DYC-1, a protein functionally linked to dystrophin in *Caenorhabditis elegans* is associated with the dense body, where it interacts with the muscle LIM domain protein ZYX-1

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Running Title: The *C. elegans dyc-1* gene
Summary

In *Caenorhabditis elegans*, mutations of the dystrophin homologue, *dys-1*, produce a peculiar behavioural phenotype (hyperactivity and a tendency to hypercontract). In a sensitised genetic background, *dys-1* mutations also lead to muscle necrosis. The *dyc-1* gene was previously identified in a genetic screen because its mutation leads to the same phenotype as *dys-1*, suggesting that the two genes are functionally linked. Here, we report the detailed characterisation of the *dyc-1* gene. *dyc-1* encodes two isoforms, which are expressed in neurons and muscles. Isoform-specific RNAi experiments show that the absence of the muscle isoform, and not that of the neuronal isoform, is responsible for the *dyc-1* mutant phenotype. In the sarcomere, the DYC-1 protein is localised at the edges of the dense body, the nematode muscle adhesion structure where actin filaments are anchored and linked to the sarcolemma. In yeast two-hybrid assays, DYC-1 interacts with ZYX-1, the homologue of the vertebrate focal adhesion LIM domain protein zyxin. ZYX-1 localises at dense bodies and M-lines as well as in the nucleus of *C. elegans* striated muscles. The DYC-1 protein possesses a highly conserved 19 amino acid sequence, which is involved in the interaction with ZYX-1 and which is sufficient for addressing DYC-1 to the dense body.

Altogether our findings indicate that DYC-1 may be involved in dense body function and stability. This, taken together with the functional link between the *C. elegans* DYC-1 and DYS-1 proteins, furthermore suggests a requirement of dystrophin function at this structure. As the dense body shares functional similarity with both the vertebrate Z-disk and the costamere, we therefore postulate that disruption of muscle cell adhesion structures might be the primary event of muscle degeneration occurring in the absence of dystrophin, in *C. elegans* as well as vertebrates.
**Introduction**

Duchenne Muscular Dystrophy (DMD) is due to mutations in the dystrophin gene. This gene encodes a 3,685 amino acid protein, which is localised under the sarcolemma of skeletal and cardiac muscles (Koenig *et al*., 1987; Ahn and Kunkel, 1993). In skeletal muscle, dystrophin is mainly localised at a rib-like lattice called the costamere, which is a specific muscle adhesion complexe linking the Z-disk to the sarcolemma (Porter *et al*., 1992). Dystrophin is not required for costameric organisation, since costameres can form in the dystrophin-deficient *mdx* mouse (Porter *et al*., 1992). However, the localisation of costameric proteins such as β-spectrin and costameric actin tends to be affected in *mdx* mice (Williams and Bloch, 1999; Rybakova *et al*., 2000). The mechanisms that slowly lead to muscle necrosis in the absence of dystrophin are not yet fully understood. Dystrophin clearly has a structural function, but there is still no definite proof as to whether the pathology observed in DMD patients is due to a mechanical weakness of the sarcolemma, or if it results from a secondary impairment of proteins displaced because of the absence of dystrophin (Dubowitz, 2000).

The nematode *Caenorhabditis elegans* has a dystrophin-like gene named *dys-1*, which shows extensive sequence similarities with its mammalian counterparts, including several similar key motifs. The DYS-1 protein is expressed in striated body-wall muscles where its localisation overlaps actin containing thin filaments (Bessou *et al*., 1998 and our unpublished results). Body-wall muscles are striated muscles required for the locomotion of the nematode and exhibit a sarcomeric organisation similar to that of vertebrate skeletal muscles (Waterston, 1988). As in vertebrates, each sarcomere is composed of myosin containing thick filaments associated with the M-line and actin containing thin filaments anchored to a specialised adhesion structure, called the dense body (Moerman and Williams, 2006).

*dys-1* mutants develop a peculiar phenotype consisting of hyperactivity, exaggerated head bending and a tendency to hypercontract (Bessou *et al*., 1998). These mutants undergo only slight muscle
degeneration (Bessou et al., 1998; Grisoni et al., 2003). However, in a sensitised \textit{hlh-1(cc561)} background, which is a mild mutation of the \textit{C. elegans} homologue of the myogenic factor \textit{MyoD}, \textit{dys-1} mutations lead to an extensive time-dependent muscle degeneration (Gieseler et al., 2000).

Interestingly other genes have been identified whose mutations produce a behavioural phenotype identical to that of \textit{dys-1} mutants: \textit{dyc-1}, \textit{dyb-1} (dystrobrevin), \textit{stn-1} (syntrophin), \textit{dgn-1} (dystroglycan), \textit{sgn-1} (sarcoglycan), \textit{slo-1} (a potassium channel) and \textit{snf-6} (an acetylcholine transporter) (Gieseler et al., 2001; Grisoni et al., 2003; Grisoni et al., 2002b; Carre-Pierrat et al., 2006; Kim et al., 2004). The similarity between the phenotypes suggests that these genes may participate in the same biological function as the \textit{C. elegans dys-1} and makes their investigation an important step in understanding dystrophin function in the nematode.

This study deals with the \textit{dyc-1} gene, which is of particular interest since its over-expression partially compensates for the absence of dystrophin in \textit{C. elegans dys-1; hlh-1} double mutants (Gieseler et al., 2000). The DYC-1 protein does not appear to have any particular motif that might help to understand its function. Its closest vertebrate relative is the CAPON protein, a putative adaptor of the neuronal nitric oxide synthase (nNOS), which is expressed in neurons and in muscle satellite cells (Jaffrey et al., 1998; Segalat et al., 2005).

We show that the \textit{dyc-1} gene encodes neuronal and muscle proteins. The \textit{dyc-1} mutant phenotypes are thoroughly characterised and shown to be due to the inactivation of the muscle isoform. We further demonstrate that mutations of \textit{dyc-1}, like that of \textit{dys-1}, lead to time-dependent muscle degeneration when introduced in the sensitised \textit{hlh-1(cc561)} mutant background.

The muscle isoform of DYC-1 was found to be associated with the dense body, which in \textit{C. elegans} is an integrin-based muscle adhesion structure functionally related to both vertebrate Z-disks and costameres (reviewed in Lecroisey et al., 2007).
The muscular DYC-1 protein physically interacts with ZYX-1, the counterpart of the vertebrate focal adhesion protein zyxin (Smith et al., 2002). ZYX-1 as well as zyxin are LIM-only proteins containing three LIM domains. In vertebrates, the LIM-only protein zyxin is present at sites of cell adhesion (Crawford and Beckerle, 1991), where it interacts with -actinin and other cytoskeletal proteins (Crawford and Beckerle, 1991), as well as with signalling proteins such as the product of the proto-oncogene Vav (Hobert et al., 1996).

We demonstrate that the ZYX-1 protein is expressed in striated muscles, where it localises, like other muscle LIM domain containing proteins of C. elegans, in the nucleus as well as at M-lines and dense bodies. Our results indicate that the interaction between ZYX-1 and DYC-1 occurs at the edges of the dense body. In addition, we show that a strongly conserved 19 amino acid stretch of the muscular DYC-1 protein is necessary for its interaction with ZYX-1 as well as for the localisation of DYC-1 at the dense body.

With respect to the functional relationship between DYC-1 and the dystrophin of C. elegans, as a whole our results suggest that the dense body is the site of the primary events of muscle degeneration occurring in the absence of dystrophin.
1) *C. elegans* strains.

*C. elegans* strains were cultured as described (Brenner, 1974). N2 Bristol strain was used as wild-type control. *MyoD/hlh-1(cc561ts)* strain (Harfe *et al.*, 1998) was obtained from B.D. Harfe (University of Florida) and A. Fire (Stanford, California). *dys-1* alleles were described in (Bessou *et al.*, 1998). *dyc-1* alleles *cx5* and *cx32* were described in (Gieseler *et al.*, 2000). *zyx-1(gk190)* and *lin-15(n765)* were obtained from the *Caenorhabditis* Genetic Center (CGC). Classical genetics methods were used to construct double and triple mutants. All strains were grown at 15°C.

2) *dyc-1:gfp* and *zyx-1:gfp* constructs and microscopy

Reporter-gene constructs were made in GFP-encoding vectors (Chalfie *et al.*, 1994) pPD95.75, pPD95.77 and pPD118.20 (kindly provided by A. Fire).

To determine the expression of the *dyc-1* isoforms, we used 3 kb of genomic sequences located upstream of each isoform transcript. These regions were amplified by PCR on N2 worm DNA and cloned into the EcoR I-Hind III site of pPD95.77.

*dyc-1:gfpVI* is a Sal I-Eag I 16.3 kb genomic fragment encompassing the *dyc-1* short transcript, and containing 3.2 kb of upstream sequences, in which the *gfp* coding sequence has been inserted at the Bsul36 I site (amino acid 781) of *dyc-1*.

*dyc-1:gfpX* is a derivative of *dyc-1:gfpVI* in which the 5’ end has been extended by 6 kb by replacing the 5’ most Sal I-BspE I fragment by a 9.2 kb Pst I-BspE I fragment.
The dyc-1 (aa 52-81):gfp construct was obtained after PCR amplification of a fragment encoding amino acid (aa) 52-81 of the muscular DYC-1 isoform. PCR was performed on cDNA clone yk259a5, (kindly provided by Y. Kohara, NIG, Japan) and the amplified fragment was cloned into the EcoR I site of pPD118.20.

The zyx-1:gfp plasmid was constructed by insertion into the Pst I and Msc I cloning sites of pPD95.75 of a 17 kb Pst I-Nco I genomic fragment, that was obtained from cosmid F42G4 and corresponds to the gene F42G4.3.

The dyc-1 (aa 52-81):gfp and the zyx-1:gfp constructs were injected in N2 wild-type animals at a concentration of 10-50 ng/μl along with marker pRF4 (150 ng/μl). All other plasmids were injected at a concentration of 1-10 ng/μl in lin-15(n765) or lin-15(n765); dyc-1(cx5) worms with wild-type lin-15 as a transformation marker. All injections were performed using standard procedures (Mello and Fire, 1995).

Observation of live animals under a fluorescence microscope (Zeiss Axioplan) was done after immobilisation of the animals on a 2% agarose pad containing 0.1% sodium azide.

3) Production and affinity purification of DYC-1-GST

The pBRV plasmid was obtained by sub-cloning a 210 bp BamH I-EcoRV fragment of the dyc-I cDNA (yk259a5) encoding aa 720-790, of the DYC-1S protein, into pGEX-3X (Pharmacia). To produce the DYC-1 (aa 720-790)-GST fusion protein, Escherichia coli (strain BL21 DE3) transformed with pBRV were allowed to grow overnight in LB medium containing 100μg/ml ampicillin. Overnight cultures were diluted 1:10 in fresh medium and grown for 1h at 37°C before adding 5mM IPTG. Cultures were incubated for 3h at 37°C. The bacteria were then pelleted, re-suspended in 1/40 culture volume of PBS 1X, 1mM PMSF, 1mM iodoacetamine, 1% Triton X-100, 1mM EDTA and mechanically lysed by French Press (about 7000psi in manual French Press).
After centrifugation at 5500 rpm for 10 min at 4°C, the supernatant was mixed with Glutathione Sepharose 4B beads (Amersham) at 4°C on a rotating platform (1 ml beads for 1 liter of initial *E. coli* culture). After adsorption for 1 h at 4°C, beads were collected by brief centrifugation at 1000 rpm and washed 3 times with 10 bead volumes of PBS1X. Elutions of the fusion protein were performed with 1 bead volume of 2 mM Tris pH 8, 5 mM reduced glutathione (Sigma) and analysed on 8% SDS PAGE. To eliminate glutathione, purified fusion proteins were then dialysed against 2 mM Tris pH 8 with 12-14 MWCO membrane (Spectrum Labs).

4) Production and purification of anti-DYC-1 antibodies

Polyclonal antibodies against the C-terminal end of DYC-1 were generated (Covalab, Lyon) in New Zealand White rabbits immunised with approximately 500 μg of DYC-1 (aa 720-790)-GST fusion protein. The protein was injected sub-cutaneously, four times at 3-week intervals.

Antibodies were affinity purified as described by (Benian *et al.*, 1993). The affinity column was prepared by coupling 15 mg of purified fusion protein to 1 ml of 50/50 mixture of Affi-Gel-10 and Affi-Gel-15 (Bio-Rad) according to the manufacturer’s directions. The column was successively washed 3 times with 3 bead volumes of each of the following solutions: 10 mM Tris pH 7.5; 1 mM ethanolamine pH 8; 10 mM Tris pH 7.5. 5 ml of the rabbit antiserum were prepared on DEAE Affi-Gel Blue Gel (Biorad) following the manufacturer’s directions. This prepared serum was pre-cleared of anti-GST antibodies on a GST coupled Affi-Gel 10-15 column. The cleared serum was then applied to the DYC-1 (aa 720-790)-GST coupled column. To remove non-specifically bound proteins the column was successively washed 4 times with 3 bead volumes of each of the following solutions: 10 mM Tris pH 7.5; 10 mM Tris pH 7.5, 500 mM NaCl. The bound antibodies were eluted by 2 bead volumes of 100 mM glycine pH 2.5, rapidly neutralised with 2 M Tris pH 8. The affinity-purified antibodies were concentrated on AMICON Ultra-15 (Millipore).
5) Immuno-histochemistry

Wild-type and dyc-1(cx32) mutant worms were analysed by immuno-histochemistry on whole-mount preparations as in (Benian et al., 1996). Monoclonal antibodies MH24 (anti-DEB-1/vinculin; (Francis and Waterston, 1985)) and affinity-purified anti-DYC-1-antibodies were used at a dilution of 1:20. Secondary goat anti-mouse (Fluoroprobes 488, green, Interchim) or goat anti-rabbit antibodies (FluoroProbes 586, red, Interchim) were used at a dilution of 1:200. Images were captured with a Zeiss ZI Imager using either X63 or X100 oil immersion objective lenses using the Metaview software. Images were processed and annotated with Adobe Photoshop CS2.

6) Immuno-electron microscopy

Animals carrying the dyc-1:gfpX transgene were fixed by high pressure freezing as previously described (Liegeois et al., 2006). For immuno-localisation analysis, freeze substitution was adapted from Müller-Reichert et al. (Muller-Reichert et al., 2003): samples were incubated in 0.01% osmium tetroxyde, 0.25% uranyl acetate, 0.25% glutaraldehyde in pure acetone for 100h at -90°C. The temperature was then raised to -50°C at a 3°/h rate and samples left for incubation for 6h. Samples were thoroughly rinsed with pure ethanol before resin infiltration, performed with graded concentration of Lowicryl HM20 monostep (E.M.S.). The resin was polymerised under UV light for 48h at -50°C and for 48h at room temperature. Ultra-thin sections were collected on carbon/formvar-coated nickel slot grids and processed for immunogold labelling on the Leica EM-IGL automate. The immunogold experiments were performed in PHEM buffer (60 mM PIPES, 25 mM HEPES, 20 mM EGTA, 2 mM MgCl2, pH 6.8) as follows: 3 rinses in PHEM; blocking in 0.1% bovine serum albumin (Sigma), 0.1% Cold Water Fish Skin Gelatin (CWFSG, Aurion) in PHEM for 30min; 1h incubation in primary antibody (rabbit anti-GFP, AbCam) diluted 1/500 in 0.1% CWFSG; 6 rinses in PHEM; 1h incubation in gold conjugated protein-A (10nm, University Medical Center, Utrecht, Netherlands); 6 rinses in PHEM; post-fixation in 1% glutaraldehyde and
extensive rinsing in distilled water. Grids were then slightly contrasted in lead citrate (15sec), uranyl acetate (1min) and lead citrate (15sec) before observation. Images were obtained with a SiS Megaview 3 ccd camera mounted on a FEI Morgagni transmission electron microscope operated at 70kV.

7) Quantification of DYC-1-GFP immuno-electron-microscopy labelling

Two independent scores of gold bead sarcomeric positions were performed on two different worm preparations, both of which were subjected to three different immuno-reactions. We determined four different sarcomeric zones: the bottom, the middle and the top of the dense body zone and a zone out of the dense body including the M-line. We also distinguished between gold beads localised on a dense body (in cross sections through the dense body, when the dense body appears in black) and beads that localised between two adjacent dense bodies (at the edge), perpendicular to the cross section plane (in cross sections between two dense bodies, when the dense body is not visible). For the first experiment, 521 gold bead positions were analysed in 20 worms; for the second experiment, 502 gold bead positions were analysed in 18 worms. The results are statistically significant. Muscle cells were taken into account only when the muscle ultra-structure was correct. The means of the two different quantifications were calculated.

8) Quantitation of locomotion

Locomotion was scored on active well-fed animals as previously described (Segalat et al., 1995). The number of body bends/min was scored for at least 20 animals per experiment. We considered a body bend to be one complete sinusoidal movement of the scored animal.
9) Yeast two-hybrid assay and screening

A Sac I- EcoR V cDNA fragment (cDNA clone yk259a5) encoding the DYC-1S protein (aa 9-793) was sub-cloned into the pAS2-1 bait vector (Clontech). The resulting construct, which was called pSEB, encodes a fusion protein composed of DYC-1S (aa 9-793) and the DNA binding domain (BD) of the yeast gal4 transcription factor. Yeast cells of the CG1945 strain were transformed with pSEB.

The C-terminal end of the DYS-1 protein (aa 2857-3674) was fused to the gal4 activation domain (AD). For this purpose, a 2.4kb dys-1 cDNA fragment (cDNA clone yk12c11) was cloned into the poly linker of pACT2 (Clontech) with respect to the reading frame. The resulting prey plasmid was transformed into the yeast strain Y187. Interaction between DYC-1S and DYS-1 was assayed as described (Grisoni et al., 2003) after mating of CG1945 yeast cells (expressing DYC-1S (aa 9-793)-BD) with Y187 yeast cells (expressing DYS-1 (aa 2857-3674)-AD). 10μl of mating cultures were deposited on minimal medium lacking Leu, Trp, and His and minimal medium lacking Leu and Trp (growth control) and incubated for 3 days at 30°C.

A two-hybrid screening was performed as described (Fromont-Racine et al., 1997) by mating CG1945 yeasts transformed with pSEB with Y187 yeasts transformed with a random primed C. elegans cDNA activation domain library (RB2) sub-cloned into the pACT vector (R. Barstead, Oklahoma Medical Research Foundation, Oklahoma City). 6.8x10⁶ diploid clones containing the pSEB bait plasmid and one prey-pACT2 plasmid (from the library) were analysed for their ability to grow on minimal medium lacking Leu, Trp, and His, after 3-day incubation at 30°C. Growth can occur only if the bait and the prey proteins interact and the HIS3 reporter gene is transactivated. 131 positive clones were obtained and prey cDNAs were sequenced. Sequence analysis was performed using BLASTn searches. 127 positive clones were found to correspond to the F42G4.3/zyx-1 gene and 4 clones to F14F3.1.
10) Mapping of DYC-1 and ZYX-1 interacting regions by yeast two-hybrid assays

A plasmid (pKG7) encoding the N-terminal region of DYC-1S (aa 9-258) fused to gal4-BD was obtained from pSEB after deletion of a Sac II-Pst I fragment encoding aa 259-793. The pKG7 plasmid was further used to introduce a deletion of an Mbo I fragment encoding aa 54-81. The resulting plasmid called pKG24 therefore encodes a fusion protein of gal4-BD and DYC-1S (aa 9-258 Δaa 54-81).

Three constructs encoding fusion proteins composed of the gal4-AD-domain and different parts of the ZYX-1 protein were obtained by sub-cloning the corresponding zyx-1 cDNA fragments (cDNA clone yk247e1) into pACT2 prey vector (Clontech). Plasmid pSNXX encodes ZYX-1 (aa 68-603)-AD, pKG12 encodes ZYX-1 (aa 384-603)-AD and pKG17 encodes ZYX-1 (aa 358-528)-AD.

Bait plasmids (pAS2-1, pSEB, pKG7 and pKG24) were transformed into the yeast strain CG1945 and prey plasmids (pACT2, pSNXX, pKG12 and pKG17) into the yeast strain Y187. Interactions between different forms of DYC-1S and ZYX-1 were assayed as described (Grisoni et al., 2003) after mating of transformed CG1945 yeast cells with the transformed Y187 yeast cells. 10μl of mating cultures were deposited on minimal medium lacking Leucine (Leu), Tryptophane (Trp), and Histidine (His) and minimal medium lacking Leu and Trp (growth control) and incubated for 3 days at 30°C.

11) GST pull-down assay

A Sac I-Sac II cDNA fragment (cDNA clone yk259a5) encoding the DYC-1S protein (aa 9-258) was subcloned into the BamH I-EcoR I cloning sites of the pGEX-2T plasmid (Pharmacia). GST and DYC-1 (aa 9-258)-GST fusion proteins were produced and purified as described above (Section 3).
The hemaglutinin (HA)-tagged ZYX-1 (aa 15-603) protein expressing plasmid was constructed by inserting a BspE I- SnaB I zyx-1 cDNA fragment (cDNA clone yk247e1) into the EcoR I- Xho I cloning sites of the pcDNA3.1/HA plasmid (kindly provided by J. Thomas and P. Lomonte, Lyon, France). 10⁶ Cos-7 cells were transiently transfected with 3μg of the ZYX-1 (aa 15-603)-HA encoding plasmid using FuGENE 6 (Roche) following the manufacturer’s directions. Transfected Cos-7 cells were grown at 37°C in Dulbecco’s Modified Eagle Medium (Invitrogen) containing 1% of penicillin (50U/ml), 1% streptomycin (50μg/ml) and supplemented with 10% foetal bovine serum.

GST pull-down assays were performed as described (Lomonte et al., 2004). Cellular extracts containing the ZYX-1 (aa 15-603)-HA were prepared 48h after transfection of Cos-7 cells with the ZYX-1-HA plasmid. Cells were washed once with PBS1X, centrifuged at 2000 rpm for 5min and the cell pellet resuspended in 300μl of a lysis buffer (15mM Tris-HCl, pH 7.5, 15mM NaCl, 2mM EDTA, 0,5mM EGTA, 0,34M sucrose, 0,01mM spermidine, 0,5% Triton X-100, 0,1M KCl and protease inhibitors (Roche Complete mini)). The suspension was incubated with shaking on ice for 45min, and the resulting lysate was centrifuged at 13000 rpm for 10min at 4°C. 50 μl of cell extracts containing ZYX-1-HA were first incubated for 1h at 4°C with continuous mixing with glutathione-sepharose beads coated with 50μg GST protein in order to reduce the background signal. The precleared extract (50μl) was then incubated for 2h at room temperature with continuous mixing with glutathione-sepharose beads coated with 50μg GST-DYC-1 (aa 9-258) protein or GST alone as negative control. Beads were then washed 3 times with 1ml of the lysis buffer. Protein complexes were eluted by boiling beads for 5min in 50 μl of Laemmlli buffer. 20 μl of these elutions were migrated on 8% SDS-PAGE. The presence of ZYX-1-HA protein was assayed by Western blotting as described earlier (Gieseler et al., 2001) using 12CA5 anti HA-1 primary antibody (Roche) at 1: 5000 and goat anti-mouse-HRP conjugated secondary antibody at 1:3000 (BioRad).
12) Quantification of muscle degeneration

For these experiments worms were grown at 15°C. Animals were fixed and stained 3 days after they reached the L4 stage. Fixation and Phalloidin-Rhodamin staining (Fluoroprobes, Interchim) were performed as described (Waterston et al., 1984). Stained body-wall muscles were observed using a Zeiss Axioscop microscope. Only the two most visible quadrants of body-wall muscles in each animal were quantified (40 cells per animal). 20 animals were scored for each genotype.

13) RNA interference

For isoform-specific RNAi experiments, an EcoR I-Sca I 350 bp fragment encompassing exons 4-5 of DYC-1L, and a Pst I-Xba I 550 bp fragment encompassing the first exon of DYC-1S, were cloned into the RNAi feeding vector L4440. The resulting constructs (RS350 and PX550) were transformed into HT115 E. coli. RNAi was performed by feeding N2 wild-type or DYC-1-GFPX expressing transgenic worms with dsRNA producing E. coli (Timmons et al., 2001).
Results

1) dyc-1 mutants exhibit dys-1-like phenotypes.

We identified the dyc-1 gene in a screen for mutations producing a dys-1-like phenotype consisting of hyperactivity, exaggerated head bending, and a tendency to hypercontract (Gieseler et al., 2000). The phenotypes of both dyc-1 alleles cx5 and cx32 are identical, and undistinguishable from that of the canonical dys-1 allele cx18 (Table 1). cx5 and cx32 were previously determined as being an early stop codon and an early frame-shift, respectively (Gieseler et al., 2000). The phenotypes of dyc-1 and dys-1 mutations are not additive: the phenotype of dys-1(cx18); dyc-1(cx5) double mutants is similar to single mutants (Table 1).

Mutation of dys-1 can lead to a progressive and severe degeneration of body-wall muscles when put in a sensitised CeMyoD/hlh-1 background (Gieseler et al., 2000). To investigate whether the same applies to dyc-1, we constructed a dyc-1(cx32); hlh-1(cc561) double mutant and observed its muscles by performing phalloidin staining. A large number of body-wall muscle cells were found to be either missing or degenerating in this double mutant, although the phenotype was not as strong as that of the dys-1(cx18); hlh-1(cc561) strain (Table 2). As in the case of dys-1, the degenerative phenotype occurring in the hlh-1(cc561) background is time-dependent and affects only adults.

2) dyc-1 encodes 2 isoforms with different expression patterns and functions

Analysis of EST tags shows that the dyc-1 gene generates at least two transcripts originating from different promoters. This gives rise to 2 proteins with a predicted length of 793 and 887 aa that we call respectively DYC-1S (short isoform) and DYC-1L (long isoform) (Figure 1). The sequences of
the transcripts and corresponding products can be found at www.wormbase.org. To investigate the tissue distribution of each isoform we produced GFP constructs. Approximately 3 kb of upstream sequence relative to each isoform was cloned in a GFP reporter vector and injected into wild-type animals. This experiment showed that the proximal promoter drives the expression of the gene in muscles (body-wall and vulval muscles), while the distal promoter drives its expression in approximately 10 of the animal’s neurons (2 lateral neurons, that we identified to be SDQL and SDQR, plus additional non-identified head and mid-body neurons, Figure 3 and data not shown). We therefore conclude that DYC-1S is the muscle isoform, and that DYC-1L is the neuronal isoform (Figure 1).

We then investigated the relationship between the phenotype of *dyc-1* mutants and the 2 isoforms. This point was addressed in two ways.

First, RNAi experiments were conducted using dsRNA specific to each of the isoforms (red bars in Figure 1). Wild-type animals were fed with bacteria producing dsRNA directed either against the muscle isoform or against the neuronal isoform. Only when the muscular isoform was inactivated did the animals display the *dys-1*-like behavioural phenotype. In order to verify the effectiveness of specific isoform inactivation, we repeated RNAi feeding experiments on animals carrying a *dyc-1:gfp* transgene, which is able to generate both isoforms (*dyc-1:gfpX*, Figure 1) and observed the GFP expression pattern by fluorescence microscopy (Figure 2). When these transgenic animals were fed with bacteria producing dsRNA directed against the muscle isoform, a drastic reduction of muscular GFP was observed, while the neuronal signal remained unaffected (Figure 2B). On the other hand, worms fed with neuronal isoform-specific RNAi exhibited a weaker but not completely absent GFP signal in neurons. In this case the muscular GFP signal seems not to be different from the control animals (Figure 2C). These observations confirm that RNAi is less effective in neurons, but also indicates that the specific inactivation of the muscular DYC-1S isoform is sufficient to induce the behavioural *dys-1*-like phenotype.
Secondly, we introduced the GFP coding sequence into a genomic fragment carrying the whole muscle isoform, but lacking the beginning of the neuronal isoform (construct $dyc-1:gfpVI$, Figure 1). The expression of this transgene is restricted to muscles thus confirming the muscle-specific expression of the short isoform. In addition, when introduced in $dyc-1$ mutants, this transgene rescues the behavioural phenotype (Table 1).

Altogether, these results show that the behavioural phenotype of $dyc-1$ mutants is of muscle origin. Similar findings were made previously for $dys-1$, since $dys-1$ null mutants can be rescued by muscle specific $dys-1$ expression (Bessou et al., 1998).

3) Subcellular localisation of $dyc-1$ isoforms in neurons and muscles

For observing the sub-cellular localisation of DYC-1 in neurons and muscles, we used the $dyc-1:gfpX$ construct which is able to generate both isoforms (Figure 1).

In neuron, this reporter gene showed a striking fluorescence pattern, consisting of a string of dots running along the axon, as shown on a mid-body lateral neuron SDQR in Figure 3. These structures might be attachment points for the axons to the extra-cellular matrix beneath them. Dots were also detected in neuron cell bodies, but it is unclear whether this corresponds to the natural localisation of the protein or a transgene artefact.

In striated muscles the $dyc-1:gfpX$ transgene displayed a repetitive fluorescent pattern reminiscent of the sarcomeric pattern (Figure 4). To identify which component of the sarcomere was labelled, we stained $dyc-1:gfpX$ expressing animals with the MH24 antibody, which recognises the DEB-1/vinculin protein, one of the main components of dense bodies (Francis and Waterston, 1985; Barstead and Waterston, 1991). The DYC-1-GFPX protein is located at the edges of MH24 labelled dense bodies (Figure 4).
4) The muscular DYC-1S isoform is localised at the edges of the dense body

To further investigate the sub-cellular localisation of DYC-1 in muscles, we produced an anti-DYC-1 polyclonal antibody. After affinity purification, this antibody was used in whole-mount immuno-cytochemistry. Wild-type animals displayed in their body-wall muscles a spotted pattern reminiscent of the repetitive sarcomere architecture, resembling that observed in reporter gene analysis. No staining was detected in dyc-1(cx32) mutants, suggesting that the antibody specifically recognises the DYC-1 protein.

We performed double-staining experiments with the anti-DYC-1 antibody and the MH24/anti-DEB-1 antibody. A detailed analysis of the pattern confirms, as observed for the DYC-1-GFPX protein, that the DYC-1 signal is located at the margins of MH24 labelled dense bodies, the C. elegans counterparts of vertebrate Z-disk (Figure 4). Therefore, DYC-1 seems to localise at the edges of dense bodies and might be associated with these muscle attachment structures.

5) Immuno-electron-microscopy confirms localisation of DYC-1 at the edges of the dense body

In order to analyse in more detail the dense body localisation of the DYC-1 protein, we used immuno-electron-microscopy. The DYC-1 antibody was not efficient enough to observe a DYC-1 signal by this approach, probably due to the low-level expression of the DYC-1 protein in wild-type worms. Therefore, DYC-1 detection was performed using a GFP antibody on dyc-1:gfpX transgenic worms. In the muscle cells of these animals we observed a strong cytoplasmic background signal, probably due to the over-expression of the transgene, as well as a signal in the sarcomeric zone. We focused on gold beads located in the contractile filaments zone of the sarcomere. Clusters of gold beads or single gold beads were frequently observed at the bottom of the dense body (Figure 5A and B). These observations indicate that DYC-1 is located in the dense body zone and primarily at the bottom of the dense body. For improved reliability, two independent scores of gold beads were performed with respect to the sarcomeric position. The results of the scores are presented in Figure
5C, and the different sarcomeric zones that were considered in these scores are indicated in Figure 5D. 72.34% of the gold beads were located in the dense body zone and only 27.66% outside this zone. In addition, gold beads were mainly located in the bottom zone of the dense body (Figure 5).

We furthermore verified whether gold beads were in the dense body or at its edges. For this purpose we compared gold-bead scoring of cross sections that went through the dense body (in this case the dense body appeared in black) with cross sections passing between two adjacent dense bodies. We found that only 4.30% of gold beads were located in the dense body (3.52% on the base of the dense body and 0.78% in the middle), while 68.04% of gold beads were located between two dense bodies (43.70% in the base zone, 12.02% in the middle zone, and 12.32% in the top zone).

Taking into account these results and the localisation of the DYC-1 antibody signalling at the margin of the MH24 antibody signal (Figure 4), we conclude that DYC-1 is mainly located between two dense bodies, meaning at the edges of the dense bodies with respect to the axis of contractile filaments, and more precisely, at the bottom of these muscle attachment structures.

6) DYC-1 binds to the zyxin-like LIM protein ZYX-1.

Yeast two-hybrid experiments were carried out in order to identify potential molecular partners of the muscular DYC-1S protein.

First, we verified in a yeast two-hybrid assay whether DYC-1S interacts directly with the DYS-1 protein. To this end the DYC-1S protein (aa 9-793) was assayed for its interaction with the DYS-1 C-terminal region (aa 2857-3674). This region of DYS-1 was chosen because it contains numerous motifs potentially implicated in protein-protein interactions (Bessou et al., 1998) and has been shown to mediate DYS-1 interaction with DYB-1 (dytrobrevin) and STN-1 (syntrophin) (Gieseler et al., 1999; Grisoni et al., 2002a). However, we detected no interaction between the DYS-1 C-terminal region and the DYS-1S protein (data not shown).
Secondly, we used the DYC-1S protein (aa 9-793) as bait in a yeast two-hybrid screen of a *C. elegans* cDNA library. Most positive clones were found to encode the LIM domain protein ZYX-1, the counterpart of vertebrate zyxin (Smith *et al.*, 2002). In vertebrate, the LIM domain protein zyxin is present at sites of cell adhesion (Crawford and Beckerle, 1991).

7) ZYX-1 and DYC-1 interact in the muscle at the edges of the dense body

To verify whether the cellular localisation of the ZYX-1 protein is compatible with a physical interaction with DYC-1S, we generated worms expressing a *zyx-1:gfp* transgene. In *C. elegans*, the *zyx-1* gene is expressed in muscles and neurons (Figure 6). In muscle cells, the ZYX-1 protein is localised in the nucleus as well as at the dense bodies and M-lines (Figure 6). This type of localisation has been reported for other muscle LIM domain proteins in *C. elegans* (reviewed in Lecroisey *et al.*, 2007).

Furthermore, we determined the localisation of the ZYX-1-GFP protein at the dense body with respect to the DYC-1S protein by staining *zyx-1:gfp* transgenic worms with MH24 and anti-DYC-1 antibodies. We observed an overlapping localisation of ZYX-1-GFP and DEB-1, while DYC-1 was located at the margins of the ZYX-1-GFP dense body dots (Figure 6).

Therefore ZYX-1 seems to be part of the dense body and interacts with DYC-1 at the edges of these muscle adhesion structures.

To investigate the function of the ZYX-1 protein and its potential functional relationship with the DYC-1S protein, we used the *zyx-1(gk190)* mutant. This mutant carries a 777 bp deletion leading to a (predicted) ZYX-1 protein lacking all of its three LIM domains.

*zyx-1(gk190)* mutants exhibit no obvious behavioural phenotype. When observed after palloidin-staining of actin filaments the striated body-wall muscles resemble those of wild-type controls (Table 2). In the same way, no significant muscle defects could be detected in *zyx-1(gk190) hlh-
$I^{(cc561)}$ or $zyx-1^{(gk190)}; dyc-1^{(cx32)}$ double-mutants. Furthermore, the $zyx-1^{(gk190)}$ mutation neither enhances nor reduces the muscle degeneration phenotype when it is introduced in a $dyc-1^{(cx32)}, hlh-1^{(cc561)}$ mutant background (Table 2). Together these results are consistent with observations described before, showing no drastic mutant phenotype for other $zyx-1$ mutants or RNAi-mediated $zyx-1$ inactivation (Smith et al., 2002). This suggests that in the absence of ZYX-1, another LIM domain containing protein might carry out the function of ZYX-1.

8) The interaction between DYC-1 and ZYX-1 involves conserved domains

To confirm the physical interaction between the DYC-1S and the ZYX-1 proteins, we produced a ZYX-1 (aa 15-603)-HA tagged protein in mammalian COS-7 cells and used cell extracts for in vitro GST pull-down experiments with DYC-1-GST fusion proteins. We were thus able to show that the N-terminal aa 9-258 of DYC-1S are involved in, and sufficient for, DYC-1 interaction with ZYX-1 (Figure 7B).

In order to determine in more detail the protein regions required for the interaction between DYC-1S and ZYX-1, we further assayed different truncated forms of both proteins for their ability to interact, using yeast two-hybrid assays.

The ZYX-1 protein possesses three LIM domains in its C-terminal half (Smith et al., 2002). We tested whether ZYX-1 LIM domains are involved in the interaction of ZYX-1 with DYC-1S. When using a portion of ZYX-1 (aa 384-603) containing all three LIM domains, interaction with DYC-1S occurs (Figure 7). However, when the third LIM domain of ZYX-1 was deleted, the remaining protein composed of aa 384-528 of ZYX-1 was no longer able to interact with DYC-1S (Figure 7). These observations indicate that the third LIM domain of the ZYX-1 protein is necessary for its interaction with DYC-1S.
Concerning the DYC-1S protein, we were able to confirm (as shown by GST pull-down assays) the importance of the N-terminal region (aa 9-258) of DYC-1S for its interaction with ZYX-1. This region interacts in yeast two-hybrid assays with ZYX-1 (aa 68-603) as well as ZYX-1 (aa 384-603) (Figure 7). Interestingly, this DYC-1 sequence contains one of the two previously identified regions of similarity to the vertebrate CAPON protein (Gieseler et al., 2000 and Figure 1). This sequence is a stretch of 19 aa (aa 58-76 of DYC-1S), which evolutionarily has been highly conserved from worms to humans (a difference of only 2 residues, Figure 7C). Since a search for variants of this sequence in protein databases did not yield any significant results, it seems likely that this motif is unique and has undergone very strong selection pressure.

In order to verify if this conserved region is involved in DYC-1S interaction with ZYX-1, we tested the N-terminal region of the DYC-1S protein (aa 9-258) in which aa 54-81 were deleted. In yeast two-hybrid assays, this DYC-1S (aa 9-258 •54-81) protein is no longer able to interact with ZYX-1 nor with ZYX-1 (aa 384-603) nor with ZYX-1 (aa 68-603) (Figure 7). This observation indicates that the conserved motif of the DYC-1S protein is required for the interaction with ZYX-1.

9) A conserved motif addresses the DYC-1 proteins to the dense body

We furthermore analysed whether the evolutionary conserved motif of the DYC-1 protein was also implicated in the sub-cellular localisation of the DYC-1 protein. We produced transgenic worms expressing a fusion protein composed of GFP and DYC-1S aa 52-81 under the control of a muscular promoter (the promoter of the myo-3 gene). In striated muscles of wild-type worms the DYC-1 (aa 52-81)-GFP protein shows a sarcomeric localisation, which resembles that of the endogenous DYC-1 protein, albeit slightly more diffused (Figure 8). Staining of transgenic animals with the MH24 anti-DEB-1 antibody revealed that the GFP signal partially overlaps that of MH24, indicating that the DYC-1 (aa 52-81)-GFP protein localises at the dense body (Figure 8). This
observation suggests that the conserved motif serves as a signalling sequence for addressing the DYC-1 protein to the dense body.
Discussion

Strategy for identifying proteins functionally related to dystrophin

Classical genetics affords the possibility for establishing the existence of functional links between genes and their products by simply studying phenotypes, without any need for pre-existing hypotheses. Using this approach to search for proteins functionally linked to dystrophin in *C. elegans* we previously identified the DYB-1 and STN-1 proteins, which are known to be partners of dystrophin in mammals (Gieseler *et al*., 2001; Grisoni *et al*., 2003). In the present study, using the same approach, we established that *dyc-1* mutants share many phenotypes with *dys-1* mutants. The DYC-1 protein, which is related to the CAPON adaptor protein, is therefore likely to functionally interact with dystrophin (DYS-1).

Sub-cellular localisation of DYC-1

The *C. elegans* dense bodies are muscle adhesion structures and the functional counterparts of the Z-disk and the costamere of vertebrate skeletal muscles, and therefore constitute the main anchoring point of sarcomeric actin. These structures have been thoroughly studied using a combination of biochemical and genetic approaches that have shed light on their composition (Waterston, 1988; Moerman and Fire, 1997). Dense bodies are themselves anchored to the muscle cell and basement membrane via specialised proteins, all of which are also known components of vertebrate focal adhesion plaques (reviewed in Cox and Hardin, 2004; Labouesse and Georges-Labouesse, 2003; Lecroisey *et al*., 2007).

The sub-cellular localisation of DYC-1 is interesting. In neurons, DYC-1 is localised in discrete and regularly spaced structures along the axons. A similar localisation has been described before for UNC-97 (Hobert *et al*., 1999). UNC-97 is one of the LIM domain containing proteins, which is
expressed in body wall muscles, where it localises, like DYC-1, at dense bodies. Because dense bodies are specialised cell adhesion structures, we think that the neuronal structures labelled either by DYC-1 or UNC-97 might be attachment points of the axons.

In the muscle sarcomere we provide clear evidence that DYC-1 is located at the edges of the dense body, where filaments of sarcomeric actin are anchored via vinculin, alpha-actinin and other proteins. The molecular mechanisms underlying the maintenance and replacement of these muscle adhesion structures are still largely unknown. However, these attachment points must have strong mechanical resistance properties since they sustain the bulk of the load when the muscle contracts.

Putative role of DYC-1 in the striated muscle

We have shown in this paper that a major binding partner of DYC-1 is ZYX-1, a LIM domain protein resembling vertebrate zyxin (Smith et al., 2002). Zyxin is an adaptor protein that localises at focal adhesion structures and binds to alpha-actinin and other cytoskeletal proteins (Crawford et al., 1992). Zyxin may also shuttle to and from the nucleus, presumably to regulate transcription (Wang and Gilmore, 2003). In C. elegans striated muscles the ZYX-1 protein is localised at dense bodies as well as in the nucleus. ZYX-1 shares this dual localisation with at least three other muscle LIM domain proteins: ALP-1, UNC-95, UNC-97 (reviewed in Lecroisey et al., 2007). Like its vertebrate counterpart, ZYX-1 might be implicated in shuttling between the nucleus and the dense body and contribute to the maintenance of the stability of the dense body and the contractile machinery. However, inactivation of the zyx-1 gene, either by RNAi depletion or the zyx-1(gk190) deletion allele, does not lead to an obvious mutant phenotype (Smith et al., 2002 and our observations), thus suggesting a functional redundancy of ZYX-1 and other muscle LIM domain proteins. This point will be a matter for future investigation.
Even if we have not yet determined the functional relationship between ZYX-1 and DYC-1, the
dissection of the protein domains required for their physical interaction allowed us to identify a
functionally important motif of the DYC-1 protein.

The DYC-1 vertebrate homologue, CAPON, is thought to be an adapter protein for nNOS, thus
enabling it to be directed to its targets such as DexRas1 (Jaffrey et al., 1998; Fang et al., 2000).
Although no homologue of nNOS has yet been found in *C. elegans*, DYC-1 may also work as an
adapter protein in *C. elegans*. CAPON and DYC-1 share two regions of homology, which
underwent tremendous selective pressure in evolution. These two regions are predicted alpha
helices, one of them carrying a motif reminiscent of a leucin zipper (Gieseler et al., 2000). It is
therefore likely that these regions serve as contact regions for binding partners, which may have
been evolutionary conserved as well.

In this paper we have provided clues as to what the function of one of these regions (aa 58-76)
might be. We have shown that this sequence is implicated in the interaction of DYC-1 with ZYX-1,
and serves as a signal for addressing the DYC-1 protein to the dense body.

The co-localisation of ZYX-1 and DYC-1 at the dense body as well as their physical interaction
suggest that both proteins participate in a common function involving a network of proteins present
in the muscle attachment structure (Figure 8).

**Functional link between DYC-1 and dystrophin**

The question of dystrophin localisation with respect to the sarcomere structure is of critical
relevance to understanding the mechanisms underlying muscle necrosis in the absence of
dystrophin. Although this has not been formally proven, increasing quantities of evidence suggest
that dystrophin may be located at, or near, the Z-disk. In vertebrate, dystrophin is enriched in
costameric region (Porter et al., 1992); muscle-specific focal attachment plaques, which encompass
the Z-disks (Pardo et al., 1983) and bind to Z-disk proteins (Ervasti, 2003). In the body-wall muscles of *C. elegans*, the DYS-1 protein localises in a broad region overlapping thin filaments and dense bodies (our unpublished results).

The similarity of the phenotypes between *dys-1* and *dyc-1* mutants is a strong argument in favour of a functional interaction between *dys-1* and *dyc-1*. In addition, it was previously reported that increasing the amount of DYC-1 is beneficial to dystrophin-deficient muscles (Gieseler et al., 2000). DYC-1 might therefore serve as a functional link between dystrophin and the dense body. Since no interactions were detected in two-hybrid assays between DYC-1 and DYS-1, it is possible that a third component is involved. Alternatively, a direct interaction might exist that did not show up in the two-hybrid assay or that implicates regions of the DYS-1 protein other than the C-terminal domain, the only domain to be assayed in this research.

Whatever the nature of the physical link between dystrophin and DYC-1, the working model that we propose from these results (Figure 9) is that DYS-1 and DYC-1 are physically linked, either directly or indirectly. The absence of DYS-1 may destabilise DYC-1. This decrease in DYC-1 function may in turn have a deleterious effect on the dense body, gradually leading to a breakdown of the sarcomere and therefore muscle degeneration.

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Resources (NCRR). The MH24 antibody developed by R.H. Waterston was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported by the Association Française contre les Myopathies (AFM), by a district (Région Rhône-Alpes) grant to L.S. and Cancéropôle Gran-Est grants to M.L.
References


**Figure legends and Tables**

**Figure 1: Structure of the dyc-1 gene and constructs used in this study**

Top. Cosmids C33G3 and C04C11 that overlap with the dyc-1 gene.

Middle. Genomic organisation of the dyc-1 gene. Rectangles indicate the different exons. The dyc-1 gene encodes two different isoforms: a short protein of 793 amino acids called DYC-1S and a long 887 amino acid protein called DYC-1L. These isoforms are generated from alternative promoters, DYC-1S is expressed in muscles and DYC-1L in neurons. Both DYC-1 proteins contain two regions of strong homology to the human protein CAPON (indicated in blue). Isoform-specific regions used in the RNAi experiments are shown as red bars. The respective positions of the dyc-1(cx5) and the dyc-1(cx32) mutations are indicated (arrows).

Bottom. Structure of two transgenes cited in this paper. Note that the dyc-1:gfpVI transgene does not contain the neuronal promoter, whereas dyc-1:gfpX contains both the neuronal and the muscular promoter.

**Figure 2: Muscular and neuronal isoform-specific RNAi experiments**

A. Control animal expressing the dyc-1:gfpX transgene fed with bacteria containing the empty plasmid (L4440). Arrow shows the muscular expression of the transgene and arrowhead indicates the cell body of a neuron expressing the transgene. B. Transgenic animals fed with muscular isoform specific RNAi. Note the strong reduction of muscular GFP signal, while the neuronal signal remains intense (arrowhead). C. Transgenic animals fed with neuronal isoform specific RNAi. The arrowhead points to a neuron. Note that the neuronal GFP signal is slightly reduced but not completely abolished. The muscular GFP signal (arrow) seems not to differ from control animals.

**Figure 3: Neuronal dyc-1 expression seen by reporter gene**
Immuno-fluorescence images of wild type animals carrying a dyc-1:gfpX transgene.

Expression is detected in approximately 10 neurons. A neuron cell body (SDQR) is visible in A (arrowhead). In A and B arrows indicate the punctate pattern along the axons.

Figure 4: **Sub-cellular localisation of DYC-1 protein in muscle cells**

Immuno-fluorescence images of wild-type animals (A-F) and dyc-1(cx32) mutants (G-I)

A, D, G: Dense body localisation of the DEB-1/vinculin protein labelled with MH24 antibody (red). B: Localisation of the dyc-1:gfpX reporter gene (green, the signal is amplified with anti-GFP antibodies). C: Merge of A and B. In the magnification of two dense bodies, the green GFP signal is seen at the edges of the red MH24 signal. E: Localisation of the DYC-1 protein labelled with purified anti-DYC-1 antibodies (green). F: Merge of D and E with a magnification of two dense bodies. Note that as in C, the green (DYC-1) signal is located at the edges of dense bodies (red MH24 signal). H: dyc-1(cx32) mutants stained with anti-DYC-1 antibodies. No specific signal can be detected. I: Merge of G and H with a detail of two dense bodies showing the absence of DYC-1 staining.

Figure 5: **Sarcomeric localisation of the DYC-1 protein in immuno-electron microscopy**

A and B: Examples of cross sections of dyc-1:gfpX expressing transgenic worms labelled with an anti-GFP antibody coupled to gold beads. Note that gold beads are preferentially located at the bottom of the dense body (arrows). Clusters of gold beads in the cytoplasm (arrowheads in A), are due to over-expression of the transgene. C: Diagram summarising quantification of gold beads according to their sarcomeric position. These results are the mean of two different counts. 72.34% of gold beads are located in the dense body region and 27.66% outside of it. Among the
gold beads which are located in the dense body zone, 4.30% are located in the dense body (3.52% at the bottom and 0.78% on the middle) and 68.04% are located at the edges (between two dense bodies), with 43.70% of them at the bottom zone, 12.02% in the middle zone and 12.32% in the top zone. D: Detail of a dense body showing the limits of different zones considered for scoring gold bead locations. In this picture the section does not cross the dense body (otherwise it would appear in black). Gold beads located at this level are therefore classified as being at the edge of the dense body.

Figure 6: Sub-cellular localisation of the ZYX-1 protein by reporter gene analysis

A-H: Transgenic animals expressing a zyx-1:gfp construct. Body-wall muscles of animals labelled with anti-DEB-1 MH24 antibody (red in A and C) or anti-DYC-1 antibodies (red in D and F). B and E show the muscle localisation of the ZYX-1-GFP protein (green, the GFP signal was amplified with an anti-GFP antibody). ZYX-1-GFP localises on dense bodies (arrowhead in B) and M-lines (arrow in B). C and F are merged images of A and B and of D and E respectively, with details of 2 dense bodies. Note in C that the DEB-1 signal co-localises with the ZYX-1-GFP signal on the dense body, and in F that the DYC-1 signal is located at the edges of the ZYX-1-GFP labelling. G: Localisation of ZYX-1-GFP in the nucleus (arrowhead) of a body wall muscle cell. H: Localisation of ZYX-1-GFP in neurons (arrowheads).

Figure 7: DYC-1S interacts with ZYX-1 through its highly conserved 19 aa region

A: Summary of results obtained with yeast two-hybrid assay. The different parts of DYC-1 and ZYX-1 proteins used in these assays are schematically indicated (top left). The table indicates the different plasmids used in these assays and the corresponding DYC-1 bait and ZYX-1 prey
proteins. Pictures show plates inoculated with diploid yeasts expressing the different DYC-1S bait and ZYX-1 prey proteins. Medium without Leu and Trp serves as a positive control showing the presence of bait and prey plasmids in the tested diploid yeasts. On medium without His, Leu and Trp, growth can occur only if the bait and the prey proteins interact. Note that negative control yeasts carrying either empty pACT2 or pAS2-1 plasmids do not grow on medium without His, Leu and Trp. Interactions occur between the bait proteins DYC-1S (aa 9-793) and DYC-1S (aa 9-258) and the prey proteins ZYX-1 (aa 68-603) and ZYX-1 (aa 384-603) (growth on medium without His, Leu and Trp). Note that no interaction is detected, neither for the bait protein DYC-1S (aa 9-258 Δaa 54-81) carrying a deletion of aa 54-81, nor for the prey protein ZYX-1 (aa 384-528) carrying only the two first LIM domains of the ZYX-1 protein (no growth on medium without His, Leu and Trp).

B: Western blotting of GST pull-down assays. HA-tagged ZYX-1 protein (aa 15-603) was detected using an anti-HA antibody after GST pull-down performed with the GST-tagged N-terminal end of DYC-1 protein (aa 9-258) but not with GST alone. The right lane shows ZYX-1-HA expression in the total COS-7 cell extract. A volume of 20μl was analysed on each lane.

C: Alignment of a DYC-1S conserved sequence. Ce: Caenorhabditis elegans, Dm: Drosophila, Ag: Anopheles, Rn: Rat, Hs: humans. Note that the region of 19 aa (aa 58-76 of the DYC-1S protein), which is strictly conserved from drosophila to humans (two conservative differences exist in C. elegans, red arrows).

Figure 8: Sub-cellular localisation of GFP fused to the highly conserved stretch of the DYC-1S protein
A: Dense body localisation of DEB-1 protein labelled with MH24 antibody (red). B: Localisation of GFP (green) fused to DYC-1S (aa 54-81) containing the highly conserved stretch of 19 aa. C: Merged image of A and B with the detail of 2 dense bodies. Note that the short sequence of the DYC-1S protein is sufficient to address GFP to the sarcomeric localisation.

Figure 9: Working model of dystrophin-DYC-1-dense body interactions

Dystrophin (in green) is known to interact with the Dystroglycan (DG)- Sarcoglycan (SG) complex (blue) by its C-terminal end. DYS-1/dystrophin interacts with DYB-1/dystrobrevin, which in turn binds STN-1/syntrophin. PAT-2/alpha-integrin and PAT-3/beta-integrin (yellow) anchors the dense body to the sarcolemma and the extra-cellular matrix. DYC-1 (purple) is located at the edges of the dense body and interacts via its conserved 19 aa stretch (small purple bead) with the third LIM domain of ZYX-1/zyxin (the three LIM domains of the Zyx-1 protein are represented as small red beads). ZYX-1 is also located in the nucleus of muscle cells where it might mediate signalling pathways. We posit that there is a direct or an indirect interaction between dystrophin and DYC-1, linking DYS-1/dystrophin to the dense body.
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<tr>
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<td>(body bends/min +/- s.e.m.)</td>
<td></td>
<td></td>
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Locomotion was scored as described in materials and methods. * : different from N2 (p<0.05).

Overbent means increased bending of the head and anterior part of the body during locomotion.

N: No, Y: Yes.
### Table 2. Muscle defects in *hlh-1; dyc-1* mutants

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<tr>
<td><em>dys-1(cx18); hlh-1(cc561)</em></td>
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<td>4.3 +/- 1.3 **</td>
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Muscle cells were observed after phalloidin staining of animals fixed 3 days after the L4-stage.

Mean +/- s.e.m. ***: different from N2 (p< 0.001).
A

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