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Roles of myosin phosphatase during *Drosophila* development

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SUMMARY

Myosins are a superfamily of actin-dependent molecular motor proteins, among which the bipolar filament forming myosins II have been the most studied. The activity of smooth muscle/non-muscle myosin II is regulated by phosphorylation of the regulatory light chains, that in turn is modulated by the antagonistic activity of myosin light chain kinase and myosin light chain phosphatase. The phosphatase activity is mainly regulated through phosphorylation of its myosin binding subunit MYPT. To identify the function of these phosphorylation events, we have molecularly characterized the *Drosophila* homologue of MYPT, and analyzed its mutant phenotypes. We find that *Drosophila* MYPT is required for cell sheet movement during dorsal closure, morphogenesis of the eye, and ring canal growth during oogenesis. Our results indicate that the regulation of the phosphorylation of myosin regulatory light chains, or dynamic activation and inactivation of myosin II, is essential for its various functions during many developmental processes.

Key words: Rac, Rho, Rho kinase, Myosin II, Myosin phosphatase, *zipper*, *spaghetti-squash*, MYPT, Dorsal closure, Ring canal growth, Eye morphogenesis, *Drosophila melanogaster*

INTRODUCTION

Myosins are a superfamily of actin-dependent molecular motor proteins involved in a variety of essential processes that include muscular contraction, cytokinesis, vesicle transport, cell movement and cell shape change (reviewed by Mermall et al., 1998; Oliver et al., 1999; Sellers, 2000; Sokac and Bement, 2000; Wu et al., 2000). Among the 17 subclasses of myosins, conventional myosins, known as myosin IIs, have been the most studied. Myosin IIs form bipolar filaments that drive contractile events by bringing together actin filaments of opposite polarity. Myosin II molecules are hexameric enzymes consisting of two heavy chains, two regulatory light chains (MRLC), and two essential light chains. They can be subclassified into four groups based on their motor domain (or tail) sequences: sarcomeric myosins, vertebrate smooth muscle/non-muscle myosins, *Dictyostelium*/*Acanthamoeba* type myosins and yeast type myosins.

The activity of smooth muscle/non-muscle myosin II is regulated by the phosphorylation of MRLC that is modulated by the antagonistic activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). MLCP is composed of three subunits: a catalytic subunit made up of protein phosphatase 1c β (also called δ), a myosin binding or targeting subunit (MYPT), and a small subunit of unknown function. MYPT binds and confers the selectivity of PP1c for myosin (Hartshorne et al., 1998).

The phosphatase activity of MLCP can be regulated in several ways (reviewed by Hartshorne et al., 1998; Somlyo and Somlyo, 2000). Rho-kinase (ROCK) phosphorylates an inhibitory phosphorylation site on MYPT and inhibits the phosphatase activity in smooth muscle. This phosphorylation may occur through ZIPK (leucine zipper interacting protein kinase)-like kinase (MacDonald et al., 2001) or integrin-linked kinase (Kiss et al., 2002). Myotonic dystrophy protein kinase phosphorylates the same inhibitory phosphorylation site (Muranyi et al., 2001), although it is not clear whether this phosphorylation event also goes through ZIPK. In addition, protein kinase C (PKC) can phosphorylate the ankyrin repeat region of MYPT, and thus attenuate the interaction of MYPT with PP1c and MRLC (Toth et al., 2000). Furthermore, CPI-17, a smooth muscle-specific inhibitor of MLCP, can also regulate the phosphatase activity of MLCP. Phosphorylation of CPI-17 by PKC, or ROCK, or protein kinase N, or p21-activated kinase (PAK) dramatically enhances the inhibition ability of CPI-17 (Eto et al., 1997; Koyama et al., 2000; Senba et al., 1999; Takizawa et al., 2002a; Takizawa et al., 2002b). Finally, MRLC can also be phosphorylated by ROCK and PAK, which itself is a substrate of Rac and Cdc42. Thus ROCK can regulate MRLC phosphorylation both through direct phosphorylation of MRLC and through inactivation of MLCP. Importantly, although the biochemistry of these phosphorylation events is well characterized, the physiological significance of these regulatory steps in vivo remains to be explored.

The in vivo function of non-muscle myosin II has been extensively analyzed in *Drosophila melanogaster*, *Dictyostelium discoideum* and *Saccharomyces cerevisiae*. *Drosophila* has a single non-muscle myosin II heavy chain encoded by *zipper (zip)*, as well as a single non-muscle myosin

II regulatory light chain encoded by *spaghetti squash (sqh)*. Analysis of the phenotypes associated with mutations in *zip* and s*qh* have revealed that non-muscle myosin II regulates cell shape changes and cell movements in multiple processes such as cytokinesis, dorsal closure and oogenesis (Edwards and Kiehart, 1996; Jordan and Karess, 1997; Wheatley et al., 1995; Young et al., 1993). In addition, mutations in both *zip* and *sqh* affect planar cell polarity during development (Winter et al., 2001).

The temporal requirement of *zip* has been studied in sqh^2 mutant animals that carry a *sqh* transgene driven by a heat shock promoter (Edwards and Kiehart, 1996). This analysis showed that *sqh* activity is needed for eye and leg imaginal discs morphogenesis. Also, during oogenesis, *sqh* is required for morphogenesis of interfollicular stalks, border cell migration, centripetal cell ingression, dorsal appendage cell migration, and rapid transport of the nurse cell cytoplasm into the oocyte. Inhibition of this transport was also observed in animals that carry homozygous sqh^1 germline clones (GLCs) (Wheatley et al., 1995).

The in vivo function of MRLC phosphorylation was determined by expression of *sqh* transgenes that contain mutated phosphorylation sites in a *sqh* null mutant background (Jordan and Karess, 1997). Embryos carrying the null mutation sqh^{AX3} die, mostly during the first larval instar, and sqh^{AX3} GLCs develop extensive defects, including failure in cytokinesis, during oogenesis. SqhA20A21, which has both the primary and secondary phosphorylation sites changed to alanine, failed to rescue sqh^{AX3}, indicating that phosphorylation of Sqh is important for myosin II function. In support of this, a change of serine 21 to glutamic acid (SqhE21), that presumably mimics constitutive phosphorylation of Sqh, substantially rescues the sqh^{AX3} oogenesis phenotype.

To gain further insight into the regulation of Zip and to define precisely the in vivo function of MLCP, we have cloned the *Drosophila* homologue of the MYPT gene (*DMYPT*). We find that *DMYPT* is essential for cell sheet movement during dorsal closure, morphogenesis during eye development, and ring canal growth during oogenesis. Our results indicate that regulation of the phosphorylation state of MRLC, and dynamic activation and inactivation of myosin II, are essential for its various functions during many developmental processes.

MATERIALS AND METHODS

Fly strains

The fly strains we used include the following: *y w* $Drok^2FRT^{19A}$*/FM7, act-p-GFP* from Liqun Luo (Winter et al., 2001), *y w;sqh[E20E21]* (Winter et al., 2001) and *w;sqh[A20A21]* from Roger Karess (Jordan and Karess, 1997), $RhoA^{720}$*/Cyo* from David Strutt (Strutt et al., 1997), *GMR-Rho/TM3,Sb*, *GMR-*Rac^{7A}, and *GMR-Cdc42* from Jeff Settleman (Nolan et al., 1998), *MKRS/1A107* (Perrimon et al., 1991), *l(3)03802/TM3,Sb*, zip^1*/Cyo*, ck^{P13} *FRT/Cyo;*kar^2ry^{506}, *Df(3L)th102, Df(3L)brm11, Df(3L)st-f13, Df(3L)st-g24, Df(3L)th117*, and *Df(3L)st-e4* from the Bloomington Stock Center, *EP(3)3727/TM6,Tb* from the Szeged P-Insertion Mutant Stock Center.

Cloning of *DMYPT*

The *DMYPT* cDNAs, AT12677, RE34228, RE63915 and AT31926 were obtained from ResGen, Invitrogen Corporation. AT12677 was sequenced completely in both directions, while the other clones were only sequenced from their most 5′ and 3′ ends. To determine the organization of the *DMYPT* locus, sequences from all clones were assembled into one contig and then compared with the *Drosophila* genome sequence. AT12677, RE34228, RE63915 contain the entire *DMYPT* open reading frame (ORF). RE34228, RE63915 include all of exon 1, AT12677 starts from the middle of exon 2, 43 bp 5′ of the start codon. AT31926 starts within exon 4. AT12677, RE34228 and AT31926 share the same 3′ end.

Generation and rescue of *DMYPT* mutations

P-element insertions were excised using the Δ2-3 transposase following conventional methods. These excision lines were then analyzed for lethality and fertility. For rescue experiments, the *DMYPT* ORF from AT12677 was cloned by PCR into CaSpeR-hs between *Not*I and *Xba*I, and injected with helper DNA (a source of Δ2-3 transposase) into w^{1118} flies to generate transgenic lines. One of the transgenic lines, *hs2-4*, is a viable insertion on the X chromosome and was used to generate *hs2-4/+;* $DMYPT^{03802}$*/TM3,Sb* animals. The numbers of rescued $DMYPT^{03802}$ mutant progeny were scored following a 1-hour daily heat-shock treatment at 37°C.

Generation of germline clones

Germline clones (GLCs) of $DMYPT^{03802}$ were generated as described previously (Chou and Perrimon, 1996), by crossing *y w hs-FLP22;* $ovo^{D1}FRT^{2A}$*/TM3,Sb* males to $DMYPT^{03802}FRT^{2A}$*/TM3,Sb* females. Third instar larval progeny were placed at 37°C for 2 hours each day for 3 days. Females with $DMYPT^{03802}$ homozygous GLCs were mated with $DMYPT^{03802}$*/TM3,actinGFP* males and allowed to lay eggs.

Cuticle and eggshell preparation

For cuticle preps, overnight egg collections were aged for 30 hours at 25°C, dechorionated with a 50% solution of commercial bleach, washed with PBST (PBS and 0.1% Triton X-100), mounted in Hoyers mounting medium with lactic acid, and heat-treated at 60°C overnight. Eggshells were prepared similarly without the bleaching step. Images were taken with a SPOT™ digital camera (Diagnostic Instruments) using phase-contrast or dark-field optics on a Zeiss Axiophot microscope and processed with Adobe Photoshop.

Dissecting and staining of egg chambers

Egg chambers were dissected in Schneider's *Drosophila* medium with 10% FBS, fixed (PBS with 0.5% Triton X-100, 6 U/ml of Texas Red phalloidin, 4% formaldehyde) for 20 minutes, washed with PBST, incubated with primary antibodies in PBS with 0.3% Triton-X-100 and 0.1 μg/μl of BSA, at 4°C overnight, washed, and incubated with secondary antibodies at room temperature for 1.5 hours. Texas Red phalloidin (2 U/ml) and DAPI (1 μg/ml) was added during the last half an hour incubation, and washed with PBST. Primary antibodies used were: anti-Kelch 1B (1:1) (Xue and Cooley, 1993), anti-hu-li tai shao RC (anti-Hts) (1:1) (Robinson et al., 1994) (both from Developmental Studies Hybridoma Bank), anti-Zip (Jordan and Karess, 1997), and anti-phosphotyrosine (4G10, 1:500, UBI). Secondary antibodies used were: Texas Red, Cy5, or Alexa Fluor 488 goat anti-mouse or anti-rabbit IgG (Jackson or Molecular Probes). Egg chambers were staged according to Spradling (Spradling, 1993). Images were captured with a Leica TCS-NT confocal microscope and a series of Z sections were stacked.

Embryo staining

In situ hybridization to embryos was performed as described previously (Patel et al., 1987). For immunofluorescence staining, embryos from *mutant/TM3,actinGFP* were fixed and stained with rabbit anti-GFP serum (1:2000, Molecular Probes) and mouse anti-fasciclin III (7G10, 1:20, DSHB) or anti-phosphotyrosine (4G10, 1:1500, UBI). Homozygous mutant embryos were identified by the absence of GFP.

Genetic interaction

Males carrying GMR-Rac^{7A} were crossed to females of the following genotypes: OreR, *Drok^{2}/FM7*, *zip^{1}/Cyo*, *ck^{P13}/Cyo*, *DMYPT$^{2\text{-}188}$/TM3,Sb Tb*, *DMYPT$^{2\text{-}199}$/TM3,Sb Tb*, *DMYPT^{03802}/TM3,Sb Tb*, *Df(3L)th102/TM3,Sb*, *y w;sqh[E20E21], w;sqh[A20A21]*, and *RhoA^{720}/CyO*. The progeny were raised at 29°C and the resulting adults were dehydrated in an ethanol series, dried in SAMDRI PVT-3B and coated with Hummer V Sputter Coater. Scanning electron micrographs were generated using a LEO 1450 VP electron microscope.

RESULTS

Structure of *DMYPT*

A BLAST search of the *Drosophila* database (http://flybase.bio.indiana.edu) with mammalian MYPT sequences reveals that the *Drosophila* genome has a single related gene, *CG5891*. *CG5891* is predicted to encode a protein with limited homology to mammalian MYPT at the N terminus (~300 aa). However, sequence analysis of several cDNAs derived from *CG5891* (see Materials and Methods) uncovered additional regions of homology between the mammalian and fly homologues suggesting that the predicted *CG5891* gene was incorrectly annotated. A representative cDNA, AT12677, encodes an ORF of 1101 amino acids (aa) that we named *Drosophila* MYPT (DMYPT) to follow the nomenclature of the mammalian protein. A comparison of the compiled *DMYPT* cDNA and genome sequences shows that the *DMYPT* locus contains 18 exons and 17 introns (Fig. 1A). The start codon lies in the second exon and the stop codon in the last. Sequence alignment shows that DMYPT shares significant homology with human MYPTs in three regions (Fig. 1B), the N terminus containing several ankyrin repeats, the C terminus, and a short peptide in the middle that contains the highly conserved inhibitory phosphorylation site (Fig. 1C) (Kawano et al., 1999; Kimura et al., 1996).

Genetics of the *DMYPT* locus

To characterize the consequences of loss of DMYPT function during development, we searched for mutations in the *DMYPT* gene. Two P-element transposon insertions in the *DMYPT* locus have been defined molecularly by recovery of flanking genomic sequence (Fig. 1A). *EP(3)3727*, in the first intron, is homozygous viable and *l(3)03802*, in the tenth intron, is associated with zygotic lethality. We also identified several deficiencies that remove *DMYPT* sequences based on genetically defined breakpoints as well as their failure to complement *l(3)03802* (Fig. 1D). *Df(3L)th102* deletes *DMYPT* entirely and thus serves as a complete loss-of-function allele for use in this study.

To determine whether the *l(3)03802* P-element insertion within the *DMYPT* locus is responsible for the lethality, and to generate new deletion alleles, we excised both *DMYPT* P-element insertions using the Δ2-3 transposase. Mobilization of each element resulted in the recovery of both viable precise excisions and lethal imprecise excisions. Among the >200 excisions derived from *l(3)03802*, over half were viable, indicating that the lethality associated with the *l(3)03802* chromosome is due to disruption of *DMYPT* and not another lethal hit. Thus *l(3)03802* is renamed as *DMYPT^{03802}* and *EP(3)3727*as *DMYPT^{3727}*. Two of the strongest embryonic lethal excision lines, *DMYPT$^{2\text{-}188}$* and *DMYPT$^{2\text{-}199}$*, like the original insert, *DMYPT^{03802}*, fail to complement *Df(3L)th102* and are described in detail below. Eleven of the 39 lethal excisions derived from *DMYPT^{3727}* failed to complement with *DMYPT^{03802}* and *Df(3L)th102*, which is consistent with the notion that they disrupt *DMYPT* activity.

To confirm that the *DMYPT^{03802}* insertion disrupts DMYPT function and that the cDNA derived from the *DMYPT* locus encodes all the functions associated with DMYPT activity, we rescued the original lethal P insertion with a transgene containing a heat shock promoter driving a *DMYPT* cDNA. Following 1-hour heat treatments daily from embryogenesis to eclosion, *hs-DMYPT* fully rescues *DMYPT^{03802}* homozygous animals to adulthood. Stopping heat treatment 1 to 2 days before eclosion lead to incomplete rescue of *DMYPT^{03802}*, with adults developing wing and leg defects similar to those noted for *zip* or *sqh* mutants partially rescued by a transgene (Edwards and Kiehart, 1996; Halsell et al., 2000) (data not shown). Stopping heat treatment 3 days prior to eclosion resulted in no rescue to adulthood. The complete rescue of the lethality associated with *DMYPT^{03802}* by the *hs-DMYPT* transgene demonstrates that loss of *DMYPT* activity is responsible for the lethal phenotype.

Loss of *DMYPT* activity during embryogenesis is associated with a dorsal closure phenotype

To assess the timing and cause of lethality associated with the *DMYPT^{03802}* insertion, embryos were collected and analyzed. Lethal phase analysis showed that 44% of homozygous *DMYPT^{03802}* animals die during embryogenesis, while the remaining 56% die during early first larval instar (485 total embryos counted). More than 80% of the dead mutant embryos displayed a failure of dorsal closure with a characteristic dorsal hole in their cuticles (Fig. 2B,C). The size of the hole in such flies is variable and is also influenced by the genetic background (data not shown). Homozygous *Df(3L)th102* embryos (Fig. 2D), as well as *DMYPT^{03802}/Df(3L)th102* embryos (Fig. 2E) also showed dorsal closure defects. The embryonic cuticle phenotype of *DMYPT^{03802}/Df(3L)th102* is more severe (more embryos displayed large dorsal holes) than homozygous *DMYPT^{03802}*, suggesting that *DMYPT^{03802}* is a hypomorphic allele. In addition, all of the embryonic lethal excision lines analyzed that were derived from *DMYPT^{03802}* (data not shown), and ten of the lethal excision lines from *DMYPT^{3727}* (Fig. 2F), produced embryos with dorsal closure defects. Altogether, these results indicate that *DMYPT* is required for dorsal closure.

Dorsal closure involves a cell sheet movement where the dorsal-lateral ectoderm on both sides of the developing embryo moves toward the dorsal midline to cover a degenerative squamous epithelium, the amnioserosa (reviewed by Knust, 1997; Noselli and Agnes, 1999; Stronach and Perrimon, 1999). This epithelial cell sheet movement encloses the embryo in a continuous protective epidermis. Genetic loss-of-function studies have identified the Jun N-terminal kinase (JNK) signal transduction cascade as one of the key modulators of dorsal closure morphogenesis (Noselli and Agnes, 1999). Transcriptional targets of JNK signaling include *decapentaplegic* (*dpp*), a secreted morphogen related to the bone morphogenetic proteins (BMPs), and *puckered* (*puc*), a dual-specificity phosphatase that mediates a negative

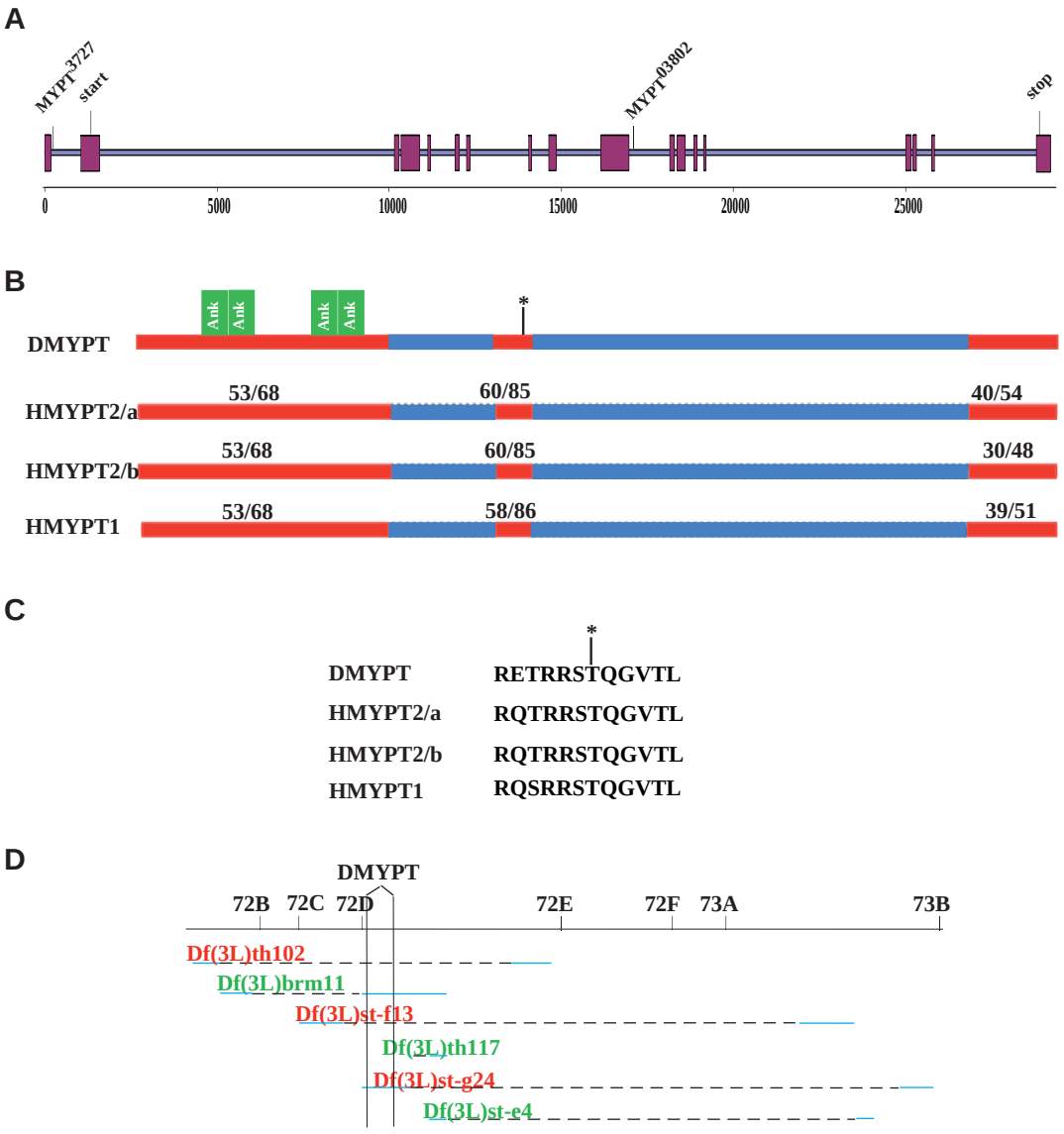

Fig. 1. The *Drosophila MYPT* gene. (A) Genomic organization of *DMYPT*. Exons are shown in purple and introns in blue. The start and stop codons of *DMYPT*, as well as the location of the two P-insertions, $DMYPT^{03802}$ and $DMYPT^{3727}$, are indicated. The sequence of *DMYPT* cDNA differs from the annotation of *CG5891* in several places. Compared to the original annotation, we find that *DMYPT* has: (1) a three base pair (bp) deletion at the beginning of exon 5 (numbered after the annotation); (2) no exon 6; (3) a 48 bp insertion after exon 10; (4) a 24 bp deletion at the end of exon 15; and (5) four additional exons at the 3′ end. At the amino acid level, DMYPT (GenBank accession number AF500094) is similar to CG5891 over the first 337 residues and is unrelated thereafter. (B) BLAST alignment of DMYPT with three human MYPT isoforms: 2/a, 2/b, and 1. Homologous regions are shown in red with percentages of amino acid identity and similarity indicated. DMYPT contains four ankyrin repeats highlighted in green at the N terminus of the protein. The overall homology is higher between DMYPT and HMYPT2 than HMYPT1. (C) Amino acid sequence around the inhibitory phosphorylation site threonine (asterisk in B and C). (D) A schematic drawing of the chromosome arm and deficiencies around the *DMYPT* locus. Deficiencies that fail to complement with *DMYPT* are shown in red and those that do complement are shown in green. Regions deleted in the deficiencies are marked with dashed line. The uncertain breakpoints are shown in blue.

feedback loop of the JNK signal transduction pathway via dephosphorylation of JNK.

To determine whether the failure of dorsal closure in *DMYPT* mutants is due to an influence on JNK signaling, we assayed for *dpp* expression in the leading cells of the ectoderm during closure. In situ hybridization revealed that the spatial and temporal expression pattern of *dpp* is normal in *DMYPT* mutant embryos (data not shown), suggesting that DMYPT does not function through the JNK pathway during dorsal closure.

To further examine the cause of dorsal closure defects in the mutants, we stained *DMYPT* mutant embryos for markers that allowed us to analyze the cell polarity and shape in the dorsal ectoderm. We observed apically localized phosphotyrosine immunoreactivity similar to wild-type flies (Fig. 3A). Moreover, there was normal basolateral fasciclin III immunostaining (Fig. 3B). Altogether, these results suggest that there are no gross defects in cell orientation or polarity. However, we did notice that older mutant embryos began to show abnormal cell shapes at the leading edge of the epidermis (Fig. 3B), which could account for the defects in dorsal closure observed in the *DMYPT* mutants.

Consistent with the late embryonic defects observed in *DMYPT* zygotic mutants, we find that *DMYPT* is maternally contributed and ubiquitously expressed during embryogenesis (data not shown). This maternal supply of DMYPT is likely the reason that the dorsal closure phenotype is variable among embryos and is influenced by genetic background. However, we cannot address this question directly since *DMYPT* is required during oogenesis (see below).

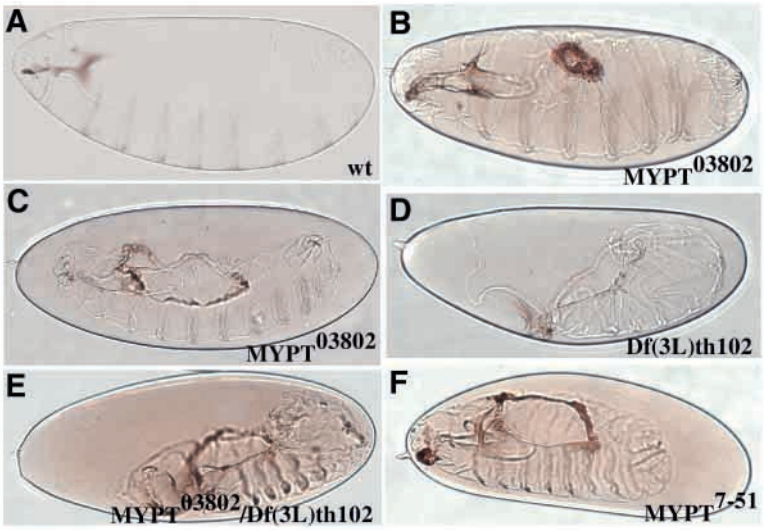

Fig. 2. Dorsal closure defects associated with *DMYPT* mutations. Cuticles of (A) wild-type embryo, (B,C) *DMYPT*^{03802} homozygous embryos with different sized dorsal holes; (D) homozygous *Df(3L)th102* embryo, (E) *Df(3L)th102/DMYPT*^{03802} embryo, and (F) homozygous *DMYPT*$^{7\text{-}51}$ embryo. *DMYPT*$^{7\text{-}51}$ is one of the imprecise excision derivatives of *DMYPT*^{3727}. Anterior is to the left and dorsal is up.

DMYPT is required for ring canal growth during oogenesis

During oogenesis, each cystoblast divides four times with incomplete cytokinesis and produces one oocyte and fifteen support nurse cells that are all connected through cleavage furrows. These cleavage furrows subsequently develop into ring canals. These open rings allow the nurse cells to transport cytoplasm into the oocyte, slowly from stage 6 to stage 10, then rapidly at stage 11. This fast phase of transport is referred to as 'dumping', and has been shown previously to require the activity of Sqh (MRLC). In *sqh* mutant germline egg chambers, dumping is blocked (Wheatley et al., 1995).

To analyze the role of *DMYPT* during oogenesis, we generated homozygous mutant germline clones (GLCs) of *DMYPT*^{03802} using the FLP-FRT/dominant female sterile technique (Chou and Perrimon, 1996). Females carrying *DMYPT*^{03802} homozygous GLCs lay few tiny eggs, about a quarter of the size of wild type eggs (Fig. 4, compare A and C), which do not develop. Examination of the mutant egg chambers revealed that the dumping of nurse cell cytoplasm to the oocyte was blocked (Fig. 4, compare B and D). This is similar to the dumpless phenotype observed with *sqh* homozygous mutant GLCs as well as for mutants in other actin binding proteins (reviewed by Robinson and Cooley, 1997).

To investigate the basis of the dumpless phenotype associated with *DMYPT*^{03802} GLCs, we stained actin filaments using Texas Red phalloidin. The most obvious defect involves the ring canals. At stage 8, wild-type egg chambers had large bagel-shaped ring canals (Fig. 5A). In contrast, the ring canals of *DMYPT*^{03802} GLC egg chambers were very small (Fig. 5B).

To determine whether the ring canals of *DMYPT*^{03802} GLCs never enlarged, or whether they grew and then collapsed, we examined the ring canals in different stage egg chambers. In wild-type egg chambers, ring canals grow from 1 μm at stage 2 to 10 μm at stage 11 (Fig. 5C) (see also, Tilney et al., 1996). In contrast, the ring canals of *DMYPT*^{03802} GLCs barely grew (Fig. 5D). Mutation of *DMYPT* in follicular cells have no effects on the ring canal growth (data not shown), suggesting that DMYPT is required in the germline for ring canal growth. Presumably, these small ring canals cannot support the fast phase cytoplasmic transport and thus cause the dumpless phenotype resulting in tiny eggs.

In addition to actin, several other proteins, including Hu-li tai shao (Hts), Kelch, and phosphotyrosine (pY)-containing proteins (Robinson et al., 1994; Xue and Cooley, 1993), are recruited to ring canals as they form. Immunolocalization experiments revealed that both Hts and Kelch were localized

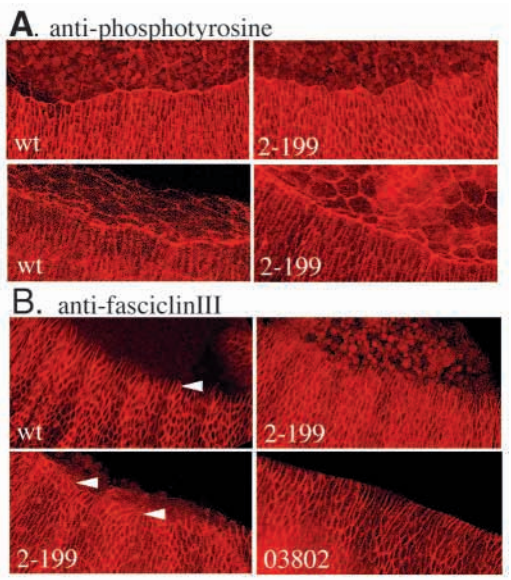

Fig. 3. Cell shape is mildly perturbed in *DMYPT* mutant embryos. (A) Phosphotyrosine immunostaining of the ectoderm and amnioserosa of wild-type (wt) and *DMYPT* mutant (*DMYPT*$^{2\text{-}199}$) embryos at an early stage of dorsal closure (top panels) and midway through dorsal closure (bottom panels). The cell shape, organization and polarity of the mutants is comparable to wild type. (B) Immunostaining for the ectodermal marker, fasciclin III, reveals orderly cell elongation at the leading edge of wild-type embryonic epidermis (arrowhead). *DMYPT* mutant embryos (*DMYPT*$^{2\text{-}199}$, *DMYPT*^{03802}) show occasional disruption of cell shape and elongation toward the end of dorsal closure (lower left panel, arrowheads). All panels are lateral views of embryos with anterior to the left.

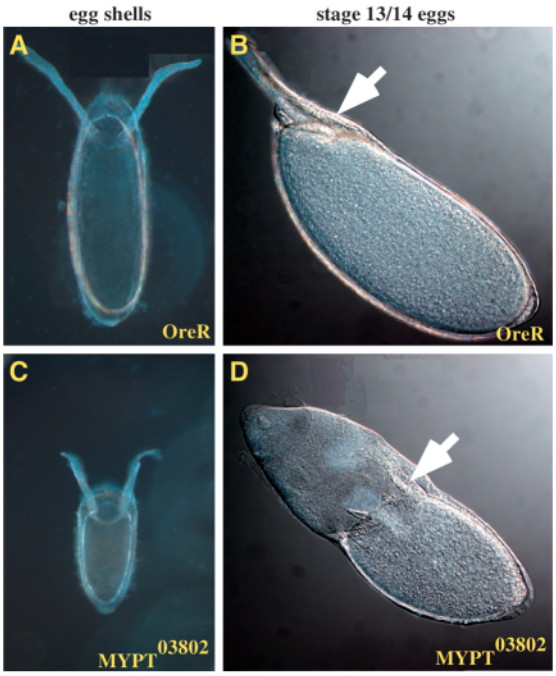

Fig. 4. *DMYPT* is required for oogenesis. (A,C) Morphology of eggs derived from *WT* (OreR) (A) and from *DMYPT*^{03802} homozygous GLCs (C) (dorsal view, anterior is up). Eggs derived from *DMYPT*^{03802} GLCs are about 25% the size of wild-type ones. (B,D) Stage 13-14 egg chambers of *WT* (B) and *DMYPT*^{03802} GLCs (D) (lateral view, anterior is up-left). Loss of DMYPT activity blocks the dumping of the nurse cell cytoplasm into the oocyte. Arrows indicate the dorsal appendages of stage 13/14 eggs.

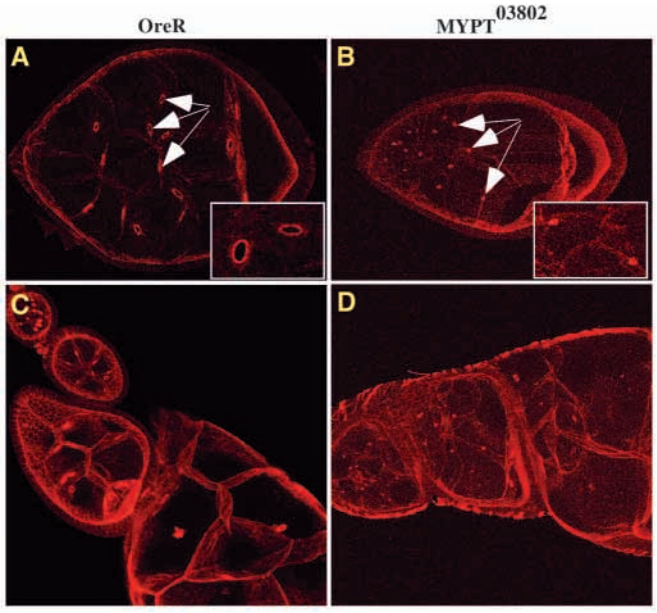

Fig. 5. *DMYPT* is required for ring canal growth. Filamentous actin staining was used to reveal ring canals of egg chambers. (A,B) Stage 8 egg chambers from (A) wild type (OreR) and (B) *DMYPT*^{03802} GLCs. Notice that the ring canals (arrows) in wild-type egg chamber are much larger than those in *DMYPT*^{03802} GLCs. The insets are higher magnification views of regions of stage 10 egg chambers. (C) Egg chambers of wild type (stages 3, 5, 7 and 9). (D) Egg chambers of *DMYPT*^{03802} GLCs (stages 3, 8 and 10). Note that in wild type, the ring canals grow as the egg chambers progress through oogenesis. This does not occur in *DMYPT*^{03802} GLCs.

to the small *DMYPT* mutant ring canals (Fig. 6A,B). Interestingly, although pY staining was present in the mutant ring canals, we also observed an ectopic accumulation of pY staining in the nurse cells (Fig. 6D arrows). The basis of this ectopic accumulation remains to be determined.

Next, we analyzed the subcellular distribution of Zip. It has been reported that mutation of Sqh caused Zip to form aggregates (Edwards and Kiehart, 1996; Jordan and Karess, 1997; Wheatley et al., 1995), thus we expected to detect an effect on Zip distribution in the absence of DMYPT. Surprisingly, no major changes in Zip distribution were detectable between wild-type egg chambers and *DMYPT* GLCs. In both cases, Zip was uniformly distributed at low level with enhanced cell cortex localization (Fig. 6C). Our observations are consistent with the result that *DMYPT* mutations have no effect on Zip localization during dorsal closure (Mizuno et al., 2002).

Interaction between *DMYPT* and the small GTPases during eye development

Previous studies have shown that the Rho family GTPases, Rac1, RhoA, and Cdc42, each play a role in dorsal closure (Glise and Noselli, 1997; Harden et al., 1995; Harden et al., 1999; Hou et al., 1997; Magie et al., 1999; Strutt et al., 1997), and may influence myosin activity through a RhoA mediated signal. Programmed overexpression of these genes by the eye-specific GMR promoter causes distinct rough eye phenotypes (Hariharan et al., 1995; Nolan et al., 1998). To pinpoint the

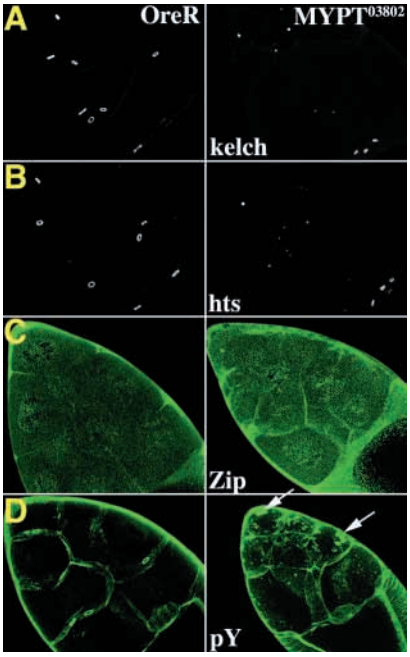

Fig. 6. Effects of *DMYPT* mutation on protein localization. Stage 10 egg chambers from (left panels) wild-type flies, and (right panels) *DMYPT*^{03802} GLCs. Immunolocalization of Kelch (A), Hts (B), Zip (C) and phosphotyrosine (D). Loss of *DMYPT* activity has little effect on the overall distribution of Kelch, Hts and Zip. Note the ectopic accumulation of phosphotyrosine-containing proteins in *DMYPT*^{03802} GLCs (arrows in D).

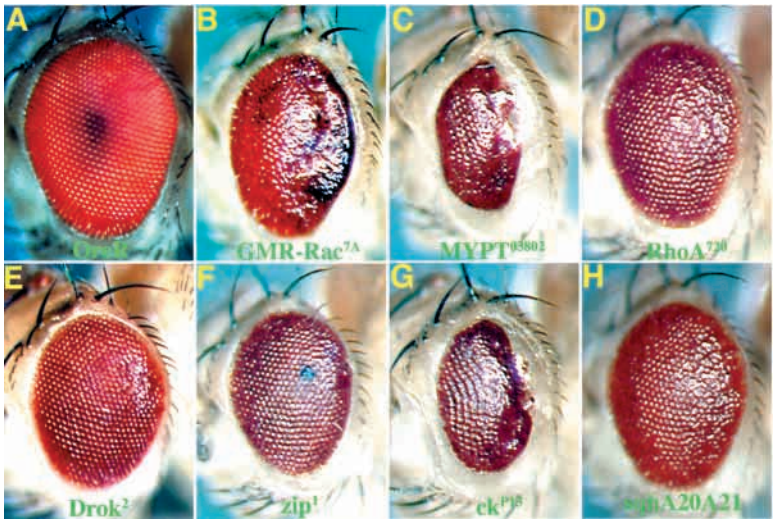

Fig. 7. *DMYPT* is a negative regulator of Rho/myosin II signaling in vivo. Heterozygosities for loss-of-function mutations in *RhoA* (D), *Drok* (E), *zip* (F), and the non-phosphorylatable *sqh* mutation (H), suppress the rough eye phenotype caused by *GMR-Rac* (B). In contrast, loss-of-function mutations in *DMYPT* (C) and *ck* (G) enhance the *GMR-Rac* phenotype. (A) OreR (wild type, +), (B) *GMR-Rac^{7A}/+*, (C) *GMR-Rac^{7A}/DMYPT^{03802}*, (D) *RhoA^{720}/+;GMR-Rac^{7A}/+*, (E) *Drok^{2}/+;GMR-Rac^{7A}/+*, (F) *zip^{1}/+;GMR-Rac^{7A}/+*, (G) *ck^{P13}/+;GMR-Rac^{7A}/+* and (H) *sqh[A20A21]/+;GMR-Rac^{7A}/+*.

relationship of *DMYPT* with these GTPases, we examined the effects of reducing *DMYPT* activity on the rough eye phenotypes. Interestingly, reduction of *DMYPT* strongly enhanced the eye phenotype caused by *GMR-Rac^{7A}* (Fig. 7 compare B and C). The eyes of *GMR-Rac^{7A}/DMYPT^{03802}* flies were much smaller, with fewer bristles and hexagonal-shaped ommatidia, than those of *GMR-Rac^{7A}/OreR* flies. Consistent with the idea that the P-insertion and the excisions are hypomorphic alleles, *Df(3L)th102* enhanced the *GMR-Rac^{7A}* eye phenotype to an even greater extent than either *DMYPT^{03802}*, *DMYPT$^{2\text{-}188}$* or *DMYPT$^{2\text{-}199}$* (data not shown). However, reduction of *DMYPT* had no effect on the size of the rough eye caused by either *GMR-RhoA* or *GMR-Cdc42*, although it did enhance the rough eye phenotype caused by *GMR-RhoA* as fewer bristles formed (Fig. 8 compare B and C). Together, these data suggest that *DMYPT* plays a role in eye development and functions downstream of, or in parallel with *Rac* and *Rho*.

DMYPT is a negative regulator of the Rho/myosin signaling pathway in vivo

RhoA functions downstream of Rac in determining ommatidia polarity in the eyes (Fanto et al., 2000). Reducing the dosage of *RhoA* enhances the effect of *sev-Rac^{N17}*, a dominant negative form of Rac driven by the *sevenless* (*sev*) enhancer-promoter in the eye, and suppresses the activity of *sev-Rac^{V12}*, which encodes a constitutively active form of Rac. Consistently, overexpression of *RhoA* (*sev-RhoA*) rescues *sev-Rac^{N17}*, while reduction the amount of *Rac* using a deficiency that uncovers *Rac* has no effect on the gain-of-function *RhoA* phenotype. Thus, similar to the Rho dependence on Rac function observed in mammalian fibroblasts, some developmental events in *Drosophila* also rely on a hierarchy of GTPase function (Nobes and Hall, 1995).

Consistent with these observations, reducing the dosage of *RhoA* partially suppresses the rough eye phenotype caused by *GMR-Rac* (Fig. 7, compare B and D). In fact, mutations of all the putative positive regulators of myosin activity (RhoA-Zip signaling pathway), including *RhoA*, *Drok* and *zip* itself, moderately suppress the rough eye phenotype of *GMR-Rac*, opposing the effect of *DMYPT* mutants (Fig. 7 compare B with D, E and F). This suggests that the RhoA-Zip signaling pathway functions downstream of *Rac*, and that DMYPT is a negative regulator of the pathway.

Importantly, replacing the phosphorylation sites of Sqh with alanine remarkably suppressed the rough eye phenotype, while replacing them with glutamic acid to mimic phosphorylation slightly enhanced the phenotype (Fig. 7 compare B with H, Fig. 8 compare D with E and F). This suggests that dephosphorylation of Sqh is important in eye morphogenesis and that DMYPT may be involved in regulating the dephosphorylation of myosin light chain in eye development.

To examine whether other myosins are also involved in this process, we tested the effect of myosin VIIA, an unconventional myosin encoded by *crinkled* (*ck*), in the same assay. Myosin VIIA was chosen because *ck* and *zip* behave antagonistically in wing hair number determination in the *Drosophila* adult wing (Winter et al., 2001). Interestingly, *ck* behaves oppositely to myosin II (Zip) during eye morphogenesis since a reduction in *ck* activity enhances the *GMR-Rac* rough eye phenotype, nearly to the same extent as a reduction in *DMYPT* (Fig. 7 compare B and G).

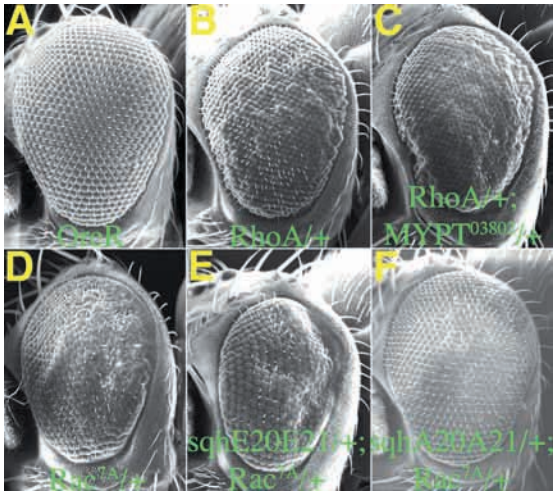

Fig. 8. Genetic interactions in the eye. Scanning electron micrographs of (A) OreR (wild type, +), (B) *GMR-RhoA/+*, (C) *GMR-RhoA/+; DMYPT^{03802}/+*, (D) *GMR-Rac^{7A}/+* , (E) *sqh[E20E21]/+;GMR-Rac^{7A}/+*, and (F) *sqh[A20A21]/+;GMR-Rac^{7A}/+*. Heterozygosity for *DMYPT^{03802}* inhibits the formation of bristles in *GMR-RhoA* eyes, but has little effect on the overall eye size. In addition, the phospho-mimicking *sqh* mutation enhances, while the non-phosphorylatable *sqh* mutation suppresses, the rough eye phenotype associated with *GMR-Rac*.

DISCUSSION

We have generated and characterized the phenotypes associated with mutations in the *Drosophila MYPT* gene. Our analyses indicate a role for this protein in various developmental processes. These include a role in dorsal closure, oogenesis and eye development. Dominant genetic interactions with *DMYPT* alleles reveal that *DMYPT* is a negative regulator of the RhoA-myosin signaling pathway, which acts downstream of, or in parallel with *Rac*.

Cell movement during dorsal closure

Several lines of evidence suggest that *DMYPT* is essential for dorsal closure. First, the *DMYPT*^{03802} P-element, which disrupts *DMYPT* activity, leads to embryos with a dorsal open phenotype that can be reverted by precise excision of the insertion. Second, deficiencies of the *DMYPT* locus, as well as a number of imprecise excisions of the P-element insertions in *DMYPT*, are also associated with embryonic lethality and dorsal closure defects. Third, the lethality associated with *DMYPT*^{03802} is rescued to adulthood using a *DMYPT* transgene.

Given that *RhoA* and *zip* are required for dorsal closure (Strutt et al., 1997; Young et al., 1993), it is not surprising that DMYPT, a regulator of myosin function presumed to act downstream of RhoA, is also implicated in dorsal closure. Like *RhoA* (Lu and Settleman, 1999; Magie et al., 1999), *DMYPT* mutations do not affect *dpp* expression suggesting that the failure of dorsal closure in *DMYPT* mutants is independent of JNK signaling. Nonetheless, it is somewhat unexpected that the loss-of-function mutants of both *zip* and its putative negative regulator, *DMYPT*, have similar rather than opposite phenotypes, each displaying late defects in cell shape and elongation (Young et al., 1993). One possibility is that the activity of myosin II has to be regulated spatially. Sqh is phosphorylated at the leading edge, indicating activation of Zip at that site (Mizuno et al., 2002). In the *DMYPT* mutant, in addition to the leading edge, phosphorylated Sqh is also localized to the dorsal boundaries of the leading edge cells. Thus this pool of mislocalized active Sqh may increase the activity of Zip where it is normally less active, ultimately interfering with dorsal cell movement. Another possibility is that dynamic regulation of Zip activity is important for cell sheet movement. Perhaps activation of Zip is required for cell shape changes in the ectoderm and for maintaining tension as the epithelial front moves forward, but concomitant inactivation of Zip is also necessary for the cells to modulate adhesion allowing forward motility. This paradoxical requirement of myosin II activity is similar to the function of cell adhesion in cell movement; some cell adhesion is necessary for cell movement, but strong adhesion inhibits cell movement.

Role of DMYPT in ring canal growth during oogenesis

Drosophila oogenesis starts with cystoblasts undergoing 4 rounds of cell division. Through an unknown mechanism, cytokinesis of the cyst cell is arrested and the cleavage furrow that separates the cells does not close completely. The cleavage furrow is then stabilized and transformed into an early ring canal, which contains only an outer rim including the actin binding protein anillin (Field and Alberts, 1995), glycoprotein mucin-D (Kramerova and Kramerov, 1999), and phosphotyrosine proteins (Robinson et al., 1994). Then, filamin (cheerio) (Li et al., 1999; Robinson et al., 1997; Sokol and Cooley, 1999), aducin-like protein hts-RC and filamentous actin are recruited to the ring canal to form an inner-rim (Robinson et al., 1994; Yue and Spradling, 1992). At the same time, phosphotyrosine proteins are also detected in the inner rim. Src64 and Tec29 are responsible for most of the phosphotyrosine staining (Dodson et al., 1998; Guarnieri et al., 1998; Roulier et al., 1998). Later, the inner rim is further stabilized by the actin bundling protein kelch (Kelso et al., 2002; Xue and Cooley, 1993).

Ring canals grow in diameter, thickness and length in two phases (Tilney et al., 1996). First, the canal increases in thickness (~6 fold) from stage 2 to 5, while its diameter and length barely grow. At the same time the number of actin filaments increase from 80 at stage 2 to ~700 at stage 6. Second, the diameter and length grow enormously, while the thickness stays the same. During the second phase, the actin filaments are changed into discrete bundles. Astonishingly, the total number and density (number of filaments per cm^2) of actin filaments remain the same.

Fluorescence recovery after photobleaching experiments have shown that the ring canal actin is highly dynamic, constantly cycling between polymerization and depolymerization (Kelso et al., 2002). This, together with the involvement of the actin-nucleating protein complex Arp2/3 in ring canal growth (Hudson and Cooley, 2002), argues that ring canals grow by de novo actin polymerization and regulated cross-linking. This model requires that the newly assembled actin filaments must slide past other bundles since there is no seam in the ring canal.

DMYPT could function at several times during ring canal formation, including cytokinesis arrest, initiation of ring canal formation, or growth of the ring canal. Since the ring canal starts as a cleavage furrow of cytokinesis, myosin II is presumably there. DMYPT may be necessary to inhibit myosin-powered contraction because in the *DMYPT* mutant we observe that the ring canals are smaller, presumably as a result of overcontraction. Secondly, the sliding of the anti-parallel actin filaments is likely to be driven by myosin. In the absence of myosin activity, such as in the *sqh* mutant GLC, ring canals are deformed, often not smooth and round, but pointed, and loosely packed (Jordan and Karess, 1997). The deformed ring canals may also contain seams. However, as during dorsal closure, the activity of myosin must be precisely regulated. Unregulated myosin II activity, for example, in the *DMYPT* mutant, may cause over-sliding of the actin filaments, thus blocking ring growth, while maintaining overall ring canal morphology. Finally, myosin II may be involved in actin filament turnover or bundling. In this case, hyperactivated myosin in the *DMYPT* mutant may cause the actin filaments to be constitutively locked, unable to incorporate new actin to promote ring canal expansion.

Loss of *sqh* activity also blocks dumping of the nurse cell cytoplasm into the oocyte (Wheatley et al., 1995). This is related in part to the inactivity of myosin II in the cell cortex in *sqh* mutants, which under normal circumstances provides the force for rapid transport of nurse cell contents to the oocyte during dumping. Currently from our analysis of *DMYPT*

GLCs, it is not clear whether *DMYPT* also regulates the activity of *zip* in the cell cortex.

Mutations in *sqh* have also pinpointed roles of myosin II in morphogenesis of interfollicular stalks, border cell migration, centripetal cell ingression, and dorsal appendage cell migration, all processes that involve the somatic tissue surrounding the germline during oogenesis (Edwards and Kiehart, 1996). Centripetal cell ingression has been compared to dorsal closure because myosin II is highly localized and forms a ring at the leading edge of the migrating cells like those at the leading edge of the ectoderm during dorsal closure. It will be very intriguing to see if *DMYPT* has functions in these somatic cells of the egg chamber.

Regulation of myosin II

The regulation of MRLC phosphorylation is essential to modulate myosin II activity and can be controled by several distinct mechanisms. For instance, RhoA can activate its effector ROCK that in turn phosphorylates MYPT, either directly or indirectly. MYPT phosphorylation inhibits the phosphatase activity of MLCP and leads to elevation of MRLC phosphorylation. Phosphorylation of MRLC can also be increased by activation of MLCK, another downstream target of RhoA (reviewed by Hartshorne et al., 1998; Somlyo and Somlyo, 2000). Thus, the antagonistic activity of kinase and phosphatase is thought to engender a delicate balance of myosin II activity modulated through the phosphorylation state of its regulatory light chain.

To assess the relationship between DMYPT regulation of myosin II and signaling via the Rho GTPase family members, we turned to the *Drosophila* eye where sensitive genetic interactions can be observed. One study has implicated *RhoA* function downstream of, or in parallel with, *Rac* during orientation of ommatidia in the eye (Fanto et al., 2000). Consistent with this, we found that reducing the amount of *RhoA*, *Drok* and *zip* partially alleviates the eye defect associated with overexpression of Rac, while reducing the dosage of a putative negative regulator of myosin enhances the rough eye phenotype. Furthermore, expression of a non-phosphorylatable form of *Sqh*, which presumably reduces the activity of Zip, dramatically rescues the phenotype, while overexpression of a phospho-mimicking Sqh mutant, which should increase the activity of myosin, exacerbates the eye defects. Taken together, these data indicate that the regulation of myosin II activity via balancing the phosphorylation level of Sqh is critical for proper morphogenesis of the *Drosophila* eye. Based on our results, we propose that it is DMYPT that mediates myosin II downregulation in this system.

Recently, Winter and colleagues have identified similar genetic interactions between *RhoA*, *Drok* and *zip* in restricting the number of F-actin based prehairs in the development of wing cells (Winter et al., 2001). Not surprisingly, the same genetic relationship holds true during dorsal closure (Mizuno et al., 2002). Overexpression of *Drok*, a positive regulator of Zip, phenocopies a mutation in the negative regulator, *DMYPT*. Moreover, a loss-of-function mutation of *zip* potently suppresses the embryonic lethality caused by mutation of *DMYPT* or over expression of *Drok*. In other developmental contexts, myosin II functions downstream of *Rho* and/or *MYPT* (Halsell et al., 2000; Mizuno et al., 2002; Piekny et al., 2000) suggesting that similar mechanisms underlie all of these very diverse biological processes. Since they all require actin cytoskeletal reorganization, it suggests that RhoA regulates cytoskeletal remodeling in non-muscle cells in vivo through the RhoA kinase-MYPT-myosin II pathway.

Interestingly, *crinkled* (myosin VIIA), an unconventional myosin, behaves antagonistically to Zip/myosin II in both eye morphogenesis (this study) and wing hair number restriction (Winter et al., 2001). This suggests that various myosins interact in different cell types to regulate reorganization of the actin cytoskeleton. It will be interesting to determine the specificity of functions of different myosins and their modes of regulation. Since there are many different myosins, and yet a single MYPT in *Drosophila*, it remains to be determined whether, and how, DMYPT interacts with other myosins.

In conclusion, we have identified the *Drosophila* homologue of mammalian *MYPT*, named *DMYPT* accordingly. *DMYPT* plays multiple roles during *Drosophila* development. Loss of *DMYPT* function leads to blockage of rapid transport of nurse cell cytoplasm, inhibition of ring canal growth, failure of dorsal closure, defects of eye morphogenesis, and other unidentified processes during pupae development. Furthermore, our data indicate that dynamic regulation of myosin II activity via regulating phosphorylation level of myosin regulatory light chain by *DMYPT* is critical for the function of myosin II.

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Note added in proof

In the course of preparation of this manuscript, Mizuno and colleagues also reported the identification of the same gene, which they called *Mbs*, and its role in dorsal closure (Mizuno et al., 2002). Their observations are consistent and complementary to ours. These authors also found that *Drok* phosphorylates Mbs in vitro.

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