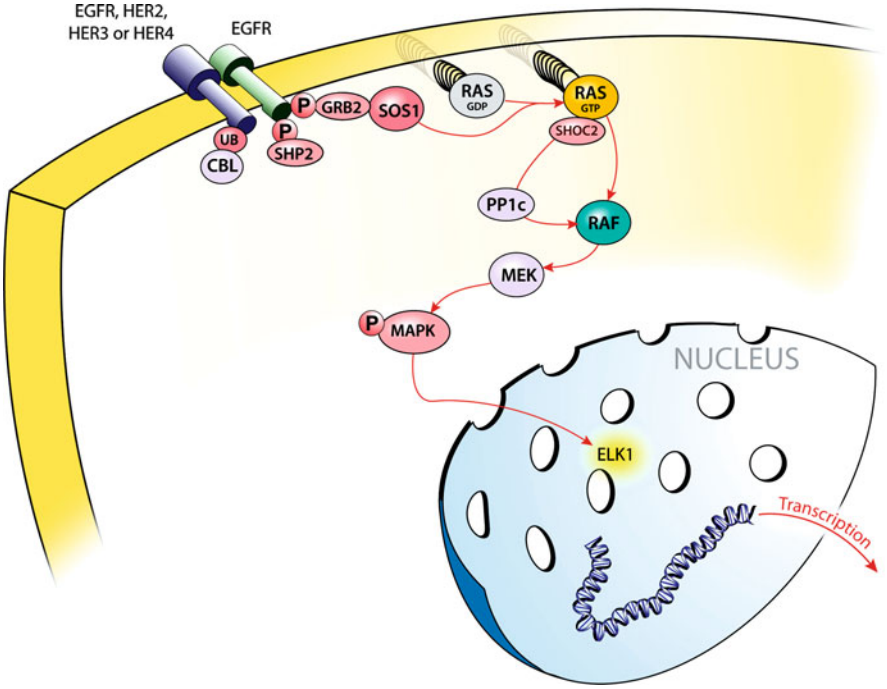

Fig. 38.4 *Ras/ERK signaling inducing transcriptional events*: Overactivation of a classic Ras downstream effector, Ras/Raf/ERK pathway, has been shown to be involved in many cancers and leads to activation of the transcription factor ELK

NF1 is an extremely large gene with a series of identified mutations with no specific hotspots having been identified (Li et al. 1995). Although neurofibromin is mostly known to be a Ras-GTPase activating proteins (Ras-GAP), it also contains a SEC14 binding domain. While yeast SEC14p is shown to be involved in regulating intracellular proteins and lipid trafficking, the function of its homologous domain in neurofibromin is unknown (Mousley et al. 2006; Trovo-Marqui and Tajara 2006). We have recently provided evidence for a new role for this protein as a deactivator of RalA activation (Bodempudi et al. 2009). Activation status of Ras is a function of balance between Ras guanine nucleotide exchange factors (Ras-GEFs) which activate Ras by transforming it to its GTP-bound (active form) and Ras-GAPs (such as neurofibromin). This induces hydrolysis of Ras-GTP back to Ras-GDP (inactive form) (Bos 1997; Cichowski and Jacks 2001; Vigil et al. 2010). Therefore, it was hypothesized that the lack of a deactivating mechanism conferred by lack of functional neurofibromin is the main leading signaling abnormality in NF1 (Harrisingh and Lloyd 2004; Katz et al. 2009). In the case of MPNST, this was proved by studying cells from MPNST patients that showed a lack of neurofibromin expression and elevated levels of Ras-GTP (Basu et al. 1992; DeClue et al. 1992). On such basis, continued efforts have been directed at inhibiting the Ras pathway as a treatment of MPNST using a series of Ras pathway pharmacological blockers including farnesyl transferase inhibitors (FTIs) (Wojtkowiak et al. 2008; Yan et al. 1995).

38.3 MAPK Pathways in MPNST Biology and Therapy

A series of studies have focused their efforts on studying the activation of MAPK pathways and their translational potentials for targeting MPNST cells. For example, a recent study showed that neurofibromin-deficient MPNST cells were shown to undergo phenotypic reversion by exposure to FTS (*S-trans*-farnesylthiosalicylic-acid) in conjunction with specific inhibitors of TGF-β and bone morphogenetic proteins (BMP) (Barkan et al. 2011). On the other hand, the blocker of Ras/Raf/ERK pathway, Sorafenib, which acts specifically on B-Raf induced inhibition of phospho-MEK and ERK resulting in cell-cycle arrest in MPNST cells (Ambrosini et al. 2008). An inhibitor of ERK pathway at the level of MEK was also found to induce selective apoptosis in three different malignant schwannoma and MPNST cells (Mattingly et al. 2006). Additionally, the transcription factor AP-1, a downstream effector of both the ERK and JNK pathways, was directly linked to neurofibromin deficiency in human MPNST cells (Kraniak et al. 2010). Our team has extensively studied the activation pattern of Ras and its downstream pathway in MPNST cells from both mouse and human origin (Farassati et al. 2008; Mashour et al. 2005; Yamoutpour et al. 2008). Distinct MPNST cells were obtained from mouse strains, which harbor inactivated *nf1* and *p53* alleles (Cichowski et al. 1999; Vogel et al. 1999). These cells could be classified in two distinct classes from the perspective of Ras pathway signaling (Farassati et al. 2008): one with enhanced activation of Ras, resulting in elevated activation of MAPK signaling pathways such

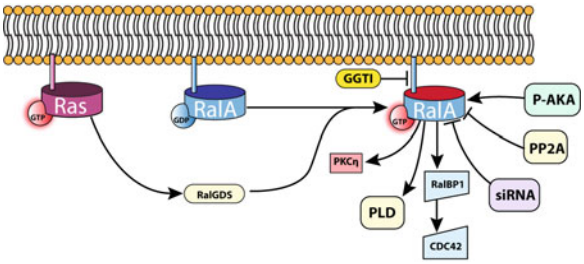

Fig. 38.5 *RalA signaling pathway*: Activation of Ras leads to the activation of RalA via mediation of RalGDS proteins. In next step, a number of pro-neoplastic downstream effectors are triggered upon activation of RalA such as PLD, RalBP1, and PKC. While P-AKA activates RalA, this small-GTP protein can be inhibited by PP2A, GGTIs, or use of inhibitory siRNA

as the ERK, JNK, p38 kinase, and PI3K pathways, and the second class contained the same level of Ras signaling activity as the nonmalignant Schwann cells (Farassati et al. 2008). Interestingly, increased activation of Ras signaling pathway in these cells was shown to result in enhanced permissiveness to G207, an oncolytic herpes mutant (Farassati et al. 2008). This was in agreement with our prior studies on the effects of Ras transformation on viral permissiveness (Farassati and Lee 2003; Farassati et al. 2001; Norman et al. 2001). However, an independent study had claimed that such a relationship was not observed for human MPNST cells (Mahller et al. 2006). However, cells in this study were not fully characterized from a Ras signaling perspective and the genetic background influencing Ras activation.

38.4 RalA Signaling Pathway and MPNST: A Novel Platform for Therapeutic Development

Ral guanine nucleotide exchange factors (Ral-GEFs) serve to transmit the activation signal from Ras to Ral, qualifying the Ral pathway as a downstream effector of Ras (Fig. 38.5) (Bodemann and White 2008; Neel et al. 2011). Ral-GEFs comprise two distinct groups of proteins: Proteins that are stimulated by Ras because of their carboxy terminal Ras binding domain (RalGDS, RGL1, RGL2), and proteins that are mainly activated by substrates of PI3K through a pleckstrin homology (PH) domain in their C-terminal (RALGPS1, RALGPS2) (Feig 2003). Ral proteins (RalA and RalB) signal through distinctly different effectors that affect gene expression and translation by interaction with ZO-1 associated nucleic acid binding protein (ZONAB) and RalA binding protein 1 (RalBP1) (Cantor et al. 1995; Frankel et al. 2005; Jullien-Flores et al. 1995). RalA is phosphorylated/activated by Aurora kinase A(AKA) (Bodemann and White 2008). RalB directly interacts with the SEC5 subunit of exocyst to facilitate the host defense response (Moskalenko et al. 2002; Sugihara et al. 2002). While being the convergence point of pro-oncogenic signaling pathways, RalBP1 functions as a CDC42/Rac-GTPase activating protein

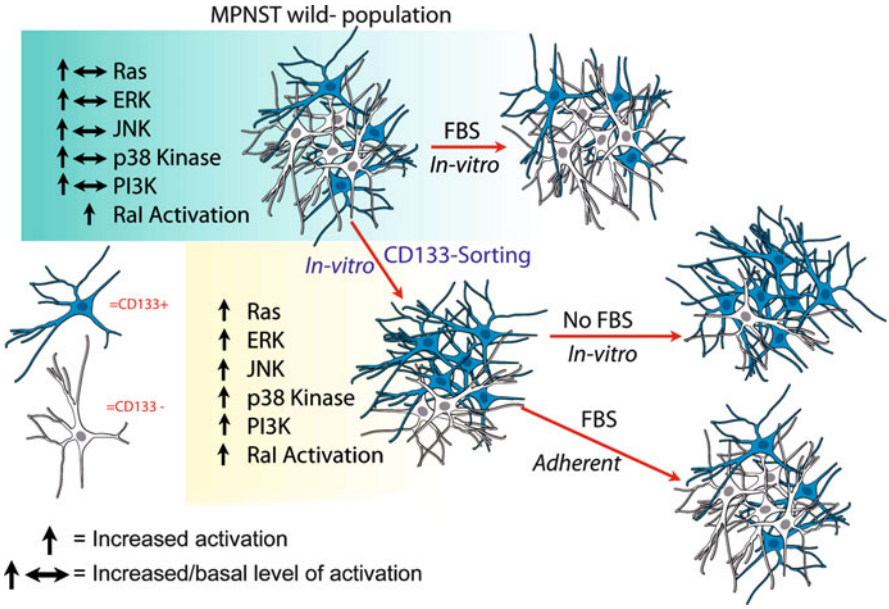

Fig. 38.6 *Ras signaling in differentiated and CSCs from MPNST origin*: When wild-type (unsorted) population of human MPNST cells are studied, the level of Ras signaling and its downstream effectors may be increased or at basal level as compared to their nonmalignant counterparts, Schwann cells. However, RalA seems to be an exemption since it was found to be constantly elevated. Once these populations are enriched for CD133 (as a marker for CSCs), Ras and its downstream effectors are found to be significantly overactivated as compared to the unsorted population

linking Ral and Rho (Jullien-Flores et al. 1995). Since the Ras-GAP function of neurofibromin is lost in these MPNST cell lines, it is noteworthy that only a fraction of MPNST cells show elevated levels of Ras while RalA remains overactive in all MPNST cell lines (Fig. 38.6). RalA pathway has been found in our work to be also overactivated in other neurological malignancies such as glioma and medulloblastoma (data not shown).

38.5 The Biological Outcome of Silencing RalA in MPNST Cells

38.5.1 Reduction in Viability and Invasiveness

Considering the lack of specific inhibitors for Ral, we utilized gene-specific silencing to evaluate the effect of inhibition of RalA on the malignant phenotype of MPNST cells (Bodempudi et al. 2009). Treatment of MPNST mouse cells with

anti-Ral siRNA caused a significant decrease in the proliferation and invasiveness of these cells. Under such conditions, the expression of a series of EMT markers was altered.

38.5.2 Reversal of "Cadherin Switch"

An important step in the process of acquiring invasive phenotype is the "E- to N-cadherin switch" in which the anti-invasion cadherin (E-cadherin) is reduced while the pro-invasion cadherin (N-cadherin) is increased. This process was also affected by RalA silencing as the levels of N-cadherin were significantly decreased while E-cadherin was not notably altered effectively reversing the E- to N-cadherin switch.

38.5.3 Loss of In Vivo Tumorigenicity

Once MPNST cells were electroporated with anti-RalA siRNA and injected subcutaneously to severe combined immunodeficient (SCID) mice, a significant retardation in the growth of tumors and a reduction in their size was observed. Extracted treated tumors showed decreased levels of RalA-GTP as compared with control siRNA-treated tumors (Bodempudi et al. 2009).

38.6 Inhibition of RalA Activation by Other Methods

In our studies, we employed two other methods to inhibit RalA activation. First, a dominant negative version of RalA (S28N) was used to inhibit RalA activation. Transient expression of S28N caused a significant reduction in RalA-GTP levels, viability, and invasiveness in human MPNST cells. We were also interested to see if inhibition of geranyl-geranylation, a posttranslational modification necessary for RalA activation, would affect the proliferation of MPNST cells. This was accomplished by the use of geranyl-geranyl transferase inhibitor 2147(GGTI-2147), a cell-permeable non-thiol peptidomimetic, that functions as a potent and selective inhibitor of geranyl-geranyl transferase I (GGTase I) (Bernot et al. 2003; Vasudevan et al. 1999). With reference to the fact that geranylation also affects other proteins including Ras, a 250 nM concentration of GGTI-2147 was used to minimize the inhibitory effects on Ras. At this concentration, loss of RalA activity and viability was observed.

38.7 Expression of GAP-Related Domain of Neurofibromin (NF1-GRD) Reduces RalA Activation

In an attempt to investigate the potential mechanisms of RalA activation, we expressed NF1-GRD in human MPNST cell lines and evaluated the outcome of such manipulation on RalA-GTP, as well as the viability and invasiveness of these cells. While the level of RalA-GTP was decreased, such treatment resulted in loss of viability and invasiveness of MPNST cells. This was taken as evidence towards a potential role for neurofibromin as a Ral-GAP protein (Bodempudi et al. 2009). However, such a role for neurofibromin needs to be further confirmed by future studies. Other effects of recruitment of expression of NF1-GRD in human MPNST cells include a decrease in the fraction of cells in G1 phase of the cell cycle and an increase in G2/S as well as elevation of apoptotic subpopulation of these cells. Such changes portray an overall growth inhibitory outcome for a decrease in RalA activation achieved by expression of NF1-GRD.

38.8 Ras Signaling Pathway in MPNST Cancer Stem Cells

According to new theories, a fraction of cells within each tumor serve to regenerate the tumor structure by acting as cancer stem cells (CSCs). The theory of CSCs was developed originally in the nineteenth century (Reya et al. 2001). However, tools to study these cells became available only about a decade ago (Bignold et al. 2006). In essence, this theory proposes the existence of a subpopulation of cells within tumors with pluripotential capabilities in terms of giving rise to all types of cells within a tumor (Akhtar et al. 2009; Costa et al. 2007; D'Amour and Gage 2002; Meeker and Coffey 1997; Mimeault and Batra 2006; Pan and Huang 2008; Rudland et al. 1980; Sagar et al. 2007; Singh et al. 2004; Spillane and Henderson 2007; Trosko 2009). CSCs are considered to be resistant to current anticancer modalities (Polyak and Hahn 2006; Singh et al. 2004). Therefore, there is a significant need to characterize CSCs from the biological and cell signaling points of view in order to find efficient methods to target them.

38.9 Markers of CSCs

A series of cell surface markers have been claimed to confer immaturity and stemness in the context of CSCs. These markers may also be found in normal tissues, usually with lower levels of expression as well as more restricted histological patterns.

Certain markers, such as CD133 (also known as prominin-1) (Corbeil et al. 2001a, b; Mehra et al. 2006; Miraglia et al. 1997), are widely accepted to be indicative of CSCs; however, the detailed characteristics of the biology of these cells including their cell signaling profile remain largely unstudied. On such a basis, we attempted to investigate the existence of CSCs in primary MPNST cells and evaluate their pro-oncogenic cell signaling features. We used nonmalignant human Schwann cells (HSCs) as the healthy counterpart of these cells for such an analysis. CD133 (Corbeil et al. 2001a, b; Mehra et al. 2006; Miraglia et al. 1997) has received a considerable level of attention due to its high expression in CSCs of a wide variety of tumor types, including medulloblastomas (Singh et al. 2004), glioblastomas (Salmaggi et al. 2006), melanoma (Monzani et al. 2007), colorectal (O'Brien et al. 2007), and prostate cancer (Collins et al. 2005). Although the function of CD133 is not clarified, it has been proposed that genes encoding the Notch and Hedgehog/Gli signaling pathways are overexpressed in CD133+ glioma cells (Clement et al. 2007; Hambardzumyan et al. 2006; Neuzil et al. 2007). It has also been reported that high levels of CD133 expression are associated with the resistance of CSCs to apoptosis (Bao et al. 2006; Frank et al. 2005; Hambardzumyan et al. 2006).

38.10 MPNST Cells and Tumor Tissues Express CSC Markers

We have reported the expression of a number of CSC markers in five different human MPNST cell lines (S805, 94.3 and S462, T530 and T532) including CD24, CD34, CD44, CD90, CD133, and EpCAM. Among these markers, CD24, CD44, and CD90 seemed to be expressed both in MPNST and HSCs, while CD133 and CD34 seemed to be preferentially expressed in MPNST cells. Furthermore, human MPNST tissues, as well as cells obtained from a mouse model for MPNST (Vogel et al. 1999), were also found to be positive for CD133.

38.11 CD133+ MPNST Cells

The functionality of CD133+ MPNST cells in conferring pluripotential characteristics has been studied by our team (Borrego-Diaz et al. 2012) and others (Spyra et al. 2011). In our studies, CD133+ MPNST cells were shown to form higher number of spheres in low-attachment conditions as compared with wild-type (unsorted) MPNST cells. Additionally, spheres formed this way were enriched in CD133 and were capable of generating CD133-negative cells once cultured in regular conditions. The work by Demestre et al. also proved the increased capability of these cells to be tumorigenic in vivo as a hallmark of their stemness (Spyra et al. 2011). In this study, clonal spheres established from a human MPNST cell line

exhibited increased expression of stem cell markers such as CD133, Oct4 (octamer-binding transcription factor 4), nestin, and NGFR (nerve growth factor receptor) and decreased expression of markers of maturity such as CD90 and NCAM (neural cell adhesion molecule).

38.12 Ras Signaling in CD133+ MPNST CSCs

By incorporating magnetic-based cell sorting for enrichment of CD133+ MPNST cells, we evaluated the activation of Ras signaling pathway in sorted and wild population of MPNST cells. A threefold enrichment of CD133+ fraction (45 % $CD133^{+}$) resulted in a significant increase not only in the level of Ras-GTP (active Ras) but also in higher levels of activation for Ras downstream effectors such ERK, JNK, PI3K, p38 kinase, and RalA as was determined by in vitro kinase and affinity precipitation assays (Fig. 38.6) (Borrego-Diaz et al. 2012). This observation is of special importance since it explains critical features in strategizing for targeting MPNST CSCs. It is logical to assume that these cells would exert a higher level of resistance to pharmacological inhibition of Ras pathway. To prove this, we exposed CD133-enriched and wild-type MPNST cells to an inhibitor of ERK pathway and measured the cell viability for up to 36 h postexposure. Expectedly, the CSC-enriched population was less affected by such therapy implying their increases resistance (Borrego-Diaz et al. 2012). In conclusion, we suggest that studies on sensitivity of MPNST cells to different therapeutic agents should also be performed on CSC-enriched populations in order to find an optimal regiment that can target this fraction efficiently.

38.13 Other Biological and Signaling Characteristics of CD133+ MPNST CSCs

38.13.1 Increased Invasiveness

Using an in vitro-based assay, CD133+ MPNST CSCs exhibited a significantly elevated level of invasiveness ($p < 0.05$) along with increased expression of β-catenin, snail, and paxillin. With attention paid to the repressing role of snail on E-cadherin (Cano et al. 2000), we expect the CD133+ cells to express lower levels of this anti-invasion protein. Furthermore, increased levels of β-catenin, either as a direct result of aberrant signaling in MPNST or as a product of reduction of E-cadherin (Kato et al. 2007), can result in the progression of EMT and enhanced expression of matrix metalloproteinases (e.g., matrix metalloproteinase 7 or MM7) as a basis for enhanced metastatic capabilities (McGuire et al. 2003; Zheng et al. 2009). The observed enhancement in invasive phenotype and molecular markers of

EMT portrays the possibility of CD133+ cells being a necessary component for the establishment of metastatic foci in MPNST. Such possibilities can be further investigated by studying CD133+ cells from tissues obtained from metastatic foci, or by exploring the existence and biology of CD133+ circulating CSCs as has been done for other models (Maheswaran and Haber 2010; Monteiro and Fodde 2010).

38.13.2 ER Stress Marker in CD133+ MPNST CSCs Cells

Accumulation of unfolded proteins induces a response, referred to as UPR (unfolded protein response), which is common between all mammalian species, as well as yeast and nematode worm (Hetz 2012). UPR is a stress response that is activated in response to the accumulation of unfolded or misfolded proteins in the lumen of the ER (endoplasmic reticulum) (Parmar and Schroder 2012). In order to remedy such a situation, UPR initiates mechanisms resulting in a decrease in protein translation as well as increasing production of chaperone proteins which can induce protein folding. The third and last approach to the UPR response is to induce apoptosis once the measures above have failed (Treglia et al. 2012).

Proteins involved in ER-stress signaling include the ER membrane and calcium-binding protein, calnexin (Bergeron et al. 1994), which retains newly synthesized glycoproteins inside the ER to undergo proper folding; the molecular chaperone proteins (BIP), which function by binding to unfolded proteins to inhibit formation of aggregates (Kohno et al. 1993); the protein disulfide isomerase (PDI), which catalyzes the formation and isomerization of disulfide bonds (Ellgaard and Ruddock 2005); the ER membrane-associated N-glycoprotein, Ero1-Lα, which assists oxidative protein folding (Cabibbo et al. 2000); the protein kinase/endoribonuclease, IRE 1α, which controls the unfolded protein response to neutralize protein misfolding (Cox et al. 1993); and the transmembrane protein, PERK, which couples ER stress signaling with the inhibition of protein translation (Harding et al. 1999; Kigawa et al. 1998). While these markers were mainly unchanged between CD133-enriched and wild populations (Borrego-Diaz et al. 2012), IRE1α was reduced in the CD133+ population. This might result in sensitizing CD133+ cells to the accumulation of misfolded proteins with potential therapeutic ramifications for agents that target the ER stress pathway.

38.13.3 Apoptosis Regulators in CD133+ MPNST CSCs

The apoptotic capabilities of cells are controlled by a series of a pro-apoptotic proteins such as Bcl-2 (B cell lymphoma 2) and anti-apoptotic proteins such as Bad (bcl-2 associated death promoter) (Munoz-Pinedo 2012). Evaluation of expression levels of these two important apoptosis regulators did not reveal any major

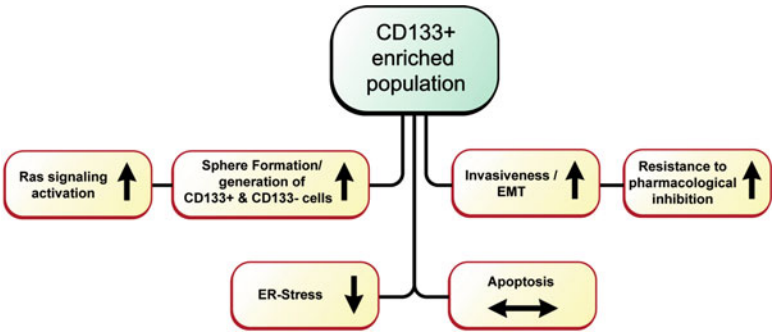

Fig. 38.7 *Biological characteristics of CD133+ human MPNST cells*: While Ras signaling, sphere formation, invasiveness, and regenerative capabilities are all enhanced, the capacity to deal with ER stress may be decreased. Expression of important apoptotic regulators remains unchanged

differences between CD133-enriched and the wild-type cell population (Borrego-Diaz et al. 2012). However, further studies on other apoptosis regulators as well as functional studies measuring the biological response to pro-apoptotic signals are needed to fully reveal the characteristics of MPNST CSCs with regard to the programmed cell death. Figure 38.7 summarizes the biological features of MPNST CD133+ CSCs.

38.14 Oncolytic Viruses Targeting MPNST

The use of oncolytic viruses (replication competent viruses capable of targeting cancer cells in a specific manner) (Borrego-Diaz et al. 2011) is currently being studied as a possibility for targeting MPNST by different groups (Farassati et al. 2008; Liu et al. 2006a, b; Mahller et al. 2006, 2007; Maldonado et al. 2010; Messerli et al. 2006; Prabhakar et al. 2010). In the realm of Ras signaling, we have shown that overactivation of Ras induces enhanced permissiveness to oncolytic herpes viruses (Esfandyari et al. 2009; Farassati and Lee 2003; Farassati et al. 2001, 2008). Additionally, we have developed oncolytic viruses capable of targeting Ras signaling pathway and CD133+ cells in a specific manner (Borrego-Diaz et al. 2011; Pan et al. 2009). Ongoing studies are being pursued to evaluate the possibility of targeting MPNST on the basis of using these anticancer viruses.

38.15 Conclusion and Future Directions

A review of information offered in this chapter portrays Ras signaling as an important platform for studying MPNST. In brief:

- Ras signaling controls important biological features of differentiated and stem cells from MPNST origin.
- Different downstream pathways of Ras are overactivated in a fraction of MPNST cells as compared with nonmalignant Schwann cells.
- Ras signaling can be considered as a therapeutic target for the treatment of MPNST cells.
- RalA leads an important signaling pathway involved at the level of differentiated and cancer stem cells from an MPNST origin.
- CD133+ cells from MPNST origin not only show CSC features but also exhibit higher levels of Ras signaling activation as compared to differentiated MPNST cells.

Future studies can involve testing the outcome of inhibition of Ras signaling pathway using specific inhibitors of downstream effectors (including RalA pathway) or gene therapy strategies utilizing replication-competent and replication-incompetent models.

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Chapter 39
MicroRNA and NF1 Tumorigenesis

Adrienne M. Flanagan and Nadège Presneau

39.1 History and miRNA Biogenesis

MicroRNAs (miRNAs) are evolutionarily conserved short (20–23-nucleotide), endogenous, single-stranded RNA molecules that regulate gene function via mRNA degradation, translation inhibition, and chromatin-based silencing mechanisms (Bartel 2004). The first miRNAs discovered were miRNAs lin-4 and let-7 in *Caenorhabditis elegans* where they were found to be involved in larval development (Lee et al. 1993). The mechanism by which they function came from the discovery that *LIN4* encodes two small transcripts (22 and 61 nucleotides in length) which contain complementary sequences to multiple conserved sites within the 3′ untranslated region (3′ UTR) of the *lin-14* mRNA, and that it is this complementarity that prevents translation of the LIN-14 protein. Since then, miRNAs have become recognised as a conserved category of molecules, which are key regulators of gene expression at the post-transcriptional level.

miRNA genes occur as single genes although ~30 % are found in gene clusters. In the latest miRNA repository miRbase version 18 (released in Nov. 2011), 1,921 unique mature human miRNAs are listed (http://www.mirbase.org). It is estimated that one-third of all protein-coding human mRNAs are susceptible to this complex miRNA regulatory network.

A detailed review of the biogenesis of miRNA is given in a number of recent reviews and will not be addressed in detail herein (Calin and Croce 2006a, b; Krol et al. 2010; Van Kouwenhove et al. 2011). The characteristic feature of miRNA genes is that their intermediate RNA transcripts fold into hairpin structures that are specifically recognised and processed by the miRNA biogenesis machinery. This process is described schematically in Fig. 39.1.

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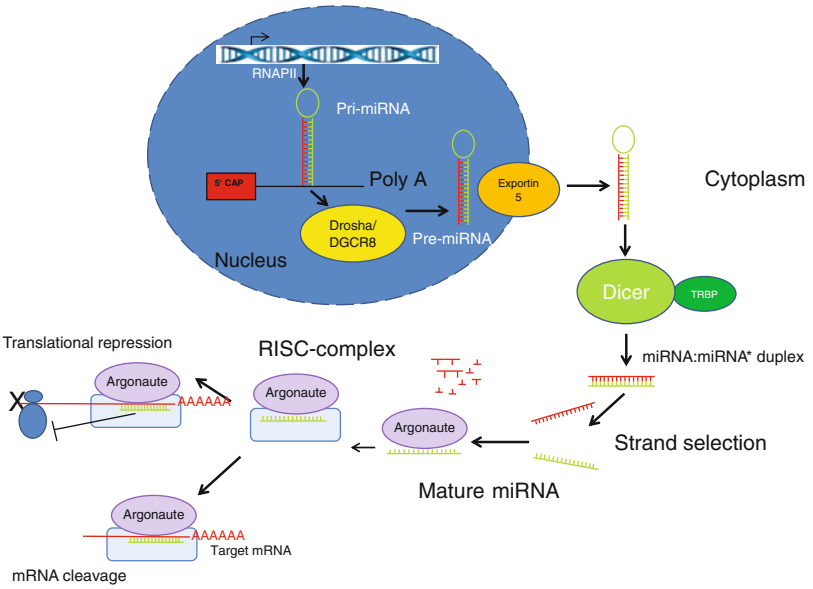

Fig. 39.1 Schematic representation of the miRNA biogenesis and function. miRNA genes are transcribed by RNAPII to produce primary-miRNAs (pri-miRNA), which have a structure of a stem–loop with a 5′ end CAP structure and poly A at their 3′ end. This stem–loop structure is recognised by the endoribonuclease Drosha, and its RNA binding partner DGCR8. Drosha crops the pri-mRNA into a miRNA precursor (pre-miRNA) (Denli et al. 2004; Gregory et al. 2004). This hairpin is then exported to the cytoplasm by exportin-5 in a Ran-GTP-dependent manner (Lund et al. 2004). It is then processed by an another enzymatic complex, Dicer, an RNase III endonuclease, together with its RNA binding protein (RBP) partner HIV-1 transactivating response (TAR) RNA-binding protein (TRBP), that cleaves the loop region of the hairpin to form the mature 20 to 23 nucleotide double-stranded RNA called the miRNA–miRNA* duplex (Hutvagner et al. 2001). The two strands are separated: one becomes the mature miRNA and the other strand is degraded. The mature strand is recognised by the Argonaute-containing complex, which generates a RNA-induced silencing complex (RISC), which is thought to bring about miRNA-mediated destabilisation (mRNA cleavage) or miRNA-mediated translational repression through recognition of target mRNA (Chendrimada et al. 2005; Filipowicz 2005; Gregory et al. 2005)

39.2 miRNA Expression Signature in Peripheral Nerve Sheath Tumours

There are only a handful of reports concerning the role of miRNA in the development of tumours in the context of neurofibromatosis type 1. The published work involves miRNA profiling studies of nerve sheath tumours, including a study comparing plexiform neurofibromas with dermal neurofibromas, and non-plexiform neurofibromas with malignant peripheral nerve sheath tumours from patients with neurofibromatosis type 1 (Chai et al. 2010; Lee et al. 2011; Pasmant et al. 2011a; Presneau et al. 2012; Subramanian et al. 2010). However, not all of these studies were confined to tumours from patients with this syndrome.

39.2.1 Inactivation of p53 Contributes to the miRNA Profile of MPNSTs

The first study of miRNA in nerve sheath tumours involved profiling 23 peripheral nerve sheath tumours including 6 MPNSTs and 11 neurofibromas and 6 schwannomas. Not all of the tumours were obtained from patients with neurofibromatosis type 1. Ten miRNAs were significantly differentially expressed between the benign and malignant tumours: five were up-regulated (miR-214, miR-377, miR-409-3p, miR-487b and miR-99b) and five were down-regulated (miR-517, miR-34a, miR-29a, miR-30e-5p and miR-27a) (Subramanian et al. 2010). The six MPNSTs selected for miRNA profiling in this study expressed a p53 inactivation gene expression signature, and miR-34a was the most significantly down-regulated miRNA in this tumour group compared to that in the benign tumours although it is not known if these tumours were from patients with neurofibromatosis type 1. As it is well documented that miR-34a is a direct target of p53 in a variety of neoplasms, including colorectal carcinoma and neuroblastoma, in which lower levels of this miRNA are associated with suppression of apoptosis, Subramanian et al. (2010) investigated its role in nerve sheath tumours (Bommer et al. 2007; Dalgard et al. 2009; He et al. 2007a, b; Hermeking 2010).

Forced expression of miR-34a in a MPNST cell line, deficient in this miRNA, resulted in increased apoptosis. Furthermore, forced expression of p53 in the same cell line resulted in a similar effect, thereby demonstrating that the expression of p53 contributes to its effect on apoptosis through miR-34a. Induction of greater expression of miR-34a in the cell line also resulted in reduced expression in a set of oncogenes, including *MYCN*, *E2F* and *CDK4*, all known to be targets of miR-34a. The knowledge that activation of these oncogenes is known to reduce cellular apoptosis adds weight to the finding that miR-34a mediates an effect on apoptosis (Hermeking 2010). The finding that these oncogenes are more highly expressed in MPNST than in neurofibromas provides evidence of their pathophysiological role in this disease (Subramanian et al. 2010; Yu et al. 2011).

Subramanian et al. (2010) also showed that the forced expression of wild-type p53 in MPNST cell lines resulted in a significant increase in the expression levels of nine miRNAs (miR-34b, miR-34c, miR-638, miR-373*, miR-492, miR-126, miR-140, miR-491 and miR-293), in addition to miR-34a, all of which are known to be induced by p53 (Raver-Shapira et al. 2007). In addition to the miR-34 family, six of these p53-induced mi-RNAs (miR-638, miR-373, miR-492, miR-126, miR-140 and miR-491) are involved in a diverse range of functions, including tumour invasion and proliferation (miR-373, miR-126) (Huang et al. 2008; Sun et al. 2010), metastasis (miR-373, miR-492) (Huang et al. 2008; von Frowein et al. 2011), suppression of neoplastic growth via the PI3K pathway (miR-126) (Guo et al. 2008), vascular integrity and angiogenesis (miR-126) via VEGF (Wang et al. 2008), proliferation, resistance to chemoresistance (miR-140) (Song et al. 2009) and apoptosis (miR-491) (Nakano et al. 2010). These findings are consistent with the knowledge that p53 is able to exert an effect on several miRNAs, which mediate a range of functions, and explains how p53 exerts its array of anti-cancer effects (Feng et al. 2011).

By exploiting the findings from the functional studies of Subramanian et al. (2010), Lee and co-workers, employing a computational systems biology approach, identified a regulatory network which involves the E2F family members, E2F7/E2F8, in several cell cycle-related gene modules (Lee et al. 2011). Since E2F transcription factors are involved in transcriptional regulation, as well as DNA repair and cell proliferation, they hypothesised that alteration in the expression of activators and suppressors of E2F7 and E2F8 transcription may play a role in the development of MPNST, similar to that reported in other cancers (Endo-Munoz et al. 2009; Reimer et al. 2007). Their findings provide evidence that a decrease in p53 and miR-34a levels contributes to the development of MPNST. However, as not all MPNST show loss of function of p53 (Verdijk et al. 2010), other mechanisms must also be involved in the malignant transformation of benign nerve sheath tumours (Subramanian et al. 2010).

A more recent miRNA profiling study using the Agilent microarray chip failed to confirm the work by Subramanian et al. (2010) which showed that miR-34a was significantly differentially expressed in ten neurofibromas compared to nine MPNST (Presneau et al. 2012). The discrepant results in the two studies may be explained on the basis that the patient groups were different. The six MPNSTs profiled by Subramanian et al. (2010) were selected on the basis of their p53 gene inactivation expression signature, whereas Presneau et al. (2012) chose their cases on the basis that the patients had neurofibromatosis type 1. It is not known if the six patients in the first study had neurofibromatosis type 1, and in the latter study gene expression profiling was not undertaken. In this context, it is of interest that p53 mutations only occur in ~25 % of MPNSTs, and that a minority of these (36 %) are found in tumours from patients with neurofibromatosis type 1 (Verdijk et al. 2010). In view of this, it is interesting to speculate that the differential expression of miR-34a in benign and malignant nerve sheath tumours is at least partly dependent on the loss of p53, which may be itself partly determined by the presence or absence of a germline mutation in neurofibromin. An alternative explanation for the discrepancy between the two studies is that different miRNA microarray platforms were used, and differences arising from this could have been compounded by the small sample numbers in the studies.

39.2.2 MiR29c Deregulation in Peripheral Nerve Sheath Tumours Progression

We identified more recently, 16 miRNAs that are significantly differentially expressed between ten neurofibromas and ten MPNST from patients with neurofibromatosis type 1 (Presneau et al. 2012). Of these, seven were down-regulated in MPNST, including miR-30e*, miR-29c*, miR-29c, miR-340*, miR-30c, miR-139-5p and let-7 g ($q = 8.48E-03$). In addition, the microarray analysis showed that most of the miR-29 family members, miR-29a, b, c*,b-1*, were also significantly

down-regulated in MPNST as compared to neurofibromas. By contrast, miR-210 ($q = 4.1E-04$) was up-regulated in MPNST compared to neurofibroma.

In silico prediction analysis identified a list of target genes for the miR-29 family including extracellular matrix (ECM) genes comprising various collagen genes and tenascin, which are involved in cell migration and invasion (Daley et al. 2008; Newby. 2006). Gene Ontology terms for the cellular component and molecular function as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis also showed that the predicted target genes represented metallopeptidase function ($p = 0.00461$), and ECM–receptor interaction ($p = 6.73E-10$).

A selection of these miR-29c target genes, including *COL1A1*, *COL1A2*, *COL5A2*, *COL21A1*, *COL4A1*, *COL4A2*, *TGFB3* and *MMP2*, were shown to be down-regulated as a result of forced expression of miR-29c with a synthetic oligonucleotide (mimic) in the MPNST cell line, sNF96.2, which shows low expression levels of endogenous miR-29c, as seen in the MPNST samples from the patients. Functional assays revealed that cell motility and invasive properties of this cell line, assessed in transwell plates and the scratch/wound healing assays, were significantly reduced when treated with miR-29c mimics. No effect on cell growth/proliferation of the sNF96.2 cells was noted following transfection with the mir-29c mimics when assessed using a variety of assays including a live-cell imaging system IncuCyte, the MTS assay, and by growing the cells in 3D culture using soft agar.

Reduced proteolytic enzyme activity of MMP2, a known target of the miR-29 family (Steele et al. 2010), contributed to the reduced migration which was brought about by the forced expression of miR-29c in the MPNST cell line. This was demonstrated by reduced levels of mRNA and protein for this miRNA (Presneau et al. 2012). In the same experiment, although there was loss of MMP9 activity, there were no significant effects on protein or mRNA expression. This is consistent with MMP9 not being a direct target on miR-29c and suggests that the miR-29c effect on MMP9 enzymatic was indirect.

Presneau et al. (2012) speculated that the low level of miR-29c in MPNST is mediated through activation of cMET/hepatocyte growth factor receptor, a protein which is commonly activated in MPNST as a consequence of gene amplification in ~20 % of cases (Mantripragada et al. 2008). Activation of cMET can also be brought about by the loss of p53 activity (Goldstein et al. 2011). This speculation is based on the work by Torres et al. (2011) who showed that high levels of expression of cMET results in increased cell motility, invasion, angiogenesis, and induced MMP2 and VEGF expression in MPNST in vitro. Silencing of cMET exerts the opposite effect in vitro, and also significantly decreases local and metastatic growth of MPNST in a mouse model (Torres et al. 2011). Furthermore, others have reported that hepatocyte growth factor inhibits collagen I and IV synthesis in hepatic stellate sells through induction of miR-29a/b (Kwiecinski et al. 2011); in addition, by forcing the expression of tumour growth factor beta in hepatic stellate cells, expression of cMET is increased and this leads to reduced expression of its ligand, hepatocyte growth factor, in addition to miR-29a/b. Consistent with this, the introduction of hepatocyte growth factor stimulates the expression of miR-29 and abolishes the effect of tumour growth factor beta.

The low level of miR-29c expression in MPNST is unlikely to be explained by chromosomal loss as the karyotype of the MPNST cell line, sNF96.2 (48, X, −X or Y, +7, add(7)(p22)x2, +8, add(9)(p24), +mar) used for studying the mechanism through which miR-29c mediates its effect, does not reveal loss of any of the loci on chromosome 1 and 7 where the miR-29 family members reside (Perrin et al. 2007). It is also unlikely that hypermethylation accounts for inactivation of miR-29c in MPNST as the methylome analysis of the neurofibromas and MPNST, which were also profiled for their miRNA by Presneau et al. (2012) revealed little difference in the methylation status of the tumour groups (Feber et al. 2011). The result of these "pooled" experiments was confirmed when the same samples were analysed separately for gene body methylation by pyrosequencing (Presneau et al. 2012).

In summary, there is evidence that the low level of miR-29c in MPNST, arising in individuals with neurofibromatosis type 1, is mediated at least in part through increased nerve sheath tumour cell migration and invasion, and this is mediated through the collagen genes and metalloproteinases, for which the respective transcripts are targeted by miR-29c (Daley et al. 2008; Newby 2006; Presneau et al. 2012).

39.2.3 MiR-214 and TWIST1 in MPNST

MiR-214 is the most highly expressed miRNAs in Subramanian et al.'s cohort of MPNSTs. Although this is not the case in the study by Presneau et al., they also found that this miRNA was expressed at a higher level in the majority of MPNST as compared to neurofibromas. This miRNA is implicated in tumorigenesis in a number of different cancers: in some it appears to act as an oncogene but in others as a tumour suppressor gene. Specifically, higher expression of miR-214 in melanoma cells results in increased metastatic behaviour of melanoma cells (Penna et al. 2011): it increases cell survival and cisplatin resistance in ovarian cancer by targeting the 3′-untranslated region of PTEN, a tumour suppressor gene, resulting in inhibition of translation, which in turn results in activation of the Akt pathway (Yang et al. 2008). Up-regulation of this miRNA is also seen in pancreatic cancer specimens where it is associated with a poor response of pancreatic cancer cells to chemotherapy (Zhang et al. 2010). By contrast, it has been reported that down-regulation of miR-214 induces cell cycle arrest in gastric cancer cells by up-regulating PTEN (Xiong et al. 2011), and others have found that reduced expression of this miRNA in breast cancer may be tumorigenic (Derfoul et al. 2011). More recently, miR-214 has been shown to suppress the proliferation, migration and invasiveness of cervical cancer cells by targeting GALNT7, findings which are supported by the suppression of cervical cancer cell proliferation, migration and invasion as a result of silencing of GALNT7 (Peng et al. 2012).

In a number of cancers, the expression of miR-214 correlates positively with the expression of TWIST1, a basic helix–loop–helix protein (Kwok et al. 2005; Lee et al. 2006, 2009), a finding consistent with this protein being expressed at high levels in virtually all MPNSTs. TWIST1 expression also correlates positively with metastatic disease in a number of other cancers including hepatocellular carcinoma (Lee et al. 2006), prostate cancer (Kwok et al. 2005; Yuen et al. 2007), breast cancer (Kallergi et al. 2011), and head and neck cancer (Ou et al. 2008). The findings that chemotaxis, a key component of the metastatic process, is inhibited in the MPNST cell line, STS26T when the expression of TWIST1 is reduced by siRNA is consistent with these findings (Miller et al. 2006). Hence, it is likely that in peripheral nerve sheath tumours, the mir-214 acts as an oncogene, although to date the genes that it targets to mediate its effect have not been identified.

39.2.4 microRNA-10b Regulates Tumorigenesis in Neurofibromatosis Type 1

The definition of a *bona fide* tumour suppressor gene such as neurofibromin (*NF1*) is its bi-allelic inactivation that is classically brought about by a mutation in one allele and chromosomal loss of the second wild-type allele in the tumour [for recent review on tumour suppressor gene inactivation see Berger et al. (2011)]. Chai and co-workers hypothesised that one of the two hits needed for complete inactivation of neurofibromin in tumours from patients with neurofibromatosis type 1 could result from silencing by a miRNA. There is good evidence that other tumour suppressor genes, including p53, are silenced in this way (Salmena et al. 2008; Swarbrick et al. 2010). By profiling miRNAs in a MPNST cell line, ST88-14, derived from a patient with neurofibromatosis type 1, and comparing it to a MPNST cell line, STS26T, derived from a sporadic tumour, the oncomirs miR-10b, miR-155 and miR-335 were found to be significantly more highly expressed in the former as compared to the latter. Furthermore, the let-7 family, and particularly let-7a and let-7b, were significantly lower in the cells derived from the patient with neurofibromatosis type 1 compared to the sporadically-derived MPNST cell line.

Functional characterisation of miR-10b, miR-155, miR-335 using antisense inhibition, and let-7 using synthetic oligonucleotide mimics, respectively in the MPNST cell line, ST88-14, revealed that miR-10b significantly decreased both cell proliferation, and migration and invasion, whereas the other miRNAs only reduced the invasive properties of the cells (Chai et al. 2010). It is noteworthy that only miR-10b, let-7a and let-7b were differentially expressed in a set of primary benign and malignant peripheral nerve sheath tumours. The discrepancy between the miRNA expression in primary tumours and cell lines may reflect that within and between MPNSTs there is histological and genetic heterogeneity (Burger and Scheithauer 2007). Only by studying a larger number of cases and correlating the miRNA profile with a variety of appearances and behaviours of the tumours will this issue be resolved.

miR-10b has also been shown to target neurofibromin, thereby providing a mechanism for the silencing of the expression of this gene in the absence of mutations and chromosomal loss. As with other miRNAs, mir-10b also targets many other genes (Thomas et al. 2010) including key molecules in the RAS signalling pathways. In this way, it is also likely to contribute to the tumorigenesis of peripheral never sheath tumours (Chai et al. 2010).

39.2.5 miRNAs in the Commonly 1.4-Mb Microdeleted Neurofibromatosis Type 1 Region in MPNST

Approximately 5 % of patients with neurofibromatosis type 1 have a microdeletion of the 17q11.2 region, which in most patients involves a germline 1.4-Mb microdeletion. Such patients have a higher risk of developing MPNST than those without this genetic aberration (Upadhyaya et al. 1998; Wu et al. 1995). The microdeletion is caused by unequal recombination between two highly homologous segments termed NF1 low copy repeats. Genome analysis shows that the microdeletion covers the complete 350-kb neurofibromin (*NF1*) gene, and at least 16 genes, 4 pseudogenes and 2 miRNAs (miR-193a and miR-365-2). The miR-193a is of particular interest because it acts as tumour suppressor gene in oral squamous cell carcinoma (Kozaki et al. 2008). It is down-regulated in oral cancer as a result of DNA hypermethylation (Kozaki et al. 2008) and is poorly expressed in melanomas containing a *BRAF* mutation (Caramuta et al. 2010). Genomic and mechanistic studies suggest that miR-193a-3p mediates up-regulation of the proapoptotic splicing form of caspase 2 in hepatocellular carcinoma 5-FU resistance through serine/arginine-rich splicing factor 2 (Ma et al. 2012).

miR-365, the second miRNA in the microdeletion, is of interest because it is down-regulated in colon cancer and inhibits cell cycle progression and promotes apoptosis of colon cancer cells, probably by targeting cyclin D1 and Bcl-2 (Nie et al. 2012).

As part of a project to test whether some of the genes in the *1.4-Mb* microdeletion interval account for the reported greater risk of developing malignant disease by acting as tumour suppressor genes, Pasmant et al. (2011a) compared the gene expression level of miR-193a and miR-365-2 in dermal neurofibromas ($n = 23$) with those in plexiform neurofibromas ($n = 13$) and MPNSTs ($n = 13$). The results were generated using real-time PCR and Human TaqMan® MiRNA assay kits, and no evidence was found for the miRNAs being differentially expressed in the different groups. Dermal neurofibromas from patients without neurofibromatosis type 1 were used as controls, as these do not transform into MPNST (Evans et al. 2002; King et al. 2000; Kleihues and Cavenee 2000; Pasmant et al. 2011a).

39.3 Role of Non-protein Coding Transcripts in Neurofibromatosis Type 1

In addition to miRNA, long non-coding RNAs (lncRNAs), a new class of non-coding RNA, are emerging. These have been implicated in the regulation of a range of biological functions, and the disruption of some of these functions, such as genomic imprinting and transcriptional regulation, plays a critical role in cancer development (Gibb et al. 2011).

Using high-resolution array comparative genomic hybridisation, Pasmant and co-workers found that the only recurrent genetic alterations in plexiform neurofibromas arising in patients with neurofibromatosis type 1 were deletions involving chromosome 9p21.3 (Pasmant et al. 2011b). The smallest common deleted region, spanned approximately 0.5 Mb and included *CDKN2A* and *CDKN2B*, and *INK-4A*/*ARF*, all of which are recognised tumour suppressor genes, and *ANRIL*, an antisense non-coding RNA located in the *INK-4A* locus, which has been shown to regulate *CDKN2A*/*B* locus transcription (Yap et al. 2010). They found a single nuclear polymorphism in *ANRIL* within intron 3 rs2151280 for which the allele T is significantly associated with a higher number of plexiform neurofibromas. They also found that this polymorphism is significantly associated with a reduced expression level of *ANRIL* mRNA, suggesting that modification of *ANRIL* expression may mediate susceptibility to developing plexiform neurofibromas. Interestingly, involvement of the 9p21.3 region in neurofibromatosis type 1-associated tumorigenesis has already been suggested because *Cdkn2a*/*Arf* deficiency leads to the formation of MPNSTs in Nf1+/− background mice (Joseph et al. 2008). These data indicate that *ANRIL* is a modifier gene in neurofibromatosis type 1. However, there are limitations to the study and interactions between *ANRIL* and neurofibromin have not been confirmed at the biological level.

39.4 Concluding Remarks: Future Directions and Perspectives: microRNAs as Therapeutic Targets in MPNST?

miRNA profiling of various cancers can be used successfully to classify tumours according to their differentiation state and cell of origin (Lu et al. 2005; Volinia et al. 2006), and in the future they may serve both as a diagnostic tool and as prognostic indicators. However, to date, such work is limited to the research laboratory and is not employed in clinical practice (Cummins and Velculescu 2006; Tricoli and Jacobson 2007; Visone and Croce 2009; Yanaihara et al. 2006).

miRNAs involved in regulating the expression of oncogenic and tumour suppressor genes are candidates for targeted therapy in the treatment of cancer (Nana-Sinkam and Croce 2011). At present, the miRNA/RNA-i therapeutic field is in its infancy but several approaches are being developed for targeting miRNA including anti-miRNA oligonucleotides (Garzon et al. 2010), and antagomiRs that

block the miRNA through complementarity (Garzon et al. 2010). Delivery of these agents has been tested through a variety of routes including intravenous and intraperitoneal injection, and inhalation (Nana-Sinkam and Croce 2011). Preliminary studies using nanoparticles modified with tumour-seeking antibodies for delivery of relevant small interfering RNA and miRNA respectively show promise *in vitro* and in animal models (Chen et al. 2010).

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Chapter 40
Translational/Clinical Studies in Children and Adults with Neurofibromatosis Type 1

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40.1 Introduction

Neurofibromatosis type 1 (NF1) is a complex disorder that stems from mutations in the *NF1* gene. Hundreds of different types of *NF1* mutations have been identified, perhaps contributing to the protean manifestations of the condition and the considerable challenges in developing effective therapies. Children and adults with NF1 are at risk for both the acute and chronic manifestations of the condition. Therapeutic interventions have to take into account the effects of treatment not only on the acute target symptoms or deficits but also the chronic effects of the disorder manifestations. The majority of NF1-related complications are not life-threatening, but can result in significant long-term discomfort and disability.

As NF1 was first conceptualized, a hallmark of the disease was tumor growth in tissues that surround nerves. Our understanding of NF1 has greatly advanced in the past two decades, with the realization that mutations of the *NF1* gene result in abnormalities in more than just Schwann cells; the same genetic defects impact brain development, as well as other organs (Zhu et al. 2002; Reilly et al. 2008). Disease manifestations range from common, chronic conditions such as cutaneous neurofibromas and learning disabilities to acute, devastating complications, including malignant peripheral nerve sheath tumors and brain tumors (Gutmann et al. 1997). All of these manifestations are potential clinical trial targets. In the past, treatments

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for children and adults with NF1 were essentially empirical, utilizing therapies available for similar disease manifestations in patients with other disorders. As example, since pruritus was a common feature of dermal neurofibromas, antihistamines were utilized in the earliest studies to relieve symptoms and possibly decrease other neurofibroma-related symptoms (Riccardi and Mulvhill 1981; Riccardi 1993). As the molecular pathogenesis of NF1 has been increasingly elucidated, innovative targeted approaches have become possible. The preclinical and translational data required to evaluate such new therapies before embarking on therapeutic studies in patients with NF1 are unsettled. Molecular analysis of human tissue has resulted in great insights, but the availability of human tissue is often limited, as is the ability to put human tissue into sustainable informative cell cultures and/or cell lines. Mouse models of various manifestations of NF1 have added to our understanding of the condition and have identified important interactions between different cell types found in tumors, the tumor microenvironment, and the factors required for the development of tumors; however, mouse modeling has its own limitations and, in many cases, has not been as informative as initially hoped.

Translating these biological advances into therapy presents other challenges. Although NF1 is a relatively common genetic (especially neurogenetic) disease, the numbers of patients available with active disease manifestations for a certain type of complication may be small, making large-scale clinical trials difficult, especially those that require control arms to prove efficacy. The manifestations of the disorder, although often quite impairing, are usually chronic, requiring cost benefit ratios that take into account not only the acute benefits of the therapy, but also the long-term potential consequences. For reasons that are only just being elucidated, specific complications of NF1 have a notoriously erratic natural history, making assessments of therapeutic efficacy difficult. Manifestations may be life-long; however, as is the case in patients with visual pathway gliomas, disease progression may occur over a discrete time window. In addition, the measurement tools required in assessing response and long-term benefits have not always been available. Even the side effects of therapy may be different in patients with NF1, as compared to the population as a whole.

Despite these limitations and challenges, the management of patients with NF1 has entered a therapeutic era. A multitude of new agents are available which could potentially benefit patients. Clinical trial infrastructure is now in place to more effectively and expeditiously study new therapies while outcome measures have become more refined and informative. As an example, through Department of Defense funding, a multicentered, multidisciplinary clinical trials consortium has been funded in the United States since 2006.

This chapter will focus on the therapeutic approaches for patients with some of the more common conditions associated with NF1, including neurofibromas (primarily plexiform neurofibromas), malignant peripheral nerve sheath tumors, gliomas (primarily visual pathway gliomas), and learning disabilities. Other complications of the disorder, that have been earmarked for treatment, will also be briefly discussed. The scientific rationale and translational approach to each of these targeted manifestations will be emphasized and evidence-based therapeutic interventions will be highlighted, whenever they exist.

40.2 General Principles of Preclinical Modeling

Preclinical testing offers the possibility of identifying potential therapeutic targets and approaches as a prelude to clinical trials in NF patients. Therapeutic approaches might include repurposing of existing drugs as well as identifying new compounds that are not currently available as drugs. Although the former could be used for clinical trials without extensive preclinical testing, preclinical validation offers a way to prescreen the most promising compounds, allowing resources to be focused on those most likely to be effective. Preclinical test systems include both in vitro and in vivo models, each offering particular strengths and weaknesses.

In vitro models include cell culture systems that reflect the various aspects of the NF phenotype as well as systems that may not be directly related to the phenotype but permit effective readout of potential targets. Cell lines can be made from human or mouse malignant tumors, especially malignant peripheral nerve sheath tumors (MPNSTs) (Fang et al. 2009; Hakozake et al. 2009; Wallace et al. 2000), and can be propagated indefinitely. Many such cell lines are available, both from humans with NF and mouse models of the disorder. Cancer cell lines can be used as standardized reagents, though the cells undergo continuous genetic evolution in vitro, so there is risk that the behavior of a cell line will not reflect the original tumor from which it was derived. Studies of nonmalignant tissues, such as fibroblasts, melanocytes, Schwann cells, and dysplastic bone, are done on primary cultures derived from human or mouse lesions. In some cases it is possible to grow cultures of Schwann cells that are heterozygous for the germline *NF1* mutation or have bi-allelic *NF1* mutations, which include the germline and the acquired somatic mutations.

A variety of readouts are used for the assessment of cell culture models. Phenotypic assessments include measurements of cell growth, survival, and motility. Biochemical readouts can include measurements of activation of signaling proteins such as RAS, MAP kinase, and mTOR. There can also be readouts at the level of gene expression, such as production of full-length protein in spite of a truncation mutation or correction of a defect in gene splicing. Cell culture systems offer relatively low cost and rapid throughput and thus lend themselves to large-scale screening of potential therapeutic compounds. This can be scaled up to screening of large chemical libraries through robotic systems, which can lead to discovery of previously unrecognized therapeutic candidates. A major disadvantage, however, is that in vitro systems may not accurately reflect the complexity of NF phenotypes, especially those that involve interactions between different types of cells. Also, cells in culture may continue to evolve, with selection for properties that favor growth in culture as opposed to growth in a patient.

Animal models present the opposite issues—higher cost and slower turnaround time for testing, but potentially more accurate reflection of the biology of the disease. With the exception of some reports of plexiform neurofibromas in cattle (Sartin et al. 1994), there are no natural animal models of NF. Genetically engineered models have been made in diverse organisms, including *Drosophila*, zebrafish, and mouse. *Drosophila* models have the advantage of low cost and the

ability to be studied extensively at the genetic level, facilitating studies of genes whose products interact with the product of the NF genes (Guo et al. 1997). Although the phenotypes of NF mutations in *Drosophila* are vastly different from their human counterparts, phenotypes can still be used as a readout for drug screening experiments. Zebrafish models are also of relatively low cost and take advantage of the short generation time and vast experience in developmental biological studies in the animal (Lee et al. 2010).

The mouse has been the most widely used model system for study of the various forms of neurofibromatosis (Brossier and Carroll 2011). The initial heterozygous mouse *Nf1* knockouts (Brannan et al. 1994; Jacks et al. 1994) did not develop an NF-like phenotype; animals died of hematological malignancies and developed pheochromocytomas, but did not develop neurofibromas. Homozygotes were found to be embryonic lethal, with cardiovascular defects. Chimeric animals having *Nf1* −/− cells on an *Nf1* +/− background did develop plexiform neurofibromas (Cichowski et al. 1999). This was one of the first demonstrations that *NF1* functions as a tumor suppressor gene. Later, conditional knockouts, with one allele under the control of a *cre* recombinase that is activated in different cell types have been shown to replicate many of the features of NF1, including plexiform neurofibromas, dermal neurofibromas, optic gliomas, skeletal dysplasias, and astrocytomas (Bajenaru et al. 2003; Kolanczyk et al. 2008; Lee et al. 2010; Parada et al. 2005; Reilly et al. 2000; Wang et al. 2011; Zhu et al. 2005). Double knockout of *Nf1* and syntenic*TP53* leads to the development of MPNST (Cichowski et al. 1999). In addition to these genetically engineered models, xenograft systems have been used to grow tumor explants for drug studies in mouse (Appenzeller et al. 1986; Babovic-Vuksanovic et al. 2004; Perrin et al. 2007).

These various model systems have provided a variety of tools in the hands of investigators for drug testing and discovery. The Children's Tumor Foundation sponsors an NF Preclinical Consortium to perform drug testing in these systems, including both benign and malignant tumors in both NF1 and NF2.

40.3 Plexiform Neurofibromas

40.3.1 Overview of Therapeutic Challenges

Plexiform neurofibromas are histologically benign nerve sheath tumors that grow along the length of nerves and involve multiple branches of a nerve (Korf 1999). They develop in up to 40% of individuals with NF—and are prime candidates for clinical trials, given the associated morbidity, including disfigurement, the potential for significant functional impairment, and great challenges in surgical resection (Canavese and Krajbich 2011; Korf 1999; Prada et al. 2012). Plexiform neurofibromas are most often noted in children and typically grow most rapidly early in life (Dombi et al. 2007; Friedrich et al. 2003). They can be visible

externally, where they may cause soft tissue overgrowth, or may be confined to deeper within the body, in which case they may not be diagnosed unless imaging is done. Orbital-facial plexiform neurofibromas can lead to facial disfigurement and loss of vision or hearing. Cervical tumors can cause airway compression. Tumors of the brachial or lumbar plexus can lead to peripheral nerve dysfunction. Mediastinal tumors can cause pulmonary restriction, and abdominal tumors can lead to intestinal obstruction. Pelvic plexiform neurofibromas may obstruct the ureter or bladder. Plexiform neurofibromas at any point along the spine can invade the neural foramen and compress the spinal cord. Plexiform neurofibromas are often associated with bony dysplasia, including defects in the sphenoid for orbital tumors, scoliosis for spinal tumors, and bony disfigurement for tumors of the extremity. In addition, there is an 8–13% lifetime incidence for degeneration to malignant peripheral nerve sheath tumors, which are characterized by a highly aggressive behavior and high mortality (Evans et al. 2002).

The only established mode of treatment for plexiform neurofibromas is surgery, but this can be difficult to perform and results are often unsatisfactory. Unless a tumor can be completely removed, recurrence is likely (Needle et al. 1997), and the large, irregular morphology of plexiform neurofibromas commonly makes complete removal impossible. Plexiform neurofibromas are highly vascular, creating a risk of hemorrhage during surgery and making it difficult to establish the boundaries of the tumor. In some cases, feeding vessels have been embolized to reduce blood loss during surgery (Canavese and Krajbich 2011; Tanaka et al. 2005).

There is a great need for the development of effective medical therapies with the goal to halt the growth of plexiform neurofibromas, to reduce their size, and decrease morbidity. Substantial progress has been made in the understanding of the pathogenesis, molecular biology, and natural history of plexiform neurofibromas. This, combined with the increasing expertise of the design of clinical trials for plexiform neurofibromas, and in the image analysis of these complex tumors, has allowed for the development of meaningful clinical trials with targeted agents.

40.3.2 Translational Aspects

Plexiform neurofibromas are composed of neoplastic Schwann cells, fibroblasts, perineurial cells, and mast cells. Neoplastic Schwann cells lack *NF1* gene expression, and loss of neurofibromin is associated with elevated levels of activated RAS (Kluwe et al. 1999; Serra et al. 2000). Activated RAS results in the initiation of a cascade of signaling events, such as activation of RAF/MEK/MAPK and PI3K/AKT and mTOR (Dasgupta et al. 2005; Johannessen et al. 2005; Lau et al. 2000). Additional cooperating events are also important for neurofibroma development and growth. Plexiform neurofibromas (PN) have increased expression of growth factors and growth factor receptors, including epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor (VEGF), which may promote tumorigenesis and progression (DeClue et al.

2000; Kawachi et al. 2003; Kim et al. 1997; Ling et al. 2005). While loss of *NF1* in the Schwann cell lineage is sufficient to generate tumors, complete *NF1*-mediated tumorigenesis requires both a loss of *NF1* in cells destined to become neoplastic as well as heterozygosity in non-neoplastic cells, indicating an important role of the tumor microenvironment in neurofibroma development (Zhu et al. 2002). This was elegantly shown in studies demonstrating a role of *NF1*+/− mast cells in neurofibroma development as a result of secretion of kit ligand by *NF1*-deficient Schwann cells (Yang et al. 2003, 2006, 2008).

Many of the potential targets identified for the treatment of plexiform neurofibromas are shared with cancers for which new drugs are being developed, such as RAS, angiogenesis, and mTOR. With the development of transgenic mouse models of spontaneous plexiform neurofibromas (Cichowski et al. 1999; Gutmann et al. 2002; Wu et al. 2008; Yang et al. 2008), the preclinical evaluation of novel agents has become feasible, will allow for validation of these models, and for rational prioritization of targeted agents for clinical trials. For example, activity of the C-kit and PDGFR inhibitor imatinib mesylate in a plexiform neurofibroma mouse model resulted in clinical development of imatinib for inoperable plexiform neurofibromas (Yang et al. 2008), and symptomatic improvement and plexiform neurofibroma shrinkage was observed in some patients (Robertson et al. 2011).

40.3.3 Clinical Trials

Initial plexiform neurofibroma trials used designs and endpoints that are similar to oncology trials, but several aspects of NF1 and PN differ from refractory cancers and require new approaches toward drug development (Kim et al. 2009; Packer et al. 2002). For example, standard methods used to measure tumor size and assess response in cancer trials (one-dimensional RECIST and two-dimensional WHO criteria) (Miller et al. 1981; Therasse et al. 2000) are inadequate to quantify clinically meaningful changes in PN (Widemann et al. 2006). Recently, methods of volumetric MRI analysis of plexiform neurofibromas have been developed (Cai et al. 2009; Poussaint et al. 2003; Solomon et al. 2004) and are used in clinical trials (Babovic-Vuksanovic et al. 2006, 2007; Jakacki et al. 2011a, b) to more sensitively and reproducibly document growth and shrinkage of plexiform neurofibromas, which limits the exposure to potentially inactive and toxic experimental therapies.

Clinical trials development has focused on the development of targeted, biologically based treatment approaches, and only one clinical trial with chemotherapeutic agents (methotrexate and vinblastine) is ongoing. Completed and ongoing clinical trials with targeted agents addressing plexiform neurofibromas are summarized in Table 40.1. Separate phase I trials for NF1 plexiform neurofibromas were performed for several agents due to the potential differences in the tolerability of agents in NF1 compared to patients with refractory cancers. Phase I trials in NF1 PN have been safe, and toxicity tolerable at comparable doses to children or adults with refractory cancers, with the exception of the pediatric

Table 40.1 Completed and select ongoing clinical trials with targeted agents in patients with NF1 related plexiform neurofibromas (PN)

Drug	Mechanism of action	Phase	*N*	Schedule	Eligibility	Endpoint	Response evaluation	Result	Ref.
Plexiform neurofibromas									
13-*cis*-retinoic acid (CRA) or interferon α-2a (IFN)	Differentiation Angiogenesis	II	57	CRA oral daily x 21d q28d IFN SC daily	Progressive PN; children and adults	Response, TTP	2D	86% of pts. On CRA, 96% IFN stable at 18 months; 3 pts. CRA and 2 pts. IFN 10–20% ↓ in PN; 3 pts. CRA and 5 pts. IFN symptomatic improvement	Packer et al. (2002)
Thalidomide	Angiogenesis	I	20	Oral continuous	Progressive PN; >5 years	Toxicity	2D	Max dose 200 mg/day; 4 of 12 pts. <25 % ↓ in PN; 8 of 12 pts. symptomatic improvement	Gupta et al. (2003)
Tipifarnib	FTase	I	17	Oral × 21d q 28d	Inoperable PN; 3–21 years	Toxicity; PK, PD	WHO	MTD 200 mg/m^2/day BID × 21d q28d; median cycle # 10; no responses	Widemann et al. (2006)
		II	60	Oral × 21d q 28d	Progressive PN; 3–25 years	TTP[a] (PN↑ ≥20 %)	3D MRI	No doubling in TTP compared to placebo	Widemann et al. (2009a, b)
Pirfenidone	Fibroblast	II	24	Oral continuous	Inoperable or symptomatic PN; adults	Response	3D MRI	7 pts. >15 % ↓ in PN; 14 pts. symptomatic or clinical improvement	Babovic-Vuksanovic et al. (2006)
		I	16	Oral continuous	Inoperable PN; 3–21 years	Toxicity; PK	3D MRI	Optimal dose 500 mg/m^2 TID; median cy # at MTD 15; no responses	Babovic-Vuksanovic et al. (2007)
		II	36	Oral continuous	Progressive PN; 3–21 years	TTP[a]	3D MRI	No doubling in TTP compared to placebo control group	Widemann et al. (2009a, b)
Peginterferon alfa-2b	Immune modulation Angiogenesis	I	30	SC q week	Inoperable PN; 1.3–21 years	MTD	Who-3D MRI	MTD 1 mcg/kg SC weekly; 11/16 pts. pain improvement, 5/17 pts. PN↓ 15–22 %	Jakacki et al. (2011a, b)
		II		SC q week	Inoperable, symptomatic, or progressive PN	TTP[a]; response[b] (PN↓ ≥20 %)	3D MRI Clinical	Ongoing	–

(continued)

Table 40.1 (continued)

Drug	Mechanism of action	Phase	*N*	Schedule	Eligibility	Endpoint	Response evaluation	Result	Ref.
Imatinib	C-Kit, PDGFR, VEGFR	II	36	Oral continuous 440 mg/m^2/day children; 800 mg/day adults	Children and adults	Response; (=>5 % ↓ cross sectional area)	2D	9/24 pts. response; 7/24 pts. Symptomatic improvement	Robertson et al. (2011)
Sirolimus	mTOR	II		Oral continuous	≥3 years; inoperable PN; radiographic progression or stable	TTP[a] for progressive PN; Response[b] (for stable PN)	3D MRI	Progressive PN ongoing; stable PN: no responses in stable PN	Weiss et al. (2010)
AZD2171	VEGFR2	II		Oral continuous	Inoperable or progressive PN; adults	Response	3 D MRI	Ongoing	–
Sorafenib	C-Raf, B-Raf, VEGFR, C-Kit, PDGFR	I	6	Oral continuous	Inoperable PN; 3–21 years	Toxicity, PK, PD, DEMRI	3D MRI	Dose-limiting PN pain at lower doses than pediatric solid tumor dose	Kim et al. (2009)
Nilotinib	C-kit	II	–	–	Adults	Response	RECIST	Ongoing	–
AZD6244	MEK	I	–	Oral continuous	12–18 years	Toxicity; PK; PD	3D	Ongoing	–
Sunitinib	VEGFR, PDGFR	II	–	–	Children and adults	Response	RECIST	Soon to open	–

Abbreviations: *d* Day, *2D* two-dimensional, *3D* three-dimensional, *TTP* time to progression, *PK* pharmacokinetics, *PD* pharmacodynamics

[a]Defined as time to ≥20 % increase in plexiform neurofibroma volume

[b]Defined as ≥20 % decrease in plexiform neurofibroma volume

phase I trial of sorafenib (Kim et al. 2009), which was intolerable in children with NF1 at lower doses compared to children with refractory cancers (Widemann et al. 2009a, b), due to severe plexiform neurofibroma pain on sorafenib treatment. Compared to children with refractory cancers, children with NF1 receive experimental agents for much more prolonged time periods and are younger when they enroll on clinical trials (Kim et al. 2009). Careful, longitudinal evaluation for chronic and potentially cumulative toxicities (Kim et al. 2009), which may not be detected in older children following shorter drug exposure or in adults, is thus mandatory for clinical trials directed at plexiform neurofibromas.

Plexiform neurofibroma shrinkage and time to disease progression defined by volumetric MRI analysis have been the primary endpoints for several phase II trials. The placebo arm of a randomized, double-blinded, placebo-controlled, crossover phase II trial of the farnesyl transferase inhibitor tipifarnib (Widemann et al. 2009a, b) has been used in several subsequent trials as control group to determine the activity of the agents under study. Tipifarnib did not result in a doubling of the time to progression compared to placebo. The median time to progression of patients with progressive plexiform neurofibromas at trial entry was 10.6 months on the tipifarnib trial, highlighting the much longer time required to document disease progression in plexiform neurofibromas compared to cancer tumors.

While no standard medical treatment has been identified to date, plexiform neurofibroma shrinkage and symptomatic improvement have been observed in several trials (Babovic-Vuksanovic et al. 2006; Gupta et al. 2003; Jakacki et al. 2011a, b; Robertson et al. 2011). With the development of the NF Preclinical and Clinical Trials Consortia, it is hoped that the development of effective treatments for plexiform neurofibromas will be greatly accelerated.

40.4 Gliomas

40.4.1 Overview of Therapeutic Challenges

Gliomas in children and adults with NF1 are not uncommon, occurring in over 20% of patients during their lifetime (Listernick et al. 1989, 1997). Arising in different areas of the brain, by far the most common are those of the visual pathway occurring in children less than 5 years of age. Such lesions often have apparent contiguous involvement of the chiasm and optic tracts and radiations. Visual pathway tumors may be found on screening examinations and there may be a mismatch between the size and extent of the tumor, as seen on MRI, and clinical manifestations, including visual acuity and visual field abnormalities. Separation of active tumor from areas of abnormal signal intensity on MRI in the visual pathway and surrounding tissue can be difficult and arbitrary. At times, MR abnormalities extend into the hypothalamus, thalamus, and other surrounding areas of brain, and it is unclear what part of the lesion should be therapeutically

targeted. Adding to the complexity of therapy is the erratic natural history of visual pathway gliomas. The majority of patients who develop tumor progression do so in the first few years of life, although late progression associated with visual deterioration has been noted.

Gliomas arising in sites other than the visual pathway occur in a less stereotyped fashion. Both focal and more diffuse glial growths seem to have proclivity to originate in the brainstem, although later in life cortical lesions, including those involving the corpus callosum, seem to occur in greater frequency. Once again, manifestations of nonvisual pathway gliomas may be subtle or absent, complicating decisions concerning the need for treatment.

The majority of NF1-associated gliomas are pilocytic astrocytomas, although grade 2 and malignant gliomas may occur (Listernick et al. 1997, 2007). Because of tumor location and the overwhelming likelihood that such tumors are pilocytic astrocytomas, surgical interventions, including biopsy, are often deemed unnecessary or too potentially harmful to vision or other neurologic functions, and patients are treated without histological confirmation. This is especially true for visual pathway and brainstem lesions (Pollack et al. 1996). Surgery is more frequently undertaken for the cortical masses in attempts to both obtain tissue for histological confirmation of grade and, in selected cases, effectively treat the tumor. This lack of human tissue limits the ability to do biological investigations. Therapeutic approaches have often been based on experience in non-NFI-related tumors, as approximately 50% of childhood visual pathway gliomas will occur in non-NF patients and are more aggressive than NF1-related lesions (Ater et al. 2011).

40.4.2 Translational Aspects

The molecular pathogenesis of sporadic pilocytic astrocytomas has been recently better elucidated (Zhu et al. 2002; Gutmann et al. 2002; Hegedus et al. 2007). Between 60 % and 90 % of sporadic tumors, histologically consistent with pilocytic astrocytomas, harbor mutations involving BRAF, which is a proto-oncogene in the RAS signaling pathway (Pfister et al. 2008). The majority of tumors are due to activating fusion mutations of *BRAF* and *KIAA*, with a smaller number being due to *BRAF* point mutations. These mutations have not been noted in the NF-associated gliomas and bi-allelic *NF1* gene inactivation is required for tumor development in those with NF1 (Gutmann et al. 2002). Loss of *NF1* gene expression does not occur in sporadic tumors. Recent genome-wide association studies have demonstrated differences in gene expression profiles of NF1-associated pilocytic astrocytomas, as compared to sporadic tumors. NF1-associated tumors display expression profiles which are similar to those of cultured normal human fetal astrocytes, while expression profiles of sporadic pilocytic astrocytoma are typically associated with oligodendroglial lineage (Lee et al. 2010).

Given the relative lack of human tissue available for study, mouse modeling has taken on a major role in the development of new molecularly targeted approaches

for the treatment of gliomas in patients with NF1, especially visual pathway gliomas (Gutmann et al. 2002; Zhu et al. 2002). Only a few knockout mouse models exist. The models demonstrate hyperplasia of optic nerves, some of which progress to optic pathway gliomas (Hegedus et al. 2009; Zhu et al. 2005). The models confirmed that bi-allelic *NF1* loss is required for tumor formation, but also disclosed that *NF1* loss alone is insufficient for astrocytoma formation and that other genetic or tumor microenvironmental factors influence NF1-associated glioma tumorigenesis (Hegedus et al. 2009; Warrington et al. 2010; Lee et al. 2010). *NF1* heterozygosity alters microglial function resulting in increased proliferation and motility and elevated production of growth-promoting molecules. Furthermore, cyclic AMP suppression induces gliomagenesis in mouse models of NF1; such suppression occurs in a developmental time window in the region of the visual pathway and may explain, in part, the timing of visual pathway tumor development in children with NF1 (Zhu et al. 2005; Warrington et al. 2010; Sun et al. 2010). A complex interaction occurs between loss of neurofibromin and the function of other growth-promoting molecules in the tumor microenvironment, such as CXCL12 (Bajenaru et al. 2002, 2003). Among other changes, this interaction affects CXCR4 signaling and induces sustained suppression of cyclic AMP levels (Dasgupta et al. 2003). Although it has been found that CXCL12 expression alone is insufficient for gliomagenesis, alterations in this pathway seem extremely important in tumor growth (Sun et al. 2010). Pharmacologic elevation of cyclic AMP with a PED4 inhibitor (rolipram) has been demonstrated to inhibit optic glioma growth.

As work continues in better elucidating the intricacies associated with visual pathway tumor development and growth, most of the molecularly targeted therapies, to date, for gliomas occurring in children with NF, have targeted the RAS pathway. This is based on the well-accepted premise, discussed earlier, that neurofibromin functions as a negative regulator of the RAS intracellular signaling pathway and reduced neurofibromin results in increased RAS pathway activation (Hegedus et al. 2007). Increased RAS signaling has been associated with increased mammalian targeting of rapamycin (mTOR) in astrocytes (Dasgupta et al. 2005). Mouse modeling has shown that loss of neurofibromin also results in proliferation of neuroglial progenitor cells during embryogenesis in a RAS and AKT-dependent fashion (Hegedus et al. 2007). mTOR/Rictor/-mediated AKT activation is a key driver of such proliferation. mTOR activation in NF1-deficient astrocytes is dependent on RAS and P13 kinase signaling.

These insights have led to some of the ongoing therapeutic trials; however, the complexity of the signaling pathway in NF1 astrocytomas and upstream and downstream biofeedback loops which may be present make therapy even more challenging. Negative feedback loops between mTOR and AKT have been found in cancer cells (Hidalgo 2012). In cell lines sensitive to mTOR inhibition, hyperphosphorylation of AKT and increased AKT kinase activity has been noted. Since AKT signaling is associated with cell survival pathways, mTOR inhibition of tumor growth may be attenuated.

A possible means to overcome escape mechanisms caused by inhibition of negative feedback loops (as is theoretically possible by mTOR inhibition) is

combination therapy, attempting to block upstream or other cellular signaling targets. Pilocytic astrocytomas have been shown to over-express epidermal growth factor receptor (EGFR) and phosphorylated EGFR, making EGFR a possible upstream target (Thorarinsdottir et al. 2008). Other potential targets include PDGFR and VEGFR (McLaughlin et al. 2003). In mouse models, dual inhibitors of mTOR and P13-kinase signaling have shown potential efficacy as tumor suppressor agents. Still another means is combining biologic agents with more standard chemotherapies to enhance efficacy.

40.4.3 Clinical Trials

Multiple clinical trials have been undertaken over the past two decades for children with pilocytic astrocytomas, including those with NF1 (Campagna et al. 2010; Gnekow et al. 2004; Gururangan et al. 2002, 2007; Hornstein et al. 2011; Jakacki et al. 2011a, b; Kadota et al. 1999; Lafay-Cousin et al. 2005; Lathier et al. 2003; Mahoney et al. 2000; Massimino et al. 2002a, b, 2010; McLaughlin et al. 2003; Mishra et al. 2010; Packer et al. 1993, 1997, 2009; Pons et al. 1992; see Table 40.2). Major impetuses for development of such trials include the young age of diagnosis of children with both NF1-associated and sporadic astrocytomas, the inability to safely and extensively resect midline tumors, and the concern that radiation therapy may cause severe, irreversible neurologic, especially neurocognitive, damage in such young patients. These concerns have been especially prominent in children with NF1-associated visual pathway gliomas and are amplified by the increased risk of NF1-related, radiation-induced mutagenesis and cerebrovascular damage. This has led most, but not all, investigators to avoid the use of radiation therapy in progressive NF1-related low-grade gliomas. A concern in the development of chemotherapy protocols for children with NF1 has been the reluctance to utilize alkylating agents, or other relatively highly mutagenic agents, because of the theoretic concern of increased mutagenesis and the development of secondary tumors, especially leukemia.

By far, the widest clinical experience in children with NF1-associated gliomas has been the use of the combination of carboplatin and vincristine (Packer et al. 1993, 1997). This drug combination, which grew out of an early experience of the use of actinomycin and vincristine in children with fibrosis, was initially empirically applied in young children, primarily those less than 5 years of age, with both newly diagnosed and recurrent visual pathway. Both limited institution trials and, more recently, prospective cooperative group studies in hundreds of prepubertal children with NF1 progressive low-grade gliomas have demonstrated an overall objective tumor response (defined differently by different studies, but requiring at least a 25% shrinkage in bi-tumoral largest diameter) of as high as 60% (Gnekow et al. 2004). Partial, defined as greater than 50% reduction in tumor bidirectional area, and complete responses have been noted in 35–40% of patients, and disease stabilization while on treatment occurs in over 90% of patients. Duration of disease

Table 40.2 Selected trials of chemotherapy for children with NF1 and low-grade gliomas

Study	NF1 patients/all patients on study	Patient status at treatment	Tumor location	Objective resp. NF/All on study	PFS or EFS/ all NF/all on study
Packer et al. (1993) Carbo/VCR	5/60	Recurrent and ND	All	NG/58 %	NG
Packer et al. (1997) Carbo/VCR	15/63	ND	All	50 %/56 %	2-year PFS 79 ± 11 %/ 75 ± 6 %
Ater et al. (2011) Carbo/VCR	127/401	Newly diagnosed	Visual pathway	NG	5-year EFS 69 %/53 %
Gnekow et al. (2004) Carbo/VCR	36/123	Newly diagnosed	All	NG	5-year PFS 80 %/53 %
Laithier et al. (2003) Procarbazine, carbo, etoposide, cyclo, VCR	23/85	ND	Visual pathway	NS/60 %	2-year PFS 82 %/51 %
Gururangan et al. (2002) Carbo	22/81	Recurrent and ND	All	NS/28 %	3-year PFS 72 %/62 %
Mahoney et al. (2000) Carbo	21/50	ND	Visual pathway	2 %/4 %	3-year PFS 67 %/52 %
Massimino et al. (2002a, b) Cisplatin/etoposide	8/34	ND	All	NG/70 %	3-year PFS 100 %/78 %
Massimino et al. (2010) Cisplatin/etoposide	7/37	ND	All	NG/64 %	3-year PFS NS/65 %
Mishra et al. (2010) Thioguanine, CCNU, VCR, procarbazine, dibromodulcitol	6/33	ND	All	NG	NG/30.3 %

Carbo carboplatin, *VCR* vincristine, *Cyclo* cyclophosphamide, *ND* newly diagnosed, *NG* not given, *NS* not significantly different, *PFS* progression-free survival, *EFS* event-free survival

control in patients with progressive, newly diagnosed low-grade gliomas seems to differ between those patients with sporadic pilocytic astrocytomas and those with NF1-associated tumors. While only approximately one-third of patients with sporadic tumors will maintain their response after 12–15 months of treatment with the carboplatin and vincristine regimen, between 3 and 5 years after initiation of treatment, nearly 70–80% of children with NF1-associated tumors will remain in disease remission (Ater et al. 2011). Responses have also been seen in patients with NF1 and brain stem lesions.

A *caveat* to these encouraging results is that although the vast majority of patients will experience disease stabilization or demonstrate tumor shrinkage while on therapy, some will continue to have visual deterioration despite the radiographic improvements (Campagna et al. 2010). It is unclear whether this is

due to a lack of efficacy of the treatment or to a delayed effect of preexisting tumor on visual function. Most of the studies to date have focused on radiographic disease response and control and have not assessed vision. Although carboplatin and vincristine treatment has been relatively well-tolerated and to date has not been associated with any apparent increased mutagenesis, the drug regimen does have inherent side effects including vincristine-related peripheral neuropathies and carboplatin-associated bone marrow suppression. A variable side effect reported in up to 20% of patients, in some series, has been carboplatin-related allergies, resulting in premature cessation of therapy.

Alternative chemotherapeutic agents and drug regimens have been utilized in patients with NF1 progressive visual pathway and other gliomas, as noted in Table 40.2. As stated previously, there has been a reluctance to utilize alkylator-based therapies such as the TPDCV protocol (6-thioguinine, procarbazine, dibromodulcitol, CCNU, and vincristine) or temozolomide for children with NF1 (Ater et al. 2011; Gnekow et al. 2004). Similarly, etoposide-containing regimens, with etoposide being used as a single agent or in combination, had been avoided. A French cooperative group has utilized aggressive chemotherapy with procarbazine plus carboplatin, etoposide plus cisplatin, and vincristine plus cyclophosphamide for patients with NF1; results were similar in those patients treated with carboplatin and vincristine (Laithier et al. 2003). The greatest experience, other than the carboplatin and vincristine regimens, has been with the use of vinblastine, especially in patients with carboplatin allergies and carboplatin alone (Lafay-Cousin et al. 2005). Both single agents demonstrate evidence of efficacy, primarily prolonged disease stabilization. Other chemotherapeutic approaches have been the use of vinorelbine and low-dose cisplatin and etoposide (Massimino et al. 2010). The latter regimen has demonstrated a high response rate, but there have been concerns of its use in children who are already visually impaired (because of *cis*platin-associated hearing loss), as well as concerns of using etoposide in the NF1 patient population.

In children with NF1-associated tumors, molecularly targeted approaches have been utilized, although most trials are in relative early development. The combination of bevacizumab and irinotecan has demonstrated an objective response rate of nearly 70% in both children with and without neurofibromatosis type 1 (Packer et al. 2009). The initial benefits in 3 children with NF1 were confirmed in a subsequent prospective national study performed by the Pediatric Brain Tumor Consortium. Interestingly, in this limited experience, not only were objective responses seen, but clinical improvement with recovery of vision and neurologic improvement were noted in 7 of 10 children. mTOR inhibitors such as rapamycin and everolimus are presently in clinical trials. The combination of rapamycin and tarceva, utilized in an attempt to overcome potential increased upstream signaling secondary to MTOR inhibition, demonstrated limited efficacy in sporadic low-grade gliomas, but prolonged stable disease in those with NF1; one NF1patient had an objective response (Packer et al. 2010).

40.5 Neurocognitive Dysfunction

40.5.1 Overview of Therapeutic Challenges

Although the predisposition to cancer is a major concern in patients with NF1, some of the more prevalent features are not directly related to tumors (Acosta et al. 2006). Cognitive dysfunction, academic difficulties, and school failure occur in 40–80% of patients with NF1 (North et al. 1997; Hyman et al. 2006; Krab et al. 2008). These complications affect the day-to-day life of children with this condition and are the largest cause of lifetime morbidity in the NF1 population (Acosta et al. 2006). These deficits also impact long-term adaptation to society and can result in poor quality of life (Acosta et al. 2006; Barton and North 2007; Krab et al. 2008).

The cognitive deficits of NF1 have only been systematically studied in the last 20 years. The early evaluations of intellectual function in the NF1 population overestimated the frequency of mental retardation due to lack of diagnostic criteria, inclusion of patients who had intracranial tumors, ascertainment bias, and the use of nonstandardized psychometric assessment. Recent studies have used larger number of patients, careful selection of cases excluding the impact of intracranial lesions, as well as careful selection of comparison controls (Hyman and North 2005; Acosta et al. 2006). More reliable phenotypes are currently being described (Acosta et al. 2006). Recent advances in the understanding of clinical and molecular aspects of the cognitive deficits in NF1, as well as developments in neuroimaging and molecular genetic techniques have the potential to lead to effective intervention in these patients (Costa et al. 2002; Li et al. 2005; Acosta et al. 2006).

The classical descriptions of cognitive deficits in this population included visuospatial learning deficits as a main feature; however, it is clear today that the cognitive phenotype of deficits in the NF1 population is more complex than previously described. As the knowledge derived from mouse models, biologically based observations, and neurocognitive testing on larger numbers of patients is combined, the focus of research has moved to those areas that may benefit from biologically driven interventions. The identification of specific cognitive deficits in patients and the application of biological and molecular models to identify a potential substrate impacting similar deficits in a mouse model is potentially a robust strategy. As a first attempt at this approach, a cholesterol lowering medication, lovastatin, proved to be useful to reverse attention deficits in a NF1 mouse model (Li et al. 2005). A phase 1 study has been completed, and promising results in this small trial (Acosta et al. 2011) have been fundamental to an ongoing randomized, double-blind, placebo-controlled clinical trial. As research in this area moves forward, it is clear that many more opportunities are available.

Clinical observations in children with NF1, such as a high frequency of ADHD symptoms, associations with autism spectrum disorders, and frequent impairments in executive function, have been followed by biological experiments in mouse models attempting to determine the molecular substrate of these clinical findings. Once these are identified, the next step is to test those results in patients, to validate the real life benefits.

40.5.2 Preclinical Research

The *NF1*+/− mouse model has been useful to test potential therapeutic interventions in several manifestations associated with this condition (Li et al. 2005; Cui et al. 2008, Hegedus et al. 2008), as well as to understand the biological basis of clinical manifestations (Brown et al. 2010, 2011). Despite the obvious differences between mice and humans, molecular and cellular cognitive studies have revealed remarkable similarities in the learning deficits caused by *NF1* mutations in the two species (Costa and Silva 2003). The *NF1* mutation would appear to affect some brain functions more than others. For example, visual–spatial learning (Silva et al. 1997) attention, and motor coordination are all impaired, whereas various forms of learning, such as classical conditioning, seem to be intact. Only 40–60% of carriers of the mutation are affected, and remedial training can alleviate the learning deficits (Silva et al. 1997). The severity of the NF1 cognitive phenotype is affected by genetic variation, which exacerbates the problem in NF1 patients without having a noticeable impact on normal siblings. Consistent with this, it was shown that a heterozygous mutation of the gene encoding the *N*-methyl-D-aspartate receptor increases the severity of the learning deficits in littermate controls (Silva et al. 1997; Costa and Silva 2003). Also, genetic background impacts the incidence of learning disabilities in *Nf1*+/− mice. These similarities have been very important to consider NF1 mouse models useful for investigating the mechanisms that underlie the learning deficits associated with NF1. Table 40.3 shows some of the cognitive phenotypes in NF1 mouse models.

40.5.3 Translational Aspects and Clinical Trials

The first translational effort in the treatment of cognitive deficits in NF1 patients was with the use of lovastatin, a cholesterol lowering medication, to reverse cognitive deficits in children with NF1. The rationale came from the observation that an increase in p21RAS activity, caused by mutations of the *NF1* gene, has been related to the learning deficits observed in NF1 mice and in humans, leading to the hypothesis that inhibition of RAS activity may reverse these cognitive deficits (Silva et al. 1997; Costa and Silva 2003). Because posttranslational farnesylation is required for p21RAS function, farnesylation has been suggested as a pharmacotherapy target for NF1 cognitive deficits. Lovastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor successfully inhibits p21RAS isoprenylation and activity (Li et al. 2005). Examining the effects of lovastatin on cognitive deficits, Li et al. reported that lovastatin effectively decreased p21RAS activity and reversed attention and learning deficits observed in *Nf1*+/− mice (Li et al. 2005).

While the results of statin therapy in mouse models are encouraging, simvastatin used in humans did not yield positive results (Krab et al. 2008). It should be noted that the simvastatin trial did not screen for selected learning disabilities, nor did it exclude

Table 40.3 Cognitive phenotypes in NF1 mice models

Mouse model	Behavioral phenotype/other biological alterations	Comparison with human symptoms	References
NF1+/−	Morris water maze Contextual fear conditioning Lateralized reaction time task Pre-pulse inhibition Delayed win-shift radial arm maze	Visual spatial learning deficits Attention deficits Working memory deficits	Silva et al. (1997) Cui et al. (2008) Li et al. (2005) Shilyansky et al. (2010)
NF1 23a−/−	Morris water maze Contextual discrimination Rota-rod	Visual spatial learning deficits Motor deficits	Costa et al. (2002)
NF1 flox/+; Synl-cre NF1 flox/+; Dlx 5/6-cre	Morris water maze	Visual spatial learning deficits	Cui et al. (2008)
NF1 flox/flox	Cortical barrel formation	Autism spectrum behaviors?	Lush et al. (2008)
NF1 −/+ OPG	Mild spatial learning/memory deficits in Morris water maze Marked defects in attention system function	Attention deficits without hyperactivity	Brown et al. (2010, 2011)

patients with mental retardation. A recently completed phase 1 open-label safety trial of lovastatin in children with NF1 and documented learning disabilities (Acosta et al. 2011) showed significant cognitive improvements between pre- and post-lovastatin treatment, suggesting that lovastatin might be effective for treating cognitive deficits in children with NF1. A multicenter, phase 2, double-blind, placebo-controlled clinical trial performed by the Department of Defense-funded NF Clinical Trials Consortium is ongoing to test these observations prospectively in a larger population of children.

Other neurocognitive disorders being explored in mouse models, which will potentially be translatable to human interventions, include ADHD-like symptoms, executive function, working memory deficits, and autism spectrum disorders. ADHD-like symptoms have been reported in as many as 60% in patients with NF1 (Brown et al. 2010), using an NF1 optic glioma (OPG) genetically engineered mice model (GEM model), reported defects in nonselective and selective attention without an accompanying hyperactivity phenotype. Similar to children with NF1, the attention system dysfunction in these mice is reversed by treatment with methylphenidate (MPH), suggesting a defect in brain catecholamine homeostasis including reduced dopamine (DA) levels in the striatum. Catecholamine homeostasis, normalized following either MPH or L-dopa administration, is believed responsible for the attention system dysfunction in NF1 GEM (Brown et al. 2010). In addition, using neuroimaging techniques, studies using [(11)C]-raclopride positron-emission tomography (PET) demonstrated a presynaptic defect in striatal dopaminergic homeostasis. While methylphenidate and L-Deprenyl corrected both striatal dopamine levels on PET imaging and defective attention system function in

Nf1 mutant mice, pharmacologic agents that target deregulated cyclic AMP and RAS signaling in these mice did not. These studies establish a robust preclinical model to evaluate promising agents for NF1-associated ADHD. These studies also support the clinical observation that stimulant medications may be useful for the treatment of behavioral manifestations associated with the diagnosis of ADHD in this population (Mautner et al. 2002) and furthermore, provide insight into the pathophysiology of these manifestations.

Autism spectrum disorders have recently been recognized as part of the cognitive spectrum in patients with NF1 (Marui et al. 2004). Lush et al. (2008) demonstrated that neurofibromin is required for the proper formation of cortical barrels. A complex network of genetic factors regulates the development of the barrel cortex. Alterations in barrel cortex have been associated with sensory integration deficits and sensory modulation processes, many of which have been correlated with autism spectrum disorders. Despite the preliminary results in this field, further exploration of the biological correlations between neurofibromin deficits and biological and clinical correlations with autism is a first step in potentially testing targeted interventions in this population. This is an area that has not been explored systematically. Clear identification and recognition of autism spectrum disorder manifestations are just recently described in systematic population-based evaluations. Behavioral problems and communication and language limitations have been described before as frequent problems in this population (Barton and North 2004). The recognition of autism spectrum disorder in this population may create the opportunity for advancement of interventions for this population and improved understanding of the biologically based alterations that may produce autistic manifestations.

Executive function deficits have been extensively described as part of the cognitive phenotype observed in patients with NF1. Identification of working memory deficits in a mouse model of NF1 has opened the door for a better understanding of the impact of the lack of neurofibromin in frontal lobe development (Shilyansky et al. 2010). Neurofibromin regulates prefrontal and striatal inhibitory networks, specifically activity-dependent GABA release, and is required for working memory performance. This mechanism is believed to underlie inhibition-dependent working memory deficits seen in NF1(+/−) mice (Shilyansky et al. 2010). These observations also have significance to a subset of patients with NF1, as working memory assessment applied during functional MRI (fMRI) studies in human subjects with NF1 revealed hypoactivation of corticostriatal networks and impaired working memory performance. These integrative mouse and human studies reveal similar molecular and cellular mechanisms contributing to working memory deficits in NF1 and may lead to interventions targeting working memory deficits.

One of the most significant challenges in the design of biologically based clinical trials targeting cognitive deficits is the use of reliable measurements. Cognitive measurements used in mouse models and the equivalent of cognitive domains in humans need to be carefully aligned. This will allow validations of the molecular target and the interventions utilized. In some cases, basic cognitive mechanisms may be comparable, such as simple attention skills, orientation, or memory. In other

cases, executive function tasks, behavioral responses, or social interactions may be difficult to compare between species. For example, measurements of attention, hippocampal-related learning, and visuospatial orientation have been correlated in animal models and correspond with some human-related functions. Other more "human-specific behaviors" such as language, social interaction, and executive functions may be more complex and difficult to compare between species. Extensive behavioral batteries have been developed to correlate behavioral testing in animal models with human behavior. As in the evaluation of potential pharmacological interventions for cognitive deficit in this and other genetic conditions, there is an ongoing need to refine behavioral measurements and provide strong validatation for interspecies comparison.

New technologies may provide a window of opportunity to observe functional changes that may be present earlier than cognitive and behavioral abnormalities can be detected. Neuroimaging can potentially advance the understanding of brain function in animal models and humans, as well as the impact of interventions before clear behavioral or cognitive testing could be used. Resting state functional connectivity (RSFC) approaches can successfully delineate functional brain networks without external stimulation (Chabernaud et al. 2012). A myriad of functional networks can be revealed through patterns of synchrony in spontaneous, low-frequency brain activity, which are remarkably similar to functional networks observed in task-based fMRI approaches. In addition, this novel technique has the potential to be used in multicenter studies using already available consortiums facilitating the data collection for clinical trials. This is the case of the recently funded trial by the National Institutes of Health Blueprint for Neuroscience Research, the Neuroimaging Informatics Tools and Resources Clearinghouse (NITRC) which facilitates finding and comparing neuroimaging resources for functional and structural neuroimaging analyses (http://www.NITRC.org).

40.6 Malignant Peripheral Nerve Sheath Tumors

40.6.1 Overview of Therapeutic Challenges

Malignant peripheral nerve sheath tumors (MPNST), also named neurogenic sarcomas, malignant schwannomas, or neurofibrosarcomas, are soft tissue sarcomas arising from peripheral nerves associated with a high risk of local recurrence and metastasis (Ferner and Gutmann 2002). They account for 10% of all soft tissue sarcomas, 50% of MPNSTs arise in individuals with NF1, and the lifetime incidence of the development of MPNSTs in NF1 is 8–13 % (Evans et al. 2002). In NF1, most but not all MPNSTs arise in preexisting plexiform neurofibromas (King et al. 2000). Early diagnosis of MPNSTs is crucial, as only complete surgical resection has been shown to be curative. The diagnosis of MPNST in NF1 may be difficult to establish or delayed because clinical indicators of malignancy such as pain, increasing mass,

and neurologic deficit may also be the features of preexisting benign plexiform neurofibromas (Kim et al. 2009; Korf 1999). MRI characteristics (Friedrich et al. 2005) and FDG-PET (Ferner et al. 2000, 2008) may aid in the diagnosis of MPNST; however, high uptake of FDG has been described for benign neurofibromas, and biopsy is required to establish the diagnosis of MPNST. Biopsies have to be carefully planned to sample tumor areas most concerning for MPNST, and several biopsies may be required. Histologically, most MPNSTs are high grade, characterized by high proliferation index, mitotic rate, and necrosis (Costa et al. 1984; Trojani et al. 1984). In individuals with NF1, high total tumor burden (Mautner et al. 2008), microdeletion (De Raedt et al. 2003; Leppig et al. 1997), and prior radiation may increase the risk for MPNST development. Large (>5 cm) tumor size, deep location, and presence of distant metastases are poor prognostic features (Scaife and Pisters 2003). In addition, poorer overall survival for NF1-associated MPNST compared to sporadic MPNST has been described in most studies, for reasons that are unknown. For example, Carli et al. (2005) reported a 5-year overall survival of 32% for 29 children with NF1-associated MPNST compared to 55% for 138 patients with sporadic MPNST. Similarly, in a more recent study, Ferrari et al. (2011) reported a 5-year overall survival of 11% for 27 children with NF1-associated MPNST compared to 48% for 44 patients with sporadic MPNST. MPNSTs carry the highest risk for sarcoma-specific death compared to other types of soft tissue sarcoma (Ferrari et al. 2011; Kattan et al. 2002).

Treatment of MPNST follows the treatment of other adult soft tissue sarcomas. Only complete surgical resection has been shown to be curative, and it remains the cornerstone of therapy (Abbas et al. 1981; Scaife and Pisters 2003). The local recurrence rate of MPNST is high and ranges from 32 % to 65 % (Gupta et al. 2008). Radiotherapy in addition to surgery has been shown to improve local control in adult soft tissue sarcomas (Pisters et al. 1996; Yang et al. 1998) and is used to improve local control for high grade MPNST >5 cm and after marginal excision (Wong et al. 1998).

The role of chemotherapy for MPNSTs has not been defied to date. In adult soft tissue sarcomas, only doxorubicin, dacarbazine, and ifosfamide were consistently associated with response rates of 20% or more, and the combination of ifosfamide and doxorubicin has produced response rates as high as 46% (Verma and Bramwell 2002). Adjuvant doxorubicin-based chemotherapy did not demonstrate statistically significant improvement in overall survival (Verma and Bramwell 2002). The response rate of MPNST to chemotherapy agents used to treat pediatric and adult sarcomas has not been determined in histology-specific trials to date. Two studies describe decreased response to chemotherapy in NF1 MPNST compared to sporadic MPNST (Carli et al. 2005; Ferrari et al. 2011). In a large retrospective analysis of 167 children with MPNST, 26 of 47 (55.3 %) patients with sporadic MPNST responded to chemotherapy compared to 3/17 (17.6 %) patients with NF1-associated MPNST (Carli et al. 2005). A second report from patients prospectively enrolled on several clinical trials for non-metastatic unresected pediatric non-rhabdomyosarcoma soft tissue sarcomas also described decreased chemotherapy response and overall survival in NF1-associated MPNST [2 of 27 patients (7 %)

responded] compared to sporadic MPNST [25 of 44 patients (34 %)] responded (Ferrari et al. 2011). Defining the response rate of sporadic and NF1-associated MPNST to standard sarcoma chemotherapy is the primary objective of an ongoing Department of Defense-sponsored clinical trial. Patients with unresectable or metastatic MPNST who have not received prior chemotherapy directed at their MPNST are stratified for sporadic or NF1-associated MPNST and receive identical chemotherapy consisting of ifosfamide, doxorubicin, and etoposide. Depending on its outcome, this trial may serve as platform for trials combining chemotherapy with targeted therapies in the future.

Based on the high incidence of MPNST in NF1, limited treatment options, and high mortality, there is a desperate need for the development of methods, which allow for earlier diagnosis of MPNST and of more effective medical treatments for MPNST.

40.6.2 Translational Aspects

Research has focused on the development of methods that allow for earlier detection of MPNST. For example, longitudinal evaluation with whole body MRI is being evaluated as a tool to monitor changes in total tumor burden, which may reflect malignant degeneration (Tucker et al. 2009). Other studies aim at the identification of biomarkers (Hummel et al. 2010), which may have utility in monitoring patients with NF1 for the development of MPNST. Another strong focus has been on the development of a better understanding of the pathogenesis of MPNST. A recent elegant study provides strong support for the development of MPNST from atypical neurofibromas through loss of *CDKN2A*/*B* as the first step in the progression to MPNST (Beert et al. 2011). In addition, multiple studies have aimed at identifying differences between plexiform neurofibromas and MPNSTs to identify targets for biologically based treatments of MPNSTs. Among others, p53, p16, and p27 (Beert et al. 2011; Perry et al. 2002), EGFR (Li et al. 2002; Ling et al. 2005), TWIST1 (Miller et al. 2006); SOX9 (Miller et al. 2006), mTOR (Johannessen et al. 2005, 2008; Johansson et al. 2008); PDGFR (Holtkamp et al. 2006); C-Kit (Holtkamp et al. 2006); TOP2A (Skotheim et al. 2003), IGF1R (Torres et al. 2011a, b; Yang et al. 2008), MET (Torres et al. 2011a, b) and HSP90 (De Raedt et al. 2011) have been implicated in the pathogenesis of MPNST. Xenograft (Johansson et al. 2008) and transgenic mouse models of MPNST (McClatchey and Cichowski 2001; Reilly et al. 2000) have become available, and preclinical trials in these models may have great utility in the rational development of clinical trials with targeted agents. As an example, mTOR inhibition demonstrated activity in transgenic (Johannessen et al. 2008) and xenograft (Johansson et al. 2008).

MPNST Models. Based on these findings, a clinical trial of an mTOR inhibitor in combination with an angiogenesis inhibitor for patients with refractory MPNST has been developed and may serve to validate the preclinical findings.

40.6.3 Clinical Trials

Several histology-specific clinical trials with targeted agents have been performed for MPNSTs and are summarized in Table 40.4. A phase II trial of the EGFR inhibitor erlotinib was the first histology-specific trial with a targeted agent to be completed in patients with NF1-associated and sporadic MPNST (Albritton et al. 2006). Twenty-four patients enrolled within 22 months from 13 institutions. Most patients experienced rapid disease progression, and erlotinib did not demonstrate activity in MPNST. This trial demonstrated that timely completion of MPNST-specific trials is feasible. Subsequently, a phase II trial of the RAF kinase and receptor tyrosine kinase inhibitor sorafenib for adults with several soft tissue sarcomas including MPNST was initiated (Maki et al. 2009). Sorafenib did not demonstrate activity in MPNST, and the median progression-free survival (1.7 months) and overall survival (4.9 months) in MPNST was shorter than in the other soft tissue sarcoma histologies studied, confirming the poor outcome of MPNST compared to other soft tissue sarcomas. In two subsequent trials for subtypes of soft tissue sarcomas including MPNST, no responses were observed to imatinib (Chugh et al. 2009) or dasatinib (Schuetze et al. 2010), and most patients with MPNST had rapid disease progression. Given the uniformly rapid progression of MPNST, future trials may include disease stability for prolonged time periods as response criteria in addition to radiographic response. Combinations of targeted agents, and possibly the addition of targeted agents to cytotoxic chemotherapy, may increase the response rate and outcome in clinical trials for MPNST. For example, recent work in a transgenic MPNST mouse model showed that the combination of an mTOR inhibitor with an HSP90 inhibitor resulted in substantial tumor shrinkage, which was not observed with administration of either agent alone (De Raedt et al. 2011). MPNST-specific trials require multiple participating sites with sarcoma experience for completion. Collaboration of the preclinical NF Consortium with the Department of Defense-sponsored NF Clinical Trials Consortium and with sarcoma-specific consortia will facilitate the timely translation of preclinical findings to the development of clinical trials with the most promising agents and the timely conduct and completion of trials for individuals with MPNST.

40.7 Skeletal Dysplasia

40.7.1 Overview of Therapeutic Challenges

NF1-associated skeletal dysplasias include sphenoid dysplasia, long bone dysplasia, non-ossifying cysts, and scoliosis. Generalized osteopenia has also been

Table 40.4 Completed and selected ongoing clinical trials with targeted agents in patients with malignant peripheral nerve sheath tumors (MPNSTs)

MPNST									
Erlotinib	EGFR	II	24	Oral continuous	Refractory disease; adults	Response	2D	19/20 pts. Progression at 2 months, 1 stable disease	Albritton et al. (2006)
Sorafenib	C-Raf, B-Raf, VEGFR2, C-Kit, PDGFR	II	12	Oral continuous	Refractory disease; adults	Response	RECIST	No responses; median progression free survival 1.7 months	Maki et al. (2009)
Imatinib	C-Kit, PDGFR, VEGFR	II	7	Oral continuous 300 mg BID	Refractory; >10 years	Response	RECIST	No responses; 1 stable disease	Chugh et al. (2009)
Dasatinib	C-Kit, SRC	II	14	Oral continuous 100–70 mg/day	Refractory disease	Response	Choi	No response or stable disease	Schuetze et al. (2010)
Bevacizumab/ RAD001	Angiogenesis/ mTOR	II	–	IV q14d/oral continuous	Refractory disease	Response	WHO	Soon to open	–

Abbreviations: *d* Day, *2D* two-dimensional, *3D* three-dimensional, *TTP* time to progression, *PK* pharmacokinetics, *PD* pharmacodynamics

reported in children and adults (Dulai et al. 2007; Kuorilehto et al. 2005; Petramala et al. 2011; Stevenson et al. 2011). Sphenoid dysplasia usually occurs in conjunction with orbital plexiform neurofibroma and contributes to facial disfigurement (Friedrich 2010). Scoliosis occurs commonly, but a small subset has a dystrophic form that requires surgical correction (Crawford and Herrera-Soto 2007). The most common long bone dysplasia involves the tibia, sometimes together with the fibula, and can lead to fracture and pseudoarthrosis formation. Non-ossifying cysts can lead to pain and fracture, but are most often asymptomatic. Osteopenia may contribute to fractures and slow healing following orthopedic procedures. The morbidity associated with these various lesions makes them appropriate targets for therapeutic development (Elefteriou et al. 2009).

40.7.2 Translational Aspects and Clinical Trials

Tibial dysplasia is usually treated by bracing with the hope of avoiding fractures. For bones that fracture, multiple different orthopedic procedures have been attempted, most often with unsatisfactory results. This has led to substantial interest in the development of novel approaches to treatment. Mouse models of long bone dysplasia have been created based on conditional knockout technology targeting bone. Although the mouse does not develop spontaneous tibial bowing or fracture, probably due to low weight-bearing, abnormal bone healing can be modeled using repair following traumatic injury to genetically modified bone. Treatment with lovastatin at high doses systemically (Kolanczyk et al. 2008) or locally has been shown to improve healing in these models. Schindeler et al. (2011) have worked with a heterozygous *Nf1* mouse model in which tibial fracture repair is monitored. They have found that the combination of bone morphogenetic protein (BMP) and the bisphosphonate zoledronic acid resulted in the union of approximately 63% of fractures compared with 25% treated with BMP alone. This led to a trial of BMP plus bisphosphonate in 7 patients with tibial pseudoarthrosis, 6 of whom had NF1. Primary healing occurred in 6 of 8 cases treated (one patient had bilateral disease).

The clinical significance of the reduction of bone mineral density in adults and children with NF1 has been difficult to interpret, in part due to small numbers of patients tested. Seitz et al. (2009) treated four NF1 patients with vitamin D and reported an increase in BMD. In another study (Brunetti-Pierri et al. 2008), eight patients were treated with vitamin D but BMD did not improve. The question of whether vitamin D will improve clinical outcome in NF1 patients therefore remains unresolved pending further clinical trials.

40.8 Dermal Neurofibroma

40.8.1 Overview of Therapeutic Challenges

Dermal neurofibromas are among the most common complications of NF1; some patients are carpeted with millions of tumors. Although not a cause of significant medical morbidity, the disfigurement associated with dermal neurofibromas can be substantial, with consequent major effects on quality of life (Wolkenstein et al. 2001). Dermal neurofibromas may protrude from the surface of the skin, be present within the dermis, or may be palpable immediately under the dermis. Dermal neurofibromas tend to arise during puberty (Huson et al. 1988) or pregnancy (Dugoff and Sulansky 1996), suggesting a possible hormonal influence on tumor growth.

40.8.2 Translational Aspects and Clinical Trials

Clinical management of dermal neurofibromas has been limited to surgical removal of tumors, with alternative approaches including laser treatment (Elwakil et al. 2007) or electrosurgery (Levine et al. 2008). No formal clinical trials have been done to assess the outcomes of these approaches. Riccardi (1993) has conducted clinical trials with the antihistamine ketotifen based on the finding of mast cells in neurofibromas. Subjective improvement in pain and itching were reported, but not objective tumor shrinkage. Clinical trials are ongoing with topical application of rapamycin or ranibizumab (based on http://www.clinicaltrials.gov, accessed 2 Dec), but no results have been published to date. Dermal neurofibromas have not been closely monitored for response in the course of clinical trials for plexiform neurofibromas, though no obvious reduction in dermal tumor burden has been reported. A mouse model of dermal neurofibromas has been developed based on ablation of the *Nf1* gene in skin-derived precursor (SKP) cells, which may facilitate future preclinical studies of dermal tumors.

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Chapter 41
The Role of NF1 Lay Foundations: Future Vision

Kim Hunter-Schaedle

41.1 Introduction

41.1.1 NF1: A Rare Disease in Perspective

Neurofibromatosis type 1 (NF1) is estimated to affect 1:3,000 people—over two million persons worldwide. NF1 is classed as a rare disease, as it affects fewer than 200,000 Americans. It is actually one of the more "common" rare diseases, but despite this, NF1 does not have a clear public "persona." Unlike rarer diseases such as cystic fibrosis, which is much more easily recognized by the public, NF1 is often unheard of. One reason for this may be that many rare diseases like cystic fibrosis have a fairly standard clinical progression. In contrast, NF1 affects individuals in a myriad of ways. Some persons with NF1 will have nerve tumors which are prominent externally as multiple "bumps" on the skin or as a larger growth on the face or elsewhere. Other persons will have internal tumors which, although often clinically devastating, are unseen. NF1 can cause bone dysplasia which can require limb amputation, often in early childhood. And two-thirds of persons with NF1 will have a form of learning disabilities, which may be mild or severe and may share features with attention deficit disorder, autism, or other well known conditions. In other words, there is no single public "face" of NF1. Moreover, as most cases of NF1 are chronic and lifelong, there are people with NF1 ranging from infancy to old age. As a result, NF1 is a challenging condition to inform the general public on; this challenge becomes an important task of the lay foundation.

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41.1.2 Why and How Lay Foundations Form

Medical lay foundations can take many forms but their fundamental goals are almost universally to serve as an advocate and point of information for those affected by a disease or medical condition. Sometimes new foundations are created to focus on a disease or condition because there is no existing foundation for the disease or condition. Sometimes new foundations are created for a disease or condition for which a foundation already exists, established by individuals who wish to take a different approach from the existing foundation. Some foundations are established by one or more persons, and intended to engage a broad constituent base. Others are established by a family, and may be solely for the purpose of philanthropy for medical research in a particular disease area or to a specific institution or investigator.

Lay foundations play a significant role for rare diseases such as NF1 where individuals diagnosed with the disorder likely don't know anyone else with NF1 and don't know where to find information. Many rare disease-focused lay foundations are established simply to provide a connection point and source of support for those with the disease. However, many disease-focused foundations have progressed from such small beginnings to a sophisticated format, particularly once the foundation begins to fund medical research. There are foundations that began with modest roots, and grow to powerful international drivers of the research agenda for particular disease areas. An example is the The Juvenile Diabetes Research Foundation (JDRF), which is focused on type 1 diabetes. JDRF's roots were as a family-driven entity founded in the 1970s. Today, JDRF is the leading international research funding entity for type 1 diabetes. JDRF has committed over a billion dollars to medical research, spearhead numerous partnerships with other entities including pharmaceutical and biotechnology companies, established international affiliates and collaborations, and is a powerful advocate for type 1 diabetes research federal funding. This illustrates the potential impact of a lay foundation, even one started as a grassroots initiative.

41.1.3 The NF1 Lay Foundation Landscape

In the United States, the two largest NF1 lay foundations are The Children's Tumor Foundation and The Neurofibromatosis Network. The Children's Tumor Foundation was established in 1978 as the National Neurofibromatosis Foundation and renamed the Children's Tumor Foundation in 2004. The Neurofibromatosis Network was established in 1988 as Neurofibromatosis, Inc., and renamed the Neurofibromatosis Network in 2011. The Children's Tumor Foundation is a national organization headquartered in New York; the Neurofibromatosis Network is an umbrella organization that includes a number of regional groups that operate with some independence. Both foundations offer information, support, and community for those

affected by NF1, and both foundations advocate for federal funding for NF research. Both foundations fund research and medical initiatives, but have evolved different approaches to this. The Children's Tumor Foundation raises funds nationally; funds are centralized in New York and disbursed to fund scientific research programs around the world and selected through a competitive application and review process. The NF Network raises funds within each region under the umbrella, and these may be disbursed locally to selected research projects. In addition to these two foundations, there are other NF1-focused foundations both in the United States and globally (List of international NF Foundations), offering support and advocacy for those affected by NF1, and in some cases independently funding research.

41.2 The Many Faces of the NF1 Lay Foundation

To examine the many faces of an NF1 lay foundation, this section will focus on the Children's Tumor Foundation: its mission and charge, resources and tools, and its recent evolution of initiatives specifically to help impact upon and accelerate NF1 research progress.

41.2.1 The NF1 Lay Foundation Roles and Responsibilities

The Children's Tumor Foundation's initiatives cover all forms of neurofibromatosis including NF1, NF2, and schwannomatosis. Here, the focus will be on the NF1 related programs of the foundation.

The Children's Tumor Foundation has four mission areas, each addressing a concern that will, at some point during the process of NF1 diagnosis and progression, be important to all individuals affected by NF1. The four mission areas, the questions they address, and how the foundation is endeavoring to answer these questions are considered below.

Mission Area 1: Offer Information and Support to Those with NF and Their Families.

Question Addressed: "What is NF1? Who else is affected?"

When a person is newly diagnosed with NF1, the most pressing issue for them and their family is usually to better understand what this diagnosis means. Access to current and easy-to-navigate NF1 resources is therefore critical. The Children's Tumor Foundation has developed a series of patient brochures (Children s Tumor Foundation Patient Brochures) that address different aspects of living with NF1. Written by NF1 clinical care experts and tailored to the affected individual and their family, these brochures address many of the most pressing questions that will arise. There is a "Guide for Educators" targeted to the teachers of the child with NF1. This brochure, and the companion "NF1 Learning Disabilities" brochure, are valuable

resources to bring to the child's school to explain what NF1 is and how it might affect the child's classroom needs.

The Children's Tumor Foundation also provides a community for those affected by NF1 to connect with other affected individuals and families. This includes a variety of online resources (Living with NF Resources) such as social media interfaces; in-person events such as regional support groups, a national NF Forum for families; an annual camp for children and young adults with NF1; and both paper and electronic bulletins including a quarterly NF Newsletter. These resources link for those affected by NF1 to information and to a new support network – both of which are critically needed during the early days following an NF1 diagnosis.

Mission Area 2: Create Better Access to Quality Health Care for NF

Question Addressed: "Where can I get clinical care for my child?"

Many children will receive their NF1 diagnosis at their pediatrician's office, or through a referral to a geneticist. The family may be fortunate that the medical facility has a physician who is knowledgeable about NF1 or is able to make a referral to a specialty clinic. However, because NF1 is rare, this expertise is not always available. Many "newly diagnosed" families contact the Children's Tumor Foundation seeking an NF1 specialist or clinic in their region. In 2007, the Foundation established the first national NF Clinic Network. The Foundation's Clinical Care Advisory Board developed a set of "Clinic Principles"—guidelines of what should be expected from a NF Clinic by patients seeking care, such as the ability to provide (or refer locally) care for all of the manifestations of NF, for both pediatric and adult patients. This is quite a demanding undertaking, since NF1 can result a myriad of brain and peripheral nerve tumor types, as well as other manifestations, but it also means that it is very important that persons with NF1 are seen by a physician who is experienced in NF1 and has access to a comprehensive care team to address specialty needs. The Foundation invited all clinics in the United States that see NF patients to apply for Affiliate Clinic status in the NF Clinic Network. Acceptance was based upon a clinic meeting the "Clinic Principles." The NF Clinic now comprises 44 Affiliate Clinics, and the Foundation is working with a number of clinics to help them reach Affiliate status. On the Foundation's Web site, individuals with NF1 and their families can access "Find a Doctor" (http://www.ctf.org/Living-with-NF/find-a-doctor.html) information on all clinics in the United States that see NF patients, and this list highlights those clinics that are NF Clinic Network Affiliate Clinics.

The NF Clinic Network has proved to be successful in consolidating information on NF clinical care, and is also a terrific way of tracking how many NF patients see a doctor annually. Each year, the Foundation requires Affiliate Clinics to submit a detailed Annual Report that includes patient volume. In 2010, over 10,000 total NF patients were reported as seen in the NF Clinic Network. Though the NF Clinic Network has a long way to go before it is fully developed, many patients and families have commended the effort for centralizing NF clinical care information in a way that was previously unavailable.

Mission Area 3: Encourage and Support NF Research Toward Finding Treatments and Cures

Question Addressed: "When will a cure be available for NF1?"

Once individuals with NF1 and their families understand the diagnosis and have established a "norm" for NF1 clinical care, they often become engaged in monitoring NF1 medical research progress and want to help with this by fund-raising to support research. The Children's Tumor Foundation is the world's leading nongovernment funder of NF research. The Foundation has funded NF research since it was first established as the National Neurofibromatosis Foundation, and since 2006 has ramped up its research programs portfolio through the development and implementation of a carefully thought out and detailed Strategic Plan that identified the areas of research in greatest need of funding to accelerate progress toward finding effective NF treatments. Those interested in supporting NF1 research through the Foundation are therefore "investing" in a Strategic Plan alongside the dollars of other fund-raisers/"investors." By supporting a centralized Strategic Plan and leveraging other donations, dollars invested can potentially have the greatest impact. The Children's Tumor Foundation research planning and funding process is described in more detail later in the chapter.

As well as directly funding research, the Children's Tumor Foundation encourages support of NF research by the federal government. The National Institutes of Health (NIH) is the largest funder of medical research in the United States. Because of the breadth of NF clinical manifestations affecting various organ systems, the NIH funds NF research through at least ten of its institutes, including the National Cancer Institute, the National Institute for Neurological Disorders and Stroke, and the National Institute for Mental Health.

Although the NIH is the largest funder of medical research in the United States, the majority of NF research since 1996 has been funded by the Congressionally Directed Medical Research Program Neurofibromatosis Research Program (CDMRP NFRP). CDMRP NFRP funding has been critical for the advancement of NF research because its goals are refreshed year to year by an Integration Panel that includes clinicians, researchers, and NF patient advocates. Each year, the Integration Panel can assess where funding is most needed to accelerate NF research most rapidly at that time, and then request funding applications in those areas. In the past, this focused approach has led to the creation of a national NF Clinical Trials Consortium comprising nine academic medical centers and to the development of genetically engineered mouse models of NF.

CDMRP NFRP funding is recommended by Congress each year for one year only, and this funding is at risk annually of being cut. The NF lay foundation and constituent base play a very important role in encouraging elected officials to support CDMRP NFRP and demonstrating how important the program is for themselves, their child, or loved one. Ensuring the continuation of CDMRP NFRP is a priority of the foundation and an area where everyone can help.

Mission Area 4: Promote Public Awareness and Acceptance of NF
Question Addressed: "Why have so many people never heard of NF1?"
Throughout their lives, those with NF1 will encounter people who are curious about the disorder and may never have heard of it. Many people with NF1 experience such inquiries, which can unfortunately extend to social difficulties and even lead to real or perceived discrimination. This can be due to a person's appearance, such as the presence of disfiguring external tumors on the body, or to communication issues, since two-thirds of persons with NF1 have learning disabilities which can make social situations difficult. It is concerning that many people have never heard of NF1. Many NF1-focused foundations endeavor to promote NF1 facts, and especially to dispel the myth that NF1 is "Elephant Man disease" (which, despite the fact that this has been demonstrated to be Proteus Syndrome, is still confused with NF1) (Legendre et al. 2011). One particularly successful independent change agent though has been Mr. Reggie Bibbs who is a young man with NF1, who through his foundation (The Just Ask Foundation) and the message "Just Ask" has invited openness and discussion about NF1. The Children's Tumor Foundation has endeavored to increase public awareness of NF through a variety of channels. However, explaining NF1 to the general public remains a tremendous challenge.

41.2.2 The NF1 Lay Foundation as a Catalyst for Progress

Even the most successful lay foundation has access to finite amounts of funding. Nevertheless, with careful planning, lay foundations can have tremendous impact on the progress of medical research, and NF1 is no exception. The Children's Tumor Foundation offers an example of how this has been accomplished. Until 2006, Children's Tumor Foundation research funding was primarily focused on supporting Young Investigator Awards (YIA). These Awards provide 2 years of salary for pre- and postdoctoral scientists focused on NF research and perceived as likely to pursue a career in NF research. This program continues to be a valuable and worthy investment which has helped to develop the careers of some of today's leading NF researchers and clinicians (Children s Tumor Foundation Young Investigator Awards). In 2006, the Foundation sought to expand its research support beyond YIA into a broader scope of programs with a goal of developing effective treatments for NF and improving the lives of those with this disorder.

The Foundation first analyzed the overall "landscape" of NF research to assess what type of NF research was being funded. The goal of this analysis was to identify research areas that were being overlooked and not funded although they might have significant impact on research progress. The Foundation analyzed NF research funded by CDMRP NFRP, by the NIH, and by the Children's Tumor Foundation, the three biggest funders of NF research. Grant funding from 1996 to 2005 was analyzed. All funded grants were allocated into "bins" dictated by the research was focused on the bench-to-bedside pathway. Over $220 M of funded NF research was "mapped" in this way to provide an NF Research Landscape. Following this

analysis, the Foundation hosted a think tank of experts from NF research, industry, from other foundations, and from the NF lay community, to examine the NF Research Landscape, identify neglected areas, and develop new research recommendations for the Foundation.

The resulting 2006 Strategic Plan (The Children's Tumor Foundation 2006) recommended a series of new initiatives be implemented, including preclinical drug testing, pilot clinical trials, and a national NF Clinic Network (described earlier in this chapter). As a result, the Children's Tumor Foundation implemented $10 M of new research investments over a 5-year period, 2006–2011. This is not a large investment considering that over that same period the CDMRP and NIH together invested over $100 M in NF research. But the new Foundation programs were catalytic, seed-funding new and somewhat risky ideas with high potential, but lacking the preliminary data required to secure federal funding.

A successful example of a Children's Tumor Foundation catalytic seed funding program is Drug Discovery Initiative (DDI) Awards (The Drug Discovery Initiative Award Program). Launched in 2006, DDI Awards offers seed funding to support pilot-stage preclinical drug testing of candidate NF drugs. The majority of DDI Awards have been $30,000 or less. Funded DDI Awards have assessed drugs in NF tumor explants, primary cells, and cell lines; in xenograft and transgenic mouse models; and in genetically engineered *Drosophila* and zebrafish models of NF. NF1 manifestations assessed have included tumors, learning disabilities, bone dysplasia, and cardiovascular defects. As for all Foundation programs, DDI Award applications are reviewed by a committee of the Foundation's Research Advisory Board. DDI Award applications do not need preliminary data, and the committee was charged to balance thorough review with risk and potential. In addition, DDI Award applications are brief—only 3 pages—and the Foundation is committed to rapid review of these. In most cases review has taken a handful of weeks, a stark contrast to the many months it takes for federal grant application review.

As the DDI Awards program is unique, the Foundation monitored a number of outcome measures to assess its success. To monitor the long-term impact of this funding, the Foundation followed up with funded investigators long after the DDI Award ended. The goal was to track whether the research continued progressing after the DDI Award ended; whether DDI Award-funded data was published; if any industry collaborations were established; and if any follow-on funding was secured from other sources to continue the research after the DDI Award ended. An assessment of the DDI Awards program over its first 5 years (2006–2011) showed that approximately 50 grants had been funded at a total investment of around $1 M. Around half of all of the DDI Awards funded showed a "positive" result, in that the drug tested impacted on the NF manifestation (e.g. the tumor shrank). Awardees published around 20 papers from their DDI research; around 20 had also formed industry (biotech or pharma) collaborations. Perhaps most remarkably, collectively the prior Awardees had secured over $5 M of follow on funding based on the data emerging from the DDI Award from diverse sources including NIH, CDMRP, institutional funds, industry grants, and foundations grants. Many DDI Awardees indicated that without the initial DDI Award funding they would probably never have got their project off the ground.

In summary, the DDI Awards program has met the criteria of success for a catalytic, seed-funding program by providing the initial funds for new and untested ideas. Overall, the DDI Awards program has helped to prime the NF preclinical drug testing pipeline and has shown that small seed amounts of funding can in fact have tremendous impact on a research field.

41.2.3 The NF1 Lay Foundation as a Broker

An important and unique role for a medical lay foundation is to engage and forge relationships with all of the different entities that might have impact on advancing the foundation's mission. Often the lay foundation is uniquely placed to forge these relationships, because it represents the voice of the patient and family community, and therefore has a clear and focused goal. Some of the areas in which the NF1 Foundation might play a brokering role are summarized below.

41.2.3.1 Building Consensus and Collaboration Between NF1 Researchers and Clinicians

The NF1 professional community includes researchers and clinicians working around the world, but this actually encompasses a fairly small number of individuals. To ensure NF1 research makes progress, it is important that the researchers and clinicians focused on this area communicate on an ongoing basis and share research advances in a timely manner. The NF1 lay foundation can play an important role in facilitating this, and the Children's Tumor Foundation has had significant success in this area. For over 25 years, the Foundation has organized an annual conference for NF scientists and clinicians from around the world, as a forum for sharing research and medical advances and for debating issues in need of attention. This event has evolved over the past few years, and with an increased focus on clinical issues in addition to the long-standing focus on discovery research, the meeting has almost tripled in size since 2005, and the NF Conference attracted over 300 attendees in 2011. For the past few years the Foundation has also professionally published the NF Conference report, thereby further increasing the professional audience that can benefit from the presentations and discussions at this important meeting (Kalamarides et al. 2012).

Sometimes an area of NF research requires a special focus in order to fully understand the status of the field and identify future priorities. The Children's Tumor Foundation has hosted a series of "Workshops"—think-tanks focused on a specific NF area. These workshops typically include 25–30 experts from the field and summarize the status of the field, the key challenges and how these should be addressed to advance research progress in that area. As with the NF Conference, the outcome reports from these workshops from the past few years have been published for the benefit of others in the NF professional community (Elefteriou et al. 2009).

41.2.3.2 Engaging Industry in the NF1 Mission

In the past, it has been challenging to engage biotechnology and pharmaceutical companies in NF1 as has been the case for many other rare diseases. This is now changing, as companies recognize the potential commercial opportunity of NF1 where there are no approved drugs and the need for lifelong treatment. However, the initial step of engaging industry remains challenging. To address this, the Children's Tumor Foundation has created research programs that provide industry an opportunity to enter into NF1 research without major cost or risk.

As described earlier, the DDI Awards program funds pilot stage preclinical drug testing. A number of DDI Awardees have been able to secure the test drug from industry. Contributing drug for a DDI study requires little commitment or risk for the company. To help fuel these collaborations, the Children's Tumor Foundation established the DDI Toolbox (The Drug Discovery Initiative Toolbox), a Web page where companies could list drugs they are willing to provide for NF research. If an investigator is interested in a specific drug, the requisite materials legal documents are signed between the company and the investigator's institution.

Taking the DDI concept to the next level, in 2008, the Children's Tumor Foundation established the NF Preclinical Consortium (NFPC) comprising six academic research centers each focused on different NF tumor types, and with validated mouse models representing those tumors. The goal of NFPC was to secure drugs from industry and test them, in parallel, in multiple NF tumor mouse models, to compare the response of different tumor types to the same drug. NFPC was overseen by an External Advisory Board comprising scientists with industrial experience. NFPC was an ambitious undertaking, at an initial cost of $4 M for the period 2008–2011 (The NF Preclinical Consortium). However, it proved attractive to industry, as it offers a comprehensive testing of a drug in NF models. NFPC has also had challenges, not least the complexity of signing legal agreements with multiple institutions for each study. In 2011, the Foundation renewed NFPC for a further 2 years, and it will be interesting to see what is accomplished overall at the conclusion.

41.2.3.3 Partnering with Other NF1 Funding Entities for Mutual Benefit

As highlighted earlier in this chapter, there are a number of NF1 lay foundations. Is there an opportunity for these entities to collaborate for the benefit of the NF1 patient population? The Children's Tumor Foundation and the Texas NF Foundation (The Texas NF Foundation) recently demonstrated that this can be done. In 2010 and 2011, respectively, the Texas NF Foundation funded an NF1 Young Investigator Award and an NF1 Drug Discovery Initiative Award through the Children's Tumor Foundation. Both projects were recommended for funding through the Children's Tumor Foundation review process. This is a great example of a tangible way in which two NF1 lay foundations can work together, and it is hoped that it lays the basis for future collaborations of this type.

41.3 Future Horizons: The Evolution of the NF1 Lay Foundation

A child newly diagnosed with NF1 today has a tremendously more hopeful outlook than a child diagnosed 10 years ago. For those with NF1, this is something of an "age of hope." NF1 clinical diagnosis and care approaches have improved, and candidate drug therapies for NF1 are being tested in the clinic. But to what extent can—and should—lay foundations offer hope to those living with NF1? Where is optimism appropriate, and where should caution be used in relaying news of progress? And are there other issues that NF1 lay foundations need to be aware of as they look to the future? And most importantly, how can NF1 lay foundations ensure that they continue to meet the needs of the affected individuals and their families?

41.3.1 NF1 Advances: Balancing Optimism and Caution in the Age of Hope

NF1 clinical diagnosis and care options have made significant advances in the past few years. There is a better understanding of the clinical progression of NF1, approaches to the early detection of NF1 manifestations (such as tumor imaging technologies) that allow persons with NF1 to receive more vigilant clinical monitoring for the earlier detection of medical issues in need of attention. Genetic testing is now widely used in diagnosis and family planning. In addition to surgical interventions, there is now a pipeline of drugs in NF1 clinical trials for treatment of NF1-related tumors, learning disabilities, and bone dysplasia. The NF1 preclinical drug pipeline is also growing, and it is likely that in the near future many more drugs will enter clinical trials. All of this means that those living with NF1 can choose from a much broader range of treatments or trials than was available just 5 years ago. It will not be long before a person with NF1 may be quite overwhelmed by the options available. However, it is important to remember that the majority of drugs entering clinical trials will ultimately not be approved, and that participating in a clinical trial will not guarantee a drug's success. If the trial is blinded and placebo controlled, the patient must accept the fact that they may not even receive active drug during the trial. Though it is certainly not exclusively the responsibility of the NF1 foundation, it is important for the foundation to inform individuals and families as much as possible about these potential issues and to provide as much current information as possible about all available modalities of treatment.

41.3.2 The Rise of "Patient Power" and Its Impact on the Future NF1 Lay Foundation

At the heart of the NF1 lay foundation are patients and families, and serving these persons is the primary responsibility of the lay foundation. Traditionally the lay foundation has served as a source and gatekeeper of information for these individuals, but the last few years have seen this change. Information about research and clinical trials is now freely available on the Internet; many patients do extensive research on their condition, and many then use this information to make decisions about selecting care and treatment regimes. Many persons newly diagnosed with NF1, or their families, are therefore often well informed when they first reach out to a lay foundation. This places the foundations under more scrutiny than in the past, and the foundation needs to be prepared for this.

Many patients and families are becoming involved in managing their own health care, particularly for rare diseases like NF1 where the clinical progression can be quite complex.

In the Unites States, the Health Insurance Portability and Accountability Act (Patients Like Me) has impacted on the ability of physicians to utilize patient information for research purposes, even though this research might lead to a better understanding of the disease or potential treatments. In contrast, patients themselves can freely share their data. At the Internet site PatientsLikeMe, individuals can share their medical history and treatment regimes and self-aggregate into groups with others who have the same condition. PatientsLikeMe includes clinical resources for patients, and serves as a connector to pharmaceutical and biotechnology companies seeking to learn about specific patient populations.

This evolution, with individuals taking a more proactive role in determining their own clinical care path, is known as "patient power." With the emerging role of genomics in clinical management, direct access to patients themselves will be tremendously valuable and could impact significantly on the rate of research progress. Lay foundations are very aware of this, and some are incorporating these changes into their own programs. For example, traditionally, lay foundations have funded the development of clinical databases which were maintained and held by clinical centers. More recently, there has been a trend instead toward patient-entered registries. The Children's Tumor Foundation will introduce such a registry in 2012. Patient-driven registries remove the restrictions of data collection through the clinic, and are anticipated to lead to faster recruitment of registry participants. This is an example of lay foundations embracing "patient power," and it may also serve as a motivational tool to engage patients more closely with the foundation.

The rise of "patient power" will also impact upon the relationships between NF1 lay foundations and those donating funds for research programs to the foundation, since many new donors wish to be closely engaged in the research that they support. As most lay foundations represent a very broad constituent base, it will be important for foundations to establish boundaries and guidelines for these evolving relationships such that they meet the needs of both the donor and the foundation.

41.4 Conclusion

The NF1 Lay Foundation plays a number of important roles for those affected by this rare disease. These roles include raising and allocating research funding to support NF1 research and clinical advances that will have the most impact on finding treatments for NF1; providing as much information as possible to those affected by NF1 so that they can make informed decisions about matters such as clinical care; and serving as an advocate and broker for NF1 when liaising with other entities that fund NF1 research, can advance the mission of the lay foundation, and ultimately will improve the lives of those with NF1. At its core, the NF1 lay foundation should embrace the role of catalyst, by supporting new ideas and concepts that have the potential to have significant impact on the NF1 landscape.

NF1 lay foundations do need to be aware of the evolution of the way that constituents access information about NF1 and make decisions about fund-raising, and be prepared to evolve with these changes.

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Chapter 42
Social Stigma in Neurofibromatosis 1

Joan Ablon

42.1 Introduction

Few clinicians who see patients with NF1 have the time or occasion to acquire any systematic knowledge of the experiences of the daily lives of their patients or the consequences of these experiences for their emotional well-being. Those scientists who devote themselves to unraveling the intricacies of genetics, molecular biology, and other technical areas around NF1 are even more removed from the life experiences of affected persons. In this chapter, I endeavor to offer a window into some of the personal and family issues around NF1 that affected individuals confront during their life course.

I will focus on one aspect of what Riccardi has called the "psychosocial burden" of NF1: "The obviously adverse affect of NF-1 on the emotional and social life of the patient" (Riccardi 1992, p. 198). Only in the past decade has a burgeoning literature appeared on "quality of life" issues in NF1 (see the review of this literature by Birch and Friedman, Chap. 8). Prior to this time, very few studies explored the psychosocial issues of life of NF1 patients. Messner and Neff Smith (1986) reported that, from an extensive review of the international literature, the adaptations of individuals and families were only "superficially mentioned." In fact, Messner and her colleagues have published most of the rare works surveying the personal emotional adaptations and the manifestations of negative self-image that typically have resulted from societal insults around their condition (Messner and Neff Smith 1986; Messner et al. 1985a, b). Several chapters in *Neurofibromatosis: A Handbook for Patients, Families and Health Care Professionals* (Rubenstein and Korf 1990) present an overview of psychosocial issues while Ablon (1999) has presented the most comprehensive exploration of these issues to date.

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While clinicians may believe that most persons with NF1 are not and will not be severely affected, Benjamin et al. (1993) found that the majority of NF1 patients in their study judged their conditions to be more serious than clinicians had judged them to be. Clinical assessments ordinarily do not factor in the *psychosocial impact* of symptoms such as even quite small external neurofibromas and learning disorders which may or may not be clinically threatening, yet may have broad implications that influence not only experiences of daily life but also major expectations, opportunities, and decisions throughout the life course. Social stigma and rejection appear to extract a far more frequent psychological and social price on subjects than do physical symptoms. Social stigma in all its forms may be a daily life companion for affected persons. Further, the specter of "The Elephant Man," long misdiagnosed as having NF1, haunted many adults throughout their childhood and teenage years.

42.2 The Concept of Stigma

In his classic work, *Stigma*, Goffman (1963) provided the first guide for addressing stigma as a social and cultural phenomenon:

> Society establishes the means of categorizing persons and the complement of attributes felt to be ordinary and natural for members of each of these categories. . .. While the stranger is present before us, evidence can arise of his possessing an attribute that makes him different from others . . . and of a less desirable kind—in the extreme, a person who is quite thoroughly bad, or dangerous, or weak. He is thus reduced in our minds from a whole and usual person to a tainted, discounted one. Such an attribute is a stigma, especially when its discrediting effect is very extensive: sometimes it is also called a failing, a shortcoming, a handicap. (pp. 2–3)

Stigma is a creation and product of society. The definitions as to what constitutes a physically normal or aesthetically acceptable person are arbitrary and are determined by cultural and social contexts. American values include significant and often rigid cosmetic and social prescriptions for "beauty," "ugliness," and "good health." These prescriptions are systematically reinforced by portrayals in the media and through selection in the social, economic, and political dimensions of life. Social success favors individual accomplishment, good physical health, external beauty, and vocational and athletic accomplishments. Negativity toward physically or mentally different individuals who may not be able to meet such prescriptions has been well documented (among many such writings are those of Goffman 1963; Eisenberg et al. 1982; Graham and Kligman 1985; Murphy 1990; Ablon 1998; Hatfield and Sprecher 1986; Grealy 1994). Conditions which result in obvious features considered as disfigurement as is often the case with NF1 may place a serious burden on affected persons. Murphy (1990) stated: "The greatest impediment to a person's taking full part in this society are not his physical flaws, but rather the tissue of myths, fears, and misunderstandings that society attaches to them" (Murphy 1990, pp. 112–113).

Goffman (1963) proposed that visible and invisible stigmas result in their bearers being *discredited* or *discreditable*. For instance, those whose stigmas are physically visible may immediately become discredited, but those who carry stigmas that are not visible during ordinary interaction are potentially discreditable if the stigmatizing features are revealed. When one's stigma is not readily perceivable, the control of information may become a chief issue for the individual. For persons whose NF1 is not readily visible, there still may exist the burden of whether or not to disclose their condition. Some of the most dramatic times for disclosure might be at the development of a new relationship or at a time of first intimacy. Managing a potentially discreditable attribute may be very anxiety-provoking and seriously affect one's definition of self. Quaid (1994) has noted:

> Genetic information is widely viewed as saying something about who the person is at some fundamental, if unarticulated, level. In that sense, especially, people appear to feel stigmatized by exposure of their genetic information, and others might be more likely to stigmatize on that basis. In some cases, even medical professionals exhibit the tendency to treat proven genetic disorders in a manner different from the way they might treat other diseases, with predictable effects on individuals at risk. (p. 6)

Although more information on NF1 is available today than ever before, on the Internet and in various media outlets, it is apparent that the major life issues confronted by children and adults with NF1 have remained the same over the years. Societal reaction to differences perceived as negative is common in most areas of life. For example, note the emergence of the newly named phenomenon of "bullying" and the books, films, and workshops in recent years dealing with this topic. Indeed, a dramatic name is now attached to age-old behavior which negatively challenges differences of many types.

42.3 Methods

The data and statements of affected individuals presented here are based on my qualitative open-ended interviews and attendance at support group meetings in California during the 1990s and follow-up interviews in recent years. I initially interviewed 54 adults with NF1 recruited from three sources (1) the population of persons attending three regional and national support groups in northern California, (2) those who had responded to notices placed in local mailing announcements of these organizations, and (3) the caseloads of the Genetics Departments of two major metropolitan hospitals. I cannot state that the subjects reported on here are representative of all persons affected with NF1, since the exact constitution of the total universe of those persons is unknown. However, I believe that these subjects are a fair representation of many of the thousands who do attend support groups, who receive announcements but do not attend, and who come to hospital programs. It should be noted, however, that these sources may attract affected persons and families who are at the more severe end of the range of affected persons.

In most cases, my interviews were carried out in subjects' homes and in a few instances in restaurants. Thirty-four subjects were interviewed one time; eleven subjects were interviewed twice; and nine others were interviewed three or more times—some five or six times over many years. Materials also were gathered at NF support group meetings and in telephone conversations. Subjects were generous in sharing with me their life experiences and attitudes toward their NF1. Interviews tended to last at least 3 hours, and many were 4 or 5 hours or longer. Many persons stated that they had never before candidly talked to another person about their condition and the ways in which they perceived it had affected their lives. For more detail on the interviews, see footnote[1] (Ablon 1999).

42.4 Stigma in NF1

Person after person spoke about their concerns around the tumors on their bodies, even if not visible when they were clothed. The brutal significance of the presence of NF1 in an affected person's life was well exemplified in this poignant statement of a young man whose numerous small tumors on his handsome face were readily apparent.

> I can deal with the surgeries and the threat of deafening, of blindness and paralysis—that's one thing. But, and it may seem vain, it's the disfigurement that bothers me. Society-wise, that is the most inhibiting thing. If I have surgery once or twice every few years people don't shun me for that. But if you look like you have some horrible skin condition, people just can really be cruel. I've had this fantasy—if I could just have twenty-four hours where I didn't have NF. Just to feel what it's like! Even if no one else could see me, and I just saw myself! It's a strange fantasy. Just to know what I would look like without it! And I try not to think how different my life would be if I hadn't had this. I try not to go on about "what if," because if I do that, I take away from my precious time now.

Despite this man's previous very serious surgeries for large internal tumors in his neck and back, his focus in our interview was on the social rejections he had experienced based on his appearance. His emphasis on appearance was a tragic irony as he died 2 months following our interview of a massive brain tumor which had been undetected.

[1] Research participants were recruited from support groups sponsored by the National Neurofibromatosis Foundation, Inc.—now renamed the Children's Tumor Foundation—and the now historical California Neurofibromatosis Network. Currently, there are also groups supported by Neurofibromatosis, Inc., California. Fifty-four affected adults were interviewed, 32 were women and 22 were men. Fifteen had a parent with NF1. Twenty had affected children. Ten persons were members of ethnic minorities: five Hispanics, three African-Americans, one Chinese, and one Filipino. Ages of subjects ranged from 19 to 70 at the time of the first interview. Educational levels varied from completion of high school to enrollment in doctoral level graduate courses. The occupations of subjects were so diverse that their number almost equaled the number of subjects. Vocations ranged through professionals, laborers, retirees, and persons living on temporary and permanent disability benefits.

42.5 The Chief Sources of Stigma

Vivid portrayals through television, films, and other public media expressing *the restrictive values of culture and society* have often created unattainable standards for beauty and good health. For persons with visible NF1, negative behavior, attitudes, and curiosity may occur in public places such as malls and during the general interactions of daily life. The flamboyance of the misdiagnosed "Elephant Man's Disease" and the haunting image of Joseph Merrick, widely popularized in print, plays, and films (Montagu 1971; Graham and Oehlschlaeger 1992) were very influential in the development of shame and depression in many persons with NF1. Likewise, the florid rhetoric used by doctors who told patients and families that this "rare" and "terrible" disease could likely result in severe disability was an important factor in the development of negative mindsets of many subjects about their condition.

A neurologist who sees many patients with NF1 stated, "It scared the hell out of patients, particularly parents who would go crazy thinking that their child would grow into this monstrosity." Said one genetic counselor, "I think The Elephant Man is the biggest disservice ever done to people with NF1. Everyone comes wondering if they will look like that." Within the context of the unpredictability of the condition, early dire statements of doctors in combination with the media depiction of The Elephant Man greatly contributed to the burgeoning of subjects' fears (Ablon 1995). A woman who described her inability to plan for her educational or vocational future told me, "I see now it was all a big mistake. I was paralyzed for years. I thought I was going to be 'The Elephant Lady.'"

The greatest traumas for parents occurred when the labeling was associated with the first diagnosis. Said one mother:

> When the doctor told me that Karen had the Elephant Man's Disease I fainted dead away on his floor. Then when I told my husband later that day I fainted again.

And another mother:

> I went right to the medical libraries at the University and at Harper Hospital.
> (What was your reaction?)
> Horror, horror.

It is a great misfortune that many doctors have continued even today to tell patients and their families that NF1 is the Elephant Man's Disease despite the fact that the misdiagnosis was clearly and publicly announced in medical journals and even popular literature some 26 years ago (Tibbles and Cohen 1986). Legendre et al. (2011) in a recent study reported that some Canadian doctors continue to confuse the Elephant Man's Disease with NF1. Further, this confusion still occurs in the media, even in a quoted dictionary entry. Stated these authors:

> Confusing NF1 with the Elephant Man's condition harms the interests of those with NF1, all the more so because it is known that NF1 sufferers experience difficulty establishing social

> ties and developing good self-esteem. . . . Having their condition misidentified as the disease the Elephant Man had, and as a result being burdened with others' perceptions that they will grow profoundly disfigured over time, can only serve to increase these difficulties and compromise their hopes of achieving a normal social life, finding employment that interests them, enjoying an enduring romantic relationship, and having children. (p. 113)

Stigma may be created and expressed to persons with NF1 by *family, school and workmates, and medical personnel*. Negative family attitudes can be particularly destructive, creating early poor self-images, and inhibiting the development of self-confidence and positive coping skills. Early school experiences can be likewise destructive. The entrance to school typically constitutes the first major confrontation of children with societal norms and public scrutiny. Taunting and discriminatory and even physical abuse by other children, now often called "bullying" behavior, may suggest to a child a preview of societal behavior to be expected for the rest of his or her life. Likewise, negative attitudes and systematic discrimination *in the workplace* by supervisors and workmates create a demoralizing work context for adults, who may already be hobbled by poor school careers due to learning disorders.

I will emphasize here that *negative interactions with medical personnel* may constitute a major source of stigma. Many persons with NF1 recounted to me statements and attitudes of doctors and other medical personnel that they found stigmatizing, some personnel openly showing disgust at their patients' appearance. One man said he always felt he was a "genetic freak," always open for viewing by students and medical colleagues, then rapidly dismissed because he could not be cured.

Many persons expressed great dissatisfaction with their doctors who they thought commonly showed little interest or concern about their own or their children's medical care. While practitioners have described patients as "compliant," "gentle," "longing for acceptance," and "not embittered by their condition" (e.g., Trevisani et al. 1982), this appearance may be created by patients' passivity based on their perceptions of a lifetime of poor medical care and low expectations for future medical interactions. Further, the effects of learning disorders and other NF1-related stress-creating factors which have adversely impacted their self-image and coping skills have created this passivity interpreted sometimes wrongly by doctors. In fact, in their conversations with me and in support group meetings, affected persons in many cases had very negative views about their care but would not express these openly in medical interactions.

Because of the unpredictability of NF1 and affected persons' fears of the multiplicity of symptoms which could develop in their lifetime, any comments which doctors may have considered as tentative or only possible predictions about their patients' conditions might have become sanctified and haunted patients for years. This hypersensitivity suggests that doctors should be aware of the potency of their statements and the particular need for clear and supportive communications. As an example, several mothers reported that pediatricians had told them that, based on the number of café au-lait spots on their infant, the child *might* have NF1, the Elephant Man's Disease, but since the diagnosis might not be known for several years, they should not worry about it. These mothers left with dread in their hearts that stayed continually with them.

42.6 Impact in Major Arenas of Life

Social stigma may seriously impact affected persons in their educational and economic experiences and through disenfranchisement from normal expectations for intimacy—sex, dating, marriage, and having children.

42.6.1 Education and Employment

Learning disorders, relatively common in NF1, are often devastating for affected persons, setting the stage for poor school careers and discrimination and debasement in school. Adults whose school experiences occurred before the current acknowledgement of widespread learning and attention deficit disorders and the development of special educational programs for these may have been particularly damaged. Many persons were told they were just "dumb." Poor school records and demoralization around school experiences often resulted in severe limitations on opportunities for gratifying or well-paying jobs. When seeking employment, individuals with special health conditions like NF1 may find themselves doubly or triply disadvantaged by their appearance, their medical problems, and the absences necessary for physicians' visits or medical treatments. Many persons I interviewed were meagerly supported by varied programs based on disability. Even individuals who were brought up in middle class or comfortable home environments found themselves as adults existing on "the edge" in marginal living situations.

42.6.2 Intimacy and Marriage

The most poignant and sensitive experiences described dealt with intimate relationships. Rigid societal prescriptions for appearance and accomplishments may discourage potential partners and engender attitudes of low self-worth in affected persons, contributing strongly to negative experiences in this most personal arena of life. Societal prescriptions for beauty and health are often linked to romantic success, despite the fact that relatively few persons in mainstream society are able to measure up to these prescriptions. Those who markedly differ may be overwhelmed by feelings of personal failure and inadequacy.

My study found women were more aggressive in pursuing opportunities for sexual encounters and dating. Men often were more discouraged by their failures and were more likely to withdraw from seeking relationships. For those persons whose NF1 was not readily apparent when clothed, issues around revealing the tumors on their body in intimate situations posed great challenges. Many individuals who did find those special persons who married them faced other challenges about decisions to have children and the possibility of passing on their

condition. Women were more likely to gamble the genetic risks of having children and also the reality that pregnancy often brought out a florescence of new tumors.

The oldest women, although quite visibly affected, were not as obviously bothered by their condition as many of the younger women. They had long and stable marriages with husbands who have accepted their NF1 as an unremarkable part of their physical persons. Younger persons today, both men and women, who are more influenced by the media and the glaring cosmetic prescriptions for beauty portrayed there, are more aware of their differences, and even the presence of a loving, accepting partner may not deaden their pain. In fact, they may be concerned that a spouse would leave them and they would be unable to find another who could tolerate their appearance or medical problems.

42.7 Factors for Successful Coping with Stigma

Interviews I have carried out with persons who have diverse genetic conditions causing physical differences (dwarfism, Ablon 1984, 1988; NF1, Ablon 1999; and osteogenesis imperfecta, Ablon 2010) reveal that in most cases those who cope successfully with stigmatized health conditions exhibit a commonality of biographical features and ideologies:

1. The most important feature is *unconditional family support* and *clear intrafamily communication*. A strong supportive family that instills confidence and security in the individual early in life, and a family system that stands stalwartly by and encourages good coping patterns to emerge after the individual is diagnosed creates a basis for coping with whatever challenges life brings. These families actively and critically seek out the very best medical care available for their children and are always available for practical and emotional support. In periods of ill health, family members accompany children or adults on doctors' visits, enter into decision making about treatment options, and are invariably at their bedsides at the time of hospitalizations. Positive parental communication is of utmost importance. Such communication appears to be critical for individuals whose parents have the same condition. If these parents make every concerted effort to educate their children about their condition and are available to talk about their fears and concerns, expressed or unexpressed, this climate will bode well for more informed and effective styles of coping.
2. *Attendance at NF support groups* has proven particularly helpful for many persons, giving them the rare opportunities to meet others with their condition and to learn about the newest clinical and scientific findings available. In sharing and discussion groups, affected persons have the opportunity to voice their concerns, fears, and problems and are often able to learn from the experiences and strategies of others. At regional and national informational events, speakers are typically clinicians, geneticists, and genetic counselors who can report on the newest treatments and scientific findings; hence, attendees have unique access to a broad range of

specialists in NF1 who can answer their questions about specific problems. Gaining sophistication about available treatment options often allays their concerns, and meeting others who have conquered their own challenges builds self-confidence in the ability to cope successfully with what the future may bring.

3. A third feature that promotes successful coping with stigmatizing conditions is *a philosophy that disavows physical difference as a basis for a stigmatized or lesser identity*. This imbues individuals with the knowledge that having a special health condition like NF1 does not detract from their innate abilities, does not make them lesser persons, and should not in any way automatically disenfranchise them from the expectations and benefits enjoyed by mainstream society. This philosophy underlies the disability rights movement which emphasizes that the locus of the chief problems of "disabilities" and special health conditions such as NF1 lies in the social, cultural, economic, and political systems that deny equal rights and opportunities to affected persons, *not* in personal differences or deficiencies of the individuals. Furthermore, these problems may be better understood as civil rights issues of prejudice toward minorities with differences poorly accepted by society. Persons who have internalized this philosophy may proceed more positively and with more confidence and sense of control because the social and cultural environment, by common effort, can and will be changed. Parents, teachers, work supervisors, and medical personnel who help develop, maintain, and exemplify this philosophy for those persons that society may mark as stigmatized will assure their possibilities for more satisfying and successful lives.

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Chapter 43
Personalized Medicine in NF1

David Viskochil

43.1 Introduction

Personalized medicine can be interpreted as the application of either clinically non-apparent or clinically unrecognized findings that, once identified, can lead to individualized management of a person's medical care. Usually, this concept applies to the population at large, but the establishment of a diagnosis in and of itself can direct medical care in a personalized manner. The diagnosis of NF1 (neurofibromatosis type 1) sets in motion a lifelong surveillance plan to identify medical issues that would not otherwise be anticipated in the general population. Thus, making a diagnosis of NF1 is an example of personalized medicine at its best; it alters the management and directs the care of those who have been diagnosed with the condition. Once diagnosed, the application of the principles driving "personalized medicine" can potentially target care for the individual with NF1.

43.2 Anticipatory Guidance for NF1

One of the most consistent concerns of individuals and families who care for a child with NF1 is when to worry and when to forget about potential medical complications. The full application of the principles of personalized medicine can lead practitioners to rely less on general anticipatory guidance for the NF1 population at large while honing in on medical concerns more likely to affect an individual who may have specific clinical, genetic, and environmental features to assist in the

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Table 43.1 Anticipatory guidance for age-related clinical expression of various NF1 manifestations (Adopted from Viskochil, D. 2010. Neurofibromatosis type 1. In: Management of Genetic Syndromes)

Age-related clinical presentation of specific manifestations anticipatory guidance
Tibial bowing—long-bone dysplasia/pseudarthrosis early childhood
Dystrophic scoliosis—early childhood
Optic nerve pathway tumor—early to mid childhood/ethnic background
Speech and language issues—early to mid childhood
Cognitive impairment—early to mid childhood
Social skills issues—childhood/adolescence
MPNSTs—late adolescence/adulthood
Dermal neurofibromas—late childhood/puberty/adolescence
Manifestations for which we are always on the "look out": plexiform neurofibromas, sarcomas, vascular anomalies, psychiatric issues ill defined

development of a customized health management plan. As an example, Table 43.1 presents a list of anticipatory guidelines for clinical manifestations of NF1 that generally arise at specific ages. Recognition of age-restricted emergence of clinical manifestations can optimize the timing of imaging or diagnostic studies as part of a personalized health-care plan, simply based on age. The variability of clinical expressivity in NF1 makes it difficult to provide more detailed anticipatory guidance. Hence, the development and implementation of markers to presymptomatically identify those at highest and lowest risk to either develop certain manifestations of NF1 or to more reliably map progression of disease could optimize care and potentially save "health-care dollars." Markers of disease in the context of personalized medicine for NF1 include a traditional recognition of genetic markers such as *NF1* genotype, functional polymorphisms, and laboratory tests as biomarkers, but also embrace imaging studies, blood pressure monitoring, clinical examination, and pedigree analysis.

One of the key questions families generally ask at the informing interview for a diagnosis of NF1, and at every visit thereafter, can be stated thus, "Is my child going to have a rough go of it, or can we relax a bit and target our worries to other things?" Regardless of the answer, letting the parents prepare and develop appropriate expectations would go a long way toward defusing the "time-bomb" stress articulated by so many patients and parents affected by NF1. The application of markers of disease to improve the definition of risks for a given individual would alleviate anxiety on the part of parents and health-care providers when confronted with the uncertainty of which clinical manifestations may affect their child: 15 % for optic nerve pathway tumor, 2 % tibial pseudarthrosis, 25 % for plexiform neurofibroma, and 10 % lifetime risk for malignant peripheral nerve sheath tumor. This alleviation of anxiety for the unknown extends to clinical manifestations that involve decisions in care, i.e., when and how often to radiographically image, the timing of therapeutic intervention, the anticipated response to therapy, to name a few.

43.3 The Application of Personalized Medicine

Personalized medicine can be viewed as the application of evidence-based medicine defined as a process in which systematic collection of evidence is critically analyzed before clinical intervention with further assessment of clinical outcome (Willard and Ginsburg 2009). This process is time consuming and takes on average 17 years to be fully implemented in clinical care (Balas and Boren 2000). Thus, a concentrated review of present evidence leading to a successful intervention for a specific manifestation of NF1 would be expected to take over a decade before integration into best practice guidelines. This would primarily apply to clinically evident manifestations. It is more difficult to envisage interventions that might prevent occurrence of such manifestation, and with the broad variability of clinical expression of NF1, it remains difficult to identify those at risk of developing any one of myriad clinical manifestations of this condition. Embedded in the practice of personalized medicine is health risk assessment to determine an individual's potential to develop a disease or in this case a manifestation of the condition (Ginsburg and Willard 2009). This risk stratification can be based on clinical recognition of an associated finding, pedigree analysis, molecular studies (biomarkers), and radiological imaging. When this information is processed through a strategy known as clinical decision support (Osheroff et al. 2007), better health care can be devised for the patient by health-care providers, which is the goal of personalized medicine.

43.4 Applications of Personalized Medicine in NF1 Care

One simple example of the application of personalized medicine in NF1 is blood pressure monitoring. A clinically invisible finding, hypertension in childhood identified by blood pressure monitoring, leads to altered health-care management. If blood pressure were elevated (compared to age, height, and sex) on three separate occasions, then imaging is indicated to identify renal artery stenosis. Thus, basic clinical information leads to effective individualized care. However, the health-care provider must determine the most effective intervention and this is where the full gamut of evidence-based medicine with health risk assessment along with clinical decision support combine to yield a set of potential recommendations as to which intervention to embrace and when. This selection will be based on a number of factors and represents the art of medicine, better still, personalized medicine.

Genotype–phenotype correlation is always an important consideration in the application of personalized medicine and is what most practitioners seem to associate with this over-used phrase. There are a few disease-causing mutations at the *NF1* locus that are predictive of potential manifestations and severity of disease. The whole-gene *NF1* deletion (Kayes et al. 1994; Pasmant et al., 2010) is suggestive of an increased risk for MPNST (De Raedt et al. 2003; Mautner et al. 2010); therefore, increased tumor surveillance is indicated. The 3-bp deletion in

exon 22 (Stevenson et al. 2006) is associated with an attenuated tumor phenotype (Upadhyaya et al. 2007); hence, decreased surveillance would be indicated, and affected individuals embrace a modified reproductive risk counseling session to reflect the lack of more severe clinical manifestations. Germ-line mutations at the *NF1* locus are distributed throughout the 60-exon gene and generally tend to result in haploinsufficiency, thereby explaining the lack of genotype–phenotype correlation in this condition. The exceptions are missense mutations that would be expected to identify specific domains involved in various aspects of neurofibromin modulation of Ras pathway activity. However, even with missense mutations in the same family, there are multiple NF1 phenotypes. Thus, by and large, *NF1* mutation screening has not provided a forum for health risk assessment.

Another application of personalized medicine in NF1 is the innovative approach to risk stratification by an assessment of tumor load accomplished by whole-body MRI (Mautner et al. 2008; Plotkin et al. 2012). This imaging protocol is a biomarker of sorts, providing a handle on subgroups within the NF1 population who may need altered surveillance for the development of MPNST (Mautner et al. 2008), both more and less surveillance, depending on the tumor volume by whole body imaging in NF1 patients. Risk factors for higher tumor volume include the number of subcutaneous neurofibromas and female gender (Plotkin et al. 2012). These data represent an early phase of evidence-based medicine in NF1, and protocol implementation has not been formally incorporated into a broad guideline for care. As clinical centers incorporate whole-body imaging studies into their respective tumor surveillance protocols, the clinical utility of NF1 population screening will need to be assessed. It exemplifies one additional component of the application of personalized medicine; an assessment of health-care costs must be included to ensure a balanced approach to maximizing health care for the individual without overburdening the remaining population with cost.

NF1 is associated with optic nerve pathway tumors, and oncologists recognize that these tumors in the context of NF1 are less aggressive than non-NF1 counterparts. This provides some insight into anticipated outcome as parents deal with the well-established protocols developed for hypothalamic-optic nerve low-grade gliomas. An interesting phenomenon that has not been adequately explained is the low incidence of optic nerve pathway tumors in individuals with NF1 of Japanese ancestry (Niimura Personal communication [~1.5 % instead of ~15 % in European and North American population Rosenfeld et al., 2010]). One can speculate about modifier genes or functional polymorphisms in the Japanese population that, once identified, could enable practitioners to apply health risk assessment algorithms for individualized risk stratification in relation to the development of OPGs, which could alter the surveillance provided by routine ophthalmologic evaluations and brain MRI for the early detection of treatable low-grade optic pathway gliomas. However, this observation of lower Japanese prevalence has not undergone the scrutiny of evidence-based medicine approaches; therefore, altered interventions have not yet been incorporated into anticipatory guidance for NF1.

Cognitive impairment is common in NF1, whereby upwards of 60 % of individuals with the condition have some form of impairment involving

speech–language difficulties, executive function problems, attention deficit, short-term memory difficulties, and problems in social integration skills, to name but a few. This recognition has led to more intense neuropsychology testing and implementation of interventions to help with school performance (Krab et al. 2008). There is great variability in the clinical presentations of cognitive impairment, and strategies for earlier diagnosis would be helpful in the development of school interventions. The use of state-of-the-art imaging such as resting state connectivity by functional MRI (Chabernaud et al., 2012) could be helpful in identifying specific cognitive impairments and response to therapies, both behavioral and medicinal interventions.

Epidemiologic studies have demonstrated associations of NF1 with increased risk for early mortality (Rasmussen et al. 2001), brain cancers (Walker et al. 2006), and breast cancer (Sharif et al. 2007). These reports have undergone evidence-based medicine review and are prime targets for the identification of biologic markers that could identify those with NF1 who might carry a higher risk for the development of MPNST (i.e., whole-gene *NF1* locus deletion), glioblastoma (i.e., functional polymorphisms in the PTEN-AKT pathway), breast cancer (i.e., epigenetic changes), or vascular disease (i.e., circulating markers of inflammation). The identification of biological markers is key to providing more accurate health risk assessment in the NF1 population to presymptomatically identify those who may develop burdensome clinical manifestations. These studies are just beginning, and the process of clinical decision support remains a long way off in the future. Nevertheless, the collection of DNA, serum and urine specimens, along with longitudinal clinical information from registries will lead the way to the application of personalized medicine for individuals with NF1.

It is important to remember that personalized medicine is not always about identifying blood or urine biomarkers or genomic changes, but can simply be clinical recognition of symptoms and signs to identify those who need different care. Rather than focusing our energies entirely on developing screens for tumors, we should also focus on pedigree information, annual medical history including review of systems, physical examination, and documentation of medical outcomes, especially when interventions are applied. Using an innovative approach to predict the presence of internal tumors, Sbidian et al. (2010) identified four variables to specify NF1 individuals with a propensity to develop internal paraspinal tumors; two or more subcutaneous neurofibromas, absence of cutaneous neurofibromas, fewer than six café-au-lait spots, and 30 years of age or younger. This NF1 score may prove useful for a subset of patients in the clinical setting to determine who should undergo closer surveillance for malignancy, and only ongoing collection of outcomes data applied as clinical decision support will alter clinical care management.

43.5 Conclusion

It is a long road, but headway in the application of paradigms in personalized medicine for specific conditions is exemplified in the treatment of asthma as outlined by Poon and Hamid (2012) where endotyping subpopulations within the

asthma population has led to the development of more effective treatments tailored to specific endotypes. As with other medical conditions, best-practice guidelines for NF1 have been developed, and continue to evolve with new knowledge, to steer families and care providers in tailored patient care specified by his or her clinical manifestations and needs. Integrating this with molecular biomarkers will provide an opportunity for biologically based interventions that can speed up the process of applying evidence-based medicine for individualized care.

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Chapter 44
Neurofibromatosis Type 1: Future Directions (Where Do We Go from Here?)

Luis F. Parada

44.1 Introduction

The successful cloning of the NF1 gene by the Francis Collins and Ray White laboratories in 1990 opened a dynamic and exciting era of research in the field of neurofibromatosis Type 1 (Ballester et al. 1990; Cawthon et al. 1990; Wallace et al. 1990). Molecular analysis quickly revealed the presence of sequences predicted to encode a putative enzymatic activity that could explain the role of NF1 as a tumor suppressor (Buchberg et al. 1990). Indeed the large encoded protein, neurofibromin, was found to contain functional Ras GTPase activating domain (GAP), leading to the model that loss of NF1 function would result in loss of negative regulation of Ras protein (Bollag et al. 1996; Martin et al. 1990; Xu et al. 1990). Today, this function of NF1 remains the most thoroughly understood, and may well account for the entirety of its activity.

The numerous chapters in this book are testament to the wide range of vigorous research and to the many advances made in better understanding this pleiotropic disease. On this firm platform, we can look forward to further advances that will add clarity but most importantly, that will have concrete impact on many of the NF1-associated clinical features.

44.2 Neurofibromin

Neurofibromin is an extremely large protein that harbors sequence conservation beyond that of its Ras-GAP domain, across many species, including mammals but extending into zebrafish and *Drosophila* (Bernards et al. 1993; Padmanabhan et al.

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2009; The et al. 1997). This strong sequence conservation has lent support to the notion that the regions outside the Ras-GAP domain retain important functions for overall protein activity. However, efforts to understand potential additional regulatory activities for neurofibromin have met with limited and sometimes controversial outcomes (Johnson et al. 1994; The et al. 1997; Tong et al. 2002). It is clear that our understanding remains insufficient and there is much to be done. The power of genetic studies in *Drosophila* and zebrafish model organisms will likely have much to contribute in this arena. Equally important is the recent successful cloning of the full-length cDNA (Frank McCormick, personal communication). Despite the cloning of the gene 22 years ago, the isolation of the full-length cDNA has been plagued by technical obstacles. As such, direct structure/function analysis of the entire coding region of the NF1 gene has remained impossible. Now, with the availability of a full-length cDNA, such approaches become possible and will certainly lead to important insight into the functions of other neurofibromin domains outside that of its Ras regulatory activity. Whether independent domains have signal transduction capability will finally be experimentally addressed by direct analysis. Certainly, additional important properties for other regions of neurofibromin will be uncovered. For example, there may well be critical protein or lipid interaction domains that may define specificity such as where, when, and how the protein can carry out its functions within the cell. Several effectors downstream of neurofibromin have been reported including the Erk, PI3-Kinase, mTOR, ral, and PKA pathways (Bodempudi et al. 2009; Bollag et al. 1996; Brown et al. 2012; Dasgupta et al. 2005; Johannessen et al. 2005; Klesse and Parada 1998). How is the relative activity of these pathways controlled in different cellular contexts? The same questions must also be resolved for the diverse pathological contexts. Thus, recent advances will permit experimentation that will have profound impact on our mechanistic understanding of neurofibromin within the cell.

44.3 Animal Models

The fact that neurofibromatosis Type 1 is a monogenic disease has greatly enhanced the ease of recapitulation of many aspects of the disease in animal models, thereby providing a distinct advantage over many other diseases and cancers that develop as a consequence of more complex mutation combinations. In a Neurofibromatosis meeting held at the Bambury Conference Center in the mid-1990s, the first reports of successful mouse modeling of NF1 tumors and of development of a conditional knockout emerged (Brannan et al. 1994; Jacks et al. 1994; Zhu et al. 2001). At that meeting, Bruce Stillman, President of Cold Spring Harbor Laboratories, declared that animal modeling of NF1 could easily become the heralding example for successful implementation of genetically engineered mouse models to recapitulate, understand, and cure a human disease. His optimism has certainly proved to have been well placed in that no area of research has more vigorously applied mouse and other species of animal models to understand the breadth and depth of the disease.

In the fruit fly, examples of learning and memory impairment have been developed (Guo et al. 2000; Ho et al. 2007) and the genetics of the system should allow continued penetration into the signaling pathway. Continued advances in these types of studies will contribute significantly. Similarly, the zebrafish is now capable of reproducing NF1-related tumors and is amenable to both genetic studies and, importantly, low- to mid-throughput therapeutic development (Lee et al. 2010). This powerful new tool will provide important contributions to the translational advancement of NF1 research.

The laboratory mouse, however, has repeatedly proved to be the experimental workhorse and merits acknowledgment as a most important contributor in past, present and future NF1 research and discovery. A multitude of laboratories are using mouse models to study a variety of the different pathological manifestations of neurofibromatosis. These include: plexiform and dermal neurofibromas, malignant peripheral nerve sheath tumors, optic gliomas, myelogenous leukemias, learning and memory deficits, bone abnormalities, and more recently, cognitive impairment associated with autism spectrum disorders (Acosta et al. 2006; Bajenaru et al. 2003; Bollag et al. 1996; Cichowski et al. 1999; Keng et al. 2012; Kolanczyk et al. 2008; Le et al. 2004; Silva et al. 1997; Vogel et al. 1999; Zhu et al. 2002, 2005). In addition, the use of mouse genetics has been applied to make entry into understanding modifiers of the *NF1* gene (Amlin-Van Schaick et al. 2012a, b). In the recent past, the successful development of such models has been followed by entry into preclinical studies using the mouse models to develop potentially new therapies (De Raedt et al. 2011; Kalamarides et al. 2012; Kolanczyk et al. 2008; Yang et al. 2008). The congregation of research groups working together to advance translation of NF1 research augurs well for the future. It is fair to say that among NF1 researchers there now exists a collective sense that the next few years will bear witness to enhancement and expansion of recent and current "proof of principle" preclinical studies that have only touched the surface of what promises to be a rapid outgrowth of new and effective therapies. These advances will greatly increase the quality of life of NF1 patients.

Mouse models permit detailed exploration into the natural history of development of the pathological state. In the case of tumors, this is of great utility because knowledge of the identity of the tumor source cells allows for detailed and direct comparison of the genetic and epigenetic changes that take place in tumors over the time of their appearance and progression in pretumorigenic and tumorigenic states. It is these direct comparisons that allow for the possible discovery of gene expression changes that emerge in pretumorigenic and in full-blown tumorigenic cells. These types of studies are naturally impossible in patients or in established cell lines. Such experimental strategies will uncover important new information about the genes and cellular metabolic and signal transduction pathways that are unique to NF1-associated tumors. Identification of these unique pathways will aid in devising targeted therapies aimed at impeding or reversing tumor development with only minimal effects on normal cells. Such studies will impact neurofibromas, optic gliomas, and malignant peripheral nerve sheath tumors, all of which develop in mouse models with remarkable fidelity to the tumors observed in patients.

What about pseudoarthrosis and developmental bone abnormalities, as well as the many lower penetrance aspects of NF1 syndrome? These areas of investigation have lagged behind the tumor research. Nonetheless, successful mouse models have for example, recently been reported for NF1-associated bone abnormalities (Elefteriou et al. 2006; Kolanczyk et al. 2008; Kuorilehto et al. 2004; Wang et al. 2011). The same complexities confronted in the tumor modeling studies will now be faced. Where do the causal mutations that originate the abnormalities lie? Understanding the "cell of origin" will allow for genomic, cellular and molecular comparisons between the normal and pathologic state. By analogy with the more advanced state of tumor studies, such continued studies will help reveal the critical phases of abnormal development and provide insight into therapeutic targets, at which point the mice will again be powerful preclinical tools.

The discussion surrounding NF1 and brain development continues to gain traction. It has been well appreciated that NF1 individuals have susceptibility to intellectual deficits and mood disorders (Acosta et al. 2006; Huson et al. 1988; Hyman et al. 2006; North et al. 1994). However, the underlying neuropathology has remained poorly understood. Apart from some imaging anomalies in NF1 brains, a causal relationship with such anomalies in specific brain regions and particular neuro-cognitive phenotypes remains ill defined. Here, mouse modeling holds great promise. Directed approaches to mutate *NF1* in specific anatomic regions of the brain have already yielded new insights and even possible therapeutic opportunities (Acosta et al. 2006). The ever more sophisticated means to temporally and spatially target loss of NF1 function, coupled with the continually maturing field of mouse behavior and analysis, holds tremendous promise for identifying susceptible cell types, systems, and regions of the brain. These advances will also lead to the execution of well-measured preclinical studies. There is much more to anticipate in this area of research.

In summary, the increasingly sophisticated and advanced physiologically relevant mouse models of diverse aspects of NF1 pathology, coupled with the convergence of clinical and basic research, hold great promise for current and future clinical trials that will enter the arena from far more sound, hypothesis-driven preclinical studies.

44.4 The Clinic

In the past, NF1 patients have relied entirely on the medical expertise, experience, dedication, and compassion of their primary caregivers, physicians, and genetic and psychological counselors. The tangible contributions of basic science to the problem, apart from genotyping and genetic counseling, are only now emerging. However, even to this nonexpert, it seems that the arena of neurofibromatosis patient care must draw substantial benefit from the assembly of basic scientists, clinical researchers, and clinicians that takes place formally among the NF community. These meetings serve to elevate the anecdotal clinical observation to the significant, and conversely to ascribe relative medical relevance and direction to the more basic scientific observations.

A case in point is the recent cooperative effort of NF1 researchers and clinicians to gather, re-examine, and analyze the incidence of autism spectrum disorders in NF1. A committed communal approach to this, spearheaded by Maria Acosta and Susan Huson, among many colleagues in the field, has served to reveal a strong incidence. The concept crystallization of this comorbidity will galvanize new studies in the clinic, and the concerted effort to develop therapies will certainly motivate the mouse modeling community to vigorously join the effort. Such advances continue all along the clinical research arena, from genotype/phenotype studies to the less frequent but nonetheless important cases of mosaicism, and appearance and treatment of less penetrant pathologies. The unity and ongoing dialog across the spectrum of NF1 research from basic to bedside continues to benefit the treatment and quality of life of patients. Ongoing clinical trials for plexiform neurofibromas are well-advanced and other tumor types will soon follow. This generation will experience a profound improvement in the quality of available therapies that will be based on anticipated advances in the molecular and animal modeling studies discussed above.

44.5 Beyond Neurofibromatosis Type 1

Germline mutations in the *NF1* gene result in a variety of defects not the least of which is a cancer predisposition for which it is a widely accepted tumor suppressor gene in the context of neurofibromatosis Type 1. The recent explosion in cancer genomics, largely fueled by the Cancer Genome Atlas Project (TCGA) and supported by animal models (Chen et al. 2012; Llaguno et al. 2008; TCGAR Network 2008; Parada et al. 2005), has moved NF1 into classification as a bona fide, general tumor suppressor gene. The high incidence of *NF1* mutations in idiopathic malignant glioblastoma places it among the most frequently mutated cancer-associated genes in this incurable tumor (Chen et al. 2012; TCGAR Network 2008). In addition, mutations in the *NF1* gene have now been detected in colon cancer, lung cancer, and pheochromocytomas (Bausch et al. 2006; Cacev et al. 2005; Ding et al. 2008). Additional associations are likely to appear as these studies are further refined now that the *NF1* gene can be considered a "usual suspect" cancer gene along with the more famous counterparts such as p53, PTEN, and Ras.

Mouse studies have also revealed and enlightened the complex interactions of the microenvironment with neurofibroma tumor cells (Zhu et al. 2002; Yang et al. 2008). Precisely how mast cells, neurons, fibroblasts, endothelial cells, and Schwann cell progenitors interact to constitute the tumor-promoting environment will be teased out with animal models and cell-based systems. These future advances will provide important insight into new potential therapeutic windows for neurofibroma treatment. The field of cancer and the microenvironment is an area of vigorous current research. It is worth speculating that the many advances in NF1 tumor-related research may also reveal important general features of tumor

cell–stromal interactions that will apply to sporadic solid tumors. Thus, all the lessons learned by the field of NF1 research in the past reach into the future, not only as the continuation of efforts to improve treatments for neurofibromatosis, but also as a broader impact into the molecular classification, prognosis, and improvement of therapies for many forms of sporadic, somatic cancers. The NF1 field has come a long way. It also has a long way to go.

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