# Supplemental Materials

## Supplemental Figure Legends

**Supplemental Figure 1. *Unplugged* full-length (FL) and Splice Variant 1 (SV1) are differentially expressed.**

(A-B) Schematic representation of AChR clustering. (A) Prior to the arrival of growth cones, migratory adaxial cells (light green) flank non-migratory adaxial or muscle pioneer cells (dark green, MP) and form a monolayer along the medial somite. Prepatterned AChR clusters (red) accumulate on the medial surface of all adaxial cells. As the first growth cones (blue) approaches, migratory adaxial fibers (light green) initiate their radial migration to the lateral surface of the somite, while fast muscle fibers (peachy) invade the space on the medial somite surface. NC, notochord. (B) Growth cones contact fast fibers and form neural *en passant* synapses. At the horizontal midline, growth cones contact muscle pioneers and incorporates prepatterned clusters into NMJs. (C-F) Confocal images of cross-sections from 20-somite stage wildtype embryos double stained for *unplugged* mRNA and F59 antibody, specific for adaxial cells. Just before the first motor axon exit from the spinal cord, *unplugged* SV1 is highly expressed in all pre-migratory adaxial cells (C-D), while *unplugged* FL is expressed at a much lower level (E-F) compared to *unplugged* SV1. Scale bar: 50 μm.

**Supplemental Figure 2. *wnt5/11* mutant analysis, *wnt11r* expression and stainings of *wnt11r* morphants.**

(A) In 27 hpf wildtype embryos, neural AChR clusters (red, α-BTX) are apposed to primary motor axons (green, znp-1). In *silberblick* (*slb*/*wnt11*, B) and *pipetail* (*ppt*/*wnt5a*, C) mutants, axon pathfinding and neuromuscular synapses are unaffected. (D-G) Cross-section images from 20-somite stage wildtype embryos double stained for *wnt11r* mRNA (green) and adaxial cells (red, F59). In caudal segments (D and E), *wnt11r* is expressed in the spinal cord and in the dorso-lateral somites, just adjacent to pre-migratory adaxial cells. In the rostral segments (F and G), after the onset of adaxial cell migration, *wnt11r* expression is increased in the spinal cord. Dashed lines indicate the spinal cord. (H and I)

Confocal images of embryos at 26-somite stage stained for CSPs (red) and motor axons (green). CSPs accumulate around the choice point (brackets) in wildtype embryos (H), but are reduced in *wnt11r* morphants (I). (J-K) Lateral views of adaxial fibers in wildtype and *wnt11r* MO-injected embryos. Scale bars: 50 μm.

**Supplemental Figure 3: Genetic interactions between *unplugged* and *wnt11r*.**
(A) Quantification of motor axon defects at 27 hpf using suboptimal *wnt11r* TL-MO dose (2 ng). *unplugged*/+ embryos are indistinguishable from wildtype embryos. The same amount of sub-optimal dose of *wnt11r* TL-MO injected into wildtype embryos resulted in 13% *unplugged*-like axonal defects, while injection into *unplugged*/+ embryos increased the phenotype to 23%. Twenty hemisegments were scored in each embryo; n=hemisegments. Results are compiled from multiple experiments as the average±S.E.M. (t-test, *$p<0.01$). (B-C) Analysis of prepatterning phenotype using optimal wnt11r TL MO concentrations (3-4 ng) . (B1-B3) Each hemisegment of embryos was scored as normal prepatterning, mild reduction or strong reduction. (C) Quantification data show prepatterning phenotype was increased in *unplugged*/+ embryos. Results were obtained from three different experiments; n= hemisegments. 3 or 4 hemisegments were scored in each embryo.

**Supplemental Figure 4: Coimmunoprecipitation of UnpFL with Wnt11r in 293T cells and analysis of the Tg(*smyhc1:UnpFLmyc)* embryos.** (A) 293T cells were cotransfected with Wnt11r-FLAG and UnpSV1-myc or UnpFL-myc. Whole cell lysates (WCL) were subjected to anti-FLAG immunoblotting (IB) to determine the expression of Wnt11r-FLAG (lower panel). The Wnt11r-FLAG binding was assessed by IB of the anti-myc immunoprecipitate (upper panel). The amount of UnpFL or UnpSV1proteins were examined by anti-myc western blotting. Wnt11r-FLAG protein coimmunoprecipitated significantly better with UnpSV1 compared to UnpFL. (B) Cross-sectional view of a 17 hpf Tg embryo (*smhyc1*:*unpFLmyc*) stained with anti-myc. (C) Expression of UnpFL in adaxial cells failed to restore prepatterned AChRs (red) in *unplugged* embryos.

**Supplemental Figure 5. Unplugged interacts with zebrafish Dishevelled.**

(A) The intracellular domain of *unplugged* /MuSK (Unpic) was cloned into a bait plasmid pGBKT7. The PDZ and DEP domains of zebrafish *dishevelled 2* and *dishevelled 3* were cloned into a prey plasmid pGADT7. Two-hybrid interactions were preformed using SD/-Ade/-His/-Leu/-Trp media to screen for reporter genes: *ADE2* and *HIS3* (+++: fast growth. ++: intermediate growth. -: no growth). (B, C): The morphology and differentiation of adaxial cells expressing Myc-Xdsh-DEP+ appears normal. 28 hpf embryo injected with *symhc1*:*myc-Xdsh-DEP+* and stained with mAb anti-Myc (red) and F59 (green, adaxial cells).

**Supplemental Figure 6. Formation of neural synapses can occur independent of AChR prepatterning.**

(A-D) Images of untreated Tg(*hsp70l:UnpSV1-myc;unplugged*) embryos (A, B), and Tg(*hsp70l:UnpFL-myc*; *unplugged*) embryos at the 20-somite stage (A, C) or at 27 hpf (B, D). AChR prepattern and neural synapses (red, α-BTX) are absent, and axons display *unplugged*-like phenotypes (green, znp-1). (E-G') High-magnification views of neural synapses in wildtype embryos (E) and 'rescued' transgenic embryos. Embryos heat-shock treated between the 26-somite stage and 27 hpf, displayed *unplugged*-like axons with neural AChRs apposed the length of the axon (G, G'). (H) Cell extracts from individual transgenic embryos, with and without heat shock treatment (38ºC for 35 min), were subjected to anti-myc immunoblotting. Scale bars: in C and D: 50 µm; in E: 20 µm.

## Supplemental Methods

### Purification and binding of Wnt11r-FLAG

HEK 293T cells (100mm dish) were transfected with 8µg of pcDNA-wnt11r-FLAG using the Effectene Transfection Reagent (Qiagen), and grown according to the manufacturer's instructions for 5 days. The supernatant was concentrated to 1ml using an Amicon ultra-centrifugal filter unit (Sigma-Aldrich), added to 20µl of anti-FLAG M2 agarose (Sigma-Aldrich) pre-washed with TBS and glycine HCL according to the

manufacturer's instructions. After overnight incubation at 4°C, the agarose was washed with TBS and Wnt11r-FLAG protein was eluted using 100µl glycine HCL pH 3.5 plus 0.5% Sodium Deoxycholate. Beads were centrifuged and the supernatant was collected and combined with 5µl of 0.5M Tris-HCl pH 7.4, 1.5M NaCl and stored at -20°C. 15 somite stage embryos were injected in the yolk sac with 7µl of purified Flag-tagged Wnt11r protein. At the 20 somite stage were fixed in 4% PFA overnight, washed 2 x 10 minutes in PBST, briefly rinsed in water and then transferred into MeOH overnight at -20°C. Embryos were transferred into PBST for 5 minutes, fixed for 20 minutes at room temperature with 4% PFA, rinsed with PBST and blocked for 4 hours at room temperature in 2% Blocking Reagent (Roche) in MABT. After overnight incubation with anti-FLAG M2 alkaline phosphatase conjugate antibody (1:1000, Sigma-Aldrich), embryos were washed in MABT, incubated with staining buffer containing BCIP and NBT.

**Plasmid construction**

SV1 and FL constructs: *unpSV1* or *unpFL* full-length cDNA was cloned into expression vector pCS2+. To make C-terminally tagged *unplugged* constructs, a 5X myc tag was cloned into the NheI site of pcS2-*SV1* or pcS2-*FL* to make pcS2-*SV1-myc* or pcS2-*FL-myc*. The endogenous signal peptide for each gene was retained. Two BsrGI sites were used to delete residues 32-173 (corresponding to the CRD) to make pcS2-*SV1*Δ*CRD-myc*. A ClaI/ApaI digestion was used to move *SV1-myc* from pcS2-*SV1-myc* to the downstream of the *hsp70l* promoter (Halloran et al., 2000) to generate pBS-*hsp70l*:SV1-myc. A BstBI/SphI digestion of pCS2-*FL-myc* was used to replace the fragment digested by BstBI/SphI in pBS-*hsp70l:*SV1-myc to generate pSK-*hsp70l*:FL-myc.

Smyhc1:SV1-myc
Primer pairs: 5'AAGGTCTAGACCTCTCGAACCATGATCAGGCCTGC3' (forward) and 5'CTCACTATAGTTCTAGAGAATTCCTCGAGG3' (reverse) were used to move SV1-myc into the XbaI site of the ISceI-*smyhc1* vector to generate *smyhc1*:SV1-myc.

Smyhc1:FL-myc

Primer pairs 5'AAAATCTAGAGTTCTGATACGAGGCTGACC3' (forward) and 5'CTCACTATAGTTCTAGAGAATTCCTCGAGG3' (reverse) were used to move FL-myc into the XbaI site of the ISceI-*smyhc1* vector to generate *smyhc1*:FL-myc.

pGEX-*UnpSV1ECD*

Primer pairs 5'AAAAGAATTCCCATGATCAGGCCTGCAGACTCTC3' (forward) and 5'AAAAGAATTCATTGAGTAGGCCGTAGACACAG3' were used to clone the extracellular domain of SV1 (residues 1-299) to the EcoRI site of pGEX-2TK (GE healthcare).

*Unplugged* SV1 specific in situ probe

Primer pairs 5'TGTATTATTGGTGTATCTGAACTTTTG3'(forward) and 5'GTGCTGCAGTAGCCGGCAT3'(reverse) were used to RT-PCR SV1 fragment (nt 1-340) from cDNA template made from 20-somite embryos and subcloned into pCR-BluntII-TOPO vector to make TOPO-*SV1US* (US: unique sequence).

*Unplugged* FL specific probe in situ probe Primer pairs 5'GCCCGTTACTATTGAAGTACAAG3'(forward) and 5'TGTGC TGCAGTAGCCGGCATGG3'(reverse) were used to move FL fragment (nt 664-1012) to pCR-BluntII-TOPO to make TOPO-*FLUS*.

<u>Wnt11r constructs</u>: Full-length *wnt11r* cDNA was cloned into the expression vector pSK+. The PCR primers 5'TGTGGAGAAATACGTCTGCAAAGGATCCTGAGCTACTGG AC3' (forward) and 5'GTCCAGTAGCTCAGGATCCTTTGCAGACGTATTTCTCC ACA3' (reverse) were used to remove the *wnt11r* stop codon and 3'UTR in pBS-*wnt11r* to generate pBS-*wnt11rNS* (*NS*: non stop). 5'TTTTGCAGGATCCCAT CGATTTAAAGCTATG3' (forward) and 5'TTTTGGATCCCTATAGTTCTAGAGGC

TCGAG3' (reverse) were used to clone a FLAG tag into BamHI site in pBS-*wnt11rNS* to form pBS-*wnt11r-FLAG*. A BstXI/ApaI fragment was then cloned into pCDNA3 to give rise to C-terminally tagged *wnt11r* construct: pcDNA-*wnt11r-FLAG*.

Dsh constructs: Primer pair:
5'AAGGTCTAGAGGATCCCATCGATTTAAAACCATG3' (forward) and
5'ACGACTCACTATAGTTCTAGAGGCTCGACGG3' (reverse) were used to add XbaI site to myc-*Xdsh+DEP* from pCS2-myc-*Xdsh-DEP+* (a gift from Dr. Peter Klein) and this PCR product was cloned into pCR-BluntII-TOPO (invitrogen). *Myc-Xdsh-DEP+* was then cloned into the XbaI site of ISceI-*smyhc1* to generate *smyhc:*myc-Xdsh-DEP+.

**In vitro GST pull-down assay**

293T cells were grown in DMEM supplemented with 10% FBS, 100ug/ml of penicillin and streptomycin, and 2mM glutamine in a 37ºC incubator with 5% CO2. Cells were seeded in 10 cm dishes from 1:10 split twenty-four hours before transfection. Cells were transfected with 8 μg of pCDNA-*wnt11r-FLAG* plasmid or pCDNA plasmid using Effectene Transfection Reagent (Qiagen) and grown for four days. The medium was then collected and concentrated using an Amicon Ultra-15 10K centrifugal filter device (Millipore). GST proteins and GST-UnpSV1ECD fusion proteins were expressed in *E.coli* and absorbed to Glutathione Sepharose 4B using the Bulk and RediPack GST purification Modules according to manufacture's instructions (GE healthcare). Sepharose coupled with GST proteins were then incubated with medium containing wnr11rFLAG or control medium (pCDNA) for 2 hours at 4ºC. Sepharose beads were washed four times with 1xPBS and bound proteins were eluted with 2x sample buffer. Eluted proteins were resolved on SDS-PAGE (10%) and blotted with anti-FLAG antibody (1:1000, Sigma) and anti-GST antibody (1:5000, Sigma). The secondary antibodies were HRP-conjugated goat anti-mouse and goat anti-rabbit (1:5000, GE healthcare). The blots were detected by ECL Plus chemiluminescent detection system (GE healthcare).

**Transient transfection, co-immunoprecipitation, and western blotting**

About $4x10^6$ 293T cells were seeded in 10 cm plate and grown for 24 hours at 37ºC. 8μg DNA were transfected using Effectene Transfection Reagent, including 4 μg of pCDNA-*wnt11r-FLAG* and 4 μg of pcS2-*SV1-myc* or pcS2-*SV1*Δ*CRD-myc*. 48 hours post transfection, cells were lysed in 1 ml ice-cold lysis buffer (25mM Tris pH7.5, 150mM NaCl, 5mM EDTA, 1% Triton-X 100, 10% glycerol, 0.1% SDS, 1mM sodium orthovanadate, protease inhibitors (Sigma) and phosphatase inhibitors II (Sigma). Cell lysates were centrifuged at 14000rmp to remove insoluble materials and incubated with 10μl anti-myc agarose (Covance) for 2-3 hours at $4^0$C. The immunoprecipitates were washed extensively four to five times using lysis buffer and eluted with 2x sample buffer and separated by SDS-PAGE (10%). Primary antibodies used were anti-FLAG(1:1000, Sigma), anti-myc (1:1000, Covance).

**Two-hybrid interaction**

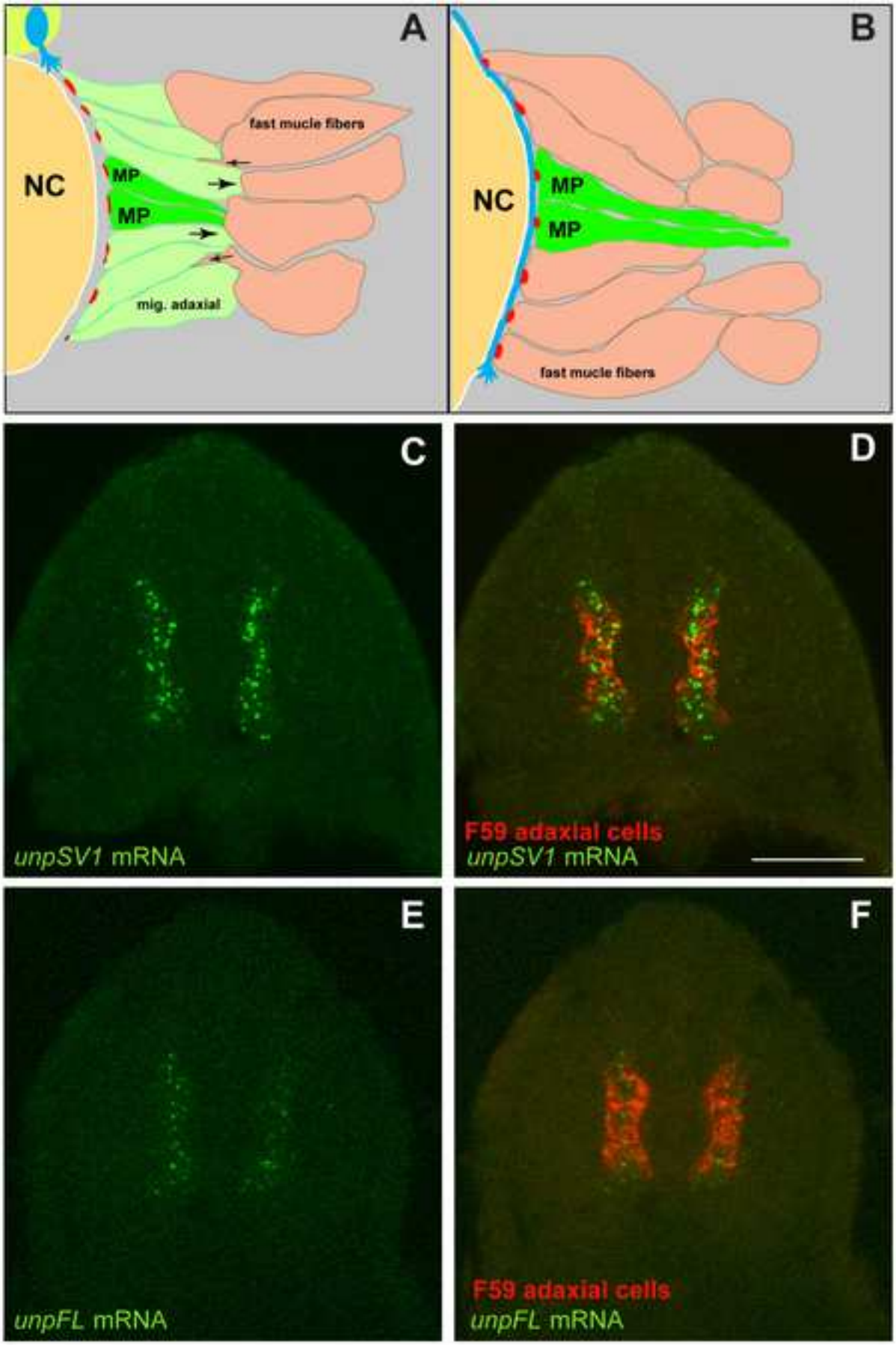
pGBKT7-*SV1IC* and pGADT7-*zDsh2/3* were cotransformed into yeast AH109 strain and plated on SD/-Ade/-His/-Leu/-Trp plates with the addition of 15mM 3-AT according to manufacture's instructions (Clonetech). The plates were incubated at 30ºC until colonies appeared.

Constructs for yeast two-hybrid assay:

5'AAAAAGGATCCGGGTGCTGGAGACACCTACATTGAC3' (forward) and 5'A AAAAGGATCCTCAGCTAGCAGAAAGACCAGATTTGAGCATC3' (reverse) to subclone *SV1* intracellular domain (IC, a.a.336-676) into pGBKT7 (Clonetech).

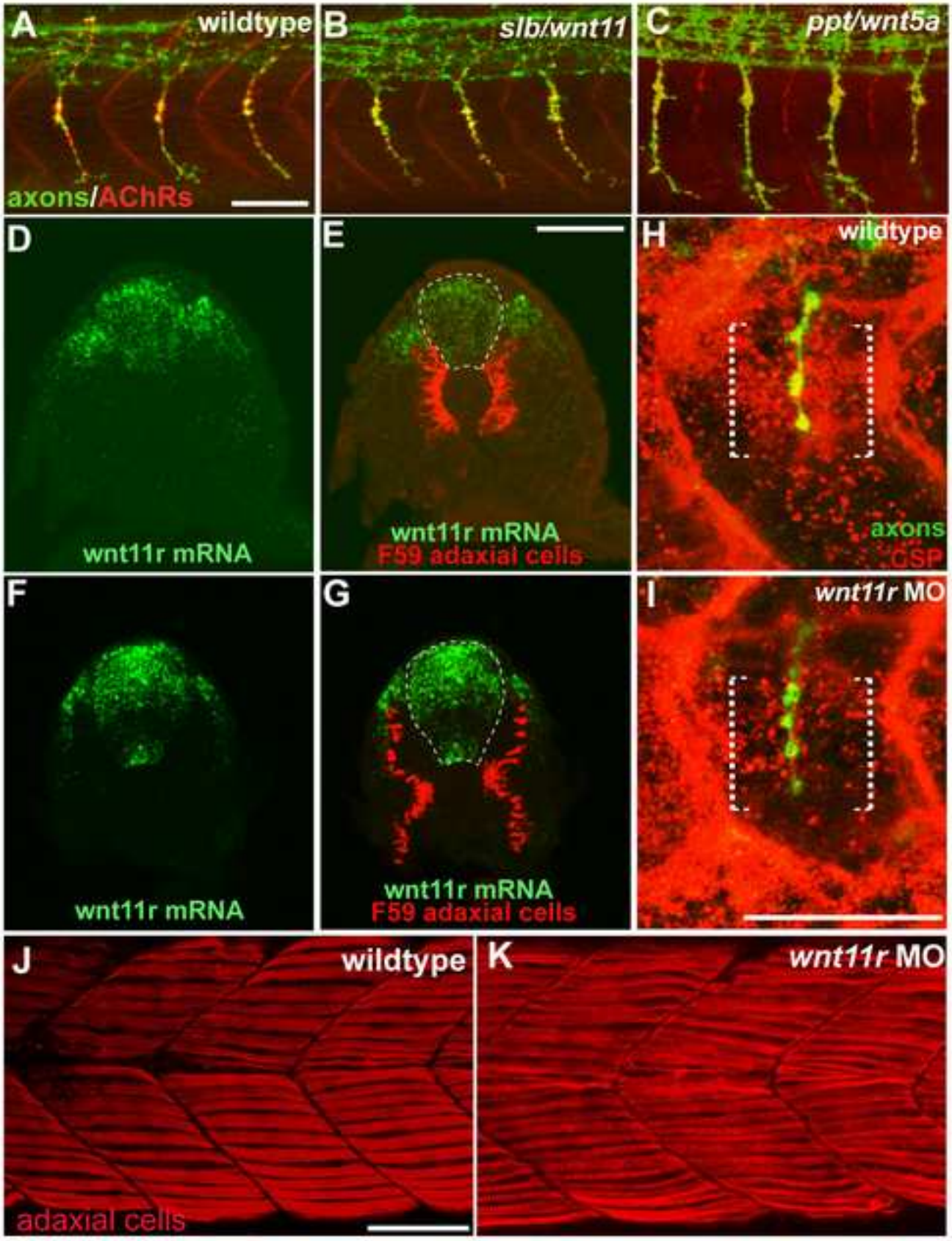
A EcoRI/KpnI digestion of pME18S-*zDsh2* (RZPD) was used to move full length of *zDsh2* to pBSSK+(Stratagene). A EcoRI/XbaI digestion of pMES18S-*zDsh3*(RZPD) was used to move full length zDsh3 to pBSSK+. Primer pair: 5'AAAAGGATCC TCATGAGCAGTGAAC3'(forward) and 5'AAAACTCGAGCATGATGTCGACG AAG3' (reverse) was used to move zDsh3 (residues:185-676, containing PDZ and DEP domain) to $pGADT_7$ (Clonetech). 5' AAAAGAATTCGTGATGAGCAGTGAA C3' (forward) and 5'AAAA ATCGATACATCACATCCACAAAAAAC3'(reverse) were used to move zDsh2 (residues: 184-747, containing the PDZ and DEP domains) to pGADT7.

**Supplemental Figure 1**



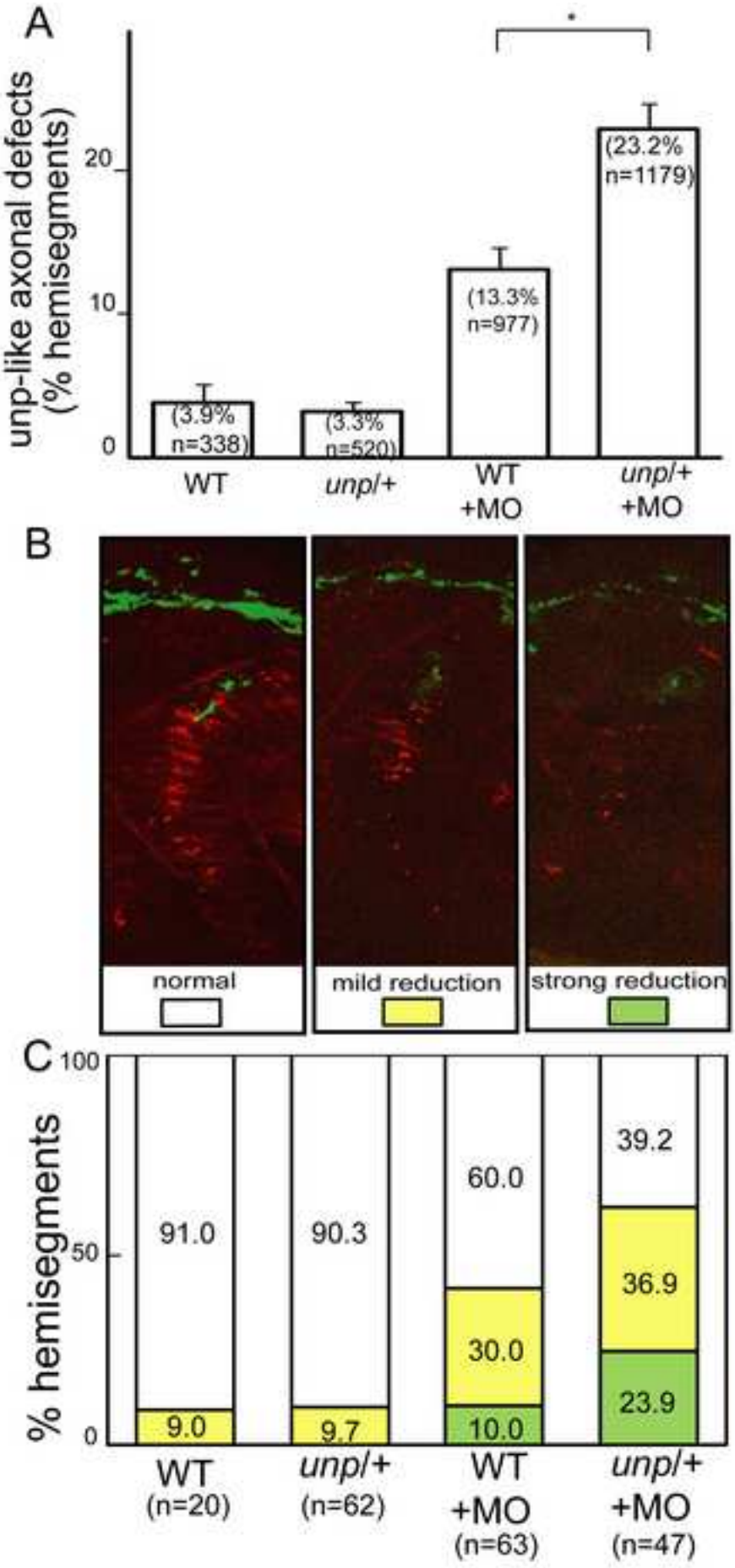

Supplemental Fig. 1 Jing et al

**Supplemental Figure 2**



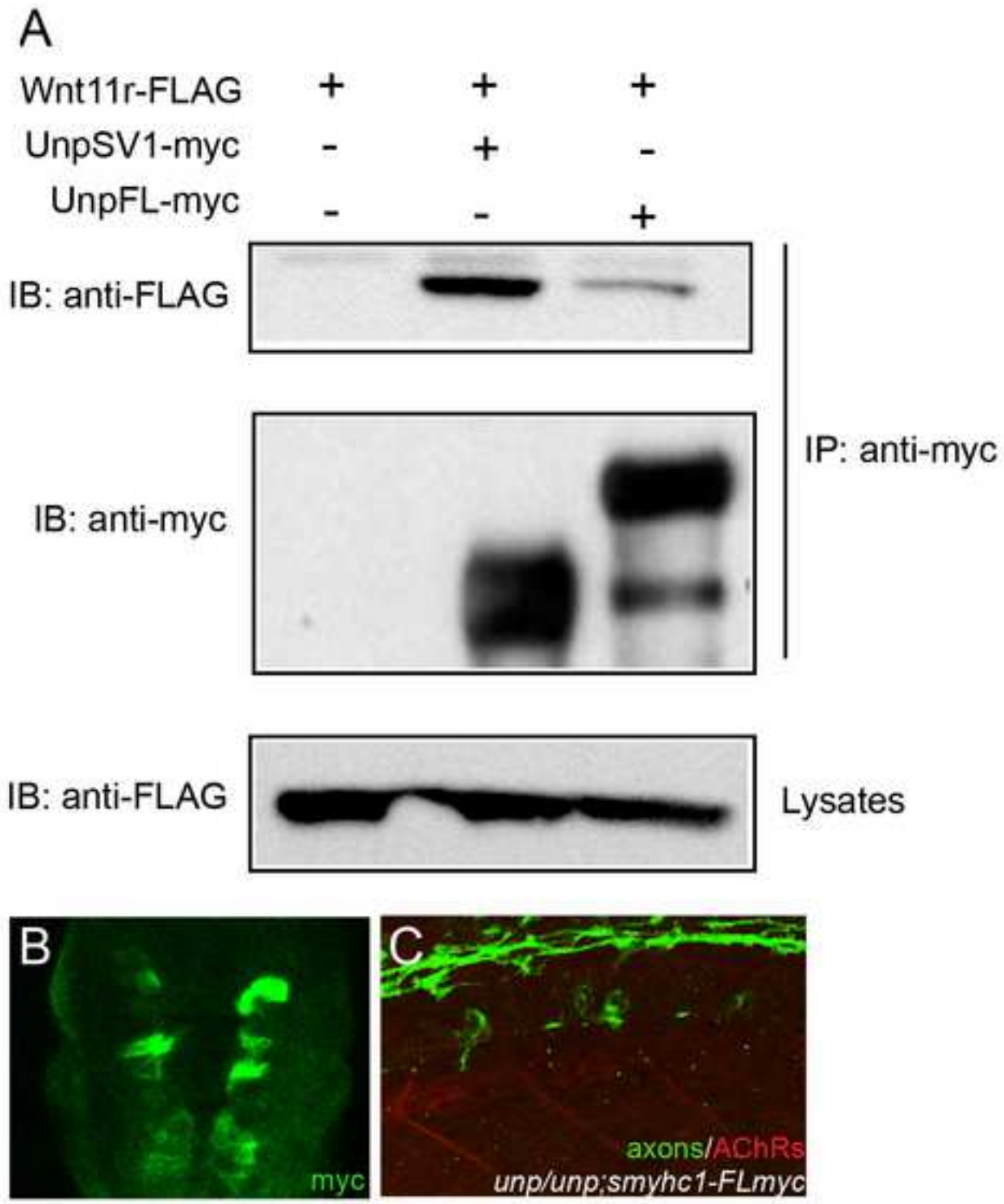

Supplemental Fig. 2 Jing et al

**Supplemental Figure 3**




Suppplemental Fig. 3 Jing et al

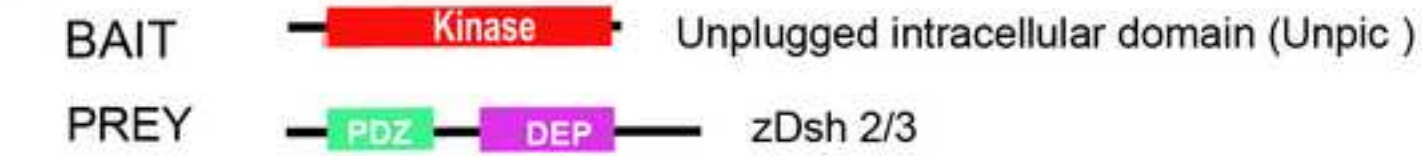
**Supplemental Figure 4**



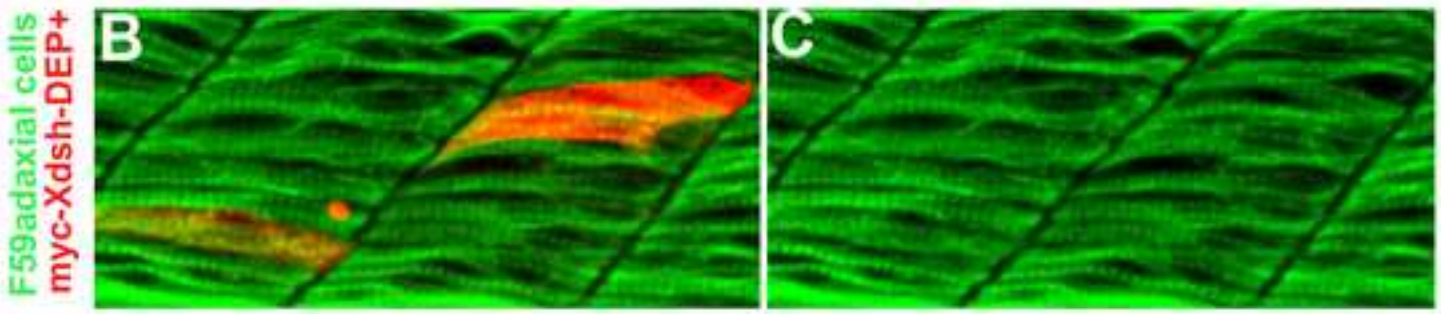

Supplemental Fig. 4 Jing et al

**Supplemental Figure 5**

A

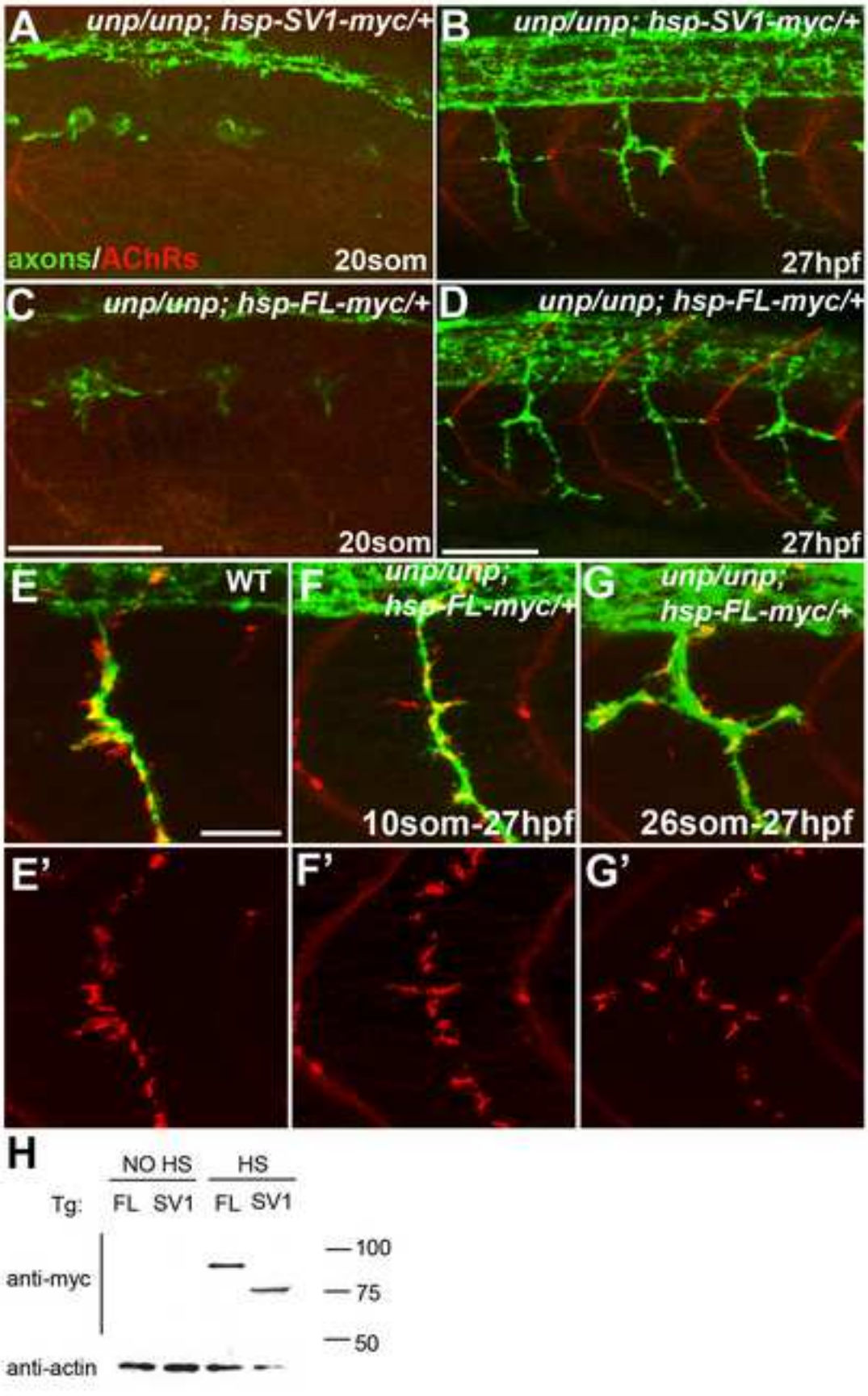



| Growth medium | pGBKT7-Unpic +pGADT7-zDsh2 | pGBKT7-Unpic +pGADT7-zDsh3 | pGBKT7-*unpic* +pGADT7 |
|---|---|---|---|
| SD/-Leu-Trp | +++ | +++ | +++ |
| SD/-Leu-Trp -Ade-His | ++ | ++ | - |




Supplemental Fig. 5 Jing et al

**Supplemental Figure 6**




Supplemental Fig. 6 Jing et al