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Actin-microtubule crosslinker Pod-1 tunes PAR-1 signaling to control synaptic development and tau-mediated synaptic toxicity

Ha-Young Kang^{a,1}, Hyung-Jun Kim^{b,1}, Kiyoung Kim^{c,1}, Seung-II Oh^a, Sunggyu Yoon^{a,d}, Jaekwang Kim^b, Sangwoo Park^a, Yeongmi Cheon^a, Song Her^a, Mihye Lee^{e,***}, Bingwei Lu^{f,**}, Seongsoo Lee^{a,*}

^aGwangju Center, Korea Basic Science Institute (KBSI), Gwangju, South Korea

^bDementia Research Group, Korea Brain Research Institute (KBRI), Daegu, South Korea

^cDepartment of Medical Biotechnology, Soonchunhyang University, Asan, South Korea

^dDepartment of Life Science, College of Natural Science, Hanyang University, Seoul, South Korea

^eSoonchunhyang Institute of Medi-bio Science, Soonchunhyang University, Cheonan, South Korea

^fDepartment of Pathology, Stanford University School of Medicine, Stanford, CA, USA

Abstract

Partitioning-defective 1 (PAR-1), a conserved cell polarity regulator, plays an important role in synaptic development, and its mutation affects the formation of synaptic boutons and localization of postsynaptic density protein Discs large (Dlg) at the neuromuscular junction (NMJ) in *Drosophila*. *Drosophila* PAR-1 and its human homolog, Microtubule affinity-regulating kinases (MARK), are also known to be implicated in Alzheimer's disease (AD) by controlling tau-mediated A β toxicity. However, the molecular mechanisms of PAR-1 function remain incompletely understood. Here we identified Pod-1, an actin-microtubule crosslinker, which functionally and physically interacts with PAR-1 in *Drosophila*. Pod-1 prominently co-localizes

*Corresponding author at: Gwangju Center, Korea Basic Science Institute (KBSI), Gwangju 61186, South Korea. Tel.: +82 62 712 4419; fax: +82 62 530 0519. soolee@kbsi.re.kr. **Corresponding author at: Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA. Tel.: +1 650 723 1828; fax: +1 650 498 6616. bingwei@stanford.edu. ***Corresponding author at: Soonchunhyang Institute of Medi-bio Science, Soonchunhyang University, Cheonan 31151, South Korea. Tel.: +82 41 413 5015; fax: +82 41 413 5006. mihyelee@sch.ac.kr.

¹These authors contributed equally to this work.

CRedit authorship contribution statement

Ha-Young Kang: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Hyung-Jun Kim:** Conceptualization, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Supervision, Funding acquisition. **Kiyoung Kim:** Conceptualization, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Supervision, Funding acquisition. **Seung-II Oh:** Methodology, Validation, Formal analysis, Visualization. **Sunggyu Yoon:** Methodology, Validation, Formal analysis, Visualization. **Jaekwang Kim:** Validation, Formal analysis, Visualization, Funding acquisition. **Sangwoo Park:** Validation, Formal analysis, Visualization. **Yeongmi Cheon:** Validation, Formal analysis, Visualization. **Song Her:** Validation, Visualization. **Mihye Lee:** Conceptualization, Methodology, Investigation, Resources, Supervision, Writing - review & editing. **Bingwei Lu:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision, Project administration. **Seongsoo Lee:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Disclosure statement

All authors declare no conflict of interest that might potentially bias this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neurobiolaging.2020.02.005>.

with PAR-1 in the postsynaptic region and regulates PAR-1 activity at the NMJ. Synaptic defects, including the reduction of boutons and delocalization of Dlg caused by PAR-1 overexpression, were rescued by Pod-1 knockdown. Conversely, the reduction of synaptic boutons in PAR-1 overexpressed NMJ was synergistically enhanced by the overexpression of Pod-1. Furthermore, Pod-1 increases the PAR-1 dependent S262 phosphorylation of tau, which is known to contribute to tau-mediated A β toxicity. In line with the change of tau phosphorylation, Pod-1 knockdown rescued tau-mediated synaptic toxicity at the NMJ. Our results suggest that Pod-1 may act as a modulator of PAR-1 in synaptic development and tau-mediated toxicity.

Keywords

PAR-1; Pod-1; Synaptic defect; Tau-mediated synaptic toxicity; NMJ; *Drosophila*

1. Introduction

Precise organization of cytoskeleton required for diverse cellular functions, such as cell division, migration, membrane trafficking, and morphogenesis, is essential for establishing functional neural networks (Kapitein and Hoogenraad, 2015). In the nervous system, cytoskeletons determine the neuronal shape and polarity and provide physical support to build up the architecture. Moreover, the cytoskeleton undergoes dynamic remodeling to regulate exploratory movement through molecular rearrangement underlying axon guidance or synapse formation (Lasser et al., 2018). Thus, to understand the role of the cytoskeleton in the development and function of the nervous system, a range of cytoskeleton binding proteins and regulators have been extensively studied, revealing that the dysfunctions of cytoskeleton-related proteins are tightly linked to developmental defects and neurodegenerative diseases (Lasser et al., 2018).

Coronins are an evolutionarily conserved family of actin cytoskeleton regulators whose functions have been studied extensively in the nervous system (de Hostos et al., 1991). All coronin homologs share the basic N terminal motif and WD repeats. They also contain a unique region of variable length conferring specific features for each coronin protein (Liu et al., 2016) (Rybakin and Clemen, 2005). They play important roles in actin-dependent processes, including cell motility, morphogenesis, and trafficking (Utrecht and Bear, 2006) (Chan et al., 2011). In mammals, 7 isoforms of Coronin have been reported, most of which show tissue-specific expression mainly in the nervous system (Chen et al., 2014) (Liu et al., 2016). In mouse neurons, Coronin 1 prevents the fusion of the retrograde signaling endosome with lysosomes and instead facilitates the recycling of the signaling endosome (Suo et al., 2014). In addition, deletion or mutation of *coronin 1* leads to neurobehavioral disabilities, including social deficits, increased aggression, and learning defects, in both mice and humans (Jayachandran et al., 2014). Coronin 6 is localized at the neuromuscular junction (NMJ) and regulates acetylcholine receptor clustering by modulating the interaction between receptors and the actin cytoskeleton in mice (Chen et al., 2014). In *C.elegans* and *Drosophila*, Coronins were also known to be involved in the control of neurodevelopment. The *C.elegans* Coronin regulates cell morphology by coordinating the actin organization in postembryonic neuroblast migration (Chen et al., 2014). The *Drosophila* homolog of

Coronin, Pod-1, which can crosslink actin and microtubule cytoskeletons, is localized to the tips of growing axons and is required for targeting of axons (Rothenberg et al., 2003). Pod-1 is also known to function in glutamate receptor cluster formation at the *Drosophila* NMJ (Liebl and Featherstone, 2008).

PAR-1, the *Drosophila* homolog of mammalian MARK, is a conserved serine/threonine kinase with multiple domains, including catalytic and protein-protein interaction domains, and was initially identified as a regulator of the microtubule cytoskeleton. The cellular functions of PAR-1 and its homologs include establishing cell polarity and asymmetric cell division in multiple cell types and organisms (Guo and Kemphues, 1995) (Penton et al., 2002). Especially in the NMJ of *Drosophila*, PAR-1 was known to affect the formation and function of the synapse (Zhang et al., 2007). PAR-1 regulates the postsynaptic scaffold protein Disc large (Dlg), the *Drosophila* homolog of mammalian PSD-95, which recruits synaptic proteins and assembles them into postsynaptic complexes. It directly phosphorylates the GUK domain of Dlg and negatively regulates the postsynaptic targeting of Dlg (Zhang et al., 2007). Additionally, PAR-1 phosphorylates tau at the S262 and S356 residues, thereby implicating dysregulation of PAR-1 in Alzheimer's disease (AD)-related tau hyperphosphorylation and toxicity (Nishimura et al., 2004) (Ando et al., 2016). The phosphorylation of T408 residue of PAR-1 by the tumor suppressor protein LKB1 is required for PAR-1 activation in vivo. Previous studies have shown that overexpression of LKB1 strongly promotes PAR-1 phosphorylation at T408 residue and enhances PAR-1-induced neurodegeneration, whereas inhibition of LKB1 has the opposite effect (Wang et al., 2007). Moreover, activated phospho-PAR-1 is a common substrate for both Skp-Cullin-F-box (SCF)-containing E3 ligase, Slimb, and the deubiquitinating enzyme, Fat facets (FAF), in synaptic development. The control of PAR-1 kinase activity through Slimb and FAF also affects tau-induced synaptic toxicity (Lee et al., 2012).

Here, we reveal another layer of regulation for PAR-1 activity. We identify Pod-1, the actin/MT crosslinker, as a novel regulator of PAR-1 in synaptic development and tau-mediated synaptic toxicity. Pod-1 physically interacts with PAR-1 and its downregulation rescues the synaptic defects caused by PAR-1 overexpression. Furthermore, we provide evidence that the downregulation of PAR-1 activity through pod-1 knockdown influences tau-induced synaptic toxicity. Our findings suggest that the modulation of PAR-1 activity through the inhibition of actin/MT crosslinker Pod-1 could be a potential target for AD therapies.

2. Materials and methods

2.1. *Drosophila* strains

The *w¹¹¹⁸* strain was used as the wild-type control. The UAS-PAR-1-WT, UAS-PAR-1-T408 A, and UAS-htauM were described previously (Lee et al., 2012). The *Mhc-GAL4* fly line was provided by Dr. T Littleton. The *FAF^{EP381}*, *UAS-Pod-1-WT*, *UAS-Pod-1-RNAi*, *UAS-Slimb-RNAi*, and *GMR-GAL4* lines were obtained from Bloomington *Drosophila* Stock Center. The flies were maintained at 25 °C on standard *Drosophila* food.

2.2. Immunohistochemistry imaging

For immunostaining, third instar larvae were dissected in PBS, fixed in 4% formaldehyde (Ted Pella) in PBS for 15 minutes and washed 4 times with 0.1% Triton X-100 in PBS. The fixed samples were incubated with primary antibodies at 4 °C overnight. The primary antibodies used were as follows: anti-PAR-1 (1:10,000), anti-Pod-1 (1:1000), and anti-Dlg (4F3) (1:50, Developmental Studies Hybridoma Bank). All secondary antibodies (Molecular Probes) and FITC-conjugated anti-HRP (Jackson ImmunoResearch Laboratories) were incubated for 2 hours at room temperature. Sample preparations were mounted in SlowFade Antifade kit (Invitrogen). Confocal images were collected from Leica TCS SP5 AOBS confocal microscopes equipped with 40 × or 100 × inverted NX oil lens, located at Gwangju Center, Korea Basic Science Institute. For analysis of the neuromuscular junction, we used the procedure described in the previous study (Lee et al., 2010).

2.3. Immunoprecipitation and western blot analysis

For coimmunoprecipitation experiments, 200 fly heads expressing *UAS-PAR-1-WT-Myc* transgene driven by *GMR-GAL4* were homogenized in lysis buffer (50 mM Tris-HCl pH 8.0%, 1% Triton X-100, 150 mM NaCl, 2 mM Na₃VO₄, 10 mM NaF, 10% glycerol, protease inhibitors), and the supernatant of lysate was obtained after centrifugation at 12,000 × *g* for 30 minutes at 4 °C. The supernatant was pre-cleared by incubation with protein A/G agarose (Pierce) for 1 hour at 4 °C. The supernatant was incubated with anti-PAR-1 antibodies for 2 hours at 4 °C and then incubated with protein A/G agarose for 12 hours at 4 °C. Beads were washed 5 times with lysis buffer or PBS and boiled in SDS sample buffer. The samples were separated by gel electrophoresis and analyzed by western blot analysis. For western blot analysis, the following primary antibodies were used: anti-PAR-1 (1:8000), anti-phospho-PAR-1 (1:1000); anti-GFP (1:2,000, Abcam); anti-Myc (1:1,000, Millipore); anti-tau (pS262) (1:2,000, Life technology); total tau (1:2,000, Dako); and anti-GAPDH (1:2,000, Abclon). All primary antibodies were diluted in 0.5% BSA in TBST (tris-buffered saline, 0.1% tween 20) and incubated with the membrane at 4 °C overnight. All secondary antibodies (Jackson ImmunoResearch Laboratories) were incubated for 1 hour at room temperature.

3. Results

3.1. Pod-1 interacts with PAR-1

PAR-1 is a critical regulator of synapse development and tau-induced synaptic toxicity. To identify potential regulators of PAR-1, we performed genetic modifier screening in the *Drosophila* eye. We found Pod-1, which encodes an actin/microtubule crosslinker, as a strong interactor of PAR-1. While overexpression of Pod-1 or PAR-1-WT alone had a mild effect on eye morphology (Fig. 1A–C), coexpression of Pod-1 and PAR-1-WT (Fig. 1D), but not PAR-1-T408A mutant (Fig. 1E and F), in which the LKB1-dependent phosphorylation is abolished (Wang et al., 2007), significantly reduced the size of the adult eye. The ubiquitin E3 ligase, Slimb, and deubiquitinating enzyme, FAF, have been known to control the stabilization of PAR-1 (Lee et al., 2012). When we induced the stabilization of PAR-1 by knockdown of Slimb or overexpression of FAF in the genetic background of Pod-1 overexpression, severe eye phenotypes were clearly observed (Fig. 1G–J). Collectively, these

data suggest that Pod-1 functionally interacts with PAR-1, as well as the PAR-1-modifying Slimb/FAF axis.

Next, to examine the molecular basis of the functional interaction between PAR-1 and Pod-1, we tested for possible physical association between them. Coimmunoprecipitation analysis was performed using fly head extracts overexpressing both PAR-1 and Pod-1-GFP-Myc. Pod-1-GFP-Myc was detected in the immunoprecipitate of PAR-1 by western blot analysis with anti-Myc or GFP antibody (Fig. 1K). These genetic and biochemical analyses provide clear evidence that Pod-1 functionally and physically interacts with PAR-1.

3.2. Pod-1 colocalizes with PAR-1

Since the role of PAR-1 has been well established for synaptic development at the NMJ (Zhang et al., 2007) (Lee et al., 2012), we sought to investigate the interaction between PAR-1 and Pod-1 at the larval NMJ in *Drosophila*. Prior to this analysis, we examined the localization of Pod-1 at the larval NMJ. Pod-1 is localized to the NMJ marked by HRP, which was consistent with the findings of a previous study (Liebl and Featherstone, 2008) (Fig. 2A–C). Next, to determine the colocalization of Pod-1 with PAR-1 in the NMJ, we carried out double-labeling immunohistochemistry experiments. Pod-1 appears largely overlapping with PAR-1 at the postsynaptic region (Fig. 2D–F), raising the possibility that Pod-1 may cooperate with PAR-1 to regulate NMJ development.

3.3. Pod-1 regulates PAR-1 function

As reported previously (Zhang et al., 2007), postsynaptic overexpression of PAR-1 results in the 27% loss of synaptic bouton number compared to control (Fig. 3A, D, and 3G). Although the overexpression or knockdown of Pod-1 alone in the postsynaptic region had no discernable effect on NMJ morphology, cooverexpression of postsynaptic Pod-1 and PAR-1 enhanced the NMJ morphological defects caused by the overexpression of PAR-1, showing the 51% reduction of bouton number compared to the control (Fig. 3B, C, E, and G). Moreover, the postsynaptic expression of Pod-1 RNAi was able to largely rescue the bouton-loss phenotype caused by PAR-1 overexpression (Fig. 3F and G). It is noted that no significant difference was found in muscle size in either genotype. In the presynaptic region where the overexpression of PAR-1 does not alter the NMJ morphology (Zhang et al., 2007), the overexpression or knockdown of Pod-1 did not show any abnormality and had no synergistic effect when combined with PAR-1 overexpression (Supplementary Fig.1).

Previous studies showed that Dlg was diffuse and less concentrated by PAR-1 overexpression, which, in turn, led to aberrant synaptic structure (Zhang et al., 2007). Since the PAR-1 overexpression effect is suppressed by Pod-1 knockdown, PAR-1-induced delocalization of Dlg might be restored by Pod-1 knockdown. To test this possibility, we examined the effect of Pod-1 knockdown on Dlg localization in a PAR-1 overexpression background. In *Mhc-GAL4>UAS-PAR-1* larval NMJ, Dlg signals were diffused at the postsynaptic compartments (Fig. 4D–F) compared to the control (Fig. 4A–C). This PAR-1-induced delocalization of Dlg was rescued by Pod-1 knockdown using Pod-1 RNAi (Fig. 4G–I) but not Pod-1-WT overexpression (data not shown). These results suggest that Pod-1

plays an important role in the postsynaptic region of the NMJ by regulating the synaptic function of PAR-1.

3.4. Pod-1 knockdown attenuates tau-induced synaptic toxicity

Next, we examined whether Pod-1 knockdown can ameliorate tau toxicity induced by PAR-1 activity. First, we examined whether Pod-1 regulates the tau-mediated synaptic toxicity in NMJ. Postsynaptic overexpression of human tau R406W mutation (htauM) leads to a strong reduction in the total number of boutons compared to the control (Lee et al., 2012) (Fig. 5A and B). Interestingly, this synaptic defect was blocked by Pod-1 knockdown (Fig. 5D and E), while overexpression of Pod-1-WT did not have an obvious effect (Fig. 5C and E). Next, to test whether the rescue by Pod-1 knockdown results from its effect on PAR-1 activity, we analyzed PAR-1 dependent phosphorylation of htau at Serine 262. The phosphorylation of htau was markedly attenuated by the knockdown of Pod-1 but increased by the overexpression of Pod-1 (Fig. 5F). The data, taken together, indicate that the downregulation of PAR-1 activity by Pod-1 knockdown may be responsible for the regulation of tau phosphorylation.

4. Discussion

The conserved kinase PAR-1/MARK has been known as a key regulator of cell polarity in diverse organisms. In *Drosophila*, PAR-1 was initially reported to regulate microtubule dynamics and/or stability during oocyte determination and differentiation (Cox et al., 2001)

(Shulman et al., 2000). More recently, PAR-1 has also been found to be involved in the synaptic development of the NMJ and dendritic pruning (Zhang et al., 2007) (Herzmann et al., 2017). Moreover, PAR-1 mediates the phosphorylation of tau, and its abnormal regulation can lead to neurodegeneration (Ando et al., 2016; Wang et al., 2007). This is consistent with the role of MARK, the mammalian homolog of PAR-1 (Yu et al., 2012). In light of the functional importance of PAR-1, regulators of PAR-1 have been pursued, and several proteins, including LKB1, FAF, and Slimb have been identified (Lee et al., 2012).

In this study, we found Pod-1, an actin-microtubule crosslinker, to be a novel regulator of PAR-1 through modifier screening. Pod-1 physically associates with PAR-1 and regulates the activity of PAR-1 in the targeting of the postsynaptic scaffold protein, Dlg, and in tau-mediated synaptic toxicity. *Drosophila* Pod-1 belongs to the conserved family of Coronin proteins involved in cell motility, morphogenesis, and trafficking. Most Coronin homologs are known to orchestrate the actin filament network by Arp2/3 based branching assembly and ADF/cofilin based disassembly. In mice, Coronin 1 and Coronin 6 were reported to interact with actin cytoskeletons or affect vesicle trafficking in synapses (Chen et al., 2014) (Suo et al., 2014). The *Drosophila* Pod-1 has the ability to directly crosslink actin and microtubule cytoskeletons (Rothenberg et al., 2003). Taken together with this molecular function of Pod-1, our data suggest that *Drosophila* Pod-1 modulates PAR-1 activity through the coordination of actin and microtubule cytoskeletons in synaptic development and toxicity. Further studies on the underlying molecular mechanisms are required to understand how Pod-1-mediated regulations of actin and/or microtubule cytoskeletons affect the activity

of PAR-1, which may help reveal the conserved molecular mechanism of Coronin homologs in synaptic development and function.

The dysregulation of PAR-1 is implicated in the pathogenesis of AD and related tauopathies. The pathogenic beta-amyloid, A β 42, induced tau phosphorylation mediated by PAR-1/ MARK, stabilizes microtubule-unbound tau and contributes to sequential phosphorylation and tau toxicity (Nishimura et al., 2004) (Ando et al., 2016). Our study revealed a novel regulatory mechanism of PAR-1-mediated tau toxicity by Pod-1. The knockdown of Pod-1 reduced PAR-1-mediated phosphorylation of tau and relieved the synaptic abnormality caused by pathogenic tau overexpression. Tau is a microtubule-associated protein, which stabilizes and promotes the assembly of microtubules, the dysregulation of which is related to neurotoxicity (Ittner and Gotz, 2011) (Burnouf et al., 2016). Meanwhile, the association of actin with tau-induced neurotoxicity has been recently unveiled, and tau has been shown to crosslink actin and microtubules (Cabrales Fontela et al., 2017). Our data support the possibility that the coordination of actin and microtubule could be critical to AD-related tau toxicity and suggest the investigation of Pod-1 in degenerative neurological diseases, including tauopathies.

Neuronal cytoskeletal impairment is strongly linked to neurodegenerative disease. Disturbance of the microtubule network accompany axonal retraction and affect axonal transport, which affects neurodegeneration processes. Indeed, alterations of microtubule modification and dysregulation of microtubule-associated proteins are involved in Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. Currently, several drugs that target cytoskeleton dysfunction, including a microtubule stabilizer, compounds targeting tubulin modification, and compounds targeting regulatory proteins of cytoskeleton dynamics, are being tested. Pod-1 could be another therapeutic target of AD as it augments tau-induced synaptic toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

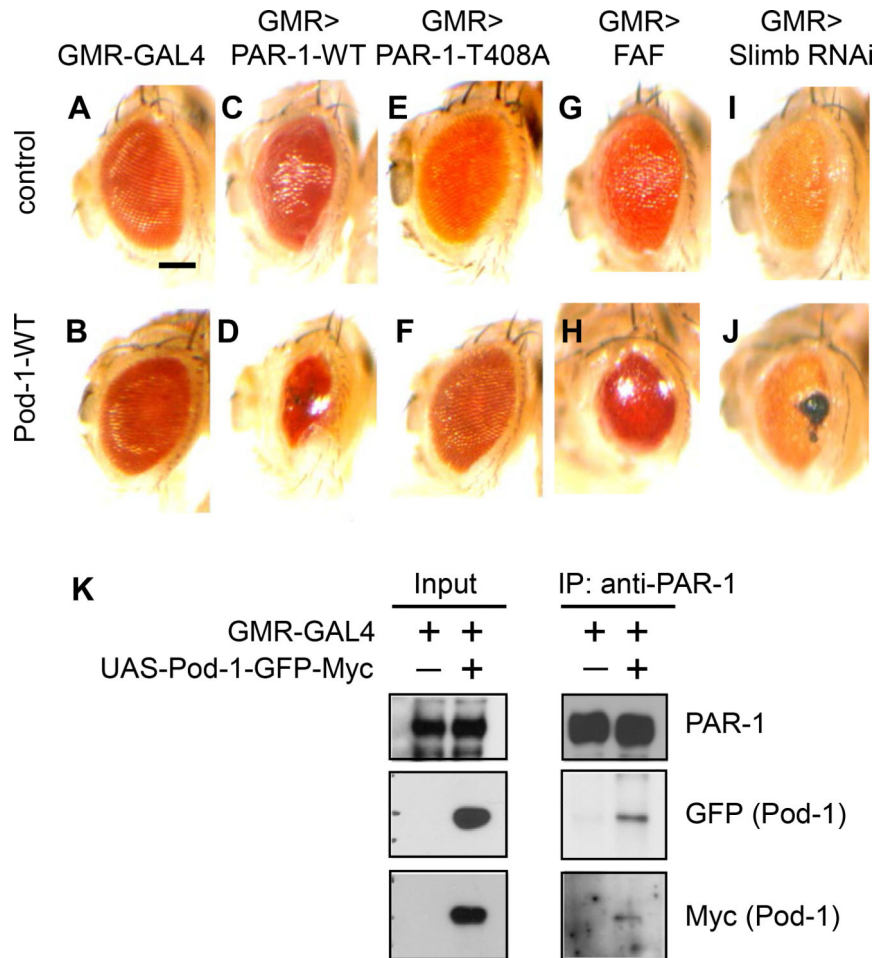
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**Fig. 1.**

Pod-1 functionally and physically interacts with PAR-1. (A–J) The interaction between PAR-1 and Pod-1 in the *Drosophila* eye. Note the severely reduced eye size in coexpressing PAR-1 and Pod-1 animal (1D). The genotypes are as follows: *GMR-GAL4* (A), *GMR-GAL4>UAS-Pod-1-WT* (B), *GMR-GAL4>UAS-PAR-1-WT* (C), *GMR-GAL4>UAS-PAR-1-T408A* (E), *GMR-GAL4>UAS-PAR-1-T408A+UAS-Pod-1-WT* (D), *GMR-GAL4>FAF^{EP381}* (G), *GMR-GAL4>FAF^{EP381}+UAS-Pod-1-WT* (H), *GMR-GAL4>UAS-Slimb RNAi* (I) and *GMR-GAL4>UAS-Slimb RNAi+UAS-Pod-1-WT* (J). Scale bar (A–J), 100 μ m.

(K) Coimmunoprecipitation of Pod-1 and PAR-1 in fly head samples. Fly head extracts from *GMR-GAL4>Pod-1-GFP-Myc* and control animals were immunoprecipitated with anti-PAR-1 antibody against PAR-1, and the presence of Pod-1-GFP-Myc in the immunoprecipitate was detected by western blot analysis with anti-Myc or GFP.

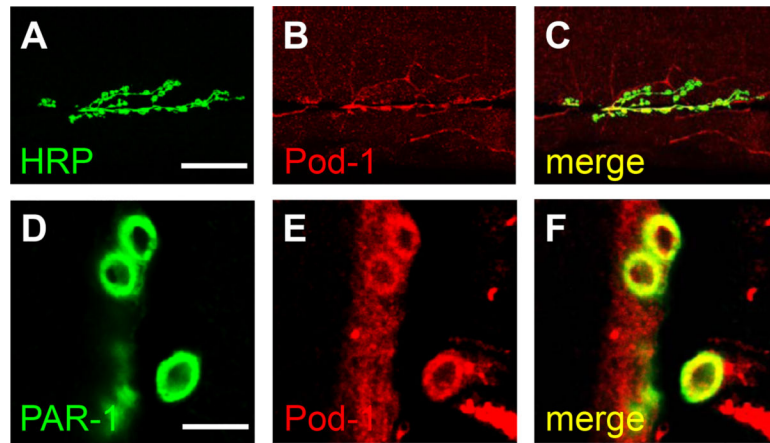


Fig. 2. Pod-1 is expressed at the *Drosophila* neuromuscular junction. (A–C) wild-type larvae stained with anti-HRP (A) and anti-Pod-1 (B) antibodies. Merged image is shown in (C). Scale bar (A–C), 50 μ m. (D–F) Pod-1 colocalizes with PAR-1. Immunostaining of the third instar larval NMJ stained with anti-PAR-1 (D) and anti-Pod-1 (E) antibodies. Merged image is shown in (F). Scale bar (D–F), 10 μ m.

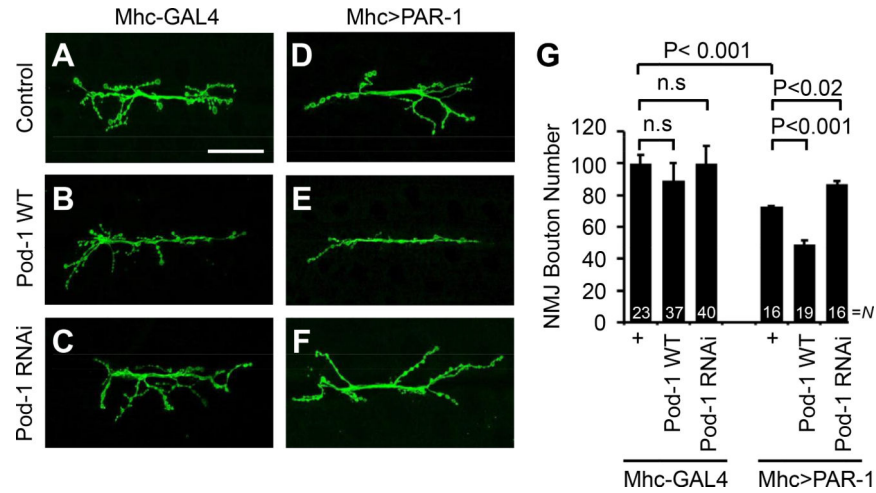


Fig. 3. Phenotypic interaction between PAR-1 and Pod-1 in *Drosophila* larval NMJ. (A–F) Representative NMJ terminals of the indicated genotypes revealed by anti-HRP immunostaining. The genotypes are *Mhc-GAL4*+/+ control (A), *Mhc-GAL4*>*UAS-Pod-1-WT* (B), *Mhc-GAL4*>*UAS-Pod-1-RNAi* (C), *Mhc-GAL4*>*UAS-PAR-1-WT* (D), *Mhc-GAL4*>*UAS-PAR-1-WT*+*UAS-Pod-1-WT* (E) and *Mhc-GAL4*>*UAS-PAR-1-WT*+*UAS-Pod-1-RNAi* (F). Scale bar (A–F), 50 μ m. (G) Quantification of data showing phenotypic interaction between PAR-1 and Pod-1 on the total number of boutons per muscle area on muscle 6/7 of A3. N indicates the number of animals analyzed in each group. Statistical significance was determined pair-wise using a two-tailed Student's *t*-test. n.s., not significant.

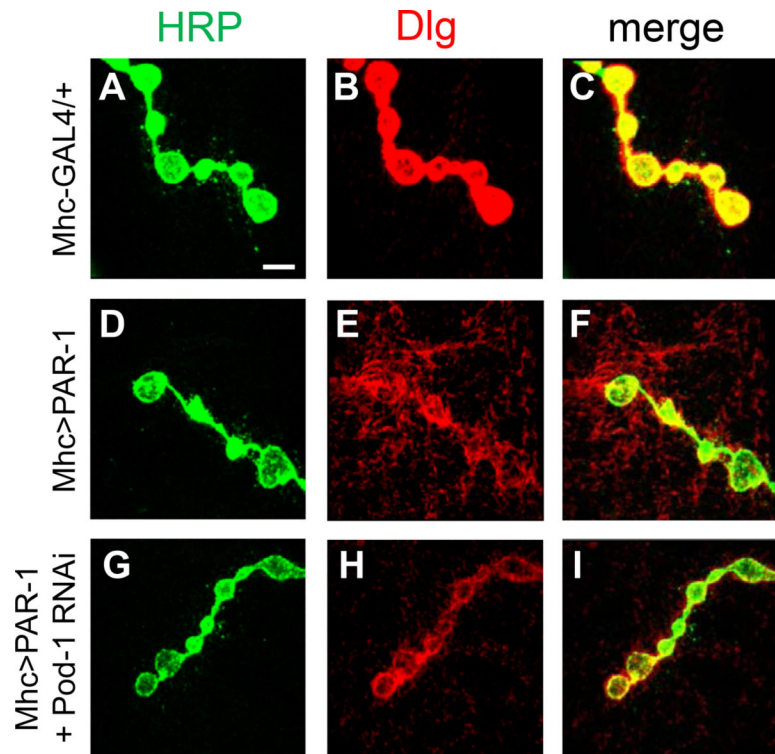


Fig. 4. Pod-1 knockdown rescues the Dlg delocalization caused by postsynaptic overexpressing PAR-1. Double-labeling at the third instar larval NMJ with anti-HRP in green, anti-Dlg in red, and merged images. The genotypes are as follows: *Mhc-GAL4/+* (A–C), *Mhc-GAL4>UAS-PAR-1-WT* (D–F), and *Mhc-GAL4>UAS-PAR-1-WT+UAS-Pod-1 RNAi* (G–I), Scale bar, 5 μ m.

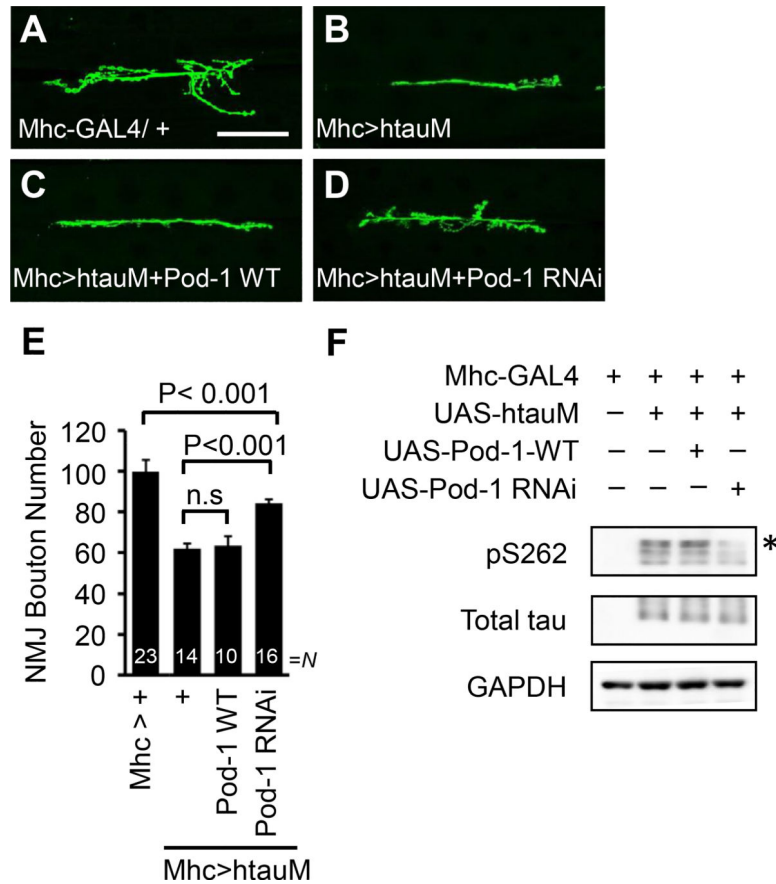


Fig. 5. Pod-1 knockdown reduces synaptic toxicity caused by htauM. (A–D) The interaction between Pod-1 and htauM in the *Drosophila* NMJ. Representative NMJ terminals of the indicated genotypes were shown by anti-HRP immunostaining. The genotypes are *Mhc-Gal4*>+ (A), *Mhc-Gal4*>*UAS-htauM* (B), *Mhc-Gal4*>*UAS-htauM*+*UAS-Pod-1-WT* (C) and *Mhc-Gal4*>*UAS-htauM*+*UAS-Pod-1 RNAi* (D). Scale bar (AeD), 50 μ m. (E) Quantification of data showing phenotypic interaction between htauM and Pod-1 on the total number of boutons per muscle area on muscle 6/7 of A3. N indicates the number of animals analyzed in each group. Statistical significance was determined pair-wise using a two-tailed Student's *t*-test. n.s., not significant. (F) Western blot analysis of pTau S262 and total tau after coexpression of Pod-1-WT and Pod-1 RNAi in an *Mhc-GAL4*>*UAS-htauM* background. The pTau S262 level in larval muscle wall extracts was increased by Pod-1-WT, whereas it was decreased by Pod-1 RNAi compared to control. pTau S262 proteins marked with an asterisk. GAPDH serves as a loading control.