# A Novel Strategy to Isolate Ubiquitin Conjugates Reveals Wide Role for Ubiquitination during Neural Development*⑤

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Ubiquitination has essential roles in neuronal development and function. Ubiquitin proteomics studies on yeast and HeLa cells have proven very informative, but there still is a gap regarding neuronal tissue-specific ubiquitination. In an organism context, direct evidence for the ubiquitination of neuronal proteins is even scarcer. Here, we report a novel proteomics strategy based on the *in vivo* biotinylation of ubiquitin to isolate ubiquitin conjugates from the neurons of *Drosophila melanogaster* embryos. We confidently identified 48 neuronal ubiquitin substrates, none of which was yet known to be ubiquitinated. Earlier proteomics and biochemical studies in non-neuronal cell types had identified orthologs to some of those but not to others. The identification here of novel ubiquitin substrates, those with no known ubiquitinated ortholog, suggests that proteomics studies must be performed on neuronal cells to identify ubiquitination pathways not shared by other cell types. Importantly, several of those newly found neuronal ubiquitin substrates are key players in synaptogenesis. Mass spectrometry results were validated by Western blotting to confirm that those proteins are indeed ubiquitinated in the *Drosophila* embryonic nervous system and to elucidate whether they are mono- or polyubiquitinated. In addition to the ubiquitin substrates, we also identified the ubiquitin carriers that are active during synaptogenesis. Identifying endogenously ubiquitinated proteins in specific cell types, at specific developmental stages, and within the context of a living organism will allow understanding how the tissue-specific function of those proteins is regulated by the ubiquitin system. *Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.002188, 1–15, 2011.*

Posttranslational modification of proteins by ubiquitin is involved in a wide range of cellular processes (1). Ubiquitination is linked to the turnover of an ever growing number of proteins; it regulates protein trafficking and is also widely used to transiently facilitate protein-protein interactions (2, 3). As the number of known ubiquitinated proteins keeps growing, the focus is turning toward identifying when, where, and how those proteins are ubiquitinated *in vivo* with the aim of understanding how protein function is being regulated within the context of a whole organism. The ubiquitin pathway is essential for brain development and function, and its failure is associated with a number of neurodegenerative diseases, including Parkinson and Alzheimer diseases (4–6). Ubiquitin conjugation is carried out by the sequential action of ubiquitin-activating (E1), -conjugating (E2), and -ligating (E3) enzymes and can be reversed by deubiquitinating enzyme (DUB)[1] proteases. The involvement of a number of those enzymes in synaptogenesis has been documented in several model systems (7–12). In *Drosophila*, for example, synaptogenesis is dependent on the E3 ligase *Highwire* and on the DUB *fat facets* (13). A few proteins involved in synaptogenesis have been shown to be ubiquitin substrates, including the postsynaptic proteins Shank, GKAP, and AKAP79/150 in cultured neurons (14) and the *Caenorhabditis elegans* synaptic protein DLK-1 kinase, which was shown to be ubiquitinated when overexpressed in HEK293T kidney cells (9). Most neuronal targets of the ubiquitin pathway, however, remain undiscovered. Yeast and HeLa cell-based proteomics approaches have failed to provide significant insights into the neuronal mechanisms regulated by ubiquitination. With the exception of a polyubiquitin affinity-based purification that successfully identified by Western blotting three ubiquitin substrates in cultured neurons (14), no proteomics approach has been described that can identify ubiquitinated neuronal proteins. Because neuronal function and activity are highly

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[1] The abbreviations used are: DUB, deubiquitinating enzyme; FDR, false discovery rate; UPS, ubiquitin-proteasome system; Hsp, heat shock protein; Ub, ubiquitin; bioUb, biotin-tagged ubiquitin; WB, Washing Buffer; DSHB, Developmental Studies Hybridoma Bank; SUMO, small ubiquitin-like modifier; NCAM, neural cell adhesion molecule; Nrt, neurotactin; fax, failed axon connections.

context-dependent, rather than working on neuronal culture, we have aimed to identify which proteins are ubiquitinated *in vivo* within the neurons of a living organism.

Herein, we describe a novel strategy for the efficient isolation of neuronal ubiquitin conjugates from flies. The approach is based on the *in vivo* biotinylation of ubiquitin by ectopically expressing the *Escherichia coli* BirA enzyme to attach a biotin molecule to a specific BirA recognition sequence (15, 16) added at the N terminus of each ubiquitin chain. With the purpose of isolating ubiquitin conjugates uniquely from the nervous system of *Drosophila melanogaster*, we used the GAL4/UAS system for tissue-targeted expression (17). To increase the biotinylation efficiency, we took advantage of the processing activity of endogenous DUBs to digest a linear polypeptide precursor containing six copies of the tagged ubiquitin and the BirA enzyme, which are then present in the same cellular microenvironment. Because of the strength and the specificity of the avidin-biotin interaction, we were able to isolate and enrich the neuronal ubiquitinated proteins from a multicellular organism up to levels not achieved previously by any other approach. This allowed us to identify by mass spectrometry those neuronal proteins that are ubiquitinated and to resolve by Western blotting whether they are mono- or polyubiquitinated. This was achieved in the absence of proteasome inhibitors; therefore, physiological ubiquitination levels are reported. We focused on identifying the proteins that are ubiquitinated within the neurons in the period from neurite outgrowth and axonal pathfinding to target recognition and synapse formation (18). For that purpose, we applied our strategy on postmitotic neurons during embryonic stages 13–17 (19), a 12-h period during which embryos undergo synaptogenesis. Our strategy could be used to isolate ubiquitin conjugates from other tissues from the fruit flies, from different developmental stages, and in different mutant backgrounds, and it is likely to be applicable to other model organisms.

EXPERIMENTAL PROCEDURES

*DNA Constructs*—BirA was amplified from *E. coli* cultures using the oligos *birAf* (AATCGTAGATCTGCTCTTCGGTATGAAGGATAACACC-GTGCCACTG) and *birAr* (ATCTAGCTCGAGCTATTATTTTTCTGCAC-TACGCAGGGATATTTCACCGCCC) and cloned into the pUAST vector between the BglII and XhoI sites to generate the control construct expressing only the BirA enzyme. The *Drosophila* ubiquitin sequence was amplified from a 0–4-h-old embryonic cDNA library using the oligos *DmUbf* (CGAGTGGCACGAGGGCAGCGGCTCGGGCATGCA-GATCTTCG) and *DmUbr* (ATTCAGGCTCTTCATACCACCACGGAGA-CGAAGAACCAAGTGAAGGG) and the biotin tag completed at the 5′-end by reamplifying this PCR product with the *bioTagf* oligo CTAATGGCTCTTCGGTATGGGTTTGAATGACATATTTGAAGCCCA-GAAGATCGAGTGGCACGAGGGC and the *DmUbr* oligo. Six copies of the tagged ubiquitin were then sequentially introduced into the BirA control vector at the SapI site 5′ of the BirA to generate the UAS-(bioUb)$_6$-birA vector.

*Fly Stocks*—Wild-type yw embryos were injected with a mixture of either of the pUAST plasmids described above (200 ng/$\mu$l) and the helper plasmid pΔ2–3 (100 ng/$\mu$l), which had been purified on a Qiagen PCR column and eluted with Injection Buffer (0.1 m$_M$ Na$_2$HPO$_4$, 0.1 m$_M$ NaH$_2$PO$_4$, 5 m$_M$ KCl, pH 7.8). Flies with the UAS-birA transgene and those with the UAS-(bioUb)$_6$-birA precursor were independently recombined with elav-GAL4 flies. UAS-FlagTRAF2 flies were obtained from the Xue laboratory (20). Elav-GAL4 flies on the second and third chromosomes (which have no GAL4 expression on the salivary glands) were obtained from the Bloomington Stock Center.

*Embryo Collections*—Fly cages were enclosed with Petri dishes containing an apple juice-rich agar layer, which was partially covered with yeast paste. Embryos were collected over a 12-h period and allowed to age a further 9 h at 25 °C, so the collected embryos were 9–21 h old. Embryos were washed with PBS containing 0.1% Triton X-100 and water, dechorionated in 50% bleach solution for 3 min, immediately washed, collected into tubes, and flash frozen in liquid nitrogen before being stored at −80 °C until required.

*Extract Preparation and Pulldowns*—About 1 g of whole dechorionated embryos (stages 13–17) expressing the Ub$^6$-BirA construct (or just BirA) under the control of the neuronal elav-GAL4 driver was homogenized under denaturing conditions by mixing it with 2.9 ml of Lysis Buffer. After clarification, the supernatant was applied to a Binding Buffer-equilibrated PD10 column (GE Healthcare), and the eluate (3.5 ml) was collected (total protein extract contained typically 32–35 mg) into 250 $\mu$l of 25× protease inhibitor mixture from Roche Applied Science and incubated with 250 $\mu$l of NeutrAvidin-agarose beads (ThermoScientific) suspension (125 $\mu$l beads) for 40 min at room temperature and a further 2 h and 20 min at 4 °C. We adjusted the amount of beads used for the pulldown to a minimum without compromising much on the amount of unbound biotinylated material, which typically was 5–10%. The beads were then washed into 10–15 ml of Washing Buffers WB1 (twice), WB2 (thrice), WB3 (once), WB4 (thrice), WB1 (once), WB5 (once), and 3× WB6 (thrice). Beads were then transferred to a 1.5-ml tube, mixed with 100 $\mu$l of Elution Buffer, and placed for 5 min on a hot plate at 95 °C. The eluted sample was separated from the beads using a Vivaclear Mini 0.8-$\mu$m PES microcentrifuge filter unit. The recovered volume for both control and experimental samples was ~130 $\mu$l. The isolated biotinylated proteins boiled off the beads gave a typical recovery yield of 20–40%. For the mass spectrometry sample, elution was performed with 40 $\mu$l, and the recovered volume was 65 $\mu$l. Buffer compositions were as follows. Lysis Buffer contained 8 $_M$ urea, 1% SDS, and 50 m$_M$ *N*-ethylmaleimide in PBS, including a protease inhibitor mixture (Roche Applied Science). Binding Buffer contained 3 $_M$ urea, 1 $_M$ NaCl, 0.25% SDS, and 50 m$_M$ *N*-ethylmaleimide in PBS. WB1 contained 8 $_M$ urea and 0.25% SDS in PBS. WB2 contained 6 $_M$ guanidine HCl in PBS. WB3 contained 6.4 $_M$ urea, 1 $_M$ NaCl, and 0.2% SDS in PBS. WB4 contained 4 $_M$ urea, 1 $_M$ NaCl, 10% isopropanol, 10% ethanol, and 0.2% SDS in PBS. WB5 contained 8 $_M$ urea and 1% SDS in PBS. WB6 contained 2% SDS in PBS. Elution Buffer contained 4× Laemmli buffer and 100 m$_M$ DTT.

*Western Blotting and Silver Staining*—We used HRP-linked anti-biotin antibody from Cell Signaling Technology at 1:100, rabbit polyclonal anti-Ub from Sigma at 1:250, FK1 anti-poly-Ub from Enzo-LifeSciences at 1:1000, rabbit polyclonal anti-Ube1 (to detect its *Drosophila* ortholog Uba1) from Abcam at 1:100, sheep polyclonal anti-UbcD4 (21) at 1:100, rabbit polyclonal anti-E2-14K (to detect its *Drosophila* ortholog Ubc-E2H) from Boston Biochem at 1:300, rabbit polyclonal anti-UbcH6 (to detect its ortholog UbcD2) from Boston Biochem at 1:300, mouse polyclonal anti-Eff (22) at 1:1000, rabbit polyclonal anti-UBE2G1 (to detect its ortholog CG40045) from Boston Biochem at 1:300, mouse monoclonal anti-neurotactin (Nrt) from the Developmental Studies Hybridoma Bank (DSHB) at 1:20, mouse monoclonal anti-Fas2 from DSHB at 1:25, rabbit polyclonal anti-Eps15 (23) at 1:250, rabbit polyclonal anti-fax from Eric Liebl at

1:1000, rabbit polyclonal anti-Rad23 (24) at 1:1000, mouse monoclonal anti-Pros54 (24) at 1:2, rabbit polyclonal anti-ArgK (25) at 1:100, mouse monoclonal anti-Hsp27 (26) at 1:20, mouse monoclonal anti-Syx1A from DSHB at 1:50, guinea pig polyclonal anti-Lqf (27) at 1:200, rabbit polyclonal anti-anaplastic lymphoma kinase (28) at 1:200, and mouse monoclonal anti-FLAG M2 (Sigma) at 1:1000. HRP-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories were used at dilutions between 1:2000 and 1:5000. Depending on the antibody, we loaded between 0.002 and 0.2% of the input samples and 3–10% of the elution samples and generally used 4–15% gradient gels (Bio-Rad). Transfers were performed using the iBlot system (Invitrogen) with PVDF membranes except for Rad23, for which we used the nitrocellulose iBlot kits. Silver staining was performed with a SilverQuest kit (Invitrogen) following the manufacturer's instructions.

*Mass Spectrometry*—Total control and biotin-tagged ubiquitin ($^{bio}$Ub) samples were separated on a short polyacrylamide-SDS gel, which was stained with Coomassie Blue. Each lane was excised into three gel bands ($<$50, 50–100, and $>$100 kDa) using the molecular mass standards as a guide. Gel bands were washed, dehydrated. and subjected to standard in-gel trypsin digestion (12.5 $\mu$g/ml trypsin). Extracted peptides were loaded onto a $C_{18}$ column (75-$\mu$m inner diameter, 10-cm length, $\sim$300 nl/min flow rate, 5-$\mu$m resin from Michrom Bioresources, Auburn, CA) and eluted during a 10–30% gradient (Buffer A: 0.4% acetic acid, 0.005% heptafluorobutyric acid, and 5% ACN; Buffer B: the same as Buffer A except 95% ACN). The eluted peptides were detected by Orbitrap (350–1500 $m/z$; 1,000,000 automatic gain control target; 1000-ms maximum ion time; resolution, 60,000 full-width half-maximum) followed by 10 data-dependent MS/MS scans in LTQ (2 $m/z$ isolation width, 35% collision energy, 5000 automatic gain control target, 200-ms maximum ion time, 30 s of dynamic exclusion) on a hybrid mass spectrometer (Thermo Finnigan, San Jose, CA). All data were converted from raw files to the .dta format using ExtractMS version 2.0 (ThermoElectron). The fly reference database (19,628 entries) was downloaded from the National Center for Biotechnology Information (July 2009) and amended by including the sequences of BSA and the $Ub_6$-BirA construct. Peptide files were searched against the concatenated target-decoy amended database using the SEQUEST Sorcerer algorithm (version 3.11, SAGE-N). Searching parameters included mass tolerance of precursor ions ($\pm$50 ppm) and product ion ($\pm$0.5 $m/z$), partially tryptic restriction, fixed modification of carboxyamidomethylated Cys (+57.0215 Da), dynamic mass shifts for oxidized Met (+15.9949 Da) and ubiquitinated Lys (114.0429), three maximal modification sites, and three maximal missed cleavages. Only b and y ions were considered during the database match. To evaluate false discovery rate (FDR), all original protein sequences were reversed to generate a decoy database that was concatenated to the original database (29). The FDR was estimated by the number of decoy matches (nd) and total number of assigned matches (nt). FDR = 2 × nd/nt, assuming mismatches in the original database were the same as in the decoy database. To remove false positive matches, assigned peptides were grouped by a combination of trypticity (full, partial, and non-tryptic) and precursor ion charge state (1+, 2+, 3+, and 4+). Each group was first filtered by mass accuracy (10 ppm for high resolution mass spectrometry (MS)) and by dynamically increasing XCorr (minimum, 1.8) and ΔCn (minimum, 0.05) values to reduce protein FDR to less than 1%. All MS/MS spectra for proteins identified by a single peptide and those modified by ubiquitin (diglycine tag) were manually inspected as described previously (30). The identified proteins/peptides are listed in the supplemental tables. If peptides were matched to multiple members of a protein family, the matched members were clustered into a single group, and only the top hit of the group is shown to reduce the redundancy.

## RESULTS

Ubiquitin proteomics studies tend to be based on polyhistidine tagging (30–35), allowing the purification to be performed under denaturing conditions to prevent protease activity, including that of the DUB proteases. Furthermore, proteins containing ubiquitin-interacting motifs should not co-purify under denaturing conditions, preventing a very large number of false positives. Yet the necessity of denaturation also presents a major drawback. Proteins with internal poly-His sequences will bind strongly to nickel affinity beads even when the sequence was not exposed in the native protein. Unfortunately and unlike for *E. coli*, the $His_6$ motif appears frequently in higher organisms. For example, in yeast, 15 proteins contain this sequence, whereas in mouse and *Drosophila* over a hundred proteins carry it. In more than half of these, the sequence extends to 9 consecutive histidines. An alternate method that works under denaturing conditions is biotin tagging. The biotin-avidin interaction is far stronger than the $His_6$-nickel interaction and allows for much more stringent washes, resulting in minimal nonspecific background. Furthermore, most organisms contain just three to four endogenous biotinylated proteins. Kaiser and co-workers (36, 37) developed a tagged ubiquitin construct containing both a $His_6$ tag and a biotin-accepting domain that is efficiently biotinylated by endogenous enzymes in cell culture. The resulting tag was larger than ubiquitin itself, so the recognition and handling by ligases, as well as DUBs, of this larger version of ubiquitin might have been seriously perturbed. We used a much shorter tag that can be biotinylated by the *E. coli* BirA enzyme *in vitro* (15) and *in vivo* (16) with very high efficiency.

*In Vivo Biotinylation of Ubiquitin*—We generated flies that express under the control of an UAS sequence $Ub_6$-BirA, a synthetic linear polyubiquitin construct with an added BirA molecule at the C terminus (Fig. 1*A*). Each ubiquitin chain in the construct had an N-terminal 16-amino acid sequence (Fig. 1*B*) that can be biotinylated by the *E. coli* BirA enzyme with very high efficiency (15, 16). We also included a 5-amino acid-long linker to provide accessibility to the tag by the BirA enzyme. This construct as well as a control construct with just the BirA enzyme was expressed in all embryonic neurons (Fig. 1*C*) by recombining it in flies carrying the embryonic panneuronal driver elav-GAL4.

In most eukaryotic organisms, ubiquitin is coded by four independent genes of which at least one codes for a polyubiquitin chain, whereas the others code for a precursor containing one ubiquitin sequence and one other protein. The individual molecules are then posttranslationally digested to release free ubiquitin (38). As far as we are aware, our design to combine a polyubiquitin sequence with an additional protein is novel. The sequential action of the endogenous DUBs to digest this precursor and of the exogenous BirA to biotinylate the free ubiquitin chains can be summarized in the following scheme.

**A**

**B** MGLNDIFEAQK*IEWHE*GSGSG***M**QIFVKTLTGKTITLEVEPSDTIENVK-
AKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG

**C** **D** α-BirA blot **E** α-biotin blot
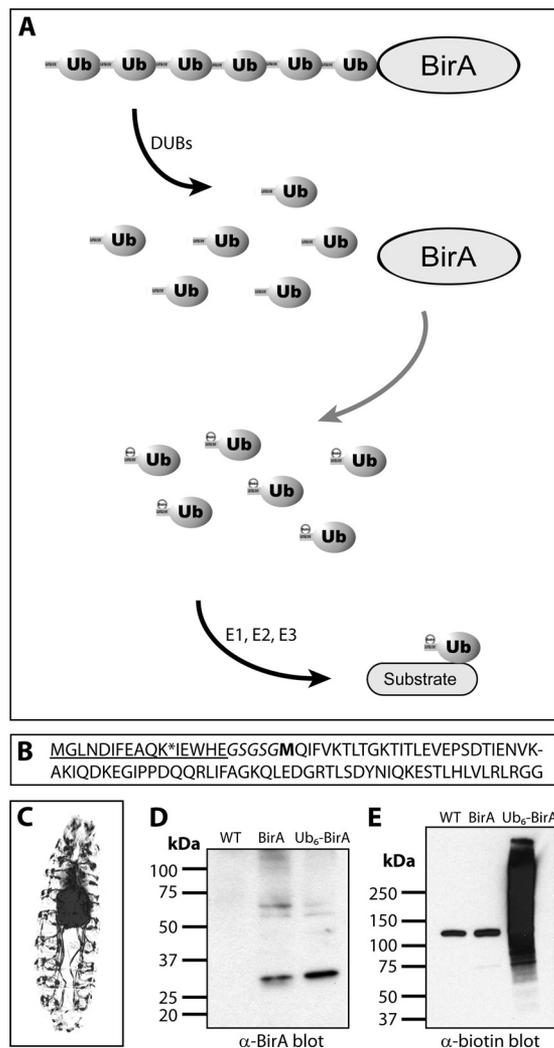
FIG. 1. **Novel strategy to isolate neuronal ubiquitin conjugates.** *A*, scheme illustrating the strategy for the *in vivo* biotinylation of ubiquitin. Digestion of the Ub$_6$-BirA precursor by endogenous DUBs is followed by BirA-mediated biotinylation of the ubiquitin chains tagged with the BirA recognition sequence. The $^{bio}$Ub can then be incorporated by endogenous ubiquitin-conjugating enzymes (E1, E2, and E3) into their neuronal substrates. *B*, sequence of $^{bio}$Ub. The BirA target sequence for biotinylation is *underlined* with the biotin-accepting lysine indicated by an *asterisk*. A linker (*italicized*) was introduced to improve the accessibility of the ubiquitin conjugates to the avidin beads. *C*, a stage 17 embryo, ready to hatch, is shown to illustrate the tissue (central and peripheral nervous systems) where the construct is expressed using the elav-GAL4 driver. *D*, comparison of wild-type (WT), BirA ((*elav-GAL4, UAS-birA*)/*TM6*) and Ub$_6$-BirA ((*elav-GAL4, UAS-(bioUb)$_6$-BirA*)/*CyO*) extracts with the anti-BirA antibody confirms full digestion of the precursor by the endogenous DUBs. The expected molecular mass of BirA is 35 kDa; the undigested Ub$_6$-BirA precursor would be expected to run at ~100 kDa. *E*, anti-biotin Western blot using the same extracts as in *D* confirms the lack of promiscuity of the BirA enzyme. One endogenously biotinylated protein is prominent both in WT and BirA extracts, but no additional bands are observed in the BirA lane. Biotinylated ubiquitin conjugates appear as a smear in the Ub$_6$-BirA lane, confirming that $^{bio}$Ub is incorporated efficiently into different substrates.

$$Ub_6\text{-BirA} \rightarrow BirA + 6\ Ub \rightarrow 6\ ^{bio}Ub$$

SCHEME 1

where $^{bio}$Ub is the *in vivo* biotinylated ubiquitin. Using specific antibodies to detect the bacterial protein BirA, we confirmed that the Ub$_6$-BirA precursor was indeed efficiently digested in the *Drosophila* nervous system by endogenous DUBs (Fig. 1*D*). Both the flies expressing the polyubiquitin precursor, which includes the BirA enzyme, and the control flies expressing only the BirA enzyme showed the same band, corresponding to the calculated molecular weight for BirA.

*In vivo* biotinylation of ubiquitin by the BirA enzyme was initially detected using doubly tagged ubiquitin, and interestingly, the biotinylation efficiency was highly tissue-specific, showing its best performance in the neuronal tissue.[2] Importantly, expression of BirA alone did not stimulate biotinylation of endogenous proteins in a nonspecific manner (Fig. 1*E*). To our knowledge, this is the first time the BirA enzyme has been used in flies for targeted *in vivo* biotinylation of proteins. Conjugation of $^{bio}$Ub to endogenous proteins was very efficient (Fig. 1*E*). Ubiquitin conjugation efficiency decreased with increasing tag sizes and even resulted in the accumulation of unconjugated polyubiquitin chains for GFP-tagged ubiquitin (data not shown), supporting our approach for using as small a tag as possible.

*Isolation and Identification of Neuronal Ubiquitin Conjugates*—To elucidate the role of ubiquitination in neuronal function, we expressed our tagged ubiquitin construct just within the nervous system using the embryonic panneuronal driver elav-GAL4. This allowed us to isolate and identify only neuronally ubiquitinated proteins using a simple one-step purification as described under "Experimental Procedures" and summarized in Fig. 2. Silver staining of a fraction of the elution samples from both the experimental and control samples revealed that the avidin-biotin-based protocol had indeed been successful in minimizing background proteins that could make the mass spectrometry analysis difficult. With the exception of one endogenously biotinylated protein (Fig. 2, *thin arrow*) and the avidin leaking from the agarose beads (Fig. 2, *arrowhead*), no further bands were visible in the silver staining of the control pulldown. In contrast, a very significant amount of high molecular weight proteins, putatively ubiquitinated, was present in the $^{bio}$Ub elution. Further characterization of the eluate with different ubiquitin antibodies confirmed that the isolated proteins were ubiquitin conjugates and also included polyubiquitinated proteins (Fig. 3, *B* and *C*).

For MS, the purified ubiquitin conjugates from control and $^{bio}$Ub samples were separated by one-dimensional SDS-PAGE, and each lane was sliced into three pieces (according to molecular weight), which were then subjected to standard trypsin digestion. Peptides were extracted and analyzed by

**Control embryos**

**Embryos with neuronal tagged ubiquitin**

BirA

Ub₆-BirA

Extract Preparation and Incubation with Avidin Beads

Stringent Washing Protocol (Urea, GdnHCl, NaCl, SDS, ...)

**Elution**

kDa

Silver Stain

250
150
100
50
25
15
10

**20**       **123**       **Proteins identified by MS**

**11 Ub carriers**
+
**Ubiquitin**
+
**SUMO**

**48 Ubiquitin conjugates (3 or more spectral counts)**

18 Proteins with known roles in synaptogenesis

12 Others

10 Heat Shock and UPS proteins

8 Uncharacterized genes

FIG. 2. **Flow chart of isolation protocol for identification of neuronal ubiquitin conjugates.** About 1 g of whole embryos (stages 13–17) expressing specifically in neuronal tissue the Ub₆-BirA construct (or the control BirA) were homogenized, clarified, and incubated with the NeutrAvidin beads. Following extensive and highly stringent washes, silver staining of the eluted material revealed in the Ub₆-BirA sample a typical smear of ubiquitin conjugates at high molecular weights that was not present in the control elution. The NeutrAvidin molecules leaking off the beads (*arrowhead*) as well as the most predominant endogenous biotin carrier (*arrow*) appear with similar intensities in both samples. MS/MS peptide analysis identified 20 proteins in the control sample and 123 in the experimental sample.

LC-MS/MS using an LTQ-Orbitrap mass spectrometer. The raw data from each LC-MS/MS run were extracted and searched against a composite target-decoy *Drosophila* database using the SEQUEST algorithm. Finally, identified proteins were filtered by matching scores to reduce the false discovery rate to less than 1%. A total of 123 proteins were identified from the [bio]Ub sample, and just 20 were identified from the control sample. All but three of the background proteins were also identified in the [bio]Ub sample (supplemental Table S1). Those three background proteins that did not appear in the experimental sample were identified by just two peptides in the control sample. For the remaining background proteins, a very similar number of unique peptides and spectral counts (a semiquantitative index that is the sum of MS/MS spectra matched to an assigned protein) were detected in both control and experimental samples (supplemental Table S1) in particular for the three endogenous biotin carriers.

Ubiquitin was identified by 15 peptides, giving 97% of the [bio]Ub sequence coverage and a total spectral count of 106, which included 28 counts corresponding to peptides modified by the diglycine signature typical of polyubiquitin chains (Table I). Although spectral count is semiquantitative and peptide ionization varies for each of the given sites, the chain linkage distribution we observed for polyubiquitin is given in Fig. 3*D*. Essentially, what is important is that we detected different chain linkages, and their relative proportions are in the same order as those found earlier in yeast (32). We also found the *Drosophila* SUMO protein, smt3, in the [bio]Ub sample with two peptides, 23% sequence coverage, and three spectral counts. We also found 11 known or predicted ubiquitin carriers in the experimental sample that are described later. From the remaining non-background proteins identified in the [bio]Ub sample, only those with three or more spectral counts were considered further (Table II). In agreement with the expected involvement of the ubiquitin system with early neuronal function, we identified by MS a very significant percentage of ubiquitin conjugates known to be involved in synaptogenesis (Table II and Fig. 2, *pie chart*). We could detect the diglycine ubiquitin signature for a few of the ubiquitin conjugates (Table I), confirming that we had isolated *bona fide* ubiquitin conjugates. We further validated by Western blotting the ubiquitination status of several of the 48 neuronal ubiquitin substrates as well as the presence in the pulldown of some of the 11 ubiquitin carriers identified by MS. Candidate ubiquitin conjugates identified by just one or two peptides were not con-

The background proteins were mostly conserved between the two samples. Those proteins in the [bio]Ub sample with three or more spectral counts, excluding SUMO, ubiquitin, and the 11 active ubiquitin carriers, are listed in Table II. The functional classification of those 48 neuronal ubiquitin substrates is shown in the form of a *pie chart*. *GdnHCl*, guanidine HCl.
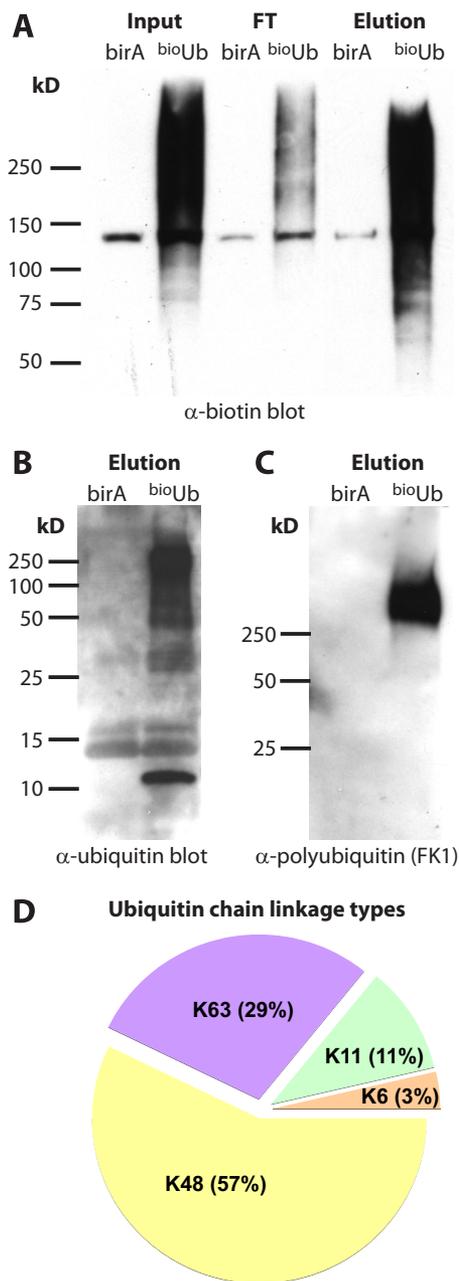
FIG. 3. **Enrichment of ubiquitin conjugates from neuronal tissue of *Drosophila* embryos.** *A*, anti-Biotin Western blotting was used to monitor the efficiency of the purification protocol. The amount of unbound biotinylated material, typically 5–10%, was estimated by comparison of the flow-through (*FT*) sample with the input sample at the same dilution. The purification yield, typically 20–40%, was estimated by comparing the intensity of the ubiquitinated material recovered in the elution relative with the input sample normalized to loaded amounts. An endogenously biotinylated protein is clearly seen in both the control and experimental samples. An anti-ubiquitin Western blot illustrates the enrichment for ubiquitin conjugates (*B*), some of which are recognized by the polyubiquitin chain-specific FK1 antibody (*C*). *D*, quantification of the branched ubiquitin peptides detected by MS according to spectral counts.

sidered further but might be confidently confirmed to be ubiquitinated once we scale up our strategy.

*Ubiquitination Substrate Conservation Across Species*—We identified several proteins for which an ortholog had been found earlier to be ubiquitinated by either proteomics or biochemical approaches. For example, we identified Eps15; Hsc70-4; ArgK; α- and β-tubulin; Src42A; the α subunit of the Na$^+$,K$^+$-ATPase pump, ATPα; both *Drosophila* 14-3-3 proteins; and the gene product of CG13349 (Table II). Most of those proteins are known to be abundant in the *Drosophila* nervous system, their involvement in synaptogenesis has already been well documented, and their orthologs have been detected to be ubiquitinated mostly in non-neuronal cell lines (Table II). Eighteen more proteins identified in this study have orthologs for which ubiquitination has been reported previously; some of them are mentioned in later sections. The conservation of specific ubiquitination pathways involving orthologs across different species underlines the value of using *Drosophila* as a model system to identify neuronal ubiquitin substrates that could then be validated in higher organisms.

One significant event of evolutionary conservation of a ubiquitin pathway was observed for Eps15, a protein involved in the recruitment of different components that are critical for intracellular trafficking (for a review, see Ref. 39) and which has been shown to have an essential role at the *Drosophila* synapse (23, 40) both for synaptic development and vesicle recycling. Two Eps15 isoforms are described in mammalian cell culture (41), but only the longest one was shown to be monoubiquitinated (42). Similarly, two Eps15 isoforms exist in *Drosophila* (23), and Western analysis of our pulldown also identified only the longer isoform to be monoubiquitinated (Fig. 4).

*Novel Neuronal Ubiquitin Conjugates with Known Roles in Synaptogenesis*—Enzymes of the ubiquitin system have been reported previously to have a role in synaptogenesis, but their targets have rarely been identified. Here, we report several novel ubiquitination substrates with well documented roles in *Drosophila* embryonic synaptogenesis. Synaptic architecture is established and stabilized through selective adhesion between presynaptic and postsynaptic partners. Homophilic cell surface glycoproteins, such as the fasciclins Fas2 and Fas3, are neural cell adhesion molecule (NCAM)-like proteins expressed on different subsets of neurons (43, 44). For example, Fas2 localizes to membranes surrounding synaptic vesicles and active zones in *Drosophila* neuromuscular junctions (45), and it is required for synaptic stabilization and growth (46). We identified by MS both Fas2 and Fas3 as being ubiquitinated in the developing embryonic brain. Nrt is also a transmembrane protein expressed at points of interneuronal cell contact and is highly expressed in the developing CNS, displaying a strong accumulation in growing axon tracts (47). In our MS analysis, Nrt was one of the most abundant proteins in the pulldown. Western blotting validation of both Nrt and Fas2 revealed that those NCAMs are monoubiquitinated in the

TABLE I

*Ubiquitin-modified peptides identified in* $^{bio}$*Ub pulldown*

Five different proteins were identified, including ubiquitin itself. The position of the diglycine signature on the ubiquitin peptides is given according to the wild-type endogenous ubiquitin sequence. Total spectral counts (TSC) are reported for each peptide. All peptides and their MS/MS spectra are given in supplemental Table S3.

| Protein | Peptide sequence[a] | Position of diglycine | TSC |
|---|---|---|---|
| Ub | G ↓ M*QIFVK(Gly-Gly)TLTGK ↓ T | Lys-6 | 1 |
| Ub/$^{bio}$Ub | K ↓ TLTGK(Gly-Gly)TITLEVEPSDTIENVK ↓ A | Lys-11 | 3 |
| Ub/$^{bio}$Ub | R ↓ LIFAGK(Gly-Gly)QLEDGR ↓ T | Lys-48 | 16 |
| Ub/$^{bio}$Ub | R ↓ TLSDYNIQK(Gly-Gly)ESTLHLVLR ↓ L | Lys-63 | 8 |
| Df31 | K ↓ K(Gly-Gly)DAVAAEEVAAEK ↓ A | 129 | 1 |
| Df31 | K ↓ LDEASTK(Gly-Gly)DEVQNGAEASEVAA ↓ - | 135 | 1 |
| Cyp1 | R ↓ SDVVPK(Gly-Gly)TAENFR ↓ A | 94 | 1 |
| 14-3-3ε | R ↓ LAK(Gly-Gly)AAFDDAIAELDTLSEESYK ↓ D | 196 | 1 |
| RpS10b | R ↓ APGGSGVDK(Gly-Gly)K ↓ G | 137 | 1 |

[a] Oxidized Met is indicated by an asterisk.

*Drosophila* neurons (Fig. 4). Another novel ubiquitin substrate found in this study was fax (failed axon connections), a protein suggested to have a role in cell-cell interactions that interacts genetically and biochemically with the Abelson tyrosine kinase (48). Fax was one of the most abundant proteins in the pulldown based on the number of peptides identified as well as on the spectral counts. Western blotting detected predominantly a monoubiquitinated band for fax (Fig. 4).

Flotillins are lipid raft-associated proteins thought to be crucial for coordinated assembly of signaling complexes, including Src family kinases (49). Within the nervous system, flotillins regulate cytoskeletal remodeling during neurite outgrowth (50), and if their function is blocked, downstream signaling molecules regulating the actin cytoskeleton are affected (51). *Drosophila* flotillin has a localized CNS expression in stages 11–16 (52). Both flotillin and flotillin-2 were identified by MS in our pulldown (Table II), but none of their mammalian counterparts have yet been reported to be ubiquitinated. Akap200, a member of the protein A kinase-anchoring protein family also identified in this work, was found in an axon guidance screen to disrupt the Fas2-containing axon bundles in the developing embryo ventral nerve cord (53). Akap200 is also required in the wiring of the antennal lobe and for axon targeting of the olfactory receptor neurons (54).

*Identification of Ubiquitin Carriers Active during Synaptogenesis*—The ubiquitin-activating E1 enzyme Uba1 as well as 10 other ubiquitin-conjugating enzymes were identified in the $^{bio}$Ub sample (see Table III). The observation of a number of ubiquitin-activating or -conjugating enzymes in our pulldown was a clear proof of them being active, that is carrying ubiquitin on their way to a substrate. The ubiquitin-proteasome system (UPS) relies on three types of enzymes, the E1, E2s, and E3s, which sequentially activate, conjugate, and ligate ubiquitin to the target protein. A cysteine at the active site of E1 and E2 enzymes is used as an attachment site for a thioester linkage to the C terminus of ubiquitin. One type of E3 ligases, those whose activity is based on a HECT domain, also use the same type of linkage, but the majority of E3

ligases interact in a non-covalent manner with ubiquitin. *Drosophila* has one E1, around 30 E2s, and over 300 E3s.

The E1 enzyme Uba1 has been shown previously to have a role in axon guidance and synaptogenesis (55) as well as in axonal pruning in mushroom body neurons during metamorphosis (7). Uba1 is reported to have two isoforms in flies (56) and in mammalian cell culture (57), the longest of which has a nuclear localization signal that is absent in the shorter isoform (58). We detected both isoforms by Western blotting (Fig. 5), suggesting that the $^{bio}$Ub construct can be conjugated to both nuclear and cytoplasmic proteins.

Among the nine E2s that were identified from the pulldown, ben, crl, and UbcD2 are known to have a restricted nerve system expression at these embryonic stages (52, 59, 60) and have also been reported to have a role on embryonic synaptogenesis. Ben is required for synaptic growth and maturation (12), Crl is required for normal development and proper activity of the CNS (60), and UbcD2 mutants showed a moderate misguidance phenotype in mushroom body neuronal clones (7). We also identified other E2s for which no role has so far been reported during embryonic neuronal development: vih, UbcD4, Ubc-E2H, and Eff. Two putative E2s, CG40045 and CG7656, whose expression patterns are unknown, were also identified. A HECT-type E3 ligase that causes in humans the neurological disorder Angelman syndrome (61) was also identified in our pulldown.

To validate the MS results, we performed Western blots of those enzymes for which antibodies were available. We could confirm that many of those proteins were indeed purifying as ubiquitin carriers. Because the thioester bond to ubiquitin was disrupted due to boiling in the Laemmli buffer, which includes the reducing agent DTT, protein bands were of identical size in the elution and the input samples for Uba1 (which has two isoforms), UbcD4, and Ubc-E2H (Fig. 5). UbcD2, Eff, and CG40045 were also detected in the $^{bio}$Ub pulldown with a corresponding band running in parallel to the input material but additionally displayed a higher band (Fig. 5). We interpret those additional bands (Fig. 5, indicated by *red arrows*) to

TABLE II

*Proteins identified in ^bioUb pulldown as ubiquitin conjugates*

Total peptides (TP) and total spectral counts (TSC) are reported for each protein. All peptides and their MS/MS spectra are given in supplemental Table S3. For those for which an ortholog has been previously found to be ubiquitinated, its name, reference (in parentheses), and cell line in which it was described are given. For those with known roles in synaptogenesis, the references where they were described are given in the last column. Seven novel ubiquitin substrates with previously reported roles in synaptogenesis are highlighted in bold. —, none.

| NCBI reference | Gene symbol[a] | Mass | TP | TSC | Ubiquitinated orthologs | Cell line | Known roles in synaptogenesis (Refs.) |
|---|---|---|---|---|---|---|---|
| | | kDa | | | | | |
| NP_788680.1 | *Hsc70-4* | 71 | 20 | 37 | HSPA8 (84) | SH-SY5Y | 85, 86 |
| NP_730196.1 | ***Nrt*** | 93 | 19 | 35 | — | — | 47 |
| NP_001014587.1 | ***Fax*** | 47 | 18 | 34 | — | — | 48 |
| NP_001027388.1 | *Df31* | 19 | 12[b] | 19[b] | — | — | |
| NP_996312.1 | *RpS7* | 22 | 10 | 13 | RPS7P10 (37) | HeLa | |
| NP_787998.1 | ***Akap200*** | 88 | 8 | 13 | — | — | 53, 54 |
| NP_651918.2 | *Rad23* | 46 | 7 | 9 | RAD23B (37) | HeLa | |
| NP_647773.1 | *CG12082* | 92 | 7 | 9 | — | — | |
| NP_523899.1 | *Hsp83* | 82 | 7 | 7 | HSP90A (64) | DA3 | |
| NP_610284.1 | *p47* | 43 | 6 | 7 | NSFL1C (37) | HeLa | |
| NP_477369.1 | *TER94* | 89 | 6 | 7 | CDC48 (31) | Yeast | |
| NP_523997.1 | *Hsp26* | 23 | 5 | 9 | — | — | |
| NP_731035.2 | *Rm62* | 63 | 5 | 9 | DDX5 (87) | COS-7 | |
| NP_524000.1 | *Hsp27* | 24 | 5 | 7 | — | — | |
| NP_648167.1 | *CG8209* | 37 | 5 | 7 | UBXN1 (37) | HeLa | |
| NP_726482.1 | *Eps15* | 132 | 5 | 7 | EPS15L1 (42) | NIH3T3 | 23, 40 |
| NP_725346.1 | *CG13349* | 42 | 5 | 6 | ADRM1 (37) | HeLa | 55 |
| NP_725476.1 | ***Flo*** | 47 | 5 | 6 | — | — | 50, 51 |
| NP_725896.1 | *βTub* | 51 | 5 | 5 | Tubulin (88) | HEK293 | 11, 89 |
| NP_649828.1 | *CG8223* | 52 | 5 | 5 | — | — | |
| NP_733453.1 | *CG1910* | 46 | 4 | 7 | — | — | |
| NP_573129.1 | *CG4420* | 51 | 4 | 6 | DDI1 (31) | Yeast | |
| NP_001014719.1 | *CG2947* | 31 | 4 | 4 | ST13 (37) | HeLa | |
| NP_729448.1 | *ArgK* | 44 | 4 | 4 | CKB (37) | HeLa | 90 |
| NP_728273.1 | *RpS10b* | 18 | 3[b] | 7[b] | RPS10 (37) | HeLa | |
| NP_732312.1 | *14-3-3ε* | 29 | 3[b] | 5[b] | YWHAE (37) | HeLa | 91 |
| NP_726912.1 | ***Fas2*** | 90 | 3 | 5 | — | — | 44, 70 |
| NP_524204.1 | *Pros54* | 43 | 3 | 4 | PSMD4 (37) | HeLa | |
| NP_524009.2 | *αTub67C* | 51 | 3 | 4 | Tubulin (88) | HEK293 | 11, 89 |
| NP_729952.1 | *Hsc70Cb* | 88 | 3 | 4 | HSPA4 (37) | HeLa | |
| NP_996250.1 | *Atpα* | 111 | 3 | 4 | ATP1A3 (92) | COS-7 | 93, 94 |
| NP_523366.2 | *Cyp1* | 25 | 3[b] | 3[b] | CPR1 (31) | Yeast | |
| NP_723647.1 | *dUTPase* | 18 | 3 | 3 | — | — | |
| NP_524003.1 | *Uch-L3* | 38 | 3 | 3 | — | — | |
| NP_727812.1 | ***Flo-2*** | 45 | 3 | 3 | — | — | 50, 51 |
| NP_723229.1 | *Hrb27C* | 45 | 3 | 3 | — | — | |
| NP_723139.1 | *eIF-4a* | 46 | 3 | 3 | EIF4A2 (37) | HeLa | |
| NP_524464.1 | *Tbp-1* | 48 | 3 | 3 | PSMC3 (37) | HeLa | |
| NP_731169.1 | *αTub84D* | 50 | 3 | 3 | Tubulin (88) | HEK293 | 11, 89 |
| NP_722724.1 | *Eno* | 54 | 3 | 3 | ENO1 (37) | HeLa | |
| NP_724107.1 | ***Fas3*** | 56 | 3 | 3 | — | — | 43 |
| NP_788888.1 | *CG7033* | 58 | 3 | 3 | CCT2 (34) | Yeast | |
| NP_724441.1 | *Src42A* | 59 | 3 | 3 | Src (95) | HEK293 | 96, 97 |
| NP_995882.1 | *pAbp* | 70 | 3 | 3 | PBAPC1 (37) | HeLa | |
| NP_725853.1 | *CG15118* | 72 | 3 | 3 | — | — | |
| NP_001014516.1 | *14-3-3ζ* | 28 | 2 | 5 | YWHAZ (37) | HeLa | 91 |
| NP_725890.1 | *hts* | 55 | 2 | 3 | — | — | |
| NP_731830.2 | *rin* | 75 | 2 | 3 | — | — | |

[a] Gene symbols given following Flybase (www.flybase.org) nomenclature.
[b] Including the peptides with the diglycine signature.
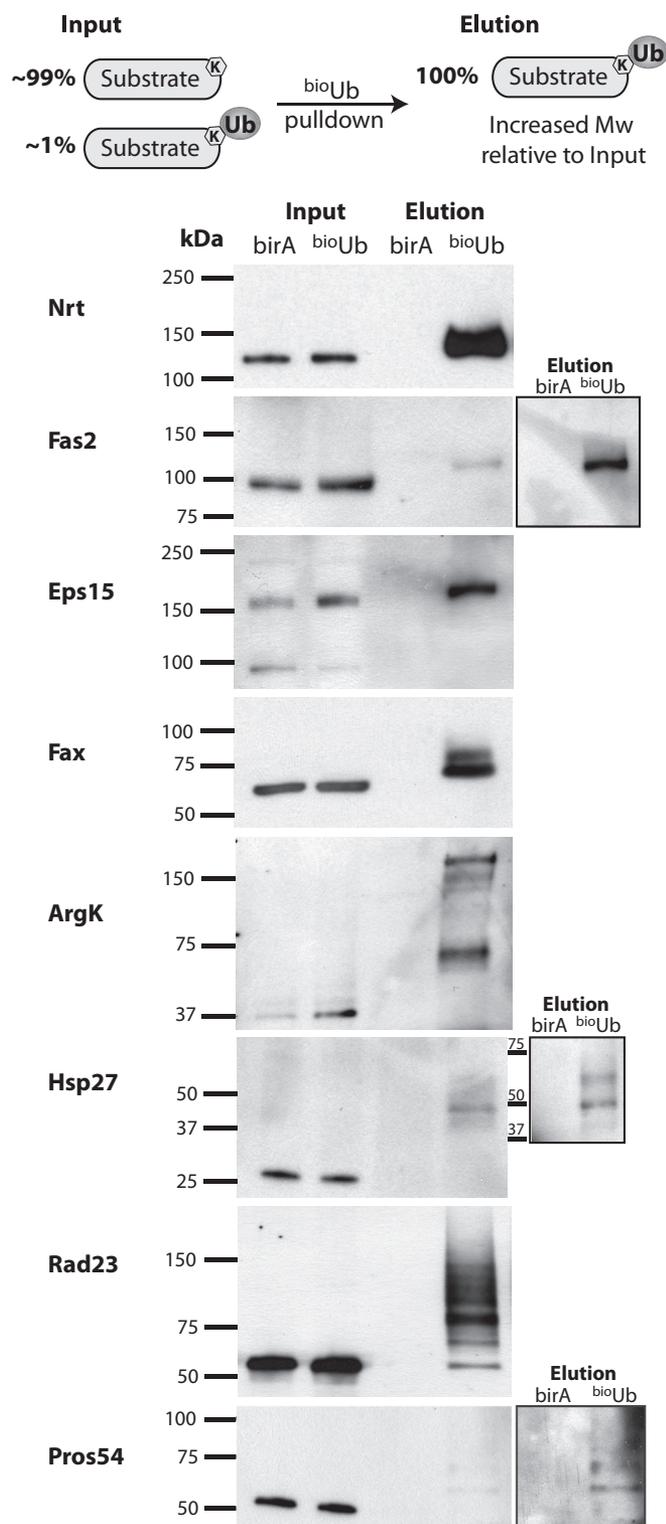
FIG. 4. **Western blot validation of identified ubiquitin conjugates.** Ubiquitination appears to target just a small percentage of the population of any of its substrates, explaining why additional ubiquitinated bands are usually not observed in whole cell extracts. With our biotin-avidin-based pulldown, we achieved a clear enrichment for ubiquitinated material, resulting in a distinct molecular mass shift being observed in the elution lanes relative to the input lanes. The

correspond to a subpopulation of the ubiquitin carriers that are ubiquitinated covalently as reported for other E2s (62). Previous ubiquitin proteomics studies seem to have missed which E2 enzymes were ubiquitin carriers at the time of the extract preparation; at least in some cases, this could be explained because they included reducing agents in the extract buffers (30, 31, 35) that would immediately reduce the thioester linkage between ubiquitin and the E2s, preventing their isolation and identification.

*UPS Components and Heat Shock Proteins Are Also UPS Substrates in Developing Brain*—In addition to the ubiquitin-conjugating enzymes, a number of other UPS-related proteins, mostly poly-Ub receptors and shuttles, were identified as being ubiquitinated in the neuronal tissue of *Drosophila* (Table II). For example, we found Rad23, a protein whose role is to shuttle polyubiquitinated proteins to the proteasome, a function that relies on its multiple ubiquitin binding domains. One possibility is that all the polyubiquitin receptors identified in our pulldown could have just co-purified with the sample enriched on ubiquitin conjugates despite the very stringent washes used during the purification protocol. However, we did confirm by Western blotting that the dominant species of the Rad23 isolated in the pulldown appeared to be ubiquitin-modified (Fig. 4), and the same was observed for Pros54, the *Drosophila* homolog of S5a/Rpn10 (Fig. 4). This is not the first time that homologs for most of these poly-Ub-binding proteins were purified together with ubiquitinated proteins using a protocol with denaturing conditions (30–37), and even MS-based validation of those proteins as being indeed ubiquitinated has been reported by their increase in molecular weight (30) as well as by detection of the diglycine signature in peptides corresponding to their sequences (31, 35, 37). Furthermore, direct observation of polyubiquitination using West-

addition of a single ubiquitin chain should result in an increase of molecular mass of $\sim$10 kDa (this could vary depending on the location of the ubiquitin along the length of the substrate polypeptide). We were able to elucidate that Nrt and Fas2 are monoubiquitinated in the *Drosophila* neurons. A longer exposure is shown to the *right* for the elution lanes of Fas2. The Eps15 protein has been described to have two isoforms. Only the longest one was detected in our pulldown, appearing to be monoubiquitinated. Fax appeared also to be mostly monoubiquitinated with a higher diffuse band that might correspond to some other type of modification. In stark contrast, ArgK appeared to be polyubiquitinated with the likely contribution of up to 10 or more ubiquitin chains. Those high order conjugates are unlikely to be targeting ArgK for degradation because no proteasome inhibition was used in this work. Two Hsp27 bands were detected in the pulldown that seem to correspond to the addition of two and three ubiquitin molecules. Elution lanes from a separate pulldown are also shown. Rad23 showed a very dense band pattern up to the equivalent of at least six ubiquitin chains. Additionally, a band corresponding to the unmodified protein was also observed. The simplest explanation is that a minor population of unmodified Rad23 survived the stringent washes non-covalently bound to the purified polyubiquitinated material. Pros54 appeared in the pulldown with up to three ubiquitin chains. A longer exposure is shown to the *right*.

TABLE III
*Proteins identified in ^bioUb pulldown known or predicted to be ubiquitin carriers*

Total peptides (TP) and total spectral counts (TSC) are reported for each protein. All peptides and their MS/MS spectra are given in supplemental Table S3. For those with known roles in synaptogenesis, the references where they were described are given.

| NCBI reference | Gene symbol[a] | Mass | TP | TSC | Enzyme | Known roles in synaptogenesis (Refs.) |
|---|---|---|---|---|---|---|
| | | *kDa* | | | | |
| NP_477310.2 | *Uba1* | 131 | 31 | 45 | E1 | 7, 55 |
| NP_001015371.1 | *CG40045* | 19 | 6 | 9 | E2 | |
| NP_524010.2 | *UbcD4* | 22 | 5 | 6 | E2 | |
| NP_511150.1 | *ben* | 17 | 4 | 5 | E2 | 12 |
| NP_730059.1 | *CG7656* | 35 | 3 | 4 | E2 | |
| NP_727234.1 | *Ubc-E2H* | 21 | 3 | 3 | E2 | |
| NP_524684.2 | *crl* | 18 | 2 | 3 | E2 | 60 |
| NP_648582.1 | *vih* | 20 | 2 | 2 | E2 | |
| NP_731941.1 | *eff* | 17 | 2 | 2 | E2 | 98 |
| NP_723616.1 | *UbcD2* | 24 | 1 | 1 | E2 | 7 |
| NP_648452.1 | *As* | 108 | 2 | 2 | E3 HECT | 99 |

[a] Gene symbols given following Flybase (www.flybase.org) nomenclature.

ern blotting has been reported for the Pros54 homolog in yeast, Rpn10 (63), as well as for the overexpressed C-terminal part of Pros54 itself in flies (24) where it has been suggested that its developmental regulation by ubiquitination is in turn used to regulate proteasomal function.

Several heat shock proteins were also found to be ubiquitinated in the fly postmitotic neurons (Table II). With specific antibodies to Hsp27, we detected two bands corresponding to ubiquitinated Hsp27 present in our pulldown (Fig. 4). The ortholog of one other heat shock protein found in the pulldown, Hsp83, has also been found to be ubiquitinated in mammalian cells (64).

*Other Neuronal Ubiquitin Conjugates*—Although we identified a whole array of known and new neuronal ubiquitin targets, some proteins escaped MS identification. We tested by Western blotting several other proteins that have been reported to be ubiquitinated in neuronal tissue in the literature and could confirm a few of them as present in our pulldown, whereas others were not. Our pulldowns included as elucidated by Western blotting (supplemental Fig. S1) the following proteins: syntaxin 1A, known to be ubiquitinated in cell culture (65); Lqf, the fly homolog of Epsin, which was shown to be ubiquitinated in imaginal discs of flies mutant to the DUB *fat facets* (27); and anaplastic lymphoma kinase, a receptor protein-tyrosine kinase reported to be ubiquitinated in *C. elegans* neurons (8). Why the MS analysis did not identify those proteins is most likely a quantitative issue in which case it should be solved by increasing the amount of starting material to detect ubiquitinated proteins of lower abundance, but it could also be due to their sequence not being optimal for MS identification.

We also expressed our tagged ubiquitin construct in combination with a FLAG-tagged version of Traf2 (20), a protein involved in TNFα-mediated activation of MAPK8/JNK and NF-κB. The mammalian homologs of Traf2 have been reported to be both Lys-48 and Lys-63 polyubiquitinated (66, 67). Western blotting on the pulldown with an anti-FLAG antibody revealed one abundant band of ubiquitin-conjugated Traf2 and some weaker bands going well above the contribution of four ubiquitin molecules (supplemental Fig. S2). No proteasome inhibition was used in these experiments, which suggest that those relatively stable higher order conjugates might have been Lys-63-linked. In addition to using our pulldown strategy to isolate endogenous proteins in cases where an antibody is not available, this technique should also be amenable to identify the ubiquitination status of tagged overexpressed proteins.

DISCUSSION

We developed a new strategy for the identification of ubiquitin conjugates from the neurons of *D. melanogaster*. We also detected a number of active ubiquitin-conjugating enzymes in our pulldown both by MS and Western blotting. The ubiquitin-activating enzyme Uba1 and some E2s, E3 ligases, and DUBs are known to be essential for correct synaptogenesis. Here, we identified the most abundant neuronal targets of the ubiquitin-proteasome system at the time of embryonic synaptogenesis. None of the proteins identified here had been reported previously to be ubiquitinated in *Drosophila*. Several of the proteins identified here have orthologs that were earlier shown to be ubiquitinated, suggesting that ubiquitination pathways are conserved across different species. All but one of those reports, however, were based in non-neuronal cell culture (Table II). Here, we found that those proteins are ubiquitinated in the neurons of a multicellular organism. We also identified 19 novel ubiquitin substrates for which no ortholog had so far been reported to be ubiquitinated. Seven of those novel ubiquitin substrates are known to be key players in synaptogenesis, implicating the ubiquitin system heavily in the formation of a functional brain.
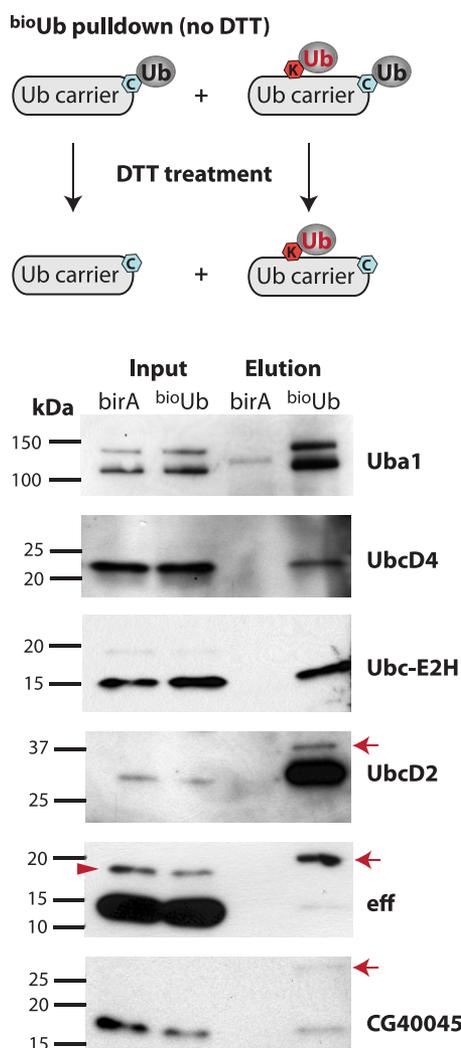
FIG. 5. **Western blot validation of identified ubiquitin carriers.** No reducing agent was used in the pulldown, allowing active ubiquitin carriers to be purified while transiently attached to ubiquitin. Both input and elution samples had DTT added before running SDS-PAGE, resulting in the loss of the ubiquitin chains linked through a thioester bond to the active site cysteine (shown in *blue*). Lysine-conjugated ubiquitins, if present, are not affected by the DTT (shown in *red*), and a distinct additional band with a higher molecular weight would be observed for the fraction of any ubiquitin carrier so modified. Anti-Ube1 was used to detect both isoforms of the *Drosophila* ubiquitin-activating enzyme, Uba1. Uba1 bands in the bioUb elution ran at the same size as the input bands (see supplemental Fig. 3S). One of the endogenous biotinylated proteins was detected by the antibody both in the control and next to the lower band in the bioUb sample. Ubc-E2H (detected using the antibody to its ortholog E2-14K; homology, 79%) and UbcD4 appeared under reducing conditions with matching molecular weights in the elution and input lanes as expected upon the loss of the active site cysteine-attached ubiquitin. UbcD2 was detected using an anti-UbcH6 antibody (homology, 73%). An additional band (*red arrow*) was interpreted as corresponding to a ubiquitin-conjugated (via lysine) fraction of the enzyme. The Western blot to Eff was particularly illustrative because it showed an additional band both in the input (*red arrowhead*) and in the elution (*red arrow*). Those two bands ran at different molecular masses because the input lane mostly contained Eff from the whole embryo (and therefore was

Because of the complexity of the sample and results, ubiquitin proteomics studies on *in vivo* tissues should be directly validated by Western blotting on at least some of the identified ubiquitin substrates. Whether Western blotting validates the MS data or vice versa, it is important to show that putative ubiquitin substrates are so by at least two independent techniques. A recent proteomics study on the ubiquitin-like modification by SUMO in *Drosophila* embryos went as far as validating the MS-identified SUMO targets by overexpressing all the necessary SUMO-conjugating enzymes as well as SUMO and the putative SUMO targets in *E. coli* (68). Often, ubiquitin candidate substrates are validated by tagged protein overexpression in cell lines not representative of their *in vivo* function. Ubiquitination is used to finely tune the activity of proteins within the cell and should therefore be validated within the context of interest and, if possible, without manipulating the protein levels. Even more importantly, the observation of specific bands with a molecular weight shift relative to the unmodified protein is a *sine qua non* to confirm that a protein is indeed ubiquitinated. For those proteins for which good enough antibodies were available, we were able to validate that they are indeed ubiquitinated within the fly neurons. The observation of discrete shifted bands in our Western blots allowed us to resolve whether those neuronal proteins are mono- or polyubiquitinated *in vivo*. All this was achieved in a wild-type background and in the absence of proteasome inhibitors, therefore reporting on physiological ubiquitination levels within the neuronal tissue of the embryos. A previous study on synaptic membranes from cultured neurons using a polyubiquitin affinity-based pulldown identified by Western blotting three synaptic proteins that appeared polyubiquitinated after proteasomal inhibition (14). In this work, we identified 48 neuronal ubiquitin substrates by MS of which eight were validated on at least two biological replicates by Western blotting and 11 ubiquitin carriers of which six were equally validated by Western blots.

Because of the denaturing conditions used in our protocol, we isolated both cytosolic and membrane proteins. In addition, we showed that this technique can also be used to isolate overexpressed tagged proteins (supplemental Fig. S2). Based on the band intensities we observed by Western blotting, we estimated the percentage for each protein that is ubiquitinated within the embryonic neurons, taking into consideration the following variables: the ratio of ectopic to endogenous ubiquitin within those cells where the tagged ubiquitin was expressed, the biotinylation efficiency of the tagged ubiquitin, the fraction for each given protein that was expressed in the neuronal tissue relative to the complete orga-

conjugated to endogenous ubiquitin), whereas the elution lane was enriched only for the bioUb-conjugated Eff (running ~2 kDa higher). CG40045 was detected using an anti-UBE2G1 antibody (antigen homology, 83%) and also displayed an additional, putatively ubiquitin-conjugated band (*red arrow*).

nism, the yield of our purification, and finally how much sample we loaded in each lane relative to the total sample. Unfortunately, we could only make a guess for some of those, but the resulting estimate was informative enough. For the E2 conjugating enzymes, we estimated that the percentage of each enzyme population within the neurons that is carrying ubiquitin is in the range of 25–100%. As for the substrates, we estimated that the percentage to which they appear to be ubiquitinated is in the range of 0.1–3% (data not shown), explaining why it is so difficult to observe ubiquitin conjugates on wild-type extracts. Only when an existing DUB activity is eliminated using a mutant fly, as in the case of the Lqf protein on $faf^{-/-}$ mutants, has there been a convincing direct proof of additional ubiquitinated bands for a *Drosophila* protein (27).

*Neuronal Ubiquitination, Much More than Degradation*— More often than not, it has been assumed that neuronal substrates of the UPS would be polyubiquitinated and targeted for degradation. Either the experiments were designed to detect the accumulation of the substrate upon the loss of function of the ubiquitin ligase (for example, see Ref. 69), or an isolation method with affinity only for polyubiquitin chains was used (14), preventing by design the identification of neuronal monoubiquitinated proteins. Here, however, half of those substrates validated by Western blotting appeared to be monoubiquitinated. One example of a protein that was expected to be a substrate of an E3 ligase to be degraded is Fas2, an NCAM localizing to membranes surrounding synaptic vesicles and active zones in *Drosophila* neuromuscular junctions (45). Fas2 levels at synapses are known to be regulated by synaptic activity (70) and by the MAPK signal transduction pathway (71), but the molecular mechanism by which Fas2 is removed from the synapse has not been elucidated so far. Both Fas2 and Hiw regulate synaptic growth and colocalize to the synaptic periactive area (46, 69), so it is not surprising that Fas2 was eventually postulated to be down-regulated by Hiw. This, however, was ruled out because total Fas2 levels appeared normal in *hiw* mutants (69). We have now confirmed that Fas2 is indeed a substrate of the UPS but only to the extent of being monoubiquitinated, presumably to regulate its trafficking away from the cell surface (72). Considering that Hiw and Fas2 display related phenotypes, it would not be surprising if Fas2 was indeed an Hiw substrate, so the strength of the synaptic contacts would be determined by synaptic levels of Fas2, which would be regulated by ubiquitination by Hiw.

In addition to the observation of monoubiquitinated proteins, we detected in our neuronal pulldown two proteins that appear to be polyubiquitinated as very high order conjugates. Those are unlikely to be targeted for degradation, or otherwise they would not accumulate in the absence of proteasome inhibitors as was the case here. It appears that a large number of synaptic proteins might be regulated through non-degradative ubiquitination as also indicated by the various ubiquitin linkage types observed (Fig. 3*D*). For this reason, it would be important to determine how ubiquitination regulates the function of those neuronal proteins.

*NCAMs and Mechanisms of Axon Growth*—Both Fas2 and Nrt are *Drosophila* cell adhesion molecules that appear to be monoubiquitinated in our pulldown (Fig. 4). Mammalian NCAMs, some of them with a known role in axon growth and navigation, have been shown to be regulated by ubiquitination and endocytosis in cell culture (73, 74). The developing axon needs to attach firmly as it navigates forward but must be capable of easing its grip at the proximal side while enforcing new attachments at the distal end. We believe this internalization could indeed define a general mechanism regulating cell migration and axon growth as proposed by others (75, 76). It has been suggested that endocytosis of the NCAM L1 must be regulated for it to be sorted into recycling endosomes and not into lysosomes (76). Because we observed that a number of NCAMs are monoubiquitinated *in vivo* during synaptogenesis, we propose that their internalization and recycling back to the synaptic surface must be regulated by monoubiquitination to allow the growth cone to move on. Internalization of plasma membrane NCAMs might also be a general mechanism regulated by ubiquitination at later stages for synaptic assembly as well as for facilitating synaptic plasticity.

*Regulation by Ubiquitination of Signaling Complexes and Signal Transduction*—Receptor and non-receptor tyrosine kinases have an important role in regulating neuronal morphogenesis (77). Src family kinases in particular are considered to have crucial roles in modulation of the actin cytoskeleton (78), the driving force behind cell shape change and cell migration. Src kinase activity has been proposed to have a dual, opposite role on L1 endocytosis: one as a positive regulator by phosphorylation of clathrin and a second role by directly phosphorylating L1 to prevent its endocytosis by disrupting L1 binding to clathrin adaptors (76). In cell culture, spatially and temporally regulated patterns of tyrosine phosphorylation by Src can control filopodial behavior downstream of guidance cues and thus the direction of neurite outgrowth (79). Ubiquitination of Src family kinases in neurons, as shown here for Src42A, could be used to regulate their localization patterns as well as to mediate interactions with the endocytic machinery. Vertebrate flotillins participate in the coordinated assembly of raft-related microdomain signaling centers in neurons (49). Those molecular complexes can include glycosylphosphatidylinositol-linked cell adhesion molecules, both flotillins, and a Src family kinase and are postulated to increase signal transduction activity, thereby regulating actin dynamics on the cytoskeleton (51). At the plasma membrane, flotillins undergo a clathrin-independent vesicular cycling after NCAM stimulation. We hypothesize that this cycling is regulated by ubiquitination because we identified both flotillins to be ubiquitinated. No literature reports describe yet any flotillin ortholog to be ubiquitinated. The $Na^+,K^+$-ATPase has also been shown to promote signal transduction as part of a

functional complex with Src (80), regulating the activity of Src itself (81) in cell culture. Na$^+$,K$^+$-ATPase endocytosis after phosphorylation of its $\alpha$ subunit (82, 83) could also be regulated by ubiquitination, but no such link has yet been reported further than its identification as a ubiquitin substrate here in the *Drosophila* neurons.

Ubiquitin modification of those Src-interacting proteins could have two alternative roles. Ubiquitin could be used as a "post-it" domain to transiently facilitate protein-protein interactions with the endocytic machinery regulating those signaling complexes to transiently deactivate them by their internalization. Alternatively, the Src-interacting proteins could be targeted for degradation to completely eliminate their activity. We have not been able to resolve by Western blotting the ubiquitin chain length of those conjugates at this stage, but we believe that once we can describe their ubiquitination status, much will be learned about the mechanism by which they are regulated.

*Conclusions*—No one had yet used proteomics to identify the neuronal targets of the ubiquitin-proteasome system. Here, we have described a novel technique to enrich and isolate ubiquitin conjugates from neurons, not in cell culture but from within a multicellular organism, up to levels that allow direct detection by MS and Western blotting. Using a relatively small sample, we identified the most abundant ubiquitin substrates in neurons, providing the first empirical overview of the involvement of the ubiquitination machinery in synaptogenesis. For some of those ubiquitin conjugates, we could even detect diglycine signatures indicative of the ubiquitin attachment sites. We could also tell whether those UPS substrates for which antibodies were available were mono- or polyubiquitinated. This was achieved in the absence of proteasome inhibitors, but it should be possible to characterize whether their ubiquitination levels change upon proteasome blockade to discern whether those ubiquitin conjugates are being targeted for degradation. The strategy described here will be used in the future to isolate ubiquitin conjugates from mutants to specific E3 ligases and DUBs, which will allow drawing E2/E3/substrate/ DUB maps of effectors and targets, and it is likely to also be applicable to other model organisms.

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