ABSTRACTS

Platform & Poster Presentations
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## Poster Abstracts

- Arranged alphabetically by presenting author
- Numbered P1, P2, etc.

## Poster Presentation Times

- **EVEN numbers** (P2, P4, etc):
  - Presenters will be at Posters **Monday 4:30pm - 6:30pm**

- **ODD numbers** (P1, P3, etc):
  - Presenters will be at Poster on **Tuesday 3:00pm - 5:00pm**

***ALL POSTERS WILL BE ON DISPLAY UNTIL TUESDAY EVENING***
Session I
Clinical & Molecular Aspects of NF
*Chair: Gareth Evans, MD*

**Speakers**

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- **Keynote** = Invited Keynote Presentation
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- **Abstract** = Selected from poster submissions to give a talk
William Kaelin, MD

Harvard University

Hereditary Cancer: Lessons from Von Hippel-Lindau Disease

Dr. Kaelin is Professor of Medicine at Harvard Medical School and a Howard Hughes Investigator. His work is focused on laying the foundation for the development of new anticancer therapies based on the functions of specific tumor suppressor proteins. He has made outstanding contributions to our present understanding of the von Hippel-Lindau tumor suppressor protein (pVHL), the retinoblastoma tumor suppressor protein (pRB), and the p53-like protein p73. His participation will bring another perspective to the NF1 and NF2 tumor suppressor proteins.

He received his MD from Duke University Medical School.
NF1 Germline and Somatic Mutation Update

During recent years, new methodological approaches have improved molecular knowledge of the NF1 mutational spectrum, both at the germline and somatic levels. The use of comprehensive overlapping techniques achieves a high mutation-detection rate, providing a general picture of the molecular pathology of the NF1 gene. We present an overview of the different techniques used together with different strategies for routine analysis. At germline level, de novo NF1 mutations arise from different mutational mechanisms, depending on parental origin. Large deletions account for around 5% of NF1 mutations and are predominantly of maternal origin. Most of the remaining mutations, mainly of paternal origin, are point mutations or small del/ins with a low proportion of single exon or multi-exon deletions. In our experience, around 50% of identified mutations affect the correct NF1 splicing. In this sense, it has been hypothesized that differences in the mutation-determined NF1-transcriptional profile could partially explain disease variability among patients bearing the same NF1 splice defect. We studied the NF1 transcriptional profile of 9 NF1 mutations in 30 patients and our results indicate that mutation per se explains the major part of profile variability among the 9 mutations studied (93.5%), while genetic background accounts for a maximum of 6.5%. In addition, an emerging field of study is the analysis through different approaches of a splicing-defect reversion as a therapeutic tool. At somatic level the selective culture of neurofibroma-derived cells facilitates the detection of the somatic mutation. LOH encompassing distinct regions of the NF1 gene and the 17q chromosome, as well as point mutations, have been described. Proportions of each type of mutation and the underlying mechanisms responsible for these are still being investigated. The genetic data obtained from such studies opens the possibility of identifying particular repair mechanisms responsible for the majority of somatic mutations found in the neurofibromas of a given patient, and thus, opening the possibility of identifying those genes that influence neurofibroma number. Lastly, the detection of LOH in neurofibromas could allow the use of this information on somatic mutation to implement indirect genetic testing in sporadic NF1 cases. A field still open to development is the establishment of genotype-phenotype correlation studies that would probably require a multi-centric effort in order to succeed.
Prevalence of Spinal Abnormalities in Neurofibromatosis Type 1

OBJECTIVE: To determine the frequency of spinal abnormalities (abnormal curvature, vertebral anomalies, and disc anomalies) and examine their statistical relationship to tumor formation in neurofibromatosis type 1 (NF1).

SUMMARY OF BACKGROUND DATA: Few data exist about the true prevalence of spinal deformity or spinal tumors in NF1. Whether the spinal deformities of NF1 are caused by adjacent spinal tumors, or are caused independently by disordered embryogenesis remains an open question.

METHODS: NF1 patients (n=344; age range 3-64 years) were reviewed for evidence of spinal abnormality. Of these, 142 had signs or symptoms of spinal pathology, of whom 120 had had sufficient radiological studies for detailed review, and were classified into these categories: abnormal curvature, vertebral anomaly, disc anomaly, canal/cord anomaly, and presence of tumor.

RESULTS:
Type of anomaly: Abnormal spinal curvature, Number: 61, % of overall: 51, % of NF1 population: 21
Type of anomaly: Vertebral anomaly, Number: 64, % of overall: 53, % of NF1 population: 22
Type of anomaly: Disc anomaly, Number: 31, % of overall: 26, % of NF1 population: 11
Type of anomaly: Canal/cord anomaly, Number: 43, % of overall: 36, % of NF1 population: 15
Type of anomaly: Spinal tumor, Number: 78, % of overall: 65, % of NF1 population: 27
Type of anomaly: Spinal tumor plus deformity, Number: 43, % of overall: 36, % of NF1 population: 12
Type of anomaly: Overall spinal deformity, Number: 97, % of overall: 81, % of NF1 population: 37

Half of patients in study had abnormal curvature, and half of those had spinal tumors. An estimated 22% of the total NF1 population had vertebral anomalies, and an estimated 21% had abnormal curvature. Fifty-six percent of those with a spinal deformity did not have an associated tumor. Over one-third of the NF1 population had some type of spinal deformity, and 14% of the population needed spinal surgery.

CONCLUSIONS: Because over half of spinal deformities were not associated with an adjacent tumor (even in adults), it is logical to conclude that NF1 has a direct effect on spine formation in utero and in early postnatal life. The high incidence of vertebral anomalies and abnormal curvature requiring surgical intervention justifies spinal screening for all NF1 patients.
NF1 Is Caused By Neurofibromin Haploinsufficiency

Since its delineation as a distinct entity by von Recklinghausen in 1882, neurofibromatosis has been classified in various ways as our pathogenic understanding has progressed. Neurofibromatosis was initially characterized as a phakomatosis or neurocutaneous syndrome, but this formulation does not fully account for the diversity of features that occurs. Bolande's insight that neurofibromas, café-au-lait spots and some other typical lesions originate in cells of neural crest origin gave rise to the concept of neurofibromatosis as a neurocristopathy, but this description is also incomplete. Identification of the NF1 gene in 1990, recognition that its protein product, neurofibromin, is involved in the ras signaling pathway, and demonstration that loss of both NF1 (or Nf1) alleles can lead to the formation of some human or mouse neoplasms resulted in the characterization of NF1 as a tumour suppressor disease. However, there is now evidence that learning disabilities, short stature, osteopenia, vasculopathy, multiple cutaneous neurofibromas and other characteristic clinical manifestations of NF1 result from neurofibromin haploinsufficiency rather than from complete loss of neurofibromin activity, as suggested by the Knudson hypothesis. The recognition that many characteristic clinical features develop in the context of retained neurofibromin function has profound implications for the prevention and treatment of these disease manifestations in people with NF1.
Molecular and Cellular Anecdotes from Analysis of NF1 Samples: Clues about Mechanisms

Dr. Wallace will provide an overview of data from examples of mutations and cell behavior from a variety of human samples.
Neurofibromatosis type 1 (NF1) is caused by defects in the NF1 tumor-suppressor gene. NF1 is a progressive disorder, notable for its phenotypic variability, with ~50% of patients presenting as de novo cases. By age 2, still ~40% of sporadic NF1 patients do not fulfill NIH criteria and the diagnosis cannot be made based on clinical findings alone. Furthermore, the diagnosis can be difficult in patients presenting with some variant forms of the disease, such as segmental NF, familial CAL-spots-only or spinal NF. A sensitive genetic test can help to define the clinical status of atypical cases and patients who do not yet fulfill the NIH diagnostic criteria, e.g. due to young age. Comprehensive mutation analysis applying RNA-based techniques including long-range RT-PCR and direct cDNA-sequencing, complemented with analysis of total gene deletion and intragenic copy number changes, achieves a mutation detection rate of 95% in familial NF1 patients fulfilling the NIH criteria (Messiaen et al., 2000). We report our experiences in analysis of over 1500 unrelated patients referred for the following reasons: 1) suspicion of NF1 in presence of only one NIH diagnostic criterion; 2) preparation for prenatal/ preimplantation diagnosis; 3) patients fulfilling NIH criteria but either very mildly or very severely affected for a given age. A completed phenotypic checklist accompanying the informed consent and the blood sample was received in >85% of the cases and allowed us to provide an individualized report on the genetic test. We identified a pathogenic mutation in 980 unrelated patients, allowing assembly of an unbiased NF1 mutation spectrum. The majority of mutations are minor lesions, including frameshift, nonsense, missense, splice site. About 2% of the mutations we identified reside deep within large introns and result in intronic sequence exonization. These mutations prove to be extremely interesting as they point to new sequences involved in splicing and further our understanding of the fundamental mechanisms that regulate the pre-mRNA splicing process. Finally, another 2% of mutations are single to multi-exon dosage alterations and ~5% are total gene deletions, mostly 1.2-1.4Mb in size. The mutations lie scattered over the entire 350-Kb gene, but a number of recurrent mutations are known. No obvious genotype-phenotype correlations have been found in NF1 so far, except for the association of the 1.4 Mb microdeletion on 17q11.2 with earlier and more severe manifestation of NF1 signs. Given the complexity and age-dependent nature of the NF1 phenotype, as well as the vast allelic heterogeneity, large cohorts of data are needed to detect additional genotype-phenotype correlations. We will present emerging genotype-phenotype correlations identified in our cohort of patients. Furthermore, the challenges associated with the interpretation of the pathogenicity of some missense alterations will be discussed.
Conservation of hotspots for recombination in LCRs associated with the NF1 microdeletion

In recent years a lot of effort has been put into mapping the variation across the Human Genome. However some of the more complex regions, like Low Copy Repeats (LCRs), have proven difficult to map accurately. This hampered the analysis of recombination rate, gene conversion rate and haplotype diversity in these regions. In a set of nuclear families we genotyped SNPs in the NF1 microdeletion breakpoint regions in 2 copies of the LCRs associated with the NF1 microdeletion and in a third copy on chromosome 19. Comparison of sequence divergence and FISH experiments in primates suggest that part of the NF1 microdeletion associated LCRs originated from the chromosome 19 copy about 8 million years ago.

We analyzed the recombination rate and tested for gene conversion in the three LCRs in the region where the microdeletion breakpoints cluster. We accurately mapped the deletion breakpoints of 60 type I microdeletions (NF1REP mediated deletions) and compared the distribution of the NF1 microdeletion breakpoints with the recombination hotspots determined by SNP analysis. The location of allelic (SNPs) and non-allelic homologous recombination hotspots (deletion breakpoints) are conserved in the three different copies of the LCRs, also in the chromosome 19 copy not involved in non-allelic homologous recombination. These results suggest that, in contrast to what is generally assumed recombination hotspots can be conserved over long periods of evolution.

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Global gene expression comparison of MPNST to Schwann cells reveals a role for TWIST1 overexpression in MPNST cell migration

Malignant peripheral nerve sheath tumors (MPNSTs) are highly invasive soft tissue sarcomas associated with the peripheral nerve that frequently metastasize and do not respond well to standard chemotherapy. Half of all MPNSTs occur in patients with Neurofibromatosis Type 1 (NF1). To identify molecular events contributing to malignant transformation in peripheral nerve, we compared global gene expression of 7 primary normal human Schwann cell samples, 8 MPNST cell lines, and 45 primary MPNSTs using Affymetrix oligonucleotide microarrays. Although MPNST lines are heterogeneous in their in vitro growth rates and exhibit diverse alterations in expression of pRb, p53, p14Arf and p16INK4a proteins, all MPNST cell lines and tumors showed a 159-gene molecular signature distinguishing MPNST cell lines and tumors from normal Schwann cells. We were unable to distinguish NF1-associated from sporadic MPNST. Confirmation of 30 genes from the MPNST signature has been carried out using quantitative real-time PCR. Expression of Schwann cell differentiation markers (SOX10, CNP, PMP22, NGFR) was down-regulated in MPNSTs, while neural crest stem cell markers, SOX9 and TWIST1, were over-expressed in MPNSTs. TWIST1 is a BHLH transcription factor important for neural crest development. Previous studies have implicated TWIST1 in apoptosis inhibition, resistance to chemotherapy, and metastasis in other systems, one or more of which could be relevant to MPNST tumorigenesis and failure of response to chemotherapy. We used TWIST1 siRNA to test relevance of TWIST1 overexpression on MPNST cell function. Reducing TWIST1 expression in MPNST cells using siRNA did not affect apoptosis or chemo-resistance. In contrast, diminishing TWIST1 inhibited cell chemotaxis by 80%. Thus TWIST1 and its transcriptional targets are attractive candidates for therapeutic strategies. We scanned the promoters of all 159 deregulated genes and identified 27 with potential TWIST1 binding sites. Our results highlight the utility of gene expression profiling in identifying genes and molecular pathways that are potential biomarkers and/or therapeutic targets for treatment of MPNST and support the use of the MPNST cell lines as a primary analytical tool.

*Full author list:
Shyra J. Miller¹, Fatima Rangwala¹, Jon Williams¹, Peter Ackerman¹, Sue Kong², Anil G. Jegga¹, Sergio Kaiser², Bruce J. Aronow², Silke Frahm³, Lan Kluwe³, Victor Mautner³, Meena Upadhyaya⁴, David Muir⁵, 5 Margaret Wallace⁶, Jussara Hagen⁷, Dawn E. Quelle⁷, Mark A. Watson⁸, Arie Perry⁸, David H. Gutmann⁵,⁹, and Nancy Ratner¹
¹Division of Experimental Hematology and ²Division of Biomedical Informatics, Cincinnati Children’s Hospital Research Foundation, University of Cincinnati College of Medicine, Cincinnati, OH; ³Department of Neurosurgery, University Clinic Hamburg-Eppendorf, Hamburg, Germany; ⁴Institute of Medical Genetics, University of Wales College of Medicine, Heath Park, Cardiff, UK; ⁵Departments of Pediatric Neurology, Neuroscience, and ⁶Molecular Genetics and Microbiology, University of Florida, Gainesville, FL; ⁷Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA; ⁸Departments of Pathology, Immunology and; ⁹Neurology, Washington University School of Medicine, St. Louis, MO
Molecular Study of Schwannomatosis

Schwannomatosis is a third major form of NF, the molecular basis for which is unknown. Our work is focused on cloning the molecular abnormality causing familial schwannomatosis with the hypothesis that this change will have implications for the more common conditions of sporadic schwannomatosis and sporadic schwannomas, and may interact with the known tumor suppressor genes NF1 and NF2.

We have previously reported the linkage of schwannomatosis to the proximal portion of chromosome 22, excluding the NF2 locus itself. We have identified 17 families in whom schwannomatosis segregates as an autosomal dominant condition with variable expressivity and incomplete penetrance. Within our previously reported candidate region of 5.2 Mb, we have developed 15 highly polymorphic micro satellite markers and reduced the candidate region to 2.5 Mb stretching from the immunoglobulin super locus to the newly developed marker LK507 at 23.908Mb. We have observed that those markers lying within the immunoglobulin super locus often show non pathogenic changes in genomic DNA derived from peripheral blood cells, presumably because of its B cell origin. We have further confirmed this region by verifying that at the embedded marker D22S303 and nearby marker AB05, all families examined share a haplotype amongst known affected individuals.

Because we and others have failed to find gross copy number changes or coding sequence abnormalities within this region, we have turned to a comprehensive sequencing approach. To further facilitate the detection of deletions, duplications or rearrangement which may be invisible in heterozygous sources of material, we have developed four somatic cell hybrid lines consisting of the affected chromosome 22 from three unrelated non founding schwannomatosis patients against two different rodent backgrounds. As controls, we have developed paired somatic cell hybrids consisting of the unaffected chromosome 22 from the same individuals. We have detected a multitude of changes in the telomeric end of the candidate region in one hybrid and its parent cell line due at least in part to the presence of chromosome 22 specific low copy repeats (LCRs) similar to those which drive DiGeorge syndrome and some cases of NF1.

Our current work is focused on identifying additional schwannomatosis kindreds which may further refine our candidate region, developing alterative sources of hemizygous material such as short term tumor cultures to sequence the immunoglobulin region, defining the structure of the LCRs which lie within the candidate region, and continuing efforts at directly sequencing the area.

*Full author list:
Lan Kluwe, Mary Ann Anderson, Andria Balogh, Jyoti Bhatia, Chelsea Boyd, James Gusella, Robert Jenkins, Nancy Ratner, and Mia MacCollin
Molecular genetic testing in NF2: a review

Since the identification of the NF2 gene in 1993, over 1,000 genetic tests have been carried out on NF2 affected individuals worldwide. The mutation detection rate in leukocyte DNA depends on which generation is tested in a family. Using sequence analysis we have identified a mutation in a member of the second generation of families with NF2 in 62/87 (71%), 17/87 (20%) had deletions or duplications detectable on MLPA; in 8 (9%) no mutation was identified. In simplex cases (i.e., a single occurrence in a family) the mutation detection rate is around 60%. About 25-30% of mutations are not detected due to mosaicism [Kluwe et al 2003, Moyhuddin et al 2003]. We have currently identified 59 mosaic patients, half by analysis of tumour, where the mutation is not detectable in blood. I will report the mutational spectrum of 440 patients with NF2 tested in Manchester and Newcastle.
## Session II
### Cell Biology/Signaling Pathways in NF
*Chair: Karen Cichowski, Ph.D.*

### Speakers

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- **Keynote** = Invited Keynote Presentation
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H-Ras, N-Ras and K-Ras are activated by point mutation in many human cancers. These proteins engage downstream effectors that act synergistically to bring about malignant transformation. Of these, the most well known are Raf kinases, the PI 3’ kinases and RalGDS proteins. A large number of candidate effectors have also been identified, many of which may also be involved in cancer and other diseases, but have not yet been validated as true effectors in normal Ras signaling or in pathogenesis. In contrast, activating mutations in B-Raf and PI 3’ kinase in human cancers validates the importance of these effectors. The Ras pathway is also implicated in other diseases, of which NF1 is clearly the best characterized. However, activating mutations in H-Ras have been described in Costello Syndrome and more, recently, activating mutations in B-Raf, MEK1 and MEK2 have been identified in Cranio-Facial Syndrome. Likewise, mutations in PTEN have been well documented in various human syndromes. Analysis of phenotypes associated with these mutations reveals interesting aspects to their role in human development and malignancy. We have generated polyclonal antibodies against recombinant fragments of the NF1 protein neurofibromin, and we are using these to search for binding partners for this large and relatively poorly understood protein. We are also using the power of yeast genetics to identify proteins that regulate neurofibromin: these results will be discussed. In addition, we have identified novel ways in which ras proteins and downstream pathways are regulated. One of these involves interference of Ras signaling by the receptor tyrosine kinase protein EphA2, which is, itself, a target of the Raf-MAPK pathway. Finally, we will describe a novel biological function associated with a cousin of the Ras proteins, M-Ras or R-Ras3. This protein recruits a scaffold protein and a novel phosphatase to the plasma membrane, resulting in activation of Raf kinase through de-phosphorylation at S259. This appears to be essential in the process of signal transduction from receptor tyrosine kinases, and offers new possibilities for therapeutic intervention in cancer and other diseases in which this pathway is activated.

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Frank McCormick, Kate Rauen, Osamu Tetsu, Pablo Rodriguez-Viciana, Madhu Macrae, Vernon Phan and Vivianne Ding. University of California Comprehensive Cancer Center, 2340 Sutter St., San Francisco 94103
Mechanisms underlying NF1-related tumorigenesis and senescence

While the NF1-encoded protein, neurofibromin, is known to critically regulate Ras, we are still elucidating the mechanisms by which NF1 mutations and Ras-dependent signals contribute to the tumorigenic process. In many cases the deregulation of Ras promotes cellular transformation. However, aberrant Ras signaling has also been shown to trigger cellular senescence in vitro, and more recently in vivo in benign human tumors. We have found that loss of NF1 results in dramatically different phenotypes in different cell types. NF1-deficiency immortalizes some cells, but triggers senescence in others. We further found that in cells that are sensitive to “oncogene-induced senescence”, the acute loss of neurofibromin activates a negative feedback signaling network that immediately terminates Ras signaling at many levels. Moreover, this negative feedback program actively participates in the senescence response. Finally, we demonstrate that this negative feedback loop functions in vivo, in a progenitor population of cells that become senescent within benign human neurofibromas. Taken together, these results suggest that senescence is likely to be critical in limiting the progression and growth of benign neurofibromas. In addition, these studies provide mechanistic insight into oncogene-induced senescence and reveal new players that may function in the process of tumor suppression.
Does the Sec14 homology region tell about neurofibromin function?

Using structural biology methodology we have previously identified a novel structural module in neurofibromin that is composed of the predicted Sec14-homology domain and a previously undetected pleckstrin homology (PH) like domain. We will give an update on the characterization of this module and discuss future strategy how to tackle the problems we are confronted with in our efforts to arrive at a full 3-dimensional structure of neurofibromin.
Hyperactivation of p21ras and PI3-K cooperate to alter murine and human NF1 haploinsufficient osteoclast functions

Three recent studies have documented that individuals with neurofibromatosis type 1 (NF1) have a high incidence of osteoporosis and osteopenia. However, understanding of the cellular and molecular basis of these sequelae is incomplete. Osteoclasts are specialized myeloid cells that are the principal bone resorbing cells of the skeleton. We found that Nf1 haploinsufficient (+/-) mice contain elevated numbers of multinucleated osteoclasts in vivo. Both osteoclasts and osteoclast progenitors from Nf1+/- mice were hyper-responsive to limiting concentrations of M-CSF and RANKL levels as compared to WT cells. Nf1+/- osteoclasts have elevated M-CSF mediated p21ras-GTP and Akt phosphorylation which is associated with gains in function in proliferation, migration, adhesion, and lytic activity (pit formation assay). To directly test the role of Class 1A-PI3-K in modulating this gain-in-function in vivo, a genetic intercross of Nf1+/- and Class1A PI3-K deficient mice (p85ÂΔ) was utilized. We found that genetic disruption of p85Δ, the regulatory subunit of PI3-K, restores elevated PI3K activity and Nf1+/- osteoclast functions to wildtype levels. To verify that the observations observed in Nf1+/- mice are recapitulated in the human system, we isolated peripheral blood monocytes, the circulating precursor cell of osteoclasts, and utilized an established methodology to differentiate these cells into osteoclasts. Similar to the murine model, we found that osteoclasts from NF1 patients display elevated Ras-PI3-K activity and increased bone lytic activity. Collectively, these data identify a novel cellular and biochemical NF1 haploinsufficient phenotype in osteoclasts that has potential implications in the pathogenesis of NF1 bone disease.

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Inter-chromosomal Associations with NF1

Gene transcription may be regulated by remote enhancer or insulator regions, which can act over long distances. Chromosome looping provides one structural framework for control, as exemplified by the long-range activity of the ICR of Igf2/H19, which is regulated by the DNA binding protein CTCF. The ICR regulates Igf2/H19 imprinting by interacting with upstream Igf2 DMRs. We devised a novel adaptation of the chromosome conformation capture (3C) technique to reveal other DNA elements, which might interact in the ICR region. We found that in three different cell lines, in addition to the ICR-DMR interaction, the maternal Igf2/H19 ICR on chromosome 7 also associated with Wsb1/Nf1 on mouse chromosome 11. CTCF binding was restricted to the maternal allele of Igf2/H19 and the paternal allele of Wsb1/Nf1. The inter-chromosomal association was confirmed by FISH analysis, which showed that the DNA segments from the 2 chromosomes were closely juxtaposed in the nucleus. Omission of CTCF protein by shRNA or deletion of the maternal but not the paternal ICR on chromosome 7 abrogated this association, and altered Wsb1/Nf1 gene expression on chromosome 11. These findings demonstrate that CTCF mediates an allele-specific association between the maternal ICR of Igf2/H19 and the paternal Wsb1/Nf1 region, perhaps directing these DNA segments to a common transcription-regulating chromosomal hub or factory. We have recently demonstrated CTCF binding sites on human NF1, and have found an interaction of NF1 with IGF2/H19. In addition, additional DNA segments that may interact with NF1 are now being characterized. These findings provide a new model for long-range parental-origin specific associations between gene regions on different chromosomes.

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JQ Ling and AR Hoffman
Strategies to image the development of plexiform neurofibromas in experimental murine models

Mutations in the NF1 tumor suppressor gene cause neurofibromatosis type 1 (NF1), a GTPase-activating protein for ras called neurofibromin. NF1 is a genetic disorder that affects approximately 250,000 individuals in the US, Europe, and Japan alone. Neurofibromas, the hallmark of NF1, are complex tumors characterized by tumorigenic Schwann cells, neoangiogenesis, fibrosis, and degranulating mast cells. Parada and colleagues established a naturally occurring tumor model that closely recapitulates the development of plexiform neurofibromas (Science, 2002). They found that nullizygosity of Nf1 in Schwann cells of conditional knockout mice (Krox20;Nf1flox/flox) was necessary but not sufficient for neurofibroma formation and that haploinsufficiency of Nf1 in lineages within the tumor microenvironment was required for neurofibroma progression. These studies are consistent with the role of the tumor microenvironment in modulating tumor progression in other experimental systems. Collectively, the data provide rationales for the use of experimental therapeutics to target the tumor microenvironment to prevent tumor progression and potentially treat existing tumors. Using proteomics arrays, siRNAs, genetic intercrosses, and adoptive transfer, we and others in the NF research community have identified a number of candidate growth factors, biochemical pathways, and lineages that regulate the haploinsufficient gains-in-function in many of the lineages within the microenvironment. A current challenge in utilizing the Krox20;Nf1flox/- model to test the specific role of these targets in vivo in tumor progression is the ability to detect the development and regression of tumors. Studies in our group have focused on developing experimental methodologies to image the development of plexiform neurofibromas in the context of delineating the role of individual lineages in tumor progression and in evaluating the role of potential key targets for experimental therapies in the clinic. Instrumentation that has been utilized includes a 9.4 T Varian MRI scanner, as well as an EVS-R9 microCT scanner in combination with a positron emission tomography imaging system developed at Indiana University. Initial studies to evaluate the development of plexiform neurofibromas have been in the context of asking an experimental question regarding the role of haploinsufficiency of Nf1 in the hematopoietic system in tumor progression. Cohorts of Krox20;Nf1flox/flox mice were transplanted with Nf1+/+ BM or WT bone marrow cells in the first month of life. Sixteen to twenty mice per cohort were evaluated. By either imaging or at postmortem, tumors were not observed in mice transplanted with WT marrow. In contrast, mice transplanted with Nf1+/− marrow had a high incidence of increased fluorinated deoxyglucose (FDG) tracer uptake beginning at approximately 6 months of age as compared to the surrounding tissue. A high association of localized FDG uptake and the formation of plexiform neurofibromas in that specific region were observed. Similar imaging results were confirmed in Krox20; Nf1flox/- mice. These studies implicate the role of the hematopoietic system in neurofibroma progression and provide the first evidence for an imaging system to detect the genesis and progression of plexiform neurofibromas. This methodology may be useful in strategies to target molecular therapies for the treatment of plexiform neurofibromas.
The neurofibromin GAP-related domain rescues endothelial but not neural crest development in Nf1-/- mice

Neurofibromatosis type 1 (von Recklinghausen's disease) is a common autosomal-dominant condition primarily affecting neural crest-derived tissues. The disease gene, NF1, encodes neurofibromin, a protein of over 2800 amino acids that contains a 216 amino acid domain with ras GTPase activating protein (GAP) activity. Potential therapies for type 1 neurofibromatosis under development and clinical testing target this activity or downstream effectors of ras signaling. Mice lacking the murine homologue (Nf1) have mid-gestation lethal cardiovascular defects due to a requirement for neurofibromin in embryonic endothelium. We sought to determine whether the GAP activity of neurofibromin is sufficient to rescue complete loss of function or whether other, potentially unidentified functions of neurofibromin might also exist. Using cre-inducible ubiquitous and tissue-specific expression, we demonstrate that the isolated GAP-related domain (GRD) rescues cardiovascular development in Nf1-/- embryos, but overgrowth of neural crest-derived tissues persists, leading to perinatal lethality. These results suggest that neurofibromin must possess activities outside of the GRD that modulate neural crest homeostasis, and that therapeutic approaches solely aimed at targeting ras activity may not be sufficient to treat tumors of neural crest origin in neurofibromatosis.

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An amplified EGF-dependent peripheral progenitor is a feature of NF1 mutant nerve

Neurofibromatosis type 1 (NF1) predisposes to benign, incurable peripheral nerve tumors. We hypothesized that expansion of a progenitor population in Nf1 mutant peripheral nerves contributes to tumorigenesis. To test this hypothesis, we studied progenitor cells from Nf1 mutant mice. We identified an embryonic dorsal root ganglia (DRG) derived cell population, which is expandable as EGF-dependent self-renewing spheres; loss of Nf1 is associated with an increase in sphere formation. Spheres from both wild type and Nf1 mutant DRG contained cells capable of glial differentiation. Spheres from Nf1 mutants contained cells with increased propensity to form neurons in vitro when compared with wild type cells. An EGFR+;p75+ cell population was previously isolated from Nf1-/- DRG cultures; here we show that these cells (Nf1-/- TXF) can be propagated as spheres at greatly (20X) increased efficiency. Consistent with a progenitor phenotype, Nf1-/- TXF cells showed migratory characteristics of neural crest stem cells in a chicken xenograft model and expressed markers (as assessed using Affymetrix GeneChips) of neural crest and at least three crest derivatives: neurons, Schwann cells, and melanocytes. Knockout of Nf1 at the embryonic Schwann cell stage of development, but not earlier or later, generated this progenitor like population, implying that loss of Nf1 at a critical stage in development causes an expansion of progenitor cells. Nf1+/- but not wild type perinatal mouse nerves contained cells expressing EGFR and p75, suggesting abnormal persistence of the progenitor population. We also prospectively identified EGFR+;p75+ cells by FACS analysis of human neurofibromas, and have derived spheres from primary neurofibroma cells in the presence of EGF. These data support the hypothesis that an EGFR+ neural progenitor is amplified in Nf1 mutants and is relevant to peripheral nerve tumorigenesis in NF1.

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DNA Repair Capacity in Schwann Cells and Sarcoma Lines Isolated from Nf1-Deficient Mice

Neurofibromatosis type 1 (NF1) exhibits variable expressivity, with both intrafamilial and interfamilial differences in phenotype and disease severity. Wiest and colleagues (2003) analyzed somatic mutation events in multiple neurofibromas, and proposed that DNA repair genes may act as modifiers of the NF1 phenotype. De novo cases of NF1 in children with inherited deficiencies in mismatch repair (Wang et al., 1999), and reduced tumor latency in Nf1+/- mice that carry targeted mutations in the Mlh1 gene (Gutmann et al. 2003), support the idea that genomic integrity influences NF1 disease outcome and severity. Moreover, Nf1+/- mice are more likely to develop therapy-induced malignant neoplasms (Chao et al., 2005), indicating that both sensitivity to DNA damage and DNA repair capacity in affected cell lineages may be important considerations in developing treatments for NF1-associated tumors.

We have used the Stratagene lacI transgenic (Big Blue) mouse to measure spontaneous mutant frequencies in vivo, and to show that a mild mutator phenotype arises in the neural crest-derived tumors and normal tissues of cisNf1+/-;p53+/- mice (Garza et al., 2006). To begin to characterize genomic instability in Nf1-deficient Schwann cells specifically, we performed single-cell gel electrophoresis (comet assay) to compare sensitivity to gamma radiation in embryonic Schwann cells, and in cell lines derived from cisNf1;p53 sarcomas. We observed a range of sensitivities for the Nf1- and p53-deficient sarcoma lines, with late passage (> 20) cells exhibiting greater sensitivity to gamma radiation-induced DNA damage than early passage cells. One sarcoma line tested to date was also delayed in repairing double strand breaks.

To identify pathways and genes that may act as modifiers of the NF1 phenotype, we crossed our cisNf1;p53 mice with animals deficient for a gene, Mrg15, encoding a chromodomain protein that promotes cell cycle progression (Tominaga et al., 2005), and that participates in histone modification during DNA repair (Kusch et al., 2004). cisNf1+/-;p53+/-;Mrg15+/- mice exhibit a slightly reduced tumor latency, and sarcoma lines derived from these animals proliferate more slowly, when compared to the original cisNf1;p53 mice and sarcoma cells. In addition, sarcoma lines derived from Mrg15-deficient mice are significantly delayed in repair of double strand DNA breaks induced by gamma radiation. Our results are consistent with the hypothesis that differences in the maintenance of genomic integrity contribute to variations in NF1 phenotype.

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Clinical trials for NF1 are complicated by the chronic, variable, and unpredictable nature of this disease. This has motivated our work to identify genetic modifiers of NF1 severity. Also motivating this search is the hypothesis that genetic modifiers control rate-limiting steps during disease development, and as such may represent good therapeutic targets. To identify modifiers we pursued a classical genetic study in Drosophila, and a probabilistic gene association study in patients. Recently, we also embarked on a combination of RNAi and small molecule synthetic lethal screens to identify specific vulnerabilities of NF1 and NF2 deficient cells. The design of the latter study and preliminary data will be presented. Our probabilistic genetic study aims to identify genetic variants associated with higher or lower than average dermal neurofibroma burden. This project consists of patient recruitment, bioinformatics, genotyping and analysis phases. To support this work we created a relational database that links all human genes to their orthologs in mice, flies, nematodes and yeast. The database also documents >180,000 interactions between genes, and was generated to facilitate the identification of candidate modifiers as well as for data handling purposes. While our original plan was to genotype individual SNPs in candidate modifiers, this approach is being superseded by less biased approaches to detect association between phenotypes and genome wide SNP haplotypes. Theoretical and practical issues with such studies will be discussed. Finally, we will summarize our latest Drosophila findings, which, contrary to results by others, continue to implicate defective GAP activity as the proximal defect responsible for NF1 symptoms.
# Session III
## Cell Biology/Signaling Pathways in NF
*Chair: Vijaya Ramesh, Ph.D.*

### Speakers

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- *Keynote = Invited Keynote Presentation*
- *Speaker = Invited Speaker*
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The Role of the Nutrient Input in TSC1/2-Rheb Mediated mTOR signaling

Recent studies have shown that many of the effects of loss of NF1 function on cell growth may be elicited through the TSC1/2-Rheb axis to mTOR and S6K1. Counter to prevailing views, recent studies from our laboratory showed that nutrient, such as amino acids (AAs), input to the mTOR/S6K1 signaling pathway is not mediated by either the tumor suppressor TSC1/TSC2 or its target, the protooncogene Rheb. In the absence of TSC1/TSC2 we found that S6K1 activation is elevated and refractile to mitogen stimulation, such as insulin, but can still be regulated by AAs. However, this is not the case for Rheb as siRNA knock-down of Rheb protein levels blocks both the insulin and AA input to S6K1. Nonetheless, withdrawal of AAs, which triggers S6K1 inactivation, has no effect on elevated Rheb-GTP levels, leading to the hypothesis that Rheb-GTP is necessary but not sufficient to drive S6K1 activation in the absence of AAs. These findings suggested that the AA input to S6K1 resided on a parallel pathway to that of the TSC1/2-Rheb axis. As earlier studies demonstrated that wortmannin, a class 1 PI3K inhibitor, blocks AA-induced S6K1 activation and AAs do not induce PKB activation, this suggested that a novel wortmannin sensitive signaling component was responsible for mediating the AA input to S6K1. These observations led us to class 3 PI3K, or hVps34, as the novel target by which these responses were mediated. In brief, ectopic expression of hVps34 drives S6K1 activation, but only in the presence of AAs, and this effect is blocked by siRNAs directed against hVps34. Moreover, stimulation of cells with AAs increases hVps34 activity as measured by the production of PI3P, the product of hVps34. PI3P mediates the recruitment of proteins containing FYVE or Px domains to endosomal membranes, with PI3P rich micro-domains acting as signaling platforms. Consistent with hVps34 mediating the AA input to S6K1, this response is attenuated by expression of a cDNA containing two FYVE domains, which bind to PI3P and block binding of proteins having either FYVE or PX domains, preventing S6K1 activation. Obviously, this novel pathway could have a large impact in stratifying NF1 patients.

George Thomas, Ph.D. is with the Genome Research Institute, University of Cincinnati, as Deputy Director of Research and holds the John and Gladys Strauss Endowed Chair in Cancer Research. He has made seminal contributions in elucidating the roles of S6K1 and S6K2 kinases in cell growth and identifications of the upstream signaling components. He is a pioneer in the field of mTOR/S6K1 signaling pathways. Given the recent finding that mTOR signaling is activated in NF1 tumors, Dr. Thomas’ research and participation will be valuable for the NF research investigators. Prior to his current position he was with Friedrich Miescher Institute for Biomedical Research (FMI) in Basel, Switzerland latterly as a Senior Group Leader. He received his Ph.D. from University of California, Santa Cruz.
The role of Merlin and the ERM proteins in membrane organization

Most tumor suppressors function intracellularly to control the cell division cycle, however, the interface between a cell and its environment also plays a central role in tumor development and metastasis. Indeed, from its primary location at the membrane:cytoskeleton interface, Merlin is poised to function in modulating the transmission of mitogenic signals from the extracellular environment. Merlin and the related ERM (Ezrin, Radixin, Moesin) proteins are thought to provide regulated linkage between the membrane and actin cytoskeleton, thereby organizing cortical domains that interface with the extracellular environment. We previously demonstrated that Merlin mediates contact-dependent inhibition of proliferation by organizing or stabilizing cell:cell junctions. In subsequent work we found that Merlin coordinates the process of adherens junction stabilization with concomitant negative regulation the Epidermal Growth Factor Receptor (EGFR) by restraining the EGFR into a membrane compartment from which it can neither signal nor be internalized - upon cell:cell contact. This activity requires precise compartmentalization of Merlin itself into a defined membrane compartment. As a consequence, physiologic EGFR activation persists in confluent \( \text{Nf2}^{-/-} \) cells, driving their continued proliferation - a situation distinct from that caused by oncogenic EGFR mutations. Our results suggest that excess EGFR signaling is critical for the hyperproliferation of \( \text{Nf2}^{-/-} \) cells and tumors and reveal a novel mechanism of tumor suppressor action. Indeed, specific EGFR inhibitors such as Gefitinib (Iressa) block the proliferation of \( \text{Nf2}^{-/-} \) cells, suggesting a therapeutic strategy for \( \text{Nf2}^{-/-} \)-mutant tumors. Our studies indicate that this model of Merlin function applies to several different cell types, including Schwann cells.

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Merlin/NF-2 in Contact Inhibition of Growth and Tumor Suppression

The cadherins mediate cell-to-cell adhesion through homophilic binding and their engagement results in growth arrest. Cancer cells are refractory to this contact inhibition of growth. We are using biochemistry and optical imaging to examine the signaling mechanisms underlying contact inhibition. We have observed that activation of PAK is sufficient to release normal endothelial cells from contact inhibition. PAK rescues endothelial cells from contact inhibition by phosphorylating and, hence, inactivating the tumor suppressor Merlin. Biochemical and imaging studies indicate that de-phosphorylated Merlin mediates contact inhibition of growth by suppressing recruitment of Rac to matrix adhesions. We suspect that inhibition of Rac is only one of multiple functions of Merlin. To understand how Merlin inhibits cell proliferation, we are using the Tandem Affinity Purification (TAP) system and the yeast two-hybrid system to isolate proteins interacting with Merlin and RNA interference to examine their function.
Upregulation and random activation of Cdc42 and Rac 1 leading to an increase of actin remodeling and protrusive structures in human primary schwannoma

Schwannoma are the hallmarks of the inherited cancer disease neurofibromatosis type 2 (NF2). These benign tumours lack both alleles for the tumour suppressor merlin, a cytoskeleton-membrane linker.

We use a model system of NF2 that is based on human primary Schwann and schwannoma cells.

RhoGTPases are involved in signalling to the cytoskeleton and cycle between an active GTP-bound and an inactive GDP-bound state. Previous studies of our group show that human primary schwannoma cells have an upregulation of active Rac1, a RhoGTPase that is responsible for lamellipodia formation, and that Rac1 can be found predominantly at the membrane, especially in actin rich protrusions.

Scanning electron microscopy now shows that the spindle-like Schwann cells have a bipolar phenotype whereas schwannoma cells are flattened and exhibit multiple protrusive structures like lamellipodia, ruffles and filopodia. Initially, immunocytochemical staining showed that Rac 1 is randomly activated throughout the whole cell periphery what is a good explanation for the multiple lamellipodia and ruffles found in schwannoma cells. We further investigated the role of Cdc42, the RhoGTPase that regulates the formation of filopodia, in the altered morphology of human primary schwannoma cells. To measure the levels of active Cdc42 we performed a pull down assay and detected an upregulation of active Cdc42. Moreover immunocytochemical staining displayed Cdc42 at the membrane all around the cell periphery and colocalises with one of its effectors phospho-PAK 2, which indicates again, that Cdc42 is activated. Phospho-ERM proteins, characteristically also involved in the formation of protrusive structures could be detected in filopodia and lamellipodia as well.

Moreover we investigated actin dynamics by transfecting pActin-EGFP into human primary Schwann and schwannoma cells and examined them by confocal life cell imaging to see the effects of an upregulation of Rac1 and Cdc42 towards actin remodelling. Schwann cells display slow actin dynamics. Schwannoma cells however, have a less coordinated, more disorganized actin dynamic especially as they show more lamellipodia with fast-moving ruffles and filopodia.

Altogether we show that human primary schwannoma cells have random activation of Rac1 as well as Cdc42 associated with increased levels of actin remodelling leading to augmented lamellipodia and filopodia formation.
Marianne James, Ph.D.*
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Modeling NF2 with human arachnoidal and meningioma cell culture systems

Meningiomas arise from arachnoidal cells of the meningeal membranes covering the brain and spinal cord and account for approximately 25% of all primary intracranial neoplasms in adults. Although slow growing and generally benign, these tumors cause significant morbidity because of their location or involvement with crucial cranial nerves or blood vessels. Mutational inactivation of the neurofibromatosis 2 (NF2) gene encoding the tumor suppressor protein, merlin, is common in NF2-associated meningiomas, as well as in approximately 60% of sporadic meningiomas. While the exact mechanism of merlin’s growth suppressive function is unclear, its similarity to the ezrin-radixin-moesin (ERM) family of plasma membrane-actin cytoskeletal linkers suggests involvement in actin cytoskeletal-based cellular functions. We show that meningioma cells lacking merlin display aberrant actin cytoskeletal organization and deregulation of cell-cell interactions. Using patient-matched meningioma and normal arachnoidal cell culture systems, together with merlin RNAi-suppressed arachnoidal cells, we identify defects in intercellular junction sites accompanied by reduced expression of core intercellular junction components. Although loss of cell contact inhibition of growth occasionally marks merlin-deficient cell cultures, merlin gene silencing did not dramatically alter initial rates of arachnoidal cell proliferation. Furthermore, during the later stages of cell culture maintenance, a premature onset of cell senescence typically is observed, reminiscent of the growth pattern that distinguishes merlin-deficient meningioma cells from normal arachnoidal and merlin-expressing meningioma cells. Together, these studies demonstrate the importance of implementing NF2 target cell types to provide insight into merlin’s tumor suppressor function(s).

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The puzzle of merlin functions in Schwann cells: putting the pieces together

Although the NF2 gene was cloned more than ten years ago, the molecular mechanisms by which its product, the merlin protein, controls cell proliferation remain largely unknown. By means of an innovative technique of cell sorting developed in our laboratory (1), we have been able to investigate the functional consequences of NF2 inactivation in primary Schwann cells (SC), one of the major cell types involved in NF2 tumorigenesis. Primary Schwann cell cultures were derived from mice harbouring a conditional Nf2 allele (2) that was subsequently inactivated by adenovirus-mediated expression of the Cre recombinase. Following loss of merlin expression, three major cellular phenotypes were observed. Firstly, the morphology of Schwann cells changed from mostly bipolar to a more fibroblastic type with a disappearance of the cortical actin cables. Secondly, Nf2-/- cells displayed increased saturation density due to a loss of contact inhibition of proliferation. Finally, in contrast to other primary cell types, loss of NF2 readily induced immortalization of primary Schwann cells in culture. Moreover, upon somatic loss of p21cip we observed subsequent clonal SC transformation. At the molecular level, loss of merlin induced accumulation of numerous proteins at the cytoplasmic membrane. Notably, we identified several receptor tyrosine kinases, including several members of the ErbB family that are essential for Schwann cell proliferation. Expression of a constitutively active ErbB2 receptor induced immortalization and transformation of rat primary Schwann cells (3). We found that reduced levels of ErbB ligand (heregulin) in the culture media restore contact inhibition in Nf2-deficient Schwann cells suggesting that loss of contact inhibition arise primarily from increased levels of ErbB receptors at the membrane. Cortical actin cytoskeleton integrity is essential for vesicular trafficking in the cell. Thus, cortical actin disruption upon loss of merlin expression could alter trafficking and membrane localisation of many cellular proteins implying that actin binding is necessary for merlin growth and tumor suppressor activity. To test this hypothesis, we have generated a merlin mutant that does not bind to actin but maintains membrane localization. As postulated, reintroduction of this mutant merlin in Nf2 deficient SC does not restore wild-type morphology and contact inhibition. Finally, in a striking similarity to the observations made in mouse Schwann cells, we found significantly increased amounts of the corresponding ortholog membrane proteins in 8 human schwannomas when compared to 3 normal human nerves. These results demonstrate that similar mechanisms of NF2-mediated tumor suppression operate in mouse and human SC. Together our work enlightens the mechanisms by which merlin controls proliferation in SC cells. It should also facilitate a more focused search for potential therapeutic targets for the treatment of neurofibromatosis type 2.

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Inhibition of MYPT-1-associated PP1 by CPI-17 prevents activation of the tumour suppressor protein merlin and causes cellular transformation

Tumour suppressor protein merlin (encoded by the neurofibromatosis type 2 gene NF2) is an important regulator of proliferation in many cell and tissue types. Merlin is activated via dephosphorylation at serine 518 (S518), which occurs upon serum withdrawal, cell-cell or cell-matrix contact. The relevant phosphatase that activates merlin’s tumour suppressor function is however unknown. Here we identify this enzyme as the myosin phosphatase (MYPT-1-PP1?). The cellular MYPT-1-PP1?-specific inhibitor CPI-17 as well as downregulation of MYPT-1 by siRNA blocked the dephosphorylation of merlin and promoted Ras activation. CPI-17 induced transformation of NIH3T3 cells, which could be reversed by the expression of constitutively active merlin-S518A showing that merlin is the decisive substrate of MYPT-1-PP1? in tumour suppression. In addition we could show that CPI-17 is elevated in several human tumour cell lines. siRNA induced downregulation of CPI-17 in two of the human tumour cell lines tested abolished the transformation phenotype in vitro and inhibited Ras dependent signalling. Taken together we identified a novel cascade of tumour suppressors whose action can be hindered in two ways, by mutation of the NF2 gene or upregulation of the novel oncoprotein CPI-17.
Merlin and Expanded cooperatively regulate receptor endocytosis and signaling

The precise coordination of signals that control proliferation is a key feature of growth regulation in developing tissues. While much has been learned about the basic components of signal transduction pathways, less is known about how receptor localization, compartmentalization, and trafficking affect signaling in developing tissues. Here we examine the mechanism by which the Drosophila Neurofibromatosis 2 (NF2) tumor suppressor orthologue, Merlin (Mer), and the related tumor suppressor expanded (ex) regulate proliferation and differentiation in imaginal epithelia. Merlin and Expanded are members of the FERM (Four-point one, Ezrin, Radixin, Moesin) domain superfamily, which consists of membrane-associated cytoplasmic proteins that interact with transmembrane proteins and may function as adapters that link to protein complexes and/or the cytoskeleton. We demonstrate that Merlin and Expanded function to regulate the steady state levels of signaling and adhesion receptors, and that loss of these proteins can cause hyperactivation of associated signaling pathways. In addition, pulse-chase labeling of Notch in living tissues indicates that receptor levels are upregulated at the plasma membrane in Mer; ex doubly mutant cells due to a defect in receptor clearance from the cell surface. We propose that these proteins control proliferation by regulating the abundance, localization and turnover of cell surface receptors, and that misregulation of these processes may be a key component of tumorigenesis.
The tumor suppressor genes NF2/Merlin and Expanded act through Hippo signaling to regulate cell proliferation and apoptosis

Merlin (Mer), the protein product of the Neurofibromatosis type-2 (NF2) gene, acts as a tumor suppressor in mice and humans. Mer is an adapter protein with a FERM domain that targets Mer to the plasma membrane and it is thought to transduce a growth regulatory signal. Despite considerable interest, the pathway through which Mer acts as a tumor suppressor is poorly understood. The function of Mer as a negative regulator of growth is conserved in Drosophila, where it functions together with Expanded (Ex), a related FERM domain protein. To define the functions of Mer and Ex in growth control and to identify the pathway downstream of Mer and Ex, we started by analyzing the phenotypes of mer;ex double mutant clones during development. We found that the phenotypes of mer;ex double mutant tissues are strikingly similar to those of mutations in hippo (hpo), warts (wts) and salvador (sav), components of a newly identified tumor suppressor pathway. We found that Mer and Ex, like Hpo pathway components, are required for cell cycle arrest and developmental apoptosis. Mutations in all these genes cause outgrowths of diverse adult structures due to extra cell proliferation and resistance to apoptotic stimuli that normally eliminate extra cells. This combination of phenotypes is not observed for mutations in other known growth control genes, and thus suggested that Mer and Ex act in the Hpo signaling pathway. We tested this hypothesis by performing genetic and biochemical epistasis experiments and found that Hpo and Wts act downstream of Mer and Ex. Taken together, our data place Mer and Ex upstream of Hpo and identify a pathway through which Mer and Ex act as tumor suppressor genes.
Session IV
Disease Models
Chair: Kevin Shannon, MD

Speakers

Venkatesh Murthy, Ph.D.  Keynote  T30
Luis Parada, Ph.D.  Speaker  T31
Mateusz Kolanczyk, Ph.D.  Abstract  T32
Vernon Phan  Abstract  T33
Karlyne Reilly, Ph.D.  Abstract  T34
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David Gutmann, MD, Ph.D.  Speaker  T37
Marco Giovannini, MD, Ph.D.  Speaker  T38
Laurence Goutebroze, MD, Ph.D.  Abstract  T39

Keynote = Invited Keynote Presentation
Speaker = Invited Speaker
Abstract = Selected from poster submissions to give a talk
Synaptic homeostasis

Neural circuits and their elements adapt to changes in their environment. One particularly adaptable element is the synapse, which is thought to be modified in many forms of learning. In recent years, however, there is an increasing realization that modification of synaptic function may also occur in, or even underlie many forms of nervous system disorders. A form of adaptation called homeostatic synaptic plasticity has become a focus of increasing attention in both basic and clinical neuroscience. Here, perturbations in overall activity in a network trigger compensatory changes in synaptic strength over a period of days. For example, a severe reduction in neural activity triggers an increase in excitatory synaptic strength and a decrease in inhibitory synaptic strength. In our laboratory, we use cell biological and biophysical tools to understand the cellular and molecular mechanisms that underlie synaptic homeostasis. In my talk, I will present some of our findings and discuss their potential relevance to nervous system disorders.

Venkatesh Murthy, Ph.D. is a Morris Kahn Associate Professor of Molecular and Cellular Biology at Harvard University, Cambridge, MA. He is a leader in the research area devoted to understanding the basic mechanisms of synaptic transmission and plasticity. The questions that he tries to address such as “how do neurons in the brain communicate with each other and how learning modify this communication?” will no doubt be valuable for understanding the learning deficits associated with NF1 children. As a basic researcher, he will be able to provide thoughtful discussions that could be very relevant for NF researchers.
He received his Ph.D. in Physiology & Biophysics at the University of Washington, Seattle.
Luis Parada, Ph.D.
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Modeling NF1 in Mice
NF1 is a Primary Bone Dysplasia - Evidence from Prx1-Cre Conditional Knockout

Neurofibromatosis type I (NF1) is a multisystem disease caused by mutations in the neurofibromin (NF1) gene. The skeleton is frequently affected in NF1, and bone abnormalities are present in approx. 50% of patients. Research into a possible role of neurofibromin in skeletal development/maintenance was hampered by the lack of an appropriate animal model. Inactivation of Nf1 in mice results in a lethal phenotype at a time point when the skeleton has not sufficiently developed, whereas Nf1+/− do not have any apparent skeletal problems. In a collaborative effort with Dr. Luis Parada (UT Southwestern, Dallas) we surmount shortcomings of a direct gene inactivation through conditional NF1 knockout in the limb bud mesenchyme. Our preliminary data indicate Prx1-Cre mediated NF1 inactivation results in a short limb dwarfism with characteristic tibia bending similar to the one observed in NF1 patients. Resulting phenotype shares some similarities with achondroplasia observed in transgenic mice expressing a constitutively active form of FGFR3 (ColII-FGFR3ach). Histological analysis reveals a reduction in the size of the prehypertrophic and hypertrophic zone as compared to littermate controls. In addition, postnatal formation of the secondary ossification centers in the epiphyses of the proximal tibia and distal femur of Prx1-CrexNF1-KO mice is delayed. Furthermore, similar to the activating FGFR3 mutation, NF1 inactivation results in down-regulation of Indian Hedgehog (Ihh) growth plate expression. In conclusion our data support the hypothesis that bone pathological changes in NF1 patients are a result of the intrinsic bone defect rather then compression and/or infiltration of bone by nearby located tumors. Importantly, Prx1-Cre mediated NF1 inactivation provides information on NF1 function in early stages of skeletal development and in the cartilage which can not be adressed using other, existing conditional NF1 knock-out mice. Based on the presented mouse model we aim to trace a detailed picture of the molecular and cellular events that account for the specific defects in cartilage and bone, resulting in malformed skeleton typical of NF1 disorders.
Neurofibromatosis type 1 is a genetic disorder with an incidence of 1 in 3500. The NF1 gene encodes the neurofibromin protein, which negatively regulates Ras activity through its GTPase activating protein (GAP) domain. Neurofibromin is a large protein, and mutations outside the GAP domain have been reported in NF1 patients; therefore we believe there are other cellular signals that interact with neurofibromin to regulate Ras signaling. The yeast S. cerevisiae has two neurofibromin homologs, Ira1 and Ira2. Similar to mammalian cells, deletions of the IRA genes result in hyperactive Ras signaling pathways. Using the yeast S. cerevisiae for a genetic screen, we have identified Ypt7, homolog of mammalian Rab7, as an effector protein that positively regulates neurofibromin activity. Ypt7 is a small GTPase belonging to the Ras protein super-family. Over-expression of Ytp7 in an ira1-deletion yeast strain rescued the temperature sensitive phenotype caused by hyperactive Ras. Further genetic epistasis studies in S. cerevisiae revealed that Ypt7 is acting upstream of Ira1 and Ira2, and Ras2 signaling pathways. Furthermore, in yeast strains that have both YPT7 and IRA1 genes deleted, cells grow poorly under normal conditions and are more sensitive at high temperature when compared to wild type, ypt7- or ira1-deletion yeast strains alone. Thus, these data strongly suggests that Ypt7 is acting upstream of Ira1, Ira2 and Ras2 proteins and that gain of Ypt7 functions is sufficient to compensate for the loss of one of the Ira protein in yeast to down-regulate hyperactive Ras activities.

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Nerve Sheath Tumor Resistance QTLs 1 and 2 Increase Tumor Resistance by Different Mechanisms in a Mouse Model of Neurofibromatosis Type 1

The purpose of this study is to identify genes responsible for variable susceptibility to tumors in neurofibromatosis type 1 (NF1) using a mouse model. NF1 is a familial disease of the nervous system with predisposition to cancer. It affects 1 in 3500 people, regardless of race or ethnicity. The clinical heterogeneity of NF1 presents a serious challenge to patients and clinicians. We are using a mouse model of NF1 (Nf1-/+;p53-/+cis mice) to understand the role of modifier genes in the malignancies associated with NF1. Nf1-/+;p53-/+cis mice develop many of the malignancies associated with NF1, in particular secondary glioblastoma and peripheral nerve sheath tumors (GEM PNSTs). We have found imprinting effects linked to chromosome 11 and strain-specific modifiers that affect the incidence of these tumors. The effect of imprinting on chromosome 11 has opposite parental effects on glioblastomas and GEM PNSTs. We recently analyzed the genetic interaction between an imprinted locus on mouse chromosome 11 and the modifier loci Nerve sheath tumor resistance QTL 1 (Nstr1) on mouse chromosome 19 and Nerve sheath tumor resistance QTL 2 (Nstr2) on mouse chromosome 15. The imprinted locus interacts epistatically with Nstr1 and Nstr2 to affect resistance to GEM PNSTs. In this current study we have extended these findings using B6.A chromosome substitution strain to examine the effect of Nstr1 and Nstr2 in isolation on GEM PNSTs. Chromosome substitution strains carrying the A/J chromosome 15 or 19 on a C57BL/6J background were crossed to the Nf1-/+;p53-/+cis mouse model on a C57BL/6J background to generate F1 mice mutant for Nf1-/+;p53-/+cis and carrying one allele of A/J on either chromosome 15 or 19. Differential effects on tumor latency and tumor penetrance are seen depending on whether the A/J chromosome 15 or 19 is present, suggesting that Nstr1 and Nstr2 act in different genetic pathways. These data confirm the location of Nstr1 on chromosome 19 and Nstr2 on chromosome 15 and further demonstrate that modifier genes affect tumorigenesis under very specific conditions. The understanding of these conditions will allow for more accurate risk assessment and genetic counseling for individuals at high-risk for cancer, and better targeting of cancer therapies based on the specific genetic and epigenetic alterations occurring within an individual tumor.

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Investigating Mutagenic and Therapeutic Effects of Anti-Cancer Treatments in Nfl Mutant Mice

The long-term goal of NF research is to develop safe and effective drugs that target molecules that are deregulated in Nf1 and Nf2 mutant cells. Until these treatments are available, patients with NF1 and NF2-associated tumors will continue to receive the conventional therapeutic modalities of surgical excision, radiation, and cytotoxic chemotherapy. Mutagen-induced secondary cancers are an important concern, particularly as individuals with NF1 and NF2 carry heterozygous germline mutations in the Nf1 and Nf2 tumor suppressor genes. We administered radiation (RAD), cyclophosphamide (CY), or RAD + CY to wild-type and heterozygous Nf1 mutant (Nf1+/−) mice and observed these animals for tumor formation. Mutagen-exposed Nf1+/− mice developed secondary cancers that are common in humans, including myeloid malignancies, sarcomas, and breast cancers. RAD cooperated strongly with heterozygous Nf1 inactivation in tumorigenesis. Most of the solid tumors showed loss of the wild-type Nf1 allele, but retained two Trp53 alleles. Comparative genomic hybridization demonstrated distinct patterns of copy number aberrations in sarcomas and breast cancers from Nf1 mutant mice, and tumor cell lines showed deregulated Ras signaling. Nf1+/− mice provide a tractable model for investigating the pathogenesis of common mutagen-induced cancers and for testing preventive strategies. Our data also have translational implications for assessing the potential risks and benefits of genotoxic modalities in persons with NF1.

In other studies, we compared the effects of CI-1040, a potent inhibitor of MEK, in a myeloproliferative disorder (MPD) initiated by inactivating Nf1 in murine bone marrow and in Nf1 mutant mice that developed acute myeloid leukemia (AML) due to retroviral insertional mutagenesis. Whereas CI-1040 showed no beneficial therapeutic index in mice with MPD, MEK inhibition induced objective regression of Nf1-deficient AMLs. These AMLs uniformly developed resistance to CI-1040 in vivo, despite equivalent biochemical inhibition of the target in paired sensitive and resistant clones. The pattern of retroviral insertions found in resistant AMLs is consistent with outgrowth of a pre-existing clone during CI-1040 administration. These studies emphasize the importance of cell context in the response to targeted agents, and establish a tractable in vivo system for identifying genes that modulate therapeutic efficacy and for probing mechanisms of acquired resistance.
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**NF1 heterozygous cells in the tumor microenvironment influence tumor development**

The Knudson’s two-hit model implies that only complete loss of a tumor suppressor gene function (inactivation of both copies of the gene) will produce a cellular defect, leading to tumor formation. However, recent studies indicate that inactivation of only one copy of tumor suppressor genes (e.g. p53, p27Kip1, and Dmp1) in certain contexts is sufficient to induce tumor formation. These observations have led to a model of haploinsufficiency in which heterozygous inactivation of a tumor suppressor gene produces functional consequences, contributing to tumor formation. One of the unique features of most familial cancers is that non-neoplastic tumor cells in the tumor microenvironment are heterozygous for a specific tumor suppressor gene. Whether loss of one copy of the tumor suppressor gene contributes to tumor formation in familial cancers remains largely unknown. We have recently developed mouse models in which a Neurofibromatosis type 1 (NF1) mutation was specifically targeted into the Schwann cell lineage. Analysis of these mouse models has revealed that the NF1 haploinsufficient tumor microenvironment contributes to neurofibroma formation. This presentation will discuss the molecular and cellular elements in the NF1 heterozygous tumor microenvironment that may play a role in neurofibroma formation. Thus, these studies may provide novel insights into the prevention and treatment of neurofibromas.
Microenvironmental influences on optic glioma formation in genetically engineered mice

Brain tumors develop in 15% of individuals with neurofibromatosis type 1 (NF1). Most of these tumors are World Health Organization (WHO) grade I astrocytomas (pilocytic astrocytomas) which arise in young children, typically in the optic nerve, chiasm, and hypothalamus. In contrast to sporadic pediatric pilocytic astrocytoma, the majority of NF1-associated gliomas in children are indolent and some tumors even regress without treatment.

In order to gain insights into the molecular and cellular pathogenesis of NF1-associated gliomas, our group has developed genetically-engineered mouse models of optic glioma. Wild-type mice lacking Nf1 expression in astrocytes do not develop gliomas, whereas Nf1+/− mice with astrocyte Nf1 inactivation develop prechiasmatic optic nerve and chiasmatic low-grade gliomas. These tumors are first detected around 8-10 weeks of age, and do not progress to high-grade gliomas. Moreover, they are readily detectable by small-animal magnetic resonance imaging (MRI), and exhibit contrast enhancement upon gadolinium administration.

Using these Nf1 optic glioma mice, we have shown that neurofibromin loss in astrocytes results in selective hyperactivation of K-RAS, and that inhibition of K-RAS in Nf1−/− astrocytes reverses the signaling and biological defects associated with Nf1 loss. Moreover, Nf1+/− mice with K-RAS, but not Ha-RAS, activation in astrocytes develop optic glioma, similar to Nf1+/− mice harboring neurofibromin loss in astrocytes. In addition, using a proteomic-based approach in collaboration with Dr. Jason Weber, we were the first to identify that neurofibromin also negatively regulates the mTOR signaling pathway in the brain, and that Nf1-deficient astrocytes and NF1-associated pilocytic astrocytomas have high levels of mTOR pathway activation. Inhibition of mTOR activation with rapamycin ameliorates the growth advantage of Nf1-deficient astrocytes. We have recently shown that deregulated mTOR pathway activation as a result of Nf1 loss leads to increased astrocyte proliferation, motility, and ribosomal biogenesis. Preclinical trials are currently underway to evaluate the efficacy of rapamycin analogs in reducing Nf1 optic glioma growth in mice.

Since astrocyte Nf1 inactivation is not sufficient for glioma formation, we next sought to determine what how the Nf1+/− microenvironment influences Nf1-deficient astrocyte growth. We have identified two microenvironmental influences on Nf1−/− astrocytes. First, since microglia infiltration is a common feature of both murine and human optic pathway gliomas, we isolated Nf1+/− brain microglia and showed that they elaborate soluble factors that stimulate the growth of Nf1-deficient astrocytes. Second, in collaboration with Dr. Joshua Rubin, we have identified a chemokine localized to the optic pathway that uniquely stimulates the growth of Nf1-deficient astrocytes. The expression of this chemokine is developmentally regulated, suggesting that Nf1 optic glioma growth might be ligand-dependent.

Lastly, in collaboration with Dr. Joel Garbow, we have recently refined our diffusion-based imaging methods to detect optic glioma in 20-30 minutes with high spatial resolution. We have also adapted visual evoked electrophysiology methods with Dr.
Milam Brantley to provide functional correlates to Nf1 optic glioma growth in mice in vivo.

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Preclinical mouse models of NF2-related tumors

To aid in identifying the ability of chemopreventive agents to inhibit tumor development, new preclinical in vivo and in vitro models have recently been developed. We have made substantial progress toward accomplishing the goal of generating models of NF2-associated tumors for biologic and preclinical therapeutic trials and of exploiting these mice to address biologic and preclinical questions. Many of the novel strains that have been developed have been shared widely with the NF research community. Some of the models contain targeted mutations capable of increasing the incidence and accelerating the onset of neoplastic lesions. These new preclinical models are assisting the analysis of genetic and environmental factors leading to neoplasia, and clinical studies to evaluate the chemopreventive efficacy of pharmacological agents in NF2 patients.

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Schwann cells schwannomin/merlin is critical for the organization of paranodal axoglial contacts

Schwannomin/merlin is the product of a tumor suppressor gene mutated in neurofibromatosis type 2 (NF2) characterized by multiple schwannomas. Although Schwannomin is ubiquitously expressed, the consequences of its mutations on cell proliferation are particularly apparent in Schwann cells. The physiological role of schwannomin in these cells is not known. We have studied peripheral nerves in mice expressing specifically in Schwann cells mutated schwannomin bearing either a deletion occurring in NF2 (SCHD39-121) or a C-terminal deletion. SCHD39-121 mice displayed some features of exuberant myelination and an increased number of Schmidt-Lanterman incisures. In both mutant lines paranodal regions ultrastructure was markedly altered with irregular, hypertrophic or atrophic loops and absence of transverse bands. The distribution of paranodin/Caspr was severely altered, with pronounced irregularities. Juxtaparanodal proteins (caspr2 and Kv1.1) were altered and limits between paranodes and juxtaparanodes poorly defined. In contrast nodal regions appeared normal and the electrophysiological properties of sciatic nerves were relatively preserved. To determine whether these abnormalities were secondary to a dominant negative effect or to a gain of function of overexpressed mutant schwannomin, we studied mice with a conditional deletion of the Nf2 gene in Schwann cells. These mice had a phenotype similar to that observed in SCHD39-121 mice, albeit more severe, with short internodes, increased number of incisures and severe paranodal alterations. In all three mutant mice abnormalities were present at 3 months. This study shows that schwannomin plays an essential role in Schwann cells and is necessary for the correct organization of paranodal axo-glial contacts.
## Session V
Therapy/Translational Research
*Chair: David Viskochil, MD, Ph.D.*

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Can Stem Cells Impact the Central Nervous System?

An intriguing, novel phenomenon with possible therapeutic dividends has begun to emerge from our observations of the behavior of neural stem cells (NSCs) in various mouse models of CNS injury & degeneration. During phases of active neurodegeneration, factors seem to be transiently elaborated to which NSCs may respond by migrating (even long distances) to degenerating regions & differentiating specifically towards replacement of dying neural cells. In other words, NSCs may “attempt” to emulate in the brain what hematopoietic stem cells do in the periphery: repopulate & reconstitute ablated regions. These “repair mechanism” may actually reflect the re-expression of basic developmental principles (particularly during particular temporal “windows” following injury) that may be harnessed for therapeutic ends. In addition, NSCs may serve as vehicles for gene delivery and appear capable of simultaneous neural cell replacement & gene therapy (e.g., with factors that might enhance neuronal differentiation, neurite outgrowth, proper connectivity, and/or neuroprotection). Intriguingly, many of these factors are produced spontaneously by the stem cells based on their state of differentiation and do not require ex vivo genetic engineering (though that technique can be used to enhance the expression of certain molecules). When combined with certain synthetic biomaterials, NSCs may be even more effective in “engineering” the damaged CNS towards reconstitution.

The ability to repair damaged tissue and reform neural connections is limited when vast amounts of parenchyma are lost. Hypoxic-ischemic (HI) brain injury is not only a critical problem in itself, but also a prototype for acquired defects (to the CNS and other organs) that are characterized by extensive tissue loss. Seeding NSCs onto a polymer scaffold that is subsequently implanted into the infarction cavities of mouse brains injured by HI not only provided a template for bridging large cystic lesions and guiding restructuring, but allowed us to observe -- in a manner heretofore unavailable, adequate substrate and vascular support having now been provided and eliminated as an experimental variable -- the multiple robust reciprocal interactions that spontaneously ensue between NSCs and the extensively damaged brain: NSCs grew exuberantly throughout the template, differentiating into a lattice of neurons and glia. As the “biobridge” became incorporated into the host brain, parenchymal loss was dramatically reduced, an intricate meshwork of many highly-arborized neurites of both host- and donor-derived neurons emerged, and some anatomical connections appeared to be reconstituted. The NSC/polymer complex, nestled within the necrotic parenchyma, altered the trajectory and complexity of host cortical neurites promoting their entrance into the matrix. In a reciprocal manner, tract tracing demonstrated donor-derived neurons extending processes into host parenchyma as far as the opposite hemisphere. Of interest was the degree to which these neurons were capable of seemingly directed, target-appropriate neurite outgrowth without specific external instructive guidance cues, induction, or genetic manipulation of host brain or donor cells. NSC/scaffold complexes appeared to unveil and/or augment a constitutive reparative response by facilitating a series of reciprocal interactions between NSCs and host CNS tissue (both injured and intact) including promoting neuronal differentiation, enhancing the ingrowth/outgrowth of neural processes, fostering the
reformation of cortical tissue, and promoting connectivity following brain injury. Monocyte infiltration and astrogial scarring was also reduced, perhaps facilitating reconstitution. A similar phenomena has been observed by us in the adult spinal cord, as well. Such biobridges, representing an interface between stem cell biology and material science, may also serve as a prototype for other multidisciplinary strategies against complex problems in the nervous and other organ systems.

Evan Y. Snyder, MD, Ph.D., is Professor and Director of the Program in Developmental & Regenerative Cell Biology (The Stem Cell Program) at The Burnham Institute. He joined the Institute after many years at Harvard Medical School. Also coordinates The Southern California Stem Cell Consortium and is on the faculty of The Department of Pediatrics at UCSD (actively involved clinically) and directs the Basic Science Program in Neonatology. His work focuses on the neural stem cells and their potential role in treatment of disease. This includes characterization by gene expression patterns and multipotency. He proposes that resident astroglia and blood-born cells play key roles in coordinating the neural progenitor cell response to brain injury by exerting direct and indirect environmentally mediated influence on neural progenitor cells. He investigates the neural progenitor-immunologic interface related to the mechanisms by which endogenous and exogenous neural progenitor cells react to brain pathology, ultimately aiding in the design of more effective therapeutic applications of stem cell biology. Dr. Snyder is also board certified in pediatrics, neurology (with special competence in children) and neonatology (newborn intensive care).

He received his MD at Harvard Medical School and Ph.D. in neurobiology from University of Pennsylvania.
Schwann cell EGFR expression drives mast cell-dependent nerve neoplasia

Benign dermal and plexiform neurofibromas characteristic of neurofibromatosis type 1 (NF1) contain mast cells that have been suggested to contribute to neurofibroma development. As some neurofibroma Schwann cells also express the epidermal growth factor receptor (EGFR), we expressed human EGFR in murine Schwann cells and caused Schwann cell hyperplasia, mast cell infiltration, collagen deposition, and loss of axon-Schwann cell interactions: reminiscent of peripheral nerve pathologies that manifest in neurofibroma. We examined the role of mast cells in these progressive nerve pathologies by genetic ablation of mast cells using W41 mutant mice, mast cell reconstitution by bone marrow transfer, and cromolyn exposure to stabilize mast cell degranulation. Remarkably, mast cell ablation in EGFR+;W41/W41 mice rescued the nerve phenotypes. Mast cell reconstitution into EGFR+;W41/W41 mice restored all nerve pathologies. Early cromolyn exposure prevented pathology development, and prevented mast cell recruitment to nerve, suggesting that EGFR+ Schwann cells activate resident nerve mast cells, which degranulate and recruit more mast cells, forming a paracrine loop. In contrast, cromolyn exposure after nerve disruption had begun arrested pathology but did not restore normal nerve morphology in EGFR+ mutants. To test if mast cell degranulation is sufficient to promote nerve pathology, we examined peripheral nerves of mouse stains with increased mast cells. We show that these pathologies are dependant on mast cells together with Schwann cell EGFR expression, as increased numbers of degranulating mast cells in peripheral nerve alone are insufficient to drive an NF1-like pathology. Using pharmacologic intervention, we show that the mast cell degranulation product histamine contributes to altered nerve homeostasis, promoting collagen deposition and progressive loss of axon-Schwann cell interactions. This work describes novel roles for mast cells in peripheral nerve homeostasis and pathology, and supports clinical treatment of human NF1 patients with mast cell stabilizers.

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PDGFRA and kit dysregulation and mutation in malignant peripheral nerve sheath tumors

Platelet-derived growth factor receptor α (PDGFRα) and c-Kit are receptor tyrosine kinases targeted by the kinase inhibitor imatinib mesylate. Mutations in these receptors have been found in gastrointestinal stromal tumors (GIST) and gliomas. PDGFRα and c-Kit has been detected in a subset of malignant peripheral nerve sheath tumors (MPNST). We therefore aimed to assess the molecular rationale for imatinib treatment of MPNST patients.

Applying single strand conformation polymorphism analysis we investigated 34 MPNST, 6 corresponding plexiform neurofibromas and 1 MPNST cell culture for sequence aberrations in PDGFRA (exons 2-21) and KIT (exons 9, 11, 13, 17). In addition we determined gene amplification and protein expression of PDGFRA and KIT using real time PCR, immunohistochemistry and western blot. Expression of PDGFRα ligands PDGF-A and PDGF-B was examined by PCR and immunohistochemistry. MPNST cells were utilized to determine the effect of imatinib in vitro.

Two MPNST carried somatic PDGFRA mutations in exons 4 and 10 leading to amino acid exchanges. Several polymorphisms were detected in PDGFRA. PDGFRA and KIT were amplified in tumors from 6 and 4 NF1 patients, respectively. Both genes were amplified in the MPNST cell culture. Expression of PDGFRα; was present in 21 of 28 (75%) MPNST patients. Focal c-Kit expression was detected in 2 of 29 (7%) MPNST patients. PDGF-A and PDGF-B was expressed in MPNST and neurofibromas indicating an autocrine loop. Imatinib treatment of the MPNST cell culture exerted a growth inhibitory effect. Ligand induced PDGFRα; phosphorylation was prevented by imatinib.

Applying multiplex ligation dependent probe amplification we currently study amplification status of genes that localize close to PDGFRA and KIT. First results point to the presence of an amplicon containing multiple genes of therapeutic interest in a subset of MPNST.

In summary our results show that dysregulation of PDGFRA and KIT occurs in MPNST and suggest that MPNST patients may benefit from imatinib treatment.

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Malignant peripheral nerve sheath tumors (MPNSTs) account for 10% of all soft tissue sarcomas, half of these malignancies arise in patients with neurofibromatosis type 1 (NF1), and the lifetime risk for the development of MPNST in NF1 is 8-13%. Surgery is the only curative treatment option. The prognosis of incompletely resected or metastatic MPNSTs is poor. In the largest reported retrospective series to date (M. Carli et al, J Clin Oncol 23:8422-8430; 2005) the 5-year overall survival for 29 NF1 associated MPNSTs was 32.14% compared to 55.12% for 138 sporadic MPNSTs (p=0.0038). Most individuals with NF1 had large, invasive, and unresectable tumors. The response rate to chemotherapy was significantly lower in patients with NF1 (17.6%, 3 of 17) compared to patients without NF1 (55.3%, 26 of 47) (p=0.007). The role of chemotherapy for adult soft tissue sarcomas including MPNSTs is not well defined, and has been confined mainly to administration after surgery (adjuvant). Dose-intensive chemotherapy prior to surgery (neoadjuvant) has become standard therapy for children and adolescents with Ewing’s sarcoma family tumors and other sarcomas and has resulted in increased long-term survival rates exceeding 50%. Using neoadjuvant chemotherapy radiographic responses have been observed in MPNSTs (5 of 6 patients) at the NCI, POB. Clinical trials specifically for MPNSTs are required to define the benefit of treatment approaches for this tumor.

A US Army sponsored multi-center phase II clinical trial will compare the clinical response rate of newly diagnosed, high grade, unresectable, or metastatic sporadic versus NF1 associated MPNSTs after neoadjuvant chemotherapy with 4 cycles of standard sarcoma chemotherapy. As the outcome for NF1 associated MPNST has been reported to be worse compared to sporadic tumors, this trial will evaluate outcome in the two groups treated with identical therapy in an attempt to determine if patients with NF1 associated MPNSTs have a worse prognosis. Secondary trial objectives include the evaluation of 18fluorodeoxy-glucose positron emission tomography and volumetric MRI analysis as tools to aid in the assessment of extent of disease and response to chemotherapy in MPNSTs, to analyze the molecular biology of MPNSTs, and to analyze if serum biomarkers can be identified, which predict for the presence of an MPNST.

This trial is the result of a collaborative effort of the MPNST-NF1 consortium (PI David Viskochil), will be coordinated by the Sarcoma Alliance for Research through Collaboration (SARC), and participating sites include NF1 and sarcoma centers. This trial will thus provide an infrastructure and serve as the platform for future more targeted treatment studies for MPNSTs.

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NF1 Consortium Operations Center: an update

The Neurofibromatosis Consortium was formed in October 2005 by the Department of Defense. Its objectives are to accelerate the clinical translation of basic NF1 research and ultimately decrease the overall impact of the disease, and to conceive, develop, and conduct collaborative pilot, phase I and II clinical evaluations of promising therapeutic agents or approaches for the management or treatment of NF1.

The Consortium is composed of the Operations Center at the University of Alabama at Birmingham, and nine clinical sites: Children’s Hospital of Boston, Children’s Hospital of Philadelphia, Children’s National Medical Center, Cincinnati Children’s Hospital, National Cancer Institute, University of Chicago, University of Utah, the University of Alabama at Birmingham, and Washington University.

Protocol development is currently proceeding in four disease-oriented areas: Plexiforms and other Neurofibromas, Neurocognitive Functioning, Malignant Peripheral Nerve Sheath Tumors, and Visual Pathways and Optic Gliomas. The structure of the Consortium and its future plans will be presented.
Double inactivation of NF1 in tibial dysplasia

Osseous abnormalities including long bone dysplasia with pseudarthrosis are associated with neurofibromatosis type 1 (NF1). The biologic basis, pathogenesis, and molecular causes of pseudarthrosis and tibial dysplasia are not known. The unilateral nature of tibial dysplasia implicates a random molecular event, which then predisposes the abnormal bone to a progressive sequence of bowing followed by fracture and subsequent poor healing that may be inherent in the bone itself.

Prospectively acquired tissue from the pseudarthrosis site of 2 NF1 individuals was utilized for immunohistochemical characterization and genotype analysis of the NF1 locus. Typical immunohistochemical features of neurofibroma were not observed. Genotype analysis of pseudarthrosis tissue using 4 genetic markers (D17S1863, GXALU, IN38, 3NF1-1) spanning the NF1 locus demonstrated loss of heterozygosity (LOH).

These results are the first to document double inactivation of NF1 in pseudarthrosis tissue, and suggest that the neurofibromin-ras signal transduction pathway is involved in this bone dysplasia in NF1.

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Neurofibromin: Ras-GAP and beyond

Loss of neurofibromin Ras-GAP activity and resultant growth promoting signals mediated by increased levels of Ras-GTP and activation of downstream signaling molecules such as MEK, are well established in NF1 associated peripheral nerve sheath tumors (PNSTs). This knowledge has provided therapeutic opportunities directed against Ras itself and its downstream effectors. Towards this end, our current pre-clinical studies are investigating the use of an orally active, small molecule MEK inhibitor in vitro and in vivo. Work to date using NF1-malignant PNST (MPNST) cell lines and explant subcutaneous xenografts are highly encouraging, with decreased overall growth resulting from anti-proliferative, pro-apoptotic and anti-angiogenic effects, with minimal side effects.

However, in addition to loss of neurofibromin in transformed Schwann cells of NF1-PNST subtype, genetic alterations likely exist, since these transformed cells vary in their biology and clinical relevance. Ongoing work in our laboratory coupling Laser Capture Microdissected (LCM) Schwann cells from NF1-PNSTs with high-resolution arrayCGH analysis, suggests the existence of additional distinct genetic alterations in the transformed Schwann cells, which are specific to the PNST subtypes. The functional relevance of these putative additional genetic alterations are being examined in Nf1-/- Schwann cells, obtained by adenoviral-Cre-recombinase excision of differentiated Skin Derived Precursor (SKPs) cells from Nf1flox/flox mice.

In addition to loss of Ras-GAP activity, evidence exists that other normal interactions, and thereby functions of neurofibromin through its other domains, also play a role in the cellular biology and clinical manifestations of NF1. We are currently undertaking mass spec (MS)-based studies on interacting proteins of neurofibromin through its Tubulin Binding Domain (TBD). Amongst the several novel TBD-interacting proteins isolated to date, we have focused on Leucine Rich Pentatrico Peptide Repeat Cassette (LRPPRC) protein, an mRNA binding mitochondrial protein, since mutations of LRPPRC results in the French Canadian variant of Leigh Syndrome (LSFC), associated with neurological developmental defects.

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Unraveling mechanisms and searching for treatments for the learning disabilities associated with NF1

Our laboratory is interested in understanding the mechanisms underlying cognitive disorders such as those underlying learning disabilities and mental retardation. This class of disorders affects one in five people worldwide, but unfortunately, currently there are no effective treatments.

Mutations in the Neurofibromatosis Type 1 (NF1) gene, encoding Neurofibromin, a p21Ras GTPase Activating Protein (GAP), causes learning disabilities and attention deficits. Our studies have shown that the learning and memory deficits of a mouse model of NF1 (nf1+/-) are caused by excessive p21Ras activity leading to enhanced GABA mediated inhibition and impairments in long-term potentiation (LTP), a cellular mechanism of learning and memory.

Recently, we identified lovastatin as a potent inhibitor of p21Ras/Mitogen Activated Protein Kinase (MAPK) activity in the brain. We found that lovastatin decreased the enhanced brain p21Ras-MAPK activity of the nf1+/- mice, rescued their LTP deficits, and reversed their spatial learning and attention impairments.

To better understand the cellular basis of the NF1 cognitive phenotype, we have derived conditional alleles with NF1 heterozygous null mutations in one of the following: astrocytes (GFAP-Cre), excitatory neurons (aCaMKII-Cre), inhibitory neurons (Dlx-Cre) or inhibitory and excitatory neurons (Synapsin I-Cre).

The results show that learning deficits result from heterozygous deletions in inhibitory neurons but not in excitatory neurons or astrocytes. Similarly, electrophysiological analysis indicates that the nf1+/- mutation leads to changes in inhibitory neurons (increase GABA release) that cause the deficits in LTP observed in excitatory neurons. These results demonstrate the cellular locus of the NF1 learning and memory deficits and they suggest that ras/NF1 signaling in inhibitory terminals regulate GABA release and modulate learning and memory.
Neurocognitive Status of Pre-School aged Children with NF-1

The neurocognitive status of children of school age has been well documented because of the high incidence of learning disabilities (LD) in that age group. However LD is a developmental disorder that begins much earlier and there are few, if any, reports of development in young children with NF-1.

The neurocognitive status of 80 children age 10-60 months (average 40 months) was assessed with age appropriate tests: Bayley Scales of Infant Development, McCarthy Scales of Children’s abilities, Test of Visual Motor Integration, and the Vineland Scales of Adaptive Abilities. Bayley Mental abilities were below average, 81.4 (16.6), while Motor abilities were well below average, 77.2 (19.7). The McCarthy general cognitive index was average, 98.3 (19.6), with Verbal, Performance, and Memory subscales only slightly below average. As in the Bayley, the Motor skills were also below average; quantitative skills were low average. Similarly, scores on the Vineland were lowest in Motor development but communication was also rated low. Daily living skills, social skills, and overall adaptive behavior composite were only slightly below average. Ability to draw line figures was also low average.

This profile in young children with NF-1 is similar to that seen in school-aged children, suggesting that poor achievers can be identified early in development and appropriate enrichment programs instituted, possibly averting academic failure later on. Potential early interventions and neuroimaging correlates will also be discussed.

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The NF2 Natural History Study: An Update

The NF2 Natural History Study has completed data collection. Data analysis continues and an update of this data will be presented. Results of this study have important implications for the basic understanding of the NF2 disease process and, therefore, should lead to improved clinical management.

This study has important implications for the management of the NF2 patient. The presentation will review the cost benefit of the Natural History Study. Specifically, data will be presented to demonstrate the health care cost savings resulting from this study.

The NF2 Natural History Study has lead to a better understanding of the NF2 disease process. Implication for basic science research will be presented. Standard recommendations regarding disease screening and disease management resulting from this study should lead to improved clinical care.

A presentation of the current and future data analysis projects will be presented. Unanswered questions regarding the clinical disease process of NF2 will be reviewed.
Poster Abstracts

Alphabetically by presenting author
Advances in neurobiology research during the last two decades, particularly the contributions of genetic engineering technology to our understanding of the molecular basis of normal and abnormal learning process have the potential to have a real impact in the daily lives of NF1 patients with learning disabilities. As we know, learning disabilities in patients with NF1 account for one of the most frequent problems in this population outnumbering the patients with NF1 that face life-threatening conditions. A review of what we know, what we do not know and what we must know to translate this knowledge into real therapies might greatly help to set up an agenda for applied research.

First, recently systematic clinical studies have recognized that learning problems in patients with NF1 are present in up to 80% of the cases. These problems imply a more challenging everyday life for most NF patients and their families. Clinical profile is more heterogeneous than it was believed before. Some molecular aspects of the pathways implicated in NF1 physiopathology have been recognized. NF1 has been one of the important models used to understand better normal and abnormal learning processes. Animal models have been very helpful to obtain this new knowledge. We are facing the convergence of molecular and clinical knowledge in NF1. However, a better understanding will come from a multidisciplinary approach that will allow us to put together our current knowledge in basic neuroscience, clinical evaluation, neuropsychological and environmental aspects.

Second, we still need to learn more about other molecular pathways affected in Neurofibromatosis. Interactions between different and very complex molecular pathways are still important part of NF1 research. Clinical characterization of the different phenotypes in learning, better descriptions of neuropsychological aspects related with learning, real impact of other complications of the disease like brain tumors in learning process, physiopathology of some clinical components of the disease like T2 hyper intensities are some of the aspects that need to be considered. Clinical impact of the Neurofibromin in brain development is clear as represented by findings as macrocephaly, higher risk of brain tumors and morphometric analysis of white-grey matter in MRI that have shown differences in the white-grey matter proportions in different part of the brain in patients with NF1 compared with normal controls. However, we still need to learn about the impact that this genetic alteration has in early brain development.

Third, we need to have a clear mapping on how to execute a translational process that provides us the opportunity to transfer our acquired knowledge. A successful process to translate animal models to clinical setting depends of our ability to understand the limitations this models, the similarities and differences in species, as well as environmental aspects that play a role in the clinical manifestation of learning problems in this population.
Patients with Neurofibromatosis clearly relay in our ability to provide a better quality of life as much as our ability to provide better treatment for those life-threatening conditions that our patients face as a result of their disease.

Treatment of learning problems has been historically limited to behavioral and occupational and school targeted interventions. Our knowledge and understanding of molecular aspects and cellular pathways implicated in memory and learning process must be seen as an opportunity to open more effective and standard therapeutic options. Many gains would result if we could pharmacologically target those altered pathways that may produce a long-term impact in learning process in this affected population.

Some medications, like stimulants, need to be proved useful for this population. Statins have been recently identified as effective components to treat learning problems in a mice model of NF1. Our NF1 community is awaiting our translation to this knowledge in the human population. We need to consider carefully benefits and limitations of this model to provide with better and more reasonable expectations for our scientific community and our patients.

We can gain from reviewing our knowledge of the learning processes in NF1 and look at this issue from different perspectives. A theoretical framework should coherently allow us to incorporate the following: 1. Normal learning in humans and mice. 2. Alterations in learning process in NF1 mice and NF1 patients. 3. Correlations and limitations of comparisons between two species. 4. Environmental factors that impact learning in humans and in NF1 population specifically. 5. Potential molecular pathways that can be targeted.

We also need to incorporate potential mechanisms targeted for these pharmacological options. A clear understanding of the limitations as well as the advantages is necessary to move forward. A Phase 1 study to use Lovastatin in Children with NF1 and Learning problems is being conducted at Children’s’ National Medical Center, preliminary results of this trial will be presented during the meeting. At the time of the submission of this abstract no results are available. International cooperation, consortiums groups and multidisciplinary teams have been established to reach our goals in NF. Needs, implications and difficulties will be discussed in this presentation.
NF1 gene has the highest mutation rate among the known human genes and 50% of patients have new mutations. Factors that may contribute to this high mutability include the large size of the gene, gene conversions mediated by pseudogenes, and the presence of repeated sequences. Therefore no clear genotype-phenotype correlation has been established except for patients with deletion of the entire NF1 gene. In addition, NF1 mutations seem to be equally distributed along the gene. However, some exons have higher mutation density in the NF1 gene and the majority of these mutations were recurrent. Therefore, we analyzed five exons (exon 4b, 16, 29, 31 and exon 37) to detect recurrent and unknown mutations in 100 NF1 patients. In this study, 496delGT, 499delTGTT, R816X, L898P, 5448insG, 5206-2A>G, Y2264X, and 6839delTAC mutations were analysed as recurrent mutations with PCR based methods and novel mutations was detected by DNA sequencing. These analyses allowed us to identify 496delGT and 499delTGTT, in exon 4b in two-family and 5866delA mutation in exon 31 which is a new mutation. LOH and large deletion were also found in the NF1 patients with tumors. It is assumed that there is no difference in terms of mutation distribution among populations. However, recurrent mutations in the aforementioned exons are not common in Turkish population. Therefore, we believed that different populations have their own NF1 gene mutation profile because of their genetic backround.

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Influence of hormones on growth of NF1-/- cells

The tumor suppressor gene, Neurofibromatosis Type 1 (NF1) is responsible for one of the most common autosomal dominant disorders, NF1. Loss of the NF1 protein product, neurofibromin, is associated with benign neurofibromas, comprised largely of Schwann Cells (SC). The number and severity of neurofibromas in NF1 patients increase during times of high hormonal activity, including puberty and pregnancy, and may regress after childbirth. Mouse embryonic stem cells (mESC) that are NF1 wild type, heterozygous, or null can be differentiated into SC-like cells, which express SC and myelination markers. Two human NF1 tumor cell lines, one from a benign plexiform neurofibroma and one from a malignant peripheral nerve sheath tumor (MPNST) cell line, have also been shown to become SC-like in culture. We have used this model system to study the proliferation of these SC-like cells expressing various levels of neurofibromin, to differing levels of hormones that increase during pregnancy: progesterone (P4), estrogen (E2), or testosterone (T), with and without their respective receptor inhibitors (RU486, ICI182780, Flutamide. We have also found that an E2 metabolite, 2-Methoxyestradiol (2ME2), which has been found to kill many types of tumor cells, is cytotoxic to the NF1-/- malignant tumor cell line, while it inhibits proliferation in the other cell lines. 2ME2 could also provide a new treatment avenue for NF1 tumors sensitive to hormones at times of greatest hormonal influence on tumor growth.
Roles of Merlin and Ezrin in Junctional Remodeling During Morphogenesis and Tumor Progression

The NF2 tumor suppressor gene was identified over a decade ago; however, the molecular function of its encoded protein, Merlin, remains elusive. Merlin and the closely related ERM (Ezrin, Radixin, Moesin) proteins localize to the membrane:cytoskeleton interface and are thought to link the actin cytoskeleton to various membrane-associated proteins. Previous work in our lab demonstrated that Nf2-deficiency in primary cells results in the loss of contact-dependent inhibition of proliferation and defective cell:cell communication. We discovered that Merlin localizes to adherens junctions (AJs) in wild-type cells and is required for their stabilization. Loss of AJs has been linked to tumor development and metastasis in humans and mice. Despite the similarity of ERM proteins to Merlin, studies suggest that ERM proteins promote cell transformation, survival, motility, and invasiveness. Indeed, recent studies have linked Ezrin expression to increased tumor metastasis, but its role in this process remains to be elucidated. Identification of molecular functions unique to any one of the mammalian ERM proteins has been impeded by their presumed redundancy. Among the ERM proteins, Ezrin exhibits the most restricted pattern of expression in vivo, and is the only ERM detected in the small intestinal epithelium. Our studies of Ezrin-mutant mice indicate that Ezrin plays an important role in junctional remodeling during intestinal villus morphogenesis. Together, our studies of Merlin and Ezrin suggest that they may play opposing roles in junctional remodeling. Here, we have begun to test this hypothesis by studying the effects of Nf2- or Ezrin-deficiency in brush border membranes isolated from the intestines of Nf2- or Ezrin-mutant mice.
Regulation of the Neurofibromatosis 2 Gene Promoter Expression during Embryonic Development

Mutations in the Neurofibromatosis 2 (NF2) gene are associated with predisposition to vestibular schwannomas, spinal schwannomas, meningiomas, and ependymomas. Presently, how NF2 is expressed during embryonic development and in the tissues affected by neurofibromatosis type 2 (NF2) has not been well defined. To examine NF2 expression in vivo, we generated transgenic mice carrying a 2.4-kb NF2 promoter driving β-galactosidase (β-gal) with a nuclear localization signal. Whole-mount embryo staining revealed that the NF2 promoter directed β-gal expression as early as embryonic day E5.5. Strong expression was detected at E6.5 in the embryonic ectoderm containing many mitotic cells. β-gal staining was also found in parts of the embryonic endoderm and mesoderm. The β-gal staining pattern in the embryonic tissues was corroborated by in situ hybridization analysis of endogenous Nf2 RNA expression. Importantly, we observed strong NF2 promoter expression in the developing brain and in sites containing migrating cells including the neural tube closure, branchial arches, dorsal aorta, and paraaortic splanchnopleura. Furthermore, we noted a transient change of NF2 promoter expression during neural crest cell migration. While little β-gal staining was detected in premigratory neural crest cells at the dorsal ridge region of the neural fold, significant expression was seen in the neural crest cells already migrating away from the dorsal neural tube. In addition, we detected β-gal expression in various NF2-affected tissues such as acoustic ganglion, trigeminal ganglion, spinal ganglia, optic chiasma, the ependymal cell-containing tela choroidea, and the pigmented epithelium of the retina. The NF2 promoter expression pattern during embryogenesis suggests a specific regulation of the NF2 gene during neural crest cell migration and further support the role of merlin in cell adhesion, cell motility, and cell proliferation during development.

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Identification of a Membrane Localization Determinant in Merlin that is Critical for Merlin-mediated Growth Suppression

Understanding the mechanism of action of any tumor suppressor protein requires a detailed understanding of its subcellular distribution. The Nf2 tumor suppressor, Merlin, is unique in that its mechanism of action lies at the membrane:cytoskeletal interface. Merlin is highly homologous to the membrane:cytoskeletal linking ERM (Ezrin/Radixin/Moesin) family of proteins. Although, Merlin and the ERMs exhibit gross localization similarities, Merlin, unlike the ERMs, is a highly insoluble protein. Fine-tuning of their localization profile must exist to distinguish the tumor suppressor activity of Merlin from the ERMs. We have identified the region of Merlin that directs its localization to the insoluble membrane compartment. Removal of this localization determinant leads to loss of Merlin enrichment in an Ezrin-deficient insoluble subapical compartment of mouse epithelial cells. Conversely, addition of this determinant to Ezrin now directs it into the insoluble membrane compartment. The consequences of this Merlin mislocalization are a reduced ability to interact with known Merlin-binding partners and confer growth suppression. Data also suggests that precise Merlin localization mediated by this determinant is critical for stable Ezrin and EBP50 interaction. Thus these results suggest that this region of Merlin recruits Merlin to an insoluble subapical compartment devoid of Ezrin, whereby it can efficiently interact with its binding partners to confer growth suppression.
[18F] Fluorodeoxyglucose positron emission tomography (FDG): High FDG uptake in benign, but growing plexiform neurofibromas in NF1

**Background:** Plexiform neurofibromas (PN) are at risk for degeneration to malignant peripheral nerve sheath tumors (MPNST). Early diagnosis of MPNST is critical as surgery is the only standard treatment option. [18F] Fluorodeoxyglucose positron emission tomography (FDG) has previously been evaluated as a tool to detect malignant degeneration of PN and standard uptake values (SUV) corrected for body weight (BW) were significantly higher in 4 biopsy proven high grade MPNST (5.9±2.4) versus 5 histologically benign (2.0±0.8) neurofibromas (Ferner et al 2000).

**Methods:** We used FDG in 4 patients (table 1) with NF1 and growth of one of their PN, or of lesions within a PN that exceeded the median growth rate (14%/year) of PN in patients (N=49) previously followed with volumetric MRI analysis at the POB. In addition, 3 NF1 patients with biopsy proven, untreated, high grade MPNST were imaged with FDG. FDG scans of the torso and areas of interest were performed on a PET/CT scan (GE Discovery LS) starting at 59 ± 4 minutes after iv injection of 0.14 mCi/kg for children and ~15 mCi for adults. Images were analyzed visually in a computer workstation and the maximum SUV (SUVₘₐₓ) per BW was calculated. Patients were fasting for ≥ 6 h and had normal glucose levels on the scan day.

**Results:** Patient characteristics and PET findings

<table>
<thead>
<tr>
<th>Pt</th>
<th>Age (yrs)</th>
<th>PN Location</th>
<th>PN Volume ml</th>
<th>↑/yr (%)</th>
<th>FDG PET SUVₘₐₓ (BW)</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Abdomen, pelvis, thigh</td>
<td>2502</td>
<td>62</td>
<td>1.5</td>
<td>PN</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>PN1: Thigh, PN2: Pelvis</td>
<td>113, 3162</td>
<td>25, -4</td>
<td>6.5, 3.3</td>
<td>Neurofibroma</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>PN1: Pelvis, PN2: Mediastinum</td>
<td>194, 349</td>
<td>190, 12</td>
<td>≥ liver*, -</td>
<td>PN</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>Neck lesion in neck PN, Neck/mediastinum PN</td>
<td>33, 2030</td>
<td>60, 2</td>
<td>5.7, 2.2</td>
<td>Not done</td>
</tr>
</tbody>
</table>

* indicates FDG scan performed at outside institution.

The rapidly growing lesions of Pts 2, 3, and 4 had higher FDG uptake than the more stable PN in the same Pts, and did not demonstrate the “bulls eye” sign characteristic for PN on MRI. Histology of these lesions was consistent with benign neurofibroma/PN in Pt1, 2, and 3. Pt4 has subsequently been followed with volumetric MRI (+5 months) and has stable disease. The SUVₘₐₓ for the 3 patients with high grade MPNST were 5.5, 7.0, and 8.3 (mean 6.9±1.4).

**Conclusions:** Rapidly growing benign PN can demonstrate high uptake of FDG. High SUV values within a growing PN are not necessarily indicating malignant transformation. Prospective study of PN with FDG PET to characterize the relationship of tumor growth and FDG uptake should be considered.

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Functional allosteric regulation of GRD by CSRD in PNS neurons

The NF1 gene encodes neurofibromin, a Ras-GAP, highly expressed in developing neural cells. The mechanism of regulation of neurofibromin as a Ras-GAP, remains poorly understood. We have recently shown that, in response to EGF, neurofibromin is in vivo phosphorylated on serine residues by PKC-alpha, in human, rat, and avian Central Nervous System (CNS) cells and cell lines. EGF-induced PKC phosphorylation was prominent in the CSR domain (CSRD) of neurofibromin, which lies in the N-terminus and upstream of the Ras-GAP domain (GRD), and this modification significantly increased the association of neurofibromin with actin in co-immunoprecipitations. In addition, we showed that Ras activation in response to EGF was significantly lowered when C62B astrocytoma cells overexpressed a construct encoding both CSRD+GRD. Moreover, when PKC-alpha was downregulated, the Ras-GAP activity of CSRD+GRD was significantly diminished, whereas overexpressed GRD alone acted as a weaker GAP and in a PKC-independent manner. Most importantly, functional Ras inhibition and EGF signaling shifts were established at the single cell level in C6 astrocytoma-derived cell lines stably overexpressing CSRD+GRD, when transient co-overexpression of Ras and PKC depletion prior to stimulation with EGF induced mitosis instead of differentiation. In order to investigate whether a similar switch in the biological output of Ras long term signaling is observed in neurons, we transfected primary Peripheral Nervous System (PNS) neurons with CSRD+GRD and studied the effect on neurotrophin long term signaling. Specifically, nodose, trigeminal and sympathetic mouse neurons were transfected, prior to plating, with Ha-Ras alone or with CSRD+GRD in the presence of neurotrophins and the rate of survival was quantitated over four days. We found that Ras overexpression had a protective role in neuronal survival, which was significantly diminished when CSRD+GRD was co-overexpressed in all types of tested neurons. Moreover, we observed that when Ras over expression reached a critical threshold, neurons after achieving a very elaborate morphological differentiation were eliminated by apoptosis; this effect was counterbalanced by overexpression of CSRD+GRD. Both events were significantly ameliorated when Ca2+-dependent PKC isoforms were downregulated with long term TPA, emphasizing the role of PKC phosphorylation in the effective function of CSRD+GRD as a Ras-GAP. Taken together, these data suggest that CSRD targets GRD in a functional manner that requires phosphorylation by PKC and that CSRD+GRD is a functional Ras-GAP in PNS neurons.
FGD-PET Imaging of Plexiform Neurofibromas: Preliminary data

The role of FGD-PET imaging for managing patients with plexiform neurofibromas (PN) is unclear because of the limited experience that exists at this time. At the present time, MRI is used as the gold standard to determine progression. The Children’s Tumor Foundation and the Joseph Stokes Research Institute at the Children’s Hospital of Philadelphia (CHOP) has funded a multi-institutional pilot study designed to determine whether FGD-PET can predict the growth rate of PN in patients with NF1. We now report preliminary findings of FGD-PET to assess the extent and the degree of disease activity in a relatively large sample of patients.

Eighteen patients (9 males, 9 females) from the Pediatric Oncology Clinic at CHOP with age range from 6 years to 27 years (mean 14.2 years; only 1 patient > 19 years) have been studied since May 2002, with the mean follow up of 20.3 months (range of 3 to 41 months; 2 patients have been lost to follow-up). All patients were clinically stable, but had PN considered at high risk for progression based on anatomical location or concern of increase in size during the previous year as noted by the patient, family, or caregivers. FGD-PET scans were obtained for baseline assessment of the disease activity within two weeks of the first MRI study. The images were interpreted with and without attenuation correction. Correlation was made to MRI on a lesion-by-lesion basis whenever possible. Standardized uptake values (SUV) per body weight have been calculated in 17 patients and 30 lesions.

The common sites of involvement by PN were the face, neck, trunk, and extremities. 16 of 18 patients (89%) showed various degrees of FGD uptake as focal abnormalities. The number of lesions ranged from one to over ten per patient. The location of FGD-PET abnormalities in these patients corresponded to that noted on the MRI scans. The maximum SUV ranged from 0.9 to 5.3 (mean 1.87). Most lesions had low SUV; only 5 lesions had maximum SUV values >2.5. Two of 18 patients (11%) did not show any identifiable abnormal focal uptake.

Our preliminary findings show that FGD-PET is useful for identifying PN. As expected, most lesions had relatively low SUV values, consistent with the benign nature of these lesions. The SUV values noted are in concordance with the SUV range (0.56 - 3.3) noted in the largest published series of PN (Ferner et al., J Neurol Neurosurg Psychiatry 2000,68:353). Further data regarding the predictive value of FGD-PET for PN growth rate is pending.

Upregulation and random activation of Cdc42 and Rac 1 leading to an increase of actin remodelling and protrusive structures in human primary schwannomas

Schwannoma are the hallmarks of the inherited cancer disease neurofibromatosis type 2 (NF2). These benign tumours lack both alleles for the tumour suppressor merlin, a cytoskeleton-membrane linker.

We use a model system of NF2 that is based on human primary Schwann and schwannoma cells.

RhoGTPases are involved in signalling to the cytoskeleton and cycle between an active GTP-bound and an inactive GDP-bound state. Previous studies of our group show that human primary schwannoma cells have an upregulation of active Rac1, a RhoGTPase that is responsible for lamellipodia formation, and that Rac1 can be found predominantly at the membrane, especially in actin rich protrusions.

Scanning electron microscopy now shows that the spindle-like Schwann cells have a bipolar phenotype whereas schwannoma cells are flattened and exhibit multiple protrusive structures like lamellipodia, ruffles and filopodia. Initially immunocytochemical staining shows that Rac 1 is randomly activated throughout the whole cell periphery what is a good explanation for the multiple lamellipodia and ruffles found in schwannoma cells. We further investigated the role of Cdc42, the RhoGTPase that regulates the formation of filopodia, in the altered morphology of human primary schwannoma cells. To measure the levels of active Cdc42 we performed a pull down assay and detected an upregulation of active Cdc42. Moreover immunocytochemical staining displayed Cdc42 at the membrane all around the cell periphery and colocalises with one of its effectors phospho-PAK 2, which indicates again, that Cdc42 is activated. Phospho-ERM proteins, characteristically also involved in the formation of protrusive structures could be detected in filopodia and lamellipodia as well. Moreover we investigated actin dynamics by transfecting pActin-EGFP into human primary Schwann and schwannoma cells and examined them by confocal life cell imaging to see the effects of an upregulation of Rac1 and Cdc42 towards actin remodelling. Schwann cells display slow actin dynamics. Schwannoma cells however, have a less coordinated, more disorganized actin dynamic especially as they show more lamellipodia with fast-moving ruffles and filopodia.

Altogether we show that human primary schwannoma cells have random activation of Rac1 as well as Cdc42 associated with increased levels of actin remodelling leading to augmented lamellipodia and filopodia formation.

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Perineural cysts are in the differential diagnosis for "paraspinous tumors" and NF1

We report two cases of isolated perineural cysts found along multiple, bilateral spinal nerve roots that were initially thought to represent “neurofibromas”. One healthy sixty year-old female had no history of trauma and the location of the cysts in a 41 year-old female did not correlate well with the trauma history. Localized congenital and post-traumatic perineural cysts have been described previously though never as extensive as the cases presented here. Dural ectasia and cystic, arachnoid dilation surrounding multiple spinal roots is most commonly seen with Neurofibromatosis-1 (NF1) or with Marfan’s. With NF1 the perineural cysts can occur at any spinal level, are associated with other clinical features for NF1, but may or may not be associated with plexiform spinal neurofibromas. Careful evaluation of MRI imaging signal characteristics is critical to first differentiate cysts from tumors in patients presenting with thickened spinal nerve roots. Those found to have perineural cysts should then be examined carefully for other stigmata of connective tissue disorders and for Neurofibromatosis-1.
Characteristics of patients with neurofibromatosis 1 (NF1) entered on treatment trials for plexiform neurofibromas (PN) at the National Cancer Institute

**Background:** With the recent availability of trials of investigational agents for PN, the goal of this study was to characterize the population of patients who enroll on these trials, at time of trial entry and during participation in trials.

**Methods:** Patients entered on ≥1 of 4 clinical trials for PN at the NCI, POB (Table 1) were included. The farnesyltransferase inhibitor tipifarnib is administered orally BID for 21 of 28 days or continuously, and the antifibrotic agent pirfenidone orally TID continuously. Patients are monitored at regular intervals for toxicities, and intake of study drug is recorded in medication diaries. PN growth (progressive disease (PD) = ↑PN volume ≥20%) is measured using automated volumetric MRI analysis every 3 to 6 months.

**Results:** Baseline characteristics: Forty-seven patients (median age 8 yrs, range 3-21 yrs; 16 female, 31, male; 2 hispanic, 6 black, 41 white) with familial (N=14) or sporadic (N=33) NF1 were entered on treatment trials. Patients had 69 PNs, and the median total PN volume was 569 ml (range 61-5629). Thirty-four patients (72%) required sedation for MRI scans. ECOG performance status was 0 (N=21), 1 (N=25), 2 (N=1). Quality of life was evaluated in 30 (64%) patients, and 23 of these (77%) had some evidence of cognitive impairment based on parent report of a learning and/or hyperactivity/attention deficit disorder. Participation in pharmacokinetic (PK) studies on phase I trials was 100%.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients (N)**</th>
<th>Tipifarnib</th>
<th>Pirfenidone</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>5 (5-16)</td>
<td>8 (3-21)</td>
<td>7 (3-19)</td>
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<tr>
<td>Dose level: mg/m²/dose (N)</td>
<td>150 (1), 200 (4), 275 (1), 375 (2)</td>
<td>200 (31)</td>
<td>250 (1), 500 (9)</td>
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<tr>
<td>Toxicty (N)</td>
<td>-</td>
<td>Rash (1), Fibr. (1), ANC (1)</td>
<td>Nausea (1), diarrhea (1)</td>
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<tr>
<td>Dose temp. held</td>
<td>Rash (2), diarrhea (1)</td>
<td>ANC (1)</td>
<td>-</td>
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<tr>
<td>Dose reduced Off study</td>
<td>-</td>
<td>Rash (1)</td>
<td>-</td>
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<td>Treatment (Months)</td>
<td>6 (1-19)</td>
<td>Phase A: 9 (3-40)</td>
<td>16 (3-24)</td>
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<tr>
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<td>PD Clinical (3)</td>
<td>PD 3D-MRI (9)</td>
<td>PD Clinical (1)</td>
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<td></td>
<td>Surgery (1)</td>
<td>Rash (1), MPNST (1)</td>
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<td>Compliance (%)</td>
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<td>98 (82-99)</td>
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Double-blinded, placebo-controlled, cross-over trial with phase “A” and “B”. **13 patients participated in 2 clinical trials. Fibr. = hypofibrinogenemia; ANC = Absolute neutrophil count decrease; 3D-MRI = volumetric MRI

**Conclusions:** Predominantly young children with good performance status enter clinical trials for PN. These young patients frequently require sedation for MRI scans. PN burden at trial entry is substantial. Most patients with NF1 receive study drug for prolonged time periods, and toxicities requiring dose interruptions, reductions, or protocol removal are infrequent. No life-threatening toxicities were observed, and all toxicities were reversible. Patients with NF1 appeared highly committed to clinical trials as evidenced by participation in a trial with placebo control, 100% PK participation, and compliance with study medication based on diary review.

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Regulation of Epidermal Development by the NF2 Tumor Suppressor Merlin

A defining feature of schwannoma cells harboring biallelic mutations of the NF2 tumor suppressor gene is fundamental actin cytoskeletal defects. Like schwannoma cells, skin derived mouse keratinocytes genetically engineered to lack the Nf2 gene display altered actin organization suggesting that this is a useful model. In addition, accumulating evidence demonstrates that Merlin plays a role in regulating cell:cell contact through adherens junction and like other cell types keratinocytes lacking Merlin do not form adherens junctions. To further investigate the role of the Nf2 encoded protein Merlin in epidermal development we have developed mice that specifically lack Nf2 in the skin. K14-cre;Nf2lox/lox mice display a drastic phenotype of having little to no initial hair growth and die of dehydration by 3-7 days following birth. K14-cre; Nf2lox/lox mice are capable of forming all of the major components of the skin as determined by histological analysis. However, Nf2 mutant mice display a pronounced compartmental expansion of a specific cell type in the epidermis and these cells have increased proliferation signatures as compared to cells of the same compartment in wild type mice, suggesting that loss of Merlin directly regulates in vivo proliferation rates of a distinct set of cells in the developing skin. This mouse model introduces new evidence that Merlin has important functions in the developing skin and provides a platform for further studies of Merlin function in normal and cancer biology.
Patients with NF1 or NF2 typically have lesions that require serial MRI or CT scans for evaluation and follow-up. Clinical reatment decisions are based on the information in these scans. Unfortunately, the current state of practice does not typically include longitudinal volumetric analysis for accurate tracking of changes in lesion volume. We are developing a centralized tumor metrics registry for making reliable, semi-automated, quantitative volumetric analyses of serial images of NF1 and NF2 lesions. There are several components of this system, including a local and wide-area network, centralized image archive, and centralized tumor volumetric analysis and review by dedicated expert staff. Images of lesions and results of volumetric analyses are presented in graphical format on a password protected secure web-based report that referring physicians can easily access by personal computer, while protecting the privacy of the patient. The tumor volumetrics service can receive MRI and CT scan images by digital transmission as well as by receipt of CD data in DICOM format. We have begun using this system for patients of the MGH neurofibromatosis clinic, and we are setting up network connections for remote sites to pilot.

To compare the sensitivity of volumetric versus linear measurements, we performed reliability studies of NF2 vestibular schwannoma (VS) measurement on 43 MRI scans from nine NF2 patients, over a range of two to seven years. Fourteen VS lesions were measured using both linear diameter and semi-automatic 3D volumetric analysis. Seven lesions from five patients showed progression on volumetric analysis but two of these did not show progression based on linear measures. Thus, 29% of lesions showed progression based on volume but not on linear measures. Volumetric measures were significantly more sensitive to change, both for total percent change and percent change per year, particularly among the progressing lesions (progressing lesions showed double the volume change vs. corresponding cubed linear measurements). We are performing a similar study with NF1 plexiform neurofibromas.

In making decisions regarding surgical intervention or evaluation of medical treatment, it is important that clinicians can determine whether a lesion is growing, and if so, at what rate. Clinical trials of a variety of potential treatments have made assessment of rate of tumor growth or shrinkage increasingly important. However, the lack of routine longitudinal tumor volumetric analysis results in difficulty in determining if individual tumors have grown in size, and can lead to delays in critical clinical decision-making. A centralized, reliable service for tracking tumor volume longitudinally will assist clinicians in making appropriate and timely care decisions.

*This research supported in part by a special grant award to Dr. Harris from the Children’s Tumor Foundation*
NF1 Expression and Tumor Susceptibility

Neurofibromatosis type 1 is the most common genetic cancer predisposition syndrome affecting the nervous system and primarily leads to the development of astrocytomas and malignant peripheral nerve sheath tumors (MPNSTs). However, familiar studies suggest that other factors affect the onset and severity of tumor development. NPCis mice carrying mutations in Nf1 and p53 on the same chromosome spontaneously develop astrocytomas and MPNSTs that closely resemble the human disease. However, NPCis mice on different genetic backgrounds have decreased or increased susceptibility to tumor development. Microarray data from the GeneNetwork database shows that some mouse strains may have different levels of Nf1 expression. The protein product of the Nf1 gene, neurofibromin, is a GTP-ase activating protein that functions as a tumor suppressor by negatively regulating the activation of oncogenic p21-ras and downstream mitogenesis. In order to determine if differences in Nf1 expression may contribute to the strain-specific effects of astrocytoma and MPNST predisposition, we examined the correlation between differences in levels of Nf1 gene expression and mouse strain susceptibility to tumor development. These data further support the hypothesis that in addition to mutations in Nf1, modifying factors present in individuals from different genetic backgrounds significantly influence the susceptibility to astrocytoma and MPNST development.
Vestibular schwannomas (VS) are posterior fossa 8th cranial nerve tumors caused by inactivation of the Neurofibromatosis Type 2 (NF2) gene. These tumors cause hearing loss, balance dysfunction, neuropathies, and brainstem compression. Genetically engineered mice with NF2 inactivation in Schwann cells develop both benign and malignant appearing tumors in various locations, but none engender lesions on their 8th nerves. Our goal was to establish a non-invasive, quantifiable human VS xenograft model in mice. First, rat malignant schwannoma KE-F11 and RT4 cells and human malignant schwannoma HMS-97 cells were implanted near the sciatic nerve in the thigh of severe combined immunodeficiency (SCID) mice. These mice developed visible tumors within two weeks. Imaging using a 4.7-tesla MRI and immuno-histopathological examination identified solid tumors in all rat KE-F11 and human HMS-97 xenografts while rat RT4 xenografts consistently developed cystic schwannomas. Additionally, human benign VS specimens were implanted in another set of SCID mice. Three-dimensional tumor volumes were calculated from MRI images over the next six months. VS xenografts demonstrate biologic variability in their growth potential. The majority of VS xenografts did not grow but persisted throughout the study, while two of 15 xenografts grew significantly. Histopathological examination and immunohistochemistry confirmed that all VS xenografts retained their original microscopic and immunohistochemical characteristics after prolonged implantation. In summary, we describe the first animal model for cystic schwannomas. We also demonstrate the use of high-field MRI to noninvasively quantify VS xenograft growth over time. The VS xenografts represent a model complimentary to NF2 transgenic and knockout mice for translational VS research. (Supported by the US Department of Defense Neurofibromatosis Research Program).

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Identification of MPNST Classes with Differential Patient Outcome

Malignant peripheral nerve sheath tumors (MPNST) are aggressive sarcomas, often frustratingly resistant to conventional therapies. Estimated five-year survival rates range from 30-50%, although considerable biological heterogeneity is encountered in clinical practice. No MPNST-associated molecular classification scheme is currently available to account for this variability in patient outcome.

Therefore, this study used a novel statistical strategy for simultaneous class discovery and class prediction to identify the molecular signatures that predict MPNST patient outcome. Two distinct groups were identified, from which a highly accurate classifier could be defined (>95% accuracy). Molecular differences among MPNST classes suggest differences in tumor cell growth and proliferation, cell death, tumor vascularization, invasiveness and therapy resistance mechanisms. By utilizing a tissue microarray from an independent cohort of MPNST patients, prognostic differences between the two MPNST classes could be confirmed at the protein level for selected biomarkers (including MYC).

Newly identified molecular signatures significantly enhance prognostic accuracy for MPNST patients. Moreover, insights into the molecular mechanisms discriminating MPNST classes may provide clues for novel targeted therapeutic approaches.
Astrocytoma initiation and progression in NPcis invtro mouse model

Higher-grade astrocytomas are among the most prevalent brain tumors and often harbor mutations in p53 gene and activation of Ras signaling. We have generated 11 primary astrocyte cultures from the brains of new born heterozygous mice, carrying germline null mutations in both Nf1 and P53 genes on the same chromosome (NPcis). These cells lose the wild type p53 allele at early passages (increasing population doublings), between the passages 3 to 6 and become tumorigenic. These cells form tumors when injected subcutaneously in nude mice.

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Carboxy-terminus regulates morphogenic activities of merlin

The mechanism by which merlin exerts its tumor suppressor function is not yet fully understood. The morphogenic properties of merlin may play a role in its tumor suppressor mechanism since cytoskeletal defects have been observed in tumor cells from patients. The aim of this study was to characterize merlin’s cell-extension activity, and to study the effect of merlin in cytoskeletal mobilization. The ability of wild-type merlin and truncated constructs to induce morphogenic alterations was studied in transiently transfected cells by morphological analysis of 293 HEK cells using immunostaining and scanning electron microscopy.

This study shows that overexpression of full-length merlin (aa 1-595) induces morphogenic changes in cells. The effect is, however, more drastic with C-terminally truncated forms of the protein. The truncated forms of merlin induced a significant increase in membrane-associated cellular projections; the effect being dependent on the site of truncation. Residues 538-568 were found to be particularly important for the cell-extension activity of merlin, and merlin 1-547 induced the most drastic phenotype with long, thin cellular processes and often a bipolar morphology. The results indicate that an internal sequence determinant for the cell-extension activity exists in this region, and that merlin is involved in the regulation of cytoskeletal organization.

Our current studies extend the findings to various patient mutations that cause preterminal truncation of merlin. In addition, we are analyzing the role of evolutionally conserved short C-terminal sequences that are located within the region that controls the morphogenic activity of merlin.

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FOXO mediates the nonautonomous effects of Ras and PI3 Kinase on peripheral nerve growth

Drosophila peripheral nerves, structured similarly to their mammalian counterparts, comprise a layer of motor and sensory axons, wrapped by an inner peripheral glia (analogous to the mammalian Schwann cell) and an outer perineurial glia (analogous to the mammalian perineurium). We found that expression specifically within the peripheral glia of the constitutively active RasV12 increases growth of the perineurial glial layer. This nonautonomous effect of RasV12 is mediated by activation of the downstream effector PI3 Kinase (PI3K) because expression within the peripheral glia of the activated PI3K-CAAX also increases perineurial glial growth, and because the growth-promoting effects of RasV12 are suppressed by loss of function mutations in PI3K or by co-expression within the peripheral glia of the dominant-negative PI3KD954A. The nonautonomous, growth-promoting effects of PI3K-CAAX are suppressed in a dose-dependent manner by loss of function mutations in Akt, the kinase downstream of PI3K, and are enhanced by co-expression within the peripheral glia of an Akt+ transgene. These observations suggest that PI3K exerts its effects via activation of Akt. Finally, we show that the growth-promoting effects of PI3K-CAAX are suppressed by co-expression within the peripheral glia of FOXO+, a transcription factor that is inhibited by Akt-dependent phosphorylation. We conclude that Ras-PI3K-Akt activity in the peripheral glia promotes growth of the perineurial glia by inhibiting FOXO. In mammalian peripheral nerves, the Schwann cell releases several growth factors that can affect the proliferative and migratory properties of neighbors. Some of these factors are oversecreted in Schwann cells defective in Nf1. Our results raise the possibility that neurofibromas might be caused at least in part by a Ras-PI3K-Akt-dependent inhibition of FOXO within Schwann cells.
Membrane lipid and protein functional binding of the SEC14 domain and subcellular targeting of neurofibromin

The role of the Nf-1 gene product, neurofibromin, in the pathogenetic mechanism of NF1 remains poorly understood. Neurofibromin is an active RasGAP, regulating growth and differentiation through Ras intracellular signaling pathway. The Ras GAP activity resides in a central 360 amino acid domain termed GAP-Related Domain (GRD). It is largely unknown whether other domains of neurofibromin regulate GRD function in vivo or in vitro. Previous work from our lab has established that the N-terminus CSRD domain of neurofibromin may confer allosteric regulation on GRD. We have now extended this analysis to include other domains with putative specific functions. Specifically, we have examined the role of SEC14, a C-terminal adjacent domain, with putative lipid/protein binding properties. Using standard cloning techniques we have generated GST and GFP fusion proteins of SEC14. After optimizing existing protocols based on sarkosyl extraction we achieved high recovery of solubilized GST fusion proteins. Furthermore, using limited trypsin proteolysis assays we found that the recovered SEC14 was properly folded. We established overlay assays and tested the lipid-binding ability of GST-SEC14 or GST, using Echelon PIP Strips. This revealed that SEC14 alone is sufficient for binding in vitro mono-phosphorylated phosphoinositides PI3P and PI5P. We then tested the potential of the GST variants as tools to study protein-binding properties of SEC14, using GST-pull-down assays and MALDI-TOF-MS-analysis. Experiments in primary cell cultures and tissue cell lysates led to identification of binding of SEC14 to intracellular proteins that regulate endocytic vesicular trafficking, namely the alpha and beta-subunits of coatomer (beta-COP), as well as to integral cytoskeleton proteins, i.e., beta-tubulin. These findings were in accordance with our concurrent studies on neurofibromin localization in different subcellular fractions. Thus, we have established fractionation protocols and begun to study stimulus-induced translocation of neurofibromin and possible association with other signaling proteins. In embryonic day 6 chick cortical neurons (E6) and COS-7 cells but not in E15 chick astrocytes, neurofibromin is almost equally present in two pools: rafts and beta-COP enriched, non-raft membranes. Both lines of experimentation suggested that SEC14 may serve as a specific targeting domain of neurofibromin. In order to study a possible localization of SEC14 in specific intracellular compartments, we proceeded to visualize GFP-SEC14 localization, using fluorescence microscopy and two dimensional time-lapse image capture and analysis. We found that GFP-SEC14 exhibited a vesicular and often tubular like-pattern, which, in combination with a prominent perinuclear distribution, was indicative of localization on the ER and Golgi network, and endocytic vesicles all depending and interacting with microtubules. Strong fluorescence was observed in clusters at the plasma membrane, shown to colocalizing with caveolin (cells fixed and analyzed with caveolin antibodies). Time lapse imaging revealed the highly dynamic nature of these GFP-SEC14-labelled vesicular structures, which displayed directional, long-range movement towards the nuclear region or the membrane. In addition, their mobility was strongly affected by nocodazole that disrupts microtubule integrity. Together, these data strongly suggest SEC14 as a specific targeting domain of neurofibromin, which may also affect or regulate the Ras GAP activity of GRD in vivo.
The HMG-CoA reductase inhibitor, lovastatin, inhibits the proliferation of neurofibromin-deficient (Nf+/-) fibroblast cells

Previous studies showed that molecular, synaptic plasticity, spatial learning, attention and sensory gating deficits of an animal model of Neurofibromatosis Type 1 (NF1) can be reversed by treating the mice with the HMG-CoA reductase inhibitor, lovastatin. The cognitive deficits of a mouse model of NF1 (nf1+/-) appear to be caused by excessive p21Ras activity. The enhanced Ras activity also contributes to the development of neurofibromas. We therefore examined the impact of lovastatin treatment on tumor characterizations of NF1 and found lovastatin inhibited the proliferation of neurofibromin-deficient (Nf+/-) fibroblast cells.

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Malignant change of the plexiform neurofibroma: a case report

A 43 years old man with NF1, the atrophy of left leg was emerged from 1993, the acmesthesia of on toes of the left foot was emerged from 1998, the muscle weakness of triceps muscle of left leg was happen from 2000, the tumour of string of beads located at a branch of the left sciatic nerve was found by using B-scan diagnostic ultrasound in 2004, and when the tumour was surgically removed, the tumorous tissue already occupy in distal 3/4 of left sciatic nerve. Postoperative pathological diagnosis of the tumor is the plexiform neurofibroma, and some pathologists believe that the tumor is converting into the malignant schwannoma. The patient refused both the amputation of limb and resection of the left sciatic nerve. One year after the operation, the tumor of right sciatic nerve was emerged, and accompany with the symptoms of anhypnia and headache. A recent diagnosis of MRI shows the tumor grow very fast and an angioma was found in brain, and patient feel the muscle convulsion in the left side of the neck, left arm and left glutea. The skin temperature of left lower limb is decreased. The fast grow of plexiform neurofibroma, evident functional impairment of the nerve, the pathological incidence indicate that the plexiform neurofibroma has transformed into malignant peripheral nerve sheath tumor in this case.
The identification of somatic mutations in neurofibromas, combined with the tracking of those mutations in cells derived from this type of tumors, allowed the identification of culture conditions for the isolation of NF1-/- Schwann cells (SC). In order to explore the functional mechanisms responsible for neurofibroma development, to better characterize neurofibromin-deficient cells and to refine established culture conditions, we are testing the proliferation and differentiation capacities of NF1-/- SC under different in vitro conditions. We are monitoring NF1 mRNA and protein expression. We are characterizing the activation state of different downstream Ras and cAMP-related signaling pathways. We are using RT-PCR and immunocytochemistry to better describe the state, in the SC lineage, of NF1(-/-) SC. We will present preliminary results on this characterization. This work will be important to improve our understanding on neurofibroma formation and will enhance current culture conditions for neurofibroma-derived cells.
Mosaic and segmental neurofibromatosis

Within segmental neurofibromatosis type 1 (NF1) different clinical subtypes emerge. Analysis of these phenotypes may provide insight into the cell types and mutational mechanisms involved in the development of particular NF1-related disease features. For this purpose three segmental NF1 patients with different clinical manifestations (pigmentary changes only, neurofibromas only and combination of both neurofibromas and pigmentary changes) were investigated at the molecular level.

The first patient presented with several café-au-lait spots within a pigmented background involving the entire left leg, hip and lower back. Combined NF1 cDNA sequencing and MLPA analysis revealed an NF1 microdeletion exclusively present in the melanocytes of the hyperpigmented area of the body. A second alteration (c.1226_1227del) in the wild type NF1 allele was only detected in the melanocytes of the café-au-lait spot.

The second patient had several small neurofibromas within a limited body region. Mutation screening on selectively cultured Schwann cells derived from two neurofibromas revealed an identical mutation (c.2041C>T (p.R681X)) in addition to two tumor specific alterations (c.1655T>G (p.L552R) and [c.603T>C, c.604_621del], respectively). Real-time quantitative PCR demonstrated the presence of the first hit in EBV (5±1%), hair follicles (2±0.6%) and fibroblasts derived from both neurofibromas (8±2% and 20±8%, respectively). Buccal smears and urine were negative (sensitivity 1/200).

The third patient had café-au-lait spots scattered over the body and several small neurofibromas located on the hand within a hyperpigmented background. Analysis of neurofibroma derived Schwann cells and melanocytes derived from both the hyperpigmentation area and a peripheral café-au-lait spot revealed an identical NF1 mutation (c.2325+1G>A) in all samples. Moreover, loss of heterozygosity was detected in the melanocytes derived from the hyperpigmented area. Real-time quantitative PCR revealed the intragenic NF1 mutation in fibroblasts derived from both the neurofibroma (5±3%) and hyperpigmented area (2±0.7%). Peripheral blood, fibroblasts derived from the peripheral café-au-lait spot, buccal smear and urine were negative (sensitivity 1/400).

These data confirm the tumorigenic properties of Schwann cells in neurofibroma development and emphasize the pivotal role of melanocytes in NF1-related pigmentary lesions since bi-allelic NF1 inactivation in this particular cell type seems to be the underlying trigger. Furthermore, these data provide for the first time molecular evidence that the clinical mosaic phenotype reflects the timing (and accordingly the cell type involved) in the somatic mutation. While mutations occurring in terminally differentiated cells (i.e. Schwann cells or melanocytes) will give rise to solitary...
symptoms somatic alterations earlier in embryonic development (i.e. common precursor of both cell types) will result in associated symptoms (neurofibromas and café-au-lait spots). Interestingly, both disease features appear to arise even within a background containing predominantly NF1+/+ cells.

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Oncolytic Herpes Simplex Virus Mutants are Efficacious Against Malignant Peripheral Nerve Sheath Tumor Xenografts

Background. Malignant Peripheral Nerve Sheath Tumors (MPNSTs) are highly aggressive and remain a significant clinical challenge. Because many patients with MPNSTs have a poor response to traditional therapies such as irradiation and chemotherapy, the development of novel therapeutics for these patients is crucial. Oncolytic viruses are lytic for many tumor types but are attenuated in their normal pathogenesis. We have previously shown MPNST cell lines to be permissive for oncolytic Herpes Simplex Virus (oHSV) mutants in culture. In the current work, we sought to create a xenograft model of MPNSTs and to test the efficacy of oHSV as a potential treatment for this disease.

Procedure. To create a MPNST xenograft model, human tumor cell lines were inoculated either subcutaneously or intraperitoneally into three strains of immunodeficient mice. Animals injected with the cell line STS26T by either route developed an aggressive disease within 1-2 weeks. Xenografts were examined microscopically for morphology, vascular density and immunohistochemical markers characteristic of MPNSTs (trichrome, S100, synaptophysin and Epidermal Growth Factor Receptor). To assess the tumor-selective nature of oHSV mediated gene transfer, we quantified systemic gene transfer following intraperitoneal dosing. Additionally, oHSV replication was measured within subcutaneous xenografts. Anti-tumor efficacy of oHSV against MPNSTs xenografts was assessed by administration of oHSV in a dose escalation fashion (3 doses of 1x105, 1x106, 1x107 pfu/animal). Anti-tumor efficacy was assessed at day 30 post-inoculation by quantification of total tumor burden and survival. As an attempt to further enhance efficacy against MPNST xenografts, we tested the combination of an EGFR inhibitor and oHSV against MPNSTs in vitro and in vivo.

Results. A highly vascular MPNST xenograft model was generated in athymic nude mice. Dosing of oHSV, G207 or hrR3, by intraperitoneal injection allowed for tumor-selective gene transfer for up to 7 days. Subcutaneous xenografts infected with oHSV showed productive virus replication. oHSV treatment decreased total tumor burden and increased overall survival by ~30%. Upon microscopic evaluation, oHSV treated xenografts showed virus-mediated tumor destruction as well as a reduction in tumor vasculature. Inhibition of EGFR phosphorylation in vitro, using erlotinib (OSI-774), enhanced oHSV mediated cytotoxic effects against MPNST cell lines in an additive fashion. Erlotinib did not enhance the anti-tumor effect of virus injection for subcutaneous tumors. Daily erlotonib by gavage at 25 mg/kg did result in a significant reduction of intraperitoneal tumor nodules alone and in combination with oHSV.

Conclusions. We have created a novel MPNST xenograft model useful for preclinical testing of experimental therapeutics. As predicted from our work using MPNSTs cell lines, oHSV mutants show significant anti-tumor efficacy against MPNSTs in vivo. These preclinical data suggest MPNST may be a suitable target for oHSV mutant therapy.

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NF2 molecular dissection across species and models: evidence for activation of the PI3K/Akt pathway, a mechanism previously correlated with Schwann cell differentiation, survival and tumorigenesis

Schwannomas develop as a result of biallelic inactivation of the NF2 gene. To assess the molecular effects of NF2-activity loss, we performed genome-wide gene expression profiling of Nf2-mutant vs. wild-type mouse Schwann cells (SC), and of SC tumors from two NF2 mouse models (over-expression in SC of a mutant NF2 protein with dominant-negative properties, POSchΔ39-121 - Giovannini et al. 1999; or Nf2 conditional biallelic inactivation in SC, POCre;Nf2flox2/flox2 - Giovannini et al. 2000). Finally, the identified mouse NF2 transcriptome signatures were compared to human schwannoma transcriptional alterations.

Transcriptomes of Nf2-/- and POSchΔ39-121 primary SC showed a 82% overlap in differentially expressed genes compared to their wild-type counterparts. Among the 517 up-regulated genes in NF2-mutant SC, we identified over-representation of myelin genes (e.g. Mpz, Mag, Mbp, Pmp22), and of most genes involved in the cholesterol biosynthesis pathway suggesting that NF2 loss induces signaling pathways involved in SC differentiation. Activation of the signals mediated by PI3K/Akt pathways has been shown to be crucial for SC differentiation, cell survival, and tumorigenesis. We found induction of PI3K/ AKT activity in NF2-mutant SC, murine and human schwannomas by western blot. However, in contrast to early passage NF2-mutant SC, schwannomas showed clear SC dedifferentiation as suggested by the down-regulation of myelin-related transcripts. Ontology analysis of differentially expressed genes in schwannomas revealed up-regulation of cell cycle, survival and protein biosynthesis-related genes (e.g. Ccnd1, Cdk4, Eif4e2, ribosomal proteins) other well-known targets of PI3K/Akt pathway.

In conclusion, combining establishment of primary SC cultures from mouse mutants with genome-wide gene expression profiling allowed us to analyze the transcriptional consequences of NF2 loss in a cell autonomous context. Extension of the analysis to NF2-related mouse and human schwannomas defined molecular signatures, such as activation of the PI3K/Akt signaling pathway that may lead to the development of novel drug-based therapies for NF2.

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Prognostic value of histopathologic grading of sporadic and NF1-associated MPNST: Need for a novel grading system?

Histopathological diagnosis of MPNST remains a challenge because of the rarity of the tumour entity, its variation in histomorphology, the lack of specific immunohistochemical markers and possible sampling errors at operation. We compared retrospectively the clinical course and histopathology of 52 sporadic and NF1-associated MPNST. 14 sporadic cases (8 female, median age at diagnosis 60.6 years) and 37 NF1 associated tumours (16 female, mean age at diagnosis 26.5 years). NF1 patients were found to be significantly younger at diagnosis (p < 0.001) and had a significantly shorter survival time as compared to sporadic cases (median survival 17 months vs. 42 months, Breslow p < 0.05). Time to local recurrence and metastatic spread were also shorter in NF1 patients (interval to local recurrence 9.4 vs. 30.0 months, p < 0.01; interval to metastatic spread: mean 9.1 months vs. 33.2 months, p < 0.001). In a subgroup of patients in whom the original histopathological data was available (22 NF1 patients, 14 sporadic cases), NF1 associated MPNST showed a significantly higher cellularity compared to sporadic tumours (p < 0.001) whereas sporadic MPNST were characterised by a significantly higher pleomorphism (p < 0.01). Cellularity and mitotic scores correlated with grading according to the French Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) only in sporadic cases. Furthermore, mitotic activity correlated with local recurrence and death in sporadic cases (p < 0.05 for both comparisons) but not in NF1-associated tumours. Indeed, in NF1 patients none of the histological parameters was found to correlate with FNCLCC grading or to have a prognostic significance for survival, interval to local recurrence, formation of metastases or metastatic spread. The poor correlation of histomorphology and grading in NF1-associated MPNST strongly suggests the need for an alternative grading system for MPNST, which takes into account the genetic background and special clinical findings in NF1 patients.

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Neurofibromin expression in mouse bone and in fracture healing

Many patients with Neurofibromatosis Type 1 display skeletal abnormalities including alterations in bone size and shape, the presence of scoliosis, and a tendency to develop pseudoarthrosis, as well as generalized skeletal osteoporosis and poor bone healing. Corrective orthopaedic intervention often fails, necessitating multiple revision surgeries followed by prolonged recovery periods. Normal bone remodeling and healing require the concerted actions of bone forming osteoblasts and bone resorbing osteoclasts. However, the cell type and pathway by which neurofibromin haploinsufficiency leads to dysregulation of normal bone remodeling and healing are unknown.

To better understand the function of neurofibromin in normal bone cell physiology and fracture healing, we used immunohistochemistry (IHC) to identify cells expressing neurofibromin protein in vivo. In addition, IHC for Cbfa-1 was used to identify osteoblast-lineage cells and histochemical staining for tartrate-resistant acid phosphatase (TRAP) identified osteoclasts. In tibiae isolated from young C57Bl6/J mice, neurofibromin-positive cells were abundant within the primary spongiosa, located below the growth plate, which is an active site for resorption of calcified cartilage and formation of trabecular bone. TRAP positive osteoclasts throughout the bone were weakly positive for neurofibromin. Bone-adherent osteoblast lineage cells uniformly expressed neurofibromin, regardless of their status for Cbfa-1 staining. This observation indicates that bone-forming osteoblasts, as well as quiescent bone-lining cells, express neurofibromin in physiological conditions. Both of these osteoblast-lineage cells function in mediating access of osteoclasts to bone surface, which is thought to regulate the rate of initiation of the bone-modeling unit and thereby determine the rate of bone metabolism.

We then examined the expression of neurofibromin across the time course of bone fracture healing using quantitative RT-PCR with RNA samples collected from a mouse model of fracture healing. Cellular events in fracture healing recapitulate those observed in endochondral ossification, with chondrocyte formation of a cartilage model, which is replaced by woven bone in primary bone formation. Woven bone is subsequently remodeled to lamellar bone, by coupled remodeling in secondary bone formation. In the mouse model of fracture healing neurofibromin mRNA expression was increased by four-fold 5 days post-fracture, with expression declining by day 14 and reduced to background levels by 21 days post-fracture. This time course of neurofibromin mRNA expression lags the immediate inflammatory response and closely parallels the endochondral-like mineralized callus formation. Elevated rates of bone resorption are typically observed in the second to fourth weeks post-fracture, during primary and secondary bone formation. Therefore, osteoclastic bone resorption coincides with the decline in neurofibromin expression. Taken together these observations indicate that neurofibromin expression is associated with bone-adherent osteoblast-lineage cells with minimal expression in osteoclasts. In addition neurofibromin expression is induced during the formation of the mineralized callus in the endochondral-like formation stage of bone fracture healing. In future experiments
characterization of the cellular and molecular events associated with bone healing in the Nf1+/− mouse fracture model will provide a model in which to test new strategies aimed at improving bone quality and healing, leading to improved outcomes and reducing the negative economic, social, and physical impact of this disorder in the primarily pediatric orthopaedic NF1 population.

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Muller cell origin of epiretinal membranes in NF2

Membranes on the inner surface of the retina (epiretinal membranes) occur commonly in the general population and have been associated with numerous ocular conditions. While epiretinal membranes peripheral to the macula are generally asymptomatic, membranes that are macular/perimacular may cause visual symptoms due to retinal distortion. Epiretinal membranes from eyes with retinal breaks or previous detachments are composed mainly of cells of retinal pigment epithelial origin, whereas astrocytes derived from the superficial retina predominate in idiopathic membranes. Curiously, epiretinal membranes occur at a high frequency in NF2 patients; however, very little is known about the pathology of these lesions. Here, we describe the first detailed histopathologic and ultrastructural examination of NF2-associated epiretinal membranes. The patient was a 34-year-old woman with NF2 who died of multiple meningiomas, and permission was granted for a complete autopsy. The membranes consisted of predominantly cuboidal cells forming occasional lumens and embedded within basement membrane material. Immunohistochemistry revealed that these cells were diffusely positive for S100 and focally positive for GFAP, consistent with glial origin. Ultrastructurally, the apical surface of the cells had short microvilli and adherens-type junctions, as are seen in epithelial cells. Pigment granules were not detected. These findings are consistent with origin from a Muller cell or Muller cell precursor. Intriguingly, Muller cells, like two of the other cell types affected in NF2 (Schwann cells and ependymal cells), are glial cells with epithelial features. Why this subset of glial cells is preferentially affected in NF2 remains to be determined.

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Pheochromocytomas and Neurofibromatosis type I: Is routine screening worthwhile?

Neurofibromatosis type I (NF 1) is a relatively common (1:3000) autosomal dominant disorder caused by germline mutations in the neurofibromin (NF1) gene. Pheochromocytomas are neuroendocrine tumors, secreting epinephrine and norepinephrine. These tumors usually diagnosed in adulthood, with mean age of diagnosis between 38 and 42 years. Most patients present with episodic hypertension, unexplained agitation, sweating and headaches. Pheochromocytomas are malignant in small proportion of patients (~10%), with tendency to metastasize into liver, lungs, bone and lymph nodes. Previous studies have reported an up to 5.7% incidence of pheochromocytoma in individuals with NF 1. However, the prevalence of these tumors at autopsy is higher, up to 13%. Plasma metanephrine screening performed as part of each patient's annual visit to the Mayo Multidisciplinary NF Clinic identified adult patients with NF 1 and previously unrecognized pheochromocytoma. Routine screening of adults with NF1 has not been endorsed by the clinical community of NF1 experts, due to the relatively low frequency of pheochromocytoma in patients with NF1. Given the morbidity and mortality associated with undetected pheochromocytoma, and the relatively cost-effective screening measures available, it may be worthwhile to reconsider annual screening in adults with NF 1 with plasma and urine metanephrine study.

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Second hit mutation analysis in melanocytes, keratinocytes and fibroblasts obtained from NF1 café-au-lait macules reveals presence of two NF1 hits in the melanocytes

Neurofibromas and café-au-lait macules (CALM) are major cutaneous manifestations of neurofibromatosis type 1. Schwann cells harbor the second NF1 mutation and this triggers the formation of neurofibromas (Serra et al, 2000). The hypothesis that alterations in the NF1 gene also play a crucial role in the pathogenesis of another benign NF1 complication, the CAL-spots, remains poorly examined. Only one study (Eisenbarth et al; 1997) addressed this question using 4 intragenic and 1 flanking polymorphic marker, but no LOH was observed in the CALM melanocytes of 11 NF1 patients.

Recently, it was shown that the melanocyte density is increased in NF1 CALM skin compared with normal skin, control normal skin and control CALM skin (De Schepper et al, 2006). This finding prompted us to revisit the abovementioned hypothesis using the more powerful comprehensive approach for NF1 mutation analysis, allowing identification of LOH as well as dosage alterations and minor lesion mutations.

We obtained CALM skin biopsies from 13 NF1 patients with known germline mutation. Using selective culture conditions, 11 fibroblast, 3 keratinocyte and 6 melanocyte cultures for further DNA and RNA-based studies were obtained. First, allelic imbalance and LOH was assessed by comparing constitutional DNA extracted from white blood cells with DNA extracted from the melanocyte, fibroblast and keratinocyte cultures. Genotype analysis was performed on amplified products using 4 intragenic microsatellite markers. No LOH was observed in any of the cell types, in agreement with previous findings. Hereafter long-range RT-PCR and direct sequencing of the entire NF1 coding region was performed. The germline mutation only was identified in the 11 fibroblast cultures as well as in the 3 keratinocyte cultures. However, in all 5 melanocyte cultures for which we had sufficient RNA for the entire analysis, a different second hit was identified: 3 were nonsense mutations, 1 was a splice mutation and finally one culture showed loss of expression of the wild type allele without identifiable mutation at the cDNA level. The gDNA alteration causing the loss of expression of the wild-type allele is currently under further investigation.

These data improve our understanding of the molecular mechanisms underlying the formation of CAL-spots and will boost further research aiming to understand the phenotypes associated with somatic mutations in melanocytes.
and their precursors during development and their association with segmental NF.

References

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Merlin interacts with microtubules in a regulated manner and affects the microtubule cytoskeleton of the mouse primary Schwann cells

Several tumor suppressor proteins, such as p53, BRCA1 and APC, interact with microtubules and regulate their function. Interaction between microtubules and these tumor suppressors is often regulated by cell cycle phase or phosphorylation. According to previous data the Neurofibromatosis Type 2 tumor suppressor protein merlin interacts with microtubules in vitro. In this work, we have further studied merlin-microtubule interaction and its functional importance. We have identified two tubulin-binding sites in merlin, one located at the beginning of the FERM-domain, and the other at the beginning of the C-terminal domain. Binding is regulated by merlin’s intramolecular association with/phosphorylation of serine 518 in merlin isoform 1. Analysis of cultured cells indicates that the association between merlin and microtubules is strictly regulated at different stages of the cell cycle; in synchronized cells merlin and tubulin colocalize only during mitosis at the mitotic spindles and during cytokinesis at the midbody. In addition to in vitro data we have studied the functional effects of this interaction. Expression of different merlin constructs in 293 cells showed that some of these (deletion and patient mutation constructs) induced the appearance of multipolar spindles, a feature commonly seen in transformed cells. Furthermore, merlin appears to affect microtubule dynamics in vitro. Finally, our recent in vivo data indicates that the lack of merlin in human mesothelioma and mouse primary Schwann cells affects their microtubule cytoskeleton. We are currently evaluating, whether the changes seen in the microtubule cytoskeleton of the primary Schwann cells plays a role in the tumor suppressor function of merlin.

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Outcomes in patients with Neurofibromatosis type 1 (NF1) and gliomas

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder with a prevalence of 1/3,500 and frequently associated with benign and malignant tumors of the central nervous system (CNS). Common manifestations of NF1 include neurofibromas, skin fold freckling, café-au-lait macules and iris hamartomas (Lisch nodules). Astrocytomas are present in 15-20% of patients with NF1 and typically represent low-grade tumors of the optic pathway. However, non-optic gliomas are also overrepresented in this patient population. Currently, little is known about the natural history of non-optic gliomas and there is no consensus on how to best manage these tumors in the setting of NF1.

We performed a retrospective analysis of 20 patients with NF1 and non-optic gliomas in the databases of Massachusetts General Hospital and Dana- Farber/Brigham and Women's Cancer Center. Out of 20 patients in this cohort, 11 were female and 9 were male. The patients ranged in age from 10 - 67 years and the mean age of our cohort was 38.1. Most of the patients were Caucasian (16/20). Sixty percent (12/20) of the patients had a family history of NF1. The most common presenting symptom was headache (8/20), followed by bulbar symptoms (5/20) and seizures (3/20). Most of the tumors were localized in the supratentorial compartment, with the second most common location in the brainstem. In imaging studies (MRI or CT), the majority of tumors (16/20) were enhanced after administration of contrast. The average age of patients who developed high-grade tumors was 49.3 compared with 34.9 for those with low-grade tumors. All patients with high-grade tumors had positive family history for NF1.

Half of the patients (10/20) received radiation therapy (XRT), mean dose 52.8 Gy. Six patients received chemotherapy (all had concurrent XRT). The indication for adjuvant treatment included either progressive clinical symptoms and/or high tumor grade. No major complications were seen with treatment, except for 1 patient who discontinued carboplatin due to an allergy. The median follow up time for our patients was 93.1 months (95%CI: 36.3, 127.9). Two deaths occurred at 2.1 and 43.6 months. Both occurred in subjects with high-grade gliomas. Our data suggests that non-optic gliomas occur in both adult and pediatric patients with NF1, and that tumor enhancement on imaging studies is not always indicative of higher tumor grade. The overall survival in this cohort of patients was excellent, although 50% of patients with high-grade tumors died during follow-up. Further analysis will be done to precisely match imaging characteristics with tumor type. Unlike in children with NF1 who predominantly suffer from brainstem and optic gliomas, adults with NF1 in our series were found to have tumors located primarily within the supratentorial compartment. Population-based epidemiological studies are needed to better characterize the incidence, natural course and preferred treatments of glioma in NF1 population.

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Gene expression profiling in Nf1 knockout mice suggests link between neurofibromin, kinesins, and axonal transport

The biological mechanisms by which NF1 gene mutation leads to cognitive deficits are not completely understood, although excessive Ras signaling and increased GABA mediated inhibition have been implicated.

To identify genes/proteins involved in the pathogenic process, gene expression analysis was performed comparing expression profiles in hippocampi of control and NF1+/− heterozygous mice. Hippocampi were dissected from NF1+/− and wild type littermates at postnatal days 10, 15, and 20 and the total RNA expression profiled on the Affymetrix Mouse genome chip (Murine 430 2.0).

These experiments identified two G protein coupled receptors that were upregulated. At postnatal day 10, a 10-fold increase was seen in the serotonin 5A receptor (5HT5A) in NF1+/− mice. A second member of the G-alpha inhibitory G protein coupled receptor family, the dopamine 3 receptor (DRD3), was also dysregulated in the Nf1+/− mice, showing a 3-fold increase in expression. Members of the kinesin family were downregulated at P15 and P20. These trends in expression levels have been validated by qRT-PCR.

Differentially regulated genes at post-natal days 10, 15, and 20 were analyzed using GeneGo network analysis software. This network analysis identified direct interactions between NF1, kinesins, integrins, filamins, and amyloid precursor protein (APP), and between integrins and DRD3. Recently, this predicted interaction between NF1 and APP has been directly confirmed in melanocytes. Our results suggest an important link between neurofibromin and transport mechanisms in axons and dendrites.

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Cognitive profile of F1 patients and molecular analysis in coding regions and 5’ UTR of OMGP gene in learning disability phenotype

Cognitive dysfunction associated with NF1 is a complicated and incompletely understood aspect of the disease. Cognitive difficulties and the frequency of learning disabilities (LD) in NF1 range 30 to 65%, which is significantly higher than the estimates of 7-10% of LD seen in the general population. The OMGP gene is located within the NF1 gene and expressed by neurons and oligodendrocytes during myelination in development and later, adult central nervous system. OMGP is also related to axon myelinization, synaptic plasticity, which are important for learning process. Therefore, OMGP gene could be a possible candidate gene for learning disability phenotype in NF1 patients. Altered expression of the OMGP gene might play a role in the clinical heterogeneity of NF1. OMGP gene is characterized by large 3’ and 5’ UTRs, where the presence of several transcription/translation regulatory elements. Thus we analyzed the coding exon and 5’UTR of the OMGP gene by direct DNA sequencing in NF1 patients with and without learning disability (n=50, n=50). Intelligence evaluation of the subjects was performed using Wechsler Intelligence Scale for Childrens- Revised (WISC-R). OMGP62 polymorphisms were also analyzed in the same groups and healthy control group (n=100). Statistically, no significant difference was observed in allele distribution. Variations in the NF1 families, whose members carrying the same mutation, but have different phenotypes in terms of LD might be significant to show the modifier effect of OMGP gene. As a result we have some supporting data for OMGP gene to be a modifier for LD.

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The Role of Drosophila Merlin in Spermatogenesis and Wg Morphogen Trafficking in the Imaginal Disc

The Drosophila Merlin protein, a homolog of the human Neurofibromatosis 2 gene product, has been shown to be important for cell proliferation in specific cell types. Previously we reported that the viable, but sterile Merlin mutant Mer3 (Met177→Ile) displayed abnormalities in cyst polarization during spermatogenesis. The Merlin protein normally localized in the acrosome of mature sperm, but this localization was altered in the Mer3 mutant. We now showed that a more severe defect in cyst polarization could be seen in the adult male pharates carrying the Mer4 allele (Gln170→stop). We also found that both the clathrin mutant Chc4, known to have abnormal cyst individualization, and the meiotic mutant ff16 displayed defective nuclei polarization and nuclear shaping. Because Merlin has been shown to associate with endocytic compartments and because mutations in the genes, such as clathrin and ff16, that are known to be important for vesicle formation and cytokinesis, also affect nuclei polarization, we examined whether Merlin is involved in the vesicular traffic in somatic tissues. We first examined potential interaction between Merlin and shibere, a dynamin participating in various microtubule-mediated processes such as cytokinesis and endocytosis. Ectopic expression of a dominant-negative mutant of shibere (shiDN), ShiK44A, by the 1096 wing pouch driver led to a disrupted wing morphology including the loss of MTR staut bristles. The shiDN wing margin phenotype was rescued by simultaneous introduction of a UAS-Mer construct carrying the Mer+ or Mer1-600 transgene. Partial restoration of the phenotype was also seen when truncated Merlin expression constructs Mer1-330 and Mer1-375 were used, while no restoration was found with Mer1-169 and Mer-f′BB. These results suggest that Merlin plays a role in the vesicular traffic and the FERM domain including the Blue Box is required for the interaction with Shibere. The MTR staut bristles are derived from the cells normally expressing wingless (Wg) morphogen whose movement through the tissue is related to the vesicular traffic. We found that the expression pattern of patched (ptc), which marks the A/P compartment border in the wing imaginal disc, was not changed in the Mer4 mutant, suggesting that the Decapentaplegic (Dpp) morphogen trafficking is likely not affected by Merlin mutation. In contrast, the stripe expression pattern of Wg at the D/V compartment border was altered in the Mer4 mutant. Intriguingly, wg-lacZ insertion in the Mer4 background revealed no change in the wg regulatory zone as compared with that in the wild-type control. While the expression of neuralized, which participates in the determination of dTR and vTR bristles, was controlled by Wg, its expression pattern was significantly deviated in the Mer4 mutant. In addition, the Wg-regulated cycE expression pattern at the D/V border was also affected by the Mer4 mutation. These results suggest that Merlin plays an important role in Wg trafficking. It has been shown that Porcupine (Porc) facilitates Wg glycosylation in the endoplasmic reticulum and the wing margin is subjected to Wg regulation. We found that both Mer3 and Mer4 mutations did not significantly affect the wing margin morphology and only caused additional sensory bristles within the row of staut bristles. While overexpression of Mer+ or Mer-f′BB did not change the wing margin pattern, overexpression of Porc led to complete disappearance of Staut bristles.
and irregularities of sensory and mechano-sensory bristles. In contrast, simultaneous overexpression of Mer and porc restored the staut bristles and normalized the arrangement of mechano- and chemo-sensory bristles. In addition, while overexpression of Mer-£BB did not affect Wg transcription and protein expression in the wing imaginal disc, porc overexpression results in complete absence of the Wg protein at the D/V border. Importantly, overexpression of both porc and Mer+ or Mer-£BB restored the Wg stripe at the D/V border. These results suggest that overexpression of Merlin may facilitates Wg secretion. Together, our data support the notion that Merlin participates in the vesicular traffic.

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Genetic alterations in the initiation and progression of NF1 deficient Schwann Cells in Peripheral Nerve Sheath Tumors (PNST)

Neurofibromatosis1 (NF1) patients harbor several subtypes of neurofibromas (nfibs), with dermal nfibs remaining benign and plexiform nfibs having a 10-15% risk of malignant transformation to a MPNST (malignant peripheral nerve sheath tumor). Besides the primary transformed Schwann cells, PNSTs consist of a number of non-transformed cell types including fibroblasts, perineurial cells, mast cells and other inflammatory cells. Limited molecular cytogenetic studies performed to date have led to an incomplete understanding of the additional genetic alterations that underlie the varying biological properties between the subtypes. Schwann and endothelial cells from patient specimens were isolated using Laser Capture Microdissection to create a high-resolution genetic alteration map using arrayCGH. In total, 5 dermal, 8 plexiform nfibs and 8 MPNSTs have been analyzed. Genetic losses were more prevalent in the plexiform nfibs, compared to MPNSTs, where gains were more common. Loss of regions on chromosomes 1, 2, 10, 13, 17 and 18 were common in both benign and malignant tumors, likely harboring genes involved early in tumor initiation. Gains on chromosomal arms 4q, 5p, 6q, 8q, 10q, 11q, 13q and 17q, were found in MPNSTs and more likely involved in progression of plexiform nfibs. The dermal nfibs reveal losses on chromosomal region 7p14, 7q11.2, 9q34 and gains on 8q11, 8q21 and 18q21-22, which are different than plexiform nfibs, indicative of varying biological behavior. Some of these results, including loss of NF1, have been confirmed using FISH analysis. Currently, we are screening for the differentially expressed transcripts for potential oncogenes and tumor suppressor genes in these chromosomal regions, with candidate genes to be screened for frequency of the alteration in a larger cohort of tumors.

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A Genomic Approach to Neurofibromin Function

Previously published work using microarray technology in neurofibromatosis type 1 (NF1) has focused on 1) tumor gene expression profiling, or 2) gene class prediction in WBCs using a case-control study design. We reasoned that gene expression profiling in lymphoblastoid cell lines (LCLs) using a single large NF1 pedigree could reduce background “noise” and limit the heterogeneity of NF1 mutations. This might increase the chance that modest expression differences in genes affected by NF1 haploinsufficiency could be observed. In particular, we hypothesized that a whole genome approach characterizing gene expression differences between NF1-affected and NF1-unaffected individuals in a large family could offer insight into the function of neurofibromin.

Materials and Methods: We obtained genome-wide expression profiles of 33 LCLs from a NF1 pedigree from the Coriell Cell Repository. Of the 33 cell lines (all blood relatives of the proband), 12 cell lines were from NF1-affected individuals and the balance were from NF1-unaffected family members. The two groups were matched for gender and age. Under standardized conditions, total RNA was isolated from the cell cultures, reverse-transcribed to cDNA and labeled with Cy3. Universal human RNA (Stratagene) was labeled with Cy5. Both were then hybridized to in-house printed microarrays containing ~36,000 oligonucleotides (Operon) representing known and putative human genes. FirstLook software was used to monitor quality of hybridization. After data filtering (removal of genes with an average quality score below 0.8 and genes with an average intensity below 250) and data pre-processing (calculating the ratio of intensities in green and red channels for each spot, and performing log2 transformation and median shift normalization), we randomly permuted sample labels 10,000 times to obtain a distribution of t-scores and a false-discovery rate (FDR) for all 25,800 genes (ArrayAnalysis.nih.gov).

Results: We analyzed 33 samples: 12 from NF1-affected and 21 from NF1-unaffected family members. Of 25,800 remaining probes on the microarrays, three corresponded to three different exons of the NF1 gene. We found that two of the three NF1 probes had the two highest t-scores and, accordingly, two lowest FDRs. The third one had a t-score in top 30 genes. These results accorded with quantitative RT-PCR data, which showed that the level of neurofibromin mRNA was decreased 1.2-1.6 times in NF1-affected individuals. Although there were no other statistically significant genes in the list, some of them are potentially biologically relevant in NF1.

Conclusions: 1) The modest changes in the level of NF1 gene expression in heterozygous NF1 LCLs were detected by microarray technology and validated by qRT-PCR; 2) genes whose expression is affected by NF1 haploinsufficiency likely require a larger sample to detect; 3) LCLs may prove to be useful and convenient tool for studying cellular and molecular mechanisms in NF1 and other genetic disorders.

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Nf1 Haploinsufficient Endothelial Cells Have An Exaggerated Angiogenic Potential/Response And A Heightened Therapeutic Sensitivity

Neurofibromatosis type 1 (NF1) is a genetic disease occurring in 1 in 4,000 births. NF1 is associated with a high frequency of benign plexiform neurofibromas and malignant peripheral nerve sheath tumors, both of which are highly vascular and can be life threatening. As with most tumors, induction of angiogenesis is thought critical for NF1 tumor development and progression. In addition, the protein product of the NF1 gene, neurofibromin, is highly expressed in endothelial cells (EC). Therefore, tumor angiogenesis and EC activation are attractive targets for therapeutic intervention in NF1. It has been previously shown that NF1 tumorigenic Schwann cells can induce angiogenesis and do so via secretion of vascular endothelial growth factor and other angiogenic factors. Recent work in our lab has shown that Nf1 haploinsufficiency in mouse EC caused an exaggerated response to angiogenic factors in vitro and in vivo. The anti-angiogenic factor endostatin can inhibit endothelial cell proliferation and migration, and induce endothelial cell apoptosis, without effecting non-endothelial cells, and appears to be particularly effective in thwarting tumor-induced angiogenesis in vivo. In testing the effects of endostatin on mouse EC utilizing typical in vitro angiogenesis assays, we see an anti-angiogenic response in both Nf1 haploinsufficient and wild type EC. Additionally, we observe an enhanced sensitivity of Nf1 haploinsufficient EC to endostatin anti-angiogenic treatment as compared to wild type EC. We plan to take a similar approach to test other anti-angiogenic agents. In addition, future work in our lab will test the in vivo effectiveness of endostatin and other angiogenesis inhibitors on reducing tumor growth in xenograft models of both NF1 plexiform neurofibroma and NF1 MPNST. We hope that these results will contribute significantly to the management and treatment of NF1 tumors by blocking the ability of these aggressive tumors to recruit the blood vessels required for their growth.

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Measurement of cutaneous neurofibroma volume: comparison between direct measurement and Fastscan laser scanning system

Background: Neurofibromatosis 1 (NF1) is a multi-system genetic disorder characterized by the development of complex tumors termed neurofibromas. Cutaneous neurofibromas (CNF) are virtually universal in adult patients with NF1 and often become a significant cosmetic burden. Surgery is the only accepted treatment for CNF but it cannot address the needs of patients with tens or hundreds of tumors. Topical administration of medications is an attractive approach to treat CNF since this route is associated with minimal systemic toxicity. Moreover, as similar cell types are involved in all neurofibromas, topical testing of CNF will serve as a rapid and relatively non-invasive means of screening drugs to prioritize them for the more complex, invasive, and expensive systemic trials. However, little has been published on the validity of endpoints for clinical trials of CNF.

Methods: We studied the reliability of direct measurement of CNF using skin calipers and compared this with semi-automated measurement using the Fastscan handheld laser scanner system (Polhemus, Colchester, VT). Three independent raters measured maximum orthogonal diameters (h, height; l, length; w, width) of 17 CNF using skin calipers. Tumor volumes were calculated using standard formulas for a rectangular prism (l x w x h). The reliability of tumor measurements was estimated using the intraclass correlation coefficient (ICC). A subset of 10 CNF was also scanned using the Fastscan laser system. Tumor volumes were determined using Delta software (ARANZ, New Zealand). This software interpolates a flat surface over the region of interest and then determines the tumor volume by comparison between the original surface (with tumor) and the interpolated flat surface (without tumor). A measure of association between tumor volumes was calculated by linear regression.

Results: Using direct measurements with skin calipers, maximum tumor dimension ranged from 0.4 cm to 3.3 cm; tumor volumes ranged from 0.035 to 11.7 cm³. The ICC for all three raters was 0.990 for maximum tumor diameter and 0.967 for tumor volume. ICC’s > 0.9 indicate excellent agreement among raters. Using the Fastscan system in a subset of 10 tumors, volumes ranged from 0.011 to 0.367 cm³. There was a significant correlation between tumor volumes calculated by direct measurement and by Fastscan (r²=0.76, p=0.001). The slope of 0.75 for the regression indicates a trend for Fastscan-based tumor measurements to be larger than the tumor volumes directly measured by calipers.

Discussion: At the present time, there is no way to assess the true volume of a CNF non-invasively since the lesion extends both above and below the surface of the skin. Given the lack of a gold standard by which to measure CNF volume, we sought to determine whether measurement by skin calipers was a convenient and reliable system of measurement for clinical trials. Our results indicate that measurement of CNF diameters and volumes by calipers is highly reproducible across raters.
addition, we measured tumor volume using the Fastscan handheld laser scanning system in the same tumors. Although there was good statistical correlation between these two volumes, the Fastscan system tended to overestimate the size of lesions.

Conclusions: Measurement of tumor volumes by skin calipers is reliable and should be considered a potential endpoint in clinical trials of CNF. Semi-automated calculation of tumor volume using a handheld laser system is promising but this system needs additional work before it is appropriate for clinical trials. Updated data on measurement of CNF tumor volume will be presented at the time of the conference.

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Approximately 30-50% of children with neurofibromatosis type 1 (NF1) have symptoms of ADHD that are relieved by stimulant medications such as methylphenidate (MPH). Symptoms of ADHD have been linked with poor working memory, the capacity to maintain information temporarily. We examined whether the neural basis of working memory in NF1 children that met criteria for ADHD differed from that of age-matched typically developing children and furthermore, whether administration of methylphenidate reduced those differences. Six children with NF1 and ADHD (mean age = 9.5 years) and six control children (mean age = 9.6 years) performed N-back tasks with increasing memory load (0-back, 1-back, 2-back) during functional magnetic resonance imaging (fMRI). Children with NF1 were imaged while on (regular dose) and off MPH (36 hour wash-out). Subjects in both groups made more errors with increasing memory load, but children with NF1 performed more poorly than controls. Performance improved on MPH in children with NF1. In control children, increasing working memory load resulted in greater activation of left dorsolateral prefrontal cortex, bilateral inferior parietal lobule, and striatal regions. In contrast, increased working memory load resulted in activation of premotor cortex, medial parietal cortex and cerebellum in children with NF1. Administration of MPH, however, revealed activation on the right dorsolateral prefrontal cortex, bilateral parietal cortex, and caudate nucleus. Thus, while the neural basis of working memory was atypical in children with NF1 and ADHD, the administration of methylphenidate enhanced recruitment of the same regions that mediated working memory in typically developing children.

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A mES cell model for Schwann cell differentiation

The Neurofibromatosis Type 1 (NF1) gene functions as a tumor suppressor gene in the autosomal dominant disorder, NF1. Loss of neurofibromin (the protein product of the NF1 gene) is associated with tumors of the peripheral nervous system, particularly neurofibromas, benign lesions in which the major cell type is the Schwann Cell (SC). We have developed an in vitro system for differentiating mouse embryonic stem cells (mESC) that are NF1 wild type (+/+), heterozygous (+/−), or null (−/−) into SC-like cells, which express SC markers and are capable of expressing myelin. Two human NF1 tumor cell lines, one from a benign plexiform neurofibroma and one from a malignant peripheral nerve sheath tumor (MPNST) cell line, both of which are NF1−/−, have also been shown to be SC-like in culture. The mESC can also be differentiated into neuron-like cells and the behavior and genetic repertoires of the cells under different developmental conditions can be compared. This system provides an ideal paradigm for studies of the role of NF1 in cell growth and differentiation.
Genomic profiling of sporadic meningiomas by microarray based comparative genomic hybridization

Meningiomas are a frequent finding in NF2 patients, causing considerably morbidity and mortality. Inactivation of the NF2 tumor suppressor gene has been shown in both NF2 related meningiomas and in a major fraction of sporadic meningiomas, though other genetic alterations may also be involved in the initiation and progression of this tumor type. In this study, we employed microarray based comparative genomic hybridization (array-CGH) to detect genome-wide genomic copy number alterations in a cohort of sporadic human meningiomas. Our goals were to identify recurrent imbalanced genomic regions that may harbor tumor suppressor genes, oncogenes or tumor progression genes and to explore the utility of whole genome profiling for tumor subclassification. We examined 65 sporadic meningiomas including 30 benign, 24 atypical and 11 malignant tumors using an Agilent human cDNA chip that contains more than 12,000 unique clones (resolution ≥200Kb). By segmentation analysis of array-CGH data, we defined recurrent regions of deletion and duplication/amplification with specific boundary information in all grades of meningiomas. Deletion events occurred frequently on chromosomes 22 (71%), 1 (40%), 14 (25%), 6 (23%), 18 (20%), 3 (17%), 9 (17%), 11 (15%), 10 (15%), 4 (15%) and X (11%), and gain events occurred on chromosome 1 (14%), 17 (14%) and 20 (12%). Unsupervised clustering analysis using array CGH genomic profiles effectively distinguish meningiomas with chromosome 22 deletions from those without. The former were further clustered into three groups that largely accorded with pathological grade. In general, high-grade tumors exhibited significantly more imbalanced chromosomal segments (ICS), but a notable subset of high-grade tumors exhibited few ICS, suggesting an alternative meningioma progression pathway. Indeed, of the 20 tumors without chromosome 22 loss half had no identifiable ICS at this resolution. The ICS detected in the remaining 10 such tumors include both loss events and amplification events, suggesting possible locations for tumor suppressor genes or oncogenes involved in sporadic meningiomas that retain NF2. The latter in particular suggest that disruption of growth factor signaling pathway plays an important role in sporadic meningioma tumorigenesis. Those tumors with no ICS are now being subjected to higher resolution analyses to identify potential initiation events. This work adds to the growing body of evidence indicating that non chromosome 22 loci participate in the genesis and progression of meningioma and suggests that array CGH can be a powerful technique to shed light on the genesis and progression of other tumor types associated with NF1, NF2 and schwannomatosis.

*Yiping Shen is a Children’s Tumor Foundation postdoctoral Young Investigator Awardee 2005 - 2007*
Role of neurofibromin in prefrontal cortex function and physiology

Neurofibromatosis type I (NF1) is a single gene disorder commonly associated with learning disabilities, and impaired attention and executive function. The Nf1 gene product (neurofibromin) regulates ras signaling, modulates hippocampal GABA inhibition and synaptic plasticity, and is required for hippocampal-dependent learning and memory in mice. Intriguingly, data from human patients with NF1 and mouse models (Nf1+/- mice) also indicate that prefrontal cortical dysfunction is a prominent phenotype associated with the disorder. Here, I present data showing that Nf1+/- mice exhibit a visuospatial attention deficit as measured by the lateralized reaction time task. Further, this deficit can be rescued by treatment with lovastatin, a HMG-CoA inhibitor which inhibits ras function. The medial prefrontal cortex is thought to play a critical role in attention processes in mice. Therefore, physiology of this area will be examined. The studies proposed here will clarify the role of ras/neurofibromin signaling in prefrontal cortex function and NF1 symptomatology.

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Are adults with NF1 at increased risk for psychiatric illness?

*Introduction:* Previously published work of a well-defined Swedish cohort found an incidence of a psychiatric diagnosis in 33% of individuals with NF1. Since then, few studies have examined psychiatric illness in NF1; none have involved a US population. Using chart review and follow-up interviews, we investigated psychiatric illness (depression, suicide, substance abuse) in the context of a general natural history study.

*Methods and Results:* Individuals living with NF1 were recruited to the NIH Clinical Center for a study on disease variability. Key eligibility criteria included age 16+ years and availability of both living biological parents; history of psychiatric disorder was not a criterion for enrollment. Twenty-three patients were evaluated by thorough review of their past medical history and physical exam. The average age of our study population was 35.7 years with a range of 17-63 years; 10 of 23 participants (43%) were male. Twelve individuals had a family history of the condition and 10 were parent-child pairs. All participants met NIH consensus criteria for NF1; one individual had two plexiform neurofibromas and no other features of the disorder and probably was mosaic NF1. There was considerable variability in skin neurofibroma burden, learning disabilities and other features in the cohort. Of the 23 individuals evaluated, 10 people (43%) reported a diagnosis of depression and 6 people (26%) were currently being treated with anti-depressant medications. Four people (17%) described suicidal ideation and two individuals (8%) had attempted suicide in the past. A significant proportion of our cohort also abused cigarettes, alcohol and other drugs.

*Discussion:* The lifetime incidence of self-reported depression in our study population was 43%. In the general population, the lifetime incidence of major depressive episode is 13%. Our observed lifetime incidence of suicidal ideation (17%) may be consistent with an increased risk of suicide in NF1. The 12-month prevalence of suicidal ideation in the general population is 3%. Given the high incidence of depression and suicidal ideation in our study participants, we hypothesize that psychiatric illness may be more common in individuals with NF1. This preliminary study is limited by the small sample size and selection bias because participants are self-referred. Many factors could contribute to psychiatric illness in NF1 including stigma related to the visibility of the condition, learning disabilities, chronic pain and perhaps an organic cause. Further investigation is required to understand the underlying etiologies, risk factors and optimal treatment regimens for depression in NF1.

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Mitotic recombination is a mechanism of NF1 loss of heterozygosity in gastrointestinal tumors (GISTs) in neurofibromatosis type 1

A variety of benign and malignant gastrointestinal tumors have been reported in NF1, including gastrointestinal stromal tumors (GISTs), leiomyomas, schwannomas, neurofibromas, ganglioneuromas, gangliocytic paragangliomas and carcinoid tumors. GISTs are the most common mesenchymal tumor of the gastrointestinal tract in the general population but still constitute less than 1% of all primary gastrointestinal tumors and have an estimated incidence of 10-20 cases/million. They are thought to arise from the interstitial cells of Cajal (pacemaker cells of the gut). In 1998 Hirota et al. reported that KIT expression in GISTs was typically accompanied by mutations in the proto-oncogene receptor tyrosine kinase (KIT); mutations in PDGFRα are also found in GISTs. We report two patients with NF1 and incidentally discovered GISTs. Both tumors were wildtype for mutations in KIT and PDGFRα. In one tumor, we report the first evidence of mitotic recombination leading to homozygosity for a germline NF1 mutation and thus subsequent loss of heterozygosity. Our review of the post-1998 literature revealed that GISTs in NF1 tend to be low risk and arise from the small intestine, especially the jejunum. There were multiple reports of hyperplasia of the interstitial cell of Cajal (ICC); such hyperplasia is probably the consequence of haploinsufficiency of NF1 and is likely to be the precursor lesion of GISTs in NF1. We hypothesize that ICC hyperplasia accounts in part for the spectrum of GI dysmotility commonly seen in NF1. With the loss of the wildtype NF1 allele (by mitotic recombination, in our case) on this background of ICC hyperplasia, GISTs can develop. This mechanism may explain the younger age of presentation and multiple GISTs observed in individuals with NF1. GISTs in NF1 can have a distinct pathogenesis and should now be regarded as a tumor associated with neurofibromatosis type 1.

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Weekly docetaxel induces partial response for pulmonary metastases of malignant peripheral nerve sheath tumor associated with NF1

Malignant peripheral nerve sheath tumor (MPNST) is known to be one of the most fatal sarcomas. It frequently metastases to the lung and may become fatal. Although a standard chemotherapy for MPNST is still not established, ifosfamide and doxorubicin have been identified as the most efficient chemotherapeutic agents, with overall response rates distinctly less than 50%. However, these agents frequently cause serious bone marrow suppression, hindering their administration in aged patients. Recently, some authors reported the cases of sarcomas successfully treated with taxanes. We report a 51-year-old woman presenting with MPNST associated with Neurofibromatosis type 1. She showed multiple pulmonary metastases, which were successfully treated with weekly docetaxel therapy. A partial response was observed after six courses of therapy without any serious side effects. In addition, weekly docetaxel therapy in outpatient clinic did not impair the patients QOL.
Antioxidation cocktail for NF-1

Neurofibromatosis type 1 (NF1), one of the most common human genetic disorder, characterized with tumor growth and learning defects, causes reduced fitness and life expectancy. Here I demonstrate that the short life span and oxidative stress vulnerability in NF1 mutant flies is related to an elevated reactive oxygen species (ROS) production and inhibited mitochondrial ATP synthesis. By feeding them with an antioxidation formula consisting superoxide scavenger such as MnTBAP and other ingredients targeting different units of ROS production, these NF1 mutants’ life expectancy was restored to normal. More surprisingly, over-expression of NF1 drastically extends animals’ life span and significantly enhances their reproductive fitness. The search for the underlying mechanisms leads us to identify mitochondrial complex I, NADH dehydrogenase, is one of the molecular targets of NF1 that mediates these events. The preliminary data in mice validating these findings will also be presented.
Vascular Disease in Neurofibromatosis-1

NF1 is associated with a vasculopathy due to the proliferation of Schwann cells, smooth muscle cells, and fibroblasts. Purpose: To assess the incidence and characteristics of symptomatic vascular disease in patients with NF1. Methods: Our database contains more than 1,000 patients with NF1 seen at the University of Chicago since 1989. The database represents approximately 40% of the patients predicted to have NF1 in our catchment area. In order to assess ascertainment bias, we divide patients into Index cases (the first patient of the family to be seen) and non-Index cases. We also record the presenting complaint for each visit. Findings: 11 patients had symptomatic cerebrovascular disease and 7 had renovascular disease. One patient had both. Two patients had small vessel thoracic aneurysms. Of the 11 patients with symptomatic cerebrovascular disease, 8 were Index cases, 6 of whom presented for evaluation of cerebrovascular disease. 3 patients had aneurysms, 7 had large vessel occlusions, and 1 patient had an undetermined cause of cortical stroke. The age of onset was birth to 51 years of age. Disease was always multifocal in patients with large vessel involvement and bilateral in 3 patients. Large vessel disease was treated with pial synangioplasty in 2 cases. Hypertension was a complicating factor in only one patient. All patients with large vessel cerebrovascular disease suffered severe permanent sequelae. Symptomatic renovascular disease was found in 7 patients. 2 patients were Index cases. Only 1 patient was specifically referred for renovascular disease. Single kidney disease was found in 2 patients but involvement of the abdominal aorta was present in all cases. Renovascular disease was multifocal in 6 of 7 cases. Age of onset was 9 months to 30 years of age. Surgical correction was attempted in 4 patients and successful in 3. 3 patients have blood pressure controlled without surgery. Severe congestive heart failure was a complicating factor in 2 cases. Large abdominal plexiform neurofibromas were present in 2 patients with extensive aortic involvement. Conclusions: Symptomatic cerebrovascular disease is uncommon in NF1 and usually associated with devastating complications. Large vessel disease was detected by Doppler studies in all affected patients and could be considered as a screening tool. Renovascular disease is rare but probably more common than symptomatic cerebrovascular disease. Surgical intervention was effective in most patients failing medical management.

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A complete absence of cutaneous neurofibromas associated with a 3-bp in-frame deletion in exon 17 of the NF1 gene (c.2970_2972 delAAT): A clinically significant NF1 genotype-phenotype correlation?

Neurofibromatosis type 1 (NF1) is characterised by café-au-lait spots, cutaneous neurofibromas, and axillary freckling. Other than whole gene deletions, no clinically useful genotype-phenotype correlations have been identified in NF1 suggesting that either unlinked modifying genes, and/or the normal NF1 allele are possibly involved in the development of specific clinical features in NF1. The presence of many cutaneous neurofibromas represent a major disfiguring feature of NF1, thus the absence of, or even a reduction in the number of dermal neurofibromas, especially of the face and arms, would represent a major improvement in the quality of life for many patients. The present study has gathered together a cohort of 19 unrelated NF1 probands, (13 familial and 6 sporadic), all of whom have the same in frame deletion AAT germline NF1 mutation, and none of whom exhibit any cutaneous neurofibromas. This mutation was not seen in over 100 normal chromosomes analysed. In one family, in one branch, the affected individuals have been reported to have neurofibromas but these patients were not available for further testing and hence we cannot testify whether their phenotype was due to the mutation c2970_2972delAAT or to a different mutation in this branch. One of the sporadic patients with this mutation only had café-au-lait spots with no other features of NF1. This study group comprised 2 patients in age range of 8-12 years, 6 in age group 12-18 years and 11 patients well above 18 years of age. Almost complete absence of such dermal neurofibromas in otherwise classical NF1 patients is highly unusual, as they are one of the cardinal features of NF1, being present in the vast majority of patients. Three of the original unrelated NF1 patients identified with this in-frame deletion mutation came from large multi-generational NF1 families who were referred for mutation analysis because of their somewhat mild clinical phenotype. Analysis identified the same 3bp deletion in exon 17 (c.2970_2972 delAAT) in each of these three patients. The additional NF1 patients in the study were obtained from scientific and medical colleagues worldwide following our request for information on other NF1 patients who had been identified with the same small deletion mutation. The in-frame deletion AAT mutation is predicted to remove one of a pair of methionines, encoded by codons 991-992, and also result in a silent change of codon 990 (ACA>ACG, Thr990Thr). Both these methionine residues show high species conservation and are thus likely to have a functional role in neurofibromin. The observation that this deletion mutation completely segregates with all 21 affected individuals in the three large NF1 families is further evidence of its likely pathogenicity. The lack of a suitable soluble peptide for the relevant protein domain containing this mutation currently precludes any further studies of this mutation’s possible effects on the 3-D structure of the mutated neurofibromin.

Thus, the present study represents a major advancement towards a better understanding of the disease processes in NF1. Furthermore, these studies may help to
ascertain the underlying biological mechanisms involved in the development of these disfiguring cutaneous neurofibromas, and help us to elucidate the reasons why patients with this specific small deletion are not prone to develop them.

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The malignant peripheral nerve sheath tumours (MPNSTs) that develop in about 5% of patients with neurofibromatosis type-1 (NF1) are usually aggressive tumours, often with a poor prognosis, and which represent a major cause of patient morbidity and mortality. The lifetime risk of an NF1 patient developing a MPNST is about 8%-13%. Affected individuals may present with complaints of increasing and persistent pain, or of a rapid increase in size of a plexiform tumour size or a change in its surface texture, or of an increasing neurological deficit. Molecular studies indicate that NF1 patients with constitutional 1.5Mb genomic deletions, that involve the entire NF1 gene, are at an increased risk of developing an MPNST. To date, only a small number of studies of the somatic mutations of the NF1 gene that occur in NF1-associated MPNSTs have been reported, or attempts made to compare this tumour-associated somatic mutational spectrum with the underlying germline mutations. A single study has reported considerable similarity between the two NF1 mutational spectra. To assess this possible mutational relationship further we analysed 30 lymphocyte- and 32 tumour-derived DNA samples from 30 NF1 patients, with a known MPNST to determine both the underlying constitutional mutations and the tumour-associated somatic mutations. The mutation analytical methods used, included SSCP, dHPLC, direct sequencing, MLPA, deletion PCR, and, more recently, high-resolution array-CGH. Germline mutations were identified in 22 lymphocyte DNA samples; these included 5 small (<20bp) deletions; 2 small (<20bp) insertions; 5 nonsense mutations; 6 missense mutations; 2 splicing mutations; a multiple-exon deletion; and a large (1.5Mb) genomic deletion. The germline mutations were confirmed in 19 of the matched tumour samples. Somatic mutations were detected in 27 tumours and included 6 small deletions, 1 small insertion and twenty large genomic deletions of variable sizes. Germline mutations were not identified in 8 patients. Four of the 7 small somatic mutations identified are novel. The predominance of MPNST-associated large genomic deletions (almost 70%), occurring as the somatic cell-based 'second hit', is notable, especially as such large deletions were found in only 20% of neurofibroma samples in our previous study. An earlier study suggested that in NF1 patients harbouring large genomic constitutional deletion, the subsequent somatic inactivation of the normal NF1 allele was not a large deletion. Our MPNST mutation analysis indicates that large genomic deletions represent the predominant 'second hits' in these tumours, the underlying germline mutations are mainly point mutations or small (<20bp) deletions and insertions. These results indicate that the MPNST-associated somatic mutational spectrum is different from that in benign neurofibromas and perhaps would suggest that abnormal intra-chromosomal recombination, mediated by flanking homologous LCR repeats, may well be causing deletions of the NF1 gene in somatic cells at mitosis. We are currently characterising the three large deletions (>2.2 Mb) that we found in MPNSTs.
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Myeloid Leukemia: Testing Cooperativity Of Myb Overexpression With Nf1 Loss In a Mouse Hematopoietic Reconstitution System

Neurofibromatosis 1 (NF1) is an autosomal dominant tumor syndrome. There is a 500-fold increased risk for children with NF1 to develop juvenile myeloid leukemia (JMML), associated with loss of the remaining NF1 allele. The Nf1 heterozygous knockout mouse is also prone to develop myeloid leukemia, at a late stage. When homozygous knockout mouse fetal liver cells are used to reconstitute the bone marrow of lethally irradiated recipient mice, the recipients develop a chronic myeloproliferative syndrome similar to JMML, with about a quarter of these progressing to acute myeloid leukemia (AML) by age 1.5 years. Together these data suggest that loss of NF1 is sufficient to cause a JMML-like condition, but additional genetic events are needed to progress to AML. A model system was previously created to find such cooperating genetic events, using the BXH2 mouse, which develops AML (age 8.5 months on average) due to insertional mutagenesis via MuLV. The Nf1 knockout mutation was previously backcrossed onto the BXH-2 background, and myeloid tumors from 67 animals were harvested. Mapping of the viral insertion sites led to the identification of a common site of integration 30-40 kb downstream of the Myb gene, which we reported in 2001. To test the hypothesis that these insertions are activating Myb and causing an oncogenic effect in cooperation with Nf1 loss, we have designed an in vivo system. Bone marrow cells from mice with one Nf1 knockout allele and one floxed Nf1 allele, and an MX1-Cre transgene, were transduced with a Myb MSCV expression vector and used to reconstitute lethally irradiated BoyJ mice. Cre activation was obtained using PIPC treatment, to force Nf1 null status in the donor cells. Two groups of mice (including controls) have been treated. Out of the first group, only one of the 3 experimental mice engrafted well with donor bone marrow, but that mouse has consistently shown an elevated WBC count (19,450-33,450) but not acute leukemia (7 months post-transplant). A non-myb control mouse also engrafted well, and only shows a slight WBC elevation (7300-13,600). A second, larger group of mice was transplanted a month ago and we are awaiting data about the success of the reconstitution. If we find that the onset of AML occurs earlier in the mice expressing myb, this will be functional data supporting the hypothesis that increased myb expression cooperates with loss of NF1 in myeloid leukemia.

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Detection and quantification of NF2 Mutation Mosaicism

Mutation mosaicism in NF2 gene is a frequent finding in sporadic NF2 patients and in the founders of NF2 families. Detecting the mutation mosaicism has very important implications for NF2 diagnosis, prognosis, offspring and prenatal testing and genetic counseling. In our DNA diagnostic lab, we have established a reliable protocol for not only detecting and confirming mosaic mutations, but also quantifying the level of the mosaicism. We have demonstrated, from our 10-year experience of NF2 testing, that SSCP followed by manual sequencing with radioactive isotope are the most sensitive methods to detect low level mosaic mutations in DNA isolated from blood. Manipulation of exposure time of autoradiograph allows the detection of very low level mosaicism of mutations. For cases in which the mutation found in patient’s tumor DNA can not be confirmed in the blood DNA despite a prolonged exposure of the autoradiograph, we have designed a sensitive assay to detect the level of mosaicism as low as 1% in patient’s blood DNA. This ligation-dependent probe amplification (LDPA) assay requires two pairs of oligos, one matching the wild type sequences and the other matching the mutant sequences based on the mutation information obtained from the tumor DNA. The level of amplification represents the load of the wild type and the mutant in the tissue tested, respectively. In order to generate a standard mutation titration curve, a series dilutions of the mutant DNA were made by mixing variable amounts of normal and mutant DNA. Using this protocol, we have quantified the mutant DNA load from various tissue types, including blood, tumor and sperm. We are currently using this protocol to confirm and quantify more than 20 extremely low mosaic mutations found in our lab. Confirmation and quantification of the mosaicism in blood DNA will enable us to deliver more accurate information and better service our NF2 patients. In addition, this will also extend our knowledge and understanding of the molecular nature of NF2 mutations and the genotype-phenotype correlation. We believe this is a significant step forward in NF2 genetic testing.

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*T = Platform Talk
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