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TITLE: A Genetic Analysis of NF1 Function in Drosophila Melanogaster (Neurofibromatosis)

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The human neurofibromatosis type 1 (NF1) protein contains a RasGAP domain and is believed to restrain cell proliferation by negatively regulating Ras-mediated signalling. We have characterized a *Drosophila* NF1 homologue encoding a protein that is 60% identical to the human NF1 protein over its entire length and generated loss-of-function mutations. Flies lacking NF1 are viable and *Ras1*-mediated signalling downstream of the *sevenless* and *torso* receptor tyrosine kinases is normal, indicating that *Drosophila* NF1 is not a crucial *Ras1* regulator. However, combined loss of NF1 and another *Drosophila* RasGAP, *Gap1*, is lethal, arguing that NF1 may be a redundant *Ras1* regulator in *vivo*. Mutants lacking NF1 are 25-30% smaller than wild type animals during all post-embryonic developmental stages and mutants also display a diminished escape response. Remarkably, the size defect is not modified by manipulating *Ras1* pathway signalling but is rescued by providing increased levels of PKA activity. Thus NF1 may modulate both PKA and Ras signalling in *vivo* and could conceivably provide a link between these signalling pathways.
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FINAL REPORT

INTRODUCTION

Neurofibromatosis type 1 (NF1) is one of the commonest human genetic diseases. Although the disease causes significant morbidity and mortality by predisposing affected individuals to benign and malignant tumors, the pathogenesis of the disease is poorly understood and no cure exists. The gene mutated in NF1 patients encodes a large protein which has domains with sequence similarity to mammalian rasGAP and the yeast IRA1 and IRA2 proteins (reviewed in (1). Although the NF1 protein can function as a rasGAP in vitro, the normal biological function of NF1 and its precise role in the pathogenesis of the disease are poorly understood.

In order to study the normal function of NF1, we chose to utilize a genetic approach. Prior to the commencement of this proposal, we had cloned a highly conserved NF1 homologue from Drosophila melanogaster. This report details our analysis of the expression and function of Drosophila NF1 using mutants that we have generated.

As outlined in the Statement of Work in our original proposal, the objectives were as follows:
Year 1 - Characterization of NF1 RNA and protein expression and a screen for lethal mutations in the vicinity of NF1. This objective has been completed. We found however, that none of the lethal mutations were lethal. We therefore needed to use an alternate approach.
Year 2 - During this year we conducted (contingency plan in our original SOW) a P-element screen to isolate NF1 mutations. This approach was successful. Mutations in NF1 were not lethal but demonstrated a surprising requirement for normal growth and neuromuscular function.
Year 3 - As stated in our original SOW, we completed phenotypic analysis of the mutants including clonal analysis. In our original SOW, we had planned to identify interacting genes and to map functional domains in the NF1 protein. However, when we found a novel link between NF1 and the PKA signaling pathway, we pursued this interaction in more detail instead. This work has led to defining a completely novel function for NF1 and a means of suppressing defects of NF1 mutants in vivo.

These results are described in this report. These results have been included in two papers which have been submitted to Nature and are currently being reviewed.


BODY

EXPERIMENTAL METHODS

Molecular biology
Most DNA, RNA and protein manipulations involved standard procedures.
Genomic clones were isolated by screening a λFIX-II Drosophila melanogaster Canton S
genomic library (Stratagene) with a probe representing the C-terminal 1598 codons of the human NF1 reading frame. The hybridization was performed at 37° C in buffer containing 5 x SSC, 25% formamide, 5% Dextran sulfate, 5 x Denhardt’s solution, 0.5% SDS, and 100 mg/ml denatured DNA. Filters were washed 6 times for 5 min in 3 x SSC at room temperature and exposed for 3 days. Drosophila NF1 cDNAs were isolated from eye disc, total disc and mixed stage embryo libraries. DNA sequences were determined by the dideoxy chain termination method, using Sequenase enzyme (U. S. Biochemical).

Drosophila embryos were collected on molasses-agar egg laying plates, dechorionated with 50% bleach, dounced in a buffer containing 20mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and centrifuged for 20 min at 14,000 xg at 4° C to remove debris. Equal amounts (100-150 mg/lane) of total protein was size separated by SDS polyacrylamide gel electrophoresis and transferred to immunobots. The latter were probed with anti Drosophila NF1 monoclonal antibodies NF1-11 or NF1-21, which had previously been generated by the MGH Cancer Center Monoclonal Antibody Core Facility. NF1 was precipitated from embryo lysates using a cocktail of four monoclonal antibodies (NF1-11, 21, 30 and 33).

Genetics
Flies were raised on cornmeal-molasses-agar-yeast medium and all crosses were carried out at 25° C. Strains, mutations and balancer chromosomes are described in (2).

F2-lethal screen
Isogenized bw; st e adult males were starved for 1 hr. and given 25 mM ethyl methane sulfonate (EMS) in 1% sucrose for 16 hr, transferred to a vial with adsorbent tissues for 1 hr, and mated to bw; TM3/TM6B virgin females. Flies were allowed to lay eggs for 24 hr and were then transferred into new bottles. After five days the parents were discarded. Resulting F1 bw; st e*TM3 or TM6B males were individually crossed to bw; Df(3R)Df(3R)bos16 st e/TM3 or TM6B virgin females (* indicates the mutagenized chromosome). Since adults homozygous for both the brown (bw) and scarlet (st) pigment genes have white eyes, the absence of white eyed flies in the F2 progeny indicates the presence of a mutation in the st e* chromosome that is lethal in trans to the Df(3R)bos16 st e chromosome. The mutagenized chromosome was recovered from siblings of the genotype bw; st e*TM3 or TM6B.

Recombination mapping:
The multiply-marked third chromosome th st cu sr e ca stock was used to map complementation group l(3)D. Males of l(3)D/TM3 were mated with virgin females th st cu sr e ca. Males th st cu sr e ca were crossed with virgin females l(3)D / th st cu sr e ca in which the recombination occurs. Since all flies from this complementation group displayed a dominant wing vein phenotype, the progeny were scored for linkage of the wing vein phenotype with one of the markers.

To map group l(3)A the stock was crossed into a white background. Males of w; l(3)A/TM3 were crossed to red eyed virgin females w; P[w]. Males w; TM3/TM6B were crossed to virgin females l(3)A/P[w]. The red eyed progeny of this cross were then tested for lethality in combination with other alleles of the l(3)A complementation group. When the lethal mutation is tightly linked to the P[w], hardly any recombination events would occur between the lethal mutation and the P[w] . The number of progeny from red eyed flies which is lethal with l(3)A would be small.

P-element screen
To generate specific NF1 mutants w; P[w] males homozygous for a P[w] inserted in 96F were crossed to virgin females Ki pD 2-3 bearing a transposase. Single F1
dysgenic males with the phenotype \( P[\text{lacZ} \cdot w] / Ki \ p^D \ \text{2-3} \) were crossed to virgin females \( \text{w}^3 \); TM3/TM6B. Single \( F_1 \) males \( \text{w}^3 \); \( P[\text{lacZ} \cdot w] / \text{TM3 or TM6B} \) were crossed to female virgins \( \text{w}^3 \); TM3/TM6B to establish single lines with stable novel P-element integrations. The red eyed progeny of this cross was analyzed in pools by inverse PCR.

**Inverse PCR screen for \( de \ novo \) P element integrations in \( NF1 \).**

Genomic DNA was prepared from pools of 40 red-eyed flies by douncing in 0.1 M Tris-HCl pH 8.5, 0.1 M EDTA, 0.1 M NaCl, and 0.5% SDS. Before homogenization, a single homozygous K33 mutant was added as a positive control. 20 ml of DNA was digested with 4 units of Sau3A for 3 hr. After 1:1 phenol:chloroform extraction and ethanol precipitation, DNA pellets were resuspended in 50 ml TE buffer. The digested DNA was then circularized by ligating 5ml in a 200 ml volume with 40 units T4 DNA ligase (New England Biolabs) at 160°C for 12-16 hr. The ligated DNA was phenol:chloroform extracted, ethanol precipitated, and resuspended in 10 ml of water. The circular products of the ligation reaction were used as a template for PCR (5 min. 85°C, followed by 40 cycles of 1 min. at 94°C, 1 min. at 58°C, 2 min. at 72°C), using a Perkin Elmer-Cetus thermal cycler. Reactions contained 10 ml of ligated DNA in a 20 ml total volume with final concentrations of 0.2 mM dNTPs (Boehringer Mannheim), 1 x PCR buffer (Promega), 1.25 mM Mg Cl2, 125 ng of primers, 1 unit of Taq polymerase (Promega) and 10 mCi [\( a^{-32}\text{P} \)] dCTP (NEN). The primers used were: CGA CGG GAC CAC CTT ATG TTA TTT CAT CAT G (P-element inverted repeat); GCC GAA GCT TAC CGA AGT ATA CAC T (5' end); GCA AGA GAC ATC CAC TTA ACG TAT GC (3' end).

DNA of phage IE8, which contains the entire \( NF1 \) gene, was digested with EcoRI. Similarly digested R5.17 phage DNA, which spans the integration site of the parental P-element, was included as a positive control. Hybridization of PCR-generated probes was allowed to proceed at 65°C for 12-16 hr in 5 x SSC, 5 x Denhardt's solution, 50% formamide, 0.5% SDS, and 150 mg/ml denatured DNA. Membranes were washed at 65°C for 2 hours with four changes of 2 x SSC, 0.1% SDS. Autoradiography was carried out for 1-5 days at -80°C using Kodak X-omat film and intensifying screens.

**Clonal analysis**

Clonal analysis was performed in a \( forke(d) \) background. Females \( f; \) \( bld \ P[f^+]/\text{TM3 Ser} \) were crossed to males \( NF1^{P1} \), \( NF1^{P2} \) or the parental K33 strain. Parents were transferred after 24 hr and the larval progeny irradiated after 48-72 hr. Adult \( F_1 \) males \( f; \) \( bld \ P[f^+]/NF1^{P1} \) or \( NF1^{P2} \) or K33 were analyzed for presence of clones in the wing and the wings were mounted in Canada balsam (Sigma). The clones were investigated with a compound microscope and 32x magnified clones were photographed. The distance of the bristles in the \( forke(d) \) \( NF1 \) mutant clone were measured and compared to the distance of the bristles in the surrounding heterozygous tissue.

**Generation of \( hsp70-NF1 \) transgenic flies**

The mammalian \( NF1 \) open reading frame is strongly selected against in E. coli. To avoid similar toxicity problems, we constructed an \( hsp70-NF1 \) mini gene by cloning a hybrid cDNA/genomic \( NF1 \) insert into the SacII and KpnI sites of the pKB176PL P element-based vector (3). Expression of this mini-gene in E. coli is prevented by the presence of three introns in the genomic part of the insert (flanked by unique MluI to Esp3I sites). The resulting P element was introduced into the germline of \( w^{1118} \) flies by injection. Administering daily 30 min 37°C heat shocks fully rescued the \( NF1 \) deficient phenotype.
**In situ hybridization to polytene chromosome**

Salivary glands were obtained from wandering third instar Canton-S larvae and polytene chromosomes were prepared for hybridization. Two non-overlapping genomic fragments were used as probes. Probes were labeled with biotin-11-dUTP by nick-translation and hybridization was performed as described (4).

**RNA in situ hybridization**

Digoxigenin-labeled sense and antisense NF1 and evenskipped RNA probes were made from linearized cDNAs using T3 or T7 RNA polymerase respectively. In situ hybridization to whole mount embryos was performed essentially as described (5), except that 4 mg/ml of proteinase K was used.

**Embryo and imaginal disc staining**

Embryo and imaginal disc staining was performed as described (6).

**Phenotypic analysis**

Scanning electron microscopy of adult Drosophila eyes and retinal sections were prepared as described (11). Wings were dehydrated in 100% ethanol and mounted in Canada balsam (Sigma).

**RESULTS**

A Drosophila NF1 homologue was identified by screening a genomic library with a human NF1 cDNA probe. Restriction mapping demonstrated that several independent clones were all derived from the same locus. A 13,295 bp region encompassing the entire homologue was fully sequenced. We also determined 9750 bp of overlapping cDNA sequence. Alignment of the genomic and cDNA sequences shows that Drosophila NF1 consists of 17 constitutive and 2 alternatively spliced exons 18a and 18b (Figure 1A). The 2764 and 2802 amino acid proteins predicted by the alternatively spliced cDNAs are 60% identical to the human NF1 protein, called neurofibromin (Figure 1B and C). Sequence similarity is observed over the entire length of the protein, including regions that fall outside the GAP catalytic domain and the more extensive segment related to yeast IRA1&2 proteins. Moreover, 11 out of 17 Drosophila NF1 splice sites map within two codons of splice sites in the human gene. No other NF1-related sequences were detected during low stringency screens of cDNA or genomic libraries, suggesting that this gene is the only Drosophila NF1 homologue.
Figure 1. *Drosophila* NF1 gene structure and comparison of the encoded protein to human neurofibromin. The top drawing (A) shows the intron-exon structure of *Drosophila* NF1 and the location of translational start and in-frame stop codons. The NF1<sup>2</sup> allele harbors a P-element at the indicated position in the first intron. The extent of the deleted segment in NF1<sup>18b</sup> is also indicated. Drawing B shows the percentage amino acid sequence identity between the indicated segments of *Drosophila* and human NF1. The GAP-related domain (GRD) and IRA-related domains are drawn as black and shaded boxes, respectively. The sequence alignment (C) compares the *Drosophila* (Dm) and human (Hs) proteins. Dashes were introduced to optimize the alignment. Amino acids encoded by the last complete codon in each exon are identified by signs. Exon numbers are indicated to the left of splice junctions. The boxed segment shows the approximate extent of the GRD. Three positions downstream of which alternatively spliced exons insert short amino acid segments in human neurofibromin, are identified by filled-in triangles. 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 2764 residue protein ending in PTDKAA.

**Expression of Drosophila NF1 RNA and protein**

For many genes, analysis of the spatial and temporal patterns of expression has provided clues as to the function of the gene. For instance, the *Drosophila* Gap1 gene, which encodes another RasGAP, is expressed in specific regions of the imaginal discs and the patterning of these same regions is perturbed in Gap1 mutants (7). We therefore examined whether NF1 was expressed at higher levels at particular stages of development or in particular tissues. A 9.5 kb mRNA was seen in all developmental stages, but this transcript was especially prominent in adult flies and in early (0-6 hr) embryos (Figure 2). Hybridization with a Ras2 probe (8), which detects several transcripts that are expressed at similar levels throughout development, showed that all lanes were equally loaded. Since little transcription occurs during the first few hours post-fertilization (9), the earliest mRNA presumably represents maternal transcripts. A less abundant 10.5 kb mRNA appears in 3-24 hr embryos and may be an alternatively spliced species. We conclude that NF1 is expressed at most stages of development.

Fig. 2. Expression of *Drosophila* NF1 is regulated during development. Each lane of the RNA blot contains 3 mg of poly(A) RNA from staged embryos, larvae, pupae, and adults as
indicated. The top panel was hybridized to a \textit{NF1} probe. The bottom panel shows the same blot after rehybridization with a \textit{Ras2} probe.

To analyze the spatial distribution of \textit{NF1} expression, we performed \textit{in situ} hybridization of whole-mount embryos with digoxigenin labeled RNA probes. Under conditions where \textit{even-skipped} (10), \textit{Rapgap1} (our unpublished data), and \textit{Rac1} (11) probes showed the expected patterns, \textit{NF1} probes showed uniform staining of all embryonic tissues. This result was obtained with two non-overlapping probes from the coding region. No staining was observed with the control sense-strand probe. This indicates that \textit{NF1} transcripts are present in most, if not all, cell types.

Monoclonal antibodies recognizing \textit{NF1} had been previously generated by the MGH monoclonal antibody facility. We have used these antibodies to stain embryos and tissues from third instar larvae and adult flies. \textit{NF1} is expressed at low to moderate levels in most, if not all cell types but is expressed at somewhat higher levels in the proliferative centers of the larval brain and in the ovaries. Flies that express no \textit{NF1} protein (see below) were used as controls and show no staining under the same experimental conditions. Since \textit{NF1} is widely expressed, it is likely that it has a function that is relevant to most cell types.

\textbf{Generation of mutants in \textit{Drosophila NF1}}

The most direct way of testing the function of a gene is to generate mutations that disrupt its function. Additional information can often be obtained by examining the phenotypic consequences of increasing the activity or the levels of expression of a gene product. We have utilized both of these approaches to study the function of \textit{Drosophila NF1} in vivo.

Fig 3. Map of the 96F region showing the approximate extent of \textit{Df(3R)boss}\textsuperscript{3} and \textit{Df(3R)boss}\textsuperscript{16} deficiencies and the location of the \textit{T(2,3)E(spl)BX21} translocation. In the schematized chromosomes filled boxes indicate intact DNA, hatched boxes regions harboring rearrangements, and open boxes deleted segments. The transcription map shows the location of \textit{boss}, an unidentified transcript \textit{D} (12), \textit{NF1}, and \textit{E(spl)}. The filled circle indicates the location of a \textit{P} element in the K33 strain. \textit{cNF1} is a cosmid; R5.17, R12, 12A, and L3 are phage clones.

In order to generate loss-of-function mutations in \textit{NF1}, we first determined the chromosomal location of \textit{NF1}. \textit{In situ} hybridization of salivary gland chromosomes mapped \textit{Drosophila NF1} to cytogenetic interval 96F-97A on the right arm of the third
chromosome. Since DNA spanning this entire region has been cloned, we mapped cosmid clones from the region and were able to determine the precise location of NF1 with respect to transcription units in the region. The Drosophila NF1 gene (Figure 3) is located between the bride of sevenless gene (12), and the Enhancer of split gene complex (13).

Our initial approach to obtaining loss-of-function mutations in Drosophila NF1 was based on the assumption that NF1 gene function was required for viability. This seemed likely since homozygous loss of murine NF1 is lethal, and because any combination of deletions that removed both copies of the NF1 gene was also lethal. We therefore screened the 96F region for lethal mutations that were uncovered by the Df(3R)boss16 chromosome (Figure 4) using chemical mutagenesis. After screening 13,500 flies we isolated 60 lethal or semi-lethal mutations in 6 complementation groups. None of these complementation groups comprised mutations in NF1. We have since confirmed that loss-of-function mutations in NF1 are indeed not lethal (see below).

Since the results of our screen suggested that mutations in NF1 did not cause lethality, we chose an alternate strategy which made no assumptions about the nature of the mutant phenotype. We chose to generate mutations by "local hopping" of P-element transposons. We identified a line (K33), which harbored a P[white] element approximately 25 kb downstream of NF1 (Figure 3). To generate hops into NF1, this transposable element was mobilized by crossing these flies to a line containing a stable source of transposase. Following the scheme shown in Figure 4, lines of flies which contained putative local hops were set up, DNA was prepared from their progeny and screened using a variation of the polymerase chain reaction (inverse PCR) to detect transpositions into the immediate vicinity of the NF1 gene (14). Of 1600 lines screened, seven had novel integrations in the immediate vicinity of NF1. Five of the integrations occurred 3' to the coding region while two integrations disrupted the 5' part of the gene. The rearrangements caused by the P-element insertions were initially characterized by Southern blotting and subsequently DNA flanking the P-element insertions was amplified by PCR. Sequence analysis of the amplified DNA enabled us to determine the precise site of integration of the P-element. In one case (allele NF1P1), the P-element insertion has been accompanied by a deletion event that has removed all but the first exon of the NF1 gene. The 3' end of the deletion is the site of integration of the original P-element in the K33 line. In the second mutant allele (NF1P2), the P-element has integrated in the first intron of NF1 in the absence of any obvious additional rearrangements.

![Diagram](image_url)

Figure 4. Scheme for the generation of "local hops"
Both mutations are viable when homozygous. However, the \textit{NF1} homozygotes are significantly (~20-30%) smaller than wild-type flies during all post-embryonic stages of the life cycle. Furthermore, each mutation has the same phenotype in trans to a deletion of the region as when homozygous and hence behaves as a null by genetic criteria. Since \textit{NF1}^P1 has deleted virtually all of the \textit{NF1} gene, it clearly represents a null allele. This argues strongly that the \textit{NF1} gene is not essential for viability. Since both mutations are viable when homozygous, we prepared extracts from homozygous mutant embryos and examined them by Western blotting using the monoclonal antibodies generated against the C-terminal part of the protein. As shown in Figure 5, no \textit{NF1} protein, which migrates at approximately 280 kDa is detectable in embryonic extracts prepared from either \textit{NF1}^P1 or \textit{NF1}^P2 embryos. This finding coupled with the observation that for either allele the hemizygous phenotype is the same as the homozygous phenotype indicates that both alleles are likely to represent a complete loss of function.

Figure 5: Lack of \textit{NF1} protein in homozygous \textit{NF1}^P1 and \textit{NF1}^P2 embryos

\textbf{Analysis of \textit{NF1} mutants}

\textit{NF1} mutants are smaller than wild type animals as larvae, as pupae and as adults (Figure 6b and not shown). Since a reduction in size might reflect a reduction in cell size or a reduction in cell numbers, we compared the wings of wild type and mutant animals. The linear dimensions of wings of \textit{NF1} mutants are 25-30\% smaller than those of wild type flies (Figure 6c and d). Since each wing epidermal cell secretes a single hair, cell densities can be determined by counting the number of hairs in a defined region. Both homozygous \textit{NF1} mutants had a 25-30\% higher wing epidermal cell density compared to the parental line (Figure 6e). However, the same is not true in other tissues, since the eyes of \textit{NF1} mutants show a reduced number of ommatidia of normal size and structure (Figure 8 and not shown). Similarly, \textit{NF1} deficient embryos are not reduced in size (not shown).
Figure 6. *NF1* mutants exhibit a size defect which is rescued by expression of a *hsNF1* transgene, and which in wings reflects a reduced cell size. Panel A shows immunoblots of embryo extracts probed with monoclonal antibody DNF-21. The arrow indicates an immunoreactive protein migrating around 280 kDa that is present in the K33 parental strain, but absent in either *NF1P1* or *NF1P2*. A transgenic strain harboring a second chromosome *hsNF1* transgene significantly over-expressed a 280 kDa immunoreactive protein even without heat shock induction (right lanes, panel A). The shorter exposure time makes the endogenous protein hard to detect in the transgenic blot. Panel B shows wandering third instar larvae (top) and pupae (bottom) of the indicated genotypes. The *hsNF1* strain was heat shocked at 37°C for 30 minutes daily. Panels C and D show wings from K33 and *NF1P1* flies, respectively. The graph in panel E shows the number of wing epidermal cells/mm² in the indicated strains.

To determine whether the reduced size of wing epidermal cells reflected a cell autonomous defect, we used X-irradiation to induce mitotic recombination in the wings of heterozygous *NF1* mutants, using the markers *bold* and *forked* to distinguish homozygous mutant clones from surrounding tissue which was heterozygous for *NF1*. No difference in the distance between wing hairs was observed between multiple *NF1-*/+ clones and surrounding cells (Figure 7). Thus the reduced size of wing epidermal cells reflects a non-cell autonomous requirement for *NF1*.
NF1 mutants also display a subtle behavioral defect characterized by a diminished escape response. Thus, in an assay that determined the number of flies that fly away within 90 seconds of release or after repeated gentle agitation at 15-second intervals between 13 to 16% of either NF1 mutant (n=200) failed to respond, as compared to 3% non-responders for the parental K33 strain. This reduced escape rate does not reflect obvious anatomical defects of the peripheral nervous system or the musculature, and the mutants scored within normal parameters in assays which measured their activity or their response to visual or olfactory stimuli. In a collaborative effort with the laboratory of Dr. Yi Zhong (Cold Spring Harbor Laboratory), we demonstrated that neuropeptide-stimulated synaptic transmission is abnormal in NF1 mutants, which may explain the diminished escape response (see below).

Interaction of NF1 with the Ras-mediated signalling pathway

Since mammalian neurofibromin is considered to be an important negative regulator of Ras in vivo, we examined for abnormalities in Ras1-mediated signalling. Surprisingly, several Ras1-mediated signalling pathways appear to function normally in NF1 mutants. The pattern of tailless expression (15) is normal (Figure 8a and b), indicating that torso-mediated signalling is not perturbed. To test for abnormalities in sevenless signalling (16, 17), we examined the retinas of mutant animals. In homozygotes of NF1P1, which also lack part of the neurogenic Enhancer of split complex, 25% of ommatidia have one or more additional photoreceptor cells (not shown). However, this phenotype is probably due to loss of some of the Enhancer of split transcripts, since the retinas of NF1Pz homozygotes, of NF1P2/NF1P1 and of NF1P2/Df(3R)boss15, a deficiency that uncovers the NF1 locus, are completely wild type (shown for the NF1Pz homozygote in Figure 8c). A particularly sensitive indicator of sevenless pathway function is the sevE4, Sos/J2/+ mutant combination (18). Only 16.4% of ommatidia in this double mutant have R7 cells and this number is very sensitive to alterations in the gene dosage of Ras1 pathway components. Flies of this genotype which are also heterozygous for NF1Pz have no significant alteration in the
percentage of R7 containing ommatidia (not shown). Thus two well defined Ras1-mediated signalling pathways are not particularly sensitive to NF1 gene dosage.

Figure 8: torso- and sevenless-mediated signalling is not affected by loss of NF1. Panels A and B show tailless expression detected by RNA in situ hybridization in K33 and NF1<sup>p2</sup> embryos, respectively. Panel C is a retinal section from a NF1<sup>p2/NF1<sup>p2</sup></sup> adult, showing regularly spaced ommatidia of normal size with the normal complement of photoreceptor and accessory cells.

To test whether NF1 might have a redundant function in regulating Ras1-mediated signalling, we attempted to generate flies that were mutant for both NF1 and for another Drosophila RasGAP homologue, Gap1 (7). Five tested viable heteroallelic combinations of four Gap1 alleles (Gap1<sup>A13p</sup>, Gap1<sup>Ae-1</sup>, Gap1<sup>Ae-2</sup>, Gap1<sup>30</sup>) were each lethal in combination with loss of NF1 function. Expression of hsNF1 rescued this lethality (not shown). Thus while either RasGAP is not essential for viability, loss of both is lethal, arguing that these proteins have redundant functions in regulating at least one essential signalling pathway.

The size defect of NF1 mutants may reflect the inability of other RasGAPs to compensate for loss of NF1 in regulating a particular Ras1-mediated pathway. If so, then reducing the gene dosage of Ras1 pathway components may influence this phenotype. However reducing the dosage of Ras1 or of Sos by 50% had no effect on the size of NF1 mutant pupae, nor did crossing in an activated Raf<sup>gef</sup> mutation (19) (Figure 9). Neither reducing nor increasing signalling through the Ras1-Raf pathway therefore modifies the NF1 phenotype. Since human neurofibromin can also stimulate the GTPase activity of R-Ras, we also tested the effects of reducing the dose of the Drosophila R-Ras homologue, Ras2. Once again no effect was observed (not shown). Thus, the small size phenotype may result from a property of NF1 independent of its function as a GAP for Ras-like GTPases.

**Interaction of NF1 with PKA-mediated signaling**

Since among RasGAPs neurofibromin is most closely related to IRA proteins, which in yeast function in a pathway in which Ras couples to adenyl cyclase, and because Ras1 and cAMP-dependent synaptic signalling is defective in Drosophila NF1 mutants (accompanying paper), we tested whether cAMP-mediated signalling might represent an alternate target for NF1 function. NF1 mutants that were also heterozygous for two different loss-of-function mutations in the protein kinase A (PKA) catalytic subunit homologue DCO did not show any alteration in their phenotype (20). However, the small
size of NF1 mutants was largely rescued by crossing in a constitutively active murine
PKA* transgene (21)(Figure 9). Heat shock induction of this constitutively active PKA
mutant resulted in lethality. However, significant rescue of the pupal size defect was
already observed in cultures grown at 25°C, presumably reflecting leaky transgene
expression. As for the size defect, the neurotransmission defect is also rescued by
manipulating cAMP levels (see below). This indicates that activation of PKA can bypass a
complete loss of NF1 function in at least two different situations in vivo.

![Graph showing pupal length](image)

**Figure 9:** Rescue of the pupal size defect of NF1 mutants by hsNF1 and activated PKA,
but not by activated raf. The graph presents the average length of pupae plus or minus the
standard deviation. The genotypes analyzed were: 1; K33/K33 (parent stock) 2;
NF1P1/NF1P1, 3; NF1P2/NF1P2, 4; hsp70-PKA*, K33/K33, 5; hsp70-PKA*;
NF1P1/NF1P1, 6; hsp70-PKA*, NF1P2/NF1P2, 7; hs-raf*, NF1P1/NF1P1, 8; hs-raf*;
NF1P2/NF1P2. Between 50 and 90 pupae were measured for each genotype.

**Role of NF1 in synaptic neurotransmission**

A potential explanation for the abnormal behavior of the NF1 mutants is that
neuromuscular function is abnormal. This seems especially likely in view of the recent
observation by Dr. Yi Zhong (Beckman Center for Memory and Learning, Cold Spring
Harbor Laboratory), that Ras1 and Raf play a crucial role in synaptic transmission at the
larval neuromuscular junction. Dr. Zhong has demonstrated that a specific synaptic current
at the neuromuscular junction requires the function of both Ras1 and Raf (22).
At the neuromuscular junction of the larval body wall, a neuropeptide related to the mammalian pituitary adenyl cyclase-activating polypeptide (PACAP38) functions as a neurotransmitter. Application of PACAP38 simulates the effects of high frequency stimulation of the motor axons. This leads to a biphasic response; a slow inward current which leads to a depolarization of the muscle membrane and subsequently a 100-fold increase in voltage-activated K⁺ conductance (Figure 10). The inward current leads to depolarization of the muscle membrane. The subsequent enhancement of K⁺ conductance probably functions either to retard the generation of action potentials or to shorten the duration of the action potential.

![Figure 10. Electrophysiological tracings showing K⁺ conductance changes after PACAP38 stimulation in wild type and Rasl mutant larvae. See (22) for details.](image)

In larvae that are mutant for either Rasl (Figure 10) or Raf, the enhancement of the K⁺ conductance is undetectable following the addition of 1mM or 10 mM of PACAP38. By examining the currents in larvae bearing gain-of-function Raf alleles, it was demonstrated that stimulation of the Ras1/Raf pathway is necessary but not sufficient for the K⁺ current. Similarly, mutants in the cAMP pathway abolish the K⁺ current but the application of cAMP analogs alone is insufficient to generate the current. Activation of both the Ras1/Raf pathway and the application of cAMP analogs is sufficient to generate the current. This suggests that signaling via both pathways is both necessary and sufficient for generating the K⁺ current (Figure 11).

![Figure 11: Proposed model for regulation of post-synaptic potential](image)
In collaborative experiments with Dr. Zhong's laboratory, we have shown that the enhancements of K⁺ conductance is completely abolished in both mutant alleles of NF1. This defect is completely rescued by crossing in an NF1 transgene under the control of the inducible hsp70 promoter. Application of two different membrane-permeable cAMP analogs, dibutyril cAMP or 8-bromo-cAMP to the larval neuromuscular preparation restores the normal response to PACAP38. cAMP analogs were effective if applied anytime prior to and within two minutes of applying PACAP38. To further test whether activation of cAMP signaling rescues the defective PACAP response of NF1 mutants, the drug forskolin, which stimulates G-protein coupled adenylyl cyclase activity was applied to the neuromuscular preparation. PACAP38 induced a normal response in NF1 mutants after forskolin incubation, similar to the effect of cAMP analogs.

Thus, as with the size defect, the defect in neuromuscular transmission can be rescued by activating the PKA pathway - in this case pharmacologically.

DISCUSSION

We have cloned and characterized a highly conserved Drosophila NF1 homologue. The high degree of sequence conservation throughout the protein as well as the conserved location of introns in the gene argue that it is indeed a true homologue. The lack of evidence for any other NF1-like genes in Drosophila despite screening by low-stringency hybridization is indicates that this gene is likely to represent the only NF1 gene in Drosophila. Analysis of the expression of NF1 RNA and protein demonstrated that the gene was expressed in most, if not all, tissues throughout development arguing that NF1 is unlikely to be a tissue specific signaling protein. In particular we found no evidence that NF1 expression was higher in neural tissues.

At the onset of this study, we considered it likely that mutations in Drosophila NF1 were likely to be lethal. This was based on the evidence that mutations in NF1 in mice cause lethality during embryonic development (23, 24). We therefore saturated the region spanning the NF1 locus for lethal mutations. However, to our surprise, none of the lethal mutations corresponded to NF1 mutations. We subsequently isolated mutations in NF1 by mobilizing P-element transposons and screening by PCR for insertions in the NF1 locus. Analysis of the mutant alleles confirmed that complete loss of function mutations are indeed viable. Thus mutations in Drosophila NF1 do not appear to compromise a number of Ras1-mediated signaling pathways that are essential for normal development and viability. We do not understand why mutations in murine NF1 are lethal while mutations in Drosophila NF1 are not. One possibility is that many of the essential functions of Drosophila NF1 can be carried out by other RasGAPs in vivo. In support of this hypothesis is our finding that the double mutant combination of NF1 and Gap1 is lethal in Drosophila.

Our studies demonstrate that NF1 is not a crucial and global negative regulator of Ras1 function in vivo. We tested for the efficacy of Ras1-mediated signaling in a variety of genetic backgrounds which are extremely sensitive to variations in signaling strength. We were unable to find any evidence of abnormalities in either torso or sevenless signaling in NF1 mutants implying that NF1 does not modulate either of these pathways significantly in vivo. Our future studies will attempt to analyse the function of these pathways in double mutant combinations (mutations in NF1 and other RasGAPs).

We have shown that NF1 is necessary for normal growth and for normal neuromuscular function in vivo. This study represents the first demonstration that NF1 is required for either of these processes. Since the effect on growth of the wing is non cell
autonomous, NF1 may be necessary in a group of cells that regulate the growth of the organism. These may be cells that secrete one of the hormones that regulates the growth of Drosophila. In the future, we propose to use the UAS-GAL4 system (25) to map the cells that require NF1 in vivo for normal growth regulation.

By far the most novel finding of our studies is the demonstration of cross-talk between NF1 and the PKA-mediated signaling pathway. We have been able to demonstrate this phenomenon in two completely different experimental contexts. Firstly, we were able to show that the growth defect could be significantly suppressed by crossing in a constitutively active PKA subunit. Secondly, the neuromuscular defect could be corrected by pharmacological activation of the PKA pathway. We presently do not understand the precise nature of the interaction between NF1 and PKA. Since activation of PKA can suppress a phenotype resulting from a complete loss of NF1 function, PKA cannot function upstream of NF1; PKA must function either downstream of NF1 or in a parallel pathway. Our future studies will employ techniques such as the yeast two hybrid system, which can identify protein-protein interactions to test potential interactions between components of the PKA pathway and NF1.

Since both the Ras and PKA pathways are highly conserved between Drosophila and humans, it is likely that human NF1 will also interact with PKA-mediated signaling. While caution is necessary in extrapolating results obtained in Drosophila to a vertebrate context, most signaling pathways studied in Drosophila appear to be essentially identical to their vertebrate counterparts. If so, agents which increase PKA activity may eventually play a role in the treatment of human type 1 neurofibromatosis.

CONCLUSIONS

1) Drosophila has a highly conserved NF1 homolog
2) Drosophila NF1 is expressed at most, if not all, stages of development in most tissues
3) Loss of function mutations in NF1 are not lethal but cause lethality in combination with mutations in Gap1
4) Many Ras1-mediated signaling pathways function normally in NF1 mutants
5) NF1 mutants show defects in organismal growth and in neuromuscular transmission
6) Both defects can be suppressed by activation of the PKA pathway

References


**Publications from this effort**


**Meeting Abstracts**


**Personnel paid from this grant**

1) Iswar K. Hariharan  P.I.
2) Andre Bernards  co-P.I.
3) S. Reginald  research technician
4) A. J. Snijders  graduate student/technician
5) S. Inge The  graduate student/technician
6) S. Brill  post-doctoral fellow
7) K. Graber  research technician

Only Drs. Hariharan and Bernards were funded for the full duration of the grant. Two other personnel were funded from this source at any time. Ms. The replaced Ms. Reginald and Dr. Brill replaced Dr. Snijders. Ms. Graber was hired during the last 5 months of the funding period to replace Dr. Brill.