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

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

Neurofibromatosis 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.1 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 NF1.3

NF1 is caused by mutations that impact the function(s) of neurofibromin, a large and evolutionarily conserved GTPase activating protein (GAP) for Ras oncoproteins.4-6 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.4-6

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,7 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 growth.8-9

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dimensions during all post-embryonic developmental stages. Mutants also have a reduced escape response (taking flight upon release), lack a neuropeptide-elicited rectifying K\(^+\)-current defect at the neuromuscular junction (NMJ), and exhibit circadian arrhythmicity, olfactory associative learning, and memory deficits. Remarkably, all defects but the circadian arrhythmicity are not particularly sensitive to genetic manipulation of Ras signaling but are suppressed by increasing cAMP/PKA 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/Galpha(S)-dependent neurotransmitter stimulated adenylyl cyclase activation and rescue of the dNf1 growth defect. 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.

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.

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. 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. 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.
cyclase activity upon loss of *Drosophila* or murine *Nf1*. Thus, we were not surprised to identify the PKA-CI 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, 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 dNf1 transgene outside of the ring gland had no effect. 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, 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-17D1 alleles reduced the number of NMJ synaptic boutons.

Studies in *C. elegans* and *Drosophila* had previously revealed roles for ALK 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. In *Drosophila*, Jeb and dAlk are both enriched at synapses, and function to control neurotransmission strength and synaptic architecture. 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), whereas others concluded that NMJ differentiation involves the activation of postsynaptic (i.e., muscle expressed) dAlk by presynaptically released Jeb. 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, and that a recent ultrastructural study found reduced curvature at concave synapses in the hippocampus of *Nf1*−/− mice. 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. 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,
the expression of Alk and NF1 in the mouse nervous system overlaps to a large extent. 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. Adding to this indirect evidence, our recent study found that shRNA-mediated suppression of NF1 expression renders human neuroblastoma cells resistant to pharmacological ALK inhibition. 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. Retroviral shRNA-mediated NF1 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. Two additional observations suggest a potential role for ALK in NF1 tumorigenesis. First, we found that human ALK is expressed in neurofibroma-derived NF1−/− 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 acts as a potent mitogen for human NF1 tumor cells. Thus, among the various phenotypic suppressors identified in our screen, we feel that ALK should be considered as a potential therapeutic target in NF1.

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No potential conflict of interest was disclosed.

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