Creation of a functional *jph-1 (Junctophilin) tagRFP* knock-in by CRISPR

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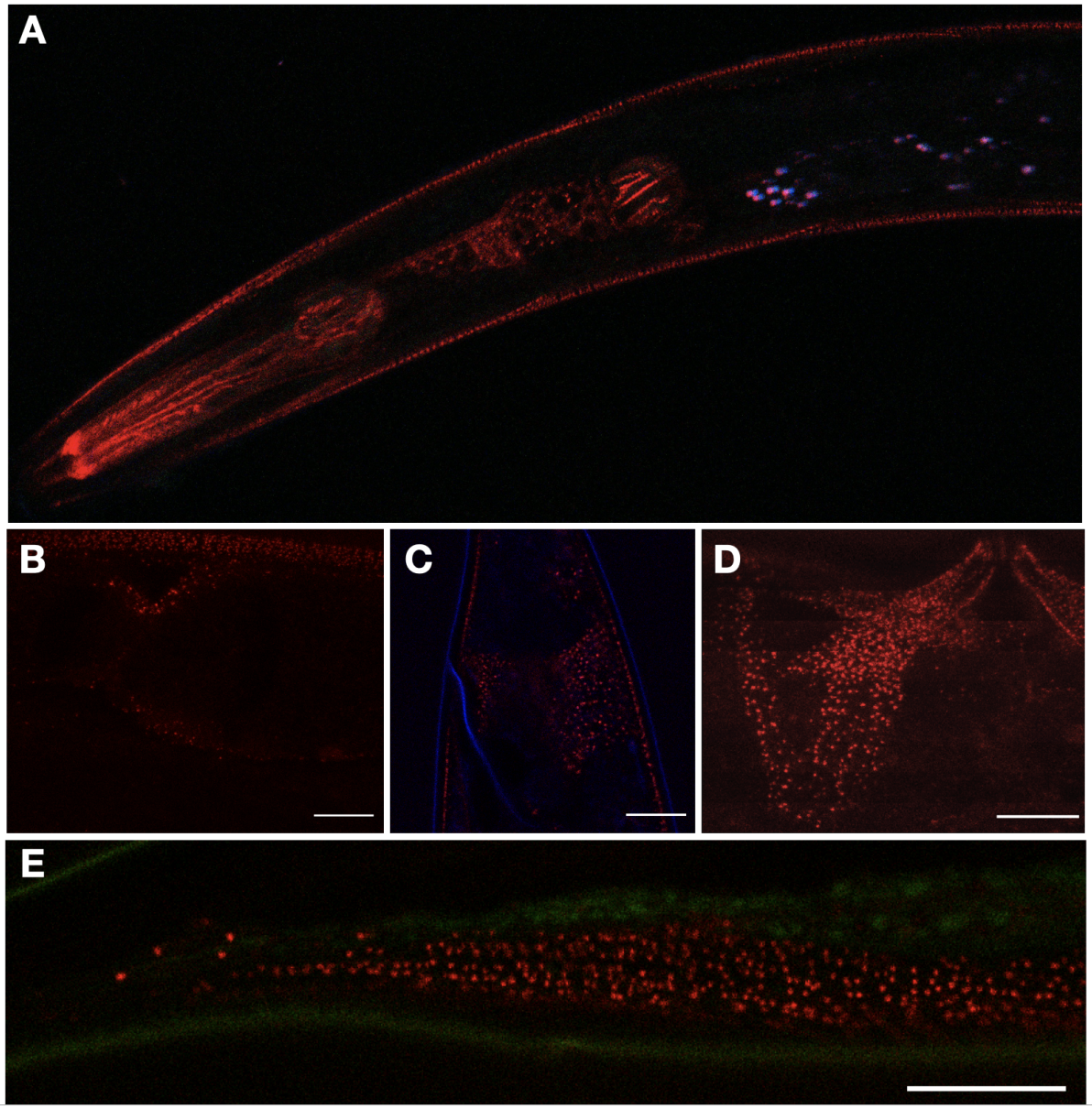
Abstract:

jph-1 localize to ER-PM contact sites and are required for regulating intracellular Ca^{2+} in both striated and nonstriated muscle. How it functions outside the muscle is poorly understood. Creating tools that will provide the complete expression pattern and subcellular localization of jph-1 is critical for future studies into the role jph-1 plays inside muscle but also for generating new hypotheses related to how jph-1 functions outside muscle. The best way to ascertain the complete and authentic expression pattern and subcellular localization is with genome editing. Specifically, by inserting a fluorescent protein gene into the endogenous locus and characterizing the phenotype. In this publication we insert tagRFP just upstream of the jph-1 stop codon. Our behavioral analyses indicate that this tag does not significantly impact function of jph-1. Moreover, we confirm that jph-1 is expressed in *all* striated and nonstriated muscle including the gonadal sheath cells, a tissue that was missed in previous expression pattern studies that relied on traditional synthetic gene approaches and thus incomplete regulatory sequences.



Methods:

Strains: vg126 is the allele code for the *jph-1::tagRFP* knock-in. The WT control (*jph-1(+) I; zdIs4 IV*) and knock in strain (*jph-1(vg126) I; zdIs4 IV*) used in the behavioral assays described in this study were a siblings created from a *jph-1(vg126)/jph-1(+); zdIs4 IV* hybrid.

Brood Size Assay: 10 L4 hermaphrodites for each genotype (*WT* and tagRFP *knock-in*) were placed one per plate and incubated at either 25°C or 20°C (40 worms total). Each adult animal was transferred 1-2 times daily as needed. Two days after a worm had been transferred off a plate, the number of animals that hatched were counted. The total number of hatched animals for each starting hermaphrodite was used to determine brood size.

Hermaphrodite Growth Rate and Body Length: 20 L4 hermaphrodites for each genotype (*jph-1(+)* and *jph-1(vg126)*) were picked to a single plate and allowed to grow to adulthood. The next day, the adults were transferred to a new plate and allowed to lay eggs for a one hour period. **48 hours (confirm time)** after the adults had been removed, the hatched worms were mounted onto a slide and photographed under a dissecting microscope. The larval stage attained by each animal was determined by vulval morphology. Absolute Body length (in microns) was measured in FIJI by drawing a segmented line through the center of the animal from the tip of the nose to the position of the anus. Measurements were calibrated using a micrometer. All incubations were done at 20°C.

Thrashing assay: Individual worms were placed on a glass slide with a 3% agar pad and about 75 ul of M9 buffer. Slides were then mounted onto a DIY microscope (original design by Kenji Yoshino) and videos were captured at 30 frames per second using an iPhone 12. To open the images in FIJI, the .MOV files were converted to an uncompressed AVI format using ff.WORKS software. The duration of each thrash was measured in FIJI by logging each frame number at which the worm completed a single body bend in one direction. Thrash duration (in seconds) was then calculated as the number of frames per body bend divided by 30 frames per second. Approximately, 1450 frames (~45 seconds) of video were reviewed for each animal. Body bends that did not fall below 150 degrees were not counted as a true body bend. When a body bend angle was suspected to be greater than 150 degrees, the angle ruler in Fiji (Image J) was used. Brief intervals where thrashing occurred along the Z-axis or the worm left the field of view were not included. The data was graphed using Plots of Differences available at https://huygens.science.uva.nl/PlotsOfDifferences/ (Joachim Goedhart, 2019).

Confocal: *jph-1(vg126[jph-1::tagRFP])* animals were anesthetized with sodium azide (10 mM) and imaged using a Leica SP8 confocal. A sequential scanning protocol was used to excite and capture various wavelengths of light in the event that we needed to distinguish autofluorescence from true jph-1::tagRFP expression. In general, Z-stacks or single focual planes were captured using the following sequential scanning protocol: Seq 1: The 488 laser was set at 2%. PMT1 (green) collected wavelengths from 494 to 544 nm and PMT2 (blue) collected wavelengths from 650 to 798 nm. Both were designed to capture autofluorescence if present. Seq 2: The 552 laser was set at 3% with the HyD detector 1 collected wavelengths from 598 to 709 nm (tagRFP and autofluorescence if present). The Hyd dectetor was critical for detecting tagRFP above background noise. The image of the vulval and uterine muscles (Figure 1) was created from a single Z-stack (68 focal planes total) using a FIJI plugin called Horizontal Chop (available upon request). This plug in splits a Z-stack into any desired number of horizontal tiles. This allowed us to create a different maximum intensity Z-stack projection for each horizontal tile. This allowed us to separate body wall muscle expression from vulval and uterine muscle. Green and blue channel images were not included in single images or Z projections if no autofluorescence was present in the images.

Reagents:

The *jph-1* sgRNA/Cas9 plasmid (pDQ1032) used to create a double strand break near the *jph-1* start codon was created by Q5 SDM (NEB) using pJW1219 as template (pJW1219 contains the sgRNA(F+E) and Peft-3::Cas9; Addgene # 61250) and the following primer pair: 5' GCAAATGGGAA**ATG**AATGGgtttaagagctatgctggaa 3' and 5' caagacatctcgcaatagga 3'. The *jph-1* target sequence is in all caps and the position of the *jph-1* START codon is in bold. The *jph-1* repair template (pDQ1031) was created by Gibson Assembly of pJW1585 (pJW1585 contains tagRFP^SEC^AID::3xFLAG and is a gift from Jordan Ward) digested with SpeI and ClaI with two *jph-1* homology arms (HA) amplified by PCR using Q5 DNA polymerase (NEB). The primer sequences used to amplify the 5' HA include ACGACGGCCAGTCGCCGGCAATGCCCCATCTTAAGCAGCA and TCCTCTCCCTTGGAGACCATTTCCCATTTGCCGTACTGCT. The primer sequences used to amplify the 3' HA include AGGATGACGATGACAAGAGAATGAATGGAGGCAGATTTGA and AACAGCTATGACCATGTTATTCGCAATTAAAAAACGGCAG. All primers used to create these plasmids were desalted only and purchased from IDT. The *jph-1(vg126[tagRFP::jph-1])* (DQ3227) worm strain was created as described by Dickinson *et al.* 2015. Given that *jph-1* null mutant alleles are small and very slow growing, we expected the heterozygous tagRFP^SEC^::jph-1::AID*::3xFLAG/+ animals carrying the self-excising casette to segregate slow growing, sickly jph-1 null mutant animals. Instead, all our rolling tagRFP^SEC^::jph-1::AID*::3xFLAG/+ isolates segregated healthy rollers and nonrollers only. To our surprise, the nonrollers turned out to be homozygous for the *tagRFP::jph-1* knock-in suggesting that spontaneous excision of the self-excising casette in the absence of heatshock was common for this strain. Successful knock-in was verified by PCR amplification, sequencing of the junction fragments and by the predicted subcellular expression pattern observed under the confocal microscope.