Chapter

Two Tails for Neurofibromin: A Tale of Two Microtubule-Associated Proteins

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Abstract

Neurofibromatosis type 1, NF-1, is a common monogenic (*NF1*) disease, characterized by highly variable clinical presentation and high predisposition for tumors, especially those of astrocytic origin (low- to high-grade gliomas). Unfortunately, very few genotype–phenotype correlations have been possible, and the numerous identified mutations do not offer help for prognosis and patient counselling. Whole gene deletion in animals does not successfully model the disease, as NF-1 cases caused by point mutations could be differentially affected by cell type-specific alternative splice variants of *NF1.* In this chapter, we will discuss the differential Microtubule-Associated-Protein (MAP) properties of NLS or ΔNLS neurofibromins, produced by the alternatively splicing of exon 51, which also contains a Nuclear Localization Sequence (NLS), in the assembly of the mitotic spindle and in faithful genome transmission. We will also commend on the major theme that emerges about NLS-containing tumor suppressors that function as mitotic MAPs.

Keywords: NLS and ΔNLS neurofibromins, astrocyte, glioblastoma, astral microtubules, spindle, Microtubule-Associated-Proteins, chromosome segregation

1. Introduction

Neurofibromatosis type 1 (NF-1) is a common, complex multisystem cancer predisposition syndrome, with a worldwide incidence at birth of 1: 2–3000 people [1] and a documented high mortality mostly due to malignancies [2]. NF-1 is caused by autosomal, dominantly inherited or de novo (50: 50 [3]) pathogenic mutations in the *NF1* gene, which encodes the large protein neurofibromin. The *NF1* gene was identified 30 years ago, yet with over 3000 different mutations identified thus far [4], only very few genotype–phenotype correlations have been postulated [5–7]. Affected individuals may present with a wide range of clinical manifestations, mostly from the Central Nervous System, CNS and the Peripheral Nervous System, PNS [1], as the *NF1* gene remains highly expressed there, whereas is downregulated in most other tissues in the adult. Thus, most NF-1 tumors are found in the CNS (gliomas) or the PNS (plexiform neurofibromas, malignant peripheral nerve sheath tumors, or the hallmark of the disease sub- and cutaneous neurofibromas), while there is increased risk for other cancers mostly of neural crest origin [8, 9].

In particular high-grade gliomas are more frequent in adults with NF-1, which have 5 times greater risk for glioblastoma (GBM) than the general population [10]. In addition, the great mutation rate of the *NF1* gene, which has also made its cloning impossible, is now recognized in ~20% of sporadic GBM [11]. In terms of specific treatments none are available for the cancers of NF-1 patients. Many drugs, like anti-angiogenic agents [12], have shown no responses, and MEK1/2 inhibitors have been approved only for plexiform neurofibromas. GBM prognosis remains dire (~2 years) even with the use of the highly cytotoxic temozolomide, while clinicians daily struggle with decisions for affected children. Unfortunately, gliomas frequently are resistant to temozolomide therapy and the candidate mechanism, for other tumors too, is the formation of tumor microtubes [13]. These recently recognized long, highly rich in actin, dynamic membrane protrusions of astrocytoma cells form a network for multicellular communication that promotes tumor growth and invasion of the brain. Therefore, understanding the cytoskeleton associations of neurofibromin is highly important in the effort to identify new therapeutic targets.

Confirmation of causative mutation with molecular diagnosis is a difficult task, as the large *NF1* gene of over 400 Kb and 57 exons has no mutational hot spots and one of the highest mutation rates in human genetic disorders, which explains the high incidence of *de novo* variants even within the same family. The complicated behavior of the gene is further highlighted from genetic manipulations of mice. When *Nf1* along with two more tumor suppressor genes (TSGs) were targeted with CRISPR/Cas in the forebrain of E13.5 mice, aggressive tumors resembling human GBMs were produced; however, whole-genome sequencing of the induced GBMs hinted to a very variable repair of CRISPR-induced double-strand breaks, potentially locus-specific [14]. Thus, mouse genetic NF-1 models have been marginally helpful in designing prognostic tests or therapies.

Even when timely, molecular diagnosis may only rarely offer help for prognosis or consultation [5, 7] and the challenge to correlate genotype–phenotype in this disease of uncontrolled cell growth and tumorigenesis remains largely unmet. It is our opinion that the impact of the various mutations will be best appreciated, once our currently limited knowledge on the functions of the distinct neurofibromin protein domains (**Figure 1**) will be expanded. As we will elaborate, these domains perform critical functions, evidently through inter- and intra-molecular interactions, most notably with tubulins, all of which are altered by cell type-specific, alternative

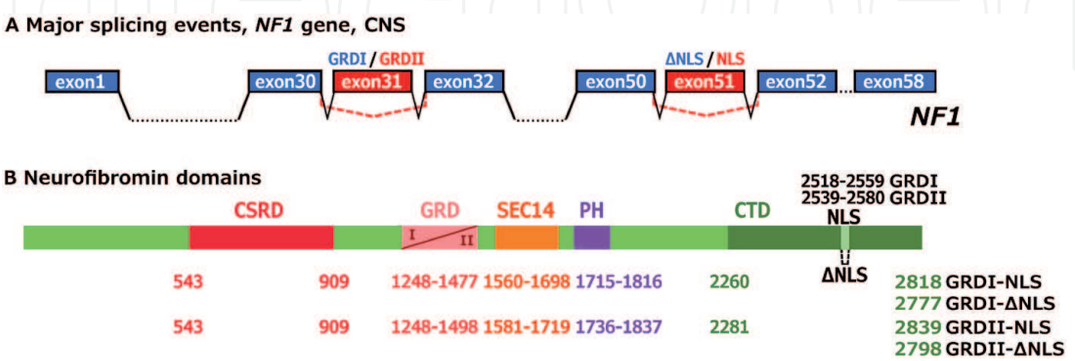

Figure 1.
Major NF1 splicing events and neurofibromin domains with functional importance in the CNS. A, the two major alternative exons in the NF1 gene, namely 31 and 51, which produce GRDI *or* GRDII *and* NLS *or* ΔNLS *transcripts, respectively. B, Neurofibromin domains of known functional importance: CSRD, Cysteine/Serine-Rich domain; GRD, GAP-related domain; SEC14, Yeast Sec14p-like domain; PH, pleckstrin homology domain; CTD, C-Terminal domain and NLS, Nuclear localization sequence. Amino acid numbers for all putative isoforms are based on GRDI-NLS neurofibromin (Ensembl transcript NF1–201).*

splicing events and post-translational modifications, with mutations adding a supra level of complexity that must be met.

Indeed, the quest for genotype–phenotype correlation is complicated by developmental stage- and tissue- or cell type-specific alternative splicing events of the *NF1* gene, which is downregulated in most tissues in the adult except CNS and PNS tissues. While several alternative exons have been described, three are common in the CNS, namely 9a/9br, 31 (former 23), and 51 (former 43) [15–17]. Addition of the small alternative exon 9a/9br correlates well with neuronal differentiation and is downregulated in oligodendrogliomas [15, 18], but no specific function assignments have been made thus far. In contrast, inclusion of the other two, namely 31 and 51, does have important functional consequences, as will be discussed next.

Skipping of alternative exon 31, which corresponds to the center of the RasGAP-related domain (GRD) of neurofibromin (**Figure 1A**) through which neurofibromin inactivates Ras, generates two variants accordingly named as GRD type I, GRDI and, if exon 31 is included, as GRD-type II, GRDII [16]. Exon 31 is mostly skipped in CNS neurons early on, whereas it is retained as the prominent transcript in astrocytes [19–21]. Due to the central role of Ras in many cellular functions and in carcinogenesis, GRDs have received high attention. Both GRDs are functional RasGAPs [22], when overexpressed in vitro [23] and as we showed in vivo [24], albeit GRDI is a much more potent RasGAP than GRDII.

Nevertheless, no significant rescue capacity of GRDs alone has been shown for NF-1 phenotypes, leading to the characterization of such phenotypes as Ras-independent by many researchers [25–27]. Along several such scientific efforts, the importance of other domains (**Figure 1B**) in the allosteric regulation of GRD has been established. Indeed, collective experimental evidence has postulated that neurofibromin domains may bind each other to form dimers [28], as well as, multiple proteins to coordinate Ras signalling ([25–27, 29, 30], reviewed in [31]). For example, in glioma cells overexpression of CSRD plus GRD -after phosphorylations by PKCε or α- binding to cortical F-actin increases and imposes a positive allosteric regulation on GRD and thus intense Ras deactivation, which is sufficient to switch the effect of EGF signalling from proliferation to differentiation [30]. While this mechanism was the first provided answer to the open question of how RasGAPs are recruited to the membrane, its clinical significance was directly postulated when large cohorts of NF-1 patients, heterozygous for nonsynonymous mutations of any of five successive amino acids in the CSRD, were found to have high, >50% predisposition to malignancies as compared to the general NF-1-affected population [5, 7]. SEC14, also reported to halt glioma cell invasion [32], is a domain that mediates binding to phospholipids [33, 34] and, as we showed, imposes, like CSRD, a positive allosteric regulation on GRD and accelerates Ras deactivation, potent enough to switch the activation of ERK from an analogous to a digital mode [24].

Exon 51 in the CTD contains the NLS, a sequence of basic amino acid clusters required for proteins of >45 kDa to dock onto the nuclear pore complex as a cargo for nuclear import. The necessary energy expenditure and the directionality of the import is provided by a gradient of RanGDP in the cytoplasm and RanGTP in the nucleus. A similar Ran gradient, generated around duplicated chromosomes during mitosis, allows the release of NLS-containing mitotic proteins that regulate spindle assembly and congression of chromosomes [35, 36]. *NF1* exon 51 may be also alternatively transcribed, producing *NLS* or *non NLS (ΔNLS)* transcripts and corresponding NLS or ΔNLS neurofibromin isoforms (**Figure 1**). We first identified in silico this bipartite NLS and documented experimentally that most neurofibromin molecules reside in the nucleus in neurons [37].

Later genetic analysis [17] revealed that in those fetal tissues, which will not retain high levels of *NF1* expression in the adult, *ΔNLS* transcripts are expressed in

higher percentages. In contrast, in tissues that *NF1* remains high in the adult, fetal expression of *ΔNLS* is very low early on and increases with development. Typical examples for the former is the liver (*ΔNLS* constitutes 25% of total *NF1* expression in the fetus and only 15% in the adult) and for the latter is the brain, where the meek expression of 1% rises by 4-fold in the adult. Thus, there is an upregulation of *ΔNLS* transcripts in the tissue most implicated in NF-1 pathology, that is the CNS [17].

Moreover, we recently addressed the pressing question of developmental regulation of exon 51 skipping/inclusion in CNS cell types, using chick embryo or the early postnatal mouse or rat brains. We find that expression of *ΔNLS* is first detected only when neurons become postmitotic with its levels rising from negligent to 10% of total *NF1* in mature neurons; in astrocytes in culture, *ΔNLS* transcripts rise along with those for glial fibrillary acidic protein (GFAP) and reach a level of ~5% of total *NF1* [19]. Thus, our studies postulate that in both neurons and astrocytes as many as four variants and neurofibromin isoforms may be expressed (**Figure 1**), while expression of *ΔNLS* transcripts and ΔNLS neurofibromins correlates with neuronal and astrocytic differentiation and underline the necessity to study the individual functions of ΔNLS and NLS neurofibromins.

The importance of NLS inclusion was totally unexplored, till we documented a few years ago that neurofibromin controls the pivotal function of chromosome congression on the mitotic spindle [38] (**Figure 2**) and then proved that depletion of NLS neurofibromins deregulates spindle assembly and positioning, leading to aneuploidy and increased micronuclei formation [39]. More importantly, these studies established the function of neurofibromins as MAPs. All currently known impacts of this function will be presented in more detail in the next section.

In concluding this introduction, we believe that the importance of exploring novel yet fundamental questions on the functions of NLS neurofibromin isoforms is tantamount for understanding patient phenotypes and designing prognostic tools for NF-1 glioma growth and NF-1 mutation-specific therapies.

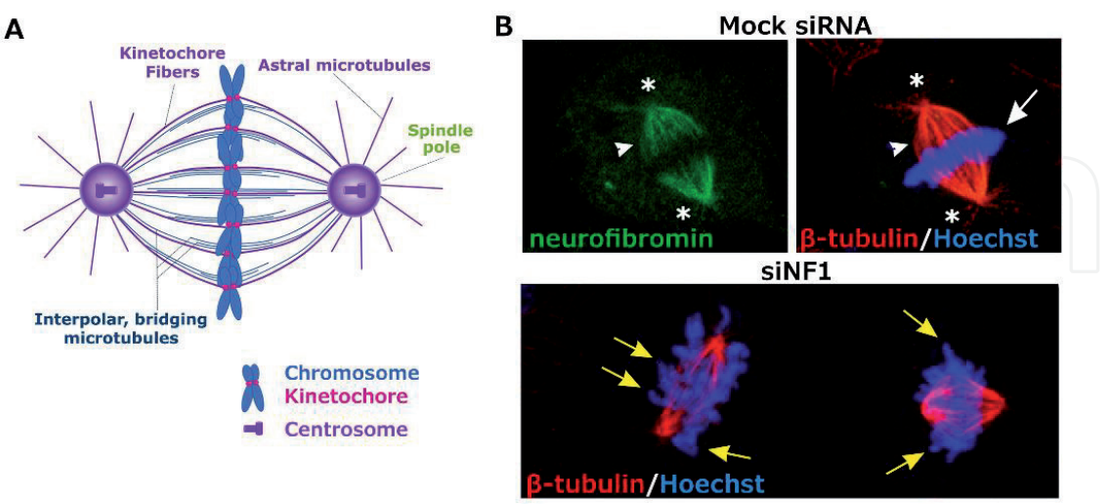

Figure 2.
Neurofibromins regulate spindle configuration and chromosome congression. A, Mitotic spindles consist of three major types of microtubules (MTs): astral MTs that radiate from the centrosomes/poles, microtubule bundles (K-fibers) to link kinetochores to poles, and interpolar bundles to separate poles, elongate the spindle, and bridge K-fibers [40]. B, SF268 glioblastoma cells, transduced with mock or NF1-specific siRNAs, are stained for neurofibromin, β-tubulin, and chromatin, as indicated. In mock siRNA-cells, neurofibromin decorates astral MTs (asterisks) and both K-fibers and interpolar MTs (arrowheads), in a symmetric spindle with properly aligned chromosomes at its equator (white arrows). Depletion of neurofibromins (siNF1) causes irregularities in the spindle geometry and chromosome congression aberrations (yellow arrows) [38]. Images are the maximal intensity projection of 0.34 μm confocal plane stacks.

Therefore, in this Chapter we will focus on novel insights on the MAP function of neurofibromins from our recent studies.

2. Neurofibromins as MAPs and their role in mitosis

Tubulins rapidly form highly dynamic noncovalent polymers, the microtubules, which execute essential functions for the constant yet ever-changing needs of all cells, such as function-coupled shapes, directed intracellular transport, migration, and, most importantly for the development of an organism, properly oriented cell divisions with accurate genomic transmission. For cell division, several types of MTs organize, elongate, and orient a bipolar spindle, through which chromosomes will position at the spindle equator for faithful sister chromatid separation and then segregation to the two daughter cells ([41–43] and **Figure 2A**).

Accordingly, multitudes of structurally different MAP proteins associate with MTs to regulate MT nucleation, polymerization, organization, bundling, and crosslinking in preparation for and completion of mitosis. The availability of mitotic MAPs is tightly regulated by coordinated transcription, as well as by cell cycle-dependent post-translational modifications, most often phosphorylations that control protein trafficking, homeostasis, and inter- or intramolecular interactions [44–46]. Aberrations in these processes may lead to aneuploidy and on to tumorigenesis, thus the ability of MAPs to alter MT dynamics is recognized for its prognostic value in cancer and as a target for cancer chemotherapies [47, 48].

Association of neurofibromin with cytosolic MTs was first established by confocal microscopy in fibroblasts and the molecule was proposed to act as a MAP, through a small segment (residues 815–834 in the CSRD) bearing in silico homology to MAPs Tau and MAP2 [49]. Since then, diverse experimental approaches, including co-immunoprecipitations, co-purifications, in vitro MT assembly, and affinity precipitations, have further documented this interaction with cytosolic [23, 24, 29, 37, 38, 50] and with mitotic MTs [38, 39].

Indeed, confocal image analysis of primary or tumor cells derived from the ectoderm or the neural crest and quadruply stained for β-tubulin, neurofibromin, F-actin and chromatin/chromosomes, shows that pools of endogenous neurofibromin colocalize with cytoplasmic MTs (e.g., rat astrocytes in **Figure 3A**, yellow arrows), as well as with F-actin, mainly at the cell cortex and lamellipodia ([37–39]; **Figure 3B**, yellow arrows). Interestingly, no association could be established with any intermediate filament in neural cells, as for example with the abundant astrocytic marker GFAP (**Figure 3C**), except for nuclear lamins [38].

In an earlier study with primary neurons, we found that neurofibromin, in addition to its colocalization with cytosolic MTs, localizes also in the nucleus and identified a bipartite NLS in the CTD (**Figure 1B**) as the probable mediator of nuclear entry [37]. Previously thought as an artifact in the skin epithelium, nuclear neurofibromin is detected with a variety of techniques, i.e., immunocytochemistry (e.g., white arrows in **Figure 3**), subcellular fractionations, or proteomics [28, 29, 37–39, 51–53] in all cells of an ectodermal origin.

We next provided evidence that the nuclear entry of neurofibromin is active, that is through interactions of its NLS with the Ran/importin system [38], as now shown in cancer breast cells [54]. Moreover, we established that a requirement for the cell-cycle regulated nuclear entry of neurofibromin is phosphorylation by Protein Kinase C (PKC) on Serine2808, a residue relatively close to the NLS [29, 38], which is retained in both isoforms. As neurofibromin expression patterns and nuclear regulation appeared to have all the attributes of a mitotic factor, in particular of a MAP, we next addressed the possibility in cells that regularly undergo mitosis.

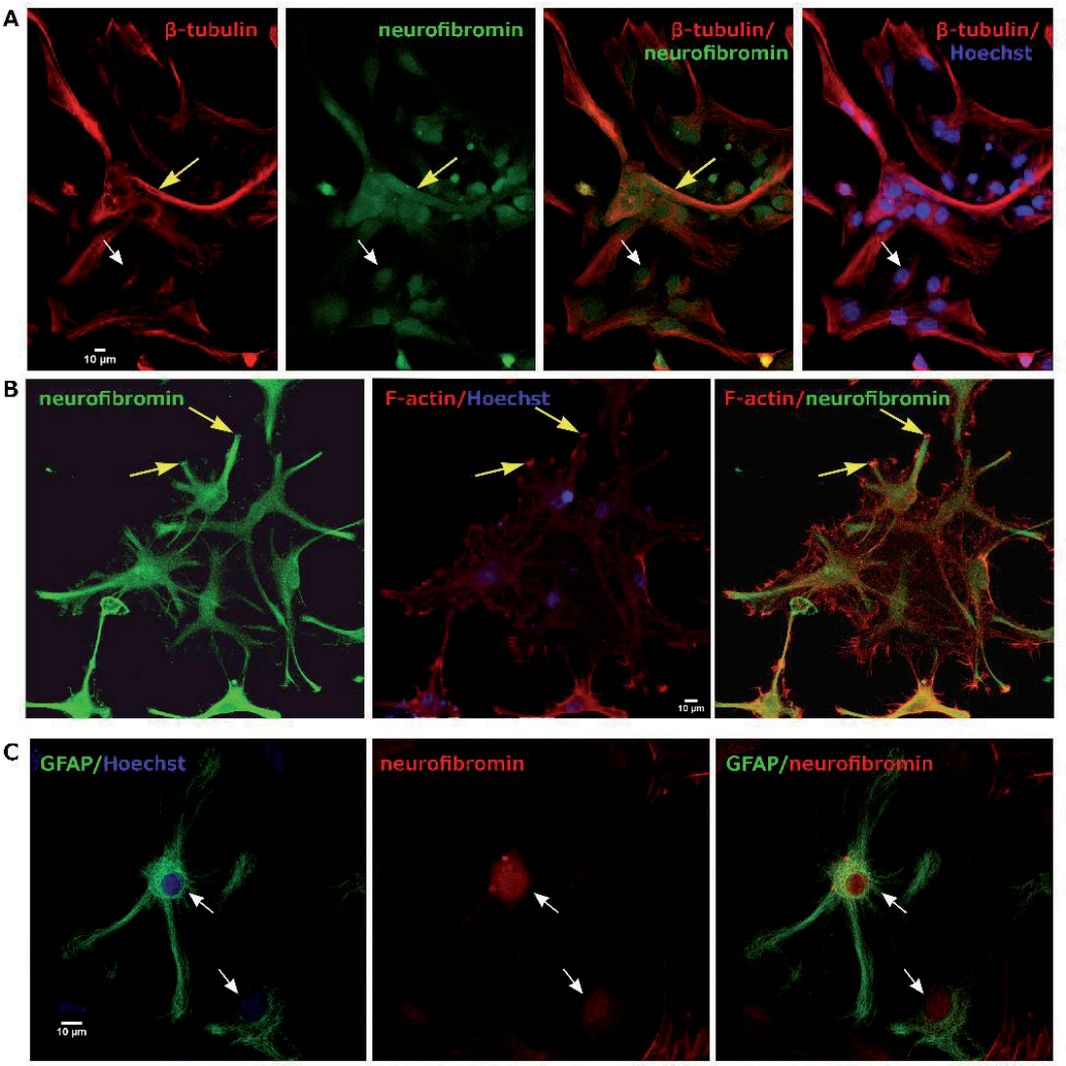

Figure 3.
Endogenous neurofibromins colocalize with both A. cytoplasmic MTs and B. F-actin, but not with C. GFAP. Rat primary astrocytes are stained for neurofibromin, chromatin (Hoechst 33258) and A, β-tubulin, B, F-actin (phalloidin) or C, GFAP. Images are the maximal intensity projection of 0.34 μm confocal planes; yellow arrows point to co-localization of neurofibromin with cytoplasmic MTs in A and actin in B; white arrows point to nuclear neurofibromin.

Thus, we have postulated that neurofibromin primarily co-localizes with β-tubulin at all stages of spindle assembly from prophase to metaphase (e.g., **Figure 2B**) and then through the transformation of the spindle to a machinery for chromosome segregation and cell division, that's is through anaphase, telophase and cytokinesis [38, 39]. Again, neurofibromin's localization onto the spindle is apparent in all cells of ectodermal origin, with no exception. More relevant for gliomagenesis, endogenous neurofibromin in primary cortical or cerebellar astrocytes colocalizes with all three tubulin classes on microtubular structures, that is with α- and β-tubulin throughout mitosis and with γ-tubulin at the centrosomes at interphase and when duplicated for entrance to mitosis [19, 38, 39].

Experimentally, at least three neurofibromin domains have been previously identified to bind tubulins, namely GRD, SEC14, and CTD. Affinities to tubulin for the first two domains were explored for regulation of neurofibromin's RasGAP activity and the third for baiting neurofibromin associated proteins or for nuclear import studies. Thus, GRDI-tubulin interactions lead to a partial inhibition of its cytosolic GAP activity [23] and certain patient mutations in GRD impair the ability of neurofibromin to associate with MTs [55], while the competition of tubulin with H-Ras for binding to SEC14 that we found in COS cells may provide an explanation

and a mechanism for neurofibromin dissociation away from cytoplasmic microtubules [24]. As for CTD, it baits the plus-end MAP Collapsin response mediator protein 2 (CRMP2) [50], while we have shown high affinity of GFP-CTD(+NLS) to α-, β- and γ-tubulins [38].

The importance of neurofibromin as a mitotic MAP was first discovered, when we showed that siRNA-depletion of all transcripts and thus of all neurofibromin isoforms leads to severe errors in chromosome congression, with chromosomes remaining unattached or randomly away from the spindle equator even at full metaphasic spindle length (**Figure 2B**, yellow arrows and [38]). Typically, the unstable lateral interactions between kinetochores and microtubules, which dominate early prometaphase, lead to the reproducible arrangement of chromosomes in an equatorial ring, or torus-like distribution on the surface of the spindle [56]. This loss of the toroidal arrangement of chromosomes with neurofibromin depletion has to be the first evidence that neurofibromin may act to stabilize microtubule for chromosome congression. Consistent with this, using overexpressions of our human CTD construct but not of other domains, abnormal bypassing of mitotic arrest was rescued in the yeast, after the yeast homologs of neurofibromin Ira1 and 2 were deleted [25].

The importance of neurofibromin isoforms as major mitotic MAPs was next discovered, when we probed the individual effects of neurofibromin isoforms that differ in the sequence of the 41 amino acids encoded by exon 51, namely of ΔNLS- and NLS-neurofibromins, on mitotic spindle assembly and faithful genomic transmission [39]. These effects will be highlighted next.

3. NLS and ΔNLS neurofibromins are different MAPS

To further address the mechanism by which neurofibromin regulates chromosome congression and considering together that a. neurofibromin accumulates in the nucleus in a Ran-dependent manner at late S/G2 and resides on the spindle throughout mitosis [38], b. the major cellular target in NF-1 for abnormal proliferation and carcinogenesis is the astrocyte [10], and c. the higher expression of NLS- over ΔNLS -*NF1* transcripts in astrocytes [19], we next evaluated separately the roles of ΔNLS- and NLS-isoforms in spindle assembly and chromosome segregation in an astrocytic cell context.

For these purposes, we have generated SF268 glioblastoma cell lines that stably express, under the control of doxycycline, shRNAs specifically designed to degrade either both GRDI- and GRDII-ΔNLS or both GRDI- and GRDII-NLS-*NF1* transcripts (referred to as NLS-cells and ΔNLS-cells, respectively). This reversible genetic modification has allowed us to pose the question of possible different functions of ΔNLS- and NLS-isoforms and dissect their roles in mitosis [39].

Confocal image analysis of cells immunostained for β-tubulin and neurofibromin and co-stained for filamentous actin, along quantitation of colocalization, postulates that ΔNLS neurofibromins are absent from the nucleus [39]. Moreover, in ΔNLS-cells association of neurofibromin with F-actin is significantly limited, especially in lamellipodia, whereas NLS-neurofibromins richly decorate them along other actin structures. Association with tubulin is not significantly reduced in ΔNLS-cells, yet microtubule organizing centers (MTOCs) are discerned with difficulty, because MTs organize a dense but non-radial network. To the contrary, NLS neurofibromin colocalization with β-tubulin is significantly enhanced, although β-tubulin intensity itself is not increased [39].

This different robustness of MTOC formation among ΔNLS-cells and the parental or NLS-cells is functionally validated with cell migration after wound (scratch) assays. In astrocytes, relocation of their major MTOC, the centrosome, between

the nucleus and the leading-edge during migration is well explained [57]. When positions of centrosomes and nuclei are observed in cells stained for γ-tubulin and Hoechst, respectively, confocal microscopy shows that, unlike parental and NLS- cells, it is readily apparent that centrosomes in ΔNLS-cells fail to position properly, remaining randomly oriented [39]. Hence, time-lapse video microscopy of cells during wound healing confirms that NLS-cells and the parental SF268 cells move with a directed, multicellular movement, while ΔNLS-cells, moving almost as fast, perform a palindromic motion and fail to repair the "scratch wound" (videos in [39]). Overall, this is the first time that neurofibromin is linked to astrocytic cell migration, and, at least the loss of NLS neurofibromins, to defective centrosome positioning and functional cell polarity [39].

Both types of neurofibromins retained colocalization with β-tubulin on the mitotic spindle, albeit colocalization levels with NLS-neurofibromin are, as also for cytosolic MTs, significantly raised (**Figure 4**, images, plots, and colocalization means; [39]). Considering together that NLS neurofibromins do not affect MT densities, whereas ΔNLS-neurofibromins inversely regulate MT densities both in the cytoplasm and the spindle [39], these data document differential MAP properties for ΔNLS-neurofibromins as compared to NLS-neurofibromins.

Examinations of colocalization with γ-tubulin on the duplicated centrosome, show a 25% decrease for NLS-neurofibromins, while ΔNLS neurofibromins show no statistical differences on this aspect. Yet, centrosomes in ΔNLS cells have larger volumes (1.8x), indicating that NLS neurofibromins may help form a more efficient mitotic centrosome, in terms of future spindle assembly [38, 39]. In human cells, the centrosome is the major MTOC for spindle MT assembly and duplicated centrosomes serve as poles to orient the spindle. More specifically, γ-tubulin and its several associated proteins form a large ring complex (γ-TuRC) that serves to

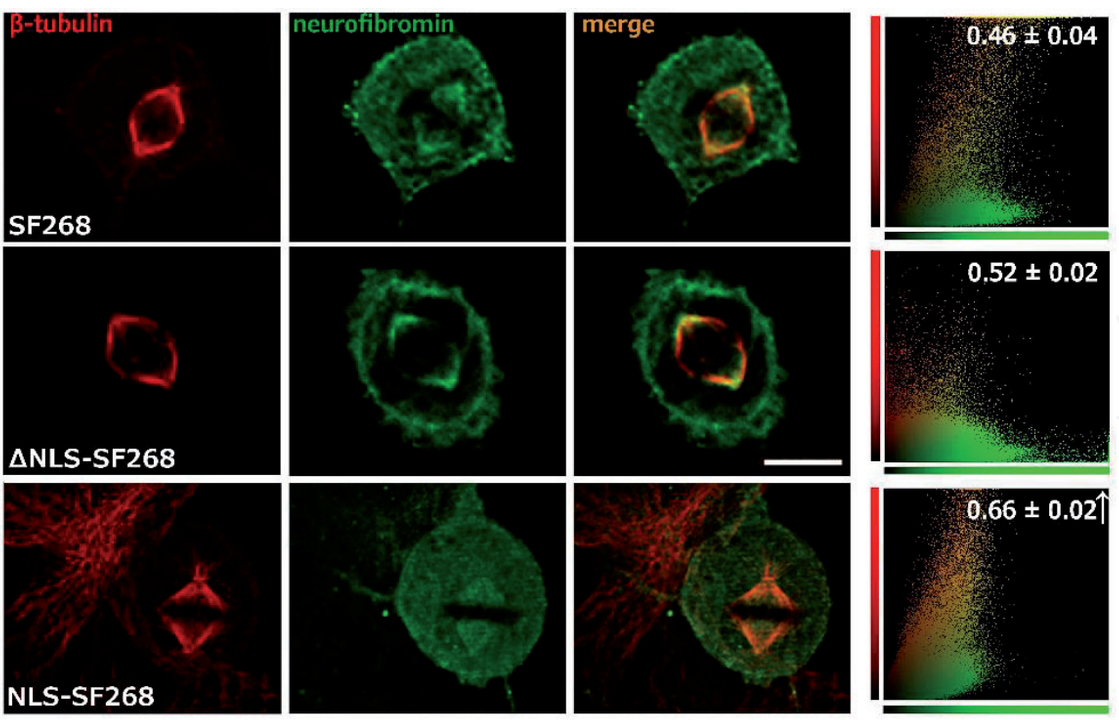

Figure 4.
NLS- and ΔNLS-neurofibromins have different affinities for mitotic MTs. Naïve, ΔNLS-, and NLS- SF268 cells are stained for neurofibromin and β-tubulin. The first two columns contain single focal 0.34 μm planes of a confocal stack and the third column their mergings. Scatter plots show signal intensity in each plane (Volocity®) and numbers are the colocalization means±SE; arrow indicates the statistically significant difference in NLS- versus ΔNLS- or parental cells.

nucleate highly dynamic MTs from the spindle, K-fibers from kinetochores, and interpolar bundles that elongate the spindle [43, 58, 59]. Because γ-TuRC is dispensable for this purpose on occasion [60], the role of MAP-dependent regulation in the nucleation to centrosomes, whether increasing [61] or inhibiting nucleation [62], has been highlighted.

The differential properties of the NLS and ΔNLS isoforms as MAPs on centrosome size are further highlighted by the effects on MT nucleation prior to nuclear envelope breakdown, when the relatively sparse microtubule formations of interphase cells transfigure into a bipolar spindle. Indeed, our studies have documented that the following parameters are greatly affected with depletion of NLS neurofibromins [39]:

3.1 Astral MT formation and spindle positioning

A most striking difference is the abnormal astral MT growth patterns with loss of NLS neurofibromins, as the average length of astral MTs in NLS-cells grows to 5.9 ± 0.33 μm over 4.0 ± 0.19 μm in parentals ($p < 0.0001$), while is robustly diminished in ΔNLS-cells (**Figures 4** and **5**, asterisks; [39]). Proper astral formation is required for spindle position and aberrations of astral MTs correlate well with spindle misorientation [41], and we too find that loss of astral MTs with loss of NLS-neurofibromin leads to statistically differential positioning of the spindle by several degrees [39]. A number of diverse families of proteins impact the timely nucleation and maintenance of astral MTs, yet few may cause loss of astral MTs. Notably, functional ablation (phosphoablating mutants) of End-binding protein 2 (EB2), an MT plus-end MAP that binds to MT lattices in a phosphorylation-dependent manner

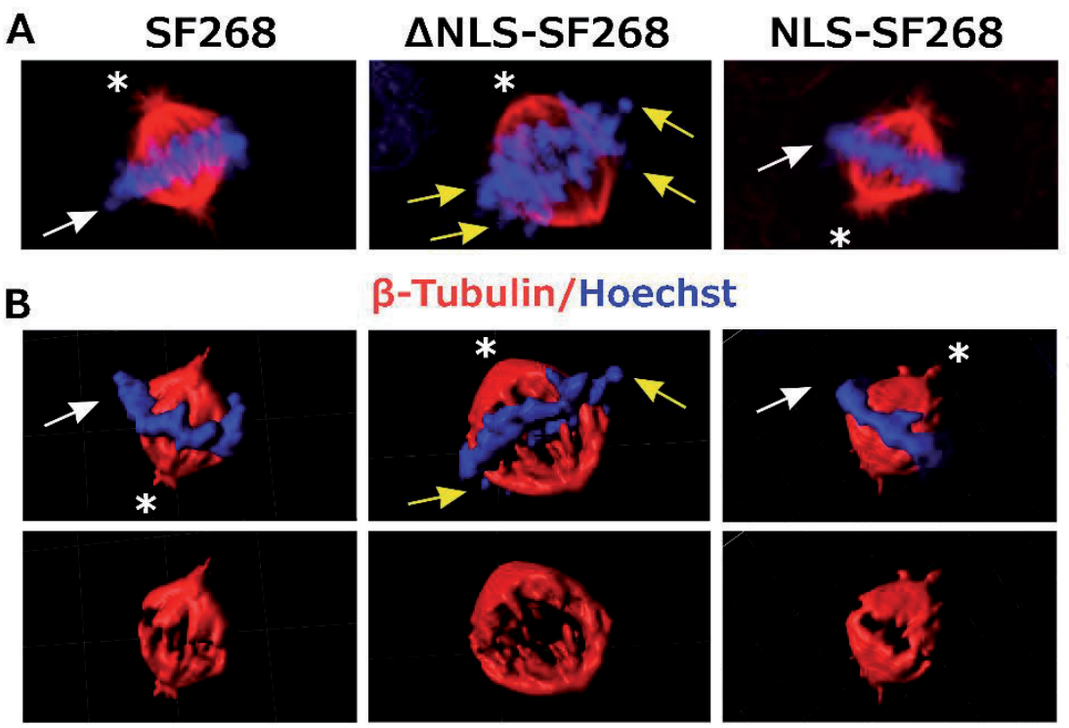

Figure 5.
Spindle configuration and chromosome congression are regulated by neurofibromin isoforms. A, Immunofluorescence confocal images of cells at metaphase stained for β-tubulin and chromatin of SF268, NLS, and ΔNLS-SF268 cells. B, 3D reconstructions (IMARIS) of the same images for better viewing show that parental and NLS-cells have a rich array of astral MTs (asterisks) and properly congressed chromosomes (white arrows). In contrast, ΔNLS cells lack astral MTs (asterisks) and display abnormal chromosome congression (yellow arrows).

during mitosis, leads to a marked delay in anaphase onset and abnormalities in chromosome congression [63]. Multifunction proteins ALIX and RACK1 acting through regulation of other MAPs [64] or motor proteins [65], are also essential for proper astral MT elongation, spindle orientation and chromosome segregation. Whether neurofibromins regulate in addition the actions of other MAPs remain to be investigated; it is clear, however, that NLS neurofibromins are essential for astral MTs formation.

3.2 Spindle length

Both parental and NLS-cells with fully developed metaphasic spindles have normally congressed chromosomes at the spindle equator (**Figure 5**, white arrows), albeit the latter have significantly shorter spindles (pole to pole distance $\bar{x}$ = 8.91 ± 0.22 μm versus 10.8 ± 0.2 μm; $p < 0.0001$ [39]). In stark contrast, cells expressing only ΔNLS-neurofibromins have very poorly aligned chromosomes at the equator (**Figure 5**, yellow arrows), although their metaphasic spindle length is significantly longer ($\bar{x}$ = 11.5 ± 0.15 μm; $p < 0.01$;). In over 50% of ΔNLS cells, the majority of chromosomes from a wide diffused ring and altogether lack the typical tight alignment seen at metaphase (**Figure 5**, yellow arrows).

3.3 Spindle geometry

When confocal z-planes of β-tubulin and Hoechst fluorescence signals are reconstructed in three-dimensions, the dramatically different spindle geometries, formed in the absence of NLS-neurofibromins, become readily apparent (**Figure 5B**). The anastral spindles of ΔNLS cells feature large hollows by the equator and chromosomes forming queues on some prominent thick K-fibers, while over half of the cells have unaligned chromosomes, or a 4-fold increase compared to control and NLS-cells [39]. In interpreting this geometry, we assume that thicker MT formations may result from upregulation of the augmin-mediated, local amplification of MTs, as augmin targets γ-TuRCs to nucleate preexisting MTs [66]; in parallel, bridging (**Figure 2A**) MTs [40] delay to develop, hence the spindle equator is almost devoid of tubulin signals. As these metaphasic patterns [39] strongly resemble those typically seen at prometaphase when unstable interactions of MTs dominate [56], the important role of neurofibromins as MAPs for mitosis is further highlighted.

3.4 Duration of mitotic phases

Abnormal positioning of the spindle often associates with altered times spent at mitotic stages. Quantification of the mitotic stage distribution for each cell type by flow cytometry validates this prediction, since loss of NLS-neurofibromin elicits an almost 50% increase in time spent at metaphase [39]. In contrast, NLS cells have significantly lower percentages in prophase and metaphase over parentals, which, combined with higher percentages in cytokinesis, reflected an overall acceleration through metaphase. Considered together, these results document for the first time that neurofibromin actively participates in the progression of mitosis. Moreover, these data further support the notion that NLS- and ΔNLS-neurofibromins may exert opposing effects during aster formation and spindle assembly, as, in parental cells, these two parameters appear to be the arithmetic sum of the results obtained with each isoform type [39].

3.5 Chromosome segregation fidelity

In parental (**Figures 2** and **5**, white arrows) and NLS-neurofibromin expressing cells (**Figure 5**, white arrows), chromosomes move in a coordinated manner towards the opposed poles and chromosome compaction is readily seen. In cells expressing only ΔNLS-neurofibromins, these parameters are again inversely regulated, namely, despite the prolonged time spent at metaphase, a significant >40% increase in cells with chromosome segregation errors mainly lagging chromosomes is documented (**Figure 5**, yellow arrows); similar delays in chromosome compaction in ΔNLS-neurofibromin cells are recorded in telophase [39]. The described effects on spindle assembly and chromosome segregation perturbations are readily traced in the high frequency of micronuclei, and a 5-fold increase in the numbers of cells with micronuclei within 10 mitotic cycles [39]. Micronuclei may facilitate rapid karyotype evolution, as their few chromosomes, unprotected from DNA damage, often undergo chromothripsis and chromoanasynthesis and then get incorporated into the genome of the host cell within just 1–2 mitoses [67].

Summarizing, these data establish for the first time that NLS- and ΔNLS-neurofibromins actively participate in the formation of mitotic asters and spindles, and efficient, error-free chromosome congression, possibly by exerting opposing effects. The question then rises about the possible mechanisms that would explain their different interactions with tubulins and microtubules. Drawing from immunoprecipitations studies with various antibodies, whereby different amounts of endogenously expressed neurofibromin is recovered from ΔNLS- or NLS-cell lysates [39], we have to reasonably presume that inclusion, or not, of the 41 amino acids of exon 51 may alter the conformation of the molecule. Numerous examples exist when one to few residues change the functional properties of a protein by imposing changes on protein conformation and post-translational modifications. Thus, an expected differential conformation of the ΔNLS or NLS neurofibromins would impact both its known intramolecular and intermolecular interactions.

In support of this argument the affinity of NLS- is higher for β- and lower for γ-tubulin when compared to ΔNLS-neurofibromins. Moreover, revisiting the question of MAP domains in the primary sequence of neurofibromin, we have identified, at higher percentages of similarity than the previously suggested [49], three other small Tau-like motifs [39], one of which corresponds to codons apposed to 50–51 or 50–52 exon junctions and could be affected by the inclusion or skipping of exon 51.

Our results show that the direction of the ΔNLS or NLS knockdown effects is most often opposite and suggest that the two functionally interact when both present. Whether this occurs through the formation of a dimer, if neurofibromins form dimers [28, 68] in eukaryotic cells at normal neurofibromin concentrations, is an intriguing question. Indeed, how the NLS and ΔNLS conformations may affect dimer formation is an interesting experimental goal.

Given the higher abundance of *NLS* transcripts irrespectively of GRD type that we observe in neurons and astrocytes [19], it is not possible to have only NLS-ΔNLS heterodimers. It is, however, possible to have homodimers only, or dimerization to be driven by properties that GRDI or GRDII attain. If any of the latter are entertained in eukaryotic cells, then an additional level of regulation is to be expected. At any rate, loss of the amino acids and the NLS encoded by exon 51 suffices to produce a different MAP. By the same token, the expression of two closely related isoforms yet with differential effects on MT structures further suggests that an extra layer of regulation on MT dynamics is thereby served by neurofibromins.

4. Conclusions: NLS-containing, tumor suppressor MAPS

A major theme that emerges from our studies and studies by others is that several MAPs have been described as tumor suppressors and correspondingly several proteins, identified as such, are found to function as mitotic MAPs. Another typical characteristic of such tumor suppressors is the presence of an NLS in their amino acid sequence, which regulates both their timely nuclear import in preparation of mitosis and their release during spindle assembly. All currently known tumor suppressor proteins with such attributes are listed in **Table 1**.

Perturbations of spindle assembly and chromosome segregation, when tumor suppressors that act as mitotic MAPs are lost or mutated, is a first step to aneuploidy. Given the usually compromised ability for DNA repair and the increased replication stress in these genetic backgrounds, the resulting aneuploidy may additionally feed chromosomal instability (CIN) and thus rapid evolution of karyotypes with clonal expansion advantages and tumorigenesis [104, 105]. Hence, the study of the regulation of NLS-containing tumor suppressors must receive high attention in the collective effort of understanding their mechanism of action and for developing better prognostic and possibly therapeutic approaches.

Protein	Gene	Functions served
APC	*APC*	MT stabilization, astral MT formation, compaction of mitotic chromatin, chromosome segregation [69–71]
ATIP3	*MTUS1*	MT stability, cell polarity and migration, centrosome number, metaphase spindle length [72–74]
BRCA1	*BRCA1*	MT nucleation, spindle assembly, centromeric cohesion [75–77]
CYLD	*CYLD*	MT polymerization and stability, especially of astral MTs; spindle positioning [78, 79]
DAB2IP	*DAB2IP*	MT stability [80]
DLC-2/STARD13	*STARD13*	MT stability, spindle positioning, chromosome segregation [81]
DLG1/ SAP97	*SAP97*	MTs polarization, centrosome positioning [57, 82]
FEZ1/LZTS1	*FEZ1/LZTS1*	MT assembly, chromosome segregation [83, 84]
FHIT	*FHIT*	MT assembly, spindle disassembly [85, 86]
NAV3	*NAV3*	MT stability [87]
Neurofibromin	*NF1*	MT polymerization, cell migration, astral MT formation, spindle assembly and positioning, chromosome segregation [19, 24, 29, 37–39]
NF2/Merlin	*NF2*	MT polymerization; actin cytoskeleton organization, signalling scaffolding at the membrane [88–90]
p53	*TP53*	Clustering of centrosomes, pole formation [91–93]
PTEN	*PTEN*	Centrosome and spindle pole motility, spindle assembly, chromosome segregation [45, 94]
RASSF1A	*RASSF1A*	MT stability, spindle organization, chromosome segregation [95–97]
RB1	*RB1*	MT dynamics, centrosome number and condensation, chromosome segregation [98–100]
VHL	*VHL*	MT stability, spindle positioning [101, 102]
WT1	*WT1*	Chromosomal segregation, mitotic checkpoint [103]

Table 1.
Tumor Suppressors with a functional NLS and established roles as MAPs.

Conflict of interest

The authors declare no conflict of interest.

Nomenclature

GFAP	glial fibrillary acidic protein
GRD	Ras-GAP related domain
MAPs	microtubule associated proteins
NF-1	Neurofibromatosis type 1
NLS	nuclear localization sequence
PKC	protein kinase C
RasGAP	Ras-GTPase activating protein
TSG	tumor suppressor gene

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