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sup-9, *sup-10*, and *unc-93* May Encode Components of a Two-Pore K^+ Channel that Coordinates Muscle Contraction in *Caenorhabditis elegans*

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Genetic studies of *sup-9*, *unc-93*, and *sup-10* strongly suggest that these genes encode components of a multi-subunit protein complex that coordinates muscle contraction in *Caenorhabditis elegans*. We cloned *sup-9* and *sup-10* and found that they encode a two-pore K^+ channel and a novel transmembrane protein, respectively. We also found that UNC-93 and SUP-10 colocalize with SUP-9 within muscle cells, and that UNC-93 is a member of a novel multigene family that is conserved among *C. elegans*, *Drosophila*, and humans. Our results indicate that SUP-9 and perhaps other two-pore K^+ channels function as multiprotein complexes, and that UNC-93 and SUP-10 likely define new classes of ion channel regulatory proteins.

Key words: C. elegans; two-pore; potassium [K^+]; channel; muscle; mutant

Introduction

The *sup-9*, *sup-10*, and *unc-93* genes of *Caenorhabditis elegans* can affect the regulation of muscle contraction. Mutants carrying semidominant gain-of-function (gf) mutations in *sup-9*, *unc-93*, or *sup-10* move sluggishly, are defective in egg-laying and defecation, and display a rubberband uncoordinated (unc) response: when prodded on the head, a mutant worm contracts and then relaxes along its entire body without moving backwards, whereas a wild-type worm contracts its anterior end and backs away (Greenwald and Horvitz, 1980, 1986; Levin and Horvitz, 1993). Although capable of muscle contraction, these mutants appear to be unable to mount a coordinated muscle response. In contrast, mutants defective in muscle structural genes are defective in muscle contraction (Waterston et al., 1980). Worms with loss-of-function (lf) mutations in *sup-9*, *sup-10*, or *unc-93* display no gross phenotypic abnormalities, suggesting that these genes have nonessential functions and may act in parallel to another gene or set of genes (Greenwald and Horvitz, 1980). Mosaic analysis of *sup-10* indicates that it functions in muscle (Herman, 1984).

Three lines of genetic evidence strongly argue that the SUP-9, SUP-10, and UNC-93 proteins physically interact as members of a protein complex. First, lf mutations in *sup-9*, *sup-10*, or *unc-93* suppress the rubberband phenotype caused by gf mutations in any of these three genes (Greenwald and Horvitz, 1980, 1986; Levin and Horvitz, 1993; De Stasio et al., 1997). Because the effects of each of these three genes are dependent on the functions of the other two genes, the gene products of all three genes must act at the same step. Second, this mutual suppression can be observed weakly in lf hetezozygotes, suggesting that a stochiometric relationship among SUP-9, SUP-10, and UNC-93 is important for their function (Levin and Horvitz, 1993). Third, certain *sup-9* alleles display gene- and allele-specific interactions with *sup-10(gf)* and *unc-93(gf)* alleles (Levin and Horvitz, 1993); such genetic interactions are a hallmark of genes that encode proteins that physically interact (Hartman and Roth, 1973).

A fourth gene, *sup-18*, was identified as an lf suppressor of the *sup-10(gf)* rubberband Unc phenotype and shown to be a partial suppressor of *sup-9(gf)* and *unc-93(gf)* rubberband Unc mutants (Greenwald and Horvitz, 1986). *sup-18* may encode a component, regulator, or effector of a SUP-9–SUP-10–UNC-93 complex. *unc-93* encodes a novel, putative, multipass transmembrane protein of unknown biochemical function (Levin and Horvitz, 1992, 1993).

Here, we report that *sup-9* encodes a two-pore K^+ channel with similarity to hTASK-1 and hTASK-3 (TASK, TWIK-related acid-sensitive K^+ channel) and that *sup-10* encodes a novel single-pass transmembrane protein. Mammalian TASK-1 and TASK-3 form a subfamily of two-pore K^+ channels that are activated by high pH, volatile anesthetics, and neurotransmitters (Duprat et al., 1997; Leonoudakis et al., 1998; Patel et al., 1999; Kim et al., 2000; Millar et al., 2000; Rajan et al., 2000; Talley et al., 2000). Our findings indicate that UNC-93 and SUP-10 associate with a SUP-9 two-pore K^+ channel and suggest that UNC-93 and SUP-10 may be regulatory subunits of this channel.

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Table 1. The paralysis caused by *sup-9*, *sup-10*, and *unc-93* gf mutations is suppressed by lf mutations in any of the three genes

Genotype	Locomotion rate (bends per min ± SEM)	Animals assayed
Wild-type	26.3 ± 0.6	*n*=16
sup-9(n1550)	Inviable	
sup-9(n1550)/+	1.3 ± 0.4	*n*=16
sup-10(n983)	5.1 ± 0.5	*n*=16
unc-93(e1500)	0.2 ± 0.1	*n*=32
sup-9(n1550n2287)	26.5 ± 0.7	*n*=16
sup-10(n983n1017)	23.5 ± 0.5	*n*=16
unc-93(e1500lr12)	24.6 ± 0.6	*n*=16
sup-9(n1550); sup-10(n983n1017)	19.2 ± 0.4	*n*=16
sup-9(n1550); unc-93(e1500lr12)	22.7 ± 0.5	*n*=16
sup-9(n1550n2287); unc-93(e1500)	24.3 ± 0.6	*n*=16
unc-93(e1500); sup-10(n1017)	23.5 ± 0.5	*n*=16
sup-9(n1550n2287); sup-10(n983)	24.7 ± 0.7	*n*=16
unc-93(lr12); sup-10(n983)	24.8 ± 0.5	*n*=16
lon-1 sup-10(n983)	5.2 ± 0.5	*n*=20
lon-1(e185) sup-5(e1464sd) sup-10(n983)	9.9 ± 0.5	*n*=20

Young adult hermaphrodites were assayed for the number of bends they make while moving on a bacterial lawn during a 1 min interval.

Materials and Methods

Strains and genetics. C. elegans strains were cultured as described previously (Brenner, 1974), except that the *Escherichia coli* strain HB101 was used, instead of OP50, as a food source. N2 was the wild-type strain (Brenner, 1974). Strains were grown at 20°C unless otherwise noted. The following mutations were used in this study: linkage group (LG) II: *lin-42(n1089)*, *sup-9(e2655gf*, *e2661gf*, *lr1*, *lr100*, *lr11*, *lr129*, *lr142*, *lr30*, *lr35*, *lr38*, *lr45*, *lr57*, *lr73*, *n180*, *n186*, *n188*, *n189*, *n190*, *n191*, *n213*, *n219*, *n222*, *n223*, *n229*, *n233*, *n241*, *n264*, *n266*, *n271*, *n292*, *n345*, *n350*, *n508*, *n659*, *n688*, *n1009*, *n1012*, *n1016*, *n1020*, *n1023*, *n1025*, *n1026*, *n1028*, *n1037*, *n1428*, *n1435*, *n1469*, *n1472*, *n1549*, *n1550gf*, *n1553*, *n1557*, *n1913*, *n1914*, *n2174*, *n2175*, *n2176*, *n2276*, *n2278*, *n2279*, *n2281*, *n2282*, *n2283*, *n2284*, *n2285*, *n2286*, *n2287*, *n2288*, *n2291*, *n2292*, *n2294*, *n2296*, *n2343*, *n2344*, *n2345*, *n2346*, *n2347*, *n2348*, *n2349*, *n2350*, *n2351*, *n2352*, *n2353*, *n2354*, *n2355*, *n2356*, *n2357*, *n2358*, *n2359*, *n2360*, *n2361*, *n3310gf*, *nP136*, *lin-31(n301)*; LGIII: *unc-93(e1500gf*, *lr12*, *n234)*, *nIs124*, *sup-18(n1030)*; LGX: *dpy-6(e14)*, *sup-10(e2127*, *n983gf*, *n183*, *n240*, *n247*, *n250*, *n251*, *n342*, *n619*, *n1007*, *n1008*, *n1017*, *n1468*, *n1626*, *n1906*, *n2297*, *n3558*, *n3564)*, *lin-15(n765ts)*. The sources of the *sup-9* and *sup-10* alleles are indicated Tables 1–4.

Cloning of sup-9. We found a 5.5 kb Tc1-containing *Eco*RI polymorphism, *nP136*, that cosegregated with the *sup-9* suppressor phenotype after nine backcrosses in *sup-9(n1428);unc-93(e1500)* mutants. We cloned 943 bp of DNA flanking the *nP136* polymorphism using a vectorette-cloning approach (Korswagen et al., 1996) with the following modifications. We used an agarose gel to purify *Eco*RI-digested genomic DNA from the *sup-9(n1428) nP136;unc-93(e1500)* strain and ligated 40 ng to 10 pmol *Eco*RI vectorette in a 100 μl volume; 3 μl was used as a template for a 100 μl PCR reaction of 30 cycles using Tc1-specific primers L2 or R2 (Zwaal et al., 1993) and the universal vectorette primer 224 (Riley et al., 1990). Nested PCRs were performed with primer 224 and the Tc1-inverted-repeat primer N412 (Korswagen et al., 1996). The PCR product generated from the L2-nested reaction was cloned, its DNA sequence was determined using an automated ABI373A DNA sequencer (Applied Biosystems, Foster City, CA), and was mapped to the overlapping cosmids, F34D6 and F19E8, using database searches of genomic sequence (*C. elegans* Sequencing Consortium, 1998). The following primers were used in PCR reactions to amplify the *sup-9* cDNA from mixed-stage cDNA: 5′-GTGTGAGCTCAGCAGCTTCT-3′ and 5′-TACTTCAAGAGATTGCAGC-3′. Rapid amplification of cDNA ends (RACE) reactions were performed using 5′ and 3′ RACE kits (Invitrogen, San Diego, CA).

Expression constructs. The *sup-9::gfp* fusion was obtained by subcloning the *Hpa*I-rescuing *sup-9* genomic fragment, which includes 2.9 kb of *sup-9* promoter sequence, into the *Eco*RV site of Bluescript (Stratagene, La Jolla, CA). A *Bam*HI site was introduced by PCR amplification immediately before the stop codon, and the genomic fragment subcloned into the *Sal*I–*Bam*HI sites of the green fluorescent protein (GFP) expression vector pPD95.77 (provided by Dr. A. Fire, Carnegie Institute of Washington, Baltimore, MD), thereby fusing *gfp* at the C-terminal end of *sup-9* to create pIP201. The *unc-54* 3′ untranslated region (UTR) from pPD95.77 was replaced with an 808 bp region of genomic sequence immediately following the stop codon of *sup-9* by standard PCR and cloning techniques. Animals carrying the *sup-9::gfp* transgenic array were treated with γ radiation to isolate a stable integrant (Fire, 1986).

The *unc-93::gfp* fusion was created by cloning a 12.9 kb *Sph*I–*Kpn*I genomic fragment from cosmid C46F11 containing 5.3 kb of *unc-93* upstream sequence, the entire coding sequence of *unc-93* and 3.2 kb of *unc-93* downstream region into the *Sph*I–*Kpn*I sites of pPD95.79 (provided by Dr. A. Fire) creating pIP314. A *Kpn*I site was introduced immediately preceding the *unc-93* stop codon using PCR, and the *Kpn*I fragment containing the *unc-93* 3′ region was excised, creating plasmid pIP321 with a GFP fusion at the C terminus of UNC-93.

The *sup-10::gfp* fusion used in colocalization studies was constructed by subcloning a 7.3 kb *Mfe*I genomic fragment from cosmid C27G6 containing *sup-10* into the *Eco*RI site of pBSKII. A 6.4 kb *Pst*I fragment was subcloned from this vector into p95.77, which contained 3.5 kb of promoter sequence and the *sup-10* coding region. Through PCR techniques, we introduced a *Sal*I site immediately preceding the stop codon of *sup-10* to create an in-frame fusion with the *gfp* coding sequence.

To make the ectopic expression constructs, the *sup-9* cDNA was amplified by PCR from the start to the stop codon, with the introduction of *Nhe*I–*Sac*I sites at the 5′ and 3′ ends, respectively, and subcloned into pPD95.86 (provided by Dr. A. Fire) or an *unc-76* promoter vector (Bloom and Horvitz, 1997) to generate *myo-3::sup-9* and *unc-76::sup-9*, respectively. The *n1550* mutation was introduced into *myo-3::sup-9* using mutagenic PCR primers.

Transgenic animals. Germline transformation experiments were performed using standard methods (Mello et al., 1991). Transformations were done using strains carrying *lin-15(n765ts)* with the coinjection marker pL15EK(*lin-15*) (Clark et al., 1994) at 100 ng/μl or with the muscle *gfp* marker pPD93.97 (provided by Dr. A. Fire) and the experimental DNA at 30–50 ng/μl. Transformants were recognized by their non-Muv phenotypes at 22.5°C or by their GFP fluorescence.

Antibodies and immunostaining. Codons 1–118 of *sup-9* were fused to the glutathione *S*-transferase (GST) gene in the vector pGEX-2T (Amersham Biosciences, Piscataway, NJ), and codons 1–110 were fused to the maltose-binding protein (MBP) gene in the vector pMal-c2 (NEB). The GST fusion protein was expressed in *E. coli*, and the insoluble protein was purified by SDS-PAGE and used to immunize rabbits. Antisera were purified against the MBP fusion protein immobilized on nitrocellulose strips and eluted with 100 mM glycine-HCl, pH 2.5.

For immunofluorescence experiments, mixed-stage worms were fixed in 1% paraformaldehyde for 2 hr at 4°C and permeabilized as described previously (Finney and Ruvkun, 1990). For colocalization studies, transgenic lines were stained with anti-SUP-9 serum at 1:150 dilution and mouse anti-GFP serum at 1:100 dilution (Quantum Biotechnologies, Montreal, Canada). A secondary goat-anti-rabbit antibody conjugated to Texas Red (Jackson ImmunoResearch, West Grove, PA) and a secondary goat-anti-rabbit antibody conjugated to FITC (Jackson ImmunoResearch) were used at a 1:150 dilution. Worms were viewed using confocal microscopy (Zeiss LSM510; Zeiss, Thornwood, NY) or epifluorescence (Zeiss Axioskop2).

Neuronal cell identifications. To aid in the identification of neurons expressing SUP-9::GFP, we constructed a *sup-9::gfp* transcriptional fusion containing the same promoter and 3′UTR region as the *sup-9::gfp* translational reporter described above but lacking any *sup-9* coding region. This reporter was brightly expressed in the cell bodies and processes of 4 of the ~15 neurons that expressed the SUP-9 translational GFP fusion. Each neuron extended a single axonal process into the nerve ring and turned posteriorly, two along the dorsal and two along the ventral cords, before ending in the posterior of the animal between the vulva and the anus. This axonal morphology was consistent with that of the four

SIA interneurons (White et al., 1986). We confirmed this identification by immunostaining SUP-9::GFP-expressing animals and UNC-93::GFP-expressing animals with antisera raised against the homeodomain transcription factor CEH-17, which is expressed in the four SIA neurons and the ALA neuron (Pujol et al., 2000).

Muscimol and rubberband Unc assays. Muscimol assays were done using 24-well plates (Costar, Cambridge, MA). A 200 mM stock of muscimol (Sigma, St. Louis, MO) was added to 1 ml of melted NGM agar (Brenner, 1974). After 4–6 hr, HB101 bacteria were streaked in each well. Two hours later, 10–12 worms were placed into each well and allowed to equilibrate for 1 hr. The rubberband Unc response was scored by touching each worm with an eyelash across its body, just posterior to the pharynx. Each worm was scored five or six times. The responses to touch were scored according to the presence of a contraction–relaxation cycle and backward movement in the following manner: 0, worms did not contract and relax but moved away from the touch; 1, worms quickly contracted and relaxed and moved away from the touch; 2, worms contracted and relaxed while concurrently generating a small backward displacement (less than one-half of body length); 3, worms contracted and relaxed but failed to move backwards; 4, worms incompletely contracted and relaxed and produced no displacement.

Results

sup-9, *sup-10*, and *unc-93* likely encode components of a protein complex

To facilitate our analyses of the interactions among *sup-9*, *sup-10*, and *unc-93*, we quantified the locomotory behavior of strains carrying mutations in one or more of these three genes (Table 1). Our data confirmed previous conclusions reached through qualitative examinations of mutant strains, including the following: (1) mutations that cause an abnormal ("altered") function of *sup-9*, *sup-10*, or *unc-93* result in a rubberband Unc paralysis and (2) the paralysis caused by an altered function mutation in any of these three genes can be suppressed by either an lf mutation in that gene or by an lf mutation in either of the other two genes. Such reciprocal suppression indicates that the activity of each of these three genes requires the functions of the other two genes and establishes that the products of the three genes act together at a single step, most likely as components of a protein complex.

sup-9 encodes a two-pore K^+ channel

We cloned *sup-9* using transposon tagging. *sup-9(n1428)* was previously isolated as a suppressor of the dominant rubberband Unc phenotype caused by the *unc-93(e1500)* gf allele in the mutagenic *mut-2* background, in which the spontaneous excision and transposition of the mariner-type transposable element Tc1 is greatly increased (Levin and Horvitz, 1992). We isolated a Tc1 insertion within the first exon of predicted gene F34D6.3 associated with the *sup-9(n1428)* mutation (see Materials and Methods). To determine whether F34D6.3 corresponded to *sup-9*, we performed transformation rescue experiments. Because *sup-9(lf)* mutants have a wild-type phenotype, they cannot be used to assay *sup-9* wild-type activity. Instead, we used *sup-9(lf);sup-10(gf)* mutants, in which the *sup-10(gf)* Unc phenotype is suppressed by the *sup-9(lf)* mutation; this suppression can be eliminated by theaddition of *sup-9* wild-type activity. Cosmid F19E8, as well as a 6.8 kb subclone of F19E8 containing the predicted gene F34D6.3, restored *sup-9* activity in transgenic *sup-9(n180);sup-10(n983)* animals (Fig. 1*A*).

The *sup-9* minimal rescuing fragment contains a single complete predicted gene. Using reverse transcriptase (RT)-PCR and 5′ and 3′ RACE, we cloned a full-length *sup-9* cDNA (Fig. 1*B*). *sup-9* encodes a predicted protein of 329 amino acids with sequence similarity to the TASK subfamily of two-pore K^+ channels (Fig. 1*C*). SUP-9 is 49–51% identical in amino acid sequence to human TASK-1(KCNK3), TASK-3(KCNK9), and TASK-5(KCNK15) channels as well as to two predicted *Drosophila* proteins. Mammalian TASK-1 and TASK-3 behave as pH-sensitive background K^+ channels when expressed heterologously in mammalian cell lines (Duprat et al., 1997; Kim et al., 1998; Leonoudakis et al., 1998; Rajan et al., 2000), whereas the properties of TASK-5 channels remain unknown. All other identified human two-pore K^+ channels, such as TREK-1(KCNK2), share <30% amino acid identity with SUP-9. Most of the amino acid identities among SUP-9 and the human TASK channels are restricted to the P domains, highly conserved loops that determine the ion selectivity of K^+ channels (Heginbotham et al., 1994), and to the first, second, and fourth transmembrane domains.

Characterization of *sup-9* mutant alleles

Suppressors of the rubberband Unc phenotypes of *sup-9*, *unc-93*, and *sup-10* gf mutants have defined many *sup-9* lf alleles. We determined the sequences of the open-reading frame and intron–exon boundaries of *sup-9* from 81 strains carrying lf mutations (Table 2). These mutations include 47 missense, 15 nonsense, and 11 splice-site mutations as well as 3 deletions, 1 three-base-pair insertion, and 1 Tc1 insertion. We did not find molecular lesions in three *sup-9* mutants; these alleles may contain mutations in the promoter region or in other regulatory elements not contained within the *sup-9* exons. The identified *sup-9* missense mutations include lesions in all four putative transmembrane domains, the M1–P1 linker, which joins the first transmembrane domain and the first P domain, and both putative P domains. *sup-9(n2282)* encodes an isoleucine instead of the initiator methionine and thus is likely to be a null allele. The mutations V41A, V44E, D58V, I61S, and A74V cause changes in the M1–P1 linker, which has been shown to act as a dimerization domain *in vitro* in the human TWIK-1 two-pore channel (Lesage et al., 1996).

The *sup-9(n1550)* gf allele that leads to the rubberband Unc phenotype causes an A236T substitution in the fourth transmembrane domain. We determined the sequences of three additional independently isolated *sup-9* gf alleles. All three, *e2655*, *e2661*, and *n3310*, contain the same nucleotide change and therefore cause the same A236T substitution (Table 3).

The partial lf alleles *n2359*, *n2360*, *n2361*, and *n2288*, which were isolated as suppressors of *sup-9(n1550)* defects (Levin and Horvitz, 1993), contain second-site mutations in addition to the A236T *sup-9 (n1550)* substitution (Table 3). *sup-9(n1550 n2360)* mutants do not display the rubberband Unc phenotype of *sup-9(n1550)* animals but rather appear wild-type. However, unlike both *sup-9(+)* and *sup-9(0)*, *sup-9(n1550 n2360)* partially suppresses the *sup-10(gf)* mutant phenotype, indicating that this allele is neither completely wild-type nor null. The *n2360* and *n2361* alleles contain a point mutation in the same codon mutated in *n1550* animals, leading to a substitution of methionine in place of the wild-type alanine and the *n1550* threonine. Two other partial lf alleles, *sup-9(n1550 n2288)* and *sup-9(n1550 n2359)*, contain missense mutations affecting the third transmembrane domain, G173E and A174T, respectively. These amino acid changes may suppress the *sup-9(n1550)* phenotype by altering an interaction between the third transmembrane domain and the mutant threonine in the fourth transmembrane domain.

sup-10 encodes a novel putative transmembrane protein

sup-10 was previously mapped to the right end of LGX near *mec-4* (Greenwald and Horvitz, 1980; A. Villenevue, personal communication to all). We analyzed restriction fragment-length polymorphisms in *sup-10* lf mutants generated by γ-ray and transpo-

Table 2. *sup-9* loss-of-function mutations

Allele	Mutation	Effect	Isolation background	Mutagen
Nonsense mutations				
n345[e]	TTA to TGA	L20 Ochre	*unc-93(e1500)*	NTG
n292[e]	CAG to TAG	Q37 Amber	*unc-93(e1500)*	SPO
n668[a]	CAG to TAG	Q42 Amber	*unc-93(e1500); sup-18(n463)*	EMS
n1012[a]	CAG to TAG	Q77 Amber	*sup-10(n983)*	EMS
n2276[c]	TGG to TAG	W78 Amber	*sup-9(n1435); unc-93(e1500)*	EMS
n2292[c]	TGG to TAG	W78 Amber	*sup-9(n1550); sup-18(n1014)*	EMS
n1549[f]	TGG to TGA	W78 Ochre	*sup-10(n983)*	EMS
lr11[b]	CAG to TAG	Q126 Amber	*unc-93(e1500)*	ENU
n1023[a]	CGA to TGA	R131 Ochre	*sup-10(n983)*	EMS
n2357[c]	TGG to TGA	W165 Ochre	*sup-9(n1550); sup-18(n1014)*	EMS
n266[e]	TGG to TAG	W165 Amber	*unc-93(e1500)*	EMS
n1037[a]	CAA to TAA	Q208 Opal	*sup-10(n983)*	EMS
lr73[b]	CAA to TAA	Q216 Opal	*unc-93(e1500)*	ENU
lr57[b]	CAA to TAA	Q216 Opal	*unc-93(e1500)*	ENU
n186[e]	CAA to TAA	Q216 Opal	*unc-93(e1500)*	EMS
Missense mutations				
n2282[c]	ATG to ATA	M1I	*sup-9(n1435); unc-93(e1500)*	EMS
n213[e]	GGA to GAA	G22E	*unc-93(e1500)*	EMS
lr129[b]	GCA to GTA	A23V	*unc-93(e1500)*	ENU
n1472[f]	GTG to GCG	V41A	*sup-10(n983)*	GR
n233[e]	GTG to GAG	V44E	*unc-93(e1500)*	DES
n2291[c]	GAC to GTC	D58V	*sup-9(n1550)*	EMS
lr35[b]	ATT to AGT	I61S	*unc-93(e1500)*	ENU
n1025[a]	GCC to GTC	A74V	*sup-10(n983)*	EMS
n1016[a]	ATC to AAC	I94N	*sup-10(n983)*	EMS
n1020[a]	GGC to GAC	G95D	*sup-10(n983)*	EMS
n2354[c]	GGC to GAC	G95D	*sup-9(n1550); sup-18(n1014)*	EMS
n190[e]	CCA to TCA	P101S	*unc-93(e1500)*	EMS
n508[g]	CCA to TCA	P101S	*sup-11(n406); unc-93(e1500)*	EMS
n2353[c]	CCA to TCA	P101S	*sup-9(n1550); sup-18(n1014)*	EMS
n2356[c]	CCA to TCA	P101S	*sup-9(n1550); sup-18(n1014)*	EMS
n2347[c]	ACA to ATA	T103I	*sup-9(n1550); sup-18(n1014)*	EMS
n1009[a]	GGA to GAA	G106E	*sup-10(n983)*	EMS
n2351[c]	GGA to AGA	G106R	*sup-9(n1550); sup-18(n1014)*	EMS
lr45[b]	TTC to TCC	F109S	*unc-93(e1500)*	ENU
n2281[c]	CCA to TCA	P119S	*sup-9(n1435); unc-93(e1500)*	EMS
n2345[c]	CCA to TCA	P119S	*sup-9(n1550); sup-18(n1014)*	EMS
lr100[b]	GGA to AGA	G121R	*unc-93(e1500)*	ENU
n264[e]	CTT to TTT	L122F	*unc-93(e1500)*	EMS
lr38[b]	TGG to CGG	W165R	*unc-93(e1500)*	ENU
n2355[c]	GGA to AGA	G172R	*sup-9(n1550); sup-18(n1014)*	EMS
n219[e]	GGA to GAA	G172E	*unc-93(e1500)*	UV
n223[e]	GGA to AGA	G172R	*unc-93(e1500)*	SPO
n2294[c]	GGA to AGA	G173R	*sup-9(n1550); sup-18(n1014)*	EMS
n2296[c]	GGA to AGA	G173R	*sup-9(n1550); sup-18(n1014)*	EMS
n2350[c]	GAA to AAA	E181K	*sup-9(n1550); sup-18(n1014)*	EMS
n2352[c]	TAC to TTC	Y190F	*sup-9(n1550); sup-18(n1014)*	EMS
n2278[f]	ACT to ATT	T195I	*sup-9(n1435)*	EMS
n2343[c]	ACT to ATT	T195I	*sup-9(n1550); sup-18(n1014)*	EMS
n191[e]	GGA to GAA	G200E	*unc-93(e1500)*	EMS
n2286[c]	GGA to GAA	G200E	*sup-9(n1550); sup-18(n1014)*	EMS
n2283[c]	GGA to GAA	G200E	*sup-9(n1435); unc-93(e1500)*	EMS
n1469[f]	GGT to AGT	G202S	*sup-10(n983)*	SPO
n2344[c]	GGT to GAT	G202D	*sup-9(n1550); sup-18(n1014)*	EMS
lr1[b]	GAC to GCC	D203A	*unc-93(e1500)*	ENU
n2346[c]	GAC to AAC	D203N	*sup-9(n1550); sup-18(n1014)*	EMS
n2349[c]	GAC to AAC	D203N	*sup-9(n1550); sup-18(n1014)*	EMS
n1557[f]	TTC to TCC	F226S	*sup-10(n983)*	EMS
n2348[c]	GGG to AGG	G230R	*sup-9(n1550); sup-18(n1014)*	EMS
n2176[c]	GGG to GAG	G230E	*sup-9(n1550)*	EMS
n189[e]	TCT to TTT	S235F	*unc-93(e1500)*	EMS
n2358[c]	TCT to TTT	S235F	*sup-9(n1550); sup-18(n1014)*	EMS
lr142[b]	GTG to ATG	V242M	*unc-93(e1500)*	ENU

Table 2. *Continued*

Allele	Mutation	Effect	Isolation background	Mutagen
Splice mutations				
n241[e]	AGgt to AGct	Intron 1 Donor	*unc-93(e1500)*	DES
n659[a]	AGgt to AGct	Intron 1 Donor	*sup-18(n463); unc-93(e1500)*	EMS
n180[e]	agAG to acAG	Intron 1 Accept	*sup-10(n983)*	SPO
n1028[a]	CGgt to CGat	Intron 2 Donor	*sup-10(n983)*	EMS
	GAT to AAT	D278N		
n2285[c]	TGgt to TGat	Intron 3 Donor	*sup-9(n1550); sup-18(n1014)*	EMS
n271[e]	AGgt to AGat	Intron 4 Donor	*unc-93(e1500)*	EMS
n1553[c]	AGgt to AGat	Intron 4 Donor	*sup-10(n983)*	EMS
n2175[c]	agGA to aaGA	Intron 4 Accept	*sup-9(n1550)*	EMS
n2174[c]	TGgt to TGat	Intron 5 Donor	*sup-9(n1550)*	EMS
n2279[c]	agAG to aaAG	Intron 6 Accept	*sup-9(n1435); unc93(e1500)*	EMS
n1026[a]	CGgt to CGat	Intron 7 Donor	*sup-10(n983)*	EMS
Insertion, deletion, and frame-shift mutations				
n1913[f]	C to GAT	Codon 4 Fs	*unc-93(e1500)*	GR
n1428[d]	Tc1 Insertion	Codon 17 Fs	*unc-93(e1500)*	SPO
n2287[c]	155 bp Del.	94–127 Del	*sup-9(n1550); sup-18(n1014)*	EMS
n1914[f]	1181 bp Del.	Codon 94 Fs	*unc-93(e1500)*	DEB
n2284[c]	561 bp Del.	Codon 95 Fs	*sup-9(n1435); unc-93(e1500)*	EMS
No mutation found				
lr30[b]	None		*unc-93(e1500)*	ENU
n188[e]	None		*unc-93(e1500)*	EMS
n229[e]	None		*unc-93(e1500)*	DES

The sequence of the *sup-9* coding region including intron–exon boundaries, but not promoter or downstream regions, was determined. Isolation background indicates the gf strain in which a *sup-9* lf allele was isolated as a suppressor. Accept, Splice acceptor; Fs, frame shift; Del, deleted; DEB, diepoxybutane; DES, diethyl sulfate; EMS, ethyl methanesulfonate; ENU, *N*-ethyl-*N*-nitrosourea; GR, gamma radiation; NTG, nitrosoguanidine; SPO, spontaneous; UV, ultraviolet light.

[a] Greenwald and Horvitz (1986).

[b] De Stasio et al. (1997).

[c] Levin and Horvitz (1993).

[d] Levin and Horvitz (1992).

[e] Greenwald and Horvitz (1980).

[f] J. Levin and H. R. Horvitz, unpublished results.

[g] I. Greenwald and H. R. Horvitz, unpublished results.

son mutagenesis using cosmid ZK54, which we found in unrelated studies to contain the *mlc-1* gene (*mlc-1* encodes a myosin light chain, and we regarded this gene as a candidate for corresponding to *sup-10*) (Fig. 2*A*). We found that *sup-10(n247)*, *sup-10(n251)*, and *sup-10(n1906)* carry allele-specific polymorphisms to the right of *mlc-1*. In transformation rescue experiments, both cosmid C27G6, which is located 23 kb to the right of ZK54, and a 7.3 kb subclone of C27G6 containing a single predicted gene suppressed the semidominant rubberband Unc paralysis of *sup-10(n983)* animals as transgenes (Fig. 2*A*). We isolated a full-length cDNA (GenBank accession number U43891) corresponding to this predicted gene by screening a mixed-stage cDNA library (Fig. 2*B*). *sup-10* encodes a predicted protein of 332 amino acids containing a putative signal sequence at its N terminus and a hydrophobic region near its C terminus, suggesting it is a type-1 transmembrane protein (Fig. 2*C*). We searched the GenBank protein database with the SUP-10 sequence but did not find any proteins sharing significant amino acid identity with SUP-10.

We identified the molecular lesions of 14 *sup-10(lf)* alleles (Table 3). *sup-10(n1008)*, *sup-10(n1017)*, *sup-10(n2297)*, and *sup-19(n1626)* contain nonsense mutations and likely represent null alleles. *sup-10(n619)* encodes a protein with a glutamate-to-lysine substitution in the predicted extracellular domain of SUP-10, whereas a second missense mutation *sup-10(n240)* causes a glycine-to-arginine substitution at residue 323 at the beginning of the predicted intracellular domain (Table 4). The gf *sup-10(n983)* allele contains an amber stop mutation at position 322, resulting in a predicted truncation of the extreme C-terminal 11 amino acids. Because these 11 amino acids span the region following the putative transmembrane domain, they may form an intracellular domain of SUP-10. Consistent with this identification, we found that the locomotory defect of *sup-10(n983)* gf mutants was partially suppressed by the amber suppressor mutation *sup-5(e1464sd)* (Table 1). We suggest that the absence of the final 11 C-terminal amino acids of SUP-10 results in altered SUP-10 function.

sup-9::gfp, *sup-10::gfp*, and *unc-93::gfp* are expressed predominantly in muscle

To study the expression of *sup-9*, we constructed a translational fusion of SUP-9 to the GFP. This fusion included 2.9 kb of *sup-9* upstream promoter sequence and 0.8 kb of *sup-9* downstream sequence (see Materials and Methods). When expressed in a *sup-9(lf);unc-93(gf)* mutant, the *sup-9::gfp* reporter could restore the rubberband Unc phenotype caused by an *unc-93(gf)* allele, indicating that this reporter fusion protein is functional.

We observed SUP-9::GFP expression along the surface of body-wall muscle cells, with a punctate stripe pattern characteristic of dense bodies (Fig. 3*A*), structures functionally analogous to vertebrate Z-lines, which connect the myofibril lattice to the cell membrane (Waterston et al., 1980). The body-wall muscle staining became apparent at the 3.5-fold stage of embryogenesis, was most apparent in late embryos and L1 stage larvae, and persisted to adulthood. The vulval muscles, predominantly the four

Table 3. *sup-9* altered-function mutations

Allele	Mutation	Effect	Isolation background	Allele type	Mutagen
n1550	GCG to ACG	A236T	Wild-type	gf	EMS
n3310	GCG to ACG	A236T	Wild-type	gf	EMS
e2655	GCG to ACG	A236T	Wild-type	gf	EMS
e2661	GCG to ACG	A236T	Wild-type	gf	EMS
n2360	ACG to ATG	T236M	*sup-9(n1550)*	Partial lf	EMS
n2361	ACG to ATG	T236M	*sup-9(n1550)*	Partial lf	EMS
n2288	GGA to GAA	G173E	*sup-9(n1550)*	Partial lf	EMS
n2359	GCA to ACA	A174T	*sup-9(n1550)*	Partial lf	EMS

The sequence of the *sup-9* coding region including intron– exon boundaries was determined. Isolation background indicates the gf mutant in which a *sup-9* allele was isolated. Alleles *n1550*, *n2360*, *n2361*, *n2359*, and *n2288* have been described previously (Levin et al., 1993). EMS, Ethyl methanesulfonate.

Table 4. *sup-10* mutations

Allele	Mutation	Effect	Isolation background	Mutagen
Loss-of-function mutations				
n1017[a]	TGG to TAG	W19 Amber	*sup-10(n983)*	EMS
n1008[a]	CAA to TAA	Q99 Opal	*sup-10(n983)*	EMS
n2297[b]	TGG to TGA	W139 Ochre	*sup-9(n1550); sup-18(n1014)*	EMS
n1626[a]	AAA to TAA	K170 Opal	*sup-10(n983)*	Gamma
n619[c]	GAG to AAG	E88K	*unc-93(e1500)*	EMS
n240[c]	GGG to AGG	G323R	*unc-93(e1500)*	DES
n1007[a]	agAT to aaAT	Exon 3 Donor	*sup-10(n983)*	EMS
n250[c]	TTgtto TTga	Exon 5 Donor	*unc-93(e1500)*	Gamma
n342[c]	TTgtto TTga	Exon 5 Donor	*unc-93(e1500)*	NTG
n3558[d]	85 bp del	Codon 16 Fs	*unc-93(e1500)*	UV-TMP
n3564[d]	99 bp del	Codon 34 Fs	*unc-93(e1500)*	UV-TMP
n183[c]	53 bp del,	Codon 128 Fs	*unc-93(e1500)*	SPO
e2127[e]	Tc1 insertion	Codon 103 Fs	Wild type	SPO
n1468[a]	Tc1 insertion	Codon 136 Fs	*sup-10(n983)*	SPO
Gain-of-function mutations				
n983[a] *gf*	TGG to TAG	W322 Amber	*Wild type*	EMS

The sequences of both DNA strands were determined for each mutant. For splice mutations, intron sequence is represented in lowercase, and exon sequence in uppercase. EMS, Ethyl methanesulfonate; SPO, spontaneous; UV-TMP, ultraviolet and trimethylpsoralen; NTG, nitrosoguanidine; DES, diethyl sulfate.

[a] Greenwald and Horvitz (1986).

[b] Levin and Horvitz (1993).

[c] Greenwald and Horvitz (1980).

[d] I. Perez de la Cruz and H. R. Horvitz, unpublished results.

[e] Levin and Horvitz (1992).

Vm1 cells, and the intestinal muscles also displayed GFP fluorescence (Fig. 3*B*,*C*), consistent with the egg-laying and defecation defects of *sup-9(gf)* mutants. We observed weaker fluorescence in the anal depressor and anal sphincter muscles (data not shown). We also observed GFP expression in 12–15 head neurons in each animal (Fig. 3*D*) (data not shown), including in the SIADL, SIADR, SIAVL, and SIAVR neurons (see Materials and Methods). Additional staining was observed in muscle arms, which project from the muscle body toward neurons to form synapses (Fig. 3*E*).

To determine whether the tissue and subcellular localization of SUP-9 is dependent on UNC-93, SUP-10, or SUP-18, we examined the expression of the *sup-9::gfp* reporter in *unc-93(lr12)*, *sup-10(n1008)*, and *sup-18(n1030)* lf mutants, all likely null alleles (De Stasio et al., 1997; I. Perez de la Cruz and H. R. Horvitz, unpublished results). We found that SUP-9::GFP was expressed and localized as in the wild type in all three mutants (data not shown), indicating that the SUP-9 K^+ channel can localize properly in the absence of UNC-93, SUP-10, or SUP-18.

To study the expression of *unc-93*, we created transgenic lines carrying an *unc-93::gfp* reporter containing 5.3 kb of *unc-93* upstream sequence, the entire *unc-93* coding region, and the *gfp* coding region fused just before the stop codon of *unc-93*. This construct restored the rubberband Unc phenotype caused by a *sup-10(gf)* mutation in an *unc-93(n234);sup-10(n983)* mutant, indicating that the fusion protein was functional. We found GFP expression in the body-wall muscle membranes and dense bodies (Fig. 3*F*), as well as in all eight vulval and intestinal muscles (Fig. 3*G*,*H*). As with the *sup-9::gfp* fusion, the *unc-93::gfp* fusion resulted in neuronal GFP expression (Fig. 3*I*) (data not shown), including in the SIADL, SIADR, SIAVL, and SIAVR neurons. We tested the expression of this reporter in *sup-9(n1913)*, *sup-10(n1008)*, and *sup-18(n1030)* null backgrounds and found that the localization of UNC-93::GFP remained unchanged (data not shown).

To study the expression of *sup-10*, we created transgenic lines carrying a *sup-10::gfp* reporter containing 3.5 kb of *sup-10* upstream sequence, the entire *sup-10* coding region, and the *gfp* coding region fused just before the stop codon of *sup-10*. This construct restored the rubberband Unc phenotype caused by a *sup-10* mutation in an *unc-93(e1500);sup-10(n183)* mutant, indicating that the fusion protein was functional. We found GFP expression in the body-wall muscle membranes and dense bodies (Fig. 3*J*), eight vulval muscles, intestinal muscles, and anal depressor muscle (Fig. 3*K*,*L*,*M*). As with *sup-9::gfp*, we observed GFP expression in muscle arms (Fig. 3*N*).

sup-9, *sup-10*, and *unc-93* function in muscles

To confirm their structure and muscle expression, we overexpressed the *sup-9*, *sup-10*, and *unc-93* cDNAs under the *myo-3*

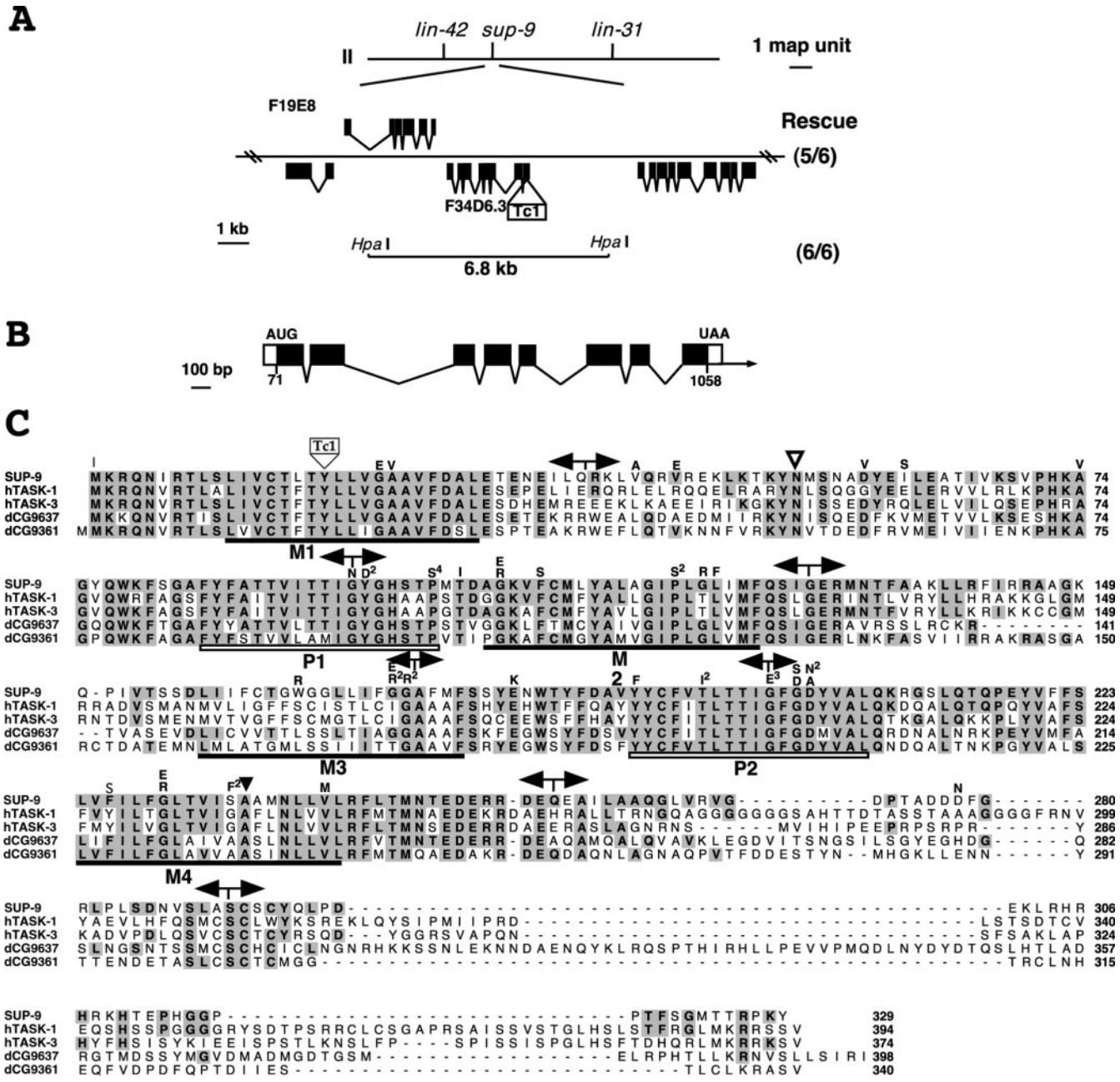

Figure 1. Molecular cloning, genomic structure, and sequence of the *sup-9* gene. *A*, Schematic representation of the *sup-9* genomic region and the Tc1 insertion found in the *sup-9(n1428)* strain. Cosmid F19E8 as well as the 6.8 kb *Hpa*I subclone rescued a *sup-9(n180);unc-93(e1500)* mutant on the basis of the appearance of the rubberband Unc response. The numbers in parentheses indicate the number of rescued lines and total transgenic lines scored. *B*, Intron–exon structure of *sup-9*. The structure of the *sup-9* gene was deduced by comparing its genomic sequence with the sequences of RT-PCR and RACE products. Black boxes indicate coding regions. White boxes indicate untranslated regions. The arrow indicates the direction of transcription. *C*, Alignment of SUP-9 with human TASK-1, human TASK-3, and *Drosophila* predicted proteins dCG9637 and dCG9361. Residues identical between SUP-9 and the other proteins are highlighted in gray. Black bars indicate proposed transmembrane domains (M1, M2, M3, and M4). White bars indicate P domains (P1 and P2). If mutations in *sup-9* are indicated by the amino acids above the aligned sequences. The superscript number indicates the number of alleles with that mutation. The black triangle indicates the site of all four gf alleles. The white triangle indicates a potential *N*-glycosylation site. The site of the Tc1 insertion from *sup-9(n1428)* is indicated. The intron–exon boundaries are indicated by a double arrow above the sequence. GenBank accession numbers are as follows: SUP-9, AY357729; TASK-1, NP_002237; TASK-3, NP_057685; dCG9637, AAF54970; and dCG9361, AAF54374.

muscle-specific promoter, which expresses in all nonpharyngeal muscle groups (Okkema et al., 1993). We tested whether the *myo-3::sup-9* transgene was capable of restoring the sluggish movement and rubberband Unc phenotype of an *unc-93(gf)* allele in a *sup-9(lf);unc-93(gf)* mutant. We found that *sup-9(n1913);unc-93(e1500)* transgenic lines expressing SUP-9 in muscle exhibited a reduced locomotory rate compared with control transgenic lines expressing only GFP (Fig. 4*A*). The rubberband Unc response was also restored to these lines but not to the control lines expressing only GFP (data not shown). The reduction of locomotory rate by *sup-9* overexpression in muscle was not caused by a nonspecific effect of transgene overexpression but rather to an interaction between *sup-9* and *unc-93(gf)*, because the *myo-3::sup-9* transgene in an *unc-93(+)* background did not cause a reduced locomotory rate (Fig. 4*B*). Likewise, we tested whether *unc-93* and *sup-10* expression in muscle would

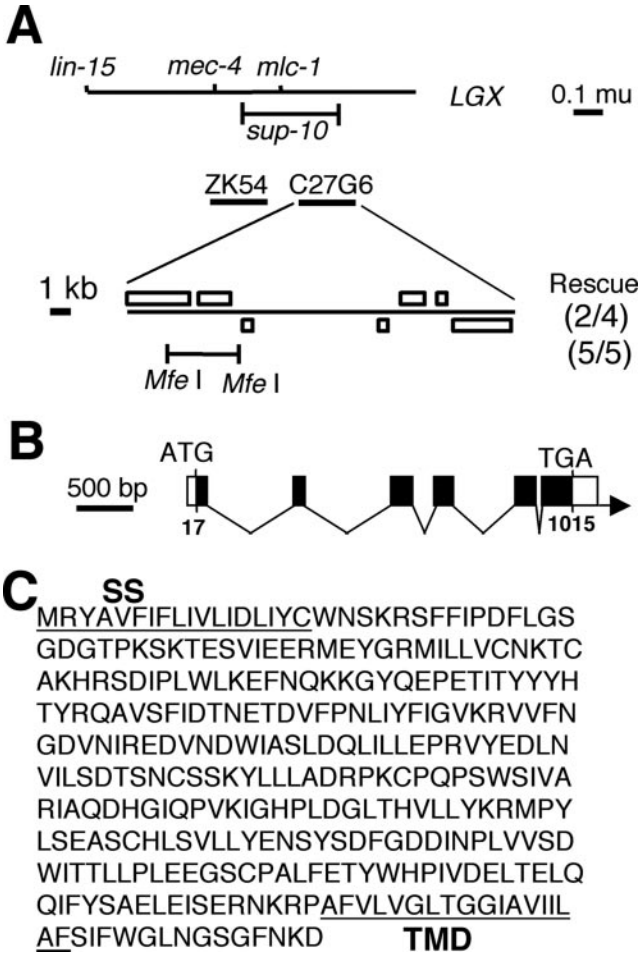

Figure 2. Molecular cloning and genomic structure of the *sup-10* gene and sequence of the SUP-10 protein. *A*, Schematic representation of the *sup-10* genomic region. Cosmid C27G6 as well as a 7.3 kb *Mfe*I subclone of C27G6 suppressed the semidominant rubberband Unc paralysis of *sup-10(n983)* gf mutants as transgenes. Numbers in parentheses indicate the number of rescued lines and total transgenic lines scored. *B*, Intron–exon structure of *sup-10*. The structure of the *sup-10* gene was deduced by comparing its genomic sequence with the sequence of cDNA GenBank accession number U43891. Black boxes indicate coding regions. White boxes indicate untranslated regions. The arrow indicates the direction of transcription. *C*, Amino acid sequence of SUP-10. The underlined residues represent the putative signal sequence (SS) and the transmembrane domain (TMD), which were determined using the PSORTII II program developed by Nakai and Horton (1999) (available at http://psort.nibb.ac.jp/).

restore the *sup-9(gf)* phenotype to a *sup-9(gf);unc-93(lf)* or a *sup-9(gf);sup-10(lf)* mutant, respectively. Lines transgenic for *myo-3::unc-93* or *myo-3::sup-10* showed a severe locomotory defect compared with control *myo-3::gfp* animals (Fig. 4*C*,*D*). We conclude that *sup-9*, *sup-10*, and *unc-93* function in muscle cells.

SUP-9, SUP-10, and UNC-93 colocalize intracellularly

If *sup-9*, *sup-10*, and *unc-93* encode components of a multisubunit K^+ channel complex, we expect them to colocalize within muscle membranes *in vivo*. To test for colocalization, we generated rabbit antibodies against SUP-9. Although these antibodies did not allow us to detect endogenous SUP-9 in whole-mount stainings of wild-type worms (Perez de la Cruz and Horvitz, unpublished observations), likely because of low expression, the antibodies did yield specific staining in transgenic worms overexpressing SUP-9 under the *myo-3* promoter (Fig. 5*A*). Double-label whole-mount stainings of fixed worms with anti-SUP-9 and anti-GFP sera revealed identical muscle membrane distributions of SUP-9 and UNC-93::GFP (Fig. 5*A*). These proteins colocalized both to the dense bodies and more diffusely throughout the cell surface. Some muscle cells contained brightly staining clusters of SUP-9 protein, which may represent mislocalized protein resulting from overexpression. UNC-93::GFP was colocalized with SUP-9 in these clusters (Fig. 5*B*). Similarly, SUP-9 and SUP-10::GFP showed identical localization patterns in double-label experiments using animals carrying SUP-9 and SUP-10::GFP transgenes (Fig. 5*C*).

We compared the localization of SUP-9 with that of PAT-3, an integrin β subunit that is a structural component of dense bodies (Gettner et al., 1995), to test whether overexpression of another muscle membrane protein also resulted in its colocalization with SUP-9. We found that although both SUP-9 and PAT-3::GFP were localized to dense bodies, their distribution patterns were only partially superimposable. Unlike PAT-3::GFP, SUP-9 was present in the spaces between adjacent dense bodies, weakly diffused throughout the muscle membrane, and primarily excluded from the M-lines (Fig. 5*D*). This result is consistent with the hypothesis that UNC-93 and SUP-10, but not PAT-3, interact with SUP-9 *in vivo*.

Muscimol can phenocopy the rubberband Unc phenotype

We hypothesized that the rubberband Unc phenotype of *sup-9(gf)*, *sup-10(gf)*, and *unc-93(gf)* animals may be caused by an inappropriately open K^+ channel. Increased K^+ efflux from muscle would lead to an accumulation of negative charge inside the cell and could result in membrane hyperpolarization and reduced muscle contraction. Electrophysiological patch-clamp recordings of *C. elegans* muscles reveal that treatment of body-wall muscle cells with muscimol, a GABA Cl^- channel agonist, leads to hyperpolarization by an inward Cl^- flux that is dependent on the GABA receptor channel UNC-49 (Richmond and Jorgensen, 1999). We tested whether pharmacologically hyperpolarizing the muscles of wild-type worms would result in a rubberband Unc phenotype similar to that of *sup-9(gf)*, *sup-10(gf)*, or *unc-93(gf)* mutants.

Worms treated with muscimol displayed a rubberband Unc response in a concentration-dependent manner (Fig. 6*A*). Saturation of the rubberband response was reached at a concentration of 2.5 mM muscimol (data not shown). The responses of wild-type animals at high drug concentrations resembled those of the strong *sup-9(n1550)* and *unc-93(e1500)* mutants, whereas lower doses resembled those of the weaker *sup-10(n983)* and *unc-93(n200)* mutants (Fig. 6*B*). Additionally, worms treated with muscimol displayed uncoordinated movement and flaccid, extended body postures similar to those of the *sup-9(gf)* and *unc-93(gf)* mutants (data not shown).

We tested whether *sup-9*, *unc-93*, *sup-10*, or *sup-18* were required for the rubberband Unc response caused by muscimol. Worms carrying lf mutations in these genes all displayed the rubberband Unc response (Fig. 6*C*), whereas, in the absence of muscimol, these lf mutants behaved identically to wild-type animals in showing no rubberband Unc responses (data not shown). Finally, we tested whether the rubberband Unc response caused by muscimol was additive with that caused by the *sup-9(gf)* mutations. We found that treatment with 1 mM muscimol enhanced the rubberband Unc phenotype of *sup-9(n1550)*/+ mutants (Fig. 6*D*). Collectively, these results establish that muscimol induces the rubberband response by a mechanism that is independent of *sup-9*, *unc-93*, *sup-10*, and *sup-18*.

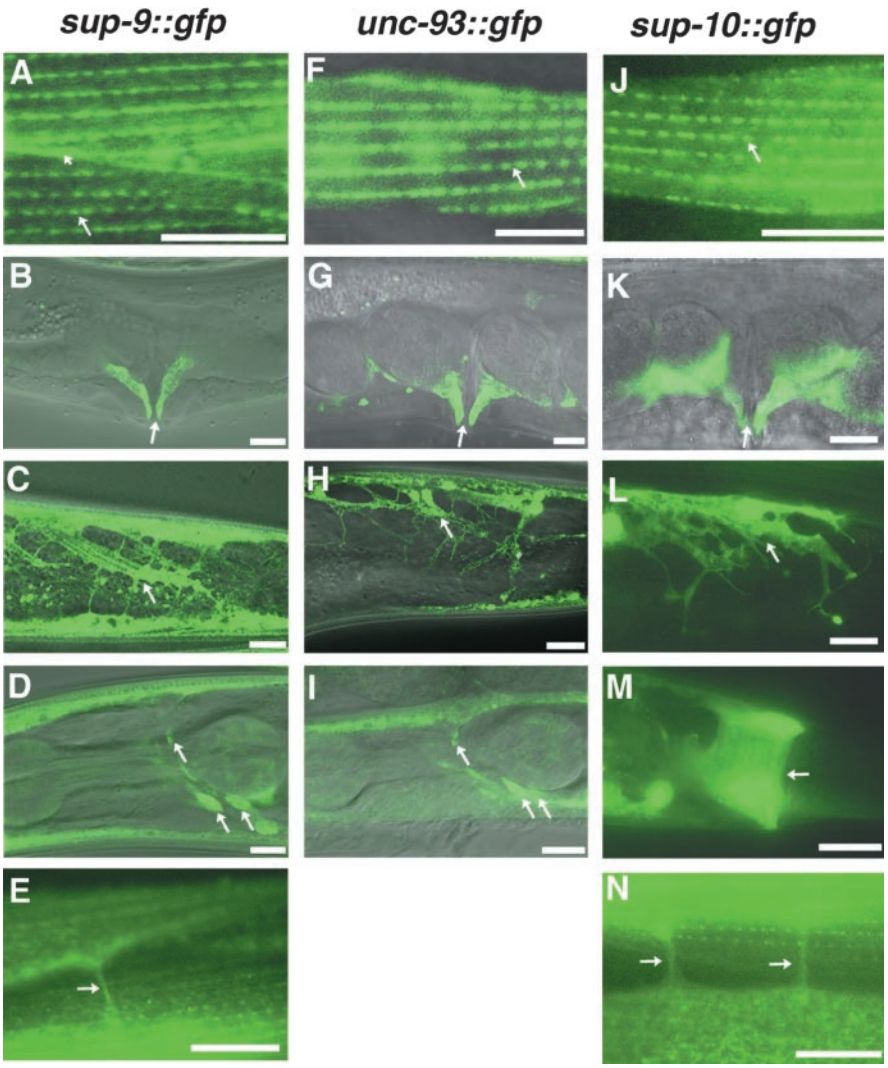

Figure 3. Expression of *sup-9::gfp*, *unc-93::gfp*, and *sup-10::gfp* fusions. *A–N*, Epifluorescence or merged Normarski and epifluorescence images of GFP expression in worms carrying a *sup-9::gfp* (*A–E*), *unc-93::gfp* (*F–I*), or *sup-10:gfp* (*J–N*) reporter transgenes. Scale bars, 10 μm. *A*, *F*, *J*, Body-wall muscle cells. The short arrow indicates the cell membranes of adjacent body-wall muscle cells; the long arrow indicates rows of dense bodies. *B*, *G*, *K*, Vulval muscle cells. Two muscle cells are visible in the focal planes shown. The arrow indicates the opening of the vulva. *C*, *H*, *L*, Intestinal muscle cells (arrows). *D*, *I*, Heads of adult animals expressing *gfp* in neurons (arrows). *E*, *N*, Muscle arms (arrows). *M*, Anal depressor muscle (arrow).

Overexpression of SUP-9(n1550gf) does not bypass the requirement for UNC-93

Why does the gf SUP-9(n1550) channel require UNC-93 to cause muscle paralysis? One possibility is that UNC-93 acts as a chaperone to increase levels of the mutant SUP-9 channel at the cell surface. Alternatively, UNC-93 may have little effect on the number of membrane SUP-9 channels but rather increase their K^+-transporting activity by stabilizing their open state. To distinguish between these models, we overexpressed the *sup-9(n1550)* gf cDNA under the control of the *myo-3* muscle-specific promoter in transgenic arrays in an attempt to bypass the requirement for *unc-93*. Three independent lines overexpressing *sup-9(gf)* in an *unc-93(lr12)* null background displayed identical locomotion rates to the wild-type strain (26.6, 26.8, and 26.0 vs 26.3 bends per minute, respectively) (Fig. 7*A*). Thus, in the absence of UNC-93, excess amounts of SUP-9(gf) channel did not hyperpolarize muscle cells. As expected, introduction of a single wild-type copy of *unc-93* into each transgenic strain resulted in a severe paralysis (0.5–1.5 bends per minute) (Fig. 7*A*). To confirm that the SUP-9(gf) channel was being overexpressed, we immunostained these transgenic lines with anti-SUP-9 antiserum (Fig. 7*B*). As expected, *sup-9(n1550)*-transgenic animals, but not their non-transgenic siblings, displayed a robust signal on their muscle surface, confirming the overexpression of SUP-9(n1550) channels in these animals. We conclude that UNC-93 likely does not function as a chaperone for SUP-9.

unc-93 belongs to a large *C. elegans* gene family and is conserved in flies and mammals

We searched the *C. elegans* genome for predicted genes with sequence similarities to UNC-93. We found that UNC-93 defines a family of 17 worm genes, of which UNC-93 is a relatively divergent member (Fig. 8). UNC-93 contains a highly charged 245 amino acid N-terminal domain followed by 5–10 putative transmembrane domains (Levin and Horvitz, 1992). This N-terminal domain is unique to UNC-93 and appears not to be conserved in the other *C. elegans* members of this family.

In addition, by searching nucleotide and protein databases, we identified four *Drosophila melanogaster* and three mouse and three human genes with predicted products with sequence similarity to UNC-93 (Fig. 8). Three pairs of mouse and human genes are likely orthologs, because they are more closely related to each other than to other UNC-93-like genes in their respective species. Of the UNC-93-like genes, the human predicted gene 366N23.1/.2 was the most similar to UNC-93, sharing 30% amino acid identity. Mouse ET8 and human ET22 are more similar to the *C. elegans* predicted gene C27C12.4 than to UNC-93. The existence of UNC-93-like genes in other organisms suggests that regulation of two-pore K^+ channels by regulatory subunits may be a common mechanism.

Discussion

The *C. elegans* genome project has identified more than 80 K^+ channels, ~50 of which belong to the two-pore structural class of background or "leak" channels (Bargmann, 1998). Mutants defective in several *C. elegans* voltage-gated K^+ channels have been characterized, including the K_v channels *exp-2* (Davis et al., 1999) and *egl-36* (Elkes et al., 1997; Johnstone et al., 1997), the HERG channel *egl-2* (Weinshenker et al., 1999) and the large-conductance Ca^{2+}-activated channel *slo-1* (Wang et al., 2001). While two-pore channels define the largest family of K^+ channels, mutations affecting only one gene in this family, *twk-18*, have been reported (Kunkel et al., 2000).

We cloned the muscle regulatory gene *sup-9* and found that it encodes a two-pore K^+ channel similar to the mammalian

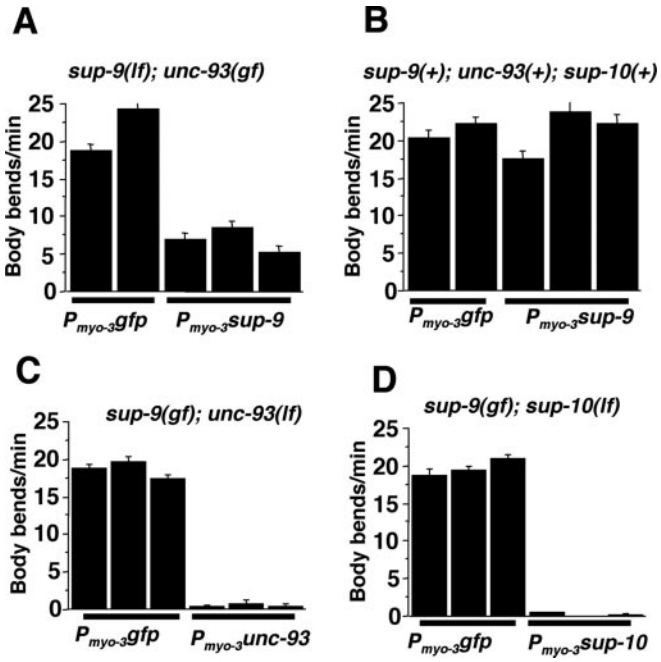

Figure 4. *sup-9*, *sup-10*, and *unc-93* cDNA rescue of locomotory defects. Locomotory rates of young adult transgenic hermaphrodites were scored on a bacterial lawn during 60 sec intervals. A body-bend is defined as a 360° sine wave. Each bar represents the mean ± SEM of an independent transgenic line. All strains carry *lin-15(n765)* and the wild-type *lin-15* gene as a transgene to aid in the identification of transgenic animals. *A*, Parental genotype: *sup-9(n1913); unc-93(e1500); lin-15(n765)*; $n = 20–24$ worms per transgenic line. *B*, Parental genotype: *lin-15(n765)*; $n = 11–13$. *C*, Parental genotype: *sup-9(e2655); unc-93(e1500 n234); lin-15(n765)*; $n = 16–17$. *D*, Parental genotype: *sup-9(n1550); sup-10(n183); lin-15(n765ts)*; $n = 16$.

TASK-1 and TASK-3 channels. Genetic evidence strongly indicates that the SUP-9 protein forms a protein complex with at least two other proteins, SUP-10 and UNC-93 (Greenwald and Horvitz, 1980, 1982; Levin and Horvitz, 1993; De Stasio et al., 1997). SUP-10 and UNC-93 are novel transmembrane proteins. SUP-9, SUP-10, and UNC-93 function in muscle and colocalize in muscle membranes. Given these findings, we propose that SUP-9, SUP-10, and UNC-93 form a multi-subunit K^+ channel complex that regulates *C. elegans* muscle contraction. We identified a large family of *unc-93*-like genes in *C. elegans* as well as related genes in *Drosophila* and mammals. We suggest that the regulation of two-pore channels by auxiliary subunits is evolutionarily conserved.

The SUP-9(gf) K^+ channel may be stabilized in an open conformation

The molecular identity of *sup-9* as a K^+ channel, together with the rubberband Unc phenotype of wild-type animals treated with muscimol, suggests that the rubberband Unc phenotype of *sup-9(gf)* mutants is the result of an increased K^+ efflux and a hyperpolarization of muscle cells. Consistent with this model, gf mutations in the muscle two-pore K^+ channel *twk-18* result in a rubberband Unc paralysis that is indistinguishable from that of *sup-9(gf)* mutants (Kunkel et al., 2000; Perez de la Cruz and Horvitz, unpublished observations). Heterologous expression of wild-type and mutant forms of TWK-18 channels in *Xenopus* oocytes reveals 30-fold greater K^+ currents of gf channels than wild-type channels (Kunkel et al., 2000). We propose that gf mutations in *sup-9* result in greater K^+ efflux and hyperpolarization of muscle cells.

What is the mechanism by which an alanine-to-threonine substitution at amino acid 236 in SUP-9 might increase K^+ channel activity? We considered three distinct mechanisms: by an increase in the number of SUP-9 channels at the cell surface, by an increase in the unitary conductance of each channel, or by an increase in the open probability of SUP-9. Because overexpression of wild-type SUP-9 under the *myo-3* promoter did not result in an Unc phenotype in transgenic animals (Fig. 4), it is unlikely that the gf mutation in *sup-9* causes an Unc phenotype by increasing the number of channels at the cell surface. We postulate that the gf mutation in *sup-9* results in either a higher unitary conductance or a higher open probability.

All four gf mutations in *sup-9* cause the same A236T amino acid substitution within the C-terminal half of the fourth transmembrane domain of SUP-9. A comparison of the crystal structures of two bacterial K_{ir}-like channels, the closed KcsA channel (Doyle et al., 1998) and the open MthK channel (Jiang et al., 2002a), suggests that during channel opening, there is a rotation of the transmembrane domains that follow the P-domain (Jiang et al., 2002b). Because the gf substitution occurs within this gating domain, we postulate that the mutant threonine may stabilize the fourth transmembrane domain in its rotated conformation, resulting in a SUP-9 channel that is constitutively open.

We determined that the second-site compensatory mutations *n2288* and *n2359*, which counteract the effects the gf A236T substitution (Levin and Horvitz, 1993), affect adjacent amino acids, G173E, and A174T, respectively, in the third transmembrane domain of SUP-9 (Table 3). In the two K^+ channels for which a crystal structure has been solved, KcsA and MthK, the transmembrane helices flanking the P-domain physically interact (Doyle et al., 1998; Jiang et al., 2002a). Thus, these second-site compensatory mutations may counteract the effects of the gf threonine substitution by directly interacting with its side chain and neutralizing its stabilization of the open state. Alternatively, these second-site mutations may interact with other residues in the fourth transmembrane domain or induce a conformational change in the third transmembrane domain that stabilizes the closed conformation of the channel. We postulate that interactions between the third and fourth transmembrane domains of SUP-9 are important in channel gating.

SUP-10 and UNC-93 may regulate SUP-9 K^+ channel gating

Although 14 mammalian two-pore channels have been cloned and characterized thus far (Goldstein et al., 2001), no two-pore channel regulatory subunits have been reported. Three cloned mammalian two-pore channels, KCNK7, KCNK13, and KCNK15, have not produced K^+ currents when expressed in a heterologous system, suggesting that association with other proteins may be necessary for their functioning (Rajan et al., 2001). Similarly, we have been unable to obtain K^+ currents from SUP-9 or SUP-9(n1550), expressed alone or together with SUP-10 and UNC-93, in *Xenopus* oocytes or human embryonic kidney (HEK) cells using whole-cell voltage-clamp configuration, although our control experiments demonstrated a robust K^+ activity from the mammalian rat TASK-1 channel expressed in both cell types (Perez de la Cruz and Horvitz, unpublished observations). Our experiments were limited to detecting pH-sensitive currents, a hallmark of TASK-1 and TASK-3 channels; we did not vary other factors such as temperature, neurotransmitters, or anesthetics. Our immunohistochemical stainings of HEK cells transfected with SUP-9, SUP-10, and UNC-93 using anti-SUP-9 antisera indicate that much of SUP-9 was not targeted to the cell membrane but instead appeared to be trapped in the endoplasmic reticulum–Golgi complex (Perez de la Cruz and

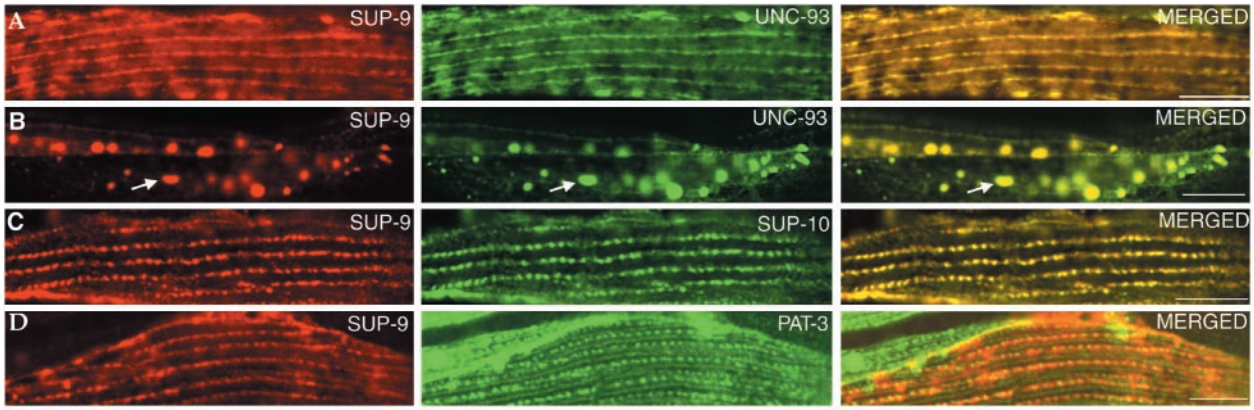

Figure 5. Subcellular colocalization of SUP-9 with UNC-93::GFP and SUP-10::GFP. Transgenic lines expressing *sup-9* driven by the *myo-3* promoter and an *unc-93::gfp* (*A*, *B*), *sup-10::gfp* (*C*), or *pat-3::gfp* (*D*) fusion was fixed and costained with anti-SUP-9 (red) and anti-GFP antisera (green). All panels display adult body-wall muscle cells analyzed by confocal microscopy for immunofluorescence. Scale bars, 10 μm.

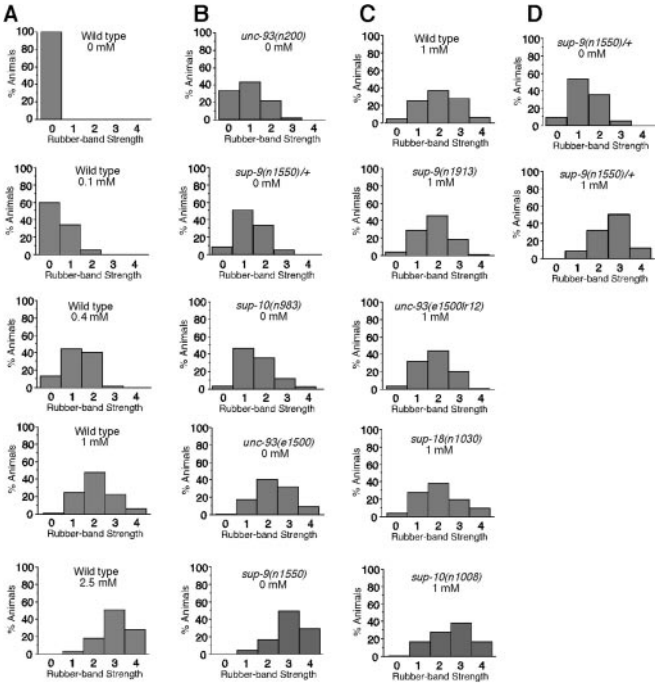

Figure 6. Muscimol phenocopies the rubberband Unc phenotype of *sup-9(gf)*, *unc-93(gf)*, and *sup-10(gf)* mutants. *A*, Wild-type (N2) worms were scored for the rubberband Unc response at the indicated concentrations of muscimol, as defined in Materials and Methods ($n = 170$ for all concentrations except 1 mM, in which case $n = 232$). *B*, Rubberband mutants were scored for the rubberband Unc response in the absence of muscimol [$n = 300$ for all genotypes except *sup-9(n1550l)*, in which case $n = 200$]. *C*, Wild-type and lf mutant worms were scored for the rubberband Unc response in 1 mM muscimol. The numbers of responses scored were $n = 979$, $n = 360$, $n = 300$, $n = 490$, and $n = 300$, respectively. *D*, Rubberband *sup-9(n1550)/+* worms were scored for the rubberband Unc response in the absence and presence of 1 mM muscimol. The numbers of responses scored were $n = 300$ and $n = 300$.

Horvitz, unpublished observations). Mutations in two additional genes, *sup-18* and *sup-11*, suppress the rubberband-Unc phenotype of *sup-9(gf)*, *sup-10(gf)*, and *unc-93(gf)* mutants and thus may encode additional regulators of the proposed channel complex. It is possible that the SUP-18 and SUP-11 proteins are required for channel activity in heterologous expression systems (Greenwald and Horvitz, 1982, 1986; Levin and Horvitz, 1993).

K^+ channel regulatory subunits play diverse roles during their association with the pore-forming α subunits of channels from other families. The cytoplasmic regulatory subunit $K_v\beta2$ associates with Shaker-like K_v channels and increases their surface expression in heterologous systems (Shi et al., 1996) while exerting small effects on channel activity (Rettig et al., 1994; Heinemann et al., 1996). In addition to regulating K^+ channels through chaperone activity or altering gating kinetics, some channel subunits, such as the SUR1 subunit of $K_{ir}6.2$ channels or the $\beta1$ subunits of Slo channels, alter the sensitivities of their associated channels to activating signals, such as voltage, ATP, or Ca^{2+} (McManus et al., 1995; Tucker et al., 1997).

Three lines of evidence suggest that SUP-10 and UNC-93 do not behave simply as chaperones to increase SUP-9 surface expression. First, we found that in both *sup-10(lf)* and *unc-93(lf)* mutants, SUP-9::GFP is expressed on the cell surface of muscle cells, suggesting that association of SUP-9 with SUP-10 or UNC-93 is not required for SUP-9 cell-surface expression. Second, overexpression of SUP-9(gf) in transgenic *unc-93* null mutants did not result in a rubberband Unc paralysis, indicating that UNC-93 is required for SUP-9 channel activity. Third, genetic analysis of gf mutations in *sup-9*, *sup-10*, and *unc-93* strongly indicates that these three genes encode subunits of a protein complex (Greenwald and Horvitz, 1980, 1986; Levin and Horvitz, 1993). Because gf mutations in the proposed channel subunits *sup-10* and *unc-93* result in a muscle paralysis similar to that found in *sup-9(gf)* channel mutants, SUP-10 and UNC-93 likely function in the regulation of SUP-9 channel activity.

Implications for K^+ channel biology

UNC-93 and SUP-10 may represent new classes of K^+ channel regulatory subunits. Sixteen other *C. elegans* genes encode proteins with sequence similarity to UNC-93. Members of this family could associate with the more than 50 *C. elegans* two-pore K^+ channels (Bargmann, 1998) to create a striking level of functional diversity. No interacting genes have been identified through genetic screens for the *C. elegans* two-pore K^+ channel encoded by *twk-18* (Kunkel et al., 2000), suggesting that unlike *sup-9*, some two-pore K^+ channels in *C. elegans* may function without regulatory subunits. Alternatively, such subunits might be either functionally redundant or essential, and for this reason have not been identified. The presence of UNC-93-like genes in the genomes of flies and humans suggests that UNC-93-like regulatory subunits represent a conserved mechanism for the regulation of two-pore K^+ channels. In contrast, we found no genes with similarity to *sup-10* in the *C. elegans* genomic sequence or in mam-

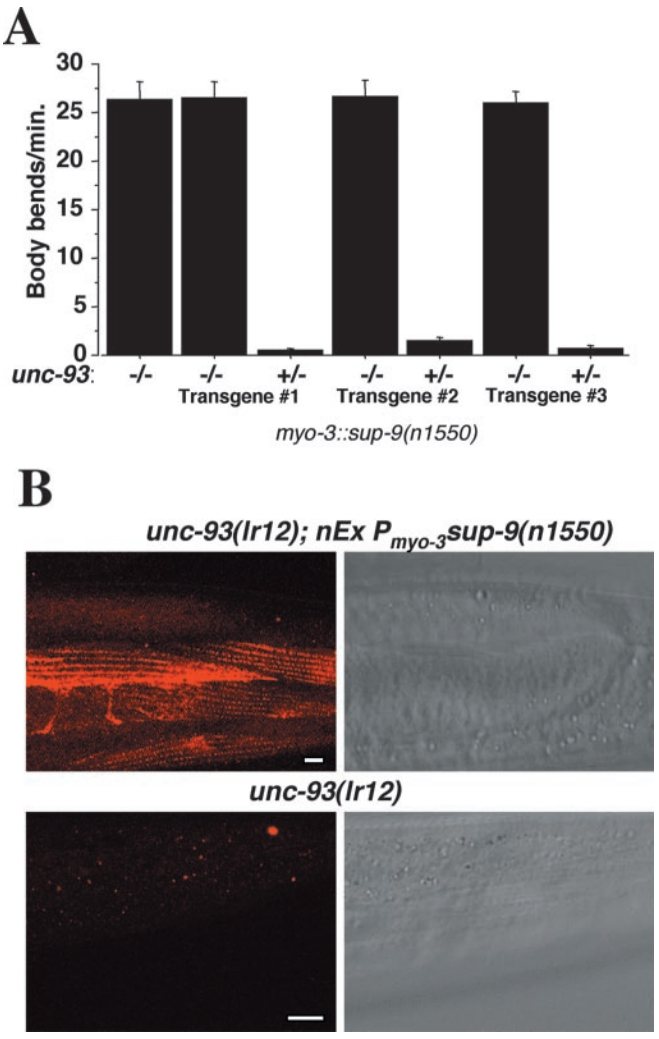

Figure 7. Overexpression of SUP-9(gf) cannot bypass the need for UNC-93. *A*, Transgenic male animals were scored on a bacterial lawn for locomotory rate. Males, rather than hermaphrodites, were used because *unc-93(lr12)* heterozygous males can be unambiguously generated. All strains contained the *lin-15(n765)* mutation to enable the identification of transgenic animals and the *unc-93(lr12)* mutation as noted previously. The independently generated transgenic lines are indicated by the numbers below the graph. Each bar represents the mean ± SEM; $n = 10$ for all lines. *B*, Confocal images of a representative adult transgenic animal (top) and a nontransgenic sibling (bottom). *unc-93(lr12)* animals with or without the *myo-3::sup-9(n1550)* transgene were fixed and immunostained with anti-SUP-9 antisera. Epifluorescence images are on the left. Normarski images on the right allow visualization of dense bodies, in which SUP-9 is expected to localize. Scale bar, 10 μm.

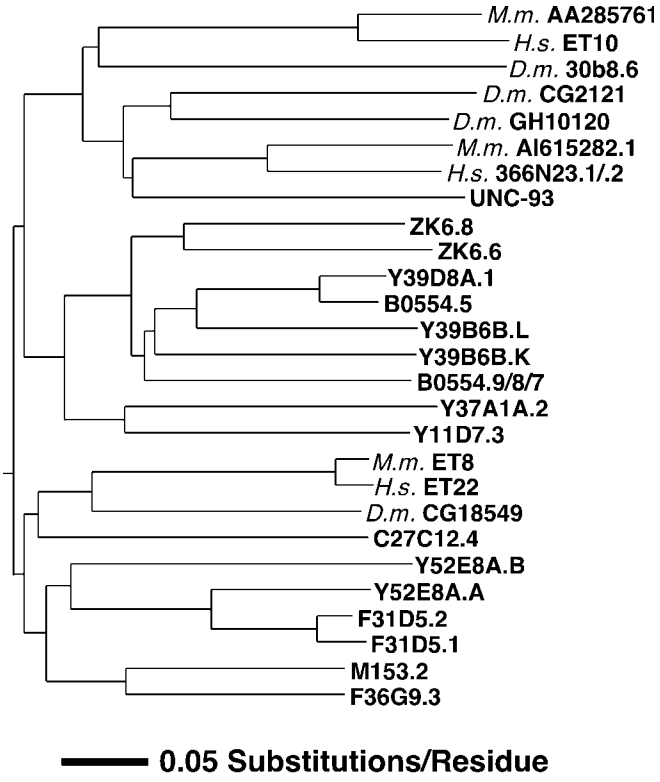

Figure 8. Phylogenetic tree of UNC-93-like genes from *C. elegans*, *Drosophila*, mouse, and human. Protein alignments were generated using the ClustalW algorithm (Thompson et al., 1994). The horizontal length between each pair of branches represents the evolutionary distance between gene pairs as measured by the number of substitutions per residue. The predicted sequences of *H.s.* dJ366N23.1 and *H.s.* dJ366N23.2 were used to create *H.s.* dJ366N23.1/.2, because we believe that these sequences likely represent the N-terminal and C-terminal segments, respectively, of a single gene. *M.m.* AI615282 and *M.m.* 285761 are encoded by ESTs and likely represent partial sequences of their respective genes. B0554.9/.8/.7 contains the combined amino acid sequences of predicted genes B0554.9, B0554.8, and B0554.7, because we believe that these sequences are likely portions of one gene. The GenBank accession numbers of the genes are as follows: *D.m.* 30B8.6, CAA15704; *D.m.* CG2121, AAF59099; *D.m.* GH10120, AF145657; *D.m.* CG18549, AAF54824; *H.s.* ET10, AF015185; *H.s.* dJ366N23.1/.2, AL021331; *H.s.* ET22, AF015186; *M.m.* AI615282, AI615282; *M.m.* ET8, AF015191; *M.m.* AA285761, AA285761; UNC-93, S23352; ZK6.8, AAC17687; ZK6.6, AAC17693; Y39D8A.1, AAC69224; B0554.5, AAB37612; Y39B6B.L, CAB60917; Y39B6B.K, CAB60916; B0554.9/.8/.7, AAB37619, AAB37618, AAB37616; Y37A1A.2, CAB16469; Y11D7A.3, CAA21581; C27C12.4, CAA93741; Y52E8A.B, AAF59523; Y52E8A.A, AAF59522; F31D5.2, AAC71105; F31D5.1, AAC71106; M153.2, CAA91944; F36G9.3, CAB04342. *H.s.*, *Homo sapiens*; *M.m.*, *Mus musculus*; *D.m.*, *Drosophila melanogaster*. All others are *C. elegans*.

malian expressed sequence tag or genomic databases, suggesting that SUP-10 may be a specialized regulatory subunit specific to the SUP-9–UNC-93 channel complex. Additional studies of the interactions between two-pore K^+ channels and their presumptive regulatory subunits should enhance our understanding of this major family of K^+ channels.

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