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Neurofibromin is actively transported to the nucleus

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Abstract Mutations in the neurofibromatosis type 1 (NF1) tumor suppressor gene predispose individuals to a variety of benign and malignant tumors. Many tumor suppressors 'shuttle' between the nucleus and the cytoplasm, thus regulating their function. By expressing different *NF1* constructs in COS-7 cells (encompassing exons 28–49 and fused to the green fluorescent protein), we identified a functional nuclear localization signal (NLS) in exon 43. Mutation of the NLS completely abolishes the nuclear entry of the NF1-derivative fusion protein. A highly expressed splice variant that lacks this NLS controls the localization and hence the function of neurofibromin. The localization of neurofibromin in the nucleus may provide novel clues to unknown functions for *NF1*.

Key words: Neurofibromatosis type 1; Neurofibromin; Nuclear localization signal; Nuclear localization; Alternative splicing

1. Introduction

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder affecting ~1 in 3000 individuals. The *NF1* gene spans ~350 kb of genomic DNA and produces a 11–13 kb transcript containing 60 exons [1–3]. Mutation of the *NF1* gene leads to diverse clinical outcomes. The most common manifestations are café-au-lait macules, neurofibromas, Lisch nodules, axillary freckling and learning disabilities [4]. The *NF1* gene is classified as a tumor suppressor gene since decrease or absence of NF1 protein is seen in malignant tumor cell lines from patients, loss of heterozygosity is seen in malignant tumors of NF1 patients and somatic mutations have been found in sporadic cancers of non-NF1 patients [5–10]. Neurofibromin, the *NF1* protein product, is a large protein of 2818 amino acids and is ubiquitously expressed, although the protein levels differ in different tissues and in developmental or functional states [11–13]. There is no absolute correlation of protein expression with the disease as neurofibromin is expressed in both affected and phenotypically unaffected tissues in NF1 patients. The pleiotropic NF1 disease manifestations cannot be explained by the current knowledge of the function of *NF1* and much remains to be elucidated about the regulation of this huge gene and the function of its protein.

Analysis of the NF1 protein sequence has revealed that a central domain is homologous to a family of proteins known as Ras-GTPase activating proteins (Ras-GAPs), which function as negative regulators for Ras proteins [14]. The Gap-related domain (GRD) spans 250–400 amino acids, representing only about 10% of the protein sequence [15–17]. While the vast majority of *NF1* research has been focused on the GRD, evidence for other important functional domains within neurofibromin is supported by the existence of pathogenic missense mutations outside the GRD [18,19], alternatively spliced *NF1* transcripts [20] and the large size of the protein.

Tumor suppressor proteins are regulated by a number of different strategies to avoid abnormal cellular growth. Over the past few years it has been shown that many tumor suppressor genes 'shuttle' between the nucleus and the cytoplasm [21]. This type of dynamic intracellular movement regulates not only protein localization, but also protein function. Neurofibromin appears to be predominantly cytoplasmic, with different cell types displaying a variable subcellular localization. Some studies have also observed neurofibromin in the nucleus [12,22–24], but the function in the nucleus and the regulation of nuclear importation remain unknown. Moreover, the scarce reports about nuclear neurofibromin, the small number of cells displaying nuclear localization, possible non-specific staining of damaged cells, as well as lack of specificity of the antibodies have cast uncertainty on the nuclear localization of neurofibromin [12,23].

Previously, we have demonstrated the existence of a highly expressed splice variant at the C-terminal end of *NF1*. This variant consists of an in-frame deletion of exon 43 (NF1-ΔE43) and lacks an in silico predicted bipartite nuclear localization signal (bipartite NLS, i.e. two basic amino acid stretches, separated by a 10–12 amino acid variable spacer) [22,25]. This variant is highly expressed in fetal and adult liver, skeletal muscle, kidney, placenta and lung [25]. In the current study we report the intracellular localization of this splice variant determined by expressing different C-terminal constructs either containing or lacking exon 43. We show that inclusion of exon 43 can target the NF1 fusion protein

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Abbreviations: NF1, neurofibromatosis type 1; GAP, GTPase-activating protein; GRD, Gap-related domain; NLS, nuclear localization signal; GFP, green fluorescent protein; FLIP, fluorescence loss in photobleaching; ROI, region of interest

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to the nucleus, whereas exclusion of exon 43 completely abolishes this nuclear localization. Hence alternative splicing of exon 43 can control the localization and the function of neurofibromin by regulating transfer to the nucleus. We characterize this NLS by site-directed mutagenesis.

2. Materials and methods

2.1. Plasmid construction

Primers were designed that amplify exons 28–49 (amino acids 1591–2805) of the *NF1* coding region. Addition of *Xma*I and *Sac*II restriction sites to the primers allowed ligation to the *Xma*I/*Sac*II-digested pEGFP-C1 vector (Clontech), resulting in an in-frame fusion to the C-terminus of the green fluorescent protein (GFP). Site-directed mutagenesis was performed using the QuickChange XL site-directed mutagenesis kit (Stratagene), using primers that specifically introduced the desired mutations (K2534A, R2535A, K2547A, R2549A and R2550A). All constructs were checked for sequence integrity on an ABI 3100 capillary system (Applied Biosystems) before and after bulk preparation of the desired plasmids.

2.2. Cell culture and transfections

COS-7 cells and PLC/PRF/5 cells (human liver hepatoma cells) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. For transient transfection experiments, cells were grown on glass coverslips in a 24 well plate to approximately 90% confluence and 0.8 μg of each GFP expression construct was transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were cultured for 24 h in serum-containing medium before subcellular localization analysis.

2.3. Subcellular localization analysis

Cells were fixed in 3% paraformaldehyde for 15 min at room temperature, washed with phosphate-buffered saline and incubated for 5 min with 300 nM DAPI (Sigma). After washing, the coverslips were mounted in VectaShield (Vector Laboratories). The subcellular localization of the GFP fusion proteins was visualized on a Blue Diode Bio-Rad Radiance 2001 confocal laser scanning microscope using a 60× oil immersion objective. For live cell imaging and photobleaching experiments, cells were plated, transfected and observed in glass bottom culture dishes (MatTek Corporation) on a Bio-Rad Radiance 2001 confocal microscopy system. A defined region of interest (ROI) of the living cells was photobleached for 5 min at 100% laser intensity using a 488 nm argon ion laser of 14 mW, measured at laser output. A single section was imaged before and after the bleaching. The thickness of the optical sections was 0.51 μm.

3. Results

We have previously identified the highly expressed NF1-ΔE43 variant which lacks the complete exon 43 [25]. To determine whether deletion of exon 43 influences the subcellular localization of neurofibromin, two expression constructs were created that respectively contained or lacked exon 43. The plasmids encoded the C-terminal half of neurofibromin (encompassing exons 28–49) fused to the C-terminus of GFP (pEGFP-NF1-E[28–49] and pEGFP-NF1-E[28–49]-ΔE43, Fig. 1A). These expression constructs were transiently transfected into COS-7 cells. As a control, the GFP expression vector alone was transfected in parallel. Subcellular localization was studied by confocal laser scanning microscopy. In the control experiment, GFP alone was distributed diffusely throughout both the cytoplasm and the nucleus (data not shown) as GFP is able to diffuse passively through the nuclear pore because of its relatively small size (27 kDa). The localization of both NF1-GFP fusion proteins differed significantly. Inclusion of exon 43 resulted in a strong nuclear localization (100% of transfected cells) in addition to a moderate

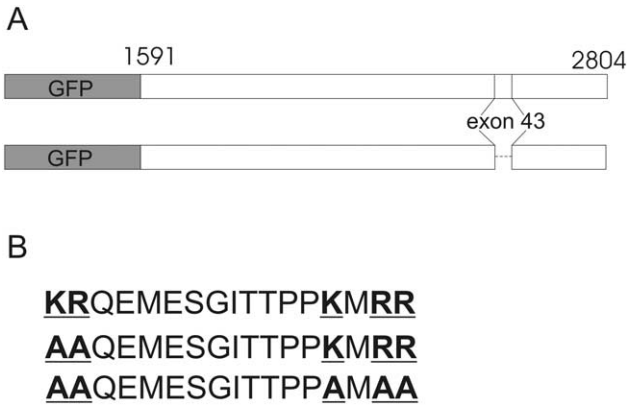

Fig. 1. A: Schematic representation of GFP expression constructs used in this study, indicating the start and the end of the *NF1* sequence and the position of the spliced exon 43. B: Wild type NLS (upper sequence), mutation of the first stretch of amino acids (middle sequence) and additional mutation of the second stretch of amino acids (lower sequence).

to faint cytoplasmic localization (Fig. 2A). In addition, small bright dots within the nucleolus were detected in cells with large and clearly visible nucleoli. Exclusion of exon 43 completely abolished the nuclear and nucleolar localization and only a strong homogeneous cytoplasmic staining was observed (Fig. 2B). Identical results were obtained by transfection of PLC/PRF/5 cells (data not shown). The same constructs were used for live cell imaging in COS-7 cells, which confirmed the localization patterns. A representative example is given for pEGFP-NF1-E[28–49] in Fig. 3, demonstrating a series of consecutive optical sections through a cell.

To further determine the amino acid residues necessary for nuclear import, we constructed mutants of pEGFP-NF1-E[28–49] and examined their subcellular localization. In a first mutagenesis experiment, the first stretch of basic amino acids of the bipartite NLS was replaced by alanines (K2534A and R2535A). In a second mutagenesis reaction also the second stretch of basic amino acids was mutated (K2547A, R2549A and R2550A) (Fig. 1B). The localization of these mutated GFP fusion proteins was examined by confocal microscopy after transfection into COS-7 cells. Mutation of the first stretch of basic amino acids resulted in a diminished ability to enter the nucleus in comparison to the wild-type fusion protein. The localization pattern was altered from the characteristic bright nuclear and faint cytoplasmic distribution of the wild-type construct to a faint nuclear and more brightly stained cytoplasmic pattern (Fig. 2C). The signal intensity of the nucleolar dots was also decreased. Mutation of the second stretch of basic amino acids completely abrogated the ability of the GFP fusion protein to enter the nucleus (Fig. 2D), resembling the localization pattern of the exon 43-deleted GFP construct. These results demonstrate that the bipartite NLS in exon 43 is necessary and sufficient to target the NF1-derivative fusion protein to the nucleus.

To determine whether the C-terminal half of neurofibromin was mobile or participated in long-lived interactions with certain cellular structures, live cell imaging was performed using fluorescence loss in photobleaching (FLIP) experiments. In FLIP, a small region of the cell is continuously bleached by a high-powered laser pulse, and loss of fluorescence in the surrounding area is followed over time. pEGFP-NF1-E[28–49] and pEGFP-NF1-E[28–49]-ΔE43 expression constructs

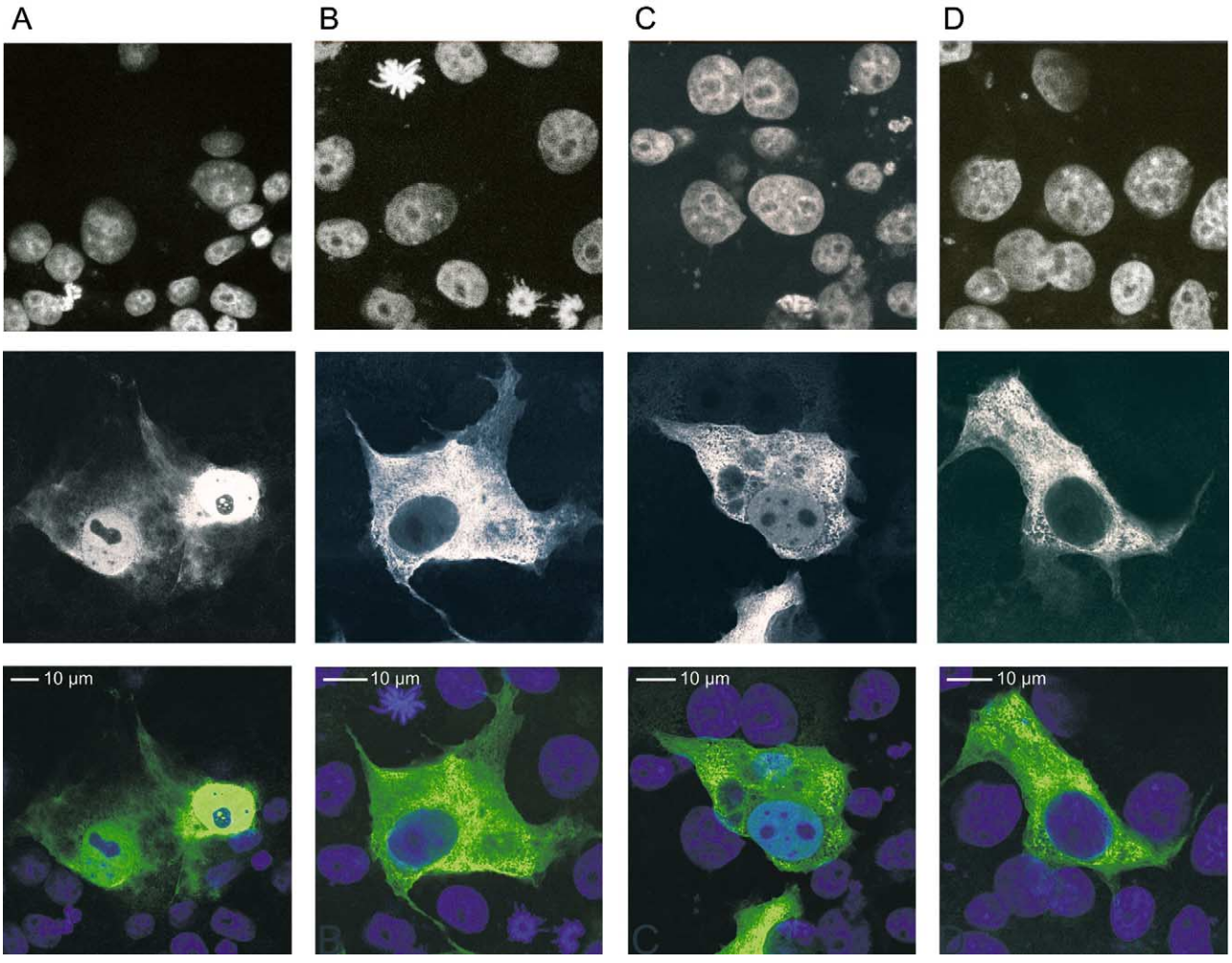

Fig. 2. Subcellular distribution of GFP-tagged NF1 constructs in transfected COS-7 cells examined by confocal laser scanning microscopy. Upper panels: DAPI staining of the nuclei; middle panels: images of GFP-tagged NF1 constructs; lower panels: merge of DAPI staining and GFP expression constructs. A: Subcellular localization of pEGFP-NF1-E[28–49]. Presence of exon 43 results in a strong nuclear localization and faint cytoplasmic localization. Small dots were detected in the nucleolus. B: Subcellular localization of pEGFP-NF1-E[28–49]-ΔE43. Absence of exon 43 completely abolishes the nuclear localization and only a homogeneous cytoplasmic staining is observed. C: Subcellular localization of pEGFP-NF1-E[28–49]-K2534A/K2535A. Mutation of the first stretch of basic amino acids results in a diminished ability to enter the nuclear compartment. D: Subcellular localization of pEGFP-NF1-E[28–49]-K2534A/R2535A/K2547A/R2549A/R2550A. Additional mutation of the second stretch of basic amino acids completely abrogates the ability of the GFP fusion protein to enter the nucleus.

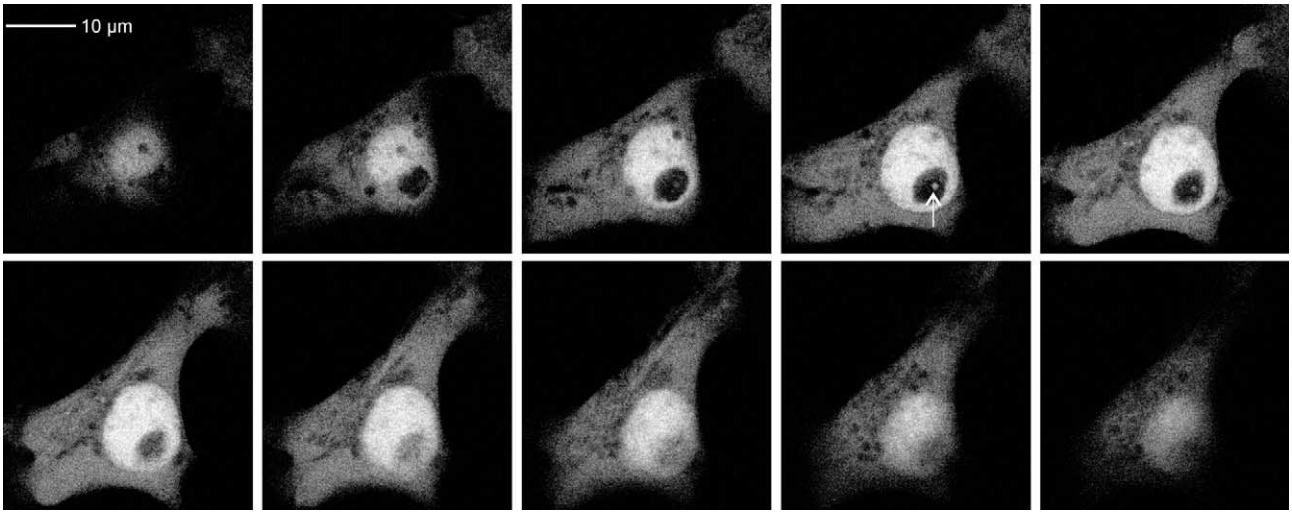

Fig. 3. Live confocal z-series through COS-7 cells transfected with pEGFP-NF1-E[28–49]. Distance between the different consecutive optical sections is 0.96 μm. A bright dot in the nucleolus is indicated by an arrow.

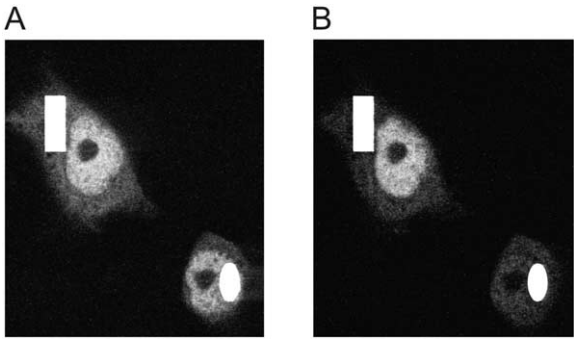

Fig. 4. FLIP analysis in COS-7 cells transfected with the pEGFP-NF1-E[28–49] construct. A cytoplasmic and a nuclear defined region (white zones) were continuously bleached for 5 min and images were made immediately before (A) and after (B) photobleaching. After FLIP a decreased fluorescence is seen outside the ROI.

were transiently transfected into COS-7 cells. When a small ROI in the cytoplasm (for both constructs) or nucleus (only for pEGFP-NF1-E[28–49]) was subjected to FLIP, part of the fluorescence in the bleached compartment (cytoplasm or nucleus) outside the ROI decreased over time. However, a part of the fluorescence signal was resistant, even after extended bleaching (Fig. 4). This indicates that part of the fusion protein moves freely through the cytoplasm or nucleus, while another part is immobilized. Bleaching a cytoplasmic region did not trigger loss of fluorescence in the nucleus, even after an extended period of bleaching, suggesting that it is difficult for the fusion protein to diffuse out of the nucleus. As a control FLIP of formaldehyde-fixed cells that were transfected with the same constructs resulted in a prominent bleached area without any bleaching outside the selected region (data not shown).

4. Discussion

Cells employ a multitude of strategies to ensure that their proliferation is highly regulated. Therefore tumor suppressor proteins are regulated at multiple levels including regulated transcription, post-translational modifications, regulated protein degradation and regulated nuclear transport. Many tumor suppressors shuttle between the nucleus and the cytoplasm. Among them are p53, p73, BRCA1, APC, VHL, Smad4, Beclin, p130 [26] and NF2 [27]. Small proteins (<45 kDa) can diffuse freely into and out the nucleus, whereas proteins with a larger molecular mass are actively transported through the nuclear pores by means of their NLS. Nuclear transport has been proven to be a fundamental and critical mechanism for regulating protein localization and function for many tumor suppressor genes. Deregulation of nuclear transport is implicated in the mislocalization and altered function of a variety of proteins and can have severe cellular consequences and can potentially lead to the initiation and progression of cancer [21].

In this study we have identified a functionally active bipartite NLS in neurofibromin which was necessary and sufficient to target a NF1-GFP fusion protein, containing the C-terminal half of NF1, to the nucleus. Mutation of this sequence results in a total exclusion from the nucleus. In order to provide insight into the dynamic properties or associations of the NF1-GFP fusion protein, live cell photobleaching studies were performed. These FLIP experiments show that part of the NF1-GFP fusion protein is mobile and moves freely in the nucleus or cytoplasm, while another part is immobilized. Cytoplasmic FLIP did not trigger loss of fluorescence in the nucleus even after extended cytoplasmic bleaching.

The presence of the NF1 fusion protein in the nucleus is very intriguing in light of the importance of nucleocytoplasmic shuttling for the function and regulation of many tumor suppressor genes. The possible function of neurofibromin within the nucleus remains unknown. Translocation to the nuclear compartment could be a mechanism to regulate the GAP function of neurofibromin by sequestration in the nucleus, as Ras is located at the plasma membrane. Sequestration of NF1 in the nucleus could have important therapeutic consequences. Exploration of the triggers that initiate nuclear export could then provide clues for targeted therapeutic intervention. Interestingly, the NF1-ΔE43 variant, unable to enter the nucleus, had a very high level of expression in lung, liver, placenta, fetal liver, kidney and skeletal muscle [25]. These tissues are not typically associated with NF1 pathology and it is tempting to hypothesize that a dosage effect due to nuclear exclusion exists in these tissues. On the other hand, neurofibromin itself may have a function in the nucleus. This hypothesis is attractive as the role of the large NF1 protein in cellular proliferation and differentiation has not yet been fully resolved.

The NF1 fusion protein was concentrated in the nucleoli as small bright dots. However, excessive amounts of this fusion protein may compromise a proper subnuclear localization and a detailed study of NF1 in its natural biological context is needed in order to interpret these foci.

The existence of a highly expressed splice variant that specifically lacks the NLS (NF1-ΔE43) indicates that the localization of neurofibromin, and hence its function, can be controlled by alternative splicing in certain tissues. This splicing is an important regulatory mechanism and may be differentially regulated at different developmental stages, in different phases of the cell cycle or by specific intra- or extracellular stimuli. Expression of the NF1-ΔE43 variant is lower in neural versus non-neural tissues [20]. This may imply that nuclear localization is important in neural tissues. So far, neurofibromin has been mainly considered as a cytoplasmic protein, with only a few studies reporting some nuclear localization. Nevertheless, nuclear staining for neurofibromin has been reported in developing neurons [22], in a subset of neurons in adult rat dorsal root ganglia [12], in a subset of NIH3T3 fibroblasts [23] and in differentiating keratinocytes [24].

Regulation of localization can occur at the level of mRNA processing, by splicing out the NLS-containing exon 43, but also at the protein level. Several mechanisms can determine the localization at the protein level. First, a protein domain, such as a nuclear export signal (NES), can mediate transport out of the nucleus. Our expression constructs contained the C-terminal half of neurofibromin and a NES situated more N-terminally might be present in neurofibromin. Second, inter- or intramolecular masking of the transport signal could regulate localization. BRCA1 has been shown to be retained in the nucleus through masking of its NES by binding of the protein BARD1 [28]. Similarly, masking of the NLS in neurofibromin may prevent its entrance into the nucleus. Third, it has been shown for some proteins that phosphorylation of sequences flanking the NLS signal can regulate the NLS-medi-

ated nuclear import [21]. NF1 contains a casein kinase II and a tyrosine kinase phosphorylation site very close to the nuclear targeting sequence and neurofibromin has been reported to be phosphorylated in vitro [29], although the specific consequences of these phosphorylations, e.g. with respect to localization, have not yet been characterized. Finally, sequestration in the cytoplasm, by association with binding partners, can also affect the localization. This form of regulation influences the nucleocytoplasmic shuttling of APC, where the C-terminus exhibits a tight association with cytoskeletal microtubules [30]. Cytoplasmic anchorage through interaction with specific binding partners (e.g. microtubules and Ras) could promote the cytoplasmic retention of neurofibromin. Proper control of nuclear import and export is likely to be an important regulator of neurofibromin function. Further examination of the regulation of localization and analysis of possible associations within this compartment may yield important clues about unknown functions of neurofibromin.

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