Chapter IV

Regulation of *duf* Expression During *Drosophila* Adult Myogenesis

**Introduction**

The characteristics of the myotubes are largely, if not completely, determined by the combination of transcription factors expressed in particular founder myoblasts. In adult flies and many vertebrates, muscles consist of many myotubes bundled together to form a contractile unit. This adds another level of complexity to the specification of muscle properties in such systems. In this case, the properties of myotubes are specified and the number of myotubes contributing to the developing muscle is set as well. So does the formation of multi-fibre arrays in the adult follow the founder-feeder model of myotube patterning as seen in embryonic myogenesis? And if so, could they form basis for setting the characteristics such as fibre number, size and final shape of individual adult muscles? Is it different from the mechanisms operating during embryonic myogenesis? The formation of all these multi-fibre muscles that function together as a contractile unit in the adult fly has been examined from the development point of view to answer some of these questions (Fernandes *et al.*, 1991; Roy and VijayRaghavan, 1999; Dutta *et al.*, 2002, 2004).

**Organization of adult *Drosophila* muscles**

In the mesothorax of the adult, the most prominent muscles are the indirect flight muscles (IFMs), whose development has been charted in some detail (Fernandes *et al.*, 1991; Roy and VijayRaghavan, 1999). The IFMs consist of the dorsal longitudinal muscles (DLMs), an array of six large fibres, and three groups of dorso-ventral muscles: DVM-I (three fibres), DVM-II (two fibres) and DVM-III (two fibres). The
mesothorax contains another large muscle, namely the tergal depressor of the
trochanter (TDT) or the jump muscle, which consists of many fibres bundled together
as a unit. The dorsal thorax also contains the direct flight muscles (DFMs) that is
involved in changing the orientation of the wing and hence located closer to the wing
hinge. The muscles in the adult abdomen are arranged as well-defined sets of fibres,
which form dorsally, laterally and ventrally in each segment (Currie and Bate, 1991).
Organization of major muscles in a Drosophila adult is shown in Fig 4.1.

Development of Adult Drosophila Muscles

rP298, a P-lacZ insertion in duf gene has been a very useful marker to chart the
development of adult muscles in Drosophila (diagrammatical described in Fig 4.2). In
the developing pupal thorax, at 7 hours after puparium formation (APF), at each
epidermal location where a DVM fiber will develop, three, two and two duf-lacZ
expressing cells are seen, corresponding, respectively, to the three, two and two fibers
of the future DVM I, DVM II and DVM III muscles. These nuclei continue to express
duf-lacZ at high levels as fusion takes place to form the syncitial fibers. The fused adult
myoblast nuclei within the syncytia show low levels of duf-lacZ expression. The level
of β-galactosidase in all DVM nuclei begins to fall by 36 hours APF and disappears by
70 hours APF. This suggest that, just as in the embryo, the formation of myotubes in
the adult may be initiated by the selection of single founder myoblasts that are
identifiable by their expression of duf-lacZ (Dutta et al., 2004).

The development of the DLMs follows a slightly different scheme. Here, the
muscles assemble on a set of pre-existing templates provided by a small set of
persistent larval fibers, the three larval oblique muscles (LOMs). Subsequently, the
three templates split to form the six fibers of the DLMs. In this case, the larval fibers
serve a founder-like function in organizing the development of the DLMs, and the
adult myoblasts aggregate on the larval fibers and fuse with them to form syncitial
myotubes (Fernandes et al., 1991). duf-lacZ is expressed between 7hr APF to 36hr APF
in the larval templates - the founder analogues of the DLMs (Dutta et al., 2004).
The abdominal muscles develop from pools of muscle-forming cells that are associated with the nerves that innervate the larval muscle field (Bate et al., 1991; Currie and Bate, 1991). These cells in turn are derived from adult muscle progenitors (AP) that arise in the embryo as the siblings of embryonic muscle FCs (Ruiz-Gomez and Bate, 1997). For example, in each hemisegment, the pool of myoblasts that will generate several myotubes that make up the ventral abdominal muscle of the adult, are all derived from a single ventral AP cell in the embryo (Ruiz-Gomez and Bate, 1997; reviewed by Baylies et al., 1998). Unlike their embryonic founder cell siblings, the adult precursors maintain twist expression and proliferate during larval life to form pools of nerve-associated, twist-expressing myoblasts. The adult precursors do not express duf-lacZ in the embryo (Ruiz-Gomez et al., 2000). Very weak duf lacZ expression is seen during early pupal stages (13-14 hours APF) but, by 24 hours APF, however, there is a clear upregulation of duf-lacZ expression in particular nuclei and by 28 hours APF, these duf-lacZ-expressing cells (also can be stained with 22C10) are positioned at the sites where individual abdominal muscle fibres will now form. These continue to express high levels of duf-lacZ within the fully formed fibres. Expression begins to diminish by 70 hours APF. This suggests that Duf is important for myoblast fusion and fibre formation during adult abdominal myogenesis too (Dutta et al., 2004).

Regulation of Adult Muscle Development

Though much has been understood about the development of adult muscles there is much to be discovered about the mechanisms that regulate development of different adult muscles. Adult muscles in Drosophila use the same developmental principles as embryonic muscles; however, the signaling mechanism used to achieve this founder-based muscle development is different. A novel interplay of components of the Fibroblast growth factor (FGF) pathway mediates founder cell choice and results in a precise pattern of FCs for each multi-fibre array of adult abdominal muscles. Developmental patterns of Heartbroken/Dof and Sprouty define the domain
and timing of activation of the Fibroblast growth factor receptor Heartless (Htl) in specific myoblasts, thereby converting them into FCs (Dutta et al., 2005).

Much of the muscle diversity and fibre number arises out of small differences in signaling events early during development. Our knowledge is poor about the role of fusion proteins and their regulation during adult myogenesis. And we have an incomplete understanding of the mechanism that results in the regularity with which individual muscles are made of specific size, shape and mass. In this chapter, the expression pattern of different duf upstream enhancer lacZ constructs is analyzed during adult muscle development. The results give some insights into the mechanisms leading to the formation of different adult Drosophila muscles and also the developmental regulation of duf expression during adult myogenesis.

**Materials and Methods**

**Fly stocks:**

rP298 (duf) lacZ (Nose et al., 1998, Ruiz-Gomez et al.,2000) has a nuclear P[lacZ] insertion into the duf locus and faithfully reproduces the expression pattern of wildtype duf. duf Gal4 (Menon & Chia) was generated by delta 2-3 assisted P[Gal4] exchange with rP298lacZ into the duf locus and expresses similar to wildtype duf. duf 7.6kb Gal4 (Mar Ruiz-Gomez, pers comm) contains sequences from -8.2kb to -0.6kb from duf start site. UAS LacZ on II and UAS GFP on III from Bloomington Stock Centre were used. duf 1.0kb lacZ, duf 1.5kb lacZ, duf 2.4kb lacZ, duf 3.0kb lacZ, duf 3.8kb lacZ, duf 4.6kb lacZ, duf 5.1kb lacZ, duf 5.35kb lacZ and duf 5.5kb lacZ are duf enhancer reporter transgenic lines generated to study duf regulation during Drosophila myogenesis.

**Tissue preparation:**

Wandering third instar larvae were collected for larval dissections. The
anterior one-third of the larvae was cut and the inverted inside out. All other larval contents were dissected and removed leaving the imaginal discs, larval brain intact with the cuticle.

For pupal dissections, white prepupae, (0 hr after puparium formation or APF) were collected on moist filter paper in a Petri dish and grown at appropriate temperatures for required time intervals. The pupa was removed out of its pupal case and dissected ventrally under a drop of cold 1X PBS and fixed with 4% Paraformaldehyde for 30min. The pupal and larval tissues were prepared for immunohistochemistry as described in Fernandes et al., (1991).

Dissected samples were permeabilized by two washes in 1x PTx (1x PBS with 0.1% Triton X-100) 15 min each, followed by blocking with 4 washes in PBTx (1x PBTx with 0.1% BSA). Samples are labelled using required dilution of primary antibody in PBTx for more than 8 hours at 4°C with constant rocking. Unbound antibody is removed by 4 washes in PBTx and labelled with secondary antibodies for 2 hrs at room temperature. Unbound antibody is removed by 3-4 washes in PTx and were mounted in 70% glycerol and stored in dark at 4°C.

**Immuno-histochemistry:**

Antibodies against β-galactosidase raised in rabbit (Molecular Probes) or in mouse (The Developmental Studies Hybridoma Bank) were used at a dilution of 1:500 and 1:50 respectively. Mouse 22C10 (The Developmental Studies Hybridoma Bank) was used at a dilution of 1:75. Anti-Twist antibody (Siegfried Roth, University of Cologne, Cologne, Germany) was first pre-adsorbed at a dilution of 1:500 to late stage (15-16) embryos and then used. Anti GFP (Molecular Probes) was used at 1:500. Goat Anti mouse or anti rabbit secondary antibodies conjugated to Alexa Fluor dyes- Alexa 488 (Green) and Alexa 568 (red) from Molecular Probes, was used.
Image Acquisition and Analysis:

Fluorescent preparations were scanned using a 20X PlanApoChromat 0.75 NA, or 40X Plan ApoChromat 1.4 NA Objectives in Ziess LSM 510Meta (Carl Zeiss GmbH). Images were taken in sequential mode for individual channels with Kalman 3 averaging. Raw images were analyzed using Ziess Image Viewer (version 3,2,0,70 Carl Zeiss GmbH). 2D projections of 3D confocal slices were processed in Adobe Photoshop CS version 8.0.

Results

Expression of duf Upstream Reporters in Larval Imaginal Discs

The pools of myoblasts from which adult myotubes will form, arise from a small number of adult muscle precursor (AP) cells in the embryo. In late third instar larvae, duf-lacZ expression can be detected at low levels in all myoblasts of the wing disc that give rise to the DLMs and DVMs (Dutta et al., 2004). Functionally, the developing myoblasts on the wing imaginal discs are the precursors for both direct and indirect flight muscles in the adult thorax. So as the first step in understanding the regulatory dynamics of duf expression pattern during adult myogenesis, duf upstream activity was assayed in wing imaginal myoblasts, which serve as the precursors for adult thoracic muscles during metamorphosis. The purpose of looking at the expression pattern of different enhancer constructs in the wing imaginal discs was to find out if there are any enhancer elements specific for the weak and uniform expression seen in the adult (imaginal) myoblasts which gets upregulated in the FCs later during development.

Late third instar larval imaginal discs were dissected from different duf enhancer constructs and double labelled with antibodies against βGal and Twi. Twi marks the AP cells in the embryo and continues to express during early stages of adult myogenesis. Twi expression fades in FCs as duf expression gets upregulated (Dutta et
al., 2004). It serves as a good marker for all the adult myoblasts. Colocalization with Twi will reveal if any of the duf enhancer constructs are expressed in developing imaginal myoblasts.

rP298lacZ and duf Gal4 driven UAS lacZ were used as positive controls for studying duf expression in wing imaginal discs. The βGal expression in rP298lacZ is very weak in the wing imaginal myoblasts and is almost non-detectable (Fig 4.3, A-C) as reported by Dutta et al., 2004. However, the expression of duf Gal4 driven UAS lacZ is seen very clearly in the notum region. Weak expression is seen in the imaginal myoblasts but very strong expression is seen as patches in the notum, antero-posterior (A/P) boundary, and central hinge region as well as in the posterior margin of the wing blade (Fig 4.4, A-C). Confocal imaging at higher magnification with thinner optical slices reveals that these cells are located closer to the surface (over the imaginal myoblasts population) and hence very likely to be epidermal. These non-mesodermal are suspected to be the domains for epidermal sensory organ precursors. With this as the backdrop, I set out to test the expression pattern of different duf enhancer constructs. The expression pattern of different transgenic lines in wing imaginal discs has revealed some surprising results.

The smallest construct duf 1.0kb lacZ is expressed very weakly in the adult myoblasts but strong and fuzzy expression is also seen in the entire notum region, which is mostly epidermal. Also expression is seen in the posterior half of the wing blade and hinge region around the A/P boundary (Fig 4.3, E, F). This expression is not similar to duf expression as revealed by rP298 or duf Gal4. All the other constructs from duf 1.5kb lacZ to duf 5.3kb lacZ have very similar expression patterns (Figs 4.3 and 4.4). They are expressed very weakly in the imaginal myoblasts but diffusely in epidermal cells of the entire notum region. The expression is strong in patches of cells in the exact locations seen with duf Gal4 and more uniformly in the rest of the notum. Also expression is seen in the central wing hinge region and three patches in the wing blade reminiscent of duf Gal4. Most interestingly, a gradient of lacZ expression is seen on either side the A/P boundary of the wing blade. This gradient is common to all the
larger duf enhancer constructs analyzed but not seen with duf Gal4 or rP298lacZ. This gradient expression in the A/P boundary appears to be an output of Dpp signaling. In general, the expression seen with the different lacZ constructs are diffuse compared to duf Gal4 (Fig 4.4).

My results from the careful analysis of the duf Gal4 and all the duf enhancer lines indicate that the expression pattern in wing imaginal discs is found mostly in the epidermal cells. Mesodermal expression is very weak and uniform in all the imaginal myoblasts. There was also no significant difference in the expression level of reporters in precursors of different muscles of the thorax.

**duf Enhancer Expression in the Adult Indirect Flight Muscles**

Among the different muscles in *Drosophila* adult thorax, DLMs comprise the largest chunk of indirect flight muscles. DLMs develop from three persistent larval muscles that act as templates in each hemithorax. These templates act analogues to founders- organizing the development of the large DLMs from the swarms of adult myoblasts that aggregate about them as metamorphosis begins. *duf* expression in these cells serves to attract the adult myoblasts to the templates, with which they then fuse to form the six fibres of the adult DLMs. So I studied the expression pattern of all the *duf* enhancer constructs during DLM development. Dutta *et al.*, 2004 find that monoclonal antibody 22C10 also stains FCs in the abdomen. 22C10 is widely used to visualize neuronal morphology and axonal projections. 22C10 recognizes the microtubule-associated protein Futsch, required for establishing proper morphology of the neuron (Hummel *et al.*, 2000). Futsch is probably a component of the microtubule machinery in the adult myotubes too and its expression gets induced soon after the founder cell initiates the formation of the myotubes (Dutta *et al.*, 2004). So expression of different *duf* enhancer lines was checked by colocalization with m22C10, which marks the larval templates also very clearly (Figs 4.5 and 4.6). rP298 (*duf*) lacZ is used a positive control which is expressed in larval templates that has split to give rise to six DLM fibres in each hemithorax. The founder nuclei are larger.
and express the reporter more strongly than the other nuclei of adult FCMs that have come into the syncytium post fusion (Fig 4.5 A-C).

Weak expression of the duf 1.0kb lacZ reporter is seen in the developing DLMs at 24hrs APF (Fig 4.5, E, F). Weak and hazy expression is also seen in the epidermis and other tissues. The level of reporter expression in DLM founders is comparable to expression in other tissues. duf 1.5kb lacZ shows reporter expression in the developing DLMs and very interestingly also in the swarms of adult FCMs that aggregate about the templates at 24 hrs APF (H, I). The level of reporter expression is higher in DLM founders than adult myoblasts or epidermal cells. duf 2.4kb lacZ (Fig 4.5, K, L) shows weak expression in DLMs similar to duf 1.0kb lacZ. duf 3.0kb lacZ (Fig 4.5, N, O) reporter expression is seen in adult FCMs that swarm about the developing DLMs. Expression of duf 3.0 kb lacZ resembles that of duf 1.5 kb lacZ but comparatively the expression in DLM fibers is weak. duf 3.8kb lacZ also shows reporter expression in the developing DLMs and very interestingly also in the swarms of adult myoblasts that aggregate about the templates at 24 hrs APF (Fig 4.6, E, F). The level of reporter expression is marginally higher in DLM founders than adult myoblasts very similar to duf 1.5kb lacZ. duf 4.6kb lacZ is not expressed in developing DLMs (Fig 4.6, H, I) but duf 5.1kb lacZ again shows weak expression in the developing DLMs at 24hrs APF (Fig 4.6, K, L) and also in the epidermis similar to duf 2.4kb lacZ. duf 5.5kb lacZ (Fig 4.6, N, O) shows weak expression in DLMs similar to duf 3.8kb lacZ.

My analysis of the duf enhancer in the developing DLMs indicate that there are elements very close to the start site in duf enhancer that is mesoderm specific and is capable of turning on weak expression in developing DLMs, but both FC- analogs and FCMs. But repressor elements located probably further upstream restrict the expression of duf specifically to adult muscle founders alone. None of the enhancer lines assayed so far have shown very strong founder specific expression of reporter in the developing DLMs. Most of these transgenic lines have shown very interesting expression pattern during embryonic myogenesis. One suggestion is that the elements regulating duf expression in the DLMs are located in regions other than the ones tested.
here. The expression pattern during early development of DVMs, DFMs and TDT at the time of founder selection was not carried out owing the lack of good markers to identify them other than rP298lacZ. duf Gal4 has a strong expression in the epidermis and hence not very useful as a marker to study these muscles.

**duf Enhancer Expression During Development of Dorsal Abdominal Muscles**

The expression pattern of duf lacZ reporters in the dorsal and lateral muscles of the abdomen was studied during different stages of development. 24hrs APF and 30hrs APF represent key stages in the development of abdominal muscles. By 24hrs APF individual myoblasts are chosen as founders for both dorsal and lateral abdominal muscle fibers. By 30hrs APF these individual founders attach to tendon cells and initiate the expression of structural genes to develop into properly patterned muscle fibers (Fig 4.2). Dutta *et al.*, (2004) find that monoclonal antibody 22C10 also stains FCs in the abdomen. Till 20hrs APF, 22C10 only stains the motor neuron, as the founders have not been chosen. By 24hrs APF, single duf-lacZ expressing founders appear and correspondingly the nascent myotubes, seeded by the founders, get labelled by 22C10. Expression persists in the muscles till later stages. Thus 22C10 serves as a robust marker for FCs in the abdomen (Fig 4.7).

At 24hrs APF, *duf* 1.0kb lacZ, *duf* 1.5kb lacZ, *duf* 2.4kb lacZ, *duf* 3.0kb lacZ, *duf* 3.8kb lacZ and *duf* 4.6kb lacZ show weak reporter expression in dorsal abdominal founders as well as the neighbouring epidermal tissues (Fig 4.7). The expression pattern is not clearly restricted to founders alone due to which the pattern is not distinguishable from the background at this stage. *duf* 5.5kb lacZ expression is seen clearly in founders of dorsal abdominal muscles and weak expression is also seen in epidermis. But the expression in dorsal abdominal founders is only slightly higher than the epidermis. This suggests that enhancers for *duf* expression in dorsal abdominal founders reside upstream of 5kb.

Slightly later during development at 30hrs APF, all the *duf* enhancer lacZ lines assayed show only weak reporter expression in the developing dorsal abdominal
muscles (Fig 4.8). The expression pattern is not clearly restricted to founders alone due to which the pattern is not distinguishable from the background i.e. epidermis at this stage. This is also true for duf 5.5kb lacZ, which expressed weakly in founders of dorsal abdominal muscles at 24hrs APF (Fig 4.8). At 30hrs APF there is no expression in dorsal abdominal muscles suggesting that 5kb fragment upstream of duf does not have necessary cis-regulatory elements for duf expression in dorsal abdominal founders. The weak expression duf 5.5kb lacZ at 24hrs APF could be due to the presence of some weak elements that initiated the expression but not sufficient to maintain it. Cis-regulatory elements probably located further upstream might strengthen this weak transcriptional signal more specifically in dorsal abdominal founders.

**duf Enhancer Expression During Development of Lateral Abdominal Muscles**

Similarly, reporter expression was assayed in developing lateral abdominal muscles in 24hrs APF and 30hrs APF pupae. At 24hrs APF, duf 1.0kb lacZ, duf 1.5kb lacZ, duf 2.4kb lacZ, duf 3.0kb lacZ and duf 3.8kb lacZ show weak reporter expression in lateral abdominal founders as well as the neighbouring epidermis (Fig 4.9). The expression pattern is not distinguishable from the background at this stage. However, duf 4.6kb lacZ and duf 5.1kb lacZ expression in lateral abdominal founders is slightly higher than the epidermis Expression in duf 5.5kb lacZ is again diffuse and not restricted to founders (Fig 4.9). This suggests that enhancers for duf expression in lateral abdominal founders reside between 3.8kb to 5.1kb upstream of duf.

At 30hrs APF, duf 1.0kb lacZ, duf 1.5kb lacZ, duf 2.4kb lacZ and duf 3.0kb lacZ show almost no reporter expression in lateral abdominal muscles (Fig 4.10). duf 3.8kb lacZ is expressed weakly in lateral abdominal muscles as well as the neighbouring epidermis. The expression pattern is barely distinguishable from the background. duf 4.6kb lacZ also shows expression in the lateral abdominal muscles but this expression is weak compared to duf 5.1kb lacZ, which is clearly expressed in lateral abdominal muscles. There is clear colocalization with 22C10 (Fig 4.10). Expression in duf 5.5kb
lacZ is again diffuse and not restricted to founders, very similar to smaller constructs (Fig 4.10). This suggests that enhancers for \textit{duf} expression in lateral abdominal founders reside in the vicinity to 5.1kb upstream of \textit{duf}.

\textbf{\textit{duf} 7.6kb Gal4 Drives Founder-Specific Expression During Adult Myogenesis}

Reporter expression pattern of the larger \textit{duf} 7.6kb Gal4 was analysed by driving UAS lacZ during adult myogenesis. Expression pattern was analyzed by standard immunohistochemistry on 24hrs and 30hrs APF dissected pupal preparations. Expression pattern in all the thoracic and abdominal muscles were studied. Interestingly, in the developing adult thorax, \textit{duf} 7.6kb Gal4 drives weak expression in the developing DLM templates, but strong expression is seen in the developing DVM founders. Expression in the DVM II is shown in Fig 4.11, B. Founder-specific expression of \textit{duf} 7.6kb Gal4 is also seen in the developing dorsal abdominal muscles (Fig 4.11, E), lateral abdominal muscles (Fig 4.11, H) and also the ventral abdominal muscles (Fig 4.11, K). No reporter expression is detected in adult FCM population. Also the non-mesodermal expression of this enhancer fragment is very weak. Some amount of randomness is also observed in the expression pattern between adjacent fibers of the same muscles. This is attributed to the defect in the vector used for cloning this fragment. The transcriptional activity of this fragment might be amplified due to Gal4-UAS system compared to lacZ reporter constructs used in this analysis.

Among all the enhancer deletion constructs analyzed during adult myogenesis, \textit{duf} 7.6kb Gal4 has shown founder specific expression in adult muscles. Specifically it is expressed in DVMs of the thorax and marks the founder cells of all the muscles in the adult abdomen. Expression in DLM founder analogs is comparatively weak. This suggests that some more \textit{cis}-regulatory elements are required for recapitulating the entire expression pattern of wildtype \textit{duf}. This fragment also does not contain 600 bases immediately upstream of the start site that might be important for complete expression. However, all the elements necessary for \textit{duf} expression in dorsal, ventral
and lateral muscle founders of the abdomen and DVMs of the thorax reside in this region of the *duf* enhancer, while elements for DLM expression might be located further upstream.

**Discussion**

**New Role for *duf* in Epidermal Differentiation**

*duf* expression and function has not been studied carefully in contexts other than myoblast fusion in *Drosophila* embryo and recently in the adult myogenesis. Most of these studies have exploited rP298 (*duf*) lacZ reporter to track *duf* expression or more importantly, the founder cell population.

On the other hand, *duf* Gal4 expression in the wing imaginal disc has revealed new expression domain for *duf*. Expression is seen in specific patches in the notum region, wing hinge region and the posterior wing blade region. *duf* Gal4 (Menon & Chia) was generated by delta 2-3 assisted P[Gal4] exchange with rP298 lacZ into the *duf* locus and expresses similar to wildtype *duf*. Given that the *duf* Gal4 faithfully reproduces expression pattern of wildtype *duf* expression during myogenesis, the epidermal expression in imaginal discs may not be an artefact. In support of this, strong *duf* enhancer reporter expression is also seen in domains where *duf* Gal4 is expressed (Figs 4.3 and 4.4). This opens up possible new roles for *duf* in the epidermal differentiation. This is especially plausible considering the molecular nature of Duf protein. Duf is a member of trans-membrane IgG proteins that are known to be involved in cell-recognition, cell adhesion and cytoskeleton reorganization. In addition to its role in muscle development, *duf* is also expressed in different tissues like the CNS and the developing eye- suggesting multiple functions in those tissues. Its homologs/ orthologs in *C. elegans* and vertebrates have very diverse function such as synaptic formation and axon guidance and kidney glomeruli formation in mouse (Shen and Bargmann, 2003; Shen 2004; Tamura *et al.*, 2005). So important epidermal
differentiation events such as sensory organ development, synapse formation and axonal path finding are some of the new areas in which role of duf should be more carefully studied.

**Transcriptional Response to Patterning Signals During Adult Myogenesis**

Analysis of duf enhancer deletion constructs during adult myogenesis has revealed some interesting results. duf enhancer lacZ lines show weak expression in the developing adult muscle precursors but expression in epidermis is significantly higher than in the mesoderm. Gradient expression in the A/P boundary of wing imaginal disc resembles of spalt (sal) expression in response to Dpp signalling (Nellen et al., 1996) and is seen in all duf enhancer constructs larger than 1.5kb. Only the shortest enhancer (1.0kb) does not show this gradient expression. This expression could be a result of presence of Dpp responsive elements such as Mad binding sites in the duf enhancer. I find two Mad binding sites in duf enhancer, one lies just -0.6kb and another -2.8kb from the start site.

Weak lacZ expression is seen in all the small constructs till 3.8kb both in dorsal as well as lateral abdominal muscles; however, epidermal expression of the reporter seems higher than in the mesoderm in these enhancer deletion lines. Larger constructs (3.8-5.1kb upstream of duf) show expression in lateral abdominal muscles. None of the constructs up to 5.5kb are expressed in dorsal abdominal muscles. This shows that region around 5kb upstream of duf has a weak module for expression in lateral muscles. duf 7.6kb Gal4 shows founder specific expression in adult muscles. It is specifically expressed in FCs of DVMs, dorsal, lateral and ventral abdominal muscles. This suggests that all the cis-regulatory elements necessary for duf expression in founders for all abdominal muscles and DVMs reside in this region of the duf enhancer. As the 5.5 kb lacZ does not show any founder specific expression in any of the adult muscles, enhancer modules for adult specific expression could therefore be narrowed down to a region between -5.5kb to -8.2kb upstream of duf.
Analysis of the sequence between -5.5kb to -8.2kb upstream of duf indicates that it may be responsive to Notch and Wg pathway effectors. There are three Su(H) binding sites, four dTCF binding sites and one Brk binding site. Individual binding sites are also seen for Tin and Mef2. There are several possible ways in which these factors can interact with each other and bring about activation or repression of reporter expression in specific cells. However, proper functional analysis of these predictions is required.

Functional Significance of duf Enhancer Expression

In late third instar larvae, duf-lacZ expression can be detected at low levels in all myoblasts of the wing disc that give rise to the DLMs and DVMs (Dutta et al., 2004). It is suspected that this initially uniform expression at a low level may reflect the origins of the adult myoblasts from lineages that generate muscle FCs in the embryo. However, uniform expression of rP298 lacZ in a population of myoblasts has no apparent functional significance. Therefore, the uniform pattern of expression is replaced by local up-regulation in a few cells that will act as founders and down-regulation in other myoblasts (FCMs), which respond to the localized Duf signal.

The expression of duf 1.5 and duf 3.8kb lacZ is similar to what could be seen with rst, which is expressed both in FCs and FCMs. As duf is a very close relative of rst both in structure and function, it could be possible that the enhancers of these two genes also share similarities. duf might have evolved from rst to perform more specialized function as myoblast attractant in FCs. Alternatively, studies by Dutta et al., (2004, 2005) on the mechanism of founder selection suggest that founders originate from the homogenous population of adult myoblasts. Intercellular signaling is involved in imparting the “founder” fate to a subset of myoblasts from within the larger domain of equivalent cells. Absence of such repressive mechanisms in the deleted enhancers could be resulting in the expression of reporter in both FCs and FCMs. Notch mediated lateral inhibition, a mechanism adopted for selection of embryonic muscle founders, is not functioning in the choice of adult founders.
Consequently, I find that duf enhancer fragments that show individual non-overlapping modular expression for different muscles of the embryo are inactive during adult myogenesis.

Signaling via Heartless (Htl), a mesoderm-specific Drosophila homologue of FGFR (reviewed in Wilson and Leptin, 2000) is the key pathway for selection of adult founders in the abdomen. Htl receptor is present in all myoblasts during pupal development, whereas Heartbroken (Hbr), a cytoplasmic protein specific for FGFR signaling (Vincent et al., 1998; Michelson et al., 1998a) is initially present in all adult myoblasts but narrows down to only the FCs. Over-expression of Htl or Hbr in all myoblasts leads to excess fibres in the abdomen, suggesting that restriction of Hbr to a few cells during normal development is critical for the specification of founders cells (Dutta et al., 2005). Htl/Hbr signalling is effected in the nucleus by Pnt P1, which activates transcription of target genes by binding to Ets sites. Interestingly, there are seven Ets binding sites in -3.8kb and another one in -5kb region (Fig 2.3), but none of them seem to be sufficient for expression in adult muscles. Enhancer region between -5.5kb to -8.2kb upstream of duf that shows founder specific expression in adult muscles does not contain a single Ets binding site (Fig 2.3).
Figures
**Figure 4.1. *Drosophila* adult muscles develop from precursor cells**

A. Schematic representation of *Drosophila* larval muscles and the arrangement of adult muscle precursors. The three thoracic segments and the pattern of abdominal segments 2-7 are shown. All the adult muscle precursors (highlighted in turquoise) in the thorax are clustered around the imaginal discs (shown in orange) but 6 adult muscle precursors are associated with neurons (black lines) in every abdominal segment. Larval muscles are shown in light green and peripheral sense organs are shown as white ellipsoid bodies. B. Lateral aspect of thoracic muscles of *Drosophila* adult seen in a sagittal section. Six fibers of dorsal longitudinal muscles (DLMs, highlighted in purple) are the largest of the indirect flight muscles (IFMs) in the thorax. C. DLMs labelled with m22C10 (red) of a 30hr After Puparium Formation (APF) wildtype pupal preparation shows 6 fibers of DLMs along with the innervating motor neurons. D. Lateral aspect of abdominal muscles of *Drosophila* adult seen in a sagittal section. Arrays of dorsal abdominal muscles are highlighted in green and lateral abdominal muscles in red. E. A 42hr APF wildtype pupa labelled with mouse 22C10 (red) shows rows of dorsal and lateral abdominal muscles. Anterior is to the top, dotted line is the dorsal midline. Scale bar: 50 µm.

Abbreviations: pd= prothoracic disc, wd= wing disc, hd= haltere disc, ld= leg disc, ISN=Intersegmental neuron.
Figure 4.2. Development of *Drosophila* adult muscles during pupal metamorphosis

A. Schematic representation of *Drosophila* larval muscle pattern (in green). The imaginal discs (yellow) with imaginal myoblasts (magenta) and nerve-associated myoblasts (orange) in the abdomen can also be seen. B. Schematic of a 12hr APF pupa showing the degradation of thoracic larval muscles except the three larval muscles in the second thoracic segment that serves as template for the DLMs. At the same time correct number of FCs (golden yellow) are chosen for every DVM fiber. The adult myoblasts (magenta) are swarming over the FCs and the templates. C. Schematic of an 18hr APF pupa in which the three larval templates have split to give rise to 6 DLM fibers each. Fusion process is going on in both DLMs as well as DVMs. At the same time, the abdominal muscles of larval origin have mostly degraded and the nerve-associated myoblasts are intact. D. Schematic of a 24hr APF pupa showing the fully separated DLMs, which are aligned longitudinally and the DVMs aligned dorso-ventrally in the thorax. In the abdomen, FCs are chosen for dorsal muscles (in orange) and lateral muscles (in blue) from the nerve associated myoblast pool. FCMs (in green) are attracted to these founders. E. Schematic of a 28hr APF pupa in which most of the thoracic muscles have achieved their final pattern and are producing structural proteins. In the abdomen, precise number of single fibers could be seen for dorsal and lateral muscles. FCMs are fusing with different founders. F. Schematic of a 36hr APF pupa in which the thoracic muscles are increasing their muscle mass and the abdominal muscles have also achieved their final pattern.
Figure 4.3. Expression pattern of *duf* upstream reporter lacZ constructs in wing imaginal discs

Confocal projections of late third instar larval wing imaginal discs double labelled with antibodies against Twi (red) and β galactosidase (green). Twi marks all the adult myoblast population (A, D, G and J) and β galactosidase shows the expression of different *duf* enhancer lacZ reporter constructs (B, E, H and K).

rP298 (*duf*) nuclear lacZ shows very weak expression in the adult myoblast pool (B, C). *duf* 1.0kb lacZ shows strong expression in the wing blade and notum region where adult myoblasts reside (E). Colocalization with Twi (F) reveals that the expression in the notum is diffuse and not restricted to myoblasts but also seen in the epidermis. Also expression is seen in the posterior half of the wing blade and hinge region around the antero-posterior (A/P) boundary. *duf* 1.5kb lacZ shows expression in the entire notum region, weakly in the adult myoblast pool (H, I). A gradient of lacZ expression is seen on either side the A/P boundary of the wing blade. Expression is also seen in the central wing hinge region and three patches in the wing blade. This expression is mostly epidermal and very weakly in the imaginal myoblasts. *duf* 3.0kb lacZ (K, L) also shows reporter expression very similar to *duf* 1.5 kb lacZ. Anterior is to the right, dorsal to the top. Scale bar: 50 μm.
Figure 4.4. Expression pattern of larger *duf* upstream reporter lacZ constructs in wing imaginal discs

Confocal projections of late third instar larval wing imaginal discs double labelled with antibodies against Twi (red) and β galactosidase (green). Twi marks all the adult myoblast population (A, D, G and J) and β galactosidase shows the expression of different *duf* enhancer lacZ reporter constructs (B, E, H and K).

*duf* Gal4 driven UAS lacZ (B, C) is seen very clearly in the notum region, weakly in the imaginal myoblasts. Strong expression is seen as patches in the notum, anterior/ posterior (A/P) boundary, and central hinge region as well as in the posterior margin of the wing blade. These cells are located closer to the surface (over the imaginal myoblasts population) and hence very likely to be epidermal and are suspected to be epidermal sensory organ precursor domains.

*duf* 3.8kb lacZ shows expression in the entire notum region, weakly in the adult myoblast pool (E, F). Expression in the central wing hinge region and three patches in the wing blade resemble *duf* Gal4 expression. A gradient of lacZ expression is seen on either side the A/P boundary of the wing blade, which is not seen with *duf* Gal4. *duf* 4.6kb lacZ (H, I) and *duf* 5.3kb lacZ (K, L) also shows reporter expression very similar to *duf* 3.8kb lacZ.

Anterior is to the right, dorsal to the top. Scale bar: 50 µm.
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**Figure 4.5. Expression of *duf* enhancers during indirect muscle development**

Confocal projections of 24hr After Puparium Formation (APF) pupal preparations of different *duf* enhancer lacZ lines double labelled with m22C10 (red) and anti β galactosidase antibodies (green) to show expression of reporters in a bundle of DLMs (6 fibers) along with the innervating motor neurons of a hemithorax.

rP298 (*duf*) lacZ is expressed in larval templates that have split to give rise to six DLM fibres in each hemithorax. The founder nuclei are larger and express the reporter more strongly than the other nuclei of adult FCMs that have come into the syncytium post fusion. In this panel, jump muscle (TDT) as well as three fibres of the DVM I are also seen (B, C). Weak expression of the *duf* 1.0kb lacZ reporter is seen in the developing DLMs at 24hrs APF (E, F). Weak and hazy expression is also seen in the epidermis and other tissues. The level of reporter expression in DLM founders is comparable to other tissues. *duf* 1.5kb lacZ shows reporter expression in the developing DLMs and very interestingly also in the swarms of adult myoblasts that aggregate about the templates at 24 hrs APF (H, I). The level of reporter expression is marginally higher in DLM founders than adult myoblasts. *duf* 2.4kb lacZ (K, L) shows weak expression in DLMs similar to *duf* 1.0kb lacZ. *duf* 3.0kb lacZ (N, O) reporter expression is seen in swarming adult FCMs and resembles that of *duf* 1.5 kb lacZ but the expression in DLM fibers is weak.

Anterior is to the top, dorsal midline to the right. Scale bar: 50 µm.
Figure 4.6. Expression of larger *duf* enhancers during indirect muscle development

Confocal projections of 24hr After Puparium Formation (APF) pupal preparations of different *duf* enhancer lacZ lines double labelled with m22C10 (red) and anti β galactosidase antibodies (green) to show expression of reporters in a bundle of DLMs (6 fibers) along with the innervating motor neurons of a hemithorax.

*rP298 (duf)* lacZ is used as positive control is expressed in six DLM fibres in each hemithorax. The founder nuclei are larger and express the reporter more strongly than the other nuclei of adult FCMs that have come into the syncytium post fusion (B, C). *duf* 3.8kb lacZ shows reporter expression in the developing DLMs and very interestingly also in the swarms of adult myoblasts that aggregate about the templates at 24 hrs APF (E, F). The level of reporter expression is marginally higher in DLM founders than adult myoblasts similar to *duf* 1.5kb lacZ. *duf* 4.6kb lacZ is not expressed in developing DLMs (H, I). Weak expression of the *duf* 5.1kb lacZ reporter is seen in the developing DLMs at 24hrs APF (K, L) and also in the epidermis. The level of reporter expression in DLM founders is comparable to other tissues. *duf* 5.5kb lacZ (N, O) shows weak expression in DLMs similar to *duf* 3.8kb lacZ.

Anterior is to the top, dorsal midline to the right. Scale bar: 50 µm.
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Figure 4.7. Expression of *duf*-enhancer lacZ in dorsal abdominal muscle founders

Confocal projections of 24hr APF pupal abdominal hemisegment of different *duf* enhancer lacZ lines double labelled with anti-β-galactosidase (green) and m22C10 (red). m22C10 marks all adult FCs and neurons, lacZ expression is driven by the length of *duf* enhancer indicated on the right panel. *duf* Gal4 > UAS-lacZ, used as positive control shows single rows of FCs expressing *duf*-lacZ are present in the region where the future dorsal abdominal muscles will form. These FCs are also labelled by m22C10.

*duf* 1.0kb lacZ, *duf* 1.5kb lacZ, *duf* 2.4kb lacZ, *duf* 3.0kb lacZ, *duf* 3.8kb lacZ and *duf* 4.6kb lacZ show weak reporter expression in dorsal abdominal founders as well as the neighbouring epidermal tissue. The expression is not clearly restricted to founders alone due to which the pattern is not distinguishable from the background at this stage. *duf* 5.5kb lacZ expression seen clearly in founders of dorsal abdominal muscles and weak expression is also seen in epidermis. But the expression in dorsal abdominal founders is only slightly higher than the epidermis. This suggests that enhancers for *duf* expression in dorsal abdominal founders reside upstream of 5kb.

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30hr APF Dorsal Abdominal Muscles

duf lacZ
duf 1.0kb lacZ
duf 1.5kb lacZ
duf 2.4kb lacZ
duf 3.0kb lacZ
duf 3.8kb lacZ
duf 4.6kb lacZ
duf 5.1kb lacZ
duf 5.5kb lacZ
Figure 4.8. Expression of *duf*-enhancer *lacZ* in developing dorsal abdominal muscles

Confocal projections of 30hr APF pupal abdominal hemisegment of different *duf* enhancer *lacZ* lines double labelled with anti-β-galactosidase (green) and m22C10 (red). m22C10 marks all adult FCs and neurons, *lacZ* expression is driven by the length of *duf* enhancer indicated on the right panel. *duf* Gal4 > UAS-*lacZ*, used as positive control shows single rows of dorsal abdominal muscles expressing *duf-lacZ* that are also labelled by m22C10.

All the *duf* enhancer *lacZ* lines assayed show only weak reporter expression in the developing dorsal abdominal muscles. The expression is not clearly restricted to founders alone due to which the pattern is not distinguishable from the background (epidermis). This is also true for *duf* 5.5kb *lacZ*, which expressed weakly in founders of dorsal abdominal muscles at 24hr APF. At 30hr APF there is no expression in founders suggesting that 5kb fragment upstream of *duf* ha some elements to turn on weak expression in dorsal abdominal founders but does not have sufficient *cis*-regulatory elements to maintain *duf* expression during myoblast fusion in dorsal abdominal muscles. Anterior is to the top, dorsal midline to the right and scale bar: 50 µm.
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**Fig 4.9 Expression of duf-enhancer lacZ in lateral abdominal muscle founders.**

Confocal projections of 24hr APF pupal abdominal hemisegment of different duf enhancer lacZ lines double labelled with anti-β-galactosidase (green) and m22C10 (red). m22C10 marks all adult FCs and neurons, lacZ expression is driven by the length of duf enhancer indicated on the right panel. duf Gal4 > UAS-lacZ, used as positive control shows single rows of FCs expressing duf-lacZ are present in the region where the future lateral abdominal muscles will form. These FCs are also labelled by m22C10.

duf 1.0kb lacZ, duf 1.5kb lacZ, duf 2.4kb lacZ, duf 3.0kb lacZ and duf 3.8kb lacZ show weak reporter expression in lateral abdominal founders as well as the neighbouring tissues such as epidermis. The expression pattern in these constructs is not distinguishable from the background at this stage. duf 4.6kb lacZ and duf 5.1kb lacZ shows expression in founders of lateral abdominal muscles and weakly in epidermis. But the expression in lateral abdominal founders is only slightly higher than the epidermis. Expression in duf 5.5kb lacZ is again diffuse and not restricted to founders. This suggests that enhancers for weak duf expression in lateral abdominal founders reside between 3.8kb to 5.1kb upstream of duf.

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**30hr APF Lateral Abdominal Muscles**
Fig 4.10. Expression of duf-enhancer lacZ in developing lateral abdominal muscles

Confocal projections of 30hr APF pupal abdominal hemisegment of different duf enhancer lacZ lines double labelled with anti-β-galactosidase (green) and m22C10 (red). m22C10 marks all adult FCs and neurons, lacZ expression is driven by the length of duf enhancer indicated on the right panel. duf Gal4 > UAS-lacZ, used as positive control shows single rows of lateral abdominal muscles expressing duf-lacZ that are also labelled by m22C10.

duf 1.0kb lacZ, duf 1.5kb lacZ, duf 2.4kb lacZ and duf 3.0kb lacZ show almost no reporter expression in lateral abdominal muscles. duf 3.8kb lacZ is expressed weakly in lateral abdominal muscles as well as the neighbouring epidermis. The expression pattern is barely distinguishable from the background. duf 4.6kb lacZ also shows expression in the lateral abdominal muscles but this expression is weak compared to duf 5.1kb lacZ that is clearly expressed in lateral abdominal muscles. Expression in duf 5.5kb lacZ is again diffuse and not restricted to founders. This suggests that enhancers for duf expression in lateral abdominal founders reside in the vicinity to 5.1kb upstream of duf.

Anterior is to the right, dorsal midline to the top and scale bar: 50 µm.
Figure 4.11. Expression of *duf* 7.6 Gal4 during adult myogenesis

Confocal projections of a 28 hr APF *duf* 7.6kb Gal4 > UAS lacZ pupa double labelled with anti-β-galactosidase (green) and m22C10 (red). m22C10 marks all adult FCs and neurons (A, D, G and J), lacZ expression is driven by -8.2 to -0.6kb fragment of *duf* enhancer (B, E, H and K). *duf* 7.6kb Gal4 drives weak expression in DLMs but strongly in DVMs. Strong staining is seen only in the DVM II (in B). In the developing abdominal muscles, founder-specific expression is seen dorsal abdominal muscles (E), lateral abdominal muscles (H) and ventral abdominal muscles (K). Expression is not detected in adult FCM population. Non-mesodermal expression of this enhancer fragment is very weak. So all the cis-regulatory elements necessary for *duf* expression in abdominal founders and DVMs reside in this enhancer fragment. Anterior is to the top and dorsal midline to the right in A-F. Anterior to the right and dorsal midline to the top in G-L. Scale bar: 50 microns.