Title:

Kon-tiki/Perdido enhances PS2 integrin adhesion and localizes its ligand, Thrombospondin, in the myotendinous junction.

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Summary statement (15 a 30 words)

The proteoglycan Kon-tiki/Perdido enhances αPS2βPS integrin adhesion and localizes its ligand, Thrombospondin. This functional interaction would increase integrin adhesion to the extracellular matrix, consolidating of the *Drosophila* muscle-tendon junction.

Summary

Cell-extracellular matrix adhesion is mediated by cell receptors, mainly integrins and transmembrane proteoglycans, which can functionally interact. How these receptors are regulated and coordinated is largely unknown and key to understand cell adhesion in development. We show that the conserved transmembrane proteoglycan Kon-tiki/Perdido (Kon) interacts with αPS2βPS integrin to mediate muscle-tendon adhesion. Double mutant embryos for *kon* and *inflated* show a synergistic increase in muscle detachment. Furthermore, Kon modulates αPS2βPS signaling at the muscle attachment, since P-Fak is reduced in *kon* mutants. This reduction in integrin signaling can be rescued by the expression of a truncated Kon protein containing the transmembrane and extracellular domains, suggesting that these domains are sufficient to mediate this signaling. We show that these domains are sufficient to properly localize the αPS2βPS ligand, Thrombospondin, to the muscle attachment, and to partially rescue Kon dependent muscle-tendon adhesion. We propose that Kon can engage in a protein complex with αPS2βPS and enhance integrin-mediated signaling and adhesion by recruiting its ligand, which would increase integrin-binding affinity to the extracellular matrix, resulting in the consolidation of the myotendinous junction.

Introduction

Cell adhesion is essential for the development and maintenance of animal tissues. It is controlled by the regulation of the binding properties of cell surface receptors and their ligands. Moreover, altered cell adhesion is a hallmark for cancer cell progression (Sahai et al., 2007). Complex tissue development requires tight control of cell adhesion between different cell layers. Cells can adhere to each other or indirectly through the adhesion to the extracellular matrix (ECM) surrounding them (Rozario and DeSimone, 2010). This is mainly achieved by the transmembrane integrin receptors (Brown, 2000; Maartens and Brown, 2015) although it can also be mediated by other receptors such as Dystroglycan (Bozzi et al., 2009) and other proteoglycan receptors (Couchman, 2010).

Integrins are major adhesion receptors, which mediate the link between the ECM and the cytoskeleton inside the cell. They are widely expressed in different tissues and conserved in evolution, and have been shown to be essential for tissue morphogenesis and homeostasis. Integrins are heterodimeric receptors composed of an α and a β subunit that bind the ECM with their extracellular domain and, they recruit many intracellular adaptors (integrin-

associated proteins) via their cytoplasmic domain, which mediate interaction with the actin cytoskeleton (Hynes, 2002). Integrins activity and regulation must be tightly controlled both in embryogenesis and in adult tissues since impaired or increased activation leads to different disorders (Legate et al., 2009; Pouwels et al., 2012). Thus, investigating the mechanisms of integrin activation and regulation are crucial to better understand integrin function. Integrins are unusual in their ability to respond both to extracellular and intracellular stimuli and thus enabling bidirectional signaling. Extracellular stimuli can induce intracellular signaling (outside-in), and intracellular signaling can cause extracellular changes (inside-out). There is a lot of information about the intracellular molecules that participate in the inside-out activation of integrins, however little is known about the molecules or mechanism by which integrins can be activated outside-in. It has been shown that growth factor receptors cooperate with integrins to mediate intracellular signaling (Ivaska and Heino, 2011). Also, cell surface proteoglycans are cell adhesion receptors that can function as co-receptors alongside high affinity growth factor receptors or adhesion receptors such as integrins (Couchman, 2010). For example, the heparan sulphate Syndecan can promote integrin-mediated adhesion in different cells (Beauvais et al., 2009; Couchman, 2010). In addition, the vertebrate chondroitin proteoglycan receptor NG2/CSPG4 has also been shown to be engaged in a protein complex with integrins and promote integrin activation in different cellular systems. However, the molecular mechanisms by which cell surface proteoglycans modulate integrin activation remain unknown (Chekenya et al., 2008; Fukushi et al., 2004; Iida et al., 1998; You et al., 2014). Here, we have used the *Drosophila* embryonic muscle-tendon adhesion sites as a model system to study the function of the *Drosophila* orthologue of CSPG4/NG2, Perdido (Kon) (Estrada et al., 2007), also named Kon-tiki, (Schnorrer et al., 2007), in the context of the αPS2βPS integrin mediated adhesion.

Muscle-tendon adhesion leads to the formation of the myotendinous junction (MTJ), which is required for the translation of the muscle contractile force into movement via tendon attachment to the cuticle. The construction of the MTJ involves the coordinated development and crosstalk between tendons and muscles (Maartens and Brown, 2015; Schweitzer et al., 2010). Different cellular processes take place in a timely manner such as cell specification, muscle migration toward tendons, muscle-tendon recognition and attachment. Tendon cells not only serve as attachment sites but also provide guiding cues for the migrating myotube. Furthermore, tendon specific gene expression and terminal differentiation depends on muscle attachment (Bate, 1990; Gilsohn and Volk, 2010; Martin-Bermudo, 2000; Volk, 1999).

Muscle-tendon attachment is a sequential process, in which the muscle first transiently comes in close contact with the epidermal cell, thereby initiating assembly of a hemiadherens-type junction. Once they are in contact, it is thought that integrins are activated, and trigger a strong attachment to the extracellular matrix, thus stabilizing the attachment prior to muscle contraction (Brown et al., 2000; Prokop et al., 1998; Tepass and Hartenstein, 1994; Yuan et al., 2010). In *Drosophila,* integrin mutant embryos contain spherical myotubes due to muscle detachment. The *Drosophila* integrin subunits present in the muscle attachment are encoded by the genes *multiple edematous wing* (*mew*, αPS1), *inflated* (*if*, αPS2), and *lethal myospheroid* (*mys*, βPS) (Bokel and Brown, 2002; Brown et al., 2000). These subunits form two different heterodimers expressed at the MTJ, αPS1βPS and αPS2βPS. αPS1βPS integrin is expressed in the tendon cells and might be involved in early events of the formation of the MTJ (Estrada et al., 2007; Roote and Zusman, 1995). And the αPS2βPS, expressed in the muscle cells, is not required for initial formation of the attachment but to form a strong muscle attachment (Wright et al., 1960). Different integrins interact with distinct types of ECM proteins: αPS1βPS interacts with laminin (Gotwals et al., 1994), whereas αPS2βPS interacts with Thrombospondin (Tsp) and Tiggrin (Bunch et al., 1998; Chanana et al., 2007; Fogerty et al., 1994; Subramanian et al., 2007). In fact, controlling the right levels of these ECM molecules is key to the formation of the MTJ (Gilsohn and Volk, 2010; Maartens and Brown, 2015; Yatsenko and Shcherbata, 2014). In particular, the role of Tsp in the MTJ has been studied in detail. Tsp is secreted from tendon cells and progressively accumulates at the junction, being essential for the biogenesis of the MTJ. In its absence, some muscles round up as a result of muscle detachment (Chanana et al., 2007; Subramanian et al., 2007). This phenotype, as well as its localization at the MTJ, is similar to the one of Kon.

Kon, is essential for the targeting and adhesion between embryonic muscles and tendons (Estrada et al., 2007; Schnorrer et al., 2007), both in the embryo and in the adult (Perez-Moreno et al., 2014; Weitkunat et al., 2014). The molecular nature of Kon and its vertebrate orthologues, CSPG4/NG2, together with some experimental data, indicates that they function as ECM receptors (Couchman, 2010; Staub et al., 2002). In addition, CSPG4/NG2 (Stallcup, 2002; Staub et al., 2002) is expressed in the sarcolemma of human postnatal skeletal muscle, as well as in regenerating myofibers (Petrini et al., 2003). In spite of this, it remains unclear what is its function in myogenesis. In *Drosophila*, loss-of-function of this gene results in rounded, detached muscles. Kon is expressed in muscles and localizes to muscle tips and to the muscle attachment site. Kon contains laminin globular extracellular

domains and a small intracellular domain with a C-terminal PDZ-binding consensus sequence. We hypothesized that Kon primes the formation of a protein complex at the myotendinous junction that would activate a signaling pathway within the muscle that is essential for myotube guidance, recognition and attachment (Estrada et al., 2007).

Here, we find that *kon* and *if* genetically interact and can form part of the same protein complex at the muscle membrane in the embryo. They together specifically promote cell adhesion both in cell culture and in the embryonic muscle-tendon junction. In addition, Kon is required for integrin dependent signaling at the muscle attachment, and together with the αPS2βPS integrin, Kon recruits the tendon secreted αPS2βPS integrin ligand, Tsp, at the MTJ. We propose that Kon mediates muscle-tendon adhesion enhancing integrin signaling and adhesion by helping to localize its ligand to the muscle membrane.

Results:

***kon* genetically interacts with *if* to mediate the muscle-tendon attachment**

In order to understand how different cell adhesion receptors may cooperate in the development of the MTJ, we have studied the genetic interactions between two receptors expressed in the muscles. These are encoded by *kon* and *if*, and are required to form a strong muscle attachment (Brown, 1994; Estrada et al., 2007; Schnorrer et al., 2007; Wright, 1960) (Fig. 1A-D). Loss of αPS2βPS integrin causes muscle detachment, although this detachment takes place after the muscles have started contracting, suggesting that αPS2βPS integrin is not involved in muscle targeting (Brown, 1994; Estrada et al., 2007). Indeed, stage 16 *if* mutant embryos show spindle shaped muscles but no muscle detachment (Fig. 1D). To analyze the relationship between *kon* and *if*, we performed a genetic interaction experiment. We compared the extent of muscle detachment in embryos hemizygous mutant for *if* (Fig. 1D), and embryos hemizygous mutant for *if* and heterozygous for *kon* (*if*/*Y*; *kon*/+) (Fig. 1E). We observed that *if* hemizygous mutants (Fig. 1D) do not present muscle detachment as expected (Brown, 1994), while *if*/*Y*; *kon*/+ embryos present a severe muscle detachment phenotype with many myospheres (Fig. 1E, G). In fact, these embryos present 37% more myospheres per hemisegment than *kon* mutant embryos (Fig. 1C, G). Moreover, we analyzed double homozygous mutant embryos for *if* and *kon* (*if*/*Y*; *kon*) (Fig. 1F) and observed the presence of 1.8 and 2.8 times more myospheres per segment than the ones found in *if*; *kon*/+ or *kon* embryos respectively (Fig. 1G). This strong genetic interaction indicates that Kon and

αPS2βPS integrin could act together in mediating the attachment of the muscle to the tendon cell, even though they may have additional independent functions.

The genetic interaction studies from our previous work between *kon* and integrins assessed by co-injection of low concentration of their dsRNAs did not show a significant interaction between *kon* and *if*, as opposed to the co-injection of dsRNAs against *kon* and *mew*, which was statistically significant (Estrada et al., 2007). These data together with the *in vivo* visualization of embryos injected with single dsRNA for these genes, suggested that αPS1βPS integrin is required earlier during the muscle guidance process, for the formation of proper projections and muscle attachment, and that the αPS2βPS integrin may participate in muscle attachment in a different manner from the inferred αPS1βPS-Kon complex, possibly by stabilizing myotendinous junctions after they have formed (Estrada et al., 2007). In this work we have further analyzed the relationship between Kon and αPS2βPS integrin by studying different genetic combinations of *kon* and *if* null alleles, which cause a complete loss of function of the genes, and where we found a clear genetic interaction between them (Fig. 1).

Kon and integrins form part of the same protein complex in embryos

Having found that *kon* interacts genetically with *if*, we wondered if the proteins encoded by these genes interact molecularly. To test this, we immunoprecipitated Kon from embryo extracts and tested if the βPS subunit co-immunoprecipitated with Kon. Indeed, we observed that the βPS subunit, co-immunoprecipitated with Kon in embryos (Fig. 2A). This result suggests that integrins and Kon form part of the same protein complex in the embryo.

Kon and αPS2βPS integrin enhance S2 cell adhesion

To further study the interaction between Kon and integrins in mediating cell adhesion, we used an S2 cell aggregation assay. The *Drosophila* S2 cell line lacks intrinsic self-adhesive properties and cells grow individually as round, non-adherent, non-aggregating cells (Bunch and Brower, 1992; Cherbas et al., 2011) (Fig. 2C). In fact, S2 cells express very little endogenous integrin and *kon* transcripts. However, they can form cell aggregates if they are transfected with cell adhesion molecules (Hortsch and Bieber, 1991). We transfected S2 cells with Kon, αPS1βPS, or αPS2βPS alone and then, Kon in combination with either of the two integrins, in order to quantify the formation of cell aggregates. We observed small number of cell aggregates when cells were transfected with αPS1βPS, αPS2βPS, or Kon alone, but the

frequency of aggregates was significantly increased when cells were co-transfected with Kon and either αPS1βPS or αPS2βPS, being significantly higher when we co-transfected with αPS2βPS (Fig. 2B, C). These aggregates were similar to the ones found in cells transfected with the known cell adhesion protein Dumbfounded (Galletta et al., 2004) (data not shown), which we used as a positive control. In addition, these aggregates contain primarily transfected cells (Fig. 2C), suggesting that the aggregates are formed by cells which co-express αPS2βPS and Kon. These results suggest that the receptors Kon and αPS2βPS specifically cooperate in mediating cell adhesion, maybe by recruiting an ECM protein that could mediate cell adhesion. This protein(s) could either be expressed by the S2 cells or be present in the culture medium.

Kon is essential for αPS2βPS integrin signaling at the muscle attachment

In the embryo, Kon and αPS2βPS integrin are both present in the muscle side of the MTJ (Estrada et al., 2007; Maartens and Brown, 2015). To gain insight into the mechanism underlying the interaction between Kon and integrin, we analyzed whether integrin function was affected in the absence of Kon. To do this, we first analyzed αPS2βPS integrin localization in *kon* mutant embryos. We found that αPS2βPS is still localized in *kon* mutants, even in detached muscles (Fig. 3A-B'', Supp. Fig. 1). Next, we studied whether signaling downstream of integrins was affected in the absence of Kon. The Focal Adhesion Kinase (Fak) is an evolutionary conserved non-receptor protein kinase involved in a myriad of cellular responses. In vertebrates, the Src-Fak complex is referred as the major hub for integrin signaling (Martin et al., 2002). Furthermore, in the *Drosophila* embryo, the phosphorylation of the Focal Adhesion Kinase (P-Fak) takes place at the muscle attachment site in an αPS2βPS integrin dependent manner (Grabbe et al., 2004) (Supp. Fig. 1B-C'). Interestingly, the expression of P-Fak is also increased in the vertebrate MTJ, highlighting the conservation of the pathway (Snow and Henry, 2009). We have studied the localization of P-Fak in *kon* mutant embryos and found that the levels of P-Fak are reduced. Since the muscle attachment is reduced in *kon* mutants we have quantified P-Fak levels normalized to the junctional area, and found that P-Fak levels are reduced by 39% at the muscle attachment (Fig. 3C-D" and Fig. 5A, D), suggesting that the αPS2βPS integrin signaling is compromised in *kon* embryos.

Finally, we tested a molecular interaction between Kon and P-Fak. Co-immunoprecipitation experiments done in embryos suggest that P-Fak forms part of a protein

complex together with Kon *in vivo* (Fig. 2A). This result further supports that Kon and integrins form part of the same protein complex in *Drosophila* embryos.

The extracellular domain of Kon is sufficient to mediate αPS2βPS integrin signaling

As we mentioned before, integrin signaling can take place inside-out and/or outside-in, regulated by a complex network of signals. In order to understand how Kon is mediating integrin signaling at the MTJ, we have studied the function of Kon extracellular and intracellular domains. Kon contains a small intracellular domain with a C-terminus PDZ binding domain. PDZ binding domains serve as a linkage to PDZ protein networks. This domain is required to bind and localize the PDZ containing protein Grip to the muscle membrane at the muscle attachment site. This intracellular signaling through Grip is conserved and is required for muscle targeting (Estrada et al., 2007; Schnorrer et al., 2007; Stegmuller et al., 2003). In order to study the function of this domain in the muscle attachment, we constructed a Kon protein where the cytoplasmic domain of Kon was deleted (KonΔcyt), containing only the transmembrane and extracellular domains (Fig. 4A). Expression of KonΔcyt, under the control of the *twist* promoter in *kon* mutant embryos, properly localized at the MTJ (Fig. 4C-D''). However we found that, as expected (due to the function of the intracellular domain in muscle targeting), it only partially rescued the muscle detachment phenotype in *kon* mutant embryos (Fig. 4C-E). In contrast, we observed the complete absence of muscle detachment in *kon* mutants expressing the full version of Kon protein (Fig. 4B, E). These results suggest that the intracellular domain is not essential for the localization of Kon to the MTJ, even though, in accordance with previous results (Estrada et al., 2007; Schnorrer et al., 2007; Stegmuller et al., 2003), it is essential for the muscle-tendon attachment.

In order to test if the cytoplasmic domain of Kon is required to mediate αPS2βPS integrin signaling in the muscle, we have analyzed if KonΔcyt is able to rescue P-Fak expression at the MTJ in *kon* mutant embryos. We found that expression of KonΔcyt in the muscles of *kon* embryos was able to rescue P-Fak levels in a similar manner as the full version of Kon protein (Fig. 5A-D).

Altogether, these results suggest that even though the cytoplasmic domain is essential to fulfill the complete Kon function, as already shown to be required for muscle-tendon targeting and interaction with the essential protein Grip (Estrada et al., 2007; Schnorrer et al., 2007), it is not essential to localize Kon to the MTJ. In addition, we show that the

cytoplasmic domain is not necessary to mediate αPS2βPS integrin downstream signaling. Thus the extracellular domain of Kon is sufficient to mediate its function in modulating the αPS2βPS integrin signaling at the MTJ.

Kon recruits the αPS2βPS integrin ligand Thrombospondin at the MTJ

We have observed that Kon is required for αPS2βPS integrin signaling and that Kon intracellular domain is not essential for this signaling. Thus, we asked how could the extracellular domain of Kon mediate integrin signaling and adhesion. The molecular nature of Kon and its vertebrate orthologues, together with some experimental data, indicates that they function as ECM receptors (Couchman, 2010; Staub et al., 2002). For this reason, we have explored if Kon could be regulating integrin signaling by localizing the αPS2βPS integrin ligands. Tsp, produced by tendons and essential for the biogenesis of the MTJ, has been suggested to be a ligand for αPS2βPS integrin, as well as being required for integrin-mediated MTJ formation (Chanana et al., 2007; Subramanian et al., 2007). In fact, Tsp levels are reduced in *mys* (Subramanian et al., 2007). In addition, we have observed that *Tsp* mutants show a similar muscle detachment phenotype to *kon* embryos (Chanana et al., 2007; Subramanian et al., 2007) (Supp. Fig. 2B), suggesting that they could be involved in the same process. Moreover, we have analyzed the levels of P-Fak in *Tsp* mutants compared to the controls and found that they are reduced by 27% (Supp. Fig. 2A-C). Thus we wondered if Kon could mediate its adhesion through Tsp at the MTJ. We first studied the localization of Tsp in *kon* mutants and found that it is reduced compared to the controls (Fig. 6A-B", E). Since Tsp has been proposed to be a ligand for the αPS2βPS integrin, it is not completely absent in *kon* mutants, and its localization is affected in *mys* mutant embryos, we wondered if the αPS2βPS integrin was also responsible for its localized expression at the MTJ. For this, we quantified the levels of Tsp in *if* mutant embryos. Indeed, we found that Tsp levels were also reduced in *if* mutants compared to the controls (Fig. 6C-C'', E). The quantification of Tsp levels showed that they were significantly more reduced in *kon* mutants (65%) than in *if* mutants (58%) (Fig. 6E). Moreover, Tsp levels were reduced by 76% in *if*; *kon* double mutants, compared to the controls (Fig. 6D-E). This significant reduction of Tsp in *if*; *kon* double mutants suggests that *kon* and *if* are both required to localize Tsp at the MTJ, and thus cooperate in recruiting Tsp.

We also studied if the overexpression of Kon in wild type muscles enhanced the accumulation of Tsp at the MTJ, and found that there are no differences in the levels of Tsp

between embryos where Kon is overexpressed compared to control embryos (Supp. Fig. 3), also suggesting that Kon is not enough to recruit Tsp to the MTJ and that it needs the αPS2βPS receptor.

We then studied if Kon extracellular domain had a role in the localization of Tsp at the MTJ. To do this, we quantified the levels of Tsp in control embryos, *kon* mutant embryos and *kon* embryos where we expressed KonΔcyt or Kon full length protein in the muscles. We found that the expression of the Kon extracellular domain partially rescued the levels of Tsp in *kon* mutants, similarly to the expression of the Kon full length protein (Fig. 7A-E). These results suggest that Kon is required to recruit Tsp at the MTJ by its extracellular domain.

Ectopic expression of Kon in tendons of *kon* mutant embryos restores Tsp and P-Fak levels and partially rescues muscle detachment

Finally, in order to test if Kon was sufficient to localize Tsp at the MTJ, we expressed Kon ectopically in the tendons of *kon* mutant embryos. We observed that Kon partially rescues Tsp localization at the junction in these embryos (Fig. 8B-B" compare with Fig. 6A-A'' and Supp. Fig. 4A), suggesting that Kon is also able to recruit Tsp at the MTJ from the tendon cell. As we have proposed that Kon could be regulating αPS2βPS integrin signaling by localizing its ligand, we studied if Kon expression in the tendons of *kon* mutants could also rescue the levels of P-Fak. We observed that P-Fak levels are restored in this genotype (Fig. 8C-D" and Supp. Fig. 4B). Thus, the expression of Kon in the tendon is sufficient to localize the αPS2βPS ligand, Tsp, and restore the αPS2βPS integrin signaling.

Moreover, the expression of Kon in the tendons of *kon* mutant embryos, also help us to study the cis and trans requirements of Kon function. In fact, the expression of Kon in the tendons of *kon* mutant embryos rescues around half of the muscle detachment observed in *kon* embryos (Fig. 8E), suggesting that the localization of Tsp and P-Fak contributes to the function of Kon in the formation of the MTJ. In addition, we have observed that the expression of KonDcyt in the tendons of *kon* mutant embryos rescues the muscle detachment in *kon* embryos in a similar way than Kon (Fig. 8E), suggesting that the extracellular domain of Kon is key out Kon function.

Discussion

Transmembrane proteoglycans can associate with other receptors, and function as co-receptors for growth factor and cell adhesion receptors, such as integrins, and affect their

function (Couchman, 2010). Understanding how integrins and transmembrane proteoglycans, are regulated and coordinated is largely unknown and key to understand cell adhesion in development and disease. Here, we find that the transmembrane receptor Kon, cooperates with the αPS2βPS integrin to mediate cell adhesion both in culture cells and in the *Drosophila* embryo MTJ. We find that embryos lacking both proteins present a stronger muscle detachment phenotype than embryos lacking either Kon or the αPS2βPS integrin alone, indicating that Kon and αPS2βPS in embryonic muscles cooperate in mediating adhesion to tendon cells. Moreover, we propose a mechanism by which this transmembrane proteoglycan can enhance integrin signaling and adhesion in the development of the MTJ, by helping to localize the integrin ligand.

The vertebrate orthologues of Kon, CSPG4/NG2, have been shown to be engaged in a protein complex with integrins and promote integrin activation in different cellular systems. For example, CSPG4/NG2 can bind directly to α4β1 integrin and enhance integrin-mediated adhesion in melanoma cells (Iida et al., 1998; Chekenya et al., 2008; Fukushi et al., 2004; You et al., 2014). How does Kon modulate αPS2βPS integrin signaling? One possibility is that Kon helps to localize integrins at the MTJ. In fact, it has been shown that Kon helps to localize integrins at the MTJ in adult flight muscles (Weitkunat et al., 2014), although this is not the case in adult abdominal muscles (Perez-Moreno et al., 2014). Here, we have found that Kon does not regulate αPS2βPS integrin localization at the MTJ in *Drosophila* embryos. However, we find that co-immunoprecipitation experiments suggest that Kon forms part of the same protein complex than the βPS subunit in the embryo. Although we have only been able to detect coimmunoprecipitation of Kon with the βPS subunit and not αPS2, we would like to suggest that Kon forms a complex with the αPS2βPS integrin, and not the αPS1βPS, because they are both expressed in the muscle membrane. In addition, we show that Kon and P-Fak, which is downstream of αPS2βPS integrin, form part of the same protein complex. This interaction is probably indirect through integrins, since the expression of a KonΔcyt rescues P-Fak localization in *kon* mutant embryos. These results suggest that the extracellular domain of Kon is key in modulating integrin-mediated signaling. In addition, our results showing that the expression of KonΔcyt in the muscles of *kon* mutant embryos only partially rescues the muscle detachment caused by the lack of Kon, suggest that the intracellular domain of Kon, while being dispensable for integrin signaling, is required for Kon-mediated MTJ formation. As this domain has been shown to interact with the PDZ protein Grip, essential to mediate muscle-tendon recognition in the embryo (Estrada et al., 2007; Schnorrer

et al., 2007; Swan et al., 2004), we would like to propose that the intracellular domain of Kon might mediate the earlier events of muscle guidance and targeting to the tendon cells but not the later requirement of Kon function, enhancement of integrin signaling. In fact, Kon lacks intrinsic enzymatic activity. Thus, we suggest that it modulates integrin signaling from the extracellular domain indirectly through the regulation of the localization or the activity of other proteins, which in turn may modulate integrin function.

Kon is an ECM receptor and its orthologues in vertebrates are involved in the recruitment of ECM components (Burg et al., 1996; Staub et al., 2002). Among the αPS2βPS ligands, Tsp has been shown to bind directly to αPS2βPS integrin in *Drosophila* S2 cells (Subramanian et al., 2007). Furthermore, here we show that Tsp levels are also reduced in *if* mutants, and that Kon is necessary to properly localize Tsp at the *Drosophila* MTJ. Moreover, the ectopic expression of Kon in tendon cells in *kon* mutants restores the localization of Tsp at the MTJ, further suggesting the essential role of Kon in localizing Tsp. But how could be Kon recruiting Tsp to the MTJ? Tsps are secreted multimeric, multidomain glycoproteins that function at cell surfaces and in the ECM, acting as regulators of cell interactions and attachments (Adams, 2001). The different conserved domains within Tsps are involved in interactions with other ECM molecules, and transmembrane proteoglycans, such as integrins, and heparan sulphate proteoglycans (Bentley and Adams, 2010). Even though, in vertebrates, Tsp has been shown to bind the heparan sulphate Syndecan (Adams and Lawler, 2004), in *Drosophila*, the heparan sulphate proteoglycan expressed in the muscles, Syndecan, does not mediate this interaction (Subramanian et al., 2007). Our studies suggest that the chondroitin sulphate proteoglycan Kon may be the adhesion receptor that localizes, together with αPS2βPS integrin, the secreted ECM protein, Tsp, to the MTJ. Kon could interact directly with Tsp through a Tsp GAG binding domain, or indirectly through another ECM molecule. In the context of Kon interaction with the ECM we also show that the presence of Kon and the αPS2βPS integrin in S2 cells significantly enhances cell adhesion in a cell aggregation assay. In this case Kon and the αPS2βPS integrin could recruit some ECM protein expressed by S2 cells, or from the culture medium to mediate adhesion. This recruitment would be similar to the function of Kon and αPS2βPS integrin in the embryonic MTJ. Although the nature of these proteins is unknown, Collagen IV, Perlecan, Laminin and Sparc are some candidates expressed by S2 cells (Cherbas et al., 2011). Moreover, it is unlikely that Tsp mediates this adhesion in the S2 cell aggregates since it is not expressed in S2 cells ((Cherbas et al., 2011) and FlyBase).

Thus, we propose that the extracellular domain of Kon modulates integrin signaling by helping to localize Tsp to the MTJ. Kon could localize Tsp and thus increase its availability to the αPS2βPS integrin, which would enhance downstream integrin signaling and adhesion. This is supported by the fact that some ECM proteins, including Tsp (Adams and Watt, 1993; Asch et al., 1991; Sun et al., 1992), present both proteoglycan and integrin binding sites promoting the formation of adhesion receptor clusters. These interactions work co-operatively to support stable cell attachments (Iida et al., 1998, Adams, 2001). Alternatively, Kon could help to localize Tsp by interacting with αPS2βPS integrin leading to integrin clustering and/or an increment of integrin ligand affinity (but not localizing the αPS2βPS integrin), which would help to localize Tsp and then enhance integrin signaling and adhesion. Our co-immunoprecipitation data together with experiments with the vertebrate orthologues (Iida et al., 1998) support this possibility too (Fig. 8F).

Moreover, the fact that the expression of Kon in tendon cells in *kon* mutants is able to localize Tsp and P-Fak at the MTJ, and can partially rescue the muscle detachment, suggest that the recruitment of Tsp and the restoration of integrin signaling are necessary for Kon function. This role of Kon in localizing Tsp and P-Fak to the MTJ would lead to increased adhesion and consolidation of the MTJ. This functional interaction between Kon and αPS2βPS integrin builds a specialized ECM microenvironment, which is essential for the development of the MTJ. Ultimately, these studies could be useful to design new drugs that prevent CSPG4/NG2 promoted integrin-dependent chemoresistance in tumor cell survival (Chekenya et al., 2008).

Methods:

<u>*Drosophila* Strains and Genetics</u>

The following stocks were used (all from Bloomington Stock Center unless stated otherwise): Strain y^1w118 as wild-type. The FTG, CTG and TTG balancer chromosomes, carrying twist-Gal4 UAS-2EGFP, were used to identify homozygous mutants (Halfon et al., 2002). Strain *perd*$^{F1-3}$ *as kon* mutants. *perd*$^{F1-3}$*/CTG* (Estrada et al., 2007). *perd*$^{F1-3}$*/ CyO, ftz-lacZ* (Estrada et al., 2007). *if*^{fg2}*/FTG. twist-GAL4. UAS-HA-kon-tiki* (*UAS-HA-kon*) (Schnorrer et al., 2007). *UAS-HA-KonΔcyt* (this work). Tsp^{pr}/CyoYFP (Subramanian et al., 2007). MD710 (*stripe-GAL4,* (Calleja et al., 1996).

<u>Cell culture and aggregation assays</u>

Drosophila S2 cells (from the *Drosophila* Genomics Resource Center) were grown and

transfected as published previously (Bunch and Brower, 1992). Transfections were done with FuGene (Invitrogen) following the manufacturer instructions. Transfection efficiencies were around 30 per cent. Aggregation assays were done as previously described (Hortsch and Bieber, 1991). Cells were transfected with different plasmids: Actin-Gal4 and UAS-HA-Kon-tiki (Schnorrer et al., 2007), pHSPSβ (*mys* cDNA under an hsp70 promoter) (Bunch and Brower, 1992), pHSPS1 (*mew* cDNA under an hsp70 promoter) (Bunch and Brower, 1992), pHSPS2 (*if* cDNA under an hsp70 promoter) (Bunch and Brower, 1992), UAS Duf-HA (Galletta et al., 2004). Positively transfected cells were identified by specific antibodies against HA (Roche), PS2 integrin specific antibody: CF.2C7 (Brower et al., 1984), PS1 integrin specific antibody: DK1A4 (Brower et al., 1984), βPS1 integrin specific antibody: CF.6G11-c (Brower et al., 1984). Each experiment consisted in the simultaneous transfection of S2 cells with all the different proteins to be compared in a six-well plate. Similar number of S2 cells were placed in each transfection plate. Experiments were run in triplicate, with two independent scorings per experiment, for a total of 6 observations per transfection. Big cell aggregates (composed of 20 or more cells) were quantified from the differently transfected S2 cells (n=6). We tested if cell cultures with different transfections differed in the number of cell aggregates they contained by means of generalized linear models with an underlying Poisson error distribution and a log link function using SAS 9.2 (SAS Institute, Cary, NC). Post hoc tests were carried out testing for differences among least square means and applying a Sidak correction of the observed p-values.

Co-immunoprecipitations

For *in vivo* Co-IPs, embryonic lysates were prepared from eight collections of stage16 w^{1118} embryos. Co-IPs were carried out as described previously (Slovakova and Carmena, 2011) except that the lysis buffer was: 50 mM Tris pH 8, 150 mM NaCl, 1% NP40, 0,3% DOC, 50 mg/ml PMSF and Complete Protease Inhibitors (Roche). Two immunoprecipitations were done in parallel, one with rabbit anti-Kon (Schnorrer et al., 2007) and one with rabbit anti-betagalactosidase (Cappel) as a control. The same amount of protein extract was loaded on the beads in each immunoprecipitation. Immunoblots were done with rabbit anti-βPS 185A-E (Gotwals et al., 1994) and rabbit anti-P-Fak (Biosource),

Embryo Immunohistochemistry and microscopy

Embryo antibody stainings were carried out as described previously (Carmena et al., 1998). All embryos were at stage 16. The following primary antibodies were used: rabbit and mouse anti-MHC (D. Kiehart), rat anti-MHC (Babraham Bioscience Technologies), rabbit and

mouse anti-betagalactosidase (Cappel, Promega), rabbit and mouse anti-GFP (Invitrogen), chicken anti-GFP (AbCam), rabbit anti-βtubulin (Leiss et al., 1988), rat anti-HA (Roche), rat anti-Tsp (Subramanian et al., 2007), rabbit anti-P-Fak (Biosource), rat anti-Tromopomyosin (Babraham Bioscience Technologies), rat anti-PS2 (Bogaert et al., 1987). We tested for differences among genotypes in the number of myospheres observed per hemisegment fitting a linear model since the dependent variable met all parametric assumptions. Confocal images were obtained using a Leica SP2 microscope and processed with Adobe Illustrator and ImageJ.

DNA construct

The construction of KonΔcyt was done as follows. DNA from plasmid pFS135 (Schnorrer et al., 2007) containing the complete Kon cDNA was PCR amplified using primers Kon-SgrA (5'-GTCACGCCGGCGTAATCTAGAGGATCTTTGTGAAG-3') and UAS-StuI (5'-GTCAAGGCCTCCCGGGTCTAGTGGATCCAG-3'). Primer Kon-SgrA introduces a stop codon before a SgrA1 site and eliminates 735 bp from the cytoplasmic domain of Kon. The amplified DNA fragment and the pFS135 plasmid were digested with SgrA1 and StuI for oriented cloning. Ligation mixtures were used to transform *E. coli* DH5α, with selection of Apr transformants on LB medium-ampicillin plates. This plasmid was used to obtain the transgenic flies.

Data Analysis

Quantification of muscle detachment was done by counting the number of myospheroid shaped muscles in stage 16 embryonic ventral muscles. Quantification of fluorescence intensity was done from maximal projections confocal stacks using ImageJ software. The quantification was done by measuring the mean grey value on manually selected regions normalized to the area of the junction. Mean grey value is the sum of the grey values of all the pixels in the selection divided by the number of pixels. Selected regions were manually selected muscle attachment sites of the ventral longitudinal muscles with ImageJ software. The background value, taken from intersegmental signal free regions, was subtracted from the mean grey value in each embryo. The quantifications data was represented as in each case in a Boxplot, where center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by dots (Spitzer et al., 2014). All statistical analyses were conducted in R (R Core Development Team). We statistically compared differences in the variables of interest (number of myospheroids or fluorescence

intensity) among the different genotypes by fitting general linear models using the *lm* function. Residuals were systematically checked for goodness of fit to a Gaussian error distribution using visual assessments of normal quantile plots and Kolmogorov-Smirnov tests (function *lillie.test* from package *nortest*). We also tested for heterokedasticity of the data using the Breusch-Pagan test with function *bptest* from package *lmtest*. In most cases variables met parametric assumptions, and when they did not, we fit the linear model on the ranked variable. Multiple comparisons were conducted by means of post-hoc Tukey tests using the *TukeyHSD* function. Asterisks over the graphs indicate significant differences, while n.s. indicate not significant differences.

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Figure legends

Figure 1. *kon* interacts genetically with *if* in the attachment of muscles to tendons. (A-F) Confocal micrographs of embryos stained for Tropomyosin. (A) Wild type embryos. (B) *kon* heterozygous mutants (*kon*/+) where there is not muscle detachment. (C) *kon* mutant embryos where many muscles (mainly ventral longitudinal) are detached. (D) *if* mutant embyos (*if*) where there is not muscle detachment, although muscles are spindle shaped (arrowhead). (E) *if*; *kon*/+ where there are many detached muscles. (F) *if*; *kon* embryos where there are more

detached muscles than in *if*; *kon*/+ (E) or in *kon* embryos (C). Arrows indicate detached muscles. (G) Quantification of muscle detachment represented by the number of myospheres per hemisegment in *kon*/+, *kon*/ *kon*, *if*; *kon*/+, and *if*; *kon* (means=0, 1.69, 2.67 and 4.72 respectively; n≥7 for each genotype).

Figure 2. Kon forms a protein complex *in vivo* with the βPS integrin subunit and P-Fak, and cooperate with αPS2βPS integrin to mediate cell adhesion in *Drosophila* S2 cells. (A) *Drosophila* embryo lysates were subject to immunoprecipitation (IP) with anti-Kon and probed on immunoblots (IB) with anti-βPS, and anti-P-Fak. (B) Quantification of S2 cell aggregates bigger than 20 cells in cells transfected with PS1, PS2, Kon, PS1 and Kon, or PS2 and Kon proteins (means=0.33, 1.57, 1.28, 9.17 and 40.71 respectively; n=6 for each experimental condition). Note that cells co-transfected with Kon and αPS2βPS integrin significantly present the highest number of aggregates. All αPS subunits were co-transfected with the βPS subunit although it is not indicated in the graphs. (C) Representative images of S2 cells co-transfected with αPS2βPS integrin and Kon, where big cell aggregates can be observed.

Figure 3. Kon is essential for normal levels of P-Fak, a downstream mediator of αPS2βPS integrin signaling, at the MTJ. Confocal micrographs of heterozygous (A, C) and homozygous (B, D) *kon* mutant embryos. (A-B") Embryos stained for Myosin Heavy Chain and αPS2 integrin, where αPS2 integrin localizes to the muscle attachment in *kon* heterozygous embryos (A-A"). In *kon* homozygous embryos αPS2 integrin is still localized to the tip of the muscle (red arrowhead), even though the morphology of the attachment is abnormal (B-B"). (C-D") Embryos stained for Myosin Heavy Chain and P-Fak, where P-Fak localizes to the muscle attachment site in *kon* heterozygous (C-C") but it shows reduced levels in *kon* mutants (red arrowhead, D-D").

Figure 4. The intracellular domain of Kon, containing a PDZ binding domain, is essential for muscle tendon attachment, although it is not required to properly localize Kon. (A) Schematic representation of the full length Kon HA-tagged protein encoded by the *UAS-kon* construct (upper) and a Kon HA-tagged protein where the cytoplasmic domain was deleted, encoded by the *UAS-KonΔcyt* construct (lower). (B-D") Confocal micrographs of

embryos stained for Tropomyosin and HA. *kon* mutant embryos that express *UAS-kon* driven by *twist-GAL4* show HA localized to the muscle attachment (B-B"). *kon* mutant embryos that express the *UAS-KonΔcyt* driven by *twist-GAL4* still show HA localized to the muscle attachment, although the morphology of the attachment is abnormal (C-D"). (E) Quantification of the number of detached muscles (myospheres) per hemisegment. While the expression of the full length form of Kon in *kon* mutants does not show any muscle detachment (mean=0; n=7), the expression of KonΔcyt in *kon* mutants partially rescues muscle detachment (mean=1.42; n=12), showing significant differences in the number of myospheres compared to *kon* mutants (mean=2.48; n=14).

Figure 5. KonΔcyt is sufficient to mediate integrin signaling. (A-C") Confocal micrographs of embryos stained for Tropomyosin and P-Fak. *kon* mutant embryos fail to localize P-Fak to the muscle attachment (arrow in A and A"). The expression of Kon or KonΔcyt in the muscles of *kon* mutants restores P-Fak localization to the attachment site (B-C"). (D) Quantification of the relative P-Fak levels normalized to the junctional area at the intersegmental region of the ventral muscles. From left to right in the boxplot, the means of the different experimental conditions are 1, 0.61, 0.97 and 1.03; n≥16 for each genotype.

Figure 6. Kon and αPS2βPS integrin are both required to localize the integrin ligand Thrombospondin at the MTJ. (A-D'') Confocal micrographs of embryos stained for MHC and Thrombospondin (Tsp). Tsp localizes to the muscle attachment in *kon* heterozygous (A-A"), but it is reduced in *kon* mutants (B-B"). Tsp is also reduced in *if* (C-C") and in *if*; *kon* double mutant embryos (D-D''). Brackets point at ventro-longitudinal muscles. (E) Quantification of the relative levels of Tsp normalized to the junctional area at the intersegmental region of the ventral muscles in *kon*/+ (mean=1; n=36), *kon* (mean=0.35; n=36), *if* (mean=0.42; n=36), and *if*; *kon* (mean=0.24; n=24) mutant embryos.

Figure 7. Kon extracellular domain partially rescues Tsp localization to the attachment site. (A-D") Confocal micrographs of embryos stained for MHC and Tsp. *kon* mutant embryos (B-B'') fail to properly localize Tsp to the muscle attachment compared to controls (A-A"). The expression of KonΔcyt in the muscles of *kon* mutants (C-C'') can restore Tsp localization to the attachment site, although the quantification of the levels of Tsp in this embryos indicate that this rescue is only partial, and similar to the levels of Tsp in *kon*

embryos where we express the full form of Kon protein (D-D''). Brackets point at ventro-longitudinal muscles. (E) Quantification of Tsp levels. From left to right in the boxplot, the means of the different experimental conditions are 1, 0.25, 0.88 and 0.8; n≥16 for each genotype).

Figure 8. Kon ectopically expressed in tendon cells is able to localize Tsp and P-Fak at the MTJ and partially rescue Kon dependent muscle detachment. (A-D") Confocal micrographs of *kon; stripe-GAL4*/+ (A, C) and *kon; stripe-GAL4*/*UAS-kon* embryos (B, D) stained for MHC and Tsp (A, B) or P-Fak (C, D). The low Tsp levels observed in *kon* mutant embryos (A'') are notably increased when Kon is expressed in tendon cells of *kon* mutants (B"). Similarly, P-Fak levels are increased in *kon* mutants expressing Kon in the tendons (D'') compared with *kon* mutants (C''). Brackets point at ventro-longitudinal muscles. (E) Quantification of the number of detached muscles (myospheres) per hemisegment. Kon (mean=1.56; n=16) or KonΔcyt (mean=1.68; n=7) expression in tendon cells of *kon* mutant embryos significantly reduced the number of myospheres compared to *kon* mutants (mean=3.37; n=16). (F) Two possible models for the function of Kon in the development of the MTJ. In model A, Kon would help to localize the integrin ligand Tsp to the MTJ. In model B, Kon would form a complex with the αPS2βPS integrin in the muscle enhancing integrin ligand affinity and the recruitment of Tsp at the MTJ. In both cases the recruitment of Tsp would enhance integrin intracellular signaling (through the phosphorylation of Fak) and adhesion to the extracellular matrix, resulting in the consolidation of the myotendinous junction.

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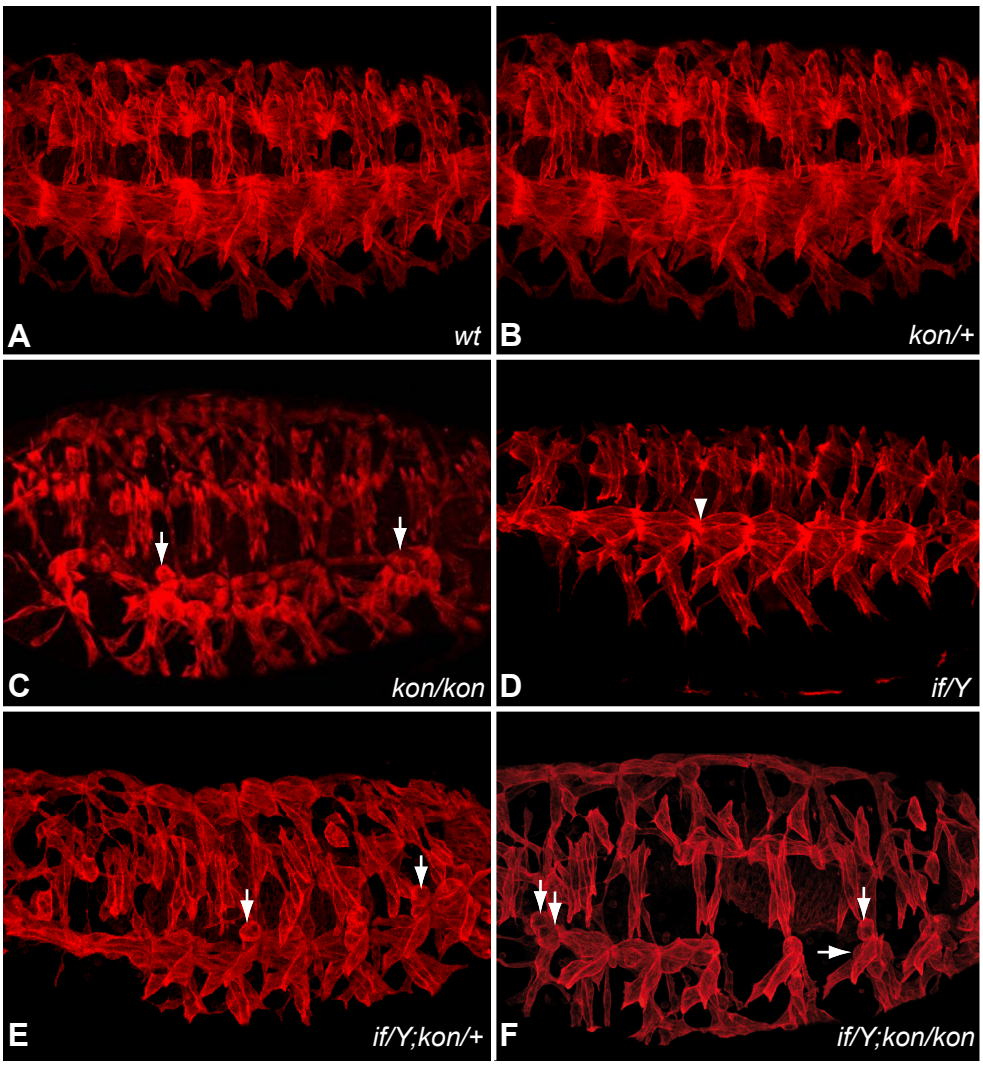
Figure 1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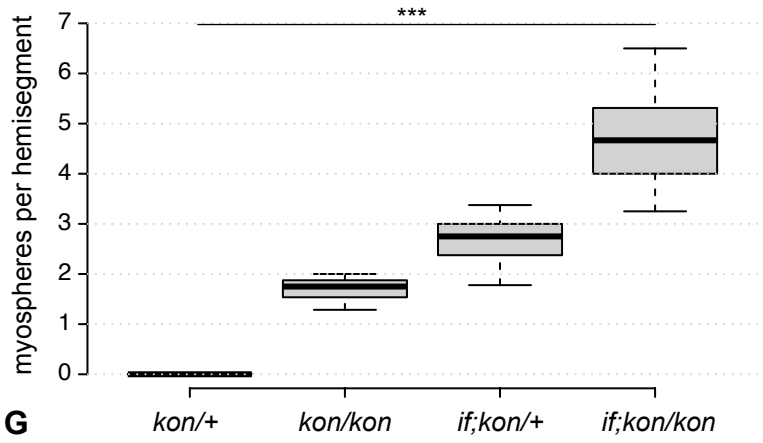
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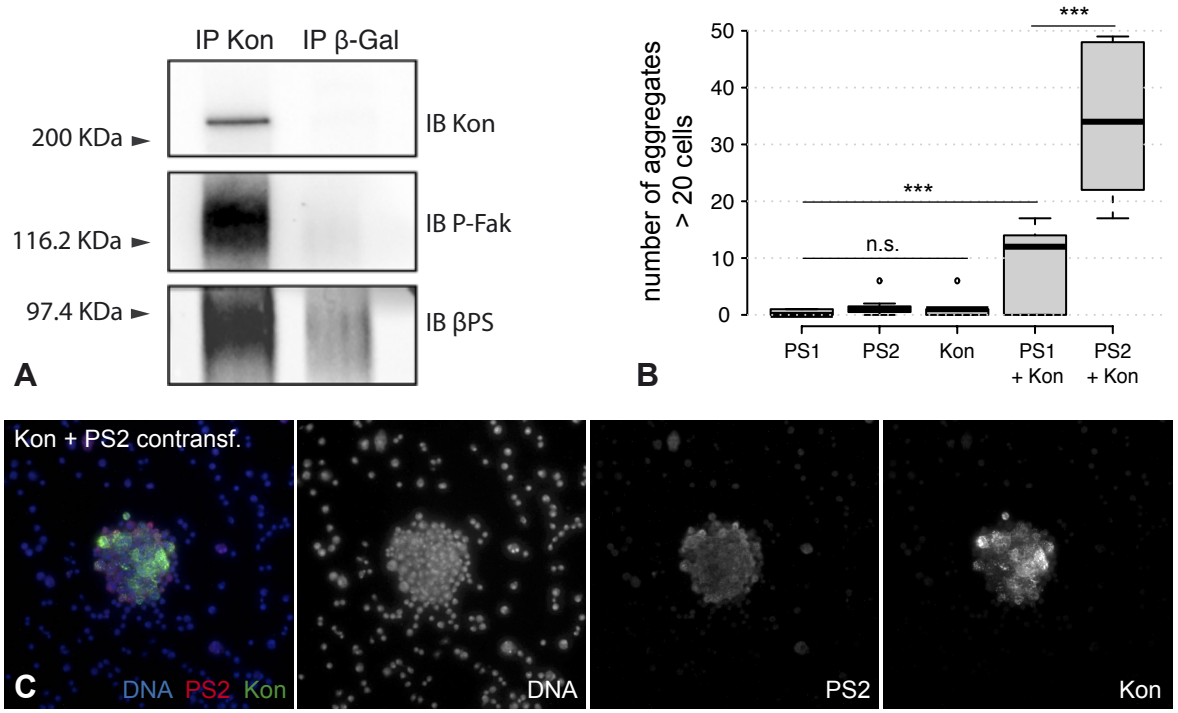
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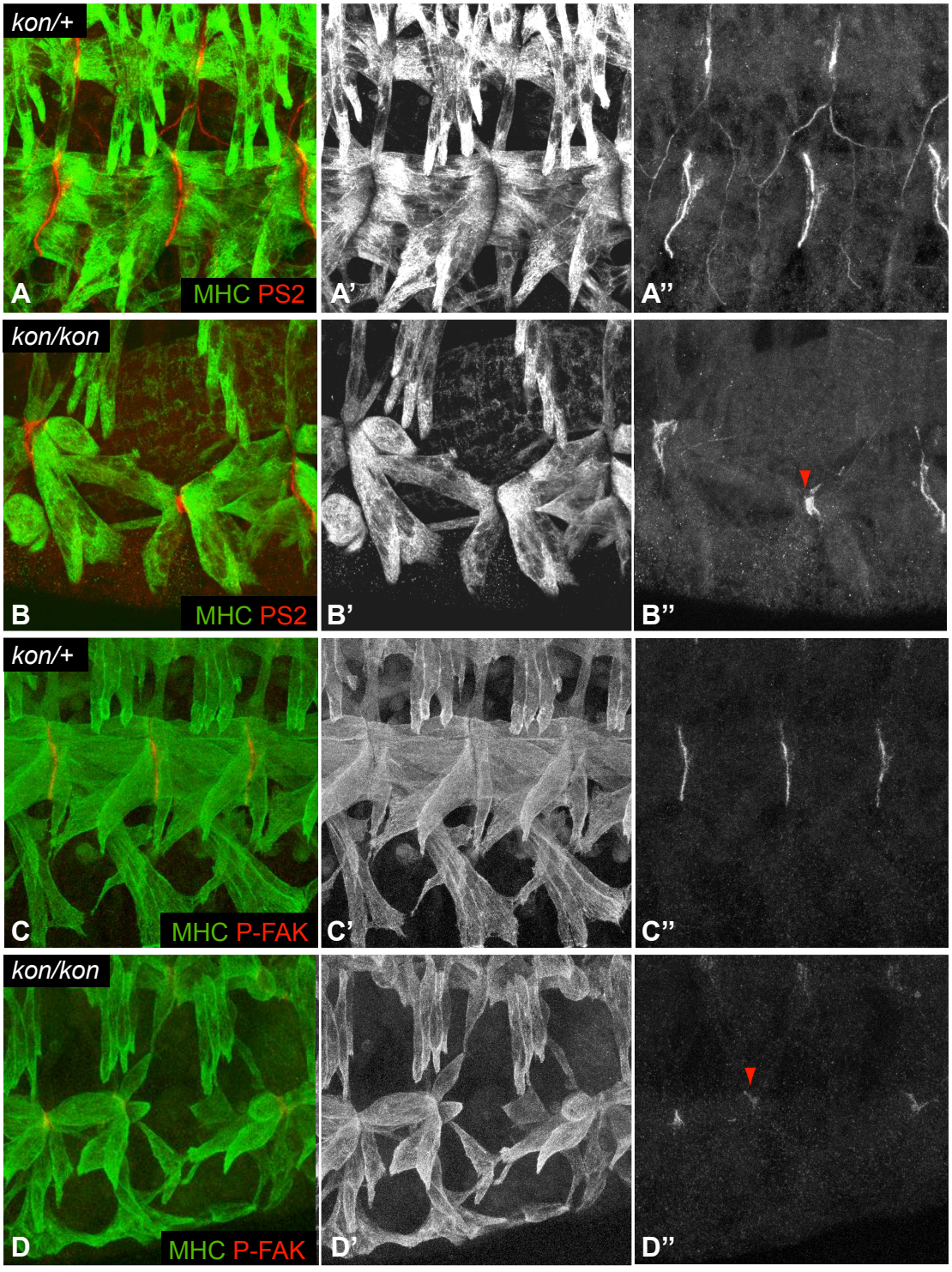
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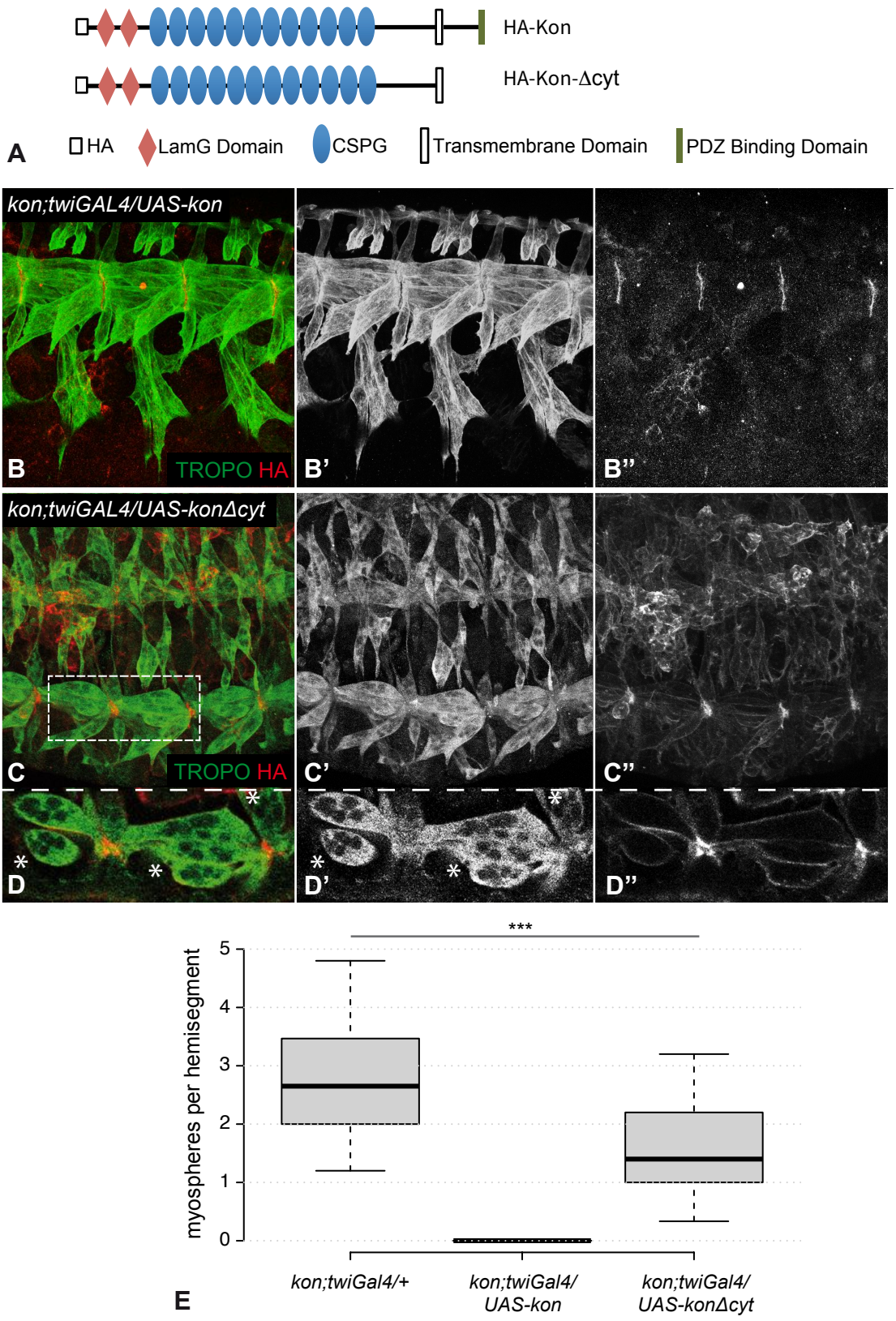
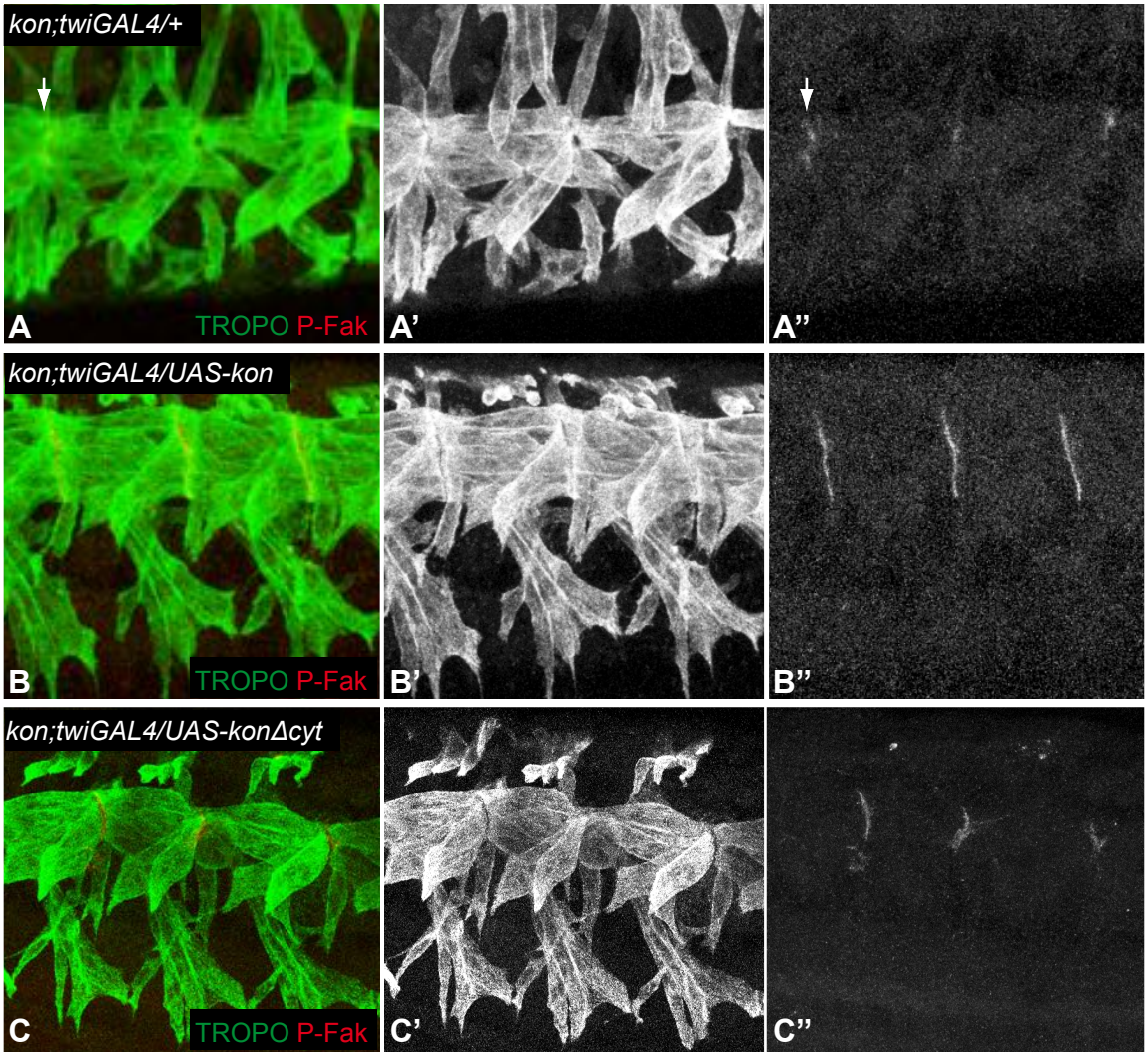

HA-Kon
HA-Kon-Δcyt
A
HA
LamG Domain
CSPG
Transmembrane Domain
PDZ Binding Domain
kon;twiGAL4/UAS-kon
B
TROPO HA
B'
B''
kon;twiGAL4/UAS-konΔcyt
C
TROPO HA
C'
C''
D
D'
D''
***
myospheres per hemisegment
0
1
2
3
4
5
kon;twiGal4/+
kon;twiGal4/
UAS-kon
kon;twiGal4/
UAS-konΔcyt
E

Figure 5


kon;twiGAL4/+
A
TROPO P-Fak
A'
A''
kon;twiGAL4/UAS-kon
B
TROPO P-Fak
B'
B''
kon;twiGAL4/UAS-konΔcyt
C
TROPO P-Fak
C'
C''

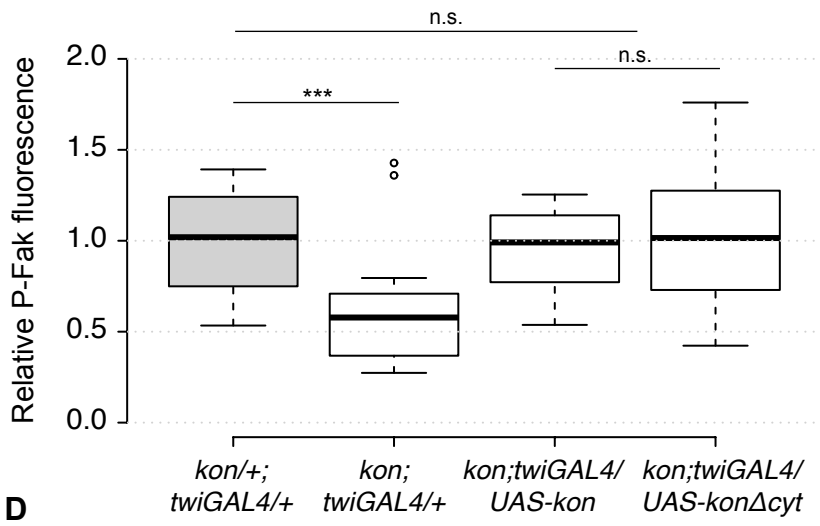
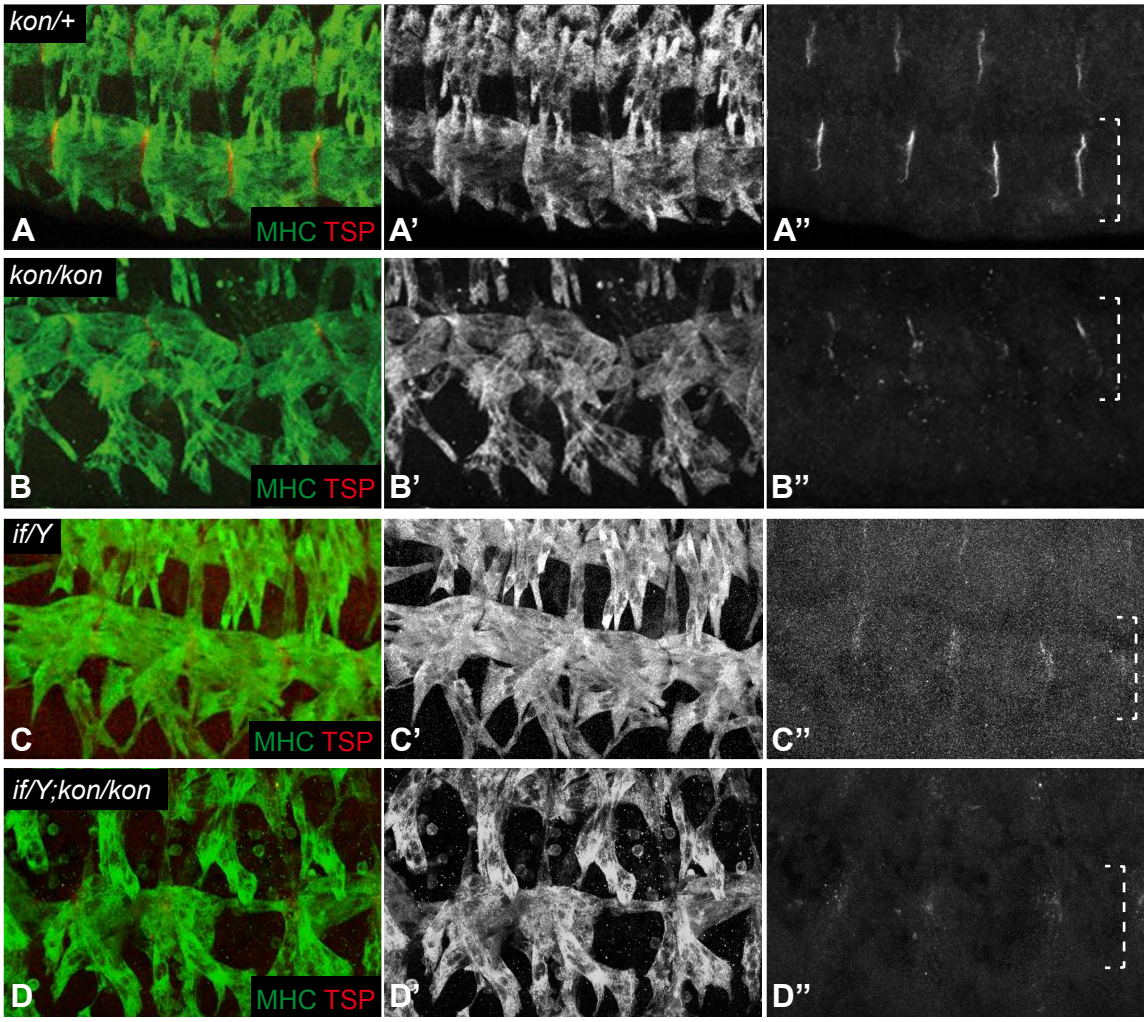

n.s.
n.s.
***
Relative P-Fak fluorescence
2.0
1.5
1.0
0.5
0.0
kon/+; twiGAL4/+
kon; twiGAL4/+
kon;twiGAL4/ UAS-kon
kon;twiGAL4/ UAS-konΔcyt
D

Figure 6


kon/+
A
MHC TSP
A'
A''
kon/kon
B
MHC TSP
B'
B''
if/Y
C
MHC TSP
C'
C''
if/Y;kon/kon
D
MHC TSP
D'
D''

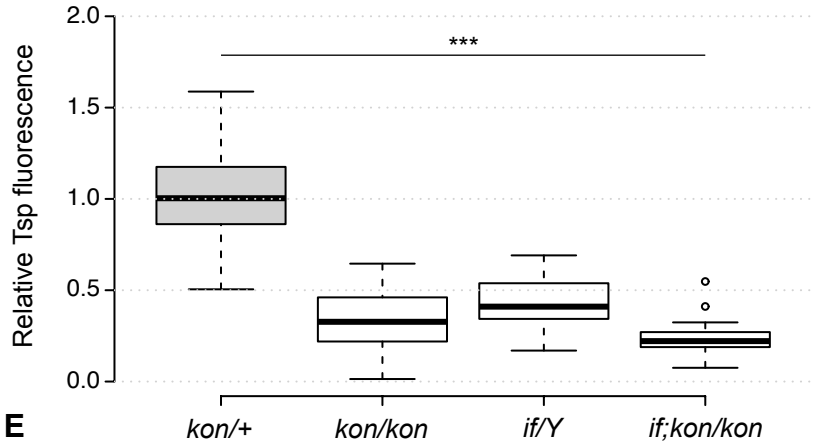
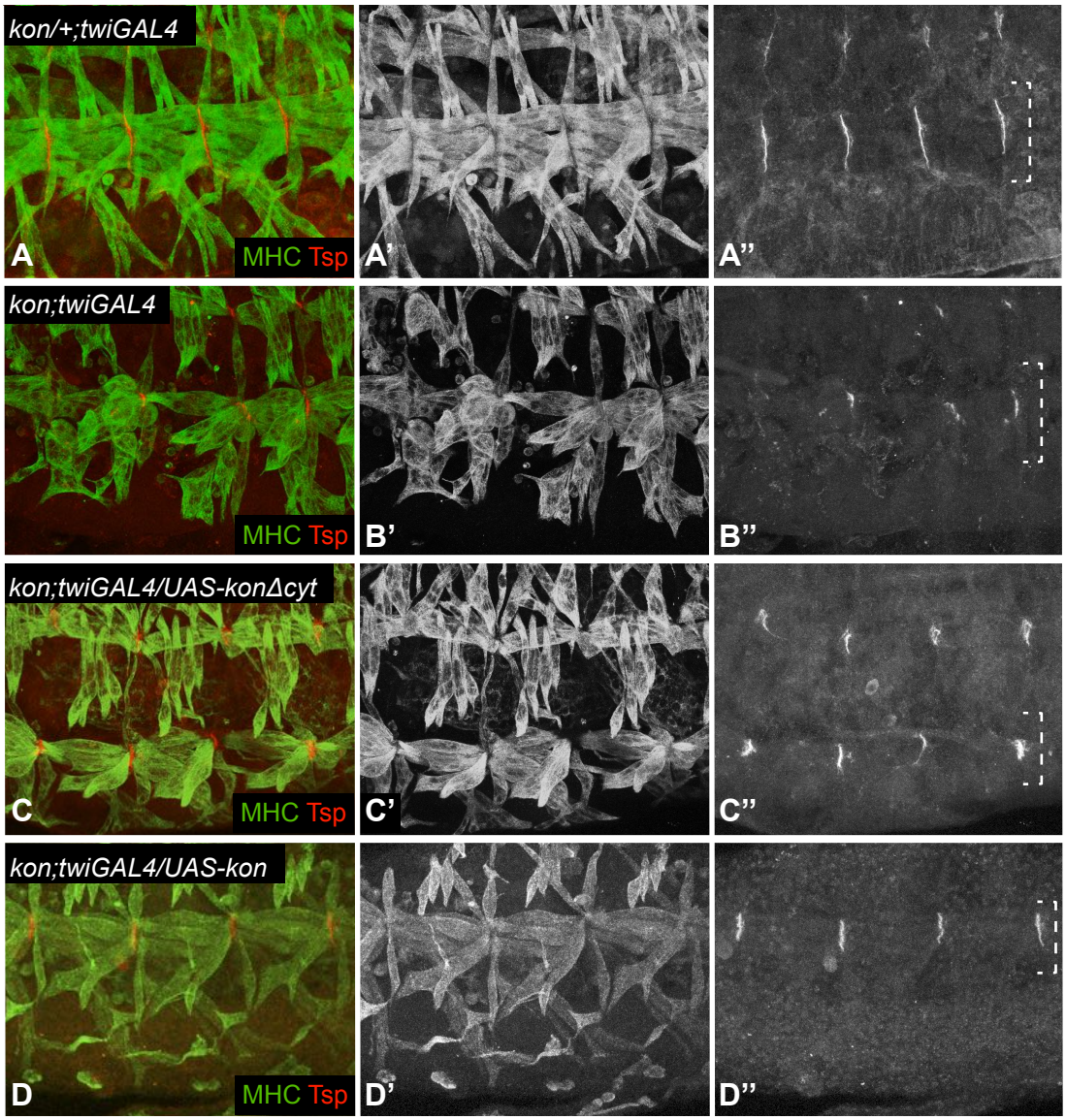

Relative Tsp fluorescence
2.0
1.5
1.0
0.5
0.0
***
kon/+
kon/kon
if/Y
if;kon/kon
E

Figure 7


kon/+;twiGAL4
A
MHC Tsp
A'
A''
kon;twiGAL4
MHC Tsp
B'
B''
kon;twiGAL4/UAS-konΔcyt
C
MHC Tsp
C'
C''
kon;twiGAL4/UAS-kon
D
MHC Tsp
D'
D''

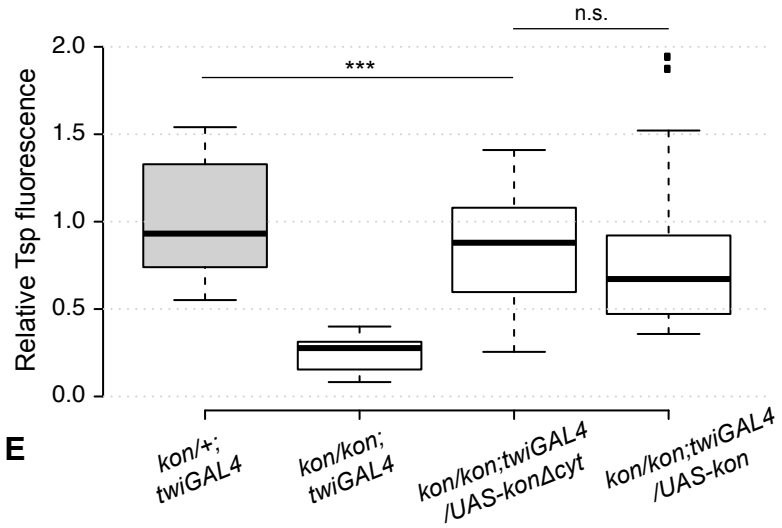
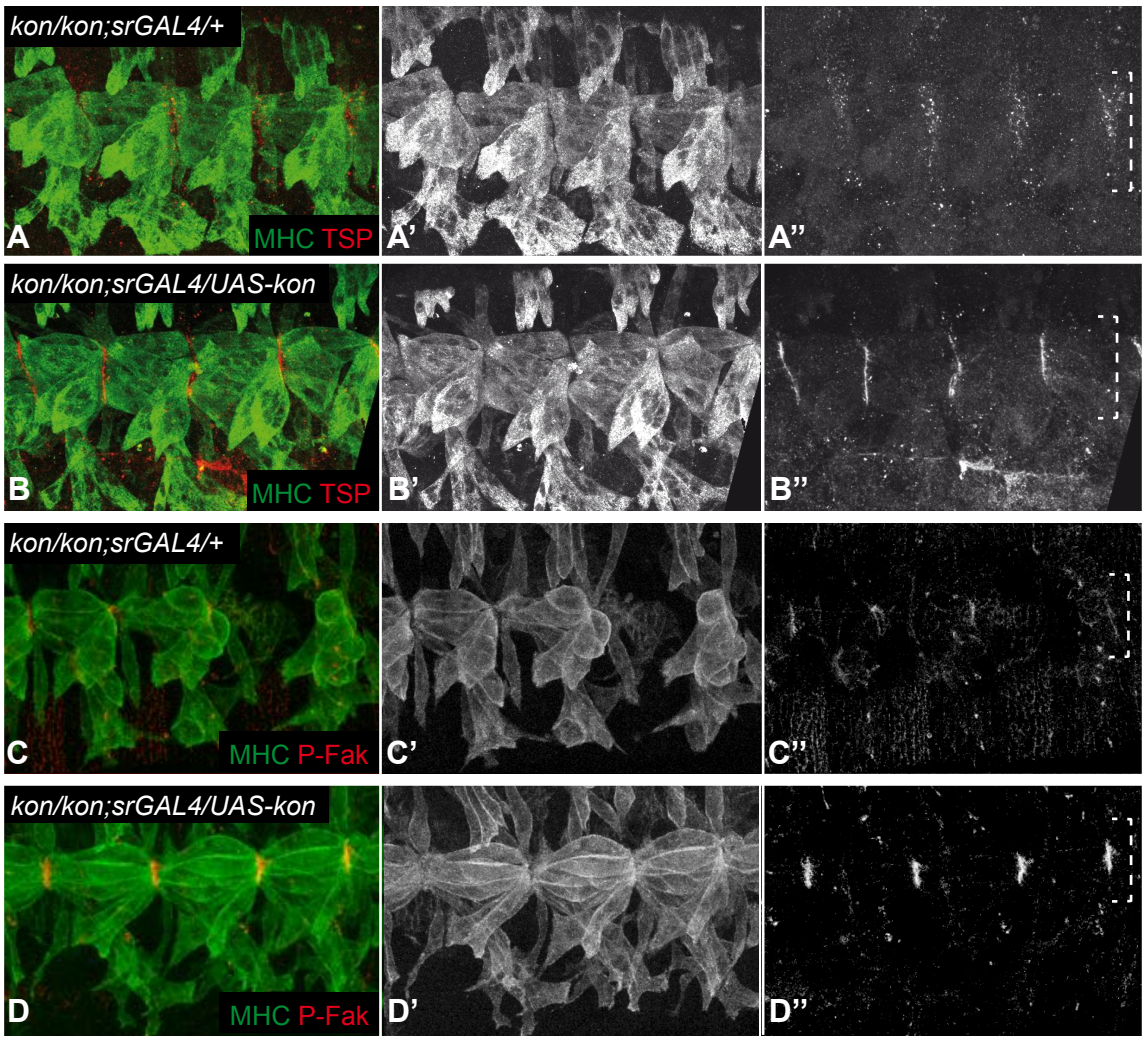
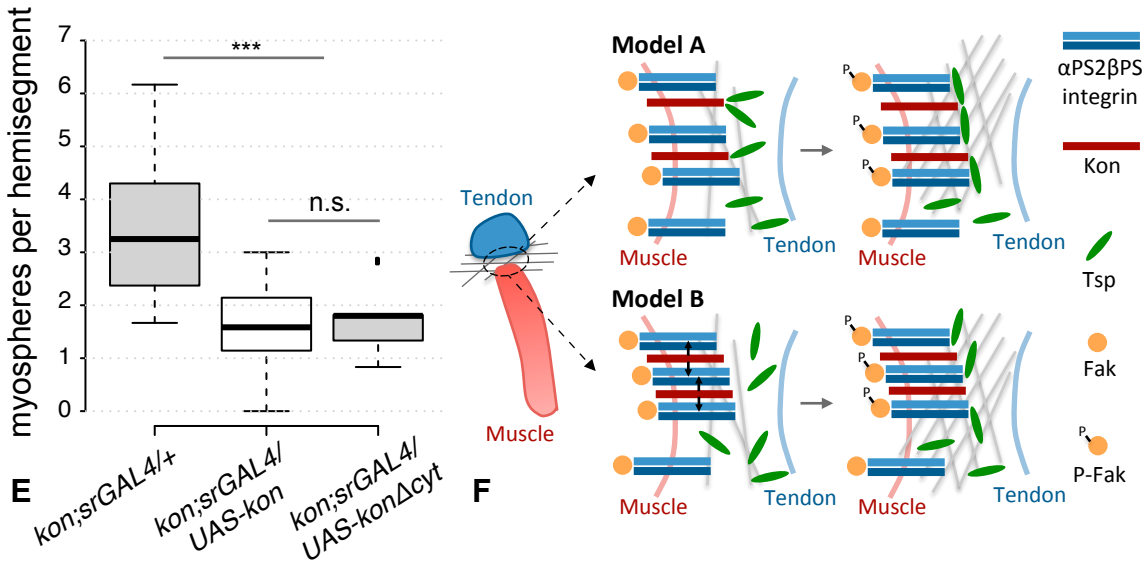

E
Relative Tsp fluorescence
2.0
1.5
1.0
0.5
0.0
***
n.s.
kon/+; twiGAL4
kon/kon; twiGAL4
kon/kon;twiGAL4 /UAS-konΔcyt
kon/kon;twiGAL4 /UAS-kon

Figure 8


kon/kon;srGAL4/+
MHC TSP
A
A'
A''
kon/kon;srGAL4/UAS-kon
MHC TSP
B
B'
B''
kon/kon;srGAL4/+
MHC P-Fak
C
C'
C''
kon/kon;srGAL4/UAS-kon
MHC P-Fak
D
D'
D''


myospheres per hemisegment
7
6
5
4
3
2
1
0
***
n.s.
kon;srGAL4/+
kon;srGAL4/ UAS-kon
kon;srGAL4/ UAS-konΔcyt
E
Tendon
Muscle
F
Model A
Muscle
Tendon
Muscle
Tendon
Model B
Muscle
Tendon
Muscle
Tendon
αPS2βPS integrin
Kon
Tsp
Fak
P-Fak