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Determination of cell fates in the R7 equivalence group of the *Drosophila* eye by the concerted regulation of D-Pax2 and TTK88

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ABSTRACT

In the developing *Drosophila* eye, the precursors of the neuronal photoreceptor cells R1/R6/R7 and non-neuronal cone cells share the same developmental potential and constitute the R7 equivalence group. It is not clear how cells of this group elaborate their distinct fates. Here we show that both TTK88 and D-Pax2 play decisive roles in cone cell development and act in concert to transform developing R1/R6/R7 into cone cells: while TTK88 blocks neuronal development, D-Pax2 promotes cone cell specification. In addition, ectopic TTK88 in R cells induces apoptosis, which is suppressed by ectopic D-Pax2. We further demonstrate that Phyllopod (Phyl), previously shown to promote the neuronal fate in R1/R6/R7 by targeting TTK for degradation, also inhibits *D-Pax2* transcription to prevent cone cell specification. Thus, the fates of R1/R6/R7 and cone cells are determined by a dual mechanism that coordinately activates one fate while inhibiting the other.

Introduction

The *Drosophila* compound eye is an excellent system to study the specification of different cell types and their integration into a highly specialized organ. It is composed of about 750 hexagonally arranged unit eyes or ommatidia. Each ommatidium consists of eight neuronal photoreceptor or R cells (R1-R8), four lens-secreting cone cells, and two primary pigment cells as well as shared secondary and tertiary pigment cells (Cagan and Ready, 1989; Ready et al., 1976; Wolff and Ready, 1993). Ommatidia begin to develop in the morphogenetic furrow, which is induced at the posterior end of the eye disc during mid third larval instar and sweeps across the disc until it reaches the anterior end during early pupal stages (Wolff and Ready, 1993). All ommatidial cells are sequentially recruited and determined, while the potential fates of the non-recruited cells are progressively reduced (Freeman, 1997; Greenwald and Rubin, 1992; Lawrence and Green, 1979; Ready et al., 1976; Tomlinson and Ready, 1987). First, the R8 founders of each ommatidium are singled out from proneural clusters in the morphogenetic furrow by a mechanism that provides through its regular spacing the basis for the hexagonal array of the ommatidia (Baker et al., 1990; Jarman et al., 1994; Tomlinson and Ready, 1987). All other ommatidial cells join each developing ommatidium through sequential recruitments within and behind the morphogenetic furrow: first R2/R5, followed by R3/R4, R1/R6, R7, the four cone cells, and finally the three types of pigment cells. After the recruitment of R3/R4, a round of mitosis, the so-called second mitotic wave (Wolff and Ready, 1993), occurs in hitherto unrecruited cells immediately behind the morphogenetic furrow.

The cells emerging from the second mitotic wave, from which the precursors of R1/R6/R7 and cone cells are recruited, share a common developmental potential and form the so-called "R7 equivalence group" (Chang et al., 1995; Crew et al., 1997; Dickson et al., 1995; Greenwald and Rubin, 1992). These cells may become R1/R6, R7, or cone cells (Hiromi et al., 1993): in the presence of the transcription factor Seven-up (Svp) and high activity of the Ras signaling pathway, these cells assume the fate of the outer photoreceptor cells R1/R6, while in the absence of Svp they become R7 and cone cells at high and low levels of Ras signaling, respectively (Begemann et al., 1995; Kramer et al., 1995). It seems important to note that the cells of the "R7 equivalence group" might not be equivalent in a strict sense, as their positions with regard to the previously recruited R cells might suffice to restrict their developmental potential within the group (Freeman, 1997; Wolff and Ready, 1993). Nevertheless, we will use the term "R7 equivalence group" to designate the group of cells from which the precursors of R1/R6/R7 and cone cells are derived.

For each cell of an equivalence group, its developmental history, which it shares with all other members of the group, and its interactions with neighboring cells play crucial roles in cell fate specification. The integration of multiple extracellular signals with intracellular factors specific for the cell group produces a spatially and temporally regulated developmental response, the expression of cell

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type-specific factors, which function coordinately to elaborate distinct cell fates (Cornell and Kimelman, 1994; Freeman, 1997; Greenwald and Rubin, 1992; Voas and Rebay, 2004). A number of cell type-specific factors have been identified in *Drosophila* eye development (Kumar and Moses, 1997; Voas and Rebay, 2004). Yet, it is still unclear how these factors function to produce particular cell fates.

D-Pax2, also known as Sparkling (Spa) or Shaven (Sv), is a transcription factor with a DNA-binding paired-domain and the closest *Drosophila* homolog of the vertebrate Pax2/5/8 subfamily (Fu and Noll, 1997). In developing ommatidia, it is expressed specifically in cone cells and primary pigment cells (Fu and Noll, 1997). Its expression in cone cells is regulated in a combinatorial fashion (Flores et al., 2000) by Lozenge (Lz), a Runt domain-containing transcription factor (Daga et al., 1996), Suppressor of Hairless [Su(H)], the major downstream effector of Notch (N) signaling (Artavanis-Tsakonas et al., 1999), and the two ETS domain proteins, Yan and Pointed P2 (PntP2), nuclear effectors of EGFR signaling (O'Neill et al., 1994; Raabe, 2000). However, the specific function of D-Pax2 in cone cell development is not known. Although expression of Lz, Su(H), and activation of the Ras/MAPK pathway are sufficient to activate D-Pax2 ectopically in the R7 precursor (Flores et al., 2000), it is not known whether ectopic D-Pax2 is also sufficient to transform R7 into a cone cell.

TTK88, the 88 kDa isoform of Tramtrack, a BTB/POZ (Broad complex Tramtrack Bric-a-brac/Pox virus and Zinc finger) domain-containing protein (Albagli et al., 1995; Harrison and Travers, 1990; Read et al., 1992) is another cone cell-specific transcriptional regulator (Xiong and Montell, 1993). TTK88, initially detected in the nuclei of the undetermined basal cells, is later restricted to cone cells (Lai et al., 1996). Ectopic TTK88 in R cell precursors represses neuronal differentiation, which suggests that the normal function of TTK88 is to block the R cell fate in developing cone cells (Li et al., 1997; Tang et al., 1997). However, it is not clear whether cone cell specification depends on TTK88.

Phyllopod (Phyl) is required specifically for R1/R6/R7 development (Chang et al., 1995; Dickson et al., 1995). Its mRNA is initially expressed in the undifferentiated cells of the morphogenetic furrow and later confined to the developing R1/R6/R7 cells. Together with Seven in absentia (Sina), a RING finger domain protein (Carthew and Rubin, 1990; Saurin et al., 1996), and the F-box protein Ebi (Boulton et al., 2000), Phyl forms an E3 ubiquitin ligase complex that targets TTK88 for degradation through a ubiquitin–proteasome pathway to counteract the inhibition by TTK88 of R1/R6/R7 development (Dong et al., 1999; Li et al., 1997; Li et al., 2002; Tang et al., 1997). Loss of *phyl* function results in the transformation of R1/R6/R7 into supernumerary cone cells, whereas ectopic Phyl in cone cell precursors transforms these into additional R7 cells (Chang et al., 1995; Dickson et al., 1995). However, it remains unclear whether failure to down-regulate TTK88 in developing R1/R6/R7 cells is sufficient for their transformation into cone cells and whether down-regulation of TTK88 in cone cell precursors is sufficient for them to adopt the R7 fate.

Here we demonstrate that both TTK88 and D-Pax2 function in normal cone cell development and that inhibition of the R cell fate by TTK88 as well as promotion of the cone cell fate by D-Pax2 are necessary for efficient transformation of R1/R6/R7 cells into cone cells. We further show that, in addition to promoting R1/R6/R7 development by down-regulating TTK88, Phyl blocks the cone cell fate by down-regulating D-Pax2 in these R cells. Therefore, it is a dual mechanism, coordinately activating one fate while inhibiting the other, that determines the fates of the neuronal R1/R6/R7 cells and non-neuronal cone cells.

Materials and methods

Genetics

The following fly stocks were used: *spa*^{pol} (Fu and Noll, 1997; Lindsley and Zimm, 1992), *ttk*^{1} (Xiong and Montell, 1993), *GMR-ttk88* (Tang et al., 1997), *UAS-ttk88* (Giesen et al., 1997), *sev-Gal4* (Ruberte et al., 1995), *phyl*^{2}, *phyl*^{4}, *2sev-phyl* (Dickson et al., 1995), and *sina*^{3} (Carthew and Rubin, 1990). Despite extensive outcrossing of the *ttk*^{1} stock, the identity of which we confirmed by analysis of its DNA, it no longer shows a large fraction, but less than 1%, of ommatidia with supernumerary R7 cells (Fig. 1H). A possible explanation might be that in the original stock a mutation closely coupled with *ttk*^{1}, but later lost, was responsible for the large fraction ($\approx$ 40%) of ommatidia with supernumerary R7 cells and reduced number ($\approx$ 20%) of outer photoreceptor cells (Lai et al., 1996; Xiong and Montell, 1993).

Constructs used for germline transformations

A 2.8 kb KpnI–XbaI fragment obtained from the *D-Pax2* cDNA, cpx1, which includes the entire open reading frame but none of the multiple polyA addition sites (Fu and Noll, 1997), was inserted into the KpnI/XbaI cloning sites of the P-element vector pSEV (Dickson et al., 1995) to generate the *sev*-D-Pax2 construct. To obtain the *GMR*-D-Pax2 construct, a 2.8 kb EcoRI–XbaI fragment of cpx1, the XbaI site of which had been filled up, was inserted into the EcoRI/StuI cloning sites of the P-element vector pGMR (Hay et al., 1994). *spa*-PC-LacZ was constructed by replacing most of the *Pax2* DNA in *spa*-PCG2 – which consists of a nearly full-length *D-Pax2*-cDNA, whose 5′ end had been extended by about 330 bp upstream of the transcriptional start site to include the promoter and whose 3′ portion downstream of the EcoRV site of exon 9 had been replaced by the corresponding 5.1 kb EcoRV–EcoRI genomic fragment to allow the production of all alternatively spliced mRNAs, cloned into the P-element vector pW6 (Fu and Noll, 1997; Fu et al., 1998) – with the coding region of the *E. coli lacZ* gene. The replaced part of the *Pax2* DNA started in the third exon immediately behind the ATG codon and ended at an EcoRI site 578 bp upstream of the 3′ end of the genomic *Pax2* DNA. The *spa*-PC-LacZ construct thus retained the most downstream polyA addition site of *D-Pax2* (Fu and Noll, 1997). The *spa*-lacZ construct was prepared by subcloning the 926 bp eye-specific enhancer of *D-Pax2* (Fu and Noll, 1997) into the polylinker adjacent to the promoter of *spa*-PC-LacZ. Transgenic lines of all constructs were obtained by P-element-mediated germline transformation according to standard procedures. 13 lines of *sev-D-Pax2*, 8 lines of *GMR-D-Pax2*, and 10 lines of *spa-lacZ* were isolated. Flies carrying one copy of the *GMR-D-Pax2* transgene show a similar and strong rough eye phenotype in all lines (data not shown), while only 2 of the 13 *sev-D-Pax2* lines show a weak rough eye phenotype with one copy, and 9 of the remaining 11 lines show a weak rough eye phenotype when present as 2 copies (data not shown).

In situ hybridization to third instar larval eye discs

In situ hybridization to third instar eye discs with a digoxygenin (DIG)-labeled *D-Pax2* antisense RNA probe was carried out as described (Tautz and Pfeifle, 1989). *D-Pax2* antisense RNA probe was prepared from a nearly full-length *D-Pax2*-cDNA (Fu and Noll, 1997) by the use of the DIG RNA Labeling Kit (SP6/T7) from Roche.

Immunohistochemistry

Late third instar eye discs were prepared and immunostained as described (Gaul et al., 1992). Rabbit anti-D-Pax2 antiserum was used as described (Fu and Noll, 1997). Rabbit anti-β-gal antiserum (Cappel) was used at 1:2000 dilution. Mouse anti-Cut MAb (2B10), used at 1:30 dilution, and rat anti-Elav MAb (7E8A10), used at 1:50 dilution, were obtained from the Developmental Studies Hybridoma Bank. Biotinylated secondary antibodies against rabbit IgG (Vector Laboratories, Inc.) were used at 1:300 dilution. For the color reaction, Vectastain ABC Kit (Vector Laboratories, Inc.) was used. Texas red- or FITC-conjugated secondary IgGs against mouse, rat, or rabbit antibodies (Jackson ImmunoResearch Laboratories, Inc.) were used at 1:200 dilution.

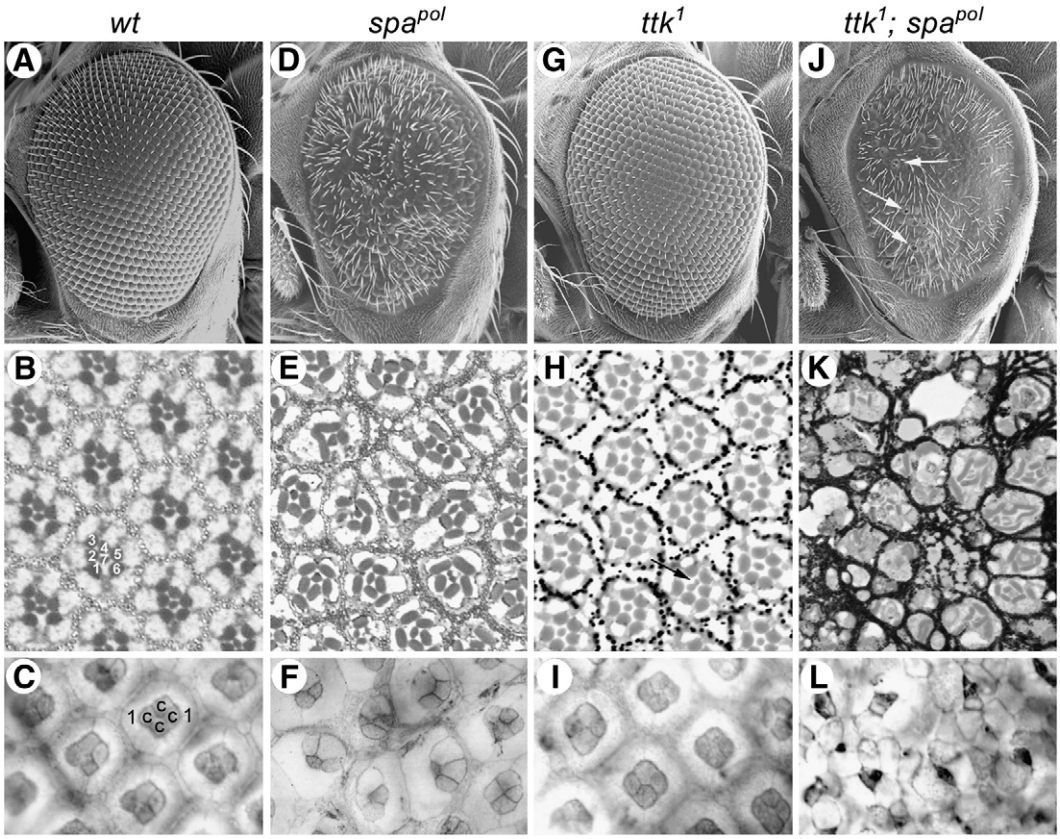

Fig. 1. D-Pax2 and TTK88 are necessary for proper development of cone cells. Scanning electron micrographs of left eyes (A, D, G, J), histological sections through adult retinas (B, E, H, K), and 45-h pupal eye discs stained with cobalt sulfide (C, F, I, L) of wild-type (A–C), spa^{pol} (D–F), ttk^1 (G–I), and ttk^1; spa^{pol} (J–L) flies are shown. The rhabdomeres of R1–R7 of an ommatidium are labeled in B. The four cone cells (c) and two primary pigment cells (1) of an ommatidium are marked in C. A black arrow points to an ommatidium with two R7 cells in H, while white arrows point to ommatidia with open holes at their apices in J.

Scanning electron microscopy and histology

Scanning electron microscopy was carried out by Thomas Gutjahr in our lab. Histological sections of adult fly eyes (Basler et al., 1991) and cobalt sulfide staining of pupal discs (Cagan and Ready, 1989) were performed as described.

To detect apoptotic cells, live discs were incubated on a slide for 5 min in *Drosophila* Ringer's solution containing 1 μg/ml acridine orange (Sigma). The staining solution was removed and the discs were washed three times in *Drosophila* Ringer's before mounting in this solution for microscopy (Neufeld et al., 1998).

Microscopy

Imaging of late third instar larval eye discs by confocal microscopy was carried out with a LEICA TCS SP microscope. Standard photomicrographs were taken with a Hamamatsu color chilled 3CCD camera, mounted on a Zeiss Axiophot microscope.

Results

D-Pax2 is necessary for cone cell development but not sufficient for transformation of R cell precursors into cone cells

During eye development, *D-Pax2* is expressed in the four cone cell precursors (in the following also called cone cells) but not in the photoreceptor cells. In spa^{pol} mutants, transcription of *D-Pax2* is abolished in cone cells because its eye-specific enhancer is deleted (Fu and Noll, 1997). Although all cone cells and photoreceptor cells are present in most ommatidia of these flies, they display a rough eye phenotype resulting from improperly shaped and arranged cone cells (compare Figs. 1D–F with Figs. 1A–C). Therefore, *D-Pax2* is necessary for proper cone cell development (Fu and Noll, 1997).

D-Pax2 is required in newly recruited cone cells for the expression of Cut (Fu and Noll, 1997), a marker specific for cone cells (Blochlinger et al., 1993), which suggests that D-Pax2 is necessary early in cone cell development. To test whether D-Pax2 expression is sufficient to convert R cell precursors into cone cells, we used two transgenes, *GMR-D-Pax2* and *sev-D-Pax2*, which express D-Pax2 ectopically in the eye disc under control of the *Glass Multimer Reporter* (*GMR*) enhancer and the *sevenless* (*sev*) enhancer, respectively. The *GMR* enhancer drives expression in all cells in and posterior to the morphogenetic furrow (Hay et al., 1994; Tang et al., 1997). Flies carrying a *GMR-D-Pax2* transgene display a rough eye phenotype (data not shown). However, most of their ommatidia exhibit four Cut-expressing cone cells, and no obvious difference in the expression patterns of the neuronal R cell marker Elav is observed between wild-type (Figs. 2A–C) and *GMR-D-Pax2*/+ third instar eye discs (Figs. 2F–H). This observation implies that ectopic expression of D-Pax2 in R cells is not sufficient to repress *elav* or activate *cut*. Consistent with this result, in *GMR-D-Pax2*/+ pupal eye discs, 78% of the ommatidia possess four cone cells, 13% have fewer, and only 9% have one supernumerary cone cell (Figs. 2J; 3A) although frequent loss of R cells and their strong deformation were observed in adult eyes (data not shown). Therefore, ectopic D-Pax2 expression driven by the *GMR* enhancer is not sufficient to convert R cell precursors into cone cells efficiently. A large number of supernumerary cells are observed between the ommatidia of *GMR-D-Pax2*/+ pupal eye discs (Fig. 2J). As an anti-apoptotic role has been reported for *D-Pax2* (Siddall et al., 2003), these cells may be the surplus cells that are not recruited into ommatidia and normally die by mid-pupal stage (Wolff and Ready, 1993), yet are maintained by ectopic D-Pax2.

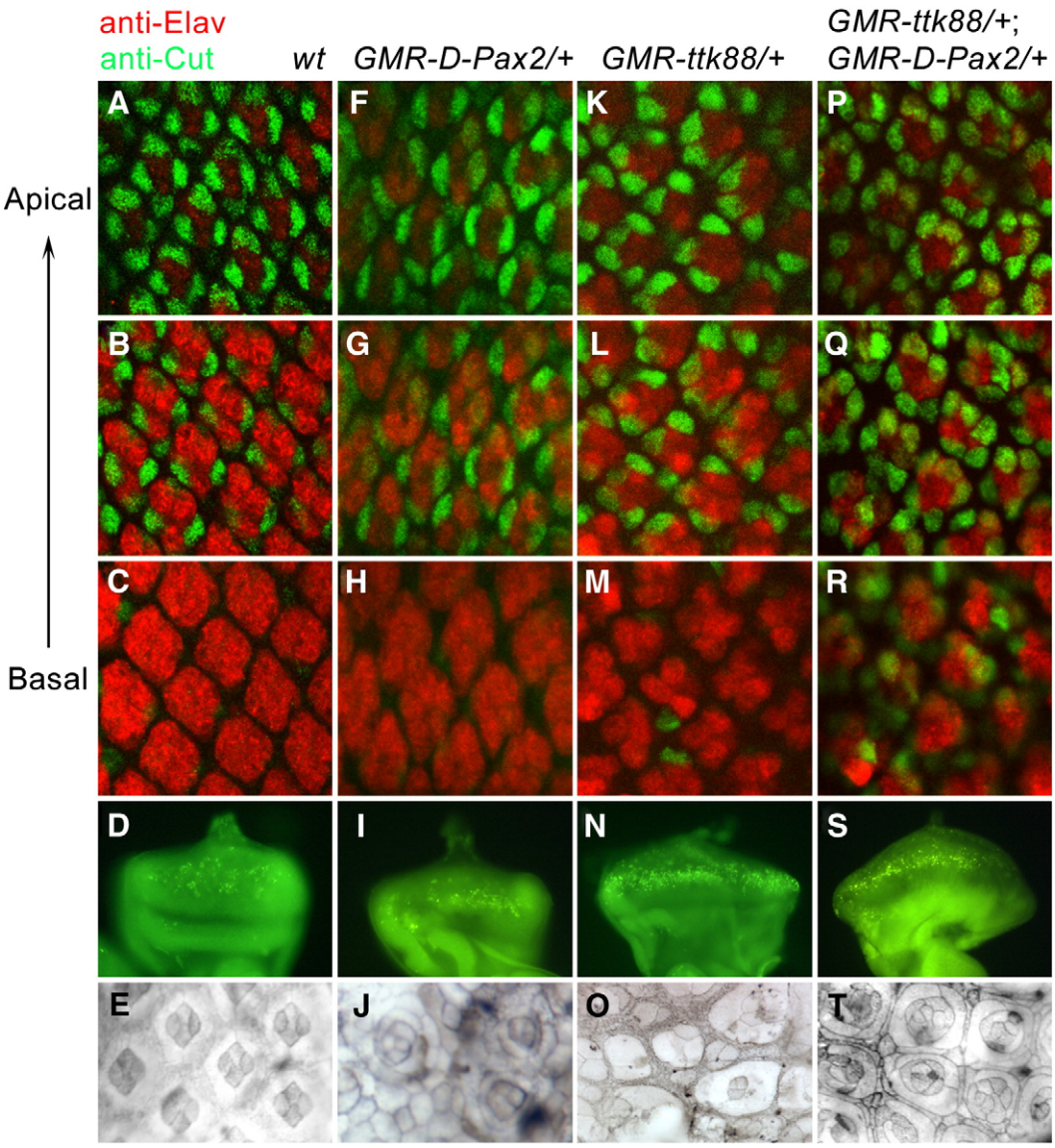

Fig. 2. Efficient transformation of R cells into cone cells depends on ectopic co-expression of D-Pax2 and TTK88. Confocal images of late third instar eye discs (A–C, F–H, K–M, and P–R) stained for Cut (green) and Elav (red), third instar eye discs stained with acridine orange to reveal apoptotic cells (D, I, N, and S), and 45-h pupal eye discs stained with cobalt sulfide (E, J, O, and T) of wild-type (A–E), *GMR-D-Pax2*/+ (F–J), *GMR-ttk88*/+ (K–O), and *GMR-ttk88*/+; *GMR-D-Pax2*/+ (P–T) larvae or pupae are shown. The *GMR-ttk88* chromosome originated from a strong *GMR-ttk88* line (Tang et al., 1997). The confocal images illustrate basal, medium, and apical sections as indicated.

Ectopic expression of D-Pax2 under the control of the *sev* enhancer, which drives expression in R3, R4, R7, and cone cells (Basler et al., 1989; Bowtell et al., 1989; Tang et al., 1997), produces results similar to those obtained with the *GMR* enhancer. Expression of Cut and Elav in *sev-D-Pax2*/+ eye discs is indistinguishable from that in wild-type eye discs (data not shown). In *sev-D-Pax2*/+ pupal eye discs, over 90% of the ommatidia have four cone cells, while only 1% contain one additional cone cell (Fig. 3A). Hence, ectopic D-Pax2 in R3, R4, and R7 is not sufficient either to promote cone cell development in these cells. It follows that, although D-Pax2 is necessary for proper cone cell development, its ectopic expression does not suffice to transform R cell precursors into cone cells.

Loss of TTK88 considerably enhances the cone cell phenotype of spa^{pol} mutants

The inhibition of the R cell fate in R cell precursors by ectopic TTK88 suggests that the normal function of TTK88 in developing cone cells is the repression of inappropriate R cell specification (Li et al., 1997; Tang et al., 1997). However, in homozygous *ttk^{1}* mutants, in which a P-element insertion prevents transcription of *ttk88* mRNA and generates supernumerary R7 cells in some ommatidia (Lai et al., 1996; Xiong and Montell, 1993; Fig. 1H; see also Materials and methods), both D-Pax2 and Cut expression appear normal in third instar eye discs (data not shown), and the number and appearance of cone cells in each ommatidium of pupal eye discs are normal as well (compare Fig. 1I with Fig. 1C). Moreover, the eyes of *ttk^{1}* (Fig. 1G) and wild-type flies (Fig. 1A) are indistinguishable in the scanning electron microscope. These observations questioned an absolute requirement for TTK88 in normal cone cell development.

To further explore the requirement for TTK88 during normal development of cone cells, we tested its influence on their fate in the absence of D-Pax2. Double mutants of *ttk^{1}* and *spa^{pol}* show a much stronger rough eye phenotype than the respective single mutants (Fig. 1). The surface of the eye of double mutants is nearly flat with irregularly arranged bristles (Fig. 1J). Only a few malformed ommatidia, many of which have open holes at their apices, are visible (Fig. 1J). Analysis of pupal *ttk^{1}*; *spa^{pol}* eye discs by staining with cobalt sulfide revealed that all cone cells were lost and only several darkly

A

Genotype \ Cone cells/ommatidium	1	2	3	4	5	6	7	8	9	10	11
GMR-ttk88/+; +/+	33 (15)	87 (40)	71 (33)	17 (7.9)	7 (3.2)	1 (0.5)					
+/+; GMR-D-Pax2/+	1 (0.7)	5 (3.6)	12 (8.6)	109 (78)	13 (9.3)						
GMR-ttk88/+; GMR-D-Pax2/+		1 (1.1)	3 (3.3)	6 (6.6)	13 (14)	22 (24)	27 (30)	9 (9.9)	6 (6.6)	3 (3.3)	1 (1.1)
sev-D-Pax2/+	1 (0.6)	0	11 (6.3)	161 (92)	2 (1.1)						
UAS-ttk88/+ or *Y; sev-Gal4/+*	1 (1.0)	3 (3.1)	84 (87)	8 (8.2)	1 (1.0)						
UAS-ttk88/+ or *Y; sev-Gal4/+; sev-D-Pax2/+*	1 (0.5)	5 (2.7)	25 (13)	78 (42)	71 (38)	5 (2.7)	2 (1.1)				

B

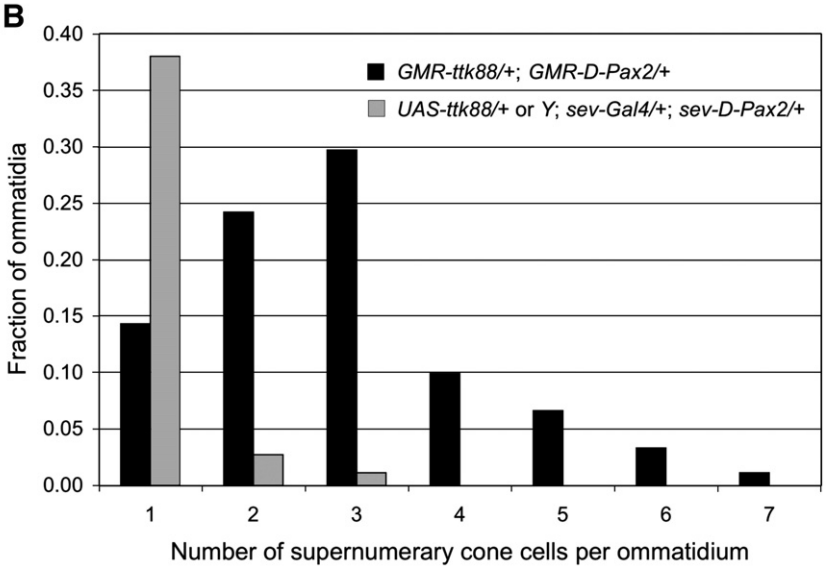

Fig. 3. Ectopic expression of both D-Pax2 and TTK88 enhances the number of cone cells per ommatidium. (A) 45-h pupal eye discs of the genotypes indicated were stained with cobalt sulfide to visualize their cone and pigment cells at the apical surface of the retina. Numbers (and percentages in parentheses) of ommatidia with indicated number of cone cells per ommatidium are shown. (B) Distribution of ommatidia with supernumerary cone cells after ectopic co-expression of D-Pax2 and TTK88 under control of the *GMR* or *sev* enhancer. The histogram was derived from the values given in A. The *GMR-ttk88* chromosome used in A and B was derived from a strong *GMR-ttk88* line (Tang et al., 1997).

stained spots remained visible at the presumptive positions of cone cells (Fig. 1L). The development of R cells is also dramatically affected in double mutants, as evident from sections of adult eyes, which show photoreceptors with strongly deformed rhabdomeres in the remaining ommatidia (Fig. 1K). However, the expression of the neuronal marker Elav in R cells of double mutants is not altered in eye discs and no supernumerary Elav-positive cells are observed (data not shown), which indicates that early R cell development is normal and cone cell precursors are not transformed into additional R cells. Since proper contact with their support cells is crucial for the development of neurons (Freeman, 2006), late R cell development may be abnormal in double mutants due to the absence of cone cells.

The absence of cone cells in ttk^1; spa^{pol} double mutants demonstrates that cone cell development strongly depends on TTK88 in the absence of D-Pax2. In the presence of D-Pax2, the absence of TTK88 is compensated by a factor redundant with it, as no transformation of cone cell precursors into R7 cells is observed in ttk^1 mutants (Fig. 1I). This factor is probably TTK69, which is synthesized from the alternatively spliced *ttk* mRNA in all four cone cells of ttk^1 mutants (Li et al., 1997; Xiong and Montell, 1993). For the same reason, cone cells are not transformed into R7 cells in ttk^1; spa^{pol} double mutants because, like TTK88, TTK69 is able to block R cell development (Li et al., 1997).

Ectopic TTK88 in photoreceptor cells is not sufficient for cone cell specification

To test whether ectopic TTK88 is sufficient to promote cone cell development in R cell precursors by blocking R cell specification, ommatidia were analyzed in eye discs that overexpressed TTK88 in developing R cells. All adult *GMR-ttk88/+* flies are devoid of photoreceptors. However, different transgenic lines of *GMR-ttk88* show rough eyes of variable expressivity (Tang et al., 1997). In weak *GMR-ttk88* lines, cone cells develop normally, as tested by staining for Cut (data not shown), and their eyes look like wild-type eyes (Tang et al., 1997). Strong *GMR-ttk88* lines produce rough and small eyes (Tang et al., 1997). Developing ommatidia of such transgenic lines lose some Elav-positive cells (compare Fig. 2M with Fig. 2C) and, in many cases, also Cut-positive cells in late third instar eye discs (compare Figs. 2K, L with Figs. 2A, B). At the same time, cell death detected by acridine orange staining is elevated in these discs (compare Fig. 2N with Fig. 2D; Tang et al., 1997). Consistent with this observation, 88% of the ommatidia lost one to three cone cells, while only 4% contain one or two supernumerary cone cells (Figs. 2O; 3A). Therefore, although R cell development is strongly suppressed in all *GMR-ttk88* flies (Tang et al., 1997), ectopic TTK88 in developing R cells is unable to transform these into cone cells. A similar result is obtained when

TTK88 is expressed ectopically under the control of the *sev* enhancer in *sev-TTK88*/+ flies: development of R3/R4/R7 is blocked (Tang, et al., 1997), but most pupal ommatidia have four cone cells (not shown). High levels of ectopic TTK88, expressed by the combination of *sev-Gal4* with *UAS-ttk88* (Giesen et al., 1997), induce the loss of at least one cone cell in about 90% of the ommatidia (Fig. 3A). We conclude that ectopic TTK88 in R cells, while blocking their fate, is not sufficient to convert them into cone cells.

Expression of both D-Pax2 and TTK88 in R1/R6/R7 precursors precedes their transformation into cone cells in phyl mutants

In the absence of Phyl, precursors of R1/R6/R7 begin to express the cone cell marker Cut instead of the neuronal marker Elav (compare Figs. 4E–H with Figs. 4A–D) and finally adopt a cone cell fate (Chang et al., 1995; Dickson et al., 1995). In third instar $phyl^2/phyl^4$ eye discs, ectopic TTK88 protein is detected in cells that correspond to R1/R6/R7 (Li et al., 1997). Because proper cone cell development depends on the activity of *D-Pax2*, we anticipated that D-Pax2 is also present in the supernumerary cone cells of these mutants. Indeed, double-staining with anti-D-Pax2 and anti-Elav antibodies confirmed that in most $phyl^2/phyl^4$ ommatidia additional D-Pax2-positive cells are present at the expense of Elav-positive cells (Figs. 4I–L), in contrast to wild-type discs where D-Pax2 is observed only in the four cone cells of each ommatidium at this stage (Fu et al., 1997; Figs. 4A–D). Double-staining with anti-Cut and anti-D-Pax2 antibodies revealed that these label the same cells in third instar $phyl^2/phyl^4$ eye discs (data not shown). Therefore, in $phyl^2/phyl^4$ mutants, D-Pax2 is also ectopically expressed in the cells that correspond to R1/R6/R7. We conclude that transformation of R1/R6/R7 into cone cells in $phyl^2/phyl^4$ mutants is preceded by the ectopic expression in the R1/R6/R7 precursors of TTK88 (Li et al., 1997) as well as D-Pax2. This raises the possibility that ectopic cone cell development of R1/R6/R7 precursors depends on the functions of both TTK88 and D-Pax2.

Transformation of neuronal R cells into non-neuronal cone cells by ectopic co-expression of D-Pax2 and TTK88

To test the hypothesis that ectopic expression of TTK88 and D-Pax2 is sufficient to promote the cone cell fate in R cell precursors, we co-expressed D-Pax2 and TTK88 under the control of the *GMR* or *sev* enhancer. In eye discs of a strong *GMR-ttk88* line that also carries a *GMR-D-Pax2* transgene, many additional Cut-positive cells appear at the expense of Elav-positive cells (Figs. 2P–R) as compared to wild-type discs (Figs. 2A–C). This effect is not observed or only marginal in eye discs that carry only one of the two transgenes (Figs. 2F–H, K–M).

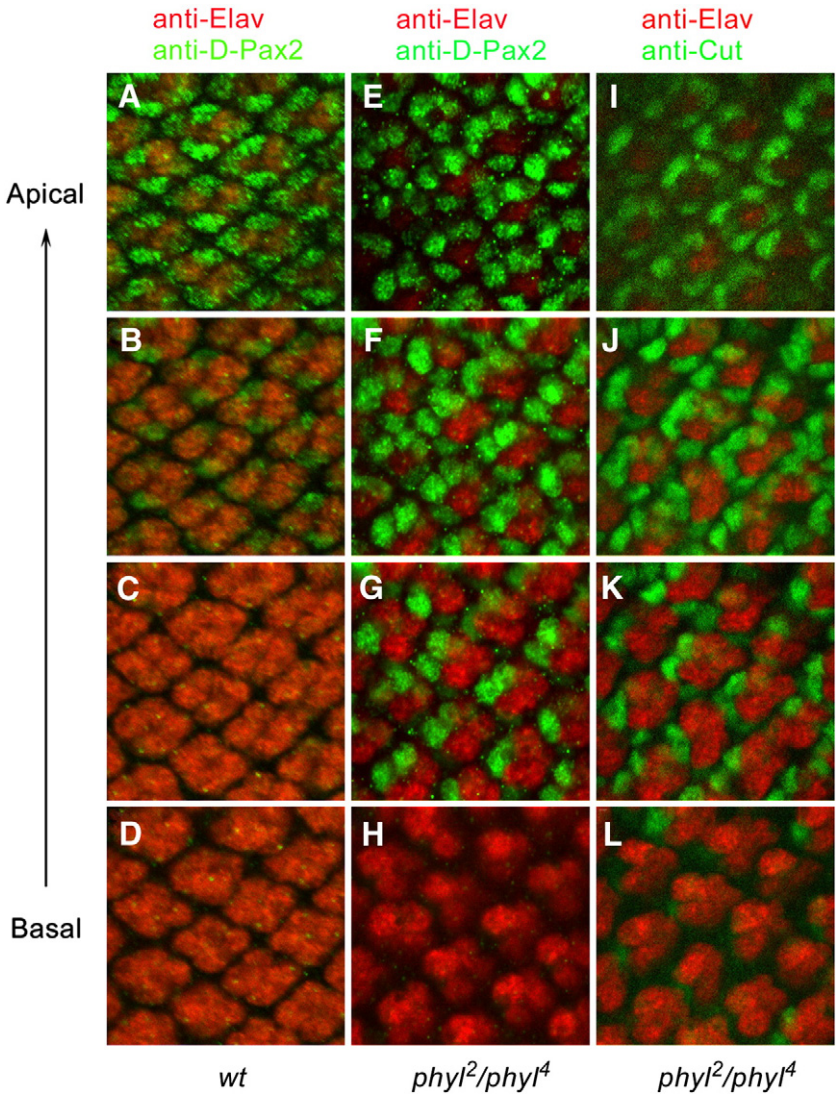

Fig. 4. Supernumerary Cut-expressing cells in $phyl^2/phyl^4$ eye discs express D-Pax2. Confocal images, corresponding to different optical sections along the basal–apical axis of a wild-type (A–D) and two $phyl^2/phyl^4$ late third instar eye discs (E–L), stained for Elav (red) and D-Pax2 (green) (A–D and I–L) or Cut (green) (E–H), are shown.

These results indicate that co-expression of TTK88 and D-Pax2 in R cell precursors frequently changes their fate to Cut-expressing cone cells. Consistent with this interpretation, many supernumerary cone cells appear in *GMR-ttk88*/+; *GMR-D-Pax2*/+ pupal eye discs (Fig. 2T), while most ommatidia possess four or fewer cone cells in *GMR-D-Pax2*/+ or *GMR-ttk88*/+ pupal eye discs (Figs. 2J, O; 3A). Indeed, ectopic expression of both *GMR-D-Pax2* and *GMR-ttk88* in photoreceptor precursors generates supernumerary cone cells in about 90% of the ommatidia of pupal eye discs, with a maximum of 11 cone cells observed in one ommatidium (Fig. 3A).

Supernumerary cone cells are also observed when D-Pax2 and TTK88 are co-expressed ectopically under the control of the *sev* enhancer: *UAS-ttk88*/+; *sev-Gal4*/+; *sev-D-Pax2*/+ pupal eye discs have additional cone cells in about 40% of their ommatidia, with a maximum of seven cone cells observed in 1% of the ommatidia (Fig. 3A). By contrast, when only one of these two genes is expressed ectopically under the control of the *sev* enhancer, about 90% of the ommatidia have the normal number of cone cells in *sev-D-Pax2* eye discs or lack one cone cell in *sev-Gal4*/*UAS-ttk88* eye discs, while in either case only 1% of the ommatidia have one supernumerary cone cell (Fig. 3A). Thus, ectopic D-Pax2 and TTK88 in developing R cells transforms them efficiently into cone cells, whereas either factor by itself does not. The generation of supernumerary cone cells depends on the use of a strong *GMR-ttk88* line or a combination of *sev-Gal4* and *UAS-ttk88* transgenes but is independent of which *GMR-D-Pax2* or *sev-D-Pax2* line is used. This indicates that for transformation of R cells into cone cells a relatively high level of TTK88, as compared to that of D-Pax2, is required to block the R cell fate efficiently.

Interestingly, ectopic co-expression of D-Pax2 and TTK88 under the control of the *GMR* enhancer produces in more than half of the ommatidia two or three supernumerary cone cells (Fig. 3). A possible explanation is that co-expression of D-Pax2 and TTK88 transforms the three photoreceptor precursors of the R7 equivalence group, R1/R6 and R7, into cone cells with a considerably higher efficiency than the remaining R cell precursors. Consistent with this interpretation, ectopic activation of *D-Pax2* and *ttk88* under the control of the *sev* enhancer, active in R3/R4 and R7, generates in most ommatidia only one supernumerary cone cell, while up to three additional cone cells in only 4% of the ommatidia (Fig. 3).

When D-Pax2 and TTK88 are co-expressed under the control of the *GMR* enhancer, most ommatidia have three supernumerary cone cells (30±6%), less have two (24±5%), while only 14±4% have one additional cone cell (Fig. 3B). This result is consistent with the assumptions that (1) R1 and R6 are both converted to cone cells more frequently than only one of them, and (2) transformation of R7 is enhanced by the transformation of both R1 and R6 as compared to the situation where only one or none of them is transformed. They are in agreement with the fact that R1 and R6 join the ommatidia in pairs shortly before R7 does (Wolff and Ready, 1993). They are further in good agreement with the reduced number of cone cells (Figs. 2O; 3A), presumably resulting from dying R and cone cells (Fig. 2N), when only TTK88 is expressed ectopically, as this observation suggests that R cells that fail to transform into cone cells in the presence of both TTK88 and D-Pax2 still die and thus cannot provide signals that may favor the transformation of R7 into cone cells.

In the strong *GMR-ttk88* line, loss of Elav-positive R cells in third instar eye discs (Tang et al., 1997; compare Figs. 2K–M with Figs. 2A–C) is accompanied by enhanced cell death (Tang et al., 1997; compare Fig. 2N with Fig. 2D). However, when *GMR-D-Pax2* is co-expressed with *GMR-ttk88*, R cells die less frequently (compare Fig. 2S with Fig. 2N) and are rather transformed into supernumerary Cut-positive cone cells (compare Fig. 2R with Fig. 2M and C), which indicates that TTK88 and D-Pax2 act in concert to transform R cells into cone cells. Thus, while TTK88 blocks the neuronal differentiation and causes cell death of the developing R cells, D-Pax2 suppresses cell death and induces these cells to adopt the cone cell fate.

In summary, we conclude that ectopic D-Pax2 and TTK88 in R cell precursors, particularly in R1/R6 and R7, act in concert to transform these into cone cells.

Down-regulation of TTK88 and D-Pax2 during transformation of cone cells into R7 cells by ectopic Phyl

Phyl is required for proper development in R1/R6 and R7. Ectopic Phyl in cone cells abolishes TTK88 (Li et al., 1997; Tang et al., 1997) and transforms them into R7 cells (Chang et al., 1995; Dickson et al., 1995). However, the absence of TTK88 protein in cone cells of ttk^1 third instar eye discs (Lai et al., 1996) does not affect the development of cone cells (Fig. 1I; cf. Materials and methods). Therefore, in addition to the down-regulation of TTK88 protein, ectopic Phyl induces other effects in cone cells that transform them into R7 cells. Since D-Pax2 is required for cone cell development, one might suspect that ectopic Phyl down-regulates D-Pax2 as well. Indeed, when Phyl is ectopically expressed in cone cells under the control of two *sev* enhancers (Dickson et al., 1995), levels of D-Pax2 protein are strongly reduced (Fig. 5B) as compared to those in wild-type eye discs (Fig. 5A). Since we have shown that D-Pax2 is crucial for the specification of the cone cell fate, its down-regulation by ectopic Phyl suggests that one role for Phyl during normal development of R1/R6 and R7 is to block cone cell specification by down-regulating D-Pax2.

Ectopic Phyl in cone cells down-regulates D-Pax2 transcription in a Sina-dependent manner

Ectopic Phyl in cone cells targets TTK88 for degradation through a ubiquitin-dependent proteasome pathway (Li et al., 1997; Tang et al., 1997). To investigate the mechanism by which ectopic Phyl down-regulates D-Pax2 in cone cells, we generated transgenic flies harboring a reporter construct, *spa-lacZ*, in which *lacZ* is under the

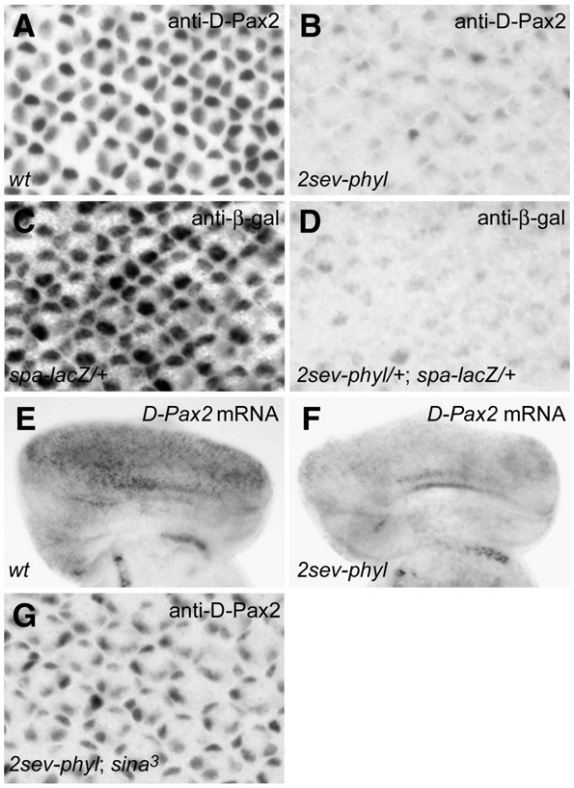

Fig. 5. Down-regulation of *D-Pax2* in cone cells by ectopic Phyl occurs at the transcriptional level and depends on Sina. Late third instar eye discs of the indicated genotypes were stained for D-Pax2 (A, B, G) or β-galactosidase (C, D), or for *D-Pax2* mRNA (E, F). The discs are shown at high (A–D, G) and low magnification (E, F).

control of the eye-specific *spa* enhancer of *D-Pax2* (Fu et al., 1998). In eye discs, this enhancer drives *lacZ* expression specifically in cone cells (Fig. 5C). In the presence of the *2sev-phyl* transgene, however, expression of *lacZ* in cone cells is dramatically reduced (Fig. 5D), which suggests that ectopic Phyl down-regulates *D-Pax2* at the transcriptional level through its *spa* enhancer. In agreement with this result, *in situ* hybridization of a *D-Pax2* antisense RNA probe to wild-type (Fig. 5E) and *2sev-phyl* eye discs (Fig. 5F) demonstrates that the level of *D-Pax2* mRNA is drastically reduced in cone cells in the presence of ectopic Phyl.

Phyl, Sina, and Ebi form an E3 ubiquitin ligase complex, which mediates the post-transcriptional degradation of TTK88 (Li et al., 1997; Li et al., 2002; Tang et al., 1997). To test whether the transcriptional down-regulation of D-Pax2 also depends on this ubiquitin-dependent proteasome pathway, we examined D-Pax2 expression in third instar eye discs of $sina^3$ mutants carrying a *2sev-phyl* transgene. As evident from Fig. 5G, ectopic Phyl cannot down-regulate *D-Pax2* expression in a $sina^3$ background, which suggests that the transcriptional down-regulation of *D-Pax2* by ectopic Phyl depends on Sina. The simplest explanation of these results is that Phyl and Sina down-regulate *D-Pax2* indirectly by targeting an activator of *D-Pax2* transcription for degradation by the ubiquitin-dependent proteasome pathway.

Discussion

Members of the R7 equivalence group have the developmental potential to become a neuronal R1/R6/R7 or a non-neuronal cone cell (Chang et al., 1995; Crew et al., 1997; Dickson et al., 1995; Greenwald and Rubin, 1992). TTK88 and D-Pax2 are both specifically expressed in developing cone cells (Fu and Noll, 1997; Lai et al., 1996). The absence of cone cells in ttk^1; spa^{pol} double mutants and their presence in ttk^1 and spa^{pol} single mutants strongly suggest that both D-Pax2 and TTK88 function in cone cell development. We have shown that (1) blocking the neuronal fate by TTK88 and (2) promoting the non-neuronal fate by D-Pax2 are simultaneous and coordinated steps in the transformation of R cells into cone cells. The TTK88 function of blocking the neuronal fate is largely redundant with that of TTK69 because ectopic Phyl, which down-regulates D-Pax2 (Fig. 5) and TTK88 as well as TTK69 (Li et al., 1997), is sufficient to transform cone cells into R7 cells (Chang et al., 1995; Dickson et al., 1995), whereas removal of only TTK88 and D-Pax2 in ttk^1; spa^{pol} double mutants results in the loss of cone cells but not their conversion into R cells. On the other hand, during R1/R6/R7 development, Phyl promotes the neuronal fate not only (1) through targeting TTK (both TTK88 and TTK69) for degradation, thereby releasing the inhibition of R cell specification (Dong et al., 1999; Li et al., 1997; Li et al., 2002; Tang et al., 1997), but also (2) by down-regulating D-Pax2 to block the cone cell fate. Therefore, we propose that the cell fates of the R7 equivalence group are determined by a dual mechanism that coordinately promotes the fate of one cell type and blocks that of the other (Fig. 6). For cone cell development, it is not sufficient to provide D-Pax2 that activates the cone cell fate, the alternative neuronal fate has to be blocked as well by TTK protein. Conversely, for R cell development, it is not sufficient to specify this fate by removing its TTK block, but inhibition of the alternative cone cell fate by preventing *D-Pax2* activation is equally important.

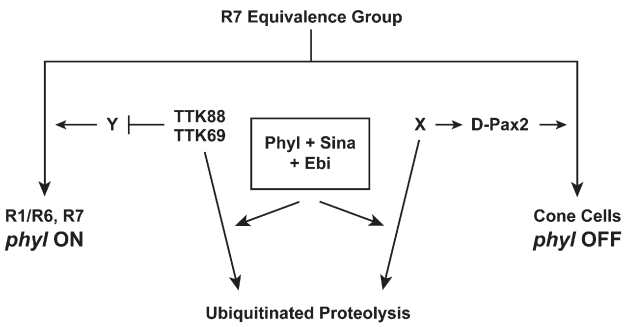

Fig. 6. A dual mechanism determines cell fates in R7 equivalence group. A dual mechanism, regulated by a binary switch that depends on the state of *phyl*, determines the fate of cells that belong to the R7 equivalence group. In R1/R6/R7 precursors, Phyl targets TTK88 and TTK69 (Li et al., 1997; Li et al., 2002), which inhibit R cell differentiation, and factor X, which is required for the transcription of *D-Pax2*, for degradation through a ubiquitin–proteasome pathway, thereby releasing the inhibition of the R cell fate and stalling the cone cell fate. Conversely, in cone cell precursors where *phyl* is OFF, TTK and X are not inactivated by degradation or inhibition (see text). Thus, the R cell fate remains blocked and the cone cell fate is activated by D-Pax2. In both cases, promotion of one cell fate and inhibition of the alternative fate are coordinated steps of equal importance to determine the cellular fate unambiguously. Note that TTK88 and TTK69 are assumed to be redundant only with respect to their ability to block R cell development. This does not imply that they repress identical subsets of the gene set Y, the expression of which is necessary for R cell development. For details, see Discussion.

In third instar larval and early pupal eye discs, TTK88 protein is initially detected in all undifferentiated basal cells, but later restricted to cone cells (Lai et al., 1996). TTK88 blocks neuronal R cell differentiation, but is unable to promote non-neuronal cone cell specification (Fig. 6). Thus, TTK88 serves as a safeguard in basal cells that maintains them in an undifferentiated state. The binary switch between R1/R6/R7 and cone cell fates is regulated by Phyl, which is present in the former but absent from the latter (Fig. 6). Activation of *phyl* depends on Svp in R1/R6, or on high levels of Ras signaling in R7 where *svp* is repressed by Lz (Daga et al., 1996). This follows from the observation that Phyl is absent from cone cells, while ectopic Svp or high levels of Ras signaling in cone cells transforms these into R7 cells in a Phyl-dependent manner (Begemann et al., 1995; Kramer et al., 1995). Moreover, it has been shown that *phyl* is activated by Sev-induced Ras signaling in R7 precursor cells (Chang et al., 1995; Dickson et al., 1995). Since Svp is absent from cone cells and Ras signaling too low because Sev is not activated, *phyl* is inactive in and its product absent from cone cells.

In R1/R6/R7 precursors, Phyl forms a complex with Ebi and Sina, activating a ubiquitin–proteasome machinery that targets TTK for degradation and hence releases the block of the neuronal fate in these cells (Dong et al., 1999; Li et al., 1997; Li et al., 2002; Tang et al., 1997). In addition to TTK, D-Pax2 must be absent from R cells because ectopic D-Pax2 in R cells of *GMR-D-Pax2* flies causes frequent loss of R cells or their strong deformation (data not shown), even though early R cell development seems normal as judged by staining for Elav (Figs. 2G, H). We propose that the same ubiquitin–proteasome machinery also targets an activator X of *D-Pax2* transcription for degradation and thus indirectly down-regulates D-Pax2 in R1/R6/R7 precursors (Fig. 6). Therefore, the cone cell fate is blocked in these cells while R1/R6/R7 specification begins.

In developing cone cells, TTK is not degraded because Phyl is absent. Strong N signaling in cone cell precursors is activated by high concentrations of the N ligand, Dl, on neighboring R cells (Tsuda et al., 2002). As a consequence, *D-Pax2* transcription is activated in cone cells by the combinatorial effect of Lz, N-activated Su(H), and the concomitant activation of PntP2 and inhibition of Yan by EGFR-activated Ras signaling (Flores et al., 2000). Thus, as TTK and D-Pax2 are both present, the R cell fate is blocked and cone cell specification is initiated (Fig. 6).

Absence of TTK88 in ttk^1 or of D-Pax2 in spa^{pol} mutants does not result in the transformation of cone cells into R7 cells (Figs. 1F, I). According to our model, efficient transformation depends on the absence of both TTK (TTK88/TTK69) and D-Pax2 (Fig. 6). This is achieved by ectopic expression of Phyl in cone cells under control of the *sev* enhancer (Chang et al., 1995; Dickson et al., 1995) or by combining the homozygous ttk^1 mutation with a heterozygous null allele of *yan* (Lai et al., 1996), which results in the derepression of *phyl* in cone cells (Dickson et al., 1995; O'Neill et al., 1994). Similarly, homozygous hypomorphic yan^P mutations combined with heterozygous strong *ttk*

alleles results in a large fraction of ommatidia with supernumerary R7 cells (Lai et al., 1996). In this case, Phyl levels induced in cone cells suffice to unblock the neuronal fate by further reducing TTK levels and to down-regulate D-Pax2 levels to an extent that cone cells are transformed efficiently into R7 cells (Fig. 6). Ectopic Phyl transforms cone cells into R7 rather than R1/R6 photoreceptor cells because Svp, which specifies the R1/R6 versus the R7 cell fate (Begemann et al., 1995; Hiromi et al., 1993; Kramer et al., 1995), is not expressed in cone cells. Thus, our model is in good agreement with earlier observations that *ttk* and *yan* mutations act synergistically to alter the fate of cone cell precursors to that of R7 cells (Lai et al., 1996). It further confirms an earlier suggestion that TTK88 functions in a pathway distinct from and parallel to Ras signaling (Lai et al., 1996).

In addition, we have shown the reverse situation to be true as well. Ectopic expression of both D-Pax2 and TTK88 in R cell precursors transforms R1/R6/R7 with a much higher efficiency into cone cells than R2-R5 (Fig. 3). For when D-Pax2 and TTK88 are expressed under the control of the *sev* enhancer, which results in their ectopic expression in R3/R4/R7 precursors, only one additional cone cell appears with high efficiency. By contrast, when ectopic expression occurs in all R cell precursors under the control of the *GMR* enhancer, only three supernumerary cone cells appear with high efficiency although up to 7 additional cone cells were observed. Moreover, ectopic co-expression in R1/R6/R7 of TTK88 (Li et al., 1997) and D-Pax2 in *phyl* mutants (Fig. 4I–K) efficiently transforms these R cell precursors into supernumerary cone cells (Chang et al., 1995; Dickson et al., 1995). Our evidence, therefore, suggests the existence of a dual mechanism regulated by a binary switch between the non-neuronal cone cell and neuronal R cell fate in the R7 equivalence group. The state of this switch depends on the presence or absence of Phyl that coordinately regulates TTK and D-Pax2 levels through ubiquitin-directed proteolysis (Fig. 6).

The model in Fig. 6 might suggest Sina as an alternative switch to Phyl. However, this possibility is excluded because Sina is expressed in photoreceptors as well as cone cells (Carthew and Rubin, 1990). Moreover, in *sina* mutants only R7 is transformed into a cone cell even though Sina is expressed in all photoreceptors (Carthew and Rubin, 1990). This observation has been explained by a redundancy of *sina* function with that of *musashi* in the down-regulation of TTK in R1 and R6 (Hirota et al., 1999). It is not known how TTK is down-regulated in photoreceptor precursors different from R1/R6 and R7. However, it appears that this mechanism is not only independent of Sina but also independent of Phyl, as Phyl is not expressed in photoreceptors different from R1/R6/R7 (Chang et al., 1995; Dickson et al., 1995).

Degradation of TTK is mediated through an E3 ubiquitin ligase complex, including Phyl/Sina/Ebi, which targets TTK to the proteasome (Boulton et al., 2000; Dong et al., 1999; Li et al., 1997; Li et al., 2002; Tang et al., 1997). Our results suggest that the same complex also functions to down-regulate *D-Pax2* transcription (Fig. 5). It is therefore conceivable that this complex targets one or several of the activators of *D-Pax2* for degradation (X in Fig. 6). It is improbable that TTK is this activator because TTK is only known to act as repressor (Brown and Wu, 1993; Read et al., 1992; Xiong and Montell, 1993), while *D-Pax2* can be activated ectopically in the absence of TTK in R7 cells by N signaling (Flores et al., 2000).

Transcription of *D-Pax2* in cone cells is regulated by the combinatorial action of Lz, N-activated Su(H), and the EGFR-regulated effectors PntP2 and Yan (Flores et al., 2000). EGFR signaling and Lz are both active in R1/R6 and R7 precursors (Daga et al., 1996; Freeman, 1996). Ectopic expression in R7 of the constitutively active intracellular domain of N, N^{IC}, activates *D-Pax2* (Flores et al., 2000). It follows that the Phyl/Sina/Ebi-dependent proteasome machinery down-regulates *D-Pax2* transcription in R7 by antagonizing N signaling. Consistent with this conclusion, it has been suggested that the E3 ubiquitin ligase complex Phyl/Sina/Ebi, targeting TTK for degradation, may inhibit the transcription-activating activity of Su(H) (Dong et al., 1999; Tsuda et al., 2002; Tsuda et al., 2006). It is thus attractive to speculate that Su(H) is the target of this complex that may not include all of its components and thus may not degrade Su(H) but only inhibit its activity required in a complex with N^{IC} to activate *D-Pax2*. Such an interpretation is consistent with our observation that Su(H) levels in R1/R6/R7 are indistinguishable from those in cone cells (data not shown). However, we cannot exclude other targets like N^{IC} which might be modified rather than degraded by the Phyl/Sina/Ebi complex since the concentration of N^{IC}, when expressed under the control of the *sev* enhancer, is independent of co-expression with Phyl (data not shown).

The mechanism by which TTK blocks neuronal development is unclear. However, since TTK encodes a transcriptional repressor (Brown and Wu, 1993; Read et al., 1992; Xiong and Montell, 1993), it may repress one or several genes (Y in the model of Fig. 6) that are required for neuronal development. Down-regulation of TTK would then derepress the Y genes, which act to specify neuronal development (Fig. 6). One of the factors encoded by Y is the transcription factor Prospero (Pros), which is required for proper development of R7 (Kauffmann et al., 1996) since ectopic expression of TTK88 in R7 precursors abolishes the elevation of Pros (Xu et al., 2000). Since loss-of-function alleles of *pros* do not affect other R cells (Kauffmann et al., 1996), other Y factors must exist.

Acknowledgments

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