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The *Drosophila* dual-specificity ERK phosphatase DMKP3 cooperates with the ERK tyrosine phosphatase PTP-ER

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SUMMARY

ERK MAP kinase plays a key role in relaying extracellular signals to transcriptional regulation. As different activity levels or the different duration of ERK activity can elicit distinct responses in one and the same cell, ERK has to be under strict positive and negative control. Although numerous genes acting positively in the ERK signaling pathway have been recovered in genetic screens, mutations in genes encoding negative ERK regulators appear underrepresented. We therefore sought to genetically characterize the dual-specificity phosphatase DMKP3. First, we established a novel assay to elucidate the substrate preferences of eukaryotic phosphatases in vivo and thereby confirmed the specificity of DMKP3 as an ERK phosphatase. The *Dmkp3* overexpression phenotype characterized in this assay permitted us to isolate *Dmkp3* null mutations. By genetic analysis we show that DMKP3 and the tyrosine phosphatase PTP-ER perform partially redundant functions on the same substrate, ERK. DMKP3 functions autonomously in a subset of photoreceptor progenitor cells in eye imaginal discs. In addition, DMKP3 function appears to be required in surrounding non-neuronal cells for ommatidial patterning and photoreceptor differentiation.

Key words: *Drosophila*, DMKP3, CL100, Eye dual-specificity phosphatase, Signal transduction

INTRODUCTION

Mitogen activated protein kinases (MAPKs) are evolutionarily conserved enzymes in signaling pathways regulating cellular fates and responses to a variety of extracellular signals. Four subgroups of the MAPK family are defined in metazoans – ERK, JNK, p38 and ERK5 (Chang and Karin, 2001). MAPKs are activated by phosphorylation of a threonine and a tyrosine residue in the so-called P-loop by dual-specificity kinases, which in turn are substrates of other kinases. This cascade-like arrangement of three kinases is predicted to make the modules sensitive to regulation and to predispose them to mediate switch-like processes (Huang and Ferrell, 1996).

A switch mechanism requires the possibility to also counteract the stimulatory activity of the dual-specificity MAPK kinases. This is achieved by phosphatases capable of dephosphorylating either the threonine residue or the tyrosine residue [serine/threonine phosphatases (STPs) or protein tyrosine phosphatases (PTPs)], or both [dual-specificity phosphatases (DSPs)] (Camps et al., 2000; Keyse, 2000). As DSPs exhibit a high specificity towards MAP kinases and within those to a subset of the family, they have also been designated MKPs (for MAP kinase phosphatases). DSPs are comprised of an N-terminal CH2 domain (for Cdc25 homology) implicated in substrate binding, which also contains a basic docking site that directly binds to the negatively charged common docking (CD) domain of MAPKs (Theodosiou and Ashworth, 2002). Upon MAPK binding the phosphatases undergo a conformational transition that stimulates the activity of the C-terminal catalytic domain (Camps et al., 1998). The prevalence of this interaction is illustrated by a dominant ERK mutation termed *Sevenmaker*, which affects the charge of the CD domain such that the physical interaction of ERK with its DSP is greatly impaired. Thereby the phosphatase activity is compromised and ERK kept in an activated state (Bott et al., 1994; Brunner et al., 1994; Chu et al., 1996). Flies carrying the dominant *Sevenmaker* mutation are viable, but display multiple phenotypes characteristic of an overactive RAS pathway, for example rough eyes because of the recruitment of extra photoreceptor cells. Numerous other studies have established the *Drosophila* eye as an excellent model to genetically dissect ERK signaling (Dickson and Hafen, 1994; Freeman, 1998).

The *Drosophila* compound eye is composed of approximately 800 ommatidia, each built up of an equivalent of 19 cells, eight of which are neuronal photoreceptor cells. Photoreceptors contain specialized microvillar stacks of membrane termed ‘rhabdomeres’. The rhabdomere of the R7 photoreceptor neuron is situated in the center of the ommatidial unit on top of that of the R8 cell. The rhabdomeres of the remaining six outer photoreceptors are arranged such that ommatidia appear in two different chiral forms. Chirality is conveyed by the R3 and R4 cells, which adopt an asymmetrical position within the ommatidium (Fig. 7A) (Wolff and Ready, 1993).

Ommatidial patterning starts in an orderly fashion at the

posterior border of eye imaginal discs in third-instar larvae. The differentiation process is accompanied by a visible indentation in the epithelium called the 'morphogenetic furrow' that sweeps across the disc. Within the morphogenetic furrow, groups of cells form 'rosette'-like clusters from which cells are singled out by lateral inhibition to become the neuronal R8 photoreceptor cell (Fig. 7A). This process requires RAS activity but appears to be independent of the receptor tyrosine kinase EGFR (Dominguez et al., 1998; Halfar et al., 2001). In a stepwise manner, whereby differentiating cells recruit undifferentiated neighbors, the ommatidia are assembled: When the R8 cell is determined it produces the TGFα-like EGFR ligand Spitz. Spitz in turn activates EGFR signaling in two adjacent cells and thereby recruits them to the cluster to form the R2/R5 pair. The new cells attract the presumptive R3/R4 pair by a similar mechanism (Freeman, 1998). Initially, one or two additional cells are incorporated into the growing cluster (Tomlinson, 1985). These so-called mystery cells are expelled from the precluster when the R3/R4 pair differentiates (Fig. 7A). A gradient in Frizzled activity originating from the dorso-ventral midline of the eye field (equator) generates a difference between the initially equivalent R3 and R4 precursors that is then amplified by a Notch-Delta interaction. The cell closer to the equator will exhibit high Delta levels and will be instructed to become a R3 cell. The more polar cell has high Notch activity and differentiates as R4 (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). Subsequently, the dorsal and ventral preclusters rotate by 90° in opposite directions thereby establishing chirality. Of the last three photoreceptor cells recruited to the precluster, the middle cell chooses the R7 fate and the two others form the R1/R6 pair.

In contrast to the R8 cell, the remaining photoreceptors are dependent on high and/or sustained Ras pathway activity (Halfar et al., 2001). Overactivation of ERK by constitutively active RAS or receptor tyrosine kinases results in severe differentiation defects (Bishop and Corces, 1988; Lesokhin et al., 1999; Lowy and Willumsen, 1993). This phenotype is mimicked by loss-of-function mutations in negative regulators of the RAS signaling pathway, like Gap1 or the ETS transcriptional inhibitor Yan. Surprisingly, apart from *PTP-ER*, mutations in genes coding for ERK phosphatases have not been identified based on a similar phenotype. It is thus possible that various phosphatases perform redundant functions on ERK. Redundancy could explain why mutants of the mouse DSP MKP1 and the *C. elegans lip-1* are fully viable (Dorfman et al., 1996; Berset et al., 2001). Likewise, HE-PTP knockout mice devoid of the ERK tyrosine phosphatase are phenotypically normal and the corresponding *Drosophila PTP-ER* mutants only exhibit slight defects (Gronda et al., 2001; Karim and Rubin, 1999).

Here we show that mammalian dual specificity phosphatases MKP3 and MKP4 and its *Drosophila* homolog DMKP3 (MKP3 – FlyBase) selectively inhibit ERK in vivo. Analysis of *Dmkp3* loss of function mutations reveals that DMKP3 performs redundant and non-redundant functions on ERK together with the tyrosine-phosphatase PTP-ER. Our results further suggest that RAS signaling is not only required within the photoreceptors to properly differentiate, but also performs a function in surrounding cells to shape the developing ommatidium. Together, we provide evidence that ERK is negatively regulated by an interplay of different phosphatases in a cell-context-dependent manner.

MATERIALS AND METHODS

Drosophila strains

The following mutant and transgenic strains have been used: *EP3142* (Rorth et al., 1998), *PTP-ER^{XE3022}* (Karim and Rubin, 1999), *DER^{elpB1}* (Baker and Rubin, 1989), *rl^{Sem}* (Brunner et al., 1994), *Df(2R)rl^{10a}* (Hilliker, 1976), *sev^{S11}* (Basler et al., 1991), *sev-raf^{torY9}* (Dickson et al., 1992), *sev-ras^{V12}* (Karim et al., 1996), *E(spl)mδ0.5-lacZ* (Cooper and Bray, 1999), *sevE(f4)-lacZ* (P. Maier and E.H., unpublished), *UAS-p35* (Hay et al., 1994), *UAS-puc* (Martin-Blanco et al., 1998) and *Δ2-3* (Robertson et al., 1988). The *GAL4* lines *sev-GAL4^{K25}* and *sev-GAL4$^{KK12/3}$* have been generated in our lab, 69B and GMR-GAL4 were obtained from the Drosophila Stock Center, and *en-GAL4^{54}* and *en-GAL4^{33}* are gifts from C. Dahmann and K. Basler. *FRT80B* stocks have been described (Xu and Rubin, 1993).

Germline transformation

MKP3, MKP4, MKP5, M3/6 and CL100 encoding cDNAs were generously provided by M. Muda and S. Arkinstall (Serono Pharmaceuticals), and *Dmkp3* full-length cDNA SD06439 was obtained from Research Genetics. The cDNAs were either subcloned into the *Drosophila* transformation vectors *pUAST* (Brand and Perrimon, 1993) or into the *sevE/hsp70P* vector pDN448 (kindly provided by D. Nellen). *Drosophila* germline transformation of the *y w^{1118}* stock was performed as previously described (Basler et al., 1991). Several independent transformant lines were established per construct.

Generation of *Dmkp3* mutations by transposase-mediated P-element mobilization and reversion mutagenesis

EP3142 was mobilized in an isogenized and phenotypically wild-type stock by *Δ2-3* transposase (Robertson et al., 1988) and mosaic males were crossed to *sev-GAL4* virgins. Individuals of the F1 progeny with rough eyes potentially bearing a reoriented EP-element were analyzed by PCR for exhibiting an inverted EP-element using a P3′ and a *Dmkp3*-specific primer. Six of 131 independent positives had an EP-insertion closer to the Start-codon, four of which had the new *EP* integrated into the 5′ UTR of *Dmkp3*. The insertion-sites upstream to the ATG are as follows: –483 bp (*Dmkp3^{4}*), –132 bp (*Dmkp3^{1}*), –128 bp (*Dmkp3^{2}*) and –11 bp (*Dmkp3^{3}*). All tested lines except for *Dmkp3^{3}*, which has undergone more complex changes (data not shown), still harbor the original EP at –1023 bp. To generate point mutations in *Dmkp3*, we treated *Dmkp3^{11}* males with 20 or 25 mM EMS according to Lewis and Bacher (Lewis and Bacher, 1968) and crossed them to *sev-GAL4* or *GMR-GAL4* females. Among 7500 F1 flies six lines transmitted and exhibited a mutation in the ORF. Mutations were first identified by DHPLC as described (Nairz et al., 2002) and then confirmed by DNA sequencing. The nucleotide changes are (compare with Fig. 3): *Dmkp3^{6}*: GCC → GTC; *Dmkp3^{7}*: CCC → CTC; *Dmkp3^{8}*: CAC → TAC; *Dmkp3^{9}*: ACA → ATA; *Dmkp3^{10}*: GGA → AGA; *Dmkp3^{5}*: AGG → AAG. As the *Dmkp3^{5}* mutation affects a splice-acceptor site (underlined in the triplet), the expected frameshift by one base was corroborated by analysis of *Dmkp3^{5}* cDNA.

Phenotypic analysis

If not indicated otherwise, all phenotypic analyses of adult flies were done in females. Histological sections of eyes and cuticle preparations of embryos were done as previously described (Basler and Hafen, 1988; Riesgo-Escovar et al., 1996). For antibody stainings, eye imaginal discs of wandering third instar larvae were fixed, permeabilized and treated with the following primary antibodies: rat anti-Elav (1:30, a gift from G. Rubin), rabbit anti-β-galactosidase

(1:2000, Cappel). Texas Red, Cy5 and FITC-conjugated secondary antibodies were used at a dilution of 1:200. Generation of clones was performed according to Xu and Rubin (Xu and Rubin, 1993) and Newsome et al. (Newsome et al., 2000). In order to increase the number of clonal boundaries for mosaic analysis, it was performed in a Minute-background (*y, w, eyFLP; FRT80B, M(3)i[55], Pw+(70C)/FRT80B, Dmkp3^{5J4}*). Non-autonomous effects were assayed in flies of the genotype *y, w, hsFLP; FRT80B, ubi-GFP/FRT80B, Dmkp3^{5}* (or *Dmkp3^{2}*) and *y, w, hsFLP; FRT80B, Pw+(70C)/FRT80B, Dmkp3^{5J4}*, respectively.

RNA in situ hybridization

In situ hybridization on eye imaginal discs was performed essentially as described (Lehmann and Tautz, 1994; O'Neill and Bier, 1994). DIG-labeled ssRNA was in vitro transcribed from *Dmkp3* cDNA subcloned into the pCRII-TOPO vector (Invitrogen) by T7 and SP6 polymerase (Roche). Samples were stained for 1 hour.

RESULTS

DMKP3 has a high specificity towards ERK in vivo

In a recent study Kim et al. (Kim et al., 2002) have shown that the *Drosophila* dual specificity phosphatase DMKP3 dephosphorylates ERK, but not JNK or p38 MAP kinases in vitro. Here we provide in vivo evidence in *Drosophila* that DMKP3 is a phosphatase specific for ERK.

We first established an assay to test for substrate preferences of DSPs in transgenic flies. Based on the assumption that overexpression of a specific inhibitor should phenocopy the loss-of-function phenotype of the target, the assay was calibrated with DSPs, whose substrate preferences have been extensively characterized. As a readout, developmental processes known to depend on JNK or ERK activity, respectively, were selected. Misexpression of the JNK and p38 dual specificity phosphatases M3/6 (from mouse), human MKP5 and the *Drosophila* JNK phosphatase Puckered in *Drosophila* embryos cause a dorsal-open phenotype akin to the *JNK/basket* loss-of-function phenotype (Fig. 1F,I and data not shown) (Riesgo-Escovar et al., 1996). However, the same phosphatases do not influence ERK-dependent developmental programs like photoreceptor and wing vein differentiation (Fig. 1D,E,G,H) (Schweitzer and Shilo, 1997). Conversely, the ERK phosphatase MKP3 (from rat) does not disrupt embryogenesis, but interferes with eye and wing development when overexpressed in *Drosophila* (Fig. 1J-L). Three conclusions can be drawn from these experiments: First, the classification of DSPs according to substrate specificity partially derived from in vitro data is qualitatively recapitulated in vivo. Second, the substrate specificity is evolutionarily conserved. Third, even high levels of an ERK- or JNK-specific phosphatase do not affect the other MAPK pathway, suggesting that these phosphatases have a very high degree of substrate specificity.

The specificity of mammalian CL100/MKP1 is controversial. Originally, CL100 was thought to be an ERK phosphatase only, but then it was shown to be specific for JNK and p38 (Camps et al., 2000). Tanoue et al. (Tanoue et al., 2001) recently observed a preference for JNK and p38 and a level-dependent anti-ERK activity. In our assay, human CL100 strongly inhibits both JNK- and ERK-dependent processes and occasionally leads to wing duplications (Fig. 1M-O and Discussion). CL100 thus behaves as a strong JNK and ERK phosphatase in vivo.

The putative *Drosophila* dual specificity phosphatase DMKP has no phosphatase activity on an artificial substrate, but JNK and ERK phosphatase activity in vitro (Lee et al., 2000). DMKP overexpression neither interferes with photoreceptor development nor causes a dorsal-open phenotype, indicating that it might have other substrates than JNK and ERK or that it is a weak phosphatase (data not shown).

Misexpression of *Dmkp3* in wing and eye imaginal discs partially inhibits vein and photoreceptor formation, but overexpression in the embryonic epidermis does not affect dorsal closure (Fig. 1P-R), classifying DMKP3 as an ERK-specific phosphatase.

Dmkp3 interacts genetically with components of the Ras pathway

To further corroborate that DMKP3 acts as a negative regulator of ERK signaling, its position in the RAS/ERK pathway was determined by genetic epistasis experiments. The *sev-Dmkp3* overexpression phenotype is dominantly enhanced by a *Ras* or a *ERK* (*rl^{10A}*) mutation (Fig. 2D,G and data not shown), indicating that RAS and ERK levels are limiting when DMKP3 is overexpressed. Conversely, high DMKP3 levels are sufficient to curb overactivation of the RAS/ERK pathway caused by activating mutations in genes coding for different pathway components. Expression of *sev^{S11}*, *Ras^{V12}* and *raf^{torY9}* transgenes in the eye cause ERK activation and formation of extra R7 photoreceptors, but co-overexpression of *Dmkp3* almost completely suppresses the eye phenotypes (Fig. 2E,H and data not shown). Finally, the interaction of *Dmkp3* with the *ERK* gain-of-function allele *Sevenmaker* (*rl^{SEM}*) was tested. In the wing, *rl^{SEM}* causes extra veins, which are not eliminated by co-overexpression of *Dmkp3*; i.e. *rl^{SEM}* is epistatic to *Dmkp3* (Fig. 2A-C). In contrast, the *rl^{SEM}* rough-eye phenotype caused by the formation of additional R7 cells is almost completely suppressed by high *Dmkp3* levels (Fig. 2F,I). It is possible that the cell context-dependent sensitivity of Rl^{Sem} to *Dmkp3* overexpression is because of different expression levels. This hypothesis is supported by data from Chu et al. (Chu et al., 1996) who showed that the mammalian Sevenmaker homologue ERK2^{D319N} is inactivated by DSPs in COS-7 cells at high, but not in NIH3T3 cells at lower expression. The genetic interactions are consistent with a function of DMKP3 between Raf and Rl^{SEM} and strongly support the interpretation that ERK is the main target of DMKP3 (Kim et al., 2002).

Isolation of *Dmkp3* loss-of-function mutations

The knowledge of a *Dmkp3* gain-of-function phenotype provided a tool to select for *Dmkp3* null mutations. First, an EP element inserted upstream of *Dmkp3* and whose Gal4-inducible promoter directs expression of the neighboring gene *MESR6* (Huang and Rubin, 2000) (Fig. 3A) was mobilized. By screening for the *Dmkp3* overexpression phenotype re-insertions pointing towards *Dmkp3* were selected. The majority of integrations occurred at the original site retaining the original EP. These are phenotypically neutral (e.g. *Dmkp3^{11}*), whereas a few independently derived integrations disrupting the 5′ UTR behave as null alleles (*Dmkp3^{1}* to *Dmkp3^{4}*). In

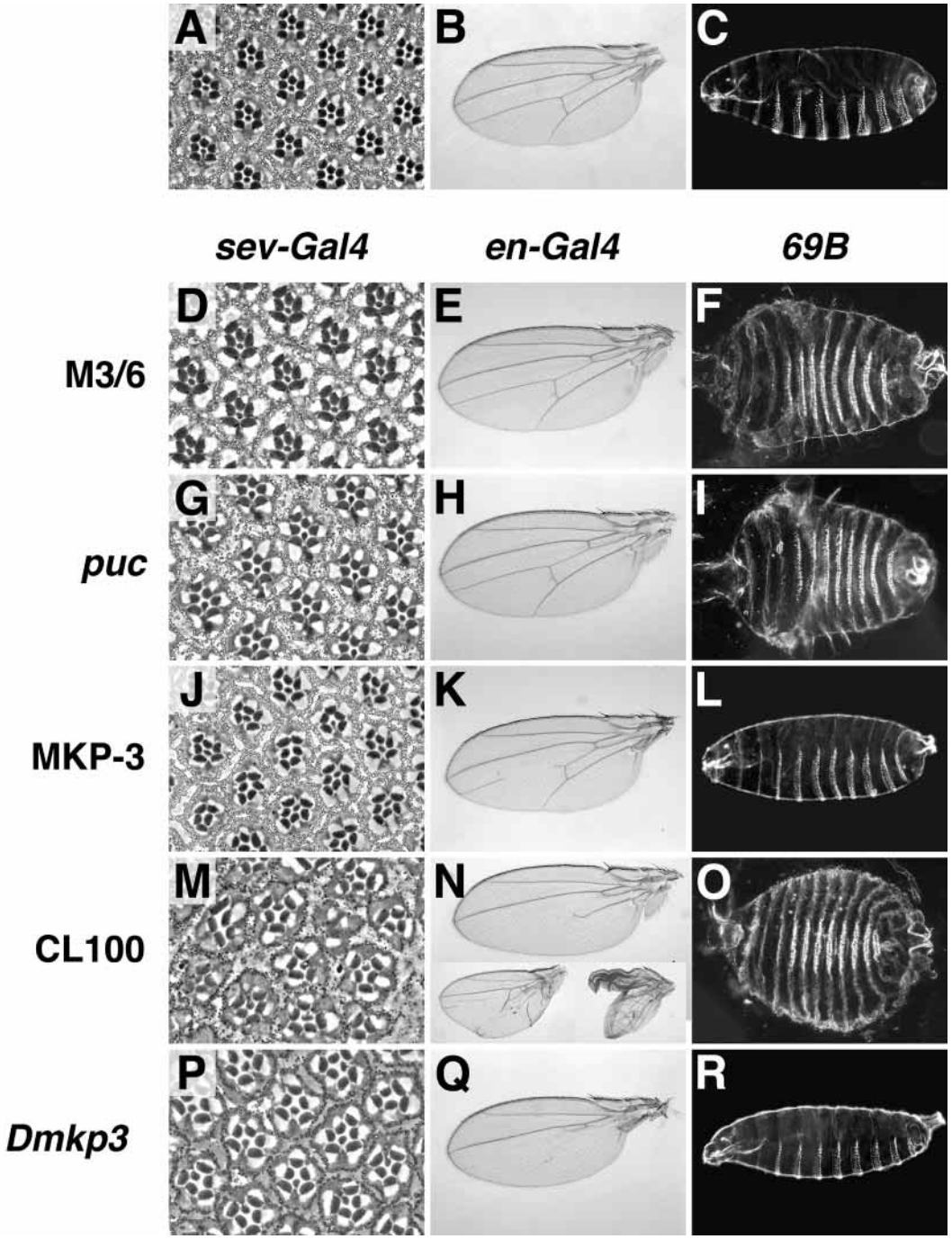

Fig. 1. DMKP3 is an ERK phosphatase. Eye sections (left), wings (middle) and cuticle preparations (right) of strains overexpressing the indicated phosphatases by *sev-GAL4*, *en-GAL4* and *69B*, respectively. Wild-type controls are shown in A-C. Eyes and wings are not affected by overexpression of mammalian M3/6 and *Drosophila puckered* (D,E,G,H), but by MKP-3 and *Dmkp3* (J,K,P,Q). The latter two do not impair embryogenesis (L,R), but the JNK phosphatases cause a dorsal closure defect (F,I). CL100 interferes with all processes (M-O) and even leads to wing duplications (N, lower). Driver lines were *sev-GAL4*^{K25} (D,G,J), *sev-GAL4*$^{KK12/3}$ (M,P), *en-GAL4*^{33} (K,N – upper wing) and *en-GAL4*^{54} (E,H,Q,N – lower wings). *en-GAL4* is expressed in the posterior wing region. The wing shown in K harbors two copies of the MKP-3 transgene and the wing in Q is derived from a male.

order to obtain mutations in the coding region, *Dmkp3*^{11} flies were mutagenized and the progeny was screened for revertants of the overexpression phenotype. Thereby the bona fide null alleles *Dmkp3*^{5} to *Dmkp3*^{10} were isolated (Fig. 3A,B). Because the w^{+} marker of the EP elements would prevent further clonal analysis, the EP elements of *Dmkp3*^{5} were precisely excised to yield allele *Dmkp3*^{5J4} (see also Materials and Methods).

DMKP3 and PTP-ER perform redundant as well as non-redundant functions on ERK

The prototypical mutation activating RAS signaling at the level of ERK is the gain-of-function *rl*^{Sem} allele. *rl*^{Sem}/+ flies are viable, but display multiple phenotypes characteristic of a pathway overactivation. These include female sterility, additional wing veins and rough eyes because of the recruitment of multiple R7 photoreceptor cells (Brunner et al., 1994) (Fig. 2B,F). *UAS-rl*^{SEM} flies possess some additional wing veins even in the absence of a *GAL4* driver, thus suggesting a subtle activation of the pathway (Fig. 4B). *Dmkp3* mutants are viable and fertile and exhibit a mild, but significant increase in wing vein material reminiscent of *UAS-rl*^{SEM} (Fig. 4A,E). In addition, they are slightly rough-eyed. A requirement for DMKP3 during eye development is consistent with its expression in third-instar eye imaginal discs posterior to the

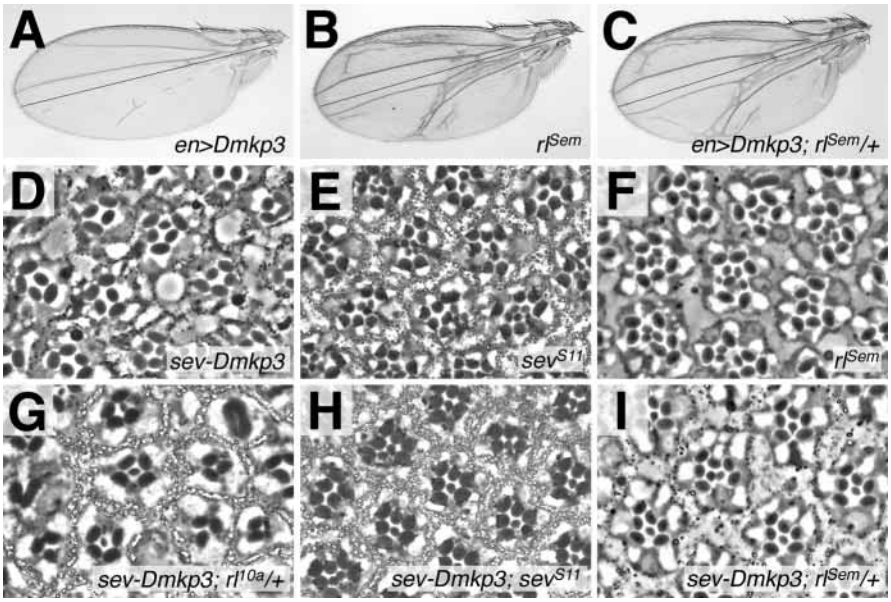

Fig. 2. Epistasis analysis of *Dmkp3* overexpression. A direct *sev-Dmkp3* transgene causes rough eyes (section in D, compare to wild-type in Fig. 1A), which are enhanced by rl^{10A} (G). Note that the direct *sev-Dmkp3* construct causes the loss of outer and central photoreceptors, whereas the *sev-GAL4* driven *Dmkp3* mainly affects the R3/R4 pair, indicating that the time-window for ERK-dependent differentiation is shorter in R7 cells than in R3/R4 cells (Fig. 1P). The construct almost completely rescues sev^{S11} (E,H) and rl^{SEM} (F,I) eyes. However, the rl^{SEM} wing phenotype (B) is unaffected by *en-GAL4*^{33}-driven *UAS-Dmkp3* (A,C). (The line marks the anterior-posterior wing boundary.) Specimens shown in A-C,F,I are from males.

morphogenetic furrow where photoreceptor differentiation occurs (Fig. 3C-E). *Dmkp3* expression is not under transcriptional control of the RAS pathway, because the expression pattern is virtually unchanged in a *sev-ras*^{V12} background (data not shown).

As expected for a specific negative regulatory role of DMKP3 in RAS signaling, the wing null phenotype is dominantly suppressed by the rl^{10A} deficiency uncovering the *ERK* locus (Fig. 4E). Alternatively, *Ellipse*, coding for an activated EGF receptor, and a mutation in *PTP-ER*, encoding an ERK tyrosine phosphatase (Karim and Rubin, 1999), dominantly enhance the *Dmkp3*$^{-}$ phenotype (Fig. 4C-E). Interestingly, *PTP-ER* mutants are viable, slightly rough-eyed, female sterile, but devoid of a wing phenotype. Hence, when combined, the *PTP-ER* and *Dmkp3* mutant phenotypes qualitatively cover the entire spectrum of the rl^{SEM} phenotype. The complementarity and the dominant interaction may indicate that these two phosphatases possess overlapping partially redundant functions. Indeed, the *PTP-ER*$^{XE\text{-}3022}$*; Dmkp3*$^{-}$ (or *PTP-ER*$^{XE\text{-}3022}$*/Df(2R)PuD17; Dmkp3*$^{-}$) double mutant genotype is synthetically lethal causing animals to die as pharate adults (Fig. 5F). Lethality could either be because of an overactivation of ERK in the absence of two negative regulators or it could merely reflect an additive effect of two weak genotypes. If the common target of DMKP3 and PTP-ER were ERK, then one would expect that reduction of ERK-levels would abrogate the developmental block imposed by increased ERK activity. An additive effect, however, would rather be enhanced than suppressed by reducing ERK-levels. *PTP-ER*$^{XE\text{-}3022}$*; Dmkp3*$^{-}$ double mutants with only one functional *ERK* allele are viable (Fig. 5G), thus indicating that PTP-ER and DMKP3 act in parallel and in a redundant fashion to negatively regulate ERK.

DMKP3 affects the R3/R4 decision whereas PTP-ER is involved in R7 development

Absence of DMKP3 function affects the R3 and R4 photoreceptors with a low penetrance (range: 3-18%), resulting in either the loss of one cell of the R3/R4 pair or in the misdifferentiation of these photoreceptors. Although R3 and R4 adopt asymmetrical positions in a wild-type ommatidium, they are often symmetrically arranged in a *Dmkp3*$^{-}$ eye. In some cases, these symmetrical ommatidia also contain an extra R7 or an extra outer photoreceptor cell. The additional cell always invades the ommatidium between the R3 and R4 cells (Fig. 5A). In ommatidial preclusters, the position between R3 and R4 precursors is occupied by the mystery cell, which retracts when development proceeds (Fig. 7A). Therefore, the extra cell is most probably a misdifferentiated mystery cell. A similar phenotype has been described for mutants affecting endocytosis or cell-adhesion (Huang and Fischer-Vize, 1996; Nguyen et al., 1997), but has not been associated with non-secreted components of ERK signaling. Strong evidence that the effect on photoreceptors is because of increased ERK activity, but not to an unrelated phenomenon, comes from the observation that reduction of ERK levels by half in the *Dmkp3*$^{-}$ background almost completely suppresses the *Dmkp3*$^{-}$ eye phenotype (Fig. 5B).

The loss of ommatidial asymmetry has been associated with an altered Notch and Delta activity in the initially equivalent R3 and R4 precursor cells. High Delta levels in both precursors would lead to R3/R3-type ommatidia and high Notch activity to facets containing two R4s (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). If *Dmkp3* participated in the Notch-Delta interaction directly (for example as a Notch target) (Berset et al., 2001), one would expect to see only ommatidia of either type. However, ommatidia exhibiting the R3/R3 and the R4/R4 shape are detected at approximately the same frequency (Fig. 5A4,A5). Moreover, in contrast to its *C. elegans* homolog *lip-1* (Berset et al., 2001), *Dmkp3* does not appear to be a transcriptional target of activated Notch (data not shown).

In order to confirm the interpretation of ommatidial shapes with a molecular marker, R4-differentiation was followed in *Dmkp3*$^{-}$ eye imaginal discs. In contrast to the wild type, occasionally two or no cells in *Dmkp3*$^{-}$ ommatidial clusters express the R4 marker *E(spl)mδ0.5* (Cooper and Bray 1999) (Fig. 5C,D), thus corroborating that both R3 and R4 are affected by the absence of *Dmkp3*. Likewise, using a R7-

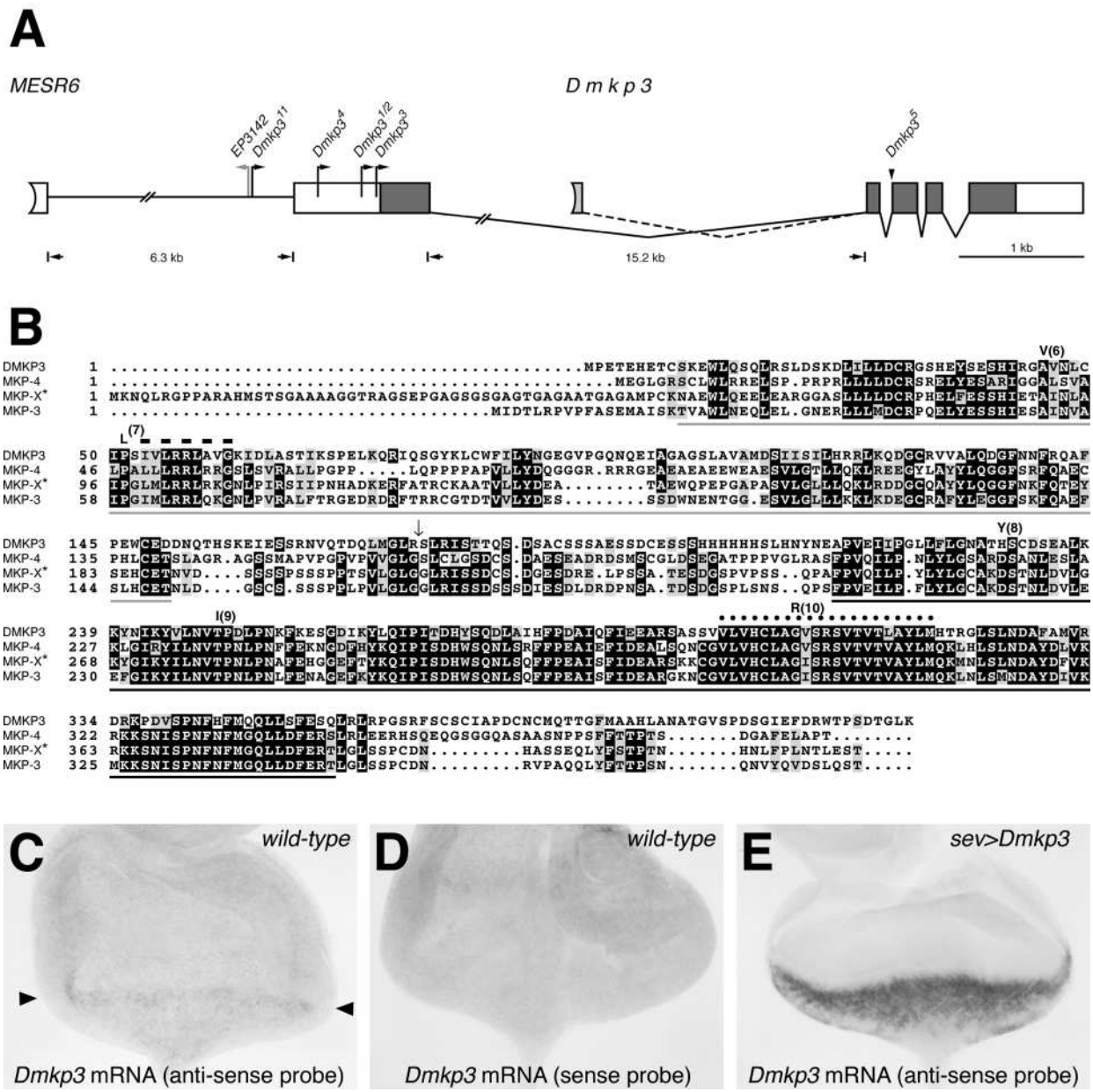

Fig. 3. *Dmkp3* genomic organization, alleles, protein structure and expression. (A) The *Dmkp3* gene consists of five exons and is characterized by a large intron and extensive 5′ and 3′ UTRs (open rectangles). A putative alternative (incomplete) transcript is denoted and the site of the *Dmkp3*^{5} splice-acceptor mutation is indicated (arrowhead). EP insertion sites and orientations are symbolized by wands. (B) The DMKP3 protein has three human functional homologs: MKP-3, MKP-4 and MKP-X, which are 52-58% similar. MKP-X sequence has been delineated from partial cDNAs. Underlined are the N-terminal Cdc25-homology (CH2) domain implicated in MAPK binding (gray) and the C-terminal catalytic domain (black). The broken line marks the ERK docking motif and the dotted line the core catalytic site. These central sites are presumably disrupted in the DMKP3^{7} and DMKP3^{10} gene products. With the possible exception of *Dmkp3*^{6}, the other alleles are null mutations as well (see amino-acid replacements and allele numbers above the alignment). The arrow marks the site of the *Dmkp3*^{5} mutation. (C) *Dmkp3* is weakly expressed just posterior to the morphogenetic furrow. Negative and positive controls are shown in D and E, respectively.

specific *lacZ* line (P. Maier and E.H., unpublished) in a *Dmkp3*$^{-}$ background, additional R7 cells are detectable (data not shown). Preclusters devoid of R4 staining very probably give rise to symmetrical R3/R3-type ommatidia (with or without an extra photoreceptor) or to ommatidia missing an outer photoreceptor. Preclusters containing two R4-positive cells will differentiate to R4/R4 ommatidia or to ommatidia containing an extra photoreceptor (Fig. 5A).

The weak rough eye phenotype associated with loss of PTP-ER function is caused by the occasional recruitment of one or more extra R7 cells, which may be accompanied by a loss of an outer photoreceptor cell. R3 and R4 cells are unaffected (Fig. 5E). The unequal *Dmkp3* and *PTP-ER* loss-of-function phenotypes suggest that the two phosphatases perform non-overlapping functions during photoreceptor differentiation. In agreement with this hypothesis, eyes of the double mutant

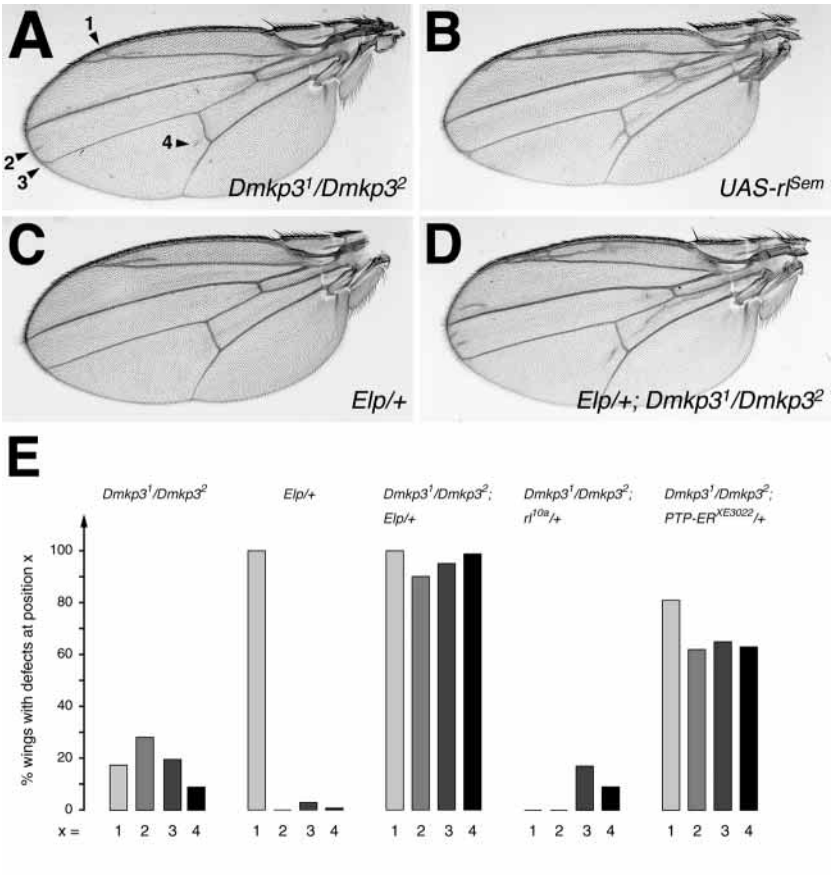

Fig. 4. The *Dmkp3* wing phenotype is sensitive to RAS pathway activity. (A) *Dmkp3* mutant wings exhibit additional vein material at four different sites marked by arrowheads. This phenotype is strongly enhanced by Ellipse (compare C to D) and it is very reminiscent of *UAS-rl*^{Sem} (B). Relative numbers exhibiting extra veins at each of the four sites are given in E. *rl*^{10A} dominantly suppresses and *PTP-ER* dominantly enhances the phenotype. Between 100 and 235 wings were counted.

pupae or eyes containing *Dmkp3*$^{-}$ clones in a *PTP-ER*$^{-}$ background feature ommatidia characteristic of either single mutant (Fig. 5F and data not shown). These results demonstrate that during eye development, DMKP3 and PTP-ER exert non-redundant functions in the specification of photoreceptor cells.

DMKP3 is required both in R3/R4 photoreceptors and outside of the ommatidial precluster

Unlike Delta and Notch, which are required specifically in R3 and R4, respectively, DMKP3 influences both R3 and R4 differentiation. DMKP3 function could thus reside in R3 and R4 cells. An alternative, but not exclusive, possibility is that DMKP3 is needed in cells, which will not differentiate as photoreceptors, but interfere with R3/R4 development. According to the model that DMKP3 is required autonomously in R3 and R4 photoreceptors, a *Dmkp3*$^{-}$ shape would always be associated with a *Dmkp3*$^{-}$ genotype in those cells. If, however, DMKP3 were not required in R3 and R4, but exhibited a non-autonomous effect, one would expect to find mosaic ommatidia featuring a *Dmkp3*$^{-}$ phenotype, but a *Dmkp3*$^{+}$ genotype in R3 and/or R4 cells.

The predictions were tested in *Dmkp3*^{5J4}-*Dmkp3*$^{+}$ mosaic ommatidia with *Dmkp3*$^{-}$ shapes. In 156 eye sections 83 mosaic ommatidia with a *Dmkp3*$^{-}$ morphology were found. Ten of them were not analyzed, because their symmetry and their location at the equator did not allow their chirality to be determined. In the unambiguous 73 mosaic ommatidia photoreceptors R6, R7, R1, R5 and R2 had a relatively high likelihood of being *Dmkp3*$^{+}$ decreasing in the listed order. Five ommatidia were *Dmkp3*$^{+}$ either for the R3 or the R4, one was wild-type for R3, R4 and the extra cell (Fig. 6A,B). In 12 of 37 mosaics exhibiting an additional inner or outer photoreceptor the extra cell was *Dmkp3*$^{+}$ (Fig. 6B). Of the *Dmkp3*$^{+}$ extra cells, which according to their location are most probably misdifferentiated mystery cells, two were outer photoreceptors and ten were R7 cells.

The reverse clonal analysis, i.e. the determination of *Dmkp3* requirement in mosaic phenotypically wild-type ommatidia, revealed that *Dmkp3* function is dispensible in any photoreceptor (data not shown).

These data and the detection of *Dmkp3*$^{+}$ cells in mutant ommatidia formally demonstrates that DMKP3 function is not absolutely essential in any of the photoreceptors and also not required in the mystery cell. However, there is a strong autonomous *Dmkp3* component in R3 and R4 cells. We thus conclude that R3 and R4 differentiation is dependent both on DMKP3 activity within the precursor cells and on DMKP3 function in cells surrounding the ommatidial precluster.

Ommatidial preclusters contain one or two mystery cells, whereas only one cell could be followed by the clonal assay. Formally, we cannot dismiss the model assigning autonomy to the mystery cell. However, it is highly unlikely. Tomlinson and Struhl (Tomlinson and Struhl, 1999) found that even the most unrelated cell pair of an ommatidial precluster, R3 and R4, has an 0.375 chance of being derived from the same clone. Assuming a similar probability for both mystery cells, the chance of undetected cells being *Dmkp3*$^{-}$ in all 12 cases is 3.55×10^{-3} [$(1\text{-}0.375)^{12}$].

Our data predict that *Dmkp3*$^{-}$ clones should manifest non-autonomous effects. The examination of *Dmkp3*^{5} and *Dmkp3*^{2} clones in eye imaginal discs indeed revealed occasional R4/R4 ommatidia outside of the clonal boundaries (Fig. 6D-H and data not shown). Only ommatidia directly bordering the clone were affected, indicating that DMKP3 action is short-range. Similarly, ommatidia exhibiting a *Dmkp3*$^{-}$ shape can be found in wild-type tissue close to *Dmkp3*^{5J4} clones in adult eye sections (Fig. 6C). These genotypically wild-type ommatidia further stress our notion that the requirement for DMKP3 in R3 and R4 cells is not absolute.

Cells outside of the ommatidial precluster are competent to respond to a Notch-Delta interaction

Although DMKP3 is very unlikely to have a function in the mystery cell(s), *Dmkp3*$^{-}$ ommatidia often contain a mystery cell having differentiated as a photoreceptor (Fig. 5A).

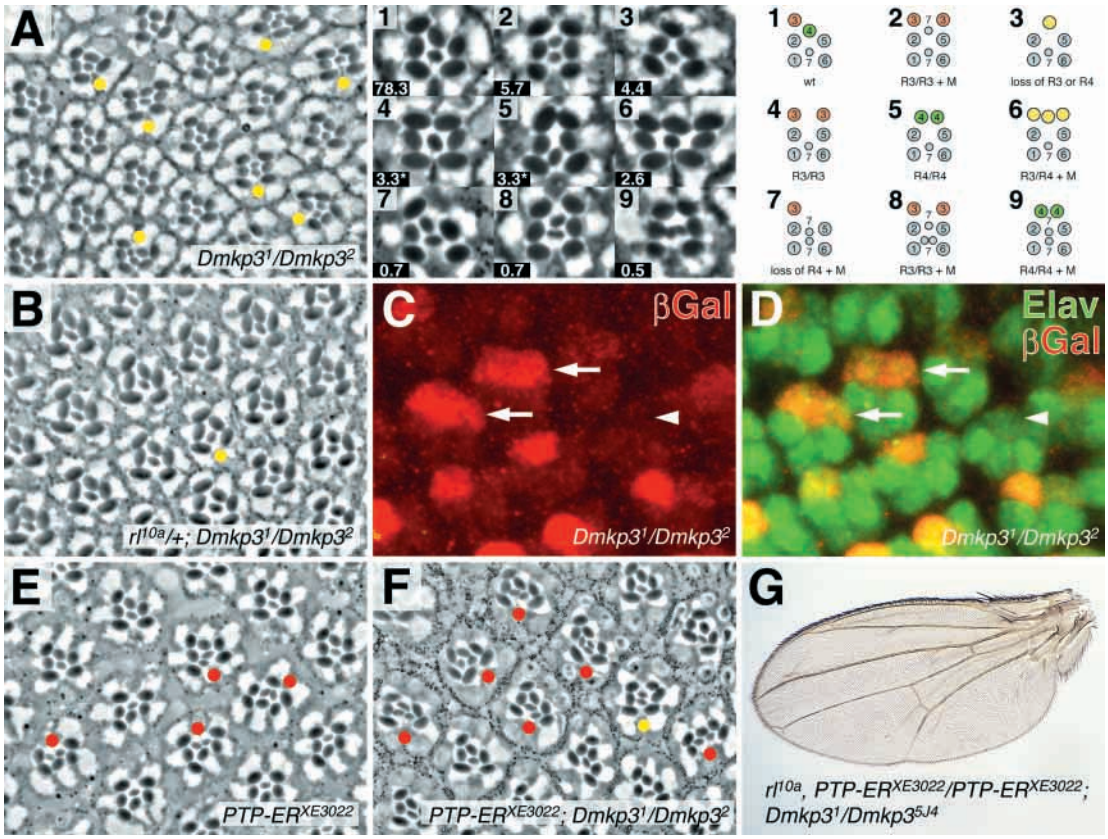

Fig. 5. DMKP3 affects different cellular decisions from PTP-ER. (A) Section through *Dmkp3* mutant eyes reveals misdifferentiations affecting the outer R3 and R4 cells, as well as a central cell that is normally not incorporated into ommatidia. Yellow dots mark mutant ommatidia and the different types and corresponding percentages are shown on the right. (609 ommatidia were analyzed, 4% of which could not be unambiguously assigned.) 1, Wild-type; 2-6,8,9, symmetrical ommatidia; 2,7-9, with an extra R7; 6, containing an extra outer photoreceptor; 2,4,8, R3/R3-type; 5,9, R4/R4 type; 3,7, devoid of an R3/R4-type photoreceptor; 4 and 5 are sometimes hard to distinguish and therefore percentages are combined. Cartoons to the right symbolize the different mutant classes with R3 rhabdomeres in red, R4 rhabdomeres in green and rhabdomeres of unclear identity in yellow. (B) The eye phenotype is almost completely suppressed by rl^{10A}. The only mutant ommatidium out of 700 is marked. (D) In *Dmkp3*$^{-}$ eye imaginal discs two (arrows) or no (arrowhead) cells per cluster (green) may differentiate into R4 cells (red, single channel in C). (E) *PTP-ER*$^{-}$ eyes are rough because of the recruitment of extra R7 cells (red dots), and *PTP-ER*$^{-}$ *Dmkp3*$^{-}$ pharate adults display ommatidia characteristic of either single mutant (yellow and red dots in F). (G) The double mutant is viable when *rl/ERK* dose is reduced and shows a wing phenotype characteristic of enhanced ERK activity.

Conceivably, R3, R4 and some surrounding cells determine the fate of the mystery cells in a DMKP3-dependent manner. For example, they could be required for their timely withdrawal from the preclusters. If this process were badly timed, the lagging cells would disrupt the Notch-Delta interactions between R3 and R4 precursors and thereby result in their own misdifferentiation. This model makes the prediction that in situations in which the mystery cell remains between R3 and R4 precursors their interaction should be inhibited, but their competence to interact should be unaffected.

The constitutively active *sevenless* allele sev^{S11} has been shown to be sufficient to reprogram mystery and cone cells to a R7 fate (Basler et al., 1991). In a sev^{S11} background the wrongly differentiating cells may frequently separate the R3/R4 precursors, which then may be free for Notch-Delta-mediated interactions with other cells. We tested this possibility by following the R4 marker in sev^{S11} eye imaginal discs and found that cells outside the ommatidial preclusters can adopt a R4 fate (Fig. 6I-K). This result can also explain why sev^{S11} ommatidia may not only contain extra R7, but occasionally also extra outer photoreceptor cells (Fig. 6L).

DISCUSSION

Elucidating substrate preferences of phosphatases in *Drosophila*

The starting point of this study was the demonstration that mammalian DSPs not only function in *Drosophila*, but also exhibit strict specificities even when overexpressed. Considering the relatively low conservation of phosphatases at the sequence level – ~50-60% similarity – this is somewhat surprising. We do, however, observe that DMKP3 is more active in flies than the mammalian ERK phosphatases MKP3 and MKP4, which was rather weak (Fig. 1K,Q and data not shown). However, different strengths of transgenes may also reflect insertion effects indicating that statements other than qualitative ones are difficult to make.

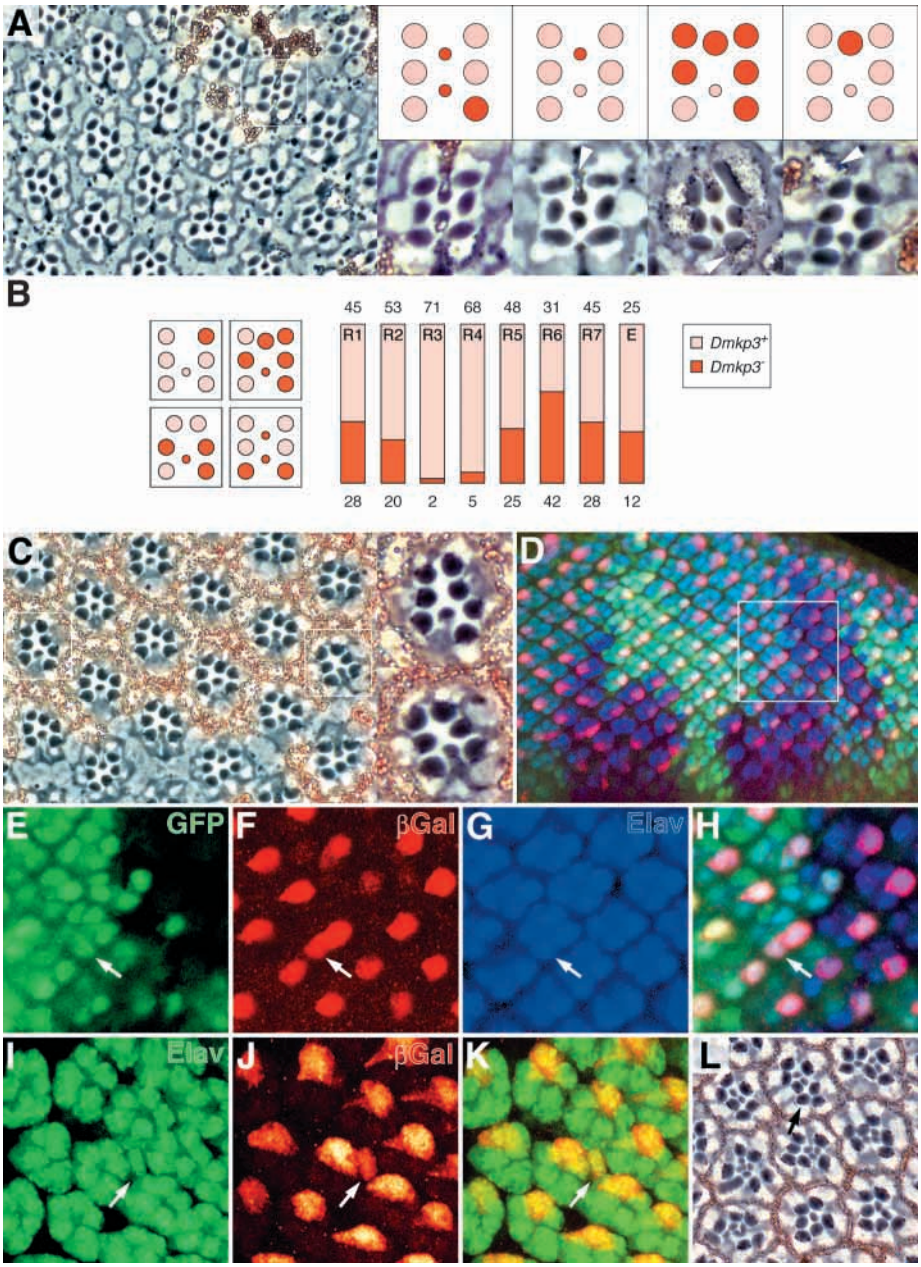

Fig. 6. DMKP3 functions both in the R3/R4 pair and outside of the photoreceptors. (A) A section through a mosaic eye and a magnification and further examples of mosaic ommatidia in insets. Pigmented cells are *Dmkp3*$^{+}$ (arrowheads), tissue devoid of pigment granules is *Dmkp3*$^{-}$. Cartoons represent the genotype with *Dmkp3*$^{+}$ photoreceptors in red and *Dmkp3*$^{-}$ cells in pink. (B) Quantitative analysis of 73 phenotypically *Dmkp3*$^{-}$ mosaic ommatidia (cartoons to the left, compare to Fig. 5A) showing both *Dmkp3*$^{-}$ (pink in the bar diagram) and *Dmkp3*$^{+}$ photoreceptors (red) at any position. Data are from both males and females and absolute numbers are given above and below the chart, respectively. Genetically wild-type ommatidia exhibiting a *Dmkp3*$^{-}$ shape can also be observed close to a *Dmkp3*$^{-}$ clone (section through a male eye in C and insets). (D) Concordantly, an additional photoreceptor (blue) can differentiate into an R4 cell (red) even outside of a *Dmkp3*$^{-}$ clone (marked by the absence of GFP, green) (higher magnification in E-H). The misdifferentiated cell is marked by an arrow. R4 cells (red) are also observed outside of ommatidial clusters (green) in a *sev*^{S11} background (arrow in I-K) providing an explanation for the occurrence of extra outer photoreceptors in *sev*^{S11} eye-sections (arrow in L).

Although some of the phosphatases tested possess anti-p38 activity in vitro, we could not compare their overexpression phenotypes with those of p38α and p38β null mutants because these null phenotypes are not known. Moreover, the p38 kinase Licorne and the p38 kinase kinase DMEKK1 loss-of-function phenotypes are very divergent (Inoue et al., 2001; Suzanne et al., 1999). We therefore cannot rule out that some aspects of the phenotypes described, such as the extreme dorsal hole or the wing-duplication effect caused by CL100 overexpression, may also be because of an effect on p38.

In our assays CL100 also caused very rough eyes and strong loss of wing veins suggesting that it is a potent phosphatase for both JNK and ERK. The wing-duplication phenotype may be the result of the downregulation of EGFR signaling because expression of a dominant negative form of RAF or RAS or reduction of EGFR and CNK dosis leads to a similar phenotype (Baonza et al., 2000). Because we have, however, never observed wing duplications by expression of DMKP3, CL100 is either more potent or has substrates other than ERK. Indeed, a wing-duplication phenotype is also observed in *twins* mutants encoding a regulatory subunit of the STP PP2A (Uemura et al., 1993).

DMKP3 is a negative regulator of the RAS/MAPK pathway

Several lines of evidence indicate that DMKP3 is an ERK-specific phosphatase and that it cooperates with PTP-ER. (1) DMKP3 dephosphorylates ERK but not JNK in vitro (Kim et al., 2002). (2) Overexpression of DMKP3 produces phenotypes resembling those of ERK but not JNK loss-of-function mutations. (3) Epistasis experiments using *Dmkp3* gain-of-function and loss-of-function alleles indicate that DMKP3 acts in the RAS/ERK pathway in the eye and the wing. (4) The

synthetic lethality of *PTP-ER⁻; Dmkp3⁻* double mutants is rescued by reducing ERK levels by half.

This interaction is reminiscent of the yeast DSP Yvh1 and the tyrosine phosphatase Ptp2, which have little effect when mutated alone, but double mutants are sporulation defective (Park et al., 1996). As there are five additional MKPs in the *Drosophila* genome (Morrison et al., 2000), negative regulation of ERK by a combinatorial network of those phosphatases will probably reveal high redundancy as well.

DMKP3 functions in R3 and R4 and in surrounding non-neuronal cells during ommatidial differentiation

In *Dmkp3* mutant eyes, both R3 and R4 cells are misspecified in a small fraction of ommatidia. DMKP3 has an autonomous and a non-autonomous role in specifying R3 and R4. The autonomous DMKP3 function derives from the high, albeit not complete correlation of a *Dmkp3⁻* phenotype and a *Dmkp3⁻* genotype in the R3 and R4 cells. Because R3 and R4 are the most distantly related cells in the precluster, the high incidence of both R3 and R4 being mutant indicates a strong requirement for DMKP3 function in these cells. The evidence for a non-autonomous function of DMKP3 comes from phenotypically mutant ommatidia in which at least one cell of the R3/R4 pair is wild-type and from phenotypically mutant and genotypically wild-type ommatidia close to *Dmkp3⁻* clones.

Non-autonomous effects on outer photoreceptors were also observed for *groucho*, *argos*, *fat facets*, *liquid facets*, *sidekick* and *atrophin* clones (Cadavid et al., 2000; Fanto et al., 2003; Fischer-Vize et al., 1992a; Fischer-Vize et al., 1992b; Freeman et al., 1992; Nguyen et al., 1997; Fanto et al., 2003). The results have been interpreted to indicate that surrounding cells participate in photoreceptor differentiation. The data presented here provide the first direct evidence that levels of RAS/ERK activity in cells surrounding the growing ommatidial cluster can influence ommatidial patterning. They may also explain why a *Ras1* gain-of-function allele dominantly enhances the *fat facets* (*faf*) loss-of-function phenotype, although *faf* function resides outside the photoreceptors (Huang and Fischer-Vize, 1996; Li et al., 1997).

A model to account for the *Dmkp3⁻* ommatidial shape

From our results we infer that the misdifferentiation of *Dmkp3⁻* ommatidia correlates with the behavior of the mystery cell (Fig. 7B). The mystery cell must leave the precluster to permit a physical interaction of R3 and R4 precursor cells to engage in a Notch-Delta-mediated specification of the R3 and R4 fate. In the absence of DMKP3 in R3 and R4 precursors and in the surrounding cell pool the mystery cell has a chance of being locked between R3 and R4, thus preventing the correct specification of its fate and that of the R3 and R4 precursors. The presence of misspecified R3/R4 cells without an intervening extra photoreceptor cells suggests that the mystery cell left the cluster too late and thus interfered with R3/R4 development.

How could cells surrounding the mystery cell be involved in eliciting its exit from the precluster? Conceivably, changes in cell adhesion, which may be regulated by an ERK signal, play a major role in expunging the mystery cells from the cluster. Upon recruitment of cells into the cluster, cell-cell contacts between photoreceptor cells are tightened. The mystery cells cannot adhere to the differentiating cells in the cluster and are expelled like melon seeds. As DMKP3 is not required in the mystery cells, it is probable that it is not the absolute value of cell-adhesive properties, but the relative amount compared with its neighbors that influences their behavior. This model implies that mutations altering cell-adhesive properties should lead to *Dmkp3⁻*-like ommatidia. Indeed, loss of *sidekick* and *atrophin*, coding for adhesion molecules, result in a very similar phenotype by affecting cells outside the cluster (Fanto et al., 2003; Nguyen et al., 1997). Furthermore, EGFR signaling and particularly ERK activity may not only influence cell fate, but also directly or indirectly influence cell adhesion. EGFR to ERK signaling has been shown to affect the adhesive properties of mammalian cells (Xie et al., 1998), and recent evidence in *Drosophila* also points to a role of EGFR in cell adhesion (Dumstrei et al., 2002). High ERK activity has also been found in migrating cells, although activated ERK per se is insufficient to influence migration (Duchek and Rorth, 2001). The possibility to modulate RAS pathway activity in

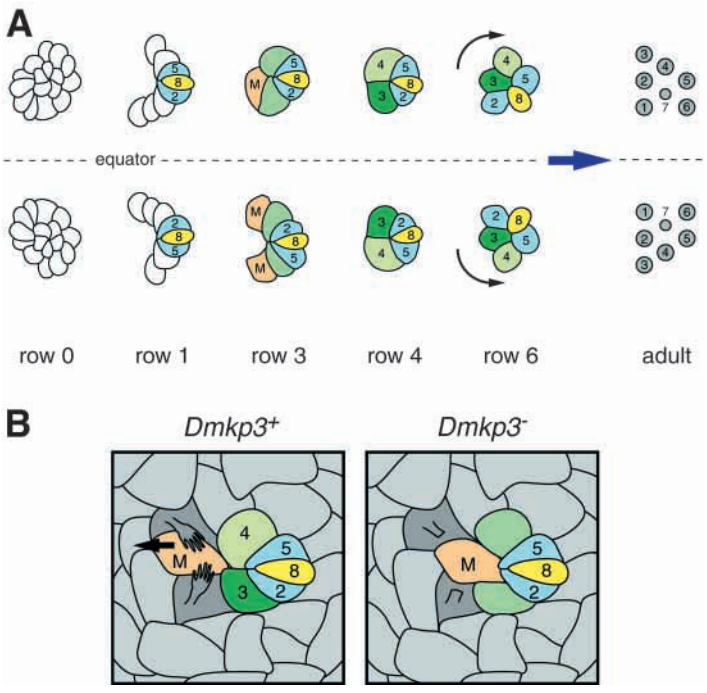

Fig. 7. Ommatidial development in the presence and absence of *Dmkp3*. (A) Schematic representation of the emergence of preclusters in successive rows posterior to the morphogenetic furrow (at row 0). Starting from a 'rosette' and 'arch' stage (rows 0 and 1), the preclusters in row 3 contain initially equivalent R3/R4 precursors and 1-2 intervening mystery cells (M). The R3/R4 equivalence group is able to sense a graded Frizzled signal from the equator such that the cell closer to the equator becomes R3, the other R4. Subsequently, the preclusters undergo a rotation and thereby achieve chirality. Ultimately, R3 and R4 adopt asymmetrical positions in the ommatidium conveying the typical trapezoidal shape (right, symbolizing the arrangement of rhabdomeres). (B) Between row 3 and row 4 the mystery cell is expelled from preclusters like a melon-seed. We propose that in the absence of DMKP3 function cells from the surrounding pool and also R3 and R4 display altered adhesive properties, thereby affecting the exit of the mystery cell(s) from the precluster.

Drosophila almost at will may establish the developing eye as an interesting system in which the connection between RAS signaling and cell adhesion within an epithelium can be further analyzed.

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