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The SCF ubiquitin ligase Slimb controls Nerfin-1 turnover in *Drosophila*

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ABSTRACT

The C2H2 type zinc-finger transcription factor Nerfin-1 expresses dominantly in *Drosophila* nervous system and plays an important role in early axon guidance decisions and preventing neurons dedifferentiation. Recently, increasing reports indicated that INSM1 (homologue to nerfin-1 in mammals) is a useful marker for prognosis of neuroendocrine tumors. The dynamic expression of Nerfin-1 is regulated post-transcriptionally by multiple microRNAs; however, its post-translational regulation is still unclear. Here we showed that the protein turnover of Nerfin-1 is regulated by Slimb, the substrate adaptor of SCF^{Slimb} ubiquitin ligase complex. Mechanistically, Slimb associates with Nerfin-1 and promotes it ubiquitination and degradation in *Drosophila S2R*$^{+}$ cells. Furthermore, we determined that the C-terminal half of Nerfin-1 (Nerfin-1^{CT}) is required for its binding to Slimb. Genetic epistasis assays showed that Slimb misexpression antagonizes, while knock-down enhances the activity of Nerfin-1^{CT} in *Drosophila* eyes. Our data revealed a new link to understand the underlying mechanism for Nerfin-1 turnover in post-translational level, and provided useful insights in animal development and disease treatment by manipulating the activity of Slimb and Nerfin-1.

1. Introduction

Caenorhabditis elegans EGL-46 as well as vertebrate INSM1/2 (also known as IA-1/2) and *Drosophila* Nerfin-1/2 (Nervous fingers 1/2) compose of the EIN protein family marked by highly conserved zinc-finger motifs [1–3]. In this protein family, the human INSM1 (insulinoma-associated 1, formerly named as IA-1) was initially identified as the founder member [4] and expressed highly in the developing nervous system and neuroendocrine tumors [5]. Recent studies indicated that INSM1 is a useful marker for pancreatic neuroendocrine tumor [6] and small cell lung cancer [7]. In *Drosophila*, Nerfin-1 expression is implicated in early axon guidance decisions and neuroblasts (NBs) terminal differentiation and maintenance [8]. Loss of Nerfin-1 led to dedifferentiation and neural tumors through either activating Myc- and TOR-mediated cellular growth in most of neurons [9], or derepressing Notch signaling in medulla neuron of the optic lobe [10]. In *Drosophila* central nervous system (CNS), the expression of Nerfin-1 was reported to be regulated post-transcriptioally by multiple microRNAs [11,12]. However, the post-translational regulation of Nerfin-1 is still unclear.

Maintaining a correct concentration is critical for protein function. The final protein amount is determined by the relative ratio of the protein synthesis and protein turnover. Particularly, the post-transcriptional and post-translational regulation empower cells responding rapidly and selectively to cellular or environmental changes [13]. Ubiquitin-proteasome system (UPS) is one of the main post-translational regulation of protein turnover. Through a series of concerted enzymatic reactions, target proteins are covalently marked with poly-ubiquitin chain(s) and directed to proteasome for degradation [14,15]. UPS-mediated protein turnover is important for protein homeostasis and involved in a multitude of cellular processes including cell growth, proliferation, differentiation and apoptosis. In the UPS pathway, ubiquitination is sequentially mediated by three enzymes: ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligases E3. The proteins with RING-domain or HECT-domain are two main types of the ubiquitin ligases E3, which is in charge of the substrate specificity [16–18]. The SCF (Skp–Cullin–F-box) complex, a well-known RING-domain containing E3 ligase, is composed of the adaptor Skp1, the scaffold protein Cullin, the RING-domain protein Roc1/Rbx1/Hrt1 and the substrate adapters F-box protein [19]. In

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Drosophila, one of the best characterized F-box protein Slimb negatively regulates multiple signaling pathways by recognizing different substrates for proteasome-mediated degradation, such as ARM in Wingless pathway, Ci in Hedgehog signaling, Dorsal in NF-κB signaling, and Ex and Smurf in Hippo signaling [20–26]. Recently, Slimb was reported to participate in the development of *Drosophila* nervous system [27,28]. In this study, we identified that Slimb associates with Nerfin-1 and promotes its UPS-dependent degradation.

2. Materials and methods

2.1. Plasmid construction

pAc-Slimb-3HA plasmid is gifted from Dr. Renjie Jiao [29]. The full-length *pUAST-attB-Nerfin-1-flag* was amplified from *Drosophila* cDNA library by PCR and ligated into the expression vector of pUAST-attB. The truncation forms of *Nerfin-1*, *pUAST-attB-Nerfin-1^{NT}-flag* (1–208 amino acid) and *pUAST-attB-Nerfin-1^{CT}-flag* (181–469 amino acid), were constructed using *pUAST-attB-Nerfin-1-flag* as template and verified by DNA sequencing.

2.2. S2R$^+$ cell culture, transfection, and western blotting

Drosophila S2R$^+$ cells were cultured in Schneider's Insect Medium (S9895, Sigma-Aldrich) with 10% fetal bovine serum at 27 °C. X-treme GENE HP DNA Transfection Reagent (6366236001, Roche) was used for transient transfection according to the manufacturer's instructions. After 36 h transfection, cells were harvested and lysed in 2 × SDS protein loading buffer (0.25 M Tris–HCl pH 6.8, DTT 78 mg/mL, SDS 100 mg/mL, 50% Glycerine, and 5 mg/mL bromophenol blue) and boiled for 5 min before western blot analysis. The following antibodies were used: mouse anti-flag (1:5000, F1804, sigma), rabbit anti-HA (1:3000, C29F4, CST), mouse anti-tubulin (1:5000, UM4003, Utibody), mouse anti-ubiquitin (1:2000, SC-166553, santa cruz).

2.3. Co-immunoprecipitation (Co-IP)

Transfected *S2R$^+$* cells were lysed with NP-40 buffer (150 mM NaCl, 1% Triton X-100, 10 mM Tris pH 7.4, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 0.5% NP-40) with protease inhibitors (1 mM PMSF; cOmplete, 4693132001, Roche) at 4 °C for 30 min. The supernatants were incubated with primary antibody at 4 °C for 2 h, followed by incubation with protein A/G beads (10080881, GE) for 1 h. Beads were washed five times with NP-40 lysis buffer and then subjected to western blotting analysis.

2.4. Immunofluorescence

S2R$^+$ cells were seeded onto coverslips in 24-well plates. 36 h after transfection, cells were fixed with 4% formaldehyde for 20 min at room temperature and permeabilized with 0.5% Triton X-100 for 20 min. After blocked by 3% BSA, cells were incubated with primary antibody over night at 4 °C. Then cells were incubated with secondary antibodies, Alexa Fluor 488 goat anti-Rabbit IgG (1:1000, Invitrogen) and Alexa Fluor Cy3 goat anti-mouse IgG (1:1000, Invitrogen), for 2 h at room temperature. The nuclei of the cells were stained with DAPI (5 mg/mL). Cells were observed under a Zeiss microscopy at 63 × magnification.

2.5. In vivo ubiquitination assay

36 h after transfection, *S2R$^+$* cells were treated with MG132 (10 mM final concentration) for 4 h and proteins were enriched by immunoprecipitation and analyzed by western blotting.

2.6. Drosophila stocks and culturing conditions

The following *Drosophila* strains were used in this study: *UAS-Slimb* (gifted from Dr. Yun Zhao), *UAS-Slimb^{RNAi}* (BL31056; obtained from Bloomington Drosophila Stock Center, Indiana University, Bloomington, Indiana, USA). *UAS-Nerfin-1^{NT}-flag* and *UAS-Nerfin-1^{CT}-flag* (generated in this study). Fly stocks were maintained at 25 °C and fed on standard diet.

3. Results

3.1. Slimb associates and co-localizes with Nerfin-1 in the nucleus

Using co-affinity purification and spectrometry analysis, the potential interaction between Nerfin-1 and Slimb was identified in *Drosophila S2R$^+$* cells [30]. To confirm the protein-protein interaction between Nerfin-1 and Slimb, co-immunoprecipitation (Co-IP) assay was performed. Our result showed that Nerfin-1 bound to Slimb when Nerfin-1-flag and Slim-3HA were co-expressed into *S2R$^+$* cells (Fig. 1A). Consistently, our immunofluorescence assay showed that Nerfin-1 and Slimb were dominantly locates to the nucleus and cytoplasm respectively when expressed each alone. However, Slimb was mainly translocated and co-localized with Nerfin-1 in the nucleus when Nerfin-1 was co-expressed (Fig. 1B). These results indicated that Slimb may functionally associate with Nerfin-1 in the nucleus.

3.2. Slimb promotes Nerfin-1 ubiquitination and degradation

Slimb binds to its substrates and promotes them degradation through UPS. Thus, we next explored whether Slimb regulates the stability of Nerfin-1. As expected, the protein level of misexpressing Nerfin-1 was dramatically reduced when Slimb was co-expressed in a gradient manner (Fig. 2A). Furthermore, Slimb-mediated degradation of Nerfin-1 was suppressed by the proteasome inhibitor MG132, but not by lysosome inhibitor BA1 (Bafilomycin A1), suggesting the degradation of Nerfin-1 is proteasome dependent (Fig. 2B). Indeed, the poly-ubiquitination of Nerfin-1 was increased upon co-expressing Slimb in our ubiquitination assay (Fig. 2C). We also observed that Slimb associates with Nerfin-2 (a paralogue of Nerfin-1 in *Drosophila*) and promotes its degradation (data not shown). Together, we provided evidences that Slimb regulates the turnover of Nerfin-1 through ubiquitination-proteasome mediated degradation.

3.3. Nerfin-1^{CT} is essential for binding to Slimb

Nerfin-1 contains three C2H2 zinc finger motifs in its C-terminal half. To identify the essential domain for binding to Slimb, two overlapping Nerfin-1 truncations, Nerfin-1^{NT} (1–208 amino acid) and Nerfin-1^{CT} (181–469 amino acid, contain three zinc fingers), were co-expressed with Slimb in *S2R$^+$* cells. Co-IP assays showed that only Nerfin-1^{CT} interacted with Slimb (Fig. 3A). Consistently, Slimb translocated and co-localized with Nerfin-1^{CT} in the nucleus, but not with Nerfin-1^{NT} (Fig. 3B). The immunofluorescence assay results also suggested that the lost interaction between Slimb and Nerfin-1^{NT} was not due to the low expression of Nerfin-1^{NT} (Fig. 3A and B). Since Nerfin-1^{CT} is sufficient to binding Slimb, we wondered to know whether Slimb regulated the stability of Nerfin-1^{CT}. Intriguing, Nerfin-1^{CT} protein level also decreased when co-express with Slimb (Fig. 3C), indicating the Slimb-mediated ubiquitination site(s) may locate in Nerfin-1^{CT}. Taken together, these results indicated Nerfin-1^{CT} is essential and sufficient for Slimb-binding and Slimb-mediated protein degradation.

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