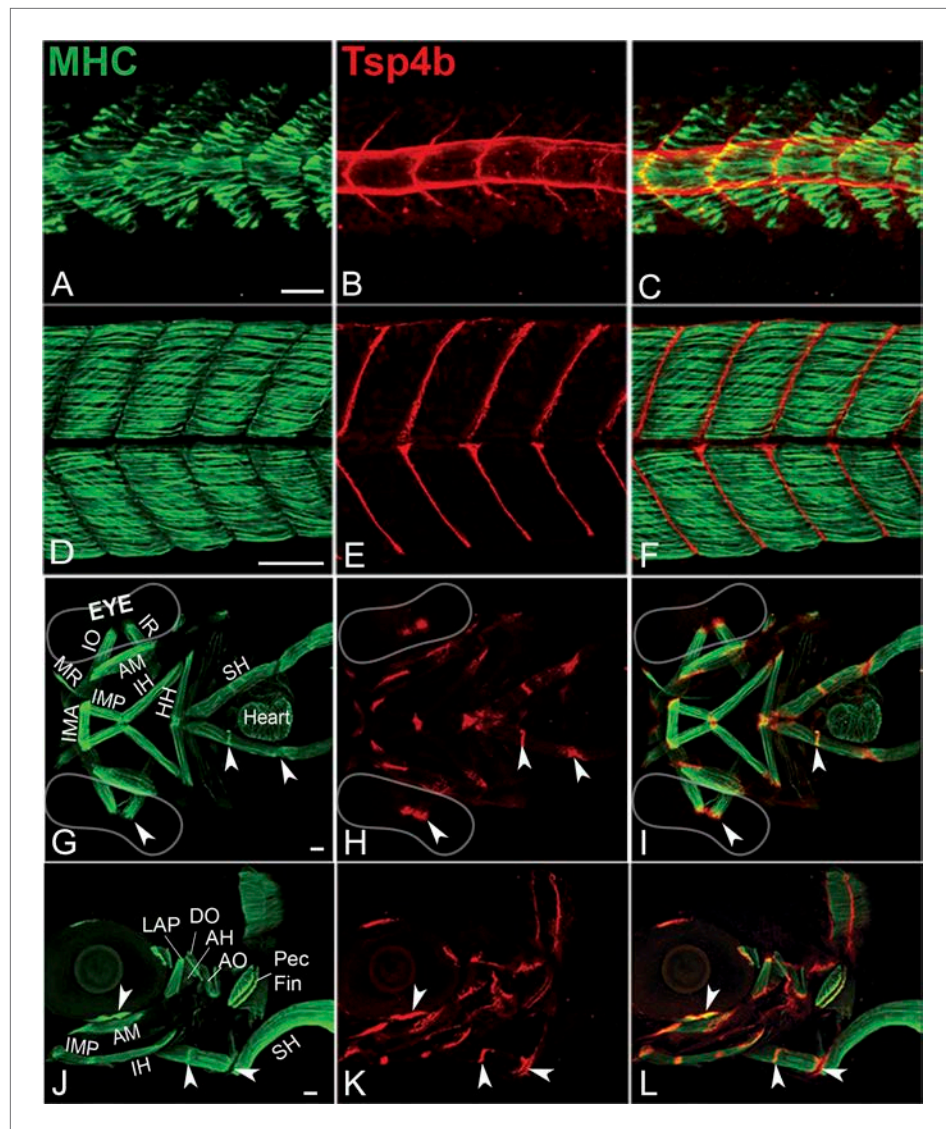


---

## Figures and figure supplements

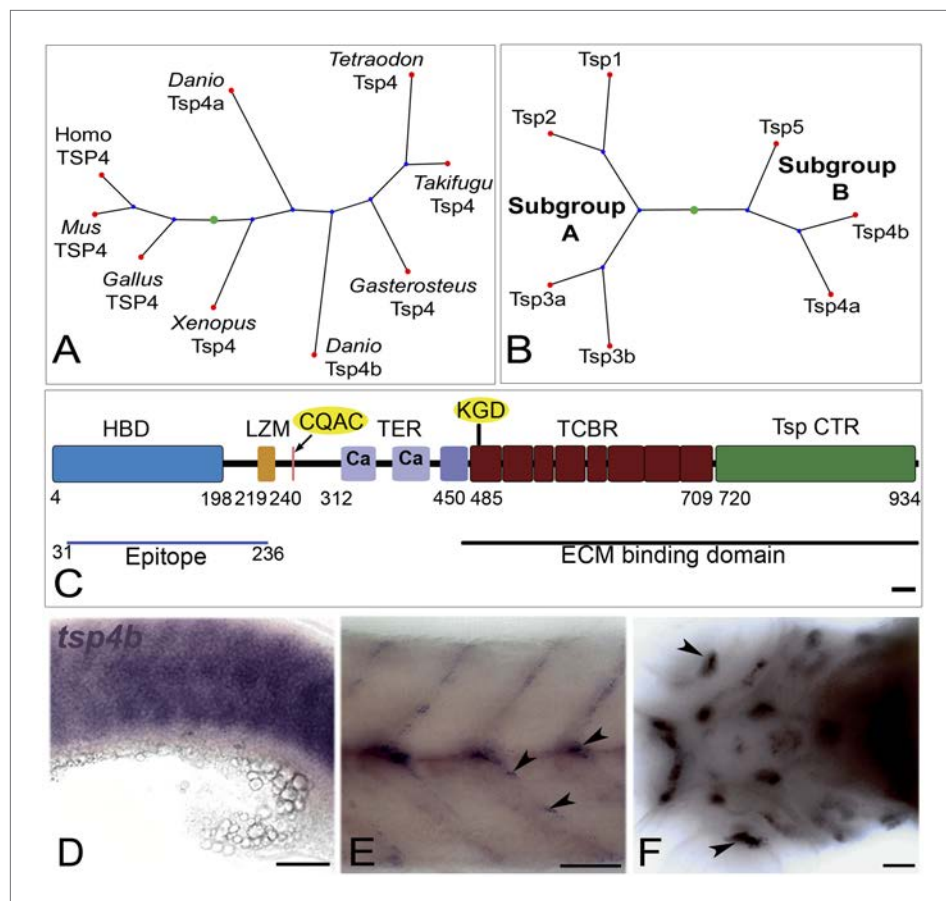
Thrombospondin-4 controls matrix assembly during development and repair of myotendinous junctions

**Arul Subramanian, Thomas F Schilling**



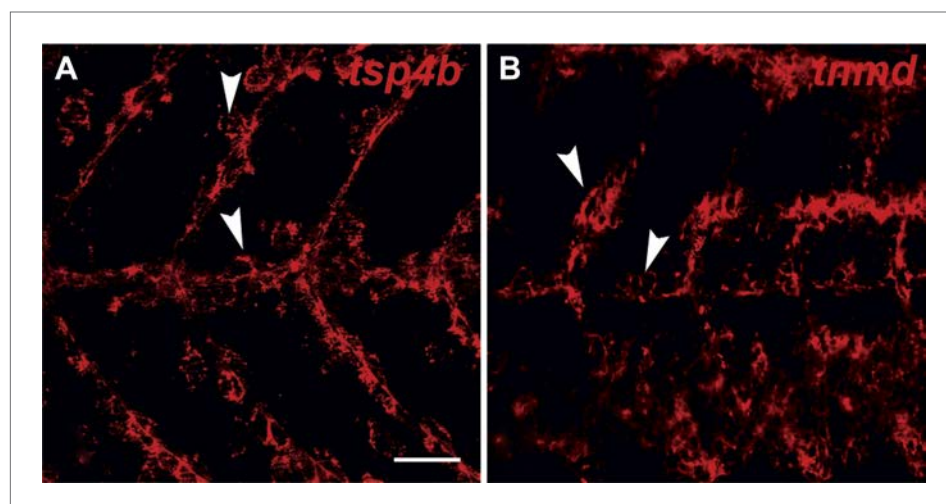
**Figure 1.** Zebrafish Tsp4b localizes to all muscle attachments. (A–L) Whole mount immunostaining of wild type embryos using anti-MHC (A, D, G, J; green) and anti-Tsp4b (B, E, H, K; red) and merged (C, F, I, L). (A–C) 20–22 hpf and (D–F) 72 hpf (lateral view) trunk showing early Tsp4b localization around notochord and medial somite boundaries (B and C) and later at somite boundaries (E and F). (G–L) Ventral (G–I) and lateral (J–L) views of 72 hpf showing Tsp4b at cranial muscle attachments. Abbreviations: AM-Adductor Mandibularis, AH-Adductor Hyoideus, AO-Adductor Operculae, DO-Dilator Operculae, HH-HyoHyal, IH-InterHyal, IMA-InterMandibularis Anterior, IMP-InterMandibularis Posterior, IO-Inferior Oblique, IR-Inferior Rectus, LAP-LevatorArcus Palatini, MR-Medial Rectus, SH-SternoHyoideus. Scale bar = 30 microns.

DOI: [10.7554/eLife.02372.003](https://doi.org/10.7554/eLife.02372.003)



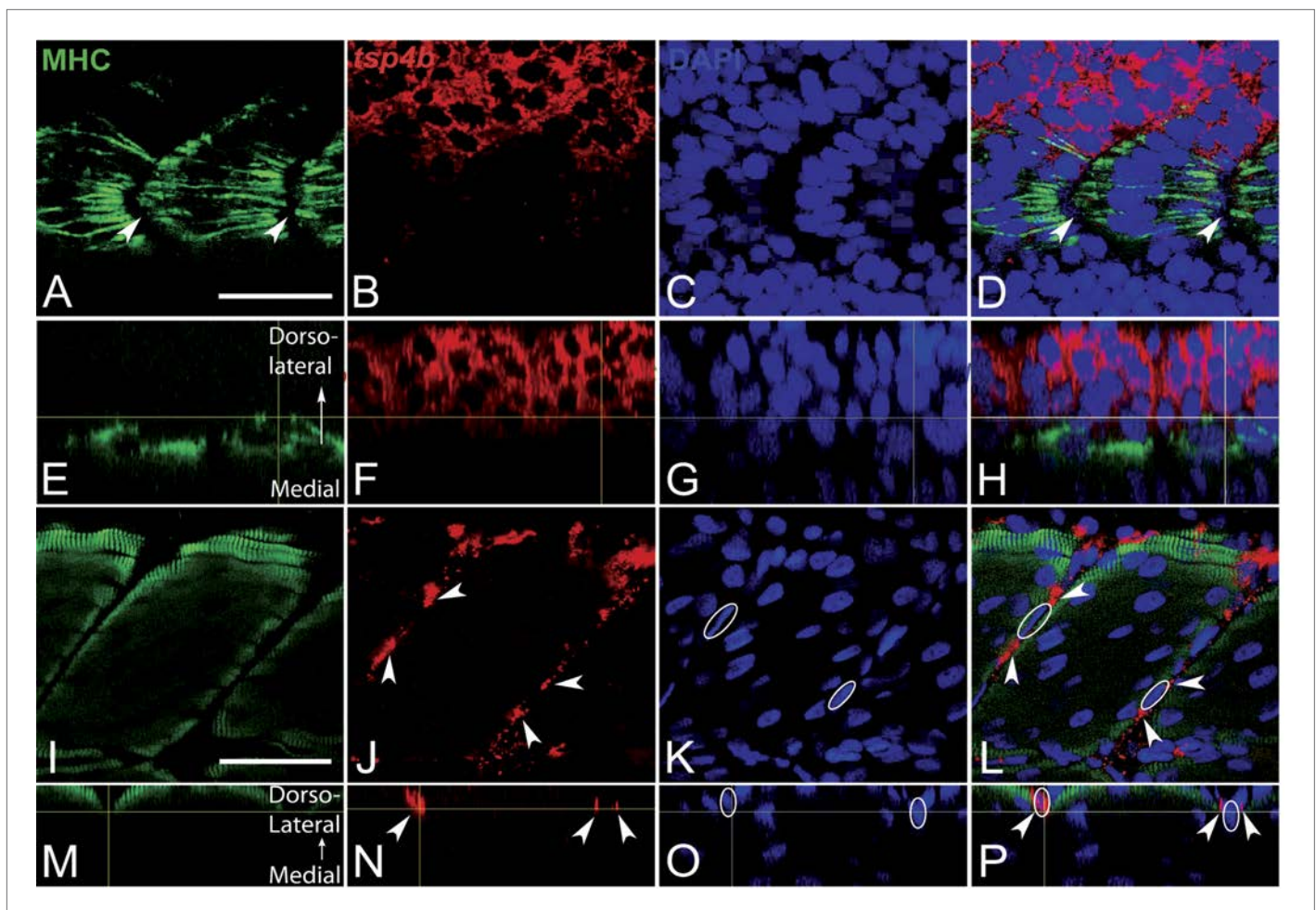
**Figure 1—figure supplement 1.** Tsp4b is expressed at muscle attachments.

DOI: [10.7554/eLife.02372.004](https://doi.org/10.7554/eLife.02372.004)



**Figure 1—figure supplement 2.** *tsp4b* and *tnmd* are expressed in tenocytes at MTJs.

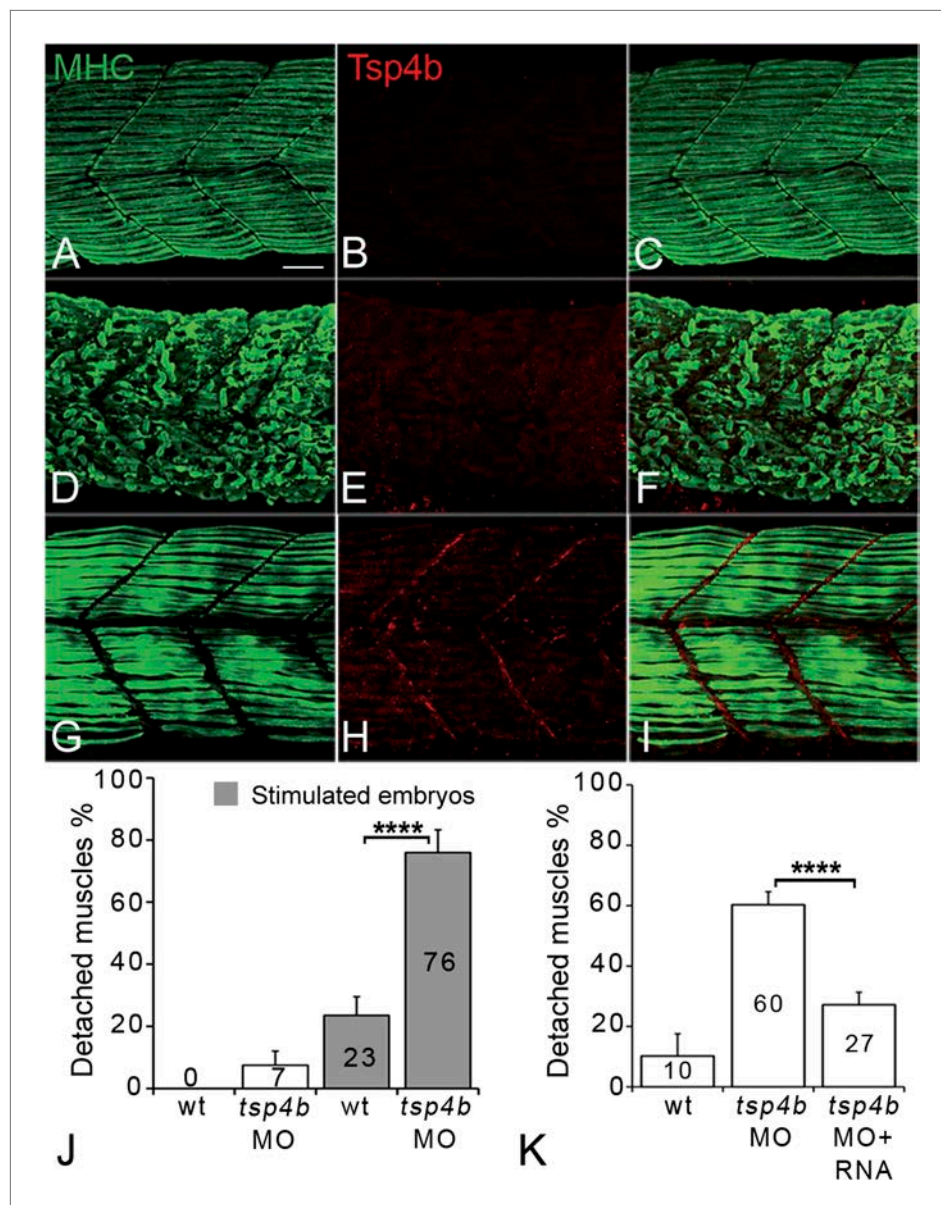
DOI: [10.7554/eLife.02372.005](https://doi.org/10.7554/eLife.02372.005)



**Figure 1—figure supplement 3.** Tsp4b expression is downregulated in myoblasts as they differentiate.

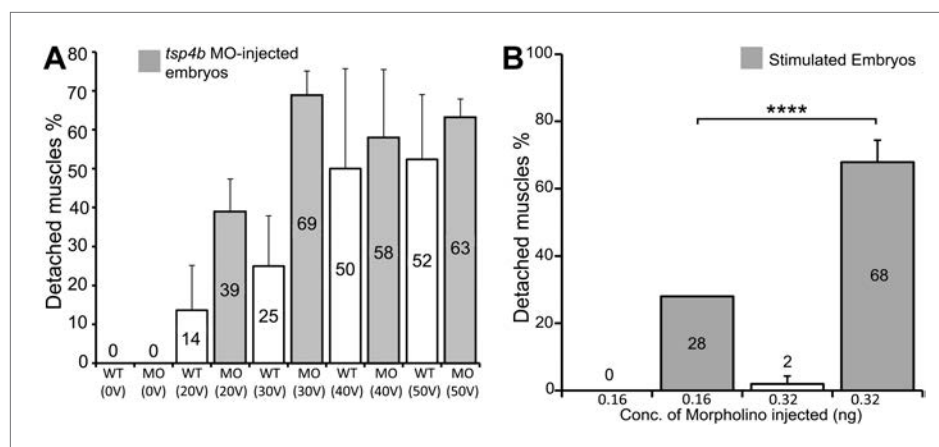
DOI: [10.7554/eLife.02372.006](https://doi.org/10.7554/eLife.02372.006)



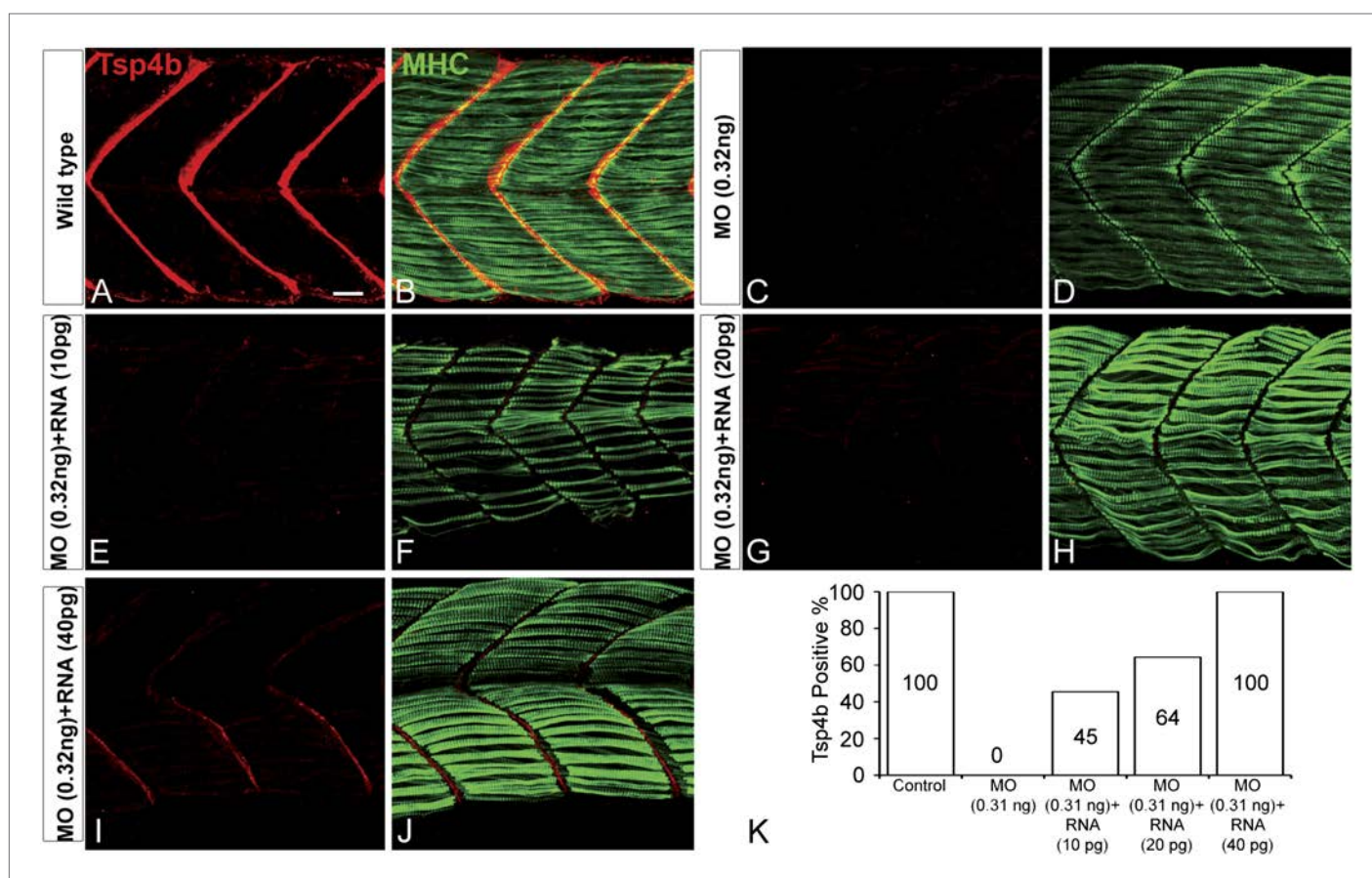


**Figure 2.** Tsp4b is required for muscle attachment. Whole mount immunostaining of 36 hpf Tsp4b-deficient embryos using anti-MHC (A, D, G; green) and anti-Tsp4b (B, E, H; red) and merged (C, F, I). (A–C) Injection of 0.32 ng *tsp4b*-MO eliminates Tsp4b protein at 72 hpf but myofibers attach. (D–F) Electrical stimulation (30 V) of these larvae causes muscle detachment. (G–I) Co-injection of *tsp4b* RNA (80 pg/embryo) rescues muscle attachment and Tsp4b localization. (J) Histogram showing muscle detachment in 76% (N = 79) of stimulated Tsp4b-deficient embryos (Chi squared test p-value<0.001). (K) Co-injection of *tsp4b* mRNA rescues muscle attachment in 67% (N = 92) of stimulated Tsp4b-deficient embryos (Chi squared test p-value<0.001). (p-value representation legend: significant \*<0.05, highly significant \*\*<0.01, extremely significant \*\*\*<0.001, extremely significant \*\*\*\*<0.0001). Scale bar = 30 microns.

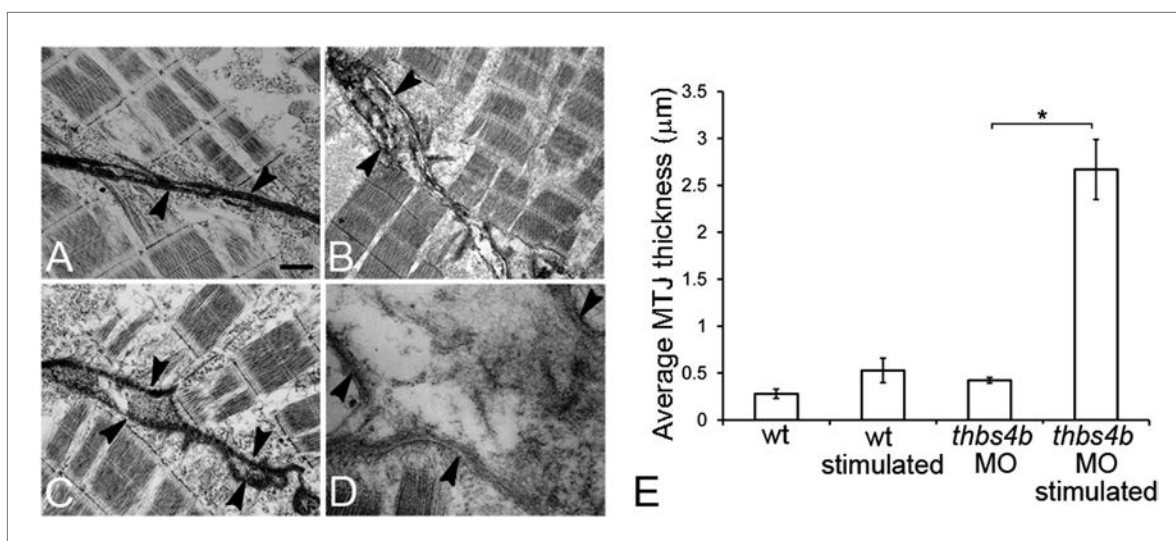
DOI: [10.7554/eLife.02372.007](https://doi.org/10.7554/eLife.02372.007)



**Figure 2—figure supplement 1.** *Tsp4b*-deficient muscles show dose-dependent detachment upon stimulation.  
DOI: 10.7554/eLife.02372.008

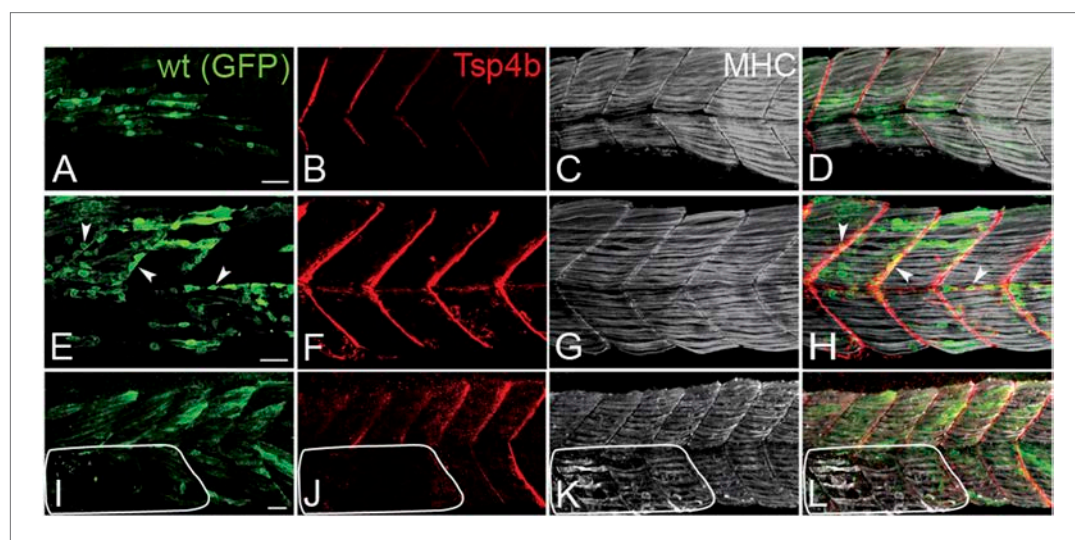


**Figure 2—figure supplement 2.** Exogenous *tsp4b* mRNA rescues Tsp4b localization in a dose-dependent manner.  
DOI: 10.7554/eLife.02372.009



**Figure 2—figure supplement 3.** Ultrastructure of MTJs in *Tsp4b* deficient embryos.

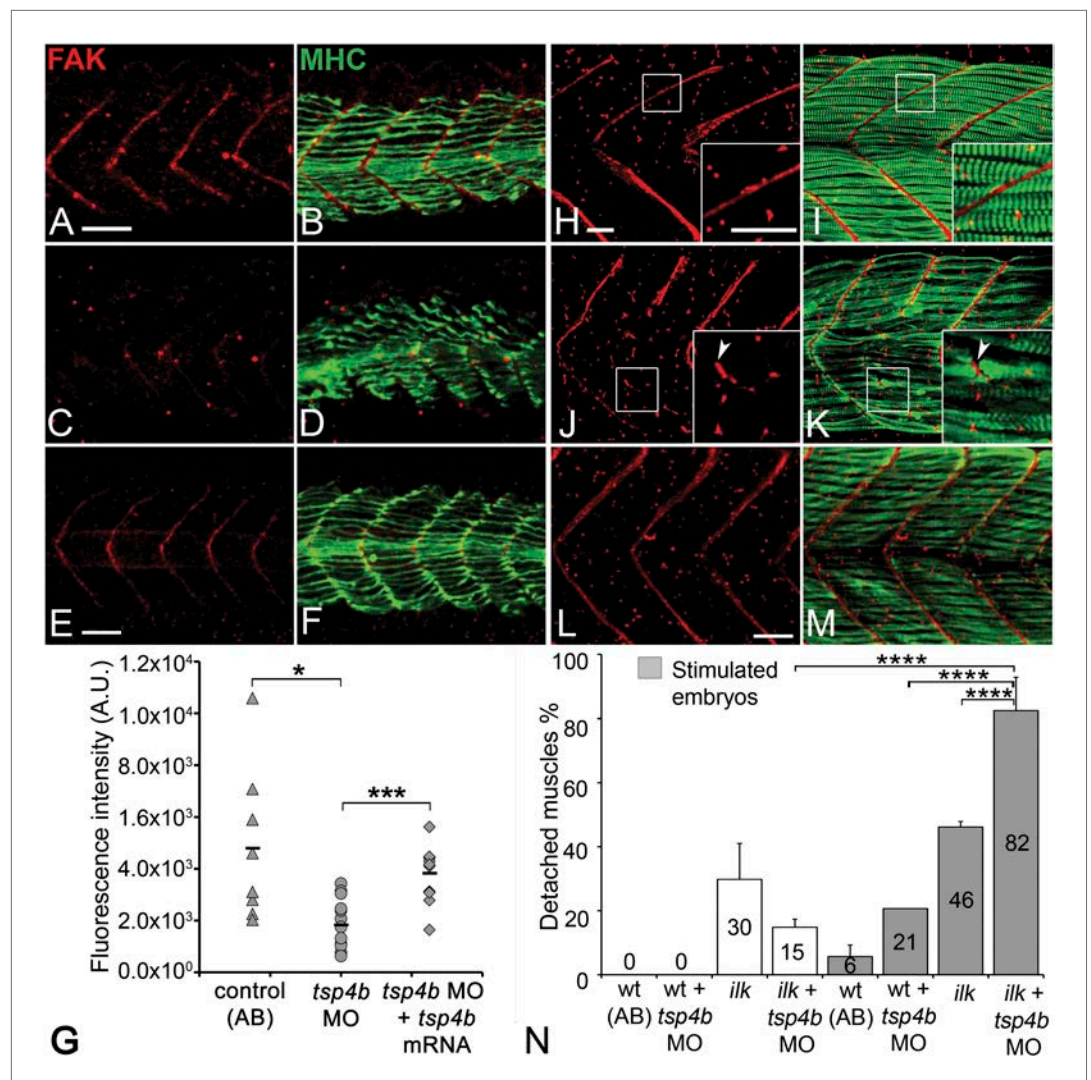
DOI: [10.7554/eLife.02372.010](https://doi.org/10.7554/eLife.02372.010)



**Figure 3.** *Tsp4b* has a non cell-autonomous function in muscle attachments. (A–L) 48 hpf (lateral views) of genetic mosaics generated by cell transplantation. GFP-labeled wild type muscle cells (green) (A and I) or putative tenocytes (arrow heads) (green) (E) were grafted into *Tsp4b*-deficient host embryos and stained with anti-Tsp4b (B, F, J; red), and anti-MHC (C, G, K; gray/white). (I–L) Transplants locally rescued muscle detachment after stimulation in regions where *Tsp4b* was restored (white line denotes region lacking *Tsp4b*). Scale bars = 30 microns.

DOI: [10.7554/eLife.02372.011](https://doi.org/10.7554/eLife.02372.011)

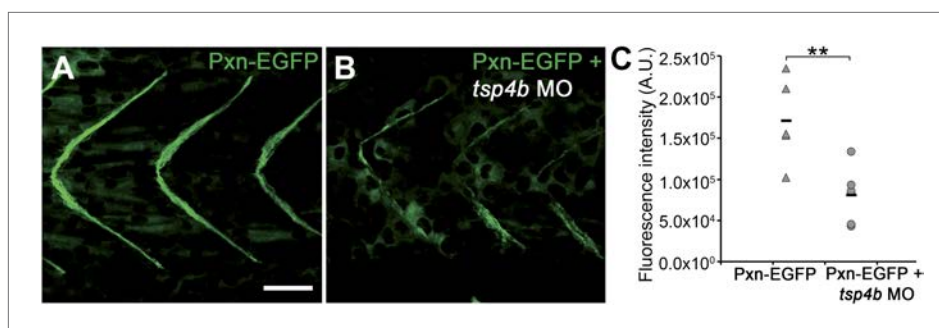




**Figure 4.** Tsp4b is required for muscle-specific integrin signaling at MTJ. (A–F) Lateral views of 20–24 hpf embryos stained with anti-phosphorylated (Tyrosine 861) FAK (pFAK; red) and anti-MHC (green). (A and B) pFAK localizes to the ends of early myofibers at wild-type somite boundaries. (C and D) Reduced pFAK levels in Tsp4b-deficient embryos. (E and F) pFAK levels are restored in Tsp4b-deficient embryos injected with full length *tsp4b* mRNA. (G) Fluorescence intensity measurements (arbitrary units [A.U.]) for pFAK staining along somite boundaries confirm significant reductions in Tsp4b-deficient embryos, and partial rescue by co-injection of full length *tsp4b* mRNA (t test: one tailed, unequal variance; p-value: wt and *tsp4b*-deficient <0.05; Tsp4b-deficient and Tsp4b-deficient + *tsp4b* RNA p<0.001). (H–M) 36 hpf (lateral views) stained with anti-pFAK (red), and anti-MHC (green). Insets show higher magnification images of white boxed areas. (H and I) pFAK localizes to muscle sarcolemma. (J and K) In Tsp4b-deficient embryos, pFAK is reduced/discontinuous at somite boundaries. pFAK associates with ectopic muscle attachments (arrowheads). (L and M) pFAK localization is restored in Tsp4b-deficient embryos injected with full length *tsp4b* mRNA. (N) Embryo percentages (N = 70 embryos) with detached muscles from an intercross between two *ilk*<sup>+/-</sup> heterozygotes, injected with sub-threshold amounts (0.16 ng) of *tsp4b*-MO and stimulated (30 V) (Chi squared test; p-value: wt+ *tsp4b*-MO (stimulated) and *ilk*+*tsp4b*-MO (stimulated) p<0.0001, *ilk* (stimulated) and *ilk*+*tsp4b*-MO (stimulated) p<0.0001, *ilk*+ *tsp4b*-MO and *ilk*+*tsp4b*-MO (stimulated) p<0.0001). Scale bar = 30 microns.

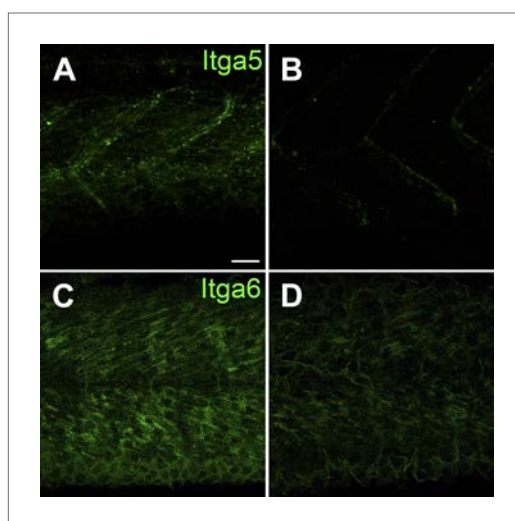
DOI: [10.7554/eLife.02372.012](https://doi.org/10.7554/eLife.02372.012)





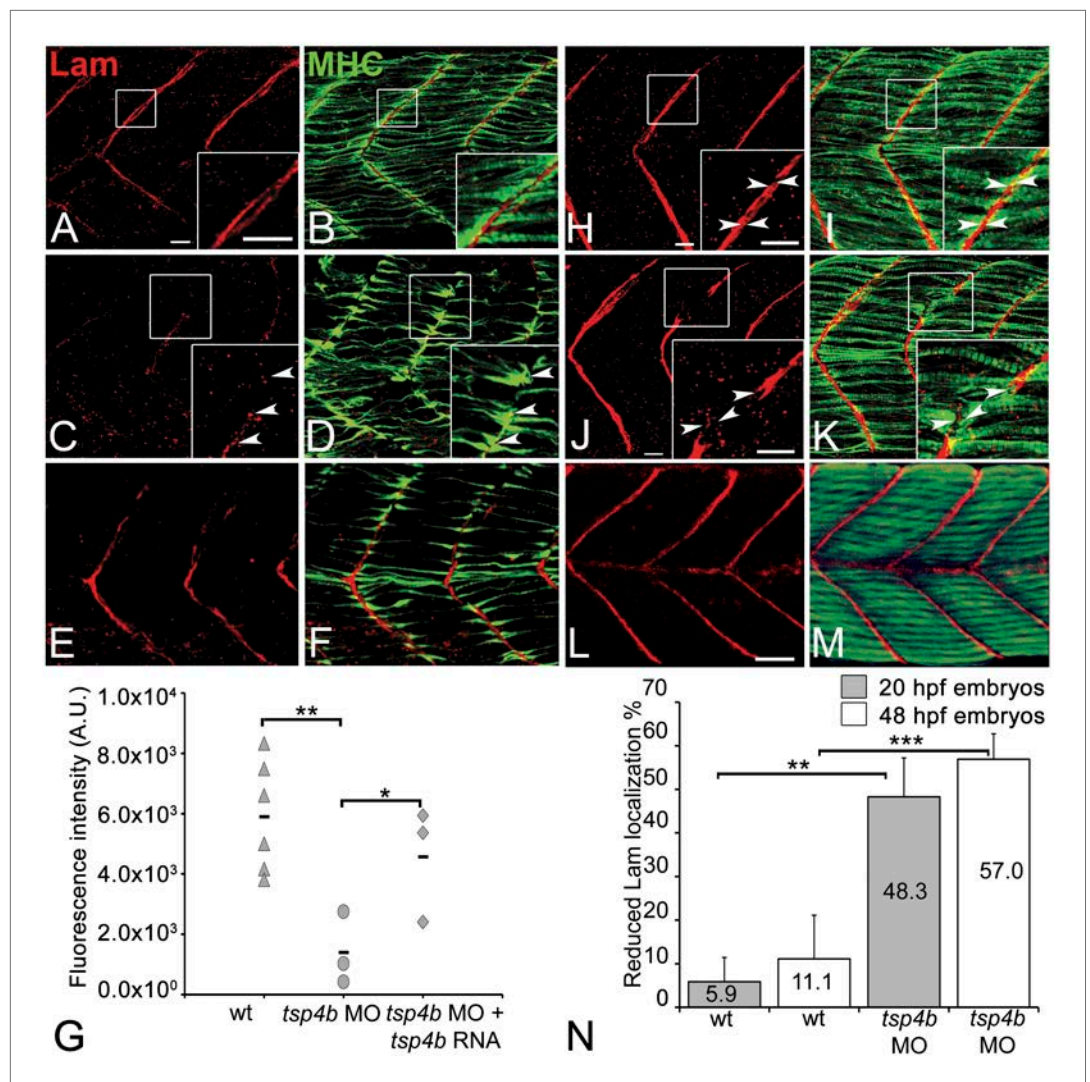
**Figure 4—figure supplement 1.** Tsp4b-deficient muscles show reduced localization of Paxillin.

DOI: [10.7554/eLife.02372.013](https://doi.org/10.7554/eLife.02372.013)



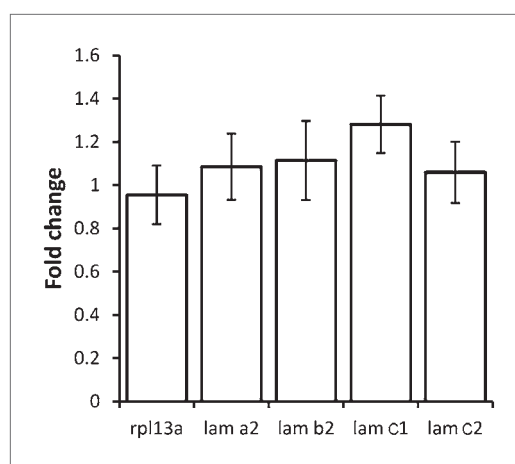
**Figure 4—figure supplement 2.** Itga5-RFP localizes to MTJs and Itga6-GFP localizes to muscle, and both require Tsp4b.

DOI: [10.7554/eLife.02372.014](https://doi.org/10.7554/eLife.02372.014)



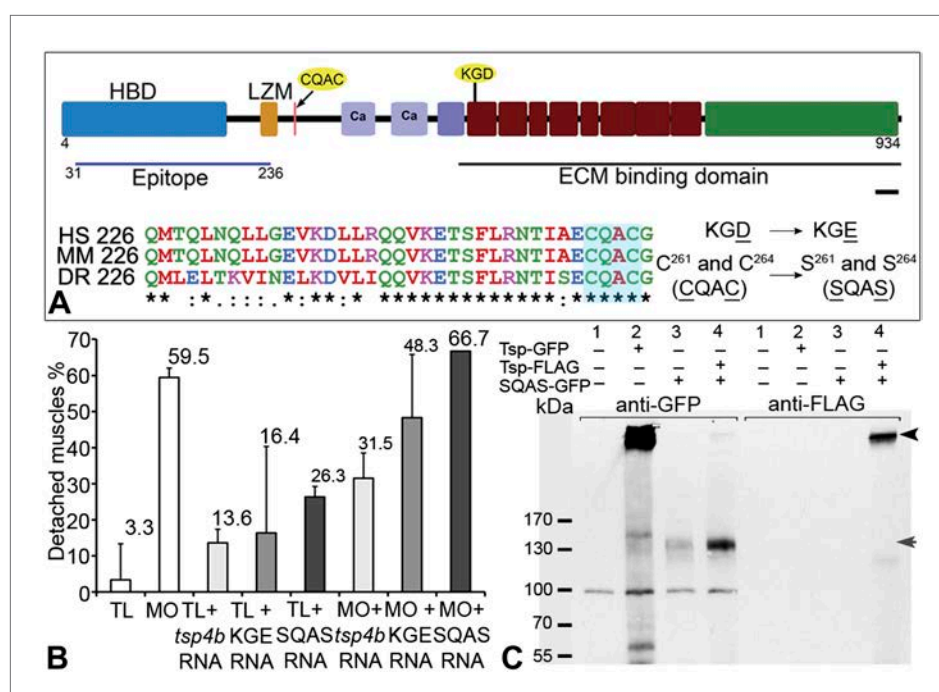
**Figure 5.** Tsp4b is required for Laminin assembly at MTJs. (A–F) Lateral views of somites in 20 hpf embryos stained with anti-MHC (green) and anti-pan-Laminin (Lam, red) antibodies. Insets show higher magnification images of the areas marked by white boxes, where Lam localizes to developing myotendinous ECM (arrowheads). (A and B) Wild-type siblings. (C and D) *tsp4b*-MO injected embryos showing local loss of Lam at 20 hpf, and ectopic muscle attachments (arrowheads) at remaining Lam foci. (E and F) Lam localization was restored in Tsp4b-deficient embryos injected with full length *tsp4b* mRNA. (G) Fluorescence intensity measurements (arbitrary units [A.U.]) at somite boundaries of anti-Lam in 20–24 hpf wild type controls versus embryos injected with *tsp4b*-MO or co-injected with *tsp4b*-MO and *tsp4b* RNA. (t test: one tailed, unequal variance; p-value: wt and Tsp4b-deficient <0.01, Tsp4b-deficient and Tsp4b-deficient and *tsp4b* RNA <0.05) Scale bar = 30 microns. (H and I) Lateral views of somites in wild type embryos at 36 hpf stained with anti-pan-Lam (red), and anti-MHC (green). Insets show higher magnification images of white boxed areas. Lam localizes to myotendinous ECM (I, arrowheads). (J and K) In Tsp4b-deficient embryos, Lam is reduced/discontinuous at somite boundaries (K, arrowheads). (L and M) Lam localization is restored in Tsp4b-deficient embryos injected with full length *tsp4b* mRNA. (N) Embryo percentages (20 hpf embryos N = 30, 72 hpf embryos N = 50) with reduced/mislocalized Lam at 20 and 48 hpf in wild type and Tsp4b-deficient embryos. (Chi squared test; p-values: 20 hpf \*\*<0.01, 48 hpf \*\*\*<0.001) Scale bar = 30 microns.

DOI: [10.7554/eLife.02372.015](https://doi.org/10.7554/eLife.02372.015)



**Figure 5—figure supplement 1.** Tsp4b depletion does not alter *lam* transcription.

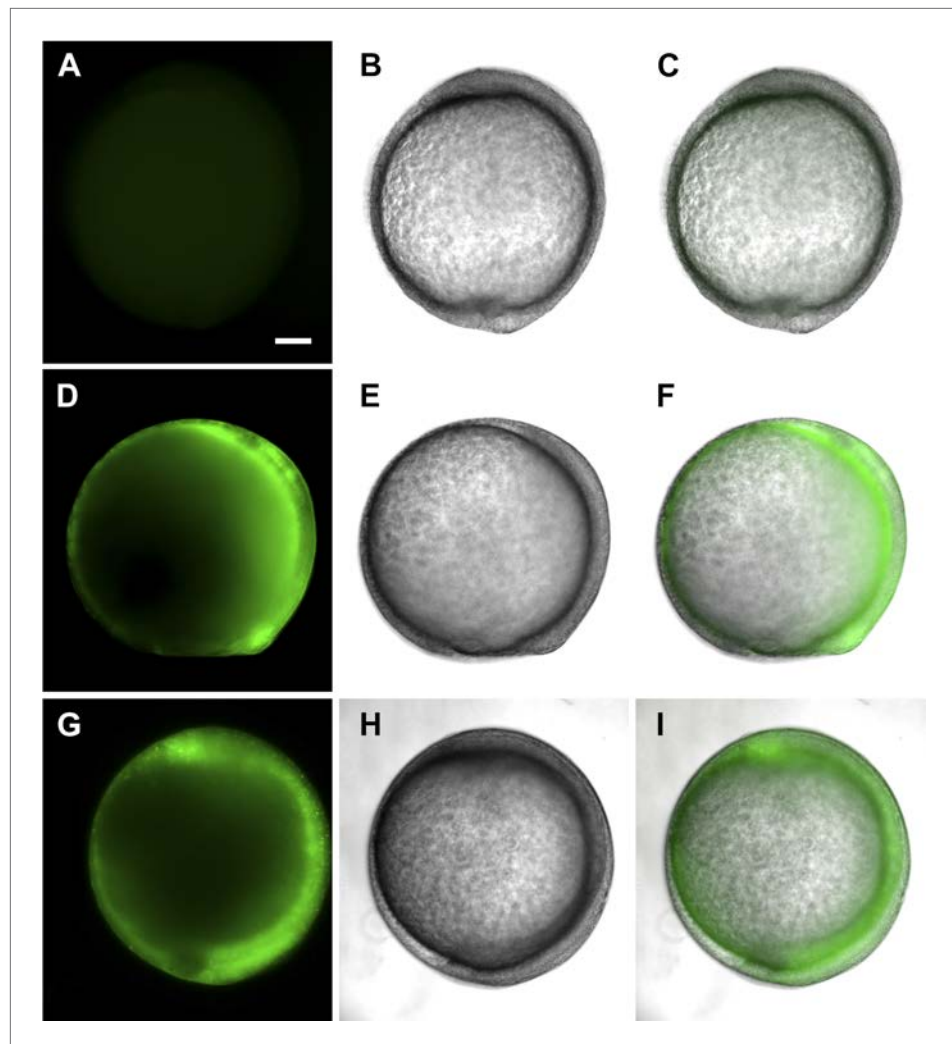
DOI: [10.7554/eLife.02372.016](https://doi.org/10.7554/eLife.02372.016)



**Figure 6.** Integrin binding and pentamerization of Tsp4b are essential for its function. **(A)** Schematic representation of Tsp4b domains showing the location of the conserved coiled-coil region with its CQAC motif (70% identical across species). Amino acid substitutions used to study the functions of KGD and CQAC motifs are underlined. **(B)** Muscle detachment frequencies in uninjected wild-type controls, wildtypes injected with *tsp4b* morpholino (MO), *tsp4b* RNA, KGE *tsp4b* mutant RNA, SQAS *tsp4b* mutant RNA, or co-injected with *tsp4b* MO and *tsp4b* RNA, KGE *tsp4b* mutant RNA, or SQAS *tsp4b* mutant RNA, after stimulation (N = 60 embryos each). **(C)** Western blot performed on whole embryo protein extract using anti-GFP and anti-FLAG antibodies under non-reducing conditions. Lanes: 1—wt (AB), 2—Tsp4b-deficient embryos injected with *tsp4b*-GFP mRNA, 3—Tsp4b-deficient embryos injected with SQAS-GFP mRNA, 4—Tsp4b-deficient embryos co-injected with *tsp4b*-FLAG and SQAS-GFP mRNAs. Pentameric Tsp4b-GFP (~663 kDa) and pentameric Tsp4b-FLAG (~535 kDa) bands (black arrow head). Monomeric SQAS-GFP (~132 kDa) (grey arrow head). The band corresponding to a 100 kDa size marker in all lanes of the blot reacted with anti-GFP is a background signal.

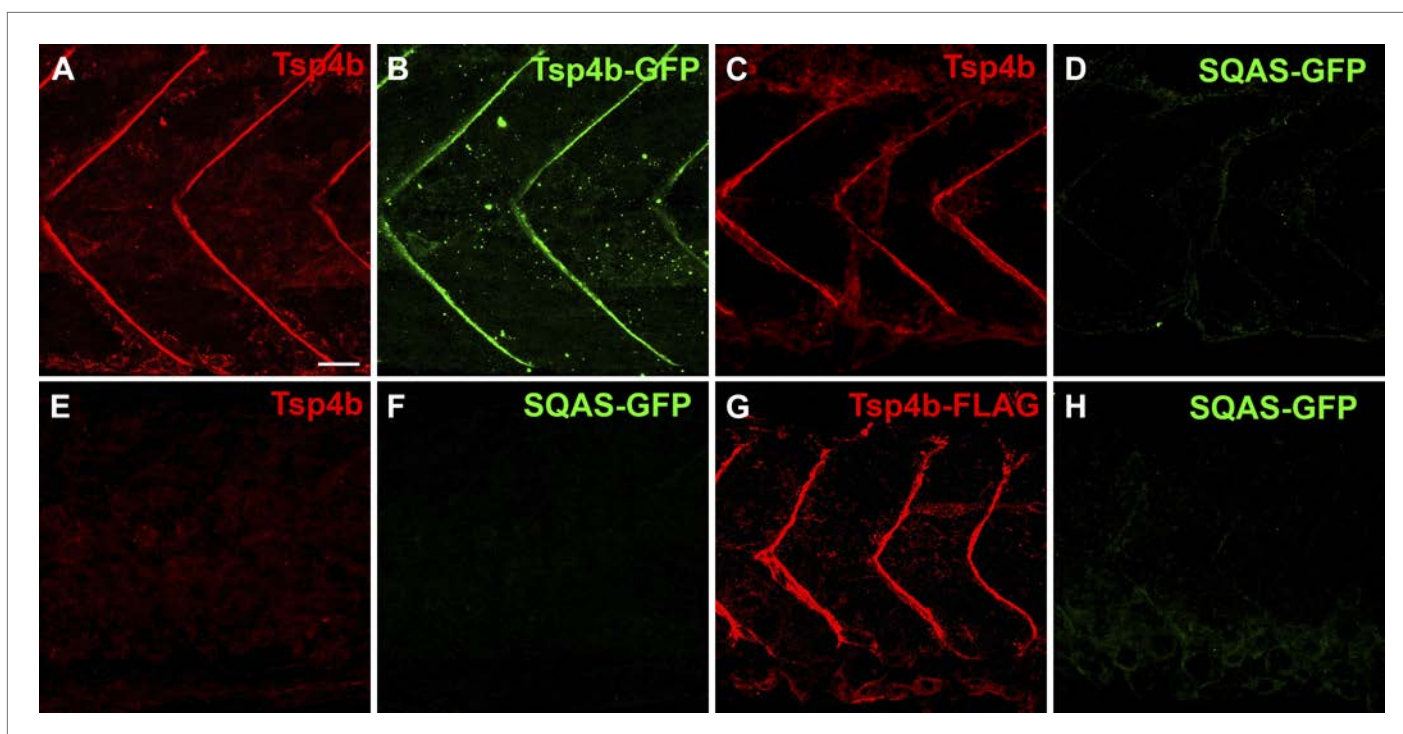
DOI: [10.7554/eLife.02372.017](https://doi.org/10.7554/eLife.02372.017)





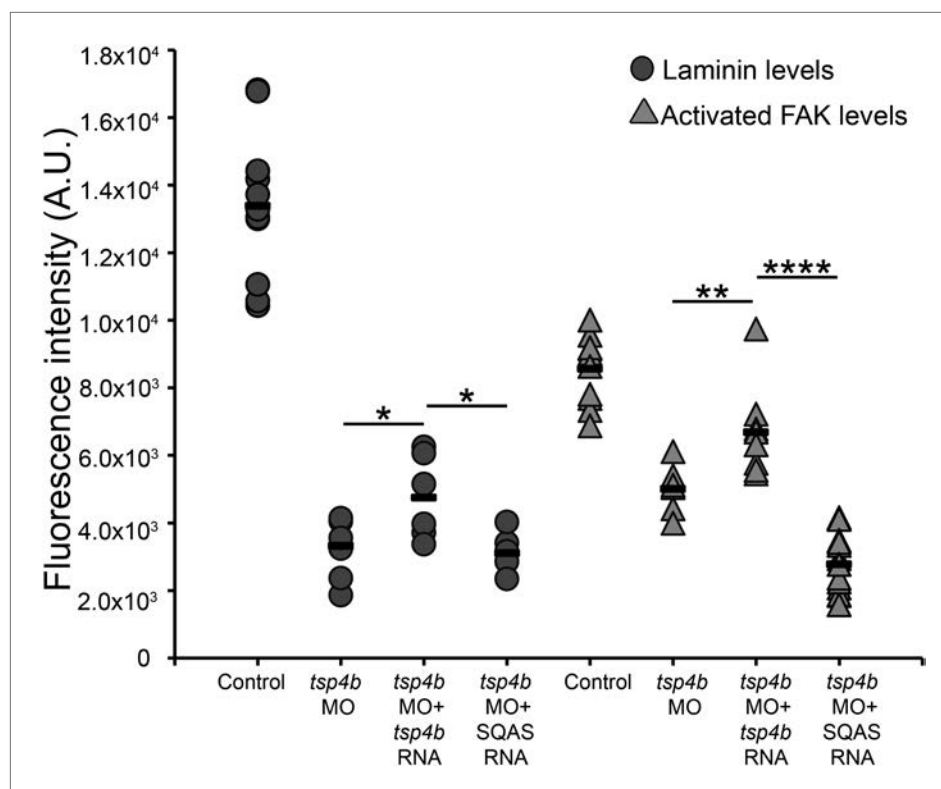
**Figure 6—figure supplement 1.** *tsp4b-SQAS-gfp* mRNA is expressed similar to wild type *tsp4b-gfp* mRNA.

DOI: [10.7554/eLife.02372.018](https://doi.org/10.7554/eLife.02372.018)



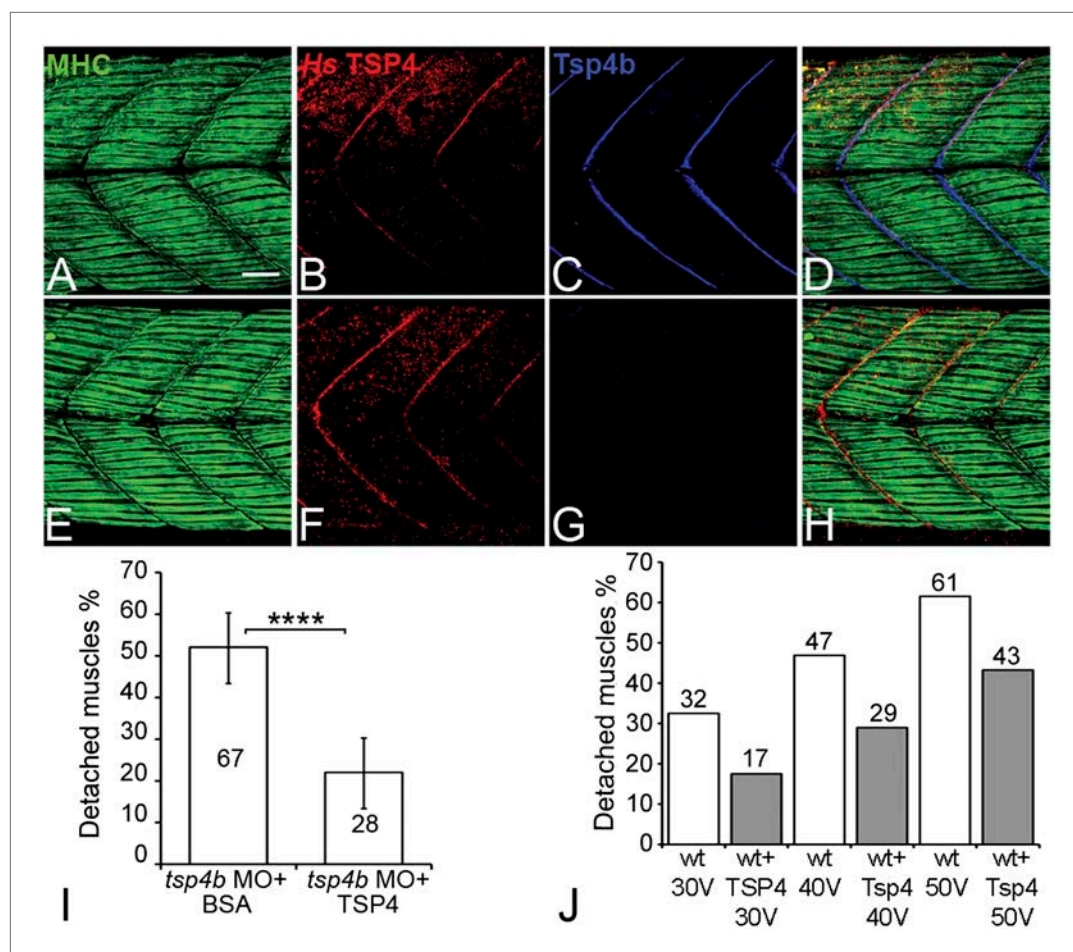
**Figure 6—figure supplement 2.** The CQAC motif is essential for Tsp4b localization and function.

DOI: [10.7554/eLife.02372.019](https://doi.org/10.7554/eLife.02372.019)

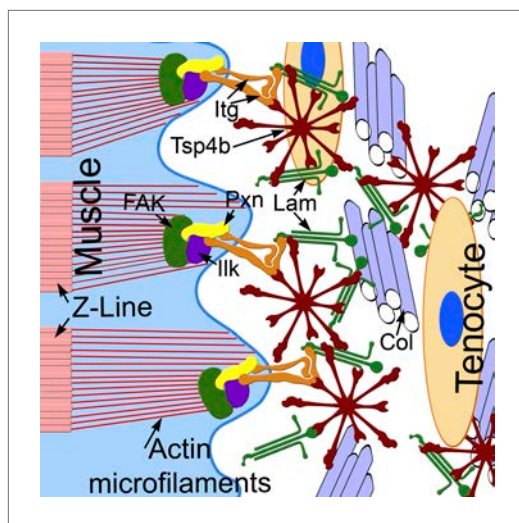


**Figure 6—figure supplement 3.** Laminin and FAK localization are dependent on pentameric Tsp4b.  
DOI: [10.7554/eLife.02372.020](https://doi.org/10.7554/eLife.02372.020)





**Figure 7.** Conserved functions for TSP4 in maintenance of MTJs. **(A–H)** Lateral views of trunk muscles in 72 hpf embryos stained with anti-MHC (green), anti-human TSP4 (red) and anti-Tsp4b (blue) antibodies. **(A–D)** Injected recombinant human TSP4 co-localizes with zebrafish Tsp4b at muscle attachments in wild type embryos. **(E–H)** Injected TSP4 localizes to somite boundaries in Tsp4b-deficient embryos. **(I)** Injected TSP4 rescues muscle attachments in Tsp4b-deficient embryos upon stimulation (N = 96 embryos) (Chi squared test, p value<0.001). **(J)** Histogram showing percentage of embryos with detached muscles in 60 hpf wt+BSA (white columns) and wt+TSP4 (shaded columns) embryos, stimulated at 30 V, 40 V and 50 V, respectively. N = 40 embryos for each sample. (Scale bars = 30 microns). DOI: [10.7554/eLife.02372.021](https://doi.org/10.7554/eLife.02372.021)



**Figure 8.** Tsp4b establishes and maintains MTJ ECM organization. A model for ECM assembly at an MTJ. Pentameric assemblies of Tsp4b (red) associate with Lam (green), Col fibrils and other ECM components. Tsp4b and Lam bind Itgs (orange) on the muscle cell surface, activating FAK (green) and recruiting Pxn (yellow) and Ilk (purple) to promote muscle specific Itg signaling and stabilize myofiber attachment.

DOI: [10.7554/eLife.02372.022](https://doi.org/10.7554/eLife.02372.022)