

Back to the Future: Proceedings From the 2010 NF Conference

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The neurofibromatoses (NF) encompass the rare diseases NF1, NF2, and schwannomatosis. The NFs affect 100,000 Americans; over 2 million persons worldwide; and are caused by mutation of tumor suppressor genes. Individuals with NF1 in particular may develop tumors anywhere in the nervous system; additional manifestations can include learning disabilities, bone dysplasia, cardiovascular defects, unmanageable pain, and physical disfigurement. Ultimately, the NFs can cause blindness, deafness, severe morbidity, and increased mortality and NF1 includes a risk of malignant cancer. Today there is no treatment for the NFs (other than symptomatic); however, research efforts to understand these genetic conditions have made tremendous strides in the past few years. Progress is being made on all fronts, from discovery studies—understanding the molecular signaling def-

icits that cause the manifestations of NF—to the growth of preclinical drug screening initiatives and the emergence of a number of clinical trials. An important element in fuelling this progress is the sharing of knowledge, and to this end, for over 20 years the Children's Tumor Foundation has convened an annual

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NF Conference, bringing together NF professionals to share ideas and build collaborations. The 2010 NF Conference held in Baltimore, MD June 5–8, 2010 hosted over 300 NF researchers and clinicians. This paper provides a synthesis of the highlights presented at the Conference and as such, is a “state-of-the-field” for NF research in 2010. © 2010 Wiley-Liss, Inc.

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INTRODUCTION: WHY “BACK TO THE FUTURE?”

As in many fields of research that have seen significant progress in the past few years, the annual international neurofibromatosis (NF) meeting has grown from its origins in the 1980s—a workshop style format, with plenty of time for discussion and hypothesis sharing—to a major, heavily attended meeting with significant competition for those wishing to make a platform presentation, but less time for informal interactions. The 2010 meeting aimed to combine elements of both the original and more recent formats and hence the theme ‘Back to the Future’ was chosen. The platform program included a combination of keynote speakers, overviews by NF laboratory leaders, panel discussions on hot topics, and submitted abstracts. Sessions were themed, and session chairs were encouraged to close with a summary of ‘What we know, what still needs to be done, and what big unanswered questions remain?’ This paper summarizes the work presented and main conclusions of the 2010 NF Conference and as such, an up to date overview of neurofibromatosis research.

THE MICROENVIRONMENT IN NF1 TUMORIGENESIS: WHAT WE KNOW—AND DON’T KNOW

Neurofibromas are complex tumors that develop in individuals with NF1 as a consequence of loss of heterozygosity (LOH) of the *NF1* gene, which encodes the tumor suppressor protein neurofibromin. Emerging studies suggest that as well as *NF1* LOH, additional important events are required in order for neurofibromas to develop. Dr. Luis Parada (University of Texas Southwestern Medical Center) and Dr. Karen Cichowski (Harvard Medical School/Brigham and Women’s Hospital) co-chaired a session that examined these “required events,” and aimed to pinpoint some of the remaining questions surrounding neurofibroma development.

Genetic studies from both NF1 human tumors and NF1 mouse models suggest that only Schwann cells need to undergo LOH in order to initiate neurofibroma formation. Furthermore, recent reports from mouse suggest that *Nf1* heterozygous (*Nf1*^{+/-}) cells, particularly bone marrow derived mast cells, play a critical role in the tumor microenvironment to promote neurofibroma development. Dr. Yuan Zhu (University of Michigan) presented his recent work looking more closely at the contribution of the *Nf1*^{+/-} microenvironment to neurofibroma formation. Zhu’s group has established a genetically engineered mouse model that develops plexiform neurofibromas throughout the peripheral nervous system as well as discrete neurofibromas in the skin. When the mouse

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model is developed on a *p53* heterozygous background, some of benign lesions that develop will then progress to malignant peripheral nerve sheath tumors (MPNSTs). Though the *Nf1*^{+/-} microenvironment is critical for neurofibroma progression, it does not appear to be required for neurofibroma initiation or for later malignant transformation to MPNSTs.

Dr. Wade Clapp (Indiana University) further defined neurofibromas as complex tumors composed of Schwann cells, endothelial cells, fibroblasts, degranulating inflammatory mast cells, and pericytes or vascular smooth muscle cells (VSMCs). He reviewed the requirement for *Nf1* haploinsufficiency in mouse models of plexiform tumors and the requirement of the *c-kit*/*kitL* signaling pathway in bone marrow derived cells, for neurofibroma formation. *Nf1* deficient, tumor prone mice were treated with imatinib mesylate, a drug that targets the *c-kit* pathway. This drug treatment resulted in a profound reduction of neurofibromas. An NF1 patient with a biopsy-proven plexiform neurofibroma was then treated with imatinib mesylate in a proof of concept study, and the neurofibroma tumor burden was reduced by approximately 70%. This observation has since been expanded, and a Phase II clinical trial is currently underway in young children, adolescents, and adults to determine the broader utility of imatinib mesylate for the treatment of plexiform neurofibromas.

Dr. Clapp further described observations from *Nf1*^{+/-} murine models and primary cells from NF1 patients that exhibit haploinsufficiency. Endothelial cells (ECs), VSMCs, and fibroblasts from mice as well as humans collectively promote neoangiogenesis and collagen synthesis, altering the extracellular matrix—a key component of the cell microenvironment. Using proteomics arrays, siRNAs and mouse genetic intercrosses based on *Nf1* mouse models [Zhu et al., 2002]. Dr. Clapp’s group has identified key growth factors and biochemical pathways that regulate haploinsufficient gains-of-function in each of the cell lineages. Through these analyses, Dr. Clapp’s group has verified that the *Nf1* mouse model closely recapitulates the NF1 human disease phenotype. These multiple *Nf1*^{+/-} cell phenotypes provide in vitro and in vivo platform model systems to test candidate NF1 drugs either alone or in combinations. It has emerged that many kinases and molecular targets that are dysregulated in neurofibromin deficient cells are also dysregulated in common cancers, providing an opportunity for preclinical testing in NF1 models those drugs developed for other cancers. Many of these drugs are in Phase I–III clinical trials for other cancers and have been extensively evaluated in other cells and animal

disease models, therefore if effective in NF1 cells and models could potentially progress rapidly to the clinic.

Finally, Dr. Clapp illustrated the use of combined positron emission tomography (PET) and computed tomography (CT) imaging for effective visualization and monitoring of the development, growth, and regression of plexiform neurofibromas in genetically engineered mice in vivo over time. PET/CT is an extremely valuable tool for monitoring drug impact in preclinical testing [further reading: Yang et al., 2008; Staser et al., 2010].

Despite a general consensus that the primary neoplastic element in neurofibromas is derived from within the Schwann cell lineage, Dr. Nancy Ratner (Cincinnati Children's Hospital Medical Center) emphasized that the precise identity of the miscreant cell that gives rise to neurofibromas remains unresolved (e.g., are they Schwann cell precursors, immature Schwann cells, and/or mature Schwann cells?) Dr. Ratner presented her group's findings showing that while most *NF1*-null Schwann cells within neurofibromas have characteristics of mature Schwann cells, there are also less differentiated cells present. This has been seen in both mouse and human neurofibromas. Dr. Ratner's group has identified a self-renewing and multi-lineage differentiating peripheral nervous system dorsal root ganglion precursor population (these cells are p75+; GFR+; and BLBP+). These cells are hypersensitive to growth factors and seem to have tumor forming capability. Neurofibromas that develop in the *DhhCre;Nf1(fl/fl)* mouse contain a progenitor population with similar growth requirements, potential, and marker expression. Furthermore, human neurofibromas contain p75+; EGFR+; and BLBP+ neurofibroma cells sphere-forming cells. Neurofibroma spheres contain glial-like progenitors that differentiate into neurons and SM/Fb-like cells in vitro and form benign neurofibroma-like lesions in nude mice. Together, this information suggests that EGFR-expressing glial precursor-like cells may contribute to neurofibroma formation [Williams et al., 2008].

Dr. Ratner also presented data supporting the hypothesis that pathogenesis of neurofibromas requires an NF1 haploinsufficient microenvironment and the presence of NF1^{-/-} Schwann cells, though this hypothesis is not relevant to the pathogenesis of sporadic neurofibromas and is inconsistent with the fact that patients who are somatic mosaics for NF1 mutations develop neurofibromas. Mice in which *Nf1* is ablated in the Schwann cell lineage via the use of a Desert hedgehog (*Dhh*)-Cre driver develop neurofibromas, despite all other cell types in the tumor microenvironment having intact *Nf1* function [Wu et al., 2008].

Transgenic mice in which a Schwann cell promoter drives expression of human EGFR [Ling et al., 2005] develop prominent peripheral nerve hyperplasia between 2 and 12 months of age. Upon microscopic examination, these tumors demonstrate characteristics of human neurofibromas, including a dissociation of non-myelinating Schwann cells from axons and accumulation of mast cells. Palpable tumors form in 20% of these mice when they are homozygous for the transgenic allele. Importantly, loss of wild-type mast cells by bone marrow transplantation or cromolyn exposure will rescue these phenotypes [Monk et al., 2007]. Together, these data support the idea that while mast cells are crucial for neurofibroma formation, in at least some model systems they may be either wild type or *NF1* mutant.

SHEDDING LIGHT ON NF2 SIGNALING

There have been some interesting reports relating to NF2 signaling in the past year. Dr. Vijaya Ramesh (Harvard Medical School/Massachusetts General Hospital) and Dr. Marco Giovannini (House Ear Institute) chaired a session focused on this area. The session began with a talk by Dr. Anthony Bretscher (Weill Institute of Cell and Molecular Biology, Cornell University) that focused on the biology and structure of ezrin-radixin-moesin (ERM) proteins, actin binding proteins and structural relatives of the NF2 protein Merlin. Dr. Bretscher asked the question what can we learn about Merlin from ERM proteins? He focused on the role of the ERM binding protein, ERM-binding phosphoprotein 50 (EBP50, also known as NHERF1) and its binding protein EPI64, in morphogenesis and in the regulation of membrane trafficking. Based on the X-ray structure, the ~300 amino FERM domain of ERM has three subdomains: F1, F2, and F3. The FERM domain is masked in the dormant form, but when activated, it opens and binds to ligands. Data from biochemical and structural studies suggest that multiples signal transduction pathways contribute to the complete opening of ERMs to activate their membrane and cytoskeleton linking activity. Functionally important intermediate states of ERM activation also appear to exist. A distinct site on the ERM-FERM domain binds to EBP50; and knockdown of EBP50 results in disappearance of microvilli, suggesting that EBP50 is required for microvilli formation. Specifically, the PDZ1 domain, but not the PDZ2 domain, of EBP50 is required for microvilli formation.

EBP50 is a substrate for many kinases, and at least three of its sites are phosphorylated by PKC. Dr. Bretscher showed that phosphorylation can regulate the accessibility of ligands to the PDZ1 domain. EPI64, a protein with TBC/RabGAP domains encoding a conserved DTYL at the C-terminal end, binds PDZ1. EPI64 is found at the base of the microvilli. An L-to-A mutant of EPI64 can no longer bind to EBP50, and when this EPI64 mutant is expressed in cells, it results in loss of microvilli. Exogenous expression of EPI64 leads to the formation of actin-covered vacuoles resembling cells expressing Arf6-GTP (Q67L). This observation led to the finding that the EPI64 TBC domain can bind Arf6-GTP, and that overexpression of EPI64 leads to increased Arf6 GTP/GDP ratio. Site-directed mutagenesis studies confirmed that TBC region of EPI64 is a functional Rab-GAP. Among the Rab proteins tested for the Gap activity of EPI64, Rab8 appears to be the key one as EPI64 (R160A) mutant increased Rab8-GTP and EPI64 (WT) decreased Rab8-GTP. Taken together, this data shows that EPI64 regulates a clathrin-independent endocytosis pathway. However, how this relates to the formation of microvilli remains unknown.

Dr. Bretscher also examined the similarities and differences between ERMs and Merlin. While ERMs exist as tightly regulated closed and open forms, the transition between closed and open forms of Merlin is not tightly regulated. ERMs are active at the cell cortex, and the active sites in Merlin are unknown. It may also have a nuclear function. While ERMs regulate membrane traffic through EPI64, and are suggested to be growth promoters, Merlin regulates endocytosis and functions as a tumor suppressor. It remains essential to understand the interaction between ERMs and Merlin [further reading: Fehon et al., 2010].

Dr. Andrea McClatchey (Harvard Medical School/Massachusetts General Hospital) reviewed her findings that Merlin localizes to cadherin-containing cell junctions known as adherens junctions (AJs) will associate with the AJ complex, and is necessary for the formation of stable AJs in several cell types [Lallemand et al., 2003]. Merlin can associate with the epidermal growth factor receptor (EGFR) and prevents its internalization and signaling in a cell contact-dependent manner [Curto et al., 2007]. These data suggested that Merlin physically coordinates the establishment of cell junctions via inhibition of EGFR signaling, providing insight into how Merlin mediates contact-dependent inhibition of proliferation. Dr. McClatchey's group has found that the ability of Merlin to block proliferation and EGFR endocytosis is dependent upon the first 17 amino acids of the protein that precede the FERM domain, and direct it to both AJs and to the insoluble, cortical cytoskeleton [Cole et al., 2008]. Dr. McClatchey's group has now used these studies as a guide to study and understand how Merlin assembles and how it more generally regulates membrane protein complexes.

Dr. McClatchey's most recent work has dissected the molecular and biochemical basis of how Merlin communicates with and stabilizes the AJ, and how Merlin controls EGFR endocytosis. Her group recently found that Merlin's ability to physically link the AJ component α -catenin to the polarity protein Par3 is necessary for organizing cell junctions in the developing skin [Gladden et al., in press]. This in turn is necessary for normal cell polarity and asymmetric division in basal epidermal cells. These studies suggest that Merlin, like other FERM-domain containing proteins, may play fundamental roles in establishing membrane asymmetry; in fact, Dr. McClatchey's group has recently found that Merlin can organize the membrane of single cells.

Dr. Helen Morrison (Leibniz Institute for Aging) has previously shown that ERM proteins can act as counterplayers in Ras activation. While Merlin is inhibitory for Ras, ERM proteins appear to enhance Ras activity. In their Ras-controlling state, these proteins are specifically targeted to their relevant sites of activity via interaction with plasma membrane proteins. Dr. Morrison's recent data demonstrates that from these plasma membrane docking sites, ERM proteins serve as essential components in the conformational regulation and activation of Son of sevenless (SOS), a major Ras guanine nucleotide exchange factor (GEF). Merlin cannot bind and regulate SOS but can antagonize this newly identified ezrin-SOS complex relevant for Ras activation. While Merlin antagonizes this ezrin function, this research has revealed an additional active role of Merlin in regulating Ras activity via GAPs. Merlin can complex and regulate p120RasGAP, an important GAPs for the downregulation of Ras activity. The functional relevance of these findings is currently being dissected in vitro and in vivo. The outcome of these experiments will address a novel role of Merlin in the regulation of p120RasGAP function during contact inhibition of growth.

Dr. Filippo Giancotti (Memorial Sloan-Kettering Cancer Center) provided new insights into Merlin's tumor suppressor function through its nuclear localization. He presented data demonstrating that Merlin specifically interacts with the E3 ubiquitin ligase CRL4^{DCAF1} in the nucleus and inhibits its activity. The closed form of Merlin accumulates in the nucleus and interacts with CRL4^{DCAF1}, while the open form of Merlin is predominantly present in the cytoplasm and does not interact with CRL4^{DCAF1}.

The FERM domain of Merlin binds to the C-terminal segment of CRL4^{DCAF1}. Expression of wild-type Merlin inhibits CRL4^{DCAF1}; however, expression of patient-derived mutations does not inhibit CRL4^{DCAF1}, strongly suggesting that Merlin is a negative regulator of DCAF1. Dr. Giancotti showed that there are three classes of NF2 mutants: (1) mutants that fail to localize to the nucleus; (2) mutants that do not interact with DCAF1; and (3) mutants that can go to the nucleus and can bind DCAF1, but fail to suppress the Ub ligase activity. DCAF1 is required for hyperproliferation of Merlin-deficient mesothelioma cells and shRNA-mediated silencing of DCAF1 in primary human schwannoma cells derived from NF2 patients suppressed the ability of these cells to progress through G1 and enter into S phase in response to mitogen. Silencing of DCAF1 decreased tumorigenicity in a xenograft model. Although the physiological substrates of DCAF1 have not been identified Dr. Giancotti proposed that Merlin functions as a tumor suppressor by controlling a wide gene expression program through inhibition of CRL4^{DCAF1}. However, how the inhibition of CRL4^{DCAF1} E3 ubiquitin ligase by Merlin relates to contact inhibition, RTK signaling and Hippo signaling where Merlin has been implicated is yet to be determined [further reading: Li et al., 2010].

NF2 signaling updates also featured heavily in a number of the selected abstracts presented, including one from Wei Li (Memorial Sloan Kettering Cancer Center) from Dr. Giancotti's group. Dr. Li described new studies to unravel further how Merlin is transported into the nucleus which has included identification of a four amino acid peptide in Merlin which appears to be essential for the protein to accumulate in the nucleus. Dr. Li also described a novel approach that allows induction of Merlin accumulation in nucleus or cytoplasm which should be useful for further elucidation of Merlin transport and function in the cell.

Meningiomas and schwannomas are the two principal types of tumor that occur in NF2 and they are, for the large part non-cancerous. Dr. Marianne James (Harvard Medical School/Massachusetts General Hospital) is focused on understanding the mechanisms that do confer these tumor types with malignant potential. Dr. James showed that the signaling mechanisms involved in this include the aberrant activation of mammalian target of rapamycin complex 1 (mTORC1) plus impaired mTORC2 signaling. Furthermore, mTORC1 and mTORC2 appear to have distinct downstream 'molecular signatures' in arachnoid cells (those from which meningiomas will arise) and Schwann cells (those from which schwannomas will arise). These findings may help inform the future development of effective treatments for the two tumor types.

Dr. Li Guo (Cincinnati Children's Hospital Medical Center) from Dr. Nancy Ratner's group described new NF2 mouse models in which Rac1 is inactivated in Schwann cells either alone, or in combination with deletion of the *NF2* gene. Rac1 activity seems to be necessary for Schwann cell myelin formation, partially through inhibition of Merlin function. Rac1 may represent a new candidate drug target for NF2 therapies.

Close to half of all Merlin protein in the cell resides in membrane rafts, and Timmy Mani (University of Cincinnati), a doctoral student in Dr. Wallace Ip's group, has been investigating how Merlin attaches to these rafts. Mr. Mani showed that Merlin binds phosphoinositides including PIP2, via a conserved binding motif in its FERM domain. Mutating this domain in Merlin blocks FERM

domain mediated PIP2 binding and association with membrane rafts showing that FERM domain mediated phosphoinositide binding is required for Merlin raft association. Mr. Mani also showed that this mutated Merlin becomes cytosolic; is much more mobile than membrane-bound Merlin and loses its growth suppressive functions; and fails to repress cyclin D1 expression as compared to wild-type Merlin suggesting a loss of ability to inhibit cell cycle progression. In summary, Mr. Mani's research shows that FERM domain mediated phosphoinositide binding and raft localization are critical for the growth regulatory function of Merlin.

Dr. Helen McNeill (Mount Sinai Hospital, Canada) works on *Drosophila* analogs of the Merlin pathway—specifically, the Hippo pathway and the large cadherin Fat, a cell surface receptor that controls growth in parallel to Merlin. The Hippo (Hpo) signaling pathway regulates organ size in both *Drosophila* and mammals. While a core kinase cascade leading from the protein kinase Hpo (Mst1 and Mst2 in mammals) to the transcription coactivator Yorkie (Yki) (YAP in mammals) has been established, upstream regulators of the Hippo kinase cascade are less well defined, especially in mammals. Dr. McNeil described how Fat controls growth with the FERM protein Expanded, and the kinase Discs Overgrown/Casein Kinase II. Full activation of the Hippo pathway requires the recruitment of Casein Kinase II to a Fat signaling complex. Loss of Fat or Casein Kinase II lead to unrestrained tissue growth, and this overgrowth was significantly increased when Merlin/NF2 was mutated, indicating synergy between these pathways.

While previous studies in *Drosophila* have implicated Merlin/NF2 as an upstream regulator of Hippo signaling, it remains to be established whether Merlin/NF2 regulates Hippo signaling in the context of normal mammalian physiology. Using conditional knockout mice, Dr. Duoja Pan (Johns Hopkins University) showed that the Merlin/Nf2 tumor suppressor and the YAP oncoprotein function antagonistically to regulate liver development. While inactivation of *Yap* led to loss of hepatocytes and biliary epithelial cells, inactivation of *Nf2* led to hepatocellular carcinoma and bile duct hamartoma. Strikingly, the *Nf2*-deficient phenotypes in multiple tissues were largely suppressed by heterozygous deletion of *Yap*, suggesting that YAP is a major effector of Merlin/NF2 in growth regulation. Dr. Pan's studies link Merlin/NF2 to mammalian Hippo signaling and implicate YAP activation as a mediator of pathologies relevant to the manifestation of clinical NF2.

UNRAVELING NF BIOLOGY THROUGH GENETICALLY ENGINEERED ANIMAL MODELS

NF research has benefited extensively over the past few years from the emergence of an array of progressively more sophisticated genetically engineered mouse (GEM) models. These models continue to provide an increasing understanding of how the manifestations of NF1, NF2, and schwannomatosis emerge, and should help to identify new candidate targets for therapeutic interventions. Several presentations at the Conference provided updates on the characterization and analysis of new NF mouse models.

A number of presentations focused on mouse models of MPNST, a relatively rare but potentially devastating malignant tumor that

can develop in NF1. Dr. Johanna Buchstaller (University of Michigan) from Dr. Sean Morrison's group examined the frequency of tumor-initiating cells from different GEM MPNST models, showing that the genotype of MPNSTs determines the frequency of tumor stem cells that occur. Dr. Eric Rahrmann (University of Minnesota) from Dr. David Largaespada's group presented a new forward genetic screening method using the *Sleeping Beauty* transposon system. Using this he has identified some potentially important genes that may play a critical role in the pathogenesis of MPNSTs. Two presentations from post-doctoral fellows of Dr. Nancy Ratner's group (Cincinnati Children's Hospital Medical Center) continued the theme. Dr. Walter Jessen performed transcriptome comparisons between human NF1 and GEM *Nf1*-driven neurofibromas and malignant peripheral nerve sheath tumors (MPNST) and showed that GEM *Nf1* mouse models recapitulate some important aspects of NF1-associated tumorigenesis in humans. Using mouse models, Jianqiang Wu showed that EGFR expression promotes malignant transformation of *Nf1*-deficient neurofibromas.

MPNST cancer stem cells were isolated and characterized from a mouse model by Dr. Faris Farassati (University of Kansas). The data showed that while mouse MPNST cells do not contain CD133+ cells, human MPNST cells contain between up to 28% CD133+ cells. This CD133-positivity in human MPNST cells has not been previously recognized.

Dr. Tilat Rizvi from Nancy Ratner's group (Cincinnati Children's Hospital Medical Center) showed that loss of the *Nf1* Ras-GAP results in altered myelination in adult mouse corpus callosum. The results implicate the importance of Ras signaling pathways in control of oligodendrocyte myelination and may be relevant to aspects of brain dysfunction in NF1.

Dr. Elena Akhmeteyeva (Nationwide Children's Hospital and The Ohio State University) from the group of Dr. Long-Sheng Chang and Bradley Welling examined the role of NF2 in neural tube closure in a newly developed an inducible *Nestin-CreER;Nf2^{flox2}/flox2* mouse model. *Nf2* inactivation in early gestation causes defects in neural tube closure and disrupts the apical adherens junctions; multiple mitogenic signaling pathways in the ventricular zone are abolished and there is a marked reduction of the progenitor pool with only moderately increased apoptosis. In contrast, when *Nf2* is inactivated in neuroprogenitor cells during mid-to-late gestation, schwannomas and lymphomas develop at a high frequency. These tumors exhibit defects in signaling of the receptor-mediated MAPK and AKT pathways as well as beta-catenin and its downstream nuclear signals. These findings point to some new potential modes of Merlin function in development and in NF2, as well as highlighting a new mouse model of NF2-related tumors.

Dr. Marco Giovannini (House Ear Institute) provided an update of the available preclinical models for NF2. These include cell lines derived from mouse models of NF2 tumors or from human NF2 tumors; NF2 tumors xenografted into immunodeficient mice; and GEM tumor models. Primary human schwannomas have proved challenging to grow, though more recently tumorigenic schwannoma and meningioma cell lines have been developed. The subsequent ability of these cell lines to grow as orthotopic xenografts has yielded animal test systems to study the growth and treatment response of engrafted tumors to specific therapeutic agents.

Two presentations described new animal models of schwannomatosis—one fly and one mouse. Dr. James Walker (Harvard Medical School/Massachusetts General Hospital) from Dr. Andre Bernards' group reported on *Drosophila* as a model to study the role of *SMARCB1*, the candidate schwannomatosis gene. He is developing a *Drosophila* model of schwannomatosis, to help elucidate the specific molecular events that give rise to this disease. T28 transgenic fly lines have been generated that express either human *SMARCB1* or its *Drosophila* ortholog, *Snr1*, under the control of the UAS/Gal4 system. Expression of *Snr1* or either of the two alternatively spliced forms of *SMARCB1* is able to compensate for loss of *Snr1* in flies. Dr. Walker also made transgenes encoding *SMARCB1* bearing mutations found in patients with familial cases of schwannomatosis. These were tested for their ability to rescue the lethality associated with the *Snr1* mutant. He hopes to uncover new functions of *SMARCB1* and *Snr1* using the *Drosophila* model system. RNA interference (RNAi) was used to knockdown the expression of *Snr1* in whole animals or in specific tissues with the UAS/Gal4 system. In the first instance, Dr. Walker is examining whether *Snr1* genetically interacts with NF2. Together these approaches using a *Drosophila* model system could improve understanding of the role of *SMARCB1* and its orthologs, providing a clearer path to tackling schwannomatosis.

Dr. Jeremie Vitte (House Ear Institute) from Dr. Marco Giovannini's group described a new mouse model of schwannomatosis. The promoter of the protein zero (P0) gene was used to knock out expression of *SMARCB1* (also known as *Snf5* or *INI1*) specifically in developing neural crest cells in a *P0Cre;Snf5/Ini1flox/flox* mouse. The resulting phenotype had reduced viability; and about 30% of the animals developed at least one of the following features between 1.5 and 4.5 months of age: tumors of the skull base, craniofacial abnormalities, and spinning behavior. On histological examination, tumors were malignant and poorly differentiated, without areas of low-grade tumor or better differentiation, and with scattered cells displaying rhabdoid features. This model will provide further mechanistic insights into schwannomatosis.

TARGETING NF1 SIGNALING

There are many challenges on the path to developing effective drug therapies for NF1. However, there has been considerable activity toward employing pharmacological inhibition as a therapeutic approach to counter the gain-of-function resulting from *NF1* LOH. A heterogeneous array of signaling pathways is involved in the causation of NF1 tumors; and within those tumors, multiple cell types are seen. Both of these facts complicate the unraveling and targeting of pharmaceutical pathways; and it is important to understand the downstream consequences of *NF1* loss.

Dr. Karen Cichowski (Harvard Medical School/Brigham & Women's Hospital) outlined these challenges and described some of the approaches that her group is taking to address them, that focus on the role of increased mTOR signaling downstream of *NF1* loss. Dr. Cichowski demonstrated that the effects of mTOR inhibitors are cytostatic and temporary in blocking tumor growth, emphasizing the importance of combination therapy that complements the cytostatic effects of mTOR inhibition. For this,

Dr. Cichowski focused on the potential value of ER stress-inducing agents and described experiments from her group looking at the synergism between the mTOR inhibitor, rapamycin, and several ER stress-inducing agent. Dramatic effects were seen when an Hsp90 inhibitor was combined with rapamycin. The therapeutic combination shows striking results in vivo against both *Nf1;p53* mutant tumors and activated *Kras;p53* mutant tumors. Dr. Cichowski's presentation raised important issues of how synergistic combinations of drugs can be identified, particularly in cases where the individual drugs act only weakly on their own.

Dr. Kevin Shannon (University of California, San Francisco) introduced the idea that although loss of *NF1* affects downstream signaling pathways, the upstream inputs, such as growth factors, are "co-conspirators" in promoting NF1 tumorigenesis. Understanding the signaling pathways downstream of these growth factor inputs and how they are remodeled during the process of tumorigenesis is important to designing more effective therapies. Dr. Shannon focused first on the MEK signaling pathway in NF1-associated leukemia and myeloid proliferative disease (MPD). He presented data on the differential response on MPD and acute myelogenous leukemia (AML) to MEK inhibition in vivo and suggested that tumors acquire "oncogene addiction" to the MEK signaling pathway as they progress. However, despite the initial response of AML to MEK inhibitors tumors eventually develop resistance. To address the issues of acquired resistance to drugs, Dr. Shannon turned to data from a mouse model of T-cell acute lymphoblastic leukemia (T-ALL) involving activating mutations in K-ras. He described that although all the leukemias derived from this mouse model carry the G12D activating K-ras mutation, the lines are heterogeneous for downstream signaling pathways. Through the introduction of second site mutations in K-ras his group has studied the effects of the Raf and PI3K signaling pathways on tumorigenesis potential. Dr. Shannon demonstrated that tumor cells undergo selective pressure to reactivate the missing signaling pathways. The ability of tumor cells to remodel their signaling pathways is critical for tumor cell growth and survival and also has important implications for drug resistance and the development of combination therapies.

Dr. Luis Parada (University of Texas Southwestern Medical Center) discussed the genesis of plexiform neurofibromas and how the cell of origin may provide a "window of opportunity" for tumor development that could have implications for the development of a preventative therapy. Dr. Parada noted that plexiform neurofibromas typically develop in childhood rather than adulthood, suggesting that the initiating cell type for these tumors may be embryonic in origin. Using mouse models that allow *Nf1* to be selectively ablated at different stages of Schwann cell development, Dr. Parada's group demonstrated that loss of *Nf1* is sufficient to induce plexiform neurofibromas in Schwann cell precursor cells and in immature Schwann cells, but not in mature Schwann cells. These results suggest that loss of *Nf1* must interact with other epigenetically regulated factors to initiate plexiform neurofibroma tumorigenesis, as mature Schwann cells lose the ability to form tumors. The identification of the factors important for tumorigenesis in Schwann cell precursors could give insights into new targets for prevention or treatment of plexiform neurofibromas.

PROGRESS IN NF PRECLINICAL DRUG TESTING

Preclinical drug testing in NF1 and NF2 has expanded significantly in the past few years, and was a focus of a number of presentations at the NF Conference. Dr. Jackson Gibbs (AstraZeneca) reviewed therapeutic targeting of the cellular-signaling pathways which have been demonstrated to be aberrantly upregulated in tumors in patients with NF1 and NF2 as a consequence of losing functional protein. The RAS-MAPK kinase pathway is activated in NF1 tumors and a variety of drugs are available to inhibit components of this pathway. Because of complex bio-feedback loops, multiple escape mechanisms exist which may render growth relatively resistant to a single drug. As a result there is significant interest in utilizing combined or multiple biologic agents in synergy to attack different parts of the pathway to overcome this means of resistance. In NF1, mTOR inhibition has been targeted by multiple approaches, and a number of molecular targets are now identified for NF2. Tolerability of the drugs is likely to become a major issue, and is already being encountered in some NF trials as described later. Given the chronic nature of many of the disease manifestations of NF1 and NF2, including plexiform neurofibromas, schwannomas and low-grade gliomas, it is unlikely that drugs with a high degree of toxicity will be acceptable to treat these chronic manifestations of disease.

Dr. Eva Dombi (National Cancer Institute) described the preclinical testing of RAD001 (a rapamycin analog) and Sorafenib (a multi kinase inhibitor) on a GEM *Nf1* neurofibroma model in which RAD001 had little or no effect while Sorafenib resulted in tumor shrinkage in some animals. The same model will be used to test molecularly targeted agents to aid in the prioritization of drugs for future clinical trials.

Dr. Janet Oblinger (Ohio State University) presented NF2 preclinical data on two novel small molecule drug candidates, HDAC42 and OSU-03012. OSU-03012 inhibits phosphoinositide-dependent kinase 1 (PKD1), which phosphorylates and activates the pro-survival protein Akt. HDAC42 is a histone deacetylase (HDAC) inhibitor that inhibits Akt activation and other mitogenic signaling pathways. Both drugs potently decrease schwannoma cell proliferation with IC₅₀ values in the low micromolar (OSU-03012) and high nanomolar (HDAC42) range. These anti-proliferative effects correlated with a strong inhibition of critical pro-survival signaling pathways. Akt phosphorylation was reduced in schwannoma cells and OSU-03012 reduced the size of HMS-97 xenograft tumors by ~55% and HDAC42 by ~58% in mice fed with drug.

Dr. C. Oliver Hanemann (Peninsula University) reviewed his studies targeting insulin-like growth factor and its binding protein (IGF/IGFBP) signaling in an in vitro model of human schwannomas. Dr. Hanemann showed over-expression/activation of PDGFR-beta and ErbB2/3 in schwannoma leading to strong activation of extra-cellular-signal-regulated-kinase 1/2 (ERK1/2) and AKT as well as increased proliferation. This was successfully inhibited by the each of the drugs Sorafenib, AZD6244, and Lapatinib. Dr. Hanemann investigated insulin-like-growth-factors-I/II (IGF-I/II) as potential additional factors and showed that IGF-I/II and IGFBP-1 are over-expressed/released from schwannoma cells and that they increase proliferation and adhesion of these cells. IGF-I-receptor is also over-expressed/activated in schwanno-

ma cells. These findings suggest that IGF/IGFBP signaling is involved in schwannoma development. Targeting IGF/IGFBP-system together with PDGFR-beta and possibly ErbB2/3 pathways, therefore represents a potential treatment approach.

Fabrice Chareyre (House Ear Institute) from Dr. Marco Giovannini's group focused on targeting aberrant EGFR activation (this is linked to Merlin deficiency) and reported that covalent pan-erbB inhibitors appear to be most effective in accomplishing this, and is one strategy that is being pursued by Dr. Giovannini's group as part of the Children's Tumor Foundation Neurofibromatosis Preclinical Consortium.

PROGRESS IN NF CLINICAL DIAGNOSIS AND MANAGEMENT

NF clinical management continues to evolve and the NF Conference has proved to be a good venue for the clinicians to share updated information. This year a variety of abstracts were presented on a range of clinical management topics. Dr. Robert Avery (Children's Hospital of Philadelphia) presented an innovative method to screen and monitor visual function in children with optic pathway gliomas. Optical coherence tomography was used to measure thickness of the retinal nerve fiber layer, which served as a biomarker for visual loss. This could potentially be used even in young children when they are sedated for an MRI scan. Dr. Michael Fisher (Children's Hospital of Philadelphia) presented a retrospective, multi-center study of visual outcomes following chemotherapy for optic pathway gliomas in NF1. Of 86 evaluable patients, visual acuity improved in 31%; remained stable in 40%; and worsened in 29%. Poor prognostic factors for progressive visual loss despite treatment included tumor location in the optic tracts and radiations, and age >5 years.

Two studies looked at the natural history of plexiform neurofibromas in NF1 children and adults. Dr. Rosa Nguyen (University Hospital Hamburg-Eppendorf) from Dr. Viktor Mautner's group reported on 195 patients with NF1 who underwent whole body MRI. They found internal plexiform neurofibromas in 48% of patients and observed that more rapid tumor growth occurred in younger patients. No new plexiform tumors were found in patients followed over time, confirming that these tumors are likely to be either congenital or to have onset in early childhood. Dr. Carlos Prada (Cincinnati Children's Hospital) reported data on 154 children with NF1 and plexiform neurofibromas; 30% were found to have plexiform tumors most commonly in head and neck, followed by extremities. Average age at detection of tumor was 5.5 years; 62% required surgical procedures, including seven patients requiring tracheostomy. Patients were more likely to benefit from surgery if the indications were to relieve airway compression or physical disfigurement.

Dr. Stéphane Goutagny (Hôpital Beaujon) reported on the natural history of meningiomas in NF2 from a study of 119 NF2 patients followed up with annual MRI. Seventy-three patients (61.3%) with intra cranial meningioma(s) were included. Three hundred and three meningiomas were followed up for an average 9 years. In adult patients, only 12 new meningiomas arose in nine patients (29–62 years old); 42 (57%) patients have been

operated on for meningioma at least once (50 tumors). Pathology showed aggressive features (grades 2 and 3) in 23%, more frequently than in sporadic meningiomas (10–15%). Dr. Goutagny concluded that meningioma development and pattern of growth in NF2 patients is unpredictable. However, meningioma burden is usually present from early adulthood. A tailored treatment should be based on the knowledge of the natural history of each individual meningioma in each patient.

Dr. David Stevenson (University of Utah) presented data on 25-OH vitamin D levels in a cohort of 109 children with NF1 compared to 218 controls matched by age, gender, and season. A significantly greater percentage of NF1 patients had 25-OH vitamin D levels in the insufficient or deficient range (50%), compared to 36% of controls. Dr. Maya Lodish (National Institute of Child Health and Development) performed DEXA scans on 34 children with NF1 and calculated bone mineral apparent density (BMAD) and whole bone mineral content/height (BMC); both measures take account of short stature. Dr. Lodish found osteopenia at any one-bone site in 48% of patients, most commonly at the lumbar spine. Two-thirds were severely or mild-moderately vitamin D deficient, but deficiency did not correlate with BMAD.

A few presentations focused on cognitive issues of NF1. Dr. Jonathan Payne (University of Sydney) examined real-world neurocognitive functioning in 216 children with NF1 and 55 sib controls and compared results with formal neuropsychological tests. They did find real-world attention and executive deficits in the children with NF1, but correlation with formal testing was inconsistent. This raises the question of whether these formal tests are good predictors of real-world functions in this population. Dr. Nadja Kadom (Children's National Medical Center) examined a potential neuroimaging cognitive function biomarker, abnormal signal in the frontal cingulate white matter. In a study of 62 children with NF1 compared with 62 age-matched non-NF1 affected controls, Dr. Kadom found that bright T2/FLAIR signal in the frontal subcortical cingulate white matter had greatest sensitivity for cognitive dysfunction in children 2–5 years of age and the greatest specificity in children >5 years of age. The need for high level of neuroradiologist experience in detecting the sign was noted. The work of Dr. Natalie Pride, presented by Dr. Kathryn North (University of Sydney) examined morphology of the corpus callosum as a marker of cognitive dysfunction. Comparison of 46 children with NF1 to 30 controls demonstrated a significantly enlarged corpus callosum in the NF1 patients and correlation of corpus callosum size with lowered IQ and several measures of cognitive dysfunction. They note that some cognitive deficits may have a structural basis and may not be reversible with pharmacological treatments, in contrast with other NF1-associated learning problems.

Moving to a focus on schwannomatosis, Dr. Miriam Smith (University of Manchester) from Dr. D. Gareth Evans' group explored the mechanism of NF2 gene inactivation using a panel of 240 schwannoma tumors: 98 NF2-related schwannomas, 104 sporadic vestibular schwannomas (VS), and 38 schwannomatosis-related schwannomas. In total, germline NF2 mutations were identified in 89% of NF2 patients and a second mutational event in 78%. Loss of heterozygosity was by far the most common form of second hit. Conventional comparative genomic hybridization

(CGH) or a combination of multiplex ligation-dependent probe amplification (MLPA) and microsatellite analysis, identified mitotic recombination (MR) as the cause of LOH in 39% of evaluable tumors. MR only accounted for 19% of sporadic VS LOH and none of the LOH in SMARCB1 patients. In contrast, five of 22 tumors from schwannomatosis patients with no known germline SMARCB1 mutation, harbored tumors exhibiting MR. High-resolution Affymetrix SNP6 genotyping revealed a range of unique recombination sites over a region of approximately 11.4 megabases. MR appears an important mechanism of second hit in NF2 related schwannomas.

DIAGNOSTIC DILEMMAS: A LIVELY DEBATE

A significant amount of controversy remains around certain NF clinical questions. Dr. Rosalie Ferner (Guys and St. Thomas's NHS Foundation Trust London) and Dr. Robert Listerick (Northwestern University) chaired a unique evening session comprising a series of debates between expert clinicians taking different perspectives. The debated topics were geared to challenge assumptions regarding common diagnostic and therapeutic problems. The discussants were assigned polarized viewpoints so as to stimulate further discussion and spirited interaction with the audience. Participants in the debates included Dr. Tena Rosser (Children's Hospital Los Angeles), Dr. Michael Fisher (Children's Hospital of Philadelphia), Dr. David Stevenson (University of Utah), Dr. Douglas Stewart (National Human Genome Research Institute National Institutes of Health), Dr. Michel Kalamarides (Hopital Beaujon), and Dr. Jaishri Blakeley (John's Hopkins University). In summary, this unique session highlighted the value of clinical discussion in determining complex areas of NF clinical diagnosis and management. Some highlights are presented below.

Question: Should all NF1 patients have whole body MRI at time of diagnosis to assess the burden of disease? The "pro" discussant presented evidence showing efficacy and relative ease with which whole body MRI can identify the burden of NF1 plexiform neurofibromas. Given that as many as 50% of NF1 patients have one at least one internal plexiform neurofibroma, whole body imaging provides the opportunity to identify and intervene when tumors are early stage. The opposing viewpoint argued that, as yet, no approved therapy for growing plexiform neurofibromas exists. In addition, the need to sedate toddlers and young children before whole body MRI suggests that the cost of whole body MRI outweighs the benefits.

Question: Should all NF1 patients should undergo genetic testing at the time of diagnosis? The "pro" discussant highlighted the value of this information because there are several known genotype–phenotype correlations in NF1 individuals: (1) whole gene deletions are associated with early appearance of large numbers of neurofibromas, more severe cognitive disabilities, and distinctive dysmorphic features and (2) a 3 bp in-frame deletion in exon 17 in which one sees multiple café-au-lait spots and intertriginous freckling but no cutaneous or plexiform neurofibromas. In addition, potential misdiagnosis of young children who actually have Legius syndrome (NF1-like syndrome with a milder phenotype) points further to the usefulness of genetic testing. The opposing viewpoint observed that genetic testing for the vast

majority of individuals with NF1 would not alter clinical care and in addition the cost would be prohibitive. Instead, targeted testing of individuals with the distinctive phenotypes as described is preferable.

Question: Is there value in inserting a sleeper auditory brainstem implant (ABI) at the time of first VS surgery in all NF2 patients? The audience watched a video of a lively, dramatized counseling session with a “patient” that underscored the positive aspects of ABI insertion at the time of the first surgery for vestibular schwannoma. From a patient perspective, ABI would provide important reassurance for the future if the hearing in the contralateral ear deteriorated, and an opportunity for early training with the ability to hear auditory sensations. Repeat surgery on the operated side may be more difficult because of scar tissue, and may not even be funded by insurance, hindering later placement of a device.

The opposing viewpoint argued that the insertion of an ABI renders subsequent MR imaging of the brain and spine difficult due to the presence of the magnet. An ABI that is not required for a lengthy period might malfunction and patients will not benefit from technical advances in ABIs in the interim. Further, sleeper ABIs do not always require activation, resulting in unacceptable cost.

A series of presentations then focused on unusual clinical cases and ‘diagnostic dilemmas’. Dr. Robert Listernick described a child with a facial plexiform neurofibroma and ipsilateral facial flushing and sweating in response to oral or olfactory stimuli since infancy (called auriculotemporal nerve syndrome or Frey syndrome). The pathogenesis of Frey syndrome is due to aberrant regeneration of parasympathetic fibers into damaged sympathetic nerve pathways, often after parotid gland surgery, leading to the development of inappropriate connections between parasympathetic secretomotor nerves and distal sweat glands and blood vessels. In the case of this NF1 patient, it was postulated that rapid plexiform neurofibroma growth had led to these abnormal neural connections.

Juvenile xanthogranulomas are seen in a small proportion of young children with NF1, occurring predominantly on the trunk and head. However, Dr. Eric Legius (University of Leuven) showed a patient with unusual ocular xanthogranuloma involving the sclera. This was treated with steroid injection and surgery. Dr. Rosalie Ferner and Dr. Susan Huson (University of Manchester) presented two patients with clinical manifestations that were benign but could be potentially mistaken for a serious NF1 complication. One patient being assessed for possible segmental neurofibromatosis had unusual linear pigmentation on the upper limb. It transpired to be a tanning lotion which left a streaky appearance on the skin! Visibly this could be easily confused with mosaic NF1. A young male was referred with NF1 and a rapidly enlarging firm swelling below the knee, erroneously thought to be an MPNST. Was this a hematoma? No—the diagnosis was prepatellar bursitis. Dr. Arvid Heiberg (Rikshospitalet Oslo) presented a difficult-to-diagnose patient with LEOPARD syndrome and cutaneous neurofibromas, lentiginos, pulmonary stenosis, spinal nerve root tumors, and a unilateral vestibular schwannoma. This patient highlighted the clinical overlap between NF1 and other RAS pathway conditions. Finally, Dr. Anat Stemmer Rachamimov (Harvard Medical School/Massachusetts General Hospital) highlighted the value of utilizing pathology in NF diagnosis, showing that the pathology of

an intrinsic cervical cord tumor, confirmed by pathology as an ependymoma, a common site for this lesion in NF2.

NF1 MODIFIER GENES: A SPECIAL FOCUS

Among the hallmarks of NF1 is a high degree of variable expressivity, a feature that has been attributed to modifier genes, and this now constitutes an emerging area of NF research. Observations that significant variability exists within NF1 pedigrees provided the first clue that unlinked modifier genes and/or environmental factors affect the severity of this disorder. Additional evidence came from a study of 175 NF1 individuals, including six monozygotic (MZ) twin pairs. High degrees of concordance between twins and first degree relatives argued that symptom-specific modifier genes are major factors contributing to clinical variability [Easton et al., 1993]. Given that modifier genes may control rate-limiting steps during disease development, they can provide clues to underlying mechanisms, and serve as therapeutic targets. Several groups continue to pursue the identification of NF1 modifiers, using a variety of approaches. In view of the important nature of this topic, at the 2010 NF Conference, Dr. Meena Upadhyaya (Cardiff University) and Dr. André Bernardis (Harvard Medical School/Massachusetts General Hospital) moderated a special discussion panel reviewing the latest progress in research on NF1 modifier genes.

Dr. Mark Daly (Massachusetts General Hospital Center for Human Genetic Research and the Broad Institute) gave a general introduction and reviewed how genome-wide association studies (GWAS) often implicate multiple loci in complex genetic disorders, such as inflammatory bowel disease. Interestingly, disease-associated genes may encode functionally related proteins, providing important clues to underlying pathways. Dr. Bruce Korf (University of Alabama at Birmingham) reminded us that while genotype–phenotype correlations are uncommon in NF1, they do exist. Patients with a recurring 1.4 Mb genomic microdeletion that removes 14 protein-coding genes including the *NF1* gene, can exhibit severe phenotypes, often having large numbers of early-onset neurofibromas [Kayes et al., 1994]. By contrast, a presumably hypomorphic single amino acid deletion in *NF1* exon 17 is associated with the absence of cutaneous neurofibromas [Upadhyaya et al., 2007], while NF1 patients presenting with bilateral spinal neurofibromas have a greater proportion of missense and splice site mutations than do classical NF1 patients [Upadhyaya et al., 2009]. Genetic mosaicism is also not uncommon in NF1 and can confound diagnosis in mildly affected patients. The recent identification of *SPRED1* mutations in patients with an NF1-like disorder [Brems et al., 2007], indicates that patients with atypical NF1-like symptoms may also be affected by mutations in other, yet to be identified genes.

Dr. Karlyne Reilly (National Cancer Institute) reviewed the use of mouse NF1 models to dissect the complex genetic and epigenetic interactions underlying cancer susceptibility [Reilly et al., 2004, 2006]. Dr. Reilly’s group is currently trying to identify imprinted modifiers involved in malignant peripheral nerve sheath tumor. Because NF1 is a complex disease involving many organ systems, it provides an opportunity to understand how genetic susceptibility affects a wide variety of phenotypes within an individual, and whether modifiers act specific to molecular pathways or specific

to organs or tissue types. Recent developments in mouse systems genetics are making it more practical to identify modifiers across multiple phenotypes using reference strain panels, such as the Collaborative Cross [Churchill et al., 2004]. Dr. Reilly proposed a collaborative effort to study modifiers of NF1 using the Collaborative Cross as a way of opening up the field of NF1 modifier research.

Dr. Douglas Stewart (National Human Genome Research Institute) described his study to identify 'quantitative trait transcripts', whose differential expression correlates with specific phenotypes. For this purpose he analyzed genome-wide gene expression in lymphoblastoid cells from 79 extensively phenotyped NF1 patients and 23 controls. Interestingly, the *MSH6* mismatch repair gene was one of three genes whose expression level significantly correlated with the number of café-au-lait spots, a phenotype that, like NF1 tumors, requires bi-allelic *NF1* inactivation. This is especially interesting in light of previous observations that children homozygous or compound heterozygous for *MSH6* mutations develop an NF1-like phenotype [Menko et al., 2004; Ostergaard et al., 2005].

Dr. André Bernards described three approaches to identify NF1 modifiers, including a GWAS of 300 persons with NF1 representing the top and bottom 15% of dermal tumor burden. Work with Dr. Eric Legius' group (Belgium) is assessing the possible involvement of haploinsufficient tumor burden modifier loci contained within the 1.4 Mb *NF1* microdeletions. A collaborative study with Dr. Efthimios Skoulakis' group (Greece) has identified that genetic or pharmacological inhibition of the neuronal Alk tyrosine kinase receptor (Alk) in *Drosophila NF1* mutants, restored organismal size, associative learning, and ERK over-activation defects, implicating human ALK as a potential rate-limiting activator of NF1-regulated Ras signaling pathways.

Dr. Pierre Wolkenstein (French Referral Centre for Neurofibromatosis) reviewed evidence supporting the idea that NF1-associated variability is indeed related to genetic modifiers. In preparation for an initial candidate gene study, and a later subsequent GeneChip 6.0 GWAS based study, to identify modifiers of NF1 phenotypes, the NF France network has collected and phenotyped 1,083 NF1 patients from 561 families [Sabbagh et al., 2009]. A second similar sized patient/control cohort will be required to validate future findings.

Dr. Elizabeth Schorry (Cincinnati Children's Hospital) presented her evaluation of phenotypic concordancies and discordancies among MZ NF1 twins. Ten pairs of MZ twins and triplets, plus 26 pairs of published NF1 MZ twins were analyzed. Highly concordant features possibly controlled by modifier genes included the overall number of café-au-lait spots and cutaneous neurofibromas, the presence of learning disabilities, ADHD and speech disorders, skeletal deformities, and Chiari I malformations. More discordant features included the presence of plexiform neurofibromas, optic gliomas, scoliosis, and MPNST. An assessment of copy number variation as a possible indicator of such discordancy is planned in NF1 MZ twins.

Overall, this session left the audience with the impression that significant progress is being made towards the identification of NF1 modifiers, and that ongoing work may reveal new insights into mechanisms underlying this complex disorder.

FUTURE OPPORTUNITIES AND CHALLENGES IN NF2 RESEARCH

Dr. Jonathan Chernoff (Fox Chase Cancer Center) chaired a panel discussion to explore new thinking in NF2 research. The panel included Dr. Andrea McClatchey and Dr. Vijaya Ramesh (Harvard Medical School/Massachusetts General Hospital), Dr. Marco Giovannini (House Ear Institute), Dr. Filippo Giancotti (Memorial Sloan Kettering Cancer Center), Dr. Gareth Evans (University of Manchester), and Dr. Oliver Hanemann (Peninsula University). A very lively discussion led to the following priorities to be addressed in order to further our understanding of the basic biology of NF2 and Merlin protein; and to advance our ability to develop effective drug treatments.

- (1) *Biochemistry*. While Merlin is similar to other ERM proteins, it also has important differences, and we do not yet have a structure of full-length Merlin. What are the structures and the functions of Merlin isoforms 1 and 2, and of their heterotypic interactions? In addition, since Merlin isoform 2 is likely in an open conformation in its active state, it is possible that the two isoforms of Merlin signal differently.
- (2) *Cell biology*. Merlin is part of a large-multi protein complex, and the nature and location of this complex may well be cell context and/or cell-type dependent. Defining the elements and context of such complexes remains an important short-term goal in NF2 research.
- (3) *Signal transduction*. There appear to be important cell membrane as well as nuclear functions for the Merlin protein. We need to know the relative contributions of Merlin at these two locations to tumor suppression and to contact inhibition, and whether the effects that Merlin has on mitogenic signaling are direct or indirect. In addition, given the genetic link between Merlin and Hippo in flies, we need to determine whether Merlin activates Hippo in mammalian cells and by what mechanism (at the membrane or further downstream), and whether this latter mechanism contributes to contact inhibition and/or tumor suppression. Finally, we also need to identify the targets and thereby the mechanism of action of the ubiquitin ligase CLR4-DCAF1.
- (4) *In vitro models*. Human in vitro models have been shown to reasonably reflect the human tumor, and to be useful for testing drugs, which can inform phase 0 trials, especially for drugs for which toxicity and PK are known. As schwannoma and meningioma cells may signal differently, it will be important to use appropriate in vitro models representing these different cell types, as well as appropriate patient-matched controls. The heterogeneity seen within tumors in vitro, as well as additional events that may occur in tumors in vivo, pose challenges for the use of these models for research and drug testing.
- (5) *Correlating genetics to clinical profile*. We still lack a good explanation for why certain mutations in the *NF2* gene produce a worse phenotype than others (e.g., truncating mutations vs. internal deletions). Is there proof of a dominant negative effect? Do some mutations in *NF2* favor or promote second hits in the remaining allele?

- (6) *Clinical.* How do we select cases for drug treatment, and which tumors do we target? Vestibular schwannomas are easiest to consider in trial design, but this ignores meningioma issues. Also, should unilateral vestibular schwannomas also be considered in clinical trials?

NF CLINICAL TRIALS: PAST, PRESENT, AND FUTURE PERSPECTIVES

Looking back only five years ago, we can state that the NF Conference was then devoid of clinical trials presentations. However, recent advances in understanding NF biology are now being translated into innovative treatment approaches that may ultimately alter the natural history of NF; and the 2010 NF Conference including a full afternoon session focusing on the current status and outlook of NF clinical trials. Dr. Brigitte Widemann (National Cancer Institute) summarized lessons learned from some of the earliest clinical trials conducted to test therapies for NF1 plexiform neurofibroma and malignant peripheral nerve sheath tumor (MPNST). The first NF1 plexiform neurofibroma clinical trial compared oral farnesyl transferase inhibitor (FTI) to placebo, in a cross-over design trial. Although drug treatment did not cause a statistically significant increase in time to disease progression compared to placebo, the study demonstrated the feasibility of utilizing three-dimensional volumetric magnetic resonance imaging (MRI) analysis to determine the primary trial endpoint. NF1 plexiform neurofibroma growth rate can be reproducibly and sensitively measured by semi-automated volumetric MRI techniques; which showed that once plexiform neurofibromas begin to grow this tends to continue at a predictable growth rate over the next few years, especially in young children [Dombi et al., 2007]. Following the FTI trial, volumetric imaging has become a more widely used approach for monitoring these tumors in clinical trials. The FTI trial also provided data from a control arm that has been used since then as a comparison arm for future single-arm studies. A variety of NF1 plexiform neurofibroma clinical trials are ongoing or have recently been completed, including Pirfenidone, Peg-Interferon Alpha-2b, Imatinib, Sirolimus, Vinblastine with Methotrexate, and photodynamic therapy.

Entry criteria and outcome measures for NF clinical trials must be carefully chosen, as some past clinical trials have proved difficult to compare to each other due to different entry criteria and outcome measures. Three-dimensional imaging is being progressively more utilized for assessment of NF1, NF2, and schwannomatosis tumor burden. Dr. Scott Plotkin (Harvard Medical School/Massachusetts General Hospital) reviewed status of an ongoing study of whole body MRI obtained in a 45-min session. Data on 183 accrued (of 300 planned) subjects age 18–97 (mean 41) were presented. The extremities—especially the legs—harbor the greatest number of tumors, followed by the thorax, pelvis, and abdomen. Ambereen Kurwa (National Cancer Institute) presented a study of whole body tumor burden in NF1 using MRI and a semi-automated body lesion detection program to measure tumor volume. Sixty-seven patients age 3–30 (median 13) have been studied to date; 65 persons had 95 measurable lesions throughout the body—the trunk being the most common site. Substantial tumor burden was observed, and spinal

neurofibromas involving all levels of the spinal cord were present in 62% of those imaged.

In summary, the breadth and quality of NF clinical trials of targeted biological therapies, which are ongoing, near completion or planned, are extremely encouraging. The implementation of the NF Phase II Clinical Trials Consortium and other multi-site collaborative efforts has helped with issues such as resource sharing and rapid recruitment. In addition, as these trials are progressing, innovative outcome measures such as imaging and biomarkers are being integrated to improve trial design. Detailed updates on NF1 and NF2 clinical trials are provided below.

NF1 CLINICAL TRIAL UPDATES

The Department of Defense sponsored NF Phase II Clinical Trials Consortium is now in the process of completing an NF1 plexiform neurofibroma trial of Sirolimus (Rapamycin) that includes patients with, or without, radiographic disease progression; an update on this trial was provided by Dr. Michael Fisher (Children's Hospital of Philadelphia). For patients with non-progressive symptomatic lesions, radiographic objective response within 6 months, as determined by volumetric analysis, was defined as being necessary for continuation of treatment. Treatment with Rapamycin was well tolerated, with little dose-limiting toxicity, but did not result in radiographic response. The treatment arm entering patients with radiographic progressive disease is ongoing. Importantly, this study has also been the first to demonstrate the feasibility of a multi-site trials consortium to perform a rapid Phase II trial utilizing real-time pharmacologic assessment from a centralized facility.

Dr. Dusica Babovic-Vuskanovic (Mayo Clinic) reviewed a trial of Cediranib, a small molecule inhibitor of VEGFR as well as c-Kit and other serine-threonine kinases. The drug was assessed in a multi-site Phase II trial of 26 adults with NF1. To date, the study has seen a mean duration of therapy of 7.8 months and no progression of disease in 10 evaluable patients. However, 13 patients withdrew from treatment due to drug toxicities, which included hypertension, diarrhea, and weight loss.

The National Cancer Institute's Pediatric Oncology Branch has undertaken a Phase I trial of Sorafenib, an oral receptor tyrosine kinase inhibitor, in children with NF1 and inoperable plexiform neurofibromas. Dr. Aerang Kim (National Cancer Institute) reviewed this trial. Data on the six children entered into this study to date were reported. The highest dose saw some grade 3 dose-limiting pain—a toxicity not previously described for Sorafenib. As a result, the dose for children with NF1 and plexiform neurofibromas has now been lowered below the recommended dose for children with malignant solid tumors.

A variety of studies have assessed therapies for MPNSTs; however, because of this tumor's relative rarity in NF1, the logistics of MPNST trials have been difficult [Widemann, 2009]. To address this, an agreement has recently been reached between the NF Clinical Trials Consortium and the Sarcoma Clinical Trials Group (SARC), who are active in developing clinical trials for MPNST. Through this agreement the two consortia will collaborate on a Phase II trial of Bevacizumab combined with RAD001 for patients with sporadic or NF1 related refractory MPNSTs. This study marks an important transition into what may be a future trend, the testing

combined therapies in NF1 tumors. Roger Packer reviewed an international study led by the Children's National Medical Center including patients in Sydney, Tel-Aviv, and Washington, DC that assessed drug combination of Tarceva and Rapamycin in 16 patients with recurrent low-grade gliomas including seven patients with NF1. All patients were treated with the drug combination for 28 days. Toxicity was mild; with the NF1 patients tumors stabilized or decreased and two patients have remained progression-free for greater than one year. Cognitive defects affect an estimated two-thirds of individuals with NF1.

Maria Acosta (Children's National Medical Center) summarized a recently completed Phase I trial of Lovastatin for children with NF1 and cognitive deficits. Twenty-four patients were treated for three months. Lovastatin was extremely well tolerated and no safety concerns arose during study period. A subgroup of patients underwent 12-hr pharmacokinetic analysis and it was found that the concentration of drug in plasma was extremely variable between participants, as were drug-related changes in cholesterol levels. Although a Phase I toxicity trial, intellectual testing of participants indicated specific areas of cognitive improvement, including memory, recall, and recognition. Interestingly, a reduction in cholesterol level was positively correlated with improved cognitive function. This information provides valuable support for the now ongoing multisite Phase II study ongoing by the NF Phase II Clinical Trials Consortium.

NF2 CLINICAL TRIAL UPDATES

Advancing NF2 clinical trials has perhaps been even more challenging than the same for NF1 trials. There are significantly fewer affected individuals; for many years, there was a lack of agreement between centers on using clinical intervention approaches surgery or radiotherapy; and until recently there were no clear candidate drug targets or agreed trial models. Recently, however, an overall consensus has been reached by the NF2 community to pursue a strategy Phase 0 and pilot Phase II clinical trials [Evans et al., 2009]. Dr. D. Bradley Welling (The Ohio State University) reviewed the status and outlook for NF2 clinical trials. New biologic insights into NF2 have brought to the fore potential therapeutic targets such as the vascular endothelial growth factor receptor (VEGFR). The NF2 clinical community is working closely together in a loose consortium to advance trials. A Phase 0 study of the EGFR and ErbB2 targeted drug Lapatinib is underway, in which patients receive the drug prior to surgical excision of the tumors. The tumors can then be analyzed to understand if the drug had a biologic effect on the tumor as monitored by decreased phosphorylation of EGFR. Lapatinib has antiproliferative activity in a preclinical NF2 vestibular schwannoma (VS model) providing rationale that it has potential in the clinic [Ammoun et al., 2010]. Dr. Matthias Karajannis (New York University Langone Medical Center) described a new Phase II Clinical Trial of Lapatinib in children and adults with NF2-related tumors. In a two-stage clinical trial design, NF2 patients older than 3 years of age with progressive VS are eligible. Lapatinib is administered continuously for 28-day courses. The primary endpoint is defined as a decrease of at least 15% in tumor volume. Enrollment of the first trial stage has been completed with nine eligible patients. Two patients discontinued protocol therapy

after three treatment cycles due to radiological progression. One of three evaluable patients to date had a 16.6% reduction in the tumor volume of his VS after three cycles. The remaining six patients continue on trial.

Dr. Scott Plotkin (Harvard Medical School/Massachusetts General Hospital) reported on the extended follow up of 29 NF2 patients treated with Bevacizumab for progressive VS, following up on the initial findings published last year [Plotkin et al., 2009]. The annual tumor volumetric growth rate prior to treatment was 80%. Four patients discontinued treatment after a median of 14 months: two stopped treatment due to disease progression (one due to hearing loss and one due to tumor growth), one died of complications related to surgery after discontinuing Bevacizumab, and one was unable to return to clinic for follow up. A number of patients continue on treatment up to 30 months sometimes on a reduced dose schedule. Fifty percent of VS showed a RECIST 20% volumetric decrease; with 70% showing some decrease in volume.

ACROSS THE RAS-MAPK PATHWAY

Over the last 5 years, multiple conditions have been shown to be caused by mutations encoding different proteins of the Ras-MAPK pathway; including Noonan syndrome, cardio-facial-cutaneous syndrome, Costello syndrome, Legius syndrome, and NF1. Shared phenotypes that were explored in this session included hypertrophic cardiomyopathy, pigmentary dysplasia, learning disabilities, and orthopedic manifestations. A session of the conference explored the shared phenotypes of these genetic syndromes collectively called the 'rasopathies'. This session was chaired by Dr. David Viskochil (University of Utah) and Dr. Maria Acosta (Children's National Medical Center).

Dr. Jonathan Epstein (University of Pennsylvania) started the session by reviewing his work on the tissue-specific knockout of murine *Nf1* in adult myocardium. Remarkably, aging mice with cardiomyocyte loss of *Nf1* developed progressive cardiomyopathy, fibrosis, dilatation and cardiac failure. Even though humans with NF1 do not generally develop cardiomyopathy, other aspects of the *Nf1* mutant cardiac phenotype in mice show the similarities to CFC and Noonan syndromes. Dr. Epstein pointed out that the phenotype could not be entirely rescued with the NF1-GRD (GAP-related domain), which led to his speculation that functional domains of neurofibromin outside of the GRD have roles in cardiomyopathy. His lab is presently attempting rescue with full-length *Nf1* to compare the resultant phenotypes.

Dr. Epstein went on to provide an elegant demonstration of a novel animal model, zebrafish. There are two zebrafish orthologs of NF1 with 90% amino acid homology (*nf1a* and *nf1b*). Each has 57 exons encoding large proteins of ~310 kDa and ~2,750 amino acids in length. There is significant conservation in the GRD and other regions, which supports the contention that other functional domains exist that either modify the role neurofibromin plays in the Ras-MAPK pathway or the existence of novel pathways contributing to the NF1 phenotype. The genes are expressed in the developing zebrafish cardiovascular system and morpholino knockdown leads to pericardial effusions, vascular patterning defects and to abnormalities in cardiac and neural crest structures. The zebrafish model now provides a way to evaluate the role of NF1

in vascular patterning, specifically primary vascular defects versus phenotypes secondary to cardiac failure. It can also be used to identify other functional protein domains outside the GRD and to perform relatively high throughput chemical screens.

Dr. Eric Legius (University of Leuven) presented a clinical update on the natural history of another RAS-MAPK pathway condition that he has identified, Legius syndrome. Dr. Legius has followed this condition in a group of families he has followed since 1990. They were identified initially as “mild NF1 cases,” and in 2007 the affected family members were shown to have mutations in the *SPRED1* gene on chromosome 15 [Brems et al., 2007]. It is currently recognized as an independent syndrome, and it is present in 1–4% of all patients with the clinical diagnosis of NF1. Legius syndrome has the appearance of ‘mild’ NF1 with cafe-au-lait spots +/- freckling, macrocephaly, and cognitive issues. Absent are optic nerve pathway tumors, neurofibromas, Lisch nodules, and the distinctive bone abnormalities of NF1 [Messiaen et al., 2009]. One child in this cohort has T2 hyperintensities on brain MRI.

An important aspect of Legius syndrome is the presence of learning disabilities. Mice and fly models have been developed to better understand the effect of *SPRED1* mutations in normal and abnormal learning. *Spred1*^(-/-) mice show decreased learning and memory performance in the Morris water maze and visual-discrimination T-maze, but normal basic neuromotor and sensory abilities [Denayer et al., 2008]. Initial studies have attempted to establish the effects of lovastatin in rescue of the learning problems in the *Spred1*^(-/-) mouse model. Those studies are still in process. There is also a proposal for the generation of an international clinical and molecular database of Legius syndrome patients so the condition can be better understood.

The session closed with a detailed description of the skeletal phenotype in syndromes of the Ras-MAPK pathway, which was presented by Dr. David Stevenson (University of Utah). He reviewed the osseous phenotype in NF1, LEOPARD, Noonan, CFC, and Costello syndromes with particular focus centered on chest wall deformities, kyphoscoliosis, short stature, osteopenia, and hip dysplasia. Key findings included the high incidence of pectus deformities in LEOPARD syndrome (~75%) and the incidence of hip dysplasia in Costello syndrome (~15%). Osteopenia is notable in Costello syndrome and NF1, but is not such an issue in LEOPARD/Noonan/CFC syndromes. Dr. Stevenson screened urine for pyridinoline and deoxypyridinoline as an indicator of bone resorption, and he showed increased dpy/pyd ratios in NF1, Costello, CFC and Noonan syndrome patients. These data suggest a role for Ras-MAPK activation in bone remodeling, but do not account for the discordant phenotypes between syndromes that all have increased ERK activation.

NEW PERSPECTIVES FOR NF: KEYNOTE PRESENTATIONS

It is increasingly clear that the mTOR pathway, a major regulator of growth in eukaryotes, is deregulated in both NF1 and NF2. Dr. David Sabatini (Whitehead Institute, MIT) delivered a keynote presentation focused on his recent work on the upstream regulation of the pathway. One of the most interesting aspects of the pathway is

that it is regulated by many upstream signals, such as growth factors, nutrients, and energy levels. How the pathway integrates these signals to set cellular growth rates is unknown. Dr. Sabatini and colleagues have recently identified the Rag GTPases and the Ragulator protein complex as essential mediators of nutrient, in particular amino acid, sensing by the mTOR pathway. The essential function of Rag-Ragulator is to mediate—in an amino acid dependent fashion—the translocation of mTOR to the lysosomal surface where it can bind to its activator Rheb. In an unknown fashion, amino acids promote the loading of the Rag GTPases with GTP and the Rag-GTP complex then serves as a docking site for mTOR on the lysosomal surface. The Ragulator, in turn, tethers the Rags to the lysosomal surface. So far we know little about how amino acids are actually sensed. In his presentation, Dr. Sabatini also showed the cryo-EM structure of mTOR Complex 1 (mTORC1) and how this structure is perturbed by the mTOR inhibitor rapamycin. This drug is increasingly thought to have potential as a treatment for various cancers, including neurofibromatosis, so an understanding of its mechanism of action is important.

Patients with NF1 frequently develop multiple benign tumors and MPNSTs, and are also predisposed to developing other neoplastic disorders, including leukemia. These predispositions result from inherited and postnatally acquired mutations of the *NF1* gene, which encodes the tumor suppressor protein neurofibromin. To date, it has been difficult to improve tumor-associated morbidity and mortality in patients with NF1 due to a lack of understanding of the genetic and environmental factors interacting with NF1 mutations. Dr. Susan Lindquist (The Whitehead Institute for Biomedical Research) gave a keynote presentation on her work relating to the role of Heat Shock Factor 1 (HSF1) in cancer. Dr. Lindquist's work has shown that inhibition of HSF1 inhibits the growth of cancer cells in vitro and in mouse models. HSF1 is known to be the major regulator of the heat-shock response in all animals. This response is one of the most ancient and highly conserved homeostatic mechanisms known. It acts to enhance survival under stressful conditions, regulates a multitude of growth responses and modulates the degenerative changes associated with aging. As a result, HSF1 is ideally situated to shape and modify the cellular landscape in which NF1 mutation operates, thereby modulating its impact on tumor formation. Immunostaining of NF1-associated MPNST specimens demonstrates HSF1 over-expression and nuclear localization consistent with its activation. Dr. Lindquist and colleagues have shown that HSF1 inhibition markedly inhibits the growth of human NF1-associated neural tumor cells, and have been exploring the use of HSF1 as a potential therapeutic target in the treatment of NF1-associated MPNSTs. Dr. Lindquist and colleagues have found that, importantly, reducing HSF1 function is well tolerated in both normal cells and in animals. They have used genetic techniques to alter HSF1 functionality as a modifier of NF1 mutated proteins, and have demonstrated that reducing HSF1 function dramatically reduces tumor formation in a mouse model of NF1. Dr. Lindquist's group will continue to validate this therapeutic strategy in NF1 in addition to focusing on the identification of promising candidates for clinical development.

Accumulating evidence indicates that genomic heterogeneity in human tumors is a critical determinant of the variable clinical response to molecularly targeted cancer therapies. Dr. Jeffrey

Settleman (Harvard Medical School/Massachusetts General Hospital) group gave a keynote presentation on his work in this area. He and his colleagues are using large panels of cancer cell lines to capture the genomic heterogeneity of human cancer and to identify clinically relevant genotype–phenotype relationships. This approach has been useful for demonstrating clear relationships between the efficacy of small molecule selective kinase inhibitors and the mutational state of genes encoding the target kinase—a finding consistent with recent clinical studies involving inhibitors of EGFR, HER2, ALK, and BRAF. This analysis has also begun to shed light on mechanisms underlying sensitivity as well as *de novo* and acquired resistance to a variety of small molecule inhibitors. Using this same platform, Dr. Settleman and colleagues have also identified a mechanism of reversible drug “tolerance” that involves a distinct chromatin state displayed transiently by small sub-populations of cancer cells. This drug-tolerant state can be disrupted by chromatin-modifying agents, potentially yielding a therapeutic opportunity to prevent or delay the development of more stable genetically conferred drug resistance mechanisms.

THE 2010 FRIEDRICH VON RECKLINGHAUSEN AWARDEE: DR. NANCY RATNER

The Friedrich von Recklinghausen Award is given annually by the Children’s Tumor Foundation to an individual researcher or physician, nominated by their peers, who has made outstanding contributions to NF research or clinical care. The Award was given historically by the Foundation in the 1980’s and 1990’s, and after a lapse, was reinstated in 2008. The recipient of the 2010 von Recklinghausen Award was Dr. Nancy Ratner, Beatrice C. Lampkin Professor of Cancer Biology at the University of Cincinnati where she heads a large and successful NF research laboratory. This is focused on understanding the role of the Schwann cell in tumor formation in both NF1 and NF2; and, utilizing a series of elegant genetic models of NF tumors which she has developed, in identifying through preclinical research candidate therapies to halt NF tumor growth. As well as her contributions individually and with her laboratory members, Dr Ratner has also been a major international leader and collaborator in coordinating and driving NF research efforts. Among these, she served as co-chair of the International Consortium on the Molecular Biology of NF1 and NF2 from 1990 to 2001; and has participated in many research consortia since, most recently through her participation in the CTF NF Preclinical Consortium. In their tributes, scientific collaborators remembered how Dr. Ratner stimulated their involvement and commitment to the field of NF research. Clinical colleagues paid tribute to her ability to communicate her work to audience’s of varying scientific knowledge and to relate laboratory findings back to the clinical picture. Finally, members of her own laboratory paid tribute to her role as a leader and mentor. Dr. Ratner’s success is evidenced by the significant representation of her group’s research on the agenda of the 2010 NF Conference which will no doubt continue to make significant advances toward understanding and identifying treatments for NF.

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