Genetic and Functional Studies Implicate Synaptic Overgrowth and Ring Gland cAMP/PKA Signaling Defects in the Drosophila melanogaster Neurofibromatosis-1 Growth Deficiency

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Published Version
doi:10.1371/journal.pgen.1003958

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:11879131

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Genetic and Functional Studies Implicate Synaptic Overgrowth and Ring Gland cAMP/PKA Signaling Defects in the Drosophila melanogaster Neurofibromatosis-1 Growth Deficiency

James A. Walker1,2, Jean Y. Gouzi1, Jennifer B. Long3, Sidong Huang4, Robert C. Maher1, Hongjing Xia1, Kheyal Khalil1, Arjun Ray1, David Van Vactor3, René Bernards4, André Bernards1,2

1 Massachusetts General Hospital Center for Cancer Research and Harvard Medical School, Charlestown, Massachusetts, United States of America, 2 Center for Human Genetic Research, Massachusetts General Hospital, Boston, Massachusetts, United States of America, 3 Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, United States of America, 4 Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Abstract

Neurofibromatosis type 1 (NF1), a genetic disease that affects 1 in 3,000, is caused by loss of a large evolutionary conserved protein that serves as a GTPase Activating Protein (GAP) for Ras. Among Drosophila melanogaster NF1 (dNf1) null mutant phenotypes, learning/memory deficits and reduced overall growth resemble human NF1 symptoms. These and other dNf1 defects are relatively insensitive to manipulations that reduce Ras signaling strength but are suppressed by increasing signaling through the 3′-5′ cyclic adenosine monophosphate (cAMP) dependent Protein Kinase A (PKA) pathway, or phenocopied by inhibiting this pathway. However, whether dNf1 affects cAMP/PKA signaling directly or indirectly remains controversial. To shed light on this issue we screened 486 1st and 2nd chromosome deficiencies that uncover >80% of annotated genes for dominant modifiers of the dNf1 pupal size defect, identifying responsible genes in crosses with mutant alleles or by tissue-specific RNA interference (RNAi) knockdown. Validating the screen, identified suppressors include the previously implicated dAlk tyrosine kinase, its activating ligand jelly belly (jeb), two other genes involved in Ras/ERK signal transduction and several involved in cAMP/PKA signaling. Novel modifiers that implicate synaptic defects in the dNf1 growth deficiency include the intersectin-related synaptic scaffold protein Dap160 and the cholecystokinin receptor-related CCKLR-17D1 drosulfakinin receptor. Providing mechanistic clues, we show that dAlk, jeb and CCKLR-17D1 are among mutants that also suppress a recently identified dNf1 neuromuscular junction (NMJ) overgrowth phenotype and that manipulations that increase cAMP/PKA signaling in adipokinetic hormone (AKH)-producing cells at the base of the neuroendocrine ring gland restore the dNf1 growth deficiency. Finally, supporting our previous contention that ALK might be a therapeutic target in NF1, we report that human ALK is expressed in cells that give rise to NF1 tumors and that NF1 regulated ALK/RAS/ERK signaling appears conserved in man.

Introduction

RASopathies, caused by mutations that activate Ras/ERK signaling, are a group of related disorders with features that include facial dysmorphism, skeletal, skin and cardiac defects, cognitive deficits, reduced growth and an increased cancer risk [1]. Neurofibromatosis type 1 (NF1; OMIM 162200), caused by loss of a RasGAP, and Noonan syndrome, caused by mutations that alter Ras/ERK pathway proteins SOS1, KRAS, NRAS, RAF1, BRAF, CBL, PTPN11, or SHOC2, are the most common members of this group, affecting 1 in 3,000, or as many as 1 in 1,000 live births, respectively [2,3]. The genetics of these disorders provides a strong argument that excess Ras/ERK signaling underlies common RASopathy symptoms, and much effort remains focused on attenuating Ras/ERK signaling as a strategy for therapeutic intervention. However, whether life-long pharmacological inhibition of Ras/ERK signaling is a viable strategy to treat the full range of often non-life-threatening, but nonetheless serious symptoms of these chronic disorders, remains an open question. This motivates our work to better understand the molecular and cellular pathways responsible for NF1 symptom development, in the hope this will identify more specific therapeutic targets.

We have been interested in using Drosophila melanogaster as a model to investigate NF1 functions in vivo, following our identification of a conserved dNf1 ortholog predicting a protein that is 60% identical to human neurofibromin over its entire 2802
Author Summary

Neurofibromatosis type 1 (NF1) is a genetic disease that affects 1 in 3,000 and that is caused by loss of a protein that inactivates Ras oncoproteins. NF1 is a characteristically variable disease that predisposes patients to several symptoms, the most common of which include benign and malignant tumors, reduced growth and learning problems. We and others previously found that fruit fly mutants that lack a highly conserved dNF1 gene are reduced in size and exhibit impaired learning and memory, and that both defects appear due to abnormal Ras and cyclic-AMP (cAMP) signaling. The former was unremarkable, but how loss of dNF1 affects cAMP signaling remains poorly understood. Here we report results of a genetic screen for dominant modifiers of the dNF1 growth defect. This screen and follow-up functional studies support a model in which synaptic defects and reduced cAMP signaling in specific parts of the neuroendocrine ring gland contribute to the dNF1 growth defect. Beyond these results, we show that human ALK is expressed in cells that give rise to NF1 tumors, and that NF1 regulated ALK/RAS/ERK signaling is evolutionarily conserved.

Moreover, the dNF1 growth defect was also suppressed by neuronal expression of the Drosophila p120RasGAP ortholog, and although we extended earlier findings by showing that heterozygous loss of dRas1 or dRas2, or of a comprehensive set of Ras effector proteins did not modify the growth defect, these mutations also did not reduce the elevated phospho-ERK level in the dNF1 central nervous system (CNS). However, some Ras/ERK pathway double mutants did suppress both defects, leading us to conclude that excess neuronal Ras/ERK signaling is the proximal cause of the non-cell-autonomous dNF1 growth defect [5]. Further supporting this notion, recent work implicated the neuronal dAlk tyrosine kinase receptor and its activating ligand jelly belly (jeb) as rate-limiting activators of dNF1 regulated Ras/ERK pathways responsible for both systemic growth and olfactory learning defects [15].

The above evidence underlies our hypothesis that loss of dNF1 increases neuronal dAlk/Ras/ERK activity, which in turn causes reduced cAMP/PKA signaling, which may or may not be cell-autonomous. Obviously, identifying additional components of dNF1-regulated growth controlling pathways followed by functional analysis might help to test this hypothesis. Here we report results of a dNF1 growth deficiency modifier screen, which identified components of tyrosine kinase/Ras/ERK and neuropeptide/cAMP/PKA pathways in addition to genes involved in synaptic morphogenesis and functioning. Further analysis showed that the requirement for dNF1 and cAMP/PKA in Drosophila growth regulation involves different tissues, with dNF1 required broadly in larval neurons, and cAMP/PKA signaling specifically in AKH-producing cells and perhaps in other parts of the neuroendocrine ring gland. These results, and the recent discovery of a novel dNF1 synaptic overgrowth phenotype [16] that is also suppressed by several genes identified in our screen, set the stage for further work to more precisely define how loss of dNF1 causes Ras/ERK and other signaling defects, the ultimate consequence of which is reduced systemic growth.

Results

Loss of dNF1 Does Not Phenocopy Starvation or Alter Developmental Timing

Animals use elaborate hormonal mechanisms to coordinate nutrient availability and feeding with changes in metabolism and overall growth. Since starvation or crowding during the larval phase of the Drosophila life cycle reduces systemic growth [17], we first examined whether the small size of dNF1 mutants reflected reduced feeding. Arguing against this hypothesis, wild-type and dNF1 larvae ingested similar amounts of dye-stained food throughout their development (Figure 1A). Unlike a pumpless (ppl) mutant [18], dNF1 larvae also showed no tendency to move away from a food source (Figure 1B). Analysis of the expression of the starvation-inducible Pepck and Lsp5 genes [18] provided further evidence that loss of dNF1 does not phenocopy starvation (Figure 1C).

Mechanisms that control Drosophila growth have been the topic of intense study and much has been learned about how an interplay between insulin-like peptide (ILP) controlled growth rate and ecdysone controlled growth duration determines overall growth [see [19] and [20] for reviews]. Arguing against an important role for ecdysone or other factors that control the length of the larval growth period, no differences in the expression of canonical ecdysone-regulated genes was found (results not shown) and no difference in developmental timing between wild-type and dNF1 mutants was detected (Figure 1D and S1). Rather, a reduced growth rate throughout larval development results in an approxi-
Figure 1. Loss of *dNf1* does not phenocopy starvation or alter developmental timing. (A) Wild-type (w*1118*) and *dNf1* larvae ingest similar amounts of food. Larvae at different stages of development were photographed after 25 minutes of feeding on dye-colored yeast paste. (B) As opposed to *ppl* mutants, wild-type and *dNf1* larvae do not wander from a food source (fraction of wandering larvae: WT 3.5% (SD 0.007), *dNf1* 2.5% (SD 0.007) and *ppl* 65% (SD 0.057)). In a similar assay, *dNf1* larvae also showed no abnormality in moving towards a food source (not shown). (C) RNA blot analysis of the starvation-sensitive genes, PEPCK and Lip3 shows that *dNf1* larvae do not show elevated levels of either mRNA under normal feeding conditions. (D) Wild-type and *dNf1* larvae show no significant differences in developmental timing, as assessed by time of pupariation after egg deposition (AED). (E) The *dNf1* growth rate, as assessed by larval weight, is reduced throughout larval development when compared to wild-type or a Ras2>UAS-*dNf1* control. (F) Two hypomorphic insulin receptor alleles, *InR05545* and *InR327*, do not modify *dNf1* pupal size. (G) ILP mRNA expression
is not obviously reduced in dNf1 larvae. H) dNf1 adult flies show no altered longevity compared to wild-type controls. I) Over-expression of Ilp2 from a hs-ilp2 transgene in dNf1 larvae results in a similar increase in size as in wild-type flies.

doi:10.1371/journal.pgen.1003958.g001

imimately 25% weight reduction of dNf1 pupae relative to isogenic controls (Figure 1E and S1).

Drosophila ILPs control systemic growth, metabolism, longevity, and female fecundity [21–24]. Among the eight Drosophila ILP genes, Ilp2, Ilp3 and Ilp5 are co-expressed in bilateral clusters of seven insulin-producing neurosecretory cells (IPCs) in the larval brain [21]. Ablation of these cells causes a severe reduction in overall size, which is rescues by inducing the expression of a hs-p70-Ilp2 transgene [22,23]. However, several results argue against a role for ILPs in the dNf1 growth defect. Firstly, two hypomorphic insulin receptor alleles, IrβE2542 and IrβE277, did not affect dNf1 pupal size (Figure 1F). Secondly, qRT-PCR analysis of RNA extracted from wandering wild-type and dNf1 third instar larvae detected no major differences in the expression of Ilp1 (not shown), Ilp2, Ilp3, Ilp5, Ilp6 and Ilp7 in fed larvae. Among the three IPC expressed ILP genes, the expression of Ilp3 and Ilp5 is reduced in response to starvation [21]. Starved wild-type and dNf1 larvae showed a similar reduction in Ilp5 expression, whereas Ilp3 showed a less pronounced response (Figure 1G). Thirdly, while certain insulin receptor or insulin receptor substrate (choi) mutants have an up to 85% increased life span [25,26], the lifespan of dNf1 mutants and isogenic controls was comparable (Figure 1H). We note that others previously reported a reduced life span for the originally identified dNf1 p-element alleles, generated in a different genetic background [27]. Finally, we previously showed that Ilp2-GAL4 driven UAS-dNf1 expression in IPCs did not rescue the dNf1 size defect [5]. Although daily heat shocking of hs-p70-ilp2 carrying larvae increased the size of dNf1 pupae, indicating that mutants do not lack the ability to respond to insulin, similar induction of this transgene, as previously noted [21], also substantially increased the size of wild-type controls (Figure 1I). Thus, reduced insulin signaling does not provide an obvious explanation for the slower dNf1 growth rate, prompting us to perform a screen to identify other genes involved in dNf1-mediated systemic growth control.

Screen for Dominant Modifiers of dNf1 Systemic Growth Phenotype

While most dNf1 defects are poorly suited for use in modifier screens, the postembryonic growth defect is robust and readily quantified during the pupal stage [4]. However, using this phenotype in a screen is complicated by the fact that organismal size is sexually dimorphic (females are larger than males) and affected by population density, feeding, environmental factors and genetic background differences. With these confounding factors in mind, we used the crossing schemes outlined in Figure 2 to test collections of isogenic 1st and 2nd chromosome deficiencies for dNf1 pupal size modifier effects or synthetic lethal interactions. For each of 139 1st and 347 2nd chromosome deficiencies from the Exelixis [28], DrosDel [29] or Bloomington Stock Center (BSC) collections, we generated Df(1)/+; Nf1E2/Neat71444 uncovers dAlk and partially overlapping suppressing Df(2R)BSC699 each uncover the gene for its activating ligand, jeh, both previously identified as dominant suppressors of dNf1 size, learning, and neuronal ERK over-activation phenotypes [15]. Other uncovered candidate modifiers, such as PKA catalytic and regulatory subunit genes, were tested in crosses with loss-of-function alleles and/or by tissue-specific knockdown using at least two independent UAS-RNAi transgenes, most of which were obtained from the Vienna Drosophila Stock Center (VDRC) [32]. For deficiencies that lacked obvious candidate modifiers, we used the UAS-RNAi approach to more broadly screen uncovered genes. Figure S3 shows examples of modifiers identified by this latter approach. Although the nutrient sensing fat body and other tissues outside of the CNS play important roles in Drosophila
candidate modifiers have only been tested by RNAi knockdown in neurons or glial cells. We focused on these cell types, because neuronal UAS-dNf1 expression sufficed to suppress the growth phenotype [5].

The dNf1 pupal size modifiers identified to date can be classified into three non-exclusive categories, the first of which consists of the previously implicated dAlk/jeb receptor/ligand pair and two not previously implicated other genes involved in Ras-mediated signal transduction. Another expected category includes genes involved in cAMP/PKA signaling, including the previously reported dnc cAMP phosphodiesterase suppressor [35], and the newly identified PKA catalytic subunit gene, PKA-C1, which acts as an enhancer.

Figure 2. Deficiency screen for dominant modifiers of the dNf1 growth defect. Isogenic 1st and 2nd chromosomes deficiencies from the Exelixis, DrosDel and Bloomington Stock Center collections were tested for their ability to alter dNf1 female pupal size. Crossing schemes to generate Df(1)/+; dNf1E2 (A) and Df(2)/CyO; dNf1E2 (B) screening stocks. The tubby-marked TM6B 3rd chromosome balancer allowed the selection of dNf1E2 homozygotes for measurements. (C) Examples of deficiencies that suppress or enhance the dNf1 size defect. Scale bar = 1 mm.

doi:10.1371/journal.pgen.1003958.g002
This group also includes the CCKLR-17D1 drosulfakinin receptor, recently implicated as a cAMP-coupled promoter of synaptic growth [36], which is particularly interesting given the recent identification of a dNf1 larval NMJ overgrowth phenotype [16].

Previously, heterozygous mutations affecting RAF/MEK/ERK kinase cascade components Drosophila tomato kinase and Drosophila MEK, or Df(1)Exel9049, did not score as modifiers (Table S2). No rI uncovering deficiencies were analyzed, but Df(1)Exel9049, which is among the stronger suppressors identified, deletes Dorsal and only two other genes, the neurogenic gene amon (amx), and CG17754, predicting a dominant suppression for either Dorsal or amx, although at least with the tested alleles, combined loss of both genes did not have a more pronounced effect (Figure 5B).

Previously, we did not observe suppression of the dNf1 pupal size defect in crosses with the DorsalE1257 allele [5]. A potential explanation may be that DorsalE1257 is a null mutant [43], whereas the molecular nature of DorsalE1257 is undetermined. Genetic background differences between these Dorsal alleles are another potential explanation for the discrepant results.

Multiple screens aimed at identifying genes involved in Drosophila tyrosine kinase/Ras signaling have been performed [44–52]. Among the genes identified, several are uncovered by 1st and 2nd chromosome deficiencies that do not modify dNf1 size. Suppressing Df(2R)BSC161 uncoveres 27 genes including connector enhancer of KSR (cnk), a scaffold protein that functions as a bimodal (both positive and negative) regulator of RAS/MAPK signaling [53,54]. Supporting a role for cnk as a dNf1 modifier, the cnkE1252 and cnkE1206 alleles act as dominant suppressors (Figure 5B), and suppression was also observed upon RNAi-mediated Cnk knockdown using Ras2-Gal4 or P(GanBE)C25-Gal4 neuronal drivers (Figure 5C). However, Df(2R)BSC154, which uncoveres cnk and only nine other genes, did not score as a modifier (Table S2).

dNf1 Size Modifiers Involved in cAMP/PKA Signaling

The dNf1 growth defect is suppressed by heat shock-induced expression of a constitutively active murine PKA catalytic subunit transgene, called PKA* [4], or by loss of the dance (dnc) cAMP phosphodiesterase [35]. Further validating our screen, two dnc uncovering deficiencies and another that removes the region

### Table 1. Deficiency screen summary.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Number screened</th>
<th>% genes uncovered</th>
<th>dNf1 Modifiers</th>
<th>Non-specific modifiers</th>
<th>dNf1 modifying loci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SUP</td>
<td>ENH</td>
<td>SUP</td>
</tr>
<tr>
<td>1</td>
<td>139</td>
<td>82.1</td>
<td>48</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>2L</td>
<td>182</td>
<td>87.7</td>
<td>14</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>2R</td>
<td>165</td>
<td>86.9</td>
<td>31</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Indicated are the number of chromosome 1, 2L and 2R deficiencies screened, the fraction of genes uncovered (based on the FB2013_03 FlyBase release), the number of dNf1 modifying deficiencies and loci identified, and the number of non-specific modifiers.

dnR Transgenes or vice-versa, that UAS transgenes had no effect in the absence of Gal4 drivers (Figure 5A and data not shown). Finally, extending previous findings and further confirming a role for jeb as a dominant dNf1 size defect suppressor, the jeb<sup>w61</sup> loss-of-function allele [42] dominantly increased dNf1 pupal size (Figure 5B).

### dNf1 Pupal Size Modifiers Involved in Jeb/dAlk/Ras/ERK Signaling

We previously reported that the dAlk receptor tyrosine kinase [40] acts as a rate-limiting activator of neuronal Ras/ERK pathways responsible for dNf1 size and learning defects [15]. Therefore, the fact that the dAlk and jeb genes are uncovered by one and two suppressing deficiencies, respectively (Table 2), validates our screen. Others recently reported that Jeb/dAlk signaling allows brain growth to be spared at the expense of other tissues in nutrient restricted Drosophila, and identified a glial cell niche around neuroblasts as the source of Jeb under these conditions [41]. To determine whether glial cells also produce Jeb involved in overall growth control under normal conditions, we used glial and neuronal Gal4 drivers to test the effect of tissue-specific jeb and dAlk knockdown. Arguing that neurons are the main source of Jeb involved in systemic growth control under non-starvation conditions, jeb knockdown with the Ras2-Gal4, C23-Gal4, and n-syb-Gal4 neuronal drivers [5] increased dNf1<sup>22</sup> pupal size (Figure 5A), whereas the Nes2-Gal4, Eaat1-Gal4 and Glu-Gal4 glial drivers had no effect (data not shown). The only glial driver that gave rise to partial rescue was the pan-glial repo-Gal4 line, although this effect was not enhanced by co-expressing UAS-Dcr2.

Control experiments showed that any driver used in these and other experiments had no effect on pupal size in the absence of
immediately upstream of the dnc coding region, all scored as suppressors (Table 2). Moreover, the Pka-R2 gene, encoding a cAMP binding regulatory PKA subunit, whose dissociation from the catalytic subunit activates the latter, is uncovered by two additional suppressing deficiencies, whereas a deficiency that uncovers the major Pka-C1 catalytic subunit gene scored as an enhancer (Table 2). Df(1)ED7261, which uncovers the rutabaga (rut) adenyl cyclase, did not score as a modifier (not shown).

Confirmation of dnc and Pka-C1 as the genes responsible for the observed effects was obtained in crosses with three dnc and three Pka-C1 loss-of-function alleles (Table 2). Pka-R2 remains an attractive candidate suppressor, but expression Pka-R2RNAi transgenes in neurons had no effect and its role as a dNf1 modifier remains unconfirmed (results not shown).

**Novel dNf1 Modifiers**

Recently, the cAMP-coupled CCKLR-17D1 drosulfakinin receptor, but not its closely related CCKLR-17D3 paralog, was
identified as a positive regulator of synaptic growth [36]. The CCKLR-17D1 gene is uncovered by three suppressing deficiencies, including Df(1)Exel9051, which uncovers only three other genes. The closely linked CCKLR-17D3 paralog is not uncovered by Df(1)Exel9051, and while Ras2-Gal4 or P(GawB)C23-Gal4 driven neuronal CCKLR-17D1 RNAi expression strongly suppressed the dNfl pupal size defect, similar suppression of CCKLR-17D3 had no effect (Figure 6A).

Beyond CCKLR-17D1, several dNfl size modifiers are expressed in brain and/or have neuronal functions. Among these, dynamin-

Figure 4. Identified deficiencies increase or decrease pupal size to different extents. Female pupal lengths for the indicated 1, 2L and 2R deficiencies. Control measurements for dNflE2 and wild-type (w1118) are in black. Colors for enhancing, suppressing and non-specific deficiencies are as in Figure 2. Pupal lengths are shown in mm, error bars denote standard deviations and are based on measurements described in Table S2. All shown deficiencies modify dNfl female pupal size with p-values<0.01.
doi:10.1371/journal.pgen.1003958.g004
Table 2. Modifying deficiencies and identification of responsible genes.

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Cytological Breakpoints</th>
<th>Modif.</th>
<th>Gene Implicated</th>
<th>Modifying allele(s) and/or RNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tyrosine Kinase/Ras signaling</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Df(2R)Exel7144</td>
<td>53C8;53D22</td>
<td>SUP</td>
<td>dAlk</td>
<td>dAlk&lt;sup&gt;a&lt;/sup&gt; (lof), dAlk&lt;sup&gt;Y&lt;/sup&gt; (lof), v11446, v107083, JF02668</td>
</tr>
<tr>
<td>Df(2R)Exel6064</td>
<td>53C11;53D11</td>
<td>SUP</td>
<td>Jellybelly (jeb)</td>
<td>Jeb&lt;sup&gt;neo&lt;/sup&gt; (lof), v103047, v30800</td>
</tr>
<tr>
<td>Df(2R)BSC199</td>
<td>48C5;48E4</td>
<td>SUP</td>
<td>connector enhancer of ksr (ckk)</td>
<td>ckk&lt;sup&gt;X&lt;sup&gt;2151&lt;/sup&gt;&lt;/sup&gt; (A), ckk&lt;sup&gt;Y&lt;sup&gt;2083&lt;/sup&gt;&lt;/sup&gt; (lof), v107746</td>
</tr>
<tr>
<td>Df(2R)BSC699</td>
<td>48D7;48E6</td>
<td>SUP</td>
<td>Dsr1 and almondex (amx)</td>
<td>Dsr1: Dsr1&lt;sup&gt;Y&lt;sup&gt;19710&lt;/sup&gt;&lt;/sup&gt; (amorph), v107276, v40026, HMS00145; amx: amx&lt;sup&gt;Y&lt;sup&gt;2265&lt;/sup&gt;&lt;/sup&gt; (hypo), v3296</td>
</tr>
<tr>
<td><strong>cAMP/PKA signaling</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Df(1)BSC710</td>
<td>3B2;3C9</td>
<td>SUP</td>
<td>dNc</td>
<td>dnc&lt;sup&gt;Y&lt;sup&gt;14&lt;/sup&gt;&lt;/sup&gt; (amorph), dnc&lt;sup&gt;Y&lt;sup&gt;43&lt;/sup&gt;&lt;/sup&gt; (amorph), dnc&lt;sup&gt;Y&lt;sup&gt;24&lt;/sup&gt;&lt;/sup&gt; (hypo)</td>
</tr>
<tr>
<td>Df(1)BSC656</td>
<td>3B3;3D2</td>
<td>SUP</td>
<td>CCK-like receptor at 17D1 (CCKLR-17D1)</td>
<td>v100760</td>
</tr>
<tr>
<td>Df(1)BSC834</td>
<td>3C11;3F3</td>
<td>SUP</td>
<td>Dynamin-associated protein 160 (Dap160)</td>
<td>Dap160&lt;sup&gt;Y&lt;sup&gt;24&lt;/sup&gt;&lt;/sup&gt; (lof), v106689, v16158, JF01918</td>
</tr>
<tr>
<td>Df(2L)Exel6024</td>
<td>30C1;30C9</td>
<td>ENH</td>
<td>cAMP-dependent protein kinase I (PKA-C1)</td>
<td>PKA-C1&lt;sup&gt;G00714&lt;/sup&gt; (leth), PKA-C1&lt;sup&gt;G00715&lt;/sup&gt; (leth)</td>
</tr>
<tr>
<td><strong>Neuronal Function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Df(1)ED447</td>
<td>17C1;17F1</td>
<td>SUP</td>
<td>CCK-like receptor at 17D1 (CCKLR-17D1)</td>
<td>v100760</td>
</tr>
<tr>
<td>Df(1)Exel9051</td>
<td>17D1;17D3</td>
<td>SUP</td>
<td>Dap160&lt;sup&gt;Y&lt;sup&gt;24&lt;/sup&gt;&lt;/sup&gt; (lof), v106689, v16158, JF01918</td>
<td></td>
</tr>
<tr>
<td>Df(1)Exel7664</td>
<td>17D1;17E1</td>
<td>SUP</td>
<td>Embryonic lethal abnormal vision (elav)</td>
<td>elav&lt;sup&gt;Y&lt;sup&gt;24&lt;/sup&gt;&lt;/sup&gt;, elav&lt;sup&gt;Y&lt;sup&gt;43&lt;/sup&gt;&lt;/sup&gt;</td>
</tr>
<tr>
<td>Df(2L)BSC2302</td>
<td>39A1;39A4</td>
<td>SUP</td>
<td>Dynamin-associated protein 160 (Dap160)</td>
<td>Dap160&lt;sup&gt;Y&lt;sup&gt;24&lt;/sup&gt;&lt;/sup&gt; (lof), v106689, v16158, JF01918</td>
</tr>
<tr>
<td>Df(2L)Exel6047</td>
<td>39A2;39B4</td>
<td>SUP</td>
<td>Nicotinic Acetylcholine Receptor alpha-30D (nAcR-30D)</td>
<td>nAcR-30D&lt;sup&gt;G00715&lt;/sup&gt; (via) nAcR-30D&lt;sup&gt;Y&lt;sup&gt;24&lt;/sup&gt;&lt;/sup&gt; (via)</td>
</tr>
<tr>
<td>Df(1)Exel46221</td>
<td>184;18B</td>
<td>SUP</td>
<td>Neutral amino acid transporter 1 (NAAT1)</td>
<td>v106027, v37380, v50063</td>
</tr>
<tr>
<td>Df(1)ED6396</td>
<td>185;18B</td>
<td>SUP</td>
<td>Casein kinase II beta (csk)-2 subunit (CkiI&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>v102633, v26915</td>
</tr>
<tr>
<td>Df(2L)BSC252</td>
<td>30C6;30E1</td>
<td>ENH</td>
<td>Serine/threonine kinase (Akt) interacting (Nikain)</td>
<td>v105893, v102018</td>
</tr>
<tr>
<td>Df(2L)Exel7043</td>
<td>30C7;30F2</td>
<td>SUP</td>
<td>Vps33/carnation (car)</td>
<td>car&lt;sup&gt;a&lt;/sup&gt; (hypo), car&lt;sup&gt;a&lt;sup&gt;Y&lt;sup&gt;43&lt;/sup&gt;&lt;/sup&gt;&lt;/sup&gt; (lof), v110756</td>
</tr>
<tr>
<td>Df(2L)Exel6025</td>
<td>30D1;30F1</td>
<td>SUP</td>
<td>Vps18/deep orange (dor)</td>
<td>dor&lt;sup&gt;a&lt;/sup&gt; (leth), v107053, v105330</td>
</tr>
<tr>
<td>Df(2L)Exel8041</td>
<td>30C9;30E1</td>
<td>SUP</td>
<td>Nicotinic Acetylcholine Receptor alpha-30D (nAcR-30D)</td>
<td>nAcR-30D&lt;sup&gt;G00715&lt;/sup&gt; (via) nAcR-30D&lt;sup&gt;Y&lt;sup&gt;24&lt;/sup&gt;&lt;/sup&gt; (via)</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Df(1)Exel6254</td>
<td>19C4;19D1</td>
<td>SUP</td>
<td>HERC2</td>
<td>v105374</td>
</tr>
<tr>
<td>Df(2L)ED3800</td>
<td>35B2;35D1</td>
<td>ENH</td>
<td>Cullin-3 (cul3)</td>
<td>cul3&lt;sup&gt;a&lt;sup&gt;Y&lt;sup&gt;43&lt;/sup&gt;&lt;/sup&gt;&lt;/sup&gt; (lof)</td>
</tr>
<tr>
<td>Df(2L)Exel1050</td>
<td>35B8;35D4</td>
<td>ENH</td>
<td>Cullin-3 (cul3)</td>
<td>cul3&lt;sup&gt;a&lt;sup&gt;Y&lt;sup&gt;43&lt;/sup&gt;&lt;/sup&gt;&lt;/sup&gt; (lof)</td>
</tr>
<tr>
<td>Df(2L)Exel1004</td>
<td>35B10;35D1</td>
<td>ENH</td>
<td>Cullin-3 (cul3)</td>
<td>cul3&lt;sup&gt;a&lt;sup&gt;Y&lt;sup&gt;43&lt;/sup&gt;&lt;/sup&gt;&lt;/sup&gt; (lof)</td>
</tr>
<tr>
<td>Df(1)BSC533</td>
<td>4F4;4F10</td>
<td>SUP</td>
<td>Heparin sulfate 3-O-sulfotransferase-B (Hst3B)</td>
<td>v110601</td>
</tr>
<tr>
<td>Df(1)Exel290</td>
<td>4F7;4F10</td>
<td>SUP</td>
<td>Heparin sulfate 3-O-sulfotransferase-B (Hst3B)</td>
<td>v110601</td>
</tr>
<tr>
<td>Df(1)Exel9068</td>
<td>1884;18B6</td>
<td>SUP</td>
<td>Neuregulin 1 (NRG1)</td>
<td>v102633, v26915</td>
</tr>
<tr>
<td>Df(2R)BSC701</td>
<td>56F15;57A9</td>
<td>SUP</td>
<td>Neuregulin 1 (NRG1)</td>
<td>v102633, v26915</td>
</tr>
<tr>
<td>Df(2L)BSC607</td>
<td>60E4;60E8</td>
<td>SUP</td>
<td>Neuregulin 1 (NRG1)</td>
<td>v102633, v26915</td>
</tr>
<tr>
<td>Df(1)BSC275</td>
<td>18C8;18D3</td>
<td>SUP</td>
<td>Neuregulin 1 (NRG1)</td>
<td>v102633, v26915</td>
</tr>
<tr>
<td>Df(1)BSC719</td>
<td>2A3;2B13</td>
<td>SUP</td>
<td>Neuregulin 1 (NRG1)</td>
<td>v102633, v26915</td>
</tr>
<tr>
<td>Df(1)BSC589</td>
<td>2B1;2B5</td>
<td>SUP</td>
<td>Neuregulin 1 (NRG1)</td>
<td>v102633, v26915</td>
</tr>
</tbody>
</table>

Modifying deficiencies for which the responsible dNf1 interacting gene has been identified. The cytological location, and the dominant effect on dNf1 pupal size (SUP - suppressor, ENH - enhancer) of each deficiency is given. The responsible genes for each modifying deficiency are shown with the mutant alleles, VDRC and TRIP RNAi lines used in their identification. Expression of RNAi transgenes was induced with the Ras2-Gal4, elav-Gal4, nyb-Gal4 and/or C23-Gal4 drivers. Abbreviations: hypo: hypomorphic; leth: lethal; lof: loss-of-function; amorph: amorphic; Δ: deletion; via: viable.

doi:10.1371/journal.pgen.1003958.t002

Associated protein 160 (Dap160) is an intersectin-related scaffold implicated in synaptic vesicle exocytosis and neuroblast proliferation [55–58]. Dap160 is uncovered by suppressing deficiencies Df(2L)Exel6047 and Df(2L)BSC302, whose region of overlap encompasses ten genes. We note that Df(2L)Exel6047 also uncovers the Drosophila Ret tyrosine kinase gene, the human ortholog of which is the receptor for glial-derived neurotrophic factor. Ret initially appeared as an especially attractive candidate suppressor, because activating RET and inactivating NF1 mutations can both lead to human pheochromocytoma [59], and because Drosophila Ret is expressed in larval brain neurons that resemble neuroendocrine cells [60]. However, among multiple lines of evidence that argue against a role for Ret in the dNf1 growth defect, UAS-dNf1 re-expression directed by a newly generated Ret-Gal4 driver that recapitulates the endogenous larval brain Ret expression pattern (Figure S4B), or

PLOS Genetics | www.plosgenetics.org
RNAi-mediated Ret inhibition, did not modify dNf1 pupal size, nor did expression of a UAS-Ret K805A kinase dead transgene. Moreover, Ret-Gal4-driven expression of UAS-Ret transgenes carrying the activating C695R mutation, which mimics a mutation found in multiple endocrine neoplasia type 2 did not phenocopy the \textit{dNf1} reduced growth phenotype, although the same transgene did produce the previously described rough eye phenotype when driven by GMR-Gal4 [60]; Figure S4C). Further arguing against a role in \textit{dNf1} growth control, Ret is uncovered by non-modifying Df(2L)BSC533. By contrast, Dap160 loss-of-function alleles (Dap160\textsuperscript{F1} and Dap160	extsuperscript{G2}; [56]), or Dap160 RNAi expression driven by three neuronal Gal4 drivers, suppressed the \textit{dNf1} pupal size defect, identifying it as the responsible modifier (Figure 6B).

The gene for the neuronal RNA binding protein elav is uncovered by suppressing \textit{Df(1)Exel6221} and \textit{Df(1)ED6396} whose region of overlap includes just three other genes. Identifying \textit{elav} as the responsible modifier, \textit{elav\textsuperscript{elavG0031}} and \textit{elav\textsuperscript{elavG0031}} alleles strongly suppressed (Figure 6C). Rab9 is a modifier uncovered by suppressing deficiency \textit{Df(2L)Exel8041}. Neuronal but not glial \textit{Rab9}\textsuperscript{Rab9\textsuperscript{C23}} expression increases \textit{dNf1} pupal size, and the same result is seen upon neuronal expression of a Rab9 dominant negative [61] mutant (Figure 6D).

\textit{NAAT1}, coding for a larval gut and brain expressed amino acid transporter with a unique affinity for D-amino acids [38], is uncovered by suppressing \textit{Df(1)Exel6290} and \textit{Df(1)BSC553} whose region of overlap includes only four other genes. Identifying \textit{NAAT1} as the responsible suppressor, three neuronal Gal4 lines driving the expression of three \textit{NAAT1} targeting RNAi transgenes suppressed the \textit{dNf1} size defect, whereas \textit{Repo-Gal4} driven glial expression had no effect (Figure 7A and Table 2).

Mammalian E3 ubiquitin ligase HERC2 controls the ubiquitin-dependent assembly of DNA repair proteins on damaged chromosomes [62]. Drosophila HERC2 is uncovered by suppressing deficiency \textit{Df(1)Ed6254}, which also uncovers the \textit{syx16}, coding for syntaxin 16. No HERC2 alleles exist, but \textit{Ras2-Gal4} driven expression of a \textit{UAS-HERC2RNAi} transgene (v105374) strongly suppressed the \textit{dNf1} pupal size defect (Figure 7A), whereas similar knockdown of \textit{Syx16} had no statistically significant effect (not shown). The gene for another E3 ligase component, \textit{Cul-3}, is
uncovered by three enhancing deficiencies, and a Cal-3 loss-of-function allele or Ras2-Gal4 driven expression of a Cal-3 RNAi transgene both enhanced the dNf1 size defect, identifying it as the responsible gene (Table 2). Suppressing Df(1)Exel9068 uncovers only four genes, including one encoding the TORC2 complex subunit Rictor. However, systematic Ras2-Gal4 driven RNAi knockdown of Df(1)Exel9068 uncovered genes identified Hs3st-B, encoding one of two Drosophila heparan sulfate 3-O sulfotransferases, as a potent dNf1 size defect suppressor (Figure 7A), whereas knockdown of Rictor had no effect (not shown). Others previously identified Hs3st-B as a positive regulator of Notch signaling [39]. However, the heparan sulfate proteoglycan substrates of Hs3st-B bind various growth factors and other ligands and have been implicated in a variety of biological processes. Exactly why loss of Hs3st-B suppresses the dNf1 growth defect remains to be determined.

Two functionally related dNf1 growth defect suppressors carnation (car/Vps33A) and deep-orange (dor/Vps18), encode subunits of the Class C Vacular Protein Sorting (VPS) complex, required for the delivery of endosomal vesicles to lysosomes [63]; Figure 7B). The Vps16A gene encodes a third member of this complex [64], but whether Vps16A located on the 3rd chromosome also acts as a dNf1 suppressor, or whether pharmacological inhibition of lysosomal degradation affects dNf1 pupal size are questions that remain to be answered.

B4/Susi is a coiled-coil protein without obvious orthologs outside of insects. It functions as a negative regulator of Drosophila class I phosphatidylinositol-3 kinase P3K92E/Dp110 by binding to its P3K21B/dP60 regulatory subunit. Homozygous B4 mutants have an increased body size [65], which may explain why Ras2-Gal4-driven RNAi-mediated suppression of B4, uncovered by suppressing deficiency Dy(2)BSC692, increased dNf1 pupal size (not shown). However, whether B4 is the responsible dominant modifier is doubtful, given that it is also uncovered by Dy(2)BSC6592, a non-modifying deficiency. Moreover, we previously found that heterozygous loss of P3K21B, or neuronal
expression of a dominant negative \(P3K92E\) transgene, did not modify \(dNf1\) pupal size [5]. Beyond \(B4\), \(dNf1\) size modifying deficiencies uncovered no genes involved in the canonical growth regulating pathways mediated by insulin and ecdysone. Indeed, several such genes were uncovered by non-modifying deficiencies. Among these genes, fat body expressed insulin-like growth factor 1\(\alpha\), which regulates larval growth in the post-feeding phase [66,67], is uncovered by two non-modifying deficiencies. A single non-modifying deficiency, \(Df(2L)BSC02606\), uncovers both the\(\alpha\) and \(\beta\) genes, whose products antagonistically control insulin-stimulated \(P3K92E/\text{Dp110}\) activity, leading to changes in body, organ, and cell size [68,69]. Among subunits of the cell growth regulating mTORC1 complex, \(raptor\) is uncovered by three and \(Tor\) by one non-modifying deficiency. Among genes implicated in ecdysone signaling, the ec dysone co-receptor \(\text{ultraspiracle}\) and the ecdysone-induced growth regulating \(DHR4\) nuclear receptor [70] are each uncovered by non-modifying deficiencies, and two such deficiencies uncover \(Pth\), coding for prothoracotropic hormone, which provides developmental timing cues by stimulating the production of ecdysone [71,72]. These results reinforce our conclusion that the canonical growth regulating pathways involving insulin and ecdysone play no obvious roles in \(dNf1\) growth control.

Manipulating cAMP/PKA Signaling in the Ring Gland Affects \(dNf1\) Systemic Growth Non-Cell-Autonomously

Several results argue that defects in Ras/ERK and cAMP/PKA signaling responsible for the \(dNf1\) growth defect involve non-overlapping cell populations. Firstly, heat shock-induced \(hsp70-PKA^*\), or \(Ras2-Gal4\) induced attenuated \(UAS-PKA^*\) transgene (see below) expression rescued the \(dNf1\) pupal size defect, but failed to reduce the elevated larval brain phospho-ERK level (Figure 8A). Moreover, several neuronal RNAi drivers that increase \(dNf1\) pupal size when driving \(UAS-dNf1\) [5], failed to modify this phenotype when driving \(UAS-\text{cnk}\) transgenes, even in the presence of the \(UAS-Dcr-2\) RNAi enhancer (Table 3). This prompted us to investigate whether genetic manipulation of cAMP/PKA signaling in cells other than \(dNf1\) requiring neurons was more effective.

To manipulate cAMP/PKA signaling tissue-specifically we used three \(UAS-dn\text{RacG}\) transgenes. We also generated a series of attenuated \(UAS-PKA^*\) transgenes using vectors with modified \(Gal4\)-inducible promoters harboring just 2, 3 or 4 \(Gal4\)-binding \(UAS\) elements (Figure 8B and C). We made the latter transgenes ubiquitously with most \(Gal4\) drivers [73]. As reported previously [74], driving \(UAS-dnNf1\) ubiquitously with \(Act5C-Gal4\), or broadly in neurons with \(elav-Gal4\), \(Ras2-Gal4\), \(C23-Gal4\), or \(3867-Gal4\) restored \(dNf1\) pupal size, whereas driving the same transgene with more restricted neuronal or non-neuronal drivers had no effect (Figure 8D and Table 3). By contrast, driving the expression of \(UAS-dn\text{RacG}\) or attenuated \(UAS-PKA^*\) transgenes with the same set of broadly expressed neuronal drivers was ineffective (Tables 3 and S5). We note that expression of the \(2\times UAS-PKA^*\) and \(3\times UAS-PKA^*\) transgenes was generally well tolerated, whereas the \(4\times UAS-PKA^*\) and the \(3\times UAS-PKA^*\) transgenes exhibited increasing levels of lethality (Tables 3 and S5). Arguing that rescue of the \(dNf1\) growth defect by manipulating cAMP/PKA signaling or \(dNf1\) expression involves different cells, strong pupal size rescue was observed by increasing cAMP/PKA signaling in adipokinetic hormone-producing cells at the base of the neuroendocrine ring gland using the \(Alkh-Gal4\) driver (Figure 8D). Rescue was also observed with the \(Feb36-Gal4\) and \(Aug21-Gal4\) ring gland drivers (Figure 8D), which give rise to expression in the corpora allata, the source of juvenile hormone, but not with the \(P0206-Gal4\) or \(Mai60-Gal4\) drivers, which express predominately in the prothoracic gland (Table 3). The tissue specificity of all \(Gal4\) drivers used in this and other experiments was verified by microscopic observation of dissected \(UAS-GFP\) expressing larvae (Table S4 and Figures 8E–H and S3).

d\(\text{Alk}, \text{Jeb, Cnk}\) and \(CCKLR-17D1\) Supress a \(dNf1\) NMJ Architectural Defect

During larval development, significant expansion of the NMJ arbor must occur, reflecting the steady muscle growth that takes place during larval life. As the NMJ grows, additional branches and boutons are added to the initial synaptic arbor that forms during late embryonic stages upon motor axon contact with its target muscle. As a result, at the wandering third instar stage, wild-type NMJs contain a highly stereotyped, segment specific number of synaptic boutons [75]. Recently, it was reported that \(dNf1\) functions presynaptically to constrain NMJ synaptic growth and neurotransmission [16]. In \(dNf1\) null mutant wandering third instar larvae, while the distribution of major presynaptic proteins is unaffected, increased overall size and synaptic bouton number is apparent at multiple NMJs, supporting a specific role for \(dNf1\) in restricting NMJ expansion [16]. Several \(dNf1\) suppressors that emerged in the current screen have also been linked to synapse morphogenesis, including \(CCKLR-17D1\), which functions as a promoter of NMJ growth [36]. As our screen identified \(CCKLR-17D1\) as a dominant \(dNf1\) size defect suppressor, we wanted to confirm the \(dNf1\) NMJ phenotype and test whether \(CCKLR-17D1\) and other suppressors affected this defect.

By quantifying bouton number at the NMJ on muscles 6 and 7, we confirmed that \(dNf1\) mutants have a significant increase in mean bouton number (Figure 9A and B). In addition, this analysis confirmed previously published phenotypes for \(d\text{Alk}, \text{Jeb}\) and \(CCKLR-17D1\) [36,76]. Importantly, the \(dNf1\) synaptic overgrowth phenotype is dominantly suppressed by \(CCKLR-17D1\), \(d\text{Alk}, \text{Jeb}\), and \(cnk\) alleles (Figure 9B), arguing that all four genes are epistatic to \(dNf1\). As a control we analyzed an allele of \(spi\), which encodes an EGF-like growth factor and is uncovered by suppressing \(Df(2L)Exel8041\). However, \(spi\) shows no genetic interaction with \(dNf1\), as loss of \(spi\) modified neither the pupal size nor the NMJ overgrowth phenotypes (Figure 9B and data not shown).

Human ALK Is Expressed in Schwann Cells and May Serve as a Therapeutic Target in NF1

The identification of \(d\text{Alk}\) as a suppressor of all hitherto analyzed \(dNf1\) defects prompted us to explore whether human ALK represents a therapeutic target in NF1. Given our hypothesis that NF1 negatively regulates ALK stimulated Ras/ERK signaling, in order to play such a role, ALK and NF1 must be co-expressed in cells that give rise to symptoms. We previously found that \(dNf1\) and \(d\text{Alk}\) expression overlaps extensively in Drosophila larval and adult CNS [15], and the expression of orthologs of both genes also overlaps in the murine CNS [77,78]. While overlapping CNS expression is compatible with a role for ALK in NF1-associated cognitive dysfunction, a causative role in another hallmark NF1 symptom, peripheral nerve-associated tumors, is less obvious. Among the near universal symptoms on NF1, benign neurofibromas, we used reverse transcription/PCR to detect the...
Figure 8. *dNf1* systemic growth related RAS/ERK and cAMP/PKA signals appear functionally and topographically distinct. (A) The elevated larval CNS pERK level of *dNf1* mutants is reduced by neuronal expression of *dNf1*, but not by neuronal or heat-shock induced ubiquitous expression of PKA*. Western blot of pERK levels in larval CNS of the indicated genotypes. In lane 6, larvae received a daily 20 min 37°C heat shock throughout development, a protocol that suppresses the *dNf1* growth defect [4]. (B) Structure of UAS-PKA* transgenes with 1 to 5 UAS elements. The 

---

**Table:**

<table>
<thead>
<tr>
<th>UAS</th>
<th>mPKA*</th>
<th>Lethality &gt; Ac5C elav</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x</td>
<td></td>
<td>††</td>
</tr>
<tr>
<td>4x</td>
<td></td>
<td>†</td>
</tr>
<tr>
<td>3x</td>
<td></td>
<td>†</td>
</tr>
<tr>
<td>2x</td>
<td></td>
<td>†</td>
</tr>
<tr>
<td>1x</td>
<td></td>
<td>†</td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th>GMR&gt;mPKA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1xUAS</td>
</tr>
<tr>
<td>2xUAS</td>
</tr>
<tr>
<td>3xUAS</td>
</tr>
<tr>
<td>4xUAS</td>
</tr>
<tr>
<td>5xUAS</td>
</tr>
</tbody>
</table>

**Figure D:**

Graph showing pupal length (mm) for the indicated genotypes: W118, dNf1E2, Ras2, Akh, Feb39, Aug21, Ras2, Akh, Feb39, Aug21, Ras2, Akh, Feb39, Aug21, v107967 dnc. The graph includes error bars and statistical significance symbols (**).
passion or absence of ALK mRNA in neurofibroma-derived NF1−/− Schwann cells and NF1−/− fibroblasts, using RNAs kindly provided by Drs. Eric Legius and Eline Beert. In these experiments, two different primer sets readily detected ALK mRNA in NF1−/− Schwann cells, but not in NF1−/− fibroblasts derived from the same tumors (Figure S6).

To test whether functional interactions between NF1 and ALK exist in human cells, we used the SK-SY5Y and Kelly neuroblastoma cells, both of which harbor constitutively active F1174L which NF1 negatively regulates ALK/RAS signaling, Crizotinib (Figures 10A, 10C and S7). Compatible with a model in resistance of both lines to ALK inhibitors NVP-TAE684 and compatibly with a role for NF1 as a negative regulator of mitogenic ALK/RAS signals, qRT-PCR verified ALK inhibition [80]. Compatible with a role for NF1 as a negative regulator of ALK/RAS signals, qRT-PCR verified ALK inhibition [80].

Discussion

The work reported here was motivated by the fact that human NF1 is a characteristically variable disease, the severity of which is controlled at least in part by symptom-specific modifier genes [81]. Thus, genetic analysis in Drosophila might not only reveal molecular pathways controlled by the highly conserved (50% identical) dNf1 protein, but also provide clues to the identity of human modifiers, which by virtue of their rate-limiting roles in symptom development might serve as therapeutic targets. The current work was also motivated by the fact that, for reasons that remain poorly understood, most dNf1 null mutant phenotypes are rescued by increasing, or phenocopied by decreasing, cAMP/PKA signaling. The identification of genetic modifiers of a cAMP/PKA sensitive defect might reveal how loss of dNf1 affects cAMP/PKA signaling, and help to resolve the long-standing controversy as to whether dNf1 affects cAMP/PKA signaling directly, independent of its role as a Ras regulator [10,27], or indirectly, secondary to a Ras signaling defect [5,15].

Table 3. Restoration of systemic growth by dNf1 and cAMP/PKA involves different tissues.

<table>
<thead>
<tr>
<th>Gal4</th>
<th>UAS-dNf1</th>
<th>dnc v107967</th>
<th>2 × UAS-PKA*</th>
<th>3 × UAS-PKA*</th>
<th>4 × UAS-PKA*</th>
<th>5 × UAS-PKA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActSC</td>
<td>Rescue</td>
<td>Rescue (pupal †)</td>
<td>SV</td>
<td>†</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td>elav</td>
<td>Rescue</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR NR</td>
</tr>
<tr>
<td>elav+Oct-2</td>
<td>Rescue</td>
<td>NR</td>
<td>n/a</td>
<td>n/a</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ras2(41)</td>
<td>Rescue</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td>Ras2(41)+Oct-2</td>
<td>Rescue</td>
<td>NR</td>
<td>N/a</td>
<td>n/a</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>C23</td>
<td>Rescue</td>
<td>NR</td>
<td>Rescue</td>
<td>†</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td>Feb36</td>
<td>NR</td>
<td>Rescue</td>
<td>NR</td>
<td>Rescue</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td>Aug21</td>
<td>NR</td>
<td>Rescue</td>
<td>Rescue</td>
<td>Rescue</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td>Akh</td>
<td>NR</td>
<td>Rescue</td>
<td>Rescue</td>
<td>Rescue</td>
<td>Rescue</td>
<td>(SV)</td>
</tr>
</tbody>
</table>

| ActSC-Gal4 driven ubiquitous dNf1 re-expression, or elav-Gal4 and Ras2-Gal4 driven neuronal re-expression rescues the dNf1 pupal size defect, whereas dnc RNAi or UAS-PKA* expression controlled by the same drivers is ineffective. By contrast, expressing dNf1 in specific parts of the neuroendocrine ring gland with the Akh-Gal4, Feb36-Gal4 or Aug21-Gal4 drivers fails to rescue, whereas using the same drivers to express dnc RNAi or attenuated UAS-PKA* transgenes does increase dNf1 pupal size. All crosses produced viable adults unless otherwise indicated.

† denotes lethality, SV sub-viable, n/a not applicable, NR non-rescue.

The data shown summarize results of a larger effort to identify the tissues in which dNf1 and cAMP/PKA affect systemic growth. Full results are shown in Table S5. doi:10.1371/journal.pgen.1003958.0003
neuroblasts as the source of Jeb under these conditions [41]. restricted Drosophila and identified a glial cell niche around growth to be spared at the expense of other tissues in nutrient deficiencies were found to be lethal in a dNf1 background (Glenn Cowley, Iswar Hariharan and A.B., unpublished), or when a pilot chemical mutagenesis screen found the reliable mapping of identified enhancer or suppressor mutations to be impracticable (Suzanne Brill, Iswar Hariharan and A.B., unpublished). Both aborted screens informed the current effort, which used precisely defined small deficiencies, isogenic crossing schemes and experimental protocols that guarded against population density differences. In total we analyzed 486 1st and 2nd chromosome deficiencies that together uncover well over 80% of chromosome 1, 2L, and 2R genes (Table 1). Among the screened deficiencies, 132 (27.2%) significantly modified dNf1 pupal size [p<0.01; two-tailed Student’s t-test]. While this is a large number, 20 deficiencies were subsequently eliminated because they also affect wild-type size. Several modifying deficiencies also uncover overlapping genomic segments, further reducing the number of dNf1 modifying loci to 76. During follow-up studies aimed at identifying responsible genes, we prioritized genes uncovered by suppressing deficiencies over those uncovered by enhancing ones, modifiers uncovered by overlapping deficiencies over those uncovered by single deficiencies, modifiers uncovered by small deficiencies over those uncovered by larger ones and stronger modifiers over weaker ones. We also limited ourselves to genes that function in the nervous system, based on the consideration that dNf1 re-expression in larval neurons is sufficient to suppress the growth defect [5].

We previously reported that dNf1 growth and learning defects are phenocopied by increasing neuronal Jeb/dAlk/ERK signaling, and suppressed by genetic or pharmacological attenuation of this pathway [15]. Validating our screen, deficiencies that uncover jeb and dAlk were identified as dominant dNf1 size defect suppressors. Others recently reported that Jeb/dAlk signaling allows brain growth to be spared at the expense of other tissues in nutrient restricted Drosophila and identified a glial cell niche around neuroblasts as the source of Jeb under these conditions [41]. However, Jeb involved in systemic growth appears of mainly neuronal origin, as RNAi-mediated jeb knockdown in neurons increased dNf1 pupal size, whereas only one of four tested glial drivers produced partial rescue (Figure 5A).

The identification of cAMP/PKA pathway modifiers dnc, PKA-C1 and tentatively PKA-R2 further validates our screen. Arguing that increased PKA activity doesn’t suppress dNf1 defects by attenuating Ras/Raf/MEK/ERK signaling, hsp70-PKA* transgene expression, using a daily heat shock regimen that suppresses the dNf1 size defect [4], does not reduce the elevated dNf1 larval brain phospho-ERK level, and neither does Ras2-Gal4 driven neuronal UAS-PKA* expression (Figure 8D). Providing further mechanistic clues, our results demonstrate that dNf1 and cAMP/PKA both affect systemic growth non-cell-autonomously, but not necessarily in the same cells. Thus, we previously showed that only relatively broadly expressed neuronal Gal4 drivers restored mutant growth when driving UAS-dNf1, whereas multiple drivers expressed in specific subsets of neurons, including several expressed in the ring gland, lacked the ability to restore dNf1 growth [5]. By contrast, using UAS-dncRNAi or a series of newly generated attenuated UAS-PKA* transgenes that avoid the toxicity associated with high level PKA expression [73], we now show that manipulating cAMP/PKA signaling with broadly expressed neuronal Gal drivers does not affect the dNf1 size phenotype, whereas the same transgenes induced with three ring gland drivers did suppress. Intriguingly, the most potent rescue was observed when UAS-dncJeb or attenuated UAS-PKA* transgenes were driven in AKH-producing cells at the base of the ring gland, whereas weaker rescue was also observed with two ring gland drivers that show overlapping expression in the juvenile hormone producing corpora allata. This suggests that the dNf1 growth deficiency involves a defect in processes controlled by one or both of these neuroendocrine hormones.

Figure 9. Several dNf1 pupal size defect suppressors also suppress a NMJ synaptiic overgrowth phenotype. (A–E) Representative micrographs of larval muscle 6/7 NMJs of the indicated genotypes. F: Mean bouton number per NMJ normalized to wild-type control. Compared to wild-type (w1118; A), dNf1 mutants (dNf1122; B) have an increased bouton number. While a cnk loss-of-function allele had no obvious NMJ phenotype, it dominantly suppressed the dNf1 NMJ defect (C). Similarly, the dNf1 NMJ phenotype was suppressed in Df(1)Exel9051 males that lack CCKLR-17D1 (D), while females heterozygous for CCKLR-17D1 (E) showed a lower level of suppression. Spitz (spi) is uncovered by a modifying deficiency but does not affect dNf1 size and was used as a negative control. In panels A–E, scale bars represent 5 μm. In panel F, error bars denote standard error of the mean.
doi:10.1371/journal.pgen.1003958.g009
As might be expected of a screen that used systemic growth as a read-out, our work identified a diverse set of potential modifiers. Notably, however, among a non-exhaustive set of 18 1st or 2nd chromosome genes implicated in various aspects of Drosophila body, organ, and/or cell size control (dAlk, B4, chico, kpo, Hr4, Hr6, jeb, Mer, mir-8, P3K21B, Ptm, Pth, SNF1A, sNPF, step, Tor, ash and yki; see Table S3 for details), only dAlk and jeb scored as dominant dNf1 pupal size modifiers, whereas the remaining 16 genes were uncovered by non-modifying deficiencies, or in the case of Pth, by two deficiencies that altered developmental timing (Table S2). Further explaining this lack of overlap, the previously implicated PI3 kinase regulator B4 act in a recessive manner and several of the above listed genes function outside of the CNS. Our screen excluded such genes, because dNf1 controls growth non-cell-autonomously by regulating neuronal Ras [5]. As previously noted, a special case is provided by insulin pathway components chico and Ptm, which affect growth antagonistically. Both genes map within 5 kb of each other on the 2nd chromosome and are uncovered by the same non-modifying deficiency.

Two newly identified dNf1 growth defect suppressors, Dap160 and CCKLR-17D1, affect synaptic architecture or functioning [36,56,57]. Because dNf1 was recently reported to function downstream of focal adhesion kinase to restrain NMJ synaptic growth and neurotransmission [16], and because the cholecystokinin receptor related CCKLR-17D1 drosulfakinin receptor stimulates NMJ growth [36], we analyzed whether this and three Ras signaling related dNf1 size defect suppressors also affected NMJ architecture. Our results confirm that dNf1 mutants exhibit synaptic overgrowth, and show that loss of CCKLR-17D1 suppresses this defect. Importantly, loss of jeb, dAlk, or cnk similarly suppresses both size and synaptic overgrowth defects, suggesting that both phenotypes may be related.

The results presented here further support our previous conclusion that excess neuronal Jeb/dAlk/Ras/MEK/ERK signaling is the root cause of the cAMP/PKA sensitive dNf1 systemic growth defect. What happens downstream of this primary defect remains less clear, although our demonstration that increasing cAMP/PKA signaling in AKH-producing cells and other parts of the neuroendocrine ring gland suppresses the size defect provides an important new clue, not only about pathways involved in the dNf1 growth defect, but also about the likely non-cell-autonomous cause of similar growth defects of PKA-C1 or dCob2 mutants [85,86]. Other questions that remain to be fully answered concern the role of the NMJ architectural defect in the dNf1 growth deficiency and the role of Jeb/dAlk signaling in the NMJ defect. We note in this respect that that C. elegans ALK ortholog, T10H9.2, has been implicated in synapse formation [87], and that recent work suggests a role for trans-synaptic Jeb/dAlk signaling in the control of neurotransmission and synaptic morphology [88]. However, while the dNf1 growth defect is due to excess dAlk signaling in neurons, NMJ synapse formation has been suggested to involve the release of presynaptic Jeb activating postsynaptic dAlk [88]. Further work will have to establish whether the suppression of the dNf1 NMJ overgrowth phenotype by jeb, dAlk and cnk involves cell autonomous roles for these genes at synapses, or non-cell-autonomous functions elsewhere in the CNS. Further work is also required to reveal the functional significance and the sites of action of other novel modifiers identified in our screen.

From a clinical perspective, perhaps the most relevant questions raised by our work are whether NF1 regulated ALK/RAS/ERK signaling is evolutionarily conserved and whether excessive ALK/RAS/ERK signaling contributes to human NF1 symptoms. Much indirect evidence hints at a positive answer to both questions. First, the expression of ALK and NF1 largely overlaps in the murine nervous system [77,78], same as it does in Drosophila [15]. Second, ALK functions as an oncogene and NF1 as a tumor suppressor in neuroblastoma [89–94]. Third, midkine, a ligand that activates mammalian ALK [95], is produced by NF1.
Schwann cells, present at elevated levels in NF1 patient skin and serum, and acts as a mitogen for NF1 tumor cell lines [96–98]. We add to this evidence by showing that shRNA-mediated NF1 knockdown renders two oncogenic ALK-driven human neuroblastoma cell lines resistant to pharmacological ALK inhibition, and by confirming that ALK mRNA is expressed in neurofibromatosis-derived NF1+/− human Schwann cells. These findings make a strong case that ALK should be explored as a therapeutic target in NF1, and that loss of NF1 expression should be considered as a potential mechanism in cases of acquired resistance to ALK inhibition [99].

Materials and Methods

Fly Stocks and Experiments

The dNf1E2 and dNf1E3 alleles have been described [5]. Exelixis, DrosDel and BSC deficiencies were obtained from the Bloomington Stock Center. Transgenic RNAi lines were obtained from the Vienna Drosophila Research Center (VDRC) and the TRIP Collection at Harvard Medical School. Eaat1SM1 and Eaat1SSM1 were provided by D. van Meyel, dALK8 and jhcol by R. Palmer, cnkE-206 by M. Thirrien, and carE by H. Kramer, ppo by M. Pankratz, hs-Ilp2 transgenic line by E. Ruhlison and US-RelB9 DN by R. Hiesinger. Flies were maintained on agar-oatmeal-molasses medium at 25°C, unless otherwise indicated.

To assess feeding, larvae at various stages of development were placed on blue food dye-stained yeast paste, removed after 20 min, washed and photographed. To analyze wandering behavior, 100 larvae (age 40–44 hr after egg deposition (AED)) were placed on an agar plate with a central blob of yeast paste, and their position after 24 hr was documented. To assess the expression of starvation-sensitive genes, larvae at 72 h AED were placed in vials with water for 16 hr, after which RNA was prepared and subjected to blot analysis. To determine developmental timing, L1 instar larvae were collected 24 hr AED using a 2 hr egg collection and reared at 140 animals per vial. The number of larvae that pupariated was scored at hourly intervals. To determine the larval weight, L1 larvae were collected 24 hr AED using a 2 hr egg collection. Larvae were reared at 140 larvae per vial and groups of 10 larvae were weighed at 8 hr intervals. Longevity was assessed by maintaining adult flies under standard conditions and counting pupariated was scored at hourly intervals. Longevity was assessed by maintaining adult flies under standard conditions and counting 10 larvae were weighed at 8 hr intervals. To induce starvation-sensitive genes, larvae at 72 h AED were placed in vials with water for 16 hr, after which RNA was prepared and subjected to blot analysis. To determine developmental timing, L1 instar larvae were collected 24 hr AED using a 2 hr egg collection and reared at 140 animals per vial. The number of larvae that pupariated was scored at hourly intervals. To determine the larval weight, L1 larvae were collected 24 hr AED using a 2 hr egg collection. Larvae were reared at 140 larvae per vial and groups of 10 larvae were weighed at 8 hr intervals. Longevity was assessed by maintaining adult flies under standard conditions and counting the number of dead flies at regular intervals. In each of these assays, genotypes were tested in duplicate. To induce hs-Ilp2 transgene expression, culture vials were placed in a circulating water bath at 37°C for 10 min once or twice a day with an 8 hr interval.

Insulin-Like Protein mRNA Quantification

The 7500 Fast Real-Time PCR System from Applied Biosystems was used to determine Ilp mRNA levels in RNA prepared from dissected larval brains or from whole wandering stage 3rd instar larvae. Results were normalized to RpL32. The following primers were used: Ilp2-Forward, GGCCAGCTCCAGCTGAAGT, Ilp2-Reverse, TCGCTGTGCGACCACCCGGGAT, Ilp3-Forward, CCAGGGCCACATGAATTTGT, Ilp3-Reverse, TTAGAAGTCTCCTGGGTCCCAA, Ilp5-Forward, TCCGCCAGGCGGCCAATCTC, Ilp5-Reverse, TAATTGCAATGCGGCCAAGGT, Ilp6-Forward, CGATGATTTTCTTACGGGACTTTC, Ilp6-Reverse, AAATGCCGTTACCTGTTCTCGGATTC, Ilp7-Forward, CAAAAGAAGCCGGCAGATG, Ilp7-Reverse, GCCATCAGGTCCCGGGTT. Expression of the distantly related Ilp8 and the midgut-expressed Ilp4 genes [21] was not analyzed.

Genetic Screening, Validation, and Statistical Analysis

The crossing schemes in Figure 2 were used to generate dNf1E2 mutants carrying 1st and 2nd chromosome deficiencies. To avoid crowding, cultures were maintained at 100–200 pupae per culture vial. Initial scoring used calipers set at the length of dNf1 female pupae, ignoring dNf1 heterozygotes recognizable by the presence of the TM6B balancer. Next, the length of individual pupae carrying candidate modifying deficiencies was measured by determining their head-to-tail length using a microscope fitted with NIS-Elements AR 3.0 imaging software. Measured pupae were then placed in 96-well plates (Falcon) to determine their gender and, if necessary, the genotype of eclosed flies. At least 40 pupae were measured for each genotype, and only measurements of female pupae were used to calculate mean values and standard deviations. Statistical significance was assessed with a two-tailed Student’s t-test. Throughout this report, single or double asterisks denote p-values<0.05 or <0.01 respectively.

To identify responsible modifiers we used specific alleles or UAS-RNAi knockdown. Alleles and UAS-RNAi lines on the 1st and 2nd chromosomes were crossed into the dNf1E2 background. UAS-RNAi lines on the 3rd chromosome were recombined with dNf1E2. UAS-RNAi lines in the dNf1E2 background were crossed to Gal4 drivers in the same background. The few deficiencies that gave rise to synthetic lethal interactions were backcrossed with dNf1E2 flies to produce Df/+; dNf1E2/dNf1E3 progeny.

To test whether genetic suppression reflected the inadvertent introduction of a wild-type dNf1 allele, we used fly DNA prepared using DNAzol (Molecular Research Inc.) in a PCR assay with AGTCAAGATTGATCTCG and GAGATCGTTGATAAGAAGT primers. The second primer introduces a penultimate single nucleotide change, which together with the E2 mutation results in the introduction of an Rsal restriction site. Rsal digestion of the PCR product gives rise to 370 and 61 bp fragments for the wild-type allele, and 348, 61 and 22 bp fragments for the dNf1E2 allele. Digests were run on 8% acrylamide gels using both wild-type (u111b) and dNf1E2 controls.

Construction of Akh-Gal4 and Attenuated UAS-PKA* Transgenes

The Akh promoter region was amplified with Akh-FORWARD (AGATCTAATTCCTCTGGAATGCGGCGAGGAGACTCTAG) and Akh-REVERSE (AGATCTAATTCCTCTGGAATGCGGCGAGGAGACTCTAG) primers. The resulting PCR fragment was subcloned into the BamHI site of a GA4 coding region containing pCaSpeR derivative. The final construct was sequenced to ensure correct orientation of the Akh promoter before being used generate transgenic flies by standard protocols.

To reduce the toxicity associated with high-level PKA expression, we generated modified pUAS-T vectors containing 1, 2, 3 or 4, rather than 5 Gal4-binding sites. The primers used to generate these vectors were: 1×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 2×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 3×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 4×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG. The resulting PCR fragment was subcloned into the BamHI site of a GA4 coding region containing pCaSpeR derivative. The final construct was sequenced to ensure correct orientation of the Akh promoter before being used generate transgenic flies by standard protocols.

To reduce the toxicity associated with high-level PKA expression, we generated modified pUAS-T vectors containing 1, 2, 3 or 4, rather than 5 Gal4-binding sites. The primers used to generate these vectors were: 1×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 2×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 3×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 4×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG. The resulting PCR fragment was subcloned into the BamHI site of a GA4 coding region containing pCaSpeR derivative. The final construct was sequenced to ensure correct orientation of the Akh promoter before being used generate transgenic flies by standard protocols.

To reduce the toxicity associated with high-level PKA expression, we generated modified pUAS-T vectors containing 1, 2, 3 or 4, rather than 5 Gal4-binding sites. The primers used to generate these vectors were: 1×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 2×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 3×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 4×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG. The resulting PCR fragment was subcloned into the BamHI site of a GA4 coding region containing pCaSpeR derivative. The final construct was sequenced to ensure correct orientation of the Akh promoter before being used generate transgenic flies by standard protocols.

To reduce the toxicity associated with high-level PKA expression, we generated modified pUAS-T vectors containing 1, 2, 3 or 4, rather than 5 Gal4-binding sites. The primers used to generate these vectors were: 1×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 2×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 3×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG; 4×UAS-FOR: AACTGCGAGGCGAGTACTGTGCTCTCGGGAAGGGAATCTTAG. The resulting PCR fragment was subcloned into the BamHI site of a GA4 coding region containing pCaSpeR derivative. The final construct was sequenced to ensure correct orientation of the Akh promoter before being used generate transgenic flies by standard protocols.
reaction with pCR2.1-UAS(2x) as template generated pCR2.1-UAS(3x) and pCR2.1-UAS(4x). The pCR2.1-UAS clones were sequenced, their inserts excised with PstI and subcloned into PstI-digested p-UAST. Correct insert orientation was verified by sequence analysis, after which the mutationally activated murine PKA* coding region [100] was subcloned into the modified vectors using XbaI and NotI.

Immunofluorescence and Analysis of NMJ Morphology

Wandering third instar larvae were dissected in Ca²⁺/Mg²⁺-free saline and fixed in 4% paraformaldehyde for 25 min at room temperature. Following fixation, larval pellets were washed three times in phosphate-buffered saline (PBS) and then blocked for one hour in PBT (PBS+0.1% Triton-X 100)+5% normal goat serum. Larvae were incubated in primary antibody solution for three hours at room temperature. Anti-HRP 568 (1:1000, Invitrogen) was used to visualize neurons and Alexa Fluor 488 phalloidin (1:500, Invitrogen) was used to visualize F-actin in the musculature. Images were collected using a Yokogawa CSU-X1 spinning-disk confocal microscope with the Spectral Applied Research (Richmond Hill, ON, Canada) Borealis modification on a Nikon Plan Apo 63x (1.4 NA) objective. The microscope was equipped with a Prior (Rockland, MA) Proscan II motorized stage. Larval samples were excited with 488-nm (for phalloidin) and 561-nm (for HRP) 100-mW solid-state lasers from a Spectral Applied Research LMM-5 laser merge module and was selected and controlled with an acousto-optical tunable filter. Emission was collected with a Hamamatsu ORCA-ER-cooled CCD camera. Hardware was controlled with MetaMorph (version 7.7.9) software (Molecular Devices, Sunnyvale, CA). Five individual animals were imaged for subsequent morphological analysis. Motor nerve terminals of muscles 6 and 7 were imaged in abdominal segments A2 and A3 and Z-stacks (0.25 μm between images) and were captured from the top to bottom of each NMJ. Morphological analysis of the NMJ was performed using NIH Image J and was assessed by quantifying the number of synaptic boutons per square micron. The number of synaptic boutons was counted as previously described [16,101] and muscle area covered by the NMJ was quantified by tracing a polygon connecting each terminal branch point [102].

Human NF1 Experiments

The retroviral RNAi vectors targeting human NF1 and expression constructs of active alleles of RAS effectors were as described previously [94]. Crizotinib (S1068) and NVP-TAE684 (S1108) were purchased from Selleck Chemicals. Antibody against NF1 was from Bethyl Laboratories (A300-140A); antibodies against p68K(s173) and AKT1/2 were from Cell Signalling; antibodies against p-ERK (E-4), ERK1 (G-16), ERK2 (G-14) and CDK4 (C-22) were from Santa Cruz Biotechnology; A mixture of ERK1 and ERK2 antibodies was used for detection of total ERK from human cell lines. Antibody against mouse PKA±cat (A-2) SC-20315 was from Santa Cruz Biotechnology, β-Tubulin E7 from Developmental Studies Hybridoma Bank.

SH-SY5Y, Kelly and Phoenix cells were cultured in DMEM with 8% heat-inactivated fetal bovine serum, penicillin and streptomycin at 5% CO₂. Subclones of each cell line expressing the murine ectopic receptor were generated and used for all experiments shown. Phoenix cells were used to produce retroviral supernatants as described at http://www.stanford.edu/group/nolan/retroviral_systems/phx.html.

To measure cell proliferation, single cell suspensions were seeded into 6-well plates (1–2 x 10⁴ cells/well) and cultured both in the absence and presence of ALK inhibitors. At the indicated endpoints, cells were fixed, stained with crystal violet and photographed. All knockdown and overexpression experiments were done by retroviral infection as described previously [103].

The 7500 Fast Real-Time PCR System from Applied Biosystems was used to determine mRNA levels. NF1 mRNA expression levels were normalized to expression of GAPDH. The following primers sequences were used in the SYBR Green master mix (Roche): GAPDH-Forward, AAGTTCAGGTCGATCTCA; GAPDH-Reverse, AATGAGGGGTGTCATGGTG; NF1-Forward, TGTCAGTGCAATAGCTTGC; NF1-Reverse, AGT-GCCATCACTCTTTTCCTGAAG; ALK mRNA levels in neurofilament-derived NF1−/− Schwann cells and NF1+/− fibroblasts were analyzed by the following two primer sets: ALK-N-Forward, GGAGTGCAGCTTTGACTTCC; ALK-N-Reverse, TGGAGTCAGCAGGTAGGTGTTG; ALK-C-Forward, GCACATCGCGTGAAGACA; ALK-C-Reverse, GCCCTTGTGAGAACAGCCAGGAG.

Supporting Information

**Figure S1** Loss of dNF1 does not alter developmental timing but reduces larval growth rate. (A) Wild-type, dNF1+/+, and dNF1/E1E2 mutants show no altered developmental timing, as judged by their rate of pupariation (also shown in Figure 1D). By contrast, larvae with phm-Gal4 driving UAS-Ras1V12 undergo accelerated development resulting in miniature pupae [104], whereas phm-Gal4 driving a dominant negative UAS-PI3KD954A transgene delayed development and produced giant pupae [71]. (B) Mouth hook length measurements (in μm) show that dNF1 larvae grow at a reduced rate. The marker represents the mean length; the upper box represents the median to Q3 value, the lower box median to Q1 value and the error bars identify the outliers. (PDF)

**Figure S2** PCR/RFLP assay for dNF1E2 mutation. (A) To make sure that stocks with putative suppressing deficiencies preserved the dNF1E2 C>T nonsense transition, we used a PCR/Restriction Fragment Length Polymorphism assay. The E2 mutation does not create or destroy a restriction site. Rather, we used a reverse primer with a penultimate A- C to transversion to amplify a 433 genomic fragment as indicated. The mutant primer creates a GTAC Ras1 restriction site when E2 genomic DNA is used as a template. (B) Ras1 digestion of PCR products gives rise to 370 and 61 bp fragments for the wild-type allele, and 348, 61 and 22 bp fragments for dNF1E2. An example of the assay is shown with both wild-type (lanes 1-11) and dNF1E2 controls (lanes 2, 3 and 4) and various deficiencies (Df) either in wild-type (Df/Y;O; +; lanes 5 and 15), dNF1 homozygous (Df/Y;O; dNF1E2; lanes 6–13) or heterozygous (Df/Y;O; dNF1E2/+; lanes 14 and 16) backgrounds. (PDF)

**Figure S3** Systematic identification for dNF1 modifiers. For deficiencies that did not uncover obvious candidate modifier genes, a systematic RNAi approach was used. UAS-RNAi lines targeting genes uncovered by a modifying deficiency were driven by Ras2-Gal4 in the dNF1E2 background and the effect on pupal size determined. (A) Identification of carnation as a dNF1 modifier uncovered by suppressing Df(1)BSC275. (B) Identification of Nai1 as the responsible gene uncovered by suppressing deficiencies Df(1)BSC533 and Df(1)Eve6290. RNAi-induced
lethality is denoted by †. Error bars show standard deviations and * indicates a p-value of <0.05. As part of the systematic identification of modifiers 385 RNAi lines were tested.

**Figure S4** The Ret tyrosine kinase is not involved in dNf1 growth control. (A) Reagents generated to analyze the involvement of Ret include Ret-Gal4 transgenic lines made by inserting a 957-bp genomic segment representing the Ret promoter region into the pChs-Gal4 vector. Other reagents include UAS-Ret transgenes harboring kinase-dead (K905A) and constitutively active (C695R) mutations made by site-directed mutagenesis. (B) Ret-Gal4 driven UAS-GFP expression recapitulates the endogenous larval brain Ret expression pattern [60]. (C) GMR-Gal4 driven UAS-Ret with a constitutively active C695R mutation produces a rough eye phenotype as previously reported [60]. (D) Ret-Gal4 driven UAS-dNf1 re-expression, RNAi-mediated Ret inhibition or expression of a UAS-Ret kinase dead transgene, all failed to modify dNf1 pupal size. Moreover, Ret-Gal4 driven expression of UAS-Ret with constitutively active C695R mutation failed to phenocopy the dNf1 size defect. By contrast, a small pupal size phenotype was observed when Ret C695R was driven ectopically with Ras2- and elav-Gal4, likely reflecting Ret-mediated activation of Ras/ERK signaling.

**Figure S5** Expression pattern of ring gland drivers. Ring gland drivers P0206-Gal4, Foh36-Gal4, Aug21-Gal4 and Alk-Gal4 were crossed to UAS-GFP. The CNS and ring glands were dissected from third instar larvae, stained with DAPI and imaged using confocal microscopy. The prothoracic gland (PG), corpora allatum (CA) and corpora cardiaca (CC) are indicated. Specimens are orientated such that the base of the brain hemispheres is at the top, indicated by a dotted line. Scale bar = 50 μm.

**Figure S6** ALK mRNA expression in neurofibroma-derived Schwann cells. Reverse transcription/PCR was used to analyze ALK expression in neurofibroma-derived NF1-/- Schwann cells and NF1+/- fibroblasts. Two primer sets, (A) ALK-N and (B) ALK-C, designed to amplify N-terminal and C-terminal ALK mRNA segments, detected ALK expression in NF1-/- Schwann cells, but not in NF1+/- fibroblasts. GAPDH primers were used as a control. To guard against positive signals due to contaminating genomic DNA, each PCR reaction was set up either with (+RT) or without (−RT) reverse transcriptase.

**Figure S7** *Nf1* suppression confers resistance to ALK inhibitors in human neuroblastoma cells. (A) Kelly cells expressing pRS and shGFP controls or shNf1 vectors were grown in the absence or presence 200 nM NVP-TAE684 or 500 nM crizotinib. Cells were fixed, stained and photographed after 14 (untreated) or 17 (NVP-TAE684 or crizotinib-treated) days. (B) Level of Nf1 knockdown assayed by qRT-PCR. Error bars denote standard deviation.

**Figure S8** Activation of RAS-RAF-MEK cascade confers resistance to ALK inhibitors in neuroblastoma cells. (A) Constitutively active KRAS, BRAF, MEK1, or MEK7 mutants confer resistance to ALK inhibitors. SH-SY5Y neuroblastoma cells expressing pBabe vector control or the indicated active RAS effector mutants were grown in the absence or presence 30 nM NVP-TAE684 or 350 nM crizotinib. The cells were fixed, stained and photographed after 12 (untreated) or 19 (NVP-TAE684 and crizotinib-treated) days. (B) Level of phosphorylated ERK and AKT in the SH-SY5Y cells described above.

**Table S1** Excluded deficiencies. Listed deficiencies were excluded for the reasons indicated. Deficiencies that failed to produce screening stocks are labeled ‘Impossible’. Unhealthy (sick) deficiencies or those that uncovered Minute mutations were also excluded.

**Table S2** dNf1 modifier deficiency screen results. All deficiencies analyzed are listed according to their relative chromosomal position. The cytological location, molecular coordinates and the dominant effect on dNf1 pupal size (NO – no interaction, SUP - suppressor, ENH - enhancer) of each deficiency is given. Female pupal length measurements for deficiencies in the dNf1 mutant background are provided, together with standard deviations and p-values. Modifying deficiencies that were subsequently found to have an effect on wild-type pupal size are indicated (Yes – indicates that a deficiency has a non-specific effect; No – no observed effect on wild-type size; No* - has an effect on wild-type size, but in the opposite direction from the effect on dNf1 mutants). Where determined, the responsible gene identified under each modifying deficiency is shown. The final column contains notes such as deficiencies that result in altered developmental timing.

**Table S3** Growth related genes uncovered by screened deficiencies. 18 cell, tissue, or systemic growth implicated genes uncovered by analyzed 1st and 2nd chromosome deficiencies. Among the deficiencies listed, only those that uncovered dAlk or jeb modified dNf1 pupal size.

**Table S4** Larval tissue expression patterns of Gal4 drivers. List of Gal4 driver lines used in this study and their expression patterns in third instar larvae as determined by crossing Gal4 drivers to UAS-GFP, or from published data. Abbreviations: Ring gland (RG), central nervous system (CNS), mushroom body (MB), prothoracic gland (PG), corpora allata (CA), corpora cardiaca (CC), neurosecretory neurons (NSNs), pars intercerebralis neurons (PI), corpora cardiaca innervating neurosecretory neuron of the medial subesophageal ganglion 2 (CC-MS 2), proventriculus (PV), fat body (FB), salivary glands (SG), imaginal discs (IDs), first instar (L1).

**Table S5** Identification of tissues that require dNf1 or cAMP/PKA signaling for growth regulation. Various Gal4 drivers in the dNf1 background were crossed to dNf1 mutants bearing attenuated UAS-Pki* transgenes or dnc RNAi lines. Rescue was assessed by measuring pupae, followed by genotyping adult flies upon eclosion. All crosses produced viable adults unless otherwise stated. † denotes lethality; NR non-escape; NR* denotes non-escape with adult eclosers with unfurled wings; n/a not applicable; n/d not determined.

**Acknowledgments**

We thank the Bloomington Drosophila Stock and Vienna Drosophila Resource Centers for deficiency and transgenic RNAi fly stocks. We are also grateful to R. Hiessinger, H. Kramer, D. van Meyel, R. Palmer and M. Thirrien for additional fly stocks, to Spyros Artavanis-Tsakonas and Doug Dardich for Exelixis deficiency stocks, and to Eline Beert and Eric Legius for NF1 tumor cell RNAs. We are grateful to the Nikon Imaging Center at Harvard Medical School for technical support for microscopy performed in this study. Transgenic flies were generated by Genetic Services Inc., Cambridge MA. We thank Iswar Harilhan, Susanne Bell and Glenn Cowley for their efforts during the early stages of this project, and our colleagues at the MGH Center for Cancer Research for valuable discussions.
Author Contributions
Conceived and designed the experiments: JAW JYG SH JBL DVV RB AB. Performed the experiments: JAW JYG SH RCM HH KK AR.

References

Analyzed the data: JAW JYG SH JBL DVV RB AB. Contributed reagents/materials/analysis tools: JAW JYG SH JBL AB. Wrote the paper: JAW JYG SH AB DVV.
76. Rohrbough J, Broadie K (2010) Anterograde Jelly belly ligand to Alk receptor


55. Roos J, Kelly RB (1998) Dap160, a neural-specific Eps15 homology and


53. Therrien M, Wong AM, Rubin GM (1998) CNK, a RAF-binding multidomain
