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

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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 *Drosophila melanogaster* Nf1 Neuromuscular junction (NMJ) overgrowth phenotype and that manipulations 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.


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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 *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 mutant [18], dNF1 larvae ingested similar amounts of dye-stained food throughout their development (Figure 1A). Unlike a panpless (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 Lih3 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 edcsyne controlled growth duration determines overall growth (see [19] and [20] for reviews). Arguing against an important role for edcsyne or other factors that control the length of the larval growth period, no differences in the expression of canonical edcsyne-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 approx-
Figure 1. Loss of dNf1 does not phenocopy starvation or alter developmental timing. (A) Wild-type (w1118) 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.

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imately 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 rescued by inducing the expression of a hsp70-ilp2 transgene [22,23]. However, several results argue against a role for ILPs in the dNf1 growth defect. Firstly, two hypomorphic insulin receptor alleles, InR<sub>05545</sub> and InR<sub>227</sub>, 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 (choio) 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 shock of hsp70-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 1<sup>st</sup> and 2<sup>nd</sup> chromosome deficiencies for dNf1 <sup>252</sup> pupal size modifier effects or synthetic lethal interactions. For each of 139 1<sup>st</sup> and 347 2<sup>nd</sup> chromosome deficiencies from the Exelixis [28], DrosDel [29] or Bloomington Stock Center (BSC) collections, we generated Df(1)/+; Nf1<sup>252</sup>/Nf1<sup>252</sup> (Figure 2A) or Df(2)/+; Nf1<sup>252</sup>/Nf1<sup>252</sup> (Figure 2B) stocks, respectively. Notably, our work identified only few synthetic lethal interactions, and in all cases tested the synthetic lethality has been specific to the chromosome carrying the Nf1<sup>252</sup> allele, and not observed when the same deficiency was tested in Nf1<sup>252</sup>/Nf1<sup>252</sup> null trans-heterozygotes [5]. To guard against size differences caused by inadvertent differences in population density or environmental conditions, each deficiency was scored at least twice using an initial rough caliper measurement of pupae attached to the side of culture vials. For each candidate modifying deficiency thus identified, microscopy combined with image analysis was used to determine the precise head-to-tail length of at least 40 pupae, which were then allowed to individually eclose in order to establish their sex. Several controls were next performed to eliminate non-specific modifiers or artificial results. First, for all suppressors the continued presence of the Nf1<sup>252</sup> nonsense mutation was confirmed by a PCR assay (Figure S2). Secondly, as a critical specificity control, all modifying deficiencies were analyzed in a wild-type background to eliminate those that affect pupal size irrespective of dNf1 genotype. Further analysis of some of these non-specific modifiers demonstrated that loss of Act57B dominantly increases pupal size, whereas heterozygous loss of the glutamate transporter Eaat1 has the opposite effect. Thirdly, because pupal size is a function of larval growth rate and duration, modifying deficiencies were monitored for obvious changes in developmental timing. Table 1 shows the number of screened chromosomes 1, 2L and 2R deficiencies, the fraction of genes uncovered and the number of dNf1 and wild-type pupal size modifying deficiencies and loci identified. Figure 2C shows the magnitude of the pupal size modification of typical enhancers and suppressors. The number of modifying deficiencies exceeds the number of identified loci, because many modifying deficiencies uncover overlapping genomic segments (Figure 3). Not unexpectedly, individual modifying deficiencies increase or decrease dNf1 pupal size to different extents (Figure 4).

Some large non-modifying deficiencies identified in our screen completely overlapped with smaller modifying ones. In such cases, stocks were re-ordered and reanalyzed. If these tests replicated the original results, genetic complementation analysis or PCR amplification using transposon and flanking sequence-specific primers was used to confirm the mapping of the deficiencies in question. This procedure identified several mismapped or mislabeled deficiencies, most of which have since been withdrawn by stock centers. Any suspect or recessive modifying deficiency, or any deficiency that uncovers genes with non-specific size phenotypes, such as Minute loci [30,31], were eliminated from further analysis. Table S1 lists these deficiencies and the reason for their exclusion.

During work to identify genes responsible for observed effects, we prioritized genes uncovered by suppressing deficiencies over those uncovered by enhancers. We also prioritized modifying loci uncovered by more than one deficiency, strong modifiers over weak ones, and genes uncovered by smaller deficiencies over those uncovered by larger ones, reasoning that effects of smaller deficiencies are more likely due to the loss of single genes. Validating the screen, suppressing Df(2R)Exel7144 uncover dAk and partially overlapping suppressing Df(2R)BSC199 and 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...
growth control [33,34], 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.

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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].

Finally, our screen also identified multiple genes whose roles in dNf1 growth control had not been anticipated and whose functional relevance remains to be established. Several genes in this group are predominantly expressed in brain or have known neuronal functions, including genes coding for the aforementioned CCKLR-17D1 receptor, the synaptic scaffold protein Dap160, the neuronal RNA binding protein elav, the neuronal Na,K ATPase interacting protein NKAIN [37], and the larval brain and alimentary channel expressed amino acid transporter NAA1T [38]. Other genes in this group include CKIIbeta[2], encoding a casein kinase regulatory subunit, the endosomal trafficking proteins deep-orange and carnation, the Notch modifier heparan sulfate 3-O sulfotransferase H3st-B [39], and the ubiquitin E3 ligases HERC2, which acts as a suppressor, and CUL3, which has the opposite effect. Table 2 lists deficiencies that modify dNf1 but not wild-type pupal size, limited to those for which the responsible gene has been identified. Table S2 identifies all analyzed deficiencies, indicates which modified dNf1 pupal size (providing female pupal sizes as a gauge of modification strength), which also altered wild-type pupal size, and which deficiencies altered developmental timing.

**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 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[22] pupal size (Figure 5A), whereas the Nvr2-Gal4, Ecot1-Gal4 and Glh-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 UAS 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 [42] loss-of-function allele dominantly increased dNf1 pupal size (Figure 5B).

Previously, heterozygous mutations affecting RAF/MEK/ERK kinase cascade components Draf (pole hole; ph), Door1/dMEK, or ERK/rolled (rl), did not modify dNf1 size [5]. In agreement, two phl-uncoupling deficiencies, Df(1)ED6574 and Df(1)ED11554, did not score as modifiers (Table S2). No rl uncoupling deficiencies were analyzed, but Df(1)Ex9049, which is among the stronger suppressors identified, deletes Door1 and only two other genes, the neurogenic gene almondex (annx), and CG17754, predicting a BTB and Kelch domain protein. Arguing that reduced Ras/ERK signaling upon loss of Door1 combined with abnormal neuronal differentiation due to loss of annx may synergistically cause the observed strong effect, Ras2-Gal4 driven UAS-RNAi transgenes targeting either gene, while causing pupal lethality at 25°C, increased dNf1 pupal size at lower temperatures (Figure 5C). Moreover, suppression of the dNf1 pupal size defect was also observed upon individual heterozygous loss of either Door1 or annx, 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[22] pupal size defect in crosses with the Door1[44–52] allele [5]. A potential explanation may be that Door1[44–52] is a null mutant [43], whereas the molecular nature of Door1[44–52] is undetermined. Genetic background differences between these Door1 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)BSC154 uncovers 27 genes including connector enhancer of KSR (cnk), a scaffold protein that functions as a bimodal (both positive and negative) regulator of RAS/ERK signaling [53,54]. Supporting a role for cnk as a dNf1 modifier, the cnk[44–52] alleles acted as dominant suppressors (Figure 5B), and suppression was also observed upon RNAi-mediated Cnk knockdown using Ras2-Gal4 or P(Gna6B)C23-Gal4 neuronal drivers (Figure 5C). However, Df(2R)BSC154, which uncovers 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

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**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>
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<td></td>
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<td>ENH</td>
<td>SUP</td>
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<td>1</td>
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<tr>
<td>2R</td>
<td>165</td>
<td>86.9</td>
<td>31</td>
<td>2</td>
<td>4</td>
</tr>
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</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.

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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 dNf1 pupal size defect, similar suppression of CCKLR-17D3 had no effect (Figure 6A).

Beyond CCKLR-17D1, several dNf1 size modifiers are expressed in brain and/or have neuronal functions. Among these, dynamin-
### 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>
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<td></td>
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<td>Df(2R)Exel7144</td>
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<td>SUP</td>
<td>dAlk</td>
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<td>Dsor1 and almondex (amx)</td>
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<tr>
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<td></td>
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<td>dnce (dnc)</td>
<td>dnce^14 (amorph), dnce^X (amorph), dnc^X (hypo)</td>
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<td>ENH</td>
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<td>PKA-C1<em>93051 (leth), PKA-C1</em>93053 (leth)</td>
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Modifying deficiencies for which the responsible dnf1 interacting gene has been identified. The cytological location, and the dominant effect on 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, nry-Gal4 and/or C23-Gal4 drivers. Abbreviations: hypo: hypomorphic; leth: lethal; lop: loss-of-function; amorph: amorphic; Δ deletion; via: viable. 
doi:10.1371/journal.pgen.1003958.002

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 an especially attractive candidate suppressor, because activating RET and inactivating NF1 mutations can both lead to human phaeochromocytoma [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
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 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 dNf1 growth control, Ret is uncovered by non-modifying Df(2R)BSC312. By contrast, Dap160 loss-of-function alleles (Dap160<sup>d1</sup> and Dap160<sup>d2</sup>; [56]), or Dap160 RNAi expression driven by three neuronal Gal4 drivers, suppressed the dNf1 pupal size defect, identifying it as the responsible modifier (Figure 6B).

The gene for the neuronal RNA binding protein elav is uncovered by non-modifying Df(1)Exel6290 and Df(1)BSC553 whose region of overlap includes only four other genes. Identifying elav as the responsible modifier, three neuronal Gal4 lines driving the expression of three dNf1 targeting RNAi transgenes suppressed the dNf1 size defect, whereas 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 Df(1)Exel6254, which also uncovers the syx16, coding for syntaxin 16. No HERC2 alleles exist, but Ret-Gal4-driven expression of a UAS-HERC2<sup>RNAi</sup> transgene v105374 strongly suppressed the dNf1 pupal size defect (Figure 7A), whereas similar knockdown of Syx16 had no statistically significant effect (not shown). The gene for another E3 ligase component, Cul-3, is

Figure 5. Validation of dNf1 modifiers involved in Jeb/dAlk/Ras/ERK and cAMP signaling. (A) Neuronal expression of dAlk RNAi using Ras2-Gal4, Ras2-Gal4+UAS-Drk, C23-Gal4 or nub-Gal4 drivers suppresses the dNf1 size defect. Expression of Jeb RNAi with the same neuronal drivers also suppresses. Weaker suppression is observed when Jeb RNAi expression is controlled by the pan-glial repo-Gal4 driver. Dark grey bars are control measurements of Gal4 drivers in the dNf1 background. Light grey bars are sizes of wild-type (w<sup>1118</sup>) and dNf1<sup>E2</sup> controls. (B) Suppression of the dNf1 size defect by the indicated Jeb, c23-Gal4 and amx alleles. (C) Neuronal cnk, Dsor1 or amx knockdown suppressed the dNf1 size defect. In the case of Dsor1 v107276 and amx, cultures were maintained at 18°C to prevent lethality observed at 25°C. Some RNAi transgene/driver combinations were lethal (†) even at 18°C. (D) Validation of dnc and Pka-C1 as dNf1 modifiers was obtained in crosses with dnc<sup>U64</sup>, dnc<sup>U65</sup>, Pka-C1<sup>6353</sup> and Pka-C1<sup>6302142</sup> loss-of-function alleles. In this and subsequent figures, * and ** denote p-values<0.05 and <0.01, respectively.

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Figure 6. Validation of dNf1 modifiers with neuronal functions. (A) Ras2-Gal4 or C23-Gal4 driven neuronal RNAi knockdown of CCKLR-17D1 but not CCKLR-17D3 suppressed the dNf1 pupal size defect. (B) Identification of dynamin-associated protein 160 (Dap160) as a suppressor of dNf1 growth. Neuronal RNAi targeting of Dap160 increased dNf1 pupal size as did two Dap160 loss-of-function alleles. (C) Two elav alleles dominantly suppress the dNf1 size defect. (D) Neuronal expression of a Rab9 RNAi transgene or of a dominant negative Rab9 mutant suppresses the dNf1 size defect.

doi:10.1371/journal.pgen.1003958.g006
uncovered by three enhancing deficiencies, and a \textit{Cal-3} loss-of-function allele or \textit{Ras2-Gal4} driven expression of a \textit{Cal-3} RNAi transgene both enhanced the \textit{dNf1} size defect, identifying it as the responsible gene (Table 2).

Suppressing \textit{Df(1)Exel9068} uncovers only four genes, including one encoding the TORC2 complex subunit Rictor. However, systematic \textit{Ras2-Gal4} driven RNAi knockdown of \textit{Df(1)Exel9068} uncovered genes identified \textit{Hs3st-B}, encoding one of two Drosophila heparan sulfate 3-O sulfotransferases, as a potent \textit{dNf1} size defect suppressor (Figure 7A), whereas knockdown of Rictor had no effect (not shown). Others previously identified \textit{Hs3st-B} as a positive regulator of Notch signaling [39]. However, the heparan sulfate proteoglycan substrates of \textit{Hs3st-B} bind various growth factors and other ligands and have been implicated in a variety of biological processes. Exactly why loss of \textit{Hs3st-B} suppresses the \textit{dNf1} growth defect remains to be determined.

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

\textit{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 \textit{Pi3K92E}/\textit{Dp110} by binding to its \textit{Pi3K21B/dP60} regulatory subunit. Homozygous \textit{B4} mutants have an increased body size [65], which may explain why \textit{Ras2-Gal4}-driven RNAi-mediated suppression of \textit{B4}, uncovered by suppressing deficiency \textit{Df(2L)BSC147}, increased \textit{dNf1} pupal size (not shown). However, whether \textit{B4} is the responsible dominant modifier is doubtful, given that it is also uncovered by \textit{Df(2L)BSC692}, a non-modifying deficiency. Moreover, we previously found that heterozygous loss of \textit{Pi3K21B}, 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 2ip6, 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)BSC206, uncovers both the chico and pten genes, whose products antagonistically control insulin-stimulated P3K92E/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 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 prothoracotrophic 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 Ras-2-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 dncRNAi 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-dnRK6 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 because a UAS-PKA* expression using the five UAS element containing standard UAS-T vector is lethal in combination with most Gal4 drivers [73]. As reported previously [74], driving UAS-dNf1 ubiquitously with Act5C-Gal4, or broadly in neurons with elavin-Gal4, Ras-2-Gal4, c23-Gal4, or 396Y-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-dnRK6 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×UAS-PKA* and 3×UAS-PKA* transgenes was generally well tolerated, whereas the 4×UAS-PKA* and the 5×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 Alk-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 predominantly 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).

dAlk, Jeb, Cnk and CCKLR-17D1 Suppress 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 dAlk, Jeb and CCKLR-17D1 [36,76]. Importantly, the dNf1 synaptic overgrowth phenotype is dominantly suppressed by CCKLR-17D1, dAlk, Jeb, and cnk alleles (Figure 9B), arguing that all four genes are epistatic to dNf1. As a control we analyzed an allele of spitz (spz), which encodes an EGF-like growth factor and is uncovered by suppressing Dpp[2L]/Exel8041. However, spz shows no genetic interaction with dNf1, as loss of spz 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 dAlk 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 dAlk 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 consist of Schwann cells, perineural fibroblasts, infiltrating mast cells, and nerve elements, with the Schwann cells sustaining the second NF1 hit [79]. To test whether increased ALK signaling in the absence of NF1 might play a role in the development of 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. (C) GMR>mPKA* larvae expressing 1x to 5x UAS-PKA* constructs. Western blot showing robust mPKA* expression in lane 1, with reduced levels in lanes 2-5, corresponding to increasing UAS element numbers. (D) Reduced pupal length of *UAS-dNf1*; *dNf1* (black bars) as compared to *3xUAS-PKA* (blue bars) and *v107967 dnc* (red bars) mutants. Error bars represent standard error of the mean (SEM), and significant differences are indicated by asterisks. (E-H) Representative images of *NF1*, *Akh*, *DAPI* stained tissues.
presence 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 neuroblasto
toma cells, both of which harbor constitutively active \( F1174L \) alleles, and both of which are highly sensitive to pharmacological \( ALK \) inhibition [80]. Compatible with a role for \( NF1 \) as a negative regulator of mitogenic \( ALK/RAS \) signals, qRT-PCR verified \( NF1 \) knockdown with two shRNA retroviral vectors increased the resistance of both lines to \( ALK \) inhibitors NVP-TAE684 and Crizotinib (Figures 10A, 10C and S7). Compatible with a model in which \( NF1 \) negatively regulates ALK/RAS signaling, \( NF1 \) knockdown resulted in elevated ERK and AKT activation (Figures 10B). Moreover, expression of activated \( KRAS \), \( BRAF \), or \( MEK \) transgenes, but not of other Ras effector transgenes, in SH-SY5Y cells conferred similar resistance to \( ALK \) inhibition (Figure S8).

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, a genetic analysis in Drosophila might not only reveal molecular pathways controlled by the highly conserved (50%) identical d\( NF1 \) 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 d\( NF1 \) 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 d\( NF1 \) affects cAMP/PKA signaling, and help to resolve the long-standing controversy as to whether d\( NF1 \) 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].

While recognizing that none of the thus far identified d\( NF1 \) phenotypes are ideally suited for use in modifier screens, we selected the pupal size defect as the phenotype to analyze in our screen for three main reasons. First, pupariation occurs at the end of the larval growth period, and pupal size is readily assessed by inspecting pupae attached to the side of culture vials, making this phenotype amenable to a large-scale screen. Second, the growth defect is among several cAMP/PKA sensitive d\( NF1 \) phenotypes. Finally, reduced growth is also a symptom of human \( NF1 \) and other RASopathies [1,82]. However, while compelling reasons support the selection of this phenotype, confounding factors include that Drosophila size is a sexually dimorphic phenotype affected by population density, feeding, environmental conditions such as temperature, and genetic background differences. Moreover, while heterozygous d\( NF1 \) mutants are marginally smaller than wild-type pupae [5], the more robust size phenotype (~15% reduction in linear dimensions, ~25% reduction in weight) used in our screen is only observed upon homozygous loss of d\( NF1 \). Thus, our screen was not designed to find modifiers that act on the d\( NF1 \) protein itself, like the recently identified SPRED proteins [83]. Finally, organism size is a function of growth rate and duration, both of which are regulated by hormonal cascades that involve cross-talk between the larval brain, the neuroendocrine ring gland, the fat body and other tissues [19,84]. Thus, a screen for modifiers of d\( NF1 \)-regulated growth may uncover genes involved in various aspects of systemic growth control.

Early attempts to identify d\( NF1 \) pupal size modifiers were abandoned when >95% of large X-ray induced 2\( nd \) chromosome lethality of these transgenes when driven with either Ac\( SC \)-Gal4 or elav-Gal4 is indicated by † whereas (−) indicates viable offspring. (C) Western blot of adult head lysates showing relative expression of G\( MF \)-Gal4-driven transgenic PKA*. Tubulin is used as a loading control. (D) Expression of PKA* or knockdown of dnc by shRNA in the ring gland rescues the d\( NF1 \) pupal size defect. In contrast, UAS-d\( NF1 \) expression with the same ring gland drivers fails to restore systemic growth. (E–H) Expression pattern of Akh-Gal4 driving UAS-GFP, co-stained with DAPI and anti-d\( NF1 \). GFP expression in the corpora cardiaca (CC) is indicated. Scale bar = 50 μm. As previously noted [74], anti-d\( NF1 \) staining is strong in the CNS, whereas staining in the ring gland is close to background.

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doi:10.1371/journal.pgen.1003958.t003

Table 3. Restoration of systemic growth by d\( NF1 \) and cAMP/PKA involves different tissues.

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<td>C23</td>
<td>Rescue</td>
<td>NR</td>
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<td>(pupal †)</td>
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<td>Feb36</td>
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<td>Aug21</td>
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*Ac\( SC \)-Gal4 driven ubiquitous d\( NF1 \) re-expression, or elav-Gal4 and Ras\( 2 \)-Gal4 driven neuronal re-expression rescues the d\( NF1 \) pupal size defect, whereas d\( nc \) RNAi or UAS-PKA* expression controlled by the same drivers is ineffective. By contrast, expressing d\( NF1 \) 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 d\( nc \) RNAi or attenuated UAS-PKA* transgenes does increase d\( NF1 \) 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 d\( NF1 \) and cAMP/PKA affect systemic growth. Full results are shown in Table S5.

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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 deletions, 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 Jbe/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 Jbe under these conditions [41]. However, Jbe 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-dncRNAi 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 10. NF1 suppression leads to ERK activation and confers resistance to ALK inhibitors in human neuroblastoma cells.** (A) NF1 knockdown confers resistance to ALK inhibitors in human neuroblastoma cells. SH-SYSY cells expressing pRS and shGFP control vectors, or shNF1 vectors were grown in the absence or presence of 50 nM NVP-TAE684 or 250 nM crizotinib. The cells were fixed, stained and photographed after 14 (untreated and crizotinib treated), or 21 (NVP-TAE684 treated) days. (B) Down-regulation of NF1 results in elevated level of phosphorylated p-ERK and p-AKT. Western blot analysis of total lysates of SH-SYSY cells expressing pRS, shGFP or shNF1 vectors. (C) The level of NF1 knockdown by each of the RNAi vectors was measured by examining the NF1 mRNA levels by qRT-PCR. Error bars denote standard deviation. doi:10.1371/journal.pgen.1003958.g010

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 growth deficiency and the role of 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 synaptic formation has been suggested to involve the release of presynaptic Jeb activating downstream of focal adhesion kinase to restrain NMJ synaptic overgrowth [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 dGoh2 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 synaptic 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 neurofibroma-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 dNf1E1 and dNf1E2 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. Eant1SM1 and Eant1SM2 were provided by D. van Meyel, dALK and jk20 by R. Palmer, cnk by M. Therrien, and cnk20 by H. Kramer, pRh6 by M. Pankratz, hs-RpL2 transgenic line by E. Ruhlison and UAS-Rab9 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 a central blob of yeast paste, and their position was documented. To assess the expression of starvation-sensitive genes, larvae at 72 h AED were placed in an agar plate with a central blob of yeast paste, and their position was scored at hourly intervals. To determine the larval starvation-sensitive genes, larvae at 72 h AED were placed in a circulating water bath at 37°C for 10 min once or twice a day with an 8 hr interval.

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 the gender and, if necessary, the genotype of eclosed flies. At least 40 pupae were measured for each genotype, and only measurements from 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 knockout. 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/dNf1E2 progeny.

To test whether genetic suppression reflected the inadvertent introduction of a wild-type dNf1 allele, we used DNA prepared using DNAzol (Molecular Research Inc.) in a PCR assay with AGTCCAGTTAATTGATCCTG and GAGATCGTTGATGAGAAAGT primers. The second primer introduces a penultimate single nucleotide change, which together with the E2 mutation results in the introduction of an RsaI restriction site. RsaI 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 (w1118) and dNf1E2 controls.

Construction of Akh-Gal4 and Attenuated UAS-PKA

Transgenes

The Akh promoter region was amplified with Akh-FORWARD (AGATCTAATCTTCTCTTTAAGCGGCGGCGGCG) and Akh-REVERSE (AGATCTATGTGGCCCTGCAACTTGATTCC) primers. The resulting PCR fragment was subcloned into the BamHI site of a Gal4 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: 1xUAS-FOR: AACTGCAAGGCGGACATCTGTGGCTTCCGGGAAGAGATCCTTAG; 2xUAS-FOR: AACTGCAAGGCGAGTACTGTGGCTTCCGGGAAGAGATCCTTAG; 3xUAS-FOR: AACTGCAAGGCGGACTGTGGCTTCCGGGAAGAGATCCTTAG; and 4xUAS-FOR: AACTGCAAGGCGGACATCTGTGGCTTCCGGGAAGAGATCCTTAG. The initial PCR fragment was subcloned into the pUAS-PKA vector for expression in transgenic flies before being used generate transgenic flies by standard protocols.
reaction with pCR2.1-UAS(2×) as template generated pCR2.1-UAS(3×) and pCR2.1-UAS(4×). The pCR2.1-UAS clones were sequenced, their inserts excised with PstI and subcloned into PstI-digested pUAST. Correct insert orientation was verified by sequence analysis, after which the mutationally activated murine PKAα 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 pelts 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 E2000 microscope (Melville, NY) Ti-E inverted microscope using a 60× Plan Apo (1.4 NA) objective. The microscope was equipped with a Prior (Rockland, MA) Proscan II motorized stage. Larval samples were excited with 405-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 Selective Chemicals. Antibody against NF1 was from Bethyl Laboratories (A300-140A); antibodies against p-ERK (E-4), ERK1 (C-16), ERK2 (C-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 PAK2-α-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 (1–2×10⁶ 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, AAGGTTAGGGCGAGGTCAACA; GAPDH-Reverse, AAGAAAGGGTGCTATGAGTGG; NF1-Forward, TGTCAGTGGCATAACTCTTTGC; NF1-Reverse, AGT-GCCCATCCTCTTCTCAGAG; ALK mRNA levels in neurofibroma-derived NF1−/− Schwann cells and NF1+/− fibroblasts were analyzed using the following two primer sets: ALK-N-Forward, GGAGTGCAGTTTGACTTCC; ALK-N-Reverse, TGGAGTCACTGTAGGTGTGTG; ALK-C-Forward, GCACATCGCTTGAAGACA; ALK-C-Reverse, GCCGTGTTGAGAGAGAAGAG.

**Supporting Information**

**Figure S1** Loss of dNF1 does not alter developmental timing but reduces larval growth rate. (A) Wild-type, dNF1d/d, and dNF1E/d2 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-Ras1D22 undergo accelerated development resulting in miniature pupae [104], whereas phm-Gal4 driving a dominant negative UAS-PI3KD954A transgene delayed development and produced giant pupae [7]. (B) Mouth hook length measurements (in μm) show that dNF1 larva 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.

**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 transversion to amplify a 431 genomic fragment as indicated. The mutant primer creates a GTAC RsaI restriction site when E2 genomic DNA is used as a template. (B) RsaI 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 (u118w) and dNF1E2 controls (lanes 2, 3 and 4) and various deficiencies (Df) either in wild-type (Df/Y; +; lanes 5 and 15), dNF1 homozygous (Df/Y; dNF1E2, lanes 6–13) or heterozygous (Df/Y; dNF1E2/++; lanes 14 and 16) backgrounds.

**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 N(A)AT1 as the responsible gene uncovered by suppressing deficiencies Df(1)BSC333 and Df(1)Exel6290. 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 (K805A) 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 els- Gal4, likely reflecting Ret-mediated activation of Ras/ERK signaling.

**Figure S5**  Expression pattern of ring gland drivers. Ring gland drivers P0206-Gal4, Fb36-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 oriented 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 or MEK1/2 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.
References


