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Drosophila globin1 is required for maintenance of the integrity of F-actin based cytoskeleton during development

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ABSTRACT

Hemoglobins (Hbs) are evolutionarily conserved small globular proteins with characteristic 3-over-3 α-helical sandwich structure that is typically known as "globin fold". Hbs have been found to be involved in diverse biological functions and the characteristic property of oxygen transportation is relatively a recent adaptation. *Drosophila* genome possesses three *globin* genes (*glob1*, *glob2,* and *glob3*) and it was previously reported that adequate expression of *glob1* is required for various aspects of development, and also to regulate the cellular level of reactive oxygen species (ROS). The present study illustrates the explicit role of *glob1* gene in *Drosophila* development. We demonstrate a dynamic expression pattern of *glob1* in larval tissues which largely concentrate around F-actin rich structures and also co-precipitate. Reduced expression of *glob1* leads to developmental abnormalities which appeared to be largely mediated by inappropriately formed F-actin based cytoskeletal structures. Our subsequent analysis in FLP/FRT mediated somatic clones establishes specific role of *Drosophila glob1* in maintenance of the integrity of F-actin based cytoskeleton during development. For the first time, we report interaction between Glob1 and actin, and propose a novel role of *glob1* in maintenance of F-actin based cytoskeleton in *Drosophila.*

1. Introduction

Hemoglobins (Hbs) could be best described as heme-containing metallo-proteins which belong to the "Globin" protein family and are characterized by the presence of the distinctive "globin fold" [1,2]. The globin fold is constituted by eight independent α helices arranged as a two-layered structure and is popularly known as "three-on-three α helical sandwich fold" [3]. Though, the evolutionarily conserved globins have been mostly studied as a group of oxygen (O_2) binding proteins those are involved in storage and distribution of gaseous O_2; however, it is increasingly clear now that *globin* genes were initially evolved to protect the prevailing primitive single-cell anaerobic organisms from the toxic effects of O_2 by sensing and scavenging it, to detoxify the nitric oxide, and to provide resistance against nitrosative stress [1,2]. With time, Hbs diversified in various organisms and acquired relatively novel and diverse functions according to the need of organisms which were evolving in different climatic conditions. Interestingly, some recent report strongly suggest that in spite of a common lineage, the evolutionary process has shaped the globin gene family to be dynamically diverse across the animal kingdom [2,4,5].

Three conserved *globin* genes (i.e. *glob1*, *glob2* and *glob3*) in *Drosophila* have been considered as typical Hbs since the protein encoded by them exhibit characteristic globin fold [6]. Occurrence of multiple *globin* genes in *Drosophila* appears to be astonishing as it has a well-developed tracheal network to accomplish the O_2 requirement for their small body and moreover, there is no obvious hypoxic stage during their life cycle [6,7]. Therefore, it is rather unfeasible that multiple *globins* in *Drosophila* are involved in O_2 management only. We have previously demonstrated that *glob1* expresses ubiquitously during embryonic and larval development with relatively higher level in physiologically active tissues [8]. Reduced expression of *glob1* resulted in manifestation of various developmental and cellular anomalies along with embryonic and larval lethality, increased level of cellular reactive oxygen species (ROS), poor musculature, locomotor and flight impairments, early aging, premature death of adult escapers etc., and several of these phonotypes seemed to be driven by pleotropic effect(s) of *glob1* mutation [8]. Therefore, we wanted to investigate the precise role of *glob1* during development, which in turn leads to various phenotypes as noted above. We demonstrate dynamic distribution pattern of *glob1* protein and its specific association with F-actin rich cellular structures. Our study for the first time suggests an explicit and indispensable role of *Drosophila glob1* in the maintenance of F-actin based cytoskeleton

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during development.

2. Materials and methods

2.1. Fly stocks and genetics

The *Drosophila* stocks used in this study were reared on standard agar/cornmeal/yeast media at 24 ± 1 °C and 12:12 h light-dark cycle. The fly stocks were obtained from Bloomington stock centre Indiana, USA. *Oregon* R^+ was used as wild type strain. A P-insertion mutant line of *glob1*, y^1w^{67c23}; + / + ;ry^{506}*P{SUPor-P}glob1*^{KG06649}*/TM3,Sb* (in subsequent text, *yw;* + / + *;P-glob1/P-glob1* has been used to designate the homozygous line) was comprehensively investigated earlier and established as loss of function allele of *glob1* [8]. *w;UAS-glob1RNAi/UAS-glob1RNAi;* + / + [9] and *w;* + / + *;UAS-glob1/UAS-glob1* [8] were utilized to achieve tissue specific RNAi mediated downregulation or upregulation of *glob1* respectively. w^{1118}; + / + *;Df(3R)Exel8162/TM6B,Tb* is a deficiency line with a defined deletion spanning the entire *glob1* genomic region (89A5;89A8) [10]. The *w;* + / + *;Act5c-Gal4/TM6B,Tb* Gal4 driver line [11] and *w;GMR-Gal4/GMR-Gal4;* + / + [12] were utilized to induce either upregulation or RNAi mediated downregulation of the desired transgene. The fly stocks *yw;* + / + *;P{ry*$^{+t7.2}$ *= neoFRT}82B, w**; + / + *;P{ry*$^{+t7.2}$ *= neoFRT}82B P{w*$^{+mC}$ *= Ubi-GFP.D}83* and w^{1118}; *P{ry*$^{+t7.2}$ *= 70FLP}10;* + / + were used to generate FLP/FRT mediated somatic clones [13].

2.2. Generation of somatic clones

Homozygous *P-glob1* mutant somatic clones were generated in third instar larval tissues via FLP/FRT mediated site specific recombination [13–15]. The *P-glob1* mutation was recombined onto *P{ry*$^{+t7.2}$ *= neoFRT}82B* carrying chromosome and somatic clones were generated by giving heat shock to desired genotype at appropriate developmental time to flip recombinase (FLP) mediated recombination. Larvae with *yw;hsFLP/* + ; *P{ry*$^{+t7.2}$ *= neoFRT}82B P-glob1/P{ry*$^{+t7.2}$ *= neoFRT}82B P{w*$^{[+mc]}$ *= Ubi-GFP.D}83* (non-tuby) genotype were dissected and examined for presence of somatic clones as per their GFP pattern [15].

2.3. Immunohistochemistry

Immunostainings were carried out as described earlier [8]. The following primary antibodies were used: rabbit anti-*Drosophila* Glob1 antibody (1:500) [16] and anti-β3 tubulin (1:5000) [17]. Secondary antibody goat anti-rabbit-Alexa-488 (A11008) (Molecular probes, Oregon, USA) was used at dilution of 1:200. TRITC-Phalloidin (1:200) (P1951, Sigma Aldrich, USA) was used to stain F-actin cytoskeleton. DAPI (4′,6-diamidino-2-phenylindole) (5 µg/ml, Roche Diagnostics, GmbH, Germany) was utilized to mark the cell nuclei.

2.4. Co-immunoprecipitation-western blot analyses

Protein isolation was performed by homogenizing third instar larval tissues in lysis buffer [150 mM NaCl, 50 mm Tris-HCl (pH 8), 1% NP-40, 5% glycerol, cocktail of Halt™ phosphatase inhibitor and cocktail of Halt™ protease inhibitor] (ThermoFisher Scientific, USA). Protein concentration was estimated by Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA). Pre-cleared 2 mg protein lysate was incubated overnight with rabbit anti-*Drosophila glob1* antibody at 4 °C. Pre-equilibrated Protein A Sepharose 4B (ThermoFisher Scientific, USA) bead slurry was added to protein lysate at 4 °C for 2 h for pull down and subsequently centrifuged at 4000 rpm for 2 min at 4 °C. The pelleted anti-*Drosophila glob1* antibody coated beads were washed five times with wash buffer [150 mM NaCl, 50 mm Tris–HCl (pH 8), 0.5% NP-40, 5% glycerol and cocktail of Halt™ phosphatase inhibitor] (ThermoFisher Scientific, USA). The bound proteins were eluted by boiling in 1XSDS dye [10% w/v SDS, 10 mM β-mercaptoethanol, 20% w/v Glycerol, 0.2 M Tris-HCl (pH 6.8), 0.05% w/v bromophenol blue] and were separated by SDS-PAGE (12% resolving and 4% stacking gel). The protein samples were transferred to nitrocellulose membrane (Millipore, USA) by overnight wet-transfer, blocked in blocking buffer followed by overnight incubation at 4 °C with anti-β-actin antibody (AC-15) (1:5000) (Pierce, ThermoFisher Scientific, USA). A reciprocal test was also carried out by incubating the lysate in anti-β-actin antibody and subsequently challenging it with anti-Glob1 antibody. Membrane was incubated with HRP conjugated secondary antibody and the blot was developed after appropriate washing using Pierce™ ECL Western Blotting Substrate kit (ThermoFisher Scientific, USA) and images were captured in Fujifilm imaging system (LAS-4000).

2.5. Microscopy and documentation

Fluorescence stained tissues were preliminarily observed under fluorescent microscope (Olympus DP71) and subsequently analysed and imaged using Leica TCS-SP5 II confocal microscope by keeping identical parameters during comparative analysis. Similar numbers of confocal optical sections were selected for constructing the projection images by Leica application suite advanced fluorescence software. Images were assembled by Adobe Photoshop CS5 software. After each staining experiment, total number of embryos/tissues with various cellular and staining defects were calculated and plotted on the graph with MS Excel 2010 software.

2.6. Statistical analysis

The error bars on figures represent the mean ± SD of all determinations. For analysis of statistical difference between two groups, Student's *t*-test was applied. Differences between two groups were considered to be significant at *p-value ≤ 0.05, **p-value ≤ 0.01 and ***p ≤ 0.001.

3. Results

3.1. Reduced expression of glob1 leads to severe cytoskeletal defects during embryogenesis

We have reported earlier that reduced expression of *glob1* in *P-glob1/P-glob1* homozygous or *P-glob1/Df(3R)Exel8162* trans-heterozygotes or *UAS-glob1RNAi/Act5c-Gal4* transgenic flies results in several identical phenotypes including fitness and flight impairments, early aging and premature death of the adult escapees [8]. In *P-glob1/P-glob1* mutant line, insertion of a P-element in the promoter region of *glob1* (at position 3R: 11,727,123) resulted in over two-fold decrease in the expression level, and *Df(3R)Exel8162* represents a deficiency line with a defined deletion spanning the entire *glob1* genomic region (89A5;89A8) [8,10]. Subsequently, due to fragile tissue tenacity in above noted genotypes, we examined the somatic musculature and cellular cytoskeletal status in *P-glob1/P-glob1* flies during development, and substantiated further in *P-glob1/Df(3R)Exel8162* and *UAS-glob1RNAi/Act5c-Gal4* lines. The F-actin based cytoskeleton (a major constituent of cellular cytoskeleton) was visualized by staining the embryos with Phalloidin-TRITC which specifically binds to F-actin and prevents depolymerization by latching the adjoining subunits together [18]. Stage 17 embryos were selected to examine the somatic musculature since the muscle development is completed by then and fully illustrated muscle pattern could be observed [19]. The muscle pattern in the embryos has been divided into six hemisegment as A2-A7 which contain the same stereotyped pattern of 30 muscle fibres with defined size, shapes, and epidermal attachment sites [20]. Interestingly, compared to distinctly organised F-actin in somatic musculature and segmentally recurring arrangement of muscle fibres in wild type embryos (Fig. 1A, n = 94), ~85% of *P-glob1/P-glob1* and ~78% of *UAS-glob1RNAi/Act5c-Gal4* embryos (n = 136) displayed loosely organised poor architecture of

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