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Running head: ADAM12-ILK interaction

Key words: ADAM12, ILK, hepatic stellate cells

Abbreviations

ADAM, a disintegrin and metalloprotease; ILK, Integrin Linked kinase; ECM, extracellular matrix; HSC, hepatic stellate cell; TGF-β, transforming growth factor-beta.

ABSTRACT

Based on its shedding and binding activities, the disintegrin and metalloprotease 12 (ADAM12) has been implicated in cell signaling. Here, we investigate the intracellular protein interaction network of the transmembrane ADAM12L variant using an integrative approach and we identify the integrin-linked kinase (ILK) as a new partner for ADAM12L cellular functions. We demonstrate that ADAM12L coimmunoprecipitates with ILK in cells and that its cytoplasmic tail is required for this interaction. In human cultured hepatic stellate cells (HSCs), which express high levels of endogenous ADAM12L and ILK, the two proteins are redistributed to focal adhesions upon stimulation of a β1 integrin-dependent pathway. We show that down-regulation of ADAM12L in HSCs leads to cytoskeletal disorganization and loss of adhesion. Conversely, up-regulation of ADAM12L induces the Akt Ser^{473} phosphorylation-dependent survival pathway *via* stimulation of β1 integrins and activation of PI3K. Depletion of ILK inhibits this effect, which is independent of ADAM12L proteolytic activity and involves its cytoplasmic domain. We further demonstrate that overexpression of ADAM12L promotes kinase activity from ILK immunoprecipitates. Our data suggest a new role for ADAM12L in mediating the functional association of ILK with β1 integrin to regulate cell adhesion/survival through a PI3K/Akt signaling pathway.

INTRODUCTION

ADAM12 is a member of the disintegrin and metalloprotease (ADAM) protein family whose members are cell-surface multi-domain proteins involved in ectodomain shedding, adhesion, and signaling activities (Edwards *et al.*, 2008). ADAM archetypes are transmembrane proteins that contain propeptide, extracellular metalloprotease, disintegrin-like, cystein-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic domains.

In humans, the expression of ADAM12 is mainly associated with adult skeletal, cardiac and smooth muscle and is dramatically increased in several pathologies, including cancer (Kveiborg *et al.*, 2008). Within tissues, ADAM12 up-regulation has been described in both cancer and stromal cells and increased urine protein levels were also reported in patients with breast or bladder tumors. Genome-wide analyses of human tumors identified ADAM12 as a new candidate cancer gene (Sjoblom *et al.*, 2006) and it is now considered as a prognosis marker for human bladder cancer (Roy *et al.*, 2004; Frohlich *et al.*, 2006) and breast cancer (Pories *et al.*, 2008; Narita *et al.*, 2010). In support of its involvement in cancer, ADAM12 was shown to regulate tumor progression in genetically modified mouse models. However, the mechanism involved remains unclear (Kveiborg *et al.*, 2005; Peduto *et al.*, 2006).

The human ADAM12 gene is expressed as two alternatively spliced transcripts that give rise to transmembrane ADAM12L and secreted ADAM12S forms, the latter lacking the transmembrane and cytoplasmic domains. Both ADAM12 variants can act as proteases and have been shown to cleave IGFBP3 and IGFBP5 (Loechel *et al.*, 2000; Shi *et al.*, 2000), EGF receptor ligands (Asakura *et al.*, 2002; Horiuchi *et al.*, 2007), Notch ligand Delta-like 1 (Dyczynska *et al.*, 2007) and placental oxytocinase (Ito *et al.*, 2004). Besides its metalloprotease activity, the membrane-anchored ADAM12L long form is involved in cell-

cell and cell-matrix interaction via its binding to cell-surface molecules that include integrins (Eto *et al.*, 2000; Kawaguchi *et al.*, 2003), syndecans (Iba *et al.*, 2000) and type II TGF-β receptor (Atfi *et al.*, 2007). However ADAM12L is not constitutively addressed to the membrane and its distribution at the cell surface is a dynamic process that requires PKCε activation (Sundberg *et al.*, 2004), recruitment of RACK1 (Bourd-Boittin *et al.*, 2008) and c-Src activity (Stautz *et al.*, 2010). All these events take place in response to external cell stimulation such as integrin engagement and depend on the intracellular domain of ADAM12L since its truncation prevents translocation of the protein (Hougaard *et al.*, 2000). The cytoplasmic tail of ADAM12 has been shown to physically interact with several signaling proteins, including the Src-non receptor tyrosine kinases, c-Src and YES (Suzuki *et al.*, 2000), the adapter proteins Grb2 (Suzuki *et al.*, 2000) and Fish (Abram *et al.*, 2003), the regulatory subunit of phosphatidylinositol 3-kinase, p85α (Asakura *et al.*, 2002), the cytoplasmic PACSIN3 phosphoprotein (Mori *et al.*, 2003), eve-1, an EEN binding protein implicated in shedding activity (Tanaka *et al.*, 2004) and two actin-related proteins, α-actinin-1 and -2 (Galliano *et al.*, 2000; Cao *et al.*, 2001). This complex protein interaction network contributes to the regulation of ADAM12L translocation and function and might in turn play a pivotal role in the coordination of cell signaling and responses to the microenvironment.

In liver cancer, we have previously demonstrated that up-regulation of ADAM12 expression was associated with the activation of hepatic stellate cells (HSCs) (Le Pabic *et al.*, 2003) and the regulation of the TGF-β signaling pathway (Atfi *et al.*, 2007). In addition, our data showed that type I collagen, the major collagen in fibrosis tissue, promoted the localization of ADAM12 to the surface of HSCs through a β1 integrin-dependent mechanism (Bourd-Boittin *et al.*, 2008). In this study, we have explored the ADAM12L protein interaction network using an integrative data-mining approach. We identify the Integrin-

Linked Kinase (ILK), a kinase associated with human malignancies, as a new potential partner for ADAM12L. Our results demonstrate that ADAM12L co-immunoprecipitates with ILK and that stimulation of HSCs by type I collagen induces the recruitment of both proteins to focal adhesion-like structures. We show that down-regulation of ADAM12L leads to a loss of cell adhesion while over-expression induces the Akt-dependent survival pathway and increases the kinase activity of ILK immunoprecipitates. We propose that ADAM12 plays a major role in adhesion-cell survival processes through enhancement of the β1 integrin/ILK/Akt signaling pathway.

RESULTS

An ADAM12 protein interaction network as part of the integrin signaling network

The interaction of ADAM12L with numerous receptors and intracellular signal mediators (listed in Table 1) suggests that it could act as a docking/signaling protein. Using the STRING database to extract direct (physical) and indirect (functional) associations between proteins, we built the ADAM12L protein interaction network shown in Figure 1. The high level of interconnections suggests strong associations with the same biological functions. The connectivity of the graph was next enriched by adding five known interactions not yet incorporated in the database, between ADAM12 and TGFBR2, GNB2L1, PRKCE and PRKCD, and between Src and TGFBR2. Interestingly, all known proteins identified as ADAM12-binding proteins were previously shown to be associated with Src. These include the four SH3 domain-containing proteins SH3D19, SH3PXD2A, YES1 and PACSIN3. In addition ADAM12 and Src both connect to the same sub-network in which the integrin signaling pathway stands out. Because engagement of β1 integrin (ITGB1) by external stimuli has been recently associated with the Src-mediated modulation of ADAM12 subcellular distribution (Stautz *et al.*, 2010), we sought to investigate a possible interaction between ADAM12 and a major functional partner of integrins, ILK. Our rationale was that ILK directly binds to integrins in response to stimulation by the microenvironment, suggesting a pivotal role in signal transduction that might implicate ADAM12.

ADAM12 interacts with the ILK integrin-linked kinase

To test this hypothesis, we first investigated whether ADAM12 interacts with ILK in Cos7 cells, which do not express endogenous ADAM12. Endogenous ILK was immunoprecipitated from extracts of cells transfected with ADAM12L. As shown in Figure

2A, both pro- and processed ADAM12, the 110 and 90 kDa forms, respectively, were expressed in Cos7 cells (left panel) and co-immunoprecipitated with ILK (center panel). The reverse immunoprecipitation was performed using a mix of ADAM12 monoclonal antibodies and blotted for ILK (Figure 2A, right panel). The co-immunoprecipitation of ILK and ADAM12 was confirmed using transfection of V5-tagged ILK in either Cos7 cells transfected with ADAM12 or in the LX2 hepatic stellate cell (HSC) line, which expresses low endogenous ADAM12 levels (Supplementary Figure S1). To show whether the interaction between ADAM12 and ILK occurs under physiological conditions, we used primary cultures of HSCs isolated from human liver tissues. Cell culture mimics the HSC activation process, which consists in the transition from a quiescent to a fibrogenic state. This is accompanied by increased expression of ADAM12 and ILK, as previously described in chronic liver diseases (Le Pabic *et al.*, 2003; Zhang *et al.*, 2006). The expression of endogenous ADAM12L in activated HSCs is shown in Figure 2B (left panel). When immunoprecipitation was performed using ILK antibodies, ADAM12/ILK complexes were clearly detected in cell extracts (Figure 2B, center panel). The reverse immunoprecipitation using ADAM12 antibodies confirmed the endogenous interaction of ADAM12 and ILK (Figure 2B, right panel).

To identify the domain of ADAM12 that is required for interaction with ILK, Cos7 cells were transfected with ADAM12L, with a truncated form lacking the cytoplasmic domain (ADAM12-Δcyt) or with the ADAM12S short form. As shown in Figure 2C, immunoprecipitation of ILK led to the recovery of high amounts of ADAM12L. In contrast, neither ADAM12L-Δcyt (90 kDa) nor ADAM12S (90 kDa and 68 kDa for the precursor and processed forms, respectively) were detected in immunoprecipitates, pointing out the implication of the ADAM12 cytoplasmic domain in the interaction with ILK. Because disintegrin and cystein-rich domains are present in ADAM12L-Δcyt and ADAM12S and have been reported to interact with integrins (Eto *et al.*, 2000; Kawaguchi *et al.*, 2003;

Thodeti *et al.*, 2003; Zhao *et al.*, 2004; Huang *et al.*, 2005; Lafuste *et al.*, 2005), the lack of recovery of ADAM12L-Δcyt and ADAM12S in ILK immunoprecipitates suggests that integrins do not mediate the interaction of ADAM12 and ILK. In addition, we investigated the presence of PINCH and parvin in ILK-ADAM12 immune complexes isolated from cell extracts as ILK has been linked to PINCH and parvin proteins in forming the IPP complex (Legate *et al.*, 2006). As expected, both proteins were detected in ILK immunoprecipitates (Figure 2D), suggesting that the interaction of ADAM12 and ILK takes place within IPP complexes.

To rule out the possible implication of additional proteins, we next investigated ADAM12/ILK interactions using recombinant proteins in *in vitro* binding assays. ILK was expressed as a GST fusion protein and purified on glutathione-agarose beads before removal of the GST tag (recILK). We then performed pull-down assays by incubating recILK with purified His-tagged ADAM12 variants. As shown in Figure 2E, recILK only bound full-length ADAM12L, demonstrating an association that is both direct and specific. These results provide strong evidence for a direct interaction of the cytoplasmic domain of ADAM12L with ILK in cells. To investigate the functional relevance of the interaction between ADAM12 and ILK, we next explored the localization of both proteins in HSCs.

ADAM12 and ILK are recruited to focal adhesions upon β1 integrin stimulation

We have previously shown that ADAM12 is stored in the cytoplasm and translocated to the membrane of HSCs upon stimulation by type I collagen (Bourd-Boittin *et al.*, 2008). To investigate the dynamics of the interaction between ADAM12 and ILK, we immunolocalized ADAM12 and ILK in HSCs stimulated or not by type I collagen. As shown in Figure 3A, immunostained ILK and ADAM12 were strongly redistributed to the cell surface when HSCs were plated on type I collagen. Similarly, in stimulated HSCs, ADAM12 co-localized with β1

integrin (Figure 3B) and vinculin (Figure 3C), a cytoskeletal focal adhesion protein involved in linkage of integrin adhesion molecules to the actin cytoskeleton. In agreement with these observations, the colocalization of ILK with β1 integrin was increased upon stimulation by type I collagen (Figure 3D). We further demonstrated the involvement of β1 integrin in the redistribution of ILK and ADAM12 to focal adhesions by using anti-β1 integrin blocking antibodies. As shown in Figure 3E, preincubation of HSCs with blocking antibodies inhibited the translocation of ADAM12 to the focal adhesions. These results suggest that activation of the integrin pathway by type I collagen induces the recruitment of both ADAM12 and ILK to focal adhesions.

ADAM12 modulates hepatic stellate cell adhesion

Because ILK and ADAM12 are recruited to focal adhesions in stimulated HSCs (Figure 3), and ILK has been previously associated with the fibrogenic phenotype of HSCs through its implication in cell adhesion (Shafiei and Rockey, 2006), we next asked whether expression for ADAM12 could affect cell adhesion. We performed ADAM12 siRNA knockdowns in human HSCs. After 48 hours of treatment, the steady-state levels of ADAM12 mRNA and proteins were significantly reduced in HSCs, indicating robust RNA interference efficiency (Figure 4A and B). Note that both the long and the short variants of ADAM12 were equally silenced while expression of type I collagen and TGF-β, the two fibrogenic markers of HSCs used as controls, was not modified. During the course of this analysis, we observed changes in cell spreading and we sought to examine the effect of ADAM12 on the cytoskeleton of HSCs by performing microscopy analysis of actin stress fibers (Figure 4C). Type I collagen induced prominent stress compared to control cultures on plastic dishes. In addition, we found that inhibition of ADAM12 expression led to a striking disorganization and retraction of stress fibers, suggesting major changes in cell spreading and adhesion processes. In line with

this conclusion, ADAM12 silencing in HSCs led to a significant decrease in adherent cells cultured on plastic dishes (26% + 2.5 and 40% +2.7 decrease at 24 and 48 hr, respectively, Figure 4D, left panel). Cultivating HSCs on type I collagen delayed this effect, probably by mobilizing more integrins. However, a significant reduction in adherent cells (-35.6% + 2.8) was observed at 48 hr under these growth conditions. In agreement with this observation, ADAM12-silenced cells plated onto either type I collagen, fibronectin or laminin, which engage different integrins, showed a reduced loss of cell adhesion compared to an albumin plating control (Figure 4D, right panel).

The effect of ADAM12 silencing on cell adhesion was maximal at 48 hours. Over longer times, expression of ADAM12 resumed in HSCs transiently transfected with siADAM12 (Supplementary Figure S2A). Note that all cells recovered in the culture supernatants following transfection with ADAM12 siRNAs no longer expressed ADAM12. In addition, cell death was unaffected in HSCs transfected with siADAM12 (Supplementary Figure S2B). This conclusion is further supported by the demonstration that overexpression of ADAM12 in CHO cells treated with staurosporine or TNF-related apoptosis-inducing ligand (TRAIL) did not significantly protect cells against death (Supplementary Figure S3). Because ILK plays an important role in the regulation of cell proliferation and migration (McDonald *et al.*, 2008), we also sought to explore how ADAM12 overexpression could modulate these effects. As shown in Supplementary Figure S4, ADAM12 induced a significant but slight increase in cell proliferation (Figure S4A) and migration (Figure S4B) suggesting a minor effect in these conditions.

Based on the physical and functional association of endogenous ADAM12 and ILK in HSCs, we next explored which signaling pathway(s) might be involved in this effect.

Expression of ADAM12 reinforces the Akt signaling pathway

Cell adhesion to the extracellular matrix is mediated by the integrin-stimulated ILK-dependent pathway, which involves Protein Kinase B (Akt) and GSK-3. To examine whether expression of ADAM12 modulates Akt and GSK-3 activation, Cos7 cells were transfected with wild-type and mutants ADAM12 constructs for 48 hours and further plated on type I collagen-coated dishes. Phosphorylation of Akt and GSK-3 was examined by western blot analysis at the times indicated in Figure 5. Overexpression of ADAM12L significantly enhanced Akt and GSK-3 phosphorylation compared to cells transfected with an empty vector (Figure 5A). Interestingly, this induction was independent of the proteolytic activity of ADAM12L since the catalytically deficient ADAM12-E351Q mutant induced Akt and GSK-3 phosphorylation to an extent similar to that of wild-type ADAM12L (Figure 5B). Neither the ADAM12-Δcyt truncated form nor the ADAM12S secreted form, which do not bind ILK (Figure 1D), could induce Akt and GSK-3 phosphorylation (Figure 5C), establishing that the effect of ADAM12L on the Akt signaling pathway is specific.

To determine whether this stimulating effect was dependent on ILK and β1 integrin, we assessed the phosphorylation status of Akt in cells either silenced for ILK or pre-incubated with anti-β1 integrin blocking antibodies. Down-regulation of ILK expression (Figure 6A) or inhibition of β1 integrin (Figure 6B) both diminished Akt phosphorylation induced by overexpression of ADAM12L, demonstrating the implication of β1 integrin and ILK in mediating the effect of ADAM12L. In line with this conclusion, adhesion on type I collagen of HSCs, which express high basal levels of ADAM12L, induced a similar phosphorylation of Akt and GSK3 (Figure 6C).

To further explore the molecular mechanisms underlying this novel modulation of the Akt pathway by ADAM12L, we next analyzed the involvement of PI3K, an upstream-acting kinase that regulates ILK activity (Delcommenne *et al.*, 1998). For this purpose, we evaluated

the effects of two PI3K inhibitors, Wortmannin and LY294002, on Akt phosphorylation. Transfected cells were pre-incubated with the inhibitors prior to plating on type I collagen for 60 min and Akt phosphorylation was analyzed by western blotting. As shown in Figure 6D, Wortmannin and LY294002 both blocked the ADAM12L-dependent induction of Akt phosphorylation, establishing the implication of PI3K activity in this phenomenon.

Providing additional support for the role of ADAM12L in the promotion of the ILK/Akt-dependent pathway, ILK immunoprecipitates from extracts of cells overexpressing ADAM12L directly phosphorylated the downstream GSK-3 crosstide peptide substrate (CT-GSK-3), while phosphorylation of CT-GSK-3 was very low in absence of ADAM12 (Figure 7). This effect was correlated with the concentration of ADAM12L in cells (Figure 7A) and was specific to ADAM12L and dependent on ILK, since the phosphorylation of CT-GSK-3 in cells overexpressing ADAM12S was similar to that in control and in ILK-silenced cells (Figure 7B). Taken together, results of these experiments demonstrate that ADAM12L expression augments the Akt/GSK3 signaling pathway in response to microenvironment stimulation via a β1 integrin/PI3K/ILK-dependent pathway.

DISCUSSION

Members of the ADAM protein family have emerged as important regulators of cell signaling, through shedding activities of membrane-anchored cytokines or growth factors. Besides ligand processing, ADAMs have been proposed to function as signal transmitters. In the present study, we used an *in silico* approach based on protein integrative annotation to explore the intracellular protein interaction network of the transmembrane variant of ADAM12, ADAM12L. The resulting highly connected graph suggests that integrin signaling plays a critical role in the ADAM12L network. In agreement with this observation, the dynamic association of ADAM12 with Src upon integrin engagement was recently suggested to exert cooperative signals for cell proliferation, migration, and invasion (Stautz *et al.*, 2010). In addition, a functional association of ADAM12 with integrin signaling has been previously implicated in the mesenchymal differentiation of adipocytes (Kawaguchi *et al.*, 2003) and myogenic cells (Lafuste *et al.*, 2005), in cell adhesion and spreading (Thodeti *et al.*, 2003; Zhao *et al.*, 2004), migration (Huang *et al.*, 2005) and invapodia formation (Albrechtsen *et al.*, 2011). However the molecular mechanism linking ADAM12 to integrin signaling has remained unclear. We now provide evidence for the interaction of ADAM12 with the integrin-linked kinase ILK and demonstrate the role of ADAM12 in enhancing the functionality of the ILK/Akt signaling pathway upon integrin stimulation. ILK is found in most cells within the IPP complex formed together with PINCH and Parvin (Legate *et al.*, 2006). Our co-immunoprecipitation experiments show that the ILK/ADAM12 interaction we report can occur within these complexes and without disrupting them, highlighting the acquisition of a new functionality for IPP as a function of ADAM12 recruitment.

Dynamic translocation of ADAM12 with ILK to focal adhesions

In response to binding of integrins to matrix components, ILK associates with adaptor proteins and is recruited to nascent focal complexes enriched in docking and signaling proteins including vinculin (Legate and Fassler, 2009). In agreement with this dynamic translocation of ILK, we have previously reported that stimulation of integrin pathways by type I collagen induces the recruitment of ADAM12 to the membrane of hepatic stellate cells (Bourd-Boittin *et al.*, 2008). We now demonstrate that, similar to ILK, ADAM12 is redistributed within vinculin-rich structures at the cell surface of HSCs stimulated by type 1 collagen. This suggests that ADAM12 contributes to the dynamics of protein-complex formation at focal adhesions in HSCs. Transiently increased levels of ADAM12/β1 integrin-containing complexes have been reported at the cell surface of pre-adipocytes (Kawaguchi *et al.*, 2003). Interestingly, this study showed that forced expression of the ADAM12-Δcyto truncated protein, which spontaneously translocates to the membrane, also led to alterations of the actin cytoskeleton, cell adhesion and survival. These observations suggest that the cytoplasmic tail of ADAM12 is required to sustain these functions. In support of this hypothesis, our data show that ADAM12 silencing induces loss of HSC adhesion and that the cytoplasmic tail of ADAM12 is required for the ILK-dependent survival pathway that correlates with cell adhesion.

Functional association of ADAM12 and ILK for Akt-dependent survival signaling

Over the past several years, the *in vivo* and *in vitro* kinase activity of ILK has been widely debated and ILK has been suggested to function as an adaptor/scaffold protein (Lange *et al.*, 2009; Maydan *et al.*, 2010; Wickstrom *et al.*, 2010; Fukuda *et al.*, 2011; Hannigan *et al.*, 2011). Nevertheless, the classical ILK-dependent pathway involves a PI3K-dependent integrin engagement that leads to the phosphorylation of downstream Akt and GSK-3 targets.

In addition, ILK properties can be modulated *in vivo* by complex protein interactions that result in activities triggered by the contextual environment, such as that found in cancer cells (Troussard *et al.*, 2006). In line with this notion, our data demonstrate that ADAM12, a protein specifically expressed in activated HSCs during chronic liver disease, enhances the activity of the ILK/Akt pathway in response to β1 integrin activation by type I collagen, the main matrix component of liver fibrosis. These results are in agreement with the reported implication of ILK pathway activity in the Akt-dependent regulation of fibroblast survival upon integrin stimulation (Nho *et al.*, 2005).

The contextual association between ADAM12 and the ILK/Akt pathway is further supported by the correlation between ADAM12 and ILK expression. ADAM12 expression is associated with the undifferentiated proliferating state of mesenchymal cells such as chondrocytes in osteoarthritis (Okada *et al.*, 2008), pre-adipocytes during high-fat diet-induced obesity (Masaki *et al.*, 2005), hepatic stellate cells in liver fibrosis (Le Pabic *et al.*, 2003), myoblasts during myogenic differentiation (Cao *et al.*, 2003) and proliferating astroglial cells in injured brain (Baertling *et al.*, 2010). It is also involved in the initial steps of pre-keratinocyte differentiation, where the absence of ADAM12 promotes migration, a differentiated feature of keratinocytes (Harsha *et al.*, 2008; Oh *et al.*, 2009). Similar to ADAM12, increased activation of the ILK/Akt pathway has been associated with cell proliferation (Persad and Dedhar, 2003). In contrast, ILK knockdown in chondrocytes (Grashoff *et al.*, 2003), keratinocytes (Lorenz *et al.*, 2007), fibroblasts (Sakai *et al.*, 2003) and cortical cells (Niewmierzycka *et al.*, 2005) is not associated with a decrease in Akt phosphorylation, suggesting that, in differentiated cells, ILK could signal through an Akt-independent pathway. Taken together, these observations suggest that the specific expression of ADAM12 in proliferating undifferentiated mesenchymal-type cells might favor ILK/Akt

pathway activity, thereby promoting the survival signaling pathway that is classically associated with cell adhesion.

Cooperative effects of ILK and ADAM12 in cancer

The ubiquitously expressed ILK is involved in tissue homeostasis. Elevated expression of ILK has been observed in tumors with poor prognosis and has been proposed as a new therapeutic target (Hannigan *et al.*, 2005). Similarly, ADAM12 expression is markedly increased in numerous human cancers (Kveiborg *et al.*, 2008) and we have previously reported the association of ADAM12 expression with liver fibrosis and cancer (Le Pabic *et al.*, 2003). In agreement with our results, ILK overexpression has also been shown to be associated with the activation of HSCs in liver fibrosis (Shafiei and Rockey, 2006; Zhang *et al.*, 2006) and induction of ILK activity has been correlated with an increased activation of Akt in hepatocellular carcinomas (Peroukides *et al.*, 2008). In line with these observations, the expression of ADAM12 in mammary-gland tumor cells was recently associated with increased cell proliferation and Akt phosphorylation levels (Frohlich *et al.*, 2011). Similar to results reported in the present study, this effect was independent of the proteolytic activity of ADAM12, suggesting that the pro-tumorigenic effect of ADAM12 is mediated by the disintegrin and cystein-rich domains which interact with integrins (Eto *et al.*, 2000; Kawaguchi *et al.*, 2003; Thodeti *et al.*, 2003; Zhao *et al.*, 2004; Huang *et al.*, 2005; Lafuste *et al.*, 2005). Interestingly we have previously shown that these same domains also interact with the TβRII receptor of transforming growth factor TGF-β (Atfi *et al.*, 2007), which affects cell survival and proliferation through a PI3K/Akt dependent pathway (Shin *et al.*, 2001; Wilkes *et al.*, 2005). These data reinforce the notion that ADAM12 might act as a scaffolding protein for integrin- and TGF-β receptor-dependent pathways, leading to the activation of PI3K/ILK/Akt signals and thereby affecting cancer cell survival.

In conclusion, results of this study, which identify ILK as a new ADAM12 partner, unravel a major aspect of the biological significance of the functional association of ADAM12 with the integrin signaling pathway. In addition, induction of the ILK/Akt pathway by ADAM12 suggests a potential role for this protein in cell adhesion and survival. According to this view, ADAM12 would act as a pivotal sensor implicated in the regulation of a microenvironment-dependent equilibrium between differentiation and proliferation.

MATERIAL AND METHODS

Cell culture and transfection

Human hepatic stellate cells (HSCs) were isolated from histologically normal specimens of partial hepatectomies from patients undergoing hepatic resection for liver metastases as previously described (Le Pabic *et al.*, 2003). HSC and Cos7 cells were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS). For all experiments, cultured HSCs were used between passages 4 and 10. When specified, cells were plated on collagen type I-coated dishes (BD Biosciences) or Permanox Lab-Tek® chamber slides™. HSCs were transfected using the AMAXA protocols and kits (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's instructions. In some experiments, cells were incubated either with PI3K inhibitors, 100 nM Wortmannin (Calbiochem, Darmstadt, Germany) or 10 μM LY294002 (Calbiochem), for 30 min or anti-ITBG1-blocking antibodies (Santa-Cruz biotechnology, CA) for 30 min at 37°C under agitation without FBS. To knock down ADAM12 and ILK expression, synthetic double-stranded ADAM12 siRNAs were purchased from Sigma. The expression vectors for ADAM12 and mutants thereof, including the complete deletion of the cytoplasmic tail (ADAM12-Δcyt) and the protease inactive mutant ADAM12-E351Q, were a gift from Dr. U. Wewer (Biotech Research & Innovation Centre (BRIC), University of Copenhagen). The expression vector for ILK was a gift from Dr. S. Dedhar (Department of Integrative Oncology, BC Cancer Research Centre, Vancouver, British Columbia, Canada).

Adhesion assays. Cell adhesion was measured using a trypsinization assay. Briefly, HSCs transfected with 2 nM non-targeted siRNA (Scr) or ADAM12L siRNA (siADAM12) were plated onto collagen type I dishes or on laminin, and fibronectin coated multiwell plates (BD

Biosciences) for indicated time in absence of serum. Cells were then treated with 0.5 ml of 0.05% trypsin-EDTA for 4 min, detached cells were collected in media containing 10% serum and manually counted. Numbers of detached cells expressed as a percentage of total were determined in three independent experiments. Controls included uncoated and albumin (10mg/mL in PBS) coated dishes.

Cell Proliferation and migration assays

Cos7 cells were transfected with ADAM12 or control vectors and cultured for 48 hours. Cells were then trypsinized, equilibrated in medium for 1 hour at room temperature and plated on collagen type I-coated dishes. For cell proliferation assay, the medium was replaced with DMEM containing 2% FCS in the presence of ^{3}[H]-thymidine and incubated for the indicated times. Cells were scraped in phosphate-buffered saline (PBS), sonicated for 10 min and treated with overnight with 30% trichloroacetic acid (TCA). Cell extracts were centrifugated and the pellets homogenized in formic acid were used for counting TCA-precipitable radioactivity. For migration assays, cells seeded on type I collagen were incubated for one hour at 37°C before addition of 2.5 µg/ml mitomycin; After 24h, the confluent monolayer was scratched using a pipette tip and images were captured at the indicated times.

Western blotting and immunoprecipitation

Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (GE Healthcare, UK). Blots were incubated for 30 min in Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk and further incubated for two hours (or overnight at 4°C) with the following antibodies: rabbit anti-ADAM12 (a gift from Dr. U. Wewer), rabbit anti-ILK (Cell Signaling Technology,

Boston, MA, USA) and monoclonal anti-β actin-peroxydase (Sigma Aldrich). Antibodies against phospho-Akt (Ser^{473}), Phospho-GSK-3β (Ser^{9}), total Akt , total GSK-3 and Alpha-parvin (D7F9) were from Cell Signaling Technology. PINCH antibodies were from BD Transduction Laboratories. Bound antibodies were visualized with horseradish peroxidase–conjugated antibodies using an enhanced chemiluminescence system (Millipore, Billerica, MA, USA). For immunoprecipitation, cell extracts were incubated with cross-linked ILK beads for 2 hrs at 4°C or preincubated for 1 hr with protein G sepharose beads (Amersham Biosciences) alone to reduce non-specific protein binding, followed by adsorption overnight to protein G sepharose beads pre-bound with 2 μg of specific or control ADAM12 rabbit IgG. The beads were washed five times and samples were analyzed by SDS-PAGE and immunoblotting.

Expression of recombinant proteins

pET102D-His-ADAM12 and pGEX-GST-ILK constructs were transformed in BL21-DE3 (Novagen) and expression was induced by 0.25 mM isopropyl 1-thio-α-D-galactopyranoside overnight at 18°C. Cells were harvested by centrifugation at 6,000 x *g* for 15 minutes and lysed either in ice-cold PBS, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) for GST-ILK, or IMAC5 (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM imidazole) for His-ADAM12, and 0.1 mM AEBSF, complete EDTA-free protease inhibitor cocktail (Roche) and 1 mg/ml lysosyme. After 1hr incubation on ice and 2 freeze/thaw cycles, the lysate was sonicated and clarified by centrifugation at 23,000 x *g* for 1 hr at 4°C. His-ADAM12 was bound to Ni-charged Mag Beads (GenScript) for 1 hour at 4°C. After washing with 10 bed volumes of IMAC20 (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 20 mM imidazole), the protein was eluted with IMAC 250 (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 250 mM imidazole) and dialysed against PBS. GST-ILK was bound to GST MagBeads

(GenScript) for 1 hr at 4°C. After washing with 10 bed volumes of WB 300 (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.02% Tween 20, 0.1 mM AEBSF), the protein was eluted with 25 mM gluthatione (Sigma Aldrich) in PBS. The GST tag was cleaved with thrombin (1 U/10 µg in 25 mM Tris-HCl pH 7.5, 1 mM DTT, 50 mM NaCl, 2.5 mM $CaCl_2$) for 2 hrs at 30°C and immediately removed by chromatography on a GST MagBeads column. All protein fractions were concentrated on Ultracel YM 30 columns (Amicon) and stored at -80°C.

Pull-down assays

His-ADAM12 in IMAC5 was bound to Ni-charged Mag-Beads (Qiagen, Hilden, Germany), for 90 min at 4°C followed by three washes with IMAC5 buffer containing 5 mM imidazole, and incubated with ILK (w/w) for a 90 min incubation at 4°C. The nickel-agarose beads were further washed five times with 10 bed volumes of IMAC5 before elution with SDS-PAGE sample buffer. Samples were used for immunoblotting.

Immunostaining and imaging

To detect ADAM12, ILK and ITGB1 in HSCs, cells were plated on Permanox Lab-Tek chamber slides for 48 hrs with 2% FBS and fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.1% Triton X-100 before incubation with rabbit anti-ADAM12 (rb122), mouse anti-ILK (Santa Cruz Biotechnology, CA, USA), rabbit anti-ILK1 (4G9) (Cell Signaling), mouse β1 integrin (Santa Cruz) or mouse anti-vinculin (Sigma, Saint-Quentin, France) antibodies, followed by Alexa Fluor® 488-conjugated anti-mouse and Alexa Fluor® 555-conjugated anti-goat antibodies (Invitrogen, Karlsruhe, Germany), respectively. The slides were washed, mounted and viewed using an automated LEICA DMRXA2 microscope.

Relative quantifications of mRNA

RT-qPCR was performed using the fluorescent dye SYBR Green methodology with the SybrTM Green I qPCRTM Core Kit (Eurogentec, Seraing, Belgium) and the ABI Prism 7700 thermocycler (Perkin-Elmer, Foster city, CA, USA) as previously described (Le Pabic *et al.*, 2003). The following primer pairs were used for the indicated target genes, ADAM12L sense: 5'-CACCATTGAAAAACTAAGGTGTGTG-3', anti-sense: 5'-GAGCCTGACAGGGTTGGAAG-3'; ADAM12S sense: 5'-CTGGGCACCTCCCTTCTGT-3', anti-sense: 5'-TGCTTCTGCTTGCCGGA-3'; Type I collagen, sense: 5'-GGTCCTGATGGAAACAATGG-3'; anti-sense: 5' TTCCACCTTGAACACCCTGT-3'; TGF-β sense: 5'-TGCGCTTGAGATCTTCAAACA-3', anti-sense: 5'-GGGCTAGTCGCACAGACCTC-3'; 18S rRNA sense : 5'-CGCCGCTAGAGGTGAAATTC-3', anti-sense: 5'-TTGGCAAATGCTTTCGCTC-3'.

In vitro kinase assays

ILK immunoprecipitates were prepared using ILK antibodies cross-linked to magnetic Protein G Dynabeads (Invitrogen, Karlsruhe, Germany). Cell extracts were incubated with cross-linked ILK beads for 2 hrs at 4°C and successively washed with extraction buffer and kinase assay buffer. Kinase assays were performed by incubating ILK immunoprecipitates with 1 μg/ml GSK-3 crosstide peptide (Cell Signaling, Boston, MA, USA) for 30 min at 30°C in reaction buffer (1 mM dithiothreitol, 10 μM ATP, 1 μCi ^{32}P-ATP, 0.01% Triton X-100, 25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$). The reaction was stopped by addition of SDS-PAGE sample buffer and samples were used for immunoblotting and autoradiography analysis.

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Gene Name	Description	Experiment Type
ACTN2	Actinin, alpha 2	*In vivo*; *in vitro*; yeast 2-hybrid
ACTN1	Actinin, alpha 1	*In vivo*; *in vitro*
GRB2	Growth factor receptor-bound protein 2	*In vivo*
SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	*In vivo*; *in vitro*
YES1	V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	*In vitro*
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	*In vivo*; *in vitro*
PRKCE	Protein kinase C, epsilon	*In vitro*
PRKCD	Protein kinase C, delta	Yeast 2-hybrid
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29)	*In vitro*
SH3PXD2A	SH3 and PX domains 2A	*In vivo*; *in vitro*
PACSIN3	Protein kinase C and casein kinase substrate in neurons 3	*In vivo*; *in vitro*; yeast 2-hybrid
SH3D19	SH3 domain containing 19	*In Vivo*; *in vitro*; yeast 2-hybrid
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	*In Vivo*; *in vitro*; yeast 2-hybrid
GNB2L1	Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	*In Vivo*; *in vitro*; yeast 2-hybrid

Table 1. List of ADAM12L-interacting proteins used as input for STRING application (http://string-db.org/).

FIGURE LEGENDS

Figure 1. The ADAM12L protein interaction network. The list of the 14 proteins that physically bind ADAM12L (Table 1) was used as input in the STRING application and the graph was generated using default parameters. The interactions between ADAM12 and PRKCE (Sundberg *et al.*, 2004), PRKCD (Asakura *et al.*, 2002), GNB2L1 (Bourd-Boittin *et al.*, 2008), TGFBR2 (Atfi *et al.*, 2007) and between TGFBR2 and Src (Galliher and Schiemann, 2007) have not been entered in the database yet and were added manually (green connecting lines).

Figure 2. ADAM12L interacts with ILK. Cell extracts from (A) Cos7 cells transfected with the long form of ADAM12, ADAM12L or (B) HSCs, were immunoprecipitated with anti-ADAM12 or anti-ILK (IP) and immunoblotted with anti-ILK or anti-ADAM12 antibodies (IB). (C) Cos7 cells were transfected either with ADAM12L or the ADAM12-Δcyto cytoplasmic tail deletion or the short ADAM12S secreted form. Cell extracts were immunoprecipitated with anti-ILK and immunoblotted with anti-ADAM12 (right panel). The left panel shows immunoblots performed on crude extracts as controls. (D) Cos7 cells transfected with ADAM12L were immunoprecipitated with anti-ILK and immunoblotted (IB) with anti-PINCH, anti parvin or anti-ADAM12 antibodies. (E) ADAM12L, ADAM12S and ADAM12-Δcyto were expressed as His-tagged fusion proteins and binding assays were performed by incubating His-ADAM12 bound to nickel-agarose with 1 μg of purified ILK. Interacting proteins were immunoblotted with anti-ILK and anti-ADAM12 antibodies.

Figure 3. ADAM12-L and ILK are recruited to focal adhesion structures upon β1 integrin stimulation. HSCs were cultured on plastic dishes (-) or on dishes coated with type I

collagen (+) overnight in medium containing 2% FBS. Cell were immunostained with antibodies against anti-ADAM12, anti-ILK, anti-vinculin and anti-ITGB1 as indicated above the panels followed by TRITC or FITC-labeled secondary IgG. Representative fields are shown. Co-localization results in yellow cellular staining. (A) Localization of ADAM12 and ILK. (B) Localization of β1 integrin (ITGB1) and ADAM12. (C) Localization of vinculin and ADAM12. (D) Localization of ILK and β1 integrin. (E) HSCs were pre-incubated with anti-β1 integrin blocking antibodies or control mouse IgG1 before seeding on type I collagen. Scale bars: 10μm.

Figure 4. RNAi-mediated decreased expression of ADAM12 in hepatic stellate cells induces actin cytoskeleton reorganization and loss of adhesion. HSCs were transfected with 2 nM non-targeted siRNA (Scr) or ADAM12L siRNA (siADAM12). After 48 hrs, Western blotting (A) and real-time PCR analyses (B) were used to confirm the efficiency and specificity of RNA interference in cell extracts. PCR controls included ADAM12S, Type I collagen (Col1) and TGF-β. (C) Transfected cells were analyzed by phase-contrast microscopy (left panels) and stained with rhodamine-conjugated phalloidin to monitor actin stress fibers (right panels). Nuclei were counterstained using DAPI (blue). (D) Cell adhesion was evaluated by counting adherent cells *vs.* cells released in the medium at 24 hr and 48 hr post-transfection. Results are expressed as the percentage of adherent cells and represented as the mean + s.d. of three independent experiments (* and #, $p<0.05$; **, $p<0.01$). Left panel: cell adhesion on plastic and collagen type I was compared 24 and 48 hrs post-transfection. Right panel, cell adhesion on different substrates was compared 24 hrs post-transfection.

Figure 5. Expression of ADAM12L augments phosphorylation of Akt and GSK-3. Cos7 cells were transfected with empty vector (control) or ADAM12 constructs and cultured for 48 hrs. The cells were then plated on dishes coated with type I collagen for the indicated times and the levels of phosphorylated Akt (Akt P-S^{473}), phosphorylated GSK-3β (GSK3β P-S^{9}), total Akt and GSK-3β were determined by western blot analysis (IB). Results are expressed as the mean + s.d. of three independent experiments (*, $p<0.05$; **, $p<0.01$; ***, $p<0.01$). (A) ADAM12L *vs.* control. (B) ADAM12L *vs.* the catalytically deficient ADAM12-E351Q mutant. (C) ADAM12L *vs.* ADAM12S and ADAM12-Δcyto (time = 60 min).

Figure 6. Induction of Akt phosphorylation by ADAM12L expression requires ILK and stimulation of integrin β1 and PI3K activity. (A) Cos7 cells were co-transfected with 2 nM non-targeted siRNA (Scr) or ILK siRNA (siILK) and empty vector (control) or the ADAM12 construct. Cells were cultured for 48 hrs, serum-starved and then plated on dishes coated with type I collagen for 1 hr. Levels of phosphorylated Akt (Akt-P-S^{473}) were determined by western blot analysis (IB), immunoblots for ILK, ADAM12 and actin are shown as controls. (B) Cos7 cells were transfected with ADAM12 constructs, cultured for 48 hrs and incubated with anti-β1 integrin blocking antibodies for 30 min before plating on plastic dishes or dishes coated with type I collagen for 1 hr. Levels of phosphorylated Akt (Akt-P-S^{473}) were determined by western blot analysis (IB), immunoblots for ILK, ADAM12 and actin are shown as controls. (C) Adhesion of HSCs on type I collagen augments phosphorylation of Akt and GSK-3. HSC cells were plated on dishes coated with type I collagen for the indicated times and the levels of phosphorylated Akt (Akt P-S^{473}), phosphorylated GSK-3β (GSK3β P-S^{9}), total Akt and GSK-3β were determined by western blot analysis (IB). Immunoblots for endogenous ADAM12 and actin are shown as controls. (D) Cos7 cells were transfected with empty vector (control) or ADAM12L constructs and cultured for 48 hrs. The cells were then

incubated with the PI3K inhibitor Wortmannin (100 nM) or LY294002 (10 μM) and further plated on type I collagen-coated dishes for 1 hr. Levels of phosphorylated Akt (Akt-P-S^{473}) were determined by western blot analysis (IB), immunoblots for ILK, ADAM12 and actin are shown as controls.

Figure 7. **ADAM12L expression induces GSK3 phosphorylation in ILK immunoprecipitates.** Cos7 cell were transfected with (A) increasing amounts of ADAM12L or (B) empty vector (control), ADAM12L, ADAM12S, non-targeted siRNA (Scr) or ILK siRNA (siILK) in the presence of ADAM12L, as indicated. *In vitro* kinase assays were carried out by incubating ILK immunoprecipitated from the corresponding cell extracts with the GSK-3 crosstide peptide substrate (CT-GSK-3). Representative autoradiographs show the amount of ^{32}P incorporation and results are expressed as the mean + s.d. of three independent experiments.

Supplementary Data

Identification of ILK as a new partner of the ADAM12 disintegrin and metalloprotease in cell adhesion and survival

Anthony Leyme,[*] Katia Bourd-Boittin,[*] Dominique Bonnier,[*] Anaïs Falconer,[†] Yannick Arlot-Bonnemains,† and Nathalie Théret[*]

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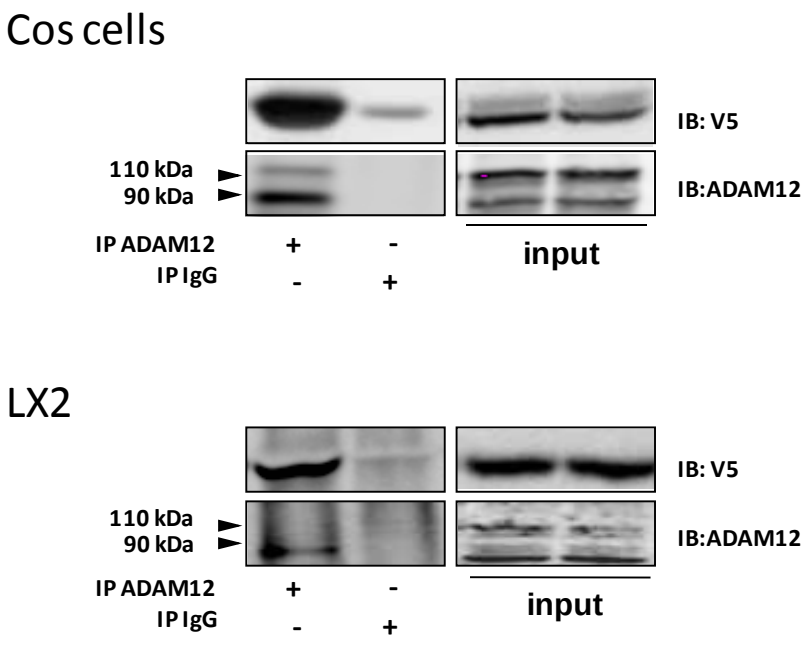
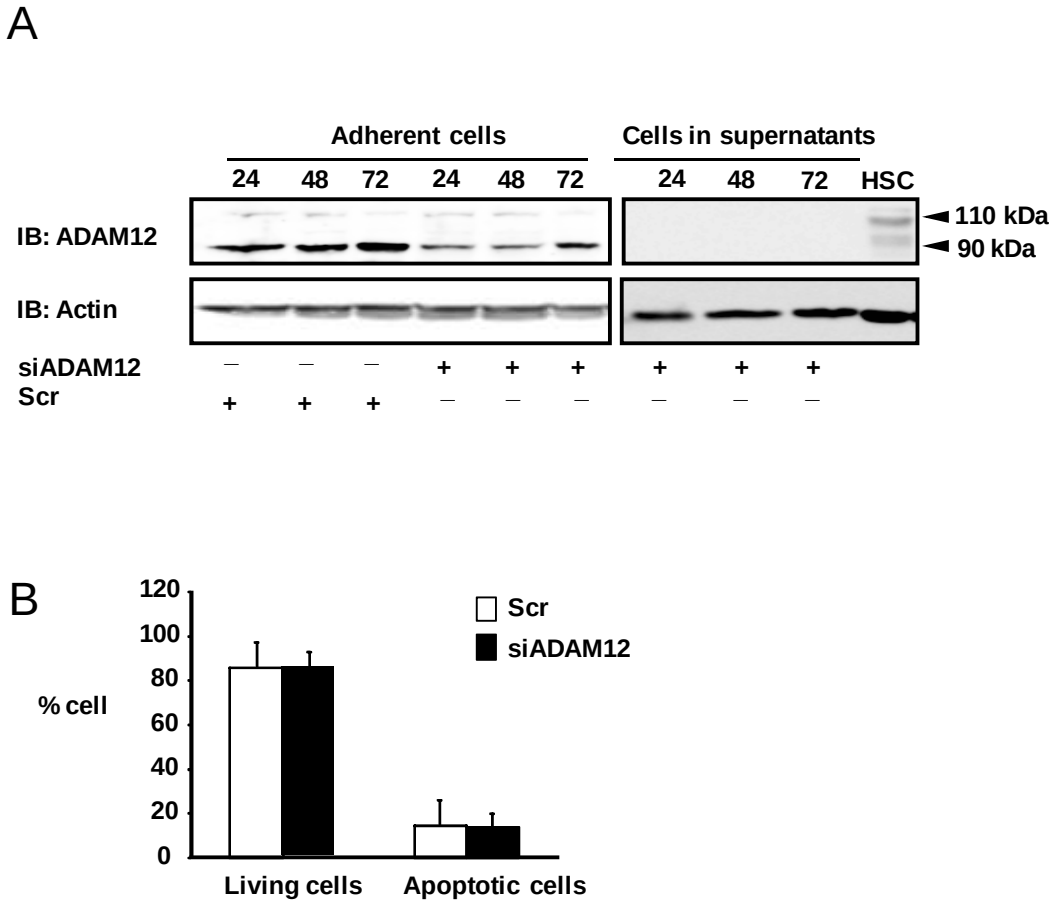
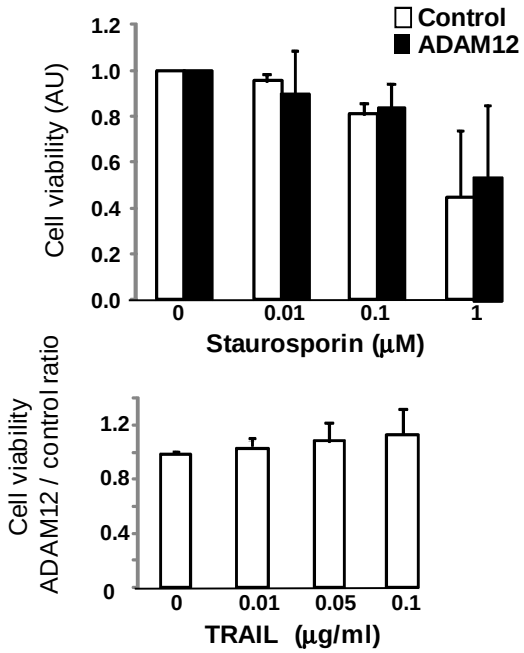
This section contains Supplementary Figure S1, Figure S2, Figure S3 and Figure S4.



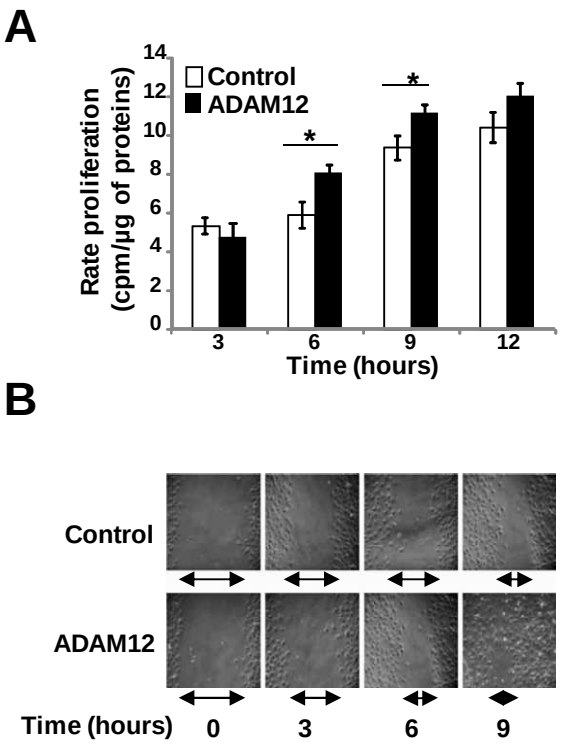
Supplementary Figure S1. ADAM12L interactions with ILK in Cos7 and LX2 cells. Cell extracts were prepared from Cos7 cells co-tranfected with the long form of ADAM12, ADAM12L and V5-tagged ILK, or from hepatic stellate LX2 cells transfected with V5-tagged ILK. Extracts were immunoprecipitated (IP) with anti-ADAM12 and immunoblotted (IB) with anti-V5. The precursor and processed (active) forms of ADAM12 correspond to the 110 and 90-kDa species, respectively, detected by the antibody used.



Supplementary Figure S2: ADAM12 silencing in HSCs is associated with loss of cell adhesion without induction of cell death. HSCs were transfected with 2 nM non-targeted siRNA (Scr) or ADAM12L siRNA (siADAM12) for the indicated times. A) Protein extracts from cells collected in culture supernatants and from adherent cells were immunoblotted with anti-ADAM12 antibodies (IB). Immunoblots for actin are shown as controls. Cell extracts from non-transfected HSCs were used as control (HSC). B) Apoptosis was measured after 48 hrs of transfection by microscopic detection using Hoechst 33342 labeling (0.5 µg/ml). Cells with apoptotic nuclei were counted relative to the total population (n = 200).



Supplementary Figure S3: Overexpression of ADAM12 does not protect cells from Staurosprine- and TRAIL-induced cell death. Chinese hamster ovary (CHO) cells were transfected with ADAM12L or an empty vector (control). At 48 hours post transfection, cells are further treated for 24 hours with A) Staurosporine (0-1 µM) and B) Flag-tagged TRAIL (0-0.1 µg/ml) from Alexis Biochemicals. A total of 2 µg/mL anti-Flag M2 (Sigma-Aldrich, Saint-Quentin Fallavier, France) was added to induce TRAIL oligomerization. Cell viability was assessed by methylene blue staining. Data represent the mean ± SD from three independent experiments performed in sextuplet.

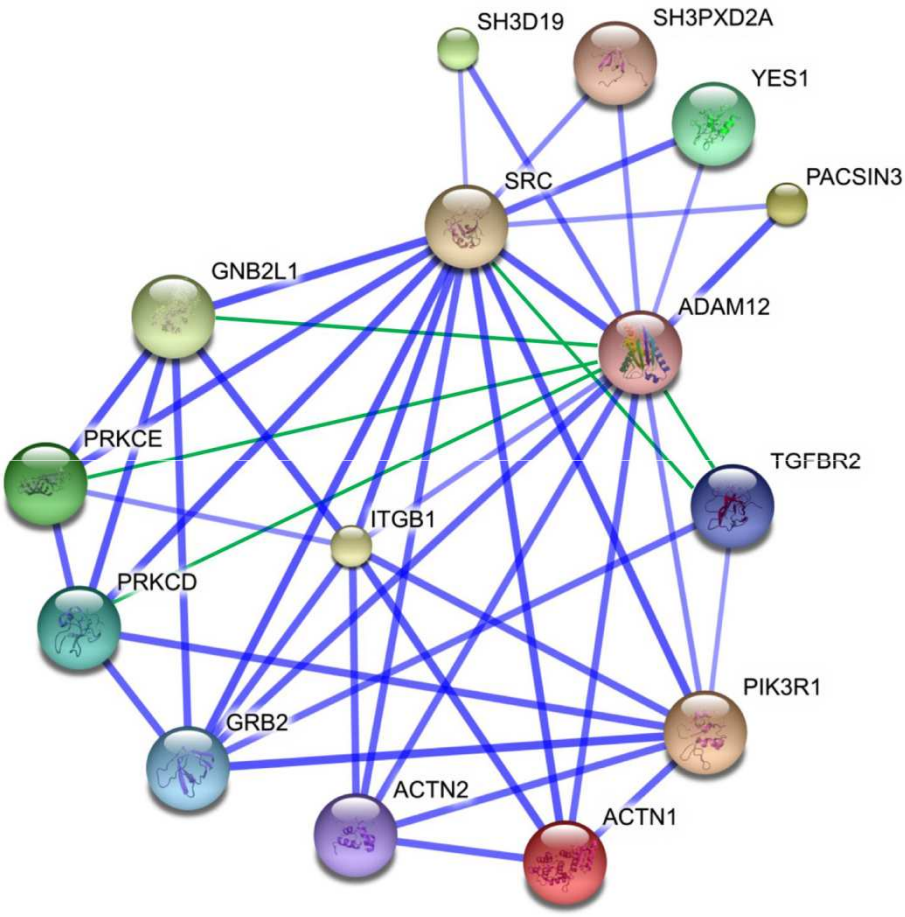


Supplementary Figure S4. **Expression of ADAM12 favors cell proliferation and migration.** Cos7 cells were transfected with empty vector (control) or ADAM12 constructs and cultured for 48 hrs. The cells were then plated on dishes coated with type I collagen for the indicated times. (A) Cell proliferation was evaluated by 3[H]-thymidine incorporation. Results are expressed as the mean + s.d. of three independent experiments (*, $p<0.05$). (B) Cell migration was evaluated by the scratch wound-healing assay, in which a confluent cell monolayer is scratched with a pipette tip and the bare area is subsequently assessed for relocation of cells. Representative data from three independent experiments are shown and arrows indicate the width of the wound.

Gene Name	Description	Experiment Type
ACTN2	Actinin, alpha 2	*In vivo*; *in vitro*; yeast 2-hybrid
ACTN1	Actinin, alpha 1	*In vivo*; *in vitro*
GRB2	Growth factor receptor-bound protein 2	*In vivo*
SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	*In vivo*; *in vitro*
YES1	V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	*In vitro*
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	*In vivo*; *in vitro*
PRKCE	Protein kinase C, epsilon	*In vitro*
PRKCD	Protein kinase C, delta	Yeast 2-hybrid
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29)	*In vitro*
SH3PXD2A	SH3 and PX domains 2A	*In vivo*; *in vitro*
PACSIN3	Protein kinase C and casein kinase substrate in neurons 3	*In vivo*; *in vitro*; yeast 2-hybrid
SH3D19	SH3 domain containing 19	*In Vivo*; *in vitro*; yeast 2-hybrid
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	*In Vivo*; *in vitro*; yeast 2-hybrid
GNB2L1	Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	*In Vivo*; *in vitro*; yeast 2-hybrid

Table 1. List of ADAM12L-interacting proteins used as input for STRING application (http://string-db.org/).

Figure 1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SH3PXD2A
YES1
SRC
PACSIN3
GNB2L1
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PRKCE
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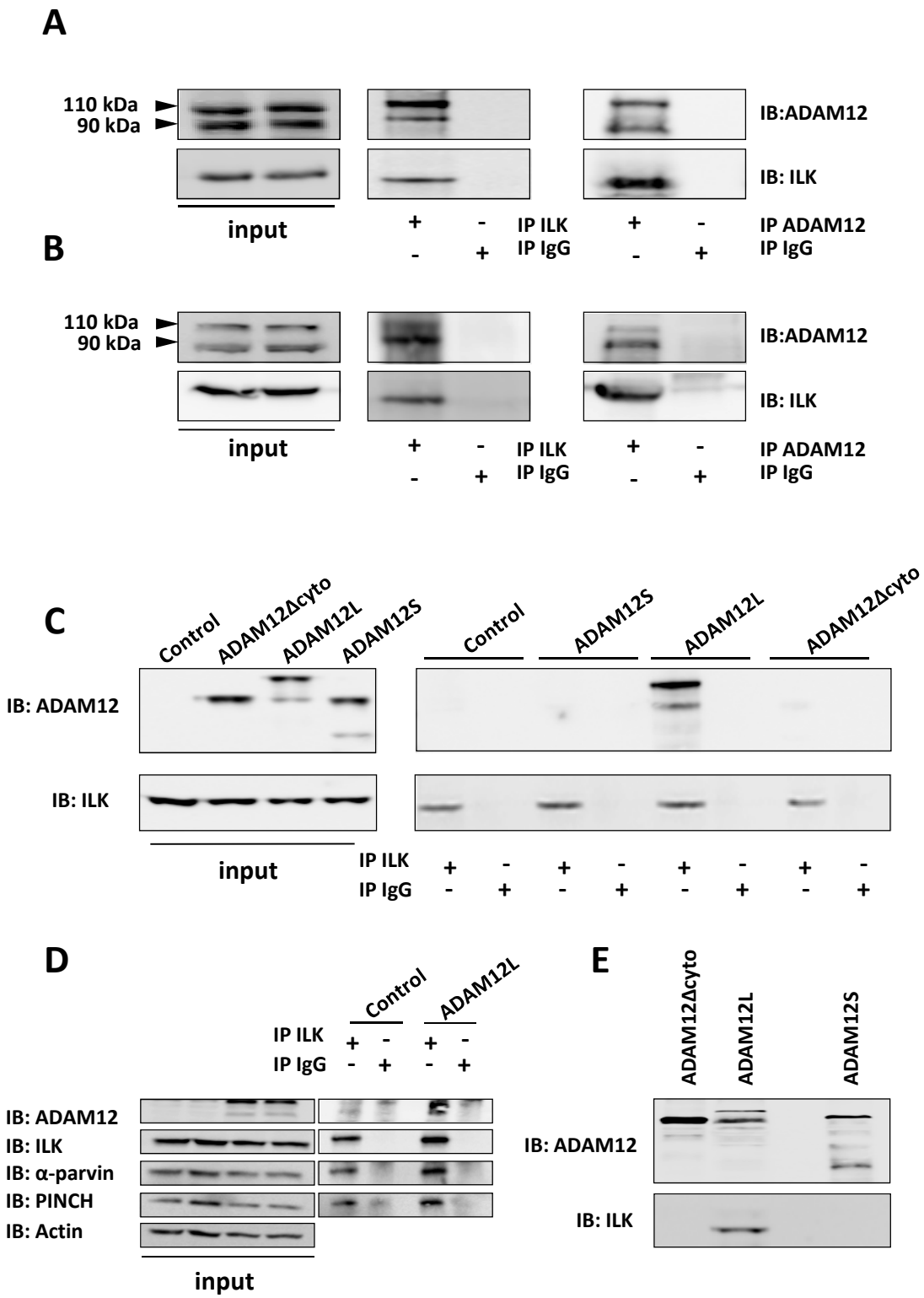
GRB2
ACTN2
ACTN1

Figure 2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IP IgG
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IP IgG
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90 kDa
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IB: ILK
input
IP ILK
IP IgG
IP ADAM12
IP IgG
C
Control
ADAM12Δcyto
ADAM12L
ADAM12S
Control
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ADAM12L
ADAM12Δcyto
IB: ADAM12
IB: ILK
input
IP ILK
IP IgG
D
Control
ADAM12L
IP ILK
IP IgG
IB: ADAM12
IB: ILK
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ADAM12S
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IB: ILK

Figure 3

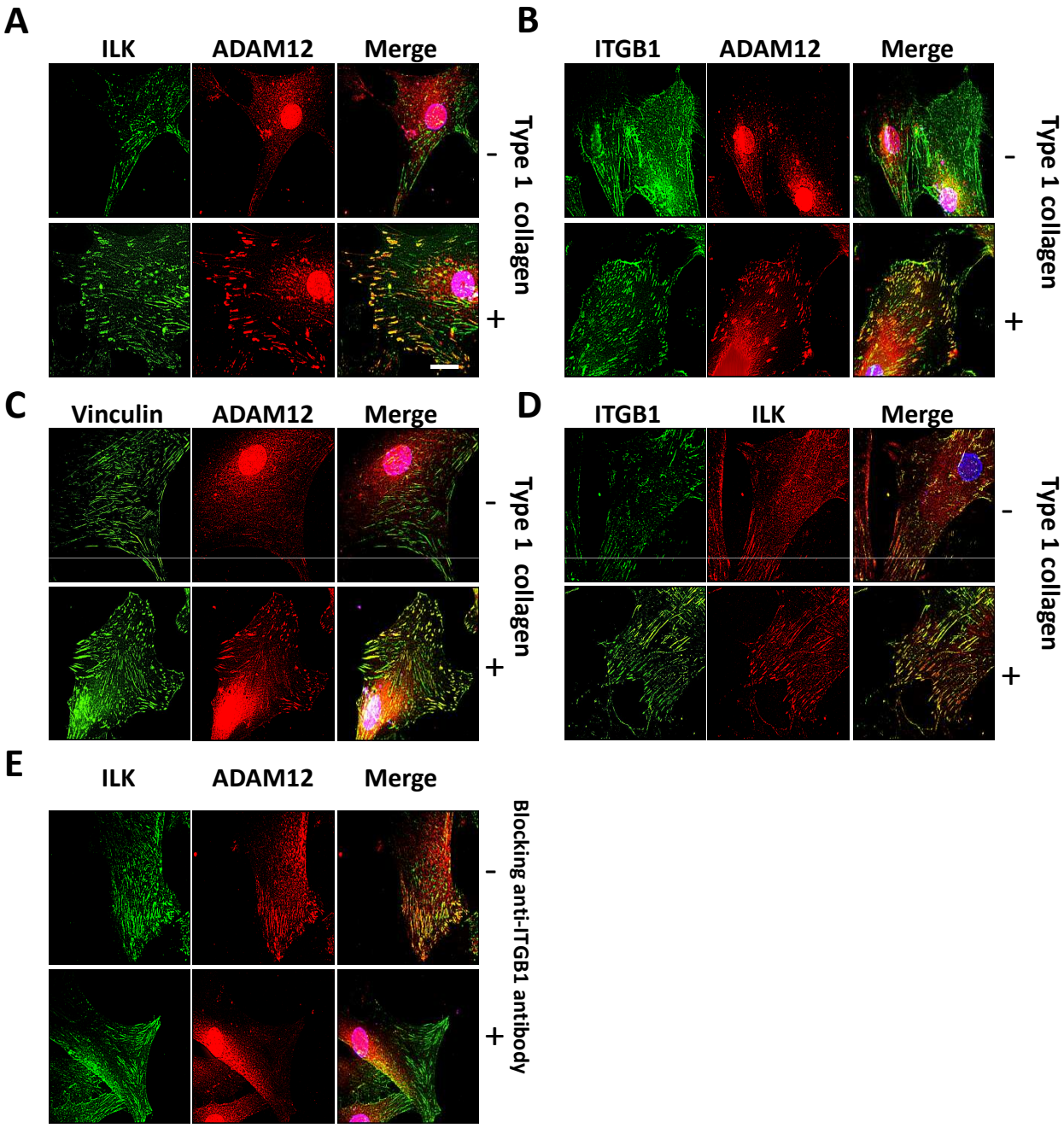
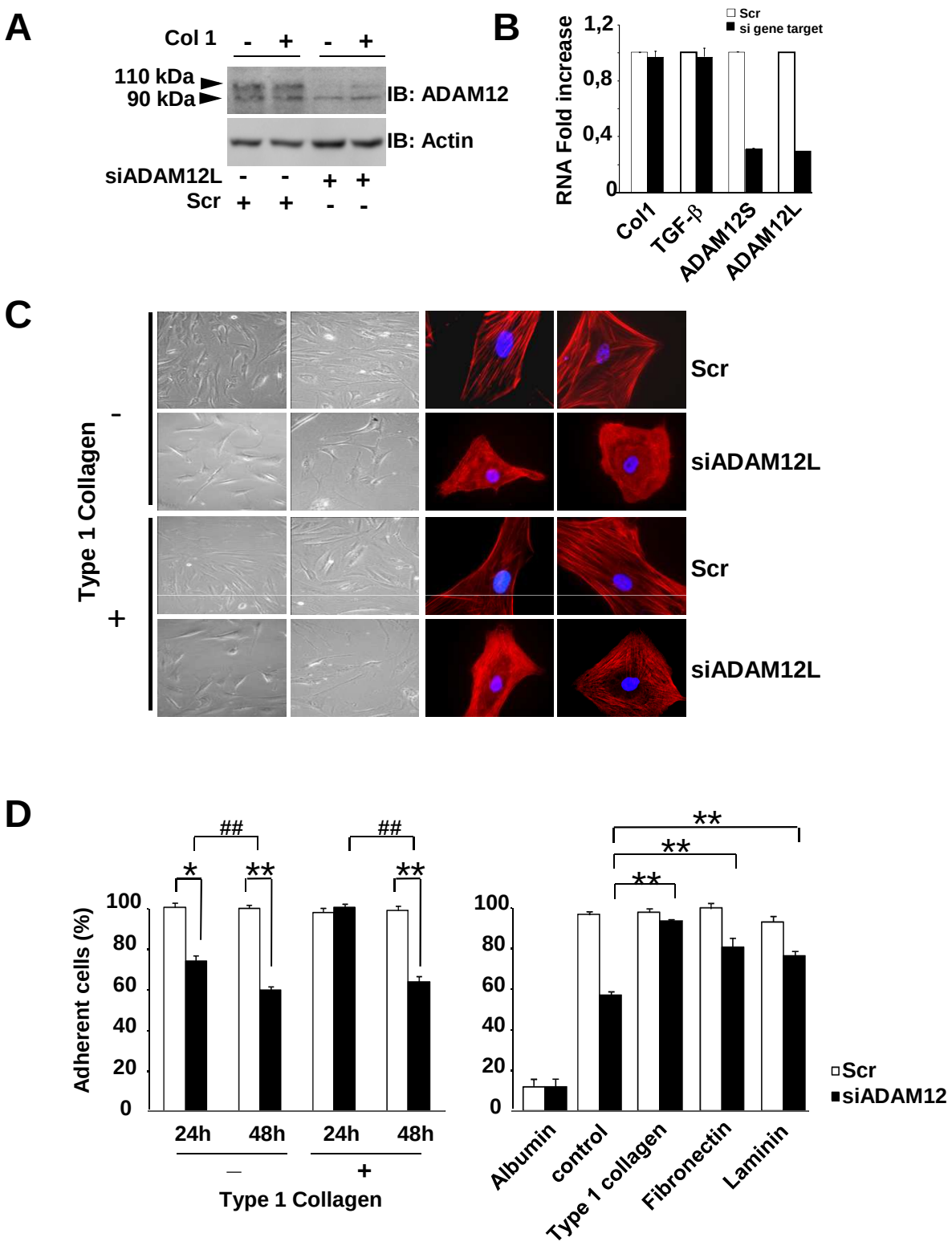

A
ILK
ADAM12
Merge
Type 1 collagen
-
+
B
ITGB1
ADAM12
Merge
Type 1 collagen
-
+
C
Vinculin
ADAM12
Merge
Type 1 collagen
-
+
D
ITGB1
ILK
Merge
Type 1 collagen
-
+
E
ILK
ADAM12
Merge
Blocking anti-ITGB1 antibody
-
+

Figure 4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Col 1 - + - +
110 kDa
90 kDa
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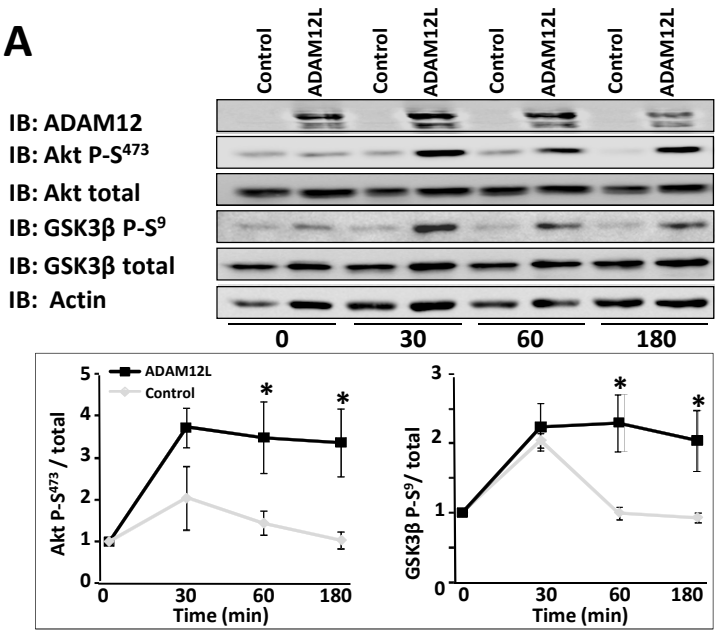
IB: Actin
siADAM12L - - + +
Scr + + - -
B
Scr
si gene target
RNA Fold increase
1,2
0,8
0,4
0
Col1
TGF-β
ADAM12S
ADAM12L
C
Type 1 Collagen
-
+
Scr
siADAM12L
Scr
siADAM12L
D
Adherent cells (%)
100
80
60
40
20
0
##
##
*
**
**
**
24h
48h
24h
48h
–
+
Type 1 Collagen
**
**
**
Albumin
control
Type 1 collagen
Fibronectin
Laminin
Scr
siADAM12

Figure 5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Control
ADAM12L
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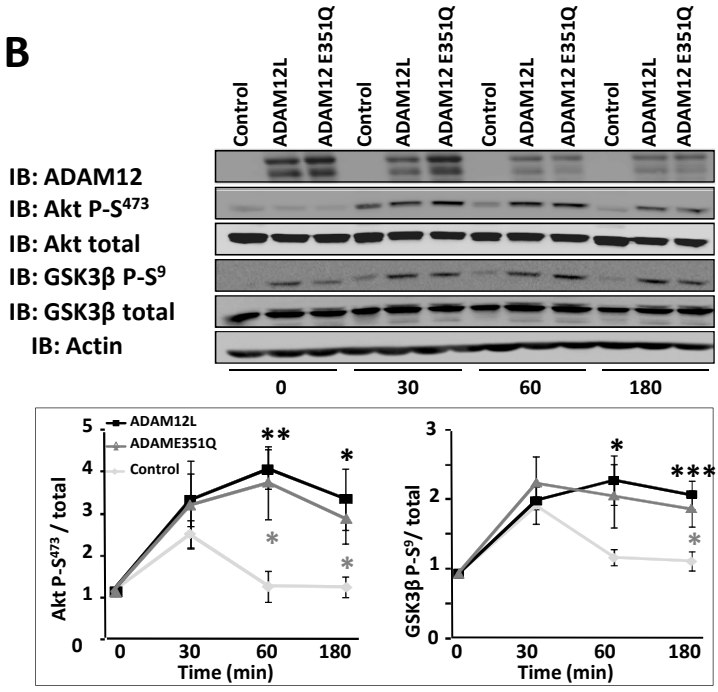
IB: Akt P-S473
IB: Akt total
IB: GSK3β P-S9
IB: GSK3β total
IB: Actin
0
30
60
180
ADAM12L
Control
Akt P-S473 / total
GSK3β P-S9/ total
Time (min)


B
Control
ADAM12L
ADAM12 E351Q
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IB: Akt P-S473
IB: Akt total
IB: GSK3β P-S9
IB: GSK3β total
IB: Actin
0
30
60
180
ADAM12L
ADAME351Q
Control
Akt P-S473 / total
GSK3β P-S9/ total
Time (min)

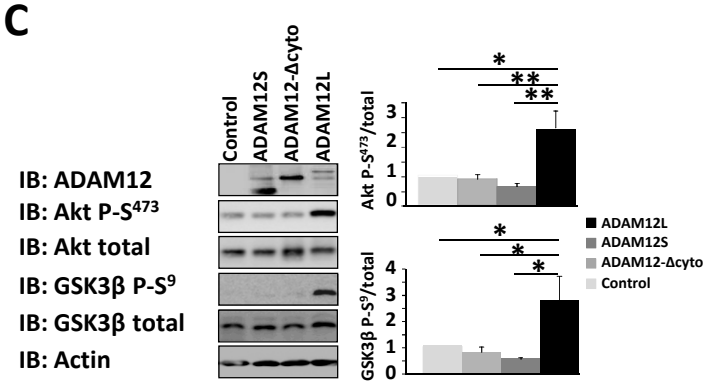
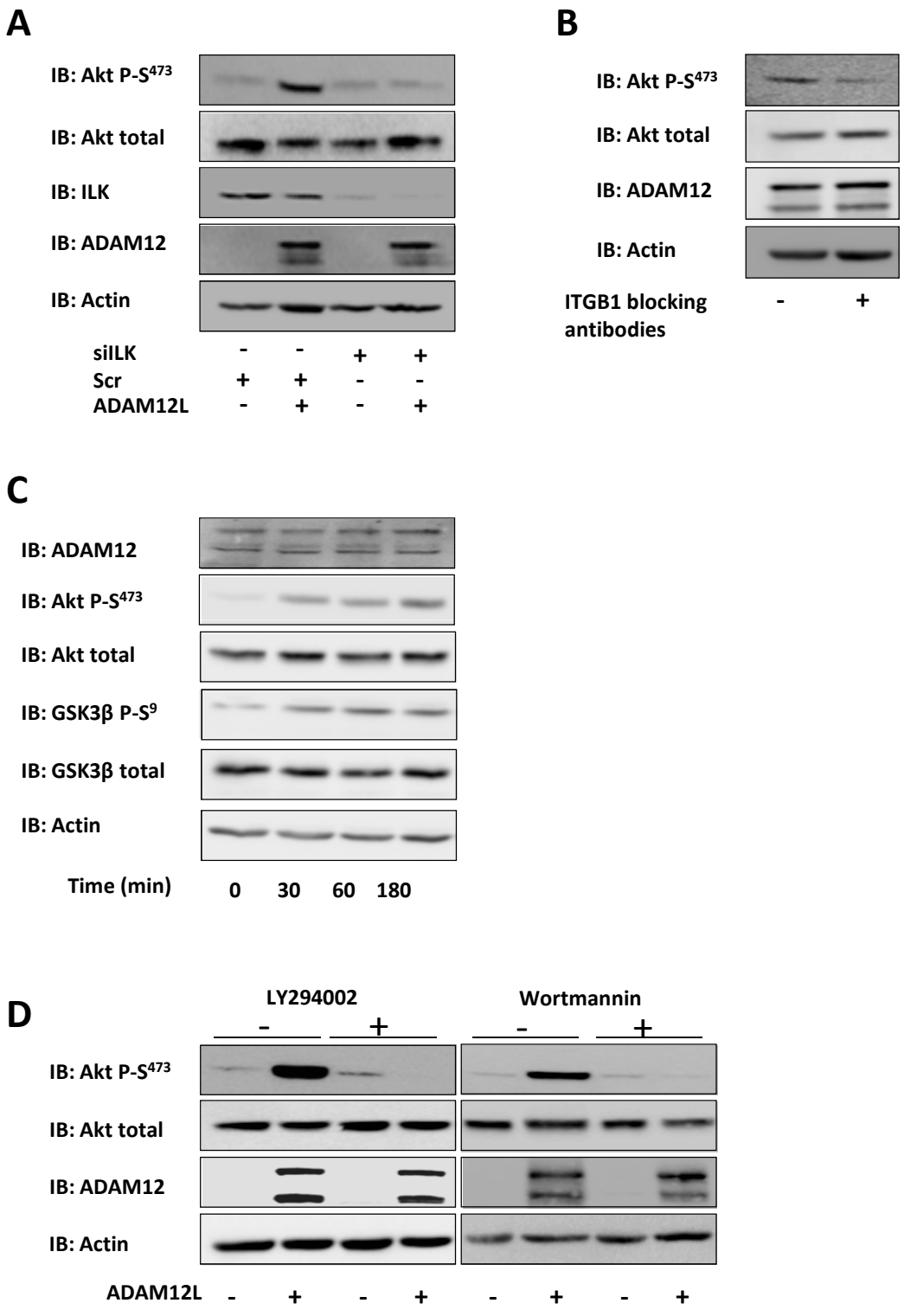

C
Control
ADAM12S
ADAM12-Δcyto
ADAM12L
IB: ADAM12
IB: Akt P-S473
IB: Akt total
IB: GSK3β P-S9
IB: GSK3β total
IB: Actin
Akt P-S473/total
GSK3β P-S9/total
ADAM12L
ADAM12S
ADAM12-Δcyto
Control

Figure 6


A
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IB: Akt total
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siILK - - + +
Scr + + - -
ADAM12L - + - +
B
IB: Akt P-S473
IB: Akt total
IB: ADAM12
IB: Actin
ITGB1 blocking antibodies - +
C
IB: ADAM12
IB: Akt P-S473
IB: Akt total
IB: GSK3β P-S9
IB: GSK3β total
IB: Actin
Time (min) 0 30 60 180
D
LY294002
Wortmannin
- + - +
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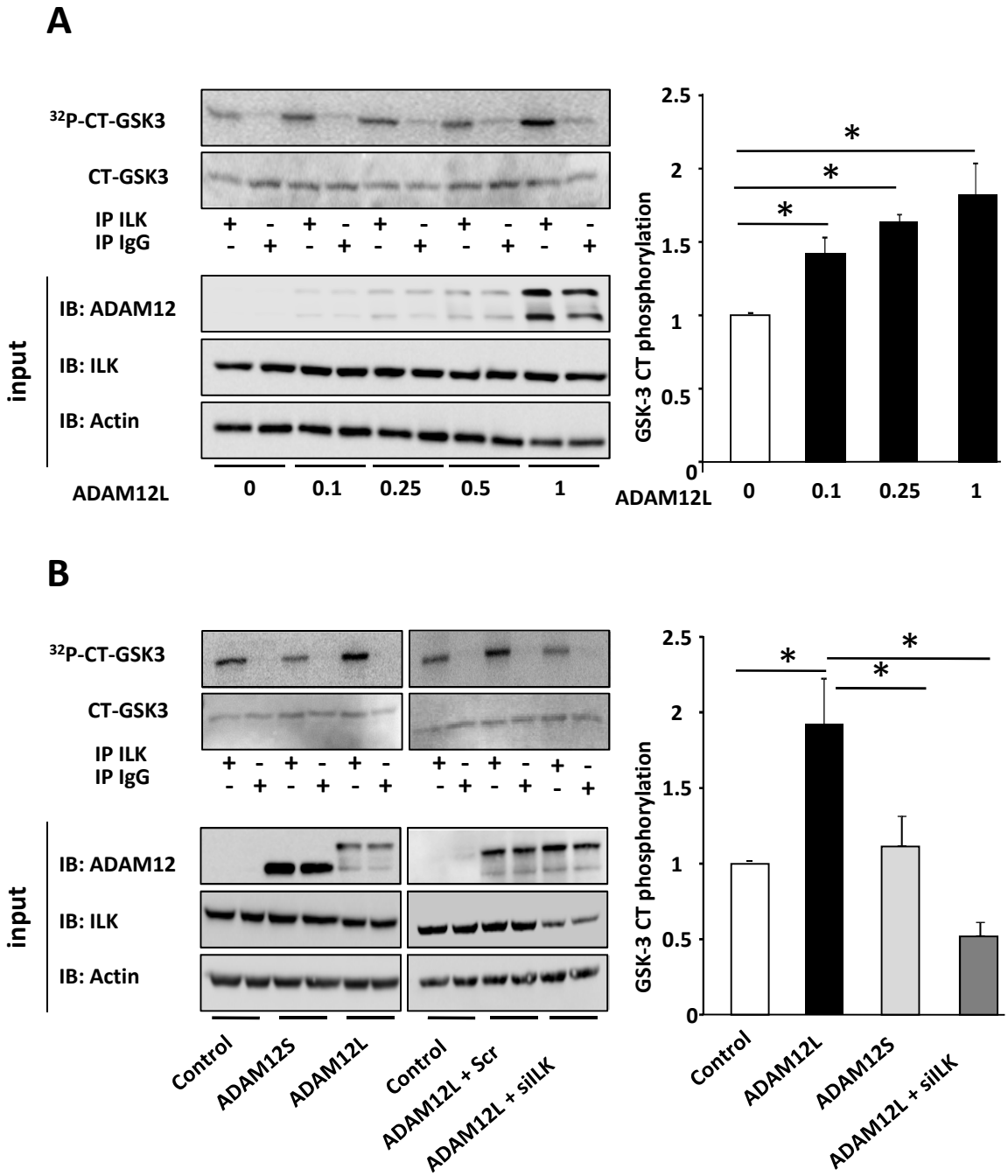
ADAM12L - + - + - + - +

Figure 7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0.25
0.5
1
GSK-3 CT phosphorylation
2.5
2
1.5
1
0.5
0
*
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CT-GSK3
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IP IgG
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Control
ADAM12S
ADAM12L
Control
ADAM12L + Scr
ADAM12L + siILK
GSK-3 CT phosphorylation
Control
ADAM12L
ADAM12S
ADAM12L + siILK

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To Dr. Carl-Henrik Heldin, Editor
Molecular Biology of the Cell

Rennes, June 10, 2012

Dear Pr. Heldin,

I wish to thank you again for giving us the opportunity to submit a suitably revised version of our manuscript entitled "Identification of ILK as a new partner of the ADAM12 disintegrin and metalloprotease in cell adhesion and survival" by Leyme et al.

We have carefully considered the points raised by the Reviewer 3 and we have addressed the three concerns, namely by making news experiments to improve microscopy pictures for Figure 4C and to reply to the questionable adhesion assays in Figure 4. These adhesion assays were clarified by adding a separated section in material and methods. Concerning the third point, the results for migration and proliferation assays, added to respond to the reviewer 2, were discussed and were now included as supplementary material.

The changes made are listed in detail below. They include a number of clarifications, underlined in the revised manuscript, as well as changes in Figure 4C and D.

We hope that our revised manuscript, which we believe brings important insights into the novel role of ADAM12 in cell survival pathway, will now be considered suitable for publication in Molecular biology of the cell.

I wish to thank you again for your time and consideration.

With all best regards,

Nathalie Théret, Ph.D.

Reviewer #3 (Remarks to the Author):

The manuscript reports the novel finding that ILK (integrin-linked kinase) associates with the cytoplasmic domain of the cell surface ADAM-12 metalloproteinase-disintegrin protein, and that these proteins co-localize in focal adhesions with β1 integrin upon binding of cells to collagen. The authors show that depletion of endogenous ADAM-12 in hepatic stellate cells (HSC) led to disorganization of actin stress fibers and affected adhesion to various matrices. Forced expression of ADAM-12 in Cos-7 cells that do not express endogenous ADAM-12 led to minor increases in cell proliferation (3H-thymidine) and cell migration (scratch wounding). ADAM-12 expression enhanced survival signalling through Akt and GSK-3 activation. All of these ADAM-12-dependent effects required the presence of the cytoplasmic domain and were independent of its metalloproteinase activity.This is an interesting, well-executed and well-written study. There is clear evidence to support the association of ILK and the ADAM-12 cytoplasmic domain, though it is not clear whether this is direct or indirect through some other shared components of the IPP or other integrin-associating proteins. It is a strength that the work uses both an over-expression model (Cos-7) and looks at the effects in a system that endogenously expresses ADAM-12. In answering the previous referees' criticisms the authors have included new experiments to analyse the effects of ADAM12 on cell proliferation and migration, and I think that these are now the weakest aspects of the revised manuscript. Overall the science is very good but there are a few specific issues remaining or introduced.

Major points:

Point 1. *The effects of ADAM-12 knockdown on cytoskeletal organization in HSCs in Figure 4C are not particularly well documented. What has happened to the DAPI staining in the central (Scr) panel? Why are there DAPI stained nuclei apparent in the upper images that do not appear to correspond to any particular cell structures? The authors should improve these images to get the message across better.*

New immune-fluorescence studies were performed to improve the quality of the pictures. These new experiments replaced the previous one in figure 2C right panel in the revised manuscript.

Point 2. *Figure 4D is also problematic. ADAM-12 knockdown affects cell adhesion to multiple matrices (as might be expected for a β1 integrin-dependent mechanism), but I think the authors should not claim that there is a "reduced loss of cell adhesion on collagen, fibronectin and laminin compared to the albumin control". The effects are very minor and modest and it is surely better to simply say that adhesion was reduced compared to the Scr treatment. On a linked matter, the Materials and Methods do not adequately explain these adhesion assays. If they are conducted in the presence of serum, then effectively there will be a coating of fibronectin from the serum present; if there is no serum then I am surprised that there is so much binding to the plastic and albumin controls. The methods should therefore be described in a separate section of the Mats and Methods.*

We fully agree with the reviewer about this point that was not correctly documented in the manuscript. In figure 4D, experimental conditions were voluntary similar to that used for observation of cytoskeleton organization in ADAM12-knockdown cells that was 2% serum (figure 4C) as described in material and methods for immunostaining studies. In such conditions and in accordance with the comment of the reviewer, the presence of serum, even low, increased the background in all conditions including albumin coated dishes. Albeit

significant, we agree that the reduced loss of adhesion on cell matrix is modest in such conditions.

To definitively demonstrate the effect of matrices in rescuing the loss of adhesion in ADAM12 silenced cells, we performed three new independent experiments in complete absence of serum. Upon that condition, there was no adhesion on albumin as expected and the effect of collagen, fibronectin and laminin was significant compared with control ($p<0.01$).

In order to homogenize our data, we also performed new kinetics of cell adhesion on type I collagen (figure 4D, left panel) using similar conditions, i.e. in free serum conditions. As expected, the loss of adhesion of knockdown ADAM12 cells compared to wild type was more stringent in absence of serum: 26%±0.25 and 40%± 2.7 instead of 15±5 and 37%±5, in presence of 2%FCS, for 24 and 48h, respectively. The type collagen again delayed this effect with a significant reduction of adhesion only after 48h (35.6±2.8 instead of 33%±5 when cells were cultured with 2% serum). Taken together, these new data did not change our conclusions described in the manuscript.

These new experiments replaced the previous one in figure 2D (left and right panels) in the revised manuscript. Material and methods were modified to include a separated section for adhesion assay (page 19).

Point 3. *In Figure 5 using the ADAM-12-transfected Cos-7 cells, I think the authors are concluding too much from these data. Both the proliferation and migration effects are very minor, and are perhaps limited by the transfection efficiency into the Cos cells. Perhaps it might be better to look at random cell migration following transfection, where ADAM-12 negative and positive cells could be followed in the same field? Likewise I think the authors need something stronger to conclude an effect on proliferation, perhaps some cell cycle analyses of cells that are ADAM-12 positive versus negative. Alternatively perhaps these aspects could be downplayed in the manuscript, as it is likely that ADAM-12/ILK interactions are not the main drivers of proliferation/migration in either model*.

The rational for exploring the effect of ADAM12 in cell proliferation and migration was based on the previous reported role of ILK in these processes. We agree with the reviewer about the minor effect observed in ADAM12 over-expressing cells but this fact was clearly indicated in the manuscript as *" a slight but significant increase in cell proliferation (Figure 5A) and migration (Figure 5B"*
In spite of repeated experiments and a high expression of ADAM12 in COS cells attesting the transfection efficiency, we never detected higher effects. However, our observations are in accordance with the literature since the effect of ADAM12L expression on cell proliferation depends on cell and culture conditions. Thus, ADAM12L expressing bronchial epithelial cell clones have been firstly shown more proliferative than wild-type cells (Rock et al., 2008), however stable ADAM12L over-expressing epithelial breast cell clones and their wild type counterpart have similar proliferation rates in presence of estrogen (Roy &Moses, 2012). More importantly, using stable expressing cell clones, Roy et al. demonstrated that unlike the short ADAM12S form, over-expression of ADAM12L did not result in significant increase in cell migration and invasion (Roy et al. 2011).

Taken together and as suggested by the reviewer, these observations support the fact that the full length ADAM12L is not *a main driver of migration* and that its role in proliferation is moderate and depends on cell type.

According to this comment, we modified the text on page 10 to downplay the role of ADAM12L in cell proliferation and migration and we included the figure 5 in supplementary data (Figure 4S) rather in the full length paper: The sentence on page 10 was modified as follow: *As shown in Figure S4, ADAM12 induced a slight but significant increase in cell proliferation (Figure S4A) and migration (Figure S4B) suggesting a minor effect in these conditions.* In addition the text was modified in discussion to perspective our own data on Cos7 cell proliferation (pages 15 and 16).

Roy R, Moses MA. Breast Cancer Res Treat. 2012 Feb;131(3):731-41.

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Roy R, Rodig S, Bielenberg D, Zurakowski D, Moses MA. ADAM12 transmembrane and secreted isoforms promote breast tumor growth: a distinct role for ADAM12-S protein in tumor metastasis.J Biol Chem. 2011 Jun 10;286(23)

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Minor points

• Scr not Src in Figure 8

Done, thanks !