RACK1 Knockdown Alleviates TDP-43 Associated Global Translational Suppression \textit{in vitro}, and Neurodegeneration \textit{in vivo}.

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INTRODUCTION

The receptor for activated C kinase 1 (RACK1) is a well-conserved scaffold protein with >100 recognized activities (1). Co-aggregation of RACK1 with TDP-43 inclusions has been observed in sporadic ALS (2), suggesting that it may be part of a pathogenic interactome involving the 2 proteins. While RACK1 knock-out is embryonic lethal in mice (3), we hypothesized that modulation of RACK1 through knockdown in mature cells and D. melanogaster (fruit flies) expressing pathogenic TDP-43 may provide a functional benefit.

DESIGN/METHODS

• **Cell culture and transfection.** RACK1 knockdown was achieved by transfecting a pool of 3 target-specific siRNA plasmids against human RACK1 using Lipofectamine RNAiMAX. HA-tagged cDNA plasmids encoding wild-type or dNLS-TDP43 (K82A/R83A/K84A) were transfected using Lipofectamine LTX.

• **Surface Sensing of Translation (SUnSET)** was performed as previously described (2), followed by immunocytochemical or biochemical detection of newly synthesized proteins using a puromycin-specific antibody.

• **Construction of RACK1 RNAi hTDP43 transgenic D. melanogaster lines.** UAS-Gal4 system was used to target expression of previously published alleles hTDP-43WT and hTDP-43Q331K (3) to retinal (GMR-driven) and motor (D42-driven) neurons (Fig. 1), cell populations widely used for their read-out of neuronal degeneration.

• **Retinal degeneration** in flies was scored according to the system published (4): 0 = normal; 1 = <25% ommatidia loss; 2 = 25-50% ommatidia loss; 3 = 50-75% ommatidia loss with small regions of necrosis (black patches); 4 = >75% ommatidia loss with massive regions of necrosis.

• **Motor function** was scored using a modification of a previously published system (3) wherein the height climbed by each fly in 10 seconds was measured every week for 28 days.

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Fig. 1: Schematic illustration for the construction of transgenic D. melanogaster lines specifically targeting retinal (GMR-driven) or motor (D42-driven) neurons.
Fig. 2: Immunohistochemical analysis of post-mortem spinal cord sections confirms that RACK1 co-aggregates with TDP-43 cytoplasmic inclusions (arrows) in sporadic ALS, as previously published (2).

Fig. 3: RACK1 KD alleviates dNLS-TDP43 (HA) aggregation. Z-stack images were acquired on a Leica TCS SP8 MP confocal microscope using a 63x oil objective to capture the full volume of cells. Statistics: Student t-test two-tailed unpaired. N=6 p-value ****: < 0.0001 Error bars: SEM.
Fig. 4 RACK1 KD leads to increased nuclear expression of dNLS-TDP43 (HA). Z-stack images were acquired on a Leica TCS SP8 MP confocal microscope using a 63x oil objective to capture the full volume of cells. Statistics: Student t-test two-tailed unpaired. 5-6 z-stacked images were acquired for each biological repeat. Error bars: SEM.

Fig. 5 SUnSET assay shows that RACK1 KD restores dNLS-TDP43 (HA) induced global translational (puromycin, PMY) suppression by immunocytochemistry (A) and Western blotting (B, C). Statistics: Ordinary one-way ANOVA Turkey multiple companions. n=4 p-value *: < 0.05; ***: < 0.001; ****: < 0.0001
Fig. 6. RACK1 KD ameliorates retinal neurodegeneration in GMR-driven hTDP43^WT and hTDP43^Q331K transgenic *D. melanogaster*. (A) Representative photos of fly eyes, transgene expression shown at adult day 1 (A1, A-D) or A6 (E-H,K,L) and un-driven controls shown at A6 (I,J). In eyes displaying mild degeneration, ommatidia are often missing from the ventral margin (arrows). Additionally, darker dots of dying ommatidia can be observed. hTDP43^WT causes mild neurodegeneration at A1 and persists to A6 (Chi^2 test, p<0.0001). This is fully rescued by RACK1-RNAi (p<0.0001). hTDP43^Q331K causes more severe neurodeneration than hTDP43^WT at A1, worsening over time (p<0.0001). In contrast, in eyes co-expressing RACK1-RNAi and hTDP43^Q331K, degeneration remains mild from A1-A6 (p<0.01). RACK1-RNAi alone (L) is comparable to control. Neurodegeneration scores are shown in the top right corner of each panel. (B) Kaplan-Meier curve shows the percentage of flies whose score remains at 1 on any given day. RACK1-RNAi significantly improves neurodegeneration caused by hTDP43^Q331K (Log-rank test: p=0.002. Error bars: 95% confidence intervals). (C) Histological analysis of retinal thickness of transgenic flies at A6 shows that retina thickness is significantly reduced by hTDP43^WT and hTDP43^Q331K, both of which are improved by RACK1-RNAi (ANOVA, p<0.05, n=4). (D) Structural organization and pigmentation of the retina are disrupted by hTDP43^WT, and markedly further by hTDP43^Q331K, both of which are improved by RACK1-RNAi. Small puncta of retinal pigment (green arrows) are sometimes observed in control and often in hTDP43^WT. Additionally, large puncta (orange arrows) are seen on expression of hTDP43^Q331K. RACK1-RNAi reduces both types of puncta in both hTDP43 transgenic fly eyes.

Fig. 7. hTDP-43^Q331K expression causes more severe motor defect than hTDP-43^WT in flies. Statistics: two-way ANOVA, p<0.0001. 83-140 flies were scored. Error bars: SD.

Fig. 8. RACK1 RNAi KD improves the motor defect caused by hTDP-43^Q331K expression in flies. Statistics: two-way ANOVA, p< 0.0001. 79-95 flies were scored. Error bars: SD.
DISCUSSION

Our results are consistent with the existence of a pathogenic interaction between TDP-43 and RACK1 in misfolded aggregates and support targeting of RACK1 to alleviate TDP-43 proteinopathy.

REFERENCES


ACKNOWLEDGEMENT

Supported by ProMIS Neurosciences