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Dominant-negative mutation in the β2 and β6 proteasome subunit genes affect alternative cell fate decisions in the *Drosophila* sense organ lineage

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ABSTRACT **In *Drosophila*, dominant-negative mutations in the β2 and β6 proteasome catalytic subunit genes have been identified as dominant temperature-sensitive (DTS) mutations. At restrictive temperature, β2 and β6 DTS mutations confer lethality at the pupal stage. I investigate here the role of proteasome activity in regulating cell fate decisions in the sense organ lineage at the early pupal stage. Temperature-shift experiments in β2 and β6 DTS mutant pupae occasionally resulted in external sense organs with two sockets and no shaft. This double-socket phenotype was strongly enhanced in conditions in which Notch signaling was up-regulated. Furthermore, conditional overexpression of the β6 dominant-negative mutant subunit led to shaft-to-socket and to neuron-to-sheath cell fate transformations, which are both usually associated with increased Notch signaling activity. Finally, expression of the β6 dominant-negative mutant subunit led to the stabilization of an ectopically expressed nuclear form of Notch in imaginal wing discs. This study demonstrates that mutations affecting two distinct proteasome catalytic subunits affect two alternative cell fate decisions and enhance Notch signaling activity in the sense organ lineage. These findings raise the possibility that the proteasome targets an active form of the Notch receptor for degradation in *Drosophila*.**

In eukaryotic cells, degradation of many proteins involves their covalent modification by conjugation with ubiquitin. Ubiquitinated proteins can be rapidly degraded by a large multisubunit complex called the 26S proteasome (1). This complex is present in the nucleus and in the cytosol of all cells. The 26S proteasome consists of a 20S core particle capped by two 19S regulatory complexes. The 20S proteasome is a barrel-shaped cylinder composed of four stacked rings of seven subunits each (2). The two external rings are composed of seven α subunits (α1–7), and the two inner rings comprise seven β subunits (β1–7) that catalyze the hydrolysis of polypeptide substrates.

In *Drosophila*, the *DTS7* and *DTS5* dominant temperature-sensitive (DTS) mutations affect the β2 and β6 proteasome subunit genes, respectively (3, 4). *DTS5* and *DTS7* heterozygous flies develop perfectly at the permissive temperature (25°C), but die as undifferentiated pupae with failures in head eversion at the restrictive temperature (29°C). The *DTS5* and *DTS7* mutations behave genetically as antimorphic mutations, and they correspond to substitutions in residues that are conserved from flies to vertebrates (3, 4). The structure proposed for the yeast 20S proteasome indicates that β2 directly interacts with β6 in the adjacent ring (2), and it predicts that the amino acids mutated in the *DTS5* and *DTS7* mutant subunits alter the β2–β6 interface (4). Consistent with this possibility, the *DTS5* and *DTS7* mutations display synthetic lethality, even at 18°C (3).

Because *DTS5* and *DTS7* mutants develop normally at the restrictive temperature until early pupal stages, the potential role of proteasome activity in regulating cell determination was examined in the adult sense organ lineage. Bristle mechanosensory organs are composed of four different cells that originate from a single precursor cell, pI (5) (Fig. 1*A*). In the notum, pI cells appear around 8–14 hr after puparium formation (APF). Each pI divides asymmetrically along the anteroposterior (a-p) axis of the fly body to generate two secondary precursor cells, a posterior pIIa cell and an anterior pIIb cell (6). pIIb divides prior to pIIa, perpendicularly to the plane of the epithelium, to generate a small subepithelial glial cell, which will later migrate away from the sense organ, and a pIIIb cell (7). pIIa then divides asymmetrically, along the a-p axis to produce the shaft and socket cells. Finally, pIIIb divides to generate the neuron and the sheath cell. At the pI, pIIa, and pIIIb divisions, distinct fates are conferred on sister cells by the unequal activation of Notch signaling that results from the asymmetric segregation of Numb (7–10). Because Numb is unequally segregated during the pIIb division, it is conceivable that Notch signaling also participates in the pIIIb/glial cell fate decision.

Here, I show that decreased proteasome activity resulted in shaft-to-socket cell fate transformations in *DTS5* and *DTS7* heterozygous pupae, and that overexpression of the β6 dominant-negative mutant subunit led to neuron-to-sheath and shaft-to-socket cell fate changes. Such fate transformations usually result from increased Notch signaling. Consistently, the *DTS5* and *DTS7* mutations were shown to strongly enhance the double-socket phenotype associated with increased Notch signaling activity. Finally, an activated form of Notch was found to be stabilized in cells that expressed the β6 dominant-negative mutant subunit. This observation indicates that this form of Notch is a target of the proteasome. These data demonstrate that a wild-type level of proteasome activity is required for the proper regulation of at least two alternative cell fate decisions during sense organ development, and that proteasome-mediated protein degradation antagonizes Notch signaling activity in this process. These findings raise the possibility that the proteasome participates in limiting Notch signaling by targeting an active form of Notch for degradation.

MATERIALS AND METHODS

Flies. The *DTS5* and *DTS7* mutations are described in refs. 3 and 4 (kind gifts from J. Belote, Syracuse Univ., Syracuse, NY). The A101 P[*lacZ*, *ry*$^+$] enhancer-trap is an allele of *neuralised* that specifically expresses nuclear β-galactosidase in pI and its progeny cells (11). The H^{E31} mutation is described

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Abbreviations: DTS, dominant temperature-sensitive; APF, after puparium formation.
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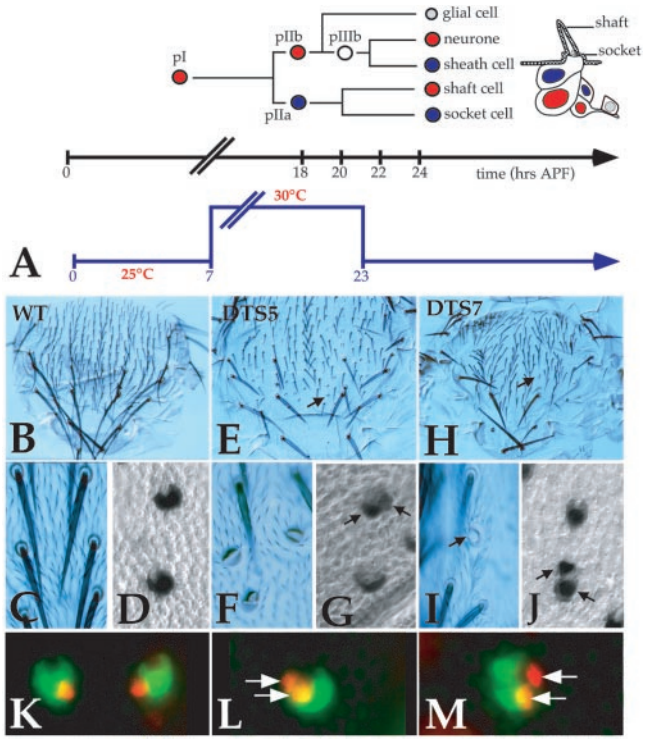

FIG. 1. Interfering with proteasome function in early pupae yields a double-socket bristle phenotype. (*A*) Diagram of the bristle lineage in the notum. pI divides asymmetrically to generate pIIa and pIIb. Notch signaling is active in pIIa (blue cell) and inhibited by Numb in pIIb (red cell). pIIb divides to generate pIIIb (white cell) and a small glial cell (gray cell) that will migrate away soon after pIIIb division. The divisions of the pIIa and pIIIb cells give rise to the four mechanosensory organ cells, schematically shown on a diagram of a differentiated microchaete. Notch signaling is active in the sheath and socket cells (in blue) and blocked by Numb in the neuron and shaft cells (in red). The approximate timing of these divisions is indicated by the black time scale below. APF, after puparium formation. The temperature shifts applied to *DTS5* and *DTS7* pupae are shown below as a blue time scale bar. (*B–J*) Cuticular preparations of dorsal thoraces from pharate adults (panels *B, C, E, F, H,* and *I*) and nota dissected from 24-hr-APF pupae stained for Su(H) immunoreactivity (horseradish peroxidase staining in *D, G,* and *J*). Wild-type (*B–D*), *DTS5*/+ (*E–G*), and *DTS7*/+ (*H–J*) were heat-pulsed at 30°C during a 7-23-hr-APF time period as indicated in *A*. No bristle phenotype was seen at the permissive temperature (not shown). Arrows point to double-socket bristles (*E, H,* and *I*) and pairs of socket cells (*G* and *J*). (*K–M*) Indirect immunofluorescence staining of nota dissected from 28-hr-APF wild-type (*K*), *DTS5*/+ (*L*), and *DTS7*/+ (*M*) pupae that were heat-pulsed at 29°C at 0–28 hr APF. Duplicated sheath cells in *DTS5* and *DTS7* mutants are shown by white arrows. Each panel correspond to the merge of images from two focal planes: the socket cells (in green: Suppressor of Hairless) were found in the epithelial plane, whereas the sheath cells (in red: Prospero) were subepithelial. These Prospero-positive cells cannot correspond to glial cells for several reasons: first, at this late stage, no glial cells accumulate Prospero; furthermore glial cells migrate away from microchaetes around 23–24 hr APF and are no longer associated with microchaetes at 28 hr APF; finally, glial cell nuclei are distinctly smaller than sheath cell nuclei (7). In all panels anterior is up.

in ref. 12. P[w^+, hs-Nintra]-I4A used to express activated Notch (ref. 13; gift from T. Lieber, Rockefeller Univ., New York) was recombined with *ptc-gal4* to generate the *ptc-gal4* P[w^+, hs-Nintra]-I4A/SM5; TM6b Tb stock.

A 1.2-kb *Bam*HI–*Nhe*I genomic fragment containing the *DTS5* allele of the β6 proteasome catalytic subunit gene *l(3)73Ai* (ref. 3; plasmid p1B176; gift from J. Belote) was subcloned into pUAS-T between the *Bgl*II and *Xba*I sites. The resulting plasmid was used to obtain 13 independent P[w^+, UAS-DTS5] transformant lines. Line 13 was used to construct a stable w[1118]; *sca-gal4*; P[w^+, UAS-DTS5]-13/TM6b Tb line.

Immunostaining. Dissected nota from 24-hr-APF pupae were processed as described previously (6). Primary antibodies were rat anti-Suppressor of Hairless [Su(H)] (1:500), mouse anti-Prospero (MR1a; 1:2), mouse anti-Notch (C17–9C6; 1:1000), rat anti-Ttk69 (1:300), and rabbit anti-β-galactosidase (Cappel; 1:500). Secondary conjugated antibodies were: Cy5- and FITC-anti-mouse (Jackson ImmunoResearch; 1:500 and 1:200, respectively); FITC- and lissamine rhodamine sulfonyl chloride (LRSC)-anti-rat (Jackson ImmunoResearch; 1:200); FITC- and LRSC-anti-rabbit (Jackson ImmunoResearch; 1:200); biotinylated anti-rat (1:1000; horseradish peroxidase staining was performed with the Vectastain Elite kit). Images were obtained on a Leica TCS 4D confocal microscope, or on a DMLB Leica microscope using a Micromax camera (Princeton Instrument). Images were processed with NIH IMAGE, MetaMorph, and PhotoShop software.

RESULTS

Shaft-to-Socket Transformation Associated with Dominant-Negative Proteasome Mutations. To investigate the role of proteasome function in cell fate determination, *DTS5* and *DTS7* heterozygous pupae were heat-pulsed at the restrictive temperature between 7 and 23 hr APF. Under these conditions, pupae developed to give pharate adults that did not emerge but showed specific bristle defects. A small number of double-socket sense organs were observed on the notum [Fig. 1 *E, F, H,* and *I*; *DTS5* and *DTS7* flies showed 8% (n = 50/621 from six nota) and 2% (n = 8/432 from five nota) of double-socket microchaetes on the notum], as well as on the abdomen and on the wing margin (data not shown). Wild-type flies subjected to the same regimen showed no defects (Fig. 1 *B* and *C*). When the restrictive temperature was applied to *DTS5* or *DTS7* mutant pupae for a longer period of time, pupal development was blocked and no pharate adults were recovered (not shown). The double-socket phenotype seen in *DTS5* and *DTS7* adult flies suggested that the shaft cell was occasionally transformed into a second socket cell. This cell fate change was confirmed by using anti-Su(H) antibodies to specifically mark the socket cells (14): a few sense organs with two Su(H)-positive cells were observed in nota from *DTS5* and *DTS7* pupae that were dissected at 24 hr APF and that had developed at the restrictive temperature as described above (Fig. 1 *D, G,* and *J*).

To examine whether the fate of the neuron and sheath cells might also be affected in *DTS5* and *DTS7* mutant pupae, Prospero was used as a sheath cell marker. Prospero, a divergent homeodomain protein, is detected in the pIIb, pIIIb, and sheath cells (7, 15, 16). It also accumulates transiently in the glial cell and in the neuron. At 28 hr APF, however, Prospero could be detected in only a single cell, the sheath cell (Fig. 1*K*). In *DTS5* and *DTS7* mutant pupae, microchaetes with two cells accumulating Prospero were occasionally observed (Fig. 1 *L* and *M*), possibly reflecting a neuron-to-sheath transformation (see below).

Shaft-to-Socket and Neuron-to-Sheath Transformations Associated with Overexpression of a β6 Dominant-Negative Mutant Subunit. To unambiguously show that the *DTS5* phenotype resulted from a defect in proteasome activity, the cDNA encoding the DTS5 mutant β6 proteasome subunit was expressed in wild-type flies by using the UAS-*gal4* system (17). When a homozygous viable *gal4* driver inserted at the *scabrous* locus (*sca-gal4*) was used, the DTS5 mutant β6 subunit was expressed in proneural clusters and in sense organ cells in third-instar larvae and early pupae. Flies carrying two copies of *sca-gal4* and one copy of the UAS-*DTS5* transgene grew normally at 25°C, but died at 29°C as pharate adults showing a strong multiple-socket phenotype [Fig. 2 *A* and *E*; 80% of the

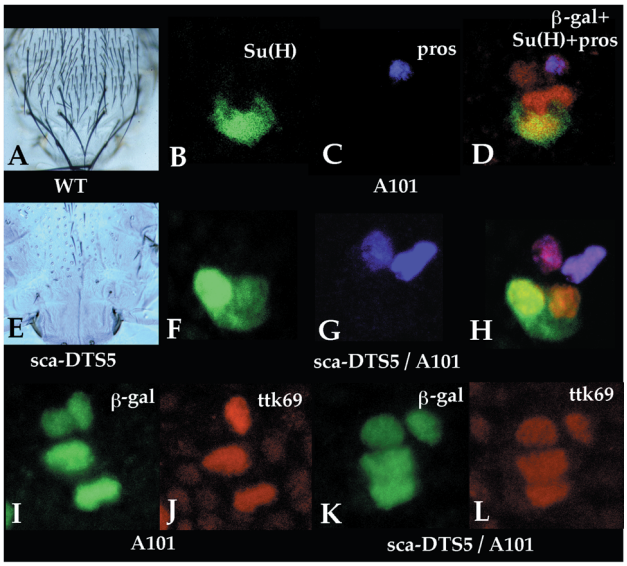

FIG. 2. Conditional expression of the mutant *DTS5* proteasome subunit results in fate transformation. (*A*) Cuticle of a wild-type adult. (*B–D*) Sense organ cells in a 24-hr-APF A101/TM6b pupa. The socket cell is marked with Su(H) in green (*B*), the sheath cell with Prospero (pros) in blue (*C*), and all the sense organ cells with β-galactosidase in red in the merged view (*D*). (*E*) cuticle of a *sca-gal4*; UAS-*DTS5*/TM6b pharate adult. The bristle phenotype exhibited by these *sca-DTS5* flies was stronger than the one observed in *DTS5* heterozygous pupae, suggesting that larger amount of mutant subunits were produced in the former ones. (*F–H*) Sense organ cells in a 24-hr-APF *sca-gal4*/+; UAS-*DTS5*/A101 pupa. Staining is as in *B–D*. (*I–L*) Sense organ cells in a 24-hr-APF A101/TM6b (*I, J*) or *sca-gal4*/+; UAS-*DTS5*/A101 pupa (*J*). Sense organ cells are marked with β-galactosidase (in green; *I* and *K*) and nonneuronal cells express a higher level of Ttk69 (in red; *J* and *L*). In all panels, anterior is up, and the notum midline is on the right side in *B–D* and *F–L*.

microchaetes of *sca-gal4*/*sca-gal4*; *UAS-DTS5*/+ flies showed a multiple socket phenotype (n = 410/511 from four nota)]. They also exhibited synthetic lethality with *DTS7*, even at 18°C (not shown). These data demonstrate that the multiple-socket phenotype specifically resulted from altered proteasome activity.

A detailed phenotypic analysis of the *sca-DTS5* phenotype was then performed. Pupae carrying a single copy of both UAS-*DTS5* and *sca-gal4* were maintained at 29°C from 0 to 24 hr APF, after which they were dissected and fixed. Sense organ cells were identified by using the A101 enhancer-trap marker, which specifically labels pI and its progeny (11). Socket and sheath cells were identified by using Su(H) and Prospero, respectively, as specific markers (6). In this genotype, the majority of sense organs appeared phenotypically wild type. The most frequently observed mutant phenotype corresponded to sense organs composed of four cells with two Su(H)-positive socket cells and two strongly Prospero-positive cells (compare *B–D* and *F–H* in Fig. 2). On the basis of its size and apical position, this second Prospero-positive cell was identified as a second sheath cell. This finding indicates that the shaft cell and the neuron were transformed into second socket and sheath cells, respectively. This neuron-to-sheath cell fate change was confirmed by using Elav as a neural marker (not shown) or Ttk69 as a nonneuronal marker (18) (Fig. 2 *I–L*). This cell fate transformation seen in *sca-DTS5* pupae suggests that the sheath cell duplications observed in *DTS5* and *DTS7* pupae (Fig. 1 *K–M*) most likely result from a similar neuron-to-sheath transformation. Thus, inhibition of proteasome activity can modify two cell fate decisions in this lineage.

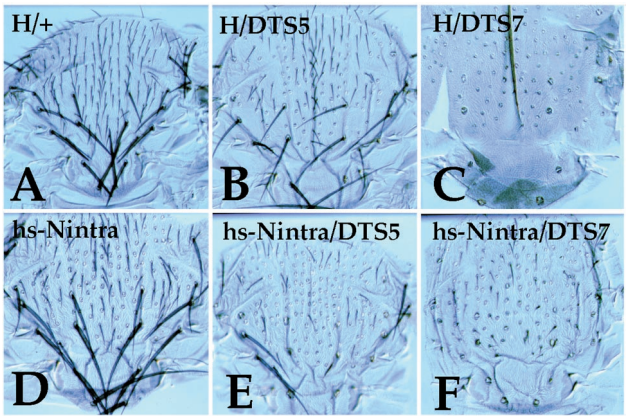

FIG. 3. *DTS5* and *DTS7* mutations enhance Notch signaling. (*A–C*) Cuticular preparations of dorsal thoraces from H^{E31} heterozygous pharate adults. Removing one dose of *Hairless* strongly enhances the effect of the *DTS5* and *DTS7* mutations in pupae heat-pulsed at 29°C during a 0- to 24-hr-APF time period: compare H^{E31}/*DTS5* (*B*) and H^{E31}/*DTS7* (*C*) with control H^{E31}/+ pharate adults (*A*). No interaction is seen at the permissive temperature (not shown). (*D–F*) Cuticular preparations of dorsal thoraces from P[w^+, hs-Nintra]-I4A pharate adults. Wild-type (*D*), *DTS5*/+ and *DTS7*/+ 0-hr-APF pupae, carrying one copy of a hs-Nintra transgene, were placed at 29°C for 24 hr and then heat-shocked at 37°C for 20 min. Expression of Nintra at 24 hr APF leads to a relatively mild double-socket phenotype. The *DTS5* and *DTS7* mutations strongly enhanced the double-socket phenotype produced by Nintra (compare *E* and *F* with D).

Genetic analysis has shown that the dominant-negative effect of *DTS5* is dose-sensitive (3), suggesting that a higher level of expression of the mutant β6 subunit might yield stronger phenotypes. Indeed, sense organs composed of four cells, with three or four Su(H)-positive cells, as well as sense organs composed of only two or three cells that were all Su(H)-positive were observed in pupae carrying 2 copies of the *sca-gal4* driver (data not shown). Similar phenotypes were seen when constitutively activated Notch was expressed in the bristle lineage (M. Gho and F.S., unpublished observations). Hence, increasing the amount of mutant β6 proteasome subunit yielded stronger phenotypes, consistent with the idea that it acts as a "poison subunit" disrupting proteasome activity in a dose-dependent manner.

Dominant-Negative Proteasome Mutations Enhance Loss of *Hairless* Function Phenotype. Neuron-to-sheath and shaft-to-socket fate transformations have previously been described as resulting from a gain in Notch signaling activity (8). To examine whether the *DTS5* and *DTS7* mutations affect Notch signaling, their ability to modify bristle phenotypes associated with gain of Notch signaling was tested.

Hairless (*H*) encodes a potent antagonist of Notch and is thought to act by sequestering Su(H) (19, 20). Heterozygosity at the *H* locus yields a dominant haplo-insufficient phenotype characterized by the appearance of a few double-socket bristles [Fig. 3*A*; on the notum of H^{E31}/+ flies, 2% of the microchaetes (n = 12/480 from four nota) showed a double-socket phenotype]. If decreasing proteasome activity up-regulates Notch signaling, then the bristle phenotype seen in *H* mutant flies should be enhanced by the *DTS5* and *DTS7* mutations. Indeed, a strong synergistic interaction was observed between *H* and *DTS5* or *DTS7* when double heterozygous pupae were heat-pulsed at the restrictive temperature (29°C) from 0 to 24 hr APF [Fig. 3 *A–C*; 58% (n = 259/448 from five nota) and 79% (n = 457/578 from five nota) of the microchaetes of *DTS5* +/+ H^{E31} and *DTS7* +/+ H^{E31} flies, respectively, exhibited a double-socket phenotype]. This genetic interaction cannot be interpreted as a result of a defect in the ubiquitin-dependent degradation of *H* mutant proteins, since a molecularly defined deficiency of *Hairless* (12) was used in this experiment. Thus,

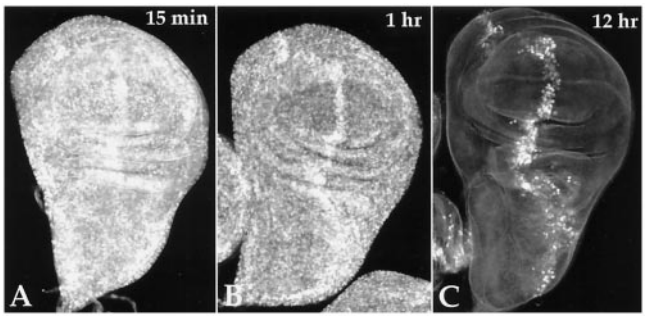

FIG. 4. Stabilization of Nintra by the mutant *DTS5* proteasome subunit. Time-course analysis of Nintra accumulation in *ptc-gal4* hs-Nintra/+; UAS-*DTS5*/+ wing imaginal discs. Fifteen minutes after heat induction, Nintra was detected in all imaginal disc cells (*A*), and gradually disappeared in cells outside the domain of expression of *ptc-gal4* (*B*) 1 hr after heat shock. Twelve hours after heat shock, Nintra was detected only in cells in the *ptc-DTS5*-expressing cells (*C*).

decreasing proteasome activity enhances Notch signaling. This conclusion suggests in turn that a component of the Notch pathway, acting positively to transduce the signal, is present in limiting amount because of its degradation by the proteasome.

Increased Activity and Stability of a Constitutively Activated Form of Notch. A candidate for this unstable component is Notch itself. To test this possibility, I examined the effect of the *DTS5* and *DTS7* mutations on the double-socket phenotype caused by an activated form of Notch, Nintra (13). The expression of Nintra was induced at 24 hr APF by a 20-min pulse at 37°C in pupae placed at 29°C at 0 hr APF. Pupae were then allowed to develop at the permissive temperature (25°C). Expression of Nintra generated a mild double-socket phenotype in wild-type pupae (Fig. 3*D*; see also refs. 19 and 21). This multiple-socket phenotype was enhanced in *DTS5* and *DTS7* pupae (Fig. 3 *E–F*). Furthermore, ectopically expressed Nintra appeared to be more stable in *DTS5*-expressing cells: Nintra was still detectable 6 hr after heat induction in double-socket sense organs in *sca-DTS5* pupae, whereas it was no longer detected in a wild-type background (not shown). Thus, these mutant proteasome subunits appear to enhance the activity of Nintra, possibly by lowering its degradation rate.

To investigate whether Nintra, ectopically expressed with heat shock, was stabilized in cells expressing the *DTS5* mutant proteasome subunit, *DTS5* was expressed in a spatially restricted manner in wing imaginal discs by using a *ptc-gal4* driver. Third-instar *ptc-DTS5* larvae were placed at 29°C for 24 hr and heat-shocked for 20 min at 37°C to induce the ubiquitous expression of Nintra. The time-course accumulation of Nintra was then followed in wing discs of larvae maintained at 29°C. Nintra accumulated in all cells after heat induction. One hour after the heat shock, Nintra accumulation was still high in *DTS5*-expressing cells, but appeared significantly weaker in cells outside the *ptc* expression domain that did not accumulate the *DTS5* mutant subunit. Twelve hours after heat induction, Nintra was detected only in the *DTS5*-expressing cells. Thus, Nintra appears to be rapidly degraded in all cells except in those expressing the *DTS5* mutant subunit (Fig. 4 *B* and *C*). In control *ptc-gal4* larvae, Nintra accumulated and disappeared uniformly after heat-induced expression, and it was no longer detectable 5 hr after heat shock (not shown). This result demonstrates that the stability of Nintra was increased in *DTS5*-expressing cells.

DISCUSSION

In this study, I used two conditional, dominant-negative mutations of the β2 and β6 proteasome catalytic subunit genes to reduce the activity of the ubiquitin-mediated degradation pathway in *Drosophila*. Lowering the level of proteasome activity in all cells at the early pupal stage occasionally resulted in shaft-to-socket cell fate transformations. Targeting the expression of the dominant-negative β6 mutant subunit in otherwise wild-type sense organ cells led to neuron-to-sheath and shaft-to-socket cell fate changes. This finding shows that a wild-type level of proteasome activity is required for the proper regulation of at least two alternative cell fate decisions during sense organ development.

These phenotypes may appear rather specific, given that the turnover of many cellular proteins is presumably reduced in cells expressing these mutant subunits, thereby altering many important cell processes. In these experiments, specificity arises in part from the conditional inactivation of proteasome function either at a particular stage or in specific cells. Also, it is important to note that proteasome activity appears to be reduced but not abolished in the mutant backgrounds studied here. Thus, the loss-of-function allele of the β6 proteasome subunit gene leads to nonconditional recessive lethality in first-instar larvae (3). The synthetic lethality observed between *DTS7* and *DTS5* (or *sca-DTS5*) at 18°C indicates that further decreasing the level of proteasome activity in *DTS5*/*DTS7* or *sca-DTS5*/*DTS7* trans-heterozygous individuals led to lethality before the third-instar larval stage even at 18°C. Therefore, the experimental strategy employed here may reveal only the cell processes that are most sensitive to a mild reduction in proteasome activity. This is in essence similar to the effect of mutations in the *Drosophila fat facet* gene. This gene encodes a ubiquitously expressed deubiquitinating enzyme that displays a highly specific phenotype in the eye. The *faf* eye phenotype can be suppressed by a reduction in proteasome activity (22). It will be of interest to examine whether, conversely, a reduction in *faf* activity can suppress the bristle defect associated with decreased proteasome activity.

The neuron-to-sheath and shaft-to-socket cell fate transformations, as well as the genetic interactions between Notch signaling components and the *DTS5* and *DTS7* mutations, indicate that decreasing proteasome activity enhances Notch signaling activity in the sense organ lineage. Notch signaling appears to involve the ligand-induced processing of the receptor at the membrane, followed by nuclear translocation of a processed intracellular fragment called NICD (23–26). In the nucleus, NICD is thought to act as a transcriptional regulator as part of a complex with Su(H) (27, 28). Therefore, the proteasome may participate in the degradation of a signal transduction component, such as Su(H) and/or NICD. Increased accumulation of Su(H) has previously been shown to also result in shaft-to-socket cell fate transformations (29). Thus, it is conceivable that a reduction in proteasome activity may lead to the stabilization of Su(H), resulting in double-socket bristles. However, accumulation of Su(H) in socket cells was found to be lower in the multiple socket cells of *sca-DTS5* pupae than that observed in socket cells of wild-type pupae (not shown). Hence, accumulation of Su(H) does not appear to correlate with shaft-to-socket fate transformation. Furthermore, a role for Su(H) in specifying the neuron/sheath decision has not yet been established (29–31). Together, these observations suggest that Su(H) may not be responsible for the neuron-to-sheath and shaft-to-socket cell fate transformations resulting from a loss of proteasome activity. Alternatively, the proteasome might act in a indirect manner to activate an antagonist of Notch signaling, like Numb and Hairless. For instance, an unstable inhibitor of Hairless or Numb might be degraded by the proteasome. However, no such inhibitors have been characterized to date. Finally, the stabilization of NICD might be responsible for the bristle phenotypes seen in the *DTS5* and *DTS7* mutant flies. Consistent with this possibility, I found that an activated form of Notch, Nintra, was stabilized in *DTS5*-expressing cells. This finding shows that the intracellular domain of Notch is a direct or indirect target of the proteasome. Whether Notch, or an activated form of Notch

such as NICD, is ubiquitinated and directly degraded by the proteasome will require additional biochemical experiments.

The model of the intracellular processing of Notch predicts that immunoreactivity against the intracellular part of Notch should be detectable in the nucleus after receptor activation. However, endogenous nuclear Notch has not yet been observed in Notch-activated cells by immunodetection methods. This negative result has been interpreted to mean that NICD accumulates to levels below immunodetection thresholds. In support of this interpretation, transfection studies have indicated that the minimal amount of nuclear Notch sufficient to activate a target gene is too small to be detected by standard immunocytochemistry (26). Consistent with this postulated low level of nuclear accumulation, the presence of a PEST sequence at its C terminus suggests that nuclear Notch may turn over rapidly. However, endogenous Notch immunoreactivity was not detected in the nucleus of *ptc-DTS5-* or *sca-DTS5*-expressing cells at the restrictive temperature (not shown), indicating that inhibition of protein degradation is not sufficient to allow for the accumulation of detectable amount of NICD in the nucleus.

Although there is, as yet, no direct evidence for the ligand-induced ubiquitination of processed Notch receptors, studies in nematodes suggest that the effect of the proteasome on Notch could be direct. Sel-10, a putative component of an E3 complex that might be involved in target recognition for ubiquitination, has been shown to down-regulate Lin-12 signaling in *Caenorhabditis elegans* and to bind to the intracellular parts of nematode Lin-12 and human Notch-3 (32). The sequence conservation of the *sel-10* genes *C. elegans*, *Drosophila* (Berkeley Drosophila Genome project/HHMI EST project, unpublished data) and humans (32) further suggests that the regulation of Lin-12/Notch signaling by ubiquitin-dependent degradation of the processed receptors may be evolutionarily conserved. An attractive hypothesis is that the proteolytic degradation of activated Notch is required to switch off Notch signal transduction.

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