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Identification of E2/E3 Ubiquitinating Enzymes and Caspase Activity Regulating *Drosophila* Sensory Neuron Dendrite Pruning

Report

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Summary

Ubiquitin-proteasome system (UPS) is a multistep protein degradation machinery implicated in many diseases. In the nervous system, UPS regulates remodeling and degradation of neuronal processes and is linked to Wallerian axonal degeneration, though the ubiquitin ligases that confer substrate specificity remain unknown. Having shown previously that class IV dendritic arborization (C4da) sensory neurons in *Drosophila* undergo UPS-mediated dendritic pruning during metamorphosis, we conducted an E2/E3 ubiquitinating enzyme mutant screen, revealing that mutation in *ubcD1*, an E2 ubiquitin-conjugating enzyme, resulted in retention of C4da neuron dendrites during metamorphosis. Further, we found that UPS activation likely leads to UbcD1-mediated degradation of DIAP1, a caspase-antagonizing E3 ligase. This allows for local activation of the Dronc caspase, thereby preserving C4da neurons while severing their dendrites. Thus, in addition to uncovering E2/E3 ubiquitinating enzymes for dendrite pruning, this study provides a mechanistic link between UPS and the apoptotic machinery in regulating neuronal process remodeling.

Introduction

The ubiquitin-proteasome system (UPS), evolutionarily conserved for the regulation of protein turnover, targets proteins for degradation via a complex, temporally regulated process that results in proteasome-mediated destruction of polyubiquitinated proteins. There are two distinct steps involved: first, the covalent conjugation of ubiquitin polypeptide to the protein substrates, and second, the destruction of tagged proteins in the proteasome complex. The transfer of ubiquitin to a target molecule slated for degradation involves at least three enzymatic modifications: ubiquitin is first activated by the ubiquitin-activating enzyme E1; ubiquitin is then transferred to a carrier protein, a ubiquitin-conjugating enzyme E2; and finally, ubiquitin is transferred to a protein substrate bound by a ubiquitin ligase E3 (reviewed in Glickman and Ciechanover, 2002). There are minor variations to this enzymatic cascade, but overall, these highly specific protein-protein interactions ensure ubiquitin targeting specificity and regulate many aspects of housekeeping protein turnover and cellular maintenance. However, with the multiple regulatory layers, different parts of this complex machinery can break down. Mutations in the UPS pathway causing accumulation of nondegraded proteins have been implicated in a variety of human diseases (reviewed in Jiang and Beaudet, 2004; Nakayama and Nakayama, 2006).

In the nervous system, aberrations in the UPS pathway have been implicated in disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and other neurodegenerative diseases (reviewed in Ciechanover and Brundin, 2003). One of the common pathological features of neurodegenerative diseases, besides neuronal loss, is local axon degeneration (reviewed in Coleman, 2005; Luo and O'Leary, 2005). For example, in the case of Wallerian degeneration in vertebrates, distal parts of a severed axon remain viable and conduct action potentials in vivo for some time before a rapid dismantling of cytoskeletal proteins and axon degeneration (Waller, 1850; reviewed in Griffin et al., 1995; Ehlers, 2004), and the initiation of this rapid axon degeneration involves the UPS pathway (Mack et al., 2001; Zhai et al., 2003). It is thought that UPS activation can lead to microtubule depolymerization and subsequent neurofilament degradation, possibly acting in conjunction with the Ca^{2+}-dependent protease calpain (Finn et al., 2000; Zhai et al., 2003). Moreover, inhibiting UPS activity in neurons prior to severing their axons can dramatically retard degradation of the severed axons (Zhai et al., 2003). These results suggest that a cell-intrinsic UPS pathway regulates axon stability and that pharmaceutical inactivation of the UPS may prevent axonal degeneration in disease states.

In *Drosophila*, the remodeling of neuronal processes during normal development closely resembles the pathological phenotypes in Wallerian degeneration. In the mushroom body γ neurons, extensive pruning of larval axons occurs during metamorphosis in a process regulated by glia engulfment (Awasaki and Ito, 2004; Watts et al., 2004; Awasaki et al., 2006; Hoopfer et al., 2006; Macdonald et al., 2006) and neuron-intrinsic UPS activity (Watts et al., 2003). Similarly, in the fly peripheral nervous system, the class IV dendritic arborization (C4da) neurons undergo complete pruning of their extensive larval dendrites during metamorphosis (Kuo et al., 2005; Williams and Truman, 2005), in a process that is also regulated by UPS activity (Kuo et al., 2005). In both of these examples, severing of neuronal processes is preceded by microtubule depolymerization and followed by cytoplasmic blebbing and degeneration, all phenotypes resembling Wallerian degeneration. Therefore, these fly neurons represent excellent systems in which to understand the roles of the UPS in regulating neuronal axon/dendrite integrity, given our rather limited knowledge of how the UPS participates in the degradation of neuronal processes. It is not known which specific E2 ubiquitin-conjugating enzyme(s) and E3 ubiquitin ligase(s) are involved in UPS-mediated remodeling/degradation of neuronal processes, or their specific downstream target(s).

We showed previously that mutations in the fly ubiquitin activation enzyme (*uba1*) and the proteasome complex (*mov34*) can prevent efficient pruning of C4da

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Table 1. Candidate E2/E3 Ubiquitinating Enzymes Tested for C4da Neuron Dendrite Pruning Defects

Gene	Protein Class	Phenotype
*cbx**	E2	none
*ubcD2**	E2	none
*ubcD10**	E2	none
*ubcD1(eff)**	E2	pruning defect
morgue	E2-like	none
*neur**	E3	none
*su(dx)**	E3	none
*ari-1**	E3	none
*ari-2**	E3	cell death[a]
*hiw**	E3	none
*lmg**	E3	none
parkin	E3	none
*diap1(th)**	E3	cell death[b]
*cul-1(lin19)**	E3-SCF	cell death[a]
*slmb**	E3-SCF	cell death[a]
grr1	E3-SCF	none

E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; E3-SCF, ubiquitin ligase complex.
* MARCM clonal analysis of C4da neurons.
[a] C4da neuron clones died during early metamorphosis.
[b] C4da neuron clones died prior to metamorphosis.

neuron larval dendrites during metamorphosis (Kuo et al., 2005). To further investigate the role of UPS in C4da neuron dendrite remodeling, we conducted a candidate gene screen to identify the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin ligase required for this process. Analysis of genetic mutants showed that UPS activation in C4da neurons likely results in UbcD1 (an E2 ubiquitin-conjugating enzyme) mediated degradation of *Drosophila* inhibitor of apoptosis protein 1 (DIAP1), an E3 ligase that antagonizes caspase activity. Degradation of DIAP1 leads to activation of caspase Dronc, which results in local caspase activation and cleavage of proximal dendrites in C4da neurons during metamorphosis. In addition to the identification of a set of E2/E3 ubiquitinating enzymes for C4da neuron dendrite remodeling—with the surprising finding that the UPS mediates degradation of the potent protease inhibitor DIAP1—this study also establishes a mechanistic link between the UPS and caspase pathways in regulating C4da neuron dendrite pruning.

Results

Identification of UbcD1 as an E2 Ubiquitin-Conjugating Enzyme Mediating C4da Neuron Dendrite Pruning

To identify the E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase mediating dendrite pruning of C4da neurons during metamorphosis, we took a candidate gene approach to systematically test the roles of known E2/E3 ubiquitinating enzymes in *Drosophila*. We assembled a set of putative E2/E3 ubiquitinating enzyme mutations and used live imaging to visualize C4da neurons carrying the E2/E3 mutation via the *pickpocket(ppk)-EGFP* marker, which specifically labels C4da neurons during *Drosophila* development (Grueber et al., 2003). We also characterized those mutants with an early lethal phase by generating mosaic analysis with a repressible cell marker (MARCM) mutant neuronal clones (Lee and Luo, 1999). Since wild-type (wt) C4da neurons during metamorphosis do not retain any larval dendrites following head eversion, as imaged 18–20 hr after puparium formation (APF) (Kuo et al., 2005; Williams and Truman, 2005), we looked for mutations that caused larval dendrite retention in C4da neurons at this stage. The candidate genes we tested mostly showed no defects in dendrite pruning or neuronal cell death (Table 1). However, one candidate, *ubcD1*, showed a modest level of larval dendrite retention at 18 hr APF (Table 1 and Figure 1).

Live imaging of wt C4da neuron MARCM clones at the start of pupariation (white pupae, WP) showed primary and secondary dendritic branching patterns typical of C4da neurons (Figure 1A). Consistent with previous reports (Kuo et al., 2005; Williams and Truman, 2005), wt C4da neurons sever their larval dendrites during early

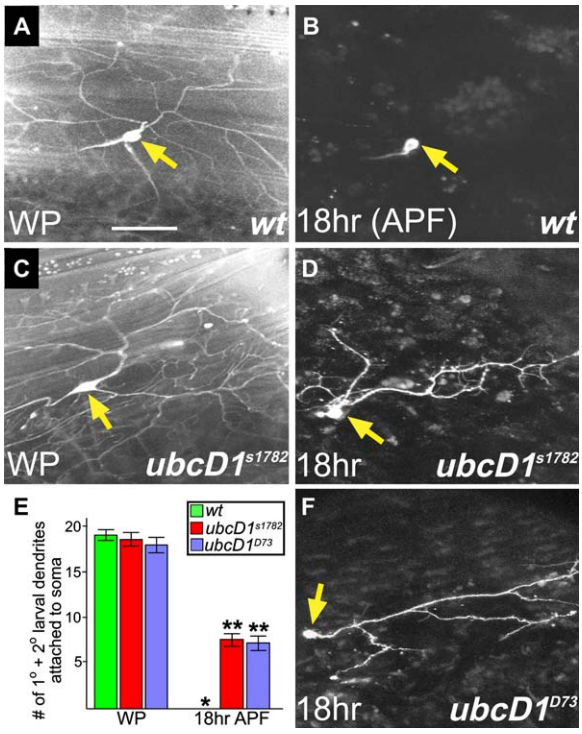

Figure 1. Mutation in *ubcD1*, an E2 Ubiquitin-Conjugating Enzyme, Leads to C4da Neuron Dendrite Pruning Defects during Metamorphosis

(A–D and F) Live confocal images of MARCM wild-type (wt) and *ubcD1* mutant C4da neuron clones during early metamorphosis. (A and B) wt MARCM clones imaged as white pupae (WP, [A]) and at 18 hr after puparium formation (APF, [B]). Note the complete pruning of C4da neuron dendrites at 18 hr APF (B). (C) *ubcD1* mutant C4da neuron MARCM clone imaged at WP stage, showing elaboration of larval dendrites. (D and F) Mutant C4da neuron MARCM clones from two independent alleles of *ubcD1* (s1782, D73) imaged at 18 hr APF. Note the persistence of larval dendrites attached to the soma of C4da neurons. In all panels, the yellow arrows point to soma of C4da neurons. Axons of C4da neurons extend into the body wall during metamorphosis and are not projected in the live Z-stack confocal images for clarity of surface dendrites. Anterior is up and dorsal is right in all images. Scale bar, 50 μm. (E) Quantitative analysis of C4da neuron dendrite pruning defects. We scored the numbers of large primary and secondary dendritic branches attached to soma (tertiary C4da neuron dendrites are difficult to assay reliably via live imaging during pupariation). n = 8 in all groups; * = 0; **p < 0.001, Wilcoxon two-sample test; error bar = SEM.

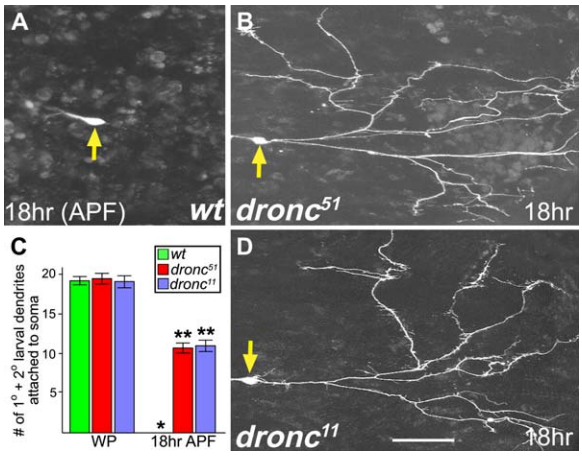

Figure 2. The *Drosophila* Caspase Dronc Is Required for C4da Neuron Dendrite Pruning during Metamorphosis

(A, B, and D) Live confocal images of MARCM wt and *dronc* mutant C4da neuron clones at 18 hr APF. (A) wt MARCM clone. Note the complete pruning of C4da neuron dendrites. (B and D) Mutant C4da neuron MARCM clones from two independent alleles of *dronc* (51, 11). Note the large number of larval dendrites attached to the soma of C4da neurons. In all panels, the yellow arrows point to soma of C4da neurons. Anterior is up and dorsal is right in all images. Scale bar, 50 μm. (C) Quantitative analysis of C4da neuron dendrite pruning defects. n = 8 in all groups; * = 0; **p < 0.001, Wilcoxon two-sample test; error bar = SEM.

metamorphosis and by 18 hr APF are devoid of any dendrites (Figure 1B). The *ubcD1* mutant C4da MARCM clones showed similar dendritic morphology to the wt clones at the onset of metamorphosis (Figures 1C and 1E). However, at 18 hr APF, the mutant clones consistently retained intact, nonsevered larval dendrites (Figures 1D–1F; 1D and 1F show phenotypes of two independent mutant alleles: $ubcD1^{s1782}$ and $ubcD1^{D73}$). Thus, the UbcD1 E2 ubiquitin-conjugating enzyme is required for proper UPS-mediated dendrite pruning in C4da neurons during metamorphosis.

The *Drosophila* Caspase Dronc Is Required for C4da Neuron Dendrite Pruning

UbcD1, encoded by the gene *effete* (Spradling and Rubin, 1982), regulates UPS-mediated degradation of the antiapoptotic protein DIAP1 (Treier et al., 1992; Wang et al., 1999; Ryoo et al., 2002). In protecting cells from apoptosis, the DIAP1 E3 ubiquitin ligase antagonizes Dronc caspase activity by regulating ubiquination and degradation of the Dronc protein (Meier et al., 2000; Muro et al., 2002; Wilson et al., 2002). Following apoptotic stimuli, UbcD1 mediates self-ubiquination and degradation of DIAP1, allowing for subsequent Dronc caspase activation (reviewed in Martin, 2002; Hay et al., 2004). The biochemical and genetic interactions between these molecules are well established. We previously overexpressed the baculovirus p35, which is commonly used to inhibit caspase activity in *Drosophila* (Hay et al., 1994), and it did not block C4da neuron dendrite pruning (Kuo et al., 2005). This may seem to make the involvement of caspases in this process unlikely; however, p35 has only limited activity against the caspase Dronc (Hawkins et al., 2000; Meier et al., 2000).

To study the effects of *dronc* mutation on C4da neuron dendrite pruning, we used two null alleles of Dronc, $dronc^{51}$ and $dronc^{11}$, previously generated in our laboratory (Waldhuber et al., 2005). MARCM analysis of *dronc* mutant clones revealed that the dendrites of mutant C4da neurons appeared normal at larval stages (Figure 2C and data not shown). However, unlike wt clones (Figure 2A), without Dronc these neurons failed to properly prune their larval dendrites during metamorphosis (Figures 2B–2D), and most showed relatively intact primary and secondary larval dendritic arbors at 18 hr APF (Figures 2B and 2D). These results show that severing of primary larval dendrites from C4da neurons during early metamorphosis requires the Dronc caspase.

Degradation of E3 Ubiquitin Ligase DIAP1 Is Required for Proper C4da Neuron Dendrite Pruning

During apoptosis, Dronc activation requires the degradation of the antiapoptotic/anticaspase protein DIAP1 (Meier et al., 2000; Muro et al., 2002; Wilson et al., 2002), which is downstream of UbcD1 (Treier et al., 1992; Wang et al., 1999; Ryoo et al., 2002). The requirement of UbcD1 for C4da neuron larval dendrite pruning during metamorphosis (Figure 1), together with our finding that Dronc caspase activity is also essential (Figure 2), raised the question of whether UPS-mediated DIAP1 degradation is a key step that allows for the severing of larval dendrites. Because loss of DIAP1 function causes C4da neuron cell death prior to the onset of metamorphosis (Table 1 and data not shown), we approached this question using a gain-of-function allele of *diap1*, $diap1^{6\text{-}3s}$, which has a single amino acid mutation that makes DIAP1 an inefficient substrate for UPS-mediated degradation (Goyal et al., 2000). We crossed *ppk-EGFP* into the gain-of-function mutant background and used live imaging to follow C4da neuron dendrite pruning during metamorphosis. The $diap1^{6\text{-}3s}$ mutation did not significantly affect the ability of C4da neurons to elaborate larval dendrites (Figure 3C and data not shown). However, unlike wt C4da neurons that completely pruned their larval dendrites by 18 hr APF (Figure 3A), C4da neurons in the $diap1^{6\text{-}3s}$ gain-of-function mutants failed to efficiently sever larval dendrites at 18 hr APF (Figures 3B–3D). These results suggest that the degradation of DIAP1 during early metamorphosis is required for proper C4da neuron larval dendrite pruning. Quantitatively, mutations in the UPS pathway that modulate Dronc activity ($diap1^{6\text{-}3s}$ and *ubcD1*) resulted in less severe dendrite pruning defects than *dronc* mutants, both in terms of total number of large dendrites attached to soma and in the length of the longest attached dendrite at 18 hr APF (Figure 3E).

Activated Caspase Activity Is Localized to C4da Neuron Dendrites during Initiation of Larval Dendrite Pruning

The UbcD1-DIAP1-Dronc pathway in apoptosis is well established. Thus, it may be necessary for C4da neurons to restrict the action of this pathway to specific cellular locations in order to prune unwanted dendrites without triggering apoptosis. To address this possibility, we looked at the subcellular distribution of DIAP1 and Dronc proteins in *ppk-EGFP* C4da neurons. During the

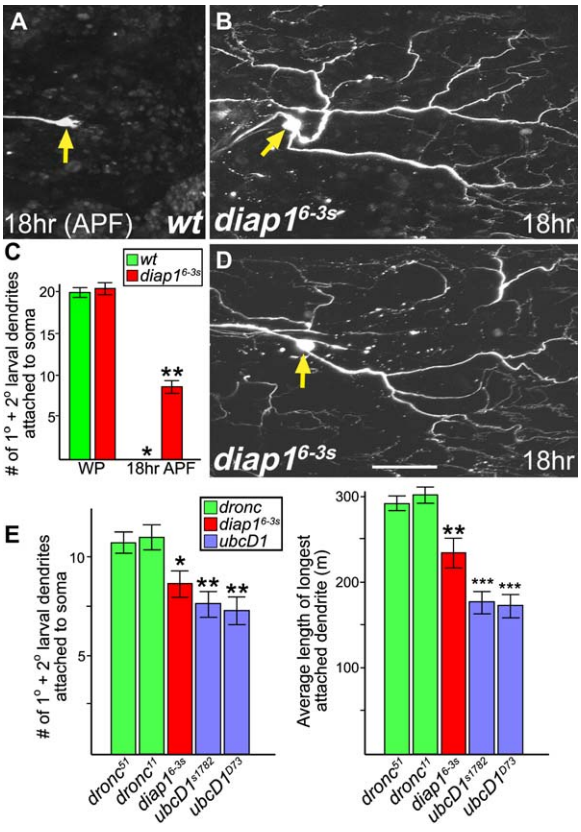

Figure 3. Degradation of the E3 Ubiquitin Ligase DIAP1, a Dronc Inhibitor, Is Required for Efficient C4da Neuron Dendrite Pruning during Metamorphosis

(A, B, and D) Live confocal images of *ppk-EGFP*-labeled wt and *diap1*$^{6-3s}$ mutant C4da neurons at 18 hr APF. (A) wt control *ppk-EGFP*-labeled C4da neuron. Note the complete pruning of C4da neuron dendrites. (B and D) Two representative *ppk-EGFP*-labeled *diap1*$^{6-3s}$ mutant C4da neurons. Note the large number of larval dendrites attached to the soma of C4da neurons. In all panels, the yellow arrows point to soma of C4da neurons. Anterior is up and dorsal is right in all images. Scale bar, 50 μm. (C) Quantitative analysis of C4da neuron dendrite pruning defects. n = 10 in all groups; * = 0; **p < 0.001. (E) Comparisons between *dronc*, *diap1*$^{6-3s}$, and *ubcD1* mutant phenotypes, based on either the number of larval dendrites attached to soma (left panel) or the average length of longest attached dendrite (right panel). For statistical analysis, we compared *diap*$^{6-3s}$ (n = 10), *ubcD1*^{s1782} (8), or *ubcD1*^{D73} (8) mutants individually to a *dronc* group that contained both *dronc*^{51} (8) and *dronc*^{11}(8). *p < 0.5, **p < 0.1, ***p < 0.01. For all quantitation we used the Wilcoxon two-sample test; error bar = SEM.

transition from third instar larvae to white pupae at the onset of metamorphosis, as well as 2 hr APF, there was a consistent induction of nuclear DIAP1 in GFP-labeled C4da neurons (see Figure S1A in the Supplemental Data available online). During the same period we detected a concurrent decrease in Dronc staining in the soma of C4da neurons, unlike those from the neighboring cells at 2 hr APF (Figure S1B). These results are consistent with previous observations that C4da neurons survive through this stage of metamorphosis (Kuo et al., 2005; Williams and Truman, 2005). However, the level of antibody staining made it difficult to monitor the distribution of DIAP1 and Dronc within the dendritic structures of the C4da neurons. Because overexpression of Dronc caused C4da neuron to undergo apoptosis prior to metamorphosis (data not shown), it was not possible to use GFP-tagged Dronc to examine its distribution in these neurons during pupariation. It was therefore necessary to search for alternative means to visualize activated Dronc or its downstream caspases.

An antibody generated against activated mammalian caspase 3 has been shown to be effective in recognizing activated caspases in *Drosophila* (Yu et al., 2002; Yin and Thummel, 2004; Awasaki et al., 2006). Whereas this antibody reportedly recognizes the *Drosophila* effector caspase Drice, it may also cross react with other activated *Drosophila* caspases such as Dronc during tissue staining, because of similarities in the sequences of these caspases in the region corresponding to the peptide used to generate this antibody (Yu et al., 2002). We therefore used this antibody to determine whether activated caspase is localized to the dendrites of C4da neurons during the initial severing event. At 4 hr APF, just prior to dendrite severing, we consistently observed antibody staining for activated caspase within the proximal larval dendrites of C4da neurons, especially within dendritic swellings (Figure 4A). In the *diap1*$^{6-3s}$ gain-of-function mutant that inhibits Dronc activity (Figure 4A), as well as in *ubcD1* and *dronc* mutant MARCM clones (Figure S2), C4da neurons did not show dendritic swellings or activated caspase staining in dendrites during early metamorphosis. Consistent with our previous observation that C4da neurons do not remodel their axons during concurrent dendrite pruning (Kuo et al., 2005), we also did not see activated caspase staining within the axons of C4da neurons during dendrite severing (data not shown). Since overexpression of p35 in these neurons did not block dendrite pruning (Kuo et al., 2005), we believe this antibody staining likely recognizes activated Dronc directly or recognizes a p35-resistant caspase that is activated by Dronc. These results show that, concurrent with the nuclear upregulation of DIAP1 in C4da neurons that prevents apoptosis, there is a local activation of caspases in the dendrites, likely as a result of UPS-mediated degradation of DIAP1 (Figure 3). The spatially restricted activation of caspases then allows the severing of proximal larval dendrites from the soma (Figure 4B).

Discussion

The ubiquitin-proteasome system is an important protein degradation pathway that regulates neuronal process remodeling and degradation in flies and mammals. The specific ubiquitin-conjugating enzymes and ligases and their downstream protein substrates that are involved in these processes are not known. We and others showed previously that the elaborate dendritic arbors of *Drosophila* C4da peripheral sensory neurons undergo complete pruning and regrowth during metamorphosis (Kuo et al., 2005; Williams and Truman, 2005). We showed that the UPS regulates pruning of larval dendrites from C4da neurons in a cell-intrinsic manner. To better understand the molecular pathways regulating UPS-mediated pruning, we conducted a candidate E2/E3 ubiquitinating enzyme screen. In this screen we uncovered an E2 ubiquitin-conjugating enzyme mutation in *ubcD1*, causing dendrite pruning defects. Taken

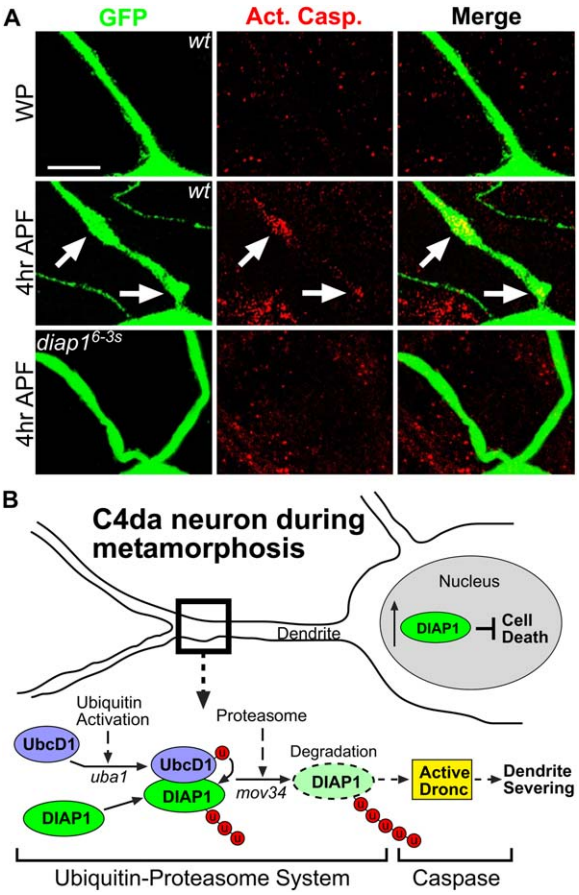

Figure 4. Activated Caspase Activity in C4da Neuron Dendrites and a Model of C4da Neuron Dendrite Pruning during Metamorphosis

(A) Antibody staining of wild-type (wt) and *diap1*$^{6\text{-}3s}$ *ppk-EGFP* pupal fillets with anti-GFP (green) and anti-activated caspase (red) antibodies at white pupae (WP) and 4 hr APF. Columns show C4da neurons labeled with anti-GFP alone, anti-activated caspase (Act. Casp.) alone, or a merge of the two images. Arrows point to colocalization of activated caspase staining within wt C4da neuron dendrite swellings at 4 hr APF, just prior to dendrite severing. Note both the lack of swellings and activated caspase staining in *diap1*$^{6\text{-}3s}$ C4da neuron dendrites at 4 hr APF. Scale bar, 3 μm. (B) Schematic representation showing C4da neuron dendrite undergoing pruning during metamorphosis. Whereas upregulation of DIAP1 in the nucleus inhibits caspase-mediated apoptosis in the soma, local activation of caspases can occur in the dendrites. We showed previously that mutations affecting ubiquitin activation (*uba1*) and the proteasome machinery (*mov34*) can inhibit dendrite pruning. Now we find that UPS-mediated dendrite pruning in C4da neurons likely acts through the UbcD1 E2 ubiquitin-conjugating enzyme to degrade DIAP1, an E3 ubiquitin ligase that inhibits the Dronc caspase.

together with the extensive biochemical characterization of interactions between UbcD1, DIAP1, and Dronc, our study suggests that in C4da neurons, UPS activation leads to UbcD1-mediated degradation of E3 ubiquitin ligase DIAP1, thereby allowing Dronc caspase activation and the subsequent cleavage of larval dendrites (Figure 4B). This work not only identifies a set of E2/E3 ubiquitinating enzymes regulating neuronal process remodeling, it also links the UPS to a hitherto unappreciated mechanism for local caspase activation in dendrites during *Drosophila* metamorphosis.

A Mechanistic Link between the UPS and Apoptotic Machinery

The mechanistic link between the UPS and caspase activity in regulating C4da neuron dendrite pruning is unexpected. Although the UPS is known to regulate remodeling and degradation of neuronal processes, it is generally believed that this process is accomplished by degradation of cellular proteins (such as microtubules and neurofilaments) that are required to keep dendrites and axons intact. However, we found that the UPS in C4da neurons is in fact causing the degradation of an E3 ligase, DIAP1, thereby allowing for subsequent dendrite pruning. In this case, UPS-mediated degradation of a protein does not in and of itself lead to a structural compromise in dendrites, but rather it leads to the activation of another protease that executes dendrite pruning. This two-step activation cascade, which involves both the UPS and the apoptotic machinery, may provide an additional level of control and flexibility that would not be possible if UPS alone regulated the pruning program. After all, these C4da neurons, which are specified during fly embryogenesis, maintain a highly elaborate dendritic field to receive sensory inputs throughout larval development, which lasts for several days. The maintenance of these dendrites over time requires a network of finely tuned cell-intrinsic and -extrinsic pathways (reviewed in Jan and Jan, 2003; Grueber and Jan, 2004). Just as important, the dendritic pruning program enables dramatic neuronal remodeling in response to profound environmental changes during metamorphosis. It is conceivable that C4da neurons evolved this dual control mechanism to prevent any accidental triggering of dendrite pruning prior to metamorphosis. We and others showed previously that initiation of C4da neuron dendrite pruning requires cell-intrinsic ecdysone signaling (Kuo et al., 2005; Williams and Truman, 2005), and ecdysone receptors have been shown to regulate Dronc expression (Dorstyn et al., 1999). It will be of interest to determine how this UPS/caspase dendritic pruning pathway is related to the ecdysone signaling cascade.

Specificity of C4da Neuron Dendrite Pruning during Metamorphosis

During metamorphosis, C4da neurons upregulate DIAP1 expression in the nucleus, which is consistent with this class of neurons surviving early stages of the metamorphosis (only one of the three C4da neurons per hemisegment, the ventral neuron, is lost at a later stage of pupariation). Remarkably, there are activated caspases within the dendrites prior to severing, and a gain-of-function *diap1* mutation can block dendrite pruning, strongly implicating a local dendritic program that can activate caspases without causing apoptosis of the neuron (Figure 4B). Although mutations in both the Dronc caspase and the UPS pathway that modulate Dronc activity (UbcD1 and DIAP1) result in retention of larval dendrites, their dendrite pruning defects differed somewhat quantitatively. Compared to *dronc* mutants, *diap1* gain-of-function and especially *ubcD1* mutants showed less retention of larval dendrites during metamorphosis (Figure 3E). This is not surprising for *diap1* gain-of-function, as it is an effective Dronc inhibitor but unlikely to be 100% efficient. UbcD1, as an E2 ubiquitin-conjugating enzyme, has wider substrate

specificity than E3 ligases. Previous study showed that UbcD1 is involved in mushroom body neuroblast proliferation (Watts et al., 2003), so it may be involved in other UPS-mediated pathways during dendrite pruning. It is also conceivable that in the absence of UbcD1 another E2 may trigger a low level of DIAP1 degradation, allowing residual Dronc activation which results in a milder dendrite pruning phenotype in *ubcD1* mutants. It is currently unclear whether UbcD1 is also required during DIAP1-mediated degradation of Dronc. However, pruning defects in the *ubcD1* mutants suggest that it may not be absolutely required, as undegraded DIAP1 continues to inhibit Dronc, presumably via interaction with another E2 protein.

How is the specificity of dendrite pruning achieved? We can imagine several possible mechanisms: first, we reported previously that C4da neurons do not change their axonal projections during dendrite pruning, so there could be dendrite-specific trafficking of components of the UPS, such as UbcD1, and/or the caspase Dronc. Of the known proteins that are preferentially trafficked to dendrites (reviewed in Horton and Ehlers, 2003; Setou et al., 2004), these molecules have not been implicated but warrant further investigation. Second, it is also possible that activated Dronc, or another p35-resistant protease activated by Dronc, could cleave a dendrite-specific substrate. Examples are now emerging from other cellular systems, such as in sperm formation and border cell migration, in which caspases can participate in cleavage of proteins not resulting in apoptosis (Arama et al., 2003; Geisbrecht and Montell, 2004; Huh et al., 2004). Third, the dendritic pruning program takes place during drastic environmental changes that include concurrent degradation and regrowth of the overlying epidermis (Kuo et al., 2005), activation of extracellular matrix metalloproteases (Kuo et al., 2005), and blood phagocytes (Williams and Truman, 2005). These environmental cues likely complement the neuronal intrinsic pruning programs, but their exact relationships are not known. Experiments addressing these and other possible mechanisms should provide us with greater insight into how the large-scale remodeling of C4da neuron dendrites is achieved.

Implications for UPS Involvement in Neuronal Process Remodeling and Degeneration

In vertebrates, the UPS pathway has been implicated in Wallerian degeneration of severed axons (Zhai et al., 2003). In the fly, mushroom body γ neurons undergo extensive remodeling of their processes during metamorphosis (Lee et al., 2000). The initial stages of axon pruning in these mushroom body neurons closely resemble Wallerian degeneration, and the UPS again plays a critical role (Watts et al., 2003). To date, the specific ubiquitin-conjugating enzymes and ligases that mediate target protein degradation have not been identified in these systems. It will be interesting to see whether the UbcD1-DIAP1-Dronc pathway implicated in C4da neuron dendrite pruning also participates in remodeling/degradation of neuronal processes in other systems. It seems likely that more than one pathway would be employed in remodeling different neurons; a previous study excluded UbcD1 as a possible ubiquitin-conjugating enzyme regulating mushroom body γ neuron remodeling (Watts et al., 2003), and we saw normal remodeling of mushroom body neuron processes in *dronc* mutant MARCM clones during metamorphosis (Figure S3).

A multilayered regulatory machinery for remodeling neurons, as uncovered in this study for C4da neurons, offers versatility and flexibility. It is conceivable that another ubiquitin ligase/caspase pair may function in an analogous UPS pathway during mushroom body neuron remodeling, potentially affording differential regulation of neuronal remodeling. Although pharmacological inhibition of mammalian caspases showed no effect on Wallerian degeneration (Finn et al., 2000), it would be important to assess the in vivo effectiveness of the inhibitors against a comprehensive panel of caspases. Moreover, a dual control mechanism, similar to what we propose for C4da neuron remodeling, may coordinately regulate UPS and another protease that executes axon degradation. Conceivably, instead of having the target of the UPS directly involved in maintaining dendrite/axon stability, the executor of neuronal process degradation may involve a different protease: in the case of C4da neurons it is the caspase Dronc, and in Wallerian degeneration the relevant protease might be the Ca^{2+}-responsive calpain. Future experiments along these lines of thinking may accelerate the identification of specific ubiquitinating enzymes involved in other areas of developmental neuronal remodeling and in diseases where the UPS pathway has been implicated. As target-specific E3 ligases are excellent candidates for pharmaceutical intervention, this approach may also help to find effective treatments for developmental and neurodegenerative diseases that involve degeneration of neuronal processes.

Experimental Procedures

Fly Stocks

The fly stocks include *ppk-EGFP*^{5} (Grueber et al., 2003); E2/E3 ubiquitinating enzymes were selected based on published literature and availability of fly mutations, and include the following: FRT alleles of *cbx*, *ubcD1*, *ubcD2*, *ubcD10*, *neur*, *su(dx)*, *ari-1*, *ari-2*, *hiw*, *Img*, *cul-1*, *slmb* (Watts et al., 2003); *ubcD1*^{D73} (Ryoo et al., 2002); *th*^{4} (Bloomington stock #5053); *parkin* (Pesah et al., 2004); *SCF*^{grr1} (CG9003, Bloomington stock #19321), *morgue* (Bloomington stock #16996). Other stocks include *dronc*^{11} and *dronc*^{51} (Waldhuber et al., 2005); *diap1*$^{6\text{-}3s}$ (Goyal et al., 2000). MARCM stocks included *FRT*^{G13}, *FRT*^{40A}, *FRT*^{82B}, *FRT*^{2A}, and *FRT*^{19A}.

MARCM Analysis

MARCM analyses were performed as previously described (Lee and Luo, 1999; Emoto et al., 2004). To generate mosaic clones, mutations recombined onto FRT chromosomes were crossed to appropriate MARCM stocks that contain *elav-Gal4*, *hsFLP*, *UAS-mCD8::GFP* and the corresponding FRT chromosome containing *tub-Gal80*. Live imaging was obtained on a BioRad MRC 600 confocal microscope.

Immunocytochemistry

For immunohistochemistry we used the following primary antibodies: rabbit anti-GFP, 1:2000 (kindly provided by Y. Hong, UCSF); chicken anti-GFP, 1:500 (Aves Labs); mouse anti-DIAP1, 1:400 (gift of B. Hay); guinea pig anti-Dronc, 1:2000 (gift of H. Steller); rabbit anti-cleaved caspase 3, 1:100 (Cell Signaling Technology). Donkey Cy2 or Rhodamine Red X-conjugated secondary antibodies were used at 1:200 (Jackson Laboratory). Fluorescence images were obtained by confocal microscopy on a Leica TCS SP2.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/51/3/283/DC1/.

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