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Review

The Sevenless signaling pathway: variations of a common theme

Thomas Raabe *

Department of Genetics, University of Würzburg, Biozentrum, Am Hubland, D-97074 Würzburg, Germany

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Abstract

Many developmental processes are regulated by intercellular signaling mechanisms that employ the activation of receptor tyrosine kinases. One model system that has been particular useful in determining the role of receptor tyrosine kinase-mediated signaling processes in cell fate determination is the developing *Drosophila* eye. The specification of the R7 photoreceptor cell in each ommatidium of the developing *Drosophila* eye is dependent on activation of the Sevenless receptor tyrosine kinase. This review will focus on the genetic and biochemical approaches that have identified signaling molecules acting downstream of the Sevenless receptor tyrosine kinase which ultimately trigger differentiation of the R7 photoreceptor cell.

1. Receptor tyrosine kinases in eye development: an overview

Each of the 800 single eye units (ommatidia) of the *Drosophila* eye contains a stereotypic arrangement of eight photoreceptor cells (R1–R8), four lens secreting cone cells and a number of accessory cells (Fig. 1A,E). During late larval and pupal stages, the fly eye develops progressively from a monolayer epithelium, the eye-antennal imaginal disc. An indentation, the morphogenetic furrow, traverses the eye disc from posterior to anterior. Ahead of the morphogenetic furrow, the cells of the eye imaginal disc are undifferentiated. The recruitment and differentiation of the different cell types of each ommatidium starts at the posterior edge of the morphogenetic furrow and follows a highly stereotypic temporal sequence. R8 is the first cell to differentiate, subsequently R2/R5 and R3/R4 are added in pairs. After the remaining undifferentiated cells have undergone a last round of mitosis, photoreceptors R1/R6, and finally R7, are recruited. Addition of the nonneuronal cone and pigment cells completes ommatidial development [1]. Differentiation of the different cell types in each ommatidium is controlled by at least two receptor tyrosine kinases (RTKs), the *Drosophila* EGF receptor (DER) and Sevenless (SEV) [2–4]. Whereas DER controls differentiation of most, if not all, cells in the developing eye and fulfills additional roles in proliferation control and cell survival [5–10], SEV is only required for specification of the R7 photoreceptor cell (Fig. 1B,F) [11]. SEV is expressed in a highly dynamic manner in a subpopulation of ommatidial cells including R1/R6, R3/R4, R7 and cone cells [12]. The SEV RTK in the R7 precursor cell becomes activated by binding to the Bride of Sevenless (BOSS) protein, a seven-transmembrane protein that is expressed in the already differentiated R8 cell at the time of R7 cell specification [13,14]. In the absence of BOSS or SEV, the R7 precursor cell develops into a nonneuronal cone cell [11,15]. These experiments provided at least for one case the mo-

* Fax: +49-931-8884452;
E-mail: raabe@biozentrum.uni-wuerzburg.de

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lecular confirmation of previous genetic experiments that suggested that inductive cellular interactions rather than cell lineage determines cellular fates in the *Drosophila* eye [16–18].

2. Genetic approaches to dissect SEV-mediated signaling

Several features make the R7 cell an attractive system to study the role of RTK signaling processes in cell fate determination. Firstly, only two cells are involved, namely the already differentiated R8 cell as the signal sender and the presumptive R7 cell as the recipient. Secondly, depending on the presence or absence of the signal, the R7 precursor cell can only choose between the neuronal R7 or the non-neuronal cone cell fate, respectively. Thirdly, the presence of the R7 cell can be easily assayed in living animals. This allows extensive genetic screens for mutations that specifically interfere with the formation of the R7 cell and, therefore, might disrupt reception, transduction, or interpretation of the inductive signal in the R7 precursor cell. Two classes of mutations are anticipated. The first class are homozygous viable mutations like *sev* or *boss* which specifically affect R7 cell development. Few isolated mutations have met the criteria of specificity. The second class of mutations are more difficult to isolate. If the SEV cascade shares components with other RTKs like DER with multiple functions during development, animals homozygous for loss-of-function mutations in the corresponding genes would presumably die prior to R7 cell commitment. Several strategies have been employed to circumvent this problem. Simon et al. [19] used a temperature sensitive allele of *sev (sev ^{ts})* which provides barely sufficient activity to allow R7 cell development. The second approach involved expression of constitutively activated versions of SEV in all *sev*-expressing cells [20,21]. This resulted in transformation of cone cells into additional R7 cells resulting in a rough eye phenotype (Fig. 1C,G). One important conclusion drawn from these experiments is that cone cell precursors are competent to respond to the inductive signal. In the wild-type situation, where they do not contact

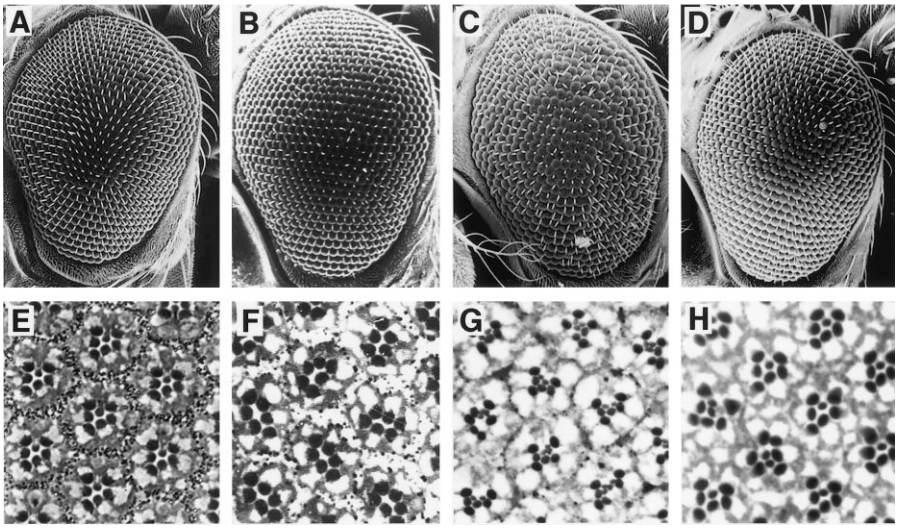

Fig. 1. Genetic interaction screens were essential for identifying mutations in genes involved in signaling downstream of the SEV RTK. The eye of a wild-type fly is a regular array of about 800 ommaditia (A). A tangential section through a wild-type eye reveals six large rhabdomeres in each ommatidium corresponding to photoreceptors R1–R6 and the centrally located small rhabdomere of the R7 photoreceptor cell (E). The rhabdomere of the R8 cell is not visible in this apical section and is located below the R7 rhabdomere. In *sev* mutant flies (B), only the R7 cell is missing, indicated by the lack of the central small rhabdomere (F). Recruitment of additional R7 cells by expression of a constitutively activated version of SEV results in disturbance of the highly regular eye architecture (C). Each ommatidium contains several small R7-like rhabdomeres (G). This phenotype is sensitive to the gene dosis of rate-limiting signaling components acting downstream of SEV. Suppression of the rough eye phenotype is observed by removal of a single copy of a positively acting component, in this case the gene encoding the multisite adaptor protein DOS (D). The number of R7 cells is greatly reduced (H).

the signal-providing R8 cell they are unable to adopt the R7 cell fate. Most importantly, in both approaches, R7 cell specification became sensitive to the gene dosage of rate-limiting components acting downstream of SEV. Hence, for a recessive lethal mutation, reducing the gene dose by half is sufficient to modify the *sev* ^{ts} or the *sev*^{S11} phenotype, which can be scored in living animals (Fig. 1D,H). Large-scale screens conducted with both systems unraveled the first steps in the SEV signaling cascade. Some of the signaling molecules identified (e.g., RAS1, RAF) were in turn used as entry points for further genetic screens [22,23]. A current model of the SEV signal transduction pathway is shown in Fig. 2.

3. Downstream of Sevenless

The immediate consequence of the BOSS–SEV interaction is the stimulation of SEV kinase activity and autophosphorylation of SEV on tyrosine residues, providing binding sites for SH2 domain containing proteins [24]. A major route by which SEV, as well as RTKs in general, transduce signals involves activation of the small GTPase RAS1. The level of the active form GTP-RAS is determined by the ratio between the activity of the RAS guanine nucleotide exchange factor Son of Sevenless (SOS) and the intrinsic GTPase activity of RAS which is enhanced by GTPase activating proteins (GAPs). A direct link between SEV and SOS is provided by the SH2/SH3 domain containing protein downstream of receptor kinases (DRK) [25,26], the *Drosophila* homologue of the vertebrate adaptor protein GRB2 [27] and the *Caenorhabditis elegans* protein SEM-5 [28]. Binding of DRK to SEV is abolished by mutating either Tyr 2546 on SEV or of conserved residues in the phosphotyrosine binding pocket of the DRK SH2 domain. The N- and C-terminally located SH3 domains of DRK mediate binding to proline-rich sequences located in the C-terminal tail of SOS [19,25,26,29,30]. The structure–function analysis of the SOS protein suggested that, beyond its adaptor function, DRK binding to SOS relieves the inhibitory effect of the SOS C-terminal domain on SOS activity [31,32].

The abundance of proteins identified in vertebrate systems as targets of RTKs and, more recently, genetic analyses in *Drosophila* strongly suggest that the SEV–DRK–RAS1 link is only part of a multiprotein complex being assembled at the SEV receptor leading to the activation of different signaling pathways. For example, the vertebrate PDGF RTK contains multiple tyrosine autophosphorylation sites that serve as docking sites for a whole variety of SH2 domain containing proteins [33]. A second strategy employed by a number of RTKs to recruit various signaling molecules is to use multisite adaptor proteins that provide additional docking sites for SH2 domain containing proteins. Vertebrate members of the multisite adaptor protein family include the Insulin Receptor Substrate (IRS) proteins, GAB1, and GAB2 [34–36].

In the case of SEV, the simple linear model of signal transduction has been questioned by the observation that a SEV protein lacking Y2546 is unable to bind to the DRK protein, yet still can transduce the signal in a RAS-dependent manner [30,37]. Further complexity was introduced by the identification of proteins that act upstream or in parallel to RAS1 to regulate signal transmission from the activated

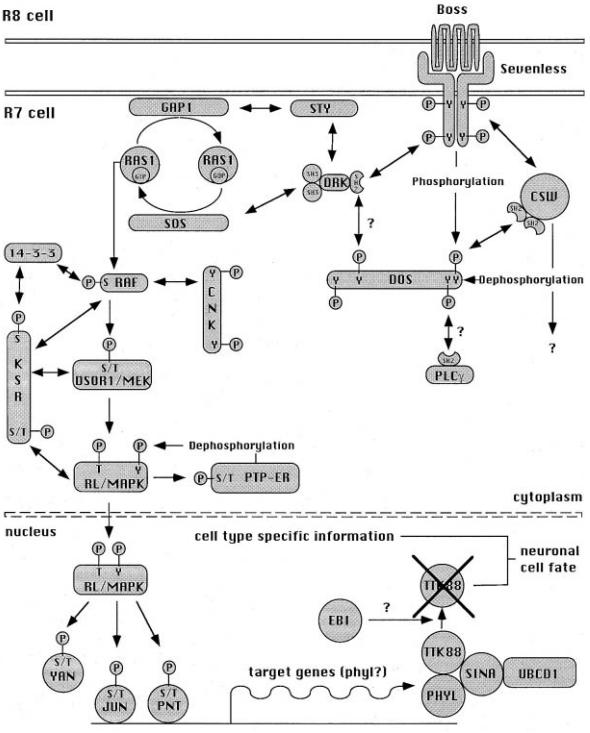

Fig. 2. A model for the SEV signal transduction pathway. For details see text.

SEV receptor. The phospholipase C-γ (PLC-γ) protein acts as a negative regulator of R7 cell development [38] while the tyrosine phosphatase Corkscrew (CSW) is a positively acting component [39,40]. Integration of these proteins into the signaling complex at the activated RTK is thought to be mediated by the Daughter of Sevenless (DOS) protein which is involved in signaling from the SEV, DER, and Torso (TOR) RTKs [37,41]. Characteristic features of DOS include a N-terminally located Pleckstrin Homology (PH) domain and multiple tyrosine residues within consensus sequences for binding of SH2 domain containing proteins. This includes putative binding sites for the DRK and SHC adaptor proteins, PLC-γ, the regulatory subunit of phosphoinositide 3-kinase (PI3-K) and the CSW tyrosine phosphatase. Thus, DOS may serve as a multisite adaptor for proteins that trigger RAS-dependent and RAS-independent signaling events upon RTK stimulation. Consistent with this model, DOS becomes phosphorylated by a constitutively activated form of SEV. Further experiments suggested a link between SEV, CSW, and DOS. While transfected SEV and CSW associate in *Drosophila* SL2 cells in a manner that does not require the CSW SH2 domains nor tyrosine phosphorylation of SEV, the CSW SH2 domains are critical for binding to tyrosine phosphorylated DOS. CSW, as well as the vertebrate homologe SHP-2, contain tandem N-terminal SH2 domains. Mutations in either CSW SH2 domain results in partial loss of DOS binding, whereas mutation of both SH2 domains completely disrupts binding to DOS [42]. In the case of IRS-1 and GAB2, two tyrosine residues were identified as binding sites of the SH2 domains of the vertebrate CSW homologue SHP-2 [36,43]. Analysis of the DOS sequence also revealed two putative binding motifs ($LQY_{801}FDL$ and $VVY_{854}RSV$) that match the consensus binding site (V/LxYxxV/L) for the CSW SH2 domains [44]. Mutational analysis proved the in vivo relevance of these tyrosine residues for DOS function and recent experiments demonstrated that these sites are indeed required for binding of CSW to DOS [45,110]. A key question toward understanding the role of CSW is the identification of CSW substrates. Although CSW is able to dephosphorylate DOS, it still remains unclear how binding of CSW to DOS generates a positive signal for R7 cell development [41,46]. Analysis of the role of CSW in TOR RTK-mediated signaling has indicated some important differences. Here, CSW binds to the TOR RTK in an SH2-dependent manner thereby regulating dephosphorylation of the RAS-GTPase activating protein (RAS-GAP) SH2 domain binding site on TOR. In addition, CSW becomes phosphorylated on sites that allow interaction with the DRK SH2 domain, a function of CSW that is dispensable in the case of SEV-mediated signaling [42,47].

One intriguing aspect of the genetic analysis of DOS was that only the CSW SH2 domain binding site is crucial for the in vivo function of the DOS protein, whereas removal of the predicted binding sites for PI3-kinase, SHC and PLCγ did not interfere with normal DOS function during development [45]. At least in the case of PLCγ, there is genetic evidence for an involvement in DER and SEV signaling [38]. A mutation in the PLCγ gene, *small wing (sl)*, results in supernumerary R7 photoreceptor cells. This indicates that PLCγ acts as a negative regulator of RTKs signaling. Further evidence was provided by the observation that *sl* mutations strongly enhance the phenotype of mutated forms of the GTPase-activating protein GAP1 [48]. The most prominent mutant phenotype of *Gap1* flies is the presence of additional R7 cells, even in the absence of a functional SEV protein [49–51]. The sequence homology of GAP1 to the mammalian GTPase activating proteins suggests that GAP1 fulfills a similar function by enhancing the intrinsic GTPase activity of RAS1 in the R7 precursor cell and thereby negatively regulate signaling. The in vivo activity of GAP1 is positively regulated by Ca^{2+} and inositol-tetraphosphate (IP4) sensitive domains adjacent to the catalytic domain [48]. Both release of Ca^{2+} from internal stores and production of IP4 are consequences of RTK induced activation of PLCγ. Hence, activation of the SEV RTK stimulates the activities of two counteracting proteins. The different kinetics of SOS and GAP1 activation in response to stimulation of the RTK might therefore determine the duration and strength of RAS activity.

Another negative regulator of DER, and most likely SEV signaling, is Sprouty (STY). Initially, STY has been described as a novel protein whose expression is induced in response to activation of the *Drosophila* FGF-like RTK Breathless and which antagonizes Breathless signaling during tracheal de-

velopment [52]. Recently, it has been shown that STY fulfills a more general role in RTK signaling and, in contrast to its nonautonomous function in tracheal development, acts in a cell-autonomous fashion in the eye to downregulate signaling from DER and SEV [53,54]. Based on the observation that STY is localized to the plasma membrane, together with experiments demonstrating the interaction of STY with DRK and GAP1 in vitro, it has been proposed that STY mediates GAP1 recruitment to the membrane and blocks the ability of DRK to interact with SOS. Since STY expression is dependent on activity of the same pathway that it regulates, this provides an efficient way to terminate or modulate RTK signaling and thereby preventing inappropriate cellular responses due to sustained signaling.

4. Downstream of Ras

Signal propagation from RAS1 to the nucleus in the SEV, DER and TOR pathways involves the sequential activation of the kinases D-RAF/RAF-1, D-SOR1/MEK and RL/MAPK [55]. The phenotypic analysis of various combinations of loss- and gain-of-function alleles of these genes has complemented and confirmed the detailed biochemical characterisation of the homologous proteins in vertebrates [56] and suggested that the same chain of phosphorylation and protein–protein interaction events described for RAF-1, MEK and MAPK in mammalian cells also exists in *Drosophila*. Although this sequence of events downstream of RAS has been well established, several important aspects have to be discussed in more detail in the context of R7 cell development. First, in addition to RAF, a number of RAS effectors have been reported including the small GTPase RAL and PI3-kinase [57]. This raises the question of whether RAF is the only effector of RAS1 required for photoreceptor cell differentiation. Expression of effector loop variants of activated $RAS1^{V12}$ that specifically blocked either the RAF, PI3-kinase, or RAL branch showed that the 'RAF-only' mutant is sufficient to specify R7 cell development [58]. Furthermore, overexpression of wild-type or mutated forms of the catalytic subunit of PI3-kinase, Dp110, did not interfere with photoreceptor cell specification but with cell growth [59].

Second, RAF activation appears to be a multistep process. It requires localization of RAF to the membrane through interaction with RAS-GTP and also requires interaction of RAF with other proteins, phosphorylation by other kinases, and oligomerization [60]. In the context of R7 cell differentiation, again genetic approaches have been highly successful in uncovering genes that regulate D-RAF activity and signal transmission to MAPK. Expression of a constitutively activated version of RAS1 ($RAS1^{V12}$) or a hybrid construct consisting of the D-RAF kinase domain fused to the extracellular and transmembrane domains of a constitutively activated TOR RTK (TOR^{4021}-RAF) under the control of the *sevenless*-enhancer result in a multi-R7 cell phenotype which have been used for modifier screens to isolate novel components of the RAF–MEK–MAPK signaling complex [22,23,61,62]. A rather complex, yet incomplete picture is beginning to emerge. Structural features of RAF include an amino-terminal regulatory domain, a carboxy-terminal catalytic domain, two RAS binding motifs in the amino-terminal part and two phosphoserine residues (S259 and S621 in RAF-1 and S388 and S743 in D-RAF) that mediate binding to proteins of the 14-3-3 family. Mutations in two 14-3-3 genes encoding different isoforms interfere with signaling from activated $RAS1^{V12}$ and TOR^{4021}-RAF in the eye [63,64]. The functional role of 14-3-3 in regulating RAF activity may be twofold [60]. In the inactive state, the RAF N-terminal regulatory domain exerts an inhibitory effect on the C-terminal catalytic domain and binding of 14-3-3 to S259 may help to maintain RAF in the inactive conformation. Consistent with this model, mutation of S259 in RAF-1 or S388 in D-RAF results in increased RAF activity and, when expressed in the developing eye, additional photoreceptor cells are recruited [65]. The successive interaction of both RAS binding domains with membrane localized RAS-GTP leads to displacement of 14-3-3 from the N-terminal binding site and relieves the inhibitory effect of the regulatory domain. Yet, 14-3-3 can still bind to RAF through interaction with the C-terminally phosphoserine binding motif. Putative functions of this binding might be stabilizing the ac-

tive conformation of RAF or recruitment of additional components to the signaling complex.

Further genetic evidence for the requirement of additional signals for full RAF activation was provided by mutational analysis in the TOR RTK signaling pathway. Complete removal of TOR or D-Raf function produces more severe mutant phenotypes in the embryo than removal of RAS1 function [66]. One of the missing links could be filled by connector enhancer of KSR (CNK), a novel RAF interacting protein [67]. In the heterozygous state, mutations in the *cnk* gene suppress the eye phenotype of activated $RAS1^{V12}$ but not of TOR^{4021}-RAF, suggesting that CNK acts downstream of RAS1 but upstream or in parallel to D-RAF. CNK has a multidomain architecture with a SAM and a PDZ domain, several tyrosine residues within consensus binding sites for binding the SH2 domains of DRK, SHC, and the regulatory subunit of PI3-kinase, as well as a PH domain that might be important for localization of CNK at the plasma membrane. Hence, CNK might act as a multisite adaptor for proteins regulating RAF activity and, in addition, it might target RAF to specific subcellular localizations. Intriguingly, overexpression of wild-type CNK strongly enhances the phenotype of $RAS1^{V12}$ and suppresses the Tor^{4021}-RAF phenotype. Together with experiments demonstrating tyrosine phosphorylation of CNK in response to RTK activation, these data suggest that CNK can only fulfill its function as a positive signaling component upon RAS1 activation.

Another critical component regulating signal propagation from RAS1 to MAPK is kinase suppressor of RAS (KSR). Consistent with a general role for KSR in RTK-mediated signaling, homologous proteins have been identified in *C. elegans*, mouse, and human [68–70]. In a similar manner as CNK, mutations in the *Drosophila ksr* gene suppress the $RAS1^{V12}$ but not the TOR^{4021}-RAF phenotype in the eye [70]. This indicated a requirement of KSR function downstream or in parallel to RAS1 but upstream or in parallel to D-RAF. Assuming that the direct interaction of D-RAF with RAS1 is not sufficient to fully stimulate D-RAF, one attractive model for KSR function would be the direct or indirect regulation of D-RAF activity. However, biochemical studies of mouse KSR expressed in *Xenopus* oocytes and in cell culture has revealed a rather complex picture. It has been reported that KSR is a ceramide-activated protein kinase that phosphorylates and activates RAF [71]. Other studies showed that neither RAF nor KSR are substrates for the kinase activity of the other protein. Instead, it appears that KSR cooperates with RAS and facilitates signal propagation through direct or indirect interaction with RAF, MEK, MAPK, and 14-3-3 proteins [72–77]. KSR might, therefore, act in part as a scaffold protein within the RAF/MEK/MAPK module. Consistent with this model, KSR translocates from the cytoplasm to the plasma membrane in RAS-dependent manner where it forms a complex with membrane associated RAF. This might provide a mechanism to localize KSR bound MEK to activated RAF at the membrane. On the other hand, KSR itself is a substrate for MAPK phosphorylation in response to RAS activation [77]. The functional consequence of this phosphorylation event remains to be determined, since mutation of the phosphorylation sites had no effect on the ability of KSR to augment RAS signaling in *Xenopus* oocytes. Phosphorylation of KSR might play an important role in regulating the kinase activity of KSR. However, the physiological substrates of KSR are still unknown. One further puzzling result was obtained upon overexpression of KSR. Even though KSR has been identified by genetic means as a positive regulator of RAS-mediated signaling, overexpression of KSR inhibits MAPK activation in tissue culture cells and blocks R7 cell formation in the eye [77]. Hence, one critical parameter for the biological functions of KSR is the expression level of the protein. This illustrates that signaling pathways cannot only be viewed as simple molecular on/off switches but as sophisticated networks of proteins whose expression/activity levels have to be tightly controlled in order to induce a cell-type-specific response. This is also exemplified in PC12 cells, where changes in the dynamics of signaling in the RAS/MAPK pathway result in different cellular outcomes [56].

Despite these complexities, the major convergence point of the RTK signaling seems to be Rolled (RL)/MAPK [78,79]. This conclusion can be drawn from the phenotypic and biochemical analysis of a dominant gain-of-function allele of *rl*, rl^{Sem}, isolated in a genetic screen for mutations that bypass the require-

ment for SEV activity [80]. The phenotypes of rl^{Sem} animals are similar to those described for constitutively activated versions of TOR, DER, and SEV RTKs. The molecular defect in rl^{Sem} is a single amino acid substitution (D334N) in the catalytic domain. The functional importance of D334 for regulating RL/MAPK activity was clarified by characterization of a novel cytoplasmic protein tyrosine phosphatase, PTP-ER [81]. Like the gain-of-function mutation rl^{Sem}, loss-of-function mutations in the PTP-ER gene locus produce ectopic R7 cells. Conversely, overexpression of wild-type PTP-ER in the developing eye blocks photoreceptor cell differentiation. The genetic and biochemical analysis of PTP-ER verified that PTP-ER associates with and dephosphorylates MAPK, thereby regulating MAPK activity. In addition, PTP-ER itself is a target for MAPK phosphorylation, suggesting that RL/MAPK might negatively regulate its own activty via phosphorylation and activation of PTP-ER. The D334N substitution renders the RL^{Sem} protein resistent to dephosphorylation and downregulation by PTP-ER without affecting the PTP-ER phosphatase activity per se. At first sight this provides a satisfactory explanation for the rl^{Sem} phenotype. However, rl^{Sem} flies display a much stronger eye phenotype than flies lacking PTP-ER function. One possible explanation could be a general resistence to other MAPK-specific phosphatases such as the phosphothreonine- and phosphotyrosine-specific phosphatases MKP-1 and MKP-3 found in vertebrate cells [82,83]. The intricate balance between the activities of RL/MAPK and different phosphatases obviously does not only regulate signal duration and intensity upon RTK stimulation but also keeps MAPK activity at a low basal level in unstimulated cells. This might explain why rl^{Sem} mutant flies have ectopic R7 cells in the absence of an activated SEV RTK.

5. The nuclear targets of RL/MAPK

Activation of RL/MAPK results in its translocation from the cytoplasm to the nucleus where it regulates the activity of transcription factors by serine and threonine phosphorylation. So far, three proteins have been identified as nuclear targets of RL/MAPK. Two of these proteins, YAN and Pointed (PNT), belong to the Ets domain family of transcription factors [84–86]. The third protein is the *Drosophila* homologue of one of the signal-responsive bZIP transcription factors of the AP-1 family in vertebrates, namely C-JUN [87,88].

The role of D-JUN in specifying photoreceptor cell fate remains elusive. Ser82, Thr92 and Thr107 on D-JUN were identified as targets for RL/MAPK phosphorylation in vitro and expression in the developing eye of a D-JUN transgene lacking the phospho-acceptor sites suppressed the differentiation of photoreceptor cells. Conversely, replacing the RL/MAPK phosphorylation sites of D-JUN with phosphorylation mimicking Asp residues promoted photoreceptor cell differentiation [89]. These experiments suggested that RL/MAPK induced phosphorylation of D-JUN is a crucial step for transcriptional regulation of target genes in the R7 precursor cell. However, the role of D-JUN has been questioned by the observation that loss-of-function mutations in the *D-jun* gene neither affect photoreceptor cell differentiation nor DER and Torso RTK-mediated signaling in the embryo [90,91]. One possible explanation for these obviously contradictory results could be that D-JUN has a redundant function during eye development. Occupancy of the JUN target sequences by mutated D-JUN proteins might cause a more severe phenotype due to interference with gene transcription.

Pointed (PNT) was originally identified as a mutation affecting the development of the embryonic nervous system and subsequently shown to be required for normal photoreceptor cell differentiation. Two alternatively-spliced transcripts are expressed, PNT^{P1} and PNT^{P2}. Both proteins share the C-terminal sequences including the Ets domain but differ in their N-terminal sequence [86,92]. PNT^{P2} has a single MAPK phosphorylation consensus site (Thr151) and, at least in vitro, PNT^{P2} is a substrate for RL/MAPK [93,94]. Using an Ets binding domain reporter construct, it has been demonstrated that PNT^{P2} becomes a strong transcriptional activator upon stimulation of the RAS/MAPK pathway [93]. As anticipated, the crucial step in the activation of PNT^{P2} is phosphorylation of Thr 151. In the eye imaginal disc, PNT^{P2} is expressed in all nondifferentiated ommatidial precursor cells and removal of

PNT^{P2} activity results in the absence of photoreceptors, including R7. This phenotype can be rescued by a transgene encoding the PNT^{P2} protein whereas a mutant version lacking the unique MAPK phosphorylation site does not rescue [93,94]. Thus, activation of the MAPK pathway is necessary for PNT^{P2} to act as a positive regulator of neuronal development.

The second Ets domain protein that acts downstream of RL/MAPK is YAN. However, the mutant phenotype of *yan* hypomorphic alleles is the converse of that described for PNT^{P2}, namely the recruitment of extra photoreceptor cells, most prominently additional R7 cells [84,85]. YAN contains eight MAPK phosphorylation consensus sites and at least some of these sites are used in vitro. Cotransfection of YAN and PNT^{P1} in tissue culture cells decreases PNT^{P1}-mediated transcription of a reporter construct containing multiple Ets binding sites. However, this repression is alleviated by coexpression of activated versions of either RAS ($RAS1^{V12}$) or MAPK (RL^{Sem}), indicating that activation of the MAPK pathway negatively regulates the ability of YAN to repress transcription, probably via phosphorylation of YAN [93]. Wild-type YAN is expressed in a number of tissues during development, including all undifferentiated cells behind the morphogenetic furrow. YAN disappears from the nuclei of ommatidial cells as soon as they start to differentiate. A mutant version of YAN lacking all the predicted MAPK phosphorylation sites remains in the nuclei of ommatidial cells when expressed in the eye imaginal disc and differentiation of neuronal and nonneuronal cells is blocked [95].

One conclusion drawn from these studies is that dephosphorylated YAN keeps cells in an undifferentiated state. RL/MAPK induced phosphorylation has a dual function: it relieves the inhibitory influence of YAN as a general repressor of differentiation and it enhances the transcriptional activity of PNT^{P2} in order to induce the expression of target genes.

6. Protein degradation: removing the block in the R7 precursor cell

The best candidate for a RAS/MAPK inducible target gene in the nucleus of the R7 precursor cell is *phyllopod (phyl)* [96,97]. Characteristic of the PHYL protein is a highly basic domain followed by a strongly acidic domain. Genetically, PHYL has been placed downstream of RL/MAPK and YAN in the SEV pathway. Whereas PNT and YAN are expressed and required in all photoreceptor cells, PHYL expression is restricted to R1, R6, and R7. In the absence of PHYL these three cells adopt the cone cell fate. Conversely, ectopic expression of PHYL is sufficient to transform cone cells into R7 cells. Activation of the RAS/MAPK pathway in the cone cell precursors is accompanied by PHYL expression whereas in *sev* mutant flies only R1 and R6 express PHYL.

Remarkably, Seven in absentia (SINA), another nuclear protein, is specifically required for R7 cell development [98], even when the RAS/MAPK pathway is constitutively activated or when PHYL is ectopically expressed in the cone cell precursors [20,61,62,80,97]. Based on their expression pattern and mutant phenotype, SINA and SEV are similar, but SINA expression does not depend on a functional SEV protein [98]. One attractive working model to account for the roles of SINA and PHYL in the R7 precursor cell is that SINA acts as a regulator of R7 cell-specific differentiation genes but only after PHYL expression has been induced by the RAS/MAPK pathway. The characterization of the Tramtrack (TTK) protein provided a mechanism how this is achieved. Two alternatively spliced variants of the *ttk* gene encode proteins of 69 kDa (TTK69) and 88 kDa (TTK88). Both TTK69 and TTK88 share a BTB/POZ domain but contain different zinc fingers in their carboxy-terminal regions [99]. Mutational analysis provided evidence that TTK acts as an inhibitor of neuronal differentiation. In the eye, removal of only TTK88 results in the formation of additional R7 cells [100]. Conversely, ectopic expression of TTK88 in all presumptive photoreceptor cells completely blocks neuronal differentiation of these cells. Simultaneous expression of PHYL counteracts the effects of ectopic TTK88 expression, but only in the presence of SINA. Expression studies verified that PHYL expression was always accompanied by the loss of the TTK88 protein and conversely, in *phyl* mutant flies, high level of TTK protein is detected in the presumptive R1, R6, and R7 cells which then differentiate as nonneuronal cone cells [101,102]. Consistent with these observations, TTK88 protein

is detected only in the nuclei of undifferentiated cells and the cone cells during eye development in wild-type animals [103]. One explanation for these results, posttranscriptional downregulation of the TTK88 protein in the presence of PHYL and SINA, was confirmed by expressing all three proteins in S2 cells. The half-life of TTK88 was greatly reduced when coexpressed with PHYL and SINA. The physical interaction between these three protein targets TTK88 for degradation by the ubiquitin/proteasome pathway [101,102]. SINA binds to the ubiquitin conjugating enzyme UBCD1, thereby providing a link between the ubiquitin-dependent proteolytic pathway and degradation of TTK88 upon binding of SINA and PHYL. Furthermore, degradation of TTK88 can be blocked by treatment of TTK88 expressing cells with proteasome-specific inhibitors.

In summary, activation of the Ras/MAPK pathway in the R7 precursor cell results in the removal of two different blocks. First, inactivation of YAN by phosphorylation allows the precursor cell to become differentiated and second, degradation of TTK88 restricts the cell to the neuronal fate. However, a number of questions remain open. The expression patterns of PHYL and SINA overlap in the R1, R6 and R7 precursor cells, yet, loss of SINA function only interferes with specification of the R7 cell. Second, in addition to the expression in the cone cells, the TTK88 protein is detected in all undifferentiated cells that have the potential to develop as photoreceptor cells [103] indicating that TTK88 degradation is a prerequisite for photoreceptor cell differentiation in general. Hence, besides SINA and PHYL, other proteins must exist that fulfill the role of targeting TTK88 for degradation in these cells. One potential candidate is EBI, a nuclear protein that belongs to the F-box/WD40 repeat-containing protein family [104]. Strikingly, *ebi* mutations interfere only with DER-mediated signaling processes during development and removal of EBI function in the eye results in the absence of photoreceptor cells. Expression of a dominant negative version of EBI during eye development delays neuronal recruitment which correlates with persistent nuclear expression of the TTK88 protein. Furthermore, TTK88 degradation upon ectopic expression of PHYL or an activated form of the DER RTK can be dominantly suppressed by *ebi* mutations. Although the precise biochemical function of EBI is not known, the involvement of other F-box/WD40 repeat-containing proteins in protein downregulation implies a similar function for EBI in DER-mediated signaling. The requirement of both SEV and DER signaling for R7 cell differentiation suggests that EBI cooperates with PHYL and SINA in TTK88 degradation in the R7 precursor cell.

7. The question of specificity

As outlined above, the RAS/MAPK pathway is required for the proper development of all ommatidial cells, via stimulation of DER and additionally SEV in the R7 precursor cell [6–8]. Hence, at some point the information for cell identity has to be integrated. In the case of the R7 cell, this could happen at different levels. At the level of receptor activation, the ligand BOSS and the receptor SEV are required only for R7 cell development. It could thus be envisaged that SEV might be able to recruit and activate a unique set of proteins in addition to the RAS/MAPK-pathway to specify R7 cell development. However, SEV function can be replaced by activated versions of other RTKs such as DER [6]. Furthermore, dependent on the stage of ommatidial assembly, activated SEV can produce photoreceptors other than R7 [21]. These experiments imply that it is not the type of RTK but the time point of signal reception which is critical for cell fate determination. However, what mechanisms restrict the developmental potential of a cell at a given time point? One potential mechanism could involve the distinct ontogenesis of the different photoreceptor cells. Photoreceptors R8, R2/R5, R3/R4 arise from cells born anterior to the morphogenetic furrow, whereas R1/R6 and R7 are derived from a second wave of mitosis posterior to the furrow. Interestingly, the expression pattern of the *phyl* gene in R1, R6, and R7 reflects the distinct ontogenesis of these cells. Nevertheless, there is the need for additional subtype-specific information in R1, R6, and R7 that allows RAS/MAPK induced expression of PHYL in these cells or prevents PHYL expression in the other photoreceptor precursors. In addition to PHYL, a number of other nuclear proteins have been described that are expressed in distinct subsets of ommatidial cells and,

when mutated, alter the developmental potential of a cell [105]. Ectopic expression of Rough (RO), a homeo domain containing protein expressed in R2/R5 and R3/R4 photoreceptors, and required in R2/R5 for correct specification of the R3/R4 cell fates, transforms the R7 cell into an R1–R6-like photoreceptor cell. Most strikingly, neuronal differentiation of this cell still depends on SEV activity [106,107]. Similar results were obtained with Seven-up (SVP), a protein which belongs to the steroid receptor family and is expressed in the R3/R4/R1/R6 photoreceptors. In *svp* mutant ommatidia, these cells differentiate as R7 photoreceptors whereas ectopic expression of *svp* specifies the presumptive R7 cell as an R1–R6 photoreceptor cell [108,109]. The presence of SVP can also explain why photoreceptors R1 and R6, despite contacting R8 and expressing SEV, SINA, and PHYL, do not develop as R7 photoreceptors. Based on these results it has been suggested that different combinations of transcription factors, such as RO and SVP, expressed in defined subsets of cells, predispose a cell to follow a specific developmental fate [105]. Clearly, along with the genetic and biochemical characterization of additional components of the SEV signal transduction cascade, one major focus of future research will be to determine how these sub-type-specific factors achieve their appropriate expression patterns.

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