The localized assembly of extracellular matrix integrin ligands requires cell-cell contact

Maria D. Martin-Bermudo and Nicholas H. Brown*

Wellcome/CRC Institute and Department of Anatomy, Cambridge University, Tennis Court Road, Cambridge CB2 1QR, UK
*Author for correspondence (e-mail: nb117@mole.bio.cam.ac.uk)

SUMMARY

The assembly of an organism requires the interaction between different layers of cells, in many cases via an extracellular matrix. In the developing *Drosophila* larva, muscles attach in an integrin-dependent manner to the epidermis, via a specialized extracellular matrix called tendon matrix. Tiggrin, a tendon matrix integrin ligand, is primarily synthesized by cells distant to the muscle attachment sites, yet it accumulates specifically at these sites. Previous work has shown that the PS integrins are not required for tiggrin localization, suggesting that there is redundancy among tiggrin receptors. We have examined this by testing whether the PS2 integrin can recruit tiggrin to ectopic locations within the *Drosophila* embryo. We found that neither the wild type nor modified forms of the PS2 integrin, which have higher affinity for tiggrin, can recruit tiggrin to new cellular contexts. Next, we genetically manipulated the fate of the muscles and the epidermal muscle attachment cells, which demonstrated that muscles have the primary role in recruiting tiggrin to the tendon matrix and that cell-cell contact is necessary for this recruitment. Thus we propose that the inherent polarity of the muscle cells leads to a molecular specialization of their ends, and interactions between the ends produces an integrin-independent tiggrin receptor. Thus, interaction between cells generates an extracellular environment capable of nucleating extracellular matrix assembly.

Key words: Extracellular matrix, Adhesion, *Drosophila*, Integrin

INTRODUCTION

When an organism is assembled during embryogenesis, the different cell layers must be appropriately connected to each other. These layers are separated by an extracellular matrix (ECM), which is a collection of secreted proteins that accumulate in the intercellular spaces (Yurchenko et al., 1994). Thus, the connection between different cell layers is often mediated not by direct cell-cell adhesion via transmembrane proteins on each cell, but instead by cell adhesion to the ECM. Many of the interactions between the cell surface and the ECM are mediated by the integrin family of cell surface receptors (Cheresh and Mecham, 1994). Changes in the composition of the ECM, particularly in the integrin ligands, will therefore substantially alter the type of cell-ECM-cell interactions that can be made. Many different forms of ECM exist, with diverse compositions, shapes and functions, including basement membranes and tendons. Therefore, the control of the assembly of extracellular matrices is an essential part of morphogenesis, yet we know little about how this is achieved.

Two processes contribute to the formation of basement membranes and fibronectin fibrils: the self assembly of matrix components, and the nucleation of assembly by cell surface matrix receptors (for reviews, see Schwarzbauer and Sechler, 1999; Timpl and Brown, 1996; Yurchenco and O'Rear, 1994). Two of the major components of basement membranes, laminins and collagen IV, can self-assemble into irregular networks. These networks are connected by nidogen, which also binds to other components such as the proteoglycan perlecan. Both integrins and another type of laminin receptor, dystroglycan, have roles in basement membrane assembly. Knocking out the mouse *dystroglycan* gene has shown that it is essential for the assembly of basement membranes (Henry and Campbell, 1998). Loss of dystroglycan does not alter the synthesis of basement membrane components such as laminin $\alpha 1$, collagen IV and perlecan, but they fail to assemble into a discrete basement membrane. The role of dystroglycan may be simply to increase the local concentration of laminin to a level high enough for self assembly to be nucleated. Alternatively, dystroglycan could play a more active role, by altering the conformation or arrangement of laminin molecules. This latter hypothesis fits with the proposed role of the other type of ECM receptor, integrins, in fibronectin fibrillogenesis: they induce a conformational change in fibronectin so that it is capable of self assembly into fibrils (Schwarzbauer and Sechler, 1999).

The role of integrins in basement membrane assembly is more modest. This has also been tested by analysing the consequences of inactivating the genes encoding integrins. Different sets of integrins are disrupted by knocking out the genes for the different α or β subunits of integrin heterodimers. In general, loss of β subunits will disrupt a larger group of integrins. Mice lacking the integrin $\alpha 3$ subunit still form basement membranes, which are less well organized and more fragile (DiPersio et al., 1997). Teratomas derived from cells

lacking the integrin β1 subunit are also still able to form basement membranes, but they are disorganized (Sasaki et al., 1998). In *Drosophila*, embryos lacking the PS integrins (equivalent to the β1 family) assemble a basement membrane, but the link between it and hemiadherens junctions is lost (Prokop et al., 1998). Therefore, dystroglycan plays an essential role by initiating the assembly of basement membranes, and integrins contribute to basement membrane formation, perhaps by recruiting additional ECM molecules or helping to order the exisiting components.

The role of integrins in organizing fibronectin-containing matrices in vivo is less clear. Experiments in cell culture showed that the fibronection receptor integrin α5β1 is required for fibronectin assembly; however, fibroblasts derived from mice lacking the α5 integrin subunit are still able to assemble a fibronectin matrix, suggesting that other integrin(s) or non-integrin receptors can also assemble fibronectin (for a review, see Brakebush et al., 1997). Inhibition of β1 integrins with antibodies blocked the formation of fibronectin fibrils on the blastocoel roof of the gastrulating amphibian embryo (Darribere et al., 1990). Therefore it seems that integrins do have an important role in the formation of fibronectin containing matrices, but there is some redundancy between integrins, and possibly with other non-integrin receptors.

Examination of ECM assembly in the context of the whole organism has revealed that the cells that synthesize ECM components can be some distance from the site where the matrix assembles. Analysis of collagen IV in *C. elegans* has shown that only a fraction of cells within the worm synthesize collagen IV subunits, which are secreted and accumulate within a subset of the basement membranes (Graham et al., 1997). An even more dramatic example is the comparison between the sites of synthesis and accumulation of an integrin ligand, tiggrin, in *Drosophila* (Fogerty et al., 1994). Tiggrin is synthesized primarily in the fat body and hemocytes, but ends up very tightly localized to the tendon matrix at the muscle attachment sites, forming a pattern of sharp stripes (e.g. Fig. 2C) that has given the protein its name. The cells that synthesize tiggrin are not close to the muscle attachment sites, and therefore tiggrin appears to be secreted into the the extracellular fluid, where is freely diffuses until it is captured at the muscle attachment sites. When the site of synthesis is changed from the fat body and hemocytes to the cells of the nervous system, tiggrin still accumulates normally in the tendon matrix (Bunch et al., 1998). This demonstrates that the site of synthesis does not play a role in determining the site of matrix assembly. This paper focuses on an examination of the important factors in the specification of the site of tiggrin accumulation.

Tiggrin is a novel ECM protein containing unique N- and C-terminal domains, linked by a region of 16 contiguous repeats, each 73-77 amino acids long (Fogerty et al., 1994). Tiggrin is a ligand for the PS2 integrin (αPS2βPS), and within the C-terminal ligand-binding domain is an RGD sequence that is essential for integrin binding (Bunch et al., 1998; Fogerty et al., 1994). The formation of the larval muscle attachment sites during *Drosophila* embryogenesis is a good model for studying the process of matrix assembly in a developing organism. At these sites, the somatic muscles and specific epidermal muscle attachment cells, called tendon cells, are linked by a specialized extracellular matrix called the tendon matrix, which contains tiggrin and other ECM proteins. Both the tendon cells and the muscles express integrins, the PS1 (αPS1βPS) and PS2 (αPS2βPS) integrins, respectively, which are essential for the maintenance of muscle attachment (for a review, see Brown et al., 2000). The initial steps in muscle attachment, where the ends of the muscles recognize and form direct cell-cell contacts with the appropriate tendon cells, occurs normally in the absence of integrins (e.g. Prokop et al., 1998). This demonstrates that rather than having a role in the initial formation of the attachments, integrins are required to strengthen them so that they can withstand the force of muscle contraction.

Within the muscles the PS2 integrin is tightly localized at the ends of the muscles, where they attach to the tendon matrix. The cytoplasmic domain of the βPS subunit confers this pattern of localization on a heterologous protein, demonstrating that intracellular signals are sufficient for integrin localization (Martin-Bermudo and Brown, 1996). Therefore the PS2 integrin is an obvious candidate for a cell surface receptor responsible for the capture of its ligand tiggrin at the muscle attachment site. However, tiggrin still accumulates at the muscle attachment sites in the absence of zygotic expression of all integrins containing the βPS subunit (Fogerty et al., 1994), including PS2, suggesting that there is redundancy among tiggrin receptors. The completion of the *Drosophila* genome sequence (Adams et al., 2000) has confirmed that the only other integrin β subunit is the βν subunit, which is not expressed at the muscle attachment sites (Yee and Hynes, 1993). This suggests either that the small amount of βPS protein deposited in the egg is sufficient for tiggrin localization, or that the other tiggrin receptors are not integrins.

Tiggrin localization is crucial for the function of the tendon matrix. The absence of tiggrin causes lethality and one type of muscle attachment site is weakened (Bunch et al., 1998). Two kinds of muscle attachment sites are formed in the developing larvae (Prokop et al., 1998). At direct muscle attachment sites, single muscles attach to tendon cells and maintain a close adhesive contact with them, with little tendon matrix accumulating between them. At indirect muscle attachment sites, multiple muscles initially contact the same tendon cells. With time a substantial quantity of tendon matrix, including tiggrin, accumulates between the ends of the muscle and the tendon cells. By experimental manipulation, it has been possible to separate this attachment into two components: muscle attachment to the tendon matrix, and tendon cell attachment to the tendon matrix. In the absence of the specification of the majority of tendon cells, muscle attachment to the matrix is maintained as a muscle-tendon matrix-muscle attachment (Martin-Bermudo and Brown, 1996). Mutations in the loci *rhea* disrupt tendon cell attachment to the matrix, without disrupting the muscle-tendon matrix-muscle attachment (Prout et al., 1997). Tiggrin is particularly required for muscle attachment to the tendon matrix, but not for epidermal attachment to the matrix or direct muscle attachments (Bunch et al., 1998).

The aim of the work presented here is to examine the mechanism by which tiggrin is localized to the muscle attachment site. The simplest model, that tiggrin is localized by binding to its PS2 integrin receptor, we confirm can be ruled out. Therefore we have attempted to answer two questions that arise from this observation. First, does the PS2 integrin have

any role in the localization of tiggrin; i.e. is it capable of recruiting tiggrin even if it is not itself necessary? Second, if PS2 is not essential for the localization of tiggrin, what are the characteristics of the putative essential tiggrin receptor? By addressing these questions we have gained insight into the general mechanisms of localized ECM assembly in developing organisms.

MATERIALS AND METHODS

The following alleles were used in these experiments (for references see Flybase; Gelbart et al., 1997): if^{B4}, tig^X, $rhea^1$, twi^{1096}, mbc^1, $Df(2R)enR$, mef^{22-21}. To ectopically express the PS2 integrin we used *UAS-βPS* line 2 (Martin-Bermudo and Brown, 1996), combined with either *UAS-αPS2c/g* line 2A, *UAS-αPS2C* line 2A, or *UAS-αPS2Δcyt* line 2 (Martin-Bermudo et al., 1997, 1998). The following GAL4 drivers were used: *69B* for epidermis (Brand and Perrimon, 1993), a combination of several GAL4 lines that drive expression in the pioneer neurons, described in the text as 'nervous system' (a gift from A. Hidalgo), and *kruppel-GAL4* for the amnioserosa (Castelli-Gair et al., 1994). All of these lines also express in the salivary glands. We expressed *UAS-SrcGFP* (Kaltschmidt et al., 2000) in the muscle using a combination of *twist-GAL4* and *24B* (see Martin-Bermudo et al., 1997). The GFP fluorescence is maintained through standard fixation, although the embryos need to be rapidly transferred from methanol to PBS, which permits labelling of the embryos with antibodies. UAS-CD2/βPS (CCβ) is described in Martin-Bermudo and Brown (1996) and was detected with OX-34 anti-CD2 monoclonal (Serotec). PS integrin expression was detected with either anti-βPS monoclonal antibody CF6G11, or anti-αPS2 monoclonal antibody PS2hc/2, as described in Martin-Bermudo et al. (1997). Tiggrin was detected with a mouse anti-tiggrin antiserum (Fogerty et al., 1994), the *stripe* enhancer trap with an anti-β-galactosidase antiserum (Amersham), and muscle myosin with the monoclonal antibody Fmm5 (a gift of D. Kiehart). Images were collected with a Spot digital camera on either a Leica DMR or Zeiss Axioplan microscope, or by confocal microscopy with a BioRad MRC 1024 system on a Nikon microscope. Images were assembled with Photoshop and labelled with FreeHand on an Apple Power Macintosh.

RESULTS

First we tested whether tiggrin is localized in the complete absence of βPS-containing integrins. We examined tiggrin expression in embryos lacking both the maternal and zygotic contribution of the βPS subunit, generated by making germ line clones of a null allele of βPS. Tiggrin is still found localized at the ends of the detaching muscles (Fig. 1). This confirms that integrins are not necessary for tiggrin localisation, and so we went on to test whether they are sufficient.

The PS2 integrin is not able to recruit its ligand tiggrin to novel locations within the embryo

The PS2 integrin is expressed primarily in the embryonic mesoderm of the developing embryo, so to investigate whether it is able to recruit tiggrin to novel sites we used the GAL4 system (Brand and Perrimon, 1993). We used different GAL4 drivers to express both subunits of the PS2 heterodimer in different tissues, and examined the expression of PS2 and tiggrin with appropriate antibodies. Although the PS2 integrin heterodimer is succesfully expressed in ectopic locations, e.g.

the epidermis (Fig. 2B), the localization of tiggrin did not change (compare Fig. 2A and C). This was also found when the PS2 integrin was expressed in either the nervous system, the salivary glands or the amnioserosa (data not shown). We also expressed PS2 heterodimers that just contain the alternatively spliced form of αPS2 that binds tiggrin with higher affinity (Fogerty et al., 1994), and again found no change in the localization of tiggrin (data not shown).

It is possible that the ectopic tissues lack intracellular factors necessary for 'inside-out' activation of the PS2 integrin (Ginsberg et al., 1992), and that this explains the lack of tiggrin recruitment. The extracellular ligand-binding activity of integrins can be activated from inside the cell, and in some cases, including PS2, this can be mimicked by a deletion of the cytoplasmic tail (Martin-Bermudo et al., 1998). Therefore we expressed a heterodimer of βPS and αPS2Δcyt in each of the ectopic locations, but still failed to recruit tiggrin to the nervous system (Fig. 2E,F), epidermis, salivary glands or amnioserosa (data not shown).

These results demonstrate that the PS2 integrin alone is not able to localize tiggrin within the developing embryo, suggesting that there is at least one other tiggrin receptor expressed at muscle attachment sites that contributes to the localization of tiggrin. To characterize which cells at the attachment site express this hypothetical receptor we manipulated the fate of the cells on each side of the muscle attachment site: the muscles and the tendon cells.

Is tiggrin localization specified by the muscles or the epidermis?

As tiggrin is localized at the point of contact between the epidermis and the muscles, either cell layer could specify the localization of tiggrin by the expression of a receptor for tiggrin or another extracellular protein that leads to the localization of tiggrin. The transcription factor Stripe is specifically expressed in tendon cells (Frommer et al., 1996; Lee et al., 1995), and Stripe could in turn activate expression of the putative tiggrin receptor. Stripe expression occurs normally even in the absence of all mesoderm, so we wished to test whether tiggrin could be localized by an epidermal receptor in the absence of muscles. However, if the mesoderm is genetically eliminated the cells that synthesize tiggrin are also eliminated, thus preventing the examination of its localization. Therefore we used embryos mutant for *myoblast city (mbc)*, which disrupts the fusion of the myoblasts into large multinucleate muscles. In this mutant, single cell muscles still form and in some cases can attach normally (Rushton et al., 1995). Fig. 3 shows that embryos mutant for *mbc* still specify their tendon cells normally, as revealed by the expression of *stripe-lacZ* (Fig. 3B), but that tiggrin localization is severely disrupted (Fig. 3D,F). This indicates that the tendon cells are not able to localize tiggrin in the absence of the muscles. If we look more carefully at the *mbc* mutant embryos, we see that small amounts of tiggrin can be detected on the surface of the unfused muscle cells, where it is concentrated at points of contact between muscle cells (arrows in Fig. 3D), or between muscle and tendon cells (arrow in Fig. 3F). This experiment strongly suggests that the putative tiggrin receptor is expressed by the muscles.

To test whether the muscles can localize tiggrin in the absence of tendon cells we altered the specification of the tendon cells. Since the majority of tendon cells are in the
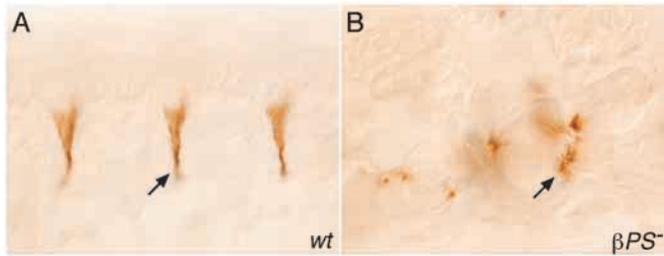
Fig. 1. Tiggrin localization in the complete absence of PS integrins. Tiggrin localization (arrows) in a wild-type embryo (A) and an embryo lacking both the zygotic and maternal contribution of the βPS subunit (B), using the null allele *mys^XG43*. In this and all subsequent figures, anterior is to the left.

posterior compartment of the segment, we disrupted tendon cell specification by eliminating the posterior compartment with a deletion of the genes *engrailed* and *invected*. In wild-type embryos, the muscles that attach to these posterior compartment tendon cells also attach to each other, end to end. In the absence of these tendon cells, many muscles retain their end-to-end attachment, particularly the band of ventral longitudinal muscles (Martin-Bermudo and Brown, 1996). Substantial amounts of tiggrin localize to these abnormal muscle-muscle attachment sites that lack a connection to the epidermis (Fig. 4B). As the PS2 integrin colocalizes with tiggrin in these mutants we also examined tiggrin in embryos lacking both PS2 and *engrailed* plus *invected* to test whether localization requires PS2 integrin in the absence of tendon cells. We found that tiggrin is still localized at sites of muscle-muscle contact (Fig. 4C; tiggrin appears as dots rather than the normal lines of expression along the ends of the rectangular muscles, due to the muscle detachment present in the PS2 mutant embryo).

As was the case in the *mbc* mutant embryos, we found that tiggrin is localized at sites of cell-cell contact, and not around the entire surface of the muscle nor at the ends of all muscles.

In particular some of the muscles in these mutant embryos have abnormal free ends, which lack tiggrin accumulation (arrows in Fig. 4B,C). To test whether it is the polarity of the muscle cell or tiggrin localization in particular that requires cell-cell contact, we examined a different marker of muscle polarity, the chimeric fusion protein CD2/βPS. We have previously shown that this protein, which contains the extracellular and transmembrane domain of the rat transmembrane protein CD2 and the intracellular domain of the βPS integrin subunit, is localized to the ends of the muscles, independently of endogenous PS integrin function (Martin-Bermudo and Brown, 1996). We also showed that the PS2 integrin is still localized to the ends of muscles that are forming attachments with other muscles in the absence of epidermal attachment cells, which we achieved with the mutation that removes both *engrailed* and *invected*. When the chimeric CD2/βPS protein is expressed in the muscles of embryos mutant for both PS2 and *engrailed* plus *invected*, it is found at the ends of all the muscles, whether they are in contact with another muscle or not (Fig. 4D). Combining this result with the others in Fig. 4 shows that in the absence of many of the tendon cells, the muscles still become polarized to make two specialized ends, but only once the ends make contact with a tendon cell or a muscle is tiggrin localized.

The *Drosophila* orthologue of the transcription factor myocyte enhancing factor-2 (MEF2) has been shown to be important for the differentiation of the somatic muscles (Lilly et al., 1995; Lin et al., 1996). We examined embryos mutant for MEF2 to see what role MEF2 has in specifying the ends of the muscles as distinct domains and the localization of tiggrin. We found that both the PS2 integrin and tiggrin are expressed in the mutant embryos and, unexpectedly, that the two proteins no longer colocalize (Fig. 5). In MEF2 mutant embryos, multinucleate muscles still form to some degree but do not attach properly and the PS2 integrin is found all around the periphery of the muscles. In contrast, tiggrin is found localized to discrete sites of muscle-muscle contact. This experiment clearly uncouples tiggrin localization from PS2

Fig. 2. Expression of the PS2 integrin is not sufficient to cause localization of tiggrin to ectopic locations. In the wild-type embryo the PS integrins are specifically expressed at the muscle attachment sites (A,D; as detected with an anti-βPS antibody). Widespread expression of the PS2 integrin in the epidermis (B) does not alter the pattern of localization of the PS2 integrin ligand tiggrin (C), a component of the tendon matrix (detected with an anti-tiggrin antibody). (B,C) Lateral views of embryos in which the GAL4

driver *69B* drives expression of *UAS-αPS2c/g* and *UAS-βPS*. Similarly, expression in the nervous system of an activated form of the PS integrin (E), lacking the cytoplasmic tail of the αPS2 subunit, does not alter tiggrin expression (F). (D-F) Ventral views of embryos with anti-βPS staining in D and E and anti-tiggrin staining in F, where multiple GAL4 lines were used to drive central nervous system expression of *UAS-αPS2Δcyt* and *UAS-βPS*.
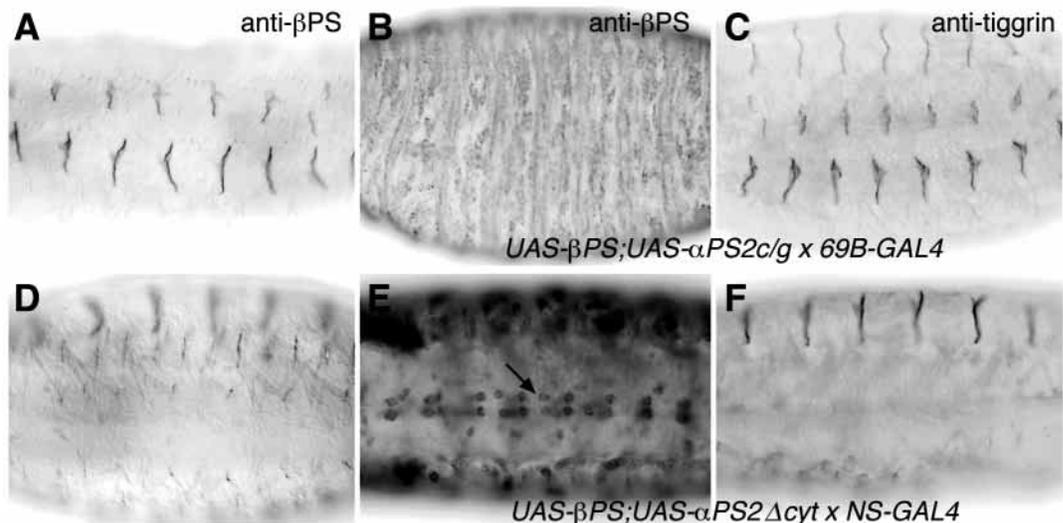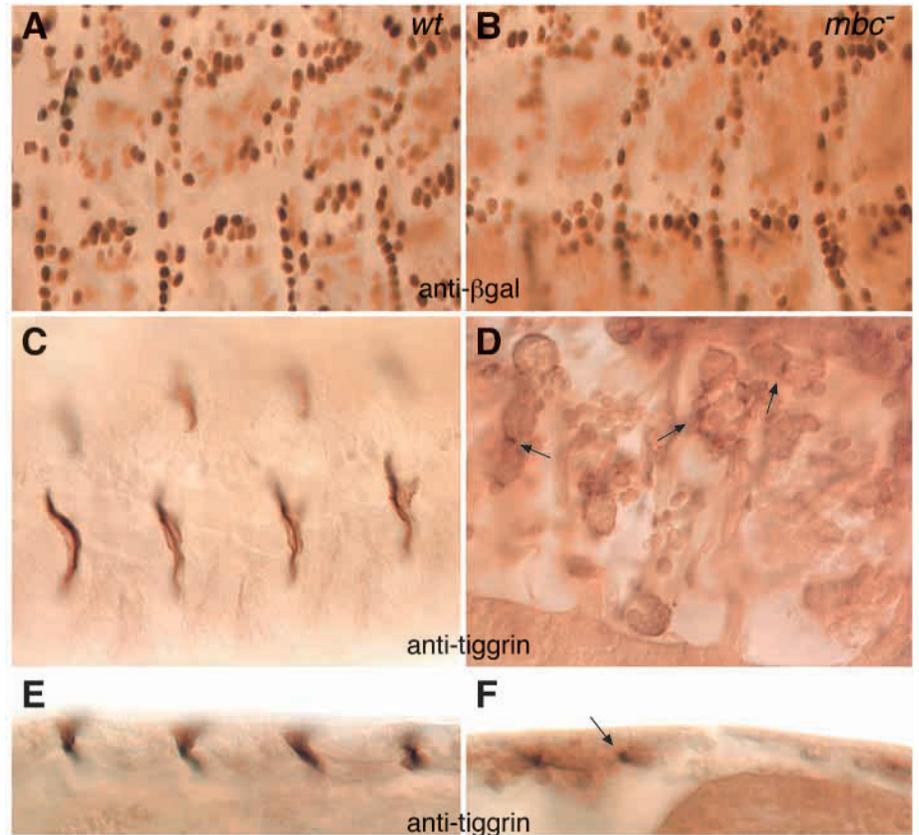
Fig. 3. Disruption of muscle development disrupts tiggrin localization. The development of multinucleate muscles was blocked by a mutation in *myoblast city (mbc)*. In this mutant background, the specification of the epidermal tendon cells is not perturbed, as indicated by the normal expression of the marker *stripe* (A, wild type, versus B, *mbc*; using a *stripe* enhancer trap and staining this with anti-β-galactosidase antibody), yet tiggrin localization is severely reduced (C,E, wild type, versus D,F *mbc*, stained with anti-tiggrin). Lateral views of embryos are shown in A-D, and horizontal views in E and F. In D, it can be seen that only small amounts of tiggrin localize at points of contact between the mononucleate muscles and the epidermis (arrows) or between muscles and tendon cells (F) compared with the wild type (C,E).

integrin localization, even when PS2 is expressed in the muscles.

Tiggrin associates with the extracellular matrix rather than the cell surface of the muscles

Since tiggrin localizes only to sites of cell-cell contact and not to the ends of unattached cells, it is possible that it becomes localized not to the cell surface but to the tendon matrix. In the wild-type embryo the muscle plasma membrane is very closely apposed to the tendon matrix, making it difficult to distinguish the two by conventional microscopy. However, our recent electron microscopic analysis of integrin mutant phenotypes (Prokop et al.,

1998) has shown that in embryos lacking the PS2 integrin the ends of the muscles detach from the tendon matrix. Therefore we examined the localization of tiggrin relative to the muscle plasma membrane in an embryo lacking PS2. To mark the surface of the membrane we expressed a chimeric SrcGFP protein consisting of the N terminus of the *Drosophila Src64* gene, which carries a signal for N-myristylation and therefore is targeted to the inner surface of the plasma membrane, fused
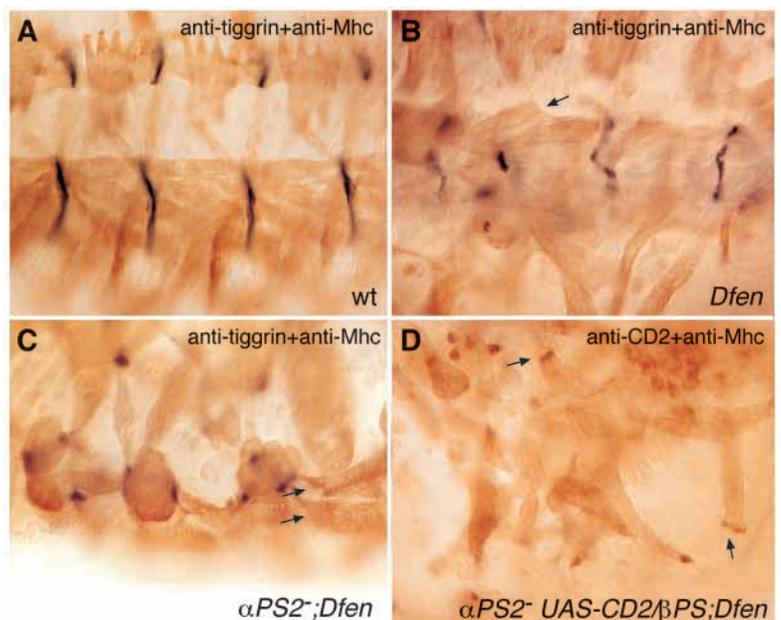
Fig. 4. Tiggrin still localizes at sites of muscle attachment in the absence of epidermal tendon cells. The specification of the majority of tendon cells was eliminated by a deletion removing the genes *engrailed* and *invected* (B; anti-tiggrin blue/black, anti-myosin brown), yet substantial amounts of tiggrin are localized at the ends of the muscles (compare to wild type in A). Even in the absence of the epidermal cells and the PS2 integrin from the muscles (C), tiggrin is still localized at points of remaining contact between the muscles (stained as in A,B). Tiggrin is only observed at points of contact between two muscles and not at free ends of muscles (arrows in B,C). In contrast, the chimeric transmembrane protein CD2/βPS, containing extracellular and transmembrane domains of the rat CD2 protein and the cytoplasmic tail of the βPS subunit (detected with an anti-CD2 antibody), is localized at all muscle ends (D), including the free ends (arrows).
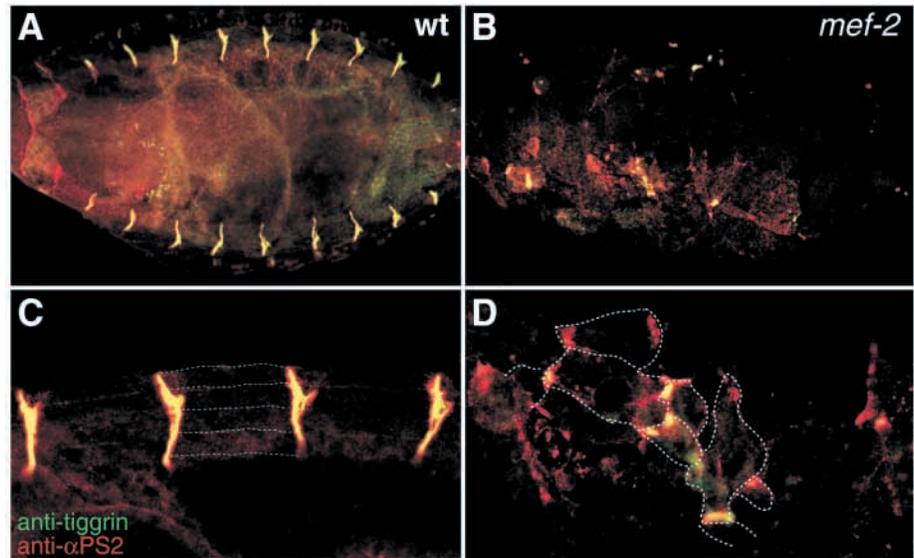
Fig. 5. Mechanisms for localization of the PS2 integrin and tiggrin at the ends of the muscle are independent. Muscle development was perturbed with a mutation in the muscle-specific transcription factor MEF-2 and the expression of the PS2 integrin (red) and tiggrin (green) was examined. Compared to wild type (A,C), in *mef-2* mutant embryos (B,D) PS2 and tiggrin no longer completely colocalize. Even though PS2 integrin is not always localized at specific points, tiggrin is found at points of muscle-muscle contact (D). The outline of muscles is dotted in C and D.
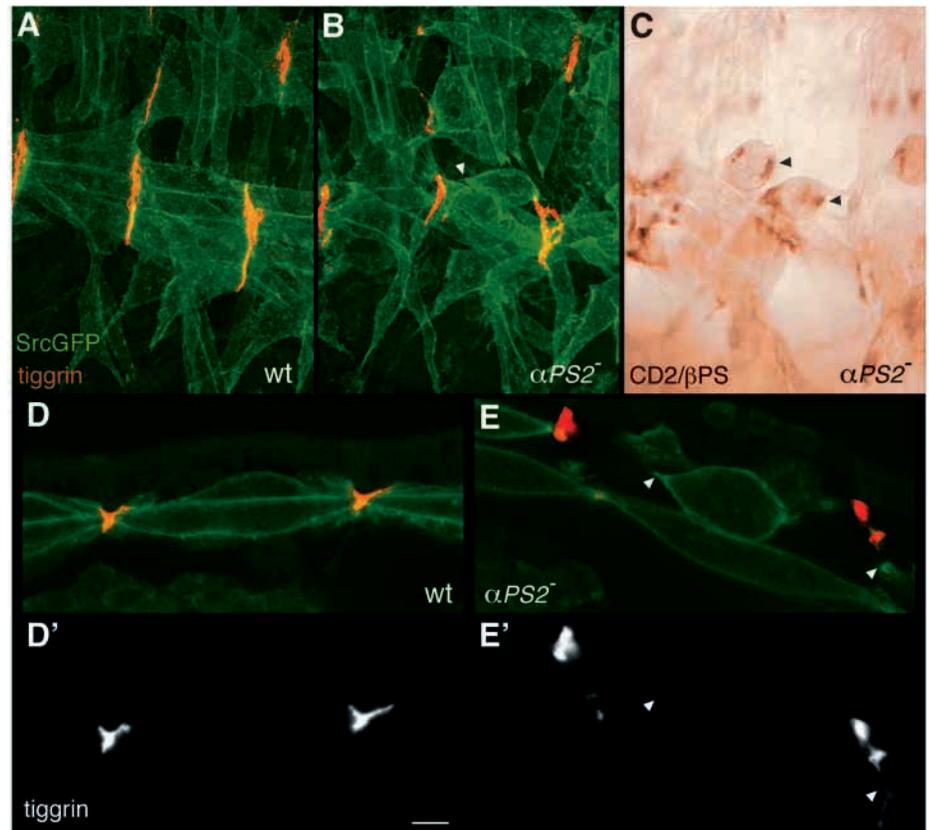
Fig. 6. In the absence of integrin-mediated muscle attachment, tiggrin is associated with the ECM and not the cell surface of detached muscles. The muscle cell surface was detected by expression of the membrane-linked myristylated Src-GFP fusion (green), and tiggrin with an antibody (red). Tiggrin is retained with the ECM and not with the cell surface of detached muscles in embryos lacking the αPS2 integrin subunit (B,E, compared to wild type, A,D). A and B are stacked collections of confocal sections, looking through the epidermis. Single horizontal sections are shown in D and E, with the tiggrin staining alone in D′ and E′. Bar in D′, 20 μm. For comparison, C shows expression of the chimeric transmembrane protein CD2/βPS, also in an embryo lacking the αPS2 subunit. The ends of the detached muscles are indicated by arrowheads.

to green fluorescent protein (Kaltschmidt et al., 2000). The SrcGFP fusion protein was expressed in the muscles using the GAL4 system, and the embryos fixed and stained with the anti-tiggrin antibody. In the wild-type embryo the tiggrin staining is very closely associated with the ends of the muscles (Fig. 6A,D). In the embryos lacking the PS2 integrin, when the muscles detach, tiggrin is not found associated with the muscle surface and remains behind with the tendon matrix (Fig. 6B,E). This can be compared to the CD2/βPS chimeric transmembrane protein, which is still associated with the ends of the detached muscles (Fig. 6C). Thus tiggrin is more strongly associated with the tendon matrix than the muscle cell surface. This result raises the possibility that localization of tiggrin is achieved by direct interaction with other components of the tendon matrix and not with a cell surface receptor at all. However, the 'first' ECM protein to be localized must be directed by the points of cell-cell contact between the ends of the muscles.
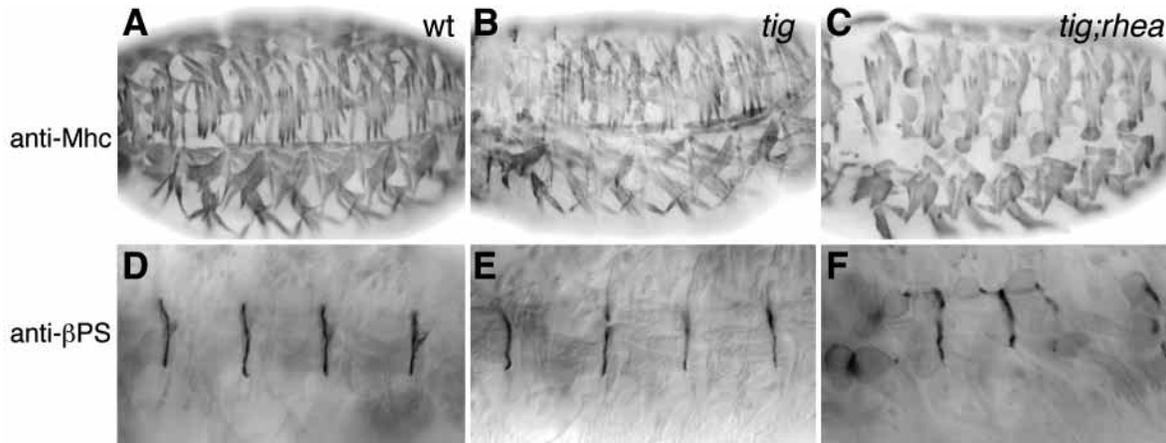
Fig. 7. Loss of *tiggrin* mediated muscle-muscle attachment and *rhea* mediated muscle-tendon cell attachment leads to complete muscle detachment. The muscles of embryos that are wild type (A,D), *tig* mutant (B,E) and *tig; rhea* double mutant (C,F) are shown by staining with an anti-muscle myosin antibody (A-C; *rhea* mutant embryos are indistinguishable from wild type, data not shown). Compared to wild-type or *tig* mutants alone, the *tig; rhea* double mutant embryos exhibit a strong muscle detachment phenotype, which is comparable to that caused by absence of PS integrins (not shown). This phenotype is not due to the loss of PS2 localization, which is detected at the end of the detached muscles (stained with an anti-βPS antibody; D-F).

Our finding that tiggrin is localized by the muscles fits very well with recent evidence suggesting that the function of tiggrin is to link the ends of muscles together, rather than in muscle-epidermis attachment (Bunch et al., 1998). The loss of tiggrin does not cause a detectable phenotype in the embryo, but in the larvae, the muscle-muscle attachments were observed to be weaker than normal. The *tiggrin* mutant phenotype is considerably milder than the phenotype caused by loss of the PS integrins from the muscle attachment site. The weaker phenotype could be due to tiggrin being one of many integrin ligands present in the tendon matrix, or there could be essentially two ligands: tiggrin, used for muscle-tendon matrix attachment, and a second ligand used for tendon matrix-tendon cell attachment. This latter linkage has been found to be specifically disrupted in embryos mutant for the gene *rhea* (Prout et al., 1997). To test whether the products of these two genes are sufficient to account for muscle detachment, we examined embryos doubly mutant for *tiggrin* and *rhea*. Compared to either single mutant, the embryos mutant for both genes have a severe muscle detachment phenotype (Fig. 7C), very similar to embryos lacking integrins. The PS2 integrin is still localized at the end of the detached muscles in these mutants (Fig. 7F), and so its synthesis and localization are not altered. The strong detachment phenotype suggests that a combination of two adhesive mechanisms, the tiggrin-dependent muscle-tendon matrix attachment and the Rhea-dependent tendon matrix-epidermal cell attachment, are necessary and sufficient for muscle attachment. The full interpretation of this result awaits the characterization of the *rhea* locus.

DISCUSSION

In this paper, we have used the muscle attachment sites and the integrin ligand tiggrin as a model system to study the mechanisms that regulate the spatial and temporal assembly of ECM during embryogenesis. We have examined how the
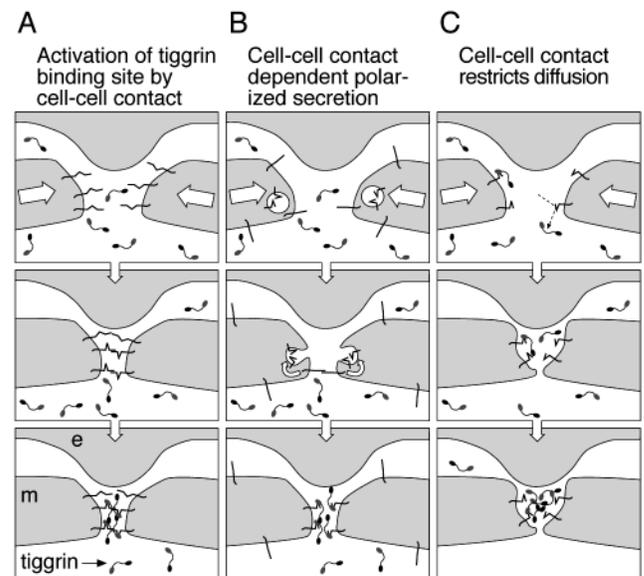
Fig. 8. Models for tiggrin localization at the muscle attachment sites. Each panel shows an epidermal cell (e, shown in bottom left panel) at the top, and two muscles (m) in the center. The different models show different ways in which the inherent polarity of the muscles (large white arrows in the top panel) lead to the localization of a cell-cell contact-dependent tiggrin receptor.

extracellular matrix protein tiggrin comes to be tightly localized at the interface between the specialized epidermal tendon cells and the ends of the muscles at the muscle attachment sites. We tested whether one tiggrin cell surface receptor, the PS2 integrin, is able to localize tiggrin to new sites within the embryo, and found that it is not. We then examined what cells are required for the localization of tiggrin and found that muscles are required, while the tendon cells are not. Unexpectedly we found that the localization of tiggrin to the end of a muscle requires contact between the muscle and another cell.

The requirement for integrins in the assembly of ECM in vivo is clearly variable. In amphibian embryos, the accumulation of fibronectin fibrils is blocked by antibodies against the integrins (Darribere et al., 1990). However, genetic elimination of integrin function has more modest effects. The initial assembly of extracellular matrices appears normal in mouse embryos lacking a variety of integrin subunits, although the matrices formed may be less stable (for references, see Brakebush et al., 1997). We have shown by ultrastructural analysis that in the absence of PS integrin function the tendon matrix still accumulates at the muscle attachment sites, although it is separated from the cell surfaces (Prokop et al., 1998). This is further supported by light microscopic findings showing that the tendon matrix protein tiggrin accumulates correctly in embryos lacking PS integrins (Fogerty et al., 1994; Figs 1 and 5). In addition, we show here that the PS2 integrin is not only not necessary for tiggrin localization but is also not sufficient. Therefore, a mechanism for the assembly of extracellular matrix at the muscle attachment sites has to be integrin-independent.

This work has posed two key questions that will have to be resolved in order to understand the mechanism of tiggrin localization: why does it require cell-cell contact, and why do some sites of cell-cell contact recruit tiggrin, while others do not. The latter point implies that something must be special about the ends of the muscles; when they make cell-cell contacts they recruit tiggrin, while other cell-cell contacts, for example between the lateral surfaces of the muscles, do not. This difference reflects the inherent polarity present within the developing muscles, which has been revealed by two separate experiments. When the rat transmembrane protein CD2 is expressed in *Drosophila* muscles it is uniformly distributed on membrane, but when its cytoplasmic tail is replaced with that from the βPS subunit, then the chimeric protein is localized to the ends of the muscles (Martin-Bermudo and Brown, 1996). This demonstrates that the βPS cytoplasmic tail is recognized inside the cell and localized to the ends of the muscles. Second, by examination of the localization of kinesin-β-galactosidase fusion proteins, it has been shown that the muscles contain a bipolar arrangement of microtubules, with the plus ends at the termini of the muscles (Clark et al., 1997). Thus the muscles clearly have an internal polarity that is able to localize molecules specifically to the ends of the muscles, and we propose that this is the first step leading to the localization of tiggrin.

There are a variety of possible models to explain why cell-cell contact is required for tiggrin localization and three will be outlined here. In the first model (Fig. 8A), the inherent polarity of the muscles leads to the localization of a transmembrane tiggrin receptor at the ends of the muscle. However, this receptor is not active until the muscle cells have made contact with another cell, such as the end of the equivalent muscle in the next segment. Following this cell contact-dependent interaction, tiggrin can bind to the receptor, and later in development binds more strongly to other proteins in the extracellular matrix, possibly by becoming crosslinked to them, so that when the muscle detaches in the PS2 integrin mutant, tiggrin remains with the extracellular matrix.

In a second model (Fig. 8B), polarization of the cells results in the specific transport of vesicles containing transmembrane receptors and or extracellular matrix components to the ends of the muscles. Then, the fusion of these vesicles with the plasma membrane, which releases the contents, requires cell-cell contact. This could be achieved by the interaction of a transmembrane receptor with a ligand on the apposing cell, which triggers an intracellular pathway leading to vesicle fusion. In this model, since the vesicles are localized, the receptor that triggers fusion does not have to be. One of the proteins in the vesicle is not freely diffusible (for example by being tethered to the membrane, as shown in the figure) and contains a binding site for tiggrin, thus recruiting tiggrin to the tendon matrix. Such polarized discharge of matrix components has been described in diverse vertebrate cells, although it has not been shown to require cell-cell contact (for a review, see Birk et al., 1991).

In these models we have focused on the muscles, since the localization of tiggrin only requires the muscles. However, both models may also be applicable to the localization by tendon cells of ECM proteins involved in tendon cell-tendon matrix attachment, such as the proteins affected by the *rhea* mutation. The main difference between the cells on the two sides of muscle attachments is that the muscles make cell-cell contacts with each other and with the tendon cells, while the tendon cells only make contact with the muscles. One of the attractive aspects to the vectorial discharge model is that the epidermal cells and muscles could secrete components that crosslink together when they interact in the extracellular space, forming a stable matrix. This would be similar to basement membrane assembly at the interface between the epidermis and the dermis, where laminin and its interacting partner nidogen are expressed in different layers (Ekblom et al., 1994). Such interaction between components secreted by the two layers may be important for the formation of a functional tendon matrix, as suggested by the *rhea* phenotype, but as we have shown here, is not required for tiggrin localization.

The two models described above involve cell contact-dependent activation of tiggrin receptors, but it is difficult to rule out the third model (Fig. 8C), where cell-cell contact has a more mechanical role and is only required to reduce tiggrin diffusion. For example the inherent polarity could produce a tiggrin receptor at the ends of the muscles that is fully active prior to cell-cell contact. The interaction of tiggrin with this receptor could be short-lived, so that it comes off again and diffuses away. The cell-cell interaction would serve to make an enclosed space or 'basket', where the resulting concentration of tiggrin would reach a high enough concentration to assemble into an insoluble matrix. However, the assembly of the tendon matrix clearly differs from the assembly of basement membranes and fibronectin fibrils, which can form on a cell surface that faces the extracellular fluid. Of course the actual mechanism of tendon matrix localization could easily involve all of these possible mechanisms.

In summary, a combination of cell polarity and cellular interaction result in the assembly of the tendon matrix in the right place. Our results have shown that in the developing embryo cell-cell contact is necessary and may be sufficient for the formation of a localized matrix, and have allowed us to formulate models that are consistent with diverse experimental results. Further characterization of the components of the tendon matrix and the transmembrane receptors on the cells should allow us to determine the mechanism of cell contact dependent localization.

REFERENCES

Adams, M., Celniker, S., Holt, R., Evans, C., Gocayne, J., Amanatides, P., Scherer, S. E., Li, P., Hoskins, R., Galle, R. F. et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-2195.

Birk, D. E., Silver, F. H. and Trelstad, R. L. (1991). Matrix Assembly. In *Cell Biology of Extracellular Matrix* (ed. E. D. Hay), pp. 221-254. New York: Plenum.

Brakebush, C., Hirsch, E., Potocnik, A. and Fassler, R. (1997). Genetic analysis of $\beta 1$ integrin function:confirmed, new and revised roles for a crucial family of cell adhesion molecules. *J. Cell Sci.* **110**, 2895-2904.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brown, N. H., Gregory, S. L. and Martin-Bermudo, M. D. (2000). Integrins as mediators of morphogenesis in *Drosophila*. *Dev. Biol.* **223**, 1-16.

Bunch, T. A., Graner, M. W., Fessler, L. I., Fessler, J. H., Scheneider, K. D., Kerschen, A., Choy, L. P., Burgess, B. W. and Brower, D. L. (1998). The PS2 integrin ligand tiggrin is required for proper muscle function in *Drosophila*. *Development* **125**, 1679-1689.

Castelli-Gair, J., Greig, S., Micklem, G. and Akam, M. (1994). Dissecting the temporal requirements for homeotic gene-function. *Development* **120**, 1983-1995.

Cheresh, D. A. and Mecham, R. P. (1994). *Integrins: Molecular and Biological Responses to the Extracellular Matrix*. London: Academic Press.

Clark, I. E., Jan, L. Y. and Jan, Y. N. (1997). Reciprocal localization of Nod and Kinesin fusion proteins indicates microtubule polarity in the *Drosophila* oocyte, epithelium, neuron and muscle. *Development* **124**, 461-470.

Darribere, T., Guida, K., Lajarva, H., Johnson, K. E., Yamada, K. M., Thiery, J. P. and Boucaut, J. C. (1990). In vivo analyses of integrin $\beta 1$ subunit function in fibronectin matrix assembly. *J. Cell Biol.* **110**, 1813-1823.

DiPersio, C., Hodivala-Dilke, K., Jaenisch, R., Kreidberg, J. and Hynes, R. (1997). $\alpha 3 \beta 1$ Integrin is required for normal development of the epidermal basement membrane. *J. Cell Biol.* **137**, 729-742.

Ekblom, P., Ekblom, M., Klein, L., Zhang, H., Kadoya, Y., Chu, M., Mayer, U. and Timpl, R. (1994). Role of mesenchymal nidogen for epithelial morphogenesis in vitro. *Development* **120**, 2003-2014.

Fogerty, F. J., Fessler, L. I., Bunch, T. A., Yaron, Y., Parker, C. G., Nelson, R. E., Brower, D. L., Gullberg, D. and Fessler, J. H. (1994). Tiggrin, a novel *Drosophila* extracellular matrix protein that functions as a ligand for *Drosophila* $\alpha_{PS2}\beta_{PS}$ integrins. *Development* **120**, 1747-1758.

Frommer, G., Vorbruggen, G., G., P., Jackle, H. and Volk, T. (1996). Epidermal egr-like zinc finger protein of *Drosophila* participates in myotube guidance. *EMBO J.* **15**, 1642-1649.

Gelbart, W. M., Crosby, M., Matthews, B., Rindone, W. P. and Chilemi, J. (1997). Flybase: a *Drosophila* database. *Nucleic Acids Res.* **25**, 63-66.

Ginsberg, M. H., Du, X. and Plow, E. F. (1992). Inside-out integrin signaling. *Curr. Opin. Cell. Biol.* **4**, 766-771.

Graham, P. L., Johnson, J. J., Wang, S. R., Sibley, M. H., Gupta, M. C. and Kramer, J. M. (1997). Type IV collagen is detectable in most, but not all, basement membranes of *Caenorhabditis elegans* and assembles on tissues that do not express it. *J. Cell Biol.* **137**, 1171-1183.

Henry, M. D. and Campbell, K. P. (1998). A role for dystroglycan in basement membrane assembly. *Cell* **95**, 859-870.

Kaltschmidt, J. A., Davidson, C. M., Brown, N. H. and Brand, A. H. (2000). Rotation and asymmetry of the mitotic spindle direct asymmetric cell division in the developing central nervous system. *Nature Cell Biol.* **2**, 7-12.

Lee, J. C., VijayRaghavan, K., Celniker, S. E. and Tanouye, M. A. (1995). Identification of a Drosophila muscle development gene with structural homology to mammalian early growth response transcription factors. *Proc. Natl. Acad. Sci. USA* **92**, 10344-10348.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B., Schulz, R. A. and Olson, E. N. (1995). Requirements of MADS domain transcription factor D-Mef2 for muscle formation in *Drosophila*. *Science* **267**, 688-693.

Lin, M. H., Nguyen, H. T., Dybala, C. and Storti, R. V. (1996). Myocyte-specific enhancer factor 2 acts cooperatively with a muscle activator region to regulate *Drosophila* tropomyosin gene muscle expression. *Proc. Natl. Acad. Sci. USA* **93**, 4623-4628.

Martin-Bermudo, M. D. and Brown, N. H. (1996). Intracellular signals direct integrin localization to sites of function in embryonic muscles. *J. Cell Biol.* **134**, 217-226.

Martin-Bermudo, M. D., Dunin-Borkowski, O. M. and Brown, N. H. (1997). Specificity of PS integrin function during embryogenesis resides in the α subunit extracellular domain. *EMBO J.* **16**, 4184-4193.

Martin-Bermudo, M. D., Dunin-Borkowski, O. M. and Brown, N. H. (1998). Modulation of integrin activity is vital for morphogenesis. *J. Cell Biol.* **141**, 1073-1081.

Prokop, A., Martin-Bermudo, M. D., Bate, M. and Brown, N. H. (1998). In *Drosophila* embryos, the absence of the PS integrins or laminin A affects the extracellular adhesion of hemiadherens and neuromuscular junctions, but not their intracellular assembly. *Dev. Biol.* **196**, 58-76.

Prout, M., Damania, Z., Soong, J., Fristrom, D. and Fristrom, J. W. (1997). Autosomal mutations affecting adhesion between wing surfaces in *Drosophila melanogaster*. *Genetics* **146**, 275-285.

Rushton, E., Drysdale, R., Abmayr, S. M., Michelson, A. M. and Bate, M. (1995). Mutations in a novel gene *myoblast city*, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development* **121**, 1979-1988.

Sasaki, T., Forsberg, E., Bloch, W., Addicks, K., Fassler, R. and Timpl, R. (1998). Deficiency of $\beta 1$ integrins in teratoma interferes with basement membrane assembly and laminin-1 expression. *Exp. Cell. Res.* **238**, 70-81.

Schwarzbauer, J. E. and Sechler, J. L. (1999). Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly. *Curr. Opin. Cell Biol.* **11**, 622-627.

Timpl, R. and Brown, J. C. (1996). Supramolecular assembly of basement membranes. *BioEssays* **18**, 123-131.

Yee, G. H. and Hynes, R. O. (1993). A novel, tissue-specific integrin subunit, β_ν, expressed in the midgut of *Drosophila melanogaster*. *Development* **118**, 845-858.

Yurchenco, P. D. and O'Rear, J. J. (1994). Basal lamina assembly. *Curr. Opin. Cell Biol.* **6**, 674-681.

Yurchenko, P. D., Birk, D. E. and Mecham, R. P. (1994). *Extracellular Matrix Assembly and Structure*. San Diego; London: Academic Press.