A Drosophila model for Duchenne muscular dystrophy

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CHAPTER 2

Embryonic expression patterns of the *Drosophila* dystrophin-associated glycoprotein complex orthologs

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Abstract

Mutations in genes encoding proteins of the human Dystrophin-associated glycoprotein complex (DGC) cause the Duchenne, Becker and limb-girdle muscular dystrophies. Subsets of the DGC proteins form tissue-specific complexes which are thought to play structural and signaling roles in the muscle and at the neuromuscular junction. Furthermore, mutations in the *dystrophin* gene that lead to Duchenne muscular dystrophy are frequently associated with cognitive and behavioral deficits, suggesting a role for *dystrophin* in the nervous system. Despite significant progress over the past decade, many fundamental questions about the roles played by Dystrophin and the other DGC proteins in the muscle and peripheral and central nervous systems remain to be answered. Mammalian models of DGC gene function are complicated by the existence of fully or partially redundant genes whose functions can mask effects of the inactivation of a given DGC gene. The genome of the fruitfly *Drosophila melanogaster* encodes a single ortholog of the majority of the mammalian DGC protein subclasses, thus potentially simplifying their functional analysis. We report here the embryonic mRNA expression patterns of the individual DGC orthologs. We find that they are predominantly expressed in the nervous system and in muscle. d*ystrophin*, *dystrobrevin*-like, *dystroglycan*-like, *syntrophin*-like *1*, and all three *sarcoglycan* orthologs are found in the brain and the ventral nerve cord, while *dystrophin*, *dystrobrevin*-like, *dystroglycan*-like, *syntrophin*-like *2*, *sarcoglycan alpha* and *sarcoglycan delta* are expressed in distinct and sometimes overlapping domains of mesoderm-derived tissues, i.e. muscles of the body wall and around the gut.

1. Results and discussion

The discovery that mutations in *dystrophin* and other genes encoding Dystrophin-associated proteins cause muscular diseases such as Duchenne muscular dystrophy and limb-girdle muscular dystrophy, led to the intense study of what is now known to be a complex of Dystrophin-associated proteins, called the Dystrophin glycoprotein complex (DGC) (Matsumura and Campbell, 1994). The DGC is composed of five protein families, Dystrophin-related proteins, including Dystrophin, Utrophin and the Dystrobrevins, the Syntrophins, the Sarcoglycans, Sarcospan and Dystroglycan. Animal models of muscular dystrophies have been complicated by the existence of related genes within each class which partially or fully substitute for an experimentally deleted gene, e.g. *utrophin* can perform at least some of *dystrophin*'s roles (Grady *et al.*, 1997). Thus, the elucidation of the basic biology of the DGC

would benefit from the study of DGC genes in animal models having less genetic redundancy. One such powerful genetic model is the fruit fly, *Drosophila melanogaster* (St Johnston, 2002). The recent elucidation of the human and fly genomic sequences indicate a high degree of conservation between these evolutionary distant organisms; importantly, some 70% of genes implicated in human genetic disorders are also found in *Drosophila*. This conservation of sequence and in many cases, function, is translating into the increasingly active use of the fly as a model system for human diseases (Fortini and Bonini, 2000).

DGC protein family	Mammalian genes	*Drosophila* ortholog	Synonyms	mRNA expression domains
Dystrophin-related proteins	DRP2 dystrophin utrophin	Dys (CG7243)	DmDYS DmDLP dmDp186 DLP1 DLP2 DLP3 dystrophin-like protein 1 dystrophin-like protein 2 dystrophin-like protein 3 dystrophin-like protein 186	dmDLP1/2: 1, 2, 4, 7, 14, 15, 16, 18, 19, 20, 21, 23, 25, 26, 28 dmDp186: 1, 3, 11, 17, 25
	a-dystrobrevin b-dystrobrevin	Dyb (CG8529)	DmDYB dystrobrevin-like	1, 2, 3, 5, 9, 10, 11, 14, 16, 18, 25
Syntrophins	a 1 b 1 b 2	Syn1 (CG7152)	DmSYN-1 syntrophin-like 1	1, 3, 8, 11, 24, 25, 26
	g 1 g 2	Syn2 (CG4905)	DmSYN-2 syntrophin-like 2	1, 15, 16, 21, 23, 25, 29
Sarcoglycans	a 1	Scgalpha (CG7851)	DmSCG-a1 sarcoglycan a	2, 3, 11, 12, 16, 25, 26
	b	Scgbeta (CG5657)	DmSCG-b sarcoglycan b	3, 5, 11, 25
	g d	Scgdelta (CG14808)	DmSCG-gd sarcoglycan d	3, 6, 11, 13, 14, 17, 18, 25, 26
Dystroglycan	Dystroglycan	Dg (CG18250)	DmDG dgn dys dg dystroglycan-like	1, 2, 4, 9, 15, 16, 18, 21, 25, 26, 27, 28, 30
Sarcospan	Sarcospan	None known	N/A	N/A

Table 1: The *Drosophila* DGC orthologs: their relationships to their mammalian counterparts and mRNA expression domains. The Berkeley *Drosophila* Genome Project names for the *Drosophila* DGC orthologs (Greener and Roberts, 2000) are given in the third column. The embryonic expression domains are indicated in the fifth column; 1, maternally contributed mRNA; 2, epidermal expression; 3, central nervous system; 4, dorsal median cells (DM cells); 5, labral sensory system; 6, specific unidentified cells in the CNS (possibly larval neuroblasts); 7, mesectodermal cells; 8, stomatogastric nervous system; 9, PNS, the atonal and chordotonal organs; 10, VUMS (ventral unpaired neurons); 11, brain; 12, supraesophageal ganglion; 13, head mesoderm; 14, somatic musculature; 15, visceral mesoderm; 16, dorsal pharyngeal musculature; 17, unidentified dorsal cell, possibly of mesodermal origin; 18, muscle fibres; 19, muscle attachment sites; 20, epidermal cells in the segmental grooves; 21, pericardial cells (dorsal vessel); 22, anlage foregut; 23, anlage midgut; 24, anlage hindgut; 25, midgut; 26, hindgut; 27, malphigian tubules; 28, stomodeum; 29, pole cells; 30, tracheal pits.

We and others have begun to study the DGC in the fruit fly, which has only a single member of each of the major DGC subfamilies (Roberts and Bobrow, 1998; Greener and Roberts, 2000, Table 1), thus simplifying their functional analyses. The *Drosophila* ortholog of *dystrophin* is, like its human counterpart, one of the largest genes in the genome and encodes at least four isoforms, three of which are expressed during embryogenesis (Neuman *et al.*, 2001). The large embryonic isoforms, dmDLP1 and dmDLP2, are predominantly found in the gut and the mesoderm, while the small isoform, dmDp186, is present at high levels in the central nervous system. Interestingly, a small isoform of human Dystrophin, Dp71, is also found to be highly expressed in the brain (Rapaport *et al.*, 1992).

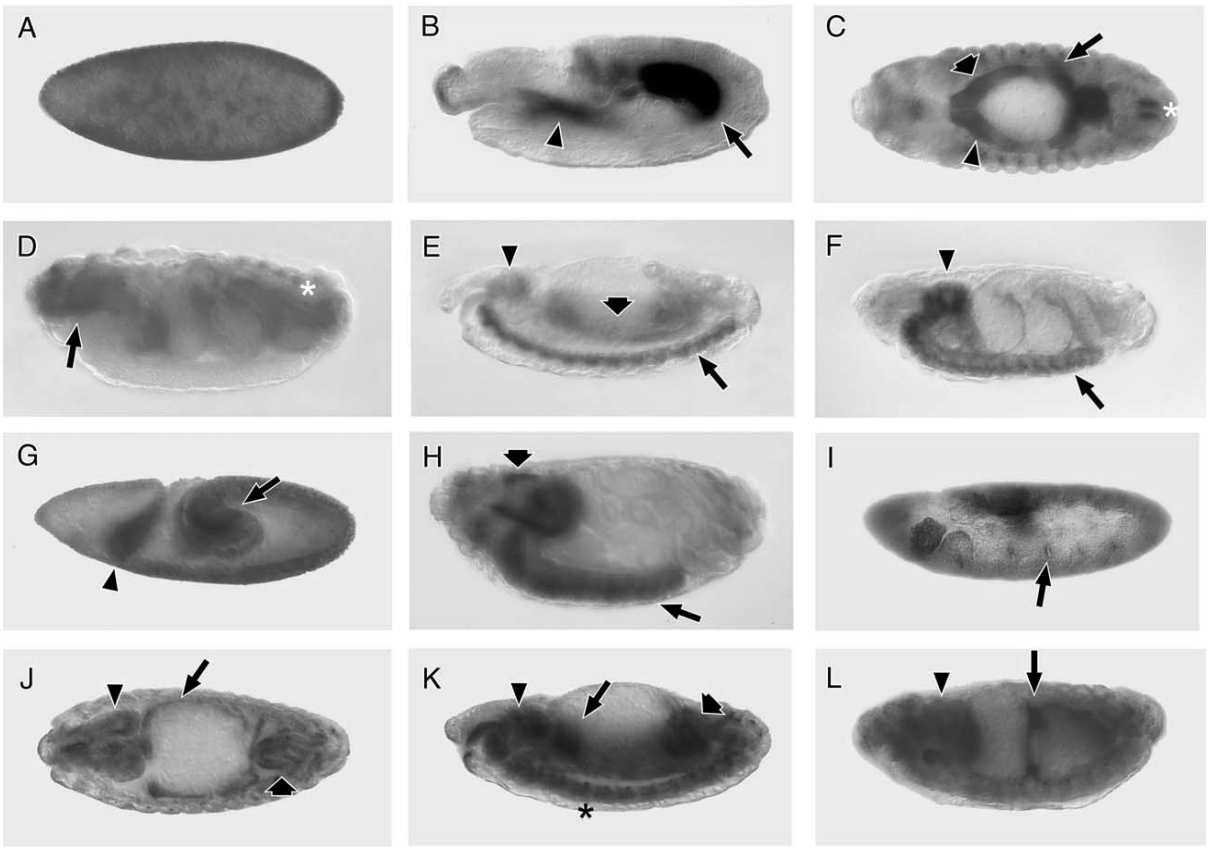

Figure 1: RNA expression patterns of *dystrophin*, *dystrobrevin*-like and *dystroglycan*-like during *Drosophila* embryogenesis. The RNA expression patterns of the *Drosophila* DGC orthologs *dystrophin* dmDLP1/dmDLP2 (**A**–**D**), *dystrophin* dmDp186 (**E** and **F**), *dystrobrevin*-like (**G** and **H**) and *dystroglycan*-like (**I**–**L**) are shown. (**A**) The large *dystrophin* isoforms dmDLP1/dmDLP2 are maternally expressed. (**B**) Later in development, at stage 11 they are expressed in the anterior (arrowhead) and posterior (arrow) midgut rudiments. (**C**) *dystrophin* dmDLP1/dmDLP2 expression is observed in the visceral mesoderm (broad arrowhead) and the anterior (arrowhead) and posterior (arrow) midgut rudiments and in the rectum of the hindgut (asterisk). (**D**) At the end of embryogenesis, expression of the large *dystrophin* isoforms is most predominant in the stomadeum and foregut (arrow) and the hindgut (asterisk). (**E** and **F**) The dmDp186 *dystrophin* isoform is expressed in the central nervous system (ventral cord (arrow) and brain (arrowhead)) and at a lower level in the midgut (broad arrowhead). (**G**) *dystrobrevin*-like is expressed during germband extension in the anterior (arrowhead) and posterior (arrow) midgut rudiments. (**H**) At a later stage *dystrobrevin*-like accumulates in the ventral cord (arrow), the brain and dorsal pharyngeal muscle (broad arrowhead). (**I**–**L**) *dystroglycan*-like RNA can be found in the tracheal pits (arrow, **I**), the brain (arrowhead, **J**–**L**), Malpighian tubules (broad arrowhead, **J** and **K**), in the visceral mesoderm (arrow, **J** and **K**), the ventral cord (asterisk, **K**) and in the midgut constrictions (arrow, **L**). Dorsal (panels (**C**, **J**) or lateral views (the remainder of the panels) of whole mount embryos of the following stages are shown: (**A**) stage 4, (**B**) stage 11, (**C**) stage 13, (**D**) stage 16, (**E**) stage 14, (**F**) stage 16, (**G**) stage 8, (**H**) stage 16, (**I**) stage 10, (**J**) stage 13, (**K**) stage 14 and (**L**) stage 15. Anterior is to the left.

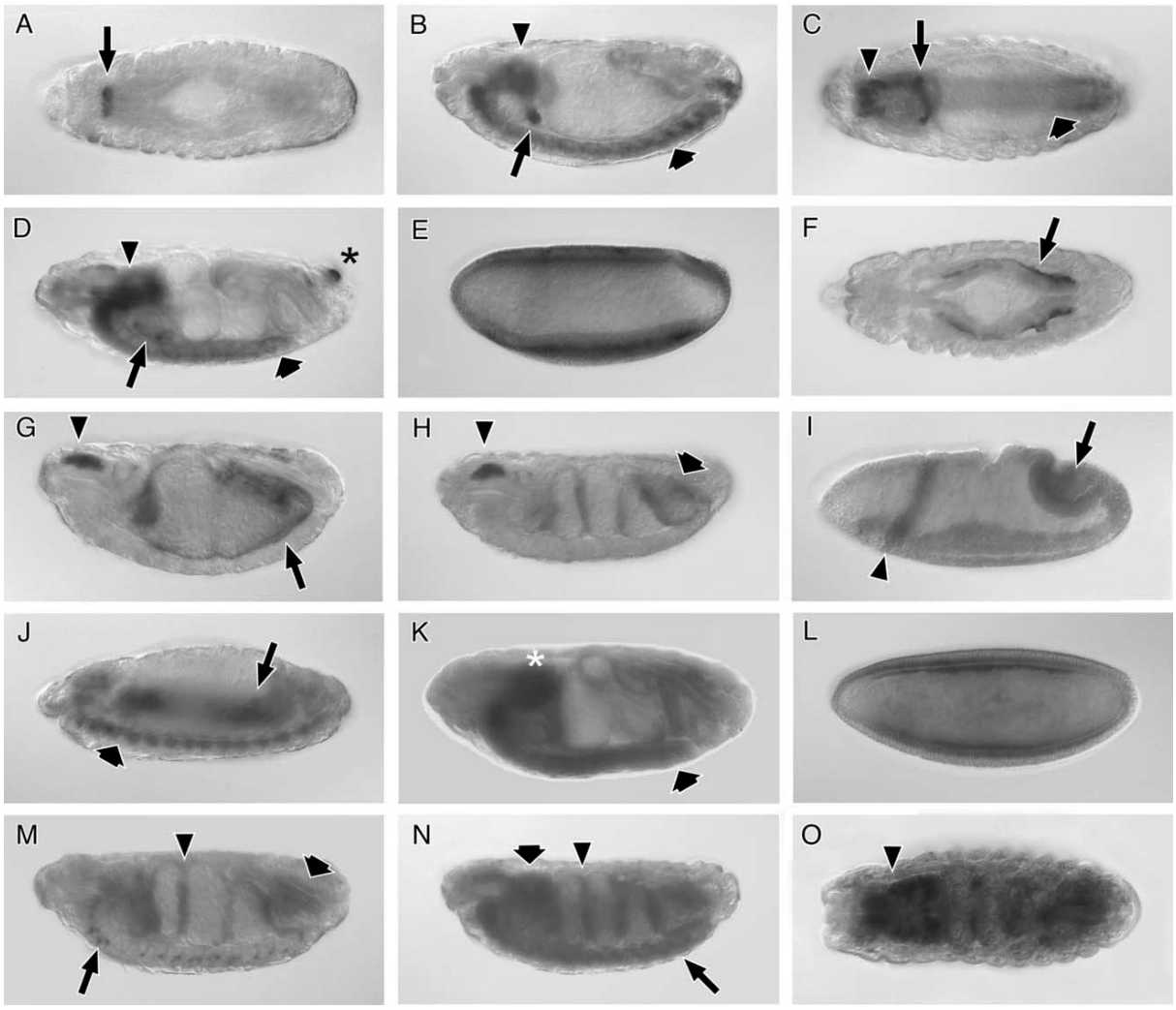

Figure 2: RNA expression patterns of *syn1, syn2, scgbeta, scgdelta* and *scgalpha* during *Drosophila* embryogenesis. The RNA expression patterns of the *Drosophila* DGC orthologs *syn1 (***A**–**D**), *syn2* (**E**–**H**), *scgbeta* (**I**–**K**), *scgdelta* (**L** and **M**) and *scgalpha* (**N** and **O**) are shown. (**A**–**D**) *syn1* is expressed in the stomatogastric nervous system (arrow), the ventral cord (broad arrow), brain (arrowhead) and the spiracles (asterisk). (**E**–**H**) *syn2* is expressed during early gastrulation (**E**) and in the visceral mesoderm (arrow), the dorsal pharyngeal muscle in the head (arrowhead) and a posterior compartment of the midgut (broad arrowhead) (**F**–**H**). (**I**–**K**) *scgbeta* is expressed during germband extension in the anterior (arrowhead) and posterior (arrow) midgut rudiments (**I**). At later stages (**J**, **K**) *scgbeta* is present in the midgut (arrow), ventral cord (broad arrowhead) and brain (asterisk). (**L** and **M**) *scgdelta* RNA is expressed in the cellular blastoderm (**L**) and later in a subset of ventral cord neurons (arrow), likely the larval neuroblasts, in the midgut constrictions (arrowhead) and the hindgut (broad arrowhead) (**M**). (**N** and **O**) *scgalpha* mRNA is present throughout the ventral cord (arrow), in the midgut constrictions (arrowhead) and in the dorsal pharyngeal muscle (broad arrowhead) (**N**). A dorsal view of the embryo shows *scgalpha* mRNA in the supraesophageal ganglia in the brain (arrowhead, **O**). Dorsal (**A**, **C**, **F** and **O**) or lateral (the remainder of the panels) views of whole mount embryos of the following stages are shown: (**A**) stage 13, (**B**) stage 14, (**C**) stage 14, (**D**) stage 16, (**E**) stage 7, (**F**) stage 13, (**G**) stage 15, (**H**) stage 16, (**I**) stage 8, (**J**) stage 14, (**K**) stage 16, (**L**) stage 5, (**M**) stage 16, (**N**) stage 15, (**O**) stage 15. Anterior is to the left.

Using RNA *in situ* hybridization on whole mount embryos, we have determined the spatial and temporal expression patterns of the other DGC orthologs during embryonic development. Probes were generated from sequence-verified expressed sequence tags (ESTs) and DGC gene expression patterns were examined throughout embryogenesis. The *Drosophila* DGC orthologs are predominantly expressed in two domains, the nervous system and the muscle (Figures 1–3 and Table 1).

Seven of the eight DGC orthologs are found at high levels in the ventral nerve cord and brain: the small *dystrophin* isoform dmDp186 (Figure 1E,F; also reported by Neuman *et al.*, 2001), *dystrobrevin*-like (Figure 1H), *dystroglycan*-like (Figure 1J,K), *syntrophin*-like 1 (*syn1*; Figure 2B,D), *sarcoglycan beta* (*scgbeta*; Figure 2K) *sarcoglycan delta* (*scgdelta*; Figure 2M) and *sarcoglycan alpha* (*scgalpha*; Figure 2N,O). A more detailed analysis of the DGC expression domains in the ventral nerve cord shows that *dystrobrevin* is also expressed at low levels by a cluster of neurons at the ventral midline whose location suggests that they are the ventral unpaired midline neurons (VUMS, data not shown), while *scgdelta* is present in superficial ventral cells throughout the ventral nerve cord (Figures 2M, 3E). Double labeling of embryos with *scgdelta* RNA and anti-Repo antibody which labels the lateral glia reveals that these cells are not of glial origin (Figure 3E). Instead, their number and location suggest that they are the larval neuroblasts, the precursors of the adult nervous system. At present, lineage-specific markers for larval neuroblasts are not available to confirm the identity of these cells. *dystroglycan*-like and *dystrobrevin*-like RNA can be detected in the cap cells that attach the atonal and chordotonal sensory organs of the peripheral nervous system to the body wall as revealed by a double labeling of embryos with *dystroglycan* RNA and the sensory neuronal marker antibody 22C10 (Figure 3C). *dystrobrevin*-like and *scgbeta* can also be detected in the labral sensory system (Figures 1H,2J), while *syn1* is highly expressed in the stomatogastric nervous system (Figure 2A–D). The large embryonic *dystrophin* isoforms dmDLP1 and dmDLP2 are expressed by the mesectodermal cells, which are derived from the presumptive ectoderm (Figure 3A). In later stages of embryonic development mesectodermal cells give rise to midline neuronal precursors and midline glial cells (Hartenstein, 1993).

The large embryonic *dystrophin* isoforms dmDLP1/dmDLP2 (Figure 3B), *dystrobrevin*-like (data not shown), *scgdelta* (Figure 3F) and *dystroglycan*-like (data not shown) are expressed in most body wall muscle fibres. Closer examination of the expression domains of the large embryonic *dystrophin* isoforms, during the stages at which the musculature is forming, indicates that they are expressed at very low levels throughout the muscle fibres, and accumulate at the muscle attachment sites (Figure 3B). *scgdelta* is seen at stage 14 at high levels in ventral muscle 13 and later throughout the musculature, including an unidentified dorsal triangular-shaped muscle (Figure 3F). *syntrophin*-like *2* (*syn2*) (Figures 2G,H), *dystrobrevin*-like (Figure 1H), *dystroglycan*-like (data not shown), the large *dystrophin* isoforms (data not shown) and *scgalpha* (Figure 2N) are expressed in the dorsal pharyngeal muscle, a large muscle in the head. *dystroglycan*-like (Figure 3D) and dmDLP1/dmDLP2 (data not shown) are also expressed in the dorsal median cells that are of mesodermal origin and lie dorsomedially on top of the ventral cord (Luer *et al.*, 1997). The dmDLP1 and dmDLP2 isoforms (Figure 1C), *dystroglycan*-like (Figure 1J) and *syn2* (Figure 2F) are expressed in the visceral mesoderm, the precursor of intestinal musculature. The large embryonic *dystrophin* isoforms, *syn2* and *dystroglycan*-like are expressed in the pericardial cells that constitute the mesoderm-derived dorsal vessel or embryonic heart (Table 1, data not shown). All DGC orthologs are detected in the midgut, although only some temporally overlap (Table 1). A summary of the domains of expression of the fly DGC genes during embryogenesis is presented in Table 1.

No studies detailing the embryonic functions of the *Drosophila* DGC orthologs have been published. However, a null allele of the *dystroglycan*-like gene has been generated and the resulting phenotype suggests a role for this protein in establishing cellular polarity in the developing oocyte and in imaginal disc epithelial cells (Deng *et al.*, 2003). We have shown here that *dystroglycan*-like and the seven other DGC orthologs in *Drosophila* are expressed in dynamic patterns throughout embryogenesis. Furthermore, partly or completely overlapping domains of expression are found for a subset of these genes in the ventral nerve cord and brain and in the somatic musculature of the body wall and in a number of smaller domains in nervous and mesoderm tissues and gut. These data suggest that, as in mammals, the *Drosophila* DGC members may form tissue-specific complexes. Further molecular and

genetic studies will provide a better understanding of the biological roles of the different members of the *Drosophila* DGC complex in the muscle and the nervous system.

2. Experimental procedures

2.1. Genetic stocks

0–24h old w^{1118} embryos were used throughout this study.

2.2. RNA *in situ* analysis and immunohistochemistry

RNA *in situ* hybridizations were performed at 52°C as described in Tautz and Pfeifle (1989). RNA and protein double labeling (Patel, 1994) and embryo staging (Wieschaus and Nusslein-Volhard, 1986) were performed as described previously. The anti-muscle myosin (Kiehart and Feghali, 1986, a gift from Corey Goodman), anti-22C10 (gift from Corey Goodman) and the anti-Repo antibodies (developed by Brad Jones, Beth Blankemeier and Corey Goodman (unpublished)) were used at a 1:5 dilution. Description of the expression patterns was performed using a standardized vocabulary (http://flybase.bio.indiana.edu/) and according to Hartenstein (1993).

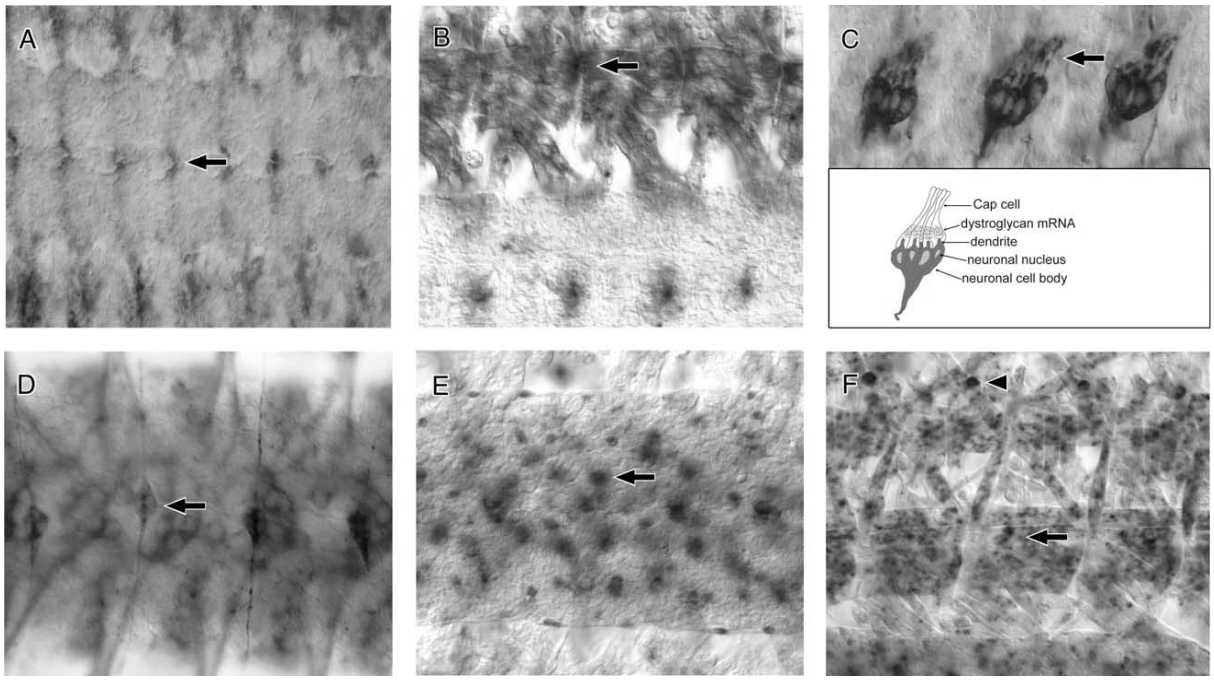

Figure 3: RNA expression of a subset of the *Drosophila* DGC orthologues in the embryonic mesoderm and derived musculature and in the nervous system. The RNA expression patterns of a subset of the *Drosophila* DGC orthologs expressed in the embryonic mesoderm and derived musculature and the nervous system are shown in dissected ventral cords and embryonic body walls: (**A**) The large *dystrophin* isoforms dmDLP1/dmDLP2 are detected in the mesectodermal cells underlying the ventral nerve cord (arrow). (**B**) At a later stage, the large *dystrophin* isoforms are expressed at the muscle attachment sites (arrow). The *dystrophin* RNA (blue) is shown relative to the muscle fibres that are labeled by the anti-muscle myosin antibody (brown). (**C**) *dystroglycan*-like RNA in blue is expressed in the accessory or cap cells (arrow) of the chordotonal sensory organs. A schematic drawing of a chordotonal organ (Campos-Ortega and Hartenstein, 1997) is shown, illustrating the relative location of the *dystroglycan*-like expression domain. *dystrobrevin*-like RNA is also expressed in the cap cells (data not shown). (**D**) *dystroglycan*-like is expressed in the dorsal-median cells (arrow). Neuronal cell bodies and axonal and dendritic processes in (**C** and **D**) are labeled by anti-22C10 (brown). (**E**) *scgdelta* RNA (blue, arrow) is expressed by subsets of ventral cord neurons, likely the larval neuroblasts, and is not present in the glial cells, here labeled by the anti-Repo antibody (brown). (F) *scgdelta* is expressed in most muscle fibres (arrow) and in a unidentified triangular-shaped cell, likely a muscle (arrowhead). The *scgdelta* mRNA expression is shown in blue, while the glial cells are labeled by the Repo antibody in brown. All panels represent dissected embryonic body walls with associated ventral cords: (**A**) stage 13, (**B**) stage 15, (**C**) stage 16, (**D**) stage 14, (**E**, **F**) stage 16. Anterior is to the left. See Appendix for color figure.

The RNA probes for *dystrophin*, *dystrobrevin*-like, *syn2* (also referred to as *g1/g2-syntrophin* (Greener and Roberts, 2000)), *scgbeta* and *scgdelta* were made from ESTs obtained from the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org). The *dystrophin* probe used is complementary to exons common to dmDLP1, dmDLP2 and dmDLP3 (Neuman *et al.*, 2001), the last reported as not being expressed during embryogenesis (Neuman *et al.*, 2001). *dystroglycan*-like and *scgalpha* probes were made from cDNAs isolated from embryonic cDNA libraries obtained from the Berkeley *Drosophila* Genome Project and the *dystrophin* dmDp186 isoform and *syn1* (also referred to as *a1/b1/b2-syntrophin* (Greener *et al.*, 2000)) probes were PCR-amplified from first-strand cDNA generated from 0 to 24 h embryonic RNA. All cDNAs were confirmed by sequence analysis.

The probe for the large *dystrophin* isoforms consists of basepairs (bps) 436–982 of the CG31175-PC mRNA (accession number **NM_169862**); for the CNS-specific *dystrophin* isoform, DLP186, bps 89–936 of the DmDP186 cDNA (**AF300294**); for *dystrobrevin*-like, bps 890–1626 of the Dystrobrevin-like protein DYB mRNA (**AF277387**); for *dystroglycan*-like, bps 400–1422 of the Dystroglycan-like protein DG mRNA (**AF277390**); for *syn1*, bps 115–1103 of the Syn1 mRNA (**AF277388**); for *syn2*, bps 137 –1287 of the Syn2 mRNA (**AF277389**); for *scgalpha*, bps 9–771 of the SCG-ALPHA/EPSILON mRNA (**AF277391**); for *scgbeta*, bps 1–1074 of the Scg-Beta mRNA (**AF277392**) plus 8 and 84 bp, respectively, of previously undescribed 30 and 50 UTR sequences; for *scgdelta*, bps 151–1319 of the SCG-Gamma/Delta mRNA (**AF277393**). The *syn2* and *scgalpha* probe sequences bear short inserts derived from genomic DNA intervening the previously described exons suggesting that they represent alternatively-spliced species.

Descriptions of the *dystrophin*, *scgbeta* and *dystroglycan*-like RNA expression domains in whole mount embryos are also available at the Gene Expression database of the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org). These descriptions include the predominant sites of expression described in this report.

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