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Behavioural Assessment of Muscle Development by Downregulating Different Types of Integrins in *Drosophila melanogaster*

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Abstract

Muscles play a major role in movement and diverse activities required for survival of an organism. In Drosophila, larval crawling and peristalsis are such kinds of rhythmic movements and are driven by muscle contractions. Larval crawling involves random walk on the solid surface whereas peristalsis involves one full body wall muscle contraction from anterior to posterior or in reverse direction in fluid. Accuracy in the development of muscles is an important factor which helps the larvae to execute such rhythmic behaviour. Integrin, the evolutionary conserved heterodimeric transmembrane protein, assists in formation of stable and functional muscles. In Drosophila, larval crawling and peristalsis have been used as a phenotype to study the genetic and developmental aspects of muscles patterning, physiology and activity. These are complex movement events controlled by various other parameters such as efficient neuromuscular junctions (NMJ) and myotendinous junction (MTJ) system etc. The present study examined the effects of downregulation of different integrin genes in growing muscle fibres and tendon cells utilizing GAL4-UAS-RNAi lines. Changes in muscle functioning was assessed through larval crawling and peristalsis behaviour. Knockdown of integrins αPS1 and αPS2 led to slowdown in larval crawling and peristalsis while αPS3 and αPS4 did not show any such significant difference. βPS downregulation in tendon cells gave weaker phenotype than muscles whereas βv knockdown showed more pronounced phenotype when downregulated in tendon cells as compared to the muscles. Our results suggest novel and crucial role of βv during muscle development in Drosophila.

Keywords: *Integrins, muscle development, Drosophila larvae, crawling and peristalsis*

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INTRODUCTION

Rhythmic behaviour in an animal is executed by complex mechanism and has implication on various systems, necessary for animal survival. These are set of activities that are carried out without any delay or hassle but with a regular pattern exhibited at regular time intervals. Such rhythmic behaviours can be seen in locomotion, nervous system, circulatory system, digestive system, respiratory system, *etc*.[1–3]. Different types of muscles develop in a specialised manner to help in executing these rhythmic behaviours. Thus, development of musculature system plays a major role in rhythmic movements, hence should be studied in detail. *Drosophila* proves to be beneficial model organism to study musculature system as it has similarity in mechanism of muscle development at molecular and cellular levels, with vertebrates [4–6]. Understanding of locomotion in *Drosophila* is one aspect which can be taken as a visual clue to study the muscle development. Life cycle of *Drosophila* has four stages—egg, larva, pupa and adult, amongst which only two, *i.e.* larva and adult can move, others are static stages. *Drosophila* larvae exhibit rhythmic peristaltic muscle contractions resulting in crawling and peristalsis movements [4]. Likewise, adult fly can walk, jump, climb and move wings. These locomotor behaviours exhibited in larvae and adults of *Drosophila* can be used as a behavioural measure to examine muscles development and functioning, as defects in muscle will affect the locomotion of fly in these stages. Quantification of such activities

can be done at organism, segment or muscle level which can be further correlated to contraction pattern of the muscles in the animal body. In case of lethal phenotypes where the examination beyond larval stage becomes difficult, larval crawling and peristalsis proves to be reliable tests of locomotion abilities during early stages of life cycle [4, 7]. Larval crawling involves random walk in the solid surface whereas peristalsis involves peristaltic movements of muscles in liquid [8].

Drosophila larvae has been used as model to study many activities such as crawling, peristalsis, learning, memory, direction finding, foraging, response to varied stimuli like photosensitive, audial, smell, chemosensory *etc*. [4, 7, 9–12]. These are complex locomotor behaviours which are performed under the influence of muscular, neuronal and other factors [4]. A particular larval movement such as peristalsis or crawling involves range of actions like spinning, wavering, swirling, burrowing, *etc.* [13–15] which are affected by many genetic and environmental parameters including differences in developmental stage, surface used for crawling, temperature *etc*. [7,16]. Many assays have been designed to do behavioural studies in fly which can be associated to human neuromuscular systems [7].

Drosophila musculature system consists of muscles, tendons as exoskeleton, extracellular matrix (ECM) and different proteins present in intra- and extracellular matrix. Amongst various proteins, integrin is one of the transmembrane proteins that assist in adhering two types of tissues embedded in ECM, *i.e*. muscles and tendons. Integrins act as glue between two different tissues *i.e*., muscles and tendons resulting into formation of hemiadheren junction called myotendinous junction (MTJ). Functional integrins are heterodimers composed of different types of two subunits that noncovalently form dimer—α (αPS1, αPS2, αPS3, αPS4) and β (βPS and βν). Structurally, integrin has small cytoplasmic tail and large extracellular domain, which binds with proteins present in ECM and intracellular protein present in cytoskeleton of muscles and tendons [17–19].

Family of integrins are involved in variety of cellular processes such as cell adhesion, transducing bidirectional signals during morphogenesis, angiogenesis, myogenesis *etc.* This superfamily of integrins has specific domains to bind with ECM ligands, ligands present at cell surface and soluble ligands which often have arginine–glycine–aspartic acid (RGD) as integrin binding motif [20]. These ligands help integrin to pass on and collect signals inside and outside the cell for their proper functioning. Integrins are known for their position specific expression rather than cell-type specific expression [21]. In human, there are 18-alpha and 8-beta integrins present that generate 24 heterodimers. Fly integrins when compared to vertebrate integrins are fewer and many different types of integrins and their associated proteins are not present in *Drosophila* [20].

Different types of integrin in *Drosophila* are involved in various developmental processes. *Multiple edematous wings* (*mew*) gene codes for αPS1 and is involved in the process of adhesion, axon guidance, musculature development, organization of organelle, epithelial cell migration, sensory perception of smell, *etc*. [20, 22–25]. αPS2 integrin is another integrin that is coded by *inflated* gene (*if*) again engaged in cell adhesion during muscle attachment [26–28] and other processes such as central nervous system development, midgut tract development, epithelial cell differentiation and structure maintenance, embryo development, and sensory perception of chemical stimulus [18, 22, 24, 29, 30].

αPS3 is also known as *scab* (*scb*) and it forms dimer with betaPS (*βPS*) and betanu (*βν*). *αPS3βPS* acts as a receptor for ECM ligand—Laminin (Lam). It has been documented that *scab* is engaged in developmental process of the salivary gland, trachea, dorsal vessel and dorsal closure [23, 31–33]. Cell adhesion, signaling, polarity, cell migration and localization of pericardial cells are some other events where *αPS3* is involved [32, 34, 35]. *αPS4* and *αPS5* integrin are supposed to have possible role in cell adhesion and oocyte growth [18, 34, 35].

Beta subunit binds with alpha subunit to form functional integrin heterodimer. *βPS* has been found responsible for adhesion of different cells and tissues during embryonic and postembryonic musculature development [27, 36–38] maintaining polarity of midgut cells with the help of cytoplasmic protein talin [39]. It is also involved in oogenesis, ommatidial organization, sex differentiation, and development of the olfactory system [40, 41].

Similar to *βPS*, *βv* is another beta integrin which helps in adhesion of cells during muscle development, cell polarity of migrating midgut cells but without talin as cytoplasmic proteins [42]. During embryonic development, phagocytic elimination is carried out by *αPS3βv* heterodimer which is supposed to activate signaling pathway [43]. *αPS3βv* together with ECM protein LamininA (LanA) mediates growth of neuromuscular junctions [40, 41].

The present study involves deciphering role of different integrin subunits in muscle development of *Drosophila* embryonic stages. We have targeted integrin genes based on the mechanism of RNA interference (*RNAi*) technology which depends on the specific sequence degradation of host mRNA. The gene-specific *RNAi* sequence has *cis* upstream activating sequence (*UAS*) elements which binds to transcription factor *GAL4* enabling the expression of downstream hairpin double stranded RNA which is further cleaved by Dicer to form the final structural element essential for gene regulation at transcriptional, translational or chromatin modification level [44]. We have done behavioural analysis such as larval crawling and larval peristalsis to check out their muscle defect in embryonic stage. Accuracy in the development of muscles is an important factor which helps the larvae to execute such rhythmic behaviour. Our results suggest that *αPS3* and *αPS4* integrin subunits may not play specific role in muscle and tendon cells though *βv* may be important for tendon cell development. *αPS1*, *αPS2* and *βPS* have already been reported for proper attachment [23, 26, 36–38] and likewise their downregulation resulted in significant reduction in larval crawling and peristaltic behaviour.

MATERIALS & METHODS

Fly Stocks and Genetics

Following integrin *RNAi* lines mentioned with their respective stock numbers were procured from Bloomington Drosophila Stock Center (BDSC), submitted under the Transgenic RNAi Project (TRiP): *αPS1* (44553), *αPS2* (38958), *αPS3* (38959), *αPS4* (44534), *βPS* (33642) and *βv* (61916). Crosses were performed to achieve muscle and tendon specific expression by using *Mef2GAL4* and *24BGAL4*, respectively which were generous gift from IISC Bangalore, India.

RNAi lines of integrins were crossed with *24BGAL4* and *Mef2GAL4* and were allowed to reproduce at 29°C to enable optimal RNAi expression in F_1 progeny of the cross.

Behavioural Tests

Larval crawling was performed on a petridish with 2% agarose on a graph of grid size 0.2 cm^2. Third instar larva with desired genotype was washed with distilled water and transferred on the petridish. Larva was allowed to acclimatize for 30 sec and then movement of larva was recorded to further count the number of grids crossed per minute. Same larva was then used for recording peristaltic movement. Larval peristalsis was done by examining number of full body wall muscle contractions of a larva placed in a cavity block filled with water. This test was then recorded for a minute and then full contractions from anterior to posterior were counted [7]. Images were taken by Olymus-SZX7 microscope using Magnus Magcam DC5 camera.

Statistics

GraphPad PRISM-5 was used to plot and analyse the graphs. Graphs were drawn using CorelDraw-11. Student's *t* tests for comparison of mean differences were done.
p-values of the *t* test were obtained and the significance of results was checked.

RESULTS & DISCUSSION

In previous studies mutation in *βPS* had led to severe defects in formation of muscles affecting different developmental steps like weak muscle attachment sites leading to detachment and rounding up of muscles from tendons [45, 46]. Similarly, in case of loss of

αPS2, muscle-specific integrin, muscles detached from their attachment [47] while absence of tendon-specific *αPS1* did not led to any severe phenotype in early stages of development [17]. These studies guided us to look for the role of remaining integrins *i.e.*, *αPS3*, *αPS4* and *βv* of *Drosophila* in development of muscles. Thus, we performed spatiotemporal knockdown by using specific *UAS-RNAi-GAL4* mechanism to study the significance of these integrins in muscle development. *24BGAL4* is an enhancer trap expressed in tendon cells [48]. Another *GAL4* driver used is *Mef2GAL4,* which expresses *Gal4* under muscle transcription factor *Mef-2* promoter in all muscle types [49]. *RNAi* lines of integrin *αPS3, αPS4* and *βv* were crossed with these *GAL4* drivers. We also performed the crosses *RNAi* of *αPS1, αPS2* and *βPS* with *24BGAL4* and *Mef2GAL4* to verify our results with the earlier findings. Knockdown of *αPS1* in tendons and muscles, *αPS2* in muscles and *βPS* in muscles and tendons have shown differential effects on larval crawling and peristalsis movement as they are crucial and directly involved in muscle development (Table 1, Figure 1).

Unlike *αPS1* and *αPS2*, downregulation of *αPS3* and *αPS4* did not show any significant effects on larval crawling and peristalsis suggesting that these integrins may not be responsible for muscle–tendon development and maintenance (Table 1).

βPS encoded by *myospheroid (mys)* locus has been reported as the binding partner of *αPS1* and *αPS2* that forms a functional heterodimeric transmembrane receptor. It has been known to participate in myotube and tendon cell attachment thus, securing the ends of myotubes and tendon cells steadily [39]. Knockdown of *βPS* in tendon cells lowered the amount of βPS protein resulting in weakening of muscle attachment sites and in return larval movement *i.e.*, crawling and peristalsis were noticeably decreased (Figure 1a and b). We got similar lowered larval peristalsis and crawling when *βv* was downregulated in tendon cells (Figure 1c and d). Though *βv* has been implicated in phagocytosis [43] and neuromuscular junction formation [40, 41], its role in tendon cell development is yet to be ascertained. Likewise, *βPS* knockdown in muscles by pan muscular driver *Mef2GAL4* led to 100% lethality at embryonic stages (Figure 2a) suggesting severe disruption of muscle formation. Downregulation of *βv* in muscles resulted in significant decrease in larval crawling (Figure 2b) and peristalsis (Figure 2c). This may be due to defective neuromuscular junction formation as reported earlier.

Table 1: *Summarised Results of Larval Crawling and Peristalsis Obtained During Down Regulation of Alpha Integrins in Muscles and Tendons of Drosophila.*

S. No.	Genotype	Controls used	Larval crawling		Larval peristalsis	
			***p*-value**	**Phenotype**	***p*-value**	**Phenotype**
1.	*+; +; UAS-αPS1-RNAi/24BGAL4*	*24BGAL4/+*	<0.0001	Significant decrease	0.0051	Significant decrease
		Mef2GAL4/+	0.0148		0.0088	
	+; +; UAS-αPS1-RNAi/Mef2GAL4	*24BGAL4/+*	0.0007	Significant decrease	0.0101	Significant decrease
		Mef2GAL4/+	0.0164		0.0018	
2.	*+; UAS-αPS2-RNAi/+; 24BGAL4/+*	*24BGAL4/+*	<0.0001	Not significant	0.0006	Not significant
		Mef2GAL4/+	0.0968		0.1805	
	+; UAS-αPS2-RNAi/+; Mef2GAL4/+	*24BGAL4/+*	0.0002	Significant decrease	0.0004	Significant decrease
		Mef2GAL4/+	0.0204		0.0005	
3.	*+; UAS- αPS3-RNAi/+; 24BGAL4/+*	*24BGAL4/+*	<0.0001	Not significant	0.0113	Not significant
		Mef2GAL4/+	0.1346		0.2368	
	+; UAS- αPS3-RNAi/+; Mef2GAL4/+	*24BGAL4/+*	0.0046	Not significant	0.6927	Not significant
		Mef2GAL4/+	0.1944		0.0001	
4.	*+; UAS- αPS4-RNAi/+; 24BGAL4/+*	*24BGAL4/+*	<0.0001	Not significant	0.0004	Not significant
		Mef2GAL4/+	0.0718		0.0004	
	+; UAS- αPS4-RNAi/+; Mef2GAL4/+	*24BGAL4/+*	0.0111	Not significant	0.7766	Not significant
		Mef2GAL4/+	0.4679		1.0000	

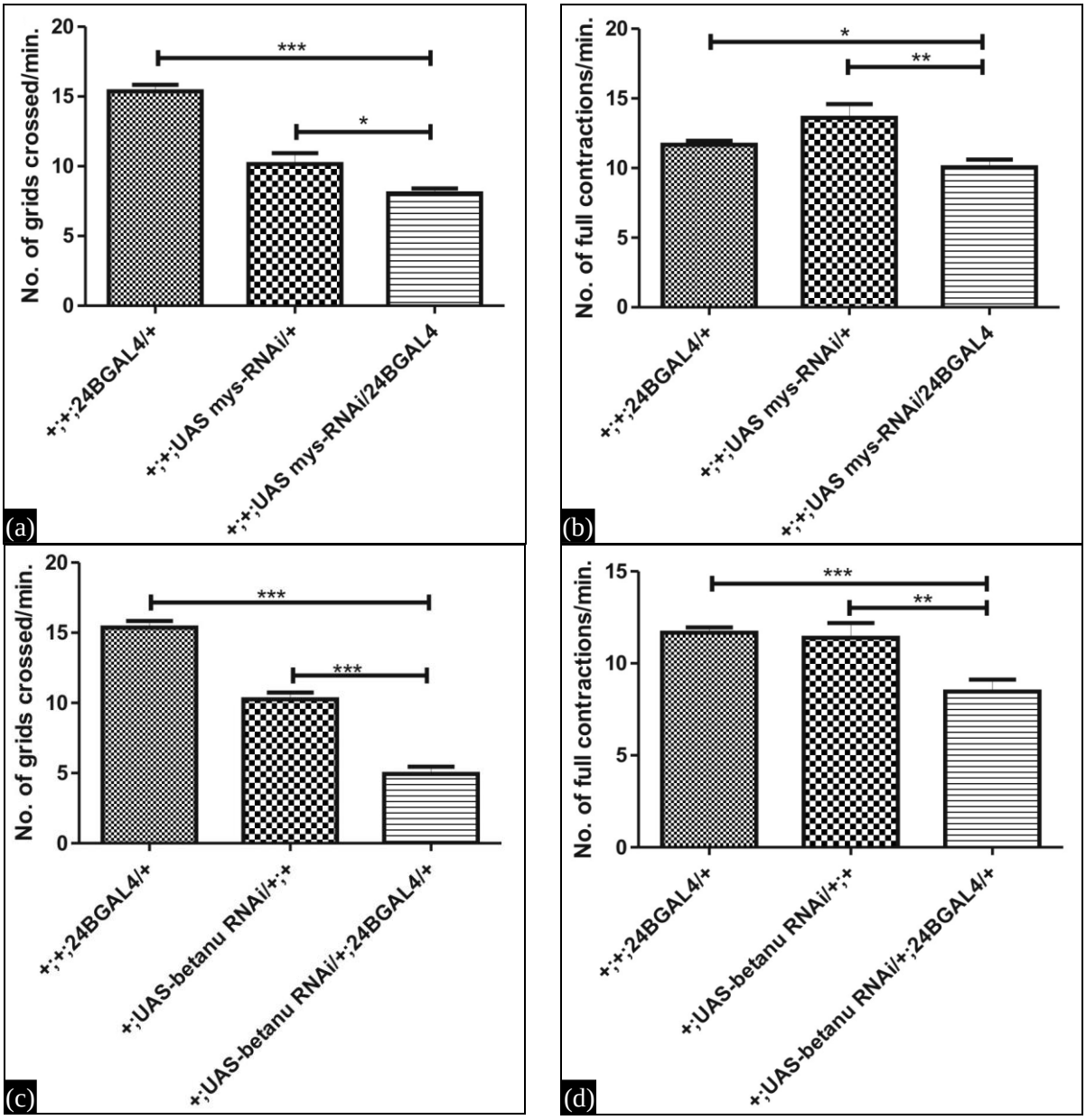

Fig. 1: *Schematic Representation Showing Results of Down Regulation of βPS and βv Integrin in Tendons. (a and b) Graph shows significant decrease in the crawling and peristalsis of larvae when βPS (mys) is downregulated in tendons; (c and d) Knockdown of βv in tendons also led to significant decrease in crawling and peristalsis of larvae.*

CONCLUSION

Study of developmental and genetic aspects of muscle physiology, patterning and functioning can been examined in larval stage by behavioural tests such as larval crawling and peristalsis. These preliminary behavioural examinations in larvae can be performed to screen for any deformity in the larval movement which in turn is dependent on proper functioning of muscles in larval body. Various studies on *αPS1, αPS2* and *βPS* deciphering their role in development of muscles in *Drosophila* have been carried out but nothing much was known about the involvement of other integrins in *Drosophila* muscle development.

αPS1 (*mew*) was reported essential for adhesion and migration of cells of many tissues such as tendon cells [17, 47], neuronal cells [50], *etc*. Therefore, when we downregulated *mew* in tendon and muscles, decrease in larval crawling and peristalsis was observed. Similarly, *αPS2* when downregulated

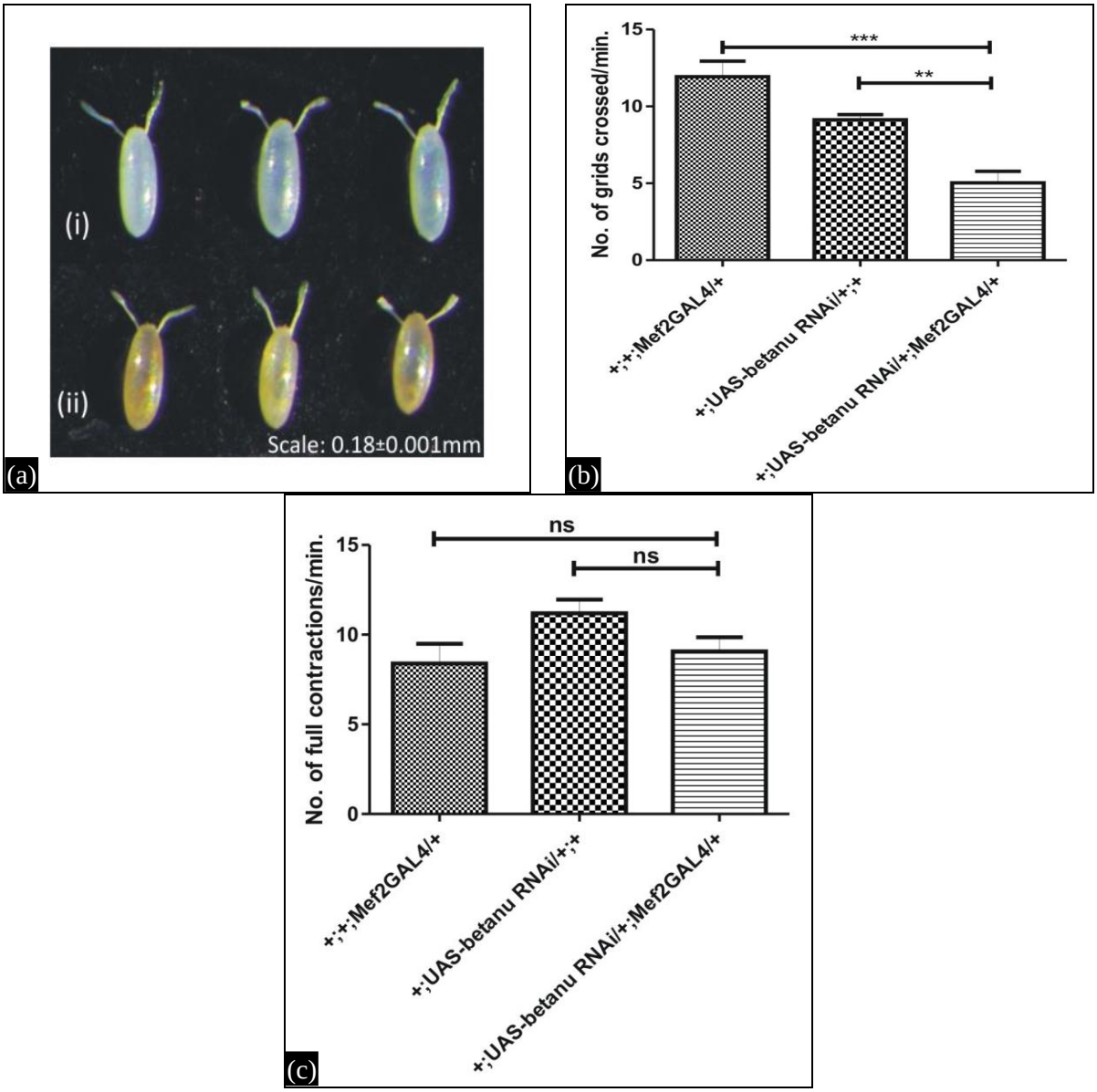

Fig. 2: *Diagrammatic Representation of Results of Knockdown of βPS and βv Integrin in Muscles. (a) Embryonic lethality due to knockdown of βPS integrin in muscles, where Figure 2(a) (i) shows control embryos and Figure 2(a) (ii) are lethal embryos. βv downregulation in muscles led to significant decline in larval crawling (b) and larval peristalsis (c).*

in muscles led to decrease in larval crawling and peristalsis. Decrease in these essential larval movements suggests importance of these integrins in the development of musculature systems. But, *αPS3* and *αPS4* knockdown in muscle and tendon cells did not affect larval crawling and peristalsis behaviour, showing that they may not be involved in muscle development. However, knockdown of *βv* in muscles and tendons resulted in slow down of larval movement providing us clues that *βv* could be involved in the process of muscle growth and tendon development thus leading to formation of stable and functional musculature system [38]. When compared to another beta integrin *i.e. βPS*, it indicates that *βPS* and *βv* have nonredundant functions and also both are important players in muscles and tendon cells development. Our results suggest that *βv* integrin could be a potentially new integrin taking part in the process of establishment of stable muscle system in *Drosophila*. Further detailed studies need to be carried out to explore role played by *βv* during muscle development.

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