Rescue of a *Drosophila NF1* Mutant Phenotype by Protein Kinase A

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The neurofibromatosis type 1 (NF1) tumor suppressor protein is thought to restrict cell proliferation by functioning as a Ras-specific guanosine triphosphatase–activating protein. However, *Drosophila* homozygous for null mutations of an *NF1* homolog showed no obvious signs of perturbed Ras1-mediated signaling. Loss of *NF1* resulted in a reduction in size of larvae, pupae, and adults. This size defect was not modified by manipulating Ras1 signaling but was restored by expression of activated adenosine 3′,5′-monophosphate–dependent protein kinase (PKA). Thus, NF1 and PKA appear to interact in a pathway that controls the overall growth of *Drosophila*.

The gene responsible for human NF1 encodes a large protein that contains a central domain related to Ras-specific guanosine triphosphatase–activating proteins (RasGAPs) (*1*). Although loss of *NF1* expression correlates with increased Ras activity in several mammalian tumor cell types (*2*), it is not known which pathways are altered to produce the diverse symptoms observed in NF1 patients, which in addition to frequent benign and infrequent malignant tumors also include short stature and learning disabilities (*1*).

We identified a conserved *Drosophila NF1* homolog (*3*). Comparison of 13,295 base pairs (bp) of genomic and 9750 bp of cDNA sequence showed that *Drosophila NF1* consists of 17 constitutive and 2 alternatively spliced COOH-terminal exons. The two cDNAs predict proteins of 2764 and 2802 amino acids that are 60% identical to the human NF1 protein, neurofibromin (Fig. 1). Sequence similarity is observed over the entire length of the proteins, including regions outside of the catalytic GAP-related domain (GRD) or the more extensive segment related to yeast inhibitor of RAS activity (IRA) proteins (*4*). No related sequences were identified during screens of several cDNA and genomic libraries, indicating that the identified gene may be the only *Drosophila NF1* homolog. RNA in situ hybridization and staining of embryos and imaginal discs with monoclonal antibodies to the *Drosophila* protein (*5*) indicated that *NF1* is widely expressed in low amounts during all developmental stages (*6*). The *Drosophila NF1* gene was mapped to cytogenetic interval 96F and subsequently localized to a 30-kb DNA segment between the *bride of sevenless* gene and the *Enhancer of split* [*E(spl)*] complex (*7*).

To isolate mutant alleles at the *NF1* locus, we mobilized a *P*-element transposon located within the *E(spl)* complex, about 15 kb downstream of *NF1* (*7*). Among 1600 lines screened by inverse polymerase chain reaction (*8*), two showed evidence of de novo transposon insertions within the *NF1* gene. One mutant allele, $NF1^{P1}$, has a deletion that removed all of the *NF1* gene except for the first exon (Fig. 1). The deletion extends from the first *NF1* intron to the site of the original *P*-element insertion and removes DNA encoding at least two *E(spl)* transcripts. The other allele, $NF1^{P2}$, contains a *P*-element in the first *NF1* intron (Fig. 1). Neither allele expresses NF1 protein (Fig. 2A).

Unlike *Nf1*-deficient mice (*9*), *Drosophila NF1* mutants are viable and fertile. Although heterozygotes (*NF1*/+) had no obvious defects, homozygotes (*NF1*/*NF1*) of either allele were 20 to 25% smaller than flies of the parental K33 strain during all postembryonic stages. This growth defect was apparent under various culture conditions, and mutant animals did not display delayed eclosion or bristle phenotypes that are observed with several *Minute* mutations (*10*). The growth defect was fully rescued by expression of a heat shock–inducible *hsNF1* (*11*) transgene (Fig. 2, A and B, and Table 1).

To determine whether reduced cell proliferation or impaired cell growth underlies the smaller size of *NF1* mutants, we compared the wings of wild-type and mutant animals. The linear dimensions of mutant wings were 20 to 25% smaller than those of wild-type flies (Fig. 2, C and D). Because each wing epidermal cell secretes a single hair, cell densities can be determined by counting the number of hairs in a defined region (*12*). Both homozygous mutants had a 30 to 35% higher cell density than flies of the parental line (Fig. 2E). To determine whether the reduced size of wing epidermal cells reflects a cell-autonomous defect, we used x-irradiation to induce mitotic recombination in the wing cells of heterozygous *NF1* mutants (*13*). No difference in cell density was observed between multiple $NF1^{-/-}$ clones and surrounding tissue (*6*). The reduced size of the wing cells therefore reflects a nonautonomous requirement for *NF1*, perhaps reflecting a hormonal deficiency or impaired nutrition or metabolism. The eyes of *NF1* mutants showed a reduced number of ommatidia of normal size and structure (Fig. 3). Furthermore, *NF1*-deficient embryos were of normal size (Fig. 3). Thus, loss of *NF1* affects the growth of various tissues in different ways.

The only known biochemical property of neurofibromin is that it negatively regulates Ras (*2*), but *Drosophila NF1* mutants did not exhibit phenotypic abnormalities associated with excess Ras1 or Ras2 activi-

Table 1. Rescue of the pupal size defect of *NF1* mutants by *hsNF1* and activated PKA, but not by activated Raf or by reduced *Ras1* gene dosage. The size of pupae is given as the average plus or minus the standard deviation. With the Mann-Whitney rank-sum test (*30*), the following populations had statistically significant differences in median pupal lengths ($P < 0.0001$): K33 and $NF1^{P1}$, K33 and $NF1^{P2}$, $NF1^{P1}$ and *hsp70-PKA**; $NF1^{P1}$ and $NF1^{P2}$ and *hsp70-PKA**; $NF1^{P2}$. The size differences between $Ras1^{e1B}$; +/+ and $Ras1^{e1B}$; $NF1^{P2}$, and between *hsp70-PKA**; +/+ and *hsp70PKA**; $NF1^{P1}$ or *hsp70-PKA**; $NF1^{P2}$ pupae were significant at the same confidence level. Rel. size, relative size.

Genotype	*n*	Pupal length (mm)	Rel. size
K33/K33 (+/+)	88	2.92 ± 0.19	1.00
$NF1^{P1}/NF1^{P1}$	53	2.43 ± 0.09	0.83
$NF1^{P2}/NF1^{P2}$	54	2.32 ± 0.15	0.79
hsNF1; +/+	79	2.98 ± 0.15	1.02
hsNF1; $NF1^{P1}/NF1^{P1}$	56	3.00 ± 0.14	1.03
hsNF1; $NF1^{P2}/NF1^{P2}$	88	3.01 ± 0.14	1.03
$Ras1^{e1B}$; +/+	64	2.83 ± 0.14	0.97
$Ras1^{e1B}$; $NF1^{P2}/NF1^{P2}$	90	2.39 ± 0.15	0.82
*hsp70-PKA**; +/+	88	3.02 ± 0.16	1.03
*hsp70-PKA**; $NF1^{P1}/NF1^{P1}$	79	2.74 ± 0.17	0.94
*hsp70-PKA**; $NF1^{P2}/NF1^{P2}$	64	2.71 ± 0.15	0.93
Raf^{gof}; $NF1^{P1}/NF1^{P1}$	67	2.39 ± 0.17	0.82
Raf^{gof}; $NF1^{P2}/NF1^{P2}$	48	2.33 ± 0.14	0.80

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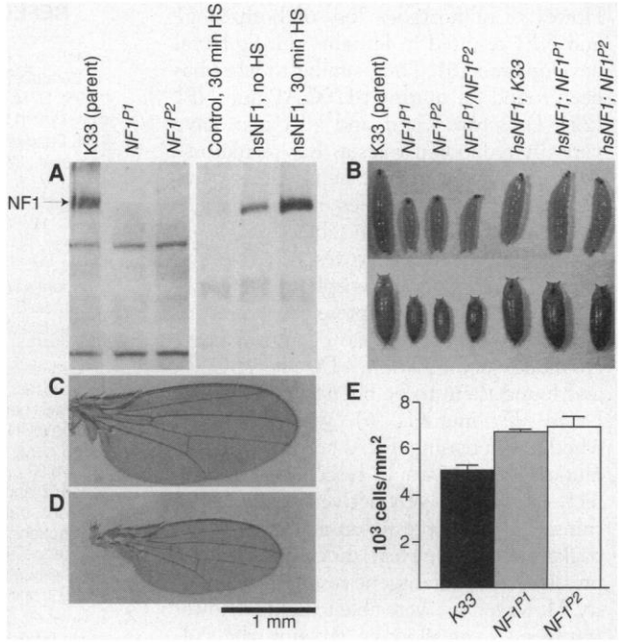
A
AUG
GRD
NF1P2
ΔNF1P1
18a 18b
B
2764/2802 aa
Identity
50%
69%
51%
C

Fig. 1. *Drosophila NF1* gene structure and comparison of the encoded protein to human neurofibromin. (**A**) Intron-exon structure and location of translational start (AUG) and in-frame stop (*) codons. The arrow indicates the location of a *P*-element in $NF1^{P2}$. The extent of the deletion in $NF1^{P1}$ is indicated by the line below the diagram. (**B**) Percentage amino acid sequence identity between the indicated segments of *Drosophila* and human NF1. The GRD- and IRA-related segments are drawn as black and shaded boxes, respectively. (**C**) Alignment of *Drosophila* (top) and human NF1 proteins. Dashes were introduced to optimize the alignment. Downward pointing arrows indicate amino acids encoded by the last complete codon in each exon. The boxed segment shows the approximate extent of the GRD. Three positions where alternate splicing inserts short segments in human neurofibromin (*28*) are identified by upward pointing arrows. One of these locations corresponds exactly to the position where *Drosophila* exon 17 is joined to either exon 18a or 18b. Exon 18b includes a translational terminator after a single codon, and cDNAs harboring this exon predict a protein ending in PTDKAA. Eleven out of 17 *Drosophila* splice sites map within two codons of splice sites in the human *NF1* gene (*29*). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Fig. 2. Size defect of *NF1* mutants: Rescue by expression of an *hsNF1* transgene and evidence of reduced cell size. (**A**) Immunoblots of embryo extracts probed with monoclonal antibody to NF1 (antibody DNF-21). The arrow indicates a 280-kD immunoreactive protein that is present in the K33 parental strain but absent in both $NF1^{P1}$ and $NF1^{P2}$. A transgenic strain harboring a second chromosome *hsNF1* transgene had an increased amount of 280-kD immunoreactive protein even before heat shock (HS) induction. (**B**) Third instar larvae (top) and pupae (bottom) of the indicated genotypes. (**C** and **D**) Wings from K33 and $NF1^{P1}$ flies, respectively. (**E**) Quantitation of the number of wing epidermal cells per square millimeter in the indicated strains.



ty. Whereas expression of activated *Ras1* or *Ras2* transgenes results in widespread developmental defects (*14*), *NF1* mutants are smaller but otherwise patterned normally. We therefore examined the regulation of Ras by *NF1* both in vitro and in vivo. We assayed GAP activity of bacterial fusion proteins containing the catalytic domains of human p120GAP, or human or *Drosophila* NF1 (*15*). All three fusion proteins stimulated the guanosine triphosphatase (GTPase) activity of human H-Ras, but not of the constitutively active $H\text{-}Ras^{Val12}$ mutant (Fig. 3A).

We also examined Ras1 function in vivo. The *Drosophila* Ras1 protein functions in signaling pathways downstream of several receptor tyrosine kinases (RTKs), including Torso and Sevenless. Because minor perturbations in Ras1 function have phenotypic consequences in each of these pathways, we examined whether loss of *NF1* perturbed Torso-controlled specification of embryonic terminal structures or Sevenless-mediated photoreceptor differentiation. The pattern of *tailless* expression, which is regulated by Torso (*16*), was normal in *NF1*-deficient embryos (Fig. 3, B and C).

To test for abnormalities in Sevenless signaling (*17*), we examined the retinas of mutant animals. Although reduced in number, the ommatidia of $NF1^{P2}$ homozygote, of $NF1^{P2}/NF1^{P1}$, and of $NF1^{P2}/Df(3R)boss^{15}$ flies (*18*) had the normal complement of photoreceptor and accessory cells (Fig. 3D). In homozygotes of $NF1^{P1}$, 25% of ommatidia had one or more extra photoreceptor cells (6). However, this phenotype may result from the deletion of genes within the neurogenic *E(spl)* complex. A particularly sensitive indicator of Sevenless pathway function is the sev^{E4}; Sos^{JC2}/+ mutant combination (*19*). Flies of this genotype that were also heterozygous for $NF1^{P2}$ had no alteration in the fraction of ommatidia that had R7 cells. Thus, at least two Ras1-mediated signaling pathways downstream of RTKs are not influenced by a reduction in *NF1* function.

Heterozygous loss of *Ras1* or *Sos* (*20*) had no effect on the size of *NF1* mutant pupae, nor did expression of an activated Raf^{gof} mutation (Table 1). Thus, neither reducing nor increasing signaling through the Ras1-Raf pathway modifies the *NF1* phenotype. Heterozygous loss of another *Drosophila* Ras-GAP homolog, *Gap1* (*21*), did not enhance the phenotype of *NF1* mutants.

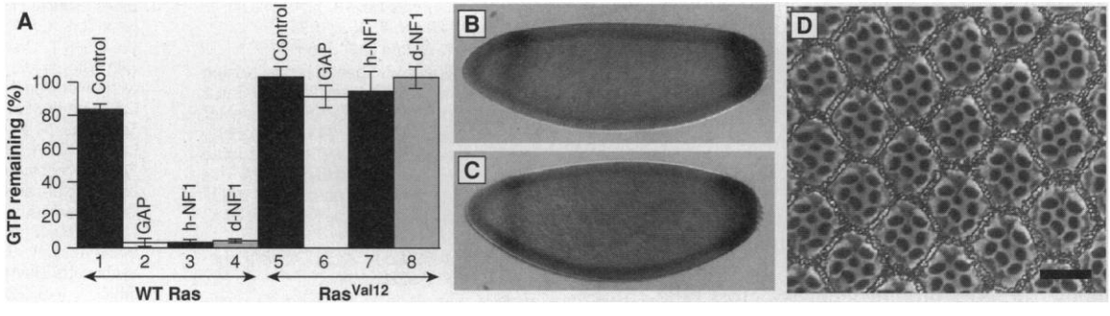


Fig. 3. Function of *Drosophila NF1* as a Ras-GAP in vitro, but not as a major regulator of Torso- or Sevenless-mediated signaling in vivo. (**A**) Radioactivity remaining on H-Ras–[γ^{32}P]guanosine triphosphate (GTP) or $H\text{-}Ras^{Val12}$–[γ^{32}P]GTP after 10-min incubations with the indicated fusion proteins (h-NF1 is the human NF1 GRD; d-NF1 is the *Drosophila* GRD; WT, wild type). (**B** and **C**) RNA in situ hybridization, showing *tailless* expression. The embryos in (B) and (C) are K33 and $NF1^{P2}$ homozygotes, respectively. (**D**) Retinal section of adult $NF1^{P2}$ homozygotes, showing ommatidia of normal size and structure. Mutant eyes have only about 550 ommatidia, compared with ~750 in K33 flies. The scale bar represents 10 μm.

However, homozygous loss of both *Gap1* and *NF1* resulted in lethality during larval development (6). Thus, similar to what has been found for murine p120GAP and NF1 (*22*), *Drosophila* Gap1 and NF1 may have partially redundant roles as Ras regulators.

Flies carrying a viable heteroallelic combination of mutant alleles of the gene encoding the PKA catalytic subunit, *DC0*, are reduced in size (*23*). To test whether the adenosine 3′,5′-monophosphate (cAMP)–PKA pathway might represent a target for NF1, we examined pupae of another heteroallelic combination, $DC0^{TW2}/DC0^{B3}$, and found them to be phenotypically similar to *NF1* mutants (6). We then tested whether increasing PKA activity in *NF1* mutant animals would rescue the size defect. A constitutively active murine *PKA** transgene was expressed in an *NF1* mutant background (*20*). Heat shock–induced expression of this transgene resulted in lethality. However, we were able to achieve lower transgene expression by growing the cultures at 28°C. Under these conditions, statistically significant rescue of the pupal size defect was observed (Table 1). In contrast to its effect on *NF1* mutant pupae, the *PKA** transgene did not modify the phenotype of *Tubby*, a mutation that results in pupae of small size (*24*). Because expression of activated PKA suppressed the phenotype of null alleles of *NF1*, PKA appears not to function upstream of *NF1* in a simple linear pathway. Therefore, PKA must either function downstream of *NF1* or in a parallel pathway.

NF1 mutants also differ from wild-type flies in an assay that determines the number of flies that fly away upon release from their containers, either spontaneously or after repeated prodding (*25*). About 15% of either *NF1* mutant ($n = 200$) failed to respond, whereas only 3% of parental K33 flies did not respond. The reduced escape rate does not reflect obvious anatomical defects of the peripheral nervous system or the musculature, and the mutants scored within normal limits in tests measuring their response to visual or olfactory stimuli (*26*). Electrophysiological studies showed that the mutants have a defect at the larval neuromuscular junction that is rescued by pharmacological manipulation of the cAMP-PKA pathway and that is insensitive to manipulation of Ras1-mediated signaling (*27*). Thus at least two *NF1*-deficient phenotypes can be rescued by increasing cAMP-PKA signaling. Further analysis of *NF1*-deficient flies may define how the NF1 protein mediates cross talk between the Ras and PKA pathways and may ultimately suggest new therapeutic strategies for the treatment of human neurofibromatosis type 1.

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3. *Drosophila NF1* clones were isolated by screening a Canton-S λFixII genomic phage library (Stratagene) in 25% formamide at 37°C with a probe representing the COOH-terminal 1598 amino acids of human *NF1*. All clones identified represented the same locus. A 13,295-bp segment representing the entire gene was sequenced. A set of 32 overlapping cDNAs was isolated from eye disc and mixed stage embryo libraries and used to determine 9750 bp of overlapping cDNA sequence. The genomic and cDNA sequences differ in multiple locations, but none of these polymorphisms affects the sequence of the encoded protein. The GenBank accession numbers for the genomic and alternatively spliced cDNA sequences are L26500, L26501, and L26502.
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6. I. The, I. K. Hariharan, A. Bernards, unpublished results.
7. The *NF1* homolog was mapped to cytogenetic interval 96F by in situ hybridization of biotinylated probes to salivary gland chromosomes [W. R. Engels, C. R. Preston, P. Thompson, W. B. Eggleston, *Focus* **8**, 6 (1986)]. We used genomic clones from this region (*18*) to sublocalize the gene to the 30-kb interval between the *bride of sevenless* and *E(spl)* loci. The K33 stock has a *P*-element inserted in the *E(spl)* complex.
8. Flies were raised on standard medium at 25°C. To generate *NF1* mutants, we crossed *w*; *P*[*w*] males (K33) to *Ki* p^{p} *Δ2-3* transposase-bearing virgin females. Single F_1 dysgenic males of genotype *P*[*lacZ w*]/*Ki* p^{p} *Δ2-3* were crossed to *w*; *TM3/TM6B* virgin females. Single F_2 males of genotype *w*; *P*[*lacZw*]/*TM3* or *w*; *P*[*lacZw*]/*TM6B* were crossed to *w*; *TM3/TM6B* virgin females to establish lines with stable *P*-element integrations. The red-eyed progeny of this cross were analyzed in pools by inverse polymerase chain reaction [B. Dalby, A. J. Pereira, L. S. B. Goldstein, *Genetics* **139**, 757 (1995)].
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13. Clonal analysis was done in a *forked* (*f*) background. Females of genotype *f* ; *bld P*[*f*+]/*TM3 Ser* were crossed to either $NF1^{P1}$, $NF1^{P2}$, or *K33* males. Parents were removed after 24 hours, and the larval progeny were irradiated [10 Gy (1 Gy = 100 rads)] after 48 to 72 hours. Adult F_1 males of genotype *f* ; *bld P*[*f*+]/$NF1^{P1}$ or *f* ; *bld P*[*f*+]/+ were analyzed.
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