Loss of tramtrack gene activity results in ectopic R7 cell formation, even in a sina mutant background

(Drosophila / eye development / Ras pathway / Sevenless)

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ABSTRACT We have screened a collection of transposable-element-induced mutations for those which dominantly modify the extra R7 phenotype of a hypomorphic yan mutation. The members of one of the identified complementation groups correspond to disruptions of the tramtrack (ttk) gene. As heterozygotes, ttk alleles increase the percentage of R7 cells in yan mutant eyes. Just as yan mutations increase ectopic R7 cell formation, homozygous ttk mutant eye clones also contain supernumerary R7 cells. However, in contrast to yan, the formation of these cells in ttk mutant eye tissue is not necessarily dependent on the activity of the sina gene. Furthermore, although yan mutations dominantly interact with mutations in the Ras1, Draf, Door1, and rolled (rl) genes to influence R7 cell development, ttk mutations only interact with yan and rl gene mutations to affect this signaling pathway. Our data suggest that yan and ttk both function to repress inappropriate R7 cell development but that their mechanisms of action differ. In particular, TTK activity appears to be autonomously required to regulate a sina-independent mechanism of R7 determination.

Determination of the R7 photoreceptor cell fate in the adult Drosophila eye is one of the best understood intercellular signaling events and serves as an excellent model system for understanding the nature and components of a physiological signal transduction process (reviewed in refs. 1 and 2). An important signal that instructs the R7 cell of its fate comes from an adjacent predetermined photoreceptor cell, R8. R8 expresses a cell surface ligand, BOSS, which contacts and activates the Sevenless (SEV) tyrosine kinase receptor on the surface of R7. Activation of SEV leads to an accumulation of the 88-kDa protein (TTKp88). Both proteins contain C-terminal Cys2His2 zinc-finger motifs and are found in the nucleus (10–12). Animals homozygous for a hypomorphic mutation (ttk1) that removes primarily the TTKp88-encoding transcript are viable, and the adult eyes contain supernumerary R7 cells. In contrast, a mutation that disrupts expression of both ttk transcripts (ttk+e11) is embryonic lethal, and attempts to generate mutant clones in the adult eye result in the formation of scars of disrupted eye tissue (9).

In this paper, we present evidence that although yan and ttk mutations both act to repress inappropriate R7 cell determination, their mechanisms of action differ. In yan mutants, the presence of supernumerary R7 cells depends on SINA function. However, in ttk mutants, supernumerary R7 cells form even when SINA activity is severely reduced. This is the only case in which significant numbers of R7 cells have been seen to develop in a sina mutant background.

MATERIALS AND METHODS

Genetics. P-element-induced lethal mutations on autosomes (950) (13) were individually used to generate yan+/yan; +/yan or yan+/yan; +/+ flies (* represents a P-element-induced lethal mutation) using standard genetic cross and recombination methods (14). The dominant effects of these P elements on the yan rough eye phenotype were initially examined under a dissecting microscope. The ttk60219 and ttk6730 alleles used in this study exhibit recessive embryonic lethality. Reversion (ttk62) and imprecise excision alleles (ttk34) of ttk6730 were produced using standard genetic methods. The ttk1 (viable) and ttk+e11 (embryonic lethal) alleles were kindly provided by Craig Montell (Johns Hopkins University School of Medicine), and Drafi0374 and sE-raf+ were generous gifts of Norbert Perrimon (Harvard Medical School) and Ernst Hafen (Universität Zürich), respectively. The ttk60219 mutation was recombined with sina3 or sina2 mutations. ttk60219 homozygous mutant cells were generated in wild-type, sev5, or sina3/sina2 mutant backgrounds by inducing mitotic recombination with x- or y-irradiation [1000 rads (1 rad = 0.01 Gy) in first-instar larvae. The transposon P[w+90E is located at polytene chromosome position 90E (15) and was used as a cell autonomous marker. ttk6730 clones were generated only at very low effi-

Abbreviations: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; FLP, FLP recombinase; FRT, FLP recombination target; Rh3 and Rh4, rhodopsins 3 and 4; CAT, chloramphenicol acetyltransferase.

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ciency using irradiation; consequently, we constructed a strain of genotype w; P[y+; hs-neo, FRT]82B, ttkM730b/TM3 and crossed males to w, hsFLP1; P[y+; hs-neo, FRT]82B, P[mini-
w+, hsπM]87E female flies to generate progeny of the genotype w, hsFLP1/w; P[y+; hs-neo, FRT]82B, P[mini-w+, hsπM]87E/P[y+; hs-neo, FRT]82B, ttkM730, ttkM730b mutant clones were generated as described (15, 16).

Histology and Immunohistochemistry. Adult eye fixation was performed and plastic sections were prepared as described (17, 18). Scanning electron microscopy was performed as described (19), except that samples were prepared by critical-point drying rather than by use of Freon. Embryos and eye discs were prepared and stained as described (7). Anti-

TTKp88 antibody was generously provided by Doug Read (Columbia University).

Assay of Rh3-CAT and Rh4-CAT Activity. The Rh3-CAT (P[Rh3-247CAT]2) and Rh4-CAT (P[Rh4-1900CAT]4) reporter constructs, which contain rhodopsin gene promoters, are both located on the X chromosome and were described previously (20). The chloramphenicol acetyltransferase (CAT) assay was also previously described (21). Extracts from 20 fly heads were used for each Rh3-CAT assay, and extracts from 10 fly heads were used for each Rh4-CAT assay.

RESULTS
yan Mutant Phenotypes Are Enhanced by Mutations in the ttk Gene. The percentage of extra R7 cells in the hypomorphic yan" mutation is very sensitive to the presence of secondary mutations (7). Such mutations are potentially disruptions of genes that encode proteins that function with YAN in the regulation of signal transduction. To identify such proteins, we screened collections of P-element-transposon-induced, recessive lethal mutations for those that dominantly modify the eye roughness of yan". From the 950 independent lines (13) examined, we identified enhancer mutations in two loci that increase the proportion of ommatidia that contain extra R7 cells. In this report, we will focus on the analysis of one locus, E(yan)100D. Flies that are heterozygous for E(yan)100D lose function of the previously described hypomorphic ttk allele, ttk1, are dominantly enhanced by a null allele of yan, yanXE18 (Table 1). Thus, a reduction in the activity of either TTK or YAN enhances the extra R7 phenotype caused by low levels of the other. This effect is synergistic and not additive, as heterozygous ttk or yan mutations have no effect on their own.

The ttk Gene Is Autonomously Required for the Suppression of Aberrant R7 Cell Development. Both ttk0219 and ttkM730 alleles are lethal to homozygotes; so, to examine ttk function in eye development, we have generated clones of cells that are homozygous for these mutations, within an otherwise heterozygous eye. These clones were generated using both x-ray-induced mitotic recombination and the FLP recombinase/FLP recombination target (FLP/FRT) recombination system using a white pigment gene marker on the right arm of the third chromosome (15, 16). The clones could be distinguished from the rest of the tissue by virtue of their lack of eye pigment (15, 16). In patches that were homozygous for ttk0219, we observed that ~67% of ommatidia were aberrant. These ommatidia contained extra R7 cells (an average of 2.2 R7 cells per ommatidium, for all ommatidia within the clone, n = 92; Fig. 1A) and occasionally lack outer photoreceptor cells (an aver-

Table 1. The percentage of ommatidia in eyes of flies of the given genotype that show the designated defects.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number scored</th>
<th>Percentage of ommatidia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With extra R7 cells</td>
</tr>
<tr>
<td>yan/+ yan&quot; ; +/+</td>
<td>1711</td>
<td>22.0</td>
</tr>
<tr>
<td>yan/+ yan&quot; ; ttk0219/+</td>
<td>1546</td>
<td>64.2</td>
</tr>
<tr>
<td>yan/+ yan&quot; ; ttkM730b/+</td>
<td>1774</td>
<td>67.1</td>
</tr>
<tr>
<td>yan/+ yan&quot; ; ttk24/+</td>
<td>1150</td>
<td>67.0</td>
</tr>
<tr>
<td>yan/+ ttkR2/+</td>
<td>1216</td>
<td>16.6</td>
</tr>
<tr>
<td>yan/+ ttk1/+</td>
<td>1037</td>
<td>52.6</td>
</tr>
<tr>
<td>+/+ ; ttk1 / ttk1</td>
<td>526</td>
<td>89.7</td>
</tr>
<tr>
<td>yanXE18/+ +/+</td>
<td>448</td>
<td>41.5</td>
</tr>
<tr>
<td>+/+ ; ttk1 / ttk1</td>
<td>838</td>
<td>72.2</td>
</tr>
</tbody>
</table>

In this table, the E(yan)100D alleles are designated ttk (see text). ND, not determined.
as above. In 37 ommatidia that were scored (only 13 R8 cells scored), the percentages of genotypically
morphologically normal ommatidia have an average of 0.9 R7 cells (Fig. 1). Mosaic ommatidia were scored for the presence of pigment in their photoreceptors (only 11 of these were scored for pigment in the R8 cell). The
performed in a sev mutant background to block the normal pathway of R7 development. A total of 36 phenotypically normal but genotypically mosaic ommatidia were scored for the presence of pigment in their photoreceptors (only 11 of these were scored for pigment in the R8 cell). The
ommatidial development, mosaic analysis was performed. Because the formation of the extra R7 cells is independent of sev, this analysis was performed in a sev mutant background to block the normal pathway of R7 development. A total of 36 phenotypically normal but genotypically mosaic ommatidia were scored for the presence of pigment in their photoreceptors (only 11 of these were scored for pigment in the R8 cell). The

age of 5.9 R1-R6 cells per ommatidium, n = 92). In contrast to ttk00219, we rarely observed large clones of homozygous ttkM730 tissue. Mitotic recombination events that were induced in first-instar larvae often resulted in the generation of a scar of disrupted tissue, although only 11% of these scars were greater than 20 ommatidia in extent. The scars were highly disrupted, although they did not contain any unpigmented photoreceptors. Xiong and Montell (9) have reported a similar phenotype associated with mitotic recombination of a choromosome bearing the ttk1211 mutation, which is a severe ttk allele lacking both TTKp88 and TTKp69 functions.

We have also observed that animals bearing certain trans-heterozygous combinations of ttk alleles are viable and that their eyes contain extra R7 cells; 4.5% of ttk0267 ttk03540 ommatidia (n = 468) and 12.3% of ttk0267/ttk05123 ommatidia (n = 356) have extra R7 cells. These phenotypes are reminiscent of that in the eyes of flies that are homozygous for ttk4 (9). To determine if the generation of supernumerary R7 cells was dependent on signaling through the SEV receptor tyrosine kinase pathway, we generated clones of ttk00219 mutant cells in sev and sina mutant backgrounds. As is the case for yan and gain-of-function mutations in sev, Ras1, Draf, and rl, R7 cells can still develop in ttk00219 mutant tissue in the absence of sev function; ommatidia within a sevΔ2; ttk00219 clone contain an average of 0.9 R7 cells (n = 48). The resulting R7 cells are always mutant for ttk (Fig. 1B). However, in striking contrast to the phenotypes of other mutations that result in extra R7 cell formation, significant numbers of R7 cells are still formed in ttk mutant tissue even when sina function is severely reduced; ommatidia within a sina2, ttk00219/sina2, ttk00219 clone contain an average of 0.9 R7 cells (n = 70). All but one of the resulting morphologically normal ommatidia have ttk mutant R7 cells (Fig. 1C); the remaining cell was presumably one of the 10% of sina2/sina4 mutant R7 cells that form even in the presence of ttk (ref. 17 and Z.-C.L., unpublished data). The formation of extra R7 cells by other ttk alleles also exhibits sina independence; the number of R7 cells per ommatidium increases from 0.10 in sina2/sina4 mutants to 0.87 in ttk1, sina2/ttk1, sina3 double mutants (Table 2).

The observation of sina-independent R7 cell formation is unprecedented. Consequently, we wished to show that these cells could be identified as R7 cells by criteria other than the small size of their rhabdomeres. Rhodopsins 3 and 4 (Rh3 and Rh4) are expressed only in R7 cells (20), as are synthetic genes in which the reporter gene CAT is regulated by the Rh3 or Rh4 promoters (Rh3-CAT and Rh4-CAT). We generated ttk and sina mutant flies that contain a single copy of one of these reporter elements, and we confirmed that Rh4 and Rh3 promoter activity is substantially reduced in a sina2/sina3 genetic background compared with wild-type promoter activity levels (Table 2). This result is consistent with a loss of R7 cells in these flies. However, in a ttk1, sina2/ttk1, sina3 genetic background, Rh4 and Rh3 promoter activity is restored to about 64% of wild-type levels (Table 2), indicating the presence of sina-independent R7 cells.

Together, our data suggest that a functional ttk gene is autonomously required within a subset of cells to repress their differentiation and that in its absence, some cells may adopt an R7 fate, in spite of a severe reduction in sev- and sina-dependent signaling. Although we observe sina independence of R7 formation in ttk mutants, our experiments show that the reduction of either sev or sina gene activity does, nonetheless, partially reduce the number of R7 cells in a ttk genetic background. To account for these observations, we suggest that the formation of R7 cells in a ttk genetic background is partly influenced by both the normal R7 developmental signals, which are sina dependent, and other signals, which are manifested only in the absence of ttk activity and which are sina independent.

To identify the developmental stage at which TTK function becomes critical, we have observed the determination of R7 cells at early developmental stages. We stained larval and pupal eye discs from ttk1 mutant animals with anti-ELAV antibody (7) to identify cells that are already determined to become photoreceptors, and we have observed some defects in ommatidial development as early as the third-instar larval stage, soon after the normal determination of R7 cell fate (data not shown). However, it is not until the 40-hr pupal stage
Table 2. R7 levels in different genetic backgrounds

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average no. of R7 cells per ommatidium</th>
<th>Rh3-CAT activity, cpm-min⁻¹ per fly</th>
<th>Rh4-CAT activity, cpm-min⁻¹ per fly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.00</td>
<td>3.10 ± 0.10 (n = 3)</td>
<td>6.9 ± 1.0 (n = 3)</td>
</tr>
<tr>
<td>sino²/sina³</td>
<td>0.10 (n = 512)</td>
<td>0.55 ± 0.10 (n = 7)</td>
<td>0.4 ± 0.1 (n = 5)</td>
</tr>
<tr>
<td>ttk¹, sina²/ttk¹, sina³</td>
<td>0.87 (n = 688)</td>
<td>2.00 ± 0.20 (n = 3)</td>
<td>4.4 ± 0.4 (n = 4)</td>
</tr>
<tr>
<td>ttk¹/ttk¹</td>
<td>1.50 (n = 448)</td>
<td>3.10 ± 0.25 (n = 7)</td>
<td>5.0 ± 0.7 (n = 6)</td>
</tr>
</tbody>
</table>

R7 levels are assessed either by counting the average number of R7 cells per ommatidium in plastic sections of adult eyes or by measuring the CAT activity (in cpm-min⁻¹ per fly) in lines that contain a single copy the rhodopsin promoter reporter constructs Prh3-247CAT[2] (designated Rh3-CAT) or Prh4-190CAT[4] (designated Rh4-CAT) on the X chromosome (omitted from the genotypes listed in the table for the sake of clarity). The Rh4 promoter construct is generally more active than the Rh3 promoter construct. In the case of R7 numbers, the wild-type genotype refers to the Canton S strain of flies, whereas for the CAT activity measurements, it refers to the genotype ttk¹/+.

The Interaction of yan and ttk with Mutations in Components of the RAS1/MAPK Pathway. To better understand the function of the ttk and yan genes, we have sought to define how they influence the SEV signal transduction pathway and, in particular, its transmission by RAS1 and the cascade of kinases that include RAF, MAPKK, and MAPK. We have used a genetic approach to this question by testing the genetic interaction of ttk and yan mutants with mutations in the genes encoding the components of the SEV signal transduction pathway. We have observed both the effect on the external eye morphology (Fig. 3) and the R7 cell phenotype in histological sections (data given in the legend of Fig. 3).

Expression of TTK Proteins in the Developing Eye. The ttk¹ allele lacks only expression of the TTKp88-encoding message, whereas the TTKp69 expression levels are normal (9). These observations suggest that inactivation of ttk does not cause ectopic R7 cell determination during larval development, as is the case for yan.

(a) that a significant number of ommatidia contain extra photoreceptors (24% of ommatidia in the most disrupted regions). Even at this stage, the percentage of mutant ommatidia is less than that seen in the adult (41.5%). These findings suggest that inactivation of ttk does not cause ectopic R7 cell determination during larval development. As is the case for yan.

Expression of TTK Proteins in the Developing Eye. The ttk¹ allele lacks only expression of the TTKp88-encoding message, whereas the TTKp69 expression levels are normal (9). These observations suggest that normal repression of R7 development depends at least partly on TTKp88 expression. Our data predict that ttk expression is autonomously required in some cells to prevent them from inappropriately adopting an R7 cell fate. Together, these observations predict that TTKp88 should be expressed in cells with the potential to become R7 cells but that in wild-type animals do not adopt that fate. We have stained eye discs from Canton S wild-type third-instar larvae with anti-TTKp88 antibody (12). The TTKp88 protein is detected in the nuclei of the undifferentiated basal cells and the cone cells (Fig. 2A and B, respectively), first in the anterior and posterior pair and subsequently in the equatorial and polar pair (Fig. 2B). Basal nuclei have the potential to adopt a photoreceptor cell fate, and upon differentiation, they rise apically. No TTKp88 stain is observed in these more apical photoreceptor precursor nuclei, consistent with the notion that TTKp88 is a repressor of photoreceptor determination and that its expression is not compatible with the adoption of this fate. In contrast, TTKp88 expression is high in the cone-cell precursors and may reflect part of a mechanism by which photoreceptor development is blocked in these cells. TTKp69 is also expressed in the cone and glial cells, like TTKp88, although we do not observe expression in the basal nuclei (data not shown).

The Interaction of yan and ttk with Mutations in Components of the RAS1/MAPK Pathway. To better understand the function of the ttk and yan genes, we have sought to define how they influence the SEV signal transduction pathway and, in particular, its transmission by RAS1 and the cascade of kinases that include RAF, MAPKK, and MAPK. We have used a genetic approach to this question by testing the genetic interaction of ttk and yan mutants with mutations in the genes encoding the components of the SEV signal transduction pathway. We have observed both the effect on the external eye morphology (Fig. 3) and the R7 cell phenotype in histological sections (data given in the legend of Fig. 3). Strong yan mutations dominantly enhance the Ras¹(112) phenotype (7), although ttk¹ and ttk¹ show only a mild enhancement of the Ras¹(112)-induced defects (data not shown). yan mutations suppress the rough-eye phenotypes of hypomorphic mutations in Draf (Draf¹[17]; Fig. 3 A and C) and Dsor1 (Dsor¹[X520], ref. 26; Fig. 3 D and F), and they enhance the gain-of-function phenotypes of Draf (sE-raf[tor]; data not shown) and sE-raf[tor] (Fig. 3 G and I). ttk mutations have no significant dominant effect on the rough eyes caused by Draf¹[17] (Fig. 3 A and B), Dsor¹[X520] (Fig. 3 D and E), or sE-raf[tor] (data not shown), although they do enhance the sE-raf[tor] phenotype (Fig. 3 G and H).

Fig. 2. TTKp88 expression in third-instar larval eye imaginal discs. (×500). (A) TTKp88 is detected in the basal nuclei of the retina. (B) TTKp88 is detected in differentiating cone cells, first in the anterior and posterior pair and then in the polar and equatorial pair. The morphogenetic furrow is at the left margin of the panels.
We have shown that extra R7 cells form in the absence of ttk gene products and that even in a sina mutant background most ttk mutant ommatidia contain R7 cells. This result contrasts with our previous finding that the formation of all R7 cells in yan mutants is sina dependent. The sina dependence of the ectopic R7 cells in yan mutants suggests that YAN functions upstream of SINA in the R7 cell determination pathway, perhaps to repress SINA activity. TTK, on the other hand, may function either downstream of SINA or in a parallel signaling pathway to regulate R7 cell fate; our genetic data do not allow us to distinguish between these possibilities.

**DISCUSSION**

We have shown that extra R7 cells form in the absence of ttk gene products and that even in a sina mutant background most ttk mutant ommatidia contain R7 cells. This result contrasts with our previous finding that the formation of all R7 cells in yan mutants is sina dependent. The sina dependence of the ectopic R7 cells in yan mutants suggests that YAN functions upstream of SINA in the R7 cell determination pathway, perhaps to repress SINA activity. TTK, on the other hand, may function either downstream of SINA or in a parallel signaling pathway to regulate R7 cell fate; our genetic data do not allow us to distinguish between these possibilities.
Our findings that mutations in the yan gene genetically interact with Ras1, Draf, Doorl, and rl mutations provide evidence that YAN functions to modulate signaling through the RAS1/MAPK pathway, which ultimately signals R7 cell determination through SINA. It is likely that YAN functions downstream of the kinase cascade, as it is phosphorylated in response to the activation of MAPK in tissue culture cells (24). Furthermore, YAN contains eight consensus phosphorylation sites for MAPK, and in tissue culture cells, the transcriptional repressor activity of YAN is abrogated by cotransfection with activated MAPK (p\text{EWM}) (24). In contrast, although mutations in ttk genetically interact with mutations of yan and rl, they do not significantly dominate the phenotypes of Ras1, Draf, and Doorl mutations. The lack of interactions with the latter mutations could be interpreted as providing evidence that TTK functions in a parallel pathway distinct from that of RAS1. However, this does not necessarily have to be the case. For example, in a screen for dominant modifiers of a hypomorphic sev phenotype, mutations were identified in the genes of components of the signaling pathway that lie within a few steps downstream of SEV (including RAS1) (25). However, no rl mutant alleles were isolated, perhaps reflecting the fact that MAPK acts further downstream in the pathway. Consequently, our interaction data do not rule out the possibility that TTK acts in the SEV pathway, downstream of SINA. This point of action is closer to MAPK and YAN than to RAS1, RAF, and MAPKK.

We have speculated that although yan and ttk genes both function to repress supernumerary R7 cell development, they act at different points in the determination process. Further evidence for this speculation is provided by our investigation of the developmental stage at which ttk activity is required; substantial defects are not apparent in ttk1 animals until the late pupal stage. This finding contrasts with the yan phenotype, which is apparent in third-instar imaginal discs soon after R7 cell determination (7). These data are consistent with a model in which yan negatively regulates the primary R7 determination pathway. Consequently, inactivation of yan is sufficient to activate the pathway. TTK may, on the other hand, prevent R7 development, but its absence may be insufficient to cause ectopic R7 cells to form without additional signals. This may explain the residual sev and sina dependence of some R7 cells in ttk mutants (Fig. 1; Table 1). The apparently sina-independent development of R7 cells in ttk mutants may result from stimulation of another pathway, the principal function of which is unrelated to normal R7 cell fate specification; this pathway functions later in eye development but, in the absence of TTK, is capable of causing R7 development. The normal function of TTK may be, in part, to repress this inappropriate signaling.

Mutations in yan and ttk do not simply increase the number of R7 cells. In both mutations, ommatidia that contain extra outer photoreceptor cells (R1–R6) are seen, and in ttk1 mutant animals, this phenotype is dominantly enhanced by a yan null allele (Table 1). These results may point to a more general role for ttk and yan in the suppression of inappropriate photoreceptor development. Indeed, YAN appears to act as a general repressor of differentiation in several tissues, including the eye (8). Surprisingly, in both mutants some photoreceptors are missing, and in yanrl mutants, this phenotype is dominantly enhanced by strong ttk alleles. We do not understand the basis for this effect, but it is possible that the ttk1 and ttk00219 missing-photoreceptor phenotype reflects partial loss of TTKp69 function, which might be expected to reduce photoreceptor cell numbers.

YAN and TTK are capable of repressing RNA transcription (24, 11), and SINA is a nuclear protein. Furthermore, the nuclear concentration of YAN is decreased upon activation of the SEV pathway (8). Therefore, it seems likely that these proteins interact to regulate the transcription of downstream genes. Clarification of the details of this model awaits an elucidation of the molecular mechanisms that underlie the identified genetic interactions.

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