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► **To cite this version:**

Nicolas Menzel, Juliane Melzer, Jens Waschke, Christof E Lenz, Heike Wecklein, et al.. The *Drosophila* p21 activated kinase Mbt modulates DE-cadherin mediated cell adhesion by phosphorylation of Armadillo. *Biochemical Journal*, 2008, 416 (2), pp.231-241. 10.1042/BJ20080465 . hal-00478988

HAL Id: hal-00478988

<https://hal.science/hal-00478988>

Submitted on 30 Apr 2010

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

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short title: Control of the cadherin-catenin complex by the PAK protein Mbt

key words: *Drosophila*, PAK protein, kinase activity, cadherin-catenin complex

total word count (including title page, figure legends and references): 8483

Abbreviations used:

Arm, armadillo; CCC, cadherin-catenin complex; CK, casein kinase; Cy3, indocarbocyanine; Cy5, indodicarbocyanine; DE-cad(herin), *Drosophila* E-cadherin; Dm, *Drosophila melanogaster*; DMEM, Dulbecco's modified Eagle's medium; DSHB, Developmental Studies Hybridoma Bank; Fc, fragment crystalline; FWHH, full width at half height; GSK-3 β , glycogen synthase kinase-3 β ; GST, glutathione S-transferase; HA, hemagglutinin; HBSS, Hanks buffered salt solution; HEK, human embryonic kidney; Hs, *Homo sapiens*; LC, liquid chromatography; Mbt, mushroom bodies tiny; NP-40, Nonidet P40; PAK, p21-activated kinase; PKA, protein kinase A; S.E.M., standard error of the mean; Xl, *Xenopus laevis*; Zw3, zeste-white 3.

Synopsis

Phosphorylations by tyrosine and serine/threonine kinases regulate the interactions between components of the cadherin/catenin cell adhesion complex and thus can influence the dynamic modulation of cell adhesion under normal and disease conditions. Previous mutational analysis and localization experiments suggested an involvement of single members of the family of p21-activated kinases (PAKs) in the regulation of cadherin mediated cell adhesion, but the molecular mechanism remained elusive. We addressed this question using the *Drosophila* PAK protein Mbt, which is most similar to vertebrate PAK4. Previous phenotypic analysis showed that Mbt has a function to maintain adherens junctions during eye development and indicated a requirement of the protein in regulation of the actin cytoskeleton and the cadherin/catenin complex. Here we show that activation of Mbt leads to destabilization of the interaction of the *Drosophila* β -catenin homologue Armadillo with DE-cadherin resulting in a decrease in DE-cadherin mediated adhesion. Two conserved phosphorylation sites in Armadillo were identified that mediate this effect. Our findings support the previous observation that activation of the human Mbt homologue PAK4 leads to anchorage independent growth and provide a functional link between a PAK protein and the cadherin-catenin complex.

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 [1-3]. The cytoplasmic tail of cadherins is anchored to the underlying actin cytoskeleton via members of the catenin family. β -Catenin and its *Drosophila melanogaster* homologue Armadillo (Arm), 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. Recent data show that α -catenin acts as a molecular switch: α -catenin in its monomeric form is a structural component of the cadherin-catenin complex (CCC) and does not interact with actin whereas α -catenin homodimers regulate actin filament organization [4]. 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/Wingless signalling [5, 6]. Degradation of cytoplasmic β -catenin/Armadillo is induced by phosphorylation of four conserved N-terminal serine/threonine residues through the combined activities of glycogen synthase kinase-3 β (GSK3 β)/Zeste-White 3 (Zw3) and casein kinase 1 (CK1). 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. Protein kinase A (PKA) and Akt kinase induced phosphorylation of serine 552 and 675 promotes the transcriptional activity of β -catenin. Phosphorylation of tyrosine 489 by the Abl tyrosine kinase upon stimulation of the axon guidance receptor Robo decreases the affinity of β -catenin for N-cadherin [7]. Beside GSK3 β and CK1, casein kinase 2 (CK2) phosphorylates β -catenin at several sites in the N-terminal region and thereby influences the stability of the protein and strengthens the binding to α -catenin [8]. Mutational analysis in *Drosophila* support the idea that Armadillo localized at adherens junctions can be phosphorylated by a serine/threonine kinase other than GSK3 β /Zw3 [9]. In this study, we addressed the question whether the *Drosophila* p21-activated kinase (PAK) Mbt can fulfil this function.

PAK proteins are a family of serine/threonine kinases that are regulated by binding of RhoGTPases such as Cdc42 or Rac1. Based on structural features, PAK proteins are classified into two subgroups, each representing three members in human (group 1: PAK1-3; group 2: PAK4-6) [10]. In *Drosophila melanogaster* (*Dm*), two PAK proteins (DmPAK and DmPAK3) belong to group 1, whereas Mbt (DmPAK2) is the only group 2 PAK. Cadherin-mediated cell adhesion 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 [3, 11, 12]. Several lines of evidence indicated a function of single group 2 PAK proteins in regulation of adhesive properties of cells. Constitutive activation of human PAK4 leads to changes in cell morphology, decreased cell adhesion and induction of anchorage independent growth [13, 14]. 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 localizes to cell-cell contact sites and regulates cell adhesion in a calcium-dependent manner [15]. Similar, *Drosophila* Mbt localizes at adherens junctions and is required for morphogenetic processes during eye development [16, 17]. Here, we provide first evidence on the molecular function of Mbt in cadherin-mediated cell adhesion.

Materials and methods

Cloning procedures

Drosophila melanogaster E-cadherin (DE-cadherin) was cloned as an Asp718/BamHI 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 EcoRI/BamHI and inserted into the pCR3 vector containing the hinge and Fc-region from human IgG1 (kindly provided by P. Schneider). The generation of stable S2R cell lines expressing Myc-Mbt, Myc-Mbt^{T525A} and Myc-Mbt^{S492N/S521E} are described in [18]. Mbt^{T525A} and Mbt^{S492N/S521E} were further cloned into the Asp718 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 *Escherichia coli*, 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 XhoI/XbaI sites of pcDNA3.1Myc. For generation of the N-terminal truncated Armadillo protein (amino acids 76-843), first an additional XhoI site was introduced by in vitro mutagenesis at base pair position 229, followed by XhoI digestion and self-ligation. Both constructs were then cloned as BamHI/XbaI fragments into the pGEX vector (GE Healthcare). For expression of hemagglutinin (HA) epitope-tagged variants of Armadillo in S2R cells, the HA-epitope encoding sequence was introduced into the KpnI site of the pAC5.1 vector to generate pAC5.1-HA. Armadillo was subcloned with BamHI/XbaI from pcDNA3.1Myc into the pBS vector (Stratagene). The QuickChange[®] site-directed mutagenesis protocol (Stratagene) was used to change the codons of serine 561 and 688 to alanine. Wild-type and mutated Armadillo constructs were then cloned with XhoI/XbaI into pAC5.1-HA. All constructs were verified by sequencing.

Cell culture and cell fractionation

Drosophila Schneider S2R and S2 cells [19] were cultured at 25°C in Schneider medium (Biowest) supplemented with 10% fetal calf 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 the calcium phosphate method. Membrane fractions were prepared as described in [20]. Human embryonic kidney (HEK) 293 cells were cultured in DMEM medium with 10% fetal calf serum. Transient transfections of HEK293 were carried out using the JetPEI Polyplus reagent (Biomol) according to the manufacturers protocol.

Immunohistochemistry and microscopy

S2R cells were grown on coverslips (if necessary induced with 0.05 µM CuSO₄), fixed with 4% (w/v) paraformaldehyde for 20 minutes, permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 minutes and incubated with rat anti-DE-cadherin (1:30, hybridoma cell line supernatant obtained from Developmental Studies Hybridoma Bank, DSHB), mouse anti-Armadillo (1:100, concentrate from DSHB), rat anti-α-catenin (1:100, concentrate from DSHB) or rabbit anti-Mbt (1:500, [17]) in PBS containing 1% (v/v) normal goat or normal donkey serum. After washing in PBS, cell were incubated with Cy3-, Cy5- or AlexaFluor[®] 488-conjugated secondary antibodies (Dianova and Molecular Probes) diluted in PBS. In some cases, a ready-to-use Hoechst stain solution (Sigma-Aldrich) was added to stain DNA. Cells were embedded in Mowiol and analyzed with a Leica DM5000B microscope using a 63x HCX PL APO oil objective. Images were taken with a Leica DFC350FX digital camera and false-colour coded with the Amira software Mercury Computer Systems). Control

stainings with single primary antibodies were performed to exclude bleed-through in other detection channels (see supplementary Figure 1).

Protein expression and purification, immunoprecipitation and immunoblot analysis

Expression of Myc-Mbt variants in Schneider S2 and S2R cells was induced by addition of 0.8 mM CuSO₄. Immunoprecipitations, SDS-PAGE and Western blot analysis were done as described previously [16] with mouse anti-Myc (Santa Cruz), mouse anti-HA (Santa Cruz), mouse anti- α -tubulin (Sigma-Aldrich), rat anti-DE-cadherin, mouse anti-Armadillo, rat anti- α -catenin (DSHB) or anti-GST (Novagen) antibodies. Expression and purification of bacterially expressed GST-Armadillo proteins were performed as described previously [16]. GST-Mbt^{T525A} and GST-Mbt^{S492N/S521E} were purified from transiently transfected HEK293 cells after lysis in lysis buffer [25 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 0.1% NP-40 plus protease inhibitor cocktail tablets (pH 7.5)] with glutathione-agarose beads (GE Healthcare). After washing with lysis buffer and PBS, bound proteins were eluted with elution buffer [10 mM glutathione, 150 mM NaCl, 50 mM Tris (pH 7.5)] and dialyzed against 20 mM Tris [pH 7.5]. For expression and purification of (DE-cadherin-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 μ g DNA. After 48 hours, the cell culture supernatant was collected, filled up to 50 ml with HBSS buffer (Roth) and stored at -80°C until further use. 30 μ l slurry of beads (Dynabeads Protein A, Invitrogen) were incubated overnight with 5 ml of (DE-cadherin-Fc)₂ in HBSS buffer. The beads were washed in HBSS (+ 300 mM NaCl, 0.1% NP-40) and taken up in 100 μ l HBSS buffer.

Kinase assays

Kinase assays (radioactive and non-radioactive) were performed as described previously [18].

Laser tweezer

The setup for the laser tweezer experiment has been described previously [21]. Beads were considered tightly bound when resisting laser displacement at 42 mW settings. A slurry of 5 μ l (DE-cadherin-Fc)₂ coated microbeads in HBSS were allowed to settle on the surface of cells in 200 μ l Schneider medium for 30 minutes. Always 100 beads were probed by the laser tweezer in a single experiment. For each cell line, five independent experiments were performed. Statistical analysis was done with Statistica. To deplete Ca²⁺, EGTA was added to the culture medium for 20 minutes. Different EGTA concentrations were tested. Consistent results were obtained for EGTA concentration equal or higher than 20 mM. After the experiment, EGTA treated cells were cultured in normal Schneider medium for several days. We did not observe a negative effect on cell viability or obvious changes in cell morphology.

Identification of phosphorylation sites

Reduction and carbamidomethylation of the Armadillo protein *in vitro* phosphorylated by Mbt was done in-gel, followed by trypsin digestion (trypsin gold from porcine pancreas, mass spectrometry grade, Promega) and an additional chymotryptic digest (chymotrypsin from bovine pancreas, sequencing grade, Roche). Peptides were extracted from the gel with 1% aqueous trifluoroacetic acid.

The samples were analyzed by nanoflow chromatography coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 Q TRAP[®] LC/MS/MS system, Applied Biosystems) equipped with a Flow NanoSpray[®] source and a heated desolvation chamber interface set to 150°C [22]. The protein digests were separated on a Tempo[™] 1D nanoLC System (Applied Biosystems) using a PepMap C18 column (75 μ m x 15 cm, Dionex) and a 45-minute linear gradient of 5% to 40% acetonitrile vs. water with 0.1% formic acid as

modifier. Samples were concentrated and desalted on a PepMap C18 capillary trap (300 μm x 5 mm, Dionex) using aqueous 2% acetonitrile/0.5% formic acid at a flow rate of 20 $\mu\text{l}/\text{minute}$ for 5 minutes prior to the analytical separation.

For standard LC/MS/MS analysis a Linear Ion Trap single MS scan over a mass range of m/z 400-1400 at 4000 amu/s was used to detect eluting peptides. Up to 5 precursors per experimental cycle were examined by an Enhanced Resolution scan at 250 amu/s to establish charge state and molecular weight. Suitable precursors were then subjected to Enhanced Product Ion scans at 4000 amu/s, with the collision energies automatically determined based on charge state and molecular weight.

For selective detection of phosphopeptides by LC/MS/MS Precursor Ion scanning for precursors of m/z 79 (PO_3^-) was performed in negative ion mode over a mass range of m/z 400-1400 at 333 amu/s, with the Q1 set to low resolution (2.0 amu FWHH) and the Q3 set to unit resolution (0.6 amu FWHH). Precursors were collisionally activated in Q2 with nitrogen at a collision energy ramp of -65 to -110 V across the m/z range. Up to two precursors per experimental cycle were examined by an Enhanced Resolution scan at 250 amu/s in negative ion mode. On detection of suitable precursors the polarity was switched to positive ion mode, and up to two precursors subjected to Enhanced Product Ion scans at 4000 amu/s. At the end of the cycle polarity was switched back to negative ion mode.

The acquired LC/MS/MS data were submitted for a database search against an NCBI nr protein sequence database using the MASCOT[®] V2.0 search engine (Matrixscience) to identify (phospho-)peptides and proteins.

Results

Expression of DE-cadherin and Mbt in *Drosophila* S2R cells

Establishment of the final retinal pattern of the *Drosophila* eye involves complex morphological rearrangements and cell sorting processes, which depend on several functional interdependent cell adhesion systems [23, 24]. To address the question, whether Mbt specifically modulates DE-cadherin mediated cell adhesion and to minimize possible secondary effects due to interactions with other cell adhesion molecules, a *Drosophila* S2R cell culture system was established. Schneider S2R cells are derived from S2 cells [19]. They express α -catenin, whereas DE-cadherin and Armadillo are only present at low amounts (Fig. 1A). Previous experiments have shown that expression of DE-cadherin in S2 cells resulted in stabilization of Armadillo at the plasma membrane [25]. Similar, expression of DE-cadherin under the actin promoter in S2R cells resulted in high Armadillo protein levels (Fig. 1A) and accumulation of DE-cadherin, Armadillo and α -catenin at the plasma membrane mainly at cell contact sites (Fig. 1B) indicating that the proteins can form a functional cadherin-catenin complex (CCC). Identical localization patterns were observed after transient transfection of the DE-cadherin construct in S2R cells (Fig. 1B) or upon generation of a stable DE-cadherin expressing S2R cell line (S2R_DEcad, data not shown). Assembly of the CCC was also verified by co-immunoprecipitation experiments with an anti-DE-cadherin antibody. Armadillo and α -catenin were co-purified with DE-cadherin from S2R_DEcad cells (see Fig. 5A).

In the next step, we combined expression of the cadherin-catenin complex and variants of Mbt in S2R cells. Stable S2R cell lines expressing Myc epitope tagged wild-type Mbt (S2R_Myc-Mbt), a kinase dead version (S2R_Myc-Mbt^{T525A}) and a constitutively activated Mbt protein (S2R_Myc-Mbt^{S492N/S521E}) under the control of the inducible metallothioneine promoter [18] were transiently transfected with the DE-cadherin construct to allow formation of the CCC at the plasma membrane followed 12 hours later by addition of Cu²⁺ to induce Mbt expression. Western blot analysis confirmed expression of the corresponding proteins (Fig. 2A). Immunocytochemical analysis revealed the presence of Myc-Mbt and Myc-Mbt^{T525A} in the cytoplasm and accumulation of the proteins at the plasma membrane, where they co-localize with DE-cadherin and Armadillo (Fig. 2B). This corresponds to the analysis in flies, where Mbt co-localizes with Armadillo at adherens junctions of the developing photoreceptor cells [17]. In the presence of the constitutively active Myc-Mbt^{S492N/S521E} protein, only in a fraction of cells clear localization of Mbt, DE-cadherin and Armadillo at cell contacts could be detected (Fig. 2B). Instead, we frequently observed accumulation of DE-cadherin and Armadillo in large vesicle-like structures within cells and enrichment of Myc-Mbt^{S492N/S521E} in the cytoplasm (Fig. 2C). The identity of these vesicles remained obscure, because staining with markers for the endocytic and exocytic pathways revealed only partial overlap (data not shown).

Activation of Mbt induces destabilisation of the DE-cadherin - Armadillo complex

To investigate, whether Mbt influences the interaction of DE-cadherin with Armadillo, we used three different approaches. In the first assay, the CCC was purified from S2R_DEcad cell lysates with the anti-DE-cadherin antibody coupled to Protein G-agarose beads. The beads with bound proteins were equally distributed and subjected to non-radioactive *in vitro* kinase assays in the presence of GST-Mbt^{T525A} or GST-Mbt^{S492N/S521E} proteins in solution, which were purified from transiently transfected HEK293 cells. After the kinase reaction, the bead fraction was directly analyzed for DE-cadherin and associated Armadillo, whereas the complete supernatant fraction was used for immunoprecipitation with the anti-Armadillo antibody to quantitatively purify Armadillo protein released from DE-cadherin during the

kinase reaction (Fig. 3A). Compared to the control reaction (Fig. 3A, lane 1), the presence of kinase inactive GST-Mbt^{T525A} protein had no influence on the amount of Armadillo bound to DE-cadherin (Fig. 3A, lane 2). In contrast, incubation with the constitutively activated GST-Mbt^{S492N/S521E} protein releases Armadillo from DE-cadherin into the supernatant (Fig. 3A, 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. 3A, lane 3). This experiment also argues against the possibility that Mbt^{S492N/S521E} simply competes out DE-cadherin binding to Armadillo.

To provide *in vivo* evidence for a role of activated Mbt on the DE-cadherin - Armadillo interaction, the stable S2R_Myc-Mbt^{T525A} and S2R_Myc-Mbt^{S492N/S521E} cells lines described above were first transfected with a DE-cadherin expression vector to allow formation of the CCC followed 12 hours later by induction of 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 amount of co-immunoprecipitated Armadillo protein compared to cells expressing the Myc-Mbt^{T525A} protein (Fig. 3B). One caveat of this experiment is that it was done with total cell lysates and this might not reflect the situation found at the plasma membrane. Therefore, we repeated the experiment but isolated membrane fractions at various time points upon induction of Myc-Mbt^{S492N/S521E} or Myc-Mbt^{T525A} and analyzed them for Armadillo, DE-cadherin and Mbt proteins. Although Myc-Mbt^{T525A} and Myc-Mbt^{S492N/S521E} show similar expression levels in total cell lysates (Fig. 3B), significantly less Myc-Mbt^{S492N/S521E} protein is found in the membrane fraction (Fig. 3C). The Myc-Mbt^{T525A} protein had no effect on the amount of membrane associated DE-cadherin or Armadillo. In contrast, the amount of Armadillo and DE-cadherin in the membrane fraction is reduced when the Myc-Mbt^{S492N/S521E} protein was expressed (Fig. 3C). In summary, the experiments support the idea that activation of Mbt disrupts the DE-cadherin - Armadillo complex.

Activation of Mbt reduces DE-cadherin dependent adhesiveness of cells

In order to directly assess changes in the adhesive properties of cells upon activation of Mbt, we applied the laser tweezer technique [21]. Paramagnetic microbeads 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. 4A). 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 [21, 26, 27]. 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 S2 cell line was established, which constitutively expresses 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 was verified by Western blot (Fig. 4B). DE-cadherin-Fc coated beads were allowed to settle for 30 minutes on the cell surface before they were probed for displacement with a laser beam (Fig. 4C). Beads were considered tightly bound when resisting laser displacement at 42 mW. In a single experiment, 100 beads were tested. On average 64 beads (± 1.67 ; n=5) resisted laser displacement when S2_DEcad cells were used whereas in the parental S2 cell line on average only 38 beads (± 5.54 ; n=5) remain attached to the cell surface (Fig. 4D). Specificity and Ca²⁺ dependency of binding of (DE-cadherin-Fc)₂-coated

beads to cellular DE-cadherin was tested by depletion of Ca^{2+} from the medium with different concentrations of EGTA. With 30 mM EGTA, the number of beads resisting displacement from S2_DEcad cells dropped to $33 (\pm 5.45; n=5)$, 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 at high levels (Fig. 4B, 3C). Compared to S2_DEcad cells, the number of (DE-cadherin-Fc)₂ coated beads that remain bound on S2_DEcad_Myc-Mbt^{T525A} cells was not significantly changed ($64 \pm 5.10; n=5$). In contrast, a significant reduction was observed upon expression of activated Mbt in S2_DEcad_Myc-Mbt^{S492N/S521E} cells. The number of beads that remain bound ($45 \pm 4.39; n=5$) was slightly higher than in the S2 and the S2_DEcad + EGTA control experiments indicating that activated Mbt strongly decreases but not completely eliminates DE-cadherin dependent cell adhesion.

Activation of Mbt results in phosphorylation of Armadillo

The experiments described so far are compatible with a model that activated Mbt mediates its effect on the DE-cadherin – Armadillo interaction by phosphorylation one of the components. Two different approaches were applied to verify this assumption. In the first assay, the CCC was immunopurified from S2R_DEcad cells with the anti-DE-cadherin antibody and subjected to *in vitro* kinase reactions with GST-Mbt^{S492N/S521E} or GST-Mbt^{T525A} proteins purified from transiently transfected HEK293 cells. The presence of DE-cadherin, Armadillo and α -catenin in the immunoprecipitates was verified by Western blot (Fig. 5A). Only in the presence of the GST-Mbt^{S492N/S521E} protein, a phosphorylation signal became visible that corresponds in size to the Armadillo protein, whereas no ³²P-incorporation into DE-cadherin and α -catenin was detected (Fig. 5A). From this experiment we concluded that Armadillo is accessible to Mbt phosphorylation in its native conformation and in complex with DE-cadherin and α -catenin. To verify Armadillo phosphorylation by Mbt, *in vitro* kinase reactions were performed with bacterially expressed GST-Armadillo fusion proteins. In addition to a full length Armadillo protein (GST-Myc-Arm), a N-terminal truncated Armadillo protein (GST-Myc-Arm [76-843]) was used. The activated Myc-Mbt^{S492N/S521E} protein, but not the kinase dead Myc-Mbt^{T525A} protein, was able to phosphorylate both Armadillo proteins to a similar degree (Fig. 5B). This indicated that Mbt phosphorylates Armadillo at sites distinct from the four N-terminal phosphorylation sites for Zw3 and CK1, which are important to target cytoplasmic Armadillo for degradation [28-30].

Identification and characterization of phosphorylation sites in Armadillo

A standard LC/MS/MS analysis of a tryptic/chymotryptic digest of Armadillo (approx. 250 fmol) resulted in a sequence coverage of 91% (Fig. 6A). There was first indication for a phosphorylation at S⁵⁶¹ (data not shown). Further phosphopeptides could not be detected. Therefore, a precursor ion scanning experiment was performed in negative ion mode for *m/z* 79 Da (PO_3^-). This allowed selective detection of phosphopeptides at high sensitivity. Intact phosphorylated precursors were then selected for MS/MS fragmentation in the positive ion mode. This experiment revealed the presence of six phosphopeptides (Fig. 6B) corresponding to two phosphorylation sites. MS/MS fragment spectra and assignment of γ - and β -type ions allowed the localization of the respective phosphorylated residues. One phosphorylation site could be assigned to S⁵⁶¹ by MS/MS experiments from three phosphopeptides resulting from missed proteolytic cleavages (Fig. 6C), and the other to S⁶⁸⁸ by MS/MS experiments of three phosphopeptides resulting from missed proteolytic cleavages and the formation of pyroglutamate from glutamate (Fig. 6D), respectively.

Both serine residues are conserved between *Drosophila* Armadillo and vertebrate β -catenins and are preceded by an arginine residue at the -2 position (Fig. 7A). Pak proteins strongly favour serine as phosphoacceptor and have a strong bias for peptides containing an arginine at

the -2 position [31, 32]. To determine whether serine 561 and serine 688 are the only sites for Mbt phosphorylation, a bacterially expressed Armadillo protein carrying the corresponding serine to alanine substitutions (GST-Myc-Arm^{S561A/S688A}) was used for *in vitro* kinase assays and compared with the non-mutated Armadillo protein. As shown in Fig. 7B, the two substitutions are sufficient to reduce Myc-Mbt^{S492N/S521E} mediated phosphorylation of Armadillo to background levels as seen with the kinase dead Myc-Mbt^{T525A} protein. Finally we tested whether mutation of serines 561 and 688 abolish the Mbt induced disruption of the DE-cadherin – Armadillo interaction. HA-tagged Armadillo^{S561A/S688A} or HA-Armadillo as a control were co-expressed together with DE-cadherin and Mbt in S2R cells. Equal amounts of HA-Armadillo and HA-Armadillo^{S561A/S688A} were co-purified with DE-cadherin in Myc-Mbt^{T525A} expressing cells (Fig. 7C). This shows that both Armadillo proteins become integrated into the CCC and argues against the possibility that mutation of both serine residues has a general influence on binding to DE-cadherin. Similar to endogenous Armadillo protein (Fig. 3B), binding of HA-Armadillo to DE-cadherin was significantly reduced in the presence of Myc-Mbt^{S492N/S521E} when compared with Myc-Mbt^{T525A} (Fig. 7C). In contrast, HA-Armadillo^{S561A/S688A} resisted Myc-Mbt^{S492N/S521E} induced release from DE-cadherin. No difference in binding of HA-Armadillo^{S561A/S688A} to DE-cadherin was observed in cells expressing Myc-Mbt^{S492N/S521E} or Myc-Mbt^{T525A}. We also tested the corresponding single mutants HA-Armadillo^{S561A} and HA-Armadillo^{S688A} in the same assay to evaluate the importance of each phosphorylation site. Unlike the HA-Armadillo and HA-Armadillo^{S561A/S688A} proteins, which behaved consistent in independent experiments, both single mutant Armadillo proteins produced variable results upon co-expression with Myc-Mbt^{S492N/S521E} in several independent experiments (data not shown). Sometimes, we observed a reduction in binding, sometimes the proteins largely resisted Myc-Mbt^{S492N/S521E} induced release from DE-cadherin. Therefore, we were unable to determine the relative contribution of each serine residue for the Armadillo – DE-cadherin interaction. In summary, the results provide evidence that destabilization of the DE-cadherin – Armadillo complex can be induced by phosphorylation of two conserved serine residues in Armadillo.

Discussion

Recent studies have implicated single group 2 PAK proteins in regulation of the adhesive properties of cells under normal and disease conditions. The human 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 [14, 33]. One of the key features during the transition from a benign to a metastatic behaviour of cells is the breakdown of adhesive bonds to allow migration to and invasion of foreign tissues. It was shown that changes in cell morphology and adhesion play an important role in the ability of an activated version of PAK4 to transform cells [13, 14]. *Xenopus laevis* X-Pak5 is expressed in regions of the embryo that undergo extensive cell movements during gastrulation. X-Pak5 localizes to cell-cell contact sites and regulates cell adhesion in a calcium-dependent manner [15]. Similar, *Drosophila* Mbt localizes at adherens junctions and is required for morphogenetic processes during eye development [16, 17]. While these studies focused on the effects of PAK activation on cell morphology and in the context of developmental processes, the underlying molecular mechanisms remained elusive. We established a cell culture system, which allowed on one hand the assembly and purification of a functional CCC upon expression of DE-cadherin and on the other hand inducible expression of variants of Mbt. We could show that in the presence of activated Mbt, Armadillo becomes phosphorylated as a free substrate but also as part of the CCC. Our results are in agreement with previous studies postulating the existence of an adherens junction localized kinase, which phosphorylates Armadillo [9] and support the idea that Mbt can fulfil such a function. We further provided evidence that it is not merely the physical presence of Mbt that weakens the DE-cadherin - Armadillo interaction and consequently DE-cadherin mediated cell adhesion, but the kinase activity of Mbt is essential to induce this effect. In this way, Mbt would differ from proteins like IQGAP1, which binds to E-cadherin and β -catenin and thereby causes disruption of the CCC [34]. One important question is whether Mbt directly binds to Armadillo. Although we could co-immunoprecipitate Armadillo and Mbt from S2R cells and also upon heterologous co-expression in human HEK293 cells, we were unable so far to reconstitute the Mbt - Armadillo interaction in cell free systems (N. M. and H.W., unpublished).

Previously we have shown that Mbt, like other PAK proteins, binds RhoGTPases in the active, GTP-bound conformation. The RhoGTPase binding domain of Mbt is essential for recruitment to adherens junctions [17]. Because Cadherin-mediated cell adhesion influences the activity of the RhoGTPases [3, 11, 12], one can envisage a model in which activation of RhoGTPases recruits Mbt to the CCC where Mbt can phosphorylate Armadillo. Studies in vertebrates indicate that a primary regulatory point for cell adhesion is at the level of Cadherin distribution at the cell membrane and in intracellular compartments [35-37]. Our observations that activated Mbt leads to accumulation of DE-cadherin and Armadillo in vesicle-like structures (Fig. 2C), reduces the amount of membrane bound DE-cadherin and Armadillo (Fig. 3C) and decreases cell adhesiveness (Fig. 4D) support the view that Mbt interferes with DE-cadherin dynamics by disruption of the DE-cadherin - Armadillo interaction. Internalization of ectopically expressed mouse E-cadherin in *Drosophila* cl-8 cells and redistribution of membrane-bound Armadillo to the cytoplasm has also been observed upon activation of the Wingless signalling pathway [20].

How can phosphorylation of Armadillo weaken the association with DE-cadherin? The Armadillo repeat domain contains twelve copies of an imperfect sequence repeat (Arm repeats), each of which consists of three helices. Structural analysis of the murine E-cadherin/ β -catenin complex revealed that the Arm repeats form a superhelix, which provides in its entire length the interaction surface for the cytoplasmic domain of E-cadherin [38]. It has been suggested that this elongated interface is suited for integration of multiple signal inputs that can modulate the overall affinity of the interaction. In this way, the mechanical properties and dynamics of adherens junctions could be modulated during morphogenetic

processes without necessarily destroying them completely. In support of this idea, CK2 and GSK-3 β mediated phosphorylation of E-cadherin increases the β -catenin - E-cadherin interaction [39]. On the other hand, Src kinase induced phosphorylation of mouse β -catenin at tyrosine 654, which is located in helix 3 of arm repeat 12, weakens the β -catenin - E-cadherin association [40]. Also in our experiments, phosphorylation of Armadillo by Mbt does not cause a complete elimination of the Armadillo - DE-cadherin interaction. One of the identified phosphorylation sites (serine 561) lies within arm repeat 10 of Armadillo, whereas serine 688 localizes in a α helix next to Arm repeat 12. Both serine residues are conserved in vertebrate β -catenins (serine 552 and serine 675). Importantly, phosphorylation of serine 552 by the protein kinase Akt also leads to dissociation of β -catenin from cell contacts and translocation to the nucleus. Mutation of serine 552 to alanine was sufficient to largely inhibit dissociation of β -catenin from the membrane [41]. We did not observe enrichment of Armadillo in the nucleus in the presence of activated Mbt. In the case of Armadillo, mutation of serine 561 and 688 to alanine was necessary to efficiently block Mbt induced release of Armadillo from DE-cadherin (Fig. 7C), whereas experiments with the corresponding single mutants gave equivocal results (data not shown). This indicates that phosphorylation of either serine residue alone only slightly interferes with the DE-cadherin - Armadillo interaction and again emphasizes the fine-tuning of the whole system. The crystal structure of β -catenin does not immediately suggest a role of these serine residues in the β -catenin - E-cadherin interaction [38, 42]. However, the structural data were obtained with unmodified β -catenin and phosphorylation could lead to conformational changes that influence the binding properties of β -catenin/Armadillo. Serine 675 and to some extent serine 552 were also identified as target sites for protein kinase A (PKA), which leads to enhanced transcriptional activity of β -catenin [43, 44]. It appears that β -catenin/Armadillo possess common phosphorylation sites for different protein kinases, which allows integration of various signalling pathways.

Although these studies provide a conceptual framework, many details of this intricate network remain elusive. In particular, the requirement of a specific phosphorylation site for regulation of diverse cadherin dependent cellular processes such as cell migration, cell shape changes and neurite extension must be verified under physiological conditions. This can be exemplified in the *Drosophila* embryo where Armadillo is the major tyrosine phosphorylated protein in the CCC [45]. In accordance with studies in vertebrates, tyrosine hyper-phosphorylation causes loss of epithelial integrity. However, no significant dissociation of Armadillo from DE-cadherin was observed and it has therefore been suggested that tyrosine hyper-phosphorylation weakens the link of the CCC to the actin cytoskeleton [45]. Another example is seen during *Drosophila* oogenesis, where a specialized group of somatic follicle cells (border cells) delaminate from the follicular epithelium, invade the nurse cell cluster and migrate to the oocyte in a DE-cadherin dependent manner. However, mutation of tyrosine 667 in Armadillo, which corresponds to tyrosine 654 of β -catenin and should therefore abrogate regulation of the DE-cadherin - Armadillo interaction, had no effect on border cell migration [46]. In the case of regulation of DE-cadherin activity by Mbt during eye development, one must take into account expression of N-cadherin in some, but not all cells of the developing eye [47, 48] and the cross talk between different adhesion systems and signalling molecules expressed in different subsets of cells [23, 49, 50].

In summary, Mbt adds to an increasing list of kinases that can regulate the function and integrity of the cadherin-catenin complex. Armadillo/ β -catenin can serve as convergence points for many kinase-mediated signals. This allows combinatorial regulation and the integration of multiple signals depending on the cellular context. Therefore, we consider Mbt as only one component that mediates fine-tuning of DE-cadherin mediated cell adhesion in morphogenetic processes.

Acknowledgements

We thank Ora Bernard, Pernille Rorth and Pascal Schneider for sending DNA constructs. The monoclonal antibodies against DE-cadherin (developed by T. Uemura), Armadillo (developed by E. Wischaus) and α -catenin (developed by M. Takeichi) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB487) to D. D. and T. R.

Stage 2(a) POST-PRINT

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Figure legends

Figure 1

Expression of DE-cadherin in S2R cells recruits α -catenin and Armadillo to the plasma membrane.

(A) Western blot analysis of total lysates from stable S2R cells expressing DE-cadherin under the actin promoter (S2R_DEcad) and the parental S2R cell line. The blot was probed with antibodies against DE-cadherin, α -catenin and Armadillo. Molecular masses in kDa are indicated. (B) S2R cells were transiently transfected with DE-cadherin and either stained with antibodies against Armadillo and DE-cadherin (top) or Armadillo and α -catenin (bottom). Non-transfected S2R cells were treated in the same way. The merge of both stainings together with DNA labelling (blue) is shown in the right column. The scale bar represents 10 μ m. Representative images of at least four independent experiments are shown.

Figure 2

Co-expression of Mbt and DE-cadherin in S2R cells.

(A) Stable S2R cells for Myc-tagged Mbt, Mbt^{T525A} or Mbt^{S492N/S521E} were transiently transfected with DE-cadherin and whole cell lysates were analyzed by Western blot before (-) and after (+) induction of Mbt expression with Cu²⁺. The blot was probed with antibodies against DE-cadherin, Armadillo, the Myc-epitope and α -tubulin as a loading control. Molecular masses in kDa are indicated. (B) Localization of Myc-Mbt, Myc-Mbt^{T525A} and Myc-Mbt^{S492N/S521E} in S2R cells. The indicated Myc-Mbt cell lines were transiently transfected with DE-cadherin followed by induction of Mbt expression with Cu²⁺. Expression of Mbt proteins was detected together with Armadillo or DE-cadherin. The scale bar represents 10 μ m. (C) Mbt^{S492N/S521E} can cause changes in the distribution of DE-cadherin and Armadillo. DE-cadherin and Armadillo, but not the Mbt^{S492N/S521E} protein, also accumulate in large vesicle structures. The scale bar represents 10 μ m. Each experiment was repeated at least three times.

Figure 3

Activation of Mbt induces destabilization of the DE-cadherin – Armadillo interaction.

(A) The anti-DE-cadherin antibody coupled to Protein G agarose beads was used for isolation of the CCC from S2R_DEcad cells (IP: anti-DE-cad). Non-radioactive *in vitro* kinase reactions were performed with equal amounts from this batch in the presence or absence of ATP and purified GST-Mbt^{S492N/S521E} or GST-Mbt^{T525A} proteins in solution. Afterwards, beads were analysed by Western blot (IB) for bound DE-cadherin and Armadillo. Armadillo protein released into the supernatant was first immunoprecipitated (IP(S)) and then detected by Western blot with the anti-Armadillo antibody. Samples from the same batches of purified GST-Mbt proteins used in the different kinase reactions were analysed by Western blot with an anti-GST antibody (Input). (B) 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. Results presented in (A) and (B) were repeated three times. (C) Analysis of membrane-associated DE-cadherin and Armadillo at different time points after induction of Myc-Mbt^{S492N/S521E} and Myc-Mbt^{T525A} expression. Two further experiments produced similar results.

Figure 4

Mbt decreases DE-cadherin mediated cell adhesion.

(A) Schematic drawing of the (DE-cadherin-Fc)₂ fusion protein that can form dimers via disulphide bridges. (B) Western blot analysis of stable S2 cell lines constitutively expressing

DE-cadherin alone or together with 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 S2R_DEcad_Myc-Mbt cells. (D) Quantification of the laser tweezer experiments. Values are means ± S.E.M. of five independent experiments. In each experiment 100 beads were tested for displacement. Pair-wise comparisons were done with Mann–Whitney U tests. Significance levels were adjusted by a Bonferroni correction to keep the experiment-wide error rate at 5%. The critical P value 0.05 is divided by the number of tests, in this case four. ** $P < 0.01$.

Figure 5

Mbt phosphorylates Armadillo as part of the cadherin-catenin complex and as a free substrate. (A) The cadherin-catenin complex was first immunopurified (IP) from S2R_DEcad cells with the anti-DE-cadherin antibody. Because Mbt^{S492N/S521E} causes dissociation of Armadillo from DE-cadherin (Fig. 3A), a second immobilization step with the anti-Armadillo antibody was necessary to avoid 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 DE-cadherin, Armadillo, α -catenin and GST-Mbt proteins (IB). Molecular masses in kDa are indicated. (B) Full-length Armadillo or an Armadillo protein lacking the N-terminal 75 amino acids were expressed as GST fusion proteins in bacteria, purified and subjected to *in vitro* kinase reactions with [γ -³²P]ATP in the presence of purified Myc-Mbt^{T525A} or Myc-Mbt^{S492N/S521E} (AR). Myc-Mbt expression was verified by Western blot (IB), GST-Armadillo proteins were visualized by Coomassie-blue staining. As noted previously [51], several GST-Armadillo fusion protein bands are observed. Full-length proteins are indicated with arrows. Presented results are representative of three independent experiments.

Figure 6

Mass spectrometric identification of Armadillo and localization of phosphorylation sites. (A) Base peak chromatogram of the LC/MS/MS run for protein identification in positive ion mode. (B) Base peak chromatogram of the LC/MS/MS run with precursor ion scanning for m/z 79 Da in the negative ion mode for selective phosphopeptide detection with annotation of the identified phosphopeptides. (C) Annotated MS/MS spectrum of the phosphopeptide RLpS⁶⁸⁸IELTN (m/z 513.27²⁺). (D) Annotated MS/MS spectrum of the phosphopeptide pyroQRSpS⁵⁶¹IATTGSQQPSAY (m/z 872.93²⁺).

Figure 7

Characterization of phosphorylation sites in Armadillo.

(A) Alignment of *Homo sapiens* (Hs) and *Xenopus laevis* (Xl) β -catenin with *Drosophila melanogaster* (Dm) Armadillo. The identified phosphorylation sites serine 561 and serine 688 in Armadillo are conserved in vertebrate β -catenins. Identical and similar residues among all proteins are highlighted in black and grey, respectively. (B) Bacterially expressed GST-Myc-Armadillo or GST-Myc-Armadillo^{S561A/S688A} fusion proteins were subjected to *in vitro* kinase reactions with Myc-Mbt^{T525A} or Myc-Mbt^{S492N/S521E} and analyzed by autoradiography (AR). Myc-Mbt expression was verified by Western blot (IB), GST-Armadillo proteins were visualized by Coomassie-blue staining. Three further experiments produced similar results. (C) Stable S2R_Mbt^{T525A} and S2R_Mbt^{S492N/S521E} cell lines were co-transfected with DE-cadherin and HA-Armadillo or DE-cadherin and HA-Armadillo^{S561A/S688A}. After immunoprecipitation with the anti-DE-cadherin antibody (IP), DE-cadherin and associated HA-Armadillo proteins were detected with the indicated antibodies (IB). Equal expression of Mbt and Armadillo proteins was verified by Western blot analysis of whole cell lysates. Presented results are representative of four independent experiments.

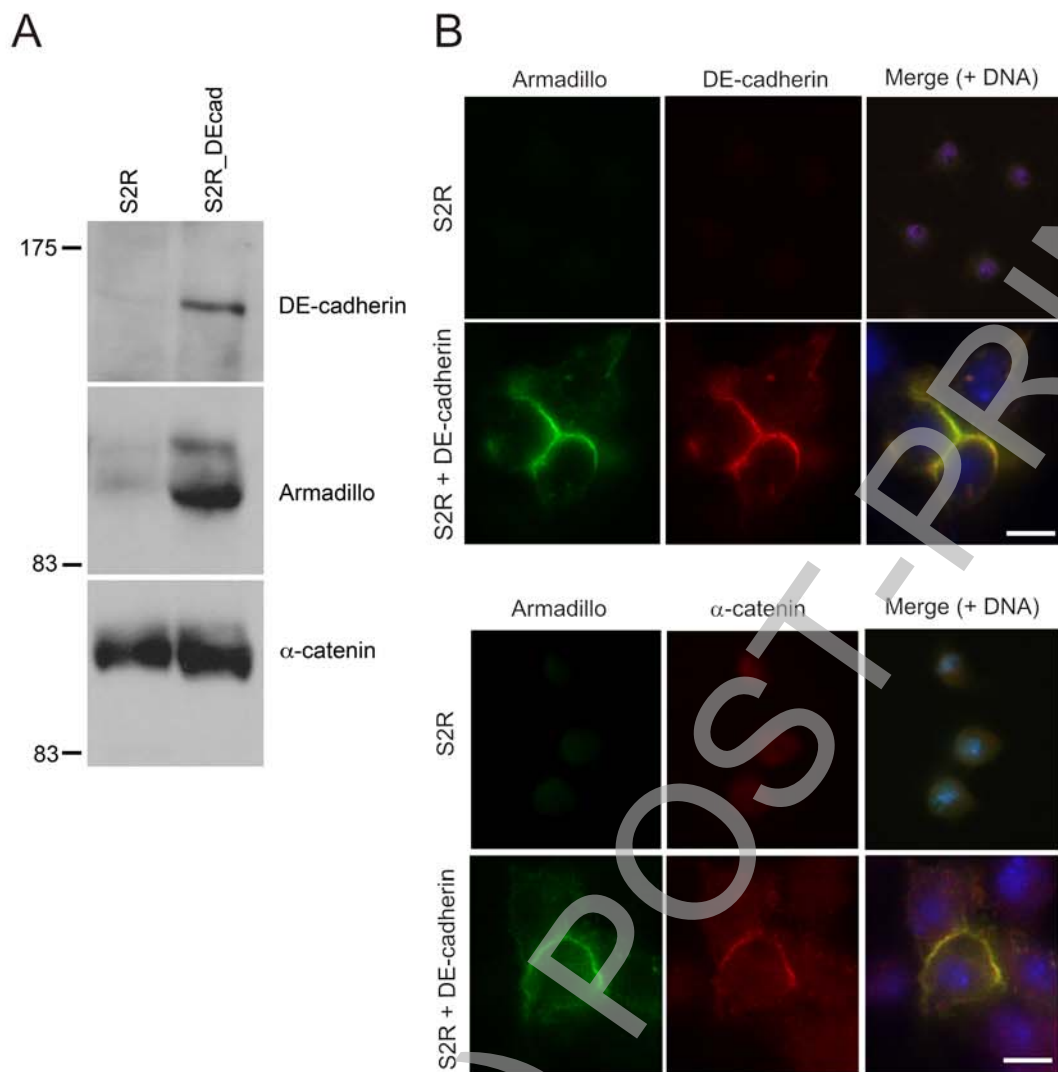


Figure 1

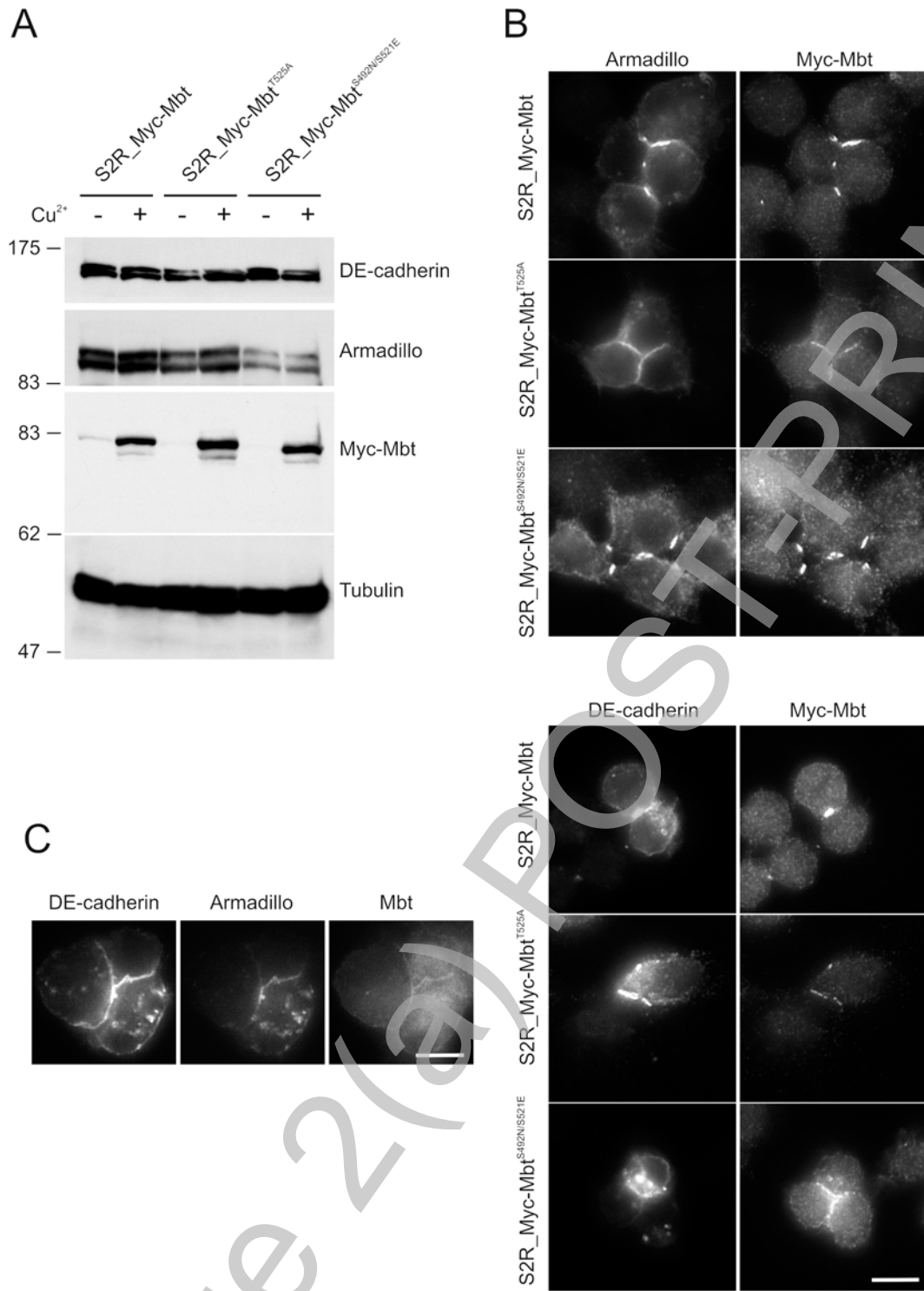


Figure 2

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20080465

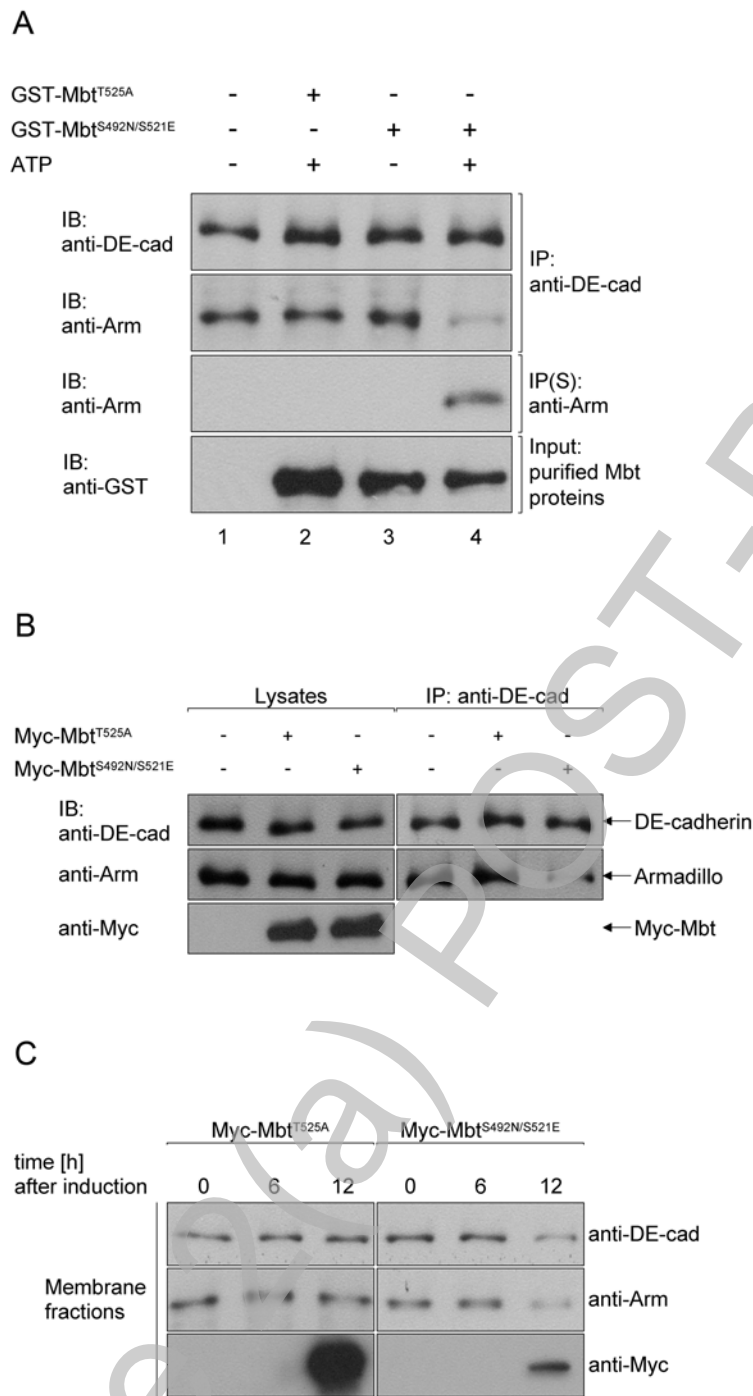


Figure 3

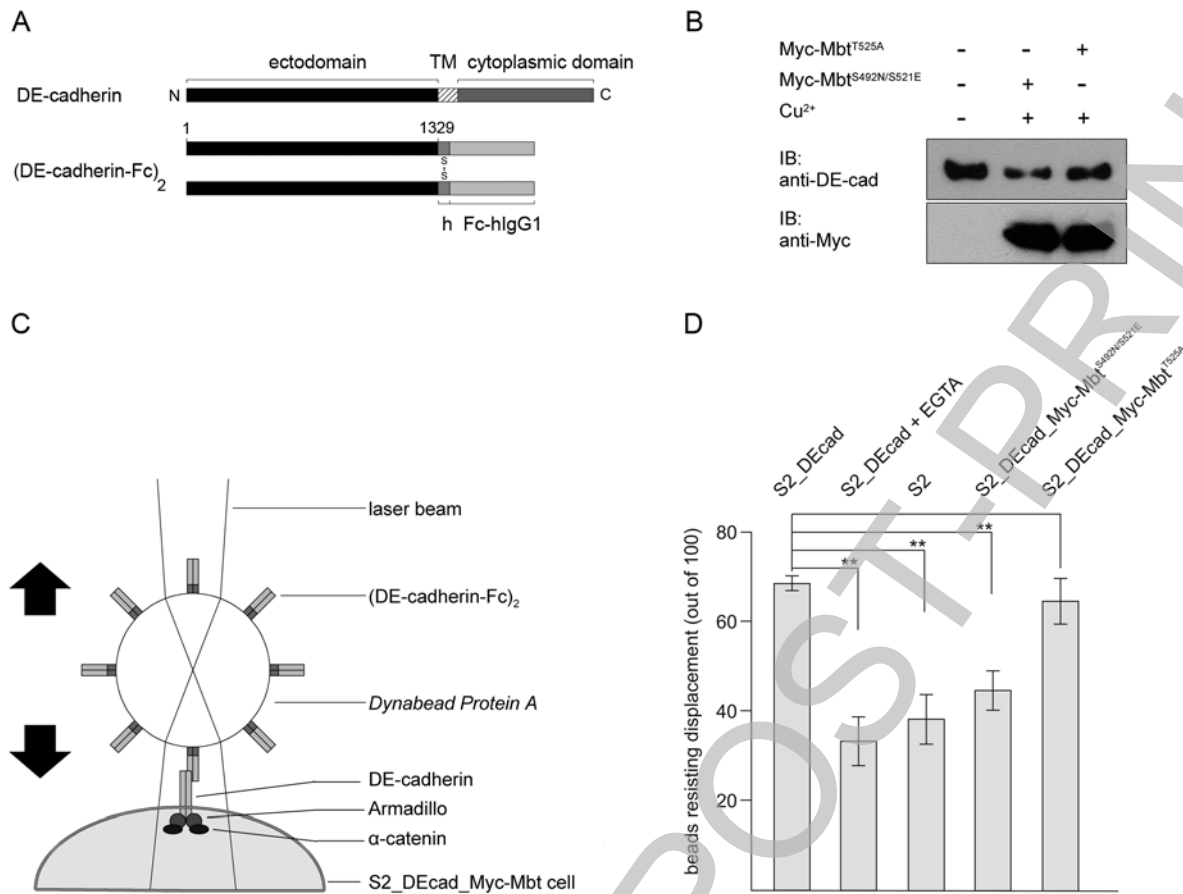


Figure 4

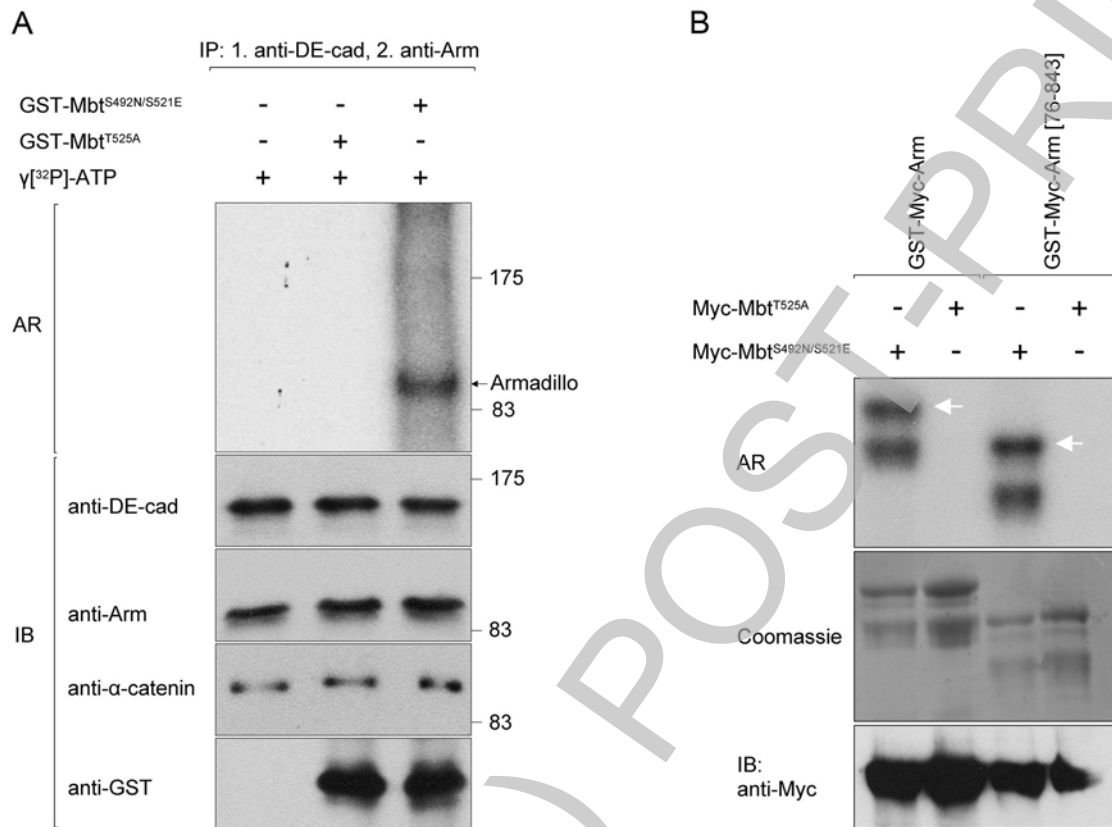


Figure 5

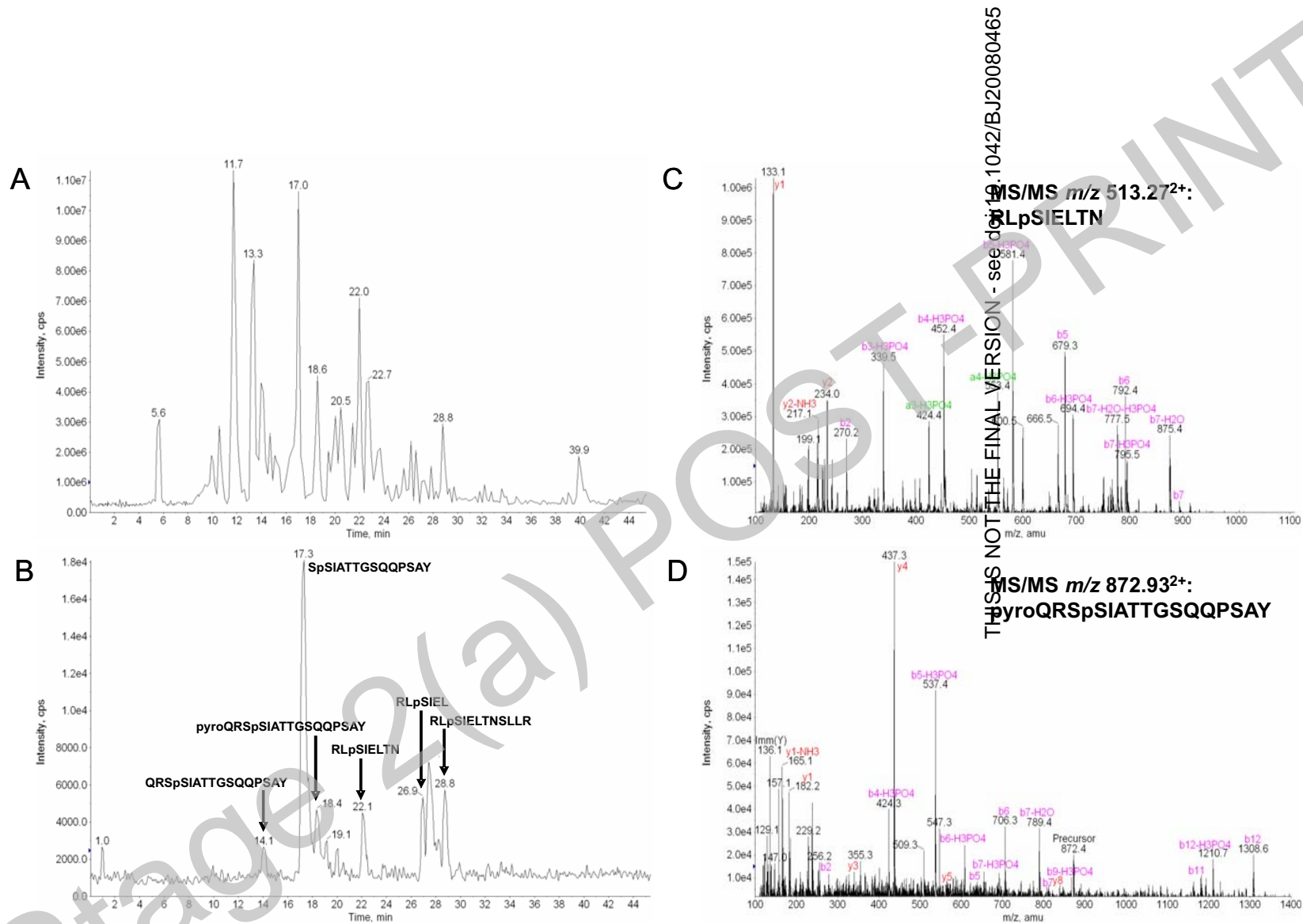


Figure 6

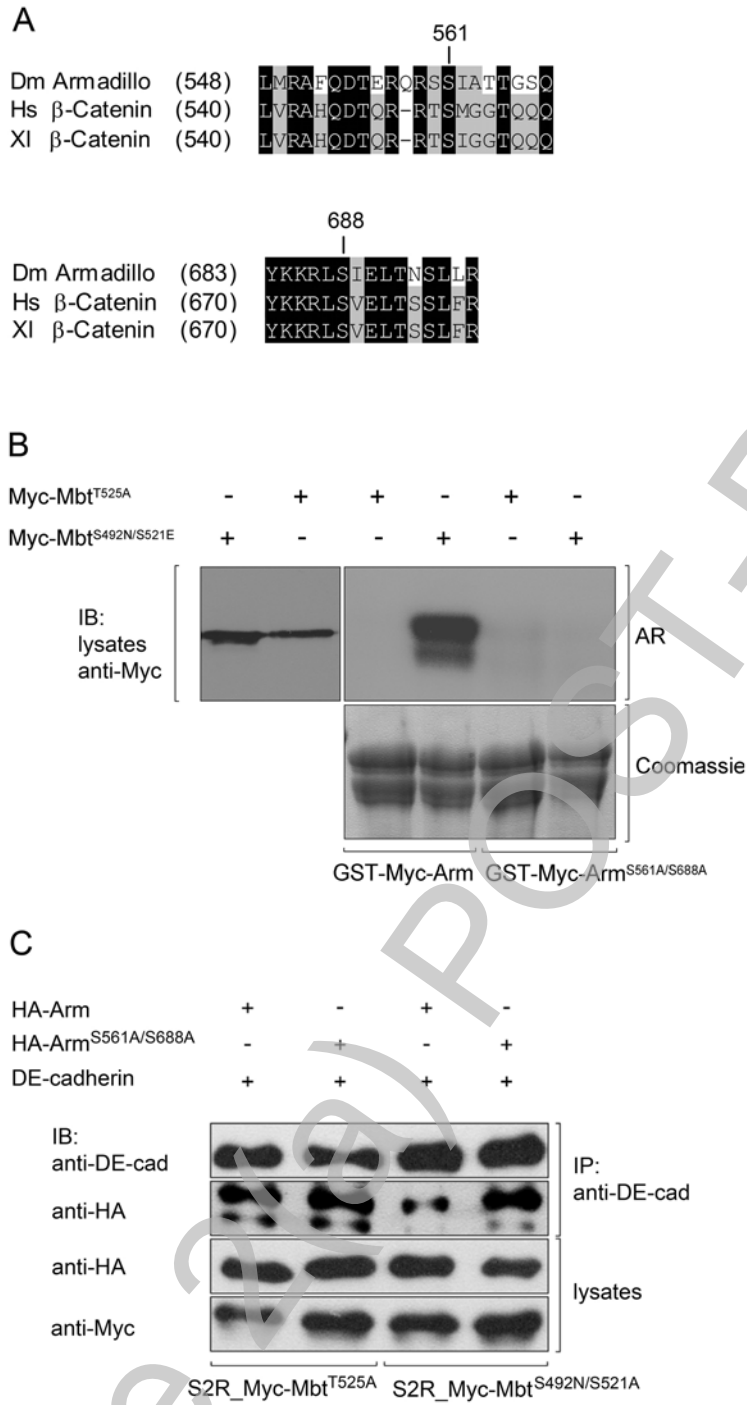


Figure 7