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Targeted Expression of Dominant Negative Proteasome Mutants in *Drosophila melanogaster*

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The major pathway for regulated protein degradation in eukaryotes is the ubiquitin/proteasome pathway (for review, see Hershko and Ciechanover, 1998). One function of this pathway is to protect cells against the harmful effects of abnormal proteins that can arise as the result of mutation or postsynthetic damage. A second important role is in mediating the regulated turnover of normal proteins. It is through this latter function that the ubiquitin/proteasome pathway plays key regulatory roles in many diverse biological processes.

The proteolytic machinery of this pathway is a large, multisubunit complex known as the 26S proteasome (Baumeister *et al.*, 1998). This complex is made up of two components: a 20S core particle composed of four stacked heptameric rings and 19S regulatory complexes capping each end. It is within the 20S particle that proteolysis of the substrate proteins takes place, while the 19S caps act to capture and unfold the ubiquitin-tagged substrates and translocate them into the inner degradative chamber.

Proteasome-mediated protein degradation has been implicated in many biological processes, including cell fate determination (Huang *et al.*, 1995; Schweisguth, 1999), circadian rhythms (Naidoo *et al.*, 1999), cell cycle progression (Hoyt, 1997), long-term memory (Chain *et al.*, 1999), metabolic regulation (Hampton and Bhakta, 1997), and programmed cell death (Jesenberger and Jentsch, 2002). One approach to investigating the role of the ubiquitin/proteasome pathway in specific processes is to use proteasome inhibitors to interfere with proteasome function and then to assess the phenotypic effects (Myung *et al.*, 2001). One drawback to this approach in intact animals is that it is not possible to target the delivery of the inhibitor only to cells of interest. Another problem is that many proteasome inhibitors lack complete specificity and side effects can be elicited, complicating the analysis. As an alternative approach to using exogenous proteasome inhibitors, we have taken advantage of the UAS/GAL4 binary system (Brand and Perrimon, 1993) to drive targeted expression of temperature-sensitive dominant negative proteasome mutants.

The two mutants used in this system were originally identified as dominant temperature-sensitive (DTS) mutants of unknown function, termed *DTS5* and *DTS7* (Holden and Suzuki, 1973). Subsequent studies (Saville and Belote, 1993; Smyth and Belote, 1999) revealed that *DTS5* and *DTS7* were missense mutations in the 20S proteasome subunits, β6 and β2, respectively, and the mutants were renamed *Pros26*^{1} and *Prosbeta2*^{1} to conform to standard nomenclature. The phenotypic effects of each mutant are similar, with heterozygotes raised at 29° dying during the pupal stage with numerous defects, including reduced imaginal disc derivatives, missing abdominal structures, and failure of head eversion. These mutants show a strong genetic interaction in that the double heterozygotes are lethal at all temperatures and display a much earlier lethal period, dying as young larvae (Smyth and Belote, 1999). Genetic and biochemical evidence suggests that each of these mutants act in a dominant negative manner to interfere with normal proteasome function (Huang *et al.*, 1995; Schweisguth, 1999; Covi *et al.*, 1999; K. Vitale and J. Belote, unpublished).

The *Pros26*^{1} genomic region was cloned from a recombinant λ phage library constructed from *Pros26*^{1}*/Df(3L)st*^{j7} mutant larvae (Saville and Belote, 1993) and placed downstream of the GAL4-responsive sequences in pUAST to give pP{w^{+}, UAS-Pros26^{1}} (Fig. 1a). The *Prosbeta2*^{1} mutant gene was PCR-amplified from *Prosbeta2*^{1}*/Df(3L)fz-M21* mutant larvae and cloned into pUAST, yielding pP{w^{+},UAS-Prosbeta2^{1}} (Fig. 1b). Both plasmids were sequenced to confirm that they were correct and transgenic lines created by injecting DNA into a host w^{1118} strain using standard procedures. Table 1 lists the lines obtained and the chromosomal linkage of the transgenes. An additional line containing both types of transgenes was generated by crossing lines P{w^{+},UAS-Pros26^{1}}2B and P{w^{+},UAS-Prosbeta2^{1}}1B.

To confirm that the UAS transgenes were functional, each line was crossed to the P{GAL4-ninaE.GMR} line that expresses GAL4 in an eye-specific manner (Freeman, 1997). Figure 2 shows the phenotypic effects. Expressing either of the dominant temperature-sensitive

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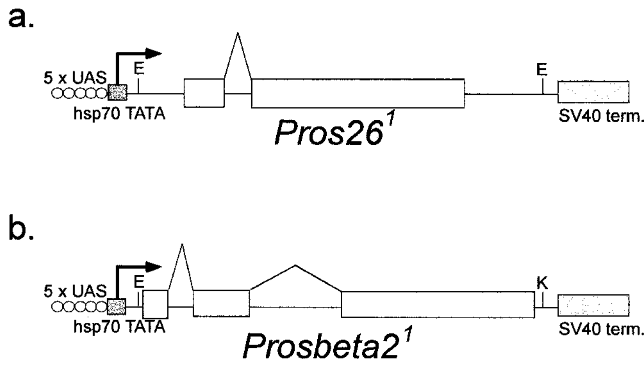

FIG. 1. UAS constructs driving expression of dominant temperature-sensitive proteasome mutant genes. **a:** A 1.2 *Bam*HI-*Eco*RI genomic fragment of *Pros26*[1] containing the entire coding region, 120 bp of DNA upstream of the translation start site and 324 bp of DNA downstream of the stop codon was made blunt-ended and subcloned into the *Sma*I site of the p34H vector (Tsang *et al*., 1991). The insert was then cut out with *Eco*R1 and ligated into the *Eco*RI site of pUAST (Brand and Perrimon, 1993) to create pP{w^+, UAS-Pros26[1]}. **b:** The *Prosbeta2*[1] mutant gene was PCR-amplified and ligated into the *Eco*RV site of pBlueScript KS (Stratagene, La Jolla, CA) using the TA cloning method (Zhou *et al*., 1995). The insert was then cut out with *Eco*RI-*Kpn*I double digestion and cloned into the *Eco*RI-*Kpn*I sites of pUAST to give pP{w^+,UAS-Prosbeta2[1]}. The pentamer of Upstream Activating Sequences (circles), the hsp70 TATA box, and the SV40 transcription terminator sequence (gray boxes) are from the pUAST vector. E, *Eco*RI; K, *Kpn*I.

Table 1
Transgenic UAS Lines With Dominant Temperature-Sensitive Proteasome Mutants

Transgenic line	Chromosome linkage
P{UAS-Pros26[1]}2B	2
P{UAS-Pros26[1]}}4A	X
P{UAS-Pros26[1]}6A	3
P{UAS-Pros26[1]}11A	X
P{UAS-Prosbeta2[1]}1B	3
P{UAS-Prosbeta2[1]}2B	3
P{UAS-Prosbeta2[1]}3B	3
P{UAS-Prosbeta2[1]}1B,4A*	3

*This line carries two P{UAS-Prosbeta2[1]} transgenes inserted into chromosome 3.

proteasome genes in the eyes leads to severely disrupted eye development, with fused facets and small spots of black, presumably necrotic, cells (Fig. 2b,c). Expressing both of the mutants at once greatly exacerbates this defect, with significant patches of necrotic tissue often occurring along the anterior edge of the eye (Fig. 2d). As expected from the temperature-sensitive nature of both proteasome mutants, the defects are severe only when the flies are reared at 29° (compare 2d,e). Since flies heterozygous for the P{GAL4-ninaE.GMR} driver have been known to exhibit a rough eye phenotype when raised at 29° (Freeman, 1997), we used as a control flies carrying P{w^{+mC}, GAL4-ninaE.GMR}12 along with a P{UAS-lacz} transgene (Fig. 2a) to confirm that overexpression of any protein in the eye at this temperature would not produce the dramatic effects seen with the proteasome mutant lines. Most of the lines in Table 1 produced effects similar to those shown in Figure 2, although the P{w^+,UAS-Pros26[1]}6A and P{w^+,UAS-Pros26[1]}11A lines showed a much weaker phenotypic effect. In another experiment, the constitutively expressed P{w^{+mC}, Actin5C-GAL4}17bF01 transgene was used to drive expression of P{w^+,UAS-Pros26[1]}1B and/or P{w^+,UAS-Prosbeta2[1]}2B. There was little if any effect at 22° but at 29° there was larval lethality.

While we have not directly shown here that the effects we see are the result of proteasome dysfunction, it is likely this is the case based on experiments carried out by Schweisguth (1999). In those experiments, transgenic lines were created carrying a construct called P{w^+, UAS-DTS5} that was similar to P{w^+, UAS-Pros26[1]} described here, and it was demonstrated using immunocytochemistry that targeted expression of this mutant proteasome subunit does inhibit degradation of an active form of Notch, a natural target of the ubiquitin/proteasome pathway. Since our results with P{w^+, UAS-Prosbeta2[1]} are virtually identical to what we see with P{w^+, UAS-Pros26[1]}, it is likely that expressing the dominant temperature-sensitive Prosbeta2[1] subunit also inhibits proteasome function.

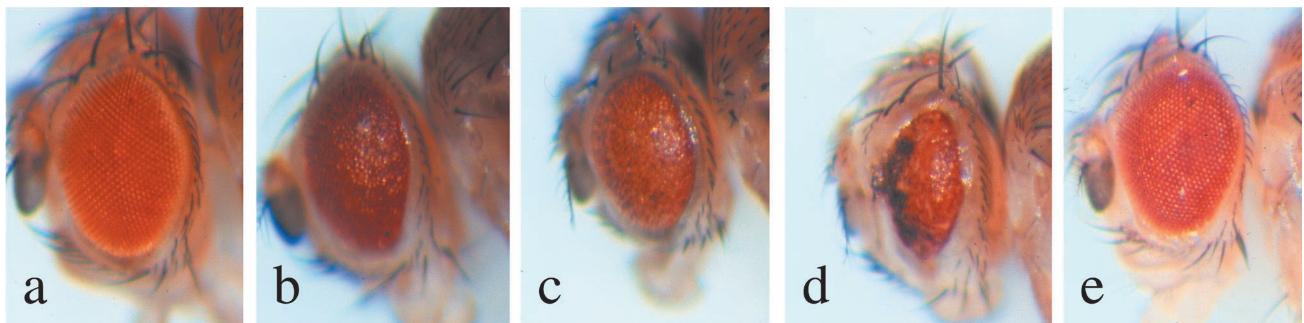

FIG. 2. Phenotypic effects of targeted eye-specific expression of dominant temperature-sensitive proteasome mutants. All flies are homozygous for w^{118} and heterozygous for the eye specific GAL4 driver, P{w^{+mC}, GAL4-ninaE.GMR}12, plus either (**a**) P{w^+,UAS-lacz}, (**b**) P{w^+,UAS-Prosbeta2[1]}1B, (**c**) P{w^+,UAS-Pros26[1]}2B, or (**d,e**) both P{w^+,UAS-Pros26[1]}2B and P{w^+,UAS-Prosbeta2[1]}1B. Flies shown in (**a–d**) were raised at 29°, while the fly shown in (**e**) was raised at 22°.

We believe the lines described here will be very useful for disrupting proteasome function in a targeted manner. The fact that this effect is cleanly temperature-sensitive means that one can not only target the effect spatially, using tissue-specific GAL4 lines, but one might also be able to direct the effect to specific stages of development using temperature-shift regimes. Moreover, the strength of the effect can also be manipulated by using either one or both of the dominant proteasome mutant transgenes in crosses with the desired GAL4 line.

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