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## A *Drosophila* screen identifies neurofibromatosis-1 genetic modifiers involved in systemic and synaptic growth

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**Keywords:** cAMP/PKA signaling, *Drosophila melanogaster*, genetic model, neurofibromatosis, Ras signaling, synaptic morphology

**Abbreviations:** NF1, Neurofibromatosis type 1; GAP, GTPase activating protein; ALK, anaplastic lymphoma kinase; CNS, central nervous system; NMJ, neuromuscular junction

**N**eurofibromatosis type 1 (NF1) is caused by loss of a negative regulator of Ras oncoproteins. Unknown genetic modifiers have been implicated in NF1's characteristic variability. *Drosophila melanogaster dNf1* phenotypes include cognitive deficits and reduced growth, both of which resemble human symptoms. We recently reported results of a screen for dominant modifiers of *dNf1* growth. Suppressors include the *dAlk* tyrosine kinase and its activating ligand, two other genes involved in Ras/ERK signal transduction, the synaptic scaffold Dap160 and the CCKLR-17D1 drosulfakinin receptor. Additional modifiers include several genes involved in cAMP/PKA signaling. Providing mechanistic insights, *dAlk*, *jeb*, and *CCKLR-17D1* also suppress a *dNf1* synaptic overgrowth defect, and increasing cAMP/PKA signaling in the neuroendocrine ring gland rescued the *dNf1* growth deficiency. Finally, among the several suppressors identified in our screen, we specifically implicate ALK as a potential therapeutic target by showing that NF1-regulated ALK/RAS/ERK signaling is conserved in human cells.

RASopathies are a group of clinically related genetic disorders caused by defects in RAS/ERK signal transduction.<sup>1</sup> NF1 is among the most common members of this group, affecting an estimated 1 in 3000 individuals in all ethnic groups. High degrees of variability and unpredictability are among the hallmarks of NF1. Patients

are predisposed to developing a variety of symptoms, the most common of which include benign but potentially highly disfiguring peripheral nerve associated tumors, termed neurofibromas. Malignant tumors, including peripheral nerve sheath tumors, are also strongly associated with NF1. Frequent non-tumor symptoms include skeletal and skin pigmentation abnormalities, reduced overall growth, and cognitive deficits, the latter seen in 50–70% of children with NF1.<sup>2</sup>

NF1 is caused by mutations that impact the function(s) of neurofibromin, a large and evolutionarily conserved GTPase Activating Protein (GAP) for Ras oncoproteins.<sup>3</sup> Neurofibromin and other RasGAPs accelerate the conversion of active Ras-GTP into inactive Ras-GDP by stimulating the low intrinsic rate of Ras-GTP hydrolysis. While excessive Ras signaling upon loss of neurofibromin is undoubtedly a major cause of NF1 defects, evidence has also been presented that neurofibromin, in Ras-dependent or Ras-independent ways, acts as a positive mediator of adenylyl cyclase activity.<sup>4–6</sup>

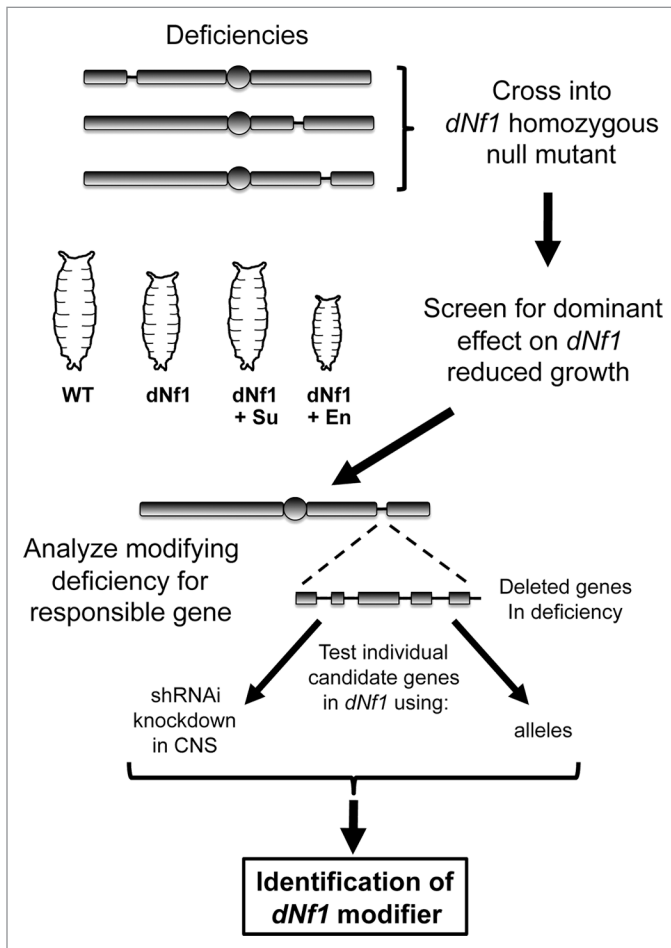
To shed light on the functions of neurofibromin, the molecular pathways involved in NF1 defects and the identity of modifier genes implicated in the characteristic variability of this disease,<sup>7</sup> we previously generated loss-of-function mutants of a highly conserved *Drosophila melanogaster dNf1* ortholog. Homozygous *dNf1* null mutants are viable and fertile, but show a 15–20% reduction in linear

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**Figure 1.** A screen for dominant *dNf1* growth defect modifiers. *dNf1* mutants are smaller than wild-type flies. To identify modifiers of this phenotype, 486 isogenic deficiencies uncovering ~80% of first and second chromosome genes were crossed into a *dNf1* null mutant background, and the length of the resulting pupal cases measured. Confounding factors include that size is a sexually dimorphic phenotype, with males being smaller than females, and that systemic growth is a multifaceted process influenced by environmental factors, such as food availability and temperature. Employing strategies to minimize these and other confounding factors, and after eliminating those deficiencies with non-specific effects on growth, candidate *dNf1* modifying deficiencies were examined by testing alleles or shRNAi lines to identify the responsible modifier genes.

dimensions during all post-embryonic developmental stages.<sup>8</sup> Mutants also have a reduced escape response (taking flight upon release), lack a neuropeptide-elicited rectifying K<sup>+</sup>-current defect at the neuromuscular junction (NMJ), and exhibit circadian arrhythmicity, olfactory associative learning, and memory deficits.<sup>8-11</sup> Remarkably, all defects but the circadian arrhythmicity are not particularly sensitive to genetic manipulation of Ras signaling but are suppressed by increasing cAMP/PKA pathway signaling or mimicked by decreasing signaling through the cAMP/PKA pathway.

While there is little doubt that loss of NF1 affects cAMP/PKA signaling, we and another group have reached contradictory conclusions about the mechanism(s) involved. Yi Zhong and colleagues reported that a C-terminal segment of human neurofibromin (that does not include the RasGAP catalytic domain) is sufficient for NF1/Gα(S)-dependent neurotransmitter stimulated adenylyl cyclase activation and rescue of the *dNf1* growth defect.<sup>4</sup> In contrast, we found that expression of a functional *dNf1* RasGAP catalytic domain is both necessary and sufficient to restore the cAMP/PKA-sensitive growth deficiency. Moreover,

*dNf1* expression during the larval growth phase is largely restricted to neurons, and expression of an unrelated *Drosophila* RasGAP in these cells sufficed to restore normal growth. Finally, although multiple Ras signaling mutants did not dominantly modify *dNf1* systemic growth, these mutants also did not reduce the elevated phospho-ERK level in *dNf1* larval brain.<sup>12</sup>

Our conclusion that neuronal Ras/ERK over-activation is the root cause of the cAMP/PKA-sensitive *dNf1* growth defect received further support from subsequent work. Neuronal overexpression of the *dAlk* receptor tyrosine kinase or of its activating ligand *jelly belly* (*jeb*) phenocopied *dNf1* growth and learning defects, while genetic or pharmacological attenuation of *Jeb/dAlk* signaling suppressed both phenotypes. Specifically implicating Ras-stimulated ERK over-activation, this study also found that neuronal expression of a constitutively active ERK mutant phenocopied the *dNf1* growth defect.<sup>13</sup>

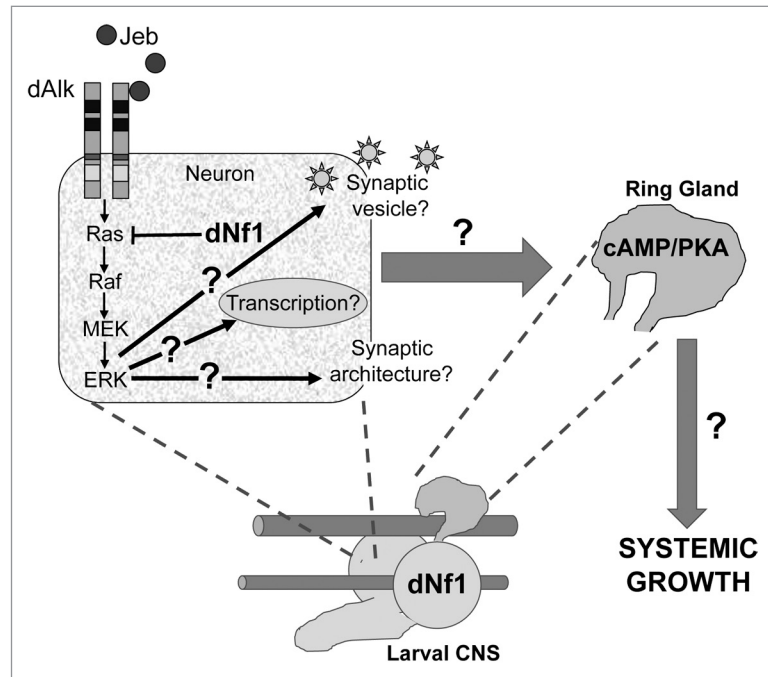
To shed further light on *dNf1*'s role in organismal growth and on the mechanistic links between *dNf1* and cAMP/PKA signaling, we recently reported results of an unbiased genetic screen for dominant modifiers of the *dNf1* growth defect.<sup>14</sup> Our screen analyzed 486 isogenic first and second chromosome deficiencies, each typically uncovering between 1 and 25 genes. The deficiencies, which together uncover close to 80% of first and second chromosome genes, were crossed into the *dNf1* null background, and modifying deficiencies were identified by measuring the length of pupal cases (Fig. 1). After eliminating deficiencies that also affect the size of wild-type pupae, responsible modifier genes were identified in crosses with available alleles, or by neuronal- or glial-specific RNAi knockdown of candidate genes.

Validating the screen, we identified *dAlk*, its activating ligand *jelly belly* (*jeb*), and the *dunce* (*dnc*) cAMP phosphodiesterase as dominant suppressors. All three genes had been identified previously as *dNf1* phenotypic suppressors.<sup>11,13</sup> Earlier work had also established that heat shock-induced expression of a constitutively active murine *PKA*\* catalytic subunit transgene normalized *dNf1* size,<sup>8</sup> whereas others found reduced brain adenylyl

cyclase activity upon loss of *Drosophila* or murine *Nf1*.<sup>15</sup> Thus, we were not surprised to identify the *PKA-C1* catalytic subunit as an enhancer, and the *PKA-R2* regulatory subunit as a yet to be fully confirmed candidate suppressor. Providing mechanistic insights, follow-up experiments indicated that growth regulation by *dNf1* and cAMP/PKA likely involves different cells. First, arguing against the idea that PKA suppresses *dNf1* defects by attenuating RAS/ERK signaling, we found that widespread or tissue-specific transgenic PKA\* expression does not reduce the elevated phospho-ERK level in *dNf1* larval brain. Second, whereas only relatively widespread neuronal *dNf1* re-expression restored the mutant growth defect,<sup>12</sup> in the current study, genetic manipulations that increased cAMP/PKA signaling in specific parts of the larval ring gland (a neuroendocrine gland analogous to the mammalian pituitary) were sufficient to restore *dNf1* growth. By contrast, expressing *dNf1* in the ring gland or widespread neuronal expression of a *dnc<sup>RNAi</sup>* transgene outside of the ring gland had no effect.<sup>14</sup> These results argue that *dNf1* controls *Drosophila* growth by non-cell-autonomously affecting cAMP/PKA signaling in the ring gland (Fig. 2). Whether a similar non-cell-autonomous neuroendocrine mechanism underlies the reduced growth of patients with NF1 or other RASopathies remains to be established.

Our screen also identified several *dNf1* growth defect suppressors with synaptic functions. Examples include the cAMP-coupled neuronal drosulfakinin receptor *CCKLR-17D1*, a positive regulator of synaptic growth, and dynamin-associated protein 160 (Dap160), an intersectin-related scaffold implicated in synaptic vesicle exocytosis and neuroblast proliferation. Because recent work identified a novel *dNf1* NMJ overgrowth phenotype,<sup>16</sup> we tested whether suppressors identified in our screen also modified the NMJ defect. Suggesting a mechanistic link between both phenotypes, loss-of-function *dAlk*, *Jeb*, and *CCKLR-D1* alleles reduced the number of NMJ synaptic boutons.<sup>14</sup>

Studies in *C. elegans* and *Drosophila* had previously revealed roles for ALK



**Figure 2.** Model of *dNf1*-regulated systemic growth. Neurofibromin functions in neurons of the larval central nervous system to regulate *Jeb/dAlk*-stimulated RAS/MEK/ERK signaling. In ways that remain poorly understood, excessive neuronal RAS/MEK/ERK signaling leads to synaptic architecture or neurotransmission defects, which appear causally linked to the reduced growth phenotype. Suggesting a neuroendocrine non-cell-autonomous mechanism, increasing cAMP/PKA signaling in specific segments of the larval brain-associated neuroendocrine ring gland suffices to suppress the *dNf1* growth defect. In contrast, only widespread neuronal *dNf1* expression restores mutant growth.

orthologs in synapse formation and neuronal differentiation. Thus, work in *C. elegans* suggested that the F-box protein FSN-1 and the RING finger protein RPM-1 form a ubiquitin ligase complex that controls synapse stability by targeting ALK ortholog T10H9.2/SCD-2.<sup>17</sup> In *Drosophila*, *Jeb* and *dAlk* are both enriched at synapses,<sup>18</sup> and function to control neurotransmission strength and synaptic architecture.<sup>19</sup> Based on our results, one might speculate that *dAlk* controls synaptic growth by activating a *dNf1*-regulated Ras/ERK signal. However, reconciling our data with these other results is less than straightforward, since *dNf1* is primarily expressed in neurons and plays its growth-related role in these cells (i.e., presynaptically),<sup>14</sup> whereas others concluded that NMJ differentiation involves the activation of postsynaptic (i.e., muscle expressed) *dAlk* by presynaptically released *Jeb*.<sup>19</sup> Although we can only speculate at this point, one potential explanation is that the growth-related role of *Jeb*, *dAlk*, and

*dNf1* involves aberrant synaptogenesis between neurons, rather than at the NMJ. It is worth noting in this respect that murine neurofibromin has been implicated in synaptic differentiation,<sup>20</sup> and that a recent ultrastructural study found reduced curvature at concave synapses in the hippocampus of *Nf1<sup>+/-</sup>* mice.<sup>21</sup>

Loss of *dAlk* or *Jeb* dominantly suppressed *dNf1* growth, associative learning, and neuronal ERK over-activation phenotypes, which, together with other results, suggests a role for *dAlk* as a rate-limiting activator of functionally important *dNf1*-regulated neuronal RAS/ERK signals.<sup>13</sup> The fact that attenuation of *dAlk* signaling rescues multiple *dNf1* defects raises important questions whether NF1-regulated ALK/RAS/ERK signaling is conserved in man, and whether ALK should be further investigated as a therapeutic target in NF1. Several observations suggest positive answers to both questions. Thus, as we previously found for *dAlk* and *dNf1*,<sup>13</sup>



the expression of Alk and Nf1 in the mouse nervous system overlaps to a large extent.<sup>22,23</sup> Supporting an evolutionary conserved functional link between both proteins, mutations that activate ALK or that block the expression of NF1 have both been implicated in neuroblastoma tumorigenesis.<sup>24,25</sup> Adding to this indirect evidence, our recent study found that shRNA-mediated suppression of *NFI* expression renders human neuroblastoma cells resistant to pharmacological ALK inhibition.<sup>14</sup> Specifically, we used two human neuroblastoma lines harboring constitutively active F1174L *ALK* alleles. Both lines are highly sensitive to pharmacological inhibition of ALK with either NVP-TAE684 or Crizotinib.

## References

- Rauen KA. The RASopathies. *Annu Rev Genomics Hum Genet* 2013; 14:355-69; PMID:23875798; <http://dx.doi.org/10.1146/annurev-genom-091212-153523>
- Korf BR. Neurofibromatosis. *Handb Clin Neurol* 2013; 111:333-40; PMID:23622184; <http://dx.doi.org/10.1016/B978-0-444-52891-9.00039-7>
- Cichowski K, Jacks T. NF1 tumor suppressor gene function: narrowing the GAP. *Cell* 2001; 104:593-604; PMID:11239415; [http://dx.doi.org/10.1016/S0092-8674\(01\)00245-8](http://dx.doi.org/10.1016/S0092-8674(01)00245-8)
- Hannan F, Ho I, Tong JJ, Zhu Y, Nurnberg P, Zhong Y. Effect of neurofibromatosis type I mutations on a novel pathway for adenylyl cyclase activation requiring neurofibromin and Ras. *Hum Mol Genet* 2006; 15:1087-98; PMID:16513807; <http://dx.doi.org/10.1093/hmg/ddl023>
- Ho IS, Hannan F, Guo HF, Hakker I, Zhong Y. Distinct functional domains of neurofibromatosis type 1 regulate immediate versus long-term memory formation. *J Neurosci* 2007; 27:6852-7; PMID:17581973; <http://dx.doi.org/10.1523/JNEUROSCI.0933-07.2007>
- Tong JJ, Schriener SE, McCleary D, Day BJ, Wallace DC. Life extension through neurofibromin mitochondrial regulation and antioxidant therapy for neurofibromatosis-1 in *Drosophila melanogaster*. *Nat Genet* 2007; 39:476-85; PMID:17369827; <http://dx.doi.org/10.1038/ng2004>
- Easton DF, Ponder MA, Huson SM, Ponder BA. An analysis of variation in expression of neurofibromatosis (NF) type 1 (NF1): evidence for modifying genes. *Am J Hum Genet* 1993; 53:305-13; PMID:8328449
- The I, Hannigan GE, Cowley GS, Reginald S, Zhong Y, Gusella JF, Hariharan IK, Bernards A. Rescue of a *Drosophila* NF1 mutant phenotype by protein kinase A. *Science* 1997; 276:791-4; PMID:9115203; <http://dx.doi.org/10.1126/science.276.5313.791>
- Guo HF, The I, Hannan F, Bernards A, Zhong Y. Requirement of *Drosophila* NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. *Science* 1997; 276:795-8; PMID:9115204; <http://dx.doi.org/10.1126/science.276.5313.795>
- Guo HF, Tong J, Hannan F, Luo L, Zhong Y. A neurofibromatosis-1-regulated pathway is required for learning in *Drosophila*. *Nature* 2000; 403:895-8; PMID:10706287; <http://dx.doi.org/10.1038/35002593>

Retroviral shRNA-mediated *NFI* knockdown increased the resistance of these cells to both inhibitors, as evidenced by continued growth and sustained MEK/ERK activation. Further, expression of activated KRAS, BRAF, or MEK transgenes, but not of other Ras effector transgenes, conferred similar resistance to ALK inhibition.<sup>14</sup> Two additional observations suggest a potential role for ALK in NF1 tumorigenesis. First, we found that human ALK is expressed in neurofibroma-derived *NFI*<sup>-/-</sup> Schwann cells, as well as in cells derived from malignant NF1 tumors. Second, others previously reported that the human ALK ligand midkine is aberrantly expressed in NF1 deficient murine Schwann cells, and

- Williams JA, Su HS, Bernards A, Field J, Sehgal A. A circadian output in *Drosophila* mediated by neurofibromatosis-1 and Ras/MAPK. *Science* 2001; 293:2251-6; PMID:11567138; <http://dx.doi.org/10.1126/science.1063097>
- Walker JA, Tchoudakova AV, McKenney PT, Brill S, Wu D, Cowley GS, Hariharan IK, Bernards A. Reduced growth of *Drosophila* neurofibromatosis 1 mutants reflects a non-cell-autonomous requirement for GTPase-Activating Protein activity in larval nervous. *Genes Dev* 2006; 20:3311-23; PMID:17114577; <http://dx.doi.org/10.1101/gad.1466806>
- Gouzi JY, Moressis A, Walker JA, Apostolopoulou AA, Palmer RH, Bernards A, Skoulakis EM. The receptor tyrosine kinase Alk controls neurofibromin functions in *Drosophila* growth and learning. *PLoS Genet* 2011; 7:e1002281; PMID:21949657; <http://dx.doi.org/10.1371/journal.pgen.1002281>
- Walker JA, Gouzi JY, Long JB, Huang S, Maher RC, Xia H, Khalil K, Ray A, Van Vactor D, Bernards R, et al. Genetic and functional studies implicate synaptic overgrowth and ring gland cAMP/PKA signaling defects in the *Drosophila melanogaster* neurofibromatosis-1 growth deficiency. *PLoS Genet* 2013; 9:e1003958; PMID:24278035; <http://dx.doi.org/10.1371/journal.pgen.1003958>
- Tong J, Hannan F, Zhu Y, Bernards A, Zhong Y. Neurofibromin regulates G protein-stimulated adenylyl cyclase activity. *Nat Neurosci* 2002; 5:95-6; PMID:11788835; <http://dx.doi.org/10.1038/nn792>
- Tsai P-I, Wang M, Kao H-H, Cheng YJ, Walker JA, Chen R-H, Chien C-T. Neurofibromin mediates FAK signaling in confining synapse growth at *Drosophila* neuromuscular junctions. *J Neurosci* 2012; 32:16971-81; PMID:23175848; <http://dx.doi.org/10.1523/JNEUROSCI.1756-12.2012>
- Liao EH, Hung W, Abrams B, Zhen M. An SCF-like ubiquitin ligase complex that controls presynaptic differentiation. *Nature* 2004; 430:345-50; PMID:15208641; <http://dx.doi.org/10.1038/nature02647>
- Rohrbough J, Broadie K. Anterograde Jelly belly ligand to Alk receptor signaling at developing synapses is regulated by Mind the gap. *Development* 2010; 137:3523-33; PMID:20876658; <http://dx.doi.org/10.1242/dev.047878>

acts as a potent mitogen for human NF1 tumor cells.<sup>26</sup> Thus, among the various phenotypic suppressors identified in our screen, we feel that ALK should with highest priority be further investigated as a potential therapeutic target in NF1.

## Disclosure of Potential Conflict of Interest

No potential conflict of interest was disclosed.

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- Rohrbough J, Kent KS, Broadie K, Weiss JB. Jelly Belly trans-synaptic signaling to anaplastic lymphoma kinase regulates neurotransmission strength and synapse architecture. *Dev Neurobiol* 2013; 73:189-208; PMID:22949158; <http://dx.doi.org/10.1002/dneu.22056>
- Hsueh YP. Neurofibromin signaling and synapses. *J Biomed Sci* 2007; 14:461-6; PMID:17370141; <http://dx.doi.org/10.1007/s11373-007-9158-2>
- Armstrong BC, Le Boutillier JC, Petit TL. Ultrastructural synaptic changes associated with neurofibromatosis type 1: a quantitative analysis of hippocampal region CA1 in a Nf1(+/-) mouse model. *Synapse* 2012; 66:246-55; PMID:22121000; <http://dx.doi.org/10.1002/syn.21507>
- Daston MM, Ratner N. Neurofibromin, a predominantly neuronal GTPase activating protein in the adult, is ubiquitously expressed during development. *Dev Dyn* 1992; 195:216-26; PMID:1301085; <http://dx.doi.org/10.1002/aja.1001950307>
- Iwahara T, Fujimoto J, Wen D, Cupples R, Bucay N, Arakawa T, Mori S, Ratzkin B, Yamamoto T. Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene* 1997; 14:439-49; PMID:9053841; <http://dx.doi.org/10.1038/sj.onc.1200849>
- Hölzel M, Huang S, Koster J, Ora I, Lakeman A, Caron H, Nijkamp W, Xie J, Callens T, Asgharzadeh S, et al. NF1 is a tumor suppressor in neuroblastoma that determines retinoic acid response and disease outcome. *Cell* 2010; 142:218-29; PMID:20655465; <http://dx.doi.org/10.1016/j.cell.2010.06.004>
- Mano H. ALKoma: a cancer subtype with a shared target. *Cancer Discov* 2012; 2:495-502; PMID:22614325; <http://dx.doi.org/10.1158/2159-8290.CD-12-0009>
- Mashour GA, Ratner N, Khan GA, Wang HL, Martuza RL, Kurtz A. The angiogenic factor midkine is aberrantly expressed in NF1-deficient Schwann cells and is a mitogen for neurofibroma-derived cells. *Oncogene* 2001; 20:97-105; PMID:11244508; <http://dx.doi.org/10.1038/sj.onc.1204026>