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Abstract: Neurofibromatosis type 1 (NF1) is an autosomal dominant tumour predisposition syndrome that is caused through loss of function mutations of a tumour suppressor gene called Neurofibromin 1. Therapeutic options are currently limited for NF1-associated tumours, where treatment is often restricted to complete surgical resection with clear margins. Herein, we discuss the multifunctional tumour suppressive role of neurofibromin, which is classically known as a GTPase activating protein (GAP) towards the RAS small GTPase. While neurofibromin inhibits proliferative growth through blockade of RAS-mediated signal transduction, neurofibromin should also be considered as a modulator of cell motility and cell adhesion. Through interfacing with the cytoskeleton and membrane structures, neurofibromin acts as a negative regulator of RHO/ROCK signalling pathways involved in cytoskeletal dynamics that are instrumental in proper neuronal development. In the context of cancer when the normal function of neurofibromin is lost via genetic mutation, heightened proliferative drive and an innate ability to migrate are key attributes that predispose NF1 patients to cancer. Malignant Peripheral Nerve Sheath Tumours (MPNST) can develop from benign neurofibromas and are the main cause of death amongst NF1 patients. Through recent research on MPNSTs, we have much more insight into the key molecular events that drive their malignancy. Advances regarding malignant drivers involved in cell migration, cell invasion and angiogenic signalling are discussed in this review, where these findings will likely influence future therapies for both NF1 and related sporadic cancers.

Neurofibromatosis Type 1: fundamental insights into cell signalling and cancer

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Abstract

Neurofibromatosis type 1 (NF1) is an autosomal dominant tumour predisposition syndrome that is caused through loss of function mutations of a tumour suppressor gene called *Neurofibromin 1*. Therapeutic options are currently limited for NF1-associated tumours, where treatment is often restricted to complete surgical resection with clear margins. Herein, we discuss the multifunctional tumour suppressive role of neurofibromin, which is classically known as a GTPase activating protein (GAP) towards the RAS small GTPase. While neurofibromin inhibits proliferative growth through blockade of RAS-mediated signal transduction, neurofibromin should also be considered as a modulator of cell motility and cell adhesion. Through interfacing with the cytoskeleton and membrane structures, neurofibromin acts as a negative regulator of RHO/ROCK signalling pathways involved in cytoskeletal dynamics that are instrumental in proper neuronal development. In the context of cancer when the normal function of neurofibromin is lost via genetic mutation, heightened proliferative drive and an innate ability to migrate are key attributes that predispose NF1 patients to cancer. Malignant Peripheral Nerve Sheath Tumours (MPNST) can develop from benign neurofibromas and are the main cause of death amongst NF1 patients. Through recent research on MPNSTs, we have much more insight into the key molecular events that drive their malignancy. Advances regarding malignant drivers involved in cell migration, cell invasion and angiogenic signalling are discussed in this review, where these findings will likely influence future therapies for both NF1 and related sporadic cancers.

Keywords: NF1; MPNST; neurofibromin; cancer; tumour suppressor

Abbreviations: CRMP-2, Collapsin response mediator protein-2; CSRD, cysteine/serine-rich domain; CTD, C-terminal domain; ERK, extracellular signal-regulated kinase; FTI, farnesyltransferase inhibitor; FAK1, Focal Adhesion Kinase 1; GAP, GTPase activating protein; GRD, GAP related domain; GEF, guanine nucleotide exchange factor; HIF, hypoxia inducible factor; LIMK, LIM domain kinase; LOH, loss of heterozygosity; MAPK, Mitogen-activated protein kinase; miRNA, microRNAs; MMP, matrix metalloproteinase; MPNST, Malignant Peripheral Nerve Sheath Tumour; mTORC1, mechanistic target of rapamycin complex 1; NF1, Neurofibromatosis type 1; OPN, Osteopontin; PH, pleckstrin homology; PKA, cAMP-dependent protein kinase; PRC2, polycomb repressive complex 2;

PTEN,Phosphatase and tensin homolog; Rheb, RAS homology enriched in brain; RB1, Retinoblastoma protein 1; ROCK, RHO-associated, coiled-coil-forming protein kinase; RTKs, receptor tyrosine kinases; STAT3, signal transducer and activator of transcription 3; TKI, tyrosine kinase inhibitor; TP53, Tumour Protein P53; VEGFR, Vascular Endothelial Growth Factor receptor.

1. Introduction

NF1 (MIM 162200) is an autosomal dominant tumour predisposition syndrome with an incidence of 1 in every 3500 births. The description of individuals with NF1 has been well-documented throughout history, going as far back as the thirteenth century. NF1 became synonymous with von Recklinghausen's disease after Friedrich Daniel Von Recklinghausen gave a classic description of Neurofibromatosis in 1882 [1]. NF1 is recognised for the variety of tissues and organs it can affect and also for its variation in disease expression within individuals. Even amongst family members, the manifestations of NF1 can vary quite markedly.

NF1 is often diagnosed early on in childhood through the presence of café-au-lait spots (observed in 99% of NF1 patients), which are dark pigmented patches on the skin, as well as inguinal/axillary freckling [2]. Other clinical features observed during childhood are benign growths called Lisch nodules that often form within the iris but do not usually impair vison. However, some affected patients can also develop optic gliomas, tumours that grow along the optic nerve to the brain, leading to reduced vison or even blindness. Other symptoms commonly associated with NF1 include hypertension, short stature, and macrocephaly. Skeletal abnormalities are also apparent, such as abnormal curvature of the spine (scoliosis). Later in adulthood, NF1 patients frequently develop neurofibromas that are benign nerve sheath tumours that are located on or just beneath the skin and can also commonly arise from nerves near the spinal cord and brain. Neurofibromas comprise of a mixture of Schwann cells (peripheral nerve support cells), perineurial-like cells and fibroblasts. There are two main types of neurofibromas called dermal neurofibroma and plexiform neurofibromas. Dermal neurofibroma can present either on the skin (cutaneous), beneath the skin (subcutaneous), or deep underneath the dermis (nodular). Plexiform neurofibromas can arises from major nerve branches from the skin [2], where these are typically congenital (effecting 60% of NF1 patients) and develop within peripheral nerves and their perineural sheaths. Plexiform neurofibromas are characteristically hyper-proliferative, leading to enlarged tumours that can affect entire body sections and can encroach on vital organs and structures. Consequently, plexiform neurofibromas can be debilitating, causing significant discomfort, disfigurement, neurological deficits and is the major cause of morbidity in NF1 patients. Surgical removal of these tumours has proven to be more difficult than other types of neurofibromas due to its infiltrative nature [3]. 10% of plexiform neurofibromas can undergo transformation to highly invasive peripheral nerve sarcomas called malignant peripheral nerve sheath tumours (MPNSTs) that grow along nerves and typically metastasise to the brain and bone as well as other locations.

It took over a decade to identify the causative gene of NF1, which posed a major challenge. In 1987, gene linkage studies initially localised the *NF1* gene to the pericentromeric region of chromosome 17. Later on, advanced mapping located the gene in a region close to the 17q11.2 [4–6], which was then further refined within 3cM of 17q11.2 [4,5,7]. Cytogenetic studies of two unrelated NF1 patients with balanced translocation provided a more definitive position of the *NF1*

gene, where the common breakpoints in both individuals was localised to the 17q11.2 region [8–10]. Finally, the *NF1* gene was identified by positional cloning experiments at 17q11.2 in 1990 [11,12].

The *NF1* gene has a high mutation rate, approximately one mutation occurring per 10,000 gametes per generation (100 times greater than many other genes). The high mutation rate in the *NF1* gene could possibly be due to its large size (350 kb with 60 exons). Within individuals affected, approximately 255 mutations have been observed, leading to protein truncation and abnormalities. As with most tumour suppressors, heterozygous loss of function mutations of NF1 is responsible for disease. NF1 patients have a higher cancer incidence compared to the general population, with increased risk of gastrointestinal tract, liver, lung, bone, thyroid, breast and ovarian cancer [13]. As well as predisposing NF1 patients to cancer, mutation of the *NF1* gene is often sporadically mutated, where mutation frequency of *NF1* occurs in 5-10% of non-NF1 associated sporadic cancers such as glioblastoma, neuroblastoma, acute myeloid leukaemia, breast cancer, lung cancer and ovarian cancer.

2. NF1 tumour suppressor and oncogenic Ras in cancer

The *NF1* gene encodes a tumour suppressor protein known as neurofibromin that is a large multi-domain protein (~250 KDa, containing 2,818 amino acids) [14]. Neurofibromin plays an upstream repressive role in the regulation of RAS small G-proteins. RAS switches between two conformation states and is active when GTP-bound or inactive when GDP-bound. This GTP/GDP switching is controlled by two opposing activities, which are governed by either GTPase activating proteins (GAPS) or guanine nucleotide exchange factors (GEFs). The GAP related domain (GRD) is central to the tumour suppressive role of neurofibromin that functions as a GAP towards RAS small G-proteins. Through this GRD, neurofibromin binds to and enhances the intrinsic GTPase activity of GTP-bound RAS, which switches RAS to an inactive GDP-bound state. Consequently, inactivating disease causing mutations of neurofibromin leads to constitutive GTP-loading and hyperactivation of RAS. There exists a large and diverse family of functional RAS GAPs, which includes not only neurofibromin, but also p120GAP, GAP1IP4BP, Ca^{2+}-promoted RAS inactivator, RAS GTPase activating-like protein RASAL, DAB2IP, nGAP, and SynGAP. In neuronal cell linages, i.e., neural crest-derived tumours such as neurofibromas, neurofibromin is thought to function as the predominant GAP towards RAS, which might help explain why NF1 patients are predisposed to these tumour types. SOS, RasGRF and RasGRP all directly oppose the action of Neurofibromin by functioning as the RAS GEF to exchange bound guanine nucleotide to GTP [15,16]. When actively GTP-bound, RAS associates with effector enzymes and results in activation of signalling pathways that control cell survival and proliferation [17]. Upon loss of neurofibromin function and RAS activation, a higher level of proliferative drive likely accounts for the large irregular tumour mass of plexiform neurofibromas as well as their cancer progression to MPNSTs. Cell proliferation would likely act as a mutation accelerant that would progress the benign plexiform neurofibromas towards malignancy.

Due to hyperactivation of RAS, NF1 is classed as a RASopathy. There are three closely related RAS small G-protein family members, HRAS, KRAS and NRAS (85% amino acid identity) [17,18]. Upon loss of NF1, transformation is driven mostly through hyper-activation of KRAS. In the context of sporadic cancers, activating mutations within KRAS is a more common early event in cancer progression (21.6%), when compared to activation mutations of either NRAS (8%) or HRAS (3.3%). One of the main downstream effectors of RAS is the protein serine/threonine kinase RAF. Studies have shown

that active GTP-bound RAS binds to and activates all three related RAF proteins (c-RAF1, BRAF and ARAF). Interaction between RAS and RAF results in re-localisation of RAF to the plasma membrane where it is then activated to drive the Mitogen-activated protein kinase (MAPK) signalling cascade. RAF does this through phosphorylation and activation of mitogen-activated protein kinase (MEK) that then subsequently phosphorylates and activates extracellular signal-regulated kinases 1 and 2 (ERK1/2) downstream in the MAPK signalling pathway (see Figure 1). Active ERK1/2 then translocates to the nucleus, where they orchestrate a transcriptional programme that drives cell proliferation [19,20].

3. Domains of neurofibromin linked to tumour formation and neuronal function

While the GRD has been researched extensively and is considered the principle domain that suppresses tumour formation by reverting RAS to an inactive GDP-bound state, the other less well-characterised domains are of equal importance for the function of neurofibromin. Key functional domains of neurofibromin also include the Sec14 and pleckstrin homology (PH) domain (referred to as the 'Sec14-PH module') as well as the C-terminal domain (CTD). The presence of multi-functional domains within neurofibromin suggests that it acts as a large scaffold to interface with multiple proteins to coordinate signalling events central to neuronal function and development (refer to Figure 1 for functional domains known to be involved in tumour suppression).

3.1 Sec14-PH module of neurofibromin is essential for tumour suppression and regulates the cytoskeletal through LIMK

In the central region of neurofibromin (proximal to the GRD) the Sec14-PH module resides, which facilitates binding of neurofibromin to membrane surfaces and vesicular trafficking within the endomembrane system. While the PH domain tethers neurofibromin to membranes surfaces through binding to phosphatidylinositol lipids on membranes, the Sec14 domain allows for active trafficking of neurofibromin between membrane structures. Spatial localisation of neurofibromin to lipid platforms on membrane surfaces within the cell is thought to proximally deactivate RAS that is also membrane tethered. Membrane anchoring of RAS is mediated via a prenylation moiety that is added post-translationally to the extreme C-terminal CAAX motif of RAS (where 'A' refers to an aliphatic residue). The Ras-GAP activity of neurofibromin within cells absolutely requires this Sec14-PH module [19,20], which implies that the correct spatial localisation of neurofibromin to membrane structures is needed for it to function as a RAS GAP. Furthermore, membrane tethering of RAS via prenylation is necessary for its transforming capacity [21-23], implying that RAS needs to be localised appropriately to membranes to relay downstream signalling. Initial investment went into drug therapies to inhibit RAS prenylation with the notion that this would block oncogenic RAS signalling. While farnesyltransferase inhibitors (FTIs) showed promise as an anti-cancer agent, it was later apparent that RAS prenylation was not exclusively post-translationally modified by farnesyltransferases, but could also be modified by geranylgeranyltransferases. This redundancy regarding RAS prenylation reduced enthusiasm to progress further with FTI therapies as a means to inhibit RAS. The observed anti-tumour activity of FTIs is likely through inhibition of other prenylated GTPases. One good GTPase candidate is Rheb (RAS homology enriched in brain), which unlike RAS is exclusively prenylated through farnesyltransferases. FTIs can sufficiently block Rheb signalling towards mechanistic target of rapamycin complex 1 (mTORC1) [24], known to drive cell growth and angiogenesis [25].

Of interest, Ozawa *et al*. showed that interfering knockdown of neurofibromin led to excessive actin stress fibre formation that was due to activation of the RHO signalling pathway involving LIM domain kinase 2 (LIMK2) [26]. LIMK2 (as well as LIMK1) are substrates of RHO-associated, coiled-coil-forming protein kinase (ROCK), which is the downstream effector of RHO. While the mechanism of action was first perceived to be through hyperactive RAS signalling, a more recent study implicated a direct interaction with neurofibromin and LIMK2. LIMK2 was found to interact with the Sec14-PH module in a yeast two-hybrid screen using the Sec14-PH module as bait. Cytoskeletal reorganisation is important for many cell processes, such as cell motility, cell adhesion, cytokinesis, and is particularly important for neurite extension. LIMK2 is a dual Serine/Threonine and Tyrosine protein kinase involved in cell cycle control, cell migration and also neuronal development. In the disease setting of NF1, LIMK2 activity is elevated, leading to actin cytoskeletal reorganisation. LIMK2 does this through phosphorylation of cofilin, which inhibits cofilin's activity to disassemble actin via depolymerisation and as a consequence lengthens actin structures. Furthermore, neurofibromin was recently shown to act as a negative regulator of the RAC1/PAK1/LIMK1/cofilin pathway independently of RAS signalling [26].

3.2 The C-terminal domain of neurofibromin acts as a protein scaffold that regulates neuronal development and growth

As well as being involved in actin rearrangements, neurofibromin interacts with the microtubular and microfilamentous cytoskeleton [27]. Through the C-terminal domain neurofibromin was initially shown to interact with Focal Adhesion Kinase 1 (FAK1, also known as protein tyrosine kinase 2) and were observed to co-localise within mammalian cells [28]. A more recent study using genetics and protein interactions within *Drosophila* models of NF1 revealed that neurofibromin is a downstream target of FAK1 (known as Fak56 in *Drosophila*), where FAK1 interacted with the N-terminal region of NF1 in a Ca^{2+}-dependent manner [29]. In this study, Tsai *et al*. showed that synaptic transmission was increased upon loss of *NF1*, indicating that NF1 is important for normal synaptic function. FAK1 is involved in cerebral cortex lamination, axon branching and pathfinding, and synapse formation.

These studies show that FAK1 can bind to both the N-terminus and C-terminus of neurofibromin. As FAK1 is a tyrosine kinase, it is tantalising to speculate that neurofibromin might be directly phosphorylated on specific tyrosine residues by FAK1 that could regulate its tumour suppressor function. Phosphorylation of neurofibromin by upstream kinases and delineation of signalling components downstream of neurofibromin has been poorly studied to date, and is currently a knowledge gap. Some advances have been made however. It is known that neurofibromin is constitutively phosphorylated within a cysteine/serine-rich domain (CSRD) as well as within the C-terminal domain. Within the C-terminal domain, a cluster of cAMP-dependent protein kinase (PKA) phosphorylation sites have been identified (Ser2576, 2578, 2580, 2813 and Thr2556), where phosphorylation leads to 14-3-3 association that impedes neurofibromin's ability to function as a RAS-GAP [30]. It is not currently known whether 14-3-3 binding to neurofibromin would impede translocation or protein-protein interactions of neurofibromin and downstream functions.

Collapsin response mediator protein-2 (CRMP-2) is another interactor of neurofibromin that binds to the C-terminal domain [31]. CRMP-2 is involved cytoskeleton remodelling required for neuronal polarity via proper axon formation and growth cone guidance. Neurofibromin was seen to co-localise with CRMP-2 at distal tips and branches of extended neurites. Neurofibromin functions as

a repressor of CRMP-2, as binding of neurofibromin to CRMP-2 represses the ability of upstream kinases (such as CDK5, GSK-3β, and ROCK1) to phosphorylate and regulate CRMP-2 [31].

4. MPNST, a model of malignancy

It is clear that neurofibromin is critically involved in neuronal development and function through a multitude of functional domains that regulates cell motility and adhesion. It is therefore unsurprising that plexiform neurofibromas are at risk of becoming malignant. Therapeutic options are currently limited for NF1-related MPNSTs, which is the major cause of morbidity in NF1 patients. MPNSTs are high grade sarcomas that typically arise from pre-existing plexiform neurofibromas and are highly invasive. MPNSTs are relatively resistant to chemo and radiation therapy and such treatments can induce secondary malignant neoplasms [32]. As overall survival is poor with chemotherapy with a 5-year survival of 14 % [33], the main treatment for MPNSTs is complete surgical removal with clear margins [34]. Surgery is typically combined with adjuvant radiation therapy that reduces rates of local disease recurrence, but however, does not appear to lessen rates of distant metastases or overall survival [35]. After radical surgical resection, only 30% of patients will remain disease free after 5 years. MPNSTs have high levels of chromosomal alteration through genetic mutation and epigenetic modification, which results in their metastatic disease progression. Consequently, it has been a real clinical challenge to treat MPNSTs with targeted mono- and combination-drug therapies, which is due in part to these MPNSTs having a high level of intra-tumoural molecular heterogeneity.

While neurofibromas are characteristically benign, additional genetic events are essential for progression to MPNSTs and appear to derive from atypical neurofibromas [36]. Atypical neurofibromas are hypercellular PNSTs that have hyperchromatic nuclei with no sign of mitosis. We can learn a lot from MPNSTs, where they could be considered as a model of malignancy. Genetic alteration, driven through loss of NF1 function and hyperproliferation and consequential genetic instability, leads to key signature events that are commonly found in other malignancies. It is likely that transformation of neurofibromas to MPNSTs involve key genomic alternations that alter the cells within neurofibromas to malignancy [36]. There are several genomic abnormalities within MPNSTs that are absent in the neurofibromas.

4.1 Amplification of receptor tyrosine kinases in MPNSTs

Revealing those ‘usual suspects’ of cancer, loss of heterozygosity (LOH) analysis linked *Tumour Protein P53* (TP53), *Retinoblastoma protein 1* (RB1) and *Phosphatase and tensin homolog* (PTEN) as key gene markers of MPNST tumour progression; where these tumour suppressor genes are commonly inactivated in sporadic cancer. Through detailed analysis of malignant progression of benign neurofibromas to MPNSTs, several key advances have been made. Most notably, high-throughput whole genome analysis with microarrays has been instrumental in determining the molecular pathophysiology of MPNSTs that revealed oncogenic signatures within MPNSTs, which are not present in benign neurofibromas [37]. MPNSTs are characteristically hyperdiploid and their genomes are highly rearranged. When looking at copy number variations, multiple receptor tyrosine kinases (RTKs) were implicated within the malignant tumours. For instance, gene amplification of the *MET*, *EGFR*, *PDGFRA*, *IL-R* genes frequently occur in MPNSTs [37-41]. Such insight into malignant progression led to both mono- and combination-RTK targeted strategies through the use of tyrosine

kinase inhibitors (TKI), which initially appeared to be an ideal targeted therapy for NF1 patients with MPNSTs.

While these initial therapies looked promising in pre-clinical studies [42], the use of mono-TKIs appeared to be less effective in NF1 patients. An open-labelled phase II study with Imatinib (also known as Gleevec®, a TKI against PDGFR) to treat MPNST patients was terminated due to no effect (ClinicalTrials.gov identifier: NCT00427583). *In vitro* cell growth and proliferation as well as MPNST growth in the xenograft models revealed that three of the six MPNST cell lines tested were highly resistant to Imatinib treatment [43]. Drug resistance (both innate as well as acquired) is a major issue when using TKIs. Drug resistance likely develops through compensatory signalling pathways at the level of RTKs, where the network of RTK allows for a high level of flexibility and redundancy that hampers targeted therapies towards RTKs. There was initial enthusiasm, when a single MPNST patient with a high RAS signalling profile showed significant clinical response to lung metastases after treatment with a multi-TKI inhibitor, Sorafenib [44] (also called Nexavar®) that inhibits Vascular Endothelial Growth Factor receptor (VEGFR), PDGFR and Raf kinases. While Sorafenib looked promising in pre-clinical studies [45], Similar to Imatinib, there was a lack of efficacy in NF1 patients with MPNSTs in larger phase II trials as a mono-therapy (ClinicalTrials.gov Identifier: NCT00245102), and also when combined with chemotherapy (ClinicalTrials.gov Identifier: NCT00837148).

4.2 Amplification of RHO signalling during malignant progression

While we previously described neurofibromin's involvement in reorganising the cytoskeleton through LIMK and FAK1, the cytoskeleton is further dysregulated during NF1 disease progression. In a recent study, RAC1-dependent signalling was observed to be further amplified during progression of benign plexiform neurofibromas to MPNSTs [46]. Upadhyaya *et al*. performed single-nucleotide polymorphism genotyping and copy number alteration; LOH, and copy number neutral-LOH analyses of DNA isolated from 15 MPNSTs and directly compared them to both benign peripheral neurofibromas and patient-matched lymphocyte DNAs. Pathway analysis showed that MPNST specifically amplified RHO-GTPase pathway genes (RAC1 and ROCK2) as well as cytoskeletal remodelling/cell adhesion genes (FAK1 and LIMK1) [37]. Interfering RNA knockdown of RAC1, ROCK2, FAK1, and LIMK1 in MPNST-derived cell lines markedly enhanced cell adhesion, while wound healing, cell migration, and invasiveness were significantly reduced [37]. Such data reveals that these RHO-regulated genes are upregulated during malignant development of benign plexiform neurofibromas and are critical drivers of malignancy.

4.3 Angiogenic signalling of MPNSTs and the involvement of STAT3

Both the infiltrative nature of MPNSTs and the success with anti-angiogenic therapies, such as Sorafenib, in pre-clinical trials is highly suggestive that MPNSTs require angiogenic signalling for their malignant development. While assessment of vascularity in the late 1980s by transmission electron microscopy did not initially implicate angiogenic signalling in MPNSTs [47], a much more recent study postulated that MPNSTs likely require an 'angiogenic switch' for their cancer progression [48]. Indeed, Gesundheit *et al*., uncovered that MPNSTs from five NF1 patients all displayed a structure of immature microvascular blood vessels that was reminiscent to 'sprouting angiogenesis'. Importantly, these vascular observations were not present within the benign peripheral neurofibromas. Through recent research on angiogenic signalling, our lab discovered that signal transducer and activator of transcription 3 (STAT3) acts as an angiogenic signalling nexus that is absolutely necessary to

orchestrate the angiogenic response through hypoxia inducible factor (HIF) and VEGF-A [49]. Within multiple MPNSTs, we identified STAT3 as a common point of convergence for many RTKs involved in the angiogenic response, where STAT3 inhibition by drug and/or knockdown was sufficient to prevent cell migration/invasion and tumour formation [49]. Of particular interest, STAT3 inhibition through knockdown completely ablated expression of HIF-1α, HIF-2α and VEGF-A. This work reveals that therapeutic strategies that target the STAT3/HIF1α pathway or pathways that converge on STAT3 might be a viable treatment option for NF1 patients. It is also possible that acquired drug resistance after STAT3 drug inhibition would be less of a concern, when compared to current TKI therapies that target VEGFR. For instance, while Sorafenib prolongs progression-free survival for advanced renal cell carcinoma, ranging from 3-6 months, some tumours show primary resistance and others acquire resistance to treatment over time. This drug resistance is likely due to activation of compensatory signalling pathways that coordinate tumour revascularisation, where a multitude of pro-angiogenic factors (such as VEGF, FGFs, EGF, and IL-8 (as well as others)) signal through a network of target RTKs to elicit the pro-angiogenic response. Consequently, angiogenic signalling is highly flexible, driven through multiple RTKs and where signalling cross-talk between receptors is also evident. This level of flexibility hampers current TKI therapies. As STAT3 is a common downstream target that is shared by many RTKs, targeting the STAT3 pathway could be a more effective anti-angiogenic therapy.

4.4 Wnt signalling and cancer progression towards MPNSTs

Comparative transcriptome analysis between neurofibromas and MPNST revealed that the β-catenin/Wnt signalling was involved in the promotion of MPNST growth [50]. By using an advanced DNA transposon-based somatic insertion mutagenesis system, termed sleeping beauty, Watson *et al*. implicated that the Wnt pathway was a malignant driver of neurofibromas [51]. Sleeping beauty is a clever forward genetic screen that is capable of identifying both oncogenes and tumour suppressors involved in malignant transformation, where insertion of the DNA transposon can either drive expression of in-frame genomic sequences (such as oncogenes) or disrupt gene expression (such as tumour suppressors). Further confirming Wnt's involvement, an independent study uncovered 20 genes involved in Wnt signalling that exhibited altered expression in MPNST biopsies and cell lines when compared to benign neurofibromas [52]. More recently, pathway analysis revealed that the canonical Wnt signalling pathway likely drives MPNST development [46]. In the context of cancer, Wnt signalling is typically activated as a consequence of either (i) over-expression of Wnt ligand genes, (ii) mutation in the β-catenin gene that causes its activation, or (iii) inactivating mutations within genes involved in the proteolytic destruction of β-catenin (such as AXIN1, glycogen synthase kinase 3 beta (GSK3β), and APC) [53]. Signalling pathways that influence β-catenin stabilization can further promote Wnt signalling. For instance, Akt can promote phosphorylation and inactivation of GSK3B, which causes stabilization of β-catenin [54]. It is important to note that loss of PTEN and hyperactivation of PI3K/Akt signalling commonly occurs in human MPNSTs [55].

Osteopontin (OPN) is a downstream transcriptional target of Wnt and is highly expressed in MPNSTs [46]. In fact, after examining copy number alterations and gene expression, OPN was observed to be the most significantly elevated differential expressed gene observed between benign and malignant tumours. OPN is a secreted into the extracellular matrix and is a small acidic phosphorylated protein that interacts with integrins. OPN is thought to be necessary for the local invasive behaviour of cancer cells that is modulated through cell attachment and signalling through

integrin association, which then facilitates tumour seeding through neovascularisation. In colon cancer, where Wnt signalling is a known driver of cancer progression, high expression of OPN was found to correlate with poor patient prognosis [56]. While Wnt targets such as OPN could be viable biomarkers for MPNSTs, they also have potential for therapy [46].

4.5 SUZ12 is an NF1 modifier gene that drives malignancy

Research into dysregulated pathways within MPNSTs has led to some major advances regarding mechanisms of malignancy. One such breakthrough was the discovery that components of the chromatin-modifying polycomb repressive complex 2 (PRC2), SUZ12 and EED are frequently inactivated through mutations within MPNSTs [57]. De Raedt *et al*. uncovered that PRC2 functions as a tumour suppressor, that when lost, causes marked epigenetic reprogramming that can lead to malignancy of plexiform neurofibromas. PRC2 is known to be dysregulated in several human tumours, including colon, ovarian, breast and liver [58,59]. *SUZ12* lies proximal to the *NF1* gene, and is considered to cooperate with NF1 to suppress tumour growth. Indeed, more seriously affected NF1 patients carry a microdeletion of 17q within their germline that contains *NF1, SUZ12* and 12 neighbouring genes [60]. Such individuals with NF1 microdeletions have a higher prevalence of benign tumours and malignancies. Highlighting this cooperative tumour suppressor function between both NF1 and SUZ12, knockdown of SUZ12 led to enhanced tumour colony growth in NF1-deficient malignant glioma cells but not glioblastomas with wild-type NF1 [61]. Furthermore, Nf1+/-; Suz12+/- mice had higher tumour burden. Loss of *Suz12* is known to modify gene-expression through reducing trimethylation of Histone 3 K27 (H3K27me3) and increasing its acetylation (H3K27Ac), which results in recruitment of bromodomain proteins and associated transcription factors. By blocking this transcriptional switch with a bromodomain inhibitor, JQ1, De Raedt *et al*. showed that they were able to restore the epigenetic status of *Suz12*-deficient cells to a more normal setting and reduced tumour size (up to 68%) in *Nf1*/*TP53*/*Suz12* heterozygous mice [57]. While this work implicates PRC2 as a tumour suppressor in the background of loss of function of NF1, others have reported that PRC2 also acts as an oncogene, where SUZ12 is upregulated in several human cancers, including lung, colon, ovarian, breast and liver [58,62,63]. De Raedt *et al*. hypothesised that loss of function of both SUZ12 and NF1 synergised to amplify oncogenic RAS signalling to drive malignancy. Like *TP53*, *SUZ12* can be considered as a modifier gene in NF1.

4.6 The role of microRNAs (miRNA) in the formation of MPNSTs

miRNAs are a type of non-coding RNA, which play a role in regulating functions associated with gene expression [64]. Since their discovery approximately 20 years ago, miRNAs have shown to play an important role in cancer progression, including involvement in the progression of NF1-associated tumours [64,65]. Approximately 50% of miRNA-coding genes have been detected within genomic regions of tumour cells [66] and have shown to be involved in tumour metastasis, invasion as well as tumour progression. They also contribute to the chemo-resistance and radio-resistance features of tumours [64], making them an attractive biomarker and therapeutic targets in cancer. Even though the role of miRNAs in NF1 tumour development is still being defined, studies have shown that a number of miRNAs could possibly play a role in NF1 tumourgenesis. A microarray analysis based study carried out by Presneau *et al.* revealed that miR-29c was the most significantly downregulated miRNA in patient MPNSTs compared to neurofibromas [67]. A list of target genes of miR-29c included matrix metalloproteinase (MMP)-2, which is known to be involved in cell migration and

invasion. To further investigate the role of miR-29c an MPNST cell lines were transfected with miR-29c which resulted in reduced cell invasion. In an earlier study, Subramanian *et al.* demonstrated that miR-34a was also downregulated in MPNST in comparison to neurofibromas. Of interest, forced expression of miR-34a in these MPNST cells resulted in apoptosis [68].

Moreover, miR-204 was shown to play a role in the growth of MPNSTs [69]. As well as NF1-associated tumours, it has also been shown that miR-204 acts as a tumour suppressor in breast, prostate and kidney cancer [70]. A study analysing differentially expressed genes between plexiform neurofibromas and MPNSTs, revelled the upregulation of a group of miRNAs (miR-301a, miR19a and miR-106b) in MPNSTs that directly target PTEN [71]. Evidently, miRNAs play an important role in tumourgenesis in the context of NF1 and may open up new aspects for future therapies.

5. Conclusion and future research directions

Since the discovery of the causative NF1 gene, many fundamental advances have been made regarding the tumour suppressor function of neurofibromin. While neurofibromin is classically known as a RAS GAP, it is clear that neurofibromin is much more multifunctional. Through interfacing with the cytoskeletal architecture and membrane structures, neurofibromin acts as a scaffold protein that relays signalling events involved in not only cell growth and proliferation, but also cell adhesion and migration. This array of attributes intimately links neurofibromin to normal neuronal development, but also makes neurofibromin a prominent tumour suppressor. Consequently, loss of *NF1* can occur in many sporadic cancers and NF1 patients have an increased risk of cancer. MPNSTs have been valuable models to uncover and characterise key signature events linked to NF1-driven malignancy. Given the mixed heterogeneity of MPNST cells, therapy has been unsuccessful to date. However, recent advances have implicated several crucial signalling pathways involved in MPNST malignant growth and have uncovered new potential drug targets. These include the use of chromatin re-modellers to restore the epigenetic status of SUZ12-deficiency as well as to block angiogenesis and cell migration that are commonly upregulated in MPNSTs, such as the STAT3/HIF and RHO/ROCK signalling pathways.

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Figure Legends

Figure 1: functional domains involved in tumour suppression of NF1 Functional domains of NF1 include the GRD, which inhibits RAS GTPases (by converting them to an inactive GDP-bound status). Consequently, loss of NF1 function causes constitutive RAS activation towards an array of downstream effectors that include RAF, PI3K, RALGDS and PLCε (as well as others). The SEC14-PH module interfaces with both the cytoskeleton and membrane structures and is necessary for NF1 to function as a RAS-GAP as well as inhibition of LIMK. NF1 is a substrate of PKA, which inhibits NF1 through phosphorylation of the CTD. At the N-terminus, CSRD is also highly phosphorylated. FAK and CRMP-2 interact with the CTD which is required for neuronal development through regulation of cell adhesion and the cytoskeleton.

Figure 2: Cancer progression of MPNSTs A summary of genetic/molecular events of MPNST cancer progression from benign neurofibromas. (i) Gene amplification and/or expression of RTKs and signalling components involved in cell migration, adhesion and invasion. (ii) Loss of function mutations to tumour suppressors such as TP53, PTEN and RB1, as well as SUZ12 (involved in epigenetic changes through chromatin remodelling). (iii) Hyperactivation of a subset of transcription factors involved in malignancy that include, STAT3/HIF as well as WNT/β-catenin. Furthermore, recent research has also implicated miRNAs in malignant progression of MPNSTs and is listed.

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Figure 1

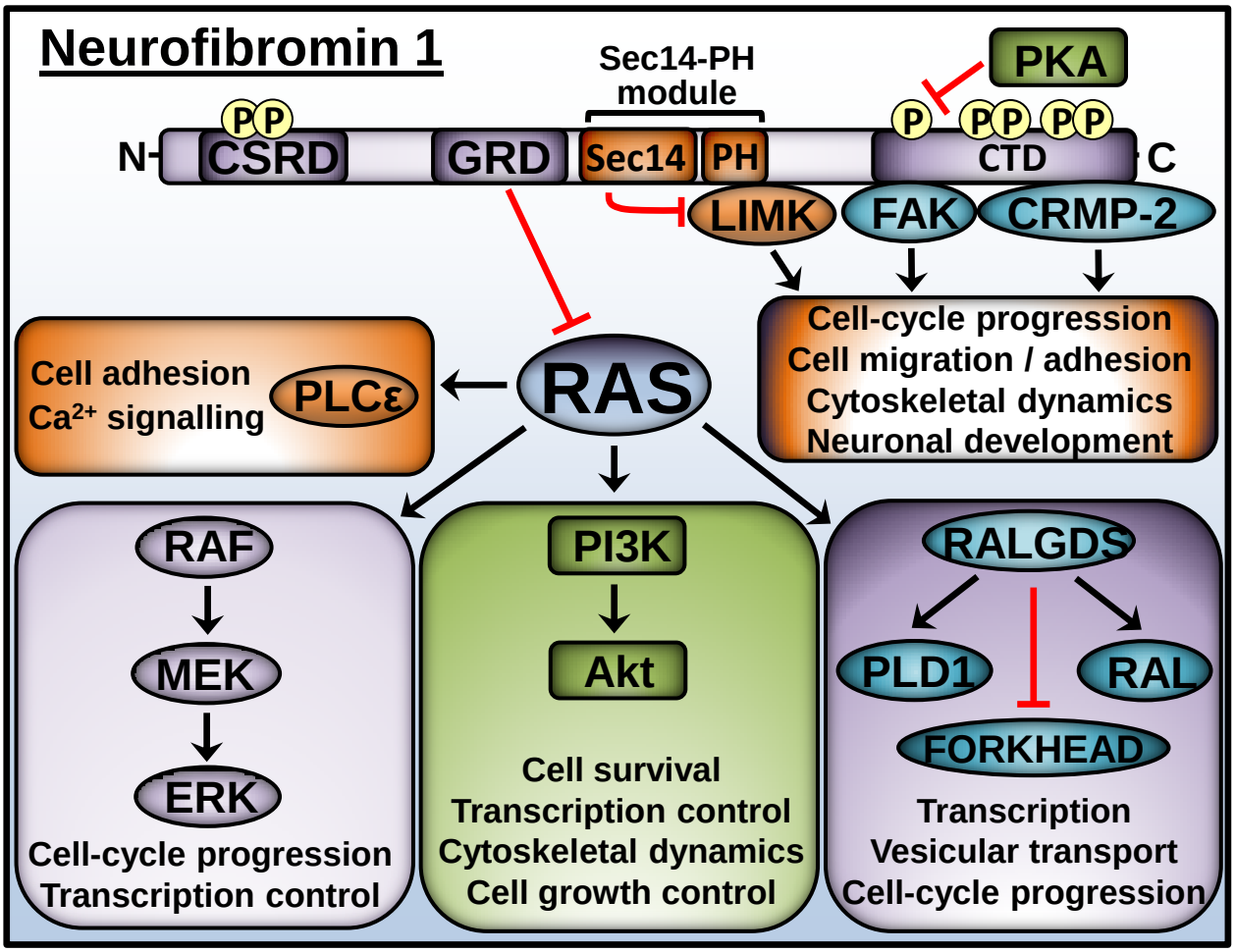
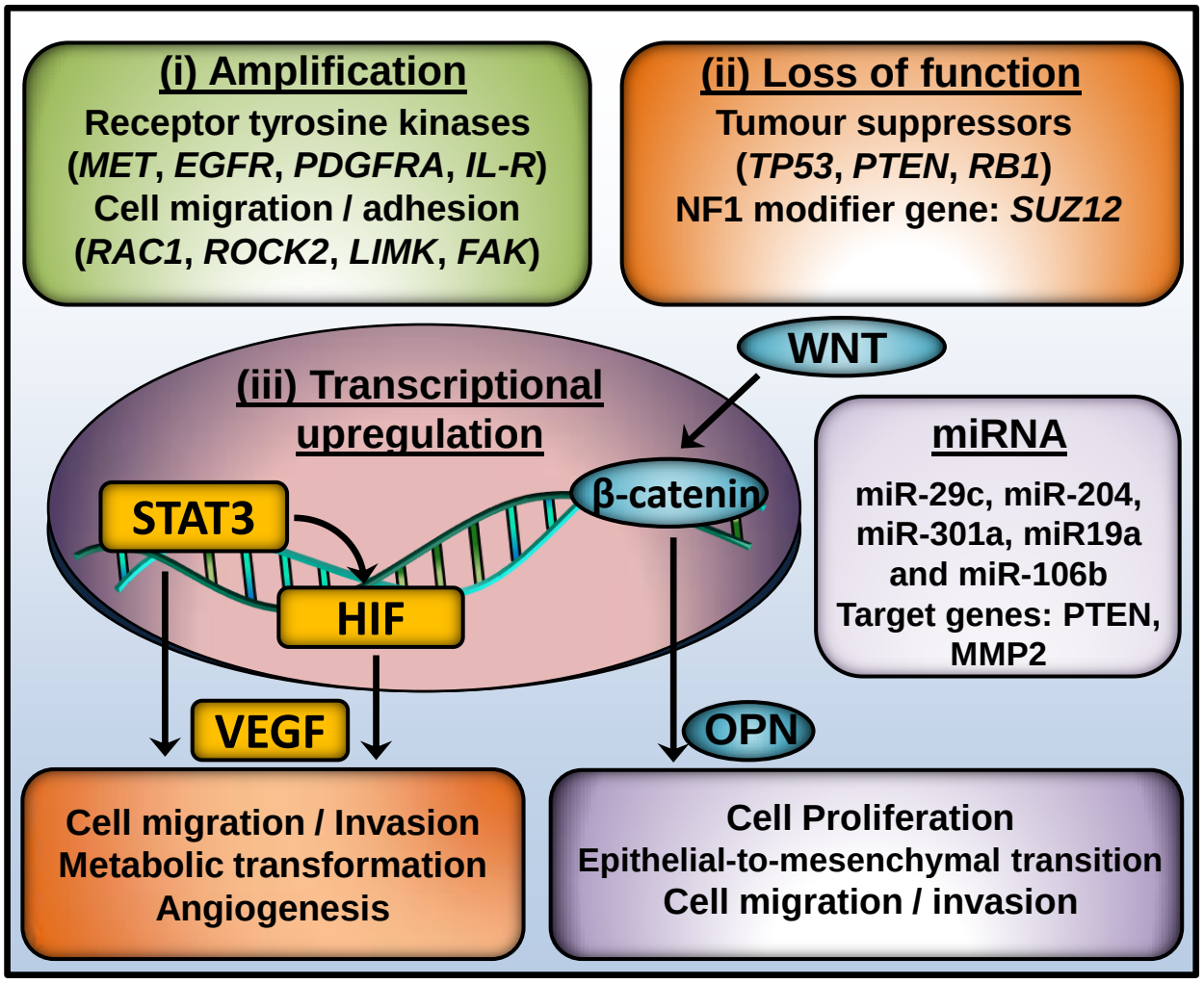

Neurofibromin 1
Sec14-PH module
PKA
P P
P P P P
N
CSRD
GRD
Sec14
PH
CTD
C
LIMK
FAK
CRMP-2
Cell-cycle progression
Cell migration / adhesion
Cytoskeletal dynamics
Neuronal development
Cell adhesion
Ca2+ signalling
PLCε
RAS
RAF
MEK
ERK
Cell-cycle progression
Transcription control
PI3K
Akt
Cell survival
Transcription control
Cytoskeletal dynamics
Cell growth control
RALGDS
PLD1
RAL
FORKHEAD
Transcription
Vesicular transport
Cell-cycle progression

Figure 2


(i) Amplification
Receptor tyrosine kinases
(*MET*, *EGFR*, *PDGFRA*, *IL-R*)
Cell migration / adhesion
(*RAC1*, *ROCK2*, *LIMK*, *FAK*)
(ii) Loss of function
Tumour suppressors
(*TP53*, *PTEN*, *RB1*)
NF1 modifier gene: *SUZ12*
WNT
(iii) Transcriptional upregulation
STAT3
β-catenin
HIF
miRNA
miR-29c, miR-204, miR-301a, miR19a and miR-106b
Target genes: PTEN, MMP2
VEGF
OPN
Cell migration / Invasion
Metabolic transformation
Angiogenesis
Cell Proliferation
Epithelial-to-mesenchymal transition
Cell migration / invasion