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MKP-3 Has Essential Roles as a Negative Regulator of the Ras/Mitogen-Activated Protein Kinase Pathway during *Drosophila* Development

Myungjin Kim,[1] Guang-Ho Cha,[1] Sunhong Kim,[1] Jun Hee Lee,[1] Jeehye Park,[1] Hyongjong Koh,[1] Kang-Yell Choi,[2] and Jongkyeong Chung[1]*

National Creative Research Initiatives Center for Cell Growth Regulation and Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701,[1] and Department of Biotechnology, Yonsei University College of Engineering, Seoul 120-752,[2] Korea

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Mitogen-activated protein kinase (MAPK) phosphatase 3 (MKP-3) is a well-known negative regulator in the Ras/extracellular signal-regulated kinase (ERK)-MAPK signaling pathway responsible for cell fate determination and proliferation during development. However, the physiological roles of MKP-3 and the mechanism by which MKP-3 regulates Ras/*Drosophila* ERK (DERK) signaling in vivo have not been determined. Here, we demonstrated that *Drosophila* MKP-3 (DMKP-3) is critically involved in cell differentiation, proliferation, and gene expression by suppressing the Ras/DERK pathway, specifically binding to DERK via the N-terminal ERK-binding domain of DMKP-3. Overexpression of DMKP-3 reduced the number of photoreceptor cells and inhibited wing vein differentiation. Conversely, *DMKP-3* hypomorphic mutants exhibited extra photoreceptor cells and wing veins, and its null mutants showed striking phenotypes, such as embryonic lethality and severe defects in oogenesis. All of these phenotypes were highly similar to those of the gain-of-function mutants of *DERK*/*rl*. The functional interaction between DMKP-3 and the Ras/DERK pathway was further confirmed by genetic interactions between DMKP-3 loss-of-function mutants or overexpressing transgenic flies and various mutants of the Ras/DERK pathway. Collectively, these data provide the direct evidences that DMKP-3 is indispensable to the regulation of DERK signaling activity during *Drosophila* development.

The mitogen-activated protein kinase (MAPK) signaling pathway is critically involved in diverse biological processes, including mitogenesis, neuronal differentiation, apoptosis, and development (3, 37, 41). Core signaling modules of the MAPK pathway consist of MAPK kinase kinase, MAPK kinase, and MAPK. MAPK kinase kinase activates MAPK kinase by phosphorylation, which, in turn, phosphorylates and activates MAPK (3, 37, 41). Subsequently, activated MAPK translocates into the nucleus, where it phosphorylates various nuclear targets leading to specific cellular processes (5, 30). In eukaryotes, three major MAPKs—extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK)—have been characterized and found to be highly conserved among various species. These three MAPK signaling cascades convey abundant information to numerous target effectors in the cell, allowing various responses to the environment.

The specific roles of Ras/*Drosophila* ERK (DERK) signaling in cell differentiation and proliferation have been intensively studied in the developing eyes and wings of *Drosophila* (21, 22, 25, 29, 51). A well-known step in the photoreceptor cell fate determination is the recruitment of the final R7 cell (2, 51, 54). In this step, Ras/DERK signaling acts as a binary switch to trigger one of the nonneuronal cone cells to differentiate into a R7 neuronal cell. This was proved by a series of experiments in which gain-of-function mutations of the components of the Ras/DERK signaling pathway induce ectopic R7 cell differentiation in the absence of upstream inducing signals (4, 6, 38, 39). For example, the rl^{sem} mutant, containing an Asp334-to-Asn point mutation in DERK, showed additional photoreceptor cells during *Drosophila* eye development. It was also demonstrated that the gain-of-function alleles of the components of the Ras/DERK pathway enhance wing vein formation (38, 42). Consistently, downregulation of the Ras/DERK signaling pathway inhibits wing vein formation (12, 15, 23). Meanwhile, it was reported that the activated form of Ras was able to drive the eye and wing imaginal discs to hyperplastic growth (27), implicating that Ras/DERK signaling is required for cell proliferation as well.

The duration and strength of the activities of Ras/MAPK signaling are tightly regulated at many different levels within the pathway (3, 37, 41). Especially, the activity of MAPK is reversibly regulated by MAPK kinase-dependent phosphorylation and MAPK phosphatase (MKP)-dependent dephosphorylation. The MKPs belong to a subclass of the dual-specificity phosphatase superfamily and dephosphorylate the critical threonine and tyrosine residues of MAPK (46, 56). Among MKPs, MKP 3 (MKP-3) tightly binds to ERK via the N-terminal ERK-binding domain (NBD) and negatively modulates ERK activities but not p38 and JNK (8, 19, 44, 45, 60). Recently, we have demonstrated that *Drosophila* MKP-3 (DMKP-3) is highly homologous to mammalian MKP-3 and plays specific and dominant roles in negatively regulating DERK activities in Schneider cells (32, 34). Therefore, understanding the function

* Corresponding author. Mailing address: Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1 Kusong-Dong, Yusong, Taejon 305-701, Republic of Korea. Phone: 82-42-869-2620. Fax: 82-42-869-8260. E-mail: jchung@kaist.ac.kr.

of DMKP-3 in Ras/DERK signaling at the organism level would provide a significant clue for deciphering the in vivo role of the Ras/ERK signaling pathway in mammals.

To better understand how MKP-3 regulate Ras/DERK signaling in a cellular and developmental context, we investigated the physiological role of MKP-3 in *Drosophila* by using highly convenient genetic and histochemical methods. In the present study, we characterized both the transgenic flies overexpressing DMKP-3 and the loss-of-function mutant flies of *DMKP-3*. The genetic analyses of these flies clearly demonstrated that DMKP-3 functions as a negative regulator of the Ras/DERK pathway by directly inhibiting DERK-MAPK. Furthermore, we demonstrated that MKP-3 plays a critical role in the ERK-MAPK-mediated developmental processes, such as cell fate determination and cell proliferation in *Drosophila*.

MATERIALS AND METHODS

Fly strains. The fly strains, *EP(3)3142*, *sev-PTP-ER*^{101}, *RafHM7*, *Raf*^{F179}, *Ras*^{e1b}, *rl*^{1}, *y/w*; *CyO*, *P{Δ2-3}/Bc Egfr*, *rho*^{AA69}, *rl*^{sem}, and UAS-*lacZ* and various GAL4 lines such as *sevenless* (*sev*)- and *apterous* (*ap*)-GAL4 (7) were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, Ind.). The *e16E*-GAL4 line (24, 59) was kindly provided by J. Kim (Korea Advanced Institute of Science and Technology). The *MS1096*-GAL4 driver line (9, 40) was a gift from M. Freeman (Medical Research Council [United Kingdom]). All *Drosophila* stocks were maintained and cultured with standard cornmeal-yeast-agar medium at 25°C, while *Raf*^{HM7} hemizygous males were maintained at 18°C.

Plasmid construction and generation of transgenic flies. To induce ectopic expression of DMKP-3, we used UAS/GAL4 system (49). A full-length of DMKP-3 cDNA was generated by reverse transcription-PCR (RT-PCR) by using the 5′ primer 5′-GCGAGATCTATGCCAGAAACGGAGCACGAG-3′ and the 3′ primer 5′-CGCCTCGAGTCAGGCCGCATCCTCATCGTA-3′ and then subcloned into the *Bgl*II-*Xho*I site of the pUAST vector. The PCR-cloned DMKP-3 was confirmed by DNA sequencing. We also generated various mutants of DMKP-3—C302A (Cys302 to Ala302), R56/57A (Arg56 and Arg57 to Ala56 and Ala57), and R56/57A/C302A—by site-directed mutagenesis (Stratagene) and cloned them into pUAST vector (32, 34). ΔN, a truncated mutant lacking the NH_2-terminal 170 amino acids, was also generated and cloned into pUAST. To obtain transgenic flies, various pUAST plasmids and a helper plasmid, pπ25.1, were microinjected into embryos prior to pole cell formation by using a microinjector model IM30 (Narishige) and an Axiovert 25 micromanipulator (Carl Zeiss).

P-element mobilization-mediated DMKP-3 mutagenesis. To generate DMKP-3 loss-of-function mutants, we used the P-element local hopping mutagenesis by crossing EP(3)3142 with *y/w*; *CyO*, *Δ2-3/Bc Egfr* flies (52), as previously described (31). We then isolated 2,000 independent lines with mobilized P elements from 2.6×10^5 screened flies generated from this mutagenesis and further narrowed the total down to three DMKP-3 loss-of-function mutants: *DMKP-3*^{P1}, *DMKP-3*^{P2}, and *DMKP-3*^{P3}. Their new P-element insertion sites were determined by PCR-based mapping and sequencing analyses (14). To isolate the homozygous *DMKP-3* mutant individuals, we used a green fluorescent protein (GFP) balancer chromosome (*TM3*, *P{ActGFP}JMR2*, *Ser*^{1}) as a tracking marker; the GFP-negative embryos were selected as homozygous *DMKP-3* mutants (10).

Analysis of eye and wing phenotypes. Scanning electron micrograph images were obtained by using a LEO 1455VP in a variable pressure secondary electron mode. Eye section experiments were performed as previously described (35). To quantify the eye phenotypes presented in Fig. 2, we calculated the percentage of ommatidia with extra R cells, the ratio of the number of ommatidia with extra R cells to the total number of ommatidia, in a single eye. In addition, the mean value of the number of photoreceptor cells per ommatidium was also calculated for each genotype. All of these experiments were independently performed by examining ~100 ommatidia per eye from 4 different flies with the same genotype.

Adult wing blades were mounted in 50% Canadian Balsam (Sigma) in methyl salicylate (Fischer). To quantify wing phenotypes, the mean number of extra veins per wing for each genotype was calculated from 110 different wings.

Feeding drugs. To test the effects of the MEK inhibitors U0126 (20) and PD98059 (1), larvae were fed a 100 μM or a 1 mM concentration of U0126 and a 250 or a 500 μM concentration of PD98059.

Immunostaing and histological analysis. Third-instar larvae were dissected in *Drosophila* Ringer's solution, and eye and wing imaginal discs were fixed in 4% paraformaldehyde phosphate-buffered saline (PBS) solution for 30 min at room temperature. After being washed with PBS–0.1% Triton X-100 (PBST), the discs were blocked for 30 min at room temperature in PBS containing 3% bovine serum albumin. The discs were further incubated with primary antibodies (mouse anti-β-galactosidase [40-1a, The Developmental Study Hybridoma Bank, University of Iowa], goat anti-Sevenless [Santa Cruz Biotechnology], rat anti-Elav [rat-Elav-7E8A10; The Developmental Study Hybridoma Bank, University of Iowa], and mouse anti-phospho-specific DERK [Sigma]) and secondary antibodies conjugated to fluorescein isothiocyanate or TRITC (tetramethyl rhodamine isothiocyanate; Jackson Laboratories). The antibody-bound samples were washed with PBS and mounted in 50% glycerol-PBS solution for confocal microscopic observations.

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was performed as previously described (35).

Propidium iodide staining experiment. Eggs collected from the female flies with *DMKP-3*^{P1} germ line clones were immersed in 50% bleach for 3 min for dechorionation and then rinsed repeatedly with PBST. The dechorionated eggs were agitated vigorously for 20 min in equal volume of 4% formaldehyde and heptane. The bottom formaldehyde phase of the solution was removed, and then methanol was added. The samples were shaken vigorously for 15 s, and the remaining heptane phase and the unsunken eggs were removed. After the samples were washed with PBST, the devitellinized eggs were incubated with 10 mg of RNase/ml at 37°C for 2 h. After several washes in PBST, 50% glycerol-PBS mounting medium with 1 μg of propidium iodide/ml was added to the eggs. The eggs in the mounting medium were transferred onto a glass microscope slide for confocal microscopic observations.

BrdU labeling experiment. Third-instar larvae cultured at 25°C were dissected in Ringer's solution and then incubated in the presence of 100 μg of 5-bromo-2′-deoxyuridine (BrdU/ml; Roche) in M3 medium for 1 h at room temperature. The samples were fixed in Carnoy's fixative (ethanol-acetic acid-chloroform [6:3:1]) for 30 min at 25°C and sequentially treated with 70% ethanol in PBST (0.3% Triton X-100 in 1× PBS), 50% ethanol in PBST, and 30% ethanol in PBST at 25°C for 3 min. Next, the samples were incubated in 2 N HCl for 1 h, and the incorporated BrdU was visualized by using an mouse anti-BrdU antibody (Roche) and Alexa 568 (Molecular Probe). The samples were observed under a confocal laser microscope (Carl Zeiss).

In situ hybridization. For in situ RNA hybridization analysis, antisense and sense digoxigenin-labeled riboprobes were prepared by using T7 and SP6 RNA polymerases and linearized T vector–DMKP-3 as a template. Digoxigenin labeling was performed according to the manufacturer's instructions (Roche Biochemicals). In situ hybridization of *Drosophila* embryos and larval tissues was conducted as previously described (16, 17).

Mitotic clonal analysis. DMKP-3 alleles were recombined into FRT79D chromosomes, and clonal analysis was performed by using the FLP/FRT system as described previously (57). For the generation of clones in the adult eye and wing, larvae of the genotype *yw hs Flp; FRT79D/DMKP-3*^{P1} *FRT79D* were subjected at 24 to 48 h after egg deposition to heat shock for 1 h at 37°C to induce mitotic recombination.

The germ line clones of *DMKP-3*^{P1} were generated by using the autosomal FLP-DFS technique (11). In brief, females with a genotype of *FRT79D DMKP-3*^{P1}*/TM6B* were crossed with males with a genotype of *y w hs-FLP/Y;;P[ovo*^{D1}*] FRT79D/Sb*. Their progenies, the larvae at first-instar stage, were heat shocked at 37°C for 2 h. The *y w hs-FLP/X;;P[ovo*^{D1}*] FRT79D/DMKP-3*^{P1} *FRT79D* females (3 or 5 days old) were selected and crossed with *w*^{1118} males to obtain *DMKP-3*^{P1} germ line clones. The lengths of eggs were determined by using the arbitrary unit scale according to the previous report (26).

RESULTS

Ectopic expression of DMKP-3 interferes with eye and wing vein development. To examine the physiological roles of DMKP-3 in vivo, we generated transgenic flies carrying *UAS-DMKP-3*. Ectopic expression of wild-type DMKP-3 in the developing *Drosophila* eye by using the *sev*-GAL4 driver resulted in roughened eye phenotypes (Fig. 1B and C), which varied from mild (Fig. 1B, *sev*-GAL4>*DMKP-3*^{15}) to severe (Fig. 1C, *sev*-GAL4>*DMKP-3*^{10}) compared to the control (Fig. 1A). We found that some ommatidia in the *sev*-GAL4>*DMKP-3*^{15} flies

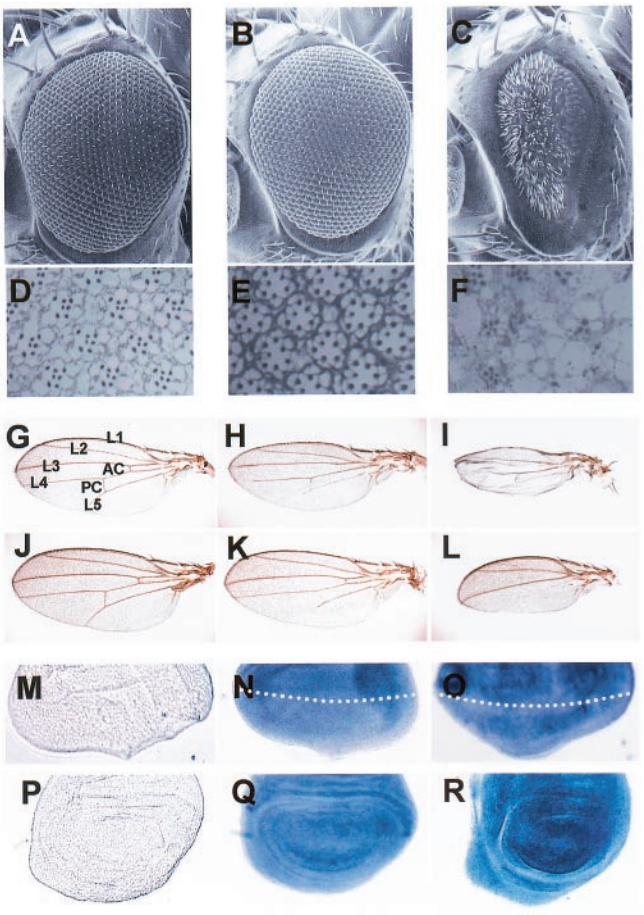

FIG. 1. Phenotypes induced by ectopic expression of DMKP-3. Scanning electron micrographs of the compound eyes (A to C) and their tangential cross sections (D to F) are shown. (G to L) Adult wing phenotypes were examined by light microscopy: L1 to L5, longitudinal veins; AC, anterior cross vein; PC, posterior cross vein. In situ hybridization analysis in eye (M to O) and wing imaginal discs (P to R): M and P, discs with sense control probe; N, O, Q, and R, discs with antisense *DMKP-3* probe. For panels N and O, DMKP-3 expression was induced by *sev*-GAL4 in the region below the dotted line. (A, D, M, and N) *sev*-GAL4/+; (B, E, and O) *sev*-GAL4/+; *UAS-DMKP-3*^{15}/+; (C and F) *sev*-GAL4/+; *UAS-DMKP-3*^{10}/+; (G, P, and Q) *MS1096*-GAL4/+; (H and R) *MS1096*-GAL4/+; +/+; *UAS-DMKP-3*^{10}/+; (I) *MS1096*-GAL4/+; *UAS-DMKP-3*^{6}/+; (J) *e16E*-GAL4/+; (K) *e16E*-GAL4/+; *UAS-DMKP-3*^{10}/+; (L) *e16E*-GAL4/*UAS-DMKP-3*^{6}.

contain six photoreceptor cells (Fig. 1E), whereas control flies normally have seven photoreceptor cells in each ommatidium (Fig. 1D). We also observed that the number of photoreceptor cells in the ommatidium of *sev*-GAL4>*DMKP-3*^{10} flies was further reduced to fewer than six (Fig. 1F), a finding which correlated with the more severe eye phenotype (Fig. 1C).

To investigate the role of DMKP-3 in wing development, wild-type DMKP-3 was ectopically expressed by using wing-specific GAL4 drivers. The wings of *MS1096*-GAL4>*DMKP-3*^{10} flies displayed a mild vein-loss phenotype (Fig. 1H) compared to the control (Fig. 1G). In addition, the ectopic expression of DMKP-3 by using a more severe transgenic allele, *DMKP-3*^{6}, caused a large reduction in the overall wing size and disruptions in the anterior cross veins and the longitudinal veins L3, L4, and L5 (Fig. 1I). When DMKP-3 was overexpressed by using the same alleles as in Fig. 1H and 1I under the *e16E*-GAL4 driver, similar phenotypes were observed: disrupted veins and reduced wing size in the wing posterior region (Fig. 1K and L, respectively).

In agreement with the observation that DMKP-3 involves in the eye and wing development, we detected ubiquitous expression of the endogenous *DMKP-3* transcripts in wild-type eye (Fig. 1N) and wing (Fig. 1Q) imaginal discs. In addition, ectopic expression of DMKP-3 using *sev*- or *MS1096*-GAL4 was also confirmed by in situ hybridization (Fig. 2O and R).

Generation and characterization of *DMKP-3* loss-of-function mutants. To further investigate the physiological roles of DMKP-3, we generated loss-of-function mutants of *DMKP-3* by using the local P-element mutagenesis method as described in Materials and Methods. By mobilizing the P element of *EP(3)3142* strain, we obtained three *DMKP-3* mutant lines with a P-element insertion within the *DMKP-3* coding region. We referred to these strains as *DMKP-3*^{P1}, *DMKP-3*^{P2}, and *DMKP-3*^{P3} (Fig. 2A). To determine the exact insertion sites of the P element in these *DMKP-3* alleles, we sequenced the flanking regions of each P element in the mutants. *DMKP-3*^{P1} and *DMKP-3*^{P3} had a P element inserted into intron 1, specifically located 1,024 and 489 nucleotides downstream from the translation start site of *DMKP-3*, respectively (Fig. 2A). On the other hand, *DMKP-3*^{P2} contained a P element at the 5′ untranslated region located 721 nucleotides upstream from the translation start site of *DMKP-3* (Fig. 2A).

To examine whether the *DMKP-3* transcripts are expressed in these mutant flies, we conducted RT-PCR analysis. We detected a weak *DMKP-3* RT-PCR signal from the homozygous *DMKP-3*^{P3} embryos and could not detect any *DMKP-3* signals from the homozygous *DMKP-3*^{P1} and *DMKP-3*^{P2} embryos (Fig. 2B, upper panel). Furthermore, these data were confirmed by in situ hybridization (Fig. 2C-F). Collectively, these results implicated that *DMKP-3*^{P1} and *DMKP-3*^{P2} are *DMKP-3*-null alleles, whereas *DMKP-3*^{P3} is a hypomorphic allele.

While analyzing *DMKP-3*^{P1} and *DMKP-3*^{P2} homozygotes, we observed that the majority (~70% [data not shown]) of them were embryonic lethal, suggesting that DMKP-3 is involved in other critical physiological functions as well as in eye and wing development. On the other hand, homozygous *DMKP-3*^{P3} flies survived up to adulthood. To confirm whether the lethality observed in both homozygous *DMKP-3*^{P1} and *DMKP-3*^{P2} mutant embryos was due to the P-element insertion, we precisely excised out the P element from the insertion loci. Resulting revertants displayed no defects on viability and fertility (data not shown). In addition, these revertants were able to fully complement *DMKP-3*^{P1} and *DMKP-3*^{P2} alleles (data not shown).

Meanwhile, homozygous *DMKP-3*^{P3} flies displayed extra photoreceptor cells in some ommatidia (Fig. 2J and S). Furthermore, in the transallelic mutants of *DMKP-3*, *DMKP-3*^{P2}/*DMKP-3*^{P3} flies, the number of ommatidia with extra photoreceptor cells increased significantly (Fig. 2M and S), demonstrating that a reduction in *DMKP-3* gene dosage correlates with extra photoreceptor cell phenotypes. Because the newly appeared extra photoreceptor cells are notably small and

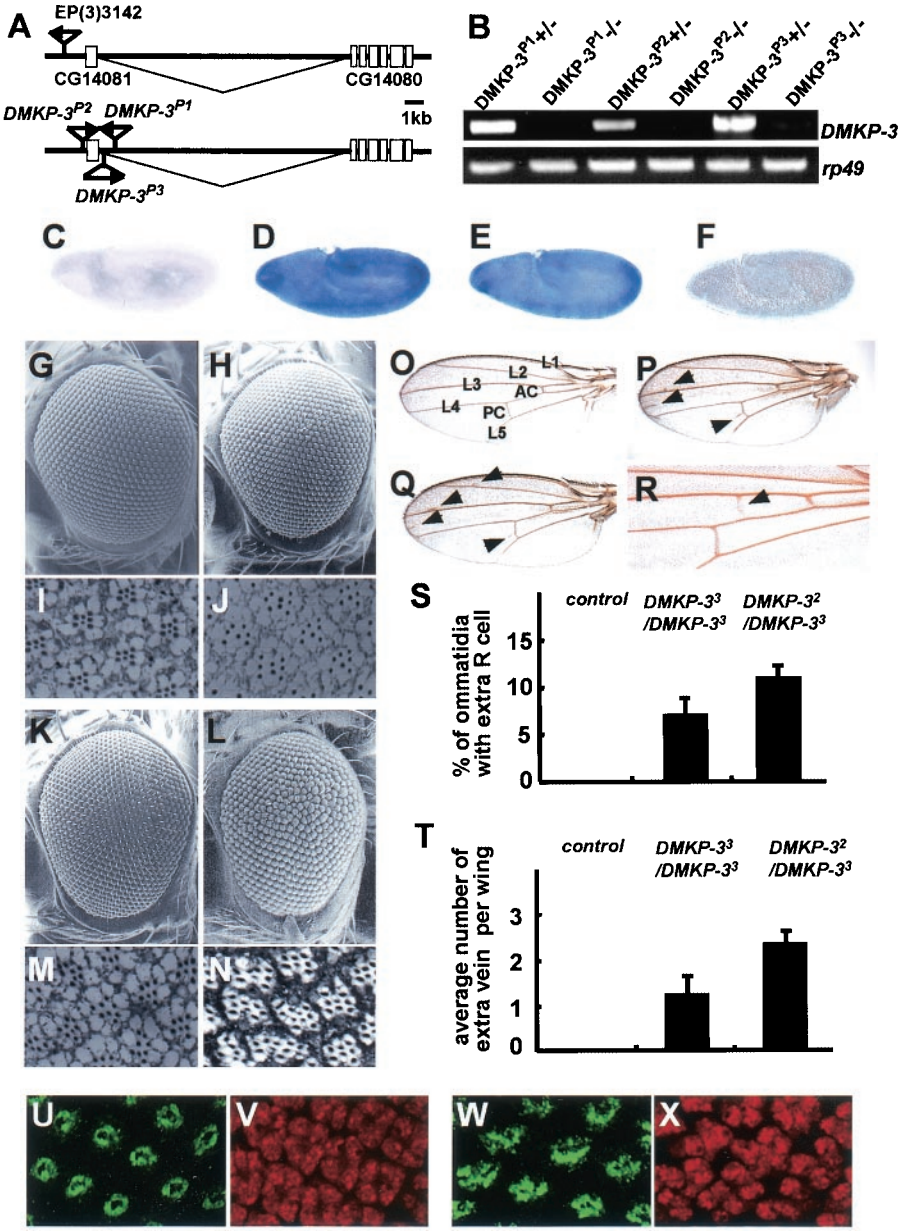

FIG. 2. Loss-of-function mutants of *DMKP-3*. (A) The insertion sites of the P element in the loss-of-function mutants of *DMKP-3*. The open boxes indicate the exons of *DMKP-3* gene, and the breaks between the boxes indicate the introns. The triangles represent the P elements, and the arrows indicate their directions. (B) DMKP-3 expression in the loss-of-function mutants of *DMKP-3*. The amount of *DMKP-3* transcripts was visualized by RT-PCR experiments from the following samples: a *DMKP-3*^{P1}/TM3, GFP, Ser (+/−) embryo; a *DMKP-3*^{P1}/*DMKP-3*^{P1} (−/−) embryo; a *DMKP-3*^{P2}/TM3, GFP, Ser (+/−) embryo; a *DMKP-3*^{P2}/*DMKP-3*^{P2} (−/−) embryo; a *DMKP-3*^{P3}/TM3, GFP, Ser (+/−) embryo; and a *DMKP-3*^{P3}/*DMKP-3*^{P3} (−/−) embryo. Rp49 transcripts were used as a control. (C to F) In situ hybridization analysis of stage 10 embryos: a wild-type embryo with sense control probe (C), a wild-type embryo with antisense *DMKP-3* probe (D), a *DMKP-3*^{P3}/*DMKP-3*^{P3} embryo with antisense *DMKP-3* probe (E), and a *DMKP-3*^{P1}/*DMKP-3*^{P1} embryo with antisense *DMKP-3* probe (F). Scanning electron micrographs of the compound eyes (G, H, K, and L) and their tangential cross sections (I, J, M, and N) are shown. (O to R) Microscopic views of the wings are shown, and the arrows point to ectopic veins. (S) Quantified data of I, J, and M. (T) Quantified data of O to Q. (U and W) R7 cells in eye imaginal discs were stained with anti-Sevenless antibody (green). (V and X) Photoreceptor cells in eye imaginal discs were stained with anti-Elav antibody (red). (G, I, O, U, and V) *w*^{1118}; (H, J, P, W, and X) *DMKP-3*^{P3}/*DMKP-3*^{P3}; (K, M, and Q) *DMKP-3*^{P2}/*DMKP-3*^{P3}; (L, N, and R) *yw hs Flp*; +/+; *DMKP-3*^{P1} *FRT79D*/*FRT79D*.

are located in the centermost ommatidium, surrounded by other photoreceptor cells, we suspected that these are extra R7 cells. To further demonstrate this, we performed immunohistochemistry with anti-Sevenless antibody, an R7-specific marker (55). As expected, multiple R7 signals were detected in *DMKP-3*^{P3} mutant ommatidia (Fig. 2W and X) but not in wild-type ommatidia (Fig. 2U and V), demonstrating that extra photoreceptor cells have R7 cell fates. However, in *DMKP-3*

eye null clones, we observed extra photoreceptor cells other than R7-type cells (Fig. 2N). Hence, we conclude that DMKP-3 is a general regulator of photoreceptor cell differentiation, including the R7 cell.

We also observed the homozygous *DMKP-3^{P3}* flies with ectopic veins in their wings (Fig. 2P and T) and more severe phenotypes in the transallelic mutants (*DMKP-3^{P2}*/*DMKP-3^{P3}*) (Fig. 2Q and T). Consistently, mitotic clonal analyses for *DMKP-3* mutants also showed an extra vein phenotype in the wings with *DMKP-3*-null clones (Fig. 2R).

Collectively, by analyzing the loss-of-function mutant flies of *DMKP-3*, we conclude that DMKP-3 suppresses the photoreceptor cell differentiation and wing vein formation. Since the Ras/DERK signaling pathway positively regulates these two developmental processes, it is likely that DMKP-3 negatively modulates the Ras/DERK pathway in vivo.

Genetic interactions between DMKP-3 and the Ras/DERK pathway. To further understand the roles of DMKP-3 in developing eyes and wings in vivo, we investigated whether DMKP-3 genetically interacts with the Ras/DERK pathway. Ectopic expression of DMKP-3 in a reduced gene dosage background of Ras (Fig. 2B and F), Raf (Fig. 2D and H), or DERK (Fig. 2J and N) resulted in a more severe rough eye phenotype with a further decreased number of photoreceptor cells than the eyes overexpressing DMKP-3 in a wild-type genetic background (Fig. 1B and E) or the control eyes in mutant genetic backgrounds (Fig. 3A, C, E, G, I, and M).

Conversely, we coexpressed DMKP-3 with constitutively active mutants of Ras (Fig. 2K and O) or DERK (Fig. 2Q and U) in the eye. The phenotype of a constitutively active Ras (*Ras^{V12}*) was almost completely suppressed by coexpression of DMKP-3 (Fig. 3L and P). However, coexpression of DMKP-3 and a constitutively active DERK (*rl^{sem}*) did not cause any changes in the phenotypes with *rl^{sem}* expression only (Fig. 3R and V). We thought that these data result from the point mutation in *rl^{sem}* gene, which abolishes the binding ability of MKP-3 to ERK (6, 8, 19).

Next, we examined the relationship between DMKP-3 and PTP-ER, which is another DERK phosphatase. Interestingly, coexpression of DMKP-3 with PTP-ER resulted in severely reduced eye size (Fig. 3T) and number of photoreceptor cells (Fig. 3X) compared to the eyes expressing DMKP-3 (Fig. 1B and E) or PTP-ER only (Fig. 3S and W) in a wild-type genetic background, suggesting the cooperative interaction between DMKP-3 and PTP-ER.

To further support these genetic interactions between DMKP-3 and DERK, we monitored the phosphorylation status of DERK in tissues by using an anti-phospho-specific antibody for ERK-MAPK (33). As shown in Fig. 4, Ras^{V12}-induced phosphorylation of DERK (Fig. 4B) was downregulated by coexpression of DMKP-3 (Fig. 4C). The result provides the direct evidence that DMKP-3 dephosphorylates DERK in vivo.

Finally, to verify that endogenous DMKP-3 affects the Ras/DERK pathway, we examined the activities of Ras/DERK signalings in a *DMKP-3* loss-of-function genetic background. Ectopic expression of either constitutively active Ras (*Rasl^{V12}*) or Raf (*Raf^{F179}*) caused roughened eye phenotypes (Fig. 5A and C, respectively) with extra photoreceptor cells in most ommatidia (Fig. 5E and G, respectively). All of these phenotypes were enhanced in *DMKP-3* hypomorphic backgrounds, and the ommatidia structures of these flies were severely disorganized and fused (Fig. 5B, D, F, and H).

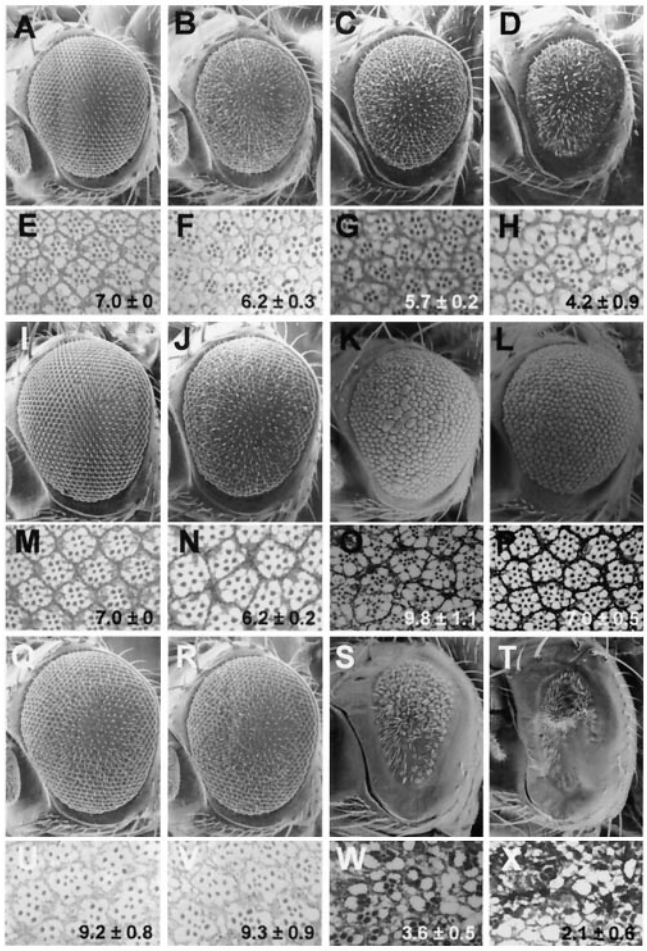

FIG. 3. Genetic interactions between DMKP-3 and the components of the Ras/DERK pathway. Scanning electron micrographs of the compound eyes (A to D, I to L, and Q to T) and their tangential cross sections (E to H, M to P, and U to X) are shown: *sev*-GAL4/+; *Ras^{e1B}*/+ (A and E); *sev*-GAL4/+; *Ras^{e1B}*/*UAS-DMKP-3^{15}* (B and F); *Raf^{HM7}*/Y; *sev*-GAL4/+ (C and G); *Raf^{HM7}*/Y; *sev*-GAL4/+; *UAS-DMKP-3^{15}*/+ (D and H); *sev*-GAL4/*rl^{1}* (I and M); *sev*-GAL4/*rl^{1}*; *UAS-DMKP-3^{15}*/+ (J and N); *sev*-GAL4/+; *UAS-Ras^{V12}*/+ (K and O); *sev*-GAL4/+; *UAS-Ras^{V12}*/*DMKP-3^{15}* (L and P); *sev*-GAL4/+; *UAS-rl^{sem}*/+ (Q and U); *sev*-GAL4/+; *UAS-rl^{sem}*/*UAS-DMKP-3^{15}* (R and V); *sev-PTP-ER^{101}*/X; *sev*-GAL4/+ (S and W); and *sev-PTP-ER^{101}*/X; *sev*-GAL4/+; *DMKP-3^{15}*/+ (T and X). The numbers shown on the bottom of eye section data (mean number of photoreceptor cells per ommatidium ± the standard deviation) were calculated as described in Materials and Methods.

If DMKP-3 does indeed negatively regulate the Ras/DERK pathway in vivo, a loss-of-function mutation of *DMKP-3* could rescue the deleterious phenotypes of loss-of-function mutations of the components in the Ras/DERK pathway. As expected, homozygous *DMKP-3^{P3}* alleles strongly suppressed the rough eye phenotype and the decreased number of photoreceptor cell phenotype of *Raf^{HM7}* hemizygote (compare Fig. 5I and K to 5J and L, respectively). In addition, hypomorphic *DMKP-3* partially rescued temperature-dependent lethality

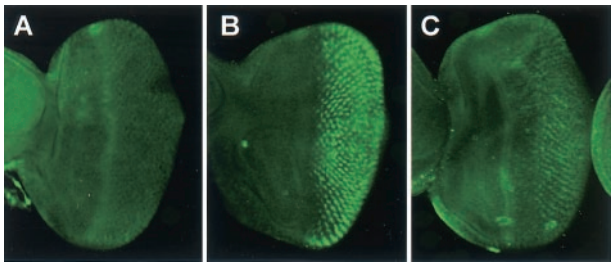

FIG. 4. Dephosphorylation of DERK by DMKP-3 in eye imaginal discs. (A) *sev*-GAL4/+; (B) *sev*-GAL4/+; *UAS-Ras*^{V12}/+; (C) *sev*-GAL4/+; *UAS-Ras*^{V12}/*DMKP-3*^{15}.

(*Raf*^{HM7} males were able to survive at 18°C, but they were lethal at 25°C at or before eclosion [43]) of hemizygote *Raf*^{HM7} males at 25°C (Fig. 5M). Collectively, these results demonstrated the indispensable roles of DMKP-3 in regulating Ras/DERK signaling during *Drosophila* development.

DMKP-3 inhibits cell proliferation. It is well established that downregulation of Ras/ERK signaling activity inhibits cell proliferation in *Drosophila* and mammals (48, 50). As shown in Fig. 1, the overexpression of DMKP-3 led to the reduction in wing size. To investigate whether this phenotype is caused by the inhibition of cell proliferation, we looked for BrdU-labeled cells in wing imaginal discs, in which compartment-specific expression of DMKP-3 can be induced by various wing-specific GAL4 drivers (Fig. 6C, F, and I). In control discs, BrdU incorporation was evenly distributed in the wing pouch except for the zone of nonproliferating cells (Fig. 5A, D, and G). In the discs overexpressing DMKP-3 by *MS1096*-GAL4, *e16E*-GAL4, or *ap*-GAL4 driver, the number of BrdU-labeled cells, however, was significantly reduced (Fig. 5B, E, and H).

The N-terminal DERK-binding domain of DMKP-3 is critical for its activity. Recently, we have reported that overexpression of a catalytically inactive form of DMKP-3 inhibits DERK function via specific DMKP-3–DERK protein-protein interactions (34). This inhibition is mediated by the NBD of DMKP-3, which in turn make DERK impossible to translocate into the nucleus (34). Since MAPK mainly exerts its activities by directly phosphorylating many nuclear targets, the restrained MAPK in the cytoplasm may result in strong inhibition of the MAPK-mediated downstream functions.

To further examine whether DMKP-3 regulates DERK activity via the NBD-mediated protein-protein interaction in *Drosophila* development, we generated various transgenic flies carrying mutated DMKP-3, such as C302A, ΔN, R56/57A, and

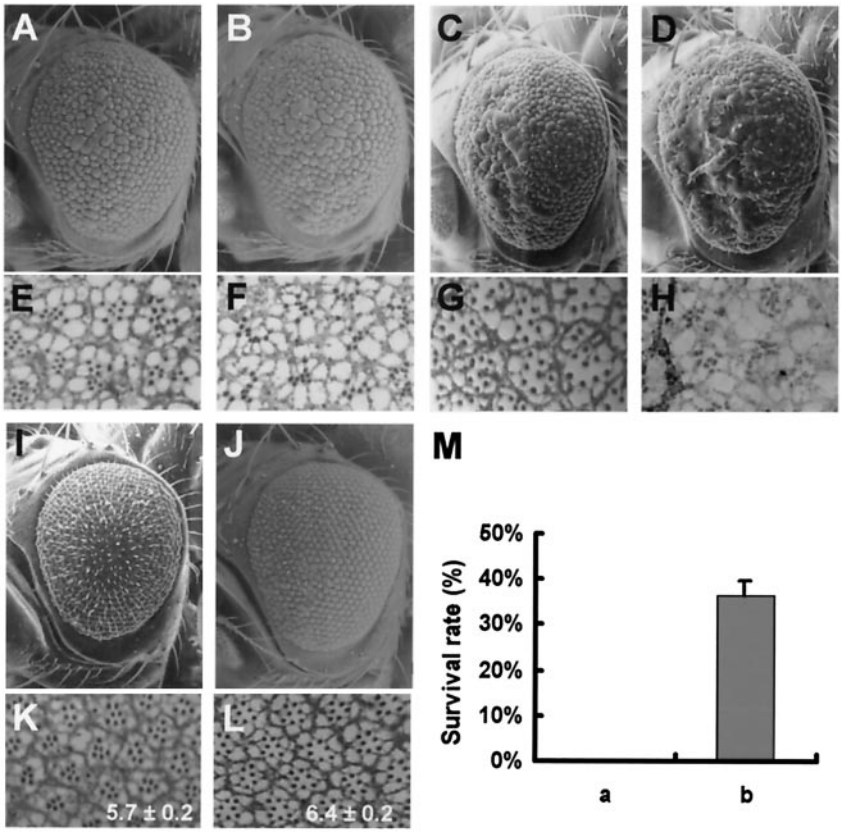

FIG. 5. Loss-of-function mutants of DMKP-3 genetically interact with the mutants in the Ras/DERK pathway. Scanning electron micrographs of the compound eyes (A to D, I, and J) and their tangential cross sections (E to H, K, and L) are shown. (A and E) *sev*-GAL4/+; *UAS-Ras*^{V12}/+; (B and F) *sev*-GAL4/+; *UAS-Ras*^{V12}/*DMKP-3*^{P3}; (C and G) *sev*-GAL4/+; *UAS-Raf*^{F179}/+; (D and H) *sev*-GAL4/+; *UAS-Raf*^{F179}/*DMKP-3*^{P3}; (I and K) *Raf*^{HM7}/Y; (J and L) *Raf*^{HM7}/Y; +/+; *DMKP-3*^{P3}/*DMKP-3*^{P3}. The numbers shown on the bottom of eye section data (mean number of photoreceptor cells per ommatidium ± the standard deviation) were calculated as described in Materials and Methods. (M) Reduced DMKP-3 expression rescued the lethality of hemizygote *Raf*^{HM7} males at 25°C culture condition. (a) *Raf*^{HM7}/Y; (b) *Raf*^{HM7}/Y; +/+; *DMKP-3*^{P3}/*DMKP-3*^{P3}.

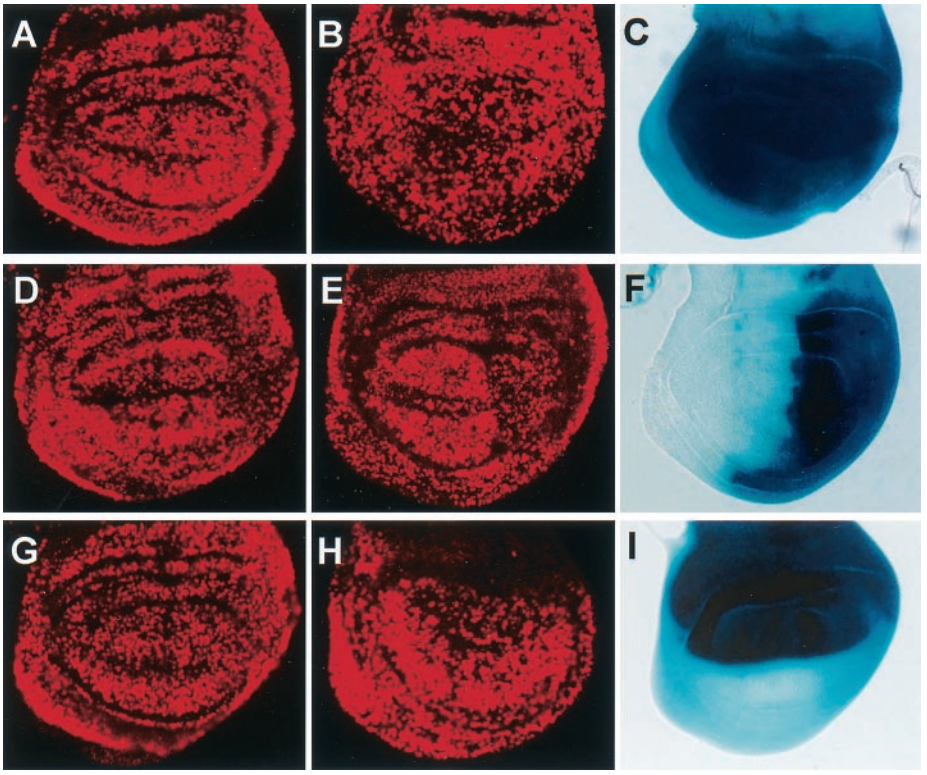

FIG. 6. Inhibited cell proliferation in the DMKP-3-overexpressing wing imaginal discs. Wing imaginal discs were stained with an anti-BrdU antibody as described in Materials and Methods. (A) *MS1096*-GAL4/+; (B) *MS1096*-GAL4/+; *UAS-DMKP-3^{6}*/+; (D) *e16E*-GAL4/+; (E) *e16E*-GAL4/*UAS-DMKP-3^{6}*; (G) *ap*-GAL4/+; (H) *ap*-GAL/*UAS-DMKP-3^{6}*. The expression pattern of GAL4 lines was tested with X-Gal staining in wing imaginal discs. (C) *MS1096*-GAL4/+; *UAS-lacZ*; (F) *e16E*-GAL4/*UAS-lacZ*; (I) *ap*-GAL4/*UAS-lacZ*.

R56/57A/C302A (Fig. 7A), as described in Materials and Methods. R56/57 is important for the specific binding between DMKP-3 and its substrate DERK, and C302 mutation can nullify the phosphatase activity of DMKP-3 (32).

Ectopic expression of ΔN (Fig. 7E and K) or R56/57A (Fig. 7F and L) in the eye by *sev*- (Fig. 7B to G) or *gmr*-GAL4 (Fig. 7H to M) driver did not show any defects compared to the driver alone (Fig. 7B and H) in spite of the presence of their intact phosphatase domain (PD), suggesting that the NBD is critical for the DMKP-3-mediated dephosphorylation and inhibition of DERK in photoreceptor cell differentiation. In addition, ectopic expression of DMKP-3 C302A (Fig. 7D) or R56/57A/C302A (Fig. 7G) under *sev*-GAL4 driver also failed to affect photoreceptor cell differentiation. However, intriguingly, when we expressed these DMKP-3 mutants using a stronger driver, the *gmr*-GAL4 driver, transgenic flies expressing DMKP-3 C302A showed a mild gain-of-function phenotype of DMKP-3 with missing photoreceptor cells (Fig. 7J).

Likewise, overexpression of DMKP-3 ΔN (Fig. 7Q), R56/57A (Fig. 7R), or R56/57A/C302A (Fig. 7S) in the wing did not display any significant phenotypic changes compared to the *MS1096*-GAL4 driver alone (Fig. 7N). However, overexpression of DMKP-3 C302A (Fig. 7P) caused several defects, including disruption of the posterior cross vein and the longitudinal vein L4, which is similar to the phenotype of overexpression of wild-type DMKP-3 (Fig. 7O).

Expression of *rhomboid* is inhibited by DMKP-3. To further confirm whether the NBD of DMKP-3 is required for the repression of DERK function, we examined the transcriptional levels of *rhomboid* (*rho*) in imaginal discs. The transcription of *rho* is directly regulated by DERK (35) and can be monitored by anti-β-galactosidase staining using the *rho^{4A69}* enhancer trap line, as previously described (13). The *rho*-lacZ expression in the eye imaginal discs was dramatically suppressed by overexpression of DMKP-3 wild type (Fig. 8B) compared to the *sev*-GAL4 driver alone (Fig. 8A). Expression of *rho*-lacZ in the eye disc overexpressing DMKP-3 C302A (Fig. 8C) or DMKP-3 R56/57A (Fig. 8D) under the control of the *sev*-GAL4 driver was, however, both quantitatively and qualitatively similar to the control eye disc (Fig. 8A). Likewise, overexpression of DMKP-3 wild type by *MS1096*-GAL4 driver strongly inhibited *rho*-lacZ expression in the primordial vein of the wing imaginal disc (Fig. 8F) compared to the *MS1096*-GAL4 alone (Fig. 8E). On the other hand, ectopic expression of DMKP-3 C302A significantly reduced *rho* expression (Fig. 8G), whereas DMKP-3 R56/57A was unable to suppress *rho* expression in the primordial vein (Fig. 8H).

From the results of domain analysis in eyes and wings, we could draw a conclusion that both NBD and PD of DMKP-3 are necessary for *Drosophila* developmental processes, which is consistent with our previous data (32, 34).

DMKP-3 is required for proper oogenesis and early embryogenesis. Because hypomorphic *DMKP-3* flies displayed a reduced fertility and *DMKP-3* transcripts were enriched in the ovary (32; data not shown), we speculated that DMKP-3 might be involved in the female reproductive system. We generated

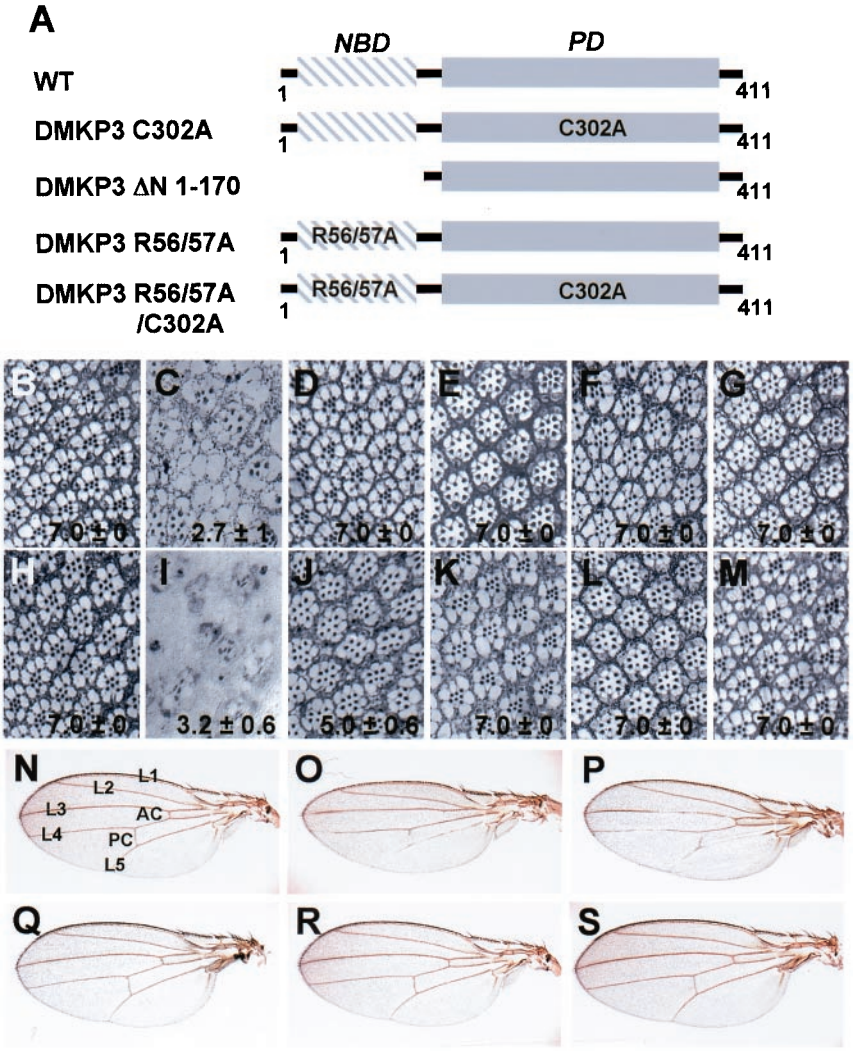

FIG. 7. The NBD and PD of DMKP-3 is required for photoreceptor cell differentiation and wing vein formation. (A) Various DMKP-3 mutants were generated in the present study as described in Materials and Methods. Functionally important amino acid residues in the NBD and PD are indicated. (B to M) Tangential cross sections of the compound eyes expressing mutant DMKP-3 are shown. (B) *sev*-GAL4/+; (C) *sev*-GAL4/+; *UAS-DMKP-3*^{10}/+; (D) *sev*-GAL4/*UAS-DMKP-3 C302A*; (E) *sev*-GAL4/*UAS-DMKP-3* ΔN; (F) *sev*-GAL4/+; *UAS-DMKP-3 R56/57A*/+; (G) *sev*-GAL4/*UAS-DMKP-3 R56/57A/C302A*; (H) *gmr*-GAL4/+; (I) *gmr*-GAL4/+; *UAS-DMKP-3*^{10}/+; (J) *gmr*-GAL4/*UAS-DMKP-3 C302A*; (K) *gmr*-GAL/*UAS-DMKP-3* ΔN; (L) *gmr*-GAL4/+; *UAS-DMKP-3 R56/57A*/+; (M) *gmr*-GAL4/*UAS-DMKP-3 R56/57A/C302A*. (N to S) Microscopic views of the wings expressing DMKP-3. (N) *MS1096*-GAL4/+; (O) *MS1096*-GAL4/+; +/+; *UAS-DMKP-3*^{10}/+; (P) *MS1096*-GAL4/+; *UAS-DMKP-3 C302A*/+; (Q) *MS1096*-GAL4/+; *UAS-DMKP-3* ΔN/+; (R) *MS1096*-GAL4/+; +/+; *UAS-DMKP-3 R56/57A*/+; (S) *MS1096*-GAL4/+; *UAS-DMKP-3 R56/57A/C302A*/+. The numbers shown on the bottom of eye section data (mean number of photoreceptor cells per ommatidium ± the standard deviation) were calculated as described in Materials and Methods.

germ line clones of *DMKP-3* by using the FLP-FRT-DFS technique (11). Interestingly, females with *DMKP-3*^{P1} germ line clone laid only a small number of eggs compared to the control flies (less than ~30%), implying severe defects in oogenesis. Even the laid eggs were abnormal: approximately 77% of the laid eggs were shorter than the normal ones (Fig. 9D; compare Fig. 9B to A), and some displayed severe defects in chorion (egg shell) formation (Fig. 9C). Furthermore, none of the *DMKP-3*^{P1} germ line clone embryos could escape the embryonic stage and progress beyond the two-nuclei stage (Fig. 9F and H) compared to the wild type (Fig. 9E and G). These results supported that DMKP-3 is required for the proper oogenesis and early embryogenesis.

DISCUSSION

We investigated in vivo the roles of DMKP-3 in *Drosophila* development. Our studies revealed novel functions of DMKP-3 as a negative regulator in a variety of developmental processes including cell differentiation and proliferation controlled by the Ras/DERK pathway. Ectopic expression of DMKP-3 in the eye strongly suppressed photoreceptor cell differentiation (Fig. 1D to F), and this phenotype was further enhanced by genetic reduction of the Ras/DERK-dependent signaling activity (Fig. 3). In addition, overexpressed DMKP-3 also caused suppression in wing vein differentiation, which is correlated with the inhibition of the Ras/DERK signaling path-

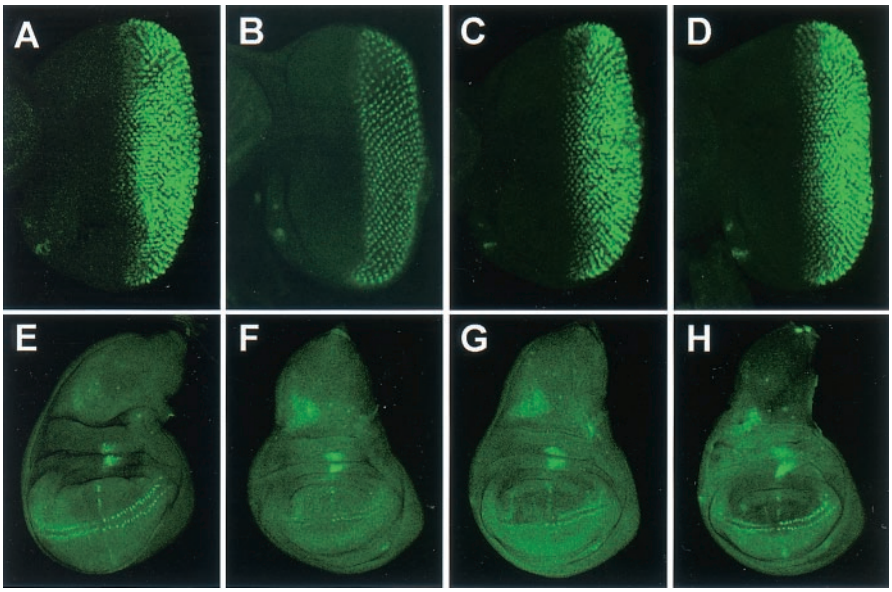

FIG. 8. Expression of *rhomboid* is inhibited by DMKP-3. Antibody staining of β-galactosidase in rho^{AA69} reveals *rho* expression. (A) *sev*-GAL4/+; rho^{AA69}/+; (B) *sev*-GAL4/+;*UAS-DMKP-3*^{10}/rho^{AA69}; (C) *sev*-GAL4/*UAS-DMKP-3 C302A*; rho^{AA69}/+; (D) *sev*-GAL4/+;*UAS-DMKP-3 R56/57A*/rho^{AA69}; (E) *MS1096*-GAL4/+; +/+; rho^{AA69}/+; (F) *MS1096*-GAL4/+; +/+; *UAS-DMKP-3*^{10}/rho^{AA69}; (G) *MS1096*-GAL4/+; *UAS-DMKP-3 C302A*/+; rho^{AA69}/+; (H) *MS1096*-GAL4/+; +/+; *UAS-DMKP-3 R56/57A*/rho^{AA69}.

way during wing development (Fig. 1G to L). Conversely, hypomorphs of *DMKP-3* (Fig. 2J and P) or *DMKP-3*-null clones (Fig. 2N and R) displayed ectopic differentiation of photoreceptor cells and wing vein cells. These results strongly suggest that DMKP-3 plays an essential role in the regulation of cell differentiation during *Drosophila* development by restraining the activities of the Ras/DERK signaling pathway.

In addition, we also observed that DMKP-3 overexpression causes a dramatic reduction in the overall size of adult wing blades (Fig. 1I and L) and larval wing imaginal discs (data not shown), as well as the defects in wing vein cell differentiation. In support of this phenotype, the active cell proliferation in wing imaginal discs, monitored by measuring BrdU incorporation, was strongly inhibited by DMKP-3 overexpression (Fig. 6). Therefore, we conclude that DMKP-3 is able to negatively modulate cell proliferation in vivo. Consistently, numerous previous results also have shown that downregulation of Ras/ERK signaling activity inhibits cell proliferation (18, 36, 50, 53). Collectively, these results provide strong in vivo evidence that DMKP-3 suppresses cellular proliferation, as well as differentiation, both of which are promoted by the Ras/DERK pathway during development.

Because the females of hypomorphic DMKP-3 mutants displayed a phenotype of decreased number of laid eggs, we suspected that there is another function of DMKP-3 in the female reproductive system. When the maternal DMKP-3 was depleted in the female germ line, the number of laid eggs was further reduced to that of hypomorphic DMKP-3 flies, and even the laid eggs showed severe morphological defects, including chorion malformation, which is known to be caused by defects in the vitellogenesis process during oogenesis (58). Although some eggs appeared to have normal shape, they failed to show any further embryonic development, just arresting at the single-nucleus or two-nucleus stage (Fig. 9E to H).

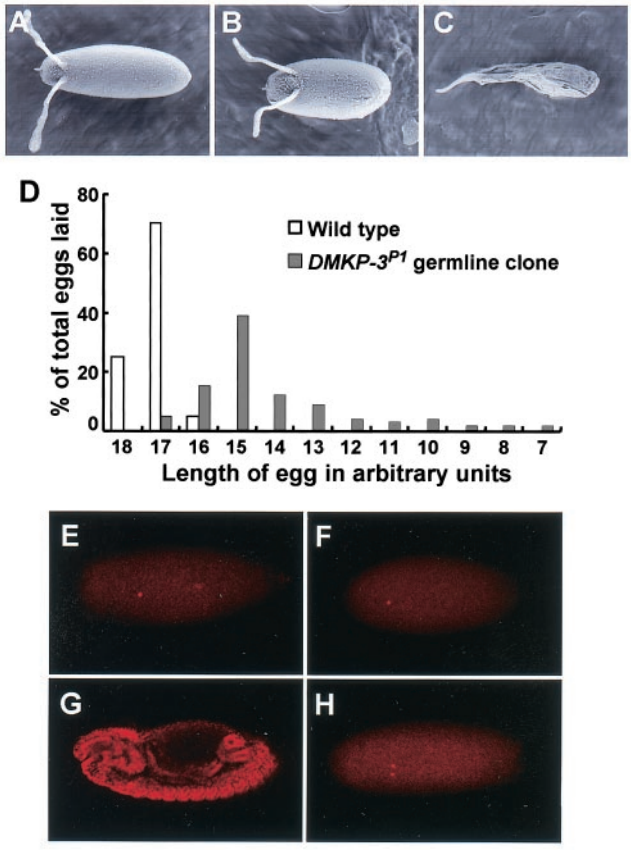

FIG. 9. Defective embryos in *DMKP-3*^{P1} germ line clones. Scanning electron micrographs of w^{1118} (A) and *DMKP-3*^{P1} germ line clone eggs (B and C). (D) Measurements of egg lengths in wild type (w^{1118}) and in *DMKP-3*^{P1} germ line clone are shown (1 arbitrary unit = 30.6 μm). (E to H) Propidium iodide-stained embryos. (E and F) Eggs 0.5 h after egg laying; (G and H) eggs 15 h after egg laying; (E and G) w^{1118}; (F and H) *DMKP-3*^{P1} germ line clones.

These results suggest that DMKP-3 is also required for proper oogenesis and early embryogenesis besides the other developmental processes, as we have described, such as eye and wing development. Interestingly, various gain-of-function mutants of DERK also have shown female sterility with severe defects in vitellogenesis in previous studies (38), demonstrating that the DMKP-3-deficient phenotypes in female germ lines may be the consequences of DERK hyperactivation.

It should be noted that another DERK phosphatase, PTP-ER, has been identified in the *Drosophila* system (28). Eye-specific overexpression of PTP-ER suppresses DERK activity, causing a reduction in the photoreceptor cell number (Fig. 3W). However, the null mutants of PTP-ER develop well and only show mild defects in photoreceptor cell differentiation (28), whereas the loss-of-function DMKP-3 mutants become lethal from the embryonic or early larval stages. Since the mammalian homologues of PTP-ER are expressed in a neuron-specific manner (28, 47), it seems that *Drosophila* PTP-ER acts specifically in neuronal photoreceptor cells. In contrast to PTP-ER, DMKP-3 was shown to be involved not only in photoreceptor cell specification but also in other developmental processes such as vein cell differentiation, oogenesis, and early embryogenesis. Moreover, DMKP-3 is ubiquitously expressed throughout all developmental stages (Fig. 1N and Q and 2D [32]). Therefore, we conclude that DMKP-3 is a general DERK phosphatase controlling various processes of *Drosophila* development.

In conclusion, we provide here firm genetic evidence to support that DMKP-3 acts as an essential antagonist against the Ras/DERK-dependent signaling pathway during *Drosophila* development. Further analyses of DMKP-3 functions in *Drosophila* may provide abundant insights for understanding the in vivo roles of the Ras/DERK-dependent signaling pathway in the context of both cell differentiation and proliferation.

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