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RESEARCH ARTICLE

Ube3a, the E3 ubiquitin ligase causing Angelman syndrome and linked to autism, regulates protein homeostasis through the proteasomal shuttle Rpn10

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Abstract Ubiquitination, the covalent attachment of ubiquitin to a target protein, regulates most cellular processes and is involved in several neurological disorders. In particular, Angelman syndrome and one of the most common genomic forms of autism, dup15q, are caused respectively by lack of or excess of UBE3A, a ubiquitin E3 ligase. Its *Drosophila* orthologue, Ube3a, is also active during brain development. We have now devised a protocol to screen for substrates of this particular ubiquitin ligase. In a neuronal cell system, we find direct ubiquitination by Ube3a of three proteasome-related proteins Rpn10, Uch-L5, and CG8209, as well as of the ribosomal protein Rps10b. Only one of these, Rpn10, is targeted for degradation upon ubiquitination by Ube3a, indicating that degradation might not be the only effect of Ube3a on its substrates. Furthermore, we report the genetic interaction in vivo between Ube3a and the C-terminal part of Rpn10. Overexpression of these proteins leads to an enhanced accumulation of ubiquitinated proteins, further supporting the biochemical evidence of interaction obtained in neuronal cells.

Keywords Ube3a · Ubiquitin · Angelman syndrome · Autism · Proteasome · Rpn10

Introduction

Angelman syndrome (AS) is a severe neurodevelopmental disorder related to autism, which presents also with intellectual disability [1]. The cause of AS is the loss of function in the brain of the ubiquitin E3 ligase coded by the *UBE3A* gene, which is located on chromosome 15q [2, 3]. Autism is a neurodevelopmental disorder characterized by social deficits, communication difficulties, stereotyped or repetitive behaviors and interests, and in some cases, cognitive delays. The prevalence for autism spectrum disorders (ASD) as a whole is six per 1,000 but in only 10 % of the cases is there a recognized genetic cause. Duplication of the 15q locus including the *UBE3A* gene is one of the most common genomic causes for autism, the preference for maternal duplications lending further support to the involvement of the maternally expressed *UBE3A* gene [4–7]. The pathogenic mechanisms that underlie autism and AS, due to the excess or lack of UBE3A activity, are still unknown. Importantly, these phenotypes could be caused by the misregulation of common ubiquitination targets of UBE3A. Interestingly, while in the cortex of ASD subjects the spine densities on pyramidal cells are greater than in controls [8], the opposite is true for *UBE3A* null AS animal models. There, reduced spine density is observed on cerebellar Purkinje cells and on pyramidal neurons in hippocampus and cortex [9, 10]. Other reports, however, have

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indicated no changes on spine density upon UBE3A overexpression [11]. How UBE3A can influence spine density and therefore neuronal function remains to be elucidated.

Ubiquitin ligases covalently conjugate ubiquitin molecules to their substrates with the support of ubiquitin activating (E1) and conjugating (E2) enzymes. This post-translational modification influences a number of biological processes, ranging from protein localization, transport, endocytosis, facilitation of protein–protein interactions or reversible inactivation, to the more widely accepted role in protein degradation. All of these processes depend on the ubiquitin chain topology that is conjugated to the substrate protein [12]. Eukaryotic organisms use hundreds of E3 ligases to ubiquitinate proteins. Each ubiquitin ligase can have a few or hundreds of substrates. This complex relationship between E3 ligases and substrates confers specificity to ubiquitin-regulated mechanisms in the cell.

Loss of UBE3A has been proposed to increase the concentration in the brain of a number of proteins, including Arc, p27, p53, and the Rho-GEF proteins Ect2 and Ephexin5 [13–17]. It remains to be tested whether those proteins are ubiquitinated in vivo by UBE3A in neurons and whether this modification has any biological relevance. For example, ubiquitination of Arc by UBE3A was validated in vitro [13], but a recent revision of this work has concluded that Arc is regulated at the transcriptional level, but is not an ubiquitin substrate of UBE3A in cells [18].

Neuronal stimulation is known to induce the transcription of hundreds of genes [19]. So, in addition to many other scenarios, changes of synaptic activity due to ubiquitination of a single UBE3A substrate could affect the protein levels of many other proteins. In order to identify biologically relevant direct substrates of UBE3A, one must identify, in vivo, proteins whose ubiquitination is UBE3A-dependent. Although attempts have been made to identify UBE3A-regulated proteins in the brains of mice and flies [20, 21], the challenge remains to identify the direct ubiquitination substrates of this E3 ligase within the brain of a living model organism. Current strategies to investigate ubiquitination are mostly based on in vitro or ex vivo assays, in which co-transfection and pull-down assays with mild washing conditions are routinely used. This leads to the identification of false positives, as proteins interacting with the target proteins can also be isolated in the absence of stringent washes.

Drosophila expresses a clear *UBE3A* ortholog with 75 % protein sequence identity, *Ube3a*, whose loss-of-function mutants cause behavioral and morphologic phenotypes highly reminiscent of AS in mammals [9, 22, 23]. Remarkably, it has been shown that ectopic human UBE3A can take over the function of the endogenous Ube3a fly protein [16]. Recently, neuronal proteins that are ubiquitin-conjugated during *Drosophila* brain development have been identified at a proteomic scale [24], establishing also that Ube3a is an active ubiquitin ligase during embryonic brain development [24]. Now we have developed an assay that, by using stringent washes, precludes the isolation of interacting proteins and therefore monitors the presence of ubiquitin conjugates just on the direct target protein. Using this neuronal cell culture approach, the ubiquitination status of any protein can be validated and the enzyme responsible be found. We have here screened those known neuronal ubiquitin substrates, some of which have been recently marked as candidate Ube3a substrates using a different proteomics approach [20], to identify direct targets of Ube3a. We have found that the proteasome-shuttling factor Rpn10, the *Drosophila* homologue of S5α/PMSD4, is directly ubiquitinated by Ube3a, targeting Rpn10 for degradation. We also identified Rps10b and the proteasome-related proteins Uch-L5 and CG8209 as direct targets for Ube3a. These findings place Ube3a as a regulator of protein homeostasis within the brain and provides new mechanistic insights for the role of UBE3A in AS and ASD.

Results

A new protocol to monitor ubiquitination in a neuronal cell culture

The E3 ligase Ube3a, the *Drosophila* ortholog of UBE3A, was previously identified as an active ubiquitin-carrier during brain development, in parallel to the identification in vivo of nearly a hundred neuronal ubiquitin substrates [24]. We have now applied a novel cell-based ubiquitination protocol for the identification within those proteins of direct substrates of Ube3a. We used BG2 neuronal cells [25], since proteins that interact with Ube3a relevant for its neuronal function might not be present in highly proliferative cell lines. We generated N- and C-terminally GFP-tagged versions for 47 neuronal ubiquitin substrates identified in vivo [24], and found distinct (nuclear, perinuclear, cytoplasmic, and membrane) localization patterns in BG2 cells (Fig. 1 and Supplementary Fig. 1).

High-affinity anti-GFP camelid antibody-coated beads (*Chromotek GmbH*) are routinely used with mild washes to prevent dissociation of interacting partners in co-immunoprecipitation experiments. However, we found that those beads also allow for extremely stringent washes. As ubiquitination is a covalent conjugation, this post-translational modification will also withstand highly denaturing washes (Fig. 2a), allowing for the elimination of all background interactions. The utility of this approach for the isolation of ubiquitinated proteins has already been shown using a different setup [26]. Capture of the GFP-tagged proteins is performed under non-denaturing conditions, but the buffer is supplemented with protease inhibitors and NEM to prevent

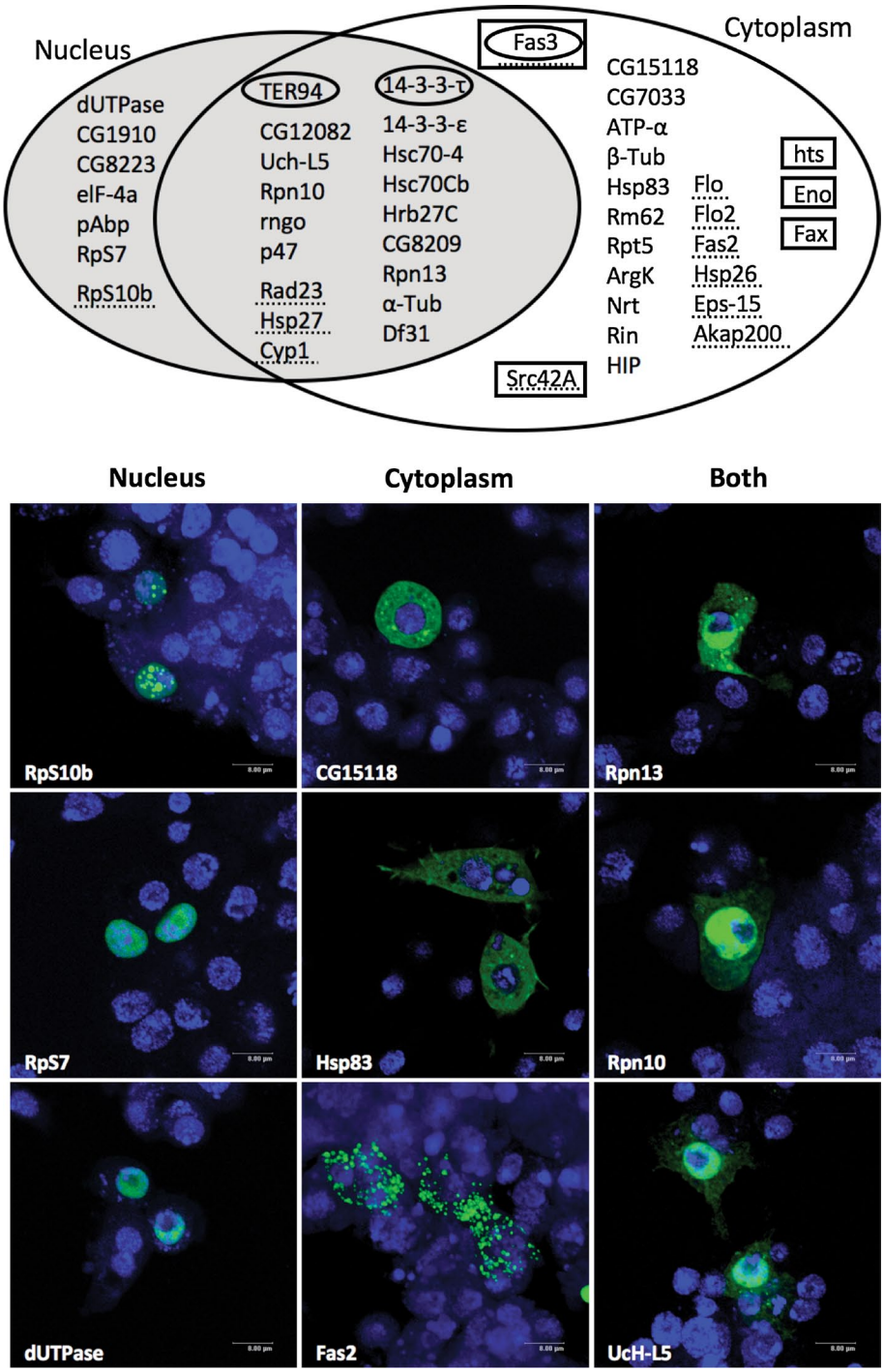

Fig. 1 Localization of 47 neuronal ubiquitination substrates in *Drosophila* BG2 cells. Forty-seven GFP-tagged genes were transfected into BG2 cells and protein localization was analyzed by confocal microscopy. Seven out of the 47 fusion proteins localized only in the nucleus, 22 localized only in the cytoplasm, and 18 both in nucleus and cytoplasm. *Dotted underlining* indicates that the protein localizes in a punctuate manner; *boxed names* indicate localization to the cell membrane; *circled names* indicate localization to the nuclear membrane. Three different examples of expression in nucleus, cytoplasm, or both are shown. *Scale bars* indicate 8.00 μm

deubiquitination. Once the GFP-tagged proteins are bound to the beads, denaturing washes (8 M urea + 1 % SDS) are applied (see "Materials and methods" for further details). In order to improve the detection of the ubiquitinated material, we co-transfected the BG2 cells with FLAG-tagged ubiquitin, which incorporated readily into conjugates.

Immunoblotting of the purified GFP-tagged proteins with FLAG antibody (red channel in Fig. 2b) confirmed that 43 out of the 47 potential substrates are indeed ubiquitinated by endogenous E3 ligases in neuronal cells, three of them showed no ubiquitination, and a fourth one was not detectable by either antibody. Each of the proteins showed their own pattern of ubiquitination. We observed lower migrating GFP reacting species (green channel) for some of the fusion proteins, corresponding to either a partial breakdown of the polypeptide chain on which the highly stable GFP is protected, or to an alternative starting codon readout. Similarly to GFP expression on its own (lane 0 of Fig. 2b), no

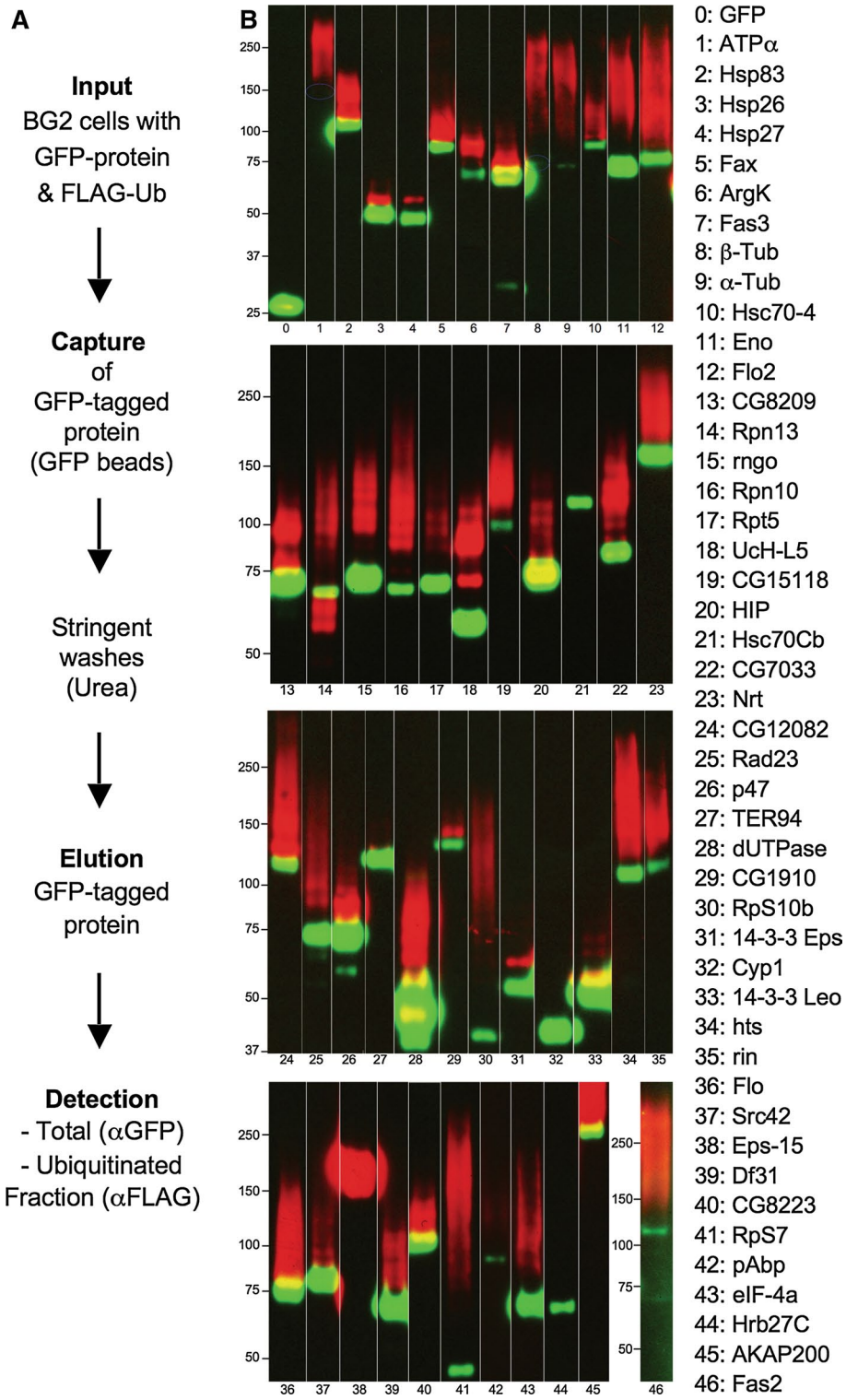

Fig. 2 Neuronal ubiquitination assay using *Drosophila* BG2 cells. **a** Scheme of the GFP pull-down protocol for monitoring ubiquitination. GFP-tagged cDNAs are co-transfected with FLAG-tagged Ub. The cell extracts were applied to GFP beads for capture of the GFP-tagged material. Detection of the ubiquitinated fraction is done with anti-FLAG antibody. **b** Ubiquitination assay for the candidate ubiquitin substrate proteins in a neuronal cell culture. No signal could be detected for Rm62. From the remaining 46 proteins, 43 were confirmed to be ubiquitinated by endogeous E3 ligases in BG2 cells (all except Hsc70Cb, Cyp1 and Hrb27C). Mouse anti-GFP antibody was used for detecting the captured proteins (shown in *green*), and HRP-conjugated anti-FLAG antibody for monitoring their ubiquitinated fraction (shown in *red*). On lane 0, GFP on its own displays no ubiquitination

ubiquitination was observed for those smaller species. In order to confirm that the ubiquitination observed is dependent on the canonical ubiquitination pathway, we tested the effect caused by silencing the endogenous ubiquitin activating E1 enzyme, Uba1, which completely abolished the ubiquitination profile of the tested proteins (Fig. 3).

Identification of direct ubiquitination substrates of Ube3a in a neuronal cell culture

We used overexpression of both wild-type (WT) or catalytically inactive Ube3a, the latter was generated by mutating the active site cysteine to a serine (C941S). UBE3A has

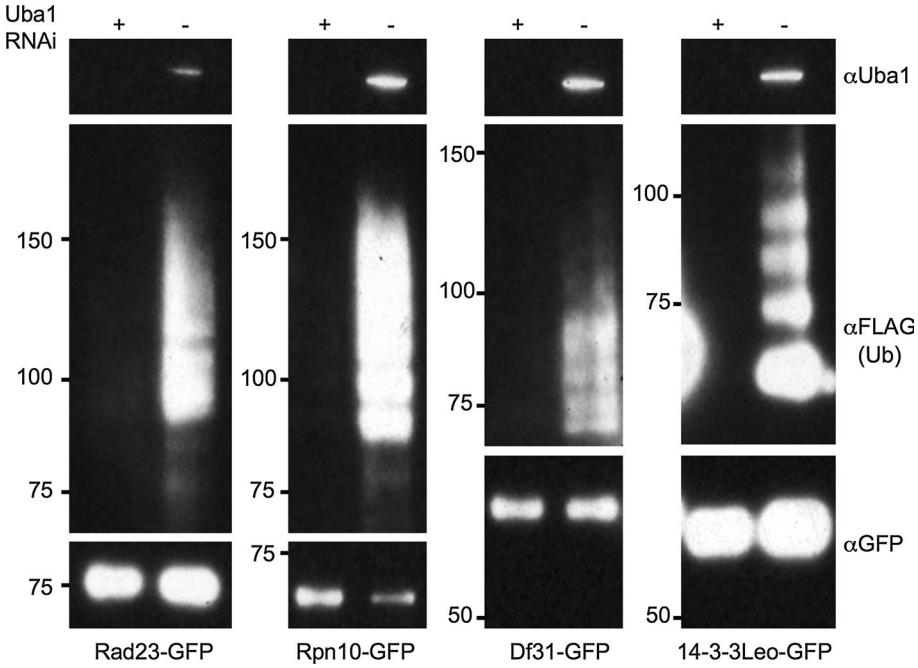

Fig. 3 Effect on substrate ubiquitination of Uba1 double-stranded RNA treatment on BG2 cells. Four proteins were chosen to elucidate the effect of Uba1 dsRNA treatment on their ubiquitination. Ubiquitination of GFP-tagged Rad23, Rpn10, Df31, and 14-3-3-Leo was analyzed by Western blot after capture of the GFP-tagged proteins. GFP-immunoblotting is shown at the *bottom* panel, while the ubiquitination signal (from FLAG-tagged ubiquitin) is shown in the *middle* panel. To detect the amount of Uba1 remaining after the dsRNA treatment, the membrane was probed with rabbit anti-Uba1 antibody

been reported to generate K48 polyubiquitin chains [27], a type of linkage that targets protein substrates for degradation. In order to identify which of those proteins ubiquitinated during brain development could be direct targets of Ube3a, we first monitored the whole cell extract from cells transfected with the GFP-tagged ubiquitin substrates upon overexpression of either Ube3a-WT or Ube3a-C941S. Upon overexpression of Ube3a-WT, we found reduced protein levels only for one out of the 47 proteins, Rpn10 (Fig. 4a and data not shown), suggesting that this protein is targeted for proteasomal degradation upon ubiquitination by Ube3a. Conversely, Ube3a-C941S overexpression seemed to exert a dominant negative (DN) effect, protecting Rpn10 from degradation.

Changes in protein levels can be caused by a number of cellular processes. Conversely, ubiquitination is not necessarily a mark for degradation. In order to obtain direct evidence of neuronal ubiquitination of Rnp10 by Ube3a, we applied the GFP pull-down protocol to cells overexpressing Rpn10-GFP and FLAG-Ubiquitin, this time by cotransfecting with either Ube3a-WT or Ube3a-C941S. Ube3a-WT induced a dramatic increase of Rpn10 ubiquitination, relative to either Ube3a-C941S or cells transfected with an empty vector (Fig. 4b) and also a significant shift of the molecular weight pattern indicating higher order of polyubiquitination. The increase in ubiquitination of Rpn10 upon Ube3a-WT overexpression was noticeable even when the total amount of GFP-tagged protein in Ube3a-WT samples was significantly decreased due to proteasomal degradation. On the other hand, ectopic Ube3a-C941S appeared to trap Rpn10 molecules ubiquitinated by endogenous Ube3a, in agreement with the idea of its acting as a DN due to the inability of the oxyester-loaded enzyme to transfer its Ub, resulting in an increase not only of total Rpn10-GFP, but also of its ubiquitinated forms.

A similar effect is observed both for N and C-terminally GFP-tagged versions of Rpn10 (Supplementary Fig. 2a). The increase in ubiquitination is masked to some degree by the downregulation of total levels of GFP-tagged Rpn10 by Ube3a-WT overexpression, but the ratio of ubiquitinated material to total GFP-Rpn10 is significantly increased. In order to compare the fraction of Rpn10-GFP that is ubiquitinated in the Ube3a-WT and Ube3a-C941S samples, we loaded in the protein gel the whole eluate for the Ube3a-WT sample and decreasing volumes of the eluted sample for Ube3a-C941S (Supplementary Fig. 2b). In lanes containing comparable levels of purified GFP-tagged Rpn10 as detected by Western blotting to GFP, ubiquitination of Rpn10-GFP is well detected for the Ube3a-WT overexpression sample, while is nearly absent in the control Ube3a-C941S sample, confirming that ubiquitination of Rpn10 is highly enhanced by Ube3a-WT.

In order to elucidate whether either of the two differentiated halves of Rpn10 are necessary or sufficient for recognition and ubiquitination by Ube3a, we cloned the two halves of Rpn10 using the same vector. The N terminal fragment (ΔC, up to residue 204) contains the von Willebrand factor A (VWA) domain for proteasome interaction, and the C terminal fragment (ΔN, starting at residue 205) contains the ubiquitin interacting (UIM) motifs (Fig. 4c). Neither of the two constructs was significantly ubiquitinated by endogenous Ube3a, but full-length Rpn10 was.

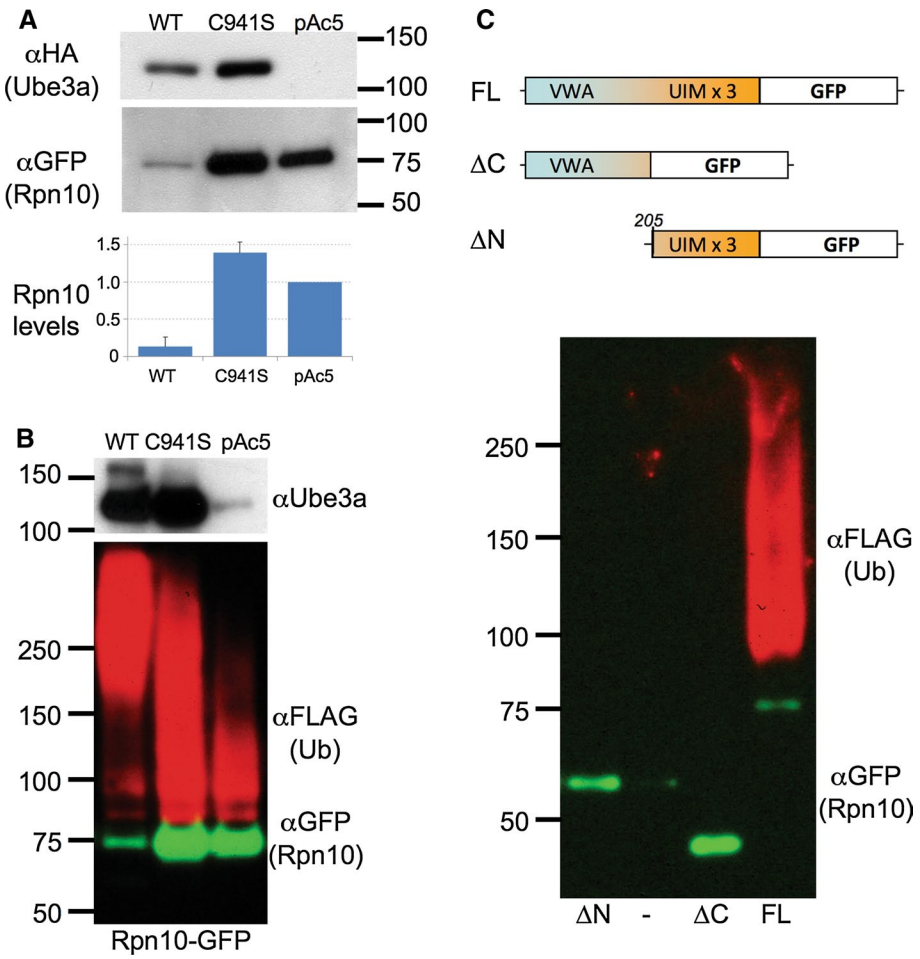

Fig. 4 Ube3a ubiquitinates and downregulates Rpn10 in BG2 neuronal cells. **a** Overexpression of HA-tagged wild-type (WT) or active site mutated (C941S) forms of Ube3a was used to monitor Rpn10 degradation by this E3 ligase. HA-tagged Ube3a was monitored here by anti-HA antibody. Relative quantification of the Rpn10-GFP levels was carried out with ImageJ software and intensities were normalized relative to the control (pAc5) on which an empty pAc5 vector was contransfected. Mean ± standard deviation of three different experiments performed independently are shown. **b** Overexpression of Ube3a-WT induced a dramatic increase of Rpn10 ubiquitination, despite the significant reduction of total Rpn10-GFP levels in the WT sample. The apparent increase of ubiquitination observed in the C941S mutant of Ube3a relative to the control (in which only endogenous Ube3a is present) is mostly due to higher levels of total Rpn10-GFP in the sample. **c** GFP-tagged Rpn10 constructs tested for ubiquitination. *VWA* von Willebrand factor A domain. *UIM* Ubiquitin interacting motif. N- and C-terminally truncated versions of Rpn10 (ΔN and ΔC, respectively) are not significantly ubiquitinated, compared to full-length (FL) Rpn10

Similarly, neither of the partial constructs was targeted for degradation upon overexpression of Ube3a-WT (data not shown).

None of the other 46 cloned ubiquitin substrates was downregulated by Ube3a, but we applied the GFP-based protocol to them in case any of those proteins would also be a direct ubiquitination target of Ube3a, with a different (non-degradative) regulation by Ube3a. By screening the full set, we identified two more proteasome-interacting proteins, the product of gene CG8209 (the *Drosophila* homologue of the ERAD component UBXN1/SAKS) and the DUB enzyme UcH-L5, to be ubiquitinated by Ube3a, as well as the ribosomal protein Rps10b (Supplementary Fig. 3). None of those three Ube3a substrates was targeted for proteasomal degradation upon ubiquitination by Ube3a-WT, indicating that polyubiquitination by Ube3a might not necessarily result in degradation of the substrate.

Ube3a and Rpn10 interaction in vivo

Our previous results indicate the interaction between Ube3a and Rpn10 in cultured cells. However, it is important to understand the biological consequences of this interaction in vivo. Rpn10 is known to shuttle polyubiquitinated proteins to the proteasome for their degradation, recognizing its client proteins using the UIM motifs in its C-terminal

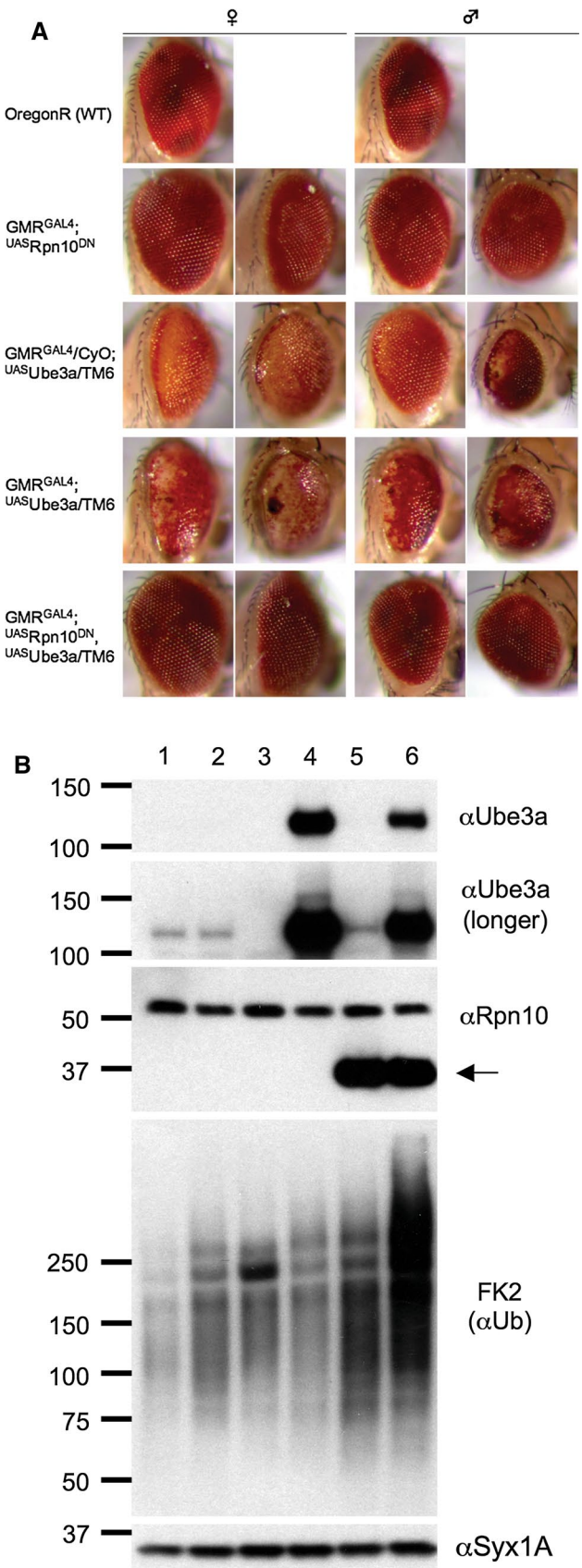

Fig. 5 Ube3a and Rpn10 interaction in vivo. **a** Overexpression of Rpn10^{DN} in the *Drosophila* eye displays an appearance similar to OregonR wild-type (WT) flies. Overexpression of Ube3a-WT on its own causes a degenerative phenotype, as well as a reduction on the eye size. Having two copies of *GMR*^{GAL4} exacerbates this phenotype. When both constructs are expressed together, using two copies of *GMR*^{GAL4}, the Ube3a phenotype is rescued, indicating a direct interaction between both proteins in vivo. Two representative female and male flies are shown per genotype. **b** Western-blot analysis of ubiquitinated proteins extracted from *Drosophila* heads using the FK2 antibody. *Lane 1*: OregonR control flies; *Lane 2*: *GMR*^{GAL4};*TM2/TM6* control flies; *Lane 3*: *If/CyO*; *15B/15B Ube3a*$^{-/-}$ null mutant flies; *Lane 4*: *GMR*^{GAL4}*/CyO*;^{UAS}*Ube3a/TM6* flies, *Lane 5*: *GMR*^{GAL4}; ^{UAS}*Rpn10*^{DN} flies. *Lane 6*: *GMR*^{GAL4}; ^{UAS}*Ube3a*,^{UAS}*Rpn10*^{DN}*/TM6* flies. Western-blot analysis of Ube3a indicates the level of Ube3a overexpression relative to endogenous Ube3a levels; Ube3a is absent on the *Ube3a*$^{-/-}$ null mutant flies (*Lane 3*). Western-blot analysis of Rpn10 is shown to illustrate the samples on which the Rpn10^{DN} construct (indicated by *arrow*) is expressed. Overexpression of Rpn10^{DN} causes an accumulation of ubiquitinated material. This is highly enhanced when WT Ube3a is coexpressed, despite the latter having not such effect on its own, indicating a direct interaction between both proteins in vivo. Syx1A western is shown as a loading control, approximately one head was loaded in each lane

part, and associating with the proteasome through the N-terminal VWA domain. Overexpression of an Rpn10 construct lacking the N-terminal VWA domain has been shown to exert a dominant negative effect, by trapping its client proteins and resulting in the accumulation of ubiquitinated material in vivo when overexpressed in various organs [28]. We have now prepared transgenic animals that express this form of Rpn10 (Rpn10^{DN}) under the control of the eye specific Glass Multimer Reporter *GMR*-GAL4 driver, for specific expression in the eye. We also prepared transgenic animals expressing the WT form of Ube3a with the same *GMR*-GAL4 driver. No such accumulation effect of ubiquitinated proteins has been reported for WT Ube3a overexpression, but instead a degeneration phenotype is observed in the eye [22] (Fig. 5a). We also generated flies with both transgenes being overexpressed by the *GMR*-GAL4 driver.

While Ube3a overexpression causes a degeneration phenotype, and overexpression of Rpn10^{DN} does not (Fig. 5a), co-expression of Rpn10^{DN} could completely suppress the Ube3a overexpression phenotype (Fig. 5a). In addition, the increase of ubiquitinated material associated with Rpn10^{DN} (Fig. 5b, lane 5) was increased upon co-expression of Ube3a-WT (lane 6), which by itself (lane 4) does not alter the levels of ubiquitinated proteins. This double synergistic effect suggests that the shuttling of polyubiquitinated proteins by Rpn10 is regulated by Ube3a. At the same time, the degeneration phenotype reported for Ube3a overexpression in the eye must be linked to the degradation of those polyubiquitinated proteins by the proteasome.

Discussion

The lack of a neuronal system for both in vivo identification and validation of ubiquitination targets has restricted many candidate ubiquitin substrates of UBE3A to be validated only in vitro. Evidence for the ubiquitination of those substrates by UBE3A in vivo, or even in cell culture, is lacking. One recent report has even redefined a previously suggested substrate as not being ubiquitinated by UBE3A when tested in cells [18]. A recent in vivo proteomic approach for the identification of Ube3a direct and indirect targets in the *Drosophila* brain did produce a list of 49 candidates for validation [20], some of which overlapped with our list of ubiquitinated proteins in the fly brain [24]. Ubiquitination by Ube3a of one out of the 49 candidates, ATPα, was validated in vitro [20], but when tested in vivo it is not ubiquitinated by Ube3a (JR, UM, unpublished results).

Here, we identified Rpn10 and other regulators of the proteasome as direct targets for Ube3a in a neuronal cell culture system, while confirming their genetic interaction in vivo. These results open a new perspective for interpretation of previous identifications of UBE3A substrates based on changes in protein levels. In this new paradigm, we could interpret UBE3A-dependent changes in the levels of proteins not as direct ubiquitination substrates of this E3 ligase, but as the result of a downstream effect caused by proteasomal regulation by UBE3A.

UBE3A has been reported to be associated in the brain both to synaptic and cytosolic proteasomes, but the nature of this association is not clear [29]. It has also been reported that UBE3A associates with proteasome complexes in HEK 293 cells, HeLa cells, and rat muscle [30–34]. Direct interaction of Ube3a with the proteasome subunit PSMD4 (RPN10) was reported as part of intact proteasomes, as well as in a smaller complex of approximately 200 kDa, interpreted as containing the shuttling pool of PSMD4 [30]. Having identified in neuronal cells that three proteasomal proteins are direct ubiquitination substrates of Ube3a, we can speculate now that one of the major roles of Ube3a is actually to regulate proteasomal function. Indeed, the suppression of Ube3a-WT overexpression phenotypes in the eye by co-expression with $Rpn10^{DN}$ (Fig. 5a) strongly supports the view of Ube3a being a key regulator of neuronal proteasome function.

Expression of *UBE3A* is induced in response to a variety of stress conditions [14], but the regulation of the proteasome itself by Ube3a has never been suggested previously. In cultured neuronal cells, we have identified four novel substrates of Ube3a, including two proteasome-interacting receptors as well as one proteasome-associated DUB. Furthermore, Ube3a seems to target protein homeostasis via ubiquitination of the ribosomal protein RpS10b. While proteasomes are ubiquitously distributed in the cell, it appears that a significant subpopulation is recruited to dendritic spines following synaptic stimulation [35]. Translocation and recruitment of proteasomes into spines seems to be highly regulated, presumably due to the requirement for coordinated proteolysis at the synapse [36, 37]. In addition, proteasomes are known to regulate dendritic spine growth, with proteasomal inhibition being reported to acutely reduce new spine outgrowth [38]. Very recently, a specific centrosomal pool of PSMD4 has been shown to regulate dendrite development in the mammalian brain [39]. If we add this to the fact that loss of maternal UBE3A in transgenic mice causes a reduction in spine density and spine length [9], while spine densities are greater on pyramidal cells in the cortex of ASD subjects than in controls [8], we can speculate that UBE3A regulation of stability or function of proteasomal components might be an essential mechanism for synaptic plasticity.

Interestingly, only Rpn10 appears to be itself targeted for degradation upon ubiquitination by Ube3a. In flies, Rpn10 null mutants are lethal, but survive until pupal stages thanks to the large amount of maternal proteasomes available from early embryonic stages. However, the brain of wandering larvae of these mutants is reduced in size [40]. *PSMD4* knockout in mice also results in early embryonic lethality [41]. Regarding the other identified Ube3a substrates, RpS10b has been identified to be differentially expressed in patients with schizophrenia [42, 43]. UcH-L5 is a proteasome-associated DUB enzyme responsible for cleavage of K48 ubiquitin chains [44], and it is essential in most cells, so it will require some further work to dissect the specific effect its misregulation would cause in brain development. CG8209 is the *Drosophila* homologue of UBXN1/SAKS, a component of the complex required to couple deglycosylation and proteasome-mediated degradation of misfolded proteins retrotranslocated into the cytosol from the endoplasmic reticulum.

The main role of Rpn10/PSMD4 is to shuttle polyubiquitinated proteins to the proteasome for their degradation (Fig. 6). Overexpression of $Rpn10^{DN}$ results in some of its client proteins being trapped, so an accumulation of polyubiquitinated material is observed (Fig. 5b). Endogenous Rpn10, however, will remain shuttling non-trapped polyubiquitinated proteins to the proteasome. When Ube3a is overexpressed, a strong eye phenotype is observed (Fig. 5a), this could be due to the persisting activity of some proteins that would otherwise have been targeted -via Rpn10- to the proteasome for degradation. Those proteins might remain ubiquitin-conjugated, or processed by DUBs into their normal forms. If both $Rpn10^{DN}$ and Ube3a are overexpressed, the reduction of shuttling endogenous Rpn10 is compounded by the trapping of its client ubiquitinated proteins by $Rpn10^{DN}$, which would no longer be processed by DUBs [45] resulting in a much higher level of

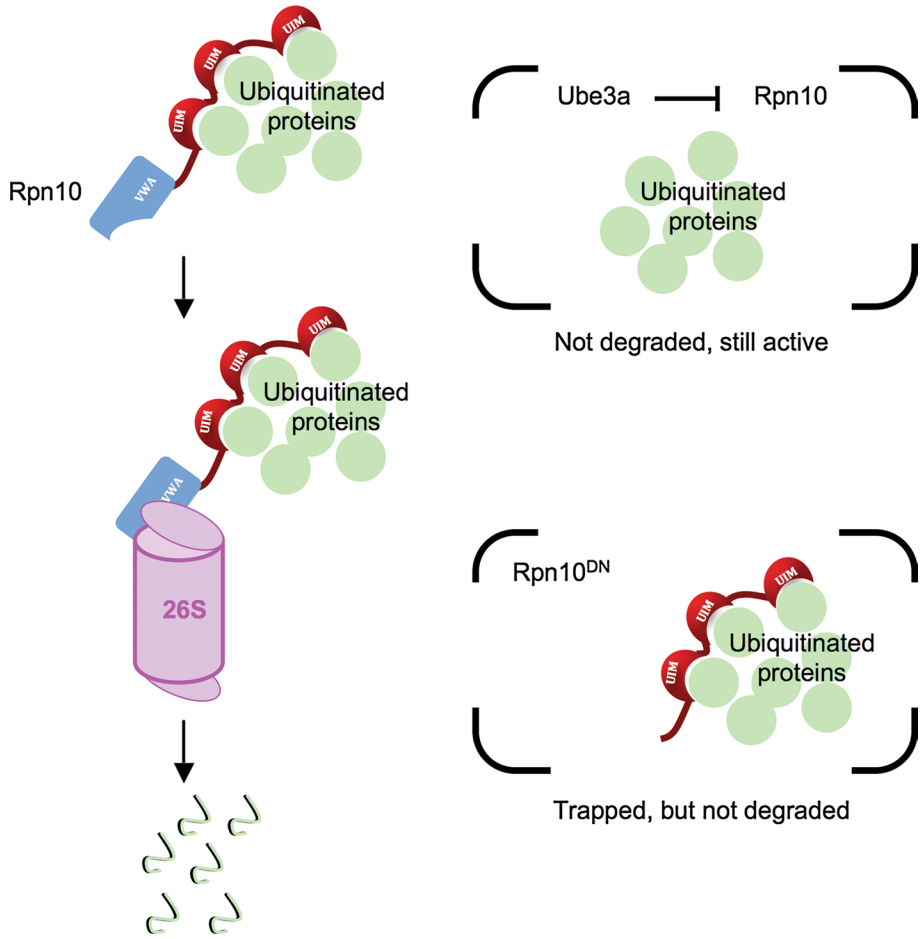

Fig. 6 Model for the interaction in vivo between Ube3a and Rpn10. The main role of Rpn10 is to shuttle polyubiquitinated proteins to the 26S proteasome for their degradation. Rpn10 recognizes its client proteins using the ubiquitin interacting motifs (UIM) in its C-terminal part, and associates with the proteasome through the N-terminal von Willebrand factor A (VWA) domain. When overexpressing a dominant negative version of Rpn10 that lacks the N-terminal VWA domain ($Rpn10^{DN}$), some its client proteins are trapped and the accumulation of polyubiquitinated material is observed. Endogenous Rpn10, however, will remain shuttling polyubiquitinated proteins to the proteasome. When Ube3a is overexpressed, a strong eye phenotype is observed. If both $Rpn10^{DN}$ and Ube3a are overexpressed, the reduction of endogenous shuttling Rpn10 is compounded by the trapping of its client ubiquitinated proteins by $Rpn10^{DN}$. The activity of those proteins trapped by $Rpn10^{DN}$ would be blocked, so the eye phenotype associated to their misregulation is no longer detectable

polyubiquitinated proteins accumulating (Fig. 5b). As those proteins are trapped by $Rpn10^{DN}$, they would not retain activity, and the eye phenotype associated with their misregulation would no longer be detectable (Fig. 5a).

By screening our previously identified neuronal ubiquitin conjugates for direct targets of Ube3a, we have found that the proteasome-shuttling factor Rpn10, the *Drosophila* homologue of S5α/PMSD4, is directly ubiquitinated and targeted for degradation by Ube3a. Further validating our findings, we observed in vivo a genetic interaction between $Rpn10^{DN}$ and Ube3a, leading to an accumulation of ubiquitinated proteins while at the same time suppressing the degeneration phenotype observed when Ube3a alone is overexpressed. These results place Ube3a as a master regulator of protein homeostasis within the brain and provide new mechanistic insights for better understanding at a molecular detail both pathologies associated to UBE3A enzyme levels in humans, Angelman syndrome, and dup15q-associated autism.

Materials and methods

Plasmid constructs

Primers are detailed in Supplementary Table 1. N-terminal and C-terminal GFP-tag was amplified with the primers C-GFP(F), C-GFP(R), N-GFP(F) and N-GFP(R), and cloned into *Drosophila* expression vector pAc5 (Invitrogen) between *Kpn*I and *Xba*I sites. The 47 substrates were cloned into this GFP-tagged pAc5 using specific primers for each of them. The Flag-tagged ubiquitin was amplified

using the primers Ub-EcoRI(R) and Ub-KpnI-Flag(F), and was inserted into the *Drosophila* expression vector pAc5 between the *Eco*RI and *Kpn*I sites.

HA-tagged Ube3a was amplified using the primers: Ube3a-XhoI HA(F) and Ube3a-XbaI(R), and inserted into the *Drosophila* expression vector pAc5 between the *Xho*I and *Xba*I sites. For the generation of the catalytic inactive HA-tagged Ube3a the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) and the primers Ube3a-C941S(F) and Ube3a-C941S(R) were used.

dsRNA synthesis

For the RNA interference experiments, double-stranded RNA (dsRNA) was synthesized by in vitro transcription of a PCR-generated DNA template containing the T7 promoter sequence on both ends with the aid of the MEGAscript kit (Ambion). Oligonucleotides to generate these templates were selected by searching the DRSC (*Drosophila* RNAi Screening Center) database. A fragment of approximately 500 bp of exon sequences was amplified for Uba1. The Uba1T7-tailed DNA was produced with the following primers: 5′-AATACGACTCACTATAGGGGCATTGGGGAAGTTCTTCAG-3′ and 5′-AATAC GACTCACTATAGGGTGACTCGAAATGGTTTATTGTC-3′. The T7-tailed dsRNA was purified with the RNeasy kit (QIAGEN) and the amount and quality of the produced dsRNA were determined by spectrophotometric analysis and agarose gel electrophoresis.

BG2 cells culture and transfection

Drosophila BG2 neuronal cells [46] were kindly provided by the DRSC. The cells were cultured in Shields and Sang M3 Insect Medium from Sigma supplemented with 10 % FBS from Invitrogen, 1:100 penicillin/streptomycin, 20 μg/ml of insulin from Sigma and 50 % conditioned medium (medium removed from cultured cells, centrifuged 5 min at 2.500 rpm) at 25 °C. Calcium phosphate transfection was used to monitor localization and ubiquitination. Effectene Transfection Reagent (QIAGEN) was used for dsRNA treatment of BG2 cells. For calcium phosphate method, 1 ml per well of BG2 cells at 1×10^6 cells/ml was seeded on 12-well plates and next day, gene constructs were transfected. Briefly, 10 μl of 2.5 M $CaCl_2$ was mixed with 2 μg of GFP-tagged genes constructs and/or 0.4 μg of Flag-tagged ubiquitin construct. To check the effect of HA-Ube3a, 0.2 μg of HA-Ube3a gene construct was added with 1 μg of Rpn10-GFP and 1 μg of Flag-Ub. H_2O was added up to 100 μl of total volume. 100 μl of 2× HEPES buffered saline (50 mM HEPES, 1.5 mM NaH_2PO_4, 280 mM NaCl at pH 7.1) was added drop-wise to DNA mix solution and incubated for 40 min at room temperature (RT) and then applied into the cell culture. After 18 h, the cells were washed with 1× PBS twice and 1 ml of supplemented M3 insect medium was added. Cells were incubated for 48 h, washed with 1× PBS three times and stored at −20 °C until further use. Effectene transfection reagent (QIAGEN) was used following the manufacturer's instructions. One milliliter per well of BG2 cells at 5×10^5 cells/ml was seeded on 12-well plates containing 800 ng of dsRNA against E1 per well and incubated for 5 days at 25 °C. After dsRNA pre-treatment, 2 μg of 47 GFP-tagged constructs and 0.8 μg of Flag-tagged ubiquitin construct were mixed with 20 μl of Enhancer. EC buffer was added up to 250 μl. The solution was incubated for 2 min at RT and then 15 μl of Effectene was added and incubated for 8 min at RT. This mix solution was co-transfected to dsRNA pre-treated cells. Seventy-two hours later cells were washed with 1× PBS three times and stored at −20 °C until further use.

GFP beads pull-down assay

Transfected cells were lysed in 300 μl of lysis buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 % Triton, 1×Proteases Inhibitor cocktail (Roche Applied Science), 0.7 % *N*-ethylmaleimide (NEM, from Sigma)] and collected for centrifugation at 13,000 × *g* for 15 min. The supernatant was mixed with pre-washed GFP beads (Chromotek GmbH) with dilution buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1×Proteases Inhibitor cocktail, 0.7 % NEM). The mixture was incubated at RT for 150 min with gentle rolling and centrifuged for 2,700 × *g* for 2 min. The supernatant was removed and the beads washed once with dilution buffer, three times with washing buffer (8 M Urea, 1 % SDS in PBS) and once with 1 % SDS in 1× PBS. The bound proteins were eluted with sample loading buffer (250 mM Tris–HCl pH 7.5, 40 % glycerol, 4 % SDS, 0.2 % BPB) heated at 95 °C for 10 min and applied into 4–15 % Tris–Glycine gels.

Western blotting

The following antibodies were used: monoclonal mouse anti-GFP antibody (Roche) at 1:1,000; monoclonal mouse anti-Flag M2-HRP conjugated antibody (Sigma) at 1:1,000; rabbit polyclonal anti-Uba1 antibody (Abcam) at 1:1,000; monoclonal mouse anti-HA antibody (Sigma) at 1:1,000; rabbit polyclonal anti-Ube3a antibody (gift from Fen-Biao Gao) [23] at 1:1,000; monoclonal mouse anti-Syx1A antibody (DSHB) at 1:50; monoclonal mouse anti-Rpn10 antibody (gift from Zoltan Lipinszki) [28] at 1:50; monoclonal mouse anti-FK2 HRP-conjugated antibody (Ubiquigent) at 1:1,000; and horseradish-peroxide (HRP) labeled secondary antibodies from Jackson ImmunoResearch

Laboratories. Western blot was performed and transferred to PVDF membrane using the iBlot system (Invitrogen). The membrane was developed using ECL kit (GE Healthcare) following the manufacturer's instructions. Dual-color westerns were prepared by assigning independent color channels to two independent westerns developed in the same membrane.

Cell staining and microscopy

The BG2 cells were transfected using the calcium phosphate method with 47 GFP-tagged genes and grown over a coverslip glass. After 72 h, cells were washed twice with 1× PBS, fixed in 2 % PFA for 30 min on ice, washed with PBT (0.1 % Triton X-100 in 1× PBS) for three times and nuclei were stained with DAPI. After staining, cells were washed with 1× PBS three times, the cover slips were mounted into slides with Vectashield Mounting Medium (Vector Labs), and samples were analyzed in a confocal microscope using LCS-Leica confocal software.

Drosophila strains

GMR^{GAL4} and OregonR flies were provided by the Bloomington Stock Center (Bloomington, IN, USA). The AS model flies, both the *Ube3a*$^{-/-}$ null mutant (15B/15B) and the *Ube3a* overexpression (^{UAS}Ube3a), were a gift from Janice Fischer [22] and the $^{UAS}Rpn10^{DN}$ flies were a gift from Zoltán Lipinszki [28].

For Western-blot experiments, six heads of 1-day-old flies were cut and homogenated in 60 µl of 4X Laemmli buffer. The samples were then centrifuged 1 min at maximum speed in a minispin and the supernatant was recovered. From each genotype, 10 µl of the supernatant was loaded into a 4–15 % gradient gel (Bio-Rad, Hercules, CA, USA). Five-day-old fly eye pictures were taken with a Leica M80 microscope using a Leica EC3 camera and the Leica Acquire Software.

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Conflict of interest The authors declare that they have no conflicts of interest.

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