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The roles of *vestigial* and *scalloped* in the embryonic muscle development of *Drosophila melanogaster*

by

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This thesis is dedicated to my parents and my family

Abstract

Vertebrate development requires the activity of multiple members of the *myocyte enhancer factor 2* (*mef2*) gene family for muscle cell specification and subsequent differentiation. Additionally, it is thought that several muscle-specific functions of MEF2 family proteins require binding additional co-factors including members of the Transcription Enhancing Factor-1 (TEF-1) and Vestigial-like protein families. In *Drosophila* there is a single *mef2* (*Dmef2*) gene as well single homologues of *TEF-1* and *vestigial-like*; *sd* and *vg*, respectively. To help clarify the role(s) of these factors, we examined the requirements for Vg and Sd during *Drosophila* muscle specification. Analysis of loss of Vg or Sd function mutations confirms that both are required for muscle differentiation, as loss of *sd* or *vg* leads to a reproducible loss of a subset of cardiac or somatic muscle cells in developing embryos. However, the requirement for Sd or Vg is cell specific, as over-expression of each of these proteins in other muscle cells also has a deleterious effect on muscle differentiation. Finally, I determined that Sd, Vg and Dmef2 can interact directly. Thus, the muscle specific phenotypes associated with loss or ectopic Vg or Sd expression may be a consequence of alternative binding of Vg and Sd to Dmef2 to form alternative protein complexes that modify Dmef2 activity.

The somatic muscles of *Drosophila* develop in a complex pattern that is repeated in each embryonic hemi-segment. Initial communication between somatic muscles and the epidermal tendon cells is critical for formation of this muscle pattern. However, later establishment of attachments between longitudinal

muscles at the segmental borders is largely independent of the muscle-epidermal attachment signals, and relatively little is known about how this event is regulated. Here I show that expression of the transcription factor Vg is required in ventral longitudinal muscles (VL1-4) to make them competent to form stable inter-muscular attachments. Further, the cell-specific differentiation events induced by Vg in two muscles fated to form attachments appear to be coordinated by *Drosophila* Epidermal Growth Factor (DER) signalling.

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List of abbreviations

A	Anterior
A/P	Anteroposterior
ac	*achaete*
act57B	*actin 57B*
ANF	Atrial natriuretic factor
bap	*bagpipe*
BE	Boundary enhancer
BMP	Bone morphogenetic protein
ChIP	Chromatin immunoprecipitation
CNS	Central nerve system
Co-IP	Co-immunoprecipitation
DA	Dorsal Acute
D/V	Dorsoventral
DIG	Digoxigenin
DO	Dorsal Oblique
DT	Dorsal Transverse
dl	*dorsal*
dpp	*decapentaplegic*
en	*engrailed*
eve	*even-skiped*
FAs	Focal Adhesions
FCM	Fusion-competent myoblast
FGF	Fibroblast growth factor
FISH	Fluorescence in-situ hybridization
GST	Glutathione S-transferase
hh	*hedegehog*
him	*hole in muscle*
htl	*heartless*
IFM	Indirect flight muscle
LL	Lateral Longitudinal

LO	Lateral Oblique
LT	Lateral Transverse
VL	Ventral Longitudinal
l'sc	*lethal of scute*
MHC	Myosin heavy chain
N	Notch
NLS	Nuclear localization signal
P	Posterior
PCR	Polymerase chain reaction
pnr	*pannier*
PS	Position-specific
QE	Quadrant enhancer
RT	Reverse transcriptase
sal	*spalt*
SBM	Segment Border Muscle
sd	*scalloped*
sens	*senseless*
SID	Scalloped interaction domain
slp	*sloppy-paired*
SM	Somatic muscle
SOP	Sensory organ precursor
sr	*stripe*
srp	*serpent*
Su(H)	Suppressor of Hairless
SUMO	Small ubiquitin-related modifier
TF	Transcription factor
tig	*tiggrin*
tin	*tinman*
TmI	*Tropomyosin*
TnI	*Troponin I*
TnT	*Troponin T*

twi	*twist*
vg	*vestigial*
vgl	*vestigial-like*
VA	Ventral Acute
VID	Vestigial interaction domain
VM	Visceral muscle
VO	Ventral Oblique
VT	Ventral Transverse
wg	*wingless*
α-CA	α-cardiac actin
β-gal	*β-galactosidase*

Chapter 1: General Introduction

A key question about development is how a specific fate is assigned to each cell during organogenesis in a coordinated way so that the mature organ contains all the required types of cells that organize in a certain pattern to perform a specific function. For example, the *Drosophil*a larval heart is a tube-like structure having 16 cells in each segment from A2-A7, with eight different types of cells formed in a certain order [1]. Although a general picture has been obtained about this process, many details are still not clear. In general, cell fate is determined progressively through the integration of external signaling with the expression of intrinsic specific transcription factors, both temporally and spatially during embryo development [1, 2].

In this thesis, I performed studies on the roles of two transcription factors, Scalloped (Sd) and Vestigial (Vg) in the development of cardiac and skeletal muscles using *Drosophila melanogaster* as the model organism. Therefore, my introduction will focus on myogenesis in *Drosophila*.

Myogenesis in *Drosophila* can be divided into three stages that happen successively. The first stage involves the origin of three different myogenic lineages: the muscles of the gut (visceral muscles), the body wall 'skeletal' muscles (somatic muscles), and cardiac muscles. They have significant differences with respect to their ultrastructure, contractile properties, and physiological functions [4]. The second stage involves diversification in each lineage: for example, each individual somatic muscle (SM) is unique in terms of its position, size, site of attachment, and patterns of innervation (Fig 1.2), although all SMs are thought to be identical physiologically [4]. The final stage involves migration and differentiation. For example, once specified, each SM needs to migrate over a certain distance to reach a specific attachment site [6]. In the meantime, myoblasts fuse to form a multinucleated myotube and structural proteins such as myosin heavy chain (MHC) and actin are produced to assemble myofibrils. These events are regulated mostly at the level of transcription, although some recent evidence shows microRNA is also involved in the

diversification process [7, 8]. Therefore, integration of transcriptional and signaling networks plays key roles in myogenesis [2, 9].

Early mesodermal subdivision-the origins of the myogenic lineages

The mesoderm in the fly is derived from the most ventral cells of the blastoderm stage embryo, which is under the control of the maternally patterning gene *dorsal* (*dl*). As the embryo gastrulates, these ventral cells invaginate and migrate dorsally to cover the inner surface of the ectoderm and form the mesoderm [10]. During migration of these cells, they maintain their relative positions in the anterior-posterior axis due to the expression of an FGF receptor, Heartless (Htl), in the cells [11, 12]. This is important, as mesoderm segmentation needs to be in register with segmentation in the ectoderm to receive proper signals from the apposing ectoderm (Fig. 1.1). The pair-rule genes and their upstream regulators have similar roles in both ectoderm and mesoderm, and their mutations cause identical alterations in the number and polarity of segments in both germ layers [13]. However, these patterning genes act through different downstream genes in mesoderm since Dorsal activates the expression of *twist* (*twi*) only in mesoderm cells before gastrulation [5]. This then turns on *tinman* (*tin*) expression in the whole mesoderm [14]. Therefore, although *even-skipped* (*eve*) and *sloppy-paired* (*slp*) have similar expression patterns in the two germ layers (Fig. 1.1), different fates are assigned to mesoderm cells [13].

In each parasegment of the ectoderm, Eve induces *hedgehog* [15] expression in the anterior (A) domain, while *wingless* (*wg*) expression in the posterior (P) domain requires *slp* activity (Fig.1.1) [16, 17]. The antagonism between *eve* and *slp*, and the reciprocal enhancement between Hh and Wg signaling leads to strong Hh and Wg signals confined to each domain [18]. In the mesoderm, a similar gene network operates at in the same time and is required to establish a prepattern of gene expression to confer the identity of A or P to mesoderm cells. However, pair-rule gene products fade away after gastrulation, so that the *hh* and *wg* products produced in mesoderm can only maintain normal A and P domains transiently. Therefore, mesoderm cells have to receive these signals from the overlying ectoderm whose segmentation is in register with the

mesoderm [13]. Wg signal from the ectoderm activates *slp* expression, which in turn is responsible for keeping a high level of *twi* expression in the P domain of the mesoderm [19]. On the other hand, a Hh signal from the ectoderm is required for *bagpipe* (*bap*) and *serpent* (*srp*) expression, which direct the development of visceral muscles (VMs) and fat body, respectively [13, 20, 21]. *bap* expression also needs Tin that is present broadly in the mesoderm at early stages, but is restricted later to the most dorsal mesoderm cells under the influence of a Decapentaplegic (Dpp) signal from the dorsal ectoderm[22, 23]. Therefore, *bap* is induced in the dorsal A domain and *srp* is activated in the ventral A domain (Fig. 1.1). The early expression of *tin* in mesoderm cells enables them competent to respond to Dpp signal, thus there is no *tin* expression in dorsal ectoderm [24]. The effect of *tin* on *bap* expression is blocked by *slp* in the P domain of mesoderm [19], making the dorsal P domain as the primordium of cardiac tissue (Fig. 1.1) [22, 23].

Another regulatory layer for subdivision of mesoderm is modulation of the *twi* expression level in the mesoderm. *twi* expression is uniform in the mesoderm at early stages but later is modulated to a relatively high level in the ventral P domain where SMs arise, and to a relatively low level in the area from which VMs and heart are derived [25]. Raising the *twi* expression level in the entire mesoderm disturbs the development of VMs and heart but has little effect on the development of SMs. On the other hand, reducing the *twi* expression level leads to derangement of SM differentiation, but VMs and heart develop normally [25]. This expression pattern is established through the alleviation of cells from the repression of Notch signaling by Wg signaling [26, 27], that act through the activation of *slp*[19].

Altogether, the combinatorial actions of the transcription factors expressed in the mesoderm and the signals from the adjacent ectoderm subdivide the mesoderm into four primordia (Fig.1.1). Cells underneath the Dpp/Hh intersect and expressing Tin and Bap are precursors of visceral muscles in the dorsal A domain. Fat body comes from cells receiving Hh signal and expressing Srp in the ventral A domain. Cells underneath the Dpp/Wg intersect and expressing Tin

become the heart precursors. Finally, somatic muscles are derived from the cells receiving Wg signal and expressing a high level of Twi in the ventral P domain.

Muscle pattern diversification-Patterning of somatic muscles

Once specified, precursors of VM and fat body invaginate to expand and coalesce to form the muscles of the gut and the mature fat body, respectively [28, 29]. However, approximately 30 SMs are produced in each abdominal segment to form a complex and repeated pattern, in which each individual SM is unique in terms of its position, size, and sites of attachment (Fig. 1.2). Again, The formation of this pattern is a result of the integration of external cues with expression of muscle-specific transcription factors [30].

After the subdivision of mesoderm, groups of cells appear at stereotypical locations expressing the proneural gene, *lethal of scute* (*l'sc*), in the region with high *twi* expression (Fig. 1.2) [31]. Only one cell will be selected from each group to continue *l'sc* expression, through lateral inhibition within the group that is mediated by the Notch signaling pathway [31]. This cell turns out to be the muscle progenitor that then divides to give rise to two founder myoblasts, or a founder and the precursor of an adult muscle [31, 32]. Each somatic myofiber derives from a founder myoblast whose *l'sc* expression disappears at this time, but it differs from surrounding myoblasts by its specific expression of potential regulatory genes, which are now called 'muscle identity genes' [2, 33, 34]. The neighboring *fusion-competent myoblasts* (FCMs) are "naive" cells that are recruited to the pattern of gene expression as they fuse with the muscle founder [33, 35]. Interestingly, in the absence of fusion, the founders can still migrate properly to their attachment sites and differentiate normally as tiny, mononucleate muscles. However, the unfused FCMs, remain round and undifferentiated [3]. Therefore, the founder myoblasts should contain the information necessary to initiate the myogenic program characteristic of the muscles whose formation they seed. It is believed that the information is provided by the muscle identity genes that are expressed selectively in each SM. These genes usually encode transcription factors and include the homeobox genes *S59* (also named *slouch*) [34, 36], *apterous* (*ap*) [37], *muscle segment homeobox* (*msh*) [38, 39] *ladybird*

(*lb*) [40], the zinc-finger encoding gene *Krüppel* (*kr*) [41], the myc-related HLH encoding gene *collier* (*col*) [35, 42], the Pax gene *Pox meso* (*Poxm*) [43], and *vestigial* (*vg*) [44]. Next, I will discuss some examples showing the roles of muscle identity genes in the diversification of SM fates.

The VA1 and VA2 muscles share the same progenitor (the big purple cell in Fig.1.2) that arises from a group of cells located at the ventral P domain and expressing *Kr* [41]. Through the lateral inhibition mechanism mediated by Notch signaling [31], *Kr* expression is restricted to the progenitor which then divides and produces two founder myoblasts [41]. Because of the asymmetrical distribution of Numb protein in the two founders, only one of them is able to maintain *Kr* expression due to the repression of Notch signaling by Numb [32, 45]. Thus, two alternative fates are assigned to the two founder myoblasts. The B cell maintains *Kr* expression and develops into the VA2 muscle through recruiting a certain number of FCMs. However, The A cell loses *Kr* expression and becomes the VA1 muscle (Fig. 1.2). Kr^{1} null mutants have VA2 transformed into VA1, while ectopic expression of Kr in VA1 switches it to VA2 [41]. Therefore, *Kr* appears to be the identity gene of VA2 and is able to initiate the myogenic program specific for VA2.

However, *Kr* is also expressed in many other SMs like DA1, LL1, and LT4 *et al.* (see Fig. 4.1 for the complete muscle pattern), and a Kr^{1} null mutation also causes defects in the development of these muscles but does not transform them into other muscles [41]. Similarly, *S59, msh*, and *Poxm* are coexpressed in VA2 and mutations in these genes all cause defects in VA2 muscle development, but not a switch of VA2 to VA1 [36, 38, 43]. Therefore, the characteristics of one SM are not determined by only one identity gene but by a combinatorial action of a group of genes. Except in some cases, a single identity gene like *Kr* may be able to act like a "master" gene to initiate the whole myogenic program in a certain context. Supporting this idea, it has been shown that maintenance of *S59* expression in VA2 requires Kr [41]. Actually, overlapping expression of different identity genes in a single muscle is common in SMs [2]. Other examples are the DA3 and DA1 muscles. DA3 specification requires both *nautilus* (*nau*) and *col*

[35, 42], while both *even-skipped* (*eve*) and *Kr* are essential for DA1 development [41, 46].

It is not always that identity genes work together to specify a single SM, since in some cases they repress each other's expression to maintain the identity of a muscle. The LO1 and VT1 muscles come from the same progenitor (big green cell in Fig.1.2) expressing *S59*, and the VT1 muscle maintains the expression of *S59* after asymmetrical division of the progenitor. Similarly, the SBM muscle and an adult muscle progenitor (AP) share the same progenitor (Big red cell in Fig.1.2) expressing *lb,* and the SBM muscle inherits the *lb* expression. In *S59* null mutants or the embryos ectopically expressing *lb*, the progenitor of LO1 and VT1 muscles expresses *lb* instead, and this causes duplication of the SBM muscle and the AP. Conversely, *lb* null mutations or ectopic expression of *S59* leads to loss of SBM muscle and the AP, but LO1 and VT1 muscles are not affected. Therefore, it is assumed that S59 is required to repress *lb* expression in order to keep the identity of LO1 and VT1 muscles [36, 40].

What is the mechanism that leads to the specific expression of these identity genes in each SM progenitor in the first place? For most of the SMs, it is not clear. However, we can get a general idea from the well studied mechanism that causes specific expression of *eve* in two pericardial cells and in the DA1 muscle [47-49].

Under the influence of both Wg and Dpp signals, a competence domain expressing *l'sc* appears and has the potential of responding to MAPK activation induced by the signaling mediated by Heartless (Htl), a fibroblast growth factor receptor tyrosine kinase (RTK) (Fig. 1.3A). Localized Htl signal enables a group of cells (cluster C2) to express *eve* within this domain (Fig. 1.3A). Dissection of the *eve* enhancer identified corresponding binding sites for Mad, Twi, Tin, dTCF, and Pnt, which assemble at the *eve* enhancer to function synergistically to promote *eve* expression (Fig. 1.3B). Wg and Dpp act upstream of Htl to prepattern cells before MAPK activation [49]. However, a Notch signal is able to repress MAPK activation and restrict the MAPK activation and *eve* expression in a single cell through lateral inhibition. This cell turns out to be the progenitor P2 that then

divides asymmetrically to form the founder cell of the DO2 muscle and a pericardial cell. This pericardial cell divides symmetrically and produces two pericardial cells expressing *eve* (Fig. 1.3A). When the MAPK activation is restricted in P2, it starts to produce Rhomboid (Rho) that is required for the secretion of active Spitz (Spi), the ligand to stimulate *Drosophila* epidermal growth factor receptor (DER) activation in neighboring cells [47]. DER activation is in turn responsible for the activation of *eve* expression in a group of cells near P2 (cluster P15). P15 then arises from this group of cells through lateral inhibition and produces the founder cell of DA1, which maintains the expression of *eve* (Fig. 1.3A).

Muscle migration and differentiation

Muscle migration guidance

SM founders start to migrate relative to each other once specified. In the mean time, neighboring FCMs fuse with the founders to make each SM grow to a certain size and shape [3]. Based on the morphology of the migrating muscles, muscle migration can be divided into three distinct phases (Fig. 1.4) [6]. In the first phase, founders are round without visible polarity [31, 45], and they move relative to each other. For example, the founder cells of muscles LO1 and VT1 move apart from each other, with LO1 migrating dorsally within the segment and VT1 traveling into the next segment in ventral-posterior direction (Fig. 1.4). In the second phase, muscle fusion occurs and myotubes are polarized, stretching to form a long axis. Therefore, migration and fusion happen at the same time and the fusion process does not appear to affect muscle migration. In this phase, each SM also produces extensive filopodia at the leading edge of the cell, searching for external cues to reach its attachment site. In the third phase, myotubes reach their target tendon cells and filopodia formation ceases. At this time, the surface of the myotube facing the tendon cells becomes smooth, and stable adhesion junctions are established between myotubes and tendon cells or between two adjacent myotubes [50].

How does each SM find the right migration path and reach the proper attachment site? Both external guidance cues and intrinsic characteristics of each

SM, which are believed to be executed by the identity genes, play a role in the migration process. For example, the founders of muscles LO1, VT1, and SBM have similar positions when they arise. They should receive similar external cues, since *S59* null mutations transform a LO1 muscle into a SBM muscle that attaches at the same sites as the normal SBM [36]. However, they migrate in three very different directions (Fig. 1.2 and Fig. 1.4).

Most muscle pathfinding factors were identified from studies of axon pathfinding and are involved in reciprocal communications between tendon cells and the migrating muscles (Fig. 1.4). Only a few molecules have been shown to serve as muscle migration guidance factors (Fig. 1.5). The only reported systematic genetic screen identified Kon-tiki (Kon) as a guidance factor involved in the migration of the VL1-4 muscles [15].

Basically, SMs either attach to segment borders (like muscles VL1-4) or attach to sites within the segment (like muscles LT1-3) (Fig. 1.5). There appear to be distinct systems to guide the migration of each type. Tendon cells at segment borders are able to secrete Slit, the ligand for Robo receptors that is expressed in muscles VL1-4 [51]. These muscles also express Grip [52] and Kon [15], two membrane associated proteins involved in migration guidance of these muscles. Grip and Kon are in the same pathway and work together [15], however there is no evidence for a direct link of Grip to Slit-Robo signaling [52]. Tendon cells within the segment do not express Slit. Also, LT1-3 muscles do not express Robos, Grip or Kon but express the atypical receptor tyrosine kinase Derailed (Drl) [53]. Thus, it is proposed that under the influence of Slit-Robo signaling and with the help of Grip and Kon, muscles like VL1-4 migrate towards segment borders (Fig. 1.4). When these muscles reach tendon cells, the Vein protein produced in muscles is able to activate the DER signaling within tendon cells leading to the final differentiation of tendon cells [54, 55], which then secretes protein Thrombospondin (Tsp) required for building stable integrin-mediated myotendinous junctions (Fig. 1.4) [56]. In *slit* or *robo* mutants, muscles VL1-4 often lose the direction of migration and attach to the sites within the segment. Also, in *grip* or *kon* mutants, these muscles produce filopodia extending in

random directions and are not able to migrate properly (Fig. 1.5) [15]. On the other hand, ectopic-expression of Robos or Grip in muscles LT1-3 makes them change their migration path and attach to segment borders instead (Fig. 1.5). Mutations in *drl,* however, often cause muscles LT1-3 to miss the appropriate attachment sites and continue to migrate downwards (Fig. 1.5). The molecular mechanisms behind these phenotypic effects are not clear and remain an open question, although Slit-Robo signaling has been shown to regulate the activity of the Rho GTPases, for example, Rac and Cdc42 during neuronal migration [57, 58], which are the central molecular switches regulating the organization of actin skeleton [59],

Muscle differentiation

Muscle structural genes, like *act57B* and *mhc*, start their expression during the second phase of muscle migration [60], but myofibril assembly begins after the muscles attach to the extracellular matrix (ECM) and stable myotendinous junctions are established [61]. The MADS-box transcription factor, myocyte enhancer factor-2 (MEF2) plays a key role in the process of muscle differentiation[62]. MEF2 recognizes a conserved A/T-rich sequence that has been identified in the control regions of nearly all skeletal and cardiac muscle genes[62]. Actually, *mef2* is expressed in all three-muscle lineages and in *mef2* loss-of-function embryos, all types of muscles are normally specified and positioned, but fail to differentiate and no MHC is present [63]. However, *mef2* is expressed in early mesoderm, long before the expression of muscle structural genes [63]. It has been shown that the Him (holes in muscle) protein represses MEF2 function during early stages of muscle development in *Drosophila* [64]. In vertebrates, there are four *mef2* genes: *mef2*a, -b, -c, and –d. However, only one *mef2* gene (*Dmef2*) exists in *Drosophila* [65], making it easy to study its function in this organism. MEF2 performs its functions by interacting with many co-factor proteins [66] and its broad expression in all three myogenic lineages suggests the existence of tissue-specific cofactors.

Coordination between the general myogenic pathway and the specific pathway in each type of muscle

Although different muscle types have distinct patterns of gene expression, they all share a general myogenic pathway: migrations of myoblasts to specific sites, proliferation, exit from the cell cycle, fusion to form multinucleated myotubes, and differentiation into mature muscle fibers. Expression of *mef2* and a set of similar structural genes in all muscle lineages also indicate the existence of a general differentiation process. However, this general pathway needs to be modified by the combinatorial actions of identity genes in order to produce diverse muscles needed by the organism. How do these identity genes execute their functions and how are the specific functions of identity genes integrated with the general myogenic pathway? Dissection of the regulatory region of *Dmef2* and many muscle structural genes such as *Troponin T* (*TnT*)[67], *Troponin I* (*TnI*)[68], and *Tropomyosin* (*TmI*) [69], provides some insights about these questions.

A complex array of enhancers controls *Dmef2* expression in mesoderm [70]. Among these enhancers, some have Tin binding sites required for its expression in heart muscles [71]; some have Twist binding sites required for its expression in somatic muscles [72], and some direct its expression in visceral muscles [70]. Therefore, *Dmef2* expression is controlled by separate regulatory elements and induced by distinct activators in each muscle lineage, leading to different expression levels of Dmef2. The expression level of Dmef2 in each lineage is critical for development [73]. For example, the visceral muscles can fully differentiate at a lower level of Dmef2 than the somatic muscles [73]. Also, different muscle cells within a muscle lineage require different levels of Dmef2. For example, VA1/2 muscles require a lower level of Dmef2 than others, e.g., LL1 muscle [73]. Correspondingly, the target genes of Dmef2 respond differently to changes in *Dmef2* activity levels: some require higher levels for their expression than others [74]. Thus, *Dmef2* may be expressed at different levels in different muscle types through the combined activities of identity genes, which probably serves as a patterning factor causing the distinct characteristics of different muscle types.

TnT, TnI, and TmI are the three important components of the Troponin complex that is involved in the regulation of calcium-mediated muscle contraction [75]. Interestingly, their regulatory regions all have two separate enhancer elements that contain clustered binding sites for a similar group of transcription factors, including Dmef2, PDP1[76], CF2[77], GATA, and Tin [67]. Both elements are required to achieve the maximal level of gene expression [67, 69, 78]. For example, deletion of one element or increasing the spacing between the two elements in the *TnI* gene is lethal [78]. *Dmef2* mutations cause the loss of *TnI* expression in all muscle lineages, indicating its requirement for all muscle types. However, *tin* mutations lead to loss of *TnI* expression only in the heart and visceral muscle. Also, *biniou* mutations prevent the expression of TnI only in visceral muscles, whereas *lameduck* (*lmd*) mutations eliminate the somatic expression [78]. Biniou is the target of *bagpipe* (*bag*) and is a key regulator of the development of visceral muscles in *Drosophila* [79], while Minc is a key transcription factor for the development of somatic fusion-competent myoblasts [80]. Therefore, in each muscle lineage, the activities of identity genes are directly involved in the production of the muscle structural proteins and they all require *Dmef2* activity.

Therefore, it appears that one way to achieve the coordination is to execute the general pathway through a distinct set of transcription factors that could activate the common set of genes involved in the general pathway of muscle development, but in different way for each muscle lineage or each type of muscle. For example, the activations may happen at different times or to different degrees. Dmef2 is one of the major components of each set of transcription factors [81] that could form transcriptional complexes on the promoters of structural genes [82, 83]. In addition, a splicing mechanism exists to make different contractile protein isoforms with specific contractile properties and functions (e.g., the rate of force generation, the relaxation rate) in different muscles[4]. This mechanism, together with the modulation of the expression levels of these proteins, leads to differential accumulations of these proteins in different muscles, which serve to establish the correct stoichiometry necessary for

the proper function of each particular muscle fiber [4, 84]. It is possible that these "identity genes" also have roles in the specific expression of the protein isoforms, which may act together with Dmef2. For example, in mouse, the cardiac-specific transcription factor GATA-4 recruits MEF2C to the promoter of cardiac α-MHC, which is an isoform of MHC specific to heart muscle cells [85].

A way to fully address these questions would be to identify all the target genes of these identity genes and understand how the transcriptional network is built for a given muscle type [86]. Recent studies have been using high-through techniques, for example, chromatin immunoprecipitation coupled with microarray analysis (ChIP on chip), to reveal the transcriptional network during muscle development [87-89]. Basically, these studies identified a so-called feed-forward circuit that temporally patterns gene expression during muscle development [90]. It is proposed that muscle identity genes directly regulate gene expression throughout the program of muscle development. Some genes are induced immediately, whereas others are induced later, and the products of early activated genes are required to cooperate with the products of identity genes to activate the later genes. Therefore, muscle identity genes do not induce the expression of all the targets simultaneously but do it step by step with different sets of genes expressed at different times [90]. Many targets of the muscle identity gene products are expressed in all muscles, but their expressions are modulated by identity gene products in order to satisfy the specific requirement for a given muscle type [86]. For example, as noted above, *lb* is the identity gene of muscle SBM, and it is able to regulate the expression of both the *if* gene, encoding an adhesion molecule, and the *CG8689/Unc93* gene, encoding a protein required for muscle contraction. These two genes are expressed in all SMs, however their expressions are modulated by Lb in SBM, probably to adjust adhesion at the muscle attachment site according to the strength of contraction produced by muscle SBM [86].

Myogenic transcription network is evolutionarily conserved between invertebrates and vertebrates

Comparisons of the heart development in *Drosophila* and vertebrates reveal that there are many parallels in the molecules and mechanisms that control the identity of cardiac cells and differentiation of cardiomyocytes (Fig. 1.6B) [1]. In both organisms, the heart develops from mesodermal cells that migrate most distally from the original place of invagination during gastrulation. The mature *Drosophila* heart is a simple linear tube that resembles the primitive heart tube of vertebrates prior to the processes of looping and septation that ultimately create a multi-chambered heart (Fig. 1.6A) [91]. Currently, five key genes have been identified forming a core regulatory network that is required for the specification and differentiation of cardiomyocytes (Fig. 1.6B) [92].

The *Drosophila* NK2 homeobox gene, *tin,* is essential for the specification of cardiac cell fates [20, 93], and is a target of combined signaling by Dpp, a member of the bone morphogenetic protein (BMP) family, FGF (fibroblast growth factor), and Wg, which belong to the Wnt superfamily [11, 22, 24, 94]. The GATA factor gene, *pannier* (*pnr*), in *Drosophila* is activated by Tin and works together with Tin to specify cardiac cells [95-99] and induce the expression of *hand* [100], T-box genes [101, 102], and *mef2* [99]. Both *hand* and T-box genes are required for normal patterning of the cardiac cell [103, 104]. The existence of autoregulatory and cross-regulatory interactions among these genes builds an efficient and stable network that serves to maintain the cardiac phenotype once it has been induced by upstream signals (Fig. 1.6B). In vertebrates, a similar network contributes to the early specification of cardiac cells [1]. For example, BMP signaling is also responsible for the activation of Nkx2.5 in the cardiogenic mesoderm [105]. Also, a mutually reinforcing positive feedback loop between Nkx2.5 and GATA factors maintains their expression in the cardiac lineage. Together with the expression of MEF2, these factors control the activation of cardiac structural genes. Thus, the differentiation of cardiomyocytes is associated with a conserved regulatory network that contains homologous transcription factors in both *Drosophila* and vertebrates.

vg is known as a "selector gene" for wing development of *Drosophila*, as loss of *vg* function leads to lack of wing tissue and ectopic expression of *vg* induces outgrowth of wing tissue [106, 107]. Vg interacts with Sd to form transcription complexes directing wing development [108], in which Sd provides the DNA binding domain and nuclear localization signal (NLS) [109, 110], whereas Vg provides the activation domain [111]. Homologues exist in vertebrates for the *Drosophila* Sd and Vg proteins and these homologues have been shown to play a role in muscle development. For example, in the mouse, ablation of TEF-1[112], the homologue of Sd, results in fetal death due to a defect in cardiac maturation[113]; and Vestigial-like 2 (Vgl-2), one of the homologues of Vg, can go into nuclei of skeletal muscle cells at the start of differentiation and augment myosin heavy chain expression [114]. Remarkably, a transgene containing human TEF-1 or Vgl-1 can partially substitute for *sd* or *vg* during the *Drosophila* wing development [111, 115], suggesting a high degree of functional evolutionary conservation among these genes.

I started this project by asking whether Sd and Vg are the cofactors of Dmef2 during the embryonic muscle development of *Drosophila*, since the homologues of them play a role in the muscle development of mouse. In chapter 3, I addressed this question by breaking it down into four parts: 1) Are *sd* and *vg* co-expressed in muscle cells? 2) Do they have functions during muscle development? 3) Are there interactions among Sd, Vg, and Dmef2 both in vitro and in vivo? 4) Are these interactions functional during muscle development? When I was testing the interactions between Sd and Vg, I noticed that there were up-shifted bands in Vg Western blots. Therefore, in chapter 2, I also examined post-translational modifications in Vg and the roles of these modifications in wing development. In chapter 4, I further study the role of *vg* in muscle development and found that Vg plays a role in the establishment of stable inter-muscular adhesions during late stages of muscle development.

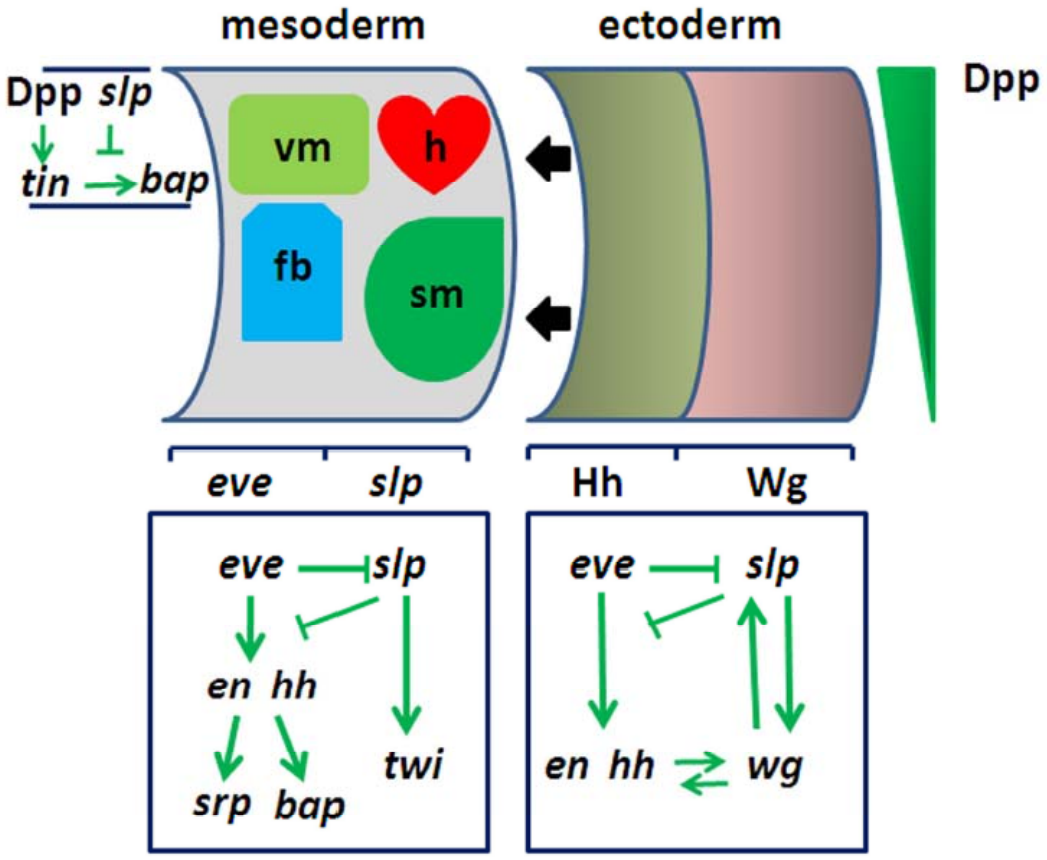

Figure 1.1: Schematic representation of the early signaling events and gene activities involved in the early mesoderm subdivision of a segment, modified from Baylies et al., and Riechmann et al. [2, 18]. Gene activities and interactions are shown according to different germ layers or different domains of the same germ layer. Positive interactions are indicated by arrows, negative interactions by lines ending in a bar; anterior is to the left and dorsal is up. See text for details.

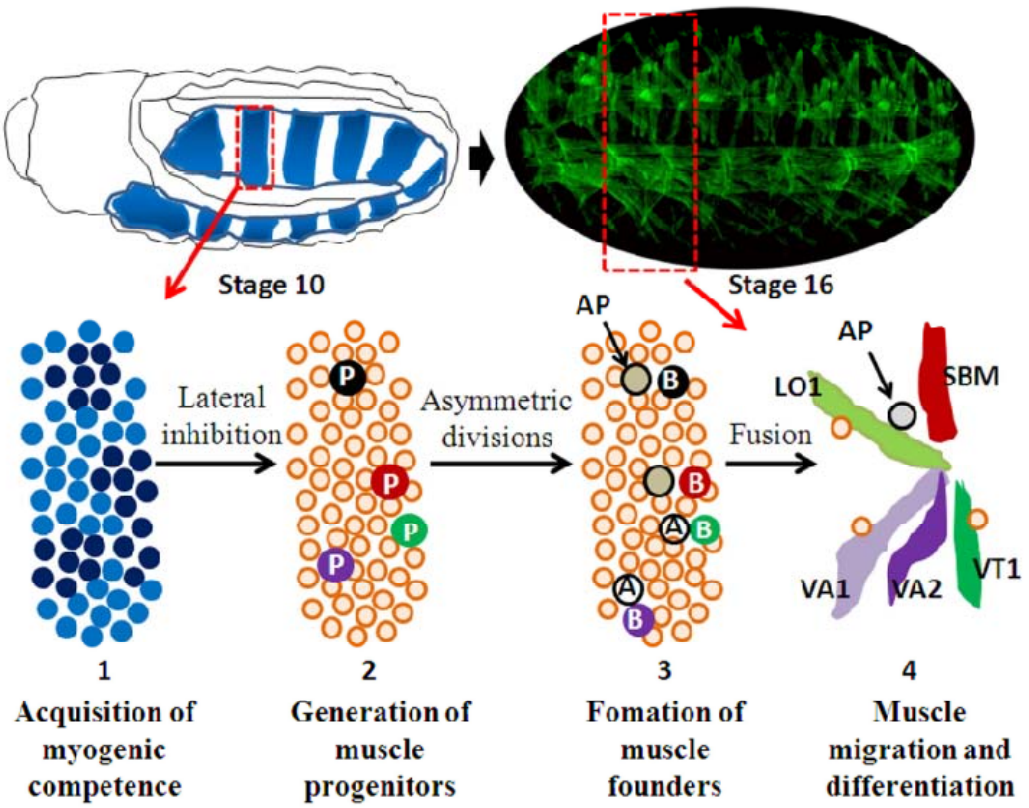

Figure 1.2: Schematic representation of the successive steps in the pattern formation of *Drosophila* SMs, adapted from Baylies et al. [2]. Muscle progenitors (P) are singled out from a group of myogenic competent cells expressing *l'sc* (dark blue) and divide asymmetrically to generate a pair of muscle founder cells or a founder and an adult muscle precursor (AP). In either case, the two daughter cells are assigned different fates (A and B or B and AP). The founders fuse with FCMs (brown) and migrate to their attachment sites in the epidermis. The muscle pattern of a stage 16 embryo was visualized by Actin staining on the top right. LO (Lateral Oblique), SBM (Segment Border Muscle), VA (Ventral Acute), VT (Ventral Transverse).

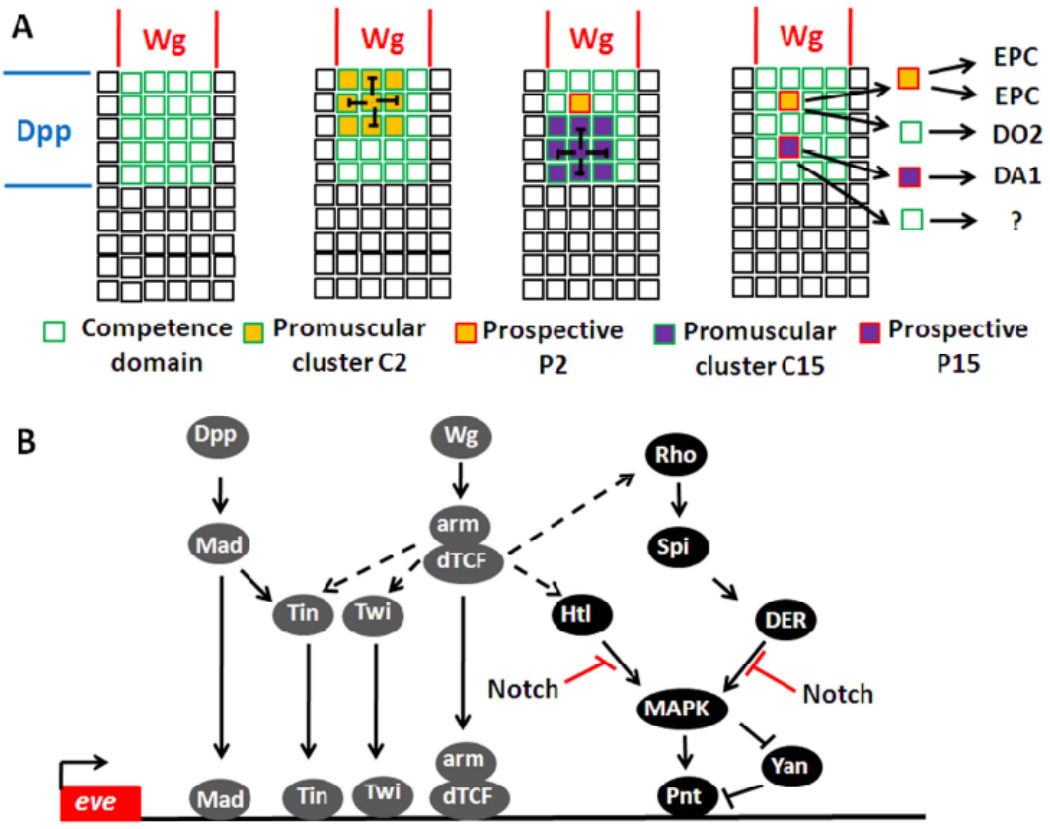

Figure 1.3: Models showing the specification of two muscle progenitors, P2 and P15 (A) and the integration of various signaling and transcription factors on a single enhancer of the *eve* gene (B), adapted from Frasch [30], and Halfon et al. [48]. See text for details.

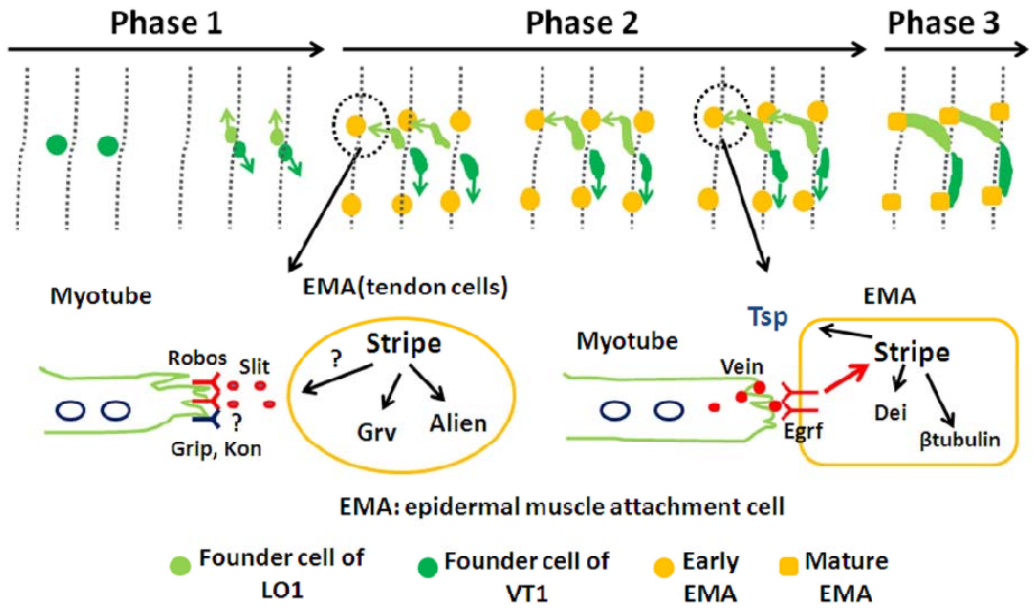

Figure 1.4: Schematic representation of the three phases of SM migration and molecules involved in the process of muscle migration and attachment, modified from Schnorrer et al. [6]. The Slit-Robos signaling works together with other muscle guidance factors (Grip and Kon etc.) to direct the migration of muscles to their attachment sites. When muscles reach the tendon cells, Vein protein secreted by muscles activates DER signaling within the tendon cells, which then become fully differentiated and start to produce Tsp used to build adhesion junctions between muscles.

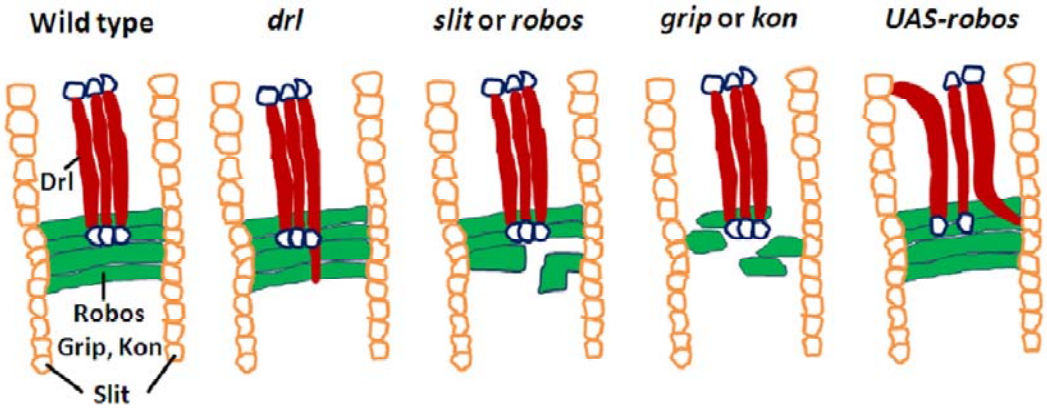

Figure 1.5: Models for control of SM migration through guidance factors, modified from Schnorrer et al. [6]. Slit is secreted only by tendon cells at segment borders (brown) and muscles VL1-4 (green) produce Robos, Grip, and Kon. Muscles LT1-3 (red) produce Drl instead and attach to tendon cells inside each segment (dark blue). See text for details.

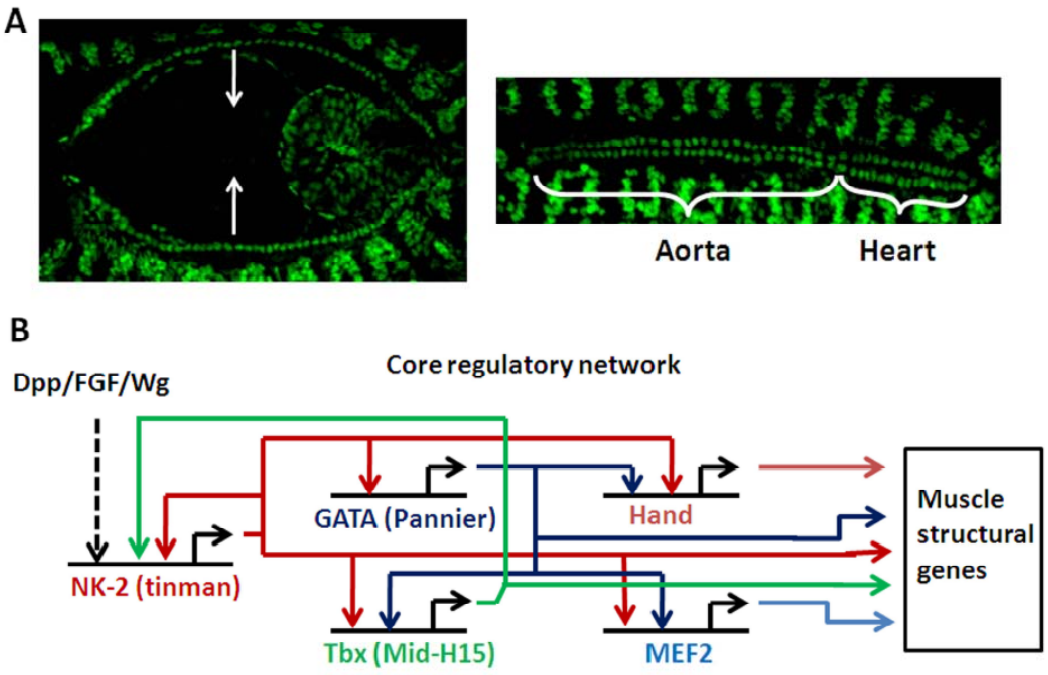

Figure 1.6: **A,** The *Drosophila* heart is a tube-like structure consisting of two lines of heart muscle cells that migrate from each side of the embryo. **B,** A simplified diagram of the core transcriptional network of *Drosophila* heart development, adapted from Olson et al. [92]. Notice the colors are assigned according to different genes. See text for details.

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Chapter 2: Vg phosphorylation and its role in wing development

Introduction

Drosophila wing development requires the combination of cells responding to external signals and the activation of internal "selector genes" which serve to define a wing identity. Selector genes usually encode transcription factors that coordinate the developmental program of a body structure from relatively undifferentiated precursor cells [1-3]. *Drosophila* imaginal discs, consisting of layers of epithelial cells, are patterned during late larval development and subsequently develop into adult tissues during pupariation. The *vestigial* (*vg*) gene is the primary selector gene for specification of the wing blade from the corresponding imaginal disc (Fig.2.1). Loss of *vg* function leads to a loss of wing tissue while ectopic expression of *vg* causes wing outgrowth [4, 5]. Two separate enhancers within the *vg* gene induce expression within the pouch area of the wing imaginal disc (Fig.2.1). The boundary enhancer (BE) directs *vg* expression along the dorsoventral (D/V) boundary and the quadrant enhancer (QE) induces the expression in the remaining areas of the wing pouch [5, 6]. The BE responds to a Notch (N) signal produced along the D/V boundary through the interactions between dorsal Apterous -expressing cells and ventral Ap-negative cells [6, 7], while QE activation requires a Decapentaplegeic (Dpp) signal from the anteroposterior (A/P) boundary and a Wingless (Wg) signal emanating from the D/V boundary [5, 8, 9]. BE activation is a prerequisite for QE activity and Vg itself is involved in the auto-regulatory activation of QE [5, 10, 11].

Vg-expressing cells at D/V border send a short-range feed-forward signal to neighbouring cells, which activates QE-dependent Vg expression in a process that also requires Wg signalling [9]. *Drosophila* Epidermal Growth Factor Receptor (DER) signalling may also be involved in this feed-forward regulation process [12]. Within the wing disc cells, Vg is responsible for activating a wing-specific combination of downstream genes. This process requires an obligate integration of Vg activity and other signalling effector proteins [2]. For example, expression of *cut* along the D/V boundary requires the physical presence of both

Vg and Suppressor of Hairless [Su(H)] (the effector protein of Notch signaling) on the regulatory region of *cut* gene. However, expression of the wing blade patterning gene, *spalt* (*sal*), requires the binding of both Vg and Mother Against Dpp (MAD, the effector protein of Dpp signaling) to the enhancer of *sal* gene [2].

The most well-known wing-specific Vg co-factor is Scalloped (Sd), a member of the TEA/ABAA domain-containing family of transcription factors [13]. Sd is responsible for transporting Vg into nuclei of cells and mediates DNA binding of the Sd/Vg complex through the TEA domain [10, 14]. Vg has two domains potential for activation and together with Sd, forms a transcriptional activator complex [15]. When the proteins form a complex, Vg binding switches the DNA-target selectivity of Sd to wing specific genes [16]. Sd also has a role in the development of external sensory organ (bristles) along the anterior wing margin [13]. This role is executed through activating *cut* expression and maintaining Wg expression along the D/V boundary by the Sd/Vg complex [2, 14, 17]. Cut is required for the development of bristles along the entire wing margin [18]. A Wg signal is necessary and sufficient for the expression of *senseless* (*sens*), a gene required for specification of the innervated bristles along the anterior wing margin [19, 20]. In addition to a role in wing development, *vg* is essential for specification of certain embryonic somatic muscles and differentiation of the adult indirect flight muscles [21, 22].

Both Vg and Sd have significant tissue specification roles other than wing identity. Therefore, there must be cell-specific modifications to their activities. The most common protein modifications involve attachment of small molecules to specific amino acids. SUMO (small ubiquitin-related modifier) is a small ubiquitin-like protein that can modify other proteins through a covalent linkage between SUMO and the lysine side chains in the target proteins, which is mediated by the SUMO conjugating enzyme Ubc9 [23]. Vg was found to be sumoylated and this has been shown to affect the activity of Vg during overall wing development [24]. However, sumoylation has not been related to any other signalling pathway in wing development. In this study, evidence is provided to

indicate that Vg undergoes a second protein modification- phosphorylation, and this affects specific cell fate changes during wing development.

Materials and methods

Cell culture and Transfections

Drosophila S2 cells were grown at 25°C in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. Transfections were carried out using dimethyldioctadecyl-ammonium [25]. Approximately 10^7 cells were transfected with 1μg of each relative expression constructs.

Drosophila Strains

All crosses were done at 25°C. The *vg*-Gal4 strain was a gift from S. Carroll and the UAS-*HA*-*vg*, UAS-*HA*-vg^{S215A}, and UAS-*HA*-vg^{S215E} strains were made in our laboratory for this study.

Plasmids

All expression vectors for transfection of S2 cells were created by Gateway Technology (Invitrogen) and the Drosophila Gateway destination vectors (Terrence Murphy, Carnegie Institute of Washington, Baltimore, MD). The *vg*::*sd* fusion gene and *vgΔSID* constructs have been described previously [14, 26]. The NLS sequence from the SV40 gene was cloned by PCR and added to the 5' end of *vg* . Inverse PCR was performed using primers that amplify specific portions of the *vg* coding region shown in Figure 4 to create deleted Vg expression constructs. Site-directed mutagenesis was performed to make point mutations in *vg* gene according to the manufacturer's directions (QuickChange Site-Directed Mutagenesis Kit, Stratagene).

Immunoprecipitations and Immunoblotting

S2 cells transfected with expression constructs containing the heat-shock promoter, and protein expression was induced by heat shocking cells for 35 min at 37°C, or alternatively transfected with expression constructs containing the *act5c* promoter. Cells were harvested 1-2 h after induction, washed one time in PBS, and resuspended in RIPA (radio-IP) buffer (50mM Tris pH 8.0, 150mM NaCl, 1.0%

NP-40, 0.5% Deoxycholic acid, 0.1% SDS, and protease inhibitor cocktail). The lysate was then incubated for 15 min at 4°C with agitation, centrifuged for 15 min at 13.2K rpm at 4°C and the supernatant was then transferred to a fresh tube. Co-IP reactions were carried out on 200µl of supernatant (600µl supernatant from 25 cm^2 flask of cell culture) using 8µl anti-FLAG M2-agarose (Sigma) in 500µl RIPA buffer. The agarose beads were incubated for one hour at 4°C with rocking, centrifuged for one min at 1.4K rpm at 4°C, and washed six times by vortexing in 500µl RIPA buffer. Primary antibodies for immunoblotting were: mouse anti-FLAG (1:1000; Sigma), rat anti-HA (1:400; Roche), Rabbit anti-Vg (1:400) [4], and rabbit anti-Myc (1:1000; Cell Signaling). Secondary antibodies were: goat anti-mouse Alexa680 or IRdye800 (1:5000; Invitrogen); goat anti-rabbit Alexa 680 or IRdye800 (1:5000; Invitrogen); goat anti-rat IRdye800 (1:5000; Invitrogen).

Fluorescence Microscopy

Wild-type and overexpression imaginal wing discs were dissected and stained with various antibodies as described previously [27]. The following primary antibodies were used at the indicated concentrations: rat anti-HA (1:200; Roche); mouse anti- Achaete (Developed by Jim Skeath and obtained from the Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa City, IA, 1:10). Corresponding Alexa488, Alexa568-conjugatged secondary antibodies (Invitrogen) were used to recognize the primary antibodies. Images were obtained using a spinning disk confocal system (Ultraview ERS; PerkinElmer) mated to a CS9100-50; camera (Hamamatsu) and an Axiovert 200M microscope (Carl Zeiss MicroImaging) using Ultraview ERS software (Version 2, PerkinElmer) and assembled with Adobe Photoshop (Version CS, Windows XP).

Results

Sd mediates Vg phosphorylation in S2 cells.

Western blot analysis of S2 cells expressing HA-tagged Vg revealed a band that ran much slower than 3xHA-Vg, and an extra slower migrating band when Sd

was co-expressed with 3xHA-Vg (Fig.1.2A). This multiple band pattern suggested there might be post-translational modifications to Vg. To determine if these modifications were caused by phosphorylation, a lysate from S2 cells expressing 3xHA-Vg was treated with λ-phosphatase which would remove all phosphates. After treatment, all slower migrating bands except one disappeared (Fig.2.2B), suggesting that Vg is phosphorylated in S2 cells. Since it was possible that the 3xHA tag affected Vg motility, a non-tagged form of Vg was similarly expressed in S2 cells. This untagged form of Vg produced the same pattern of slower migrating bands on a western blot as 3xHA-Vg (Fig.2.2C). Since the slower migrating Vg band became much more prominent in the presence of Sd, we tested if interaction with Sd was required to enhance Vg phosphorylation.

Vg requires Sd for entry into nuclei [10, 26]. Therefore, it was possible that the requirement for Sd during Vg phosphorylation is through a nuclear localized kinase. To test this possibility, the well characterized Nuclear Localization Signal (NLS) peptide of SV40 was added to the N-terminal of Vg. Nls-Vg is no longer dependent upon Sd for concentration within the nuclei (data not shown). Despite being able to move into the nucleus independently of Sd, Vg still requires the presence of Sd for Vg phosphorylation (Fig.2.2C). The TEA domain of Sd is the critical region required for Vg phosphorylation, as a fusion protein where full length Vg is fused to the TEA domain of Sd was modified even in the absence of Sd (Fig.2.2C). Confirming a role for Sd in Vg phosphorylation, a truncated form of Vg without the Sd interaction domain (SID) did not show significant modification even in the presence of Sd with or without a NLS (Fig.2.2C). Therefore, a physical binding with Sd is required for Vg phosphorylation, and a potential kinase interaction is enhanced by the presence of the Sd TEA domain.

Vg sumoylation requires prior phosphorylation

It has been shown previously that sumoylation of Vg enhances Vg function and sumoylated Vg can be detected in S2 cells [24]. We confirmed that the phosphastase resistant slow-migrating band represents the sumoylated isoform by co-expressing 3xFLAG-SUMO and 3xHA-Vg in S2 cells. The phosphatase resistant Vg band is recognized by both anti-FLAG and anti-HA antibodies

(Fig.2.3A); Further, 3xFLAG-SUMO co-immunoprecipiatated (CoIPed) with Vg (Fig.2.3B); Finally, the SUMO conjugating enzyme Ubc9 was found to colocalize with Vg in S2 cells (Fig.2.3C). However, it seems that sumoylation of Vg does not need the interaction with Sd.

Sumoylation happens at a non-consensus site in Vg, making it difficult to identify the actual site [24]. Seven Vg deletions were made to investigate the modification sites in Vg. If a deleted form of Vg does not show the slower migrating band on Western blot, it is probable that the deleted region contains the modification site (Fig.2.4A). Analysis of these Vg deletions revealed that only one (VgΔ5) produces a single band (Fig.2.4B), suggesting that the deleted region in VgΔ5 may contain multiple modification sites. There is one conserved domain containing a consensus phosphorylaiton site for MAP kinase (serine 215) and one lysine (K257) site in this region (Fig.2.4C). Therefore, each of these amino acids was mutated. Surprisingly, mutagenesis of the serine at position 215 to alanine led to loss of both slower migrating bands, while mutagenesis of the lysine at position 257 to arginine led to the same pattern of band migration as seen in wild type (Fig.2.4D). Serine 215 was also switched to glutamic acid to mimic phosphorylated serine (Vg^{S215E}). The electrophoresis mobility of Vg^{S215E} was the same as wild type Vg (Fig.2.4C), suggesting that the slow-migrating band in a Vg^{S215E} Western blot may represent both phosphorylated and sumoylated Vg.

Vg^{S215A} causes ectopic growth of sensory bristles along the posterior wing margin.

To investigate the functional consequence of Vg phosphorylation during development, HA tagged Vg^{S215A} was expressed via *vg*-Gal4 in wild type wing discs. This Gal4 driver is under the control of the *vg* boundary enhancer and shows expression solely in cells immediately along the D/V boundary of the wing disc eight hours after the beginning of second larval instar [6]. Expression of Vg^{S215A} in these cells of the wing disc, that also express wild type Vg, led to adult wings with only mild defects including loss of some sensory bristles along the anterior margin. This phenotype mirrors what happens when unmodified HA-tagged Vg is expressed in the same cells. To assay the function of Vg^{S215A} in the

absence of endogenous Vg, the UAS-*HA-vg*^{S215A} transgene was then expressed via *vg*-Gal4 in homozygous *vg*^{null} flies that produce no detectable amounts of Vg [28]. Vg^{S215A} was able to significantly rescue the wing loss phenotype caused by the *vg*^{null} mutation. However, the resulting wings had ectopic sensory bristles along the posterior margin (Fig.2.5D-D^2). This phenotype seems to be solely the result of Vg^{S215A} expression, as similar expression of UAS-*HA-vg* or UAS-*HA-vg*^{S215E} via *vg*-Gal4 led to adult wings that had only a few sensory bristles growing along the anterior margin (Fig.2.5B-C). In addition, in flies where the *vg*^{null} mutation was rescued by expression of UAS-*HA-vg* or UAS-*HA-vg*^{S215E}, the resulting wings had a similar vein pattern to wild type (Fig.2.5A-C). However, in flies where the *vg*^{null} mutation was rescued by *vg*^{S215A}, the adult wings developed only two veins in the wing blade (Fig.2.5E).

To determine when a potential fate change was occurring, developing third instar wing imaginal discs were stained with an antibody that specifically recognizes Achaete (Ac) [29], a marker for sensory organ precursor (SOP) cells that will develop into the sensory bristles in adult wings. Wild type third-instar wing imaginal discs have two lines of SOPs along the anterior margin of the wing pouch region (Fig.2.6A). Expression of either UAS-*HA-vg* or UAS-*HA-vg*^{S215E} via *vg*-Gal4 in *vg*^{null} wing discs produces similar results with rare SOPs appearing along the entire margin of presumptive wing pouch region of the wing disc (Fig.2.6B and D). This correlates with the adult wing bristle phenotype produced by these transgenes (Fig.2.5B-C). However, expression of UAS-*HA-vg*^{S215A} via *vg*-Gal4 in *vg*^{null} wing discs had SOPs growing only along the anterior margin of the wing pouch (Fig.2.6C). The apparent induction of sensory bristle precursors at the anterior margin at this early stage does not match the phenotype of adult wing bristles along both margins when this transgene is expressed (Fig.2.5D).

The kinase p38b interacts with Sd and inhibition of p38b activity decreases Vg phosphorylation .

Deletion analysis of Vg identified one region that is potentially phosphorylated. This region contains a Pro-Asp-Ser-Pro sequence that matches the consensus sequence (Pro-Xaa-Ser/Thr-Pro) for MAP kinase phosphorylation

[30]. *Drosophila* p38b has been shown to be a MAP kinase involved in Decapentaplegic (Dpp) signal transduction during wing morphogenesis [31]. Decreasing p38b expression in wing discs produced a similar wing phenotype to *dpp* mutants in which veins in the wing blade fused together leading to only two major veins [31]. Since expression of UAS-*HA*-vg^{S215A} via *vg*-Gal4 in vg^{null} flies also produces adult wings with only two veins (Fig.2.5E), p38b was tested as the most likely kinase that induces Vg phosphorylation. The kinase activity of p38b can be inhibited in S2 cells by using SB2030580 (4-(fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole), a specific inhibitor of p38 MAPK [32, 33]. SB2030580 inhibition of p38b decreased Vg phosphorylation in S2 cells in a dose-dependent manner (Fig.1.7A). Since Sd mediates Vg phosphorylation (Fig.2.2), we further examined if there is interaction between Sd and p38b. CoIP analysis showed that these two proteins interact in S2 cells (Fig.1.7B).

A Vg::SdTEA fusion protein is significantly modified in the absence of additional Sd expression (Fig.2.2C), indicating the kinase acting on Vg may interact with Sd TEA domain. MAP kinases usually bind with their target substrate through a docking domain (D domain) with a consensus sequence of $(R/K)^{2-3}$-X^{1-6}-Φ_A-X-Φ_B (Φ_A and Φ_B are hydrophobic residues, X is any residue) [34]. Vg does not contain any region similar to the D domain consensus. Interestingly, Sd has two domains corresponding to this consensus sequence within the TEA domain (Fig.2.8A). Thus, it appears that Sd is recruiting p38b to the Vg/Sd complex. Sd deletions and mutants were then tested for their effects on Vg phosphorylation (Fig.2.8A-B). SdΔ1 and SdΔ2 (Fig.2.8A) delete distinct regions of the TEA domain with SdΔ2 removing the two potential D-domains. Vg and the NLS-Vg fusion were then co-expressed with SdΔ1 and SdΔ2 (Fig.2.8B). In cells expressing SdΔ1 or SdΔ2, there is a significant reduction in the slower migrating bands that have been shown to be due to Vg phosphorylation (Fig.2.8B). SdΔ2 was not able to induce phosphorylation of Vg even if Vg is forced to enter the nucleus by fusion to a NLS (Fig.2.8B). Sd mutants where the RK or IQ consensus amino acid pairs were mutated to AA or SN were also tested. Only

Sd158RK-SN showed an appreciable decrease of the slower migrating band shown to be due to Vg phosphorylation (Fig.2.8B). Fluorescent protein fusions of SdΔ1, SdΔ2, and Vg were co-expressed in S2 cells to check the localization of these proteins. In cells expressing GFP-SdΔ1, RFP-Vg can be seen to form foci in the nucleus whereas in S2 cells expressing GFP-SdΔ2, RFP-Vg does not appreciably appear in the nucleus (Fig.2.8C). Therefore, it appears that p38b is recognizing the second D-domain consensus within the Sd TEA region for docking and phosphorylation of Vg (Fig.2.8D).

Discussion

Vg is a primary selector for wing development and its activity needs to be regulated carefully to direct the development of this complex tissue [2]. Post-translational modifications (*e.g.* phosphorylation and sumoylation) are used commonly in cells to differentially control the activity of a protein within a specific subset of cells in response to extracellular signals. In this study, phosphorylation of Vg via interaction with Sd appears to be required for specification of bristles along the wing margin and TGF-β pathway (Dpp)-based definition of the venation pattern. Replacing endogenous Vg with the non-phosphorylable Vg^{S215A} specifically caused an ectopic appearance of sensory bristles along the posterior margin during wing development, indicating that phosphorylation of Vg is required to help define cell response based on their position within the imaginal disc.

Ser 215 was identified as the likely p38b phosphorylation site in Vg. Mutating Ser215 to Ala led to a coincident loss of sumoylation in Vg (Fig.2.4 and A.8). This would suggest strongly that phosphorylation at this site is also required for subsequent Vg sumoylation. CoIP analysis showed that all forms of Vg were pulled down with SUMO, including non-modified Vg (Fig.2.3B). This heterogeneity is likely due to the known formation of heterotetrameric Sd/Vg complexes in *Drosophila* cells [16]. Previous phosphorylation at serine adjacent to a lysine has been shown to be necessary for sumoylation in some proteins [35, 36]. However, it appears that this simple model is not the case for Vg, as mutation

of the two lysine sites close to S215, K180 and K258, indicates that they are not sumoylated [24]. It is possible that protein folding brings an alternative sumoylated lysine adjacent to S215. It appears that the sumoylated lysine is within the VgΔ5 deletion, as this form of Vg was not sumoylated (Fig.2.4). VgΔ5 removes only one lysine, K258. However, mutating K258 to Arg failed to block Vg sumoylation as detected by western blot (Fig.2.4C). It is possible an alternative SUMO E3 ligase links SUMO to the non-consensus site in Vg [23]. Thus, while our data confirmed the previous result that Vg was sumoylated in S2 cells [24], we were not able to verify the amino acid site within Vg where this modification occurs.

Replacing endogenous Vg activity with Vg^{S215A} produced ectopic sensory bristles along the posterior wing margin, suggesting a role for Vg phosphorylation in cell fate determination in this region. Since Vg^{S215A} is neither phosphorylated or sumoylated (Fig.2.4), it is difficult to discern which modification (or both) is required for this Vg function. However, previous studies of Vg sumoylation showed that Vg activity was primarily enhanced during wing development and no fate transformation phenotype was seen in flies heterozygous for mutations in *vg* and genes in the sumoylation pathway [24]. Therefore, it is likely that loss of Vg phosophorylation is the cause of the ectopic bristle phenotype. Conversely, when Vg is replaced with Vg^{S215E} in cells along the wing margin, sensory bristles are not seen along the anterior or posterior. The Vg^{S215E} mutation would mimic phosphorylated Vg and Western blot analysis indicated it was sumoylated in S2 cells. Since Vg^{S215E} produced a phenotype similar to that of overexpressing wild type Vg, this would suggest that Vg is alternatively modified by phosphorylation during wing development, but there must be a time window when unmodified Vg is required for the specification of sensory organ precursors (SOPs) at the anterior margin.

One pitfall of using the GAL4/UAS system for expressing transgenes is the slight lag between expression of the GAL4 protein and subsequent activation of the UAS-transgene. Therefore, it is possible that expression of a transgene via *vg*-Gal4 may miss a critical time window, so that there is no proper specification of

SOPs, whereas forced expression of Vg^{S215A} via *vg*-Gal4 would not miss this time window. The relative level of Vg expression may also be a factor affecting the phenotype caused by expression of these different *vg* transgenes, as balanced activity between Vg and Sd is necessary for proper wing development [10], and Sd is required for sensory organ differentiation [13]. Therefore, expression of UAS-*HA*-*vg* or UAS-*HA*-vg^{S215E} via *vg*-Gal4 may break the balanced activity between Vg and Sd leading to loss of sensory bristles, whereas expression of UAS-*HA*-vg^{S215A} did not. However, it is unlikely that small changes in the level of Vg expression can cause the differences in phenotypes observed between the vg^{S215E} and vg^{S215A} transgenes. Expression of UAS-*vgRNAi* or UAS-*vg* via various Gal4 drivers failed to cause ectopic growth of sensory bristles along posterior wing margin [37]; suggesting that differences in Vg activity level is not causing the fate transformation caused by Vg^{S215A}.

The fate transformation induced by UAS-*HA*-vg^{S215A} appears to be occurring later than third larval instar. Wing discs isolated from larvae expressing vg^{S215A} do not have Ac expression along the posterior D/V boundary. Since Ac is the early marker for SOPs, the requirement for non-phosphorylated Vg in this process is likely downstream of initial Ac/Sc specification during the development of sensory bristles. The primary determinant of posterior cell identity of wing is expression of the homeobox gene *engrailed* (*en*) [38]. DER signaling has been shown to cooperate with En in the specification of posterior identity, as decreasing DER signaling in the posterior wing compartment results in the posterior to anterior transformation of the wing margin [39]. It is possible that Vg is phosphorylated by the MAPK downstream of DER signalling. However, knocking down the expression of *rolled,* the *Drosophila* homologue of Erk-1*,* failed to decrease Vg phosphorylation in S2 cells using RNAi (data not shown). During later differentiation of anterior and posterior bristles, only the former are innervated [40]. Posterior bristles do have the potential to generate neurons, but the neuron cells or their precursors undergo apoptosis during normal wing development [20]. Alternatively, it is possible that the requirement for

phosphorylated Vg is to regulate apoptosis in developing neurons during the posterior bristle development.

There is a clear difference in the requirement for modified or un-modified Vg in wing discs. Expression of UAS-*HA-vg* or UAS-*HA-vg*^{S215E} via *vg*-Gal4 in *vg*^{null} wing disc produces wings that have vein patterns similar to wild type, whereas expression of UAS-*HA-vg*^{S215A} produces wings with vein pattern similar to *dpp* mutants. p38b has been shown to work downstream of Dpp signaling during wing morphogenesis [31]. Thus, the data supports a model where p38b is a primary kinase for Vg phosphorylation via interaction between Sd and p38b, which is supported by the observation that inhibiting p38b activity decreases Vg phosphorylation in S2 cells. However, inhibiting p38b activity by expressing a dominant-negative allele of p38b or applying SB203580 in wing disc failed to produce a fate transformation phenotype [31]. It is possible that there is still residual p38b activity in these wing discs or there is an additional Vg kinase active in the cells along the posterior D/V boundary.

We have identified a phosphorylated site within Vg and the potential kinase (p38b) that regulates this event. The p38b kinase needs to interact with Sd to get access to the phosphorylation site in Vg (Fig.2.8D). The TEA domain of Sd is found to be necessary for Vg phosphorylation and a potential docking site for MAP kinase in this region is important for Vg phosphorylation. Thus, the role of Sd is not only to act as a binding partner of Vg supplying a DNA interaction domain but also plays a direct role in Vg modification. Since the data indicate that the development of sensory bristles seems to require non-phosphorylated Vg, it is possible that binding of Sd to Vg is specifically regulated at the D/V boundary during wing development.

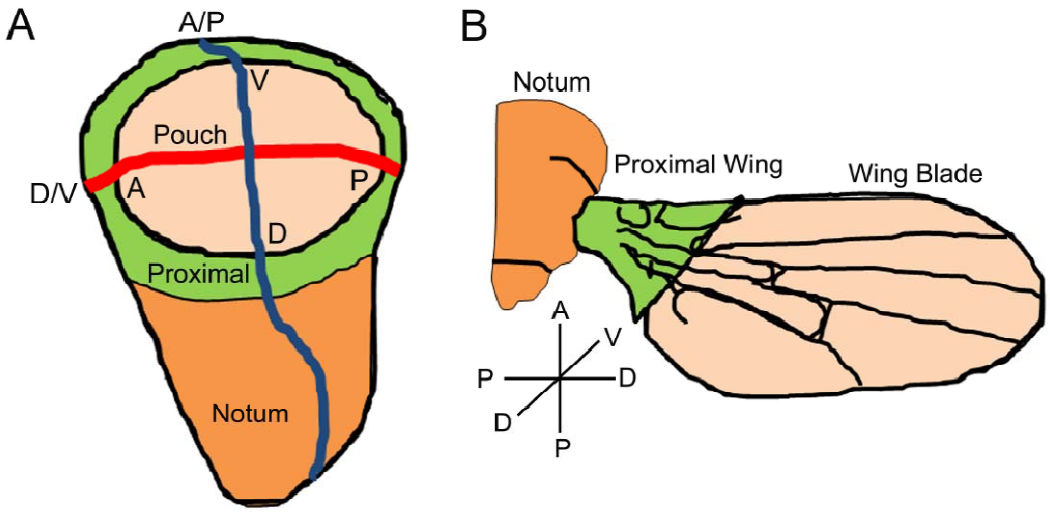

Figure 2.1. A, Fate mapping indicates the regions of the third larval instar wing disc that give rise to the adult wing; colors are coordinated to show the approximate origins of the wing. Compartment boundaries, wing pouch, proximal wing, and notum regions are also indicated. A/P, anterior-posterior compartment boundary; D/V, dorsal-ventral compartment boundary. **B,** A diagrammatic representation of an adult wing indicating the three main domains of the P/D axis: the notum, the proximal wing, and the wing blade. P/D, proximal-distal axis.

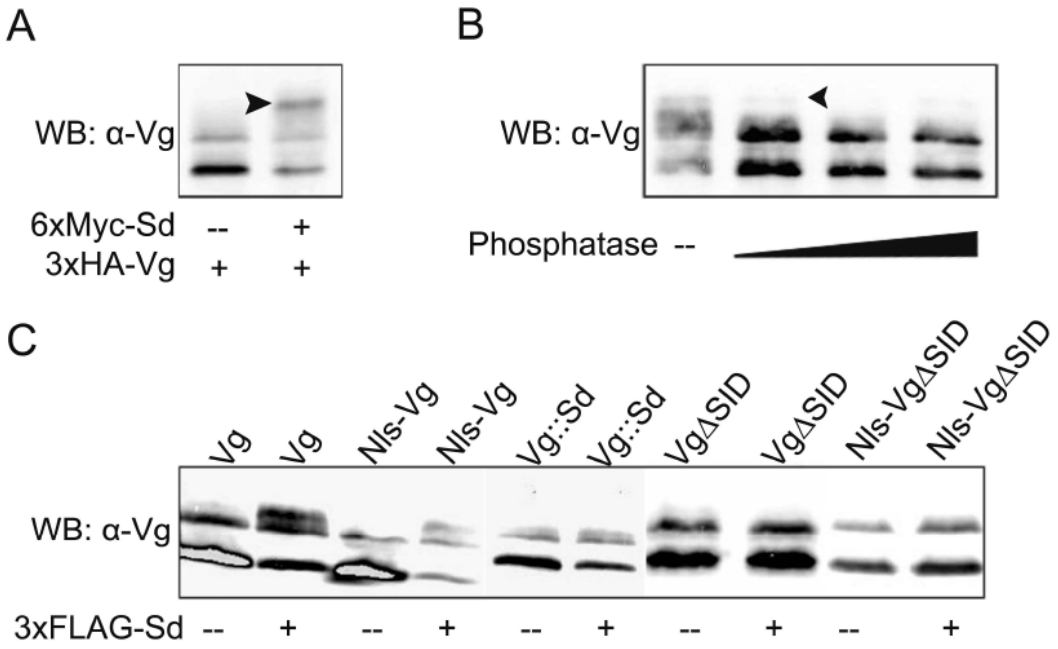

Figure 2.2. Sd is required for Vg phosphorylation in S2 cells. **A,** When expressed in S2 cells, a prominent slower-migrating band is observed on western blots when Sd was co-expressed with epitope-tagged Vg (arrowhead). **B,** The slower migrating band disappeared proportionally to increasing concentrations of non-specific phosphatase (arrowhead). **C,** Full length Vg or modified Vg were expressed in S2 cells with or without the co-expression of FLAG-tagged Sd. When Vg is fused to a nuclear localizing signal (NLS) peptide, Sd is still required for the presence of the slower migrating (phosphorylated) band. Vg::Sd, a fusion protein of Vg with the TEA domain of Sd has a prominent slower-migrating band even in the absence of Sd. However, when the SID, Sd interaction domain is deleted from Vg, slower migrating band is no longer present, even if a NLS peptide is added.

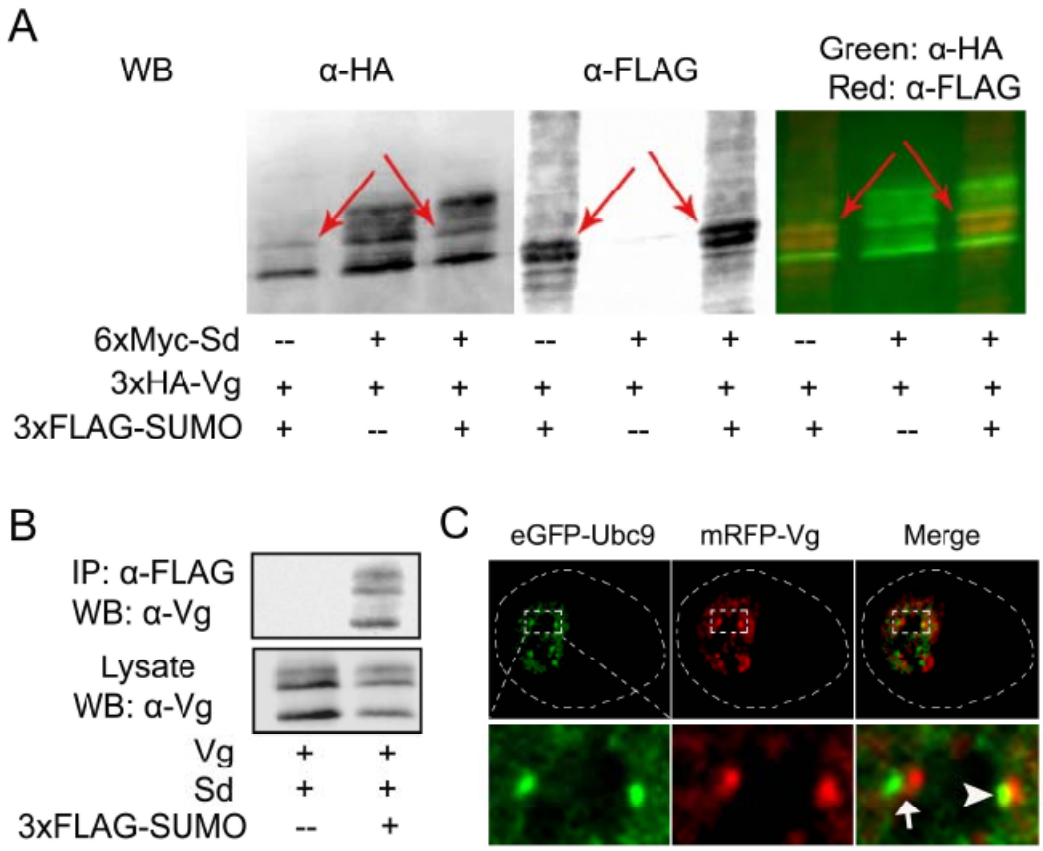

Figure 2.3. Vg is sumoylated in S2 cells independently of Sd. **A,** 3xHA tagged Vg and 3xFLAG tagged SUMO were detected on the same membrane using anti-HA and anti-FLAG antibody respectively. The first slower migrating band was strongly stained through both anti-HA and anti-FLAG antibodies (arrows). **B,** Immunoprecipitation of FLAG-tagged SUMO from S2 cells also co-immunporecipitated Vg. **C,** S2 cells were transfected with eGFP tagged Ubc9 and mRFP tagged Vg. Confocal imaging of these cells revealed that these two protein formed separate foci in the nuclei, and these foci are either adjacent (arrow) or co-localized (arrowhead). Broken lines show the edge of a cell and the pictures in the bottom are the close-ups of the boxed area.

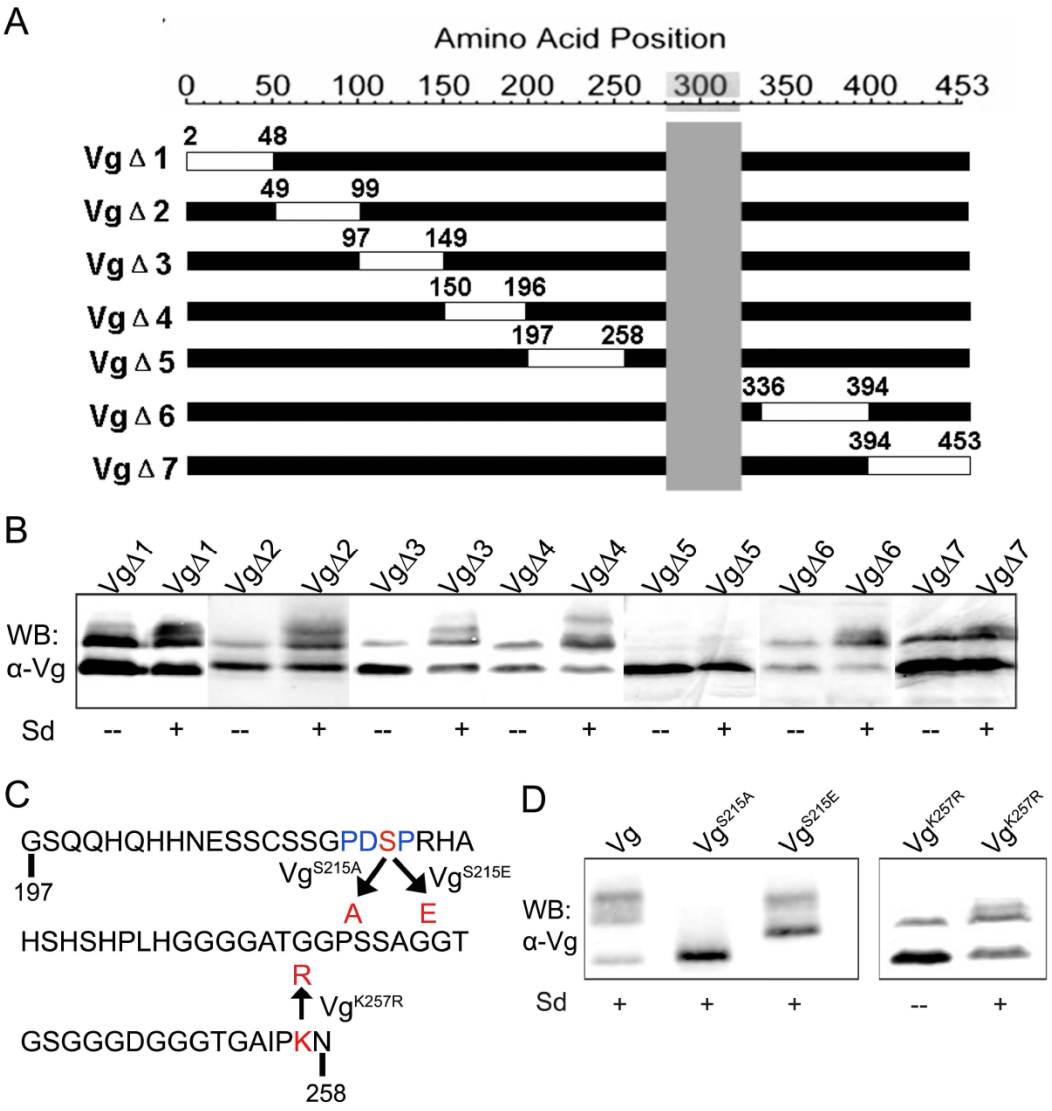
A
Amino Acid Position
0 50 100 150 200 250 300 350 400 453
Vg Δ 1
2 48
Vg Δ 2
49 99
Vg Δ 3
97 149
Vg Δ 4
150 196
Vg Δ 5
197 258
Vg Δ 6
336 394
Vg Δ 7
394 453
B
VgΔ1 VgΔ1 VgΔ2 VgΔ2 VgΔ3 VgΔ3 VgΔ4 VgΔ4 VgΔ5 VgΔ5 VgΔ6 VgΔ6 VgΔ7 VgΔ7
WB:
α-Vg
Sd -- + -- + -- + -- + -- + -- + -- + -- +
C
GSQQHQHHNESSCSSGPDSPRHA
197
Vg^S215A
Vg^S215E
A
E
HSHSHPLHGGGGATGGPSSAGGT
R
Vg^K257R
GSGGGDGGGTGAIPKN
258
D
Vg
Vg^S215A
Vg^S215E
Vg^K257R
Vg^K257R
WB:
α-Vg
Sd + + + -- +

Figure 2.4. Vg is phosphorylated at serine at position 215. **A,** A schematic diagram shows the Vg deletions used to identify potential modification sites. Numbers indicate the amino acid position in Vg and empty boxes represent deleted regions in Vg. The gray shaded region indicates the Sd interaction domain (SID) previously identified within Vg. **B,** Each of these Vg deletions was expressed in S2 cell with or without the co-expression of Sd. Western blot patterns of these deletions were revealed using anti-Vg antibody. VgΔ5 does not produce any of the slower migrating bands shown to be due to sumoylation or phosphorylation. All of the other deletions produced a similar pattern of three or more additional slower migrating bands as full length Vg. **C,** The amino acid sequence deleted in VgΔ5. Ser 215 was replaced with ala in Vg^{S215A}, ser 215 with glu in Vg^{S215E}, and lys 257 with arg in Vg^{K257R}. These different Vg mutants were expressed in S2 cells with or without the co-expression of Sd. **D,** VgS215A is not modified in the presence of Sd. VgS215E produces one slower migrating band that corresponds to sumoylation. VgK257R also produces one slower migrating band that corresponds to sumoylation in the presence or absence of Sd. However, when Sd is present, VgK257R is also phosphorylated.

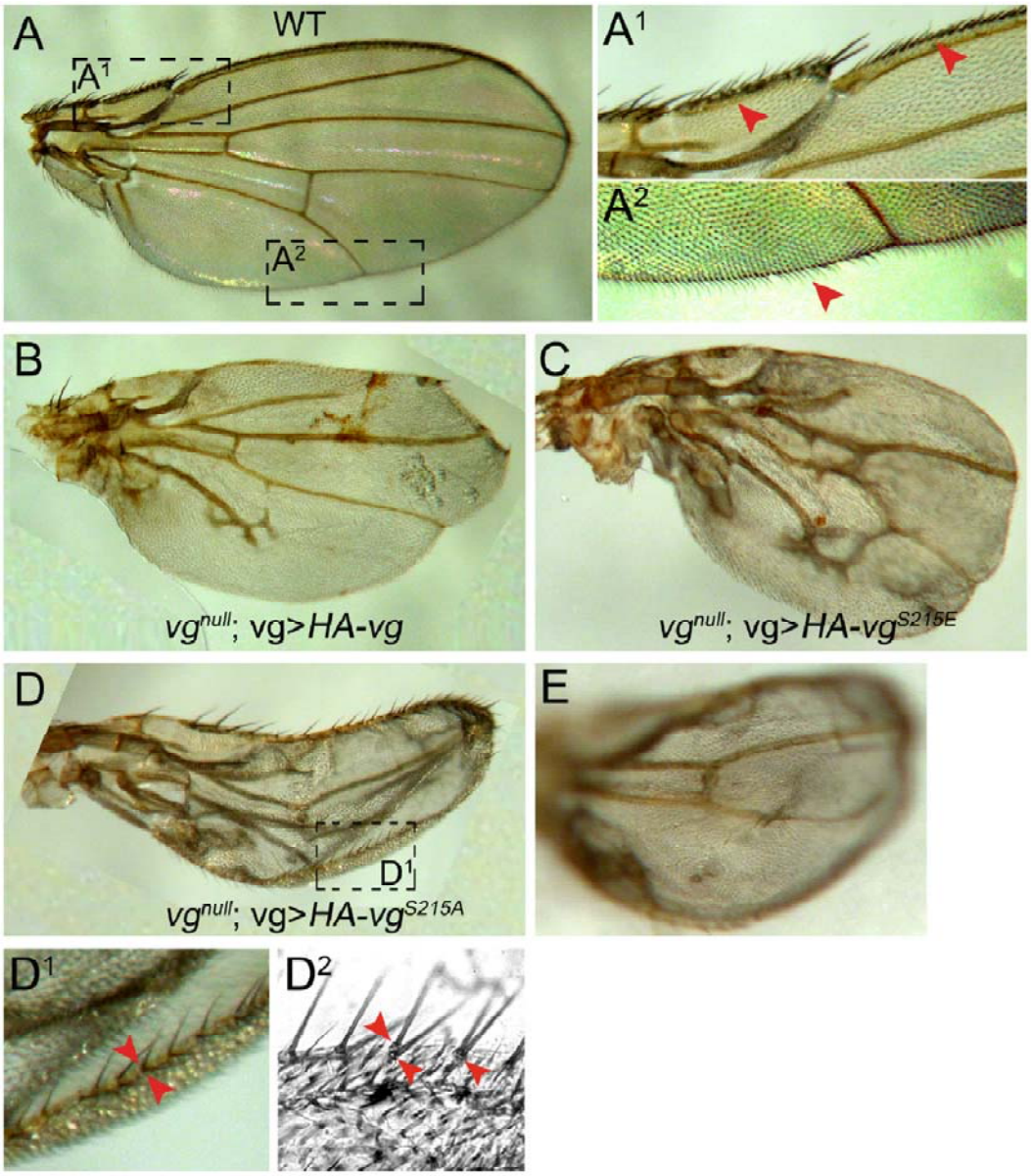
A
WT
A1
A2
A1
A2
B
vgnull; vg>HA-vg
C
vgnull; vg>HA-vgS215E
D
D1
vgnull; vg>HA-vgS215A
E
D1
D2

Figure 2.5. Replacement of endogenous Vg with Vg^{S215A} during wing development caused ectopic growth of sensory bristles along the posterior margin of wing. **A-A^2,** Wild type wings have sensory bristles growing only along the anterior margin of wing (arrowheads in the close-up shown in A^1). A^1 and A^2 are the close-ups of the framed area in A. **B,** Replacement of endogenous Vg with HA-tagged wild type Vg produced wings with a wing blade surface that were significantly larger than that in vg^{null} flies. These wings had few bristles along the entire wing margin. However, vein patterns in these wings are similar to wild type. C) A similar pattern of wing development was seen when Vg^{S215E} was used to replace endogenous Vg. **D,** Replacement of endogenous Vg with HA-Vg^{S215A} produced wings that had sensory bristles growing along the entire margin of wing. D^1 is the close-up of the framed area in D. Two lines of sensory bristles could be seen along the posterior margin of Vg^{S215A} wings (arrowhead). D^2 is a lateral view of posterior margin of the same wing shown in D^1. It shows that these bristles have sockets like those normally found along the anterior margin (arrowheads). **E,** Vg^{S215A} wings have only two veins within the wing blade.

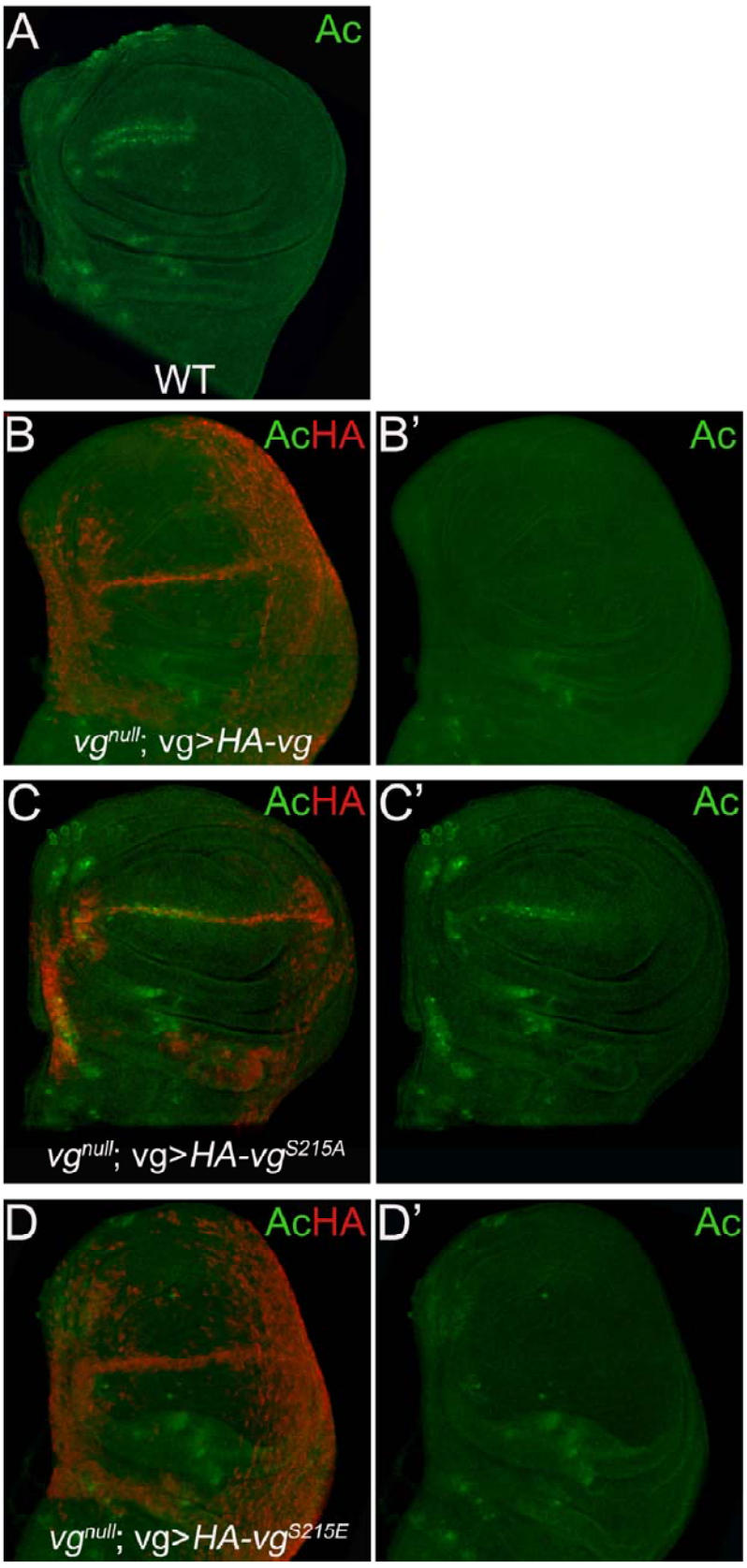
A
Ac
WT
B
AcHA
vg^null; vg>HA-vg
B'
Ac
C
AcHA
vg^null; vg>HA-vg^S215A
C'
Ac
D
AcHA
vg^null; vg>HA-vg^S215E
D'
Ac

Figure 2.6. Replacement of endogenous Vg with Vg^{S215A} in cells along the dorsal ventral margin does not appear to affect early differentiation of neurons in imaginal discs. **A,** In wild type third instar imaginal wing discs, Ac (green) is present in two rows of cells along the anterior margin. **B-B',** Normal *ac* expression (green) along the anterior margin is not seen when HA-tagged wild type Vg (red) was expressed along the entire wing margin via *vg*-GAL4 in a vg^{null} genetic background (B' is the single green channel, the same as C' and D'). **C-C',** When HA-tagged Vg^{S215A} (red) was expressed along the DV margin in vg^{null} wing discs, expression of *ac* (green) along the anterior margin was similar to wild type. **D-D',** Expression of HA-tagged Vg^{S215E} through *vg*-GAL4 had similar effects on expression of Ac as HA-tagged Vg in vg^{null} wing discs.

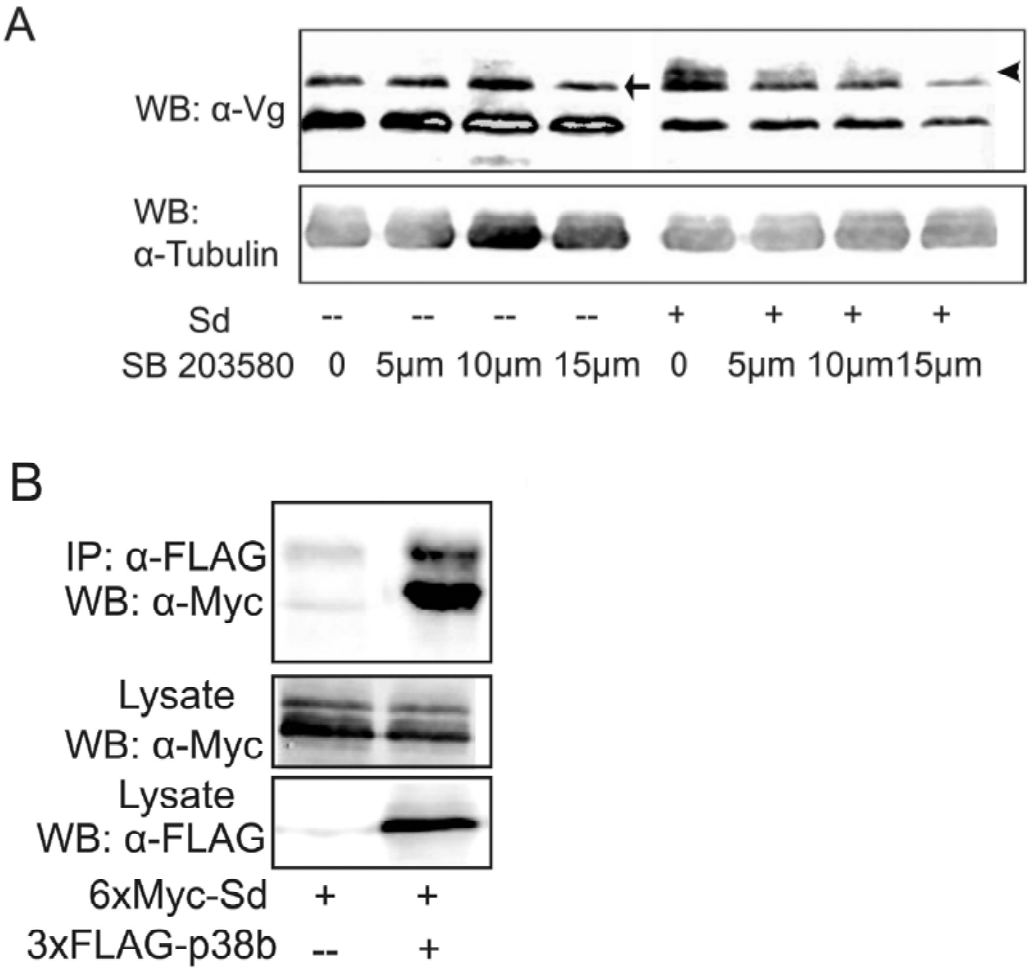

Figure 2.7. p38b requires interaction with Sd to phosphorylate Vg in S2 cells. **A,** Vg was expressed in S2 cells with or without the co-expression of Sd. A specific p38b inhibitor (SB203580) was added in the culture medium in increasing concentrations. Increased concentrations of the inhibitor lead to a progressive loss of the slower migrating Vg band (arrowhead) relative to the concentration of the most rapid migrating band (arrow). The relative amount of each Vg isoform was constant when Sd was not co-expressed. Tubulin staining shows that each lane is relatively equally loaded. **B,** CoIP of FLAG tagged p38b from S2 cell lysate shows that Sd is in a protein complex with p38b.

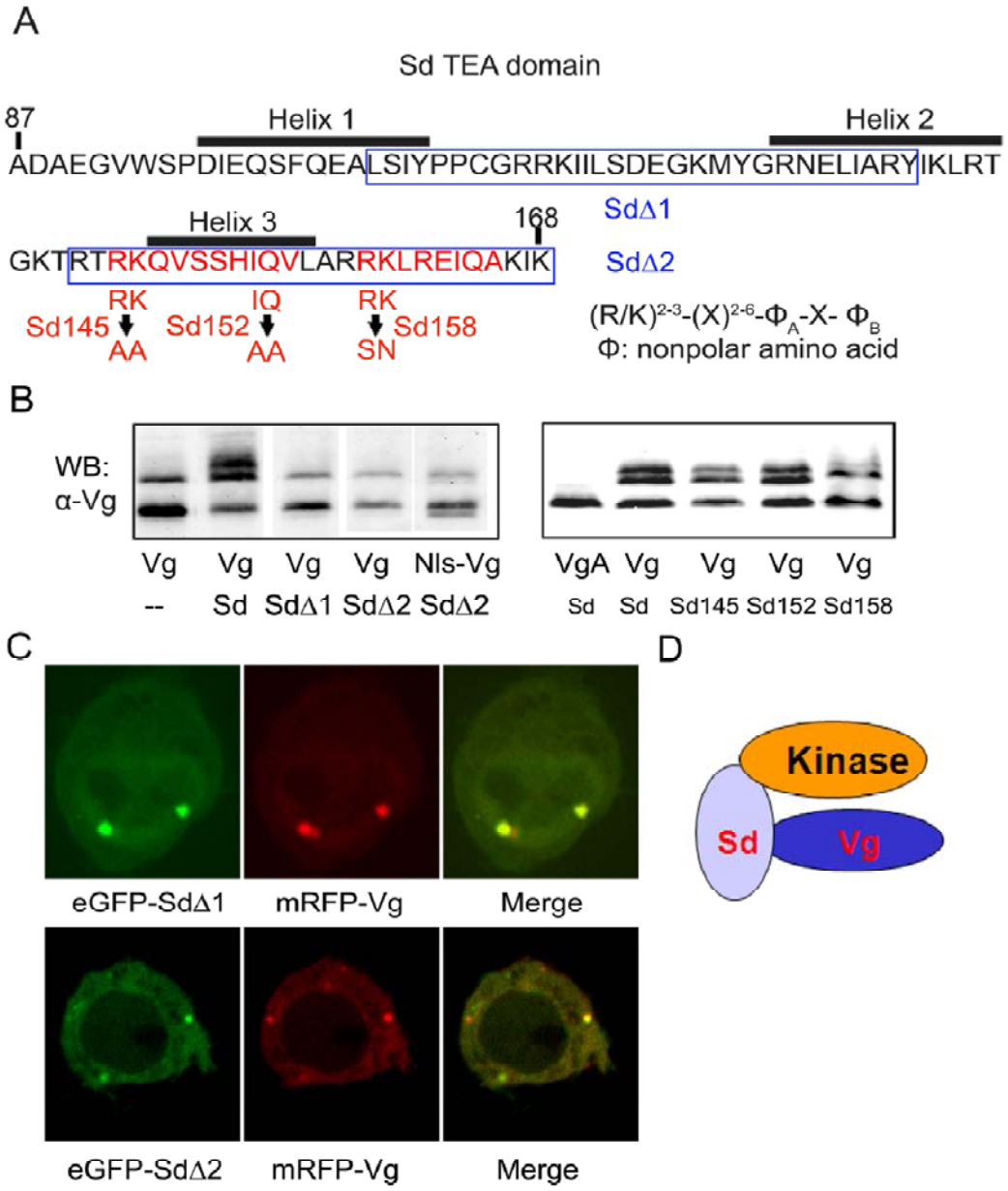
A
Sd TEA domain
87
Helix 1
Helix 2
ADAEGVWSPDIEQSFQEALSIYPPCGRRKIILSDEGKMYGRNELIARYIKLRT
Helix 3
168
SdΔ1
GKTRTRKQVSSHIQVLARRKLREIQAKIK
SdΔ2
RK
IQ
RK
Sd145
Sd152
Sd158
AA
AA
SN
(R/K)2-3-(X)2-6-ΦA-X- ΦB
Φ: nonpolar amino acid
B
WB:
α-Vg
Vg
Vg
Vg
Vg
Nls-Vg
--
Sd
SdΔ1
SdΔ2
SdΔ2
VgA
Vg
Vg
Vg
Vg
Sd
Sd
Sd145
Sd152
Sd158
C
eGFP-SdΔ1
mRFP-Vg
Merge
eGFP-SdΔ2
mRFP-Vg
Merge
D
Kinase
Sd
Vg

Figure 2.8. Mutation of the potential p38b kinase docking domains within Sd strongly reduced Vg phosphorylation in S2 cells. **A,** The amino acid sequence of the Sd TEA domain is shown to have two consensus docking domains for P38b kinase (red letters). The two regions with blue boxes show the portions of the TEA domain deleted in SdΔ1 and SdΔ2 respectively, and the amino acid replacements are shown for each Sd mutants. **B,** Co-expression of SdΔ1 or SdΔ2 with Vg failed to produce the slower migrating band indicative of phosphorylated Vg, while co-expression of $Sd^{RK158SN}$ with Vg lead to only weak Vg phosphorylation. **C,** S2 cells were transfected with mRFP-Vg and eGFP-SdΔ1 or eGFP-SdΔ2. Confocal imaging showed that both Sd deletions co-localized with Vg to form foci in the cells. However, SdΔ1 was able to bring Vg into the nucleus wheras SdΔ2 was not. **D,** A model for potential interaction among Sd, Vg, and the p38b kinase.

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Chapter 3: Alternative requirements for Vestigial, Scalloped and Dmef2 during muscle differentiation in *Drosophila melanogaster*.

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Introduction

Specification and differentiation of both vertebrate and invertebrate muscles requires a conserved cohort of transcription factors [1, 2]. Among these, myocyte enhancer factor-2 (MEF2) plays a key role in specification and subsequent differentiation of all muscle types (skeletal, smooth and heart muscle) [3, 4]. There are four different known vertebrate *mef2* genes: *mef2-a*, *-b*, *-c* and *–d* [3]. These four genes produce several different MEF2 isoforms involved in differentiation of all muscle types. In addition, it has been proposed that MEF2 proteins have a requirement for tissue-specific cofactors to confer additional specificity. For example, during mammalian heart development, GATA-4 [5] helps to recruit MEF2 to the promoters of cardiac specific genes including atrial natriuretic factor (ANF) and *α-cardiac actin* (*α-CA*) [6]. MEF2 also interacts with another transcription factor, HAND1, during activation of ANF in cardiac cells [7]. This complex interplay between MEF2 proteins and co-factors is not restricted to cardiac muscles as during skeletal muscle development, MEF2 interacts with MyoD during activation of specific structural genes [8, 9].

In terms of MEF2 protein family activity, muscle differentiation in *Drosophila* is relatively less complex as there is only a single homologue *mef2*, *Dmef2* [10]. Like vertebrates, *Drosophila* Dmef2 isoforms activate muscle specific genes [3, 8] and also seems to interact with a conserved cohort of interacting proteins for muscle specification, including cardiogenesis. These include: *tinman* [11], dHAND [12], and the gene encoding the GATA factor Pannier (Pnr) [13]. Dissection of the regulatory region of the muscle specific structural genes, TroponinT [14], *TroponinI* (*TnI*), and *tropomyosin* (*TmI*) indicates that co-factors work together with Dmef2 during cardiogenesis in *Drosophila* [15-17]. However, relatively little is known about the Dmef2

interacting partners during differentiation of somatic muscles (analogous to mammalian skeletal muscles) versus cardiac muscle cells. We have focused on the muscle-specific role of two proteins Scalloped [18] and Vestigial (Vg) that have been shown previously to be potent activators of fate specification in several non-muscle cell types. There is considerable functional conservation in the activities of TEF-1/Sd and Vgl/Vg as mammalian TEF-1 can functionally substitute for Sd [19] and Vgl-2 can partially substitute for Vg during Drosophila development [20].

Sd is the only member of the Transcriptional Enhancer Factor-1 (TEF-1) family of proteins in *Drosophila* (Campbell *et al.*, 1992), and together with an activating co-factor, Vg, induce formation of the wing. In fact, ectopic expression of Vg, in the cells of the developing eye that also express Sd, lead to a re-specification of these cells to a wing phenotype [21, 22]. Vg has two domains that influence transcriptional activation activity [23] and Vg requires Sd for nuclear localization [21, 22, 24]. Both TEF-1 and Sd bind DNA via a conserved TEA domain, although like TEF-1, Sd does not exhibit significant transcriptional activation ability on its own. Vg interacts directly with Sd to form a transcription factor (TF) complex required for wing specific gene expression [21]. There is also evidence that TEF-1 acts in concert with other transcription factors. For example, YAP65 has been identified as a powerful transcriptional co-activator of TEF-1 in mouse [25].

After identification of the Sd-interaction domain of Vg [21], several mammalian genes encoding Vestigial-like proteins with homologous domains were identified. These include Vestigial-like 2 [26], which interacts with TEF-1 in skeletal muscle to augment myosin heavy chain (MHC) expression [26, 27]. Vestigial-like 4, which is enriched in heart muscle also functionally interacts with TEF-1 [28]. Similarly, the Sd homologue, TEF-1 is a MEF2 interacting protein expressed in all muscle types [29, 30]. The phenotype of a TEF-1 mouse-knockout suggests a role in cardiac maturation [31], but TEF-1 is also required for skeletal and smooth muscle gene expression [32]. However, TEF-1 cannot

activate transcription alone [33], and over-expression of TEF-1 results in repression of transcription [34].

In terms of muscle development, mammalian TEF-1 has been shown to interact with MEF2 and this interaction interferes with MEF2-dependent activation of the β-Myocyte heavy chain (β-MHC) promoter [35]. Other known MEF2 co-factors include: Poly (ADP-Ribose) Polymerase (PARP) on the cardiac TnT gene [14]; Max on the cardiac α-myosin heavy chain gene [36]; and serum response factor on the skeletal α-actin gene [37]. Given the multiplicity of interactions between these proteins, it is possible that MEF2 and TEF-1 function within a larger complex of TFs that includes additional proteins, like members of the Vgl family, and that alternative composition of these various complexes may provide cell-specific gene activation during muscle differentiation.

Although Dmef2 has a clear role in *Drosophila* muscle differentiation, specific functions for Vg or Sd in muscle cells has not yet been well characterized. To test the role for a complex of MEF2, TEF-1 and the Vgl-family of proteins in the differentiation of muscle cells led us to probe the combinatorial activities of each of these proteins during *Drosophila* embryonic muscle specification. There is some precedence for a role for Vg in muscle development as it had been reported to be required for late-stage development of indirect flight muscles (IFMs) derived from the wing disc-associated myoblasts [38]. In wing discs isolated from flies with null *vg* mutations, myoblasts proliferate, migrate and fuse normally but further differentiation fails to occur [39], a phenotype similar to that associated with mutations in *Dmef2* [40, 41]. While it is possible that this phenotype is due to the well known wing-specification role previously ascribed to Vg, it is equally possible that this represents a muscle specific activity for Vg and Dmef2 and further suggests that these two proteins may functionally interact.

To clarify the role of Sd and Vg during embryonic muscle development, in addition to the IFM precursors, we have looked at all of the developing muscles in *sd* and *vg Drosophila* mutant embryos, and found consistent defects in both the cardiac and somatic musculature. Additionally, we have shown that *sd* is expressed in at least some *Drosophila* embryonic muscles. Further, we have

tested protein interactions between *Drosophila* Dmef2, Sd and Vg and found that these proteins do interact both *in vitro* and *in vivo*. Finally, we have tested the specific combinatorial requirement for the presence or absence of Vg or Sd in certain muscle types as elevated expression of each causes significant defects in the specification or differentiation of specific muscle cell types.

Materials and Methods

Cell culture and transfections

Drosophila S2 cells were grown at 25°C in Schneider's *Drosophila* Medium (Invitrogen) supplemented with 10% fetal bovine serum. Transfections were carried out using dimethyldioctadecyl-ammonium [42].

Drosophila strains

Ectopic-expression of Gal4-UAS transgenes [43] was performed using: Dmef2-Gal4 [44], sd-Gal4 [45] and *5053-gal4* [46]. All other UAS-transgene animals were made in our laboratory for this study.

Plasmids

GST-Sd and GST-Dmef2 vectors were created by insertion of full-length *sd* and *Dmef2* into the *Bam*HI and *Sal*I sites of pGEX-4T1 (GE Biotech), respectively. Vg deletions (Fig. 5) in pET16b (Novagen) were as described previously [21]. Expression vectors for transfection of S2 cells (Fig. 4) were created by Gateway Technology (Invitrogen) and the *Drosophila* Gateway destination vectors (Terrence Murphy).

Fluorescent *in situ* hybridization

Anti-sense Digoxigenin (DIG, Roche) RNA probes targeting *sd* were made by creating a double stranded PCR product with a T7 polymerase binding site incorporated into the 3' primer. The primers used were 5'-gaacaacctgagctgcagcgagttgg and 5'-taatacgactcactatagggagacagcacttggatgtgcg. Embryo fixation and hybridization of the probes and detection of the fluorescent signal were performed using the method of Hughes and Krause [47], including the modifications outlined in [48].

GST Pull-down assays

GST fusion proteins were expressed in *E. coli* [Rosetta 2(DE3), Novagen] and purified according to the manufacturer's directions (GE Biotech). Probe proteins were S^{35} labeled in vitro using the TNT-coupled in vitro transcription-translation system (Promega). For the in vitro binding assay, 3-6µl of S^{35} -labeled probe proteins were incubated with 2µg of immobilized GST fusion proteins in 500µl of buffer (20mM Tris pH 7.6, 100mM NaCl, 0.5mM EDTA, 10% glycerol, 1% Tween-20) containing 0.25% bovine serum albumin (BSA) and protease inhibitor cocktail for 2h at 4°C. The beads were washed six times in 500µl of the same buffer and the bound proteins were resolved by SDS-PAGE and analyzed by autoradiography.

Immunoprecipitations and immunoblotting

S2 cells were transfected with relevant expression constructs containing the heat-shock promoter and protein expression was induced by heat shocking cells for 35 min at 37°C. Cells were harvested one hour after induction, washed one time in PBS, and re-suspended in RIPA (radio-IP) buffer (50mM Tris pH 8.0, 150mM NaCl, 1.0% NP-40, 0.5% Deoxycholic acid, 0.1% SDS, and protease inhibitor cocktail). The lysate was then incubated for 15 min at 4°C with agitation, centrifuged for 15 min at 13.2K rpm at 4°C and the supernatant was then transferred to a fresh tube. Co-IP reactions were carried out on 200µl of supernatant (600µl supernatant from 25 cm^2 flask of cell culture) using 8µl anti-FLAG M2-agarose (Sigma) in 500µl RIPA buffer. Agarose beads were incubated for one hour at 4°C with rocking, centrifuged for one min at 1.4K rpm at 4°C, and washed six times by vortexing in 500µl RIPA buffer. Primary antibodies for immunoblotting were: mouse anti-FLAG (1:1000; Sigma), rat anti-HA (1:400; Roche), and rabbit anti-Myc (1:1000; Cell Signaling). Secondary antibodies were: goat anti-mouse Alexa680 or IRdye800 (1:5000; Invitrogen); goat anti-rabbit Alexa 680 or IRdye800 (1:5000; Invitrogen); goat anti-rat IRdye800 (1:5000; Invitrogen). Nitrocellulose membranes were scanned and analyzed by Odyssey Infrared Imaging System (LI-COR).

Reverse transcriptase PCR (RT-PCR)

Total RNA from stage 12-15 wild type embryos and over-expression embryos was isolated with Trizol reagent (Invitrogen) and treated with *DNase I* (Ambion). Reverse transcription was carried out using 2μg of total RNA, *SuperScript* II reverse transcriptase (SS II, Invitrogen) and gene specific first-strand primers. Subsequent amplification of the resulting cDNA was performed using *Taq* DNA polymerase (Invitrogen) and one pair of nested primers for each gene. Primers for the control *rp49* cDNA were: first-strand primer, 5'-cttcttgagacgcaggcga; nested primers, 5'-agcatacaggcccaagatcg and 5'-agtaaacgcgggttctgcat. Primers for *Act57B* cDNA amplification were: first-strand primer, 5'-gcaggagacaggtgagtagacc; nested primers, 5'-ctccggcatgtgcaagg and 5'-gcaacacgcagctcgttg. Primers for *mhc* cDNA amplification were 5'-agaaggctgaggaactgc and 5'-gttcaagttgcggatctg. The primers were designed for *rp49* and *mhc* to span an intron and the forward primer for *Act57B* cDNA to span the conjunction of two exons. To make sure the RT-PCR was in the linear range of amplification, we performed PCR reactions at increasing cycle numbers (15, 20, 25, and 30), and similar results were observed. The RT-PCR was performed on two different mRNA isolations, and repeated three times, with consistent results.

Fluorescence microscopy

Wild type and over-expression embryos were fixed and stained with various antibodies as described previously [47]. The following primary antibodies were used at the indicated concentrations: mouse anti-FLAG (1:1000; Sigma); rat anti-HA (1:200; Roche); rat anti-Myosin (1:500; Abcam); mouse anti-Myc (1:300; Cell Signaling); rabbit anti-Dmef2 (1:1000; from B. Paterson); rabbit anti-Tinman (1:1000); mouse anti-βPS-integrin (Developed by Danny Brower and obtained from the Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa City, IA, 1:500); mouse anti-β-Gal (Promega, 1:500). Alexa488, Alexa568, Alexa594, Alexa647-conjugatged secondary antibodies (Invitrogen) were used to recognize the primary antibodies. Muscle actin was stained by Alexa546-conjugated phallodin (Invitrogen, 1:25). Images were obtained using a spinning disk confocal system (Ultraview ERS;

PerkinElmer) mated to a CS9100-50; camera (Hamamatsu) and an Axiovert 200M microscope (Carl Zeiss MicroImaging) using Ultraview ERS software (Version 2, PerkinElmer) and assembled with Adobe Photoshop (Version CS, Windows XP).

Results

sd is expressed in a subset of developing somatic and cardiac muscle cells.

We used two independent methods to examine various aspects of *sd* expression in *Drosophila* embryos. We first performed fluorescent in-situ hybridization (FISH) to detect *sd* mRNA. While it appears that *sd* mRNA is expressed at low levels in most cells in developing embryos requiring extensive signal intensification, the specificity of our *sd* probe was verified by examining wing imaginal discs where elevated levels of sd mRNA are seen in the dorsal-ventral region (Fig. 3.1A). In developing embryos, elevated levels of *sd* mRNA were found mostly in the heart region of the dorsal vessel and in SMs of stage 13 wild type embryos (Fig. 3.1 B-C) although we could no longer detect elevated sd transcript levels in SMs of wild type embryos at early stage 16 compared to the staining observed in other tissues (i.e. salivary glands, Fig. 3.1D).

We also tested the muscle specific expression of two different well-characterized reporter constructs that express *β-galactosidase* (*β-gal*) under the control of *sd* enhancers. sd^{ETX4} animals have an enhancer-trap (*β-gal)* P-element construct inserted into the 5' regulatory region of the *sd* locus and have been used extensively to obtain the pattern of *sd* expression in embryo and other tissues [19, 49-51]. Similar to what we observe with FISH detection of the *sd* signal, in sd^{ETX4} embryos, significant levels of *β-gal* can be detected in some muscle cells (Fig. 3.2 A). This includes cardiac cells at stage 13 (data not shown), and by stage 16 *β-gal* is expressed in almost all cardiac cells in the heart region of the dorsal vessel (Fig. 3.2A).

We also tested a second *sd*-GAL4 enhancer trap reporter line with the P-element inserted into the first intron of the *sd* locus which matches the wing-specific expression of sd^{ETX4} [45]. An advantage to this GAL4 based reporter is

that we could use it to induce expression of a UAS-*3xFLAG-sd* transgene. This allows the examination of a FLAG-tagged Sd under the control of an endogenous *sd* enhancer, closely mirroring the cellular produrance of the endogenous Sd protein. This consideration is important as *sd* expression driven by *sd*-GAL4 appears to be very dynamic. At stage 13, it drives expression in 31% cells of SMs and several cardiac cells (Fig. 3.2B, C). At early stage 16, it drives expression in all SMs but not in heart cells (Fig. 3.2D). The expression appears to drop during late stage 16, and is restricted to only some ventral SMs (Fig. 3.2E). It appears that *sd*-GAL4 induced expression of *3xFLAG-sd* does not cause any dominant-negative changes to the somatic or cardiac muscle specification in embryos (Fig. 3.2B-F). We also analyzed the expression of UAS- *β-gal* driven by *sd*-GAL4. The β-Gal protein is extremely stable, which although makes interpretation of the dynamic nature of sd-GAL4 expression difficult in earlier stages, allowed us to confirm that expression is restricted to ventral SMs, the same as that of *3xFLAG-sd* at late stage 16.

_vg_ is expressed in embryonic SMs but not heart muscle.

We used an anti-Vg antibody to correlate *vg* expression with that of the *sd*-reporters in embryonic muscles (Fig. 3.2C-E). *3xFLAG-sd* expression does not affect the expression of *vg* in muscle cells, since *vg* has the same expression pattern as in wild type. Vg is first detected at stage 11 in the progenitors of ventral SMs, VL1-4 (data not shown, see Fig. 3.2 for the diagram of each muscle identity). Then it is present in the muscles, LL1 and DA1-3, at stage 13 (Fig. 3.2C). Vg is also present in VL1-4, LL1 and DA1-3 when 3xFLAG-Sd appears in all SMs of early stage 16 embryos (Fig. 3.2D) and when the expression of *3xFLAG-sd* fades and is restricted to some ventral SMs at late stage 16 (Fig. 3.2E). After late stage 17, Vg cannot be reliably detected in muscle cells, confirming what has been reported previously [1].

Both the sd^{3L} and vg^{null} mutations cause defects in embryonic muscle development.

The X-linked, recessive, sd^{3L} allele is hemizygous lethal. Sequencing of sd^{3L} identified a T-A substitution producing a premature stop codon [52]. The sd^{3L}

likely represents a strong loss-of-protein-function allele as some hemizygous male animals do hatch and survive as feeble larvae with behavioral abnormalities that maybe result from muscle defects [53]. For example, recently hatched wild type larvae have characteristic contraction waves that pass from the posterior to anterior and are responsible for locomotion. We found that the waves of contraction are much slower in sd^{3L} hemizygotes, taking approximately three times as long to pass from posterior tip to anterior tip as compared with wild type. Notably, examination of the embryonic muscles of sd^{3L} hemizygotes revealed defects in both heart and somatic muscle development (Fig. 3.3A-G). Many of these embryos (30%) have less than the wild type number of cardiac cells (Fig. 3.3C) and many of the remaining cardiac cells have nuclei larger than normal (Fig. 3.3A-B). In many of the mutant embryos we also see somatic muscle defects, most often the ventral SMs (VO4-6) get lost or have defects in development (Fig. 3.3D-E). Actin staining also revealed that the VO4-6 muscles disappeared in some segments (Fig. 3.3F-G).

The vg^{null} mutant is homozygous viable but with severely reduced viability compared with wild type flies including female sterility [39]. vg^{null} larvae showed similar muscle contraction defects to those associated with sd^{3L}, taking approximately two times as long for the contraction waves to reach the anterior tip as compared to wild type. Actin staining showed that the VL2 muscle was often missing in vg^{null} embryos (Fig. 3.3F, H) with no detectable defects in other muscles. This phenotype is enhanced in *Dmef2* RNAi background (Fig. 3.S2F), indicating a functional interaction between these two genes.

Sd, Vg and Dmef2 can form alternative complexes

As some interaction of the mammalian homologues of Sd, Vg and Dmef2 has been reported previously, co-immunoprecipitation (co-IP) was performed from S2 cell lysates expressing epitope tagged Vg, Sd or Dmef2. These three proteins appear to form a tri-partite complex as any two could be co-IPed with the third (Fig. 3.4A). For example, co-IPs of 3xFLAG-Sd also could detect 3xHA-Vg and 6xMyc-Dmef2 (Fig. 3.4A). Similar results were observed when we used 3xFLAG-Vg or 3xFLAG-Dmef2 to co-IP the other two proteins. The interactions

between any two of these three proteins appear to be independent of the third, as co-IP of any two does not require the co-expression of the third (Fig. 3.4B, D). To further test for the possibility that Vg is required for the interaction between Sd and Dmef2, sequential IPs were performed by first isolating 3xFLAG-Sd and 6xMyc-Dmef2 or 3xFLAG-Dmef2 and 6xMyc-Sd and then testing for the presence of Vg. In either case, Vg was not detected (Fig. 3.4C). Time-course IPs were also performed to test the specificity of the interaction between Vg and Dmef2 (Fig. 3.4D). This interaction appears to be highly specific, as the amount of 6xMyc-Dmef2 IPed by 3xFLAG-Vg increases with time.

Vg interacts with Dmef2 and Sd at different sites.

Since Vg, Sd and Dmef2 interact, it is possible that they bind alternatively to the same sites or simultaneously at different locations. To map the region within Vg that interacts with Dmef2, Glutathione *S*-transferase (GST) pull-down assays [54] were performed. We first confirmed that Vg can bind directly with Dmef2 (Fig. 3.5A). We then tested deleted or truncated forms of Vg and found only one Vg deletion, Vg3-9, did not interact with Dmef2 (Fig. 3.5B-D). This indicates that at least two independent domains within Vg participating in binding to Dmef2: one within amino acids 1-187 and the other within amino acids 279-336 (Fig. 3.5C). The Sd interaction domain of Vg has been mapped to amino acids 279-336 [21]. We note that there is some residual binding of one of the deletions (Vg 1-4) with the GST control. However, the much stronger signal obtained when GST-Dmef2 is present makes us confident that this residual binding is due to weak, non-specific "stickyness" of the N-terminal region of Vg. Since Vg8-9, a deletion that removes the Sd interacting region, still interacts strongly with Dmef2 (Fig. 3.5B), Vg could potentially interact with Sd and Dmef2 at the same time via different domains.

Altering Vg and/or Sd levels affects the expression of known muscle differentiation genes.

We next tested the functional consequence of altering the levels of Vg or Sd in various differentiating muscle types. Dmef2 is highly expressed in the developing midgut (Fig. 3.6A). Elevated expression of Vg (Fig. 3.6B) in these

tissues does not have any obvious effect on muscle differentiation. However, elevating the expression of Vg and Sd together or Sd alone causes the loss of tissue specific constrictions (Fig. 3.6C). This over-expression does not affect the expression level of Dmef2 (Fig. 3.6C) and cause a phenotype similar to a *Dmef2* deletion mutant [4]. In the somatic muscles, expression of a known Dmef2 target, myosin is affected when Sd is present and this effect is enhanced when both Vg and Sd are present (Fig. 3.6D-F). This phenotype is similar to that of embryos over-expressing a Dmef2 inhibitor, Him in SMs [55]. We observed a similar reduction in Actin staining when Vg and Sd were over-expressed in cells also expressing Dmef2 (Fig. 3.6G-H). We then tested the mRNA levels of two known Dmef2 target genes, *actin57B* (*act57B*) and *myosin heavy chain* (*mhc*). *Drosophila* Act57B is the major myofibrillar actin expressed in skeletal, visceral and cardiac muscle during embryogenesis and there is a conserved Dmef2 binding site within the promoter that is necessary for the full expression of *act57B* in embryos [56]. Similarly, *mhc* expression is significantly reduced in *Dmef2* mutants [4]. When elevated levels of Sd and Vg are present in *Dmef2* expressing cells, a significant reduction in both *act57B* and *mhc* mRNA are observed (Fig. 3.6I). To confirm that the effect we see on suppression of actin or myosin is specific to the presence of Vg and Sd, we also expressed these transgenes in a single muscle using a Gal4 driver specific for VL1 (Fig. 3.6K). We see a corresponding reduction in actin or myosin expression in the VL1 muscle with increased expression of *sd* and *vg* (Fig. 3.6 J-K). These reductions lead to the missing of myofiber in SMs (Fig. 3.6H, K). Altogether, these data indicate that Sd and Vg repress Dmef2 function during muscle differentiation.

Altered expression of Sd and/or Vg leads to abnormal SM development.

As increased expression of Vg and Sd leads to reduction of muscle specific markers like *act57B* and *mhc*, we next examined the fate of these cells (Fig. 3.7). Since the mammalian homologues of Sd, Vg and Dmef2 have a role in terminal muscle differentiation[4, 26, 31], we used *Dmef2*-GAL4 to express transgene(s) in all mesodermal cells of late stage 7 embryos and later in cardiac cells of the heart, visceral and somatic muscle cells until the end of

embryogenesis [44]. The pattern of the muscle system in wild type embryos is shown in Fig. 3.7A-B. An *act57B*-LacZ transgene was used to visualize muscle fibers (Fig. 3.7A) and to monitor for gross alterations in the expression level of *act57B* in muscle cells.

Expressing additional Vg in the somatic muscles seems to cause a complete loss of the VO4-6 muscles although the overall organization of the remaining muscles appears normal (Fig. 3.7C). However, in embryos expressing UAS-*3xFLAG-sd,* the normal precise organization of muscle fibers is lost (Fig. 7D and Fig. 3.S2C). Specifically, we repeatedly see that muscle LL1 does not develop well or gets lost, and muscle VL1-2 often disappear in many segments (Fig. 3.7D). Unlike what is seen with ectopic Vg, the ventral muscle VO4-6 is still present but ectopic Sd expression seems to cause defects in migration with more projections than what is seen in wild type (Fig. 3.7A, D and Fig. 3.S2C). Co-expression of UAS-*3xFLAG-sd* and UAS-*3xHA-vg* together in SMs causes repression of *act57B* in most cells (Fig. 3.7E). In these embryos, the organization of muscle fibers is completely disrupted (Fig. 3.7E and Fig. 3.S2D). Actin staining of these embryos failed to visualize the muscle fibers, probably because there is little myofibril formation (Fig. 3.6H). Expression of UAS-*3xFLAG-sd* in the *Dmef2* RNAi background produced similar phenotype to that of co-expression of UAS-*3xFLAG-sd* and UAS-*3xHA-vg* (Fig. 3.S2E*),* further conforming that Sd and Vg repress Dmef2 function during muscle differentiation.

We further examined the organization of the developing muscles by staining for βPS-integrin, one of the major integrins, acting as a transmembrane protein that stabilizes attachments between two neighbor muscles and that between muscles and epidermis along the segment border [57, 58]. In wild type embryos, muscle cells attach at characteristic positions relative to segment borders (Fig. 3.7H). However, in embryos expressing UAS-*3xFLAG-sd* and UAS-*3xHA-vg*, the organization of muscle cell attachment is severely disrupted (Fig. 3.7I). Embryos ectopically expressing UAS-*3xHA-vg* in SMs exhibit a different phenotype. SMs still have a highly organized pattern (Fig. 3.7C) and muscle cells do not lose their positions (data not shown), but the migration of VO4-6 muscles

seem to be inhibited or redirected (Fig. 3.7F-G). However, expression of UAS-*vg 3*-9, the Vg deletion that loses interaction with Dmef2 (Fig. 3.5C), produced wild type phenotype (data not shown). As a control, we examined embryos over-expressing UAS-*6xMyc-Dmef2* and this does not cause any obvious defect in SMs (data not shown).

Altered expression of Sd, Vg and Dmef2 causes defects in cardiac cell development.

In wild type, there is a single row of Dmef2-positive cardiac cells on each side of the embryo (Fig. 3.8A) and four Tin-positive cardiac cells per hemisegment with some Tin-positive pericardial cells (Fig. 3.8B, see Fig. 3.2F for a diagram of each cardiac cell fate). It was reported previously that the *Dmef2*-GAL4 driver was not active in pericardial cells [44]. But, based on our analysis of the pattern of *β-gal* expression driven by *Dmef2*-GAL4, it also drives expression, at least transiently, in pericardial cells (Fig. 3.8A'). We used this driver for examining the effect of elevated levels of Vg or Sd on cardiac cell differentiation.

Ectopic expression of UAS-*3xHA-vg* does not affect the Dmef2-positive cardiac cells (Fig. 3.8C), but causes two additional Tin-positive cardiac cells per hemisegment (Fig. 3.8D). *Dmef2*-GAL4 induced expression of UAS-*3xFLAG-sd* causes two extra rows of Dmef2-positive cardiac cells (Fig. 3.8E) while the total number of Tin-positive heart cells is similar to wild type (Fig. 3.8F). However, this pattern of the differentiating cardiac cells becomes disorganized with some Tin-positive cells in the SM region (Fig. 3.8F). Elevating the levels of Dmef2 using a UAS-*6xMyc-Dmef2* combination produces one extra row of Dmef2-positive cardiac cells (Fig. 3.8G), but does not affect the Tin-positive cardiac cells (Fig. 3.8H). Ectopic expression of UAS-*3xHA-vg* and UAS-*6xMyc-Dmef2* together has a synergistic effect, as there are two extra rows of Dmef2-positive cardiac cells (Fig. 3.8I) and six Tin-positive cardiac cells per hemisegment with many more Tin-positive pericardial cells around (Fig. 3.8J). Expression of UAS-*3xFLAG-sd* and UAS-*6xMyc-Dmef2* together causes a similar phenotype to embryos expressing UAS-*3xFLAG-sd* alone (Fig. 3.8K-M). However, the pattern of heart cells is more organized (Fig. 3.8M, compared to F), indicating a partial

rescue of the phenotype. Expression of UAS-*3xFLAG-sd* and UAS-*3xHA-vg* together leads to loss of almost all Dmef2-positive cardiac cells (Fig. 3.8N) and dislocation of all Tin-positive cells in the SM region (Fig. 3.8O). Finally, expression of the three transgenes together causes the same phenotype as that of expression of UAS-*3xFLAG-sd* and UAS-*3xHA-vg* together (data not shown). With the exception of embryos expressing UAS-*6xMyc-Dmef2,* all embryos died at the end of embryogenesis.

Discussion

We have shown that there is an analogous requirement for the TEF-1 homologue, Sd, in the late-stage specification or differentiation of muscle cells. The cardiac phenotype associated with the sd^{3L} mutation (Fig. 3.3) confirms that like mammalian TEF-1, there is a clear role for Sd in cardiac muscle differentiation. Expression of *sd* seems to be driven by the same elements that induce elevated *sd* expression in other tissues like the wing, as both the sd^{ETX4} and *sd*-GAL4 reporters which were previously shown to faithfully report *sd* expression in wing imaginal discs, indirect flight muscles and nervous system [49, 59] match the *sd* expression pattern in embryonic muscles revealed by *sd in situ* (Fig. 3.1). We have also determined that Vg, the first known TEF-1 family co-factor, has a role in SMs but not cardiac muscles (Fig. 3.3H), and in fact, elevating Vg expression in the developing dorsal vessel has a negative effect on differentiation of this organ.

Our results show several lines of evidence supporting a model whereby Sd and Vg, in a complex with Dmef2, help to regulate late-stage *Drosophila* embryonic muscle development. One prediction of this model is that altering the relative levels of these proteins will have significant effects on specific sub-sets of muscle cells. The effect of the relative levels of each of these proteins can be observed in various cell types: a) *sd* and *Dmef2* are co-expressed in the cardiac cells (Fig. 3.2A-B), where *vg* is not expressed; b) Starting at stage 11, *vg* and *Dmef2* are co-expressed in the progenitors of some SMs (Fig. 3.2C-E), where *sd* is expressed at a later stage (Fig. 3.2C-E); c) These three genes are co-expressed

in SMs, DA1-3, LL-1 and VL1-4 at early stage 16 (Fig. 3.2D), but by late stage 16 the co-expression is restricted to some ventral SMs (Fig. 3.2E). We have also shown that: a) Sd is able to interact with Dmef2 without the presence of Vg (Fig. 3.4B, C); b) Vg is able to interact with Dmef2 without the presence of Sd (Fig. 3.4D); c) It is also possible for Sd, Vg and Dmef2 to form a tri-partite complex (Fig. 3.4A). Given that Vg appears to bind Dmef2 at two different sites, it may be that Vg could be the bridge protein connecting Sd and Dmef2, since Vg can bind each of them via a different domain (Fig. 3.5B-C). Finally, a requirement for the presence of Sd and Vg appears to be specific to differentiation of specific muscle types as mutations in *sd* and *vg* cause defects in different muscles (Fig. 3.3); whereas alterations in the relative expression levels of any of these three genes in developing muscles of *Drosophila* caused specific alterations in both SM and cardiomyoctes (Fig. 3.7 and 2.8).

Since our data show that Vg can bind Dmef2 independently of Sd, it is possible that Vg may modify Dmef2 activity in the absence of Sd. We noted that the *vg* and *Dmef2* genes are co-expressed in some SMs (DA1-3, LL1 and VL1-4) before Sd is present in those muscles (Fig. 3.2C-E). They are also co-expressed in the progenitors of muscle VL1-4. We also show that functional interactions exist between Vg and Dmef2, as co-expression of them in heart cells has a synergistic effect on increasing the numbers of Dmef2-positive and Tin-positive cardiac cells (Fig. 3.8I-J), and the phenotype of vg^{null} mutant is enhanced in a *Dmef2* deficiency background (Fig. 3.S2F). Previous studies of *vg* have almost exclusively focused on its function as a wing identity gene. However, there is now mounting evidence that Vg also defines the cellular identity of a subgroup of embryonic SMs (Fig. 3.3H, Fig. 3.7C, G; [1, 38] although the functional role of Vg in the development of these muscles is not clear. *Dmef2* is considered to be a "differentiation gene" playing a role in the final stages of muscle differentiation. Thus, this begs the question: what is the significance of the interaction between these two proteins that apparently have roles at different developmental stages? A recent study showed that Dmef2 not only binds to regulatory regions of muscle structural genes but also binds many muscle "identity genes" and genes involved in early

signal pathways of muscle development [60] indicating a role of *Dmef2* in early muscle development. Therefore, Vg may act together with Dmef2 to specify those SMs in which Vg is expressed. Our data support this idea, since vg^{null} mutants often lose muscle VL-2 (Fig. 3.3H), and over-expression of Sd leads to either poor development or loss of muscle LL1 and VL1-4 (Fig. 3.7D^1-D^3) where Vg is present. Considering the strong functional interaction that is known to occur between Vg and Sd [21], over-expression of Sd may interfere with the function of Vg in those muscles.

Just as Vg and Dmef2 may interact in the absence of Sd, a Sd/Dmef2 complex may exist in muscle cells where *vg* is not expressed significantly; *i.e.* cardiac cells in the heart region and some somatic muscle cells (Fig. 3.2). Expression of UAS-*6Myc-Dmef2* via *Dmef2*-GAL4 results in one extra row of Dmef2-positive cardiac cells (Fig. 3.8G). This phenotype is not unexpected as *Dmef2*-GAL4 is also active in pericardial cells that surround cardiac cells (Fig. 3.8A'). However, it is unexpected that expression of UAS-*3xFlAG-sd* also produces extra rows of Dmef2-positive cardiac cells (Fig. 3.8E). These results indicate that Sd could activate the expression of Dmef2 in the pericardial cells. Since the pattern of expression directed by the enhancer of *Dmef2* in muscle cells is very complicated [40] it has been proposed that there is an autoregulation mechanism to maintain its expression in differentiated muscles [61]. Therefore, Sd might be required to act with Dmef2 to maintain expression of Dmef2 in cardiac cells at late stages. The ability of Dmef2 to partially rescue the heart phenotype caused by expression of UAS-*3xFLAG-sd* (Fig. 3.8K-M) also suggests a functional interaction between Sd and Dmef2, since Sd itself does not have transcriptional activation ability and over-expression of Sd can lead to repression of transcription [21].

Mutation and ectopic-expression analysis also revealed that Sd has a role in both heart muscle and SMs development (Fig. 3.3). Recently, Sd was shown to be the target of the Hippo (Hpo) signaling pathway that governs cell growth, proliferation and apoptosis [62]. Inactivation of Sd diminishes Hpo target gene expression and reduces organ size, whereas a constitutively active Sd promotes

tissue overgrowth [62]. We see that in sd^{3L} mutants there are fewer heart cells and the VO4-6 muscles appear to have defects in their differentiation (Fig. 3.3A-F), whereas over-expression of Sd in VO4-6 produces more projections (Fig. 3.7D). These phenotypes would suggest a role of Sd in both growth and proliferation of muscle cells. Conversely, ectopic-expression of Vg in VO4-6 muscles leads to a similar phenotype as sd^{3L} (Fig. 3.7F-G). Thus, it appears that ectopic-expression of Vg in those muscles interferes with the function of Sd.

We observed that co-expression of UAS-*3xFLAG-sd* and UAS-*3xHA-vg* for extended times via *Dmef2*-GAL4 causes significant defects in muscle differentiation, including significant alterations in their sites of attachment. In cardiac muscles, Tin-positive heart cells end up in the SM region (Fig. 3.8O); by the end of muscle development, the stereotyped patterning of SMs is totally disrupted (Fig. 3.7E). This phenotype may be a result of the apparent dynamic expression we observe of the *sd* reporters in SMs (Fig. 3.2). Thus, any Sd-Vg complex that would be formed in developing muscles would be transient, freeing each potential co-factor to interact with Dmef2 independently.

It is interesting that Sd-Vg complex represses Dmef2 function without affecting Dmef2 expression during muscle development (Fig. 3.6 and Fig. 3.S2E). The protein Him (Holes in muscle) was also shown before to repress Dmef2 function during muscle differentiation and the authors argue that a balance of positive and negative inputs controls muscle differentiation [55]. Our data support this idea and may reveal another layer of negative input, the Sd-Vg complex, in muscle differentiation, since over-expression of Sd or Sd and Vg produces similar phenotype to that of over-expression of Him in developing SMs (Fig. 3.6E-F), and also to that of *Dmef2* RNAi embryos. The repression we see of *act57B*, the product of which is primarily required during muscle differentiation may be a normal occurrence during late stage 16 when most SMs are presumably fully differentiated, having finished migration and reached their attachment sites [63]. At this time, some SMs in different segments contact each other and specific extracellular matrix (ECM) contacts between muscles form [64, 65]. *act57B* is initially expressed in SMs at stage11, and by stage16 there is already high levels

of *act57B* transcript in SMs [56]. Specifically, these SMs would slow myofibril growth by repressing the expression of *act57B*, especially in those SMs that contact with neighboring muscles, like LL1 and VL1-4, and the presence of Vg in these muscles (Fig. 3.3H) may be mediating this repression.

The most significant repression of Dmef2 function appears to require the presence of both Sd and Vg (Fig. 3.6F and Fig. 3.S2D). However, this is at odds with the presumptive activating function mediated by an Sd/Vg complex that occurs in other tissues like the wing imaginal disc where an Sd-Vg complex binds and activates the *vg* boundary enhancer [22]. The differential activities of these proteins in muscle versus wing development may reflect a requirement for yet additional proteins within a presumptive Vg/Sd/Dmef2 complex to modify its activity in a tissue-specific manner. Alternatively, post-translational modifications to Vg, Sd (or both) may modify their activity. Interestingly, the yeast Sd homologue, Tec1 is phosphorylated and then degraded during the mating pheromone response [66]. In mammals, TEF-1 is phosphorylated responding to cAMP/PK-A signaling [67]. However, there is, as yet, no clear indication that Sd is phosphorylated in *Drosophila* cells.

Finally, although we have shown that Sd, Vg and Dmef2 interact directly, similar to their mammalian homologues, our data suggest potential new functions of Sd, Vg during muscle specification. For example, Vg seems to have role in the specification of Ventral muscles VL1-4, and Sd has a role in the development of muscle VO4-6, especially in the development of their projections. In addition, the Sd-Vg complex represses Dmef2 function, which is at odds with the known activities of their mammalian homologues. However, this repression only happens in certain muscles (e.g. VL1-4) that need to contact neighboring muscles.

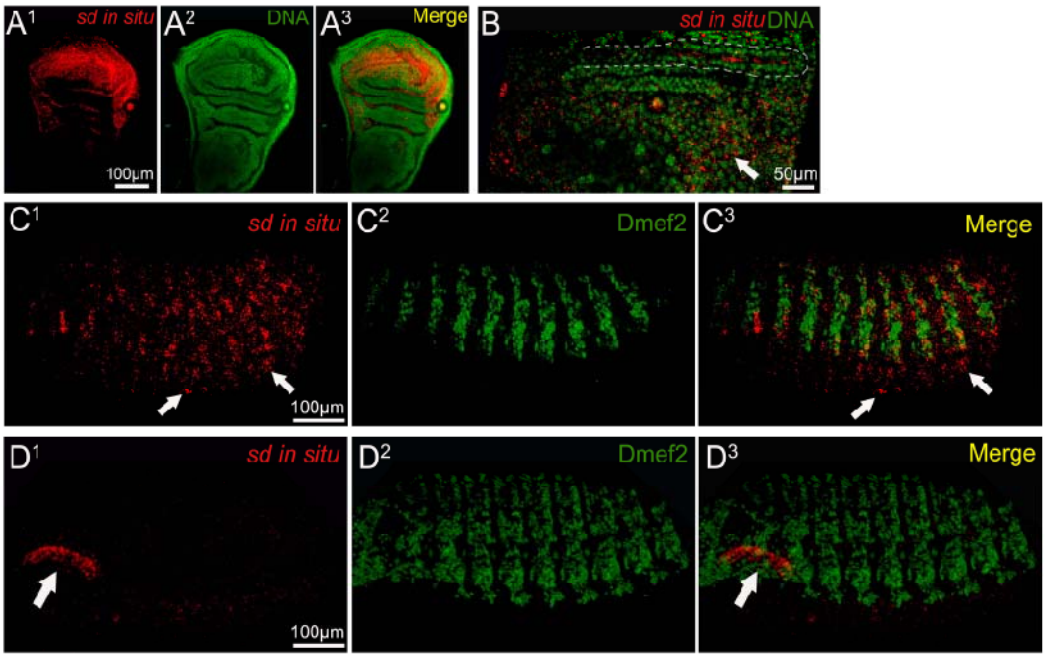
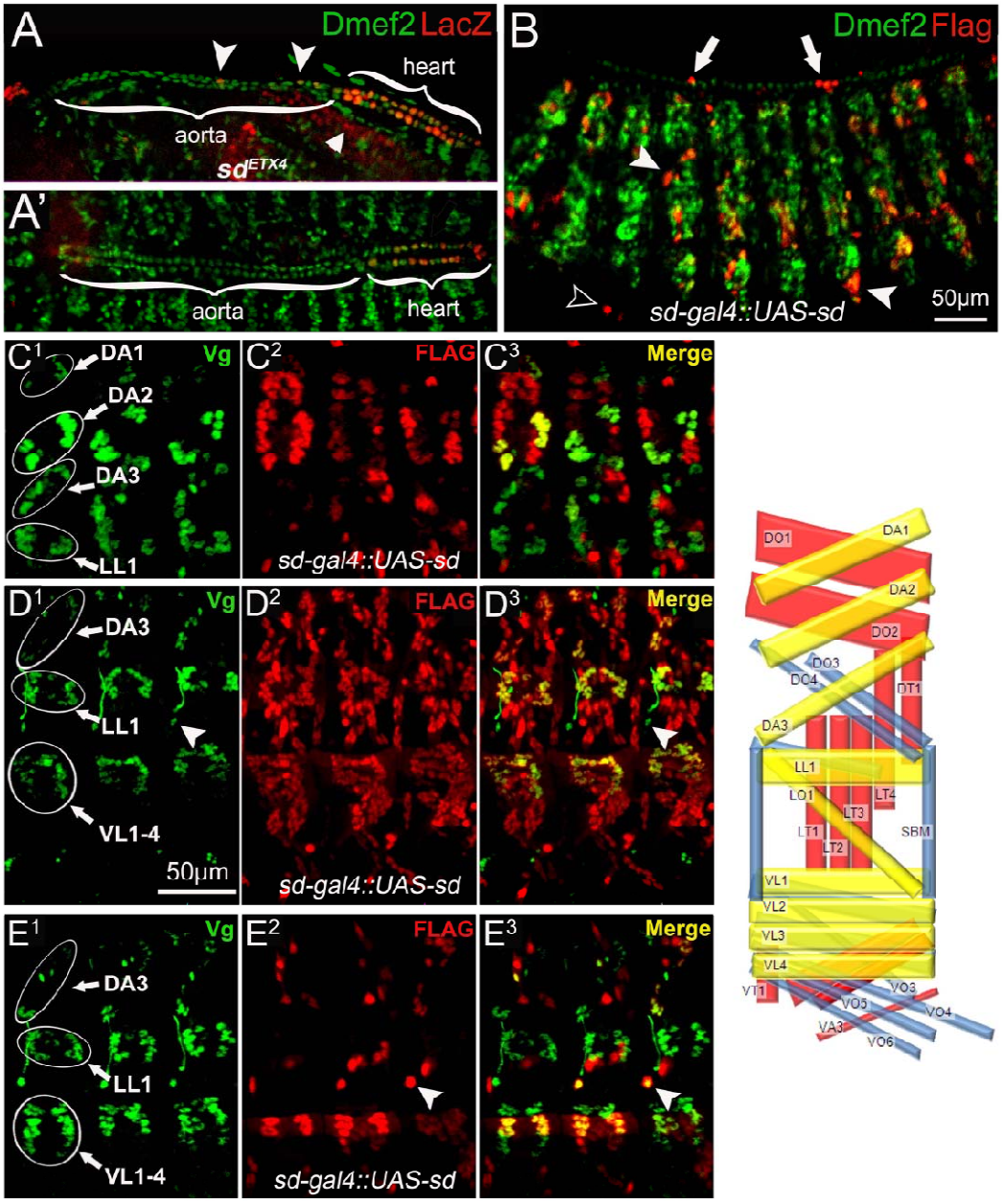


Figure 3.1: *sd in situ* reveals the expression of *sd* in both SMs and dorsal vessel. **A$^{1\text{-}3}$,** The specificity of the *sd* probe for *in situ* was tested on wing disc, and *sd* expression pattern in wing disc was accurately revealed by this probe. **B,** *sd* transcript was found mostly in the heart region of the dorsal vessel (dashed line). It also appears in the hind gut (arrow). **C$^{1\text{-}3}$,** *sd* transcript was detected in SMs of stage 13 wild type embryos. SMs are visualized by Dmef2 staining. *sd* transcript was also detected in the CNS cells (arrows). **D$^{1\text{-}3}$,** It failed to detect *sd* transcript in SMs of wild type embryos at early stage 16. High expression of *sd* was found in salivary gland at this stage (arrow).


A
Dmef2 LacZ
heart
aorta
sd^ETX4
A'
aorta
heart
B
Dmef2 Flag
sd-gal4::UAS-sd
50μm
C1
DA1
Vg
DA2
DA3
LL1
C2
FLAG
sd-gal4::UAS-sd
C3
Merge
D1
DA3
Vg
LL1
VL1-4
50μm
D2
FLAG
sd-gal4::UAS-sd
D3
Merge
E1
DA3
Vg
LL1
VL1-4
E2
FLAG
sd-gal4::UAS-sd
E3
Merge
DO1
DA1
DA2
DO2
DO3
DO4
DT1
DA3
LL1
LO1
LT4
LT3
LT1
LT2
SBM
VL1
VL2
VL3
VL4
VT1
VO3
VO5
VO4
VA3
VO6

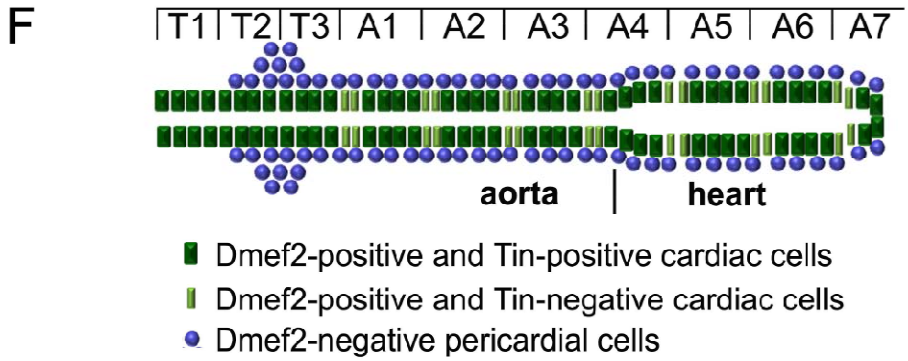

F
T1 T2 T3 A1 A2 A3 A4 A5 A6 A7
aorta
heart
Dmef2-positive and Tin-positive cardiac cells
Dmef2-positive and Tin-negative cardiac cells
Dmef2-negative pericardial cells

Figure 3.2: *sd, vg* and *Dmef2* are co-expressed in embryonic muscles. To facilitate double staining, *sd* expression was detected by examining sd reporter constructs ($sd^{ETX4,}$ A- A'), or using 3xFLAG-Sd driven by *sd*-GAL4 (B-E). Muscle cells are marked with anti-Dmef2 (green in A-B) and Vg is labeled by anti-Vg (green in C-E). 3xFLAG-Sd and LacZ are visualized with anti-FLAG and anti-β-Gal (red), respectively. **A,** In stage 16 embryos the sd^{ETX4} reporter is activated in the heart region of the dorsal vessel and in some cardiac cells in the aorta region (arrowheads). It is also expressed in the hind gut, underneath the visceral muscles (VMs, small arrowhead). A shows a dorsal-lateral view and A' a dorsal view. **B,** *sd*-GAL4 drives expression of 3xFLAG-Sd in several cardiac cells (arrows) and about 31% cells of somatic muscles (SMs, arrowheads) at stage 13. Note that *sd*-GAL4 is also activated in cells of central neuron system (CNS, empty arrow). Dmef2 is present in all muscle cells. $\mathbf{C^1}$-$\mathbf{C^3}$ shows the dorsal SMs where *vg* is expressed at stage 13. 3xFLAG-Sd can be detected in some SMs. $\mathbf{D^1}$-$\mathbf{D^3}$ shows that *vg* is expressed in the DA3, LL1 and VL1-4 muscles when 3xFLAG-Sd appears in all SMs at stage 16. Vg also appears in some neuronal cells (arrowheads). DA1-2 are not shown because they are out of the field of view. $\mathbf{E^1}$-$\mathbf{E^3}$ shows that *vg* is still expressed in the DA3, LL1 and VL1-4 muscles when the expression of 3xFLAG-Sd fades in SMs and appears only in some ventral SMs at late stage 16. At this stage, 3xFLAG-Sd appears with Vg in the neuron cells shown above (arrowheads). **F,** A schematic drawing of a stage 16 embryonic dorsal vessel (Dorsal view, anterior to the left). Heart cells include two parallel rows of Dmef2-positive cardiac cells in the middle with four Tinman-positive cardiac cells per hemisegment starting from T1. Dmef2-negative pericardial cells surround the cardiac cells. On the right is a schematic representation of the embryonic SMs in each abdominal hemisegment A2-A7 (lateral view with anterior up) using the nomenclature of Crossley [68]. Inner, middle, and outer muscle layers are shown in red, blue, and yellow, respectively [69].

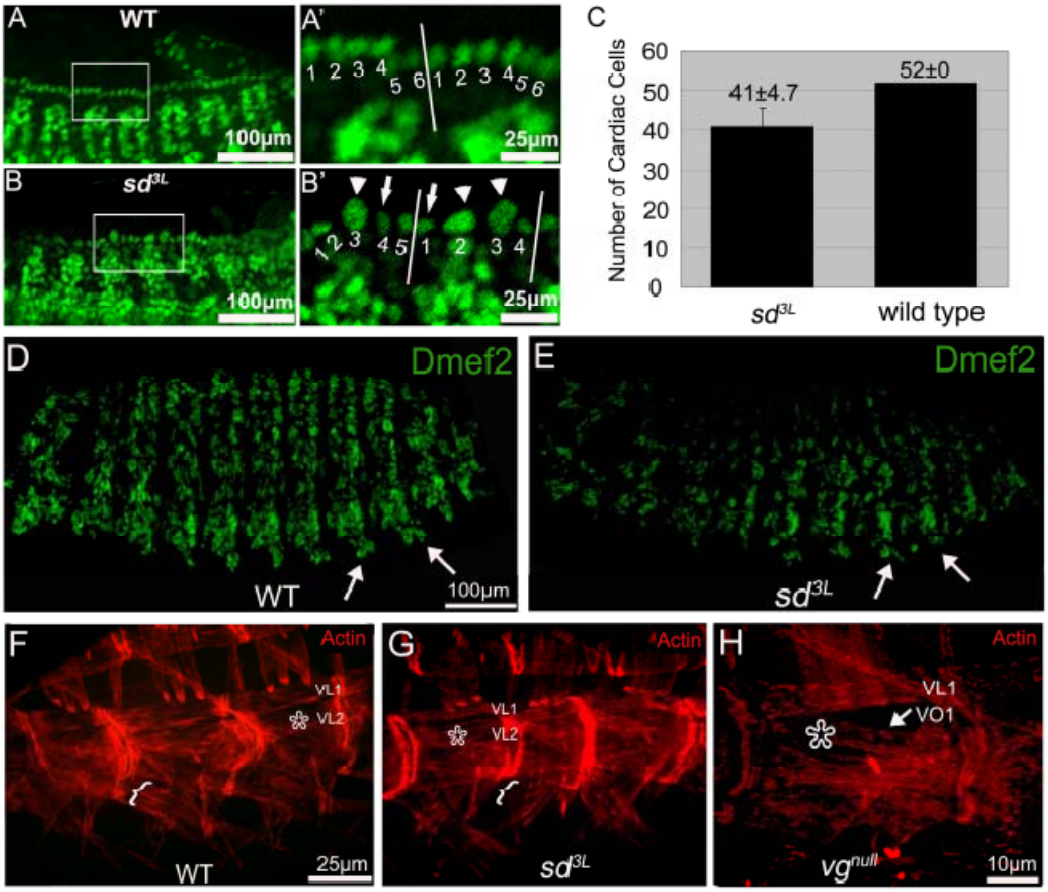

A
WT
100µm
A'
1 2 3 4 5 6 1 2 3 4 5 6
25µm
B
sd3L
100µm
B'
1 2 3 4 5 1 2 3 4
25µm
C
Number of Cardiac Cells
60
50
40
30
20
10
0
41±4.7
52±0
sd3L
wild type
D
Dmef2
WT
100µm
E
Dmef2
sd3L
F
Actin
VL1
VL2
WT
25µm
G
Actin
VL1
VL2
sd3L
H
Actin
VL1
VO1
vgnull
10µm

Figure 3.3: The *sd*^{3L} and *vg*^{null} mutants have defects in embryonic muscle development. Muscle cells are marked with anti-Dmef2 (green) and muscle fibers are visualized by phalloidin staining (red). Anterior is to the left. **A,** In stage 13 wild type embryos (A' is the close-up of the boxed area in A), there are six cardiac cells per hemisegment (A'). **B,** In stage 13 *sd*^{3L}mutant embryos (B' is the close-up of boxed area in B), there are many cardiac cells with enlarged nuclei (arrowheads) relative to neighboring cells (arrows) and there are fewer cardiac cells per hemisegment (compare A' with B'). **C,** The number of cardiac cells on one side of *sd*^{3L} embryos (41±4.7, mean ±SD, n=8) is less than that of wild-type (52±0, n=10). **D** shows the SMs of a wild type embryo at early stage 16. **E** shows the SMs of a *sd*^{3L}mutant embryo at the same stage. Many ventral SMs (VO4-6, see Fig. 2) have severe developmental defects or are absent entirely (arrows, compare E with D). **F,** Actin was stained by phalloidin in a stage 16 wild type embryo. The VO4-6 muscles are indicated by a bracket the VL2 muscle is demarked by *. **G,** In the *sd*^{3L} mutant, the VO4-6 muscles is absent in some segments (bracket). **H,** In the *vg*^{null} mutant, the VL2 muscle is absent in some segments (star) and the VO1 muscle underneath can be seen (arrow).

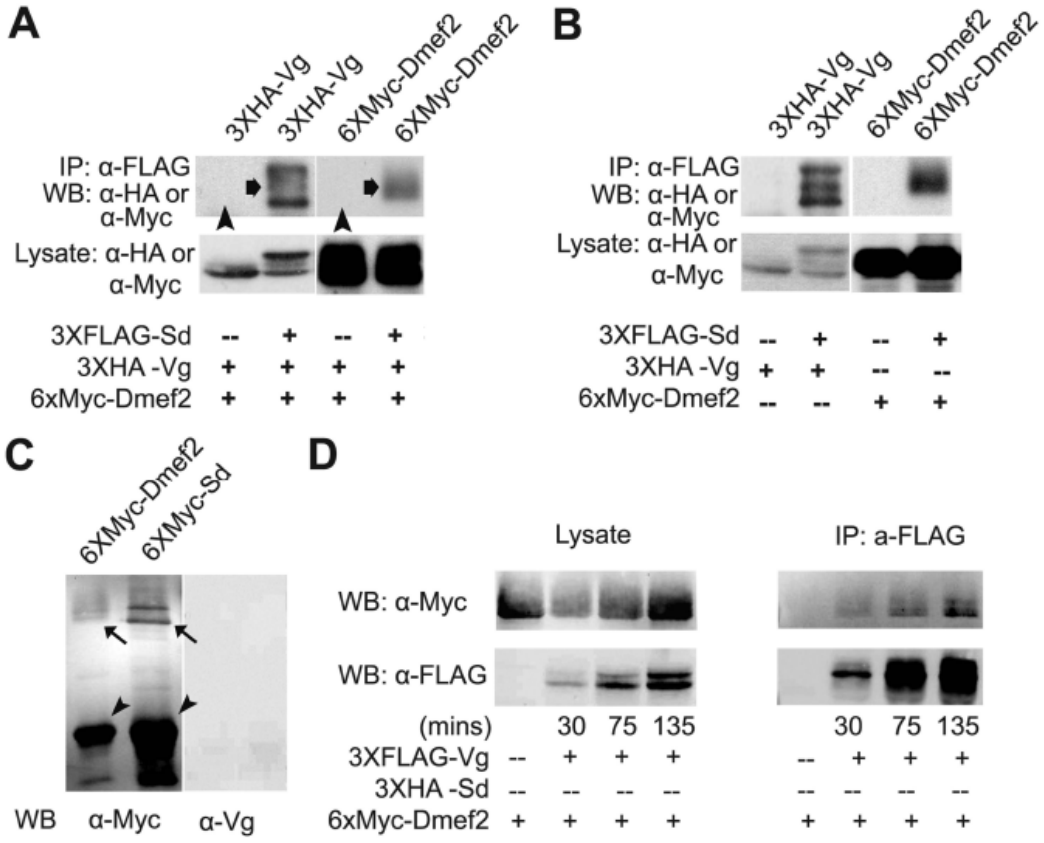

A
3XHA-Vg
3XHA-Vg
6XMyc-Dmef2
6XMyc-Dmef2
IP: α-FLAG
WB: α-HA or α-Myc
Lysate: α-HA or α-Myc
3XFLAG-Sd -- + -- +
3XHA -Vg + + + +
6xMyc-Dmef2 + + + +
B
3XHA-Vg
3XHA-Vg
6XMyc-Dmef2
6XMyc-Dmef2
IP: α-FLAG
WB: α-HA or α-Myc
Lysate: α-HA or α-Myc
3XFLAG-Sd -- + -- +
3XHA -Vg + + -- --
6xMyc-Dmef2 -- -- + +
C
6XMyc-Dmef2
6XMyc-Sd
WB α-Myc α-Vg
D
Lysate
IP: a-FLAG
WB: α-Myc
WB: α-FLAG
(mins) 30 75 135 30 75 135
3XFLAG-Vg -- + + + -- + + +
3XHA -Sd -- -- -- -- -- -- -- --
6xMyc-Dmef2 + + + + + + + +

Figure 3.4: Interactions between Sd, Vg and Dmef2 can be shown by co-immunoprecipitation (Co-IP) assays. Indicated proteins with different tags were co-expressed in S2 cells and Co-IP was performed using anti-FLAG beads in both control and experiment samples. Proteins coming down with the beads and the relative expression level of the proteins in the lysate were detected with corresponding tag antibodies. **A,** Dmef2 and Vg were co-IPed simultaneously with Sd (arrows). In the control, tagged Vg and Demf2 did not come down with the beads (arrow heads). There are additional bands for Vg and Dmef2, likely because of post-translational modifications. **B,** Sd co-IPed Vg or Dmef2 without co-expression of Dmef2 or Vg, respectively. **C,** The anti-FLAG bead-purified IP complex of 3xFLAG-Sd and 6xMyc-Dmef2 and that of 3xFLAG-Dmef2 and 6xMyc-Sd were immunoblotted with anti-Myc and anti-Vg antibodies. Significant levels of Vg could not be detected in these complexes. Arrows show the proteins coming down with the beads and arrowheads show the primary antibody bands. **D,** Co-IP was performed on S2 cells transfected with the indicated proteins at different times, following heat shock. The relative amount of co-IPed Dmef2 (right) increased with the expression level of Vg and Dmef2 (left).

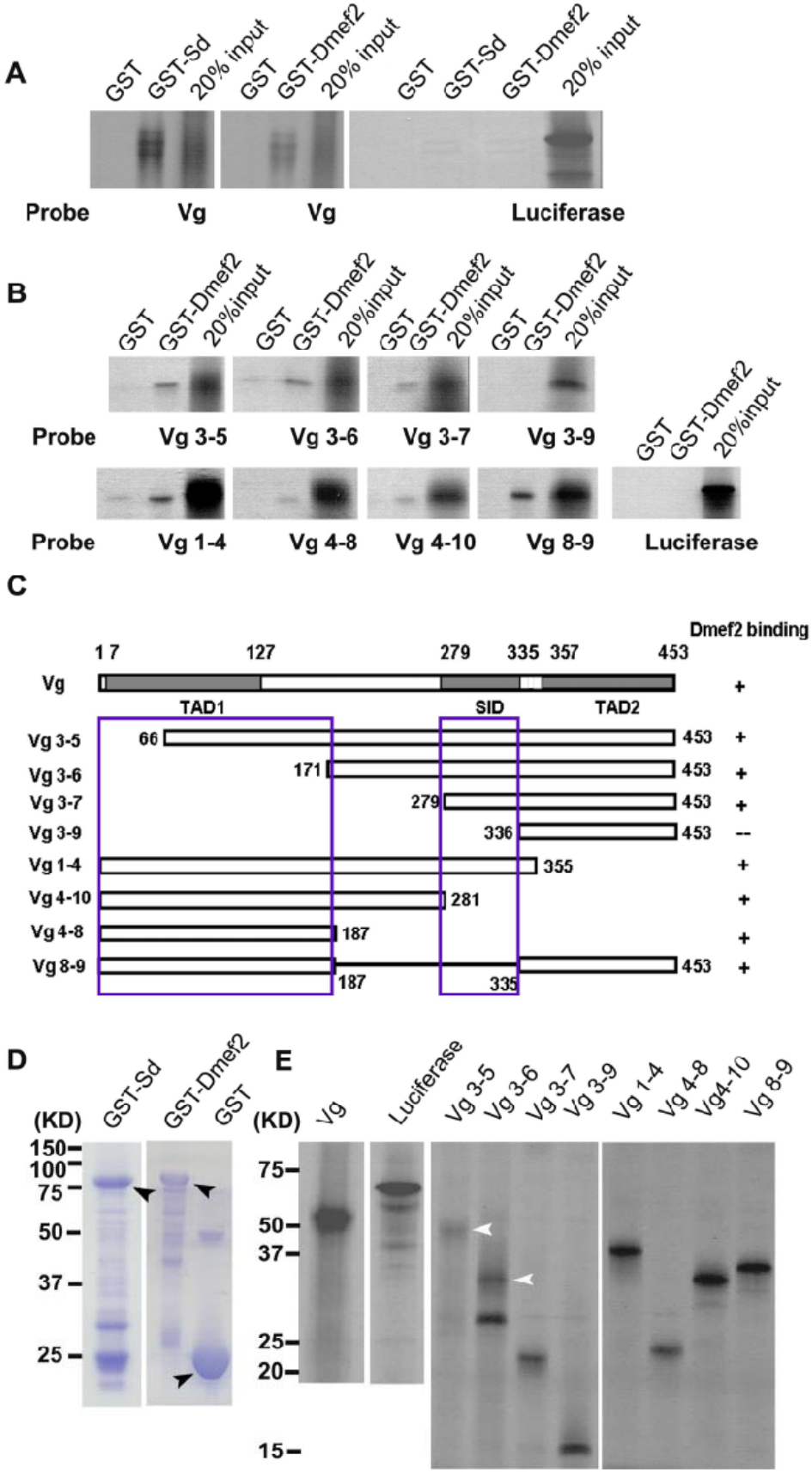

A
GST
GST-Sd
20% input
GST
GST-Dmef2
20% input
GST
GST-Sd
GST-Dmef2
20% input
Probe
Vg
Vg
Luciferase
B
GST
GST-Dmef2
20%input
Probe
Vg 3-5
Vg 3-6
Vg 3-7
Vg 3-9
Vg 1-4
Vg 4-8
Vg 4-10
Vg 8-9
Luciferase
C
Dmef2 binding
1 7
127
279
335 357
453
Vg
TAD1
SID
TAD2
Vg 3-5
Vg 3-6
Vg 3-7
Vg 3-9
Vg 1-4
Vg 4-10
Vg 4-8
Vg 8-9
66
171
279
336
355
281
187
187
335
453
D
(KD)
GST-Sd
GST-Dmef2
GST
150
100
75
50
37
25
E
(KD)
Vg
Luciferase
Vg 3-5
Vg 3-6
Vg 3-7
Vg 3-9
Vg 1-4
Vg 4-8
Vg4-10
Vg 8-9
75
50
37
25
20
15

Figure 3.5: Vg can interact with Dmef2 at a different site than it interacts with Sd. **A,** A positive control shows an interaction with a known Vg binding partner (GST-Sd). A similar robust interaction is detected between Vg and GST-Dmef2. Luciferase serves as a negative control. **B,** Two separate domains (illustrated with purple boxes in **C**) in Vg are capable of interacting with Dmef2. All deletions except Vg3-9 interact with GST-Dmef2 since at least one of the two domains is intact in all other deletions tested. **D and E,** Gel analysis confirming expression of proteins used in pull-down assays (arrowheads). Protein size is indicated on the left. SID (Sd interaction domain); TAD (transcription activation domain).

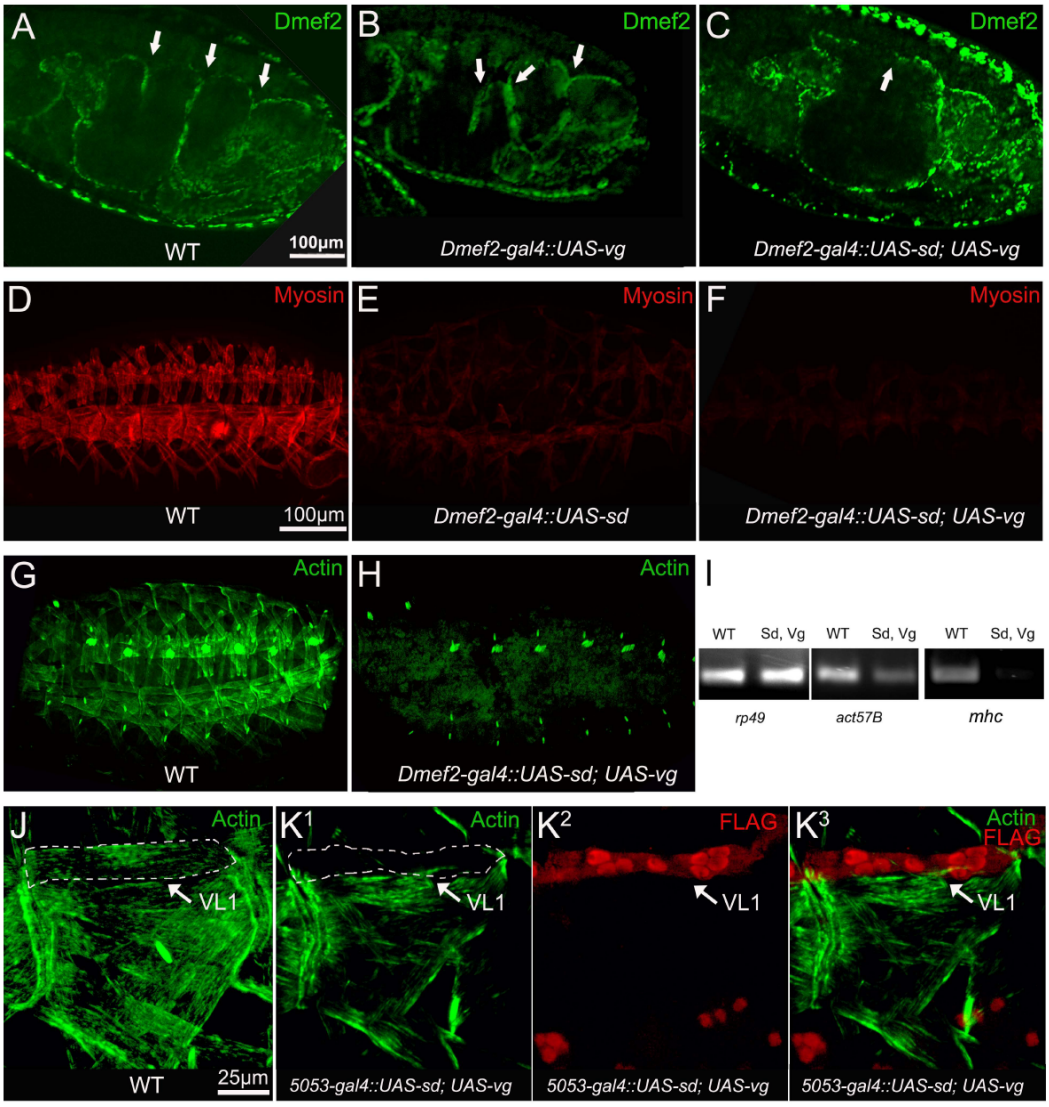

A
Dmef2
WT
100μm
B
Dmef2
Dmef2-gal4::UAS-vg
C
Dmef2
Dmef2-gal4::UAS-sd; UAS-vg
D
Myosin
WT
100μm
E
Myosin
Dmef2-gal4::UAS-sd
F
Myosin
Dmef2-gal4::UAS-sd; UAS-vg
G
Actin
WT
H
Actin
Dmef2-gal4::UAS-sd; UAS-vg
I
WT
Sd, Vg
WT
Sd, Vg
WT
Sd, Vg
rp49
act57B
mhc
J
Actin
VL1
WT
25μm
K1
Actin
VL1
5053-gal4::UAS-sd; UAS-vg
K2
FLAG
VL1
5053-gal4::UAS-sd; UAS-vg
K3
Actin
FLAG
VL1
5053-gal4::UAS-sd; UAS-vg

Figure 3.6: The Sd-Vg complex represses Dmef2 function during muscle differentiation. Embryos (stage 16) are shown as lateral views and dorsal up with anterior to the left. **A,** The three constrictions that subdivide the midgut into four chambers are shown with arrows in a wild type embryo. **B,** Ectopic expression of Vg in visceral muscles of embryos via *Dmef2*-GAL4 does not affect the formation of these constrictions. **C,** Ectopic co-expression of Sd and Vg in visceral muscles leads to the repression of Dmef2 function in these muscles and all three constrictions disappear. **D-F** shows the Myosin staining of wild type embryos (D) and the embryos over-expressing Sd or Sd and Vg (E-F). The apparent level of myosin staining is reduced when Sd is over-expressed in *Dmef2* expressing muscles (E) and even more reduced when Vg and Sd are present (F). **G-H,** Actin staining by phalloidin failed to show the formation of myofibers in muscles of embryo over-expressing Sd and Vg. **I,** the results of RT-PCR from stage 12-15 wild type embryos or embryos over-expressing Sd and Vg. The relative amount of *act57B* and *mhc* mRNA in embryos over-expressing Sd and Vg is much lower than wild type. *rp49* mRNA was used as loading control. **J,** Ventral SMs in one segment are visualized by Actin staining in a wild type embryo, and VL1 is shown by the dashed frame. **K^{1-3},** Specific over-expression of Sd and Vg in VL1 via *5053*-GAL4 leads to the missing of myofiber in this muscle. (Sd and Vg are 3xFLAG tagged and 3xHA tagged respectively).

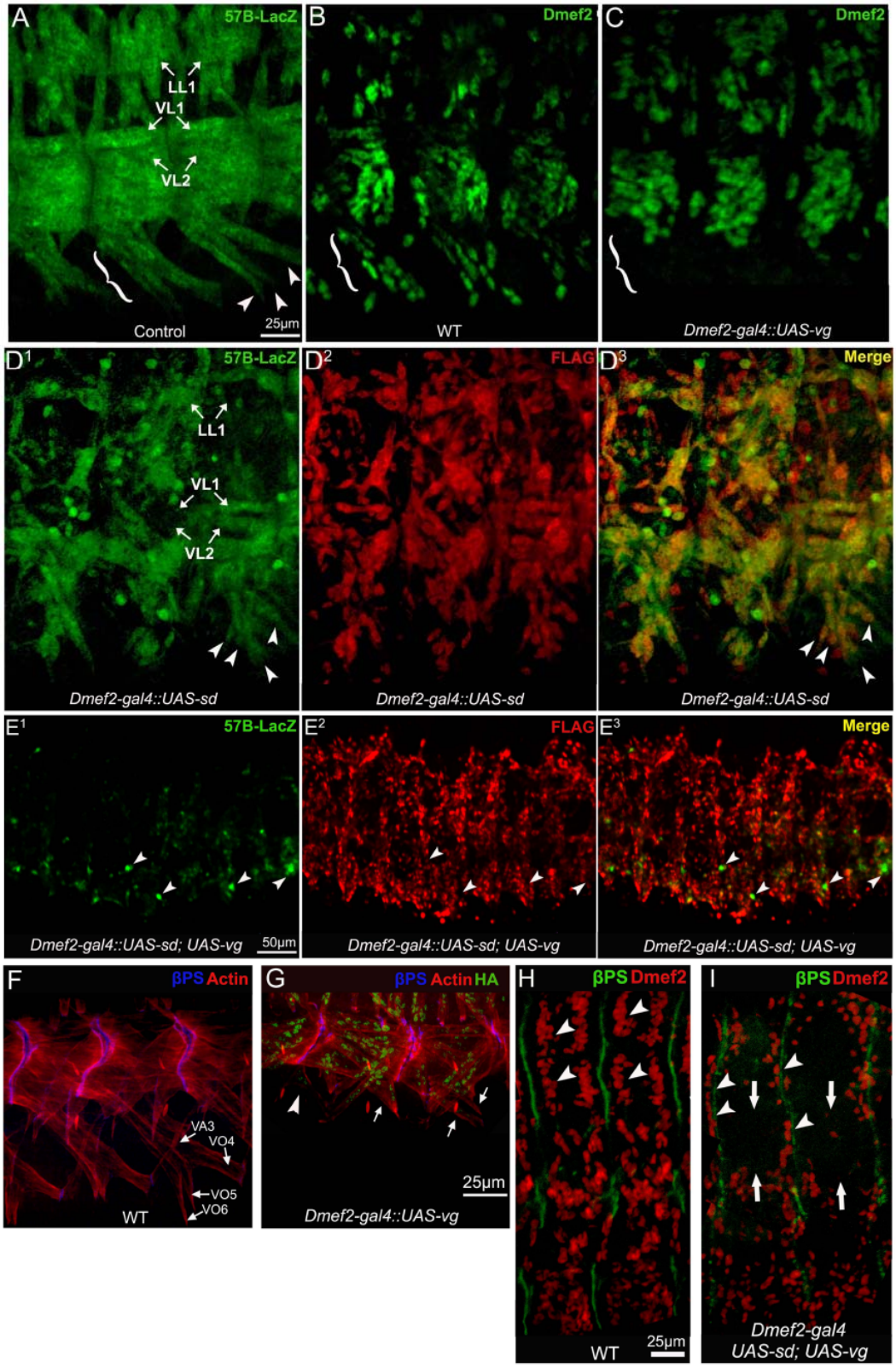

A
57B-LacZ
LL1
VL1
VL2
Control
25μm
B
Dmef2
WT
C
Dmef2
Dmef2-gal4::UAS-vg
D1
57B-LacZ
LL1
VL1
VL2
Dmef2-gal4::UAS-sd
D2
FLAG
Dmef2-gal4::UAS-sd
D3
Merge
Dmef2-gal4::UAS-sd
E1
57B-LacZ
Dmef2-gal4::UAS-sd; UAS-vg
50μm
E2
FLAG
Dmef2-gal4::UAS-sd; UAS-vg
E3
Merge
Dmef2-gal4::UAS-sd; UAS-vg
F
βPS Actin
VA3
VO4
VO5
VO6
WT
G
βPS Actin HA
25μm
Dmef2-gal4::UAS-vg
H
βPS Dmef2
WT
25μm
I
βPS Dmef2
Dmef2-gal4
UAS-sd; UAS-vg

Figure 3.7: Ectopic-expression of Sd and/or Vg via *Dmef2*-GAL4 leads to abnormal development of somatic muscles. Embryos (stage 16) are shown as lateral views and dorsal up with anterior to the left. **A,** Muscle fibers are visualized by anti-β-Gal (green) in control embryos expressing LacZ in cytoplasm under the control of the *act57B* promoter shown in Fig. 7A. Arrows point to muscle LL1, VL1 and VL2, and bracket shows the Ventral Oblique muscles (VO4-6, see Fig. 3D). VO4-6 muscles produce three projections that expand posterior-ventrally. **B,** Muscle cells are marked with anti-Dmef2 (green) in wild type embryos. Bracket shows muscle VO4-6. **C,** In embryos ectopically-expressing 3xHA-Vg, the extension of VO4-6 is lost (arrows), but the DMs are still highly organized (compare B with C). **D^1-D^3,** Embryos (*act57B-lacZ)* ectopically-expressing 3xFLAG-Sd (red) have disorganized somatic muscles. LacZ staining shows the whole muscle and FLAG staining shows the muscle nuclei. Muscle LL1, VL1 and VL2 do not develop well or disappear in some segments (arrows). The ventral muscle VO4-6 can still produce projections that expand ventrally, but there are more projections than wild type and some projections expand anterior-ventrally (arrowheads, compare D^1 with A). **E^1-E^3,** Embryos (*act57B-lacZ)* ectopically-expressing both 3xFLAG-Sd (red) and 3xHA-Vg have disorganized SMs, and the extension of VO4-6 is also lost (arrows). The expression level of LacZ is generally very low compared with the control, and cells with high expression levels usually do not express 3xFLAG-Sd or have low expression (arrowheads). Staining of 3xHA-Vg is not shown, since 3xFLAG-Sd and 3xHA-Vg always appear in the same muscle cells. **F,** Wild type ventral SMs (arrows) are labeled by phalloidin and the segment border is labeled with anti-βPS-integrin. Arrows point to muscle VO4-6 and VA3. **G,** In embryos over-expressing 3xHA-Vg, VL1-4 muscles are not affected, but ventral SMs are severely affected. It seems that these muscles are still there, but their migrations are either inhibited (arrowhead) or directed in a different path (arrows), which lead to no long extensions of muscle fiber. HA staining (green) shows the nuclei of muscles. **H,** Wild type SMs are labeled with anti-Dmef2 (red) and the segment border is labeled with anti- βPS-integrin. All muscle cells have their proper

positions relative to the border (arrowheads). **I,** In embryos over-expressing both 3xFLAG-Sd and 3xHA-Vg, muscle cells lose their positions and appear to cluster along the segments border (arrowheads). Large gaps are seen within each segment (arrows).

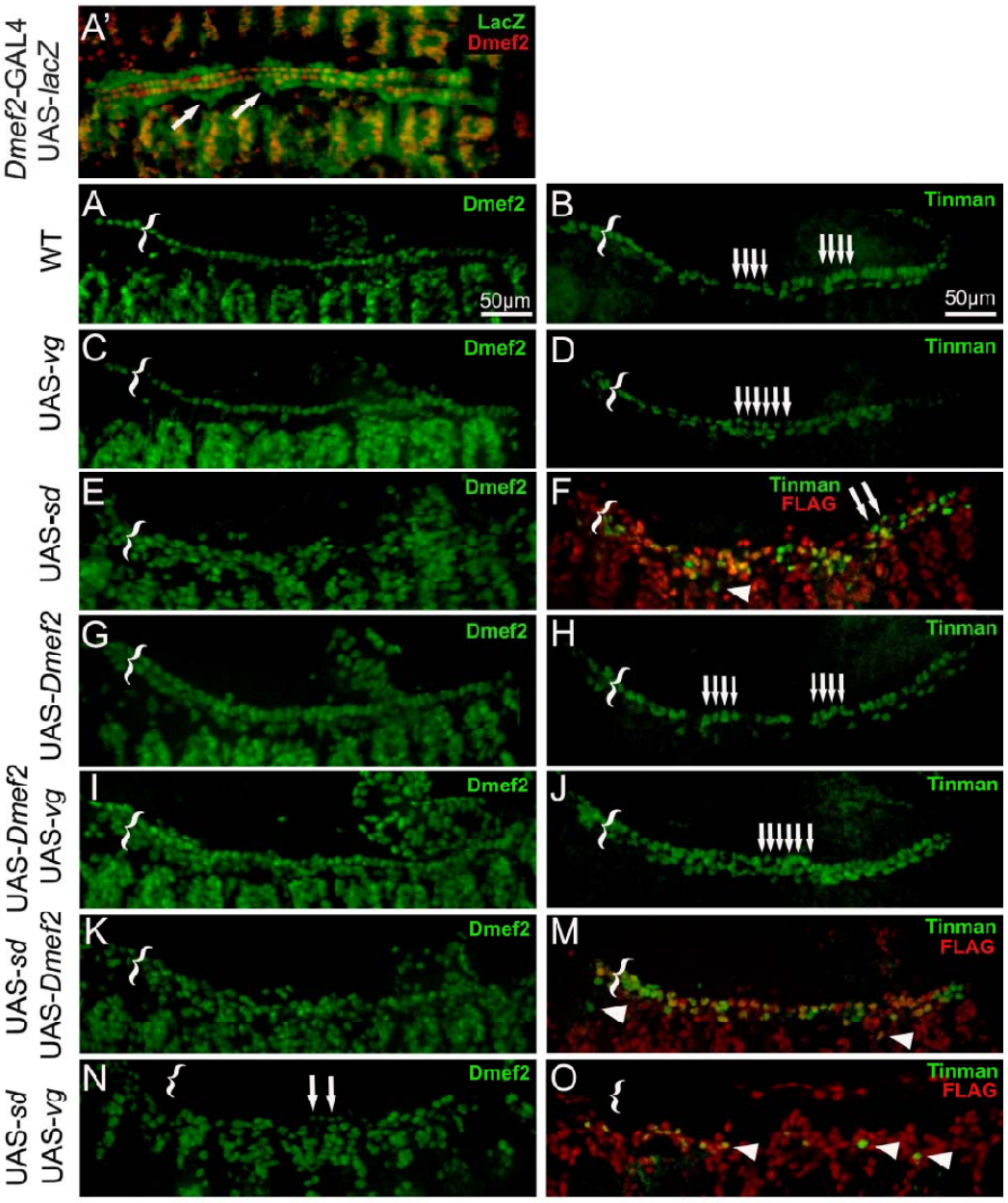

Dmef2-GAL4
UAS-lacZ
A'
LacZ
Dmef2
WT
A
Dmef2
50μm
B
Tinman
50μm
UAS-vg
C
Dmef2
D
Tinman
UAS-sd
E
Dmef2
F
Tinman
FLAG
UAS-Dmef2
G
Dmef2
H
Tinman
UAS-Dmef2
UAS-vg
I
Dmef2
J
Tinman
UAS-sd
UAS-Dmef2
K
Dmef2
M
Tinman
FLAG
UAS-sd
UAS-vg
N
Dmef2
O
Tinman
FLAG

Figure 3.8: Functional interactions between Sd, Vg and Dmef2 can be shown by ectopic-expression of various combinations of Sd, Vg and Dmef2 in heart cells. Embryos shown as lateral views and dorsal up with anterior to the left are at stage 14 and stained with antibodies as indicated by the colored lettering. Brackets show the area where the heart cells are located. A', *Dmef2*-GAL4 drives the expression of LacZ in all SMs and in both cardiac cells and pericardial cells (arrows). **A-B,** Wild-type. There is one row of Dmef2-positive cardiac cells (A) and four Tin-positive cardiac cells per hemisegment (B, arrows). **C-D,** Embryos ectopic-expressing 3xHA-Vg have the normal one row of Dmef2-positive cardiac cells (C), but now have six Tin-positive cardiac cells per hemisegment (D, arrows). **E-F,** Embryos ectopic-expressing 3xFLAG-Sd have two to three rows of Dmef2-positive cardiac cells (E). Tin-positive heart cells become disorganized: sometimes, you see only two Tin-positive cardiac cells in one hemisegment (F, arrows) and sometimes, you see Tin-positive cells appear in the region of the SMs (F, arrowhead). **G-H,** Embryos over-expressing 6xMyc-Dmef2 have two rows of Dmef2-positive cardiac cells (G), but there are four Tin-positive cardiac cells per hemisegment like wild type (H, arrows). **I-J,** Embryos over-expressing both 6xMyc-Dmef2 and 3xHA-Vg have two to three rows of Dmef2-positive cardiac cells (I) and six Tin-positive cardiac cells per hemisegment (J, arrows). Many more Tin-positive heart cells also appear (compared J with D and H). **K-M,** Embryos over-expressing both 3xFLAG-Sd and 6xMyc-Dmef2. The phenotype is similar to E-F, but heart cells are more organized (compare M with F). Arrowheads show the Tin-positive cells mixed with SMs. **N-O,** Embryos over-expressing both 3xFLAG-Sd and 3xHA-Vg have only a few Dmef2-positive cardiac cells left (N, arrows) and all the Tin-positive cells appear in the region of SMs (O, arrowheads).

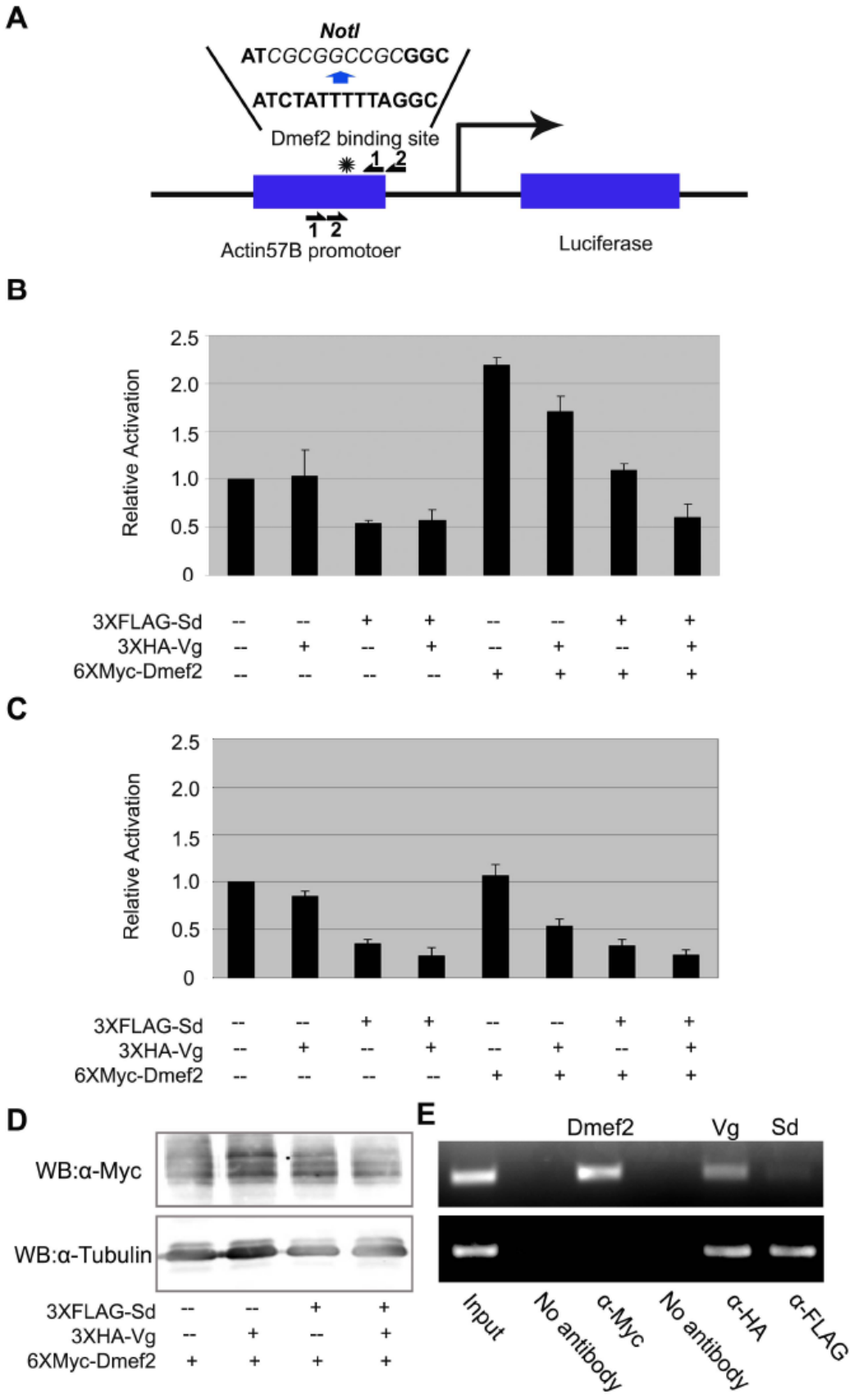

A
NotI
ATCGCGGCCGCGGC
ATCTATTTTTAGGC
Dmef2 binding site
1 2
1 2
Actin57B promotoer
Luciferase
B
Relative Activation
2.5
2.0
1.5
1.0
0.5
0
3XFLAG-Sd -- -- + + -- -- + +
3XHA-Vg -- + -- + -- + -- +
6XMyc-Dmef2 -- -- -- -- + + + +
C
Relative Activation
2.5
2.0
1.5
1.0
0.5
0
3XFLAG-Sd -- -- + + -- -- + +
3XHA-Vg -- + -- + -- + -- +
6XMyc-Dmef2 -- -- -- -- + + + +
D
WB:α-Myc
WB:α-Tubulin
3XFLAG-Sd -- -- + +
3XHA-Vg -- + -- +
6XMyc-Dmef2 + + + +
E
Dmef2
Vg
Sd
Input
No antibody
α-Myc
No antibody
α-HA
α-FLAG

Figure 3.S1: The Sd-Vg complex binds and regulates the activity of Dmef2 on the *act57B* promoter. **A,** A diagram of the reporter construct with a conserved Dmef2 binding site. The locations of two pairs of primers used in ChIP assays (small arrows) and the mutation of the Dmef2 binding site to a *Not*I site are shown. **B,** Luciferase activities of lysates from S2 cells expressing the indicated proteins were analyzed 40h after transfection. The averages of three independent experiments plus standard deviations are shown and error bars indicate standard deviations. Co-expression of Sd and Vg decreased the transcription activity of Dmef2 to half of the basal activity of *act57B* promoter in S2 cells. **C,** The same experiment as in **B** was done on the reporter construct with the Dmef2 binding site mutated. Co-expression of Dmef2 did not change the repression level of Sd and/or Vg on the transcription of *Act57B* promoter in S2 cells. **D,** Western blotting shows that the expression levels of Dmef2 in different luciferase assay samples are similar to each other. **E,** In the up panel, ChIP of a 3xHA-Vg and 6xMyc-Dmef2 complex, co-purifies with the *act57B* promoter. 3xFLAG-Sd also seems to interact with the *act57B* promoter, although more weakly. The first pair of primers was used to do ChIP on the construct with wild type *act57B* promoter. In the bottom panel, the second pair of primers was used to do ChIP on the construct with mutant *act57B* promoter, and ChIP of a 3xFLAG-Sd and 3xHA-Vg complex, co-purifies with the mutant promoter. 6xMyc-Dmef2 does not bind to the mutant promoter any more.

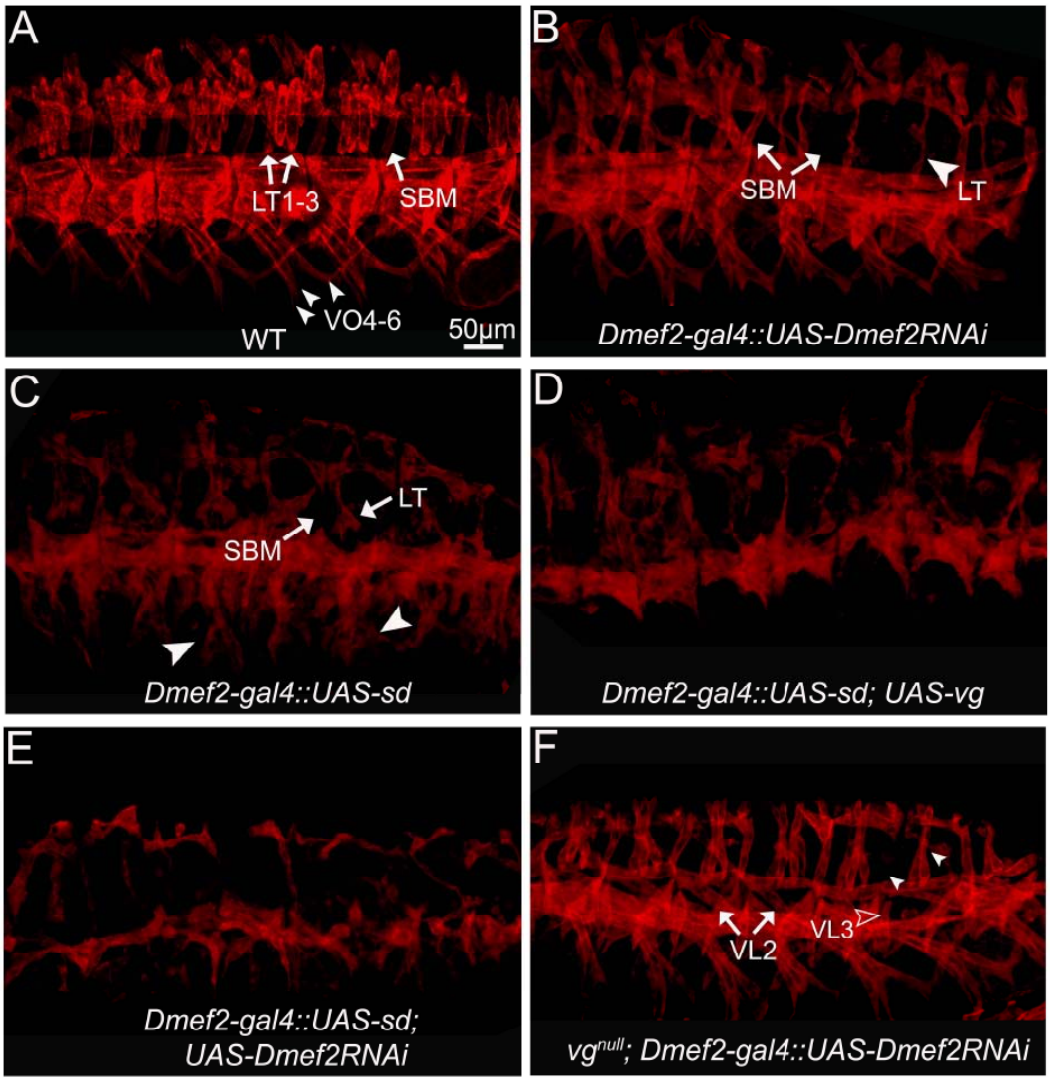

A
LT1-3
SBM
VO4-6
WT
50µm
B
SBM
LT
Dmef2-gal4::UAS-Dmef2RNAi
C
LT
SBM
Dmef2-gal4::UAS-sd
D
Dmef2-gal4::UAS-sd; UAS-vg
E
Dmef2-gal4::UAS-sd;
UAS-Dmef2RNAi
F
VL2
VL3
vg^null; Dmef2-gal4::UAS-Dmef2RNAi

Figure 3.S2: Muscle phenotypes of embryos over-expressing Sd and *vgnull* mutants are enhanced in *Dmef2* deficiency background. SMs are visualized by Myosin staining in all panels, and embryos shown as lateral views and dorsal up with anterior to the left are at stage 16. **A,** A wild type embryo is shown with some SMs labeled. **B,** The *Dmef2* RNAi line used to turn down Dmef2 expression produced a mild phenotype with muscle SBM (arrows) and LT1-3 (arrowhead) lost in some segments. The remained LT muscles are thinner than wild type (arrowhead). **C,** Over-expression of Sd produced a similar phenotype as muscle SBM and LT1-3 are lost in some segments (arrows) and the remained LT muscles are thinner. Muscle VO4-6 are still there but disorganized. **D,** When both Sd and Vg are present, these muscles are lost in almost all segments and big holes are observed. **E,** Over-expression of Sd in *Dmef2* RNAi background produced a similar phenotype to that of over-expressing Sd and Vg. **F,** *vgnull* mutant phenotype is enhanced in *Dmef2* RNAi background. About 70% segments start to lose muscle VL2 (arrows) compared with about 30% segments in only *vgnull* mutant. Also, some segments start to lose muscle VL3 (empty arrowhead). Note this embryo also has a similar phenotype to that in B (arrowhead).

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Chapter 4: Vestigial is required during late-stage muscle differentiation in *Drosophila melanogaster* embryos.

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Introduction

Embryonic muscles are first specified as founder cells within the embryonic mesoderm. The specification of diversity among muscle founder cells has been linked to differences in expression of a combination of transcription factors known as muscle identity genes including: *slouch*, *apterous*, *ladybird*, *vestigial* (*vg*), *nautilus* and *even-skipped* [1]. Surrounding fusion-competent myoblasts are recruited to founder cells with corresponding patterns of expression of these factors and fuse with them. This initial formation of a syncytial muscle is followed by a precise series of events whereby each muscle migrates to a specific location, interacting with neighbouring cells to form attachments allowing for coordinated movement.

During later stages of *Drosophila melanogaster* embryonic development, somatic muscles (SMs) organize into a complex pattern in each abdominal hemi-segment from A2 to A7 (Fig. 4.1A-B). Formation and maintenance of this pattern requires both internal differentiation events and intercellular signalling to direct a precise pattern of migration and attachments. After migration, SMs form two different types of attachments: to epidermal cells (tendon cells) and intermuscular adhesions diagrammed in Fig. 4.1C. Ultrastructural analysis reveals intermuscular attachments contain extensive extracellular matrix consisting of fuzzy electron dense fibres while muscle-epidermis attachments contain only a thin line of extracellular electron-dense material [2]. Muscle-tendon cell interactions guide the initial stages of migration and attachment [3, 4]. Similar to its role in axon pathfinding [5], the guidance protein Slit is secreted from tendon cell precursors at the segment borders and the corresponding receptor Robo is found on the surface of ventral-longitudinal muscles (VLs). In muscles, Slit has a bi-functional role, repelling myotubes during early development, but attracting them later. In

slit mutant embryos, VLs aberrantly cross the midline due to the lack of a repellent Slit source along the midline. If *slit* is expressed only in midline cells, VLs stop crossing the midline but fail to reach their normal attachment sites due to the lack of an attractive Slit source at the segment borders [6].

Developing myotubes also secrete Vein, a ligand for the *Drosophila* epidermal growth factor receptor (DER), which activates the Ras pathway in the tendon cells, leading to the final differentiation of tendon cells through elevating expression of *stripe* (*sr)* [3, 7]. Sr, in turn, induces expression of the secreted protein Thrombospondin (Tsp), which is required for building stable integrin-mediated junctions by binding the αPS2βPS (PS2) integrin receptor [8]. In *sr* mutant embryos, myotubes fail to make attachments with epidermis, losing their elongated morphology and becoming rounded in appearance [9, 10].

Formation of the junctions between muscles or muscle and tendon cells is largely mediated by integrins. Integrins are heterodimeric single-pass transmembrane receptors that mediate attachment to the extracellular matrix (ECM) [11]. The two major *Drosophila* integrins PS1 (αPS1βPS) and PS2 (αPS2βPS) have a complementary pattern of expression with PS2 concentrated at the ends of SMs and PS1 accumulating on the tendon cells [12, 13]. PS1 cannot substitute for PS2 at the muscle attachments and vice versa [14]. The integrin-mediated myotendinous adhesions are established between muscles and tendon cells (Fig. 4.1 B-C) [13]. The process of muscle specification appears to be independent of actual formation of the adhesions as absence of one or more of the adhesion proteins, including PS integrins, does not affect initial specification, fusion, and attachment of SMs. Rather, these muscles detach and round-up upon first contraction due to the lack of strong adhesion.

Several lives of evidence have established that formation of muscle-muscle adhesions is a distinct process from that involved in specification of muscle-tendon attachments. Muscle-tendon cell signalling is blocked by mutation of both *engrailed* and *invected*. In these embryos, Tig and βPS remain localized at the end of muscles in contact with each other. This suggests that initial localization of the ECM component Tig at the segment borders is independent of

tendon cells and integrin, but requires muscle-muscle contact [15]. Mutations in *rhea* (encoding Talin) cause the specific disruption of the muscle tendon cell attachments but not muscle-muscle attachments (Fig. 4.1C) [16]. Conversely, mutations in *tiggrin* (*tig*) lead to weak muscle-muscle attachments but muscle tendon cell attachments are not affected significantly [17]. Notably, there appears to be significant redundancy in this process as embryos with mutations in either gene do not manifest a severe muscle detachment phenotype. However, in embryos with both *tig* and *rhea* mutations, SMs detach and round-up due to the disruption in both types of attachments [15]. Thus, embryos homozygous for *rhea* mutations are an excellent sensitised genetic background for studying the role of factors that influence establishment of intermuscular attachment.

Vg was first identified as a key 'selector' gene that specifies wing identity during *Drosophila* development [18]. Vg does not have a DNA binding domain but contains two domains important for gene activation [19] and thus, must partner with additional proteins that bring it to the DNA. In ectodermal cells of the wing imaginal disc, Vg interacts with Scalloped [20], which has a conserved DNA binding domain and a well characterized Vg interaction domain [20-22]. These two proteins form a wing-specific transcription factor complex that directs wing development in any ectodermal cell type where it is expressed [21, 22]. This aspect of *vg* function is well known as many mutations in *vg* have been recovered that eliminate all adult wing formation but are otherwise viable. However, there are strong hypomorphic and dominant *vg* alleles that have phenotypes affecting other tissues. During pupal development, *vg* has been shown to be a muscle identity gene for specific flight muscles [23]. For these muscle cell specific activities, Vg appears to require interaction with *Dmef2*, a key myogenic gene required for specification and subsequent differentiation of all muscles [24, 25].

To further clarify the role of Vg during embryonic muscle development, we performed both loss-of- and gain-of-*vg*-function analysis. Our results revealed a role of Vg in the establishment of stable inter-muscular myotendinous junctions. Further, we show DER signaling may mediate the inter-muscular communication

and muscles expressing Vg become competent to respond to this communication by building a stable inter-muscular junctions.

Materials and Methods

Drosophila strains

The *rhea*[1], *robo*[1], and *slit*[2] mutations and w^{1118} used as the untransformed reference strain were obtained from the Bloomington Stock Center; sd^{3L} [26], vg^{null} [27], and $dgrip^{ex36}$ [28] have been described previously. Ectopic transgene expression was performed using the Gal4-UAS system [29] using the following lines: *Dmef2*-Gal4 [30], *twi*-Gal4 [31], *sd*-Gal4 [32], C23-Gal4 (from Bloomington Stock Center), UAS-*robo* [6], UAS-*vg* [24], UAS-*DN-egfr* [3], UAS-*λ-egfr* [33], UAS-*sdΔTEA* [34], and UAS-*lacZ* (Bloomington).

Immunohistochemistry and microscopy

Embryos were formaldehyde fixed [35] and the following primary antibodies were used at the indicated dilutions: mouse anti-FLAG (1:1000; Sigma); rat anti-HA (1:200; Roche); rat anti-Myosin (1:500; Abcam, Cambridge, MA); mouse anti-βPS-integrin (developed by Danny Brower and obtained from the Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa City, IA, 1:500); mouse anti-β-Gal (1:500; Promega); anti-muscle myosin heavy chain MAb FMM5 (1:10; from D. Kiehart, Duke University, Durham, NC); rabbit anti-Vg [18]; rat anti-Thrombospondin [8]; mouse anti-Talin [36]; rabbit anti-PINCH [37]; rabbit anti-Kon [38]. Donkey Alexa488-, Alexa568-, Alexa594-, and Alexa647-conjugated secondary antibodies were used (1:4000; Invitrogen). Muscle actin was stained using Alexa546-conjugated phalloidin (1:25; Invitrogen). Images were obtained with a Zeiss LSM510 or Ultraview ERS confocal microscope (Perkin Elmer, Norwalk, CT) and assembled using Adobe Photoshop (Ver. CS, San Jose, CA).

Tiggrin antibody production

A cDNA fragment encoding the C-terminal 270 amino acids including the RGD (Arg-Gly-Asp) domain of Tig [39] was cloned into pDEST17 bacterial expression vector (Invitrogen), expressed in *Escherichia coli* [BL21(DE3),

Stratagene] and purified using Ni-NTA according to the manufacturer's protocol (QIAGEN). Purified fusion protein was injected into rabbits (Pocono Rabbit Farm and Laboratory Inc. Canadensis, PA). Specificity of the rabbit polyclonal serum was determined by testing it against purified Tig and fixed *Drosophila* embryos confirming the localization pattern was the same as published for Tig previously [39].

Results

vg^{null} but not *sd*^{3L} mutant embryos show muscle detachment in a *rhea*^{1} background.

Antibody staining of wild type embryos showed that *vg* is expressed at relatively high levels in muscles making both intermusclar and muscle tendon cell attachments at the segment border (Fig. 4.2A). To determine if there is a significant role for Vg regulation of the migration or attachment function of these late-stage embryonic SMs, we examined the muscle phenotypes of embryos homozygous for the *vg*^{null} mutation. Some flies with a homozygous *vg*^{null} genotype do survive to adulthood but they are invariably unable to produce progeny and have significant defects in the adult musculature [40]. In homozygous *vg*^{null} embryos, VL2 muscles were completely absent in at least 30% of segments (Fig. 4.2B-C^{1}). This muscle-loss phenotype appears to be VL2 cell-specific as VL1 muscles were present in all segments (Fig. 4.2C and Fig. 4.S2 C-D). However, the loss of Vg does not seem to block initial formation of adhesions between two differentiating muscles, as similar to stage 16 wild type embryos (Fig. 4.2B-B^{1}), in homozygous *vg*^{null} , pairs of VL muscles formed tight adhesions between their corresponding VL muscle in the next segment (Fig. 4.2C-C^{1}).

To further dissect the requirement for Vg during formation of adhesions between SMs we paired mutations in *vg* with those in *rhea* (talin deficient) which would make small changes in attachments induced by loss of *vg* more apparent. In *rhea*^{1} mutant embryos, junction formation between pairs of VL muscles appears normal (Fig. 4.2D-D^{1}). At early stages (14) the VL muscles showed no significant migration or attachment defects in *vg*^{null}*; rhea*^{1} double mutant embryos (Fig.

4.2E-E[1]). However, at later stages (16+) several muscles, most noticeably VL1 can be seen detaching from their normal location (Fig. 4.2F[1]-H[1]). This muscle detachment phenotype appears to be due solely to lack of Vg activity as it can be rescued by expression of wild type Vg in muscle cells (Fig. 4.2I-I[1]). As Sd is the known binding partner for Vg function during wing development [22], the muscle phenotype of *sd*^{3L}; *rhea*^{1}double mutants was also examined. Mutants homozygous for a loss-of-function *sd*^{3L} allele [26, 41] and *rhea*^{1} produce only a mild SM phenotype described previously [24]. Otherwise, they are indistinguishable from *rhea*^{1} mutants (Fig. 4.2J).

Expressing a form of Sd that can bind Vg but not DNA, causes a phenotype similar to *tig* null mutants.

In cells of the wing imaginal disc, Vg forms a complex with Sd to localize to the nucleus and bind chromatin via a conserved TEA binding domain within Sd [21, 22]. Previously a form of Sd that removes the TEA DNA binding domain, (*sdΔTEA*) has been shown to bind Vg, but as the resulting complex cannot bind DNA acts to inhibit Vg activity in imaginal discs [34]. During experiments to confirm that tissue-specific expression of a UAS-*sdΔTEA* transgene can specifically inhibit the gene activation function(s) of Vg in the wing disc we noted that expression of *sdΔTEA* by *sd*-GAL4 also produced elongated pupae and adults (Fig. 3.3A and C). A database search produced only one other mutation that produces elongated body, muscle spacing and semi-lethality phenotypes, a null mutation in *tig* , required for intermuscular junction formation [17].This phenotype was also notable as *sd*-GAL4 would lead to only weak activation of *sdΔTEA* in tissues such as embryonic SMs from stage13 to stage16 [24]. Given the similarity of interfering with Vg function via *sdΔTEA* and *tig* mutations, we tested the effect of *sdΔTEA* in muscle cells using a driver that is expressed at much higher levels (*Dmef2*-GAL4) (Fig. 3.3B). This combination was semi-lethal, with 78.7% of pupae (n=619) failing to eclose. The larvae hatched from embryos over-expressing *sdΔTEA* via *Dmef2*-GAL4 had obvious gaps between the DA1 and DA2 muscles that were larger than wild type (Fig. 4.3D-D') suggesting the elongated body type was caused by muscle defects. However, the requirement for

Vg function was stage specific as expression of *sdΔTEA* in muscle progenitor cells at earlier embryo developmental stages (7-11) using *twist*-GAL4 [31] did not lead to elongated adults and significant lethality (Fig. 4.3C). Finally, to ensure that the phenotype we observed was caused by inhibition of Vg through formation of a non-functional Vg/*sdΔTEA* complex and not through over-expression of Sd, we over-expressed a form of Sd that cannot bind Vg (SdΔVID). This combination produced flies with no detectable defects in developing muscles.

VL muscle migration and initial adhesion occurs normally in the absence of Vg.

The progressive muscle detachment phenotype we observed when Vg activity is reduced could be caused by several events, including: improper muscle specification, a failure of muscles to migrate to the attachment site, or select the appropriate target site, or the inability to form a strong connection that can resist the force of muscle contraction at later stages. To determine if Vg was required for initial establishment and maintenance of the VL cell lineage, we used a marker that would be activated solely in the VL1 muscle and would persist during later development (*5053*-GAL4 and UAS-*lacZ*) [38]. The VL1 muscle is initially specified correctly in all segments in both vg^{null} and wild type embryos (Fig. 4.S2C-D). Similarly, development of tendon cells was not affected in vg^{null}; $rhea^{1}$ double mutants (Fig. 4.S2A-B).

As early specification of VL1 was unaffected when Vg is absent, we next assayed for changes in formation of junctions between two muscles at the segment border. There are three known major components of muscle-muscle junctions [13]: 1) PS2 integrin; 2) ECM containing PS2 integrin ligands: Tig [39], and Tsp [8] and 3) Talin and its associated proteins including PINCH [37]. Together, they link PS2 integrin to the muscle myofiber, forming a tight adhesion junction or integrin complex holding muscles together (Fig. 4.4A-A^3). In the vg^{null}; $rhea^{1}$ double mutant embryos, βPS and Tig (Fig. 4.4B-B^3), PINCH and βPS (Fig. 4.4D-D^3), and Tsp (Fig. 4.4E-E^2), were all concentrated at the ends of detaching VL1 muscles and connected to myofibers, similar to wild type (Fig. 4.4A-A^3 and G-G^3) or $rhea^{1}$ single mutant embryos (Fig. 4.4C-C^3). Thus, the affinity of PS2

integrin for its ligands did not appear to be affected and the integrin complex was still largely intact in muscles of the double mutant embryos, prior to detachment.

Since the major adhesion proteins were being localized correctly at the end of VL1 muscles in vg^{null}; $rhea^{1}$ mutant embryos, we examined the process of VL1 migration. The transmembrane protein Kon-tiki (Kon) is localized to the tips of VL muscles and is required for formation of filopodia and proper migration of developing muscles [38, 42].The expression and localization of Kon in the vg^{null}; $rhea^{1}$ double mutants was indistinguishable from wild type embryos (Fig. 4.4G-H^{3}). In addition, the direction of VL1 muscle migration was unaffected. VL1 muscles normally migrate from the posterior border of each segment to the anterior border and then attach to both [38]. If muscles in embryos with vg^{null}; $rhea^{1}$ mutations migrated inappropriately then they would fail to attach to the anterior border and round up at the posterior side of each segment. However, in the double mutants, more than half of the VL1 muscles detached from the posterior borders remaining attached to the anterior (Fig. 4.4D-F), indicating they already reached to their attachment sites.

Ectopic-expression of Vg induces abnormal muscle-muscle attachments.

The phenotype associated with vg^{null} mutants strongly suggested that Vg expression induces cell-specific changes in VL muscles for them to be competent to form inter-muscular attachments at the segment border. If this hypothesis is correct, then Vg should be able to induce additional attachments when expressed ectopically in muscles where it is not normally found at high levels. The LT1-4 muscles migrate vertically within each segment and normally make only muscle tendon cell attachments in wild type embryos (Fig. 4.5A). Ectopic-expression of Vg within the LT muscles caused them to migrate towards the segment borders and attach there, producing muscle-muscle attachments between LT and VA muscles, where they normally do not occur (Fig. 4.5B-D). Notably, this ectopic expression does not appear to alter the initial muscle identities of LT-2, LT-4, and VA2 (Fig. 4.S2E-F). The ectopic muscle-muscle adhesions induced by ectopic expression of Vg in the LT muscles appeared to be functional, as muscle VA1 was often located away from its normal position due to tension produced by

abnormal attachment to the LT muscles (Fig. 4.5C). We confirmed that this phenotype was Vg specific by expressing both *vg* and *sdΔTEA*. This combination showed fewer ectopic muscle attachments (24.5%, compared with 81.8% when Vg is expressed alone, Fig. 4.5E-F). Similarly, we saw a correlation between the severity of the ectopic LT muscle attachment phenotype and increasing the expression of ectopic Vg (Fig. 4.5F). Finally, ectopic expression of Vg in both cells is required to induce them to form ectopic attachments. Expression of Vg in only a single muscle group failed to build ectopic adhesion sites between LTs and VAs (Fig. 4.5G-H).

Ectopic adhesion sites were also induced between VT1 and LTs (Fig. 4.6A^3) or between SBM and LO1, (Fig. 4.6B^3, see Fig.1 for legend showing muscle identities) by higher levels of ectopic Vg expression. While expression of Vg clearly induced formation of extra attachments between muscles, it is possible that these attachments were not functional. Assembly of intermuscular junctions can be perturbed at several steps. A chimeric mimic of activated integrins can recruit Talin in embryonic muscles, but not other integrin-associated proteins like PINCH [43]. Similarly, in mammalian cells, activation of high affinity αVβ3 integrin produced ectopic integrin clusters that recruit Talin but not other integrin-associated protein [44]. We determined whether each of these integrin-associated components (Tig, Tsp, Talin and PINCH) was recruited to the additional adhesion sites associated by ectopic/over-expression of Vg (Fig. 4.6A^1-C^3). More importantly, the internal muscle myosin-actin fibre was connected to the ectopic integrin clusters (Fig. 4.6A^4). This suggests that a functional intermuscular attachment was induced in cells that migrate abnormally due to ectopic Vg.

Vg is required for the ectopic attachments formed between ventral midline-crossing muscles in *slit*^{2} mutants.

Although expression of Vg can induce the formation of muscle-muscle junctions, the contribution of signals from the surrounding tendon cells may be influencing this effect. Therefore, we examined the effect of blocking Vg function in *slit*^{2} mutant embryos, where the VL muscles cross dorsally over the CNS meeting those from the other side to form ectopic muscle attachments along the

ventral midline (Fig. 4.7A). This allows us to test the effect of loss of Vg on muscle-muscle attachments independently, as there are no tendon cells within this region [45]. Blocking Vg function via *sdΔTEA* in VL muscle cells that abnormally migrate along the midline led to fewer and smaller muscle-muscle adhesions (Fig. 4.7B). Conversely, increasing the expression of Vg produced more and larger adhesion sites (Fig. 4.7C-D).

Embryonic muscles expressing Vg require DER signalling to form attachments.

As a transcriptional activating "selector gene", the role of Vg is assumed to be induction of cell-specific changes in gene expression [21, 22]. However, since we observed that Vg expression is required in both cells forming a muscle-muscle adhesion, this would suggest additional coordination via cell-cell communication. The *Drosophila* epidermal growth factor receptor (DER), is ubiquitously expressed within the mesoderm [46]. Notably, one DER ligand, Vein, is enriched at the segment borders where intermuscular junctions are formed [3], making it the prime candidate for coordination of muscle-muscle adhesions in this region. Therefore, we tested if DER signalling is required for Vg mediated establishment of intermuscular attachments. Mesodermal expression of dominant-negative DER (*DN-Egfr*, [47] did not affect the specification of VL muscles that were crossing the midline, although the specification of muscle LL1 and VO4-6 were affected as described previously (Fig. 4.S2G-H) [47]. Increasing Vg expression in the SMs of $slit^2$ mutants greatly enhanced the adhesion level between SMs (Fig. 4.8A-B'), while reducing the activity of DER signalling decreased the size and/or number of adhesion sites caused by ectopic Vg (Fig. 4.8C-C' and E). Mesodermal expression of DN-Egfr alone in $slit^2$ mutants also decreased the size and number of the ectopic intermuscular adhesion sites along the midline (Fig. 4.8D and E).

Mesodermal expression of constitutively active λ-Egfr [33] disrupts normal migration of SMs in the mesoderm (Fig. 4.9A). There was no obvious adhesion between these muscles (arrowheads in Fig. 4.9A). However, ectopic-expression of Vg in this same genetic background caused disorganized muscles to

produce a large number of small adhesion sites between them (arrowheads in Fig. 4.9B). Similarly, mesodermal expression of λ-Egfr in *slit*^{2} mutants produced many small adhesion sites between midline-crossing VL muscles (Fig. 4.9C). Finally, this ectopic attachment phenotype was shown to be Vg specific as interfering with Vg function by co-expression of *sdΔTEA* resulted in fewer of these adhesion sites (Fig. 4.9D), while these adhesion sites become much bigger with increased Vg expression (Fig. 4.9E).

Vg induces ectopic adhesion between SM cells that make contact with each other through filopodial extensions

Both loss of Vg and *tig* function produce a unique elongated body phenotype associated with defects in the embryonic musculature (Fig. 4.3). One of the predicted roles of Tig is to induce formation of filopodia, and this may be required for muscle migration [17]. We noted that migrating SM cells seeking attachment targets extend filopodia at their leading edges. LTs muscle extend filopodia to the segment border and filopodia from migrating SM cells can be observed contacting each other between developing LT and ventral acute (VA) muscles (Fig. 4.10A^{1} -A^{2}). We did not observe filopodial contact between LT, VL or VL and VA muscles. Correspondingly, ectopic adhesion sites were produced between LTs and VAs, but not between LTs and VLs or between VLs and VAs when ectopically expressing Vg (Fig. 4.10 B and D). Additional filopodia or integrin localization was not observed at the leading edge of muscles expressing elevated levels of Vg (Fig. 4.10C-C^{1}). Thus, Vg induced formation of muscle-muscle adhesions requires close filopodial contact between migrating muscles.

Discussion

When two migrating somatic muscles come into close contact, there must be a cell-intrinsic mechanism to determine whether or not to build a stable adhesion junction. This would require coordinate regulation of this activity in each type of muscle to prevent inappropriate adhesions. We have shown that the transcriptional activator Vg is a key factor regulating this event in embryonic VL1-4 muscles. While expression of Vg in muscle cells makes them competent to

form intermuscular junctions, this process requires DER signalling to coordinate formation of attachments (Fig. 4.8-9). Finally, this process is associated with contact between filopodia from each of the cells expressing Vg (Fig. 4.5-6 and Fig. 4.10).

We used three independent methods to test the requirement for Vg: null mutations, interfering with Vg function using *sdΔTEA,* and ectopic Vg expression. When Vg function was blocked, adhesion between VL muscles was disrupted. This effect was enhanced in a $rhea^{1}$ mutant background while formation of intermuscular adhesions was reduced in $slit^{2}$ mutants. Conversely, adhesion between VL muscles in $slit^{2}$ mutants was enhanced when increasing *vg* expression in the muscles that normally express *vg*. It appears that formation of muscle-mucle attchments is directly related to the relative level of Vg in both cells as ectopic attachments failed to form if *vg* is expressed only in a single muscle (Fig. 4.5G-H).

Blocking Vg function by using a vg^{null} mutation or over-expressing *sdΔTEA* produced similar phenotypes (Fig. 4.2 and Fig. 4.S1). The one paradoxical difference between these methods was that the surviving pupae and adult vg^{null} mutants were less elongated compared to those over-expressing *sdΔTEA* (Fig. 4.2). The *sdΔTEA* transgene may block the functions of Vg-containing transcription factor complexes that do not normally include Sd, which would explain the more severe phenotype. Our data supports this conclusion, as vg^{null}*;* $rhea^{1}$ double mutants have an identical phenotype to that caused by expression of *sdΔTEA* in $rhea^{1}$ mutants (Fig. 4.S1). We can confirm that Vg function is blocked specifically when *sdΔTEA* is expressed in SMs, as over-expressing *sdΔTEA* via *Dmef2*-GAL4 was able to significantly rescue the LT muscle re-routing phenotype caused by over-expression of Vg, while expression of a transgene that deleted only the Vg interaction domain produced a wild-type phenotype.

Ectopic expression of Vg in LT muscles redirects their migration to the segmental borders (Fig. 4.5). This phenotype is similar to that caused by ectopic expression of Robo or Grip [6, 28]. Slit-Robo signalling provides an important

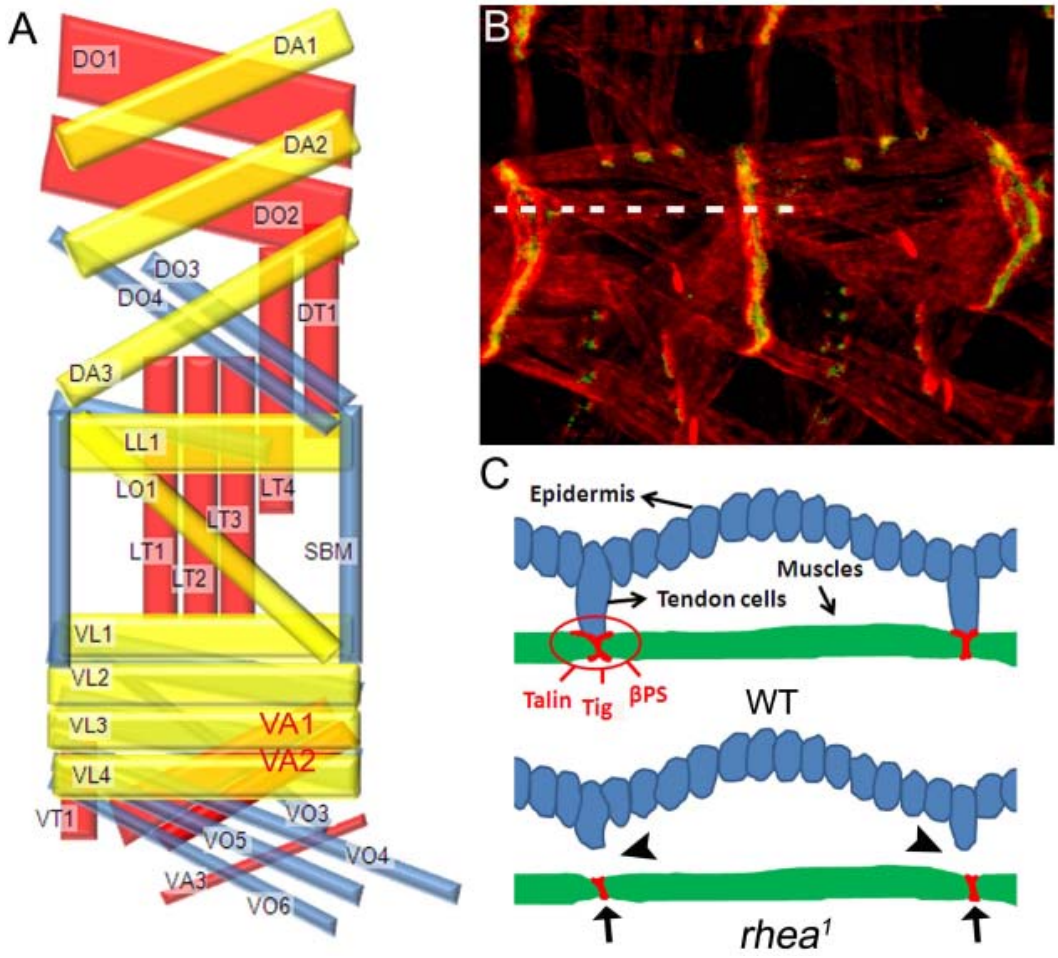
external cue to guide Robo-expressing muscles like VL1-4 to the segment border [6]. The PDZ domain protein Grip also plays an important role in the migration of VL muscles [28]. However, the aberrant muscle migration phenotype caused by ectopic Vg is independent of Slit-Robo signalling or Grip (Fig. 4.S3). Rather, our results suggest that Vg induces cell competence to form attachments. Thus, in muscle expressing ectopic Vg, formation of extra attachments may induce abnormal migration.

The mechanical connections of muscle-muscle attachments are thought to be primarily mediated by integrin and its associated adhesion proteins [13]. However, there must be a corresponding cellular regulation that determines if it is appropriate for two muscles coming into contact form specific types of attachments or not. Examination of the proteins representative of the three major components of the integrin complex showed that they were all present at the termini of VL muscles in vg^{null}; $rhea^{1}$ mutant embryos (Fig. 4.4), suggesting that the integrin complex was established properly. Thus, the role of Vg is clearly not during initial establishment of the junctions. However, muscular junctions are relatively dynamic, and may require cellular coordination to maintain their structure. The affinity of integrin to its ligands can change under different conditions and Talin binding to the integrin β integrin cytoplasmic tail represents the final common step in integrin activation [48]. When integrin affinity to ECM is low due to the loss of interaction of Talin with the β subunit cytoplasmic domains, the diffusible protein Tig does not co- localize with integrin at the end of detaching muscles [49], However, this phenotype is not what we observed in vg^{null}; $rhea^{1}$ double mutants (Fig. 4.4). Hyperactive PS2 integrins can be made when the cytoplasmic domain of α_{PS2} subunit is deleted, and mesodermal expression of this mutant integrin is able to produce ectopic intermuscular attachment [50]. Even so, the phenotype produced by hyperactive PS2 integrins is far milder than what we observed when ectopic sites are induced between VA and LT muscles where Vg is over expressed. Therefore, our results suggest a role for Vg during the establishment of intermuscular attachment that is permissive rather than directly altering the affinity of integrin within the junctions.

We have shown previously that *vg* has a role in the specification of embryonic muscle VL2 together with *Dmef2* [24]. However, in vg^{null} mutant embryos, examination of molecular markers unique to the VL1 muscle (*i.e. Kon*) showed no apparent change in identity compared to wild type. It retained a VL1 identity, and migrated to the correct location, making appropriate initial intermuscular attachments in most segments (Fig. 4.S2C-D). However, these same VL1 muscles detached from each other in vg^{null}*; rhea*^{1} double mutants. It would seem that *vg* is required to make a subset of muscles competent to establish intermuscular attachments. However, the cell-intrinsic role of Vg must be paired with a differential response to cell-cell communication. In cultured fibroblasts, EGFR signalling was shown to play a role in the establishment of mature focal adhesions (FAs) [51]. Knocking down EGFR signalling induces fast turnover of focal adhesions and produced small FAs, suggesting EGFR is involved in focal adhesion stabilization [52]. FAs are integrin-mediated structures closely related to the myotendinous junctions formed by skeletal muscle cells [53]. While the establishment of muscle-muscle attachments is a complex process and the mechanism behind this process is not clear [54, 55]. We observed that the relative level of Vg activity directly affected the number and size of the intermuscular adhesion sites induced by ectopic λ-Egfr (Fig. 4.9). Thus, Vg might be responding to external signalling to induce as yet uncharacterized muscle-specific genes that regulate turnover of intermuscular attachment and its stabilization. Alternatively, Vg may inducing expression of genes that are required for specific morphological changes in a subset of migrating muscles such as filopodia at the leading edge (Fig. 4.10), which may be required for making initial contacts with neighbouring cells to determine if a muscle-muscle junction is to be formed.

The later stages of muscle migration and attachment are remarkably similar in both *Drosophila* and vertebrates [56]. Our finding of a role for Vg in embryo SM development makes *Drosophila* Vg more analogous to the related Vestigial-like (Vgl) proteins in mammals [57, 58]. Among them, Vestigial-like 2 (*Vgl-2*) is expressed in skeletal muscle and is able to augment MyoD-induced

myosin heavy chain (Mhc) expression in 10T1/2 cells [58]. In addition to the known roles in adult wing and flight muscle development, our results reveal a novel cell-autonomous role for Vg in somatic muscle development. Two muscle cells expressing Vg communicate via DER signalling to coordinate production of intermuscular attachments.


A
DO1
DA1
DA2
DO2
DO3
DO4
DT1
DA3
LL1
LO1
LT4
LT3
LT1
LT2
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VL1
VL2
VL3
VA1
VA2
VL4
VT1
VO3
VO5
VO4
VA3
VO6
B
C
Epidermis
Muscles
Tendon cells
Talin
Tig
βPS
WT
rhea1

Figure 4.1. A) A schematic representation of the somatic muscles (SMs) in each abdominal hemisegment A2-A7 of the developing embryo (lateral view with anterior left and dorsal up) using the nomenclature of Crossley (1978). Inner, middle, and outer muscle layers are shown in yellow, blue, and red, respectively (Bate and Rushton, 1993). Dorsal Oblique (DO), Dorsal Acute (DA), Dorsal Transverse [28], Lateral Longitudinal (LL), Lateral Oblique (LO), Lateral Transverse [44], Segment Border Muscle (SBM), Ventral Longitudinal (VL), Ventral Acute (VA), Ventral Transverse (VT), Ventral Oblique (VO). VA1 and VA2 are highlighted in red. **B)** Muscle-muscle and muscle-tendon cell junctions in wild type embryos visualized by staining developing muscle cells with Actin [48] and βPS integrin (green). **C)** Diagrams showing a cross-sectional view along the broken line in B. The adhesion proteins (Talin, βPS, and Tig etc.) all concentrate at the end of SMs and are involved in forming stable muscle-muscle or muscle tendon cell adhesions in wild type embryos. In *rhea*^{1} mutant embryos, the muscle tendon cell connections are broken (arrowheads) but the muscle-muscle connections remain (arrows).

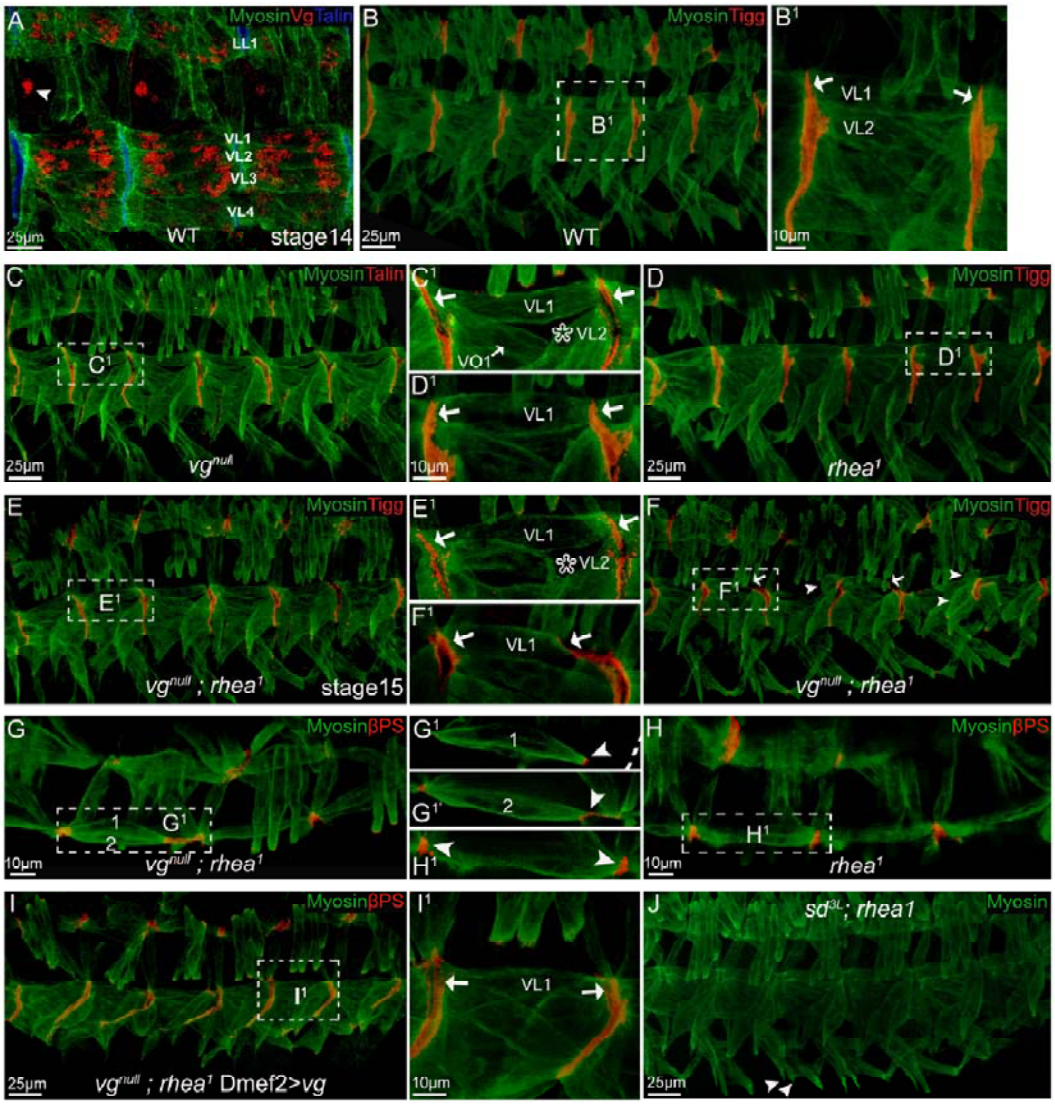
A
MyosinVgTalin
LL1
VL1
VL2
VL3
VL4
25µm
WT
stage14
B
MyosinTigg
B1
25µm
WT
B1
VL1
VL2
10µm
C
MyosinTalin
C1
25µm
vgnull
C1
VL1
VL2
VO1
D1
VL1
10µm
D
MyosinTigg
D1
25µm
rhea1
E
MyosinTigg
E1
vgnull ; rhea1
stage15
E1
VL1
VL2
F1
VL1
F
MyosinTigg
F1
vgnull ; rhea1
G
MyosinβPS
1
G1
2
10µm
vgnull ; rhea1
G1
1
2
H1
H
MyosinβPS
H1
10µm
rhea1
I
MyosinβPS
I1
25µm
vgnull ; rhea1 Dmef2>vg
I1
VL1
10µm
J
sd3L; rhea1
Myosin
25µm

Figure 4.2. SMs were detached in vg^{null}; $rhea^{1}$ embryos but not in sd^{3L}; $rhea^{1}$ embryos. Embryos (stage 16 or a specified stage) are shown as lateral views, with dorsal up, and anterior to the left. Staining is color-coded and indicated on each panel. B^{1}-I^{1} are the close-ups of the framed area in B-I. **A)** *vg* is expressed in muscle LL1 and VL1-4. The arrowhead points to a neuronal cell also expressing *vg*. Compared to **B)** wild type embryos, **C)** vg^{null} or **D)** $rhea^{1}$ single mutation, or **E)** vg^{null}; $rhea^{1}$ double mutant embryos in early stages (before stage 15) all produced a muscle pattern similar to wild type embryos, except that a vg^{null} mutation caused muscle VL2 to be missing in about 30% of segments (star in C^{1} and E^{1}). Notice VL muscles (e.g. VL1) all formed tight adhesions between each other (arrows in B^{1}-E^{1}). **F-F^{1})** By late stage 16 when muscles start to contract, muscle VL1 or other VL muscles detached from the attachment sites only in the vg^{null}; $rhea^{1}$ double mutant embryos (arrows in F^{1}). Arrows in F indicate detaching muscles and arrowheads indicate detached muscles. **G-H^{1}**) An overview of the vg^{null}; $rhea^{1}$ double mutant embryos (stage 16, G-$G^{1'}$) compared with $rhea^{1}$ embryos (H-H^{1}). Both VL1 and VL2 are retracting from their normal attachment sites (arrowhead in G^{1}-H^{1}). G^{1} and $G^{1'}$ are two different confocal sections and the broken line in G^{1} indicates the segment border. **I-I^{1})** The muscle detachment phenotype of vg^{null}; $rhea^{1}$ embryos can be rescued by expression of Vg via *Dmef2*-GAL4. Notice VL1 muscles built tight adhesions between each other (arrows in I^{1}). **J)** sd^{3L}; $rhea^{1}$ embryos did not have a muscle detachment phenotype. Some muscles do not develop well (VO4-6, arrowheads) in these embryos but this mirrors the phenotype seen in sd^{3L} single mutants.

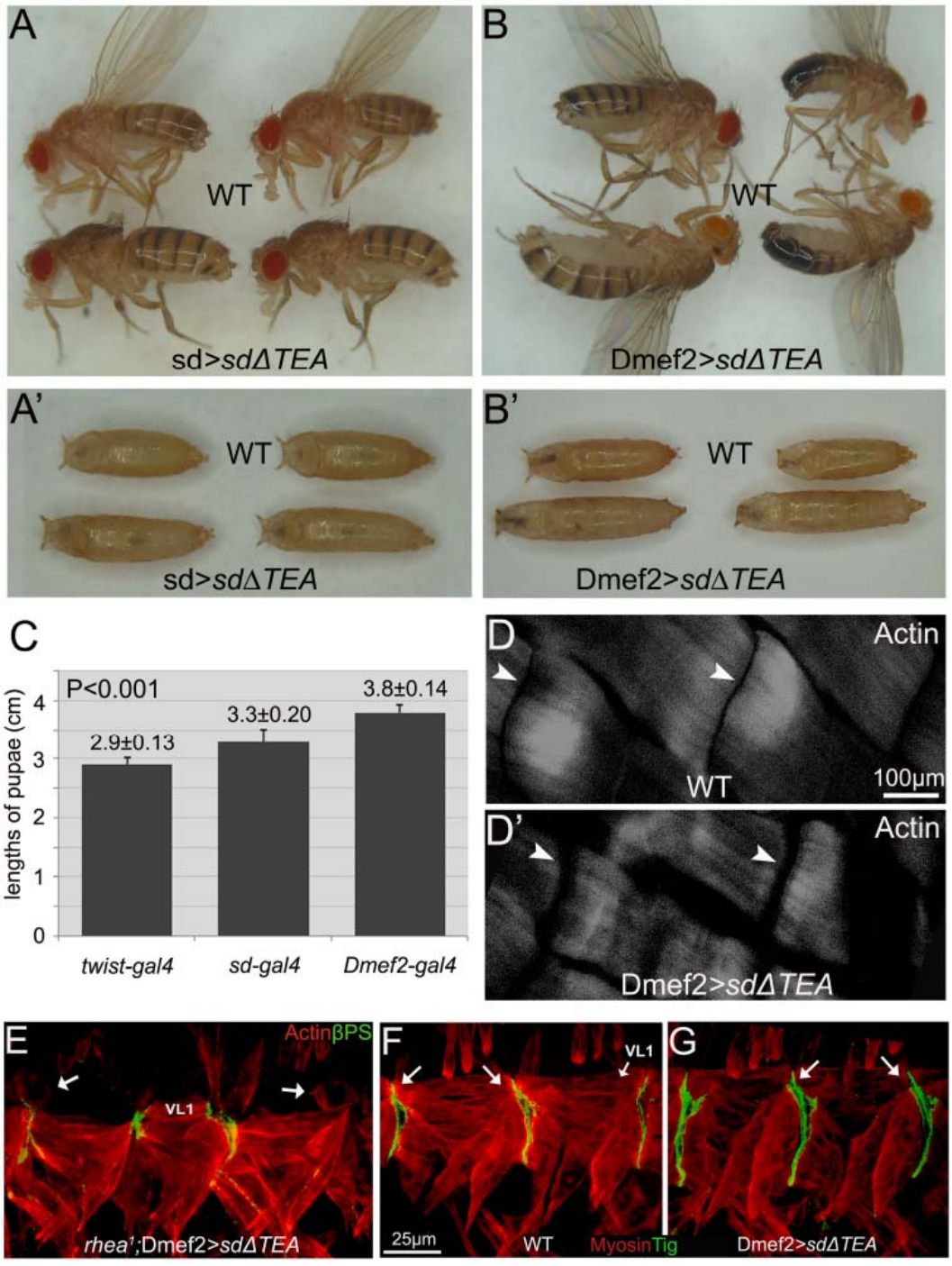
A
WT
sd>sdΔTEA
B
WT
Dmef2>sdΔTEA
A'
WT
sd>sdΔTEA
B'
WT
Dmef2>sdΔTEA
C
P<0.001
2.9±0.13
3.3±0.20
3.8±0.14
lengths of pupae (cm)
0
1
2
3
4
twist-gal4
sd-gal4
Dmef2-gal4
D
Actin
WT
100μm
D'
Actin
Dmef2>sdΔTEA
E
ActinβPS
VL1
rhea1;Dmef2>sdΔTEA
F
VL1
25μm
WT
MyosinTig
G
Dmef2>sdΔTEA

Figure 4.3. Tissue-specific expression of *sdΔTEA* interferes with Vg function and produced elongated larvae and adults. **A)** Expression of *sdΔTEA* via *sd*-GAL4 (sd>*sdΔTEA*) in the wing disc caused loss of the adult wing by interfering with Vg. However, we also noted that the pupae (**A'**) and adult flies were elongated compared to wild type (WT) siblings. **B)** This phenotype was caused by interfering with Vg in the muscle cells as these effects were seen in pupae (**B'**) and adults when UAS-*sdΔTEA* was expressed exclusively in muscle cells via *Dmef2*-GAL4 (Dmef2>*sdΔTEA*). **C)** Quantification of pupal length in animals over-expressing *sdΔTEA* using the indicated GAL4 drivers (mean ± SD, n=23). Student T tests show significant difference between any pairs of these experiment groups ($P<0.001$). The pupal length of twist-GAL4>*sdΔTEA* animals was not statistically different from wild type. **D-D')** Larvae expressing *sdΔTEA* in the muscles (*Dmef2*-GAL4) (D') had a larger gap (arrowhead) between muscle DA1 and DA2 than wild type (D). **E)** Expression of *sdΔTEA* in developing muscle cells in embryos that are homozygous for the $rhea^{1}$ mutation produced a muscle detachment phenotype where the majority of the VL1 cells became rounded (arrows). **F)** Tig protein localizes to the tips of muscles forming junctions including VL1 (arrows). **G**) Embryos expressing *sdΔTEA* in muscles show the same pattern of Tig localization (arrows). In all panels ventral muscles in two or three segments are shown in embryos (stage 16) presented as lateral views, with dorsal up, and anterior to left.

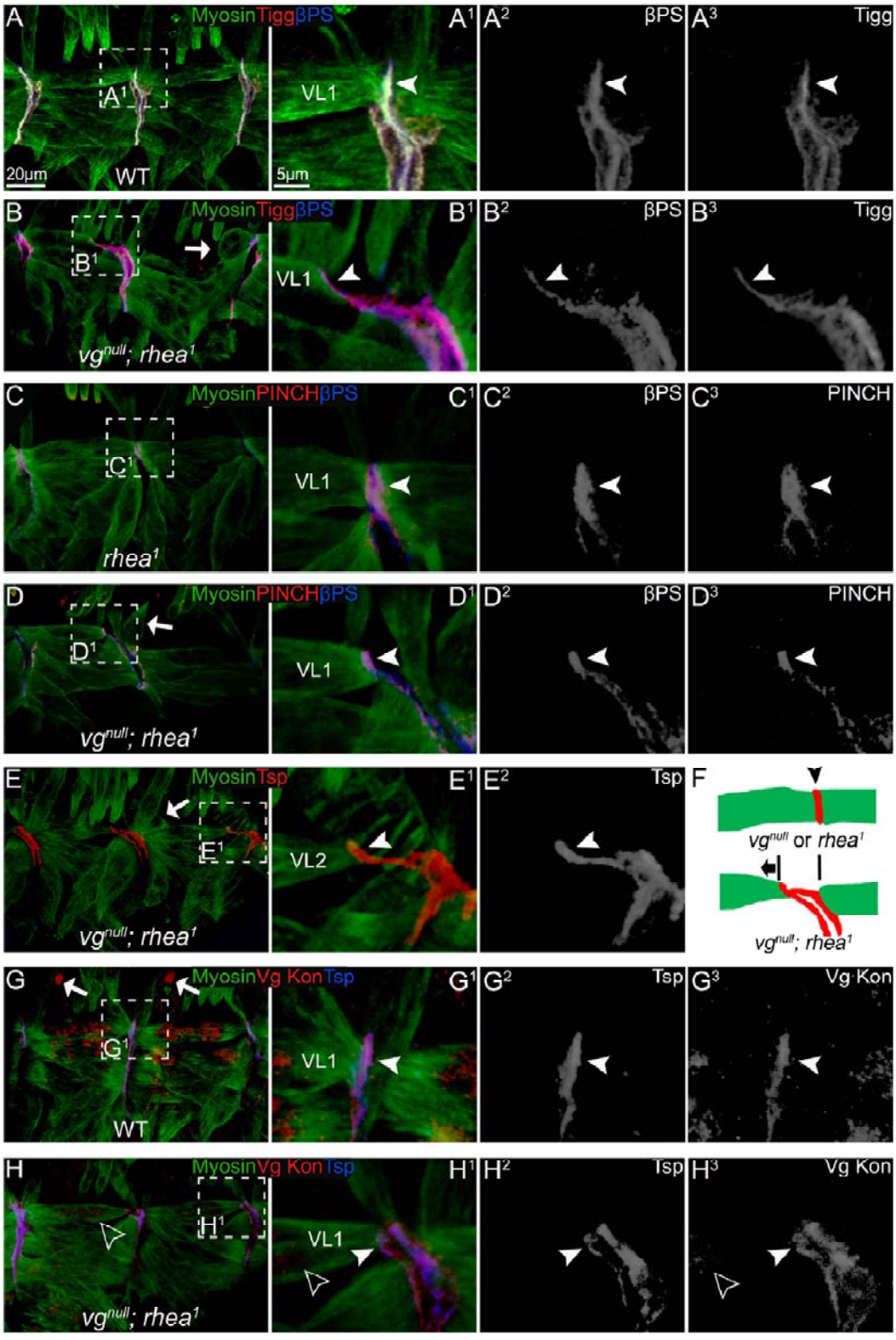
A
MyosinTiggβPS
A1
A2 βPS
A3 Tigg
A1
VL1
20μm
WT
5μm
B
MyosinTiggβPS
B1
B2 βPS
B3 Tigg
VL1
vgnull; rhea1
C
MyosinPINCHβPS
C1
C2 βPS
C3 PINCH
VL1
rhea1
D
MyosinPINCHβPS
D1
D2 βPS
D3 PINCH
VL1
vgnull; rhea1
E
MyosinTsp
E1
E2 Tsp
F
VL2
vgnull; rhea1
vgnull or rhea1
G
MyosinVg KonTsp
G1
G2 Tsp
G3 Vg Kon
VL1
WT
H
MyosinVg KonTsp
H1
H2 Tsp
H3 Vg Kon
VL1
vgnull; rhea1

Figure 4.4. The muscle detachment phenotype observed in vg^{null}; $rhea^{1}$ embryos was not due to lack of localization of integrin or its known ligands, nor an obvious muscle migration defect. **A)** In wild type embryos, βPS and Tig can be seen localized normally at the junctions between two VL muscles (arrowhead). **B)** In vg^{null}; $rhea^{1}$ double mutant embryos, the VL muscles were either detaching (arrowheads) or were already detached (arrows). However, βPS and Tig remain concentrated at muscle termini and followed the detaching muscles (arrowheads). **C)** In $rhea^{1}$ mutant embryos, the adhesion proteins PINCH and βPS formed tight junctions between VL muscles (arrowheads). **D)** Similar to βPS and Tig, in detaching muscles in vg^{null}; $rhea^{1}$ embryos, PINCH and βPS remain concentrated at muscle termini and followed the detaching muscles (arrowheads). Many muscles appeared to be detaching from the posterior border of each segment. **E)** In the vg^{null}; $rhea^{1}$ embryos, Tsp shows the same localization to the end of detaching muscles as PINCH, βPS and Tig. **F)** A diagram of the localization of adhesion proteins (red, arrowhead) in vg^{null} or $rhea^{1}$ mutant embryos and the direction (anterior, arrow) in which VL muscles are moving after they detach. **G)** In wild type embryos, Kon, the major migration guidance protein for VL muscles, normally found at the end of muscle cells (arrowhead). **H)** In vg^{null}; $rhea^{1}$ embryos, some residual (maternally supplied) Vg protein can still be seen in VL1 muscle (empty arrowheads). These muscles still had a detachment phenotype, but Kon is localized properly (arrowhead). A^{1}-H^{1} are the close-ups of the framed area in A-H. A^{2}-H^{2} and A^{3}-H^{3} show each confocal channel separately.

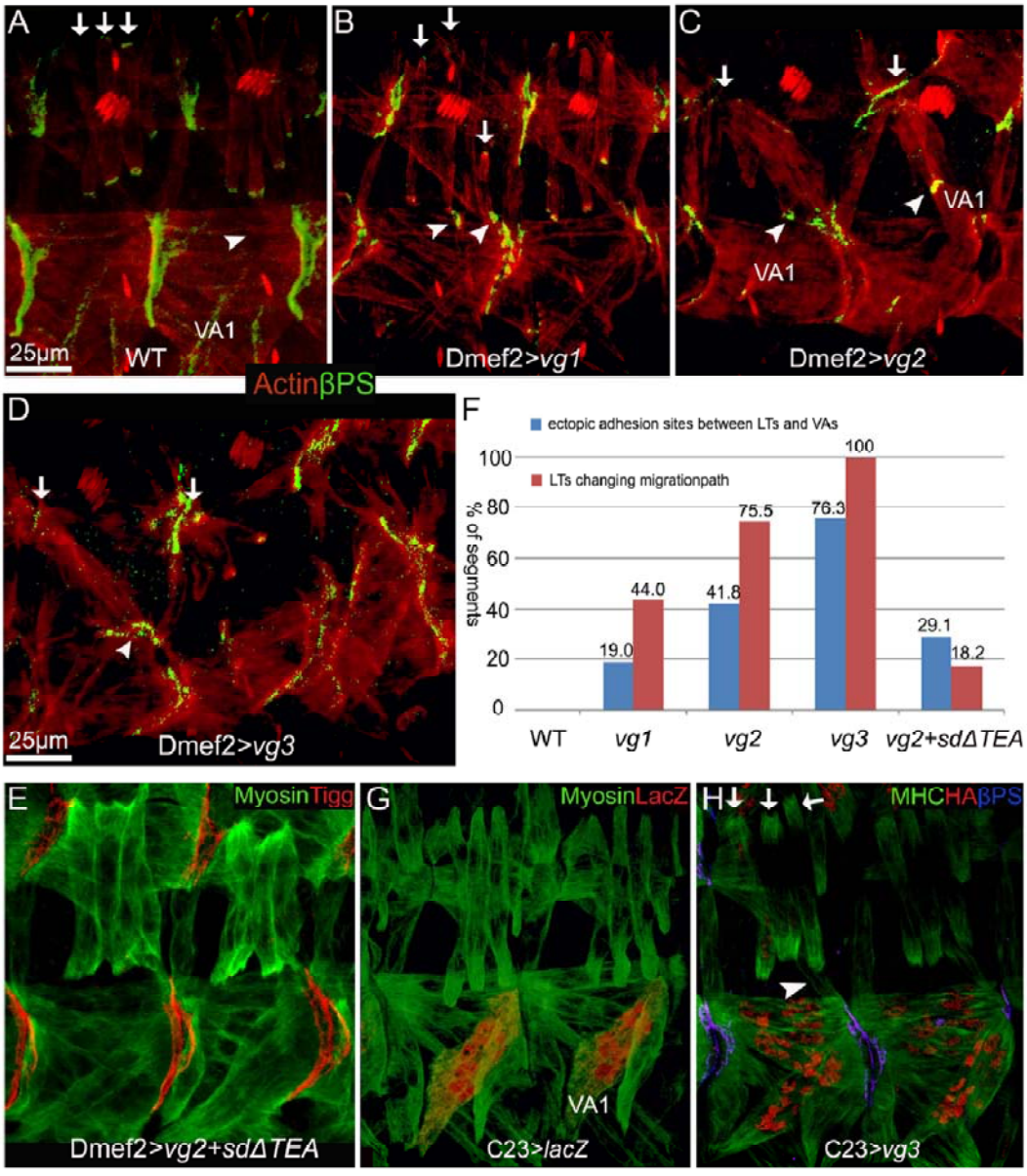
A
B
C
VA1
VA1
VA1
25μm
WT
Dmef2>vg1
Dmef2>vg2
ActinβPS
D
25μm
Dmef2>vg3
F
ectopic adhesion sites between LTs and VAs
LTs changing migrationpath
% of segments
100
80
60
40
20
0
19.0
44.0
41.8
75.5
76.3
100
29.1
18.2
WT
vg1
vg2
vg3
vg2+sdΔTEA
E
MyosinTigg
Dmef2>vg2+sdΔTEA
G
MyosinLacZ
VA1
C23>lacZ
H
MHCHAβPS
C23>vg3

Figure 4.5. Ectopic expression of Vg in the developing embryonic SMs produced ectopic inter-muscular attachments. The transgenic lines *vg1*, *vg2* and *vg3* express relatively higher levels of Vg respectively as verified by western blotting. **A)** In wild type embryos, LT1-4 muscles that stain brightly with muscle specific Actin (red, arrows) are seen passing left to right over the VA1 muscle and form βPS mediated attachments (green) at intrasegmental sites. Normally no adhesions form where the LT and VA muscles are adjacent (arrowhead). **B-D)** Ectopic expression of progressively higher levels of Vg in all muscles via *Dmef2*-GAL4. B) Ectopic expression of relatively lower levels of Vg (vg1) in SMs caused the LT muscles to abnormally form attachments at the segment borders; **C)** Expression of relatively higher levels of Vg (vg2) cause the formation of abnormal attachments at the segment borders (arrows). Further, ectopic muscle-muscle attachments were observed between LT and VA muscles (arrowheads). In some cases, muscle VA1 was observed deviating from its original position (arrwoheads) **D)** This number of abnormal and ectopic attachments becomes even more severe when a transgene (vg3) expressing relatively highest levels of Vg is used. **E)** Quantification of the percentage of segments having LTs with abnormal migration (red columns) or ectopic adhesion sites between LT and VA muscle cells (blue columns) for each indicated over-expression line (n=110). **F)** Ectopic expression of Vg with *sdΔTEA* led to a partial rescue of the phenotype caused by ectopic expression of Vg from the UAS-*vg2* transgene. **G)** The C23-GAL4 line induces expression at high levels in VA1 but relatively low expression in LTs as detected by an UAS-*lacZ* reporter. **H)** Ectopic attachments are not formed when Vg is present at relatively high levels in VA1 cells.

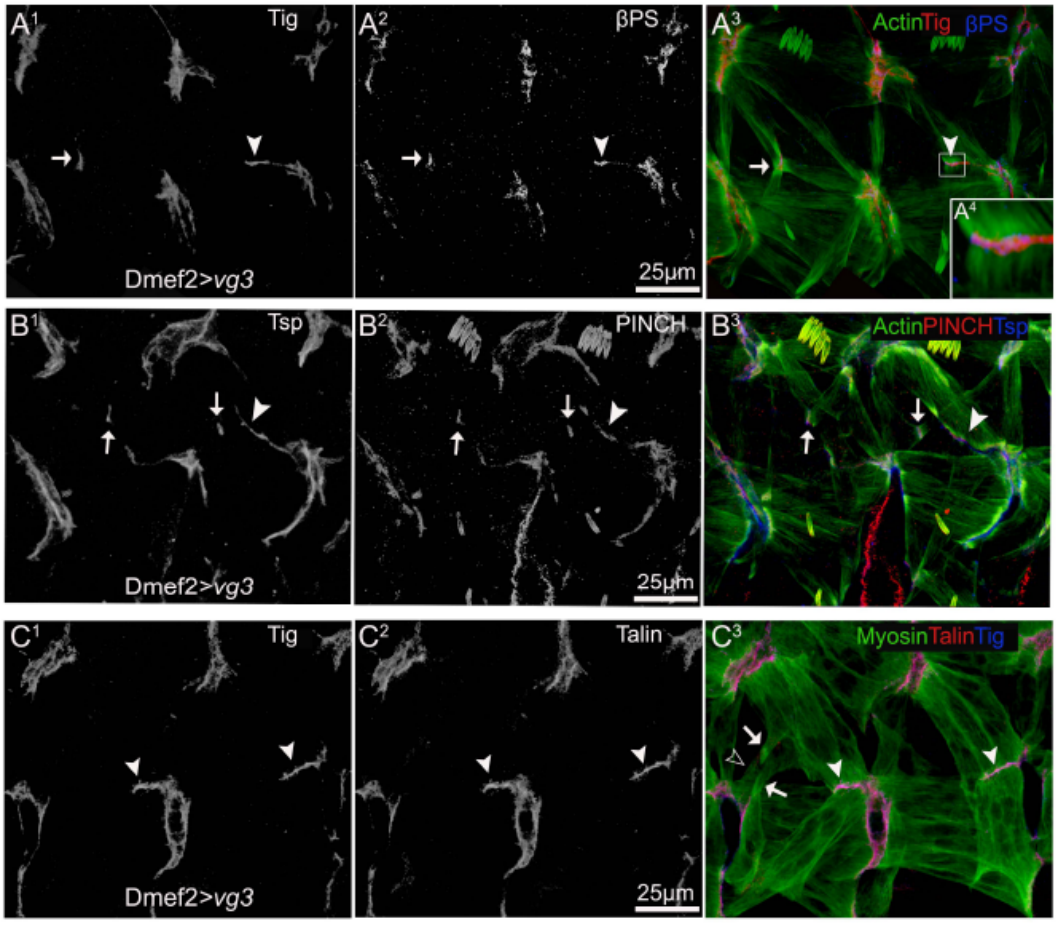
A1
Tig
A2
βPS
A3
ActinTig βPS
A4
Dmef2>vg3
25μm
B1
Tsp
B2
PINCH
B3
ActinPINCHTsp
Dmef2>vg3
25μm
C1
Tig
C2
Talin
C3
MyosinTalinTig
Dmef2>vg3
25μm

Figure 4.6. The muscle attachments induced by ectopic Vg include βPS integrin and its associated cytoplasmic linker proteins, PINCH and Talin. **A)** Ectopic expression of Vg by *Dmef2*-GAL4 caused additional attachments to form between muscles stained with muscle specific Actin (green). These ectopic attachments (arrowheads) contained Tig (red, A^1), an extracellular ligand for PS2 integrin (blue, A^2). Ectopic muscle attachments were also produced between muscle cells other than LTs and VAs, which also contained Tig (arrows). Individual myofibers were linked to the new adhesion sites through integrin complexes (A^4, is a close-up of the boxed area in A^3). **B)** These ectopic attachments also contain Tsp (blue, B^1) and PINCH (red, B^2). **C)** Talin is also localized to the ectopic muscle attachments (red, C^2). Note the processes emerged from the lateral surface of muscle LTs (arrows in C^3) and VT1 (empty arrowhead in C^3).

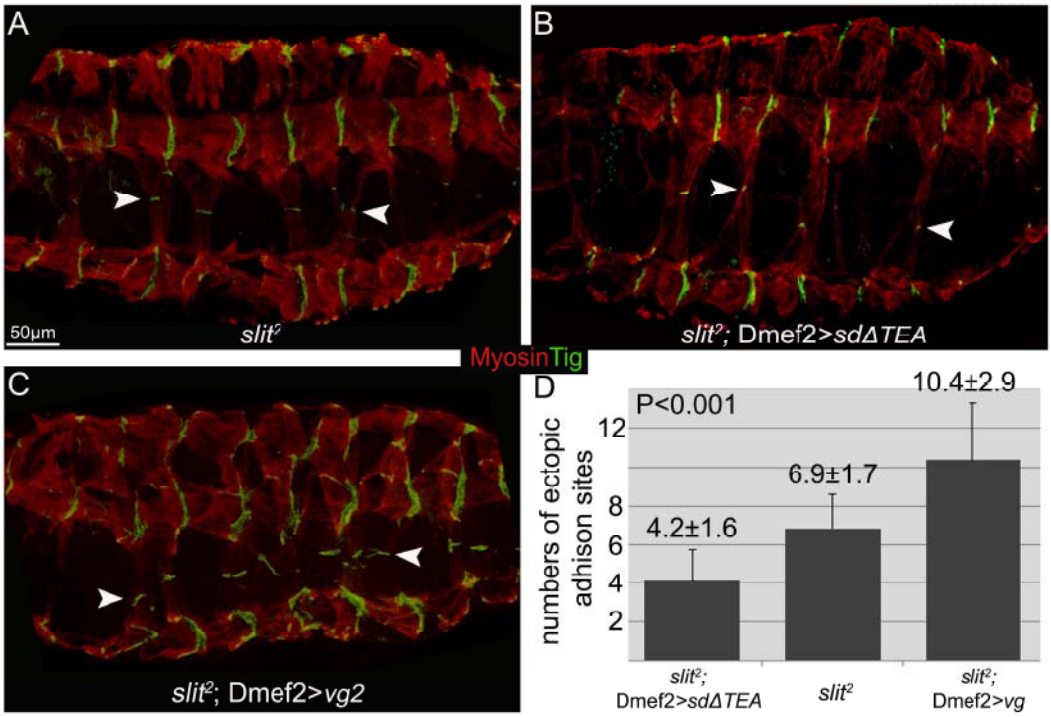

Figure 4.7. Altered levels of Vg function regulate ectopic intermuscular attachments independently of signalling from tendon cells in VL muscle cells. A) In slit2 mutant embryos, VL muscle cells migrate dorsally over the CNS from the lateral sides of the embryo meeting near the midline to form muscle-muscle adhesions (arrowheads) in a region of the embryo devoid of tendon cells. B) Interference with Vg function by expression of sdΔTEA in these slit2 mutant embryos led to fewer and smaller adhesion sites (arrowheads). C) Over-expression of Vg in slit2 mutant embryos produced more and larger adhesion sites (arrowheads) in the abnormally positioned VL muscles. D, Quantification of the number of VL cell adhesion sites formed in slit2 mutant embryos with varying levels of Vg activity (mean ± SD, n≥15).

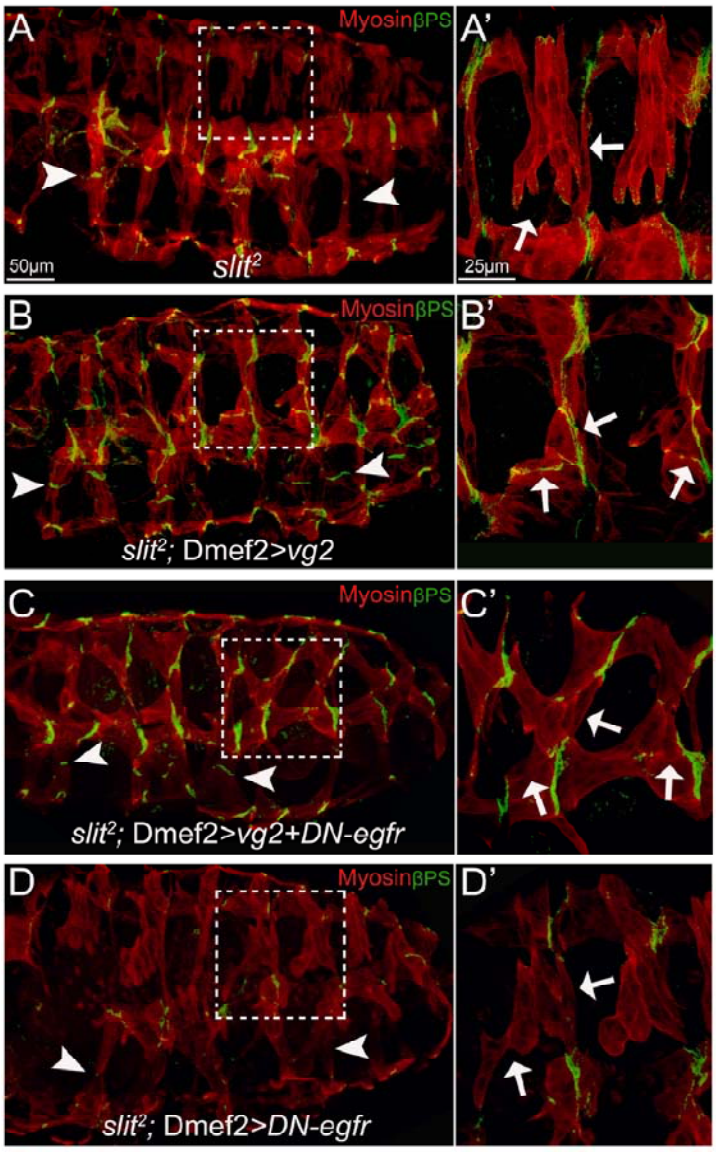
A
MyosinβPS
A'
50μm
slit2
25μm
B
MyosinβPS
B'
slit2; Dmef2>vg2
C
MyosinβPS
C'
slit2; Dmef2>vg2+DN-egfr
D
MyosinβPS
D'
slit2; Dmef2>DN-egfr

Figure 4.8. DER mediated cell-cell communication is required for the production of inter-muscular attachments between Vg expressing cells in *slit*^{2} mutant. **A)** In *slit*^{2} mutant embryos, VL muscle cells stained with Myosin [48] meet abnormally near the midline to form ectopic muscle-muscle adhesions identified by βPS staining (green, arrowheads). **A')** A magnified view of the boxed region in A, showing that few βPS localizing adhesions sites were seen in the peripheral muscles in *slit*^{2} mutant embryos. **B)** Over-expression of Vg in *slit*^{2} mutant embryos increased the size and number of βPS marked adhesion sites formed between ectopic VL muscles at the midline and long adhesion sites appeared between lateral muscles (arrows in B'). **C)** Co-expression of a dominant negative form of DER (DN-egfr) as well as Vg in the developing muscles of a *slit*^{2} mutant embryo reduced the size and number of ectopic adhesions marked by βPS (arrowheads in C and arrows in C'). When DER signaling is inhibited, the majority of the long adhesion sites induced by ectopic Vg disappear (arrows in C'). **D)** Expression of DN-egfr in *slit*^{2} mutant embryos reduces the overall size and number of βPS marked muscle-muscle adhesion sites (arrowheads in D and arrow in D^{1}).

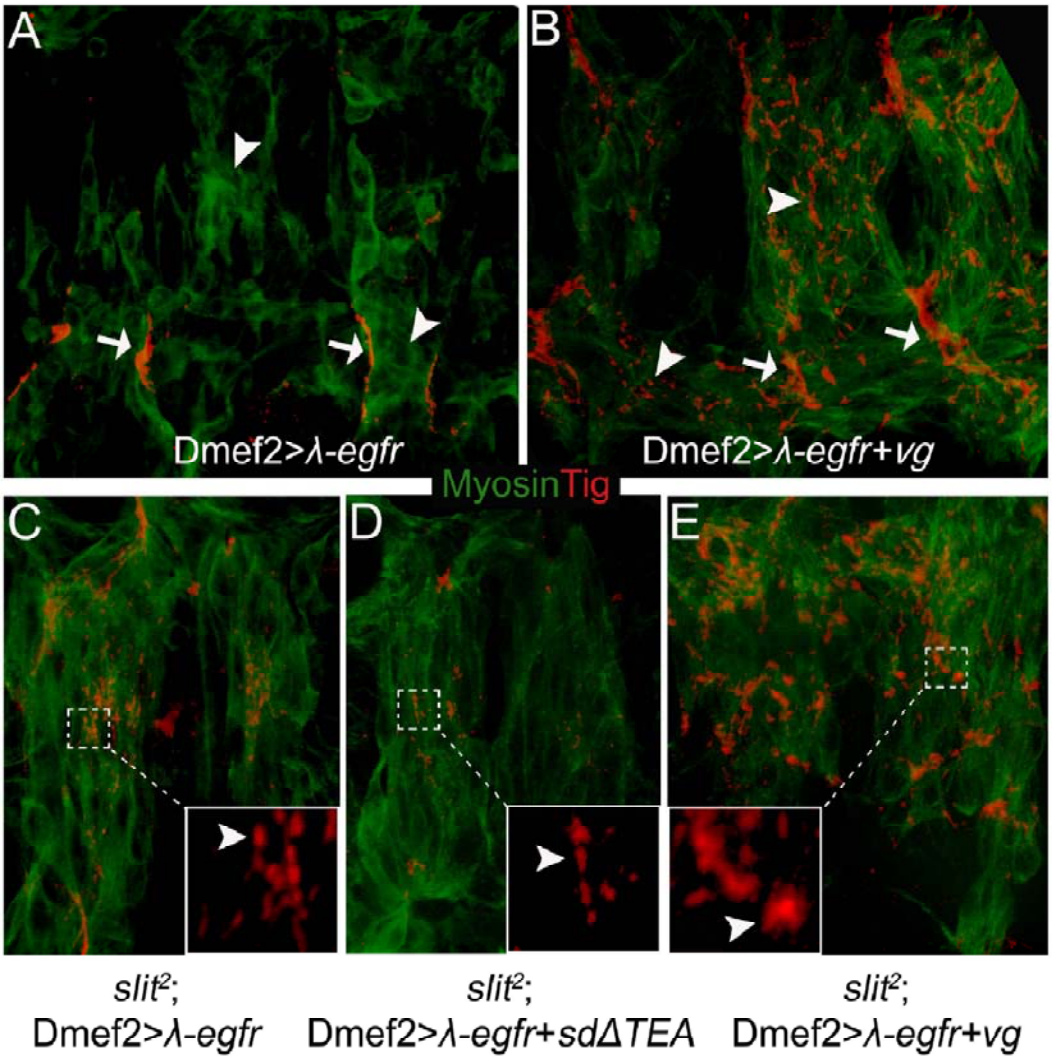
A
B
Dmef2>λ-egfr
Dmef2>λ-egfr+vg
MyosinTig
C
D
E
slit²;
Dmef2>λ-egfr
slit²;
Dmef2>λ-egfr+sdΔTEA
slit²;
Dmef2>λ-egfr+vg

Figure 4.9. Changes in muscle-muscle adhesion caused by expression of a constitutively active DER (λ-egfr) are sensitive to the presence of Vg. **A)** Expression of λ-egfr in developing embryonic SM cells marked by muscle specific Myosin (green) disrupted the normal SM pattern. However, these muscles still form attachments at the segment borders marked by Tig staining (red, arrows). When cells are expressing λ-egfr, no adhesion sites were established between muscles that are in abnormal locations (arrowheads). **B)** Over-expression of Vg in SMs expressing λ-egfr significantly increased the size and number of adhesion sites marked by Tig staining [48]. Additional sites of adhesion sites were seen between abnormally localized muscles (arrowheads, arrows indicate segment borders). **C)** Ectopic adhesion sites were formed between the midline-crossing muscles in *slit*^{2} mutant embryos (arrowhead) when λ-egfr is expressed in developing muscle cells (Dmef2>λ-egfr) (arrowhead, inset). **D)** Expression of λ-egfr in developing muscle cells where Vg function was inhibited by *sdΔTEA* produced fewer ectopic adhesions (arrowhead, inset). **E)** Relatively more and larger ectopic adhesions were formed when embryonic muscle cells were over-expressing both λ-egfr and Vg (arrowhead, inset). Insets are close-ups of the area framed by the dotted lines.

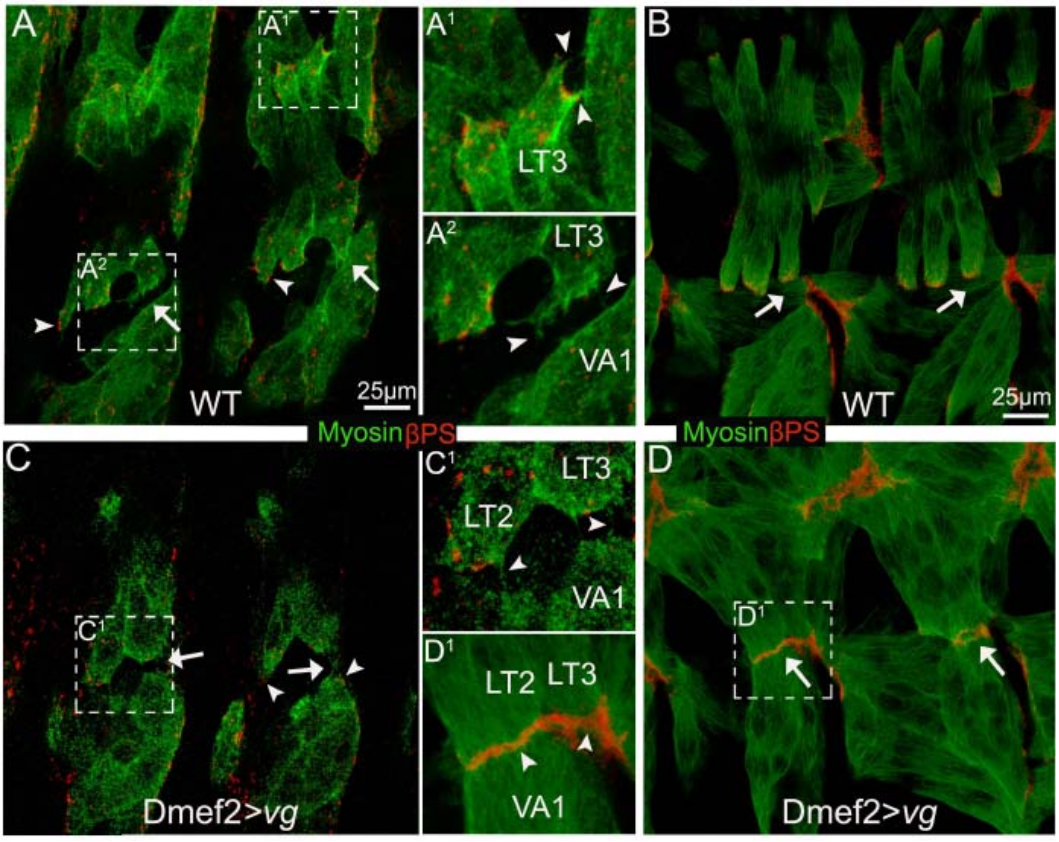

Figure 4.10. Filopodial contact between muscles forming junctions are not affected by changes in Vg expression. **A)** In wild type embryos (stage 14), the leading edge of LT muscles produce filopodia that contact with corresponding filopodia protruding from the lateral edge of VAs or other muscles (arrows in A and arrowheads in A^1 -A^2. A^1 and A^2 are magnified photos of the framed areas in A). βPS integrin accumulates at the leading edge of the myotube (arrowhead). **B)** At developmental stage16, LTs normally find their attachment sites and form stable adhesion inside each segment. There are no connections between LTs and VAs (arrows in B). **C)** Muscles in embryos that were expressing Vg ectopically (stage 14) produced similar number of filopodia compared to wild type (arrows in C and arrowheads in C^1). Also, βPS integrin accumulated at the leading edge of muscles (arrowheads in C) in the same way as wild type muscles. **D)** At stage 16, LT muscle cells have formed stable attachments with muscle VAs (arrows in D and arrowheads in D^1).

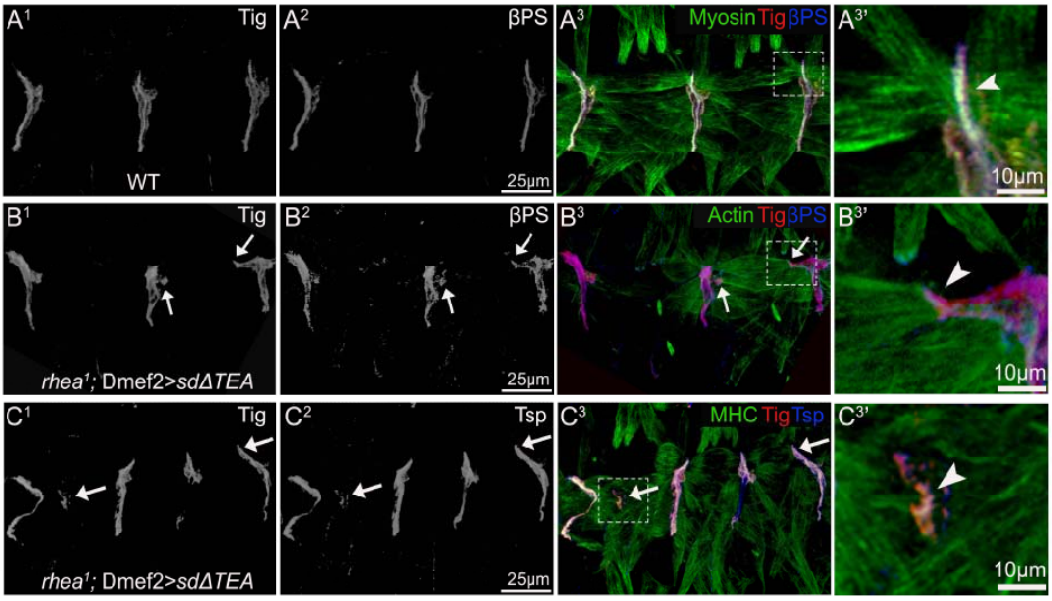

Figure 4.S1. The muscle detachment phenotype observed when the dominant-negative *sdΔTEA* was expressed in *rhea*^{1} mutants was not due to the lack of localization of integrin or its known ligands. **A)** In wild type embryos, Tig (A^{1}, red) and PS2 integrin (A^{2}, blue) containing complexes forms a tight junction holding muscles together. (arrowhead in A$^{3'}$). **B)** Tig (B^{1}, red) and PS2 integrin (B^{2} blue) remained concentrated at the muscle cell termini and followed the detaching muscles in *rhea*^{1} embryos where Vg activity is inhibited by *sdΔTEA* (arrows, arrowhead in B$^{3'}$). **C)** Tig (C^{1}, red) and Tsp (C^{2} blue) showed the same localization and retention at the end of detaching muscles in *rhea*^{1} embryos where Vg activity is inhibited (arrows, arrowhead in C$^{3'}$). A$^{3'}$, B$^{3'}$, and C$^{3'}$ are magnifications of the framed areas in A^{3}, B^{3} and C^{3} respectively.

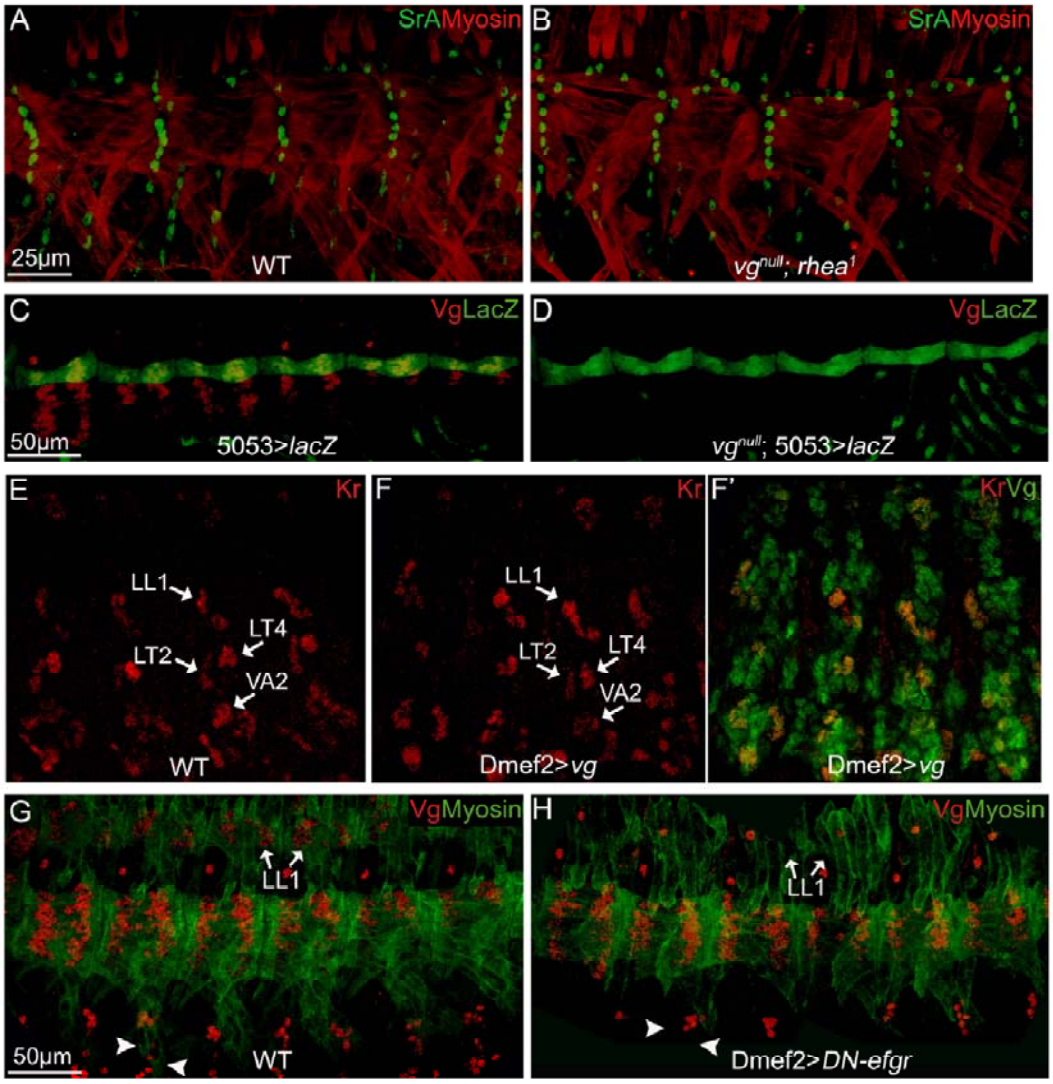
A
SrAMyosin
25µm
WT
B
SrAMyosin
vg^null; rhea^1
C
VgLacZ
50µm
5053>lacZ
D
VgLacZ
vg^null; 5053>lacZ
E
Kr
LL1
LT4
LT2
VA2
WT
F
Kr
LL1
LT2
LT4
VA2
Dmef2>vg
F'
KrVg
Dmef2>vg
G
VgMyosin
LL1
50µm
WT
H
VgMyosin
LL1
Dmef2>DN-efgr

Figure 4.S2. Loss of Vg or expression of DN-egfr or over-expression of Vg does not alter tendon cell or specific muscle cell identity. **A)** The pattern of muscles were marked by Myosin [48] and tendon cells in wild type embryos was visualized by staining of SrA (green), a marker for fully differentiated tendon cells. **B)** $vg^{null};rhea^{1}$ double mutants did not have detectable defects in tendon cell development. **C)** The identity of VL1 muscle can be specifically marked by using a *5053*-GAL4, UAS-*lacZ* marker. **D)** In vg^{null} mutants, VL1 muscles were properly specified at all stages of embryonic development. **E)** The *krüppel*-expressing muscle progenitor cells can be revealed through Kr staining in stage 13 wild type embryos (arrows). **F)** These progenitor cells still express Kr when Vg was over-expressed in SMs via *Dmef2*-GAL4 (arrows). **G)** In wild type embryos, the overall pattern of embryonic muscles can be observed via staining with Myosin (green). The VL1-4 muscles were identified by their position and presence of Vg [48], LL1 muscles are marked with arrows. **H)** Expression of DN-egfr in the muscles of developing embryos did not alter the specification of the VL muscles but muscles LL1 (arrows) and VO5-6 (arrowheads) were missing in several segments.

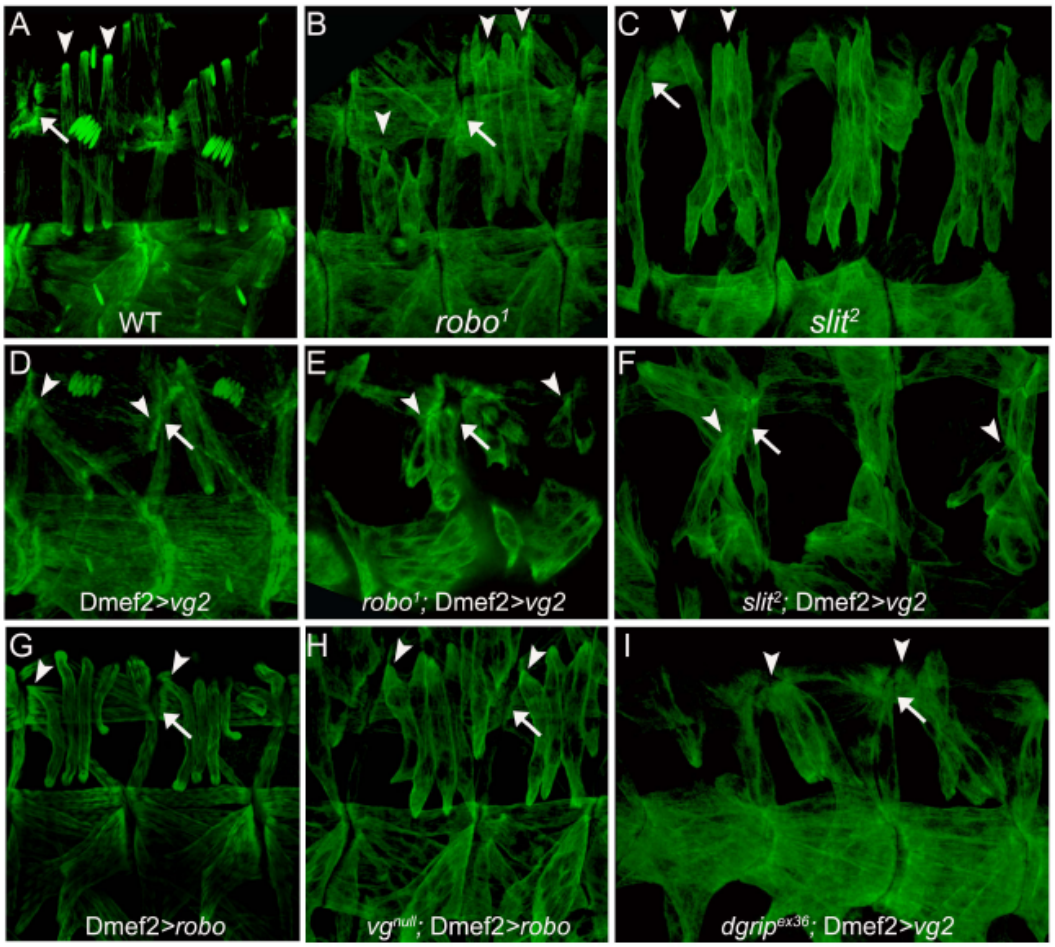
A
WT
B
robo1
C
slit2
D
Dmef2>vg2
E
robo1; Dmef2>vg2
F
slit2; Dmef2>vg2
G
Dmef2>robo
H
vgnull; Dmef2>robo
I
dgripex36; Dmef2>vg2

Figure 4.S3. The altered migration of LT muscles caused by ectopic-expression of Vg is independent of the Slit-Robo guidance signals and Grip. **A)** The normal pattern of wild type muscles visualized by Actin staining (green). **B)** In embryos with the *robo*^{1} mutation, LT muscles migrated to intrasegmental sites (arrowheads) distant from the segment border (arrow). **C)** A similar pattern of LT muscle migration to intrasegmental sites (arrowheads) distant from the segment border (arrow) as was seen in embryos with the *slit*^{2} mutation. **D)** Ectopic-expression of Vg in wild type embryos caused LTs (arrowheads) to change their apparent migration path and attach to the segment borders (arrows). **E-F)** The *robo*^{1} or *slit*^{2} did not alter this apparent aberrant migration. **G)** In some segments, ectopic-expression of Robo1 produced a phenotype similar to ectopic Vg, with LT1 muscles (arrowheads) moving to the segment borders (arrowheads). **H)** Expression of Robo1 in all developing muscle cells in *vg*^{null} mutant embryos produced a similar phenotype. **I)** Removing *grip* function in muscles cells ectopically expressing Vg did not significantly alter the phenotype induced by over-expression of Vg alone compared to (D).

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Chapter 5: General discussion and conclusions

Part 1: Vg phosphorylation and its role in wing development

To examine if there are post-translational modifications in Vg protein, we checked the Western blot patterns of Vg under different conditions (Chapter 2). This project started when several shifted bands were found in Vg Western blots. This research led to several conclusions:

Vg can be phosphorylated and sumoylated in S2 cells. Phosphorylation happens on serine at position 215, and this modification occurs only when Sd is present. The sumoylation site has not been identified in Vg, and this modification does not require the presence of Sd. Sumoylation appear to occur before phosphorylation, but mutagenesis of the phosphorylation site also gets rid of sumoylation, suggesting that the 3-D structure around the phosphorylation site is essential for Vg sumoylation.

p38b is a potential kinase for Vg phosphorylation. Inhibiting p38b activity decreases Vg phosphorylation. Sd interacts with p38b, and the TEA domain of Sd contain two conserved p38b docking domains, only one of which is important for Vg phosphorylation. Therefore, it appears that Sd recruits p38b to the Sd-Vg complex, and then p38b phosphorylates Vg.

Ectopic bristles are produced along the posterior margin of wing tissue when endogenous Vg is replaced with the non- phosphorylated Vg mutant. However, this phenotype does not show up when using the wild-type Vg or the phosphorylation-mimicking Vg mutant. Vg phosphorylation might be regulated during late-stage bristle development.

A key question arises from this study is: when and where is Vg phosphorylated during wing development? To address this question, a specific antibody again phos-Vg needs to be produced. Effort has been taken to make this antibody, but no good antibody was produced. Another way to approach this question is to examine Sd activity during wing development, as the presence of Sd is required for Vg phosphorylation.

Part 2: Sd, Vg, and Dmef2 form alternative complexes during muscle development.

To understand the roles of *sd* and *vg* in the development of *Drosophila* embryonic muscles, we first examined the interactions among Sd, Vg and Dmef2 (Chapter 3). This project was inspired by the previously identified interactions among the homologues of these proteins in mice and their functions in the muscle development of mice [1-7]. The results of these experiments led to several conclusions:

sd and *vg* have different roles in muscle development. First, both their expression patterns and the time windows for their expression are different. *sd* is expressed in the heart region of the dorsal vessel and has a short period of expression in all SMs before stage 16, while *vg* is only expressed in SM DA1-3, LL1, and VL1-4, starting at stage 9 until stage 17, when it is hard for the antibody to penetrate the embryos. Secondly, *sd* and *vg* mutations cause distinct muscle phenotypes. sd^{3L} mutants do not have enough cardiac muscle cells and have defects in the development of muscles VO4-6. vg^{null} mutants, on the other hand, are missing muscle VL2.

Sd, Vg and Dmef2 may form alternative transcription complexes with other unknown proteins during muscle development (Fig. 5.1). Vg has one potential domain that binds Dmef2 independently of the Sd interaction domain, indicating the possibility of a transcription complex containing all of these proteins. This is important for muscle diversification, since *Dmef2* is expressed in all types of muscles and distinct cofactors are required in different muscles to modify its activity or recruit it to different muscle genes.

Vg and Dmef2 work together during the specification of VL2-3 muscles and a Sd-Vg complex suppresses Dmef2 function during muscle differentiation. The interaction between Vg and Dmef2 is functional, since the missing muscle phenotype caused by a vg^{null} mutation is enhanced when the expression level of *Dmef2* is decreased. Sd, Vg, and Dmef2 could form a transcription complex at the promoter of *act57B*, but the activity of Dmef2 is suppressed by Sd and Vg at this

promoter. Sd alone is able to suppress Dmef2 activity but less efficiently when Vg is not present.

The relative expression levels of Sd, Vg, and Dmef2 are important during muscle development. Increasing *sd* activity leads to defects in the development of many SMs including VL1-4, and produces more cardiac muscle cells and a disarranged dorsal vessel. Ectopic expression of Vg in other muscles causes severe defects in the development of the VO3-6 muscles. Increasing both *sd* and *vg* activity in SMs leads to a complete disruption of the normal muscle pattern.

Based on these conclusions, there are several key future areas that would need to be addressed:

If Sd, Vg, and Dmef2 could form alternative transcription complexes, then what would be the targets of these complexes? To fully understand the roles of *sd* and *vg* in muscle development, it is necessary to know what genes are regulated by them. ChIP-on-chip has been used to predict spatio-temporal *cis*-regulatory activities of five transcription factors including Dmef2, in order to know what genes are regulated by these proteins and at what time-point [8]. Unfortunately, Sd is a weak antigen and we are not able to make a good antibody. In addition, Vg itself can not bind to DNA and must rely on Sd to recognize targets during wing development [9, 10]. Since *sd* and *vg* have different functions in muscle development, there should be a different partner for Vg in muscle development. Dmef2 may be the partner considering the interaction between them. We have performed a microarray analysis on the embryos that over-expressed Vg in SMs and found that hundreds of genes were upregulated (data not shown). It would be interesting to compare these genes with the indentified target genes of Dmef2.

How is transcription of *vg* and *sd* regulated during muscle development? There are two separate enhancers directing *vg* expression in different areas of the wing disc [11, 12]. However, there is no evidence to show that these enhancers also control *vg* expression during muscle development [13]. No regulatory elements have been identified directing *sd* expression. During wing development, *sd* and *vg* have the same expression pattern [14, 15] and are both required for the auto-regulation mechanism responsible for keeping their expression high in the

wing disc [9, 16]. However, *sd* is also expressed in other imaginal discs like eye, leg, where *vg* is not expressed [15, 17], suggesting the existence of another independent element controlling *sd* expression. *sd* and *vg* have different expression patterns from each other during muscle development, also indicating different regulatory elements are used to control their expression in muscles, instead of the ones for expression in wing discs. A possible way to identify these elements is to make constructs that hook up the upstream DNA fragments of *sd* or *vg* gene to a reporter gene like *lacZ,* and then examine the expression pattern in transgenic flies with these constructs.

What other cofactors are in the same complex as Sd or Vg? Mutations in *sd* or *vg* produced only a mild muscle phenotype with about 30% of the segments having affected SMs, suggesting the existence of redundant genes. This phenomenon is quite common during embryonic muscle development of *Drosophila* [18-20], which makes it important to identify those unknown proteins working together with Sd or Vg. We have made transgenic flies with constructs expressing FLAG or HA tagged Sd or Vg, which can be used to pull down whole complexes containing Sd or Vg from developing embryonic muscles. These complexes can be separated in SDS-PAGE gels and subjected to mass spectrometry (MS) analysis.

Part 3: Vg has a role in the establishment of muscle-muscle attachment.

A phenotype similar to *tig* null mutants was observed when Vg function was interfered with by over-expression of SdΔTEA in SMs. Tig is an adhesion protein that is specifically located in muscle-muscle attachments [21], which indicates Vg might have a role in the establishment of muscle-muscle attachments. Loss-of and gain-of-function analyses were performed to further test this hypothesis (Chapter 4). This data showed several key findings:

Loss-of-Vg-function analyses reveal that Vg is required for the establishment of inter-muscular attachments. Vg function is disrupted by mesodermal expression of SdΔTEA or when a vg^{null} mutation is introduced in $rhea^{1}$ or $slit^{2}$ mutant background in which VL muscles build only muscle-muscle

attachments. In these cases, the inter-muscular attachments are broken or weakened, suggesting that Vg is required for the establishment of this type of adhesion. The *sd*^{3L} mutation was also introduced in *rhea*^{1} mutants, but no muscle detachment phenotype was produced. Therefore, Sd may not have a role in the building of inter-muscular attachments. This further confirms that Sd and Vg have different roles during embryonic muscle development. Vg affects the establishment of inter-muscular attachments not through modifying the affinity of intergrin to ECM, but probably through affecting the turnover and stabilization of the attachment.

Gain-of-Vg-function analyses show that ectopic-expression of Vg is able to produce ectopic inter-muscular attachments. Ectopic attachments are established between muscles that contacted each other through filopodia. Production of this phenotype depends on the expression level of ectopic Vg and requires expression of Vg in both attaching muscles. These ectopic attachments contain all the major components of an integrin-mediated adhesion complex.

Vg works downstream of, or parallel to DER signaling in the production of inter-muscular attachments. Decreasing DER signaling through mesodermal expression of DN-Egfr greatly decreases the size and number of the ectopic attachments between the midline-crossing VL muscles in *slit*^{2} mutant embryos. Increasing the expression level of Vg in this background "rescues" this phenotype and makes the size and number of the ectopic attachments similar to a *slit*^{2} mutant. Increasing DER signaling through mesodermal expression of λ-Egfr produces many small adhesion sites between midline-crossing VL muscles. However, interfering with Vg function in this background led to a much smaller number of this type of adhesion site, with increasing the expression level of Vg produces more and larger adhesion sites.

The phenotype caused by ectopic Vg has nothing to with Slit-Robos or Grip guidance signals. Ectopic expression of Vg in muscle LT1-3 makes them change migration paths and attach to segment borders, which is similar to that caused by ectopic expression of Robos or Grip in muscle LT1-3. However, removing Slit, Robo1, or Grip fails to rescue the phenotype caused by ectopic Vg,

suggesting Slit-Robos signaling or Grip is not involved in the muscle-path changing phenotype.

A key question arises from these findings is: How does Vg affect the establishment of inter-muscular attachments? In *Drosophila*, *vg* is considered as an identity gene for muscle VL1-4 based on its expression pattern in SMs [22]. Identity genes can affect many aspects of muscle development, for example, specification, migration, and adhesion etc. [23]. Examining the muscle phenotype of vg^{null} mutants identified the specification role of Vg in muscle VL2 as this muscle is missing in many segments. However, the identity of VL1 muscles is not affected by the vg^{null} mutation, and they are detached from each other in vg^{null}; $rhea^{1}$ double mutants, suggesting that Vg directs expression of some genes that play a direct role in the establishment of inter-muscular attachments. DER signaling has been shown previously to play a role in the turnover and stabilization of Focal Adhesions (FAs), which is an adhesion structure similar to the muscle-muscle adhesions [24, 25]. The results herein show that there are interactions between Vg and the DER pathway in the production of muscle adhesions. Therefore, to address the question above, it is necessary to identify the targets of Vg and the components of DER pathway that are involved in the turnover of muscle adhesions. Then, it may be possible to find the link between Vg activity and DER signaling. Alternatively, it is possible that Vg works downstream of the DER pathway in production of muscle adhesions. Further studies are necessary to determine this.

A second question that arises is: What is the partner of Vg during muscle development? As discussed in the first part, Sd may not be the partner of Vg during muscle development as it is during wing development. However, Vg relies on the physical interaction with Sd to move into nucleus during wing development (Fig. 5.2) [9, 26]. If Sd was not the partner of Vg during muscle development, how could Vg move into the nuclei of muscle cells? Although Dmef2 is a possible partner of Vg during muscle development, it is not able to bring Vg into the nucleus as Sd does in S2 cells (Fig. 5.2C). In addition, SdΔTEA is able to hold Vg outside the nucleus in S2 cells but fails to do so in developing

muscles (Fig. 5.3C). Therefore, there is likely an unknown protein responsible for transferring Vg into the nucleus in muscles.

One of the primary targets of the Sd/Vg complex during wing development is *vg* itself, and both Sd and Vg are required for the auto-regulation mechanism to keep expression of Vg high in wing disc [9, 27]. A similar mechanism may also exist in developing muscles, as over-expression of SdΔTEA leads to a lower expression of Vg in muscles and produces a phenotype similar to the vg^{null} mutants (Fig. 5.3). However, Sd may not be involved in this process in muscles, as sd^{3L} mutants do not produce a similar phenotype as the vg^{null} mutants. A recent study identified an enhancer directing *vg* expression in adult flight muscles, and Dmef2 works together with Twist and Sd in the activation of this enhancer [13]. Therefore, the activation of *vg* expression in embryonic muscles may require Dmef2 and other unknown proteins that may be also possible for bringing Vg into nucleus.

Part 4: A comparison of muscle development between *Drosophila* and vertebrate.

Vertebrate skeletal muscle progenitors arise from somites [28], which is first patterned into two distinct compartments (sclerotome and dermomyotome) in response to signals from surrounding tissues (Fig. 5.4). As the somite matures, cells delaminate from the dermomyotome lips (DML and VLL) and migrate underneath to form a third compartment, the myotome, which contains skeletal muscle precursors (Fig. 5.4B). The epaxial myotome will differentiate into the back muscles, and the hypaxial myotome will either give rise to the body wall and abdominal muscle, or migrate to the limb bud to form limb muscle (Fig. 5.4B) [29]. Similar to *Drosophila*, Sonic hedgehog (Shh, the homologue of Hh in mammals), and Wnts (the homologues of Wg in mammals) signals are essential for specification of these skeletal muscle precursors. However, unlike in *Drosophila*, where there is a clear distinction between muscle founder cells and fusion competent myoblasts; in vertebrate, there does not appear to be such distinction among the muscle precursors, all of which resemble the fusion

competent myoblasts of *Drosophila* in that they are unrestricted in their fate [30]. The coordinated activities of Shh and Wnts lead to expression of *Pax3* and *Myf5* in myotome, both of which is able to activate the expression of *MyoD* [31]. Similar to the *Drosophila* muscle identity genes, *Myf5* and *MyoD* work redundantly to initiate the whole differentiation program for skeletal muscles [32, 33]. However, this activity of MyoD or Myf5 is inhibited until the muscle precursors migrate to the right place, exit cell cycle, and fuse with each other to form multinucleated myofibers [34]. Therefore, the diversification of vertebrate skeletal muscles happens much later in development, in the case of limb muscles, this happens only when they reach the final target area [35]. Local signals from the target area, for example, from the tendon cells might be responsible for fate decision of these migrated muscle precursors [30, 35], but very little is known about the mechanisms about the regulations of the morphogenesis of individual muscles and their associated tendons [36]. However, in *Drosophila*, this information is provided by a combinatorial expression of a specific group of muscle identity genes in each muscle founder cell, whose fate is decided from the birth (Chapter 1). Thus, there is a greater degree of plasticity in vertebrate muscle development, which may be necessary for the generation of a much more complex muscle pattern in vertebrate. Notably, Nautilus, the MyoD homolog in flies, is only expressed in subset of SMs and required for the specification of these muscles [20].

Despite these differences in the way muscles acquire their fate, the late stages of muscle differentiation, like migrating toward tendon cells, the reciprocal interactions between muscle and tendon cells, and the construction of junctions between muscle and tendon cells are similar in *Drosophila* and vertebrate[35]. Therefore, *Drosophila* serves as a good model to study these processes. Our data reveal the roles of Vg and the DER signal during late-stage muscle differentiation in *Drosophila*, especially in the establishment of stable inter-muscular junctions. These researchs should be able to provide some insight on how muscle pattern is built in vertebrate. In addition, we identified p38b as a potential kinase for Vg phosphorylation, and p38 signaling is a key pathway involved in the final

differentiation of skeletal muscle in vertebrate [37]. A future direction of study would be to examine the role of p38 MAP kinase in the establishment of inter-muscular junctions.

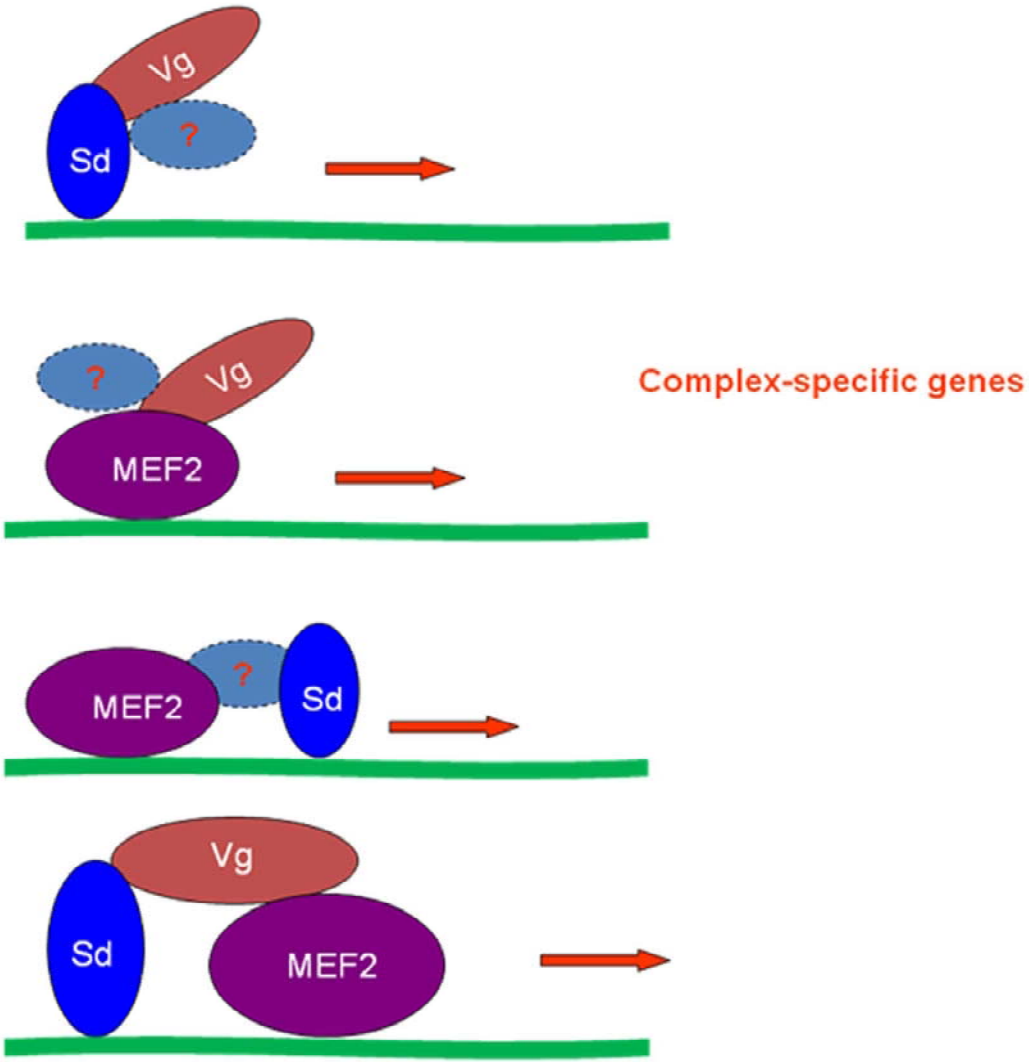

Figure 5.1: Possible combinations for Sd, Vg, and Dmef2 to form a transcription complex during muscle development.

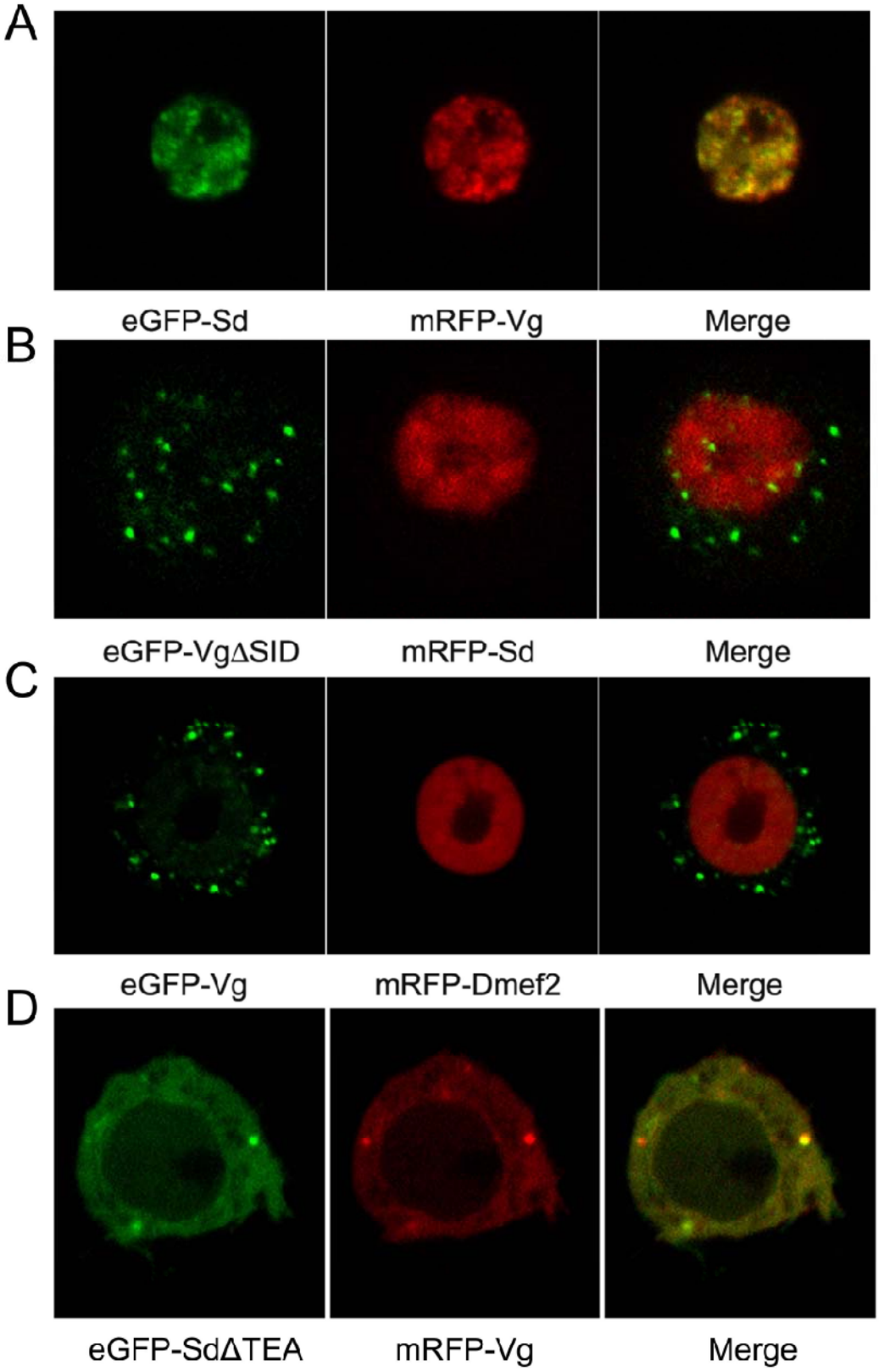
A
eGFP-Sd
mRFP-Vg
Merge
B
eGFP-VgΔSID
mRFP-Sd
Merge
C
eGFP-Vg
mRFP-Dmef2
Merge
D
eGFP-SdΔTEA
mRFP-Vg
Merge

Figure 5.2: Dmef2 fails to bring Vg into nucleus. **A,** Sd is co-localized with Vg in the nucleus. **B,** The Vg deletion without Sd interaction domain (SID) is not able to move into nucleus where Sd is located. **C,** Dmef2 fails to bring Vg into nucleus. **D,** SdΔTEA can hold Vg outside nucleus.

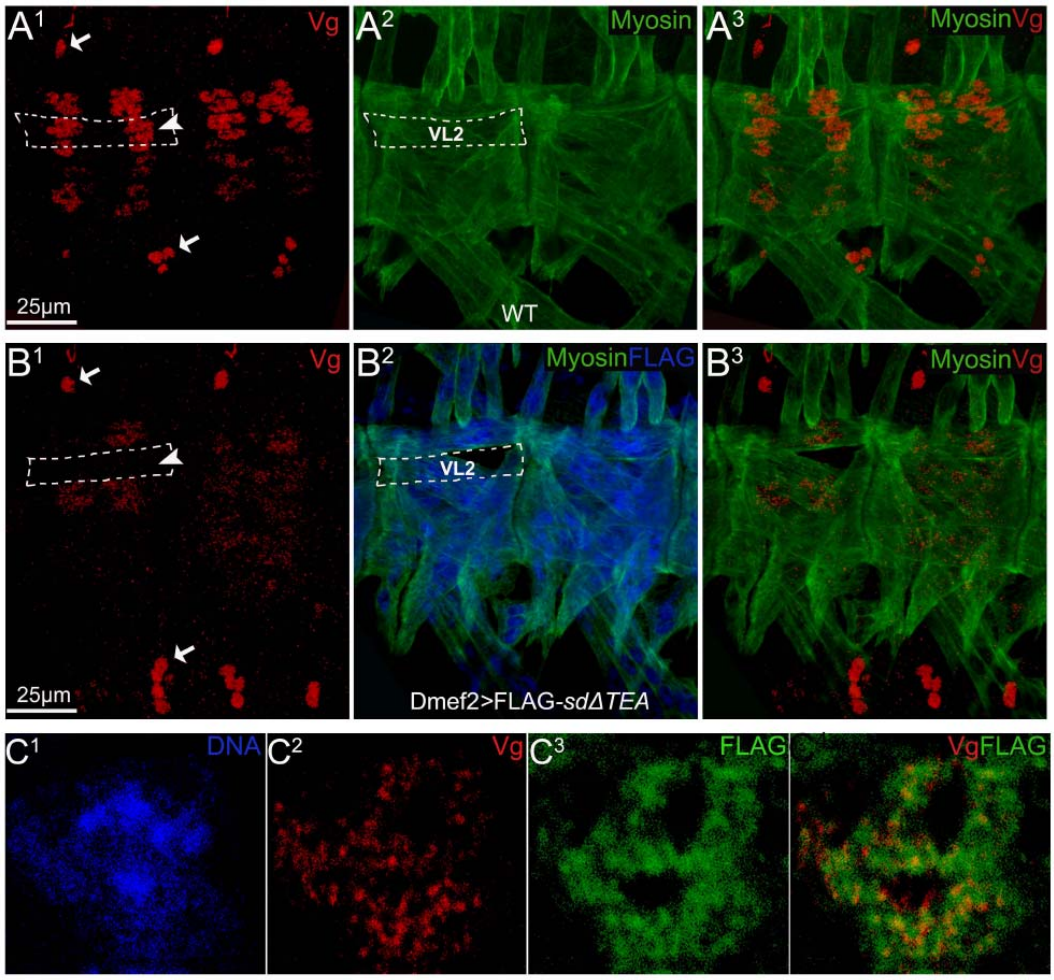

Figure 5.3: Over-expression of SdΔTEA produces a similar phenotype to *vg*^{null} mutants**. A^{1}-B^{3},** Over-expression of SdΔTEA led to low expression of Vg in SMs and produced a phenotype similar to *vg*^{null} mutants. Vg expression was much lower in SMs of the over-expression embryos (B^{1}) than the wild type (A^{1}). As a control, the expression level of Vg was the same in neuronal cells of both embryos (arrows in A^{1} and B^{1}). Note that over-expression of SdΔTEA caused almost loss expression of Vg in muscle VL2 of some segments (arrowheads in A^{1} and B^{1}), and led to loss of this muscle in about 20% segments (A^{2} and B^{2}). **C^{1}-C^{4},** a single confocal projection revealed the co-localization of FLAG- SdΔTEA with endogenous Vg in one nucleus of the embryo over-expressing FLAG- SdΔTEA.

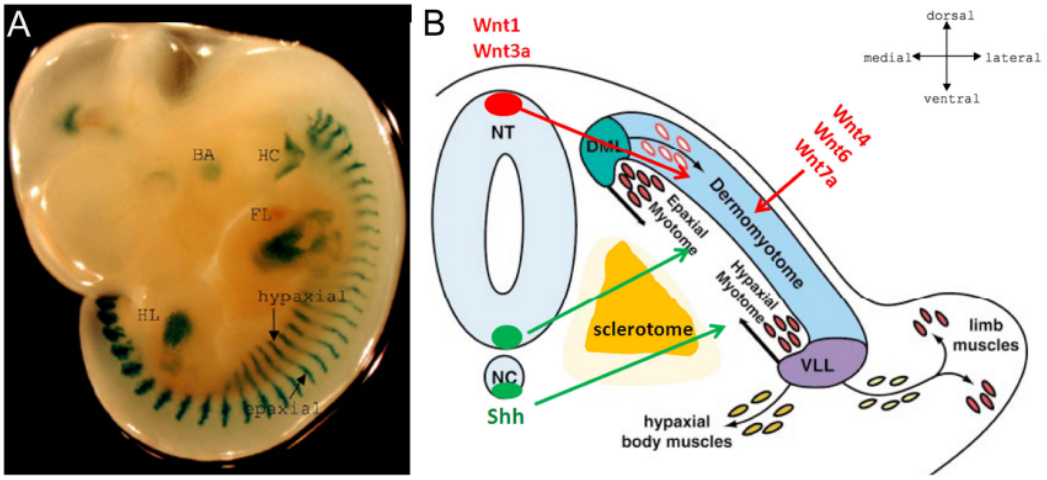

Figure 5.4: Muscle development in vertebrate. **A,** Myogenic progenitors in the mouse embryo at stage 11.5E, as visualized through expression of the *Myf5* Enhancer/*LacZ* reporter. **B,** Schematic representation of a cross section of the embryo showing the somite origins of myogenic progenitors for epaxial, hypaxial, and limb muscles in mouse embryos. Signal cues involved in muscle specification are also indicated. Adapted from Pownall et al.[38]. Abbreviations used: NT neural tube; NC notochord; DML dorsomedial momyotome lip; VLL ventro-lateral lip.

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