

ORIGINAL ARTICLE

 α -Catulin, a Rho signalling component, can regulate NF- κ B through binding to IKK- β , and confers resistance to apoptosisC Wiesner¹, G Winsauer¹, U Resch¹, M Hoeth¹, JA Schmid¹, J van Hengel^{2,3}, F van Roy^{2,3}, BR Binder¹ and R de Martin¹¹Department of Vascular Biology and Thrombosis Research, Medical University of Vienna, Vienna, Austria; ²Department for Molecular Biomedical Research, Ghent, Belgium and ³Department of Molecular Biology, Ghent University, Ghent, Belgium

Rho GTPases regulate diverse cellular functions including adhesion, cytokinesis and motility, as well as the activity of the transcription factors NF- κ B, serum response factor and C/EBP. α -Catulin, an α -catenin-related protein that shares structural similarities with cytoskeletal linker proteins, facilitates Rho signalling by serving as a scaffold for the Rho-specific guanine nucleotide exchange factor Lbc. We report here that α -catulin also interacts with a key component of the NF- κ B signalling pathway, namely the IKK kinase (IKK)- β . In co-immunoprecipitations, α -catulin can bind IKK- β and Lbc. Ectopic expression of α -catulin augmented NF- κ B activity, promoted cell migration and increased resistance to apoptosis, whereas knockdown experiments showed the opposite effects. Together, these features suggest that α -catulin has tumorigenic potential.

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Introduction

The nuclear factor- κ B (NF- κ B) family of transcription factors plays an essential role in the regulation of genes involved in a broad range of biological functions, including innate and adaptive immune responses, tissue differentiation and apoptosis (Baeuerle and Baltimore, 1996; Ghosh *et al.*, 1998; Baldwin, 2001). In mammals, the five cognate NF- κ B members, RelA (p65), RelB, c-Rel, p50 (NF- κ B1) and p52 (NF- κ B2) bind to DNA target sequences as homo- and heterodimers (Siebenlist *et al.*, 1994) to mediate gene expression. A variety of signals activate NF- κ B in different cell types, including proinflammatory, physical and microbial stimuli (Pahl, 1999; de Martin *et al.*, 2000). NF- κ B-regulated target genes include chemokines, cytokines, adhesion molecules and its own inhibitors I κ B α and I κ B β . They also include anti-apoptotic genes that are required for

preventing tumour-necrosis factor (TNF)- α -induced cell death (Beg and Baltimore, 1996; Stehlik *et al.*, 1998), as well as ‘anoikis,’ a form of apoptosis that occurs upon loss of contact during cell migration (Frisch and Francis, 1994).

Different extracellular stimuli activate the I κ B kinases IKK- α and - β , which phosphorylate I κ B α . This leads to ubiquitin-dependent degradation of I κ B α and translocation of NF- κ B to the nucleus. IKK- α and - β contain leucine zipper domains that mediate their homo- and heterodimerization, and HLH domains that physically interact with their kinase domains to function as intramolecular regulators (Zandi *et al.*, 1997). However, the IKK HLH domains may also interact with other proteins, and biochemical evidence indicates that indeed a variety of additional proteins may be loosely or transiently associated with the IKK complex (Bouwmeester *et al.*, 2004).

α -Catulin is a 73-kDa protein that has similarities to human vinculin and α -catenin, especially in the N-terminal region, which contains binding sites for β -catenin, talin and α -actinin (Janssens *et al.*, 1999). Amphipathic helices in the C-terminal homology region corresponding to α -catenin contain potential binding sites for the tight junction protein ZO-1 and the actin cytoskeleton (Janssens *et al.*, 1999; Park *et al.*, 2002), suggesting that α -catulin may act as a cytoskeletal linker protein. Recently, α -catulin was identified as a part of the Rho signalling pathway, serving as a scaffold protein for Lbc (Park *et al.*, 2002), a member of the dbl family of Rho guanine nucleotide exchange factors (GEFs) (Cerione and Zheng, 1996; Dutt *et al.*, 2004). Rho family GTPases play important roles during organization of the actin cytoskeleton and formation of focal adhesions (Ridley and Hall, 1992). α -Catulin was shown to interact with the N-terminal region of Lbc and facilitate Lbc-induced Rho signals (Zheng *et al.*, 1995; Park *et al.*, 2002).

In an attempt to identify novel proteins that modulate NF- κ B signalling or mediate its crosstalk with other pathways, we performed a genetic screen using the regulatory C-terminal HLH domain of IKK- β as bait, and identified α -catulin as an IKK-interacting protein. α -Catulin augments activation of NF- κ B after stimulation with the inflammatory stimuli TNF- α or IL-1, as well as activation of the Rho signalling pathway. This, together with its ability to bind both IKK- β and Lbc,

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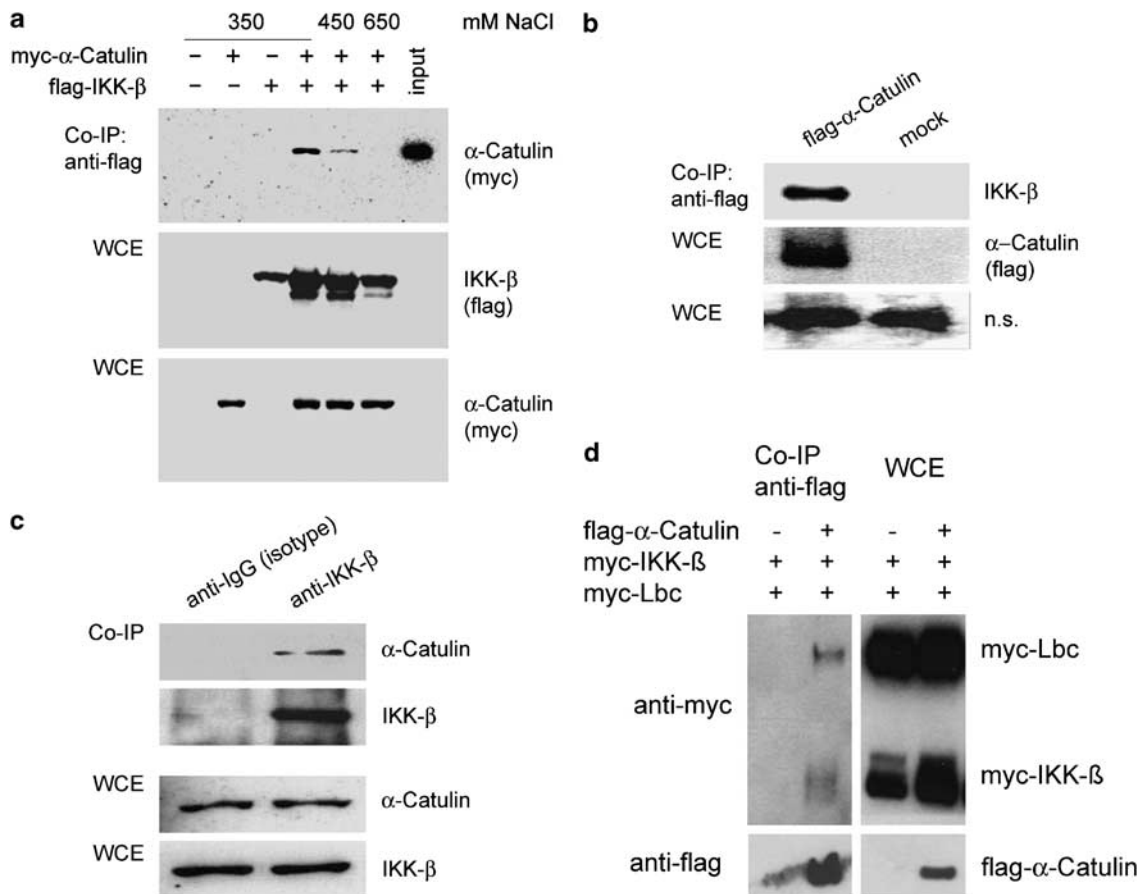


Figure 1 Co-immunoprecipitations of α -catulin with IKK- β . (a) Myc- α -catulin and flag-IKK- β were transfected into HEK293 cells, and IKK- β precipitated with anti-flag antibody followed by detection of α -catulin with anti-myc antibody. Different NaCl concentrations were used to probe for the stringency of the interaction. Input in the upper panel is 10% of whole cell extract (WCE) from α -catulin and IKK- β transfected cells. In the lower two panels, WCE from all samples were probed for IKK- β and α -catulin ('input' lane was not loaded again). (b) Semi-endogenous co-immunoprecipitation: after transfection of flag- α -catulin, immunoprecipitation was performed with anti-flag antibody, and co-precipitated endogenous IKK- β detected with an anti-IKK- β antibody. The lower panels show the presence of the transfected α -catulin in WCE, and a nonspecific band (ns; also in WCE) serving as loading control. (c) Co-immunoprecipitation of endogenous proteins: HeLa cell extracts were incubated with either anti-IKK- β antibody or an isotype control, and precipitated proteins analysed by western blotting using the 2E10 anti- α -catulin monoclonal antibody. Analysis of WCE is shown in the lower two panels. (d) Co-immunoprecipitation of Lbc and IKK- β with α -catulin. Cells were transfected with expression vectors as indicated, flag- α -catulin precipitated and myc-Lbc and myc-IKK- β detected by western blotting. The lower panels show the presence of α -catulin in the precipitate (left) and WCE (right).

suggests that α -catulin serves as a link between the two pathways. The biological consequences thereof are the promotion of cell migration and the protection of cells from apoptosis, features that are hallmarks of a gene with tumorigenic potential.

Results

Identification of α -catulin as an IKK- β interacting protein

Because it has been postulated that the HLH domain of IKK- β possesses a regulatory function, we used this region (amino acids 466–756) as a bait in a yeast two-hybrid screen. Screening of a human liver cDNA library yielded ~5.8 million transformants, of which 92 grew on selection plates. Novel interactions included the granulocyte macrophage colony-stimulating receptor α chain,

and a protein termed IKIP (Ebner *et al.*, 2003; Hofer-Warbinek *et al.*, 2004). Eight clones contained different C-terminal parts of α -catulin (CTNNAL1). Subsequently, the full-length cDNA was isolated from human umbilical vein endothelial cells (HUVEC) by reverse transcription (RT)-PCR. The open reading frame of α -catulin encodes a protein of 734 amino acids as described (Zhang *et al.*, 1998; Janssens *et al.*, 1999). The smallest clone contained the last 87 C-terminal amino acids (corresponding to positions 647–734), thereby revealing the site of interaction with IKK- β .

To confirm the binding of α -catulin to IKK- β , tagged constructs were transfected into HEK293 cells, and immunoprecipitations were carried out using increasing NaCl concentrations to determine the strength of the interaction. As shown in Figure 1a, α -catulin interacted with IKK- β in up to 450 mM NaCl, demonstrating strong binding. We further tested the interaction between

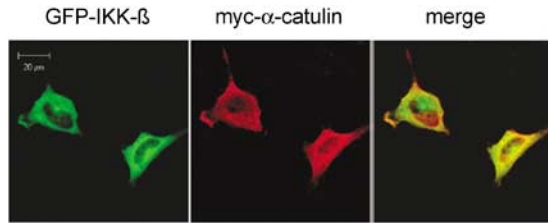


Figure 2 Co-localization of α -catulin and IKK- β . Myc- α -catulin was co-transfected together with a GFP-IKK- β fusion construct into HeLa cells. α -Catulin was detected by immunostaining with anti-myc and Alexa568-conjugated secondary antibodies, and viewed by confocal microscopy. Bars represent 20 μ m.

α -catulin and IKK- β using endogenous proteins. Both semi-endogenous (transfected α -catulin and endogenous IKK- β) and endogenous co-immunoprecipitations demonstrated the interaction of α -catulin and IKK- β (Figures 1b and c). Furthermore, binding of Lbc and IKK- β to α -catulin could be demonstrated (Figure 1d).

To test for co-localization of α -catulin with IKK- β , we co-transfected myc- α -catulin with an enhanced green fluorescent protein-IKK- β fusion construct (GFP-IKK- β), followed by immunofluorescence detection by confocal microscopy. IKK- β partially co-localized with α -catulin in the cytoplasm and at the plasma membrane (Figure 2).

Subcellular distribution of α -catulin

Using an antibody raised against recombinant α -catulin, we performed immunostaining of HUVEC cells. α -Catulin was distributed throughout the cell, including cytoplasm, cell membrane and nucleus (Figure 3a), with some variations between individual cells. We then generated a set of α -catulin mutants (Figure 3b), and analysed their subcellular distribution. Because in the yeast two-hybrid screen the C-terminal region containing the HLH domain of α -catulin was found to interact with IKK- β , we generated truncated forms of the protein. The deletions were of the entire N-terminal part except the IKK- β binding region (Δ 1–647; Δ N), the IKK- β binding region itself (Δ 647–734; Δ C1), or the extended binding domain including two potential tyrosine phosphorylation sites (Δ 404–734; Δ C2) (Figure 3b). Further, these potential tyrosine phosphorylation sites were mutated to Ala (Y534/536 \rightarrow AA). All α -catulin mutants contained an N-terminal myc tag. Due to the sequence similarity of α -catulin with α -catenin and vinculin, we envisaged that it might bind to cytoskeletal structures and therefore stained cells with phalloidin to reveal cellular structures and possible co-localization with actin.

In HUVEC, wild-type (wt)- α -catulin and its phosphorylation mutant (not shown) were again localized all over the cytoplasm, but they were also partially associated with the plasma membrane. In contrast to the endogenous protein, no significant nuclear staining could be observed. Although the reason for this discrepancy is not clear, it might be that overexpression of α -catulin favours the cytoplasmic localization, especially since it contains no apparent nuclear localization signal

and would therefore presumably depend on endogenous proteins for nuclear transport. Cells transfected with mutant Δ C1 and even more so with Δ C2, showed increased membrane staining with dots on the surface. The small C-terminal part, α -catulin- Δ N, however, was distributed both at the plasma membrane and in the cytoplasm, where small dot-like structures could be observed. Moreover, a number of cells expressing Δ N were small and exhibited membrane blebbing, an observation that prompted us to perform more specific studies to reveal a possible involvement of α -catulin in apoptosis (see below). A similar picture was observed in HeLa cells (not shown).

α -Catulin enhances NF- κ B activity after activation by inflammatory mediators and by the Rho signalling pathway

We first asked whether α -catulin as an IKK- β interacting protein would influence NF- κ B activity. HeLa cells were transfected with an NF- κ B luciferase reporter gene ($5 \times$ NF κ B-Luc) together with either wt- α -catulin or a control vector, and 24 h later they were treated for different times with TNF- α or IL-1. Increased luciferase levels were first observed after 3 h of treatment. From then on, α -catulin augmented both TNF- α - and IL-1-induced NF- κ B activation; the largest increase was seen 6 h after stimulation (Figure 4a).

We then transfected the different α -catulin constructs (wt, Δ N, Δ C1, Δ C2 and an antisense construct) together with IKK- α or IKK- β into HeLa cells, and analysed NF- κ B activity 24 h later. α -Catulin stimulated NF- κ B about 3.5-fold in the presence of IKK- β , but had only minor effects on basal levels and in IKK- α transfected cells, suggesting that IKK- β is the relevant I κ B kinase to mediate α -catulin functions towards NF- κ B. In contrast, all α -catulin mutants either suppressed NF- κ B activation (especially the Δ N mutant), or showed no effect (Figure 4b). In order to analyse in more detail the effects of inhibiting α -catulin, we generated two siRNA constructs in the vector pSuppressor, namely si-catu-1 and -2. Each of these constructs could reduce α -catulin mRNA and protein levels by 70–80%, but their combination had no additional effect (Figure 4c). They could also reduce the TNF- α -stimulated NF- κ B activation by \sim 30% (Figure 5c).

Since NF- κ B activation by Rho has been well documented (Perona *et al.*, 1997), we asked whether Lbc, a GEF for Rho that interacts with α -catulin (Park *et al.*, 2002) could activate NF- κ B and if so, whether modulation of α -catulin levels would influence Lbc-stimulated NF- κ B activation. Co-transfection of α -catulin increased NF- κ B activity \sim 3.5-fold in cells transfected with Lbc or Lbc plus RhoA, whereas α -catulin mutants again had little or no effect (Figure 5a). Also, co-transfection with RhoA alone showed a very minor effect. Activation of the Rho signalling pathway by lysophosphatidic acid (LPA) stimulated NF- κ B in the presence of α -catulin, whereas the α -catulin antisense construct (Figure 5b) or knock down by RNA interference (Figure 5c) diminished this activation. This demonstrates that α -catulin is

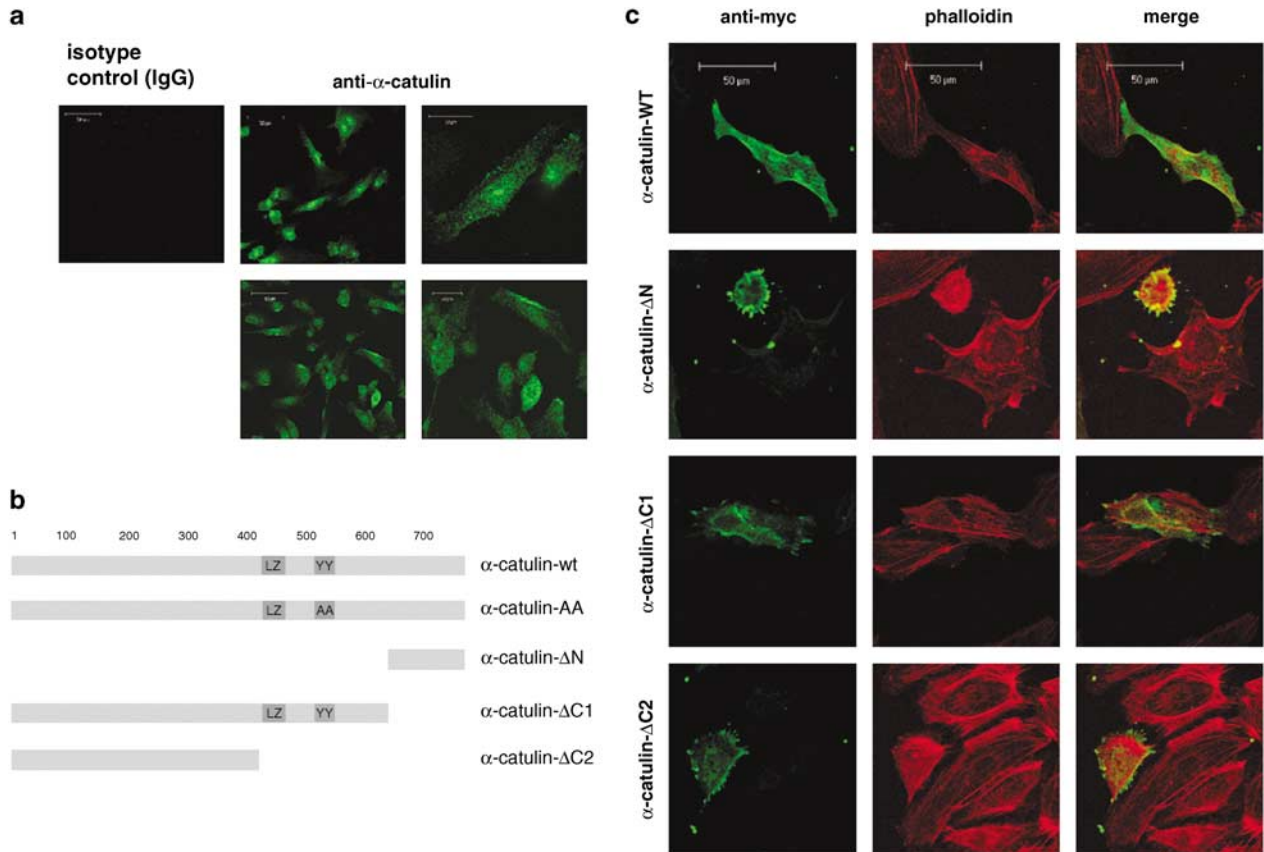


Figure 3 Subcellular localization of α -catulin. **(a)** Human umbilical vein endothelial cells (HUVEC) were stained with either control or anti- α -catulin monoclonal antibody 2E10, and Alexa488-conjugated anti-mouse secondary reagent, and viewed by confocal laser scanning microscopy. Different sections and magnifications are shown; bars represent 50 (left, middle) and 20 μ m (right panels). **(b)** Schematic representation of α -catulin mutants. LZ (leucine zipper) and YY (two Tyr) residues were mutated to Ala. The Δ N mutant comprises the IKK- β interaction domain. **(c)** Localization of α -catulin relative to actin. HUVEC cells were transfected with the respective constructs as indicated on the left and immunostained for α -catulin using Alexa488-conjugated secondary antibody, and for actin with rhodamine-phalloidin. Bars represent 50 μ m.

involved in the activation of NF- κ B not only by the classical inflammatory mediator TNF- α , but also by stimulation via the Rho signalling pathway. Moreover, knock down of α -catulin had a more pronounced effect on NF- κ B activation by LPA than by TNF- α , suggesting that it is more important for stimulation through Rho activators.

It has been suggested that α -catulin acts as a scaffolding protein for Lbc (Park *et al.*, 2002). Scaffolding proteins can affect signalling in a dose-dependent manner, facilitating signalling at low concentrations, but disrupting the pathway at high concentrations (Levchenko *et al.*, 2000). We therefore performed dose-response experiments by transfecting increasing amounts of α -catulin, and determined NF- κ B activity after stimulation with TNF- α or LPA. As shown in Figure 5d, low amounts of α -catulin enhanced NF- κ B activity, whereas high amounts inhibited it. This dose-dependency is consistent with the notion that α -catulin may act as a bridging molecule that links IKK- β to the Rho-Lbc complex.

To be consistent with such a role, α -catulin would also be expected to relay signals in the other direction,

namely from the NF- κ B to the Rho signalling pathway. We therefore assayed for Rho activation following inflammatory stimulation. Using a mutant SRE-Luc reporter gene where the TCF site has been deleted and that can serve as a read-out for Rho activity (Dutt *et al.*, 2004), knock down of α -catulin diminished not only the LPA-, but also the TNF- α -induced Rho activation (Figure 5e).

α -Catulin protects against apoptosis induced by serum starvation or TNF- α

We noted in some experiments that the α -catulin- Δ N mutant led to increased cell death 2 days after transfection. To determine whether this was due to apoptosis, we transfected cells with wt- α -catulin, Δ N- α -catulin and the siRNA construct si-catu-1, and analysed them by annexin V staining. To assay for both pro- and anti-apoptotic effects, cells were either left unstimulated, serum starved or treated with TNF- α plus cycloheximide, a combination that promotes apoptosis (Hsu *et al.*, 1995). Knock-down of α -catulin or expression of the α -catulin- Δ N mutant enhanced the number of apoptotic cells,

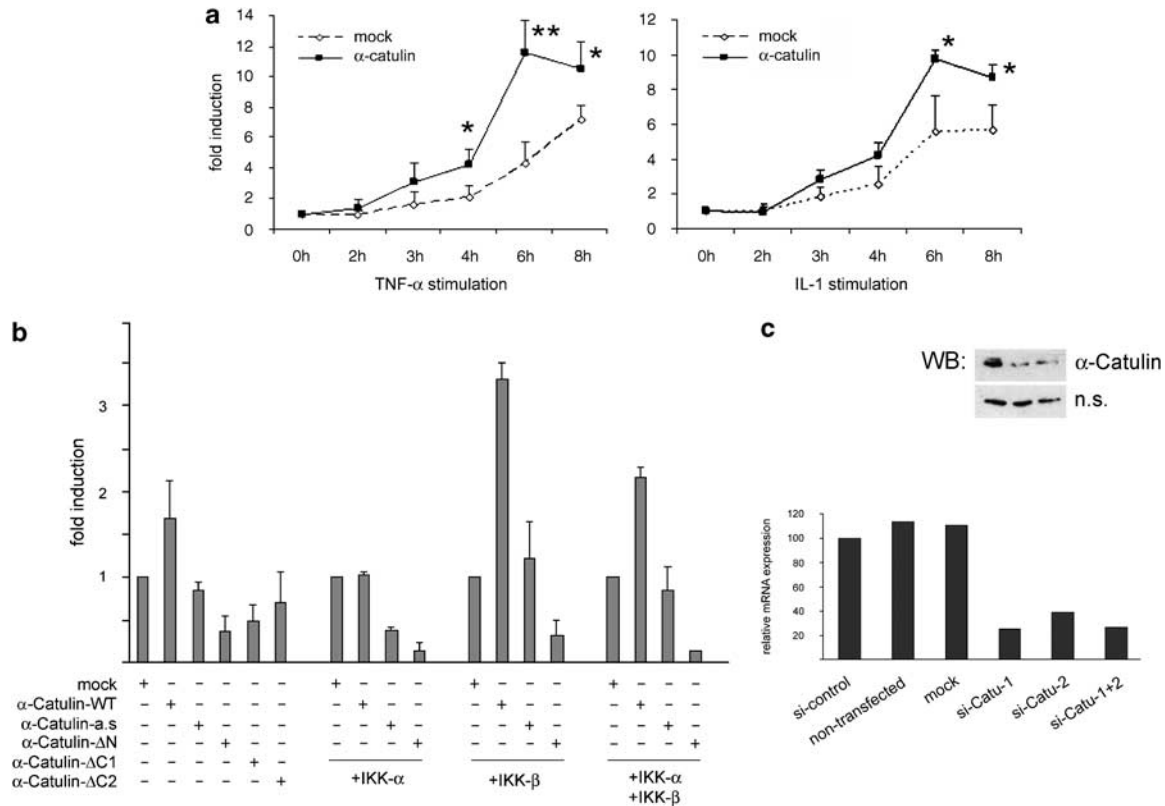


Figure 4 α -Catulin augments inflammatory NF- κ B activation. (a) A $5 \times$ NF- κ B-luc reporter gene was co-transfected into HEK293 cells together with α -catulin. After 24 h cells were stimulated for the indicated times with tumour-necrosis factor (TNF)- α (left) or IL-1 (right panel). Luciferase levels were normalized for a co-transfected Renilla luciferase control. *Indicates $P < 0.05$, ** $P < 0.01$. (b) Expression vectors for wt and mutant α -catulin (described in Figure 2), or an α -catulin antisense construct, were co-transfected together with IKKs and the $5 \times$ NF- κ B-Luc reporter as indicated. Luciferase values were determined 24 h later and normalized for co-transfected Renilla values. (c) Cells were transfected with two different vector-based siRNA constructs directed against α -catulin (si-catu-1, si-catu-2; Materials and Methods), and α -catulin mRNA and protein levels were analysed by real-time PCR (bars) and western blotting (inset), respectively. A representative experiment is shown.

whereas the wt protein (and the other mutants, not shown) had no effect (Figure 6a). These results were confirmed in a more detailed time-course experiment (Figure 6b). However, when extending the assay to longer time periods (24 and 36 h), a significant protective effect of α -catulin could be observed (Figure 6c). Concomitantly, the expression of several anti-apoptotic genes was induced by transfected α -catulin (Figure 6d); however, cyclin D1, which has been previously reported to be inhibited by α -catulin (Merdek *et al.*, 2004), was not affected. Together, these results demonstrate that α -catulin has anti-apoptotic properties, but that at least in this cell type and at earlier time points the basal levels of α -catulin (and of the anti-apoptotic genes) are sufficient for protection.

α -Catulin promotes cell migration

Since Rho family proteins, including Cdc42, Rac1 and RhoA, regulate signalling pathways that mediate actin cytoskeleton changes required for both cellular motility and cell-cell adhesion, we tested the effects of altered α -catulin expression on cell migration. HeLa cells were transfected with either α -catulin or the corresponding siRNA expression constructs, and assayed for migration

in a Transwell system. As shown in Figure 7a, α -catulin increased the number of cells migrating towards higher serum concentrations, whereas the siRNA constructs diminished it. When re-assaying the subcellular distribution of α -catulin in sparsely seeded HeLa cells, we also found a partial co-localization with actin at the leading edge of migrating cells, regions that correspond to lamellipodia (Figure 7b), indicating that α -catulin is involved in this process.

Discussion

There is ample evidence that both the NF- κ B and RhoA signalling pathways play multiple roles in tumorigenesis, of which cell migration, invasion and escape from apoptosis are important aspects (Schmitz *et al.*, 2000; Basseres and Baldwin, 2006). The finding that α -catulin comprises a part of these two signalling pathways and influences their respective cellular functions is therefore of considerable interest.

The multitude of cellular functions of NF- κ B, the complexity of its regulation, and the demonstrated crosstalks with other signalling pathways suggest that

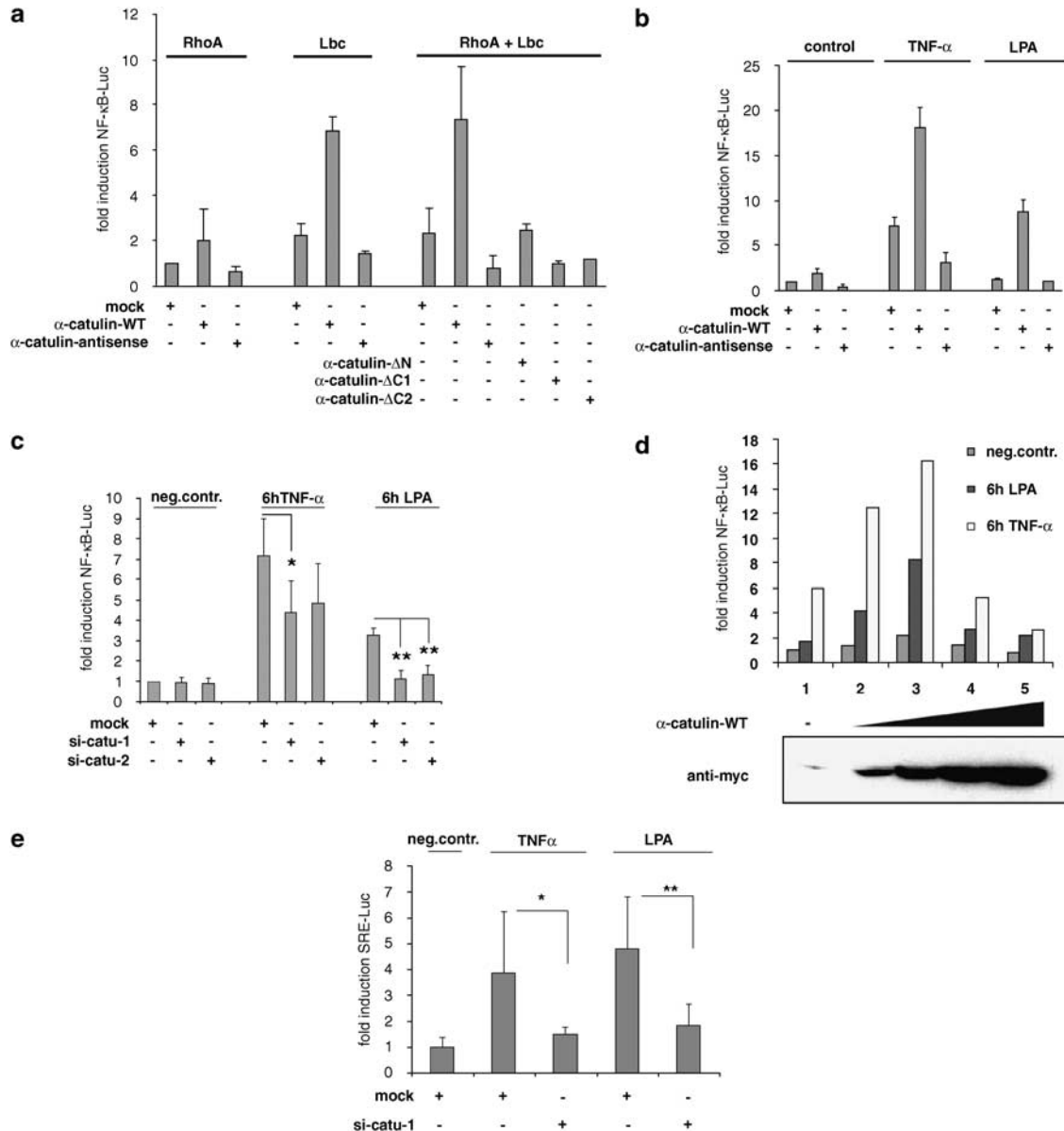


Figure 5 α -Catulin relays RhoA mediated NF- κ B, and tumour-necrosis factor (TNF)- α mediated Rho activation. (a) HEK293 cells were transfected with RhoA and Lbc expression vectors, and with wt, antisense and mutant α -catulin constructs as indicated. NF- κ B activity was determined using the 5 \times NF- κ B-luc reporter. (b) Comparison of NF- κ B activation induced by TNF- α or lysophosphatidic acid (LPA). Cells were transfected with wt or antisense α -catulin, and 24 h later stimulated with TNF- α or LPA for 6 h. (c) Same as in (b), but using RNA interference instead of the antisense construct. *Indicates $P < 0.05$ and ** $P < 0.01$. (d) α -Catulin in low concentrations facilitates NF- κ B activation, but inhibits it in high concentrations. Increasing amounts of the myc- α -catulin expression vector were transfected, and NF- κ B activity was measured with and without TNF- α or LPA stimulation using the 5 \times NF- κ B-reporter. Levels of myc- α -catulin in the extracts were determined by western blotting with anti-myc antibody and are shown below. A representative experiment is shown. (e) Knock-down of α -catulin diminishes activation of Rho by LPA or TNF- α . After transfection with the siRNA construct directed against α -catulin, cells were stimulated for 5 h with TNF- α or LPA, and assayed for Rho activation using a TCF-mutated SRE-Luc reporter (Dutt *et al.*, 2004).

many other regulators of this signalling pathway remain to be identified. We have therefore set out to explore such novel regulators, starting our studies with IKK- β , which is a central part of this pathway. We concentrated on the C-terminal domain of IKK- β , which has been demonstrated previously to have regulatory functions.

Evidence that α -catulin binds to IKK- β is provided by co-immunoprecipitations with transfected and

endogenous proteins, as well as by (partial) co-localization in the cytoplasm and at the cell membrane. The isolation of different clones from the yeast two-hybrid screen enabled us to narrow down the interaction region in α -catulin to its C-terminal 87 amino acids. In different transfection experiments, α -catulin augmented NF- κ B activity, and its knock down reduced it. Thereby both the basal as well as the TNF- α - and IL-1-induced activation

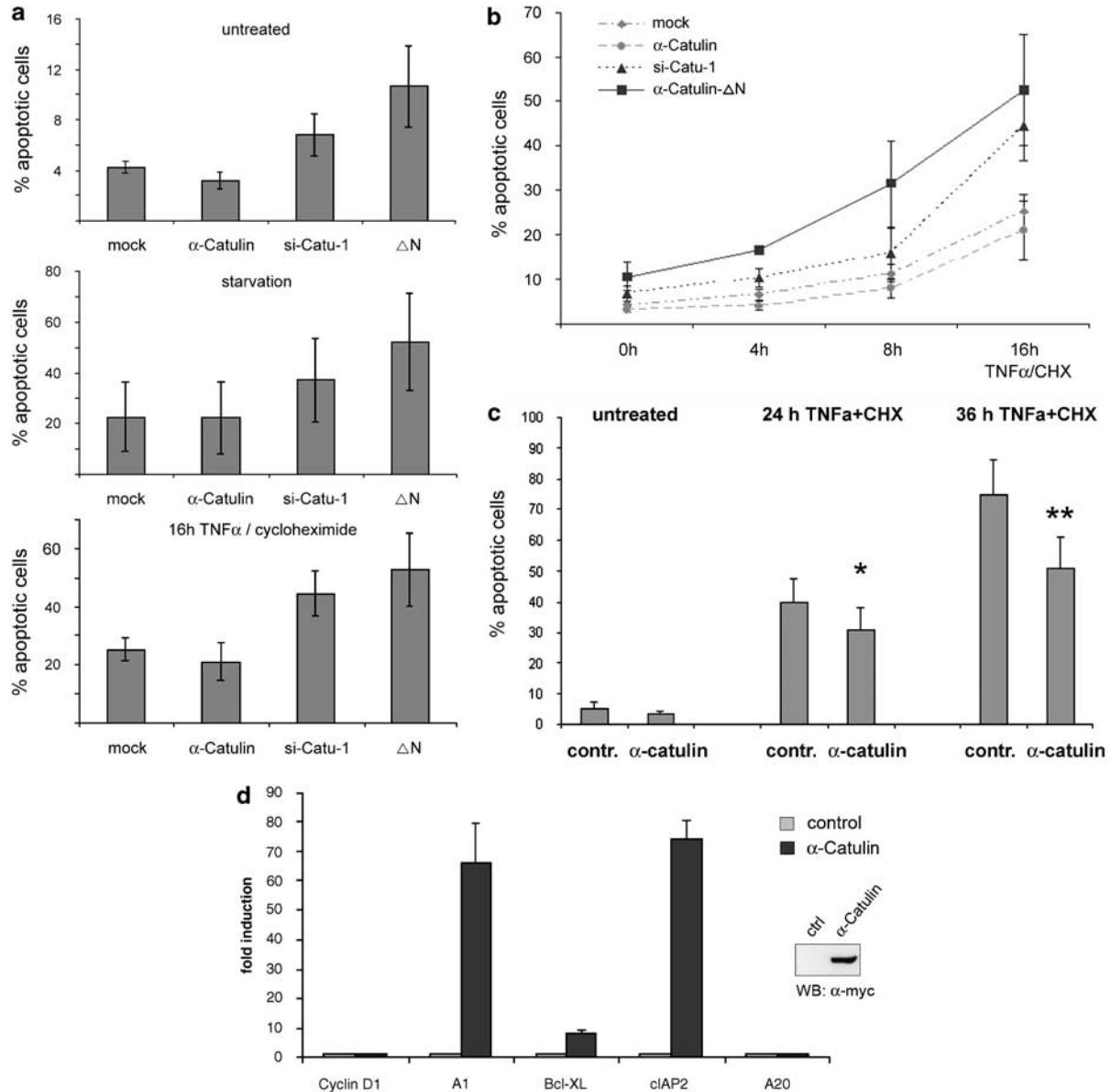


Figure 6 (a) Expression vectors for control (mock), α -catulin, its N-terminal deletion mutant (Δ N), or an siRNA construct (si-catu-1) were transfected into HEK293 cells. After 48 h, cells were either left untreated (upper panel), serum-starved for 16 h (middle panel), or treated with a combination of TNF- α and CHX for 16 h (lower panel). The percentage of apoptotic cells was determined by FACS after annexin V staining. (b) Cells were treated with TNF- α and cycloheximide (CHX) as in (a), but were analysed at different time points as indicated. (c) Cells were transfected with control or α -catulin expression vectors and assayed after 24 and 36 h as in (a). *Indicates $P < 0.05$, ** $P < 0.01$ as compared to control transfected cells at the same time point. (d) α -Catulin enhances the expression of anti-apoptotic genes. Myc- α -catulin was transfected into HEK293 cells, and the expression of a set of anti-apoptotic genes and of cyclin D1 was analysed 24 h later by real-time PCR. The inset shows a western blot to confirm expression of the transfected α -catulin.

were affected. Although we also observed binding to IKK- α (data not shown), IKK- β seems to be the relevant kinase for α -catulin mediated NF- κ B activation (Figure 4b).

Despite its first description in 1999 (Janssens *et al.*, 1999), no clear function has been assigned to α -catulin yet. Surpili *et al.* (2003) found that it interacts with NEK, a poorly characterized member of a family of kinases involved in cell cycle regulation. The group of Toksoz found that α -catulin inhibits Ras-mediated

signals to the cyclin D1 promoter (Merdek *et al.*, 2004). α -Catulin shares a number of structural similarities with cytoskeleton-associated proteins, including homologies to vinculin, α E-catenin and α N-catenin (Janssens *et al.*, 1999; Park *et al.*, 2002). α -Catenin is a component of the cadherin-catenin complex involved in cell-cell contacts and focal adhesions (Aberle *et al.*, 1996). It links the adhesion complex to the actin cytoskeleton by binding several actin-binding proteins including α -actinin, vinculin and ZO-1 (Itoh *et al.*, 1993;

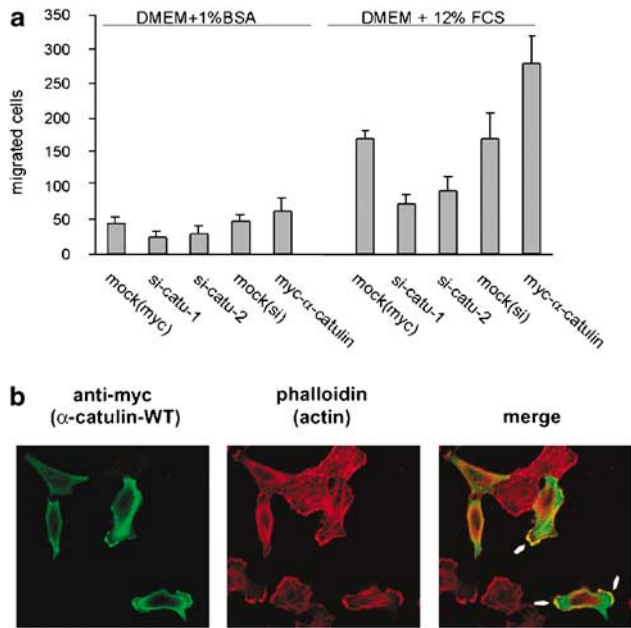


Figure 7 α -Catulin promotes cell migration and is localized at the leading edge of migrating cells. (a) HeLa cells were transfected with myc- α -catulin, α -catulin siRNA expression constructs (si-catu-1, -2), control vector (mock(myc)) or control (mock(si)). Migration was assayed using the Transwell system. Cells in the upper chamber were seeded in a medium containing 1% bovine serum albumin (BSA), while the lower chamber contained medium with either 1% BSA (left) as control, or 12% fetal calf serum (FCS) (right). (b) Myc- α -catulin was transfected into sparsely seeded HeLa cells, and 24h later stained for α -catulin with anti-myc, followed by Alexa488-conjugated secondary antibody, and for actin with rhodamine-phalloidin.

Knudsen *et al.*, 1995; Watabe-Uchida *et al.*, 1998). α -Catenin has been proposed to modulate actin polymerization states locally at cadherin-mediated cell-cell junctions (Drees *et al.*, 2005; Yamada *et al.*, 2005). Furthermore, α -catenin itself can directly bind to actin filaments (Rimm *et al.*, 1995). In α -catulin, the amphipathic helices corresponding to the N-terminal region of α -catenin have binding sites for β -catenin, α -actinin, and vinculin, and are conserved. The C-terminal region of α -catulin also contains the putative binding site for the ZO-1 cytoskeletal linker and the actin cytoskeleton. Together, these conserved structural elements suggest that α -catulin may bind to signalling and/or cytoskeleton complexes at the cytoplasmic membrane.

One important aspect for our studies that is in line with these inferred functions is the finding that α -catulin binds to Lbc, a member of the Dbl family of Rho GEFs (Park *et al.*, 2002). We therefore hypothesized that, by binding to Lbc and to IKK- β , α -catulin could serve as a scaffold protein for both proteins, thereby enabling crosstalk between the two corresponding signalling pathways. Indeed, a number of studies have reported crosstalk between the RhoA signalling pathway and NF- κ B (Perona *et al.*, 1997; Montaner *et al.*, 1998, 1999; Hippenstiel *et al.*, 2002). In addition, members of the transforming protein family Dbl of Rho GEFs efficiently activate transcription from NF- κ B-responsive

elements (Whitehead *et al.*, 1999), but the molecular basis for this activation remained elusive.

We performed a series of experiments to study the hypothesis that α -catulin links the two signalling pathways. First, activation of the Rho pathway, either by co-transfected Lbc or RhoA plus Lbc, or by LPA, resulted in increased NF- κ B activity. Second, this activation could be reduced at least partially by interfering with α -catulin expression. Vice versa, knock down of α -catulin diminished Rho activity after stimulation with the inflammatory mediator TNF- α , demonstrating signal transduction in both directions. Third, dose-response experiments demonstrated an α -catulin-dependent increase of NF- κ B activity at low doses, but inhibition at higher amounts (Figure 5d). This is consistent with the role of a bridging molecule that facilitates the connection of its binding partners at low concentrations, but disrupts it at higher concentrations due to out titration (Levchenko *et al.*, 2000). It is also consistent with the finding (from the yeast two-hybrid screen) that α -catulin binds IKK- β via its C-terminus, whereas its interaction with Lbc occurs at the N-terminal part (Park *et al.*, 2002), which would allow simultaneous binding of both proteins.

Concerning the cellular function of α -catulin, the Rho family GTPases have been recognized as key regulators of actin cytoskeleton reorganization, formation of focal adhesions and several aspects of cell shape remodelling, such as the one occurring during cell migration (Paterson *et al.*, 1990; Ridley and Hall, 1992). To assess whether α -catulin is involved in these processes, we performed a cell migration assay. Ectopic expression of α -catulin promoted, whereas RNA interference impaired migration of HeLa cells (Figure 7). The partial localization of α -catulin to the leading edge of migrating cells, regions corresponding to lamellipodia, could also indicate a functional association with Rac1, through either mutual influence or even physical association, which we have not tested. It is, however, interesting to note that already more than a decade ago Rac1 been found to activate NF- κ B (Sulciner *et al.*, 1996) and, more recently, that it is the balance between Rho and Rac activity that controls lamellipodia extension (Brock and Ingber, 2005).

In addition, and based on our initial observations that cells expressing certain α -catulin mutants were prone to cell death, we examined its involvement in apoptosis. Again, interfering with α -catulin expression by RNA interference or by expression of the Δ N mutant promoted apoptosis, whereas expression of the wt protein provided protection at later time points (Figure 6). In line with this, α -catulin enhanced the expression of the anti-apoptotic genes A1, Bcl-x1 and cIAP2 (but not A20). Although the reason for the lack of A20 induction is not clear, the common feature of these four genes is their regulation through NF- κ B. Also, we could not confirm the reported inhibition of cyclin D1 by α -catulin (Merdek *et al.*, 2004), possibly due to differences in the experimental system.

In summary, these data indicate that α -catulin, through its binding to both IKK- β and Lbc, may function to

facilitate the crosstalk between the NF- κ B and Rho signalling pathways. In situations where cells lose contact with the extracellular matrix, such as during cell division or migration, and become susceptible to apoptosis (anoikis) (Frisch and Francis, 1994), coordinated activation of NF- κ B and Rho signalling and the expression of anti-apoptotic genes could help to protect them from undergoing cell death. Increased cell migration and resistance to apoptosis could also be relevant under pathological conditions such as transformation events that result in elevated α -catulin levels.

Materials and methods

Cell culture

HeLa and HEK293 cells were obtained from American type culture collection (ATCC) and maintained in Dulbecco's modified Eagle's medium with 25 mM HEPES, antibiotics, glutamine and 10% fetal calf serum (FCS). Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in M199 medium supplemented with 20% FCS, antibiotics, endothelial cell growth supplement and Heparin as described (Zhang *et al.*, 1995).

Expression vectors

The full-length cDNAs of α -catulin and Lbc were isolated by RT-PCR from HUVEC and cloned into pCMVmyc (Clontech, Saint-Germain-en-Laye, France). The antisense construct contained the full-length cDNA in the opposite orientation. Mutations of the potential tyrosine phosphorylation sites of α -catulin (substitutions of Y534 and Y536 to Ala) were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands). The α -catulin mutants lacking the N-terminal 647 amino acids (Δ N) or the C-terminal 86 or 330 amino acids (Δ C1 or Δ C2) were generated by restriction digest with PvuII/EcoRI (Δ N) or by partial PvuII/NotI (Δ C1 or Δ C2), blunting and religation. To generate α -catulin siRNA expression constructs, double-stranded oligonucleotides were cloned into the XhoI-XbaI sites of the vector pSuppressor (Imgenex, San Diego, CA, USA). The sequences of all the primers are given in the Supplementary data. Expression vectors for flag-IKK- β and GFP-IKK- β were kindly provided by M Karin, and those for RhoA by W Graier. The 5 \times NF- κ B-Luc and Renilla luciferase reporter constructs were from Stratagene and Clontech, respectively, the SRE-Luc vector containing a mutated TCF binding site for measuring Rho activity was provided by D Toksoz (Dutt *et al.*, 2004).

Yeast two-hybrid screen

A C-terminal part of human IKK- β (aa 466–756) containing the HLH domain was cloned in the bait vector pAS2-1 (Clontech), and transformed into the PJ69 yeast reporter strain (Gietz *et al.*, 1997). No autoactivation was detected after transformation with an empty library vector. A cDNA library from human liver with a complexity of 3×10^6 independent clones (Clontech) was used for screening. From ~ 5.8 million transformants, 92 grew on selection plates, and were further tested for β -galactosidase activity. Inserts were isolated from yeast colonies by PCR and sequenced. Back-transformation of the clones was carried out to exclude activation by interaction of the prey with the empty bait vector.

Transfections and reporter assays

HEK293 or HeLa cells were seeded into six-well (35-mm diameter) plates at 5×10^5 cells per well and transfected by the calcium

phosphate precipitation method using 4 μ g of DNA in total. They were stimulated 24 h later with TNF- α at 20 ng ml⁻¹ or IL-1 α at 10 ng ml⁻¹ (both from Biosource, Vienna, Austria). Oleoyl-L- α -lysophosphatidic acid (Sigma, Vienna, Austria) was used at a final concentration of 50 μ M, and cells were serum-starved for 12 h prior to stimulation. For luciferase reporter assays, cells were lysed and assayed for luciferase and Renilla-luciferase activity according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega, Mannheim, Germany). Reporter gene assays were performed in triplicates, and mean and s.d. values are shown. Significance was calculated by the Student's paired *t*-test.

Co-immunoprecipitation and western blotting

HEK293 cells were transfected in six-well plates. For western blotting, proteins were extracted 24 h later on ice in a buffer containing 0.5% Nonidet P-40, 1 mM EDTA and 10% (v/v) protease inhibitors (Roche, Vienna, Austria). For co-immunoprecipitation, this buffer was supplemented with 50 mM HEPES (pH 7.4), 350 mM NaCl, 20 mM β -glycerophosphate, 1 mM benzamide, 50 mM NaF, 1 mM Na₃VO₄, 5 mM para-nitrophenyl phosphate and 2 mM dithiothreitol. Cellular debris was removed by centrifugation at 10 000 *g* for 10 min, and protein concentrations were determined by the Bradford assay. Equal amounts of total protein extracts were separated by 7.5–12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond C nitrocellulose membranes (Amersham Pharmacia Biotech, Vienna, Austria). Membranes were blocked with 5% nonfat milk in phosphate-buffered saline (PBS), and immunodetection was carried out using the antibodies described below.

Flag-tagged proteins were immunoprecipitated with anti-flag M2 antibody (Sigma) at 4 °C for 3 h; immunoprecipitates were washed 3–5 times in lysis buffer, resuspended in SDS-PAGE sample buffer, and subjected to SDS-PAGE, followed by transfer to nitrocellulose membranes and immunodetection. The monoclonal anti-myc antibody (clone 4A6) was kindly provided by E Ogris, Vienna Biocenter, and can be purchased from Upstate Biotechnology (Vienna, Austria). The monoclonal anti-IKK- β antibody was from Imgenex. As secondary reagents, horseradish peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit antibodies (Amersham Pharmacia Biotech) were used and detected by chemiluminescence (West Pico, Pierce, Rockford, IL, USA).

Generation of monoclonal antibodies against α -catulin

An RT-PCR product comprising the complete open reading frame of human α -catulin was cloned in the prokaryotic expression vector pLX32TR-X-E, providing a C-terminal E-tag together with an N-terminal thioredoxin-tag (vector and construct were made by the VIB Protein Expression & Purification Service Facility, Ghent, Belgium). Monoclonal antibodies were generated by injection of 30 μ g purified fusion protein mixed with Titermax gold (Sigma) into C57Bl/6 mice. Boosts were given in 4-week intervals, and sera were tested by ELISA until a titre of 1:10 000 without loss of reactivity was obtained after three months. Hybridomas were generated by fusion of spleen cells with Sp20_Ag14 myeloma cells. Supernatants of hybridoma cell lines that were positive in ELISA were tested by Western blotting and immunofluorescence for recognition of either endogenous α -catulin or transfected myc tagged α -catulin. Different hybridoma cell lines were cloned; 2E10 was used in all experiments reported here.

Real-time PCR

Total RNA was extracted from HEK293 cells using the High Pure RNA Isolation Kit (Roche). 1 μ g of total RNA was

reverse-transcribed with SuperScript TM II using random primers as specified by Invitrogen (Lofer, Austria). Real-time PCR was performed in a Light Cycler (Roche) with SYBR Green for detection, according to the manufacturer's instructions. Oligonucleotides were designed to avoid amplification of potential contaminating genomic DNA. Primer sequences are given in the Supplementary data.

Transient transfection and immunostaining

HeLa cells or HUVEC were seeded into fibronectin-coated ($10 \mu\text{g ml}^{-1}$; Sigma) chamber-slides, and transfected with either myc- α -catulin-wt, myc- α -catulin- Δ N, myc- α -catulin- Δ C1, myc- α -catulin- Δ C2, EGFP-IKK- β or EYFP-IKK- α using JetPEI-RGD (Polyplus-transfection, Illkirch, France) as described by the manufacturer. After 24 h, cells were fixed with 4% paraformaldehyde in PBS for 15 min at 25 °C, and permeabilized with 0.1% Triton X-100 in PBS for 7 min. Proteins were immunodetected with a monoclonal anti-myc antibody, followed by incubation with Alexa488- or Alexa568-conjugated rabbit anti-mouse antibodies (Molecular Probes, Lofer, Austria). Chamber slides were mounted onto coverslips in 60% glycerol-Tris-buffered saline, and viewed under a confocal microscope (LSM 510, Zeiss, Vienna, Austria).

Cell migration assay

HeLa cells transfected with either α -catulin-wt or the siRNA expression constructs were trypsinized 48 h later, labelled with CMFDA (Molecular Probes), and resuspended in Dulbecco's

modified Eagle's medium (DMEM) per 1% bovine serum albumin (BSA). $100 \mu\text{l}$ containing 10 000 cells were placed in the upper chamber of a transwell (Costar, Cambridge, MA, USA) with $8 \mu\text{m}$ pore size, and $500 \mu\text{l}$ DMEM containing either 1% BSA or 12% FCS was placed in the lower chamber. After incubation for 3 h, cells on the lower part of the filter were harvested and counted by fluorescence-activated cell sorting (FACS) analysis.

Abbreviations

HUVEC, human umbilical vein endothelial cells; I κ B, inhibitory subunit of NF- κ B; IKK- β , I κ B α kinase- β ; NF- κ B, nuclear factor- κ B; wt, wild-type.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).