The *C. elegans* UNC-23 protein, a member of the BCL-2-associated athanogene (BAG) family of chaperone regulators, interacts with HSP-1 to regulate cell attachment and maintain hypodermal integrity

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Mutations in the *unc-23* gene in the free-living nematode, *Caenorhabditis elegans* result in detachment and dystrophy of the anterior body wall musculature and a bent-head phenotype when grown on solid substrate. We have determined that the *unc-23* gene product is the nematode ortholog of the human BAG-2 protein, a member of the Bcl-2 associated athanogene (BAG) family of molecular chaperone regulators. We show that a functional GFP-tagged UNC-23 protein is expressed throughout development in several tissues of the animal, including body wall muscle and hypodermis, and associates with adhesion complexes and attachment structures within these 2 tissues. In humans, the BAG protein family consists of 6 members that all contain a conserved 45 amino acid BAG domain near their C-termini. These proteins bind to and modulate the activity of the ATPase domain of the heat shock cognate protein 70, Hsc70. We have isolated missense mutations in the ATPase domain of the *C. elegans* heat shock 70 protein, HSP-1 that suppress the phenotype exhibited by *unc-23(e25)* mutant hermaphrodites and we show that UNC-23 and HSP-1 interact in a yeast-2-hybrid system. The interaction of UNC-23 with HSP-1 defines a role for HSP-1 function in the maintenance of muscle attachment during development.

**Introduction**

Mechanical forces play a critical role in morphogenesis, tissue formation and the maintenance of cellular integrity throughout development. An excellent example of the requirement for mechanical force during morphogenesis is the development of the *C. elegans* embryo, which involves the transformation of a round ball of cells into a long thin worm. This transformation entails large-scale cell shape changes, cell spreading and cell migrations, all of which require mechanical forces (for a review see ref. 1). The requirement for mechanical forces during embryogenesis is not limited to the *C. elegans* embryo. The process of gastrulation, which is common to multicellular embryos, requires mechanical forces that cause some cell layers to fold outwards while others bend and invaginate 2 (for a review see ref. 3).

Tissue formation also involves mechanical forces. In order to bear different types of mechanical stresses such as tension, compression or shear, tissues produce highly specialized matrices such as skin, bone, and cartilage. 4 Cells are able to control the deposition, composition and maintenance of molecules that make up their surrounding extracellular matrix (ECM), based on the type and magnitude of the mechanical stresses that act on the tissue. 5 Cells under mechanical stress have also been shown to respond by changing the distribution of the structural components that associate with the cytoskeleton and anchoring junctions. 6 The compositions of the biopolymers (i.e. microfilaments, microtubules, and intermediate filaments) that make up the cytoskeleton are tailored to accommodate the mechanical demand that is put on the cell. 7 The ability to rearrange the ECM and the cytoskeleton in the face of mechanical stresses is an essential response, one that allows the cell to maintain its shape and withstand forces that compromise cellular integrity. To keep their shape and maintain their integrity cells within a tissue must be capable of adaptive responses to various types of mechanical stresses.
We are interested in elucidating some of the mechanisms and molecules involved in the maintenance of cell-ECM adhesion in tissues under physiological stress due to muscle attachment and movement. Movement in the free-living nematode *Caenorhabditis elegans* involves transmission of the contractile force of the body wall muscle cells to the cuticle. This transmission is achieved via a continuous physical linkage between the body wall muscle and the cuticle through the basement membrane and hypodermis. Detailed anatomical studies on *C. elegans* have revealed 2 major extracellular matrices that are involved in motility: the basement membrane and the cuticle. The basement membrane is a specialized ECM that covers the pharynx, intestine, gonad, and body wall muscle cells, and is required for myofilament assembly in the body wall muscle.\(^8,9\) The cuticle is required for embryonic morphogenesis, and provides the exoskeleton that covers the outside of the animal (for a review see ref. 10). In between the basement membrane and the cuticle is the hypodermis, which is the nematode’s equivalent of skin. The hypodermis not only plays an important role in transmission of force from the muscle to the cuticle, it is also responsible for deposition of the cuticle and perhaps some basement membrane proteins.\(^8,10,11\) Hemidesmosomes, which are the cell-ECM anchoring junctions for the intermediate filaments, are located at both the basal and cuticular membranes of the hypodermis.\(^8,11\) Hemidesmosomes provide the structural and physical linkage between the basement membrane and the cuticle and are therefore direct mediators of muscle to cuticle attachment. The association between the apical and basal hemidesmosomes is made by intermediate filaments, which not only connect the hemidesmosomes but also provide cellular integrity during force transmission.\(^8\) Since the hypodermis is directly involved in the transfer of contractile force from muscle to cuticle, it affords a great opportunity to study the maintenance of cell-ECM attachment during muscle contraction.

To better understand the mechanism involved in the maintenance of adhesion and cellular integrity during mechanical stress, several mutations that affect this pathway have been identified in *C. elegans*.\(^12,13\) These mutations have a very distinct phenotype, which manifests itself in early larval development and involves the progressive detachment of muscle cells from the underlying hypodermis. At least some of the genes defined by this class of mutation appear to be involved in cell-ECM adhesion including *unc-23*,\(^13 mua-1, 2, 3, 4, 5, 6, 7, 9, 10, vab-10, let-805, mup-4, ifb-1 and pat-12.\(^12,14-20\)

Mutations affecting the *unc-23* gene were first isolated in a forward genetic screen designed to identify animals with visible mutant phenotypes.\(^21\) Among the original 619 mutants that were identified, only Unc-23 mutant animals exhibited a progressive dystrophy of the head musculature resulting in a bent-head phenotype. A detailed analysis of this unique phenotype revealed that mutations in the *unc-23* gene result in loss of hypodermal integrity and detachment of the muscle cells from the hypodermis during larval development in *C. elegans*.\(^13\) These observations suggest that the *unc-23* gene encodes a structural or a regulatory protein that is involved in the maintenance of muscle cell attachment to the hypodermis.

In this paper we describe the identification and characterization of the *unc-23* gene product as the nematode ortholog of the human BAG-2 protein, a member of the Bcl-2 associated athanos-ene (BAG) family of molecular chaperone regulators. We also provide evidence that the UNC-23 protein interacts with the heat shock cognate protein 70, HSP-1 to maintain intracellular adhesion strength.

### Results

The *unc-23* gene encodes a protein with a domain similar to mammalian Bcl-2-associated athanogene 2 (BAG-2), a chaperone regulator.

The *unc-23* gene maps to a region of chromosome V that is uncovered by several deficiencies. We correlated the physical and genetic maps for this region and localized *unc-23* to a region of 12 cosmids defined by the left breakpoint of *mDf1* and the right breakpoint of *sDf71*. Genetic crosses using the *unc-23*(e25) allele and transgenic strains carrying cosmids and fosmids from this region of the genome revealed that *unc-23* resides on the sequenced fosmid H14N18. We have determined that the *unc-23* gene corresponds to the H14N18.1 open reading frame\(^22\) (data available from GenBank/EMBL/ DDBJ under accession #AF068719), by identifying the sequence alterations corresponding to 6 *unc-23* alleles. Two of the mutations identified are deletions of 117 (ra801) and 155 (ra806) bp and a third mutation (rh192) is a 4 bp insertion (Table 1 and Fig. 1). The remaining 3 EMS-induced mutations are GC – AT point mutations. The *unc-23*(e25) allele is a missense mutation that changes the Glu297 codon to a Lys codon. The *e988* and *e324* mutations introduce stop codons into exons 2 and 5, respectively (Table 1 and Fig. 1).

Our sequencing results for the yk293b4 cDNA obtained from the *C. elegans* cDNA project (Y. Kohara, pers. comm.) confirmed the gene structure predicted for the H14N18.1 ORF by the

### Table 1. Molecular lesions associated with *unc-23* mutations.

| Allele | Type of mutation | Residue changes | Exon affected |
|--------|------------------|-----------------|--------------|
| e25    | Missense         | GAA (glu) to AAA (lys) | 3            |
| e324   | Nonsense         | CAA (gln) to TAA (stop) | 5            |
| e988   | Nonsense         | CGA (arg) to TGA (stop) | 2            |
| ra801  | Deletion         | 117 bp in frame deletion. New Leucine made at site of deletion. | 2            |
| ra806  | Deletion         | 155 bp out of frame deletion. | 2            |
| rh192  | Insertion        | GGAA insertion. Frame shift and termination near end of exon | 2            |
The partial sequences of several other cDNA clones are available at www.wormbase.org, and these data reveal the presence of mRNA species encoding alternative translation start sites (see for example yk1007e10 and yk1745g09). The unc-23 gene consists of 5 exons (Fig. 1) and, based on the cDNA sequence data, can potentially encode 3 different protein isoforms of 458, 457 and 399 amino acids, respectively. A search of the database using the BLAST server at NIH identified a 211 amino acid human protein called BAG-2 with significant sequence similarity to the UNC-23 protein (Fig. 2). Human BAG-2 is a member of the Bcl-2 associated athanogene (BAG) family of molecular chaperone regulators. The members of this protein family contain a conserved 45 amino acid region, the BAG domain, near their carboxy-termini.

**Figure 1.** The unc-23 gene consists of 5 exons and potentially encodes 3 proteins. The intron and exon structure of the unc-23 gene is shown in the above diagram. Also shown are the locations of the 3 potential start codons identified by cDNA analysis as well as the locations of the alleles that have been identified by sequence analysis. The gene spans 2660 bp from the start codon in exon 1 to the stop codon in exon 5. Scale bar is 150 bp.

**Figure 2.** The sequence alignment of UNC-23 and BAG-2 shows homology between the 2 proteins. The human BAG-2 protein is most similar to the carboxy-terminus of the UNC-23 protein. The BAG homology domain of UNC-23 starts at residue 427 and extends to the end of the protein (arrows). The BAG domain in the human BAG-2 protein starts at residue 164 and ends at residue 196. C. elegans UNC-23 and human BAG-2 share 40% amino acid identity (shown as boxed residues) and 62% amino acid similarity (highlighted residues) over this region. The N-terminal region of UNC-23 is not homologous to any sequence available in the database and has no predicted structure. The stubby arrow points to the start of the short UNC-23 isoform. The amino acid residues affected by the e988, e324 and e25 point mutations are indicated by circles. The first 2 mutations introduce stop codons at residues 186 and 397, respectively, and e25, the most severe allele of unc-23, changes glutamic acid residue 297 to a lysine. This glutamic acid residue is conserved in UNC-23 and BAG-2.
which binds Hsp70/Hsc70 and controls their chaperone activity. To date, 6 members of this family, BAG-1 to BAG-6, have been identified in humans and 2 members have been identified in nematodes. The region of similarity between mammalian BAG-2 and UNC-23 is confined to the carboxy-terminus of UNC-23. The amino-terminus of UNC-23 is not similar to any sequence available in the database and has no predicted structure. The BAG domain of UNC-23 starts at residue 427 of the longest sequence available in the database and has no predicted structure.

UNC-23. The amino-terminus of UNC-23 is not similar to any BAG-2 and UNC-23 is confined to the carboxy-terminus of the BAG domain and its upstream region.

The UNC-23 protein is present in both body wall muscle and hypodermis.

The unc-23 gene potentially encodes 3 protein isoforms that differ at their amino-termini. To ensure that the transgene we constructed for microinjection and expression analysis could produce all 3 isoforms as GFP-tagged proteins, we amplified the entire unc-23 gene, including 2166 bp of promoter, from genomic DNA and inserted the GFP encoding sequences at the 3’ end of the gene upstream of the stop codon. All UNC-23 protein isoforms made from this construct will have the GFP attached to their C-terminal. Animals carrying the raEx20 [unc-23::GFP; rol-6(mg1006)] transgenic array produce at least one functional GFP-tagged UNC-23 protein (shown in Fig. 3) that fully rescues the bent-head phenotype of unc-23(e25) mutant animals (see Fig. 4). GFP fluorescence in animals carrying this array is found throughout development appearing first at the 1.5-fold stage of embryogenesis in a few unidentified cells. Expression of the GFP-tagged UNC-23 protein continues during embryogenesis and by the 3-fold stage, it has expanded to include muscle, hypodermal and pharyngeal cells. In adult hermaphrodites GFP fluorescence is expressed in the pharynx, body wall muscle cells, hypodermis, vulva, and H cells, as well as some unidentified neurons and touch cells. In the hypodermis, UNC-23::GFP is distributed throughout the entire tissue including some nuclei and nucleoli, and in addition, is localized in a pattern reminiscent of the intermediate filaments (shown in Fig. 3A). In body wall muscle cells, UNC-23::GFP is localized to the dense bodies and M lines (Fig. 3B) in a pattern similar to that observed for several proteins including PAT-3/B-integrin, UNC-112, UNC-97/ PINCH, and PAT-4/ILK.

The unc-23 gene product is required for muscle cell attachment to the hypodermis.

Mutations in the unc-23 gene result in the progressive detachment of the anterior body wall muscle cells from the hypodermis. During embryonic development, muscle cells are capable of myofilament assembly, growth, contraction, and attachment to the hypodermis in the absence of the unc-23 gene product. However, during mid-larval development body wall muscle cells in the head region of Unc-23 mutant hermaphrodites begin to separate from the hypodermis, resulting in adults with heads that are noticeably thinner than wild type animals. The resulting loss of control of the head causes a bent-head phenotype when the animal tries to move forward. Detachment of the body wall musculature from the hypodermis begins with the anterior-most cells and is usually confined to the head of the animal (compare Fig. 4A and 4B). Other phenotypes observed in Unc-23 mutants include the occasional protrusion of the vulva, egg laying defects, mild constipation due to interruption of the defecation cycle and rarely, small blisters at the tip of the nose. The pharynx of Unc-23 mutant animals functions normally. The extent of muscle detachment is highly variable among different mutant strains, ranging from severe to almost wild type (see Table 2).

Four of the 6 unc-23 mutations that have been identified at the sequence level are likely to affect all 3 predicted UNC-23 protein isoforms. The two nonsense mutations should result in either the complete absence of any UNC-23 proteins, or perhaps, the presence of truncated proteins that are missing all, or most of the BAG-2 homologous region of UNC-23. Thus, the severe muscle detachment phenotype exhibited by animals homozygous for these mutations most likely results from the complete absence of unc-23 gene function. Since hermaphrodites homozygous for the unc-23(e25) missense mutation also exhibit a severe phenotype, this mutation is likely to drastically reduce unc-23 gene function.

Figure 3. The spatial distribution of a GFP-tagged UNC-23 protein in rescued homozygous unc-23(e25) hermaphrodites. The UNC-23::GFP protein is present in a variety of tissues including the hypodermis (A) and the body wall muscle cells (B) in unc-23(e25); raEx20[rol-6; unc-23::GFP] hermaphrodites. Images are from the mid-section of the animals. Scale bars are 20 microns. (A) A view of UNC-23::GFP localization in the hypodermal syncytium is shown. The UNC-23 protein is present throughout the whole of the hypodermis including some, but not all, nuclei and nucleoli. In addition, the GFP-tagged UNC-23 protein is localized in a pattern identical to that seen for intermediate filaments. The closed head white arrow in the hypodermis points to a nucleus that exhibits UNC-23::GFP expression. The open arrow-head arrow points to the in the intermediate filament-like expression pattern. (B) A view of UNC-23::GFP localization in 2 body wall muscle cells is shown. The GFP-tagged UNC-23 protein localizes with the dense bodies and M-lines at the cell membrane. The closed head white arrow points to a region of dense bodies and M-lines, while the open arrow-head arrow points to the intermediate filament-like expression pattern in the overlying hypodermis.
as well. In these mutants, muscle detachments extend just beyond the terminal bulb of the pharynx and involve both the dorsal and ventral quadrants (shown in Fig. 4B). The upper tissues completely collapse on top of the pharynx, resulting in complete loss of head movement and a true bent-head phenotype. The GFP-tagged UNC-23 protein(s) produced by the raEx20 transgenic array completely suppress this muscle detachment phenotype in unc-23(e25) homozygous hermaphrodites (shown in Fig. 4C).

In mutants exhibiting a mild phenotype, the detachments do not extend past the anterior bulb of the pharynx. Only the tip of the nose is affected and, for the most part, no head dragging is observed. The sequence alterations responsible for the mild phenotype exhibited by strains carrying either unc-23(ra801) or unc-23(ra806) are known. Neither of these mutations is predicted to result in the complete loss of unc-23 gene function. The ra801 mutation is an in frame deletion that eliminates amino acid residues 17 to 55 of the longer protein isoforms, but has no effect on the shortest UNC-23 protein. The ra806 mutation is an out of frame deletion near the 5' end of the unc-23 gene that should result in the complete absence of the longer UNC-23 proteins. However, the shortest UNC-23 protein should still be present in animals homozygous for this mutation.

The e25 missense allele of unc-23 is the reference allele of unc-23(e25) and D. L. Riddle (pers. comm.) observed that unc-23(e25) mutant animals grown in liquid culture fail to exhibit the bent-head phenotype. Suppression of the muscle cell detachment phenotype lasts up to 48 hours after animals are placed on solid medium. Here we have examined the effect of this temporary suppression of muscle detachment on the arrangement of the myofilament lattice and on muscle cell positioning. In liquid grown adult worms, anterior muscle cells are positioned correctly and the myofilaments appear normal when observed under polarized light (shown in Fig. 4D). However, these muscle cells are easily detached by mechanical stress. Application of external force, such as putting worms under a coverslip and gently rolling them, can result in the detachment of muscle quadrants from the underlying hypodermis. Subjecting wild-type animals to a similar treatment does not result in any muscle cell detachment. In fact, wild type animals can withstand a great deal more force than the unc-23(e25) mutant animals.

The temporary muscle detachment suppression observed with the liquid grown unc-23(e25) mutants and the manifestation of this phenotype after transfer to agar plates, suggests there are different forces acting on muscle and hypodermal attachments in these 2 growth conditions.

**Figure 4.** Polarized light images of wild type and unc-23(e25) homozygous animals. Polarized light images of a wild type hermaphrodite (A), a plate grown homozygous unc-23(e25) adult hermaphrodite (B), a plate grown unc-23(e25); raEx20(rol-6; unc-23::GFP) hermaphrodite (C) and a liquid grown homozygous unc-23(e25) adult hermaphrodite (D). The closed head white arrow points to regions of muscle birefringence and the open arrow-head arrows point to the terminal bulb of the pharynx. Anterior is to the right in all panels. Scale bars are 60 microns. (A) Polarized light image of a wild-type adult hermaphrodite. In C. elegans, the body wall muscle cells extend from the tip of the nose to the posterior end of the animal. The myofilament lattice in the muscle sarcomeres is seen as a repeating pattern of light and dark birefringence. The pharynx, which is the grinding apparatus of the worm, can be seen in this image. The bulbous structures are the anterior and posterior bulbs of the pharynx. (B) Polarized light image of a plate grown unc-23(e25) homozygous adult hermaphrodite. The most anterior muscle cells have detached from the tip of the nose and retracted posteriorly. Note that the head in this animal is thinner than in the wild type animal. The left closed head white arrow point to the region where the muscle cells have stopped detaching and the right closed head white arrow points to a region where the muscle cells have detached. Posterior to the site of detachment, the light and dark pattern of thick and thin filaments can clearly be seen. (C) Polarized light image of a plate grown unc-23(e25) homozygous adult hermaphrodite carrying the raEx20 transgenic array. The GFP-tagged UNC-23 protein produced by the transgenic array suppresses the muscle detachment seen in the unc-23(e25) homozygous mutant animals. As shown in this image, muscle cells are positioned correctly and extend all the way to the tip of the nose. The light and dark pattern, resulting from the thick and thin filament organization of the myofilament lattice in the sarcomeres, are well organized and show no defects. (D) Polarized light image of an unc-23(e25) homozygous animal grown in liquid culture. The muscle quadrants extend to the tip of the nose and no detachment is observed. Animals grown in liquid culture are usually thinner than plate grown animals. The worm shown here is, therefore, thinner than its plate grown counterparts. As shown in this image, muscle cells are positioned correctly and extend all the way to the tip of the nose. The light and dark pattern, resulting from the thick and thin filament organization of the myofilament lattice in the sarcomeres, are also well organized and show no defects.
Table 2. Phenotypes associated with Unc-23 mutant animals.

| Allele   | Phenotype * | Protruding Vulva | Blister at tip of nose | Induced by |
|----------|-------------|------------------|------------------------|------------|
| e25      | Severe (n = 100) | 30               | 2                      | EMS (Brenner) |
| e255     | Mild (n = 52)    | 0                | 0                      | EMS (Brenner) |
| e324     | Severe (n = 26)  | 15               | 0                      | EMS (Brenner) |
| e462     | Mild (n = 33)    | 0                | 0                      | EMS (Brenner) |
| e478     | Mild/Wild-type (n = 66) | 0       | 0                      | EMS (Brenner) |
| ra801    | Mild/Wild type (n = 40) | 0       | 0                      | TMP (VRGCF) |
| ra806    | Mild/wild type (n = 45) | 0       | 0                      | TMP (VRGCF) |
| e988     | Severe/Mild (n = 55) | 11      | 0                      | EMS (Brenner) |
| e1182    | Mild (n = 48)    | 0                | 0                      | EMS (Brenner) |
| e2154    | Mild/Wild-type (n = 70) | 0       | 1                      | Mutator strain (Mancillas and Waterston, pers. Comm.) |

*Based on the severity of the bent-head phenotype. The Mild designation indicates that only the tip of the nose was affected and the animals observed did not exhibit a bent-head phenotype. The Severe designation indicates that the whole head region was affected and the animals observed exhibited a bent-head phenotype.

1Brenner (1974) 2Vancouver Reverse Genetics Core Facility

Basement membrane and hypodermal components associate with detached muscle cells in unc-23 mutants.

The unc-23 mutant phenotype reflects a defect in muscle cell attachment to the hypodermis rather than a defect in muscle cell positioning or myofilament assembly and is seen almost exclusively in the head region even though the UNC-23 protein is present throughout the whole animal. It begins during larval development likely in response to mechanical forces exerted while the animals are foraging for food. In Unc-23 mutant worms, muscle cells detach whole from the underlying tissues. No detachment, fracturing, shearing, or separation of muscle cells from each other is observed. A previous study by Waterston et al. used electron microscopy to examine hypodermal-muscle junctions in unc-23(e25) mutant animals, and these authors observed delamination and local ruptures within the hypodermis. Their result indicates that the hypodermis is the site of tissue fragility and detachment in these mutants. To determine the effect of muscle cell detachment on the surrounding tissues and the location of proteins involved in attachment, both plate grown and liquid grown unc-23(e25) homozygous hermaphrodites were examined using antibodies that recognize components of the basement membrane or attachment structures within the hypodermis (Fig. 5).

UNC-52/perlecan is a basement membrane component that plays an essential role in myofilament lattice assembly. This protein is distributed over the whole of the basement membrane between the body wall muscle and hypodermis and is concentrated over muscle cell dense bodies and M-lines (shown in Fig. 5A). Examination of plate grown unc-23(e25) homozygous animals with the GM1 polyclonal serum revealed that UNC-52/perlecan is completely absent from the areas where muscle cells have detached (see Fig. 5B). The distribution of UNC-52/perlecan in the rest of the animal ranges from normal to slightly disorganized in areas of stress such as the vulva. As expected, liquid grown unc-23(e25) mutant hermaphrodites stained with GM1 show the wild type pattern of UNC-52/perlecan localization (shown in Fig. 5C).

In C. elegans, structures called hemidesmosomes anchor the intermediate filaments within the hypodermis and are direct mediators of muscle to cuticle attachment. The VAB-10/plakin protein in the hemidesmosomal complex can be visualized by the MH5 monoclonal antibody. In wild type animals the staining pattern of this protein appears as a series of small dots that cover the muscle quadrants with more intense staining in areas where muscle cells contact each other, but in unc-23(e25) homozygous animals this pattern is disrupted as was observed for perlecan (our unpublished results). Intermediate filaments are recognized by the MH4 mAb and appear as repeating and equally spaced bands that run circumferentially from one edge of a muscle quadrant to the other (shown in Fig. 5D). Examination of MH4 staining in unc-23(e25) homozygous animals grown on plates revealed that there is a good deal of variability in intermediate filament organization (see Fig. 5E). The disruption in MH4 staining observed in affected areas can range from mild to full detachment. Homozygous unc-23(e25) liquid grown mutants stained with either MH5 (our unpublished data) or MH4 (Fig. 5F) show no disorganization or detachment and resemble wild type animals. These data show that at least some components of the basement membrane and hypodermis remain attached to the body wall muscle cells after they separate from the hypodermis. This result is consistent with the earlier conclusion of Waterston et al. that the body wall muscle cells likely detach because of a weakness in the hypodermis.

Mutations in the hsp-1 gene suppress the muscle detachment phenotype of unc-23(e25) mutant hermaphrodites.

We have isolated 7 independent homozygous strains that carry mutations capable of suppressing the bent head phenotype of homozygous unc-23(e25) hermaphrodites. All seven of these strains segregate Unc-23 mutant animals when outcrossed, and are presumed to carry linked suppressors of the unc-23(e25) mutation. The two suppressor mutations that have been characterized, ra807 and ra808, are alleles of the non-inducible heat shock protein 70, hsp-1 gene on linkage group IV. Both of these mutations are dominant and fully suppress the muscle detachment phenotype of unc-23(e25) homozygous animals. No muscle detachment is observed in either heterozygous (unc-23(e25); hsp-1(ra807 or ra808)+)
or homozygous (unc-23(e25); hsp-1(ra807 or ra808)) suppressed animals. The remaining 5 mutations that were isolated in this screen are all recessive suppressors and have not been mapped to a chromosome.

During our genetic characterization of the suppressor strains it became apparent that hermaphrodites homozygous for either hsp-1(ra807) or hsp-1(ra808) in the unc-23(e25) background exhibit a temperature sensitive phenotype. These animals are healthy when grown and maintained at 15°C, but die as L3 larvae when grown at 25°C. The majority (over 95%) of the homozygous suppressor animals either arrest development as L3 larvae or become sterile adults when grown at 20°C. When removed from the unc-23(e25) genetic background hsp-1(ra807) or hsp-1(ra808) homozygous hermaphrodites appear wild type and are healthy when grown at either 15°C or 25°C. Similarly, homozygous unc-23(e25) hermaphrodites are viable when grown and maintained at either 15°C or 25°C. Thus, it appears that the temperature sensitive phenotype occurs only in animals homozygous for the unc-23(e25) mutation and either hsp-1(ra807) or hsp-1(ra808).
The F26D1.3/hsp-1 gene in *C. elegans* encodes a 640 amino acid protein with 79% identity to the mammalian Hsc70 protein (Fig. 6; data available from GenBank/EMBL/DBJ under accession #Z80223). The first 386 amino acids of the HSP-1 protein comprise the ATPase domain, while amino acids 384 to 543 are involved in substrate binding. The sequence alterations corresponding to the ra807 and ra808 suppressor mutations have been identified and both are GC to AT point mutations that result in amino acid substitutions in the ATPase domain of the HSP-1 protein. The ra807 mutation changes the conserved Ala379 residue to a Val residue. The ra808 mutation replaces the conserved Asp233 residue with an Asn residue.

Suppression of the muscle detachment phenotype of unc-23(e25) by hsp-1(ra807) is allele specific. Animals homozygous for the e324 null allele of unc-23 still exhibit a severe bent head phenotype when either heterozygous or homozygous for the hsp-1(ra807) suppressor mutation. This result suggests that the ra807 mutation does not eliminate the requirement for HSP-1 interaction with UNC-23. Rather, the unc-23(e25) missense mutation, E297K, most likely results in a conforma-
tional change in the tertiary structure of UNC-23, so that its interaction with the normal HSP-1 is severely affected. The hsp-1(ra807) missense mutation, A379V, presumably alters the HSP-1 ATPase domain enabling it to now interact with the E297K variant of UNC-23.

The UNC-23 protein binds to the HSP-1 protein in the yeast 2-hybrid system.

The region of shared similarity between the mammalian BAG-2 protein and UNC-23 is confined to the carboxy-terminus of UNC-23 (see Fig. 2). Using this region of the UNC-23 protein as bait in a yeast 2-hybrid screen we identified the hsp-1 gene product as a potential UNC-23 interacting protein. Our primary screen identified 36 clones expressing proteins that could potentially interact with the UNC-23 protein. Only ten of these showed an interaction with UNC-23 when reintroduced into...
yeast and screened for a second time. When sequenced, all 10 positive clones were found to contain overlapping DNA sequences encoding the HSP-1 protein. The minimal region of the HSP-1 protein present in all of the clones extends from amino acid 189 through to amino acid 419 (see Fig. 6). This region is part of the ATPase domain of HSP-1 and includes the amino acids affected by the ra807 and ra808 suppressor mutations. Thus, our data reveal that the carboxy-terminus of UNC-23 potentially interacts with the ATPase domain of HSP-1. This result is in agreement with the mammalian results that identify BAG-2 as an Hsc70 interacting protein and the more recent results demonstrating the direct interaction of these 2 nematode encoded proteins in vitro.

Discussion

The unc-23 gene encodes the nematode ortholog of the human BAG-2 protein, a member of the Bcl-2 associated athanogene (BAG) family of molecular chaperone regulators. In humans this protein family consists of 6 members that all contain a conserved 45 amino acid BAG domain near their C-termini, while in C. elegans there are 2 members of the BAG family, UNC-23 and the F57B10.11/bag-1 gene product. The most intensively studied member of the human BAG family is BAG-1 which was first identified as a Bcl-2 binding protein that increases the anti-apoptotic ability of cells. Since its initial identification, BAG-1 has been implicated in many cellular interactions, including the binding of hepatocyte growth factor receptors, glucocorticoid receptors, retinoic acid receptors, and Raf-1 kinase, as well as targeting to the proteasome. While human BAG-1 is not known to have a role in muscle, BAG-3 has been shown to regulate filamin levels in muscle and several genetic myofibrillar myopathies have been associated with BAG-3 and filamin.

The BAG proteins bind to and modulate the activity of the ATPase domain of the heat shock cognate protein 70, Hsc70 thus inhibiting its chaperone activity. The protein structure of Hsc70 is conserved and can be divided into 3 functional segments, an N-terminal ATPase domain, a central substrate binding domain, and a C-terminal region that may act as a lid to the substrate-binding cleft (for a review see ref. 43). Substrate binding to Hsc70 is regulated by the binding, hydrolysis and exchange of ATP nucleotides. The ATP bound form of Hsc70 has a low overall affinity for its substrate while the ADP bound form of Hsc70 firmly binds to its substrate. The cycling of Hsc70 between these 2 different nucleotide states is regulated by several chaperone co-factors that bind Hsc70 and modulate its ATPase activity. BAG-1 stimulates the release of ADP from the Hsc70 chaperone and, as a result, promotes the release of the bound substrate.

Two independent in vitro studies on the effects of BAG-1 on Hsc70 agree that BAG-1 is probably a negative regulator of Hsc70. BAG-1 interaction with Hsc70 in vivo however, can result in both inhibition and enhancement of the chaperone-dependent events, depending on which substrates are involved. This suggests that the in vivo function and effect of BAG-1 on Hsc70 may depend on the specific substrate and the cellular context under which the 2 proteins are interacting. Therefore, the effect of BAG-1 regulation on the chaperoning activity, chaperone-substrate complex, and the folding state of released substrate remains unclear. Takayama et al. showed that the BAG domain and the upstream region of the BAG-2 and BAG-3 proteins also interact with the ATPase domain of Hsc70 in vitro and in vivo. Some of the functional perplexity of the BAG family members may be resolved if these proteins could be studied in a model organism.

Studying the UNC-23 protein in C. elegans offers an opportunity to examine the possible functions of a BAG-2 ortholog within a biologically relevant context. We have shown that UNC-23 interacts with the ATPase domain of the heat shock 70 protein, HSP-1 in vivo and in the yeast 2-hybrid system, whereas Papsdorf et al. have shown a direct interaction between these 2 proteins in vitro and also have demonstrated that UNC-23 acts as a nucleotide exchange factor. The results of our genetic suppression screen, which identified missense mutations in HSP-1 as suppressors of the unc-23(e25) muscle detachment phenotype, indicate a direct interaction between the BAG-2 homologous region of UNC-23 and HSP-1. The two dominant HSP-1 suppressor alleles, hsp-1(ra807) and hsp-1(ra808) are missense mutations that affect the ATPase domain of the protein, and are both located within the minimal region required for the yeast 2-hybrid interaction of HSP-1 with UNC-23.

Several groups have used RNA interference (RNAi) to determine the null phenotype of the hsp-1 gene with varying results. The most severe phenotype observed was arrest during embryogenesis. Hermaphrodites homozygous for either of the hsp-1 suppressor alleles appear wild type in the absence of the unc-23 (e25) mutation suggesting they do not result in the complete loss of HSP-1 activity. Rather, they most likely alter the ATPase domain of HSP-1 allowing it to interact with the E297K mutant variant of the UNC-23/BAG-2 protein without drastically affecting its interaction with other binding partners or with the wild type UNC-23 protein.

Recently Papsdorf et al. demonstrated that loss of the DNJ-13 protein in C. elegans acts as a suppressor of the muscle detachment induced by the loss of the UNC-23 protein. They also show that in vitro DNJ-13 promotes the binding of Hsc70 to its substrate while the BAG protein, UNC-23 promotes the release of Hsc70 from its substrate. Thus, DNJ-13 and UNC-23 have opposite effects on the Hsc70 chaperone and presumably HSP-1. When UNC-23 is missing but DNJ-13 is still present the system is out of balance and the muscle cells detach from the hypodermis. Apparently, in the absence of both UNC-23 and DNJ-13, the HSP-1 protein remains functional, albeit unregulated, and the muscle cells remain attached. Papsdorf et al. have also found that overexpressing the hsp-1 gene in wild type animals leads to a muscle detachment phenotype similar to that observed in Unc-23 mutant animals.

The association of UNC-23 in C. elegans with adhesion complexes within muscle cells and the attachment structures within the hypodermis suggests that UNC-23 acts in some manner to
regulate cell attachment and maintain hypodermal integrity. In
Unc-23 mutant worms, muscle cells detach whole from the
underlying tissues. No detachment, fracturing, shearing, or sepa-
ration of muscle cells from each other is observed.12,13 A previous
study by Waterston et al.13 used electron microscopy to examine
hypodermal-muscle junctions in unc-23(e25) mutant animals, and
these authors observed delamination and local ruptures
within the hypodermis. Our antibody staining results revealed
that the hemidesmosomes and the intermediate filament net-
works in the hypodermis are disrupted when muscle cells detach
in unc-23(e25) mutant hermaphrodites. Although UNC-23 is
present at the dense bodies and M lines in body wall muscle cells,
no weakness or detachment is observed at these sites in detached
muscle cells. In fact, restricted expression of UNC-23 to the mus-
cle cells alone is not sufficient to rescue the bent-head phenotype
of Unc-23 mutant animals (our unpublished results). Taken
together these observations suggest that UNC-23 expression is
required in the hypodermis to maintain the integrity of muscle
cells attachments.

Based on the different phenotypes of unc-23(e25) homozygous
animals grown in liquid or on plates, it is reasonable to predict
that the interaction of UNC-23 with HSP-1 is required in biologi-
cal pathways that respond to increasing contractile force by rein-
forcing the density and integrity of the hypodermal attachment
structures. Muscle cells in liquid grown mutant hermaphrodites
are capable of myofilament assembly, growth, contraction, and
attachment. However, this attachment is not stable since applied
eXternal force or movement of worms on a solid substrate results
in muscle detachment. Animals grown in liquid culture do not
experience the shear force that plate-grown animals experience as
they forage for food. Once animals are transferred to a solid sub-
strate, they use their head muscles extensively to test the environ-
ment and forage for food. The use of the anterior muscle cells and
the shear force exerted during movement initiates the muscle
detachment and loss of hypodermal integrity in Unc-23 mutant
hermaphrodites. This suggests that the integrity of the hypodermis
is not challenged in liquid grown worms because there is less
mechanical stress. The HSP-1 complex including UNC-23 and
DNJ-13 must respond to cues involving mechanical stress. What
those cues might be is currently unknown but may include
RacGTPase, PAK-1, PIX-1 and GIT-1.53

New proteins may be required in response to increasing con-
tractile force to build up existing or newly formed attachment
structures in the hypodermis. HSP-1 might be involved in fold-
ing, assembling, and chaperoning these newly made proteins to
their locations. Likewise, in response to an increase in contractile
force, certain proteins essential for the maintenance of cell-ECM
attachments might be produced in larger amounts. Hsc70 chaper-
one has been shown to interact with several cytoskeletal proteins
including intermediate filaments and microtubules.54-57 Production of large amounts of intermediate fila-
ments, for example, would be required at newly made hypoder-
mal attachment sites and for the reinforcement of existing
attachment structures to help maintain the integrity of the hypo-
dermis. HSP-1 might bind the intermediate filaments to prevent
their aggregation and chaperone them to their proper location.

The chaperone functions of HSP-1 are not limited to interaction
with intermediate filaments. Membrane bound receptors and
structural proteins that make up the attachment structures and/or
bind the intermediate filaments are other possible substrates
for HSP-1. If UNC-23 functions as a negative regulator of HSP-
1, in the same manner as BAG-2, then one might predict that
the interaction of UNC-23 with HSP-1 is required for the release
of the substrate from the binding cleft of HSP-1. In the absence
of UNC-23, HSP-1 is unable to release its substrate in response
to mechanical stresses experienced by the hypodermis. The sub-
cellular localization of UNC-23 in the hypodermis is pertinent
to this type of model, since its distribution is reminiscent of the
distribution of the intermediate filaments within the hypodermis.
The predicted role of UNC-23 as a regulator that promotes
HSP-1 substrate release, agrees with the localization pattern
observed in the hypodermis.

It is important to note that the UNC-23/HSP-1 interaction is
not necessary for the initial assembly and localization of attach-
ment structures during embryogenesis, or for the maintenance
of attachment structures within the body wall muscle cells. Given
the number of biological pathways that HSP-1 is involved it is
not surprising that loss of hsp-1 gene function by RNA interfer-
ence results in embryonic lethality.51,52 Mutations in the unc-23
gene do not result in a similar phenotype; they only affect
the hypodermal integrity and muscle cell attachment of the anterior
body wall muscle cells. This suggests that the interaction of
UNC-23 with HSP-1 provides specificity for HSP-1 function to
the biological pathway(s) in the hypodermis that are involved in
the maintenance of muscle attachment during development.
UNC-23 as a chaperone regulator thus refines the amount of
proteins deposited at adhesions junctions in developing and
growing C. elegans animals.

Materials and Methods

Nematode strains
The unc-23 mutant strains used in this study were provided by
Jonathan Hodgkin at the MRC-LMB (Cambridge, England), the
Caenorhabditis Genetics Center (CGC) at the University of Min-
snesota (Minneapolis, Minnesota), the C. elegans Reverse Genetics
Core Facility at the University of British Columbia (Vancouver,
British Columbia), and Edward Hedgecock at Johns Hopkins
University (Baltimore, Maryland). Strains carrying the sDf71 and
mDf11 deficiencies were obtained from David Baillie at Simon
Fraser University (Burnaby, British Columbia). Strains carrying
the ZC196, B0507, C01B7, W09B7, ZC411, K06C4,
H14N18, T19A5, F59E11 and R03H4 cosmids were kindly
provided by Michael Nonet at Washington University School of
Medicine (St. Louis, Missouri). The DM5110: sdF71 (e224) V and DM5111: mDf11 (e224) V strains were con-
structed for this study.

Growth in liquid culture
Worms were grown in liquid culture in 250 ml flasks contain-
ning 50 ml 5% (W/V) OP50 in S medium at 20°C and aerated by
shaking at 200 rpm. Adult animals were washed from plates, then transferred to liquid culture and allowed to grow for at least one generation. The liquid grown animals were spun gently in glass tubes to remove the culture medium and then washed twice in M9 buffer before antibody staining. Hermaphrodites for polarized light microscopy were grown in micro-titer wells containing 150 ml of 5% (W/V) E. coli X1666 in S medium. Two adult animals were placed in each well. The adult F1 or F2 progeny were removed from the wells with a pipette, placed on a plate and then transferred to 11.5 ml of M-9 buffer on a glass slide.

Mapping deficiency breakpoints by PCR
The following PCR strategy was used to determine if a particular cosmid was deleted by either Df/71 or Df/1. Arrested embryos homozygous for the deficiency were treated with proteinase K, and the DNA obtained was used in PCR reactions as previously described. The genomic sequence of the cosmid in the region of unc-23 was obtained from the Caenorhabditis elegans Genome Sequencing Consortium, and a series of primers were designed that would amplify specific DNA fragments from these cosmid. If a fragment of the correct size was amplified using DNA isolated from the arrested mutants, then that region of the cosmid was not deleted by the deficiency. If the correct fragment was not obtained then the region covered by the primers was deleted by the deficiency. Approximately 4–6 arrested sDf/71 or Df/1 homozygous embryos, obtained from the DM5110 and DM5111 strains, were used per PCR reaction. Polymorphisms associated with particular unc-23 mutations were identified in a similar manner using primer sets designed to amplify the H14N18.1 ORF.

Sequence analysis of unc-23 and hsp-1
The Nucleic Acid/Protein Service (NAPS) unit at the University of British Columbia did all of the sequencing for this study. The full-length unc-23 cDNA, YK293b4 was obtained as a λZapII clone from the C. elegans cDNA project (kindly provided by Dr. Y. Kohara, National Institute of Genetics, Mishima, Japan). The bluescript plasmid containing the cDNA insert was excised and transformed into XL1 blue cells following the protocol provided by Stratagene (PDI Bioscience Inc., Aurora, Ontario). Plasmid DNA was prepared for sequencing using the Qiagen miniprep kit (catalog # 27106. QIAGEN Inc. Mississauga, Ontario). The entire cDNA insert was sequenced using the Qiagen primer set designed to independently confirm the exon/intron boundaries predicted by the Genefinder program.

Three overlapping DNA fragments were amplified from mutant hermaphrodites homozygous for the e25, e324, e988, ra801, ra806, and rh192 alleles of unc-23 using the polymerase chain reaction as described by Rogalski et al. The 3 primer sets used cover all of exons 2, 3, 4, and the majority of exon 5 (except for the last 25 bps) of the H14N18.1/unc-23 gene. Overlapping DNA fragments that span the whole of the hsp-1 gene were amplified from adult hermaphrodites homozygous for the hsp-1 (ra807) and hsp-1(ra808) mutations. All PCR fragments were prepared for sequencing using the QIAquick PCR purification Kit (Catalog #28104. QIAGEN Inc. Mississauga, Ontario).

Proteins with sequence similarity to UNC-23 were identified using the NCBI blast search algorithm. The Clustal W program (MacVector 6.0, Oxford Molecular Group) was used for protein sequence alignment and comparison.

Immunofluorescence staining and microscopy
Adult hermaphrodites were stained as described by Finney and Ruvkin with some minor modifications. Incubation times were overnight at 15°C for both the primary and secondary antibodies. The mouse monoclonal antibodies MH4 and MH5 were diluted 1:200, while the rabbit polyclonal sera GM1 was diluted 1:50. The secondary antibodies, FITC-labeled donkey anti-mouse IgG F(ab’)2 and TRSC-labeled donkey anti-rabbit IgG F(ab’)2 (Jackson ImmunoResearch Laboratories; West Grove, PA, USA) were diluted 1:200.

Confocal images were captured on a BioRad MRC 600 system, using the CoMOS 7.0a application. Optical sections of adult worms were collected at 0.2μm intervals (Z steps) for the full depth of the specimen. Z-series were imported to NIH image 1.60 for viewing and projections. For final images, projections were imported into Adobe Photoshop (Versions 5.0 and 7.0).

Worms examined under polarized light were removed from NGM plates, placed in a drop of M9 buffer on a slide, and then immobilized by placing a coverslip over them. Polarized microscopy was done on a Zeiss Axioshot Photomicroscope (Carl Zeiss D-7082 Oberkochen).

Construction of DNA fragments for microinjection
A DNA fragment containing the unc-23 promoter and coding region (minus the TAG stop codon) fused to the green fluorescent protein (GFP) was constructed using the technique described by Cassata et al. and Hobert et al. The GFP coding and 3’ untranslated sequences were derived from the pPD95.75 Fire LabVector Kit available at http://www.addgene.org/pgve1? f-c andcmd-showcol andcold=1 ). The unc-23 promoter that was used starts at -2166 upstream of the ATG start codon. This region contains all of the 5’ upstream region of the unc-23 gene plus a portion of the 3’ UTR of the upstream H14N18.2 gene. The DNA fragments obtained were purified for microinjection using the QIAquick PCR purification Kit (Cat. # 28104. QIAGEN Inc. Mississauga, Ontario).

Microinjections of C. elegans
Microinjections of adult hermaphrodites were performed as described by Mello and Fire. The unc-23::GFP DNA fragment (~10ng/μl) was co-injected with the pPD95.75 GFP plasmid (~33ng/μl) and the pRF4 rol-6 plasmid (~78ng/μl) into the gonad syncytium of wild type hermaphrodites. The pPD95.75 GFP plasmid was included in the injection mix because it has homology to the unc-23::GFP PCR fragment. F1 Rol hermaphrodites were selected and any that produced Rol progeny were kept as transgenic strains. The following transgenic strain was obtained: DM7020: +/+; raEx20[pRF4 rol-6;isu1006dm] + unc-23::GFP + pPD95.75(GFP).

Strains homozygous for unc-23(c25) and carrying the raEx20 array were constructed by mating +/+; raEx Rol hermaphrodites with unc-23(c25) / + males. Single F1 Rol hermaphrodites with

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the genotype unc-23(e25) / +; raEx20 were selected and Rol hermaphrodites that were homozygous unc-23(e25) were obtained from among the progeny of these animals.

Isolation and mapping of suppressor mutations of unc-23 (e25)

Mutagenesis was done using 0.05 M ethyl methanesulfonate (EMS) in M9 buffer as described by Sulston and Hodgkin. Dominant and recessive revertants were obtained by screening both the F1 and F2 progeny of approximately 1000 mutagenized unc-23(e25) homozygous hermaphrodites for well-moving animals that lacked the bent-head phenotype. Individual wild type worms were transferred to new plates and their progeny were screened for the presence of the Unc-23 and wild type phenotypes. Homozygous suppressor strains were established by picking wild type animals that failed to segregate Unc-23 progeny. All of the strains obtained segregated Unc-23 animals in the F2 generation after outcrossing to wild type males, indicating that the suppressor mutations were not closely linked to the unc-23 gene.

The ra807 and ra808 suppressor alleles were mapped to linkage group IV in the following manner. Males of genotype unc-23 (e25) / +; dpy-13(e184) /+ IV were mated with homozygous suppressors hermaphrodites (unc-23(e25)V; hsp-1(ra807or8)IV). F1 progeny with the genotype unc-23(e25)V; dpy-13(e184) +/+ hsp-1(ra807or8)IV were selected and allowed to reproduce. In the case of the ra807 and ra808 dominant suppressor mutations only Wild and DpyUnc F2 progeny were observed. The absence of Dpy nonUnc and Unc nonDpy F2 progeny indicated that the suppressor mutations were linked to dpy-13(e184) IV. The recessive suppressor mutations have not been mapped.

The hsp-1(ra807) IV; unc-23(e324)V strain was constructed by mating unc-23(e324) / + V; GFP(cv24) IV males with hsp-1 (ra807) IV; dpy-11(e224) V hermaphrodites. Progeny with the genotype GFP(cv24) + / + hsp-1(ra807); unc-23(e324) / dpy-11(e224)V were identified and allowed to reproduce. The presence of Unc nonGFP hermaphrodites (genotype: hsp-1(ra807) IV; unc-23(e324) V) indicated that the e324 allele of unc-23 is not suppressed by the hsp-1(ra807) mutation.

Construction of clones for yeast 2-hybrid screens

The pDM#220 (pGBDU-C3 + unc-23 bait) and pDM#221 (pGAD-C3 + unc-23 bait) plasmid clones were constructed as follows. A 614 bp DNA fragment was amplified from an unc-23 cDNA clone using the PWO DNA Polymerase kit (Roche Molecular Biochemicals, Catalog #1644 947, Mannheim, Germany), inserted into a pCR-BLUNT vector (Invitrogen, Carlsbad, California) as described by the manufacturer (http://www.invitrogen.com) and transformed into DH5α competent cells (Life Technologies, catalog #18265–017. Burlington, Ontario). The unc-23 encoding DNA fragment was released from the pCR-BLUNT vector and re-inserted into the Eco RI sites of the pGBDU-C3 and pGAD-C3 vectors. The orientation and sequence of the cloned unc-23 encoding DNA fragments were determined by PCR analysis and sequencing. The yeast strain PJ69–4A was transformed with the pDM#220 and pDM#221 clones as described by James et al.65 to obtain the yDM#220 and yDM#221 yeast strains.

The yDM#220 strain was used as the bait to screen a mixed stage C. elegans cDNA yeast 2-hybrid library (kindly provided by Bob Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK) as described by James et al.65 Briefly, colonies obtained on His’ selection plates were transferred to adenine minus (Ade’) plates to be re-screened for their positive interaction. The established colonies on Ade‘ plates were transferred to a third selection plate containing 5-Fluoroorotic Acid (FOA) to promote loss of the bait plasmid. The prey plasmid was recovered and re-transformed into yDM#220 cells to confirm the initial positive interaction. DNA was isolated from the plasmids that showed an interaction with yDM#220 and sequenced by the NAPS unit at the University of British Columbia.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Authors’ Contributions
Conceived and designed experiments: PR, DM. Performed the experiments: PR, TR. Analyzed the data: PR, TR, DM. Wrote the paper: PR, TR, DM.
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