Materials and Methods

Positional cloning and molecular analysis of *anc-1*

Mutant alleles *e1802*, *e1753*, and *e1873* (*S1*) were provided to us by the *Caenorhabditis* Genetics Center or from Jonathan Hodgkin's collection at the MRC. *anc-1(e1753, ku367 rev)* was isolated as a spontaneous wild-type revertant. We mapped *anc-1* using the strain HR 735 (provided by Paul Mains, University of Calgary) to create *unc-73(rh40), let-502(ca201), bli-4(e936)/anc-1(e1753)* heterozygotes from which Unc non-Let recombinants were picked. Eight of the 20 recombinants segregated Anc animals, placing *anc-1* at -1.66 of chromosome I (Fig S1A).

Injections of cosmids in this region failed to rescue the Anc phenotype. We therefore performed single nucleotide polymorphisim (SNP) mapping. SNP mapping was done as previously described (*S2*) except that we used the divergent wild type strain from Hawaii CB4856 (*S3*). The following SNPs were identified: *kuP2* is a C in N2 and a G in CB4856 at 12616 of cosmid C18E3, *kuP5* is a T in N2 and an A in CB4856 at 35884 of cosmid C43E11, *kuP6* is a T in N2 and a G in CB4856 at 30648 of cosmid ZK973, and *kuP3* has an inserted T in CB4856 at 10255 of cosmid C41D11. An *unc-73(rh40), anc-1(e1753), dpy-5(e61) I* strain was created and crossed to CB4856. Eighty-eight recombinant chromosomes with crossovers between *unc-73* and *dpy-5* were isolated and analyzed with respect to the SNPs. Six of the 72 recombination events between *anc-1* and *dpy-5* were mapped left of the SNP *kuP3*; one of these crossovers occurred to the left of *kuP6* (Fig. S1B). 16 recombination events between *unc-73* and *anc-1* were found to be right of *kuP2*; three of these crossovers were right of *kuP5* (Fig. S1B). These data placed *anc-1* within an absolute physical region of about 110 kb between *kuP5* on cosmid C43E11, and *kuP6* on cosmid ZK973. Furthermore, three-point mapping data using the SNPs placed *anc-1* on the cosmids T03A1 and ZK973.

Expressed sequence tags (ESTs) were identified from the predicted open reading frame using The Intronerator software (*S4*). ESTs were kindly provided by Yuji Kohara at the National Institute of Genetics, Mishima, Japan. The sequences of the cDNAs represented by ESTs yk25a11, yk32c4, and yk7e10 were determined. In addition, RT-PCR products were isolated representing the first 18 exons of *anc-1* and connecting what was previously predicted to be three separate open reading frames (ZK973.6, T03A1.4 and T03A1.3) from the predicted 5' end of the gene using the ThermoScript RT-PCR system (Life Technologies, Rockville, MD) from total *C. elegans* RNA produced using the RNeasy kit (Qiagen, Valencia, CA) and cloned into pT-Adv (Clontech, Palo Alto, CA). An in-frame stop codon was detected three base pairs upstream of the predicted ATG initiation codon suggesting that the 5' end of the transcript was identified.

dsRNA for RNAi experiments was made from genomic PCR product templates with overhanging T7 promoters using T7 RNApol and injected into N2 adult hermaphrodites (*S5*). Progeny were scored for Anc defects under Nomarski optics three days after injection. RNAi templates were genomic DNA PCR fragments. For genomic Southern analysis, DNA was digested with *Bgl*II and probed with yk25a11.

Actin binding assay

The actin binding protein biochem kit (Cytoskeleton, Denver, CO) was used following the supplied protocol. [^{35}S]methionine-labeled ends of ANC-1 were made using the TNT coupled reticulocyte lysate system (Promega, Madison, WI). The 2.1 kb 5' RT-PCR product was used for the NH2-terminal template. For the COOH-terminal template, *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used to amplify the final 346 residues of ANC-1 (everything downstream of

the end of the tandem repeats) with overhanging *Nde*I and *Not*I sites and an NH2-terminal FLAG epitope tag. This product was cloned into pET28a, which provided an NH2-terminal start site and a His epitope tag. Relative amounts of [^{35}S] in the supernatant versus the pellet were quantified using a Storm PhosphorImager and ImageQuant software (Amersham Pharmacia Biotech, Piscataway, NJ).

Antibody production, western analysis, and immunofluorescence

The 3.2 kb cDNA represented by EST yk25a11, which contains an entire tandem repeat of ANC-1, was amplified by *Pfu* DNA polymerase with overhanging *Bam*H1 restriction enzyme sites. The product was then cloned into the *Bam*H1 site of pGEX-2T (Amersham Pharmacia Biotech) to create a construct encoding an ANC-1 repeat/GST fusion protein. The fusion protein was expressed in *E. coli* strain BL21 codon plus (Stratagene) and purified on glutathione sepharose 4B beads (Amersham Pharmacia Biotech) according to the protocol supplied by the manufacturer.

Polyclonal antibodies were raised in three 200 g female Sprague Dawley rats (Harlan, Indianapolis, IN) injected four times with 200 µg of purified fusion protein in Freund's adjuvant at three week intervals. Nine days after the final boost, the rats were bled to produce 3-4 ml of crude serum. 1 ml of serum from the best responder (rat #3) was affinity purified against 10 mg of ANC-1 repeat/GST fusion protein on a 1 ml Affi-Gel 15 (Bio-Rad, Hercules, CA) column (*S6*). Immunoblot analysis was done on a 5% acrylamide gel as previously described (*S7*) with affinity purified antibody used at a dilution of 1/500.

For immunofluorescence with the ANC-1 antibody, N2 or *unc-84* mutant young larvae and adults were placed on poly-lysine coated slides; in some cases, internal tissue was extruded by slight pressure from a coverslip. Animals were permeabilized by the freeze-crack method, fixed for 30 minutes in 4% paraformaldehyde in PBS and then 15 minutes in -20°C methanol before being blocked in PBS-T +5% dry milk (*S8*). The fixed specimens were stained as described (*S8*). Affinity purified primary antibody was used at a 1/250 dillution. Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1/200 in PBS-T was used as the secondary antibody. DNA was visualized by staining for five minutes with 100 ng/ml of 4,6-diamidino-2-phenylindole (DAPI; Sigma, St Louis, MO) in PBS-T. To recognize the HA epitope tag specimens were fixed as above and stained with monoclonal antibody HA.11 (Covance, Princeton, NJ) at a 1/2000 dilution and Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) diluted 1/200 as the secondary antibody. Actin was recognized with TRITC labeled phalloidin (Sigma) was used at 0.2 µg/ml in animals fixed as above. Images were collected using an Axioplan2 microscope (Carl Zeiss Inc, Thorton, NY) and a Hamamatsu C4742-95 CCD camera (Hamamatsu Photonics KK, Bridgewater, NJ). Images were deconvolved and analyzed using Openlab 2.0.7 (Improvision, Lexington, MA).

ANC-1 end domain fusion constructs and mitochondrial GFP constructs

A genomic region representing the COOH-terminal 346 residues of ANC-1 (everything downstream of the end of the tandem repeats) was amplified by *Pfu* DNA polymerase with overhanging *Nco*I sites and an NH2-terminal start codon and HA tag. The product was then cloned into the *Nco*I site of pPD49.78 under control of the heat inducible promoter of *hsp16-2* (supplied by A. Fire, Carnegie Institute) to create pDS73. A construct encoding residues 1087-2974 of ANC-1 (pDS76) was made in an analogous manner. A 9.7 kb *Pst*I / *Nhe*I genomic fragment including the endogenous *anc-1* promoter and the first 566 residues of ANC-1 was

cloned in frame to a COOH-terminal GFP in pPD95.77 (supplied by A. Fire) to create pD81. Finally, the first 566 residues of ANC-1 were taken from the RT-PCR product and cloned into the *Nhe*I site of pPD49.78 under control of the *hsp16-2* promoter. GFP or a nine myc tag, each with a stop codon, were then fused in frame after the 566 residues of ANC-1 to create pD98 and pD97 respectively. Lines with extragenic arrays were made using *sur-5::GFP* or *rol-6(su1006)* as expression markers (*S9, S10*). Transgenic animals were heat shocked at 33°C for two hours or at 30°C for 16 hours, GFP positive animals were examined under Nomarski optics as above.

To visualize mitochondria, GFP with a mitochondrial localization signal expressed in body wall muscle by the *myo-3* promoter (pSAK4; *S5*)was co-injected with a dominant *rol-6* marker (pRF4; *S10*) into a variety of genetic backgrounds to make lines with extragenic arrays. GFP-positive mitochondria were observed as above.

Supporting Text

ANC-1 was found to have a homologous structure to *Drosophila* and human proteins

The *anc-1* gene product is a highly repetitive protein. ANC-1 contains six tandem repeats, spanning exons 20 to 26 (Fig. 2D). Each repeat is 903 amino acids and is nearly 100% identical to the other repeats. Even the 123 bp intron within each repeat is identical. The second of the six repeats has an unconfirmed insert of 51 amino acids, of which five residues are unique; the rest are repetitive with the 46 residues preceding the insert. The sixth repeat is only two thirds as long as the other five. Using genomic Southern analysis, we confirmed that the tandem repeats are over 18 kb long. Not only were the full repeats present in wild-type worms from England (N2), but they were also found in divergent populations of *C. elegans* from Hawaii (CB4856), Australia (AB4), and Germany (RC301). The length of the repeat region may thus be maintained because of a selective advantage to keep ANC-1 large. There is a shorter repetitive region in exon 18 where 10 repeats of 58 amino acids were found (Fig. 2D). However, these repeats are only between 35% and 89% identical to one another at the amino acid level.

Most of ANC-1, including all the repetitive regions, is predicted to be highly alpha-helical with interspersed regions of likely coiled-coil domains. This suggests that ANC-1 may fold in an elongated myosin tail-like structure. Electron microscopy studies have shown that the 1500 amino acid coiled-coil domain of myosin heavy chain tail extends about 150 nm (*S11*). If ANC-1 forms a similar coiled-coil structure, it could easily extend four to five times that distance. This would allow a single ANC-1 molecule to extend over 0.5 μm, long enough to stretch between the nucleus and the actin cytoskeleton.

BLAST searches identified a conserved domain at the COOH-terminal 60 residues of ANC-1 that was 40% identical (55% similar) and 50% identical (74% similar) to the COOH-termini of the *Drosophila* protein Klarsicht and a protein represented by EST CK00024 respectively (Fig. S2). ANC-1 was also found to be 55%-60% identical (75%-76% similar) to the COOH-termini of the mammalian proteins Syne-1 and Syne-2 (Fig. S2). ANC-1 was found to contain two approximately 100 amino acid stretches of homology that are 44% and 31% identical (64% and 52% similar) to the calponin domains of human alpha-actinin, 51% and 38% identical (65% and 63% similar) to domains of human Syne-2, and 42% and 31% identical (54% and 50% similar) to domains of Msp-300 (Fig. S2).

Three previous groups working on Syne-1 and Syne-2 (also called myne-1 and -2 or nesprin-1 and -2) identified only the C-terminal portion of the proteins (*S12-S14*). We collected DNA sequences from various sources to compile predicted, full-length cDNA sequences of *syne-*

1 and *syne-2.* A 21.8 kb human *syne-2* cDNA was recently identified and named NUANCE (*S15*). Subsequently, we constructed a likely full-length human *syne-1* sequence of 8739 amino acids consisting of a cDNA sequence located just upstream of the partial *syne-1* gene. The N-terminal 60% of human-Syne-1 was constructed using two existing cDNA sequences of 1001 and 1363 bp (GeneBank AB051543 and AB033088) and a protein structure predicted by Genescan (ctg2.111; http//genome.ucsc.edu). We also identified a 3612bp cDNA sequence corresponding to the C-terminal portion of the mouse *syne-2* gene (GenBank accession number BC010723).

ANC-1 localization at the nuclear envelope in mutant backgrounds
ANC-1 localization was tested in eleven mutant strains. ANC-1 antibodies localized normally in animals that were *N2* wild-type, *unc-84* class four alleles that disrupt the NH2 part of UNC-84 (*n322* and *e1411*), and the null allele *unc-83(e1408)*. ANC-1 was not seen at the nuclear envelope in animals that were *unc-84* null (*n369*), or *unc-84* class two or three alleles with mutations in the SUN domain (alleles *n323*, *n371*, *sa61*, and *n399*). Finally as a control, ANC-1 failed to localize in the three *anc-1* alleles tested (*e1753*, *e1802*, and *e1873*).

Overexpression of the NH2-terminus of ANC-1 disrupts muscle function
When expressed long-term (by heat shock at 30°C for 16 hours or from multiple copies of the endogenous promoter in an extragenic array), the NH2-terminal ANC-1::GFP localized more broadly in a pattern similar to actin, including strongly in the pharynx and at the cortex of many cells including gut cells (Fig. S3). Ultimately, the long-term overexpression of NH2-terminal ANC-1::GFP blocked muscle development. In the most severe cases, when overexpressed in the embryo from the endogenous *anc-1* promoter, NH2-terminal ANC-1::GFP ectopically blocked proper muscle formation and lead to a paralyzed and arrested elongation at twofold (Pat) phenotype where embryos failed to elongate due to deformed muscles (Fig S3; *S16*). Analogous constructs with a nine myc tag in place of GFP gave similar localization patterns and phenotypes. The Pat phenotype seen here is the same as has been previously described for mutations in a number of different muscle components (including myosin heavy chain A, tropomyosin, and troponin) and in components required to attach muscles to the body wall (such as α- and β-integrins, perlecan, and vinculin; *S16, S17*). This neomorphic effect suggests that the N-terminal of ANC-1 interferes with the function of a different protein. Likely candidates are one of the other calponin-containing proteins, of which *C. elegans* has at least twelve. A particularly strong candidate is α-actinin, an actin-binding protein that is a major component of the dense body and functions to attach muscle to the hypodermis (S*18*).

Fig. S1. (A) The *anc-1* genomic region of chromosome I is depicted. Genetic mapping data places *anc-1* at approximately –1.66 between *unc-73* and *let-502*. (B) The physical region around *anc-1* is shown. Thick lines represent cosmids sequenced by the genome consortium. The physical location of SNPs, *kuP2*, *kuP5*, *kuP6*, and *kuP3*, are marked with arrows and dotted guidelines. The genetic positions of *anc-1*, from three-point mapping with *kuP2*, *kuP5*, and *anc-1*, or with *anc-1*, *kuP6*, and *kuP3*, are similarly marked. 88 recombinants between *unc-73* and *dpy-5* were characterized to determine more precisely where the crossover events took place. The numbers of recombination events that occurred between each marker is shown in large numbers at the bottom. A 20 kb scale bar is shown.

Fig. S2. The top row is a ClustalW alignment of the C-terminal KASH domain of ANC-1 as compared to homologous proteins. The predicted trans-membrane region is underlined. The second and third rows show ClustalW alignments of the two calponin-like domains of ANC-1 as compared homologous proteins. Residues identical in at least two thirds of the compared proteins are boxed.

Fig. S3. GFP fluorescence (A) and DIC (B) images of an embryo arrested at the twofold stage because of overexpression of the N-terminal ANC-1::GFP fusion protein from a multi-copy extragenic array from the endogenous *anc-1* promoter. Anterior is top left. Scale bar is 10 μm.

Fig. S4. ANC-1 and UNC-84 function to control nuclear anchorage. ANC-1 (the dark and light blue coiled structure) could extend up to 1 μm in length. The COOH-terminal KASH domain of ANC-1 (yellow) is targeted to the outer nuclear membrane (ONM) through an interaction with the SUN domain (red) of UNC-84 (yellow). It is unknown if the ANC-1/UNC-84 interaction is direct. UNC-84 could localize to either the outer or inner nuclear membrane (INM) in this model. While genetics suggests that ANC-1 is required for positioning of mitochondria, we do not have experimental evidence that ANC-1 associates with the mitochondrial membrane. Presumably, the COOH-terminal end of ANC-1 interacts with an unknown component (yellow oval) of the outer mitochondrial membrane. The NH2-terminal two calponin-like domains of ANC-1 (red) attach to actin microfilaments (green) to effectively anchor nuclei and mitochondria in the cytoplasm.

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