Molekulare Analyse der zellulären Funktionen der p21-aktivierten Kinase Mushroom bodies tiny von *Drosophila melanogaster*

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ERKLÄRUNG

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1. Hiermit erkläre ich ehrenwörtlich, daß ich die vorliegende Dissertation selbstständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.
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Würzburg, den 22. April 2007

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1 Einleitung

1.1 Signaltransduktion durch p21-aktivierte Kinasen

Signaltransduktionen auf inter- und intrazellulärer Ebene erlauben den Zellen, untereinander zu kommunizieren, um den strukturellen und funktionellen Anforderungen des vielzelligen Gesamtorganismus gerecht zu werden. Eine der zentralen chemischen Reaktionen stellen reversible posttranslationale Phosphorylierungen der Aminosäuren Serin, Threonin und Tyrosin von Proteinen dar, die durch diese Modifikation innerhalb einer Signalkaskade aktiviert oder inaktiviert werden. Die für die Phosphatgruppen-Übertragungsreaktionen verantwortlichen Enzyme bezeichnet man als Kinasen, ihre Antagonisten als Phosphatasen. Der Ablauf von Signalketten verlangt eine strikte Koordination und setzt neben Phosphorylierung/ Dephosphorylierung eine Spezifität der Interaktionen zwischen den Molekülen voraus, die über die Bindung an komplementäre Proteindomänen von Enzymen und nicht-katalytischen Adapterproteinen erreicht wird.

1.1.1 Aufbau von p21-aktivierten Kinasen

Protein-Interaktionen in Signalkaskaden erfolgen über konservierte Proteinmotive, deren Vielfalt durch Variabilitäten in den Aminosäuresequenzen bestimmt wird. Im allgemeinen ergeben sich daraus entweder Kopplungsmöglichkeiten durch Multivalenz mit mehreren Modulen in einem Protein oder gleichen Modulen mit unterschiedlichen Bindungsaffinitäten.

Die p21-aktivierten Kinasen (Paks) gehören zur Klasse der Serin/Threonin-Kinasen und sind ursprünglich als Bindungspartner der RhoGTPasen Rac und Cdc42 gefunden geworden (Manser et al., 1994). Unterschiedliche Domänenstrukturen und biochemische Eigenschaften führten zur Gliederung der Pak-Proteine in zwei Untergruppen (s. Fig. 1-1): Gruppe I (Säugetiere: Pak1-3; *Drosophila*: D-Pak, D-Pak3) und Gruppe II (Säugetiere: Pak4-6; *Drosophila*: Mushroom bodies tiny (Mbt)). Obwohl beiden Gruppen eine C-terminale Kinasedomäne und N-terminale Bindedomäne PBD (PBD: *p21-binding domain*) für die GTP-beladenen RhoGTPasen Rac und Cdc42 gemein sind, unterscheiden sich die Mitglieder beider Gruppen in Bezug auf Regulation und Bindemotive für Interaktionspartner (Bagrodia and Cerione, 1999; Bokoch, 2003; Jaffer and Chernoff, 2002). So tragen Pak1-3 und D-Pak ein prolinreiches PXXP-Motiv (P: Prolin, X: beliebige Aminosäure), das zum Verankern an SH3-Domänen (SH3: *Src-homolgy3*) des Adapterproteins Nck (*Drosophila*: Dock) dient, um über Rezeptortyrosinkinasen morphogenetische Prozesse wie axonales Wachstum einzuleiten (Bokoch et al., 1996; Hing et al., 1999; Lu et al., 1997; Zhao et al., 2000). Zwischen der PBD und Kinasedomäne befindet sich ein Bereich zur Bindung von GEFs (GEF: *guanine nucleotide exchange factors*) der PIX/Cool-Familie (Manser et al., 1998). Die GEFs regulieren den GDP-GTP Austausch von RhoGTPasen und wirken dadurch aktivierend auf Pak-Proteine (Daniels et al., 1999; Feng et al., 2002). In *Drosophila* können ganze Entwicklungsprozesse davon abhängen wie das Beispiel Trio GEF zeigt, das über Rac das Gruppe I Pak-Protein D-Pak aktiviert und auf diese Weise die Wegfindung von Photorezeptoraxonen steuert (Newsome et al., 2000). Der äußerste C-Terminus

von Gruppe I Paks verfügt außerdem über die GBD-Sequenz (GBD: *G-protein binding domain*) für $G_{\beta\gamma}$-Untereinheiten heterotrimerer G-Proteine (Leberer et al., 2000; Leeuw et al., 1998), nach deren Bindung die Kinaseaktivität von humanem Pak1 gehemmt (Wang et al., 1999), beim Homolog STE20 aus *Saccharomyces cereviviae* hingegen erhöht wird (Leeuw et al., 1998). Unter den Gruppe II Pak-Proteinen konnte bisher kein definiertes Bindemotif für GEFs wie für Gruppe I gefunden werden. Dennoch bindet Pak4 an PDZ-RhoGEF (PRG) und reguliert dadurch negativ die Aktivierung von Rho-Proteinen (Barac et al., 2004).

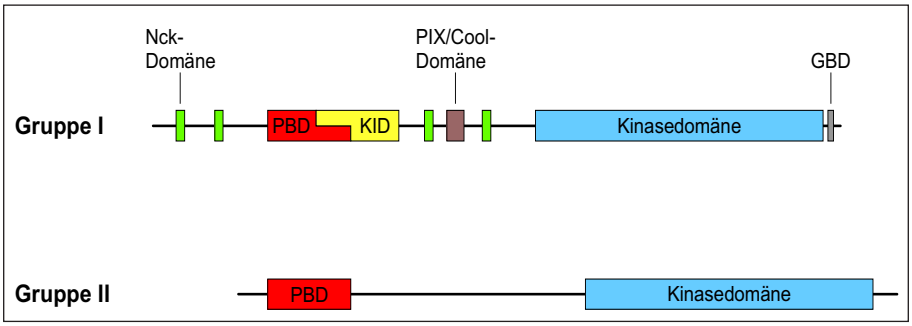

Fig. 1-1. Aufbau von p21-aktivierten Kinasen.

1.1.2 Regulation von p21-aktivierten Kinasen

Strukturelle und biochemische Daten haben dazu beigetragen, den Aktivierungsmechanismus von Gruppe I PAKs im Detail aufzuklären (Gizachew et al., 2000; Hoffman et al., 2000; Leeuw et al., 1998; Lei et al., 2000; Morreale et al., 2000). Dem Modell nach liegt Pak1 zunächst als Homodimer inaktiv vor, wobei die Kinasedomäne eines Moleküls jeweils *in trans* durch die autoinhibitorische Domäne KID (KID: *kinase inhibitory domain*) des anderen blockiert wird (Lei et al., 2000; Parrini et al., 2002). Erst die Bindung einer aktivierten RhoGTPase führt zur Konformationsänderung (Knaus et al., 1998), verbunden mit exogener Phosphorylierung von T423 an Pak1 durch die Proteinkinase PDK1 (King et al., 2000). Das Dimer trennt sich voneinander und beide Monomere führen über ihre Kinasedomänen Autophosphorylierungen bis zum katalytisch aktiven Status durch (Zenke et al., 1999). Eine Deaktivierung der Kinaseaktivität durch Phosphatasen wie POPX1, POPX2 und PP2A wurde für verschiedene Zellinien beschrieben (Koh et al., 2002; Westphal et al., 1999; Zhan et al., 2003). Neben dem Mechanismus über RhoGTPasen gibt es auch noch unabhängige Aktivierungen wie die Caspase-3 bedingte proteolytische Spaltung von Pak2 (Rudel and Bokoch, 1997) und die Bindung von Shingolipiden an die PBD, die dadurch den gleichen Effekt wie GTP-beladene RhoGTPasen hervorrufen (Bokoch et al., 1998).

Für Gruppe II Paks konnte bislang kein Zusammenhang zwischen RhoGTPase-Bindung und Erhöhung der Kinaseaktivität beobachtet werden, allerdings konstatierte eine Studie mit Pak5 eine gesteigerte Autophosphorylierung nach Cdc42-Bindung (Ching et al., 2003). Die Assoziation mit RhoGTPasen, die Gruppe II Paks präferentiell mit Cdc42•GTP eingehen (Abo et al., 1998; Schneeberger and Raabe, 2003), dient im wesentlichen zur Lokalisierung innerhalb der Zelle. Da Mitglieder der Gruppe II Paks keine KID tragen, bleiben sie nach exogener- und

Autophosphorylierung konstitutiv aktiv (Bagrodia and Cerione, 1999). Vor kurzem ermöglichten kristallographische Daten einen Einblick in den komplexen Akivierungsmechanismus von Gruppe II PAKs. Demnach werden strukturelle Regionen der Kinasedomäne, nämlich die αC-Helix, eine Glycin-Schleife und das Aktivierungssegment unter Ausbildung von Wasserstoffbrücken und Ionenwechselwirkungen durch Konformationsänderung in unmittelbare Nachbarschaft gebracht. Die Kinasedomäne vollzieht dadurch eine molekulare Plastizität, wie sie bisher bei keiner anderen Proteinkinase beobachtet wurde. Erst die koordinierte Anordnung der drei Strukturelemente führt zur Hydrolyse von ATP und zum Austausch von ADP. Der Aufbau dieser Triade kann durch die Bindung von Kofaktoren reguliert werden (Eswaran et al., 2007).

1.1.3 Allgemeine Funktionen von p21-aktivierten Kinasen

In der Zelle fungieren Gruppe I und II Paks als wichtige Regulatoren bei einer Vielzahl von Prozessen wie Proliferation, Transkription, Apoptose sowie der Organisation des Cytoskeletts und Motilität der Zelle (Bokoch, 2003; Kumar et al., 2006) (s. Fig. 1-2).

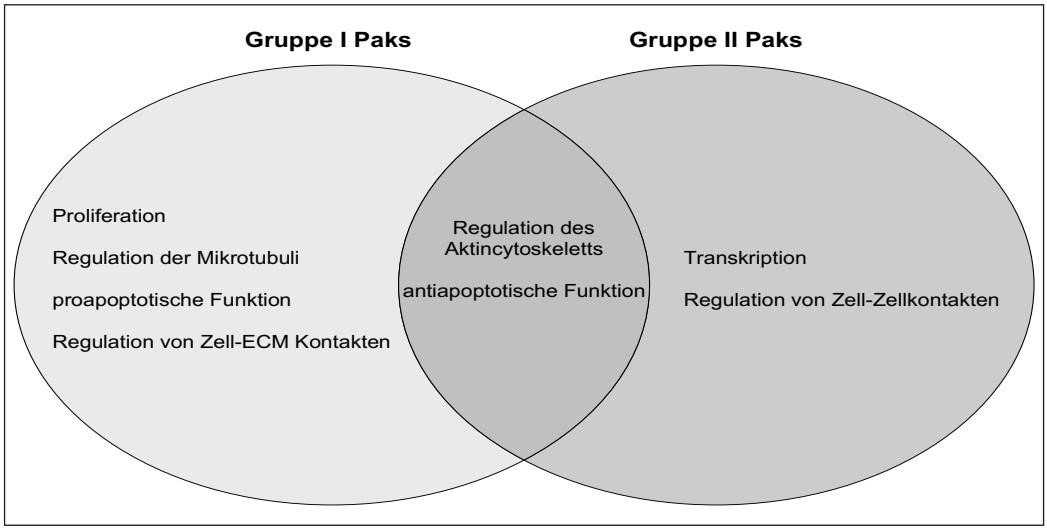

Fig. 1-2. Funktionen von Pak-Proteinen in der Zelle.

In Bezug auf Transkription konnte bisher nur für Pak6 eine Rolle identifiziert werden. Pak6 nimmt durch Bindung an den aktivierten Androgenrezeptor (AR) Sun Einfluß, indem es vom Cytoplasma in den Zellkern rekrutiert wird und dort AR-gesteuerte Genexpressionen reprimiert (Yang et al., 2001). Im Zusammenhang mit Proliferation wurde Pak1 als phosphorylierende Kinase von MEK und Raf1 im MAPK-Signalweg gefunden, über den im wesentlichen Differenzierungsprozesse der Zelle gesteuert werden (Coles and Shaw, 2002; Frost et al., 1997; Zang et al., 2002). Als Regulator in Zellteilungs-abhängigen Vorgängen ist ebenfalls Pak1 etabliert, da es Histon H3 für die Chromatin-Kondensation und die für die Ausrichtung der Centrosomen verantwortliche Aurora A Kinase phosphoryliert (Li et al., 2002; Zhao et al., 2005). Für den geordneten Aufbau des Spindelapparats inaktiviert Pak1 ferner den Mikrotubuli-Depolymerisationsfaktor Op18/Stathmin (Daub et al., 2001; Wittmann et al., 2004). Neben diesen

Aufgaben wurden Pak-Proteine auch in Verbindung mit pro- und antiapoptotischen Funktionen gebracht. So phosphoryliert Pak1 die Proteinkinase Raf1, die wiederum den proapoptotischen Faktor BAD inaktiviert. Ebenfalls konnten direkte Phosphorylierungen von BAD durch Pak1, Pak2, Pak4 und Pak5 gezeigt werden (Cotteret et al., 2003; Gnesutta et al., 2001; Jakobi et al., 2001; Schurmann et al., 2000; Tang et al., 2000). Vor kurzem wurde Pak1 mit dem Überleben der Zelle in einen neuen Zusammenhang gebracht. Pak1 interagiert und phosphoryliert DLC1 (DLC1: *dynein light chain 1*), eine Komponente des Dynein-Motorproteinkomplexes (Vadlamudi et al., 2004a). Unter apoptotischen Stimuli geht das proapoptotische BIML, das BCL2 inaktiviert (Puthalakath et al., 1999), eine Dimerisierung mit DLC1 ein. DLC1 wird aus dem Dynein-Komplex entlassen, beide Moleküle werden von Pak1 phosphoryliert und dadurch für den proteasomalen Abbau markiert (Vadlamudi et al., 2004a). Während einzelne Pak-Proteine spezifisch pro- oder antiapoptotisch wirken können, bildet Pak2 eine Ausnahme, da es in Abhängigkeit vom zellulären Kontext in beide Richtungen reagiert: Als antiapoptotischer Faktor phosphoryliert es BAD (Schurmann et al., 2000), die Abspaltung seiner Kinase-Domäne durch Caspase-3 erfolgt hingegen proapoptotisch (Rudel and Bokoch, 1997; Walter et al., 1998).

1.1.4 Funktionen von p21-aktivierten Kinasen: Regulation des Aktin-Cytoskeletts

Unter den zahlreichen Aktivitäten beider Pak-Gruppen spielt die Regulation des Aktincytoskelets in Verbindung mit morphologischen Änderungen der Zelle eine der wichtigsten Rollen. In Studien mit aktivierten Mutanten von Pak1 konnte gezeigt werden, daß Pak1 an der Ausbildung von Lamellipodien sowie der Auflösung von Aktin-Zugfasern und Fokalkontakten beteiligt ist (Bokoch, 2003; Dharmawardhane et al., 1997; Sells et al., 1997). Alle drei Prozesse finden während der Zellwanderung in polarisierten Zellen statt. Im Zuge dessen kann Pak1 in Lamellipodien P41-ARC, eine Untereinheit des Aktin-Nukleation vermittelnden Arp2/3-Komplexes, durch Phosphorylierung aktivieren (Vadlamudi et al., 2004b). Die zusätzliche Bindung des F-Aktin Vernetzungsproteins Filamin A an die PBD-Region von Pak1 verstärkt dort dessen Aktivität (Vadlamudi et al., 2002). Am führenden Ende der Zelle phosphoryliert Pak1 seinen Effektor Lim-Kinase (Limk) (Edwards et al., 1999), der dann den Aktin-Depolymerisationsfaktor Cofilin durch Phosphorylierung eines Serinrestes an Position 3 hemmt (Arber et al., 1998; Yang et al., 1998). Als Bindungspartner der Limk konnte die gleiche Wirkung für aktiviertes Pak4 bestätigt werden (Dan et al., 2001). Diese Interaktion ist mit einer Effektivitätssteigerung der Limk verbunden, da Pak4 die Phosphatase Slingshot (Ssh-L1) als Aktivator des Cofilin zusätzlich durch Phosphorylierung inaktiviert (Soosairajah et al., 2005). Im mittleren Teil der wandernden Zelle sind Aktin-Zugfasern lokalisiert und haben als kontraktiles Element Myosin II gebunden, dessen Interaktion mit F-Aktin erst durch Phosphorylierung mittels MLCK (MLCK: *myosin light chain kinase*) ermöglicht wird. Pak1 kann nun die MLCK hemmen und so die Zelle in ihrer Motilität beeinflussen (Sanders et al., 1999). Für die Zelldynamik spielt ferner der temporäre Kontaktverlust zwischen Zelle und extrazellulärer Matrix (ECM) eine entscheidene Rolle. Fokaladhäsionen bestehen aus Multiproteinkomplexen wie Integrinen, die die Zelle mit der ECM verankern. Pak1 kolokalisiert nicht nur mit Fokalkontakten (Frost

et al., 1998; Manser et al., 1997; Sells et al., 2000), sondern löst diese im aktivierten Zustand durch einen noch nicht vollständig geklärten Mechanismus auf (Sells et al., 1997). Auch in *Drosophila* ist das entsprechende Homolog D-Pak mit Fokalkomplexen assoziiert und sorgt mit *Drosophila* RacA unter Ausbildung von Aktinnetzwerken für die dorsale Schließung des Embryos (Harden et al., 1996).

1.2 Cadherin-vermittelte Zelladhäsion

Zelladhäsion ist die Vorraussetzung für den Aufbau und die Aufrechterhaltung von dreidimensionalen Gewebestrukturen in Organismen. Adhäsive Elemente von Zellen können generell in drei Hauptgruppen klassifiziert werden:

1. Kommunikationskontakte wie *gap junctions* oder chemische Synapsen zum Austausch von Molekülen zwischen Zellen,
2a. Verbindungskontakte wie Cadherin-Catenin Komplexe (Zell-Zell Kontakte) und Fokalkontakte (Zell-Matrix Kontakte), die beide mit Aktinfilamenten assoziiert sind,
2b. Verbindungskontakte wie Desmosomen (Zell-Zell Kontakte) und Hemidesmosomen (Zell-Matrix Kontakte), die mit Intermediärfilamenten verbunden sind,
3. Verschlußkontakte wie *tight junctions*, die die Abgrenzung benachbarter Epithelien zum Abtrennen von Reaktionsräumen ermöglichen.

1.2.1 Aufbau von Cadherin-vermittelten Zellkontakten

In epithelialen Zellen stellen Cadherin-vermittelte Zellkontakte spezialisierte Regionen der Plasmamembran dar, deren Komponenten aus Calcium-abhängigen transmembranen Glykoproteinen bestehen (Hyafil et al., 1981; Hyafil et al., 1980) und als *cis*-Dimere Bindungen *in trans* mit Cadherinen von Nachbarzellen eingehen (s. Fig. 1-3). Je nach Art des Gewebetyps werden die klassischen Cadherine vom Typ I als E- (epithelial), P- (plazental) und N- (neuronal) Cadherine bezeichnet. Der extrazelluläre Teil von Cadherinen besteht aus jeweils 5 Domänen (EC1-5), von denen die EC1-Domäne die Zelladhäsion und Spezifität der Zellsortierung durch homophile Interaktion mit Nachbarzellen determiniert (Nose et al., 1990). Obwohl diesbezüglich auch den anderen EC-Motiven eine Funktion zugesprochen wurde (Chappuis-Flament et al., 2001), kam eine aktuelle Studie zu dem Ergebnis, daß bei Prozessen wie der Zellsortierung die ECs weder Bindungs- noch Adhäsionswirkung haben (Niessen and Gumbiner, 2002). Im Cytoplasma sind zahlreiche Moleküle mit der konservierten C-terminalen Domäne von Cadherin assoziiert. Darunter fallen das für die laterale Bündelung von Cadherinen verantwortliche p120-Catenin (p120ctn) (Yap et al., 1998), sowie β-Catenin (*Drosophila*: Armadillo) und γ-Catenin (Plakoglobin), die beide wiederum mit α-Catenin interagieren. In diesem Adhäsionskomplex geht α-Catenin indirekte Kontakte mit dem Aktincytoskelett über α-Actinin, Vinculin, ZO-1 und anderen Molekülen ein (Wheelock and Johnson, 2003). Auf die genaue Funktion von α-Catenin als Bestandteil des Cadherin-Catenin Komplexes und als Regulator der Aktinpolymerisation wird in den Arbeiten von Drees und Yamada (2005) im Abschnitt 1.2.2 eingegangen.

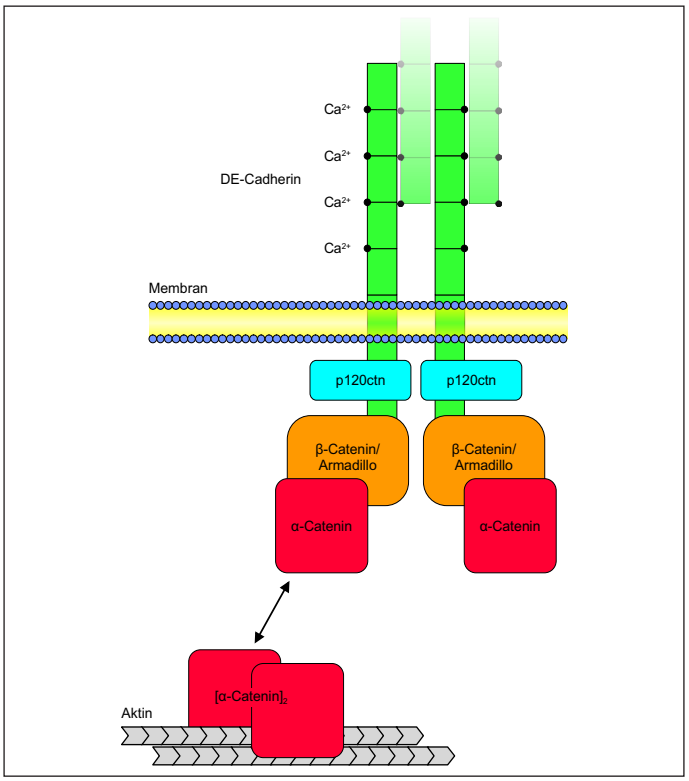

Fig. 1-3. Aufbau von Cadherin-vermittelten Zellkontakten.

1.2.2 Regulation von Cadherin-vermittelten Zellkontakten

Cadherine vermitteln interzelluläre Adhäsion auf unterschiedliche Weise: Sie erhalten einerseits als statische Elemente stabile Verbindungen zwischen epithelialen Zellen aufrecht und können andererseits während der Morphogenese zur Zellwanderung veranlaßt werden. Solche Situationen spiegeln eine Dynamik wieder, der es streng kontrollierter Mechanismen vom Auf- und Abbau molekularer Komplexe bedarf. Vorgänge dieser Art reguliert die Zelle durch Phosphorylierung/Dephosphorylierung und Assoziation/Dissoziation von Komponenten des Adhäsionskomplexes, deren Wirkungen entsprechend mit einer Zu- oder Abnahme der Adhäsionsstärke korrelieren.

Die Proteinkinase Src phosphoryliert beispielsweise E-Cadherin in MDCK-Zellen, was zur Bindung der E3-Ubiquitin-Ligase Hakai und Endocytose von E-Cadherin führt (Fujita et al., 2002). Wesentlich abundanter als die erwähnte Cadherin-Phosphorylierung sind Ser/Thr- und Tyr-Phosphorylierungen von β-Catenin (s. Fig. 1-4). FER- und Fyn-Kinasen phosphorylieren β-Catenin an Y142, vermindern dadurch die Bindungsaffinität zu α-Catenin und fördern gleichzeitig die Bindung an den Trankriptionsfaktor BCL9-2 (Brembeck et al., 2006; Brembeck et al., 2004; Piedra et al., 2003; Rosato et al., 1998). Dagegen führen Phosphorylierungen an T489 durch die Abl-Kinase (Rhee et al., 2002) und S654 durch c-Src zur Ablösung von E-Cadherin (Lilien et al., 2002). Den gegenteiligen Effekt leitet CKII (CKII: *Casein Kinase II*) ein, die S29, T102 und T112 phosphoryliert und damit die Assoziation zu α-Catenin steigert (Bek and Kemler, 2002). Den unterschiedlichen Kinasen stehen als Antagonisten die Tyr-Phosphatasen PTP1B und LAR gegenüber, die Adhäsionseigenschaften und Phosphorylierungsstatus von β-Catenin

zusätzlich beeinflussen (Balsamo et al., 1998). Verläßt β-Catenin nach Phosphorylierung seine Membranständigkeit und geht ins Cytoplasma über, so wird es vom Proteasomenkomplex in Abwesenheit eines Wnt- (*Drosophila*: *wingless*, Wg) Signals degradiert (Aberle et al., 1997; Brembeck et al., 2006; Prunier et al., 2004; Willert and Nusse, 1998). Dieser Prozeß wird über sequentielle Phosphorylierungen an S45, T41, S37 und S33 durch CKI (CKI: *Casein Kinase I*) und GSK3 (GSK3: *Glykogen Synthase Kinase 3*) induziert (Liu et al., 2002). Unter der Wirkung eines Wnt-Liganden an den *frizzled*-Rezeptor wird aber β-Catenin als Effektormolekül des Wnt-Signalwegs im Cytoplasma stabilisiert (Kennerdell and Carthew, 1998; Takada et al., 2005) und kann in diesem Zustand nach CKII-Phosphorylierung von T393 eine erhöhte cotranskriptionelle Aktivität leisten (Song et al., 2003). Desweiteren wird β-Catenin nach Phosphorylierung von S675 durch die Kinase PKA (cyclische AMP-abhängige Protein Kinase) vor Ubiquitin-bedingtem Abbau geschützt (Hino et al., 2005) und stimmuliert nach Phosphorylierung von S675 und S552 seine TCF/LEF-abhängige Gentranskription in HEK293- und COS7-Zellen (Taurin et al., 2006).

Neben Cadherinen und β-Catenin sind auch dem p120ctn wichtige Eigenschaften in der interzellulären Adhäsion zu Teil. Es konnte gezeigt werden, daß p120ctn durch Src und FER phosphoryliert wird und dadurch von E-Cadherin dissoziiert, verbunden mit einer anschließenden endosomalen Internalisierung von E-Cadherin (Kim and Wong, 1995; Reynolds and Roczniak-Ferguson, 2004; Reynolds et al., 1989). Obwohl p120ctn zu den elementaren Bestandteilen in der Aufrechterhaltung von Cadherin-vermittelten Zellkontakten zählt (Davis et al., 2003; Ireton et al., 2002; Xiao et al., 2003), zeigte eine Studie mit *Drosophila* Null-Allel Mutanten, daß p120ctn zwar ein positiver, aber nicht-essentieller Regulator der Adhäsion ist (Myster et al., 2003).

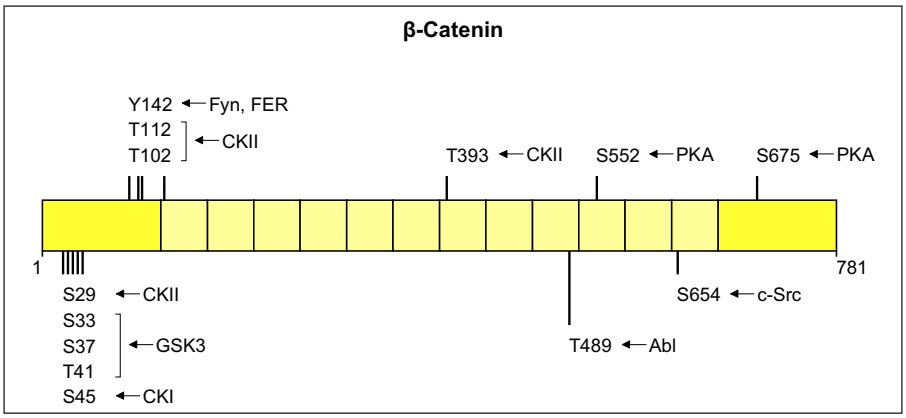

Fig. 1-4. Übersicht der Phosphorylierungsstellen und entsprechenden Kinasen von β-Catenin.

Für den Aufbau von Cadherin-Catenin Komplexen in verschiedenen Zelltypen bedarf es auch der Rekrutierung von nicht-katalytischen Molekülen wie RhoA, Cdc42 und Rac1 (Braga, 2000; Braga et al., 1997). Eine erhöhte Stabilität des Cadherin-Catenin Komplexes resultiert demnach aus der Bindung zwischen aktivierten Cdc42 und Rac1 mit IQGAP1, das ohne die beiden Proteine nach Interaktion mit β-Catenin die Dissoziation von α-Catenin initiieren würde (Fukata et al., 1999; Kuroda et al., 1998). Für die Bildung von Adhärenzkomplexen

benötigen Zellen ferner eine koordinierte Aktinpolymerisation. RhoA fördert diesen Prozeß über sein Effektorprotein mDia (*Drosophila*: diaphanous) (Sahai and Marshall, 2002), während Cdc42 und Rac1 die Proteine WASp bzw. Scar/WAVE aktivieren (Eden et al., 2002; Rohatgi et al., 2000). Das α-Catenin kann entsprechend seiner Eigenschaften als Monomer, Homo- und Heterodimer Einfluß auf die Zellkontakt- und Aktin-regulierten Vorgänge an der Zellmembran nehmen. Entgegen der bisherigen Vorstellung ist α-Catenin einem neuen Modell nach zu keinem Zeitpunkt mit dem Cadherin/β-Catenin Komplex und dem Aktincytoskelett simultan verknüpft. Als Heterodimer bindet α-Catenin an den Cadherin/β-Catenin Komplex während es als Homodimer mit dem Aktincytoskelett assoziiert ist. Zwischen diesen Zuständen liegt α-Catenin als Monomer vor und kann in Abhängigkeit seiner Oligomerisierung regulatorisch in beide Richtungen wirken (Drees et al., 2005; Yamada et al., 2005). Als Homodimer inhibiert es beispielsweise den Aktin-Nukleationsfaktor Arp2/3 (Drees et al., 2005) und sorgt in Koordination mit Cortactin und Mitgliedern aus der Ena/VASP-Familie für den Aufbau von Adhäsionskomplexen am führenden Ende der Zelle (Helwani et al., 2004; Scott et al., 2006). Die transiente Interaktion von α-Catenin mit dem Cadherin/β-Catenin Komplex hat hingegen eine Art „Speicherfunktion“, nach der α-Catenin entlassen wird und als Homodimer wieder die Aktinpolymerisation steuern kann (Drees et al., 2005; Gates and Peifer, 2005).

1.2.3 Funktionen von Cadherin-vermittelten Zellkontakten

Genetische und biochemische Studien haben in verschiedenen Organismen gezeigt, daß Adhärenzkomplexe weitaus diversere Funktionen besitzen als der ausschließliche Erhalt der epithelialen Integrität. Morphogenetische Prozesse sind durch Rearrangements von Zellen während der Entwicklung gekennzeichnet und verlangen nach einer den Zellbewegungen entsprechenden Dynamik. In *Drosophila* führen zygotische Nullmutanten von DE-Cadherin in einigen Epithelien wie der ventralen Epidermis zu schweren Defekten, in anderen wie der dorsalen Epidermis jedoch nicht. In letzteren reicht vermutlich maternales DE-Cadherin aus, während die Bildung der ventralen Epidermis auf zygotische Expression von DE-Cadherin angewiesen ist (Tepass et al., 1996; Uemura et al., 1996). In der Augenentwicklung von *Drosophila* müssen die Photorezeptorzellen R3 und R4 zur ommatidialen Rotation polarisiert werden. Der Auslöser dafür ist die differentielle Expression von DN-Cadherin in R3 und R4 in Kombination mit DN-, DN2- und DE-Cadherinen auf den Nachbarzellen (Tepass and Harris, 2007). In Prozessen wie asymmetrischen Zellteilungen von sensorischen Vorläuferzellen in *Drosophila* wird die apikal-basale Polarität ebenfalls von DE-Cadherin bestimmt (Le Borgne et al., 2002). Bei einigen Säugern und *Drosophila* vermitteln Cadherine die Adhäsion zwischen somatischen und Zellen der Keimbahn (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998; Jenkins et al., 2003; Newton et al., 1993). Diese Art der Interaktion konnte während der *Drosophila*-Oogenese beobachtet werden, in der die Oozyte am posterioren Pol der Eikammer positioniert wird (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998). An der Entwicklung von Nervensystemen sind N-Cadherine maßgeblich beteiligt: *In vitro* fördert N-Cadherin das Auswachsen von Neuriten (Neugebauer et al., 1988; Tomaselli et al., 1988), *in vivo* sind N-Cadherine im optischen Tectum vom Hühnchen (Treubert-Zimmermann et al., 2002)

und in *Drosophila* (Iwai et al., 1997) für die axonale Wegfindung verantwortlich. Ebenso ist N-Cadherin in die Etablierung synaptischer Kontakte involviert (Bruses, 2000; Huntley, 2002).

Unter pathologisch relevanten Aspekten manifestieren sich Störungen der Adhäsion in vielen humanen epithelialen Tumorzellinien. Während der Tumorprogression wird die Transkription von E-Cadherin oder Cadherin-13 herunterreguliert und gleichzeitig die Expression von N-und R-Cadherinen erhöht (Islam et al., 1996; Shimazui et al., 1996). Bei diesem Wechsel gelangen die Zellen in den Zustand der epithelialen-mesenchymalen Transition (EMT), der von einer gesteigerten Invasivität begleitet wird. Veränderungen der Zelladhäsion in Richtung onkogener Transformation wurden auch für humanes Pak4 gefunden. In den betroffenen Zellen war entweder der *Pak4*-Genlokus amplifiziert oder Mutationen führten zu unkontrollierbaren Kinaseaktivitäten (Callow et al., 2002; Qu et al., 2001). Eine Studie in *Xenopus* mit XPak5 unterstützt die Beteiligung von Gruppe II Paks an der Regulation Calcium-abhängiger Adhärenzverbindungen (Faure et al., 2005). Mutationen, die β-Catenin während des Wnt-β-Catenin Signalwegs im Cytoplasma stabilisieren, begünstigen in vielen Fällen die nukleäre Akkumulation von β-Catenin (Munemitsu et al., 1995). Die Konsequenzen sind aktivierte Gentranskriptionen, veränderte Zellmigration und Zellpolarität (Cottrell et al., 1992; Morin et al., 1997; Polakis, 2000). *Loss-of-function* Mutationen in α-Catenin führen ferner zu bestimmten Formen von Lungen-, Ovarien- und Prostatatumoren (Perez-Moreno and Fuchs, 2006).

2 Zielsetzung der Arbeit

In einer vorrausgegangenen genetischen Studie wurde die *Drosophila* Nullmutante des Gruppe II Pak-Proteins Mushroom bodies tiny (Mbt) phänotypisch in Bezug auf Struktur und Funktion charakterisiert. Der adulte Augenphänotyp dieser Nullmutante zeichnete sich durch „rauhe Augen“ und Defekte der Photorezeptorzellen in Bezug auf Anordnung und Morphologie aus. Es stellte sich heraus, daß Mbt als Regulator während der Differenzierung von Photorezeptorzellen und als Interaktionspartner der RhoGTPase Cdc42 fungiert, nach deren Bindung Mbt an Zelladhärenzverbindungen rekrutiert wird (Schneeberger and Raabe, 2003). Ausgehend von den Befunden in der Fliege sollten im Rahmen der vorliegenden Arbeit die molekularen Ursachen der Mbt-Nullmutante *in vivo* und *in vitro* erforscht werden, um Mbt in das Netzwerk intrazellulärer Signalwege einzuordnen.

Ein erstes Ziel sollte dabei sein, Interaktionspartner von Mbt mit Hilfe eines genetischen Interaktionsscreens zu finden und aufzuklären, ob Mbt mit den entsprechenden Proteinen an der Regulation des Aktincytoskeletts beteiligt ist. Einen solchen Zusammenhang ließen insbesondere die mißgestalteten Zellstrukturen der Mbt-Nullmutante vermuten.

Einem zweiten Projekt galt es, die Kolokalisierung zwischen Mbt und Zellkontakt-assoziiertem Armadillo (*Drosophila* β-Catenin) zu analysieren. Es wurde die Hypothese aufgestellt, nach der Mbt die Adhäsionseigenschaften des *Drosophila* DE-Cadherin-β-Catenin/Armadillo-α-Catenin Komplexes moduliert und folglich als Effektor für Umgestaltungsprozesse von Photorezeptorzellen in Frage kommt. Zur Überprüfung sollte ein Zellkultursystem in *Drosophila* Schneiderzellen etabliert werden, das es ermöglicht, Adhärenzverbindungen aus DE-Cadherin-β-Catenin/Armadillo-α-Catenin *in vivo* zu rekonstituieren. Unter dem Einfluß verschiedener Kinase-Varianten von Mbt sollte dieser Komplex *in vivo* und nach Isolierung *in vitro* auf Veränderungen unter Anwendung von immunologischen Techniken und Verfahren zur Kraftmessung mittels Laserpinzette untersucht werden.

Im Rahmen des dritten Projekts ergab sich die Fragestellung, ob Mbt durch die Identifizierung unbekannter Phosphorylierungssubstrate in den Kontext weiterer Mbt-regulierter Prozesse gestellt werden kann. Zu diesem Zweck sollte ein kombiniertes Verfahren aus einer radioaktiven *in vitro* Phosphorylierungsreaktion und Proteinaufreinigung entwickelt werden, das es zum einen erlaubt, Mbt-spezifische Phosphosignale aus einem Schneiderzellextrakt zu detektieren und zum anderen die entsprechend *bona fiden* Proteine über Massenspektrometrie zu identifizieren.

3 Wissenschaftliche Arbeiten

3.1 The *Drosophila* p21 activated kinase Mbt regulates the actin cytoskeleton and adherens junctions to control photoreceptor cell morphogenesis

Nicolas Menzel, Daniela Schneeberger, Thomas Raabe

Mechanisms of Development 124 (2007) 78–90

3.2 The *Drosophila* p21 activated kinase Mbt modulates DE-cadherin mediated cell adhesion by phosphorylation of Armadillo

Nicolas Menzel, Jens Waschke, Detlev Drenckhahn, and Thomas Raabe

Manuskript eingereicht

3.3 A 5'-fluorosulfonylbenzoyladenosine based method to identify physiological substrates of a *Drosophila* p21 activated kinase

Nicolas Menzel, Ashwin Chari, Utz Fischer, Monica Linder, and Thomas Raabe

Manuskript eingereicht

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The *Drosophila* p21 activated kinase Mbt regulates the actin cytoskeleton and adherens junctions to control photoreceptor cell morphogenesis

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Abstract

The p21 activated kinase (Pak) family of protein kinases are involved in many cellular functions like re-organisation of the cytoskeleton, transcriptional control, cell division and survival. These pleiotropic actions are reflected in a plethora of known interacting proteins and phosphorylation substrates. Yet, the integration of a single Pak protein into signalling pathways controlling a particular developmental process are less well studied. For two of the three known Pak proteins in *Drosophila melanogaster*, D-Pak and Mbt, distinct functions during eye development have been established. In this study we undertook a genetic approach to identify proteins acting in the Mbt signalling pathway during photoreceptor cell morphogenesis. The genetic screen identified the actin depolymerisation factor Twinstar/Cofilin as one target of Mbt signalling. Twinstar/Cofilin becomes phosphorylated upon activation of Mbt. However, biochemical and genetic experiments question the role of the LIM domain protein kinase (Limk) as a major link between Mbt and Twinstar/Cofilin as it has been suggested for other PAK proteins. Constitutive activation of Mbt not only disturbs the actin cytoskeleton but also affects adherens junction organisation indicating a requirement of the protein in cell adhesion dependent processes during photoreceptor cell differentiation.

Keywords: Drosophila eye, photoreceptor cells, morphogenesis, adherens junctions, actin cytoskeleton, RhoGTPases, p21 activated kinases, D-Limk, Twinstar

1. Introduction

Morphogenesis is a fundamental process during the development of a multi-cellular organism, which not only requires the specification of the various cell types, but also their assembly into complex tissues by means of cell shape changes, cell movement, sorting processes and elimination of surplus cells. Many of these events depend on precisely controlled modulation of cell-cell or cell-extracellular matrix contacts (Gumbiner, 2005; Lecuit, 2005).

Adherens junctions are specialized membrane structures that mediate adhesion between epithelial cells and provide a link to the actin cytoskeleton (Perez-Moreno et al., 2003). The central component of adherens junctions is the cadherin-catenin complex. Cadherins constitute a family of transmembrane proteins that mediate Ca^{2+}-dependent cell-cell adhesion. The intracellular domain of cadherin binds to β-catenin (*Drosophila*: Armadillo), which in turn can associate with the actin binding protein α-catenin. However, the prevailing dogma that cadherins at adherens junctions are linked to

the actin cytoskeleton through β-catenin and α-catenin in a stable quaternary complex has recently been questioned by the finding that α-catenin is associated in a mutually exclusive manner with either cadherin-β-catenin or with actin (Drees et al., 2005; Yamada et al., 2005).

More recently, a role of adherens junctions as a focal point for intracellular signalling has emerged. Notably, the formation of cadherin-mediated cell contacts influences the activity of the RhoGTPases Cdc42, Rac and Rho (Braga, 2002; Jaffer and Chernoff, 2004). These proteins function as molecular switches, cycling between an active GTP-bound conformation and an inactive, GDP-bound state. In its active form, RhoGTPases bind to a vast number of downstream effector proteins and one of the major effects is the reorganisation of the actin cytoskeleton.

A family of proteins, which are influenced by RhoGTPases in its activity and localisation, are the p21-activated kinases (Pak). Pak proteins are characterised by a C-terminal serine/threonine kinase domain and a N-terminal p21-binding domain (PBD) required for binding of Rac- or Cdc42-like RhoGTPases. Based on additional structural features, which determine the regulation of the kinase activity and the binding to other proteins, Pak proteins can be classified into two subgroups. From the six Pak proteins known in *Homo sapiens* (Hs), three (HsPak1-3) have been assigned to group 1, whereas HsPak4-6 belong to the group 2 (Bokoch, 2003; Hofmann et al., 2004). Similar, the three Pak proteins found in *Drosophila melanogaster* can be classified as group 1 (D-Pak, D-Pak3) and group 2 (Mbt/D-Pak2) members, respectively (Mentzel and Raabe, 2005). The role of Pak-proteins as regulators of the cytoskeleton, cell morphology and cell motility is well established. Different Pak proteins can induce the formation of lamellipodia, filopodia, and membrane ruffles as well as the dissolution of stress fibers and the disassembly of focal adhesions (Bokoch, 2003; Hofmann et al., 2004). More recently, a function of Pak proteins at the centrosomes of dividing cells has been described (Zhao et al., 2005). Deregulation of Pak protein activity can result in oncogenic transformation (Kumar et al., 2006). The pleiotropic functions of Pak proteins are reflected in the plethora of known interacting proteins and phosphorylation substrates (Bokoch, 2003; Kumar et al., 2006). This obviously raises the question of differential activation of effector pathways by different Pak proteins in a cell-type specific manner.

We are using the developing *Drosophila* eye as a model system to study the role of the group 2 Pak protein Mbt in tissue morphogenesis. The adult eye with its 800 single eye units (ommatidia), each containing eight photoreceptor cells as well as non-neuronal cone, pigment and bristle cells, arises from a monolayer epithelium, the eye imaginal disc. Differentiation of the different cell types of the eye is initiated in third instar larvae, when an indentation known as the morphogentic furrow starts to move from posterior to anterior across the eye disc. Anterior to the furrow, cells continue to proliferate, posterior to it, the photoreceptor cells and lens-secreting cone cells become specified in a sequential manner through a series of inductive cell-cell interactions (Voas and Rebay, 2004; Wolff and Ready, 1993). At pupal stage, pigment and bristle cells are added to complete the ommatidium structure and surplus cells are eliminated by programmed cell death. After recruitment and determination, photoreceptor cells undergo massive morphological changes to form the rhabdomeres, the light-sensitive structures. Rhabdomeres are apical membrane

specializations. Alignment of rhabdomeres to the ommatidial optical axis is achieved by involution of the apical domains and adherens junctions into the epithelial layer followed by their massive expansion in distal-proximal direction.

Loss of function studies of the Pak proteins Mbt and D-Pak provided evidence for distinct localisation and function of these proteins during photoreceptor cell development. D-Pak is required in growth cones to control guidance of photoreceptor cell axons, whereas recruitment of Mbt to adherens junctions is dependent on a functional RhoGTPase binding site (Hing et al., 1999; Newsome et al., 2000; Schneeberger and Raabe, 2003). In *mbt* mutant animals, recruitment of the photoreceptor cell appears largely normal, but their final differentiation is disturbed. The adherens junctions become highly disorganized and rhabdomeres fail to elongate properly leading to the suggestion that Mbt acts as a downstream effector of activated RhoGTPases at adherens junctions to regulate photoreceptor cell morphogenesis (Schneeberger and Raabe, 2003). We show that activation of Mbt disturbs the co-ordinated assembly of the ommatidium structure by interfering with the actin cytoskeleton and adherens junctions. Given the multitude of potential candidate targets of Mbt, we established a genetic screen to identify components that act downstream of Mbt to control photoreceptor cell morphogenesis. This screen identified the actin depolymerisation factor Twinstar as one target of Mbt signalling. Yet, biochemical and genetic experiments question the role of LIM domain protein kinase (Limk) as a major link between Mbt and Twinstar.

2. Results

2.1. A genetic screen to identify components of the Mbt signalling pathway

Previous studies showed that Mbt is required for normal eye and brain development (Melzig et al., 1998). A null allele of *mbt,* mbt^{P1}*,* causes a rough eye phenotype (Fig. 1A, B). Mutant ommatidia contain a variable number of photoreceptor cells, which have misshaped rhabdomeres (Fig. 1D, E). The phenotypic analysis indicated that the mbt^{P1} phenotype does not result from a defect in the recruitment of photoreceptor cells but from a defect of these cells to adopt their final morphology (Schneeberger and Raabe, 2003). This can obviously also lead to a loss of the cell. In order to identify signalling components that are relevant for Mbt function during eye development, we established a genetic screen for mutations that dominantly modify the rough eye phenotype caused by mutations in *mbt.* The strategy was to use a hypomorphic *mbt* mutation that shows only a slight rough eye phenotype and to screen for second site mutations that either enhance or suppress the rough eye phenotype already in the heterozygous state. This is of importance because it was expected that at least some proteins working in Mbt signal transduction be also required by other Pak proteins. Therefore mutations in the corresponding genes are most likely homozygous lethal.

In our screen for mutations affecting the *Drosophila* brain structure we identified a third *mbt* allele, mbt^{P3}, which causes a weak rough eye phenotype in homozygous females and hemizygous males (Fig. 1C). Tangential sections of the eyes uncovered mild defects in the arrangement and morphology of the rhabdomeres. In some cases, single photoreceptor cells are missing (Fig. 1F). The

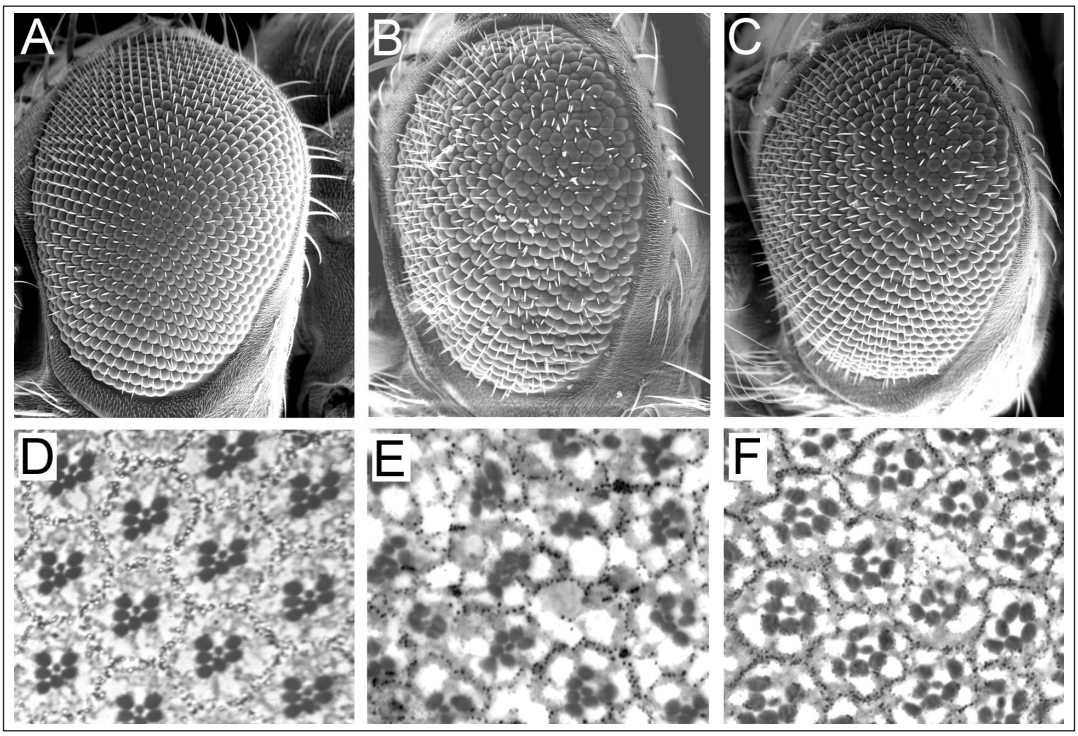

Fig. 1. Adult eye phenotypes of different *mbt* alleles. Scanning electron micrographs and tangential sections of adult eyes of the following genotypes are shown: (A, D) wild-type; (B, E) mbt^{P1}/Y, (C, F) mbt^{P3}/Y. In apical sections of wild-type ommatidia (D), the large rhabdomeres of photoreceptor cells R1-R6 and the central rhabdomere of the R7 cell are visible. In mbt^{P1} (B), the regular arrangement of ommatidia and their morphology is disturbed. Ommatidia contain a variable number of photoreceptor cells with misshaped rhabdomeres (E). Mbt^{P3} causes a slight roughening of the eye surface (C) with mild defects in ommatidial structure and rhabdomere morphology (F).

P-element insertion in mbt^{P3} was mapped to the 5′ untranslated region of the *mbt* transcription unit 265bp upstream P-element insertion in mbt^{P3} was mapped to the 5′ untranslated region of the *mbt* transcription unit 265bp upstream of the ATG translation start codon (Fig. 2A). To confirm that mbt^{P3} is indeed a hypomorphic allele, we performed Western blot analysis and immunohistochemical studies using an antiserum raised against the Mbt protein (Schneeberger and Raabe, 2003). On Western blots of lysates from wild-type flies, a signal of 68-69kd was detected, which corresponds to the predicted molecular weight of the Mbt protein (Fig. 2B). Mbt protein is absent in lysates from mbt^{P1} animals and markedly reduced in mbt^{P3} (Fig. 2B). These results were confirmed by staining of eye imaginal discs of 3rd instar larvae. The Mbt protein localizes at adherens junctions throughout all stages of eye development (Schneeberger and Raabe, 2003). The photoreceptor cells, which become sequentially recruited posterior to the morphogentic furrow to build up the ommatidial clusters express high levels of Mbt at adherens junctions (Fig. 2C). In mbt^{P1} eye imaginal discs, no staining is observed (Fig. 2D), whereas in mbt^{P3}, weak staining can be detected (Fig. 2E). At higher laser power it became evident that the Mbt expression pattern observed in mbt^{P3} is very similar to the pattern seen in wild-type eye discs (Fig. 2F). In summary, these data provided evidence that mbt^{P3} animals express, in terms of localization

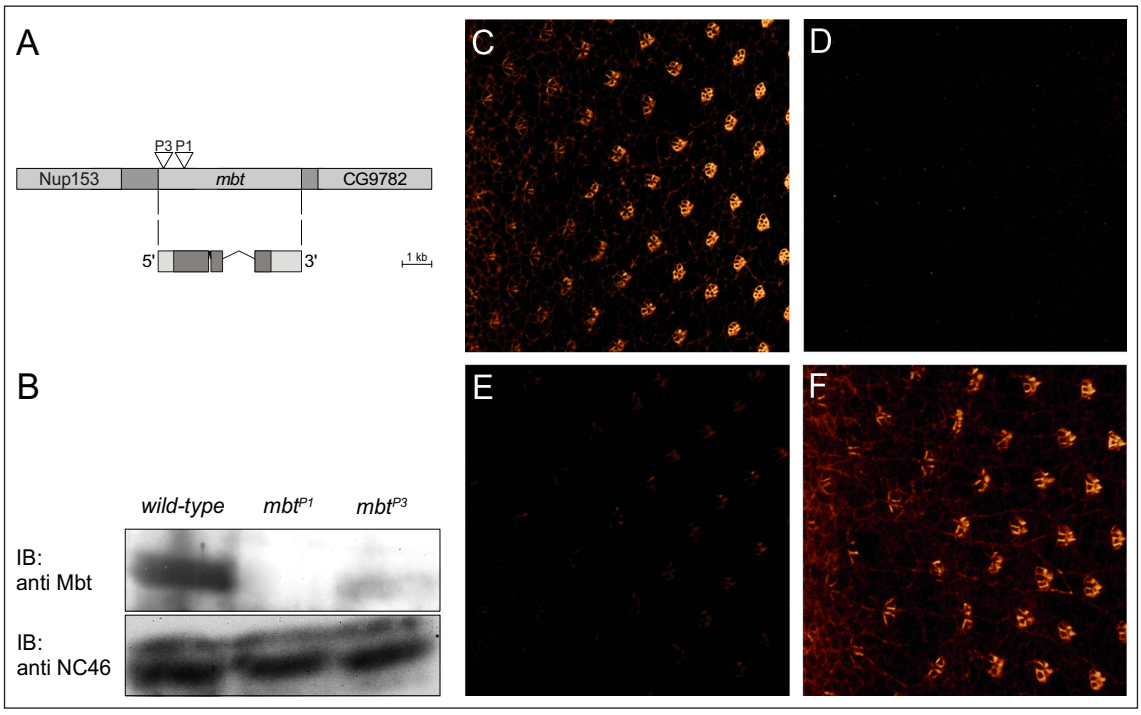

Fig. 2. Characterisation of *mbt*^{P3}. (A) Schematic representation of the genomic region of the *mbt* gene, the exon-intron structure of the *mbt* transcription unit with the open reading frame boxed in grey and localisation of the *mbt*^{P1} and *mbt*^{P3} P-element insertions. (B) Western blot analysis of lysates of wild-type, *mbt*^{P1} and *mbt*^{P3} flies. The Western blot was probed with the anti-Mbt antiserum and the monoclonal antibody NC46 (Reichmuth et al., 1995) to detect Sap47 as a loading control. (C) Expression of Mbt in third instar eye imaginal discs. Shown are apical to basal projection views of a region posterior to the morphogenetic furrow (left side). The eight photoreceptor cells of become stepwise recruited in a single ommatidium and show pronounced apical Mbt staining (Schneeberger and Raabe, 2003). No staining is seen in *mbt*^{P1} (D), whereas in *mbt*^{P3} low levels of the Mbt protein are expressed (E). At higher laser power it becomes evident that the localisation pattern of Mbt in *mbt*^{P3} animals does not deviate from the wild-type pattern (F).

and protein size, low level of functional Mbt protein, which is barely sufficient to promote normal eye development. Thus, reducing the gene dosage of a rate limiting component acting in the Mbt signal transduction pathway should lead to suppression or enhancement of the *mbt*^{P3} rough eye phenotype.

Males from available collections of homozygous lethal P-element insertions lines (Bloomington and Szeged *Drosophila* Stock Centers) were crossed to *mbt*^{P3} females and the male progeny was screened for enhancement or suppression of the *mbt*^{P3} eye phenotype. Out of 2000 crosses, 11 candidate lines were identified, each of which hits a different gene. For some genes, the observed genetic interactions could be confirmed with available independent alleles. Three of the identified genes have known functions in re-organisation of the actin cytoskeleton. Mutations in the *canoe* gene *(cno*^{009105} and *cno*^{2}*)* enhance the *mbt*^{P3} phenotype (Fig. 3A). *Cno* encodes a PDZ domain protein, which localizes to adherens junctions during eye development (Matsuo et al., 1999). It is the homologue of the actin binding protein AF-6/Afadin in vertebrates. Mutations in *twinstar (tsr*^{k05633} and *tsr*^{N96A}*)* and *slingshot (ssh*$^{1-11}$ and *ssh*$^{1-63}$*)* act as suppressors

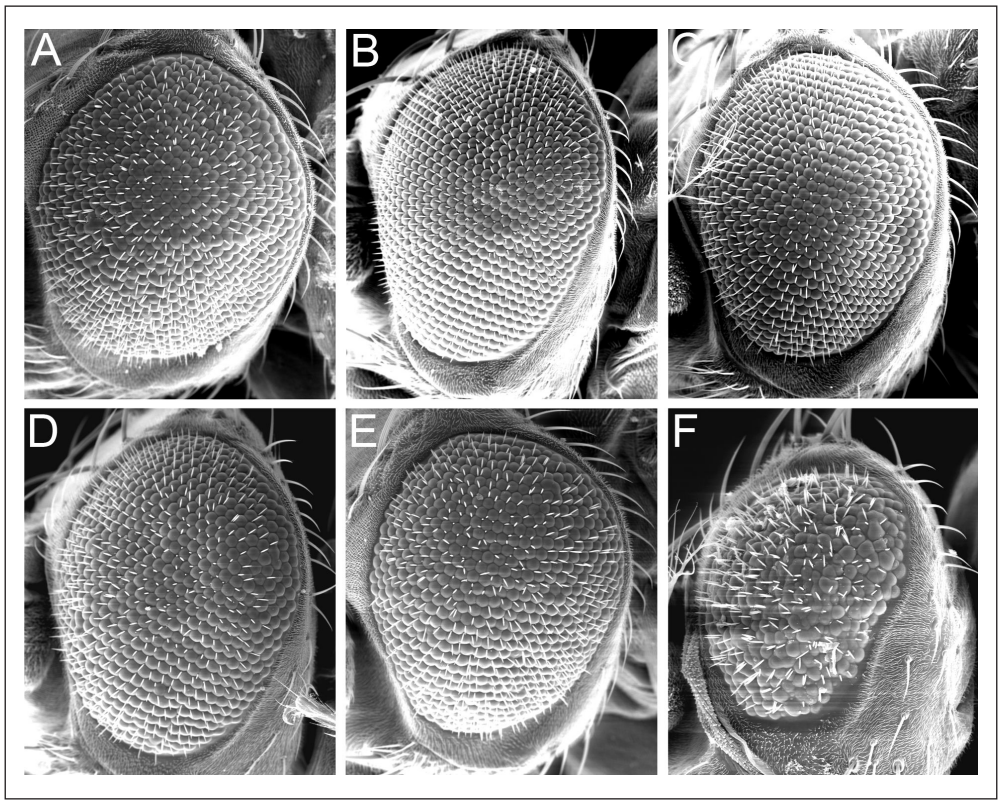

Fig. 3. Genetic interaction of *mbt*^{P1} and *mbt*^{P3} with *cno*, *tsr*, *ssh* and *D-Limk* mutations. Shown are eyes of males of the following genotypes: (A) *mbt*^{P3}*/Y; cno*^{2}*/+,* (B) *mbt*^{P3}*/Y; ssh*$^{1\text{-}11}$*/+,* (C) *mbt*^{P3}*/Y; tsr*^{N96}*/+,* (D) *mbt*^{P1}*/Y; tsr*^{N96}*/+,* (E) *mbt*^{P3}*/Y; Limk*^{2}*/+,* (F) *mbt*^{P1}*/Y; Limk*^{2}*/+.*

of the *mbt*^{P3} phenotype (Fig. 3B, C). Due to the slight rough eye phenotype of *mbt*^{P3}, the suppression effect of *tsr* and *ssh* mutations is more difficult to detect. Therefore, *tsr* and *ssh* alleles were also tested in the *mbt*^{P1} null mutant background. A pronounced suppression of the *mbt*^{P1} phenotype was observed in *mbt*^{P1}*/Y; tsr/+* males (Fig. 3D). Twinstar is the *Drosophila* homologue of Cofilin, which mediates turnover of actin filaments by severing actin filaments and by stimulating actin subunit disassembly from the pointed ends. The activity of Cofilin is negatively regulated by phosphorylation through Limk and TES-kinases (Bamburg and Wiggan, 2002). The *slingshot* gene product is a protein phosphatase, which dephosphorylates and thereby down-regulates Limk activity and at the same time up-regulates Cofilin activity by dephosphorylation thus resulting in an increase in actin filament turnover (Endo et al., 2003; Niwa et al., 2002; Soosairajah et al., 2005).

2.2 D-Limk is not a major mediator of Mbt signalling

The genetic screen suggested that Mbt could regulate the activity of the *Drosophila* Cofilin homologue Twinstar. To test whether Mbt is able to induce Twinstar phosphorylation, HA-epitope tagged kinase defective Mbt (Mbt^{T525A}, Schneeberger and Raabe, 2003) or constitutively activated Mbt were purified from transiently transfected HEK293 cells and used in *in vitro* kinase assays in the presence of bacterially expressed GST-Twinstar. To constitutively activate Mbt, we followed the same strategy as described for human Pak4 (Qu et al., 2001). In this case, strong

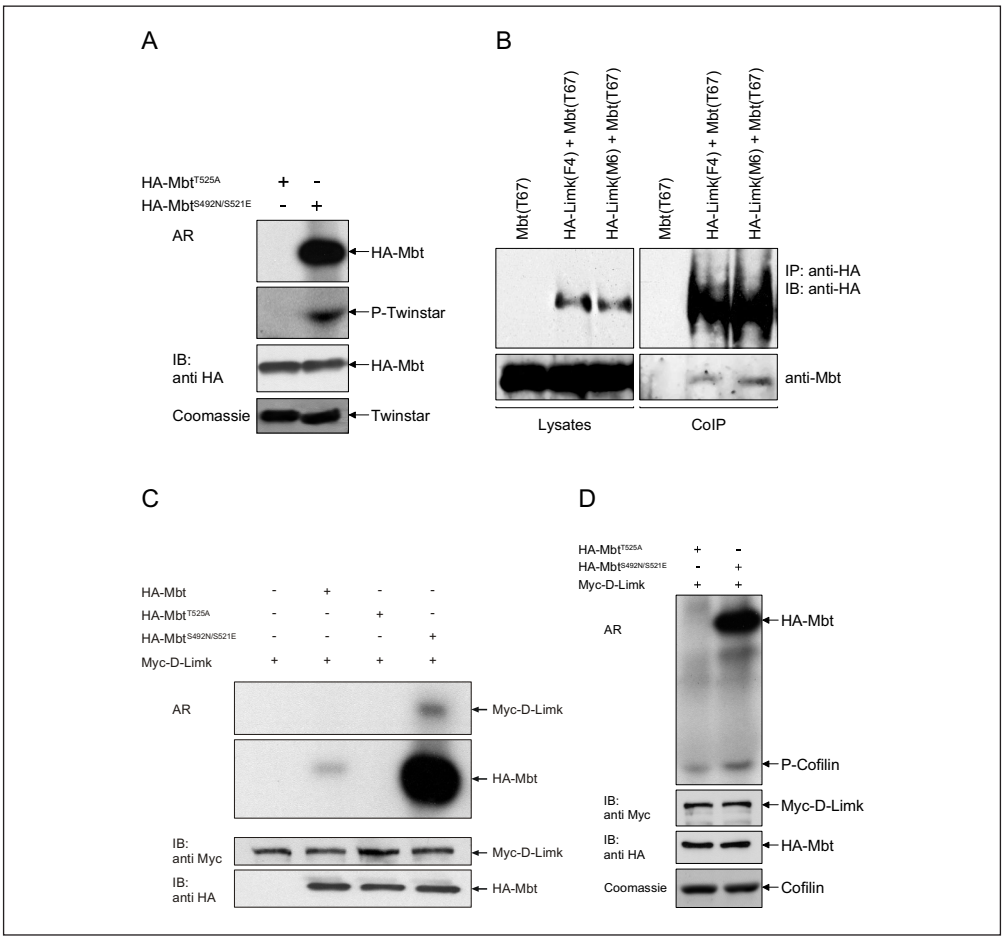

Fig. 4. (A) Mbt induces phosphorylation of Twinstar. HA-Mbt^{T525A} and HA-Mbt^{S492N,S521E} were purified from transiently transfected HEK293 cells and used in *in vitro* kinase assays in the presence of GST-Twinstar. Autophosphorylation of Mbt and GST-Twinstar phosphorylation was analysed after SDS-PAGE by autoradiography (AR). Expression of the HA-Mbt proteins was verified by Western blot analysis (IB), the presence of equal amounts of GST-Twinstar was assessed by coomassie-blue staining. (B) Mbt associates with D-Limk. Flies bearing the *P[UAS:mbt(T67)]* transgene alone or in combination with the *P[UAS:HA-D-Limk]* transgene (*F4* or *M6;* Niwa et al., 2002) were crossed with the *hs-Gal4* driver line. Total lysates from heat-shocked progeny flies were subjected to immunoprecipitation with the anti-HA antibody (IP). Purified complexes were analysed by SDS-PAGE and Western Blot with the anti-HA antibody and the anti-Mbt antiserum (IB). Expression of the proteins was verified by Western blot of total lysates (Lysates) with the same antibodies. (C) D-Limk becomes phosphorylated by Mbt. Myc-D-Limk and the indicated HA-Mbt proteins were immunopurified from separately transfected HEK293 cells and incubated in kinase buffer in the presence of [γ-^{32}P]ATP. Autophosphorylation of Mbt and D-Limk phosphorylation was analysed after SDS-PAGE by autoradiography (AR). Expression of the various HA-Mbt proteins and of Myc-D-Limk was verified by Western blot analysis of cell lysates using anti-Myc or anti-HA antibodies (IB). (D) Effect of Mbt on D-Limk activity. Myc-D-Limk was purified from HEK293 cells co-transfected with HA-Mbt^{S492N,S521E} or HA-Mbt^{T525A} followed by *in vitro* kinase reactions with GST-Twinstar (AR). Expression of the HA-Mbt proteins and Myc-D-Limk was verified by Western blot analysis (IB), the presence of equal amounts of GST-Twinstar was assessed by Coomassie-blue staining.

stimulation of kinase activity was achieved by simultaneous mutation of a predicted autophosphorylation site (S474) to glutamate and a serine residue at position 445 to asparagine, which should stabilize the catalytic loop. Substitution of the corresponding serine residues in Mbt ($Mbt^{S492N,S521E}$) resulted in strong autophosphorylation and GST-Twinstar phosphorylation, whereas the Mbt^{T525A} protein lacks any kinase activity (Fig. 4A). This indicated that activated Mbt controls phosphorylation of Twinstar directly or indirectly through activation of an associated kinase.

In vertebrates, members of both Pak subgroups, namely Pak1 and Pak4, phosphorylate and thereby activate the protein kinase Limk-1. Pak1 and Pak4 enhance the Limk catalysed phosphorylation of serine 3 of Cofilin, which is essential for down-regulation of Cofilin activity (Dan et al., 2001; Edwards et al., 1999). *Drosophila* Limk (D-Limk) shares characteristic features with vertebrate Limk, including two LIM-domains, a PDZ domain, and a kinase domain. A threonin residue at position 569 in D-Limk corresponds to threonin 508 in human Limk, which is a known phosphorylation site for Pak and Rock kinases. D-Limk differs from vertebrate Limk by a long C-terminal extension of about 600 amino acids with unknown function. It has also been shown that D-Limk is able to phosphorylate Twinstar (Ohashi et al., 2000). To test whether Mbt interacts with and phosphorylates D-Limk to regulate Twinstar activity, we performed co-immunoprecipitation and *in vitro* kinase experiments. Due to the lack of specific antibodies that could be used for immunoprecipitation studies in flies, lysates from transgenic flies expressing Mbt and a HA-tagged version of D-Limk (HA-D-Limk, Niwa et al., 2002) under the heat-shock (hs) promotor were subjected to immunoprecipitation with an anti-HA antibody. Mbt was co-immunoprecipitated specifically with D-Limk but not from control lysates of flies expressing Mbt alone (Fig. 4B). The interaction of both proteins was also verified by *in vitro* association experiments using lysates from HEK293 cells, which were transiently transfected with D-Limk and Mbt constructs (data not shown). To test whether Mbt phosphorylates D-Limk, HA-tagged Mbt, Mbt^{T525A} or $Mbt^{S492N,S521E}$ were immunopurified from transiently transfected HEK293 cells and the kinase activity was analysed after incubation with [γ^{32}P]ATP in the presence of Myc-tagged D-Limk isolated from transfected HEK293 cells. As shown in Fig. 4C, strong autophosphorylation of the HA-$Mbt^{S492N,S521E}$ protein and D-Limk phosphorylation can be detected. The wild-type Mbt protein showed weak autophosphorylation and after longer exposures also D-Limk phosphorylation became visible. No phosphorylation signal was detected in the presence of Mbt^{T525A} protein. To verify, whether Mbt is able to enhance D-Limk catalysed phosphorylation of Twinstar, HEK293 cells were co-transfected with constructs for Myc-D-Limk and HA-$Mbt^{S492N,S521E}$ or HA-Mbt^{T525A}. Myc-D-Limk was purified with an anti-Myc antibody and *in vitro* kinase assays were performed with bacterially expressed GST-Twinstar. A phosphorylation signal that corresponds in size to the Mbt protein is seen in the reaction with HA-$Mbt^{S492N,S521E}$, but not with HA-Mbt^{T525A} (Fig. 4D). This confirms the association D-Limk with the activated Mbt protein. D-Limk induced phosphorylation of Twinstar is visible in the reaction with HA-Mbt^{T525A} and a slightly stronger signal is seen in the reaction with HA-$Mbt^{S492N,S521E}$. A similar weak increase in Cofilin phosphorylation has been reported for

Pak4 and Limk in an identical experimental set-up (Dan et al., 2001). However, these experiments do exclude the possibility that D-Limk associated Mbt is able to directly phosphorylate Twinstar.

The biochemical studies raised the question on the role of D-Limk as a mediator of Mbt signalling *in vivo*. Genetic experiments performed with a recently generated null allele of *D-Limk* (*D-Limk*^{2}, Ang et al., 2006), which localizes on the X-chromosome, argue against a major role of D-Limk as a downstream target of Mbt. First, *D-Limk*^{2} males are viable with no obvious external morphological defects (Ang et al., 2006). Second, when the *D-Limk*^{2} null mutation was recombined onto the *mbt*^{P3} chromosome, only a slight enhancement of the *mbt*^{P3} eye phenotype was observed in *mbt*^{P3}, *D-Limk*^{2}/Y males (Fig. 3E). Importantly, the eye phenotype of *mbt*^{P3}, *D-Limk*^{2}/Y males is less severe than in *mbt*^{P1}/*Y* males (Fig. 1B). Both observations provide evidence that Mbt signalling is still working to a major degree even in the complete absence of D-Limk function. On the other hand, complete removal of both Mbt and D-Limk function in *mbt*^{P1}, *D-Limk*^{2}/Y males resulted in a strong synergistic enhancement of the *mbt*^{P1} phenotype (Fig. 3F) indicating a co-operative function of both proteins in eye development.

In summary, the genetic and biochemical experiments supports the idea that despite its binding to Mbt, D-Limk is not the major mediator in the Mbt signalling pathway. Nevertheless, D-Limk, together with Mbt, has a function during eye development, which appears to be redundant.

2.3. Mbt activation disturbs the actin cytoskeleton and adherens junctions

Our data suggested that Mbt is able to control F-actin dynamics through regulation of the F-actin-depolymerisation factor Twinstar. To test for a specific effect of Mbt on the actin cytoskleleton in the developing photoreceptor cells, we compared the phenotypic consequences of over-expression of the wild-type Mbt protein or the activated Mbt^{S492N,S521E} protein using the Gal4/UAS system (Brand and Perrimon, 1993). Several independent transgenic lines for both constructs were established and crossed to the neuron specific *elav-Gal4* driver line. The eyes of animals over-expressing the wild-type Mbt protein either had normal size and morphology (Fig. 5A) or showed only minor irregularities with missing bristles or misshaped ommatidia (Fig. 5B). Also in tangential sections, only few ommatidia with irregular structure were observed (Fig. 5C). In contrast, expression of Mbt^{S492N,S521E} in the developing photoreceptor cells caused a severe rough eye phenotype. Depending on the transgenic line analysed, eyes with ommatidia of different size and shape are observed (Fig. 5D), in other lines also the overall eye size is reduced and the eyes appear glazed in some parts (Fig. 5E). The external eye phenotype reflects the disturbed ommatidial structure seen in tangential sections. Ommatidia contain a variable number of photoreceptor cells and the rhabdomeres of these cells are misshaped (Fig. 5F). The eye phenotype became even stronger, when the *GMR-Gal4* driver line was used to express Mbt^{S492N,S521E} in all cells posterior to the morphogenetic furrow. In this case, small glazed eyes with no discernable ommatidial structure were observed (data not shown).

To determine whether and at which stage Mbt^{S492N,S521E} influences the actin cytoskeleton, eye imaginal discs were stained at different developmental stages with rhodamine-conjugated phalloidin. In addition, we used an antibody directed against the neuronal

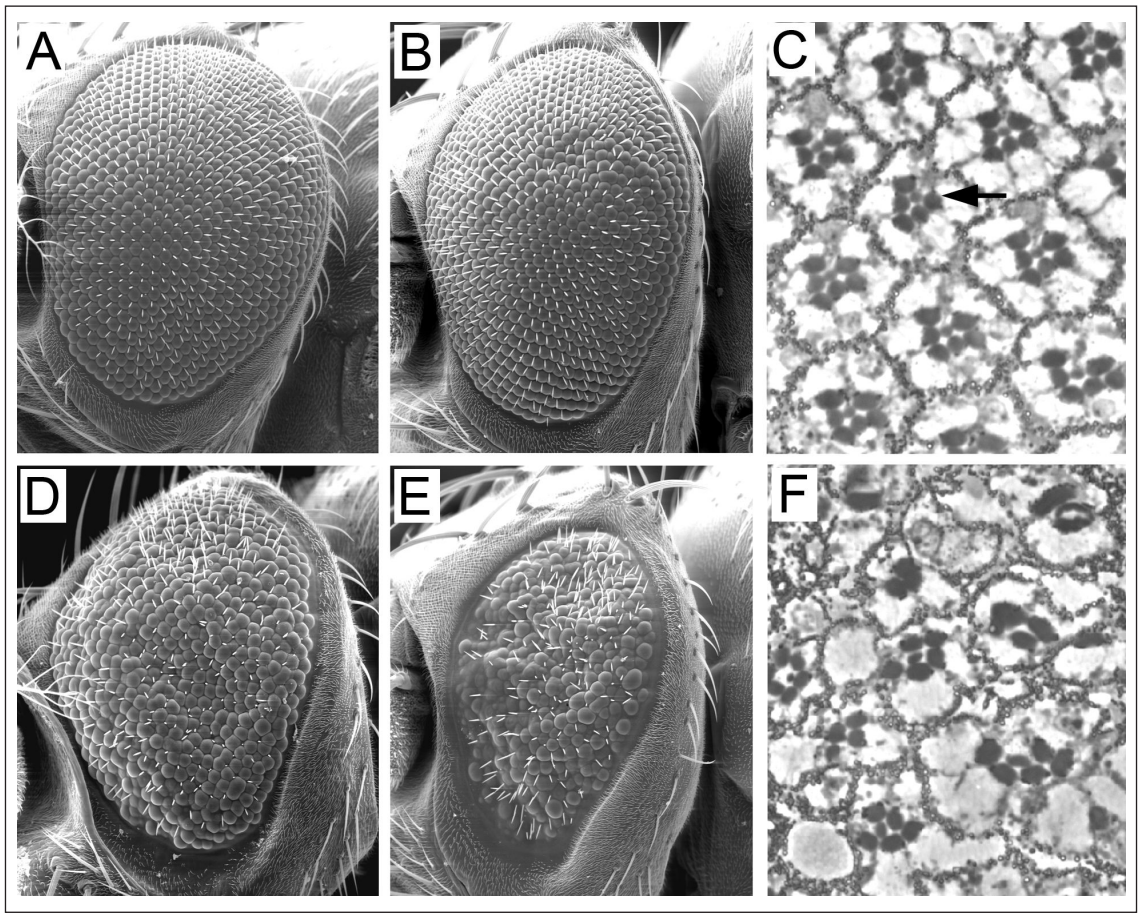

Fig. 5. Overexpression of Mbt and Mbt^{S492N,S521E}. Scanning electron micrographs and tangential sections of adult eyes from females of the following genotypes are shown: (A) *elav-Gal4/+; UAS:mbt (T26)/+*, (B, C) *elav-Gal4/+; UAS:mbt(T67)/+*, (D) *elav-Gal4/+; UAS:mbt^{S492N,S521E} (DM3),* (E, F) *elav-Gal4/+; UAS:mbt^{S492N,S521E} (DM8b)/+*. Expression of the non-mutated Mbt protein in photoreceptor cells causes no (A) or only slight disturbances of the eye morphology caused by misshaped ommatidia or missing bristle cells (B). Only few ommatidia lack photoreceptor cells (C, arrow) or have defects in the morphology of rhabdomeres. The morphology of the rhabdomeres appears more irregular than in the wild-type (compare to Fig. 1B). Overexpression of Mbt^{S492N,S521E} causes severe eye defects. Dependent on the transgenic line, also the overall eye size is reduced (D, E). Most ommatidia contain fewer photoreceptor cells and the rhabdomeres are misshaped.

marker Elav (Robinow and White, 1991) to stain the nuclei of all eight photoreceptor cells as soon as they begin neural differentiation. This process is accompanied by migration of the nuclei to more apical positions. In wild-type eye imaginal discs of 3rd instar larvae, F-actin accumulates at the constricted apical domains of the photoreceptor cells (Fig. 6A). Elav-positive photoreceptor cells arise in a defined sequence (Fig. 6B, Tomlinson and Ready, 1987). At pupal stage, photoreceptor cells undergo morphological changes to adopt their final structure (Wolff and Ready, 1993). The F-actin rich apical domains of the photoreceptor cells involute to point to the centre of the ommatidium and elongate in proximal-distal direction to form the rhabdomeres (Fig. 6C). Phalloidin staining also outlines the surrounding pigment and bristle cells (Fig. 6C). Expression of the wild-type Mbt construct in the photoreceptor cells with the *elav-Gal4* driver did not cause any

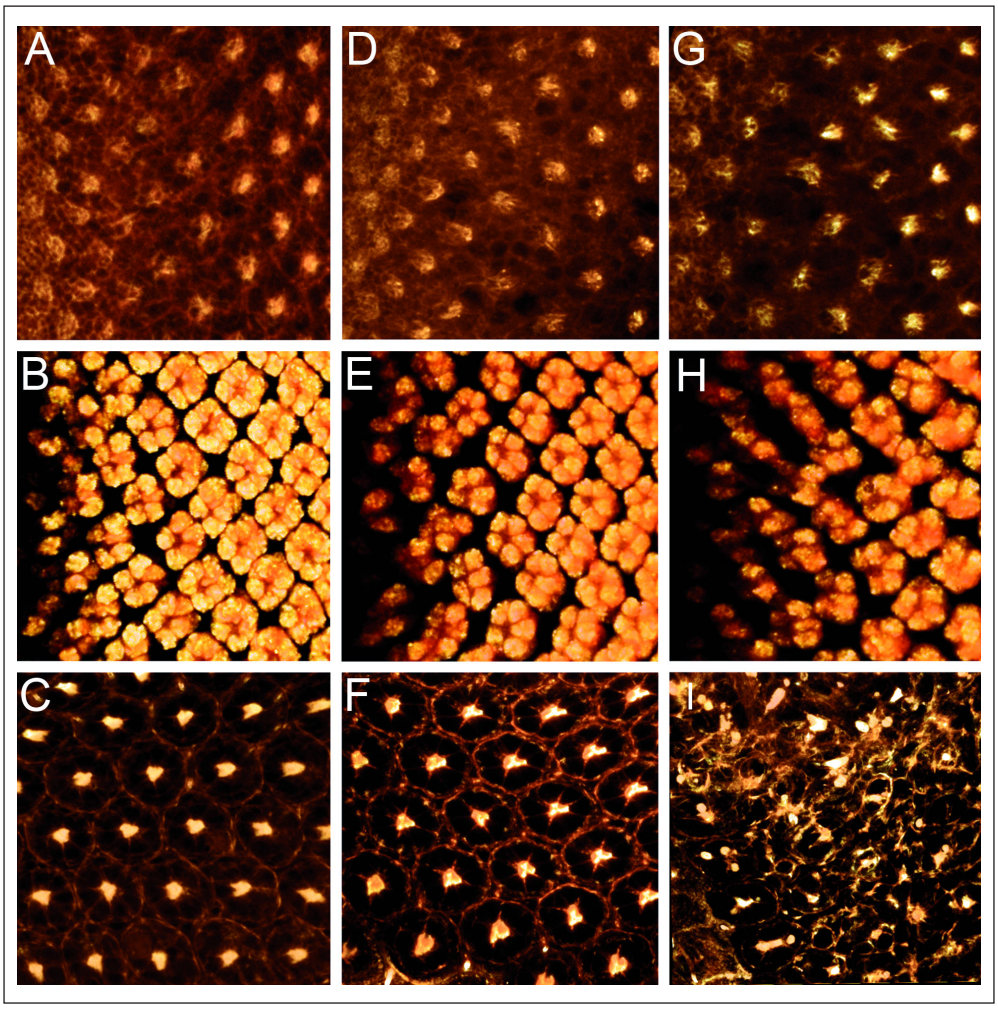

Fig. 6. Effect of Mbt activation on the actin cytoskeleton. Shown are apical to basal projection views of 3rd instar eye imaginal discs stained with rhodamine-phalloidin (first row) or an anti-Elav antibody (middle row) and proximal to distal views of eye discs at 40% pupal development (p.d.) stained with rhodamine-phalloidin (last row). In the wild-type, F-actin accumulates apical in the developing photoreceptor cells (A). They also start to express the neuronal marker Elav in the nuclei upon their sequential recruitment into the ommatidial clusters (B). In pupal eye discs, F- actin remains localized at apical membranes, which now point to the center of the ommatidium and start to elongate in proximal-distal direction (C). F-actin staining also outlines the surrounding cone, pigment and bristle cells. Ectopic expression of the *UAS:mbt (T67)* transgene in photoreceptor cells with the *elav-Gal4* driver line does not change the F-actin pattern (D, F) or neuronal differentiation (E) in transheterozygous animals. In *elav-Gal4/+; UAS:mbt*^{S492N,S521E} *(DM8b)*/+ animals, severe defects in F-actin organisation are observed primarily at pupal stage (I). Also, the localisation of the photoreceptor cell nuclei at apical levels is disturbed (H).

significant changes in F-actin organisation or neuronal differentiation (Fig. 6D-F). We noticed that many eye discs bulged around the equator, which had no effect on the final eye morphology. In contrast to the severe eye defects seen in the adult, expression of the activated Mbt^{S492N,S521E} protein in photoreceptor cells had rather mild effects on F-actin before pupal stage (Fig. 6G). F-actin staining appears to be more irregular. Occasionally, the spacing of the developing ommatidial clusters is disturbed or the apical F-actin cluster in each

ommatidium splits into separate clusters. Anti-Elav staining revealed that the nuclei of many photoreceptor cells that normally lie in the apical region now occupy various positions along the apical-basal axis, thereby disturbing the highly regular arrangement seen in wild-type eye discs (Fig. 6H). At pupal stage, the ommatidial structure is completely disturbed. Instead of the central F-actin rich core in each ommatidium, several large aggregates of F-actin can be seen in many cases. The size and shape as well as the localisation of these actin clusters along the proximal-distal axis are highly variable (Fig. 6I). In summary, our data show that the activated $Mbt^{S492N,S521E}$ protein interferes with the actin cytoskeleton during photoreceptor cells morphogenesis.

In order to find out whether activation of Mbt also disturbs the organisation of adherens junctions, DE-Cadherin staining of pupal eye imaginal discs were performed. The DE-Cadherin staining pattern in wild-type animals reflects the complex arrangement of adherens junctions in the pupal eye (Fig. 7A-C, Longley and Ready, 1995; Wolff and Ready, 1993). With a few exceptions, which correlate to the weak phenotypes seen in adult eyes (Fig. 5B, C), the pattern remains unchanged upon *elav-Gal4* driven expression of wild-type Mbt (Fig. 7D-F). In some cases, the arrangement of adherens junctions of the photoreceptor cells (Fig. 7D) and the regular spacing of bristle cells is disturbed (Fig. 7D, E). Despite neuronal expression of transgenic Mbt, we also observed few ommatidia with additional or missing cone cells (Fig. 7D). DE-Cadherin staining of eye imaginal discs expressing the activated $Mbt^{S492N,S521E}$ protein uncovered multiple defects. The number and arrangement of photoreceptor, cone, pigment and bristle cells are disturbed (Fig. 7G, H). At the proximal side, rod-like clusters of DE-Cadherin of unknown origin appear (Fig. 7I). The phenotypes indicate that neuronal expression of activated Mbt protein interferes with adherens junction mediated cell-cell adhesion. In this way, processes like motility, sorting or survival of all retinal cells can be disturbed, which are dependent on the integrity of adherens junctions (Grzeschik and Knust, 2005; Hayashi and Carthew, 2004; Mirkovic and Mlodzik, 2006).

3. Discussion

The view of adherens junctions as static structures that maintain epithelial integrity appears to be at odds with the requirement of dynamic cell shape changes during development. The assembly of the ommaditia of the *Drosophila* eye from an initially unpatterned epithelium provides an example. Photoreceptor cells become specified during 3rd larval instar, but their final morphology is established at pupal stage. Simultaneously, the non-neuronal cone, pigment and bristle cells must form a precise cell lattice that requires cell shape changes, movements and elimination of surplus cells. In spite of these dynamic processes, the integrity of adherens junctions as a basic feature of epithelia cells has to be maintained throughout eye development. The prevailing view that E-Cadherin is stably linked via β-Catenin and α-Catenin to the actin cytoskeleton has recently been questioned. It was shown that monomeric α-Catenin binds to E-Cadherin/β-Catenin, whereas dimeric α-Catenin does not and instead binds with high affinity to actin to influence Arp2/3-mediated actin polymerisation (Drees et al., 2005; Yamada et al., 2005). If it is not α-Catenin that provides a physical link between adherens junctions and the actin cytoskeleton, what other proteins could mediate such a link?

In this report, genetic and biochemical

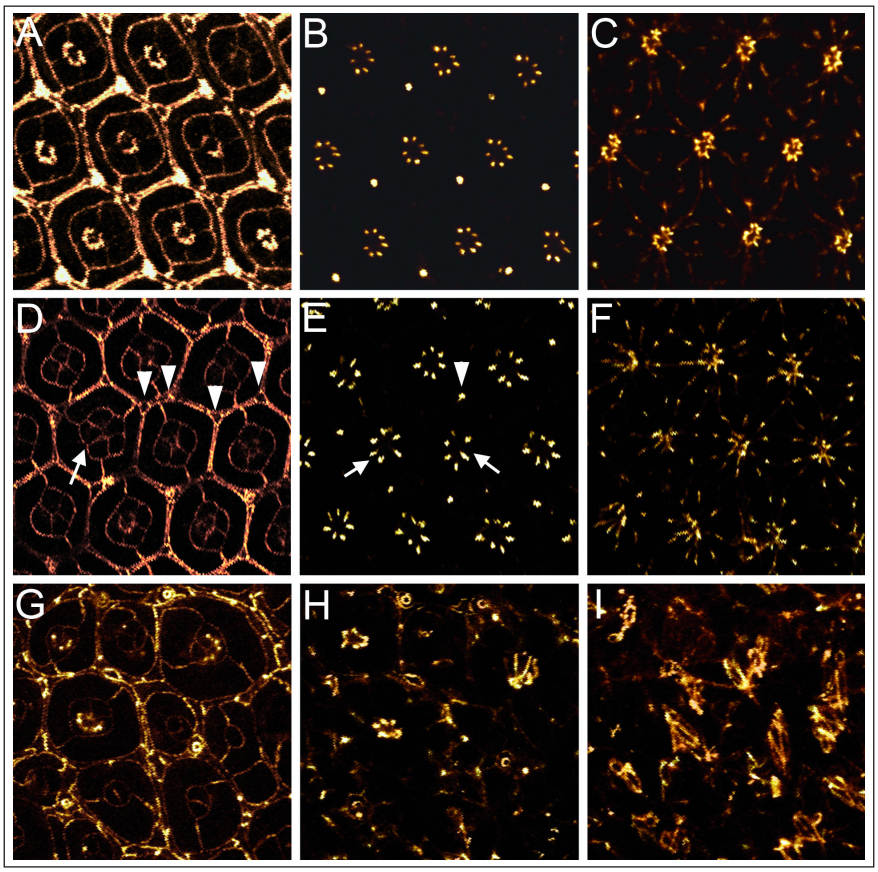

Fig. 7. Effect of Mbt activation on adherens junctions. Staining of pupal eye imaginal discs (40% p. d.) with anti DE-Cadherin. (A-C) wild-type. At distal levels (A), DE-Cadherin outlines the four cone cells, the two surrounding bean-shaped primary pigment cells and a lattice of secondary and tertiary pigment cells and bristle cells. More proximally (B), pronounced DE-Cadherin expression is seen in the bristle cells and in seven dots representing adherens junctions of the photoreceptor cells. Photoreceptor apical surfaces become anchored to the retinal floor via the cone cell plate and are surrounded by a petal like arrangement of pigment and bristle cells (C). (D-F) DE-Cadherin staining of pupal retina of *elav-Gal4/+; UAS:mbt(T67)/+* animals. Few defects in the number of cone cells (arrow in D), spacing of bristle cells (arrowheads in D, E,) and the arrangement of adherens junctions in photoreceptor cells (arrows in E) are observed. (G-I) DE-Cadherin staining indicates a complete deorganisation of adherens junctions in *elav-Gal4/+; UAS:mbt^{S492N,S521E} (DM8b)/+* animals.

evidence is presented that the Pak protein Mbt, which localizes in a RhoGTPase dependent manner to adherens junctions, could provide a link to the actin cytoskeleton during photoreceptor cell morphogenesis. Constitutive activation of Mbt in photoreceptor cells causes clustering of F-actin, which finally leads to complete disruption of the adult eye morphology. In addition, the positioning of nuclei at apical levels is disturbed. Our finding that mutations in *twinstar* and *slingshot* act as modifiers of the eye phenotype of a hypomorphic *mbt* allele suggest that these proteins work together to control photoreceptor cell morphogenesis. Additional evidence comes from the phenotypic analysis of the corresponding mutants. In *twinstar* mutants, elevated levels of F-actin, disruption of the ommatidial architecture and misshapen rhabdomeres are observed (Delalle et al., 2005). Loss of Slingshot function results in increased F-actin levels and defects in the apical movement of photoreceptor cell

nuclei (Rogers et al., 2005). So far, we have no evidence for a direct interaction between Slingshot and Mbt. In vertebrates, Pak4 inactivates Slingshot and activates Limk by phosphorylation. Slingshot plays a dual role. It dephosphorylates and thereby down-regulates the activity of Limk to phosphorylate Cofilin at serine 3 and it directly dephosphorylates Cofilin at serine 3 (Soosairajah et al., 2005). Thus Pak4 activation results in a decrease of Cofilin activity and actin filament turnover. A major role of D-Limk in Mbt signalling is questioned by our biochemical and genetic experiments. We found that activated Mbt is able to induce Twinstar phosphorylation. However, despite binding and phosphorylation of D-Limk by activated Mbt, we did not see a strong increase of D-Limk activity. Furthermore, genetic experiments with a recently identified null mutation in *D-Limk* (Ang et al., 2006) show that Mbt signalling is not completely blocked in the absence of D-Limk function. Yet, the strong synergistic effect upon complete removal of Mbt and D-Limk function indicates the requirement of both proteins in eye development. If it is not Mbt, what other proteins could be involved in regulation of D-Limk function during eye morphogenesis? Recent experiments implicate a role of the Par3 protein as a regulator of vertebrate Limk-2 activity towards Cofilin (Chen and Macara, 2006). Interestingly, the *Drosophila* Par3 homologue Bazooka localizes to and is required for maintenance of adherens junctions during photoreceptor cell morphogenesis (Nam and Choi, 2003).

It is also evident that regulation of Twinstar activity by Pak proteins is not restricted to eye development. Mutations in *twinstar* lead to proliferation defects of central brain neuroblasts and the neurons derived from these neuroblasts show axon growth defects (Ng and Luo, 2004). In this study, genetic evidence was presented that Twinstar function in axon growth is activated by Slingshot and inhibited by D-Limk, which in turn is regulated by D-Pak and Rock-kinases. In vertebrates, Limk and Slingshot control growth cone motility and morphology via Cofilin phosphorylation (Endo et al., 2003). Knock-out of *Limk* in mice leads to changes in Cofilin phosphorylation and the actin cytoskeleton resulting in abnormalities in spine morphology and in synaptic function (Meng et al., 2002). A dominant-negative version of mouse Pak1 causes changes in spine number and synaptic morphology (Hayashi et al., 2004). Thus, different Pak proteins might not only act as general regulators of Cofilin activity, but they could also provide spatial and temporal control even in a single cell.

In addition to the role in re-organisation of the actin cytoskeleton, our overexpression experiments indicate that Mbt is also able to influence the adhesive properties of cells. Differential adhesion is crucial to establish the final retinal pattern. It has been demonstrated that an apical protein complex consisting of Crumbs, Stardust and DPATJ controls assembly of adherens junctions and is essential for rhabdomere maintenance (Izaddoost et al., 2002; Nam and Choi, 2006; Pellikka et al., 2002; Richard et al., 2006). Differential adhesion, by expression of DE-Cadherin alone or together with DN-Cadherin has been suggested as a mechanism that controls cell shape changes. Expression of DN-Cadherin in cone cells -in addition to DE-Cadherin, which is expressed in all cells in an ommatidium- confers on them specific adhesion properties in order to adopt a cell shape that minimizes surface contacts with surrounding cells (Hayashi and Carthew, 2004). Integration of other proteins into the adhesive complex could be another mechanism to modify adhesive contacts.

Hibris and IrreC/rst, transmembrane proteins of the immunoglobulin superfamily, become integrated into the adhesion complex and form an intercellular link only at the interface between the presumptive primary pigment cells and secondary/tertiary pigment cells to control proper cell sorting during pupal eye development. Interference with DE-Cadherin function blocks proper localisation of IrreC/rst and results in cell sorting and survival defects (Bao and Cagan, 2005; Grzeschik and Knust, 2005). It is therefore conceivable that neuronal expression of activated Mbt influences DE-Cadherin-mediated cell adhesion of photoreceptor and bristle cells with the surrounding pigment and cone cells. In this way, motility, sorting or survival of all retinal cells can be affected. Evidence for a function of other group 2 PAK proteins in regulation of cell adhesion comes from studies with human PAK4 and *Xenopus* X-Pak5. Expression of an activated version of human Pak4 leads to anchorage-independent growth of fibroblasts (Qu et al., 2001). X-Pak5 is expressed in regions of the embryo that undergo extensive cell movements during gastrulation. It was shown that X-Pak5 localises to cell-cell junctions and regulates in a calcium-dependent manner cell-cell adhesion. An activated version of X-Pak5 decreased cell adhesiveness, while kinase-dead X-Pak5 increased adhesion (Faure et al., 2005). However, in all cases the molecular targets of the PAK proteins to exert these effects remain to be identified.

4. Experimental procedures

4.1. Genetics

Flies were raised at 25°C on standard cornmeal food. The isolation of the mbt^{P1} allele was described previously (Melzig et al., 1998). Mbt^{P3} was identified in the same histological screen for viable structural brain mutants on the X chromosome. Collections of stocks bearing *P[lacW]* element insertions on the 2nd or 3rd chromosome were obtained from the Szeged and Bloomington stock centres (Deak et al., 1997; Spradling et al., 1999). Mbt^{P3} females were crossed to males carrying *P[lacW]* element insertions and the male progeny was analysed for enhancement or suppression of mbt^{P3} eye phenotype. For selected lines, the genetic interaction was verified by precise excision of the *P[lacW]* element using the $P[ry^{+}, \Delta 2\text{-}3]$ transposase and by independent mutant alleles. The P-element insertion in cno^{009105} localizes 433bp downstream of the first exon of the *cno* gene, $ssh^{1\text{-}11}$, $ssh^{1\text{-}63}$ (Niwa et al., 2002) and tsr^{N96A} (Ng and Luo, 2004) are null alleles generated by imprecise P-element excision. The $Limk^{2}$ mutation is described in Ang et al. (2006) and was kindly provided by Huey Hing. Standard genetic techniques were used to establish the $Limk^{2}$, mbt^{P1} and $Limk^{2}$, mbt^{P3} strains. *P[UAS:mbt]* transgenic lines were generated by injecting Qiagen-purified plasmid DNA together with into w^{1118} embryos. The *P[UAS:HA-D-Limk]* transgenic line is described in Niwa et al. (2002). Transgenes were expressed using the Gal4-UAS system (Brand and Perrimon, 1993) with the *P[elav-Gal4]* or *P[heat shock-promotor (hs)-Gal4]* driver lines.

4.2. Molecular Biology

The P-element insertion sites of the different *P[lacW]* element lines isolated in the genetic screen and of mbt^{P3} were determined by inverse PCR. Briefly, genomic DNA of 30 flies was isolated according to the DNeasy Tissue kit protocol (Qiagen), digested with different restriction enzymes, precipitated and self-ligated overnight. After inverse PCR reaction with P-element specific primers, the PCR product was purified using the PCR purification kit (Qiagen) and sequenced. A cDNA clone encoding D-Limk was obtained from the Berkeley *Drosophila* Genome Project (Stapleton et al., 2002) and used as a template to amplify and subclone the *D-Limk* open reading frame as a XbaI-Bsp120I fragment into a pcDNA3 vector (Invitrogen) 3´ to a 6x Myc epitope sequence. The plasmids used

for expression of HA-tagged Mbt or Mbt^{T525A} in HEK293 cells have been described (Schneeberger and Raabe, 2003). To generate an activated version of Mbt, the following oligonucleotides were used to change the codons of serine 492 to asparagine and of serine 521 to glutamate by the QuickChange™ site-directed mutagenesis kit (Stratagene): 5′ CA-TCAAGTCGGACAACATTCTGCTGG for S492N, 5′ GAAGCGCAAGGAGCTGGTTGG for S521E. The open reading frame was then cloned as an EcoRI/ApaI fragment into pcDNA3-HA1 vector (Invitrogen). For generation of transgenic flies, the corresponding *mbt* constructs were cloned as Asp718/NotI fragments into the pUAST vector (Brand and Perrimon, 1993). The open reading frame of *twinstar* was amplified by PCR from plasmid pQE60D (a gift from K. Ohashi) and cloned as an EcoRI-XhoI fragment into pGEX-KG. All constructs were verified by sequencing.

4.3. Expression and purification of GST-Twinstar

Drosophila Twinstar was expressed as a GST fusion protein in BL21 Star™ (DE3) (Stratagene). Bacteria were grown to an optical density of 0,6 at 600 nm and protein expression was induced with IPTG (f.c. 0,8 mM). After 4 h at 30 °C, cells were lysed by sonification in TPE (1x PBS, 1% Triton X 100, 100 mM EDTA) in the presence of protease inhibitors. The supernatant was passed over a PolyPrep-column (Biorad) loaded with glutathione-agarose beads (Pharmacia) and washed in wash buffer (50 mM Tris [pH7,5], 100 mM EDTA, 0,1% Tween 20). GST-Twinstar was eluted with 50 mM Tris [pH 7,5], 10 mM glutathione, 150 mM NaCl and dialysed against 20 mM HEPES [pH 7,4], 20 mM $MgCl_2$, 150 mM NaCl.

4.4. Immunoprecipitation and Western blot analysis

To induce expression of the *P[UAS:mbt]* and *P[UAS:HA-D-Limk]* transgenes by the *P[hs-Gal4]* driver, flies were given a 37°C heat shock for 1h and were subsequently kept at room temperature for additional 5h prior to homogenisation in lysis buffer (25 mM Tris [pH7.5], 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 0,1% NP-40, 5 µg/ml Antipain, 1 µg/ml Aprotinin, 0,5 µg/ml Leupeptin, 0,7 µg/ml Pepstatin, 100 µg/ml PMSF). Total protein concentrations were determined by Bradford assay. For immunopreciptiations, lysates (1,5 mg) were pre-cleaned with 10 µl G-protein agarose (Roche) and then mixed overnight at 4°C with 30 µl G-protein agarose and 5 µl monoclonal anti-HA antibody (Santa Cruz). Immunoprecipitated proteins were washed three times in lysis buffer, separated by SDS-PAGE and analysed by immunoblotting.

4.5. In vitro kinase assays

HA-Mbt constructs and Myc-D-Limk were transiently transfected into human embryonic kidney (HEK) 293 cells according to the manufacturer's protocol (PolyFect Transfection Reagent, Qiagen). After 48 h cells were harvested in 1x PBS and lysed for 40 min at 4°C in lysis buffer. Immunoprecipitations were done with 300 µg of total cell lysate as described above using G-protein agarose and either a monoclonal anti-HA antibody (Santa Cruz) or a monoclonal anti-Myc antibody (Santa Cruz). Immunoprecipitates were incubated for 30 min at 30 °C in kinase buffer (20 mM HEPES [pH7.4], 20 mM $MgCl_2$, 20 mM PNPP, 10 mM ß-glycerol phosphate, 2 mM DTT, 1 mM Na_3VO_4) containing 5 µCi of [γ-^{32}P] ATP (3000 Ci/mmol). To test for phosphorylation of Twinstar, 50 µg of GST-Twinstar were added to the reaction mixture. Reactions were terminated with SDS-PAGE loading buffer, followed by SDS-PAGE and autoradiography.

4.6. Immunohistochemistry, histology and microscopy

Eye imaginal discs from late 3rd instar larvae or pupae (40% of pupal development) were dissected in PBS and fixed in 4% paraformaldehyd/PBS for 20 min on ice. After blocking in PBT (PBS supplemented with 0.3% Triton)/ 3% normal goat serum, eye discs were incubated with purified anti-Mbt antiserum

(Schneeberger and Raabe, 2003) or rat anti-Elav (1:100) and rat anti-DE-Cadherin (1:200) antibodies (both from the Developmental Studies Hybridoma Bank), followed by washing in PBT and incubation with Cy3 conjugated secondary antibodies (Dianova). F-actin was visualized by incubation of eye discs with rhodamine-conjugated phalloidin (1:50, Molecular Probes). Eye discs were mounted in Vectashield and analysed with a Leica TCS laser confocal microscope. Images were processed with the Amira software (Mercury Computer Systems).

For histological eye sections, heads were fixed in OsO4 as described previously (Basler et al., 1991) and embedded in EPON. Eye sections (1µm) were stained with toluidine blue/borax solution and analysed with a Leica DM microscope. For scanning electron microscopy, adult flies were in fixed in 6.25% glutardialdehyd over-night, end-point dried and coated with palladium/gold.

Acknowledgements

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The *Drosophila* p21 activated kinase Mbt modulates DE-cadherin mediated cell adhesion by phosphorylation of Armadillo

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Abstract

Phosphorylation by tyrosine and serine/threonine kinases regulates the interactions between cadherin, β-catenin and α-catenin and thus can influence the dynamic modulation of cell adhesion under normal and disease conditions. Previous studies suggested an involvement of p21-activated kinases (PAKs) in the regulation of cadherin mediated cell adhesion, but the molecular mechanism remained elusive. We have addressed this question using the *Drosophila* PAK protein Mbt, which has a function to maintain adherens junctions during eye development. Activation of Mbt results in phosphorylation of the *Drosophila* β-catenin (Armadillo), which in turn leads to destabilization of the DE-cadherin-Armadillo interaction. Laser tweezer experiments revealed a decrease in DE-cadherin mediated adhesion in the presence of activated Mbt. These findings support the previous observation that activation of the human Mbt homologue PAK4 leads to anchorage independent growth and provide the first molecular link between a PAK protein and the cadherin-catenin complex.

1. Introduction

The establishment, maintenance and modulation of cell-cell contacts is of crucial importance during the development of multicellular organisms to built complex tissues by means of cell sorting processes, cell movement and changes in cell morphology. Consequently, aberrant changes in the cells´ adhesive properties can result in pathological conditions such as cancer formation. Adherens junctions provide the mechanical strength to cell-cell contacts and consist of classic cadherins, which undergo homophilic, Ca^{2+} dependent connections between cells (Gumbiner, 2005; Perez-Moreno and Fuchs, 2006; Perez-Moreno et al., 2003). The cytoplasmic tail of cadherins is anchored to the underlying actin cytoskeleton via members of the catenin family. β-Catenin and its *Drosophila* homologue Armadillo, in addition to their role in the Wnt (Wingless in *Drosophila*) signalling pathway, serve as molecular adaptors between cadherins and α-catenin, which is linked directly or indirectly to the actin cytoskeleton. However, recent data question the existence of a stable quaternary complex and instead show that α-catenin acts as a molecular switch: monomeric α-catenin is a structural component of the cadherin-catenin complex (CCC) and does not interact with actin whereas dimeric α-catenin regulates actin filament organization (Drees et al., 2005; Yamada et al., 2005). On the other hand, β-catenin/Armadillo is subject to a complex (and not completely deciphered) pattern of phosphorylation by tyrosine and serine/threonine kinases to regulate its diverse function in cell adhesion and Wnt/Wg signalling. Degradation of cytoplasmatic β-catenin/Armadillo is induced by

phosphorylation of four conserved N-terminal serine/threonine residues through the combined activities of glycogen synthase kinase-3β (GSK3β)/Zeste-White 3 (Zw3) and casein kinase I (CKI). Phosphorylation of tyrosine 654 in β-catenin by Src kinases leads to a loss of binding to cadherin, whereas phosphorylation of tyrosine 142 by Fer and Fyn kinases disrupts binding to α-catenin but promotes binding to the co-factor BCL9-2 to induce gene transcription (Brembeck et al., 2006; Lilien and Balsamo, 2005; Nelson and Nusse, 2004). In addition, casein kinase II (CKII) phosphorylates β-catenin in the N-terminal region and thereby influences the stability of the protein and strengthens the binding with α-catenin (Bek and Kemler, 2002). Importantly, these authors provided evidence for the existence of another serine/threonine kinase distinct from GSK3β and CKII. Furthermore, mutational analysis in *Drosophila* support the idea that Armadillo localized at adherens junctions can be phosphorylated by a serine/threonine kinase other than GSK3β/Zw3 (Pai et al., 1997).

Cadherin-mediated cell adhesion also influences the activity of the RhoGTPases Cdc42, Rac and Rho, which in turn can also modulate the assembly and stability of the cell adhesion complex (Braga and Yap, 2005; Jaffer and Chernoff, 2004; Perez-Moreno et al., 2003). Several lines of evidence indicated a requirement of p21-activated kinases (PAKs) as downstream effectors of the RhoGTPases Rac and/or Cdc42 in regulation of adhesive properties of cells. PAK proteins can be divided into two subgroups, each representing three members in human (group 1: PAK1-3; group 2: PAK4-6). In *Drosophila*, two PAK proteins (D-PAK and D-PAK3) belong to group 1, whereas Mbt (D-PAK2) is the only group 2 PAK (Bokoch, 2003; Hofmann et al., 2004; Mentzel and Raabe, 2005). Constitutive activation of human PAK4 leads to changes in cell morphology, decreased cell adhesion and induction of anchorage independent growth (Callow et al., 2002; Kumar et al., 2006; Qu et al., 2001). In *Xenopus laevis*, the group 2 PAK protein X-Pak5 is expressed in regions of the embryo that undergo extensive cell movements during gastrulation. X-Pak5 localises to cell-cell contact sites and regulates cell adhesion in a calcium-dependent manner (Faure et al., 2005). Similar, *Drosophila* Mbt localizes at adherens junctions and is required for morphogenetic processes during eye development (Menzel et al., 2007; Schneeberger and Raabe, 2003). However, the underlying molecular mechanisms are unclear. In this study we examined, whether *Drosophila* Mbt is the proposed kinase that phosphorylates Armadillo at adherens junctions (Pai et al., 1997). We provide first evidence on the molecular function of a group 2 PAK protein in cadherin-mediated cell adhesion.

2. Results and discussion

2.1. DE-cadherin expression in Drosophila S2R cells leads to the formation of the cadherin-catenin complex at the plasma membrane

In order to analyze whether Mbt acts at adherens junctions to modulate cadherin mediated cell adhesion, we established a *Drosophila* S2R cell culture system. S2R cells, like S2 cells, express moderate levels of α-catenin mainly in the cytoplasm (Yanagawa et al., 1998) whereas DE-cadherin and Armadillo are present at very low amounts (Fig. 1A). Based on the previous observation that expression of DE-cadherin in S2 cells results in stabilization of Armadillo at the plasma membrane (Wang et al., 2004), we established a stable S2R cell line expressing

DE-cadherin under the actin promotor (S2R_DEcad). Staining for DE-cadherin, Armadillo and α-catenin revealed the accumulation of these proteins at the plasma membrane mainly at cell contact sites (Fig. 1A) indicating that these proteins form a functional cadherin-catenin adhesion complex (CCC). This assumption was further verified by co-immunoprecipitation experiments with the anti-DE-cadherin antibody. Armadillo and α-catenin were co-purified with DE-cadherin (Fig. 1B).

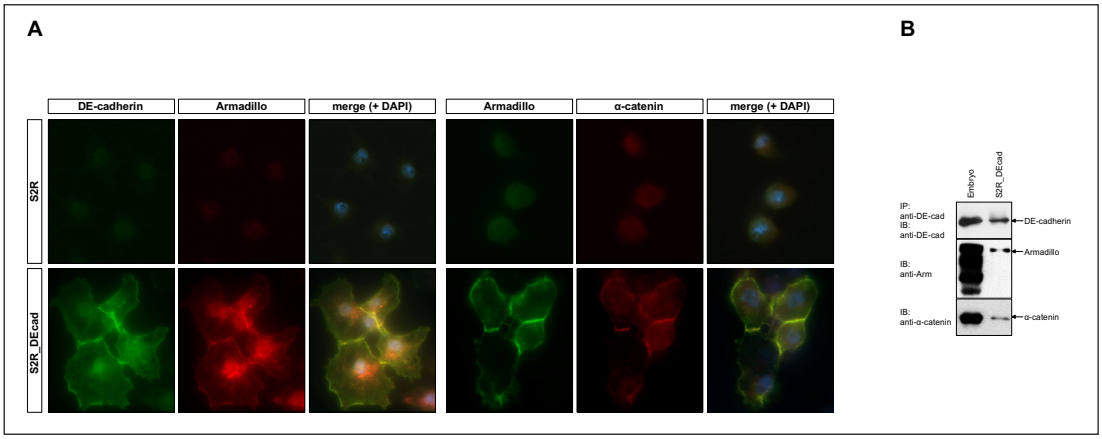

Fig. 1. (A) Stable S2R cells expressing DE-cadherin under the actin promotor (S2R_DEcad, lower panel) and the parental S2R cell line (upper panel) were stained with antibodies against DE-cadherin, α-catenin and Armadillo. (B) DE-cadherin, α-catenin and Armadillo form a complex that can be immunoprecipitated (IP) with the anti-DE-cadherin antibody from S2R_DEcad cells or from *Drosophila* embryos (0-12 hours). In embryos, Armadillo is expressed as a set of differently phosphorylated isoforms. Scale bar: 10μm.

2.2. Binding of Mbt to Armadillo

Mbt co-localizes with Armadillo at adherens junctions of the developing photoreceptor cells (Schneeberger and Raabe, 2003). The absence of detectable levels of endogenous Mbt protein made S2R cells suitable for ectopic expression of Mbt variants. Upon transient transfection of S2R_DEcad cells with a GFP-tagged version of Mbt (GFP-Mbt), co-localization of DE-cadherin and Mbt was observed at the plasma membrane, although Mbt additionally accumulates in the cytoplasm (Fig. 2A). Similar to flies (Schneeberger and Raabe, 2003), the localization pattern remained unchanged upon expression of a kinase-dead version of Mbt in S2R_DEcad cells (GFP-Mbt^{T525A}). Remarkably, expression of a constitutively activated version of Mbt (GFP-Mbt$^{S492N/S521E}$) resulted in a nearly complete absence of plasma membrane-localized Armadillo and also the GFP-Mbt$^{S492N/S521E}$ protein can hardly be detected at the plasma membrane (Fig. 2A). To exclude apoptotic cell death, cells were additionally evaluated for DNA fragmentation by *Hoechst 33258* staining (data not shown). To provide evidence for an interaction of Mbt and Armadillo, stable S2R cell lines expressing Myc epitope-tagged versions of Mbt, Mbt^{T525A} and Mbt$^{S492N/S521E}$ under the control of the inducible metallothionine promotor were established (S2R_Myc-Mbt, S2R_Myc-Mbt^{T525A} and S2R_Myc-Mbt$^{S492N/S521E}$). These cell lines were transiently transfected with a DE-Cadherin construct to allow stabilization of Armadillo as part of the CCC at the plasma membrane followed 12 hours later by addition of Cu^{2+} to induce Mbt

expression. Immunoprecipitation experiments with the anti-Armadillo antibody showed that Myc-Mbt$^{S492N/S521E}$ and to a lesser degree Myc-Mbt and Myc-Mbt^{T525A} were co-purified with Armadillo (Fig. 2B). Association of Mbt and Armadillo was also obtained upon heterologous expression of both proteins in human embryonic kidney (HEK) 293 cells (data not shown).

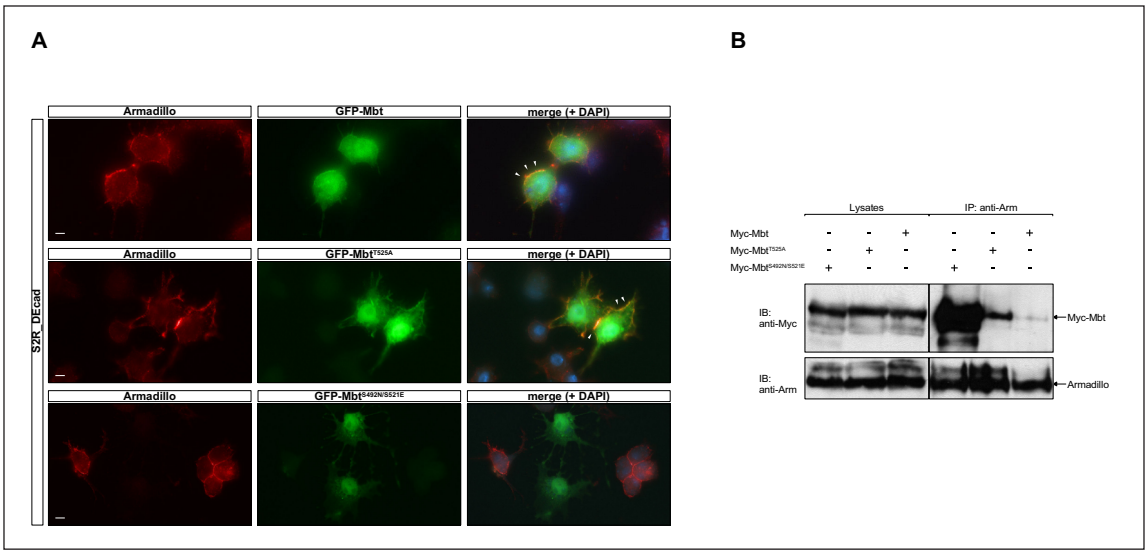

Fig. 2. (A) S2R_DEcad cells transiently expressing GFP-tagged Mbt, Mbt^{T525A} or Mbt$^{S492N/S521E}$ were co-stained for GFP and Armadillo 36 hours after transfection. Arrowheads indicate co-localization of Armadillo with GFP-Mbt and GFP-Mbt^{T525A} at the cell periphery. Scale bar: 10µm. (B) Mbt associates with endogenous Armadillo. Stable S2R cell lines, which allow inducible expression of Myc-Mbt, Myc-Mbt^{T525A} and Myc-Mbt$^{S492N/S521E}$, were transiently transfected with a DE-cadherin construct to allow formation of the CCC. 12 hours after transfection, Mbt expression was induced by addition of Cu^{2+}. Immunoprecipitations were done with the anti-Armadillo antibody (IP), the purified complexes were analyzed by SDS-PAGE and Western blot with the anti-Myc and anti-Armadillo antibodies (IB). Expression of the proteins was verified by Western blot of whole lysates (Lysates) with the same antibodies.

2.3. Activated Mbt phosphorylates Armadillo as free substrate and as a component of the cadherin-catenin complex

To test whether Armadillo is also a phosphorylation substrate of Mbt, two different approaches were applied. In the first assay, we used recombinant GST-Armadillo fusion proteins as a substrate. In the second approach, Armadillo was purified from cells as part of the CCC. The bacterially expressed GST-Armadillo fusion proteins are depicted in Fig. 3A. Besides a full-length Armadillo construct (GST-Myc-Arm) we generated (1) a N-terminal- truncated construct comprising the central Armadillo repeats and the C-terminal domain (GST-Myc-Arm [76-843]), (2) a N-terminal construct (GST-Arm [1-76]) which includes the four regulatory Ser/Thr phosphorylation sites for Zw3 and CK1, which are important to target Armadillo for degradation (Matsubayashi et al., 2004; Peifer et al., 1994; Yanagawa et al., 2002) and (3) a corresponding construct where the four phosphorylation sites were mutated to alanine (GST-Arm (S/T->A)[1-76]). The *in vitro* kinase assays showed that the activated Myc-Mbt$^{S492N/S521E}$ protein is able to phosphorylate Armadillo within the first 76 amino acids but also in the remaining

protein segment whereas the kinase-dead Myc-Mbt^{T525A} protein showed no phosphotransferase activity. Interestingly, mutation of the ZW3 and CK1 phosphorylation sites had no influence on Mbt-induced phosphorylation (Fig. 3B, C). These results suggested a complex phosphorylation pattern of Armadillo by Mbt. One major caveat to this analysis was that purified recombinant Armadillo proteins were used. Therefore, not all phosphorylation sites might be accessible to Mbt when Armadillo is in its native conformation and in complex with DE-cadherin and α-catenin. To address this issue, the CCC was first immunopurified from S2R_DEcad cells with the anti-DE-cadherin antibody followed by a second immobilization step with the anti-Armadillo antibody. Complexes were subjected to *in vitro* kinase reactions with purified GST-Mbt$^{S492N/S521E}$ or GST-Mbt^{T525A} proteins from transiently transfected HEK293 cells and analyzed by autoradiography. Only in the presence of the GST-Mbt$^{S492N/S521E}$ protein, a phosphorylation signal became visible that corresponds in size to the Armadillo protein (Fig. 3D), whereas no ^{32}P-incorporation into DE-cadherin and α-catenin were detected (not shown). From this experiment we conclude that the association of Mbt with the CCC results in phosphorylation of Armadillo.

2.4. Activation of Mbt induces destabilization of the cadherin-catenin complex

So far, the experiments did not address the question of the biological relevance of Armadillo phosphorylation by Mbt at the plasma membrane. Based on the phenotypic analysis of *mbt* mutations in flies (Menzel et al., 2007; Schneeberger and Raabe, 2003), one function could be the modulation of the CCC. To specifically analyze binding of Armadillo to DE-cadherin under the influence of Mbt, the CCC was purified from S2R_DEcad cell lysates with the anti-DE-cadherin antibody. The immunoprecipitated proteins were equally distributed and subjected to non-radioactive *in vitro* kinase assays in the presence of purified GST-Mbt^{T525A} or GST-Mbt$^{S492N/S521E}$. Compared to the control reaction (Fig. 4A, lane 1), the presence of kinase-inactive GST-Mbt^{T525A} protein had no influence on the amount of co-purified Armadillo (Fig. 4A, lane 2). In contrast, incubation with the constitutively activated GST-Mbt$^{S492N/S521E}$ protein leads to a reduction of DE-cadherin bound Armadillo (Fig. 4A, lane 4). This effect is dependent on a phosphotransferase reaction, because the same experiment performed in the absence of ATP resulted in no obvious reduction in DE-cadherin bound Armadillo (Fig. 4A, lane 3). To provide *in vivo* evidence for a role of activated Mbt on the DE-cadherin - Armadillo interaction, we used the stable S2R_Myc-Mbt^{T525A} and S2R_Myc-Mbt$^{S492N/S521E}$ cells lines described before. These cells were first transiently transfected with a DE-cadherin expressing vector to allow formation of the CCC followed 12 hours later by addition of Cu^{2+} to induce Mbt expression. After immunoprecipitation with the anti-DE-cadherin antibody, the amount of DE-cadherin bound Armadillo was analyzed by Western blot. The presence of the Myc-Mbt$^{S492N/S521E}$ protein resulted in a markedly reduced co-immunoprecipitated Armadillo protein compared to cells expressing the Myc-Mbt^{T525A} protein (Fig. 4B). From these results we concluded that activation of the Mbt protein is required to destabilize the DE-cadherin - Armadillo interaction. However, the experiments were done with total cell lysates, which may not reflect the situation found at the plasma membrane. To address this issue, Myc-Mbt$^{S492N/S521E}$ and Myc-Mbt^{T525A} expression was induced upon transient expression of the DE-Cadherin construct as described before. At

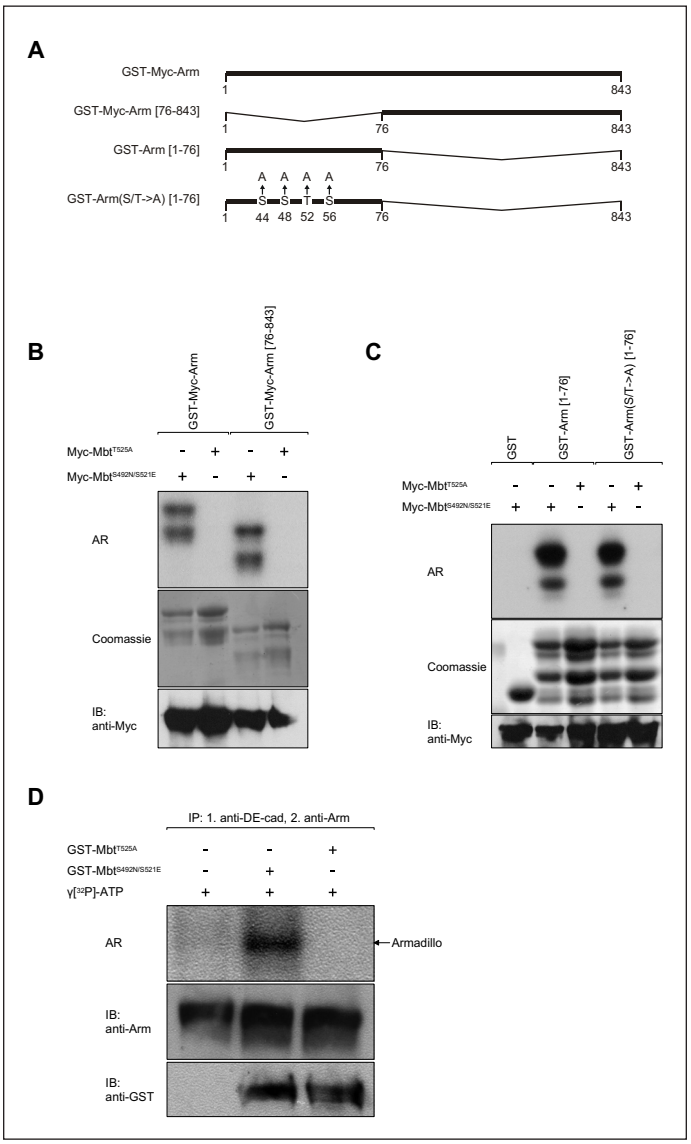

Fig. 3. Mbt phosphorylates Armadillo as a free substrate. (A) Schematic drawings of bacterially expressed GST-Armadillo proteins. (B, C) The different indicated GST-Armadillo proteins or GST as a control were subjected to *in vitro* kinase reactions in the presence of purified Myc-Mbt^{T525A} or Myc-Mbt$^{S492N/S521E}$ and analyzed after SDS-PAGE by autoradiography (AR). Myc-Mbt expression was verified by Western blot (IB), GST-Armadillo proteins were visualized by Coomassie-blue staining. As noted previously (Pai et al., 1996), several fusion protein bands are observed. Full-length fusion proteins are indicated with arrowheads. (D) Mbt phosphorylates Armadillo as part of the cadherin-catenin complex. The CCC was first immunopurified (IP) from S2R_DEcad cells with the anti-DE-cadherin antibody and then stabilized with the anti-Armadillo antibody to avoid potential loss of the Armadillo protein from the complex during the *in vitro* kinase reaction with GST-Mbt$^{S492N/S521E}$ or GST-Mbt^{T525A}. Phosphorylation of Armadillo was detected by autoradiography (AR). Western blot analysis verified the presence of Armadillo and GST-Mbt proteins (IB).

various time points upon induction of Myc-Mbt$^{S492N/S521E}$ or Myc-Mbt^{T525A} expression, membrane fractions were isolated and analyzed for Armadillo, DE-cadherin and Mbt proteins. Although Myc-Mbt^{T525A} and Myc-Mbt$^{S492N/S521E}$ show similar expression levels in whole cell lysates (Fig. 4B), significantly less Myc-Mbt$^{S492N/S521E}$ protein is found in the membrane fraction (Fig. 4C). The Myc-Mbt^{T525A} protein had no effect on the amount of membrane-associated DE-cadherin

or Armadillo (Fig. 4C). In contrast, the presence of the Myc-Mbt$^{S492N/S521E}$ protein resulted in a reduction of Armadillo but also of DE-cadherin levels in the membrane fraction. The cell fractionation experiments correlate with the immunohistochemical analysis of S2R_DEcad cells expressing the Mbt variants (see Fig. 2A). To exclude the possibility that the decrease of membrane associated Armadillo is due to transcriptional downregulation, Northern Blot analysis was performed. The expression of the different Mbt proteins had no visible effect on the steady state levels of Armadillo mRNA (Fig. 4D). In summary, the experiments support the idea that Mbt-mediated phosphorylation of Armadillo results in disruption of the DE-cadherin -Armadillo complex. It is not merely the physical association of Mbt with Armadillo that weakens the DE-cadherin - Armadillo interaction but Mbt kinase activity is essential to induce this effect. In this way, Mbt differs from proteins like IQGAP, which binds to β-catenin and thereby causes disruption of the CCC (Kuroda et al., 1998).

2.5. Activation of Mbt reduces cadherin-dependent adhesiveness of cells

The biochemical studies indicated that Mbt is able to modulate cadherin-dependent cell adhesion by destabilizing the CCC complex. In order to directly assess changes in the adhesive properties of cells upon activation of Mbt, we applied the laser tweezer technique (Baumgartner et al., 2003). Paramagnetic beads were coated with a recombinant chimeric protein comprising the extracellular domain of DE-cadherin fused to the hinge-Fc domain of human IgG1 (DE-cadherin-Fc)$_2$ (Fig. 5A). In the case of vertebrate N-, VE- and E-cadherin it has been shown that the corresponding chimeric proteins can assemble as *cis* dimers through intermolecular disulphide bridges and are able to form adhesive (*trans*) complexes with cadherins expressed at the cell surface (Baumgartner et al., 2003; Drees et al., 2005; Lambert et al., 2000). For cellular expression of DE-cadherin alone or in combination with Mbt variants, we chose Schneider S2 cells because they are semi-adherent and have, in contrast to S2R cells, a lower tendency to self aggregate, which makes them more suitable for adhesion assays. To apply the laser tweezer technique it was necessary to identify single cells expressing the appropriate Mbt constructs, which turned out to be very inefficient by transient transfections. Therefore, a stable cell line was established, which constitutively expressed DE-cadherin (S2_DEcad). By using a double selection strategy, this cell line was further modified to allow additional inducible expression of Myc-epitope tagged Mbt$^{S492N/S521E}$ (S2_DEcad_Myc-Mbt$^{S492N/S521E}$) or Mbt^{T525A} (S2_DEcad_Myc-Mbt^{T525A}). Expression of DE-cadherin and the Mbt variants in the different cell lines were verified by Western blot (Fig. 5B). DE-Cadherin-Fc coated beads were allowed to settle for 30 min on the cell surface before they were probed for displacement with a laser beam (Fig. 5C). Beads were considered tightly bound when resisting laser displacement at 42 mW. 70% of the (DE-cadherin-Fc)$_2$ beads remained bound on S2Cad cells compared to only 38% on S2 cells. Specificity and Ca^{2+} dependency of binding of (DE-cadherin-Fc)$_2$ beads to cellular DE-cadherin was tested by depletion of Ca^{2+} from the medium with EGTA. Under these conditions, the percentage of beads resisting detachment dropped to 30%, which corresponds to the value determined with S2 cells. The effect of kinase-dead Myc-Mbt^{T525A} and activated Myc-Mbt$^{S492N/S521E}$ was tested 12 hours after induction with Cu^{2+}, when Mbt proteins accumulated

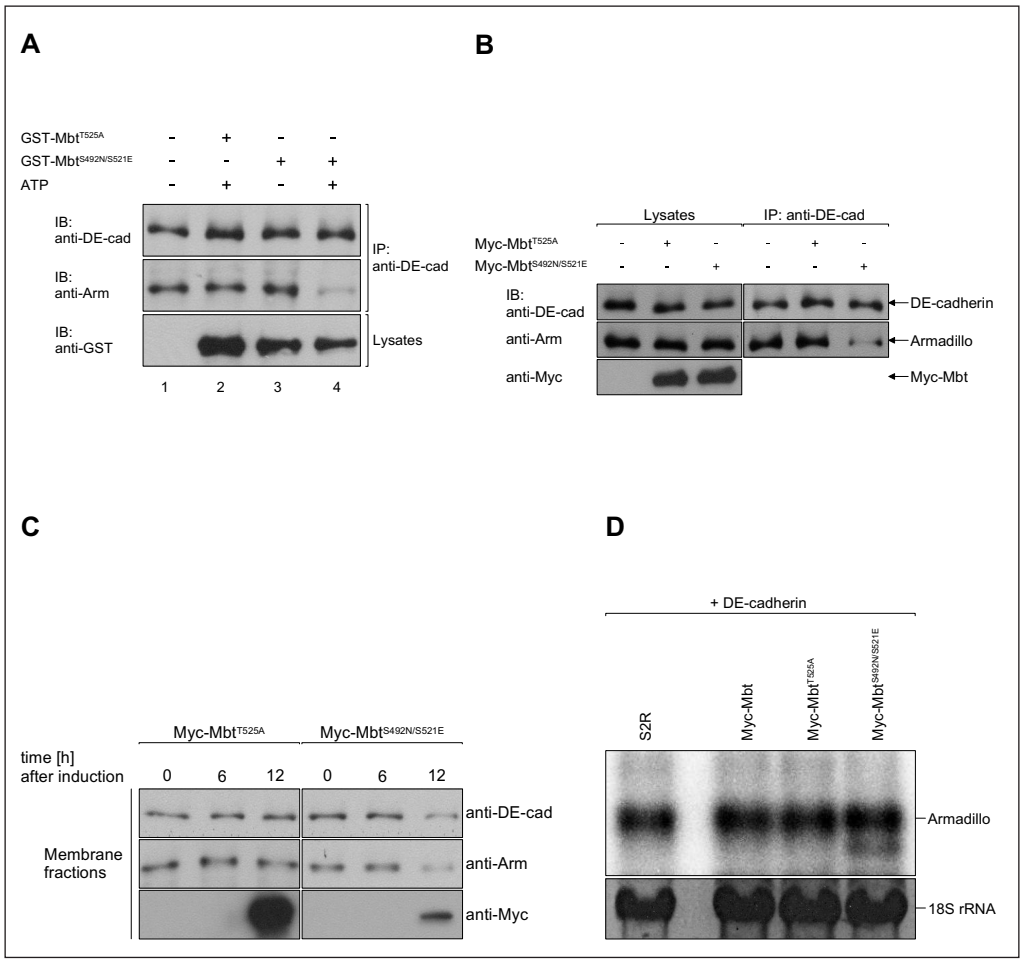

Fig. 4. Mbt phosphorylation induces destabilisation of the CCC. (A) The anti-DE-cadherin antibody was used to purify the CCC from S2R_DEcad cells (IP). Non-radioactive *in vitro* kinase reactions were performed with equal amounts from this batch in the presence or absence of ATP and soluble GST-Mbt$^{S492N/S521E}$ or GST-Mbt^{T525A}. The reactions were subjected to Western blot analysis with anti-DE-cadherin and anti-Armadillo antibodies (IB). Expression of the GST-Mbt proteins was confirmed with the anti-GST antibody. (B) Expression of Mbt$^{S492N/S521E}$ decreases the amount of Armadillo bound to DE-cadherin *in vivo*. DE-cadherin was immunoprecipitated from stable S2R_Myc-Mbt^{T525A} and S2R_Myc-Mbt$^{S492N/S521E}$ cells transiently transfected with DE-cadherin (IP: anti-DE-Cad) before and upon induction of Myc-Mbt^{T525A} or Myc-Mbt$^{S492N/S521E}$ expression and analyzed for association with Armadillo (IB). The corresponding lysate controls are shown on the left half of the Western Blot. (C) Analysis of membrane-associated DE-cadherin, Armadillo and Mbt at different time points after induction of Myc-Mbt$^{S492N/S521E}$ and Myc-Mbt^{T525A} expression. (D) Northern blot analysis of total RNA isolated from S2R and S2R_Myc-Mbt, S2R_Myc-Mbt^{T525A} or S2R_Myc-Mbt$^{S492N/S521E}$ cell lines after transient expression of DE-cadherin and induction of Mbt expression.

at high levels (Fig. 5B and 4C). Compared to S2Cad cells, the percentage of $(DE\text{-}cadherin\text{-}Fc)_2$ beads that remain bound on S2_DEcad_Myc-Mbt^{T525A} cells was not significantly changed. In contrast, a 30% reduction was observed upon expression of activated Mbt in S2_DEcad_Myc-Mbt$^{S492N/S521E}$ cells. The percentage of beads that remain bound is still higher than in the S2 and the S2_DEcad + EGTA control experiments indicating that activated Mbt only weakens and not completely eliminates cadherin-dependent cell adhesion. We therefore consider Mbt as only one component that mediates fine-tuning of cadherin- mediated cell adhesion. One model

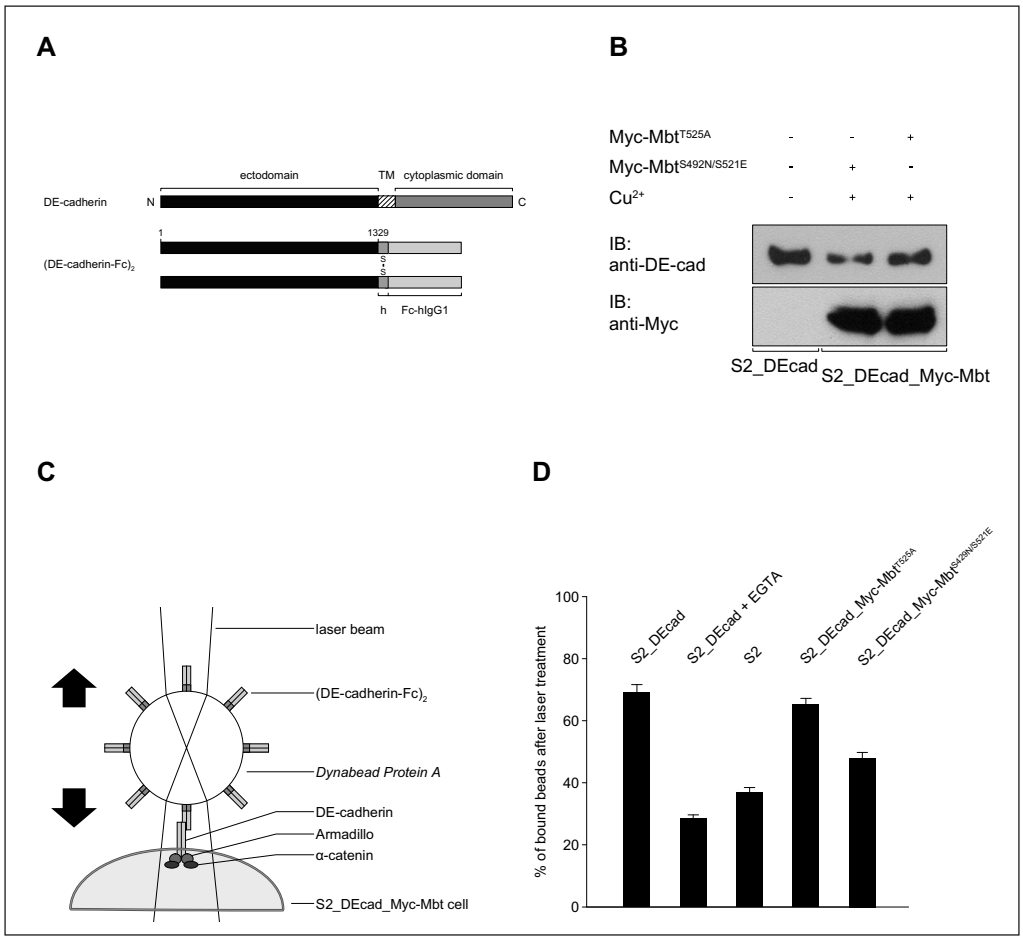

Fig. 5. Mbt decreases cadherin-mediated cell adhesion. (A) Schematic drawing of the DE-cadherin -Fc fusion protein that forms dimers via disulphate bridges. (B) Western Blot analysis of stable S2 cell lines constitutively expressing DE-cadherin and inducible Myc-Mbt^{T525A} or Myc-Mbt$^{S492N/S521E}$. (C) Set-up of the laser tweezer to test for displacement of (DE-cadherin-Fc)$_2$ coated beads from S2_DEcad_Myc-Mbt cells. (D) Statistical analysis of the laser tweezer experiment. Each data set represents six independent experiments with the indicated cell line each counting 100 beads. Paired student *t*-test P<0.05.

suggests that *cis*-dimerisation or clustering of cadherins as well as the engagement of cadherins in homophilic binding are influenced by the interaction of β-catenin to cadherins (Gumbiner, 2005). Besides Mbt, Armadillo could serve as a convergence point for other regulatory signals. Hyperphosphorylation of Armadillo in response to activation of the Wingless signalling pathway leads to a redistribution of membrane-bound Armadillo to the cytoplasm resulting in a loss of E-Cadherin from the cell surface and reduced cell adhesion (Wodarz et al., 2006). In vertebrates, phosphorylation of β-catenin and E-cadherin by CK2 increases stability of the CCC (Bek and Kemler, 2002; Lickert et al., 2000), whereas tyrosine phosphorylation of β-catenin by Fer, Fyn, Met and Src weakens the interaction with E-cadherin or α-catenin (Brembeck et al., 2006; Lilien and Balsamo, 2005; Nelson and Nusse, 2004). Thus, phosphorylation of Armadillo/β-catenin by several kinases allows combinatorial regulation and the integration of multiple signals depending on the cellular context. Evidence for an *in vivo* function of Mbt at adherens junctions came from localization experiments and from loss and gain-of-function studies during eye development (Menzel et al., 2007; Schneeberger and Raabe, 2003). Differential adhesion

mediated by DE- and DN-cadherin plays an important role in controlling cell shape changes, establishment of planar cell polarity, cell sorting during pigment and bristle cell formation and cell death in eye development (Tepass and Harris, in press). Because formation of cadherin-mediated cell contacts influences the activity of the RhoGTPases Cdc42, Rac and Rho and also of Rap1 GTPase (Braga and Yap, 2005; Jaffer and Chernoff, 2004; Van Aelst and Symons, 2002) one can envisage a model where local activation of RhoGTPases at adherens junctions controls recruitment of Mbt, which in turn feeds back to the CCC by phosphorylation of Armadillo. It will be a challenging task to describe the complex interplay between these different components in tissue organization.

3. Materials and methods

3.1. Cloning procedures

Drosophila E-cadherin (DE-cadherin) was cloned as an *Asp*718/*Bam*HI fragment from pRm-DE-cadherin (kindly provided by P. Rorth, Heidelberg) into pAc5.1 and pIZ/V5 (*Invitrogen*). To generate a chimeric IgG-fusion protein, the coding region of the extracellular domain of DE-cadherin (bp 1 to 3987) was amplified by linker-PCR, cut with *Eco*RI and *Bam*HI and inserted into the pCR3 vector containing the hinge and Fc-region from human IgG1 (kindly provided by P. Schneider). For expression of Myc-Mbt variants in S2 cells, Mbt, Mbt^{T525A} and Mbt$^{S492N/S521E}$ (Menzel et al., 2007) were cloned 3′ to a 6x *myc*-tag sequence and then inserted into the *Asp*718/*Bsp*120i sites of pMT/V5. GFP-tagged variants of Mbt were generated by cloning *Eco*RI/*Bsp*120i fragments into the pAc5.1 vector 3′ to the GFP sequence. Mbt^{T525A} and Mbt$^{S492N/S521E}$ were further cloned into the *Asp*718 site of pEBG, a GST-expression vector for HEK293 cells (a gift of O. Bernard). For expression of a full length GST-Armadillo fusion protein in bacteria, the coding region of *Armadillo* (cDNA clone LD33342, obtained from the *Drosophila Genome Resource Center*) was amplified by linker PCR and first cloned into the *Xho*I/*Xba*I sites of pcDNA3.1Myc and then subcloned into the *Bam*HI/*Xba*I sites of pGEX-KG. A full length *Armadillo* construct carrying serine to alanine substitutions at position 44, 48, 56 and a threonine to alanine substition at position 52 was a gift from S. Yanagawa (Yanagawa et al., 2002). Both full-length constructs were modified by *in vitro* mutagenesis at base pair position 229 to introduce an additional *Xho*I site. For expression of amino acids 1-76 of Armadillo, *Xho*I fragments were cloned into pGEX6-P1. After self-ligation of the *Xho*I cut construct, a *Bam*HI/*Xba*I fragment was cloned into pGEX-KG to the express the C-terminal part of Armadillo (amino acids 76-843).

3.2. Cell culture and cell fractionation

Drosophila Schneider S2R and S2 cells (Yanagawa et al., 1998) were cultured at 25°C in Schneider medium (*Biowest*) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin (*Gibco*) and if necessary with 100 µg/ml hygromycin (*Calbiochem*) and 400 µg/ml zeocin (*Invitrogen*) for establishment and maintenance of stable cell lines. Transfections of S2R and S2 cells were performed with calcium phosphate. Human embryonic

kidney 293 (HEK293) cells were grown at 37°C with 5% CO_2 in *Dulbecco's modified Eagle's medium* (DMEM) containing 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin. Cells were transfected according to the manufacturer's protocol (JetPEI Polyplus, *Biomol*). Cell fractionation experiments were performed as described elsewhere (Wodarz et al., 2006).

3.3. Protein expression, immunoprecipitation and immunoblot analysis

Expression of Myc-Mbt variants in S2 and S2R cells was induced by addition of 0.8 mM $CuSO_4$. Immunoprecipitations were done with mouse monoclonal anti-Myc (*Santa Cruz*) or rat anti-DE-cadherin (*Developmental Studies Hybridoma Bank, DSHB*) antibodies followed by SDS-PAGE and Western blot analysis as described previously (Menzel et al., 2007). For the expression and purification of $(DE\text{-}Cadherin\text{-}Fc)_2$ nine 1.5 cm diameter dishes were seeded with HEK293 cells in DMEM medium supplemented with IgG-depleted fetal bovine serum (*Gibco*). Each dish was transfected with 6 µg DNA. After 48 hours, the supernatants were collected, filled up to 50 ml with HBSS buffer (*Roth*) and stored at -80°C until further use. 30 µl slurry of beads (*Dynabeads Protein A*, *Invitrogen*) were incubated overnight with 5 ml of $(DE\text{-}Cadherin\text{-}Fc)_2$ in HBSS buffer. The beads were washed in HBSS (+ 300 mM NaCl, 0.1% NP-40) and taken up in 100 µl HBSS buffer. Expression and purification of bacterially expressed proteins were performed as described previously (Menzel et al., 2007).

3.4. Kinase assays

Kinase assays were performed as described previously (Schneeberger and Raabe, 2003).

3.5. Northern Blot analysis

Total RNA was isolated with the *RNeasy (Qiagen)* kit, separated by gel electrophoresis and transferred on a *Hybond-N* membrane (*Amersham*). The hybridization probe was made using the *Random Primed DNA labeling Kit* (*Roche*) and a *Xho*I/*Xba*I fragment derived from *Armadillo* in pcDNA3.1Myc (see above) as a template. Hybridization and washing of the membrane were done according to standard procedures followed by autoradiography.

3.6. Laser tweezer

The setup for the laser tweezer experiments has been described previously (Baumgartner et al., 2003). The laser intensity was adjusted to 42 mW. A slurry of 5 µl $(DE\text{-}Cadherin\text{-}Fc)_2$ coated microbeads in HBSS were allowed to settle on the surface of cells in 200 µl Schneider medium for 30 min. To deplete Ca^{2+}, 30 mM EGTA was added to the culture medium. Each set of data consisted of six independent experiments each counting 100 beads for detaching or resisting on cells.

3.7. Immunohistochemistry and microscopy

Cells were fixed with 4% paraformaldehyd for 20 min, permeabilized with 0.2% Triton X100 for 5 min, and incubated with mouse anti-Armadillo (1:20, *DSHB*), rat anti-DE-cadherin (1:30, *DSHB*), rat anti-α-catenin (1:50, a kind gift of H. Oda) or rabbit anti-GFP (1:1000, *MoBiTec*) and the corresponding Cy3- or Alexa488-conjugated secondary antibodies (*Dianova*). Cells were embedded in Mowiol and analyzed with a *Leica* DM5000B microscope using a 100x NA 1.3 plan Neofluar oil objective. Images were taken with a *Leica* DFC350FX b/w digital camera and false color coded with the FW4000 software.

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A 5'-fluorosulfonylbenzoyladenosine based method to identify physiological substrates of a *Drosophila* p21 activated kinase

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Abstract

Nearly all processes in cells are regulated by the coordinated interplay between reversible protein phosphorylation and dephosphorylation. It is therefore a great challenge to identify all phosphorylation substrates of a single protein kinase to understand its integration into intracellular signalling networks. In this work, we developed an assay that holds the promise as being useful for the identification of phosphorylation substrates of a given protein kinase of interest. The method relies on irreversible inhibition of endogenous kinase activities with the ATP-analogue 5'-fluorosulfonylbenzoyladenosine (5'FSBA). 5`FSBA treated cell extracts are then combined with a purified activated kinase to allow phosphorylation of putative substrate proteins followed by a two-step purification protocol and identification by fingerprint mass spectrometry. Specifically, we have applied this method to identify new phosphorylation substrates of the *Drosophila* p21 activated kinase (PAK) protein Mbt. Among candidate proteins identified by mass spectrometry, the Dynactin complex subunit Dynamitin has been verified as a *bona fide* Mbt phosphorylation substrate and interaction partner suggesting an involvement of this PAK protein in the regulation of Dynactin dependent cellular processes.

1. Introduction

P21-activated kinases (PAKs) are a family of serine/threonine kinases that are regulated through binding of Cdc42 and/or Rac-like RhoGTPases. By this means, they can act as central nodes in a variety of RhoGTPase controlled cellular processes such as proliferation, migration, polarisation and reorganisation of the cytoskeleton (1, 2). Based on structural features, PAK proteins are classified into group I and II. Representative members of group I are human PAK1, 2 and 3 and *Drosophila* D-PAK and D-PAK3, group II members include human PAK4, 5 and 6 and *Drosophila* Mbt. Members of both subgroups share a N-terminal binding site for Cdc42 and/or Rac GTPases and a C-terminal kinase domain, but they differ in the regulation of their kinase activity and the presence of additional protein interaction sites, which allow for integration into different signalling pathways at distinct subcellular sites (3, 4). This can be exemplified for *Drosophila* D-PAK and Mbt, which are found in growth cones and at adherens junctions of developing photoreceptor cells, respectively (5-7).

Group II PAKs have recently received considerable interest because of their involvement in morphogenetic processes. Constitutive activation of PAK4 in cultured cells leads to decreased cell adhesion, cell rounding and induction of anchorage-independent growth. The PAK4 transcription unit localizes to a genomic region on chromosome 19 amplified in several tumours, and increased PAK4 activity can be detected in cell lines derived from many different

cancers (8, 9). Studies in *Xenopus laevis* have revealed a role of X-PAK5 as a regulator of blastomeric adhesive properties by modulating calcium dependent cell-cell adhesion (10). These findings have been further corroborated by studies of *Drosophila* Mbt, which is involved in morphogenesis of photoreceptor cells (7). In contrast to group I PAKs, only few phosphorylation targets of group II PAKs have been described so far (3, 11). Cytoskeletal changes regulated by PAK4 are mediated by LIM-kinase 1 (Limk1) and the phosphatase Slingshot (Ssh), which act in an antagonistic manner on the actin depolymerisation factor Cofilin (12, 13). Constitutive activation of *Drosophila* Mbt also induces changes of the actin cytoskeleton, yet the phenotypic observations made in transgenic flies implicate that additional proteins are subject to regulation by Mbt (14).

In this work, we set forth to identify novel phosphorylation substrates for Mbt. This has been achieved by the supplementation of cell lysates with an activated Mbt variant after irreversible inhibition of endogenous kinase activities. Candidate substrate proteins were identified by peptide fingerprint mass spectrometry and verified by *in vitro* kinase assays and co-immunoprecipitation studies. This method has identified the *Drosophila* orthologue of the Dynactin complex subunit Dynamitin as a novel substrate of Mbt. In summary, this work has established a generic method for the identification of *bona fide* substrates of known kinases and implies a function of group II PAKs in the regulation of Dynactin in cells.

2. **Materials and methods**

2.1. Cloning procedures

For expression of Myc-Mbt variants in S2R cells, the coding sequences of Mbt, Mbt^{T525A} and Mbt$^{S492N/S521E}$ (14) were cloned 3´ to a 6x myc-tag sequence and then inserted into the Asp718/Bsp120i sites of pMT/V5 (*Invitrogen*). The cDNAs for *Drosophila* CG1532 (LD36566), CG4802 (GM01885), Dynamitin (LD07994), Fimbrin (LD05347), Junctophilin (GH05993) and Thioredoxin-Reductase 1 (LD21729) were obtained from the *Drosophila Genomics Resource Center* (DGRC). The coding sequences of CG1532, CG4802 and Dynamitin were amplified by PCR and cloned as NcoI/NotI fragments into the GST-fusion vector pETM-30 (a gift from A. de Marco, EMBL, Heidelberg). Fimbrin, Junctophilin and Thioredoxin-Reductase 1 were ligated as EcoRI/XbaI, EcoRI/XhoI and BamHI/XbaI fragments into pGEX-KG. All constructs were verified by sequencing.

2.2. Expression and purification of recombinant proteins

Bacterial expression and purification of GST-fusion proteins were performed as described (14).

2.3. Cell culture and immunoprecipitation

Drosophila S2R cells (15) were cultured at 25°C in *Schneider Drosophila medium* (*Biowest*) supplemented with 10% foetal calf serum and 1% penicillin-streptomycin (*Gibco*). Cells were detached with a cell scraper in PBS buffer. Stable S2R cell lines expressing Myc-Mbt, Myc-

Mbt$^{S492N/S521E}$ and Myc-Mbt^{T525A} were generated by transfection of the constructs with calcium-phosphate and selection with 100 µg/ml hygromycin-B (*Calbiochem*). Myc-Mbt protein expression was induced by addition of 0.8 mM $CuSO_4$ for 36 h. Cells were homogenized in lysis buffer (25 mM Tris [pH7.5], 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 0,1% NP-40, 5 µg/ml Antipain, 1 µg/ml Aprotinin, 0,5 µg/ml Leupeptin, 0,7 µg/ml Pepstatin, 100 µg/ml PMSF). Total protein concentrations were determined by Bradford assay. For immunopreciptiations, lysates were mixed overnight at 4°C with 30 µl G-protein agarose (*Roche*) and 8 µl monoclonal anti-Myc antibody (9E10, *Santa Cruz*). Bound proteins were washed with lysis buffer prior to SDS-PAGE and then transferred to a nitrocellulose membrane. Membranes were probed with anti-Myc, anti-Mbt (7) or anti-Dynamitin (*BD Bioscience Pharmingen*) antibodies. Detection was done with HRP-conjugated secondary antibodies and ECL (*GE Healthcare*).

2.4. In vitro kinase assays

For *in vitro* kinase assays, immunoprecipitated Mbt proteins were washed in kinase buffer (20 mM HEPES [pH7.6], 150 mM NaCl, 20 mM $MgCl_2$) and incubated with cell lysate fractions in kinase buffer containing 5 µCi of [γ-^{32}P]ATP (3000 Ci/mmol) for 30 min at 30°C. To test for phosphorylation of GST-fusion proteins, 30 µg of the corresponding GST protein was added to the reaction mixture. Reactions were terminated by boiling in SDS-PAGE loading buffer, followed by SDS-PAGE and autoradiography.

2.5. Preparation of ATPγS and 5'-fluorosulfonylbenzoyladenosine (5'FSBA) treated S2R cell extracts

Confluent S2R cells from fourteen 10 cm diameter dishes were washed three times with PBS and harvested. The cleared lysate was dialysed against kinase buffer. To this extract ATPγS or 5'-fluorosulfonylbenzoyladenosine (5'FSBA, *Sigma*) were added to 30 mM and incubated for 45 min at 30°C. Excess ATPγS was removed by dialysis, 5'FSBA was inactivated by addition of 5 mM DTT.

2.6. Fractionation of cell extracts

5 ml of 5'FSBA-treated cell extract in kinase buffer was applied to a 1 ml HiTrap Q HP column (*Amersham*). After washing the column with 10 ml kinase buffer, proteins were eluted with a 3-step linear gradient against kinase buffer containing 1 M NaCl collecting 1 ml fractions (gradient: 0.15 – 0.35 M NaCl in 4 ml, 0.35 - 0.65 M NaCl in 19 ml, 0.65- 1 M NaCl in 4 ml). *PHOS-Select^{TM} Iron Affinity Gel* (*Sigma*) chromatography was performed according to the manufacturer's instructions with minor modifications. In brief, following anion exchange chromatography and the radioactive kinase assay, the fraction containing putative substrate proteins was adjusted to 250 mM acetic acid and 30% acetonitrile (final pH: 2.5) and incubated with 80 µl *PHOS-Select^{TM} Iron Affinity Gel* resin for 45 min at 4°C on a rotator. Unbound proteins were removed by washing the beads twice with 500 µl of the same solution and once with 500 µl water. Phosphorylated proteins were eluted by addition of 200 µl of 150 mM,

250 mM and 400 mM ammonium hydroxide, respectively, and precipitated with TCA. Protein pellets were resuspended in SDS-PAGE loading buffer and subjected to SDS-PAGE, Coomassie staining and autoradiography.

2.7. In-gel digestion and protein identification by MALDI-TOF mass spectrometry

Bands were excised from the gel, proteins were carbamidomethylated and digested in-gel using mass spectrometry-grade trypsin "Trypsin gold" (Promega, Madison, USA) in 25 mM NH_4HCO_3. Mass spectra of resulting tryptic peptides were recorded using an Ultraflex TOF/TOF (*Bruker Daltonics*, Bremen, Germany) in the positive-ion reflectron mode with dihydroxy benzoic acid, 20 mg/mL in 50% acetonitrile/0.85% o-phosphoric acid, as matrix. Peptide mass profiles were analyzed with Mascot (www.matrixscience.com) using the *National Center for Biotechnology Information* (NCBI) database.

3. **Results**

Our goal was to devise a strategy with high selectivity that allows the identification of new substrates of the PAK protein Mbt from total lysates of *Drosophila* cells. The general strategy was to irreversibly block all endogenous kinase activities and then to perform *in vitro* kinase assays in the presence of exogenously provided Mbt. First, we searched for a cell line with no detectable Mbt expression. We reasoned that although Mbt is absent form these cells, at least a subset of substrates would be expressed and be present in a hypo-phosphorylated form. On the other hand, such a cell line would allow ectopic expression of Mbt variants without possible interference by the endogenous protein. S2R cells (15) fulfil these criteria. As shown by Western Blot with an anti-Mbt antibody (7), S2R cells do not express detectable levels of endogenous Mbt (Fig. 1A). Stable cell lines were established, which allowed inducible expression of Myc epitope tagged wild-type Mbt (Myc-Mbt), constitutively activated Mbt (Myc-Mbt^{S492N,S521E}) and kinase-dead Mbt (Myc-Mbt^{T525A}), the latter one was used as a control in Figure 1A.

Next, we determined conditions to inhibit endogenous kinases present in the extract. Untreated cell extracts show high endogenous kinase activities as detected by *in vitro* kinase assays with [γ-^{32}P]ATP (Fig. 1B, lane 1). Pre-treatment of the extract with the slowly hydrolysable ATP-analogue ATPγS resulted in a strong reduction of endogenous kinase activities, however, the residual activities were still too high for a reproducible and unambiguous identification of Mbt substrate proteins (Fig. 1B, lane 2). Therefore we replaced ATPγS by the adenine nucleotide analogue 5'- fluorosulfonylbenzoyladenosine (5'FSBA), which targets a critical lysine residue in the ATP binding cleft and forms a sulfonamide resulting in irreversible inhibition of the enzyme's ATPase activity (16). In the presence of 30 mM 5`FSBA, endogenous kinase activities are completely blocked (Fig. 1B, lane 3). After inactivation of unbound 5'FSBA, *in vitro* kinase reactions were performed either with purified Myc-Mbt^{S492N,S521E} or Myc-Mbt^{T525A}. In the presence of Myc-Mbt^{S492N,S521E}, several phosphorylation signals became visible. (Fig. 1B, lane 5). The lack of any phosphorylation signal after supplementation with Myc-Mbt^{T525A} (Fig. 1B, lane 4) confirms the dependence on Mbt kinase activity and argues against the possibility that a co-purified kinase performs the phosphorylation reaction.

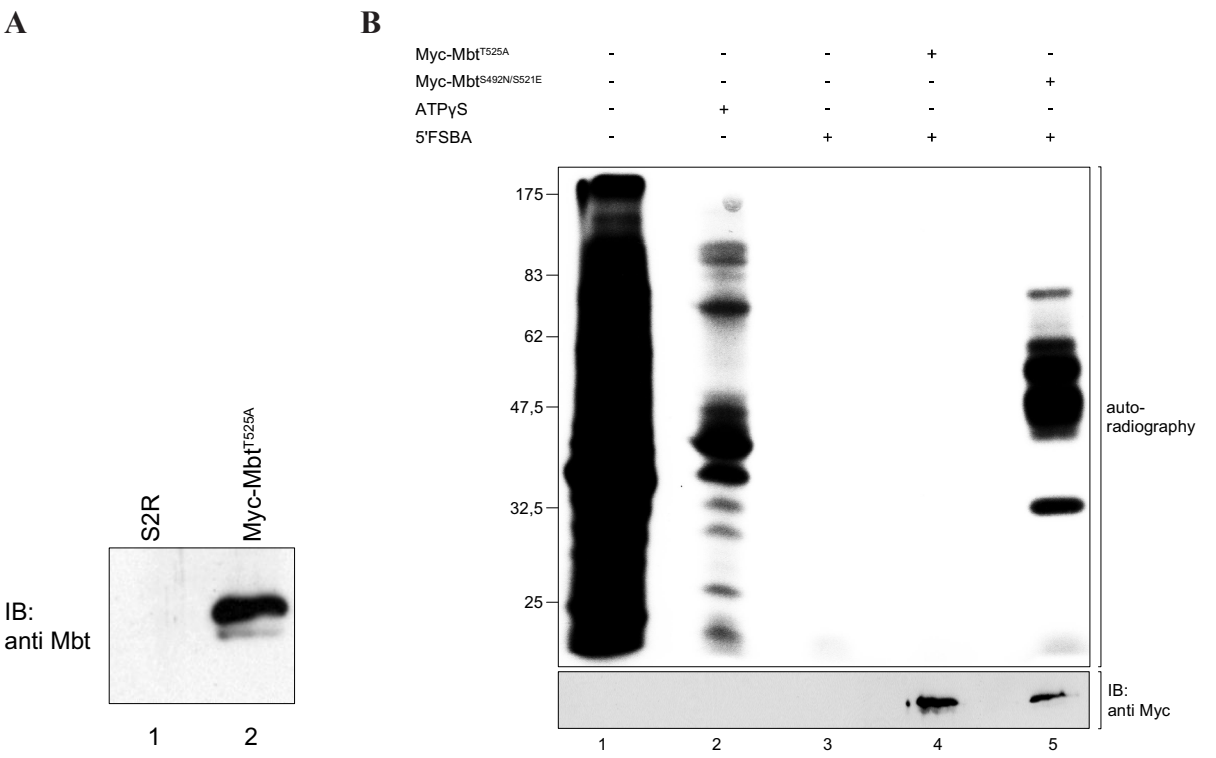

Fig. 1. (A) Total lysates of S2R (lane 1) and S2R cells expressing Myc-Mbt^{T525A} as control (lane 2) were analysed for the presence of Mbt with an anti-Mbt antibody (lanes 1 and 2). (B) *In vitro* kinase assays of S2R cell extracts without (lane 1) or after pretreatment with ATPγS (lane 2) or 5'FSBA (lane 3). Addition of purified activated Myc-Mbt^{S492N,S521E} (lane 5) but not of kinase-dead Myc-Mbt^{T525A} protein (lane 4) to 5'FSBA-treated cell extracts results in the appearance of several distinct phosphorylation signals. The presence of the respective Myc-Mbt proteins was verified by Western blot (IB) with an anti-Myc antibody. Molecular weight markers are indicated at the left side.

Next we tried to reduce the complexity of proteins in the sample by fractionation of the 5'FSBA treated extract on an anion exchange column. Proteins were eluted by applying a salt gradient and the collected fractions (Fig. 2A, upper panel) were subjected to *in vitro* kinase reactions in the presence of Myc-Mbt^{S492N,S521E} (Fig. 2A, lower panel). Note the enrichment of Mbt phosphorylated proteins from fraction 25 to 27. In a further fractioning step, we performed affinity chromatography with the *PHOS-Select^{TM}* resin. This method is based upon the ability of Fe(III) to chelate phosphate groups on amino acid residues under denaturating conditions at pH 2. Elution of proteins from the column is achieved by the addition of increasing concentration of $NH_4(OH)$. Fraction 25 from the anion exchange column (Fig. 2A) was first subjected to an *in vitro* kinase reaction with Myc-Mbt^{S492N,S521E} and then applied to the *PHOS-Select^{TM}* resin (Fig. 2B). Note the enrichment of several Mbt phosphorylated polypeptides in the first elution fraction (Fig. 2B, autoradiography panel).

Gel slices from the indicated positions (A-E, Fig. 2B) were excised and candidate proteins were identified by fingerprint mass spectrometry. Figure 2C shows the list of identified proteins. To verify phosphorylation by Mbt, all candidate proteins - with exception of BSA, which was considered as a contaminant- were overexpressed as GST-fusion proteins in *E.coli* and subjected to *in vitro* kinase assays with purified Myc-Mbt^{S492N,S521E} or Myc-Mbt^{T525A} (Fig. 3A). Only in

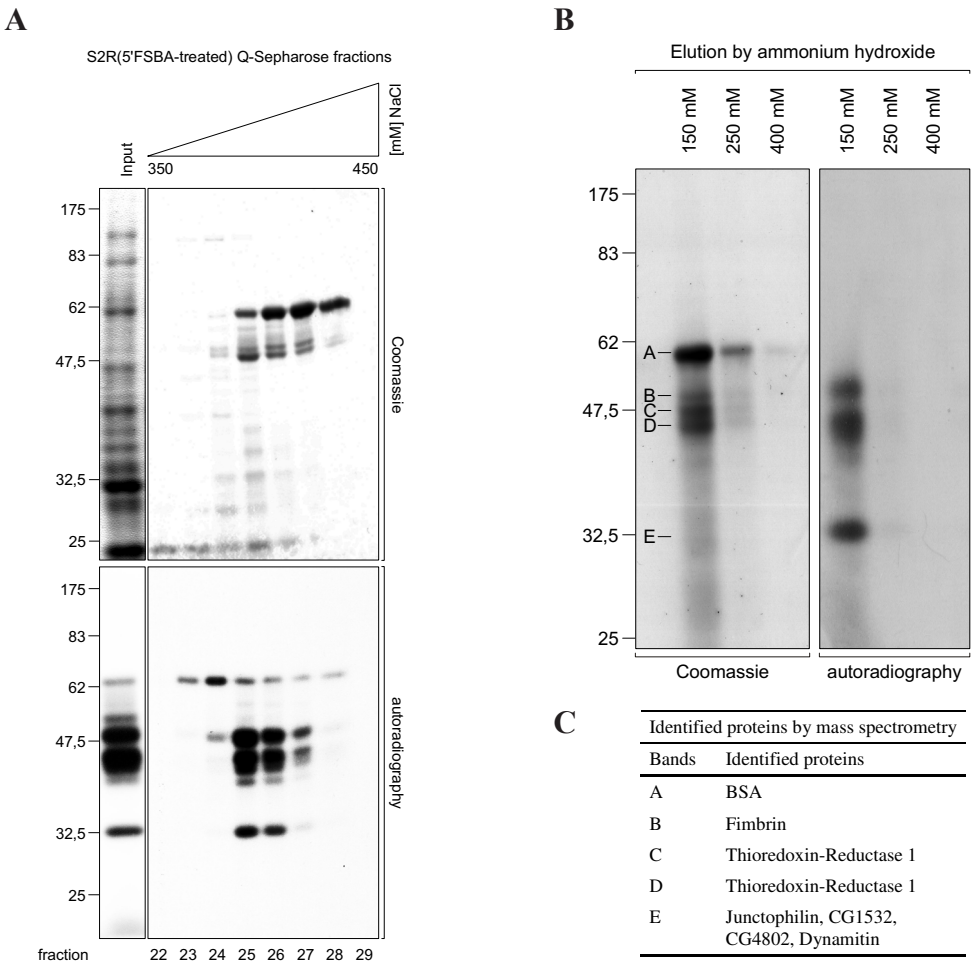

Fig. 2. (A) The 5'FSBA-treated S2R extract was loaded onto a HiTrap Q HP column and eluted in sixty 1 ml fractions by applying a sodium chloride gradient (see Material and Methods). 30 µl of each fraction were analysed for the presence of Mbt substrate proteins in radioactive kinase assays with purified Myc-Mbt^{S492N,S521E} (autoradiography) and in parallel for total protein content by Coomassie blue staining after SDS-PAGE. Only fractions 22-29 are shown. Molecular weight markers are indicated. (B) Enrichment of Mbt-phosphorylated proteins by *PHOS-Select^{TM} Iron Affinity Gel* chromatography. Fraction 25 (Fig. 2A) was supplemented with Myc-Mbt$^{S492N/S521E}$ and [γ-^{32}P]ATP to label Mbt substrate proteins and then passed over the *PHOS-Select^{TM}* matrix. After washing, phosphorylated proteins were eluted with increasing concentrations of ammonium hydroxide, separated by SDS-PAGE and analysed by autoradiography and Coomassie blue staining. Bands A-E were cut out for subsequent mass spectrometry analysis. (C) List of candidate proteins identified by mass spectrometry.

the case of GST-Dynamitin efficient phosphorylation by Myc-Mbt^{S492N,S521E} was observed. We further noticed a weak but non-reproducible phosphorylation of Thioredoxin-Reductase 1 in some experiments leaving open the possibility that this protein might be a substrate for Mbt. Kinase reactions in the presence of Myc-Mbt^{T525A} failed to yield any signal upon autoradiography, confirming dependence of the phosphorylation reaction on intrinsic Mbt kinase activity.

Finally, we investigated whether Mbt and endogenous Dynamitin are associated with each other in cells. This was assessed by co-immunoprecipitation experiments using the cell lines stably expressing Myc-Mbt, Myc-Mbt^{T525A} or Myc-Mbt^{S492N,S521E}. Endogenous Dynamitin was co-purified with Myc-Mbt and Myc-Mbt^{S492N,S521E} (Fig. 3B, lanes 4 and 6) but not with the catalytically inactive mutant Myc-Mbt^{T525A} (Fig. 3B, lane 5). In summary, these results

demonstrate that Dynamitin is a *bona fide* substrate for the group II PAK protein Mbt.

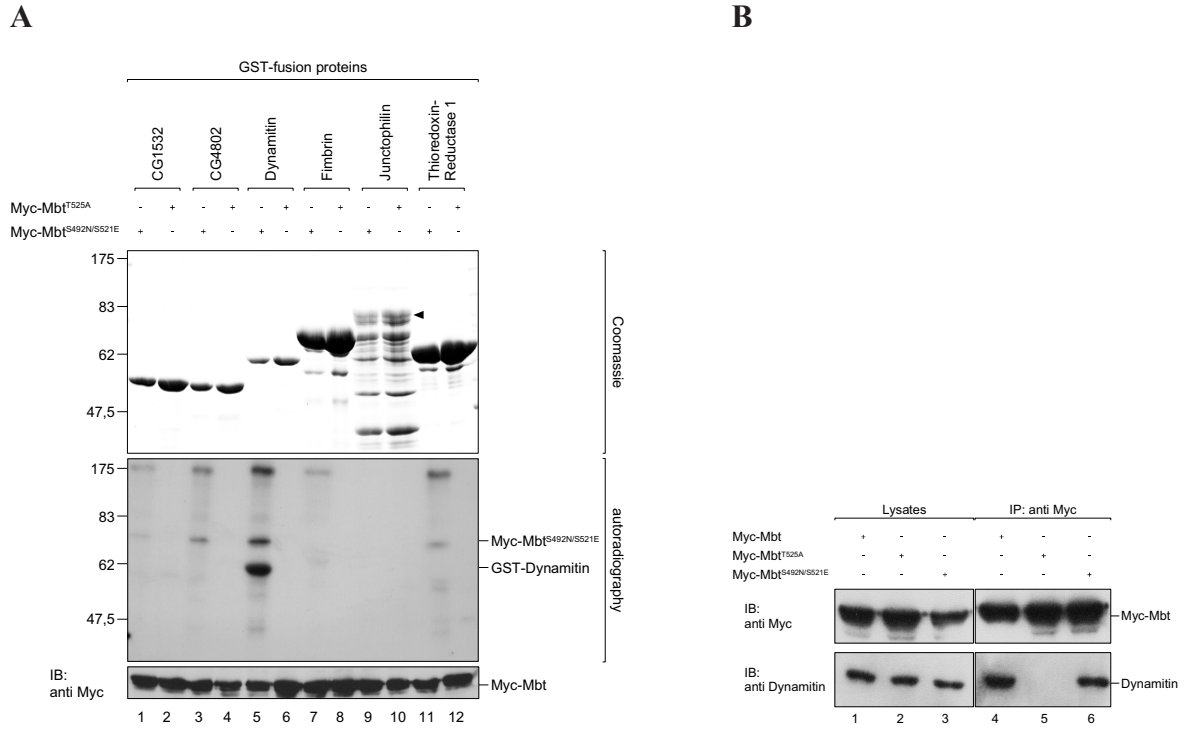

Fig. 3. Mbt phosphorylates and interacts with Dynamitin. (A) The indicated candidate proteins were expressed as GST fusion proteins and used in kinase assays together with Myc-Mbt^{S492N,S521E} and Myc-Mbt^{T525A}. Reactions were subjected to SDS-PAGE and analysed by autoradiography. The presence of the GST proteins was verified by Coomassie blue staining, Myc-Mbt proteins were detected by Western Blot analysis with the anti-Myc antibody (IB). GST-Junctophilin is subjected to degradation, the expected full length protein is indicated by an arrowhead. Note Myc-Mbt^{S492N,S521E} autophosphorylation. (B) Mbt interacts with endogenous Dynamitin. S2R cells stably expressing Myc-Mbt, Myc-Mbt^{T525A} and Myc-Mbt^{S492N,S521E} were induced for expression of Mbt proteins. Immunopreciptaions from cell lysates were done with the anti-Myc antibody (IP). Immunoprecipitates were analysed by SDS-PAGE and Western Blot with the anti-Myc and anti-Dynamitin antibodies (IB). The corresponding lysate controls are shown in lanes 1-3.

4. Discussion

In this study we have developed a highly stringent *in vitro* kinase assay to identify physiological phosphorylation substrates of a kinase of interest in cell extracts, exemplified by the identification of Dynamitin as novel substrate protein of the *Drosophila* kinase Mbt. In general, incubation of native cell extracts with [γ-^{32}P]ATP and a kinase of interest are of limited value to identify substrate proteins, because the presence of multiple endogenous kinase activities results in high "background" phosphorylation thereby masking kinase-specific phospho-signals. KESTREL, a method that has successfully applied to several protein kinases, tries to reduce the background problem by a combination of short incubation times, high concentrations of the added kinase, depletion of the endogenous ATP pool, the use of Mn^{2+} instead of Mg^{2+} as a co-factor and an chromatography step to separate endogenous kinases from substrate proteins (17, 18). Another approach uses synthetic ATP derivates, which can only used by mutated forms of protein

kinases (19). We improved the signal-to-noise ratio by pre-incubation of the cell lysate with the nucleotide analogue 5'FSBA, which was formerly used in enzyme kinetic studies and for affinity labelling (16). Because most ATP-hydrolyzing enzymes contain an evolutionary conserved motif for nucleotide binding and 5'FSBA reacts covalently we expected a broad spectrum of inactivated ATP-, ADP-, NAD- and NADH-dependent proteins (16). Indeed, *in vitro* kinase assays with 5'FSBA treated cell extracts showed complete blockage of endogenous kinase activities. The ATP-analogue ATPγS turned out to be less effective, most probably because it slowly hydrolyses and then dissociates from the kinase. The assay requires the availability of a purified, activated kinase, which in our case was isolated from a stably transfected cell line. One potential problem might be the co-purification of other kinases. Control experiments with a kinase-dead variant of the kinase of interest or the comparative analysis of several closely related kinases should help to distinguish between background signals and the physiological relevant phosphorylation signals. For the identification of the proteins by peptide fingerprint mass spectrometry, it was necessary in our hands to reduce the complexity of the protein sample first by anion-exchange chromatography and second by a phosphoprotein-selective affinity matrix. For general use of this strategy, we recommend first the inhibition of endogenous kinase activities by 5'FSBA, followed by fractionation with an ion-exchange column and third affinity purification using a phospho-protein selective resin. While we have been successful with anion-exchange chromatography, other applications might warrant the replacement of this step by cation-exchange chromatography.

In the case of Mbt, analysis by mass spectrometry identified six candidate proteins from which only Dynamitin could be verified as a true phosphorylation and interaction partner. Interestingly, only the wild-type and the constitutively active version of Mbt were found to be associated with Dynamitin suggesting that this interaction is dependent on Mbt's kinase activity. Our results are also in line with a recent protein network analysis of the human Pak1-βPix-GIT-Paxillin complex, which among many other proteins identified two components of the Dynactin complex: Dynamitin and $p150^{Glued}$ (20).

The multi-subunit complex Dynactin is composed of two structural units: a rod-like unit consisting of an Arp1 polymer and actin capping proteins that provides the interface for cargo binding and a projecting unit formed by a dimer of $p150^{Glued}$ that mediates binding to dynein motor proteins and microtubules. Both units are connected by Dynamitin (p50). The main function of the Dynactin complex is to allow the attachment of Dynein motor proteins to their cargos but it also contributes to the binding of Dynein to centrosomes, the nucleus and the cell cortex (21). Dynactin acts also independent of Dynein to anchor microtubules at centrosomes and $p150^{Glued}$ is required to organize radial microtubule arrays (22, 23). Components of the Dynactin complex have also been implicated in regulation of adhesive structures of cells. Dynamitin interacts with MacMARCKS (Macrophage-enriched myristoylated alanine-rich C kinase substrate) and both microtubule and MacMARCKS are part of the cytoskeletal constraint on β2 Integrin molecules that regulate Integrin molecular mobility and activation (24). Interference with the function of *Drosophila* $p150^{Glued}$ induces defects in the organisation of adherens junctions during morphogenesis of photoreceptor cells and mislocalisation of Armadillo (β-catenin) as a central component of the Cadherin-Catenin complex (25). How $p150^{Glued}$ exerts its effect on adherens

junctions is not known, however, there are some striking similarities to the adherens junction defects observed in *mbt* mutant photoreceptor cells (7). It is therefore tempting to speculate that Mbt and Dynactin act at least in part synergistically to control adherens junction organisation during photoreceptor cell morphogenesis. The phosphorylation of Dynamitin by Mbt might control assembly, localisation or function of the Dynactin complex at adherens junctions. Further studies will focus on the identification of the phosphorylation sites to investigate their physiological role in developmental processes.

Acknowledgements

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4 Zusammenfassende Darstellung der Ergebnisse und Diskussion

In einer vorrausgegangenen genetischen Studie zur zellulären Funktion der Proteinkinase Mushroom bodies tiny (Mbt) in *Drosophila melanogaster* konnte Mbt als Regulator während der Differenzierung von Photorezeptorzellen identifiziert werden (Schneeberger and Raabe, 2003). Morphogenetische Umgestaltungsprozesse sind durch Zellbewegungen gekennzeichnet, die statischen und dynamischen Wechselwirkungen unterliegen. Daher wurde in dieser Arbeit der Einfluß von Mbt auf die Reorganisation des Aktincytoskeletts, Zell-Zelladhäsion und der Integration von Mbt in weitere Signalwege untersucht.

Im ersten Projekt wurde zunächst ein genetischer Interaktionsscreen durch Kreuzungen eines Stammes mit einem hypomorphen *mbt*-Allel (mbt^{P3}) und *Drosophila*-Stämmen mit Einzelgenmutationen durchgeführt, wobei Mutationen in den Aktin-regulatorischen Proteinen Cofilin (*Drosophila*: Twinstar) und Slingshot als Modifikatoren des mbt^{P3}-Phänotyps gefunden wurden. Cofilin fungiert als Aktin-Depolymerisationsfaktor und steht am Ende einer Kette von Signalübertragungswegen, die von Pak4 (*Drosophila*: Mbt) über die Lim-Kinase (Limk) bis zum Cofilin geleitet werden und schließlich dessen Aktivität durch Limk vermittelte Phosphorylierung hemmen. Die Phosphatase Slingshot stellt den Antagonisten der Limk und den Aktivator von Cofilin dar. In genetischen und biochemischen Ansätzen konnte gezeigt werden, daß aktiviertes Mbt mit Twinstar und *Drosophila* Limk (D-Limk) assoziiert ist und die Phosphorylierungen beider Moleküle induzieren kann. Genetische Ergebnisse stellen die Bedeutung der D-Limk als zentralen Mediator zwischen Mbt und Twinstar in Frage und stehen damit in Widerspruch zur Situtation von Pak4 in Vertebraten. Eine Fragestellung bleibt, ob in Photorezeptorzellen das Mbt-Signal direkt an Twinstar, über Slingshot oder unbekannte Kinasen geleitet wird (s. Fig. 4-1). Neben der Funktion von Mbt als Regulator des Aktincytoskeletts lieferten immunohistologische Färbungen gegen *Drosophila* DE-Cadherin und Armadillo (Vertebraten: β-Catenin) Hinweise auf die Mbt-abhängige Modulation des DE-Cadherin-β-Catenin/Armadillo-α-Catenin Komplexes von Zell-Zellkontakten (Menzel et al., 2007).

Das zweite Projekt knüpfte an diese Beobachtungen an, jedoch wurden die Analysen nicht in Fliegen sondern mit *Drosophila* Schneiderzellen durchgeführt, da es aufgrund der pleiotropen Funktionen während der Entwicklung schwierig ist, mit DE-Cadherin und Armadillo in Fliegen zu arbeiten. Das dazu entwickelte Zellkultursystem ermöglichte die Bildung vollständiger DE-Cadherin-β-Catenin/Armadillo-α-Catenin Komplexe *in vivo* und schaffte die Vorraussetzungen für Untersuchungen in Zellen und *in vitro* mit Kinase-aktiven und -inaktiven Varianten von Mbt. Es stellte sich heraus, daß Mbt mit dem DE-Cadherin-β-Catenin/Armadillo-α-Catenin Komplex interagiert und Armadillo/β-Catenin an den Aminosäuren S561 und S688 phosphoryliert. Infolgedessen wird die Bindung zwischen DE-Cadherin und Armadillo/β-Catenin destabilisiert, deren Wirkung sich in einer verminderten Adhäsion der Zellkontakte zwischen Zellen äußert. Unter Anwendung der Laserpinzetten-Technik zur relativen Kraftmessung konnte dieser Effekt bestätigt werden (Kapitel 3.2, Manuskript eingereicht).

Ein drittes Projekt befaßte sich mit der Suche nach bisher unbekannten Phosphorylierungssubstraten von Mbt, um weitere von Mbt regulierte Signalkaskaden beschreiben zu können. Zu diesem Zweck wurde eine stringente, radioaktive *in vitro* Phosphorylierungsreaktion

entwickelt, dessen Prinzip auf der Mbt-spezifischen Phosphorylierung von potentiellen Proteinpartnern in einem nativen Extrakt aus *Drosophila* Schneiderzellen beruhte. Vor der radioaktiven Kinasereaktion mit aufgereinigtem Mbt wurde das hergestellte Lysat mit dem ATP-Analogon 5'-Fluorosulfonylbenzoyladenosin (5'FSBA) vorbehandelt, um sämtliche endogenen Kinasen irreversibel zu inhibieren. In zwei Stufen wurden dann die modifizierten Proteine aufgereinigt, wobei die erste aus einer allgemeinen Fraktionierung mit Hilfe einer Anionenaustauschersäule bestand während die zweite Stufe Mbt-phosphorylierte Proteine chromatographisch über eine Phosphopeptid-spezifische Matrix isolierte. Die eluierten radioaktiven Proteine wurden nach Auftrennung im SDS-Gel durch Massenspektrometrie identifiziert. Von den 6 gefundenen Kandidaten konnte in den Kinaseansätzen mit GST-Fusionsproteinen und Immunopräzipitationsexperimenten das *Drosophila* Dynamitin als Interaktionspartner und Phosphorylierungssubstrat von Mbt verifiziert werden (Kapitel 3.3, Manuskript eingereicht).

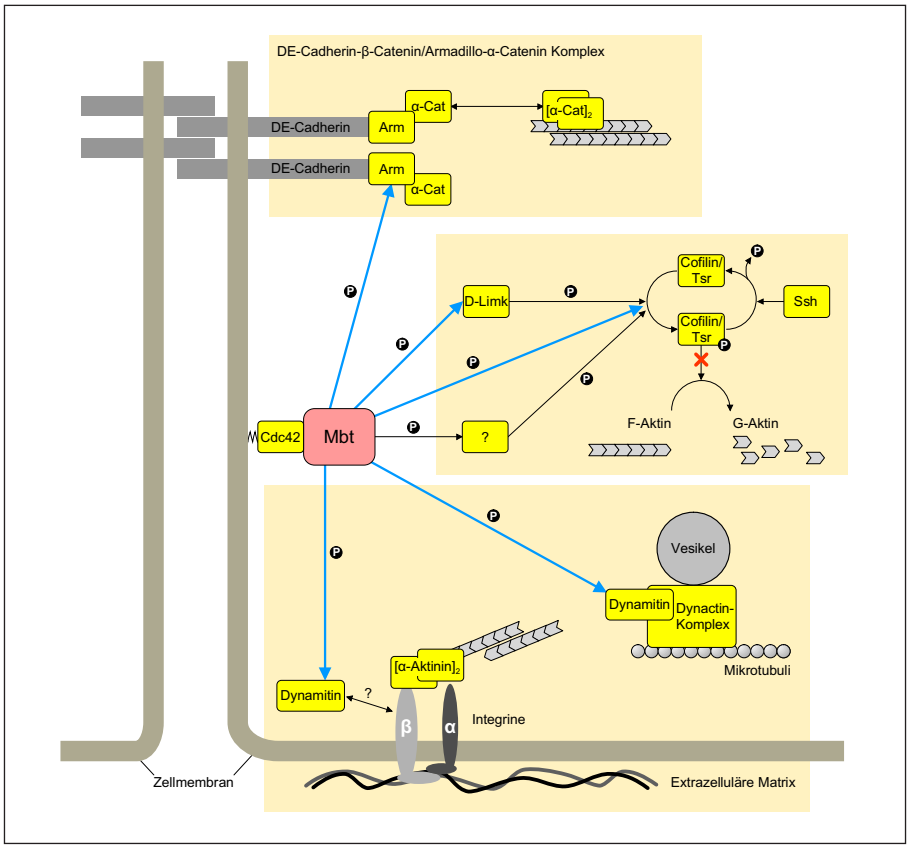

Fig. 4-1. Übersicht der in dieser Arbeit gefundenen Signalwege, an denen Mbt beteiligt ist.

Das Komplexauge von *Drosophila melanogaster* ist ein etabliertes Modellsystem für morphogenetische Prozesse von Zellen. Ein charakteristisches Merkmal sich entwickelnder Photorezeptorzellen sind dynamische Umgestaltungsprozesse, deren molekulare Ursachen in der regulierten Adhäsion von Zellen und der Reorganisation des Aktincytoskeletts begründet sind (Tepass and Harris, 2007). Viele der molekularen Wechselwirkungen sind im Hinblick auf Photorezeptorzellen nicht untersucht, daher beziehen sich im folgenden die zitierten

Arbeiten auf Untersuchungen in Vertebraten. Die Bildung von Cadherin-Catenin vermittelten Zell-Zellkontakten wird am führenden Ende der Zelle durch Rac- und Arp2/3-abhängige Aktinnukleation initiiert (Gavard et al., 2004; Yap and Kovacs, 2003). Der Aufbau eines kurzen, dicht verzweigten Aktinnetzwerks wird außerdem von den Faktoren Cortactin, Fer und p120-Catenin gefördert (El Sayegh et al., 2005; El Sayegh et al., 2004; Gavard et al., 2004; Helwani et al., 2004; Xu et al., 2004; Yap and Kovacs, 2003).

Im weiteren Verlauf muß die Zelle einen Wechsel von kurzen, gewinkelten Filamenten zu langen, parallelen Aktinbündeln betreiben, um kontraktile Elemente aufbauen zu können, die später in die Struktur von Lamellipodien eingehen werden. Als Teil dieser Umstellung inhibieren Ena/VASP-Proteine sowie homodimere α-Catenine die Aktivität des Arp2/3-Komplexes (Drees et al., 2005; Scott et al., 2006; Yamada et al., 2005), während gleichzeitig Aktinfasern durch die Interaktion von Vinculin und α-Catenin stabilisiert werden (Janssen et al., 2006).

Aufgrund der phänotypischen Analyse konnte Mbt im Zellcortex eine Rolle als regulatorische Komponente des Aktincytoskeletts zugeordnet werden. Nach der Bindung von Mbt an die RhoGTPase Cdc42 lokalisiert es an die Membran zum DE-Cadherin-β-Catenin/Armadillo Komplex (Menzel et al., 2007; Schneeberger and Raabe, 2003). Die Membranständigkeit von Mbt nach Cdc42-Rekrutierung ist zudem für dessen Funktion essentiell (Schneeberger and Raabe, 2003). Als aktivierte Kinase inhibiert Mbt durch Phosphorylierung den Aktin-Depolymerisationsfaktor Cofilin/Twinstar und verursacht eine Akkumulation des filamentösen Aktins (Menzel et al., 2007). Auf diese Weise könnte Mbt Vorraussetzungen schaffen, die zur Ausbildung kontraktiler Aktinstrukturen für die Motilität der Zellen notwendig sind. Die Motilität von Photorezeptorzellen ist ferner von einer ommatidialen Rotation geprägt und fördert im Zuge dieses Vorgangs die Etablierung der planaren Zellpolarität. Obwohl die mechanistischen Aspekte noch weitgehend unbekannt sind, lieferten Untersuchungen in Fliegen Hinweise darauf, daß die koordinierte, differentielle Expression von DN- und DE-Cadherinen teilweise für die Prozesse verantwortlich ist (Mirkovic and Mlodzik, 2006). In diesem Kontext könnte Mbt auch als Modulator von Adhärenzverbindungen eine wichtige Rolle spielen, allerdings stehen entsprechende Untersuchungen in Photorezeptorzellen noch aus. Aus den durchgeführten Experimenten mit *Drosophila* Schneiderzellen folgt ein Modell, nach dem Mbt spezifisch β-Catenin/Armadillo im DE-Cadherin-β-Catenin/Armadillo-α-Catenin Komplex an den Aminosäuren S561 und S688 phosphoryliert und dadurch die Verbindung von β-Catenin/Armadillo zu DE-Cadherin destabilisiert (Kapitel 3.2, Manuskript eingereicht). Als Folge sinkt die Adhäsion gegenüber DE-Cadherinen *in trans*. Ein solcher Effekt konnte in Laserpinzettenexperimenten verifiziert werden und implizierte eine Reduktion und nicht die vollständige Eliminierung der DE-Cadherin-Catenin vermittelten Zelladhäsion (Kapitel 3.2, Manuskript eingereicht). Zusammengefasst nimmt die Proteinkinase Mbt bei der Entwicklung eine zentrale Stellung innerhalb der Gewebeorganisation ein: Sie fördert die interzelluläre Motilität, indem sie synergetisch das Aktincytoskelett reorganisiert und als Regulator von Cadherin-abhängigen Zell-Zellkontakten fungiert (s. Fig. 4-1).

Eine offene Frage bleibt, nach welchem mechanistischen Prinzip die Cadherin-Catenin Funktion durch Mbt moduliert wird. Einem Modell nach führt die *cis*-Dimerisierung der Cadherine nach Phosphorylierung von β-Catenin/Armadillo zu einer verminderten adhäsiven

Konformation (Gumbiner, 2005), während eine andere Hypothese die Endocytose von DE-Cadherin an der Zellmembran favorisiert wie sie für die Proteinkinase Src und E-Cadherin in MDCK-Zellen beschrieben wurde (Fujita et al., 2002). Eine weitere offene Frage betrifft die Auswirkungen für das phosphorylierte Armadillo. Es müßte zunächst geklärt werden, ob Armadillo nach Phosphorylierung durch Mbt den DE-Cadherin-Catenin Komplex verläßt und nach Übergang ins Cytoplasma als Signalmolekül zum cotranskriptionellen Faktor des Wnt/Wg-Signalwegs wird oder proteasomal degradiert wird (Siegfried et al., 1994; Stadeli et al., 2006; Thompson, 2004; van de Wetering et al., 1997; Wodarz et al., 2006; Yanagawa et al., 2002). Einen wichtigen Hinweis dazu würde die ausstehende Identifizierung der Phosphorylierungsstellen im N-Terminus von Armadillo geben. In der Literatur sind die Auswirkungen der beiden Aminosäuren des humanen β-Catenin beschrieben worden, die den in dieser Arbeit ermittelten Aminosäuren S561 und S688 im C-Terminus von Armadillo entsprechen. Danach führen die Phosphorylierungen zum Schutz vor Ubiquitin-bedingtem Abbau (Hino et al., 2005) und nach Bindung an den LEF (*lymphoid enhancer factor*)-Transkriptionsfaktor zur verstärkten cotranskriptionellen Aktivität des β-Catenin (Taurin et al., 2006).

Die dargestellten Funktionen machen Mbt als kritische Komponente bei der Morphogenese von Photorezeptorzellen verantwortlich. Die Identifizierung von Dynamitin als Interaktionspartner und Phosphorylierungssubstrat erweitert die Kenntnis von Mbt-integrierten Signalwegen und läßt auf eine weiterführende Rolle von Mbt in entwicklungsrelevanten Prozessen schließen (Kapitel 3.3, Manuskript eingereicht). Dynamitin als Teil des Multiprotein-Komplexes Dynactin ist zusammen mit Dynein-Motorproteinen am Vesikeltransport entlang der Mikrotubuli beteiligt (Schroer, 2004). Mbt könnte durch Phosphorylierung von Dynamitin den Auf- und Abbau oder die Lokalisierung dieser Proteinkomplexe steuern. Desweiteren interagiert Dynamitin mit MacMARCKS (*Macrophage-enriched myristoylated alanine-rice C kinase substrate*) und reguliert so die laterale Mobilität von β_2-Integrinen, die als Bestandteil von Fokalkontakten Zellen mit der extrazellulären Matrix (ECM) verbinden (Jin and Li, 2002; Jin et al., 2001). In diesem Zusammenhang könnte Mbt über Dynamitin als Knotenpunkt zwischen Cadherin- und Integrin vermittelten Signalwegen auftreten.

Bisher wurden solche Signale in jeweils nur eine Richtung beschrieben. So führt das Entfernen von Fibronektin in Speicheldrüsenzellen, ausgehend von Integrinen, zur fehlerhaften Lokalisierung von Cadherinen an Zell-Zellkontakten (Sakai et al., 2003). In Follikelzellen von *Drosophila* supprimiert Talin, ein Verbindungsmolekül von Integrinen mit dem Aktincytoskelett, die Transkription von DE-Cadherin (Becam et al., 2005). In pankreatischen Krebszellen besteht ein Einfluß von Integrinen auf Cadherine durch die Proteinkinasen Src und Fak. Sobald die Zellen an Collagen Typ I kontaktieren, wird Src aktiviert, gefolgt von Phosphorylierung und Rekrutierung von Fak an E-Cadherin Komplexe. Fak löst dann durch Phosphorylierung die Cadherin-Catenin Verbindungen auf (Koenig et al., 2006).

Im umgekehrten Fall existiert auch die Regulation von Integrinen durch Cadherine. VE-Cadherin vermittelt über das Transduktionsmolekül PECAM1 die Aktivierung der Rezeptorkinase VEGFR2, die wiederum PI3-Kinase und Integrin αvβ3 beeinflußt (Tzima et al., 2005). Mbt könnte sowohl auf Cadherin- als auch auf Integrin-abhängige Signalwege wirken und bidirektionell die Kontakte beider Klassen von Adhäsionsmolekülen beeinflussen, um die

entwicklungsrelevante Zellmotilität während der Morphogenese zu fördern.

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6 Zusammenfassung

In *Drosophila melanogaster* wurde der p21-aktivierten Proteinkinase Mushroom bodies tiny (Mbt) eine wichtige Rolle als Regulator während der Differenzierung von Photorezeptorzellen zugeschrieben. Da morphologische Umgestaltungsprozesse der Photorezeptorzellen von dynamischen Zellbewegungen begleitet und die molekularen Details größtenteils noch ungeklärt sind, wurden in dieser Arbeit die Funktionen von Mbt in Bezug auf veränderte Zelladhäsionseigenschaften, Reorganisation des Aktincytoskeletts und die Beteiligung an weiteren Signalwegen analysiert.

Im ersten Projekt wurde ein genetischer Interaktionsscreen mit Hilfe eines hypomorphen *mbt*-Allels (mbt^{P3}) durchgeführt, um zu untersuchen, in welche zellulären Signalwege Mbt einzuordnen ist. Die Identifizierung des Aktin-Depolymerisationsfaktor Cofilin (*Drosophila*: Twinstar) und der Phosphatase Slingshot bestätigte, daß Mbt in Prozesse involviert ist, die die Aktindynamik kontrollieren. In Vertebraten phosphoryliert und inaktiviert die Proteinkinase Pak4 (*Drosophila* Homolog zu Mbt) die Lim-Kinase (Limk), die wiederum Cofilin durch Phosphorylierung hemmt. Dieser Effekt kann nach Dephosphorylierung des Cofilin durch die Phosphatase Slingshot wieder aufgehoben werden. In *Drosophila* konnte gezeigt werden, daß aktiviertes Mbt mit Twinstar und *Drosophila* Limk (D-Limk) assoziiert ist und die Phosphorylierungen beider Moleküle induzieren kann. Zusammen mit genetischen Experimenten stellen die Ergebnisse entgegen der Situation in Vertebraten die Funktion von D-Limk als Vermittler zwischen Mbt und Twinstar in Frage und lassen vielmehr auf einen Verlauf des Signals von Mbt direkt an Twinstar, über Slingshot oder unbekannte Kinasen schließen (Menzel et al., 2007).

Ein zweites Projekt beschäftigte sich mit dem Einfluß von Mbt auf die DE-Cadherin-β-Catenin/Armadillo vermittelte Zelladhäsion. Dazu wurde ein Zellkultursystem in *Drosophila* Schneiderzellen etabliert, welches es erlaubte, den DE-Cadherin-β-Catenin/Armadillo-α-Catenin Komplex vollständig zu rekonstituieren. Die Resultate zeigten, daß Mbt mit dem Komplex interagiert und β-Catenin/Armadillo an den Aminosäuren S561 und S688 phosphoryliert. Die Phosphorylierung bewirkt eine Destabilisierung der Bindung zwischen DE-Cadherin und β-Catenin/Armadillo und vermindert die Adhäsion der Zellkontakte zwischen Zellen (Kapitel 3.2, Manuskript eingereicht).

Im dritten Projekt ging es um die Suche nach unbekannten Phosphorylierungspartnern und der Integration von Mbt in weitere Signalwege. Dazu wurde eine stringente, radioaktive *in vitro* Phosphorylierungsreaktion entwickelt, die die Detektion von Mbt-spezifischen Phosphorylierungssubstraten aus einem Extrakt von *Drosophila* Schneiderzellen ermöglichte. In einer Vorstufe wurde dieses Extrakt mit dem ATP-Analogon 5'-Fluorosulfonylbenzoyladenosin (5'FSBA) vorbehandelt, um sämtliche endogenen Kinasen irreversibel zu inhibieren und die nachfolgende Phosphorylierungsreaktion mit aufgereinigtem Mbt spezifisch für Mbt zu machen. Nach Auftrennung und Identifizierung der potentiellen Phosphoproteine durch Massenspektrometrie wurde das *Drosophila* Dynamitin als neuer Interaktions- und Phosphorylierungspartner von Mbt gefunden (Kapitel 3.3, Manuskript eingereicht).

7 Summary

In *Drosophila melanogaster*, the p21 activated kinase Mushroom bodies tiny (Mbt) fulfils a critical role in the differentiation of photoreceptor cells during development. Differentiation of photoreceptor cells is accompanied by morphogenetic rearrangement, however, the underlying molecular mechanisms are not completely understood. Therefore in this work the function of Mbt was analysed with respect to the modulation of cell-cell adhesion, reorganization of the actin cytoskeleton and the integration of Mbt in different signalling pathways.

In the first project a genetic screen with a hypomorphic *mbt* allele (mbt^{P3}) was performed to identify components of Mbt signalling pathways. The identification of the actin depolymerization factor Cofilin (*Drosophila*: Twinstar) and the phosphatase Slingshot confirmed that Mbt is involved in actin regulating processes. In vertebrates, the p21 activated kinase Pak4 (*Drosophila* homolog of Mbt) acts as an upstream activator of the Lim-Kinase (Limk) which in turn inactivates the actin depolymerization factor Cofilin by phosphorylation. This effect can be reversed by the phosphatase Slingshot. In *Drosophila* activated Mbt is not only associated with Twinstar and *Drosophila* Limk (D-Limk) but also induces phosphorylation of both molecules. Together with genetic experiments these results are in contrast to the situation of Pak4 in vertebrates and question the role of D-Limk as a major mediator in signalling between Mbt and Twinstar. Instead we propose that Mbt can either directly act on Twinstar or regulates Twinstar phosphorylation through Slingshot or other unknown kinases (Menzel et al., 2007).

In the second project, the influence of Mbt on DE-cadherin mediated cell adhesion was investigated. Therefore a *Drosophila* Schneider cell line was established which allowed the reconstitution of a functional DE-cadherin-β-catenin/Armadillo-α-catenin complex. The results revealed an interaction between Mbt and this complex and further showed that Mbt specifically phosphorylates β-catenin/Armadillo on amino acids S561 and S688. As a consequence the binding between β-catenin/Armadillo and DE-cadherin is destabilized leading to a reduced adhesion between cells (Chapter 3.2, manuscript submitted).

The third project was to devise a strategy that allows with high selectivity the identification of phosphorylation substrates of Mbt from total lysates of *Drosophila* Schneider cells. The general idea was to irreversibly block endogenous kinase activities by the ATP-analog 5'-fluorosulfonylbenzoyladenosine and then to perform a radioactive *in vitro* kinase assay in the presence of exogenously provided Mbt. The potential phosphoproteins were purified and identified by mass spectrometry. The *Drosophila* Dynamitin was found as a novel interaction and phosphorylation partner of Mbt (Chapter 3.3, manuscript submitted).

Wissenschaftliche Konferenzen

Oktober 2003: *Drosophila* Research Conference, Göttingen, Deutschland

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Veröffentlichungen

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The *Drosophila* p21 activated kinase Mbt modulates DE-cadherin mediated cell adhesion by phosphorylation of Armadillo

Nicolas Menzel, Jens Waschke, Detlev Drenckhahn, and Thomas Raabe

Manuskript eingereicht

A 5'-fluorosulfonylbenzoyladenosine based method to identify physiological substrates of a *Drosophila* p21 activated kinase

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