Quantitative proteomics reveals neuronal ubiquitination of Rngo/Ddi1 and several proteasomal subunits by Ube3a, accounting for the complexity of Angelman syndrome

Juanma Ramirez [1], Benoit Lectez [1], Nerea Osinalde [2], Monika Sivá [3, 4, 5], Nagore Elu [1], Kerman Aloria [6], Michaela Procházková [7], Coralia Perez [8], Jose Martínez-Hernández [1, 9], Rosa Barrio [8], Klára Grantz Šašková [3, 4], Jesus M. Arizmendi [1] and Ugo Mayor [1, 9, *]

[1] Department of Biochemistry and Molecular Biology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), 48940 Leioa, Spain

[2] Department of Biochemistry and Molecular Biology, Faculty of Pharmacy (UPV/EHU), 01006 Vitoria-Gasteiz, Spain

[3] Department of Genetics and Microbiology, Charles University, 12843 Prague, Czech Republic

[4] Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, 16610 Prague, Czech Republic

[5] First Faculty of Medicine, Charles University, 12108 Prague, Czech Republic

[6] Proteomics Core Facility-SGIKER, University of the Basque Country (UPV/EHU), 48940 Leioa, Spain

[7] Institute of Molecular Genetics of the Czech Academy of Sciences, Czech Centre for Phenogenomics and Laboratory of Transgenic Models of Diseases, Division BIOCEV, Vestec, Czech Republic

[8] Functional Genomics Unit, CIC bioGUNE, 48160 Derio, Spain

[9] Ikerbasque, Basque Foundation for Science, 48013 Bilbao, Spain

* corresponding author: ugo.mayor@ehu.eus, Phone: +34946015908

Abstract

Angelman syndrome is a complex neurodevelopmental disorder caused by the lack of function in the brain of a single gene, *UBE3A*. The E3 ligase coded by this gene is known to build K48-linked ubiquitin chains, a modification historically considered to target substrates for degradation by the proteasome. However, a change in protein abundance is not proof that a candidate UBE3A substrate is indeed ubiquitinated by UBE3A. We have here used an unbiased ubiquitin proteomics approach, the ^{bio}Ub strategy, to identify 79 proteins that appear more ubiquitinated in the *Drosophila* photoreceptor cells when Ube3a is over-expressed. We found a significantly high number of those proteins to be proteasomal subunits or proteasome-interacting proteins, suggesting a wide proteasomal perturbation in the brain of Angelman patients. We focused on validating the ubiquitination by Ube3a of Rngo, a proteasomal component conserved from yeast (Ddi1) to humans (DDI1 and DDI2), but yet scarcely characterised. Ube3a-mediated Rngo ubiquitination in the fly neurons was confirmed by immunoblotting. Using human neuroblastoma SH-SY5Y cells in culture, we also observed that human DDI1 is ubiquitinated by UBE3A, without being targeted for degradation. The novel observation that DDI1 is expressed in the developing mice brain, with a significant peak at E16.5, strongly suggests that DDI1 has biological functions not yet described that could be of relevance for Angelman syndrome clinical research.

Introduction

Angelman syndrome (AS) is a rare neurodevelopmental disorder (OMIM #105830), with an estimated incidence of 1/15,000 births, characterised by a severe developmental delay, language impairment, ataxic movements, epilepsy, sleep disturbances and episodes of frequent laughter (1). In contrast to other complex syndromes that are caused by large genetic duplications/deletions, the underlying cause for AS is the loss of maternal expression in neurons of the brain of one single enzyme, UBE3A (2, 3), a HECT-type ubiquitin E3 ligase (4–6). Although the deficiency of this paternally imprinted gene (7) is commonly originated by maternally-inherited deletions on the 15q11-q13 chromosomal region (8), the syndrome is also caused by mutations affecting exclusively the *UBE3A* gene (9). Some of these UBE3A mutants lack the ubiquitin-ligase activity when tested *in vitro* (10, 11), indicating that AS is caused by the lack of the ubiquitin ligase activity of UBE3A in neurons. Ubiquitinated substrates of UBE3A are therefore likely to be the effecting pathways of the resulting brain connectivity and/or function alterations. Interestingly, excess ligase activity of UBE3A has also been associated with autism spectrum disorders (12–14).

Ubiquitin E3 ligase enzymes catalyse the covalent attachment of ubiquitin to the lysine residues on target proteins. According to *in vitro* studies, UBE3A catalyses the preferential attachment of K48-linked poly-ubiquitin chains (15), presumably targeting its substrates for proteasomal degradation. Based on this premise, several UBE3A putative substrates (AIB1, Bak, Blk, Mcm7, Pbl) whose levels changed in the presence/absence of this enzyme were reported (16–20). Nevertheless, ubiquitination of those proteins by UBE3A has not yet been described. Similarly, the ubiquitination of other proposed neuronal UBE3A substrates was only validated *in vitro* (Arc, Na^+/K^+ ATPase, p27, Ring1B, Adrm1, Rpt5) or using non-denaturing immunoprecipitation approaches (Annexin A1, HHR23A, PSMD2,

Ephexin5, p53) (21–29). We developed an ubiquitination assay for neuronal cell culture using a highly denaturing protocol, and showed that proteasome regulating proteins Rpn10 and Uch-L5 are ubiquitinated by Ube3a (30), both being reported to be essential for mammalian brain development (31, 32). Recently, evidence is accumulating in regards to UBE3A regulating proteasomal activity (28–30, 33), suggesting that UBE3A might indirectly be affecting the regulation of many other proteins targeted to the proteasome by other E3 ligases.

Interaction between an ubiquitin ligase and its substrates **is transient and hard to capture** in vivo. Additionally, the low stoichiometry at which ubiquitin modified proteins are found within the cell hinders the identification of ubiquitination substrates *in vivo*. Antibodies that specifically recognise the ubiquitin diGlycine (diGly) signature have been employed to isolate, and subsequently identify putative ubiquitination sites by mass spectrometry (MS) (34–39). Such methodology has also been recently used in a screen for putative UBE3A substrates in HEK293 cells (29). However, this approach requires digestion of proteins by trypsin prior to their isolation, preventing any orthogonal validation, which is essential, as the diGly signature is also a remnant of other ubiquitin-like modifications, such as Nedd8 or ISG15 (35), and can even be an experimental artefact under certain conditions (40). We have developed in our lab two methodologies that have proven to be suitable for the *in vivo* analysis of ubiquitinated proteins (41). The ^{bio}Ub strategy, based on the *in vivo* biotinylation of ubiquitin (42), has recently been used in combination with quantitative shotgun proteomics to identify substrates of the E3 ligase Parkin involved in Parkinson's disease (43). The second strategy favours the isolation of GFP-tagged proteins under denaturing conditions, and was first used to screen for *Drosophila* Ube3a substrates (30).

In the present study, we have combined the ^{bio}Ub strategy with the over-expression of Ube3a to identify 79 putative neuronal Ube3a substrates. Amongst those, we noted the presence of 13 proteasome subunits or proteasome interacting proteins. We validated that Ube3a ubiquitinates the proteasomal ubiquitin receptor Rings lost (Rngo) in *Drosophila* photoreceptor neurons *in vivo*. Furthermore, we have confirmed that ubiquitination of DNA damage-inducible protein 1 homologue 1 (DDI1), the human orthologue of Rngo, is enhanced upon UBE3A over-expression in neuroblastoma cells, without being targeted for degradation. The observation that DDI1 is highly expressed in the developing mice brain suggests that this protein has a yet uncharacterised biological function in neuronal development,.

Results

Unbiased identification of Ube3a substrates in Drosophila *neurons* in vivo

In order to identify by MS analysis the proteins whose ubiquitination depends on Ube3a, we used the following fly lines: BirA, ^{bio}Ub, ^{bio}15B and ^{bio}A3. ^{bio}Ub flies express the $(^{bio}Ub)_6$-BirA precursor (**Figure 1A)** in the *Drosophila* photoreceptor neurons under the control of the eye-specific GMR-GAL4 driver, which has been shown to be the most suitable neuronal(-like) driver for identifying low abundance proteins, and for optimizing reproducibility across samples (44). BirA control flies express just the bacterial biotinylating enzyme BirA (44). ^{bio}A3 flies are ^{bio}Ub flies over-expressing the Ube3a E3 ligase (**Figure 1B**), as confirmed by immunoblotting (**Figure 1C**). On the other hand, ^{bio}15B flies are ^{bio}Ub flies carrying a loss of function *Ube3a* deletion (*Ube3a^{15B} allele*) in heterozygosis. Homozygous mutant *Ube3a^{15B}* flies lack any detectable Ube3a protein (**Supplementary Figure 1A**), but flies carrying this allele could not be expanded in homozygosis. The *Ube3a^{15B}* heterozygous ^{bio}15B flies used for this study only show a partial reduction of Ube3a protein levels (**Figure 1C**). Free BirA, indicating appropriate processing of the $(^{bio}Ub)_6$-BirA precursor, was observed for all three genotypes (**Figure 1D**); no undigested forms of the precursor were found above the expected molecular size of BirA (35 kDa). Biotin immunoblotting confirmed biotinylation and incorporation into conjugates of the GMR-GAL4-driven ectopic biotin-tagged ubiquitin in all ^{bio}Ub, ^{bio}A3 and ^{bio}15B flies (**Figure 1E**). Expression of the ^{bio}Ub construct in those three fly lines did not significantly alter total ubiquitin levels, when compared to the BirA control (**Supplementary Figure 1B**).

Comparison of the ubiquitinated proteome of ^{bio}A3 flies and ^{bio}Ub flies should allow the identification of proteins whose ubiquitination is enhanced by Ube3a. Conversely, we would expect to have a reduction on the ubiquitination of Ube3a substrates on ^{bio}15B flies in

respect to ^{bio}Ub flies (**Figure 2A**). Biotinylated ubiquitin conjugates formed within the fly photoreceptor neurons were isolated using neutravidin beads, those pulldowns being performed on three biological replicates for each of the three conditions. Despite collecting whole heads, the isolated material is expected to originate just from the GMR-GAL4 expressing cells. Similar amounts of ubiquitinated proteins were eluted from the three genotypes (**Figure 2B** and **Supplementary Figures 2A and 2B**). After fractionation by SDS-PAGE,each gel lane, corresponding to one sample, was cut into several slices as indicated in **Supplementary Figure 2B**). Protein loads from each individual gel slice were in-gel digested with trypsin and subsequently analysed by LC-MS/MS.

Similar number of ubiquitinated proteins and a high correlation of the Label Free Quantification (LFQ) intensity values were detected, both between replicas and across the different genotypes (**Supplementary Figures 3A and 3B**). Random LFQ values from a distribution meant to simulate expression below the detection limit (45) were imputed to those proteins for which LFQ values were not reported by the MaxQuant software on that given experiment (**Supplementary Figure 3C**). The experimental design was successful as evidenced from the LFQ intensity values obtained for Ube3a, which appeared highly enriched in the ^{bio}A3 sample, and significantly reduced in the ^{bio}15B sample (**Figure 2C**). Western blot analysis confirmed those results and revealed that Ube3a is mostly isolated in an unmodified form (**Figure 2D**), as previously reported (44).

We compared the LFQ intensities of ubiquitinated proteins on the ^{bio}A3 sample with the corresponding ^{bio}Ub values and plotted their fold-changes (X-axis) and significance p-values (Y-axis) as a Vulcano plot (**Figure 3**). As expected, most of the proteins detected in this study displayed a ratio close to one, including endogenously biotinylated proteins Acetyl-CoA Carboxylase (ACC), Pyruvate Carboxylase (PCB) and CG2118 (46) (shown in grey in

Figure 3), indicating that the pulldown process was equally efficient in the different samples. From the 751 protein groups identified across all genotypes, 79 were significantly ($p < 0.05$) enriched at least two-fold in the ^{bio}A3 sample relative to the ^{bio}Ub control, and can therefore be defined as putative Ube3a substrates (**Supplementary Table 1**). In order to focus on the highest confidence Ube3a substrates, the 79 proteins regulated by Ube3a were analysed at the peptide level to comply with the following requirements: 1) Average ratio between common peptides identified in both conditions should be at least two after the subtraction of the standard error of the mean (SEM); 2) Individual peptide intensities should globally follow the same tendency as the protein LFQ intensity and show in average a two-fold enrichment. Out of the 79 proteins identified as enriched at the protein level, 39 candidate Ube3a substrates (labelled with green dots in **Figure 3**) appeared homogenously enriched also at the peptide level (**Supplementary Figure 3D**), most of them having human orthologues (**Table 1**). Among those 39 high confidence candidate Ube3a substrates, 8 proteins regulate protein degradation through the ubiquitin-proteasome system, including proteasomal subunits Prosα1, Prosα3, Rpt2, Rpt4, Rpn3 and Rpn8, and the proteasomal shuttling proteins Rngo and Rpn10, the latest being previously identified as target of Ube3a in neuronal cell culture (30). Besides, two proteins related to autophagy, Atg8a and Ref(2)P (47), and three chaperone proteins, CCT3, CCT7 and CCT8 (48), were also found as high confidence substrates of Ube3a. In addition, proteasomal subunits Prosα4, Prosα7, Rpn2, proteasome activator REG, and the proteasome associated deubiquitinating (DUB) enzyme Uch-L5 were significantly enriched according to LFQ values, but did not pass the requirements at peptide level.

On the other hand, a total of 55 proteins also appeared to be less ubiquitinated in ^{bio}A3 sample due to the over-expression of Ube3a (**Supplementary Table 1**), displaying a two-fold

significant reduction in their abundance compared to ^{bio}Ub (^{bio}A3/^{bio}Ub < 0.5; p < 0.05). Following the same peptide analysis described before (**Supplementary Figure 3E**), we concluded that 14 proteins are clearly less ubiquitinated when Ube3a is over-expressed, as compared to the control sample (labelled with red dots in **Figure 3).** Interestingly, we reliably identified Calcium/calmodulin-dependent protein kinase II (CaMKII) –which in *Drosophila* is coded by a single gene- as being less ubiquitinated upon over-expression of Ube3a.

We also compared the LFQ intensities of the ^{bio}15B sample with the ^{bio}Ub values; but, using the same criteria as above, Ube3a itself did not appear significantly enriched with high confidence in the ^{bio}Ub sample relative to the ^{bio}15B ubiquitinated material. However, three proteins (Rdhb, Map2015 and Axo) appeared significantly less ubiquitinated in the ^{bio}15B sample, barely above the defined thresholds (data not shown).

Ubiquitin LFQ values and ubiquitin chain linkages indicate proteasomal perturbation upon Ube3a over-expression

Based on the experimental design, we expected ubiquitin levels to be unaltered by Ube3a over-expression. Furthermore, no significant differences were observed on the hard to quantify smears of the silver stained gels (**Figure 2B**), as well as ubiquitin (**Supplementary Figure 1B**) and biotin (**Supplementary Figure 2A**) blots. However, based on LFQ values, ubiquitin levels were quantified to have a significant increase of 1.66-fold in the ^{bio}A3 flies, relative to the ^{bio}Ub sample (**Supplementary Table 1**), this is, a >60% increase of ubiquitin was detected on the collected ubiquitinated material of ^{bio}A3 flies. In order to elucidate the type of ubiquitin chains enriched upon over-expression of Ube3a, we compared the intensity of all detected diGly containing peptides across the samples. Digestion of a complex mixture

of ubiquitinated proteins is expected to result in a complex mixture of ubiquitin chain linkages. Even though the isolated ubiquitinated material is composed of substrates of hundreds of ligases, this analysis indicated that K48 and K63 linkages were significantly more abundant on the ^{bio}A3 sample, while K33 linkages were reduced (**Supplementary Figure 3F**). The 60% increase on ubiquitin levels together with the significant changes in chain linkages suggests a global perturbation of the ubiquitin proteasome system upon Ube3a over-expression.

Rngo is ubiquitinated by Ube3a in vivo *in the* Drosophila *neurons*

Amongst the highest confidence candidates as Ube3a substrates (**Table 1**), eight of the identified proteins are proteasome integral or regulatory subunits. We decided to focus on Rngo, a predicted proteasomal shuttling factor for which working antibodies were available (49), and which was already observed in our first neuronal ubiquitome studies (42). According to the label free quantitative MS-based analysis performed, Rngo is 5-fold more enriched in Ube3a over-expressing flies in respect to control flies. In agreement with that, Western blot analysis showed that ubiquitination of Rngo is enhanced when Ube3a is over-expressed and reduced in its absence (**Figure 4A**), making Rngo the first Ube3a substrate validated in any type of neuron *in vivo*. Interestingly, Rngo total levels were not altered, suggesting that it is not being targeted for degradation. As a control, the same membrane was used to detect the presence of Fax, a protein that we found by MS to be less ubiquitinated upon Ube3a over-expression (**Figure 3**). Indeed, Fax displayed the opposite trend, and its ubiquitination was dramatically reduced by the over-expression of Ube3a (**Figure 4A**), indicating that the increase ubiquitination seen for Rngo is specific to the protein and not to a more efficient general isolation of proteins in the ^{bio}A3 sample. We also

analysed by Western blot the ubiquitination of another proposed UBE3A substrate (27), the Na^+/K^+-ATPase α-subunit (Atpα), but as with the MS analysis (**Figure 3**), Western blot did not show any enhancement by Ube3a over-expression on its ubiquitination (**Figure 4B**).

Ddi1 and Ddi2 are expressed throughout development in the mouse brain

Mammalians have two protein homologues to *Drosophila* Rngo: DNA damage-inducible protein 1 homologue 1 (DDI1) and homologue 2 (DDI2). Partial expression data for DDI2 are available at the Human Protein Atlas (www.proteinatlas.org), but information about mammalian DDI1 is very scarce. We tried to assess the expression of DDI1 using commercially available antibodies, but, since DDI1 and DDI2 proteins have very high amino acid sequence identity (72%), all the commercially available antibodies recognize DDI2 protein, or do simply not work (data not shown). We therefore tested whether mouse Ddi1 and Ddi2 are expressed in the brain. Analysis of Ddi1 gene expression in mice brain at different ages performed by qRT-PCR revealed a drastic and significant increase of Ddi1 mRNA levels at embryonic stage E16.5 (**Figure 5A**). The Ddi1 mRNA expression peak was rapidly reduced from E17.5, returning to basal levels at E19.5-P1, remaining relatively stable during all the tested adult time points. In contrast, Ddi2 mRNA levels, which were also detectable in the mice brain, only fluctuated slightly during development (**Figure 5B).**

Additionally, we analysed the expression profile of Ddi1 in the developing brain by performing RNA *in-situ* hybridization experiments in CD-1 mouse embryos at four different stages of development – E9.5, E10.5, E14.5 and E16.5. As shown in **Figure 6**, Ddi1 is expressed in all parts of developing brain (telencephalon, diencephalon, mesencephalon and rhombencephalon) at the two younger developmental stages. Similar Ddi1 expression pattern was observed for these two stages in both whole mount samples (**Figure 6A, 6C**) and

sagittal paraffin sections (**Figure 6B, 6D**). At the stage E14.5, the expression is located in neurons of mesencephalic and telencephalic structures, with signal accumulation in upper hill (colliculus tectum) and ventricular zone of pallium (**Figure 6E, 6F, 6G**). At further developmental stage E16.5, which shows highest mRNA level expression according to our qRT-PCR screen, Ddi1 is expressed in particular telencephalon parts, mainly the ventricular layer and cortical plate of isocortex and the ventricular layer of olfactory bulb (**Figure 6H, 6I**). This could represent the dividing neuroblasts and their migration towards the superficial layer of isocortex. The positive staining of neuronal cells in the isocortex (**Figure 6J**) is clear evidence of Ddi1 expression in neuronal tissue, however, up to date no single report has described what the role DDI1 could exert in the brain.

Human DDI1 is ubiquitinated by UBE3A, but is not targeted for degradation.

Once identified and confirmed that Ube3a ubiquitinates Rngo in flies, we aimed to test whether its homologs DDI1 and DDI2 are substrates of human UBE3A. For that purpose, we employed an *in cellulo* ubiquitination assay (30, 50) in SH-SY5Y neuroblastoma cells. Wild type UBE3A ($UBE3A^{WT}$) induced ubiquitination of DDI1-GFP in SH-SY5Y cells (**Figure 7** and **Supplementary Figure 5A**), but did not seem to increase the ubiquitination status of DDI2-GFP (**Supplementary Figure 5B**). We also confirmed the specificity of UBE3A-dependent DDI1 ubiquitination by proving that Parkin, the E3 ligase involved in Parkinson's disease, could not mediate ubiquitination of DDI1 (**Supplementary Figure 5B**). Altogether, the data presented here demonstrates for the first time that Rngo human homologue DDI1 is a UBE3A substrate. Despite earlier reports suggested that UBE3A generates degradation-leading K48 ubiquitin linkages *in vitro* (15), at least in SH-SY5Y neuroblastoma cells, UBE3A-mediated ubiquitination of human DDI1 does not lead to its degradation, as indicated by the intensity of the DDI1-GFP bands, which is independent of the activity of UBE3A (**Figure 7**).

Discussion

Our unbiased proteomic analysis for the identification of differentially ubiquitinated proteins in *Drosophila* photoreceptor neurons upon Ube3a over-expression has resulted in a list of 79 putative Ube3a substrates, out of which proteasomal proteins appear very highly enriched. To our knowledge, this is the first time a list of candidate Ube3a substrates, whose ubiquitinated fraction is enhanced upon Ube3a over-expression, is reported in neurons *in vivo*. Several of the putative Ube3a substrates identified (Arc1, Chc, Gclc, GlyRS, Path, Tig and SesB) play a role either in axon and dendrite morphogenesis (51–54) or synaptic transmission (55–57). Interestingly, upon Ube3a over-expression we also found a significant reduction of the ubiquitination of CaMKII, a key kinase known in humans to regulate neurotransmitter synthesis and release, modulation of ion channel activity, neurite extension, synaptic plasticity, learning and memory (58). Reduced activity and protein levels of CaMKII at the postsynaptic density have been described in a mouse model of AS (59). It still remains to be explained how a reduction of UBE3A levels enhances the ubiquitination and/or degradation of CaMKII.

Analysis of the putative Ube3a substrates by G:Profiler analysis of GO:Terms and KEGG pathways indicate a highly significant enrichment of the proteasome (data not shown). Furthermore, comparison of the data obtained in this work to the dataset of Parkin substrates (43), further confirms a deregulation of the proteasome upon Ube3a over-expression. Indeed, UBE3A is known to be a proteasome-associated protein (28, 60), as well as being capable of ubiquitinating several proteasomal subunits in cell culture (28–30). However, it is controversial whether UBE3A inhibits (28) or stimulates (33) the proteolytic activity of the proteasome. Ube3a over-expression results in an increase in total ubiquitin levels, while over-expression of Parkin did not. Having identified several proteasomal

subunits as ubiquitinated by Ube3a, the simplest explanation is that Ube3a-driven proteasomal deregulation results in the accumulation of substrates ubiquitinated by Ube3a or other E3 ligases. This would also explain the reduction observed here in the ubiquitination of many other proteins. It is known that the specific deregulation of the proteasome by over-expressing a dominant negative Rpn10 subunit reduces the ubiquitination levels of Fax and other mono-ubiquitinated proteins (44). Deubiquitination of mono-ubiquitinated proteins is also seen when inhibiting the proteasome pharmacologically (35), and is explained by a reduction of the free ubiquitin available pool concomitant to the accumulation of proteasome targeted poly-ubiquitinated proteins (36, 61–63). The decrease on Fax ubiquitination upon Ube3a over-expression, detected here by both MS (**Figure 3**) and immunoblotting (**Figure 4A**), could therefore be caused by an Ube3a-induced proteasomal inhibition. Significant changes in global ubiquitin chain linkages (**Supplementary Figure 3F**) are also in line with this interpretation.

In this work, we have validated by Western blot that Rngo is a direct Ube3a substrate in photoreceptor neurons. Rngo contains both an Ubiquitin-like (UBL) and an Ubiquitin-binding domain (UBD), a hallmark of proteasomal shuttles (49), but can also bind directly another proteasomal shuttle protein, Rpn10 (49). The ubiquitination of Rpn10 is also increased in Ube3a over-expressing flies (**Figure 3**), and was already identified as an Ube3a substrate in *Drosophila* cells, as well as being shown to interact genetically with Ube3a in neurons *in vivo* (30). Ubiquitination of such proteasomal regulators by Ube3a can be predicted to severely interfere with proteasomal function. Considering that the proteasome regulates dendritic development (32), long term potentiation (64), long term depression (65), synaptic plasticity (66), synaptic strengthening (67), memory consolidation (68), circadian rhythms (69) and many other aspects of neuronal function, an UBE3A-dependent

proteasomal regulation could easily explain how a single E3 ligase mutation can cause a disorder as complex as AS. And indeed, it should be noted that UBE3A has been shown to regulate most aspects of neuronal function listed above (70–74). Nondegradative ubiquitination of proteasomal receptors could alter their function to a similar extent as their degradation, since the activity of those receptors is dependent on their ubiquitin binding domains. An ubiquitinated ubiquitin-binding subunit is likely to prioritirily bind its own ubiquitin moieties, therefore blocking its normal function. Our prediction would be that – based on the increased ubiquitination of those subunits upon UBE3A over-expression- proteasome activity should be increased in the brain of AS patients, on which UBE3A levels are reduced.

Interestingly, Rngo is also the *Drosophila* homologue of yeast Ddi1/Vsm1, a protein that binds to several Snc-interacting t-SNAREs (75), negatively regulates exocytosis (76) and also regulates protein secretion (77). Similarly, the *C. elegans* homologue DDI-1/VSM-1 has also been proposed to regulate synaptic function, with *vsm-1* mutants displaying a significant increase in synaptic density along the dorsal nerve cord (78). Thus, Rngo/DDI1 may have an additional role in synaptic transmission by controlling SNARE mediated exocytosis. In fact, over-expression of Ube3a, but not its ligase dead form, has been reported to alter neurotransmission at the neuromuscular junction in *Drosophila* (79).

Two mammalian Rngo homologues have been described, DDI1 and DDI2, but neither of those proteins have to date been functionally characterised. We have found that both are expressed in the developing brain (**Figure 5** and **6**). Structurally, both proteins contain the characteristic Retroviral Protease-like domain that was recently reported to cleave/activate the Nrf1 transcription factor under proteasome inhibition (80, 81). Further, they both contain an additional helical HDD domain (82, 83) and an N-terminal UBL domain, but lack

the well-defined Ubiquitin-associated (UBA) domain. Although, a weak Ubiquitin-binding motif (UIM) is present at the C-terminus of DDI2 (82), no such a motif can be found in DDI1. It is thus feasible that the DDI1 UBL domain, which in yeast Ddi1 is capable of binding ubiquitin (84), could substitute the role of the UBA domain, a mechanisms that would be facilitated by DDI1´s homodimeric conformation.

Having confirmed the ubiquitination of Rngo in photoreceptor neurons by the *Drosophila* Ube3a and the expression of its mammalian homologues in the brain, we then tested whether any of Rngo´s human orthologues are ubiquitinated by UBE3A. We confirmed DDI1 to be an ubiquitination substrate of UBE3A in SH-SY5Y neuroblastoma cells. Different controls indicated that ubiquitination of DDI1 by UBE3A is specific, but does not lead to a reduction of DDI1 protein levels. It is not the first time that regulation of protein activity, even with formation of K48-linked chains, has been reported to be proteolysis-independent (85). A Ubiquitin-binding domain (UBD) found in Met4 was proposed to cap its own K48-linked ubiquitin chain, inactivating the protein and protecting it from degradation (86), and something similar could be happening with Rngo/DDI1. Further work is required to elucidate what the functional role of DDI1 ubiquitination might be and whether the presence of UBD/UIM/UBA/UBL in ubiquitination substrates might interfere into the canonical role of K48 ubiquitin-linked chains.

Up to now, no candidate ubiquitination substrates of UBE3A had been directly validated in neurons *in vivo*. We had earlier identified a proteasomal shuttling factor, Rpn10, to be regulated by Ube3a in *Drosophila* cells (30), which we now confirmed *in vivo* by MS. Further, we have validated that in *Drosophila* photoreceptor neurons Ube3a ubiquitinates another proteasomal shuttling factor, Rngo, becoming the first Ube3a substrate to be identified and validated *in vivo* in neurons within a whole organism. More importantly,

UBE3A regulates the orthologue DDI1 protein in human neuroblastoma cells. Since UBE3A ubiquitination appears to be regulating several proteasomal-associated subunits, as already indicated by ourselves and others (29, 30, 33), it would not be surprising to see that a highly significant number of proteins regulated downstream the proteasome will display significant changes on their abundance upon UBE3A mutation or over-expression. It is now a challenge to elucidate which Ube3a substrates are direct, in addition to the proteasomal proteins themselves.

DDI1 gene has been reported to be affected in siblings of a familial neurodegenerative disorder characterised clinically as a variant of Alzheimer's disease (87). Having now described for the first time the temporal and spatial expression of Ddi1 in the mouse brain, we next need to perform a functional characterization to uncover the neuronal role of DDI1, as this might as well bring light to our understanding of how AS is regulated. Based on studies in yeast and *C. elegans*, it is likely that DDI1 is involved in both regulation of synapses and proteasomal function (75–78, 84). Given the complexity of neuronal function, and previous work aiming to identify the mechanisms regulated by UBE3A, it is likely that both processes are actually misregulated during the genesis of AS. Finally, if we take into account that Ube3a regulates the proteasome, and that UBE3A expression declines with age (88), it would not be surprising that this E3 ligase has a further role in proteostasis not yet characterised.

Material and Methods

Drosophila stocks and sample collection

We have used in this work the BirA, ^{bio}Ub, ^{bio}A3 and ^{bio}15B flies, all of which express their corresponding constructs in the *Drosophila* photoreceptor neurons under the control of the GMR-GAL4 driver. BirA and ^{bio}Ub flies, expressing respectively the BirA enzyme alone and the $(^{bio}Ub)_6$-BirA precursor, have been previously described (44). Their genotypes are respectively *GMR-GAL4/CyO;UAS-BirA/TM6* and *GMR-GAL4,UAS-(^{bio}Ub)$_6$-BirA/CyO*. Ube3a gain of function (*UAS-Ube3a^{A3}*) and loss of function (*Ube3a^{15B}*) flies (89) were a gift from Professor Janice Fisher. Both *UAS-Ube3a^{A3}* and *Ube3a^{15B}* fly lines were independently mated to *GMR-GAL4,UAS-(^{bio}Ub)$_6$-BirA/CyO;TM2/TM6* flies to generate *GMR-GAL4,UAS-(^{bio}Ub)$_6$-BirA/CyO;UAS-Ube3a^{A3}/TM6* and *GMR-GAL4,UAS-(^{bio}Ub)$_6$-BirA/CyO;Ube3a^{15B}/TM6* lines. The *Drosophila* Ube3a mutants (*Ube3a^{15B}*) had been reported to be viable and fertile in homozygosis (89). When combined with ^{bio}Ub flies, it was, however, required to grow them in heterozygosis, as null Ube3a flies were viable but not fertile at 25 °C (J. Ramirez and U. Mayor, unpublished data). GMR-GAL4 (BL 1104) and *OregonR* (BL 2376) flies were provided by the Bloomington *Drosophila* Stock Center (Bloomington, IN, USA). The ^{bio}Ub abbreviation is used throughout the text to refer to the *GMR-GAL4,UAS-(^{bio}Ub)$_6$-BirA/CyO* flies, and the ^{bio}A3 and ^{bio}15B (for *Ube3a^{A3}* and *Ube3a^{15B}* alleles) to refer to *GMR-GAL4,UAS-(^{bio}Ub)$_6$-BirA/CyO;UAS-Ube3a^{A3}/TM6* and *GMR-GAL4,UAS-(^{bio}Ub)$_6$-BirA/CyO; Ube3a^{15B}/TM6* flies, respectively.

Flies were grown at 25 °C in 12 hours light-dark cycles in standard *Drosophila* medium (0.9 % agar, 7.5 % dextrose, 6 % corn flour, 8.5 % yeast, 2.5 % Nipagin, 0.4 % propionic, 0.02 % benzalkonium chloride in distilled H_2O). 2-5 days old mixed-sex flies were flash-frozen in liquid nitrogen and shaken while still frozen to sever the heads. Frozen fly heads were

then separated from the remaining body parts using a pair of sieves with a nominal cut-off of 710 and 425 µm, and then stored at -80 °C. Head collections were typically performed in the morning.

Plasmids

Commercial pEGFP-N1 vector (Clontech) was used to generate DDI1-GFP and DDI2-GFP vectors (see Cloning procedures). *FLAG-UBE3A-pCMV* ($UBE3A^{WT}$) and *FLAG-UBE3A^{LD}-pCMV* ($UBE3A^{LD}$) plasmids, expressing N-terminally FLAG-tagged versions of the wild type and catalytically inactive human UBE3A protein (90), were a gift from Dr. Vjekoslav Tomaić. FLAG-tagged ubiquitin (30) in pCDNA3.1 vector (FLAG-Ub) was generously provided by Dr. Jose Antonio Rodriguez Pérez (University of the Basque Country-UPV/EHU, Spain). Untagged human Parkin plasmid has been previously described (43). Empty pCDNA3.1 vector (Invitrogen) was used as control.

Cloning procedures

DDI2-pEGFP-N1 (DDI2-GFP) plasmid was generated by amplifying *DDI2* gene (Uniprot Q5TDH0) from DDI2-pET16b plasmid (82) with DDI2-Fw (5'-*AAGGTACCATGCTGCTCACCGTG-3'*) and DDI2-Rv (5'-*AAGGATCCCCTGGCTTCTGACGCTCTGC-3'*) primers and inserted between *KpnI* and *BamHI* restriction sites of pEGFP-N1 vector (Clontech). Gene for human DDI1 protein (Uniprot Q8WTU0) was synthesised by GenScript and further amplified using the *DDI1-Fw* (5'-*TATAGGTACCATGCTGATCACCGTG-3'*) and *DDI1-Rv* (5'-*TATAACCGGTATGCTCTTTTCGTCC-3'*) primers and inserted between the *Acc65I* and *AgeI* sites of the DDI2-pEGFP-N1 vector, after the *DDI2* gene had been removed using the same restriction enzymes. All PCR reactions were carried out with Phusion High-Fidelity DNA polymerase (Thermo Scientific). PCR product gel extractions and plasmid purifications were

performed with the QIAGEN Gel Extraction Kit and QIAGEN plasmid mini and midi kits, respectively. Correct sequence for all plasmids was confirmed by sequencing either by the GATC Biotech Company (Köln, Germany) or the SGIKER Unit of Sequencing and Genotyping at the University of the Basque Country (Leioa, Spain).

Western blotting and Silver staining

Both 4-12 % Bolt Bis-Tris Plus pre-cast gels (Invitrogen) and 4-12 % NuPAGE Bis-Tris gels (Invitrogen) were used for SDS-PAGE, then proteins were transferred to PVDF membranes using the iBlot system (Invitrogen). Following primary and secondary antibody incubation, membranes were developed with an ECL kit (Biorad Clarity). Dual-colour westerns were prepared by assigning independent colour channels to two independent westerns developed in the same membrane. The amount of material loaded for Western blot analysis varied according to the tissue and the antibody employed. In the case of material obtained from fly biotin pulldowns, between 0.001 % and 0.2% of the input samples and 5-10 % of the elution samples were loaded. However, when material purified from cells was used, between 10-20 % of inputs and 10-40 % of the elution samples were loaded.

The following primary antibodies were used: goat anti-biotin-horseradish peroxidase (HRP) conjugated antibody (Cell Signalling; catalogue number 7075) at 1:1000; chicken polyclonal anti-BirA antibody (Sigma; catalogue number GW20013F) at 1:1000; mouse monoclonal anti-GFP antibody (Roche Applied Science; catalogue number 11814460001) at 1:1000; mouse monoclonal anti-FLAG M2-HRP conjugated antibody (Sigma; catalogue number A8592) at 1:1000; mouse monoclonal anti-Syx1A antibody (Developmental Studies of Hybridoma Bank; DSHB; catalogue number 8C3) at 1:100; rabbit polyclonal anti-Fax antibody, a gift from Eric Liebl (Deninson University, OH, USA) at 1:1000; mouse monoclonal anti-Atpα antibody (DSHB; catalogue number α5) at 1:50; rabbit polyclonal anti-Ube3a

antibody (91) for the detection of *Drosophila* Ube3a protein at 1:1000; rabbit polyclonal anti-Rngo antibody (49) at 1:500; rabbit polyclonal anti-ubiquitin antibody (Sigma; catalogue number U5379) at 1:100; mouse monoclonal anti-UBE3A (clone E6AP-300) antibody (Sigma; catalogue number E8655) for the detection of human UBE3A protein at 1:1000. The following secondary antibodies were used: goat anti-mouse-HRP-labelled antibody (Thermo Scientific; catalogue number 62-6520) at 1:4000; goat anti-rabbit-HRP labelled antibody (Cell Signalling; catalogue number 7074) at 1:4000 and donkey anti-chicken-HRP labelled antibody (Jackson ImmunoResearch; catalogue number 703-035-155) at 1:2000.

About 10 % of the neat elution samples were used for silver staining analysis. Gels were fixed for 1 h at room temperature with 40 % methanol and 10 % acetic acid containing solution and then were stained using the SilverQuest kit from Invitrogen according to manufacturer's instructions.

Biotin pulldown

Biotin pulldowns (42) from *Drosophila* heads were performed as previously described (43, 44, 92). About 500 mg of 2-5 days old fly heads of each genotype were homogenised in 2.9 mL of Lysis buffer (8 M Urea and 1% SDS in PBS) supplemented with 50 mM N-ethylmaleimide (Sigma) and a cOmplete protease inhibitor cocktail (Roche Applied Science). Lysates were centrifuged for 5 min at 16000 *g* at 4 °C and supernatant applied to a PD10 desalting column (GE Healthcare) previously equilibrated with 25 mL of Binding buffer (3 M Urea, 1 M NaCl, 0.25 % SDS and 50 mM N-ethylmaleimide). Eluates, except 50 µL that were kept for monitoring the inputs, were then incubated with 250 µL of NeutrAvidin agarose beads suspension (Thermo Scientific). Unbound material (flow through) was separated by spinning the beads at 230 *g* for 2 min. Beads were then subjected to stringent washes with

six different washing buffers (WB): twice with WB1 (8 M Urea, 0.25 % SDS), thrice with WB2 (6 M Guanidine-HCl), once with WB3 (6.4 M Urea, 1 M NaCl, 0.2 % SDS), thrice with WB4 (4 M Urea, 1 M NaCl, 10 % Isopropanol, 10 % Ethanol, 0.2 % SDS), once with WB1, once with WB5 (8 M Urea, 1 % SDS) and thrice with WB6 (2 % SDS). All buffers were prepared in PBS. Beads were then heated at 95 °C for 5 min in 125 µL of Elution buffer (250 mM Tris–HCl pH 7.5, 40 % glycerol, 4 % SDS, 0.2 % BPB, 100 mM DTT) and centrifuged for 2 min at 16000 *g* in a Vivaclear Mini 0.8 µm PES micro-centrifuge filter unit (Sartorius) to recover the eluted proteins. Finally, eluates were concentrated in Vivaspin 500 centrifugal filter units (Sartorius).

Cell culture and transfection

Human neuroblastoma SH-SY5Y cells were cultured under standard conditions (37 °C, 5 % CO_2) in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) with GlutaMAX (Thermo Scientific), supplemented with 10% Fetal Bovine Serum (Thermo Scientific), 100 U/mL of penicillin (Invitrogen) and 100 µg of streptomycin (Invitrogen). SH-SY5Y cells (3 x 10^5 cells) were seeded in 6 well-plates for transfection experiments. Overnight incubation under serum starvation was routinely performed prior to transfections. The following day OptiMEM serum-free medium (Thermo Scientific) was replaced by fresh DMEM/F-12 and cells were co-transfected with 1 µg of *FLAG-Ub* and 1 µg of *DDI1-GFP*, or *DDI2-GFP*, for 72 h using Lipofectamine 3000 transfection reagent (Invitrogen) according to manufacturer's instructions. 1 µg of either *pcDNA3.1* (control), *UBE3A*^{WT}, *UBE3A*^{LD} or *Parkin* plasmids were additionally added to the transfection mixture to check the effect of UBE3A in DDI1 and DDI2 ubiquitination. Cells were washed twice in PBS and stored at -20 °C until required.

GFP beads pulldown assay

Transfected SH-SY5Y cells were lysed with 500 µL of Lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 % Triton, 1X Protease Inhibitor cocktail from Roche Applied Science and 50 mM N-ethylmaleimide from Sigma) and centrifuged at 14000 *g* for 10 min. Supernatants were mixed with 25 µL of GFP-Trap-A agarose beads suspension (Chromotek GmbH), which had been previously washed twice with a Dilution buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1X Protease Inhibitor cocktail, 50 mM N-ethylmaleimde). The mixture was then incubated at room temperature for 150 min with gentle rolling and centrifuged for 2700 *g* for 2 min to separate the beads from the unbound material. GFP beads were subsequently washed once with the Dilution buffer, thrice with Washing buffer WB5 (8 M Urea, 1% SDS in PBS) and once with 1% SDS in PBS. Bound GFP-tagged proteins were eluted in 25 µL of Elution buffer (250 mM Tris–HCl pH 7.5, 40 % glycerol, 4 % SDS, 0.2 % BPB, 100 mM DTT) by heating at 95 °C for 10 min.

In-gel trypsin digestion and peptide extraction

Eluates from biotin pulldown assays were resolved by SDS-PAGE using 4-12 % Bolt Bis-Tris Plus pre-cast gels (Invitrogen) and visualised with Colloidal Blue following manufacturer's instructions (Invitrogen). When processing biotin pulldown samples, each gel lane was cut into 7 slices (see **Supplementary Figure 2B**). Based on earlier experiments and the BirA control, we excluded the intense bands corresponding to avidin monomers, dimers and an endogenously biotinylated protein from further analysis. The remaining 4 slices were subjected to in-gel digestion as described previously (93). Briefly, proteins were reduced and alkylated by incubating with DTT and chloroacetamide, respectively. Protein digestion was performed by saturation of the gel pieces with trypsin and overnight incubation at 37 °C. Resulting peptides were extracted from the gel, dried down in a vacuum centrifuge and

stored at -20 °C. Peptide mixture was resuspended in 0.1 % formic acid previous to the LC-MS/MS analysis.

LC-MS/MS analysis

Mass spectrometric analyses were performed on an EASY-nLC 1000 liquid chromatography system interfaced with a Q Exactive mass spectrometer (Thermo Scientific) via a Nanospray Flex ion source. Peptides were loaded onto an Acclaim PepMap100 pre-column (75 µm x 2 cm, Thermo Scientific) connected to an Acclaim PepMap RSLC (50 µm x 15 cm, Thermo Scientific) analytical column. Peptides were eluted from the column using a linear gradient of 2 to 40 % acetonitrile in 0.1 % formic acid at a flow rate of 300 nL min^{-1} over 45 min. The mass spectrometer was operated in positive ion mode. Full MS scans were acquired from *m/z* 300 to 1850 with a resolution of 70,000 at m/z 200. The ten most intense ions were fragmented by higher energy C-trap dissociation with normalised collision energy of 28 and MS/MS spectra were recorded with a resolution of 17,500 at m/z 200. The maximum ion injection time was 120 ms for both survey and MS/MS scans, whereas AGC target values of 3 x 10^6 and 5 x 10^5 were used for survey and MS/MS scans, respectively. In order to avoid repeat sequencing of peptides, dynamic exclusion was applied for 45 s. Singly charged ions or ions with unassigned charge state were also excluded from MS/MS. Data were acquired using Xcalibur software (Thermo Scientific).

Data processing and bioinformatics analysis

Acquired raw data files were processed with the MaxQuant (94) software (version 1.5.3.17) using the internal search engine Andromeda (95) and searched against the UniProt database restricted to *Drosophila melanogaster* entries (release 2015_11; 43712 entries). Spectra originated from the different slices corresponding to the same biological sample

were combined. Carbamidomethylation (C) was set as fixed modification whereas Met oxidation, protein N-terminal acetylation, and Lys GlyGly (not C-term) were defined as variable modifications. Mass tolerance was set to 8 p.p.m. and 20 p.p.m. at the MS and MS/MS level, respectively. Enzyme specificity was set to trypsin, allowing for cleavage N-terminal to Pro and between Asp and Pro with a maximum of 2 missed cleavages. Match between runs option was enabled with 1.5 min match time window and 20 min alignment window to match identification across samples. The minimum peptide length was set to 7 amino-acids. The false discovery rate for peptides and proteins was set to 1 %. Normalised spectral protein Label Free Quantification (LFQ) intensities were calculated using the MaxLFQ algorithm.

Data analysis and statistical tests

MaxQuant output data was analysed with the Perseus module (version 1.5.6.0) (45). Initially, proteins only identified by site, contaminants, reverse hits and proteins with no unique peptides and/or no intensity were removed. Missing LFQ intensity values were replaced with values from a normal distribution (width 0.3 and down shift 1.8), meant to simulate expression below the detection limit (45). To determine statistically significant changes in protein abundance, as well as in ubiquitin diGly peptides, two-tailed Student's t test was used.

In the analysis of the biotin pulldowns, two comparisons were carried out: ^{bio}A3 *vs* ^{bio}Ub (i.e. Ube3a gain of function *vs* control) and ^{bio}15B *vs* ^{bio}Ub (i.e. Ube3a loss of function *vs* control). Proteins displaying a LFQ fold change bigger than 2 with a p-value smaller than 0.05 were selected for further analysis. The selected proteins were further filtered based on the intensity pattern observed for their peptides. Statistical significance in Western blotting semi-quantification was evaluated using an analysis of variance (ANOVA) complemented by

Tukey's honest significance difference test (Tukey's HSD) performed in GraphPad PRISM software.

RNA extraction, reverse transcription, and Real-Time qPCR

Total RNA was isolated from 30 mg of brain from embryos, young and adult wild type mice from different ages. RNA was extracted and further purified by using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. Contaminating genomic DNA was removed by treatment with deoxyribonuclease I (QIAGEN), and cDNAs were synthesised from 1 µg RNA using the AffinityScript Multi Temperature cDNA Synthesis Kit (Agilent Technologies). Quantitative RT-PCR was performed on cDNA in the presence of Power SYBR Green PCR Master Mix (Applied Biosystems) containing preset concentrations of deoxynucleotide triphosphates and with specific primers, using the ABI Prism 7900 sequence Detection System (Applied Biosystems). PCR parameters were 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 sec, and 60 °C for 1 min. The purity of the PCR products was assessed by dissociation curves. The amount of target cDNA was calculated by the comparative threshold (Ct) method and expressed by the $2^{-\Delta\Delta Ct}$ method according to Applied Biosystems' instructions, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Expression of GAPDH mRNA was not affected by age, and the ratio of ΔCt value did not vary with the amount of cDNA. Each primer set was used at its optimal concentration (300 nM) with maximal efficacy. It was verified that one single specific product was amplified as shown by analysis of its melting temperature value.

Primers GAPDH: Forward-5'-ACCACAGTCCATGCCATCAC-3'

Reverse- 5'-TCCACCACCCTGTTGCTGTA-3'

Primers DDI1: Forward- 5'-TCACTGTGTATTGTGTGCGTAG-3'

Reverse- 5’-AGCTGTTCCATGTAAACGATCTG-3’

Primers DDI2: Forward- 5’-CCTCTCCGAGGTGACCTTTTC-3’

Reverse- 5’-GGCCTTTCTGCATAGACAATCT-3’

RNA *in situ* hybridization

CD-1 mice were mated overnight, and the presence of a vaginal plug indicated embryonic day (E) 0.5. Unsexed embryos at E9.5 and E10.5 (the age 9.5 and 10.5 days post coitum) were fixed in 4 % PFA and used for *in situ* hybridization, both in whole mount and in paraffin sections. Heads of 14.5 and 16.5 days post coitum old CD-1 embryos were fixed in 4 % PFA and further processed for paraffin sections. Embryo samples for whole mount ISH were frozen in methanol at -20 °C prior tissue hydration, proteinase K treatment, acetylation and the prehybridization and hybridization procedures. The embryos and heads used for sectioning were dehydrated, embedded in paraffin, cut to 7 µm sections and rehydrated prior further treatment. The experimental procedures were performed according to standard protocols (96).

Murine DDI1 coding sequence (NM_027942.1) was synthesised by GenScript and cloned into pGEM-T® easy plasmid. Linearized plasmid was purified with PCR Purification Kit (QIAGEN) and used for generation of digoxigenin labelled riboprobes with digoxigenin RNA labelling kit (Roche Applied Science) by *in vitro* transcription according to the provided manual. Probes were further cleaned by RNeasy Mini kit (QIAGEN) following RNA cleanup manual. Hybridization was performed overnight at 70 °C with all probes for both whole mount and sectioned embryo samples. The DIG labelled probes were detected with anti-DIG antibody conjugated with alkaline phosphatase and BM purple AP substrate precipitating solution (Roche Applied Science) was used for signal development. All samples were

postfixed in 4 % PFA and the slides were mounted in Aquatex. Images were taken using Zeiss ApoTome microscope.

Cell staining and microscopy

SH-SY5Y cells in Supplementary Fig 5C were transfected using Lipofectamine 3000 transfection reagent (Invitrogen) and grown over a coverslip glass. After 48 h, cells were washed twice with 2× PBS, fixed in 4 % PFA for 20 min at room temperature, washed with PBST (0.1 % Triton X-100 in 1× PBS) for three times and nuclei were stained with NucBlue Fixed Cell Stain Readyprobes reagent (Invitrogen). After staining, cells were washed with 1× PBS three times, the cover slips were mounted into slides with ProLong Diamond antifade reagent (Invitrogen), and samples were analyzed in an inverted microscope ECLIPSE TS2-FL (Nikon).

Funding

This work was supported by March of Dimes [Research Grant 1-FY15-339]; and Spanish MINECO [grants SAF2013-44782-P and SAF2016-76898-P to UM, BFU2014-52282-P and BFU2017-84653-P to RB]. UM is also part of Proteored, ISCIII and COST action Proteostasis. This work was also partially supported by Fondation Jérôme Lejeune grant 1381-MU2015A to UM. This work was supported by the Severo Ochoa Excellence Accreditation [grant SEV-2016-0644] and Consolider Programs [grant BFU2014-57703-REDC] to RB. Support was also provided from the Government of the Basque Country (Etortek Research Programs) and the Bizkaia County (Innovation Technology Department). This work was also supported by the Ministry of Education, Youth and Sports of the Czech Republic within the National Sustainability Program II [Project BIOCEV-FAR LQ1604] and by LM2015040 Czech Centre for Phenogenomics by MEYS, CZ.1.05/1.1.00/02.0109 Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University in Vestec (BIOCEV) by MEYS and ERDF, and CZ.1.05/2.1.00/19.0395 – Higher quality and capacity for transgenic models by MEYS and ERDF.

Acknowledgements

The authors are grateful of the technical support provided by UPV/EHU SGIker and Animal house staff, which are supported with European funding (ERDF and ESF). In particular we thank Arantza Alejo and the Animal Unit at UPV/EHU. We thank Janice Fisher, Fen-Biao Gao, Eric Liebl, Jose Antonio Rodríguez Pérez, Vjekoslav Tomaić and Andreas Wodarz for antibodies, flies and plasmids.

Conflict of Interest Statement

The authors declare no potential conflict of interest.

References

1. Buiting,K., Williams,C. and Horsthemke,B. (2016) Angelman syndrome - insights into a rare neurogenetic disorder. *Nat. Rev. Neurol.*, **12**, 584–593.

2. Kishino,T., Lalande,M. and Wagstaff,J. (1997) UBE3A/E6-AP mutations cause Angelman syndrome. *Nat. Genet.*, **15**, 70–73.

3. Matsuura,T., Sutcliffe,J.S., Fang,P., Galjaard,R.J., Jiang,Y.H., Benton,C.S., Rommens,J.M. and Beaudet,A.L. (1997) De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. *Nat. Genet.*, **15**, 74–77.

4. Huibregtse,J.M., Scheffner,M., Beaudenon,S. and Howley,P.M. (1995) A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc. Natl. Acad. Sci. U. S. A.*, **92**, 2563–2567.

5. Scheffner,M., Huibregtse,J.M., Vierstra,R.D. and Howley,P.M. (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell*, **75**, 495–505.

6. Scheffner,M., Nuber,U. and Huibregtse,J.M. (1995) Protein ubiquitination involving an E1–E2–E3 enzyme ubiquitin thioester cascade. *Nature*, **373**, 81–83.

7. Rougeulle,C., Glatt,H. and Lalande,M. (1997) The Angelman syndrome candidate gene, UBE3A/E6-AP, is imprinted in brain. *Nat. Genet.*, **17**, 14–15.

8. Gentile,J.K., Tan,W.H., Horowitz,L.T., Bacino,C.A., Skinner,S.A., Barbieri-Welge,R., Bauer-Carlin,A., Beaudet,A.L., Bichell,T.J., Lee,H.S., *et al.* (2010) A neurodevelopmental survey of Angelman syndrome with genotype-phenotype correlations. *J. Dev. Behav. Pediatr. JDBP*, **31**, 592–601.

9. Sadikovic,B., Fernandes,P., Zhang,V.W., Ward,P.A., Miloslavskaya,I., Rhead,W., Rosenbaum,R., Gin,R., Roa,B. and Fang,P. (2014) Mutation Update for UBE3A variants in Angelman syndrome. *Hum. Mutat.*, **35**, 1407–1417.

10. Nawaz,Z., Lonard,D.M., Smith,C.L., Lev-Lehman,E., Tsai,S.Y., Tsai,M.J. and O'Malley,B.W. (1999) The Angelman Syndrome-Associated Protein, E6-AP, Is a Coactivator for the Nuclear Hormone Receptor Superfamily. *Mol. Cell. Biol.*, **19**, 1182–1189.

11. Cooper,E.M., Hudson,A.W., Amos,J., Wagstaff,J. and Howley,P.M. (2004) Biochemical analysis of Angelman syndrome-associated mutations in the E3 ubiquitin ligase E6-associated protein. *J. Biol. Chem.*, **279**, 41208–41217.

12. Urraca,N., Cleary,J., Brewer,V., Pivnick,E.K., McVicar,K., Thibert,R.L., Schanen,N.C., Esmer,C., Lamport,D. and Reiter,L.T. (2013) The Interstitial Duplication 15q11.2-q13 Syndrome Includes Autism, Mild Facial Anomalies and a Characteristic EEG Signature. *Autism Res. Off. J. Int. Soc. Autism Res.*, **6**, 268–279.

13. Noor,A., Dupuis,L., Mittal,K., Lionel,A.C., Marshall,C.R., Scherer,S.W., Stockley,T., Vincent,J.B., Mendoza-Londono,R. and Stavropoulos,D.J. (2015) 15q11.2 Duplication Encompassing Only the UBE3A Gene Is Associated with Developmental Delay and Neuropsychiatric Phenotypes. *Hum. Mutat.*, **36**, 689–693.

14. Yi,J.J., Berrios,J., Newbern,J.M., Snider,W.D., Philpot,B.D., Hahn,K.M. and Zylka,M.J. (2015) An Autism-Linked Mutation Disables Phosphorylation Control of UBE3A. *Cell*, **162**, 795–807.

15. Kim,H.C. and Huibregtse,J.M. (2009) Polyubiquitination by HECT E3s and the determinants of chain type specificity. *Mol. Cell. Biol.*, **29**, 3307–3318.

16. Kühne,C. and Banks,L. (1998) E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box. *J. Biol. Chem.*, **273**, 34302–34309.

17. Thomas,M. and Banks,L. (1998) Inhibition of Bak-induced apoptosis by HPV-18 E6. *Oncogene*, **17**, 2943–2954.

18. Oda,H., Kumar,S. and Howley,P.M. (1999) Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination. *Proc. Natl. Acad. Sci. U. S. A.*, **96**, 9557–9562.

19. Mani,A., Oh,A.S., Bowden,E.T., Lahusen,T., Lorick,K.L., Weissman,A.M., Schlegel,R., Wellstein,A. and Riegel,A.T. (2006) E6AP mediates regulated proteasomal degradation of the nuclear receptor coactivator amplified in breast cancer 1 in immortalized cells. *Cancer Res.*, **66**, 8680–8686.

20. Reiter,L.T., Seagroves,T.N., Bowers,M. and Bier,E. (2006) Expression of the Rho-GEF Pbl/ECT2 is regulated by the UBE3A E3 ubiquitin ligase. *Hum. Mol. Genet.*, **15**, 2825–2835.

21. Kumar,S., Talis,A.L. and Howley,P.M. (1999) Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. *J. Biol. Chem.*, **274**, 18785–18792.

22. Mishra,A. and Jana,N.R. (2008) Regulation of turnover of tumor suppressor p53 and cell growth by E6-AP, a ubiquitin protein ligase mutated in Angelman mental retardation syndrome. *Cell. Mol. Life Sci. CMLS*, **65**, 656–666.

23. Margolis,S.S., Salogiannis,J., Lipton,D.M., Mandel-Brehm,C., Wills,Z.P., Mardinly,A.R., Hu,L., Greer,P.L., Bikoff,J.B., Ho,H.Y.H., *et al.* (2010) EphB-mediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. *Cell*, **143**, 442–455.

24. Shimoji,T., Murakami,K., Sugiyama,Y., Matsuda,M., Inubushi,S., Nasu,J., Shirakura,M., Suzuki,T., Wakita,T., Kishino,T., *et al.* (2009) Identification of annexin A1 as a novel substrate for E6AP-mediated ubiquitylation. *J. Cell. Biochem.*, **106**, 1123–1135.

25. Greer,P.L., Hanayama,R., Bloodgood,B.L., Mardinly,A.R., Lipton,D.M., Flavell,S.W., Kim,T.K., Griffith,E.C., Waldon,Z., Maehr,R., *et al.* (2010) The Angelman Syndrome

protein Ube3A regulates synapse development by ubiquitinating arc. *Cell*, **140**, 704–716.

26. Zaaroor-Regev,D., de Bie,P., Scheffner,M., Noy,T., Shemer,R., Heled,M., Stein,I., Pikarsky,E. and Ciechanover,A. (2010) Regulation of the polycomb protein Ring1B by self-ubiquitination or by E6-AP may have implications to the pathogenesis of Angelman syndrome. *Proc. Natl. Acad. Sci. U. S. A.*, **107**, 6788–6793.

27. Jensen,L., Farook,M.F. and Reiter,L.T. (2013) Proteomic Profiling in Drosophila Reveals Potential Dube3a Regulation of the Actin Cytoskeleton and Neuronal Homeostasis. *PLoS ONE*, **8**, e61952.

28. Jacobson,A.D., MacFadden,A., Wu,Z., Peng,J. and Liu,C.W. (2014) Autoregulation of the 26S proteasome by in situ ubiquitination. *Mol. Biol. Cell*, **25**, 1824–1835.

29. Yi,J.J., Paranjape,S.R., Walker,M.P., Choudhury,R., Wolter,J.M., Fragola,G., Emanuele,M.J., Major,M.B. and Zylka,M.J. (2017) The autism-linked UBE3A T485A mutant E3 ubiquitin ligase activates the Wnt/β-catenin pathway by inhibiting the proteasome. *J. Biol. Chem.*, **292**, 12503–12515.

30. Lee,S.Y., Ramirez,J., Franco,M., Lectez,B., Gonzalez,M., Barrio,R. and Mayor,U. (2014) Ube3a, the E3 ubiquitin ligase causing Angelman syndrome and linked to autism, regulates protein homeostasis through the proteasomal shuttle Rpn10. *Cell. Mol. Life Sci. CMLS*, **71**, 2747–2758.

31. Al-Shami,A., Jhaver,K.G., Vogel,P., Wilkins,C., Humphries,J., Davis,J.J., Xu,N., Potter,D.G., Gerhardt,B., Mullinax,R., *et al.* (2010) Regulators of the proteasome pathway, Uch37 and Rpn13, play distinct roles in mouse development. *PloS One*, **5**, e13654.

32. Puram,S.V., Kim,A.H., Park,H.Y., Anckar,J. and Bonni,A. (2013) The Ubiquitin Receptor S5a/Rpn10 Links Centrosomal Proteasomes with Dendrite Development in the Mammalian Brain. *Cell Rep.*, **4**, 19–30.

33. Tomaić,V. and Banks,L. (2015) Angelman syndrome-associated ubiquitin ligase UBE3A/E6AP mutants interfere with the proteolytic activity of the proteasome. *Cell Death Dis.*, **6**, e1625.

34. Xu,G., Paige,J.S. and Jaffrey,S.R. (2010) Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling. *Nat. Biotechnol.*, **28**, 868–873.

35. Kim,W., Bennett,E.J., Huttlin,E.L., Guo,A., Li,J., Possemato,A., Sowa,M.E., Rad,R., Rush,J., Comb,M.J., *et al.* (2011) Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell*, **44**, 325–340.

36. Wagner,S.A., Beli,P., Weinert,B.T., Nielsen,M.L., Cox,J., Mann,M. and Choudhary,C. (2011) A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. *Mol. Cell. Proteomics MCP*, **10**, M111.013284.

37. Na,C.H., Jones,D.R., Yang,Y., Wang,X., Xu,Y. and Peng,J. (2012) Synaptic protein ubiquitination in rat brain revealed by antibody-based ubiquitome analysis. *J. Proteome Res.*, **11**, 4722–4732.

38. Wagner,S.A., Beli,P., Weinert,B.T., Schölz,C., Kelstrup,C.D., Young,C., Nielsen,M.L., Olsen,J.V., Brakebusch,C. and Choudhary,C. (2012) Proteomic Analyses Reveal Divergent Ubiquitylation Site Patterns in Murine Tissues. *Mol. Cell. Proteomics MCP*, **11**, 1578–1585.

39. Sarraf,S.A., Raman,M., Guarani-Pereira,V., Sowa,M.E., Huttlin,E.L., Gygi,S.P. and Harper,J.W. (2013) Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. *Nature*, **496**, 372–376.

40. Nielsen,M.L., Vermeulen,M., Bonaldi,T., Cox,J., Moroder,L. and Mann,M. (2008) Iodoacetamide-induced artifact mimics ubiquitination in mass spectrometry. *Nat. Methods*, **5**, 459–460.

41. Ramirez,J., Elu,N., Martinez,A., Lectez,B. and Mayor,U. (2017) In Vivo Strategies to Isolate and Characterize the Neuronal Ubiquitinated Proteome. In *Current Proteomic Approaches Applied to Brain Function*, Neuromethods. Humana Press, New York, NY, pp. 179–189.

42. Franco,M., Seyfried,N.T., Brand,A.H., Peng,J. and Mayor,U. (2011) A novel strategy to isolate ubiquitin conjugates reveals wide role for ubiquitination during neural development. *Mol. Cell. Proteomics MCP*, **10**, M110.002188.

43. Martinez,A., Lectez,B., Ramirez,J., Popp,O., Sutherland,J.D., Urbé,S., Dittmar,G., Clague,M.J. and Mayor,U. (2017) Quantitative proteomic analysis of Parkin substrates in Drosophila neurons. *Mol. Neurodegener.*, **12**, 29.

44. Ramirez,J., Martinez,A., Lectez,B., Lee,S.Y., Franco,M., Barrio,R., Dittmar,G. and Mayor,U. (2015) Proteomic Analysis of the Ubiquitin Landscape in the Drosophila Embryonic Nervous System and the Adult Photoreceptor Cells. *PLoS ONE*, **10**, e0139083.

45. Tyanova,S., Temu,T., Sinitcyn,P., Carlson,A., Hein,M.Y., Geiger,T., Mann,M. and Cox,J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat. Methods*, **13**, 731–740.

46. Chandler,C.S. and Ballard,F.J. (1986) Multiple biotin-containing proteins in 3T3-L1 cells. *Biochem. J.*, **237**, 123–130.

47. Nezis,I.P., Simonsen,A., Sagona,A.P., Finley,K., Gaumer,S., Contamine,D., Rusten,T.E., Stenmark,H. and Brech,A. (2008) Ref(2)P, the Drosophila melanogaster homologue of mammalian p62, is required for the formation of protein aggregates in adult brain. *J. Cell Biol.*, **180**, 1065–1071.

48. Pavel,M., Imarisio,S., Menzies,F.M., Jimenez-Sanchez,M., Siddiqi,F.H., Wu,X., Renna,M., O'Kane,C.J., Crowther,D.C. and Rubinsztein,D.C. (2016) CCT complex restricts neuropathogenic protein aggregation via autophagy. *Nat. Commun.*, **7**, 13821.

49. Morawe,T., Honemann-Capito,M., von Stein,W. and Wodarz,A. (2011) Loss of the extraproteasomal ubiquitin receptor Rings lost impairs ring canal growth in Drosophila oogenesis. *J. Cell Biol.*, **193**, 71–80.

50. Min,M., Mayor,U. and Lindon,C. (2013) Ubiquitination site preferences in anaphase promoting complex/cyclosome (APC/C) substrates. *Open Biol.*, **3**, 130097.

51. Stevens,A. and Jacobs,J.R. (2002) Integrins regulate responsiveness to slit repellent signals. *J. Neurosci. Off. J. Soc. Neurosci.*, **22**, 4448–4455.

52. Chihara,T., Luginbuhl,D. and Luo,L. (2007) Cytoplasmic and mitochondrial protein translation in axonal and dendritic terminal arborization. *Nat. Neurosci.*, **10**, 828–837.

53. Lin,W.Y., Williams,C., Yan,C., Koledachkina,T., Luedke,K., Dalton,J., Bloomsburg,S., Morrison,N., Duncan,K.E., Kim,C.C., *et al.* (2015) The SLC36 transporter Pathetic is required for extreme dendrite growth in Drosophila sensory neurons. *Genes Dev.*, **29**, 1120–1135.

54. Mercer,S.W., La Fontaine,S., Warr,C.G. and Burke,R. (2016) Reduced glutathione biosynthesis in Drosophila melanogaster causes neuronal defects linked to copper deficiency. *J. Neurochem.*, **137**, 360–370.

55. Trotta,N., Rodesch,C.K., Fergestad,T. and Broadie,K. (2004) Cellular bases of activity-dependent paralysis in Drosophila stress-sensitive mutants. *J. Neurobiol.*, **60**, 328–347.

56. Montana,E.S. and Littleton,J.T. (2006) Expression profiling of a hypercontraction-induced myopathy in Drosophila suggests a compensatory cytoskeletal remodeling response. *J. Biol. Chem.*, **281**, 8100–8109.

57. Kasprowicz,J., Kuenen,S., Miskiewicz,K., Habets,R.L.P., Smitz,L. and Verstreken,P. (2008) Inactivation of clathrin heavy chain inhibits synaptic recycling but allows bulk membrane uptake. *J. Cell Biol.*, **182**, 1007–1016.

58. Yamauchi,T. (2005) Neuronal Ca2+/calmodulin-dependent protein kinase II--discovery, progress in a quarter of a century, and perspective: implication for learning and memory. *Biol. Pharm. Bull.*, **28**, 1342–1354.

59. Weeber,E.J., Jiang,Y.H., Elgersma,Y., Varga,A.W., Carrasquillo,Y., Brown,S.E., Christian,J.M., Mirnikjoo,B., Silva,A., Beaudet,A.L., *et al.* (2003) Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *J. Neurosci. Off. J. Soc. Neurosci.*, **23**, 2634–2644.

60. Martínez-Noël,G., Galligan,J.T., Sowa,M.E., Arndt,V., Overton,T.M., Harper,J.W. and Howley,P.M. (2012) Identification and Proteomic Analysis of Distinct UBE3A/E6AP Protein Complexes. *Mol. Cell. Biol.*, **32**, 3095–3106.

61. Dantuma,N.P., Groothuis,T.A.M., Salomons,F.A. and Neefjes,J. (2006) A dynamic ubiquitin equilibrium couples proteasomal activity to chromatin remodeling. *J. Cell Biol.*, **173**, 19–26.

62. Groothuis,T.A., Dantuma,N.P., Neefjes,J. and Salomons,F.A. (2006) Ubiquitin crosstalk connecting cellular processes. *Cell Div.*, **1**, 21.

63. Kaiser,S.E., Riley,B.E., Shaler,T.A., Trevino,R.S., Becker,C.H., Schulman,H. and Kopito,R.R. (2011) Protein standard absolute quantification (PSAQ) method for the measurement of cellular ubiquitin pools. *Nat. Methods*, **8**, 691–696.

64. Fonseca,R., Vabulas,R.M., Hartl,F.U., Bonhoeffer,T. and Nägerl,U.V. (2006) A balance of protein synthesis and proteasome-dependent degradation determines the maintenance of LTP. *Neuron*, **52**, 239–245.

65. Forrest,C.M., Darlington,L.G. and Stone,T.W. (2013) Involvement of the proteasome and caspase activation in hippocampal long-term depression induced by the serine protease subtilisin. *Neuroscience*, **231**, 233–246.

66. Dong,C., Bach,S.V., Haynes,K.A. and Hegde,A.N. (2014) Proteasome modulates positive and negative translational regulators in long-term synaptic plasticity. *J. Neurosci. Off. J. Soc. Neurosci.*, **34**, 3171–3182.

67. Zhao,Y., Hegde,A.N. and Martin,K.C. (2003) The ubiquitin proteasome system functions as an inhibitory constraint on synaptic strengthening. *Curr. Biol. CB*, **13**, 887–898.

68. Figueiredo,L.S., Dornelles,A.S., Petry,F.S., Falavigna,L., Dargél,V.A., Köbe,L.M., Aguzzoli,C., Roesler,R. and Schröder,N. (2015) Two waves of proteasome-dependent protein degradation in the hippocampus are required for recognition memory consolidation. *Neurobiol. Learn. Mem.*, **120**, 1–6.

69. van Ooijen,G., Dixon,L.E., Troein,C. and Millar,A.J. (2011) Proteasome function is required for biological timing throughout the twenty-four hour cycle. *Curr. Biol. CB*, **21**, 869–875.

70. Jiang,Y.H., Armstrong,D., Albrecht,U., Atkins,C.M., Noebels,J.L., Eichele,G., Sweatt,J.D. and Beaudet,A.L. (1998) Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron*, **21**, 799–811.

71. Miao,S., Chen,R., Ye,J., Tan,G.H., Li,S., Zhang,J., Jiang,Y. and Xiong,Z.-Q. (2013) The Angelman Syndrome Protein Ube3a Is Required for Polarized Dendrite Morphogenesis in Pyramidal Neurons. *J. Neurosci.*, **33**, 327–333.

72. Gossan,N.C., Zhang,F., Guo,B., Jin,D., Yoshitane,H., Yao,A., Glossop,N., Zhang,Y.Q., Fukada,Y. and Meng,Q.J. (2014) The E3 ubiquitin ligase UBE3A is an integral component of the molecular circadian clock through regulating the BMAL1 transcription factor. *Nucleic Acids Res.*, **42**, 5765–5775.

73. Pignatelli,M., Piccinin,S., Molinaro,G., Di Menna,L., Riozzi,B., Cannella,M., Motolese,M., Vetere,G., Catania,M.V., Battaglia,G., *et al.* (2014) Changes in mGlu5 receptor-dependent synaptic plasticity and coupling to homer proteins in the hippocampus of Ube3A hemizygous mice modeling angelman syndrome. *J. Neurosci. Off. J. Soc. Neurosci.*, **34**, 4558–4566.

74. Pastuzyn,E.D. and Shepherd,J.D. (2017) Activity-Dependent Arc Expression and Homeostatic Synaptic Plasticity Are Altered in Neurons from a Mouse Model of Angelman Syndrome. *Front. Mol. Neurosci.*, **10**, 234.

75. Marash,M. and Gerst,J.E. (2003) Phosphorylation of the Autoinhibitory Domain of the Sso t-SNAREs Promotes Binding of the Vsm1 SNARE Regulator in Yeast. *Mol. Biol. Cell*, **14**, 3114–3125.

76. Lustgarten,V. and Gerst,J.E. (1999) Yeast VSM1 encodes a v-SNARE binding protein that may act as a negative regulator of constitutive exocytosis. *Mol. Cell. Biol.*, **19**, 4480–4494.

77. White,R.E., Dickinson,J.R., Semple,C.A.M., Powell,D.J. and Berry,C. (2011) The retroviral proteinase active site and the N-terminus of Ddi1 are required for repression of protein secretion. *FEBS Lett.*, **585**, 139–142.

78. Guthmueller,K.L., Yoder,M.L. and Holgado,A.M. (2011) Determining genetic expression profiles in C. elegans using microarray and real-time PCR. *J. Vis. Exp. JoVE*, 10.3791/2777.

79. Valdez,C., Scroggs,R., Chassen,R. and Reiter,L.T. (2015) Variation in Dube3a expression affects neurotransmission at the Drosophila neuromuscular junction. *Biol. Open*, 10.1242/bio.20148045.

80. Lehrbach,N.J. and Ruvkun,G. (2016) Proteasome dysfunction triggers activation of SKN-1A/Nrf1 by the aspartic protease DDI-1. *eLife*, **5**.

81. Koizumi,S., Irie,T., Hirayama,S., Sakurai,Y., Yashiroda,H., Naguro,I., Ichijo,H., Hamazaki,J. and Murata,S. (2016) The aspartyl protease DDI2 activates Nrf1 to compensate for proteasome dysfunction. *eLife*, **5**.

82. Sivá,M., Svoboda,M., Veverka,V., Trempe,J.-F., Hofmann,K., Kožíšek,M., Hexnerová,R., Sedlák,F., Belza,J., Brynda,J., *et al.* (2016) Human DNA-Damage-Inducible 2 Protein Is Structurally and Functionally Distinct from Its Yeast Ortholog. *Sci. Rep.*, **6**, 30443.

83. Trempe,J.F., Šašková,K.G., Sivá,M., Ratcliffe,C.D.H., Veverka,V., Hoegl,A., Ménade,M., Feng,X., Shenker,S., Svoboda,M., *et al.* (2016) Structural studies of the yeast DNA damage-inducible protein Ddi1 reveal domain architecture of this eukaryotic protein family. *Sci. Rep.*, **6**, 33671.

84. Nowicka,U., Zhang,D., Walker,O., Krutauz,D., Castañeda,C.A., Chaturvedi,A., Chen,T.Y., Reis,N., Glickman,M.H. and Fushman,D. (2015) DNA-damage-inducible 1 protein

(Ddi1) contains an uncharacteristic ubiquitin-like domain that binds ubiquitin. *Struct. Lond. Engl. 1993*, **23**, 542–557.

85. Flick,K., Ouni,I., Wohlschlegel,J.A., Capati,C., McDonald,W.H., Yates,J.R. and Kaiser,P. (2004) Proteolysis-independent regulation of the transcription factor Met4 by a single Lys 48-linked ubiquitin chain. *Nat. Cell Biol.*, **6**, 634–641.

86. Flick,K., Raasi,S., Zhang,H., Yen,J.L. and Kaiser,P. (2006) A ubiquitin-interacting motif protects polyubiquitinated Met4 from degradation by the 26S proteasome. *Nat. Cell Biol.*, **8**, 509–515.

87. Alexander,J., Kalev,O., Mehrabian,S., Traykov,L., Raycheva,M., Kanakis,D., Drineas,P., Lutz,M.I., Ströbel,T., Penz,T., *et al.* (2016) Familial early-onset dementia with complex neuropathologic phenotype and genomic background. *Neurobiol. Aging*, **42**, 199–204.

88. Williams,K., Irwin,D.A., Jones,D.G. and Murphy,K.M. (2010) Dramatic Loss of Ube3A Expression during Aging of the Mammalian Cortex. *Front. Aging Neurosci.*, **2**, 18.

89. Wu,Y., Bolduc,F.V., Bell,K., Tully,T., Fang,Y., Sehgal,A. and Fischer,J.A. (2008) A Drosophila model for Angelman syndrome. *Proc. Natl. Acad. Sci. U. S. A.*, **105**, 12399–12404.

90. Tomaić,V., Pim,D. and Banks,L. (2009) The stability of the human papillomavirus E6 oncoprotein is E6AP dependent. *Virology*, **393**, 7–10.

91. Lu,Y., Wang,F., Li,Y., Ferris,J., Lee,J.A. and Gao,F.B. (2009) The Drosophila homologue of the Angelman syndrome ubiquitin ligase regulates the formation of terminal dendritic branches. *Hum. Mol. Genet.*, **18**, 454–462.

92. Ramirez,J., Min,M., Barrio,R., Lindon,C. and Mayor,U. (2016) Isolation of Ubiquitinated Proteins to High Purity from In Vivo Samples. *Methods Mol. Biol. Clifton NJ*, **1449**, 193–202.

93. Osinalde,N., Sánchez-Quiles,V., Akimov,V., Blagoev,B. and Kratchmarova,I. (2015) SILAC-based quantification of changes in protein tyrosine phosphorylation induced by Interleukin-2 (IL-2) and IL-15 in T-lymphocytes. *Data Brief*, **5**, 53–58.

94. Cox,J. and Mann,M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.*, **26**, 1367–1372.

95. Cox,J., Neuhauser,N., Michalski,A., Scheltema,R.A., Olsen,J.V. and Mann,M. (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.*, **10**, 1794–1805.

96. Wilkinson,D.G. and Nieto,M.A. (1993) Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.*, **225**, 361–373.

Figure Legends

Figure 1. *Drosophila* Ube3a mutant and over-expressing flies expressing the biotin-tagged ubiquitin.

A. Schematic representation of the (^{bio}Ub)$_6$-BirA construct, which is expressed as a poly-ubiquitin chain fused to BirA. This precursor polypeptide is digested by endogenous DUBs, so the ubiquitin moieties and the BirA enzyme are released. Each ubiquitin bears a 16 amino-acid long biotinylatable motif at their N-terminal part (bio) that is recognised by BirA. The sequence for biotinylation added to each ubiquitin is shown underlined, followed by the five amino-acid linker (italicised). The lysine where the biotin is attached is highlighted in red. **B.** Schematic representation of the domain structure of *Drosophila* Ube3a. Only two domains have been characterised for Ube3a so far: the AZUL domain (Amino-terminal Zn-finger of Ube3a E3 Ligase), which is thought to play a role in substrate recognition, and the HECT domain (Homologous to the E6-AP Carboxyl Terminus) that provides the E2-binding platform and a catalytic cysteine residue (C941) to which ubiquitin associates via a thioester linkage. Flies over-express Ube3a without any tag and under the control of a UAS sequence. **C.** Anti-Ube3a immunoblot on head extracts. The specific band for Ube3a is indicated with an arrow. Unspecific bands are indicated with arrowheads. **D.** Anti-BirA immunoblot on head extracts. Appropriate processing of the (^{bio}Ub)$_6$-BirA precursor was observed for all three genotypes (^{bio}Ub, ^{bio}A3, and ^{bio}15B). Flies over-expressing just BirA were used as control. **D.** Anti-Biotin Western blot performed for each of the genotypes. In the control sample (BirA) only endogenously biotinylated proteins are detected.

Figure 2. Isolation of candidate Ube3a substrates in *Drosophila* neurons.

A. Workflow for the identification of *Drosophila* Ube3a substrates. Flies over-expressing the (^{bio}Ub)$_6$-BirA precursor in the photoreceptor cells under the control of the GMR-GAL4 driver

(^{bio}Ub) were combined with heterozygous Ube3a mutant (^{bio}15B) or with Ube3a gain of function flies (^{bio}A3) in order to identify proteins whose ubiquitination increases in a Ube3a dose dependent manner. Fly heads of each of the genotype were subjected to biotin pulldown and mass spectrometry analysis. Proteins whose ubiquitination is regulated by Ube3a should be found in more abundance in ^{bio}A3 flies, as compared to ^{bio}Ub and ^{bio}15B controls. **B.** Silver staining of the eluted material from biotin pulldowns. Only endogenously biotinylated proteins are detected on the BirA control sample. **C.** LFQ intensities of Ube3a obtained from MS analysis of eluted samples. One asterisk indicates p-value < to 0.05; three, p < to 0.0001. **D.** Western blot to Ube3a indicates that it is mostly purified in its unmodified form (arrow), which is bound to the avidin beads due to ubiquitin bound to its active-site cysteine. This ubiquitin is removed from the active site by DTT-treatment of the samples on the elution step. A small fraction of Ube3a was also found conjugated to ubiquitin (asterisk). A non-specific band is observed in all input samples (arrowhead).

Figure 3. Identification of candidate Ube3a substrates in *Drosophila* neurons.

Comparison of the abundance, determined by their LFQ intensities, of the ubiquitinated proteins identified by mass spectrometry upon Ube3a over-expression relative to ^{bio}Ub flies. The Vulcano plot displays the LFQ ^{bio}A3/^{bio}Ub ratios in $\log_2$ scale (*x* axis) and the *T-test p-values* in $-\log_{10}$ scale (*y* axis), determining the statistical significance ($p<0.05$, horizontal grey lane) of the fold changes, for each protein. Labelled green dots represent high confidence proteins found more ubiquitinated in the ^{bio}A3 sample than in the ^{bio}Ub sample. Labelled red dots are those found less ubiquitinated in ^{bio}A3 sample. Endogenously ubiquitinated proteins (ACC, CG2118 and PCB) are shown with grey dots, and ubiquitin in blue. The earlier reported putative Ube3a candidate Atpα, is shown in brown. ^{bio}Ub: *GMR-GAL4,UAS-(^{bio}Ub)$_6$-BirA/CyO*; ^{bio}A3: *GMR-GAL4,UAS-(^{bio}Ub)$_6$-BirA/CyO;UAS-Ube3a^{A3}/TM6*.

Figure 4. Rngo is ubiquitinated by Ube3a in *Drosophila* photoreceptor neurons.

A. Immunoblot with anti Rngo-antibody confirmed the increase ubiquitination detected by MS in ^{bio}A3 flies as compared to ^{bio}Ub flies, and more significantly to ^{bio}15B flies. This same membrane was reprobed with anti-Fax. Levels of ubiquitinated Fax were found reduced in ^{bio}A3 flies, which corroborated the MS results and confirmed that the increase ubiquitination seen for Rngo is specific to the protein and not to a more efficient general isolation of proteins in the ^{bio}A3 sample. Putative mono-, tri- and tetra-ubiquitinated forms are indicated with asterisks and unmodified or cysteine-ubiquitinated forms are indicated with an arrow. Equal levels of Avidin bands, which are non-specifically detected by Rngo antibody, are also shown. **B.** Atpα is not a substrate of Ube3a in *Drosophila* photoreceptor cells. Western blot performed with anti-Atpα showed that its ubiquitination is not regulated by Ube3a, as levels of mono-ubiquitinated Atpα are similar, or even lower in ^{bio}A3 than in ^{bio}15B flies. The unmodified proteins are indicated with an arrow. Ubiquitinated forms are indicated with asterisks.

Figure 5. Ddi1 and Ddi2 gene expression temporal profile in the mouse brain.

Changes in expression of Ddi1 (**A**) and Ddi2 (**B**) mRNA levels in brains of C57BL/6J mice during aging. RNA was isolated from the brains of E13.5 to 9-month-old mice and subjected to qRT-PCR. A significant increase of Ddi1 mRNA level was observed at embryonic stage E16.5. Ddi1 and Ddi2 mRNA levels were determined and adjusted by the signal intensity of GAPDH, and the average results (n=3) were calculated and expressed with respect to the values obtained in E13.5.

Figure 6. Spatial profile of Ddi1 gene expression in the mouse brain.

Ddi1 is expressed in central nervous system during embryonic development according to RNA *in-situ* hybridization study on CD-1 mouse embryos. **A.** and **B.** Expression profile of Ddi1 in whole mount and paraffin sections of E9.5 embryo, respectively. **C.** and **D.** Ddi1 expression mapping in whole mount and paraffin section of E10.5 embryos. **E.** Sagittal section of E14.5 embryonic brain with clearly located expression of Ddi1 in colliculus midbrain tectum and pallial part of telencephalon. **F.** Detail of pallium: Ddi1 is expressed in isocortex and olfactory bulb. **G.** Midbrain and hindbrain tissue shows expression of Ddi1 in culliculus tectum. **H.** and **I.** Ddi1 is specifically expressed in isocortex and ventricular layer of olfactory bulb of E16.5 mouse brain. **J.** High resolution detail of the scan shown in **I**. Red stars highlight tubular structures – probably capillaries of the central nervous system. Abbreviations: T=telencephalon; D=diencephalon; M=mesencephalon; R=rhombencephalon; C=cerebellum; CMT=colliculus midbrain tectum; TL=thalamus; P=pallium; SP=subpallium; HT=hypothalamus; BG=basal ganglia; OB=olfactory bulb; IC=isocortex; NC=nasal cavity.

Figure 7. Human DDI1 is ubiquitinated by UBE3A in SH-SY5Y cells.

A. DDI1-GFP showed a significant increase in its ubiquitinated fraction in the presence of wild type UBE3A ($UBE3A^{WT}$), as illustrated by Western blot to FLAG-tagged ubiquitin, compared to control (pCDNA3.1) or to ligase dead UBE3A ($UBE3A^{LD}$). The non-modified form of DDI1 was detected with anti-GFP antibody (green). The bottom panel shows levels of human UBE3A protein in the whole cell extract before the isolation of the GFP-tagged proteins. **B.** Quantification of the ubiquitination of DDI1 was performed by calculating the FLAG:GFP ratio in panel A with Image-J. Statistical significance differences (***, $p < 0.001$ (mean ± SEM, n = 5)) were observed for the $UBE3A^{WT}$ sample relative to both control (pCDNA3.1) and $UBE3A^{LD}$ samples.

Fly protein		MS data			Human orthologue[b]		
Name[a]	MW (kDa)	Fold Change	p-value	Unique Peptides	Gene symbol[c]	Gene name[c]	CC[e]
Increased ubiquitination upon Ube3a over-expression							
Ube3a	**108**	**434,43**	**4,1E-05**	**24**	**UBE3A**	**Ubiquitin protein ligase E3A**	
CG12065	71	53,55	1,3E-03	11	-	-	-
Gclc	81	21,61	1,9E-05	10	GCLC	Glutamate-cysteine ligase catalytic subunit	
CG7896	134	15,12	4,9E-02	8	IGFALS	Insulin-like growth factor binding protein acid labile subunit	
Gapdh2	35	13,16	1,4E-03	3	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	
CG32640	15	5,15	9,9E-04	7	-	-	
Rngo	**51**	**5,00**	**1,5E-04**	**11**	**DDI1/2**	**DNA damage inducible 1 homolog 1/2**	
CCT7	59	4,97	1,3E-03	4	CCT7	Chaperonin containing TCP1 subunit 7	
Rpn3	**56**	**4,76**	**4,0E-04**	**11**	**PSMD3**	**Proteasome 26S subunit, non-ATPase 3**	
Copia\GIP	48	4,26	1,6E-03	16	-	-	-
Msr-110	69	4,20	2,6E-03	11	-	-	-
Prosα3	**29**	**4,18**	**4,8E-02**	**4**	**PSMA4**	**Proteasome subunit alpha 4**	V
Prosα1	**27**	**4,05**	**2,4E-04**	**4**	**PSMA6**	**Proteasome subunit alpha 6**	
Chc	191	3,88	1,4E-02	12	CLTC/-L1	Clathrin heavy chain/Clathrin heavy chain like 1	V
GlyRS	76	3,47	2,6E-03	4	GARS	Glycyl-tRNA synthetase	
Ref(2)P	65	3,43	3,0E-04	9	SQSTM1	Sequestosome 1	V
V		3,40	7,4E-04	6	EIF4A1/2	eukaryotic translation initiation factor 4A1/2	
CG9990	84	3,34	1,5E-03	5	ABCA1-13	ATP binding cassette subfamily A member 1-13	V
eEF1α1	50	3,29	3,2E-04	3	EEF1A1/2	eukaryotic translation elongation factor 1 alpha 1/2	
Gp150	106	3,17	1,6E-03	21	TLR3	Toll like receptor 3	ER/E
CG3036	54	3,11	3,8E-02	4	SLC17A5	Solute carrier family 17 member 5	
Ple	66	3,06	2,0E-03	9	TH	Tyrosine hydroxylase	
CCT3	59	3,02	5,6E-03	11	CCT3	Chaperonin containing TCP1 subunit 3	
AnxB11	33	2,90	1,7E-02	5	ANXA11	Annexin A11	
Emp	53	2,87	1,3E-02	9	SCARB2	Scavenger receptor class B member 2	
Jafrac1	20	2,86	8,9E-03	5	PRDX1/2	Peroxiredoxin 1/2	
SerRS	56	2,83	8,3E-03	6	SARS	Seryl-tRNA synthetase	
Atg8a	14	2,80	3,2E-02	4	GABARAP	GABA type A receptor-associated protein	V
Awd	17	2,77	2,8E-03	6	NME1	NME/NM23 nucleoside diphosphate kinase 1	
Rpn10	**43**	**2,75**	**3,0E-02**	**5**	**PSMD4**	**Proteasome 26S subunit, non-ATPase 4**	
Cad99C	185	2,69	2,7E-02	19	PCDH15	Protocadherin related 15	S
CCT8	59	2,67	1,4E-05	7	CCT8	Chaperonin containing TCP1 subunit 8	
Rpt4	**45**	**2,54**	**3,5E-04**	**9**	**PSMC6**	**Proteasome 26S subunit, ATPase 6**	
Rpt2	**49**	**2,51**	**3,4E-02**	**6**	**PSMC1**	**Proteasome 26S subunit, ATPase 1**	
Hsp26	23	2,34	1,4E-03	7	HSPB1/2	Heat shock protein family B (small) member 1/2	
Cad87A	218	2,27	1,6E-04	13	CDH23	Cadherin related 23	
Actn	104	2,27	1,3E-02	9	ACTN2	Actinin alpha 2	CT
Shrb	25	2,16	2,1E-02	3	CHMP4B	Charged multivesicular body protein 4B	
CG34417	190	2,14	5,4E-03	14	SMTN/-L1	Smoothelin/Smoothelin like 1	CT
Rpn8	**38**	**2,11**	**4,7E-02**	**3**	**PSMD7**	**Proteasome 26S subunit, non-ATPase 7**	
Reduced ubiquitination upon Ube3a over-expression							
Fax	47	1/2,19	6,6E-04	29	FAXC	Failed axon connections homologue	
Zw	60	1/2,24	1,1E-02	11	G6PD	Glucose-6-phosphate dehydrogenase	
Bark	349	1/2,35	6,8E-03	20	LOXL3	Lysyl oxidase like 3	
Wat	60	1/2,81	4,0E-02	15	FAR1/2	Fatty acyl-CoA reductase 1/2	P
Prom	138	1/3,41	1,4E-02	14	PROM1/2	Prominin 1/2	ER
Yps	37	1/3,42	1,6E-02	7	YBX1-3	Y-box binding protein 1/2/3	
Goe	100	1/3,71	4,1E-02	12	PHEX*	Phosphate regulating endopeptidase homolog X-linked	
CG10254	154	1/3,79	2,7E-02	8	UBE2O	Ubiquitin conjugating enzyme E2 O	
Cmb	184	1/3,96	5,5E-04	10	PCM1	Pericentriolar material 1	CT
CaMKII	58	1/4,27	9,9E-04	5	CAMK2D	Calcium/calmodulin dependent protein kinase II delta	
DAAM	162	1/4,64	5,6E-03	18	DAAM1	Dishevelled associated activator of morphogenesis 1	
Pdh	30	1/4,81	5,4E-05	15	HPGD	15-hydroxyprostaglandin dehydrogenase	
Arp3	47	1/5,90	2,5E-03	10	ACTR3	ARP3 actin related protein 3 homolog	CT
CG43078	274	1/6,87	3,1E-02	6	-	-	-

[a] Given according to Flybase nomenclature
[b] Orthologues with the best Flybase score are provided
[c] Given according to HUGO Gene Nomenclature Committee
[e] Given according to Human Protein Atlas (www.proteinatlas.org) and Uniprot (www.uniprot.org)
*Other M13 metallopeptidases are also considered orthologues (see Supplementary Table 1)

Table 1. High confidence proteins whose ubiquitination is dependent on Ube3a over-expression. Proteins whose abundance in the pulldowns is significantly altered by Ube3a, both at protein and at peptide level, are shown. The complete data set is available as Supplementary Table 1. Proteasomal proteins are highlighted in bold. Cellular localization (CC) of the human proteins are indicated by colours (blue: nuclear; green: cytoplasmic; pink square: plasma membrane). If a more specific localization within each compartment has been reported it is further indicated with text (CT: cytoskeleton; E: endosome; ER: endoplasmic reticulum; P: peroxisomes; S: secreted; V: vesicles)

Abbreviations

AS: Angelman syndrome

^{bio}15B: ^{bio}Ub flies carrying a loss of function *Ube3a* deletion in heterozygosis.

^{bio}A3: ^{bio}Ub flies over-expressing the Ube3a E3 ligase

^{bio}Ub: Flies expressing the $(^{bio}Ub)_6$-BirA precursor in the photoreceptor cells.

DDI1: DNA damage-inducible protein 1 homologue 1

DDI2: DNA damage-inducible protein 1 homologue 2

DTT: Dithiothreitol

DUB: Deubiquitinating enzyme

ECL: Enhanced chemiluminescence

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

HECT: Homologous to the E6-AP Carboxyl Terminus

HRP: Horseradish peroxidase enzyme

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LD: Ligase dead

LFQ: Label free quantification

MS: Mass spectrometry

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

UBA: Ubiquitin associated domain

UBD: Ubiquitin-binding domain

UBL: Ubiquitin-like domain

UIM: Ubiquitin interacting motif

WT: Wild type