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Poly(ADP-ribose) glycohydrolase and poly(ADP-ribose)-interacting protein Hrp38 regulate pattern formation during *Drosophila* eye development

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ABSTRACT

Drosophila Hrp38, a homolog of human hnRNP A1, has been shown to regulate splicing, but its function can be modified by poly(ADP-ribosyl)ation. Notwithstanding such findings, our understanding of the roles of poly(ADP-ribosyl)ated Hrp38 on development is limited. Here, we have demonstrated that Hrp38 is essential for fly eye development based on a rough-eye phenotype with disorganized ommatidia observed in adult escapers of the *hrp38* mutant. We also observed that poly(ADP-ribose) glycohydrolase (*Parg*) loss-of-function, which caused increased Hrp38 poly(ADP-ribosyl)ation, also resulted in the rough-eye phenotype with disrupted ommatidial lattice and reduced number of photoreceptor cells. In addition, ectopic expression of DE-cadherin, which is required for retinal morphogenesis, fully rescued the rough-eye phenotype of the *hrp38* mutant. Similarly, *Parg* mutant eye clones had decreased expression level of DE-cadherin with orientation defects, which is reminiscent of DE-cadherin mutant eye phenotype. Therefore, our results suggest that Hrp38 poly(ADP-ribosyl)ation controls eye pattern formation via regulation of DE-cadherin expression, a finding which has implications for understanding the pathogenic mechanisms of Hrp38-related Fragile X syndrome and PARP1-related retinal degeneration diseases.

1. Introduction

Drosophila Hrb98DE/Hrp38 gene, a member of the hnRNP family of proteins, has two RNA-binding motifs and one glycine-rich region, having highest identity with human hnRNP A1 (Haynes et al., 1990). The *hrp38* gene has been shown to regulate alternative splicing, both *in vivo* and *in vitro*, using the S2 cell line (Blanchette et al., 2009; Borah et al., 2009; Shen et al., 1995). Alternative splicing is used extensively to produce the different mRNA isoforms of a gene to increase the complexity of the transcriptome in higher eukaryotic genomes (Nilsen and Graveley, 2010). It is generally believed that two groups of RNA-binding proteins, hnRNPs and serine-arginine-rich splicing factor (SR protein), regulate alternative splicing by binding with exonic and intronic splicing silencers (ESSs and ISSs) and enhancers (ESEs and ISEs), respectively (Matlin et al., 2005). However, emerging evidence suggests that hnRNPs can also bind with ESEs and ISEs to enhance splicing (Blanchette et al., 2009; Borah et al., 2009; Kiesler et al., 2005). In addition, the genome-wide analysis of the mRNA bound by hnRNP and SR proteins revealed that *Drosophila* hnRNPs regulate quite different sets of genes compared with SR proteins in *Drosophila* cell lines, challenging the current model that hnRNP and SR proteins have an antagonistic effect on splicing regulation (Blanchette et al., 2005).

As an alternative to changing the concentration of splicing factors in different tissues or developmental stages (Matlin et al., 2005), regulating the splicing activities of hnRNPs and SR proteins may be accomplished by post-translational modification via poly(ADP-ribosyl)ation (Gagne et al., 2003; Ji and Tulin, 2009, 2010; Malanga et al., 2008). Specifically, Hrp38 is modified at the post-translational level by poly(ADP-ribosyl)ation through the activity of poly(ADP-ribose) polymerase 1 (PARP1) in *Drosophila* (Ji and Tulin, 2009). In addition, protein poly(ADP-ribosyl)ation can be reversed by poly(ADP-ribose) glycohydrolase (PARG), which degrades poly(ADP-ribose) polymer (Hanai et al., 2004; Tulin et al., 2006). Consequently, Hrp38 is highly poly(ADP-ribosyl)ated in the *Parg* mutant (Ji and Tulin, 2009). Furthermore, it appears that poly(ADP-ribosyl)ation inhibits the RNA-binding ability of hnRNPs and can modulate the alternative splicing pathways (Ji and Tulin, 2009). Our recent study suggested that poly(ADP-ribosyl)ation regulates Hrp38-dependent translation of DE-cadherin by the inhibition of Hrp38 binding to 5′UTR of DE-cadherin mRNA (Ji and Tulin, 2012). Based on this evidence, it could be reasonably concluded that post-translational modification of hnRNPs by poly(ADP-ribose) is a novel mechanism that regulates such hnRNP-dependent pathways as splicing and translation.

Abbreviations: PARG, poly(ADP-ribose) glycohydrolase; PARP1, poly(ADP-ribose) polymerase 1; pADPr, poly(ADP-ribose); hnRNP/Hrp, heterogeneous nuclear ribonucleoproteins; SR protein, serine-arginine-rich protein; ESSs, exonic splicing silencers; ISSs, intronic splicing silencers; ESEs, exonic splicing enhancers; ISEs, intronic splicing enhancers; GSC, germline stem cell; 5′UTR, 5′ untranslated region; DE-cadherin, *Drosophila* epithelial cadherin; Df, deficiency; UAS, upstream activation sequence; rd1, retinal degeneration 1; RP, retinitis pigmentosa.

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Therefore, we have been further assessing whether hnRNP poly(ADP-ribosyl)ation regulates gene expression during development. In our previous study, we have demonstrated that Hrp38 poly(ADP-ribosyl)ation controls germline stem cell (GSC) self-renewal and oocyte localization during *Drosophila* oogenesis by regulating DE-cadherin translation (Ji and Tulin, 2012). Importantly, we note that DE-cadherin-mediated adherens junctions are required for retinal morphogenesis by organizing photoreceptor cell patterns and regulating ommatidial rotation (Tepass and Harris, 2007). Accordingly, in the present study, we further found that both Hrp38 loss-of-function and its poly(ADP-ribosyl)ation cause a rough-eye phenotype displaying disorganized ommatidia. As expected, the rough-eye phenotype in the *hrp38* mutant was rescued by overexpression of DE-cadherin in the eye, while the *Parg* mutant eye clones showed decreased expression of DE-cadherin. These results suggest that Hrp38 poly(ADP-ribosyl)ation plays a role during eye pattern formation by regulating DE-cadherin expression.

2. Materials and methods

2.1. Drosophila strains

Flies were cultured on standard cornmeal-molasses-agar media at 22 °C. *Hrb98DE/hrp38* GFP trap line (ZCL588) (Ji and Tulin, 2009; Morin et al., 2001), *P{w[+mC] = GAL4-ninaE.GMR}12* (stock number: 1104) and *P{w[+mC] = longGMR-GAL4}2* (stock number: 8605) were from the Bloomington *Drosophila* Stock Center. A P-element insertion of the *hrp38* gene (w^{*},*P[XP]*^{d05172}/*TM6B, Tb*^{1}), a *hrp38* region deficiency line (w^{1118}; *Df(3R)Exel6209, P{XP-U}Exel6209/TM6B, Tb*^{1}) and the UAS-Hrp38:RFP transgenic line were previously described (Ji and Tulin, 2012). The UAS-DE-cadherin:GFP (UAS-DEFL) transgenic line is a gift from the laboratory of Dr. Yamashita (Inaba et al., 2010). *Tubulin-DE-cadherin* transgenic line is a gift from Dr. Mark Van Doren (Mathews et al., 2006). Two *Hrp38* RNAi lines (w^{1118}; *P{GD14939}v29523* and w^{1118}; *P{GD14939}v29524/CyO*) were from the Vienna *Drosophila* RNAi Centre.

2.2. FRT/FLP clonal analysis

The *Parg* female heterozygotes (*Parg*$^{27.1}$/*FM7a,w*^{a}) (Hanai et al., 2004; Tulin et al., 2006) were crossed with w^{1118}, *sn*^{3}, *P{neoFRT}19A/Y* (Xu and Rubin, 1993) to generate the FRT-bearing *Parg* mutations (*Parg*$^{27.1}$, *P{neoFRT}19A/FM7a,w*^{a}) by genetic recombination. To induce the adult *Parg* mutant and wild-type eye clones, *Parg*$^{27.1}$, *P{neoFRT}19A/FM7a,w*^{a} or w^{1118}, *sn*^{3}, *P{neoFRT}19A/Y* was crossed with *P{GMR-hid}SS1, y*^{1} *w** *P{neoFRT}19A; P{GAL4-ey.H}SS5, P{UAS-FLP1.D}JD2* using the ey-Gal4/UAS-FLP/GMR-hid method (Stowers and Schwarz, 1999). To induce the *Parg* mutant eye disc clones in the third-instar larvae stage, *Parg*$^{27.1}$, *P{FRT(w*^{hs}*)101}*/FM7a,w^{a} (Ji and Tulin, 2012) was crossed with Ubi-GFP, *P{FRT(w*^{hshs}*)101}/Y*; *P{GAL4-ey.H}SS5, P{UAS-FLP1.D}JD2* to select the GFP mosaic eye imaginal disc.

2.3. Western blotting

Total protein (50 μg) from the wild-type fly, mutants (non-GFP homozygotes) at the wandering third-instar larvae stage, and the head and body of the *Parg* mutant mosaic adult was isolated and measured as described previously (Ji and Tulin, 2009). The proteins were then resolved in SDS–PAGE and transferred to nitrocellulose membrane (0.45 μm, Bio-Rad). The blot was incubated with rabbit anti-pADPr antibody (Calbiochem) at 1:1000 dilution. The signals were detected with horseradish peroxidase-conjugated secondary antiserum and ECL™ reagents (GE Healthcare). The blots were stripped and detected with mouse anti-α-tubulin antibody (DM1A, Sigma) at 1:1000 dilution.

2.4. Immunohistochemistry

The eye imaginal discs of the third-instar larvae were dissected in Grace's insect medium and fixed in 4% paraformaldehyde + 0.1% Triton X-100 in PBS for 20 minutes. Afterwards, the discs were incubated with mouse anti-Elav (1:10; DSHB) and Alexa Fluor 488 goat anti-mouse antibody (1:400; Invitrogen), respectively. The nuclear DNA was stained with DRAQ5 dye (Biostatus). The pupal retinae at 44 hours after puparation were dissected and stained with Alexa Fluor 633 phalloidin (1:40; Invitrogen) for 30 minutes. The adult eyes were dissected and stained with anti-rhodopsin (4C5) antibody (1:10, DSHB) based on the published protocol (Williamson and Hiesinger, 2010). All the images were visualized using the Leica TCS-NT confocal microscope.

2.5. Electron microscopy

For scanning EM, the dissected heads were fixed as for TEM, postfixed in 1% OsO4 for 3 hours, dehydrated in ethanol and critical point dried as described (Anderson, 1951). The samples were viewed on an Autoscan scanning electron microscope (ETEC, Hayward, CA). For ultrastructural analysis by transmission EM, the heads were dissected, fixed with 2% formaldehyde/2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) in 0.1% Triton X-100 overnight, postfixed for 1 hour with osmium tetroxide, dehydrated in ethanol and propylenoxide, and embedded in EMbed-812 (EMS, Fort Washington, PA) in flat molds. After polymerization for 60 hours at 65 °C, 70 nm sections were cut on a Leica UC6 microtome (Leica, Austria), placed on formvar/carbon-coated grids, and stained with 2% uranyl acetate/lead citrate. Sections were viewed on a Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR).

3. Results

3.1. Loss-of-function of the hrp38 gene causes the rough-eye phenotypes

Our previous study suggested that the *hrp38* gene is important for fly development because a full 75% of the *hrp38* hemizygotes (*hrp38*^{d05172}/*Df*) died before the pupa stage (Ji and Tulin, 2012). Interestingly, we observed that the adult escapers of the *hrp38* hemizygotes showed rough-eye phenotype with disorganized ommatidia and bristles (Figs. 1D and D′), while the wild-type fly had no defects (Fig. 1A). Occasionally, loss of one photoreceptor cell was observed in the ommatidia (Fig. 1E) compared to the wild type (Fig. 1B). In addition, Hrp38 expression was observed in the photoreceptor cells in the ommatidia in a Hrp38:GFP protein trap line (Fig. 1C). We further examined the larval eye disc stained with anti-Elav (a neuron marker) antibody in the wild-type and *hrp38* mutant. It appears that both *hrp38* mutant cells (Fig. 1H) and wild type (Fig. 1F) had normal specification of the photoreceptor fate. However, the pattern of photoreceptor cells in the *hrp38* mutant cells was irregular (Fig. 1H′) compared to the wild-type cells (Fig. 1F′). Moreover, the *hrp38* mutant pupal eye showed a disrupted ommatidial lattice (Fig. 1I) compared with the wild-type eye (Fig. 1G). Based on this evidence, we concluded that the rough-eye phenotype caused by Hrp38 loss-of-function results from disrupted pattern formation shown as early as at the larval stage.

3.2. Expression of Hrp38:RFP transgene in the eye rescued the rough-eye phenotype of the Hrp38 mutant

In addition, we used RNAi to knock down *hrp38* expression in the eye using two eye-specific GAL4 drivers (ninaE.GMR-Gal4 and longGMR-Gal4). While ninaGMR-Gal4 heterozygotes, as the control, had the wild-type eye phenotype (Figs. 2A and A′), *hrp38* RNAi induced by ninaE.GMR-Gal4 driver also caused the rough-eye phenotype with disrupted ommatidia and bristle organizations (Figs. 2B and B′). However, hrp38 knockdown by longGMR-Gal4 driver did