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FBXL5 interacts with p150^{Glued} and regulates its ubiquitination

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Abstract

The microtubule motor cytoplasmic dynein and its activator dynactin drive vesicular transport and mitotic spindle organization. p150^{Glued} is the dynactin subunit responsible for binding to dynein and microtubules. The F-box proteins constitute one of the four subunits of ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box), which governs phosphorylation-dependent ubiquitination and subsequent proteolysis. Our recent study showed that the proteolysis of mitotic kinesin CENP-E is mediated by SCF via a direct Skp1 link [D. Liu, N. Zhang, J. Du, X. Cai, M. Zhu, C. Jin, Z. Dou, C. Feng, Y. Yang, L. Liu, K. Takeyasu, W. Xie, X. Yao, Interaction of Skp1 with CENP-E at the midbody is essential for cytokinesis, Biochem. Biophys. Res. Commun. 345 (2006) 394–402]. Here we show that F-box protein FBXL5 interacts with p150^{Glued} and orchestrates its turnover via ubiquitination. FBXL5 binds to p150^{Glued} *in vitro* and *in vivo*. FBXL5 and p150^{Glued} co-localize primarily in the cytoplasm with peri-nuclear enrichment in HeLa cells. Overexpression of FBXL5 promotes poly-ubiquitination of p150^{Glued} and protein turnover of p150^{Glued}. Our findings provide a potential mechanism by which p150^{Glued} protein function is regulated by SCFs.

Keywords: Ubiquitin; F-box proteins; FBXL5; p150^{Glued}; Dynactin; CENP-E

The ubiquitin–proteasome system is responsible for the degradation of a large number of cellular proteins, and thus has an important role in many different cellular processes, such as cell cycle regulation and neuron degeneration [1,2]. Target proteins are labeled with small soluble protein ubiquitins through a multi-step reaction which is catalyzed sequentially by an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3) [3]. Then poly-ubiquitinized proteins are transported into proteasomes where they are finally reorganized and destroyed [4]. One most well characterized E3 ubiquitin ligase is the SCF (Skp1-Cullin-F-box protein) complex, whose substrate specificity depends on its component F-box proteins (FBP) [5]. F-box proteins are reported to bind Skp1 and cullins through their F-box domain, and recognize and recruit substrate proteins to the core SCF through their Leucine-rich repeat (LRR) domain, WD-40 repeat (WD) domain or other protein–protein interaction domains [6]. FBXL5 is an F-box protein containing a conserved F-box domain at its N-terminal first three and four predicted Leucine-rich repeats (Fig. 1A), identified and nominated by Jin et al. [7].

Dynactin is a large multi-subunit protein complex that binds motor dynein and activates dynein's long-range trafficking of diverse vesicles and organelles along microtubules [8]. Among all the dynactin subunits, p150^{Glued} (DCTN1), first identified and cloned from

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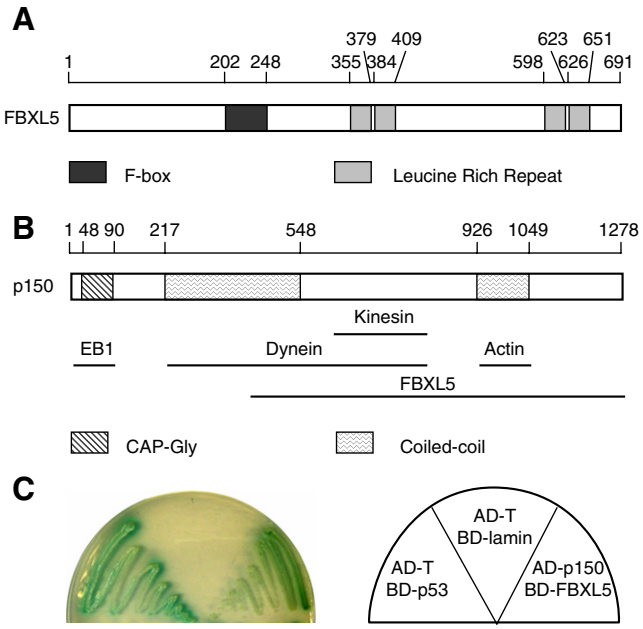

Fig. 1. Yeast two-hybrid screen identified a FBXL5-binding protein, p150^{Glued}. (A) Schematic diagram of FBXL5 protein structure. FBXL5 contains an F-box domain and four Leucine-rich repeats. (B) Schematic diagram of p150^{Glued} structure features. p150^{Glued} contains CAP-Gly and two coiled-coil domains. Beelines show the p150^{Glued} interaction fragment with indicated proteins. The lowest line represents the p150^{Glued} fragment binding FBXL5, which was decided by the yeast two-hybrid data. (C) FBXL5 interacts with p150^{Glued} in AH109 yeast cells. AH109 cells co-transformed with indicated plasmids were streaked on SD/–Ade/–His/–Leu/–Trp/X-α-Gal medium. Specific interaction between FBXL5 and p150^{Glued} was visually detected by X-α-Gal.

Drosophila melanogaster [9], is the largest subunit and has a particularly important function in both dynein's binding and its activation [10–12]. p150^{Glued} comprises a CAP-Gly (Cytoskeleton-Associated Protein, Glycine-rich) [13] motif in its extreme N-terminal fragment, and two coiled-coils (Fig. 1B). p150^{Glued} is diffusely distributed in the cytoplasm, and conspicuously accumulates at microtubule plus ends and centrosomes [14,15]. Defects in degradation of p150^{Glued} may cause an increase of dynein and dynactin aggregation accompanied by enhanced cell death, which underlies extensive neuron degeneration diseases [16]. However, the molecular mechanism of p150^{Glued} degradation is unclear.

In this study, we performed yeast two-hybrid screen and identified p150^{Glued} as a binding partner of FBXL5. In addition, we utilized immunoprecipitation and GST pull down assays and validated that FBXL5 forms a complex with p150^{Glued} both *in vivo* and *in vitro*. In addition, our study showed that FBXL5 and p150^{Glued} both co-localize to the cytoplasm. More importantly, our study demonstrated that p150^{Glued} is a poly-ubiquitination substrate of FBXL5, and the existence of FBXL5 significantly increases the instability of p150^{Glued}, implying that FBXL5 may play a role in neuron degeneration diseases.

Materials and methods

Reagents. Mouse monoclonal antibody to p150^{Glued} was purchased from BD Biosciences Pharmingen (San Diego, CA). Mouse monoclonal antibody to GST was purchased from Cell Signaling (Beverly, MA). Mouse monoclonal antibody to ubiquitin was purchased from Chemicon (Temecula, CA). Anti-FLAG antibody, anti-FLAG-conjugated agarose beads, 4,6-diamidino-2-phenylindole (DAPI), MG132, and Cycloheximide (CHX) were purchased from Sigma Chemicals (St. Louis, MO). Glutathione Sepharose was purchased from Amersham Biosciences (Piscataway, NJ). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA).

DNA construction. Human FBXL5 was obtained from a human testis cDNA library by a standard polymerase chain reaction using primers 5′-ATG GCG CCC TTT CCT-3′ (forward) and 5′-TCA TTC GCC AGA GCG-3′ (reverse), and constructed into pGBKT7, pET-28a, pGEX-6p-1, pcDNA 3.1-flag, and pEGFP-C1 vectors. Full-length rat p150^{Glued} was a generous gift from Dr. Xiaojiang Li, and was inserted into pcDNA 3.1-flag vectors. Human p150^{Glued} fragment containing amino acids 489–1278 was screened out from human testis cDNA library by yeast two-hybrid assay.

Yeast two-hybrid. A yeast two-hybrid screen was performed essentially as previously described [17]. In brief, the full-length human FBXL5 was inserted into the BamHI restriction endoenzyme site of pGBKT7 and transformed into yeast strain AH109 along with a human testis cDNA library constructed in pACT2. The transformants were first plated on SD/–His/–Leu/–Trp medium and then streaked to SD/–Ade/–His/–Leu/–Trp/X-α-Gal medium for further selection. Positive clones, including AD-p150^{Glued}, was co-transformed with BD-FBXL5 into AH109 and streaked to SD selection medium again to verify the specificity of interaction.

Cell culture. HeLa and 293T cells, from American Type Culture Collection (Rockville, MD), were cultured as subconfluent monolayers in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS (Hyclone, Logan, UT) and 100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) with 10% CO_2. Plasmids were transfected using Lipofectamine 2000 reagent. For proteasome inhibitor MG132 treatment, cells were exposed to MG132 (20 mM) 2 h before collection. For cycloheximide (CHX) treatment, 24 h after transfection, cells were treated with cycloheximide (20 μg/ml) and harvested at recording time points. Densitometric data was calculated by Quantity One 4.4.0 software (Bio-Rad, Hercules, CA).

Immunoprecipitation. Human 293T cells were co-transfected with GFP-FBXL5 and Flag-p150^{Glued}. Thirty-six hours after transfection, cells were harvested and lysed in ice cold PBS with 1% Triton X-100. Anti-Flag antibody-conjugated agarose beads were mixed with cell lysate's supernatant at 4 °C for 2 h, and washed in ice cold PBS with 1% Triton X-100 for three times followed by another PBS wash without Triton X-100. Following separation on 10% SDS–PAGE and transfer to nitrocellulose membrane, precipitates were detected with antibody and visualized by an ECL kit (Pierce, Rockford, IL).

Protein expression. The GST-FBXL5 recombinant protein was expressed in *Escherichia coli* BL21 (D3), and purified by affinity chromatography using Glutathione Sepharose, as previously described [18].

GST pull-down assay. GST-FBXL5 was purified and conjugated to Glutathione Sepharose. Human 293T cells were transfected with Flag-p150^{Glued} and lysed by ice cold PBS with 1% Triton X-100. Supernant of cell lysate was incubated with GST-FBXL5-conjugated sepharose at 4 °C for 2 h. Resultant sepharose was washed three times in ice cold PBS with 1% Triton X-100 followed by ice cold PBS. The proteins bound by sepharose were then fixed in SDS–PAGE sample buffer, subjected to 10% SDS–PAGE, transferred to the nitrocellulose membrane, probed with antibody, and finally developed with an ECL kit.

Immunofluorescence. Human HeLa cells were transfected with GFP-FBXL5 and cultured for an additional 24 h. Transfected cells were then fixed in −20 °C methanol for 10 min, and washed three times in PBS, followed by blocking with 0.1% BSA in PBS. After incubation with primary antibody against p150^{Glued} (diluted 1:200) for 1 h and three washes

with PBS with 0.1% Triton X-100, cells were labeled with rhodamine-conjugated goat anti-mouse antibody to visualize endogenous p150^{Glued}, and counterstained with DAPI to label nuclei. Images were obtained on a Zeiss Axiovert 200 inverted fluorescence microscope using Axiovision 3.0 software.

Results and discussion

p150^{Glued} is a novel FBXL5 binding partner

The ubiquitin-dependent proteolysis pathway regulates the degradation of various proteins and thus influences cellular dynamics and plasticity. To further understand ubiquitin's role in cellular dynamics, we conduct a search for novel substrates of F-box proteins using a yeast two-hybrid assay. The full-length of FBXL5 (Fig. 1A) cDNA was used as bait to screen a human testis cDNA library by using GAL4 yeast two-hybrid system. Nucleotide sequencing revealed that one of these interactors encodes the C-terminus of p150^{Glued} (amino acids from 489 to 1278; Fig. 1B). Its sequence matches human p150^{Glued} (GenBank™ Accession No. Q14203). As in Fig. 1C, BD-FBXL5 activated the *lacZ* reporter gene when it was co-transformed with AD-p150^{Glued}, indicating that these two proteins can interact. AD-T and BD-lamin were co-transformed as negative control. p150^{Glued}, an intermediate chain of cytoplasmic dyein complex (Fig. 1B), has been shown to interact with several cytoskeletal regulators including EB1, dynein, kinesin, and actin [19–22]. However, no F-box proteins have been identified as binding partners of p150^{Glued}.

FBXL5 binds to p150^{Glued} in vivo and in vitro

To validate the interaction between FBXL5 and p150^{Glued} observed in yeast, we asked whether these two proteins interact *in vivo* and tested if FBXL5 forms a complex with the p150^{Glued}. To this end, extracts from GFP-FBXL5 and FLAG-p150^{Glued} co-transfected cells were incubated with anti-FLAG antibody-conjugated agarose beads, and the immunoprecipitate was subjected to Western blot and probed with anti-GFP antibody. As shown in Fig. 2A, we observed that FBXL5 was precipitated by Flag immunoprecipitation of p150^{Glued}, whereas GFP alone was unable to bind p150^{Glued}, which demonstrated an association of FBXL5 and p150^{Glued}. Thus, we conclude that the interaction between FBXL5 and p150^{Glued} is specific.

We next confirmed p150^{Glued}'s ability to directly bind FBXL5 by adopting a GST pull-down assay. FLAG-p150^{Glued} translation product was incubated with GST-FBXL5 as well as GST control, which were both expressed in *E. coli* and purified by Glutathione Sepharose. Then the isolated complexes were fractionated on SDS–PAGE and probed by immunoblotting with epitope tag antibodies. We found efficient pull-down of FLAG-p150^{Glued} only in the sample where GST-FBXL5 was present (Fig. 2B), indicating that the interaction between FBXL5 and p150^{Glued} is physically direct.

Therefore, these results show that the interaction of FBXL5 and p150^{Glued} observed in yeast, was also detectable by co-immunoprecipitation and GST pull down assay, implying that p150^{Glued} is able to bind FBXL5 directly both *in vivo* and *in vitro*.

FBXL5 is co-localized with p150^{Glued}

Previous studies suggest that p150^{Glued} is conspicuously concentrated at microtubule plus ends and centrosomes as well as spread throughout the cytoplasm. To determine the subcellular distribution of FBXL5, and further verify the spatio-temporal possibility that FBXL5 and p150^{Glued} might interact in mammalian cells *in vivo*, we utilized immunofluorescence microscopy on HeLa cells using antibodies specific for human p150^{Glued}. HeLa cells were transfected with GFP-FBXL5 to visualize FBXL5, and anti-p150^{Glued} monoclonal antibody was used to detect endogenous p150^{Glued}. As shown in Fig. 2C, FBXL5 was distributed through out the cytoplasm of interphase cell, which was overlapped with the localization of most endogenous p150^{Glued}. This immunocytochemical data show that FBXL5 and p150^{Glued} are co-distributed in HeLa cells.

FBXL5 promotes p150^{Glued}'s ubiquitination

All of the results presented above substantiate the direct binding between FBXL5 and p150^{Glued} *in vivo* and *in vitro*. Given the fact that one of the most important function of F-box proteins is to recognize substrate proteins for the ubiquitin–proteasome system and induce ubiquitination of substrates, we hypothesized that FBXL5 might target p150^{Glued} for ubiquitination.

We began by examining the possibility that p150^{Glued} is ubiquitinated, and FBXL5 promotes this ubiquitination using an ubiquitin assay. To this end, we transfected FLAG-p150^{Glued} into human 293T cells along with GFP-FBXL5 or GFP vector, followed by treating the cells with or without the proteasome inhibitor MG132. MG132 can block the degradation of poly-ubiquitinated proteins by inhibiting proteasome activity, and thus visualizes poly-ubiquitinated protein. As in Fig. 3A, p150^{Glued} in cells treated with MG132 clearly show upward protein migration, suggesting that p150^{Glued} is ubiquitinated *in vivo* and p150^{Glued} is conjugated to poly-ubiquitin. Furthermore, we observed substantial poly-ubiquitination of p150^{Glued} in cells co-transfected with FBXL5, even without the presence of MG132, indicating that FBXL5 is a powerful promoter of p150^{Glued}'s poly-ubiquitination.

To further confirm this hypothesis, we performed an immunoprecipitation assay by co-transfecting human 293T cells with FLAG-p150^{Glued} and GFP-FBXL5 or GFP vector. The protein samples were resolved on a 6% SDS–PAGE gel, and probed with an anti-Flag antibody and anti-ubiquitin antibody. As shown in Fig. 3B, FBXL5

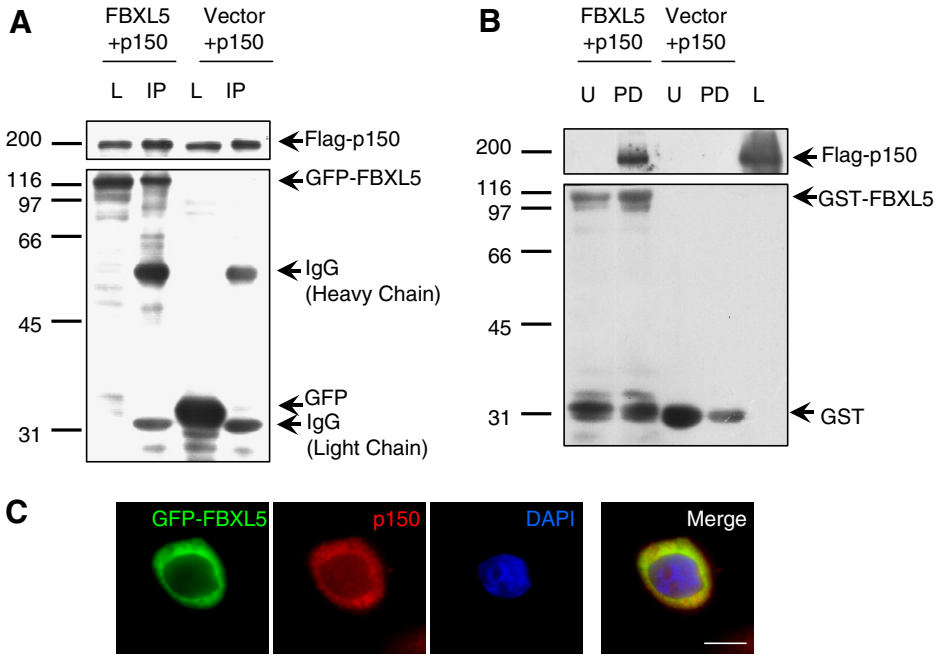

Fig. 2. Biochemical experiments prove FBXL5–p150^{Glued} interaction. (A) FBXL5 was co-immunoprecipitated with p150^{Glued} *in vivo*. Human 293T cells were co-transfected with GFP-FBXL5 and Flag-p150^{Glued}, lysed and immunoprecipitated by anti-Flag antibody, using GFP as control. The fraction of cell lysate (L) and immunoprecipitate (IP) were resolved by SDS–PAGE and subjected to immunoblotting assay with an anti-Flag antibody (upper panel) and an anti-GFP antibody (lower panel). (B) p150^{Glued} was pulled down by FBXL5 *in vitro*. GST-tagged construct of FBXL5 was expressed in *E. coli*, purified by GST affinity column, and applied with Flag-p150^{Glued} transfected human 293T cell extract, using GST as control. The GST-FBXL5 alone (U), GST-FBXL5 bound fraction (PD), and cell extract (L) were separated by SDS-PAGE and probed with an anti-FLAG antibody (upper panel) and an anti-GST antibody (lower panel). (C) FBXL5 and p150^{Glued} were co-distributed in the cytoplasm. Twenty-four hours after transfection with GFP-FBXL5, human HeLa cells were fixed, blocked, and stained for endogenous p150^{Glued}. Both of FBXL5 (GFP-FBXL5, green) and p150^{Glued} (red) localize to cytoplasm. Nucleus was visualized by staining with DAPI (blue). Bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

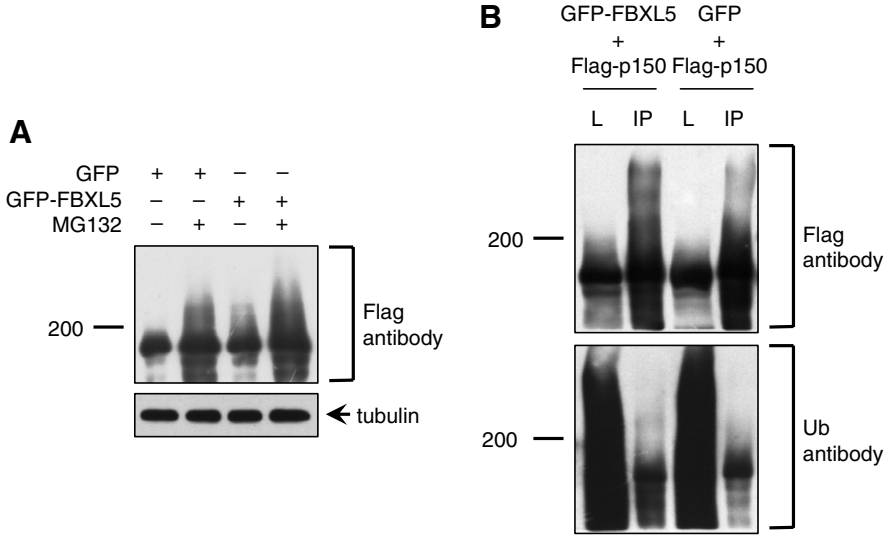

Fig. 3. FBXL5 promotes the ubiquitination of p150^{Glued}. (A) Ubiquitination of FLAG-p150^{Glued} was maximized upon pretreatment of MG132 or overexpression of FBXL5. FLAG-p150^{Glued} transfected human 293T cells were co-transfected with GFP-FBXL5 or GFP, and treated with or without MG132 2 h before cell harvest. Cell lysates were analyzed by SDS–PAGE, and immunoblotted with an anti-Flag antibody (upper panel) and an anti-tubulin antibody (lower panel). (B) Immunoprecipitated FLAG-p150^{Glued} was conjugated by poly-ubiquitin more efficiently when GFP-FBXL5 was co-expressed. Human 293T cells expressing FLAG-p150^{Glued} along with GFP-FBXL5 or GFP, were immunoprecipitated by an anti-FLAG antibody, resolved by 6% SDS–PAGE, and detected with indicated antibody. L indicates cell lysate fraction; IP indicates immunoprecipitated fraction.

promotes both ubiquitination and the degradation of FLAG-p150^{Glued}, validating our premise that p150^{Glued} is poly-ubiquitinated via FBXL5.

FBXL5 accelerates p150^{Glued}'s degradation

Most poly-ubiquitinated proteins are destined to be degraded in proteasomes; therefore, we next explored whether FBXL5–p150^{Glued} interaction may be responsible for p150^{Glued} degradation. If FBXL5 is responsible for p150^{Glued} degradation, overexpression of FBXL5 would minimize p150^{Glued} protein accumulation.

To this end, we co-transfected human 293T cells with equal amounts of FLAG-p150^{Glued}, and an increasing amount of GFP-FBXL5. The transfected cells were harvested after 36 h, and cell lysates were subjected to SDS–PAGE and immunoblotting. As shown in Fig. 4A and B, overexpression of FBXL5 resulted in a dose-dependent reduction of p150^{Glued} protein levels as a function of FBXL5 plasmid transfected. Thus, we conclude that FBXL5 promotes the degradation of p150^{Glued}.

To further substantiate FBXL5's function in p150^{Glued}'s degradation, we next examined whether the half-life of p150^{Glued} was shortened by FBXL5. We co-transfected human 293T cells with FLAG-p150^{Glued} and GFP-FBXL5 or GFP vector, and monitored the stability of p150^{Glued} after the addition of cycloheximide which stops new protein synthesis. As shown in Fig. 4C and D, p150^{Glued}'s half-life decreased when co-transfected with GFP-FBXL5. In contrast, the stability of an unrelated protein tubulin was not influenced, indicating that FBXL5 specifically helps p150^{Glued}'s turnover *in vivo*. Thus, we conclude that FBXL5 interacts with and governs the turnover of p150^{Glued}.

F-box proteins function as substrate recruiters in the ubiquitin–proteasome system [5], but the target for FBXL5 has not been reported before. Our work recognizes p150^{Glued} as a potential binding partner of the F-box protein FBXL5 and validates that this binding is physically direct both *in vivo* and *in vitro*. Moreover, we demonstrated that p150^{Glued} is poly-ubiquitinated *in vivo*; over-expression of FBXL5 promotes this poly-ubiquitination, oppresses the abundance of p150^{Glued}, and shortens the half-life of p150^{Glued}. These findings reflect the functional consequence of FBXL5 and p150^{Glued} interaction: FBXL5 targets p150^{Glued} for poly-ubiquitination and thus promotes p150^{Glued}'s turnover by proteasome.

p150^{Glued} has been shown to play important roles in the dynein–dynactin complex, and remains at a high level throughout the cell cycle [8]. Previous study showed that p150^{Glued} is cleaved by caspase during apoptosis [23]. However, the regular degradation mechanism of p150^{Glued} in most cells is still unclear. In our study, we offered a potential mechanism by which p150^{Glued} are degraded in normal mammalian cells. Through the binding of FBXL5, cytoplasmic p150^{Glued} is recruited by SCF E3 ubiquitin ligase,

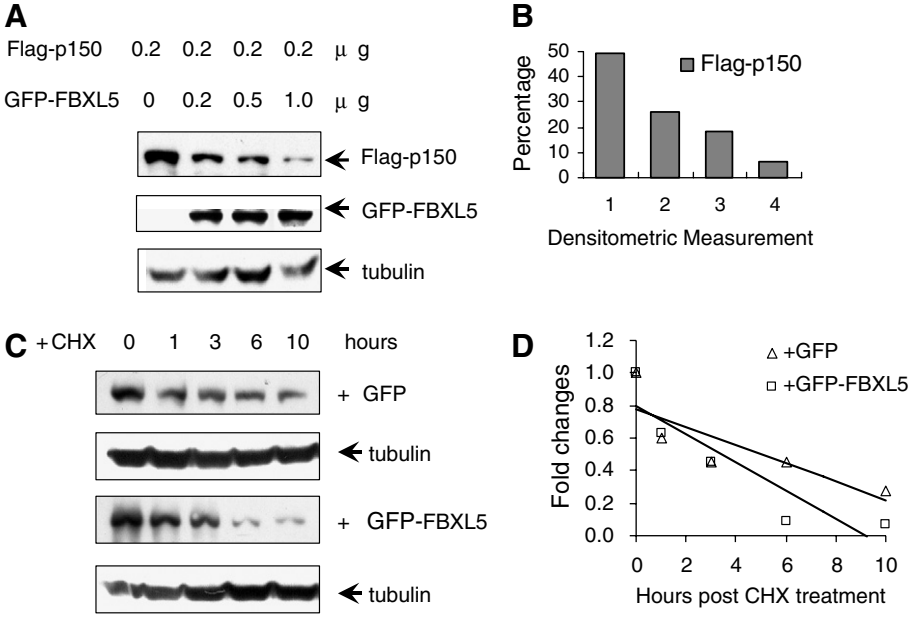

Fig. 4. FBXL5 accelerates the degradation of p150^{Glued}. (A,B) Expression of p150^{Glued} is reduced in a FBXL5 dose-dependent manner. Human 293T cells were co-transfected with FLAG-p150^{Glued} and an increasing amount of GFP-FBXL5. Thirty-six hours later, cells were lysed and subjected to immunoblotting assay with an anti-Flag antibody (upper panel), anti-GFP antibody (mid panel), and anti-tubulin antibody (lower panel). Tubulin level was used as a loading control. The percentage of p150^{Glued} quantifications in each fraction, as shown in the histogram (B), decreased when GFP-FBXL5 level was augmented. (C,D) FBXL5 promoted p150^{Glued} degradation. Twenty-four hours after co-transfection with Flag-p150^{Glued} and GFP-FBXL5 or vector, Human 293T cells were treated with cycloheximide (CHX, 20 μg/ml) for indicated times, followed by immunoblotting assay with an anti-Flag antibody or an anti-tubulin antibody, using tubulin as a loading control. Densitometric quantification of FLAG-p150^{Glued} is shown graphically (D), with the quantification of FLAG-p150^{Glued} at 0 h after CHX treatment defined as 1, upon which fold change was calculated. The reduction of p150^{Glued} sped up when the cells were co-transfected with FBXL5, indicating FBXL5 accelerates p150^{Glued} degradation.

decorated by poly-ubiquitin, transported into proteasomes and goes through proteolysis there. Recent reports suggested that $p150^{Glued}$ aggregates in neuron cells and leads to neuronal degeneration diseases [16]. One possible explanation for $p150^{Glued}$'s aggregation is a defect in $p150^{Glued}$'s turnover. Our findings that FBXL5 governs the turnover of $p150^{Glued}$ provide a regulatory mechanism underlying $p150^{Glued}$ dynamics.

Taken together, we have established a novel interrelationship between FBXL5 and $p150^{Glued}$, and demonstrated a critical role FBXL5 in the poly-ubiquitination $p150^{Glued}$ and its subsequent degradation, which illustrates a potential mechanism for regulating $p150^{Glued}$'s turnover in mammalian cells.

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