

Rab3 and Synaptic Release in *Caenorhabditis elegans*

**Undergraduate Honors Thesis
University of California at Berkeley**

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RAB 3 AND SYNAPTIC RELEASE IN *C. elegans*.

I. Project Summary

Over the last ten years, a great deal of progress has been made in our understanding of the chemical synapse. Despite this progress large deficits remain in our knowledge of the precise molecular mechanisms involved in synaptic release and its regulation. The aim of my project is to elucidate the function of Rab3, one of the genes involved in synaptic release.

Rab3 is a ras-like small GTP binding protein localized to synaptic vesicles. Different members of the Rab gene family are found on many different types of secretory vesicles. The protein is most likely involved in maintaining unidirectional targeting of synaptic vesicles to the active zone. I have used a reverse genetic approach to determine the function of Rab3 in *C. elegans* synaptic release.

Mike Nonet, a post-doc in the Meyer Lab, used degenerate PCR primers designed from conserved regions of the gene to obtain two *C. elegans* cDNA's. The *C. elegans* gene product shares 73% amino acid identity with human Rab3a. The gene is located on the left arm of chromosome II between *lin-4* and *bli-2*. Nonet made a polyclonal antibody directed against the *C. elegans* protein. Wild-type animals show staining exclusively in the nerve processes of the nervous system. I generated animals containing extrachromosomal arrays of the Rab3 homologue and showed that they overexpress the gene and exhibit staining of nerve cell bodies, which is never observed in wild-type animals. Overexpressing Rab3 causes no gross phenotype.

To elucidate the function of Rab3 in the worm, I generated two site-directed mutations based on mutations shown to act dominantly in Sec4 and Ras p21. One mutation is a Thr to Asn substitution at position 36, analogous to Asn17 in Ras p21. The second mutation is an Asn to Ile substitution at position 135, analogous to Ile133 in Sec4. Transgenic animals with either mutant gene are resistant to aldicarb, an inhibitor of

acetylcholine esterase. Presumably the resistance is due to decreased synaptic efficacy, which prevents the hyperstimulation and death aldicarb causes in wild-type animals. Rab3 - T36N confers no other visible phenotype.

Rab3 - N135I prevents localization of both mutant and wild-type protein to nerve processes and causes staining of only cell bodies. Staining with an antibody to synaptotagmin, a synaptic vesicle associated integral membrane protein, showed that synaptic vesicle production and transportation is normal. The Rab3 - N135I array caused animals to be uncoordinated, with 15% of animals never developing past L1. Simultaneously overexpressing both Rab3 - N135I and wild-type Rab3 proteins restores staining of nerve processes, but does not rescue the Unc phenotype. Thus it seems likely that it is the dominant action of the mutant protein that disrupts nerve function and not simply non-specific interference with subcellular localization. I am utilizing the aldicarb resistance of Rab3 mutants to selectively screen for chromosomal Rab3 mutants. The screen consists of mating mutagenized males into a strain with a deficiency that covers Rab3 and testing the F1's for aldicarb resistance.

II. Introduction to Rab3a

Neurons are unique in their ability to communicate rapidly over long distances. The synapse is the vital link between nerve cells. Synaptic vesicles at the presynaptic nerve terminal fuse and dump neurotransmitter into the synaptic cleft. Neurotransmitter receptors at the post-synaptic nerve terminal respond to the chemical signal and initiate action potentials that can travel the length of the nerve. Synaptic release is a crucial component of animal nervous systems. Proper regulation of neurotransmitter release is vital to maintaining the integrity of the system as well as allowing for the plasticity needed to adapt to a changing environment. Over the last ten years, a great deal of progress has been made in understanding the functioning of the chemical synapse (Kelly, 1993). In spite of this

progress, large deficiencies remain in our knowledge of the precise molecular mechanisms involved in synaptic release and its regulation.

Rab3 and the small GTP-binding family

Rab proteins are members of the ras superfamily of small GTP-binding proteins and are involved in the control of several types of vesicular transport. Membrane-bound Rab3 in the brain is associated exclusively with synaptic vesicles (Fischer v. Mollard et al., 1990). Rab3 is also expressed in exocrine tissue, where it is located on microvesicles along with other synaptic vesicle proteins. The Rab3 protein is attached to the synaptic vesicle via a polyisoprenylated carboxy-terminal post-translational modification. Like most small GTP-binding proteins the Rab3 protein is present in soluble and membrane-bound pools, approximately 70% is membrane-bound under normal circumstances. Rab3 is not associated with any of the early stages of the secretory pathway (i.e. the Golgi complex) (Matteoli et al., 1991). This demonstrates that Rab3 associates with synaptic vesicles only after they are formed by the Golgi Complex. After synaptic vesicle exocytosis in synaptosomes (pinched off nerve terminals), Rab3 is lost from synaptic vesicle membranes. This suggests that Rab3 cycles between its bound and unbound forms as the synaptic vesicle membrane cycles between exocytosis and endocytosis. If endocytosis is blocked by removing extracellular calcium, massive exocytosis of synaptic vesicles results in Rab3 localization on the plasma membrane.

Model for Rab3 function in synaptic vesicle trafficking

The present working model for Rab3 action is based on the model developed for Sec4, a homologue of Rab3 in yeast (Walworth et al., 1989). Sec4 is an essential gene required for the last stage of secretion in yeast. Sec4 mutants cause a build up of secretory vesicles. The hydrophobic modification has been shown to be required for Sec4 function. Sec4 - Ile133 is a dominant mutation, which results in a mutant protein that does not detectably bind GTP. The model proposed to explain this data is as follows (see figure 1): Sec4 - GDP is in a soluble form in the cytoplasm. When the GDP is exchanged for GTP the

N.C.Walworth *et al.*

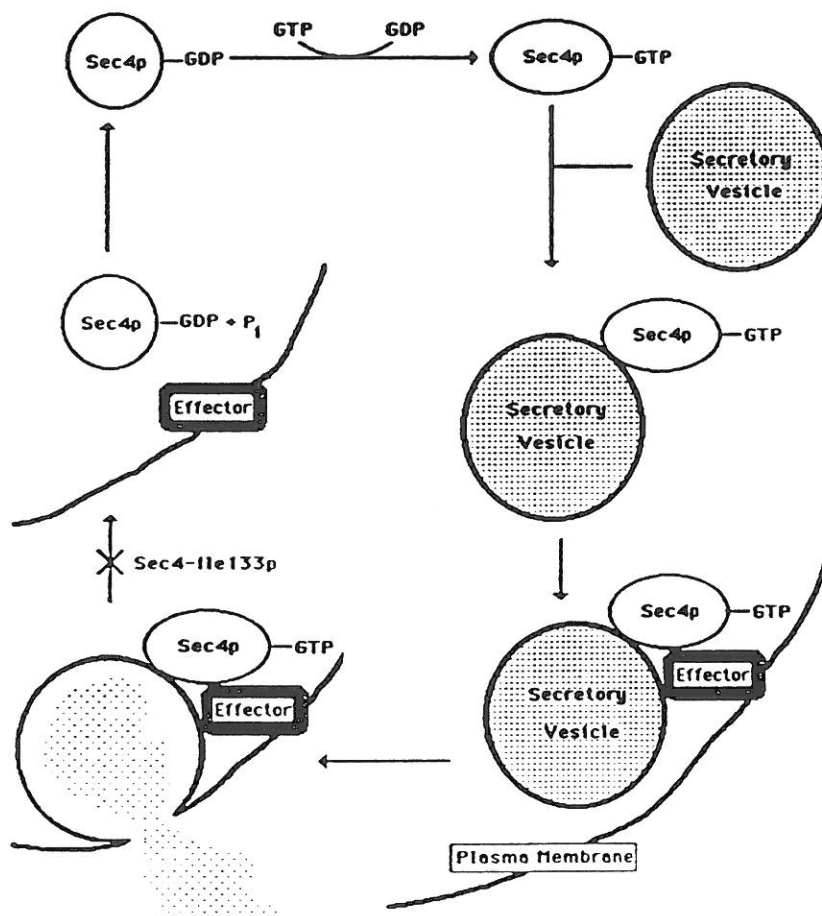


Figure 1. Model of Sec4 Action.

Sec4 - GTP protein is attached to the secretory vesicle. The vesicle then becomes docked at the plasma membrane. When the vesicle fuses with the plasma membrane, or soon afterwards, the GTP is hydrolyzed into GDP. The Sec4 - GDP then dissociates from the membrane and once again is soluble Sec4 - GDP in the cytoplasm. Presumably there are many regulatory factors that influence the Sec4 cycle. Many of these factors have been studied genetically, but there is little biochemical evidence for specific roles.

Regulation of Rab3

Three factors have been partially characterized biochemically that appear to specifically interact with Rab3. A Rab3 GDP dissociation inhibitor (GDI) has been purified and cloned (Matsui et al., 1990). The Rab3 GDI inhibits the dissociation of GDP and subsequent replacement with GTP. Rab3 GDI also inhibits the attachment of GDP bound Rab3 to synaptic membranes, but does not inhibit the binding of GTP bound Rab3 to synaptic membranes. Thus Rab3 GDI appears to stabilize the soluble GDP-bound form of Rab3. An activity, called guanine nucleotide-releasing protein, (GNRP) has been shown to accelerate by ten fold the release and subsequent binding of guanine nucleotides (Burnstein and Macara, 1992). Finally a protein called rabphilin-3a has been purified and cloned (Shirataki et al., 1993). Rabphilin-3a complexes specifically with the GTP bound form of Rab3, but not with the GDP bound form. Rabphilin-3a has two repeats homologous to the C2 domain of protein kinase C. These same domains are found in synaptotagmin, which is localized on synaptic vesicle membranes, and are presumably involved in calcium-dependent binding of phospholipids. Figure 2 incorporates these factors into the model proposed for Sec4.

C. elegans is a powerful organism in which to study nerve function because it combines well developed genetics with a simple nervous system of only 302 neurons. The worm is particularly well suited for studying severe mutations affecting the nervous system because the majority of the nervous system is not required for viability and fertility under laboratory conditions.

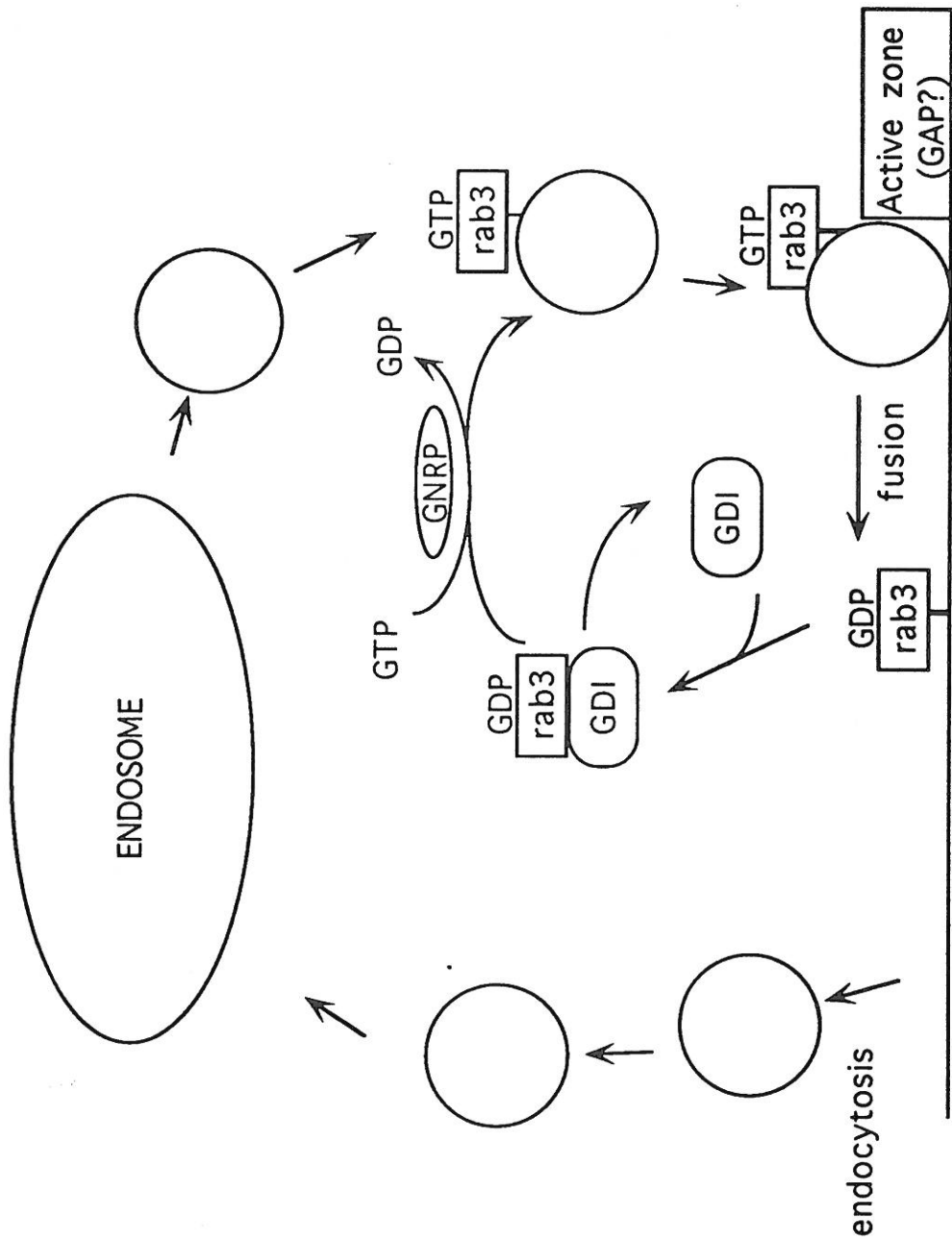


Figure 2. Model of Rab3 Action.

III. State of Project before I started

A partial cDNA of the *C. elegans* Rab3 homologue (See figure 3) was obtained by Mike Nonet, a post-doc in the Meyer lab, using degenerate PCR primers designed from highly conserved regions of the gene. The cDNA was missing only the extreme 5' end. RACE-PCR was used to obtain two alternatively spliced cDNA's that include the 5' end of the coding region (see figure 4). The two 5' alternative splices do not alter the coding region in any way. The *C. elegans* Rab3 homologue shares 73% amino acid identity with human Rab 3a and 76% identity with *Drosophila* rab (see figure 5). The Rab3 coding region minus the first four amino acids was expressed and used to generate mouse polyclonal antibodies against the protein. This antibody stains only the nerve processes of the *C. elegans* nervous system (see figure 6). The three areas that stain are the nerve ring (the region of the nervous system with the highest density of synaptic connections), the dorsal and ventral nerve cords and the pharyngeal nervous system. *Unc-104* encodes a kinesin-like motor believed to be used in axonal transport of synaptic vesicles. Severe *Unc-104* mutants accumulate synaptic vesicles in the neuronal cell bodies. Staining *Unc-104* mutants with the Rab3 antibody resulted in staining of neuronal cell bodies with very little staining of the nerve processes. Mike used the worm physical map to localize the Rab3 homologue to two overlapping cosmids, T09H9 and F11G1 (see figure 7). These cosmids are located on the left arm of chromosome II between *clr-1* and *lin-4*.

IV. My Honors Thesis Project

Localization of the Rab3 homologue to chromosome II

I narrowed the interval within which Rab3 could be located using PCR amplification of dead homozygous deficiency eggs to determine which of two deficiencies in the region,

Figure 3. *C. elegans* Rab3 gene (0.9kb probe is underlined).

1	ggtttaattacccaagtttgaggcggcggaaacgaagaagaccatgaataatcaacaggc	60
61	tgccattgcatccgccagaagtcgaatggcggctggcggacaacctcaaggcgctacacc	120
	M A A G G Q P Q G A T P	
121	<u>gggacaacccgatcagaactttgactacatgttcaagctcctgataatcggaattcatc</u>	180
	G Q P D Q N F D Y M F K L L I I G N S S	
181	<u>agttggaaaaacatcattcctcttccgttactgtgatgattcattcacttctgccttcgt</u>	240
	V G K T S F L F R Y C D D S F T S A F V	
241	<u>ctctactgtcggaaatcgattttcaaagtgaaaactgtgttccgtggagacaaacgagtcaa</u>	300
	S T V G I D F K V K T V F R G D K R V K	
301	<u>acttcaaactctgggataccgcccggacaggagaggtaccgtaccatcaccaccgcctacta</u>	360
	L Q I W D T A G Q E R Y R T I T T A Y Y	
361	<u>tcgtggagcaatgggattcattctgatgtatgacatcactaatgaagagtcttttaatag</u>	420
	R G A M G F I L M Y D I T N E E S F N S	
421	<u>tgttcaggattgggtgcactcaaactcaagacatactcatgggaaaatgctcaagttgtttt</u>	480
	V Q D W C T Q I K T Y S W E N A Q V V L	
481	<u>ggttggaaataaatgtgatatggactctgaaagagttgtatctatggataggggacgcca</u>	540
	V G N K C D M D S E R V V S M D R G R Q	
541	<u>acttgctgatcaacttggtttgggaattcttcgaaacatcagccaaggagaacattaatgt</u>	600
	L A D Q L G L E F F E T S A K E N I N V	
601	<u>aaaggcagtttttgagaagttggaggagattatttgtgataagatggcagagagtttggg</u>	660
	K A V F E K L V E I I C D K M A E S L D	
661	<u>taaggacccacagcaacagccaaaaggacagaagctcgaagcgaatccgacccaaaagcc</u>	720
	K D P Q Q Q P K G Q K L E A N P T Q K P	
721	<u>tgctcaacagcaatgcaattgctaaaaattctacgttctattatactatctataaaaact</u>	780
	A Q Q Q C N C	
781	<u>caaaattttcccaaattccaaacgcaataatattaattttctaaaagcgcgtagctctca</u>	840
841	<u>gttttccctctctcaacatctaggcaccagatatatggatcgtaaatctaacttataaatc</u>	900
901	<u>gaatttctatgattttttggaaagtaagtttttttaaatctaacttattttatttttagg</u>	960
961	<u>gcgtagttggtttaattatcttttcaaactccttttactgtcacttattaagttttcatt</u>	1020
1021	<u>cagcgctatttttattaatatttttaacttattccaacggttcaatcatattccccatttgt</u>	1080
1081	aaaaaatcaacaattttctctcaacacttccaaacaattcccagagctgtgttttctaact	1140
1141	aatcatagtcgcctattattattattttattttattgtatttctctatttcccttattt	1200
1201	cctagtgacttcccagttcccaatgattacaccaccccgctgaaagattgattttggagg	1260
1261	aaattattttcgaggcataataaattattttattacaaagagataaaaaaaaaaaaa	1314

Figure 4. Two alternatively spliced 5' ends of Rab3 cDNA.
The underlined region of B is spliced out of A.

A.)

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1              31
ga tTC TAG AAT TCC GCg gtt taa tta ccc aag ttt gag atg gcg gct ggc gga caa cct
                                     met ala ala gly gly gln pro
61              91
caa ggc gct aca ccg gga caa ccc gat cag aac ttt gac tac atg ttc aag ctc ctg ata
gln gly ala thr pro gly gln pro asp gln asn phe asp tyr met phe lys leu leu ile
121             151
atc gga aat tca tca gtt gga aaa aca tca ttc ctc ttc cgt tac tgt gat gat tcA TCG
ile gly asn ser ser val gly lys thr ser phe leu phe arg tyr cys asp asp ser ser
181
AAT TCC TGC
asn ser cys

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B.)

```

1              31
ccg atT CTA GAA TTC CGC ggt tta att acc caa gtt tga ggc ggc gga aac gaa gaa gac
61              91
cat gaa taa caa cag gct gcc att gca tcc gcc aga agt cga atg gcg gct ggc gga caa
                                     met ala ala gly gly gln
121             151
cct caa ggc gct aca ccg gga caa ccc gat cag aac ttt gac tac atg ttc aag ctc ctg
pro gln gly ala thr pro gly gln pro asp gln asn phe asp tyr met phe lys leu leu
181             211
ata atc gga aat tca tca gtt gga aaa aca tca ttc ctc ttc cgt tac tgt gat gat tcA
ile ile gly asn ser ser val gly lys thr ser phe leu phe arg tyr cys asp asp ser
241
TCG AAT TCC TGC
ser asn ser cys

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rab3 sequences

Human A	MASATDSRYGQKESDQNFDMFKILIIIGNSSVGKTSFLFRYADDSFTPAFVSTVGIDFKVKTIIYRNDKRIKLIQIWDTACQERYETITTAYRGAMGFILMYDITNEESF	110
Human B	...V...GKH·V·DA·	110
Drosophila	...GGDPK-W·DAA·	109
C. elegans	..AGGQPQGATPGQP·	110
Human A	NAVQDWSTQIKTYSWDNAQVLLVGNKCDMEDEVVSSERGRQLADHLGFEFFFEASAKONINVKQTFERLVDVICEKMSSESLDTADP-AVTCAKQGPQLSDQ-QVPPHQDCAC	220
Human BA·	219
Drosophila	·S·	220
C. elegans	·S·	219

* marks residues commonly conserved among a majority of small GTP-binding proteins of the ras supergene family.

% identity	Human A	Human B	Droso	C. elegans
Human rab3A	100	77.3	77.3	73.2
Human rab3B	-	100	75.9	69.1
Drosophila rab	-	-	100	76.3
C. elegans rab	-	-	-	100

Figure 5. Comparison of human, fly, and worm Rab3 homologs.

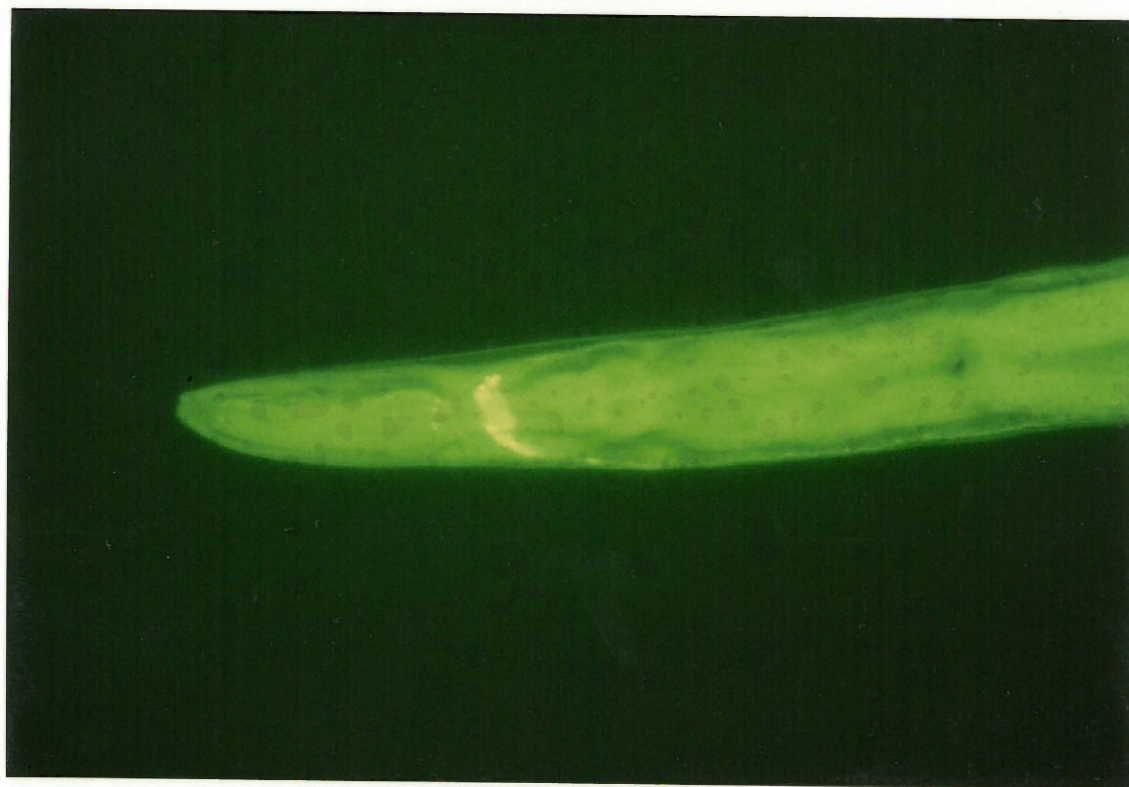
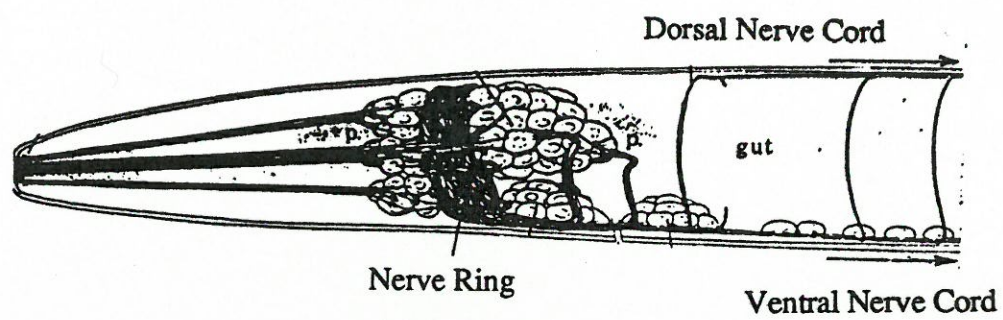
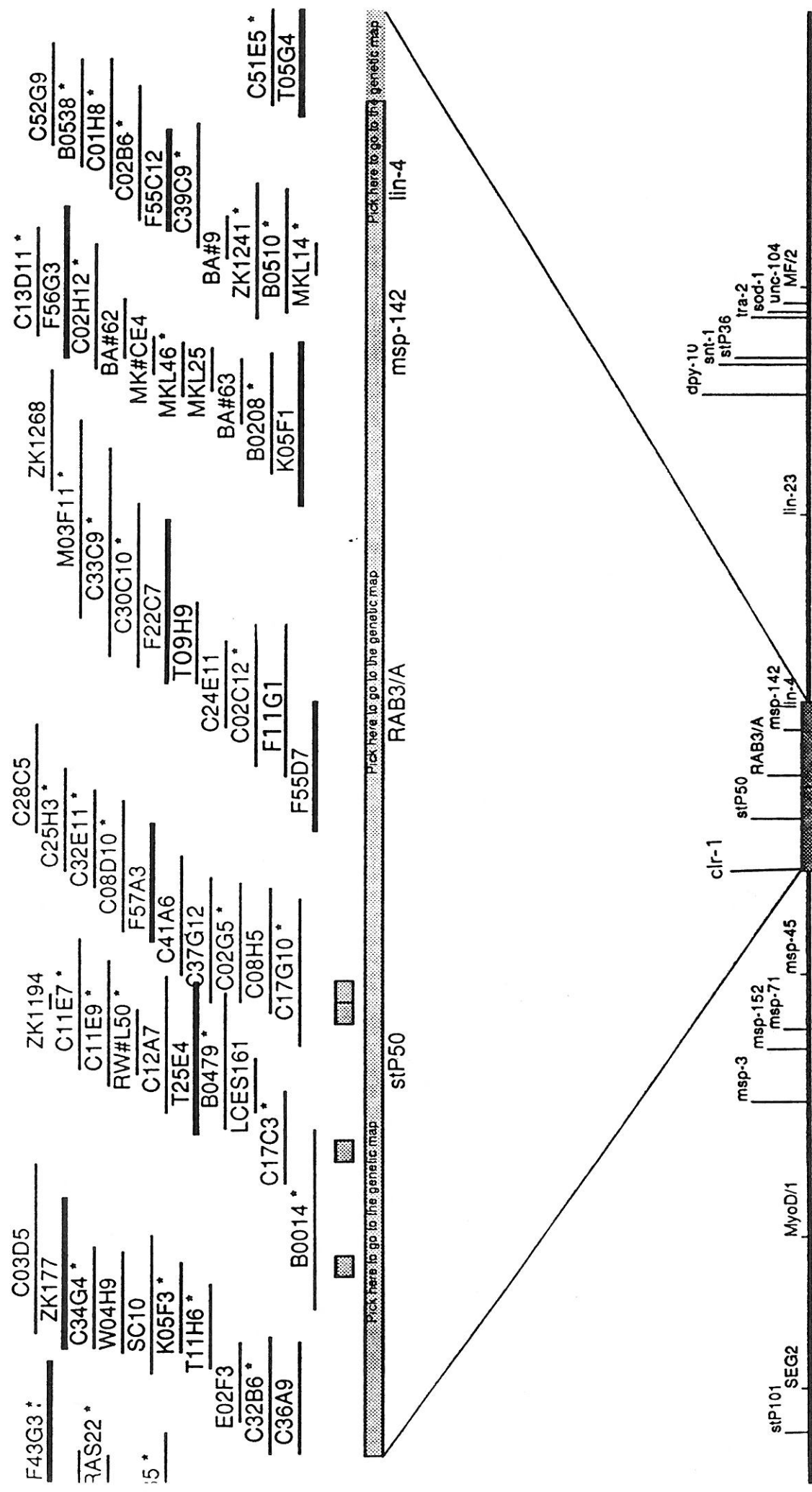


Figure 6. Wild-type animal stained with Rab3 antibody.

Figure 7. Physical Map. Rab3 is on the two overlapping cosmids, T09H9 and F11G1. *Clr-1* is on F43G3 and *Lin-4* is on C02B6.



ccDf5 and maDf4, eliminates the Rab3 homologue. The gene is deleted by maDf4 and not by ccDf5. Thus the *C. elegans* Rab3 homologue is located between *bli-2* and *lin-4* (see figure 8). There are no previously isolated mutations in this region that are reasonable candidates to be Rab3 mutants.

Cloning of Rab3 Homologue

My first priority when I began the project was to create a genomic clone of Rab3 that I could use to express wild-type and mutant Rab3 proteins in transgenic worms. I used the larger cDNA to probe Southern blots of digests of the two cosmids. I cloned two fragments that I thought would contain the entire gene. The two subclones are called pMK1, a 6.2 kb Hind III fragment, and pMK2, a 4.5 kb Bgl II fragment (see figure 9). The two fragments were ligated into pBluescript II in different orientations so that I could generate single-stranded DNA of either strand for the majority of the coding region for single-stranded sequencing. These two fragments contained 2 kb of sequence upstream of the probe. The majority of *C. elegans* introns are very small, about 50-100 bases in length. Unfortunately, this gene has a 2-3 kb intron at the extreme 5' end, which I only detected when I reprobated the Southern blots with the 5' end cDNA. Since no single restriction digest fragment contained the entire gene I added additional fragments to pMK2 to generate pMK3 and pMK4, 9.5 kb and 13 kb (excluding vector) respectively.

Because it was my long term goal to use site-directed mutagenesis to generate dominate-acting mutations in Rab 3, I needed to know the genomic sequence containing the coding region. I used primers designed using the sequence of the cDNA's to sequence the genomic region containing the Rab3 coding region, except for the most 5' five amino acids (see figure 10).

Expression of the Rab3 Homologue in *C. elegans*

To convince myself that the subclones I had made contained the entire gene and associated regulatory sequence, I generated transgenic worms that carried the Rab3 genomic clone to demonstrate that the gene was expressed using immunocytochemistry.

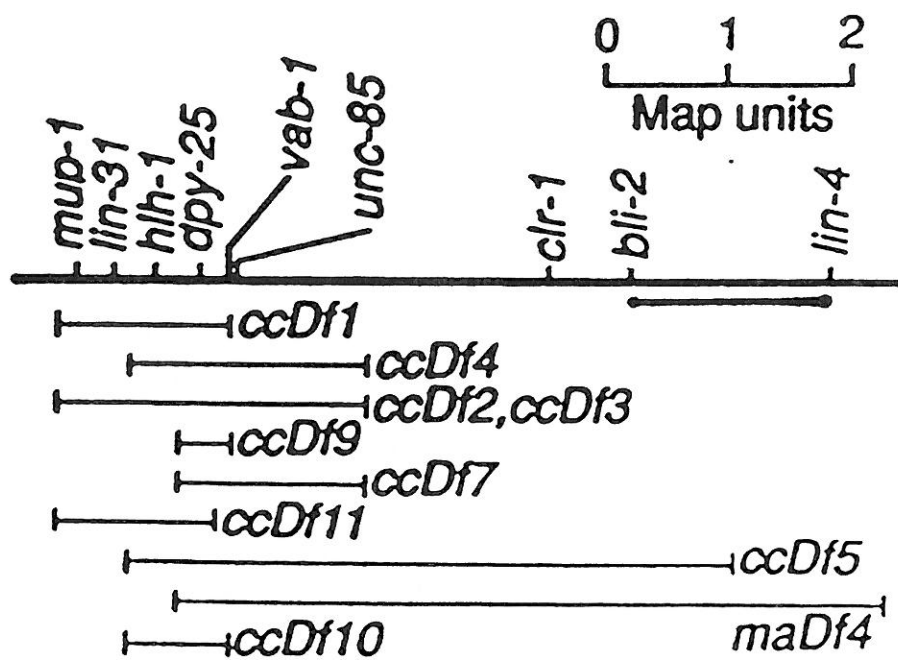


Figure 8. Physical mapping techniques require the *C. elegans* Rab3a homolog to fall within the colored region.

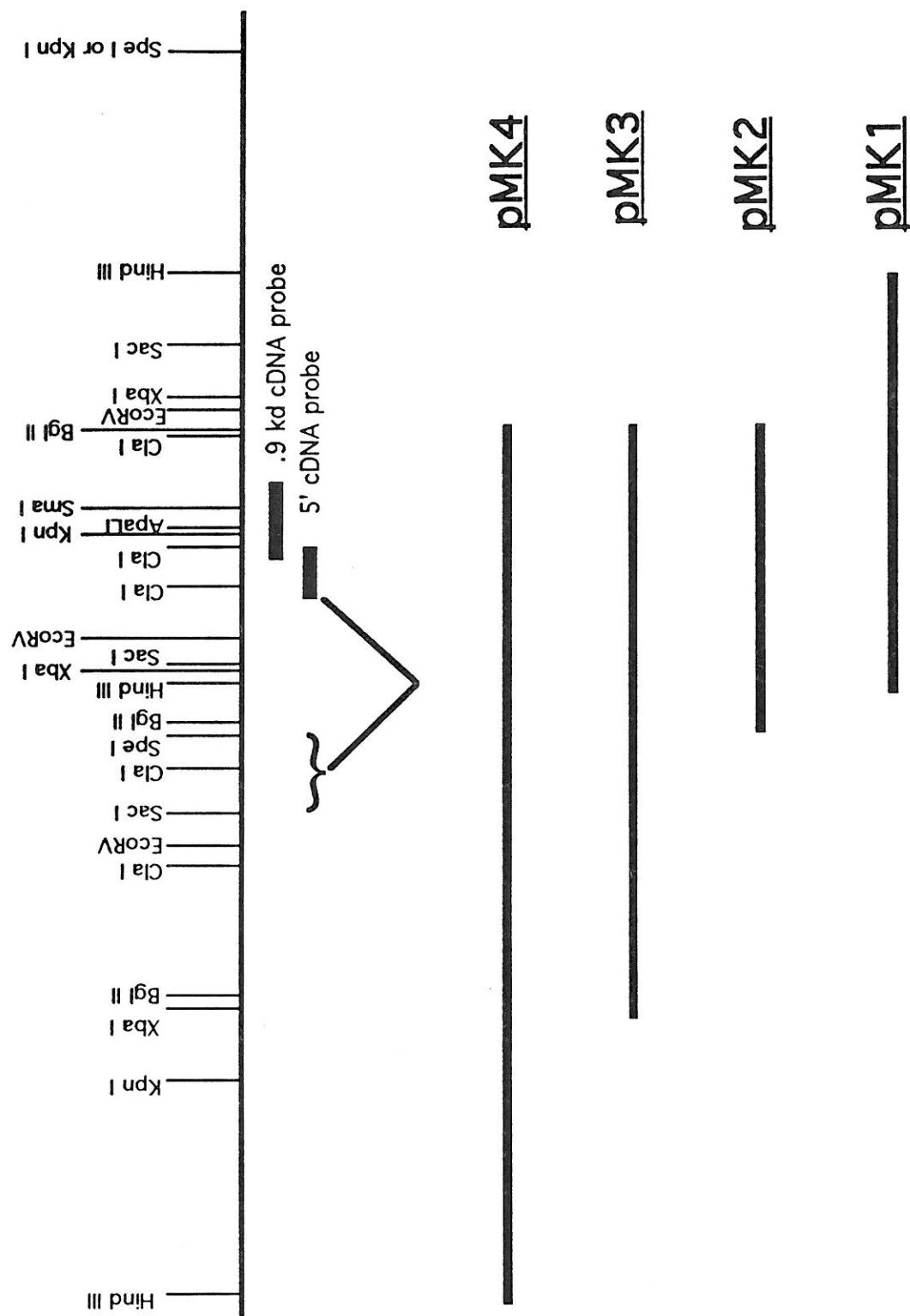


Figure 9. Restriction map of the genomic region containing the *C. elegans* Rab3a homolog. (1cm = 1kilobase.)

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      20      40      60      80
*      *      *      *
AAAACTAAT TGGCGGGGAA TCTGAAGATT GGGATAAATT TTATTGACTG TGAAAGTGAC AATTACTCAA TTTGCAAAAC TTATCTTAGG
TTTTTGATTA ACCGCCCTT AGACTTCTAA CCTATTTTAA AATAACTGAC ACTTCTACTG TTAATGAGTT AAACGTTTTG AATAGAATCC

100      120      140      160      180
*      *      *      *      *
TTTCAAATAT ATATATATAT ATATATATAT ATATATATAT ATTTTATCTT AGGTTTCAAA TACCTAAATT TGAACATTTT TGGAAATGAT
AAAGTTTATA TATATATATA TATATATATA TATATATATA TAAATAGAA TCCAAAGTTT ATGGATTTAA ACTTGTAATA ACCTTTACTA

200      220      240      260
*      *      *      *
AGCAATACTC CAAATTCAAC AAAAATTCOA ATCTTGAAG GGTTCAAATT TAATGTTTAC TTTTTTTTGG AATGTTATGA GAGTATTTAT
TCGTTATGAG GTTTAAGTTG TTTTAAAGGT TAGAAGCTTC CCAAGTTAAA ATTACAAATG AAAAAAACC TTACAATACT CTCATAAATA

280      300      320      340      360
*      *      *      *      *
AAAAATTCOA GCAGACAATA CTTCGCCTTT GATAGATAAG AAACCGCAAA AATTGCCCAA TCCTAAATTT TTATCCTAAT TTTTTCAGCT
TTTTTAAGGT CGTCTGTTAT GAAGCGGAAA CTATCTATTC TTTGGCGTTT TTAACGGGTT AGGATTTTAA AATAGGATTA AAAAAGTCGA

380      400      420      440
*      *      *      *
CCTGATAATC GGAATTCAT CAGTTGGAAG AACATCATTC CTCTCCGTT ACTGTGATGA TTCATTCACT TCTGCCTTCG TCTCTACTGT
GGACTATTAG CCTTTAAGTA GTCAACCTTT TTGTAGTAAG GAGAAGGCAA TGACACTACT AAGTAAGTGA AGACGGAAGC AGAGATGACA

460      480      500      520      540
*      *      *      *      *
CGGAATCGAT TTCAAAGTGA AAATGTGTT CCGTGGAGAC AAACGAGTCA AACTTCAAAT CTGGGATACC GCGCGACAGG AGAGGTACCG
GCCTTAGCTA AAGTTTCACT TTTGACACAA GGCACCTCTG TTTGCTCAGT TTGAAGTTTA GACCCTATGG CGGCCTGTCC TCTCATGGC

560      580      600      620
*      *      *      *
TACCATCACC ACCGCCTACT ATCGTGGAGC AATGGGATTC ATTCTGATGT ATGACATCAC TAATGAAGAG TCTTTTAATA GTGTTTCAGGA
ATGGTAGTGG TGGCGGATGA TAGCACCTCG TTACCCTAAG TAAGACTACA TACTGTAGTG ATTACTTCTC AGAAAATTAT CACAAGTCCT

640      660      680      700      720
*      *      *      *      *
TTGGTATGTT GAGCATGGTT TTAAGAAAAT ATGTATCTTG ATTCAATTCA GGTGCACTCA AATCAAGACA TACTCATGGG AAAATGCTCA
AACCATACAA CTCGTACCAA AATTCTTTTA TACATAGAAC TAAGTAAAGT CCACGTGAGT TTAGTTCTGT ATGAGTACCC TTTTACGAGT

740      760      780      800
*      *      *      *
AGTTGTTTTG GTTGGAAATA AATGTGATAT GGAAGTCTGAA AGAGTTGTAT CTATGGATAG GGGACGCCAA CTGTCTGATC AACTTGGTAA
TCAACAAAAC CAACCTTTAT TTACTATATA CCTGAGACTT TCTCAACATA GATACCTATC CCCTGCGGTT GAACGACTAG TTGAACCATT

820      840      860      880      900
*      *      *      *      *
GTATTTTGAG AAAAAATTGT GCTGAAAAAC GAATTTAATT TTAGGTTTGG AATTCTTCGA AACATCAGCC AAGGAGAACA TTAATGTAAA
CATAAACTC TTTTAAACA CGACTTTTGT CTTAAATTAA AATCCAAACC TTAAGAAGCT TTGTAGTCGG TTCCTCTGTG AATTACATT

920      940      960      980
*      *      *      *
GGCAGTTTTT GAGAAGTTGG TGGAGATTAT TTGTGATAAG ATGGCAGAGA GTTTGGATAA GGTATGGTTT TGAATTTGTA GATTTTAA
CCGTCAAAA CTCTCAACC ACCTCTAATA AACACTATTC TACCGTCTCT CAAACCTATT CCATACCAA ACCTTAACAT CTAATAAATT

1000      1020      1040      1060      1080
*      *      *      *      *
CTTGTTTTTA TTTTGAAAA AACGTAAATT TATGTATATA GAAAATGGCA TGATAGAGAT TATATTCTAA CGGTCTCATG GGCCTCATGA
GAACAAAAT AAAAATTTT TTGCATTAA ATACATATAT CTTTACCCT ACTATCTCTA ATATAAGATT GCCAGAGTAC CCGGAGTACT

1100      1120      1140      1160
*      *      *      *
TTTCTGCAT CTGAAATTT TAGTTCGCTT CAAAACCCGG GGCATTATCT TTTTGTGAAA AACATTGCAA TTTTGTAAA CAAATTATAA
AAAGGACGTA GACTTTTAAA ATCAAGCGAA GTTTTGGGCC CCGTAATAGA AAAAACTTT TTGTAACGTT AAAAACTTT GTTTAATATT

1180      1200      1220      1240      1260
*      *      *      *      *
TTTACAATT ATGTTAGTTT ATAAAACACT TCCTCTTAAT AAAAACTTA TTAAGTAGT TTTTTCAGG ACCCAGAGCA ACAGCCAAAA
AAATGTTAAA TACAATCAA TATTTGTGA AGGAGAATTA TTTTAGAAT AATTTTCATC AAAAAAGTCC TGGGTGTCGT TGTGCGTTTT

1280      1300      1320      1340
*      *      *      *
GGACAGAAGC TCGAAGCGAA TCCGACCCAA AAGCCTGCTC AACAGCAATG CAATGCTAA AAATTCTACG TTCTATTATA CTATCTATAA
CTGTCTTCG AGCTTCGCTT AGGCTGGGT TCGGACGAG TTGTCGTTAC GTTAACGATT TTAAGATGC AAGATAATAT GATAGATATT

AAACTCAA
TTTGAATT

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Figure 10. Genomic sequence of *C. elegans* Rab3 homolog missing 15bp of 5' coding region (Coding region underlined in red.)

The method used to generate transgenic worms is fairly straightforward. The transforming plasmid is microinjected into the hermaphrodite gonad along with a dominant marker, *rol-6(su1006)*, a collagen mutant that results in a defective cuticle that causes animals to roll about in circles (Mello et al., 1991). In the gonad the DNA is taken up by mitotically dividing gamete precursor cells. The injected DNA assembles into large tandem arrays that are maintained extrachromosomally. These extrachromosomal arrays are lost at a fairly high rate. This loss causes a significant portion of the array-bearing animals to be mosaic for the array. I injected both pMK3 and pMK4 at two different concentrations, 100 µg/ml and 20 µg/ml, and coinjected with 100 µg/ml of *rol6(su1006)*.

When I stained the transgenic animals it was clear that the gene was being overexpressed. In wild-type animals, the dorsal and ventral nerve cords and the nerve ring (a region of the nervous system rich in synaptic connections) stain. In these animals overall staining is much more intense compared with wild-type and the nerve cell bodies are stained with the antibody (see figure 11). It is formally possible that the clone has a mutation in it that causes nerve cell body localization, but I think this is extremely unlikely. The transgene appears to be expressed throughout the worm nervous system and nowhere else. The cell bodies that stain are located in the three expected places. First, the ventral nerve cord has neuronal cell bodies located along its length, while the dorsal nerve cord has no cell bodies along it. Second, the regions just anterior and posterior of the nerve ring contain the cell bodies of the nerve ring neurons. Finally the cell bodies of the pharyngeal nervous system are located around the pharynx. In wild-type animals the cell bodies never stain, because normally the vast majority of the Rab3 protein is at the nerve terminals. Animals with either of the two concentrations of pMK3 or pMK4 have identical staining. The transgenic animals exhibit no gross phenotype as a result of overexpressing Rab3.

Generation and Characterization of Two Dominant Mutations in the Rab3 Homologue

Since there were no previously isolated Rab3 mutants, I decided to use site-directed mutagenesis to generate mutations I hoped would act in a dominant manner. The two

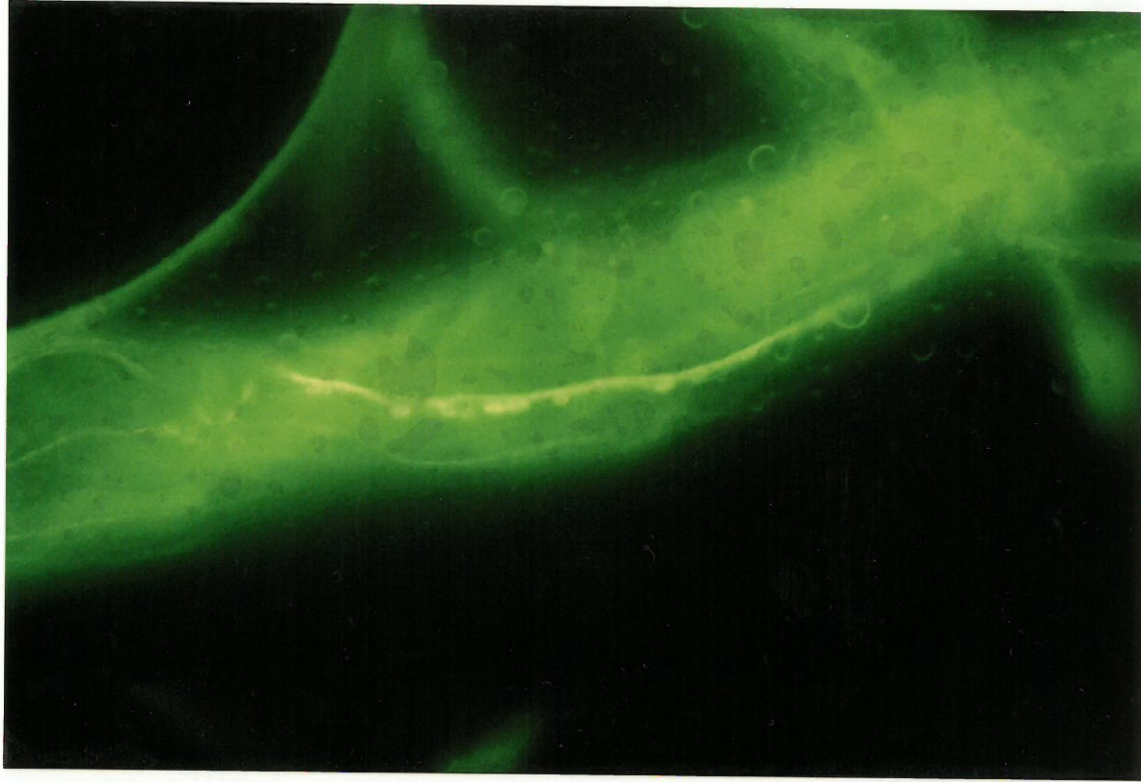


Figure 11. Overexpression of Rab3, stained with Rab3 antibody.

mutations I made are analogous to mutations that have been shown to act dominantly in Ras p21, Sec4, Rab4 and Rab5 (Walworth et al., 1989; Grand and Owen, 1991; Bucci et al., 1992). I tried to do the mutagenesis on both the complete genomic clone pMK4 and the partial genomic clone pMK2. I was unable to get the mutagenesis to work using pMK4, presumably due to its large size. The pMK2 mutagenesis worked and I rebuilt pMK4 from the two mutant pMK2s. One of the mutations I made was a threonine to asparagine substitution at position 36 (see figure 12). This mutation is analogous to the Ras p21 Asn17 mutation and the rac Asn17 mutation. This mutation is in region I (phosphate binding loop) and inhibits the conversion from the GDP-bound form to the GTP-bound form. This mutation acts as a dominant inhibitory mutation. The second mutation I made was an asparagine to isoleucine substitution at position 135. This mutation is analogous to Ile 116 in Ras p21, Ile 133 in Sec4, Ile 121 in Rab4, and Ile 133 in Rab5. This mutation is in region III (guanine specificity) and results in no detectable binding of GTP, presumably because the turnover is extremely fast. Because the vast majority of cellular guanine nucleotide is GTP, this presumably results in a mutant protein that is never inactivated and so acts as a dominant activating mutation. In yeast the mutation is a dominant secretory mutant, resulting in accumulation of secretory vesicles and death. Expressing the Rab5Ile133 protein in baby hamster kidney cells results in a 50% reduction in the rate of endocytosis without significantly affecting the rate of vesicle recycling.

Rab3 Associated Dominant Uncoordination and Lethality

I established lines of transgenic animals with each of the mutant genes. I injected with concentrations of 15 micrograms/ml of mutant DNA and 100 micrograms/ml of *rol6(su1006)*. The animals bearing the Rab3 - N135I transgene are egg-laying defective and severely uncoordinated (or Unc). Up to fifteen percent of the animals bearing the transgene never develop past the first larval stage. This L1 arrest uncoordinated (or Luc) phenotype is typical of animals that have complete or near complete loss of nervous system function. Null mutations in *cha-1* (choline acetyltransferase), *unc-17* (acetylcholine vesicular

Th \longrightarrow

Asn 135 to Ile

- * marks residues commonly conserved among a majority of small GTP-binding proteins of the *ras* supergene family.

Figure 12. Amino acid substitutions made to generate dominant mutations.

transporter), and *unc-104* (a kinesin-like motor used for axonal transport of synaptic vesicles) all exhibit this Luc phenotype. It is likely that the animals that survive to adulthood are mosaic for the transgene and thus retain some nervous system function. It has been shown that only a small subset of the *C. elegans* nervous system is required to be able to eat enough to be viable and fertile. Of the 302 neurons in the hermaphrodite nervous system only three neurons, the M4 neuron required for feeding and the two CAN neurons possibly necessary for proper osmoregulation, have been shown to be essential for viability (Avery and Horvitz, 1989). Thus it seems reasonable to speculate, given the fact that the extrachromosomal array is easily lost, that 85% of the array-bearing animals are viable because they lack the array in those crucial neurons necessary for survival.

Further evidence consistent with the lethality caused by the Rab3 - N135I transgene comes from my inability to stably integrate the array into the worm genome. Gamma ray irradiation of array-bearing animals causes the transgene to integrate into a worm chromosome at an average rate (as reported by other members of the lab) of one integrant progeny per 10-15 irradiated P₀ animals so long as the array to be integrated does not affect viability. After irradiating more than forty P₀ animals and following all of their progeny I was not able to find any animals that did not lose the array. It is likely that any animals that had the array inserted into their genomes arrested at the first larval stage.

The Rab3 - T36N animals exhibited no gross phenotype.

Resistance to Aldicarb, an Inhibitor of Acetylcholinesterase

It has been shown that several mutations in *C. elegans* that interfere with synaptic transmission (i.e. synaptotagmin and choline acetyltransferase) are resistant to aldicarb, an inhibitor of acetylcholinesterase (Nonet et al., 1993). Acetylcholinesterase is responsible for the breakdown of acetylcholine in the synaptic cleft (see figure 13). Wild-type animals die on aldicarb due to massive overstimulation of cholinergic synapses. Resistance is presumably imparted by decreased synaptic efficacy, which lowers the amount of neurotransmitter released into the cleft and prevents hyperstimulation. Both mutant

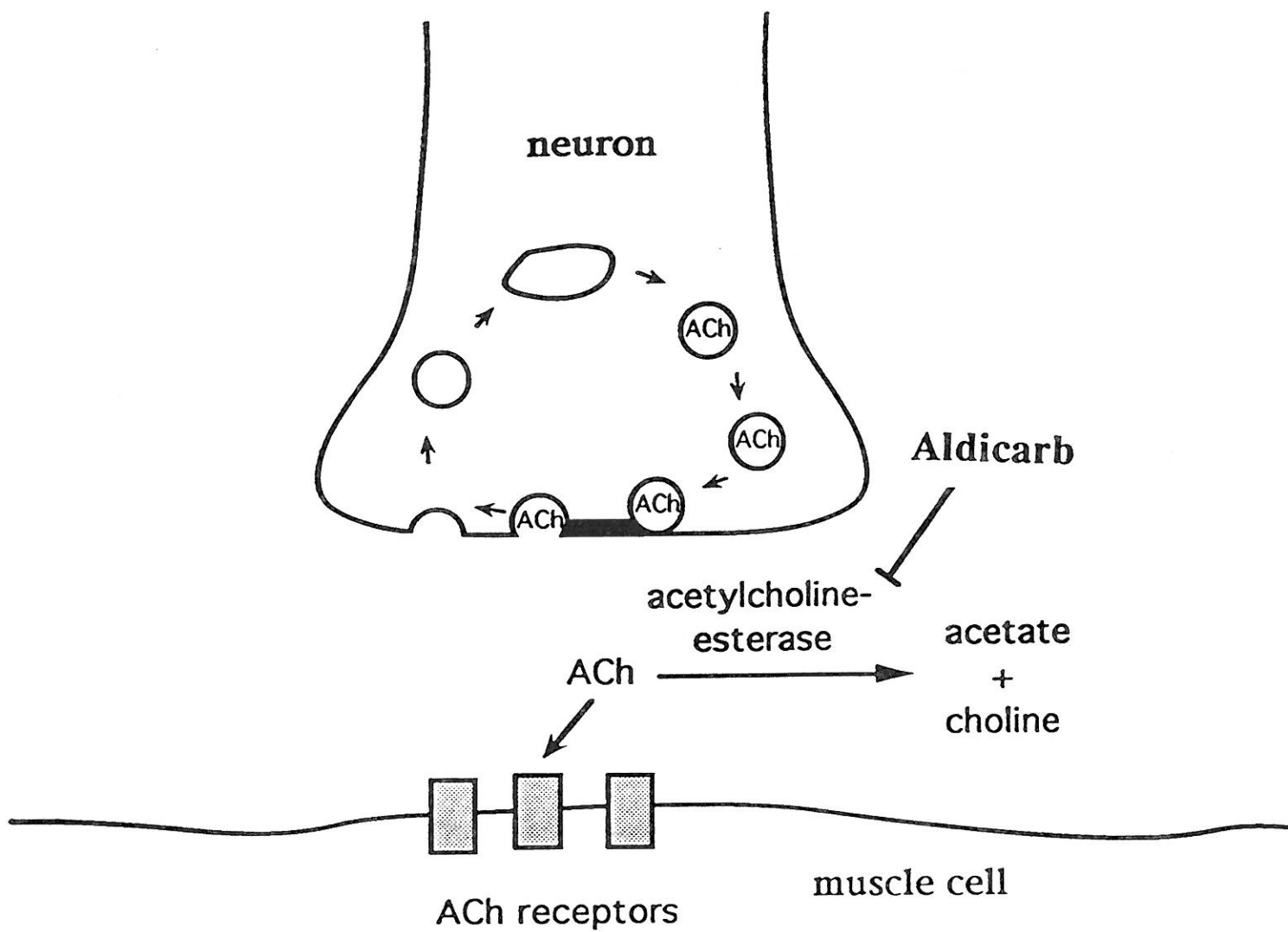


Figure 13. Aldicarb, Inhibitor of Acetylcholinesterase.

transgenes Rab3 - T36N and Rab3 N135I caused the animals carrying them to be resistant to aldicarb. This phenotype is abbreviated as Ric, resistant to inhibitors of cholinesterase. The majority of previously identified mutant genes that confer aldicarb resistance also cause uncoordination, although it is also possible to have a Ric mutants that is able to move properly. It appears that mutations in Rab3 can result in either Ric Unc or Ric non-Unc mutants. Simply overexpressing wild-type Rab3 does not confer resistance to aldicarb.

Effects of Mutant Transgenes on Cellular localization of Rab3

When stained with the rab3 antibody, animals bearing arrays with Rab3 - T36N look exactly like animals overexpressing the wild-type rab3 protein (see figure 14). The Rab3 - N135I transgene, however, resulted in a startling staining pattern when stained with Rab3 antibody. In these animals only neuron cell bodies light up and the nerve processes appear completely devoid of Rab3 protein (see figure 15). It is not surprising that the mutant protein might be mislocalized, but it is surprising that the wild-type protein, presumably also present in these animals, is not properly localized. To determine if the mutant protein disrupts general cellular transport, these animals were stained with an antibody to synaptotagmin, a synaptic vesicle associated integral membrane protein, which showed that synaptic vesicle production and axonal transport are normal (see figure 15b). Thus it appears that the Rab3 - N135I transgene causes disruption of only Rab3 protein localization.

Elucidating the Dominant Action of Rab3 - N135I

Given the lack of Rab3 protein at the nerve terminals of Rab3 - N135I animals, it was unclear whether the dominant action of the transgene was caused by the improper cellular localization of the protein or an actual dominant acting mechanism at the nerve terminal itself. To sort this out, I simultaneously overexpressed both the wild-type and mutant proteins, by generating lines bearing an array with a 10 : 1 ratio of wild-type to mutant DNA. These animals were still uncoordinated, but now exhibited staining of both nerve processes and nerve cell bodies. I have not tested whether these animals throw Luc animals. The Rab3 staining is identical to that seen in animals overexpressing only wild-

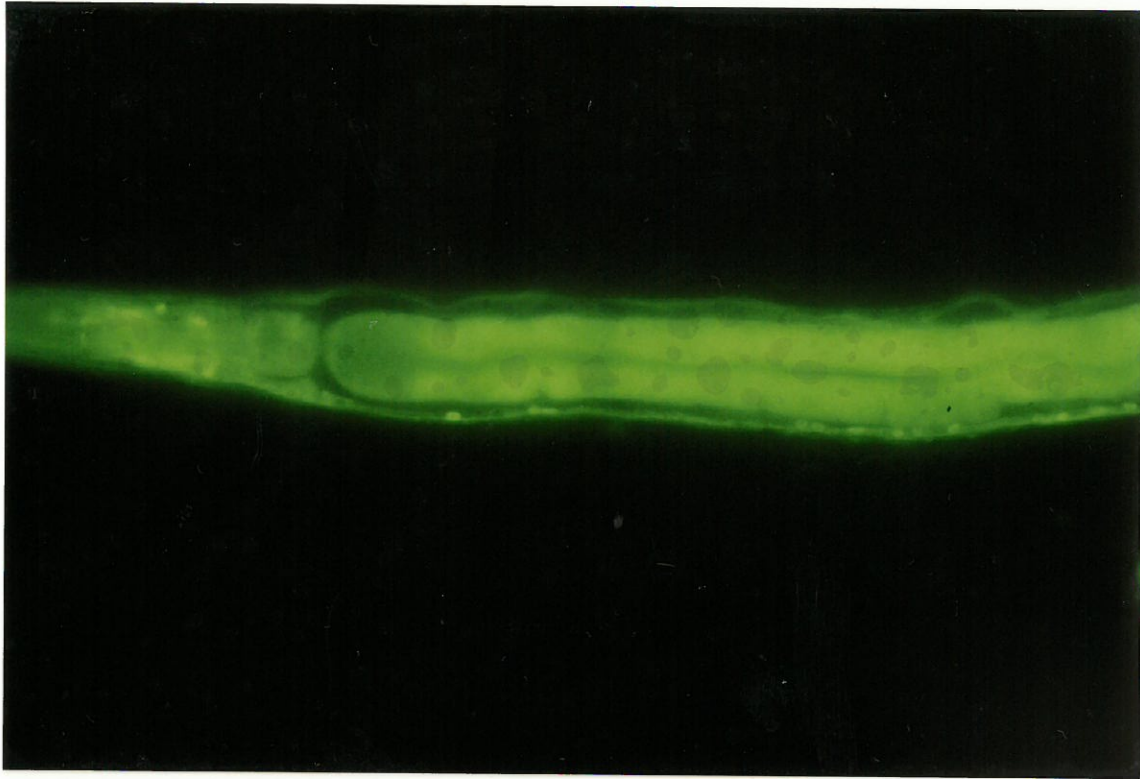
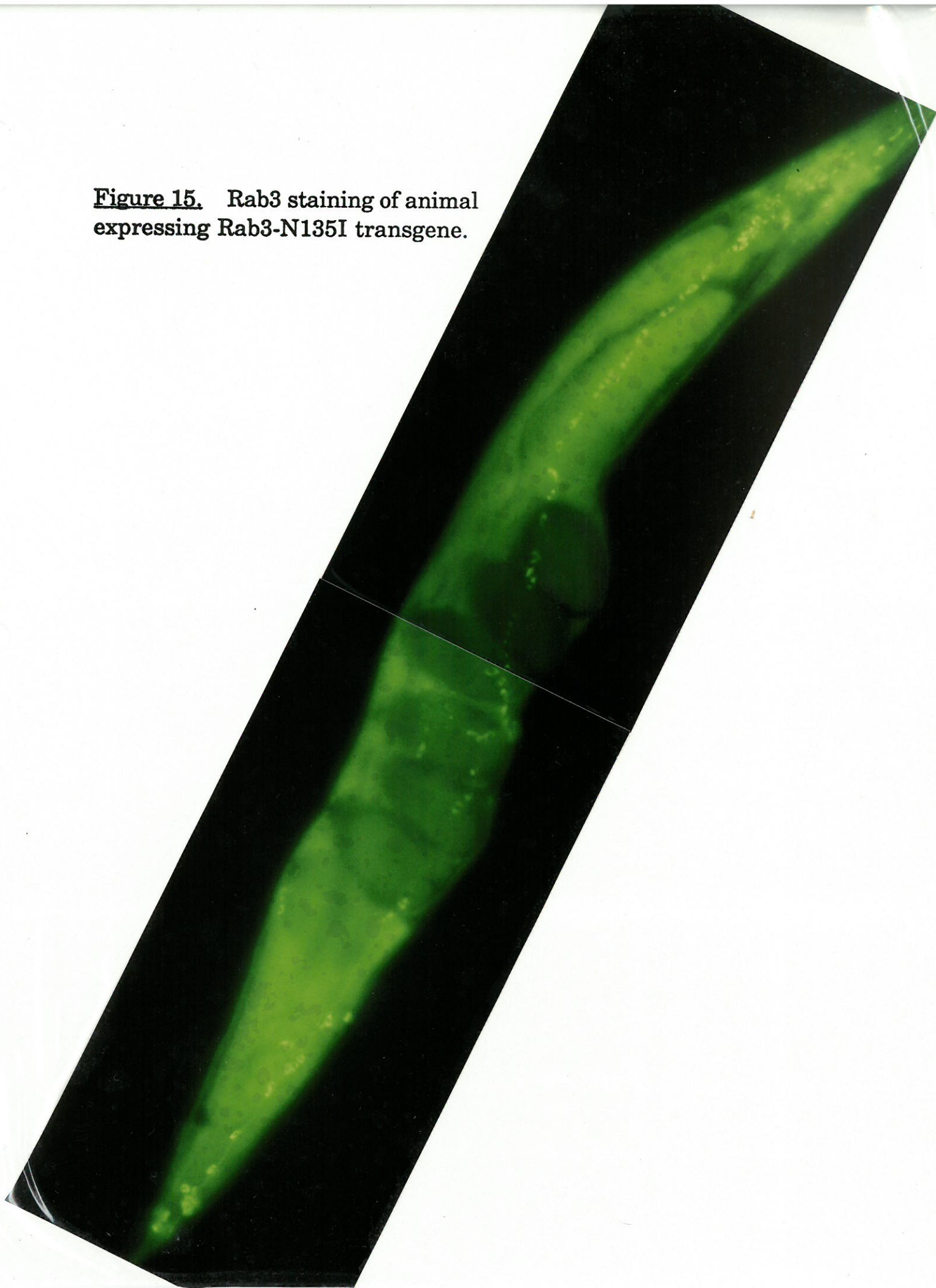


Figure 14. Rab3 staining of animal expressing Rab3-T36N transgene.

Figure 15. Rab3 staining of animal expressing Rab3-N135I transgene.



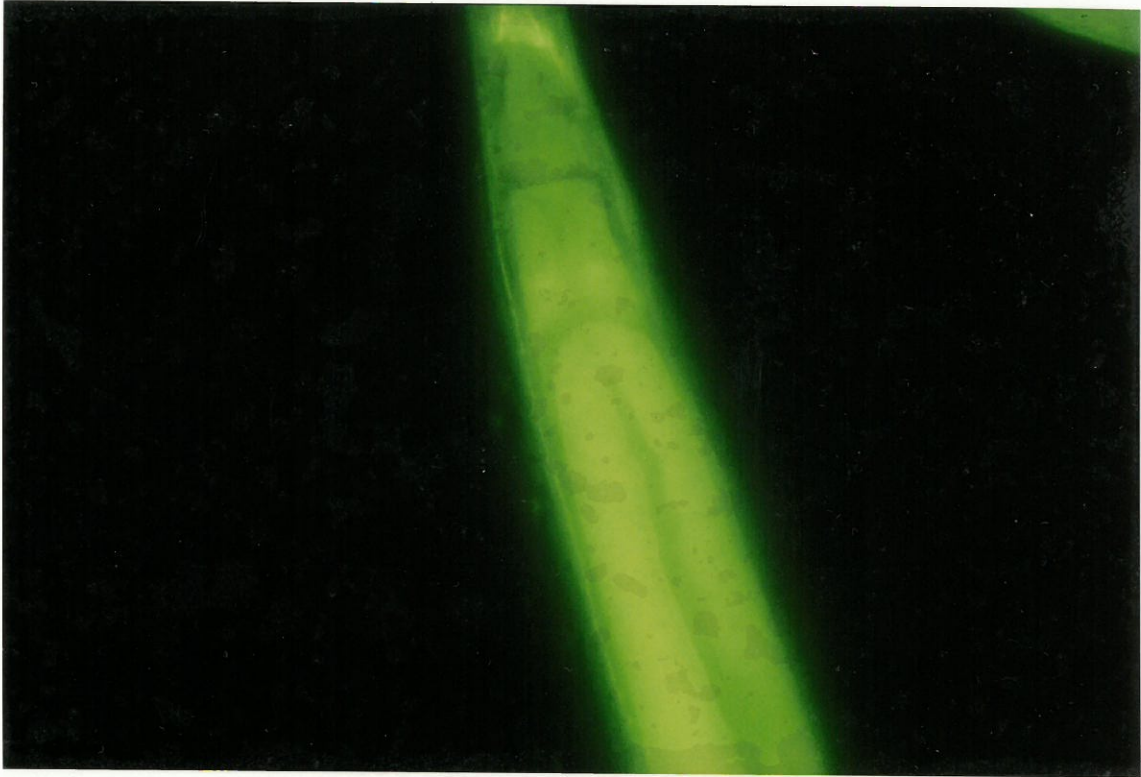


Figure 15b Rab3 - N135I animal stained with an antibody to synaptotagmin. This staining is identical to wild-type synaptotagmin staining.

type Rab3 protein. From this result I conclude that it is the dominant action of the mutant protein at the nerve terminal that disrupts nerve function and not simply non-specific interference with subcellular localization.

Genetic Screen for Mutations in the Rab3 homologue

Because the extrachromosomal dominant mutations are lost at fairly high rates and cannot be integrated, I decided to try to recover chromosomal mutations to use for further characterizing the function of the Rab3 homologue in *C. elegans*. The dominant mutants I created made it clear that it is possible for Rab3 mutants to be both uncoordinated and resistant to aldicarb. This is the phenotype I decided to screen for.

There are at least twenty genes that can be mutated to generate a Ric phenotype (Jim Rand, personal communication). In order to avoid sorting through large numbers of non-Rab3 Ric mutants I made use of the *maDf4* deficiency that deletes Rab3 to design an F1 screen that could uncover both dominant and recessive Rab3 mutations. The screen is fairly straightforward. I mutagenize wild-type males with EMS (ethyl methyl sulfonate) and mate them into a strain with the deficiency *maDf4* balanced with *dpy-25(e817)* (see figure 16). The animals are allowed to mate and lay eggs overnight. The parents are then transferred to a fresh plate and aldicarb, an acetylcholinesterase inhibitor, is added to the plate with the progeny, to a final concentration of .5mM. The aldicarb kills all of the progeny before they reach adulthood, except for those that are resistant because of a dominant mutation, or a recessive mutation in trans to the deficiency. The *dpy-25(e817)* gene is present for three important reasons. First *dpy-25* balances the deficiency. The *e817* allele is semi-dominant. Animals homozygous for *dpy-25(e817)* are extremely dumpy and have few progeny. Animals with only one copy of the *dpy-25(e817)* gene (even in trans to a deficiency of the gene) are only semi-dumpy, much healthier and much more fertile. The second reason to use this mutation is because it increases the mating efficiency of wild-type males, since the hermaphrodites are slow moving. Finally the semi-dumpy animals are hypersensitive to

Genetic Screen for Rab3 Mutants

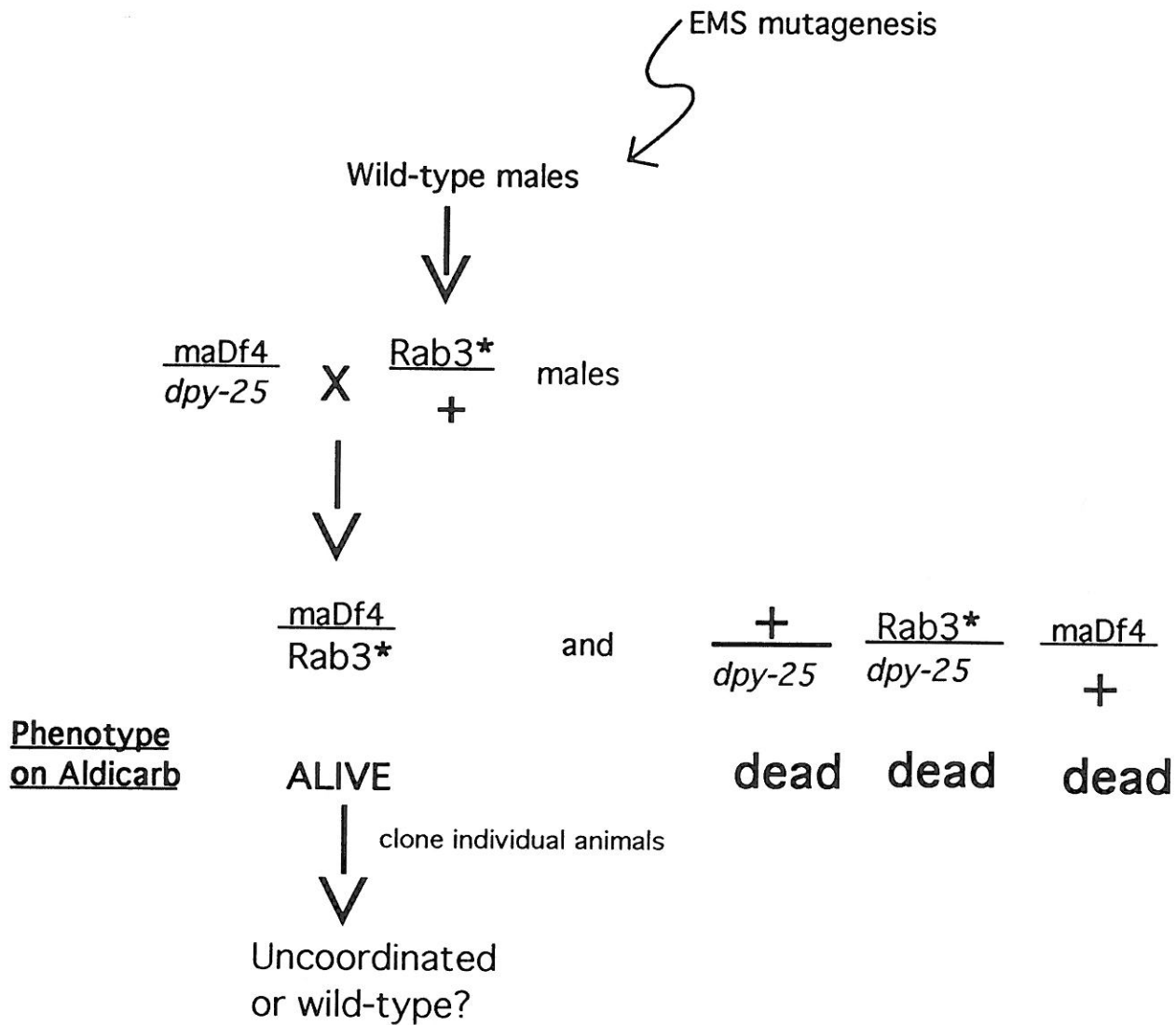


Figure 16 F1 screen for Rab3 mutants.

Parents are allowed to mate and lay eggs overnight.

Then the parents are removed and aldicarb is added to the progeny.

aldicarb and die much more easily than wild-type. This hypersensitivity selects against both self-progeny and those animals that do not receive the deficiency chromosome.

To date I have screened roughly 150,000 F1 animals. I have isolated 75 independent lines that are resistant enough to aldicarb to eat all of the bacteria on an aldicarb-treated plate. Of these 75 lines, 19 lines are uncoordinated homozygotes. I am in the process of sorting out which, if any, of these lines is a mutant in Rab3. After retesting for aldicarb resistance, I have been crossing males heterozygous for each mutant back into the original deficiency strain and looking for uncoordinated progeny. When I tried to generate heterozygotic males, four mutant lines had uncoordinated heterozygous or hemizygous male progeny. Three of the four are X-linked Unc mutations. The fourth is a dominant Unc that may or may not be Rab3. I have tested 9 of the 15 recessive autosomal Ric Unc lines and none of them segregate uncoordinated mutant over deficiency animals. If the mutations are hypomorphic the uncoordination should be at least as severe over deficiency as the homozygote is. If the mutation was dominant the heterozygous males would have been uncoordinated. Unless the mutation is an unusual neomorph, it is highly unlikely that any of the Ric Unc lines I have tested are mutant in Rab3.

There are two possible explanations for the large numbers of non-Rab3 mutants surviving the screen. The first explanation is that recessive mutations made it to the F2 generation because a few non-resistant animals somehow make it to adulthood and laid a few eggs before they died. Some of these eggs would be homozygous for the non-Rab3 Ric mutation. This is certainly possible, but I think the number of these F2 eggs laid from non-resistant animals is small enough that I would not expect 75 lines of resistant animals to arise. The second explanation is that mutations arise that cause some dominant resistance to aldicarb. Only one of the 13 autosomal Ric Unc lines tested was uncoordinated when heterozygous. It seems quite possible that mutations could arise which when heterozygous impair synaptic function just enough to provide some aldicarb resistance without causing uncoordination, but when homozygous are uncoordinated and more resistant to aldicarb. It

is also possible that a large portion of the 56 Ric non-Unc mutants I have isolated are Rab3 mutants which exhibit a phenotype similar to that caused by the Rab3 - T36N. That is highly resistant to aldicarb, but well coordinated. It is difficult to test if I have isolated mutants of this type because aldicarb resistance is hard to score, since a significant fraction of aldicarb resistant animals still die when put on aldicarb.

V. Discussion

Conclusions

In conclusion I feel that my research has shown that proper Rab3 function is vital for nerve function in *C. elegans*. Rab3 Ile135 disrupts proper Rab3 localization to synaptic vesicles. Rab3 Ile135 acts in a dominant manner at the synaptic terminal to prevent the proper functioning of the synaptic release machinery. Both Rab3 Asn36 and Ile135 impart resistance to aldicarb, presumably by decreasing synaptic efficacy. Finally I have demonstrated possible Rab3 mutant phenotypes, which has made the design of a screen to recover Rab3 chromosomal mutations possible.

Results are consistent with Rab3 model of synaptic vesicle targeting

I feel that the results presented in this thesis are consistent with a model of Rab3 action similar to that proposed for Sec4. The most likely explanation for the presence of Rab3 protein in the nerve cell bodies of animals overexpressing wild-type Rab3 protein is that the Rab3 regulatory proteins involved in specifically localizing Rab3 to synaptic vesicles are overwhelmed by the amount of Rab3 in the nerve cells. Normally a significant proportion of Rab3 protein exists as freely soluble protein in the cytoplasm. It is reasonable to speculate that Rab3 might flow back into the cell body if there was not enough regulatory proteins to continually return it to the synaptic terminal. It is also possible that synaptic vesicles (or the proteins on them that allow for Rab3 localization to the synaptic vesicles)

may become saturated with Rab3 protein. In this case the Rab3 protein would remain in the cell bodies because there is no other place to localize the excess Rab3.

Effects of Rab3 - N135I on localization of Rab3 protein

The most likely explanation for the mislocalization of Rab3 protein caused by Rab3 - N135I is that after the post translational hydrophobic modification has been added to the Rab3 protein, the protein attaches to the nearest membrane. In a wild-type animal this may not be a problem because Rab3 GDP dissociation inhibitor (GDI) probably removes the Rab3 protein from the membrane and allows localization to synaptic vesicles. Rab3 GDI has been shown to induce the disassociation of the GDP-bound form of Rab3 from membranes. In the Rab3 - N135I mutant animals, the mutant Rab3 protein probably never changes to the GDP-bound conformation. It has been suggested that removal of Sec4 from membranes requires the GDP-bound conformation. It is likely that the GDP-bound form is also required for removal of Rab3 by its regulatory factor. In vitro soluble GTP-bound Rab3 does not detectably complex with Rab3 GDI (Matsui et al., 1990). It is possible that the GDI, or some other factor responsible for localizing the Rab3 protein to synaptic vesicles, can bind Rab3 in the GTP-bound conformation, but gets stuck when it cannot switch the Rab3 - N135I conformation from GTP-bound to GDP-bound. Once all of the regulatory factor is bound to mutant Rab3 protein the localization of wild-type protein will also be disrupted. Rab3 - N135I must get to the nerve terminal in order to account for its dominant effect on the synaptic release machinery located there. Presumably any Rab3 - N135I protein that randomly attached to a synaptic vesicle after its hydrophobic modification is added would be transported to the nerve terminal. This small amount of Rab3 - N135I protein would most likely be undetectable using immunohistochemical techniques, but appears to be enough to cause its dominant effect even in the presence of overexpressed wild-type Rab3 protein.

Differences between Rab3 - T36N and Rab3 - N135I

Superficially one might think that either of the two mutations I generated would have the same effect on synaptic vesicle trafficking, since they each disrupt the Rab3 cycle. The evidence shows, however, that the differences are significant. It has been proposed that the mutation in Sec4 analogous to Rab3 - N135I causes the mutant protein to be locked in an active conformation (Walworth et al., 1989). It is quite possible that the release of Rab3 from an effector at the active zone requires the change from the active to inactive forms. If Rab3 - N135I cannot make this change the effector will be blocked, unable to guide any more synaptic vesicles to the active zone. Once all of the effectors are blocked the worm would be unable to make proper use of its neurons and would become severely uncoordinated. The animals would not hypercontract in response to aldicarb because very little neurotransmitter could be released and muscle cells would therefore not be overstimulated.

The clear differences in the phenotype caused by the two mutant transgenes suggests that the Rab3 - T36N mutant protein has a very different mode of action. If the mutation causes the rate of GDP for GTP exchange to be dramatically slowed, the mutant protein may not cycle at the right pace, but it is not clear that this would significantly effect the action of the wild-type Rab3 protein also present in the nerve cells. The key point is that in the transgenic animals I generated, the mutant protein was not only present, it was probably greatly overexpressed with respect to wild-type Rab3 levels. This overexpression would result in a large concentration of GDP-bound Rab3 protein, which could titrate out the Rab3 guanine disassociation inhibitor. The Rab3 GDI has been proposed to stabilize the soluble GDP-bound form of Rab3. Lowered levels of GDI available for wild-type Rab3 protein could cause a general decrease in the rate at which synaptic vesicles could be trafficked to the active zone.

Under normal synaptic release conditions this may not cause a visible phenotype (i.e. uncoordination), because synaptic vesicle trafficking to the active zone occurs at rate that is able to keep up with synaptic release. In the presence of an inhibitor of

acetylcholinesterase, however, synaptic release conditions may be far from normal. Overstimulation of a cholinergic motoneuron by a cholinergic interneuron will cause a much elevated rate of synaptic release (see figure 17). The rate of recycling of synaptic vesicles in a wild-type worm is so fast that nerve cells can release huge amounts of acetylcholine onto muscle cells, resulting in hyperstimulation and death. In Rab3 - T36N mutants, trafficking of synaptic vesicles to the active zone may not be able to keep up with demand since Rab3 GDI is in very short supply. When worms are put on aldicarb it appears to take a few hours for aldicarb levels within the animals to be high enough to cause hyperstimulation. It is during this time that the synaptic vesicle store of the Rab3 - T36N animals are depleted. As a result of the decreased stores of synaptic vesicles and the inability to quickly replace them, the Rab3 - T36N mutant releases much less acetylcholine compared to a wild-type animal. This prevents muscle hyperstimulation and death. This explanation fits precisely with the phenotype of the Rab3 - T36N.

Predictions based on the model of Rab3 action

This model based on the results of my project allows for the following predictions . First a loss of function mutation in the Rab3 effector gene hand is likely to have a phenotype similar to that caused by Rab3 N135I: a partial loss of function should cause severe uncoordination and aldicarb resistance, and a true null should result in a Luc phenotype. A second prediction is that a partial loss of mutation in the Rab3 GDI gene would result in a phenotype similar to that of Rab3- T36N, aldicarb resistance without uncoordination. This model also predicts that in the Rab3 - T36N mutants the ratio of Rab3 that exists in the membrane-bound pool compared to the soluble pool would be shifted from the wild-type ratio. (The ratio of membrane-bound to soluble Rab3 is 7:3 in vertebrate nerve cells). Membrane prep and cytoplasm preps could be made of Rab3 - T36N worms and the relative amounts of Rab3 determined using the Rab3 antibody. It is likely that the overexpression of a Rab3 - T36N mutant protein that cannot convert to the GTP-bound form would cause the majority of Rab3 to be located in the cytoplasm. This experiment is not trivial, because

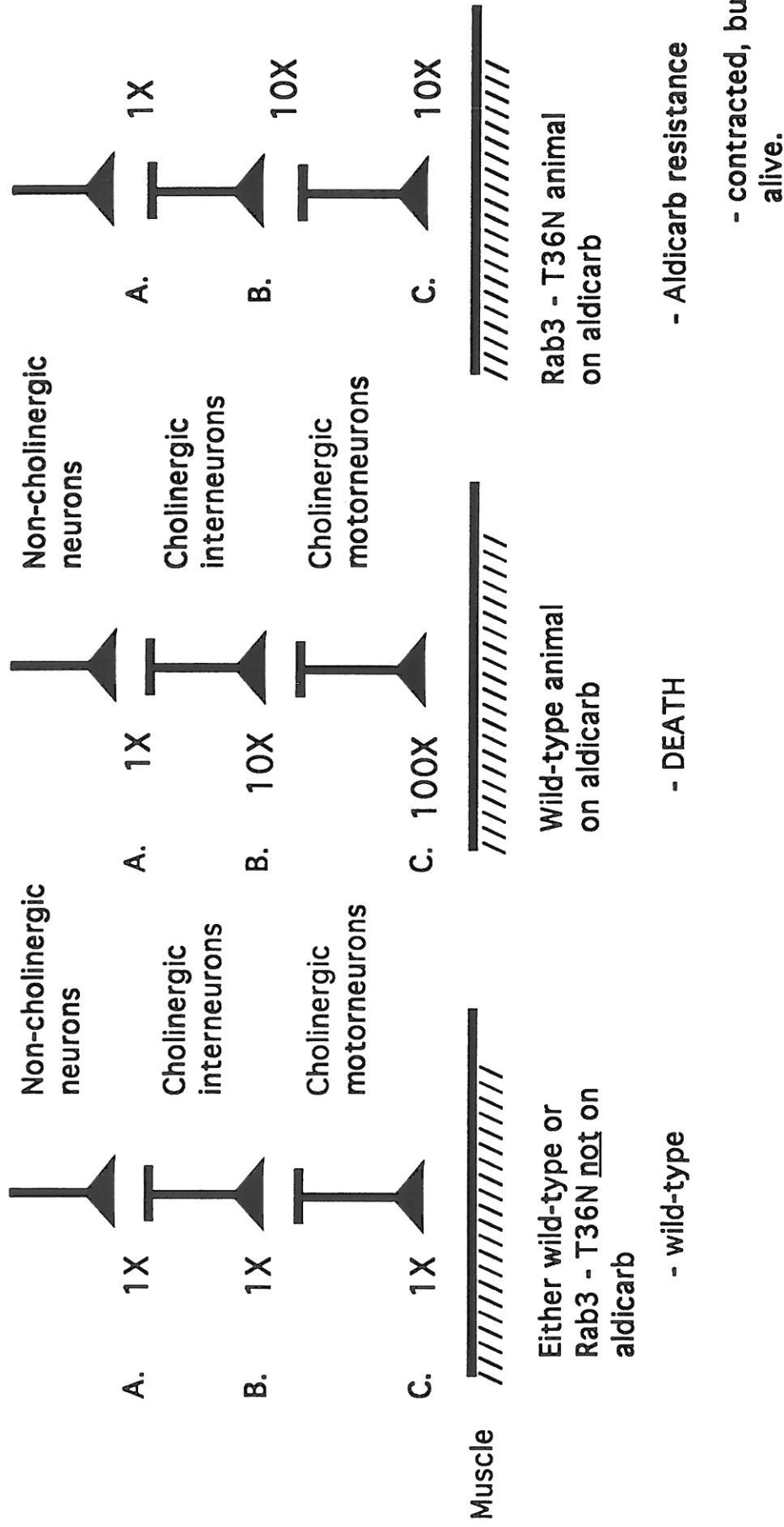


Figure 17. Model to explain Ric phenotype of Rab3 - T36N

1X is the normal amount of transmitter released from nerves. In this model aldicarb increases the amount of transmitter in the synaptic cleft by ten fold. In Rab3 - T36N mutants synaptic recycling proceeds at a maximum rate of 10X so the synapse labeled C cannot release transmitter at the rate a wild-type animal (100X). This prevents Rab3-T36N mutants from hypercontracting which results in death.

optimizing immunohistological techniques may prove difficult. Finally, the model predicts that overexpressing the Rab3 GDI would rescue the Rab3 - T36N phenotype by providing so much GDI that the large amount of mutant GDP-bound protein is not longer able to tie up all of the GDI. This would allow enough GDI for the wild-type Rab3 protein to function normally and restore the ability of the nerve terminal to rapidly recycle synaptic vesicles, making the worm sensitive to aldicarb.

Future Plans

Once I have recovered what I believe to be an uncoordinated mutant in Rab3 I will inject the animal with wild-type Rab3 DNA and look for rescue of the uncoordinated phenotype. In other words, generating Rab3 mutants with a wild-type Rab3 transgene should result in animals that are wild-type or at least significantly more healthy. It is possible that overexpressing Rab3 could rescue a synaptic defect caused by a mutation in a gene other than Rab3. Thus mapping and sequencing of the Rab3 gene of the mutant will also be required to confirm that it is mutant in Rab3. I would also like to make a site-directed mutation in the Rab3 gene that would delete the Cys-X-Cys site of hydrophobic modification and check to see if this mutant protein is still able to rescue the Rab3 mutant phenotype. I hope to do a non-complementation screen in order to find a null Rab3 mutant. Eventually the Rab3 mutant will be used to do a suppression screen to look for mutants in Rab3 regulatory factors.

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