A RASopathy gene commonly mutated in cancer: the neurofibromatosis type 1 tumour suppressor

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Abstract | Neurofibromatosis type 1 (NF1) is a common genetic disorder that predisposes affected individuals to tumours. The *NF1* gene encodes a RAS GTPase-activating protein called neurofibromin and is one of several genes that (when mutant) affect RAS–MAPK signalling, causing related diseases collectively known as RASopathies. Several RASopathies, beyond NF1, are cancer predisposition syndromes. Somatic *NF1* mutations also occur in 5–10% of human sporadic cancers and may contribute to resistance to therapy. To highlight areas for investigation in RASopathies and sporadic tumours with *NF1* mutations, we summarize current knowledge of NF1 disease, the *NF1* gene and neurofibromin, neurofibromin signalling pathways and recent developments in NF1 therapeutics.

Neurofibromatosis type 1 (NF1; Online Mendelian Inheritance in Man (OMIM) database 162200) is a common inherited tumour predisposition syndrome that affects approximately 1 in 3,000 individuals worldwide[1–3]. The history of NF1 research and NF1 diagnostic criteria are described in FIG. 1. Some manifestations of NF1 are observed in early childhood, whereas others present later in life (FIG. 2).

Almost all patients with NF1 develop cutaneous neurofibromas, which are benign peripheral nerve tumours[4,5]. Some patients also develop benign plexiform neurofibromas, which can cause substantial morbidity and can degenerate to form peripheral nerve sarcomas known as malignant peripheral nerve sheath tumours (MPNSTs). These tumours are key contributors to reduced life expectancy in NF1 (REF. 6). Another common tumour in patients with NF1 is optic pathway glioma (OPG)[7]. In 1988, a US National Institutes of Health (NIH) consensus conference defined the currently used NF1 diagnostic criteria[8,9]. Notably, these criteria include neurofibroma and OPG but do not include malignant disease. Rarer tumours that develop in patients with NF1 are juvenile myelomonocytic leukaemia (JMML)[10], benign or malignant pheochromocytoma[11], gastrointestinal stromal tumour (GIST)[12], glomus tumours[13], juvenile xanthogranuloma, rhabdomyosarcoma[14] and lipoma[15]. Cloning of the *NF1* gene (OMIM 613113) led to the identification of biallelic *NF1* mutations in patient-derived tumours, which in turn immediately led to classification of *NF1* as a tumour suppressor gene[16–18]. All NF1-related tumours show biallelic inactivation of the *NF1* gene[19,20]. Patients with NF1 may also be at an increased risk of developing secondary cancers following radiation exposure, and it is important to consider this risk in the treatment of this predisposed population[21]. In addition, patients with NF1 have an increased risk of developing several adult cancers[22]. An analysis of UK death certificates found that patients with NF1 may also be at an increased risk of cancers of the gastrointestinal tract, liver, lung, bone, thyroid, breast and ovary[22]. Risk of malignant melanoma, non-Hodgkin lymphoma and chronic myeloid leukaemia might also be increased.

Early studies identified the protein encoded by *NF1*, neurofibromin, as having homology to the yeast proteins Ira1 and Ira2, which are inhibitory regulators of the RAS–cyclic AMP pathway[23–25]. In yeast, Ira proteins negatively regulate Ras by converting it from the active GTP-bound form to the inactive GDP-bound form. This is required to reduce levels of cAMP under nutrient-limiting conditions and to mediate membrane association of adenylyl cyclase. Neurofibromin is a GTPase-activating protein (GAP) that regulates RAS (RASGAP). It binds to GTP-bound RAS through its GAP-related domain (GRD) to dramatically augment its intrinsic GTPase activity[26]. Neurofibromin thereby functions as an off signal for all of the vertebrate RAS GTPases, including HRAS, NRAS, KRAS, MRAS, RRAS and RRAS2 (also known as TC21)[27]. Therefore, loss of *NF1* activates signalling through the RAS pathway, which is a key driver of cancer. GTP-bound RAS

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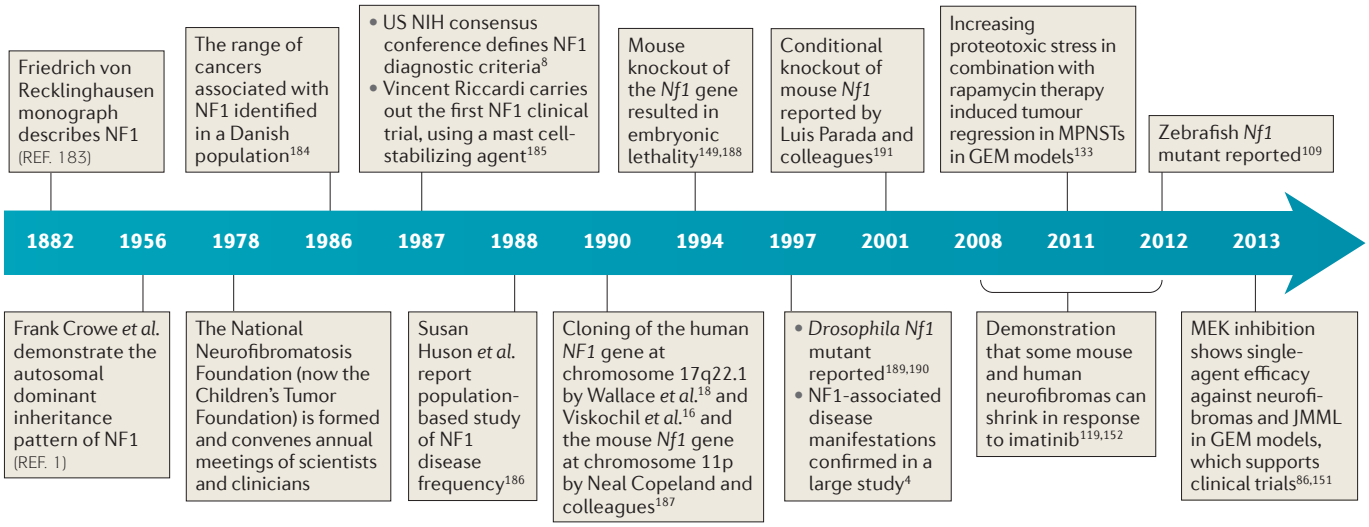


Figure 1 | **Neurofibromatosis type 1 historical developments.** From the development of diagnostic criteria to the development of ongoing clinical trials, the neurofibromatosis type 1 (NF1) field has been aided by close clinician–scientist interactions, which have been facilitated by the Children's Tumor Foundation. Currently accepted diagnostic criteria include six or more café-au-lait macules with a minimum diameter of >5 mm in pre-pubertal subjects; two or more neurofibromas of any type or one plexiform neurofibroma; freckling in the axillary or inguinal region; optic pathway glioma; two or more Lisch nodules (iris hamartomas); a distinctive osseous lesion, such as sphenoid dysplasia or thinning of long bone cortex with or without pseudarthrosis; and a first-degree relative with NF1 according to these criteria. GEM, genetically engineered mouse; JMML, juvenile myelomonocytic leukaemia; MPNSTs, malignant peripheral nerve sheath tumours; NIH, National Institutes of Health.

activates multiple effector pathways, including the RAS–MAPK pathway, in which GTP-bound RAS activates the RAF–MEK–ERK cascade (reviewed in REF. 28) (FIG. 3).

Mutations in many other genes that encode components of the RAS–MAPK pathway also predispose patients to partially overlapping sets of manifestations (known as RASopathies), which can include tumours[29] (BOX 1). Patients with Noonan syndrome have a 4% risk of developing cancer by the age of 20 years, with JMML predominating and rhabdomyosarcoma, neuroblastoma and low-grade glioma occurring at lower incidences[30]. Patients with Costello syndrome have a 15% risk of cancer by the age of 20 years, with rhabdomyosarcoma developing in 9% of patients, and neuroblastoma and bladder cancer developing in 1% of patients[30]. Patients with Legius syndrome develop lipoma[31]. The fact that all known RASopathy mutations affect the RAS–MAPK signalling pathway supports the idea that the MAPK pathway downstream of RAS, and not other pathways downstream of RAS, is the crucial driver of tumorigenesis in patients with RASopathies. 'RASopathy clinics' are beginning to study and compare patients with RASopathies. It is hoped that some or all RASopathy manifestations will respond to therapies such as MAPK inhibitors.

Importantly, sequencing of tumour exomes and genomes has revealed that somatic *NF1* mutations are present at incidences from 2.5% to 11.8% in sporadic, predominantly adult, tumour types such as lung cancer[32], glioblastoma[33], ovarian cancer[34], breast cancer[35] and acute myeloid leukaemia (AML)[36]; however, they are also present in the paediatric tumour rhabdomyosarcoma.

NF1 genetics: mutations and modifiers

Structure of the* NF1 *gene and mutational analysis. The *NF1* gene contains 60 exons and generates multiple alternatively spliced isoforms[37]. More than 1,400 mutations in the *NF1* gene have been reported in the Human Gene Mutation Database, most of which are clearly loss-of-function alleles. These include splice site, nonsense and missense mutations, as well as deletions, insertions, frameshifts and translocations[38]. Notably, several patient missense mutations that affect the neurofibromin GRD selectively diminish GAP activity, which supports the notion that the regulation of RAS has a crucial role in NF1 disease[39]. Identification of *NF1* mutations in patients remains difficult owing to the large gene size and structure, as well as the large range of mutations that have been identified[40]. For many years, 95% of patient mutations were identified using a combination of complementary methods, including protein truncation, fluorescence *in situ* hybridization, heteroduplex, Southern blot and cytogenetic analyses[38]. A preliminary report applied next-generation sequencing to samples from patients with NF1 (REF. 41), and DNA-based sequencing is now being offered as a clinical test for diagnostic purposes (see the website of University of Alabama at Birmingham Medical Genomics Laboratory). Thus, *NF1* mutation analysis can assist in diagnosing cases of NF1 in which a clinical diagnosis cannot be established with certainty.

The variability in manifestations in patients from a single family with the same *NF1* mutation does not support a major role for genotype–phenotype correlations in NF1 (REF. 42), although there are several important exceptions. Germline splice site mutations occur in 30%

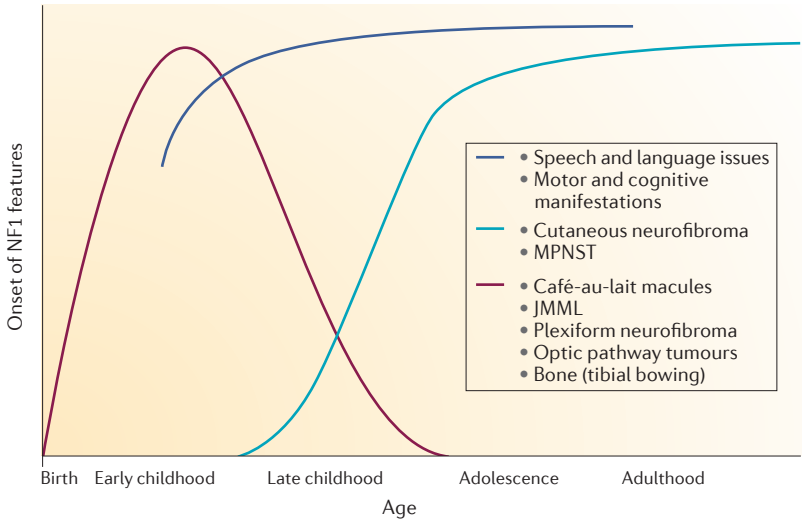


Figure 2 | **Disease manifestations in patients with neurofibromatosis type 1: epochs in which they develop.** Most plexiform neurofibromas are present at a very young age but, depending on the tumour location, may not be diagnosed until later in life if whole-body magnetic resonance imaging is not performed[192]. Multiple hyperpigmented skin lesions (café-au-lait macules) are an early sign of neurofibromatosis type 1 (NF1) and are observable in children under 3 years of age[193]. Young children may also present with bone dysplasia[194], delayed speech[195] and delayed acquisition of motor skills[196]. Young children with NF1 are at an increased risk of developing juvenile myelomonocytic leukaemia (JMML)[10] and optic pathway glioma (mean age of 5 years[197]). Later in childhood, cognitive issues surface[198]. If the features labelled in burgundy do not occur early, they will not develop later in life. The dark blue line shows that speech, language, motor and cognitive changes are detected, as children would normally develop specific skills. Cutaneous neurofibromas typically begin to grow during puberty[5]. Although malignant peripheral nerve sheath tumours (MPNSTs) may occur in childhood, they are most common in adult patients with NF1 over 30 years of age[124]. Beyond cancer, it is now appreciated that generalized or specific cognitive impairment is observed in >50% of patients with NF1 (REF. 198), and many have attention deficit hyperactivity disorder. Features of autism spectrum disorder may occur[199], and vascular defects are common[200]. On the basis of cloning of the *NF1* gene, understanding of the related disorders and clarification of age-of-onset of individual disease manifestations, it has been suggested (since 2007) that the diagnostic criteria may need revision[201].

Café-au-lait macules
Hyperpigmented spots on the skin of patients with neurofibromatosis type 1 (NF1). They are used as an NF1 diagnostic criterion, particularly in young children.

Polycomb repressive complex 2
A complex that regulates epigenetic silencing of chromatin and includes the subunits SUZ12, EED, EZH1 or EZH2 and RBAP48. It also has histone methyltransferase activity.

Astrocytes
The most abundant type of glial cell in the central nervous system. Astrocytes regulate the extracellular neuronal environment.

of patients with NF1, and these patients may have an increased overall tumour risk[43]. Mutations that delete the *NF1* gene, and several flanking genes, occur in up to 10% of patients with NF1. This class of mutations predisposes affected individuals to an increased risk of intellectual disability, to greater numbers of cutaneous neurofibromas and to MPNSTs[44]. Another genotype–phenotype correlation is of a very rare 3-bp deletion in patients with NF1 who lack neurofibromas[45].

Modifier genes in NF1. Given the paucity of NF1 genotype–phenotype correlations, it was proposed that modifier genes underlie the variable penetrance of NF1. Monozygotic twins with NF1 showed a high degree of concordance for cutaneous neurofibroma tumour burden and numbers of café-au-lait macules, supporting the idea that modifier genes contribute to these features[46,47]. The large polygenic deletions (mentioned above) indicate that modifier genes might be linked to *NF1* (REF. 44). Recently, the gene encoding the chromatin remodelling complex Polycomb repressive complex 2 subunit SUZ12, which lies within this region, has been shown to be a cooperating tumour suppressor in mouse models and in human tumours[48–50]. Studies in mouse models also support roles for modifier genes in NF1. Astrocytoma resistance alleles were recently identified as spinal cord resistance to astrocytoma modifier 1 (*Scram1*) and astrocytoma resistance locus in males 1 (*Arlm1*) loci[38,51,52]. It is unclear whether disease modifier genes in NF1 are also relevant in *NF1*-mutant sporadic tumours.

In support of a role for modifier genes in NF1 is OPGs; these tumours have a decreased prevalence in the African-American population compared with other races[53]. Sex-linked factors may modify prognosis; males are at an increased risk of sporadic high-grade glioma, but NF1 females with low-grade OPG have a worse prognosis than males with NF1-related OPG[54]. In addition, male *Nf1*$^{-/-}$ mouse astrocytes expressing dominant-negative p53 show increased tumorigenesis and inactivation of the RB protein compared with cells derived from females[55]. An imprinting control region remotely interacts with an intergenic sequence between *Nf1* and *Wsb1* on chromosome 11 to regulate *Nf1* transcription, and mutations in this intergenic sequence could also potentially modify NF1 disease[56].

NF1 ***mutations in sporadic cancers.*** The advancement of whole-genome sequencing has resulted in the identification of *NF1* mutations in various non-NF1-associated sporadic cancers, including glioblastoma[33,57], neuroblastoma[58], AML[36], lung cancer[32], ovarian cancer[34] and breast cancer[59]. We anticipate that the identification of tumours that contain *NF1* mutations will continue to increase with future sequencing efforts. A comprehensive study that analysed somatic mutation patterns in more than 1,500 cancer-related genes in a large panel of lung, breast, ovarian, pancreatic and prostate tumours identified *NF1* (mutation frequency >5%) as one of ten genes that are mutated most often in these types of tumours. In comparison, the point mutation frequency was 33% for *TP53*, 7% for *KRAS* and 5% for cyclin-dependent kinase inhibitor 2A (*CDKN2A*)[59]; however, deletions in *CDKN2A* are much more common than deletions in *NF1*. It is as yet unknown whether biallelic loss of *NF1* is common or whether only hemizygous loss of *NF1* contributes to tumour progression in sporadic disease. Consistent with the latter possibility, mouse cells hemizygous for *Nf1* mutations show abnormal growth and invasion[60–62]. Hemizygous *NF1*-mutant cells might show lower levels of GTP-bound RAS than cells with complete inactivation of *NF1* and/or develop mutations in additional RAS–MAPK pathway genes to affect tumour properties. Although sporadic tumours with *NF1* mutations are largely exclusive of those tumours that harbour mutations in MAPK kinase 1 (*MAP2K1*) or *NRAS*, on the basis of our analyses of somatic co-mutation patterns in The Cancer Genome Atlas data sets (cBio Portal for Cancer Genomics), 11 of 114 melanomas with *NF1* mutations also show mutations in *BRAF*, *NRAS* or *RAF1*. Thus, there may be subcategories of tumours, and perhaps cells, in which *BRAF*, *NRAS* or *RAF1* are co-mutated with *NF1*.

Imprinting control region
A regulatory element (a segment of DNA) that is modified by methylation to regulate gene expression.

In tumours with *NF1* mutations, the order of mutations seems to affect tumour grade in specific cell types; for example, initial loss of *NF1* in nerve glial cells triggers neurofibroma. In this case, oncogene-induced senescence occurs, and inactivation of p53 bypasses this response for progression to MPNSTs[63]. Similarly, benign grade 1 astrocytomas develop in genetically engineered mice (GEMs) when *Nf1* is lost first[64]. By contrast, aggressive gliomas form when *Trp53* is co-mutated with *Nf1* (REF. 65). Indeed, in human glioblastomas, half of the tumours with *NF1* mutations also harbour *TP53* mutations[66]. Mutational order may explain why patients with NF1 are not predisposed to certain sporadic tumours, such as lung tumours, whereas >10% of sporadic lung cancers[32] have *NF1* mutations, which are probably acquired late in tumorigenesis.

The NF1 protein: neurofibromin

Neurofibromin is a large multi-domain 2,818 amino acid protein[67]. Exon 23 encodes part of the GRD, which is the RAS regulatory domain of neurofibromin. An exon 23 splice variant inserts an alternative exon 23a, which decreases neurofibromin RASGAP activity[68]. In addition to this central GRD, neurofibromin contains other functional domains, most of which are of uncertain importance in the tumour suppressor function of neurofibromin (FIG. 4).

Interactions of neurofibromin. In 1991, Bollag and McCormick[69] reported that the lipids arachidonate, phosphatidate and phosphatidylinositol-4,5-bisphosphate inhibit neurofibromin GAP activity, but no definitive *in vivo* role for lipid–neurofibromin interaction through the SEC14 and pleckstrin homology (PH) domains has so far been identified. Neurofibromin is also implicated in connecting RAS signalling to activation of RHO-family GTPases, which results in modulation of the cytoskeleton.

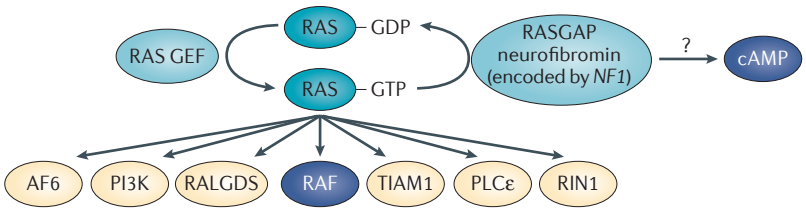


Figure 3 | **Neurofibromatosis type 1 signalling pathways.** In the absence of negative regulation of RAS proteins, resulting from loss of neurofibromatosis type 1 (*NF1*, which encodes neurofibromin), GTP-bound RAS levels are increased. Therefore, signalling pathways downstream of RAS that are normally activated by receptors — including receptor tyrosine kinases, integrins and ion channels — show enhanced activation. RAS signalling pathways include the MEK–ERK signalling cascade downstream of RAF and also many other potential RAS effectors, including AF6, an F-actin and RAP1-binding protein; RAL guanine nucleotide dissociation stimulator (RALGDS), a guanine nucleotide exchange factor (GEF) for the RALA and RALB GTPases; T lymphoma invasion and metastasis-inducing protein 1 (TIAM1), an exchange factor for the GTPase RAC1; phospholipase Cε (PLCε), an isoform of the phospholipase C family; and RAS and RAB interactor 1 (RIN1), which is a RAS effector and RAB5 GEF. In addition, loss of *NF1* results in deregulation of cyclic AMP levels in affected cells through poorly characterized mechanisms that may be independent of RAS and/or result from crosstalk between RAS and heterotrimeric G protein signalling. RAF and cAMP are the only effector pathways currently shown to have therapeutic potential in NF1 disease. GAP, GTPase-activating protein.

Therefore, it is intriguing that the neurofibromin phospholipid-binding SEC14 and PH domains interact with LIM domain kinase 2 (LIMK2) and thereby inhibit activation of LIMK2 by RHO-associated protein kinase, which is known to modulate the actin cytoskeleton[70]. Some neurofibromin domains may function as scaffolds that target neurofibromin to specific intracellular locations. Indeed, neurofibromin interacts with sprouty-related, EVH1 domain-containing protein 1 (SPRED1). This interaction results in localization of neurofibromin to membranes, which enables neurofibromin to downregulate GTP-bound RAS[71]. Interestingly, *SPRED1* is also a RASopathy gene. Unlike mutations in *NF1*, mutations in *SPRED1* do not predispose affected individuals to neurofibromas, gliomas or MPNSTs but rather to lipomas. Distinct features of each disorder may arise from cell type-specific use of components of the MAPK pathway.

Neurofibromin is the main RASGAP in neuronal dendritic spines[72], where the molecular chaperone valosin-containing protein (VCP) interacts with the leucine-rich repeat domain of neurofibromin. In mice, mutations in *Vcp*, like loss of *Nf1*, reduce spine density[73]. Importantly, VCP functions downstream of neurofibromin, and expression of VCP rescues spine defects in $Nf1^{+/-}$ neurons. VCP is now under investigation as a cancer target. Dimethylarginine dimethylaminohydrolase 1 (DDAH1), which degrades the endogenous nitric oxide inhibitor asymmetric dimethylarginine (ADMA), was identified as another neurofibromin interaction partner. Knockdown of *Nf1* rescued the decrease in cell proliferation caused by knockdown of *Ddah1* in mouse endothelial cells[74,75], and this is of special interest because the modulation of nitric oxide is an attractive therapeutic target.

Neurofibromin regulation and signalling

Neurofibromin protein levels can also be affected by mechanisms beyond *NF1* mutation. One category of neurofibromin-interacting proteins is ubiquitin ligases, which ubiquitylate and cause degradation of neurofibromin, thus sustaining RAS signalling. The ubiquitin ligase SAG (sensitive to apoptosis gene protein; also known as RNF7 and RBX2) was reported to interact with neurofibromin and affect vascular development[76], whereas the ubiquitin ligase cullin 3 degraded neurofibromin in glioblastoma cells in which neurofibromin was not mutated[76,77]. It remains to be determined whether these and/or other ubiquitin ligases only degrade neurofibromin in specific settings. Downregulation of neurofibromin may also result from the methylation of *NF1* in cancer cells[78,79]. *NF1* is also a target of microRNAs (miRNAs); expression of miR-128 in neurons and miR-193b in head and neck squamous cell carcinoma cells decreased the levels of *NF1* mRNA and neurofibromin[80,81].

Modulation of RAS signalling by neurofibromin. The absence of neurofibromin leads to slowed hydrolysis of GTP-bound RAS, which sustains RAS signalling. Nevertheless, upstream receptors are required to activate RAS, and several receptors have been implicated upstream of neurofibromin in particular cell types. For example,

genetic and biochemical evidence support a necessary role of granulocyte–macrophage colony-stimulating factor (GM-CSF) and activation of the GM-CSF receptor to maintain JMML in *Nf1*-mutant mice[82]. This receptor may also have a role in neurofibroma formation after nerve injury[83]. By contrast, activation of the KIT receptor by stem cell factor (also known as KIT ligand) has a key role in neurofibroma formation in mice[62]. In *Drosophila melanogaster* expressing mutant *Nf1*, the loss of the receptor tyrosine kinase Anaplastic lymphoma kinase (Alk) rescues the small size of the flies and ERK activation[84]. Other receptors probably function as upstream regulators of neurofibromin in specific cell types.

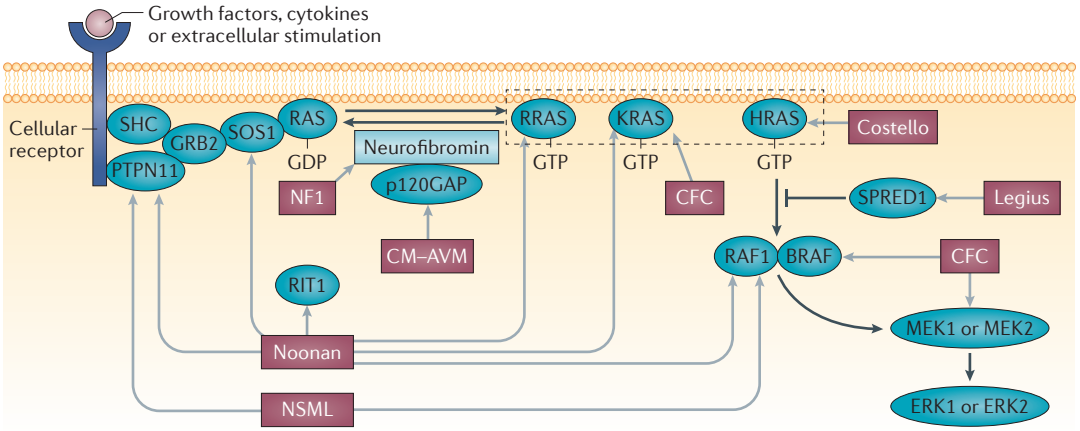
Neurofibromin and downstream signalling. Of the many putative downstream effectors of RAS signalling — RAL guanine nucleotide dissociation stimulator (RALGDS), PI3K, phospholipase Cε, T lymphoma invasion and metastasis-inducing protein 1 (TIAM1), RAS association domain family protein (RASSF), RAS and RAB interactor 1 (RIN1), RIN2, RIN3, AF6 (also known as afadin and MLLT4), impedes mitogenic signal propagation (IMP; also known as BRAP2), RAS-associated and PH domains-containing protein 1 (RAPH1), growth factor receptor-bound protein 7 (GRB7), and PDZGEF1 (also known as RAPGEF2) (FIG. 2) — only a few have been studied in the context of NF1. The best studied is the neurofibromin–RAS–MAPK pathway. Loss of neurofibromin results in the activation of this pathway in multiple types of tumour[85,86]. Heat shock response[87] and MAF regulation of mTOR signalling[88] also occur downstream of *NF1* loss and subsequent activation of ERK. RAL guanine nucleotide exchange factors have also been implicated downstream of RAS in MPNSTs[89].

Several studies in mice support important roles for PI3K signalling when loss of *Nf1* drives tumour formation, and several pathways have been implicated downstream of PI3K. For example, PI3K–mTOR signalling is enhanced in *Nf1*-mutant astrocytes and MPNSTs[90,91]. PI3K–AKT signalling downstream of *Rras2* has a role in the initiation of neurofibromas[92]; activation of the PI3K pathway through loss of *Pten* in mice promotes the transformation of *Nf1*-driven neurofibromas to MPNSTs[93]. Sustained activation of AKT in MPNST cells requires calcium and calmodulin[94]. On the basis of these results, it will be important to consider cell type-specific neurofibromin–RAS effector pathways when attempting to identify therapeutic targets. In addition, simultaneously targeting multiple RAS effector pathways may provide enhanced effects.

NF1 and cAMP. In yeast, the *NF1* homologues *IRA1* (REF. 95) and *IRA2* (REF. 96) regulate both Ras and cAMP signalling. Ira1 and Ira2 each simultaneously bind to Ras2 and adenylyl cyclase, thus regulating both pathways[97]. It is accepted that increased levels of cAMP inhibit the proliferation of most cell types, and it has been proposed that altered levels of cAMP contribute

Box 1 | **RASopathy genes and syndromes**

RASopathies commonly predispose patients to short stature, developmental delay and cardiac abnormalities[29]. The syndrome (or syndromes) associated with each gene (or genes) is indicated by light grey arrows in the figure. RASopathies include the common disorders neurofibromatosis type 1 (NF1; which affects 1 in 3,000 individuals) and Noonan syndrome (which affects 1 in 2,000 individuals) and rare conditions, such as Costello syndrome. Mutations in several RAS–MAPK genes can cause Noonan syndrome, Noonan syndrome with multiple lentigines (NSML; previously known as LEOPARD syndrome) and cardio-facio-cutaneous (CFC) syndrome. Other RASopathies, including NF1, are associated with a single gene mutation. Legius syndrome is associated with mutations in sprouty-related, EVH1 domain-containing 1 (*SPRED1*), and capillary malformation–arteriovenous malformation (CM–AVM) is associated with mutations in *RASA1* (which encodes p120GAP). RASopathy genes include protein tyrosine phosphatase non-receptor type 11 (*PTPN11*), *CBL*, son of sevenless homologue 1 (*SOS1*), *RAF1*, *HRAS*, *NRAS*, *KRAS*, *BRAF*, MAPK kinase 2 (*MAP2K2*) and *SHOC2*. New RASopathy genes continue to be identified; newly identified genes include *RRAS*[180], RAS-like without CAAX 1 (*RIT1*)[181], *MAP2K1* and *RASA2* (REF. 182). Many RASopathies predispose to tumours, including juvenile myelomonocytic leukaemia, rhabdomyosarcoma or neuroblastoma[29]. GRB2, growth factor receptor-bound protein 2; SHC, SRC homology 2 domain-containing.



Schwann cells
Glial cells derived from neural crest cells that ensheathe and myelinate axons in the peripheral nervous system.

Oligodendrocytes
Glial cells derived from neuroepithelial cells that ensheathe and myelinate axons in the central nervous system.

NG2 cells
Oligodendrocyte progenitor cells that may have additional functions in the mature brain.

to cancer. Mouse[98], *D. melanogaster* [99] and zebrafish[100] cells expressing mutant *Nf1* all show deregulated cAMP levels. In *D. melanogaster* lacking *Nf1* cAMP levels are low, but it remains unclear whether this results from increased Ras activity or whether it occurs independently of Ras[98,99]. By contrast, cAMP levels are increased in mouse Schwann cells (peripheral nerve support cells) or human MPNST cells lacking *NF1* (REF. 101). Many different RAS–cAMP pathway interactions may occur in specific *NF1*-mutant cell types. In mouse neurons, atypical protein kinase C-mediated β-adrenergic receptor kinase 1 (ADRBK1)-driven inactivation of the G protein $G\alpha_s$ modulated RAS activity[98,102]. In another system, the protein kinase A-activated transcription factor cAMP-responsive element-binding protein (CREB) bound to the *mir-9* promoter, which repressed expression of *NF1* and encouraged cell migration[103]. Interfering with cAMP signalling may present a therapeutic opportunity in several manifestations of NF1, as was proposed for *NF1*-driven brain tumours[104]. Blocking RAS–MAPK signalling and increasing cAMP levels may be useful therapeutically; for example, reversing one zebrafish brain defect required blockade of MEK, whereas reversing another defect required an increase in cAMP levels[100].

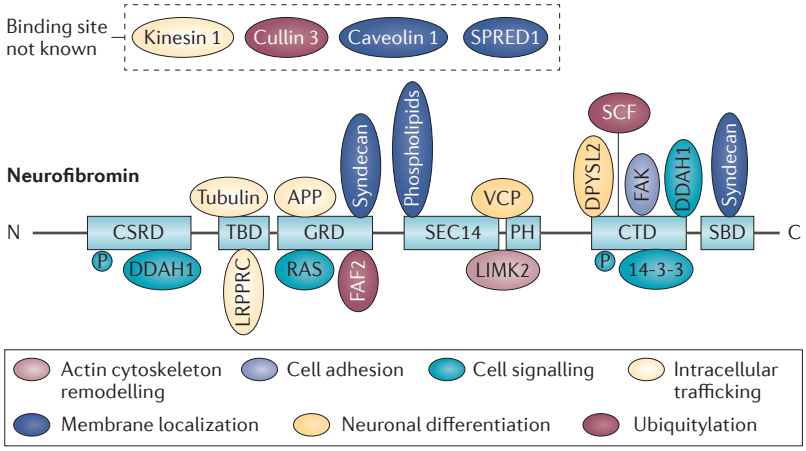


Figure 4 | **Neurofibromin protein structure and interacting proteins.** Neurofibromin contains multiple domains (light blue). These include a cysteine–serine-rich domain (CSRD), a tubulin-binding domain (TBD), a central GTPase-activating protein-related domain (GRD), a SEC14 domain[202,203], a pleckstrin homology (PH) domain, a carboxy-terminal domain (CTD) and a syndecan-binding domain (SBD). The SEC14 and PH domains bind to phospholipids, and have been studied structurally[204]. Proteins identified as neurofibromin-interacting proteins and phospholipids (ovals) are shown associated with functions ascribed to them, including intracellular trafficking (light yellow); neuronal differentiation (dark yellow); membrane localization (dark blue); actin cytoskeleton remodelling (light pink); ubiquitylation (dark pink); cell adhesion (purple) and cell signalling through nitric oxide via dimethylarginine dimethylaminohydrolase 1 (DDAH1) and RAS (turquoise). Each interacting protein is shown bound to the domain of neurofibromin with which it is believed to interact. Some proteins are known to interact with neurofibromin, but the binding site is unknown. Phosphorylation (P) sites implicated as protein kinase A substrates are shown. Descriptions of each interacting protein, binding domains and literature references are shown in Supplementary information S1 (table). APP, amyloid-β (A4) precursor protein; DPYSL2, dihydropyrimidinase-related protein 2; FAF2, FAS-associated factor 2; FAK, focal adhesion kinase; LIMK2, LIM domain kinase 2; LRPPRC, leucine-rich pentatricopeptide motif-containing protein; SCF, Skp, Cullin, F-box-containing complex; SPRED1, sprouty-related, EVH1 domain-containing protein 1; VCP, valosin-containing protein.

NF1 tumorigenesis and tumour cells of origin

Many tumours in patients with germline *NF1* mutations are neural crest cell-derived tumours (pheochromocytomas, neurofibromas and MPNSTs) or neuroepithelial cell-derived tumours (pilocytic astrocytomas). Non-neural-crest-related cells are also predisposed to tumorigenesis (JMML and rhabdomyosarcoma). It is not known why specific cell types are sensitive to loss of *NF1*. Affected populations do not express increased levels of neurofibromin compared with other cells; it has been speculated that these populations critically rely on neurofibromin rather than other GAPs.

Low-grade astrocytoma. At least 15% of patients with NF1 develop OPGs, which are mainly grade I pilocytic astrocytomas[7]. These tumours are defined as benign, generally have a favourable prognosis and rarely progress[105]. In contrast to NF1-related pilocytic astrocytomas, pilocytic astrocytomas in patients without NF1 are typically more aggressive, although they have RAS pathway mutations, including *BRAF* duplications or activating point mutations in *BRAF* or *KRAS*[106]. In GEM models of grade 1 astrocytoma resulting from loss of *Nf1*, many neurons in the brain and most macroglial cells are $Nf1^{-/-}$ owing to use of glial fibrillary acidic protein (*Gfap*)–Cre or brain lipid-binding protein (*Blbp*; also known as *Fabp7*)–Cre drivers, which excise *Nf1* in most brain stem and progenitor cells[64,98,107]. In this model, other cells of the body are $Nf1^{+/-}$ (REF. 64). Evidence indicates that when progenitor cells of the developing third ventricle lack *Nf1*, they develop into aberrant astrocytes, which are characteristic of pilocytic astrocytoma[108]. Consistent with this interpretation, loss of *Nf1* in other brain cell types (such as oligodendrocytes or NG2 cells) failed to generate astrocytomas in zebrafish or mice[109,110].

Cutaneous neurofibroma. All neurofibromas contain nerve Schwann cells (with biallelic *NF1* mutations[111]), as well as *NF1* wild-type or heterozygous fibroblasts, mast cells, macrophages, perineurial cells and endothelial cells. The percentage of each cell type varies from tumour to tumour. Neurofibromas in the human dermis or epidermis (known as cutaneous or dermal neurofibromas) are benign and do not transform; however, they can cause a substantial cosmetic burden in patients. It has been a source of confusion that some plexiform neurofibromas, which are more aggressive than cutaneous neurofibromas, also develop in the skin and subcutaneous tissue; alternative nomenclature has been discussed but not defined by consensus. There are no model organisms at present in which cutaneous neurofibromas spontaneously develop. However, after growth *in vitro* and transplantation into $Nf1^{+/-}$ syngeneic hosts, skin hair follicle-derived $Nf1^{-/-}$ precursors (SKPs) formed tumours that resembled dermal neurofibromas[112]. Transplantation of $Nf1^{-/-}$ SKPs into pregnant female mice increased growth of neurofibromas in the mouse model[112], and cutaneous neurofibromas can develop and grow during puberty[5] and can increase in size and number during pregnancy[113]. However, it remains unclear whether and which hormones or other factors increase

the growth of human cutaneous neurofibromas. It is also unclear which factors limit the growth of most dermal neurofibromas in humans, and why they are resistant to transformation.

Plexiform neurofibroma. Plexiform neurofibromas are complex tumours that can weigh kilograms and can compress vital structures. Therefore, they are of considerable interest as targets of therapy. Plexiform neurofibromas develop within peripheral nerves and their perineurial sheaths. However, plexiform neurofibromas can invade adjacent tissue by disrupting the perineurium, remaining non-metastatic and clinically 'benign' but accounting for substantial morbidity and an increased risk of mortality when symptomatic[114].

Extensive modelling of plexiform neurofibromas has been carried out in mice. Plexiform neurofibroma models all have biallelic loss of *Nf1* in the Schwann cell lineage[115–118], which is driven by *P0* (also known as myelin protein zero (*Mpz*))–Cre, desert hedgehog (*Dhh*)–Cre, tamoxifen-inducible proteolipid protein (myelin) 1 (*Plp1*)–Cre, or *Krox20* (also known as *Egr2*)–Cre. Each of these drivers knock out expression in Schwann cell progenitors, with differences in precise timing, location and the number of cells affected. Some models, probably those with fewer cells showing recombination, require additional hemizygous inactivation of *Nf1* in haematopoietic cells to generate neurofibromas[119]. In all cases, the tumours (grade 1 neurofibromas) in GEM models resemble those of humans. In humans, plexiform neurofibromas primarily develop very early in life. Unexpectedly, plexiform neurofibromas can be induced even in adult mice, although in one model these are rare[116,117]. Unfortunately, this result does not resolve the uncertainty around the plexiform neurofibroma cell of origin, as adult mouse dorsal root ganglia and dorsal roots contain stem-like cells, mature Schwann cells and satellite cells, all of which have been proposed as possible neurofibroma-initiating cells[115–118,120]. Epidermal growth factor receptor (EGFR)-expressing Schwann cell precursor-like cells are present in mouse and human neurofibromas, and form neurofibroma-like lesions under the skin[120] or in peripheral nerves[121] of immunocompromised mice. Extensively passaged Schwann cells from neurofibromas can also form neurofibromas in immunocompromised mice[122]. Thus, de-differentiated Schwann cells and/or Schwann cell precursors may each be cells of origin for neurofibromas.

MPNSTs. MPNSTs are nerve-associated sarcomas, most of which arise in pre-existing plexiform neurofibromas in patients with NF1 (REF. 123), and are aggressive tumours that typically metastasize to the brain, bone and other sites[124]. Half of all MPNSTs arise sporadically, and the other half occur in patients with NF1 (REF. 125). Genetic alterations that affect the RAS pathway, including *NF1*, *BRAF*, *NRAS* or *KRAS* mutations, have been identified in some sporadic MPNSTs[126,127], and NF1 and sporadic MPNSTs share a gene signature[128]. MPNSTs are also typically hyperdiploid, and their genomes — like genomes in most sarcomas — are highly rearranged[129]. Mutations associated with the transformation from benign plexiform neurofibromas to malignant MPNSTs include early mutations in *CDKN2A*[129] and later mutations in *TP53* (REFS 130–132) and *SUZ12* (REF. 133). Loss of the tumour suppressor gene *RB1* (which encodes RB) is found in 25% of MPNSTs[134,135], and monosomy for the *PTEN* locus is observed in 50% of MPNSTs[126,134,136,137]. Low-level amplification of growth factor receptor genes, including *EGFR*, is also common[126].

Human MPNSTs and MPNST cell lines contain $CD133^+$ cells that may be stem-like cells[138,139]. GEM models of MPNSTs include combined loss of *Nf1* and *Trp53* (REFS 140,141) or *Nf1* and *Cdkn2a*[142,143]. MPNSTs from these models and human MPNST cell lines contain stem-like cells that propagate disease[144]. Comparative oncogenomics and insertional mutagenesis screens have been used to identify candidate drivers of MPNST formation, including MEK, β-catenin and embryonic stem cell-expressed RAS (ERAS)[86,145,146]. In zebrafish, ribosomal gene mutations predispose to the formation of MPNSTs, and resulting tumours show loss of p53 translation[147].

Other tumours in patients with NF1. Pheochromocytomas[11], rhabdomyosarcomas[14], glomus tumours[20,148] and GISTs[12] are present at increased incidence in patients with NF1 and show biallelic inactivation of *NF1*. Of these, only pheochromocytoma has been successfully modelled to date. Pheochromocytomas form in 15% of $Nf1^{+/-}$ mice[149]; however, this model has not been used in preclinical tests, probably owing to low tumour incidence. JMML is a rare paediatric manifestation of NF1 (REF. 10) and a RAS-driven haematopoietic stem cell disorder. Thus, patients with NF1 and patients with RASopathies who have mutations in *NRAS*, *KRAS*, *CBL*, protein tyrosine phosphatase non-receptor type 11 (*PTPN11*; also known as *SHP2*) or son of sevenless homologue 1 (*SOS1*) are predisposed to this low-grade leukaemia[150], which is recapitulated in an *Mx1–Cre*;$Nf1^{fl/fl}$ model[151].

Therapeutic implications

Preclinical testing. Importantly, the available mouse models of JMML, OPG, plexiform neurofibroma and MPNST are currently used for preclinical testing. Consistent with a major role for RAS–MAPK signalling in JMML, preclinical testing showed significant response to inhibition of MEK[151]. Disease burden was markedly reduced, although mutant stem cells persisted. The finding that the multi-receptor tyrosine kinase inhibitor imatinib or inhibition of MEK can shrink plexiform neurofibromas in mouse models led to clinical trials[86,119,151]. In the imatinib trial, 6 of 36 patients, primarily young children with small plexiform neurofibromas, responded to treatment; the target kinase (or kinases) affected in these patients is not yet known[152]. In a Phase I trial of the MEK inhibitor selumetinib (presented at the 2014 American Society of Clinical Oncology meeting), 11 of 18 patients with plexiform neurofibromas, some up to kilograms in weight, shrank by ≥20% in response to therapy, and many showed prolonged response[153]. These positive results not only support continued use of mouse

Aurora kinase
A serine/threonine kinase that functions during mitosis and is required for correct function of centrosomes.

Bromodomain inhibitors
A new class of epigenetic modulators of gene expression.

models to guide clinical testing in human neurofibromas but also emphasize the finding that these benign tumours can respond to single-agent therapy for up to 3 years without showing resistance.

Complete surgical resection is required to cure MPNSTs, and no single-agent or combination tested to date has cured MPNSTs in any model system (reviewed in REF. 154). Consistent with the idea that combination therapy will be necessary to treat these aggressive cancers, the mTOR complex 1 (mTORC1) inhibitor rapamycin, together with agents that enhance oxidative stress, shrank MPNSTs[133]. In MPNST xenografts, prolonged responses have been observed with Aurora kinase inhibition and bromodomain inhibitors, but these have not yet been tested in mouse models of MPNSTs or in patients with NF1 (REFS 155,156). In some MPNSTs, autocrine chemokine (C-X-C motif) ligand 12 (CXCL12)–CXC receptor 4 (CXCR4) signalling activates β-catenin through the AKT–glycogen synthase kinase 3β–β-catenin stabilization pathway[157]. Transposon mutagenesis confirmed WNT pathway signalling as a driver of the transformation to MPNSTs, and many β-catenin pathway genes are deregulated in neurofibroma and MPNSTs[158]. Although other links between NF1 signalling and β-catenin pathway activation remain to be identified, these data and analysis of human *NF1* Schwann cells support inhibition of the β-catenin pathway as a possible therapeutic target in *NF1*-driven tumours[159].

NF1 ***loss as a drug resistance marker.*** MPNSTs in patients with NF1 are notoriously resistant to chemotherapy and radiation therapy. Recently, a large-scale study showed almost no benefit of chemotherapy or radiotherapy for NF1 MPNSTs, and a worse outcome has been reported for NF1 than for sporadic MPNSTs[124]. These data must be interpreted with caution, as late diagnosis of MPNSTs arising in plexiform neurofibromas in patients with NF1 may account for differences between the groups in treatment outcome[160]. However, although reasons might differ as to what causes resistance in sporadic tumours, *NF1* has been identified as a gene that confers resistance to targeted therapy, including inhibition of kinases and the RAS pathway, in sporadic neuroblastoma[58], lung carcinoma[161] and melanoma[162]. In lung cancer models, resistance to EGFR therapy was mediated by *NF1*, and blocking MEK restored the response[161]. Melanoma develops in mice with mutant *Braf*, and loss of *Nf1* blocked *Braf*-driven oncogene-induced senescence. *Nf1*-mutant and *Braf*-mutant tumours are resistant to BRAF inhibitors; however, they are sensitive to combined inhibition of MEK and mTOR[162]. Loss of *NF1* was also identified as a crucial mediator of resistance to BRAF inhibitors in melanoma cells[163,164]. These studies indicate that blocking *NF1* pathways — for example, by targeting MEK — might enhance the therapeutic response in those tumours with *NF1* mutations. There are probably also other resistance pathways downstream of *NF1*. In a neuroblastoma model, resistance was mediated by *NF1* through *ZNF423*, which encodes a zinc-finger transcription factor[58].

It is increasingly clear that the recruitment of mast cells, macrophages and other stromal cells in the tumour microenvironment can elicit tumour cell resistance to therapy[165]. *NF1*$^{-/-}$ Schwann cells upregulate major histocompatibility complex class II mRNA and protein levels, which may influence tumour–immune cell interactions[166]. Neurofibromas and MPNSTs contain blood-derived mast cells[167] and macrophages[168]. The stroma in NF1 neurofibromas has been recently reviewed[169]. These haematopoietic cells have a crucial role in the formation and growth of neurofibromas. In some GEM models, the haematopoietic cells must express mutant *Nf1* for neurofibromas to form[119]. Treatment with PLX3397 — which targets both KIT signalling to prevent mast cell recruitment to tumours, and CSF1 receptor signalling to prevent macrophage recruitment to tumours — had two effects on neurofibromas. It increased their size when given during tumour initiation and enabled tumour regression in some mice when given after tumour establishment. Therefore, macrophages may protect against developing tumours and later become permissive to tumour formation[168]. However, wounding peripheral nerves in *Nf1*-mutant mice, which recruits macrophages, facilitates neurofibroma formation, implying that macrophages may promote tumour formation in this context[83,170]. OPGs that arise in *Nf1*-mutant mice also contain CX3CR1-expressing microglial cells (brain-resident macrophages). *Cx3cr1*$^{-/-}$ mice show delayed optic glioma formation, which supports interference with the microenvironment as a possible therapy in patients with NF1 (REF. 171). In an MPNST xenograft, PLX3397 treatment resulted in macrophage depletion and substantially delayed MPNST growth. The effect was enhanced by the mTORC1 inhibitor rapamycin and correlated with enhanced depletion of macrophages[172].

Discovering new drug targets for **NF1-*mutant cells.*** Screening a library of 200,000 small molecules on mouse *Nf1*-mutant MPNST cells identified compound 21, with selectivity towards *NF1*-mutant cells and efficacy in xenografts[173]. Another library-screening study identified UC1 as a small molecule that targets *NF1*$^{-/-}$ versus sporadic MPNST cell lines. Budding yeast in which *IRA2* was deleted validated selectivity of UC1 for *NF1*-mutant cells[174]. Direct targets of these compounds are not known. Gene expression, methylome and copy number changes on several sample sets are publically available for neurofibromas and MPNSTs, and are being used to identify pathways and targets for drug discovery[175]. Preliminary assessments of miRNAs and serum biomarkers have also become available. Several proteins were identified either at increased levels in patients with NF1 but without neurofibromas (interferon-γ, interleukin-6 and tumour necrosis factor) or at increased levels in patients with NF1 and MPNST (insulin-like growth factor-binding protein 1, C-C motif chemokine 5 (CCL5) and adrenomedullin)[176,177]. Expression of miR-801, miR-214 and miR-24 can distinguish patients with both NF1 and MPNST from patients with NF1 but without MPNST[178]. However, no NF1 biomarker has yet been tested clinically.

Successes and future challenges

The NF1 community has been very successful in identifying the *NF1* gene and developing animal models for plexiform neurofibroma, MPNST and JMML. The community has also succeeded in identifying plausible therapeutic strategies and advancing them from preclinical testing to clinical trials, through preclinical and clinical testing consortia and a group developing end points for clinical trials[179]. However, some *NF1*-driven cancers still lack model systems or have models that are difficult to use for preclinical testing. Although the neurofibromin protein has been studied, many questions remain concerning the relevance of possible interaction partners and functions of neurofibromin protein domains. Although it is now clear that the RAS–MAPK pathway is crucial for mediating *NF1*-mutant tumour growth, other pathways downstream of RAS signalling are likely to be relevant and may be cell type dependent. In particular, an important goal of the next few years will be to better understand altered non-RAS–cAMP signalling downstream of *NF1* loss. Identification of inhibitors of cell type-specific pathways that synergize with blockade of MEK, including inhibitors that target other RAS effector pathways, could be attempted. We anticipate that therapies successful in treating NF1 disease manifestations will also be successful in the treatment of other RASopathies and hope that these therapies, in combination with other drugs, will also be useful in treating sporadic *NF1*-mutant cancer.

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Competing interests statement
The authors declare no competing interests.

DATABASES
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FURTHER INFORMATION
cBio Portal for Cancer Genomics: http://www.cbioportal.org
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University of Alabama at Birmingham Medical Genomics Laboratory: http://www.uab.edu/medicine/genetics/medical-genomics-laboratory

SUPPLEMENTARY INFORMATION
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