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Supplementary Information for

Molecular determinants of homo- and heteromeric interactions of Junctophilin-1 at triads in adult skeletal muscle fibers

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SI Materials and Methods

Live imaging in PI(4,5)P_2 depletion experiments

Acquisition of live imaging in HeLa cells was performed with a spinning-disc confocal microscope (SDC), using the UltraVIEW VoX system (PerkinElmer) including an inverted microscope (Ti-E Eclipse; Nikon) equipped with Perfect Focus (PFS), temperature-controlled stage, 14-bit electron-multiplying charge-coupled device camera (C9100-50; Hamamatsu Photonics), and spinning disc-confocal scan head (CSU-X1; Yokogawa Corporation of America) controlled by Volocity software (PerkinElmer). All images were acquired through a 63x oil objective (1.4 NA, CFI Plan Apochromat VC). Green fluorescence was excited with a 488-nm/50-mW diode laser (Coherent) and collected by a band pass (BP) 527/55-nm filter. Red fluorescence was excited with a 561-nm/50-mW diode laser (Cobolt) and collected by a BP 615/70-nm filter. Near-infrared fluorescence was excited with a 640-nm/50-mW diode laser (CVI Melles Griot) and collected by a BP 705/90-nm filter. Multicolor images were acquired sequentially (1). 10 μM Oxotremorine (Oxo-M, Tocris Bioscience or Sigma Aldrich) was added after 1-2 minutes of acquisition followed by 50 μM Atropine (Tocris Bioscience or Sigma Aldrich) during live imaging (2). Image analysis was performed using Volocity and ImageJ (National Institutes of Health) softwares. Alternatively, experiments on muscle fibers electroporated with plasmid constructs encoding a voltage-sensitive phosphatase (VSP) and mRFP-JPH1ΔTMD were performed following procedures described in (3). We used a plasmid encoding *C. Intestinalis* VSP (Ci-VSP) in a bicistronic p-IRES-EGFP expression vector, provided by Y. Okamura (University of Osaka, Japan). Single fibers isolated from the electroporated FDB and interosseous muscles were voltage-clamped using the silicone-clamp technique (4). The extracellular solution contained (in mM) 140 TEA-methanesulfonate, 2.5 $CaCl_2$, 2 $MgCl_2$, 1 4-aminopyridine, 10 HEPES and 0.002 tetrodotoxin. The intracellular-like solution used in the voltage-clamp pipette contained (in mM) 120 K-glutamate, 5 Na_2-ATP, 5 Na_2-phosphocreatine, 5.5 $MgCl_2$, 15 EGTA, 6 $CaCl_2$, 5 glucose, 5 HEPES. Both

solutions were adjusted to pH 7.20. An RK-400 patch-clamp amplifier (Bio-Logic, Claix, France) was used in whole-cell voltage-clamp configuration. Command voltage pulse generation was achieved with an analog-digital converter (Digidata 1440A, Axon Instruments, Foster City, CA) controlled by pClamp 9 software (Axon Instruments). Analog compensation was adjusted to further decrease the effective series resistance. Confocal fluorescence imaging on the voltage-clamped fibers was conducted with a Zeiss LSM 5 Exciter confocal microscope equipped with a 63x oil immersion objective (numerical aperture 1.4). mRFP-JPH1ΔTMD fluorescence was collected above 560 nm with excitation from the 543 nm line of a HeNe laser. Ci-VSP activation was triggered by stepping the holding voltage to values larger than +100 mV during 15 s, from a resting level set at either -80 or -30 mV. Image processing and analysis were performed using Image/J (NIH, USA) and Microcal Origin (Microcal Software Inc., Northampton, MA). In order to assess statistical significance of the changes in triadic localization of the various probes tested in response to T-tubule $PI(4,5)P_2$ depletion, the following procedure was used. In each fiber, an x,y image of the probe fluorescence was taken in the control situation (before $PI(4,5)P_2$ depletion), during the $PI(4,5)P_2$ depletion protocol and then upon recovery of $PI(4,5)P_2$ in the T-tubule membrane. The test image was acquired 6 s after the beginning of the depolarizing step, and the recovery image was acquired 60 s after returning the holding voltage to its resting value. As the extent of the effect of $PI(4,5)P_2$ depletion on the triadic localization of each tested probe was, to a variable extent, corrupted by photo-bleaching, statistical assessment of a change in triadic localization was achieved by simply measuring the standard deviation (SD) of pixels intensity in the region of interest (ROI) divided by mean value of pixels intensity in that same ROI (SD/mean) in the fluorescence images acquired before $PI(4,5)P_2$ depletion, during $PI(4,5)P_2$ depletion and upon subsequent recovery of t-tubule $PI(4,5)P_2$. Rationale for using the SD/mean ratio is that a simultaneous decrease and increase in triadic and cytosolic fluorescence, respectively, translates into a reduced image contrast and thus a reduced average SD of pixel intensity. This analysis makes no use of the spatial information provided by the triadic profiles (SI Appendix and Fig. S5, B), but we found it to be very reliable to follow the loss and recovery of triadic fluorescence (see SI Appendix, Fig. S5, C). Statistical analysis was then achieved using a t-test. For this, in each fiber, the SD/mean values calculated during

$PI(4,5)P_2$ depletion and upon $PI(4,5)P_2$ recovery were normalized to the corresponding SD/mean value calculated from the control image. A t-test was then used to determine whether the mean SD/mean values from all fibers during $PI(4,5)P_2$ depletion and upon $PI(4,5)P_2$ recovery, differed from 1. Paired t-tests using the 3 non-normalized values of SD/mean in each fiber gave essentially identical results in terms of statistical significance. Image processing and analysis was performed using Image/J (NIH, USA), Microsoft Excel software or Prism GraphPad software.

1. Nández R, Balkin D-M, Messa M, Liang L, Paradise S, Czapla H, Hein M-Y, Duncan J-S, Mann M, De Camilli P (2014) A role of OCRL in clathrin-coated pit dynamics and uncoating revealed by studies of Lowe syndrome cells. *Elife* 3:e02975. doi: 10.7554/eLife.02975.

2. Giordano F, Saheki Y, Idevall-Hagren O, Colombo S-F, Pirruccello M, Milosevic I, Gracheva E-O, Bagriantsev S-N, Borgese N, De Camilli P (2013) PI(4,5)P2-dependent and Ca^{2+}-regulated ER-PM interactions mediated by the extended synaptotagmins. *Cell* 153(7):1494-1509. doi: 10.106/j.cell.2013.05.026.

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4. Jacquemond V (1997) Indo-1 fluorescence signals elicited by membrane depolarization in enzymatically isolated mouse skeletal muscle fibers. *Biophys J* 73(2):920-928.

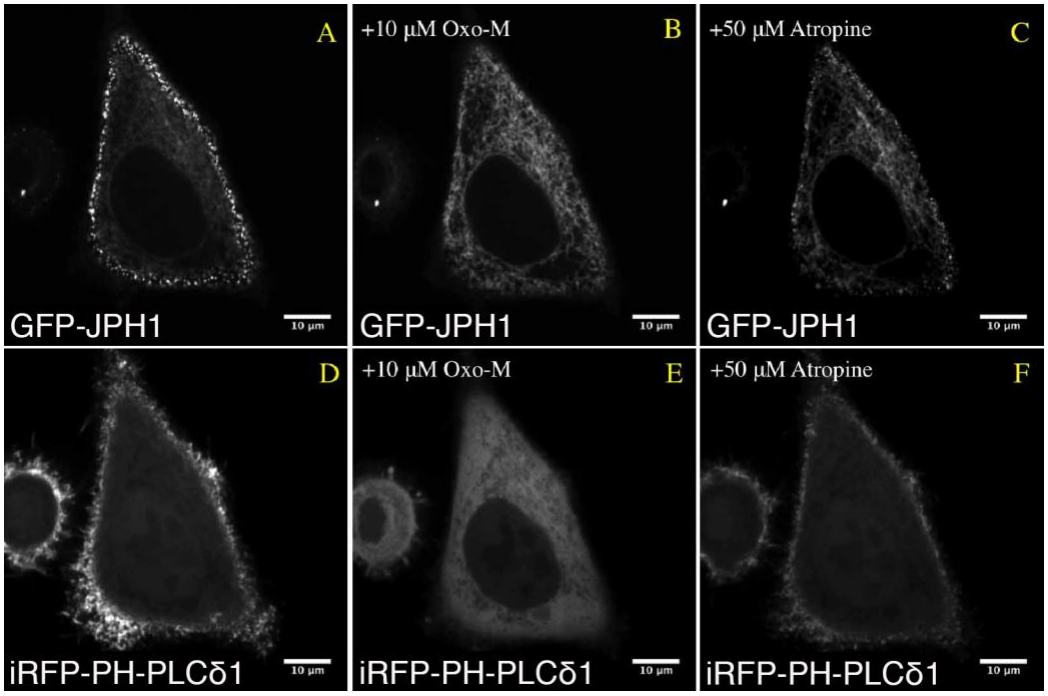

Fig. S1. PI(4,5)P_2 depletion by Oxo-M and Atropine assay in HeLa cells. Spinning-disc confocal images of single HeLa cells co-transfected with the untagged M_1 muscarinic acetylcholine receptor (M_1R) and either GFP-JPH1 **(A)**, or the PI(4,5)P_2 probe iRFP-PH-PLCδ1 **(D)**. Cells were treated with 10 μM Oxo-M in Tyrode Buffer (**B** and **E**, 10 μM Oxo-M). Cells were imaged every 1 minute. After 3 minutes after Oxo-M addition, cells were treated with 50 μM Atropine (C and F, 50 μM atropine). Bar = 10 μm.

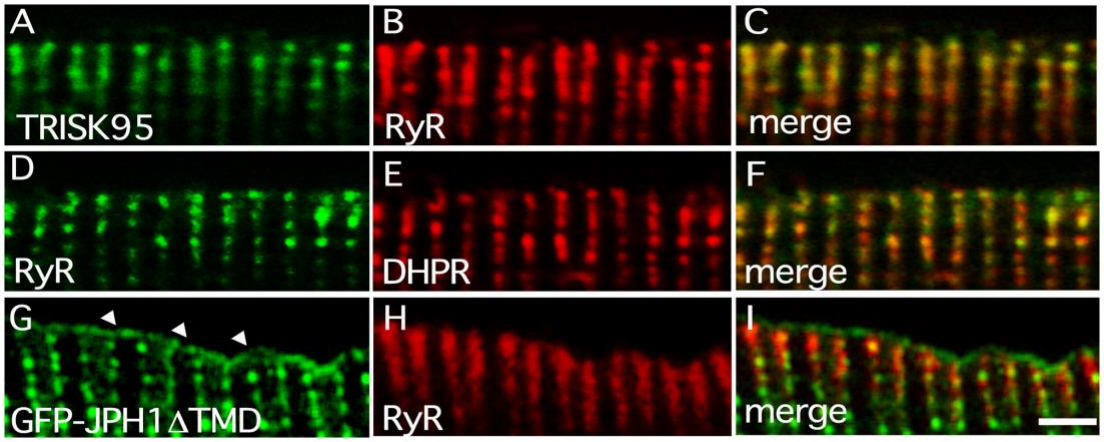

Fig. S2. Expression of endogenous triadic proteins and recombinant GFP-JPH1ΔTMD mutant in adult mouse FDB muscle fibers. Confocal images of adult mouse FDB muscle fibers decorated with primary polyclonal antibodies against TRISK95 (A), monoclonal (B) or polyclonal (D) antibodies against RyR or monoclonal antibodies against the DHPR alpha-1 subunit (E). Immunoflurescence signals were revealed by AlexaFluor488 or Cy3-conjugated secondary antibodies. FDB muscle fibers were electroporated with plasmids encoding the GFP-JPH1ΔTMD mutant (G) and counterstained with polyclonal antibodies against RyR (H) and anti-rabbit Cy3-conjugated secondary antibodies. Arrowheads indicate the surface sarcolemma. Bar = 3 μm.

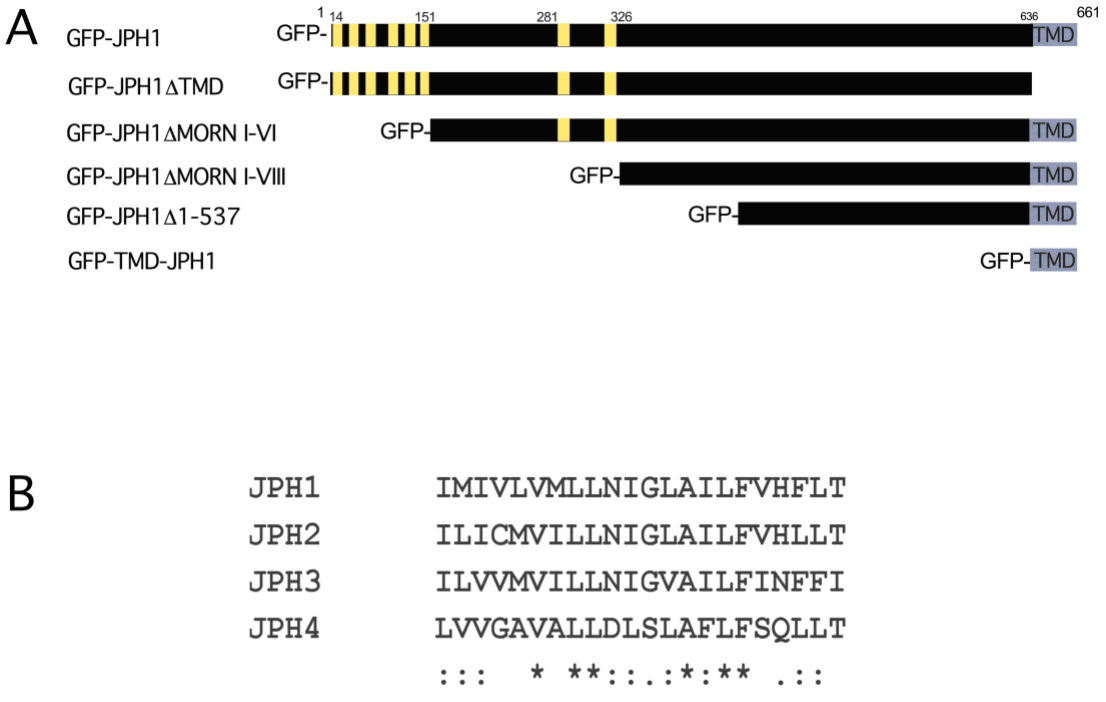

Fig. S3. A. Schematic representation of N-terminal and C-terminal deletion mutants of JPH1. Full-length and deletion mutant of JPH1 are represented. Numbers indicate amino acid positions in the JPH1 full-length sequence. Yellow bars indicate MORN motifs, grey blocks at the C-terminus indicate the transmembrane domains (TMD). **B. Sequence alignment of the TMDs of human JPH isoforms.** The symbol (*) indicates positions which have a single, fully conserved residue. The symbol (:) indicates conservation between groups of strongly similar properties. The symbol (.) indicates conservation between groups of weakly similar properties.

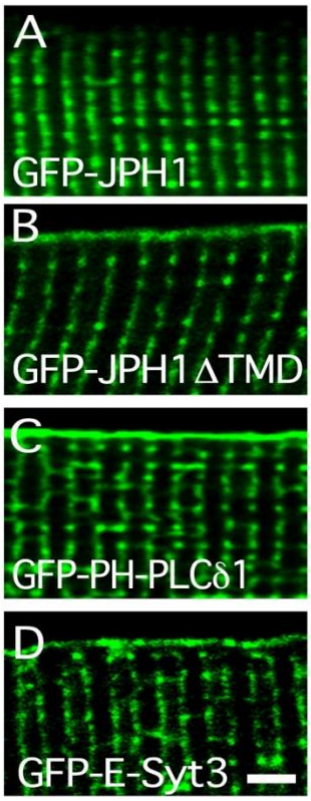

Fig. S4. Expression of GFP-JPH1, GFP-JPH1ΔTMD, GFP-PH-PLC δ1 and GFP-E-Syt3 in adult mouse FDB muscle fibers. Confocal images of adult mouse FDB muscle fibers in vivo electroporated with plasmids coding for GFP-JPH1 (A), GFP-JPH1ΔTMD (B), GFP-PH-PLC δ1 (C) and GFP-E-Syt3 (D). Bar = 2.5 μm.

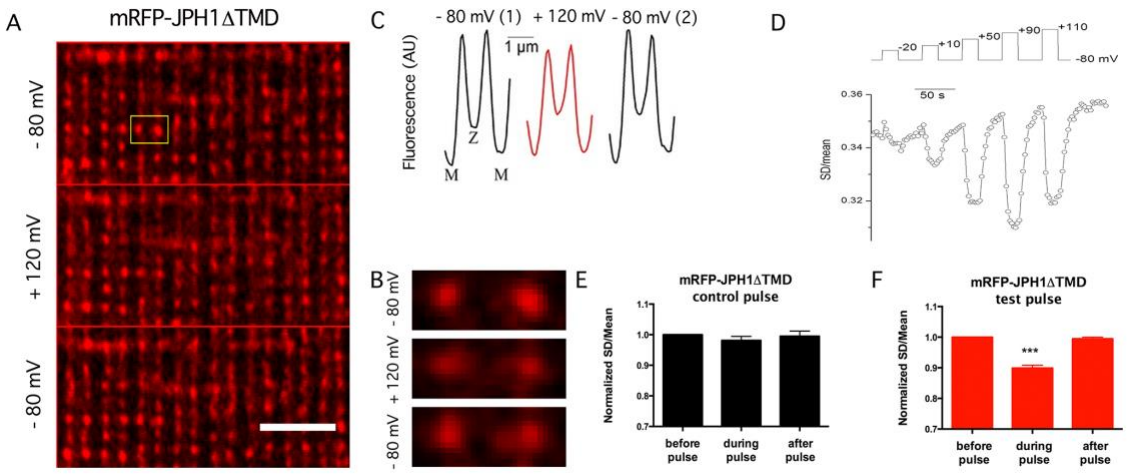

Fig. S5. PI(4,5)P_2 depletion in isolated FBD muscle fibers using the voltage-sensing phosphatase (VSP). A. Confocal frames of the mRFP fluorescence from an isolated FDB mouse muscle fiber electroporated with mRFP-JPH1ΔTMD and a voltage sensitive phosphatase (VSP) (bar = 5 µm). From top to bottom, fluorescence frames were successively acquired with the voltage clamped at -80 mV, +120 mV and -80 mV again, respectively. **B**. Enlarged view of the region delimited by the yellow box in panel A that highlights two clusters of mRFP-JPH1ΔTMD in two triadic regions. **C**. Typical changes in the triadic fluorescence profile of mRFP-JPH1ΔTMD upon VSP activation. M and Z refer to the approximate position of the M-line and Z-disk. **D.** Changes in SD/mean pixel intensity in a fiber expressing mRFP-JPH1ΔTMD stimulated by voltage pulses of increasing amplitude. **E-F.** Mean values for the normalized SD/mean pixel intensity ratio before, during and after application of a depolarizing pulse in fibers expressing mRFP-JPH1ΔTMD. Data are from 5 fibers expressing mRFP-JPH1ΔTMD and challenged by a control (non-VSP activating) pulse (E) and from 13 fibers expressing mRFP-JPH1ΔTMD and challenged by a test (VSP-activating) pulse (F). Error bars indicate the standard deviation. *** $p \leq 0.001$ using a paired t-test.

Table S1. Bimolecular fluorescence complementation assay (BiFC) on HeLa cells.

VN-173-TMD-JPH1/ VC-155-TMD-JPH1	VN-173-TMD-JPH1/ VC-155-TMD-JPH4	VN-173-TMD-JPH4/ VC-155-TMD-JPH4
11.85%±2.71	**1.44%±1.44 ****	**0.167%±0.167 ****

Table S1. The percentage of positive cells was calculated in six independent experiments. Total number of cells analyzed was as follows: pBiFC-VN173-JPH1-TMD and pBiFC-VC155-JPH1-TMD =1252; pBiFC-VN173-JPH1-TMD and pBiFC-VC155-JPH4-TMD = 1367; pBiFC-VN173-JPH4-TMD and pBiFC-VC155-JPH4TMD = 1241. Numbers indicate the average of positive cells ± standard deviation. Statistical analysis was performed using the GraphPad Prism 6.0 software, using the Kruskal-Wallis test. ** = $p \leq 0.01$.

Movie S1: PI(4,5)P_2 depletion in isolated HeLa cells. Spinning-disc confocal microscope (SDC) movie of HeLa cells co-transfected with the PI(4,5)P_2 probe iRFP-PH-PLCδ1 and M_1 untagged muscarinic acetylcholine receptor (M_1R). Cells were imaged every 30 seconds. After 3 to 5 minutes after Oxo-M addition, cells were treated with 50 µM Atropine.

Movie S2: PI(4,5)P_2 depletion in isolated HeLa cells. Spinning-disc confocal microscope (SDC) movie of HeLa cells co-transfected with GFP-JPH1 and M_1 untagged muscarinic acetylcholine receptor (M_1R). Cells were imaged every 30 seconds. After 3 to 5 minutes after Oxo-M addition, cells were treated with 50 µM Atropine.

Movie S3: PI(4,5)P_2 depletion in isolated HeLa cells. Spinning-disc confocal microscope (SDC) movie of HeLa cells co-transfected with GFP-JPH2 and M_1 untagged muscarinic acetylcholine receptor (M_1R). Cells were imaged every 30 seconds. After 3 to 5 minutes after Oxo-M addition, cells were treated with 50 µM Atropine.