

Mbt, a *Drosophila* PAK protein, combines with Cdc42 to regulate photoreceptor cell morphogenesis

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SUMMARY

The *Drosophila* gene *mushroom bodies tiny* (*mbt*) encodes a putative p21-activated kinase (PAK), a family of proteins that has been implicated in a multitude of cellular processes including regulation of the cytoskeleton, cell polarisation, control of MAPK signalling cascades and apoptosis. The mutant phenotype of *mbt* is characterised by fewer neurones in the brain and the eye, indicating a role of the protein in cell proliferation, differentiation or survival. We show that mutations in *mbt* interfere with photoreceptor cell morphogenesis. Mbt specifically localises at adherens junctions of the developing photoreceptor cells. A

structure-function analysis of the Mbt protein in vitro and in vivo revealed that the Mbt kinase domain and the GTPase binding domain, which specifically interacts with GTP-loaded Cdc42, are important for Mbt function. Besides regulation of kinase activity, another important function of Cdc42 is to recruit Mbt to adherens junctions. We propose a role for Mbt as a downstream effector of Cdc42 in photoreceptor cell morphogenesis.

Key words: *Drosophila*, Eye, Adherens junctions, Mbt/Cdc42

INTRODUCTION

The p21-activated kinases (PAKs) are a family of proteins that were originally identified as binding partners of the small GTPases Rac and Cdc42 (reviewed by Bagrodia and Cerione, 1999; Daniels and Bokoch, 1999). At present, six PAK isoforms have been described in mammals (PAK 1-6). In *Drosophila*, two PAK proteins, D-PAK (PAK – FlyBase) and Mushroom bodies tiny (Mbt), have been analysed at the molecular and phenotypic levels; a third PAK protein (D-PAK3) is predicted from the genome sequence. Based on their distinct molecular structure, PAK 1-3 and D-PAK are classed together as the group I PAKs, whereas PAK4-6 and Mbt constitute the group II PAKs (reviewed by Dan et al., 2001b; Jaffer and Chernoff, 2002; Pirone et al., 2001).

All PAK proteins share a C-terminal kinase domain and a N-terminal binding domain for proteins of the Rho family of small GTPases (p21-binding domain, PBD). The group I PAKs show some additional structural features that are missing in group II PAKs. Most importantly, the PBD of group I PAKs is C-terminally flanked by the kinase inhibitory domain (KID), which negatively regulates kinase activity through interaction with the kinase domain. Binding of GTP-bound forms of Cdc42 or Rac releases this intramolecular association, resulting in autophosphorylation and full activation of the kinase (Buchwald et al., 2001; Chong et al., 2001; Lei et al., 2000). Group I PAKs also possess several proline-rich sequences that bind to SH3 domain-containing proteins. Interaction with the SH2/SH3 domain adaptor proteins Nck and the corresponding

Drosophila homologue Dock provides a link to cell-surface receptors (Galisteo et al., 1996; Hing et al., 1999; Lu et al., 1997; Lu and Mayer, 1999; Zhao et al., 2000). SH3 domain-mediated binding to Cool/PIX proteins can positively or negatively regulate PAK kinase activity (Bagrodia et al., 1999; Bagrodia et al., 1998; Manser et al., 1998).

Two major functions of PAK proteins have emerged: regulation of signalling cascades, which influence cell proliferation, differentiation, or survival; and organisation of the cytoskeleton. PAK proteins can regulate the activity of the mitogen-activated protein kinase (MAPK) signalling cascade by direct phosphorylation of Raf-1 and MEK (King et al., 1998; Sun et al., 2000). Recently, it has also been shown that PAK6 associates with the Androgen Receptor (AR) and translocates to the nucleus to repress AR-mediated transcription (Yang et al., 2001). Different PAK proteins can also exert pro- and anti-apoptotic effects (reviewed by Jaffer and Chernoff, 2002). A function of PAK proteins in regulation of the cytoskeleton was first implicated by localisation of PAK1 to polymerised actin (Sells et al., 1997). The morphological changes that have been attributed to different PAK proteins include formation of lamellipodia, filopodia and membrane ruffles, as well as the disassembly of focal adhesions (reviewed by Bagrodia and Cerione, 1999; Daniels and Bokoch, 1999; Jaffer and Chernoff, 2002). Activated PAK4 can also induce loss of cell adhesion and anchorage-independent growth, characteristic features of oncogenic transformation. Expression of PAK4 is elevated in many tumour cell lines (Callow et al., 2002; Qu et al., 2001).

Localisation of PAK proteins to distinct cellular compartments appears to be an important mechanism to control their function in the reorganisation of the cytoskeleton. For example, activated Cdc42 causes the redistribution of PAK4 to the Golgi compartment followed by actin polymerisation (Abo et al., 1998). *Drosophila* D-PAK colocalises with actin structures present in leading edge cells, which elongate in the process of dorsal closure during embryogenesis (Harden et al., 1996; Harden et al., 1999). Localised activation of D-PAK in the growth cone of photoreceptor cell axons appears to be controlled by membrane recruitment of D-PAK through binding to the Nck homologue Dock and by the guanine exchange factor Trio, which controls Rac1 activity (Hing et al., 1999; Newsome et al., 2000).

We have analysed the function of the *Drosophila* group II PAK protein Mbt in photoreceptor cell morphogenesis. In contrast to *D-PAK* mutations, mutations in *mbt* do not cause axonal guidance defects, but result in the frequent absence of photoreceptor cells in the eye and other neurones in the brain (Hing et al., 1999; Melzig et al., 1998). This suggests a role of Mbt in cell proliferation, differentiation or cell survival. The *Drosophila* eye develops from a monolayer epithelium: the eye-antennal imaginal disc (Wolff and Ready, 1993). As is typical for polarised epithelial cells, contacts between cells in the developing eye are mediated by adherens junctions (AJs), specialised membrane structures that also play important roles in separating apical and basolateral membrane domains and in coordination of cell shape changes (reviewed by Tepass et al., 2001). Photoreceptor cells undergo massive morphological changes during terminal differentiation. We show that Mbt localisation at AJs is required for normal photoreceptor cell development. Mbt preferentially binds to GTP-loaded Cdc42. A structure-function analysis revealed the importance of the Cdc42 binding domain and the kinase domain for the *in vivo* function of Mbt. Besides regulation of Mbt kinase activity, binding of Cdc42 to Mbt is required to recruit Mbt to AJs. Our results provide evidence for a role of Mbt as a downstream effector of Cdc42 in photoreceptor cell morphogenesis.

MATERIALS AND METHODS

Plasmid construction

For expression of a HA-tagged Mbt protein in HEK293 cells, the *mbt*-coding sequence (Melzig et al., 1998) was amplified by PCR and cloned as an *EcoRI/ApaI* fragment into the pcDNA3-HA1 vector (Invitrogen). The cDNAs for Cdc42 (LD24752), Rac1 (LD34268) and Rho1 (LD22066) were obtained from the Berkeley *Drosophila* Genome Project (BDGP). The coding sequences of Cdc42, Rac1 and Rho1 were amplified by PCR and cloned as *EcoRI-EcoRV* fragments into a modified pcDNA3 vector 3' to the 6xMyc epitope sequence. Mbt^{T525A}, Mbt^{H19,22L}, Cdc42^{G12V}, Rac1^{G12V} and Rho1^{G14V} were constructed by using the site-directed mutagenesis kit (Stratagene). In order to generate transgenic flies, the various *mbt* constructs were cloned as *NotI-Asp718* fragments into the pUAST transformation vector (Brand and Perrimon, 1993).

Generation of a Mbt antiserum

A *mbt* cDNA-fragment coding for amino acids 71-349 was amplified by PCR and cloned as a *EcoRI/XhoI* fragment 3' to the His-tag encoding sequences of the pET-28c expression vector (Novagen). Expression and purification of the protein was carried out according

to the manufacturer's protocol. The purified protein was used for immunisation of rabbits by a commercial supplier. The specificity of the antiserum was tested by immunohistochemistry and western blot analysis.

Immunohistochemistry, histology and microscopy

Eye imaginal discs from third instar larvae or pupae were stained as described by Gaul et al. (Gaul et al., 1992), except that Cy3 or Alexa488-conjugated secondary antibodies were used. The following primary antibodies were used: rabbit anti-Mbt (1:500), mouse anti-Elav (1:10, provided by E. Hafen), mouse anti-Armadillo (1:50, E. Knust), mouse anti-Crumbs (1:10, E. Knust), rabbit anti-Canoe (1:500, D. Yamamoto), rabbit anti-Discs large (1:1000, A. Wodarz), rat anti-mCD8 (1:100, Caltag) and anti-HRP-FITC (1:500, Cappel). Eye discs were mounted in VectaShield and analysed with a LeicaTCS confocal microscope. Image processing was carried out with the AMIRA software (TGS). For histological eye sections, heads were fixed with OsO₄ as described previously (Basler et al., 1991) and embedded in Epon. Eye sections (1 µm) were stained with Toluidine Blue/borax solution for microscopy.

Genetics, clonal analysis

Transgenic lines were generated by injecting Qiagen-purified plasmid DNA into *w¹¹¹⁸* embryos. To analyse whether the wild-type or the mutated *mbt* transgenes can rescue the *mbt^{P1}* eye phenotype, males carrying the appropriate *UAS:mbt* transgene were crossed to *mbt^{P1}/mbt^{P1}; Gal4:238Y/Gal4:238Y* females. Eye imaginal discs from the male progeny were dissected and analysed by immunohistochemistry. Adult males were scored for rescue of the *mbt^{P1}* eye phenotype. For each *mbt* construct, at least two independent insertion lines were tested. The *UAS:Cdc42^{G12V}* and *UAS:Cdc42^{T17N}* flies were provided by Liqun Luo. To analyse the effect of *Cdc42³* and *Cdc42⁴* mutations (flies provided by Richard Fehon) on Mbt

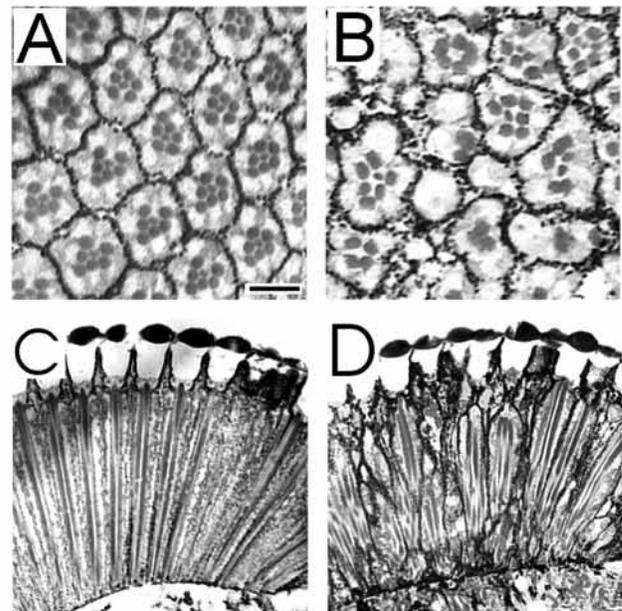


Fig. 1. Adult *mbt* eye phenotype. Tangential sections through wild-type (A) and *mbt^{P1}* (B) eyes. In the wild type, each ommatidium contains eight photoreceptor cells. R1-R6 have large rhabdomeres, the central R7 and R8 (not seen in apical sections) rhabdomeres are small. Longitudinal sections through wild-type (C) and *mbt^{P1}* (D) eyes show the defects in proximodistal rhabdomere extension in the mutant. Scale bar: 10 µm.

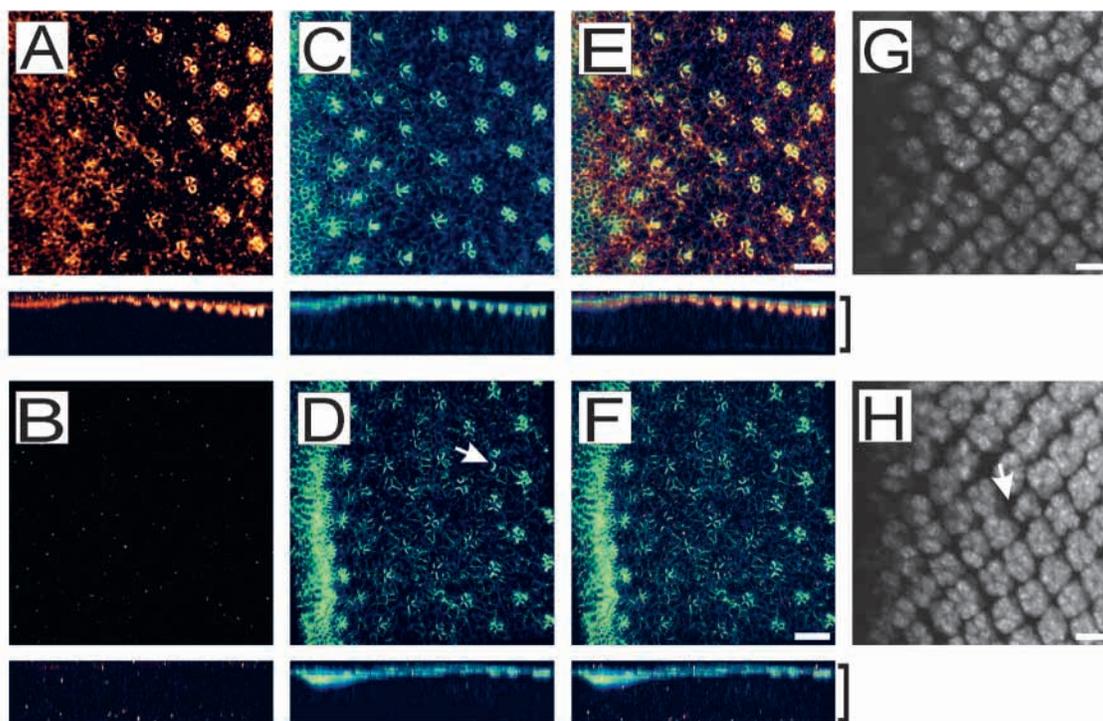


Fig. 2. The *mbt* phenotype in third instar eye imaginal discs. Shown are apical-to-basal projection views of wild-type (A,C,E) and *mbt^{P1}* (B,D,F) eye imaginal discs stained with anti-Mbt (A,B) or anti-Arm (C,D). A merge of both stains is seen in E,F. Below each projection view, an apical-to-basal cross-section is shown (the bracket indicates the apical-basal extension of the eye disc). Photoreceptor cell differentiation starts in the morphogenetic furrow and involves the step-wise recruitment of eight photoreceptor cells in a single ommatidium. In the wild type, Mbt and Arm colocalise at the AJs of all photoreceptor cells as soon as they become integrated into the ommatidial clusters (E). Despite the absence of detectable levels of Mbt protein in *mbt^{P1}* mutant eye discs (B), AJs between photoreceptor cells do form in the mutant, although some irregularities are already evident (arrow in D). (G,H) Anti-Elav staining of wild-type (G) and *mbt^{P1}* mutant eye discs (H). Occasionally, *mbt^{P1}* ommatidia contain fewer differentiating photoreceptor cells (arrow). The morphogenetic furrow is on the left-hand side in all figures. Scale bar: 10 μ m.

localisation, the mosaic analysis with a repressible cell marker (MARCM) system was used (Lee and Luo, 1999). Homozygous *Cdc42³* or *Cdc42⁴* mitotic clones were induced 72 hours after egg laying by two 60 minutes heat shocks at 37°C of animals of the following genotype: *Cdc42^{3/4}, FRT19A/tubP-Gal80, hs-Flp, FRT19A; GMR-Gal4/UAS:mCD8-GFP*. Clonal tissue was visualised by staining eye imaginal discs with an anti-mCD8 antibody.

Cell culture, Immunoprecipitation and western blot analysis

Human embryonic kidney (HEK) 293 cells were grown at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% foetal calf serum (FCS), 1% penicillin-streptomycin and 1% L-glutamine. For transient transfections, 6×10⁵ HEK293 cells were seeded in six-well plates and transfected 24 hours later with 2 μ g of the indicated DNA using the PolyFect reagent (Qiagen). Twenty-four hours after transfection, cells were serum starved for 36 hours in DMEM medium containing 0.3% FCS, harvested in PBS and lysed at 4°C for 30-40 minutes in lysis buffer (25 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA 10% glycerol, 0.1% NP-40 supplemented with 5 μ g/ml Antipain, 1 μ g/ml Aprotinin, 0.5 μ g/ml Leupeptin, 0.7 μ g/ml Pepstatin, 100 μ g/ml PMSF). Total protein lysate (100-250 μ g) was incubated with 8 μ l of a monoclonal anti-HA antibody (clone12CA5) for 10 minutes, followed by incubation with protein-G-Agarose beads (Roche) for 2 hours. Proteins were resolved by SDS-PAGE and after transfer on nitrocellulose membranes probed with an anti-HA antibody (1:1000, Roche) or a

1:2000 dilution of the anti-Myc antibody (clone 9E10, Santa Cruz). Proteins were detected using the ECL kit (Amersham Pharmacia Biotech).

Protein kinase assay

HEK293 cells were grown after transfection in DMEM containing 10% FCS for 24 hours and then grown at low serum conditions (0.3% FCS) for additional 20-24 hours. After immunoprecipitation, the protein-G-Agarose beads were washed twice with lysis buffer, once with 0.5 M NaCl and twice with kinase buffer (20 mM HEPES pH 7.6, 20 mM MgCl₂, 10 mM β -glycerophosphate, 20 mM p-nitrophenylphosphate). The kinase reaction was performed in kinase buffer containing 0.5 mM Na₃VO₄, 2 mM DTT, 50 μ M ATP, 5 μ Ci [γ -³²P]ATP together with 5 μ g myelin basic protein (MBP, Sigma) as a substrate for 30 minutes at 30°C. Proteins were visualised after SDS-PAGE by autoradiography.

GST Pull-down assay

The coding region of *Cdc42* was cut out of the *pcDNA3-myc-Cdc42* construct with *EcoRI/NotI* and cloned into the pGEX vector (Amersham). The GST-Cdc42 fusion protein was expressed and glutathion-sepharose purified according to standard procedures. After elution with 100 mM glutathione, the protein was dialysed with 50 mM Tris pH 7.5. GDP/GTP loading of the fusion protein and the pull-down assay were performed as described previously (Pandey et al., 2002) with cell lysates transiently expressing HA-tagged Mbt.

RESULTS

Localisation of Mbt to adherens junctions in developing photoreceptor cells

Mbt mutant flies display a rough eye phenotype (Melzig et al., 1998). Tangential sections through adult eyes revealed the absence of a variable number of photoreceptor cells in many ommatidia (Fig. 1A,B). The rhabdomeres of the remaining photoreceptor cells show morphological defects. The cross-section profiles of the rhabdomeres are enlarged and neighbouring rhabdomeres often contact each other, which is not seen in the wild type. Longitudinal sections revealed that the rhabdomeres are twisted, fragmented and do not extend from the pseudocone to the floor of the retina (Fig. 1C,D). These phenotypes suggested that Mbt is required for recruitment and/or proper differentiation of photoreceptor cells.

To analyse the function of Mbt during eye development, we generated a polyclonal antiserum and stained eye-antennal imaginal discs from third instar larvae and pupae. Differentiation of the cells that comprise the single eye units (ommatidia) occurs in a step-wise fashion and is initiated in the morphogenetic furrow, which moves across the eye disc from posterior to anterior (Wolff and Ready, 1993). Staining of third-instar eye-imaginal discs with the Mbt antiserum revealed an accumulation of the Mbt protein at apical membrane sites of the photoreceptor cells as soon as they become recruited to the ommatidial clusters and initiate differentiation (Fig. 2A). Low levels of Mbt protein were detected at the membranes of undifferentiated cells. Staining was completely absent in eye discs derived from *mbt^{P1}* mutant larvae (Fig. 2B), demonstrating the specificity of the antiserum and confirming our previous notion that *mbt^{P1}* is a complete loss-of-function allele (Melzig et al., 1998). To determine the subcellular localisation of Mbt more precisely, we co-stained eye discs with anti-Armadillo (Arm, *Drosophila* β -Catenin) antibodies, a marker for adherens junctions (AJs). Staining for both largely overlaps in the photoreceptor cells (Fig. 2C,E). From apical to basal cross sections it became evident that Mbt is less abundant in the most apical domain of Arm expression in the photoreceptor cells.

The final architecture of the ommatidia is established during pupal development and is accompanied by major morphological changes (Longley and Ready, 1995; Wolff and Ready, 1993). At 37% of pupal development (p.d.), the apical domains of the photoreceptor cells have involuted. Thus, the apical domains of the photoreceptors point towards the centre of the ommatidial cluster. After involution, the apical membranes of the photoreceptor cells start to expand to form the rhabdomeres. Each rhabdomere is surrounded by the stalk membrane, which connects it to the zonula adherens. As shown by anti-Arm staining (Fig. 3), the AJs span the whole proximal-to-distal length of the photoreceptors at 50% p.d. Mbt remains

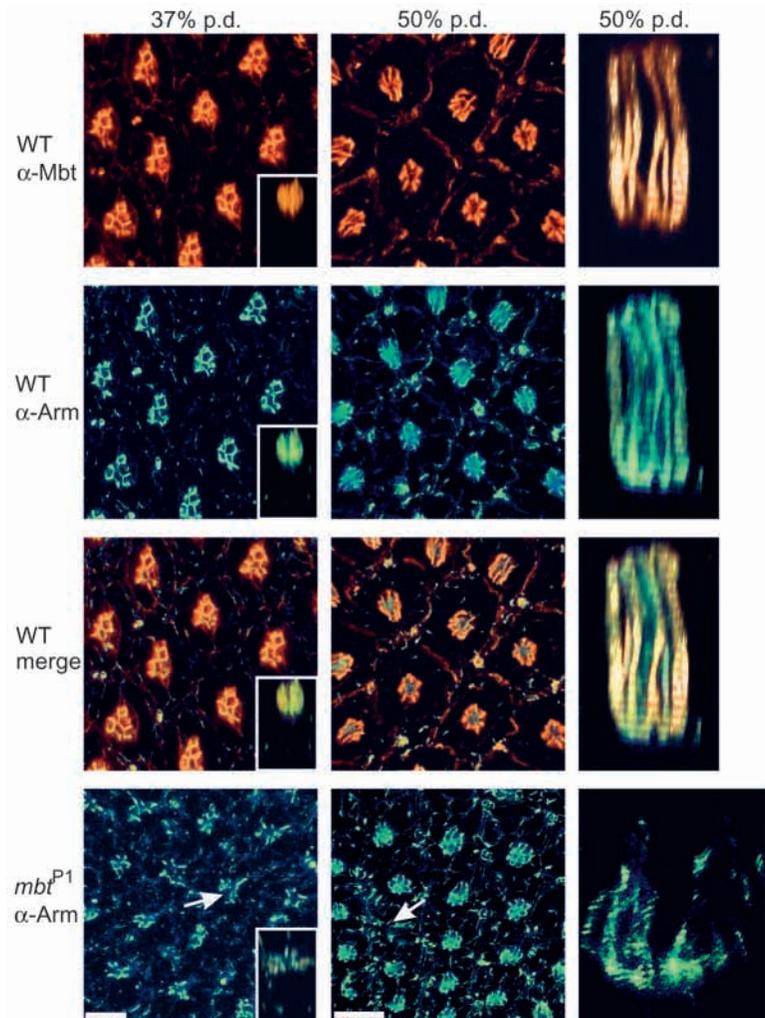


Fig. 3. *Mbt* mutations disrupt adherens junction integrity during pupal photoreceptor cell morphogenesis. Shown are proximal-to-distal projection views of wild-type (WT) eye imaginal discs at 37% p.d. (left column) and 50% p.d. (middle column) stained with anti-Mbt and anti-Arm antibodies. Staining of both overlap in the developing photoreceptor cells and also in the surrounding pigment and bristle cells (WT merge). Three-dimensional reconstructions of single ommatidial clusters at 37% p.d. (left column, insets) and 50% p.d. (right column) illustrate the massive distal (top)-to-proximal (bottom) extension of the AJs during pupal development. Anti-Arm staining of *mbt^{P1}* pupal eye discs (*mbt^{P1}*, α -Arm) shows the disorganisation of the AJs. In the proximal-to-distal projection view, lateral extensions are evident (arrow) and the AJs fail to extend (inset). At 50% p.d., Armadillo accumulates in patches (right picture). Scale bars: 10 μ m.

colocalised with Arm at AJs of pupal photoreceptor cells at different stages of their development (Fig. 3). Higher levels of Mbt expression can also be seen in the future bristle cells, whereas cone and pigment cells express low levels of Mbt. A 3D reconstruction of a wild-type ommatidial cluster stained with anti-Arm and anti-Mbt antibodies shows the colocalisation of both proteins at AJs of the photoreceptor cells along the whole proximodistal length (Fig. 3). In summary, these data provide evidence that Mbt is localised at AJs of photoreceptor cells from the initial recruitment to their final differentiation.

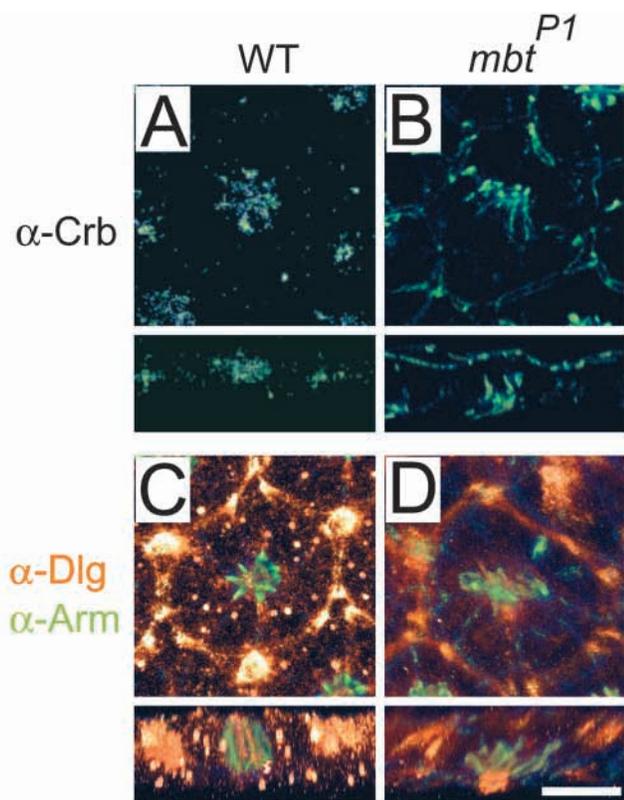


Fig. 4. Staining of wild-type and *mbt^{P1}* ommatidia (50% p.d.) with anti-Crb (A,B) and anti-Dlg (C,D) antibodies. Below each proximal-to-distal projection view, the corresponding proximal-to-distal cross-section is shown. Scale bar: 5 μ m.

Mutations in *mbt* interfere with photoreceptor cell morphogenesis

The observed phenotypes in *mbt^{P1}* eyes could result from a defect in cell proliferation, photoreceptor cell recruitment or differentiation. To determine whether *mbt* mutations affect recruitment or early neuronal differentiation of photoreceptor cells, wild-type and *mbt^{P1}* third instar larval eye discs were stained with an antibody against the neuronal differentiation marker Elav. Only rarely did *mbt^{P1}* ommatidia contain fewer Elav-positive cells than wild-type clusters (Fig. 2G,H). This result was confirmed by using HRP as an independent differentiation marker (data not shown). This suggests that a failure in recruitment of photoreceptor cells is not the major cause of the *mbt* phenotype.

The specific localisation of Mbt at AJs prompted us to look for AJ defects in *mbt^{P1}* third instar and pupal eye imaginal discs with an anti-Arm antibody. In third instar eye discs, the AJs of the developing photoreceptor cells appear disorganised. Frequently, the AJs extend laterally (Fig. 2D,F). The AJ defects become much more pronounced at pupal stages (Fig. 3). At 37% p.d., AJs fail to extend in proximodistal direction. At 50% p.d., AJs are fragmented and form patchy and disorganised structures. To verify these results and to exclude the possibility that *mbt^{P1}* disturbs only Arm localisation without affecting AJs, *mbt^{P1}* eye imaginal discs were co-stained with anti-Canoe antibodies as an independent AJ marker (Matsuo et al., 1999). Canoe and Arm remain colocalised in *mbt^{P1}* eye discs (data not

shown). In addition, we stained wild-type and *mbt^{P1}* pupal eye discs with antibodies against the apical determinant Crumbs (Crb) and the Discs large (Dlg) protein, which is a marker for septate junctions in epithelial cells (reviewed by Tepass et al., 2001). Recently it has been shown that Crumbs is essential to maintain AJ integrity during photoreceptor cell morphogenesis and is localised at the stalk membrane between AJs and the rhabdomeres (Izaddoost et al., 2002; Pellikka et al., 2002). Compared with wild-type ommatidia, Crb (Fig. 4A,B) and Dlg (Fig. 4C,D) are de-localised in *mbt^{P1}* mutant cells. In summary these data suggest that Mbt function is required in the developing photoreceptor cells to undergo their morphological changes.

Mbt interacts with Cdc42

To gain insight into the molecular mechanisms that control Mbt function, we next tested the binding of Mbt to Rho-type GTPases. Group I PAKs have been shown to interact via the p21-binding domain (PBD) with GTP-loaded Rac and Cdc42 but not with Rho, whereas the group II PAK proteins PAK4 and PAK5 preferentially bind to GTP-bound Cdc42 (Abo et al., 1998; Dan et al., 2002; Pandey et al., 2002).

Myc-tagged versions of the *Drosophila* homologues of Cdc42, Rac1 and Rho1 were co-expressed with HA-tagged Mbt in HEK293 cells. Co-immunoprecipitation experiments revealed a nearly exclusive binding of Cdc42 to Mbt (Fig. 5A). Rac1 showed only a very weak interaction whereas no binding of Rho1 to Mbt was detected. The specificity of the interaction between Cdc42 and Mbt was tested by mutation of two conserved histidine residues in the PBD (Fig. 5C) to leucine (*Mbt^{H19,22L}*). The mutant Mbt protein was unable to bind to Cdc42 (Fig. 5A). Thus, the interaction between Cdc42 and Mbt is indeed mediated by the PBD. To determine whether activation of Rho-type GTPases influence binding to Mbt in vivo, the constitutively activated variants *Cdc42^{G12V}*, *Rac1^{G12V}* and *Rho^{G14V}* were co-expressed with Mbt in HEK293 cells. *Cdc42^{G12V}* showed an enhanced interaction with Mbt when compared to wild-type Cdc42 (Fig. 5A). This result indicated that only the active, GTP-bound form of Cdc42 binds to Mbt. To verify this result, Cdc42 was expressed as a GST-fusion protein in bacteria and used in pull-down experiments upon loading with GDP or GTP. Mbt selectively binds to GTP-loaded Cdc42 but not to unloaded or GDP-loaded Cdc42 (Fig. 5B). Thus, the preference for binding GTP-bound Cdc42 appears to be a common feature among group II PAKs.

Regulation of Mbt kinase activity in vitro by Cdc42 binding

The PBD of group I PAKs is C-terminally flanked by the kinase inhibitory domain (KID). Binding of activated Cdc42 and Rac relieves the inhibitory influence of the KID on PAK kinase activity (Buchwald et al., 2001; Chong et al., 2001; Lei et al., 2000). In addition, group II PAKs share significant sequence homology C-terminal to the PBD, but the sequences differ significantly from the group I PAK KID (Fig. 5C).

To analyse the influence of Cdc42 binding on Mbt kinase activity we used the Cdc42 binding-deficient *Mbt^{H19,22L}* construct. A second Mbt construct used in this study bears a mutation in the kinase domain. This mutation (T525A), located in the linker region between subdomains VII and VIII,

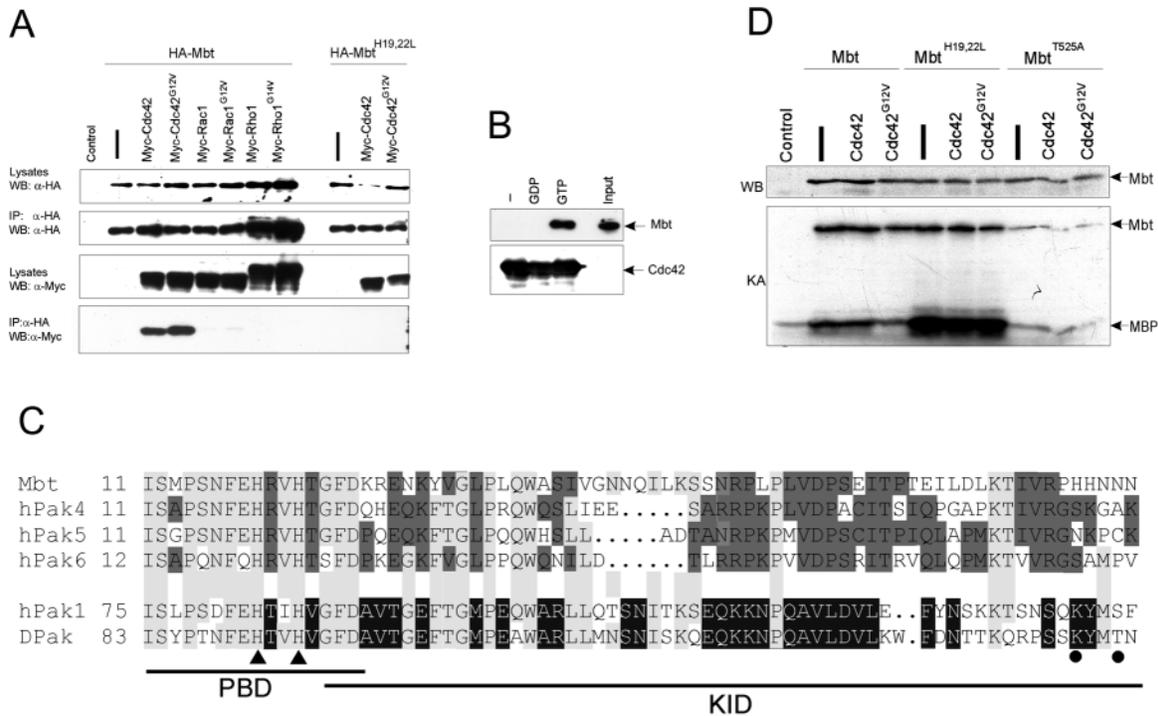


Fig. 5. (A) Binding of Mbt to Rho-type GTPases. HA-tagged Mbt or Mbt^{H19,22L} were transiently expressed in HEK293 cells alone or co-expressed with Myc-tagged Cdc42, Rac1 or Rho1, or the corresponding activated forms. Cell lysates (Lysates) were analysed using western blot (WB) with anti-HA or anti-Myc antibodies to indicate expression of the corresponding proteins. For co-immunoprecipitation, cell lysates were incubated with an anti-HA antibody and GTPases bound to immunopurified Mbt were detected by western blot using the anti-Myc antibody (IP; α -HA; WB, α -Myc). The presence of Mbt in the immunoprecipitates was verified by western blot (IP, α -HA; WB, α -HA). The binding of Cdc42 and Cdc42^{G12V} to Mbt is disrupted by mutation of the PBD (Mbt^{H19,22L}). (B) Mbt interacts with Cdc42 in a GTP-dependent manner. The protein extract from HEK293 cells transiently transfected with the *pcDNA3-HA-Mbt* construct was incubated either with unloaded, or with GDP- or GTP-loaded GST-Cdc42 protein. The GST protein and any bound cellular proteins were recovered by precipitation with glutathione-sepharose beads and separated by SDS-PAGE. Mbt protein was detected by western blot using the anti-HA antibody. As a control, total lysates from *pcDNA3-HA-Mbt* transfected HEK293 cells are shown (input). The presence of equal amounts of the GST-Cdc42 was verified with an anti-GST antibody. (C) Sequence alignment of the p21-binding domain (PBD) and the kinase inhibitory domain (KID) of human PAK1 and D-PAK (group I PAKs) with the corresponding sequences of human PAK4, PAK5, PAK6 and Mbt (group II PAKs). Highly conserved amino acids are indicated in light grey, amino acids only conserved between PAK1/D-PAK or PAK4-6/Mbt are shown in black and dark grey, respectively. The serine 144 autophosphorylation site and lysine 141 (black circles), which are involved in regulating PAK1 kinase activity are not present in PAK4-6/Mbt. The histidine residues that were mutated to create a Cdc42 binding-deficient Mbt protein are indicated by triangles. (D) Regulation of Mbt kinase activity. HEK293 cells were transfected with either an empty *pcDNA3* vector (control), with HA-tagged Mbt, Mbt^{H19,22L} or Mbt^{T525A} alone or in combination with Myc-tagged Cdc42 or Cdc42^{G12V}. Expression of Mbt was verified by western blot (WB). Mbt proteins were immunopurified from cell lysates using an anti-HA antibody and incubated in kinase buffer with myelin basic protein (MBP) as a substrate in the presence of [γ -³²P]ATP. Autophosphorylation of Mbt and MBP phosphorylation were analysed by autoradiography.

corresponds to the T777A mutation in the *Saccharomyces cerevisiae* PAK protein Ste20p and has been found to disrupt autophosphorylation and catalytic activity of Ste20p (Wu et al., 1995). HEK293 cells were transfected with HA-tagged wild-type or the presumptive kinase-dead version of Mbt and the immunopurified protein complexes were incubated with kinase buffer and [γ -³²P]ATP together with myelin basic protein (MBP) as a substrate. Compared with wild type Mbt, the T525A mutation strongly reduced autophosphorylation and substrate phosphorylation (Fig. 5D). Co-expression of Cdc42 with Mbt did not increase autophosphorylation or MBP phosphorylation when compared with cells transfected with Mbt alone. Importantly, co-expression of Mbt and the constitutively activated Cdc42^{G12V} construct slightly reduced rather than enhanced the ability of wild-type Mbt to phosphorylate MBP. Conversely, the Cdc42-binding defective

Mbt^{H19,22L} protein showed a moderate increase of MBP phosphorylation independent of co-expression with Cdc42 or Cdc42^{G12V} (Fig. 5D). Autophosphorylation was not affected by removal of the Cdc42-binding site. These results fit with previous observations that kinase activity of PAK4, 5 and 6 is not upregulated upon Cdc42 binding, whereas deletion of the PBD can lead to enhanced kinase activity (Abo et al., 1998; Pandey et al., 2002; Yang et al., 2001). Thus, group II PAKs appear to differ from group I PAKs in their mechanism to regulate kinase activity.

Requirement of the Cdc42 binding domain and the kinase domain for Mbt function in vivo

In order to test the requirement of the Cdc42-binding domain and the kinase domain for Mbt function in vivo, we expressed wild-type or mutated Mbt proteins during eye development in

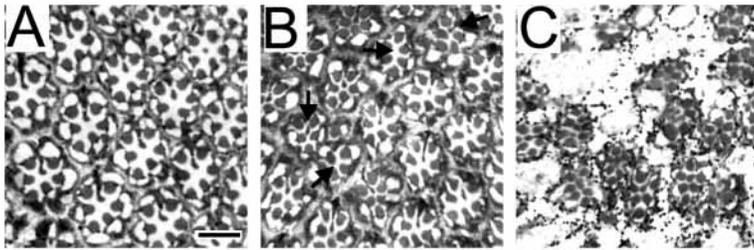


Fig. 6. Rescue of the *mbt* eye phenotype by transgenic constructs. Tangential sections of eyes of the following genotypes: (A) *mbt^{P1}/Y; UAS:mbt/+; Gal4:238Y/+*; (B) *mbt^{P1}/Y; UAS:mbt^{T525A}/+; Gal4:238Y/+*; and (C) *mbt^{P1}/Y; UAS:mbt^{H19,22L}/+; Gal4:238Y/+*. Expression of the *mbt* cDNA with Gal4 line 238Y in a *mbt^{P1}* mutant background results in a complete rescue of the *mbt^{P1}* eye phenotype (A, compare with Fig. 1B). Mbt^{T525A} can only partially substitute the loss of endogenous Mbt function (B); occasionally photoreceptor cells are missing (arrows). No rescue of the *mbt^{P1}* phenotype is observed upon expression of the *mbt^{H19,22L}* construct (C). Scale bar: 10 μ m.

the absence of the endogenous Mbt protein. Northern blot analysis (Melzig et al., 1998) and antibody staining (Fig. 2B) indicated that *mbt^{P1}*, which carries a P-element insertion in the protein encoding sequence, is a complete loss-of function allele. Previously we have shown that Gal4:238Y (Tettamanti et al., 1997)-driven expression of a *mbt* cDNA in the brain is sufficient to rescue the *mbt^{P1}* brain phenotype (Melzig et al., 1998). We found that Gal4:238Y is also expressed in the eye-antennal imaginal disc in a manner that closely resembles the expression pattern of the endogenous Mbt protein (see below). Consistent with this observation, the eye phenotype of *mbt^{P1}* flies was completely rescued by Gal4:238Y-driven expression of the wild-type *mbt* cDNA (Fig. 6A). By contrast, the Cdc42-binding deficient Mbt^{H19,22L} protein was unable to rescue the *mbt^{P1}* eye phenotype (Fig. 6C), whereas the kinase-defective Mbt^{T525A} construct partially rescued the *mbt^{P1}* eye phenotype (Fig. 6B).

In summary, these experiments have verified the importance of the Cdc42-binding domain and the kinase domain for the in vivo function of Mbt during eye development. The partial rescue ability of the Mbt^{T525A} construct indicated that some functions of Mbt are independent of kinase activity. The differences observed in the rescue ability of the kinase defective Mbt^{T525A} and the Cdc42 binding-deficient Mbt^{H19,22L} proteins also suggested that Cdc42 binding to Mbt influences Mbt function in a kinase-independent manner. One possibility we investigated was the proper localisation of the Mbt protein to AJs.

Localisation of Mbt depends on Cdc42

Group II PAKs lack the N-terminal binding site for the Nck/Dock adaptor protein, which could provide a link to membrane-bound proteins. To investigate whether the Cdc42-binding domain is responsible for the observed localisation of Mbt to AJs, we expressed wild-type and mutated *mbt* cDNAs with the Gal4:238Y driver line in a *mbt^{P1}* mutant background and analysed the subcellular localisation of the corresponding Mbt proteins in pupal eye discs. The expression pattern of the transgenic, non-mutated Mbt protein in the eye imaginal disc closely resembled the expression pattern of the endogenous Mbt protein. High levels of transgenic Mbt accumulate at the AJs of the developing photoreceptor cells (Fig. 7A-C). Consistent with the complete rescue of the adult *mbt^{P1}* eye phenotype (Fig. 6A), no morphological abnormalities were observed when pupal eye discs were stained with anti-Mbt (Fig. 7A) or anti-Arm (Fig. 7B) antibodies. An identical localisation pattern was observed when the kinase-defective Mbt^{T525A} protein was expressed in the *mbt^{P1}* mutant

background (Fig. 7D-F), indicating that eliminating kinase activity does not influence the subcellular distribution of the Mbt protein. However, as revealed by co-staining with an anti-Arm antibody, the transgenic Mbt^{T525A} protein only partially rescued the AJs defects of *mbt^{P1}* animals (compare with Fig. 3). The AJs extend to some degree in proximal-to-distal direction but still do not have a regular architecture (Fig. 7D-F). This result correlates with the partial rescue observed in adult eyes (Fig. 6B). By contrast, the Cdc42 binding-deficient Mbt^{H19,22L} protein did not accumulate at AJs but instead was distributed within the cytoplasm (Fig. 7G). Anti-Arm staining revealed that the Mbt^{H19,22L} protein is unable to rescue the AJs defects seen in *mbt^{P1}* mutant eye discs (Fig. 7H,I). Thus, there is an absolute requirement of the Cdc42 binding domain for localisation and function of the Mbt protein during eye development. To exclude that the failure of the Mbt^{H19,22L} protein to localise at AJs is not a secondary effect of the *mbt^{P1}* phenotype itself, we also expressed the Mbt^{H19,22L} protein in a wild-type background (Fig. 7K-M). Although endogenous and transgenic Mbt protein cannot be distinguished in this case, two observations were made. First, the Mbt^{H19,22L} protein did not cause any obvious AJs defects when expressed in a wild-type background (Fig. 7L). Second, Mbt protein was found at AJs and in the cytoplasm (Fig. 7K). Because no cytoplasmic Mbt protein was detected upon expression of the non-mutated Mbt protein in a wild-type background (data not shown), we conclude that it is the Mbt^{H19,22L} protein, which localises in the cytoplasm.

To show that Cdc42 and not another protein bound to the PBD of Mbt is responsible for localisation of Mbt to AJs, we wanted to analyse animals that either lacked Cdc42 function or ectopically expressed mutated versions of the Cdc42 protein. Because removal of Cdc42 function causes lethality (Genova et al., 2000), homozygous mutant *Cdc42³* or *Cdc42⁴* cell clones were generated using the MARCM system (Lee and Luo, 1999). Only those cells that are homozygous for the *Cdc42* mutation express the membrane localised mCD8 marker. Most of the *Cdc42³* or *Cdc42⁴* clones obtained in the eye disc contained only a few mCD8-positive cells. Consistent with previous findings that *Cdc42* mutant cells can initiate their differentiation into photoreceptor cells (Genova et al., 2000), the majority of mCD8-positive (*Cdc42* mutant) photoreceptor cells analysed extend an axon. In Fig. 8, an apical to basal projection view of an anti-Mbt (A) and an anti-mCD8 (B) stained eye-imaginal disc with a single *Cdc42* mutant photoreceptor cell is shown. From single apical sections (Fig. 8D-F) and apical-to-basal cross-sections (Fig. 8G-I) it is evident that Mbt is localised at the apical side, whereas the

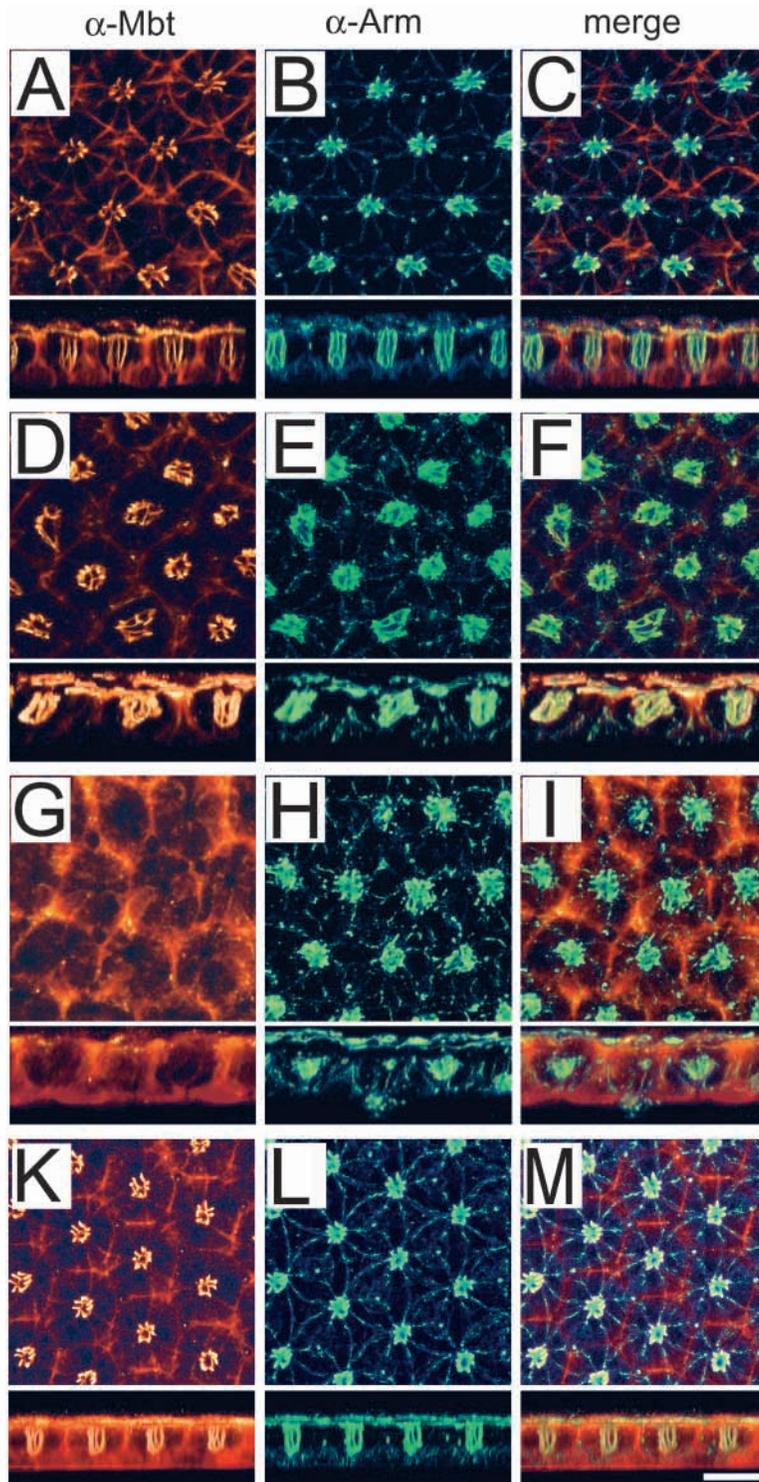


Fig. 7. The Cdc42-binding domain is required for AJ localisation of the Mbt protein. Pupal eye discs (50% p.d.) from *mbt^{P1}* animals expressing transgenic Mbt (A-C), Mbt^{T525A} (D-F), or Mbt^{H19,22L} (G-I) with Gal4:238Y were co-stained with the anti-Mbt (A,D,G) and anti-Arm (B,E,H). A merge of the staining for both is seen in C, F and I. Below each proximal to distal projection view, a proximal-to-distal cross-section is shown. The transgenic Mbt and Mbt^{T525A} proteins colocalise with Arm at AJs of the photoreceptor cells (A-C and D-F, respectively). In contrast to the non-mutated Mbt protein, the Mbt^{T525A} protein is unable to rescue the *mbt^{P1}* AJ defects fully (compare B with E). Mutation of the Cdc42-binding site leads to cytoplasmic distribution of the Mbt^{H19,22L} protein (G). As revealed by anti-Arm staining, the Mbt^{H19,22L} protein cannot rescue the *mbt^{P1}* AJs defects fully (H). (K-M) Expression of Mbt^{H19,22L} in a wild-type background. Note that the Mbt antibody recognises both the endogenous and the Mbt^{H19,22L} protein in this preparation. Scale bar: 10 μ m.

with the Gal4:238Y driver line resulted in embryonic lethality, the eye-specific GMR-Gal4 driver line was used. Consistent with our finding that Mbt only binds to GTP-loaded Cdc42, expression of Cdc42^{T17N} had only minor effects on Mbt localisation and the AJs morphology (Fig. 8K,L). By contrast, Cdc42^{G12V} caused a dramatic change in the Mbt and Arm expression pattern (Fig. 8M,N). Mbt accumulates at membrane sites of all cells. Arm expression can only be seen at early stages of photoreceptor cell recruitment, indicating that Cdc42^{G12V} completely disrupts the integrity of AJs in the developing photoreceptor cells.

DISCUSSION

PAK proteins are involved in the regulation of cellular processes such as gene transcription, cell morphology, motility and apoptosis (Bagrodia and Cerione, 1999; Daniels and Bokoch, 1999; Jaffer and Chernoff, 2002). Our results illustrate that different PAK proteins fulfil distinct functions in the same cell. In the developing photoreceptor cells, D-PAK is required in growth cones to control axon guidance (Hing et al., 1999) whereas Mbt is localised at AJs and is required for cell morphogenesis.

One major difference between group I and group II PAKs is the regulation of kinase activity. For group I PAK proteins it has been shown that binding of GTP-bound Cdc42 or Rac releases the inhibitory effect of the KID on catalytic activity (Buchwald et al., 2001; Chong et al., 2001; Lei et al., 2000). The lack of an obvious KID in group II PAKs (Fig. 5C) is reflected by their distinct biochemical properties. In contrast to group I PAKs, a slightly reduced rather than enhanced kinase activity is observed upon co-expression of Mbt and a constitutively active variant of Cdc42 in serum starved cells. A Cdc42 binding-deficient Mbt protein showed enhanced kinase activity in vitro. Similar results have been reported for other group II PAKs. Kinase activity of PAK4 was not further enhanced upon co-

mCD8 marker labels the whole cell surface of the *Cdc42* mutant photoreceptor cell, including the axonal projection. Loss of Cdc42 function is accompanied by the loss of apical Mbt protein.

Finally, we analysed the influence of the constitutively activated (GTP-loaded) Cdc42^{G12V} and of the dominant-negative (GDP-loaded) Cdc42^{T17N} protein (Luo et al., 1994) on Mbt localisation. Because expression of these constructs

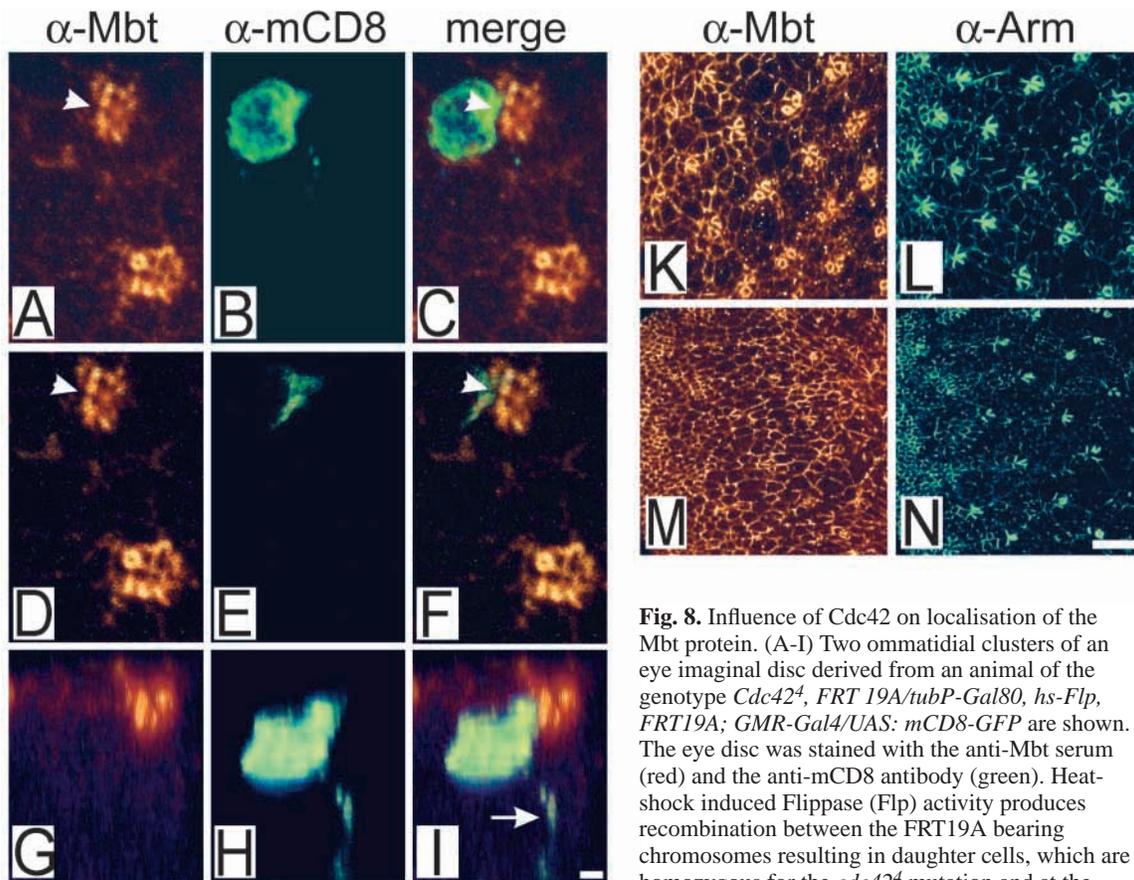


Fig. 8. Influence of Cdc42 on localisation of the Mbt protein. (A-I) Two ommatidial clusters of an eye imaginal disc derived from an animal of the genotype *Cdc42^Δ, FRT 19A/tubP-Gal80, hs-Flp, FRT19A; GMR-Gal4/UAS: mCD8-GFP* are shown. The eye disc was stained with the anti-Mbt serum (red) and the anti-mCD8 antibody (green). Heat-shock induced Flippase (Flp) activity produces recombination between the FRT19A bearing chromosomes resulting in daughter cells, which are homozygous for the *cdc42^Δ* mutation and at the same time lack the Gal4 suppressor Gal80. This

allows expression of the GMR-Gal4-driven mCD8-GFP reporter gene in homozygous *cdc42^Δ* cells. Apical-to-basal projection views of an eye disc containing a single *cdc42^Δ* mutant photoreceptor cell are shown in A (anti-Mbt), B (anti-mCD8) and C (merge); D-F focus on the most apical domain; G-I are apicobasal cross-sections. The *cdc42^Δ* mutant cell is devoid of detectable levels of Mbt protein (arrowheads), while the other photoreceptor cells express high levels of apical localised Mbt protein. The arrow indicates to the axonal projection. (K-N) Localisation of Mbt and Arm in third instar eye imaginal discs upon expression of *Cdc42^{T17N}* (K,L) and *Cdc42^{G12V}* (M,N). Scale bars: 5 μ m in I; 10 μ m in N.

transfection of activated Cdc42 but deletion or mutation of the PBD of PAK4 and PAK6 resulted in enhanced kinase activity (Abo et al., 1998; Callow et al., 2002; Yang et al., 2001).

From these data, the question remains of what role activated Cdc42 plays in regulating the functions of group II PAKs. Our genetic studies have verified the importance of the kinase domain and the PBD for the in vivo function of Mbt. Despite increased kinase activity in vitro, a construct lacking the PBD was unable to rescue the *mbt^{P1}* mutant phenotype in the eye. However, a kinase-dead Mbt protein partially rescued the *mbt^{P1}* phenotype. This indicated that Cdc42 binding to Mbt fulfils some additional essential functions that are independent of kinase activity. Localisation studies showed that one major function of the PBD is to recruit Mbt specifically to adherens junctions. These data are also supported by our observation, that localisation of a PBD-deficient Mbt protein to the cellular membrane by fusing it to a general membrane targeting sequence is not sufficient to restore the wild-type function of the protein (T. R. and D. S., unpublished). We therefore propose that Cdc42 has a dual function: specific recruitment of Mbt to AJs and regulation of the catalytic activity of Mbt. The

importance of proper targeting of PAK proteins to distinct subcellular compartments for their in vivo function is also evident from other studies. PAK4 recruitment to Golgi membranes by activated Cdc42 is dependent on an intact PBD (Abo et al., 1998). Activated Rac and Cdc42 also promote the relocalisation of a recently described group II PAK protein in *Xenopus laevis*, X-PAK5, from microtubule networks to actin-rich regions (Cau et al., 2001). In the case of group I PAKs, autophosphorylation of the Nck and PIX SH3 domain binding sites has been suggested as a mechanism to control cycling between different cellular compartments (Chong et al., 2001; Zhao et al., 2000). Also, D-PAK function in the photoreceptor axons and growth cones is dependent on the interaction with the *Drosophila* Nck homologue dreadlocks (Dock), which binds to the tyrosine phosphorylated axon guidance receptor DSCAM through its SH2 domain (Hing et al., 1999; Schmucker et al., 2000).

Rho GTPases are important regulators of the actin cytoskeleton and are involved in many developmental processes that require morphological changes of epithelial and neuronal cells (reviewed by Luo, 2000; Van Aelst and Symons,

2002). Each GTPase regulates a diverse range of effector molecules and thus induces multiple defects when misregulated. For this reason, it is difficult to reconcile all the data obtained with the loss-of-function *Cdc42* alleles and the various ectopically expressed *Cdc42* variants (Eaton et al., 1995; Genova et al., 2000; Harden et al., 1999; Luo et al., 1994; Riesgo-Escovar et al., 1996), but a number of conclusions can be drawn with respect to the function of Mbt and D-PAK. First, activated *Cdc42* has an effect on the levels of D-PAK accumulating at the dorsal most ends (leading edge) of epidermal cells flanking the amnioserosa (Harden et al., 1999). This is consistent with our result that overexpression of activated *Cdc42* in the eye disc leads to accumulation of Mbt at the membrane. Second, the *mbt* and the *Cdc42* mutant phenotypes in the eye display some similarities. In the eye disc, cells devoid of endogenous *Cdc42* or Mbt function can initiate their differentiation into photoreceptor cells (Genova et al., 2000) (Fig. 2H). In the adult eye, loss of *Cdc42* function also causes the loss of photoreceptor cells and defects in rhabdomere morphology of the remaining photoreceptor cells (D. S. and T. R., unpublished). At first sight the similarities in the loss-of-function phenotypes contradict the biochemical data implying a negative role for *Cdc42* in kinase activation. In addition, the *Cdc42* binding-deficient Mbt protein, despite enhanced kinase activity *in vitro*, does not cause visible phenotypes in the eye even when expressed in a wild-type background (Fig. 7). There are several possibilities to reconcile the data. In all cases, Mbt is either not present or not localised to AJs. As discussed above, some functions of Mbt might be independent of kinase activity. Alternatively, the moderate increase in kinase activity of the Mbt^{H19,22L} protein might not be sufficient to induce dominant phenotypes. A more detailed analysis of Mbt kinase activity *in vivo* requires the identification of physiological substrates.

The described roles of PAK proteins and RhoGTPases in regulating the actin cytoskeleton and the results presented in this study imply that Mbt localised at AJs mediates signals to the cytoskeleton to ensure proper photoreceptor cell morphogenesis. Because Mbt interacts only with GTP-loaded *Cdc42*, recruitment of Mbt to AJs would require localised *Cdc42* activation. Although we have no direct evidence so far for a selective *Cdc42* activation at AJs in photoreceptor cells, studies in mammalian epithelial cell lines have demonstrated *Cdc42* activation by the AJ protein E-cadherin (Kim et al., 2000). In addition, the molecular link between Mbt and the actin cytoskeleton remains to be defined. Similar than reported for PAK4, the *Drosophila* homologue of LIMK could provide a link between Mbt and the actin cytoskeleton (Dan et al., 2001a; Ohashi et al., 2000). Based on the similar mutant phenotype, the PDZ domain protein Canoe could be another interaction partner of Mbt. Canoe is localised at AJs and the mammalian ortholog Afadin has been shown to bind to actin filaments (Matsuo et al., 1999). Unravelling the precise molecular functions of Mbt in cell morphogenesis awaits the identification of interaction partners and physiological substrates.

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